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Photon correlation spectroscopy of synthetic phospholipid vesicles during osmotic swelling

Rutkowski, Christopher Anthony, Ph.D.

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PHOTON CORRELATION SPECTROSCOPY OF SYNTHETIC PHOSPHOLIPID VESICLES DURING OSMOTIC SWELLING

by

CHRISTOPHER ANTHONY RUTKOWSKI

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.

1991

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

PHOTON CORRELATION SPECTROSCOPY OF SYNTHETIC PHOSPHOLIPID VESICLES DURING OSMOTIC SWELLING

by

Christopher Anthony Rutkowski

Advisor: Professor Thomas H. Haines

Osmotic swelling experiments were conducted on a variety of preparations of "uniform", unilamellar vesicle systems. The synthetic lipid preparations included both vesicles produced by extrusion through polycarbonate ultrafiltration membranes and vesicles produced by the pH adjustment method. The vesicles were monitored by photon correlation spectroscopy during swelling as the osmolarity of the external solution was decreased. Large unilamellar vesicles produced from acidic lipids using the pH adjustment technique were highly polydisperse and did not swell in a manner that permitted the computation of an elastic modulus, presumably due to the polydispersity of the preparations. Analysis of osmotic swelling of extruded unilamellar vesicles has allowed us to assign elastic moduli for bilayers of dioleoylphosphatidyl choline and dioleoylphosphatidyl glycerol, in the range 5×10^8 to 12×10^8, and 3×10^8 to 6×10^8 dyn/cm^2, respectively. Membranes derived from bovine submitochondrial particles and rye plasma membrane did not produce evidence of swelling when subjected to similar protocols.

ACKNOWLEDGEMENTS

This thesis is dedicated to the memory of family and friends who have died during the years I have been preoccupied with this thesis: my brother John, uncle Stanley, Richard Buffum, Richard Daskowski, Faith Erlich, Esparanza Simon, Donald Sloan and Phil Yacos.

I would like to thank my family and friends for their support during the last eight years. Both thanks and apologies are due to you all. I realise how difficult this has been for you; this extra burden you could have well done without.

I thank the members of my thesis committee, Professors Herman Z. Cummins, Peter Lipke, Charlotte S. Russell, and Horst Schulz for their assistance, encouragement and advice. A number of researchers have provided samples and services which have been essential for this study: Theodore Axenrod, Robert Bittman, Ilya Glezer, Ronald Gordon, Barry Lentz, David Lynch, Lawrence Mayer and Harvey Penefsky. There are many colleagues at the City College of New York to whom I want to express my thanks. I make special mention of Diogenes Aybar-Batista, Dean Cuebas, Gregory Linn, Charlotte Martin, Martin Muschol, and Jing-Yi Syz. My deepest thanks and appreciation goes to Lloyd Williams whose fellowship and collaboration was essential to the sucessful completion of this thesis.

Most of all I would like to thank my research mentor, Professor Thomas H. Haines for his guidance and his years of encouragement and advice.

I would also like to acknowledge the financial support obtained from the Chemistry Department of the City College of New York, the Biochemistry Department of the City University of New York Medical School, the Polaroid Corporation, Louis and Samuel Rover, and Marvin Winston.

"Is this the nation that made Washington?

It made him a cripple.

Protector of liberty.

Whose, Hamilton's?-to harness the whole, young, aspiring genius to a treadmill? Paterson he wished to make capital of the country because there was waterpower there which to his time and mind seemed colossal. And so he organised a company to hold the land thereabouts, with dams and sluices, the origin today of the vilest swillhole in christendom, the Passaic River; impossible to remove the nuisance so tight had he, Hamilton, sewed up his privileges unto kingdomcome, through his holding company, in the State legislature. *His* company. *His* United States: Hamiltonia- the land of the company.

You violate your own concept of what history should be when you speak so violently.

The pendulum must swing. Is it not time it swung *back?*

But was Burr really better who founded Tammany?

Child's play.

So you have raised the point that once the Revolution over the New World instead of being freed slipped into a tyranny as bad as or worse than the one it left behind; that, of this tyranny, Hamilton was the agent; and that-perhaps- in Burr reposed the true element, liberty, which a party in power tried to smother. What basis, other than the one adopted under the Constitution, could the new Government have taken firmer? Burr proposed none. This is the charge against him: that he proposed nothing yet refused to abide loyally by the established order."

William Carlos Williams

The Virtue of History

"In the American Grain"

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ABBREVIATIONS

BLM, black lipid membrane;

DMPC, 1,2-dimyristoyl-*sn*-3-phosphatidylcholine;

DOPC, 1,2-dioleoyl-*sn*-3-phosphatidylcholine;

DOPG, 1,2-dioleoyl-*sn*-3-phosphatidylglycerol;

EM, electron microscopy;

EPC, egg phosphatidyl choline;

FID, free-induction decay;

GMO, glycerol mono-oleate;

GPC, gel permeation chromatography;

HDTAC-C, hexadecyl-trimethylammonium chloride - cholesterol;

LEH, liposome encapsulated hemoglobin;

LUV, large unilamellar vesicles;

LUVET, large unilamellar vesicle (produced by) extrusion techniques;

MDO, membrane derived oligosaccharides;

MV, membrane vesicle;

NMR, nuclear magnetic resonance;

PC, phosphatidyl choline;

PCS, photon correlation spectroscopy;

rpm, revolutions per minute;

SMP, sub-mitochondrial particles;

SUV, small unilamellar vesicles;

TEM, transmission electron microscopy;

Tris-HCl, tris(hydroxymethyl)aminomethane Hydrochloride.

INTRODUCTION

An amphipathic lipid bilayer makes cellular organization possible. The underlying molecular structure of the membrane was first described by Gorter and Grendel (1925). For procaryotes this membrane separates the inside, cytosol, from the outside of the cell. The membrane functions as a barrier which sequesters the biochemical systems of life from the outside world. It thus sets the stage for vectorial transport, creating a barrier which establishes chemical, ionic, and electrical gradients. Bilayer membranes that are composed of phospholipids show remarkabe permeability to water, gasses, and uncharged organic molecules with just a few carbons (Collander and Barlund, 1933; Danielli and Davson, 1935). Lipophilic (fatty) compounds and organic solvents enter the membrane rapidly although there is a tendency for these molecules to remain within the hydrocarbon region of the bilayer. Salts, macromolecules and charged or hydrophilic organic molecules do not readily cross the hydrocarbon core of the bilayer (Bangham et al., 1974; Deamer and Bramhall, 1986). In an environment enclosed by a phospholipid bilayer, water readily passes throught the membrane so that solute concentration differences between the internal aqueous compartment and the external medium approach equilibrium. As such, the membrane represents an example of a semi-permeable membrane and the volume encapsulated by the membrane increases or decreases (Blok et al., 1976; Carruthers and Melchior, 1983; Hantz et al., 1986) in response to the net flow of water. In the case of volumetric expansion, the surface area of the membrane expands to accommodate the increased volume.

In biological membranes, the semi-permeable nature of the membrane leads to the problem of osmotic stress. In plant cells the extent of volumetric expansion is limited by the presence of the cell wall. In cells not equipped with a cell wall other systems are required for an adequate response to osmotic stress. The biological membrane is a semi-permeable lipid bilayer into which proteins are embedded. These proteins provide specific transport, highly specific gating and recognition systems (receptors)(cf: Singer and Nicholson, 1972). Through its transport systems the membrane is the principal energy processor of living cells. It is a barrier which the organism utilises to sequester chemical and electrostatic gradients, providing energy to drive biochemical processes including ATP synthesis. The efflux of protons may be coupled to the export of anions such as acidic fermentation products (Raven and Smith, 1982), or it may be translated into other cationic gradients such as sodium and potassium by exchange proteins, etc. Obviously these cation and anion transport systems operate in an osmotically sensitive environment since the bilayer is semipermeable. It appears that one of the universal features of living organisms is that potassium is accumulated and sodium is extruded from the interior (Wilson and Lin, 1980; Harold, 1986). The active pumping of sodium from the organism relieves a portion of the osmotic stress on the membrane. However, a perpetual osmotic imbalance is present in all organisms.

As a micro-environment including a domain with low polarity in an otherwise highly polar environment, the amphipathic bilayer is a good candidate for the site of original organization of much of the fundamental chemistry of life. Recent advances in lipid biochemistry have provided us with the structures of many

membrane-forming compounds of the archaebacteria, which are models of primordial lipids (Kates and Kushwaha, 1976; Lo et al., 1989). The properties of these lipid systems provide us with insight into the adaptability of life to extreme environments. Other findings of bilayer formation by fatty acids (Gebicki and Hicks, 1973, 1976) and aliphatic sulfates (Hargreaves and Deamer, 1978) have shown that a variety of surfactant-like molecules may form biological (Mayers and Haines, 1967; Mayers et al., 1969) as well as bio-mimetic membranes.

Studies of the physical properties of liposomes and vesicles will help us understand the role of the membrane in the function of subcellular organelles. Liposomes can be constructed with either encapsulated materials, or with integral membrane proteins. As such they represent simple models of subcellular organelles. Variation of lipid composition, temperature, osmotic strength and ionic strength of the media can be investigated in these simple systems as a means of probing their effects on more complex biological systems. Likewise, exploration of the basic physical properties of liposomes may aid in the development of advanced therapeutic and diagnostic systems. Among these physical properties are: size and distribution of sizes of a particular vesicle preparation, permeability of solute, elasticity and resistance to bursting. Liposomes as models of biological membranes have been constructed so that functional proteins are incorporated within the membrane (Scotto and Zakin, 1985, 1986; Scotto et al., 1987) as well as covalently bound to intrinsic membrane compounds (Loughrey et al., 1990). Thereby, liposomes may contain encapsulated materials as well as surface-bound recognition or catalytic agents. As the technology of liposome formation and encapsulation techniques are perfected their utility in medicine will expand.

Though the techniques are relatively new, significant gains have been made in the utilization of liposomes in medicine. The utility of liposome therapy is based on the delivery of therapeutic substances to target cells or the sustained release of a substance from the liposomal bilayer or its encapsulated aqueous compartment. Mechanisms by which liposomal contents are delivered include: intermembrane transfer, contact release, fusion and phagocytosis/endocytosis (New, 1990). Fusion has been shown by Miller and Racker (1976) to be enhanced by osmotic swelling. The encapsulation of hemoglobin into liposomes, as an emergency blood substitute, is under development (Gaber et al., 1983; Farmer and Gaber, 1987). The inclusion of allosteric effectors (such as 2,3-diphosphoglycerate) of oxygen binding to hemoglobin may increase the efficiency of oxygen transport capabilities of liposome encapsulated hemoglobin. Likewise enzyme therapy may be effected by the delivery of enzymees encapsulated by liposomes (cf. Gregoriadis et al., 1971; Finkelstein and Weissman, 1978). The technique may also be applied to drug therapy using peptides and other macromolecules since serum half-lives of these potentially therapeutic substances (peptides) are often on the order of minutes upon direct injection. Chelation therapy may also benefit from the incorporation of metal chelators within liposomes (cf: Rahman and Wright, 1975). Cancer therapy with a variety of agents encapsulated within liposomes are under investigation (cf: Ellens et al., 1982 (arabinofuranosylcytosine); Gabizon et al., 1983 (adriamycin); Fidler and Schroit, 1984 (muramyl dipeptide); Supersaxo et al., 1988 (5-fluoro-2-deoxyuridine); Bally et al., 1990 (doxorubicin)). Likewise liposome encapsulated agents have been applied to the treatment of bacterial (Fountain et al. 1985 (aminoglycosides)), fungal (Lopez-Berenstein et al., 1984 (amphotericin B)),

and viral (Koff and Fidler, 1985) infections. Liposome therapy has progressed to the level of human trials, both for therapy of ovarian cancer (cf: Delgado et al., 1989; Mayer et al., 1989 (doxorubicin); as well as for the therapy of immuno-compromised cancer patients with *Candida albicans* infections (Lopez-Berenstein et al., 1987 (amphotericin B)). Additional applications include their use as *in vitro* (Martin and Kung, 1987; Fiechtner et al., 1989) diagnostic agents in which ligand-bearing liposomes are used in particle agglutination assays. These systems provide enhanced responsiveness and specificity for the detection of rheumatoid factor, anti-streptococcal antibodies and anti-human erythrocyte subgroup antibodies. Their use as *in vivo* diagnostic agents (Caride et al., 1982) includes their use as contrast agents for computed tomography (Cat-Scan) of the liver and spleen. In this particular system, liposomes containing brominated phosphatidyl cholines are directed to the elements of the reticulo-endothelial system (RES) in the liver and spleen upon intraveneous administration. Lastly immune therapy has been effected using liposome encapsulated DNA (cf. Nicolau et al, 1983). In this system a recombinant plasmid encoding rat preproinsulin I was encapsulated in liposomes and administered intraveneously. Uptake of liposomes by the macrophages of the reticulo-endithelial system directed the liposomes primarily to the liver and spleen. The preproinsulin I gene is expressed and correlates with the insulin level in blood and with glycemia. Liposomes composed of synthetic cationic lipids mediate DNA-transfection in vitro (Felgner et al., 1982). These liposomes interact with DNA spontaneously, fuse with cells in tissue cultures and provide enhanced delivery of functional DNA to the nucleus. While the lack of reliable data on the osmotic properties of liposomes has not halted progress in the application of

liposomes to the problems of medicine, a greater understanding of these properties may spur the development of novel methods of drug therapy utilising liposomes.

It was Bangham and his group (Bangham et al., 1965, 1967a, 1967b; Bangham, 1968) that first demonstrated that synthetic phospholipids could spontaneously assemble into liposomes (Bangosomes) that displayed osmotic activity. The assay that permitted Bangham to determine that these dispersions encapsulated an aqueous compartment was microscopic observation of osmotic swelling. Once it was observed that these multilamellar phases (smectic mesophases) were semi-permeable, analysis using electron microscopic techniques permitted the latter to be used as the assay for liposome formation. However it was the osmotic swelling that convinced the research community that these liposomes corresponded to biological membranes.

Liposomes of sub-optical sizes had been synthesised prior to Bangham's demonstration of osmotic activity in liposome preparations. These early preparations envisioned the use of phospholipid "emulsions" as carriers of lipophilic therapeutics (Saunders et al., 1962) without realising that the preparations they had made were actually liposomes which encapsulated an aqueous compartment as well. The development of preparations of unilamellar vesicles was an extension of this work (Huang, 1969). Huang's method of extreme sonication produced curvature limited vesicles that seemed unlikely to behave like osmometers. Furthermore their sub-optical dimensions precluded a direct investigation of their osmotic properties by microscopic techniques. It was apparent that the demonstration of osmotic activity in sub-optical sized preparations could be best addressed using synthetic phospholipid bilayers organised as unilamellar liposomes (Bangham et

al., 1974, Blok et al., 1976) which displayed uniform size, without the restriction of a curvature limited surface.

Generally small unilamellar vesicles (SUV) are considered those with sizes (diameters) less than 100 nanometers, large unilamellar vesicles (LUV) are considered those with sizes above 100 nanometers (Szoka & Papahadjopoulos, 1980). Frequently small vesicles refer to terminally sonicated vesicles (15-30 nm diameter) and anything larger than these are considered large vesicles. Therefore the terms SUV and LUV must be qualified according to the actual size of the vesicles or the preparation technique. Uniform preparations of unilamellar vesicles are produced using a variety of techniques. Huang's vesicles (Huang , 1969; Huang et al., 1973) were produced by extensive sonication of multilamellar vesicles (MLV, Bangosomes) (Bangham et al., 1965, 1967a; Bangham, 1968). Extensive sonication yielded vesicles that were limited by curvature of the particle. In the inner leaflet the area per phospholipid headgroup was 0.61 nm^2, on the outer leaflet the area per phospholipid headgroup was 0.71nm^2 (Chrzeszcyck et al, 1977). The size of these vesicles (SUV) were on the order of 15-30 nm diameter.

Limiting the extent of sonication allows one to produce vesicles of varying size, but since multilamellar vesicles are the usual starting material and small, curvature- limited vesicles are the endpoint of sonication, preparations of sonicated vesicles of any intermediate size usually contain significant amounts of both types of vesicles. Sonicated vesicles with 100 nm mean diameter (as determined by PCS) are surprisingly highly polydisperse relative to terminally sonicated vesicles or to similarly sized vesicles produced by alternative means.

Besides sonication, unilamellar phospholipid vesicles can be produced by: (a) detergent dialysis (Parente & Lentz, 1984; Petri et al., 1980; Milsmann et al., 1978); (b) evaporation of the organic solvent from a ternary system: organic solvent:phospholipid:aqueous buffer, and subsequent dialysis (Szoka & Papahadjopoulos, 1980; Barenholtz et al., 1979; Deamer and Bangham, 1976; Gruner et al., 1985b); (c) solubilization from a physically constrained support (Lasic et al., 1987); and (d) extrusion of MLV's through polycarbonate filters (submicron) at high pressure (Hope et al., 1985; Mayer et al., 1986; Nayar et al., 1989). Additionally, deposition of a protonated film of acidic lipids, hydration, rapid addition of base, and neutralization (pH-jump) yields a polydisperse mixture of phospholipid vesicles (Hauser & Gains, 1982; Aurora et al., 1985; Hauser, 1989), from which a fraction of small vesicles can be obtained by centrifugation or gel filtration. The majority of preparative techniques produce polydisperse preparations of vesicles that would provide data of limited usefulness in laser light scattering (PCS) measurements of osmotic swelling for several reasons. First, the larger vesicles scatter light with significantly greater intensity than smaller vesicles, so that their properties would be expected to dominate the statistically-averaged results. The measurements would therefore primarily reflect the swelling properties of the larger vesicles. Lastly, we have noted that the mean size obtained from PCS measurements is not a reliable indication of the actual mean size of a highly polydisperse distribution of vesicles as there are wide variations in the mean size of samples withdrawn from stock solutions.

Synthetic vesicle preparations do preclude interference from transbilayer porting systems present in natural membrane isolates (Rivers and Williams, 1990;

Miyamoto et al., 1988). Additionally, solute permeability data is available for many phospholipid membranes. Information on the elasticity and osmotic properties of pure phospholipid bilayers in unilamellar liposomes first appeared in the data presented by Hantz et al. (1986), Sun et al. (1986), and Kwok and Evans (1981).

Osmotic properties of phospholipid bilayers have become an increasingly important issue in our understanding of the behavior of transmembrane proteins. Recent description of a mechanicosensitive ion channel (Gustin et al., 1988) has indicated that there are stress-activated ion channels in yeast plasma membranes. A system of stress-activated osmotic relaxation occurs in erythrocytes and amphibian oocytes (Yang & Sachs, 1987). Furthermore, changes in the elastic properties of bilayers have been attributed to the activation of glucose transport systems in brush border membranes (Miyamoto et al., 1988). Recent studies on rye plasma membrane (Lynch and Steponkus, 1987, 1989) propose a compositional adaptation of the plasma membrane to sub-freezing temperatures in order to maintain osmotic activity.

Not only must osmotic integrity be maintained by the cell, but the cell must resist the influx of potentially harmful solutes or respond to such an influx. Failure to adapt to a high salt environment would lead to intracellular salt concentrations which would normally be deleterious to cytoplasmic proteins. *Halobacteria* are committed to a life in high salt. They have apparently made this adjustment by producing proteins that function in a medium of high ionic strength. Other procaryotes regulate osmolarity exquisitely with a variety of genetic control systems (Higgins et al., 1987). Among the operons of gram-negative bacteria that

regulate osmolarity are the *kdp* operon, the products of which regulate the high-affinity potassium transport in response to changes in osmolarity; the *proU-proP* operon, the products of which regulate betaine transport systems; the *OmpC-OmpF* operons which products (porins) regulate the aperture size of the outer membrane; the *mdo* (membrane-derived-oligosaccharides) operon which products influence the the ionic strength of the intermembrane space; and *malB*, the maltose transport system. This wide variety of osmotic-sensitive controls displays the importance of osmolarity to the life of the cell. At high osmotic strength potassium sequestration is halted and betaine (N,N,N-trimethyl glycine) is accumulated when betaine is provided in the culture medium. Betaine is accumulated to increase the cytoplasmic ionic strength because at high potassium concentration normal enzyme function is inhibited. Betaine replaces solutes in the hydrated "shell" surrounding an enzyme and so can restore activity in salt inhibited enzyme systems (Yancey et al., 1982; Hand and Somero, 1982). Betaine (and to a lesser extent proline and trehalose) is the principal osmoticant compatible with enzymatic activity in *E. coli* and *S. typhimurium.* Betaine is scavanged from the external medium and accumulated to molar concentrations in the cytoplasm. This is accomplished by *proU* , the high-affinity betaine transport system. This genetic locus is apparently activated solely by cytoplasmic potassium levels (Higgins et al, 1987) as opposed to activation of an existing transbilayer protein in response to tension. The *kdp* operon of *E. coli* alters the potassium ion concentration in its protoplasm in response to osmolarity changes in the external medium (Laimins et al., 1981). The interesting feature of this system is that the authors have proposed that the kdpD protein acts as a "mechanogenetic" controller. At low turgor

pressure the conformation of the kdpD regulator changes and binds with the *kdp* promoter. Regulation of the *mdo* operon influences the osmolarity of the periplasmic space between inner and outer membranes by the increased synthesis of polyanionic oligosaccharides: the membrane-derived-oligosaccharides (MDO) (Weissborn & Kennedy, 1984). In each of these cases the response of the organism to osmotic stress is the activation of specific genetic loci. In the case of the MDO this results , secondarily, in alteration of the membrane composition. In the case of the *kdp* operon Laimins et al., (1981) proposed the coupling of membrane tension to expression. In the case of the *proU* operon, translation activation is influenced by cytoplasmic potassium levels and the response is independent of any membrane processes or compositional modification.

The regulation of liver cell volume is also osmolarity-related; it is directly associated with metabolism (Watford, 1990). Cell volume changes, as occur during hypoosmotic perfusion, affect metabolic activities of rat liver such as glycolysis, glutamine catabolism and urea production (Graf et al., 1988; Haussinger et al., 1990).

Establishment of ionic and osmotic gradients on cell membranes should result in varying degrees of swelling or at least varying degrees of tension on the membrane. These stresses may well be transmitted to the hydrophobic domains of membrane proteins. Until very recently investigations on membrane proteins did not emphasise osmotic stresses simply because there was a paucity of information on the elastic properties of sub-cellular particles and lipid bilayers. The characterization of a membrane protein which couples ionophore activity to membrane tension in yeast plasma membrane (Gustin et al., 1988) heralds a field in which it is

recognised that the mechanical forces on the membrane will be recognised as a controlling force relating osmolarity and ionic strength to transmembrane flow.

The Measurement of Elastic Constants in Bilayers

Until 1986, approaches to the experimental quantitation of the elastic constants of bilayer membranes had been either capacitance measurements of black lipid membranes (Alvarez and Latorre, 1978) or micropipette aspiration of giant phospholipid vesicles (Kwok and Evans, 1981). The elastic constants are the Young's modulus, M_e, and the area compressibility modulus, K, describe the force required to expand the surface area of a material by a relative degree. The Young's modulus describes the stress/strain relationship for a given material. In this case the material is a phospholipid bilayer. A Young's modulus of 3.5×10^5 dyn/cm^2 was found from capacitance measurements (Crowley, 1973; White 1974). A reinvestigation of membrane capacitance (Alvarez and Latorre, 1978) produced a direct measurement of membrane capacitance as a function of voltage from which a Young's modulus (or thickness compressibility modulus) on the order of 10^8 dyn/cm^2 was inferred. The variation in Young's moduli found by this technique indicated that an improved method to determine the Young's modulus was required.

