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Lipid-Polypeptide Interactions in Bilayer Lipid Membranes

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Summary. The modifications of the electrical properties of bilayer lipid membranes (BLM) composed of cholesterol and an ionic surfactant upon interaction with charged polypeptides were studied. The addition of 10^{-8} M polylysine (Ps^+) to one side of anionic cholesterol dodecylphosphate BLM increases the specific membrane conductance over 1000-fold (from 10^{-8} to 10^{-5} mho/cm^2) and develops a cationic transmembrane potential larger than 50 mV. This potential is reverted by addition of polyanions such as RNA, polyglutamic or polyadenilic acid to the same side on which Ps^+ is present, by addition of Ps^+ to the opposite side, or by addition of trypsin to either side. Both conductance and potential changes are hindered by increasing the ionic strength or by raising the pH of the bathing medium, disappearing above pH 11.5 where it is known that Ps^+ folds into an α-helix. The interaction of polyglutamic acid (PGA) with a cationic cholesterol-hexadecyltrimethylammonium bromide BLM results in increased membrane conductance and development of an anionic transmembrane potential which is reverted by addition of polycations to the same aqueous phase where PGA is present. Addition of either Ps^+ or PGA to one or both sides of a neutral BLM composed of 7-dehydrocholesterol induces no significant change. The observations suggest the formation of a lipid polymer membrane resultant from the interaction, predominantly electrostatic, of the isolated components. The implications of these results are discussed in terms of the current models of membrane structure.

Model systems of membrane structure consisting of phospholipid liquid-crystals, either in the form of a vesicle (*cf.* Bangham, 1968; Huang, 1969) or an isolated ultra-thin film (*cf.* Mueller & Rudin, 1969*b*; Thompson & Henn, 1970) have provided considerable insights into the mechanisms underlying exquisite membrane functions, such as excitability (Mueller & Rudin, 1963, 1968), ionic selectivity (Mueller & Rudin, 1967; Skulachev, Sharaf & Liberman, 1967; Finkelstein & Cass, 1968; Hopfer, Lehninger & Thompson, 1968), light-induced phenomena (*cf.* Tien, 1970) and several others (for an authoritative recent account *see* Mueller & Rudin, 1969*b*).

In these studies, conferral of the specific membrane function to the lipid system has been attained either by adsorption of complex and unidenti-

fied proteinaceous materials or by the incorporation of relatively well defined cyclic peptides, antibiotics, fungistatics, lipid-soluble weak acids and pigments among others (*cf.* Mueller & Rudin, 1969*b*).

At the realm of these reconstitutions lies the fundamental problem of the nature of lipid-protein interactions. In this paper, an attempt is made to describe a model-system for lipid-protein associations in membranes in which the predominant factor determining the interaction is coulombic. For this purpose, use has been made on one hand, of anionic, neutral and cationic isolated bilayer lipid membranes (BLM)[1] composed of cholesterol and an ionic surfactant, since Lawrence (1961) has described the formation of smectic mesophases in a ternary system consisting of an ionic and a non-ionic amphiphile in water, and Tien and Diana (1967*a*) have shown that smectic mesophases, in the form of isolated bilayer membranes, develop when a system of an ionic (DAP or HDTAB) and a non-ionic amphiphile (cholesterol) in oil (*n*-dodecane) is spread under water over an orifice in a teflon partition separating two aqueous phases, and on the other hand, of anionic or cationic polypeptides with well defined ionic properties in solution (Doty, Imahori & Klemperer, 1958; Applequist & Doty, 1962; Katchalsky, Sela, Silman & Berger, 1964). The relevance of these observations to the problem of biological membrane structure and function will be discussed.

Similar experiments have been recently reported on other model membranes by Shah (1969, 1970) using stearic acid monolayers and polylysine, and by Hammes and Schullery (1970) and Kimelberg and Papahadjopoulos (1971*b*) with phosphatidyl serine vesicles and polylysine.

Materials and Methods

The lipid bilayers were formed and studied as originally reported by Mueller, Rudin, Tien and Wescott (1962) and recently described in detail by Mueller and Rudin (1969*a*). The membrane cell consisted of two concentric chambers, the inner one being a teflon cup 2 cm in diameter, hereafter designated as the inside, and the outer one a 5 cm × 2 cm lucite box. The wall of the inner chamber is pierced by a hole, 1.1 mm in diameter in which the BLM is formed. The volume of the inside compartment is 4 cc and is continuously stirred by means of a miniature magnetic spin-bar. The volume of the unstirred outside compartment is 8 cc. The membranes were continuously observed with a stereo-microscope under reflected light. The area of the membrane was taken to be that of the opening in the teflon partition (1 mm^2) and no corrections were made for the negligible torus obtained with this type of BLM. The membrane solutions employed were: (1) 1%

1 Abbreviations used: BLM Bilayer lipid membrane; DAP Dodecyl acid phosphate; HDTAB Hexadecyltrimethylammonium bromide; PGA Polyglutamic acid; Ps^+ Polylysine; *Vm* Membrane Potential; *Rm* Membrane Resistance; *Gm* Membrane Conductance.

cholesterol (Sigma, St. Louis, Missouri) and 0.1% dodecyl acid phosphate (Hooker Chemical Co., Niagara Falls, N.Y.) in *n*-dodecane, which is anionic (Tien & Diana, 1967*a*); (2) 1% 7-dehydrocholesterol (Sigma) in *n*-decane, which is uncharged (Tien, Carbone & Dawidowicz, 1966); and (3) 1% cholesterol (Sigma) in *n*-dodecane with 0.001% Hexadecyltrimethylammonium bromide (HDTAB) included in the aqueous phase, which is positively charged (Tien & Diana, 1967*a*, *b*). The aqueous phase consisted of NaCl buffered with Tris-chloride at the concentration and pH indicated in figure and table legends. To control the pH changes introduced by the addition of the adsorbates, the pH was adjusted before and tested after every experiment. No significant deviation from the adjusted buffer value was detected. The temperature was 25 ± 1 °C. The electrical circuits are analogous to those described by Mueller and Rudin (1969*a*) and Tien and Diana (1967*b*). For the measurements of the DC membrane resistance a selected potential *Vi* was applied from a calibrated source (pH millivolt testbox, Model EUA-20-12, Heath Co., USA) to the input resistor *Ri*, and the membrane resistor *Rm*. With the use of this instrument *Ri* could be varied from 10^5 to 10^9 Ω and *Vi* from 0 to 1.4 V. The potential appearing across the membrane *Vm*, was measured with a high input impedance electrometer (Keithley, Model 610 R, Keithley Instruments, Inc., Cleveland, Ohio) between two saturated calomel electrodes (Beckman 39270). The inner compartment electrode was the active one, attached to the electrometer; the outer compartment electrode was connected to ground as a reference electrode. The output of the electrometer was connected to a strip chart recorder (VOM 6-2E, Bausch and Lomb, N.Y., or 906T Visicorder Oscillograph, Honeywell, Denver, Colorado). Since *Vi* and *Ri* are known and *Vm* is measured, *Rm* can be calculated by means of the following equation:

$$Rm = \frac{Vm}{Vi - Vm} \cdot Ri.$$

It should be noted that the membrane potential was usually less than 1 mV when the aqueous solutions were identical, so that electrode asymmetry potentials and junction potentials were considered negligible when compared to the magnitude of the potentials induced by the adsorbates under study. Transference numbers were calculated from membrane potential measurements assuming that only the two ions of highest concentration were permeable, that is $t^{\text{anion}} + t^{\text{cation}} = 1$ using the following equation (McInnes, 1961):

$$t^{\text{cation}} = \frac{Vm}{2V\text{max}} + 0.5$$

where t is the transference number, *Vm*, the measured membrane potential and *V*max, the Nernst potential, corresponding to the maximum potential across a permselective membrane and given by the following equation:

$$V\text{max} = -\frac{RT}{F} \cdot \ln \frac{a}{a_{\text{ref}}}$$

where R = gas constant, T = absolute temperature, F = Faraday equivalent, a = salt activity in the compartment designated as the inside and a_{ref} = salt activity in the compartment designated as the outside.

