INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9315507

Proton entrapment in acidic lipid membranes: Intramolecular vs. intermolecular hydrogen bonding

Syz-Chin, Jing-Yi, Ph.D.

City University of New York, 1993

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

PROTON ENTRAPMENT IN ACIDIC LIPID MEMBRANES

— INTRAMOLECULAR vs. INTERMOLECULAR HYDROGEN BONDING

by

Jing-Yi Syz-Chin

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

1993

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.

11/13/92
Date

Thomas H. Haines
Chair of Examining Committee

November 13, 1992
Date

Horst F Schulz
Executive Officer

Charlotte S Russell
William Sweeney
Lesley Davenport
Horst F Schulz
Supervisory Committee

The City University of New York

ABSTRACT

PROTON ENTRAPMENT
IN ACIDIC LIPID MEMBRANES
— INTRAMOLECULAR vs. INTERMOLECULAR
HYDROGEN BONDING

by
Jing-Yi Syz-Chin
Advisor: Professor Thomas H. Haines

Proton-pumping across biological membranes is a major energy transduction activity of all living cells. At present there has been no suggestion that lipids participate in this activity. Many proton-pumping membranes are constituted primarily of anionic (together with uncharged) lipids, e. g., chloroplasts, archaebacteria. All biological membranes have excess negative charges on their membrane surfaces. The pK's of anionic groups are altered by their local environment in macromolecular systems. Three known microenvironments that alter anion pk's are (1) insertion into a hydrophobic medium or domain, (2) the proximity of neighboring anions (acid-anion formation) or (3) the proximity of neighboring cations in three dimensional space. In this work an example of each of the first two is explored in detail. The example of the first is the chlorosulfolipid system found in all fresh-water algae and best explored in *Ochromonas danica.* This system is an example of protonated alkyl sulfates assembled by intermolecular hydrogen bonds involving acidic protons. The

second is a unique acid-anion found in cardiolipin. Cardiolipin is present in membranes such as the mitochondrial inner membrane and also the inner membrane of certain gram-negative bacteria (e. g., *E. coli*) where proton-pumping is a central energy-transducing activity.

Ochromonas danica, a fresh water phytoflagellate, grows optimally at pH 4.5. The plasma and flagellar membranes of this organism are mainly composed of 1,14-docosane-disulfates with from 1-6 chloro groups replacing hydrogens on an otherwise saturated chain (at least 15 of them). These chlorosulfolipids constitute over 90% of the polar lipids of the membrane, with sterols (~15% total lipid) and free fatty acid (~12% total lipid) as the other principal lipid constituents. *O. danica* also extrudes "extracellular vesicles" which consist of the above lipids in the same proportion.

In order to explore the basis for stable bilayer formation by chlorosulfolipids the chlorosulfolipid mixture was isolated and purified from *O. danica* and reconstituted into vesicles using oleic acid and cholesterol replacing free fatty acid and sterol as a comparable model system. Bilayer reconstitution required prior protonation of the sulfatides. This occurred at pH 2.0 or less. A rapid jump to pH 8 provoked bilayer vesicle formation. Examination of the reconstituted vesicles by EM (electron microscopy) and PCS (photon correlation spectroscopy) indicated that these vesicles are stable at pH 7. However, they are unstable below pH 6.5 and tend to aggregate or fuse producing lipidic aggregates. The above data are interpreted as further evidence that the secondary sulfate of the alkyl disulfate lipids is in the protonated form in the low dielectric. They confirm the earlier observations of Ezzat Elmaraghy that the extracellular vesicles, on repeated suspension in deionized, distilled water are gradually dissolved releasing a ratio of protons and sulfatides of 1:1 through ten resuspensions.

The second part of this thesis is devoted to another type of hydrogen bonding of lipids involving acidic protons. Such bonding is referred to as acid-anion formation (intramolecular hydrogen bonding) and might explain the properties of the headgroup of cardiolipin (CL). Formation of an acid-anion for cardiolipin would require the participation of the hydroxyl on the central carbon of the glycerol connecting the two phosphates on the molecule. To explore the possibility of acid-anion formation for this lipid the CL and its deoxy analogue (dCL), in which the central hydroxyl of the gylcerol is replaced by a hydrogen atom, were prepared as bilayers and titrated against 1 and 2 equivalents of KOH and examined by ^{31}P NMR. In addition the two molecules were titrated directly. Both the titration curves and the ^{31}P NMR showed that CL has two pK's ($pK_1 = 2.8$; $pK_2 = 7.5$), while the deoxy analogue of CL has two different pK's ($pK_1 = 2.8$; $pK_2 = 3.8$). This is interpreted to mean that the central hydroxyl group of CL forms an intramolecular hydrogen bonding to one of the phosphate groups forming a unique acid-anion altering the second pK. The second phosphate if protonated, forms a hydrogen bond with a pair of electrons on the hydroxyl. The hydroxyl thus participates in two hydrogen bonds, one as a donor and one as an acceptor, this permits the single proton to resonate between the two phosphates. CL displays a single pK when it is dissolved in an organic solvent as has been known for many years. These results suggest that CL traps a proton via intramolecular hydrogen bonding in bilayers or organic solvents. It is found in this state in natural membranes. The second pK of CL is sufficiently high (and changes to higher levels as the titration proceeds) so that its role may be to participate in proton-pumping by providing a reservoir for protons at unusually high pH's.

We therefore conclude that both intermolecular hydrogen bonding and intramolecular hydrogen bonding of acidic protons are important in bilayers.

ACKNOWLEDGEMENTS

This thesis is dedicated to my parents and husband whose love, patience and encouragement have helped to bring this thesis to its successful completion.

My special thanks to the full support of Dr. Thomas H. Haines, my mentor, who, despite all manner of uncontrollable obstacles, has been gracious with his time and has given invaluable assistance in formulating the ideas presented here. His guidance has helped me probe deeper into the issues and has motivated me to think critically.

I am especially indebted to Dr. Morris Kates for his advice and providing the cardiolipin and deoxycardiolipin for these studies. These experiments would not have been completed without his help. I greatly appreciate the help of Dr. Theodore Axenrod who obtained the NMR spectra used in this study. I thank Dr. David Gosser for the computer program that permitted me to obtain the pK's from the titrations. I also thank Dr. Laura Tres who obtained some of the EM pictures used in this study. I would like to give a special thanks to Dr. Christopher Rutkowski for his moral support.

TABLE OF CONTENTS

A. INTERMOLECULAR HYDROGEN BONDING: THE CHLOROSULFOLIPIDS

INTRODUCTION

EXPERIMENTAL PROCEDURES

RESULTS

B. INTRAMOLECULAR HYDROGEN BONDING OF CARDIOLIPIN

INTRODUCTION

EXPERIMENTAL PROCEDURES

RESULTS

DISCUSSION

CODA:

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS:

ATP, adenosine 5'-triphosphate; CL, cardiolipin; 18:0-CL, stearic-cardiolipin; CSA, chemical shift anisotropy; CSL, chlorosulfolipids; dCL, deoxy-cardiolipin; 16:0-dCL, palmitic-deoxy-cardiolipin; DNA, deoxyribonucleic acid; dPGP, deoxy-phosphoglycerophosphate; EM, electron microscopy; H_{II}, hexagonal II phase; NMR, nuclear magnetic resonance; PAPS, 3'-phospho-adenosine-5'-phospho-sulfate; PC, phosphatidylcholine; PCS, photon correlation spectroscopy; PE, phosphatidyl-ethanolamine; PG, phosphatidyl-glycerol; PGP, phosphoglycerophosphate; PS, phosphatidyl serine; SDS, sodium dodecyl sulfate; SOS, sodium octadecyl sulfate; TEM, transmission electron microscopy; TLC, thin layer chromatography; T_m, transition temperature.

PREAMBLE

Living organisms are surrounded by negatively charged membranes. The negative charges are derived from an excess of both acidic lipids and acidic proteins. The biological reason for this is not known. There are three classes of lipids, namely zwitterionic (PC, PE, etc.), glycolipids (or uncharged) and anionic lipids (cationic lipids are extremely rare in nature). Some zwitterionic lipids such as PS are rendered negative by virtue an extra carboxyl. Some glycolipids such as gangliosides and sulfated cerebrosides are also rendered negative by an extra carboxyl or sulfate.

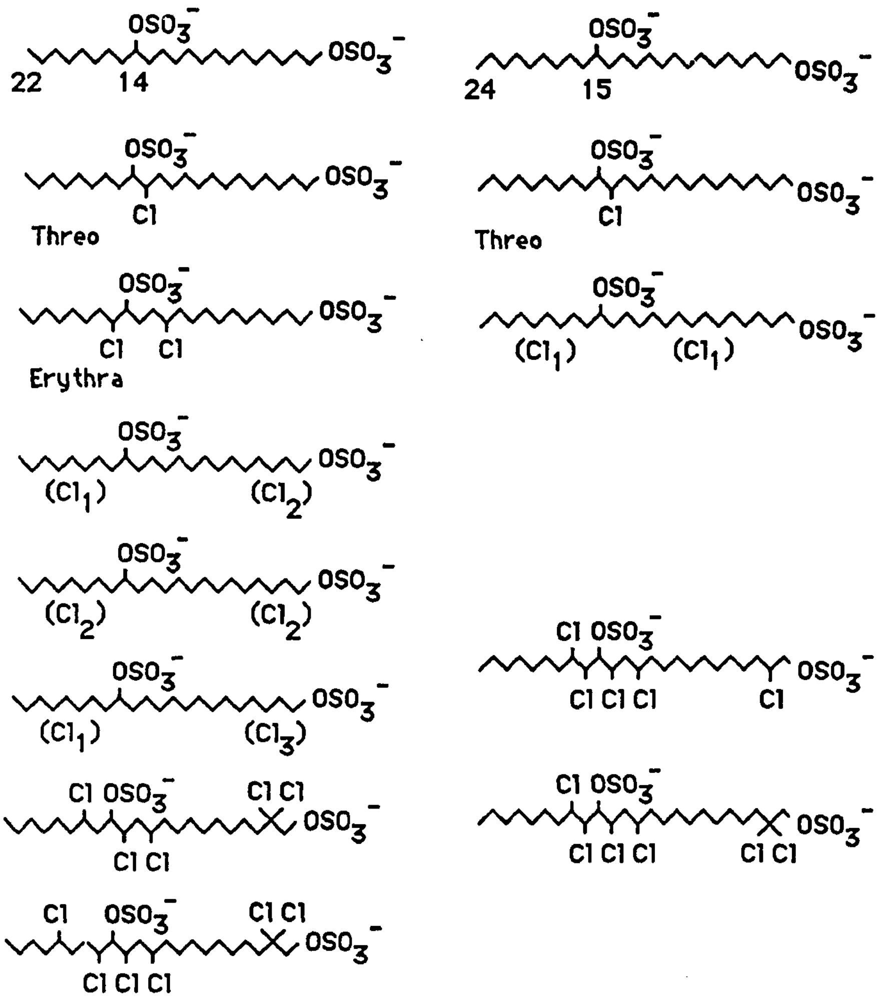
One consequence of negatively charged bilayer lipids is that they concentrate solution cations to the surface of the bilayer (Gouy-Chapman effect) including especially protons at low ionic strength. A second possible consequence of the presence of negative lipids is suggested by the present work, namely proton entrapment. Two examples of this phenomenon are illustrated by this work. The first is an example (chlorosulfolipids) in which the entrapped acidic protons are engaged in hydrogen bonds in the low dielectric (aliphatic domain) of the bilayer. One function of the entrapped protons appears to be the stabilization of the bilayer since the chlorosulfolipids will not form a bilayer unless they are protonated.

The chlorosulfolipids (CSL) of fresh water algae, are derivatives of 1,14-docosane disulfate, 1,15-tetracosane disulfate and 1,14-tetracosane disulfate with chloro-groups replacing hydrogens on an otherwise saturated chain (Haines et al., 1969; Elovson and Vagelos, 1969 & 1970). The first two have been identified in *Ochromonas danica* (Haines, 1973) (Figure 1) and the latter in *O. malhamensis* (Mercer and Davies, 1979). Examples of CSL have been characterized in some 11 species of algae (Mercer & Davies, 1974, 1975, 1979). For a review see Haines, 1972.

Neither *O. danica* nor *O. malhamensis* have a cell wall or matrix to protect the membrane. Yet each is a fresh water organism which implies the bilayer must resist osmotic bursting. A hydrogen bonding network in the low dielectric of the bilayer (possibly including the intermolecular participation of protonated sulfates and chlorogroups) appears to provide an osmotic resistant membrane bilayer. This is analogous to the the hydrogen bonds in double-stranded DNA which are embedded in a hydrophobic interior of the helix. Indeed many enzymes have critical functional groups of amino acids (e. g., glutamate or aspartate) that have pK's which are unexpected due to their location in a hydrophobic pocket. An outstanding example is lysozyme (Phillips, 1966).

A curious use for the entrapped protons in the CSL system is pH-adjustment of the culture medium. *O. danica* is cultured at pH 4.5 for optimal growth. It was found by El Maraghy (1982) that

Figure 1. Disulfatides of *Ochromonas danica*

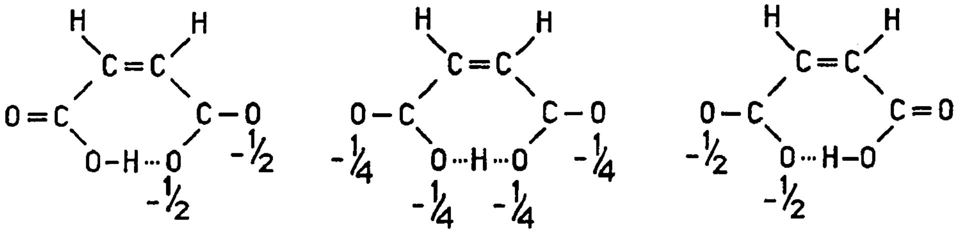


extracellular vesicles were formed when the organism is suspended in media at pH 7. When these extracellular vesicles (which are made of plasma membrane) are repeatedly resuspended in deionized water there is a release of CSL and protons in a ratio of 1:1 until the solution pH is adjusted to 4.5. Since the organism is a fresh water organism and since it grows optimally at pH 4.5, another use of the entrapped protons is to adjust the local environment of the fresh water phytoflagellate.

A second type of pK-shifting is observed for specific anions proximal to charged groups in three-dimensional space. If one anion is proximal to the second by virtue of molecular shape, conformation, or molecular assembly it may be protonated and form a hydrogen bond with the second. Such a hydrogen bond is stabilized by resonance. Maleic acid is an example (Figure 2). Consider an electron on the anionic hydrogen-bond acceptor which moves to the protonated acidic group (donor). The two acidic groups switch roles. The acidic proton simply vibrates as the electron pair is delocalized among the oxygens of both carboxyls. This phenomenon, described as an acid-anion, was first described by Westheimer (Westheimer and Benfey, 1956). The acid-anion is based on the proximity (by ring formation) of the two carboxyls of the dicarboxylic acid. The two pK's of maleic acid ($pK_1 = 1.9$; $pK_2 = 6.3$) are determined by the geometry of the molecule. The pK's of fumarate ($pK_1 = 3.1$; $pK_2 = 4.6$), which carboxyls are *trans* compared to those of maleate (*cis*) are

Figure 2. Diagram of an Acid-Anion

This figure is based on CNDO/2 calculations (Morita and Nagakura, 1972) describing acid-anion formation of maleate. The calculation indicates that the hydrogen atom vibrates between two oxygens, one from each of the two carboxylates. When the hydrogen is equidistant from the two oxygens, all four oxygen atoms share the single negative charge. The calculation suggests that each oxygen contains 1/4 of the negative charge (B). When the hydrogen is closer to one oxygen than the other (A and C) there is a covalent bond between one oxygen and the hydrogen. The CNDO/2 calculations are consistent with the x-ray crystal structure and Raman spectra.



17½%
(A)

59%
(B)

17½%
(C)

illustrative of how acid-anion formation is important in understanding the unique pK's of maleate's carboxylates.

If cardiolipin could form a double-ring system, it would be capable of forming an acid-anion as shown in Figure 3. The deoxy derivative is an analogue that is not capable of forming the double-ring structure (Figure 4). It would therefore be useful to compare the pK's of the deoxycardiolipin to those of CL. It should be noted that cardiolipin (in eukaryotes) is only present in the mitochondrial inner membrane and that it is also found in aerobic prokaryotes. It is absent from anaerobes. These two CL-containing membranes, the mitochondrial inner membrane and the prokaryote cell membranes, have two interesting features in common. They both engage in proton pumping and both participate in the coupling of redox reduction with ATP synthesis via proton pumping. Incidentally, all membranes that contain CL also lack cholesterol. The evidence presented herein suggests that cardiolipin might play a role in maintaining protons available for proton pumping at high pH's when the proton concentration would be low. That would be possible if the pK of the second proton of CL were high (>pH 7.0). In this context one might examine mutants of *E. coli* that lack cardiolipin. Such mutants grow by substituting PG for CL (Gally, et al., 1980).

In summary, this thesis describes the entrapment of protons by anionic lipids in a bilayer due to

Figure 3. The Resonance Forms of Cardiolipin
The figure illustrates the two resonance forms of an acid-anion involving the phosphates and the central free hydroxyl of CL.

