from the P.H.L.S. Centre for Applied Microbiology and Research, Porton Down, United Kingdom.
12. The enzyme (~2.2 μ*M*) and various concentrations of inhibitor (0.5 to 5.0 μ*M*) were incubated together. Small samples were withdrawn at suitable times and assayed spectrophotometrically (232 nm) against benzylpenicillin for β-lactamase activity.
13. Under second-order conditions, the fraction X of residual activity at time t is given by $X = (I_0 - E_0)/(I_0 \exp[(I_0 - E_0)k_i t] - E_0)$, where E_0 and I_0 are the initial concentrations of enzyme and inhibitor, respectively, k_i the second-order rate constant for the inactivation. Experimental data was fitted to this equation by a nonlinear least-squares procedure.
14. The hydrolysis of cephalothin (2.46 m*M*), catalyzed by the *E. cloacae* P99 β-lactamase (2.3 n*M*), was followed spectrophotometrically at 292 nm in the presence of **3** (0.5 to 2.5 m*M*).
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Molecular Mechanisms and Forces Involved in the Adhesion and Fusion of Amphiphilic Bilayers

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The surface forces apparatus technique was used for measuring the adhesion, deformation, and fusion of bilayers supported on mica surfaces in aqueous solutions. The most important force leading to the direct fusion of bilayers is the hydrophobic interaction, although the occurrence of fusion is not simply related to the force law between bilayers. Bilayers do not need to "overcome" some repulsive force barrier, such as hydration, before they can fuse. Instead, once bilayer surfaces come within about 1 nanometer of each other, local deformations and molecular rearrangements allow them to "bypass" these forces.

THE FUSION OF AMPHIPHILIC (surfactant and lipid) monolayers or bilayers arises in both colloidal and biological systems (*1*). However, an understanding of interbilayer forces, the mechanisms of fusion, and the relation between the two is still far from clear. Some of the forces between amphiphilic surfaces have only recently been discovered. Thus, in addition to the expected attractive van der Waals forces and repulsive electrostatic double-layer forces (*1–3*), the existence and importance of repulsive hydration and attractive hydrophobic forces (*2–6*), short-range attractive ion-correlation forces (*7*), and medium- to short-range repulsive undulation (or fluctuation) forces (*8*) are only now being recognized. Their role in fusion has not yet been investigated. The origin of the so-called "hydration" and "hydrophobic" forces is still far from clear. Second, various models of the fusion process have been suggested, mainly based on electron micrographs of small vesicles or membranes (*1, 9, 10*) or of optical, capacitance, and conductance measurements of large fusing vesicles or "black lipid membranes" (BLMs) (*1, 11*). However, the precise molecular events and rearrangements accompanying the fusion process are still unknown. We present results of measurements of the interactions between various adsorbed bilayers in aqueous electrolyte solutions using the surface forces apparatus (SFA) technique.

The SFA technique allows forces and pressures to be measured between two curved or flattened molecularly smooth mica surfaces (*12*), or between monolayers or bilayers deposited on such surfaces (*5, 6, 13*), with a distance resolution at the angstrom level. Surface deformations accompanying adhesion (*14*) and fusion (*15*) can be directly visualized in real time. We note the good agreement in the measured forces between lecithin bilayers using the SFA and osmotic pressure technique (*16*).

We used two methods for coating mica surfaces with bilayers: (i) adsorption from solution and (ii) for insoluble lipids, controlled deposition using a Langmuir-Blodgett (LB) trough. Single-chained bilayers of CTAB (recrystallized in 9:1 ethanol:ether) were adsorbed onto mica surfaces at concentrations at or above their critical micelle