We define the sign of the measured membrane potential developed by addition of the adsorbates as cationic to a potential which is positive with respect to the side of the partition to which the adsorbate is added; in other words, that the measuring electrode in the inner compartment records a negative potential. The opposite convention applies for an anionic potential.

All data presented are representative and have been reproduced in a minimum of 3 different BLM. Processed data points represent means of at least 3 observations in 3 different BLM. Poly-L-lysine HBr of defined molecular weights were obtained from Sigma (St. Louis, Missouri) and Miles-Yeda Ltd. (Kiryat Weizmann, Rehovoth, Israel). Cytochrome *c* Type III, polyglutamic, polyadenilic and ribonucleic acids were from Sigma, L-lysine from Mann Research Laboratories, N.Y., and other chemicals were of the highest purity commercially available. Glass-redistilled water was used throughout.

Results

Effects of Polylysine on Cholesterol-Dodecyl Acid Phosphate BLM

The specific membrane conductance, defined as $Gm = 1/Rm$, of the unmodified cholesterol-DAP BLM is of the order of 1×10^{-8} mho/cm^2 (Table 1) (Tien & Diana, 1967*a*). As illustrated in Fig. 1*A*, after the formation of the black film is complete, the membrane conductance is tested giving a value of 1.2×10^{-8} mho/cm^2. Thereafter, 1.25 μg/ml of polylysine (mol wt 127,000) are added to the inner compartment; approximately 25 sec after the addition of polylysine, a cationic membrane potential starts to develop reaching a steady state value of 58 mV after about 5 min. Simultaneous to the potential generation, the membrane conductance increases attaining a value of 4×10^{-6} mho/cm^2 after about 9 min. The onset time of these changes varies inversely with the polylysine concentration and, above 2.5 μg/ml, a transient potential precedes the steady state changes described in Fig. 1*A*. An example, of the two types of potentials measured after addition of 25 μg/ml of polylysine (mol wt 280,000) to a cholesterol-DAP BLM is presented in Fig. 1*B*. *Rm* is 0.5×10^8 Ω cm^2. Immediately after addition of polylysine a transient potential of 71 mV can be measured; it decays in less than 30 sec to a value of 25 mV and then gradually increases

Table 1. Influence of the degree of polymerization of polylysine on the induced modifications of the electrical properties of cholesterol-DAP BLM [a]

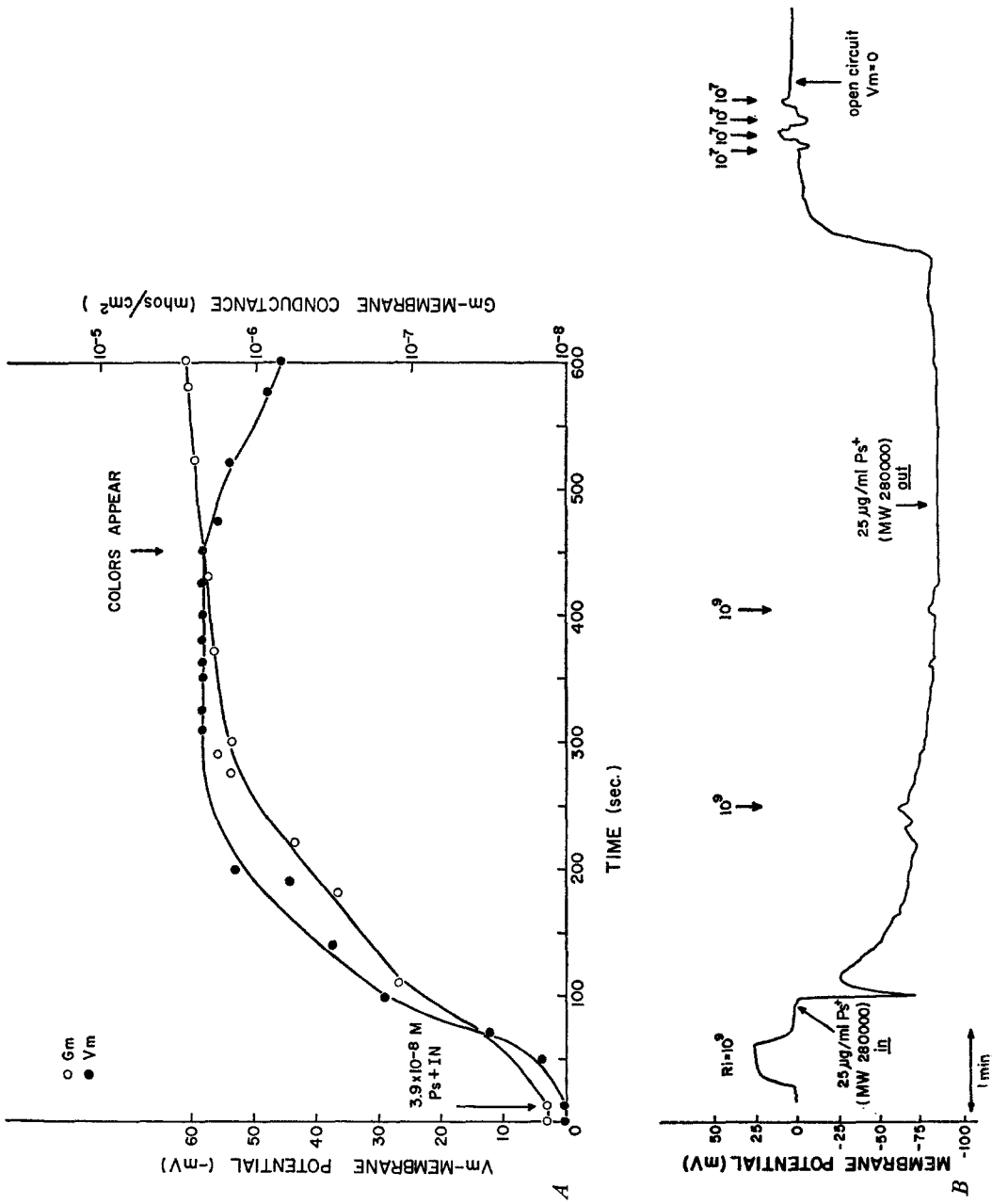
Polylysine (mol wt)	Final concentration (10^{-8} M)	*Vm* (−mV)	*Rm* (Ω cm^2)
None	—	—	10^8
18,000	2.8	69	1×10^6
127,500	2.0	71	7×10^5
195,000	1.3	75	9×10^5
280,000	1.6	80	1×10^5

[a] Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.6.

to reach a steady state value of 86 mV at 5 min with an *Rm* of 8.7×10^5 Ω cm^2. Six min after the addition of Ps^+ to the inner compartment, 25 μg/ml of Ps^+ are added to the outer compartment and it can be noted that in less than 3 min the potential reverts to the zero base line (as judged by opening the circuit) and *Rm* now has the value of 3.3×10^4 Ω cm^2. It should be pointed out, that whenever a BLM is formed in a solution containing Ps^+ on one compartment the potential and conductance changes are immediately and reproducibly monitored, i.e., that the overall modifications of conductance and potential are independent of the order of membrane formation and addition of polylysine, but kinetically the steady state values are attained faster by addition of Ps^+ prior to BLM formation.

The steady state potential and conductance changes are stable for several minutes, usually between 15 and 30 min, but occasionally the potential slowly decays, in a way reminiscent of the potentials elicited by adsorption of cationic detergents to BLM composed of total brain lipids and *E. coli* total lipids described by Seufert (1965), and Tien (1970), respectively.

