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**The preparation, elasticity and proton/hydroxyl permeability of
uniform, unilamellar vesicles of anionic phospholipids**

Li, Wei, Ph.D.

City University of New York, 1988

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**THE PREPARATION, ELASTICITY AND PROTON/HYDROXYL
PERMEABILITY OF UNIFORM, UNILAMELLAR VESICLES
OF ANIONIC PHOSPHOLIPIDS**

by
WEI LI

**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.**

1988

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

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ABSTRACT

THE PREPARATION, ELASTICITY AND PROTON/HYDROXYL
PERMEABILITY OF UNIFORM, UNILAMELLAR VESICLES
OF ANIONIC PHOSPHOLIPIDS

by

Wei Li

Advisor: Professor Thomas H. Haines

A general procedure for the preparation of large unilamellar uniform vesicles of selected sizes (200 - 3000 nm) has been developed. The procedure consists of dissolving the lipid in organic solvent, washing with mild acid, removing the solvent, adding saline and adjusting the pH up to 10 and immediately back to 7.5. Vesicle size varies with the lipid structure, the composition of the lipid mixture and the ionic strength of the medium. The technique of dynamic light scattering has been used for measuring the elastic response of vesicle bilayer membranes to an applied osmotic pressure. The vesicles swell uniformly upon dilution. The observed change in size is not due to any change in the shape of the vesicles. The amplitude of the swelling is initially linearly proportional to the osmotic pressure difference across the bilayer. It was found that the elastic properties of the bilayer vary by a factor of ten in electrolyte media as compared to isoosmolal non-electrolyte media and that this variation appears to be dominated by the ionic strength of the media. Measurements of the proton/hydroxyl permeability across the bilayers of vesicles prepared by the pH adjustment procedure have been made. Membrane conduc-

tivity was assayed by the concentration of acridine orange into vesicles during the imposition of a pH gradient of 2.5 to 3.0 units (neutral range, inside low pH) across the bilayer. The effects of the lipid head-groups, the incorporation of cholesterol into the vesicle bilayer and detergents on the membrane permeability were studied.

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TABLE OF CONTENTS

LIST OF TABLES	xi
LIST OF FIGURES	xiii
ABBREVIATIONS	xv
INTRODUCTION	1
MATERIALS	15
EXPERIMENTAL	17
Preparation of the Vesicles	17
Characterization of the Vesicles	18
(1) Electron Microscopy	18
(2) Sepharose Column Extrusion	18
(3) Trapped Volume Determination	18
(4) Photon Correlation Spectroscopy (PCS)	19
Osmotic Dilution of Vesicle Suspensions	26
(1) Osmotic Dilution of Synthetic Vesicle Suspensions	26
(2) Osmotic Dilution of Sub-mitochondrial Particles	27
H ⁺ /OH ⁻ Permeability Measurements	28
CALCULATIONS	31
RESULTS	42
Preparation and Characterization of the Vesicles	42
(1) Characterization of the Vesicles	42

(2) Preparation of Uniform Vesicles comprised of Anionic Lipids	48
(3) Vesicle Preparations in Media of High Ionic Strength	59
(4) Vesicle Preparations in Media of Low Ionic Strength	59
(5) Vesicles Prepared with Lipid Mixtures Containing Anionic Lipid	64
Membrane Elastic Property Measurements using Osmotic Swelling	64
(1) DOPA Vesicle Swelling in 0.15 M KCl Solution	66
(2) DOPA Vesicle Swelling in 0.25 M Sucrose Solution	70
(3) Sub-mitochondrial Particles Swelling in 0.25 M Sucrose Solution	70
(4) Vesicle Bursting and Reswelling	70
(5) Aqueous Media Effects on Membrane Elastic Properties	75
(6) Lipid Structure Effects on Membrane Elastic Properties	82
(7) Thickness Changes of Bilayer on Osmotic Swelling	85
H ⁺ /OH ⁻ Currents Across Lipid Bilayers	90

(1) Permeability of Anionic Straight-Chain Phospholipid Vesicles	90
(2) The Effect of Sterol and Phytanyl Chains on Permeability	92
(3) The Effect of Detergents on Proton/Hydroxyl Permeability	92
DISCUSSION AND CONCLUSION	97
Preparation and Characterization of the Vesicles	97
(1) Vesicle Preparations of Anionic Phospholipids and Lipid Mixtures Containing Anionic Lipids	99
(2) Vesicle Preparations in Non-Saline Media	102
(3) Unilamellar Vesicles Obtained from Single-Chain Lipids	103
Membrane Elastic Property Measurements	
by Osmotic Swelling	104
(1) Measurement of the Membrane Modulus by Osmotic Swelling	104
(2) Measurement of the Elastic Limit	108
(3) Effects of Media, Ionic Strength and Lipid Headgroups on Elastic Properties of Vesicles	114
(4) Curvature and the Membrane Modulus	116
(5) Bilayer Thickness Changes during Osmotic Swelling of Vesicles	118
(6) Membrane Modulus of Biological Membranes	118

(7) Comparison of Membrane Elastic Properties of Synthetic Lipid Bilayers	124
H ⁺ /OH ⁻ Currents Across Lipid Bilayers	125
(1) Lipid Structure Effects	126
(2) Detergent Effects	131
BIBLIOGRAPHY	132

LIST OF TABLES

TABLE	PAGE
I. Variation of vesicle size and polydispersity with lipid headgroups made of monounsaturated glycerol lipids	49
II. Variation of vesicle size and polydispersity with lipid headgroups	50
III. Vesicle preparations with fatty acids	51
IV. DOPG vesicle preparations made in solution of different salt concentration	60
V. Vesicle preparation of DOPA and DOPG in low ionic strength media	63
VI. DOPG/DOPC vesicle preparation in KCl and pentaerythritol	65
VII. A typical set of osmotic swelling data	67
VIII. The experimental and calculated mean vesicle sizes after rupture and reswelling of DOPA vesicles	76
IX. Observed membrane moduli, the surface area increase and the pressure difference of DOPA vesicles	77
X. Observed membrane moduli, the surface area increase and the pressure difference of DOPA and DOPG vesicles in different aqueous media	81
XI. Observed membrane moduli, the surface area	

increase and the pressure difference of DOPG vesicles in the mixture of KCl and pentaerythritol solution	83
XII. Observed membrane moduli, the surface area increase and the pressure difference of vesicle preparations constituted of different lipids	84
XIII. H^+/OH^- permeability of vesicles with different lipid structure	91
XIV. H^+/OH^- permeability of DOPG/octyl glucoside and DOPG/cholate vesicles	95
XV. Membrane modulus of biological membranes	120
XVI. Comparison of the membrane modulus, the pressure difference across the membrane at bursting and the surface expansion of synthetic lipid bilayers	121

LIST OF FIGURES

FIGURE	PAGE
1. Photon correlation spectroscopy (PCS) experimental set-up	20
2. Light scattering intensity autocorrelation curve for polystyrene spheres and a DOPG vesicle preparation	23
3. H^+/OH^- current assay	29
4. Illustration of an osmotic swelling experiment	32
5. Theoretical model for the modulus calculation	34
6. Theoretical curves for choosing vesicle sizes in the osmotic swelling experiment	38
7. Standard deviation of DOPA vesicle sizes based on negative staining electron microscopy	43
8. Elution profile of DOPA vesicles from a Sephacrose 4B column	45
9. DOPA/cholesterol vesicles in KCl solution	52
10. DOPG/sterol vesicles in sucrose solution	54
11. Negative stained electron micrographs of oleic acid vesicles	57
12. DOPA vesicle size in KCl/sucrose solution mixture	61
13. Osmotic swelling curves of DOPA and DOPA/cholesterol vesicles in KCl solution	68
14. Osmotic swelling curves of DOPA and DOPA/cholesterol vesicles in sucrose solution	71

15. Osmotic swelling curves of sub-mitochondrial particles in sucrose solution	73
16. Osmotic swelling curves of DOPG vesicles	79
17. Osmotic swelling curves of vesicles made from different lipids	86
18. X-ray diffraction study of thickness changes of the blayer on osmotic swelling	88
19. Effect of cholesterol on membrane H^+/OH^- permeability	93
20. Transbilayer pressure difference during osmotic swelling of DOPA/cholesterol vesicles	110
21. Transbilayer pressure difference during osmotic swelling of oleic acid vesicles in 150 mM KCl solution	112

ABBREVIATIONS

BLM, black lipid membrane; DLPA, 1,2-dilauroyl-*sn*-3-phosphatidic acid; DMPC, 1,2-dimyristoyl-*sn*-3-phosphatidylcholine; DMPE, 1,2-dimyristoyl-*sn*-3-phosphatidylethanolamine; DOPA, 1,2-dioleoyl-*sn*-3-phosphatidic acid; DOPC, 1,2-dioleoyl-*sn*-3-phosphatidylcholine; DOPE, 1,2-dioleoyl-*sn*-3-phosphatidylethanolamine; DOPG, 1,2-dioleoyl-*sn*-3-phosphatidylglycerol; DOPS, 1,2-dioleoyl-*sn*-3-phosphatidylcholine; D Φ PC, 1,2-diphytanoyl-*sn*-3-phosphatidylcholine; D Φ GLS, 1,2-diphytanyl-*sn*-3-glycerolglycolipid sulfate; D Φ PGP, 1,2-diphytanyl-*sn*-3-phosphatidylglycerol phosphate; D Φ PG, 1,2-diphytanyl-*sn*-3-phosphatidylglycerol; D Φ PGS, 1,2-diphytanyl-*sn*-3-phosphatidylglycerol sulfate; EM, electron microscopy; EPC, egg phosphatidylcholine; FRAP, fluorescence recovery after pulse; HDTA, hexadecyltrimethylammonium chloride; LUV, large unilamellar vesicles; MDO, membrane-derived oligosaccharides; P, proton/hydroxyl permeability; PC, phosphatidylcholine; PCS, photon correlation spectroscopy; PE, phosphatidylethanolamine; PI, (bovine liver) phosphatidyl inositol; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol; SDS, sodium dodecyl sulfate; SOPC, 1-stearoyl-2-oleoyl-*sn*-3-phosphatidylcholine; SUV, sonicated (small) unilamellar vesicles; TLC, thin layer chromatography; Tris-succinate, tris(hydroxymethyl)aminomethane succinate.

INTRODUCTION

Ever since the classic experiments of Chapman (1958) which clearly described the transition temperature in lamellar sheets of membrane lipids, investigators have been seeking methods of making model lipid bilayers that resemble natural biological membranes. Bangham (1968) first succeeded in making lipid preparations (multilayered liposomes) that entrapped aqueous compartments and were osmotically active. An important requirement was that the liposome be above the transition temperature of lipids. A unilamellar liposome (vesicle) of uniform size (30 nm) was soon prepared by Huang (1969) using extensive sonication. Huang's vesicles were curvature limited with each phospholipid head group on the exterior surface occupying 0.74 nm^2 and on the interior surface, 0.61 nm^2 (Chrzeszczyk et al., 1977). In recent years methods have been developed for preparing large "uniform" vesicles (LUV's) which are generally made at solvent-water interfaces (Batzri & Korn, 1973; Barenholz et al., 1979; Saunders et al., 1962; Papahadjopolous & Miller, 1967; Deamer & Bangham, 1976) or by detergent removal methods (Parente & Lentz, 1984; Mimms et al., 1981; Zumbuehl & Weder, 1981; Petri et al., 1980; Enoch & Strittmater, 1979). In a careful review of these procedures (Parente & Lentz, 1984), Parente and Lentz found those made at solvent-water interfaces to be mixed with multilamellar vesicles and not reliably reproducible. They found that cholate-prepared vesicles left significant amounts of cholate as contaminant in the preparation, and octylglucoside-prepared vesicles required long dialysis (3 days) in order to produce a uniform preparation of detergent-free large unilamellar vesicles. Special mention should be made of the method

developed by P.R.Cullis and co-workers (Hope et al., 1985; Mayer et al., 1986). It is an ingenious method that uses filtration extrusion with a variety of pore sizes for making vesicles with selected diameters. This method has an advantage over the procedure we have developed which requires anionic lipids, in that any lipid can be used to make the vesicles. However the retention of a large number of small vesicles by the extrusion procedure can be a problem. Vesicle preparations that are not monodisperse LUV's are not appropriate for precise osmotic swelling experiments because the forces are different in different sizes in the same suspension (Barenholz et al., 1979).

In surprising contrast to attempts to make LUV's with low polydispersity, several vesicle preparations derived from biological membranes are uniform in size. The preparations of mitochondrial inner membrane vesicles (submitochondrial particles) (Penefsky et al., 1960) or bacterial membrane vesicles (Kabalak, 1968) turned out in later work to be relatively uniform in size. Although submitochondrial particles are uniformly 100 nm, the latter preparation contained size ranging from 500 to 1500 nm in diameter. Both are inside-out in orientation of the bilayer with respect to the biological system. Prokaryote cells are approximately 2 microns in length whereas eukaryote cells are 20-30 microns. The properties (including size) of cell-derived vesicles are presumably dominated by the proteins in the membrane since each preparation has its unique size range and the isolated lipids do not spontaneously form the same size vesicles. Uniform size is important for many physical studies and transport studies and innumerable attempts have been made to prepare monodisperse vesicles in the size range obtained from biological

sources.

We have developed a simple, quick procedure for making uniform preparations of unilamellar vesicles by a pH adjustment method. Its origins can be traced to the method developed by Hauser and Gains (Hauser & Gains, 1982; Hauser et al., 1983; Gains & Hauser, 1983) for obtaining small unilamellar vesicles. The procedure requires that anionic lipids be at least one component of the vesicle bilayer. In addition to producing a narrow standard deviation in size, the method allows the size of the vesicles to be selected by varying the composition, the structure of the component lipids, the ionic strength of the medium and the conditions used for making the vesicles.

Vesicles of pure phospholipids have been used as models for an understanding of the properties of biological membranes since the early experiments of A. D. Bangham and his coworkers who showed that synthetic phospholipids could form bilayers spontaneously (Bangham et al., 1965; Bangham, 1968, 1972) with osmotic properties (Bangham et al., 1967). Most of this work has been conducted on multilayered liposomes which are a complicated system for studying osmotic swelling as has recently been shown by comparing the osmotic properties of standard preparations of open-ended rolls to multilamellar closed concentric spheres made by a new preparation procedure (Gruner et al., 1985b). Very recently osmotic studies have been conducted on unilamellar vesicles including giant vesicles (10 μm) (Kwok & Evans, 1981) of DOPC, sonicated (SUV) vesicles (40-60 nm) (Milon et al., 1986; Sun et al., 1986) of DMPC and on large (LUV) vesicles of DOPC or DMPC (160-180 nm) (Hantz et al., 1986).

The significance of osmotic pressure effects on membranes can hardly be understated since ion transport is central to bioenergetics. Most eukaryotic cells are 10 μm in diameter and the organelles in which the energy is transduced are about 200-600 nm in size. Osmotic stress on membranes has also been of practical interest in the cryobiology of plants (Steponkus & Wiest, 1978) and the minimization of hemolysis after the storage of frozen erythrocytes (Takahashi et al., 1986).

Osmotic swelling of biological membranes has been a subject of investigation since Leeuwenhoek recognized the erythrocyte shape in 1675. The literature on erythrocyte swelling is voluminous [for a recent review see Mohandas et al., (1983)]. As Mohandas et al. state, "most of deformation occurring in vivo, as well as most observed in vitro, involve no increase in surface area. The normal cell can undergo large linear extension of up to 230% of its original dimension, but an increase of even 3% or or 4% in surface area results in lysis." A subtle distinction that comes forward in this summary of the elasticity of the erythrocyte membrane is that the observation on erythrocyte swelling are by and large observations on the cytoskeleton of the erythrocyte. The 3 to 4% area increase appears only after this spectrin network has been disrupted so that it is no longer in intimate contact with the lipid bilayer. This distinction of bilayer's expansion as a material as opposed to increased erythrocyte volume was experimentally sorted out by Evans et al. (Kwok & Evans, 1981; Evans & Waugh, 1980; Evans et al., 1976). Bilayer tension has been studied in other biological membranes including sea urchin eggs (Cole, 1932; Michison & Swann, 1954), giant algal cells (Kozuge & Ta-

zawa, 1979), neutrophils (Sung & Chien, 1978; Sung et al., 1982) and isolated rye protoplasts (Wolfe & Steponkus, 1981; 1983).

The capacity of bacteria to deal with osmotic swelling has also come to the attention of biochemists with the recent identification of at least three operons in *E. coli* that are specifically regulated by osmotic changes in the culture media: the *kdp* operon which responds to osmolarity changes by altering the potassium ion concentration in the cell (Laimins et al., 1981); the *omp* operon which, among other responses regulates the size of the pore formed by the protein, porin, in response to changes in osmolarity (Comeau et al., 1985); and the *mdo* operon which initiates the biosynthesis of MOD (membrane-derived oligosaccharides), which are polyanionic oligoglucosides that regulate osmolarity in the periplasmic space protecting the inner membrane (Weissborn & Kennedy, 1984). Several bacteria that are halotolerant respond to an increase of the salinity of the culture medium by increasing the anionic component of their lipid composition. This includes both halophilic gram negative bacteria (Ohno et al., 1976; Ohno et al., 1979; Kogut & Russell, 1984; Hanna et al., 1984) and halotolerant gram-positive bacteria (Kanemasa et al., 1972; Komaratat & Kates, 1975; Miller, 1985). A well investigated eukaryote, *Dunaliella salina*, has a unique osmoregulatory system that allows it to survive in a wide range of salinities by synthesizing glycerol in response to increased salt in the culture medium (Ben-Amotz & Avron, 1983) so that the glycerol content may exceed 50% of the total dry weight of the organism.

Membrane elasticity and curvature are both features of the response of a bilayer to osmotic stress and information about both may be obtained from

studies on osmotic swelling. Several groups have begun in recent years to develop a picture of membrane elasticity and curvature. A number of reviews and theories from different points of view are now available (Helfrich, 1973, 1974; Scheetz & Singer, 1974; Deuling & Helfrich, 1976; Israelachvili et al., 1976; Petrov et al., 1978; Evans & Hochmuth, 1978; Evans & Skalak, 1979; Haines, 1979; Doniach, 1979; Israelachvili et al., 1980; Dimitrov, 1981; Gruner et al., 1985a). Most of reviews and theories focus fundamental features of the biological membrane as a material sheet. Osmotic swelling of sized unilamellar vesicles permits the study of both elasticity and curvature. These are related in turn to the area per lipid molecule in the membrane, which is a fundamental parameter in the statistical mechanical models that have been proposed for bilayers (Nagle, 1973, 1980; Marcelja, 1974; Jacobs et al., 1975; Berde et al., 1980; Scott, 1977; McCammon & Deutch, 1975; Jackson, 1976; Pink, 1982; Kirk et al., 1984; Gruner et al., 1985a). Many of these models can be probed by imposing an enforced increase in the area per molecule by membrane stretching during osmotic stress. Quantitation of the stressed state may permit measurement of the intermolecular forces of lipids and the relationship of lipid structure and those forces.

One would expect, for example, that lipid dynamics would be altered by osmotic swelling. Several studies have been conducted on the effect of osmotic stress on "fluidity" (chain motion) in the bilayer using both fluorescent and esr probes. With two exceptions (Rigaud et al., 1974; Nune, 1981), it was found to increase with an increased osmotic pressure difference across the bilayer (Borochoy and Borochoy, 1979; Surewicz, 1983; Curtain et al., 1983; Barkai et

al., 1983; Takahashi et al., 1986). Araki and Rifkind (1981) have shown that the rate limiting step of hemolysis of erythrocytes correlates with membrane fluidity. The T_m (transition temperature) of the bilayer decreases as both osmotic pressure and external pressure are applied (Barkai et al., 1983). In all of these studies relatively large osmotic gradients (>300 mosM), which we have shown to be well in excess of that necessary to burst vesicles (Li et al., 1986), have been imposed on the bilayers.

Cation transport across biological membranes plays a major role in the handling of energy by cells. The mechanical properties of membranes are presumed to play a significant role in the transport of ions across membranes. Such properties will vary with lipid and protein composition. Membrane elasticity may change when cells undergo carcinogenic transformation, since the lipid, protein and ionic composition of cells change during transformation. Recently, Haines (1979,1982) has proposed a mechanism for the electrical and mechanical coupling of cation transport activities such as transport in mitochondria during oxidative phosphorylation. In this mechanism, the movement of an ion through a cell membrane produces a compaction which in turn produces a standing compaction wave. In order to study the affect of stress developed in the membranes during active cation transport, a knowledge of membrane elasticity is required (Parsegian, 1975).

Many experiments on ion transport and bioenergetics have been conducted on closed unilamellar vesicles derived from biological sources or synthetic phospholipids. Ion transport in such vesicles necessarily results in osmotic swelling (or shrinking) of the vesicles. The stresses placed on a bilayer during

osmotic swelling will presumably disturb the conformation and dynamics of both the transport proteins and the lipids. Deformations of this sort presumably effect the transport rate and other properties of membrane proteins. Furthermore, living cells must experience such deformations during transport, cation (such as calcium or proton) signaling, etc. At present, investigators in this field do not generally consider osmolarity in bioenergetic studies on vesicles primarily because accurate measurements on the deformations and/or the elastic limit of bilayers have not been available.

Two methods have been widely used to measure the elastic constants of bilayer membranes. In the first method, membrane capacitance was measured as a function of voltage impressed across it. This was assumed to produce an electrocompression of the membrane. Crowley (1973) and White (1974) reported a thickness compressibility modulus of 10^6 dyn/cm². It was, however, pointed out by Alvarez and Latorre (1978) that the mechanisms by which a voltage-dependent capacitance could arise in black lipid membranes include: a) formation of new membrane from the surrounding torus region of the membrane, b) exclusion of the solvent from the membrane into the torus which leads to the formation of microlenses, and c) thinning of membrane at a constant volume of the membrane. Only the third mechanism appears to have some relevance since biological membranes do not contain solvents and are not in equilibrium with a torus region. They measured the membrane capacitance in membrane systems in which the solvent flow effects were minimized. In their experiment, a direct measurement of the membrane thickness as a function of electric field indicated a thickness modulus in the order of 10^8

dyn/cm². The second method consists of micropipet aspiration of a small portion of a membrane and measurement of the ensuing change in area. This method has been applied at first to red blood cell membranes (Rand, 1964) and more recently to PC vesicles (Kwok & Evans, 1981). The membrane modulus measured by this method was found to vary through the range 7.3×10^6 to 2×10^9 dyn/cm². In their review article, Evans and Hochmuth (1975) concluded that the best experiments involving micropipet aspiration yield a thickness compressibility modulus of about 4.5×10^8 dyn/cm² for a 10-nm thick membrane. In this method, a fairly large deformation is required to obtain good measurements.

Attempts to study the elasticity of a model bilayer by the osmotic swelling of unilamellar vesicles in the size range of organelles or whole cells (prokaryote) have been frustrated by two problems. The first is that most vesicle preparations, even when they appear to be uniform in size, have sufficient size variation (standard deviation of size) that will not permit elasticity measurements during osmotic swelling that are useful. Furthermore, preparations of unilamellar vesicles having a size uniformity that might be considered appropriate for such studies are obtained by sonication according to the method of Huang (1969). Such vesicles are curvature limited (Chrzeszczyck et al., 1977; Huang & Mason, 1978). Since curvature fixes their size and is the presumed basis for their uniformity, it is not possible to use these vesicles for osmotic swelling measurements of the membrane modulus.

Secondly, a method for obtaining precise and rapid measurements of vesicle size to an accuracy of within a few Angstroms has not been available.

Photon correlation spectroscopy (PCS) is a laser light scattering technique capable of measuring the size and shape of particles in solution with high accuracy (Cummins & Pike, 1973; Berne & Pecora, 1976; Chu, 1974). It has been applied by several investigators to the determination of the size and size distribution of both membrane and phospholipid vesicles (Dahneke, 1983; Goll, et al., 1982; Sornette & Ostrowsky, 1981; Norisuye & Yu, 1977). Taking advantage of PCS and the method that allowed for the preparation of highly uniform LUV with sizes between 200- and 800-nm diameters, we have studied osmotic swelling of phospholipid vesicles.

The significance of the impermeability of the inner mitochondrial membrane to protons (Mitchell and Moyle, 1967) has been well established. It was therefore somewhat surprising to discover (Nichols et al., 1980) that phospholipid bilayers leaked proton/hydroxyl with a net permeability coefficient P (10^{-4} cm/s) that was several orders of magnitude greater than that (10^{-12}) of sodium and other metal cations (Hauser et al., 1972) for an equivalent gradient. Since this finding, the proton/hydroxyl flux through lipid bilayers has been the subject of considerable interest (Nichols & Deamer, 1980; Kell & Morris, 1980; Biegel & Gould, 1981; Clement & Gould, 1981; Nozaki & Tanford, 1981; Gutknecht & Walter 1981a; 1981b; Pohl, 1982; Rossignol et al., 1982; Deamer & Nichols, 1983; Elamrani & Blume, 1983; Cafiso & Hubbell, 1983; Krishnamoorthy & Hinkle, 1984; Gutknecht, 1984; O'Shea et al., 1984a; 1984b; Perkins & Cafiso, 1986; Dencher et al., 1986; Grzesiek & Dencher, 1986). The observed P values range from 10^{-3} to 10^{-9} cm/s. This shows that many factors contribute to it. Because of this high flux, it is necessary to

correct the measurements of P/O ratios in mitochondria for the bilayer proton/hydroxyl flux under the experimental conditions used (Hinkle & Yu, 1979; Berry & Hinkle, 1983) in order to obtain accurate ratios.

