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Structure of planar membrane formed from liposomes

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Lipid vesicles with incorporated ion channels from polyene antibiotic amphotericin B were used to investigate structures of planar membranes formed by Shindler's techniques. A planar membrane assembled on the aperture in a lavsan film from two layers generated at the air-aqueous liposome suspension interface is not a simple bilayer but a bimolecular membrane containing numerous partly fused liposomes. A complete fusion of liposomal membranes with the planar bilayer is an unlikely event during membrane formation. A planar bimolecular lipid membrane without incorporated liposomes can be made by a method consisting of three stages: (1) formation of a lipid layer on the air-water interface of a suspension containing liposomes, (2) transfer of this layer along the surface of the solution into a chamber containing a solution without liposomes where a lipid monomolecular layer forms gradually (within about 20 min) at the air-water interface, (3) assembling of the planar bilayer membrane from this monolayer. The knowledge of the planar membrane structure may be useful in experiments on incorporation of membrane proteins into a planar lipid bilayer.

Introduction

Reconstitution of membrane proteins in a planar lipid membrane is a very important development in membrane protein studies because it allows the electric properties of such proteins to be characterized in detail even if the 'patch clamp' method [1] cannot be used. Recently, a great deal of progress has been made by H. Schindler and coworkers towards developing the reconstitution technique [2–4]. According to the procedure described in Refs. 2–4, the planar membrane was formed from lipid-protein vesicles in the following way. A suspension of vesicles was put into two compartments of a Teflon cell to just below the aperture in the membrane frame. After some time, lipid-protein layers spontaneously appeared at the air-water interfaces. The planar bilayer membrane was formed by raising the water level above the aperture.

It is obvious that the thermodynamic conditions are different for proteins in the bimolecular lipid membrane and in the lipid monolayer at the air-water interface. Therefore a question arises, i.e., whether membrane proteins do migrate from proteoliposomes to the phospholipid monolayer at the air-water interface and if so whether they remain native? We can assume that membrane proteins are translocated from proteoliposomes to bimolecular lipid membranes without being incorporated into the monolayer as follows: first, proteoliposomes partially fuse with the phospholipid monolayer at the air-water interface, as shown in Fig. 1a; then complete implantation of this vesicles into lipid bilayer takes place during bilayer formation, as shown in Fig. 1d.

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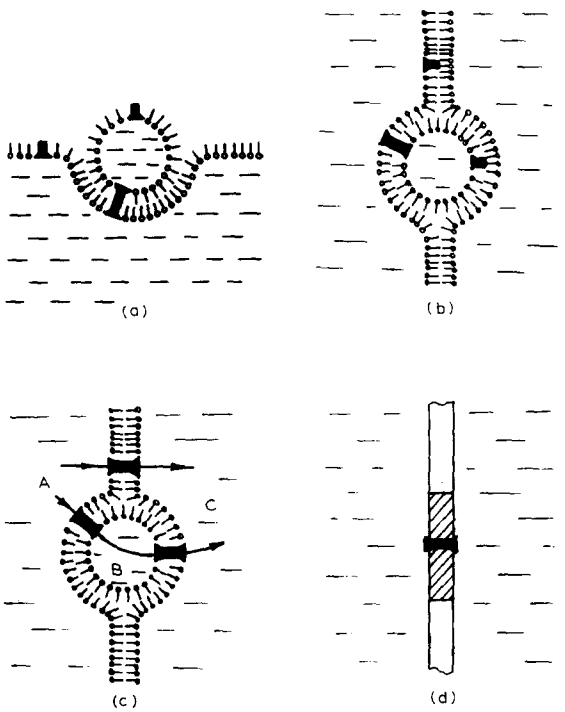

Fig. 1. Liposome structures. (a), a hypothetical structure of a liposome partially fused with a monolayer. (b), the structure of a liposome partially fused with planar lipid bilayer. The membrane was formed by method 1 of two different monolayers. One cell compartment contained liposomes with amphotericin B, the other without amphotericin B. Amphotericin B channels composed of one 'semipore' and of two 'semipores' are shown. (c), the structure of liposome partially fused with planar lipid bilayer. The membrane was formed by method 1 of two identical layers. Both compartments contained liposomes with amphotericin B. Amphotericin B channels assembled of two 'semipores' are shown. Arrows indicate current flowing across planar membrane (I_{AC}) and current through partially fused liposome (I_{ABC}). (d), a liposome implanted completely in planar lipid bilayer (dashed portion of the membrane).

There is evidence for the above assumption. In Ref. 5 a lipid layer at the air-water interface of a liposome solution has been studied by surface pressure-area measurements. The author concluded that numerous vesicles are present just beneath the lipid monolayer at the solution surface. The outer lipid layer of the liposomes is involved in the lipid transport to and from the monolayer at the surface. A question arises, i.e., what is the mechanism of such an exchange? It can be assumed that liposomes situated just beneath the lipid monolayer as shown in Fig. 1a. A part of these liposomes appears partly fused with bimolecular lipid membrane as shown in Fig. 1b, c. The other part of vesicles can be completely implanted into lipid bilayer during membrane formation as shown in Fig. 1d.

In the present work we investigated the possibility of such an implantation. For that purpose we used liposomes with incorporated ion channels formed by polyene antibiotic amphotericin B. Another aim of this study was to estimate how many closed liposomes can be incorporated into Schindler's membrane (Fig. 1b, c). The knowledge of that is essential for the analysis of the properties of proteins incorporated into a planar membrane. Liposomes with amphotericin B were used for that as well.

