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ASYMMETRIC MEMBRANES RESULTING FROM THE FUSION OF TWO BLACK LIPID BILAYERS

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SUMMARY

When two black lipid bilayers are brought into close contact in the presence of divalent ions they interact, a process termed "fusion". The resulting structure is suggested to be another bilayer with a multitude of intercalated solvent lenses and micelles. This is corroborated by the observation that gramicidin A can induce unit conductance increments with parameters identical to those formed in normal planar black lipid bilayers. Moreover, the fused membrane has a specific capacitance which amounts to 75–80 % of the value found with normal membranes. Asymmetric membranes can be produced by the fusion of two membranes of different lipid composition.

INTRODUCTION

Liberman and Nenashev [1, 2] and Badzhinyan et al. [3, 4] have described the process of fusion* that takes place when two black lipid membranes are brought into close contact. They studied this phenomenon under various ionic conditions and related it to the fusion and adhesion of biological membranes. Their results were discussed in terms of the interaction of two charged surfaces [5] by analogy to stability considerations in colloid chemistry [6]. This implies the greatly enhanced effectiveness of divalent ions in initiating fusion as compared to monovalent ions.

In this paper the nature of the fused membrane is further characterized. The process of fusion is used to produce membranes with an asymmetric lipid composition.

MATERIALS AND METHODS

Experimental setup

Two black lipid bilayers were formed simultaneously on nozzle-shaped tips of two polypropylene vessels (see Fig. 1a for chamber geometry). The vessels were

* Liberman and Nenashev [1, 2] used the term "adhesion". "Fusion" is preferred here because it is clear from the present investigation that a new structure results at least over part of the membrane, which is different from just two bilayers in close contact.

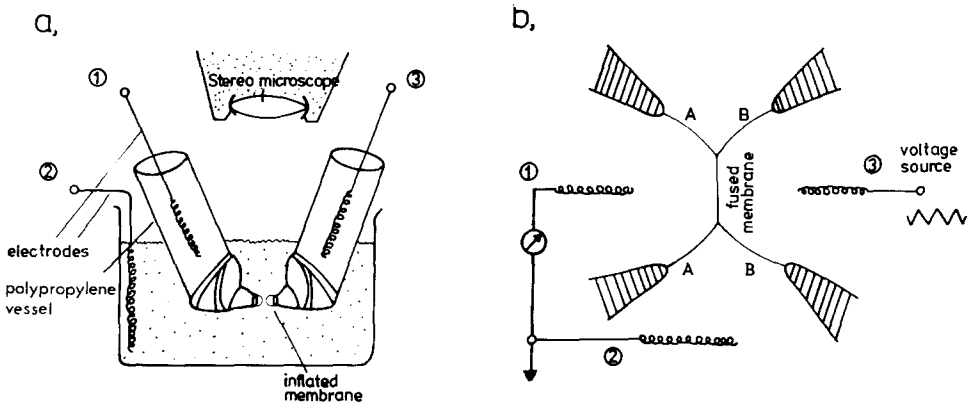

Fig. 1. (a) Overall view of the arrangement before the two inflated bilayers are brought into contact. (b) Schematic view of the membranes, the membrane supports, and the electrode configuration after fusion. A and B designate membrane regions to the left and right of the fused zone, respectively. The electrodes are connected for measuring electrical characteristics of the fused membrane.

held by micromanipulators, and their tips, together with the membranes, were viewed under dark field illumination (Fig. 1a). The membranes were inflated either by raising the vessels or by raising the water level inside the vessels. This resulted in membranes shaped like half-spheres with diameters ranging from 0.3 to 0.8 mm. During the thinning process membranes showed the typical appearance indicated in Fig. 3a. When they were brought into contact, they remained separate for some time (Fig. 3b) depending on the electrolyte composition. During this time they showed the appearance of two rubber spheres pressed against each other. The membranes were able to slide on each other and could be readily separated. In the presence of calcium the membranes fused after a time interval ranging from seconds to minutes. This resulted in a structure illustrated in Fig. 3c.

