Chemistry (PhD), CH81 [redacted]

ABSTRACT

GLYCERYL MONOOLEATE BILAYER MEMBRANES--
CHARACTERISTICS AND APPLICATIONS

By

Sechoing Lin

The capacitance and thickness of bilayer membranes formed from dissolving glyceryl monooleate in various hydrocarbon solvents have been studied using the charge injection technique. The effects of different individual solvents, varying the mole fraction in binary solvents, and lipid concentration on the capacitance and thickness of membranes have been included in this study. The capacitance values obtained in single hydrocarbon solvents are in quite good agreement with values obtained by previous workers who used either ac or other dc techniques. The capacitance values are found to be independent of lipid concentrations for membranes in the squalene system. Both squalane and paraffin oil have been developed into suitable solvents for the formation of membranes with little or no solvent. Binary solvents such as n-decane/n-hexadecane or n-decane/squalene provide a way to conveniently synthesize membranes with a wide and continuous range of thicknesses.

The effects of benzyl alcohol and cholesterol on the

capacitances of two different membrane systems have been studied. The adsorption of benzyl alcohol into the bilayer causes a decrease in the membrane capacitance for n-hexadecane-containing membranes, while this trend is not observed for membranes with little or no solvent. The incorporation of cholesterol increases the capacitance for the n-decane-containing membranes and conversely, a decrease in the capacitance is observed for membranes with little or no solvent. An attempt to develop a bilayer membrane-based molecular sensor for polycyclic aromatic hydrocarbons is unsuccessful.

The stability of bilayer membranes formed from dissolving glyceryl monooleate in n-alkanes or squalene has been found to increase drastically with the addition of a small amount of ferric chloride to the aqueous phases. In the presence of ferric chloride, the lifetime of this membrane formed on an aperture of 1.5 mm diameter is prolonged from a few minutes to more than 24 hours, and an accompanying substantial increase in the dielectric breakdown voltage of this membrane is observed.

The effect of a recently synthesized compound, namely 1,1,2-tris-(1H-benzimidazole)ethane (TBIE) on the membrane conductance has been studied. The membrane conductance is found to increase with the addition of a small amount of TBIE to the aqueous phases. Further enhancement of the membrane conductance occurs with the addition of copper(II) chloride, but not with zinc(II), cobalt(II), and nickel(II)

chlorides.

A ferric(III)-stabilized bilayer membrane has been tested as a sensor. The change in resistance of this membrane caused by the selective transport of $(Ph)_4As^+$ is found to correlate with the concentration of $(Ph)_4AsCl$ in a linear fashion over a limited concentration range. This property is utilized in a membrane-based sensor to indicate the equivalence point in the titration of unknown mercury(II) with standard $(Ph)_4AsCl$ in 3 M NaCl solution. The results have been quite promising; an average accuracy of 5.5 % is obtained from five trials.

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INTRODUCTION

Historical

In 1899, Overton (1) first observed that cell membranes were very permeable to lipids and thus suggested that cell membranes contained lipids. Langmuir (2), in 1917, emphasized that lipids tended to form structured monolayers when placed in contact with water and measured the surface pressure exerted by this monolayer film on water surface. Gorter and Grendel (3), in 1925, found that lipids extracted from red cell membranes would spread on water to a thickness just about half that of the membrane itself. Assuming in accord with Langmuir that the lipid layer was one molecule thick, they concluded that the membrane was essentially a double layer of lipid molecules. Davson and Danielli (4) in 1943, on the basis of data on the low surface tensions of membranes, advanced the model to include the proteins for the outer membranes of animal cells. The membrane is depicted as a lamellar bimolecular lipid layer with the polar head groups of the lipids oriented toward the high dielectric aqueous medium which is both inside and outside the cell. This configuration allows the hydrocarbon chains of the lipids to be in their own low dielectric environment. Proteins present in the membrane are believed to be adsorbed on the surface of the lipid bilayer. This model was widely

accepted by biologists at the time.

The first in vitro preparation of a bilayer lipid membrane was successfully performed by Mueller et al. (5) in 1962 by brushing a complex mixture of brain lipids, n-tetradecane, silicone fluid and mineral oil across a small aperture on a Teflon sheet between two aqueous compartments. Rapid progress has been made since then. Techniques of bilayer lipid membrane formation have been improved and synthetic amphiphiles have been found that provide systems more amenable to fundamental physicochemical investigation. A schematic configuration of this model membrane with a bilayer structure on the center and a relatively thick torus region surrounding it is shown in Figure 1-1. This bilayer lipid membrane is seen to be akin to the classical model of Gorter-Grendel, and is similar to the so-called smectic soap mesophase (6). In this liquid crystalline structure, the polar groups of the lipids are thought to orient in a plane which is perpendicular to the membrane.

Formation of Bilayer Lipid Membranes

General

Bilayer lipid membranes are formed from solutions of one or more lipids in nonpolar solvents. However, the number of suitable lipids and nonpolar solvents is quite limited. The use of water-soluble solvents such as chloroform and ethanol is not precluded provided a significant portion of

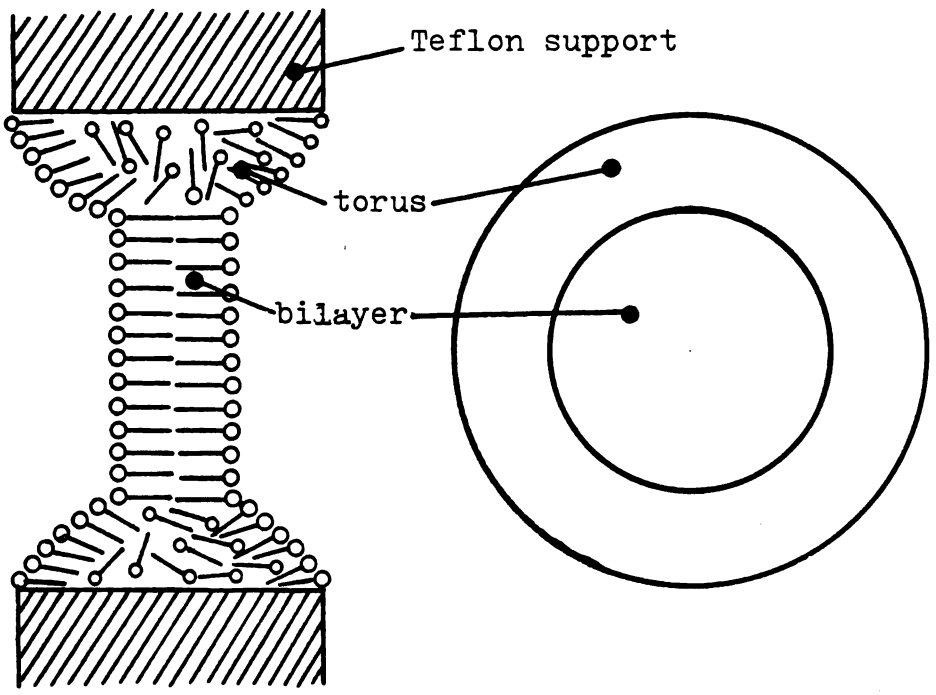


Figure 1-1. Schematic of a physical model of a bilayer lipid membrane.

a water-insoluble solvent is also present. The main disadvantage of this kind of mixed solvent system is that one or more of the water-soluble solvents may dissolve out of the membrane, thus a true equilibrium structure of the membrane can not be obtained. As a consequence, the properties of the membrane such as composition, thickness, and tension may be time-dependent. Lipids should be strongly surface active, because the rupture of the membrane under the pressure of the London-van der Waals and other thinning forces is prevented by a lipid component which must not be readily desorbed. Clearly, the more powerful the affinity of the lipid for the interface, the more effective it is likely to be as a stabilizer.

Choice of Lipid

The lipids known to form stable membranes include many of the naturally occurring phospholipids and some monoglycerides (7). Among those lipids suitable for bilayer lipid membrane studies, the nature and stability of the resulting membranes vary enormously. Therefore, the choice of lipid is strongly dictated by the kinds of studies being investigated.

There are significant differences between the behavior of phospholipids and monoglycerides in bilayer lipid membrane systems and these differences are listed below.

Characteristics of phospholipids:

1. Phospholipids do not usually dissolve molecularly in hydrocarbons but rather exist as large aggregates which

may nevertheless remain dispersed for long periods.

2. The adsorption process is more complicated and takes time.
3. Monolayers are "insoluble" and are in equilibrium only with the monolayers on the adjacent bulk phase interfaces.
4. Phospholipids have strong affinity. Unless the lipid and hydrocarbon solvent are carefully dried, the black films obtained are liable either to be abnormally thick or to contain abnormally thick patches, and to exhibit a curious "spider's web" appearance.

Characteristics of monoglycerides:

1. Monoglycerides yield molecular solutions in hydrocarbons and form micelles in the millimolar region in a manner similar to that observed for many water-soluble surfactants.
2. At several millimoles per liter, the maximum adsorption is attained in a matter of seconds or less.
3. Monolayers are always in equilibrium with bulk lipid solution.

Careful examination of the characteristics for these two classes of lipids indicates that monoglycerides possess certain advantages over phospholipids and these advantages can become especially important in determining the composition of the membrane and in studying certain physicochemical phenomena using this lipid system.

Choice of Solvent

The requirement for selecting a solvent for a bilayer

lipid membrane is that the solvent itself should be both nonpolar and nonvolatile. Appreciable water-solubility and volatility will lead to membranes that are not at equilibrium and are therefore poorly defined. If a single solvent is to be used, the most satisfactory are the alkanes from n-octane to n-hexadecane. The most remarkable differences within this series of alkanes is the membrane thickness change attributed to a variation in the adsorption of the alkanes into the membrane which depends upon the chain length. As the solvent chain length increases, the increased exclusion of the solvent leads to a thinner membrane. In the case of glyceryl monooleate, as the solvent is varied from n-decane to n-hexadecane, the thickness of the membrane decreases by about 16 Å.

Mechanism for The Formation of Bilayer Lipid Membranes

A dynamic mechanism has been advanced by Snyder et al. (8) to explain the formation of bilayer lipid membranes. The mechanism is viewed as a two-step process; (a) initial thinning, and (b) surface spreading. In the first step, as shown in Figure 1-2, material flows from the center of the membrane (bilayer) to the border area (torus) near the edge of the supporting aperture. As with the flow of fluids in channels, a pressure gradient is required to maintain the thinning process. The pressure difference can be traced to the curvatures of the thinning film and the border area. The variations in surface curvature can produce bulk flow within the membrane and cause it to thin.

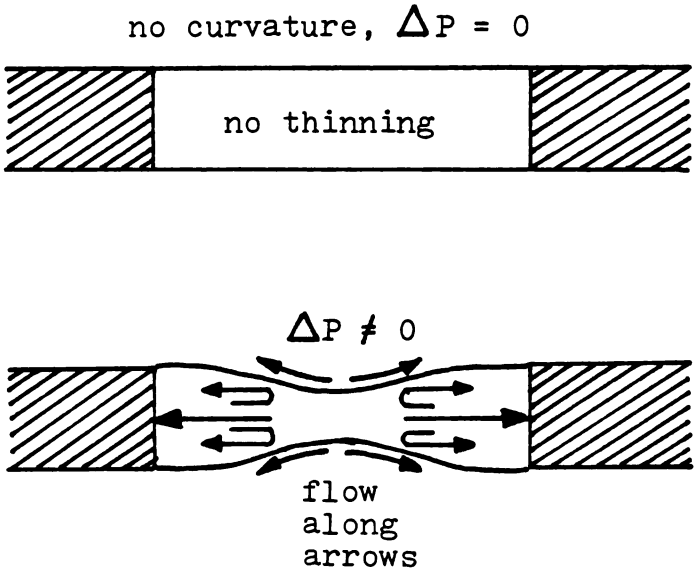


Figure 1-2. Schematic diagram of the thinning process for bilayer lipid membranes (initial thinning process).

The rate of thinning is controlled by the bulk viscosity of the membrane solution.

As thinning proceeds, the thickness of the membrane is reduced until it reaches the molecular dimensions where van der Waals forces can take over and produce local areas of bimolecular layers.

Once the spots of local bimolecular layers occur, the formation mechanism changes from a simple hydrodynamic flow process to a surface spreading phenomenon. The major driving force, at this time, is the change in bifacial tension across the torus/bilayer border. The force resulting from the pressure gradient has a negligible influence on the surface spreading process. The rate of spreading is mainly determined by "surface viscosity" if other factors are fixed.

Electrical Properties of Bilayer Lipid Membranes

Unmodified Bilayer Lipid Membranes

A bilayer lipid membrane so formed has a very high resistance for small inorganic ions. This is attributed to the large difference in the dielectric constants between both media-- aqueous solution (ca. e = 80) and hydrocarbon interior of the membrane (ca. e = 2.1). In order for ions to cross the relatively low dielectric hydrocarbon interior of a membrane, a great amount of energy is required (9), and this leads to the observed high resistance. If one tries purposely to lower the

dielectric constant of hydrocarbon interior of a membrane by using a more polar solvent which is supposedly present in the bilayer, a decreased resistance is expected. This is indeed the case as observed by Dilger et al. (10) who employed 1-chlorodecane as solvent for a membrane-forming solution. Moreover, as mentioned previously, the composition of a bilayer lipid membrane can be varied via the changes of either lipid or solvent or both. For example, a bilayer membrane formed from an oxidized cholesterol/n-decane solution has a resistance in the order of 10^8 ohm-cm^2, while the resistance of a bilayer membrane formed from glyceryl monooleate/n-decane solutions is in the order of 10^6 ohm-cm^2. The two-order of magnitude greater resistance of the membrane formed from an oxidized cholesterol/n-decane solution is apparently due to the stronger hydrophobic interaction between the nonpolar portion of oxidized cholesterol in the membrane interior.

On the one hand, the intrinsically high resistance of a bilayer lipid membrane makes it very dissimilar to biological membranes although the two systems are analogous in many other aspects (11,12). On the other hand, the high resistance of a bilayer lipid membrane facilitates the study of the effects of doping with various modifiers. The addition of certain modifiers can drastically change the resistance of a bilayer lipid membrane. This has stimulated many studies of biologically associated phenomena.

Modified Bilayer Lipid Membranes

The intrinsic properties of a bilayer lipid membrane can be modified by introducing modifiers into the membrane system. These modifiers can be either ions or molecules. According to the mechanism by which the modifiers affect the resistance of a bilayer lipid membrane, they can be grouped into five categories; (a) those which facilitate direct transport, (b) those which act as ion carriers, (c) those which form ion-conducting channels, (d) those which vary the internal energy barriers of the membrane interior, and (e) those which interact through a receptor mechanism.

Lipophilic ions (or fat-soluble ions) such as tetraphenylborate, tetraphenylarsonium, tetraphenylphosphonium, etc. belong to category (a). The delocalization of charge among the phenyl groups makes these ions very fat-soluble. When these ions are added to the aqueous solution, they diffuse across the membrane by the direct transport mechanism. In the presence of tetraphenylborate anions, the membrane resistance decreases about three orders of magnitude even at a concentration of only 10^{-7} M (13) in the aqueous phase. In category (b), a representative example is valinomycin - a cyclic compound which possesses polar carbonyl groups around its center and a nonpolar portion as well. When residing at a membrane-aqueous interface, the polar groups orient toward the aqueous phase and are responsible for the selective formation of complexes with ions. Once present inside the membrane

interior, the portion of ion and polar groups are surrounded by the nonpolar portion of valinomycin molecule via a conformation change so that the whole complex is very hydrophobic. The ion transport across the membrane is through the ion-molecule complex acting as a recycling carrier. This process produces a drop in the membrane resistance (13,14). Molecules which fall into category (c) are polyene antibiotics such as nystatin and amphotericin B, or polypeptides such as gramicidin. Once added to the membrane, these molecules form polar channels through which small ions can travel. The decrease in the membrane resistance by way of the channel formation mechanism is generally greater than that from either of the other two mechanisms mentioned above (13,14). Molecules classified in category (d) are small, polar organic compounds such as phloretin (15) and pentachlorophenol (16,17). These molecules are adsorbed at the membrane-aqueous interface. This adsorption, in turn, varies the ion transport energy barrier of the membrane interior through the effect of the dipolar property of the adsorbed molecule. Finally, in category (e), a typical example of antigen-antibody is human serum albumin diazotized with sulfanilic acid as antigen and corresponding immune serum as antibody (18). Antigen is usually reconstituted into the membrane and the addition of antibody would make an antigen-antibody interaction take place. This mode of interaction is very specific and the resulting change in membrane resistance is

transient.

A Preview of This Work

There is no doubt that knowledge about biomembranes has been further advanced as a result of extensively using bilayer lipid membranes for studies of biologically relevant phenomena. Moreover, the accumulation of this knowledge may suggest many applications in nonbiologically related areas. To mention a few examples: (a) The bilayer lipid membrane itself is a new type of interfacial adsorption phenomena. Therefore, it can be a useful tool for the understanding of physics and chemistry of amphipatic compounds and will be relevant to the further development of interfacial chemistry and colloid science; (b) A bilayer lipid membrane can be employed as a membrane matrix and the relatively selective, sensitive interaction of compounds with or on this membrane matrix often causes the change of electrical property of the membrane. Taking advantage of this approach, bilayer lipid membranes may be developed as sensitive sensors and expected to find some applications in the bioanalytical area.

This study makes of monoglycerides and emphasizes the more nonbiological applications. It begins with the characterization of the glyceryl monooleate bilayer membrane system in various solvents and lipid concentrations with the goal to better characterize this membrane system. A study of stabilizing effect of this membrane system in

the presence of ferric chloride follows. The conductance of this membrane system to Cu(II), Co(II), Ni(II), and Zn(II) ions is studied when a new compound, 1,1,2-tris-(1H-benzimidazole)ethane, is present in the aqueous phase. Finally, this membrane system which is stabilized by ferric chloride is applied as a sensor in the determination of Hg(II).

Measurements of Capacitance and Conductance of Bilayer Lipid Membranes

General

The characterization of the properties of unmodified bilayer lipid membranes is most often performed using methods of electrical measurement. Moreover, electrical measurements can provide information about the changes resulting from the interactions between the bilayer lipid membrane and its modifiers. Both dc and ac techniques have been employed for the measurement of various electrical parameters. The typical arrangement of a cell for studies of electrical properties is generally represented below:

Nonpolarizable Electrode	Aq. Soln. I	BLM on Teflon Support	Aq. Soln. II	Nonpolarizable Electrode

Cell Assembly and Electrodes

An electrochemical cell is usually set up as shown in Figure 1-3. The cell consists of two rectangular Teflon chambers. Each chamber has a volume of approximately 45 ml and is separated by a thin Teflon sheet with a thickness of 0.25 mm. A circular aperture with a diameter between 1.0 to 2.0 mm is punched in the Teflon sheet. The Teflon sheet is made dismountable to allow for the alteration of the aperture size by the replacement of the Teflon sheet. Both chambers have 9.5 mm holes in the center of the faces which contact the Teflon sheet. These holes are surrounded on the outside face by Viton "O" rings (2.54 cm O.D.) inset in the Teflon with 0.12 mm protrusion. When the cell is assembled, the Teflon sheet is sandwiched by the "O" rings between the two cell chambers which are in turn held together by clamps.

On the face of each chamber opposite the side sandwiching the Teflon sheet there is a 2.54 cm hole. A circular glass window with 2.54 cm diameter is pressed fit into each chamber. This permits the membrane formation to be viewed with a microscope through the front window when illuminated through the rear window.

The electrodes used for the electrical measurements of membranes should be nonpolarizable to ensure that the voltage measured (or applied) mainly occurs across the membrane. The two types of electrodes most commonly employed are silver-silver chloride and calomel.