Addition of 10^{-8} M concentrations of polyanions such as polyglutamic, polyadenilic or ribonucleic acids to the inside, after the potential induced by Ps^+ has attained a steady state, results in its complete reversal, without significant modifications of the membrane conductance. It is relevant to note that polyanions *per se* do not modify the electrical properties of anionic BLM. (*See* Table 3). Fig. 1*C*, illustrates that reversal of the Ps^+-induced potential can also be obtained by a proteolytic enzyme such as trypsin. This particular membrane with an initial *Rm* of 1.1×10^8 Ω cm^2 broke after addition of 2.9×10^{-8} M Ps^+ to the inside; a new membrane is formed in the same solution, and as shown in Fig. 1*C* a steady state potential of 65 mV is readily measured, the membrane conductance being of the order of 2×10^{-6} mho/cm^2. The membrane was left in this situation for over 30 min and no significant changes of either *Vm* or *Gm* were detected. Thereafter, 100 μg/ml of trypsin are added to the outside; 20 sec elapsed between the trypsin addition and the reversal of potential of approximately 54 mV, bringing the conductance down to a value of 2×10^{-7} mho/cm^2. The circuit is opened and a residual transmembrane potential of 18 mV is recorded. The membrane is kept under these conditions for 10 min when 100 μg/ml of trypsin are added to the inside; as can be noted, there is a further reversal of the potential with a small overshoot which slowly decays to the zero potential line. No further modifications of *Gm* were detected. The time span of the experiment was over 60 min; the BLM dielectric breakdown voltage was 200 mV. It should be added that, if trypsin is added only to the polylysine side, a complete reversal is readily obtained, and no


Gm-MEMBRANE CONDUCTANCE (mhos/cm²)
10⁻⁵
10⁻⁶
10⁻⁷
10⁻⁸
COLORS APPEAR
○ Gm
● Vm
3.9x10⁻⁸ M Ps+IN
Vm-MEMBRANE POTENTIAL (-mV)
0
10
20
30
40
50
60
100
200
300
400
500
600
TIME (sec.)
A
10⁷ 10⁷ 10⁷ 10⁷
open circuit Vm=0
10⁹
10⁹
25 µg/ml Ps⁺ (MW 280000) out
Ri=10⁹
25 µg/ml Ps⁺ (MW 280000) in
MEMBRANE POTENTIAL (mV)
50
25
0
-25
-50
-75
-100
1 min
B

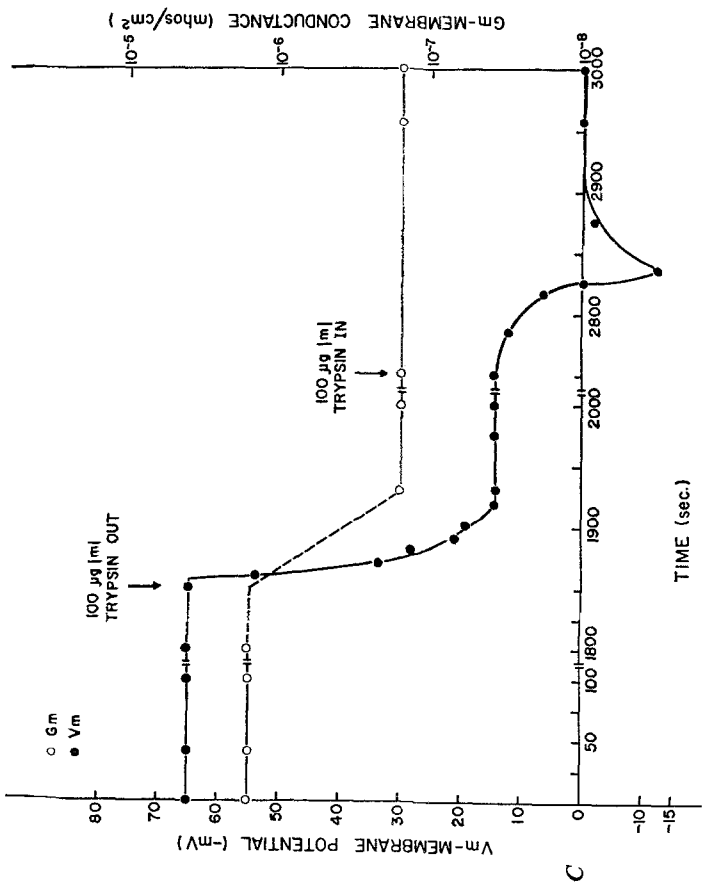


Fig. 1. Effects of polylysine on the electrical properties of cholesterol-DAP BLM. (*A*) Reaction mixture: 6 mM NaCl (unbuffered), pH 5.5; (*B*) Reaction mixture: 6 mM NaCl, 6 mM Tris-Cl, pH 8.4, $Vi = 30$ mV, $Ri = 10^9$ or 10^7, as indicated; (*C*) Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.0. This membrane, with an initial Rm of $1.1 \times 10^8\,\Omega\,cm^2$, broke after the addition of 2.9×10^{-8} M polylysine and a new BLM was immediately formed in the same solution

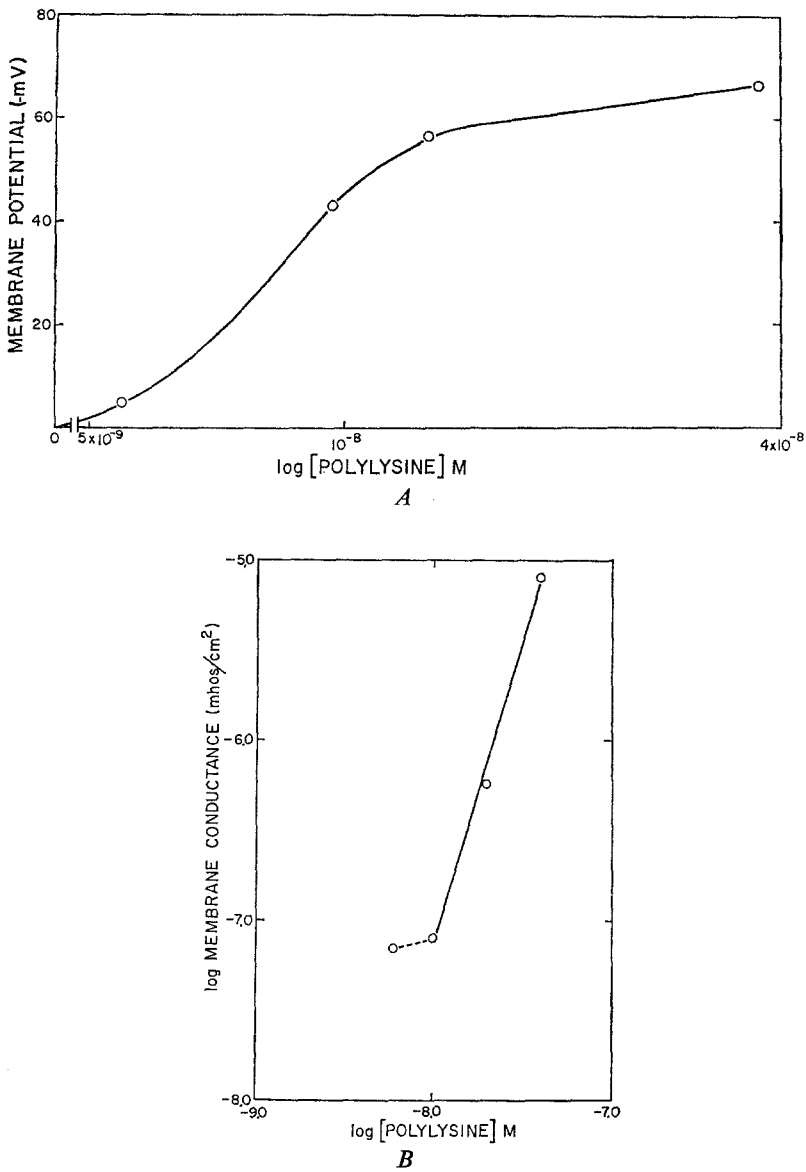


Fig. 2. (*A*) Relation between membrane potential developed on anionic BLM and polylysine concentration in the aqueous phase. Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.0; (*B*) Relation between the membrane conductance of anionic BLM and polylysine concentration in the aqueous phase. Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.0

effects of trypsin on the unmodified BLM are detected. Moreover, it has been determined by standard assay techniques that under the conditions of these BLM experiments, hydrolysis of polylysine by trypsin is indeed occurring.

