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Formation of Bimolecular Membranes from Lipid Monolayers and a Study of Their Electrical Properties

(membrane structure/membrane reconstitution/asymmetric membranes/lipid bilayers)

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ABSTRACT Bimolecular membranes are formed from two lipid monolayers at an air-water interface by the apposition of their hydrocarbon chains when an aperture in a Teflon partition separating two aqueous phases is lowered through the interface. Formation of the membrane is monitored by an increase of the electrical capacity, as measured with a voltage clamp. Electrical resistance of the unmodified membrane is analogous to that of conventional planar bilayers (black lipid membranes) prepared in the presence of a hydrocarbon solvent, i.e., 10^6-10^8 ohm cm^2; the resistance can be lowered to values of 10^3 ohm cm^2 by gramicidin, an antibiotic that modifies the conductance only when the membranes are of bimolecular thickness. In contrast to the resistance, there is a significant difference between the capacity of bilayers made from monolayers and that of hydrocarbon-containing bilayers made by phase transition; the average values are 0.9 and 0.45 μF cm^{-2}, respectively. The value of 0.9 μF cm^{-2} approximates that of biological membranes. Assuming a dielectric constant of 2.1 for the hydrocarbon region, the dielectric thickness, as calculated from a capacity of 0.9 μF cm^{-2}, is 22 Å. This value is 6-10 Å smaller than the actual thickness of the hydrocarbon region of bilayers and cell membranes, as determined by x-ray diffraction. The difference may be due to a limited penetration of water into the hydrocarbon region near the ester groups that would lower the electrical resistance of this region and reduce the dielectric thickness. Asymmetric membranes have been formed by adjoining two lipid monolayers of different chemical composition.

Lipid bilayers, which are thought to be the basic structural element of cell membranes, account for many of their properties. They can be assembled from lipids either as small vesicles (1) or as single planar structures that separate two aqueous phases (2). Both models complement each other, and each has its own advantages and shortcomings. The spherical bilayers allow flux measurements with relative ease, and the absence of hydrocarbon solvent may be a factor aiding the incorporation of membrane proteins for functional reconstitutions (3-5). However, their inner compartment is small and inaccessible to chemical manipulation and electrical measurements. In planar bilayers, both compartments are easily accessible, but their mode of formation and the presence of hydrocarbon solvent may be responsible for reported failures to incorporate large membrane proteins. In addition, their electrical capacity is considerably lower than that of cell membranes, implying a different structure or thickness of the dielectric region.

For these reasons the formation of planar bilayers without the aid of a hydrocarbon solvent would be desirable. We report here the formation of planar bilayers separating two aqueous phases, in the absence of hydrocarbon solvent, by the hydrophobic apposition of two lipid monolayers at an air-water interface, by a modification of the method used by Takagi, Azuma, and Kishimoto (6) to form "rhodopsin membranes." It will be shown that the electrical capacity of these bilayers exactly matches that of biological membranes, and that the system allows the formation of asymmetric membranes; eventually, this technique may aid in the incorporation of membrane proteins into the lipid bilayer.

MATERIALS AND METHODS

The following chemicals were used: glyceroldioleate (Armak Chemicals Division, Philadelphia, Pa. and Applied Science Laboratories, Inc., State College, Pa.), oleyoyl acid phosphate (Hooker Chemical Co., Niagara Falls, N.Y.), egg lecithin, bovine cardiolipin, and plant phosphatidylinositol (Applied Science Laboratories, Inc.), gramicidin and cholesterol (Sigma Chemical Co., St Louis, Mo). Analytical grade reagents were used throughout. White-matter lipid from brain was extracted according to Mueller *et al.* (7) or by the method of Leuzinger and Schneider (8).