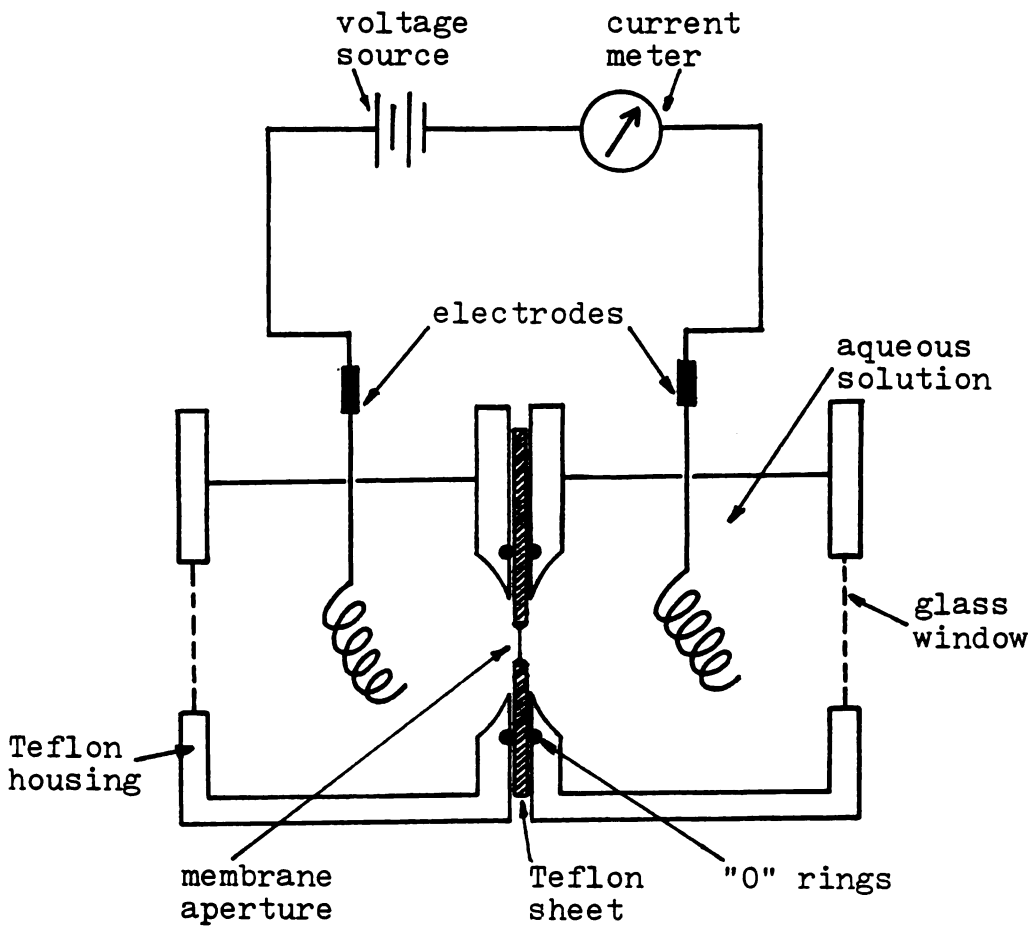


Figure 1-3. Cell for the electrochemical study of bilayer lipid membranes.

Membrane Conductance

Membrane conductance is usually measured by impressing a small voltage across a bilayer lipid membrane via a pair of nonpolarizable electrodes. Under a low applied voltage (about 50 mV), Ohm's law is generally applicable. Therefore, the membrane conductance can be evaluated from the known applied voltage, V in volts, and the current, I measured in amperes. In equation form, this is expressed as

$$\text{Conductance} = 1/R = I/V$$

Experimentally, a simple set up, as shown in Figure 1-3, is most often used to attain an accurate, fast determination of the current at a known value of the potential across the membrane.

The solution and electrode resistance (approximately 10^4 ohms with an aperture in place) is usually negligible with respect to the membrane resistance which is in the range of 10^6-10^8 ohm-cm^2. This means that essentially the entire voltage applied to the pair of nonpolarizable electrodes appears across the membrane. Because the area of a bilayer lipid membrane is often small (about 0.01 cm^2), even with a membrane resistance of 10^4ohm-cm^2 the error is approximately 1 % (assuming a value of 10^4 ohms for the resistance of both solution and electrodes). In some cases, the membrane resistance is much greater than 10^8 ohm-cm^2 (such as membranes formed from oxidized cholesterol/n-decane solution). The current through these membranes is extremely

small which requires the use of an extremely sensitive current meter. Generally speaking, the current through any of the membranes studied under the condition when the Ohm's law is applicable is very small. Thus an electronic picoammeter designed around an electrometer amplifier with a very high input impedance is generally required for the current readout.

Membrane Capacitance

An unmodified bilayer lipid membrane can be regarded as a three-layered structure with a hydrocarbon portion in the center and two polar layers adjacent to it on both sides. The analysis of its capacitive behavior can therefore be carried out in terms of a model of three capacitors in series. Because of the relatively large dielectric constant and the small thickness of the polar regions (19-23), it is true in most cases that the central hydrocarbon region is the only factor which contributes significantly to the membrane capacitance (20). A simplified, equivalent circuit for an unmodified membrane as shown in Figure 1-4 is thus useful.

A simplified schematic of the ac bridge designed by White et al. (24) for the measurement of the membrane capacitance is given in Figure 1-5. At balance, the membrane capacitance can be evaluated using the expression.

$$C_m = (R_1/R_2)\, C_k$$

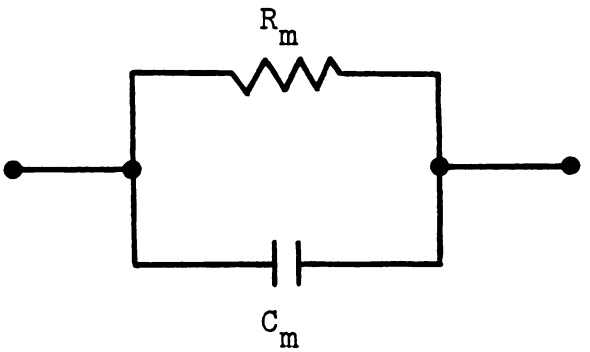


Figure 1-4. A simplified, equivalent circuit of an unmodified bilayer lipid membrane.

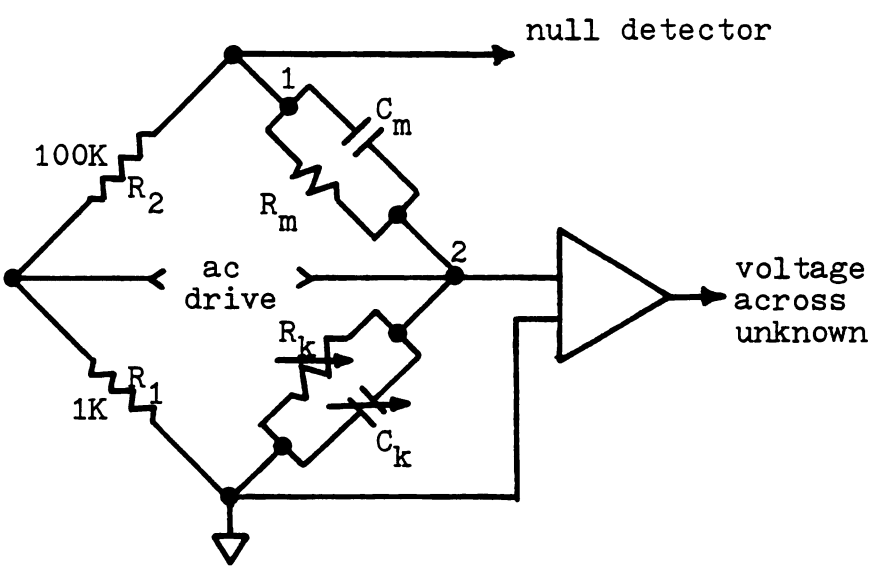


Figure 1-5. A simplified ac bridge circuit (by S. H. White) for the measurement of membrane capacitance.

Where R_1 = 1 kΩ, R_2= 100 kΩ, and C_k is the balance value. Also at balance, terminal 1 is at a virtual ground so that the amplifier at terminal 2 measures the ac signal appearing across the membrane. The bridge is excited via a photocoupled isolator which introduces minimal stray impedances and acts as a nearly ideal transformer. This ac bridge for the measurement of the membrane capacitance has been reported capable of obtaining a value of the membrane capacitance with a nominal accuracy of 0.05 % between the frequencies of 100 Hz and 10 KHz (24). The required voltage across the membrane is only 7 mV (rms).

A dc transient technique used by Montal et al. (25) for the measurement of the membrane capacitance is shown in Figure 1-6.

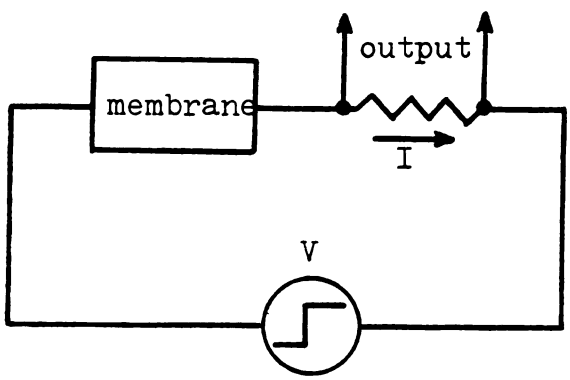


Figure 1-6. A schematic circuit for the measurement of the membrane capacitance by the dc transient method.

A potential step is applied across the series combination of the membrane and a resistor, and the voltage across the latter (proportional to the current) is monitored by means of an oscilloscope. Thus

$$C_m = q/V = \frac{1}{V}\int_0^\infty I\ dt$$

Where q is the charge which is stored in the membrane capacitor under the applied potential, V. The integral $\int_0^\infty I\ dt$ is obtained by the graphical estimation of the area under the value of the current versus time.

Although easy and relatively cheap to set up, the dc transient method tends to be less accurate than the ac method. Thus unless signal averaging techniques are employed, the integration procedure, or the evaluation of the relaxation time of the current transient, introduces uncertainties which can be greater than 1 %.

Membrane Thickness

The thickness of the membrane is an important piece of information in membrane studies. The measurement of the membrane capacitance permits the calculation of the membrane thickness according to the equation given below.

$$C_m = C_t/A_m = e_o e_m/d_m$$

Where C_m is the specific membrane capacitance, C_t is the membrane capacitance measured, e_o is 8.854×10^{-14} F/cm, e_m is the membrane dielectric constant, d_m is the membrane thickness, and A_m is the area of the bilayer portion of the membrane. A measurement of the membrane area within

1 % accuracy can be obtained if the circular membrane diameter is greater than 1 mm. The membrane dielectric constant can be approximated without a significant error by using that of an appropriate hydrocarbon, which is about 2.1 (26).

Measurement of the Membrane Capacitance by a Computer-controlled Charge Injection Technique

Principles

An unmodified bilayer membrane has a resistance value in the range of 10^6-10^8 ohm-cm^2. Therefore, charge injected onto the membrane capacitance will leak off very slowly if the conductance through the membrane is the only discharge pathway. This property makes it possible to measure the membrane capacitance by placing an external precision resistor between the electrodes, charging the membrane, and determining the time constant of the voltage decay through the known external resistance.

When an external resistor is placed between the electrodes, an equivalent circuit can be depicted as shown in Figure 1-7(a). A charge pulse (see Figure 1-7(b)) is injected into the membrane through both the electrode and solution resistances (R_f + R_s) in a very short time, t_p (usually 50-100 ns). The resulting voltage decay (see Figure 1-7(c)) is followed. The data is then linearized to acquire the slope which is equal to $-1/RC_m$ (see Figure 1-7(d)). Since the discharge pathway is through

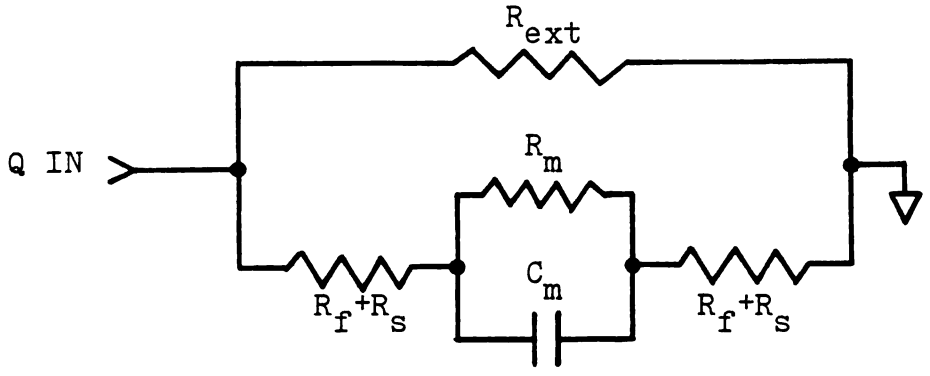


Figure 1-7(a). Equivalent circuit when an external resistor is placed across the membrane between two electrodes.

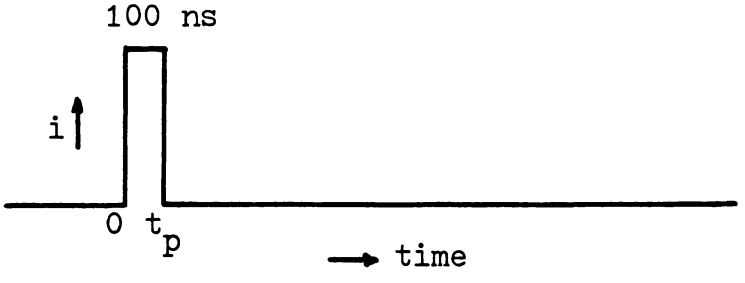


Figure 1-7(b). A short constant current pulse. $Q_{total}=it_p$.

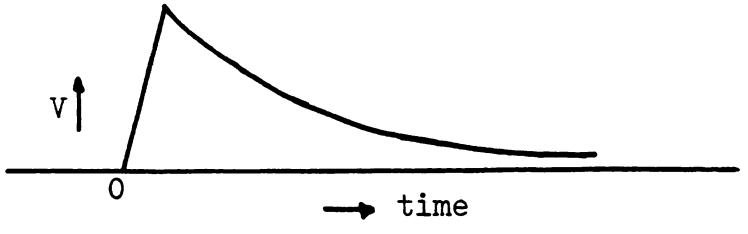


Figure 1-7(c). An idealized voltage decay.

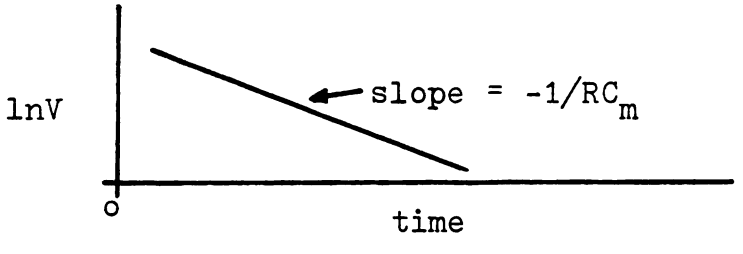


Figure 1-7(d). A lnV versus t straight line. If $(R_f+R_s) \ll R_{ext}$, $R = 2(R_f+R_s) + R_{ext} \approx R_{ext}$ and the membrane capacitance can be obtained from the slope.

$R_f + R_s + R_{ext}$, $(R_f + R_s)$ must be small relative to R_{ext} in order for the simplification to be made (i.e. $R = R_{ext}$ if $R_{ext} \gg (R_f + R_s)$). This can be accomplished through the use of high concentration of salt solution and large area electrodes.

Instrument

A commercial pulse generator (Chronetics PG-33 model) was used for this study. This pulse generator has an output voltage compliance of 12 volts, and a rise time of 6 ns maximum. The device is capable of driving 200 mA into 50 ohms, and can be triggered externally.

A schematic of the cell amplifier is shown in Figure 1-8. The amplifier is placed as close to the electrodes as possible in order to minimize capacitance and stray signal pickup in the connecting wires. The input signal comes from the pulse generator via a coaxial cable. The charge pulse travels through the solution and charges the membrane which is situated between the two electrodes. The voltage transient generated is amplified by the LH0032 FET input amplifier to obtain a voltage excursion in the range of 0-4 volts at the output. The diodes (IN 914 and FD300 in series) prevent the voltage at the input of the amplifier from exceeding approximately 0.7 volts which in turn, avoids the saturation of the amplifier during the pulse application. The 10 kΩ resistor was included to ensure that the major current pathway for the charge pulse remains the membrane pathway. The diodes used for input protection

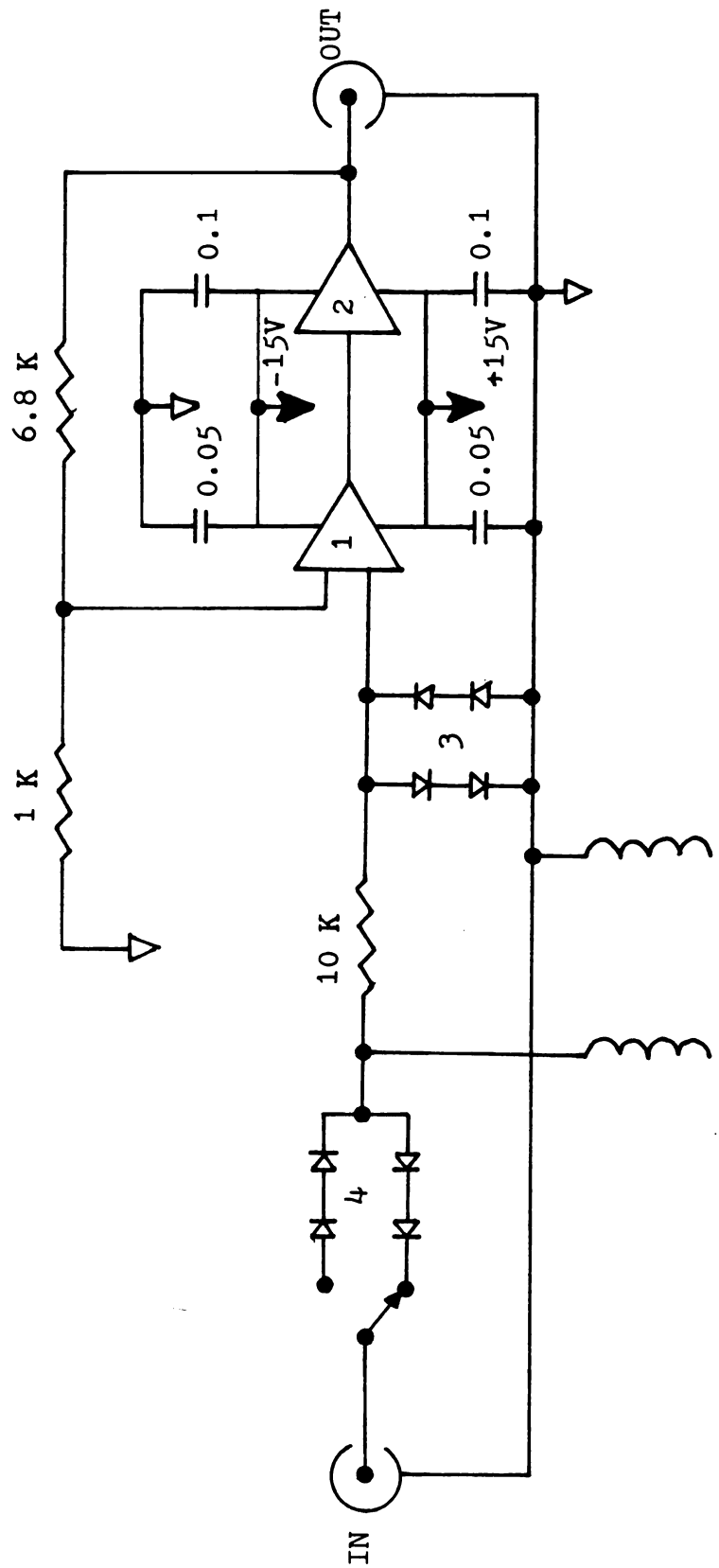


Figure 1-8. Schematic of cell amplifier.
1: LH0032, 2: LH0063, 3: 2x IN914 & FD300 series,
4: 2x In5317 & FD300 in series. Capacitances are in uF.

as well as reverse blocking were chosen in combination to yield fast switching characteristics as well as low leakage when in the "OFF" state. The output signal is buffered by an LH0036 cable driver (terminated with 50 ohms) to the transient recorder.

