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AN ABSTRACT OF THE THESIS OF Martin Mense for the Master of Science in
Physics presented July 30, 1993.

Title: Comparison of Ion Adsorption to Phosphatidylcholine/Phosphatidylserine
and Sarcoplasmic Reticulum Membranes

APPROVED BY THE MEMBERS OF THE THESIS COMMITTEE:

Pavel Smejtek, Chair

Jonathan J. Abramson

Erik Bodégom

Stan Hillman

Artificial lipid membranes have been used in biophysical studies as well defined models of biological membranes. In the present work we studied adsorption of ions to artificial lipid membranes, composed of phosphatidylcholine (PC) and phosphatidylserine (PS), and to biological membrane from sarcoplasmic

reticulum (SR). The studies of ion adsorption and electrokinetic characterization of membranes were done by means of microelectrophoresis of PC/PS liposomes and SR vesicles. The ions of interest were positively charged potassium (K), calcium (Ca), tetraphenylarsonium (TPAs), tetraphenylphosphonium (TPP) and negatively charged pentachlorophenol (PCP).

Electrophoretic mobility of PC/PS liposomes and SR vesicles has been measured as a function of pH, ionic strength and the concentration of adsorbing ions. From the data we have determined the isoelectric point and the density of electric charge of the SR membrane. We have shown that the mobility of PC/PS liposomes and SR vesicles can be understood in terms of electrostatic screening of membrane surface charge by the diffuse double layer of counterions and by ion adsorption. The experimental results, with the exception of Ca and SR membranes, have been found consistent with the adsorption model based on Langmuir adsorption isotherm and Grahame's equation relating the membrane electric potential and the membrane surface charge.

The adsorption of ions to membranes have been characterized by the ion association constant and the membrane surface area of adsorption site. These quantities have been obtained from the fit of the adsorption model to the electrophoretic mobility data and the results are reported in the thesis.

The following two findings are most interesting and important. First, the adsorption of K and lipophilic ions TPAs, TPP, and PCP to both the SR

membrane and the artificial PC/PS membrane can be well characterized by the the adsorption model. The theoretical predictions of the adsorption model agree with the experimental results for a single (although different for PC/PS and SR membranes) set of ion adsorption constant and adsorption site area. This indicates a similarity between the biomembrane and the artificial lipid membrane. In contrast, such similarity was not found for calcium. Adsorption properties of SR membrane and artificial lipid membrane for Ca were found to be very different. Whereas the adsorption of Ca on PC/PS membranes could be described by one set of adsorption sites, the results for the SR vesicles indicate the presence of more than two types of Ca adsorption sites in the SR membrane. This finding is of physiological significance since the SR membrane regulates calcium transport in the muscle cell. Second, the adsorption affinity of SR membrane to all ions has been found to be significantly smaller compared to that of the lipid bilayer. This indicates that membrane proteins in the SR membrane reduce ion adsorption. This effect cannot be due to electrostatic interactions because the artificial lipid membranes had similar surface charge density as the biological membrane and the reduced adsorption was observed for both the positively charged TPAs and TPP as well as for the negatively charged PCP.

COMPARISON OF ION ADSORPTION TO
PHOSPHATIDYLCHOLINE / PHOSPHATIDYLSERINE
AND SARCOPLASMIC RETICULUM MEMBRANES

by

MARTIN MENSE

A thesis submitted in partial fulfillment of the
requirements for the degree of

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CHAPTER I

INTRODUCTION

Life could not exist without membranes.

Membranes are essential in regulating and controlling physiological processes. Life as we know it, would be impossible without membranes. Each cell or organelle is wrapped by a membrane. Properly working membranes ensure that cells function optimally. To function cells need to maintain certain concentration gradients of positively and negatively charged ions between inside and outside with the corresponding potential across the membrane. Cells need to release or uptake certain ions for specific actions, such as communication and metabolic regulation; common example for this are transmembrane movement of Na^+ and K^+ in nerves (action potential), and release and uptake of Ca^{2+} ions in muscle contraction.

Distribution of ions across membranes and transmembrane electric potential difference, ΔV , are closely related. In the simplest case the ratio of concentrations is given by Boltzmann factor

$$\frac{C_{in}}{C_{out}} = e^{\frac{-q\Delta V}{kT}} \quad (1)$$

In addition to the transmembrane potential difference the concentration of ions at the membrane surface is critical for the operation of membrane-bound enzymes. The surface concentration of ions is controlled by the electric potential difference between the membrane surface and the bulk aqueous compartment, V_s ,

$$\frac{C_s}{C_{aq}} = e^{\frac{-qV_s}{kT}} \quad (2)$$

The surface electric potential is in turn determined by the density of charge at the membrane surface and ion composition of aqueous solution. The surface charge of membrane depends on the presence of electrically charged lipids and ionized groups of membrane-bound proteins. Adsorption of ions to membranes changes the distribution of electric potential.

Biological membranes have specific carriers in the form of enzymes or channels as membranes are permeable to specific ions and molecules. They are generally impermeable to small ions. The impermeability is due to the lipids which represent a significant portion of biological membranes. Owing to its hydrophobic nature the lipid matrix repels hydrophilic ions. In contrast, there are lipophilic ions, environmental pollutants such as chlorinated phenols and many drugs that adsorb to membranes. They are biologically active by operating as carriers for other ions or by altering the transmembrane or surface electrical potential.

Knowledge of adsorption and related phenomena as well as mechanisms of adsorption of drugs is important in medicine. All drugs work through adsorbing to membranes or to specific protein receptors and therefore understanding of their beneficial effects depends upon their adsorption properties.

One of the novel concepts combines drugs and membranes in an interesting new way. Papahadjopoulos and others (1991) investigated the use of liposomes loaded with particular drugs for brain tumor therapy. In their research they compared the blood clearance kinetics of different sterically stabilized liposomes in order to investigate their lifetime in the body. Moreover, they found that antitumor drugs, like doxorubicin and epirubicin, when encapsulated in liposomes are substantially more efficient when injected into infected mice than injections of the pure drugs.

The focus of this study was the comparison of ion adsorption to artificial and biological membranes. Electric potential at the surface of membranes is affected by ions in two ways: (1) by electrostatic screening of charged membrane surface by ions in the aqueous solution, and (2) by ion adsorption which alters the membrane surface density of electric charge. Past studies have been done with artificial lipid membranes, and association constants and binding site densities have been determined for a large number of adsorbing species. Experiments were done with various monovalent, divalent, and multivalent ions. Differences in adsorption of lipophilic and hydrophilic ions have been also

investigated (McLaughlin 1976 and 1981, Ermakov 1990, Smejtek 1990a, Tatlivan 1988).

Understanding of ion adsorption to biological membranes is limited. One interesting aspect of exploring ion adsorption to biological membranes is to discover how well the models developed for the artificial membranes, fit "real life", the biological membranes. Biological membranes are far more complex than artificial membranes. They invariably consist of more than one type of lipids and a large number of enzymes and proteins. From the differences in adsorption characteristics of biological membranes and model membranes one may conclude how the membrane-bound proteins affect the adsorption process. (Conrad and Singer 1979 and 1981)

In this project sarcoplasmic reticulum (SR) membrane has been selected as the biological membrane and its adsorption characteristics have been compared to those of phosphatidylcholine/phosphatidylserine (PC/PS) membranes. SR is at the center of current research interest (Abramson 1988 and 1993, Liu and Oba 1989 and 1990 among others), since it plays a major role in muscle contraction. Its biological function is clearly defined, it controls the calcium concentration in both skeletal muscle and cardiac muscle.

We have studied the effect of several ions: potassium, calcium, tetraphenylarsonium (TPAs), tetraphenylphosphonium (TPP), and pentachlorophenol (PCP).

Potassium and calcium were chosen because of their major importance in biological processes. Potassium is concentrated in the cell interior and calcium plays an important role in mitochondria and in muscle cells (Lehninger 1970).

Although TPAs and TPP have no environmental or biological significance they have been used as membrane permeability probes in several previous membrane studies (Liu and Oba, Flewelling and Hubbel among others). These ions are of interest because they have high affinity to lipid membranes and an identical structure.

Pentachlorophenol is the most toxic member of the family of chlorinated phenols. It is environmentally important due to its presence in the food chain and in humans (Ahlborg 1980, Hattemeyer-Frey et al. 1989). The toxicity of PCP is associated with its presence in membranes and uncoupling of oxidative phosphorylation.