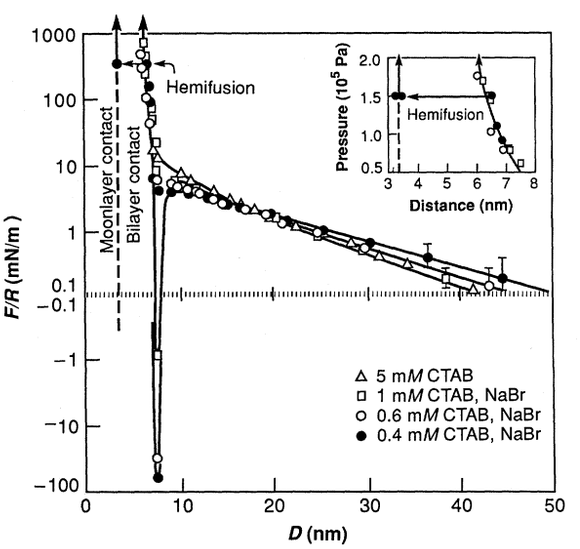

Fig. 1. Forces (F/R, force to radius) as a function of distance D between CTAB bilayers 3.2 to 3.6 nm thick adsorbed onto mica surfaces from CTAB solution; $D = 0$ corresponds to mica-mica contact in water, in contrast to Fig. 4, where it corresponds to bilayer contact. The concentration of CTAB + NaBr of the solution in the chamber was progressively diluted below the CMC (about 1 m*M*), until hemifusion occurred at and below 0.4 m*M*. The long-range forces are repulsive electrostatic "double-layer" interactions. The measured Debye lengths were 11 nm at 0.4 m*M*, 9 nm at 0.6 m*M*, and 8 nm at both 1 and 5 m*M*. [The similarity of the latter two Debye lengths arises because above the CMC the micelles and their bound counterions no longer contribute to the Debye length (*17*).] The adhesion forces at the minima are for bilayers that had been in contact for 1 hour. At high values of F/R, the surfaces flatten elastically, and the pressure can be measured, as shown in the inset. Hemifusion (see Fig. 2) occurred at 0.4 m*M* at a pressure of 1.5 atm.

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concentration (CMC) (*17–19*). Bilayers of "insoluble" double-chained lipids such as lecithins were deposited with the LB technique, with the aqueous bathing medium saturated with lipid at the CMC as previously described (*13*).

The apparatus chamber could be progressively diluted with pure water, thereby reducing the concentration of the bathing medium below the CMC. Desorption of lipids occurred, causing a thinning of the adsorbed bilayers that resulted in a new equilibrium with a larger hydrophobic area (chain segments) per molecule exposed to the aqueous phase (*13*). Thus the "hydrophobicity" of these "depleted" bilayers was increased in those experiments devoted to studying the effects of the hydrophobic interaction on adhesion and fusion.

Bilayers composed of pure lipids do not fuse if the bilayers are "fully developed" or "saturated," that is, if the surfactant or lipid concentration in the bathing medium is at or above the CMC so that it acts as a saturated reservoir for the bilayer lipids to exchange with. The absence of fusion between such bilayers was noted for all of the single- and double-chained lipids, both above and below the critical temperature, T_c, and up to compressive pressures exceeding 100 atm. However, fusion could be induced by depleting bilayers of their lipids, which renders them more hydrophobic.

When fully developed bilayers interact in water, their hydrophobic regions are effectively shielded from the aqueous phase, and there is no hydrophobic contribution to the attraction between them. For example, the adhesion between lecithins and other neutral bilayers such as MGDG and DGDG is well accounted for by the Lifshitz theory of van der Waals forces (*13*). Between two purely hydrocarbon surfaces in salt solutions, the hydrophobic force is long range and exceeds the van der Waals force by one to two orders of magnitude at distances up to 8 nm (*6*). With increasing depletion (hydrophobicity) of the bilayers, both the range and magnitude of the attractive forces increase and fusion occurs with increasing ease.