At present it is not known whether H^+ or OH^- is the permeant species at neutral pH, nor is the mechanism of transport known. The only specific mechanism suggested to date has been that of Nichols & Deamer (1980), who suggested a transmembrane array of water molecules forming a "proton wire". One implication of the suggestion is that the proton flux should correlate with water movement across the bilayer, although water movement across the bilayer could be related to the flux by other specific mechanisms including, for example, bilayer defects (e. g., hexagonal II phase, etc.). Most investigators seem to believe that proton/hydroxyl permeability is associated with water transport and several of the above investigators have interpreted their results as support for the Nichols & Deamer mechanism solely because the flux correlates with water transport (Rossignol et al., 1982; Elamrani & Blume, 1983; Gutknecht, 1984; and Perkins & Cafiso, 1986). Factors established in the above-cited work that affect the proton/hydroxyl permeability of phospholipid vesicles include: (1) small, unilamellar, curvature-limited, sonicated vesicles are 5-10-fold less permeable than large unilamellar vesicles (Perkins and Cafiso, 1986) (This is consistent with the lower permeability of these vesicles to water.) (2) the magnitude of the pH gradient has a large impact on permeability (Deamer & Nichols, 1983; Elamrani & Blume, 1983) (P is rate-limited by counterion diffusion.), (3) movement of uncharged HCl at the nanomolar level (Gutknecht & Walter, 1981a)] below pH 7 and of hydroxyl ion at high pH

(Gutknecht & Walter, 1981b) both contribute to P outside of the neutral range, (4) the flux is directly proportional to membrane "fluidity" as measured by fluorescence polarization using perylene as a probe (Rossignol et al., 1982) (This is not surprising since the probe simply measures the *motion* of the chains and the motion would be expected to be in proportion to any transport activity across the bilayer.), and (5) the proton/hydroxyl conductivity in PC bilayers is proportional to the degree of unsaturation of the fatty acid chains (Perkins & Cafiso, 1986). The latter finding was actually predicted by Nichols et al. (1980) on the speculation that proton/hydroxyl permeability may be related to water permeability in bilayers which should have its correspondence to water solubility in bulk hydrocarbons. They pointed out that Black et al. (1948) had found water to be more soluble in hydrocarbons containing double bonds.

Cholesterol is a well-known inhibitor of permeability of both ions and neutral molecules (Demel et al., 1972; Papahadjopoulos et al., 1972; Clejan et al., 1979; Hermetter & Paltauf, 1981). It inhibits the permeability of water (Cass & Finkelstein, 1967; Finkelstein & Cass, 1967) which was the first permeability inhibition to be reported. Its effect on P had been checked by Nichols & Deamer (1980) in one experiment and found to have a slight inhibitory effect in PC vesicles.

Grzesiek & Dencher (1986) have developed a method for measuring the buffering capacity of acidic phospholipids via the P using the established linear relation between pH-relaxation rates and the buffering power of the internal acidic headgroups. This method was specifically developed to test the theory

(Haines, 1983) that acidic lipids trap protons thereby creating a proton pool in the headgroup domain of proton pumping membranes. Their method utilizes sonicated (15-30 nm) vesicles which have a high surface-to-internal-volume ratio. Because their vesicles are sonicated and very small compared to those used for studying proton transport proteins, and because sonicated vesicles have reduced permeability compared with LUV's, they have less value for workers studying proton transport in reconstituted systems.

While developing an assay for an H^+/Na^+ exchange protein in membrane vesicles derived from the halotolerant alga, *Dunaliella salina*, (Katz et al., 1986) we sought a model membrane system that would minimally leak protons under the assay conditions. A comparison of the leakage of sonicated PC vesicles with those derived from the cells (Sheffer et al., 1986) showed that the PC vesicles displayed a substantially higher P. We have used pH-adjustment procedure to make vesicles of acidic lipids. This method permits knowledge of the vesicle size with some precision while it eliminates impurities such as organic solvents and detergents. In order to aid workers in the selection of lipids for model vesicle preparation when studying transport and at the same time seek insight into the features of natural membranes that reduce proton/hydroxyl permeability, we have explored the effects of various phospholipid headgroups, phytanyl chains, and the mole fraction of cholesterol and detergents on proton/hydroxyl permeability.

Dynamic light scattering provides a rapid and accurate technique for determining the diffusion coefficients of vesicles in solution (Goll et al., 1982; Pownall et al., 1978; Sut et al., 1978; Chen et al., 1977). From diffusion

coefficients, one can easily determine the vesicle size. PCS measures the digital correlation function of photoelectric pulses. As a digital technique it is well suited to computer analysis. The techniques of dynamic light scattering have been extensively discussed in the literature (Cummins & Pike, 1973).

MATERIALS

DOPA, DOPG, DOPE, DOPS, DOPC, bovine liver PI, *E. coli* PE and *E. coli* lipids were procured from Avanti Polar Lipids, Inc. (Birmingham, AL). They were checked for purity by TLC on Silica gel 60 plates (EM Laboratories, Elmsford, NY) using $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (65:35:5 v/v/v) as a solvent system. Ultrapure sucrose was purchased from Schwarz/Mann (Orangeburg, NY). Cholesterol (98% pure) was procured from Aldrich Chemical Company, Inc. (Milwaukee, WI). Lanosterol was obtained from Mann Research Laboratories, Inc. (New York, NY). 5α -Cholestan- 3α -ol (epicholestanol) was purchased from Research Plus Laboratories, Inc. (Denville, NJ). 5β -Cholestan- 3α -ol (epicoprostanol) was from Steraloids, Inc. (Wilton, NH). The purity of these four sterols was checked by TLC on Silica gel 60 plates (EM Laboratories, Elmsford, NY) using hexane/ether/acetic acid (80:20:1 v/v/v) as a solvent system. Pentaerythritol, trehalose and acridine orange were purchased from Sigma (St. Louis, MO). D Φ GLS, D Φ PGP, D Φ PG and D Φ PGS were isolated from *Halobacterium cutirubrum* by Ms. Laura Stewart in Dr. Kates' laboratory, Ottawa, Canada, using the procedure described previously (Kates, 1978). Dodecanesulfonic acid (sodium salt) was purchased from Aldrich (Milwaukee, WI). Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Vitamin B₁₂ was procured from Sigma (St. Louis, MO). Formvar solution (0.5 %) in ethylene dichloride was obtained from Ladd Research Industries, Inc. (Burlington, VT). The uncoated copper grids were procured from Polaron Equipment Ltd. (Watford, England). Cholic acid was obtained from E. Merck Darmstadt (Germany), and octyl- β -D-glucopyranoside was obtained from Calbiochem (La

Jolla, CA). The submitochondria particles derived from the inner membrane of bovine heart mitochondria were kindly provided by Dr. H. S. Penefsky. All chemicals were reagent grade unless otherwise indicated. All solvents were redistilled before use.

EXPERIMENTAL

Preparation of vesicles

The pH adjustment procedure for making vesicles is as follows. The phospholipid (5-10 mg) was dissolved in 2 ml of chloroform. In order to remove sodium (or ammonium) ions, this solution was washed 4 times with 2 ml of chloroform/methanol/0.2 M HCl (3:48:47 v/v/v). The upper phase was discarded each time. The resulting lower phase was washed with a 2 ml mixture of chloroform/methanol/ water (3:48:47 v/v/v). When vesicles were prepared from lipid mixtures, the lipid solutions were mixed in solvent at this point. The lower phase was then rotary evaporated in a round bottom flask to form a phospholipid film. The phospholipid film was dried under a water aspirator at room temperature for about 10 min. The dried film (total sample of 5-10 mg) was then dispersed in 3.0 ml of the desired medium for vesicle formation. The mixture was stirred with a stirring bar for 30 min at room temperature in order to suspend the lipid film in the solution. This suspension was titrated with 0.1 N NaOH to a pH of 7.55-11.0 and the pH then was immediately adjusted to 7.55 with 0.1 N HCl.

The lipid concentration in the vesicle suspension was about 1.5 mg/ml. This suspension (3.0 ml) was diluted with the same solution as the vesicles were made in to obtain a total volume of 15.0 ml. In order to see the effect of vesicle size on the elastic properties of bilayer, larger vesicles were required. Larger vesicles could be made by a slight change of the procedure. When the phospholipid suspension was brought to a high pH in the pH adjustment procedure, it was allowed to stay at the high pH for up to 3 min; as a result of

which larger vesicles were formed. The entire experiment was conducted at $20 \pm 0.2^\circ\text{C}$. In order to check for the presence of any phase transition in the membrane, we studied the vesicle sizes between $15 - 25^\circ\text{C}$ without conducting any dilution.

Sonicated vesicles were made by adjusting the pH of the crude lipid suspension which was obtained by adding the desired medium to a dried lipid film to pH 7.55 with 0.1 N NaOH. The resulting suspension was then sonicated with a Branson sonicator at a sonication power of 50 Watts.

Characterization of the Vesicles

(1) *Electron microscopy.* Vesicles were studied with a Philips 300 EM at 60 kV. Formvar coated grids were prepared with a 0.5% formvar solution on uncoated grids. A drop of solution containing phospholipid vesicles was placed on the Formvar grid. The excess solution was adsorbed with a No. 1 filter paper after 15 sec. A drop of uranyl acetate solution (5%) was placed on the grid. After 5 sec, the excess was absorbed with filter paper. The grid was then allowed to dry in air for 10 min prior to examination with the EM.

(2) *Sepharose Column Extrusion.* The original vesicle (1.5 mg lipids/ml) suspension in the buffer solution (1.0 ml) was loaded onto the Sepharose 4B column (47 x 0.9 cm). Fractions (0.3 ml) were collected at an elution rate of 18-20 ml/h at room temperature using the same medium as that in which the vesicles were made. The void volume was 12 ml and the total volume was about 33 ml.

(3) *Trapped Volume Determination.* The trapped volume of the vesicles

was determined by preparing the vesicles in the presence of vitamin B₁₂ (7.0 x 10⁻³ M in 0.15 M KCl solution). External vitamin B₁₂ was removed by passage over a Sepharose 4B column. The total trapped marker was determined on a spectrophotometer at 361 nm ($\epsilon = 2.81 \times 10^4$). The internal volume was calculated from the amount of trapped vitamin B₁₂, its concentration in the initial medium and the amount of phospholipid. The latter was assayed by the Ames and Dubin procedure (1960). A DOPA molecule was assumed to occupy 60 square Angstroms on both the inner and outer surface of the bilayer.

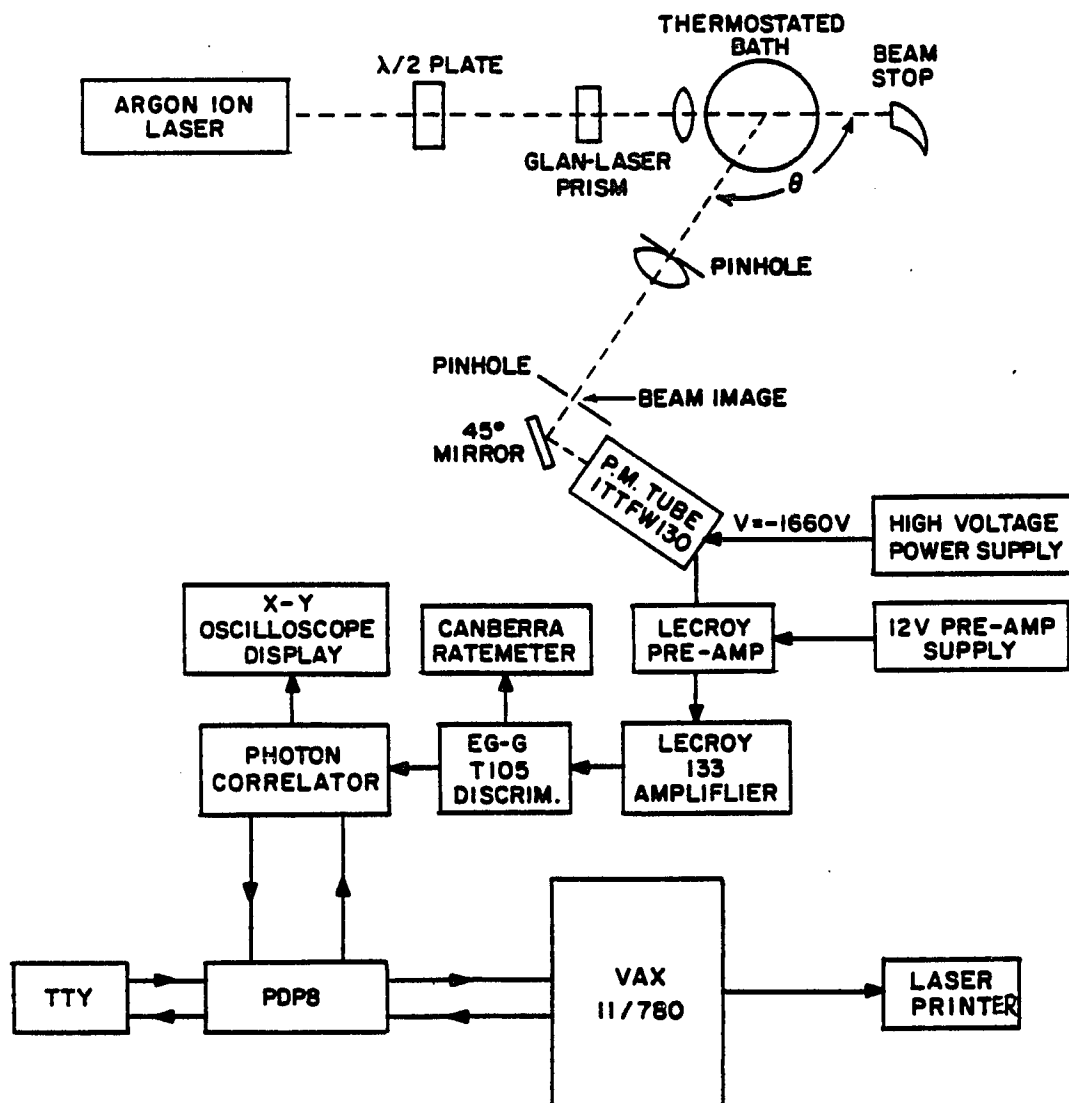
(4) *Photon Correlation Spectroscopy (PCS)*. The size of the vesicles was determined by using photon correlation spectroscopy. The apparatus used was as described by Hwang and Cummins (1982) and illustrated in Figure 1. Light from an argon ion laser at 488 nm was focused onto the vesicle sample in a glass cuvette maintained at constant temperature (20+/-0.2°C) by a Lauda water circulator. The intensity $I(\tau)$ of the scattered light was detected at 90 degrees to the incident beam with a Hamamatsu (Middlesex, NJ) photomultiplier tube (PMT).

The radius of the vesicles in a sample is calculated from the intensity correlation function $C(\tau) = \langle I(\tau)I(\tau+t) \rangle_\tau$. In PCS, one measures the normalized second order correlation function $g^{(2)}(\tau)$, $g^{(2)}(\tau) = C(\tau) / (\langle I(\tau) \rangle)^2$, where τ is the delay time. For particles undergoing translational Brownian motion, the correlation function decays exponentially with the delay time according to the following expression:

$$g^{(2)}(\tau) = B(1 + A.e^{-\Gamma \cdot \tau})$$

where B is the base line, Γ is the inverse correlation time and the A is a con-

Figure 1. Photon correlation spectroscopy (PCS) experimental set-up.



stant between 0 and 1 determined by the optical system. Γ is given by $2Dq^2$, where D is the translational diffusion coefficient and q is the scattering vector. D is related to the particle radius (R) by the Einstein- Stokes expression:

$$D = kT/6\pi\eta R$$

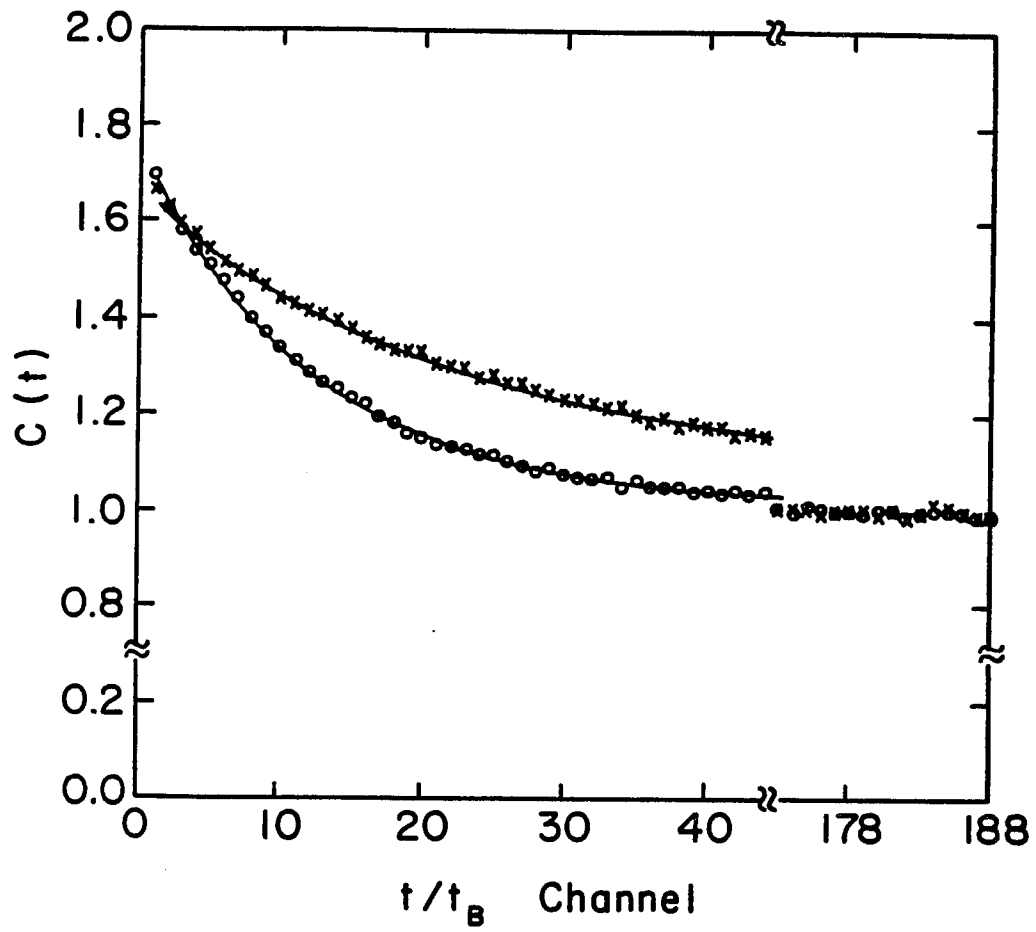
k = Boltzmann's constant, η = viscosity of the solution and T = absolute temperature. By fitting a single exponential through the correlation data, one can determine the diffusion coefficient, D and from this the particle radius (R). A typical set of data showing autocorrelation is displayed in Figure 2. Data for polystyrene spheres and a DOPG vesicle preparation are shown.

If the vesicles in a sample have a distribution of sizes, the correlation function does not exhibit the simple exponential decay indicated above, but is a superposition of exponentials instead. It is then possible to extract the Z-average decay time Γ_o and μ_2 , and the second moment of Γ about Γ_o . By a cumulants analysis technique, the average radius (actually the z-average radius) is found from Γ_o while the ratio μ_2/Γ_o^2 is an index of polydispersity. For vesicles whose size distribution is Gaussian with a mean radius R_o and a standard deviation σ , the fractional width of the distribution is related to the index of polydispersity (μ_2/Γ_o^2) by :

$$\sigma/R_o = x \mu_2/\Gamma_o^2$$

where x is an empirical number between 1 and 4 determined experimentally. Our comparison of electron micrograph measurements of the diameter and standard deviation of uniform polystyrene spheres (Dow Chemical Co., Indianapolis, IN) with the polydispersity obtained from PCS on samples having diameters 85 nm, 126 nm and 557 nm yield $x=4$, 3, and 1, respectively. For

Figure 2. Light scattering intensity autocorrelation data for polystyrene spheres (dia = 126.8 nm) in water (○) and for a DOPG vesicle preparation (dia = 456.3 nm) in 150 mM KCl (×). The solid lines are single exponential fits of the data to $C(t) = B(1 + a e^{-t/t_c})$. The correlation time, t_c , found from the fits is related to the translational diffusion constant, D , by $t_c = 1/2Dq^2$ where q is the scattering vector. There is a 128-channel delay between channels 44 and 172. The background, B , was found from the delayed channels. The bin times, t_b , for the two experiments were 20 μ sec.



DOPA vesicles having diameters 50 nm, and 500 nm the values for x are 1.5 and 3, respectively. Several workers have developed techniques for extracting the complete molecular weight distribution of polydisperse samples from correlation data by sophisticated computer analysis, but these techniques are still controversial. The cumulants method, on the contrary, is generally reliable and has been widely applied (Pike & Cummins, 1977).

The correlation function was accumulated in a 60 channel photon autocorrelator and initially displayed on an oscilloscope. An A/B ratio > 0.5 indicates a good signal to noise ratio. The sampling time was adjusted to obtain exponentially decaying correlation functions which were collected by a DEC PDP-8 minicomputer and subsequently analyzed by the technique of multiple linear regression (cumulants analysis) on a VAX 11/780 mainframe computer (DEC, Maynard, MA).

The average diffusion coefficient and standard deviation were calculated by averaging eleven successive autocorrelation functions, each with the same bin (sampling) time. The count rate was essentially constant in most of the samples which implies that the vesicle sample was not seriously contaminated by dust or aggregates. Occasional samples with widely fluctuating count rates were discarded. In addition to the diffusion coefficient, the analysis yields an index of polydispersity of the sample which was determined by the cumulants analysis method (Pike & Cummins, 1977).

The scattering vector $q = 4\pi n \sin(\theta/2)/\lambda$, where n is the refractive index of the solution, θ is the scattering angle and λ is the vacuum wavelength of the incident light. To determine D and R from the measured Γ , it is thus

necessary to accurately determine n and η . Refractive indices of sucrose and KCl solutions were determined by measuring the angle of minimum deviation at a wavelength of 488 nm. The refractive index was measured at various concentrations and the desired value was evaluated from a linear fit through the data. The viscosity, η , of sucrose and KCl solutions were directly determined with an Ostwald viscometer using water as a standard.

The light scattering apparatus was calibrated with a standard, monodisperse sample of polystyrene latex spheres (diameter=85 nm). Whether or not samples were chromatographed prior to examination by light scattering, they were diluted 10 times to bring the photon count rate within the range of 5000 to 10000 counts per second. They were filtered through Millipore (Bedford, MA) membrane filters with a pore size of 3 μm . Sucrose and KCl Tris buffers were prefiltered through a 0.25 μm pore size filter.

Osmotic Dilution of Vesicle Suspensions

(1) *Osmotic Dilution of Synthetic Vesicle Suspensions.* Initially the vesicle dilution was carried out by two methods. In the first method, 1.0 ml of vesicle suspension was poured into each of a number of cuvettes. Each vesicle suspension was then diluted by adding 1.0 ml of a buffer solution. Thus buffer solutions of different concentration were used for dilution in order to obtain the desired concentration of the vesicle suspension. In this method of dilution, the number of vesicles remained the same for all the cuvettes. In the second method of dilution, 1.0 ml of vesicle suspension was placed in a cuvette and different amounts of the same diluted buffer were added to it (either 0.15

M non-saline solution or 0.086 M KCl solution). In this second method of dilution, the vesicle concentration changed during the process of dilution. In our later work, the second method was adopted since it was more convenient and we did not detect any difference in results obtained by the two methods.

The dilution was carried out very slowly in order to prevent the vesicles from experiencing osmotic shocks. A syringe pump (Razel, Stamford, CT) was employed for conducting slow dilution of vesicle suspensions (0.025 ml/min). The vesicle suspension was continuously stirred with a magnetic stir bar during dilution and was kept in a closed cuvette (with a small hole in the Teflon stopper for the syringe needle) to prevent evaporation of water, which might give rise to size change due to changes in the solution concentration. The diluent was added to the vesicle suspension at the rate of 0.025 ml/min. The vesicle suspension and the buffer solutions were filtered through 1.0 and 0.3 μm pore size filter paper (Nuclepore; Pleasanton, CA), respectively, prior to dilution.

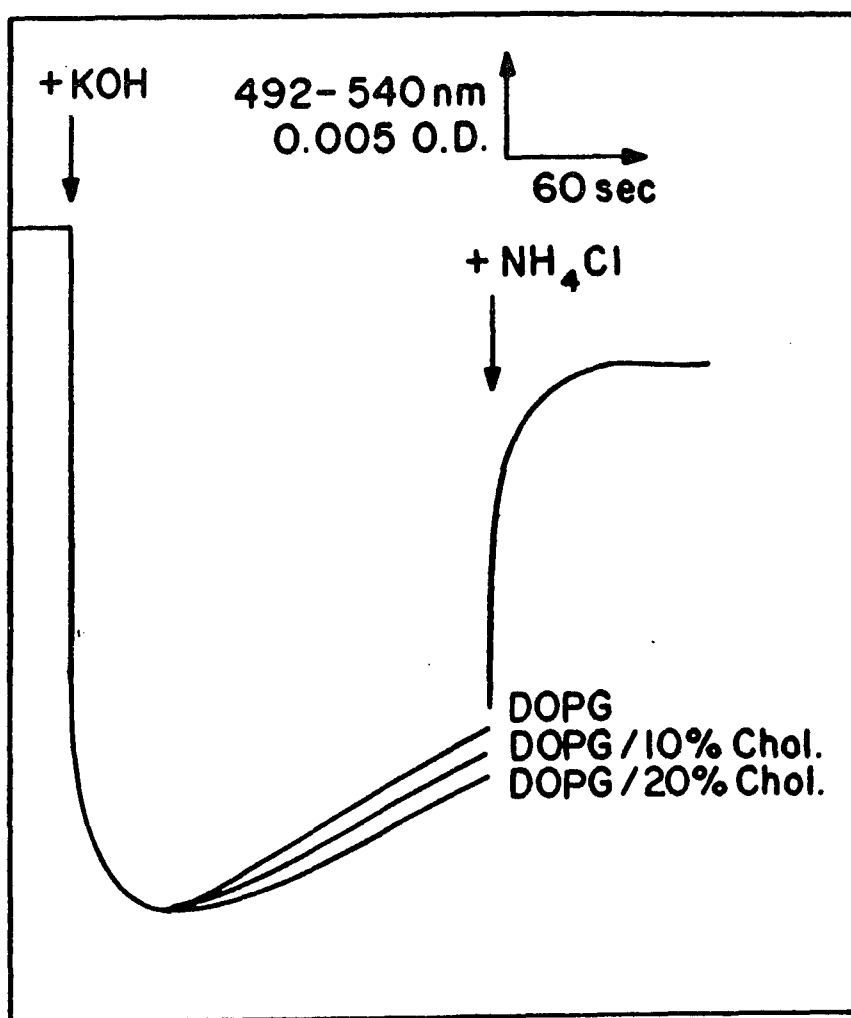
(2) *Osmotic Dilution of Sub-mitochondrial Particles.* The isolation procedure for bovine heart sub-mitochondrial particles derived from the inner mitochondrial membrane is that of Knowles and Penefsky (1972). The original particle suspension in 0.25 M sucrose-Tris buffer (0.16 mg protein/ml, pH 7.5) was diluted 40 times by adding 0.25 M sucrose-Tris (2.0 mM, pH 7.55) buffer, then filtered with 1.0 μm pore size filter paper (Nuclepore; Pleasanton, CA). The dilution procedure was the second one described above. Comparable studies on osmotic swelling in KCl solutions are not possible because the nature of the protein composition of the particles would change on exposure to 0.15 M

KCl (Huang, et al., 1973).