In spite of extensive research done on the function of sarcoplasmic reticulum very little is known about the electrokinetic properties of SR and the electric potential at the SR membrane. Some of these aspects have been addressed by Rooney and Lee (1983) and by Arrio (1984):

Rooney and Lee compared the binding of a number of commonly used hydrophobic drugs to lipid bilayers with their binding to $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase from SR. These workers also obtained adsorption constants for egg-PC membranes. The focus of their studies was the relationship between the presence of drugs

and the ATPase activity. Arrio on the other hand only explored the electrokinetic properties of the SR vesicles without comparing his results to simple membranes, such as lipid membranes. In his study the adsorption of Na^+ , Ca^{2+} , and Mg^{2+} is described qualitatively, without any quantitative adsorption parameters. Only a very narrow range of ionic strength and corresponding salt concentrations was used in that study.

This work is based on the measurements of electrophoretic mobility of SR vesicles and liposomes. (i) It is shown how the mobility of SR vesicles changes with the concentration of hydrogen ions which provides information on the dependence of surface potential and the charge density at the surface of SR membranes as a function of pH. (ii) We have imitated the surface charge properties of SR vesicles by liposomes prepared from the mixture of two lipids: electrically neutral egg-phosphatidylcholine (PC) and negatively charged phosphatidylserine (PS). (iii) We have measured the dependence of electrophoretic mobility of SR vesicles and liposomes as a function of concentration of ions of interest and have analyzed the data in terms of a model of membrane-water interface which takes into account both screening of the membrane charge and ion adsorption.

Our major findings are: (1) The Langmuir-Stern-Grahame model introduced by McLaughlin (1976) describes the adsorption of monovalent metal cations and monovalent hydrophobic cations to SR membrane as well as the

adsorption to artificial lipid membranes. In contrast it was impossible to explain the calcium concentration dependence of mobility of SR vesicles with one set calcium adsorption parameters indicating that more than one kind of adsorption site for calcium are present.

(2) We have confirmed results of Conrad and Singer (1979 and 1981) that adsorption of ions and amphipathic molecules to biological membranes is significantly smaller compared to that of lipid membranes. Conrad and Singer proposed a hypothesis that a large internal pressure in biological membranes caused by the presence of proteins inhibits the adsorption of hydrophobic molecules. Their hypothesis was controversial and a consequence of possible experimental artifacts. It is conceivable that our results confirming lower adsorption affinity of SR membrane obtained by a very different method may revive the discussion and stimulate interest in the origin of adsorption site in biomembranes and artificial membranes.

CHAPTER II

MEMBRANES AND ION ADSORPTION TO MEMBRANES

This chapter will provide concepts and background pertinent to this study:

- discussion of phospholipids and lipid membranes as a model for biological membranes
- the selection of sarcoplasmic reticulum, as a representative of biological membranes
- adsorption theory
- the method of microelectrophoretic mobility

PHOSPHOLIPIDS

A major part of all biological matter consists of membranes. Plant cells, animal cells, and organelles are all wrapped by membranes. These membranes are composed of lipids which are divided into three classes: sterols, glycolipids and phospholipids. All of these lipids share the common characteristic that they are amphipathic. They are composed of a polar headgroup and hydrocarbon tails, two fatty acid chains, which usually contain 14 to 24 $-\text{CH}_2-$ groups (Stryer 1974).

Phosphatidyl choline

Phosphatidyl serine

Figure 1. Molecular structure of phosphatidylcholine and phosphatidylserine. The "R"s at the left side of the molecule stand for the two hydrocarbon chains.

This study used two phospholipids, the electrically uncharged phosphatidylcholine (PC) and the negatively charged phosphatidylserine (PS) (Figure 1). Both the PC and PS have a polar headgroup with a negatively charged phosphate group esterified to the head alcohol, which may also have an electrical charge. In the case of PC the alcohol is choline, whereas for PS it is the hydroxyl amino acid serine (Lehninger 1970).

Figure 2. A lipid bilayer vesicle.

Due to their amphipathic nature, hydrophilic headgroup and hydrophobic tail, phospholipids in aqueous solution form structures in which they shield their tails from H_2O by their headgroups. The structures strongly favored by our method of sample preparation are spherical particles consisting of several bilayer of lipids (Figure 2). The thickness of the bilayer membrane is ≈ 5 nm.

Since in biological membranes such as SR, lipids constitute 20 - 80 % of the mass and a large fraction of the surface, the artificially prepared lipid membranes are often used as a model for adsorption measurements. (Smejtek 1987, Tatulian 1987, McLaughlin 1978, and others)

The focus of this study was the comparison of adsorption properties of artificial phospholipid membrane with that of actual biological membrane (SR). In our study we used a mixture of electrically neutral PC and negatively charged PS for our model membranes since the biological membranes are usually negatively charged. Most biomembranes contain about 10 % of negatively charged PS, the content of PS in SR membrane is 11.4% (MacLennan 1971). Furthermore, the majority of the proteins present in biological membranes are also negatively charged.

SARCOPLASMIC RETICULUM

Sarcoplasmic reticulum (SR) has been chosen as a representative biological membrane in this project because it is relatively simple and well characterized membrane and is frequently used in studies of calcium transport and muscle contraction. (Abramson, MacLennan, and Oba among others)

As mentioned above, a high percentage of the mass in biological membranes results from lipid content. For SR the lipid/protein ratio is about 1:1. Among the SR proteins, the calcium pump protein, ATPase (70 % of the protein composition), predominates. 10 - 15 % of the remaining mass is Calsequestrin. Considering an average mass of 115 000 Daltons for the calcium pump protein (Hymel, Fleischer 1983) and assuming that other proteins are of similar mass,

and further using an average mass of 750 g/mol for a PC molecule, we estimate that the molar percentage of phospholipids is about 99 %.

*Figure 3. The fluid mosaic model of membrane structure.
(After S. Singer and G. Nicolson)*

Surface topography of biological membranes is expected to be complex: proteins can either protrude out of the membrane on both sides, protrude out of one side, like the calcium pump, or simply be attached to the surface as extrinsic proteins (Figure 3). The surface of SR vesicles is not known and may not be as smooth as the surface of liposomes.

ADSORPTION THEORY

The theoretical description of the adsorption of ions to membranes and the issue of electrostatic potentials at the membrane-solution interface has been

of great concern. A first theory of charged surface applicable to membranes was developed by Gouy (1910) and Chapman (1913), when they introduced the concept of the diffuse double layer. Their model required modifications and extensions. The most important modification was the combination of the earlier existing Helmholtz-Perrin model and the new Gouy-Chapman model made by Stern (1924). The so called Stern Model was later modified and extended by Grahame (1947) and others, and finally became the Langmuir-Stern-Grahame Adsorption Model. Its application to studies of adsorption to lipid membranes was developed by McLaughlin (1976).

Below we outline the adsorption theory and the final model as used in this study. A detailed discussion of the electrically charged surface can be found in the textbook by Bockris and Reddy (1973).

The first model applicable to membranes (Helmholtz-Perrin Model, 1879) regarded the interface as a parallel plate condenser, by assuming a layer of oppositely charged ions attached to the surface of the electrode or, in our case, the membrane. Gouy and Chapman proposed a different model in which ions in the aqueous solutions would form a diffuse cloud in the vicinity of the charged surface due to their thermal energy.

The Gouy-Chapman Model explains the spatial electrostatic potential that arises at the interface between the membrane and the electrolyte due to an inhomogeneous ion distribution in the electrolyte. The origin is the electrostatic

interaction between the membrane surface charge and the ions, as well as the diffusion of charge towards regions of lower concentration (McLaughlin 1977).

The Boltzmann equation describes the equilibrium state:

$$n^{\pm}(x) = n(\infty) \exp\left(\mp \frac{ze\Psi(x)}{kT}\right) \quad (3)$$

where $n(x)$ is the volume density of the ions, and $\Psi(x)$ the electrostatic potential at a distance x from the membrane. z is the ion valence and e the unit charge, k is Boltzmann's constant and T is the absolute temperature.

