ASYMMETRIC PHOSPHOLIPID BILAYER MEMBRANES: FORMATION AND ELECTRICAL CHARACTERIZATION

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Summary: Asymmetric bipolar phospholipid membranes were formed by juxtaposition of two distinct lipid monolayers sharing a common interfacial phase. The resulting bimolecular films were stable for periods of up to two hours and displayed both normal and anomalous rectification behavior. Both membrane stability and rectification were dependent on the concentration of supporting electrolyte in the aqueous phases. Studies with calcium indicated it played an important role in determining the I-V characteristic of the film. The Katchalsky theory for membrane elements arrayed in series adequately described the system's electrical response.

The two most commonly assumed characteristics of natural membrane systems are that a lipid bimolecular leaflet serves as the structural core and that asymmetry or "sidedness" is ubiquitous (1,2). Many workers have attempted to introduce the asymmetry feature in model phospholipid bilayer systems by the addition of extraneous additives or by chemical modification of preformed symmetrical films (3-5). The principal difficulties with these methods are the resulting uncertainties in structural perturbations (deformation of the bileaflet to an undefined form), and possible induction of other modes of operation (channels, carriers, etc.). The latter problem makes the design of meaningful testing procedures difficult since many suspected mechanisms are not always mutually exclusive. In order to circumvent these problems, we have used a modification of the method described by Tsofina, et al., (6) to form asymmetric bipolar lipid bilayers by hydrophobic coalescence of two distinct phospholipid monolayers. The resulting film separating two aqueous compartments was easily accessible for electrical characterization.

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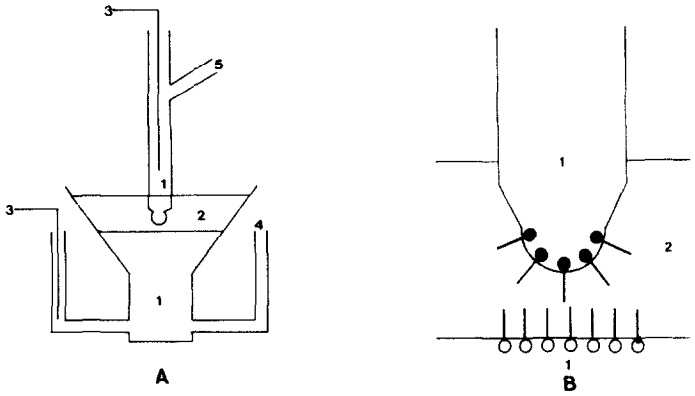
Materials and Methods

Egg phosphatidyl choline, chromatographically pure, and phosphatidyl serine, Folch fraction III, were purchased from General Biochemicals. The lecithin was used without further purification. The phosphatidyl serine (PS) was chromatographed on a silicic acid-silicate column according to Rouser, et al., (7). The collected PS gave one spot on TLC plates exposed to I_2, ninhydrin and rhodamine 6G. Both phospholipids were stored as 2% solutions in n-decane at -20°C in evacuated ampoules until used. Silicic acid (Unisil) was purchased from Clarkson Chemical Company and used without further treatment. The hydrocarbon solvents n-decane (Gold Label) and n-nonane (A R grade) were purchased from Aldrich Chemicals; inorganic salts were A R grade. Aqueous solutions were prepared from double distilled water having a specific conductance less than 1 μmho/cm.

Experimental

Figure 1 shows the experimental arrangement for the formation of asymmetric membranes. In general, the following procedure was used: a monolayer of lecithin was formed at the interface of an aqueous subphase and an upper hydrocarbon phase of 1:1 n-nonane and n-decane. A similar monolayer film of phosphatidyl serine was then brought down through the organic phase to the lecithin monolayer by means of a mechanical stage. At the surface of contact, the two monolayers coalesced through their hydrophobic ends to form a bimolecular leaflet separating two aqueous compartments. Two Ag/AgCl electrodes were used to make electrical connections on each side of the bilayer. Current-voltage curves were obtained by applying a dc voltage to the film and measuring the current as a voltage drop across a precision resistor in series with the membrane, using a Keithley 600A electrometer. The cell potential (membrane and series resistor) was measured with a Fischer 310 pH meter and the membrane voltage calculated by difference. Capacitance discharge curves were recorded on a Tektronix 549 oscilloscope by taking an output signal from the electrometer which was proportional to