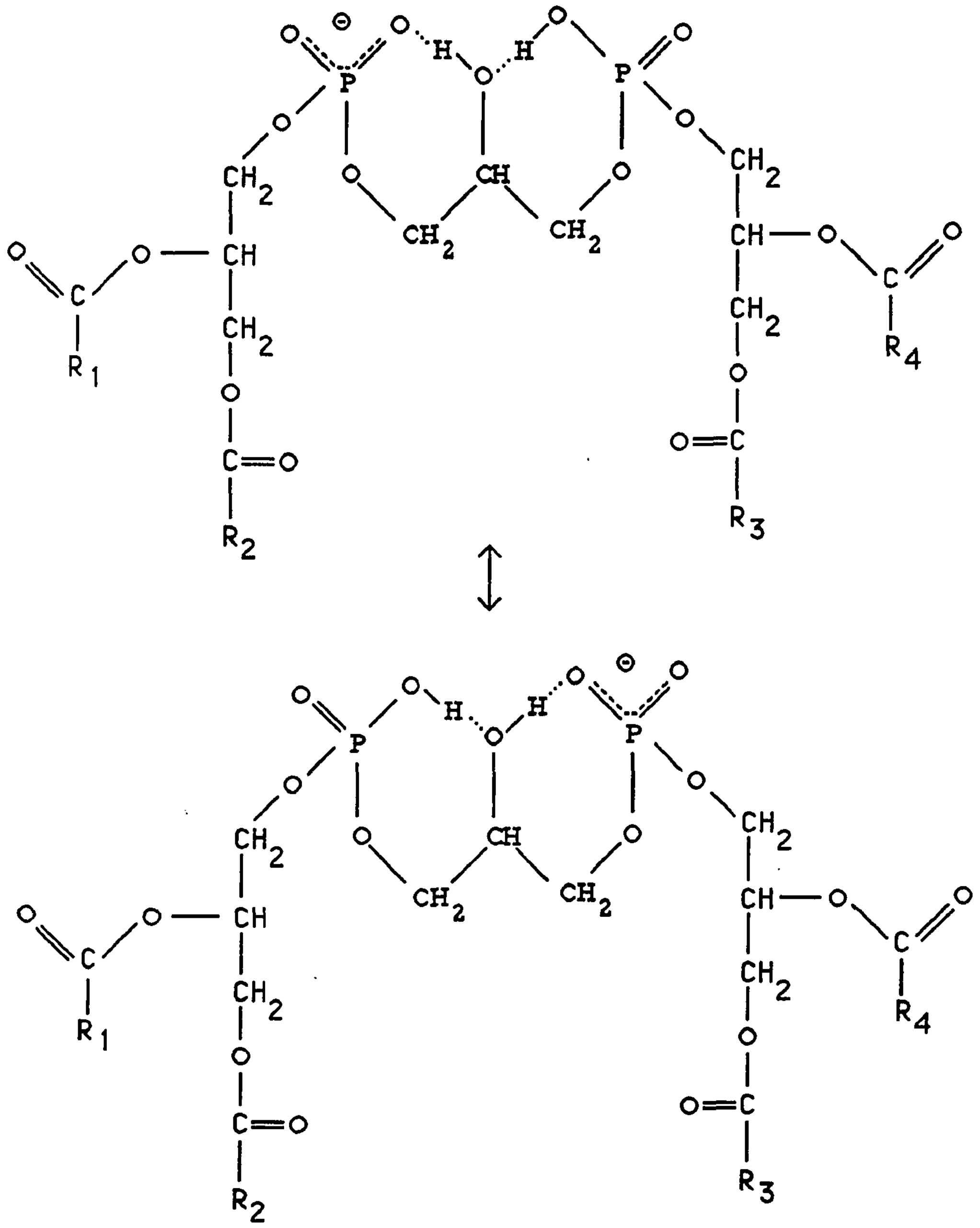
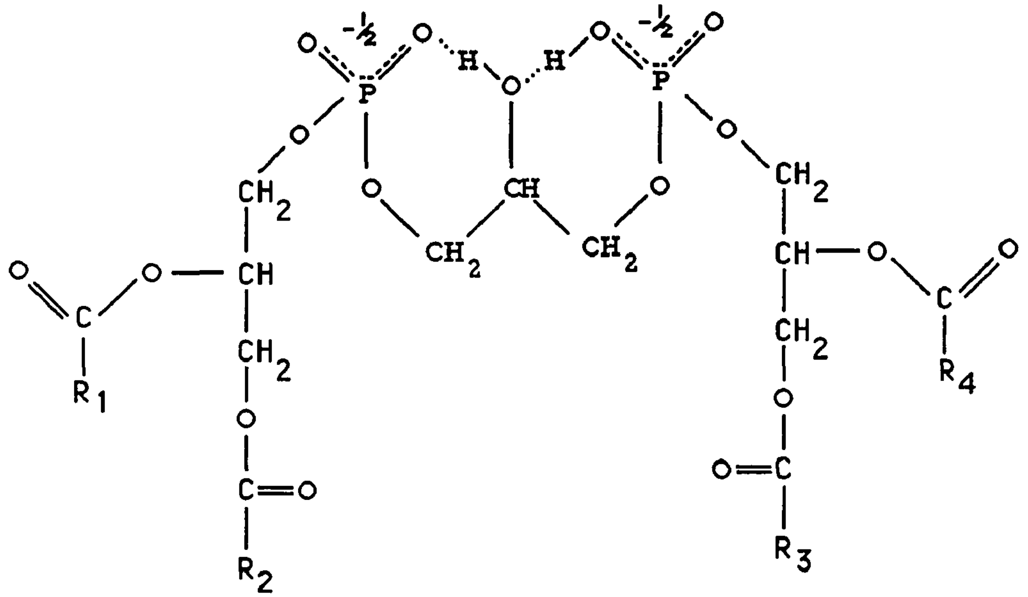
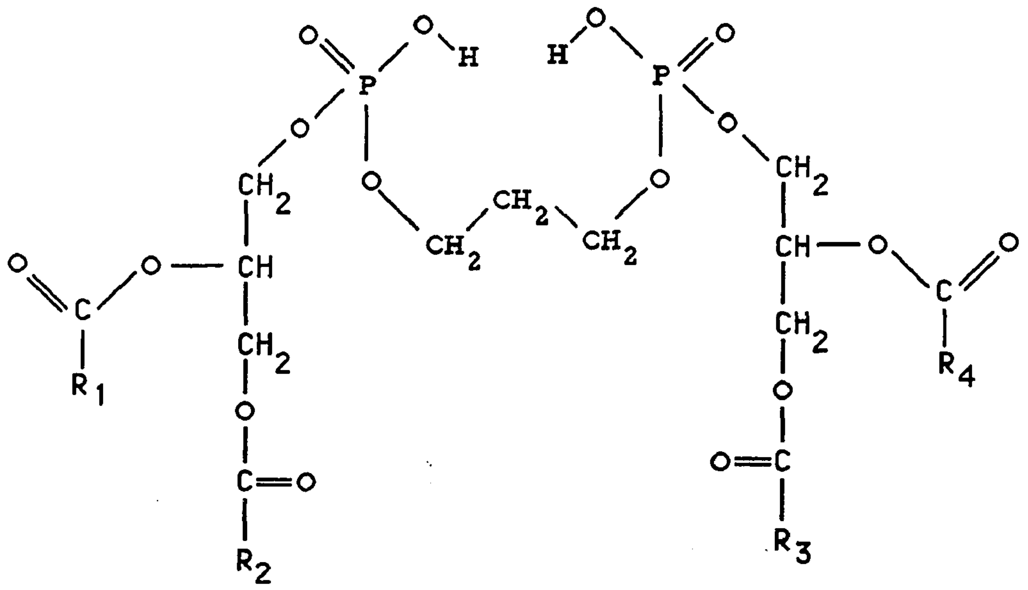


Figure 4. Structures of Cardiolipin (CL) and Deoxycardiolipin (dCL)

Deoxycardiolipin cannot form a bicyclic conformation since it lacks the hydroxyl.



CARDIOLIPIN (CL)



DEOXYCARDIOLIPIN (dCL)

(a) intermolecular hydrogen bonding deep in the bilayer. (This is exemplified by the chlorosulfolipids which may ultimately facilitate strengthening of the bilayer laterally, as well as adjustment of the medium pH in cultures of *O. danica*), and

(b) intramolecular hydrogen bonding in cardiolipin when it is in bilayers (where it may serve as a reservoir for protons in proton-pumping membranes at high pH).

A. INTERMOLECULAR HYDROGEN BONDING: THE CHLOROSULFOLIPIDS

INTRODUCTION

Most membrane investigations are performed on phospholipids and glycolipids because they are the major components of known biological membranes. These polar lipids have two chains per headgroup and spontaneously form bilayers in water with their polar components facing the aqueous surfaces. Nevertheless, chain hydrocarbons containing a terminal primary sulfate as a head group have been characterized in several natural membranes (Haines, 1972). Likewise sterol sulfates are known to function as important membrane constituents in algae (Kates et al., 1978) and throughout the mammalian system (Ishizuka et al., 1978; Sweeley and Siddiqui, 1977). “Sulfolipid” is used as the general term; it includes steroidal and other lipoidal sulfate esters as well as sulfonic acids, sulfocholines, etc. (Haines, 1965, 1972; Radin, 1983). The term "sulfatide" is restricted to those sulfolipids that contain ester sulfate including both the hydrocarbon sulfates and glycolipid sulfates.

Chlorosulfolipids as Membrane Lipids:

In recent years several different aliphatic and alicyclic single chain (or steroid) lipids have been characterized that suggests the existence in the biosphere of a different kind of membrane than the phospholipid membrane (Hatanaka et al., 1976). These lipids are alkyl sulfates with chains that vary from 18 to 30 carbon atoms (Liem and Laur, 1976b) though typically contain 22 to 24 carbon atoms (Mercer and Davis, 1979). All of these alkyl sulfates, with one exception (Liem and Laur, 1976b), have 2 sulfates on the chain: a primary sulfate and a secondary sulfate. The latter may be positioned 6 to 18 carbon atoms down the chain from the primary sulfate.

Most of the 22 algal species that contain these sulfatides carry chloro groups on their alkyl chains (Mercer et al., 1974). The chloro groups are generally located near the sulfate groups. There is at present no explanation for this. Another unusual quality of these membranes is that they always contain free fatty acids. Free fatty acids are rarely found in phospholipid membranes. Indeed membranes, such as diatoms (Anderson et al., 1978a, b) and the brush border membrane, both of which have significant quantities of sterol sulfate, also contain a significant amount of free fatty acids.

This "alkyl sulfate" membrane has not been previously described. The remarkable feature of it is the presence of secondary sulfates down the chain and presumably in the low dielectric of the bilayer.

Mercer and Davies (1979), who screened 30 marine and fresh water organisms for alkyl sulfates, found them to be absent from all 8 of the marine organisms but present in all 22 of the fresh water organisms. Only 2 algae (*Ochromonas danica and Ochromonas malhamensis*) had alkyl sulfates in large quantity. Alkyl sulfates were first recognized to be the major flagellar membrane lipids (92% of the polar lipids) by Chen et al. (1976) and later by Stern (1978) , Poncz (1978) and El Maraghy (1982). They were shown to be present in the same proportion in the plasma membrane and the extracellular vesicles of *Ochromonas danica.* Fatty acids constituted approximately 15% of the total lipid (including both polar and non-polar), sterols around 10%, and the chlorosulfolipid some 70%.

Ochromonas danica a fresh water facultative phagotrophic phytoflagellate, has several membrane characteristics that violate the general rules of membrane stability of the biosphere. Furthermore it has no cell wall or envelope that normally protect the organisms from osmotic bursting in deionized water. Secondary, the organism lacks a protective protein, polysaccharide, glycoprotein or other polymeric coat which normally shields microbes from lipolytic enzymatic hydrolysis of other organisms. Third, if the cells are suspended in pH 7.0 media it sheds extracellular vesicles from its membrane. These vesicles adjust the medium pH to 4.5 - its optimal growth pH.

The organism lacks phospholipids (except in its chloroplast and mitochondria). Its principal lipids consist of the chlorosulfolipids of the flagella and plasma membranes (Figure 1), an unusual zwitterionic glycerolipid that lacks phosphate in its headgroup, and containing N-trimethyl homoserine (Elovson, 1974a and 1974b). This is the dominant lipid in the chloroplast envelope, Golgi, and other intracellular membranes.

In *O. danica* alkyl sulfates constitute 15% of the total lipids and 3% of the dry weight of the cells (Chen and Haines, 1976; Chen et al., 1976). Analysis of the flagellar membrane preparations (Chen and Haines, 1976; Chen et al., 1976) from the organism found that the preparations contained no phospholipids. The flagellar membrane is continuous with the plasma membrane. Alkyl sulfate was established as 90% of the polar lipid; accompanying a small percentage of glycolipid (Chen et al., 1976), sterols (Gershengorn et al., 1968), and fatty acids (Chen et al., 1976; Haines et al., 1962). Investigations of *O. danica* plasma membrane (Patni et al., 1974; Aaronson, 1980) and extracellular vesicles (membrane vesicles excreted from all over the surface of the organism) (Kahan et al., 1978; El Maraghy 1982) reveals a lipid composition which is essentially that of the flagellar and plasma membranes.

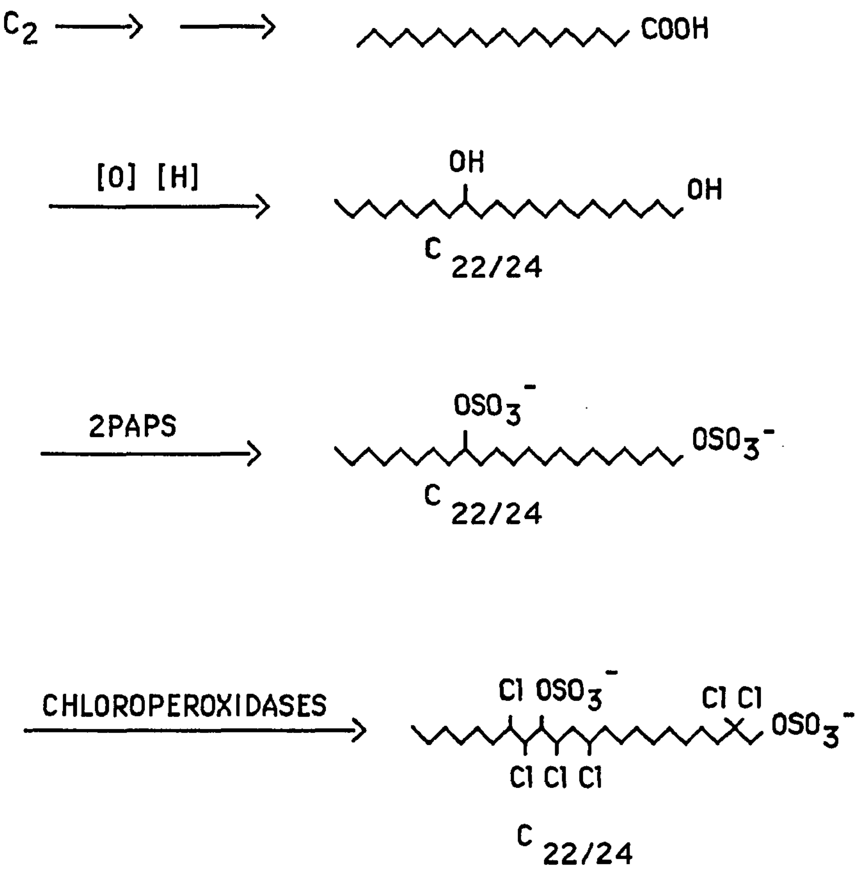
In this alga two families of chlorosulfolipids are present. The first is based on n-docosane-1,14-diol disulfate and the second on n-tetracosane-1,15-diol disulfate. These sulfatides may have from zero to six chlorine atoms replacing hydrogens on the saturated chain. The 13-chloro-, the 11,15-dichloro-, the 2,2,11,13,15-pentachloro- and the 2,2,11,13,15,16-hexachloro-derivatives of *n*-docosane-1,14-diol disulfate have been identified along with other tri- and tetrachloro derivatives. The 14-chloro-, the 1,12,14,16,17-pentachloro-, and the 2,2,12,14,16,17-hexachloro- derivatives of *n*-tetracosane-1,15-diol disulfate also have been identified (Elvoson and Vagelos, 1969 & 1970; Haines el at., 1969; Mayers and Haines, 1967; Mayers et al., 1969) (see Figure 1).

It was found that these sulfatides are biosynthesized through hydroxylation via direct oxidation of the saturated chain. This reaction uses molecular oxygen (Elovson, 1974a), sulfation, and subsequent chlorination (Mooney et al., 1972; Mooney and Haines, 1973). The aliphatic chain is synthesized from acetate through the usual fatty acid synthesis system. The fatty acid is reduced to the chain alcohol. The oxygen of the secondary hydroxyl group is derived from molecular oxygen. PAPS (phosphoadenosine-phospho-sulfate) is the intermediate used to synthesize the sulfatides in vitro. The alkyl chain is then chlorinated presumably by a chloroperoxidase, with from one to six chlorine atoms to give the final product (Mooney and Haines, 1973) (Figure 5).

The alkyl sulfate membrane has several distinct chemical differences from ordinary phospholipid membranes:

a. The membrane lipids do not contain a glycerol moiety; alkyl sulfates are the sole polar lipids.
b. The density of the head group anions is twice that of the phospholipids, i. e., one headgroup sulfate per chain.

Figure 5. Biosynthetic Pathway for the Chlorosulfatides of *Ochromonas danica.*



c. The chloro groups near the sulfates presumably reduces the pK of the sulfates by induction as occurs with the substitution of chloro for the hydrogen on the acetic acid. For the hexachlorosulfatide there are two chloro groups next to each sulfate.

d. Present evidence indicates that the secondary sulfate (C-14 or C-15) of the chlorosulfolipids is located in the hydrophobic region of the bilayer. The model building indicates that the distance of the secondary sulfates from the headgroup, perpendicular to the plane of the bilayer, is approximately that of the *cis*-double bond of the oleic acid-containing phospholipids. This is the same point at which the C-17 side chain of sterols (such as cholesterol) is attached to the fused alicyclic ring system in the bilayer.

e. Typical membrane lipids spontaneously form bilayers when mixed with water at pH 7. Surprisingly, the CSL do not.

One can argue that the secondary sulfate is anionic and on the surface with the chain folding at the seventh or eighth carbon atom so that the secondary sulfate is exposed to water. In this model the 2 negatively charged headgroups are in the water and the lipid has three very short hydrocarbon chains. This model is not in accordance with the experimental studies of alkyl sulfate analogs (Hargreaves and Deamer, 1978; Kunitake and Okahata, 1977) nor with the theoretical arguments of Tanford (1978) that chains with less than eight carbons may not form bilayers. Electrostatic considerations do not allow a charged sulfate to exist in a low dielectric region, therefore, the sulfate must be neutralized. The proton was found to be the counterion for these membrane lipids (Stern, 1982).

It was the last of the above distinctions between the phospholipids and the sulfolipids that provoked the present study. If the CSL did not spontaneously form bilayers, then how could they be membrane lipids? As might be expected from the title of this thesis the conditions under which CSL were found to form spontaneous bilayers included very low pH. This is not what might have been expected since sulfate esters are commonly hydrolyzed under these conditions.

Experimental Problems Associated with CSL Bilayer Reconstitution:

The purpose of the first part of this thesis is to determine the conditions under which the reconstitution of the CSL form bilayers. In addition to the inability of the CSL to form spontaneous bilayers at pH 7 there were two experimental problems associated with their reconstitution: (a) Contrary to the extraction of phospholipids into the lower phase of the Bligh and Dyer procedure, the CSL were soluble in the methanol-water (upper) phase. (b) Phospholipids are easy to assay on the microgram level whereas sulfolipids are not. In 1982, Heller had successfully made liposomes from sodium dodecyl sulfate and oleyl sulfate by including fatty acids and cholesterol. El Maraghy (1982) found that high pH increases the solubility of SL provoking the formation of small vesicles. Therefore the pH adjustment procedure (Aurora et al., 1985) was used. This method involves the formation of vesicles by first protonating the anionic lipids and then quickly raising the pH.

EXPERIMENTAL PROCEDURE

Materials:

All chemicals were Reagent Grade (Fisher Scientific, Fairlawn, NJ). All solvents were redistilled before use. SDS (sodium dodecyl sulfate) was purchased from Sigma (St Louis, MO). Sodium octadecyl sulfate (SOS) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). The purity of the sulfates was monitored with thin-layer chromatography (TLC) on Silica gel 60 plates (EM laboratories, Elmsford, NY) using $CHCl_3/CH_3OH$ (75:25, v/v) as a solvent system. Cholesterol (98% pure) was procured from Aldrich Chemical Company, Inc. (Milwaukee, WI). Oleic acid was purchased from Sigma. Cholesterol and oleic acid were determined by TLC to be at least 99% pure. ORION Research Model 601A digital ionalyzer (Cambridge, Mass) and pH Microelectrode Model MI-410 from Microelectrodes, Inc. (New Hampshire) were used for all pH measurements.

Carbon coated formvar grids for electron microscopy were purchased from Polysciences, Inc. (Warrington, PA). Uncoated copper grids were purchased from Ladd Research Industries (Burlington, VT.). Formvar solution (0.5%) in ethylene dichloride was obtained from Ladd Research Industries. Uranyl acetate was purchased from Ladd Research Industries.

Cultures:

O. danica was grown in the chemically defined medium at pH 4.5 and 23°C according to Aaronson and Baker (1959) in relative darkness (to prevent the formation of chloroplasts). Inoculations were done in ambient light. The cultures were harvested according to Haines and Block (1962) and deflagellated according to Chen and Haines (1976).

Isolation of Sulfolipids:

Fifty milliliters of *O. danica* cells with a minimum amount of water was suspended in 1000 mL solvent ($CHCl_3/CH_3OH$, 2/1 v/v). The suspension was centrifuged in a Beckman Ultracentrifuge (Model L2-65B) or SORVALL superspeed (RC2-B) Automatic Refrigerated Centrifuge (Norwalk, Conn.) at a speed of 10000 rpm for 20 minutes (Temperature = 10-15°C). The pellet was resuspended in another 1000 mL organic solvent and centrifuged under the same condition. At this point, the centrifugate contained a small amount of particles. If the particles floated a small amount of water (a few mL) was added to congeal the precipitate. Deionized-distilled water (250 mL per 1000 mL of organic solvent) was added to the combined centrifugate. The resulting solution was placed in a separatory funnel, gently swirled, and left at room temperature overnight.

A 2-phase system developed. Fresh solvent was prepared and set aside for repeated extraction. The upper phase of the extract solution was washed four times with fresh lower phase to separate CSL from other membrane lipids including fatty acids, sterols, N, N, N-Trimethyl-homoserine diglyceride (Elovson, 1974b), and mitochondrial lipids. Examination of the CSL by TLC in a series of upper phase extractions showed that the bulk of the material was recovered in the first two upper phase washes. It was necessary after each partitioning to adjust the pH to 7.0 by the addition of 0.1N NaOH.

The methanol-water layer (upper phase) of the Folch solvent system contained CSL along with diols (partially hydrolyzed CSL due to the acid produced by the extraction). Separation of CSL from the diol was done by washing the methanol-water phase 4 times with the fresh upper (chloroform) Folch phase. Centrifugation was done to speed up the phase separation. After the fourth wash, the methanol-water phase contained the sodium salt of sulfatides without traces of diols (examined by TLC). The upper phase was concentrated to a small volume and stored below 0°C. Persistent clouding of the chloroform phase was solved by centrifugation and/or drying the chloroform phase briefly with Na_2SO_4.