A subsequent method used for the determination of the bilayer elastic modulus is the method of micropipette aspiration. The first measurements using this method were conducted on erythrocytes (Rand, 1964). More recently this technique has been applied to giant liposomes (Kwok and Evans, 1981). Rand (1964) obtained Young's moduli for erythrocytes ranging from 7×10^6 to 3×10^8

dyn/cm^2. Waugh and Evans (1979) claimed that the contribution of spectrin and phospholipid membrane to the area compressibility modulus could be resolved by the detachment of the membrane from the spectrin network at elevated temperatures. Prior to the expansion of the erythrocyte surface the membrane may be deformed with relative ease. The erythrocyte membrane can be bent, and major portions of the erythrocyte can be sucked into the bore of a micropipette. The surface extension at constant area (shear) deformation of the erythrocyte membrane displayed a surface shear modulus of 6 x 10^{-3} dyn/cm. These linear extensions of the membrane occur without any corresponding increase in the erythrocyte surface area (strain). Consequently, bending of the erythrocyte membrane from its initial "dimpled" shape to a sherical shape requires little energy imput. Once a spherical form is attained (with a "tail" extended into the bore of the micropipette) the surface of the erythrocyte can be expanded approximately 3%, for which an area compressibility modulus of 450 dyn/cm or a Young's modulus of 9.0 x 10^8 dyn/cm^2 (assuming 40 Å bilayer thickness) was obtained (see Kwok and Evans, 1981). Expansion of the membrane surface area greater than 3% results in lysis. This latter calculation of the area compressibility modulus does not resolve the contribution of spectrin and phospholipid membrane and so the area compressibility modulus cited results from both the elasticity of the membrane as well as that of the underlying spectrin network. As opposed to the erythrocyte, vesicles have no underlying spectrin network. Giant vesicles ideally have no surface shear modulus, as there is no resistance to the flow of the liquid phospholipid material above the transition temperature of the membrane. However, the phospholipid membrane of vesicle resists area dilation and an area compressibility modulus may

be obtained. The swelling of giant (10 μm) DOPC vesicles (Kwok and Evans, 1981) produced an elastic area compressibility modulus K = 140 dyn/cm, or, with d = 40 Å, a Young's modulus of 3.5 x 10^8 dyn/cm^2. Here, surface area expansion was limited to 2-3%. More recent experiments (Needham and Nunn, 1990) show a variation in the area compressibility which correlates with phospholipid component and the cholesterol content of the bilayer. Additionally, the surface are expansion at bursting varies from 2-5 % again depending on the composition of the membrane.

These results have shown that the magnitude of the Young's modulus of biological membranes is on the order of that of that of phospholipid bilayers (see Table I). Presumably, this is the modulus of the material (bilayer) within which mechanicosensitive channels will be embedded. It is useful to determine the extent to which the Young's modulus of bilayers is determined by the structure of the phospholipids that make up the bilayer as well as other components of the bilayer (sterols, etc.) At present this measurement (for both membranes and synthetic phospholipid bilayers) has exhibited a fairly narrow range of values (10^8 - 10^9 dyn/cm^2). The variation within this range may be attributed to the lipid headgroup, to the nature of the acyl chains or to both.

While the micropipette aspiration studies performed on erythrocytes (Rand, 1964), giant liposomes (Evans and Kwok, 1981) and rye plasma membranes (Wolf and Steponkus, 1983; Wolf et al., 1986) utilise preparations that are virtually the same size as most eucaryotic cells (10 μm), their use as models for the representation of the osmotic behavior of subcellular organelles, or for diagnostic and therapeutic liposomes (0.1 - 5 μm) remains unclear. The micropipette

aspiration technique cannot be applied to preparations of this size range. It is therefore important to seek methods for evaluating membrane elasticity in small vesicles.

An entirely different approach for examining the elastic properties of bilayers of phospholipids emerged in 1986. The development of photon correlation spectroscopy (PCS) (c.f. Cummins and Pike, 1973; Chu, 1974; Berne and Pecora, 1976; Pike and Cummins, 1977) provided a method by which submicron particles could be sized very precisely. This technique has been applied to the sizing and distributional analysis of unilamellar vesicles (Schurtenberger and Hauser, 1984; Sun et al., 1986; Hantz et al., 1986; Woodle and Martin, 1988; Kojro et al., 1989, Woodle and Papahadjopoulos, 1989; Nayar et al., 1989; Ruf et al., 1989) and to biological membranes (Warashina, 1981; Miyamoto et al. 1988; Fujime et al., 1988; Miyamoto and Fujime, 1990).

The combined osmotic-swelling-PCS approach to measuring bilayer elasticity in vesicles was developed by three groups: Sun et al., (1986); Hantz et al., (1986) and our group (Aurora et al., 1985; Li et al., 1986; Li and Haines, 1986; Li, 1987; Haines et al., 1987). Sun used vesicles that had been fractionated by gel-exclusion chromatography (Sephacryl S-1000); Hantz employed ultrafiltration (0.45 μm) to restrict the size of their vesicles. Our group initially utilised vesicles formed by the pH-manipulation technique (Hauser and Gains, 1982; Aurora et al., 1985), then used vesicles produced by extrusion through polycarbonate ultrafiltration membranes (Hope et al., 1985). All three groups gave brief theoretical calculations for obtaining a modulus from the observed swelling as measured by PCS.

The procedure (and calculations) is based on the use of small vesicles with minimal size polydispersity as models for subcellular organelles. The majority of experiments are conducted on vesicles of approximately 100-120 nanometers diameter. The initial requirement for PCS studies of osmotic swelling was therefore, a preparation of vesicles with moderate size (100-500 nanometers dia. versus 10,000 nanometer diameter of the giant DOPC vesicles) and minimum polydispersity. Preparations of small sonicated vesicles (30 nanometers diameter) were monodisperse but did not expand in response to osmotic pressure (Johnson and Buttress, 1973). These extremely small "curvature limited" vesicles did not release entrapped radioactivity or show changes in their hydrodynamic radius upon two-fold osmotic dilution. Only dialysis against distilled water produced loss of entrapped radioactive label. Size increases in small vesicles, in response to osmotic swelling, would probably be below the resolution of PCS techniques.

Of the three osmotic swelling-PCS experiments published in 1986, each group synthesized vesicles by different methods. Sun (Sun et al., 1986) utilized the ether-injection method of Kremer et al., (1977). This procedure yielded small unilamellar DOPC vesicles of 60-120 nm diameter, which were obtained after extensive dialysis and gel-exclusion chromatography. The polydispersity of the vesicle preparation was "nearly as monodisperse" as a latex polystyrene standard 110.0 +/- 3.0 nm diameter. Hantz (Hantz et al. 1986) utilized the reverse phase evaporation technique of Szoka & Papahadjopoulos (1980) which produced large unilamellar DOPC vesicles of diameter 160-180 nm. This preparation yielded a polydispersity index of 0.15-0.25 after centrifugation and filtration. The polydispersity index, Q, is a measure of the deviation of a sample from a

monodisperse size; in limited cases it is a measure of the width of gaussian distributions of vesicles (see THEORY: *Polydispersity*). Li (Li et al., 1986) utilized "pH-jump" vesicles produced by the modified technique of Aurora (Aurora et al., 1985). These large vesicles were approximately 300-400 nm diameter. Polydispersity indices were in the range 0.36-0.44. No fractionation other than filtration through 1.0 micron polycarbonate filters was used. In the current work osmotic swelling was observed only in preparations made by the extrusion method of Cullis. This technique produced LUV (LUVET) of 105-120 nanometers diameter; with polydispersity indices in the range 0.07-0.12 .

Protocols for the light scattering experiments have been developed for vesicles (Kremer et al. 1977; Aurora et al.,1985; Milon et al. 1984, 1986; Hantz et al. 1986; Sun et al., 1986); as an alternative to analytical ultracentrifugation (Huang, 1969) and freeze fracture electron microscopy (Watts et al., 1978; Hope et al., 1985) as an indicator of size and distributional characteristics. The method bypasses potential fusogenic events encountered in the freezing of vesicles (Crowe et al., 1985) and is extremely rapid. Similarly it reduces the liklihood of shrinkage which is a common effect of fixation and dehydration for samples analysed using transmission electron microscopy (Aurora et al, 1985; McCracken & Sammons, 1987)

Early light scattering experiments on synthetic vesicles did not involve osmotic swelling. They were conducted on small unilamaellar vesicles composed of DMPC (Mishima, 1976; Ostrowski & Hesse-Bezot, 1977) at and near the lipid phase transition. The authors noted a rapid increase in the size of vesicles upon warming through the transition temperature. This increase in size continued after

cooling and was presumed to indicate fusion. The change in the vesicles radius was on the order of 1.5 times the initial radius. This study was followed by a more precise measurement of the size of DMPC vesicles at or near the phase transition (Milon et al., 1984). The notion of assessing the monodispersity of a distribution of vesicle sizes by using the index of polydispersity of the sample and the inclusion of control experiments to confirm the integrity of initial samples provided a means of attacking the question of osmotic swelling in small vesicles.

What followed was a virtually simultaneous publication of osmotic swelling data from three groups. A joint MIT-Pasteur Institute group (Sun et al., 1986), including authors of the preceding study (Milon et al., 1984), observed differential swelling of DMPC vesicles according to their size, smaller vesicles, i.e., less than 80 nm in diameter did not swell greater than 1% at 25 ^{o}C, slightly larger vesicles (106 nm) swelled 5% of their initial size . Total swelling was approximately 1 to 4 nanometers, depending on the initial size of the vesicles. This confirmed the earlier studies (Johnson and Butress, 1973) indicating that small unilamellar vesicles (approximately 30 nanometers dia.) produced by sonication, were insensitive to osmotic shock. Sun's study suggested that the membrane modulus rapidly decreased as the initial size of the vesicles increased (Table I). Although the initial values are within the range that reinforce the concept of an osmotically inelastic small vesicle, the elasticity of the larger vesicles (106 nm dia.) suggests expansion that our studies cannot confirm.

A study by the second group (Hantz et al., 1986) reports a modulus comparable to Sun et al., (1986) for the larger vesicles but low compared to ours (Rutkowski et al., 1991) (Table I). The third experimental group observed osmotic

swelling (Li et al, 1986) in a variety of pure phospholipid and natural membrane preparations. This group introduced the concept of swelling to an elastic limit, followed by bursting and resealing. Li's results could not be reproduced, our current experiments experiments (Rutkowski et al., 1991) indicate that the Young's moduli of DOPC and DOPG bilayers are on the order of 5 x 10^8 dyn/cm^2.

Submitochondrial particles (Penefsky et al., 1960) and rye plasma membrane vesicles (Wolf and Steponkus, 1983; Wolf et al., 1986; Lynch and Steponkus, 1987) were analysed by PCS . Numerous attempts to observe swelling in isolated sub-mitochondrial preparations and plasma membrane preparations have not produced swelling consistent with previously conducted micro-pipette aspiration studies (Wolf and Steponkus, 1983; Wolf et al., 1986; Lynch and Steponkus, 1987). In all cases these sub-cellular preparations are osmotically insensitive or the apparent osmotic insensitivity is an artifact of the preparation or our experimental technique. The initially reported pattern of swelling and bursting of vesicles is not supported by the majority of the experiments conducted during the course of this investigation. Without confirmation of swelling to a burstpoint and independent corroboration of rupture, the determination of a maximum percentage surface increase and transbilayer osmotic difference is impossible for either sub-cellular particles or pure phospholipid vesicles. Present data indicates only linear expansion in the range of osmotic dilution we have imposed. In this region we can calculate the membrane modulus and we can assess the extent of surface expansion without conjecturing upon the limit to expansion of the surface.

Beyond a review of currently available information on osmotic swelling of phospholipid vesicles, the thesis includes our latest assignment of Young's moduli for DOPC and DOPG vesicles in equiosmolar sucrose and KCl. The swelling data also provided information on the maximum *observed* surface area expansion. In order to bolster our sizing claims, negative-staining electron microscopy was used to compare the mean size obtained by the former to that obtained by PCS. We attempt to resolve the inconsistencies in the accounts of osmotic swelling of phospholipid vesicles and biological membrane preparations. A re-analysis of the pH-adjustment ("pH-jump") procedure, in relation to the size characterization of vesicles produced by this method, has been conducted.

Since current literature accounts do not always incorporate rigorous use of control experiments, adequately discuss the statistical confidence of their measurements, or provide an adequate assessment of the distribution of sizes of the vesicles used, these issues will be discussed. Each of these factors become significant when considering that osmotic swelling manifests itself as an increase which is on the order of a 1.5-2.5 nanometers in the mean diameter of a distribution of vesicles with a mean diameter of approximately 110 nanometers. When considering such slight effects it is important to disassociate these effects from temporal effects, (i.e., changes in the mean size during the duration of the experiment), and the uncertainty in the mean size as a consequence of the experimental technique.

The current picture of liposomes, that are sensitive to osmotically induced release of contents (Li et al., 1986; Haines et al., 1987; Li, 1987) requires revision. Likewise, the limited surface area expansion noted in the studies of Kwok and Evans (1981) needs examination. The picture emerging from this study is

that liposomes can withstand twofold osmotic dilution and surface area increases beyond 5%, without bursting.

THEORY

Photon Correlation Spectroscopy

Introduction

The average size and the polydispersity of the vesicles was determined by PCS. PCS is a technique that was developed to monitor intensity fluctuations of light scattered by fluids, or by macromolecules and particles in suspension. We observed the scattering produced by phospholipid vesicles or liposomes, which undergo Brownian motion as a supramolecular structure (assembly) rather than as individual molecular units. The scattering of light by polymers, particles and vesicles has been reviewed extensively (cf.: Cummins and Pike, 1973; Phillies, 1986; Pike and Cummins, 1977). The intensity fluctuation of the scattered light permits us to assess the translational (Brownian) motion of the vesicles. The application of a few simple assumptions permits us to gauge their size.

Temporal fluctuations in intensity may be observed in the light scattered by suspensions of large particles with the naked eye. For smaller particles the "flicker" rate of the scattered light is too rapid to discern. The digital autocorrelator allows us to compare the intensity observed in one five microsecond period (bin time) to previous periods. These comparisons are performed simultaneously on the preceeding 128 values of the intensity. From this data a correlation function is obtained. The fluctuations in the intensity can be characterized by the correlation time (τ_c). From τ_c the diffusion coefficient (D_T) can be measured, and hence the average diameter calculated. The calculation requires knowledge of the refractive index and viscosity of the medium, the wavelength of the incident

radiation, and the geometry of the light scattering apparatus.

Scattering Theory

The initial task is the description of light as an electromagnetic wave with a vacuum wavelength of λ. For an incident beam this corresponds to a wave vector $k_i=2\pi n/\lambda$ (n is the refractive index of the liquid surrounding the vesicle). The amplitude of the electric component of the electromagnetic radiation propagating in the direction k_i can be written as:

$$E_i = E_i^0 e^{i(\omega t - k_i r)} \tag{1}$$

where ω is the frequency of the electromagnetic radiation in radians per second. The electric field vector is restricted to the plane normal to the propogation of the electromagnetic radiation and has positive and negative components. The intensity, I_i, of the incident light is related to its electric field, E_i, by the equation:

$$I_i = \varepsilon | E_i |^2 \tag{2}$$

where ε is the electric permitivity of the medium.

Light scattering occurs when a substance absorbs energy from the incident electromagnetic radiation and re-emits this electromagnetic radiation. In the case of elastic scattering, existing dipoles in the substance are induced to vibrate at the frequency of the incident electromagnetic radiation. This energy is re-emitted as electromagnetic radiation at the same frequency as the incident radiation. Since all matter undergoes translational motions, the concept of purely elastic scattering is unrealistic. Rather, elastic scattering is a special case of quasielastic light scattering. In quasielastic scattering the frequency of light is changed by the

translational motions of the sample. Samples undergoing motion (such as Brownian motion) do not emit light at precisely the same frequency as the incident radiation, but rather at at a range of frequencies centered on that of the incident electromagnetic radiation. The changes in the internal energy of the sample are not associated with particular transitions in the electronic or vibrational energy levels of the sample (Phillies, 1986). The frequency shifts in the scattered light occur along with fluctuations in the intensity of the scattered light. Assessment of the of molecular motion in the sample is possible by either analysis of the frequency spectrum of the scattered light or by analysis of the intensity fluctuations of the scattered light. Analysis of the frequency can be accomplished using optical mixing techniques. The intensity fluctuations which occur in the scattered radiation occur on a time scale which is convenient for conventional measurement techniques and contain information identical to that found in the spectrum of the scattered light, namely information on the molecular diffusion of the scattering sample.

The scattered radiation, E_s, at the detector is described using the same form as was used to describe the incident radiation, thus:

$$E_s = E_s^0 e^{i(\omega t - k_s r)} \tag{3}$$

where k_s is the wavevector of the scattered light. As in the case of the incident radiation, the intensity of the scattered radiation is proportional to the square of the electric field so: $I_s = \varepsilon E_s^*(t) E_s(t)$. In this case the intensity is expressed as the product of the electrical field (as a function of time) and its complex conjugate.

The scattered light is detected by a photomultiplier tube. In the PCS technique the spectrum of the photodetections is measured (via the correlation function). The photodetector (photomultiplier) output follows the modulations in the intensity of the scattered light caused by the Brownian motion. The relationship between the photocurrent spectrum and the optical spectrum depends on the statistics of the signals and detection process but if we assume Gaussian statistics, the relationship is straightfoward.

The probability of emmission of a photocount by the PMT is given by:

$$\omega^{(1)}(t)= \alpha E_s^*(t)E_s(t)= aI(t) \tag{4}$$

where $[\alpha,a]$ are suitably defined quantum efficiencies of the photodetection system. PCS measures the correlation function:

$$C(\tau)=<I(t)I(t+\tau)> \tag{5}$$

$$=\varepsilon^2<E^*(t)E(t)E^*(t+\tau)E(t+\tau)>$$

$$=\varepsilon^2<E^*(t)E(t)>^2 g^{(2)}(\tau)$$

where:

$$g^{(2)}(\tau)=\frac{<E^*(t)E(t)E^*(t+\tau)E(t+\tau)>}{<E^*(t)E(t)>^2} \tag{6}$$

This is the normalized intensity autocorrelation function. Thus:

$$C(\tau)=\varepsilon^2<I(t)>^2 g^{(2)}(\tau). \tag{7}$$

For a Gaussian process the Siegert relation applies and we can relate $g^{(2)}(\tau)$, the measured normalized intensity correlation function, to $g^{(1)}(\tau)$, the normalized autocorrelation function of the electric field of the light as follows:

$$g^{(2)}(\tau)=1+|g^{(1)}(\tau)|^2 \tag{8}$$

For vesicles undergoing Brownian motion $g^{(1)}(\tau)$ has the form (Berne and Pecora, 1974):

$$g^{(1)}(\tau)=e^{-i\omega_0\tau}e^{-\Gamma\tau} \tag{9}$$

where ω_0 is the frequency of the incident laser light. Thus:

$$C(\tau)=\alpha<I(t)>^2[1+e^{-2\Gamma\tau}] \tag{10}$$

In equations 9 & 10, the decay constant Γ is given by:

$$\Gamma=D_T q^2 \tag{11}$$

where q is the scattering vector:

$$q=\frac{4\pi}{\lambda}n\sin\frac{\theta}{2} \tag{12}$$

and D_T is the diffusion constant for the Brownian motion of the vesicles. Using the Stokes-Einstein equation we can write:

$$D_T=\frac{kT}{3\pi\eta d} \tag{13}$$

and thus extract the diameter of the vesicles. Equation (10) assumes that the illuminated area of the photocathode is less than the coherence area of the scattered light. In practice we write (using equation 11) :

$$C(\tau)=B[1+Ae^{-2D_T q^2\tau}] \tag{14}$$

where B is related to the scattered intensity and A is a constant between 0–1 that depends on the coherence property of the optical system.

Polydispersity

One of the central assumptions in the above analysis was that all the scattering particles were the same size. Upon consideration of the problem of a distribution in size of the scatterers the paramount difference in this analysis from the preceeding one is that the correlation function is no longer an exponential but rather a superposition of exponentials corresponding to the distribution of vesicle sizes in the sample.

The correlation function $g^{(1)}(\tau)$ is now:

$$|g^{(1)}(\tau)| = \int_0^\infty F(\Gamma) e^{-\Gamma\tau} d\Gamma \tag{15}$$

where $F(\Gamma)d\Gamma$ is that portion of the total correlated signal contributed by vesicles whose diffusion constants correspond to Γ's in the range Γ to $\Gamma+d\Gamma$. In order to analyse this superposition of exponential decays we utilise the method of cumulants (Koppel, 1972).

According to the method of cumulants $g^{(1)}(\tau)$ can be fit (to 2^{nd} order) to:

$$\ln|g^{(1)}(\tau)| = -<\Gamma>\tau + \mu_2 \frac{\tau^2}{2} + \cdots \tag{16}$$

Where Γ and μ_2 are the first and second moments of the cumulant expansion, respectively. The first cumulant, Γ is found to give the diffusion constant (using equation 11), the second cumulant is a measure of the width of the distribution (Cummins and Pike, 1973). Thus our correlation function can be expressed as:

$$C(\tau) = B[1 + A|g^{(1)}(\tau)|^2] \tag{17}$$

$$= B[1 + Ae^{-2\Gamma\tau + \mu_2\tau^2 + \cdots}]$$

A polydispersity index, Q, is then defined where:

$$Q=\frac{\mu_2}{\langle\Gamma^2\rangle} \tag{18}$$

This represents the degree to which the size distribution of the vesicles deviates from being monodisperse. An example of the relation of polydispersity, Q, to the normalized variance, σ_n^2 in the sizes of vesicles will be shown, for extruded vesicles of 110 nm size, in this work. A comparison of the polydispersity of a fraction of vesicles, obtained from light scattering experiments is compared with size data obtained using negative-staining Electron Microscopy.

The Determination of Elastic Modulii.

The principle of the osmotic swelling experiments presented herein have been published previously (Li et al. 1986). The swelling experiment is depicted in Figure 1. The vesicle is treated as an ideal system in which an impermeant in a homogeneous aqueous solution is entrapped by the vesicle, a thin shell (bilayer) of phospholipid (Gorter and Grendel, 1925; Bangham et al., 1967a, 1967b; Finer et al., 1972; Blaurock, 1982).

The vesicles are prepared in aqueous solutions containing C_i (moles/litre) impermeant. Buffered water or dilute solutions of the impermeant are then added to the sample, lowering the concentration of the impermeant in the non-enclosed aqueous compartment. Lowering the concentration of impermeant institutes an osmotic pressure difference across the bilayer. Water flows through the bilayer (Bangham et al.,1967a; Blok et al., 1976; Trauble, 1971) and the internal aqueous compartment expands until the osmotic pressure difference across the bilayer is balanced by the elastic resistance of the bilayer to further expansion. Assuming

that the bilayer is impermeable to the solute (Harold, 1986; Li et al., 1986) then the total amount of the solute contained by the phospholipid bilayer remains a constant. Since the volume of the internal aqueous compartment has expanded, the concentration of impermeant within it is reduced, to C_1 as the pressure rises to P_1. For dilute solutions the osmotic pressure is proportional to the concentration of solute so:

$$(P_1-P_0)=K(C_1-C_f) \tag{19}$$

where P is the pressure on the bilayer and K is an experimentally determined proportionality constant. Using the assumption that there is no efflux of solute, the product of concentration and volume is the same:

$$C_i V_0=C_1 V \tag{20}$$

given that the diameter of the initial preparation is d_0 and the final diameter d, the volume relationship is $V=\frac{\pi d^3}{6}$ and therefore: $C_i\ d_0{}^3=C_i\ d^3$ the proportionality equation (19) becomes:

$$(P_1-P_0)=K\left\{ C_i\frac{d_0^3}{d^3} - C_f \right\} \tag{21}$$

This can be most easily understood using two extremes of elasticity for the membrane: In the case of a rigid membrane (effectively inelastic) the final radius equals the initial radius for any and all dilutions. In this situation the implication is that the size of the vesicle does not increase, and since water is relatively incompressible, it would suggest that this membrane would exclude any influx of water. The basic permeability of the membrane would be unchanged but water influx would be effectively halted. In the second extreme, the membrane is

infinitely flexible, that is there is no resistance to the increase in the surface area of the membrane and the vesicle will expand until the inner pressure equals the outer pressure:

$$d=d_0(\frac{C_i}{C_f})^{1/3} \tag{22}$$

In the case of synthetic phospholipid vesicles there is a finite resistance to the area dilation of the membrane.