The membranes were very liable to rupture during these manipulations. Polypropylene was found to be a more suitable supporting material in this respect than polytetrafluoroethylene, especially when the cut ends of the nozzles had been smoothed by gentle heating. Polypropylene vessels of the shape indicated in Fig. 1a were easily fabricated from disposable syringes. The material can be drawn into capillaries and can be welded after gentle heating over a flame. Experiments were performed at room temperature (19–22 °C).

Materials

The salt solutions were prepared from Merck Suprapur reagents and were buffered to pH 7.6–7.9 with 5 mM Tris–HCl. Phosphatidylserine and dioleoyl-L-α-lecithin were obtained from Koch–Light and Supelco, respectively. Valine gramicidin A and monazomycin were kindly supplied by Dr Grell from this institute and by Dr Yonehara from Tokyo University. *N*-Decane (reference substance for gas chromatography) was obtained from Merck.

Electrical measurements

Ag–AgCl electrodes were used to establish electrical connections to the two

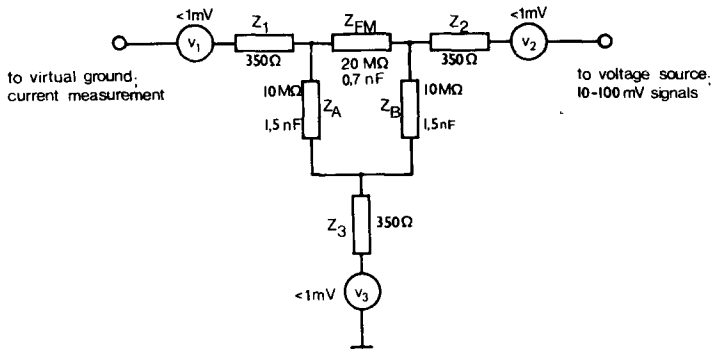

Fig. 2. Equivalent circuit for the electrode arrangement of Fig. 1b. Z_1, Z_2 and Z_3 represent the electrodes and the corresponding electrolyte spaces (maximum values). Changes in electrode polarization (v_1, v_2, v_3) were measured to be less than 2 mV, if currents 10 times higher than the experimental ones were passed for comparable periods. The impedance values for the three membrane sections Z_A, Z_B, Z_{FM} (FM means fused membrane) are representative for a monazomycin experiment (Fig. 6; minimum values; phosphatidylserine-containing membrane is membrane A). It is evident that (a) more than 99.9 % of the voltage applied drops at Z_{FM}, (b) the voltage drop at Z_A is negligible, since Z_B and Z_3 provide a powerful voltage divider, and (c) the time constants of the RC-networks in the shunt paths are in the μs range.

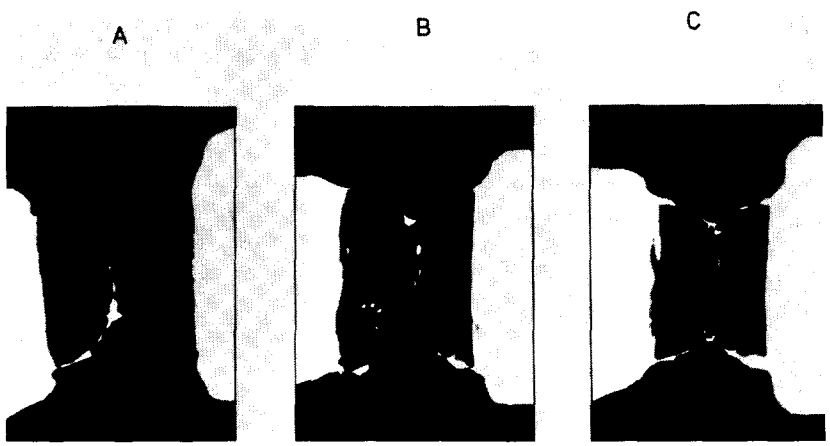

Fig. 3. Micrographs of inflated bilayers under dark field illumination. a, Two separated bilayers (the left one has not yet completely thinned); b, two bilayers in contact before fusion; c, two fused bilayers. An annulus of "colored" membrane is left next to the supports. Normally the ring delineating the fused region is very faint and would not be visible on the micrograph. In this case solvent lenses had coalesced with it, making it clearly visible. Diameter of left bilayer sphere: 0.51 mm.