The Poisson equation for a planar surface is given by

$$\frac{d^2\Psi(x)}{dx^2} = \frac{-\rho(x)}{\epsilon_0\epsilon_r} \quad (4)$$

where $\rho(x) = \sum_i z_i e n_i(x)$ is the charge density at a distance x , ϵ_0 is the permittivity of free space, and ϵ_r the dielectric constant of the solution. z_i is the valence including the sign of the charge, "+" for positive charge and "-" for negative charge. A combination of equations (3) and (4) leads to the Poisson-Boltzmann equation

$$\frac{d^2\Psi(x)}{dx^2} = \left[\frac{2ze n(\infty)}{\epsilon_0\epsilon_r} \right] \sinh\left(\frac{ze\Psi(x)}{kT}\right) \quad (5)$$

Throughout this approach it assumed that the charges are uniformly "smeared" over the membrane surface.

Applying the appropriate boundary conditions, which are $\Psi = 0$ and $\frac{d\Psi}{dx} = 0$ at infinity, and the potential at the surface, $\Psi(0)$, defined, the equation (5) can be solved. The $\Psi(x)$ obtained through integration is a rather complicated

expression but can be simplified by linearization, if the potential is small, so that $\exp (ze\Psi(0) / 2kT)$ can be approximated by $1 + ze\Psi(0) / 2kT$. (Bockris and Reddy 1973) The linearization yields exponential decrease of potential with distance from the charged surface

$$\Psi(x) = \Psi(0) e^{-\kappa x} \quad (6)$$

$1/\kappa$ is the Debye length, defined by

$$\kappa = \sqrt{\frac{2}{\epsilon_0 \epsilon_r kT} \sum_i (z_i e)^2 n(x)_i} \quad (7)$$

where $n(x)_i$ stands for the volume number density of ions.

In order to obtain a relationship between the surface charge density of the membrane σ and the surface potential $\Psi(0)$, further assumptions other than that of the "smeared" surface charge have to be made: Ions are considered to be point charges, the dielectric constant of the electrolyte constant up to the membrane surface, and image charge effects at the electric boundary are neglected.

The result can be obtained by taking into account that electroneutrality implies $\sigma = -\int_0^\infty \rho(x) dx$. This approach yields Grahame's equation (McLaughlin 1977).

$$\sigma = \text{sgn}(\Psi(0)) \sqrt{((2 \epsilon_0 \epsilon_r kT) \sum_i n_i(\infty) \left[\exp\left(-\frac{z_i e \Psi(0)}{kT}\right) - 1 \right])} \quad (8)$$

A biologically important consequence of the accumulation of counterions at the charged membrane surface is a local change in pH. Since the membrane is

negatively charged the concentration of protons at the membrane surface is higher than in the bulk.

The above description is known as Gouy-Chapman-Grahame Theory. The striking improvement was introduced by Stern, the combination of the Helmholtz-Perrin Model and the Gouy-Chapman Model. Stern's extension resulted in a layer of counterions not directly attached to the membrane but a certain distance away, referred to as excess charges. Beyond the excess charge layer was the region of the diffuse double layer. (Bockris and Reddy 1973)

In this study we used in part the model introduced by McLaughlin and Harary (1976). The model combines the Langmuir adsorption isotherm with the Boltzmann relation for the local concentration of adsorbing species at the membrane-solution interface and Grahame's equation, equation (8).

The Langmuir adsorption isotherms for monovalent metal cations, monovalent lipophilic cations and divalent cations are respectively:

$$(M^+)A_M = K_M[M^+](1 - (M^+)A_M - (L^+)A_L - (D^{2+})A_D) \quad (9 \text{ a})$$

$$(L^+)A_L = K_L[L^+](1 - (M^+)A_M - (L^+)A_L - (D^{2+})A_D) \quad (9 \text{ b})$$

$$(D^{2+})A_D = K_D[D^{2+}](1 - (M^+)A_M - (L^+)A_L - (D^{2+})A_D) \quad (9 \text{ c})$$

where (M^+) , (L^+) , and (D^{2+}) are the membrane surface densities of adsorbed ions, $[M^+]$, $[L^+]$, and $[D^{2+}]$ are the concentrations of the molecules in the aqueous phase at the interface, A_M , A_L , and A_D are the adsorption site areas, and K_M , K_L , and K_D

are the association constants. $(1 - (M^+)A_M - (L^+)A_L - (D^{2+})A_D)$ represents the fraction of free adsorption sites.

The membrane surface densities of the adsorbed ions can be calculated using the Boltzmann relation where $[M^+]_0$, $[L^+]_0$, and $[D^{2+}]_0$ are the bulk concentrations of the adsorbing species:

$$[M^+] = [M^+]_0 e^{\frac{-e\Psi(0)}{kT}} \quad (10 \text{ a})$$

$$[L^+] = [L^+]_0 e^{\frac{-e\Psi(0)}{kT}} \quad (10 \text{ b})$$

$$[D^{2+}] = [D^{2+}]_0 e^{\frac{-2e\Psi(0)}{kT}} \quad (10 \text{ c})$$

A combination of the Langmuir adsorption isotherm, Grahame's equation and the Boltzmann relation for the membrane surface densities of the adsorbed ion allows the computation of the *adsorption parameters* K and A for known surface potentials. When applying Grahame's equation one must consider that the total surface charge density σ is the sum of the initial surface charge density of the membrane and the surface charge density due to adsorption.

The applicability of the Langmuir-Stern-Grahame models similar to that above has been shown several times. (McLaughlin 1976, Puskin 1980, Lau 1981, and others). McLaughlin also showed that the model, although originally developed only for monovalent ions, is very capable of describing the adsorption of divalent (1981) and trivalent ions.

Limitations of this theory arise from discrete charge effects. In his study on adsorption of divalent ions to phosphatidylglycerol membranes McLaughlin (1981) found a negligible discrepancy between calculated and measured surface potentials. A more thorough investigation of discrete charge effects in lipid bilayers (Winiski 1986) showed that the "smeared" charge hypothesis explains the measurements far better than a discrete charge theory. In his review (1977) McLaughlin states that the discrete charge effect is largest for low surface charge densities and high ionic strengths. In this work the membrane charge is assumed to be "smeared".

MICROELECTROPHORETIC MOBILITY

Microelectrophoresis is a method of determination of membrane surface potentials which makes it possible to study the adsorption characteristics of ions and membranes. The advantage of microelectrophoresis to other methods like NMR and fluorescence probes is that the potential can be obtained directly without any probe. Use of probes results in higher errors since the determination of surface potential is indirect. Furthermore, probes are not always absolutely specific. Consider, for example, fluorescence or electron spin probes which are used to monitor ion transport across the membranes and membrane surface potentials. Interpretation of experimental results is based on special assumptions

of probe response. In contrast the electrophoretic mobility method is more direct and probably mor reliable.

Electrophoresis uses an electric field applied across a suspension of charged particles in an electrolyte. Due to a balance between the electrostatic and viscous forces a charged particle moves with a constant velocity V . The electrophoretic mobility μ of the particle as velocity per unit electric field E is defined through the relation

$$\mu = \frac{V}{E} \quad (11)$$

It is assumed that the electrolyte is a Newtonian fluid, which implies that the viscous forces dominate inertial forces while the fluid flows past the particle. The electrophoretic mobility is related to the potential at the surface of shear of the particle, the so called " ζ -potential" in a simple way if the particle radius is large compared to the Debye length. This relationship is given by the Helmholtz-Smochulowski equation

$$\zeta = \frac{\eta}{\epsilon_0 \epsilon_r} \mu \quad (12)$$

where η is the viscosity of the fluid.

The microelectrophoretic mobility data are connected to the Langmuir-Stern-Grahame adsorption model (McLaughlin, Smejtek, Tatulian) by relating the ζ -potential to the membrane surface potential $\Psi(0)$.

$$\zeta = \Psi(0) e^{-s\kappa} \quad (13)$$

where s is the shear plane distance. However, this relation only holds if s is much smaller than κ . Smejtek (1987) found the position of the shear plane as a function of ionic strength by measuring the electrophoretic mobilities of liposomes with a known surface charge density. A. McLaughlin (1983) determined the shear plane distance by combining ζ -potential and surface potential. Typically $s \approx 0.2$ nm.