Quantitative Analysis of Sulfolipids:

Thin layer chromatograms (TLC) of sulfatides were developed in $CHCl_3/CH_3OH$ (75/25, v/v). Both SDS and SOS were used as standards. Plates were visualized by spraying with 25 percent (w/v) sodium bisulfite in 3 percent (v/v) sulfuric acid followed by charring at 450°C. Different sulfatides were identified with

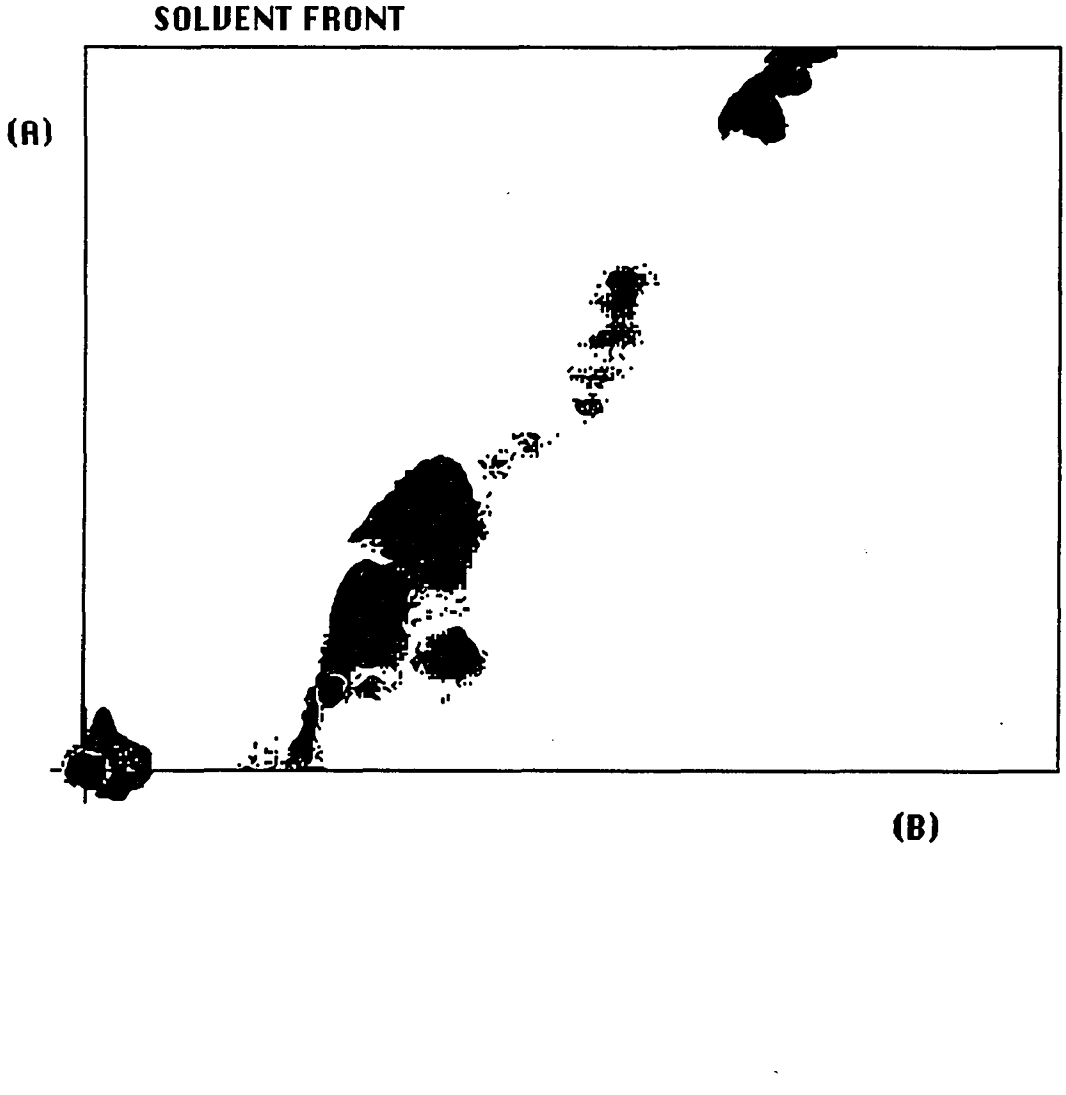
Rouser's solvent system (Rouser et al., 1966). The solvent in the first dimension was $CHCl_3/CH_3OH/NH_4OH$ 65:35:5, v/v/v and $CHCl_3/CH_3COCH_3/CH_3OH/CH_3COOH/H_2O$ 50:20:10:10:5, v/v/v/v/v in the second dimension (Figure 6).

The presence of sulfatides in the upper Folch phase was checked by means of the Kean assay (1968). Aliquots (10µL to 100µL) of samples (2mM to 5mM) were pipetted into screw cap test tubes. All the samples in these test tubes were blown dry under nitrogen. The following solvents were added to each test tube accordingly:

a. 5.0 mL of $CHCl_3/CH_3OH$ 1/1 (v/v)
b. 5.0 mL of 0.05 N H_2SO_4
c. 1.0 mL Azure A solution (40mg Azure A in 5.0mL of 0.05 N H_2SO_4 diluted to 100mL with water)

The tubes were vortexed (with caps on) for 30 seconds and centrifuged at 300g for 5 minutes (International desk top centrifuge). The lower phase was withdrawn and its absorbance was measured at 645nm (Gilford Instrument #2600, Oberlin, OH) to obtain the concentrations of sulfatides with SDS as standard. The timing of this procedure is critical for accurate results (the absorbance increases with time). A standard curve (5 samples) is assayed concurrently each time the assay is used to account for differences in timing on a giving run.

FIGURE 6. Two-Dimensional TLC of the Chlorosulfatides
Two-dimensional TLC of the sulfolipids (second Folch wash methanol-water (upper) phase) from *O. danica*.
Solvents: (A) $CHCl_3/CH_3OH/NH_4OH$ (conc.): (65/35/5)
(B) $CHCl_3/CH_3OH/CH_3COCH_3/HC_2H_3O_2/H_2O$: (50/10/20/10/5, in volume) (Rouser et al., 1966)



Preparation of Alkyl Sulfate Vesicles:

Vesicles with different ratios of dodecanol and SDS (2:1, 1:1, 1:2) were prepared by suspending the samples in deionized-distilled water (20 mg/mL) and sonicated at 50 watts for 15 minutes (on ice) with Branson Sonifier (Branson Sonic Power Co., Danbury, CN.) from Heller's (1982) procedure. Centrifugation (bench top; Model CL, International Equipment, Needham, MA) at 3000g for 20 minutes removed titanium chips from the sonicator tip during sonication of the liposomes. Some vesicles were prepared by using the cup horn sonication method (Barenholtz et al., 1979). This procedure bypasses the necessity of removing titanium fragments.

Preparation of Chlorosulfolipid Vesicles:

Aqueous CSL (sodium salt, 4.5 mL, 6.5 μmol) was added to 5.0 mL of chloroform. Immediately 0.25 mL of concentrated (18 M) sulfuric acid was added. Both *n*-propanol (3.2 mL) and sodium sulfate (35 mg) were added to the mixture. The protonated sulfolipids extracted into the lower phase (chloroform/*n*-propanol). Although the acidified upper phase separated readily from the lower chloroform phase, without the sodium sulfate an intractable interfacial emulsion formed. The mixture was vortexed (1 minute) and centrifuged for 5 minutes with a speed of 1000g in the bench top centrifuge. The upper phase was carefully removed above the interface and the lower phase withdrawn from underneath avoiding the interface. The upper phase was washed twice with chloroform/propanol (5:3, v/v).

The combined lower phases (total = 20.3 mL) were collected into a screw-cap vial and stored at 5°C.

Cholesterol and oleic acid were added to the combined lower phase so that the final molar ratio of sulfolipids to cholesterol and oleic acid was 3-4:1:1. The lower phase solvent was then removed by rotary evaporation in a round bottom flask to form a thin sulfolipid film. The lipid film was not dried completely in order to prevent further hydrolysis of the sulfolipids. The filmy sheet was then dispersed in 5.0 mL of 150 mM NaCl solution. The suspension was stirred with a small (5 mm) magnetic stirrer for 5 minutes at room temperature in order to break up the lipid film in the solution.

At this point, the modified pH adjustment procedure was used (Aurora et al., 1985). The pH of the vesicles was monitored with an Orion (Cambridge, MA) digital pH meter equipped with a micro-pH probe. The initial pH of the solution was about 2 (± 0.5). With a Hamilton micro-syringe, NaOH (0.2 N in 150 mM NaCl) was injected into the vesicle solution with constant stirring until the pH was about 8. When a lower pH was desired for the final vesicle preparation, HCl (0.2 N in 150 mM NaCl) was then injected with constant stirring to the solution in order to lower the pH below 8. The vesicle solution was then filtered through a 2.0 μm Nucleopore polycarbonate membrane (Pleasanton, CA).

Characterization of Vesicles:

To determine the percent composition of chlorosulfatide in each vesicle preparations, a portion (1.0 mL, ~2.1μmol) of the vesicle preparations was centrifuged in Sorvall RC70 ultracentrifuge (T865.1 rotor) at 45,000 rpm for one hour at 4-8°C. Quantitative analysis of the CSL were performed via TLC and the Kean assay.

The average size of the vesicles in each preparation was determined by using photon correlation spectroscopy (PCS). All the samples were filtered through a 2.0 μm Nucleopore polycarbonate membrane prior to size measurements to remove dust. The apparatus used was that described by Hwang and Cummins (1982). Light from an argon ion laser (Spectra Physics Model 165, Palo Alto, CA) at 488 nm was focussed onto the vesicle sample in a glass cuvette (1.0 cm x 1.0 cm, Hellma, Jamaica, NY) maintained at constant temperature (20°C) by a Lauda water circulator. Variations in the intensity of the scattered light was detected at 90° to the incident beam with a Hamamatsu (Middlesex, NJ) photomultiplier tube. The radius of the vesicles in a sample was calculated from a correlation function (see below). Uniform polystyrene spheres (Dow Chemical Co., Indianapolis, IN) with diameter of 126 nm were used as a standard.

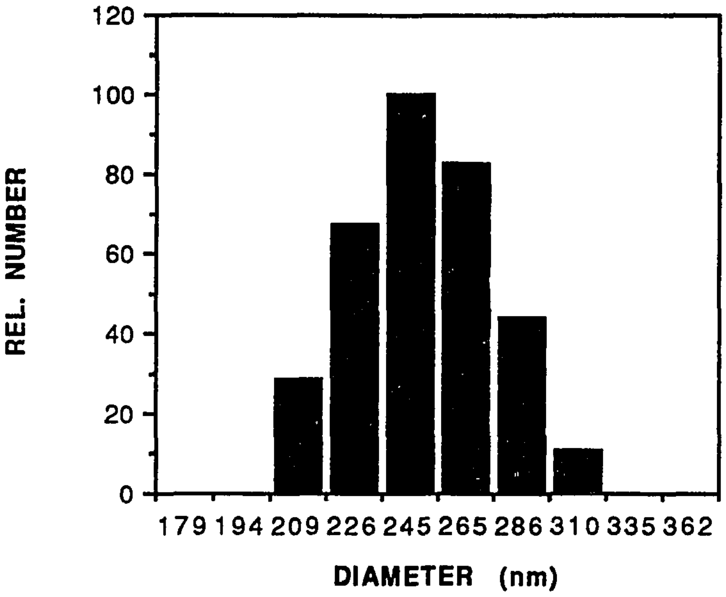
The correlation function was accumulated in a 60 channel photon autocorrelator and initially displayed on an oscilloscope. An A/B ratio (the ratio of first channel counts to the baseline counts) greater then 0.5 indicated a good signal to noise ratio. The signals were collected by a PDP-11 (Plessy) minicomputer or Langly-Ford (Amherst, MA) Model 1096 Digital Autocorrelator (128 channels with 16 baseline channels, 1096 delayed channels) and subsequently analyzed (cumulants analysis) on a VAX 11/780 mainframe computer (Digital Equipment Corporation, Maryland, MA).

The average diffusion coefficient and standard deviation were calculated by averaging nine successive autocorrelation functions for PDP-11 or 3 to 5 for the Langly-Ford, each with the same bin (sampling) time. The count rate was essentially constant for most of the samples (~10,000 cps, the greatest drift of the count rate during sizing was 10%). The analysis also gives an index of polydispersity (this is a term to indicate the size distributions of the vesicles) of the sample which was determined by the cumulants analysis method (Rutkowski et al., 1991).

In addition the polydispersity was assessed using a NICOMP (Model 370) Submicron Particle Sizer (version 4.0) (Nicomp Instruments Inc., Goleta, CA.) (Figure 7). Both our PCS instrument and the NICOMP measurements gave similar results for dodecyl sulfate/dodecanol (50 mol%) vesicle sizes.

The samples were also examined using electron microscopy (EM). Formvar coated grids either prepared with a 0.5% formvar solution in amyl acetate on uncoated 3 mm copper grids or

FIGURE 7. Size Distribution of SDS/Dodecanol Vesicles in 150mM NaCl.



Distribution of the sizes of equimolar SDS-dodecanol vesicles obtained using PCS with the NICOMP. The vesicles were prepared in 150 mM NaCl according to the modified "pH-jump" method. The mean size of the vesicles was 260 ±10 nm.

purchased carbon coated formvar on 3 mm copper grids were used. Each unfiltered sample (5 μL aliquot) was placed on the Formvar coated grid and the excess solution was absorbed with Whatman No. 1 filter paper. A drop of uranyl acetate solution (0.5%, w/v) was placed on the grid and the excess was again absorbed with the filter paper. The grid was allowed to air dry for at least 30 minutes. The grids were studied by transmission EM with a Philips 300 at 60 KV. Kodak Electron Image film was used. Kodak papers were used for the enlargements. Magnifications were calibrated with a diffraction grating of known wavelength.

The standard used for EM was the vesicles containing only cholesterol and oleic acid (1/1, mol/mol) and prepared as described for chlorosulfolipid/cholesterol/ oleic acid.

A Zeiss photomicroscope with Zeiss-Nomarski differential interference equipment for transmitted light microscopy (Carl Zeiss, Inc., NY, NY) was used to examine the vesicles.

RESULTS

Bilayer Vesicles of Alkyl Sulfates and Alkanols:

The preparation of SDS vesicles was used as a model system for the reconstitution of chlorosulfolipid membrane. An advantage of using SDS-derived vesicles as a standard for developing a procedure for the preparation of CSL vesicles is that the aqueous *acidified* CSL are not stable for an extended period. This is especially due to the lability of the secondary sulfate.

Sulfatides may hydrolyze on standing in water when the pH of the solution is lower than 7. The hydrolysis of sulfate esters is autocatalytic (acid produced during hydrolysis accelerates the reaction). This reaction is enhanced by solvents. Indeed traces of water in nucleophilic organic solvents provoke rapid solvolysis of all aliphatic sulfates (Burstein and Lieberman, 1958; Haines, 1971).

SDS vesicles were used to explore the optimum concentration of NaCl needed for vesicle formation and stability (Table I). Absence of Na^+ was demonstrated by the flame test to assure fully protonated dodecyl sulfate.

Hargreaves and Deamer (1978) and Heller (1982) had earlier pointed out that on visual inspection an opalescent solution is an indication of liposome formation (diameter of 1 to 10 μ) of the

TABLE I. Influence of NaCl Concentration on the Size of SDS/dodecanol Vesicles:

pH	[NaCL] (mM)	Vesicle Size (nm)	Polydispersity	Residual supernatant SDS (%)	NOTE
7.07	0.0	350	0.10	44.0%	
7.28	25.0	224	0.07	14.7%	ppt after 3 hours
7.14	50.0	235	0.08	13.7%	ppt after 3 hours
7.03	150.0	260	0.09	0.9%	

This table illustrates the effect of NaCl concentrations on the sizes of protonated dodecyl sulfate/dodecanol vesicles (50 mole%). The purity of the alkyl sulfates was confirmed by TLC. All vesicles were prepared by the modified "pH jump" method (Aurora, et al., 1985). PCS was used to measure the size of the vesicles The suspension was measured by PCS daily for a week; the size of vesicles remained constant (± 5nm) with a constant polydispersity (0.09±0.01). The percent SDS solubilized from vesicles of each vesicle preparation was determined as follows: Amount of sulfate in each vesicle preparation was determined by the Kean assay (1968). Each sample was then centrifuged at 1000g for 15 minutes to obtain a pellet. The amount of sulfate in the supernatant was determined by the Kean assay.

lipid dispersion whereas a milky solution indicates oil droplet formation (diameter >10 μ). Table I shows that 150 mM NaCl is appropriate for preparing uniform vesicles with minimum loss of sulfatide to the solution. The vesicles do not aggregate or precipitate for at least a week (Table I). The size distribution of these vesicles is shown in Figure 7.

The mole fraction of dodecanol was varied to determine its affect on the size and stability of SDS/dodecanol liposomes (Table IIA , Figure 8). The largest vesicles (1300 nm) were formed from pure protonated dodecyl sulfate. At 50 mol% the most uniform and smallest SDS/dodecanol vesicles (260 nm) were obtained. These are also the most stable (Figure 8B). For the octadecyl sulfate/octadecanol vesicles the most uniform and smallest vesicles contained a mole fraction of 0.33 (Table IIB, Figure 9).

Rutkowski et al., (1991) reported that PCS measurements of the sizes of the phospholipid vesicles prepared from the pH-adjustment procedure with low polydispersities (≤0.1) may be considered "uniform" whereas the vesicle preparations with polydispersities greater than 0.2 give a fairly high normalized variance (>.45). The relationship between polydispersity and normalized variance is expressed by the formula, $\sigma_n^2 = \sigma^2/d^2$, where σ_n is the normalized variance and d is the diameter. This formula may be used to calculate the standard deviation (σ) for each vesicle preparation. This was done for the samples and is displayed as error bars in Figures 8 and 9. The formula assumes

Table II. Vesicle Size Versus the Concentration of Alkanol in Protonated Alkyl Sulfate/Alkanol Vesicles.

A.

Mole Fraction of Protonated SDS/Dodecanol	Days After Vesicle Preparation	Mean Size (nm)	Polydis-persity	[NaCl] (mM)	pH
1.0	*	1300	0.16	134.0	7.00
0.85	*	1000	0.13	133.5	7.16
0.85	9	1100	0.13	133.5	7.16
0.67	*	340	0.10	134.4	7.00
0.67	9	680	0.18	134.4	7.00
0.50	*	260	0.09	128.9	7.03
0.50	9	540	0.13	128.9	7.03
0.33	*	650	0.20	134.4	7.14
0.33	9	580	0.18	134.4	7.14

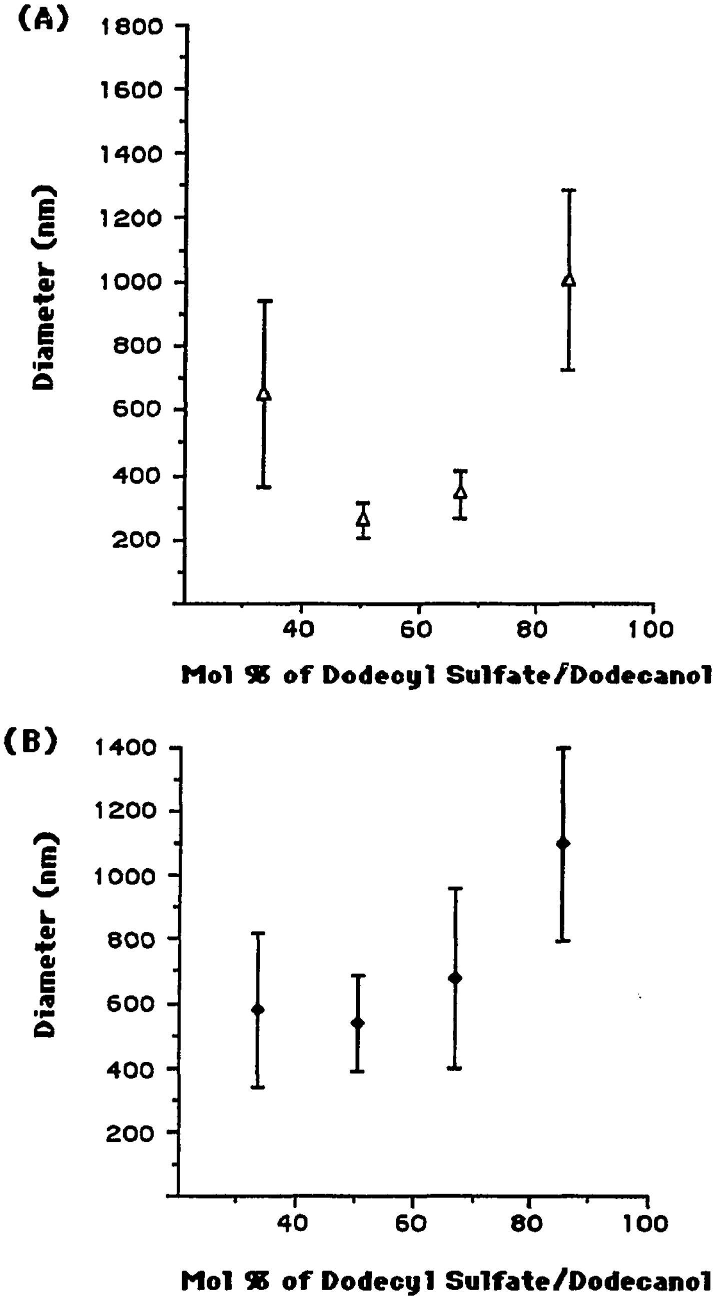
B.

