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DIRECT MEASUREMENT OF THE FORCE BETWEEN TWO LIPID BILAYERS AND OBSERVATION OF THEIR FUSION

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The force between two phosphatidylcholine bilayers is measured as a function of their separation, showing a strong hydration repulsion at short range, as previously reported by LeNeveu et al. (LeNeveu, D.M., Rand, R.P., Parsegian, V.A. and Gingell, D. (1977) (Biophys. J. 18, 209–230). The experimental technique also allows direct observation of the molecular process by which two bilayers fuse into one.

The process (or processes) by which biological membranes fuse is not well understood at the molecular level. A reasonable approach to the problem is to study the fusion of lipid bilayers [1–7]; first, because they constitute the simplest model of a membrane and we have some hope of understanding what happens in this system, and second, because there is evidence that even in real biological membranes fusion occurs in bilayer regions devoid of membrane proteins [8–14]. Clearly, fusion of two bilayers will only occur if

(i) they approach very closely, which requires overcoming a repulsive force between them, and

(ii) the bilayers rupture and somehow reform into a single bilayer. In this article I describe an experiment in which both these steps are monitored directly by bringing together two bilayers of phosphatidylcholine each adsorbed onto a curved mica surface.

The experimental technique is that described by Israelachvili and Adams [15], in which the force (F) between two crossed cylindrical surfaces of mica immersed in a liquid is measured as a function of the distance (D) between them. The mica surfaces are molecularly smooth, and have a radius of curvature R of approx. 1 cm. The force is measured by a spring deflection method with an accuracy of 10^{-7} N, and the distance is determined by an optical interference technique [16] with a resolution of 0.1–0.2 nm. The optical method involves visual observation of fringes of equal chromatic order [17] at the exit port of a spectrometer. The shape of the fringes reflects the shape of the curved mica surfaces, so that any deformation of the surfaces is immediately observed.

To measure the force between lipid bilayers in aqueous solutions, a bilayer is deposited onto each mica surface. This is achieved, at least for phosphatidylcholines, simply by immersing the micas in water and subsequently adding a dispersion of sonicated lipid vesicles to a final concentration of 0.1 mg/ml. A bilayer then adsorbs spontaneously on each mica surface within 3h, as ascertained by noting that the mica surfaces no longer come into contact ($D = 0$) as they did in water, but encounter a steep repulsion at a separation of 9–10 nm, the thickness of two phosphatidylcholine bilayers with a thin layer of water between them. The

Abbreviations: DLPC, dilaurylphosphatidylcholine; egg-PC, egg-yolk phosphatidylcholine (egg lecithin).

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precise mechanism by which these adsorbed bilayers form is not known, though it is thought that small unilamellar vesicles must be breaking open and adhering following collisions with the mica surfaces. If vesicles were simply adhering to the surfaces and remaining intact, a force would be detected at larger mica separations corresponding to one or two vesicle diameters, but no such force is observed. In an experiment in which only phosphatidylcholine monomers were present in the water, no measurable adsorption occurred in 36 h. An alternative method to form bilayers is the Langmuir-Blodgett deposition technique in which the micas are first withdrawn from an aqueous phase with a monolayer of lipid at its surface, then reimmersed [18,19]. When this is done, comparable results are obtained for the force between the adsorbed bilayers (Marra, J., this laboratory, personal communication).

Experiments were performed with dilaurylphosphatidylcholine (DLPC) obtained from Avanti, and egg-yolk phosphatidylcholine (egg-PC) from Sigma, both used without further purification. Of the synthetic phosphatidylcholines available, DLPC was chosen because its hydrocarbon chains are fluid at room temperature, 21 ± 1°C, at which measurements were made. Longer chain phosphatidylcholines would have their hydrocarbon regions in the gel state, which is not the normal physiological condition.

Fig. 1 shows the force measured between adsorbed bilayers of DLPC and egg-PC, plotted on a logarithmic scale against the mica-mica distance D. The force between curved surfaces of radius R can be simply related to the interaction energy E_f between two flat surfaces at the same separation by the Derjaguin approximation [20,21], $E_f = F/2\pi R$. For this reason the force shown in Fig. 1 is normalised by R, and the energy E_f is indicated on the right-hand ordinate. There is a strong short-range repulsion between the adsorbed bilayers, which is called hydration repulsion and is attributed to the work required to remove water of hydration from the polar PC headgroups as bilayers are forced together. It does not vary when sodium or calcium is present in the water up to physiological concentrations. These results confirm the findings of Rand, Parsegian and coworkers [22–25], who measured the hydration repulsion between bilayers in multilamellar phases of phosphatidylcholine and other phospholipids using an osmotic stress technique combined with X-ray diffraction.