One may divide the osmotic swelling of the membrane into two stresses: a tangential stress σ_T which is isotropic in the plane of the membrane and a radial stress which is considered negligible due to the negligible thickness of the membrane. The tangential stress (σ_T) in the membrane has been (Li et al., 1986) described as:

$$\sigma_T=\frac{d}{4t}(P_1-P_0) \tag{23}$$

where t is the thickness of the bilayer. The tangential stress can also be derived (Li et al., 1986) by considering a plane bisecting a vesicle. The osmotic pressure forces the hemispheres apart with a force equal to $\frac{\pi d^2}{4}(P_1-P_0)$ The force is distributed over the surface of the vesicle abutting the plane which is πdt. The tangential stress is

$$\sigma_T = \frac{\frac{1}{4}\pi d^2(P_1-P_0)}{\pi dt} = \frac{d}{4t}(P_1-P_0) \tag{24}$$

The in-plane longitudinal strain, e, equals the fractional increase in the radius, $(d-d_0)/d_0$ and is related to the tangential stress, σ_T (Li et al., 1986) by:

$$e = \frac{\sigma_T}{2M_e} \tag{25}$$

where M_e is the elastic modulus. Combining equations (21), (24), & (25) this gives:

$$(d-d_0)=\frac{d_0^2K}{8tM_e}[C_0(\frac{d_0}{d})^3-C_f] \tag{26}$$

The data from an osmotic swelling experiment was fit using a two-parameter non-linear least squares fit in which the initial diameter and the membrane modulus were the fitting parameters. The error indicated in the modulus indicates a 90% confidence limit on the parameter.

The bilayer is considered highly permeable to water (Trauble, 1971; Reeves and Dowben, 1970; Finkelstein, 1976, 1987; Fettiplace and Haydon, 1980) but relatively impermeable to solutes such as KCl, LiCl, NaCl and sucrose (Hauser et al.,1972; Papahadjopoulos et al., 1971, 1973; Papahadjopoulos and Kimelberg, 1974) and are thus osmotically active (Bangham et al. 1967a; Reeves and Dowben, 1970; Bittman and Blau, 1972). In our experiments sucrose and potassium chloride are used as impermeants. Based upon permeability data (K^+ permeability is approximately 1.5 x 10^{-12} cm/sec) potassium chloride may be justifiably considered an ideal impermeant. Although there is a difference in the permeability of the bilayer of two orders of magnitude between potassium and chloride, one may not pass through the bilayer because of the high energy barrier preventing charge separation. The applicability of this assumption of impermeability of sucrose is valid (Papahadjopoulos et al., 1973) because sodium and sucrose have equivalent permeabilities at 20 ^{0}C for DOPG membrane vesicles (Na^+

permeability is approximately 3 x 10^{-12} cm/sec). However most other sugars have permeabilities far greater than ionic solutes in pure lipid systems (Jung, 1971; Papahadjopoulos and Kimelberg, 1974; Bangham et al., 1974; Gruner, 1988). Serious objections to the assumption of solute "impermeability" have entered the literature lately (Rivers and Williams, 1990) and alternative analyses of osmotic swelling data have been presented (Hantz et al., 1986). Nevertheless, we argue that the observation of vesicle swelling in response to osmotic pressure leads to the extraction of bilayer elastic modulii; for synthetic phospholipid vesicle systems.

Area Compressibility Modulus

The elasticity of the membrane is essentially the resistance of the membrane to dilation of the membrane surface. Generally the stretching of a surface due to strain yeilds a thinning of the surface material and the problem of the elastic modulus is approached by determining an elastic area compressibility modulus (Evans and Waugh 1977, Kwok and Evans, 1981).

In the course of a micropipette aspiration study (Evans and Waugh, 1977; Kwok and Evans, 1981) an elastic area compressability modulus, K, is defined as $T=K\Delta\alpha$ where $\Delta\alpha$ is the fractional change in the vesicle surface area and T is the membrane isotropic tension. T is related to the tangential stress, σ_T, by $T=\sigma_T t$, where t is the membrane thickness. The in-plane strain e_1 is related to the in-plane stress, σ_T by $e_1=\sigma_T/2M_e$ (Li et al, 1986). Since $\Delta\alpha=2e_1$,$\sigma_T=M_e\Delta\alpha$ or $T=tM_e\Delta\alpha$. The elastic modulus M_e, can be compared with the elastic area compressibility modulus, K, by the relation

$$M_e = \frac{K}{t} \tag{27}$$

in order to compare elastic modulii calculated from osmotic swelling experiments to the elastic area compressibility modulus calculated from micropipette aspiration studies. This relation would allow a comparison of our experimental data with the data derived from the micropipette aspiration studies, if a thickness could be assumed. In order to make the comparison, we use the DOPG bilayer thickness of 42 Å found by Schwarz and Paltauf (1977) for Table I. This value was also used by Hantz et al. (1986) in the computation of their elastic modulus. Hantz et al. (1986).

MATERIALS

DOPG, and DOPC were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Ultrapure sucrose was purchased from Schwarz/Mann (Orangeburg, NY). Formvar solution (0.5 %) in ethylene dichloride,uranyl acetate and carbon coated copper grids were obtained from Ladd Research Industries, Inc. (Burlington, VT). Electron image film, Polycontrast paper, Dektol and HC-110 developers were purchased from Eastman Kodak Company (Rochester, NY). All chemicals were reagent grade unless otherwise noted. All solvents were redistilled before use. Water was distilled from a Corning (Corning, NY) Mega-pure MP 3A system after passing through Illinois Water Treatment Company (Rockford, ILL) universal and research ion- exchange resins. Electron microscopy was performed using a Phillips Model 300 electron microscope and a JEOL JEM 100CX electron microscope. Freeze-Fracture Electron Microscopy was performed at the Mount Sinai Medical School under the direction of Dr. Ronald Gordon of the Pathology Department. Platinum-Carbon replica were made with a Balzers 301 Freeze Fracture Appratus Electron micrographs were analysed utilising the Microcomp Integrated Image Analysis System, Southern Micro Instruments, Inc. (Atlanta, GA) driving the Planar Morphometry software package. Vesicle diameters derived from video images of the electron micrographs were stored in an IBM X-T personal computer and were transferred to the VAX for further analysis.

The submitochondrial particles derived from the inner membrane of bovine heart mitochondria were kindly provided by Dr. H. S. Penefsky of the New York City Public Health Research Institute. Rye plasma membranes were provided by D. Lynch of the Department of Agronomy, Cornell University. Extruded vesicles

were manufactured in a Lipidex (Vancouver,Canada) extruder and in a homebuilt apparatus designed by Dr. Barry Lentz, of the University of North Carolina, consisting of a Millipore (Bedford, MA) high pressure filter holder. Stainless steel tubing, adapters and Swagelock quick-connects were supplied by R. S. Crum & Co. (Mountainside,NJ). Polycarbonate filters for vesicle extrusion and buffer filtration were supplied by Nucleopore Corporation (Pleasanton, CA).

Photon Correlation Spectroscopy utilised a Spectra Physics (Mountain View, CA) Model 165 Argon Ion Laser/ Model 265 Exciter equipped with a Model 589 air-spaced Etalon for single frequency operation. Scattered light was detected with a Hamamatsu (Middlesex, NJ) R464 photomultiplier tube. High voltage for the photomultiplier was supplied from a Power Designs (Westbury, NY) Model 2K20 High Voltage Calibrated D.C. Power Source. Signal voltage for the photomultiplier was provided by an EG & G (Oak Ridge, TN) NIMBIN power supply. Photomultiplier tube output was directed to either a homebuilt digital autocorrelator or a Langly-Ford (Amherst, MA) Model 1096 digital autocorrelator equipped with a Model PAD-1 pulse amplifier descriminator. Data transfer from digital autocorrelator to mainframe computer utilised either a Digital Equipment Corporation (Maynard, MA) PDP-8 or a Plessy Peripheral Systems (Irvine, CA) model LSI-11. Temperature in the sample cells was maintained with a Lauda RM6 refrigerated recirculating constant temperature bath supplied by Brinkmann (Westbury, NY). Stoppered 10 mm x 10 mm fluorescence cuvettes were supplied by Hellma Cells, Inc. (Jamacia, NY).

Phosphorus 31 Nuclear Magnetic Resonance Spectroscopy was performed by Dr. Lawrence D. Mayer of the Department of Biochemistry, University of British

Columbia utilising a Bruker WP-200 spectrometer operating at 81 MHz and by Dr. T. Axenrod of the Department of Chemistry, City College of New York utilising an IBM NR-200 200MHz spectrometer.

EXPERIMENTAL

Preparation of Vesicles (pH-Jump)

The pH adjustment procedure for making vesicles is a modification of the procedure of Hauser and Gains (1982) described by Aurora et al. (1985). The anionic phospholipid (DOPG) (10 mg) was dissolved in 2 ml of redistilled chloroform. In order to remove sodium (or ammonium) ions and protonate the lipid this solution was washed twice with 2 ml of chloroform/methanol/0.2 M HCl (3:48:47 v/v/v). The upper phase was completely discarded each time. The resulting lower phase was washed with a 2 ml mixture of chloroform/methanol/ water (3:48:47 v/v/v). Although the acid washes separate readily from the lower chloroform phase, the water/methanol upper phase frequently forms an emulsion between the upper and lower phases. This can usually be avoided by performing the wash procedure in a test tube with an inside diameter 20mm or greater and by mixing the upper and lower phases gently. Centrifugation at ca. 1000 rpm in a benchtop clinical centrifuge also clarifies the emulsion in 3-5 minutes. The upper phase was completely removed and the lower phase (chloroform:protonated lipid) was withdrawn from the bottom, avoiding any interfacial material, and transfered to a 50 ml round bottom flask. This solution was then rotary evaporated to form a thin phospholipid film on the lower third of the flask. The phospholipid film was dried under a water aspirator at room temperature for 30 min. Vacuum from a mechanical pump (100 microns Hg) was then applied to insure removal of chloroform. 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 swirled gently by hand

for 3-5 minutes at room temperature in order to suspend the lipid film in the buffer solution. The dispersion was transferred to a small vial and stirred vigorously with a small magnetic stir bar. The pH was monitored using an Orion (Cambridge MA) digital pH meter equipped with a micro pH probe. Where an ionic buffer was used it was 150 mM KCl, 2 mM Tris 0.02% and NaN_3 at pH 7.55 (0.276 Os). Where a non-ionic buffer with equivalent osmolarity was used the solution was 250 mM sucrose, 2 mM Tris and 0.02% NaN_3 at pH 7.55 (0.272 Os). These solutions were pre-filtered through 0.1 micron polycarbonate filters (Nucleopore; Plesanton, CA; or Schneider & Schuell; Keene, NH). This suspension was rapidly titrated with 100 mM NaOH solution in the same buffer. The pH was adjusted to 10.0-10.5 by injecting a predetermined aliquot of the alkaline solution. It remained at high pH with vigorous mixing for 1-2 minutes. The pH was back-adjusted to 7.55 with another pre-determined aliquot of 0.1 N HCl in the appropriate aqueous medium. For buffer solutions of sucrose this alkaline or acid solution was freshly made.

The lipid concentration in the vesicle suspension was about 3.3 mg/ml. This suspension (3.0 ml) was diluted with the same medium as that in which the vesicles were made to a total volume of 15.0 ml. This suspension is referred to as the diluted stock vesicle solution. The vesicles were then filtered through a prewashed 25mm Nucleopore (Pleasanton, CA) polycarbonate 1.0 micron filter.

Preparation of Vesicles (Extruded)

Extruded vesicles (LUVETs) were made from multilamellar vesicles following the procedure of Cullis (Mayer et al., 1986; Hope et al., 1985. One milliliter of a solution of phospholipid in chloroform (10 mg/ml) was rotary evaporated in a screw cap test tube under vacuum from a water aspirator so that a thin film covered the lower 3-4 cm of the tube. After all visible traces of the chloroform were removed (approximately 30 minutes), the samples were placed in a vacuum desiccator. Vacuum (about 100 microns Hg) was applied for an additional 3-4 hours to assure removal of chloroform. The phospholipid film was dispersed in 3 ml of the appropriate buffer. This step is facilitated by adding 1-2 1/8" glass beads per milliliter buffer (typically 6 beads for 3ml) to the tube and vortexing the solution for 5-10 minutes. The pH of the suspension of multilamellar vesicles (MLV) suspension was adjusted to pH 7.55 and vortexed for an additional minute.

The resulting suspension was then subjected to the extrusion process. Extrusion was accomplished using a homemade apparatus constructed according to a design graciously provided by Dr. Barry Lentz. This device is depicted in Figure 2. This device is identical in principle to the original Cullis apparatus (Mayer et al, 1986, Hope et al., 1985). The procedure is as follows: Two 25 mm polycarbonate (Nucleopore, Pleasanton, CA) ultrafiltration membranes with pore size of 0.1 micron are stacked atop a 25 mm polyethylene drain disc (Nucleopore, Pleasanton, CA) and clamped into a Millipore (New Bedford, MA) High Pressure filter holder. The solution of MLV is placed in the stainless steel reservoir attached to the filter holder inlet. High pressure nitrogen (approx 200-400 psi) is used to force the lipid solution at high speed through the filter. The turbid

solution becomes opalescent on the first pass through the membrane (approx. 15-30 seconds for 3ml of a 3.3 mg/ml solution). The apparatus is disassembled, cleaned and loaded with fresh membranes and drain disc and the apparatus is washed with buffer. This procedure is repeated 9 times with the second set of polycarbonate filters for a total of ten passes. The resulting opalescent solution is diluted 5 times with buffer previously filtered through a 0.1 micron Nucleopore filter. The dilution is conducted slowly using a Razel (Stamford, CT) syringe pump as the solution is stirred gently with a magnetic stir bar.

Electron Microscopy (Negative Staining)

Vesicles were studied by negative staining electron microscopy with a JEOL JM-100CX at 60 kV. Magnifications were calibrated with a diffraction grating of known wavelength. Formvar coated grids were prepared with a 0.5% Formvar solution in amyl acetate on uncoated 3mm copper grids (Ladd Industries, Burlington VT). Otherwise carbon coated Formvar on 3mm copper grids were used (Polysciences, Warrington PA). The Formvar grid was placed upon a drop of solution containing phospholipid vesicles. The excess solution was adsorbed with Whatman No. 1 filter paper after 15 sec. The grid was then placed on a drop of uranyl acetate solution (1%). After 15 sec, the excess was absorbed with the filter paper. The grid was then allowed to dry in air for 10 min prior to examination. Electron micrographs were recorded on Kodak (Rochester, NY) Electron Image Film, developed in HC-110 developer and fixed in Kodak Rapid Fixer. Enlargements of the 2 1/4 x 3 1/4" negatives were made including a ruled scale to determine additional magnification.

Electron micrographs were analysed directly from negatives utilizing a Microcomp Integrated Image Analysis System (Southern Micro Instruments, Atlanta, GA), a semi-automatic interactive system which measures vesicle sizes from video images of electron micrographs displayed on a computer monitor. Distributional information was collected utilizing the Microcomp Planar Morphometry software.

Freeze fracture electron microscopy was graciously performed by Dr. Ronald Gordon of the Mount Sinai Medical School, Department of Pathology. Vesicles were dropped on a gold die and frozen in freon slush (Gordon et al., 1986). In order to assess true distributions without sacrificing the actual size of the vesicles, no cryo-protectant (glycerol) was used. The samples were fractured and replicated with a Balzers BAF 400D appratus. Micrographs of the replica were taken using a JEOL JM100CX electron microscope. Only unilamellar vesicles were noted in the extruded preparation. Fracture planes were too small to assess the distributional characteristics of the vesicles. Thus, attempts to observe the size distributions by freeze fracture is probably not possible without the introduction of significant amounts of cryo-protectant (25% glycerol) or specialized freezing techniques (freeze-spray).

Nuclear Magnetic Resonance (^{31}P-NMR)

Phosphorus 31-NMR experiments were conducted to determine the extent of unilamellarity of vesicles produced by the extrusion and pH-jump procedures. Mn^{++} added to a vesicle preparation broadens the ^{31}P signal of phospholipid molecules that are in contact with the manganous solution. The ^{31}P NMR signal

of phospholipids of vesicles in which the inner leaflet of the vesicles is not in contact with the manganous ion is not broadened. In non-curvature-limited unilamellar vesicles 50% of the phospholipid is in the inner leaflet. In such vesicles 50% of the signal remains after the addition of manganous ion. A 50 mM $MnCl_2$ solution in the 150 mM KCL, 2 mM Tris and 0.2 % NaN_3 (pH 7.55) buffer was prepared. Extruded vesicles of DOPC and DOPG as well as pH-jump DOPG vesicles were prepared at a concentration of 80 mg/ml. The spectra was obtained for the phospholipids without manganous ion, whereupon the sample was withdrawn from the NMR spectrometer and an aliquot of the manganous solution was added to produce a final Mn^{++} concentration of 5 mM. An additional spectrum, with an equivalent number of scans was acquired and the signal intensities were compared. NMR measurements were made by Dr. Lawrence Mayer of the University of British Columbia, Vancouver.

A Bruker WP-200 spectrometer operating at 81 MHz was used, the FID corresponding to 2000 transients was accumulated. An 11 μsec rf pulse at 47^0 with a 1 second interpulse delay was employed with gated proton decoupling using a 20 KHz sweep width. Exponential multiplication corresponding to 20 Hz linebroadening was applied prior to Fourier Transform.

Photon Correlation Spectroscopy (PCS)

The average size of the vesicle preparations was determined by using photon correlation spectroscopy. A schematic of the experimental apparatus is shown in Figure 3a. The beam of a Spectra Physics (Palo Alto, CA) Model 165 argon ion laser equipped with a single wavelength pass etalon at 488 nm was directed into

the vesicle sample in a 10 mm x 10 mm stoppered fluoresence cuvette (Hellma Jamaica, NY) maintained at constant temperature (20+/-0.1°C) by a Lauda water circulator. Scattered light at 90 degrees was collimated by two pinholes 200 μm and 660 μm diameter and focussed into the photomultiplier tube. The intensity $I_s(t)$ of the scattered light was detected with a Hamamatsu (Middlesex, NJ) photomultiplier tube. Signal from the photomultiplier is directed to either of two correlator units: a Langley-Ford Model 1096 Digital Autocorrelator (Langley-Ford Instruments, Amhearst, MA) or a homebuilt unit referred to as the Pulse Sequence Analyser II. The latter apparatus was described by Hwang and Cummins (1982) The calculations used for sizing with this apparatus are described in the theoretical section. A representative correlation function is depicted in Figure 3b. Output of the VAX analysis of the correlation function is depicted in Figure 3c. The laser was operated at 20 Amps and thermostatting baths were stabilized for at least 30 minutes before use. Polystyrene standards were run during this period to insure proper A/B ratios. The ratio of the first channel counts to the baseline counts (A/B ratio) was observed. An A/B ratio > 0.60 indicates an acceptable signal to noise ratio. This ratio may be maximized by modifying any of the following parameters: optical alignment of the path of scattered light, adjustment of the laser etalon for obtaining monomodality of the incident laser beam (this indicates a single frequency of 488 nm in the incident beam), and adjustment of the signal to noise ratio of the incident beam (fluctuations in the intensity of the incident rather than the scattered light). The sampling (bin) time was adjusted to obtain exponentially decaying correlation functions. Correlation data were collected by a DEC PDP-8 minicomputer (Digital Equipment Corporation, Maynard, MA) or a

Plessy PDP-11 and subsequently analyzed by the technique of multiple linear regression (cumulants analysis) on a VAX 11/780 mainframe computer (Digital Equipment Corporation, Maynard, MA). The majority of the experiments performed for this work were accumulated on a Langley-Ford Model 1096 Correlator using 128 channels with an additional 16 delayed channels to obtain the baseline. Experiments in which osmotic swelling was observed were carried out as follows: A sample of vesicles was diluted with an equal volume of buffer. The sample, in a cuvette, was inserted into the apparatus and five minutes were allowed for thermal equilibration. The count rate was adjusted to 10,000 to 20,000 cps when using the Pulse Sequence Analyser II. Count rates of 30,000-50,000 cps were used when photomultiplier output was directed to the Langly-Ford Correlator. Ten or eleven runs of 180 seconds duration were performed. This initial sample, representing unswollen vesicles, was retained and repeatedly rerun during the experiment in order to assess changes in the sample as a function of time. Subsequent samples were swollen and then thermally incubated in the recirculator bath as measurements on previous samples occurred. The duration of an experiment involving 11 separate samples with 2-3 additional measurements of the initial sample was approximately 12-14 hours.

The average diffusion coefficient and standard deviation were calculated by averaging ten or eleven successive autocorrelation functions, each with the same bin (sampling) time and experimental duration. The count rate typically drifted approximately 5-10% in the course of an experiment. In addition to the diffusion coefficient, the analysis yields an index of polydispersity of the sample which was determined by the cumulants analysis method (cf, Koppel, 1972; Pike &

Cummins, 1977).

To determine the diffusion constant and thereby the radius from the first cumulant, Γ it is necessary to accurately determine the scattering vector. The scattering vector $q=4\pi\frac{n}{\lambda}\sin\frac{\theta}{2}$ where n is the refractive index of the solution, θ is the scattering angle and λ is the vacuum wavelength of the incident light; varies with the refractive index of the medium. Refractive indices of sucrose and KCl solutions were determined experimentally using a refractometer and a fluorescent light source filtered with a Kodak (Rochester, NY) Wratten 45A filter. The refractive index was measured at various concentrations at 20^0 C and was evaluated from a linear fit through the data. Distilled water was used as a standard. The viscosity, η, of sucrose and KCl solutions were calculated from literature equations (Stokes and Mills, 1965 ; Barber, 1966) and compared to values experimentally derived by Aurora et al. (1985). There was excellent agreement between our correction factors for swelling in sucrose solutions with those of Aurora et al. (1985). Our analysis of swelling in KCl solutions includes a correction for viscosity and refractive index that was not used by Aurora et al. (1985). Sizes calculated by the Langley-Ford correlator and cumulants analysis on the VAX normally utilize values for the refractive index and viscosity of distilled water. The radius provided by these analyses must therefore be corrected to reflect the viscosity and refractive index of sucrose and KCl solutions.

