TENSIONS AND FREE ENERGIES OF FORMATION OF "SOLVENTLESS" LIPID BILAYERS

Measurement of High Contact Angles

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ABSTRACT A method is described for the accurate measurement of the interfacial tension of lipid bilayer membranes containing little or no solvent. The tensions were obtained from the interfacial tensions of the equilibrium film-forming solution in the Plateau-Gibbs border, measured by conventional techniques, and the contact angle between the border and the bilayer. The contact angles in these systems are large ($>10°$) and were estimated by a new method that involved the injection of small known volumes of lipid solution into the bilayer so as to form a lens. Results have been obtained for monoolein-triolein, monoolein-squalene, and monoolein-squalene-decane systems. Half bilayer tensions in these systems were up to ~ 1 mN m^{-1} less than the single interface tensions. Although bilayer tension tended to increase with bilayer thickness, the interdependence of these quantities varied with the alkane solvents present. In the monoolein-squalene-decane systems, small concentrations of decane have a larger effect on tension than on thickness. Free energies of formation of the near-solventless bilayers were much greater than estimated from the simple application of Lifshitz theory.

INTRODUCTION

The adsorption of small organic molecules, such as *n*-alkanes and *n*-alkanols, into lipid bilayers has been shown to influence both the steady-state and kinetic properties of the pore-forming polypeptide gramicidin A (1–7). There are, moreover, some interesting parallels between these findings and the influence of the alkanes and alkanols on the sodium and potassium channels of the squid giant axon (8–10). Although relatively little is yet understood about how these small nonspecific molecules act, there is evidence that both the thickness and tension of the lipid bilayer are of some importance (1–12). Techniques for the estimation of bilayer thickness from the measurement of electrical capacitance (13, 15) or optical reflectance (14, 15) are well-established, but the accurate measurement of bilayer tension has so far been achieved only for relatively thick (hydrocarbon region $\gtrsim 32$ Å) black films. In these systems, the tensions were obtained from the combination of the interfacial tension of the membrane-forming lipid solution against the appropriate aqueous solution, with the contact angle measured by an interference method (16, 17). Thus, fringes arising from the illumination of either the thick lipid solution of the Plateau-Gibbs border or of a lens of lipid solution trapped in the black film, indicate the profile of the lipid-water interface and, hence, from extrapolation to the notional contact line, enable the contact angle to be calculated. For thinner films, however, and especially as the solvent adsorption approaches zero, the contact angles become progressively larger, the spacing of the interference fringes become too small for adequate resolution, and the method breaks down. Such systems are, nevertheless, of interest partly because all levels of adsorption (and low ones, particularly) are likely to be important biologically, but also because of the need to test theoretical models of the bilayer. Recent calculations of free energies of formation of bilayers, based on a mean-field model (18, 19), have yielded values for the near-solventless structures; it is important to compare these with experimental data.

A conceptually simple and effective technique was therefore developed to measure the large contact angles expected in the thin, solventless bilayers. Like the interference method, it involves a lens of bulk lipid solution trapped in the black lipid film, but the contact angle is determined from a knowledge of the base radius and volume of the lens. When viewed in transmitted light, the edge of the lens is clearly visible, especially for large contact angles and bulk solutions of high refractive index; and, when the lens is produced from a spherical drop of bulk film-forming solution of known volume, delivered from a micropipette, the lens volume is accurately obtained.

Results have been obtained for black films formed from monoolein and squalene (20) together with varying proportions of *n*-decane (21). Such films have a range of thicknesses and tensions and, for the thinner structures, exhibit

free energies of thinning that are much larger than predicted from simple Lifshitz calculations (22).

EXPERIMENTAL

Apparatus

The apparatus consisted essentially of an inner (polytetrafluoroethylene [PTFE]) and outer (perspex) cell mounted on a microscope stage, with the whole being positioned on a vibration-damped table (Fig. 1). A camera, mounted separately, allowed both drop and lens to be photographed.

