BRANCHED BIMOLECULAR LIPID MEMBRANES

H. SCHINDLER *and* G. FEHER

From the Department of Physics, University of California, San Diego, La Jolla, California 92093. Dr. Schindler's present address is University of Basel, Biozentrum, CH-4056 Basel, Switzerland. Address reprint requests to Dr. Feher.

ABSTRACT Branched bimolecular lipid membranes separating three and four aqueous phases have been formed. The procedure is based on the technique of Montal and Mueller generalized to three and four lipid surface films spanning an appropriate aperture. The technique to produce Teflon structures for the mechanical support of branched bilayers is presented. The existence of the branched bilayer was established by measuring the specific capacity, specific resistance, and the gramicidin-induced single channel conductance of each branch. These structures should facilitate the study of transport properties of ionophores and other molecules and may also serve as model systems for the study of cell fusion.

Since the introduction of the Mueller-Rudin technique of forming lipid bilayers (1), several modifications have been introduced to form planar lipid membranes separating *two* aqueous phases (2–5). In order to facilitate the study of transport properties in membranes (e.g., the lateral diffusion and the flip-flop process of ionophores and other molecules), it seemed advantageous to form lipid bilayers separating *three* or more aqueous phases. In this communication we report the production and testing of such a branched lipid bilayer.

The experimental approach is based on the technique of Montal and Mueller (3) generalized to three or more lipid surface films spanning an appropriate aperture that allows for the formation of a branched lipid bilayer. The method used to produce the structure (septum) for the support of a three-branch bilayer is illustrated in Fig. 1. Three TFE teflon sheets (Saunders Corp., Los Angeles, Calif.), with dimensions 6 μm × 1 cm × 2 cm, are sandwiched between quartz blocks (Fig. 1 *a*) that have carefully ground sharp edges. The assembly is put into a springloaded clamp and heated for 10 min in an oven set to a temperature of 490°C. This produces a T-septum (Fig. 1 *b*) that is perfectly fused including the important region where the three branches join. After folding one of the branches over, a hole is punched with a sharpened stainless steel tube (Fig. 1 *c*). Hole diameters between 0.15 and 0.35 mm have been used. The hole-punching operation is performed under a microscope with the septum being placed on a polished aluminum surface and the tubing guided in a plexiglas mount. By carefully sharpening the punch, irregularities around the perimeter of the hole were routinely reduced to 2 μm or less. In order to make the septum more rigid, it is sandwiched between three sheets of FEP teflon (Saunders Corp.) with dimensions 50 μm ×

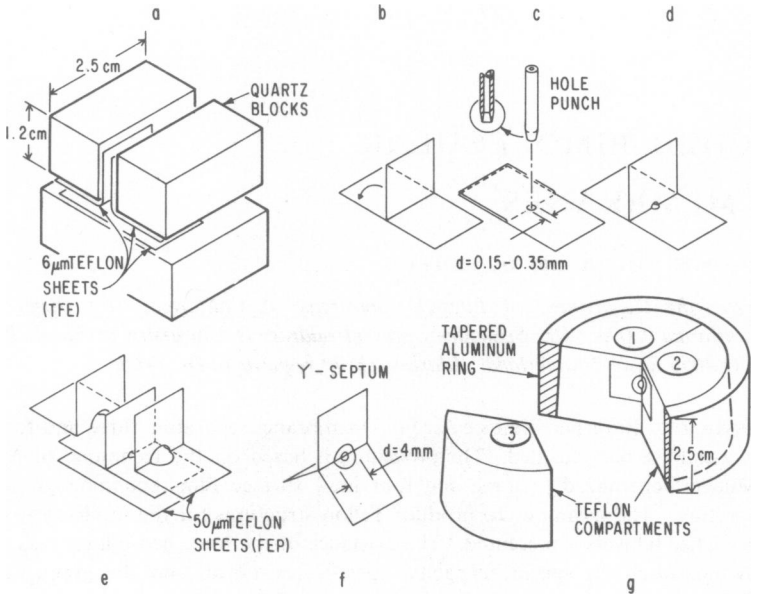

FIGURE 1 Schematic representation of the assembly of a septum for the support of a branched lipid bilayer (*a-f*) and the three-cell compartments (*g*). Step *e* is not absolutely necessary but helps to produce a rigid, easily handable structure that insures watertight contact between the cell compartments. An X-septum was also constructed by using four identical quartz blocks. That septum was used in a four-compartment Teflon cell.

1.2 cm × 2.5 cm, each having a 4 mm diam hole (Fig. 1 *e*). Clamping the assebmly between quartz blocks as before and exposing it for 5 min to 490°C results in a fused structure that can be bent into a Y-shape (Fig. 1 *f*) and trimmed to the desired size. An additional advantage of this procedure is that it stretches the Teflon around the aperture to form near perfect planar hole boundaries. The assembly of the septum in the three-compartment Teflon cell is illustrated in Fig. 1 *g*. A slight downward pressure of the tapered metal ring over the tapered cell insures a watertight contact between septum and cell compartments (without the use of grease or Vaseline). A bilayer septum separating four aqueous phases (X-membrane) has also been successfully made but was not studied in detail.