Osmotic Dilution of Synthetic Vesicle Suspensions

The vesicle solution (withdrawn from the diluted stock solution) is diluted with a fixed volume of diluent. If the initial volume is one ml and the diluent vol is 1ml; a two-fold dilution of vesicles and buffer concentration is thus obtained. Likewise if the initial volume of the vesicle solution is 1ml and the diluent volume is 2ml a three-fold dilution of vesicles and buffer concentration is obtained. Our current protocols call for a two-fold dilution of vesicles in both KCl solution and sucrose solution. The pH-manipulated vesicle suspension and the buffer solutions were filtered through 1.0 and 0.1 micron pore size filter membranes (Nuclepore; Pleasanton, CA), respectively, prior to dilution. This step was not required for extruded vesicles because they are the product of serial filtration at the level of 0.1 micron. Buffer sulutions were filtered through a 0.1 μm pore size filter (Nucleopore; Pleasanton, CA). In the case of vesicles in sucrose solutions: eleven 1 ml aliquots of vesicles in 250 mM Sucrose, 2 mM Tris, and 0.02% NaN_3 at pH 7.55 are diluted with eleven aliquots of buffer containing 2 mM Tris, 0.02% NaN_3 (pH 7.55) with the following sucrose concentrations: 250 mM, 225 mM, 200 mM, 175 mM, 150 mM, 125 mM, 100 mM, 75 mM, 50 mM, 25 mM, 0 mM. The diluent is injected at the rate of 0.05 ml/min by a Razel (Stamford, CT) syringe pump. This slow dilution procedure was used in an attempt to specifically reproduce the original data (Li et al., 1986). Experiments were not conducted to verify the validity of slow dilution. The final sucrose concentrations are: 250 mM, 238 mM, 225 mM, 213 mM, 200 mM, 188 mM, 175 mM, 163 mM, 150 mM, 138 mM, 125 mM. Likewise, vesicles prepared in 150 mM KCl, 2 mM Tris and 0.02% NaN_3 (pH 7.55) are diluted with eleven aliquots

of buffer (2 mM Tris and 0.02% NaN_3 pH 7.55) with the following KCl concentrations: 150 mM, 135 mM, 120 mM, 105 mM, 90 mM, 75 mM, 60 mM, 45 mM, 30 mM, 15 mM and 0 mM. The final KCl concentrations in the diluted samples are: 150 mM, 143 mM, 135 mM, 128 mM, 120 mM, 113 mM, 105 mM 98 mM, 90 mM, 83 mM and 75 mM. The vesicle suspensions were stirred continuously with a magnetic stir bar during dilution and were kept in a closed cuvette (with a small hole in the Teflon stopper for the syringe needle) to prevent evaporation of water. The diluted samples were incubated in the same recirculating bath used to maintain the PCS sample at 20 ^{o}C in order to achieve rapid thermal equilibrium prior to data acquisition.

During the course of an osmotic swelling experiment the sample was divided into 11 fractions. The first sample was diluted with one volume of the initial concentration buffer. This provides a two-fold dilution of the number of vesicles without any osmotic dilution. The number of vesicles per unit volume was held constant throughout the experiment to prevent the dilution series introducing error due to variation in the intensity (count rate) of the samples. The subsequent ten samples are diluted with one volume of buffers with decreasing osmotic strength. This procedure produces eleven samples from which the osmotic swelling is observed. Each sample is analysed with 10 or 11 correlations of 180 seconds duration. The mean size and polydispersity from each correlation function was obtained and averaged. The standard deviation of the mean size was calculated and was divided by the square root of the number of correlations (10 or 11). Typically this reduces the standard deviation by a factor of approximately 3. The "error" thus assigned represents $\frac{\sigma}{n^{1/2}}$. This quantity is a measure of the

"confidence" of a value. The sizes are plotted against the final external buffer concentration for each sample. These data are presented as a "swelling curve." Each graphic representation (Figure 4) of the data consists of the actual data points and a two parameter non-linear least squares fit (NLLS, Bell Laboratories) from which the modulus is calculated from equation (26)(p. 29). As in any experiment, the data scatters about this "fit". No single point is considered as an absolute indication of the actual size of the vesicles. Swelling appears not as a linear incremental increase in the size of the vesicles but rather as a trend in the data. As such, no data has been eliminated from any of the sizing data. The first sample indicates the unstressed size of the vesicles; it is also used as the experimental "control". This sample is monitored at the beginning of the experiment and at subsequent times during the experiment. The control established that the swelling was not independent of the osmotic dilution. That is, the vesicles do not "swell" or "shrink" during the course of the experiment unless the solute concentration is altered.

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). Samples were kindly supplied by Dr. Harvey Penefsky as deep brown pellets frozen at -78 ^{o}C. The samples were thawed in a micro test tube immersed in water at 20 ^{o}C. The original particle suspension in 250 mM sucrose-Tris buffer (0.24 mg protein/ml, pH 7.5) was diluted 40 times with 250 mM sucrose, 2 mM Tris, and 0.02% NaN_3 at pH 7.55 using a syringe pump to avoid osmotic shock.

The diluted solution was filtered through a pre-wetted 1.0 micron polycarbonate filter (Nuclepore; Pleasanton, CA). The samples were diluted by buffers prefiltered through a 0.1 micron filter to a final volume of 2 ml. The final concentrations of the buffers decreased as described above. PCS measurements were taken at 20 ^{0}C within 24 hours of thawing. The control consisted of a time-course study of the size of submitochondrial particles, carried out over the course of 72 hours. In this experiment, vesicles were maintained in 250 mM sucrose and the size was monitored as a function of time.

Osmotic dilution of Rye Plasma Membrane Vesicles

Cold-acclimated and non-acclimated plasma membrane of *Secale cereale* (rye seedlings) were isolated by D. Lynch as described by Lynch and Steponkus, (1987). The original particle suspension in 250 mM sucrose, 2 mM Tris, and 0.02% NaN_3 at pH 7.55 was diluted 25 times and filtered through a pre-wetted 1.0 micron polycarbonate filter (Nucleopore; Pleasanton, CA). The samples were diluted with an aliquot of pre-filtered (0.1 micron) buffer to a final volume of 2ml. The concentration of sucrose was varied in order to effect osmotic dilution as described above. Samples were analysed by PCS within 48 hours of isolation and were neither frozen nor thawed. PCS was conducted at 20^0 C.

RESULTS

Extruded Vesicles (PCS)

The initial size of the vesicles varied from sample to sample in the size range 105 - 120 nm diameter. Measurements were made to a confidence of 0.3% uncertainty in a mean of ten measurements. The polydispersity indices of the vesicles were between 0.07-0.12. Vesicles extruded in sucrose solutions were slightly less polydisperse than those extruded in KCl solutions, this may be due, in part, to the higher viscosity of the solutions producing greater shear in the extrusion process.

Swelling of DOPC and DOPC vesicles produced by extrusion through polycarbonate ultrafiltration membranes (LUVET) was examined. A consistent pattern of swelling was noted in ionic as well as non-ionic solutions. The swelling noted was not an incremental increase in the size of the vesicles but rather a trend towards larger sizes. This swelling is depicted in Figure 4. A control experiment was established in which the vesicle size was monitored as a function of time. No swelling was observed in the control during the course of an experiment (approximately 12 hours). The swelling was attributed solely to the dilution of the osmoticant (solute) in the external medium. Swelling proceeds with an increase in the mean size of the preparation. During the course of the experiments values for the "mean" size of the vesicles deviate, from the non-linear least squares fit of the data. Outlying datapoints are not discarded. Trends in subsets of the data are also not attributed to any other factor. In that this experiment monitors the size of the preparations we cannot attribute minor deviations in the "elastic" behavior of

the vesicles to bursting, fusion or any other potential outcome. Without confirmatory evidence of entrapped solute release or bilayer fusion these alternative explanations cannot be supported. These potential outcomes may be dismissed only by citing the unpublished personal communication of others (Barbara Mui, 1991, personal communication). These deviations from elastic behavior have been previously attributed to bursting (Li et al., 1986; Miyamoto et al., 1988). In the former case these data are not reproducible, in the latter, the deviation of the size data is so minor that we would attribute the deviation to scatter in the experimental data.

In each experiment the elastic modulus was obtained by fitting the osmotic swelling curves to the elasticity equation derived in Li et al., (1986). The data was fit using a two parameter non-linear least-squares algorithm in which the initial diameter and the membrane modulus were the parameters. The moduli so extracted are given in Table I. The errors indicate a 90% confidence limit on the parameter. It is noted that as the elastic modulus increases, the error in our determination of the elastic modulus increases. This is also noted in the graphical representation of the data (Figure 4). It is noted that the experiments which have relatively slight deviation of the data from a pattern of incremental swelling are those with a greater degree of total swelling. As the degree of swelling decreases, the number of of datapoints which deviate from the "incremental" pattern of swelling. From the diameter was obtained the increase in the surface area of the vesicles as well as their increase in volume. The former is presented in Table I. Deviations from elastic behavior were not significant enough to indicate "bursting" or the attainment of an elastic limit. We observed continuous swelling of the vesicle preparations without a "plateau region" as was reported by Li et al., (1986)

and Haines et al., (1986). In so far as we no longer observe the phenomenon of swelling to a burst point, followed by a region of no increase in vesicle size ("plateau") (Li et al., 1986) we cannot derive an elastic limit for the vesicles in the region of osmotic gradients imposed on the vesicle bilayers. Osmotic swelling experiments performed by other groups (Hantz et al., 1986; Sun et al., 1986) confirm this account of osmotic swelling.

The elastic modulus of the DOPC and DOPG appears slightly lower in sucrose than in KCl. However the error in the determination of the elastic modulus of the bilayers in KCL (particularly for DOPC) overlaps the error of those obtained in sucrose solutions. As such, a definite statement as to a reduction in the Young's modulus in low ionic strength medium cannot be made without further experiments.

Extruded Vesicles (EM)

In order to provide supporting evidence of the uniformity of the extruded vesicle preparations, we have conducted an electron microscopic distributional analysis of extruded vesicle preparations. Figure 5 indicates the size distribution of vesicles produced by extrusion as monitored by negative-staining electron microscopy. Table II compares the mean size and normalized variance as derived from EM to the mean size and polydispersity derived from PCS of a preparation of extruded DOPG vesicles. These were in good agreement with previously published accounts of this technique (Hope et al., 1985). There was also good agreement in the mean size obtained from EM with the mean size obtained from light scattering experiments.

This agreement between the light scattering diameter and the mean diameter from the EM measurements was not observed in the pH-jump data (see below). For the extruded vesicles each data set showed reasonable consistency in the mean and normalized variance from one grid to another. The histogram of all the extruded files was illustated in Figure 5. The normalized variance and mean diameter $< d>$ can be related to the polydispersity Q, in the case of the extruded vesicles. Light scattering experiments indicate that the mean vesicle size of the preparation was 110 nm and the polydispersity of the vesicles was 0.09 . Electron micrographs of an identical preparation yielded a mean vesicle size of 106 nm and a normalized variance of 0.11 This corresponds to a normalized standard deviation of 0.33. This indicates that there is excellent agreement between the sizes obtained from electron microscopy and light scattering. Recent experiments by Cullis (Nayar et al., 1990) investigated the size data obtained from light scattering experiments and freeze-fracture EM. These experiments noted a great variation in the PCS sizing of the vesicles which was dependent on the analysis algorithm employed. Freeze-fracture electron micrographs indicated the mean size of the preparations were significantly smaller than that of the pores in the ultrafiltration membranes, in contrast to our results.

One of the most significant points to be made from these investigations is that the polydispersity is not equivalent to the standard deviation of the preparation. Some light scattering instruments (NICOMP) use these terms interchangeably, which may lead to misleading claims in the literature. In our experiments the normalized variance and the polydispersity were compared. This issue has been addressed more fully in a simulation study developed by Lloyd Williams in

Rutkowski et al (1991). Briefly, the size distribution of vesicles obtained from negative staining EM and computer-derived Gaussian size-distributions of vesicles were used to produce synthetic correlation functions. These synthetic correlation functions were generated using a solution to the Mie problem of a hollow sphere found by Aragon and Elwenspoek (1982). The correlation functions were then analysed as though they were experimental data. This enabled us to extract both a diameter and polydispersity. The values given by the computer modeling were compared with the light scattering and EM values in Table II. Agreement was found for the diameters for all three results and the polydispersity found from the computer modeling agreed with the PCS results.

This same procedure was used to determine the relationship between the polydispersity index and the normalized variance for Gaussian distributions of vesicles. A series of synthetic correlation functions, representing a series of distributions with increasing normalized variance was analysed in a manner identical to the experimentally obtained correlation functions. The results of the analysis of the analysis yield polydispersity indices corresponding to the various Gaussian distributions. In Figure 6 we plot the polydispersity, Q, against the normalized variance of the vesicle sizes $\sigma_n^2=\sigma^2/d^2$ (where d is the average vesicle diameter). For small values of normalized variance, the polydispersity and the normalized variance may be compared, if the vesicles are assumed to have a Gaussian distribution. In cases where the the normalized variance exceeds 0.40, the polydispersity eventually saturates at a polydispersity index of approximately 0.20. Therefore preparations of vesicles in which the polydispersity index exceeds 0.20 have sizes distributions in which the normalized variance is equal to or exceeds 0.40. These

results also illustrate the limitations of the PCS technique for samples with polydispersities greater than approximately 0.2.

Extruded vesicles (^{31}P-NMR)

P^{31} NMR spectroscopy was performed on suspensions of extruded and pH-jump vesicles in order to determine the extent of unilamellarity in the preparations. Measurements were made by Dr. Lawrence D. Mayer at the University of British Columbia. The spectra shown in Figure 7 indicate that approximately 50% of the signal produced by the phospholipids are quenched (linebroadened beyond detection) upon introduction of 5 mM Mn^{++}. Control light-scattering experiments indicated that fusion is not provoked by the introduction of this divalent cation. This indicated that 50% of the phospholipid headgroups in both DOPC and DOPG extruded vesicle preparations were inaccessible to the manganese in solution. A similar result was obtained for pH manipulated DOPG preparations. Furthermore the spectra (Figures 7 & 8) obtained by both Mayers and myself, in conjunction with Dr. Theodore Axenrod, are consistent with published spectra of unilamellar vesicles of 100 nanometer diameter (Burnell et al., 1980; Smith and Ekiel, 1984). Due to the inherent symmetry of the spherical vesicles and their high tumbling rate the phosphorus chemical shift displays a relatively narrow spectral linewidth although it is wider than that of triphenylphosphite (Figure 8).

pH-Jump Vesicles and Submitochondrial Particles

The PCS measurements indicated an initial mean vesicle size in the range 200 - 300 nm for the pH-jump vesicles. The polydispersity index was 0.35-0.45, four times larger than that of the extruded vesicles. The sub-mitochondrial particles displayed a mean vesicle size range of 140 - 160 nm with a polydispersity index of ~ 0.35.

Experiments on pH-manipulated vesicles indicated that imposition of an osmotic gradient does not result in swelling , as appeared when extruded vesicles (LUVETs) of identical composition were used. This finding contradicts accounts of vesicle swelling previously described by our group (Li et al., 1986; Haines et al., 1987, Li, 1987). In contrast we note a decrease in the mean radius of the distribution of vesicle sizes.

Similarly, submitochondrial particles from bovine heart prepared by Dr. Harvy Penefsky produced no swelling in contrast to previous results (Li et al., 1986). Figure 9 displays the effect on vesicle diameters of osmotic dilution. As in the case of pH-manipulated vesicles the mean size decreases, in contradiction to previously documented size increases. In the case of submitochondrial particles, this size loss is solely a function of incubation at 20 ^{o}C (Figure 9). The reduction in the size of the vesicles was shown to be completely independant of the osmotic dilution. This reduction in the size of the vesicles was a function of time, the majority of the shrinking occurs in the time usually used for the osmotic swelling experiments. Since temporal shrinking dominates the sizes of the particles under the conditions used, further investigation of the osmotic swelling of these particles was abandoned.

EM measurements were made on the pH-jump vesicles. Attempts to ascertain the size distribution of the pH-jump vesicles proved inconclusive. This was primarily due to the inhomogeneity in the sample. The mean size and standard deviation showed considerable variation from grid to grid. The general picture was that of a sample with the majority of the vesicles in the 50 - 150 nm range, with a fraction of the preparation displaying sizes in the 300 -600 nm range. However, it seems that the average size of particle is smaller than we obtained from light scattering; with some grids giving mean diameters as low as 80 nm compared to the light scattering value of 250 nm. The contribution of each vesicle to the correlation function is weighted by the amount of light that it scatters. Larger vesicles scatter disproportionately more light. Thus, in a system like the pH-jump system, the few larger vesicles may dominate the light scattering properties of the proparation. Information gained from light scattering on these preparations would not be expected to accurately reflect the mean size including the contribution of the small vesicles. This observation underscores the importance of making PCS measurements on systems with a low (~0.10) index of polydispersity.

Rye Plasma Membrane Vesicles

Rye plasma membrane vesicles, (Wolf and Steponkus, 1983; Wolf et al., 1986; Lynch and Steponkus, 1987) suspended in 250 mM sucrose, were subjected to swelling protocols identical to those applied to the bovine submitochondrial particles. Swelling was not noted in plasma membrane vesicles derived from either native or cold-acclimated winter rye seedlings *(Secale cereale)*. Initial plasma membrane vesicle sizes were approximately 195 nm diameter with a polydispersity

of 0.12-0.14. Throughout the swelling experiment, the sizes of these particles did not change significantly. This trend of osmotic insensitivity was noted equally in both classes of plasma membrane vesicles (native and cold-acclimated). The resuts of these experiments may be explained in light of the recent simulation studies of Rivers and Williams (1990).

DISCUSSION

In these experiments we have measured the Young's modulus of phospholipid bilayers, organized into vesicular structures under conditions of non-ionic and ionic aqueous solvation. This was achieved by using PCS to follow their osmotic swelling. The swelling measurements are sufficiently precise to follow the increase in the mean size of the vesicle preparation. Throughout the swelling no increase in the polydispersity of the preparations were noted. This leads us to conclude that the size increases noted are the result of swelling of the entire ensemble of vesicles, rather than the fusion of a sub-set of the population or the swelling of a sub-set of the population. We have calculated an elastic modulus for DOPC and DOPG bilayers and found them to be on the order of 5×10^8 to 12×10^8 and 3×10^8 to 6×10^8 dyn/cm^2, respectively. Within the limits of error in our experimental system, a difference in the elasticity of each individual type of bilayer is not observed with respect to ionic or non-ionic solvation. Rather it appears that the variation in the elasticity of these bilayers is a function of the structure of the lipids or the properties of the bilayer implied by those structures. It is the function of this discussion to illuminate those points which support and those which challenge our claim.

A valid question is whether or not PCS accurately measures the size of bilayer vesicles. The mean size and size distribution of the vesicle preparation prior to swelling was investigated using electron microscopy and photon correlation spectroscopy. Our comparison of the vesicle size distribution obtained from negative-stained electron microscopy with computer-modelled light scattering data

has allowed us to relate the polydispersity of a preparation of vesicles with the normalized variance in the measured (EM) size of the same preparation. Our comparison of PCS sizing with negatively stained EM of DOPC and DOPG LUVET agree with a portion of the PCS data presented by Cullis for a preparation of EPC LUVET (Nayar et al., 1989). Negative-staining EM provides a closer agreement to PCS results than freeze-fracture EM (although Cullis analyses the light scattering data using an algorithm intended for solid spheres (i.e. polystyrene latex spheres) rather than thin spherical shells (i.e. vesicles) in order to achieve a correspondence of the freeze-fracture EM and PCS results). Thus, the sizing of the vesicles by PCS is a reasonable source of size data for the calculation of the elastic (Young's) modulus. Furthermore, the results of P^{31} NMR are consistent with the claim that the preparation is one of unilamellar vesicles only. Therefore, the elasticity calculated is characteristic of single bilayer vesicles under osmotic stress. The swelling was shown not to be a temporal function of the vesicles but rather a function of dilution of the "impermeant" solute bathing the vesicles (see Figure 4). The moduli we report are in excellent agreement with those reported by groups using micro-pipette aspiration and photon correlation spectroscopy (Kwok and Evans, 1981; Hantz et al., 1986; Sun et al., 1986). Our analysis of the swelling data appears to be adequate for the analysis of vesicles composed of pure phospholipids which display a high degree of resistance to diffusion of the "impermeant" used to generate the osmotic pressure gradient.

The use of extruded vesicles composed solely of synthetic phospholipids has eliminated potential artefactual increases in the mean size of the vesicles. A fraction of small, sonicated vesicles are known to fuse spontaneously (Lentz et al.,

1987). These fusion events are probably due to lipid packing defects in the small curvature-limited vesicles: hydrocarbon exposure to the aqueous medium is limited by fusion. Liposomes which contain ion channels such as porin and nystatin are known to fuse with planar phospholipid bilayers when the vesicles are subjected to osmotic stress (Cohen et al., 1989; Niles et al., 1989). These fusion events are not noted when the ion channels are not incorporated in the vesicles. Various other vesicle forming techniques may leave residual amounts of fusogens in a vesicle preparation: detergent dialysis methods may leave small amounts of residual surfactant; pH-manipulation techniques may result in the formation of lysolecithin. Since the vesicles used in this study are extruded 0.1 μm pure DOPC and DOPG phospholipid vesicles, fusion resulting from either a small fraction of curvature-limited vesicles, artefactual incorporation of "fusogens" or vesicles with ion channels which increase the the permeability of the "osmoticant" (Cohen's terminology) is avoided. Further study concerning vesicle-vesicle fusion during osmotic swelling is warranted; presently, control experiments indicate that there is no fusion independant of osmotic swelling during the course of the experiments. Osmotic swelling of 0.1 μm EPC LUVET (Mui et al., 1991) proceeds without vesicle-vesicle fusion. The adaptation of current protocols to include relaxation of the imposed osmotic gradient (and a return of the swollen vesicles to their initial mean size) would completely rule out this potential interference by indicating increases in the mean size of the vesicle preparation that were not a function of osmotic pressure differences across the bilayer.

There are additional objections to our findings which must be considered. We infer a surface area increase on the order of 3-6% in our experiments.

Previous osmotic swelling-PCS experiments indicate surface area expansion as high as 25% (Hantz et al., 1986; Sun et al., 1986)(See Table I) and surface area expansivity beyond 3% are unremarkable in these experiments. This degree of swelling far surpasses the surface area expansion of giant phospholipid vesicles or erythrocytes (Kwok and Evans, 1981) directly observed in the microscopic studies on these systems. The major objection to the use of osmotic-swelling/light-scattering experiments comes from the groups which utilize these micropipette aspiration techniques. In experiments using the micropipette aspiration techniques, vesicles are made by gently swirling lipid with a buffer solution. Giant vesicles are then mechanically manipulated under a video-linked microscope during the experiment. The giant vesicles co-exist with numerous small microscopic and sub-microscopic vesicles. Aspiration of these giant vesicles yield size data which can be used to calculate K, the membrane area compressibility modulus, as well as the elastic shear modulus and the limit to surface area expansion (prior to bursting). K may readily be converted to M_e, the Young's Modulus, if the thickness of the bilayer is known or a reasonable value assumed. The experimental data lead to a wide range of moduli for a given preparation of vesicles. The average modulus is calculated from this wide set of individual moduli. In fact the membrane area compressibility moduli found for giant DOPC vesicles (Kwok and Evans, 1981: see fig 7) is a bimodal distribution. The major group of moduli have an average of 140 dyn/cm. Another group of moduli have an average modulus of 227 dyn/cm. This outlying set are *a posterori* dismissed as being due to "bilamellar vesicles" and are not included in the calculation of the average modulus. Their objection to osmotic swelling experiments is that from their

experience liposomes are present in wide, typically multimodal preparations and that bilamellar and multilamellar vesicles often escape detection. Calculation of the elastic modulus from the ensemble vesicle population such as these would not effectively screen out the influence of these components. While the micropipette aspiration technique may be applied to additional problems regarding the materials property of phospholipid bilayers (shear modulus, limit to area dilation) its utility is restricted to micron sized systems. Unfortunately, phospholipid vesicles of these dimensions do not display the temporal stability associated with submicron sized vesicles. Micropipette aspiration is superior to the osmotic swelling-PCS technique in those systems where the membrane is associated with an underlying cytoskeletal framework, such as is found in the erythrocyte, assuming adequate size of the particles under study. Additionally, osmotic swelling probably could not be resolved in vesicle preparations sized on the order of 0.1 μm using our current osmotic swelling-PCS protocols were the Young's modulus greater than 2-3 X 10^9 dyn/cm^2. This is due to the fact that the degree of swelling would be extremely slight and the trend towards "swelling" would be contained within the error of observation.

