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Mutual adhesion of lecithin membranes at ultralow tensions

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(Reçu le 4 octobre 1988, accepté sous forme définitive le 13 décembre 1988)

Résumé. — On présente une étude par microscopie optique de l’adhésion mutuelle de doubles couches de lécithine d’œuf ou dimyristoyl dans des échantillons très gonflés. Les tensions latérales permettant l’adhésion, en dessous de $3 \times 10^{-4}$ dyn cm$^{-1}$, sont déterminées à partir de la courbure de la membrane à proximité de la zone de contact. L’angle de contact maximal des deux membranes soumises à des tensions égales a une valeur maximale de 45°. En dépit de quelque dispersion, cet angle ne semble pas dépendre de la tension jusqu’à sa valeur observable la plus faible, $3 \times 10^{-6}$ dyn cm$^{-1}$. L’angle de contact maximal d’une membrane isolée adhérant à un ensemble de membranes est proche de 70°. Les données indiquent que l’adhésion mutuelle est toujours induite par la tension latérale. La valeur constante des angles de contact semble compatible avec la théorie des ondulations, mais cette valeur nous pousse à postuler une rugosité de la membrane, non détectable optiquement, qui servirait de réserve d’aire.

Abstract. — We present an extensive light-microscopic study of mutual adhesion of egg (and dimyristoyl) lecithin bilayers in highly swollen samples, including adhesion brought about by osmotic inflation of vesicles. The lateral tensions associated with adhesion, if below ca. $3 \times 10^{-4}$ dyn cm$^{-1}$, could be read from a rounding of the membrane next to the contact area. The contact angle of symmetric adhesion, i.e. of two membranes under equal tension, had a maximum of 45°. While showing some scatter, it did not seem to depend on tension down to $3 \times 10^{-6}$ dyn cm$^{-1}$, the lowest tension observed. The maximum contact angle of single membranes adhering to bundles of membranes was near 70°. The data indicate that mutual adhesion of lecithin membranes is always induced by lateral tension. The constancy of the contact angles appears to be compatible with undulation theory, but their magnitude compels us to postulate an unknown, optically unresolvable roughness of lecithin membranes which serves as an area reservoir.

Introduction.

There has been much progress in the last fifteen years in the understanding of interactions between phospholipid membranes in water. The forces at short distances were first investigated in the pioneering work of Rand, Parsegian et al. [1, 2]. They studied dispersions of phospholipids in water, measuring by X-ray diffraction membrane separations as a function of osmotic, water vapour, or hydraulic pressure. For fluid membranes of lecithin, an electrically neutral material, they found a maximum spacing of about 3 nm which they interpreted as an equilibrium between repulsive hydration and attractive van der Waals
forces. The adhesion energies as calculated from their work are 0.01 erg cm\(^{-2}\) for 1,2-dimyristoyl-3-sn-glycerophosphocholine (DMPC) and 0.02-0.08 erg cm\(^{-2}\) for egg lecithin (EL). Horn [3] and Marra and Israelachvili [4], coating mica cylinders with phospholipid layers and measuring interaction energies at distances below [3, 4] and above [4] the energy minimum, obtained qualitatively similar results with fluid DMPC. However, the equilibrium spacing [3, 4] of 2 nm is smaller and the adhesion energy [4] of 0.1 erg cm\(^{-2}\) larger than the values found with the other method.

The interaction between the membranes of giant unilamellar lecithin vesicles (diameters > 10 \(\mu\)m) in 120 mM NaCl solution was studied by Evans \textit{et al.} with the micropipet aspiration technique. They varied the mechanical lateral tension in one of the vesicles and thus its contact area with a tightly stretched, practically stiff partner. Measuring in a range of tensions above \(10^{-2}\) dyn cm\(^{-1}\), they found an adhesion energy of 0.015 erg cm\(^{-2}\) for EL [5] and 0.01-0.015 erg cm\(^{-2}\) for other fluid lecithins including DMPC [6].

The adhesive behaviour of tension-free and, later, slightly stressed lecithin membranes has been investigated with optical microscopy in our group. In a large excess of water, lecithins above the main transition temperature always swelled and usually disintegrated, forming giant, often unilamellar vesicles in the process [7-10]. Furthermore, vesicles displaying well visible undulations (up to 10\% of the radius for spheres) were never seen to adhere on collisions [9]. The use of 0.5 M NaCl solutions instead of pure water slowed down the swelling, but vesicles still emerged and did not adhere to each other [11]. Well-ordered multilayer systems of EL extending from top to bottom of the sample cell were also found to swell indefinitely in the presence of excess water, their mean membrane spacings reaching 20 nm in the course of roughly a week [12]. An indirect check of the repulsive force at large spacings indicated that any electrostatic contribution was negligible at 2 or 3 nm where equilibrium could have been expected. All this points to the existence of a repulsive force between electrically neutral membranes which overcomes van der Waals attraction at large spacings where hydration forces are ineffective.

The new mechanism of repulsion has been attributed to the thermal undulations of unstressed fluid membranes [13]. These give rise to a steric interaction which is inversely proportional to the bending rigidity of the membranes. The undulation forces are comparable in range and strength to van der Waals attraction. We assumed them to be just strong enough in the case of lecithins to prevent adhesion. Evans and Parsegian [14] recently adopted undulation forces to account for the differences in equilibrium spacing and adhesion energy between membranes immobilized on mica [3, 4] and membranes in dispersions [1, 2] or around vesicles [5, 6]. However, they maintained the idea of an equilibrium spacing of lecithin bilayers.