The membranes were formed initially with a modified version of the apparatus described by Takagi (9) (see Fig. 1*a*). It consists essentially of two monolayer troughs (18 × 12 cm) separated by a vertically movable septum containing in its center a hole of 1.0 cm (diameter) covered by a thin Teflon (tetrafluoroethylene) film (25 μm thick), obtained from Yellow Springs Instruments, Yellow Springs, Ohio (Membrane Kit no. 5937); this film contains a small aperture (see Fig. 1*a*). The septum is sealed with silicone grease to the walls of the trough and insulates the two water compartments electrically. It can be moved by a motor at a preset speed downwards, so that the aperture moves from above to below the water surface. The troughs and septum were made from Teflon. The aperture in the thin Teflon film was formed either by an electrically heated platinum wire, which was ground to a sharp point, or by a punch made from a tuberculin-syringe needle by beveling its wall. In a simplified version of this method, the thin Teflon film with the aperture was clamped between two halves of a trough and kept stationary. The membrane was formed by filling the two compartments with water or saline to below the aperture and, after spreading a lipid monolayer on each side, raising first one, then the other water level slowly above the aperture by gravity flow.

It is important that the film containing the aperture have a hydrophobic surface; this favors contact with the lipid hydrocarbon chains. Of several materials tested, only Teflon and

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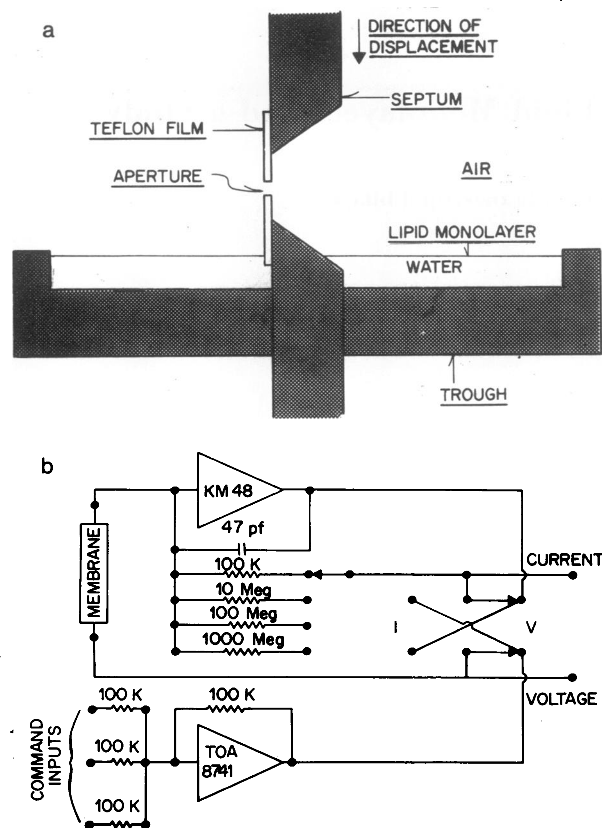

FIG. 1. (*a*) Simplified diagram of the apparatus for membrane formation. Dimensions are not drawn to scale. (*b*) Circuit diagram of the current and voltage clamp. The two operational amplifiers KM48 and TOA 8741 were obtained from Polytron Devices, Inc., Patterson, N.J. and Transitron, Wakefield Mass., respectively. The circuit can be switched between the constant-current and constant-voltage modes as indicated. Current and voltages were controlled by pulses from a pulse generator that were applied to the command inputs. Output current and voltage were displayed on a dual-beam storage oscilloscope. The scope sweeps were synchronized with the command pulses.

Parafilm (American Can Co., Neenah, Wis.) performed satisfactorily. More hydrophilic materials such as Saran (Dow Chemical Co.) can be used provided their surface is preconditioned by wiping it with a 2% solution of petroleum jelly in pentane or ferric stearate in chloroform–methanol.

High ionic strength (>0.1 M) in the water phase and also a high humidity of the room atmosphere seem to have adverse effects on membrane stability during formation. All experiments were performed at room temperature.

The monolayers were spread on a clean surface as condensed films at their own lens pressure from lipid solutions in ethanol, chloroform, pentane, or hexane; the solutions were applied to the side of the trough near the septum so that the monolayer swept along the septum and removed any surface impurities. The effects of monolayer surface pressure on membrane formation have not been evaluated. Mechanical compression of the monolayer during membrane formation had no beneficial effects.