Films were formed in a conventional, horizontal PTFE pot (17), mounted on a larger perspex outer cell by three leveling screws. Film-forming solution was delivered to the 1-mm hole in the PTFE pot by a feeder tube arrangement employing a 1-ml Hamilton gas tight syringe (No. 8700; Hamilton Bonaduz Ag., Bonaduz, Switzerland) and interconnecting PTFE and stainless-steel tubing.

Drops of film-forming solution were produced from a micropipette situated directly underneath the 1-mm hole. The pipette was connected, via a PTFE tubing sleeve, to the driving fluid delivery tube which was mounted in a PTFE rod, held in a micromanipulator head. This allowed accurate positioning of the pipette in the horizontal x, y plane. Vertical movement was facilitated by a coarse cogged drive on the support stand, which was clamped to the movable microscope stage.

The optical system was based on that used in the interference technique (17). An incident light microscope provided the basis of the system. Films, drops, and lenses were viewed by using a $\times 10$ eyepiece and $\times 6.5$ and $\times 13$ long-working-distance (17 mm) air objectives. Photographs, on Polaroid Type 47 film (Polaroid Corp., Cambridge, MA), were taken by means of a trip mirror, which allowed the image to be directed into a polaroid camera mounted so as to be mechanically isolated from the microscope.

Films were illuminated by vertically incident light by means of an epi-illuminator in which a tungsten lamp provided a suitable beam of white light focused through a series of adjustable apertures, lenses, and full and half-silvered mirrors. Transmitted light, from a similar tungsten lamp, turned through 90° by a small mirror underneath the outer cell, was directed up into the microscope, so illuminating the micropipette, drop, film, and lens. For high contact angle systems, the vertically incident light is not reflected back from the edge of the film or lens. However, the rapidly thickening bulk solution of the border or lens provides a sufficient change in refractive index to allow these edges to be readily observed in transmitted light. Transmitted light was also very useful for observing the condition of the hole prior to film formation, when the incorporation of water bubbles, arising from the use of the feeder tube, could easily be detected and then removed with a Pasteur pipette. The vertical displacement of, and drop production from, the micropipette was observed in transmitted light from a tungsten lamp by means of a Wild dissecting microscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland).

Small droplets of lipid solution were produced as shown in Fig. 1. The tubing and 1-ml syringe could be flushed through with double distilled water from the 2-ml reservoir syringe to fill and remove any air bubbles from the system. A tap then isolated this large syringe, and drop production was controlled by the micrometer-driven, 1-ml syringe.

EXPERIMENTAL PROCEDURE

Micropipettes were made from 1-mm external diameter glass capillary tubing (Clark Electromedical Instruments, Pangbourne, Reading, England) using a conventional horizontal pipette puller. Tip diameters ranging from 5 to 20 μm were routinely produced by cutting at the appropriate point on the long shoulder of the pipette with a diamond knife (Diatome SA, Bienne, Switzerland) giving uniformly flat tips. The pipette was back-filled with lipid solution; during this procedure the solution sometimes emerged from the tip and wetted the external surface