Several years ago we noticed by chance that a slight osmotic inflation of egg lecithin vesicles in contact with each other gave rise to mutual membrane adhesion as manifested by well-developed contact areas [9, 15]. Subsequently, we started to give attention to the very rare adhesive contacts to be recognized in samples of swollen EL. From the geometry of a fluctuating tubular vesicle sticking to a tightly stretched membrane we estimated a lateral tension of \(10^{-4}\) dyn cm\(^{-1}\) in the tube membrane [16]. This was possible because a small rounding of the membrane next to the contact area permitted us to evaluate the tension. Contact rounding, which was not seen in the osmotic experiments, is due to the bending stiffness which opposes a sharp bend of the membrane. It should be optically resolvable at tensions \(\approx 3 \times 10^{-4}\) dyn cm\(^{-1}\), as will be derived again below. Using the Young equation for interfaces, like Evans \textit{et al.} [5, 6], we calculated the adhesion energy from contact angle and tension of the tube membrane. A search of the many photographs of swollen lecithin taken in
our group revealed additional adhesions with the membranes involved always being under some tension. Considering only the extremely rare cases of symmetric adhesion, i.e. nearly equal contact angles, of single membranes, we noted that the contact angle, while scattering below a maximum of 45°, appeared to depend rather little on lateral tension over two orders of magnitude of the latter [15]. On the basis of the Young equation this implies that in the same range the adhesion energy per unit area is roughly proportional to the tension and, in addition, almost equal to it. The proportionality was shown to agree with a scaling law of tension-induced adhesion which follows from a simplified model involving only van der Waals and undulation forces.

The original aim of the present work was to furnish more data on accidental adhesion and to analyse them more fully than previously in conference proceedings [15]. Our many additional observations verify the tension-independence of the contact angle of symmetric adhesion down to $3 \times 10^{-6}$ dyn cm$^{-1}$ and confirm the maximum of 45°. Moreover, we checked that using NaCl solutions instead of pure water had no noticeable effect. We extended the study to single membranes adhering to bundles of other membranes, finding a maximum contact angle of ca. 70° which also seemed not to depend on tension. Finally, the evaluation of lateral tensions was improved and some photographs are shown again in a larger magnification to bring out necessary detail.

Being analysed as previously, the new multitude of data looks like definitive proof that the separation of unstressed membranes is due to undulation forces. However, a novel argument employing the stretching energy of the free membrane shows eventually that the adhesion energy is a hundred times too large to permit this conclusion. In order to explain our data we are compelled to postulate an optically unresolved roughness of lecithin membranes which «absorbs» much more area than do the undulations. The Young equation has to be modified by introducing a variable ratio of real to projected membrane area.

Materials and methods.

EL and synthetic DMPC purchased from Sigma (Munich) were used without further purification. In most cases the egg lecithin contained a significant amount of 3sn-glycerophosphoethanolamine (ca. 25%) as judged from thin-layer chromatography. However, several samples which were pure with respect to the choline head group gave equal results. In a typical experiment a small quantity (some 0.3 μg) of the material was placed on an object slide and covered with ion-depleted water, the sample thickness being ca. 100 μm. Usually 50 μM Na$_2$N were dissolved in the water to prevent bacterial contamination. After mounting the cover glass the sample was sealed with a glue to stop evaporation. A few experiments were done with DMPC ca. 5 °C above its main transition temperature at 23 °C and some others in NaCl solutions, as will be described below.

The sample was put under a phase-contrast microscope (Leitz) equipped with a video system (Grundig) and a camera (Olympus). Membranes are visible in the object plane where they are parallel to the optical axis of the microscope. After one hour or more of swelling, the search was started for adhesive contacts of single membranes which were very rare and found only in a few samples. In general, single bilayers were easy to recognize from their weak and uniform optical contrast. The lamellarity could also be checked photometrically [17, 18]. We concentrated on states of approximately symmetric adhesion for evaluation.

Osmotic experiments, only with EL, were carried out in a round chamber 2 mm high and 1 cm wide which permitted the exchange of the solution. The water was driven by a peristaltic pump, a practically complete exchange taking about 3 min. After swelling the lecithin in stationary NaCl or glucose solution, we lowered the osmolarity to induce adhesion. Some vesicles sticking to the object slide remained in place during the exchanges. The clusters or
rare pairs of those vesicles were watched for the effects of osmotic inflation and deflation if they appeared to be unilamellar.

Lateral tensions were calculated or, at the limit of optical resolution, estimated from the contact rounding. Smooth mergings of the membrane contours instead of sharp angles are enforced by the bending rigidity of the membrane. The extension of the rounding reveals the angular correlation length, i.e. the correlation length of orientation, of a membrane under lateral tension. The method allowed us to measure very small tensions of ca. $3 \times 10^{-4} \text{ dyn cm}^{-1}$ and less which at the present time cannot be controlled by the micropipet aspiration technique.