For the electrical measurements, the two troughs were connected with calomel electrodes (Beckman no. 39270) to the circuit shown in Fig. 1*b*. The formation of the membrane was followed by rapid and repeated (3–5 sec^{-1}) measurements of the membrane capacity by application of a constant voltage pulse, and display of the capacitative current on a storage oscilloscope. After formation, the membrane resistance was measured by the application of a calibrated constant-current pulse and display of the resulting membrane potential.

The clamping circuit shown in Fig. 1*b* allows switching between constant current and constant voltage modes and adjustment of the current measuring time constant in the constant voltage mode.

The capacity was calculated from the current records by:

$$C = \frac{\int_0^\infty I dt}{\Delta V},$$

where I is the current and ΔV the amplitude of the constant voltage pulse. The consistency of the method was checked by replacing the membrane by a calibrated *RC* circuit. In the constant voltage mode the time constant and gain of the current record depends on the value of the feedback resistor in the current measuring amplifier. Because the capacity currents are small, the feedback resistance was kept about 100 kΩ. The corresponding amplifier time constant introduces a phase shift between the current record and the membrane potential such that the record (but not the actual membrane current) outlasts the rise time of the potential, which is determined only by the membrane capacity and the series resistance of the electrodes and the aqueous phase ($\sim 3 \times 10^4$ Ω) and was typically 5–10 μsec. The amplifier time constant also causes a deviation of the recorded initial peak current from its theoretical value of V/R (V = applied potential step; R = series resistance). Instead, the peak current increases nonlinearly with the capacity. However, these effects do not alter the recorded current integral and do not invalidate the capacity measurements.

RESULTS

Membrane formation and electrical properties

When the aperture descends below the water level, the two monolayers come in contact and begin to form a bilayer by joining their hydrocarbon chains. The process is presented diagrammatically in Fig. 2. At the same time, the capacity, measured by voltage clamp, increases progressively above the background level of the thin Teflon film and reaches a final stable value once the aperture is entirely submerged (Fig. 3*a*). The rate at which the membrane forms is usually faster than the mechanical rate of submersion of the aperture. For example, in Fig. 3*a*, where the aperture diameter was 0.25 mm, the final capacity was reached within 4 sec, whereas the rate of submersion was 0.5 mm/min. Visual observation shows that this discrepancy is caused by a rapid rise of the water level due to capillarity that occurs often but not always when the level reaches the lower rim of the aperture. During membrane formation, the relative speed between aperture and water surface should not be much larger than 0.5 mm/min. The membranes are most prone to breakage during the first few seconds after their formation. Once stable, they have been observed to last for several hours. The upper and lower limits of the aperture diameter with which stable membranes can be formed have not been explored.

For a given lipid, the final value of the membrane capacity depends only on the area of the aperture, and has a typical specific value of 0.9 ± 0.1 μF cm^{-2} for the lipids studied thus far. This value is almost twice as large as the capacity of

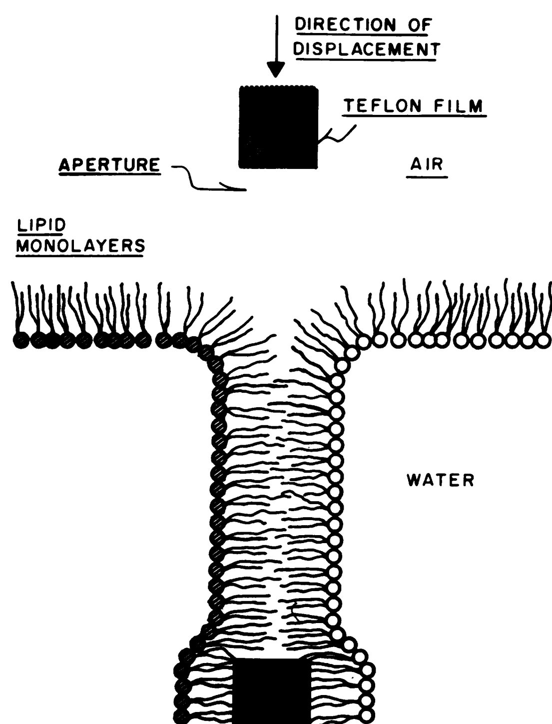

FIG. 2. Principle of bilayer formation by the apposition of two lipid monolayers at an air-water interface. The *shading* of the polar-head groups of one monolayer indicates a different chemical composition, and the consequent formation of an asymmetric bilayer. Not drawn to scale.