H⁺/OH⁻ Permeability Measurements

Proton uptake by vesicles was assayed by monitoring changes in absorption of the aminoacridine probe, acridine orange (Schuldiner et al., 1972) in an Aminco DW-2C dual wavelength spectrophotometer at 492-540 nm after imposition of a Δ pH (interior acid) across the vesicle bilayer. Initial reaction mixtures contained 150 mM KCl, 10 mM magnesium chloride, 20 mM Tris-succinate, pH 5.2, 2.5 μ M acridine orange, and 10 μ l of vesicles (1.5 mg/ml) in a total volume of 3 ml. Samples were incubated for 10 min at 10°C to allow pH equilibration, and a premeasured amount of unbuffered Tris was added to create a transmembrane pH gradient of 2.5-3.0 units which decayed at varying rates at 10°C ($t_{1/2}$ for decay > 20 min) depending on the preparation. A typical set of proton/hydroxyl permeability assay data is shown in Figure 3. Permeability calculations are essentially those of Nichols & Deamer (1980; see also Cafiso & Hubbell, 1983). Because this work is strictly on large unilamellar vesicles, criticisms of the use of acridine dyes on small vesicles do not apply (Dencher et al., 1986). To check counterion limitation with this pH gradient (Deamer & Nichols, 1983) controls were run on the P of *D. salina* vesicles with and without valinomycin (5 μ M) in KCl (50 mM). No change in the net flow was observed for the valinomycin-exposed vesicles.

Figure 3. H^+/OH^- -permeability of vesicles was assayed by monitoring changes in the absorption of the aminoacridine probe, acridine orange (Schuldiner et al., 1972) at 492-540 nm after imposition of a ΔpH (interior acid) across the vesicle bilayer by adding 0.5 N KOH to a vesicle preparation. Initially the mixtures contained 150 mM KCl, 2.5 μM acridine orange, and 10 μl of vesicles (1.5 mg lipid/ml). Samples were incubated for 10 min at 10⁰C to allow pH equilibration.



CALCULATIONS

The principle of an osmotic swelling experiment is illustrated in Figure 4 and the theoretical model for modulus calculation is shown in Figure 5. Vesicles with initial inner radius a_0 (Figure 5) are prepared in an aqueous solution containing C_i (moles/liter) of solute (sucrose, KCl, etc.). Water is then added to the sample, reducing the final external concentration to C_f , thus producing an osmotic pressure difference across the vesicle wall. Assuming that the vesicle is permeable to water but not to the solute, water will flow into the vesicle which expands until the final internal concentration C_1 is reached with final radius a , at which point the osmotic pressure difference $P_1 - P_2$ is just balanced by the elastic forces produced by the expansion. Since for dilute solutions the osmotic pressure is proportional to the concentration,

$$(P_1 - P_2) = K (C_1 - C_2) = K (C_1 - C_f) \quad (1)$$

where K is the experimentally determined proportionality constant.

Since the total solute content of a vesicle is conserved, the product of concentration and volume is constant. Thus $C_1 a^3 = C_i a_0^3$ or $C_1 = C_i (a^3 / a_0^3)$, and eq (1) becomes

$$(P_1 - P_2) = K \left\{ \frac{C_i}{(a / a_0)^3} - C_f \right\} \quad (2)$$

Two limiting cases can be analyzed immediately. (1) If the membrane is infinitely rigid, the vesicle will not expand, so $a = a_0$ for all C_f . (2) If the membrane is infinitely flexible, the vesicle will expand until the inner and outer pressures are equal, so

Figure 4. Illustration of an osmotic swelling experiment.

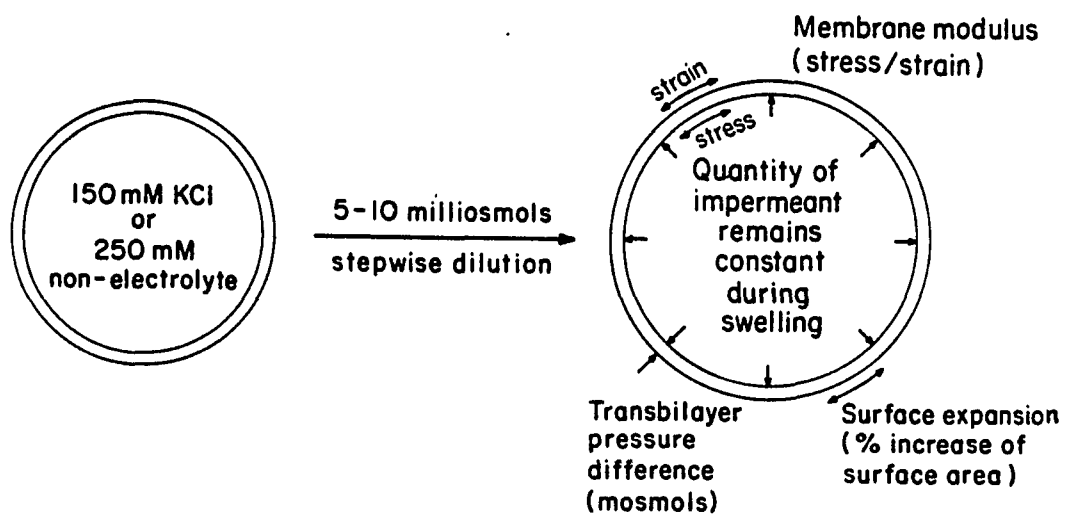
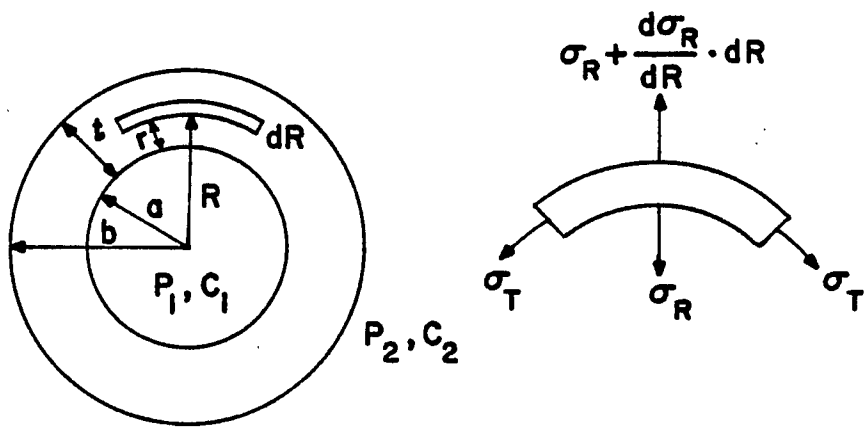


Figure 5. Theoretical model for modulus calculation.



$$a = a_0 \left(\frac{C_i}{C_f} \right)^{1/3} \quad (3)$$

For real vesicles, the final radius a will increase as C_f is decreased by dilution, but not as rapidly as in the limit of equation (3). To compute a as a function of C_f , we need to consider the elastic forces in detail.

In Figure 5, a small segment of the vesicle wall is shown on the right. It is located at a distance r from the inner wall (or $R = a+r$, from the center) and has thickness dR . By symmetry, the segment is subjected to two stresses: a tangential stress σ_T which is the same in any direction in the plane of the membrane, and a radial stress σ_R which varies with r from P_1 at the inner surface to P_2 at the outer surface. The requirement that all such elements be in static equilibrium leads to the result (Timoshenko & Goodier, 1951)

$$\sigma_R = \frac{1}{R^3(a^3 - b^3)} \left\{ P_2 b^3 (R^3 - a^3) + P_1 a^3 (b^3 - R^3) \right\} \quad (4a)$$

$$\sigma_T = \frac{1}{2R^3(a^3 - b^3)} \left\{ P_2 b^3 (2R^3 + a^3) - P_1 a^3 (b^3 + 2R^3) \right\} \quad (4b)$$

For vesicles with thin walls, $b = a+t$, $t \ll a$, and eqs (4) reduce to

$$\sigma_T = \frac{a}{2t} (P_1 - P_2) \quad (5a)$$

$$\sigma_R = \frac{1}{t} \left\{ P_1 (t - r) + P_2 r \right\} \quad (5b)$$

Note that $\sigma_R/\sigma_T \sim t/a$ and σ_R can therefore usually be neglected. Equation 5a can be derived simply by considering a plane bisecting the vesicle. The net force pushing the two hemispheres apart is $\pi a^2 (P_1 - P_2)$. This force is distributed over the intersection of the plane and the wall, an area of $2\pi at$. The tangential stress is therefore

$$\sigma_T = \frac{\pi a^2 (P_1 - P_2)}{2\pi a t} = \frac{a}{2t} (P_1 - P_2)$$

in agreement with eq (5a) (Southwell, 1941).

Here we present the central conclusions obtained by assuming that the membrane is isotropic in its plane, and that the elastic response is linear. The in-plane longitudinal strain e which equals the fractional increase in radius $(a - a_0)/a_0$ is related to the tangential stress σ_T by

$$e = \frac{\sigma_T}{2M_e} \quad (6)$$

where the vesicle elastic modulus M_e is related to the elastic constants C_{ij} by

$$M_e = \left(\frac{1}{2}\right) (C_{11} + C_{12} - \frac{2C_{13}^2}{C_{33}}). \quad (7)$$

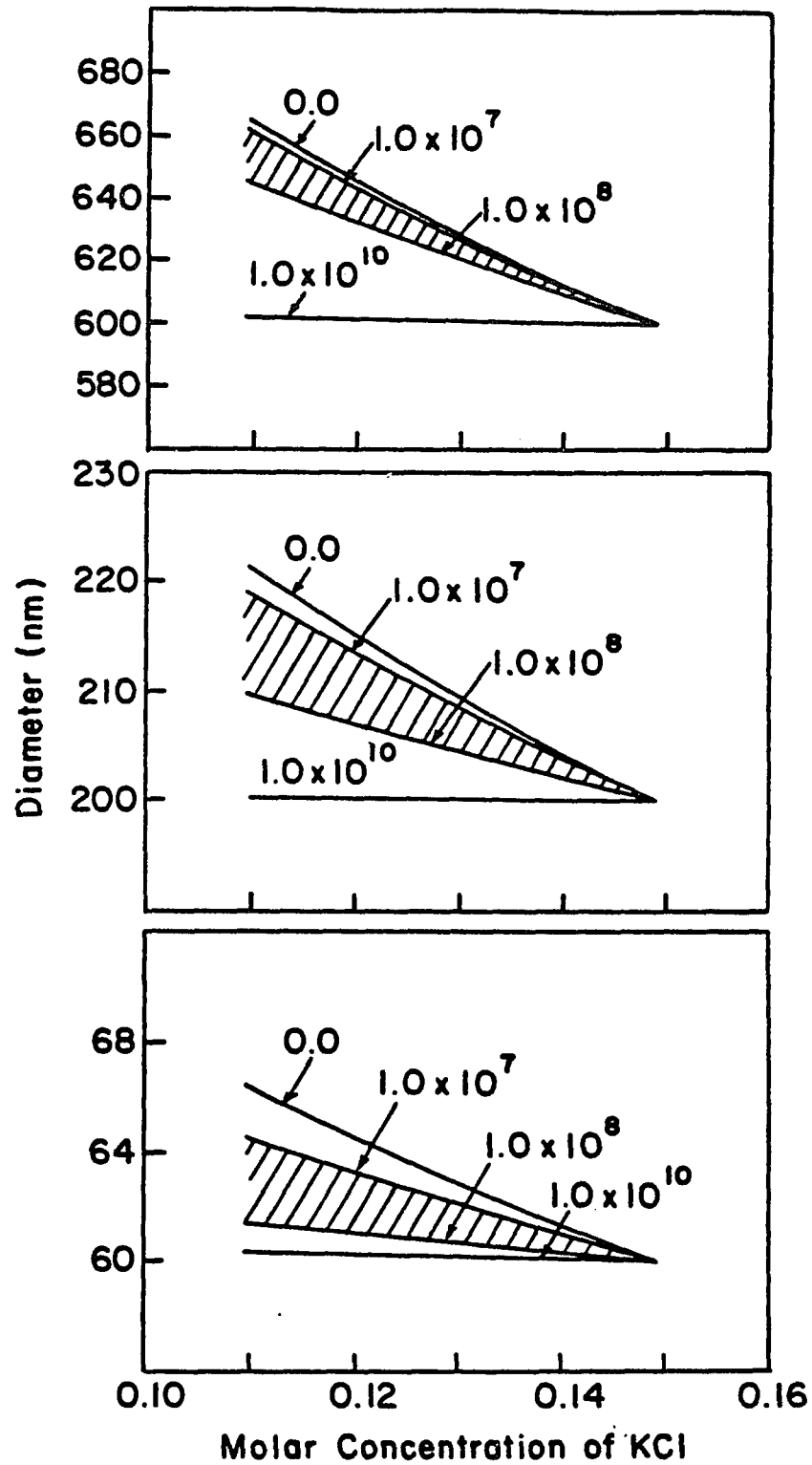
Combining equations (2), (5a) and (6), and replacing the final radius a by R_f , we obtain

$$e(1+e)^3 = \left(\frac{R_f K}{2t}\right) \left(\frac{1}{2M_e}\right) [C_i - C_f (1+e)^3] \quad (8)$$

which can be solved numerically as a function of C_f for different assumed values of M_e , as shown in Figure 6.

Theoretical curves of the variation of vesicle radii with the concentration of the solution are shown in Figure 6. These curves are obtained using eq (8) for different values of the membrane modulus. It is seen from these curves that the percent change in vesicle radii is larger for vesicles which have a large initial radius for a given amount of dilution. The vesicles with radii close to 50 nm are harder to swell. The situation is similar to that of blowing up a balloon, in the sense that it is harder to blow it up when it is small but as the radius increases it becomes comparatively easier to blow it up further.

Figure 6. Theoretical curves for choosing vesicle size in osmotic swelling experiment. The variation of vesicles diameter with the change of concentration of KCl solution (e.g. change of osmotic pressure) is shown. These are calculations from equation (8). The elastic membrane moduli (M_e) are indicated on the curves as dyn/cm².



Vesicles with radii of about 200 nm are seen to be better suited for osmotic swelling studies where the expected vesicle elastic modulus is in the range of 10^8 dyn/cm². For this reason we chose vesicles in this size range for our studies.

The total membrane modulus contains three additional contributions besides the purely elastic part of Eq. (7): one is a surface tension effect due to the increase in surface area (and hence surface energy) as the vesicle swells (Evans & Simon, 1975); the second is a bending term due to the decrease in curvature as the vesicle swells (Evans & Hochmuth, 1978); the third one is double layer contribution due to the diffused layer on the negatively charged vesicle surface. The total membrane modulus M_{total} which replaces M_e in Eq. (7), is then

$$M_{total} = M_e + \frac{\gamma}{2t_o} + \frac{10B}{t_o R_o^2} + \frac{1}{16\pi t_o} \left(\frac{\partial^2 A}{\partial R_o^2} \right) \quad (9)$$

where t_o and R_o are the initial thickness and radius of the vesicle, and A is Helmholtz free energy of diffuse double layer around vesicle surface.

Numerical estimates show that the bending term is $\sim 3 \times 10^5$ dyn/cm², while both the surface tension term and the double layer contribution are $\sim 10^8$ dyn/cm². The values of the observed membrane modulus we found to be in the range of 1×10^7 to 1×10^{10} dyn/cm², so the bending term can be neglected. But the surface tension term and double layer contribution must be retained, and plays a more important role than the elastic term in determining the total swelling of vesicles in response to osmotic pressure changes.

Evans (Evans & Waugh, 1977; Kwok & Evans, 1981) reported an elastic area compressibility modulus, K , for red cell membranes of 450 dyn/cm at 25°C. Their K is defined by $T = K \Delta\alpha$, where $\Delta\alpha$ is the fractional change in

area and T is the membrane isotropic tension. T is related to the in-plane stress σ_T by $T = \sigma_T t$, where t is the thickness of the membrane bilayer. The in-plane strain e_1 is related to the in-plane stress σ_T by $e_1 = \sigma_T / 2M_{\text{total}}$, where M_{total} is the total membrane modulus determined from osmotic swelling experiments. Since $\Delta\alpha = 2e_1$, $\sigma_T = M_{\text{total}}\Delta\alpha$ or $T = t M_{\text{total}}\Delta\alpha$. Therefore our total membrane modulus M_{total} is just Evans' area compressibility modulus K divided by the membrane thickness t . For $t = 3.0$ nm, $K = 450$ dyn/cm corresponds to $M_{\text{total}} = 1.5 \times 10^9$ dyn/cm², slightly larger than the largest values in our experiments (Table X).

RESULTS

Preparation and Characterization of the Vesicles

(1) *Characterization of the Vesicles.* Negative-stained electron micrographs of vesicles made either by sonication or by pH adjustment are shown in Figure 7. The uniformity of the vesicles is apparent in both pictures. Below each micrograph is a distribution of the vesicle sizes as measured from the photomicrographs. The standard deviation of the size is shown above the bar graph. Both the sonicated vesicles and the pH adjusted vesicles are shown to have standard deviations representing approximately 20% of the size. This approach was checked by examining polystyrene spheres of 85 nm, 126 nm, and 557 nm size. We found the same standard deviation as those provided by Dow Chemical Co., who also used electron microscopy to measure the size and determine the standard deviation. The size of the vesicles found in the electron micrographs is smaller than those obtained by light scattering. This difference is presumably due to shrinkage during negative staining.

The pH adjustment method of Hauser and Gains (1982) yielded uniform small unilamellar vesicles of dilauroylphosphatidic acid (DLPA) of about 30 nm diameter. These vesicles were eluted from a Sepharose 4B column as the major peak while some larger vesicles of varying sizes appeared in the void volume.

In our experiments (Figure 8) the pH adjustment vesicles produced from DOPA also yielded 2 peaks. The principal one, however, came off the column near the void volume. The SUV peak was minor. Three methods were used to quantitate the results. First, phosphate analysis (Figure 8) was used to

Figure 7. Negative-stained electron micrographs of vesicles made by sonication (A) and pH adjustment (B). The DOPA vesicles were both made in 0.15 M KCl-Tris buffer. Maximum pH for the vesicles made by pH adjustment was 9.00. Sonication time for the sonicated vesicles was 30 min. Grids were examined on a Philips 300 EM at the magnification 33,000 and 13,000 for A and B respectively. Uranyl acetate solution (5%) was used for negative staining. C and D are vesicle size distributions for A and B respectively. They were the measurements of vesicle size on the electron micrographs. The size of sonicated vesicles on electron micrograph (A) were smaller than those obtained from PCS presumably because the negative staining procedure shrunk the vesicles slightly. The standard deviation (σ) of the vesicle size is shown above the bar graph.

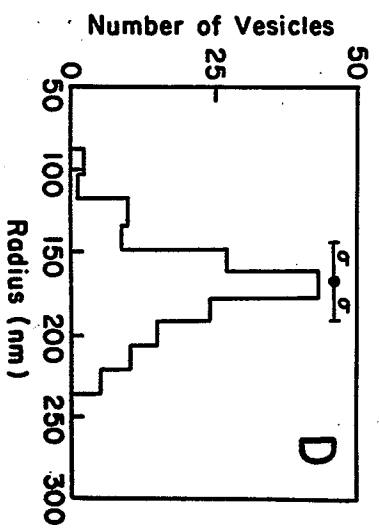
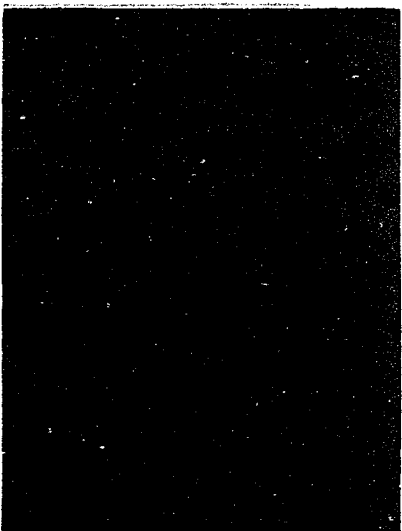
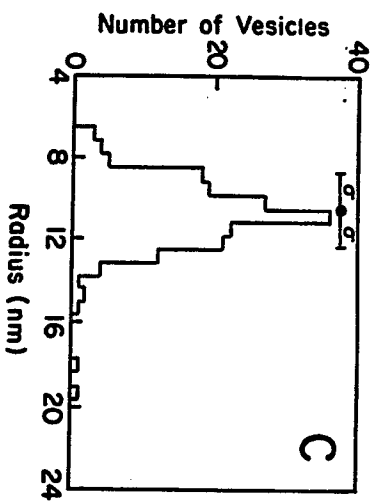
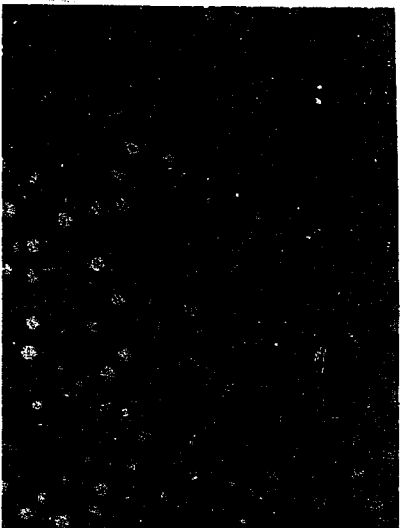
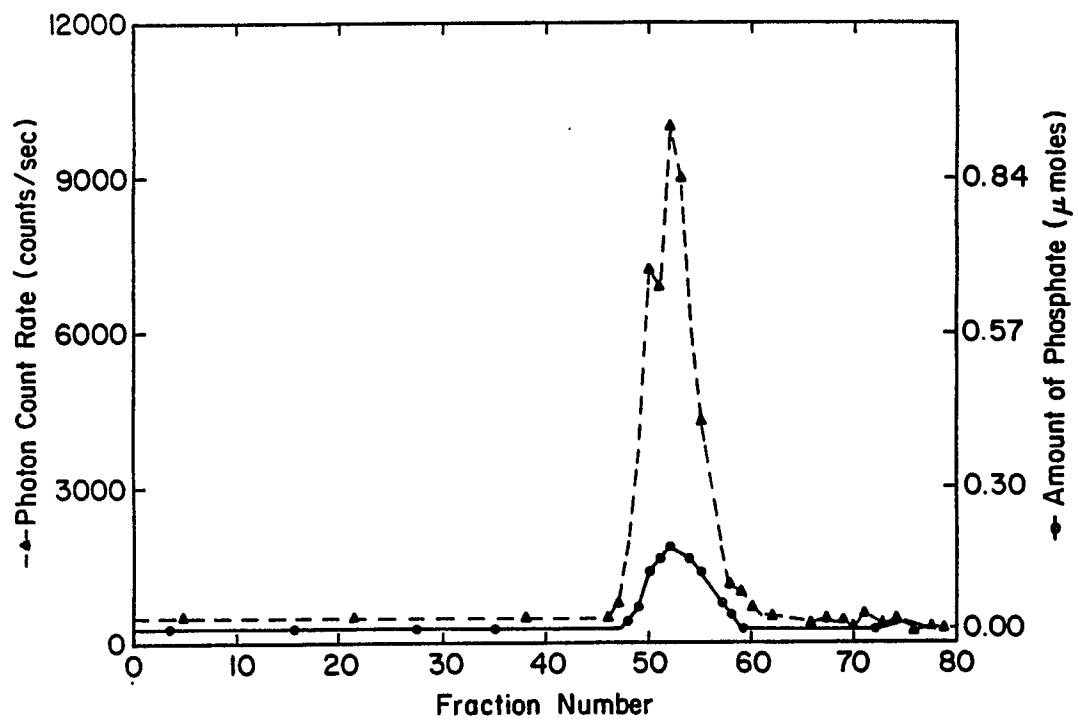


Figure 8. Elution profile of DOPA vesicles from a Sepharose 4B column (47 x 0.9 cm). Phospholipid was assayed by the method of Ames and Dubin (1960) and total counts were measured by PCS. The vesicles were made by the pH adjustment method (maximum pH 9.00) in 0.15 M KCl Tris buffer. The original vesicle suspension in the buffer solution (1.0 ml) was loaded onto the column. Fractions (0.3 ml) were collected at an elution rate of 18-20 ml/h at room temperature. The void volume is approximately at fraction 40, the total volume is off scale at about fraction 110.



determine the amount of phospholipid per fraction (Ames & Dubin, 1960). Second, the total counts per fraction (Figure 8) was measured with PCS. Third, the absorbance of vitamin B₁₂ at 361 nm (not shown) was measured during the entrapped volume determination. All results gave the same pattern (Figure 8), which shows the utility of light scattering for assaying vesicles in column fractions (as well as measuring vesicle radii) since it is so rapid and convenient.

Huang (1969) and others (Hauser & Gains, 1982; Hauser et al., 1983; Gains & Hauser, 1983; Schurtenberger & Hauser, 1984) have found that the passage of phospholipid vesicles through a Sepharose 4B column results in adsorption of phospholipids onto the column. It was suggested that this problem might be avoided by first saturating the column with phospholipids. In order to explore the nature of this adsorption, we examined vesicles that were passed through a column twice. The vesicle size was found to be smaller for vesicles prepared in sucrose after a second passage through the column (data not shown) and the polydispersity was found to increase. This suggested that the adsorbed phospholipids may be derived directly from the vesicles reducing their average diameter. For vesicles prepared in a KCl-Tris buffer, the size did not change on passing the sample twice through the column. This result may also be due to a difference in surface pH between the two preparations.

For internal volume determination, vesicles were prepared in cyanocobalamin (vitamin B₁₂). The sizes of vesicles entrapping cobalamin do not significantly differ from those without cyanocobalamin as measured by PCS. DOPA vesicles prepared in the presence of cyanocobalamin displayed a mean di-

ameter of 208.8 ± 1.4 nm (compared to a control of 220.4 ± 0.6 nm) with an index of polydispersity of 8. The trapped volume determination yielded a mean radius of 231.4 nm which is comparable to that obtained by light scattering. Vesicles containing cyanocobalamin were found to be unstable over several hours.