There are limits for the applicability of the above model. The surface of the particles needs to be sufficiently smooth, otherwise the shear plane distance becomes unknown. An example of complex surface are red blood cells which have a number of protruding sugars on their surface. These sugars have a large impact on the position of shear plane and introduce additional hydrodynamic drag. In addition the distribution and location of charges within the surface layer complicates the analysis of electrophoretic mobility. The resulting discrete charge effects in combination with a "rough" surface are beyond the limits of this study. Models taking these facts into account have been proposed by Donath and Pastushenko (1979), and by Wunderlich (1981).

CHAPTER III

MATERIALS AND METHODS

CHEMICALS

Tetraphenylphosphonium chloride (TPP), tetraphenylarsonium chloride hydrate (TPAs) and pentachlorophenol (PCP) were all bought from Aldrich Chemical Company, Milwaukee, WI. The phospholipids, egg-phosphatidylcholine (PC) and L- α -phosphatidyl-L-serine (PS), were both from Avanti Polar Lipids, Birmingham, AL. They came dissolved in chloroform, the concentration was 20 mg/ml for PC and 1 mg/ml for PS, both were 99 % pure. Potassium phosphate dibasic trihydrate, boric acid, calcium chloride dihydrate (all Mallinckrodt Chemical Works, St. Louis, MO), Hepes, that is N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (Research Organics Inc., Cleveland, OH), potassium chloride (J.T. Baker Chemical Co., Phillipsburg, NJ) and chloroform (American Burdick and Jackson, Muskegon, MI) were of reagent or higher purity grade. They were all used without further purification.

All solutions were prepared with deionized water.

SAMPLE PREPARATION

a) Liposomes

PC and PS stock solution was diluted with chloroform in a 50 ml round bottom flask. The ratio of PC to PS was 30 : 1 for all preparations except for one calcium adsorption series, when it was 10 : 1. The flask containing lipids dissolved in chloroform was connected to a flash evaporator (Buchler Instruments, Fort Lee, N.J.) and the chloroform evaporated under rotation. In order to speed up the process there was a temperature gradient established by cooling the collector flask with ice water and heating the chloroform flask with warm water (30°C). The result was a thin layer of lipids in the flask. To eliminate the contents of chloroform in the lipid layer as much as possible, a rotary pump was then used to pump on the flask for at least one hour. Finally a buffer solution was added and the flask was gently shaken to obtain liposomes. The pH-adjusted solutions contained as a buffer either 5 mM Hepes (with KOH adjusted to pH 7.3) or 0.002 M phosphate, 0.002 M citrate and 0.005 M borate at different pH-values (titrated with KOH or HCl). Concentration of KCl was 0.009 M. To achieve a liposome size similar to the size of the SR vesicles, the liposomes were sonicated for 5 min by placing the round bottom flask, that was used for the preparation, into an ultrasonic bath.

b) SR vesicles

The SR vesicles were prepared in Dr. Jon Abramson's lab (Buck 1993 and MacLennan 1970). The batch used for all experiments was prepared in a solution of 20 mM Hepes and 100 mM KCl with a protein concentration of 11 mg/ml. For the measurements, the SR stock solution was diluted 30-fold in either the Hepes or the phosphate-citrate-borate buffer.

To reduce the sedimentation of vesicles within the electrophoretic cell to a minimum, both the SR samples and the liposome samples were centrifuged for five minutes with an Adams Sero-Fuge (Clay Adam, Parsippany, NJ) after the final preparation of the suspension, before the measurement.

Prepared liposome stock solutions were not used longer than two days because of the instability of the surface charge of the membranes on a longtime scale. For similar reasons the SR vesicle stock solution was stored in volumes of about 1 ml at liquid nitrogen temperature and was kept on ice for the duration of the experiments.

The solutions for Ca^{2+} , TPAs and TPP adsorption measurements and those for ionic strength dependence determination were all prepared by dissolving appropriate salts in the Hepes buffer solution. For the pH-dependence and the PCP adsorption, the solutions were prepared in the phosphate-citrate-borate buffer due to the better buffering capacity of this buffer in a wide pH-range. PCP was obtained from a 6 mM stock solution (in KOH, pH 12).

The pH-adjustments were done with a digital pH-meter, Jenco model 6072 (Jenco Electronics, Ltd., Taipei, Taiwan). The electrode was an Orion Ag/AgCl glass electrode (Orion Research Inc., Boston, MA) filled with a 4 M KCl solution that was saturated with AgCl (Corning, Medfield, MA). The meter was calibrated before each measurement with pH 7.00 and pH 4.00 or pH 10.00 standard solution (VWR Scientific, Portland, OR).

DETERMINATION OF ELECTROPHORETIC MOBILITIES

The DELSA (Doppler Electrophoretic Light Scattering Analyzer) model 440 by Coulter Electronics, Inc. (Hialeah, FL) was used to measure electrophoretic mobility of SR vesicles and liposomes.

The DELSA uses the Doppler shift of He/Ne-laser light scattered from particles drifting in an external electric field.

The Electrophoretic Cell

The major parts of the electrophoretic cell (Figure 4) are two silver electrodes with a quartz glass block and a thermistor. The purpose of the thermistor is to monitor and reduce the temperature gradients across the cell. The two electrodes each contain a hemispherical cavity connected by a channel 1 mm high. These cavities and the channel are filled with the suspension. The cell is filled through plastic filling tubes and the filling process has to be executed very

carefully to avoid presence of any air bubbles because they have an enormous impact on the accuracy of the measurements.

Figure 4. The electrophoretic cell. (From DELSA manual.)

For measurements the cell is mounted in a stage that allows vertical positioning of the laser beam within the channel.

The cell height and the beam position is determined with a digital gauge with resolution of 1 μm (Mitutoyo Digimatic Indicator IDC 543 by Mitutoyo Corp., Tokyo, Japan).

Flow Profile

Since an electric field is applied to measure across the cell the mobility of the vesicles and the potential of all walls is different from that in the channel, the inhomogeneous ion distribution within the channel causes electroosmotic flow of the electrolyte. The velocity of the electrolyte is dependent on the position. The geometry of the channel, which has a rectangular cross-section, produces a parabolic flow profile of the electrolyte since the cell is a closed system and the net flow therefore has to be zero. Consequently there are two layers, symmetric to the axis of the channel, where the velocity of the electrolyte is zero. These layers are called "stationary layers." Outside of the stationary layer the measured velocity is always a superposition of the velocities of the electrolyte and that of the charged particles. Therefore, for the above reason, to measure the true mobility of the particles it is absolutely necessary to have the laser beam positioned at the stationary layer.

Theory predicts the position of stationary layers is at 16 % of the cell height off the channel top and bottom. However, the position of these layers may change due to the precipitation of charged particles within the channel. Therefore, it is preferred to measure the mobility at the upper stationary layer because its position is less affected by precipitation.

Light Scattering

A 5 mW He/Ne laser generates a laser beam (wavelength ≈ 633 nm) that is horizontally polarized. The beam intensity can be adjusted manually. A diffraction grating is used to split the beam into five rays, of which the main beam carries 80% of the intensity. The remaining 20% are split equally among the four reference beams. A converging lens makes the beams to intersect within the channel of the electrophoretic cell. The position of the intersection can be observed through a 24x microscope and has to be manually centered within the channel. The reference beams that cross the main beam at angles of 7.5° , 15° , 22.5° and 30° are detected by separate detectors.

Light of the main beam that is scattered by the vesicles is collinear with the respective reference beam at the detector. The movement of the particles causes a frequency shift of the scattered light as follows:

$$\Delta\nu = 2nV \cos(\alpha) \sin(\theta/2)/\lambda \quad (14)$$

with n being the index of refraction of the medium, V the velocity of the vesicles referred to the detector, α the scattering angle, θ the angle between the scattering vector and the particle velocity and λ the wavelength in vacuum (Figure 5).

Figure 5. The scattering vector. (From DELSA manual.)

The scattering vector $\mathbf{K}$ is given through

$$\mathbf{K} = \mathbf{k}_1 - \mathbf{k}_0 = 4\pi n \sin(\theta/2) / \lambda \quad (15)$$

where $\mathbf{k}_1$ and $\mathbf{k}_0$ are the wave vectors before and after the scattering.

Signal Processing

The signal at the photodetector results from the interference of the scattered main beam and the unscattered reference beam. The amplitude of the signal is varying sinusiodally with a frequency of $\Delta\nu = \nu_1 - \nu_2$. This oscillation of the light intensity is transformed into a detector current

$$I(t) = \sum_i A_i \sin(2\pi \Delta\nu_i t) \quad (16)$$

where i is the index of the different scattering species.

