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Keyhole Limpet Hemocyanin (KLH)– Lipid Bilayer Membrane (BLM) Interaction

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Summary. The effect of keyhole limpet hemocyanin (KLH) on bimolecular lipid membranes formed in different buffers and at varying pH values was studied. The KLH was added after forming the black film. Upon its addition, the electrical resistance of the film decreased by as much as 6 orders of magnitude. The maximum change was observed in membranes bathed in "C" media at pH 7.1.

The KLH-induced bilayer showed a voltage-dependent negative resistance characteristic. This effect is similar to that reported by Mueller and Rudin (1968, *Nature* **217**:713) in EIM-induced lipid bilayers.

The properties and functions of cellular membranes appear to be based upon the interaction of their lipid and protein moieties. The development of techniques allowing for the formation of stable bimolecular lipid film between aqueous phases affords an opportunity to study artifical membrane systems which resemble cell membranes; i.e., thickness, electrical capacitance, dielectric strength, water permeability and surface tension [1, 5–7, 11, 17]. The resistance of thin bilayer lipid film is several orders of magnitude greater than that of cell membranes; however, it can be decreased to physiologic levels by a number of substances [2, 9, 10, 12–15, 18, 19]. Thus, the similarities between lipid bilayer membranes and biological membranes suggest that the lipid membranes may provide an accurate model for studying the interrelationship between cell membranes and certain biologically active substances.

It has been suggested that immunologic and enzymatic reactions on cell surfaces often result in large changes in membrane permeability. It has also been shown, that the addition of excitability inducing material (EIM), a proteineous compound, produces an increase in bilayer conductance and induces action potential [3, 14]. Little, however, is known about the protein-lipid bilayer interaction and the relationship between the increase in perme-

ability and the increase in conductance [15]. In an attempt to illucidate this interaction, experiments were performed to discern whether the fixation of keyhole limpet hemocyanin (KLH), a large copper-containing protein, to lipid bilayer membranes would influence their electrical resistance. KLH is among the most immunogenic of proteins.

Materials and Methods

Bilayer lipid membranes were made from the following solutions: (1) oxidized cholesterol prepared by bubbling oxygen through a 4% solution of cholesterol in *n*-decane at its fluxing temperature; (2) a mixture of 0.35% dodecyl acid phosphate and 0.39% cholesterol in *n*-dodecane; and (3) phospholipid extracts from pig red blood cells. The bilayers were formed according to the method of Mueller, Rudin, Tien and Wescott [14], while the red blood cells (RBC) were prepared as described by Dodge, Mitchell and Hanahan [4]. Using double-distilled octane as a solvent, the membranes were formed across a 1-mm diameter hole in a 10-ml teflon cup which was immersed in the desired solution. The temperature was regulated with a glass coil located outside the teflon cup and was varied with a Haake temperature bath. The bilayers were formed by spreading the lipid solutions over the hole with a Hamilton microsyringe. Initially, the membrane was thick; however, depending on the nature of the membrane material, it thinned down in 2 to 20 min.

Commercially prepared Key Hole Limpet Hemocyanin (KLH) was purchased from Calbiochem. Suspensions of KLH were prepared in physiologic saline, allowed to stand and then the insoluble material was removed by filtering. This material was then used to activate the lipid bilayer by adding varying amounts of the solution, e.g., 10 to 50 ml to the buffers bathing the membrane. This stock solution was generally stable and effective for weeks if refrigerated at 4 °C.

Various types of buffer solutions were used to bathe the bilayers. Among these were: (1) standard buffers of pH 10.0, 7.0 and 4.0 (Mallinckrodt Co.), (2) 0.85% saline (pH 7.2), (3) phosphate buffers (pH 6.8) and (4) "C" media (pH 7.2). The latter material is a nutrient media used for growing viruses.

The electrical properties of the membrane were measured as described by Pant and Rosenberg [15]. The potential was applied from a mercury battery with a tenturn helipot. The current and potential were measured with a Keithley 610BR electrometer in current and voltage mode. The electrodes consisted of two identical calomel electrodes with saturated salt bridges. The applied potential was measured with another Keithley 610BR electrometer in voltage mode.

Results

The addition of KLH caused a dramatic change in the electrical properties of the bimolecular lipid membrane. Prior to KLH addition, the current-voltage curve for the membrane with 0.85% saline on both sides of the membrane was linear and had a conductance of approximately 10^{-8} ohms^{-1} $\times$ cm^{-2}. After KLH was added, the conductance increased by five orders of magnitude within 5 to 20 min. KLH-induced conductance was voltage-dependent, giving a negative resistance. The survival time of the membrane

Table 1. Effect of KLH on electrical conductivity of oxidized cholesterol bimolecular lipid membrane with varying pH values and solutions