planar bilayers formed from lipids in hydrocarbon solvents, and identical to the typical value reported for cell membranes. Fig. 3*b* and *c* show a comparison of the capacity currents of two membranes formed on the same aperture from the same lipid mixture (glyceroldioleate–cholesterol-oleyoyl acid phosphate), either by brushing an octane solution of this lipid over the aperture (Fig. 3*b*) or by the apposition of two monolayers (Fig. 3*c*).

In contrast to the different capacities, the resistances of the two membranes are identical and range between 10^6 and 10^8 ohm cm^2, as illustrated in Fig. 4*a* and *b*. Membranes formed from brain-lipid monolayers tend to show the highest resistances. The dielectric breakdown occurs at voltages between 150 and 500 mV.

Evidence that these membranes are of bimolecular thickness is derived not only from the magnitude of the capacity, but also from the observation that the addition of 0.1 μM gramicidin to one aqueous compartment lowers the resistance to 10^3 ohm cm^2 (Fig. 4*c*); in the presence of a transmembrane ion-gradient, 0.1 μM gramicidin generates a cationic transmembrane potential as it does in hydrocarbon solvent-containing bilayers and cell membranes. Gramicidin is known not to act on membranes that are thicker than one bilayer (10, 11).

We have also formed membranes from two monolayers of different composition and charge, e.g., glyceroldioleate–cardiolipin, glyceroldioleate–phosphatidylinositol, glyceroldioleate–mixed brain lipids. No significant differences of resistance or capacity (Fig. 3*d*) have been observed, nor did these membranes show rectification or asymmetrical capacities.

Table 1 summarizes the properties and characteristics of the bilayers derived from monolayers, and those of cell membranes and hydrocarbon solvent-containing bilayers.