The intensity distribution then is Fourier transformed into a power spectrum, separately for each angle.

$$S(\nu) = \sum_i a_i [\delta(\nu - \Delta\nu_i) + \delta(\nu + \Delta\nu_i)] \quad (17)$$

a_i stands for the integrated amplitudes, $\delta(\nu - \Delta\nu_i)$ and $\delta(\nu + \Delta\nu_i)$ are the delta functions at the characteristic Doppler shift.

If the signal processing were just like that described above one could not distinguish between the positive and negative mobility, since the detectors do not distinguish positive and negative frequency shifts. To overcome this problem DELSA is equipped with a frequency shifter. The frequency is shifted one half of the width of the selected frequency range (see below).

The computations of the power spectrum are done with an IBM Personal System/2 Model 50 Z Computer, that also controls the instrument. The power spectrum is converted either into a frequency, a mobility or a zeta-potential spectrum. The conversion from the frequency shift into the mobility is done using the relationship between mobility, particle velocity and applied electric field, equation (11) Mobility and zeta-potential are related by the Helmholtz-Smoluchovski equation, equation (12).

The Brownian motion of the particles is the origin of the so-called diffusion broadening of the peaks. Since the broadening is inversely proportional to the particle size, this provides the means to measure average particle size when no electric field is applied. Furthermore, the angular dependence of the Doppler

shift obtained from the different detectors makes it possible to distinguish between real signals and instrumental artifacts. Only the true signals have frequency shifts proportional to the detector angle (approximated for small $\theta/2$).

The complete data analysis is performed by software, the operator only has to decide what part of the spectrum should be included in the analysis.

Parameters

Prior to any measurement the following parameters have to be defined : the temperature, the cell constant, the frequency range, the viscosity, the refractive index, and the dielectric constant of the solution, the time intervals for "field on" , "field off" time periods, and the run time, and the cell current .

In all experiments the temperature of the particle suspension was 25°C. The cell constant was calibrated using the conductivity of a standard with $1000 \pm 5 \mu\text{S/cm}$ (Yellow Springs Instruments Company, Inc., Yellow Springs, OH).

The particle mobility and the desired resolution determine the frequency range. Six different frequency ranges are available, from 50 to 1000 Hz. DELSA works as a multichannel analyzer with 256 channels, so the resolution is given by the frequency range divided by 256.

Except for very few data points with mobilities around zero all measurements were conducted with a frequency range of 500 HZ, because the signals at higher resolution were usually too noisy.

Neglecting the effect of the electrolyte, the values for viscosity (0.00890 poise), refractive index (1.3330) and dielectric constant (78.360) were set to the values of water according to the Handbook of Chemistry and Physics.

"Field on" time was usually set to 2 s, "field off" time to 0.5 s and the run time was 30 s. Run time was only increased in case of weak and noisy signals and the ratio of "field on" time to "field off" time was decreased if heating of the sample occurred.

During the measurements the electric field was applied for the "field on" time, followed by a "field off" time which allowed the sample and the electrodes to recover. The polarity of the electric field for the next "field on" time was reversed to avoid artifacts due to electrode polarization. Our choice of the "field on" time of 2 s also assured that the "field on" time was long enough not to affect the frequency resolution, since the frequency resolution depends inversely on the "field on" time.

The current was normally chosen to achieve a frequency shift between the different angles of at least 10 Hz. However, this was not possible for high conductivities and particle mobilities around zero because the current needed for the 10 Hz shift would cause overheating and convection inside the channel and some electrochemical reaction affecting irreversibly the mobility of particles.

Determination of the Stationary Layer

As mentioned above, the theory predicts that stationary layers are at 16% of the channel height above the channel bottom and below the channel top. However, this theoretical prediction was not always applicable due to the sedimentation of charged particles. The sedimentation affected the parabolic flow profile and consequently the position of the stationary layers shifted.

We found that the mobility standard supplied by the instrument manufacturer is not suitable for the calibration of position of the stationary layer due to a fairly high error margin in the mobility.

The following procedure has been developed to determine the stationary layer: After centrifuging the sample to reduce precipitation to a minimum, the sample was inserted into the cell and the cell height was measured. This was done by varying the vertical cell position. The upper and the lower edge of the channel are strongly scattering the light so that a rather sharp minimum in intensity occurs if the laser beam falls exactly on the edge. The vertical distance between the minima was used to determine the cell height.

Since there was still some residual sedimentation present, the cell height varied throughout all experiments within a range of 20 μm . Within a single series of measurements it varied within 10 μm . After the measurement of the cell height the cell was positioned so that the laser beams penetrated through the sample at 16 % of the cell height below the channel top. The accuracy of the

positioning was 0.5 μm . Measurements then were conducted at two different current levels. Usually the current for the second measurement was 20 to 25 % higher than that for the first measurement. If the mobilities obtained from these measurements were within the normal scattering range of the mobility values (0.08 mobility units), it was assumed that the measurements were done in the stationary layer.

This procedure was based upon the fact that the particle velocity off the stationary layer is a superposition of the particle electrophoretic velocity and the velocity of the electrolyte. Only for the stationary layer the mobility values for different currents were the same. In case of a greater discrepancy between the mobility results obtained for two different currents the cell position was changed until the mobility values were close enough to fall within the scattering range.

Other Procedures

After inserting the sample solution into the cell, the temperature of the sample has to reach the preset temperature (25°C), which usually takes up to ten minutes. During this time the cell position was adjusted to the theoretical stationary layer. (See determination of stationary layer)

Furthermore, before the first measurement of a series of measurement took place the beam intersection was checked (see above) and the intensity of the laser beam adjusted. The intensity of the main beam was 2 to 30 times greater than the intensity of the reference beam, as recommended.

Finally, the current was set and the measurement done. The conditions for the first run were set for the current corresponding to ~120 % (in mA) of the square root of the conductivity. If the signal was satisfactory then a second run was conducted with a current increased by 20 to 25 %. (See determination of the stationary layer.) If the mobility values for both runs were within the scattering range usually one more run was done at one of the current settings for mobility confirmation. In case of disagreement of the mobility values the cell position was adjusted and the above procedure repeated.

In any case the cell position was checked before each run and readjusted if necessary (fluctuations of $\pm 2 \mu\text{m}$ occurred). The reproducibility was within a micron. After each measurement the cell was rinsed three times with deionized water and flushed with air for at least five minutes to dry it. Then the cell was filled with the next sample solution.

For every ion adsorption study the concentration of the significant ion varied within the range of at least 3 orders of magnitude. The first data point taken was always the control point, it was the mobility in the absence of adsorbing ions.

Computer Models for Electrophoretic Mobility

The theoretical electrophoretic mobility of particles have been computed with a FORTRAN 77 program which has been developed for this purpose. The program is base on equations in section 2, it includes both screening and

adsorption of ions. The routines are very rudimentary and include calculation of Debye length, shear distance for given electrolytes, buffer and pH, and calculation of theoretical mobilities from the initial surface charge density and adsorption parameters. In the modeling process the first step was to determine the initial surface charge density from the mobility control point data. (sample prepared without adsorbing species.) After that the adsorption parameters were varied by a trial and error until a satisfying fit was obtained. Consequently the adsorption parameters given in chapter four have to be considered as good estimates.

Two values of mobility have been calculated: one from the surface potential and one from the zeta potential. The model using the zeta potential breaks down at ionic strength greater than about 1 M.

For analysis of mobility of liposomes and SR vesicles in the presence of negatively charged PCP we used a more sophisticated routine developed in this lab earlier. This routine uses the Marquardt-Algorithm for the fit of nonlinear functions to experimental data. (Bevington 1969). Chi-square is minimized as a function of adsorption site area and association constant.