The properties of amphotericin B induced ion channels were described in Refs. 6–9. The high conductivity of the membrane arose after addition of amphotericin B at both sides of the lipid bilayer due to the anion channels strongly bound to the membrane. It was suggested that these anion channels consisted of two 'semipores'. After adding amphotericin B at one side of the membrane, single 'semipore' channels were formed, the bimolecular lipid membrane conductivity in this case was very small and cation selective.

Materials and Methods

Chemicals. Tris, EDTA and sucrose were purchased from Serva. Tetraethylammonium chloride was from Fluka. Bovine brain phospholipids were purified according to Folch et al. [10].

Preparation of liposomes. Liposome suspension was prepared as described [3]. The water solution contained KCl/sucrose (in some of the experiments)/1 mM EDTA 10 mM Tris-HCl (pH 7.35), 35 °C. Salt and sucrose concentrations were the same inside and outside the vesicles, they are given in figure legends. The lipid concentration in the liposome suspension was 6 g/l. Amphotericin B channels were incorporated into a liposomal membrane by means of an ultrasonic disperser USDN-1 (22 kHz, 50 W/cm^2). A mixture of the liposome suspension (5 ml) and amphotericin B (5 μM) was sonicated for 4 min. The prepared suspension was stored for 1 h at 35 °C and then put into a Teflon cell. The size and structure of vesicles

were determined using an electron microscope JEM-7A [12]. This preparation was a mixture of closed single-bilayer vesicles and of a small amount of closed multi-bilayer vesicles with a radius of about 70 nm.

Membrane formation. Method 1. The Teflon cell had two compartments separated by a lavsan film with an aperture of 100 μm in diameter. The aperture was pretreated with hexadecane. The suspension of vesicles with amphotericin B was added to the two compartments of the Teflon cell to just below the aperture in the lavsan film. The presence of phospholipid at the air-water interfaces was checked by surface pressure measurements [5,11]. The equilibrium surface pressure was 45 ± 2 mN/m. The equilibrium surface pressure of the layer formed by liposomes without amphotericin B was the same. The planar membrane was formed by raising the water level above the aperture.

Membrane formation. Method 2. Fig. 2 shows schematically the construction of the cell used for bilayer formation. The surface areas of compartments 1, 4 and 2, 3 were 1.5 cm^2 and 1 cm^2, respectively. Electrolite (without vesicles and antibiotic was filled into compartments 2 and 3 to just below the aperture in the lavsan film (6). After that, amphotericin B was added into compartment 3 up to a concentration of 50 nM. The suspension of vesicles with incorporated amphotericin B channels was added to compartment 1. The solutions in compartments 1 and 2 communicated with each other via a curved etched glass of 1.5×1 cm^2 surface area. Compartment 4 was empty. A layer of phospholipid with amphotericin B was spontaneously formed at the air-water interface in compartment 1. This layer spread over the wet surface of the curved glass (5) to compartment 2 [5]. The surface pressure in compartment 2 reached its constant value of 43 ± 2 mN/m within about 3 min after adding the vesicle suspension into compartment 1 (Fig. 3).

The phospholipid monolayer at the air-water interface in compartment 3 was formed by another method. A small amount of the hexane solution of phospholipids was distributed over the surface of the electrolite. A monomolecular layer of phospholipids was formed at the surface of the electrolite after evaporation of hexane.

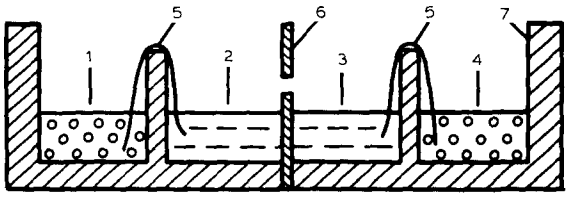

Fig. 2. The scheme of the measuring cell. (1, 4, aqueous suspension of liposomes; 2, 3, electrolyte; 5, a curved glass with a wet surface; 6, lavsan film with an aperture of 100 μm in diameter at which a planar membrane was formed; 7, Teflon.

The planar membrane was formed by raising the water level in compartments 2 and 3 above the aperture in the lavsan film within a definite time, t, after addition of liposome suspension into compartment 1.

Membrane formation. Method 3. The cell construction was the same as in method 2 (Fig. 2). The electrolite (without vesicles and antibiotic was filled into compartments 2 and 3 to just below the aperture. Curved etched glasses (5) connected the compartments 1 and 2, 3 and 4. The suspension of vesicles with amphotericin B was added to compartments 1 and 4. Layers of phospholipid with amphotericin B were spontaneously generated at the solution surface in compartments 1 and 4. Layers spread over the wet surfaces of the curved glasses to compartments 2 and 3. The presence of phospholipid layers at the air-water interface was checked by surface pressure measurements.

The planar membrane was formed by raising

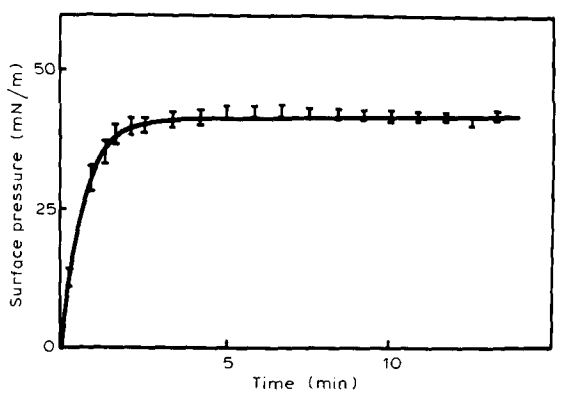

Fig. 3. Increase in surface pressure in compartment 2 with time. The time registration was started from addition of liposomes to compartment 1.

the water levels in compartments 2 and 3 above the aperture in the lavsan film within 30 min after addition of liposome suspension into compartments 1 and 4.