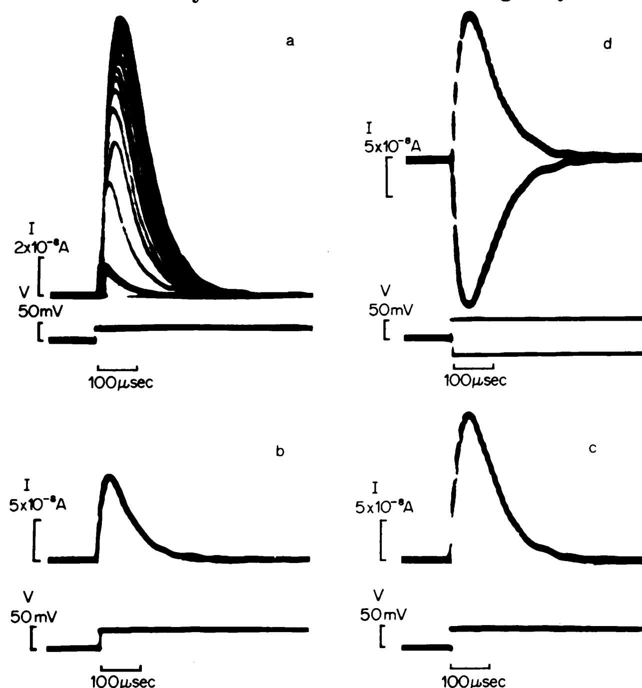

FIG. 3. (*a*) Capacity currents in response to a voltage step of 37.5 mV during the formation of a bilayer membrane. *Lower trace*, voltage; *upper trace*, current. The voltage trace represents the potential at the input to the electrodes and not the true membrane potential which, neglecting the relatively small series resistance of the electrodes and aqueous phase, has the same amplitude but a longer rise time ($\sim$10 μsec.). For reasons discussed under *Methods*, the current outlasts the rise time of the potential. Pulses of 10-msec duration were applied at 300-msec intervals, and the resulting traces were superimposed on an oscilloscope. The time progresses from the smallest current record, which corresponds to the capacity of the submerged portion of the thin Teflon film, to the largest, which results from the capacity of the completed (fully formed) membrane. The intermediate traces are the capacity currents of the partially formed membrane when the aperture is not yet completely submerged, as illustrated in Fig. 2. The measured value of the capacity was 1.0 μF cm^{-2}. This particular membrane was formed from two monolayers of different composition; one monolayer contained glycerol dioleate and cholesterol, in a molar ratio of 1.0 to 0.26. The other monolayer was formed from beef-heart cardiolipin. The aqueous phase contained 0.01 M NaCl (pH 5.5). The aperture area was 0.048 mm^2. (*b*) Capacity currents from a hydrocarbon solvent-containing bilayer, which was formed by brushing a glycerol dioleate-cholesterol-oleyoyl acid phosphate solution in octane (molar ratios 1.0:0.26:0.02) over the submerged aperture. The oleyoyl acid phosphate was added to increase the rate of membrane thinning. The aperture area was as in *a*. The time integral of this current corresponds to a capacity of 0.47 μF cm^{-2}, which is a typical value for this type of membrane. Composition of the aqueous phase as in *a*. (*c*) Capacity currents from a bilayer formed by adjoining two monolayers spread from a mixture of lipids identical in composition to that in *b*, except that the octane was replaced by pentane, which evaporated before the membrane was formed. The capacity of this membrane is 1.04 μF cm^{-2}. Composition of the aqueous phase as in *a*. (*d*) Capacity currents for voltage pulses of opposite polarity from an asymmetric membrane. The composition of the monolayers and aqueous phase was as in *a*. There is no asymmetry of the capacity currents.