The procedures used for cleaning the cell, pretreating the septum, spreading the lipids in hexane, and raising the water levels have been described in detail by Montal (4). In addition, the modification introduced by Benz et al. (5) of raising the water levels immediately after spreading the lipid was also used. With both methods, three-branch membranes were formed. The success rate of forming branched membranes by the method of Benz et al. was higher and comparable with the success rate obtained with unbranched membranes. Once the membrane was formed, its mean lifetime was of the order of $\frac{1}{2}$–1 h; the longest observed lifetime was 10 h.

In order to prove the existence of a branched lipid bilayer as sketched in Fig. 2 *a*, the specific capacity, specific resistance, and the gramicidine-induced conductance changes

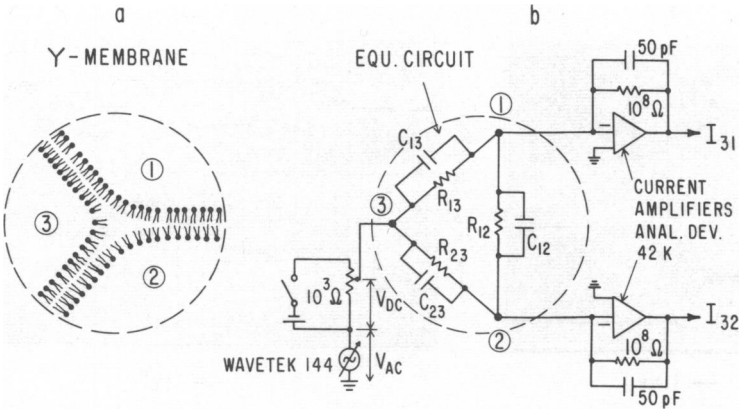

FIGURE 2 Schematic representation of a branched lipid bilayer membrane (*a*) and its approximate equivalent circuit, together with the voltage source and current amplifiers. In order to measure the electrical characteristics of all three branches, connections 1 and 3 or 2 and 3 were interchanged. The output of the current amplifiers is further amplified 100 times and fed into an oscilloscope or recorder (see Fig. 3).

of each branch were measured. The equivalent circuit of the membrane, together with the current measuring circuitry, are shown in Fig. 2 *b*. Two identical current amplifiers (Analog Devices, Norwood, Mass.) are connected to Ag/AgCl electrodes placed in cell compartments 1 and 2. The voltage is applied to the electrode in compartment 3. It is made up of a sinusoidal AC source (Wavetek, San Diego, Calif.) set to a frequency $\nu = 10$ Hz in series with a variable DC source (battery). The low input impedance of the amplifiers insures the independence of the two current measurements. By exchanging the connections between terminals (e.g., 1 and 3, or 2 and 3), all three currents in the cells were determined. When the branch of the membrane whose current is not being monitored (e.g., 1–2, in Fig. 2) breaks, the current amplifiers saturate (clamp); this provides an additional, independent check of the intactness of the membrane. From the measured DC voltage and current, the conductance was obtained. Since, at 10 Hz, $1/(2\pi\nu C) \ll R$, the AC current is predominantly capacitive. Consequently, its value was used to determine the capacitance of the membrane branches. The procedure was checked and calibrated by replacing the membrane with a 100 pF precision capacitor.

The experimental results obtained with a branched bilayer membrane of soy bean lecithin (Sigma Chemical Co., St. Louis, Mo.), purified according to Kagawa and Racker (6), is shown in Fig. 3. The membrane started to form about 2 min after the water levels were simultaneously raised (5); its formation was monitored by displaying the AC current (Fig. 3 *a*). The specific resistance of each of the branches in the absence of gramicidin was found to be $\sim 10^8$ Ω-cm^2 and the specific capacitance 0.8–0.9 μF/cm^2. These values are in agreement with those reported for unbranched artificial lipid bilayers (4, 5).

The single channel conductance steps (7–9) observed after the addition of gramicidin