Rivers and Williams (1990) argue that osmotic swelling experiments may not be valid for vesicles derived from natural membranes because the vast majority of biologically derived bilayers probably do not satisfy the basic requirement of non-permeability to solute. These authors simulated osmotic swelling of brush border membrane vesicles using an algorithm which accounts for an efflux of impermeant during the experiment. In the case of intestinal brush border membranes, the "lipid bilayer" is composed of a plethora of membrane transport proteins which

may permit permeabilities to many solutes far in excess of their permeabilities in pure lipid bilayers.

The presence of transbilayer and membrane-embedded transport proteins may alter the bilayer permeability of many solutes (Stevens et al., 1982, 1984), particularly when osmotic gradients are encountered (Laimins et al., 1981; Weissbom and Kennedy, 1984; Higgins et al., 1987). Additionally, tension on the biological membrane has been shown to alter ion permeability (Yang and Sachs, 1987; Gustin et al., 1988) so that at least two potential pathways for relaxation of the osmotic gradient are potentially present in biological systems under osmotic pressure. Osmotic swelling requires dilution of an "impermeant" in the external medium associated with the influx of water to the entrapped aqueous compartment. The time scale in which PCS experiments are conducted is far greater than those using the micropipette aspiration technique, thus, leakage of an impermeant may reduce the extent of swelling observed. Singer (1981) demonstrated that the permeability of the membrane is greatest at the transition temperature. In order to avoid gel-phase (L_β) to liquid-crystalline (L_α) phase transition our experiments were conducted on lipids with di-monounsaturated acyl chains (DOPC and DOPG) whose transition temperatures (-20 ^{o}C) are well below ambient temperatures. Experiments by Borochov and Borochov (1979) suggest that the fluidity within the membrane increases upon osmotic swelling. This contention is supported by the recent report by Cullis (Mui et al., 1991) in which changes in lipid packing which were induced by osmotic swelling of 0.1 μm EPC LUVET were studied using fluorescent membrane probes. Osmotic swelling results in a less ordered membrane, and a decrease in the packing order of the lipids. These

results indicate that only the gel to liquid-crystalline phase transition would occur as a result of osmotic swelling. Any contention that osmotic swelling leads to a phase transition in lipids that are already in the liquid-crystalline is effectively ruled out. However vesicles that are already near their transition temperature may be induced to go through a transition to liquid state upon osmotic swelling and this potential phase transition cannot be ruled out in other lipid systems.

Beyond the issues concerning the effects of osmotic swelling on the integrity of the liposome as an ideal osmometer, are other practical objections to the osmotic swelling-PCS determination of the Young's modulus. In the course of an aspiration experiment, the vesicle is sucked into the bore of a micropipette, this produces a spherical vesicle with a "tail" sucked into the pipette. Suction is applied to the vesicle and the surface area increase is monitored. The time scale of each experiment is on the order of seconds. Since there is little time for equilibration, the membrane is tensed, it expands and eventually bursts. In contrast, the time course of an osmotic swelling experiment is such that the vesicles are diluted slowly (over 20 minutes) and allowed to thermally equilibrate (5 minutes) prior to analysis (35 minutes). The slow dilution prevents osmotic shock (this group's previous experiments (Li et al., 1986) suggested this was an experimental problem). During the period of dilution the membrane is subjected to increasing osmotic stress, which is theoretically maintained during the latter portion of the experiment. The "back diffusion" of impermeant from the swollen vesicle to the dilute external medium would provide relaxation of the osmotically-induced swelling of the membrane.

These objections (Rivers and Williams, 1990) call into question the applicability of osmotic swelling as a tool to study the bilayer elastic modulus. In general terms, these authors are correct: the determination of the elastic modulus from size data derived during the osmotic swelling of naturally derived bilayer membranes is suspect. This was confirmed by repetition of earlier experiments on bovine submitochondrial particles conducted by Li (Li et al., 1986; Li, 1987) and also in subsequent experiments conducted on rye plasma membranes supplied by Steponkus. These membranes do not swell when subjected to protocols similar to those which produce evidence of swelling in synthetic phospholipid vesicles. In fact the submitochondial particles lacked the stability to retain their size during the protocols. The rye plasma-membrane, cold-acclimated vesicles had previuosly been shown to undergo a large surface area expansion on the order of 60% (as opposed to 20% for non-acclimated membrane). This expansion is not elastic until the last 2-3% of surface expansion. Wolf (Wolf and Steponkus, 1983; Wolf et al., 1986) compares this elastic surface expansion to that obtained by Evans (Kwok and Evans, 1981) for the erythrocyte. The elastic modulus for the rye plasma membranes (cold acclimated, K= ~ 225 dyn/cm) is half that of the erythrocyte (K= 450 dyn/cm). During our experiments, no swelling was noted upon imposition of osmotic gradients on these rye plasma-membrane isolates. Unfortunately, the size stability of these particles was not investigated separately from the osmotic swelling experiments. Our qualified conclusion drawn from these results is that osmotic swelling was ineffective in bringing about swelling in a membrane which can be readily expanded in micropipette-aspiration experiments. Though qualified by the potential interference of the polydispersity of the sample

or an inherent instability of the mean size of the particles, our results may provide impetus to the notion that biologically-derived membranes may not accurately reflect the resistance of the membrane to an imposed osmotic pressure. Rather, impermeant efflux throught the bilayer, transbilayer ports or other as yet undetermined processes dominate the response of these particles to imposed osmotic gradients.

Another objection to the use of osmotic-swelling / PCS computation of the elastic modulus in biological samples considers the mechanical coupling of the membrane to structures embedded within the membrane. These membrane-intrinsic structures, and their response to tension in the membrane may dominate the response of biological systems to osmotic swelling (Green, 1990). The degree of swelling observed may not reflect the materials property of the lipid bilayer but rather the response of the membrane, as an ensemble lipid-protein structure, to the imposed tension. The elastic modulus calculated would reflect the elasticity of the membrane-protein composite, from which the contribution of the phospholipid bilayer could not readily be resolved.

Rivers and Williams (1990) are particularly critical of Miyamoto and Fujime's (1988) measurements of a modulus for brush border membranes using mannitol as an impermeant. They note that mannitol has a higher permeability than would reasonably be permitted for making reliable calculations of the elastic modulus. Miyamoto and Fujime used (Miyamoto et al., 1988; Fujime et al., 1988; Miyamoto and Fujime, 1990) experimental protocols similar in principle to those described herein, but they were conducted on a much abbreviated time scale. The osmotic swelling occurs on the order of 300 seconds (as opposed to 1200 seconds

in our experiments). Swelling is immediately followed with the aquisition of ten correlation functions from which the mean size data is calculated. Rivers and Williams (1990) may have mis-interpreted Fujime's experimental protocol in their paper. Rivers alleges 120 seconds of data acquisition following osmotic swelling in Miyamoto's experiments; however we interpret Miyamoto's procedure to indicate that ten 120 second correlations are acquired for a total of 1200 seconds of data acquisition. This is more in line with the duration of the light scattering portion of our experiments: eleven 180 second correlations. However, if Miyamoto's experiments were conducted on the brief time scale described by Rivers and Williams, then Miyamoto may well show swelling in osmotic swelling experiments whereas a similar experiment, conducted under our protocols, might not. The duration of our experiment for each data point is on the order of 50 minutes (20 minutes swelling, approx 5-10 minutes thermal equilibration and 33 minutes of data acquisition). The revelation of the temporal particulars of the experiments conducted by Miyamoto (Miyamoto et al., 1988), in the report presented by Rivers and Williams (1990), if correct, also brings into the question the ability of Fujime's group to accurately measure the mean size of these polydisperse preparations. Under the conditions of our light scattering experiment, 120 seconds of data acquisition would not produce sufficient data to precisely measure the mean size of phospholipid vesicles. How such brief data acquisition periods supply sufficient data to calculate mean sizes with such precision of a vesicular system which is more polydisperse than extruded preparations raises further questions as to the number of correlations acquired, the photon count rate, and the determination of error in Miyamoto and Fujime's exerimental system. Nevertheless

Miyamoto and Fujime's (Miyamoto et al., 1988; Miyamoto and Fujime, 1990) claims are the sole claims that osmotic swelling is observable in isolated (biological) vesicular preparations and that size data derived therein allows one to determine the elastic modulus.

Hantz (Hantz et al., 1986) has formulated an elasticity equation in which the permeability of solute is factored into the determination of the elastic modulus during osmotic swelling. Hantz notes the extreme variability of values for permeability of phospholipid bilayers to several solutes. Upon consideration of the magnitude of permeability of chloride and potassium presented in the literature (Hauser et al., 1972; Bangham et al., 1974a; Papahadjopoulos and Kimelberg, 1974; Gruner, 1988) we infer that the effect of solute diffusion is minimal in the ionic aqueous solution, 150 mM KCl. Although chloride is several orders of magnitude more permeable than most monovalent cations, excess chloride diffusion from osmotically stressed vesicles is prevented by the concomitant buildup in the electrical potential across the membrane. Thus, the chloride diffusion from the vesicles is dominated by the potassium diffusion. Our assumption that the bilayer is impermeable to solute is valid in this case. Values for sucrose permeability across bilayers (Papahadjopoulos et al., 1971, 1973) show that it is more permeable than monovalent cations such as potassium and sodium but not significantly so. Currently, we are not able to discern a significant difference in the elasticity of phospholipid vesicles in sucrose or KCl solutions because of an overlap of the error in our measurements. This "equivalence" may reflect the similarity of KCl and sucrose permeabilities, an intrinsic insensitivity of the membrane to the ionic strength of the medium (osmotic strength dominating the degree of swelling of the

membrane) or both. The faint differences may be real but are not significant due to the limited number of experiments performed. Difference in the Young's modulus in sucrose and KCl solutions may reflect permeability differences of the solutes, both in regard to differences of KCl and sucrose permeability in one individual lipid, and permeability differences between DOPC and DOPG bilayers. The potential failure of synthetic phospholipids vesicles to swell to the limit which would have been observed had these bilayers satisfied the (Rivers and Williams, 1990) condition of "impermeability" may preclude any meaningful discussion as to the relative elasticities of phospholipid and mixed phospholipid/sterol, etc. bilayers. Clearly a larger base of data is required before any significant interpretation of the results can be accomplished.

The results presented here are preliminary results, the experimental protocols were focussed upon the reproduction of this group's previous experiments. In order to increase our confidence in the present results, the observation of osmotic swelling using PCS techniques requires modification of the present protocols in order to increase the magnitude of osmotic pressuree differential across the bilayer. This will produce a larger amount of swelling in the vesicle systems investigated, as well as allow the analysis of vesicle systems where the the magnitude of the Young's modulus may approach 5×10^9 dyn/cm^2 (Needham and Nunn, 1990). Recent, as yet unpublished, studies indicate that extruded vesicles can withstand 600 mOsm pressure differentials across the bilayer (Mui et al., 1991). This approach appears to be readily applicable to the present problem. Another approach may involve the synthesis of large unilamellar vesicles with sizes on the order of 200-400 nanometers.

The results of the present study indicate that the pH manipulation technique is one of many procedures which produce highly polydisperse vesicle preparations. Highly polydisperse vesicle preparations may not be used with osmotic swelling to determine a bilayer elastic modulus. The results of this study indicates that the determination of the elastic properties of phospholipid bilayer, in submicron vesicle systems, requires a preparation of minimal polydispersity. The extrusion method appears adequate for obtaining suitably uniform vesicles with sizes on the order of 100 nanometers. The results of computer modeling of liposome size distributions (Rutkowski et al., 1991) indicate that the polydispersity of the preparation should be on the order of 0.1 or less. When the polydispersity of the preparation approaches 0.2 this term no longer adequately describes the width of a gaussian distribution of vesicle sizes. Vesicles used for these determinations should be characterized in terms of the following parameters: extent of unilamellarity, permeability of solute through the lipid bilayer, and size distribution characteristics. The current study indicates the significance of each of these parameters as well as the consequences of ignoring any of these factors.

Presently, the polydispersity of most vesicle preparations and biological systems limits the reliability of PCS sizing. This in turn limits our ability to discern osmotic swelling in these systems. While extrusion through polycarbonate ultrafiltration membranes was sufficient to produce a preparation of vesicle which does swell, this technique may not be applicable for all vesicle systems (biological isolates). Therefore improved methods of size fractionation, or vesicle formation are needed. The formation of vesicles by dialysis of detergent from phospholipid/detergent mixed micelles has been shown to produce narrow size

distributions (Jamshaid et al., 1988). This method of vesicle formation also permits the incorporation of membrane-bound proteins into the vesicles. Vesicles produced by any one of a number of techniques may be further fractionated in order to reduce polydispersity by techniques such as sedimentation field flow fractionation (Kirkland et al., 1982; Dreyer et al., 1988) or dialysis against sized ultrafiltration membranes (Bosworth et al., 1982). Reduction of the polydispersity in preparations of biological origin may allow the computation of the elastic modulus upon osmotic swelling. Unlike phospholipid vesicles, strong evidence will be required to prove that observed size increases, if they occur, are the result solely of osmotic dilution and are not the result of fusion initiated by osmotic swelling.

We have presented an experimental system which provides a reproducible method for swelling and measuring mean size changes in the vesicle preparations. The analysis used in this course of study, requires specific assumptions, which we claim have been met. Nevertheless the claim that these assumptions have been met are testable. Objections to the applicability of osmotic swelling in the determination of elastic modulii of vesicular preparations presented by Rivers and Williams (1990) though generally correct, are of minimal significance in pure phospholipid vesicles swollen with effective impermeants such as KCl and sucrose (additionally LiCl and NaCl). The size changes in the vesicles and the elastic modulii derived from these may be tested by alternative methods (i.e.: fluorescence resonance energy transfer between surface bound fluorescent probes, decrease in fluorescence quenching in probes contained in the entrapped aqueous compartment) thus supporting or contradicting the thesis posited. The data

presented in the thesis suggests that the surface expansion of small unilamellar vesicles exceeds that measured from giant vesicles during micropipette aspiration studies. In the case of pure lipid or surfactant based vesicles of submicron size, in which the diffusivity of solute through the bilayer is known (or can be tested) osmotic swelling coupled with photon correlation spectroscopy provides a rapid, non invasive technique towards the determination of elastic modulii. This system provides a means of producing a stable preparation of swollen vesicles for analysis by PCS and potentially by a variety of spectroscopic techniques. Thereby, this experimental protocol may be adapted to probe the dynamics of lipid bilayers during osmotic swelling. The influence of vesicle polydispersity, solute permeability, and the temporal stability of the size of biological preparations, when properly accounted for, may be minimized and the combined osmotic swelling-PCS experiment may be of use in the determination of the Young's modulus in sub-micron sized biological systems. For sub-optical sized particles it is currently the only technique from which the elastic (Young's) modulus may be obtained.

CONCLUSIONS

These experiments supersede those conducted in the following papers (Aurora et al., 1985; Li and Haines, 1986; Li et al., 1987; Haines et al., 1987) and these results alter the previously reported conclusions as follows:

1) The pH-adjustment method for obtaining a uniform preparation of vesicles does not produce a uniform preparation of vesicles; they are highly polydisperse. PCS measurements of this preparation during dilution of the solute

do not permit the determination of a Young's modulus.

2) There was no "plateau" region in the osmotic swelling curves and therefore no evidence for vesicle bursting. Therefore there is no evidence for an elastic limit of vesicles.

3) The elasticity of phospholipid bilayers in ionic solutions is not measurably different than it is in nonionic solutions. Thus the finding that the elastic properties of acidic phospholipid bilayer vesicles depend on the ionic strength of the solute is a spurious result.

4) The PCS method has not been used successfully here to explain osmotic swelling of vesicles derived from natural membranes, namely submitochondrial particles.

TABLE I

author	membrane	solution	K_c (dyn/cm)	d (cm) X 10^8	M_0 (dyn/cm^2) X10^{-8}	maximum observed $\delta A/A$	vesicle diam. (nm)
Wobschall	HDTAC-C	40 mM KCl	150-300	N.A.	N.A.	N.A.	N.A.
Alvarez & Latorre	GMO	1. 0 M NaCl	N.A.	N.A.	1.4	N.A.	N.A.
Kwok & Evans	EPC	100 mM NaCl	140	40	3.5	2-3% [b]	10,000
Sun *et. al.*	DMPC	350 mM LiCl	660	34	19.4	2.8%	85.2
	DMPC	350 mM LiCl	305	34	8.7	6.4%	89.0
	DMPC	50 mM LiCL	68	34	1.9	9.7%	106
Hantz *et. al.*	DOPC	150 mM NaCl	63	42	1.5	25%	160-200
	DMPC	150 mM NaCl	51	34	1.5	12.5%	160-200
Miyamoto *et. al.*	Brush Border MV	200 mM Mannitol with (0 mM Glucose)	150	N.A.	N.A.	5.5%	320-340
		(10 mM Glucose)	80	N.A.	N.A.	6.4%	320-340
This work	DOPC	150 mM KCl	340	42	8.2 ± 1.4	2.9%	116
	DOPC	250 mM Sucrose	240	42	5.5 ± 0.7	4.6%	118.5
	DOPG	150 mM KCl	220	42	5.2 ± 0.5	4.5%	107.5
	DOPG	250 mM Sucrose	165	42	3.9 ± 0.6	5.6%	108.7

Vesicle Elastic Constants and Surface Area Expansion

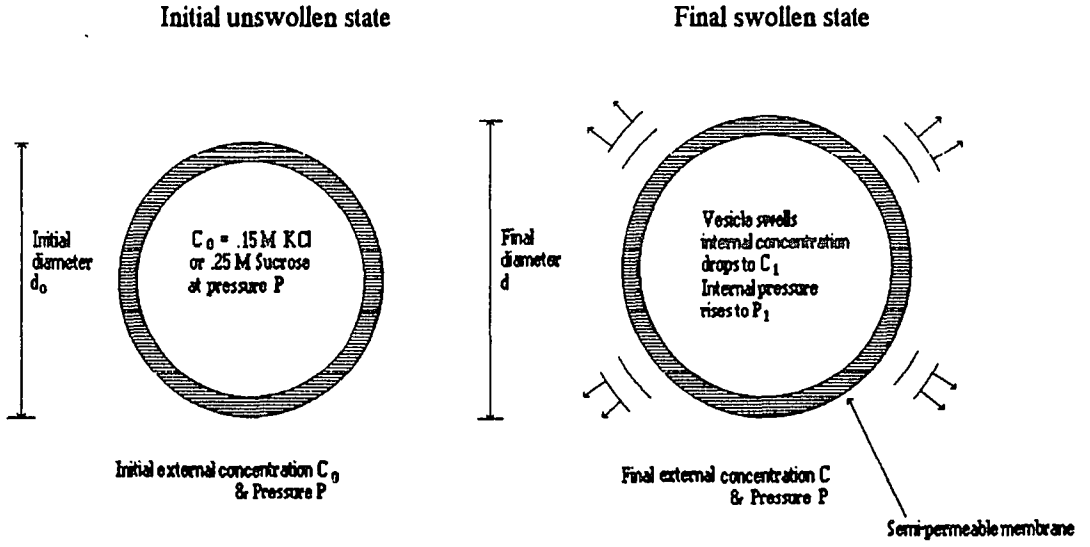
(from Rutkowski et al., 1991)

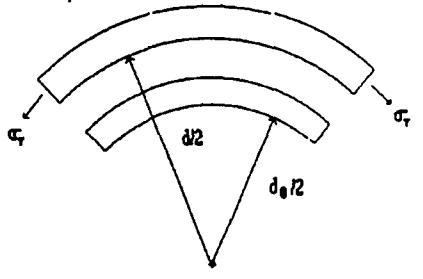
TABLE II

	mean diameter (nm)	normalized variance (v) or polydispersity (p)
electron microscopy	106.2	0.11 (v)
photon correlation spectroscopy	110.0	0.09 (p)
computer modeling	111.0	0.09 (p)

Mean Size, Normalized Variance and Polydispersity of extruded DOPG vesicles; A Comparison of PCS and Negative-Staining EM Techniques with a Computer-Modeled Distribution of Vesicles.