A fast transient recorder (27), which employs a temporary analog storage register serves for the recording of a very fast transient signal. The analog signal is then converted into digital form and stored in the memory of a PDP 11/40 minicomputer. The transient recorder is capable of operating at a 10 MHz sampling rate.

The program handling the data collection, baseline subtraction, and data storage in the floppy diskette called "MEMBRN.FTN" was developed by Last (28) from this laboratory. The corrected data was then fit into another program called "EXPFIT.FTN" to obtain the membrane capacitance. The program, "EXPFIT.FTN", essentially linearizes the experimental data, calculates the proper weighing coefficients, and then calls a weighted linear least squares fitting subroutines.

CHAPTER II

EFFECT OF SOLVENTS AND LIPID CONCENTRATIONS ON THE CAPACITANCE AND THICKNESS OF GLYCERYL MONOOLEATE BILAYER MEMBRANES

Introduction

Glyceryl monooleate yields well defined systems which are in many ways easier to study quantitatively than phospholipids (7,29). Membrane capacitance is an important electrical property and its measurement provides us with information about the thickness, and indirectly, about the composition and structure of bilayer lipid membranes. Studies of the thickness of glyceryl monooleate bilayer membranes by means of capacitance measurement constitute part of this work.

Experimental

Materials

Unless otherwise stated, the membrane-forming solution was made by dispersing the proper amount of glyceryl monooleate (K & K) into appropriate solvents. N-decane, n-octane and n-hexadecane (Aldrich), n-dodecane, n-tetradecane and squalene (Sigma), Squalane (Matheson, Coleman & Bell), paraffin oil (J. T. Baker), and benzyl alcohol (Fisher Scientific) were used without further purification. Cholesterol (Fisher Scientific) was recrystallized twice from absolute alcohol. The aqueous solution was 3 M

lithium chloride which was of analytical grade. The water used to prepare the aqueous solutions was deionized.

Methods

The membrane was supported by a 1.5 mm diameter hole drilled in a Teflon partition which was clamped between two symmetric Teflon chambers containing the aqueous solutions. The membrane was formed across the aperture by forming a bubble of lipid at the end of a Pasteur pipette under the surface of the aqueous solution and applying the bubble to the aperture. The membrane size was determined using a microscope reticule. The data were taken 10 minutes after the completion of thinning. The membrane capacitance was measured using a charge injection technique through a pair of nonpolarizable silver/silver chloride electrodes. This technique has been employed for study of electrochemical systems in the measurement of electrical double layer capacitance on the electrode (30) as described in the "INTRODUCTION" chapter. All measurements were performed at temperature $24 \pm 1\ ^{o}C$.

Results and Discussion

Capacitance and Thickness of Glyceryl Monooleate Bilayer Membranes in a Single Solvent System

Glyceryl monooleate, when dissolved in n-alkanes, forms membranes although glyceryl monooleate itself does not form bilayer membranes in water. The extent of the adsorption

of the alkane in the membrane interior depends on the chain length of the alkanes. Since the area per molecule of the lipids is not appreciably affected (31,32), the adsorption of n-alkane would mainly affect the thickness of the hydrocarbon region of the bilayer. Therefore, the thickness of a bilayer membrane reflects the volume of solvent within a bilayer membrane. The thickness of a bilayer membrane can usually be estimated by the measurement of the bilayer specific capacitance using the equation, $d = e_o e_m / C_m$, where e_o is 8.85×10^{-14} F/cm, e_m is estimated to be 2.1 (the dielectric constant of lipid acyl chain equivalent to hydrocarbon (26)), and C_m is measured experimentally.

Both the capacitance and thickness of bilayer membranes formed from glyceryl monooleate dissolved in various n-alkanes have been systematically studied by Fettiplace et al. (7) and White (33). For the purpose of comparison, their results along with ours are given in Table 2-1. As noted, our results are generally quite in good agreement with those obtained by previous workers. Furthermore, the volume fraction of lipid in a bilayer membrane can be estimated using the equation, volume fraction (VF) = $2V_a/dA_a$, where V_a is the partial molar volume of the acyl chain in the bilayer, d is the thickness of the bilayer membrane, and A_a is the area of each lipid molecule at the interface. The density of the components in the bilayer may similarly be interpolated from those for the bulk hydrocarbon. The

Table 2-1. Capacitance and thickness of bilayer membranes formed from glyceryl monooleate dissolved in various n-alkanes and squalene.

solvents	*Fettiplace (32) C_m, nF/cm^2	d, Å	*White (33, 35) C_m, nF/cm^2	d, Å	***This Work C_m, nF/cm^2	d, Å
n-heptane	389 ± 3	47.8±0.3	--	--	414 ± 4	44.9 ± 0.4
n-Octane	--	--	396 ± 3	46.9±0.4	406 ± 8	45.8 ± 0.4
n-decane	383 ± 3	48.5±0.4	385 ± 3	48.3±0.4	405 ± 6	45.9 ± 0.5
n-dodecane	--	--	388 ± 4	47.9±0.5	419 ± 6	44.4 ± 0.4
n-tetradecane	465 ± 4	40.0±0.4	422 ± 3	44.0±0.4	474 ± 8	39.2 ± 0.4
n-hexadecane	580 ± 4	32.0±0.3	543 ± 4	34.2±0.3	595 ± 5	31.2 ± 0.3
n-octadecane	--	--	641 ± 7	29.0±0.3	--	--
squalene (at 24 °C)	--	--	777 ± 5	23.9±0.2	780 ± 8	23.8 ± 0.2

*Membranes were formed from a 3.5 mg glyceryl monooleate per ml of alkane solution in 0.1 M NaCl and at 21 °C.
**Membranes werw formed from a 10 mg glyceryl monooleate per ml of alkane solution in 0.1 M NaCl and at 30 °C.
***Membranes were formed from a 10 mg glyceryl monooleate per ml of alkane solution in 3 M LiCl and at 24 °C.

main justification is that, in micelles of alkyl chain surfactants, the hydrocarbon interior has effectively the same density as in the bulk (34). Therefore, were 1-heptadecene assumed to be equivalent to the acyl chain of glyceryl monooleate, the partial molar volume of lipid acyl chain could then be evaluated from the equation, $V_a = M/\rho N$, to attain a value of 475×10^{-24} cm^3 (31,32); where M is the molecular weight of 1-heptadecene, ρ is the density of 1-heptadecene in the bulk, and N is the Avogadro's number. The area of lipid molecule at the interface, A_a, has been estimated using the adsorption isotherm technique to obtain values between ca. 37.5 and 39.5 $Å^2$ in various alkanes (31,32). More recently, White (36) obtained a value of 38.3 $Å^2$ for bilayer membranes formed from glyceryl monooleate dissolved in squalene. All these values are considered to be quite close; we have chosen to use 38.5 $Å^2$ in our calculations for the volume fractions. Both the volume fraction of lipid and solvent are given in Table 2-2. The values for the volume fraction (VF) of the solvent is obtained by 1 - VF of lipid. A zero volume of mixing of solvent and lipid chains is also assumed, but this is unlikely to introduce a significant error (7). Figures 2-1 & 2-2 again indicate that both the thickness of the bilayer and the volume of solvent in the bilayer drastically decrease as the number of carbon atoms for the solvent increases. Therefore, it should be expected that a bilayer membrane with little or no solvent can be formed by the conventional Mueller-Rudin

Table 2-2. Estimates of volume fraction (VF) of lipid and various solvents in glyceryl monooleate bilayer membranes. The volume fraction of lipid in the bilayer is calculated by using the equation, $VF = 2V_a/dA_a$, where $V_a = 475 \times 10^{-24}$ cm^3 and A_a is assumed to be 38.5×10^{-16} cm^2.

solvents	C_m, nF/cm^2	d, Å	VF (lipid)	VF (solvent)
n-heptane	414	44.9	0.55	0.45
n-octane	406	45.8	0.54	0.46
n-decane	405	45.9	0.54	0.46
n-dodecane	419	44.4	0.56	0.44
n-tetradecane	474	39.2	0.63	0.37
n-hexadecane	595	31.2	0.79	0.21
n-octadecane from White(35)	641	29.0	0.85	0.15
squalene	780	23.8	1.04	~0.00

*Membranes were formed from a 10 mg per ml of alkane solution in 3 M aqueous phase at 24 ± 1 °C.

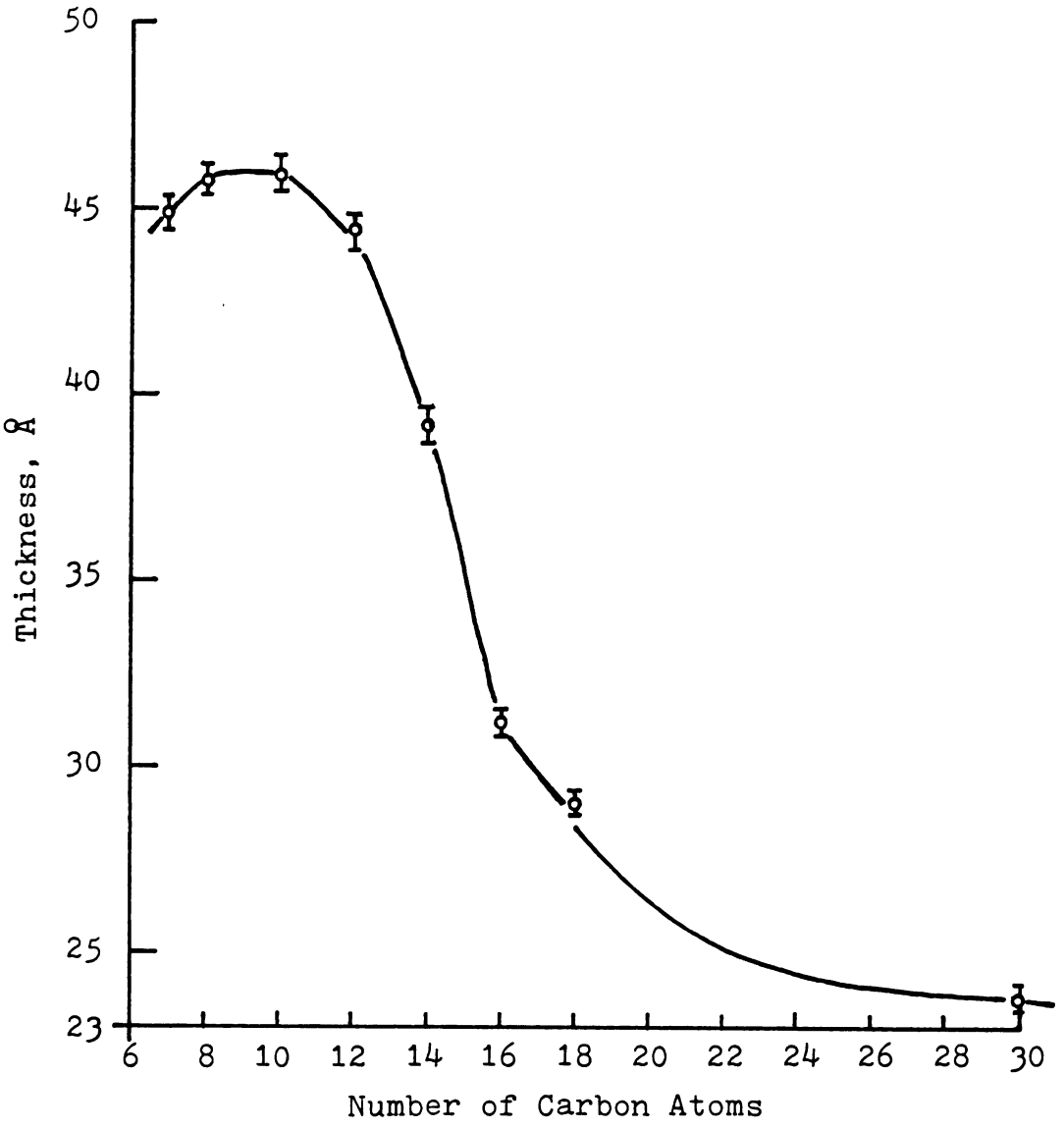


Figure 2-1. Capacitance and thickness of bilayer lipid membranes formed from glyceryl monooleate dissolved in various n-alkanes and squalene.

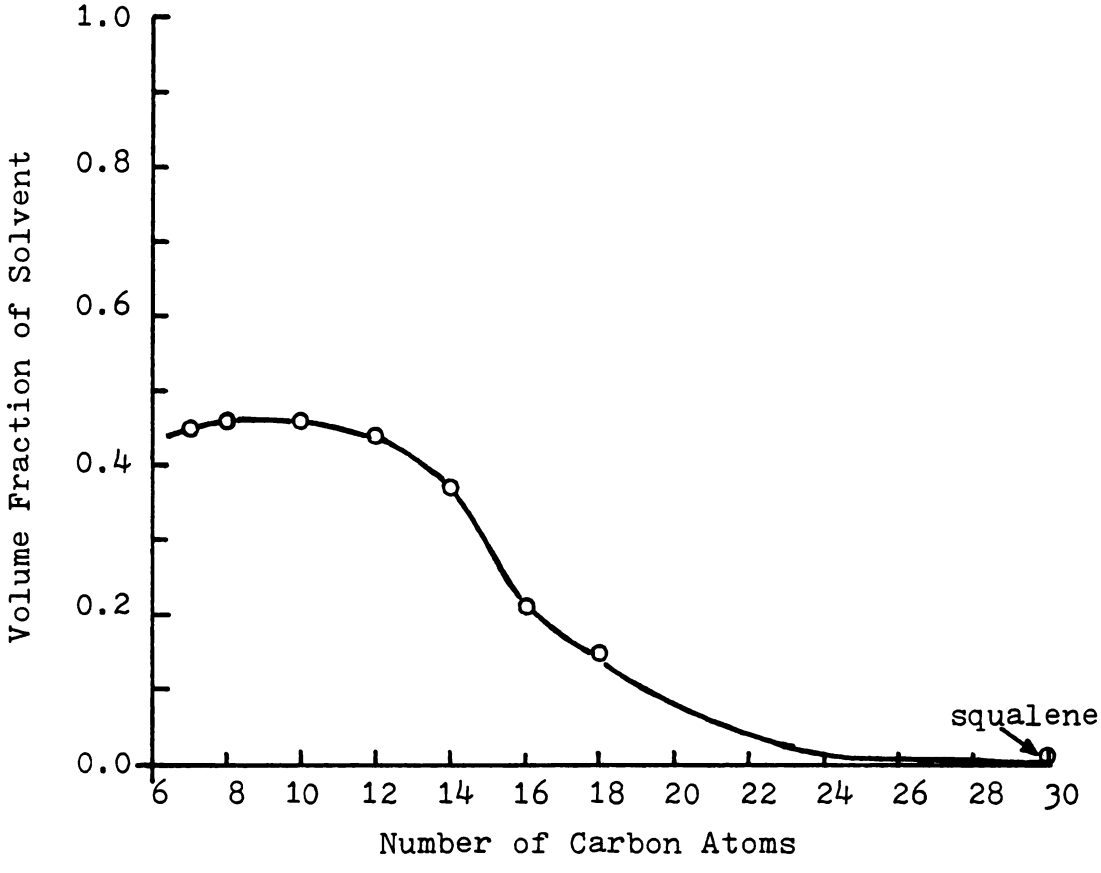


Figure 2-2. Volume fraction of the alkane solvent and squalene which is present in the bilayer region. Note that the volume fraction decreases drastically to very low value at high carbon numbers of alkane.

technique if a hydrocarbon solvent with a high enough carbon number can be found which is still a liquid under normal experimental conditions.

Effect of Lipid Concentration on the Capacitance and Thickness of Glyceryl Monooleate Bilayer Membranes

Because of relatively short thinning time in the formation of bilayer membranes, earlier workers made very dilute membrane-forming lipid solutions near the critical micelle concentration (for example, 1.75 mM for n-decane) above which the lipid content of the bilayer membrane is expected to remain constant (31). Recently, Waldbillig et al. (36) measured the capacitances of membranes formed from glyceryl monooleate dissolved in n-hexane and n-decane, respectively, at various lipid concentrations. They observed a linear dependence of membrane capacitance on the lipid concentration and further took advantage of this property to form solvent-depleted bilayer membranes by preparing a very highly concentrated membrane-forming solution.

It is well-established that the volume of solvent within the bilayer can be estimated by the measurement of the membrane capacitance (32,37-39) and this is also demonstrated in the previous section. This approach is employed here to evaluate the distribution of solvent between the bilayer and torus at various lipid concentrations. The solvent systems chosen in the work by Waldbillig et al. (36) are both n-hexane and n-decane. As one may notice, the

membranes formed from glyceryl monooleate dissolved in both solvents contain relatively large amounts of solvent. This study extends the observation of the solvent distribution to include membrane systems that include less solvent. The three solvent systems studied are n-decane, n-hexadecane and squalene (see Figure 2-3 for the structure). With a concentration of 10 mg/ml of glyceryl monooleate for each solvent, the volume fractions of solvent that remained in the bilayer are 0.46 for n-decane, 0.21 for n-hexadecane, and about 0.00 for squalene. As seen in Figure 2-4, in all three solvent systems a linear relationship of membrane capacitance versus lipid concentration is observed, while the rate of change of membrane capacitance versus lipid concentration (i.e. the sensitivity) appears to be different. The concentration effect is greater in the case of n-decane

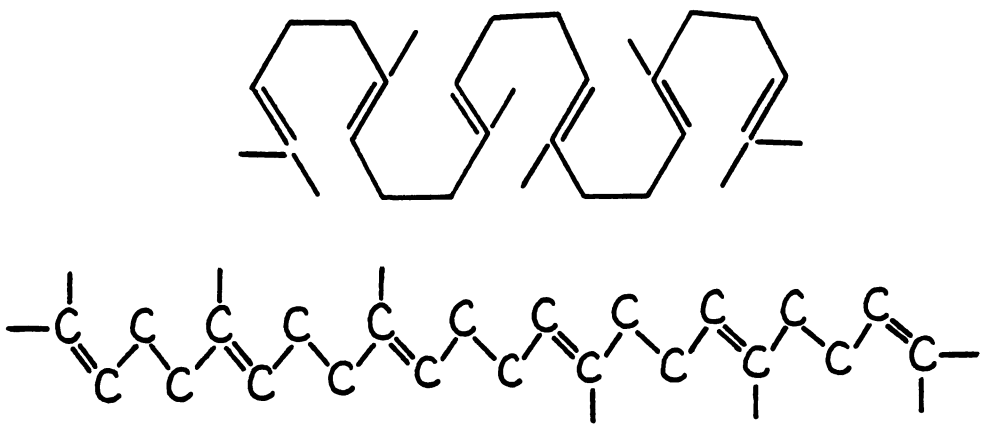


Figure 2-3. A picture of the molecule squalene. Squalene ($C_{30}H_{50}$) is a liquid at room temperature. It has a density of 0.8584 g/cm^3 and an extended length of about 29 Å. Unlike cholesterol, squalene lacks a hydroxyl group, which may anchor it to the interface. Note that the rings are not closed.