The specific capacity of the membranes formed by all three methods was the same, 8 ± 1 mF/m^2. The transmembrane current was measured by means of a 'Keithley 301' electrometer amplifier. In the measurements of current-voltage characteristics, the transmembrane voltage was increased at a constant rate of 3.0 mV/s. The conductance of the unmodified membranes (without antibiotic) was below 1 pS.

A peristaltic pump (LKB) was used to change solutions in the cell. This procedure did not destroy the membrane.

Results and Discussion

Implantation of vesicles into Schindler's membrane as shown in Fig. 1d

The following experiment was done in order to solve the question whether the implantation of vesicles into bilayer (as shown in Fig. 1d) takes place during membrane formation. The planar membrane was assembled from two different layers by Method 1. The *cis*-layer was formed by vesicles with incorporated amphotericin B channels, the *trans*-layer was formed by liposomes without amphotericin B. Fig. 4 shows the short-circuit current through such a membrane. For more than 2 h the membrane conductance persisted below 13 pS. The membrane was cation selective.

These observations suggest that during such membrane formation the liposomes did not fuse completely with the planar bilayer as it has been shown in Fig. 1d. When amphotericin-enriched liposomes are implanted in membrane (Fig. 1d) anion selectivity and a high membrane conductance should be expected because channels consisting of two 'semipores' (Fig. 1d) are strongly bound to the lipid bilayer [19]. However, in our experiment the conductance was as low as 8 ± 5 pS and the selectivity was cationic. The low cation conductance of the membrane appears to be due to the partial fusion of liposomes with membrane, as seen in Fig. 1b. In this case amphotericin B is present at one side of membrane only. In Refs. 6 and 8 it was shown that the presence of the polyene antibiotic only at one side of the lipid bilayer causes a low cation conductance of membrane.

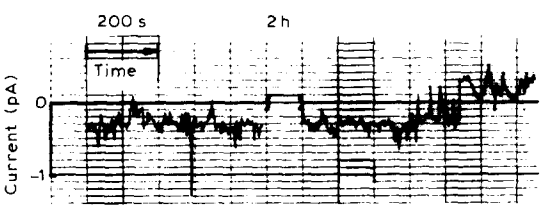

Fig. 4. Short-circuit current across a planar membrane. At the *cis*-side of the membrane, liposomes with amphotericin B (0.25 M KCl) 0.4 M sucrose; at the *trans* side, liposomes without amphotericin B (0.05 M KCl). The membrane was assembled of two layers at the first trial. 2 h after membrane formation zero current potential was 36 ± 3 mV ($P_{K^+} > P_{Cl^-}$).

Fig. 4 also indicates that under the given conditions the implantation of liposomes in membrane directly from the solution (as shown in Fig. 1d) is hardly likely. It is seen that the current changed in a jump as late as 2 h after membrane formation, the selectivity being changed from a potassium to chloride one. Such a jump of current indicates that a single liposome with incorporated amphotericin B channels has been implanted in the planar bilayer, as shown in Fig. 1d.

At the same time, there is evidence [13] that the implantation of liposomes in a lipid membrane containing a solvent (heptane + decane) is more likely than it was under the conditions of our experiment. The fact that no essential quantity of liposomes was implanted into membrane directly from the solution in our experiment can probably be explained by the use of membrane without solvent. Evidence for such an explanation is provided by the results of Ref. 14 where fusion of two planar bilayers without solvent has been studied. It has been shown that such membranes do not fuse in the absence of Ca^{2+} in the solution and at a membrane voltage below the puncture one. Therefore it can be assumed that under the conditions of our experiment the implantation of liposomes into a planar lipid bilayer without solvent cannot occur (Fig. 1d).

According to Ref. 15, the presence of 0.4 M sucrose only in one compartment inside and outside the liposomes facilitates their incorporation into lipid membrane with solvent (decane +

heptane). But in our case such an implantation under the same conditions was very unlikely.

Probably analogous conclusions will be valid also for liposomes with various membrane proteins incorporated. If no direct implantation of proteoliposomes in lipid membranes occurs, the proteins are expected, when incorporated into lipid bilayer in such a way, to remain in the phospholipid layer at the water-air interface for some time.

To make sure that liposomes contain a sufficient number of amphotericin B channels, the layers in both compartments were formed of liposomes with incorporated amphotericin B channels. The layers were treated by Method 1 to assemble a planar membrane. Fig. 5 shows the short-circuit current occurring with time after membrane formation. The ratio of KCL concentrations at opposite sides of the membrane was 5. The potential of zero current was equal to -27 mV and did not vary in time ($P_K + < P_{Cl^-}$).

The measured potential of zero current coincides with the value known for an amphotericin B channel assembled from two semipores, which should be expected, as amphotericin B was present at both sides of the membrane. In this case (Fig. 5) the membrane conductance was more than three orders higher than that in the preceding experiment (Fig. 4) and equalled approx. 20 nS, which indicated a great number of amphotericin channels incorporated into liposomes. The rapid increase of conductance just after membrane formation seen in Fig. 5 seems to be due to the amphotericin B channels formed by the semipores being initially situated in opposite phospholipid monolayers at the air-water interface.