In order to derive the theoretical contour of a single membrane next to its contact area, we start from the total energy per unit area of membrane, $g_{\text{total}}$. With $k_c$ being the bending rigidity and $\sigma$ the lateral tension, we may write

$$g_{\text{total}} = \frac{1}{2} k_c \left( \frac{\text{d}\phi}{\text{d}s} \right)^2 + \sigma (1 - \cos \phi)$$

(1)

where $s$ is the length of the contour measured from the edge of the contact area and $\phi$ is the local angle the contour makes with its asymptotic direction (see Fig. 1). The Euler-Lagrange formalism yields the shape equation of the free membrane

$$\frac{\text{d}^2\phi}{\text{d}s^2} = \frac{\sigma}{k_c} \sin \phi$$

(2)

which can be approximated for small $\phi$ by

$$\frac{\text{d}^2\phi}{\text{d}s^2} = \frac{\sigma}{k_c} \phi.$$  

(3)

Accordingly, the natural definition of the angular correlation length is

$$\xi_\sigma = \left( \frac{k_c}{\sigma} \right)^{1/2}.$$  

(4)

In calculating the lateral tension from $\xi_\sigma$ we always use $k_c = 2 \times 10^{-12} \text{ erg}$ as measured by our group for EL [7, 19] and DMPC [19] bilayers. Values between $2 \times 10^{-12}$ and $9 \times 10^{-13} \text{ erg}$ were obtained by others [20-23]. With an optical resolution slightly better than
1 μm this means that tensions are measurable up to $3 \times 10^{-4}$ dyn cm$^{-1}$. The same upper limit should approximately apply to the visibility of membrane fluctuations in our samples.

The differential equation (2) can be integrated to obtain a function $y = y(x)$ of the membrane contour in cartesian coordinates. Taking the $x$ axis to coincide with the asymptote for the free membrane, one finds the convenient parametrized representation

$$
\begin{align*}
\frac{x}{\xi} &= 2 \cos \phi / 2 + \ln \tan \phi / 4 + c \\
\frac{y}{\xi} &= 2 \sin \phi / 2.
\end{align*}
$$

(5)

Fig. 2. — Universal contour of contact rounding (solid line) and its exponential asymptote (broken line). The angular correlation length $\xi$ serves as unit length. Some of the angles $\phi$ the contour makes with the asymptotic direction, i.e. the $x$ axis, are indicated.

The integration constant $c$ may be chosen such that the universal form of the membrane contour given by equation (5) asymptotically coincides with the exponential function $y/\xi = \exp(x/\xi)$ for small $\phi$. Both curves are shown in figure 2. The deviation of the exact solution from the exponential is seen to be insignificant for angles up to 30°. To determine the angular correlation length from the contour we measured the contact angle and $y_0$, i.e. the distance of the edge of the contact area from the asymptote to the free membrane. This quantity is an attractive measure, being the largest simply defined characteristic length of the visible curve. However, the asymptotic direction was difficult to define in the presence of stationary deformations other than contact rounding. Moreover, fluctuations strongly disturbed the contours at the lowest tensions.
Adhesion energy densities $g_a$ were calculated from the lateral tension and contact angles (see Fig. 1) by using the conditions of mechanical equilibrium which hold for fluid interfaces [9, 15]. For two membranes in adhesion with tensions $\sigma_i$ and contact angles $\Psi_i$, they are the Young equation

$$g_a = \sigma_1(1 - \cos \Psi_1) + \sigma_2(1 - \cos \Psi_2)$$

and the balance of the normal components of the tensions

$$\sigma_1 \sin \Psi_1 = \sigma_2 \sin \Psi_2.$$  

The two-dimensional analysis should be sufficient whenever the angular correlation length is smaller than the cross section of the contact area. In the case of symmetric contact ($\Psi_1 = \Psi_2$) the two equilibrium conditions reduce to

$$g_a = 2 \sigma (1 - \cos \Psi).$$

Results.

In the osmotic experiments the decrease of osmolarity, while ranging from 0.2 to 16%, was typically 0.5 to 2%. All these changes which may be expected to increase the vesicle volume by 0.2 to 19% produced mutual adhesions of vesicles that had been in touch prior to osmotic inflation. Points of contact developed into areas which were not seen without inflation. The membranes of adhering vesicles displayed no visible fluctuations or contact roundings. This suggests that their lateral tensions were at least $10^{-3}$ dyn cm$^{-1}$. Actually, the limiting tension may have been somewhat lower since membrane visibility was poor in those very thick samples. Most of the immobile vesicles occurred in clusters rather than dimers and were situated at the bottom of the sample cell. The maximum contact angle of apparently unobstructed symmetric adhesion was 45°. The contact areas used to vanish in the course of minutes or hours, but could be produced again by further lowering the osmolarity of the medium. In a few cases we were able to increase and decrease a contact area several times and reproducibly by alternating deflation and inflation of the vesicles. An example with an exceptionally large expected volume increase of 19% is shown in figure 3. Here adhesion was turned on three times and turned off intermittently by alternating between 156 and 131 mOsm. In all the osmotic experiments the vesicles were never seen to rupture unless the theoretical increase in volume was much larger than 20%. The actual increases in volume and, especially, area were uncertain because of the uncontrollable attachment of the vesicles to the glass.

In addition to osmotically induced adhesion we studied the rare accidental contact areas that occur as transitory but in general long-lived states (hours or days) during the swelling of lecithin. Figure 4 depicts one of two experiments where it was possible to follow an interesting, rather rapid evolution. One sees two bilayers of DMPC (not EL as stated earlier [15]) belonging to complex vesicular structures and forming a large contact area. In the first picture (a) the right membrane looks like the segment of a stiff sphere while the left one exhibits large contact angles and visible angular correlation lengths. Within minutes the adhesion became symmetric with contact angles of 45° and no resolvable contact rounding (b). The next picture was taken 90 min later when the correlation length was again visible on the left and the contact angle still discernible on the right (c). It permits us to compute $\sigma_1$ (left) with equation (4) and then $\sigma_2$ (right) with equation (7). From this configuration and two similar ones, photographed before (b) and after (d), we invariably obtained $\sigma_2 = 1.1 \times 10^{-3}$ dyn cm$^{-1}$. Provided $\sigma_2$ is indeed constant all the time, we read from (b) the
Fig. 3. — Osmotically induced adhesion of EL vesicles attached to glass. The same vesicles (a) in 156 mOsm NaCl prior to osmotic inflation, (b) in 131 mOsm after first inflation, and (c) in 131 mOsm after third inflation and spontaneous ceasing of adhesion. (Before the last picture, adhesion was turned on 3 times and intermittently turned off by changing between 131 and 156 mOsm.) The bar indicates 10 μm.
Fig. 4. — Evolution with time of adhesion between DMPC membranes. See text for details. The bar indicates 10 μm.