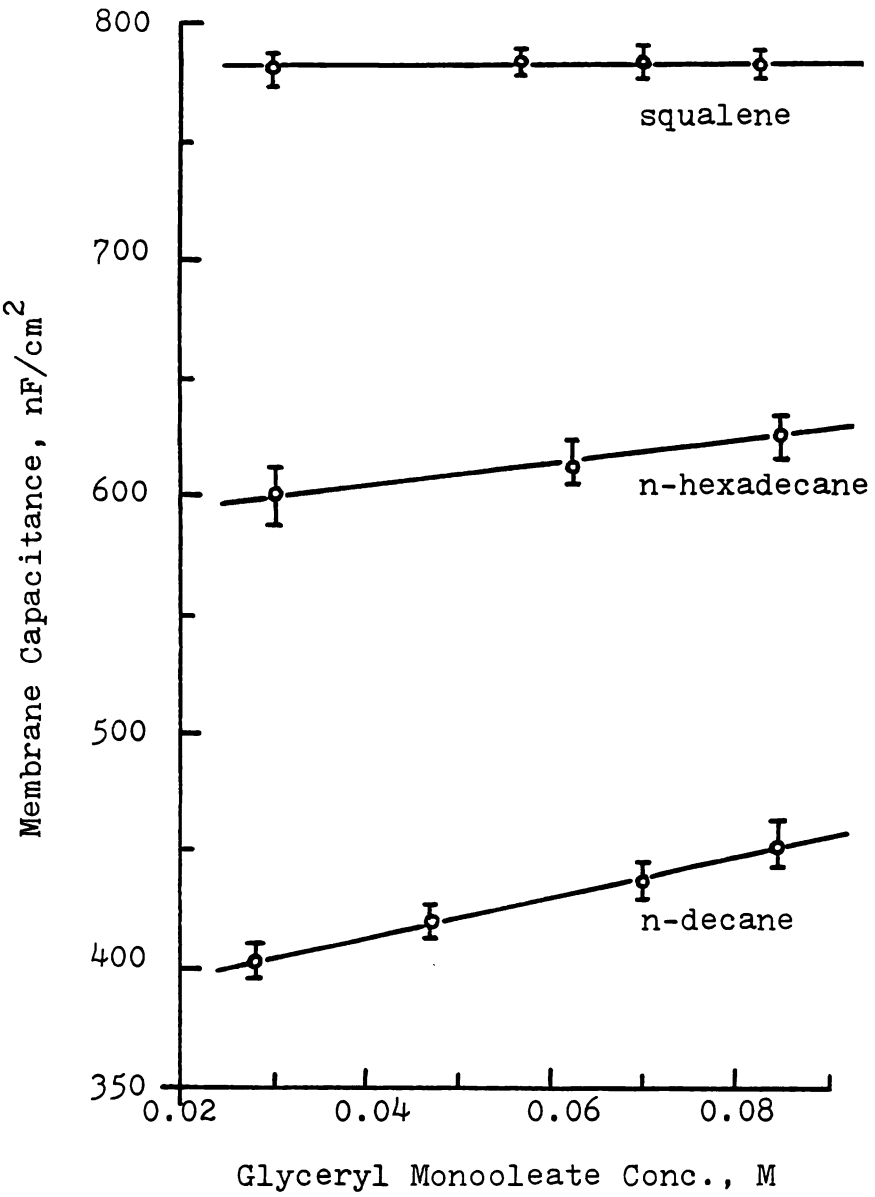


Figure 2-4. Capacitances of bilayer lipid membranes formed from appropriate amounts of glyceryl monooleate dispersed in n-decane, n-hexadecane and squalene, respectively, as a function of glyceryl monooleate concentration. The aqueous solution was unbuffered 3 M LiCl and the temperature was 24 ± 1 °C. Note that in squalene system the membrane capacitance is independent of glyceryl monooleate concentration.

than in n-hexadecane. In the case of squalene, the membrane capacitance remains constant and does not increase with increasing lipid concentration, i.e. the sensitivity equals zero. As in n-decane and n-hexadecane systems, the increase of membrane capacitance with lipid molarity implies that membranes formed from concentrated lipid solutions are thinner and thus contain less solvent. Apparently, the solvent content of the bilayer is primarily determined by the solvent content of the membrane-forming solution. The linearity of membrane capacitance on lipid concentration allows us to synthesize different thickness of membranes conveniently by preparing the appropriate concentrations of lipid solutions. The different sensitivities indicate that the effect of lipid concentration on the membrane capacitance depends on the volume of solvent in the bilayer. For the n-hexadecane system, the volume of solvent is less than that for the n-decane system and therefore, a lower sensitivity in n-hexadecane system is observed. For the squalene system, the membrane capacitance is essentially solvent-free (35) and thus the membrane capacitance is independent of lipid concentration.

In summary, it appears to us that the linear dependence of membrane capacitance on the lipid concentration apparently is a characteristics for those membrane systems with appreciable amounts of solvent remaining in the bilayer. Moreover, the linear dependence of membrane capacitance on the lipid concentration such as n-decane and n-hexadecane

systems provides a very convenient way to form membranes with different thicknesses by preparing lipid solutions of the appropriate concentrations. For a membrane system with little or no solvent such as membranes formed from lipid dissolved in squalene, the membrane capacitance is independent of lipid concentration.

Development of Nearly Solvent-free Glyceryl Monooleate Bilayer Membranes by Conventional Mueller and Rudin Technique (5)

The solvents commonly used in the formation of bilayer membranes are normally not present in biological membranes (40). The presence of the solvent does not prevent the use of such membrane systems for studies of physicochemical activities in many cases. However, some problems arising from the presence of solvents in the bilayer have been reported (41). In an effort to remove the difficulty caused by the presence of solvents in the bilayer. Montal et al. (42) modified the technique by Takagi et al. (43) to form bilayer membranes from lipid monolayers. This method suffers the disadvantages of (a) a complicated formation procedure; (b) the use of a smaller hole (ca. 0.1-0.2 mm in diameter) (7); and (c) the requirement of a nonpolar solvent such as petroleum jelly to form the torus in order to stabilize the membrane (44,45). Although this method has disadvantages, it should still be credited for being able to form asymmetric membranes which are assembled by two different monolayers from different kinds of lipids.

Further, White (46) made solvent-free membranes using a solvent "freeze-out" technique by lowering the temperature to below the melting point of the solvent so that the solvent is frozen out into the torus while the bilayer still remains at liquid-crystalline state. Waldbillig et al. (36) made solvent-depleted membranes using very highly concentrated lipid solutions. More recently, the property of large molecule insolubility in the bilayer (47) has been employed to form solvent-free glyceryl monooleate bilayer membranes (35). Apparently, this latest technique offers the easiest way to form bilayer membranes with little or no solvent.

The amount of the solvent in the bilayer decreases drastically with increasing number of carbon atoms of the alkanes used as solvents in the preparation of membrane-forming solutions as demonstrated in the previous section. This approach can be advantageous in forming nearly solvent-free bilayer membranes by selecting a large, long-chain, and nonpolar hydrocarbon as the solvent for a membrane-forming solution. Hydrocarbons which are large, long-chain, and simultaneously exist as liquid states at normal conditions are rare. After a careful examination, we decided to investigate squalane ($C_{30}H_{62}$), a saturated hydrocarbon with six methyl groups at side chains, and paraffin oil, a long chain hydrocarbon mixture, as solvents for glyceryl monooleate bilayer membranes.

At a concentration of 10 mg glyceryl monooleate per ml

of the solvent, the capacitance values of the membranes are 750 nF/cm^2 when squalane is the solvent, and 790 nF/cm^2 in the case of paraffin oil. As noted in Table 2-3, these capacitance values are comparable with those nearly solvent-free membranes formed from other techniques. The high value of the capacitance suggests that both squalane and paraffin oil can be used as solvents in yielding membranes which are virtually solvent-free.

Moreover, the capacitances of bilayer membranes formed by dissolving lipid in either squalane or paraffin oil at various lipid concentrations have been studied. The results, as seen in Table 2-4 and Figure 2-5, do not show a lipid concentration dependence. This independence of membrane capacitances versus lipid concentration is consistent with the observation made from studies earlier -- a bilayer lipid membrane with the presence of solvent in the bilayer region, such as in the case of lipid/n-decane, gives a linear, increasing dependence of membrane capacitances versus a increase of lipid concentrations, while a bilayer membrane with little or no solvent such as in the case of lipid/squalene does not. One may also note in Figure 2-5, there are differences in the membrane capacitances among three solvent systems. One possible, but not unlikely explanation is that the membrane formed from these solvent system may not actually be entirely solvent-free. The difference in capacitance values may be due to the undetectable trace amount of solvent remaining in the bilayer and this amount

Table 2-3. Comparison of capacitance values for virtually solvent-free glyceryl monooleate bilayer membranes formed using various techniques.

methods	capacitance, uF/cm^2	reference
monolayer	0.75-0.81	Montal et al. (42)
solvent freeze-out	0.790	White (46)
dispersed in squalene	0.777	White (35)
dispersed in paraffin oil	0.790	this work
dispersed in squalane	0.750	this work
dispersed in squalene	0.780	this work

Table 2-4. Capacitance values of glyceryl monooleate (GMO) bilayer membranes at various lipid concentrations in paraffin oil, squalane, and squalene solvent systems, respectively.

Paraffin oil		Squalane		Squalene	
GMO conc., M	C_m, nF/cm^2	GMO conc., M	C_m, nF/cm^2	GMO conc., M	C_m, nF/cm^2
0.029	790 ± 5	0.028	752 ± 7	0.030	781 ± 8
0.056	790 ± 7	0.056	750 ± 6	0.057	783 ± 7
0.072	790 ± 8	0.070	750 ± 7	0.070	780 ± 7
0.084	790 ± 6	0.084	749 ± 8	0.083	779 ± 6

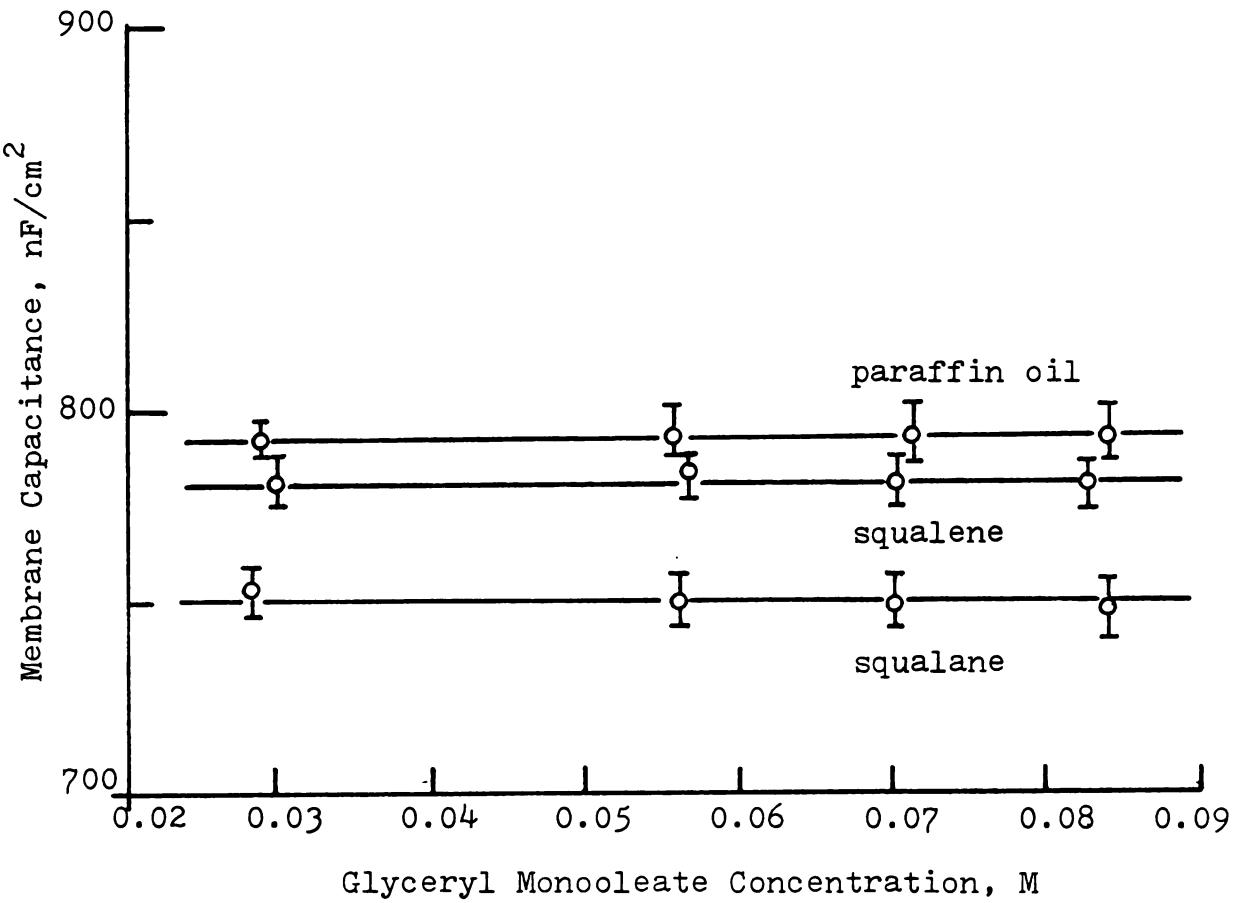


Figure 2-5. The capacitances of bilayer lipid membranes formed from appropriate amounts of glyceryl monooleate dispersed in squalene, squalane, and paraffin oil, as a function of glyceryl monooleate concentration. The aqueous solution was unbuffered 3 M LiCl and the temperatures were 24±1 °C. Note that in all three solvent systems, membrane capacitance is independent of glyceryl monooleate concentration because of the absence of solvent in the bilayer.

of solvent may depend upon the solvent chosen, thus affecting the capacitance values to a different degree. However, these differences in membrane capacitances are within 6 % at maximum.

In summary, a large, long-chain, and nonpolar hydrocarbon can be employed in the membrane-forming solution. A bilayer membrane with little or no solvent is then able to be formed using this lipid solution by the conventional Mueller and Rudin technique. We have extended these solvents to include squalane and paraffin oil. Again, as in squalene (35), it always takes a much longer time to begin the thinning process with these solvents than with the analogous short alkanes. This is attributed to the increased bulk viscosities that these solvents possess and the resulting slower development of the pressure gradient which is responsible for the initiation of the thinning process.

Capacitance and Thickness of Glyceryl Monooleate Bilayer Lipid Membranes in Binary Solvent Systems

The hydrocarbon solvents present in synthetic bilayer lipid membranes are not present in biological membranes (40). Since the use of a single solvent in the formation of bilayer lipid membranes is already troublesome, what merit would the study of bilayer lipid membrane in binary solvents have ? The interesting behavior observed in the following studies gives the best answer to this

question.

Two binary solvent systems employed in the preparation of membrane-forming solutions are n-decane/n-hexadecane and n-decane/squalene. When membrane capacitances versus bulk solvent volume fractions are plotted, two very different kinds of results are observed as seen in Figure 2-6. For n-decane/n-hexadecane system, a linear relationship is obtained, while the membrane capacitance versus bulk solvent volume fraction for n-decane/squalene system is non-linear. This difference may be explained in terms of closeness of molecular size, structure and the underlying solubility in the bilayer for both n-decane and n-hexadecane relative to those for both n-decane and squalene.

The use of n-decane/n-hexadecane offers a way to make any desired thickness of membranes ranging from 3.1 nm to 4.6 nm. An empirical equation can be derived for the convenience of membrane preparation using these binary solvents.

$$C_m = C^o_{m,d} V_d + C^o_{m,h} V_h$$

$$= C^o_{m,d} V_d + C^o_{m,h} (1 - V_d)$$

$$= C^o_{m,h} + (C^o_{m,d} - C^o_{m,h})\, V_d$$

where $V_d + V_h = 1$

apply $C = e_o e/d$

$$e_o e/d_m = e_o e_{m,h}/d_{m,h} + (e_o e_{m,d}/d_{m,d} - e_o e_{m,h}/d_{m,h})\, V_d$$

since $e_m = e_{m,h} = e_{m,d}$

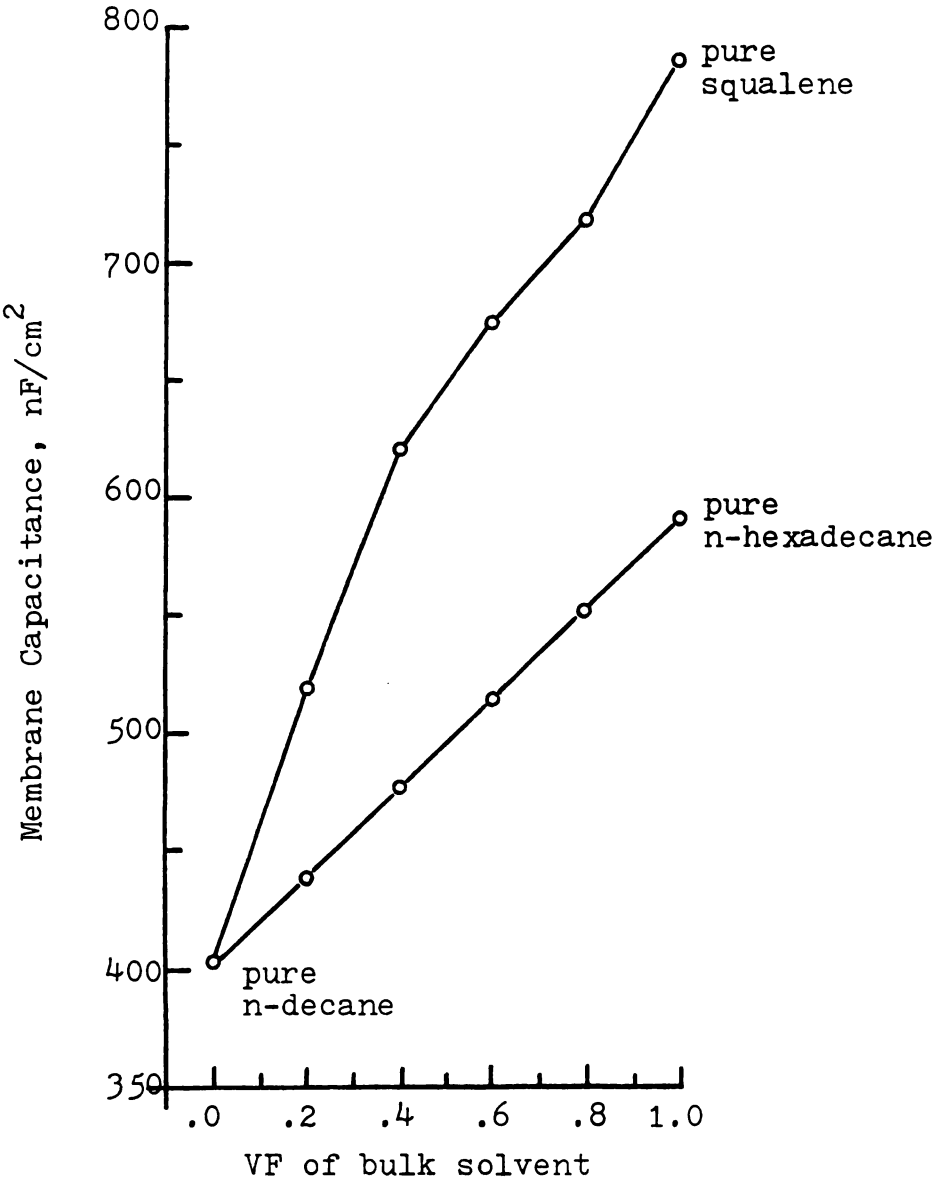


Figure 2-6. Capacitances of bilayer lipid membranes formed from dispersing 100 mg glyceryl monooleate in 10 ml mixture solvents. As noted, a linear dependence of membrane capacitance upon the volume fraction is observed for the n-decane/ n-hexadecane system while a nonlinear relationship is seen for the n-decane/squalene system.

$$1/d_m = 1/d_{m,h} + (1/d_{m,d} - 1/d_{m,h})\ V_d$$

where V_d : bulk volume fraction of n-decane.