In Fig. 1 we show this phenomenon for two CTAB bilayers. These singly chained, positively charged surfactants were first adsorbed onto mica from a micellar solution above the CMC (of ~1 m*M*) where the bilayers are fully developed, and there was no adhesion or fusion (see top curve for 5 m*M* CTAB). As the CTAB concentration in the chamber was progressively decreased below the CMC, the long-range part of the double-layer forces changed by a small amount as expected theoretically, but at closer distances the bilayers unexpectedly jumped into an adhesive minimum from a separation of 1.8 to 2.5 nm. In addition, the depth of the adhesive minimum increased dramatically, both in magnitude and with the time in contact. These phenomena point to the emergence of an attractive force that is far too large to be explained in terms of van der Waals forces. Eventually, below a certain concentration (see Fig. 1), fusion occurred spontaneously with the two bilayers coming together and fusing straight into one bilayer. Similar effects were found with the double-chained lipids DLPC and DMPC, as described below.

For all of our adsorbed bilayers, fusion always commenced with two bilayers fusing into one bilayer, commonly referred to as "hemifusion" or "monolayer fusion" (*1*), which is essentially the same as found by Horn (*15*) for the fusion of egg-PC bilayers. Video recordings of the changing fringe pattern during hemifusion (Fig. 2) show that the two bilayers do not have to first come into contact to fuse; indeed, with the PC bilayers, fusion occurred spontaneously when the bilayer surfaces were more than 1 nm apart. The crucial first breakthrough step starts by a highly localized lateral parting of the head groups on opposite sides of the bilayers, thereby exposing or "opening up" hydrophobic-hydrocarbon regions (Figs. 2C and 3A) that are shielded from the aqueous phase in the isolated fully developed bilayers. Because of the long-range nature of the hydrophobic interaction (*6*), these apposing hydrophobic regions become unstable and spontaneously jump together, or break through (Fig. 2, steps C to D, and Fig. 3, steps A to B) across the gap and fuse. The two outer monolayers of the locally hemifused bilayers now slide radially outwards from the fusion site until only one bilayer remains (Figs. 2F and 3C). Such hemifused bilayers have previously been observed as stable or metastable states during the fusion of large unilamellar vesicles in solution (*1, 10, 11*). The fusion of adsorbed lecithin bilayers did not progress beyond the hemifusion stage (see Fig. 1). The second stage, from "hemifusion" to "full-fusion,"