CHAPTER IV

RESULTS AND DISCUSSION

EFFECT OF PH ON MOBILITY OF SR VESICLES AND LIPOSOMES

We determined the mobility dependence on the bulk pH for liposomes and SR vesicles at an ionic strength of 10 mM using the Phosphate-Borate-Citrate Buffer. The effect of pH on the mobility is shown in Figure 6 (corresponding data in Table I) where we compare the experimental results for SR vesicles and PC/PS liposomes. The major feature of the SR mobility isotherm is that at low pH the mobility is positive and changes polarity with the increasing pH. The mobility levels off above pH 6.5 at 1.44 ± 0.06 ($\mu\text{m/s})/(\text{V/cm})$. These results indicate that at $\text{pH} < 5$ the surface of SR membrane becomes positively charged due to the protonation of membrane proteins. At progressively higher pH the membrane surface is deprotonated and becomes negatively charged.

In contrast, the polarity of surface charge of PC/PS liposomes did not change with the pH range 3-10. It remained negative although there is also a strong evidence for the protonation of liposome membrane, presumably of

Figure 6. Mobility dependence on bulk pH for liposomes and SR vesicles. At ionic strength 10 mM.

TABLE I
PH DEPENDENCE OF ELECTROPHORETIC MOBILITY

Liposomes		SR Vesicles	
pH	Mobility [[$\mu\text{m/s}$]/(V/cm)]	pH	Mobility [[$\mu\text{m/s}$]/(V/cm)]
3.10	- 0.12	3.60	1.90
3.90	- 0.63	4.60	0.94
4.70	- 1.40	5.10	0.43
5.50	- 1.53	5.80	- 0.82
6.20	- 1.81	6.30	- 1.25
6.90	- 1.78	6.50	- 1.38
7.60	- 1.83	7.15	- 1.37
8.40	- 2.09	8.00	- 1.53
9.20	- 2.11	9.40	- 1.45
10.00	- 2.13	10.30	- 1.45

negatively charged PS lipids at low pH. It appears that there are two mobility plateaux for PC/PS liposomes, one at pH 6.2-7.6, and one at pH 8-10.

The pH-dependence of mobility of SR vesicles is qualitatively similar to that found for erythrocytes (Heard and Seaman 1960 and 1961, Meszaros 1988). The mobility of erythrocytes also levels off for pH higher than 6 (Furchgott and Ponder 1940, Heard and Seaman 1960).

In this work the isoelectric point for SR membrane was determined at pH 5.3 +/- 0.2. It has not been measured earlier, there were only indications that it was below pH 6 (Arrio 1984). There was no comparable data for erythrocytes available.

The surface charge density of SR membrane $\sigma_m = -6.1 \times 10^{-3}$ Coulomb/m² (error < 5%) corresponding to 0.038 unit charges per nm² was determined from Grahame's equation.

$$\sigma_m = \text{sgn}(\Psi(0)) \sqrt{(2\varepsilon_0\varepsilon_r kT) \sum_i n_i(\infty) \left[\exp\left(-\frac{z_i e \Psi(0)}{kT}\right) - 1 \right]} \quad (8)$$

where the membrane surface potential $\Psi(0)$ was set equal to ζ -potential obtained from Helmholtz-Smoluchovski equation,

$$\Psi(0) = \zeta = \frac{\mu\eta}{\varepsilon_0\varepsilon_r} \quad (12)$$

In his study Arrio (1984) obtained a surface charge density for SR membrane of -5.4×10^{-3} Coulomb/m². Liu and Oba (1990) determine -9.2×10^{-3} Coulomb/m².

EFFECT OF IONIC STRENGTH ON MOBILITY OF SR VESICLES AND LIPOSOMES

The dependence of mobility on ionic strength was conducted in a 5 mM Hepes buffer at pH 7.3 by varying the concentration of KCl in the solution. It was very difficult to obtain reliable data at high ionic strengths since the mobility values, especially for SR, were close to zero and the necessary currents for the electrophoretic measurements caused overheating of the sample. Furthermore, it was observed that already after two runs with a total "field-on" time of only 48 s the mobility shifted towards more negative values. Our explanation for this phenomenon is that electrochemical reactions must occur and irreversibly alter the membrane surface. For these reasons we omitted all data at ionic strengths higher than 250 mM. Figure 7 (a) and (b) illustrate a very similar ionic strength dependence for liposomes and SR vesicles. (The corresponding data is presented in tables II, III (a) and (b)).

In fact the relation between ionic strength and mobility for liposomes and SR vesicles is even more similar than the two graphs might suggest. One has to consider that the initial surface charge density for liposomes was 0.12 unit charge / nm² whereas for SR it was only 0.038 unit charge / nm².

Figure 7. Mobility isotherm in dependence of ionic strength. (a) Liposomes and (b) SR vesicles, at pH 7.3.

This accounts for the lower more negative mobility for liposomes. When the different surface charges are considered both curves could be reproduced by the adsorption model with very similar association constants for potassium. These were 0.04 M^{-1} for SR and 0.02 M^{-1} for the artificial PC/PS membrane.

The difference between association constants for SR and liposomes is within the experimental errors. It can be concluded that adsorption properties of SR and PC/PS membranes for potassium ions are very similar and that potassium ions do not adsorb under physiological conditions.

TABLE II
LIPOSOME MOBILITY ISOTHERM

Ionic strength [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
6.74		- 5.67	- 5.05
9.00	- 4.37	- 5.13	- 4.52
14.00	- 3.97	- 4.42	- 3.83
16.00	- 3.37	- 3.46	- 2.94
38.00	- 2.59	- 2.95	- 2.47
74.00	- 1.97	- 2.17	- 1.78
122.00	- 1.53	- 1.69	- 1.38
242.00	- 1.07	- 1.17	- 0.97
403.00		- 0.87	- 0.75
603.00		- 0.67	- 0.61

TABLE III
MOBILITY ISOTHERMS FOR SR VESICLES

(a) Experimental Data

Ionic strength [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility [($\mu\text{m/s}$)/(V/cm)]
8.20	- 2.00	- 2.00
12.00	- 1.53	- 1.58
22.00	- 1.13	- 1.23
32.00	- 0.94	- 0.89
62.00	- 0.64	- 0.61
104.00	- 0.53	- 0.58
202.00	- 0.53	- 0.47

(b) Theoretical Data

Ionic strength [mM]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
5.74	- 2.51	- 2.25
7.74	- 2.18	- 1.93
10.74	- 1.86	- 1.63
18.74	- 1.41	- 1.21
32.74	- 1.04	- 0.88
52.74	- 0.79	- 0.66
82.74	- 0.60	- 0.49
102.70	- 0.52	- 0.42
202.70	- 0.30	- 0.25
302.70	- 0.19	- 0.16

CALCIUM ADSORPTION

We have found the same features in the mobility data in the presence of calcium (Figure 8 (a) and (b), Table IV (a) and (b)) as obtained earlier by McLaughlin (1981) in their studies of Ca^{2+} adsorption on PC/PS liposomes with different mixture ratios of PC to PS. In the present study we measured the effect of Ca^{2+} on mobility of liposomes with two different mixture ratios of PC:PS, 30:1 and 10:1. Our results are also in good agreement with data published by Tatulian (1988). However, there are significant differences in the adsorption constants determined in the three studies. McLaughlin, who determined the adsorption constant for Ca^{2+} to PS to be $K_{\text{Ca}} = 8 \text{ M}^{-1}$ assumed that one divalent cation binds to one lipid molecule. Tatulian on the other hand introduced the concept of different adsorption site areas for different lipids and different adsorbing species. He determined the adsorption constants K_{Ca} for PC to be 42 M^{-1} and 12 M^{-1} PS. Our results are consistent with K_{Ca} equal to 155 and 80 M^{-1} for the 30:1 and the 10:1 liposomes respectively and our analysis also assumed variable adsorption site areas.

We have observed that the presence of PC increased adsorption of calcium. The large discrepancies between the association constants in the above studies is not understood.

Figure 8. Mobility isotherm for Ca^{2+} adsorption to liposomes. (a) For PC:PS (10:1) liposomes and (b) for PC:PS (30:1) liposomes, at pH 7.3.