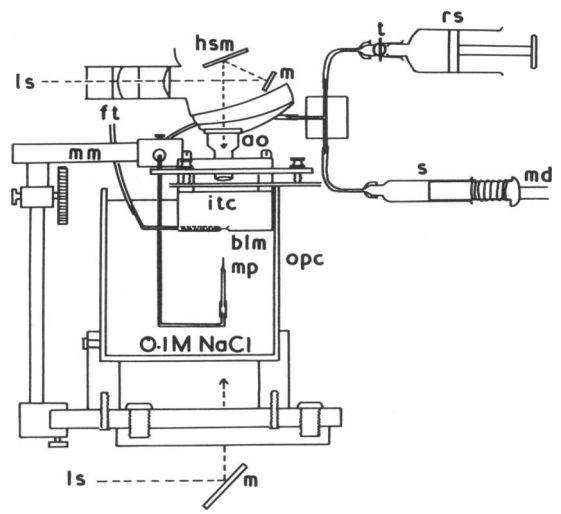

FIGURE 1 Diagram of the apparatus for measurement of high contact angles: *ao*, air objective; *blm*, black lipid membrane; *ft*, feeder tube; *hsm*, half silvered mirror; *itc*, inner PTFE cell; *ls*, light source; *m*, mirror; *md*, micrometer drive; *mm*, micromanipulator; *mp*, micropipette; *opc*, outer perspex cell; *rs*, reservoir syringe (2 ml); *s*, syringe (1 ml); *t*, tap.

with oil. This extraneous lipid solution was removed by carefully dipping the pipette in freshly prepared chromic acid solution, which was then washed off in double distilled water. The tip and external surfaces of the pipette were thus made hydrophilic and became water-wetted in use. The loaded pipette was then mounted in the PTFE sleeve in which the level of driving fluid had been slightly withdrawn so that an air bubble separated the two liquids.

With the pipette in position, 0.1 M NaCl was added to the outer cell. Any air trapped at the pipette tip was driven out and a series of drops of lipid solution were produced and viewed from the side by the dissecting microscope to make sure that they emerged evenly from the tip and did not creep back down the shoulder. The essence of the technique lies in producing a drop of known, constant volume so that the volume of the subsequent lens is also accurately known. Any wetting of the external shoulder surface would be undesirable and would introduce large errors into the method. Also, if the drop remained in contact with the column of lipid solution in the pipette, then small leaks, slowly equilibrating pressure gradients or thermal motion, might cause movement of the driving fluid and lipid solution, thereby randomly changing the drop size. The problem was circumvented by carefully wetting the first millimeter of the inside tip surface with the aqueous phase which caused, upon production of a drop of desired size, the column of lipid solution to recede, leaving the drop isolated on the tip.

If satisfactory drops were produced, the PTFE pot was assembled in its support ring and metal holder, film-forming solution was flushed through the feeder-tube to pre-wet the hole, and was then placed in position on the outer cell. Salt solution was added to outer and inner compartments to a depth in the pot at which the microscope had been previously calibrated by using a 1-mm, 1-μm per division stage micrometer placed on the hole.

During the ~1 h required to ensure thermal equilibrium, several films were made; the pot was leveled and the pipette was carefully centered ~2 mm underneath the hole. The pipette was slightly angled from the vertical so that the tip and drops could be clearly seen in transmitted light and were not masked by lower, out-of-focus parts of the tubing.

Black lipid films were formed as described elsewhere (17). With a black film in position an isolated drop of desired size was produced on the tip of the pipette, positioned ~2 mm below the center of the film, as viewed from the side. The long-working-distance $\times 13$ air objective then allowed the drop to be viewed from above, through the film, in transmitted light alone. It was then brought into sharp focus, and photographed (Fig. 2*a*). The edge of the film as viewed from above was brought into