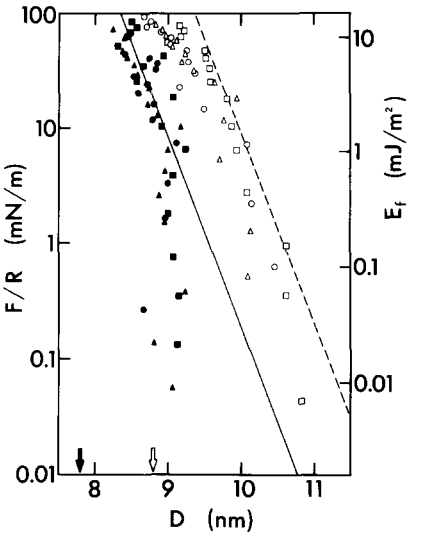
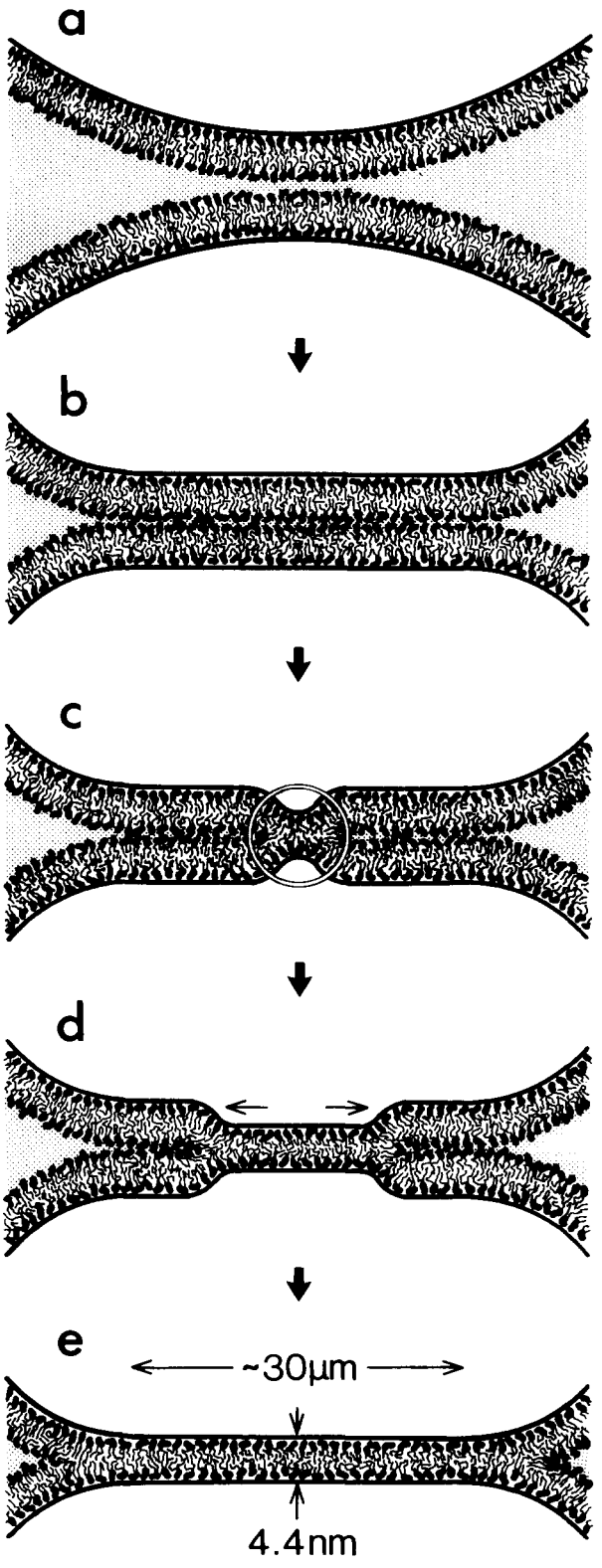


Fig. 1. The force F between phosphatidylcholine bilayers adsorbed onto curved mica surfaces, normalised by the radius of curvature R, plotted as a function of the mica-mica separation D. The corresponding interaction energy between flat surfaces, E_f, is indicated on the right-hand ordinate. Solid symbols are for DLPC, open symbols for egg-PC, with different symbols representing different experiments. The lines show the empirical fits of Lis et al. [25] to their data obtained by an entirely different method, assuming that the egg-PC bilayers (dashed line) would 'contact' at $D = 8.8$ nm (open arrow), and DLPC (solid line) would 'contact' at 7.8 nm (solid arrow) as discussed in the text. At larger separations F/R (and E_f) become negative due to van der Waals attraction between the bilayers.

There are, however, difficulties in making an exact comparison between the results of the two experiments. First there is the perennial problem of defining the zero of separation between bilayers, since it is impossible to tell from the force curve when, if ever, the headgroups of opposing bilayers come into 'contact'. Rand et al. have an operational definition based on the volume fraction of water present in their multilamellar system, which would underestimate the bilayer thickness because it assumes that no part of the headgroup penetrates into the water layer. In the present case the simplest operational definition is to equate the bilayer thickness to the mica-mica separation measured when there is only one bilayer between the



surface (one monolayer per surface, as described below) then subtract twice this value from the mica-mica distance D. For egg lecithin this is 4.4 nm, so an estimate of the spacing between the bilayers is obtained by subtracting 8.8 nm from the values of D shown in Fig. 1. An accurate measurement was not obtained for DLPC, but according to Lis et al. [25] each DLPC bilayer is 0.5 nm thinner than an egg-PC bilayer, so in that case we subtract 7.8 nm from D to obtain the inter-bilayer spacing. Using these values, the empirical fits of Lis et al. [25] to their data are included in Fig. 1, showing that the two different experiments are in reasonable agreement, at least for large forces.