vessels and to the outer compartment (see Figs 1a and 1b). By different ways of connecting the three electrodes to ground, to a voltage source and to a current meter, respectively, the current–voltage relations of the three membrane sections (designated as A, B, and "fused membrane" in Fig. 1b) could be measured separately. Fig. 1b shows the electrode connections used to measure the current–voltage characteristics of the fused membrane region. In this case a voltage signal applied to Electrode 3 cannot produce a change in current flow in the membrane section A, since it is shorted

TABLE I

ELECTRODE CONNECTION

Each column gives a connection scheme for measurement on a specific membrane region. Electrode designation as in Fig. 1b.

	Membrane to the left (A) without contact area	Membrane to the left (A) with contact area	Contact area alone	Membrane to the right (B) without contact area	Membrane to the right (B) with contact area
Voltage signal	2	2 3	3	2	2 1
Current meter	1	1	1	3	3
Ground	3		2	1	

by Electrode 2 (see also Fig. 4). This holds for all values of membrane resistance and electrolyte concentrations used in this study, as exemplified in the equivalent circuit of Fig. 2.

Current–voltage relations of the other membrane regions could be determined by connections as specified in Table I. For current measurements a low drift, low noise operational amplifier (Function Modules Inc., 380 K) in a standard feedback circuit was used, which measured current and at the same time clamped the potential at its input to ground.

Steady-state properties were measured by applying long duration voltage pulses or steps; capacitance was measured by applying triangular waveforms of ± 10 mV amplitude and a frequency of 20 Hz. In the latter case the amplitude of the resulting rectangular waveform is proportional to the membrane capacitance. If the membrane has a high conductance (e.g. due to gramicidin A) a superposition of a rectangular pulse and a triangular waveform is obtained. In this case the fast rising part of the signal is proportional to the capacitance and the slowly rising vamp is proportional to the conductance. Thus, both conductance and capacitance can be measured within one half cycle of the triangular waveform.

Current and voltage records were stored on FM-magnetic tape (Hewlett–Packard 3960) and later replayed for analysis.

RESULTS

The fusion process

When two membranes were brought close to each other during the application of a triangular waveform to Electrode 3, no current was recorded, provided the membranes were apart (Fig. 4a; the electrode configuration of Fig. 1b was used). When they touched each other, a small current appeared which was the doubly differentiated input signal (Fig. 4b). This was to be expected for a series combination of two capacitors shunted to ground via a low impedance path. Subsequently, the time constant of the current transients increased, a process which reflects the gradual decrease in thickness of the water layer between both membranes (Fig. 4c). When fusion occurred, a purely capacitive current (rectangular) appeared, and approached a steady state within about 500 ms (Fig. 4d). When the fused membrane region was viewed under reflected light it had a silvery appearance as opposed to a "black lipid membrane".