V_h : bulk volume fraction of n-hexadecane.

C_m : membrane capacitance in n-decane/n-hexadecane.

$C^o_{m,d}$: membrane capacitance in pure n-decane.

$C^o_{m,h}$: membrane capacitance in pure n-hexadecane.

e_o : 8.854×10^{-14} F/cm.

e_m : membrane dielectric constant, ca. 2.1.

$e_{m,d}$: dielectric constant of pure n-decane.

$e_{m,h}$: dielectric constant of pure n-hexadecane.

d_m : membrane thickness in binary solvents.

$d_{m,d}$: membrane thickness in pure n-decane.

$d_{m,h}$: membrane thickness in pure n-hexadecane.

By varying the bulk volume fraction of n-decane, V_d, different thicknesses of membranes can be made. On the other hand, although no simple relationship between membrane capacitance and bulk solvent volume fraction can be derived for n-decane/squalene system, the preparation of any desired thickness of membranes between 2.4 nm to 4.6 nm is demonstrated.

In summary, the use of binary solvents permits the synthesis of membranes of any desired thickness, instead of the fixed, intermittent values of thickness obtained by varying the hydrocarbon chain length from n-octane to n-hexadecane in single solvent systems.

Future Work

The ion transport rate across the bilayer lipid membrane is an interesting topic that has been extensively studied (13). The effect of membrane thickness on the electrical conductance has been reported in studies such as (a) ion transport using the pore mechanism such as o-pyromellithyl-gramicidin channels (48); (b) ion transport using the carrier mechanism such as valinomycin carriers (49-52); and (c) direct ion transport such as dipicrylamine (53,54), tetraphenylborate,tetraphenylarsonium, and tetraphenylphosphonium ions (55). In all these experimental results, it is observed that the conductance has decreased as membrane thickness increased, although discrepancies exist between theoretical and experimental results.

The thickness of the hydrocarbon core of the membrane has been changed in two different ways; (a) varying the lipid chain length; and (b) varying the solvent chain length. The membrane thickness obtained from both methods would be expected to be fixed and intermittent, instead of continuous. Furthermore, the discrepancies of experimental results mentioned above led Hladky (56) to conclude that membrane thickness is not the only variable of importance in electrical conductance. Apparently, both ways of changing membrane thickness would drastically change the orderliness of the membrane core due to different structures of either lipids or solvents as observed by McIntosh et al. (57), and this would reasonably be expected

to lead to changes in electrical conductance by changing the mobility or the solubility of the ions in the membrane core in both carrier and direct ion transport cases.

The results from membrane thickness values in binary solvent systems (for example, squalene/n-decane) in this study suggest that membrane thickness could be changed to obtain any desired thickness of membranes between 2.4 nm to 4.6 nm. It should be very interesting to test the electrical conductance and membrane thickness correlation using these membrane systems.

A new series of phosphonium salts are found to be effective as anti-trypanosoma cruzi (58,59) and anti-schistosoma mansoni (60) drugs. These phosphonium cations are lipophilic and selectively transport across the membrane. How these drugs act is still not known. Studies of membrane conductances caused by these drugs may permit us to gain information such as the dependence of drug activity on the magnitude of membrane conductances. Furthermore, the effects of membrane thickness on the conductance of these phosphonium salts would also be interesting.

CHAPTER III

APPLICATIONS OF MEMBRANE CAPACITANCE MEASUREMENT ON SOLVENT-CONTAINING (N-ALKANES) AND NEARLY SOLVENT-FREE (SQUALENE) GLYCERYL MONOOLEATE BILAYER MEMBRANE SYSTEMS

(A) Effect of Benzyl Alcohol on Both Solvent-containing (GMO/n-hexadecane) and Nearly Solvent-free Bilayer Lipid Membranes

The anesthetic potency of n-alkanes is closely related to an increase in absorption of the alkane into bilayer membranes (61,62). The expansion of membrane thickness is proposed to explain the blockage of the nerve impulse by n-alkanes in axons. By incorporating a local anesthetic molecule, benzyl alcohol, into a n-tetradecane-containing bilayer membrane, a similar result is observed and this simultaneously led Ashcroft et al. (63,64) to arrive at the same hypothesis for the action of benzyl alcohol. Since the membrane-forming solvent may modify the benzyl alcohol adsorption parameters or conversely, benzyl alcohol may modify the amount of solvent in the membrane, we decided to make membrane capacitance measurement while incorporating benzyl alcohol into both solvent-containing and nearly solvent-free glyceryl monooleate bilayer membranes. These measurements may be a suitable test of the hypothesis for the mechanism proposed for the anesthetic action of benzyl alcohol.

Equal amounts of benzyl alcohol were added into each aqueous phase. The result in Figure 3-1 shows that benzyl alcohol actually increases the capacitance of the bilayer membrane which is essentially solvent-free. The membrane capacitance in the absence of benzyl alcohol is 0.78 uF/cm^2, which corresponds to a membrane thickness of 23.8 Å. The capacitance increase is about 6 % at 75 mM of benzyl alcohol. Conversely, the membrane capacitance decreases substantially in the presence of benzyl alcohol when n-hexadecane is present in the membrane. This behavior is qualitatively similar to the observation obtained by Ashcroft et al. (63, 64) and apparently is not unique to a particular lipid-solvent combination when the solvent is present in the bilayer.

The remarkable differences of the membrane capacitances in the absence and presence of solvents infer an interesting and contrasting action of benzyl alcohol on these two kinds of glyceryl monooleate bilayer membranes. The specific capacitance of a bilayer membrane is given by $C_m = e_o e_m / d$, where C_m is the specific capacitance of the membrane, e_o is 8.85×10^{-14} F/cm, e_m is the dielectric constant of the membrane interior, and d is the membrane thickness. Because the dielectric constant of benzyl alcohol (e = 13.1 at 20 °C) is much greater than that of hydrocarbon (e = 2.1), the incorporation of benzyl alcohol into the bilayer would make one expect that the change in dielectric constant of the membrane interior, e_m, may be greatly affected and this change would, in turn, affect the membrane capacitance.

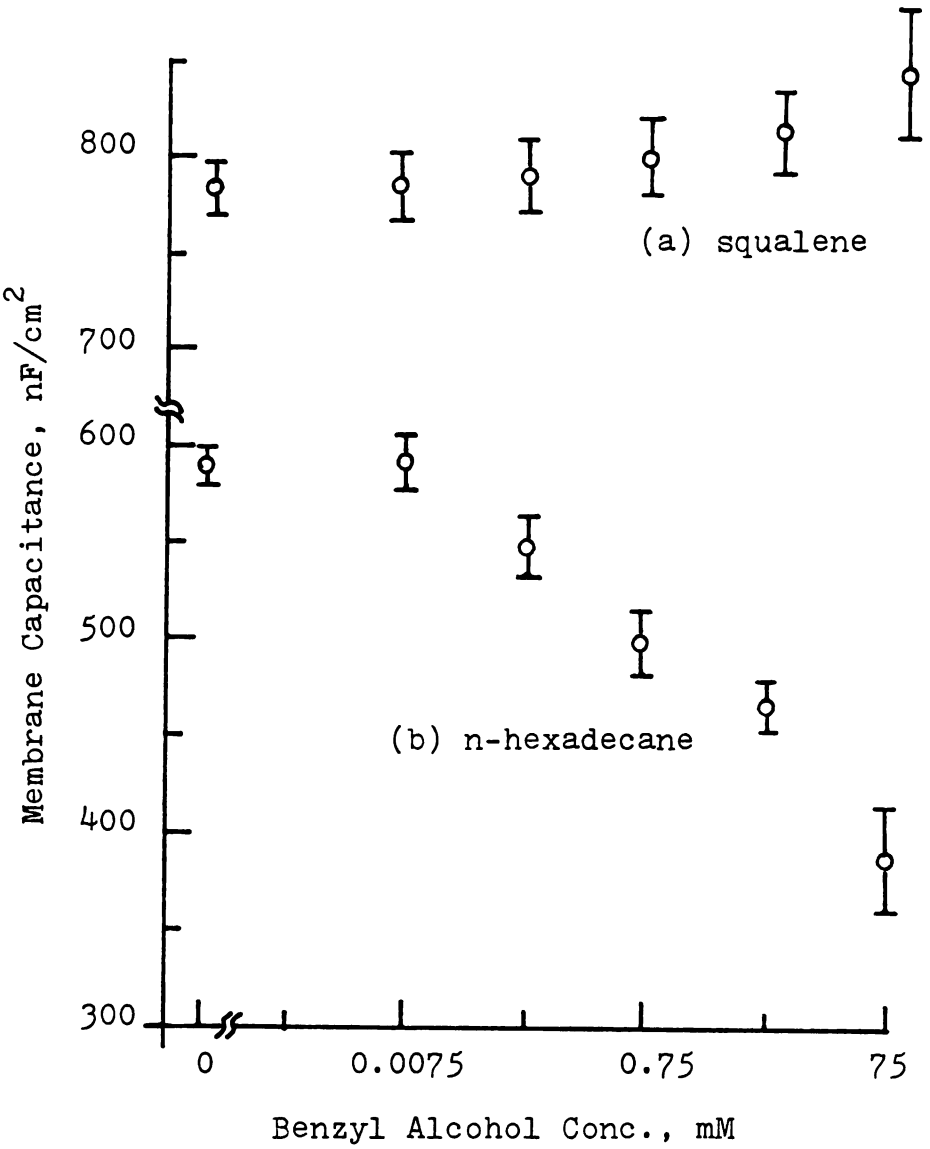


Figure 3-1. Membrane capacitance as a function of benzyl alcohol concentration. In (a), the membrane-forming solution is glyceryl monooleate dispersed in squalene (10 mg/ml). The membrane formed from this solution is essentially solvent-free. In (b), the membrane-forming solution is glyceryl monooleate dispersed in n-hexadecane (10 mg/ml). The membrane formed from this solution contains significant amount of solvent. The aqueous solution was unbuffered 3 M LiCl and the temperatures were 24 ± 1 ^{o}C.

However, this dielectric constant effect appears to be relatively small. Since the membrane capacitance changes were primarily due to increases in dielectric constant of the membrane interior via the incorporation of benzyl alcohol, the membrane capacitances should increase for both solvent-containing and nearly solvent-free bilayer membranes. Clearly, this is not the case. There are two most probable reasons to account for the small benzyl alcohol-induced change in dielectric constant. First, the effective dielectric constant of benzyl alcohol in the membrane interior is probably much lower than 13.1. The presence of hydroxyl group on benzyl alcohol is the key factor in the high bulk dielectric constant of benzyl alcohol relative to toluene which has a dielectric constant of 2.38 at 21 ^{o}C. However, if the hydroxyl group is oriented to the interface, it will be unlikely to contribute appreciably to the membrane's dielectric constant. Second, owing to the relatively low concentration of benzyl alcohol employed in the aqueous phase, the partition of benzyl alcohol into the membrane interior would be relatively small. Thus, the observed changes in membrane capacitance can't be due primarily to changes in dielectric constant. Therefore, they must be due, in large part, to changes in membrane thickness, d.

If the effect of benzyl alcohol on the change in dielectric constant of the membrane interior is small, then what causes the increase in membrane thickness when n-hexadecane is present in the bilayer as we observed ?

As previously noted, both solvent-containing and nearly solvent-free planar bilayer membranes are in equilibrium with a torus. It is likely that the manner in which solvent partitions between the torus and the bilayer region of the membrane will be a factor of considerable importance. In view of the observation made by Hui et al. (65), that alcohol may associate in nonpolar liquids at these concentration ranges, it seems plausible to consider the activity effect of benzyl alcohol in the membrane. The effect of benzyl alcohol on the n-hexadecane-containing bilayer membrane may therefore be rationized as follows: the incorporation of benzyl alcohol into the bilayer region may be responsible for the decrease in chemical potential of n-hexadecane (and lipid) in this region. The decrease of this chemical potential would cause the redistribution of solvent molecules to reach a new equilibrium. As a consequence, an increased partition of solvent molecules is in favor of the membrane interior. Since n-hexadecane has been observed to lie parallel to the acyl chain of the lipid in the bilayer region (57), this amount of increased n-hexadecane may further straighten out the acyl chains of glyceryl monooleate and thus increase the membrane thickness. In contrast, for nearly solvent-free membrane system, the slight increase in the membrane capacitance is likely due to the increase in the dielectric constant of the membrane interior owing to the partition of benzyl alcohol into the bilayer region. Therefore, the membrane thickness is

unlikely to be much changed. This conclusion is qualitatively consistent with the result obtained by Turner et al. (66) who used high-field deuterium nuclear magnetic resonance spectroscopy and calculated a thickness reduction of 2 Å for liquid crystalline state bilayers of dimyristoyllecithin in the presence of large amount of benzyl alcohol (3 to 1 benzyl alcohol/lipid mole ratio).

In summary, the incorporation of a third component such as benzyl alcohol in these studies acts to vary the chemical potential of n-hexadecane, thus resulting in an increase in membrane thickness. However, this behavior does not occur in nearly solvent-free membranes.

(B) Effect of Cholesterol on Both Solvent-containing and Nearly Solvent-free Bilayer Lipid Membranes

Cholesterol (see Figures 3-2(a) & 3-2(b) for the structure) is widely distributed in animal membranes and is found in larger amounts in plasma membranes of cells rather than in the intracellular membranes. The incorporation of cholesterol into bilayer membranes leads to the rigidification of the membrane (67-72) and simultaneously to an increase in the stability of the membrane (12).

The capacitance of bilayer membranes has been observed to increase with the incorporation of cholesterol into the membrane (32,52,54,73) when the membrane contains n-decane. The contribution due to the dielectric constant of cholesterol alone does not account for this increase (74). Therefore, a reduction in the average extension of the chain length is invoked to explain the observed result (32,74).

The difference in the capacitance and thickness of the bilayer membrane formed from dispersing glyceryl monooleate in n-decane and in squalene is significant. The critical comparison of the capacitances of these two kinds of membranes with cholesterol incorporated in them would facilitate us to further examine the solvent effect on the membrane structure.

When incorporated into the bilayer membrane, cholesterol molecules orient to an interface with hydroxyl groups oriented toward the aqueous solution while both the ring and the branching chain at the 17 position is directed

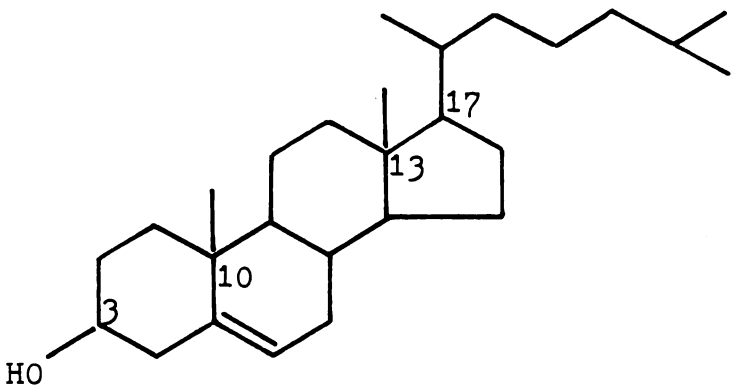


Figure 3-2 (a). Structure of cholesterol with polar hydroxyl group at 3 position and a branched hydrocarbon side chain at 17 position.

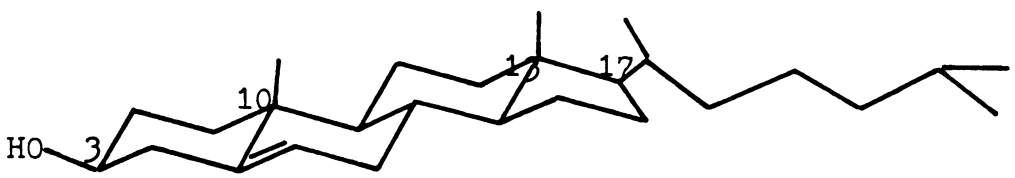


Figure 3-2 (b). Side view of structure of cholesterol. The steroid nucleus is a fused, bulky, reduced tetracyclic ring system that is hydrophobic and stereochemically rigid. The length of the steroid nucleus along the long molecular axis is 9 Å.

toward the membrane core. The actual composition of glyceryl monooleate and cholesterol in the membrane remains unclear.

As seen in Table 3-1, for n-decane-containing membranes, the capacitance increases and the thickness decreases with the amount of cholesterol in membranes. This result is consistent with that obtained by the earlier workers (32,52, 54,73). At a 1 to 1 molar ratio of glyceryl monooleate and cholesterol in the membrane-forming solution, a 29 % decrease of membrane thickness is observed. The largest estimate for the dielectric constant of cholesterol is 2.27 (32). Such a dielectric contribution for cholesterol would have less than 3 % effect on the overall dielectric constant of the membrane considered. This would not explain the large difference observed above. The n-decane-containing membrane has a thickness of 48 Å which is greater than twice of fully extended length of glyceryl monooleate (about 44.6 Å). Experimental evidence (33,57) supports that n-decane molecules reside in the center zone of the bilayer. The decrease of the membrane thickness in the presence of cholesterol would then be rationalized as (a) the exclusion of more n-decane molecules from the bilayer into the torus region. This may be caused by the stronger interactions between cholesterol and the acyl chains of glyceryl monooleate, which then provides the driving force for the flow; (b) subsequent kinking and bending of the acyl chain toward the branching alkyl group of cholesterol to maximize

Table 3-1. Effect of cholesterol on the capacitance and thickness of glyceryl monooleate (GMO) bilayer membranes.

n-decane			squalene		
GMO/cholesterol (molar ratio)	capacitance nF/cm^2	*thickness Å	GMO/cholesterol (molar ratio)	capacitance nF/cm^2	*thickness Å
GMO only	405	45.9	GMO only	780	23.8
1:0.25	409	45.4	1:0.25	736	25.2
1:0.5	410	45.3	1:0.5	710	26.2
1:1	571	32.5	1:1	682	27.2

*Thickness is calculated based on e = 2.1 (26).

the interaction (75) so that there will not be any empty space in the center of the bilayer. This kinking would decrease the membrane thickness. The significantly shorter extended length of cholesterol (about 17.5 Å) relative to that of glyceryl monooleate (about 22.3 Å) along with the greater fluidity in the center of the bilayer (74,75) makes this kinking possible.

On the other hand, the nearly solvent-free membrane as formed from dispersing glyceryl monooleate in squalene has a thickness of 23.8 Å. This value is much smaller than twice of extended length of glyceryl monooleate (about 44.6 Å). The large discrepancy infers that the acyl chains of lipid molecules must be highly deformed. As cholesterol is incorporated into the membrane, both the stronger interaction between the ring portion of cholesterol and the acyl chains of glyceryl monooleate, and the intrinsically rigid ring structure of cholesterol would prevent the bending of acyl chains of glyceryl monooleate. In order to accommodate the bulky branching group of cholesterol at C-17 position, the acyl chains of glyceryl monooleate have to be straightened to a significant extent. This explains the increase of the membrane thickness as seen in Table 3-1 for membranes formed from squalene solvent. The extension of the acyl chain here is the dominant factor for the observed capacitance decrease with the presence of cholesterol in the membrane. Were the dielectric contribution of cholesterol significant, the capacitance would increase instead of

decrease. One additional interesting point to note from Table 3-1 is that even at 1 to 0.25 molar ratio of glyceryl monooleate and cholesterol, the decrease of the thickness for the n-decane-containing membrane is less than 1 % while the increase is about 6 % for the nearly solvent-free membrane.