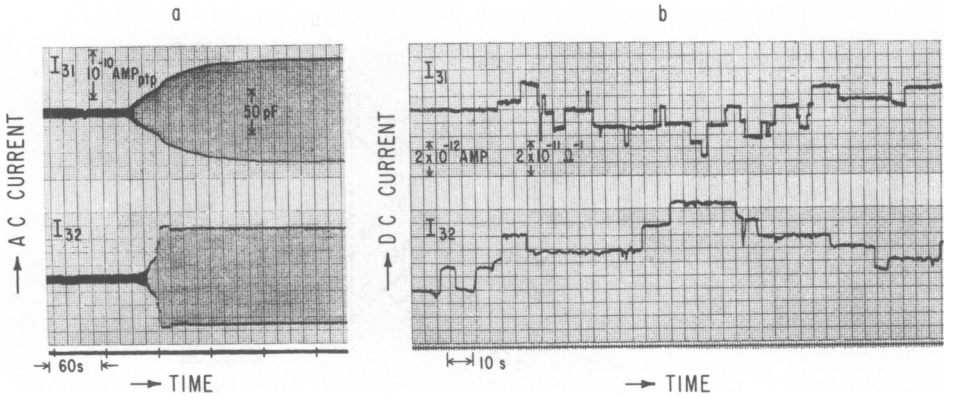

FIGURE 3 The electrical characteristics of a branched lipid bilayer membrane formed across an aperture having a diameter of 0.18 mm. The membrane was formed by starting with the level of the NaCl (1 M) solution below the aperture, introducing one drop of lecithin in hexane (0.2–0.5% wt/vol) into compartments 1, 2, and 3 and raising the levels immediately and simultaneously above the top of the aperture. T = 23°C. In *a*, an AC voltage (V_{ptp} = 30 mV, ν = 10 Hz) was applied as shown in Fig. 2. From the value of the current the capacitance was determined. In *b*, a DC voltage (V_{DC} = 100 mV) was applied and gramicidin was added to all compartments. The characteristic, discrete conductance steps are seen.

(Sigma) to *each* compartment are shown in Fig. 3*b*. The most probable conductance change per step was found to be 1.2×10^{-11} Ω^{-1} (in 1 M NaCl), in agreement with the value found by Bamberg and Läuger (8). The independence of the conductance steps of the two branches of the membrane (see top and bottom traces of Fig. 3*b*), together with the fact that gramicidin does not change the conductance of membranes that are thicker than one bilayer (10), constitutes additional proof that a branched bilayer had been formed. The absence of capacitance fluctuations after the branched membrane was formed indicates the structural stability of the region where the branches join.

In a set of preliminary experiments, gramicidin was added to one compartment only and the time course of the conductance changes across the opposite membrane branch was measured for different hole sizes. A detailed analysis of experiments of this kind is expected to yield information about the transport properties of ionophores and other molecules in the plane of the membrane and between its polar surfaces. Another possible application of the model branched lipid bilayer system is the study of cell fusion (see, for instance, ref. 11) and the concomitant transfer of molecules from one cell to another.

We are indebted to M. Montal for introducing us into the intricacies of forming lipid bilayers and to M. Weissman and R. Isaacson for helpful discussions and technical assistance.

The work was supported by grants from the National Science Foundation (DMR 74-24361), the National Institute of Health (GM-13191), and by a postdoctoral fellowship to H. Schindler from the Deutsche Forschungsgemeinschaft.

Received for publication 28 May 1976.

REFERENCES

1. MUELLER, P., D. O. RUDIN, H. TI TIEN, and W. C. WESCOTT. 1962. Reconstitution of excitable cell membrane structure in vitro. *Circulation.* **26**:1167.
2. VAN DEN BERG, H. J. 1965. A new technique for obtaining thin lipid films separating two aqueous media. *J. Mol. Biol.* **12**:290.
3. MONTAL, M., and P. MUELLER. 1972. Formation of biomolecular membranes from lipid monolayers and a study of their electrical properties. *Proc. Natl. Acad. Sci. U.S.A.* **69**:3561.
4. MONTAL, M. 1974. Formation of biomolecular membranes from lipid monolayers. *Methods Enzymol.* **32**:545.
5. BENZ, R., O. FROHLICH, P. LÄUGER, and M. MONTAL. 1975. Electrical capacity of black lipid films and of lipid bilayers made from monolayers. *Biochim. Biophys. Acta.* **394**:323.
6. KAGAWA, Y., and E. RACKER. 1971. Partial resolution of the enzyme catalyzing oxidative phosphorylation. *J. Biol. Chem.* **246**:5477.
7. KLADKY, S. B., and D. A. HAYDON. 1972. Ion transfer across lipid membranes in the presence of gramicidin A. *Biochim. Biophys. Acta.* **274**:294.
8. HAYDON, D. A., and S. B. HLADKY. 1972. Ion transport across thin lipid membranes: a critical discussion of mechanisms in selected systems. *Q. R. Biophys.* **5**:187.
9. BAMBERG, E., and P. LÄUGER. 1974. Temperature-dependent properties of gramicidin A channels. *Biochim. Biophys. Acta.* **367**:127.
10. MUELLER, P., and D. O. RUDIN. 1967. Development of $k^+ - Na^+$ discrimination in experimental bimolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* **26**:398.
11. FARQUHAR, M. G., and G. E. PALADE. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375; and SATIR, B., C. SCHOOLEY, and P. SATIR. 1973. Membrane fusion in a model system. *J. Cell Biol.* **56**:153.