contact angle of symmetric adhesion for the comparatively high tension of $1 \times 10^{-3}$ dyn cm$^{-1}$. The last photograph (d) represents a state of rather low tension of the left membrane. The slight inflections of its contour in (d) as well as in (c) are presumably due to a very small force
tending to pull the membranes apart. Table I lists for all states of figure 4 the lateral tensions and the adhesion energies calculated by means of the Young equation, i.e. equation (6) or equation (8).

Table I. — Data extracted from figure 4: Height $y_0$ of edge of contact area above asymptote of membrane on the left ($i = 1$), lateral tensions $\sigma_i$ of both membranes, and adhesion energy $g_{ae}$ as obtained from equation (6).

<table>
<thead>
<tr>
<th>$y_0/\mu\text{m}$</th>
<th>$\sigma_1/\text{dyn cm}^{-1}$</th>
<th>$\sigma_2/\text{dyn cm}^{-1}$</th>
<th>$g_{ae}/\text{erg cm}^{-2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1.26</td>
<td>$1.2 \times 10^{-4}$</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>b</td>
<td>—</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>c</td>
<td>0.81</td>
<td>$3.1 \times 10^{-4}$</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>d</td>
<td>1.20</td>
<td>$1.4 \times 10^{-4}$</td>
<td>$1.1 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

Figure 5 shows a sequence of pictures capturing the separation of two membranes that initially are in roughly symmetric adhesion. The evaluation of the tensions of the membranes is impaired by deformations other than contact rounding (and probably accompanied by

Fig. 5. — Gradual breakup of an adhesive contact between EL membranes. (The contact is in the center.) The bar indicates $10\mu\text{m}$.
pressure differences), but measuring a single tension is enough in principle since the rest can be obtained by means of the equilibrium condition (7). A case of symmetric adhesion with exceptionally large, well resolved angular correlation lengths is shown in figure 6. The irregularities in the membrane contours are due in part to the large fluctuations associated with the lowest tensions. The majority of the new data were obtained from « branched » structures in which single membranes run from one bundle of membranes to another. At the end of a bundle one often finds symmetric adhesion. An example is given in figure 7. The contours were usually seen in the middle between top and bottom of the sample cell.

Fig. 6. — Symmetric adhesion of EL membranes with large angular correlation lengths. Some of the distortions are due to thermal fluctuations. The bar indicates 10 μm.

Fig. 7. — A « branched » structure displaying symmetric adhesions and adhesion of single membranes to bundles of membranes. The bar indicates 10 μm.
The lateral tension of a curved membrane is, of course, associated with a pressure difference $\Delta p$ across it. In general, $\Delta p = \sigma (1/R_1 + 1/R_2)$ where $R_1$ and $R_2$ are the principal radii of curvature. In the branched structures the membranes seem to form semicircles which usually span the sample cell. A straight contour then indicates $\Delta p = 2 \sigma/D$, $D$ being the sample thickness. Note that contact rounding does not involve pressure. The exact membrane shapes apart from the visible contours are unknown. However, we assume them not to affect what is seen since $D$ (ca. 100 $\mu$m) is much larger than $\xi_0$ and the contours of symmetric adhesion do not reveal to which side the two membranes turn.

Adhesions of single membranes were regarded as symmetric and used for further evaluation if the two contact angles differed by no more than 10$^\circ$. Of the 40 contacts satisfying this criterion and exhibiting rounding, only four had one or two angles below 30$^\circ$. It is remarkable that in seven cases both angles reached within 1$^\circ$ the maximum of 45$^\circ$. The contact angles appeared to be independent of lateral tension in the covered range from $3 \times 10^{-6}$ to ca. $1 \times 10^{-3}$ dyn cm$^{-1}$. Any remaining dependence of the angles on tension should be weaker than their scatter.

Adhesion energies of symmetric adhesion were calculated with the Young equation (8) from the pair averages of angles and tensions. The data are compiled in figure 8 where adhesion energy is plotted versus lateral tension. According to (8), an invariable contact angle means proportionality of the two variables and, therefore, a straight line of slope unity in the doubly logarithmic plot. Since contact roundings were not always visible and the maximum contact angle of osmotically induced adhesion also was 45$^\circ$, we think that at least for egg lecithin the proportionality extends to some tension above $10^{-3}$ dyn cm$^{-1}$. Note that errors in evaluating the angular correlation length, even a wrong value of the bending rigidity, do not affect the relationship of lateral tension and adhesion energy except that they shift the data points parallel to it.