The continuous observation of the membrane has permitted the detection of changes in its appearance during the course of an experiment. When the changes induced by Ps^+ reach a steady state, the BLM is no longer completely black, but minuscule lenses of colored membrane appear and tend to progressively occupy all the black area. In most of the experiments a "silvery" appearance dominates the area of the membrane (Mysels, Shinoda & Frankel, 1959).

Fig. 2*A* presents a concentration dependence of the potential changes elicited by polylysine (mol wt 127,000) and Fig. 2*B* is a log polylysine concentration *versus* log membrane conductance plot. From Fig. 2*B* a slope of 6 can be obtained.

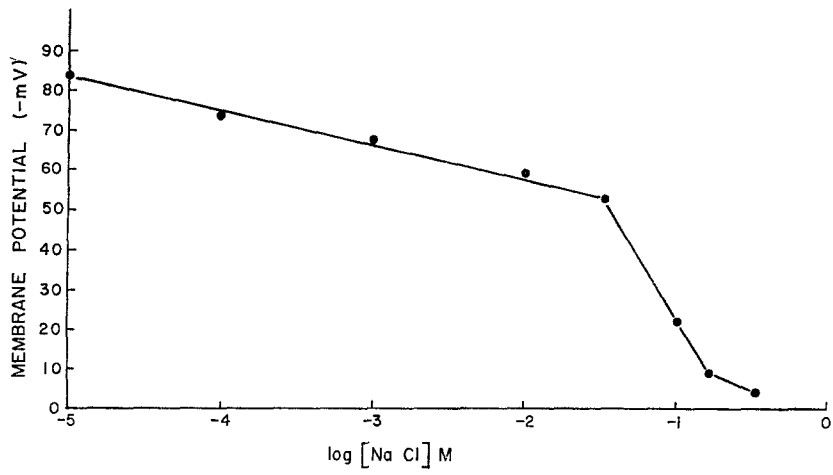
No significant differences in the extent of *Rm* or *Vm* changes are detected when polylysines of 18,000, 127,500, 195,000 and 280,000 mol wt are tested (Table 1).

Influence of Ionic Strength and pH on the Polylysine-Induced Modifications of Cholesterol-DAP BLM

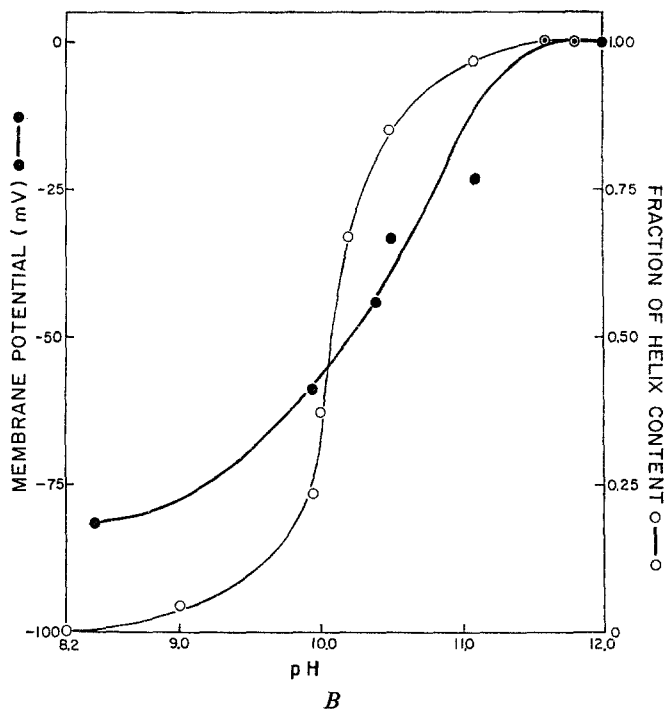
As shown in Fig. 3*A* the potential generated by addition of Ps^+ to anionic BLM is considerably diminished by raising the ionic strength of the aqueous solution prior to the addition of polylysine.

Fig. 3*B* illustrates that while the Ps^+-induced potential at pH 8.4 can be as high as 84 mV, it is null above pH 11.6 (closed circles). The data of Applequist and Doty (1962) on the variations of the fraction of helix content of polylysine solutions with pH have been replotted in Fig. 3*B* (open circles), indicating that the Ps^+-induced potential is absent at those pH's where Ps^+ exists predominantly as a helix and the membranes maintain their initial high *Rm* ($0.5-1\times10^8\ \Omega\ cm^2$) over considerably long periods, even at very high polylysine concentrations.

Mueller and Rudin (1968) observed that in the presence of alamethicin and an ionic-gradient, adsorption of basic polymers on sphingomyelin BLM developed an anionic membrane potential. The installation of an ionic gradient on cholesterol-DAP BLM once the modifications induced by the addition of Ps^+ to the inner compartment have reached completion, results in the development of a membrane potential of anionic sign as demonstrated in Fig. 4*A*. The transference number for anions of the unmodified cholesterol-DAP membrane is less than 0.42 and becomes between 0.86–0.98 after adsorption of Ps^+. Furthermore, the membrane potential varies with the log of the salt activity gradient across the membrane as illustrated in Fig. 4*B* where the line represents the Nernst potential, and the symbols illustrate the

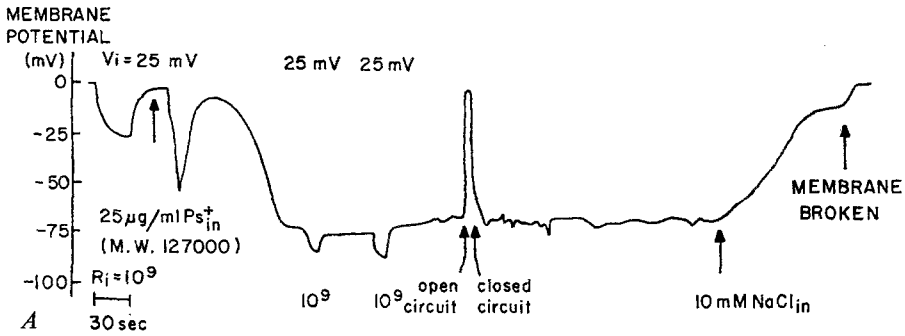


A



B

Fig. 3. (*A*) The effect of ionic strength, which was increased by addition of NaCl, on the membrane potential developed on anionic BLM in the presence of polylysine. Immediately after the addition of polylysine to the inner compartment, the BLM was formed. Reaction mixture: NaCl at the molarity indicated; 1 mM Tris-Cl, pH 7.6, 1.59×10^{-8} M polylysine (mol wt = 280,000); (*B*) Effect of pH on the polylysine-induced membrane potential on anionic BLM (closed circles) and the fraction of helix content of polylysine solutions, [data of Applequist and Doty, 1962] (open circles). Reaction mixture: 6 mM NaCl, 6 mM Tris-Cl at the indicated pH, 8.9×10^{-8} M polylysine (mol wt = 280,000)