1A: Cell design used for generating bipolar lipid membranes and measuring their electrical behavior. 1, aqueous phase; 2, 1:1 n-nonane + n-decane phase; 3, Ag/AgCl electrodes; 4, aqueous reservoir and piston drive; 5, air piston drive.

1B: Diagramatic representation of monolayer orientation resulting in bilayer formation by coalescence: 1, aqueous phase; 2, 1:1 n-nonane + n-decane; ● = phosphatidyl serine, ○ = lecithin.

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the discharge current through the series resistor. Direct visualization of the film was achieved by a simple mirror-lens system which caught light reflected off the bilayer from a source passing upward through the bottom of the cell. Membrane diameters were measured by incorporating a reticle in the lens eyepiece and calibrating prior to each membrane formation.

Results and Discussion

Figure 2a shows a typical current-voltage curve for a 1:1 mixed symmetrical phosphatidyl serine-lecithin bilayer. Generally these films had specific resistances between 5×10^7 ohm-cm^2 and 1.0×10^9 ohm-cm^2, which agrees well with values reported by Hendrickson and Scattergood (4) and Gitler and Montal (8), for mixed films of similar composition. Values for specific capacitance were more consistent from film to film and averaged 0.31 mfd/cm^2. In both symmetric and asymmetric systems, membranes were stable up to two hours after formation. Films which did rupture tended to do so in the first few seconds after formation. Membrane rupture

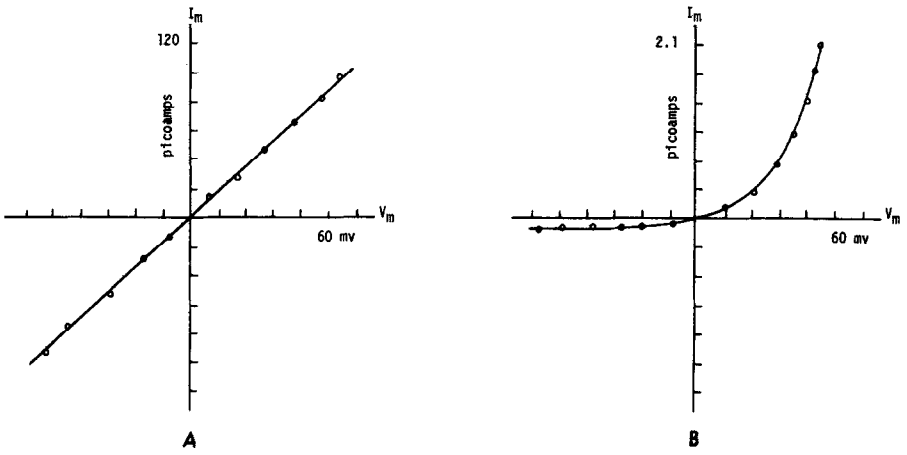

Figure 2

2A: I-V curve for 1:1 mixed symmetric membrane. Aqueous phase consisted of 50 mM NaCl + 0.5 mM $CaCl_2$, unbuffered at pH 5.5. Circles are experimental points, solid line is a linear least-squares fit.

2B: I-V curve for phosphatidyl serine-lecithin asymmetric membrane. Aqueous phase consisted of 50 mM NaCl + 0.5 mM $CaCl_2$, unbuffered at pH 5.5. Circles are experimental points, solid line is a theoretical curve for the Katchalsky model with: $(t^+_{ps} - t^+_{lecithin}) = 0.38$; $R = 7.2 \times 10^8\ \Omega\ cm^2$.