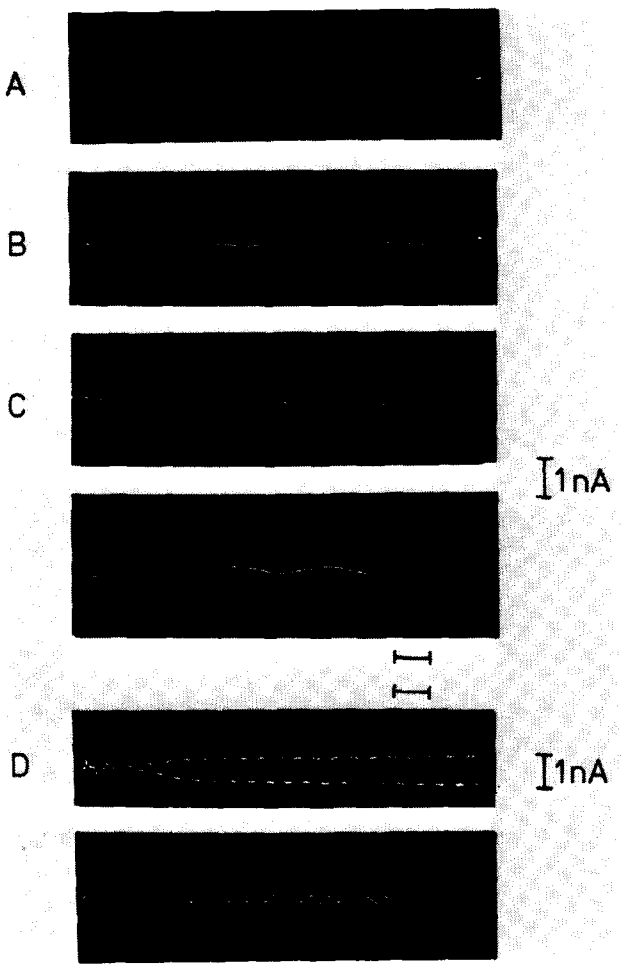

Fig. 4. Current recorded by the instrument, when a triangular waveform is applied to Electrode 3 (configuration of Fig. 1b). a, the membranes are separate; b, immediately after contact; c, 2–3 min later; d, during fusion. The triangular waveforms represent the voltage signals for a, b, c, (upper one) and d(lower one). Note the different time scale for d. Calibration: 20 ms for a, b, c and 100 ms for d. Dioleoyl-L-α-lecithin; 100 mM KCl.

Symmetric membranes; thickness of the fused region

The specific capacitance of a membrane which had been produced by the fusion of two dioleoyl-L-α-lecithin membranes was determined 2–5 s after the onset of fusion and at various times afterwards. Immediately after fusion, it was $0.3 \pm 0.02\ \mu F/cm^2$ which is 75–85 % of the value characteristic for a newly formed planar black lipid bilayer under the same conditions (dioleoyl-L-α-lecithin; $c = 0.39 \pm 0.02\ \mu F/cm^2$). In the following 5–10 min the specific capacitance rose by 5 to 15 %. It remained, however, lower than the value for normal planar bilayers.

Gramicidin A is known to be a very sensitive indicator of the membrane thickness. The average lifetime of the unit conductance channel changes by approx. 20 %

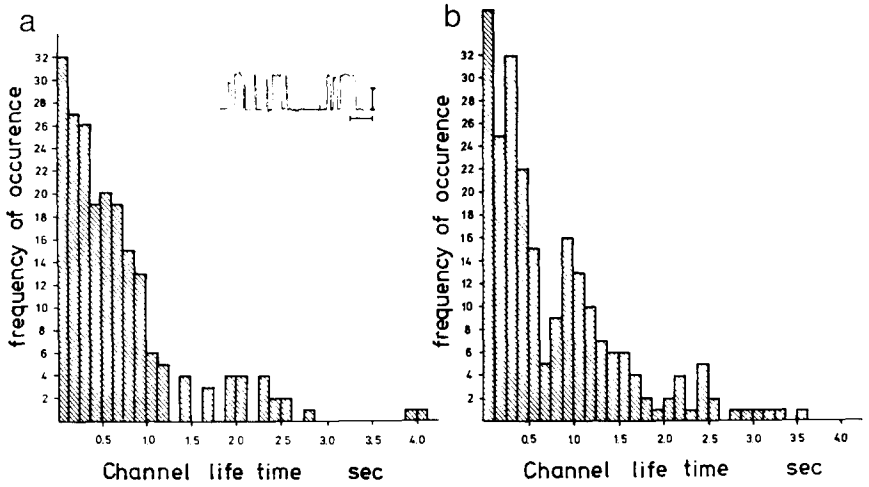

Fig. 5. Histograms of unit channel life times in the fused membrane (a) and in the normal membrane (b). Insert in (a) is an example of the original current record from the fused membrane. Applied voltage: 100 mV. Calibration: 1,25 pA; 5 s. 1 M KCl. Dioleoyl-L-α-lecithin.