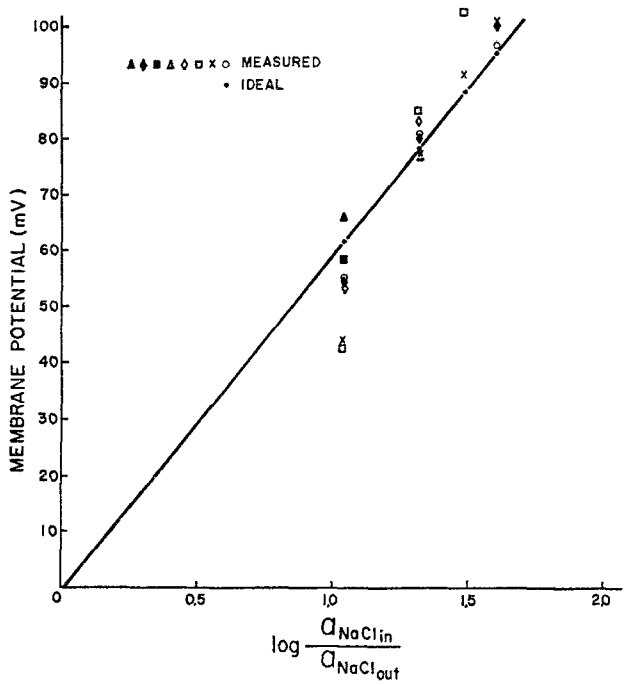


Fig. 4. Conferral of anion selectivity to cholesterol-DAP BLM by adsorption of polylysine. (*A*) Reversal of the Ps^+-induced potential on cholesterol-DAP BLM by installation of a NaCl gradient across the membrane. Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.6. After the formation of the black film is complete *Rm* is tested with $Vi = 25$ mV and $Ri = 10^9$, giving a value of $2.4 \times 10^8\ \Omega\ cm^2$. 25 μg/ml of Ps^+ (mol wt = 127,000) are added to the inner compartment giving rise to a *Vm* of about −75 mV and to a decrease of *Rm* to a value of approximately $2 \times 10^6\ \Omega\ cm^2$. After approximately 7 min of the addition of Ps^+, a 10 μliter aliquot of a 4 M NaCl solution is added to the inside to give a final concentration of NaCl in the inner compartment of 11 mM. A reversal of potential of 58 mV is measured. (*B*) Membrane potential as a function of the log of a NaCl concentration gradient across a cholesterol-DAP BLM after interaction with polylysine. Initial reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.6 and 9.8×10^{-8} M polylysine on the inner compartment. The line represents the Nernst potential calculated as mentioned in the methods section introducing $\frac{2.3\,RT}{F} = 59.2$ mV at 25 °C. Each symbol represents values obtained from a single membrane

experimental results. In other words, the adsorption of Ps^+ has conferred to the membrane the property of an anion-selective system; this type of effect has been described by Sollner (1969) for protamine-collodion membranes that exhibit anionic transference numbers greater than 0.95 and have been designated as membranes of highest ionic selectivity or permselective.

Effects of Cations Other than Polylysine on the Electrical Properties of Anionic BLM

To assess the specificity of the polylysine-induced modifications of anionic BLM, several types of cations were studied and the results of the prototype cations are summarized in Table 2. L-lysine and cytochrome *c*, a basic protein containing a high proportion of lysine residues, modified the membrane to a negligible extent (*see also* Mueller and Rudin, 1969*b*). It should be noted that though the molar charge of cytochrome *c* is about one-tenth that of Ps^+ (mol wt = 18,000), concentrations of the former over 10^3-fold that of Ps^+ did not affect the BLM. HDTAB, modified the mem-

Table 2. Effect of different cations on the electrical properties of cholesterol dodecylphosphate BLM[a]

Cation	Concentration	Molar charge	Resistance decrease	Cationic potential developed	
				Transient (mV)	Steady (mV)
Polylysine	2.8×10^{-8} M	83	10^2–10^3	Only at higher concentrations (*see* text)	50–70
L-lysine[b]	1.8×10^{-3} M	1	none	none	none
Cytochrome *c*[b]	3.7×10^{-5} M	9	less than 10^1	Less than 10 (variable)	none
HDTAB[b]	6.85×10^{-6} M	1	10^1–10^2	40–90	20–90 (variable)
$CaCl_2$	2 mM	2	less than 10^1	25–30	8–12
NaCl (KCl or C_sCl)	25 mM	1	none	20–25	none
HCl	10^{-6} M	1	none	40	none

[a] Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.0; all additions are made only to one of the compartments separated by the membrane.
[b] The concentration presented is the maximal attainable without inducing BLM breakdown.

brane in a manner similar to that elicited by Ps^+, although variable steady state *Rm* and *Vm* changes were detected. A divalent cation such as Ca^{++}, and monovalent cations such as Na^+, K^+, Cs^+ or H^+ induced only transient cationic potentials but no steady alterations were developed.

Effects of Polycations and Polyanions on Cationic Cholesterol-HDTAB-BLM

Tien and Diana (1967*a*) observed that pure cholesterol in *n*-dodecane would not form stable BLM but in the presence of a surfactant such as HDTAB in the aqueous phase, at the concentration of 0.008% (ω/v), stable BLM could be obtained. More reproducible results have been obtained in this laboratory using 0.001% (ω/v) HDTAB in the aqueous phase. At this concentration of surfactant there should be over 10^{14} $HDTA^+$ ion/cm^2 adsorbed on the cholesterol film (Ter Minassian-Saraga and Wietzerbin, 1970) conferring to the surface of the membrane a considerably high positive charge. Indeed, addition of different concentrations of Ps^+ to one or both sides of the membrane induce no modification of either *Rm* or *Vm* of the cationic BLM even when studied for considerably long experimental periods. However, as shown in Fig. 5, polyanions, as typified by polyglutamic acid (mol wt = 40,000 to 100,000) drastically alter the electrical

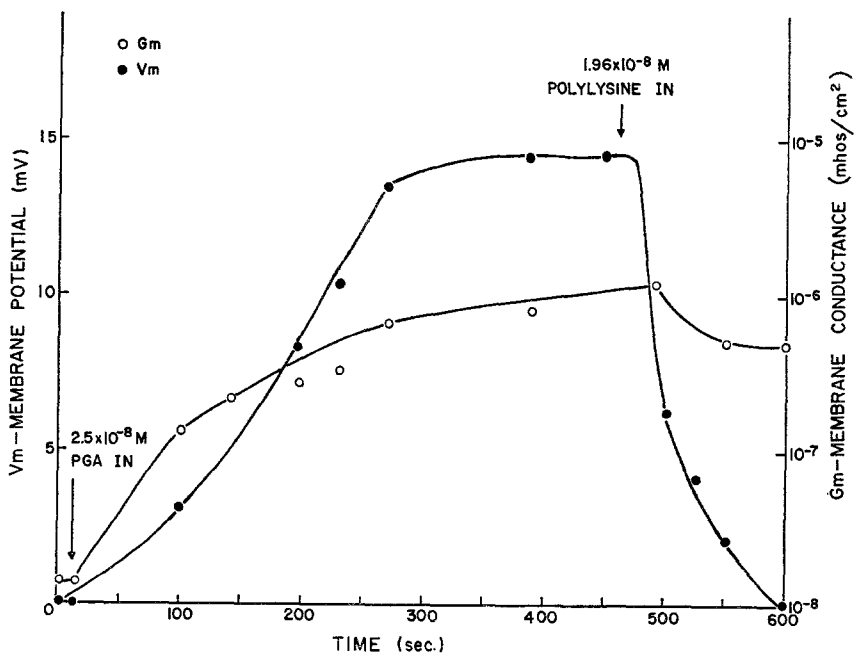


Fig. 5. Effect of polyglutamic acid on the electrical properties of cholesterol-HDTAB BLM. Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.0, 0.001% HDTAB

properties of cationic BLM; the addition of 2.5×10^{-8} M PGA to the inner compartment results in a progressive increase in conductance simultaneous to the development of an anionic transmembrane potential attaining values after 7 min of 10^{-6} mho/cm^2 and 14.5 mV, respectively. After 450 sec, 1.96×10^{-8} M Ps^+ is added to the inside and as a consequence, a reversal of potential is monitored, though no significant changes in conductance are detected. In general terms, the phenomenology of the interaction of polyanions with cationic BLM is the mirror image of that described in certain detail for the interaction of polylysine with anionic membranes. The fact that in the case of cationic BLM the surfactant is present both in the membrane and the solution may explain the low value of the membrane potential developed, by the existence of detergent-polypeptide interaction at the membrane-water interface as well as in solution.