Occasionally, as in figure 5, we saw membranes with very small contacts suddenly separate and relax into new equilibrium positions. Assuming the contact areas to be circular (which need not be correct) we estimated the total adhesion energies just before separation to be a

![Fig. 8. — Plot of adhesion energy versus lateral tension for symmetric contacts. Adhesion energies were calculated by means of equation (8). The point at $10^{-3}$ dyn cm$^{-1}$ represents DMPC, all others EL. The open boxes indicate the results of Evans and Metcalfe, which were measured with the more flaccid membrane being between $10^{-2}$ and $10^{-1}$ dyn cm$^{-1}$, and those of Rand et al. (lower $g_\sigma$) and Marra and Israelachvili (higher $g_\sigma$), which we arbitrarily put at $\sigma = 1$ dyn cm$^{-1}$. Note that in all three cases the experiments were different from ours. The data are shown only for comparison.](image)
few $k_B T$, i.e. in the range of thermally accessible energies. In branched structures such as that of figure 5 several membranes join each other one by one to form a bundle. The lateral tension varied among bundles and, to a lesser extent, among the membranes composing a bundle. We refrained from a systematic study of tensions as the bundles blurred the contact roundings of the single membranes. The contact angles of the bilayers used to increase with the number of membranes already in the bundle up to a limiting value of $(70 \pm 5)^\circ$ which was reached with 5-10 membranes.

Accidental adhesion was also studied in samples containing 30 or 100 mM NaCl solution instead of pure water. These concentrations are low enough to avoid a severe slow-down of swelling. On the other hand, they are high enough to be visible in phase contrast if the solution borders pure water. (This was verified in separate experiments in which vesicles formed in salt solution were exposed to pure water.) We saw no signs of water penetrating into the lecithin without the salt. Branched structures similar to that shown in figure 7 were also found in salt solution, but their dimensions were reduced and most of the lateral tensions were too large to be measurable. The contact angles of nine symmetric adhesions displayed the same maximum of 45° and a similar scatter as in the samples without salt. Swelling of DMPC even in pure water did not produce any branched structures suitable for evaluation.

Discussion.

The experiments demonstrate that the contact angle of symmetric mutual adhesion of egg lecithin membranes does not significantly depend on lateral tension between $3 \times 10^{-6}$ and ca. $1 \times 10^{-3}$ dyn cm$^{-1}$. If there is spontaneous adhesion, its energy would have to be $10^{-6}$ erg cm$^{-2}$ or less. Since electrostatic repulsion can be ruled out for several reasons (see below), we may conclude with more confidence than ever before that unstressed lecithin membranes are driven apart by steric interaction and that any adhesion in our samples is induced by lateral tension. The same conclusion can be drawn for the upper limit of the contact angle of symmetric adhesion at 45° since spontaneous adhesion would permit angles $\geq 90^\circ$. This argument can be extended from pairs to stacks of membranes where an upper bound of ca. 70° again signals the absence of spontaneous adhesion.

In the following quantitative discussion we first attempt to analyse the induced mutual adhesion of lecithin membranes in the current theoretical framework. Specifically, we assume steric interaction to originate alone from the thermal undulations of the membranes. On the basis of a simplified model we then rederive the proportionality of adhesion energy and lateral tension which agrees so well with experiment. A subsequent comparison of forces raises doubts if the undulation forces are strong enough to separate unstressed membranes, but by itself is not conclusive. However, the adhesion energies as calculated with the Young equation for interfaces turn out to be a hundred times larger than is allowed if the free membrane absorbs area, i.e. increases real area over projected area, solely by means of its undulation. In order to avoid a glaring contradiction we will postulate an optically unresolvable roughness of the lecithin membranes. The new roughness may take up so much area that the Young equation for interfaces has to be replaced by a novel generalization for membranes.

The total force per unit area, $f_{\text{tot}}$, between membranes may be divided into four contributions,

$$f_{\text{tot}} = f_h + f_v + f_q + f_u,$$

representing hydration, van der Waals, electrostatic, and undulation forces, respectively. The first three are called « direct » [24] and refer to a uniform membrane spacing. The last one, arising from membrane undulations, can be defined only for a mean spacing. The
superposition of this force on the others is, of course, naive and may at best be regarded as an approximation.

The repulsive hydration force is in general expressed [2] by an exponential,

\[ f_h = f_h^0 \exp(-z/z_h), \]  

(10)

where \( z \) is the membrane spacing and \( z_h \) a characteristic length of a few 0.1 nm. This force dominates below the equilibrium spacing of 2 nm for immobile lecithin membranes but is negligible at greater distances. However, for fluctuating membranes at large mean spacings it should make itself felt as an effective thickening of the bilayers by 1 nm on each side.

Electric forces, also repulsive, vanish in the case of lecithin unless the membranes contain ionic impurities or absorb impurity ions. Any electrostatic repulsion in our samples must have been weaker than van der Waals attraction over the range of lateral tensions (and associated membrane spacings) covered by our observations of induced adhesion. (See Ref. [12] for an analysis of electrostatic repulsion at zero lateral tension.) Furthermore, the fact that we obtained consistent data over several years and with lecithins from many different bottles strongly suggests that there was no electrostatic interaction involved. A direct proof of its absence is provided by the samples containing NaCl solution instead of pure water. The contact angles of accidental (and osmotically induced) adhesion were not noticeably affected by concentrations of 100 or 30 mM reducing the Debye screening length to 1.0 nm or 1.8 nm, respectively. Electrostatic forces will therefore be omitted in the following. Debye screening might still be expected to weaken van der Waals attraction by suppressing its zero-frequency contribution, but within experimental accuracy we noted neither an increase nor a decrease of the contact angles due to salt.

The attractive van der Waals force per unit area between parallel membranes is expressed by the half-space approximation,

\[ f_v = -\frac{A_h}{6 \pi z^3}, \]  

(11)

\( A_h \) being the Hamaker constant. Since the bilayers have a finite thickness of ca. 4 nm, the \( 1/z^3 \) law is gradually superseded by a \( 1/z^5 \) dependence [2] at spacings larger than that (and finally by a \( 1/z^6 \) law because of retardation). Other details of van der Waals interaction will be mentioned when we consider numbers.