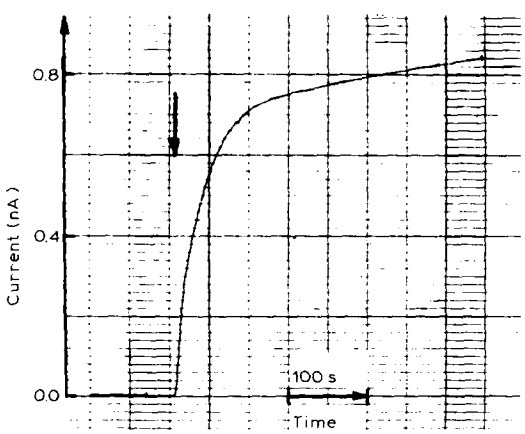

Fig. 5. Short-circuit current across a planar membrane in the presence of amphotericin B at both sides of the membrane. The arrow indicates the moment at which membrane was formed. Zero current potential after membrane formation was -27 ± 2 mV ($P_K + < P_{Cl^-}$) and did not change with time. At the *cis*-side of the membrane, liposomes with amphotericin B/0.25 M KCl; at the *trans*-side, liposomes with amphotericin B/0.05 M KCl.

Partial fusion of liposomes with a membrane formed by Method 1

In studying the behaviour of membrane proteins incorporated into lipid membrane by Schindler's technique, it is essential to know how many liposomes fuse partially with the planar membrane to form a structure shown in Fig. 1b, c. This

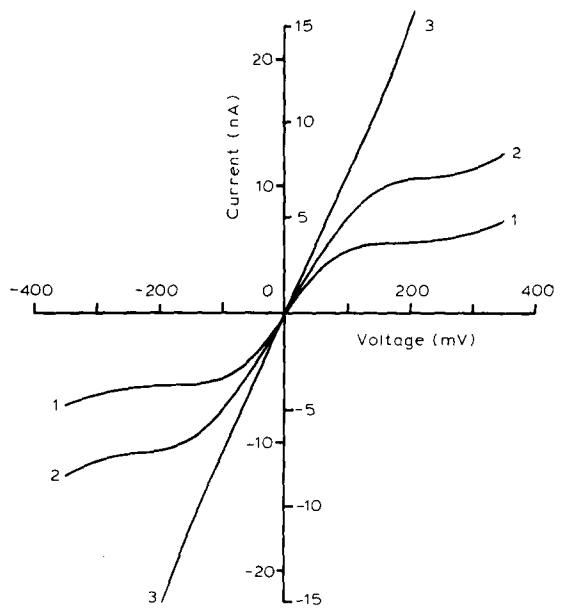

Fig. 6. Current-voltage characteristic of the membrane 1 h after its formation by method 1 from amphotericin B-containing liposomes: 1, in the presence of 2 mM tetraethylammonium inside and outside the liposomes; 2, after replacing the solutions in the cell with tetraethylammonium-free ones, the membrane not being destructed by replacing procedure; 3, in the absence of tetraethylammonium both inside and outside the liposomes, the current was not blocked. Curve 3 is well approximated by a straight line, as distinct from curves 1 and 2. The scale of current for curves 1 and 2 is shown on the ordinate to the right and for curve 3 to the left. In both cell compartments 0.25 M KCl was present.

problem was investigated qualitatively.

For this purpose, amphotericin B containing liposomes with tetraethylammonium inside and outside were used. Tetraethylammonium is a blocker of amphotericin B channels [16]. From such a suspension layers were formed, and a membrane was formed by Method 1. Prior to measurements, the membrane was allowed to stand for 1 h so that surface densities of amphotericin B channels in partly fused liposomes and planar bilayer equilibrated. The plot 1 in Fig. 6 shows the current-voltage characteristic of a membrane thus formed. It can be distinctly seen that the plot deviates from the tangent through the origin towards the abscissa with increasing membrane voltage. Such a deviation takes place due to the blocking of transmembrane current by tetraethylammonium. Then the suspensions in both compartments were replaced with solutions of the same ionic composition but without tetraethylammonium. As seen from plot 2 of Fig. 6, the current blocking decreased after removal of tetraethylammonium, although it did not entirely disappear.

This result gives evidence that a sufficient quantity of liposomes has been partially fused with planar bilayer. If planar membrane contained no liposomes partially fused with it, the current blocking would not occur after removal of tetraethylammonium, since it has been shown that this blocker can be easily washed off from amphotericin channels in membrane (Silbershtein, A.Ya., unpublished data). By contrast, if planar membrane contains numerous partially fused liposomes the transmembrane current is a sum of two components: the current through the channels in planar bilayer and that through amphotericin channels in partially fused liposomes (Fig. 1c).

After the removal of tetraethylammonium, the blocking of the current through amphotericin channels in planar bilayer must disappear, whereas that of the current through amphotericin channels in partially fused liposomes must only slightly fall but not disappear. This is caused by tetraethylammonium remaining inside partially fused liposomes after replacing the solutions.