In summary, the effect of cholesterol on both n-decane-containing and nearly solvent-free membranes is very different. The incorporation of cholesterol causes the thickness to decrease for the n-decane-containing membrane through the exclusion of the n-decane into the torus mostly, and conversely, the thickness to increase for the nearly solvent-free membrane through the stretch of the acyl chains of lipids in order to accommodate the bulky branching group of cholesterol at C-17 position.

(C) Attempt to use Bilayer Lipid Membranes as a Molecular Sensor for the Detection of Potential Carcinogens-- Polycyclic Aromatic Hydrocarbons

Introduction

The interaction of small molecules with bilayer lipid membranes has attracted recent interest since many well-characterized small molecules are toxicants or drugs (76). The small molecules of interest could be either polar ones such as pentachlorophenol (17,77), 2,3,5,6-tetrachlorophenoxyacetic acid (78), and 2,4-dichlorophenoxyacetic acid (79-81), or nonpolar ones such as n-alkanes, benzene, adamantane, and so on. Most of polar molecules, due to their high hydrophilicity in nature, are believed to interact with polar head groups of lipid molecules at the membrane-aqueous interface to cause a lowering of the dipolar field in the membrane interior and a change of underlying ion transport rate. In contrast, the nonpolar molecules often go into the membrane interior and interact with the acyl chains to lead to the variation of either membrane thickness, the motion of the acyl chains, or both.

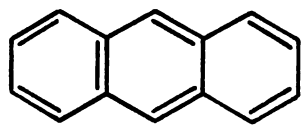
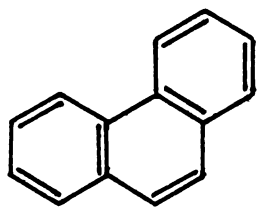
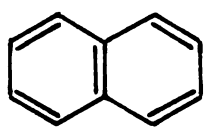
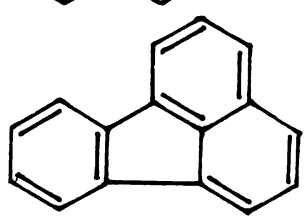
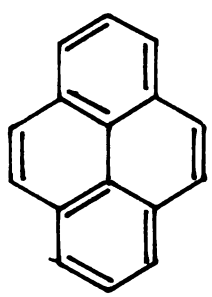
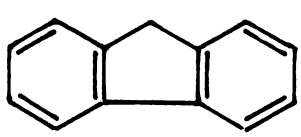
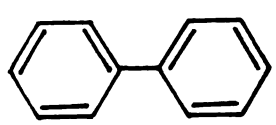
Benzene, adamantane and their derivatives have been found to increase the motion of the acyl chains in the membrane interior (82), while n-alkanes (61-63,83,84) and p-di-t-butylbenzene (85) are responsible for the decreased capacitance and the increased thickness of the membrane. Bilayer membranes possess considerable internal order by virtue of the packing arrangement of their fatty acyl

chains. The inherent anisotropy of the bilayer structure places certain constraints on the interaction of small molecules with these membranes (86). The perturbation of this peculiar structure via molecule-membrane interactions, if they take place, may find some applications. Based on the results of earlier work and an awareness of the imporatnce of carcinogens in living tissues, it appeared that the development of a molecular sensor for the detection of carcinigens would be possible and worthwhile. In this part of work, the measurement of the capacitance of the bilayer membrane with a group of potential carcinogens incorporated, namely polycyclic aromatic hydrocarbons, are examined to test the feasibility of using a bilayer membrane as a molecular sensor.

Experimental Design

The compounds employed in this study are listed in Figure 3-3. Pyrene, anthracene (both from Eastman), phenanthrene (Mallinckrodt), fluoranthene and fluorene (both from Aldrich) were recrystallized twice from ethanol. The individual compound was then saturated in the membrane-forming solution which was prepared by dispersing 300 mg of glyceryl monooleate in 10 ml of squalene. Saturation was intentional to ensure the maximum incorporation of polycyclic aromatic hydrocarbon into the membrane interior. In other words, we were seeking the maximum possible change of capacitance. The membrane formed from this squalene-based membrane-forming solution is essentially solvent-free

Figure 3-3. Structure and chemical names of polycyclic aromatic hydrocarbons.

chemical names	structure
anthracene	
phenanthrene	
naphthalene	
fluoranthene	
pyrene	
fluorene	
biphenyl	

and the acyl chains in the membrane interior are believed to be highly deformed (35). This membrane system has the advantage of avoiding the interference of solvent molecules, while facilitating the maximum interaction between polycyclic aromatic hydrocarbon molecules and acyl chains of the membrane. The membranes were formed in 3 M NaCl aqueous solution and all the measurements were performed at $23 \pm 1\ ^{o}C$.

Results and Discussion

As seen in Table 3-2, essentially no change of membrane capacitance or thickness was observed within experimental error. Two possible explanations may account for this observation: First, in order for these potential carcinogens to cause the appreciable difference in membrane thickness, the potential carcinogens present in the membrane interior must reach a certain level of concentration. A low concentration of potential carcinogens in the membrane interior may not alter the membrane thickness at all. Second, the concentration of potential carcinogens in the membrane interior may be appreciable, but these molecules may perfectly fit into the void volume between acyl chains without causing the extension of highly deformed length of acyl chains.

The lipophilicity of these potential carcinogens is intrinsically high and this property makes these compounds very soluble in the membrane core (87). The partition of these molecules into the membrane interior by the method

Table 3-2. The membrane capacitance and thickness. The polycyclic aromatic hydrocarbon was saturated in 300 mg of glyceryl monooleate and 10 ml of squalene.

compounds	*membrane capacitance, $nF\text{-}cm^{-2}$	**membrane thickness, A
--	780±6	23.8±0.2
anthracene	779±12	23.8±0.4
fluorene	780±13	23.8±0.4
fluoranthene	781±10	23.8±0.3
naphthalene	781±8	23.8±0.3
phenanthrene	780±5	23.8±0.2
pyrene	780±8	23.8±0.3
biphenyl	780±7	23.8±0.3

*Membrane capacitance is the average of eight membranes in each case.

**Membrane thickness is calculated by assuming dielectric constant equal to 2.1 (26).

employed in this work would be expected to be greater than the physiologically active concentration. Since no chamge in the membrane capacitance or thickness is observed, we would conclude that the utilization of a bilayer membrane as a molecular sensor for polycyclic aromatic hydrocarbons via the change of the membrane capacitance or thickness is not feasible.

CHAPTER IV

STABILIZATION OF GLYCERYL MONOOLEATE BILAYER MEMBRANES IN THE PRESENCE OF FERRIC CHLORIDE

Introduction

Many applications using a glyceryl monooleate bilayer membrane as a model membrane in physicochemical investigations have appeared in the literature (29,48,88-96) although the membrane made from this lipid has limited stability (97-99). This requires workers who use this model membrane to either form the membrane on a very small aperture (97) or dope the membrane with a stabilizer such as cholesterol. The former method suffers the disadvantage of decreased accuracy and sensitivity toward the phenomena of interest, while the latter rigidifies the membrane and drastically changes the resistance.

Calcium ion is known to stabilize phospholipid bilayer membranes (100,101). Uranyl ion has been reported to stabilize both phospholipid (98,102) and glyceryl monooleate bilayer membranes (98). Lanthanum ion has been shown to stabilize oxidized cholesterol bilayer membranes (103). These ions are believed to interact with the polar head groups of the lipid molecules in some way that increases the stability of the membrane. Furthermore, Snyder et al. (8) examined the relative stability of cholesterol-oxidized cholesterol bilayer membranes in lithium

chloride, sodium chloride and potassium chloride solutions, and found that the membrane formed in lithium chloride solution has greater stability than in the other two. They related that the lack of the stability of the membrane in both potassium and sodium chloride solutions is due to the disruption of the electric double layer as well as to interference with the internal van der Waals interaction by these two cations because of their lesser ability to arrange water molecules around the interface region. All these studies demonstrate that the composition of the ionic solution affects the stability of the membrane.

Ferric(III) ion has been observed to produce a large change in bilayer resistance (104,105) and it has been shown to be adsorbed on the polar head groups of lipid molecules (105). However, no stabilizing effect from ferric(III) ion has been reported. This highly charged ion, according to Frank et al. (106), should be categorized as having properties similar to lithium and thus is expected to play some kind of role in the region at the membrane-aqueous interface. Qualitative observation indicated that glyceryl monooleate bilayer membrane exhibits a decreasing stability in the alkali ionic solutions in the order LiCl< NaCl< KCl. The trend in stability is consistent with that in the chloroplast-oxidized cholesterol membrane system. This report describes the results of a study of the stabilizing effects of ferric chloride for glyceryl

monooleate bilayer membranes in terms of the membrane lifetime, the dc resistance and the dielectric breakdown voltage.

Experimental

Materials

The membrane-forming solution was made by dispersing 100 mg of glyceryl monooleate (K & K) into squalene (SIGMA) to make 10 ml of solution in a volumetric flask. The aqueous solution comprised 0.10 M potassium chloride to which various amounts of hydrochloric acid or potassium hydroxide were added to adjust the pH. All inorganic salts were analytical grade and the water used to prepare aqueous solutions was twice distilled.

Methods

The membrane was supported by a 1.5 mm diameter hole drilled in a Teflon partition which was clamped between two symmetric Teflon chambers containing the aqueous solutions. The membrane was formed across the aperture by forming a bubble of lipid at the end of a Pasteur pipette under the surface of the aqueous solution and applying the bubble to the aperture. The addition of ferric chloride solution was done prior to the formation of the membrane. The selection of glyceryl monooleate in squalene made this method of formation practical since the membrane-thining process is extremely rapid as soon as thinning is triggered. Thinning was often begun through the aid of slight tapping.

The membrane formed from this lipid solution, according to White (35), is essentially solvent-free.

In all cases the dc resistance of the membrane was found to be ohmic up to about 50 mV. Earlier work indicated that the conductance occurs through the bilayer region (12, 100,104,105). The conductance in this work was measured by applying a 20 mV potential between a pair of silver/silver chloride electrodes and using a 610 A Keithley electrometer to monitor the current. This was done 10 minutes after the membrane became completely black. The measurement of the membrane area was performed using a microscope (Bausch & Lomb) with a reticule.

Dielectric breakdown voltages were determined by increasing the impressed potential until membrane rupture occured. Membrane rupture was indicated by a sudden surge of current through the electrometer. All measurements were performed at a temperature of 24 ± 1 °C.

Results and Discussion

The formation of the membrane was attempted in 0.10 M potassium chloride solution over a broad range of pH. No membrane could be formed at all above pH 8.5. In the pH range of 3.0 to 8.0, the average membrane lifetime was only a few minutes and the maximum lifetime achieved was about 15 minutes.

The stability of the membrane is drastically increased by the addition of ferric chloride to the aqueous solution in both chambers prior to the formation of the membrane.

A comparison of the membrane lifetime with and without ferric(III) ions is seen in Table 4-1. The stabilizing effect on the membrane through the presence of 3.33×10^{-5} M ferric chloride occurs over a pH range of 4.0 to 6.0 with somewhat greater stability at the lower pH. The factors which are responsible for the increased stability are still speculative. According to Atkinson et al. (107), ferric ions undergo extensive hydrolysis and for relatively dilute solution ($< 10^{-3}$ or 10^{-4} M) only the following hydrolysis reaction, $Fe^{+3} + H_2O = FeOH^{+2} + H^+$, is important. The dissociation constant for this hydrolysis reaction is 0.90×10^{-3} at 25 °C. Therefore, the predominant species at pH = 4.28 is $FeOH^{+2}$ at about 97.3 % while the remainder is Fe^{+3}. Thompson et al. (105) have shown that ferric ions specifically bind to lipid polar groups at the membrane-aqueous interface and this binding decreases as the pH of the aqueous solution increases. When the binding of ferric ions at the membrane-aqueous interface occurs, the adjacent water molecules would be very ordered since these bound ions are highly charged. This would, in turn, prevent the water from reaching the hydrocarbon region of the membrane to emulsify and eventually produce membrane rupture. The greater stability at lower pH's may be due to increased concentration of ferric ions present and thus increased binding. However, the ratio of bound ferric ions to lipids must be low to avoid the increase of destabilizing repulsive force between adjacent bound ferric ions. As observed by

Table 4-1. Effect of 3.33×10^{-5} M ferric chloride on membrane stability in 0.10 M potassium chloride solution at pH = 4.28.

conditions	control	ferric chloride
lifetime	15 minutes	>12 hours
resistance	*$(3.18 \pm .06) \times 10^6$ ohm-cm^2	$(1.68 \pm .39) \times 10^6$ ohm-cm^2
number of experiments	25	5

*Average dc resistance from a few successfully-formed membranes.

Table 4-2. Effect of pH on both membrane resistance and dielectric breakdown voltage in the presence of 3.33×10^{-5} M ferric chloride in 0.10 M potassium chloride solution.

pH	*dielectric breakdown voltage, mV	*resistance, ohm-cm^2
3.88	290±10	$(1.59 \pm .18) \times 10^6$
4.28	290±8	$(1.68 \pm .39) \times 10^6$
4.98	>300	$(1.02 \pm .42) \times 10^5$
5.38	>300	$(1.88 \pm .51) \times 10^4$
6.32	280±8	$(1.98 \pm .63) \times 10^6$
6.72	285±7	$(2.88 \pm .49) \times 10^6$

*The values are the averages of at least four membranes.

Thompson et al. (105), the lipids involved in binding have been indeed shown to be only a few tenths of a percent in the case of lecithin dispersion. Therefore, it would be expected that most of 2.7 % of Fe^{+3} at pH = 4.28 resides in the aqueous solution.

It is also observed that even with the presence of 3.33×10^{-5} M of ferric chloride, it is difficult to form a stable membrane below pH = 3.5 and this may suggest that additional factors such as proton competition are involved since the concentration of ferric ions is high at such a low pH. However, the stability of the membrane (below pH = 3.5) is again observed at increased ferric chloride concentration which provides sufficient strengthening of the membrane to overcome the destabilizing forces. But it has to be mentioned that the thinning rate is relatively slow when the concentration of ferric chloride goes above 3.32×10^{-4} M.

The resistance of the membrane, as seen in Table 4-2, is essentially constant below pH = 4.5 and starts to drop between pH = 4.8 and pH = 6.0. When the pH is raised to above 6.2 the resistance of the membrane remains constant again. This unusual property has been discussed in detail with the bilayer membrane formed from lecithin/cholesterol in methanol/n-decane in 0.10 M sodium chloride solution at pH = 4.0 to pH = 6.5 (105). The resistance drop of the membrane within this range is due to the abnormal conduction of chloride anions across the membrane (105).

The dielectric breakdown voltage of the membrane in the presence of ferric chloride is also remarkable. It is about two to three times the value in the absence of ferric chloride which is measured to be in the range of 120 to 140 mV (see Table 4-2).

Other features of glyceryl monooleate bilayer membrane in the presence of ferric chloride include; (a) increased stability and relative insensitivity to mechanical vibration which make it possible to form membranes in a larger aperture. We have formed very stable membranes in a 2 mm diameter aperture without difficulty; (b) the stabilization of the alkane-containing glyceryl monooleate bilayer membrane (as n-decane is used for solvent) is marked. But the addition of ferric chloride is advised to immediately follow the formation of the membrane in order to remove the problem of the slowing of the thinning process; and (c) stabilization of glyceryl monooleate bilayer membranes in other salt solutions such as sodium chloride, lithium chloride, ammonium chloride, sodium acetate, calcium chloride and so on is also realized.

In summary, the stability of glyceryl monooleate bilayer membrane in the presence of ferric chloride in 0.10 M potassium chloride solution is reported. The lifetime of glyceryl monooleate bilayer membrane formed on an aperture of 1.5 mm in diameter increases from a few minutes to more than 24 hours. At pH = 4.28, the membrane has an average dc resistance of 1.68×10^6 ohm-cm^2 and an

average dielectric breakdown voltage of 290 mV.

CHAPTER V

THE EFFECT OF METAL(II) IONS ON THE MEMBRANE CONDUCTANCE IN THE PRESENCE OF 1,1,2-TRIS-(1H-BENZIMIDAZOLE)ETHANE

Introduction

In accordance with the chemiosmotic hypothesis, weak acids that transport protons across bilayer lipid membranes act as uncouplers of oxidative photophosphorylation in mitochondria, bacteria and chloroplast. Among such acids, the substituted benzimidazoles have been attractive choices for studies of this phenomenon. The compound, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), has been widely studied in lipid bilayers (108-114), biological membranes (115-119) and reconstituted systems (120) while 5,6-dichloro-2-trifluoromethylbenzimidazole (DTFB) has been recently employed in lipid bilayers (114,121).

In view of the extensive interest in using weak acids as uncouplers for proton transport across the bilayer lipid membrane, we have investigated a new compound, namely 1,1,2-tris-(1H-benzimidazole)ethane (TBIE) as shown in Figure 5-1 for its effects on membrane conductance. Qualitative results from uv spectra indicate that this compound complexes Cu(II), Ni(II), Co(II) and Zn(II) with decreasing formation constant in the order given. Therefore, studies of the effects of these metal ions on the membrane conductance in the presence of TBIE are also included.

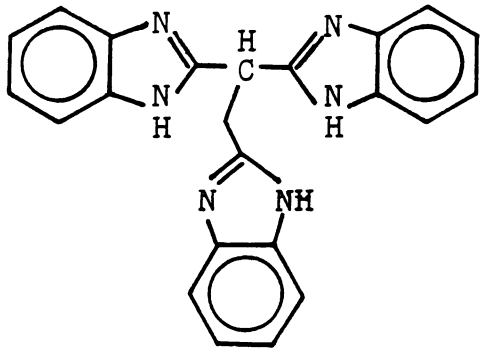


Figure 5-1. Structure of 1,1,2-tris-(1H-benzimidazole)ethane (TBIE).

Experimental

Materials and Methods

The membrane-forming solution was made by dissolving 100 mg of glyceryl monooleate (K & K) into n-hexadecane (Aldrich) to make 10 ml of solution in a volumetric flask. The aqueous solution comprised 0.10 M sodium chloride in 0.10 M acetate buffer at pH = 5.0. All inorganic salts were analytical grade and the water used to prepare aqueous solutions was twice distilled.

The membrane was supported by a 1.5 mm diameter hole drilled in a Teflon partition which was clamped between two symmetric Teflon chambers containing the aqueous solutions. The membrane was formed across the aperture by forming a bubble of lipid solution at the end of a Pasteur pipette under the surface of aqueous solution and applying the bubble to the aperture.

The membrane conductance was measured by applying a 20 mV potential across a pair of Ag/AgCl electrodes and using a 610 A Keithley electrometer to monitor the current. The measurement of the area of the bilayer membrane was performed using a microscope with a reticule. All measurements were carried out at a temperature of 24 ± 1 °C.

Preparation of 1,1,2-tris-(1H-benzimidazole)ethane (TBIE)

Phenylenediamine (3.24 g) was suspended in 10 ml of 1,3,4-trichlorobenzene. After the solution was heated to 170 °C with stirring, 1,1,2-tricarbethoxyethane was added drop by drop at a rate of 30 seconds per drop. As soon as the addition was complete, the temperature was further brought up to 185-190 °C. Following refluxing for 2 hours, the reaction mixture was cooled down to room temperature. The product was then separated by suction and washed with ether and alcohol. A light yellow solid (2.45 g) was obtained with a yield of 65 %. Both the mass spectrum (m/z = 378; m/z = 247) and the nmr spectrum (δ = 4.67, doublet, two methylene protons; δ = 6.57, triplet, one methine proton; δ = 7.50, four aromatic protons; δ = 7.60, eight aromatic protons) confirmed the structure of 1,1,2-tris-(1H-benzimidazole)ethane. The compound was mass-spectrometrically pure. Further checks on purity were carried out with a melting point at 310 °C and the observation of one spot with thin layer chromatography.