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was most frequent when very small amounts of phospholipid were applied to either interface. This may suggest bilayer formation was correlated to monolayer surface pressure, however, the relationship was not elucidated in detail.

The I-V characteristic for an asymmetric membrane (PS on one side, lecithin on the other) is shown in Figure 2b. Positive current is defined as cation flux out of the PS side of the bilayer. The experimental data is in excellent agreement with the equation given by Katchalsky for bipolar films (9):

$$V = \frac{2RT}{F}(t^+_{ps} - t^+_{lecithin})\ln(1 + \frac{I}{I_0}) + IR$$

where V and I are the membrane voltage and current, respectively; t^+ is the cation transport numbers for the components of the film; I_0 is the reverse saturation current; and R is the ohmic resistance defined as the sum of the two monolayer resistances.

Membrane currents were linearly related to the monovalent salt concentration in the aqueous phases. The presence of Ca^{++} ions strongly altered the I-V response in the opposite direction, i.e., decreasing membrane currents. A possible explanation is that Ca^{++} interacts with the phosphatidyl serine monolayer, forming a more condensed phase, and also raises the volta potential for cation flux through the film. This interpretation is supported by the results of studies on the role of divalent ions in bilayer film stability and monolayer surface pressure-area measurements (10,11).

The ratio of limiting conductances between the forward bias and reverse bias states, for aqueous phases comprised of 50 mM NaCl + 0.5 mM $CaCl_2$, varied from 60 to 133. In essence, this ratio reflects the apparent difference in transport numbers of the PS and lecithin surfaces of the film for a given ion. Application of the Katchalsky model for membrane elements arrayed in series gave values for $\{t^+_{ps} - t^+_{lecithin}\}$ between 0.15 and 0.38. The maximum obtainable value for this system would be around 0.4 based on the transport numbers reported for alkali metal halides in lecithin bilayers by Miyamoto and Thompson (12). Negative deviations from this value indicates varying degrees of mixing between the two monolayers prior to coalescence. No significant changes were seen in the I-V curves for stable membranes up to two hours after formation; hence, it seems unlikely that mixing occurred in the bilayer state by either inversion or lateral diffusion mechanisms. Further, spin-label studies of Kornberg and McConnell (13) with vesicle systems have shown these processes to be slow compared to the membrane lifetimes reported here.

DC capacitance measurements on bipolar films under conditions of reverse bias did not show the asymmetric capacitance expected of typical solid state rectifying devices. In all cases, a single time constant accurately described the discharge curves initiated at all negative voltages down to the punch-through potential. Either of two different effects could

produce this result: the intrinsic hydrocarbon capacitance completely masked any space-charge effects which possess a voltage dependent capacitance; or assuming neither term is dominant, the ion distribution gradient within the film was linear. The second alternative infers the constant field assumption of Goldman and would result in a voltage independent space-charge capacitance (14,15). However, in the absence of small ac signal analysis data no distinction can be made between these possibilities.

In the presence of salt gradients of NaCl up to 5:1, the bipolar films rectified in the direction of decreasing concentration, thus exhibiting anomalous rectification. Furthermore, for a given gradient ratio, inversion of the gradient direction altered the magnitude but not the sign of the diffusion potential, demonstrating a non-Nernst behavior. The Katchalsky model predicts both effects for bipolar films of relatively high resistance as reported here. In addition, both facts support the conclusion that the rectification behavior arises from differences in the transport properties of the PS and lecithin components forming the bilayer and not from the nature of the surrounding aqueous phases.

The collective studies show that stable asymmetric phospholipid bilayers can be formed by coalescence of individual monolayers. The resulting films show many of the electrical properties displayed by excitable membrane systems under steady-state voltage clamp conditions. In addition, the membrane's ion transport behavior can be accurately explained by existing theories on model systems with intrinsic asymmetry features. The system thus offers a good basis for further investigation on the role of asymmetric lipid-lipid and protein-lipid distributions in membrane function.

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