In order to arrive at another independent argument for the occurrence of incorporated vesicles in the membrane formed by Schindler's technique, an additional experiment was made. Curve 1 in

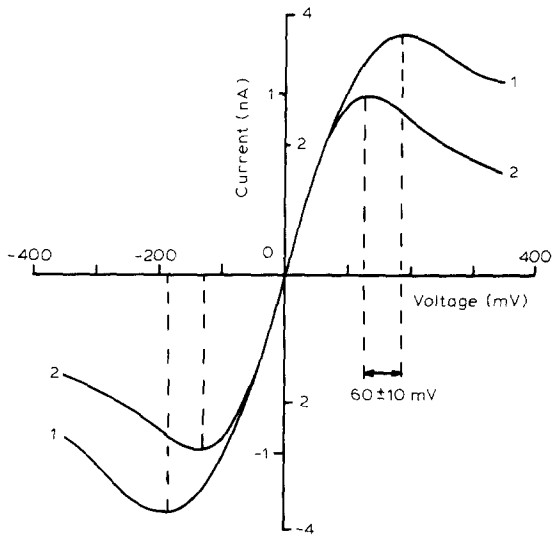

Fig. 7. Blocking of current with tetraethylammonium for membranes formed by Shindler's and Montal-Mueller's techniques in the presence of amphotericin B. 1, current-voltage characteristic of a membrane formed by method 1 1 h after its formation from amphotericin B-containing liposomes in the presence of 10 mM tetraethylammonium inside and outside liposomes; 2, current-voltage characteristic of planar bilayer formed by Montal-Mueller's technique in the presence of 50 nm amphotericin B/10 mM tetraethylammonium. The current scale for curve 1 is shown on the ordinate axis to the right, for curve 2 to the left. The cell contained 0.15 M KCl.

Fig. 7 presents the current-voltage characteristic of the membrane 1 h after its formation by method 1 from liposomes with amphotericin B, 10 mM tetraethylammonium being outside and inside. For comparison, curve 2 in Fig. 7 present the current-voltage characteristic of the bimolecular lipid membrane formed by Montal-Mueler's technique from monomolecular lipid layers while 50 nM amphotericin B and 10 mM tetraethylammonium were present at both sides of the membrane. The current scales for curves 1 and 2 had been chosen so that the tangents to both curves coinside at origin. Curves 1 and 2 are seen to be different in form. The current-voltage characteristic for curve 1 reaches its maximum at 125 ± 5 mV, whereas that for curve 2 at 185 ± 5 mV. The extremum shift of 60 ± 10 mV is a reliable, well reproducible value. Besides, though the tangents of curve 1 and curve 2 coincide at the origin, curve 1 runs above curve 2 at voltage exceeding 50 mV. This indicates that for the voltages given the coefficient of block-

ing (α) for Montal-Mueller's membrane is greater than that for Schindler's membrane ($\alpha = I_1/I$ where I_1 and I are blocked and resting currents, respectively).

Such a difference in current-voltage characteristics can be explained, like in the above case, by the fact that Schindler's membrane contains greater amounts of partially fused liposomes compared to Montal-Mueller's membrane. Indeed, let us consider the current passing through a liposome partially fused with planar bilayer versus the voltage between the two cell compartments, V_{AG} (Fig. 1c). In this case the voltage falls on both liposome membrane, $V_{AC} = V_{AB} + V_{BC}$. Thus, the voltages on amphotericin B channels incorporated into partially fused liposomes, V_{AB} and V_{BC}, are less than that on amphotericin B channels incorporated into planar bilayer V_{AC}. Because the blocking coefficient rises with increasing membrane voltage and is independent of the number of amphotericin B channels [18], the coefficient of blocking of current through the liposome, I_{ABC}, at a given voltage, V_{AC}, is less than that of current through planar bilayer I_{AC}. Therefore, the blocking coefficient is greater for that membrane in which the partially fused liposomes are less in number.

Thus, two independent experiments made us conclude that Schindler's membrane contains a sufficient quantity of liposomes partially fused with it. It is of interest to estimate the membrane area that is occupied by partially fused liposomes. In Appendices 1 and 2 such estimation is made for two different experiments, the results of which are presented in Figs. 6 and 7. Both estimates give equal results: about 50% of the membrane area is occupied by partially fused liposomes.

A question arises as to what way such a great number of liposomes can partially fuse with planar bilayer in the case of a membrane formed by method 1. It can be assumed that liposomes first partly fuse with the phospholipid monolayers at the air-water interface (Fig. 1a), then after membrane formation they become partially fused with planar bilayer (Fig. 2b, c). A different mechanism is also possible. The liposomes in the solution directly fuse with already formed planar bilayer. To clarify the possibility of the second mechanism an additional experiment was performed.

Partial fusion of liposomes with Montal-Mueller's membrane

Aqueous solution containing 0.15 mKCl/10 mM tetraethylammonium was in the cell. The planar bilayer was formed by Montal-Mueller's technique [17]. Then 1.05 g/l liposomes with incorporated amphotericin B channels were added at both sides of the bimolecular lipid membrane. Inside and outside the liposomes 10 mM tetraethylammonium and 0.15 M KCl were present. 2 h after adding liposomes the current-voltage characteristic was measured. It appeared that by changing the scale on the current axis, the plot of this characteristic can be made coincide (within the experimental error) with that for the Montal-Mueller membrane at both sides of which 50 nM amphotericin B was present and liposomes were absent (Fig. 7, plot 2). In both cases the current-voltage characteristics reached their extrema at voltages of ± 125 (± 5) mV.

Such coincidence of current-voltage characteristics indicates that no bulk partial fusion of liposomes with Montal-Mueller's membrane takes place. The partial fusion of liposomes with planar bilayer would produce a shift in the extremum position toward greater absolute values of voltage, as shown above. The estimations made by the method described in Appendix 1 showed (with an accuracy of 10%) that there were no partly fused liposomes in the Montal-Mueller membrane.

To summarize:

1. Complete fusion of liposomes with the planar bilayer, as shown in Fig. 1d, was not observed during membrane formation from liposomes by method 1. Therefore, an assumption can be made that an analogous implantation of proteoliposomes into planar bilayer is also hardly likely. A number of membrane proteins seem to stay for some time in the phospholipid monolayer at the air-water interface, when being incorporated into planar lipid membrane by this method.