Mole Fraction of Protonated Octadecyl Sulfate/Octa-decanol	Days After Vesicle Preparation	Mean Size (nm)	Polydis-persity	[NaCl] (mM)	pH
0.67	*	376	0.16	134.0	7.06
0.50	*	356	0.13	135.0	7.16
0.33	*	223	0.13	133.5	7.16

PCS measurement of the sizes of vesicles constituted of different mole fractions of protonated dodecyl sulfate/dodecanol (A) and protonated octadecyl sulfate/octadecanol (B). All vesicles were prepared by the modified "pH-jump" method. The purity of the protonated sulfates was checked by TLC and the Kean assay (1968). According to Rutkowski, et al. (1991), polydispersities between 0.1 and 0.2 may be significantly altered in their mean size by relatively few oversized vesicles.

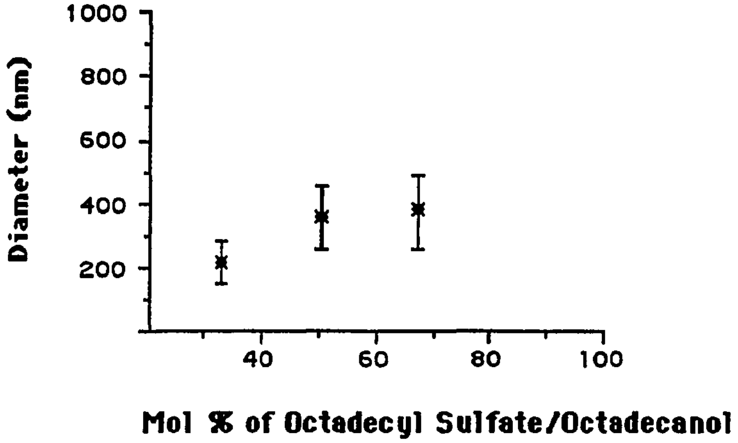
* = measured immediately after preparing the vesicles.

FIGURE 8. The Sizes of Dodecyl Sulfate:Dodecanol Liposomes



Liposomes sizes were measured immediately after preparation at pH 2.0 and adjustment to 7.0 (A) and 9 days later (B). Error bars represent the standard deviation (σ) in liposome size.

FIGURE 9. The Sizes of Octadecyl Sulfate:Octadecanol Liposomes



Liposomes sizes were measured immediately after preparation at pH 2.0 and adjustment to 7.0. Error bars represent the standard deviation (σ) in liposome size.

the diameters of the vesicles possesses a normal Gaussian distribution.

Heller (1982) indicated that liposomes suspended in solutions generally appeared turbid. Dodecyl sulfate:dodecanol liposomes prepared by the pH-adjustment methods were turbid 7-9 days later. These liposome preparations showed "particles" settling down in the bottom of the vials and the resulting supernatants appeared to be much less turbid. PCS measurements showed that the sizes of these liposomes were larger than those measured 9 days earlier (Figure 8, Table IIA). However examination with TEM showed that aggregation, and not swelling, occurred during the 9 days.

The sulfatides of *O. danica* are docosanol (C_{22}) disulfates. Therefore vesicles of octadecyl (C_{18}) sulfate/oleyl alcohol were prepared (Figure 9, Table IIB). The sizes of the C_{18} liposomes were not significantly different from the C_{12} liposomes (Figure 8, 9, and Table II) when the mole % ratios are similar. It was concluded that mol% plays a relatively larger role than alkanol chain length in determing the vesicle size and stability.

Reconstitution of Chlorosulfolipid Vesicles:

The above preliminary experiments probed the stability and sizes of vesicles constituted of alkyl sulfates and alkanols. These studies were done to determine the appropriate conditions to be used for reconstituting chlorosulfolipids of *O. danica* into bilayers.

Heller (1982) had shown that it is possible to prepare liposomes of sodium oleyl sulfate with cholesterol only when the mixtures were between 40 to 70 mol%.

The natural membrane of *O. danica* contains 70 mol% chlorosulfatides, 15 mol% sterols and 12.5 mol% polyunsaturated fatty acids. It was therefore deemed appropriate to use 3/1/1 as the molar ratios of the three components: chlorosulfolipids / cholesterol /oleic acid.

An electron micrograph of a preparation of CSL vesicles is shown in Figure 10. The vesicles, prepared in the range of pH 4.0 to 7.0, were examined with PCS and EM over a 3 day interval. They are described in Table III and Figure 11.

The unsonicated dispersions at pH 7.0 yielded average sized vesicles of 220 ± 62 nm as determined by PCS. In general these vesicles seemed to be stable (they neither aggregate nor precipitate) for several days. Vesicles prepared below pH 6.5 formed aggregates which precipitated during the first 24 hours. Additionally, the supernatant above these precipitates contained vesicles with sizes smaller than those prepared initially. The supernatants became less turbid on standing. After day 3, there was no visible turbidity in the supernatants. Adhesion is shown in the EM of the precipitate (Figure 12) not unlike that of the phospholipid vesicles at low pH obtained by Verkleij (1984). It can be seen from Figure 11 and Table III that chlorosulfatide

Figure 10. Electron Micrograph of Negatively Stained Protonated Sulfatide Vesicles Showing Bilayer Formation

The magnification of the micrograph was 280,000. The pH of the protonated sulfatide preparation was 5.7.

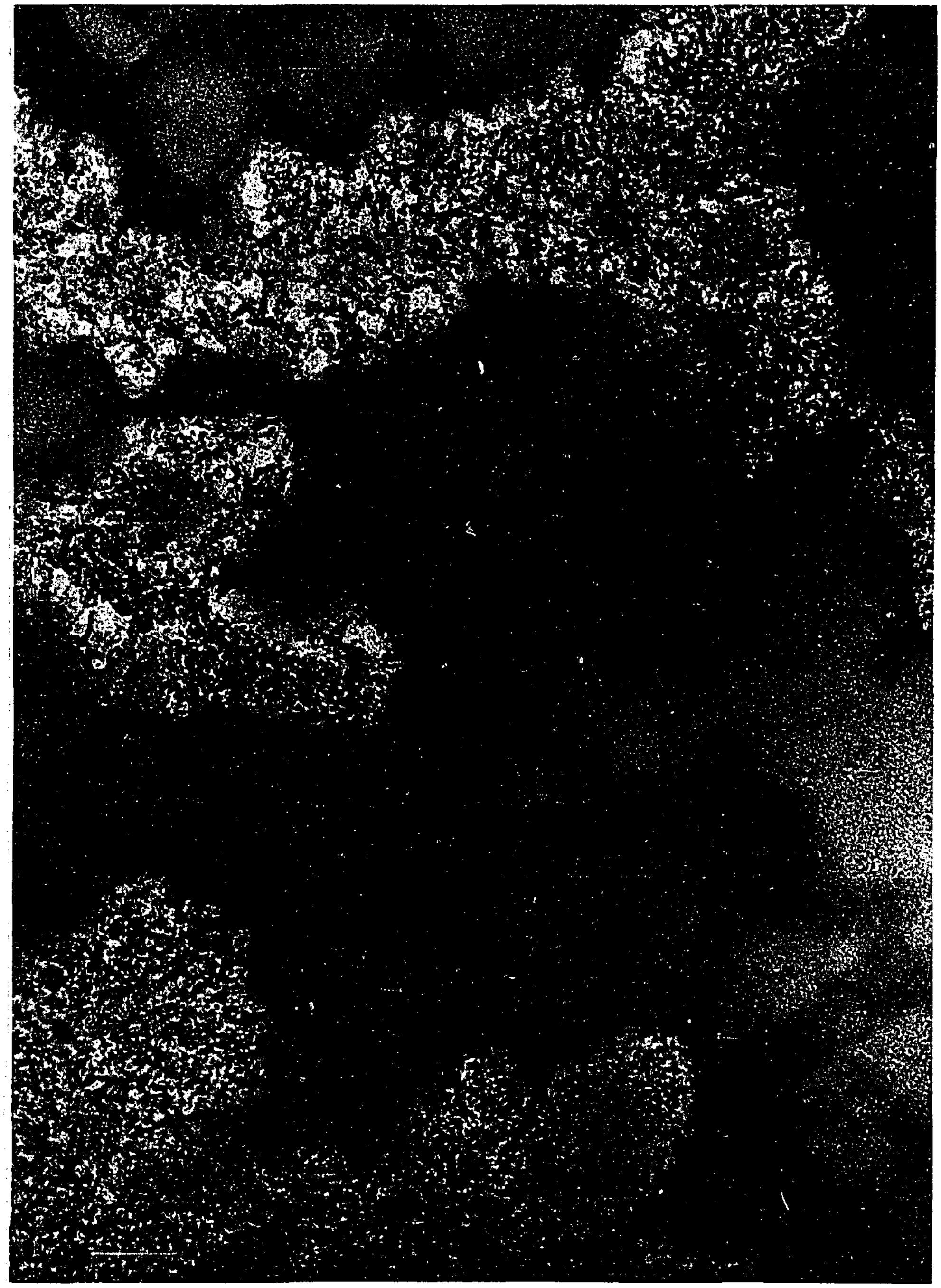
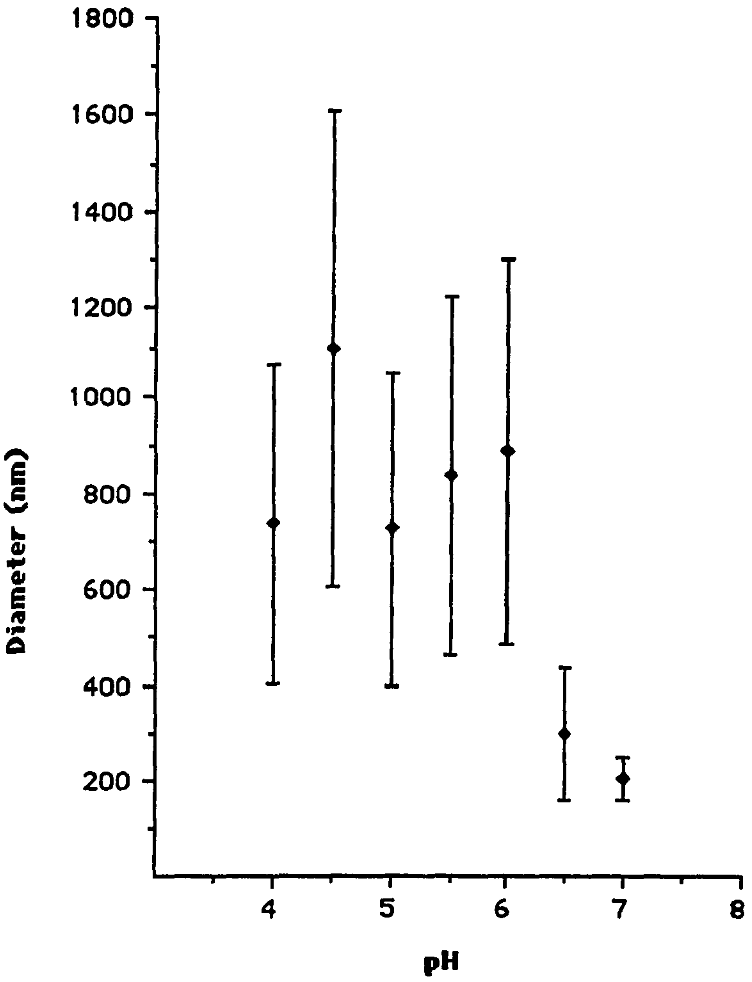


TABLE III. CSL: Cholesterol : Oleic Acid Vesicles.

pH	% CSL Solubilized Initially	Ratios of Chlorosulfolipids/ Cholesterol/ Oleic acid	DIAMETER (nm)		
			initial	24 hours	48 hours
4.0	18.9	3/1/1	2300	1700	740
4.5	9.2	3/1/1	2200	1600	1100
5.0	13.5	3/1/1	3100	1600	730
5.5	0.8	3.5/1/1	3100	1400	840
6.0	1.8	3.5/1/1	1800	1200	890
6.5	12.5	3/1/1	470	430	300
7.0	5.2	3/1/1	220	220	210

Chlorosulfolipid:cholesterol:oleic acid vesicles were prepared with an initially high pH which was then adjusted to final pH ranging from 4.0 to 7.0 in 150 mM NaCl. Diameters were determined by Photon Correlation Spectroscopy (PCS). The polydispersity of the 48-hour samples was less than 0.35. The samples were highly polydisperse. Each sample, ~3mL, contained 2.5-3.0 µmol of chlorosulfolipids. The vesicles were larger for samples with a final pH below pH 6.5. Since the vesicles tended to aggregate below pH 6.5, the precipitate that formed during the three day experiment was ignored. As can be seen the sizes of the residual vesicles in the supernatant decreased as the more dense aggregates settled in the low pH range. This left a comparatively more uniform preparation in the supernatant. Only the pH 6.5 and pH 7.0 samples could be considered "uniform".

FIGURE 11. pH Versus Size for CSL:Cholesterol:Oleic Acid Vesicle



Graph displaying the sizes of the liposomes of chlorosulfolipids/cholesterol/oleic acid in the proportion 3/1/1. The liposomes were prepared from protonated chlorosulfolipids by raising the pH quickly to 8 and then dropping the pH to the pH indicated. The preparation was maintained thereafter at the same pH. The liposomes are described in Table III.

Figure 12. EM of Aggregated Liposomes
The magnification of the liposomes was 70,000. The pH of the preparation was 7.0.

vesicles preparations between pH 6.5 and 7.0 are more uniform than those prepared below pH 6.5 presumably because of the observed adhesion at the lower pH.

DISCUSSION

The constituent lipids of natural biomembranes are diverse presumably because they exhibit a wide variety functions in the membranes that contain them. Reconstitution of lipid bilayers (Bangham and Horne, 1964; Bangham et al., 1965) was a major advance that opened the possibility of studying molecular interactions in model membranes. Today the reconstitution of active proteins combined with the isolation of actively transporting liposomes from living membranes provides a model experimental system for enhancing our understanding of biological membrane systems.

No reported lipids are as unexpected in structure as those of *O. danica*. Reconstituting bilayers from these lipids was essential in order to demonstrate that the lipids could form a viable membrane. This had been impossible because the lipids are water soluble! The use of pH as an approach to understanding the problem arose from the efforts of several workers (El-Maraghy, 1982; Heller, 1982 and Winikov, 1986). A procedure developed by Hauser and Gaines (1982) and Aurora, et al. (1985), a pH-jump method for preparing vesicles from acidic phospholipids, proved productive in this work. We report here a model for alkyl sulfate vesicle preparation using as a key, protonated alkyl sulfates. This method was then applied to the chlorinated aliphatic disulfates isolated from *O. danica* .

Protonation of Headgroups of Acidic Lipids:

As early as 1910 (Gouy) it was recognized theoretically that the extent to which polyanionic surfaces sequestered cations from the bulk phase (solution) was dependent upon the ionic strength of the bathing solution. Later theoretical work by Chapman (1913) and Stern (1924) laid out a framework for predicting (McLaughlin, et al., 1971) that the pH at a maximally charged polyanionic surface could be as much as 3 pH units below that of the bulk phase. The theory was elegantly demonstrated by Bedzyk et al. (1990) using X-ray standing waves to visualize the diffuse-double layer at a phospholipid surface bathed in dilute zinc chloride. The Debye length of the layer containing zinc varied from 0.3 to 5.8 nm in thickness. The Debye length for protons (McLaughlin et al., 1971) is 9.0 nm. At low ionic strength the aggregated cations are protons.

Significance of the protonated sulfatides for forming bilayers of chlorosulfolipids:

Previous attempts to reconstitute the chlorosulfolipid membrane have been unsuccessful. In 1978, David C. Deamer and T. H. Haines (unpublished experiments) attempted to reconstitute the membrane with a mixture of chlorosulfolipids: oleic acid: cholesterol = 3:1:1 at pH 7.0. This reconstitution appeared successful when examined with light microscopy and EM. An analysis of the isolated vesicles, however showed they were devoid of sulfolipids (which were soluble in the water at pH 7.0) and consisted instead of oleic acid and cholesterol. How the organism *O. danica* is able to put together these constituents and make a viable plasma and flagellar membrane remained mysterious since it could not be reconstituted in the laboratory.

The issue became clearer from the work of Stern (1978) who showed that no counterion existed in the membrane. The putative presence of the secondary sulfate seemed to require a resident cationic counterion. His results showed that the only viable counterion was the proton.

An important observation of Hargreaves and Deamer (1978) was that single chain anionic lipids (soaps or detergents) form liposomes about 3 pH units above the pK of the anion. They suggested that there must be "spacer" molecules in the plane of the bilayer to separate the charged head groups. This pH effect was explained by Haines (1983) as the formation of acid-anions between adjacent fatty acid headgroups. It was shown by Heller (1982) and Haines (1983) by ^{13}C-NMR that protonation of the headgroup fatty acids could range from 20% to 80% and still form stable fatty acid bilayers. In a sense, the protonated fatty acids are being utilized as "spacer" molecules separating the anions. Such protonated fatty acids are uncharged and occupied an area of the bilayer equivalent to a lattice unit occupied by a chain. The concept of "spacers" is not unrelated to the fact that all bilayer forming phospholipids have two chains per headgroup.

Heller (1982) indicated that sterols (such as cholesterol) also can act as "spacer" molecules in forming bilayers. In bilayer systems, cholesterol exhibits a well-characterized ability to inhibit formation of the crystalline (gel) state and to decrease the permeability of liquid crystalline lamellar systems. Cholesterol has an area of 37-38 $Å^2$/molecule. It acts as "spacer" between neighboring molecules and at the same time it presents a rigid, planar surface that decreases the local mobility of neighboring acyl chains. It also inhibits the permeability of ions and neutral molecules including H_2O (Finkelstein, 1976).

Analysis of the lipid composition of *O. danica* showed that approximately 70 molar per cent of the lipid in its membrane is the mixture of chloro-sulfatides and 25 molar percent is a mixture of sterols and free fatty acid (Chen and Haines, 1976; Chen et al., 1976). These substances constitute over 90 mol per cent of the membrane and was assumed to be on both sides of the bilayer. *O. danica* is cultured best at a pH of about 4.5. Thus the pH of the bulk medium is 4.5 and the surface pH of the membrane may be as low as 1.5. This suggests that most if not all of the free fatty acids are protonated and may participate as "spacers". Since the pK of an alkyl sulfate is about 1.5 (Jencks, 1976) a portion of the chlorosulfolipids (CSL) may be also in the protonated state.

Stern (1978) showed that the sulfatides of *O. danica* were not present in the membrane bilayers as divalent cation salts. He proposed that the secondary sulfate in the hydrophobic region of the membrane was held together by two hydrogen bonds. The sulfatides contain one to four chloro groups near the secondary sulfate (Haines et al., 1969 ; Elovson and Vagelos, 1969 & 1970). These chloro groups are sterically the size of methyl groups and would be expected to raise the T_m of the membrane. This is analogous to the *cis* double bonds of natural unsaturated fatty acids. The chloro groups also have a high electronegativity as well as unpaired electrons and thus can act as acceptors for the hydrogen-bonds for protonated secondary sulfates. Since such hydrogen bonds would be parallel to the bilayer they might provide greater stability at high pH (6-7).

All of these considerations suggest that the sulfatides, which would not form bilayers at pH 7.0 and indeed would not even enter the fatty acid/cholesterol bilayers, might form stable bilayers once they were protonated.

Isolation and purification of protonated sulfatides:

Isolation of sulfolipids from *O. danica* cells was performed by following the modified Folch procedure (1957) in which the methanol phase of the solvent system was collected instead of the commonly used chloroform phase. These CSL are generally discarded in lipid extracts because they are exclusively in the methanol phase and are therefore much more widespread in all fresh water algae (Mercer and Davies, 1979) than is commonly known.