TABLE IV
MOBILITY ISOTHERM FOR Ca^{2+} ADSORPTION TO LIPOSOMES

(a) PC:PS (10:1) Liposomes

Ca^{2+} concentration [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
0.500	- 1.39	- 1.54	- 1.38
0.800	- 1.08	- 1.18	- 1.05
1.500	- 0.58	- 0.69	- 0.60
3.000	- 0.12	- 0.16	- 0.14
7.000	0.330	0.410	0.350
10.000	0.450	0.620	0.520
25.000	0.870	1.030	0.850
50.000	0.950	1.190	0.970
80.000	1.030	1.200	0.990
150.000	0.960	1.080	0.950
300.000	0.870	0.810	0.850
500.000	0.770	0.690	

(b) PC:PS (30:1) Liposomes

Ca^{2+} concentration [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
0.500	- 0.75	- 0.85	- 0.76
0.800	- 0.55	- 0.57	- 0.51
1.500	- 0.15	- 0.18	- 0.16
3.000	0.320	0.230	0.200
7.000	0.630	0.680	0.580
10.000	0.810	0.850	0.710
25.000	1.040	1.190	0.970
50.000	1.070	1.340	1.090
80.000	1.120	1.370	1.140
150.000	1.080	1.310	1.150
300.000	0.990	1.110	1.110
500.000	0.890	0.910	

It might be due to the different modifications of the Langmuir-Stern-Grahame Model as well as errors in calibration of the calcium concentration. The adsorption site area for calcium obtained in this work, 4.1 nm^2 , was the same for both 30:1 and 10:1 PC/PS membranes.

The mobility isotherm of SR vesicles in the presence of calcium is different from that for liposomes (Figure 9, data in Table V). While the liposome mobility reaches a maximum at about $0.06 - 0.08 \text{ M Ca}$, the SR mobility continues to increase with calcium concentration up to 1 M Ca^{2+} , the end of the calcium concentration range. Furthermore, it was impossible to fit the data with the adsorption model using a single set of adsorption parameters. The association constants obtained from the partial fits were $K_{\text{Ca}} = 70 \text{ M}^{-1}$ for low concentrations and $K_{\text{Ca}} = 6 \text{ M}^{-1}$ for high concentrations using the adsorption site area of 4.1 nm^2 equal to that for liposomes.

TETRAPHENYLARSONIUM (TPAs) AND TETRAPHENYLPHOSPHONIUM (TPP)

In contrast to the calcium isotherms, the TPAs and TPP mobility isotherms for liposomes and SR vesicles are also very similar. Both SR and artificial membranes show the same major characteristics: (1) The mobility of the vesicles is only slightly affected at low concentrations of TPAs ($<10^{-4} \text{ M}$) (Figure 10 (a) and (b) and Table VI (a) and (b)).

Figure 9. Mobility isotherm for Ca^{2+} adsorption to SR vesicles. At pH 7.3.

TABLE V

MOBILITY ISOTHERM FOR Ca^{2+} ADSORPTION TO SR VESICLES

Ca^{2+} concentr. [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mob. Surf. low [($\mu\text{m/s}$)/(V/cm)]	Mob. Zeta low [($\mu\text{m/s}$)/(V/cm)]	Mob. Surf. hi [($\mu\text{m/s}$)/(V/cm)]	Mob. Zeta hi [($\mu\text{m/s}$)/(V/cm)]
0.16	- 1.33	- 1.46	- 1.29		
0.32	- 1.03	- 1.16	- 1.02		
0.65	- 0.77	- 0.80	- 0.70		
1.10	- 0.52	- 0.50	- 0.44		
1.90	- 0.39	- 0.18	- 0.16	- 1.22	- 1.06
3.50	- 0.23	0.17	0.14	- 0.93	- 0.80
5.60	- 0.09	0.42	0.36	- 0.71	- 0.61
8.10	- 0.05	0.61	0.51	- 0.54	- 0.46
16.00	0.04	0.91	0.75	- 0.25	- 0.20
29.00	0.12			- 0.02	- 0.01
48.00	0.22			0.15	0.12
65.00	0.23			0.24	0.20
130.00	0.29			0.40	0.35
258.00	0.42			0.51	0.49
484.00	0.52			0.54	
710.00	0.55			0.53	
970.00	0.58			0.50	

This is particularly true for the SR vesicles for which the measured mobility remains unchanged within the limits of the accuracy over a concentration range from 1.6 - 400 μM . (2) At higher concentrations, above 1×10^{-4} M for liposomes and 4×10^{-3} M for SR vesicles the absolute magnitude of the mobility becomes significantly smaller. With the further increase of the lipophilic cation concentration we observe a reversal of the polarity of electrophoretic mobility. The reversal takes place between 2×10^{-3} and 3×10^{-3} M for the liposomes and between 2×10^{-2} and 4×10^{-2} M for SR vesicles. At higher TPAs concentrations the mobility reaches a maximum and decreases with the further increase of concentration.

The dependence of mobility on the concentration of TPAs ions is understood, the observed changes are a combination of screening effects of the electrolyte and adsorption of TPAs. The onset of mobility decrease for both the liposomes and SR vesicles is associated with the adsorption of positively charged TPAs. The mobility decrease at TPAs concentrations above that corresponding to the mobility maximum is due to screening of positively charged membrane surface by negatively charged counterions. The solid and broken curves illustrate the predictions of the model.

Figure 10. Mobility isotherm for TPAs adsorption. (a) To Liposomes, $K_{TPAs} = 280 \text{ M}^{-1}$, and (b) to SR vesicles, $K_{TPAs} = 20 \text{ M}^{-1}$, at pH 7.3.

TABLE VI
MOBILITY ISOTHERM FOR TPAs ADSORPTION

(a) Liposomes

TPAs concentrat. [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
1.6 x 10 ⁻³	- 3.53	- 3.59	- 3.27
3.2 x 10 ⁻³	- 3.26	- 3.54	- 3.23
7.2 x 10 ⁻³	- 3.03	- 3.50	- 3.18
10.4 x 10 ⁻³	- 3.11	- 3.48	- 3.14
0.02	- 2.85	- 3.38	- 3.04
0.06	- 2.83	- 3.03	- 2.73
0.10	- 2.45	- 2.78	- 2.50
0.20	- 2.15	- 2.32	- 2.09
0.40	- 1.60	- 1.74	- 1.56
0.80	- 0.89	- 1.07	- 0.96
2.00	- 0.38	- 0.12	- 0.11
3.00	0.22	0.29	0.25
7.00	0.90	1.03	0.90
10.00	1.15	1.26	1.09
20.00	1.39	1.54	1.31
30.00	1.42	1.57	1.30
70.00	1.26	1.39	1.14
100.00	1.06	1.24	1.02

TABLE VI
MOBILITY ISOTHERM FOR TPAs ADSORPTION
(continued)

(b) SR Vesicles

TPAs concentrat. [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
1.6 x 10 ⁻³	- 2.07	- 2.12	- 1.87
3.2 x 10 ⁻³	- 2.08	- 2.12	- 1.87
7.2 x 10 ⁻³	- 1.94	- 2.11	- 1.87
10.4 x 10 ⁻³	- 2.01	- 2.11	- 1.87
0.024	- 2.04	- 2.11	- 1.87
0.060	- 1.99	- 2.09	- 1.85
0.10	- 2.02	- 2.08	- 1.84
0.20	- 1.96	- 2.04	- 1.80
0.40	- 2.03	- 1.96	- 1.73
0.80	- 1.77	- 1.83	- 1.62
2.00	- 1.79	- 1.52	- 1.34
4.00	- 1.39	- 1.18	- 1.03
7.00	- 1.05	- 0.84	- 0.73
10.00	- 0.60	- 0.62	- 0.53
20.00	- 0.01	- 0.19	- 0.16
40.00	0.21	0.16	0.13
70.00	0.47	0.35	0.29
100.00	0.41	0.43	0.35
194.00	0.19	0.49	0.40
300.00		0.47	0.39
400.00		0.44	0.38

The observations that the reversal of mobility and mobility maximum for liposomes occur at concentrations significantly lower than those for SR vesicles demonstrate lower adsorption affinity of the SR membrane relative to the artificial phospholipid membrane. The concentration dependence of mobility of liposomes and SR vesicles can be satisfactorily fit by the adsorption model for the following association constants: K_{TPAs} (liposome) = 280 M⁻¹ and K_{TPAs} (SR) = 20 M⁻¹, and adsorption site areas: A_{TPAs} (liposome) = 7.2 nm² and A_{TPAs} (SR) = 9.6 nm².

The experimental results for liposomes and SR vesicles in the presence of TPP are similar (Figure 11 (a) and (b), Table VII (a) and (b)). The data can be satisfactorily fit by the adsorption model for the following association constants: K_{TPP} (liposome) = 230 M⁻¹ and K_{TPP} (SR) = 20 M⁻¹, and adsorption site areas: A_{TPP} (liposome) = A_{TPP} (SR) = 9.6 nm².