2. A membrane thus formed contains numerous closed liposomes partially fused with the planar bilayer (Fig. 1b, c). Therefore, it can be assumed that a membrane formed from proteoliposomes by the above technique also contains a great number of proteoliposomes partially fused with the planar lipid bilayer. Some membrane proteins can migrat to bimolecular lipid mem-

branes from such proteoliposomes.

3. The incorporation of such a great number of closed liposomes to Schindler's membrane appears to occur as follows: liposomes first partially fuse with the phospholipid monolayer at the air-water interface (Fig. 1a) (or they interact with the phospholipid monolayer by another way). Then, after membrane formation, these closed liposomes are found incorporated into planar bilayer (Fig. 1b, c).

The complex structure of such model membranes makes the investigation of natural membrane proteins incorporated into it difficult. A technique is needed to form a planar membrane without incorporated closed vesicles. We present such a technique (method 3), using liposomes containing amphotericin B ion channels. But first let us consider the electrical properties of a membrane formed by Method 2.

Partial fusion of liposomes with membrane formed by method 2

The measurements carried out on membranes formed by method 2 showed: if a membrane was made 3.5 or 5.5 min after introduction of liposomes into compartment 1, its conductivity was 1600 ± 700 or 90 ± 50 pS (0.1 KCl/10 mM tetraethylammonium), respectively. At the same time, the conductivity of a membrane formed 30 min after introduction of liposomes into compartment 1 was as low as that of unmodified lipid membrane (below 1 pS) and did not increase throughout the time of observation (1 h).

The high conductivity of membranes formed in the first period after addition of liposomes to compartment 1 can be most probably explained by two arguments.

First, as stated above, the phospholipid monolayer at the air-water interface (compartment 1) contains numerous liposomes which are partially fused with it (Fig. 1a). In our case, such a layer spontaneously propagates from compartment 1 to the surface of solution in compartment 2. Certainly, in the first period the monolayer in compartment 2 also contains a great number of partially fused liposomes. Hence, the planar membrane formed from such a layer will include the incorporated liposomes and the transmembrane ion current will flow through amphotericin B channels situated in incorporated liposomes as shows in Fig. 1c. Therefore, the membrane conductance must be great.

In order to show the presence of liposomes incorporated into planar membrane let us compare the current-voltage characteristic of a membrane formed by method 2 5.5 min after addition of liposomes to compartment 1 (Fig. 8, plot 1) with that of Montal-Mueller's membrane [17] in the presence of 35 nM amphotericin B and 10 mM tetraethylammonium at both sides of the membrane (Fig. 8, plot 2). It follows from plots 1 and 2 (Fig. 8) that the maximum current for the membrane formed by method 2 is shifted by 40 ± 10 mV towards greater voltages as compared to that for Montal-Mueller's membrane. Such a maximum current shift was observed for all membranes formed by method 2 5.5 min after addition of liposomes to compartment 1. The shift of the maximum current to greater voltage indicates, according to the above reasoning, the presence of a great number of liposomes incorporated to planar bilayer (Fig. 1c).

The second reason for the high conductivity of the membrane formed in the initial period after addition of liposomes to compartment 1 can be the presence of amphotericin B semipores in the monolayers of compartments 2 and 3. After assembling of the membrane, the amphotericin B semipores situated at the opposite sides of the membrane form ion channels in the membrane by joining to each other, which results in an increase of conductivity. That such a conductance increase takes place does not contradict the above argument that membrane formed by method 2 contains incorporated liposomes.

Let us consider the factors responsible for the low conductance of a membrane formed by method 2 30 min after addition of liposomes to compartment 1.

Amphotericin B semipores found in the phospholipid monolayer at the air-water interface in compartment 2 appear to migrate rapidly to the solution of the compartment from where amphotericin B was previously absent. This assumption is based on the evidence that amphotericin B semipores are rapidly (in about 1 min) washed out from lipid membrane to the solution [19]. Thus, the second reason for the high membrane conduc-

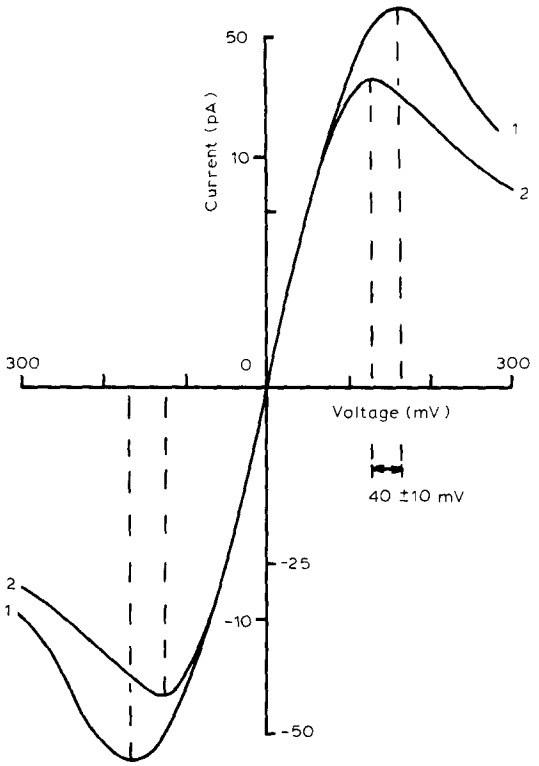

Fig. 8. Comparison of the blocking of transmembrane current with tetraethylammonium for membrane formed by method 2 with that for membranes formed by Montal-Mueller's technique in the presence of amphotericin B. 1, current-voltage characteristic of the membrane formed by method 2 5.5 min after introduction of liposomes to compartment 1. 10 mM tetraethylammonium were present in the solution of the cell (inside and outside of the liposomes); 2, current-voltage characteristic of planar bilayer formed by Montal-Mueller's technique in the presence of 35 nM amphotericin B and 10 mM tetraethylammonium at both membrane sides. The current scale for plot 1 is given on the ordinate axis to the left, for plot 2 to the right. Both characteristics were measured 1 h after membrane formation.

tance is valid only when the membrane is formed shortly enough after addition of liposomes to compartment 1.