The chlorosulfatides may be hydrolyzed to form diols in an acidic environment. During a typical extraction the pH of the extract was found to be acidic ($pH > 4$). The addition of NaOH was required in order to prevent hydrolysis of the sulfatides during isolation. The sulfatides isolated from *O. danica* were thus in the sodium salt form.

The sodium salt form of the CSL was protonated according to the acid Bligh and Dyer (1959) procedure prior to liposome preparation to minimize hydrolysis. The pH-jump method provided quick liposome formation. Throughout the preparation of the liposomes, the degree of hydrolysis was minimized and was quantitatively monitored by TLC and the Kean (1968) assay.

To examine whether CSL was incorporated with cholesterol and oleic acid as lipid bilayers, the following experiment was done. The liposome suspension (in 135 mM NaCl) was centrifuged (Sorvall Instrument, Model # RC70, Wilmington, Delaware) at 4^oC and 45,000 rpm for one hour to obtain a liposome pellet. Both the centrifugate and the pellet (resuspended in the fresh salt solution) were examined for CSL by TLC and the Kean assay. The results of these examinations showed that >90% of CSL was indeed in the pellet presumably as bilayers with cholesterol and oleic acid.

Model Sulfatide vesicles:

There are several advantages to using SDS as a model for making sulfate ester bilayers. First, sodium dodecyl sulfate (SDS) contains the ionic sulfate group as does the chlorosulfatides of *O. danica*. Heller (1982) had characterized vesicles made from SDS and cholesterol or dodecanol. Since SDS has only a primary sulfate it does not hydrolyze as easily as does the chlorosulfatides which also contain a more labile secondary sulfate. Last, but not least, SDS is readily available!

Preliminary experimental methods and the laboratory manipulation of liposomes made with sulfate as the head group had been explored by Heller (1982). These included cholesterol sulfate, oleyl sulfate, and SDS. He showed that the addition of dodecanol to dilute SDS solutions resulted in the spontaneous formation of liposomes. The average diameter of these liposomes increased with the mole percent of dodecanol. Cholesterol (up to 70mol%) is a good "spacer" between sulfate headgroups for the preparation of vesicles of longer chain alkyl sulfates (such as oleyl sulfate). A combination of oleyl sulfate and cholesterol (after sonication) resulted in liposomes with an average diameter (800Å). They appeared to be stable for up to four weeks.

It was noted that the SDS/dodecanol liposomes prepared by sonication (Heller, 1982) gave similar results to those prepared by the pH-jump method. The latter method appears to produce liposomes quickly. We therefore prepared CSL liposomes via the pH-jump method.

Hargreaves and Deamer (1978) utilized a Zeiss photomicroscope with Nomarski differential interference equipment for transmitted light microscopy to distinguish the different types of lamella. We used light microscopy to establish and distinguish between the formation of both unilamellar vesicles and mutilamellar vesicles in water. Multilamellar vesicles are highly refractive whereas unilamellar vesicles display ghost-like borders.

Chlorosulfatide Liposomes:

Vesicles of SDS with dodecanol, and protonated dodecyl sulfate with dodecanol are successfully made with minimum loss of dodecyl sulfate (0-2% loss, Table IIA). Attempts to make the potassium and sodium salts of chlorosulfolipids with oleic acid and/or cholesterol vesicles were successful judging from the appearance of the preparations. The protonated chlorosulfolipids together with oleic acid and/or cholesterol forms liposomes. Greater than 90% of the sulfolipids incorporated into the bilayers when the preparations are promptly centrifuged. The latter appears to be necessary to prevent redissolution or degradation of the CSL.

The measurement of the liposome sizes was primarily performed via PCS. The count rate drifted about 10% in the course of an experiment. Occasionally, samples had widely fluctuating count rates. Large, erratic count rates usually imply occasional large particles that are probably aggregates. Electron microscopy and light microscopy showed fairly large aggregates occurred. The size of the aggregates may be larger than 1000nm in diameter (Figure 12). This explains the apparent decrease in the size of the vesicles as the pH changes form 4.0 to 6.5 as measured by PCS.

pH and ionic strength studies of the stability of the chlorosulfolipid liposomes:

Figure 11 shows that the liposomes prepared between pH 4.0 and 6.0 display a large polydispersity, but are approximately in the same average size range. Above pH 6.0, however, the liposomes become significantly uniform and are small.

At pH 7.0 the size of the liposomes are smaller (217 nm in diameter). The electron microscopy was consistent with PCS experiments which are shown in Table III. It should be noted that the PCS experiments were not optimal since the calibration of the instrument was done with smaller particles (polystyrene, diameter, 126 nm). Under these conditions the error increases as the size of the particles increases. As the particle size approaches 488nm (the wavelength of the laser) the measurement loses validity. These factors were of marginal importance because the accuracy of the diameter was not critical to our analysis.

As shown in Figure 11, the vesicles aggregate at a final pH of 4.0 to 6.5. Due to the Gouy-Chapman relationship, the surface pH of the vesicles is near the pK of the sulfate groups (1.5). Consequently, the sulfatides are extensively protonated. The adhesion is shown in the EM pictures and is similar to corresponding pictures of phospholipids at low pH obtained by Verkleij (1984, Verkleij et al., 1980; Verkleij et al., 1985). The light scattering determines average liposome sizes. It is less useful due to the relatively high polydispersity and the aggregation.

B. INTRAMOLECULAR HYDROGEN BONDING OF CARDIOLIPIN

INTRODUCTION

Hydrogen Bonding of Water with Phospholipids:

The hydrogen-bonding of water at the phospholipid interfaces of bilayers has several components. These include hydration, the hydrogen bonding of hydronium ions sequestered at the headgroup domains of acidic phospholipids and possibly the entrapment of anhydrous hydrogen ions between acidic headgroups; i. e., the formation of acid-anions.

With regard to hydration, there is direct evidence (Blume et al., 1988) using infrared spectroscopy for the hydrogen bonding of water to the *sn-2* carbonyl groups of neutral phospholipids but not the *sn-1*. In contrast, these investigators showed that both the *sn-1* and *sn-2* carbonyl groups of acidic phosphatides are hydrated. Furthermore, they found that the percent hydration at the *sn-1* and *sn-2* carbonyl groups of DMPC is different from that of DMPG. This suggested that the conformation of DMPC should be different from that of DMPG. Such a difference was observed on x-ray studies of the crystals (Pascher, et al., 1987). The significance of hydration forces between apposed phospholipid bilayers has been explored by Rand et al. (1988). Hydration is important on many levels.

For anionic polar head groups of phospholipids interactions with cations obey the Gouy-Chapman-Stern theory. In low ionic strength media, the hydronium ions will aggregate in proportionally high concentration in the Stern layer as compared to the bulk solution. McLaughlin et al. (1971) indicated that the maximum theoretical pH difference between bulk phase and the surface (with solely anionic lipid head groups), based on a single charge per chain, is 3 pH units. That is, the apparent pK of an anion at a negatively charged surface is not the same as that anion in the bulk phase. Similar pH differences were observed by Heller (1982) and Haines (1983) for sodium dodecyl sulfate micelles (ΔpH = 2.30) and for sodium oleyl sulfate (ΔpH = a range from 2.35 to 1.65). These observations were obtained by using the fluorescent probes, alkyl 2-aminocoumarin and alkyl 2-hydroxycoumarin as described by Fernandez and Fromherz (1977).

Many natural membranes are mixtures of zwitterionic and anionic, and/or uncharged lipids. To the extent that the lipids are anionic these membranes exhibit a low surface pH. Membranes constituted of solely anionic lipids are called "acid membranes". The aggregation of anionic phospholipid headgroups on a surface (e. g. PG) requires that the groups be proximal. The situation is analogous to the acid-anion of maleic acid (Figure 2). If one carboxylate is protonated and forms a hydrogen-bond with its neighbor then the resonance of the resulting acid-anion traps a dehydrated proton between the lipid headgroups. This was proposed by Heller (1982) and Haines (1983) as an explanation for the titration curve they obtained for fatty acids in water which formed bilayers only between pH 9.45 and 7.15. It should be noted that the oleic acid remained in bilayer form throughout the range of 80% to 20% dissociation of the carboxyl.

Acid-anions of phospholipids were proposed by Haines (1983) and Heller (1982) consistent with their data on the fatty acid bilayers. These proposals had the following features:

1. The putative phospholipid acid-anions are stabilized by resonance throughout a wide range of pHs. They consist of two phosphates from adjacent lipids bound by a hydrogen ion (sharing one charge). Protonation of this species will result in two protonated monomers which may protonate other neighboring acid-anions. This allows proton switching from one head group to another (rapid migration of protons).
2. This activity requires that the headgroups reside in a thin sheet which is in consistent with the studies of Lazzati et al. (1968).

The above discussion implies that there are two species of hydrogen ions at the surface of the liposomes: (a) H_3O^+ (hydrated proton) in the bulk phase near the headgroups of the lipids (McLaughlin et al., 1971) and (b) dehydrated H^+ (protonated carboxylates) in the headgroups with putative acid-anion formation.

Evidence for Lateral Proton Conductance at Anionic Lipid Bilayer: Both direct (fluorescent probe and surface potentials) and indirect (changes in surface pressure) measurements on lipid systems from several laboratories suggested proton movements along the anionic monolayers (Prats et al., 1986, 1987; Teissie et al., 1985, 1990; Gabriel et al., 1991). They concluded that lateral H^+ transfer along the interface is possibly due to the existence of a H-bond network involving the polar head groups and the interfacial water molecules. They also indicated that protons can diffuse along the surface of a lipid monolayer faster than that in the bulk phase.

Amongst these studies, Teissie provided evidence for lateral proton conduction in the monolayers of certain anionic phospholipids (Teissie el al., 1985, 1990). These include the analogues of the major anionic phospholipids of the extreme halophiles, diphytanylglycerol phosphatidyl glycerol phosphate and its dihexadecylglycerol analogue synthesized by Kates (Stewart et al, 1988, 1990; Stewart and Kates, 1989). These phospholipids have structures similar to that of cardiolipin except that they lack the diacyl glycerol attached to the terminal phosphate.

Sakurai and Kawamura (1987, 1989) studied electrical conductance along PC monolayers in water and D_2O. They concluded that the lateral electrical conductivity of a surface monolayer of PC depends upon the order and arrangement of the polar head network within the monolayer, as well as on the mobility of protons.

In addition to monolayers studies on models of membranes some evidence for local proton current in the chloroplast may involve lipids. Measurements with and without uncouplers such as nigericine (Allnutt et al., 1989) showed that chloroplast thylakoid membranes when de-energized and in the dark retain H^+ within metastable sequestered domains. These researchers suggested that the H^+ is at the membrane interface domain, possibly in the acidic lipids. Chloroplasts have no zwitterionic lipids only anionic.

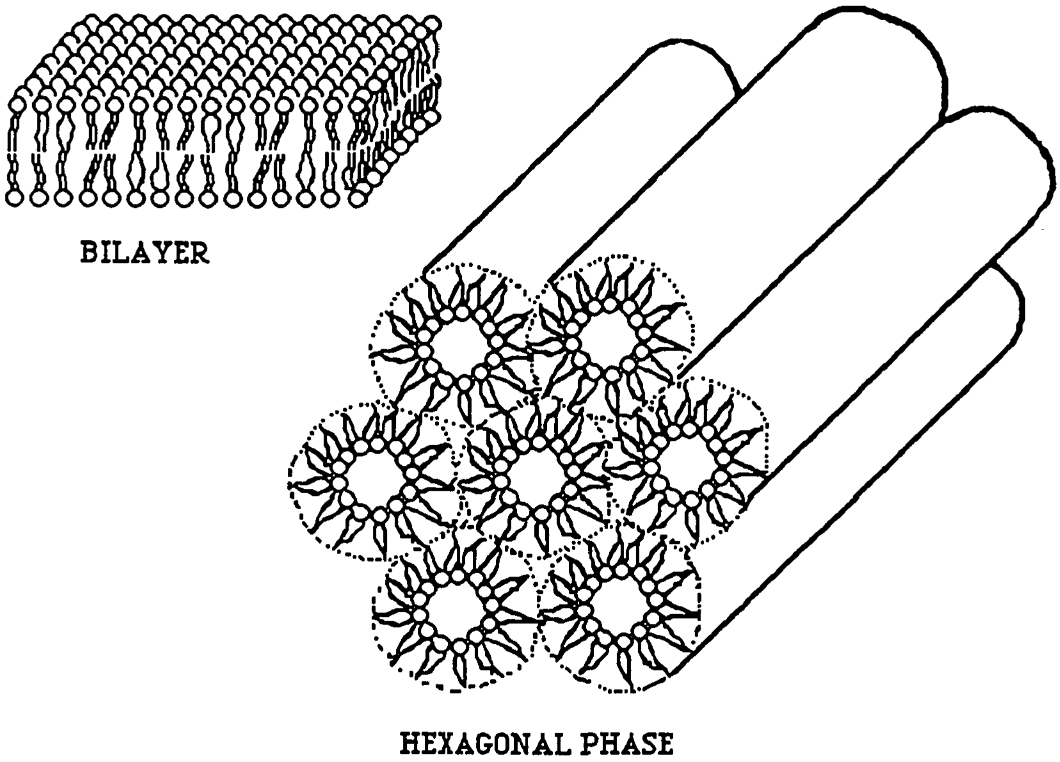
The relationship between transbilayer proton movement and ATP synthesis in biological membranes either between the bulk aqueous compartments or within the bilayer has been debated for some thirty years. Mitchell (1961) proposed the redox-driven proton pumps of the mitochondrion are coupled via proton translocation to ATP synthesis by proton flow and backflow between two separate aqueous compartments through the membrane. At the same time, Williams (1961) proposed that a pH gradient across the membrane may not be the primary energy source in proton-pumping, energy-transducing membranes. He suggested that the protons remain within the membrane during energy transduction (ATP synthesis). He acknowledged that such protons would be in rapid equilibrium with the bulk phase on both sides of the bilayer.

Cardiolipin:

CL is found in all aerobic eubacteria (it is not present in archaebacteria) and in the inner mitochondrial membrane of all aerobic eukaryotic cells (Holland and Stevenson, 1990; Hostetler et al., 1971; Hovius et al., 1990; Ioannou and Golding, 1979; Krebs et al., 1979; Sperka-Gottlieb et al., 1988; Hoch, 1992) where it is the only major negatively charged phospholipid (about 20 mol% of the total lipid, thus 33% by weigh). CL isolated from mammalian sources are highly unsaturated, specifically enriched in linoleic acid (Gray and MacFartlane, 1958; Holland and Stevenson, 1990; Schlame et al., 1988; Schlame and Rustow, 1990). CL is unique in its structure among membrane lipids: It contains four fatty acids with two phosphates. CL has three glycerol moieties; the two glycerol backbones and one bridging the two phosphate groups. Until this work, it has been presumed that CL has 2 negative charges associated with the head group at neutral pH.

At neutral pH and in the absence of divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+}, saturated and unsaturated CL adopts a lamellar phase. Calcium (above 1mM) can reduce inter-head group electrostatic repulsion and has the ability to induce H_{II} (hexagonal II) phase (Cullis et al., 1978; deKruijff, 1982; Verkleij, 1984). H_{II} phase (Figure 13) first defined from x-ray scattering (Luzatti, et al., 1968) is easily characterized by ^{31}P-NMR (Battenburg et al., 1987). This phase is described as a hydrocarbon matrix penetrated by aqueous channels (~20Å

Figure 13. Lipid Bilayer vs. Hexagonal II phase
Schmatic drawing of the lipid bilayer and hexagonal II phase.



diameter) surrounded by a bilayer of lipid with polar head groups oriented to the external aqueous medium. Dehydration of the phospholipid headgroup is a common feature of the formation of H_{II} phase. Such dehydration can be experimentally affected by protonation of the CL at low pH or by divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+} (Cullis et al., 1978; Verkleij, et al., 1985). In the presence of Mn^{2+} and Ca^{2+}, phosphatidylcholine/ cardiolipin (PC/CL) liposomes phase separate permitting the CL to form H_{II} phase (Verkleij et al., 1980). Lipidic particles (80 to 130 Å) are intrabilayer inverted micellar structures which may be used to identify the presence of H_{II} phase (Verkleij et al., 1980, 1985).

Several laboratories have isolated and/or studied *cls*-mutants of *E. Coli* (Pluschke et al., 1978; Nishijima, et al., 1988; Hwang, 1984). In general the only observable phenotype is that the bacterium compensates the absence of CL by producing sufficient anionic lipids (PS and PG) to maintain a fixed ratio of anionic to zwitterionic lipids. Additionally Hwang (1984) in the laboratory of B. Tropp has established that *E. coli* growth is suppressed for the *cls*-mutant (as compared to the wild type) above pH = 8.0.

The pK's of Cardiolipin:

The question before us is whether CL has 2 charges per headgroup or does trap a proton to form an acid-anion. The model for such an acid-anion is shown in Figure 3. The central hydroxyl on the glycerol facilitates acid-anion formation but does not participate in the resonance. The acid-anion is present only if one phosphate is protonated. This protonation makes the molecule symmetrical on the one hand and traps a proton on the other. The pK of the trapped proton is determined by the forces that hold the groups proximal. The pK of such a novel acid-anion is not predictable.

A search of the literature showed that there had never been a measurement of the second pK of cardiolipin because all investigators have assumed that the two phosphates had the same pK (Few et al.,1960; Sedden et al., 1983). Measurement of the pk's of CL required that the CL be in the bilayer or lamellar phase. Since CL constituted polyunsaturated fatty acids forms H_{II} phase at extreme pH's, it was necessary to saturate the chains of the beef heart cardiolipin.

In order to ascertain that the second pK measurement is a consequence of the proposed acid-anion model (Figure 3), a titration was conducted on an analogue of cardiolipin lacking the hydroxyl on the glycerol connecting the two phosphates. The synthesis was conducted by Dr. Morris Kates. The titration curves of both CL and deoxycardiolipin (dCL) were compared.

A summary of the literature review, the affinity of cardiolipin for mitochondrial proteins, is presented at the end of the Discussion. This documentation suggested that the pH-dissociation characteristics of cardiolipin may be of interest in connection with its structure-function relationships. I therefore examined the pH potentiometric titration of cardiolipin and found that, instead of titrating as a simple dibasic acid, it displayed two widely separated pK's, one at 2.8 and a second, anomalous pK above physiological pH. It is conceivable that the unusually high second pK is due to a unique particulate form of the lipid. To test the proposed acid-anion explanation the deoxy analog of cardiolipin was provided by Dr. Morris Kates and used in our laboratory. In this molecule, the intramolecular hydrogen-bonding of CL which is due to the central free hydoxyl group is not possible since it is replaced by a hydrogen atom (Figure 4). Deoxy-cardiolipin titrates as a typical dibasic acid. A comparison of the two titrations suggests that the free hydroxyl plays a role in stabilizing the monoprotonated form of the cardiolipin molecule.

Physical Measurement — ^{31}P NMR

^{31}P-NMR has been useful in determining the head group structure of phospholipids in membranes. Electrons exert a "screening" effect on a nucleus placed in a magnetic field, which causes the "shift" in the signal compared to a standard. Chemical shifts can be affected by hydrogen-bonding and ionization. The electron distribution surrounding a phospholipid phosphorus nucleus is anisotropic and thus in a magnetic field the phosphorus experiences an orientation-dependent "shielding" termed the "chemical shift anisotropy" (CSA). For lipids in a vesicle with a large radius (≥ 200nm) which implies a liquid crystal the CSA is partially averaged on the NMR time scale by the rotational axial motion of the molecule. In the presence of proton-decoupling, this results in a characteristic asymmetric ^{31}P NMR lineshape with a low-field shoulder and high-field peak, exhibiting an effective CSA (measured from the low field shoulder to the high field peak) of approximately -40 to -50ppm, depending upon the lipid species, temperature, or other factors (Battenburg et al., 1987)

For a lipid in the hexagonal II phase (H_{II}) additional motional averaging is experienced due to diffusion of the lipids around the cylinders (Figure 13). This results in a two-fold reduction in the effective CSA and a reversed asymmetry. Thus, the relative intensities of the low- and high-field shoulders are reversed compared to the lamella system (Battenburg et al., 1987). If the rate of diffusion of the lipid molecules around the cylinder is slow relative to the spectroscopic timescale ($\sim 10^{-5}$ sec), then no additional averaging effects will be observed. In a system where molecular reorientation occurs over all possible orientations (isotropic motion) in times on the order of the NMR timescale or less, motional averaging results in a single narrow ^{31}P NMR resonance. This occurs for lipids in solvent, small vesicles, micelles, and inverted micelles. Furthermore, ionization of the phosphate head group may lead to a conformation change of the head group and may thus introduce a phosphorus chemical shift.