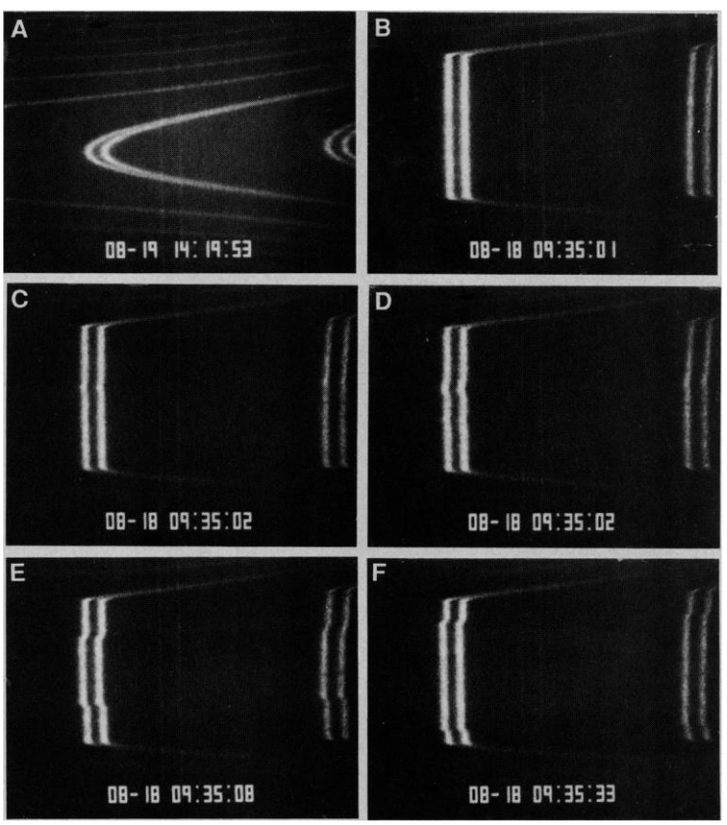

Fig. 2. Fringe patterns showing the various stages of hemifusion of two partially depleted DMPC bilayers in the fluid state under a pressure of 50 atm. The shapes of the fringes accurately reflect the macroscopic shapes of the surfaces after noting that the horizontal (gap thickness) magnification is about 10^4 times the vertical (lateral) magnification. (**A**) Curved surfaces separated before or after contact. (**B**) Surfaces flattened under a pressure of ~50 atm. The diameter of the contact zone is ~95 μm (seen as the flat vertical parts of the fringes) and the distance between the bilayer surfaces is ~1 nm. (**C**) One second later: breakthrough. (**D**) A small fraction of a second later. (**E**) Six seconds after breakthrough: the hemifused area has spread out over a distance of ~50 μm. (**F**) Thirty-two seconds after breakthrough. The spreading out is still continuing, but at a slower rate. The externally applied pressure remained unchanged throughout these experiments.

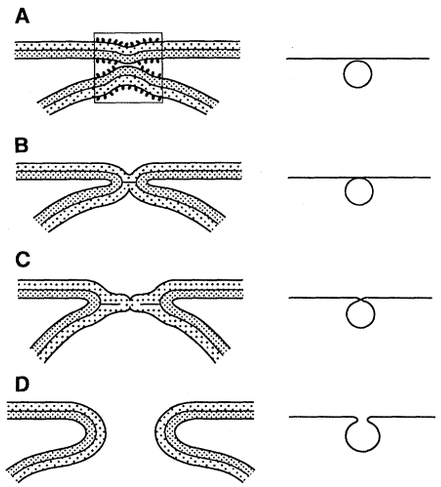

Fig. 3. (**Left**) Deformations and instabilities associated with the fusion of two supported bilayers in the fluid state through a metastable "hemifused" state as ascertained from video recordings of the changing optical fringe pattern with time (Fig. 2). (**A** and **B**) Breakthrough, corresponding to Fig. 2, C and D; (**C**) spreading out, corresponding to Fig. 2, E and F; and (**D**) separation. (**Right**) Proposed fusion mechanism of a vesicle with a planar membrane going through the same intermediate stages.

involves the total removal of the central bilayer. This process is shown in Fig. 3 as steps C to D and is characterized by a second critical breakthrough.

In Fig. 4 we show results obtained for uncharged DLPC and DMPC bilayers. The large inward jump from point J for the partially depleted bilayer is a clear indication of a much increased attraction [these are well-known instabilities that occur whenever the gradient of a force exceeds the spring constant (*12*)]. As in the case of CTAB, the magnitude, range, and distance dependence of the attractive interaction between these lipid-depleted bilayers cannot be attributed to van der Waals forces and must therefore reflect the additional long-range hydrophobic attraction originating from newly exposed hydrocarbon areas. Likewise, the fusion between the lipid-depleted CTAB, DLPC, and DMPC bilayers must be related to the hydrophobic interaction and cannot be attributed simply to the small reduction in the repulsive hydration force between them [compare with the many other experiments between fully developed bilayers in which no fusion was observed even though the hydration forces were much weaker, the adhesion stronger, and the bilayers forced together under much higher pressures than in Figs. 1 and 4 (*13, 18, 20*)]. We conclude that the hydrophobic interaction must be primarily responsible for both the increased adhesion and fusion of these depleted bilayers and that other attractive forces, such as van der Waals, "ion correlation," and "ion binding" forces, may enhance the adhesion but not necessarily the fusion of planar bilayers. Note that attractive electrostatic ion-binding and ion-correlation forces (*7, 18, 20*) act between the surface head groups, whereas hydrophobic forces act between the interior parts of bilayers.

Our two main conclusions are: first, up to the point where two approaching bilayers fuse, the force law between them is no indicator that fusion is about to occur. Fusion can occur spontaneously between repelling bilayers when they are still at a finite distance from each other without their having to "overcome" the repulsive force barrier (such as hydration) between them. Highly localized molecular rearrangements allow this to happen by a process of "bypassing" these forces, leading to fusion through spontaneous instabilities or "breakthrough" mechanisms. The fusion mechanism we have observed is thus analogous to a first-order phase transition, such as a liquid to solid transition, where up to the point of solidification there are no indications in any of the thermodynamic properties that a major change is about to happen.