for a 2 % change in hydrocarbon thickness (Haydon, D. A. and Hladky, S. B. [7]). Unit conductance steps were, therefore, recorded from the contact region as well as from a planar bilayer in order to determine whether the thickness of the fused membrane differs from that of the normal membrane. Figs 5a and 5b show histograms of channel lifetimes from fused and normal membranes, respectively. In the insert of Fig. 5a an example of the original fused membrane record is given.

The mean lifetime was calculated as $\tau_1 = 0.79 \pm 0.05$ s for the fused membrane, and $\tau_2 = 0.77 \pm 0.03$ s for the normal membrane. This difference is not significant. Correspondingly, the analysis of the current noise from the fused region at higher levels of conductance [8] did not reveal any statistically significant differences in the kinetics of channel formation as compared to normal membranes. Moreover, the amplitudes of the unit conductance steps were the same with both fused membranes and conventional bilayers.

Asymmetric membranes

When two membranes of different lipid composition are brought into contact, asymmetric membranes should result after fusion. Experiments were performed with dioleoyl-L-α-lecithin (neutral) on one side and phosphatidyl-serine/dioleoyl-L-α-lecithin (charged; 1 : 1 mixture in the membrane-forming solutions) on the other side. The resulting fused membrane was very fragile. Frequently it broke immediately after fusion, resulting in a membrane cylinder connecting both nozzles. Nevertheless in some cases successful electrical measurements could be made.

The conductance produced by monazomycin was used as an indicator for asymmetry. This antibiotic induces a conductance which is very strongly dependent on voltage. If a voltage smaller than a certain threshold value is applied, there is a negligible current flow. If the voltage is increased above this value an e-fold rise in conductance can be observed for every 3–4 mV change in potential (Müller and Finkel-