^{31}P-NMR and pK measurements of CL, as well as its deoxy analog (dCL), vesicles with up to two equivalents of base were performed. The pK's of various analogous phosphate esters were measured using the same two procedures.

EXPERIMENTAL PROCEDURE

Materials:

All chemicals were Reagent Grade (Fisher Scientific, Fairlawn, NJ). All solvents were redistilled before use. Cardiolipin (bovine heart) as the disodium salt was purchased from Avanti Polar Lipids Inc. (Birmingham, AL). Beef heart cardiolipin was hydrogenated by Dr. Morris Kates (Ottawa, Canada) and provided as the diammonium salt. The purity of the cardiolipin preparations were checked by thin layer chromatography in $CHCl_3/CH_3OH/ H_2O$, 65:25:3.5, (v/v/v). The deoxy analog of CL (dCL) as the diammonium salt was provided by Dr. Morris Kates. L-α-glycerophosphate di(monocyclohexlyammonium) salt (95%), DL-glyceraldehyde-3-phosphate diethylacetal (monobarium salt), diphenyl phosphate, α– & β-glycerophosphate disodium salt, and phenyl phosphate disodium salt were all purchased from Sigma Chemical Co. (St Louis, MO). Dibarium-1,3-diphosphopropanediol was also provided by Dr. Kates. Dowex 50W was purchased from Fisher Scientific.

Protonation of phosphates:

Approximately 3 grams of Dowex-50W (a cation exchanger) was placed in a screw capped test tube with enough deionized-distilled water to cover the beads. The beads were protonated by the addition of 0.2M HCl dropwise until the pH was less than 3 (pH meter).

Aliquots of deionized-distilled water were used to wash the acidified resin. The wash was repeated at least 3 times until the pH of the eluent was 7.0.

In order to remove the cations from the water-soluble phosphates (di-monocyclohexylammonium-α-glycerophosphate, barium DL-glyceraldehyde-3-phosphate diethylacetal, diphenyl phosphate, disodium α– & β-glycerophosphate, disodium phenyl phosphate, dibarium-1,3-diphosphopropanediol), 25mg of each was dissolved in 2mL of deionized-distilled water and mixed with a spatula of protonated Dowex-50W in a screw cap test tube. The test tube was centrifuged in a table top centrifuge at 300 g for 10 minutes. The supernatant containing protonated phosphates was placed in a screw cap vial (5 mL capacity) and titrated with various concentrations of KOH.

To protonate a water insoluble phosphate such as glyceraldehyde-3-phosphate (monobarium salt), the phosphate (~25 mg) was placed in 2 mL deionized-distilled water with spatula-full of Dowex-50W in a screw cap test tube and heated in a water bath at about 55°C with constant stirring until completely dissolved. The test tube was chilled in ice-water bath for one minute and centrifuged in a desk top centrifugator at 300 g for 10 minutes. The supernatant containing protonated phosphates was titrated with 0.034 N KOH. Any significant hydrolysis during this procedure would have been observed in the subsequent procedure, titration or NMR. None was observed.

Preparation of protonated CL and deoxy-CL:
Protonation of cardiolipin and deoxy analog of cardiolipin was conducted according to the acidified Bligh and Dyer procedure (Bligh and Dyer, 1959). The lipid (~25 μg) was dissolved in 2mL $CHCl_3$. Methanol (3mL) and 0.2N HCl (2.7mL) were added to the lipid solution. The mixture was vortexed and centrifuged for 10 minutes in a bench top centrifuge (at 300 g). Because this biphase system contains several solvents, washing and back extracting each phase requires the fresh preparation of a Bligh and Dyer mixture of the solvents. The upper (methanol-water) phase was washed two times with 2 mL fresh lower phase of Bligh and Dyer (1959). All the lower layers including the washes were collected in the same screw cap test tube. A small amount (1 mL) of benzene was added to the suspension in order to dehydrate it.

To prepare protonated CL or dCL liposomes two different procedures were used. (A) The lipid suspension was rotary evaporated to dryness to obtain a lipid film. The dried lipid film was then dispersed in 2 mL of either (1) deionized-distilled water, (2) methanol and 150 mM KCl solution, or (3) 150 mM KCl solution with constant stirring for 30 minutes at room temperature. (B) Alternatively, the suspension was sonicated in a cup horn sonicator (Ultrasonics, Inc., W-385, Plain-view, NY) using a Lauda K-2/R water circulator (Brinkmann Instruments) to maintain temperature control at 50°C.

Potentiometric titration:

The pH measurement for all the titrations was done with an Orion Digital pH Meter (Model 601A, Cambridge, MA) equipped with a microelectrode (Microelectrodes, Inc., model # MI-410, Londondery, NH). The pH microelectrode was found to have maximum accuracy in the range of pH 2 to 10, therefore all titrations were terminated around pH 10. Potassium diphthalate (0.411 N) (Baker Chemical Co., Phillipsburg, NJ) was prepared and used to standardize an 0.851 N KOH solution (Fisher; Fairlawn, NJ). Various concentrations of KOH were prepared by volumetric dilutions of standardized KOH with deionized-distilled water.

Phosphoric acid, sodium biphosphate, sodium dihydrogen phosphate, and various protonated phosphates were titrated with KOH as follow: With a Hamilton syringe (2 mL capacity), various concentrations of KOH (depending on the concentrations of phosphates) were delivered to the phosphate dispersions graduately with a Razel syringe pump (Razel Scientific Instruments, Stamford, CN.). The titrations were followed either by a recorder (Beckman Instruments, Kipp and Zonen Model # BD40, Palo Alto, CA.) or by periodic notation of the pH which was fed into a computer program.

Liposomes of CL or deoxy CL were prepared in protonated form from the modified Bligh and Dyer procedure (1959) described above. Titration of protonated CL and deoxy CL vesicles was performed according to the procedure described as for protonated phosphates. Repeated titrations were conducted by re-isolating the lipids using the acidified Bligh and Dyer (1959) procedure after each titration.

Titration curves were computer-simulated using the general equation for either monoprotic or diprotic systems (Harris, 1987).

Physical measurements:

CL (hydrogenated beef heart, 6.7 mg), dCL (12.3 mg), and 1,3-diphosphopropane (28.4 mg) in the salt form were each protonated using the acidified Bligh and Dyer (1959) procedure as described above. The protonated CL (or dCL) suspended in water (approximately 0.5 mL) was then sonicated in a cup horn style sonicator at 50°C for 3 hours. The final volume of the sample was adjusted to 1 mL with D_2O. ^{31}P-NMR (proton decoupled) spectra of the sonicated CL and dCL samples and a solution of 1,3-diphosphopropane were measured on a Bruker WP-200 spectrometer (operating at 81 MHz), kindly performed by Dr. Theodore Axenrod. All samples were measured in D_2O/H_2O (1/1, v/v) in 5 mm NMR tubes. Between 400 to 2,000 scans were collected. Phosphoric acid (85%) was used as an internal standard. The signals were locked to the D_2O solvent. The calculated 2 equivalents of KOH were added to the protonated CL and dCL. Three equivalents were added to the protonated 1,3-diphosphopropane.

RESULTS

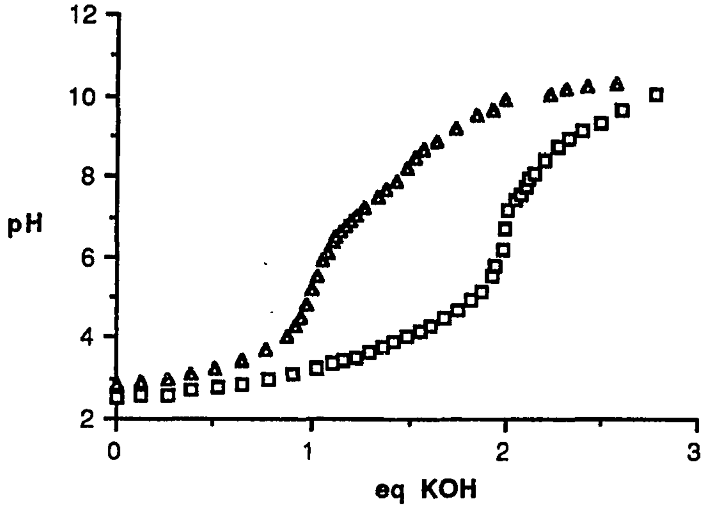
Potentiometric Titration:

In order to find a suitable solvent for titrating unhydrogenated beef heart CL, it was titrated in: (1) 100% methanol, (2) 0.05 M KCl in methanol/water (1/1, v/v), and (3) 0.150 M KCl in methanol. The second solvent was found to be the best solvent.

The hydrogenated beef heart cardiolipin (18:0-CL) formed bilayer phases throughout the pH range in either sonicated aqueous dispersion or in methanol-water (1:1) (Hubner et al., 1991). On titration (Figure 14) 18:0-CL showed the presence of one strong acid group, accounting for 1 equivalent of acid per mole, and a weak acid group with a surprisingly high pK accounting for a second equivalent of acid. The titration curve of the unhydrogenated beef heart cardiolipin in 0.150 M KCl was different than that of hydrogenated CL (Figure 15). This may be due to the formation of H_{II} phase when dispersed in water at low pH (Hubner et al., 1991). In contrast, 16:0-dCL titrated as a normal dibasic acid in sonicated aqueous dispersion (Figure 14) at any pH. It titrates 2 equivalents of base with an apparent pK of about 3.

For comparison, the pK values of several organic phosphates, PGP, and dPGP (1,3-diphosphopropanediol) were obtained by titration (Table IV).

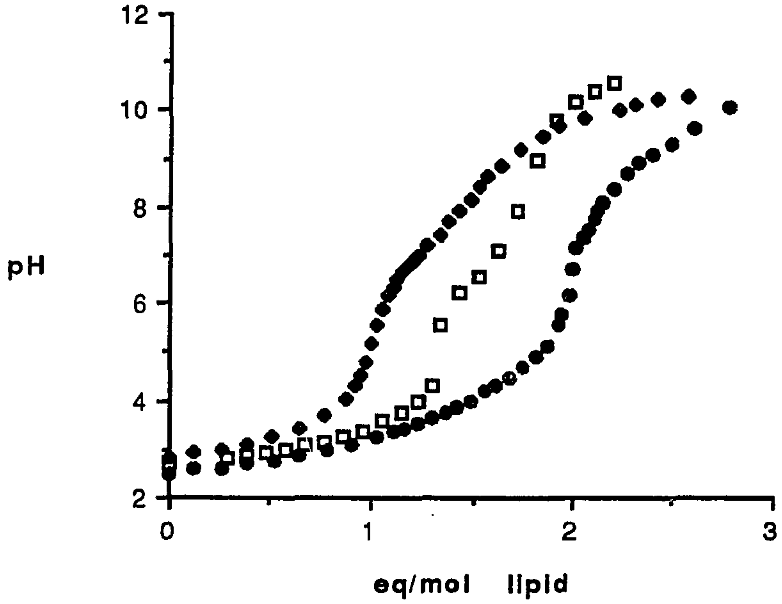
Figure 14. Titration Curves of Cardiolipin and Deoxycardiolipin Vesicles



Hydrogenated beef heart cardiolipin (4.63 μmol, 6.7 mg) was protonated using the acidified Bligh and Dyer (1959) procedure and dispersed in 50 mM KCl (Δ). The dispersion was then titrated against 52 mM KOH.

Synthetic $C_{16:0}$ deoxycardiolipin (11.4 μmol, 15.2 mg) was protonated according to acidified Bligh and Dyer (1959) procedure and dispersed in 50mM KCl (□). The dispersion was then titrated against 50mM KOH.

Figure 15. Titration Curves of Hydrogenated Cardiolipin, Natural Cardiolipin, and Deoxycardiolipin



Titration of both protonated dCL (•) and hydrogenated CL (♦) were described in Figure 13. Bovine heart (unhydrogenated) CL (□) (25 mg, 16.86 μmol) was protonated using the acidified Bligh and Dyer (1959) procedure and dispersed in 150 mM KCl. This dispersion was then titrated against 0.162 N KOH.

Table IV. pK Values of Phosphates, Cardiolipin and Deoxycardiolipin

Phosphate esters	pK_1	pK_2	pK_3
H_3PO_4	2.48 ± 0.05 2.12*	7.05 ± 0.14 7.21*	11.76 ± 0.17 12.32*
α-glycerolphosphate	2.19 ± 0.13 1.40*	6.50 ± 0.17 6.44*	
β-glycerolphosphate	2.28 ± 0.14 1.37*	6.35 ± 0.04 6.34*	
Glyceraldehyde-3-phosphate	2.50 ± 0.18 2.10*	6.73 ± 0.20 6.75*	
phenyl phosphate	2.02 ± 0.10	6.51 ± 0.04	
diphenylphosphate	1.76 ± 0.16		
1, 3-diphosphoglycerol	2.25/2.35**	6.3/7.1**	
1,3-diphosphopropanediol	2.25/2.35**	6.3/7.1**	
cardiolipin (beef heart)	2.8***	7.5***	
deoxycardiolipin (C16:0)	2.8***	3.8***	

* Values obtained from CRC Handbook of Biochemistry, second ed., 1970.

** Values provided by Dr. Morris Kates.

*** Values obtained from computer analysis.

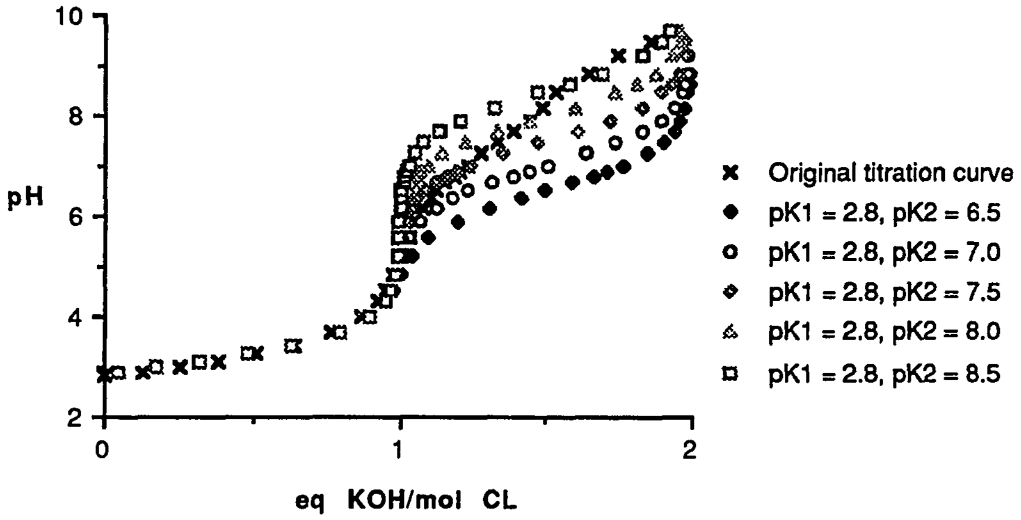
For hydrogenated CL, it was apparent that a single value for pK_2 could not be obtained. Therefore, an attempt was made to obtain a more precise pK_2 of 18:0-CL by computer-simulation (Harris, 1987). The computer-simulated curves were graphed according to the Henderson-Hasselbalch equation. Assuming the titration was for a diprotic system.

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

The curves fit best when the pK_1 was assumed to be 2.8. As is shown in Figure 16 the experimental data did not fit a distinct pK_2. It crossed through a family of theoretical curves corresponding to values varying from 7.0 to 9.5. It can be seen that the experimental curve differs from the theoretical curves by a "flattening" of the experimental curve. The second pK of cardiolipin appears to follow the formula: $pK_2 = 7.5 +$ (fractional equivalent/2), approximating a linear increase in its pK value between 7.5 and about 9.0.

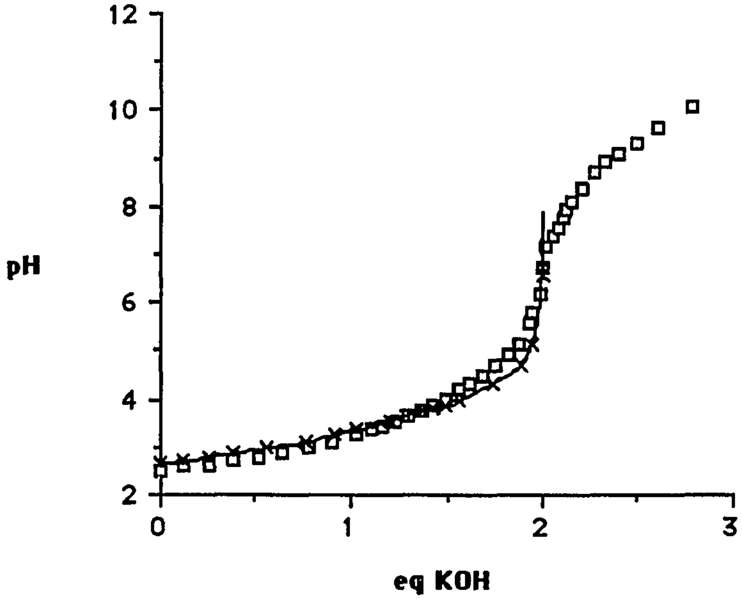
In contrast the titration of 16:0-dCL can be computer-simulated (Figure 17) by a theoretical curve for a typical dibasic acid having two strongly acidic groups with a pK_1 of 2.8 and a pK_2 of 3.8. The "flattening" of the experimental curve here might also be accounted for by the surface charge effects discussed by Tanford and Kirkwood (1957). Unlike the cardiolipin titration which displays an apparent shift in the pK_2, dCL exhibits only a slight flattening of its titration curve (Figure 17). This shows that

Figure 16: Computer Simulation of CL Titration Curves



All simulated curves (Harris, 1987) are generated according to the Henderson-Hasselbalch equation where the first pK is set at 2.8 while the second pK is varied from 6.5 to 8.5 with an interval of 0.5 pH unit to determine the best pK_2 value for the hydrogenated beef heart CL. The titration curve for the hydrogenated beef heart CL (x) is included for comparison.

Figure 17: Computer Simulated and Experimental Titration Curves of dCL



Computer simulated curve of dCL (X) is generated according to the Henderson-Hasselbalch equation where the first pK is set at 2.8 and the second pK is at 3.8. The titration curve for the deoxy analog of CL (□) is included in this figure for comparison.

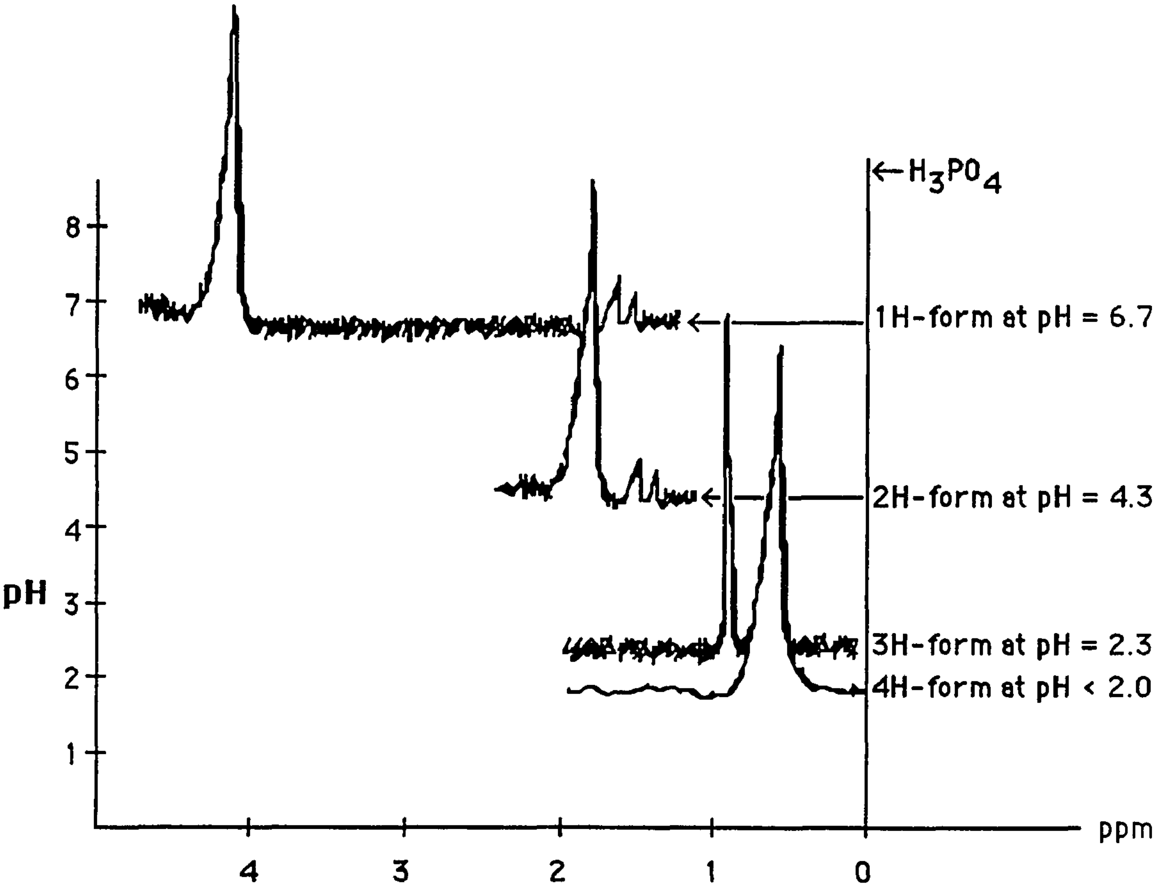
the Gouy-Chapman effects are insufficient to provoke the dramatic pK_2 shift observed with cardiolipin (Figure 15). Nor do they explain the "flattening" of the inflection point.