The repulsive undulation force per unit area was predicted to be, in a stack of undulating membranes [13],

\[ f_u = \frac{3}{64} \pi^2 \frac{(k_B T)^2}{k_c z^3}. \]  

(12)

Here \( k_c \) is the bending rigidity and \( z \) the mean membrane spacing. Equation (12) is based on the assumption of vanishing direct interaction. Note that both van der Waals and undulation forces are inversely proportional to the cube of the (mean) membrane spacing. More than the direct forces, undulation forces will depend on the number of membranes in the system. The difference between two (a pair), a few or several (a bundle), or a quasi-infinite number (a stack) of membranes is largely neglected in our discussion.

The total interaction of membranes that arises from the interplay of undulations and direct forces can be calculated rather straightforwardly and accurately if the distribution function of membrane spacing is roughly Gaussian [14, 25]. Otherwise, one has to take recourse to functional renormalization [24], an analogy to wetting in two dimensions [26], or computer simulation [27]. All three methods have been used in dealing with the unbinding transition of
membranes. However, the first and second suffer from large uncertainties of the numerical factors and results of the third are not yet available.

Here we consider the simplifying case of «ideal» competition, taking into account only undulation forces and the half-space approximation of van der Waals attraction. We are interested in some scaling laws of this special case which has the advantage of enabling us to incorporate lateral tension. There are two characteristic lengths in the model: the correlation length \( \xi_\sigma \) of membrane orientation is the presence of tension as given by (4) and the lateral correlation length \( \xi_l \) of membrane spacing [24],

\[
\xi_l = \left( \frac{k_c}{k_B T} \right)^{1/2} \bar{z}.
\]

(Apart from a numerical factor near unity, the latter length is that of the edges of a square piece of membrane just large enough so that its undulations fill an interval of width \( \bar{z} \).

Lateral tension suppresses long-wavelength undulations and thus weakens their forces at large mean spacings. The reduction factor should depend only on the ratio of the two characteristic lengths or, more conveniently, on

\[
\frac{\xi_l^2}{\xi_\sigma^2} = \frac{\sigma \bar{z}^2}{k_B T}
\]

which is independent of \( k_c \). (This holds for \( n_\sigma^2 + n_l^2 \ll 1 \), where \( n \) is the surface normal of the undulating membrane, i.e. usually for \( k_c > k_B T \).) The forces at large mean spacings have been predicted to drop exponentially with \( \bar{z}^2 \sigma / k_B T \) (mean field [16]) or \( \bar{z} (\sigma / k_B T)^{1/2} \) (renormalization [28]) in the exponent. No new length enters if there is van der Waals attraction in addition to steric repulsion since both forces vary with the same power of spacing. Therefore, we may write down the following ansatz for the total force per unit area,

\[
f_{\text{tot}} = 3 \frac{\pi^2 (k_B T)^2}{64 k_c \bar{z}^3} F \left( \frac{A_h k_c}{(k_B T)^2}, \frac{\sigma \bar{z}^2}{k_B T} \right).
\]

The reduction factor \( F \) depends on the length ratio (14) and the ratio of the strengths of the pure interactions. In the case \( \sigma = 0 \), it equals unity for \( A_h = 0 \) and will come down to zero when \( A_h \) reaches a critical value \( A_{h,c} \). The order of magnitude of \( A_{h,c} \) should be given by

\[
A_{h,c} = \frac{9}{32} \frac{\pi^3 (k_B T)^2}{k_c},
\]

where the right-hand side is the value obtained from (11) and (12) if the superposition principle is taken to be valid.

The membranes collapse into each other for \( A_h > A_{h,c} \) as the model lacks the hydration barrier. For \( 0 < A_h < A_{h,c} \), the total force is positive at all \( \bar{z} > 0 \). (A pressure is needed to keep the system at these spacings.) In the case of interest, \( \sigma > 0 \) and \( A_h < A_{h,c} \), the total force becomes zero, i.e. equilibrium is reached, at a certain \( \sigma \bar{z}^2 / k_B T \). Evidently, the equilibrium spacing \( \bar{z}_{eq} \) satisfies the scaling law

\[
\bar{z}_{eq}^2 \sim 1 / \sigma
\]

for a given system and temperature. Since the interaction energies go with \( 1 / \bar{z}^2 \), the adhesion energy may be expected to obey \( g_a \sim 1 / \bar{z}_{eq}^2 \) which, because of (17), results in

\[
g_a \sim \sigma.
\]
This is the proportionality which we observed. It was derived previously in terms of a special model [15].