Medium	Electrical conductivity ($ohm^{-1}\ cm^{-2}$)	
	With KLH	Without KLH
"C" media, pH=7.1	10.50×10^{-3}	5×10^{-8}
Phosphate buffer, pH=6.8	6.50×10^{-3}	5×10^{-8}
Saline solution, pH=7.0	2.05×10^{-3}	5×10^{-8}
Standard buffer, pH=7.0	6.00×10^{-3}	5×10^{-8}
Standard buffer, pH=10.0	12.00×10^{-8}	5×10^{-8}
Standard buffer, pH=4.01	15.00×10^{-8}	5×10^{-8}

decreased in the presence of KLH, but the membrane appeared clean and regular. Table 1 shows the conductance of the oxidized cholesterol bilayer at different pH values, both in the presence and absence of hemocyanin. The conductance in the absence of hemocyanin was the same at all pH values. In the presence of KLH, the maximum increase in conductance was noted when the bilayer was bathed in "C" media at physiologic pH. With the other buffers, only slight changes were noted at this pH. At pH 4.01 the conductance decreased to near the bare membrane value. This was interpreted as being caused by denaturation of the protein at this pH. At higher pH values, i.e. pH 10.0, the conductance decreased by a factor of 10^3 which may have been caused by denaturation, dissociation of the protein molecules, or minimization of the protein-bilayer interaction. The conductance increase of the bilayer has been observed when double-distilled water was used as the bathing solution, but the membrane was poorly stable under these conditions. Increases in bilayer conductance have also been noted when buffers at different pH values have been placed on either side of the membrane, i.e. pH 4.01 on one side and pH 7.0 on the other. The rectifying behavior of the current-voltage (I/V) curve, however, was not affected in these instances.

Fig. 1, curve *a* shows the I/V curve of oxidized cholesterol bilayer film in the presence of KLH in saline. This KLH-induced conductance was voltage-dependent and showed negative resistance. Current increased up to 25 mV of applied potential, but further increases in potential caused decreases in current. This decrease continued until 45 mV were applied; however, above this value, the current increased linearly. All current values were steady state. At each 5-mV increment, readings were taken when the current reached a stable value. Similar results were observed when using pig red blood cell membranes (Fig. 1, curve *b*). The nature of the I/V curve in

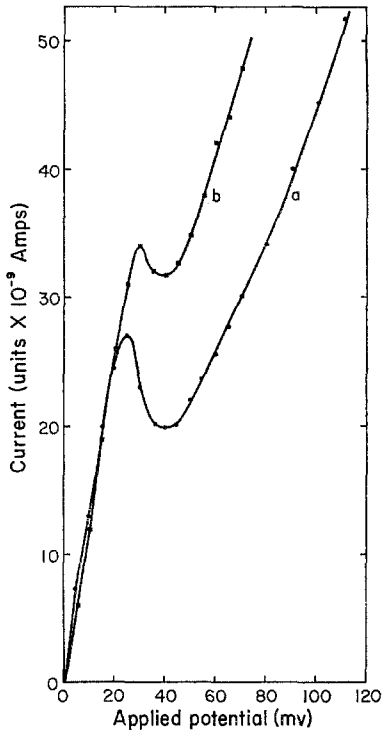

Fig. 1. Curve *a:* current-voltage (I/V) curve of oxidized cholesterol bimolecular lipid membrane in presence of keyhole limpet hemocyanin (KLH) in 0.85% saline as bathing solution. Curve *b:* I/V curve of pig red blood cell (RBC) bimolecular lipid membrane in presence of (KLH) in 0.85% saline as bathing solution

KLH-induced bilayer was dependent on the nature of the bathing solution. In "C" media, the current started decreasing after 100 mV of applied potential. In the presence of KLH, voltage-dependent oscillations have also been observed. The amplitude and frequency of oscillations were largest at the value of the applied potential which corresponded to the minimum conductance in the I/V curve. The conductance measurements were also made within a short range of different temperatures, e.g. 23 to 35 °C. The range was limited since the bilayer films were not stable above 35 °C. Within this short range of temperature, conductivity varied exponentially with temperature.

At higher concentrations of KLH (1 ml saturated solution) it was not possible to achieve a stable low resistance bilayer, since the bilayer was punctured at this concentration. No appreciable emf was found on adding hemocyanin on one side of the bilayer in open circuit. The maximum self emf created by the protein was ± 1.0 mV.

Discussion

The large conductance increase of the bilayer lipid membranes in the presence of KLH may be caused by the interaction between the bilayer material and protein. The possibility of the formation of channels and/or pores during this interaction, as has been suggested by Bean, Shepherd, Chan and Eichner [3] in EIM-induced lipid films, cannot be ignored. The most important observation was the effect of pH on conductance in the presence of protein. This observation suggests that the protein interacts with the lipid bilayers only under certain specific conditions. The lack of activity at pH 4.01 may be caused by denaturation of the protein. This same effect at a higher pH, i.e. 10.0, may be caused by denaturation or dissociation, since Bartel and Campbell have shown that KLH dissociates at pH 8.5 in borate buffer [2]. Further studies in this area will hopefully illustrate the nature of the lipid-protein interaction.

Although this present study is only a superficial examination of lipid-protein interaction, it appears that lipid bilayer membranes may represent an excellent experimental model for the study of cell wall interaction with biologically active substances.

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