1,3-diphosphoglycerol and 1,3-diphosphopropanediol, model compounds for the polar headgroups of 18:0-CL and 16:0-dCL, respectively, both titrated as tetrabasic acids having two strong acid groups with pk 2.25 and 2.35, respectively, and two weak acid groups with pk 6.3 or 7.1, respectively (Table IV). These results show that the free hydroxyl on the 1,3-diphosphoglycerol does not alter the titration characteristics of the phosphate groups for these water-soluble compounds. It suggests the aberrant CL titration may be due to the fatty alkyl chains.

^{31}P-NMR:

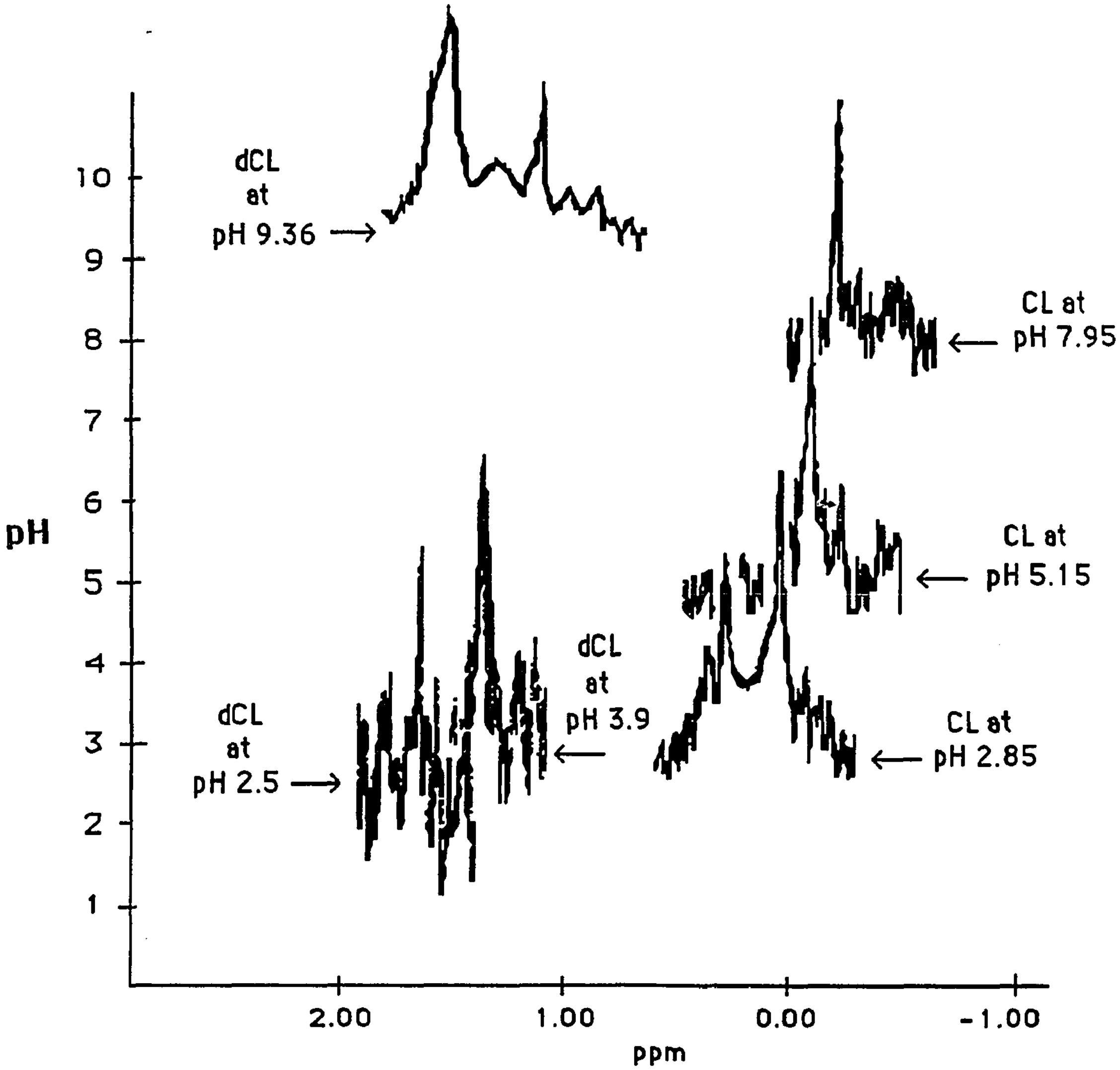
The ^{31}P-NMR spectrum of 1,3-diphosphopropane is shown in Figure 18. Each of the protonated forms of 1,3-diphosphopropane gave a single well-resolved signal, in the range +0.8 to +4.4 ppm. Proton decoupled ^{31}P-NMR spectra in D_2O/H_2O (1/1) of sonicated, fully protonated hydrogenated CL and its deoxy analog (dCL) are summarized in Figure 19. Both CL and dCL samples appeared to be milky when suspended in D_2O. The partially protonated CL (pH 2.85) showed a strong signal at 0.00 ppm, relative to H_3PO_4 (85%), and a weaker one at 0.33 ppm. The monoprotonated CL (pH 5.15) showed a signal at -0.08 ppm. The unprotonated CL (pH 7.95) showed one major signal at -0.17 ppm. The two weak signals at -2.76 and -3.23 ppm were later

Figure 18. **Proton-decoupled ^{31}P-NMR Spectra of 1,3-propanediphosphate**



Proton-decoupled ^{31}P-NMR spectra were recorded at 81MHz on a Brucker Model Spectrometer. Tetraprotonated 1,3-propanediphosphate (4H) was suspended in D_2O/H_2O (1/1, v/v) and the spectra were measured immediately after the protonation (see procedure), and after the addition of one (3H), two (2H), and three (1H) equivalents of KOH. Chemical shifts are relative to 85% H_3PO_4 (-55.4777 ppm).

Figure 19. Proton-decoupled P-31 NMR Spectra of Sonicated Beef Heart CL and dCL



Proton-decoupled ^{31}P-NMR spectra were recorded at 81MHz on a Brucker Model WP200 Spectrometer. Both hydrogenated (beef heart) CL (6.7 mg) and the deoxy analog of the CL (12.3 mg) were suspended in D_2O/H_2O (1/1, v/v) and sonicated for 3 hours. The spectra were measured immediately after the sonication, after the addition of one equivalent of KOH, and after the addition of two equivalents of KOH. Chemical shifts are relative to 85% H_3PO_4 (-55.4777 ppm).

identified as artifactual breakdown products (water soluble glycerol phosphates). Fully protonated and monoprotonated dCL spectra each showed a single strong signal at +1.62 or +1.30 ppm, respectively, whereas the deprotonated dCL showed a strong signal at +1.53 ppm accompanied by a weaker one at +1.07 ppm. These results show that the ionized state of CL or dCL influences the chemical shift of the ^{31}P-NMR signals. This must be considered when interpreting spectra of cardiolipin.

Many investigators have reported double peaks for the ^{31}P-NMR spectra of cardiolipin (e. g., Henderson et al., 1974; Torregrossa et al., 1977; Cullis et al., 1978; Battenburg et al., 1987) although others have reported single peaks (e. g., London and Feigenson, 1979; Mogelson et al., 1980; Beyer and Klingenberg, 1985). On the assumption that the double peaks are twins, as found by some investigators (e. g., Henderson et al., 1974), Powell and Jacobus (1974) have proposed that the two phosphorus atoms are in a different environment due to the chiral centers on the three glycerols. The twin peaks have not been found in any other phospholipid. The presence of two different protonated forms in the same solution at neutral pH (or extracted from a solution at neutral pH), or even a closed bicyclic ring structure (see Figure 3) having two different protonation states with a slow exchange rate between them might explain the twin peaks. The interpretation of cardiolipin's ^{31}P-NMR spectra is far from settled.

DISCUSSION

Knowledge of the roles of individual membrane lipid components is required to establish appropriate characteristics of the membrane proteins associated with them. The activity of these proteins is expected to be modulated by the surrounding lipid bilayer. A number of bilayer features may be considered, such as viscosity (or lipid packing density), the thickness of the bilayer, variations in the structures of the chains, and the charge density at the lipid-water interface. All these parameters are determined by the structures of the bilayer lipids. There are few reports that address the question of how alterations in the lipid environment of a membrane protein may affect the activities of membrane proteins.

Titration of Cardiolipin:

Cardiolipin was first isolated and characterized by Mary Pangborn (1942, 1947). It was so named because heart was the best source for this antigen that produced antibodies used to diagnose syphilis. She provided Few of Cambridge, England (1960) with homogenized samples with which he measured the the pK of the lipid. Few had developed an electrophoretic method for measuring the pK's of groups on the surface of living bacteria. His titration of cardiolipin showed a pK of 1.75. The titration continued an upward drift as the pK increased. This drift which is not unlike the present titration was assumed at that time to be an artifact.

It is likely that Few's sample of unhydrogenated beef heart mitochondria at a pH below 1.0 was in the H_{II} phase. Neither the H_{II} phase nor CL's tendency to form it was known at that time. Nevertheless, the upward drift of the titration curve obtained by Few et al. (1960) appears to be consistent with the titration in Figure 14.

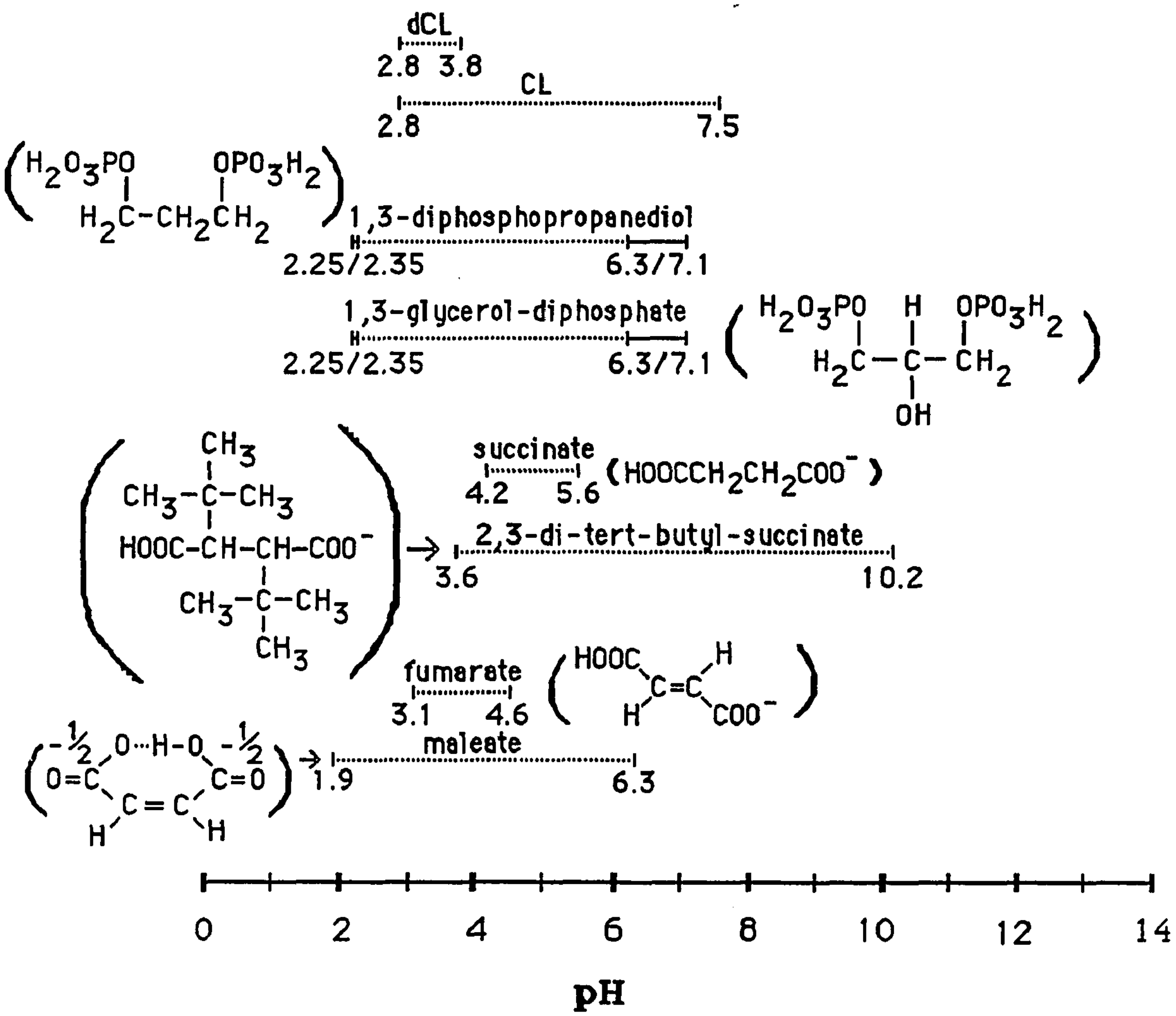
A second attempt to measure the pK of cardiolipin (Coulon-Morelec et al., 1962) was made in ethanol solution using indicator dyes. In this case a single pK was reported to be below 4.0 although a precise number could not be assigned.

A third measurement of CL's pK's was that of Seddon et al. (1983) who reported a pK below 2.8 based upon the pH dependence of phase changes. This measurement could not be expected to detect a second pK since CL displayed only one phase change. These investigators assumed that there was only one pK and that cardiolipin above that pK is dibasic (Marsh, 1990).

The titration of cardiolipin presented in this thesis is apparently the first quantitative electrometric titration of cardiolipin. Unlike previous attempts to titrate cardiolipin, the present measurement specifically examines the number of equivalents titrated at each pK. This titration yielded two significant and surprising results. First, there are two pK's with the second pK being above physiological pH even at its lowest value. The second unexpected result is that the pK_2 shifts rather dramatically as the titration proceeds. In view of the comparison of CL titration to that of the deoxycardiolipin these unexpected results are due to the presence of a free hydroxyl group on the connecting glycerol in cardiolipin. That this hydroxyl group can significantly affect the pK of CL is best explained by the formation of an intramolecular hydrogen bond with one of the P-OH groups (Figure 3). Such pK-related intramolecular hydrogen bonding is equivalent to the acid-anions first described by Westheimer and Benfey (1956) and applied to lipids by Haines (1983).

In their initial proposals on the anomalous pK's of mono-protonated diacids Westheimer and Benfey (1956) explained the unusually high second pK in terms of the resonance stabilization of the single electron. In their examples the negatively-charged groups were locked in the intramolecular hydrogen-bond. The acid-anions they discussed formed rings, trapping protons as described in Figure 20. Any factor contributing to a tighter ring would be expected to increase the pK_2. Such factors may include insertion of the chains into a bilayer, an increase in the charge density of the bilayer surface, binding of the headgroup to a protein, etc. Maleate and fumarate differ only by the orientation of the carboxyls which forces the acid-anion formation of maleate. Addition of bulky groups such as two *t*-butyl groups at both the second and the third carbon of succinate provokes acid-anion formation. Meanwhile the water soluble headgroup moieties, 1,3-glycerol-diphosphate and 1,3-propane-diphosphate, do not display a significant difference in titration.

Figure 20: pK's of Selected Acid-anions



Illustrations of acid-anion formation. Fumarate and maleate differ by the orientation of the double bond; maleate is *cis* forcing the carboxyls to be proximal. Both pK's of maleate are affected. Di-*tert*-butyl succinate is an acid-anion up to pH 10.2 whereas succinate is not. In aqueous solution neither 1,3-glycerol-diphosphate nor 1,3-diphosphopropanediol forms an acid-anion. In contrast cardiolipin, which has the same headgroup structure, forms an acid-anion.

In contrast lipids with glycerol-phosphate headgroups have been shown to contain internal hydrogen-bonds. The existence of such an intra-molecular hydrogen bond (acid-anion) has been demonstrated by FTIR spectroscopy in PGP (phosphatidyl-glycerophosphate) (Stewart et al., 1988, 1990), as well as in cardiolipin (Hubner et al., 1991). This data suggests that the difference between the titrations of 18:0-CL and 16:0-dCL may be due to the conformation of the headgroup of CL allowing the alignment of the C-OH group and P-OH groups. In the language of Westheimer, it is proposed that resonance stabilizes the monoprotonated CL species at high pH (Figure 3). The delocalization of the electron on the two phosphates is presumed to be facilitated by vibrating protons on the monoprotonated species as is the case for the maleic acid anion. This latter feature may explain why the pK_2 of CL increases during the titration from 7.5 to 9.5 (Figure 14).

The pK_2 of CL apparently shifts as the charge density increases. The titration of deoxycardiolipin displays a pK_2 only slightly higher than its pK_1. This represents a good control for the suppression of ionization by the increased charge density on the surface as predicted by Tanford and Kirkwood (1957). In contrast, cardiolipin displays a shifting pK_2 as the titration proceeds. This suggests that the pK_2 of cardiolipin is sensitive to the charge density in two ways, first in the same way that deoxycardiolipin is, and second by some conformational effect relating to the H-bonded double ring structure. Presumably the second effect is significantly greater than the first. Not only does a small increase in charge density provide a large increase in pK_2, but as the titration proceeds it appears to shift the pK.

^{31}P-NMR:

Proton decoupled ^{31}P-NMR of fully protonated CL vesicles shows an intense high-field edge and shallow low-field shoulder which are typical of line shapes recorded from bilayers of hydrated phospholipids. The spectrum of deprotonated CL shows two peaks at -0.00 and 0.33 ppm (relative to H_3PO_4) which is similar to that of Henderson et al. (1974). The peaks they observed in chloroform were at -1.0 and -0.8 ppm.

^{31}P-NMR chemical shift values have been used to measure pH in cells. Gradient changes of up to 3.5 pH units have been measured in cell envelope vesicles (Kouyama et al., 1987) following light-induced proton translocation.

For the hydrogenated CL, the spectra display shifts after the addition of 1 and 2 equivalents of KOH which are far less than 5ppm. After the addition of 2 equivalents of KOH, a precipitate was observed and the ^{31}P-NMR spectrum displayed two upfield peaks at -2.76 and -3.23 ppm. This suggested some hydrolysis of the sample (Henderson et al., 1974) which is not surprising since the samples were left overnight in D_2O/H_2O. Proton decoupled ^{31}P- NMR displayed in a singlet for deoxy-CL. The two signals of the spectra for dCL with 2 equivalents of KOH may represent the difference in spectra between the lipid molecules in the outer and that on the inner monolayer of the bilayer or the existence of H_{II} phase in the sample. Proton decoupled P-31 NMR spectra of 1,3-propanediphosphate (Figure 18) show chemical shifts of the signal after the addition of 1, 2, and 3 equivalents of KOH which implies the chemical shift of the two phosphates average on a timescale faster than that of the spectral analysis.

The Affinity of Cardiolipin for Specific Membrane Proteins:
CL has been found to be specially associated with many proteins. In general they can be categorized into two areas (A) mitochondrial-related proteins and (B) other than mitochondrial proteins.