Finding the same scaling law in theory and experiment looks like the final proof that the membranes are driven apart solely by undulation forces. However, the following examination of numbers will eventually compel us to abandon this attractive picture. We begin with a comparison of forces, inserting material parameters into the force laws (11) and (12). A calculation of the undulation forces from equation (12) carries more weight today than previously since the numerical factor in the equation seems to have recently been confirmed within 10% in experiments with very flexible membranes [29, 30]. Using \( A_h = 6 \times 10^{-14} \text{erg} \) (a typical value [2, 31]), \( k_c = 2 \times 10^{-12} \text{erg} \) (measured in our group), and \( k_B T = 4 \times 10^{-14} \text{erg} \) (room temperature), we obtain \( f_v = -3.1 \times 10^{-15} \text{erg/}z^3 \) and \( f_u = 3 \times 10^{-16} \text{erg/}z^3 \), which would mean that undulation forces are too weak for separation by an order of magnitude. The gap is closed if one takes for the Hamaker constant and the binding rigidity the smallest values reported, \( A_h = 1.3 \times 10^{-14} \text{erg}^{4} \) and \( k_c = 9 \times 10^{-13} \text{erg} \) [20-23]. They give \( f_v = -6.9 \times 10^{-16} \text{erg/}z^3 \) and \( f_u = 8.2 \times 10^{-16} \text{erg/}z^3 \), i.e. a slight preponderance of undulation forces. However, the inevitable distortion of the distribution function of the membrane spacing by direct forces lowers the total free energy, which is likely to reverse the advantage in a complete theory. On the other hand, hydration forces and the accelerated drop of van der Waals attraction at distances beyond ca. 4 nm, both disregarded in our model, promote membrane separation. (They also raise questions about the range of validity of \( \sigma_a \sim \sigma \) which we ignore.) The effect of an impenetrable hydration layer can be more or less compensated if the apparent plane of origin of van der Waals attraction is shifted towards the water. A shift of this sort extending 0.5 nm on each side of the lecithin bilayer was recently reported [4], but a new theory of van der Waals interaction identifying a special contribution from the molecular headgroups interprets these data differently [31]. On the whole, it seems that experimental and theoretical uncertainties are still considerable. While the mutual adhesion of unstressed lecithin membranes may appear more probable than their separation by undulation forces, a reliable prediction is as yet not possible.

We now turn to another argument which is less direct but shows very clearly that the large contact angles of induced adhesion are not compatible with the weak undulations of lecithin membranes. Considering for simplicity only the symmetric case, we first define the following quantities: \( \sigma_f \) and \( \sigma_l = \sigma_f \cos \Psi \) are the lateral tensions of the free and the adhering membrane, respectively, which are now distinguished by subscripts; \( \alpha_f \) and \( \alpha_l \) are the ratios of projected to real membrane area for the two states; \( g_f \) and \( g_l \) are the respective free energies of membrane stretching per unit projected area. We will neglect the dependence of real area on tension, so that stretching is equivalent to a flattening of undulations or other roughness of an otherwise unstretchable membrane. The ratios \( \alpha < 1 \) of projected to real area, introduced here for the first time, can be omitted in the following formulas if they are close enough to unity. The adhesion energy \( g_a \), also referring to unit projected area, obeys the equations

\[
\alpha_l \ g_a = 2(\alpha_f - \alpha_l \cos \Psi) \sigma_f \tag{19}
\]

and

\[
\alpha_l \ g_a = 2(\alpha_f g_f - \alpha_l g_l) \tag{20}
\]

The first is a extension of the Young equation (8) to membranes and the second is an obvious relationship between energies. Whenever \( \sigma_f > 0 \) we have \( g_f > 0 \) and, if there is no spontaneous adhesion, \( \sigma_l > 0 \) and \( g_l > 0 \). Accordingly, equation (20) implies the inequality

\[
g_a < 2 \frac{\alpha_f}{\alpha_l} g_f \tag{21}
\]
The statistical mechanics of undulations predicts, for egg lecithin, $\alpha_f$ and $\alpha_I$ to be unity within a few percent, as shown previously [16] and rederived in the Appendix. The energy of stretching, i.e. the energy of undulation flattening, has also been calculated [6]. For membranes as stiff as lecithin bilayers it should be

$$g_f = \frac{k_B T}{8 \pi k_c} \sigma$$

(22)

to a very good approximation (see Appendix). Inserting $k_B T = 4 \times 10^{-14}$ erg and $k_c = 2 \times 10^{-12}$ erg yields $g_f = 0.8 \times 10^{-3}$ erg cm$^{-2}$. The adhesion energies calculated from (19) for $\alpha_f \approx \alpha_I \approx 1$ obey $g_a = (1/2) \sigma_f$ and thus exceed the upper limit imposed by (21) together with (22) and $\alpha_f \approx \alpha_I \approx 1$ by more than a factor of 100. It is interesting to note that if this bound were valid it would, because of (19), imply very small contact angles $\Psi \leq 2^\circ$.

The enormous discrepancy between the experimental adhesion energies and their theoretical upper bounds cannot be explained away by errors in measuring the bending rigidity. We are thus forced to postulate that an optically unresolvable roughness of lecithin membranes absorbs much more area than do the undulations without destroying the stiffness of the bilayers. Primitive estimates of the minimum hidden area of the unstressed membrane needed to permit $g_a \approx (1/2) \sigma_f$ over two orders of magnitude lead to $\alpha_f = 0.5$. (They suppose that $\alpha$ goes from this value to almost unity in the range of our experiments.) However, ratios $\alpha$ markedly below unity require use of the generalized Young equation (19). As it is reasonable to expect $\alpha_f > \alpha_I$, i.e. a stronger flattening if the roughness where the membranes are in adhesive contact, the factor $(\alpha_f/\alpha_I - \cos \Psi)$ may be significantly less than $(1 - \cos \Psi)$ and the true adhesion energy correspondingly smaller. If this is the case, there are two important consequences. First, $\alpha_f$ for $\sigma = 0$ need not be as small as 0.5. Second, the compilation of results in figure 8 has to be viewed with caution. The plot of adhesion energy versus lateral tension which is shown there may eventually have to be replaced by some lower-lying curve, and the intriguing proportionality of the two quantities could be an artefact.