Results and Discussion

The results of membrane conductance as a function of TBIE concentration are given in Figure 5-2. The membrane conductance is observed to increase linearly up to a TBIE concentration of 2×10^{-6} M and then start to level off. The levelling-off may be caused by a saturation of the adsorption of TBIE at the membrane-aqueous interface above 2×10^{-6} M TBIE in the aqueous solution. If so, the levelling-off at such a low concentration of TBIE suggests that the binding of TBIE at the membrane interface could be strong. In the absence of TBIE, all four divalent metal ions tested do not significantly affect the membrane conductance over a wide range of concentrations. This is shown in Figure 5-3.

In the presence of a TBIE concentration of 4×10^{-4} M, the addition of metal(II) ions into both aqueous solutions gives very different results as shown in Figure 5-4. For Co(II), Ni(II) and Zn(II), the membrane conductance does not change appreciably with the addition of metal(II) at a concentration of about 3.0×10^{-6} M. In contrast, the membrane conductance is enhanced one order of magnitude by Cu(II) at the same metal(II) concentration. The data also indicates that the enhancement of membrane conductance caused by TBIE is inhibited at elevated concentrations of Ni(II) although the inhibition mechanism is not known. The membrane conductance, on the other hand, is further enhanced linearly with the increase of Cu(II) concentration.

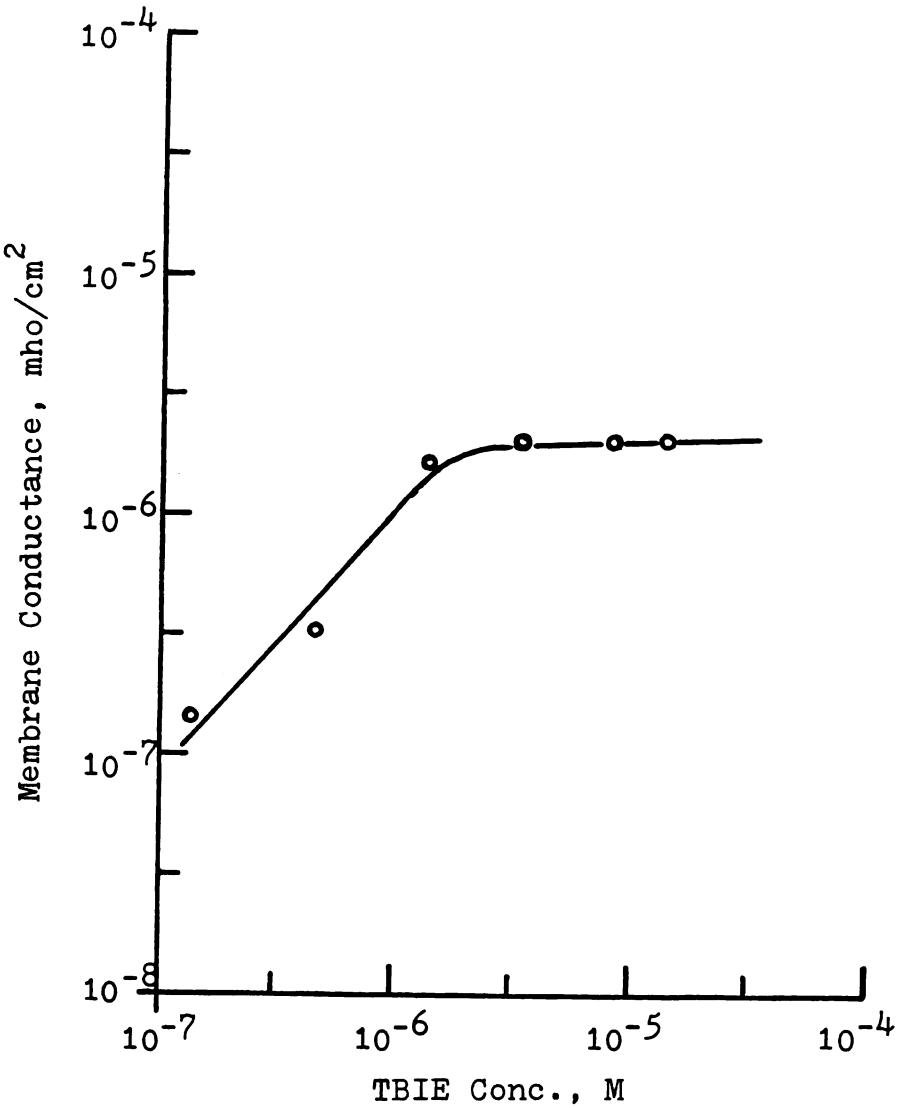


Figure 5-2. The control experiment shows that TBIE alone increases the membrane conductance. The membrane-forming solution is 10 mg glyceryl monooleate per ml n-hexadecane solution. The aqueous solution is 0.1 M NaCl + 0.1 M NaAc buffered at pH = 5.0.

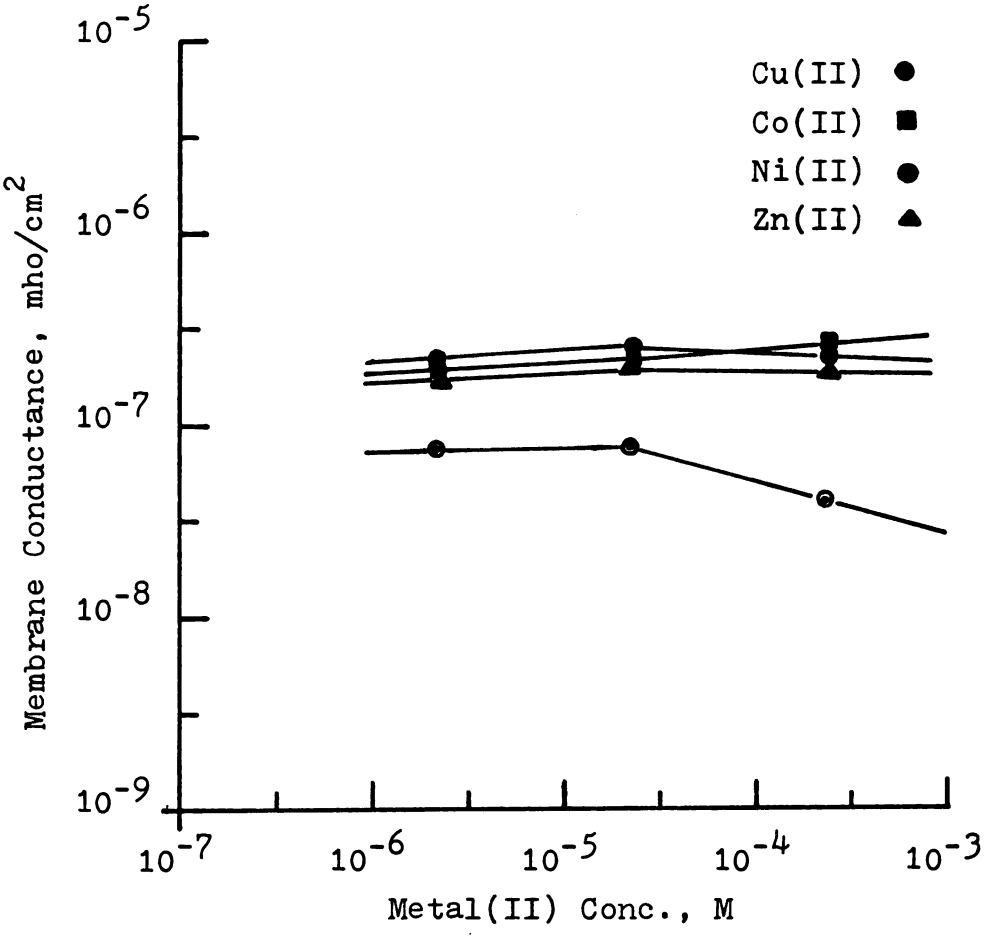


Figure 5-3. The control experiment. The addition of metal(II) chloride does not show any significant change in the membrane conductance. The membrane-forming solution is 10 mg GMO/ml n-hexadecane. The aqueous solution is 0.1 M NaCl + 0.1 M NaAc buffered at pH = 5.0.

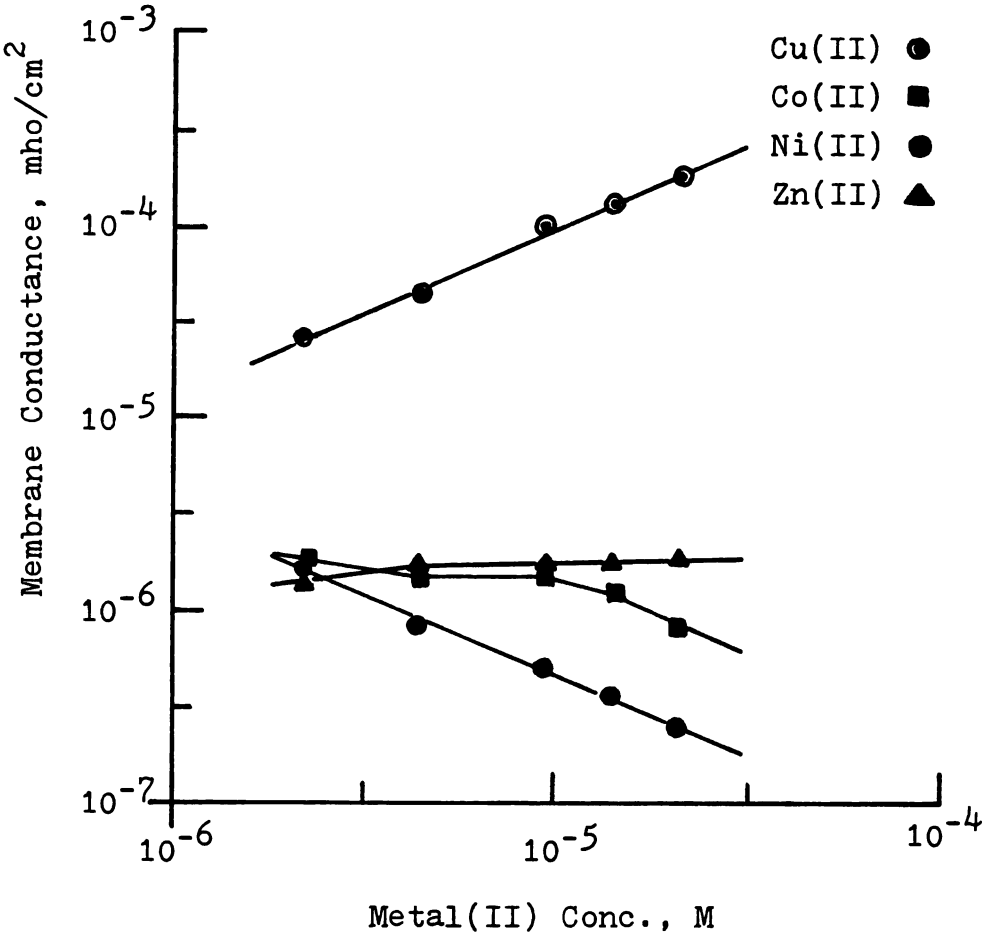


Figure 5-4. The effect of various metal(II) ions on the membrane conductance in the presence of 4×10^{-4} M of TBIE in the aqueous solution. The membrane-forming solution is 10 mg GMO/ml n-hexadecane. The aqueous solution is 0.1 M NaCl + 0.1 M NaAc buffered at pH = 5.0.

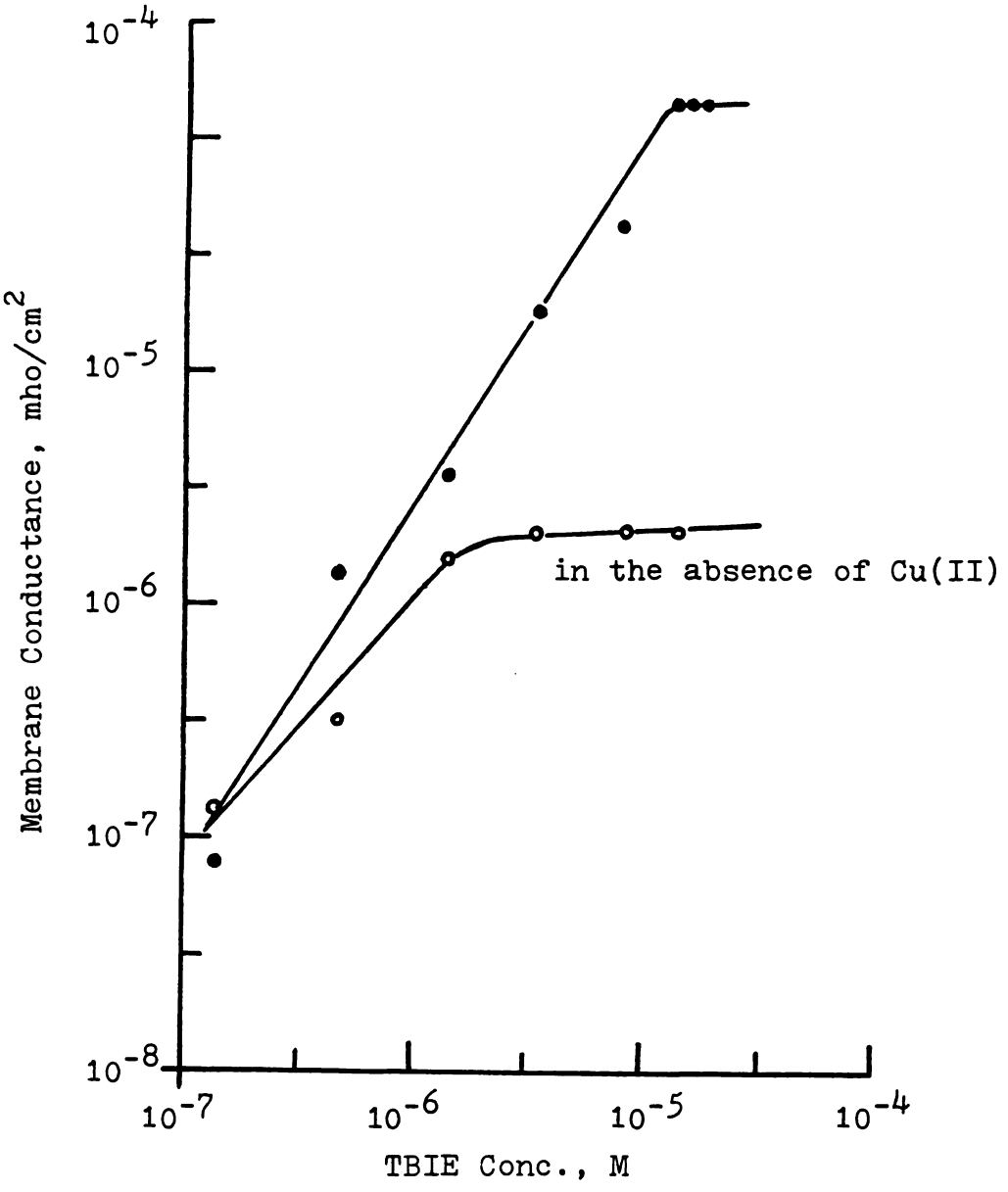


Figure 5-5. The membrane conductance is enhanced in the presence of 1.15×10^{-5} M of copper(II). The membrane-forming solution is 10 mg glyceryl monooleate per ml n-hexadecane solution. The aqueous solution is 0.1 M NaCl + 0.1 M NaAc buffered at pH = 5.0.

In Figure 5-5, the linearly enhanced membrane conductance is again observed with the increase of TBIE concentration at a Cu(II) concentration of 1.15×10^{-5} M. The saturation effect in the presence of Cu(II) occurs only when TBIE concentration is in excess of that required to complex the Cu(II) (about 1×10^{-5} M in Figure 5-5).

A tentative, but plausible explanation is advanced which accounts for the enhancement of membrane conductance observed by Cu(II) in the presence of TBIE. TBIE alone would be protonated in the acidic solution, and the increased membrane conductance is attributed to these protonated-TBIE species passing through the membrane. When Cu(II) is added, TBIE would undergo a conformational change to complex with Cu(II) through the lone electron-pairs of three tertiary nitrogens. The increased hydrophobicity (i.e. lipid solubility) of the resulting complex may be responsible for the further enhancement of the membrane conductance.

Ion transport is an important process in biological systems. It has been shown in the present work that TBIE can be used to modify the biological membrane process and may help in elucidating the mechanism involved in the membrane process. Furthermore, in view of the existence of loosely bound Cu(II) in biological systems (122,123), the enhancement of membrane conductance by Cu(II) in the presence of TBIE may be biologically significant.

Future Work

The preliminary work above presents some interesting possibilities. The results obtained thus far lead us to propose that future work should direct to studies of (a) the equilibrium dissociation constant of TBIE, which would be useful in characterizing the mechanism of ion transport induced by TBIE; (b) the kinetics aspects of this mechanism which can be obtained through relaxation studies. The dependence of the relaxations on the voltage, the TBIE concentration, and the pH of the aqueous solution should all be included.

CHAPTER VI

AN ATTEMPT TO USE A BILAYER LIPID MEMBRANE AS A SENSOR AND APPLICATION TO THE DETERMINATION OF MERCURY (II)

Introduction

A bilayer lipid membrane is a good model membrane which has been utilized to study biologically associated phenomena since it was first formed in 1962 (5). Its unique electrical properties have been employed in clinical analysis (124,125) and more recently, in the assay of antibiotics (126). As a result of the extensive interest in using tetraphenylarsonium ion as a probe for studies of direct ion transport across membranes, the following facts have generally been established; (i) the membrane is sensitive and selective to tetraphenylarsonium ions in terms of the increase in membrane conductance. An increase in membrane conductance is observed even at a concentration of 10^{-5} M of tetraphenylarsonium ion; (ii) the step in which tetraphenylarsonium ion is transported across the membrane determines the conductance; (iii) there is a linear dependence of the conductance upon the concentration of tetraphenylarsonium ion in solution in certain concentration ranges (55,127).

On the other hand, tetraphenylarsonium chloride is a good analytical reagent for the determination of mercury(II)

in 1 to 3 M NaCl solution (128,129). In the presence of chloride, Hg^{+2} exists in the $HgCl_4^{2-}$ form and the addition of tetraphenylarsonium chloride solution causes Hg^{+2} to be quantitatively precipitated out as $((C_6H_5)_4As)_2HgCl_4$. However, because of the lack of a suitable washing solution, the gravimetric method does not give satisfactory results. Instead, the determination of Hg^{+2} is usually carried out by titrating potentiometrically an excess of tetraphenylarsonium chloride with standard iodine solution in the presence of iodide.

The application of a conventional conductometric titration (130) would also be very difficult, if not impossible, in a solution of relatively high salt concentration such as is the case with the titration of small amount of Hg^{+2} by standard tetraphenylarsonium chloride in 1 to 3 M NaCl solution. This difficulty is mainly due to the intrinsic non-specificity of conductometry.

In this study, an attempt was made to use a bilayer membrane as a sensor by taking advantage of the property of selective conductivity of this membrane system toward tetraphenylarsonium ion, and eventually, to use the membrane sensor in the titration of small amount of an unknown Hg^{+2} with a standard tetraphenylarsonium chloride solution. Before applying this membrane system for such purposes, two other important considerations regarding the membrane have to be made: First, the lack of durability of the membrane; this can be partially overcome through the

addition of a small concentration of ferric ions to the aqueous phases on both sides of the membrane as demonstrated in a previous study. Second, the non-reproducibility from membrane to membrane; this would not be expected to impose any problem at all so long as the membrane is stable during the period of titration.