FIGURE 1





Dilute solutions $(P_1 - P) = K(C_1 - C)$

Stress $\sigma_T = \frac{d}{4t}(P_1 - P)$

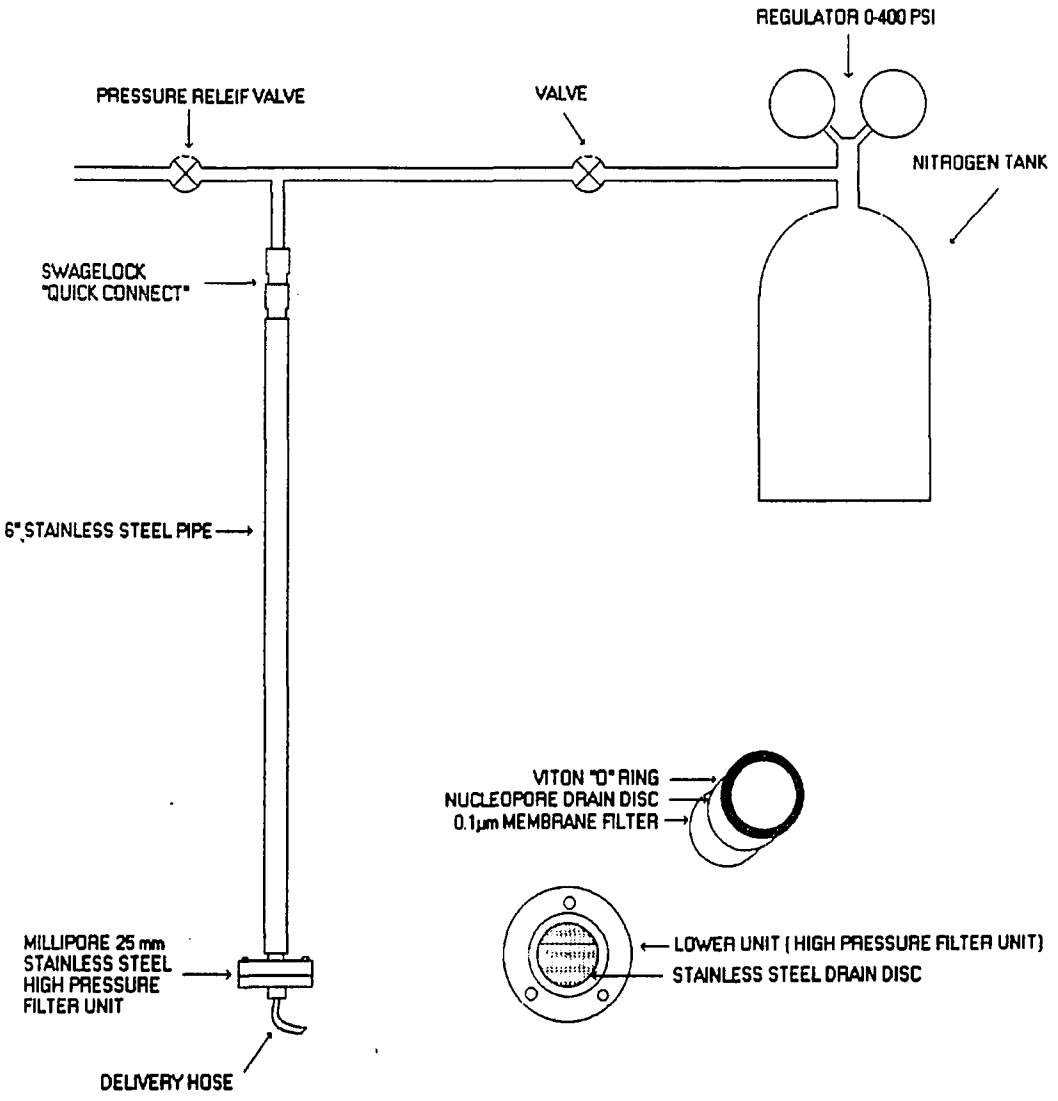
Strain $e = (d - d_0)/d_o$

Elastic modulus $M_e = \frac{\sigma_T}{2e}$

Elasticity equation $e(1+e)^3 = \left(\frac{dK}{8tM_e}\right)[C_0 - C(1+e)^3]$

Osmotic Swelling Theory

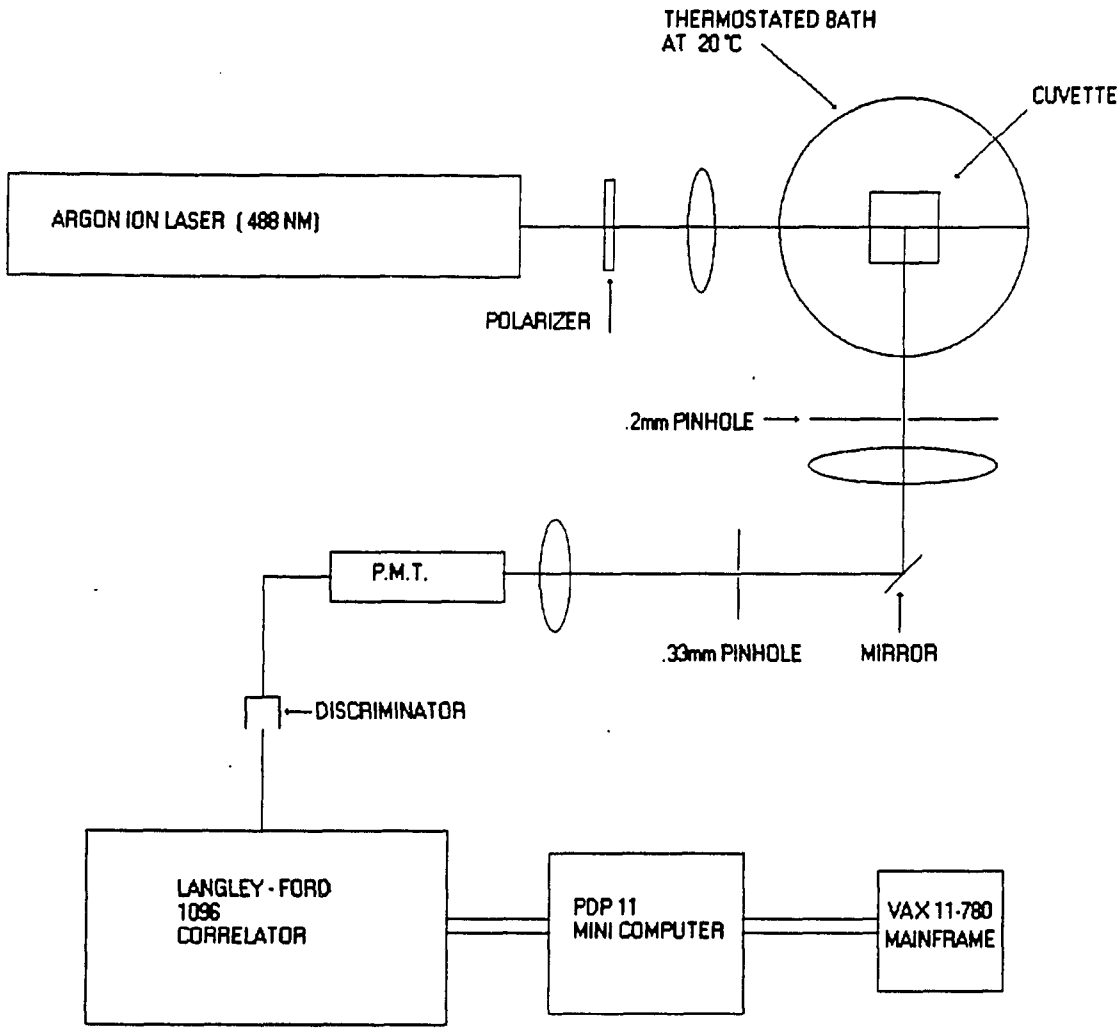
FIGURE 2



Vesicle Preparation Apparatus.

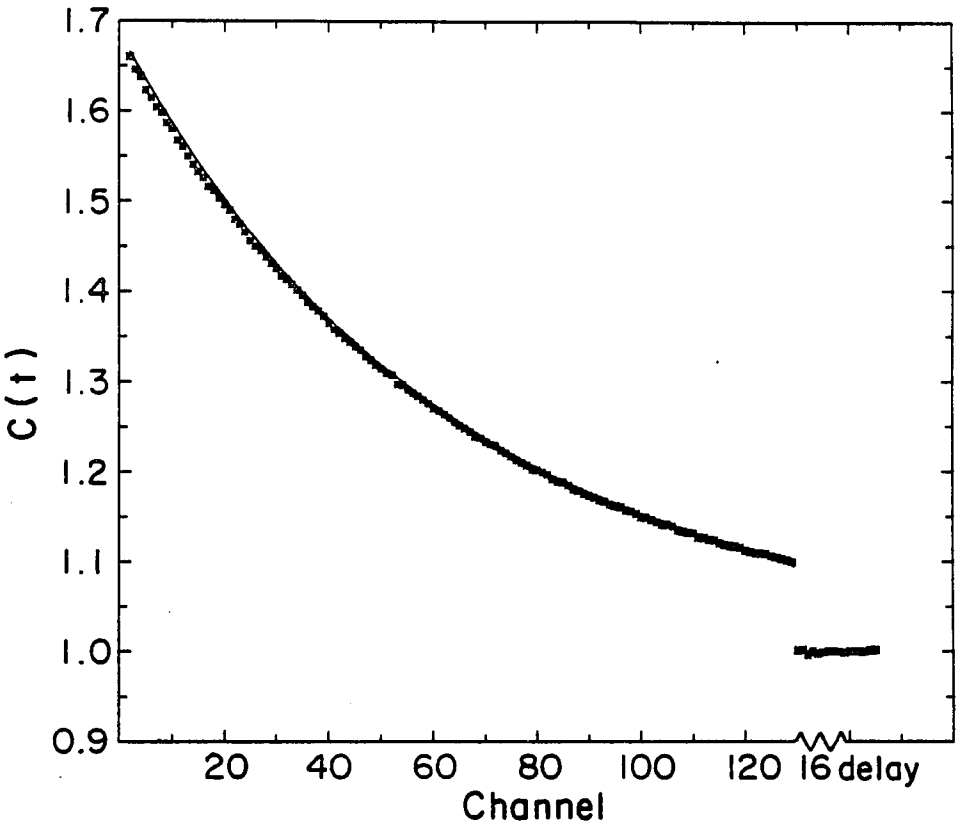
The design of this appratus was graciously provided by Dr. Barry Lentz of the University of North Carolina, Chapel Hill.

FIGURE 3a



Light Scattering Apparatus

FIGURE 3b



Correlation Function $C(\tau)$ obtained from Langley Ford Model 1096 Digital Auto-correlator. Correlation function consists of 128 channels of data and 16 delayed channels from which the background is calculated. Channel width (bin time) 4.0 μsec. Sample: DOPG in 250 mM sucrose.

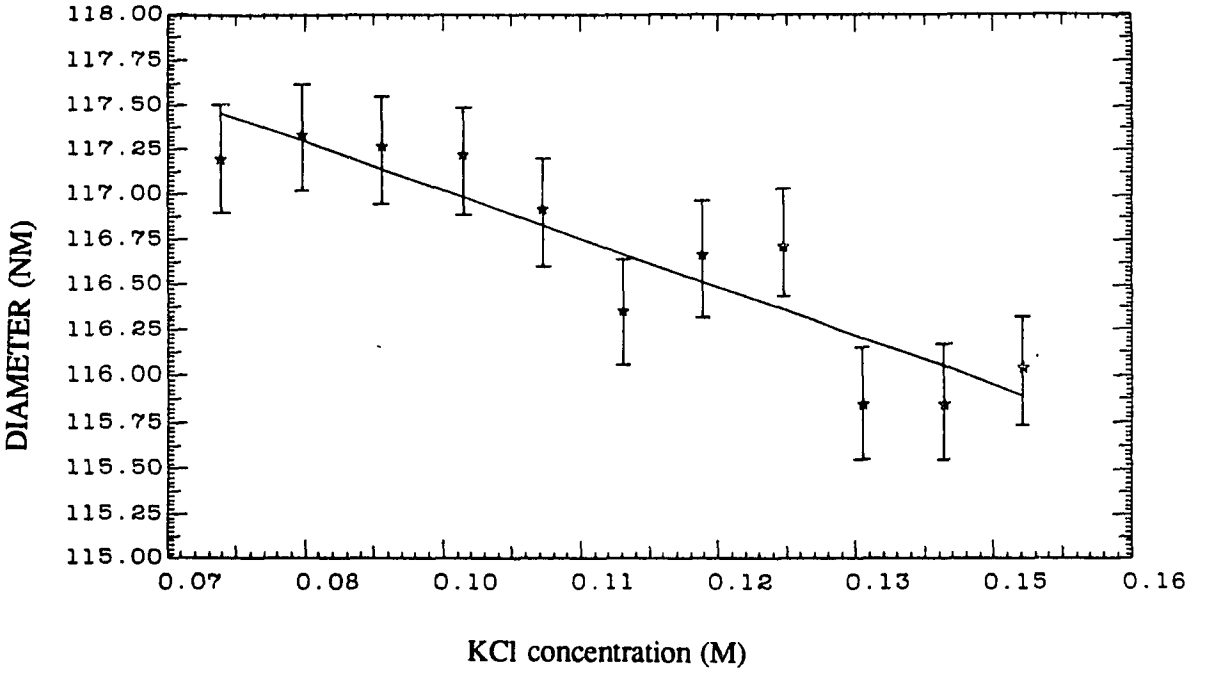
FIGURE 3c

N	NU	B(KCT)	RATE	A/B	TC	D(CM2/S)	%ERR	RADIUS	$K2/K1^2$	%ERR
1	128	1045.0	38.10	0.68	254.	0.3218e-07	0.5	667.25	0.119	12.
2	128	1070.6	38.52	0.73	253.	0.3230e-07	0.6	664.86	0.116	15.
3	128	1076.4	38.69	0.68	255.	0.3199e-07	0.5	671.31	0.119	13.
4	128	1054.1	38.27	0.66	252.	0.3244e-07	0.7	661.94	0.130	15.
5	128	1053.0	38.28	0.66	255.	0.3204e-07	0.6	670.21	0.124	14.
6	128	1044.5	38.11	0.66	253.	0.3224e-07	0.5	666.03	0.134	10.
7	128	1043.9	38.10	0.64	254.	0.3220e-07	0.6	666.94	0.112	14.
8	128	1042.4	38.07	0.63	252.	0.3242e-07	0.5	662.26	0.118	11.
9	128	1089.5	38.87	0.70	256.	0.3194e-07	0.7	672.33	0.065	29.
10	128	1082.6	38.81	0.65	254.	0.3221e-07	0.5	666.65	0.101	14.

<D>=0.3219e-07 %VAR= 0.5 <R>= 666.98 <K2/K1**2>= 0.11 %VAR= 17.1

Depiction of the computer output of the program lfcor2 run on a VAX mainframe computer. The correlation data was transferred from Langley Ford Model 1096 Correlator to VAX. The output indicates: N, the correlation number; Nu, the number of channels per correlation; B(kct), the background counts; Rate, the countrate (counts/sec); A/B, the A/B ratio; TC, the correlation time (μsec); D, the diffusion constant (cm^2/sec); Radius, the radius in angstrom; $K2/K1^2$, the Polydispersity, Q.

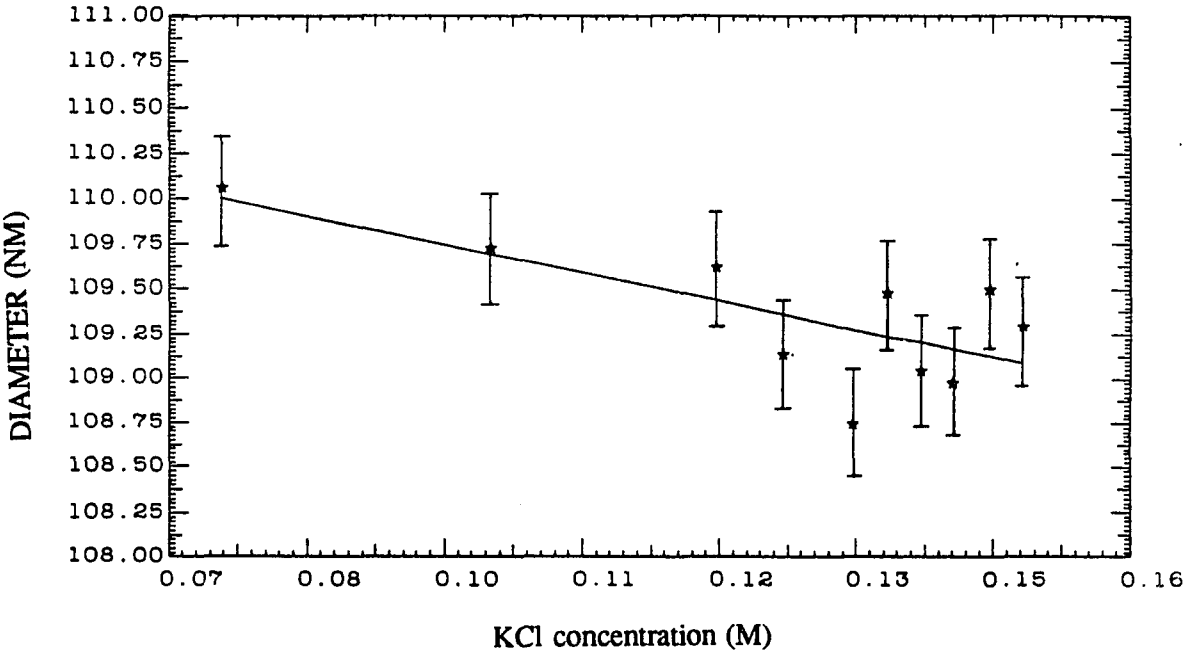
FIGURE 4a



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPC vesicles in 150 mM KCl were subjected to a two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of size measurements for each point (confidence). Modulus (8.2 (+/- 1.4) X 10^8 dyn/cm^2) is calculated from equation (26).

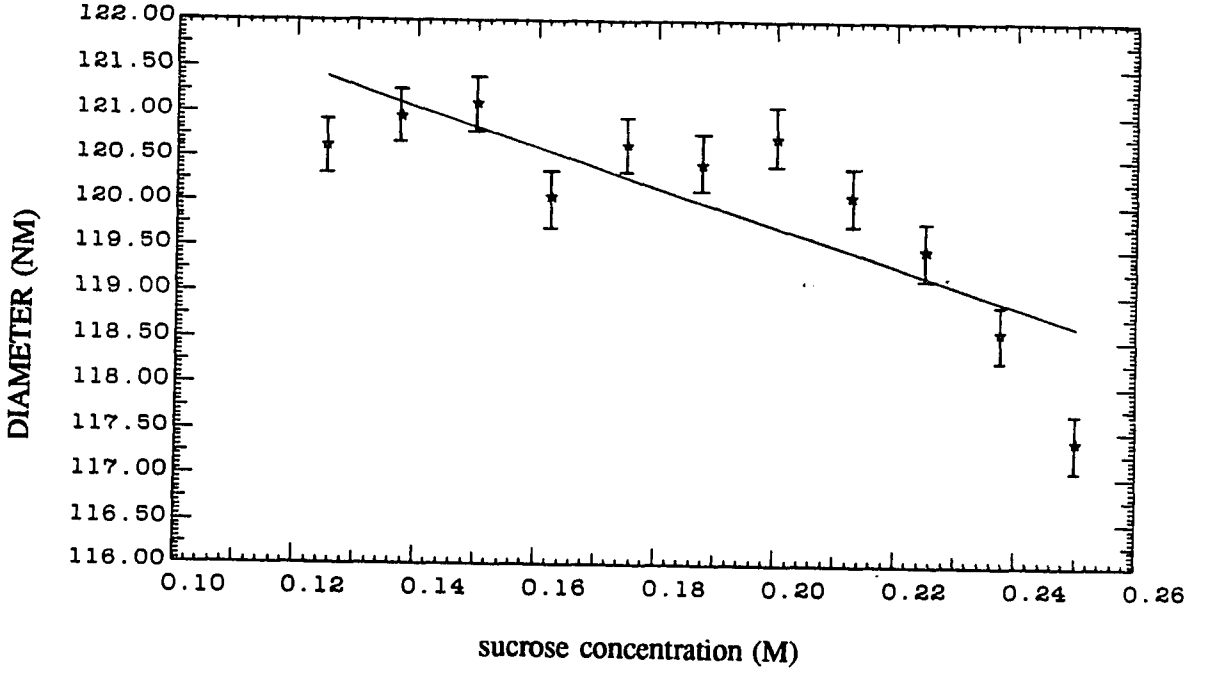
FIGURE 4b



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPC vesicles in 150 mM KCl were subjected to a two-fold dilution of Osmotic impermeant. The datapoints indicate the the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of size measurements of each point (confidence). Modulus (12.9 (+/- 4.0) X 10^8 dyn/cm^2) is calculated from equation (26).

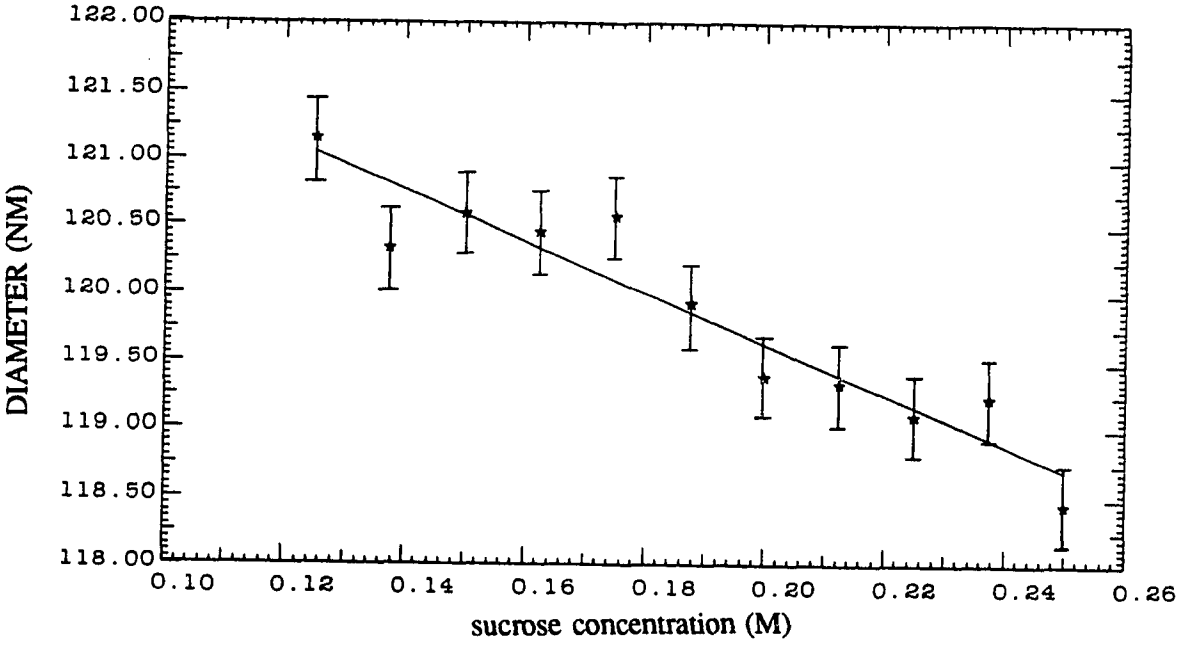
FIGURE 4c



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPC vesicles in 250 mM sucrose were subjected toa two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of size measurements of each point (confidence). Modulus (4.8 (+/- 1.4) X 10^8 dyn/cm^2) is calculated from equation (26).

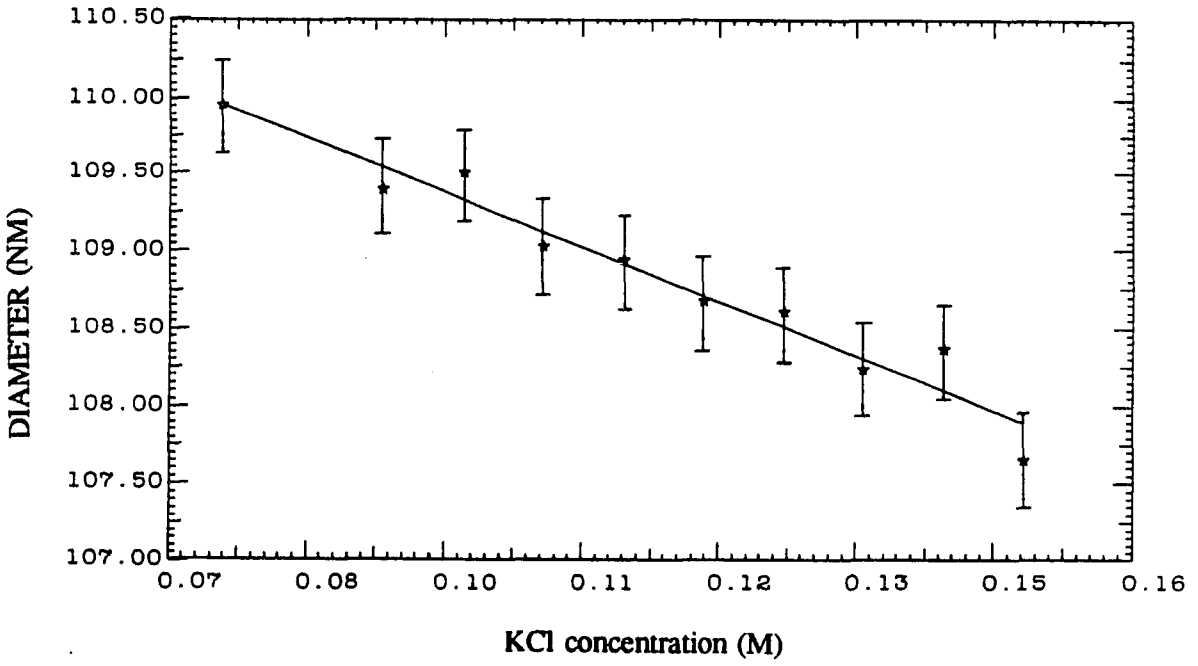
FIGURE 4d



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPC vesicles in 250 mM sucrose were subjected to a two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of size measurements of each point (confidence). Modulus (5.7 (+/- 0.7) X 10^8 dyn/cm^2) is calculated from equation (26).