Second, the interbilayer forces that enhance bilayer adhesion are quite different from those that promote fusion. The major force that leads to fusion is the hydrophobic attraction between internal hydrocarbon chain groups that have become exposed to each other across the aqueous phase. However, fusion is not promoted by attractive forces between the surface head groups or because of a weakly repulsive hydration force between head groups, although these forces increase the adhesion between bilayers. Attractive van der Waals forces play a negligible role in fusion, although again, they increase bilayer adhesion.

Perhaps our most important conclusion is that the major attractive force between bilayers that leads to direct bilayer-bilayer fusion is the hydrophobic interaction, which acts between the interiors of membranes, suggesting that attractive forces between the (exterior) surfaces of membranes should only lead to adhesion. Previous SFA studies on the attractive forces between adsorbed bilayers with negatively charged head groups in the presence of divalent cations such as Ca^{2+} (*20*) indicate that the attraction only favors adhesion but not fusion, although Ca^{2+} and Mg^{2+} ions are well-known fusogens of free bilayers. However, when Ca^{2+} ions are introduced "asymmetrically" into only one side of a negatively charged free bilayer or between two such bilayers, the bilayers weaken, break, and fuse; but if the Ca^{2+} is introduced "symmetrically" into both sides, the bilayers become stiffer, more stable, and adhere to each other, but do not fuse (*1, 21, 22*). Divalent cations can cause strong adhesion of biological membranes that does not lead to fusion (*2*). We suggest that any fusion resulting from such ionic interactions must operate indirectly and through a completely different mechanism, such as vesicle rupture or asymmetric bilayer stresses, that first destabilizes the bilayer.

We cannot say whether the basic mechanism we have observed for our adsorbed or "supported" surfactant and lipid bilayers is the same as generally occurs between "free" bilayers, vesicles, or biological membranes.

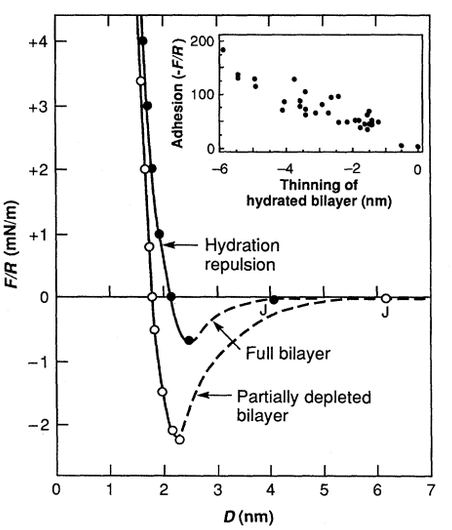

Fig. 4. Induction of fusion between two LB-deposited DLPC monolayers (each on a solid DPPE monolayer) by increasing the hydrophobic attraction between the fluid DLPC monolayers. (●) Forces between fully developed DLPC surfaces in water saturated with DLPC monomers at 22°C showing a van der Waals attraction beyond 2.5 nm and hydration repulsion below 2.5 nm. The van der Waals attraction causes the bilayers to jump into adhesive "contact" from the point J at $D = 4.2$ nm. No fusion was observed even up to very high F/R values of 1000 mN/m, corresponding to a pressure of 40 atm. (○) Forces between two depleted DLPC monolayers in a partially saturated DLPC solution where the bilayers had thinned to about 85% of the original thickness (that is, where the head group areas were about 15% larger than at equilibrium). The two surfaces jumped into contact from a greater distance (point J at $D = 6.2$ nm). The bilayers spontaneously fused into one bilayer when the pressure between them reached 3 atm. For thinner bilayers, the attractive forces were even greater in range and magnitude, and fusion occurred as soon as the bilayers came into contact. Adhesion of depleted DLPC and DMPC bilayers [on solid DPPE monolayers (*13*)] as a function of the thinning of the "hydrated" bilayer below the value for the fully developed bilayers is shown in the inset. Bilayer thicknesses were determined from the force minima, that is, from the positions the surfaces jumped apart relative to the positions of the hemifused bilayers at $D = -5.8$ nm.