Experimental

Reagents

Tetraphenylarsonium chloride (Tridom Chemical Inc., "P.A." grade) was dried in the oven at 100 ^{o}C for several hours to remove water residue and then made into a 0.05 M of titrant solution. Mercuric chloride (Fisher Scientific Co., "Fisher certified" reagent) was made into 0.1 M of solution. Sodium chloride (Matheson, Coleman & Bell, "A.C.S." reagent) and ferric chloride (Allied Chemical Co.) were made into solutions of the appropriate concentrations. All aqueous solutions were prepared with twice-distilled water. The membrane-forming solution was made by dissolving 100 mg of glyceryl monooleate (K & K) in 4 ml of squalene and 6 ml of n-hexadecane (both were purchased from Sigma Chemical Co.).

Apparatus

The membrane was supported by a 1.5 or 1.2 mm diameter hole drilled in a Teflon partition which was clamped between two symmetric Teflon chambers containing the aqueous

solutions. The membrane was formed across the aperture by forming a bubble of lipid solution at the end of a Pasteur pipette under the surface of aqueous solution and applying the bubble to the aperture. The measurement of the diameter of the membrane was carried out using a microscope with a reticule. The steady-state current was measured with an electrometer (model 610 A, Keithley Instruments) through a pair of silver/silver chloride electrodes as 20 mV of external voltage was impressed. Under such low-field conditions, the resistance, R, can be calculated according to the equation, $R = V/I$, where I is the current in amperes and V is the voltage in volts. The addition of titrant to the chamber in which the positive potential is applied was carried out with a 10 µl automatic pipette. At the same time, an equal volume of 0.1 M of NaCl was added to the other chamber to keep the pressure symmetric with respect to the membrane. Stirring was provided by a magnetic stirrer in each chamber. The stirring motor was separated from the Teflon titration chambers by an aluminum plate connected to ground. The high resistance of the bilayer membrane necessitated careful shielding where appropriate.

Procedure

Each Teflon chamber was filled with 30 ml of 3.0 M of sodium chloride solution. Glyceryl monooleate solution was then introduced into the orifice with a Pasteur pipette to form the membrane. Once the membrane was completely thinned, 0.1 ml of 0.1 M ferric chloride was added to each

chamber. After waiting for about 10 minutes for the membrane to stabilize, 30 μl of 0.1 M mercuric chloride was added to the chamber to which the positive potential is applied. By doing so, the transport of tetraphenylarsonium ions across the membrane is accelerated in the direction of the electric field. The addition of mercuric chloride did not change the membrane resistance. The titration with a 10 μl of aliquot of 0.05 M of standard tetraphenylarsonium chloride solution was started about 5 minutes after the addition of mercuric chloride. The voltage was applied after two minutes of stirring of the mixture for each addition of tetraphenylarsonium chloride solution and then the steady-state current was taken. All titrations were performed at ambient temperature which was 24 ± 1 °C.

Results and Discussion

Electrical Properties of the Membrane

The current-voltage relationship of a thinned and stable bilayer membrane is shown in Figure 6-1. The behavior of the membrane is ohmic up to about 50 mV; hence all analytical work was carried out within this voltage range.

The membrane resistance was followed for the first two hours after its formation in the presence of ferric chloride (0.558 mg). As seen in Figure 6-2, the membrane is quite stable throughout this time range.

The conductance of the iron(III)-stabilized membrane versus the concentration of the titrant was studied and the

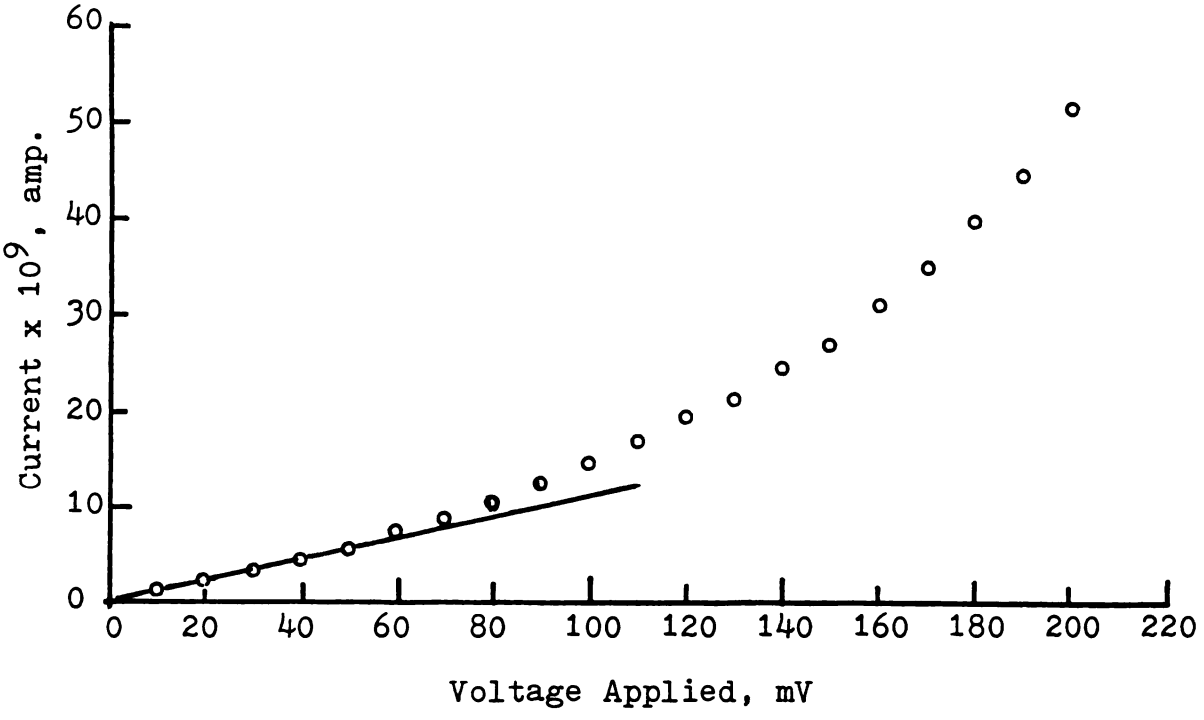


Figure 6-1. A typical current versus applied voltage curve. Note that the linearity is up to about 50 mV.

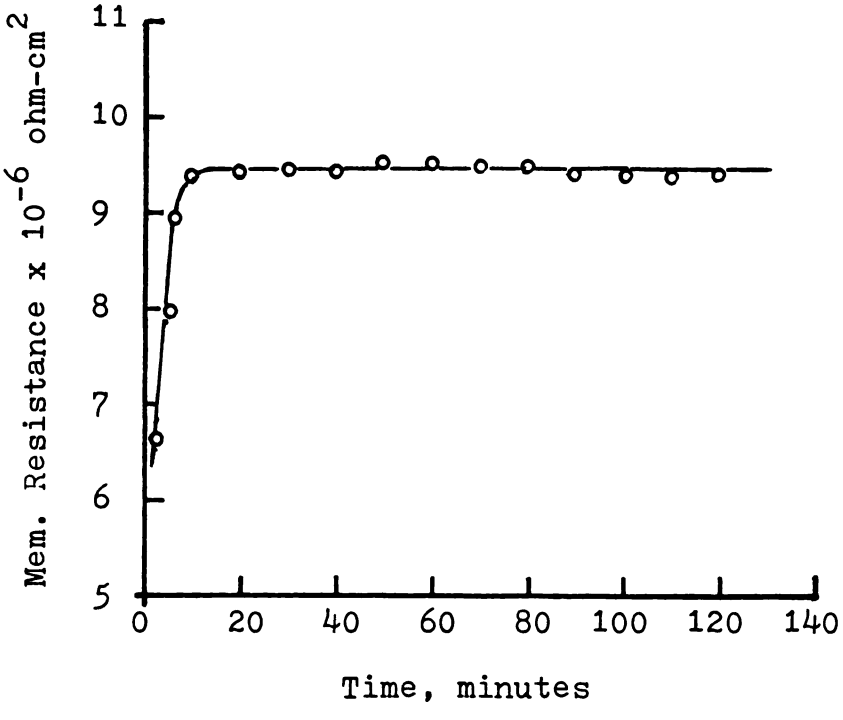


Figure 6-2. A typical membrane resistance versus time curve.

results obtained are shown in Figure 6-3. A linear relationship is observed over the concentration range employed. Here the conductance is expressed in terms of the current and the concentration of the titrant is expressed in terms of the volume of the titrant. The small volume of the titrant used relative to the total volume of the solution did not contribute any significant dilution effect.

Determination of Mercury(II)

A typical titration curve is given in Figure 6-4. Provided that excess mercury(II) is still present in the solution, tetraphenylarsonium ions added are consumed by the reaction. As seen in the earlier portion of the titration curve, the conductance increases slowly indicating that the reaction between $HgCl_4^{2-}$ and $(Ph)_4As^+$ species occurs, but not to completion. When the equivalence point has passed, the addition of further amounts of tetraphenylarsonium ions causes a marked increase in the conductance and this is seen in the later portion of the titration curve. The result from the titration of 5 samples of 0.1 M of mercuric chloride with sample size of 30 μl is given in Table 6-1. An accuracy of 5.5 % has been achieved.

Interference Problems

Due to the highly selective transport of tetraphenylarsonium ion over small inorganic species such as alkali metal ions, alkaline earth metal ions, NH_4^+, NO_3^-, SO_4^{2-}, CO_3^{2-},

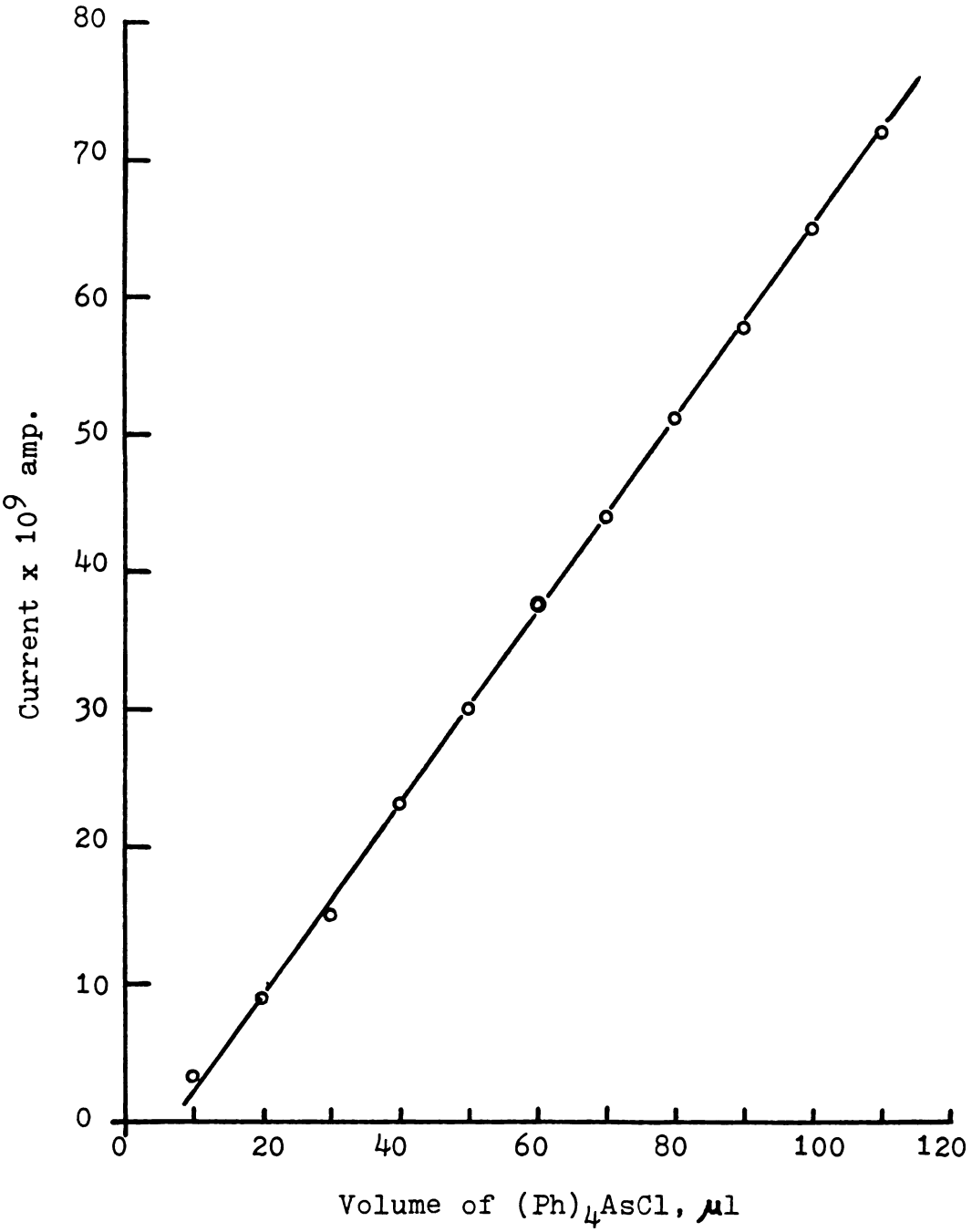


Figure 6-3. A typical current versus volume of titrant, $(Ph)_4AsCl$, curve. Note the linear relationship.

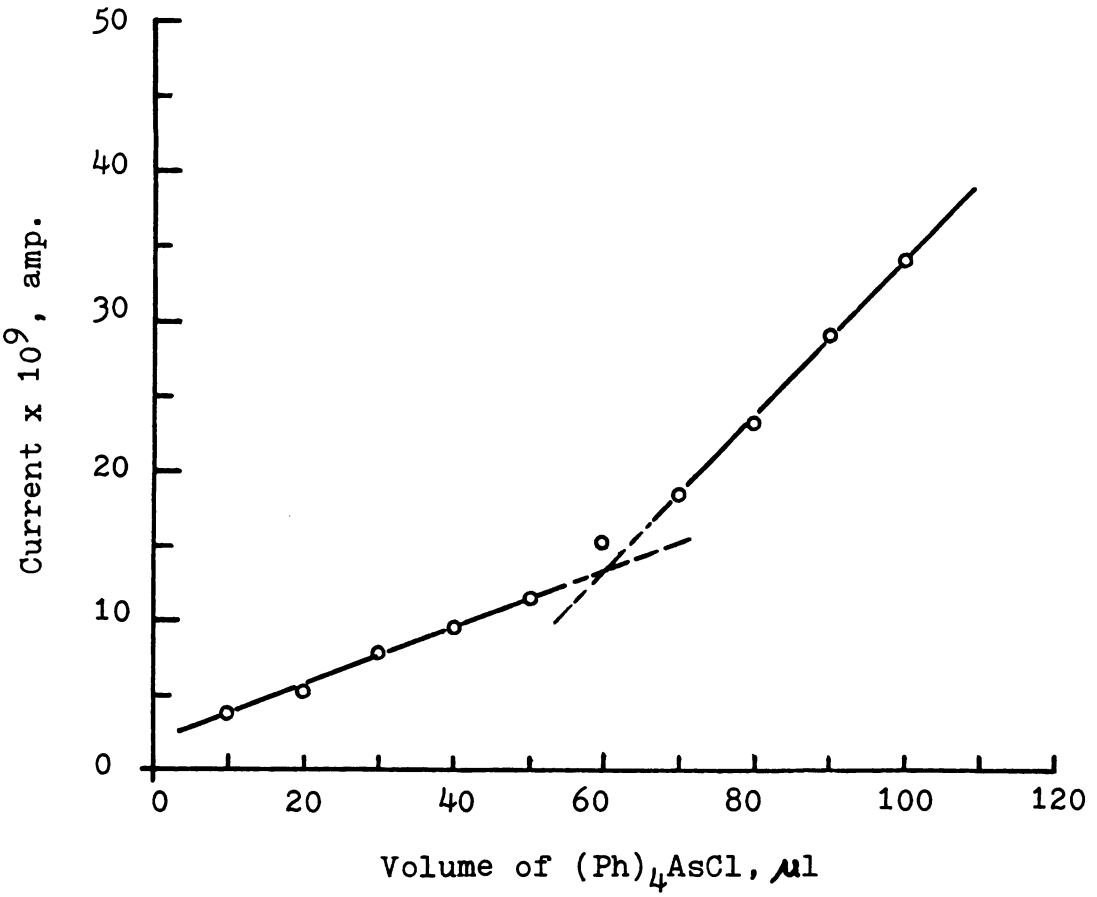


Figure 6-4. A typical titration curve. 30 µl of 0.1 M mercury(II) chloride in 30 ml of 3.0 M NaCl solution was titrated with 0.05 M of standard tetraphenylarsonium chloride solution.

Table 6-1. Analytical results; 30 µl of 0.1 M mercury(II) chloride in 30 ml of 3.0 M NaCl solution was titrated with 0.05 M of standard tetraphenylarsonium chloride solution.

trial no.	mercury(II) found mg	mercury(II), actual mg	deviation mg	s. d. mg
1	0.632	0.602	+0.030	±0.033
2	0.642		+0.040	
3	0.572		−0.030	
4	0.602		+0.000	
5	0.570		−0.032	

$C_2O_4^{2-}$ and ClO_3^-, the interference from these small ions is generally negligible in this method. However, those ions forming precipitates with tetraphenylarsonium ions as discussed by Willard (131) still interfere since this method is not designed to eliminate this problem. These ions include MnO_4^-, CrO_4^{2-}, WO_4^{2-}, MoO_4^{2-}, IO_4^-, ClO_4^-, ReO_4^-, I^-, Br^-, F^-, CNS^-, and the halide complexes of Bi^{3+}, Sn^{4+}, Pt^{4+}, Au^{3+}, Tl^{3+}, Zn^{2+}, Cd^{2+}, and Fe^{3+}. The Fe^{3+} ions employed to stabilize the membrane in this case might thus be expected to cause a problem in determining the mercury(II). However, no precipitates were observed, even with a microscope, in the solution in the absence of mercury(II) while precipitates were immediately seen in the presence of mercury(II) when tetraphenylarsonium ions were introduced into the solution.

On the other hand, two advantages resulting from this method worth of mentioning; (a) the use of the membrane makes the conductometric titration in the high salt concentration possible, and (b) all interferences resulting from the use of iodine and iodide are certainly eliminated.

Conclusion

The potential use of a bilayer membrane as a sensor and its application to the determination of mercury(II) in this study have been proved promising. However, the durability of the membrane, although stabilized by the presence of ferric chloride, has been reduced to about an hour or so

with the presence of increased amounts of tetraphenylarsonium ions. Therefore, a more durable membrane which possesses properties analogous to a bilayer membrane, when developed, should be expected to increase the analytical applicability.

Future Work

The bilayer lipid membrane has been demonstrated as a useful detection device in the conductometric titration to determine the concentration of inorganic species such as mercury(II).

The development of membrane-based sensor for the analysis of organic species might also be feasible. This is especially true for the *in vivo* detection of trace amounts of organic substances such as drugs and their metabolites. Conventionally powerful tools such as the chromatographic technique encounter difficulty in this area of chemical analysis because of the very complicated sample matrix.

An overview of mechanisms proposed for functions such as olfaction indicates that the electrogenic process at the cell membrane provides an interesting model for the development of electrochemical sensor. Bilayer lipid membranes were originally synthesized to mimic the cell membrane. The incorporation of organic species into the membrane and the underlying elucidation of membrane interaction has been, at least, partially successful.

The changes of bilayer membrane properties resulting from the interaction of membranes with antigen-antibodies (18,124, 125), enzyme-substrates (124,125), macrocyclic antibiotics (132), polyene antibiotics (133), acetylcholine receptors (134), and odorant-olfactory receptors (135) are a few examples.

The specificity of these interactions and the low concentration required to elicit a response suggest a possible application in analytical chemistry and the analogy of bilayer lipid membrane with natural systems further suggest the suitability for in vivo analysis. Future work along this direction will certainly be interesting.

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