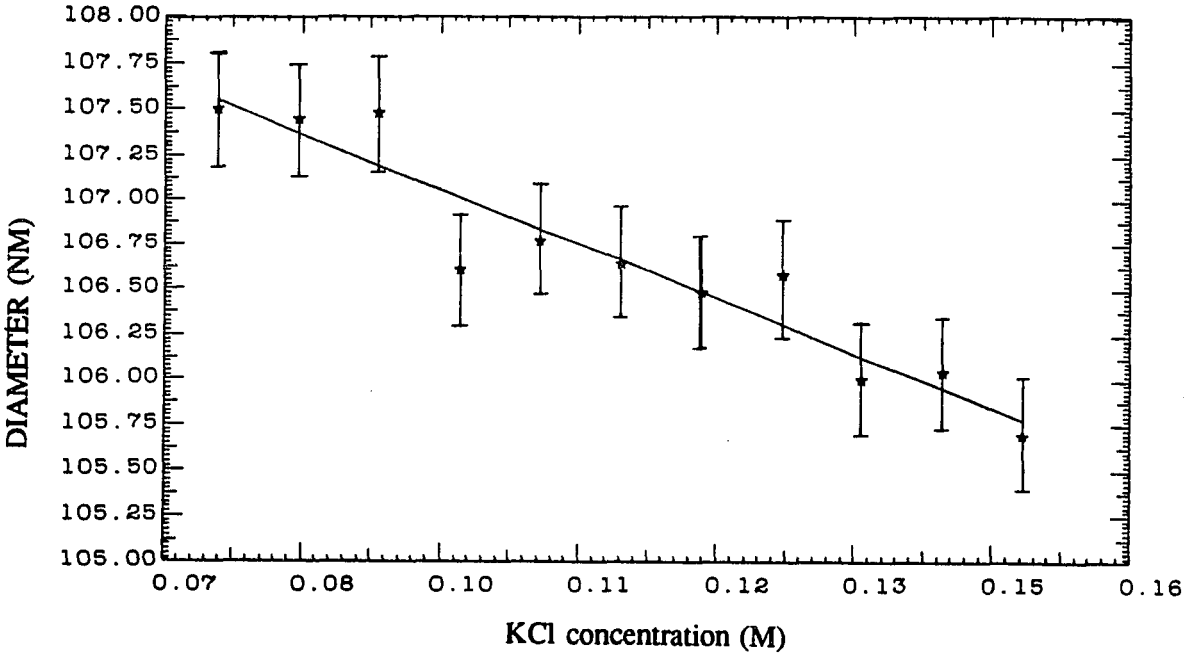
FIGURE 4e



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPG vesicles in 150 mM KCl were subjected to a two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of size measurements of each point (confidence). Modulus (5.2 (+/- 0.5) X 10^8 dyn/cm^2) is calculated from equation (26).

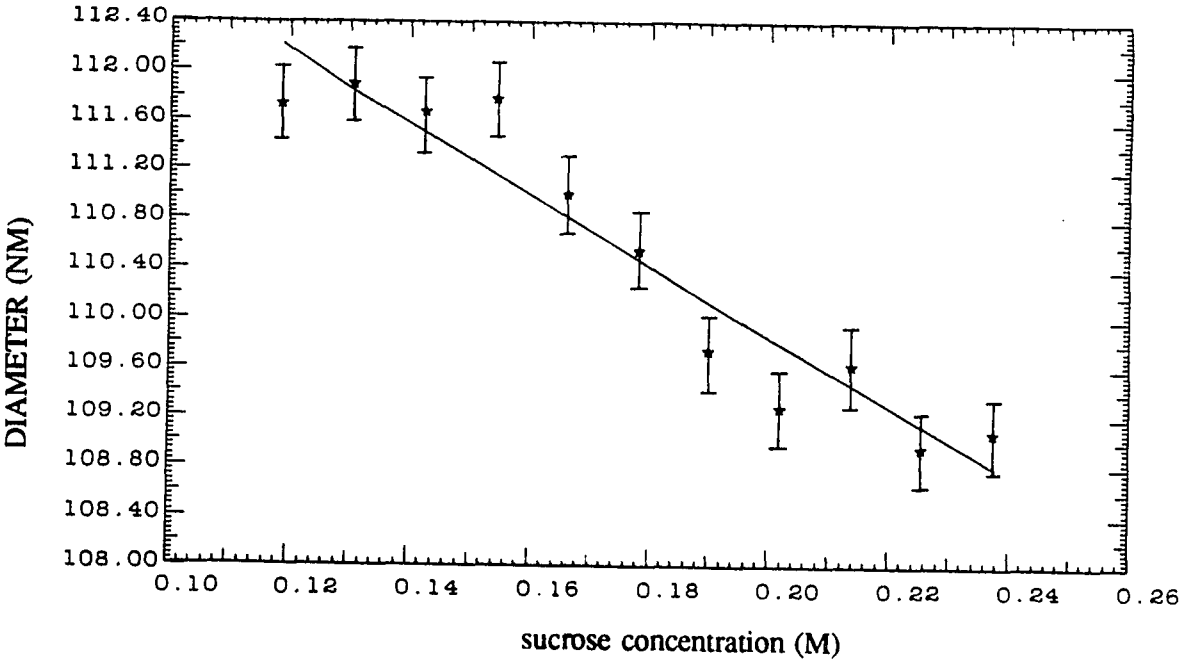
FIGURE 4f



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPG vesicles in 150 mM KCl were subjected to a two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of size measurements (confidence). Modulus (6.0 (+/- 0.7) X 10^8 dyn/cm^2) is calculated from equation (26).

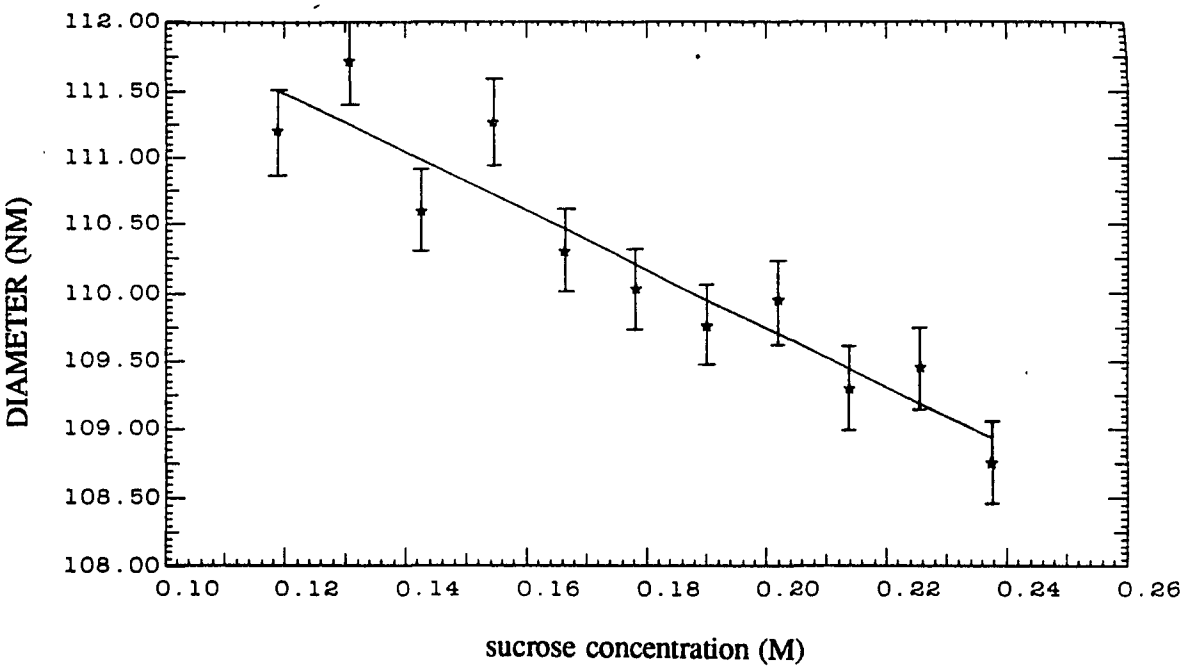
FIGURE 4g



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPG vesicles in 250 mM sucrose were subjected to a two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of measurements of each point (confidence). Modulus (3.0 (+/- 0.4) X 10^8 dyn/cm^2) is calculated from equation (26).

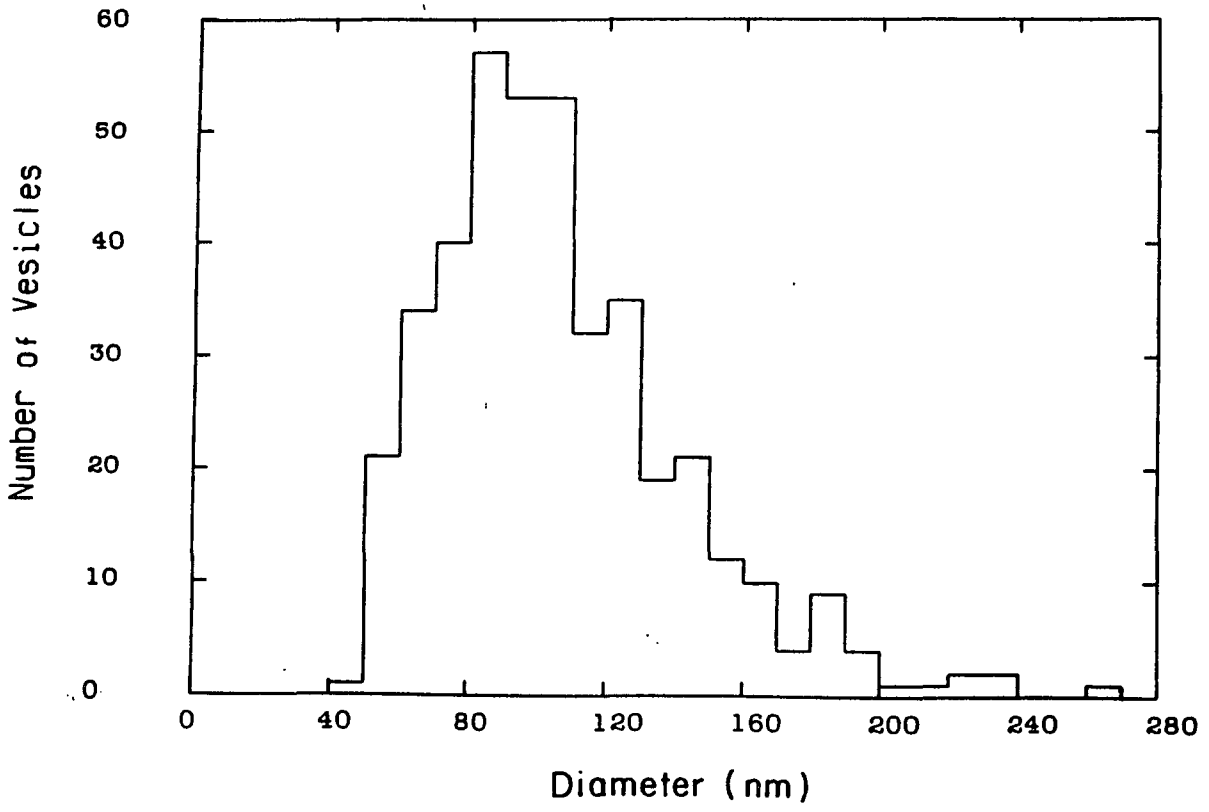
FIGURE 4h



Osmotic Swelling Extruded Phospholipid Vesicles

Extruded (0.1 micron) DOPG vesicles in 250 mM sucrose were subjected to a two-fold dilution of osmotic impermeant. The datapoints indicate the mean of 11 measurements. Error bars indicate the standard deviation of the size measurements divided by the square root of the number of measurements of each point (confidence). Modulus (4.1 (+/- 0.6) X 10^8 dyn/cm^2) is calculated from equation (26).

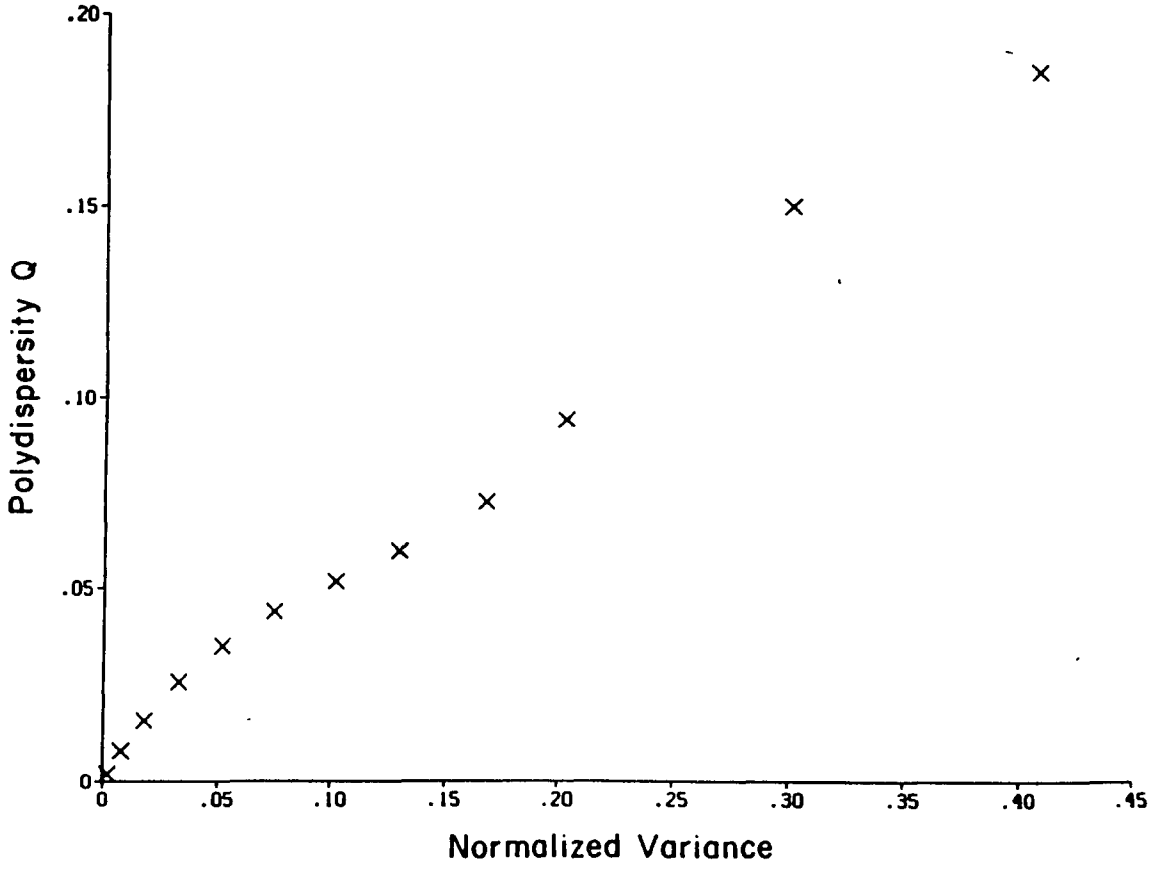
FIGURE 5



DOPG Vesicle Sizes Determind by Negative-Staining EM.

Extruded (0.1 μm) DOPG vesicles in 150 mM KCl were negatively-stained and examined with a JEOL JM-100CX electron microscope at 60 kV. Electron micrographs were analysed using the Microcomp Integrated Image Analysis System distributional information was collected using Microcomp Planar Morphometry Software.

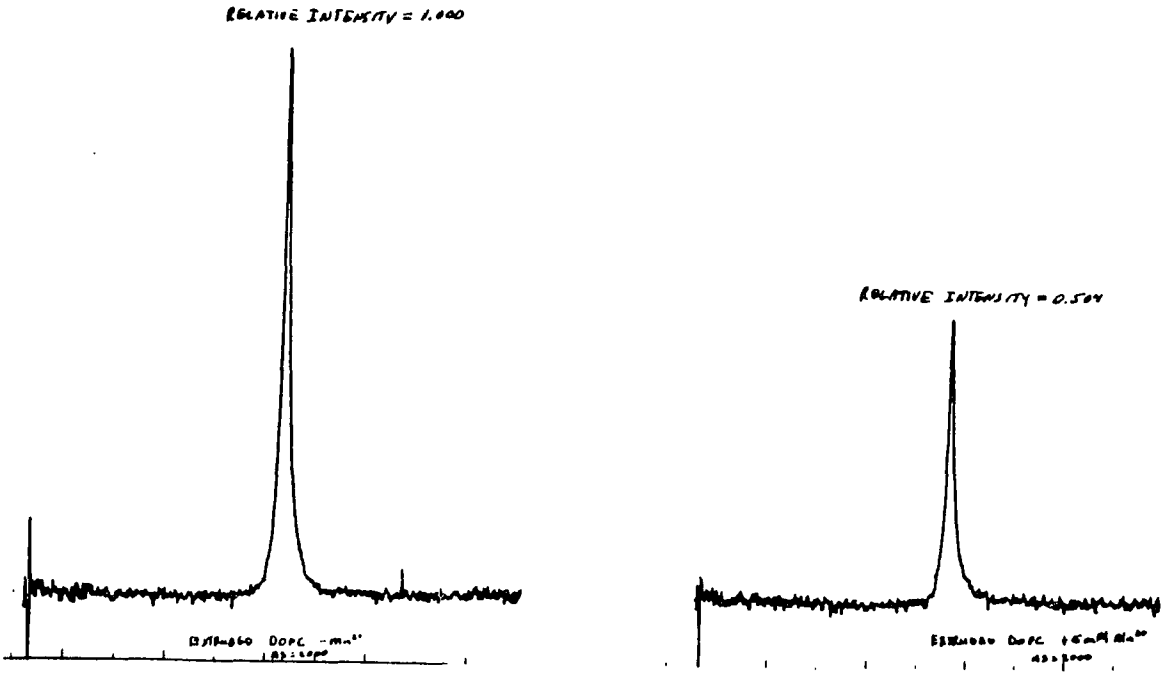
FIGURE 6



Polydispersity Versus Normalized Variance.

Polydispersity, Q is plotted against the normalized variance $(\sigma_n)^2$ for a series of computer-modeled Gaussian distributions of 100 nm vesicles from which correlation functions were generated. The correlation functions were analysed in a manner identical to experimental PCS data and the respective polydispersity indices were obtained (From Rutkowski et al., 1991).

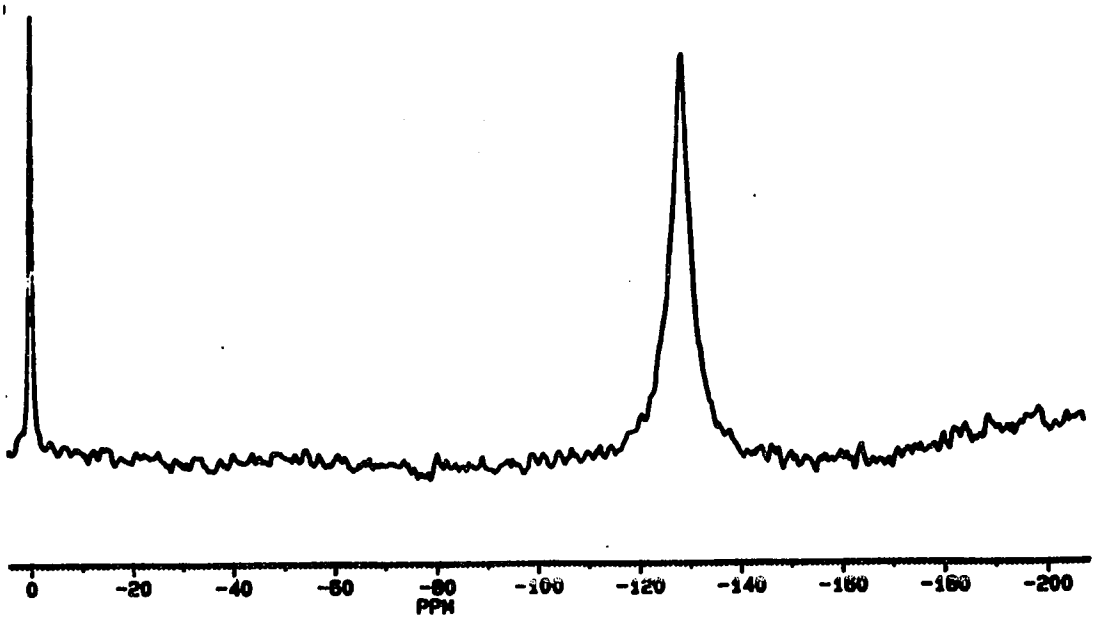
FIGURE 7



Determination of Unilamellarity in DOPC vesicles by NMR Spectroscopy.

^{31}P MNR spectroscopy was conducted on extruded (0.1 micron) DOPC vesicles in 150 mM KCl by Dr. Lawrence Mayer at the University of British Columbia on a Bruker WP-200 spectrometer. The left spectrum is that of the vesicles in KCl prior to the addition of Mn^{++}. The right spectrum is the identical preparation after addition of Mn^{++} to a final concentration of 5 mM. The protocols employed were identical to those described in Mayer et al. (1986). The residual signal intensity after addition of the manganous solution was 50.4%.

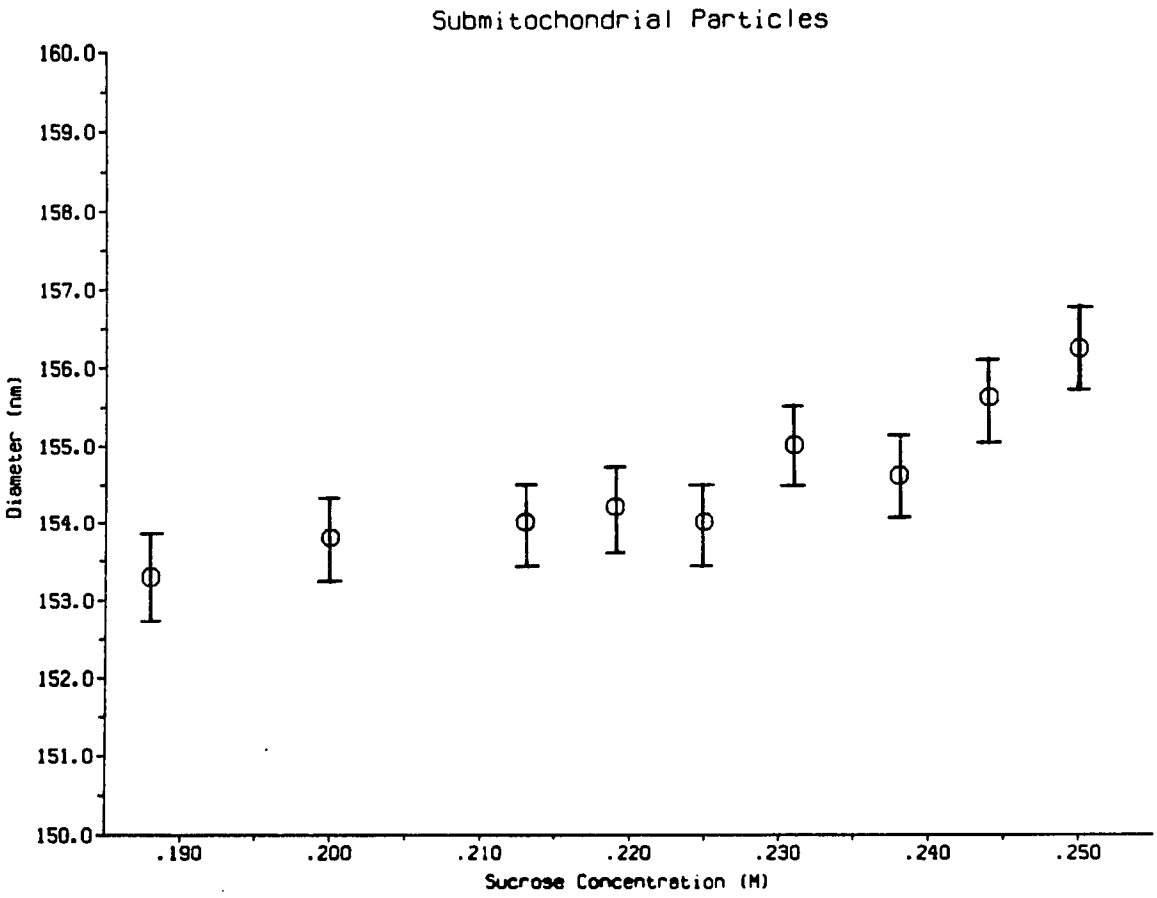
FIGURE 8



NMR Spectroscopy, Chemical Shift.