(2) *Preparation of Uniform Vesicles Comprised of Anionic Lipids.* A variety of phospholipids were examined for their capacity to form uniform, unilamellar vesicles. The results of these experiments are summarized in Tables I-III. Attempts to make vesicles with DOPC alone were unsuccessful.

Table I illustrates that monounsaturated lipids (or in the case of *E. coli* lipids, a mixture of monounsaturated and cyclopropane lipids) that contain anionic headgroups may be used to make vesicles in 200 to 700 nm diameter size range.

In contrast, lipids containing the bulkier phytanyl chains (Table II) yield sizes in the 400 to 1000 nm range. The finding that increased bulk in the hydrocarbon domain of the bilayer results in larger-sized vesicles is consistent with that vesicle size is directly proportional to the mole fraction of sterols in DOPA and DOPG vesicles (Figure 9 & 10). These two observations suggest that the changes in the average distance between the head groups relates to the curvature of the vesicles prepared by this procedure.

Table II shows several features of our procedure that should be pointed out. First, the polydispersity of these vesicles is significantly higher than those of the straight-chain lipids. This means that these vesicles are not uniform. Second, a comparison of the initial pH of D Φ PGP vesicles made in 0.15 M KCl

Table I. Variation of vesicle size and polydispersity with lipid head-group for vesicles made of monounsaturated glycerol lipids. All preparations are in 0.15 M KCl solution. In the preparation obtained *E. coli* lipids/DOPA, the mole ratio of two components was 1:1. PI refers to bovine liver phosphatidyl inositol. All vesicle preparations were readjusted to pH 7.55 after exposure for maximum pH, except that DOPE and *E. coli* PE were adjusted to pH 8.60. The error represents error in measurement and not the standard deviation on the vesicle size.

Lipids	Initial pH	Maximum pH	Vesicle size Diameter (nm)	Index of polydispersity
PI	5.17	10.0	226.3+/-0.9	0.09
DOPS	4.41	8.50	277.8+/-1.2	0.07
DOPA	4.52	10.0	318.1+/-2.0	0.07
<i>E. coli</i> lipids/DOPA	4.49	9.03	445.5+/-2.6	0.10
DOPG	4.10	10.0	456.0+/-2.3	0.11
<i>E. coli</i> lipids	6.94	9.50	517.1+/-3.1	0.09
DOPE	5.94	10.65	544.3+/-2.3	0.06
<i>E. coli</i> PE	5.36	10.05	716.0+/-4.5	0.12

Table II. Variation of vesicle size and polydispersity with lipid head-groups. Vesicle size was measured by PCS. Φ =phytanyl; *Halobacterium* lipids were the whole lipid extract from *Halobacterium cutirubrum*. The error represents error in measurement and not the standard deviation on the vesicle size.

Lipids	Suspension	Initial pH	Maximum pH	Vesicle size Diameter (nm)	Index of polydispersity
<i>Halobacterium</i> lipids	0.15 M KCl	2.73	9.70	440.1+/-3.5	0.12
D Φ PGP	0.15 M KCl	3.60	10.0	631.7+/-3.6	0.13
D Φ PGP	H ₂ O	6.85	6.92	632.6+/-3.3	0.13
D Φ PG	0.15 M KCl	6.55	9.51	749.5+/-4.8	0.11
D Φ GLS	0.15 M KCl	6.04	9.68	759.2+/-4.8	0.17
D Φ PGS	H ₂ O	6.85	6.90	1045+/-6	0.15

Table III. Vesicle preparations with fatty acids. Vesicle size was measured by PCS. In the preparation of oleic acid/oleyl alcohol (oleyl alc.) and $C_{12}\text{-SO}_3/C_{12}\text{-OH}$, the mole ratio of two components was 1:1. The error represents error in measurement and not the standard deviation on the vesicle size.

Fatty acids	Suspension	Initial pH	Maximum pH	Vesicle size Diameter (nm)	Index of polydispersity
Oleic acid	0.15 M KCl	5.84	10.0	383.2+/-1.7	0.07
Oleic acid	0.15 M KCl	5.30	7.00	601.7+/-3.8	0.07
Oleic acid	4.0 M KCl	4.50	7.00	2450+/-20	0.12
Oleic acid	Sea water (30 ppt)	6.29	6.29	1690+/-14	0.12
Oleic acid	Sea water (35 ppt)	6.28	6.28	2800+/-26	0.12
Oleic acid	Sea water (36.5 ppt)	6.48	6.48	1356+/-12	0.11
Oleic acid/oleyl alc.	0.15 M KCl	6.15	7.00	355.0+/-1.5	0.08
$C_{12}\text{-SO}_3/C_{12}\text{-OH}$	0.15 M KCl	6.75	6.75	3000+/-24	0.09

Figure 9. DOPA/cholesterol vesicles in KCl solution. The vesicles were made by the pH adjustment method in 0.15 M KCl-Tris buffer (maximum pH 9.00). The initial pH was 4.50. Chloroform containing the cholesterol was added to the lower phase after acid washing of the disodium salt of DOPA. The acidified phosphatidic acid:cholesterol mixture was dried in vacuo, and the buffer was added to the dried film. The size was measured with PCS. Standard deviation refers to the error in measurement, not the vesicle size.

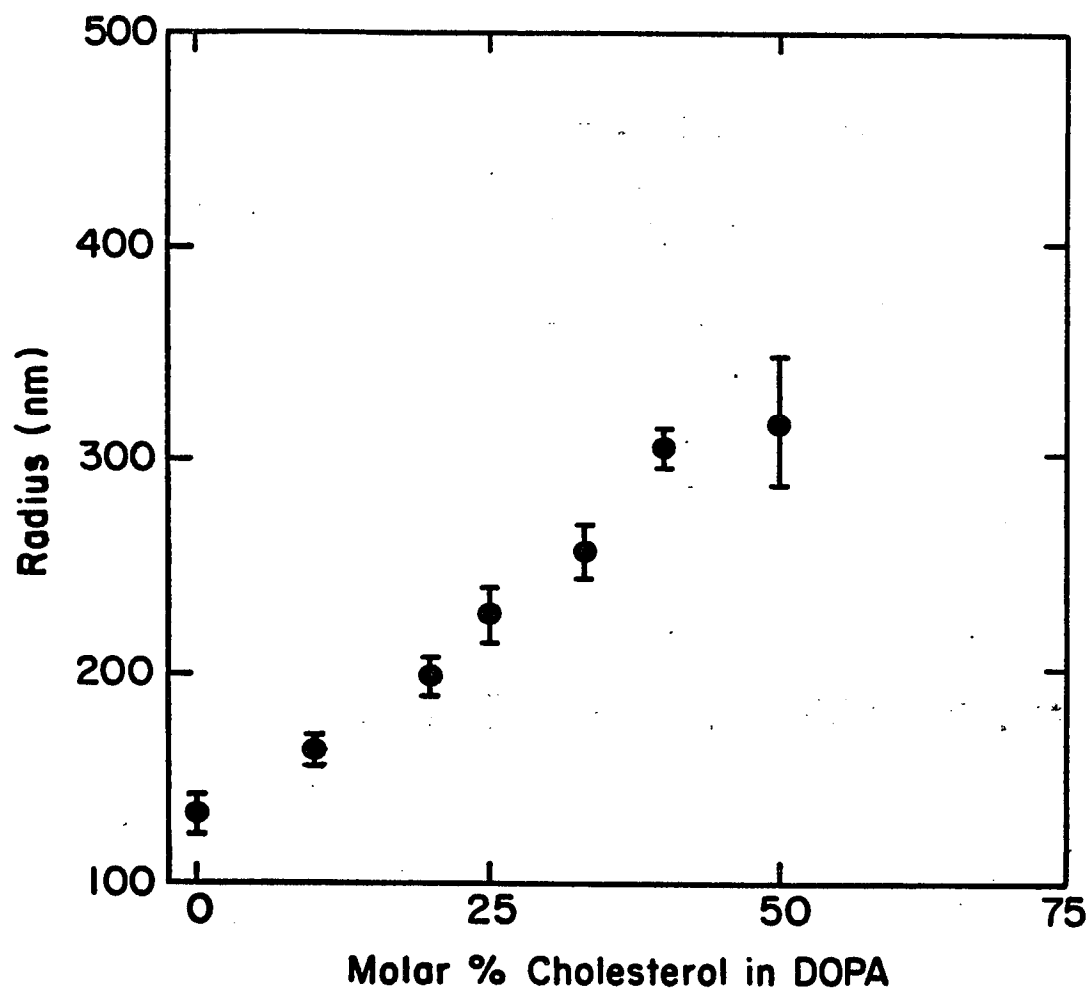
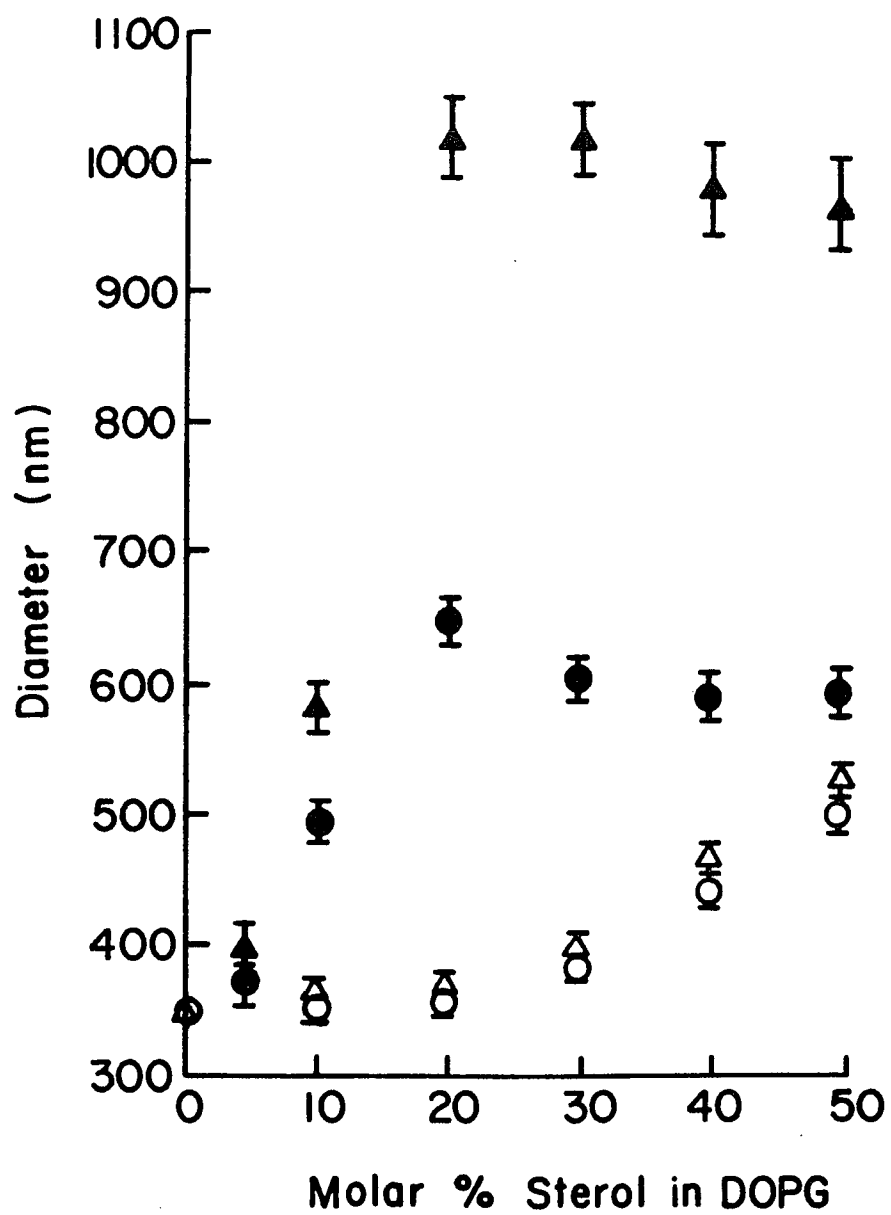


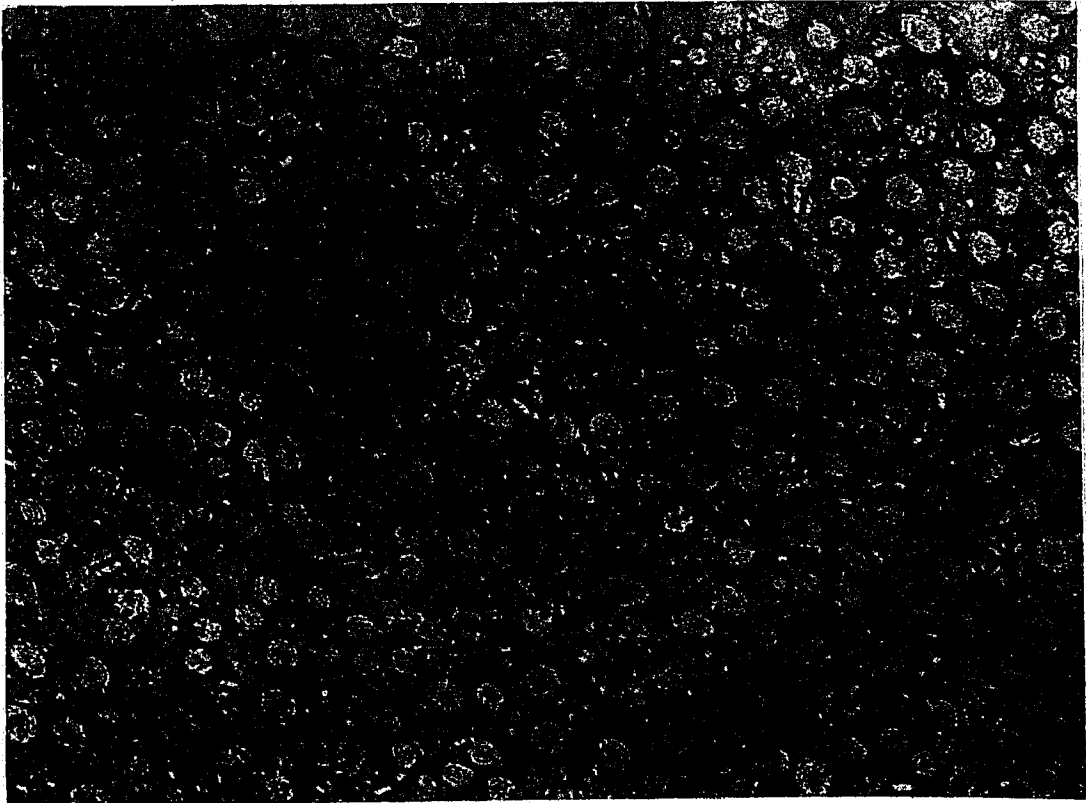
Figure 10. DOPG/sterol vesicles in sucrose solution. The DOPG/sterol vesicles [DOPG/cholesterol (○), DOPG/lanosterol (△), DOPG/epicoprostanol (●) and DOPG/epicholestanol (▲)] were made by the pH adjustment method in 250 mM sucrose-Tris buffer (2.0 mM, pH 7.55). Chloroform containing the sterol was added to the lower phase after acid washing of the sodium salt of DOPG. The acidified DOPG/sterol mixture was dried in vacuo, and the buffer was added to the dried film. The size was measured with PCS. Standard deviation refers to the error in measurement, not the vesicle size.



to those made in H_2O illustrates the extent to which acidic protons are sequestered at the polyanionic membrane surface in water. This does not occur in salt solution. When the lipids are suspended in water (as compared to saline), the surface pH is lower, consequently the bulk-phase pH (initial pH) is higher. Because the headgroup has two phosphates, the protonated form would be expected to yield an unusually low initial pH. A similar statement might be made about D Φ PGS but this substance was only available in limited supply. The possibility of solvolysis of the sulfate esters (Mayers et al., 1969) discouraged a thorough acid wash of this sample.

Table III shows that vesicles can be made with free fatty acids and with dodecyl sulfonic acid/dodecanol (1:1) mixtures. The latter results show a sharp size dependence on the ionic strength and on the maximum pH. The formation of fatty acid vesicles is unique since only fatty acid liposomes (oligolamellar) and not unilamellar vesicles have been obtained from free fatty acids (Gebicki & Hicks, 1976; Hargreaves & Deamer, 1978; Haines, 1983). The formation of unilamellar vesicles from free fatty acids in sea water may have its implications to the origin of the life on prebiotic earth. The vesicles of oleic acid are surprising because they are significantly larger than those of phospholipids and because they are uniform in size. Figure 11 shows a negatively stained electron micrograph of oleic acid unilamellar vesicles made in 0.15 M KCl. The size of the vesicles in the electron micrograph are much smaller than those obtained by PCS (Table III). This difference is presumably due to shrinkage during negative staining. In order to establish that the vesicle preparations of oleic acid are unilamellar, the osmotic properties (membrane

Figure 11. Negatively stained electron micrographs of vesicles made by pH adjustment with oleic acid in 0.15 M KCl. The maximum pH was 10.0. Grids were examined on a Philips 300 EM at a magnification of 55,000. Uranyl acetate solution (5%) was used for negative staining. The vesicle size obtained from EM is smaller than those measured by PCS, possibly because the negative staining procedure shrunk the vesicles.



modulus, maximum surface area increase and transbilayer pressure difference at vesicle bursting) were studied on the vesicle preparations. The data are shown in Figure 21 (see DISCUSSION AND CONCLUSION).

It may be seen in Table III that restricting the maximum pH to 7 produced substantially larger vesicles than were obtained at pH 10. The large value of polydispersity in high salt seems to be a characteristic of the method of vesicle preparation.

(3) *Vesicle Preparations in Media of High Ionic Strength.* The size of DOPG vesicles was found to vary with the salt concentration in which vesicles were made as shown in Table IV. It can be seen that the size increases up to 2.0 M KCl and then remains relatively constant. The very large sizes seen in Table IV are accompanied by a substantial increase in the polydispersity. In the last three of these experiments the protonated phospholipid film are suspended in 0.15 M KCl with or without base (as an alternative to conducting a pH adjustment *after* suspension) are approximately the same size but display a higher polydispersity.

Figure 12 shows the variation of the sizes of the vesicles obtained when DOPA vesicles were prepared in mixtures of 0.25 M sucrose and 0.15 M KCl. It can be seen that the vesicle size increases linearly with salt concentration in the range 0 to 0.15 M KCl.

(4) *Vesicle Preparations in Media of Low Ionic Strength.* Table V shows the preparation of vesicles of DOPA and DOPG in a variety of media with low ionic strength. The preparation of each in 0.15 M KCl is included for comparison. It may be seen that the size of the resultant DOPG vesicles in-

Table IV. DOPG vesicle preparations made in solutions of different salt concentrations. Vesicle size was measured by PCS. For the pH of last three DOPG vesicle preparations, the pH was adjusted by addition of an appropriate concentration of NaOH to the suspending medium of the phospholipid film. No pH adjustment was made after the film was suspended. The vesicle size was measured at that pH. The error represents error in measurement and not the standard deviation on the vesicle size.

Lipids	Suspension	Initial pH	Maximum pH	Vesicle size Diameter (nm)	Index of polydispersity
DOPG	H ₂ O	3.07	7.20	162.4+/-0.8	0.06
DOPG	0.15 M KCl	4.10	10.0	419.7+/-2.8	0.09
DOPG	0.50 M KCl	2.91	9.95	560.0+/-3.2	0.11
DOPG	1.00 M KCl	2.92	9.93	1170+/-7	0.12
DOPG	1.50 M KCl	2.93	9.96	2240+/-16	0.10
DOPG	2.00 M KCl	2.93	9.98	2920+/-19	0.18
DOPG	3.00 M KCl	2.73	9.94	3100+/-23	0.20
DOPG	4.00 M KCl	2.73	10.15	2990+/-23	0.16

Figure 12. DOPA vesicle size varies with the volume fraction of 0.15 M KCl in 0.25 M sucrose, this mixture was used as a suspension medium during the synthesis of vesicles. Vesicles were prepared using the pH adjustment method. Vesicle diameters were obtained by using PCS.

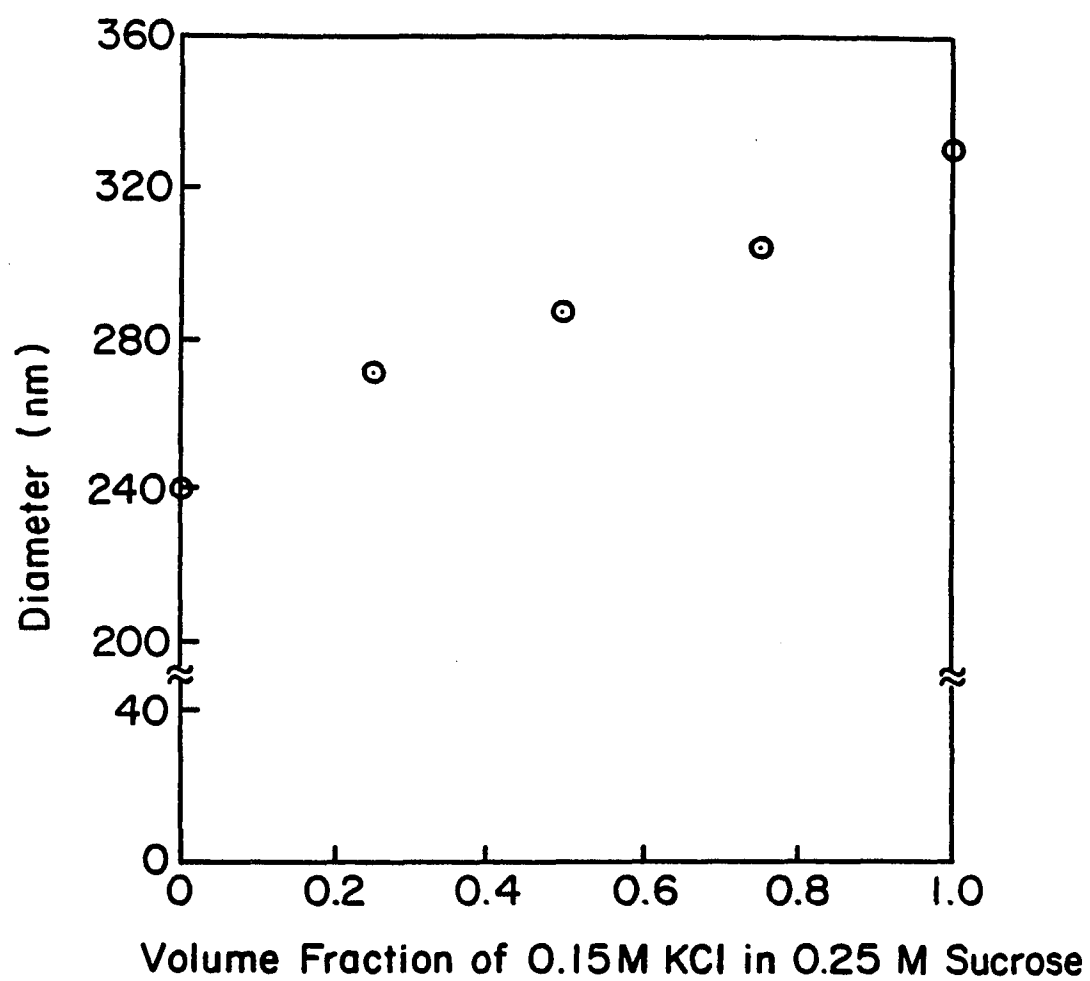


Table V. Vesicle preparations in low ionic strength media. Vesicle size was measured by PCS. The preparations in KCl/sucrose means the media used for making vesicles was 1:1 volume ratio mixture of 0.15 M KCl and 0.25 M sucrose solution. The error represents error in measurement and not the standard deviation on the vesicle size.

Lipids	Suspension	Initial pH	Maximum pH	Vesicle size Diameter (nm)	Index of polydispersity
DOPA	0.25 M pentaerythritol	5.10	10.1	177.7+/-0.6	0.08
DOPA	0.25 M sucrose	4.80	10.0	231.3+/-1.0	0.08
DOPA	0.25 M trehalose	5.52	10.1	290.7+/-1.6	0.09
DOPA	KCl/sucrose	4.59	10.1	291.4+/-2.0	0.09
DOPA	0.15 M KCl	4.82	9.85	325.5+/-1.9	0.08
DOPA	0.05 M KP_i	7.37	7.55	535.8+/-3.4	0.12
DOPG	H ₂ O	3.07	7.20	162.3+/-0.8	0.06
DOPG	0.25 M pentaerythritol	4.90	10.02	202.4+/-0.9	0.07
DOPG	0.25 M trehalose	5.32	9.96	268.2+/-1.7	0.08
DOPG	0.25 M sucrose	4.94	10.0	335.6+/-1.4	0.10
DOPG	KCl/sucrose	4.38	9.90	390.5+/-2.5	0.11
DOPG	0.15 M KCl	4.10	10.0	419.6+/-2.8	0.10
DOPG	0.05 M KP_i	7.30	7.50	536.9+/-3.2	0.12

creases in the following order of the suspending medium: water, pentaerythritol, trehalose, sucrose, KCl, potassium phosphate. A similar comparison for DOPA vesicle sizes yields essentially the same progression except that the effects of trehalose and sucrose are reversed. These data show that there is no special relationship between the phospholipid headgroups and the sugars in dilute solution in this system. Such a relationship has been suggested in the literature (Crowe & Crowe, 1984).

(5) *Vesicles Prepared with Lipid Mixtures Containing Anionic Lipid.*

Table VI shows the sizes of vesicles made of mixtures of DOPG and DOPC in 0.15 M KCl and in 0.25 M pentaerythritol. The studies show that the vesicle size increases as the mole fraction of DOPC increases. This statement applies to vesicles made in both media, although vesicles made in 0.15 M KCl were larger and displayed greater polydispersity.

Membrane Elastic Property Measurements using Osmotic Swelling

Several preliminary experiments were conducted to establish the parameters of the vesicle preparation. The number of vesicles in a given sample was calculated from the amount of phospholipid in the sample which could be obtained either by phosphate analysis or from the total counts per fraction measured on PCS and the vesicle radii which were determined from light scattering. It was found to be about 10^{12} /ml in both cases. The two methods of dilution (slow dilution with a low gradient and more rapid dilution with a high gradient) yielded the same results for the diffusion coefficient (and hence the vesicle radius) of a given sample. Since the two methods yield different con-

Table VI. DOPG/DOPC vesicle preparations in KCl and pentaerythritol (penta.). Vesicle size was measured by PCS. The error represents error in measurement and not the standard deviation on the vesicle size.