Another difficulty in comparing these results with those of Rand, Parsegian and co-workers is that with their osmotic stress technique the bilayers actually thicken as water is removed from the multilamellar system, whereas in the present case the two bilayers are free to get thinner as they are forced together, so the work done in bringing bilayers to a certain separation is not exactly the same in the two experiments.

Finally, we note that here the two bilayers are constrained by the mica surfaces, but in a multilamellar phase bilayers would have more freedom to undulate. Such movements could give rise to a steric hindrance between bilayers, leading to an additional repulsion [26,27]. The absence of this phenomenon in the present experiment may account for the steeper fall-off of the force at larger separations (particularly evident for DLPC around $D = 9$ nm) compared with the results of Lis et al. Indeed, my results would not be well fitted by a single exponential function. In the absence of any detailed theory of the hydration force I prefer not to attempt any empirical fit to the data, nor integration of any force law to obtain the interaction energy between curved bilayers.

As the separation increases the force becomes attractive due to van der Waals interactions (not shown on the log scale of Fig. 1). The sum of

Fig. 2. The process by which two adsorbed bilayers fuse into one when they are forced together, as monitored by direct observation of optical interference fringes. See text for a description. Note that the horizontal scale is 10^3-times larger than the vertical scale.

attractive and repulsive contributions to the force results in an adhesive minimum of $F/R = -0.50 \pm 0.05$ mN/m at $D = 10.7 \pm 0.2$ nm for egg lecithin, and -0.55 ± 0.05 mN/m at 9.3 ± 0.2 nm for DLPC. These values correspond to minima in the energy between flat bilayers of -0.08 and -0.09 mJ/m^2, respectively.

One consequence of the fact that in this experiment the two lipid bilayers can thin as they are squeezed together is that they can, under certain circumstances and a sufficiently large force, rupture and fuse together into a single bilayer. Observation of the interference fringes shows that this fusion process follows the sequence depicted in Fig. 2. The force required is so large that the solid surfaces on which the bilayers are adsorbed begin to flatten (Fig. 2b). Then the mica surfaces pinch through at the centre of the flattened region to a separation corresponding to just one bilayer between them (Fig. 2c); for egg-PC this was measured at 4.4 ± 0.2 nm. As soon as this occurs the central region begins to grow radially (Fig. 2d), taking a few seconds to sweep out half of each bilayer until a single bilayer (one monolayer on each surface) occupies the entire flat region, whose diameter is typically 30 μm (Fig. 2e). It has never been possible to observe rupture and removal of the final bilayer, because with the surfaces now almost perfectly flat it is impossible to apply enough pressure at one point to initiate the process. The surfaces adhere strongly when there is only one bilayer between them, and on separating the mica sheets they jump apart spontaneously to a large distance [15] so it is not possible to observe the process in reverse.

The event depicted in Fig. 2 did not occur in every experiment. It was observed more often in egg-PC than in the synthetic and somewhat purer DLPC, which suggested that impurities in the bilayer might help trigger fusion by facilitating the initial rupture. This idea was substantiated by the observation that fusion occurred readily when a small amount of *n*-hexane was deliberately incorporated in the bilayers. Additional alkane in the bilayers would promote the concave curvature required in the regions indicated by the circle in Fig. 2c. Other authors have suggested a requirement for this kind of curvature in the fusion of lipid bilayers [3,4,12,28,29]. The intermediate molecular arrangements they propose differ from the one observed here, but all agree that fusion follows a local destabilisation of the normal bilayer arrangement.

Even in the presence of some destabilising agent the force shown in Fig. 1 was not measurably affected, and a large force had to be applied to overcome the hydration repulsion before the bilayers fused. With the surfaces flattening as shown in Fig. 2b it was possible to estimate the applied pressure, and fusion commonly occurred under a few tens of atmospheres. However, this does not represent any critical pressure required for fusion of pure lecithin bilayers, because with DLPC pressures at least 10-times higher than this were frequently applied without fusion occurring. Clearly the factors which determine whether fusion will occur are related not so much to the forces between bilayers as to the forces within bilayers which give them their cohesive strength.

Although the fusion event described here is incomplete, in that the final bilayer is not broken, it may parallel the intermediate processes occurring in the membrane fusion required in certain secretory systems. Thin-section electron micrographs [29–31] show fusing membranes giving first a pentalaminar image, corresponding to two closely-apposed bilayers (devoid of proteins) as in Fig. 2b, then a trilaminar image corresponding to a single bilayer, as in Fig. 2e. The final step to complete fusion must involve rupture of that bilayer.

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