Moreover, the low conductance of a membrane formed by method 2 30 min after addition of liposomes to compartment 1 indicates that such a membrane presents a planar bilayer without incorporated liposomes. Probably the phospholipid layer at the surface of the solution in compartment 2 30 min after introduction of liposomes to compartment 1 presents a monomolecular phospholipid layer without incorporated liposomes. Now consider the opposite: let a phospholipid monolayer with incorporated liposomes be situated in compartment 2 at the air-water interface 30 min after addition of liposomes to compartment 1 (Fig. 1a). Since amphotericin B channels assembled from two semipores, according to the control data, are very slowly washed from the lipid bilayer (time constant > 1 h) a fused liposome would contain amphotericin B ion channels. In this case, the conductance of a membrane formed by method 2 would be unambiguously higher than that of an unmodified lipid membrane (see above). However, the experiments showed that the conductance of such a membrane is as low as that of unmodified lipid membrane. Therefore: (a) the planar membrane does not contain incorporated liposomes; (b) the phospholipid layer at the air-water interface in compartment 2 30 min after addition of liposomes to compartment 1 is a monomolecular layer without incorporated liposomes.

The latter is in agreement with the evidence of Schindler who studied the surface pressure under analogous conditions [5].

Partial fusion of liposomes with bimolecular lipid membranes formed by method 3

Based on the above, we may assume that a planar membrane formed by method 3 30 min after introduction of liposomes to compartments 1 and 4 presents a bilayer without incorporated closed liposomes. The experiments showed that the conductivity of such a membrane was as low as that of unmodified lipid membrane (less than 1 pS) throughout the time of measurement (40 min).

In some studies [20–22] the researches formed proteolipid layers by applying a very small amount of proteoliposomes to the air-water interface, proteoliposomes being absent in the aqueous solution before. Our findings make us conclude that if a very small amount of proteoliposomes is applied to the electrolyte surface, 30 min after that a proteolipid monomolecular layer is formed. Such monolayers can be used to reconstruct a bilayer without incorporated closed proteoliposomes.

Appendix 1

Estimation of membrane area occupied with partially fused liposomes based on the experimental evidence presented in Fig. 7

By the Kirchof law, the transmembrane current is as follows:

$$I = I_{AC} + I_{ABC} \tag{A-1}$$

where I_{AC} and I_{ABC} are currents passing through planar membrane and partially fused liposomes, respectively, as shown in Fig. 1c. The expressions for I_{AC} and I_{ABC} are obtained assuming that: (1) a partially fused liposome is a sphere situated symmetrically about the membrane surface; (2) the surface density of amphotericin B channels n is the same over the whole surface of partially fused liposomes and in bimolecular lipid membranes.

$$I_{AC} = i_{AC} n (1 - K) S$$

where i_{AC} is current passing through a single amphotericin B channel in membrane, S is membrane area, K is the ratio between membrane area occupied by liposomes and total membrane area.

$$I_{ABC} = i_{ABC} n N \frac{S_1}{2}$$

where i_{ABC} is current passing through a single amphotericin B channel in liposome, N is total number of liposomes partially fused with planar bilayer, S_1 is surface area of liposome.

By the assumptions made, $S_1 = 4S_0$ where S_0 is area occupied by liposome in planar membrane.

Hence:

$$I_{ABC} = i_{ABC} n \, 2KS$$

Now substitute the expressions for I_{AC} and I_{ABC} into Eqn. A-1 and obtain:

$$I = [i_{AC} n (1 - K) + i_{ABC} n \, 2K] S \tag{A-2}$$

Let us find expressions for currents i_{AC} and i_{ABC} passing through single channels. It follows from Refs. 16, 19 that the current through a single amphotericin B channel in the presence of tetraethylammonium at one membrane side is well approximated by the following function:

$$i = \frac{\xi_0 sh(\eta V)}{1 + 10^{\beta V + \gamma}}, \tag{A-3}$$

where V is membrane voltage: ξ_0, η, β, γ are parameters. Moreover, at sufficiently high voltages such an approximation must be satisfactory also if tetraethylammonium is present at both membrane sides.

It was shown in Ref. 16 that the plot for the current-voltage characteristic of a single amphotericin B channel coincides well with that of a membrane with numerous amphotericin B channels when the scale on the current axis is changed. Therefore, parameters η, β, γ are chosen so that the function (A-3) should best approximate the experimental current-voltage characteristic of membrane with numerous amphotericin B channels. Assume that the Montal-Mueller's membrane contains no partially fused liposomes. Then the parameter η, β, γ may be chosen based on the experimental plot 2 in Fig. 7. Indeed, at voltages above 80 mV the current-voltage characteristic 2 (Fig. 7) is well approximated by the function A-3 at the following parameter values: $\eta = 3.3 \pm 0.3$ V^{-1}; $\beta = 4.5 \pm 0.2$ v^{-1}; $\gamma = 0.04 \pm 0.03$; $\xi_o = 12$ nA. The chosen values of η, β, γ are true for the current-voltage characteristic of a single amphotericin B channel (Eqn. A-3) in the presence of 10 mM tetraethylammonium at both sides of the membranes, $V > 80$ mV.