DOPC in DOPG (molar %)	Suspension	Initial pH	Maximum pH	Vesicle size Diameter (nm)	Index of polydispersity
0	0.15 M KCl	4.10	10.0	456.4+/-2.3	0.11
15	0.15 M KCl	4.80	10.05	500.7+/-1.8	0.12
30	0.15 M KCl	4.84	10.05	560.3+/-2.4	0.13
50	0.15 M KCl	5.15	10.03	624.7+/-3.2	0.13
75	0.15 M KCl	6.15	10.06	808.2+/-3.7	0.16
0	0.25 M Penta.	4.90	10.02	199.5+/-0.7	0.07
15	0.25 M Penta.	5.00	10.0	218.9+/-0.9	0.07
30	0.25 M Penta.	5.02	10.25	245.6+/-1.1	0.08
50	0.25 M Penta.	6.10	10.0	280.4+/-1.4	0.08
75	0.25 M Penta.	6.15	10.08	454.7+/-1.7	0.11

centrations of vesicles, this indicates that the diffusion coefficient does not depend on the vesicle concentration in the concentration range at which this work was carried out. The vesicle radii did not change with temperature over the range 15 - 25°C confirming the absence of any gel to liquid crystal phase transition (Evans & Kwok, 1982) at the temperature this work was conducted.

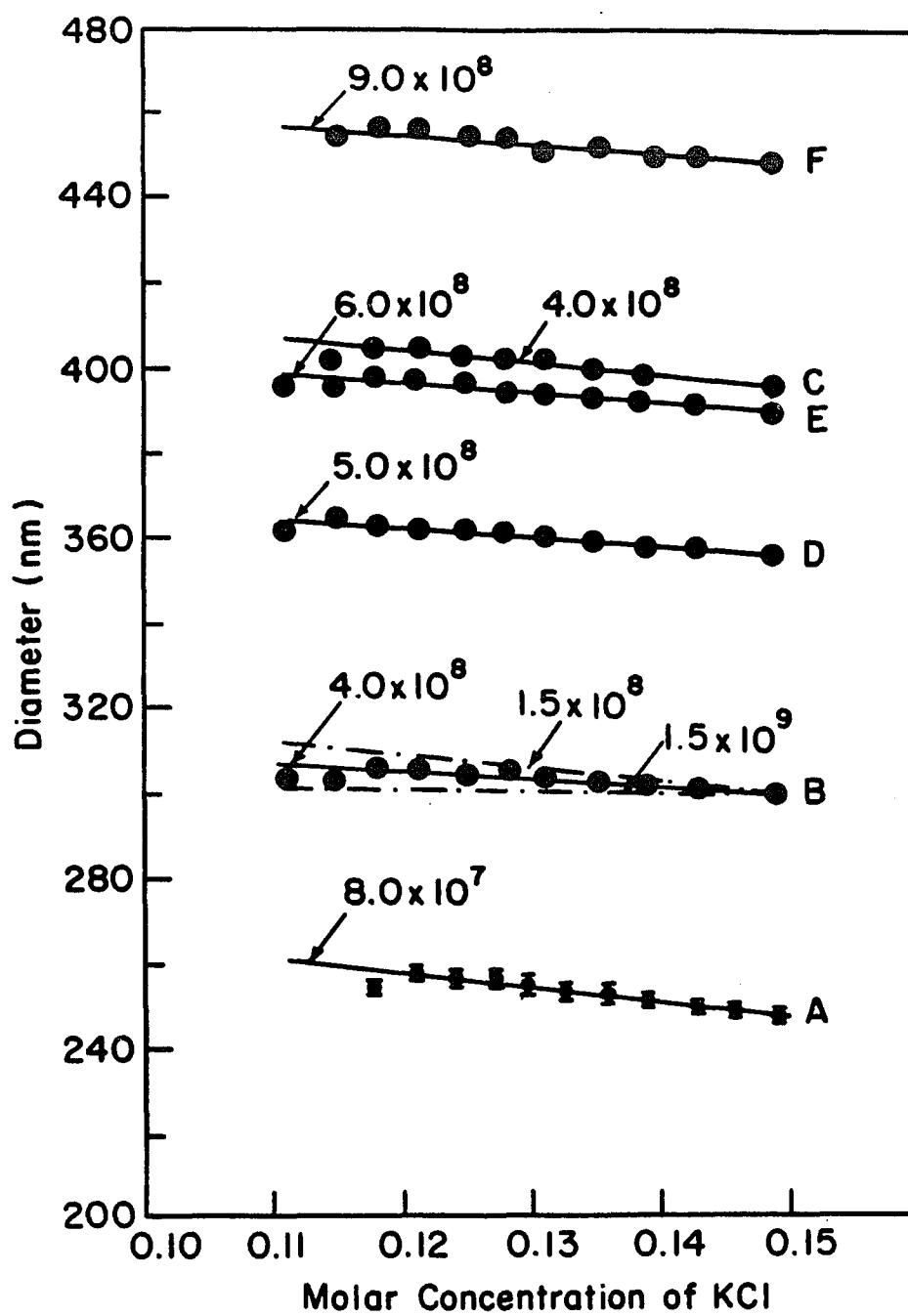
(1) *DOPA Vesicle Swelling in 0.15 M KCl Solution.* A typical set of osmotic swelling data is given in Table VII. It is seen that the vesicle radius steadily increases on dilution. For dilution steps of about 0.004 M KCl each, the swelling is linear for 8-10 dilutions. Vesicle mean radii increase for each step of the dilution although the error of some measurements are over half of the total expansion. The fact that the initial swelling is persistently linear in all of our experiments gives us confidence in the expansion measurements. The index of polydispersity does not change significantly and did not display a trend that related to the swelling in any of our studies.

Figure 13 shows typical results obtained for osmotically swelling vesicles made from DOPA and from DOPA-cholesterol in a solution of 0.15 M KCl. In the DOPA-cholesterol vesicles, the amount of cholesterol varies from 10 to 25 mole percent. For DOPA vesicles (curves A, B and C), the observed membrane modulus is found to be in the range $0.8-4.0 \times 10^8$ dyn/cm² for vesicles in the size range 110-200 nm. The addition of cholesterol to DOPA increases slightly the observed membrane modulus (curves D, E and F); however, addition of cholesterol also increases the size of the vesicles. Two calculated membrane moduli are shown in Curve B to exhibit the error made by this method.

Table VII. A typical set of osmotic swelling data. The DOPA-cholesterol (15%) vesicles were prepared using the pH adjustment method in 0.15 M KCl-Tris buffer. Dilution was made by adding 0.086 M KCl solution at the rate of 0.025 ml/min. KCl concentration in the table refers the concentration outside the vesicle after dilution. The vesicle radii and the index of polydispersity were measured with PCS. These data are also presented in Figure 13, curve E and in Figure 20.

KCl concentration (M)	Vesicle diameter (nm) after swelling	Index of polydispersity
0.150	389.8 +/-5.6	0.08
0.143	390.8 +/-3.0	0.08
0.139	391.6 +/-4.4	0.08
0.135	392.4 +/-5.6	0.08
0.131	393.0 +/-6.4	0.08
0.128	393.8 +/-4.0	0.08
0.125	395.2 +/-4.6	0.08
0.121	396.0 +/-4.0	0.09
0.118	397.0 +/-4.0	0.08
0.115	396.0 +/-4.6	0.08
0.111	395.6 +/-4.2	0.08

Figure 13. Osmotic swelling curves of DOPA (A, B and C) and DOPA-cholesterol (D, E and F) vesicles in 0.15 M KCl-Tris (2.0 mM) buffer (pH 7.55). The vesicles were made by the pH-adjustment method. The DOPA vesicles with diameter of 395.0 nm (C) were made by increasing the period of exposure to high pH during vesicle formation. D, E and F were 10, 15 and 25 % mole percent cholesterol (based on mixing mole fractions) in DOPA respectively. Dilutions were made by adding 0.086 M KCl-Tris (2.0 mM) buffer (pH 7.55) to each sample at the rate of 0.025 ml/min at room temperature. Before dilution, the vesicle suspensions and buffer solutions were filtered through the 1.0 and 0.3 μ m (pore size) filter paper, respectively. Solid lines are the "observed" membrane modulus calculated from the osmotic swelling curve using equation (8). The error bars (shown on Curve A only) represent the error of measurement using PCS and not the standard deviation in vesicle size. The calculated dashed lines on Curve B show two calculated membrane moduli so that error in this calculation can be estimated. The heavy line drawn through the first 8-10 dilution steps of each dilution was calculated from equation (8). The subsequent dilution steps were not included in the fit as they fall below the line and are assumed to represent burst vesicles (see Table IX). The dashed lines (shown in curve B only) permit an estimate of the error involved in assigning a "best fit" value to the modulus.



(2) *DOPA Vesicle Swelling in 0.25 M Sucrose Solution.* The results of osmotic swelling of vesicles in a sucrose-Tris buffer (pH 7.55) is shown in Figure 14. The observed membrane modulus is found to be of the order of 10^7 dyn/cm² and is persistently an order of magnitude smaller than that measured in the KCl-Tris buffer (pH 7.55).

(3) *Sub-mitochondrial Particles Swelling in 0.25 M Sucrose Solution.* The variation of the size of sub-mitochondrial particles on dilution is shown in Figure 15. The observed membrane modulus is found to be about 3×10^7 dyn/cm² for the particles which have average of diameter 200 nm. These particles are also found to rupture after an expansion of 5% of initial diameter (see below).

(4) *Vesicle Bursting and Reswelling.* In Figures 13, 14 and 15 it may be seen, that the vesicles swell according to the observed membrane modulus (eq (8)) to a maximum, at which point the size no longer increases but appears to plateau (or slightly decline). Once the vesicle has reached its elastic limit then it would be expected to burst, release solution and return to the unstressed state of the bilayer which is the original size. The amount of solution released by the vesicle can be estimated by the difference between the vesicles' volume at the maximum (bursting) size and its original (unstressed) volume. The concentration of the interior at its maximum size is presumably equal to the concentration of the released fluid. We may therefore calculate the concentration difference (inside vs outside) and estimate the osmolarity of the vesicle when it reseals at the original unstressed size. Having resealed at a new osmolarity we find that the vesicle reswells to a new size that may be

Figure 14. Osmotic swelling curves of DOPA (A and B) and DOPA-cholesterol (C and D) vesicles in 0.25 M sucrose-Tris (2.0 mM) buffer (pH 7.55). The vesicles were made by the pH-adjustment method. The DOPA vesicles with diameter of 300.8 nm (B) were made by increasing the period of exposure to high pH during vesicle formation. C and D were 10 and 15 % mole percent cholesterol (based on mixing mole fractions) in DOPA respectively. Dilutions were made by adding 0.15 M sucrose-Tris (2.0 mM) buffer (pH 7.55) to each sample at the rate of 0.025 ml/min at room temperature. Before dilution, the vesicle suspensions and buffer solutions were filtered through the 1.0 and 0.3 μm (pore size) filter paper respectively. Solid lines are the "observed" membrane modulus calculated from the osmotic swelling curve using equation (8). The error bars (shown on Curve A only) represent the error of measurement using PCS and not the standard deviation in vesicle size.

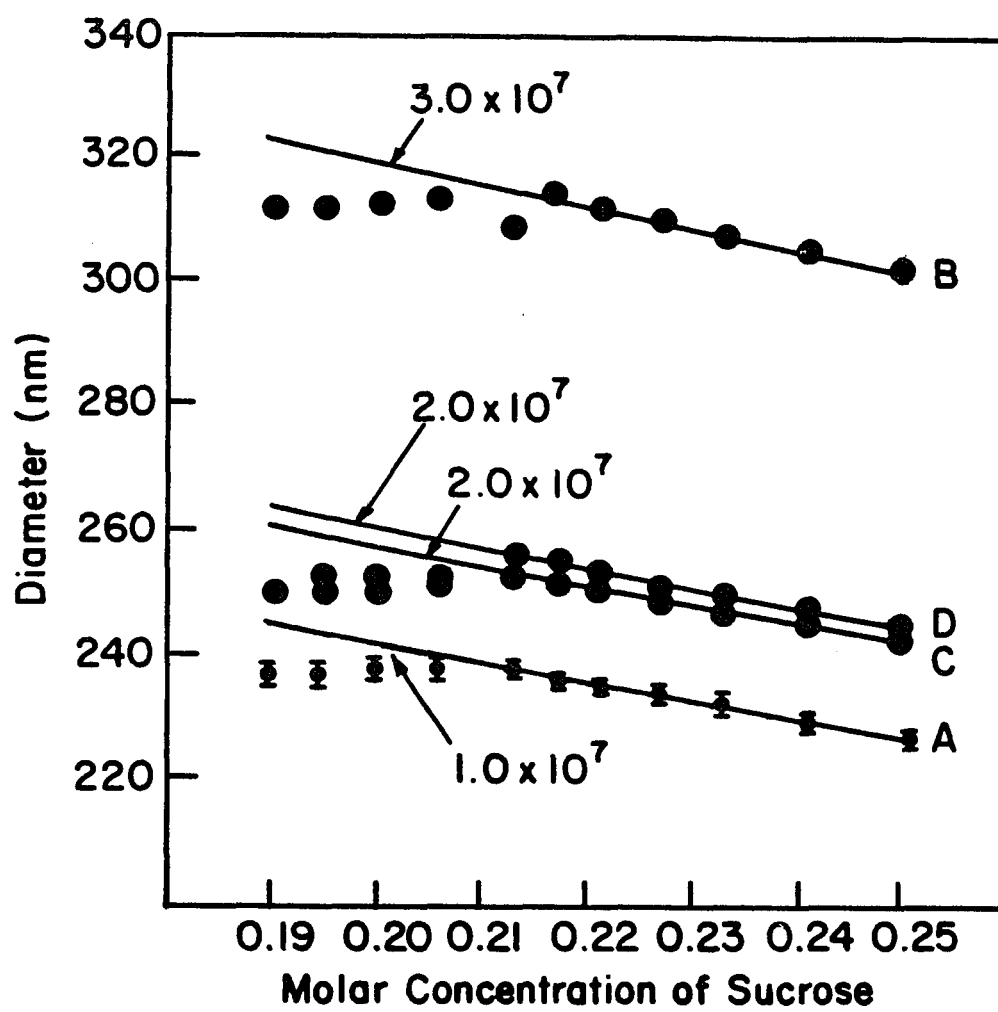
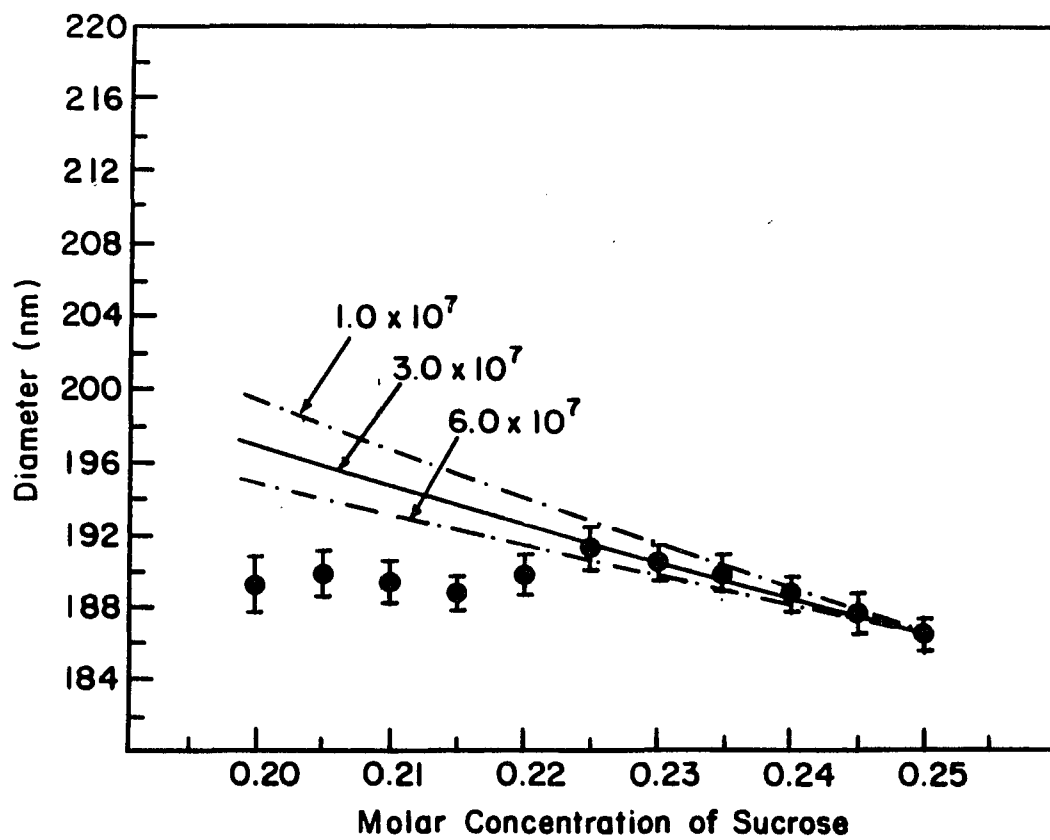


Figure 15. Osmotic swelling curve of sub-mitochondrial particles derived from the inner membrane of bovine heart mitochondria in 0.25 M sucrose-Tris (2.0 mM) buffer (pH 7.0). The particles were a gift of Dr. H. Penefsky. Before osmotic dilution, the original particle suspension was diluted 40 times (volume) in 0.25 M sucrose-Tris (2.0 mM) buffer (pH 7.55), and then filtered through a 1.0 μm (pore size) filter. Concentration dilutions were made by adding 0.15 M sucrose-Tris (2.0 mM) buffer (pH 7.55) (filtered through 0.3 μm (pore size) filter) to each sample at the rate of 0.025 ml/min at room temperature. The solid line is the "observed" membrane modulus calculated from the osmotic swelling curve using equation (8). The broken lines give an estimation of the error in the observed membrane modulus. The error bars represent the error of measurement using PCS and not the standard deviation in vesicle size.



calculated from the observed membrane modulus. Let C_i and R_i be the initial concentration (both sides of the bilayer) and initial vesicle radius respectively. When the vesicle swells during dilution, the radius increases until the maximum radius R_f obtains. The concentration outside the vesicles C_f at the maximum vesicle swelling state is known from the amount of diluted solution added. C_{vi} , the concentration inside the vesicles at this state is given in the equation:

$$C_i \left(\frac{4\pi R_i^3}{3} \right) = C_{vi} \left(\frac{4\pi R_f^3}{3} \right) \quad (10)$$

This gives:

$$C_{vi} = C_i \left(\frac{R_i}{R_f} \right)^3 \quad (11)$$

The vesicle reswells according to its observed membrane modulus using C_{vi} as the initial concentration (C_i , eq (8)) for the reswelling. The calculated radius after vesicle reswelling for each first point on the plateaus in Figures 13, 14 and 15 are shown in Table VIII. It can be seen that for all but 2 of the eleven preparations the vesicles reswell to within 1 nm of the calculated size. The agreement between the calculated and the observed size may be taken as evidence that the plateau begins at the elastic limit of the vesicles.

Table IX shows the elastic limit in terms of increased surface area for all of the experiments reported here. Vesicles swelled in sucrose display substantially larger elastic limits than those in KCl. This includes both the synthetic phospholipid vesicles and the submitochondrial particles.

(5) *Aqueous Media Effects on Membrane Elastic Properties.* Our previous work on DOPA vesicles had suggested that the concentration of the salt

Table VIII. The experimental and calculated (cal.) mean vesicle sizes after rupture and reswelling. Sizes are expressed in nm and are only significant to 3 figures.

Vesicle preparation	Initial vesicle diameter	Maximum vesicle swelling diameter	Vesicle diameter after reswelling	Calculated vesicle diameter after reswelling	Size difference (found-cal.)
Fig. 15	186.6	191.4	189.8	188.0	-1.8
Fig. 14 A	226.0	237.4	233.0	229.8	-3.2
Fig. 14 C	242.0	252.6	247.6	248.0	+0.4
Fig. 14 D	243.2	254.0	248.0	244.4	-3.6
Fig. 13 A	247.0	257.4	254.0	251.8	-2.2
Fig. 13 B	299.0	305.8	303.0	303.0	0
Fig. 14 B	300.8	313.0	308.0	306.4	-1.6
Fig. 13 D	355.4	363.4	362.4	306.8	-1.6
Fig. 13 E	389.8	397.0	396.0	395.0	-1.0
Fig. 13 C	395.0	404.4	401.2	401.6	+0.4
Fig. 13 F	448.2	456.0	455.2	453.8	-1.4

Table IX. Observed membrane moduli, the surface area increase and the pressure difference (ΔP) across the membrane at bursting of vesicle preparations in KCl and sucrose solution.

Vesicle preparation	Diluent	Initial mean diameter (nm)	Mole % cholesterol	% Surface increase	Observed modulus ($\times 10^{-7}$ dyn/cm ²)	ΔP [mosmols] ($\times 10^{-6}$ dyn/cm ²)
Fig. 13 F	KCl	448	25	3.5	90	[43.9] (1.12)
Fig. 13 C	KCl	395	-	4.8	40	[41.0] (1.04)
Fig. 13 E	KCl	390	15	3.7	60	[43.2] (1.10)
Fig. 13 D	KCl	355	10	4.6	50	[45.6] (1.16)
Fig. 14 B	sucrose	301	-	8.4	3	[4.2] (0.11)
Fig. 13 B	KCl	299	-	4.7	40	[40.0] (1.02)
Fig. 13 A	KCl	247	-	8.6	8	[22.6] (0.57)
Fig. 14 D	sucrose	243	15	9.0	2	[5.9] (0.15)
Fig. 14 C	sucrose	242	10	9.0	2	[6.4] (0.16)
Fig. 14 A	sucrose	226	-	10.3	1	[4.0] (0.10)
Fig. 15	sucrose	186.6 *	-	*	3	*

* The initial size of the submitochondrial particles does not permit a calculation of the % surface increase as they may already have been in a partially swollen state at the beginning of a swelling experiment.

might have a significant effect on the modulus, the elastic limit, and the trans-bilayer pressure at bursting. As a test for this concept we conducted a series of experiments swelling vesicles of DOPG in isoosmolar solutions of 150 mM KCl, 250 mM sucrose, and a 50:50 mixture of each solution (Figure 16). It can be seen by inspection of the slopes as well as by the calculated moduli (straight lines) that the modulus increases significantly with the salt concentration between zero and 75 mM KCl whereas the difference in the modulus at 75 mM KCl and 150 mM KCl is minor.

In order to establish whether it was the sucrose or the ionic strength that was altering the modulus, we used 250 mM trehalose and 250 mM pentaerythritol as impermeants in our swelling experiments. The results are shown in Table X for both DOPA and DOPG vesicles which have essentially the same pattern. It is interesting to note the initial sizes of the vesicles using our pH adjustment method show a rather wide range for the non-electrolyte impermeants. That vesicles made in 250 mM trehalose have nearly the same diameter as those made in 150 mM KCl and yet display a dramatically different modulus, elastic limit and ΔP shows that these properties are not dependent on the radius of the vesicles but primarily on the ionic strength. Nonetheless, a slight increase in the modulus for trehalose as compared to sucrose and pentaerythritol suggests that the radius might have a small but noticeable effect on the elastic properties.

Having established that the sucrose was not acting in a unique way to alter the properties of the bilayer during osmotic swelling, we then have examined more closely the alteration of the modulus with ionic strength (Table

Figure 16. Osmotic swelling curves of DOPG vesicles in 250 mM sucrose (A), 125 mM sucrose/75 mM KCl (B) and 150 mM KCl (C) Tris-buffer (2 mM, pH 7.55). The vesicles were made by the pH-adjustment method. Dilutions were made by adding 150 mM sucrose (A), 75 mM sucrose/43 mM KCl (B) and 86 mM KCl (C) Tris-buffer (2.0 mM, pH 7.55) to the sample at the rate of 0.025 ml/min at room temperature. Before dilution, the vesicle suspensions and buffer solutions were filtered through the 1.0 and 0.3 μm (pore size) filter paper respectively. The error bars (shown on Curve A only) represent the error of measurement using PCS and not the standard deviation in vesicle size. The heavy line (Modulus) drawn through the first 7-10 dilution steps of each dilution is a "best fit" calculated from equation (8). The subsequent dilution steps were not included in the fit as they fall below the line and represent burst vesicles. Curve B shows two calculated moduli in addition to the "best fit" to permit estimation of the error in the "best fit".

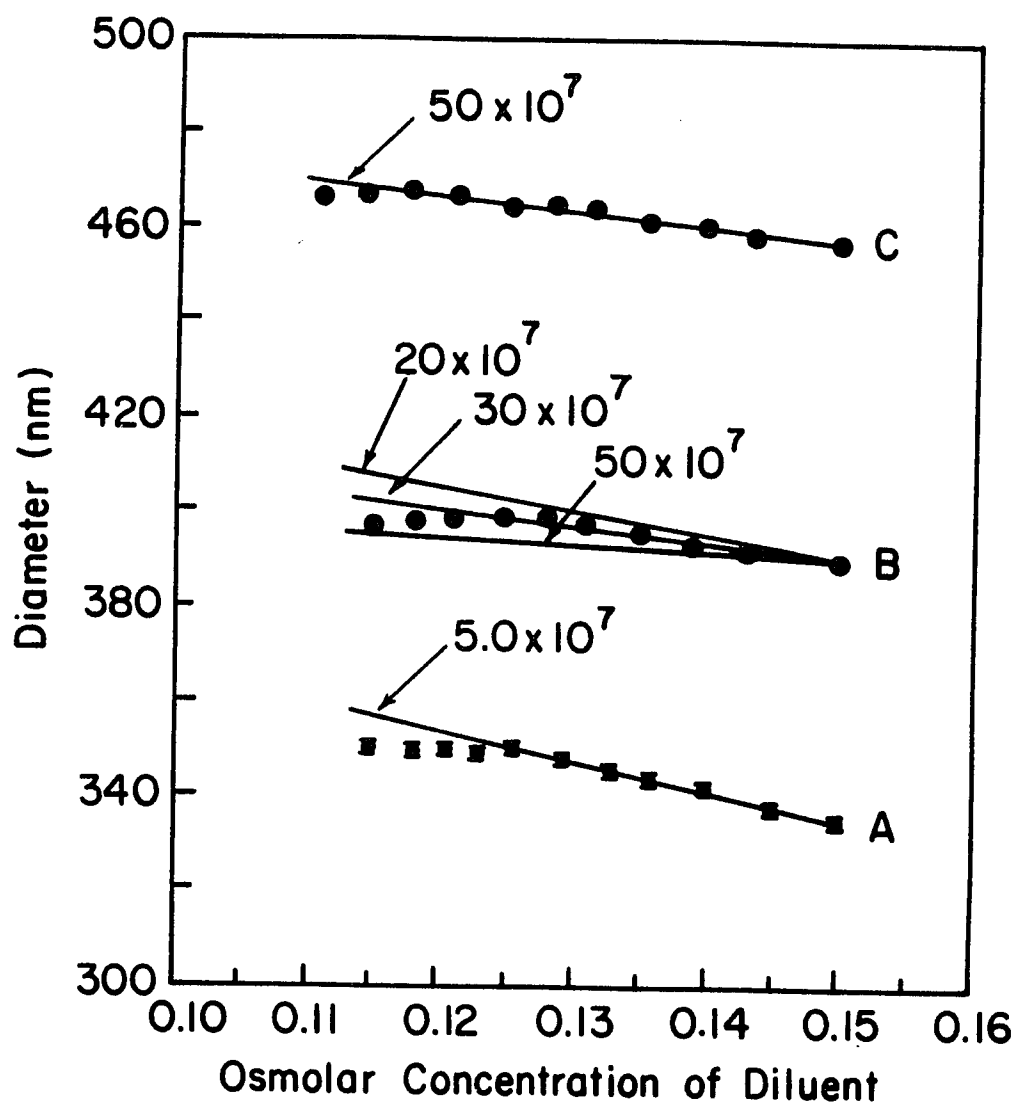


Table X. The observed membrane moduli, surface area increase and pressure difference across the membrane at bursting (ΔP) of DOPA and DOPG vesicle preparations in 150 mM KCl, 125 mM sucrose/75 mM KCl, 250 mM sucrose, 250 mM trehalose, and 250 mM pentaerythritol.