First, the lipids in free bilayers may be expected to be able to undergo elastic and other deformations even more easily than our supported bilayers. Second, although we "depleted" our bilayers to expose hydrophobic groups, in the case of vesicles and biomembranes such exposed areas would arise from certain stresses, that is, from inhomogeneous ionic or osmotic stresses, or local packing stresses induced by integral membrane proteins. Indeed, both lipid vesicles and biological membranes may fuse by going through similar stages as depicted in Fig. 3 (*1, 10*). Such a molecular mechanism is very simple and does not involve any complex intermediate nonbilayer or inverted micellar structures.

Recent studies have increasingly implicated the hydrophobic interaction in the adhesion and fusion of membranes (*23–26*) through mechanisms not unlike the ones we have found for supported bilayers, namely, through membrane conformational changes involving the exposure of hydrophobic "domains" (*24*), "pockets" (*25*), or "segments" (*26*). Our force measurements are not inconsistent with these observations on membrane adhesion and fusion, and—more generally—shed new light on the forces and mechanisms that govern such interactions.

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17. The surfactants and lipids were of the highest purity available (>99%) from Sigma (St. Louis, Missouri) or Avanti (Birmingham, Alabama) and were used without further purification. The water was distilled, filtered in a Labconco purification unit, and then redistilled. The NaBr from Mallinckrodt (Paris, Kentucky) was analytical grade. Abbreviations for lipids: CTAB, hexadecyltrimethylammonium bromide; DGDG, plant digalactosyldiglyceride lipids; MGDG, plant monogalactosyldiglyceride lipids; DLPC, L-α-dilaurylphosphatidylcholine; DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; egg-PC, egg-yolk phosphatidylcholine; DPPE, L-α-dipalmitoylphosphatidylethanolamine.
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Cognate DNA Binding Specificity Retained After Leucine Zipper Exchange Between GCN4 and C/EBP

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Both C/EBP and GCN4 are sequence-specific DNA binding proteins that control gene expression. Recent evidence implicates C/EBP as a transcriptional regulator of genes involved in lipid and carbohydrate metabolism. The C/EBP protein binds avidly to the dyad symmetric sequence 5′-ATTGCGCAAT-3′; GCN4 regulates the transcription of genes that control amino acid biosynthesis in yeast, and binds avidly to the dyad symmetric sequence 5′-ATGA(G/C)TCAT-3′. Both C/EBP and GCN4 bind DNA via the same structural motif. This motif has been predicted to be bipartite, consisting of a dimerization interface termed the "leucine zipper" and a DNA contact surface termed the "basic region." Specificity of DNA binding has been predicted to be imparted by the basic region. As a test of this hypothesis, recombinant proteins were created wherein the basic regions and leucine zippers of GCN4 and C/EBP were reciprocally exchanged. In both of the recombinant polypeptides, DNA binding specificity is shown to track with the basic region.

A NEWLY RECOGNIZED CLASS OF SEQUENCE-SPECIFIC DNA binding proteins has been described (*1*) that includes biochemically defined DNA binding proteins such as C/EBP and AP1 (*2*), genetically defined regulatory proteins such as GCN4 (*3*), and transforming proteins encoded by proto-oncogenes such as *fos* and *jun* (*4*). These proteins have been proposed to interact with DNA through a common, bipartite DNA binding motif (*5*). One component of the motif is a dimerization interface termed the leucine zipper. The zipper is thought to form as a result of the intermolecular association of two amphipathic α helices. Helix association is believed to result, at least in part, from hydrophobic interactions generated by aliphatic amino acids in a manner analogous to the coiled-coil intertwining of filamentous, structural proteins (*6*). As a result of dimerization, highly basic polypeptide regions located on

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