The above proposals suggest that V_{AC} on membrane is 2-times higher than the voltage on the liposome membrane $V_{AB} = (1/2)V_{AC}$. Taking this into consideration, from Eqns. 2 and 3 we obtain the transmembrane current in the presence of 10 mM tetraethylammonium inside and outside of partially fused liposomes at membrane voltages above 0.16 V:

$$I \approx \xi n S \left[\frac{(1 - K) \cdot sh(\eta V_{AC})}{1 + 10^{(\beta V_{AC} + \gamma)}} + \frac{2K \cdot sh(\frac{1}{2}\eta V_{AC})}{1 + 10^{(\frac{1}{2}\beta V_{AC} + \gamma)}} \right] \tag{A-4}$$

Let us substitute the chosen values of η, β and γ into Eqn. A-4, differentiate the right side of A-4 with respect to voltage and equate the obtained derivative to zero. From the obtained equation

$I'_{V_{AC}}(V_{AC}, K) = 0$ and from the maximum of plot 1 in Fig. 7 we find $K = 0.45 \pm 0.15$.

Note that at $\xi nS = 32$ nA and with the chosen β, γ, η, K values the plot of Eqn. A-4 coincides rather well with the experimental curve 1 in Fig. 7.

Appendix 2

Estimation of membrane area occupied with partially fused liposomes based on the experimental evidence presented in Fig. 6

The additional experiment showed that the current-voltage characteristic of the Montal-Mueller's membrane with a great number of incorporated amphotericin B channels in the presence of tetraethylammonium at one side of the membrane is approximated by Eqn. A-3. Tetraethylammonium concentration of 2 mM the parameter values are taken as follows:

$$\eta = 3.3 \pm 0.3\ \mathrm{V}^{-1},\ \beta = 4.5 \pm 0.2\ \mathrm{V}^{-1},\ \gamma = -0.7 \pm 0.02, \quad \text{(B-1)}$$

the value of ξ_o is proportional to the number of amphotericin B channels in bimolecular lipid membrane. 2 mM tetraethylammonium being present at both membrane sides, the experimental current-voltage characteristic is quite well approximated by Eqn. A-3 with the same parameter values (Eqn. B-1) but at voltages whose values are above 0.08 V.

Using the same method as in Appendix 1, we find that the experimental current-voltage characteristic in Fig. 6 at $V_{AC} > 0.2$ V can be expressed by Eqn. A-4 at η, β and γ values given in Eqn. B-1).

Now find the expression for the transmembrane current after replacing the solution in the cell with a tetraethylammonium free one, (I_2). The blocking of current through a single channel in a planar bilayer disappears [19]:

$$i_{AC} = \xi \cdot sh(\eta V_{AC}) \quad \text{(B-2)}$$

where i_{AC} is current through a single channel in planar bilayer. Since after replacing the solution tetraethylammonium remains inside partially fused liposomes at the initial concentration [13] then, according to the properties of the amphotericin B channel [16]:

$$\begin{cases} i_{AB} = \dfrac{\xi \cdot sh(\eta V_{AB})}{1 + 10^{(\beta V_{AB} + \gamma)}} \\ i_{BC} = \dfrac{\xi \cdot sh(\eta V_{BC})}{1 + 10^{(\beta V_{BC} + \gamma)}} \end{cases} \quad \text{(B-3)}$$

where i_{AB} and i_{BC} are currents through single amphotericin B channels in a liposome, i_{AB} is current from region A to region B, i_{BC} is current from region B to region C (Fig. 1c), V_{AC}, V_{AB}, V_{BC} are voltages at respective membranes (Fig. 1c). Based on the assumptions made in Appendix 1, $i_{AB} = i_{BC}$. Hence, the following is true:

$$\begin{cases} \dfrac{sh(\eta V_{AB})}{1 + 10^{(\beta V_{AB} + \gamma)}} = \dfrac{sh(\eta V_{BC})}{1 + 10^{(\beta V_{BC} + \gamma)}} \\ V_{AC} = V_{AB} + V_{BC} \end{cases} \quad \text{(B-4)}$$

Now from Eqn. B-4 find V_{BC} as a function of V_{AC}. Taking into account Eqns. A-2, B-2 and B-3, find:

$$I_2 = \xi nS \left[sh(\eta V_{AC})(1 - K) + \frac{2K \cdot sh(\eta V_{BC})}{1 + 10^{(\beta V_{BC} + \gamma)}} \right] \quad \text{(B-5)}$$

It follows from Eqns. A-4, B-1 and B-5 that

$$\frac{I_2}{I} = \frac{(1 - K) \cdot sh(\eta V_{AC}) + \dfrac{2K \cdot sh(\eta V_{BC})}{1 + 10^{(\beta V_{BC} + \gamma)}}}{\dfrac{(1 - K) \cdot sh(\eta V_{AC})}{1 + 10^{(\beta V_{AC} + \gamma)}} + \dfrac{2K \cdot sh\left(\frac{1}{2}\eta v_{AC}\right)}{1 + 10^{(\frac{1}{2}\beta V_{AC} + \gamma)}}} \quad \text{(B-6)}$$

where I is transmembrane current in the presence of 2 mM tetraethylammonium at both sides of Schindler's membrane formed of amphotericin-containing liposomes.

Substituting the experimental values of I_1/I from plots 1 and 2 (Fig. 6) at different V_{AC} ($V_{AC} \approx$ 0.2 V) into Eqn. B-6 we find $K = 0.5 \pm 0.2$.

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