Lipids	Diluent	Initial size (nm)	Maximum size (nm)	% Surface increase	Modulus ($\times 10^{-7}$ dyn/cm ²)	ΔP [mosM] ($\times 10^{-6}$ dyn/cm ²)
DOPA	KCl	299.0	305.8	4.8	40.0	[40.0] (1.02)
DOPA	sucrose/KCl	294.0	308.4	10.0	10.0	[26.9] (0.68)
DOPA	sucrose	226.0	237.4	10.3	1.0	[4.0] (0.10)
DOPA	trehalose	290.0	302.0	8.4	4.0	[9.2] (0.23)
DOPA	pentaerythritol	176.8	184.8	9.3	1.0	[6.5] (0.16)
DOPG	KCl	456.3	467.2	4.8	50.0	[39.3] (1.00)
DOPG	sucrose/KCl	389.8	400.0	5.3	30.0	[30.0] (0.72)
DOPG	sucrose	334.5	348.4	8.5	5.0	[9.1] (0.23)
DOPG	trehalose	268.3	280.0	8.8	3.0	[7.8] (0.20)
DOPG	pentaerythritol	201.9	211.8	10.0	1.0	[3.9] (0.10)

XI). The table shows a sharp distinction in the three properties in the narrow range between the absence of KCl and its presence at the 5 mM level. It might be noticed from Table XI that the initial vesicle size increases with the ionic strength, a feature of this method of making vesicles. Indeed the size differences are greater in the higher ionic strength range (see Table XI) whereas the mechanical property differences are the greatest in the low ionic strength range.

(6) *Lipid Structure Effects on Membrane Elastic Properties.* In order to assess the significance of lipid structure on the modulus we examined several lipids containing different headgroups and measured the membrane modulus, the % surface increase and ΔP at bursting. Table XII shows measurements comparing DOPS, DOPE, DOPG, and oleic acid vesicles to DOPA and DOPG. It should be noticed that DOPE vesicles cannot be made below pH 8.6 by this method so that measurements on that lipid were made at that pH although all of the other measurements were made at pH 7.55. DOPS vesicles cannot be made if the pH is raised above pH 8.6 so that the vesicles were not made in the usual way. This may explain why the DOPS vesicles are smaller than the others although they only slightly smaller than the DOPA vesicles. The method of preparation is unlikely to have affected our measurements on the DOPS vesicles. They display a remarkably low modulus for anionic vesicles in salt. These vesicles are easily stretched (low modulus) and yet require a moderately high ΔP at bursting, going so at a low surface expansion. In contrast, DOPE displays the largest modulus we have obtained with any lipid bilayer, the lowest % surface expansion at bursting and the highest ΔP at burst-

Table XI. The observed membrane moduli, surface area increase and pressure difference across the membrane at bursting (ΔP) of DOPG vesicles at constant osmolarity and in variable ionic strength in mixtures of KCl and pentaerythritol (penta).

Diluent	Initial size (nm)	Maximum size (nm)	% Surface increase	Modulus ($\times 10^{-7}$ dyn/cm ²)	ΔP [mosM] ($\times 10^{-6}$ dyn/cm ²)
KCl (150 mM)	456.3	467.2	4.8	50.0	[39.3] (1.00)
sucrose/KCl (125/75 mM)	389.8	400.0	5.3	30.0	[30.0] (0.72)
penta/KCl (167/50 mM)	358.9	372.2	7.4	15.0	[24.4] (0.61)
penta/KCl (201/30 mM)	337.2	352.2	9.1	13.0	[24.6] (0.62)
penta/KCl (242/5 mM)	218.4	228.1	9.1	5.0	[19.2] (0.49)
pentaerythritol (250 mM)	201.9	211.8	10.0	1.0	[3.9] (0.10)

Table XII. The observed membrane moduli, surface area increase and pressure difference across the membrane at bursting (ΔP) of vesicle preparations constituted of different lipids. All vesicles were prepared with the pH-adjustment method in 150 mM KCl except for the *E. coli* lactose carrier reconstituted vesicles (*E. coli* recons.) which were prepared with the detergent dilution procedure in 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7.5). The vesicles were made by adjustment of pH to maximum pH 10 except for DOPS which was adjusted to pH 8.5. All vesicles were restored to pH 7.55 except DOPE which was restored to pH 8.60.

Lipids	Diluent	Initial size (nm)	Maximum size (nm)	% Surface increase	Modulus ($\times 10^{-7}$ dyn/cm ²)	ΔP [mosM] ($\times 10^{-6}$ dyn/cm ²)
DOPS	KCl	277.0	292.6	5.6	5.0	[16.6] (0.42)
DOPE	KCl	544.0	553.8	3.6	80.0	[41.8] (1.06)
Oleic acid	KCl	308.7	329.4	13.0	1.0	[5.9] (0.15)
D Φ PG	KCl	656.4	665.6	2.8	50.0	[16.0] (0.41)
<i>E. coli</i> lipids	KCl	537.4	550.4	5.8	60.0	[30.2] (0.77)
<i>E. coli</i> recons.	$\text{KP}_i/\text{K}_2\text{P}_i$	400.8	410.2	4.7	90.0	[70.8] (1.78)

ing. The effect of reconstituting the lactose carrier in *E. coli* lipids seems to have a significant impact on the three properties of the membrane. The reconstituted vesicles display an even greater modulus than that of DOPE, a low surface expansion and the highest ΔP at bursting of any bilayer system tested to date. The data from Table XII were also shown on Figure 17.

(7) *Thickness Changes of The Bilayer on Osmotic Swelling.* Changes on the bilayer thickness would be expected based on the assumption that when the vesicles swell the volume of the hydrophobic domain of the bilayer remains constant. Lateral expansion of the bilayer necessarily implies thinning of it. Another way to explain how the expanded state would not result in a thinning of the bilayer is to assume that water molecules enter the bilayer thereby increasing its volume. The latter would not result in changes of the bilayer thickness. In order to distinguish between these two possibilities, the thickness of the vesicle bilayer on the expanded state was measured using x-ray diffraction (Gruner et al., 1982). This was done at Princeton University and at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratories by D. Turner and S. Gruner of Princeton University. The vesicle preparations of DOPG in 250 mM pentaerythritol were provided by our laboratory. The data is shown on Figure 18. All samples except for the first one were in the swollen state, but had not burst. The swollen vesicles were checked by PCS measurement in our laboratory after the x-ray results were obtained on a portion of the sample. As can be seen the data show no clear trend was obtained within the error of the measurement. Since the error for each point is 3 to 4 Angstroms, thinning of less than 3 Angstrom can not be

Figure 17. Osmotic swelling curves of DOPS (A), oleic acid (B), *E. coli* reconstituted (C), *E. coli* lipids (D), DOPE (E), and D Φ PG (F) vesicles in 150 mM KCl-Tris buffer (2.0 mM, pH 7.55). The vesicles were made by the pH-adjustment method. Dilutions were made by adding 86 mM KCl-Tris buffer (2.0 mM, pH 7.55) to each sample at the rate of 0.025 ml/min at room temperature. Before dilution, the vesicle suspensions and buffer solutions were filtered through the 1.0 and 0.3 μ m (pore size) filter paper respectively. Solid lines are the "observed" membrane modulus calculated from the osmotic swelling curve using equation (8). The error bars (shown on Curve A only) represent the error of measurement using PCS and not the standard deviation in vesicle size.

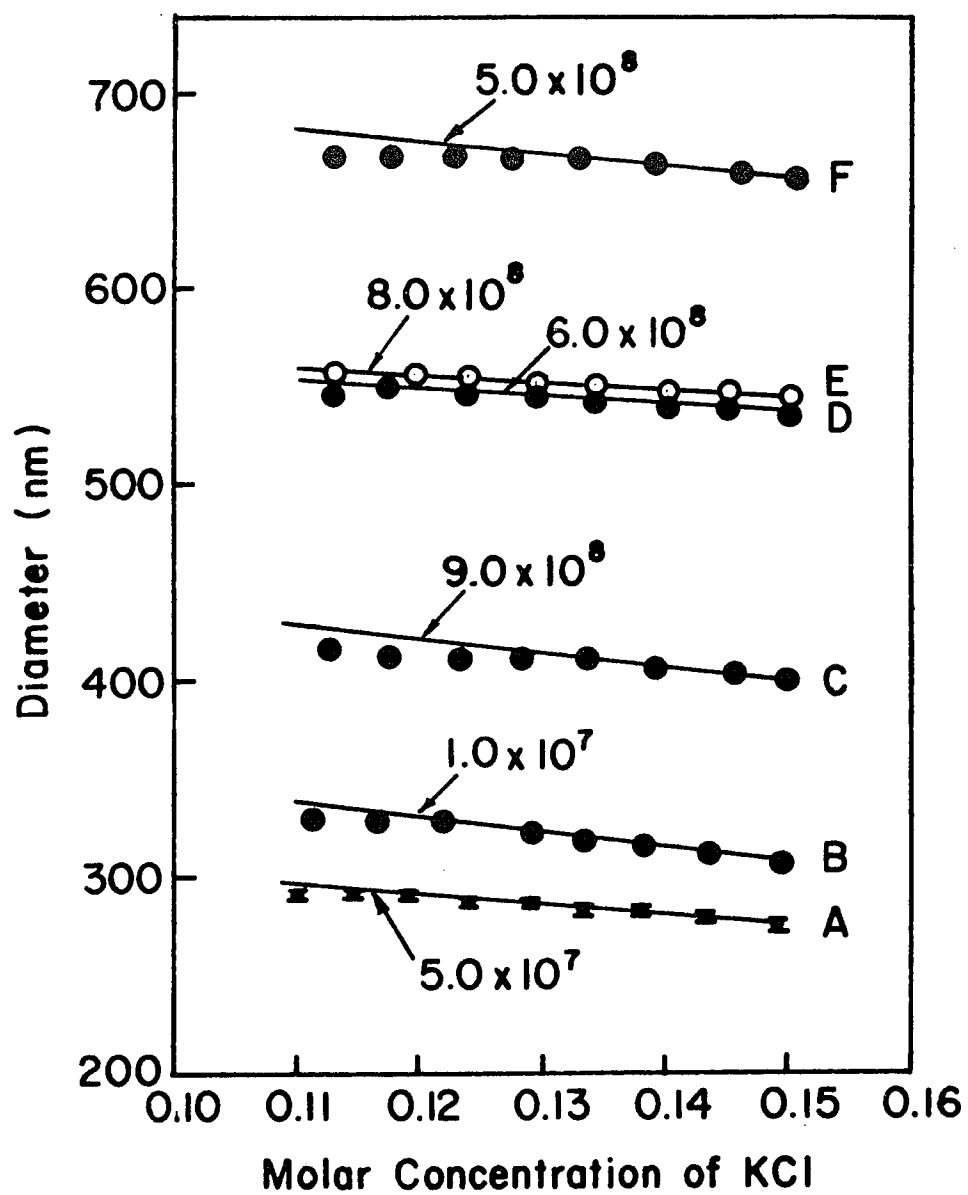
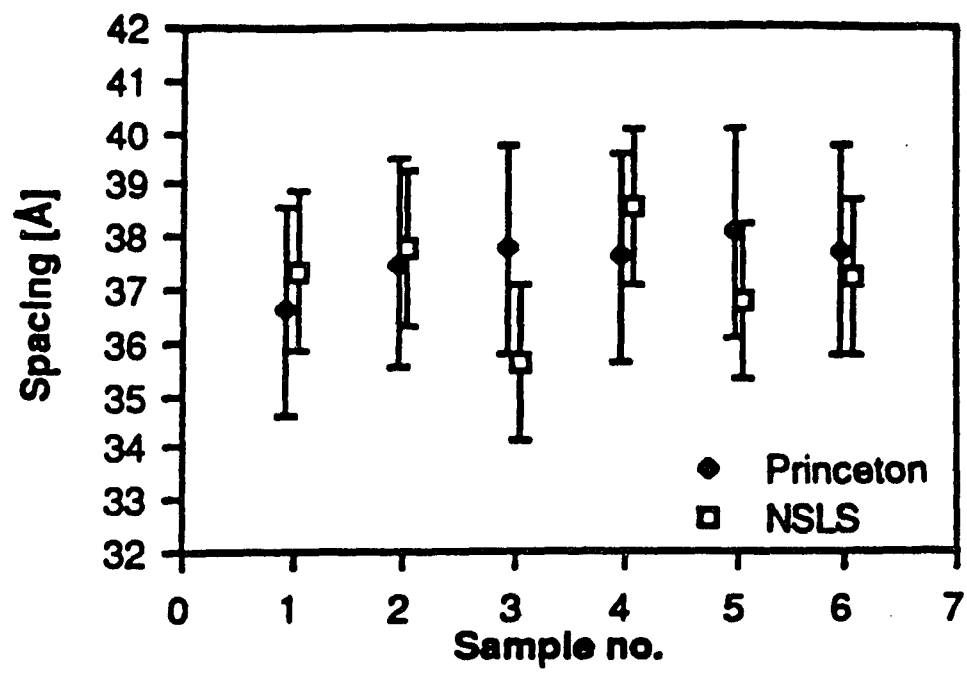


Figure 18. X-ray diffraction study of thickness changes of the bilayer on osmotic swelling. This was done at Princeton University (◆) or at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratories (□). DOPG vesicles were made by the pH adjustment procedure in 250 mM pentaerythritol-Tris solution (2.0 mM, pH 7.55). The bilayer thickness is expressed as spacing in Angstroms in the figure. Vesicles were swollen by the addition of diluent [150 mM pentaerythritol-Tris solution (2.0 mM, pH 7.55)] to the outside. In each of the five dilution steps (expressed as sample number) vesicles were exposed to about 5 mosM transmembrane osmotic pressure by the addition of diluent. After 5 dilution steps an 8 % surface area expansion was found by the size measurement with PCS. The swollen vesicles were stable at least for two weeks. Concentration of the vesicles by centrifugation into a pellet followed by dilution into a solution of the same osmolarity resulted in no change of the vesicle size.



ruled out.

H⁺/OH⁻ Currents Across Lipid Bilayers

(1) *Permeability of Anionic Straight-Chain Phospholipid Vesicles.* The proton/hydroxyl permeability of vesicles made of phospholipids with anionic headgroups is shown in Table XIII along with the permeability of DOPC vesicles made by sonication. Although DOPC vesicles display greater permeability than all of the other preparations (except the 50:50 mixture of DOPG and DOPE), DOPC may not properly be used as a standard of comparison in this assay because the method of vesicle preparation is different, the vesicles are more polydisperse and smaller, and the use of acridine orange for the study of proton/hydroxyl permeability of PC vesicles may not be comparable to the others (Dencher et al., 1986). It should be noted that vesicles made of DOPE can only be made above pH 8.6 by this method and that vesicles of DOPS cannot be made if the pH is raised above pH 8.6 during the pH adjustment. We were therefore unable to compare the proton/hydroxyl permeability of the vesicles made of pure DOPE to those made of other lipids.

DOPA would be expected to display a greater surface charge density than DOPG since non-aggregated DOPA has two charges per headgroup and DOPG in the non-aggregated state has only one. This statement is consistent with many experimental observations regarding the charge densities of these lipids (Boggs, 1987). In general the data suggest P may be lower (Table XIII) for vesicles with a higher surface charge density.

The greatest permeability we observed is that of a 50:50 mixture of

Table XIII. H^+ -permeability (P) of vesicle preparations. Vesicles were prepared using the pH-adjustment method except the DOPC and *D. salina* sample. DOPC vesicles were prepared by sonication with a Branson sonicator at 35 watt for 30 min. The *D. salina* sample was natural plasma-membrane vesicles isolated from *D. salina* cells (Katz et al., 1986). Vesicle size was determined by PCS. The errors indicated for the diameter of vesicle preparations are errors of measurement and not the standard deviation of the vesicle sizes.

Lipid	Vesicle size (Diam. nm)	P $\times 10^6$ (cm/s)
DOPG	433.6 $\pm$ 2.1	40 $\pm$ 5
DOPA	301.0 $\pm$ 2.5	5.5 $\pm$ 0.7
DOPS	298.6 $\pm$ 1.9	100 $\pm$ 12
DOPC	36.4 $\pm$ 0.8	170 $\pm$ 20
D Φ PG	714.8 $\pm$ 3.6	4.1 $\pm$ 0.5
<i>D. salina</i>	Not examined	6.5 $\pm$ 0.8
<i>E. coli</i> lipids	492.7 $\pm$ 3.4	41 $\pm$ 5

DOPE and DOPG. The addition of DOPE to the DOPG raised the proton/hydroxyl permeativity from 40×10^{-6} cm/s to 250×10^{-6} cm/s. That this is not due to the increased size of the vesicles is shown by the fact that the much larger DPG vesicles display low permeability.

(2) *The Effect of Sterol and Phytanyl Chains on Permeability.* Figure 19 shows the decreased permeability associated with increased cholesterol concentration in DOPG vesicles. It may be seen that there is little inhibition of the proton/hydroxyl flux at low levels of cholesterol but as the concentration of the cholesterol approaches 30 mole% the level of permeability decreases to about one tenth of that of DOPG. This lower permeability corresponds to that of the vesicles of *D. salina* membrane (Table XIII) which contain sterols in approximately the same molar concentration (Sheffer et al., 1986).

Permeability is also significantly lower in vesicles made of DΦPG which differs from DOPG only with respect to the acyl chains. The permeability of DΦPG vesicles is approximately that of vesicles of *D. salina* plasma membrane and of the DOPG vesicles containing cholesterol at the 2:1 (phospholipid:cholesterol) ratio.

(3) *The Effect of Detergents on Proton/Hydroxyl Permeability.* Because detergents are commonly used in the reconstitution of transport proteins into vesicles it seemed appropriate to examine P in vesicles exposed to the low levels of detergents commonly used for membrane reconstitution. Table XIV shows the effect of such low levels of detergents. It can be seen that at low levels detergents do not enhance leakage but, if anything, inhibit it. At higher levels they do not appear to break down the bilayer barrier. Octyl glucoside

Figure 19. Effect of cholesterol on membrane H^+/OH^- -permeability. Vesicle preparations were made using the pH-adjustment method. Chloroform containing the cholesterol was added in the lower phase after acid washing of the sodium salt of DOPG. The DOPG:cholesterol mixture was dried in vacuo and the buffer was added to the film. The H^+/OH^- -permeability (P) was assayed at 10° by monitoring changes in absorption of acridine orange at 495 nm after imposition of a ΔpH (interior acid) across the vesicle bilayer.

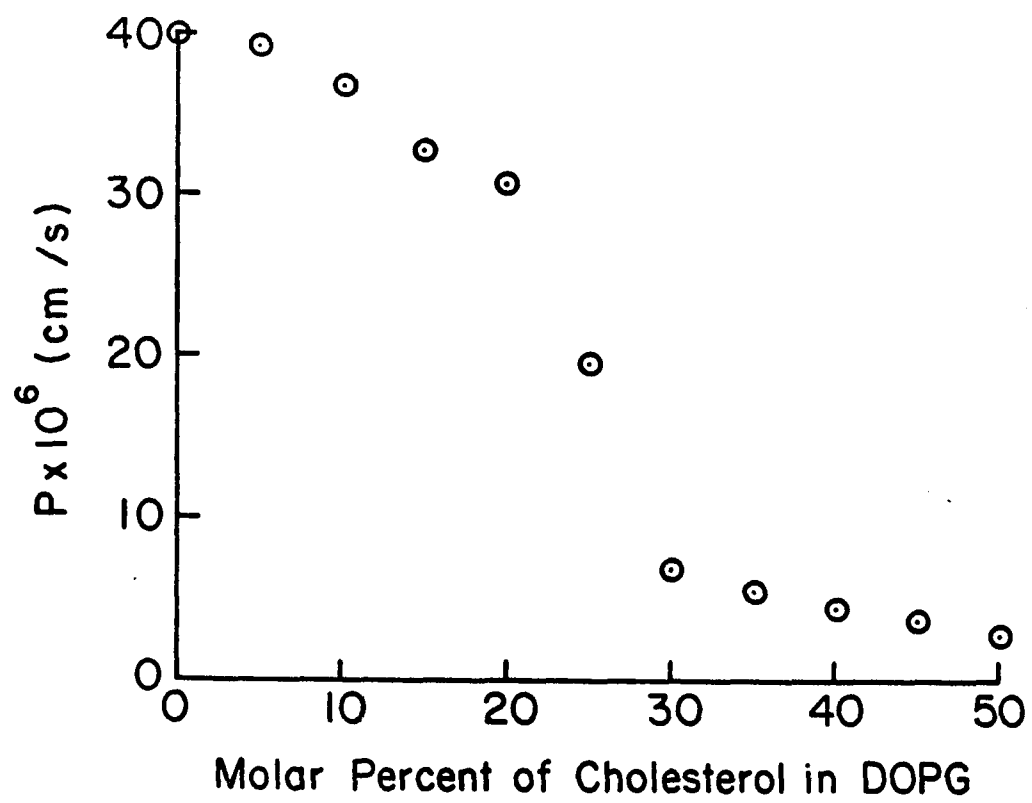


Table XIV. H^+ -permeability (P) of DOPG, DOPG with octyl glucoside and DOPG with cholate vesicle preparations. Vesicles (5 mg lipid and detergent) were prepared using the pH-adjustment method and suspended in 3 ml saline buffer. The mole fraction of the detergent in the DOPG vesicle preparation is indicated. The detergent concentrations expressed as molarity or weight percent of the aqueous solution are for octyl glucoside (8.34×10^{-3} to 0.714 mM; 0.024 to 2.1 %, w/v) and for cholate (8.34×10^{-3} to 0.714 mM; 0.034 to 2.9 %, w/v). Vesicle size was determined by PCS. The errors indicated for the diameter of vesicle preparations are errors of measurement and not the standard deviation of the vesicle sizes.

DOPG plus (+)	Vesicle size (Diam. nm)	P x10 ⁶ (cm/s)
Control	433.6+/-2.1	40+/-5
0.005 octyl glucoside	465.6+/-2.3	34+/-4
0.015 octyl glucoside	457.8+/-2.5	30+/-4
0.1 octyl glucoside	487.6+/-2.8	18+/-2
0.3 octyl glucoside	493.8+/-3.1	13+/-2
0.005 cholate	452.1+/-2.7	42+/-5
0.015 cholate	478.4+/-3.1	44+/-5
0.1 cholate	508.3+/-4.0	160+/-19
0.3 cholate	513.5+/-4.8	180+/-22

continues to inhibit permeability at high molar ratios whereas cholic acid at high concentration behaves like the hexagonal-II-phase-forming lipids DOPS and DOPE.

DISCUSSION AND CONCLUSION

Preparation and Characterization of the Vesicles

The formation of monodisperse large unilamellar vesicles of a desired size between 200 and 3000-nm diameter is described. The method is a further development of the pH adjustment technique of Hauser and Gains (1983). Important differences allowed us to make vesicles more than 10 times larger. These differences include variations of the salt concentration, the choice of lipid structure and lipid composition, and the use of sterol to further separate the headgroups, which we believe to be largely acid-anions (Haines, 1983) during vesicle formation.

The vesicle sizes and polydispersity were determined by several methods which gave approximately the same results. We were able to verify the size and standard deviation of polystyrene spheres by *both* EM and PCS. Comparison of the sizes of curvature-limited SUV's measured by PCS to those obtained by EM showed excellent agreement (Grzesiek & Dencher, 1986) whereas large unilamellar vesicles (~ 240 nm) show slightly larger hydrodynamic diameters in PCS than in EM probably due to slight shrinkage during the negative staining (Aurora et al., 1985). These give us confidence in the use of PCS as a more reliable method for the determination of vesicle size than EM for the measurement of vesicle radii. The measurement of internal volume of vesicles by entrapment of B_{12} and calculations of vesicle size based on the assumptions about the area occupied by a phospholipid molecule are useful in that it validates the size range of the vesicles. These estimates are slightly larger than that measured by PCS. PCS was shown in these studies to yield mean radii, measure approximate polydispersity and compare relative amounts of vesicles in fractions (Figure 1) in a few minutes for each sample.

Studies of the osmotic properties (membrane modulus, maximum surface area expansion and transbilayer pressure difference at vesicle bursting) on our vesicle preparations gave very strong evidence these vesicle preparations are unilamellar bilayer. X-ray diffraction studies on DOPG vesicles confirmed this result (D. Turner & S. M. Gruner, 1987, personal communication).