The chemical shift of extruded (0.1 micron) DOPC vesicles in 150 mM KCl was conducted by Dr. T. Axenrod at CCNY using an IBM NR-200 spectrometer. Triphenyl phosphite (1% in methanol) contained in a concentric capillary tube was used as a reference (0.004 ppM). ^{31}P NMR spectroscopy was conducted as per Mayer et al. (1986). The chemical shift of phosphorus was 128.824 ppm upfield of triphenyl phosphite.

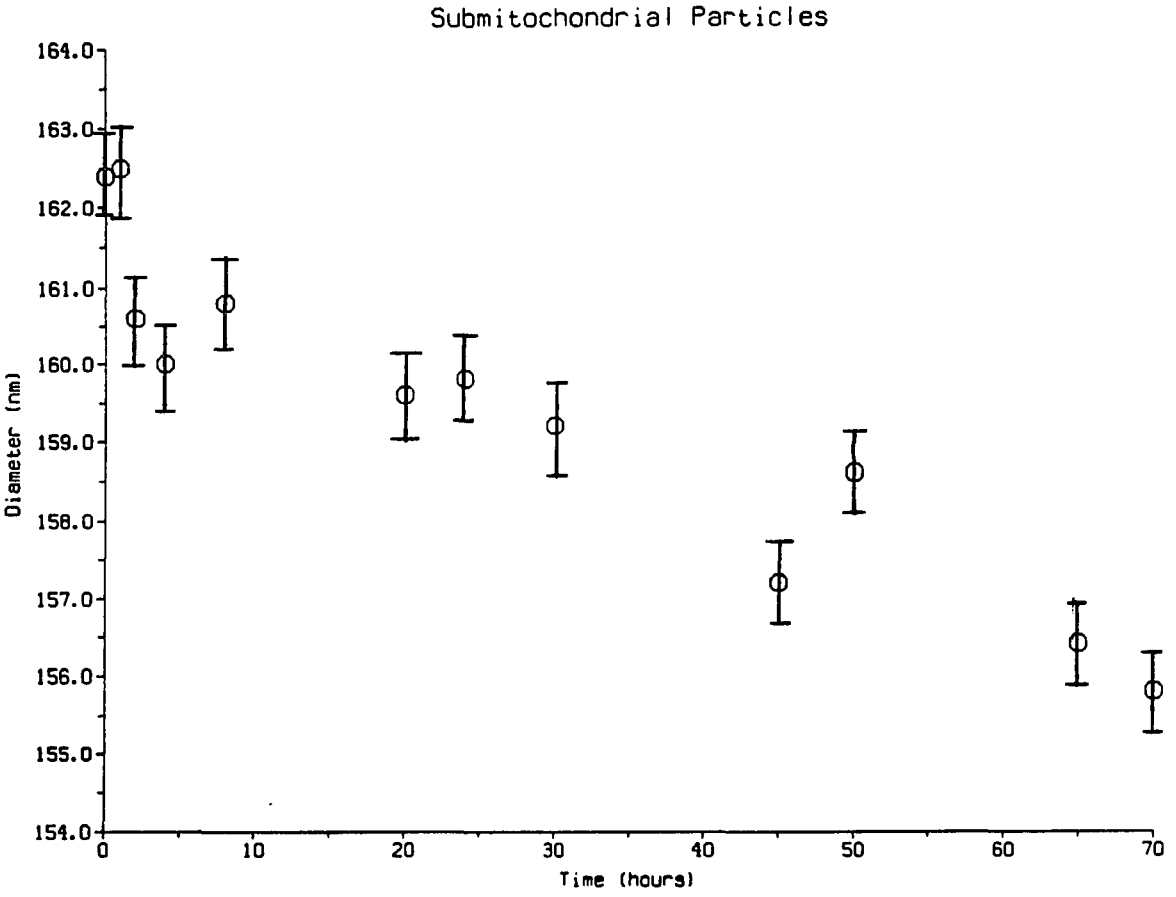
FIGURE 9a



Submitochondrial Particles: Osmotic Swelling

Submitochondrial particles (suspended in 0.250 M sucrose) were observed during osmotic swelling experiments. The mean size decreased during the dilution. Consequently, it was impossible to determine the elastic modulus of this preparation.

FIGURE 9b



Submitochondrial Particles: Time-Course Studies

The size of submitochondrial particles (suspended in 0.250 M sucrose) was observed as a function of time. No osmotic dilution was performed. This study indicated that the mean size of these particles was unstable and the preparation was unsuitable for further osmotic swelling studies.

REFERENCES

Alvarez, O. and Latorre. R. (1978) *Biophys. J. 21,* 1-17.

Aragon, S. R. and Elwenspoek, M. (1982) *J. Chem. Physics 77,* 3406-3413.

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

Bally, M. B., Nayar, R., Masin, D., Hope, M. J., Cullis, P. R., and Mayer, L. D. (1990) *Biochim. Biophys. Acta 1023,* 133-139.

Bangham, A. D. (1968) *Prog. Biophys. Mol. Biol. 18,* 29-95.

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

Bangham, A. D., de Gier, J., and Greville, G. D. (1967) *Chem. Phys. Lipids 1,* 225-246.

Bangham, A. D., Standish, M. M., Watkins, J. C., and Weissmann, G. (1967) *Protoplasma 63,* 183-187.

Bangham, A. D., Hill, M. W., and Miller, N. G. (1974) in *Methods Membrane Biology 1,* ed. Korn, E. D., Plenum, New York, 1-68.

Barber, E. J. (1966) *J. Nat. Cancer Inst. Monographs 21,* 219.

Barenholz, Y., Amselem, S. and Lichtenberg, D. (1979) *FEBS Lett. 99,* 210-214.

Berne, B. and Pecora, R. (1976) "*Dynamic Light Scattering*" (Wiley, New York).

Bittman, R. and Blau, L. (1972) *Biochemistry 11*, 4832-4839.

Blaurock, A. E. (1982) *Biochim. Biophys. Acta 650*, 167-207.

Blok, M. C., Van Deenen, L. L. M. and De Gier, J. (1976) *Biochim, Biophys. Acta 433* , 1-12.

Borochov, A. and Borochov, H. (1979) *Biochim. Biophys. Acta 550*, 546-549.

Bosworth, M. E., Hunt, C. A. and Pratt, D. (1982) *J. Pharm. Sci. 71*, 806-812.

Burnell, E. E., Cullis, P. R. and deKruijff, B. (1980) *Biochim. Biophys. Acta 603*, 63-69.

Caride, V. J., Sostman, H. D., Twickler, J., Zacharis, H., Orphanoudakis, S. C., and Jaffe, C. C. (1982) *Invest. Radiol.. 17*, 381-385.

Carruthers, A and Melchior, D. L. (1983) *Biochemistry 22*, 5797-5807.

Chu, B. (1974) "*Laser Light Scattering*" (Academic Press, New York).

Chrzeszczyk, A., Wishnia, A. and Springer, C. S. Jr. (1977) *Biochim. Biophys. Acta 470*, 161-169.

Cohen, F. S., Niles, W. D., and Akabas, M. H. (1989) *J. Gen. Physiol. 93*, 201-210.

Collander, R. and Barlund, H. (1933) *Acta Botanica Fennica 11*, 1.

Crowe, L. M., Crowe, J. H., Rudolph, A., Womersley, C. and Appel, L. (1985) *Arch. Biochem. Biophys. 242*, 240-247.

Crowley, J. M. (1973) *Biophys. J. 13*, 711-724.

Cummins, H. Z and Pike, E. R. Eds. (1973) "*Photon Correlation Spectroscopy and Light Beating*" (Plenum Press, New York).

Danielli, J. F. and Davson, H. (1935) *J. Cell. Comp. Physiol. 5*, 495.

Deamer, D. and Bangham, A. D. (1976) *Biochim. Biophys. Acta 443*, 629-634.

Deamer, D. W. and Bramhall, J. (1986) *Chem. Phys. Lipids 40*, 167-188.

Delgado, G., Potkul, R. K., Treat, J. A., Lewandowski, G. S., Barter, J. F., Forst, D., and Rahman, A. (1989) *Am. J. Obstet. Gynecol. 160*, 812-819.

Dreyer, R., Hawrot, E., Santorelli, A. C. and Constantinides, P. P. (1988) *Anal. Bioch. 175*, 433-441.

Ellens, H., Rustum, Y. M., Mayhew, E. and Ledsesma, E. (1982) *J. Pharm. Exp. Therap., 222*, 324-330.

Evans, E. A. and Kwok, R. (1982) *Biochemistry 21*, 4874-4879.

Evans, E. A. and Waugh, R. E. (1977) *Biophys. J. 20*, 307-313

Farmer, M. C. and Gaber, B. P. (1987) *Methods Enzymology 149*, 184-200.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrup, J. P., Ringold, G. M., and Danielsen, M. (1982) *Proc. Natl. Acad. Sci. 84*, 7413-7417.

Fettiplace, R. and Haydon, D. A. (1980) *Physiol. Rev. 60*, 510-550.

Fidler, I. J. and Schroit, A. J. (1984) *J. Immunol., 133*, 515-518.

Fiechtner, M., Wong, M., Bieniarz, C., and Shipchandler, M. T. (1989) *Anal. Biochem. 180*, 140-146.

Finer, E. G., Flook, A. G., and Hauser, H. (1972) *Biochim. Biophys. Acta 260* , 49.

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

Finkelstein, A. C. (1987) *Water Movement Through Lipid Bilayers, Pores and Plasma Membranes; Distinguished Lecture Series of the Society of General Physiologists V.4*, Wiley Interscience, New York.

Finkelstein, M. and Weissman, G. (1978) *J. Lipid Res. 19*, 289-303.

Fountain, M. W., Weiss, S. J., Fountain, A. G., Shen, A., and Lenk, R. P. (1985) *J. Infectious Dis. 152*, 529-535.

Fujime, S. Takasaki-Ohsita, M. and Miyamoto, S. (1988) *Biophysics J. 53*, 497-503.

Gaber, B. P., Yager, P., Sheridan, J. P. and Chang, E. L. (1983) *FEBS Lett. 153*, 285-288.

Gabizon, A., Goren, D., Fuks, Z., Barenholz, Y., Dagan, A. and Meshorer, A. (1983) *Cancer Res., 43*, 4730-4735.

Gebicki, J. M. and Hicks, M. (1973) *Nature 3243*, 232-234.

Gebicki, J. M. and Hicks, M. (1976) *Chem. Phys. Lipids 16(2)*, 142-160.

Gordon, R. E., Shaked, A. A., and Soland, D. F. (1986) *Am. J. Pathol. 125*, 585-600.

Gorter, E. and Grendel, F. (1925) *J. Expt'l. Med. 41*, 439-452.

Graf, J., Haddad, P., Haussinger, D. and Lang, F. (1988) *Renal Physiol. Biochem 35*, 200-220.

Green, D. P. L. (1990) *Biophys. J. 58*, 1557-1558.

Gregoriadis, G., Leathwood, P. D. and Ryman, B. E. (1971) *FEBS Lett. 14*, 95-99.

Gruner, S. M. (1988) *Liposomes*, Ostro, M. J., Ed. (Marcel Dekker, New York) 1-38.

Gruner, S., Lenk, R, P., Janoff, A. S., and Ostro, M, J. (1985) *Biochemistry 24*, 2833-2842.

Gustin, M. C., Zhou, X-L., Martinac, B. and Kung, C. (1988) *Science 242*, 762-765.

Haines, T. H., Li, W., Green, M. and Cummins, H. Z. (1987) *Biochemistry 26*, 5439-5447.

Hand, S. C. and Somero, G. N. (1982) *J. Biol. Chem 257*, 734-741.

Hantz, E., Cao, A., Escaig, J. and Taillandier, E. (1986) *Biochim. Biophys. Acta 862*, 379-386.

Hargreaves, W. R., and Deamer, D. W. (1978) *Biochemistry 317*, 3759-3768.

Harold, F. M. (1986) *A Study of Bioenergetics* W. H. Freeman & Co., NY, 311.

Hauser, H. (1989) *Proc. Nat. Acad. Sci., USA 86*, 5351-5355.

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

Hauser, H., Phillips, M. C. and Subbs, M. (1972) *Nature 239*, 342-344.

Haussinger, D., Lang, F., Bauers, K. and Gerok, W. (1990) *Eur. J. Biochem. 188*, 689-695.

Higgins, C. F., Cairney, J., Stirling, D. A., Sutherland, L. and Booth, I. A. (1987) *Tren. Bioch. Sci. 12*, 339-344.

Hope, M. J., Bally, M. B., Webb, G. and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55-66.

Huang, C. H. (1969) *Biochemistry 8*, 344-351.

Huang, C. H., Keyhani, E., and Lee, C. P. (1973) *Biochim. Biophys. Acta 305*, 455-473.

Jamshaid, M., Farr, S. J., Kearney, P. and Kellaway, I. W. (1988) *Int J. Pharmaceutics 48*, 125-131.

Johnson, S. M. and Buttress, N. (1973)*Bioch. Biophys. Acta 307*, 20-26.

Jung, C. Y. (1971) *J. Membrane Biol. 5*, 200-214.

Kates, M. and Kushwaha, S. C. (1976) *Lipids* Paoletti, R. *et al.* (eds), Raven Press, NY, Vol 1: Biochemistry, p267.

Kirkland, J. J., Tau, W. W., and Szoka, F. C. (1982) *Science 215*, 296-298.

Koff, W. C. and Fidler, I. J. (1985) *Antiviral Res. 5*, 179-190.

Kojro, Z., Lin, S.- Q., Grell, E. and Ruf, H. (1989) *Biochim. Biophys. Acta 985*, 1-8.

Koppel, D. E. (1972) *J. Chem. Physics 57*, 4814-4820.

Kremer, J. H. M., van der Esker, M. W. J., Pathmamanoharan, C. and Wiersema (1977) *Biochemistry 16*, 3932-3935

Kwok, R., and Evans, E. (1981) *Biophys. J. 35*, 637-652.

Laimins, L. A., Rhoads, D. B. and Epstein, W. (1981) *Proc. Natl. Acad. Sci. USA 78*, 464-468.

Lasic, D. D., Kidric, J. and Zagorc, S. (1987) *Biochim. Biophys. Acta 896*, 117-122.

Lentz, B. R., Carpenter, T. J. and Alford, D. R. (1987) *Biochemistry 26*, 5389-5397.

Li, W. (1987) *Doctoral Thesis CUNY Dissertation Abst. Intl. B; Science and Engineering, 49*, no. 8, p. 3165B, #DA8821099.

Li, W. and Haines, T. H. (1986) *Biochemistry 25*, 7477-7483.

Li, W., Aurora, T. S., Haines, T. H. and Cummins, H. Z. (1986) *Biochemistry 25*, 8220-8229.

Lo, S. L., Montague, C. E. and Chang, E. L. (1989) *J. Lipid Res. 30*, 944-949.

Lopez-Berenstein, G., Bodey, G. P., Frankel, L. S. and Mehta, K. (1987) *J. Clin. Oncol. 5*, 310-317.

Lopez-Berenstein, G., Hopfer, R. L., Mehta, R., Mehta, K., Hersh, E. M. and Juliano, R. L. (1984) *J. Infect. Dis. 150*, 278-283.

Loughrey, H. C., Wong, K. F., Choi, L. S., Cullis, P. R. and Bally, M. B. (1990) *Biochim. Biophys. Acta 1028*, 73-81.

Lynch, D. V. and Steponkus, P. L. (1987) *Plant Physiol. 83*, 761-767.

Lynch, D. V. and Steponkus, P. L. (1989) *Biochim. Biophys. Acta 984*, 267-272.

Martin, F. J. and Kung, V. T. (1987) *Meth. Enzymology 149*, 200-213.

Mayer, L. D., Hope, M. J. and Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161-168.

Mayer, L. D., Tai, L. C., Ko, D. S. C., Masin, D., Ginsberg, R. S., and Bally, M. B. (1989) *Cancer Res. 42*, 4734-4739.

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

Mayers, G. L, Pousada, M. and Haines, T. H. (1969) *Biochemistry 38*, 2981-2986.

McCracken, M. S. and Sammons, M. C. (1987) *J. Pharm. Sci. 76*, 56-59

Miller, C. and Racker, E. (1976) *J. Membr. Bioch. 30*, 283-300.

Millsman, M. H. W., Schwendener, R. A., and Weder, H-G. (1978) *Biochim. Biophys. Acta 512*, 147-155.

Milon, A., Ricka, J., Sun, S-T., Tanaka, T., Nakatani, Y. and Ourrisson, G. (1984) *Biochim. Biophys. Acta 777*, 331-333.

Milon, A., Lazrak, T., Albrecht, A.-M., Wolff, G., Ourisson, G. and Nakatani, Y. (1986) *Biochim. Biophys. Acta 859*, 1-9.

Mishima, K. (1976) *J. Phys. Jap. 41*, 2139-2140.

Miyamoto, S. and Fujime, S. (1990) *Biophys. J. 57*, 615-620.

Miyamoto, S., Maeda, T. and Fujime, S. (1988) *Biophys. J. 53*, 505-512.

Mui, B. L-S., Madden, T. D. and Cullis, P. R. (1991) *Biophys. J. 59*, 499a.

Nayar, R., Hope, M. J., and Cullis, P. R. (1989) *Biochim. Biophys. Acta 986*, 200-206.

Needham, D. and Nunn, R. S. (1990) *Biophys. J. 58*, 997-1009.

New, R. R. C. (1990) *Liposomes: A Practical Approach, IRL Press, Oxford.*

Nicolau, C., Lepape, A., Soriano, P., Fargette, F., and Juhel, M. F. (1983) *Proc. Nat. Acad. Sci. USA 80*, 1068-1072.

Niles, W. D., Cohen, F. S. and Finkelstein, A. (1989) *J. Gen. Physiol. 93*, 211-244.

Ostrowsky, N. and Hesse-Bezot C. (1977) *Chem. Phys. Lett. 52*, 141-144.

Papahadjopoulos, D. and Kimelberg, H. K. (1974) *Prog. Surf. Sci. 1* , 141-232.

Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) *Biochim. Biophys. Acta 311*, 330-348.

Papahadjopoulos, D., Nir, S. and Okhi, S. (1971) *Biochim. Biophys. Acta 266*, 561-583.

Parente, R, A. and Lentz, B. R. (1984) *Biochemistry 23*, 2353-2362.

Penefsky, H. S., Pullman, M. E., Datta A. and Racker, E. (1960) *J. Biol. Chem. 235*, 3330-3336.

Petri, W. A., Jr., Estep, T. N., Pal, R., Thompson, T. E., Biltonen, R. L., and Wagner, R. R. (1980) *Biochemistry 19*, 3088-3091.

Phillies, G. D. J (1986) In *Treatise on Analytical Chemistry 2nd ed.,* Elving, P. J., Ed. (John Wiley & Sons, New York); Part I, Vol. 8, Section 8.

Pike, E. R. and Cummins, H. Z., Eds: "*Photon Correlation Spectroscopy and Velocimetry*" (Plenum Press, New York, 1977).

Rahman, Y. E. and Wright, B. J. (1975) *J. Cell Biol. 65*, 112-122.

Rand, R. P. (1964) *Biophys. J. 4*, 303-316.

Raven, J. A. and Smith F. A. (1982) *Biosystems 15*, 13-26.

Reeves, J. P. and Dowben, R. M. (1970) *J. Membr. Biol. 3* , 123-141.

Rivers, R. L. and Williams J. L. (1990) *Biophys. J. 57*, 627-631.

Ruf, H., Georgalis, Y. and Grell, E. (1989) *Meth. Enzymol. 172*, 364-390.

Rutkowski, C. A., Williams, L. M., Haines, T. H. and Cummins, H. Z., (1991) *Biochemistry, in press.*

Saunders, L., Perrin, J. and Gammack, D (1962) *J. Pharm. Pharmacol. 14*, 567-572.

Schurtenburger, P. and Hauser, H. (1984) *Biochim. Biophys. Acta 778*, 470-480.

Schwarz, F. T. and Paltauf, F (1977) *Biochemistry 16*, 4335-4339.

Scotto, A. W. and Zakim, D. (1985) *Biochemistry 24*, 4066-4075.

Scotto, A. W. and Zakim, D. (1986) *Biochemistry 25*, 1555-1561.

Scotto, A. W., Goodwyn D. and Zakim D. (1987) *Biochemistry 26*, 833-839.

Singer, S. J. and Nicholson, G. L. (1972) *Science 175*, 720-731.

Singer, M. (1981) *Chem. Phys. Lipids 28*, 253-267.

Smith, I. C. P. and Ekiel, I. H. (1984) *Phosphorus-31 NMR* Gorenstein ed. Academic Press, NY, Ch. 15, 447-475.

Stevens, B. R., Kaunitz, J. D., and Wright, E. M. (1984) *Ann. Rev. Physiol. 46*, 417-433.

Stevens, B. R., Ross, H. J. and Wright, E. M. (1982) *J. Membr. Biol. 66*, 213-225.

Stokes, R. H. and Mills, R. (1965) "*International Encyclopedia of Physical Chemistry and Chemical Physics*" Volume 3 Topic 16, Pergamon, New York.

Sun, S-T., Milon, A., Tanaka, T., Ourisson, G. and Nakatani, Y. (1986) *Biochim. Biophys. Acta 860*, 525-530.

Supersaxo, A., Rubas, W., Hartman, H. R., Schott, H., and Hengartner, H. (1988) *J. Microencapsulation 5*, 1-11.

Szoka, Jr, F. and Papahadjopoulos, D. (1980) *Ann. Rev. Biophys. Bioeng. 9*, 467-508.

Trauble, H. (1971) *J. Membr. Biol. 4* , 193-208.

Warashina, A. (1981) *Biochim. Biophys. Acta 672*, 158-164.

Watford, M. (1990) *Tren. Bioch. Sci. 15*, 329-330.

Watts, A., Harlos, K., Maschke, W. and Marsh, D. (1978) *Biochim. Biophys. Acta 510*, 63-74.

Waugh, R. and Evans, E. A. (1979) *Biophys. J. 26*, 115-132.

Weissborn, A. C. and Kennedy, E. P. (1984) *J. Biol. Chem. 259*, 12644-12651.

Wilson, T. H. and Lin, E. C. C. (1980) *J. Supramolec. Str. 13*, 421-446.

Wolf, J. and Steponkus, P. L. (1983) Plant Physiol. 71, 276-285.

Wolf, J., Dowgert, M. F., and Steponkus, P. L. (1986) J. Membrane Biol. 93, 63-74.

Woodle, M. C. and Martin, M. J. (1988) *Biophys. J 53*, A122.

Woodle, M. C. and Papahadjopoulos, D. (1989) *Meth. Enzymol. 171*, 193-217.

White, S. H. (1974) *Biophys. J. 14*, 155-158.

Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982) *Science 217*, 1214-1222.

Yang, X. C. and Sachs, F. (1987) *Biophysics J. 52*, 252a