(A) mitochondrial-related proteins :

- (a) Addition of CL to cytochrome c oxidase results in stimulation of the enzymatic activity (Abramovitch et al., 1990; Awasthi et al., 1971; Dale and Robinson, 1988; Fry and Green, 1980; Powell et al., 1985; Vik et al., 1981).
- (b) CL stimulates the enzyme activity of mitochondrial NADH dehydrogenase (Fry and Green, 1981).
- (c) CL binds with the F_0 - F_1 ATPase of the inner mitochondria membrane (four moles of CL are tightly bound with one mole of F_0 - F_1 ATPase) which regulates the hydrolysis the ATP (Kagawa, et al., 1973; Eble et al., 1990).
- (d) CL is tightly bound to ATP - ADP carrier protein where it helps the transport function of the protein (Horvath et al., 1990).

(e) CL was found to be specifically hydrogen-bonded to cytochrome c (Caffrey et al., 1991; Spooner and Watts, 1990a and b; Soussi et al., 1990).

(f) CL stimulates the binding of cholesterol to the mitochondrial cytochrome P-450scc (Holland and Stevenson, 1990; Lambeth et al., 1982; Tuckey and Stevenson, 1985) which protein is involved in hormone production in the corpus luteum, a major steroid hormone producing cell.

(g) CL binds to mitochondrial isoenzyme of heart creatine kinase which is located at the outer leaflet of the inner membrane (Cheneval and Carafoli, 1988).

(h) CL may regulate DNA replication in mitochondria since CL strongly inhibited the activity of all 3 eukaryotic DNA polymerases α, β, and γ as well as the terminal deoxynucleotidyl transferase. The strong inhibition by CL of DNA polymerase γ, the polymerase specifically associated with mitochondrial DNA synthesis was well characterized (Yoshida, S., et. al. 1989).

(B) Other proteins:

(a) CL can bind to nucleotide-free *dna* A protein of *E. coli* which prevents ATP binding and initiation of *oriC* (chromosome) replication (Sekimizu and Kornberg, 1988).

(b) CL modifies the interaction of human serum carboxylesterase (the enzyme that hydrolyzes the long-chain fatty acid esters of triolein) with substrates in such a way as to facilitate its interaction with hydrophobic substrates (Shirai et al., 1988).

Considering the above data one might suggest that CL plays an important role in proton transport.

CODA
PROTON ENTRAPMENT BY ACIDIC LIPIDS IN BILAYERS

From the data reported in this paper, one may conclude that both intermolecular and intramolecular hydrogen-bonding of acidic lipids may be important in membrane structure and dynamics. In the case of CL an intramolecular hydrogen-bond traps a proton. This might explain the biological significance of cardiolipin in oxidative phosphorylation, namely to bind protons at relatively high pH and provide them to proteins within the membrane. Thus cardiolipin might buffer protons providing a reservoir for protons at high pH. These statements run counter to the suggestion that cardiolipin could only provide protons at low pH (Seddons et al., 1983).

For the chlorosulfolipids it appears that the secondary sulfates are protonated in the low dielectric of the bilayer. These protons neutralize the anionic sulfates stabilizing the bilayer. Under these circumstances the protonated sulfates may create unusually strong hydrogen-bonds in the low dielectric not unlike those in the double helix of DNA.

REFERENCES

Aaronson, S. (1980) In *Biochemistry and Physiology of Protozoa,* second edition, *vol. 3,* M. Levandowsky and S. H. Hubner, eds. Academic Press, N. Y., 117-170.

Aaronson, S. and Baker, H. (1959) *J. Protozool.* 6, 282-284.

Abramovitch, D. A., Marsh, D. and Powell, G. L. (1990) *Biochim. Biophys. Acta* 1020, 34-42.

Allnutt, F. C. T., Atta-Asafo-Adjei, E. and Dilley, R. A. (1989) *J. Bioenergetics and Biomembranes,* 21, 535-551.

Anderson, R., Kates, M., and Volcani, B. E. (1978a) *Biochim. Biophys. Acta* 528, 89.

Anderson, R., Livermore, Kates, M., and Volcani, B. E. (1978b) *Biochim. Biophys. Acta* 528, 77.

Aurora, T. S., Li, W., Cummins, H. Z., Haines, T. H. (1985) *Biochim. Biophys. Acta* 820, 250-258.

Awasthi, T. C., Chuang, T. F., Keenan, T. W., and Crane, F. L. (1971) *Biochim. Biophys. Acta* 226, 42-52.

Bangham, A. D. and Horne, R. W. (1964) *J. Mol. Biol.* 8, 660-668.

Bangham, A. D., Standish, M. M. and Watkins, J. C. (1965) *J. Mol. Biol.* 13, 238-252.

Barenholz, Y., Amselen, S. and Lichtnegerg, D. (1979) *FEBS lett.* 99, 210-214.

Battenburg, A. M., Hibbein, J. C. L., and de Kruiff, B. (1987) *Biochim. Biophys. Acta* 903, 155-165.

Bedzyk, M. J., Bommrito, M., Caffrey, M., and Penner, T. L. (1990) *Science,* 248, 52-56.

Beyer, K. and Klingenberg, M. (1985) *Biochemistry* 24, 3821-3826.

Bligh, E. A. and Dyer, W. J. (1959) *Can. J. Bichem. Biophysiol.* 37, 911-917.

Blume, A., Hubner, W. and Messner, G. (1988) *Biochemistry,* 27, 8239-8249.

Boggs, J. M. (1986) *Biochem. Cell Biol.* 64, 50-57.

Boggs, J. M. (1987) *Biochim. Biophys. Acta* 906, 353-404.

Burstein, S. and Lieberman, S. (1958) *J. Am. Chem. Soc.* 80, 5235.

Caffrey, M. S., Daldal, F., Holden, H. M., and Cusanovich, M. A. (1991) *Biochemistry* 30, 4119-4125.

Chapman, D. L. (1913) *Phil. Mag.* 25, 475.

Chen, L. L. and Haines, T. H. (1976) *J. Biol Chem.* 251, 1828-1834.

Chen, L. L., Pousada, M., and Haines, T. H. (1976) *J. Biol. Chem.* 251, 1835-1842.

Cheneval, D. and Carafoli, E. (1988) *Eur. J. Biochem.* 171, 1-9.

Coulon-Morelec, M. J., Faure, M., and Marechai, J. (1962) *Bull. Soc. Chim. Biol.* 44, 171-183.

Cullis, P. R., Verkleij, A. J. and Ververgaert, P. H. J. Th. (1978) *Biochim. Biophys. Acta* 513, 11-20.

Dale, M. P. and Robinson, C. (1988) *Biochem.* 27, 8270-8275.

de Kruijff, B., Verkleij, A. J., Leunissen-Bijvelt, J., Van Echteld, C. J. A., Hille, J. and Rijnbout, H. (1982) *Biochim. Biophys. Acta* 693, 1-12.

Eble, K. M., Coleman, W. B., Hantgan, R. R., and Cummingham, C. C. (1990) *J. Biol. Chem.* 265, 19434-19440.

El Maraghy, E. (1982) Ph. D. Thesis, The City University of New York.

Elovson, J. (1974a) *Biochemistry,* 13, 2105-2109.

Elovson, J. (1974b) *Biochemistry,* 13, 3483-3487.

Elovson, J. and Vagelos, P. R. (1969) *Proc. Natl. Acad. Sci., U. S. A.,* 62, 957.

Elovson, J. and Vagelos, P. R. (1970) *Biochemistry,* 9, 3110-3126.

Esmann, M., Powell, G. L. and Marsh, D. (1988) *Biochim. Biophys. Acta* 941, 287-292.

Fernandez, M. S. and Fromherz, P. (1977) *J. Phys. Chem.* 81, 1755-1781.

Few, A. V., Gilby, A. R., and Seaman, G. V. F. (1960) *Biochim. Biophys. Acta* 38, 130-136.

Finkelstein, A. (1976) *J. General Physiol.* 68, 127-135.

Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) *J. Biol. Chem.* 226, 497-509.

Fry, M. and Green, D. E. (1980) *Biochem. Biophys. Res. Commun.* 93, 1238-1246.

Fry, M. and Green, D. E. (1981) *J. Biol. Chem.* 256, 1874-1880.

Gabriel, B., Prats, M., and Teissie, J. (1991) *Biochemistry* 30, 9359-9364.

Gains, N. and Hauser, H. (1983) *Biochim. Biophys. Acta* 731, 31-39.

Gally, H. U., Pluschka, G., Overath, P., and Seelig, J. (1980) *Biochemistry* 19, 1638-1643.

Gershengorn, M. C., Smith, A. R., Goulston, G., Goad, L. J., Goodwin, T. W., and Haines, T. H. (1968) *Biochem.* 7, 1698-1701.

Gouy, M. (1910) *J. Phys. (Paris)* 9, 457-486.

Gray, G. M. and MacFarlane, M. G. (1958) *Biochem. J.* 70, 409-425.

Haines, T. H. (1965) *J. Protozool* 12, 655-659. (isolate SL)

Haines, T. H. (1971) *"The Chemistry of The Sulfolipids"* In *Progress in The Chemistry of Fats and Other Lipids, Vol 11*, Holman, R.T. Ed., Pergamon, N. Y., 299-349.

Haines, T. H. (1972) CRC Handbook of Microbiology, *Vol. V*, 115-123.

Haines, T. H. (1973) *Annual Review of Microbiology, Vol. 27*, 403-411.

Haines, T. H. (1983) *Proc. Nat. Acad. Sci.* 80, 160-164.

Haines, T. H., Aaronson, S., Gellerman, J., and Schlenk, H. (1962) *Nature* 194, 1282-1283.

Haines, T. H. and Block, R. J. (1962) *J. Protozool.* 9, 33-38.

Haines, T. H., Pousada, M., Stern, B., and Mayer, G. L. (1969) *Biochem. J.* 113, 565-567.

Haines, T. H. and Kates, M. (1992) in preparation.

Hargreaves, W. R. and Deamer, D. W. (1978) *Biochem.* 17, 3759-3768.

Harris, D. C. (1987) *Quantitative Chemical Analysis*, Second Ed., W. H. Freeman and Co., N. Y.

Hatanaka, H., Egami, F., Ishizuka, I., and Nagai, Y. (1976) *Biochim. Biophys. Acta.* 438, 176-185.

Hauser, H. and Gaines, N. (1982) *Proc. Natl. Acad. Science U. S. A.* 79, 1683-1687.

Heller, M. (1982) Ph. D. Thesis, The City University of New York.

Henderson, T. O., Glonek, T., and Myers, T. C. (1974) *Biochemistry* 13, 623-628.

Hoch, F. L. (1992) *Biochim. Biophys. Acta* 1113, 71-133.

Holland, J. W. and Stevenson, P. M. (1990) *Biochim. Biophys. Acta* 1022, 401-407.

Horvath, L. I., Drees, M., Beyer, K., Klingenberg, M., and Marsh, D. (1990) *Biochemistry* 29, 10664-10669.

Hostetler, K. Y., Van den Bosch, H., and Van Deenan, L. L. M. (1971) *Biochim. Biophys. Acta* 239, 113-119.

Hovius, R., Lambrechts, H., Nicolay, K., and DeKruijff, B. (1990) *Biochim. Biophys. Acta* 1021, 217-226.

Hubner, W., Mantsch, H. H., and Kates, M. (1991) *Biochim. Biophys. Acta* 1066, 166-174.

Hwang Y-W (1984) Ph. D. Thesis, The City University of New York.

Hwang, J. S. and Cummins, H. Z. (1982) *J. Chem. Phys.* 77, 616-621.

Ioannou, P. V. and Golding, B. T. (1979) *Prog. Lipid Res.* 17, 279-318.

Ishizuka, I., Inomata, M., Ueno, K and Yamakawa, T. (1978) *J. Biol. Chem.* 253, 898-907. (rat brain)

Jencks, W. P. (1976) In *Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data*, third edition, *vol. I.* G. D. Fasman Ed., CRC Press, Cleveland, Ohio.

Kagawa, Y., Kandrach, A., and Racker, E (1973) *J. Biol. Chemistry* 248, 676-684.

Kahan, D., Oren, R., Aaronson, S., and Behrens, U. (1978) *J. Protozool.* 25, 30-33.

Kates, M. (1975) In *"Techniques of Lipidology" of "Laboratory Techniques in Biochemistry and Molecular Biology"*, T. S. Work gand E. Work eds., North-Holland/American Elsevier Co., INc., New York., 424-427.

Kates, M., Tremblay, P., Anderson, R., and Volcani, B. E. (1978) *Lipids,* 13, 34-41.

Kean, E. L. (1968) *J. Lipid Res.* 9, 319.

Krebs, J. J. R., Hauser, H., and Carafoli, E. (1979) *J. Biol. Chem.* 254, 5308-5316.

Kunitake, T. and Okahata, Y. (1977) *J. Am. Chem. Soc.* 99, 3860.

Lambeth, J. D., Kitchen, S. E., Farooqui, A. A., Tuckey, R. C., and Kamin, H. (1982) *J. Biol. Chem.* 257, 1876-1884.

Liem, P. Q. and Laur, M-H (1976a) *Biochimie.* 58, 1367-1380.

Liem, P. Q. and Laur, M-H (1976b) *Biochimie.* 58, 1381-1396.

London, E. and Feigenson, G. W. (1979) *J. Lipid Res.* 20, 408-412.

Luzatti, V., Gulik-Krzywicki, T. and Tardieu, A. (1968) *Nature* 218, 1031-1034.

Marsh, D. (1990) *Handbook of Lipid Bilayers*, p. 85, CRC Press, Boca Raton, Florida.

Mayers, G. L. and Haines, T. H. (1967) *Biochem.* 6, 1665-1671.

Mayers, G. L., Pousada, M., and Haines, T. H. (1969) *Biochem.* 8, 2981-2986.

McLaughlin, S. G. A., Szabo, G., and Eisenman, G. (1971) *J. Gen. Physiol.* 58, 667-683.

Mercer, E. I. and Davies, C. L. (1974) *Phytochem.* 13, 1607-1610.

Mercer, E. I. and Davies, C. L. (1975) *Phytochem.* 14, 1545-1548.

Mercer, E. I. and Davies, C. L. (1979) *Phytochem.* 18, 457-462.

Mercer, E. I. Thomas, G., and Harrison, J. (1974) *Phytochem.* 13, 1297-1302.

Mitchell, P. (1961) *Nature* 191, 144-148.

Mogelson, S., Wilson, G. E. and Sobel, B. E. (1980) *Biochim. Biophys. Acta* 619, 680-688.

Mooney, C. L. and Haines, T. H. (1973) *Biochem.* 12, 4469-4472.

Mooney, C. L., Mahoney, E. M., Pousada, M., and Haines, T. H. (1972) *Biochem.* 11, 4839-4845.

Morita, H. and Nagakura, S. (1972) *Theor. Chim. Acta* 27, 325-338.

Nishijima, S., Asami, Y., Uetake, N., Yamagoe, S., Ohta, A. and Shibuya, I. (1988) *J. Bacteriology* 170, 775-780.

Pangborn, M. (1942) *J. Biol. Chem.* 143, 246-256.

Pangborn, M. (1947) *J. Biol. Chem.* 168, 351

Pascher, I., Sundell, S., Harlos, K., ad Eibl, H. (1987) *Biochim. Biophys. Acta* 896, 77-88.

Patni, N. j., Billmire, E., and Aaronson, S. (1974) *Biochim. Biophys. Acta* 373, 347- 355.

Phillips, C. D. (1966) *Sci. Amer.* 215, 78-90.

Pluschke, G., Hirota, Y., and Overath, P. (1978) *J. Biochem.* 253, 5048-5055.

Poncz, L. (1978) Ph. D. Thesis, The City University of New York.

Powell, G. L. and Jacobus, J. (1974) *Biochemistry* 13, 4024-4026.

Powell, G. L., Knowles, P., and Marsh, D. (1985) *Biochim. Biophys. Acta* 816, 191-194.

Prats, M, Tocanne, J. F., and Teissie, J. (1987) *Eur. J. Biochem.* 162, 379-385.

Prats, M., Teissie, J., and Tocanne, J. F. (1986) *Nature* 322, 756-758.

Radin, N. S. (1983) *"Sulfatides"*, In *Handbook of Neurochem. vol. 3*, Lajtha, A., ed., Plenum Publish, 163-177.

Rand, P. R., Fuller, N., Parsegian, V. A., and Rau, D. C. (1988) *Biochemistry* 27, 7711-7722.

Rintoul, D. A., Welti, R. and Song, W. (1988) *Biophys. J.* 53, 126a.

Rouser, G., Fleischer, S. and Yamomoto, A. (1969) *Lipids* 5, 494.

Rouser, G., Siakotos, A. N., Fleischer, S., (1966) *Lipids* 1, 85-92.

Rutkowski, C. A., Williams, L. M., Haines, T. H., and Cummins, H. Z. (1991) *Biochemistry* 30, 5688-5696.

Sakurai, I. and Kawamura, Y. (1987) *Biochim. Biophys. Acta* 904, 405-409.

Sakurai, I. and Kawamura, Y. (1989) *Biochim. Biophys. Acta* 985, 347-350.

Schlame, M., Rabe, H., Rustow, B., and Kunze, D. (1988) *Biochim. Biophys. Acta* 958, 493-496.

Schlame, M. and Rustow, B. (1990) *Biochem. J.* 272, 589-595.

Seddon, J. M., Kaye, R. D., and Marsh, D. (1983) *Biochim. Biophys. Acta* 734, 347-352.

Sekimizu, K. and Kornberg, A. (1988) *J. Biological Chem.* 263, 7131-7135.

Shirai, K., Ohsawa, I., Saito, Y. and Yoshida, S. (1988) *Biochim. Biophys. Acta* 962, 377-383.

Soussi, B., Bylund-Fellenius, A-C, Schersteu, T., and Angstrom, J. (1990) *Biochem. J.* 265, 227-232.

Sperka-Gottlieb, C. D, M., Hermetter, A., Paltauf, F., and Daum, G. (1988) *Biochim. Biophys. Acta* 946, 227-234.

Spooner P. J. R. and Watts, A. (1990a) *Biochemistry* 30, 3871-3879.

Spooner P. J. R. and Watts, A. (1990b) *Biochemistry* 30, 3880-3885.

Stern, A. (1978) Ph. D. Thesis, The City University of New York.

Stern, O. (1924) *Z. Eleckrochem* 30, 508-516.

Stewart, L. C. and Kates, M. (1989) *Chemistry and Physics of Lipids,* 50, 23-42.

Stewart, L. C., Kates, M., and Smith, I. C. P. (1988) *Chemistry and Physics of Lipids* 48, 177-188.

Stewart, L. C., Kates, M., Yang, P. W., and Mantsch, H. H. (1990) *Biochem. Cell Biol.* 68, 266-273.

Sweeley, C. C. and Siddiqui, B. (1977) In *Biochemistry of Mammalian Glycoprotein and Glycolipids*, Pigman, W. W., and Horowitz, S. T. eds. Academic Press, N. Y.

Tanford, C. (1978) *The Hydrophobic Effect,* Wiley Interscience, N. Y., Chap. 9.

Tanford, C., and Kirkwood, J. G. (1957) *J. Am. Chem. Soc.* 79, 5333-5339.

Teissie, J., Prats, M., LeMassu, A., Stewart, L. C. and Kates, M. (1990) *Biochemistry* 29, 59-65.

Teissie, J., Prats, M., Soucaille, P. and Tocanne, J. F. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3127-3221.

Torregrossa, R. E., Makula, R. A., and Finnerty, W. R. (1977) *J. Bacteriol.* 131, 486-492.

Tuckey, R. C. and Stevenson, P. M. (1985) *Eur. J. Biochem.* 148, 379-384.

Verkleij, A. J., Humbel, B., Studer, D. and Muller, M. (1985) *Biochim. Biophys. Acta* 812, 591-594.

Verkleij, A. J., Van Echteld, C. J. A., Gerritsen, W. J., Cullis, P. R. and Dekruijff, B. (1980) *Biochim. Biophys. Acta* 600, 620-624.

Verkleij, A. T. (1984) *Biochim. Biophys. Acta* 779, 43-63.

Vik, S. B., Georgevich, G., and Capaldi, R. A. (1981) *Proc. Natl. Acad. Science U. S. A.* 78, 1456-1460.

Westheimer, F. H. and Benfey, O. T. (1956) *J. Am. Chem. Soc.* 78, 5309-5311.

Williams, R. J. P. (1961) *J. Theor. Biol.* 1, 1-13.

Winikov, I. (1986) Ph. D. Thesis, The City University of New York.

Yoshida, S., Tamiya-Koizumi, K. and Kojima, K. (1989) *Biochim. Biophys. Acta* 1007, 61-66.