Parente and Lentz (1984) have prepared LUV's in the 800-1000 Angstrom diameter size range by using octyl glucoside dialysis. Their preparation was made from many synthetic lipids—all of them containing only saturated fatty acyl groups. Their results have clearly demonstrated the difficulties associated with removing trace impurities from detergent dialysed sample. In the present method the principal contaminants expected to be introduced would be in the original solvent. The lipids were found to be unhydrolyzed by TLC after vesicle formation; including acid-washing.

Attempts to enhance the size of the LUV and maintain uniformity was based on a combination of principles derived from two theoretical views on the hydrocarbon domain (Haines, 1979; 1982) and the headgroup domain (Haines, 1983) of lipid bilayers. In general it was reasoned that a large vesicle would be derived from a more *planar* array of phospholipids. Such an array would be maximized by balancing the average attractive and repulsive interactions between neighboring headgroups. Attractive interactions are considered to be acid-anion dimer interactions (acidic H-bonding) (Haines, 1983) which average on a rapid timescale over the entire bilayer and repulsive interactions are due to adjacent anions. According to this view, the pK's of phospholipids in the bilayer may not be predicted from pK's measured in the absence of a bilayer. The pK of such headgroups of phospholipids in the bilayer results not only from the functional group's measured pK's but also from the forces that

hold adjacent groups in close proximity allowing acid-anion formation. Thus a membrane lipid containing two negative charges (like PA) would display pK's including those of both acid-anions (Haines, 1983).

The inner monolayer headgroup interactions determine the curvature of a vesicle. A shift toward greater attractive interactions between adjacent headgroups results in sharper (greater) curvature and therefore smaller vesicles. Enhanced repulsive interactions provokes a more planar bilayer and therefore larger vesicles. If protons bridge headgroups as attractive forces then enhanced pH (which removes protons) gives a larger vesicle because of greater repulsion between headgroups. According to this model, any branching on the fatty acyl chain or the insertion of cholesterol which separates headgroups would reduce the attractive interactions and therefore increase the vesicle size.

The separation of the headgroups provides, in this view, a favorable balance of attractive and repulsive forces for planar formation because less hydrogen bonding (on the average) occurs. The addition of sterol was reasoned to even further spread the headgroups and reduce the H-bonding during the pH adjustment.

(1) *Vesicle Preparations of Anionic Phospholipids and Lipid Mixtures Containing Anionic Lipids.* All biological membranes contain an excess of negatively charges in their lipids. The technique which we described here is therefore useful for creating analogues of biological membranes. Using the pH adjustment procedure we were unable to make unilamellar vesicles from DOPC alone. This work reports that the method can be used to make uniform unilamellar vesicles of virtually any anionic lipid(s) including all of the common anionic phospholipids either alone or in combination with zwitterionic lipids (Table VI) found in natural membranes. The lower the mole fraction of

the anionic lipid the larger the vesicle.

Special emphasis in this study is directed toward the lipids of *Halobacterium cutirubrum* and *E. coli*. *Halobacteria*, extreme halophiles, lack zwitterionic lipids. Their charged lipids are solely anionic lipids. Additionally, their lipids contain exclusively phytanyl chains in ether linkage with the glycerol backbone (Kates, 1978). These phytanyl ethers which contain methyl groups extending from the chain on every fourth carbon, are bulkier than the straight oleyl chains of the other lipids we have examined. These methyl groups increase the average distance between adjacent headgroups and therefore yield larger vesicles. It is possible that the increased average distance between the headgroups decreases the hydrogen bonding (acid-anion formation). The hydrogen bonds represent putative attractive forces between the headgroups (Haines, 1983). A decrease in attractive interactions necessarily (because the headgroups are anionic) increases the repulsive interactions. We propose that the balance between attractive vs. repulsive interactions of the inner monolayer (of the forming vesicle) fixes the curvature of the vesicles. Increased repulsion between adjacent headgroups results in larger vesicles; increased attraction (H-bonding) results in smaller vesicles. Phytanyl chains with their bulky methyl groups provoke the formation of larger (750-nm diameter for D Φ PG; Table II) vesicles than does the straight chain lipids (450-nm diameter for DOPG; Table I). It should be noted that the mixture of total lipids of *Halobacterium* yields smaller vesicles than vesicle preparations made from each of the pure anionic lipids. This is presumably due to the fact that the organism contains neutral, uncharged glycolipids (Kates, 1978) as well as anionic lipids. No attempts were made to make vesicles from the glycolipids of *Halobacteria*.

E. coli lipids were also investigated (Table I). In this study we examined analogues of the principal lipids of this organism, namely DOPG, DOPE and DOPS. Each of these yielded uniform vesicles preparations, however DOPS formed remarkably small (30-nm diameter) vesicles above pH 9.0 (data not shown), so that it could not be used to make large unilamellar vesicles above pH 8.5. In surprising contrast DOPE formed a precipitate below pH 8.6, although an excellent preparation of vesicles was obtained at pH 10.6. We did not examine the PE vesicle preparation pattern in the intermediate pH range. The pH sensitivity of these two lipids is presumably due to the protonation of the amino group, which is presumably involved in headgroup-headgroup interactions.

PE from *E. coli* contains largely cyclopropane fatty acids with some monounsaturated fatty acids, esterified to the glycerol (Goldfine, 1982). In the context of the above discussion on the bulkiness of the phytanyl chains, we suspect that the larger vesicles of the *E. coli* PE result from a greater average distance between the headgroups (compared to DOPE) caused by the slightly bulkier of the cyclopropane chains compared to that containing a double bond.

The observation of that increasing the mole fraction of cholesterol yields increasing vesicle sizes in 150 mM KCl suggested that the separation of anionic headgroups provoked larger vesicles. In order to explore this possibility we chose three structural analogues of cholesterol (lanosterol, epicoprostanol and epicholestanol) to conduct an analogous study. The lateral separation of the headgroups would be expected to be the greatest for epicholesterol, which displays a significant tilt (Murari et al., 1986) in PC bilayers. This is due to the fact that the 3-hydroxyl is on the α -face and that there are no other protuberances from the α -face on the sterol nucleus. Lanosterol has methyl

groups on the α -face but its 3-hydroxyl is β . Its capacity to separate headgroups would presumably be slightly greater than that of cholesterol. Epicooprostanol, however, has *both* an α -hydroxyl at position 3 *and* a *cis* fusion between rings A and B. This double distortion would be expected to straighten out the sterol but separate lipids more than lanosterol and thus give intermediate sizes. This is what is observed in Figure 10 for the three sterols. It might be noted in the figure that the two episterols show dramatically enhanced sizes (emphasizing *headgroup* separation as a feature for adjusting vesicle size in the method for preparing vesicles) up to 20 mole percent. Increasing the mole fraction beyond that level does not increase the vesicle size suggesting either that the sterols interact with other sterols at high mole fractions or that the sterols were not incorporated into the vesicles. The latter appears likely since the size steadily increase (Figure 10) for the lanosterol- and cholesterol-containing vesicles at least up to 50 mole percent.

Thus an analysis of vesicle sizes in the presence of selected cholesterol analogues is consistent with the interpretation given in this analysis of the relationship between vesicle size and the area per molecule occupied by the lipids.

(2) *Vesicle Preparations in Non-saline Media.* It can be seen from the data in Tables IV, V and VI, and displayed in Figure 12, that the salt concentration has a significant impact on the size of the vesicles prepared by the pH adjustment method.

We have generally found smaller vesicles in preparations made in non-ionic impermeants and so we decided to compare the sizes of vesicles made in mixtures of sucrose and KCl solutions (Figure 12). It may be seen that the vesicle size increases in a linear manner with the KCl component of the KCl-

sucrose mixtures. This does not, however, establish that it is salt alone that establishes the size difference. It is entirely possible that sucrose interacts in some specific way with the phospholipid headgroups. Such interaction has been proposed for trehalose (Crowe & Crowe, 1984). For this reason we used 0.25 M pentaerythritol to conduct experiments on DOPG vesicle synthesis. The shape of this molecule makes it unlikely that it interacts uniquely with phospholipid molecules. The results of these experiments (Table V) and of experiments using trehalose, indicate that the use of any non-ionic membrane impermeant for adjusting the osmolarity yields vesicles that are smaller than those produced in 0.15 M KCl. Indeed vesicles synthesized in pentaerythritol are nearly as small as those produced in water.

(3) *Unilamellar Vesicles Obtained from Single-chain Lipids.* Vesicles were also prepared from two single-chain amphiphiles: pure oleic acid and an equimolar mixture of dodecyl sulfonate and dodecanol. Although these "lipids" are not natural membrane lipids, they have been used in several attempts to make unilamellar vesicles from them. This work is largely motivated by the discovery of a class of chlorosulfolipids (alkyl disulfate lipids) that constitute the flagellar membrane of the phytoflagellate, *Ochromonas danica* (Chen & Haines, 1976; Chen et al., 1976; Haines, 1973).

Liposomes (oligolamellar particles entrapping water) were first made of oleic acid by Gebicki and Hicks (1973,1976). Hargreaves and Deamer (1978) elaborated and further characterized these liposomes and showed that similar liposomes could be made from alkyl sulfates and alcohols. The liposomes are generally made by adjusting the pH from the micellar pH range (above pH 10) down to neutral pH. Haines (1983) explained the basis for stability of these liposomes on the basis of acid-anion formation and rapid exchange of the acid-

anions. Acid-anion pairs are uncoupled, in this view, by protonation of the pair, each protonated carboxyl protonates a neighboring acid anion. The timescale for these events would be faster than 10^{15} per second so that the statistical consequences of acid-anion formation is that acidic H-atoms (not hydronium ions) provide attractive forces (via unique H-bonding) between the negatively charged headgroups. The attractive interactions are therefore due to the presence of non-hydrated protons in the headgroup domain (Haines, 1983). The preparation of unilamellar fatty acid vesicles is novel, as is the preparation of unilamellar vesicles from the dodecyl sulfonate:dodecanol mixture. We used a different approach to making the vesicles than that used by Hargreaves and Deamer (1978), in that we first made a film of protonated fatty acids and then suspended it in saline while adjusting the pH. In 0.15 M KCl the result is a uniform preparation of large vesicles wherein the size depends upon the salt concentration (Table III). It had earlier been shown (Gebicki & Hicks, 1976; Hargreaves & Deamer, 1978; Haines & Heller, 1982; Haines, 1983) that the liposomes are only stable between pH 7.15 and pH 9.45. We therefore sought vesicles only in this pH range.

The fact that vesicles could be made in the 4 M KCl (Table III) suggested that it might be possible to make vesicles in sea water. Earlier attempts to make liposomes of free fatty acids in sea water failed presumably because the calcium in the sea water precipitates the fatty acids at high pH. Our attempts in three different sea waters each resulted in vesicles although these preparations, like that in 4 M KCl displayed high polydispersity.

Membrane Elastic Property Measurements by Osmotic Swelling

(1) Measurement of the Membrane Modulus by Osmotic Swelling. Liv-

ing cells transport ions as a major component of their energy transduction. Such ion movements necessarily generate concentration gradients with their resultant osmotic pressure deformations of the entrapping membrane. Knowledge of the elasticity of lipid bilayers and of their elastic limit is necessary for a full understanding of ion transport in cells. Measurement of the membrane modulus had previously been undertaken by two methods, membrane capacitance measurements (Alvarez & Latorre, 1978) and micropipet aspiration of a small portion of a membrane (Rand, 1964). The latter method has greater applicability to stress in living cells but a large deformation is required in order to obtain the measurement. The Rand approach has been used by Kwok and Evans (Kwok & Evans, 1981; Evans & Kwok, 1982; Evans & Needham, 1986) on large (10-20 μ) vesicles made from DMPC (sometimes mixed with DMPE). These investigators use the expression "compressibility modulus".

Osmotic swelling of vesicles is a useful method for studying the membrane modulus because (1) it has immediate applicability to the membranes of living cells; (2) bilayers made of synthetic lipids can be used to explore the impact of specific molecular features on the membrane modulus, elasticity and its linearity; (3) solution conditions (ionic strength, range of osmolarity, pH, etc.) can be varied to establish their impact on elasticity; (4) the impact of stress on lipid (and protein) conformation and dynamics can be studied using physical methods on uniform preparations; and (5) many fundamental features of *membrane* structure and dynamics can be explored.

Osmotic pressure has only recently been considered from the point of view of its effect on membranes (Gruner et al., 1985). It is so important to living cells that *E. coli* regulates its osmolarity in the periplasmic space by a

group of membrane-derived oligosaccharides (MDO) (Kennedy, 1982) which in turn regulate phospholipid turnover (van Golde et al., 1973).

Obtaining the membrane modulus by osmotic swelling requires a uniform preparation of unilamellar vesicles in a size range appropriate for such studies. Calculations showed (Figure 6) that the appropriate vesicle size for measuring a modulus of 10^7 or 10^8 is in the 100 to 600-nm diameter range. Uniform preparations of unilamellar vesicles have only been available in much smaller sizes (20-100-nm diameter). We recently developed a procedure for making uniformly sized unilamellar vesicles in a range of choice from 200 to 3000-nm diameter.

The vesicle size was found to increase linearly in discrete increments during 8 to 10 steps of concentration dilution (Figures 13, 14, 15, 16 and 17). The observed membrane moduli shown in each of these dilution experiments are calculated from the experimental results using equation (8). They were chosen because they align closely with the data points ("best fit"). It is possible, nonetheless, that the increase in the average size of the vesicles during dilution is not due to the stress of swelling but to a shape variation which results in spheres at its maximum size. Such shape changes were suggested during an osmotic swelling study of vesicles in the size range of 40-nm diameter (Lichtenberg et al., 1981). It should be noted that the KCl concentration range used in that study was of the order of 10 times that of this study. Furthermore, the elastic limit as established in the present study appears to be exceeded in the Lichtenberg et al. study. Finally, the vesicle size they used was so much smaller than that of the present work that their conclusions may not be applicable. Kwok and Evans (1981; Evans & Kwok, 1982) found a surface area increase of 2-3% for DMPC vesicles in the 10-20 μ size range. We find

the surface area increase to be 3-5% for the DOPA and DOPG (anionic lipid) vesicles that are 0.2-0.6 μ size range in 0.15 M KCl. The vesicles in the same size range show a 5-10% surface area increase in 0.25 M non-saline medium. It should also be noted that the observed membrane modulus increases with the size of the vesicle in our study and % surface area increase decrease with the increasing vesicle size. All of these data are consistent with the work of Evans and Kwok.

Before concluding that the linear increments in observed size are due to surface expansion, and not due to a non-spherical equilibrium configuration that become spherical upon dilution of the KCl, we examined the polydispersity during swelling. In the typical results of a swelling experiment shown in Table VII it can be seen that the polydispersity remained constant throughout the experiment. Identical results were obtained in all of the swelling experiments. Had large changes occurred in shape of the vesicles upon dilution, the apparent polydispersity would have changed. We therefore conclude that the vesicles were spherical throughout the experiments. This is consistent with our observations in electron microscopy (Figure 11). The observed increase in internal volume is therefore assumed to be due to stretching of the bilayer.

Experiments designed to examine the effect of cholesterol on the observed membrane modulus in both KCl (Figure 13) and sucrose (Figure 14) preparations showed that the presence of cholesterol at concentrations up to 25 mol % had no significant effect. Because vesicle size in KCl was dependent upon cholesterol content, a special preparation of vesicles (Figure 13, experiment C) was made (400-nm diameter) in the size range of cholesterol-containing preparations as a control, these exhibited an only slightly smaller observed membrane modulus than the cholesterol-containing preparation (Figure 13, ex-

periment E) of the equivalent size. Likewise cholesterol-containing vesicles made in sucrose displayed the same observed membrane modulus (Figure 14) as those lacking cholesterol.

(2) *Measurement of the Elastic Limit.* After 8 to 10 size measurements during dilution in which stepwise swelling was observed each of the samples displayed a plateau in the swelling curve. The vesicles appear to have been ruptured at the break but they did not return to their original unstressed size. Measurement of the sizes at each point on the plateau allowed us to calculate the size expected if the appearance of the plateau did indeed represent the elastic limit, the calculation is based on the following assumptions:

(1) That the initial quantity of diluent (KCl or non-saline diluent) that was trapped in the vesicles remained there throughout the swelling.

(2) That the vesicle is ruptured at its elastic limit and returned to its unstressed (initial) size.

(3) That the solution ejected at the rupture contained the same concentration of diluent as was present in the vesicle at the time of the rupture.

(4) That the amount of solution ejected is equal to the change in volume from the swollen state (just before the elastic limit) to the unstressed vesicle state.

(5) That the vesicle reseals immediately at the unstressed state and re-swells to the appropriate size predicted by its observed membrane modulus.

The results of this calculation are shown in Table VIII. We find that the amount of ejected solution is insufficient, at the inside concentration of the diluent, to equilibrate the vesicle with the external concentration. Each of the points in Table VIII represents a ruptured vesicle preparation that swells beyond its elastic limit. The calculated sizes based on the above assumptions,

all of which are reasonable, show remarkable agreement with the experimental data. We have therefore taken the last dilution on the observed membrane modulus as the size of the vesicle at its elastic limit.

Figure 20 uses the sample data in Table VII to display the surface expansion and the transbilayer pressure difference during an expansion of the vesicles. The transbilayer pressure difference is calculated using the assumption that the internal quantity of KCl remains that which was trapped in the vesicles at the start of the experiment although it is diluted during the vesicle expansion. The surface expansion reaches a maximum at the transbilayer pressure difference of 43 mosmols. We have assumed that the burst vesicles release trapped KCl (see Table VIII and discussion above) in order to obtain the decreased transbilayer pressure difference (equation (2)) shown in the full circles. The dashed circles assume no KCl has been removed from the vesicles, but the decreased size of the vesicles suggest that this is not the case. The same kind of data was calculated and shown on Figure 21 in order to establish that the vesicle preparation of oleic acid are unilamellar (see RESULTS, (2) Preparations of Uniform Vesicles with Anionic Lipids).

Table IX shows the elastic limit in terms of increased surface area for all of the experiments reported here. Vesicles swelled in sucrose display substantially larger elastic limits than those in KCl. This includes both the synthetic phospholipid vesicles and the submitochondrial particles. By and large the elastic limit (maximum area increase before bursting) was found to decrease with increasing initial size while the observed membrane modulus was found to increase with increasing size. Therefore the elastic limit varies inversely with the observed membrane modulus. The stiffer the vesicle the smaller the expansion before it bursts, (i.e., a large modulus implies a smaller elastic limit).

Figure 20. Transbilayer pressure difference (○) during the osmotic swelling of the vesicles described in Table VII (also Figure 14, curve E). KCl trapped inside the vesicles at the start of the swelling experiment was assumed to remain in the vesicle as it expanded. The concentration difference is used to calculate the transbilayer pressure difference using Equation 2, the dashed circles assume that the vesicle did not burst whereas the corresponding solid circles are calculated based on the assumptions used for the Table VIII bursting and reswelling. The surface area expansion (Δ) suggests by the decrease in size at 43 mosmols that the vesicles have burst. The vesicles (390 nm) are DOPA containing 15 mole % cholesterol.

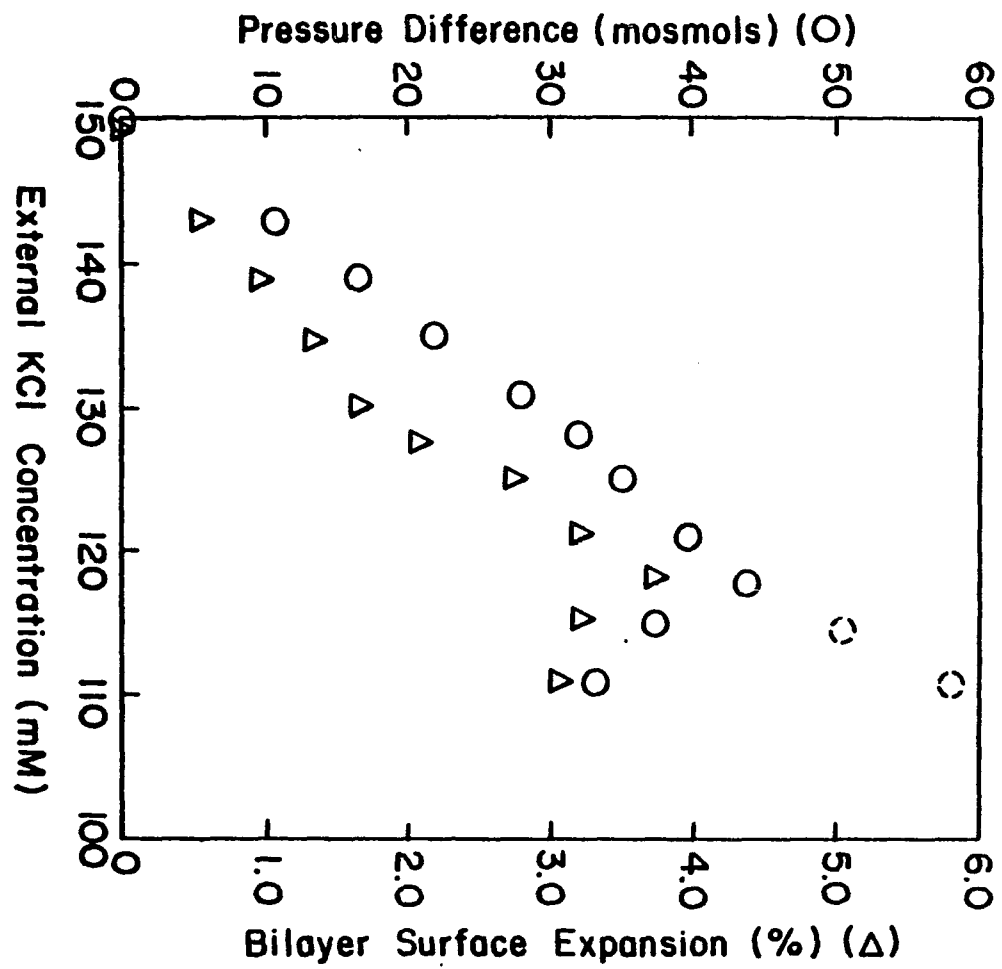
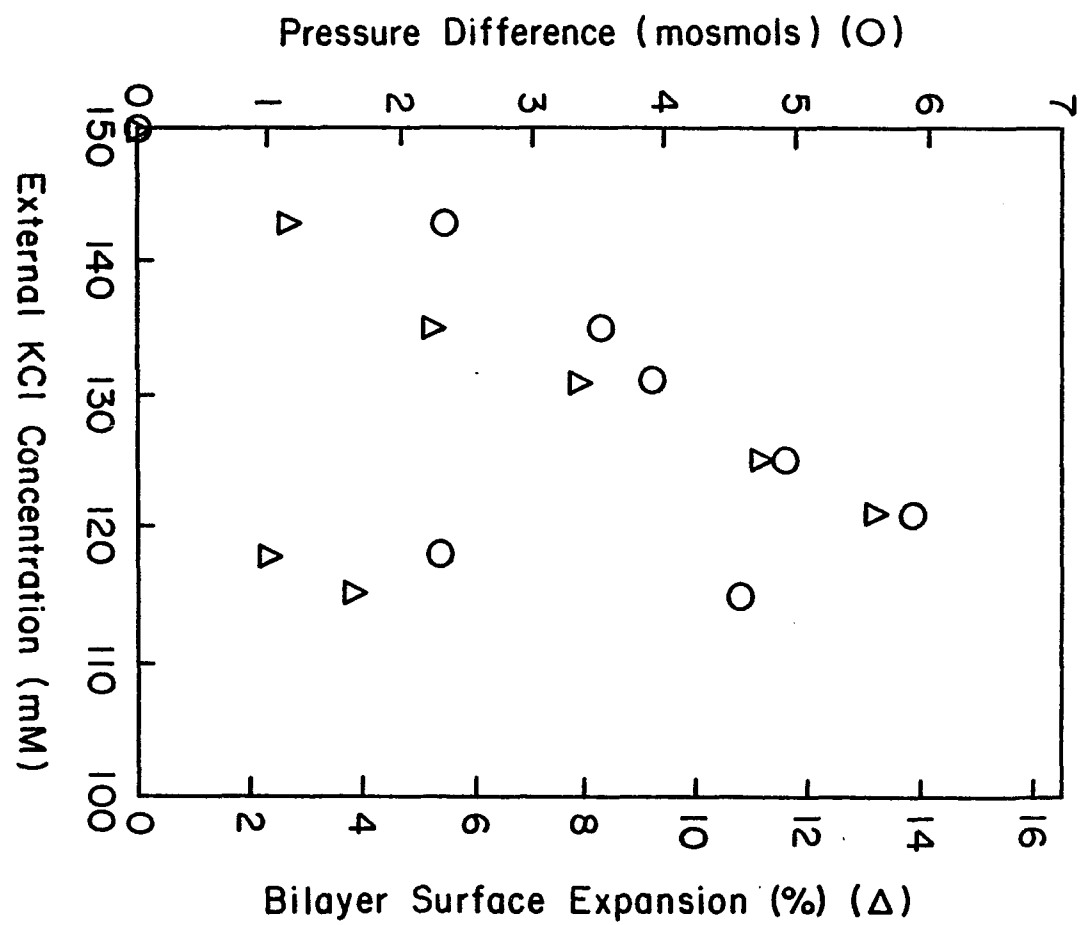


Figure 21. Transbilayer pressure difference ($\bigcirc$) during the osmotic swelling of oleic acid vesicles (309 nm) in 0.15 M KCl. KCl trapped inside the vesicles at the start of the swelling experiment was assumed to remain in the vesicle as it expanded. The concentration difference is used to calculate the transbilayer pressure difference. The surface area expansion (Δ) suggests by the decrease in size at 5.9 mosmoles that the vesicles have burst.



The transbilayer pressure difference is significantly greater for vesicle swollen in KCl than for those swollen in non-saline medium.

The studies of "flicker phenomena" on synthetic vesicles by Servuss et al. (1976) first yielded a bilayer bending modulus for vesicles from which an elastic modulus could be obtained. The recent work of Evans (Kwok & Evans, 1981; Evans & Kwok, 1982; Evans & Needham, 1986) has expanded this work and applied it to zwitterionic model membrane systems. Our work cannot properly be compared to the work of Evans since our vesicles are not made of zwitterionic lipids. This factor appears to dominate the modulus in KCl. The equivalent membrane modulus obtained for ~ 10 micron phosphatidyl choline vesicles by Kwok and Evans (1981) ranges from 5×10^8 dyn/cm² to 2×10^9 dyn/cm² for DOPC and SOPC with or without cholesterol (assuming the membrane is 3.0-nm thick) whereas our largest polyanionic vesicles (DPPG, 0.66 micron) are 5×10^8 dyn/cm². The data show that the ranges are not inconsistent. The present method has the disadvantage of being restricted to negatively charged lipids, while it has the advantages of speed of obtaining results with the choice of vesicle sizes up to the range of cells, organelles and synthetic ion-pumping systems.