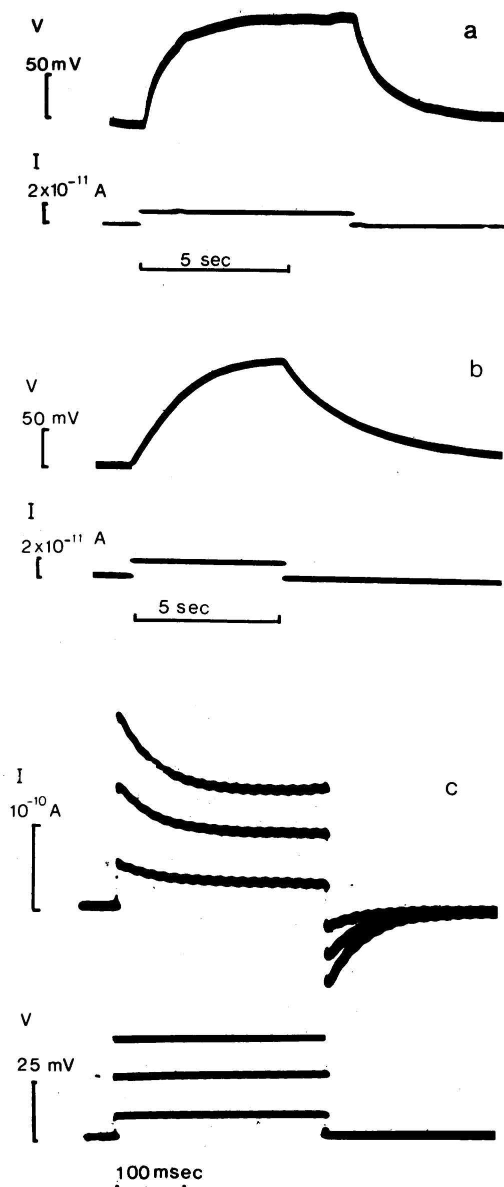

FIG. 4. (*a*) Membrane potential records as a function of applied constant currents (20 pamp) of a hydrocarbon-containing bilayer of composition as in Fig. 3*b*. From the steady-state value of this potential, the membrane resistance was calculated to be 4.8×10^6 ohm cm^2. The aperture area was 0.048 mm^2. The aqueous phase contained 0.01 M NaCl at pH 5.5. (*b*) As in *a*, except that the membrane was made from monolayers. The lipid composition was identical to *a*, except that octane was replaced by pentane. The aperture area was 0.039 mm^2 and the calculated membrane resistance is 3.4×10^6 ohm cm^2. The time constant of the current is larger than in *a* because of the larger specific membrane capacity. (*c*) Membrane currents at constant voltage in the presence of gramicidin. The membrane was formed as in Fig. 3*c* from two monolayers. 0.1 μM Gramicidin was then added to one of the aqueous compartments. The aperture area was 0.035 mm^2. Three successive voltage pulses of different amplitude are superimposed. From these records, the membrane resistance was calculated as 4.4×10^3 ohm cm^2. Composition of the aqueous phase as in *a*. In this record, the time constants of the transient capacity currents are longer than in Fig. 3 because the clamping circuit was set to a longer time constant.

DISCUSSION

Membrane resistance and capacity

One of the arguments against the bilayer model of cell membranes has been the low capacity of the membranes formed from lipid solutions in hydrocarbon solvents. This difficulty is resolved by the present experiments. As summarized in Table 1, the properties of the bilayers formed from monolayers are identical in every aspect to those of biological membranes, except for the membrane resistance, which is generally much lower in cell membranes—and is there attributed to the presence of special translocators that locally modify the bilayer. However, the resistance of the experimental membranes can also be lowered to that of the cell membrane by the insertion of translocator peptides and proteins (12, 13).

The high values of the capacity cannot be accounted for solely on the basis of the thickness of the hydrocarbon region as determined by x-ray diffraction. The effective dielectric thickness can be estimated from

$$C = \frac{\mathcal{E}A}{4\pi d}$$

where C is the membrane capacity, $\mathcal{E}$ the dielectric constant, A the membrane area, and d the dielectric thickness. With 2.1 as an average value for the dielectric constant of long-chain hydrocarbons, the dielectric thickness for a capacity of 0.9 μF cm^{-2} would be only 21 Å, whereas the actual thickness of the hydrocarbon region in lipid bilayers and cell membranes lies between 28 and 35 Å (see Table 1). Since there is no reason to assume that the dielectric constant of the hydrocarbons in the membranes is different from that of bulk hydrocarbons of the same chain-length and saturation, we have to conclude that the effective dielectric thickness of the hydrocarbon region is less than its physical dimension. A possible explanation for this effect would be a limited penetration of water into the hydrocarbon region, aided by the presence of the oxygen of the ester linkages and dynamic fluctuations of the free volume in their vicinity, that may extend perhaps as far as the first three methylene groups. The dissociation of this water, albeit less than in bulk, would lower the electrical resistance of this region considerably and, therefore, reduce the thickness of the dielectric. The penetration of water might go as far as 3–4 Å beyond the plane of the glycerols, reducing the dielectric thickness by 6–8 Å, to a value sufficient to account for the observed capacities. This suggestion is supported by the evidence of water penetration into the hydrocarbon region of detergent micelles as determined by nuclear magnetic resonance (NMR) spectroscopy (14–17). It may also explain the abnormally high capacity values (3–4 μF cm^{-2}) of small lipid vesicles (18), which for geometrical reasons have a lower packing density and higher mobility of their outer lipid layer (19). The larger free volume would allow a deeper penetration of water into the hydrocarbon region and decrease its average dielectric thickness. A concomitant lower activation energy of water permeation could also be expected and has been observed (19).

The lower capacities of the hydrocarbon-containing bilayers may be attributed to a steric exclusion of this water by the intercalated hydrocarbon solvent, although an increased membrane thickness due to the presence of the hydrocarbon cannot be excluded. No x-ray data are available from such membranes, but light-reflectance measurements (20) indicate a membrane thickness of 62 Å for lecithin in decane.