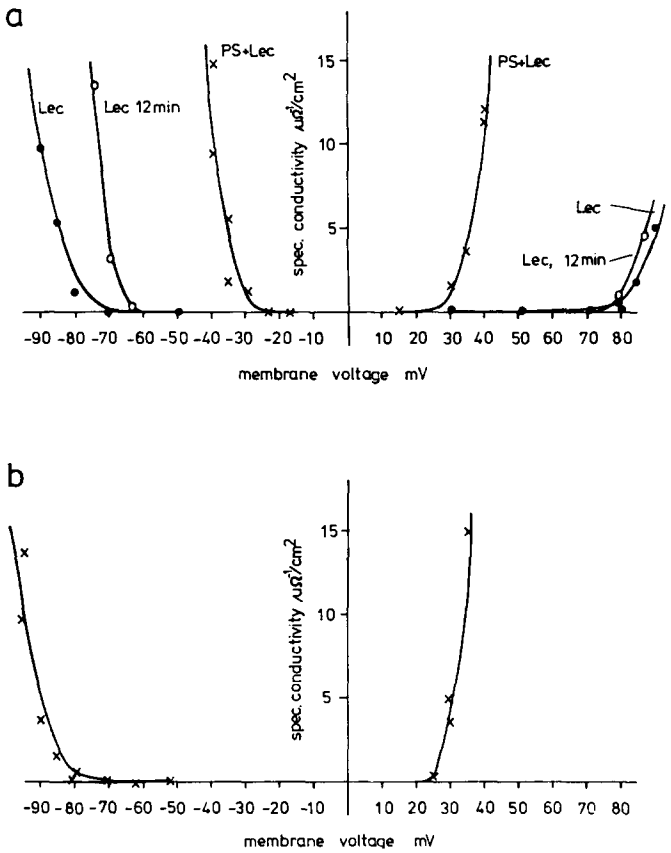

Fig. 6. (a) Conductance–voltage relations of symmetric membranes in the presence of 0.8 μg/ml monazomycin. LEC, Dioleoyl-L-α-lecithin membrane before fusion; PS-LEC, membrane made from a mixture of phosphatidylserine and dioleoyl-L-α-lecithin (1:1, molar ratio in the membrane-forming solution) before fusion; LEC-12 min, dioleoyl-L-α-lecithin membrane 12 min after fusion. (b) Asymmetric membrane. Values given are isochronous measurements at 10 s after the application of a voltage pulse. Polarity is positive when the uncharged side of the fused membrane or the outside of original bubble is positive. Electrolyte: 100 mM NaCl, $5 \cdot 10^{-5}$ mM $CaCl_2$, 5 mM Tris-HCl, pH 7.8.

stein [10]). If monazomycin is present on one side of the membrane, this occurs only if the voltage polarity is such that the positively charged monazomycin molecule is "driven" into the membrane. The value of the threshold voltage depends on the monazomycin concentration, on the surface charge density of the membrane, and on the composition of the electrolyte [10].

For control purposes, current–voltage relations of the original membranes were measured prior to fusion in the presence of 0.8 μg/ml monazomycin in all electrolyte compartments. Symmetric current–voltage relations resulted as shown in Fig. 6a.

In the case of the fused membrane, however, an asymmetric conductance–voltage relation was obtained (Fig. 6b). This is to be expected from the results of Müller and Finkelstein [10] if it is assumed that the fused membrane consists of one monolayer of charged lipid and another of uncharged lipid. In a few cases the fused

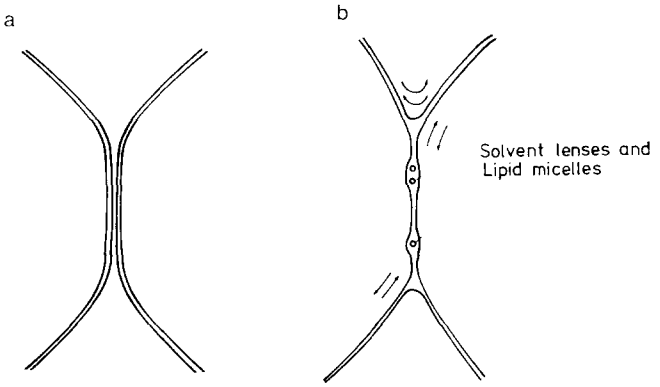

Fig. 7. Scheme of two membranes in contact before (a) and after (b) fusion.

membranes were sufficiently stable in order to observe their electrical parameters for periods from 30 to 60 min. No decay of asymmetry could be observed. The conductance–voltage relation of the charged membrane adjacent to the fused membrane remained unchanged during this period, whereas the threshold of the uncharged membrane at the negative polarity (outer side of the sphere, Fig. 1b, negative) slowly decreased. This may reflect mixing of the lipids by lateral motion at the border line (see arrows in Fig. 7).

DISCUSSION

Despite the silvery appearance, which differs from that of normal black lipid membranes, the fused membrane showed electrical properties characteristic for bimolecular lipid membranes (high capacitance, gramicidin and monazomycin conductance). It is, therefore, suggested that the silvery appearance is caused by microscopic solvent lenses or lipid water micelles which are entrapped when the two central layers of the originally four-layered structure collapse, and the two outside layers form a new bilayer (Fig. 7). The proposed mechanism of fusion is analogous to the collapse of the two inner detergent layers when two soap bubbles are brought into contact [11].