Effects of Polycations and Polyanions on Neutral 7-Dehydrocholesterol BLM

Tien *et al.* (1966) described the formation of stable BLM with 7-dehydrocholesterol in *n*-decane. Neither polylysine, nor polyglutamic acid, at the concentrations at which they interacted with anionic or cationic BLM, respectively, notably modified the electrical and optical properties of 7-dehydrocholesterol neutral BLM.

Table 3. Effect of cationic and anionic polypeptides on the specific membrane conductance and transmembrane potential of anionic, neutral and cationic cholesterol bilayer lipid membranes [a]

Additions	Cholesterol-DAP BLM (anionic)		7-dehydrocholesterol (neutral)		Cholesterol-HDTAB BLM (cationic)	
	Specific membrane conductance (mho/cm^2)	Potential developed (mV)	Specific membrane conductance (mho/cm^2)	Potential developed (mV)	Specific membrane conductance (mho/cm^2)	Potential developed (mV)
None	10^{-8}	–	$2\text{–}4 \times 10^{-7}$	–	10^{-8}	–
Polylysine	10^{-5}	50–70 (cationic)	$2\text{–}4 \times 10^{-7}$	less than 5: transient (cationic)	10^{-8}	–
Polyglutamic acid	10^{-8}	–	$2\text{–}4 \times 10^{-7}$	–	10^{-5}–10^{-6}	10–20 (anionic)

[a] Reaction mixture: 1 mM NaCl, 1 mM Tris-Cl, pH 7.0, and, when indicated, 2.5×10^{-8} M polyglutamic acid, 1.59×10^{-8} M polylysine.

As a summary, the effects of polylysine and polyglutamic acid on the specific membrane conductance and transmembrane potential of anionic, neutral and cationic cholesterol BLM are presented in Table 3. Thus, Ps^+ increases by more than 10^2 the specific membrane conductance and develops a cationic potential of 50 to 70 mV on anionic BLM while no significant alterations are detected on neutral or cationic membranes. PGA, while increasing the specific membrane conductance by more than 10^2 and developing an anionic potential of 10 to 20 mV on cationic BLM, induces no notable modifications on neutral or anionic membranes.

Discussion

The interaction of charged polypeptides with ionic cholesterol membranes results in drastic modifications of the electrical and structural properties of the lipid bilayers. The notably high membrane resistance of unmodified cholesterol-ionic surfactant BLM ($0.5-1\times10^8\ \Omega\ cm^2$) is lowered by more than 10^2, thus bringing this value to a range characteristic of several biological membranes (*cf.* Mueller & Rudin, 1969*b*) and comparable with the resistance of phosphatidylserine vesicles reacted with polylysine, as calculated from the isotopically determined ionic fluxes reported by Kimelberg and Papahadjopoulos (1971*b*). A steady membrane potential, of the same sign as the polypeptide molecule itself and of varying magnitude, is developed and maintained for considerably long periods. With respect to the nature of the species carrying the current, two possibilities appear consistent: either an outward directed positive current supplied by Ps^+ *per se* or an inward directed negative current arising from the extraction of DAP from the BLM upon complexation with Ps^+, followed by replenishment from the material present in the torus; however, it remains difficult to precisely establish the source.

Accordingly, as it would be expected if the phenomena were due to surface charge, the screening of this charge by increasing the ionic strength of the solution with indifferent monovalent electrolytes, (Fig. 3*A*), or the induction of the random-coil-helix transition by raising the pH of the solution and consequently removing the charge of Ps^+ (Applequist & Doty, 1962; Bradburry, Crane-Robinson, Goldman & Rattle, 1968) (Fig. 3*B*) significantly hinders (Fig. 3*A*) and even nullifies (Fig. 3*B*) the interaction. Other examples of bilayer conductance changes dependent on surface charge have been previously described by Bangham, Standish and Watkins (1965) for vesicles and by Hopfer, Lehninger and Lennarz (1970*a*, *b*) and McLaughlin, Szabo, Eisenman and Ciani (1970) for isolated thin films.

As a result of the interaction, notable, macroscopic changes of the structure of the membrane are detected. The membranes are completely "black" (i.e., no light is reflected) (*cf.* Mysels *et al.*, 1959; Mueller & Rudin, 1969*b*), when the bimolecular configuration is attained. After interaction with the polypeptides, light is reflected from the membrane giving a "silvery" appearance to the previously "black" area, thus indicating that the thickness of the film is now comparable to "a quarter of the wavelength of the average color" ($\sim$1000 Å; Mysels *et al.*, 1959).

The drastic electrical and structural modifications of the membranes resultant from the complex formation suggest that significant phase transitions are occurring in both lipid and polypeptide components. With regard to the lipid phase, transition from bilayer to micelle (Luzzati & Husson, 1962; Ohki & Aono, 1970), or microemulsification in discrete regions of the bilayer, as observed with lysolecithin on lecithin-cholesterol bilayer vesicles (Bangham & Horne, 1964) or with HDTAB on lecithin-cholesterol isolated bilayers (Ter Minassian-Saraga & Wietzerbin, 1970), could constitute the initial membrane rearrangement which would be manifested electrically being optically silent due to the isotropic and hence transparent nature of micelles and microemulsions (*cf.* Bangham, 1963). This would be followed by more complex aggregation phenomena resulting in a thick, light-reflecting film; the high-order dependence of membrane conductance on Ps^+ concentration would be consistent with this suggestion. These observations are reminiscent of the formation of complex coacervates (dicomplexes) following the precipitation of colloid cations with colloid anions so thoroughly studied by Bungenberg de Jong (1949).

As far as the polypeptide is concerned, Hammes and Schullery (1970) using circular dichroism, optical rotatory dispersion and nuclear magnetic resonance recorded the transition from random coil to α-helix of polylysine upon its interaction with phosphatidyl serine vesicles. Since charge repulsion is a major factor in destabilizing the helix, and since the effects of an electric charge are far more pronounced at a surface than in the bulk (Davies & Rideal, 1961), this phase transition of the polypeptide would be expected to occur upon interaction with anionic BLM. In contrast, cytochrome *c*, which affected anionic BLM to a negligible extent, has been reported to undergo no change in optical rotation upon interaction with mixed beef heart phospholipids (Ulmer, Vallee, Gorchein & Neuberger, 1965) when in the form of an isooctane-soluble complex (Das & Crane, 1964), thus suggesting that the protein is already stabilized. However, it has been recently demonstrated by Kimelberg and Papahadjopoulos (1971*a*, *b*) that cytochrome *c* increased up to 3 orders of magnitude the $^{22}Na^+$-permeability

of phosphatidylserine but not phosphatidylcholine vesicles and increased the surface pressure of phosphatidylserine monolayers at the air-water interface. In line with this report, Gulik-Krzywicki, Schechter, Luzzati and Faure (1969) detected remarkable differences between the CD spectra of phosphatidyl-inositol-cytochrome *c* complex in water and free cytochrome *c* in solution. To the best of our knowledge, the effect of cytochrome *c* on phosphatidylserine or phosphatidyl-inositol black membranes has not been studied in detail and no comparison between the 2 models is yet available; however, these findings do indicate that, provided adequate experimental conditions are encountered, the interaction of cytochrome *c* with anionic BLM may be obtained.