(3) *Effects of Media, Ionic Strength and Lipid Headgroups on Elastic Properties of Vesicles.* The finding that the membrane modulus of bilayers constituted of anionic phospholipids is dominated by the ionic strength of the bathing solution is unexpected. It offers an explanation as to why microbes which are tolerant to high concentrations of salt and increase the anionic lipid mole fraction when exposed to the higher salt concentration (Kanemasa et al., 1972; Komaratat & Kates, 1975; Ohno et al., 1976; Thirkell & Summerfield, 1977; Ohno et al., 1979; Hiramatsu et al., 1980; Kogut & Russell, 1984; Hanna

et al., 1984; Miller, 1985). *E. coli*, at the moderate increases of salinity that are permissible, showed no change in the phospholipid headgroups, but an increase in the cyclopropane content of the hydrocarbon chains (McGarrity & Armstrong, 1975).

Why would microbes increase the anionic lipids fraction of the plasma membrane when faced with an increase in salt concentration? The increase in the charge density of the bilayer surface would, according to the data presented here, restrict the expansion of the bilayer (reduce the increase of the surface area per lipid molecule during osmotic stress) and at the same time increase the transbilayer pressure necessary to burst it. Both are important for retaining the integrity of the cell. At the present it is not known if a bilayer thins during expansion by osmotic stress. Whether or not it does, an expansion of its surface area may be expected to alter the conformation of proteins embedded in it. Resistance to expansion by altering the modulus is a defense against the distortion of protein conformation in the plasma membrane. Obviously the simultaneous increase in the ΔP resulting from the greater surface charge density implies that greater transbilayer pressure differences are necessary to burst the bilayer and leak proteins from the cell. It might also be noted that a high content of negatively charged lipids is generally found in halophilic bacteria (Peleg & Tietz, 1971; Stern & Tietz, 1973) including extreme halophiles (Kates et al., 1966; Hancock & Kates, 1973; Evans et al., 1980).

Another surprising result we obtained is the unusually high membrane modulus of DOPE vesicles. This lipid is not normally considered an anionic lipid. The unusual modulus could result from H-bonding between headgroups (Hitchcock et al., 1974; Plates et al., 1986). DOPE does not form vesicles above pH 8.6, which implies a pK in that region and that below this pH

the headgroup is protonated. In the studies just cited on lipid compositional changes in the membranes of gram negative bacteria exposed to high salinity, cardiolipin was increased at the expense of PE (Kanemasa et al., 1972; Ohno et al., 1976; 1979; Hiramatsu et al., 1980; Kogut & Russell, 1984; Hanna et al., 1984; Miller, 1985).

(4) *Curvature and the Membrane Modulus.* There seemed to be a trend shown in the data (Table IX and XII) that the observed membrane modulus varies inversely with the curvature. Although the trend is by no means linear it is apparent in both the 150 mM KCl and the 250 mM non-saline medium swelling experiments. We have attempted to explain this data in the theory section considering the elastic modulus, the bending elastic modulus and surface tension. Tolman (1949) showed that the surface tension of a spherical surface varies with its curvature. He showed the relationship to be

$$\frac{\sigma}{\sigma_0} = \frac{1}{1 + \frac{2\delta}{R}}$$

where σ is surface tension, δ is a constant and R is the radius of the sphere. To obtain a lowering of surface tension of say 4%, a value of 0.02 is necessary for δ/R , thus δ is of the order of magnitude of 10^{-8} cm, too small to explain our observations. The application of Tolman's theory to our system therefore cannot explain our data on the variation of the membrane modulus with curvature. Nonetheless our data suggest that the magnitude of the observed membrane modulus is best explained by surface tension and that, surprisingly, it is not due to the inherent elastic modulus nor to the bending energy.

The energy per unit area at the surface (surface tension) includes both hydrophobic interactions and electrostatic contributions due to the surface charges. All of our measurements are conducted on negatively charged lipid

systems. (Although the submitochondrial particles contain both zwitterionic and anionic lipids and proteins, the net surface charge is negative.) The fact that the observed membrane modulus is substantially (an order of magnitude) higher in 150 mM KCl than in 250 mM non-saline medium suggests that charges may dominate the magnitude of the modulus we are observing. The suggestion that anionic charges have much to do with membrane elasticity is consistent with the observations of Kogut and Russell (1984) that the adaptation of *Vibrio costicola* to high salt medium is associated with an increase in the PG component of the membrane phospholipids at the expense of PE, a zwitterionic lipid. They also noted that reduction in the salinity reversed this adaptation. Furthermore all of the obligate halophiles contain only anionic lipids, no zwitterionic. A halotolerant Gram-negative organism, *Planococcus* sp., increases its anionic lipids component in response to increased osmolarity (Miller, 1985). This paper contains many references to other work indicating similar observations.

A second area of interest with respect to cell osmolarity and negative charges on the surface is the MDO system of *E. coli* (Weissborn & Kennedy, 1984; Kennedy, 1982). A decrease in the osmolarity of the culture medium (from 300 mosmols, which is the osmolarity of the cytosol (Stock et al., 1977) to 50 mosmols) provokes *E. coli* to produce polyanionic oligosaccharides on the periplasmic side of the plasma membrane to the extent that they are attached to the lipids they could stiffen the membrane. Comparisons of the membrane modulus of lipid vesicles with different surface charge densities and variations in the ionic strength and surface pH may help to explain the contributions to the surface energy of hydrophobic interactions, double layer effects, spherical field effects and headgroup interactions. Such experiments will require sys-

tematic studies.

(5) *Bilayer Thickness Changes during Osmotic Swelling of Vesicles.*

The fact that a vesicle expands on swelling leaves at least two alternative molecular descriptions of the expanded state. In one, the bilayer thins as a consequence of its expansion retaining a constant membrane volume. This is the basis of much of the calculations of Evans and Hochmuth (1978) and is suggested by the very small thickness changes found in bilayers under pressure (Lis et al., 1982 and references therein) and in the capacitance measurements of bilayers (Alvarez & Latorre, 1978). Thinning the bilayer means that the distance between the headgroup sheets decreases. This is only possible if there is either an increase in the tilt of the chains, which would increase the order, or an increase in the statistical number of *t-g* isomerizations per chain which implies an enthalpy increase. The latter would be expected to decrease the order parameter of each methylene. This latter description is consistent with an increase in the fluidity (see INTRODUCTION) during vesicle expansion while maintaining a constant membrane volume (thinning the bilayer). A second possible consequence of vesicle expansion is that the bilayer does *not thin* but remains at constant thickness. In this case, the bilayer increases its volume as a response to osmotic swelling of a vesicle. This would be possible if water entered the bilayer. There is no other apparent way for the bilayer volume to increase. This is also consistent with the increase in fluidity that accompanies lateral expansion of the bilayer. Our x-ray data of membrane thickness during osmotic swelling of the vesicles support the second possibility.

(6) *Membrane Modulus of Biological Membranes.* Mechanical meas-

urements had been conducted on erythrocyte membranes (Seifriz, 1926; Norris,

1939) and on the membrane of sea urchin eggs (Cole, 1932; Mitchison & Swann, 1954) using ingenious methods and what have turned out to be reasonable assumptions. Only recently, however, it has been recognized that many of the membrane's properties that were being examined were actually derived from the cytoskeleton rather than from the bilayer (Evans & Waugh, 1980; Mohandas et al., 1983).

In a biological membrane the bilayer not only includes the lipids but also includes those proteins (transport proteins, receptors, etc.) that occupy significant space in the membrane continuum. Studies of the mechanical properties of biological membranes in which the cytoskeleton is not considered but only the membrane bilayer is examined, are comparable to studies on bilayer vesicles made of synthetic or extracted lipids as in the present work. This can be seen from observed membrane moduli obtained for a number of biological membranes (Table XV) using variety of techniques and comparing these data to those in Table XVI which were obtained with synthetic bilayers. All of these studies report moduli in the range of 1 to 90×10^7 dyn/cm². This consistency is striking because Waugh and Evans (1979) found the elastic bilayer modulus for the erythrocyte to be 4 orders of magnitude higher than the elastic shear modulus of the cell which is a measure of the deformation of the cytoskeleton during osmotic and mechanical stress.

The elastic modulus of the bilayer has been measured in a variety of ways. The kinetics of osmotic swelling just prior to hemolysis was used by Katchalsky et al. (1960) to calculate a modulus. An elastimeter (drawing a membrane into a micropipette under a microscope) was developed by Mitchison and Swann (1954). The device was used on the erythrocyte by Rand (1964; Burton & Rand, 1964), Evans et al. (1976), Sung & Chien (1978), and

Table XV. Membrane Modulus of Biological Membranes.

Membrane examined	Method used	Modulus as ($\times 10^{-7}$) dyn/cm ²	Reference
Erythrocytes	Kinetics of osmotic swelling	2.4	Katchalsky et al. (1960)
Erythrocytes	pipette aspiration	22.0	Evans et al. (1976); Waugh & Evans (1979)
Erythrocytes	pipette aspiration	22.0	Dimitrov et al. (1978)
Rye protoplasts	pipette aspiration	12.0	Wolfe & Steponkus (1983)
Submitochondrial particles	osmotic swelling (PCS)	3.0	This work
Brush border vesicles	osmotic swelling (PCS)	20.0-29.0	S. Miyamoto (personal communication)
<i>E. coli</i> Lac permease vesicles	osmotic swelling (PCS)	90.0	This work

Table XVI. Comparison of the membrane modulus, the pressure difference (ΔP) across the membrane at bursting and the surface expansion of synthetic lipid bilayers.

Method	Bilayer	Medium	Modulus ($\times 10^{-7}$ dyn/cm ²)	ΔP mosmoles	% surface expansion	Reference
Stressed BLM	chol-HDTA	40 mM KCl	7.5-15.0	-	-	Wobschall (1971)
BLM Capacitance	PS	100 mM NaCl	10-15	-	-	Alvarez & Latorre (1978)
	PE	1 M KCl		-	-	
Pipette Aspiration 10 μ vesicles	EPC	100 mM NaCl	7	-	2-3	Kwok & Evans (1981)
426 nm vesicles PCS	DMPC	350 or 150 mM LiCl	33.0	-	< 1	Sun et al. (1986)
53.0 nm vesicles PCS	DMPC	350 or 150 mM LiCl	3.4	-	> 5	Sun et al. (1986)
PCS	DMPC DOPC	150 mM KCl	15	-	-	Hantz et al. (1986)
PCS	DOPA	150 mM KCl	40-90	40-46	3-5	This work
PCS	DOPA	250 mM Sucrose	1-3	4-6	8-10	This work

Dimitrov et al. (1978). It was used on cancer cells by Weiss (1968), on neutrophils by Sung et al. (1979; 1982), on giant algal cells by Kosuge & Tazawa (1979), and on rye protoplasts by Wolfe & Steponkus (1981; 1983). Only those studies that reported a modulus in a manner that could be compared to the others are reported in Table XVI. Special analysis of the surface undulations (flicker phenomenon) of the erythrocyte has been used by Brochard & Lennon (1975) to measure their curvature elastic properties, including a study under osmotic stress (Fricke & Sackmann, 1984). The curvature elasticity can be converted to the elastic modulus by making a few reasonable assumptions (Evans & Hochmuth, 1978). This method has been used to study phospholipid bilayers (Servuss et al., 1976) which displayed precisely the same modulus as that of the intact erythrocytes. The latter finding is striking since the live erythrocyte's membrane deformability (elastic shear modulus) appears to be dominated by its contact with the cytoskeleton and yet the undulations observed by Brochard & Lennon (1975; Fricke & Sackmann, 1984) on erythrocytes appear to be due to the bilayer alone because they are equivalent in frequency to the synthetic lipid bilayers of Servuss et al. (1976). Deformation of the erythrocyte during osmotic swelling may have to do exclusively with the spectrin network and have nothing to do with the contact between the cytoskeleton and the bilayer. Such a model has been suggested by Stokke et al. (1986).

Osmotic swelling of the erythrocyte membrane allowed Katchalsky et al. (1960) to estimate the modulus as 2.4×10^7 dyn/cm² as quoted in Table XV. This value is lower than the other values reported in the table except for the sub-mitochondrial particles. The latter may be due to the fact that the sub-mitochondrial particles are isolated in 250 mM sucrose which consistently

gives a lower modulus. All of other results given in the Table XV were obtained in 100-150 mM salt. The values obtained in these studies are intermediate between our results for non-electrolyte impermeants 1 to 5×10^7 dyn/cm² and the values of 30 to 80×10^7 dyn/cm² that we get for vesicles swollen in 150 mM KCl. [The exceptions to this statement are DOPS (measured at high pH) and oleic acid.] Furthermore, from the calculation of Evans & Hochmuth (1978), the quantitative agreement of Brochard & Lennon (1975) and Frick & Sackmann (1984) both using the flicker phenomenon approach enhances the general agreement. Finally, the statistical mechanical theory of Marcelja (1974) predicts an elastic modulus of 7.5 to 10×10^7 dyn/cm², and Israelachvili et al. (1976) derive from surface tension a modulus of 10×10^7 dyn/cm². (These values are recalculated to dyn/cm².)

The studies of Wolfe & Steponkus (1983) on the modulus and mechanical properties of rye protoplasts have been followed by an interesting series of papers (Wolfe et al., 1985; 1986) on the time-dependent response of the membrane bilayers to osmotic stress. Special emphasis in the most recent paper (Wolfe et al., 1986) has been on the greater percent surface expansion observed from cold-acclimated plants. Protoplasts derived from non-acclimated plants display a surface expansion of between 2 and 3 %, whereas protoplasts derived from acclimated seedlings allows a 6 to 8 % surface expansion before bursting. The combination of the greater expansion, the fact that the kinetics of the expansion is different, and the irreversibility of the slower expansion, provoke them to conclude that there exists in the protoplasts a reservoir of lipids that enter the bilayer during osmotic stress only. Some of this data could be interpreted, in the context of our results, as being due to a change in the ionic strength of the medium due, for example, to accumulation of sugar in the

medium of the cold-acclimated protoplasts, and/or a change in the lipid head-group composition.

The membrane modulus in Table XV is recalculated to dyn/cm^2 for comparison. Studies on membrane tension that are not included are Mitchison and Swann (1954) on sea urchin eggs; Weiss (1968) on transformed cells; Rand (1964; Burton & Rand, 1964) on erythrocytes; Sung et al. (1983) on neutrophils; and Kosuge & Tazawa (1979) on giant algal cells. All of these were conducted with the pipette aspiration technique of Mitchison & Swann (1954) but none reported values that could be directly converted (without making assumptions) to a comparable membrane modulus. Neither ΔP or % surface expansion is obtainable by osmotic swelling experiments with biological-membrane-derived vesicles since the initial interior osmolarity of any such vesicle preparations can not be known with precision. Evans et al., (1976) found 2-4% surface expansion for the erythrocyte and Wolfe & Steponkus (1983) found 2% for rye protoplasts. Both groups used the pipette aspiration technique. Shigeaki Miyamoto prepared vesicles of rat small intestine brush border membranes using 0.2 mM MgSO_4 in 200 mM mannitol buffer (1 mM Hepes) at pH 7.4. Surface expansion (4-6%) of these vesicles was observed at bursting.

(7) *Comparison of Membrane Elastic Properties of Synthetic Lipid Bilayers.* Measurements on the elastic constants of bilayers prepared from synthetic or extracted lipids shown in Table XVI include those of Wobschall (1971) (changes in capacitance of a BLM as a function of time during sinusoidal expansion), Servuss et al. (1976) (flicker phenomenon calculated from the curvature elasticity as indicated), Kwok & Evans (1981), Sun et al. (1986), (Measurements of the modulus for vesicles of five different sizes have

been made; only the 48.5 and 53.0-nm diameter vesicles which appear not to be curvature-limited are reported.) Hantz et al. (1986), and our group. All of these studies yielded the same range of values as that found for the natural membranes or membrane vesicles as in Table XV for natural membranes. Wobschall (1971) obtained the result using the detergent, HDTA, in combination with cholesterol. Thus the modulus of natural membranes seems indeed to reflect the lipid bilayer. It is therefore useful to examine the bilayer modulus to learn about the energetics of a lipid bilayer under stress and to seek information about lipid dynamics and lateral headgroup interactions.

H⁺/OH⁻ Currents Across Lipid Bilayers

The permeability studies were conducted on uniform preparations of vesicles that are in the size range of 300 to 700-nm and of vesicles (liposomes) obtained from natural membranes. The latter are generally in the same size range and relatively uniform. Since the original finding of a high H⁺/OH⁻ permeability by Nichols et al. (1980) there have been some 17 studies on the "proton currents" of synthetic bilayers, all cited above. Three were on black lipid membranes (BLM's) and another five were on sonicated vesicles (SUV's) which Perkins & Cafiso (1986) have shown that they display lower rates of transport than the vesicles of sizes comparable to those isolated from natural sources. Of the remaining studies about half were conducted on vesicles which had been exposed to low concentrations of detergents (octyl glucoside, cholate, etc.).

From the point of view of studying proton pumps in synthetic vesicles the problem of a large Δ pH is often inherent in the experimental design. This is because the rate of pumping for each protein is of the order of 1 msec/proton pumped or 1000 protons/sec per protein under optimal conditions.

This, taken together with the small volume into which the protons are being pumped creates a substantial proton gradient. In the studies of Hinkle et al. (Krishnamurthy & Hinkle, 1984; Hinkle & Yu, 1979; Berry & Hinkle, 1983) in which synthetic vesicles were used as controls for mitochondrial membrane gradients, the ΔpH was 0.5-4.0 pH units. Our studies were therefore designed to examine this ΔpH range although it is known that in this range there can be a considerable limitation on the rate due to limited counterion diffusion (Deamer & Nichols, 1983; Elamrani & Blume, 1983).

From the combination of the many studies cited above a picture of the parameters of H^+/OH^- permeability is emerging. At first, it appeared that there was a large range of permeabilities being reported (Nozaki & Tanford, 1981), but if one eliminates the rate-limiting counterion diffusion problem (Deamer & Nichols, 1983) by working with a small ΔpH the results of a large number of laboratories (Nichols & Deamer, 1980; Rossignol, et al., 1982; Deamer & Nichols, 1983; Elamrani & Blume, 1983; Krishnamurthy & Hinkle, 1984; Gutknecht, 1984; O'Shea, et al., 1984b; Perkins & Cafiso, 1986; Grzesiek & Dencher, 1986) are in agreement within a rather narrow range of observed permeabilities (10^{-4} to 10^{-5} cm/s). The permeabilities reported herein are slightly higher, possibly due to the magnitude of the ΔpH which is the magnitude used for studying proton pumping in vesicles this size. The importance of the data with respect to the structure of the lipids is in the *relative* values under the conditions stated rather than in the absolute permeabilities.

(1) *Lipid Structure Effects.* Haydon and Myers (1973) have shown that ion permeability across bilayers is decreased by surface charge. It seems not unreasonable, therefore, that lower conductance of protons/hydroxyl ions would occur in bilayers constituted solely of anionic lipids. A direct comparis-

on of the H^+/OH^- flux in anionic phospholipid bilayers to those of zwitterionic lipids has not been made in this study because the vesicle sizes were not comparable and because the pH adjustment method can only be used to make large, uniform, unilamellar vesicles containing *acidic* lipids. However, we have found lower permeability in DOPG vesicles than in DOPC vesicles. This result may be colored by the observations of Dencher et al., (1986) suggesting that 9-aminoacridine is ineffective for measuring the H^+/OH^- permeability of vesicles constituted of PC because its successful use requires acidic lipids. Since we did not determine the H^+/OH^- permeability with any other technique, so this criticism may therefore be valid. Other results we have obtained suggests that a higher charge density may decrease the H^+/OH^- permeability. This is seen in a comparison of DOPA permeability to that of DOPG. The permeability of DOPA vesicles is smaller by an order of magnitude, whereas DOPA is known (Eibl & Blume, 1979) to have a substantially greater charge density at the surface than DOPG (Watts et al., 1978). Furthermore, dilution of the charge density of DOPG with DOPE (Table XIII) likewise increased the H^+/OH^- permeability by an order of magnitude. Finally, the comparison of DOPS with the other data shows a trend in the same direction.

Since cholesterol inhibits water transport through bilayers (Finkelstein & Cass, 1967; Cass & Finklestein, 1967) and if water is indeed the carrier of the charge (H^+/OH^-) as proposed by Nichols and Deamer (1980) then cholesterol would be expected to inhibit its transport. It is interesting that although we have found a relationship between the permeability inhibition and the cholesterol content of the bilayer, the relationship is by no means linear. It seems that the permeability inhibition is lower than DOPG at 20 mole % cholesterol (Figure 19). This observation is consistent with the phase separa-

tion of cholesterol in phospholipid bilayers. Evidence for the formation of 2:1 (phospholipid: cholesterol) domains that coexist with pure phospholipid domains [see Presti et al. (1982)] has been obtained by x-ray diffraction (Engelman & Rothman, 1972), permeation (Tsong, 1975; Blok et al., 1977), complement fixing activities at membrane surfaces (Humphries & McConnell, 1975), surface pressure measurements (Tajima & Gershfield, 1978), and lateral diffusion (FRAP) measurements (Rubenstein, et al., 1979). Several observations that relate to a break in the behavior of cholesterol-containing bilayers at 20 mole % are explained by Monte Carlo simulations (Snyder & Freire, 1980) which show that the 2:1 domains have a maximum area of boundary phospholipid at 20 mole % total cholesterol content. Tsong (1975), for example, found that a change in permeability occurred at 17 mole %, which is consistent with our shift at 20 mole %. We cannot explain the gradual decrease in permeability above 30 mole % cholesterol.

An important observation in this study is that H^+/OH^- permeability is the same as that of the natural membrane vesicles when the synthetic vesicles contain 30 mole % cholesterol. It should be noted that this is the mole % found in the *D. salina* vesicles although the sterols are 7-dehydroporiferasterol and ergosterol (5%) and their peroxides (27%) (Sheffer et al., 1986) rather than cholesterol.

A direct comparison of the H^+/OH^- permeability of membrane containing phytanyl chains as compared to those containing straight chain lipids has not been made. Nor has such a comparison been made to our knowledge with respect to water permeability. This is a little unusual in view of the ready availability of purple membrane and the widespread investigation of this system. Two of the above cited studies on H^+/OH^- permeability used lipids with

phytanyl chains in the course of their work (Cafiso & Hubbel, 1983; Grzesiek & Dencher, 1986). Grzesiek & Dencher used DMPC as a control for their studies on acidic lipids which did not contain phytanyl chains, whereas Cafiso & Hubbell were comparing D Φ PC to EPC which is rich in unsaturated fatty acids. The data show a ten-fold decrease in the proton/hydroxyl permeability of D Φ PG as compared to that of DOPG. Perkins & Cafiso (1986) found a ten-fold greater permeability for DOPC than they did for POPC. Unfortunately, PC and PG cannot be directly compared in our system and the analogous POPG was not available. Nonetheless, we feel the permeability we find for D Φ PG is surprising especially in view of the results in Figure 19. The P of vesicles of D Φ PG as compared to those of DOPG shows the same inhibition as those of DOPG:cholesterol = 2:1 compared to DOPG.

In conclusion, we have found that in addition to the various experimental conditions cited above that are now known to affect P including the increase observed in PC vesicles containing double bonds (Perkins & Cafiso, 1986), other features of phospholipid vesicles affect P. It is decreased in the presence of sterol to a lower level at 30 mole %, it is likewise decreased to the same level by phytanyl chains, it appears to be decreased by the surface charge density, it is decreased by octyl glucoside and by low levels of cholate, whereas higher levels of cholate increase the proton/hydroxyl permeability.

Both DOPE and DOPS characteristically form hexagonal II phase under conditions that DOPC, DOPG and other membrane lipids do not (Cullis & de Kruijff, 1979; Gruner et al., 1985). The "high permeability" results for these lipids might be explained by the occurrence of hexagonal II phase domains, fleeting or stable, in the vesicle bilayer. Considering the uncertainty of the DOPC results, the only high permeability results we obtained were on vesicles

consisting of the two hexagonal-II-forming lipids and the vesicles containing a high molar ratio of cholate. It would not be surprising if there were two different mechanisms for H^+/OH^- movement across the bilayer, one for high permeability and another for "medium" permeability that is reduced by sterol to low permeability.

The correspondence between the inhibition of water permeability by cholesterol and the inhibition of proton/hydroxyl permeability as shown in Figure 19 suggests not only that the two processes are related (as others have found) but it suggests that studies of proton/hydroxyl permeability might also be used to examine the effect of lipid structure on water permeability. One of the possible implications of the decrease of permeability observed for D Φ PG vesicles compared to DOPG vesicles is that the alkyl groups decrease both the water and the proton/hydroxyl permeabilities in the prokaryote membranes that contain them. Likewise the *E. coli* lipids contain cyclopropane groups instead of double bonds as are found in the DOPG and DOPE mixture. One possible function of such alkyl group modifications by prokaryotes is that they alter the P of both protons/hydroxyl ions and water. It should be noted in this context that sterols are generally absent from prokaryotes and Figure 19 suggests that one role for sterols may be to decrease the permeability of protons/hydroxyl ions and/or water. Prokaryote lipid chains typically contain alkylated modifications (Goldfine, 1982) such as the cyclopropane group, phytanyl chains, *iso*-, and *anteiso*- fatty acids [which have a branch at the end of the chain (Kaneda, 1977)] and many others. It is also interesting to note that such modifications are always present in the chains of extreme alkalophiles and acidophiles. One outstanding example is the lipids of the acidothermophilic bacterium, *Bacillus acidocaldarius*, 80% of which contain a cy-

clohexane ring at the end of the fatty acid chains (Oshima & Ariga, 1975; Endo et al., 1982).

(2) *Detergent Effects.* Much has been made of detergents as disruptive agents in studies of permeability. We therefore examined the effect of four different solution concentrations of octyl glucoside and cholate on H^+/OH^- permeability. These levels are in the range often used to isolate membrane proteins and reconstitute them into vesicles. Octyl glucoside was found to decrease the proton/hydroxyl permeability of DOPG vesicles in proportion to its concentration (Table XIV). The permeability in the presence of cholate was not significantly affected by the lowest levels but did increase at higher levels of the detergent (Table XIV).

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