This view is consistent with the finding that there are bilayer regions of normal thickness in the contact area through which gramicidin-induced conductance can occur. The value of the specific capacitance c allows a rough estimate of the proportion α which is in the black membrane state. If we designate c_1 and c_2 as the specific capacitances of the bilayer region and of the thicker region, respectively, then c is calculated to be

$$c = \alpha \cdot c_1 + (1-\alpha)c_2 \tag{1}$$

If it is further assumed that all of the lipid and solvent material of the original structure has to be accommodated in the region of contact, and that the thick region has uniform thickness, then

$$\frac{2}{c_1} = \alpha \cdot \frac{1}{c_1} + (1-\alpha)\frac{1}{c_2} \tag{2}$$

since $1/c_1$, $1/c_2$ are proportional to thickness. Using Eqns 1 and 2 and values for c between 0.75 c_1 and 0.8 c_1 it can be calculated that 66–75 % of the total area is made up of black lipid membrane.

The low values of specific capacitance reported by Badzhinyan and Chailakhyan [9] could not be reproduced by the measurement technique employed here. A bilayer structure with numerous intercalated micelles and lenses does not contradict the results of Badzhinyan et al. [3, 4], who found that the fused membrane has a higher specific conductance both for the unmodified membrane [3] as well as for the membrane modified by the uncoupling agent TTPB [4]. In both cases the authors report that their membranes showed increased conductance in the border region. Since there are many lenses in the contact zone, most of it may have properties similar to the border region.

The results on monazomycin may be taken as a further indication, that true bilayer regions are formed on the contact zone. If there were just two bilayers in close contact, the voltage drop across the whole membrane would be divided into two parts. One would expect that the sum of the threshold voltages of individual bilayers has to be applied in order to produce current across the whole structure. The experiment shows that a highly asymmetric current–voltage relation is obtained when two membranes of different lipid compositions are fused. The threshold voltages obtained differ widely for both polarities. Each of the two threshold values can be measured individually on the half-spheres before they are brought into contact. Obviously, in that case the threshold behavior for either membrane is symmetric.

It is concluded that the observed asymmetry of fused membranes originates from an asymmetric lipid distribution, which arises when one layer of the original phosphatidylserine-containing bilayer and one layer of the phosphatidylserine-deficient bilayer form a new membrane. From the work of Müller and Finkelstein [10] it may be anticipated that an asymmetric current–voltage characteristic of the observed kind should occur for a membrane with an asymmetric surface charge distribution. Membranes of asymmetric lipid distribution have so far been described by Mormann and Kuhn [11], Träuble and Grell [12] and recently by Montal [13].

In principle it should be possible to measure the exchange of phospholipids across the bilayer (a process called "'phospholipid flip-flop" by McConnel and coworkers [14, 15] by observing the equilibration of the membrane asymmetry. In the present experiment no decay was observed within 1 h, which is in accordance with the half time of the flip-flop process, given by Kornberg and McConnell [14] as 6.5 h. However, it must be considered that in the present investigation both layers of the fused membrane are in contact with adjacent membranes of different composition. Lateral diffusion across the borderline would tend to maintain the asymmetry. Therefore, from this investigation it may only be concluded that the diffusion across the bilayer (flip-flop) is slower than the lateral motion of phospholipids over a distance of the order of 0.5 mm.

Recently, efforts were taken to incorporate dyes or proteins into black lipid bilayers by the fusion of liposomes with bilayers [16, 17]. It was claimed that a unidirectional current flow can be generated in a bimolecular lipid membrane due to partial reconstitution of the respiratory chain in liposomes [18] and subsequent fusion of liposomes and black lipid membranes [17]. The authors discussed their data in terms of liposomes sticking to the planar bilayer and modifying the underlying

bilayer region. Two closely spaced membranes are assumed to exist in close contact. It should be noticed that all of the data reported in ref. 17 also can be explained if the liposomes fused with the membranes as reported in this investigation. A very simple explanation would be offered by the decreased stability of asymmetrical membranes. On attempts to fuse membranes of different lipid composition the following sequence of events was observed most frequently: contact-fusion-rupture of the contact zone. By analogy this process would result in the liposomal membrane being incorporated into the bilayer.

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