It is of interest to note that similar modifications of the electrical characteristics of anionic BLM were induced by Ps^+ and by the cationic detergent HDTAB whose interaction with the membrane is predominantly hydrophobic. In 1935, Danielli and Harvey and Danielli and Davson suggested and presented some evidence regarding the surface-active nature of membrane proteins. Shah (1969, 1970) has studied the interaction of polylysine with stearic acid monolayers and detected decrease of the surface potential as well as expansion of the monolayers upon interaction with Ps^+; moreover, Ps^+ exhibited surface activity between pH 10 and 11 where coil-helix transformation occurs. With regard to the observations on anionic BLM, the profound structural and functional alterations of the membrane after interaction with Ps^+ suggest a surfactant tendency of the polypeptide.

The observations on the effects of trypsin on lipid-polypeptide interactions deserve particular comment. While it should be clear why addition of trypsin to the side where Ps^+ is present leads to reversal of the potential it is not readily apparent why addition of trypsin to the side opposite to that where Ps^+ is present would exert approximately the same effect, unless one conceives a profound rearrangement of the structure of the membrane. These observations are reminiscent of the inhibition of the excitability phenomena induced by EIM (excitability-inducing material) on BLM by protease addition to the compartment opposite to that where EIM is present (*cf.* Mueller & Rudin, 1969*b*) and the antigen-antibody reaction occurring when the components are present on opposite sides of the membrane, provided complement is supplied (Barfort, Arquilla & Vogelhust, 1968). This trypsin-sensitivity of the electrical changes induced by Ps^+ on anionic BLM, taken together with the expansion of stearic acid monolayers in the presence of Ps^+ (Shah, 1969, 1970) and the nuclear magnetic resonance evidence of the Ps^+-induced immobilization of the fatty acid chains of phosphatidylserine vesicles (Hammes & Schullery, 1970) indicates that the

"polypeptide phase" extends across the lipid film and suggests that while the initial force determining the interaction is predominantly electrostatic, the overall complex formation and membrane reassembly involves important hydrophobic interactions between the lipid and polypeptide components.

On account of these observations one is tempted to envision the structure of a biological membrane as a lipid protein mosaic, with a lipid bilayer as its core, which is penetrated, micellized or simply modified at discrete regions and to a varying extent by the protein, which confers to it the transport and enzymatic functions so characteristic of living systems (Gorter & Grendel, 1925; Danielli & Davson, 1935). This proposal explains most of the observations made so far on natural and artificial systems; moreover, it provides a physical basis on which the dynamicity and plasticity of biological phenomena can be understood in terms of phase transitions of lipid and protein components.

I subscribe to the opinion of Mueller and Rudin (1969*b*) that "attempts to reformulate or abandon the ... (bilayer) ... theory (Green & Purdue, 1966; Korn, 1966; Green *et al.*, 1967) lack comprehensiveness and consistency with the experimental observations. In some cases, e.g., (Lenard and Singer (1966)) (*see also* Glaser, Simpkins, Singer, Sheetz & Chan, 1970), the proposal that the insertion of protein regions into lamellar lipid layers presents a new alternative to the bilayer theory results simply from a misunderstanding of the more general aspects of that theory".

It is relevant to note that, in line with the polybase induced modifications of the permeability and structural characteristics of anionic model membranes are the modifications that follow the interaction of negatively charged viruses, bacteria, fungi and blood cells with positively charged polypeptides (*cf.* Katchalsky *et al.*, 1964) as well as the major influence of charged polypeptides on the uptake of macromolecules at the cell surface (*cf.* Ryser, 1968).

Finally, it is worth noting the possibility of constructing asymmetrically charged membranes by the selective adsorption of charged polypeptides or surfactants on ionic BLM; this might give rise to electrically active membranes of the type described by Shashoua (1969) and might give some insights into the problem of symmetry and function of biological membranes. It is also to be expected that the comprehension of the fundamental factors determining model lipid-polypeptide interaction in BLM will orient our efforts toward a rational reconstitution of more complex membrane functions such as transport ATPases (Jain, Strickholm & Cordes, 1969; Red-

wood, Muldner & Thompson, 1969; Mueller & Rudin, *personal communiaction*; Montal & Racker, *unpublished observations*).

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References

Applequist, J., Doty, P. 1962. α-helix formation in poly ε-carbobenzoxy-L-lysine and polylysine. *In:* Polyaminoacids, Polypeptides and Proteins. M. A. Stahmann, editor. p. 161. The University of Wisconsin Press, Madison, Wisconsin.

Bangham, A. D. 1963. Physical structure and behaviour of lipids and lipid enzymes. *In:* Advances in Lipid Research. R. Paoletti and D. Kritchevsky, editors. Vol. 1, p. 65. Academic Press Inc., N.Y.

Bangham, A. D. 1968. Membrane models with phospholipids. *In:* Progress in Biophysics and Molecular Biology. J. A. V. Butler and D. Noble, editors. Vol. 18, p. 29. Pergamon Press, N.Y.

Bangham, A. D., Horne, R. W. 1964. Negative staining of phospholipids and their structural modification by surface-active agents, as observed in the electron microscope. *J. Mol. Biol.* **8**:660.

Bangham, A. D., Standish, M. M.,Watkins, J. C. 1965. Diffusion of univalent ions across the lamella swollen phospholipids. *J. Mol. Biol.* **13**:238.

Barfort, P., Arquilla, E. R., Vogelhust, P. O. 1968. Resistance changes in lipid bilayers: immunological applications. *Science* **160**:1119.

Bradburry, E. M., Crane-Robinson, C., Goldman, H., Rattle, H. W. E. 1968. Proton magnetic resonance and optical spectroscopic studies of water soluble polypeptides: poly-L-lysine HBr, poly(L-glutamic acid), and copoly (L-glutamic acid^{42}, L-lysine HBr^{28}, L-alanine^{30}). *Biopolymers.* **6**:851.

Bungenberg de Jong, H. G. 1949. Complex colloid systems. *In:* Colloid Science. H. R. Kruyt, editor. Vol. 2, p. 335. Elsevier Publishing Co. Inc., N.Y., Amsterdam, London, Brussels.

Danielli, J. F., Davson, H. 1935. A contribution to the theory of permeability of thin films. *J. Cell Physiol.* **5**:495.

Danielli, J. F., Harvey, E. N. 1935. The tension at the surface of mackerel egg oil, with remarks on the nature of the cell surface. *J. Cell Physiol.* **5**:483.

Das, M. L., Crane, F. L. 1964. Proteolipids I. The formation of phospholipid cytochrome *c* complexes. *Biochemistry* **3**:696.

Davies, J. T., Rideal, E. K. 1961. Interfacial Phenomena. Chap. 2. Academic Press Inc., London.

Doty, P., Imahori, K., Klemperer, E. 1958. The solution properties and configurations of a polyampholytic polypeptide: Copoly-L-lysine-L-glutamic acid. *Proc. Nat. Acad. Sci.* **44**:424.

Finkelstein, A., Cass, A. 1968. Permeability and electrical properties of thin lipid membranes. *J. Gen. Physiol.* **52(1, pt. 2)**:145*s*.

Glaser, M., Simpkins, H., Singer, S. J., Sheetz, M., Chan, S. I. 1970. On the interactions of lipids and protein in the red blood cell membrane. *Proc. Nat. Acad. Sci.* **65**:721.

Gorter, E., Grendel, F. 1925. On bimolecular layers of lipoids on the chromocytes of the blood. *J. Exp. Med.* **41**:439.