PENTACHLOROPHENOL

In contrast to previous ions, ionized pentachlorophenol molecules are negatively charged. The studies of electrophoretic mobility of liposomes and SR vesicles in the presence of PCP were done at pH 9.2. At this pH PCP is fully ionized (aqueous $pK_a=4.8$).

Figure 11. Mobility isotherm for TPP adsorption. (a) To liposomes, $K_{TPAs} = 230 \text{ M}^{-1}$, and (b) to SR vesicles, $K_{TPAs} = 20 \text{ M}^{-1}$, at pH 7.3.

TABLE VII
MOBILITY ISOTHERM FOR TPP ADSORPTION

(a) Liposomes

TPP concentration [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
0.500	- 2.14	- 1.80	- 1.62
0.700	- 1.46	- 1.51	- 1.35
1.000	- 1.23	- 1.18	- 1.06
2.000	- 0.68	- 0.53	- 0.47
3.000	- 0.46	- 0.15	- 0.13
7.000	0.440	0.530	0.460
10.000	0.610	0.740	0.640
20.000	1.040	1.000	0.850
50.000	0.900	0.950	0.790

(b) SR Vesicles

TPP concentration [mM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	Mobility (Surf. pot.) [($\mu\text{m/s}$)/(V/cm)]	Mobility (Zeta pot.) [($\mu\text{m/s}$)/(V/cm)]
0.500	- 1.80	- 1.93	- 1.70
0.700	- 1.73	- 1.86	- 1.64
1.000	- 1.71	- 1.77	- 1.56
2.000	- 1.61	- 1.52	- 1.33
3.000	- 1.59	- 1.33	- 1.17
7.000	- 0.82	- 0.84	- 0.73
10.000	- 0.62	- 0.62	- 0.53
20.000	0.010	- 0.05	- 0.04
50.000	0.360	0.250	0.200
90.000	0.340	0.430	0.350

The mobility isotherms of liposomes and SR vesicles are shown in Figure 12 (a) and (b) (Table VIII (a) and (b)). The change of mobility with PCP concentration is opposite to that observed for the positively charged ions. The mobility of both liposomes and SR vesicles becomes more negative with the increasing concentration of PCP indicating an increase of the density of negative surface charge at the membrane surface. A 100-fold increase of PCP concentration resulted in mobility change of about 2 mobility units for liposomes compared to about 0.5 mobility units for SR vesicles. This observation suggests that adsorption of ionized PCP to PC/PS bilayer is greater compared to the SR membrane.

The adsorption parameters obtained from the chi-square fit are as follows, association constant: K_{PCP} (liposome) = $(3.8 \pm 0.2) \times 10^4 \text{ M}^{-1}$ and K_{PCP} (SR) = $(5.0 \pm 0.1) \times 10^3 \text{ M}^{-1}$; adsorption site area: A_{PCP} (liposome) = 3.8 nm^2 and A_{PCP} (SR) = 29.6 nm^2 . The theoretical mobility curves (Figure 12 (a), (b)) illustrate the model predictions.

This has been the first study of adsorption of ionized PCP on negatively charged membranes. Uncharged lipid membranes have been used in all previous adsorption studies with PCP because of the anticipated reduction of adsorption of negatively charged PCP due to the repulsion from the negatively charged membrane surface.

Figure 12. Mobility isotherm for PCP adsorption. (a) To liposomes, $K_{PCP} = (3.8 \pm 0.15) \times 10^4 \text{ M}^{-1}$, and (b) to SR vesicles, $K_{PCP} = (5.0 \pm 0.03) \times 10^3 \text{ M}^{-1}$, at pH 9.2.

TABLE VIII
MOBILITY ISOTHERM FOR PCP ADSORPTION

(a) Liposomes

PCP concentration [μM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	theor. Mobility [($\mu\text{m/s}$)/(V/cm)]
5.00	- 2.74	- 2.81
7.00	- 2.48	- 2.88
10.00	- 2.94	- 2.98
20.00	- 3.14	- 3.21
60.00	- 3.74	- 3.72
100.00	- 4.14	- 3.88
200.00	- 4.46	- 4.43
500.00	- 4.78	- 4.96

(b) SR Vesicles

PCP concentration [μM]	Mobility [($\mu\text{m/s}$)/(V/cm)]	theor. Mobility [($\mu\text{m/s}$)/(V/cm)]
3.20	- 1.44	- 1.34
6.40	- 1.46	- 1.35
11.00	- 1.35	- 1.36
22.60	- 1.35	- 1.41
64.50	- 1.45	- 1.47
100.00	- 1.49	- 1.49
260.00	- 1.66	- 1.63
645.00	- 1.77	- 1.79
970.00	- 1.86	- 1.84

The value of association constant obtained for PC/PS liposomes, $(3.8 \pm 0.2) \times 10^4 \text{ M}^{-1}$, is similar to that found for structurally similar but uncharged

phospholipid, K_{PCP} of dipalmitoylphosphatidylcholine in fluid state was found to be earlier $(4.5 \pm 0.9) \times 10^4 \text{ M}^{-1}$ (Smejtek and Wang, 1990).

The most important result is the finding of significantly smaller adsorption constant for a negatively charged lipophilic ion, ionized PCP, for the SR membrane compared to that for phospholipid membrane.

CONCLUSIONS

In this project we determined the electrophoretic mobility of SR vesicles and PC/PS liposomes as a function of pH and concentration of ions with different adsorption affinity to lipid membranes. These were positively charged K, Ca, TPAs, TPP and negatively charged PCP.

We have shown that electrophoretic mobility of PC/PS liposomes and SR vesicles can be understood in terms of electrostatic screening of charged membrane surface by counterions and by ion adsorption. All mobility isotherms with the exception of Ca and SR vesicles can be fit by the Langmuir-Stern-Grahame model assuming competitive adsorption of ions.

The mobility results indicate very weak adsorption affinity of both PC/PS and SR membrane for potassium and chloride ions since the mobility changes with salt concentration can be explained by screening. The results are summarized in Table IX.

TABLE IX
ADSORPTION PARAMETERS OBTAINED FROM COMPUTER MODELING

Ion	Liposomes		SR Vesicles	
	K, (M ⁻¹)	A, (nm ²)	K, (M ⁻¹)	A, (nm ²)
Ca	155 (30:1)	4.1	n. a.	n. a.
	80 (10:1)	4.1		
TPAs	280	7.3	20	9.6
TPP	230	9.6	20	9.6
PCP	$(3.8 \pm 0.2) \times 10^4$	3.9	$(5.0 \pm 0.1) \times 10^3$	29.3

The fact that it has not been possible to fit the mobility data of SR vesicles in the presence of Ca with one set of adsorption parameters strongly indicates the presence of more than one type of calcium adsorption sites on the SR membrane. Considering the physiological importance of calcium and the physiological function of the SR membrane our finding is very interesting. Our data and analysis suggests that within the given calcium concentration range, Ca adsorption is dominated by two types of sites: one with association constant of $K_{Ca} \approx 70 \text{ M}^{-1}$ for low Ca concentrations and $K_{Ca} \approx 6 \text{ M}^{-1}$ for high concentrations. It is also possible that there is a continuous, rather than discrete distribution of Ca adsorption sites in the SR membrane.

Perhaps the most interesting aspect of our findings is the significantly lower adsorption of both positively charged lipophilic ions and negatively charged PCP to SR membranes compared to that to lipid membranes.

Qualitatively similar results were published earlier only by Conrad and Singer (1979 and 1981). They used a filtration method called hygroscopic desorption and measured the solubility of amphipathic molecules in biological and lipid membranes. Their finding was about 10^3 -fold lower adsorption to biological membranes than to lipid bilayers. To explain their findings they introduced a hypothesis of existence of a large "internal pressure" in biological membranes which would inhibit the adsorption of amphipathic molecules. Conrad's and Singer's hypothesis was later dismissed as being due to an experimental artifact.

This work contributes to efforts to understand binding of ions and neutral molecules to lipid membranes and biomembranes. We have demonstrated by a different method, without using probes, that the adsorption of lipophilic ions is suppressed in SR membrane compared to the phospholipid bilayer.

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