It may be relevant in this context that we never saw vesicles burst during osmotic inflation unless the theoretical increase in volume was very large ($\geq 20\%$). For spherical vesicles the increase in projected membrane area $\Delta A/A$ is $2/3$ of that in volume. Therefore, volume increase of $4\%$ or less should lead to tensions above $3$ dyn cm$^{-1}$ and thus to membrane rupture, if $\Delta A/A$ is due to the flattening of undulations and the usual stretching elasticity (see Appendix). The fact that no rupture was observed suggests the presence of a reservoir of real area which may be provided by the envisaged roughness. The few experiments of osmotic deflation after inflation seem to confirm the presence of a reservoir since they did not result in nonspherical, flaccid vesicles.

**Conclusion.**

The postulated submicroscopic roughness would have the attractive feature of increasing the steric interaction of the membranes at small spacings. Because of the smallness of the undulation forces it might be this contribution which permits the total steric forces to overcome van der Waals attraction.

There need be no contradiction between our data and those of E. Evans et al. [5, 6] who found an invariable adhesion energy of $0.015$ erg cm$^{-2}$ for egg lecithin. In their experiments, one of the membranes was tightly stretched, forming a stiff spherical surface, and the other kept at tensions $\approx 10^{-2}$ dyn cm$^{-1}$. We suspect that most of the postulated roughness is flattened when the tensions are raised to these orders of magnitude.
If nature makes use of induced adhesion between and within biological cells, it should prefer contact areas that are well-defined and large enough to be thermally stable. Contact angles around $45^\circ$ seem much more suitable for these purposes than the very small angles permitted by undulation theory. In this laboratory, efforts are under way to obtain more evidence for the conjectured roughness and to detect at least fingerprints of its structure. A tentative model to explain the roughness is being proposed elsewhere [33]. It invokes a possible instability of the bilayer with respect to deformations containing very strong saddle curvature.

Acknowledgment.

Part of this work was supported by the Deutsche Forschungsgemeinschaft through SFB 312.

Appendix.

The statistical mechanics of the single undulating membrane under lateral tension has been considered previously [16]. In the following we rederive the formulas which are needed in the Discussion. The undeformed fluid membrane is taken to coincide with the $xy$ plane and its actual shape is described by $u = u(r)$ where $r = (x, y)$ and $u^2/2$. Assuming periodic boundary conditions and a quadratic base of area $A$, we write down the Fourier expansion

$$u(r) = \sum (a_q \cos (q \cdot r) + b_q \sin (q \cdot r))$$

where $q = (q_x, q_y) = 2 \pi A^{-1/2}(m, n)$, $m$ and $n$ being integers. Half the $q$ plane may be assigned to cosine modes, the other to sine modes. The equipartition theorem gives

$$\langle a_q^2 \rangle = \langle b_q^2 \rangle = \frac{2 k_B T}{A(\sigma + k_c q^4)} .$$

The mean relative change per mode of projected area is

$$\delta A_q / A = -\frac{1}{4} q^2 \langle a_q^2 \rangle$$

for a membrane conserving its real area. The sum over all modes yields the total relative change of projected area, $\Delta A / A$. We replace the sum by an integral according to

$$\sum_q \rightarrow \frac{A}{(2 \pi)^2} \int_{q_{\min}}^{q_{\max}} 2 \pi q dq$$

and put $q_{\min} = \pi / A^{1/2}$ and $q_{\max} = \pi / a$, $a$ being a molecular length. Carrying out the integration leads to

$$\frac{\Delta A}{A} = -\frac{k_B T}{8 \pi k_c} \ln \frac{\pi^2 / a^2 + \sigma / k_c}{\pi^2 / A + \sigma / k_c} .$$

(A.1)

This formula is good only for $|\Delta A / A| \ll 1$. For

$$k_c \frac{\pi^2}{A} \ll \sigma / k_c \ll k_c \frac{\pi^2}{a^2}$$
it simplifies to
\[
\frac{\Delta A}{A} = -\frac{k_BT}{8\pi k_c}\ln\frac{k_c\pi^2}{\sigma_t a^2}.
\]

Adding now to the flattening of undulations the effect of regular stretching elasticity, we may write
\[
\frac{\Delta A}{A} = -\frac{k_BT}{8\pi k_c}\ln\frac{\sigma_t a^2}{k_c\pi^2} + \frac{\sigma_t}{\lambda}
\]
where \(\lambda\) is the stretching rigidity. The free energy of stretching, \(g_t\), obeys
\[
dg_t/d\sigma_t = \sigma_t \frac{d(\Delta A/A)}{d\sigma}
\]
which leads to
\[
\sigma_t = \frac{k_B T}{8\pi k_c} \sigma_t + \frac{1}{2\lambda} \sigma_t^2
\]
for \(\sigma_t \ll k_c \pi^2/a^2\). Kwok and Evans [32] measured \(\lambda = 140 \text{ dyn cm}^{-1}\) for egg lecithin membranes. It is easily seen that with \(k_B T = 4 \times 10^{-14} \text{ erg}\) and \(k_c = 2 \times 10^{-12} \text{ erg}\) the contributions of regular stretching to \(\Delta A/A\) and \(g_t\) should be negligible whenever \(\sigma_t \lesssim 10^{-2} \text{ dyn cm}^{-1}\). For \(\sigma_t = 0\), equation (A.1) takes the form
\[
\frac{\Delta A}{A} = -\frac{k_BT}{8\pi k_c}\ln\frac{A}{a^2},
\]
which represents the largest possible relative decrease of projected area. For \(a = 5 \times 10^{-8} \text{ cm}\) and \(A = 4\pi(10^{-3})^2 \approx 10^{-5} \text{ cm}^2\) one finds \(\Delta A/A = -1.9 \times 10^{-2}\). Accordingly, we may expect \(\alpha = 0.98\) for \(\sigma_t = 0\) and values even closer to unity in the presence of tension.

References


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