Green, D. E., Allmam, D. W., Bachmann, E., Baum, H., Kopaczyk, K., Korman, E. F., Lipton, S., Mac Lennan, D. H., McConnell, D. G., Purdue, J. F., Rieske, J. S., Tzagoloff, A. 1967. Formation of membranes by repeating units. *Arch. Biochem. Biophys.* **119**:312.

Green, D. E., Purdue, J. F. 1966. Membranes as expressions of repeating units. *Proc. Nat. Acad. Sci.* **55**:1295.

Gulick-Krzywicki, T., Schechter, E., Luzzati, V., Faure, M. 1969. Interactions of proteins and lipids: Structure and polymorphism of protein-lipid-water phases. *Nature* **223**:1116.

Hammes, G. G., Schullery, S. E. 1970. Structure of macromolecular aggregates. II. Construction of model membranes from phospholipids and polypeptides. *Biochemistry* **9**:2555.

Hopfer, U., Lehninger, A. L., Lennarz, W. 1970*a*. The effect of the polar moiety of lipids on the ion permeability of bilayer membranes. *J. Membrane Biol.* **2**:41.

Hopfer, U., Lehninger, A. L., Lennarz, W. 1970*b*. The effect of the polar moiety of lipids on bilayer conductance induced by uncouplers of oxidative phosphorylation. *J. Membrane Biol.* **3**:142.

Hopfer, U., Lehninger, A. L., Thompson, T. E. 1968. Protonic conductance across phospholipid bilayer membranes induced by uncoupling agents for oxidative phosphorylation. *Proc. Nat. Acad. Sci.* **59**:484.

Huang, C. 1969. Studies on phosphatidylcholine vesicles. Formation and physical characteristics. *Biochemistry* **8**:344.

Jain, M. K., Strickholm, A., Cordes, E. H. 1969. Reconstitution of an ATP-mediated active transport system across black lipid membranes. *Nature* **222**:871.

Katchalsky, E., Sela, M., Silman, H. I., Berger, A. 1964. Polyaminoacids as protein models. *In:* The Proteins. Composition, Structure and Function. H. Neurath, editor. Vol. 2, p. 406. Academic Press Inc., N.Y.

Kimelberg, H. K., Papahadjopoulos, D. 1971*a*. Phospholipid-protein interactions: Membrane permeability correlated with monolayer "penetration". *Biochim. Biophys. Acta* **233**:805.

Kimelberg, H. K., Papahadjopoulos, D. 1971*b*. Interactions of basic proteins with phospholipid membranes. Binding and changes in the sodium permeability of phosphatidylserine vesicles. *J. Biol. Chem.* **246**:1142.

Korn, E. D. 1966. Structure of biological membranes: The unit membrane theory is reevaluated in light of the data now available. *Science* **153**:1491.

Lawrence, A. S. C. 1961. A new type of interfacial interaction. *In:* Symposium on Membrane Transport and Metabolism. A. Kleinzeller and A. Kotyk, editors. p. 35. Czechoslovak Academy of Sciences, Prague.

Lenard, J., Singer, S. J. 1966. Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. *Proc. Nat. Acad. Sci.* **56**:1828.

Luzzati, V., Husson, F. 1962. The structure of the liquid crystalline phases of lipid-water systems. *J. Cell Biol.* **12**:207.

Mac Innes, D. A. 1961. The Principles of Electrochemistry. Chap. 13. Dover Publications, Inc., New York.

McLaughlin, S. G. A., Szabo, G., Eisenman, G., Ciani, S. 1970. Surface charge and the conductance of phospholipid membranes. *Proc. Nat. Acad. Sci.* **67**:1268.

Mueller, P., Rudin, D. O. 1963. Induced excitability in reconstituted cell membrane structure. *J. Theoret. Biol.* **4**:268.

Mueller, P., Rudin, D. O. 1967. Development of $K^+ - Na^+$ discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* **26**:398.

Mueller, P., Rudin, D. O. 1968. Action potentials induced in bimolecular lipid membranes. *Nature* **217**:713.

Mueller, P., Rudin, D. O. 1969*a*. Bimolecular lipid membranes: Techniques of formation, study of electrical properties and induction of ionic gating phenomena. *In:* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stampfli, editors. p. 141. Springer-Verlag, Berlin, Heidelberg, N.Y.

Mueller, P., Rudin, D. O. 1969*b*. Translocators in bimolecular lipid membranes: Their role in dissipative and conservative bioenergy transductions. *In:* Current Topics in Bioenergetics. D. R. Sanadi, editor. Vol. 3, p. 157. Academic Press Inc., N.Y.

Mueller, P., Rudin, D. O., Tien, H. T., Wescott, W. C. 1962. Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. *Nature* **194**:979.

Mysels, K. J., Shinoda, K., Frankel, S. 1959. Soap Films: Study of Their Thinning. Pergamon Press Inc., N.Y.

Ohki, S., Aono, O. 1970. Phospholipid bilayer-micelle transformation. *J. Colloid Interface Sci.* **32**:270.

Redwood, W. R., Muldner, H., Thompson, T. E. 1969. Interaction of a bacterial adenosine-triphosphatase with phospholipid bilayers. *Proc. Nat. Acad. Sci.* **64**:989.

Ryser, H. J. P. 1968. Uptake of protein by mammalian cells: An underdeveloped area. *Science* **159**:390.

Seufert, W. D. 1965. Induced permeability changes in reconstituted cell membrane structure. *Nature* **207**:174.

Shah, D. O. 1969. Lipid-protein interactions in monolayers. Effect of conformation of poly-L-lysine on stearic acid monolayers. *Biochim. Biophys. Acta* **193**:217.

Shah, D. O. 1970. Lipid-polymer interactions in monolayers: Effect of conformation of poly-L-lysine on stearic acid monolayers. *In:* Surface Chemistry of Biological Systems. M. Blank, editor. p. 101. Plenum Press, N.Y.

Shashoua, V. E. 1969. Electrically active protein and polynucleic acid membranes. *In:* The Molecular Basis of Membrane Function. D. C. Tosteson, editor. p. 147. Prentice-Hall, Inc., Englewood Cliffs, N.J.

Skulachev, V. P., Sharaf, A. A., Liberman, E. A. 1967. Proton conductors in the respiratory chain and artificial membranes. *Nature* **216**:718.

Sollner, K. 1969. The electrochemistry of porous membranes, with particular reference to ion exchange membranes and their use in model studies of biophysical interest. *J. Macromol. Sci. Chem.* **A3(1)**:1.

Ter Minassian-Saraga, L., Wietzerbin, J. 1970. The action of hexadecyl-trimethylammonium bromide on bilayer lipid membranes. *Biochem. Biophys. Res. Commun.* **41**:1231.

Thompson, T. E., Henn, F. A. 1970. Experimental phospholipid model membranes. *In:* Membranes of Mitochondria and Chloroplasts. E. Racker, editor. p. 1. ACS Monograph 165. Van Nostrand Reinhold Co., N.Y.

Tien, H. T. 1970. The effect of modifiers on the intrinsic properties of bilayer lipid membranes (BLM). *In:* Surface Chemistry of Biological Systems. M. Blank, editor. p. 135. Plenum Press, N.Y.

Tien, H. T., Carbone, S., Dawidowicz, E. A. 1966. Formation of "black" lipid membranes by oxidation products of cholesterol. *Nature* **212**:718.

Tien, H. T., Diana, A. L. 1967*a*. Some physical properties of bimolecular lipid membranes produced from new lipid solutions. *Nature* **215**:1199.

Tien, H. T., Diana, A. L. 1967*b*. Black lipid membranes in aqueous media: The effect of salts on electrical properties. *J. Colloid Interface Sci.* **24**:287.

Ulmer, D. D., Vallee, B. L., Gorchein, A., Neuberger, A. 1965. Optical rotatory dispersion of cytochrome *c* phospholipid complexes. *Nature* **206**:825.