TABLE 1. *Comparison of the electrical properties of bimolecular lipid membranes in the presence and absence of hydrocarbon solvent with those of cell membranes*

Electrical property	Bilayer (with hydrocarbon solvent)	Bilayer from monolayers (no hydrocarbon solvent)	Cell membranes
Membrane resistance (ohm cm²)	10^6–10^8 (12, 13)	10^6–10^8	$<10^6$ (39)
Effect of gramicidin on membrane resistance (ohm cm²) at 0.1 μM	10^3 (12, 13)	10^3	$<10^3$ (40) (inferred)
Membrane capacity (μF cm⁻²)	0.45 ± 0.05 (12, 13, 21)	0.9 ± 0.1	Range: 0.8–1.2 (21) Squid giant axon 0.9–1.1 (41, 42) *Nitella* 0.94 (43)
Thickness of hydrocarbon region obtained from:			
(*a*) Capacity data*	42 Å	22 Å	16–24 Å
(*b*) X-ray data	—	29 Å (44†, 45)	28 Å (Photoreceptor) (46)‡ 33 Å (red cell) (47) 35 Å (*Mycoplasma*) (48, 49)

* A dielectric constant of 2.14 (hexadecane) is assumed (see *text*).
† Extracted from the reported diffraction patterns.
‡ These values are obtained by substraction of 12 Å (corresponding to the polar-head thickness) from the reported repeat spacings.

Fettiplace, Andrews, and Haydon (21) have formed membranes from a lecithin solution in hexadecane that exhibit capacities of 0.6–0.7 μF cm⁻², and that were assumed to exclude the solvent. For these membranes, they calculated a thickness of the hydrocarbon region of 30 Å and concluded that cell membranes, due to their higher capacities of 1.0 μF cm⁻², must have a hydrocarbon thickness of 20 Å, and a correspondingly less-dense packing of their polar groups, effects that were attributed to the interaction of the hydrocarbon chains with protein. The fact that pure lipid bilayers have the same capacity as cell membranes makes these assumptions unnecessary. Furthermore, the available x-ray data do not give evidence for such a thin hydrocarbon region.

Membrane asymmetry

One of the attractive possibilities offered by this method is the formation of asymmetric membranes by the apposition of two monolayers of different composition, because cell membranes with asymmetrically arranged lipids may play a role in redox and photo-energy transductions (22–24). Several methods to accomplish this have been proposed (25–28), but no experimental results have been published. The asymmetric membranes as studied thus far have not shown any asymmetries of capacity and resistance, and none can be expected unless the field induces a change of the membrane structure or unless the lipids form asymmetrically charged pores by local micellization.

There is the possibility that the two lipid types mix during membrane formation, but in light of the data of Kornberg and McConnell (29)—who reported half-times of many hours—this process would be much too slow. Nevertheless, until the asymmetry is established by other means, such as differences in the adsorption of multivalent ions (30), this question must be kept open.

Reconstitution of membranes

Introduction of small peptides and membrane-active antibiotics into planar bilayers containing hydrocarbon solvents has led to functionally active membranes that display ion selectivity and electrical excitability (12). In contrast, attempts to introduce large and defined membrane proteins in a functionally active state have met with considerable difficulties (31, 32), although recently there have been reports of some promising advances (8, 33–35). The failures may either be attributed to the presence of hydrocarbons or to the inability of the proteins to appropriately interact with a preformed bilayer, especially since membrane proteins are only soluble in the presence of detergents [for a review see Razin (36)].

The present method allows, at least in principle, the simultaneous assembly of lipid and protein into a bilayer after both components are spread as a monolayer at an air–water interface, or after the proteins are allowed to penetrate an expanded monolayer. This simultaneous assembly may resemble more closely the natural formation of cell membranes, in which the membrane proteins are probably inserted into the lipid matrix during their synthesis. However, possible adverse effects of surface denaturation still must be evaluated. The method would also allow the asymmetric distribution of the proteins with respect to the bilayer.

The fact that bilayers can be formed from monolayers also suggests that this principle might have played a role in prebiotic evolution, as suggested by Goldacre (37), since long-chain fatty acids have been synthesized under abiotic conditions (38).

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