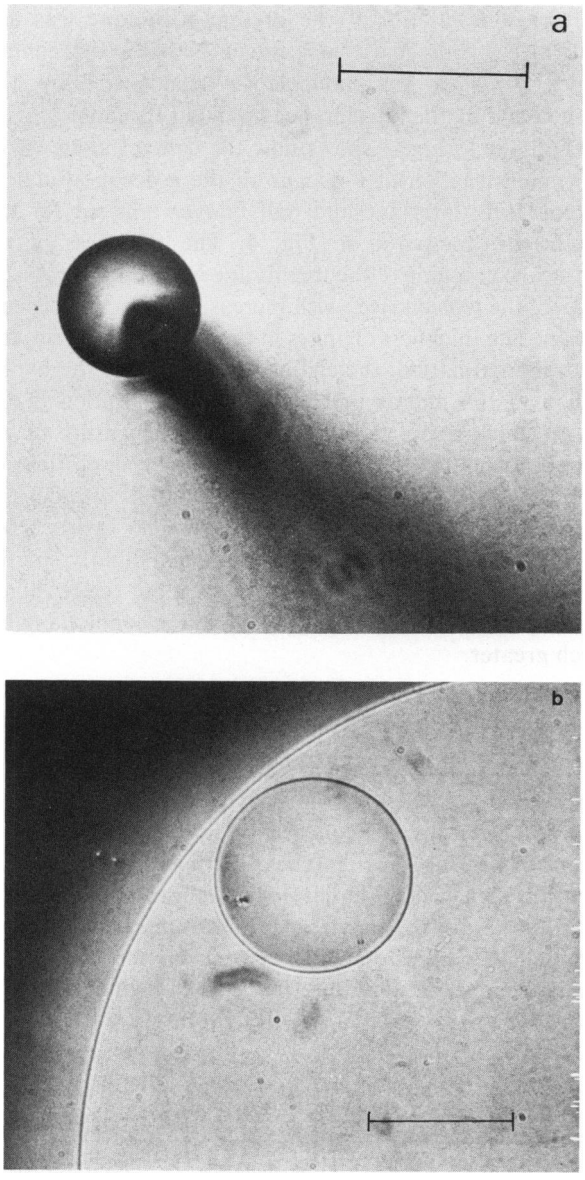

FIGURE 2 The droplet and resulting lens. (*a*) A droplet of monoolein 8.4 mM in squalene under 0.1 M NaCl on the end of a pipette of tip external diameter 13.6 μm. (*b*) The lens was formed by touching the droplet to a black lipid film formed from the same solution. Bar equals 100 μm.

focus and the pipette was slowly raised until the drop just touched the underside of the bilayer. Coalescence with the film spontaneously formed a lens that remained stationary in the film if, upon detaching from the pipette, it had not acquired momentum, and if the film was level and free from contaminants. Slow lens movements could be followed by moving the whole apparatus on the stage. A few seconds were allowed for the lens to attain its equilibrium profile and it was then photographed (Fig. 2*b*).

This procedure was repeated with several films and a suitable range of drop sizes to give lens radii of 50–180 μm for each lipid solution. Drop and lens radii were measured from the photograph by traveling microscope (Pye Scientific Instruments, Cambridge, England; No. 6147). Interfacial tensions between bulk lipid solutions and the aqueous phase (0.1 M NaCl) at 25°C were determined by the drop-volume technique (23). The densities of the monoolein solutions in *n*-decane-squalene mixtures were obtained by direct measurement. The membrane capacitances were determined by an AC bridge technique (13) at 500 Hz.

Materials

Monoolein and triolein were obtained from Nu-Chek Prep., Inc., Elysian, MN, and were used without further purification. Puriss grade *n*-decane from Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, England and Grade I squalene from the Sigma Chemical Co., St. Louis, MO, were both passed through an alumina column to remove polar impurities. The water was doubly distilled and sodium chloride was roasted overnight at 700°C.

Calculation of the Contact Angle, Membrane Tension, and Free Energy of Formation

The droplets on the micropipette were effectively spherical and hence the volume, V, of the lens in the black film could be calculated readily from the radius, r, of the droplet. From the lens volume and the base radius, r', the lens height, d, could be obtained; i.e.,

$$V = \frac{\pi}{3}(3dr'^2 + d^3). \quad (1)$$

This involves the assumption that the lens consists of two identical spherical segments, a point that was established in an earlier paper for systems with low contact angles (17) and that appears from calculations and from Fig. 3 (see below) to be valid for the present systems. From the lens height and the base radius, the radius of curvature, R, of the lens is calculated, and the contact angle, θ, is then given by

$$\sin\theta = \frac{r'}{R}. \quad (2)$$

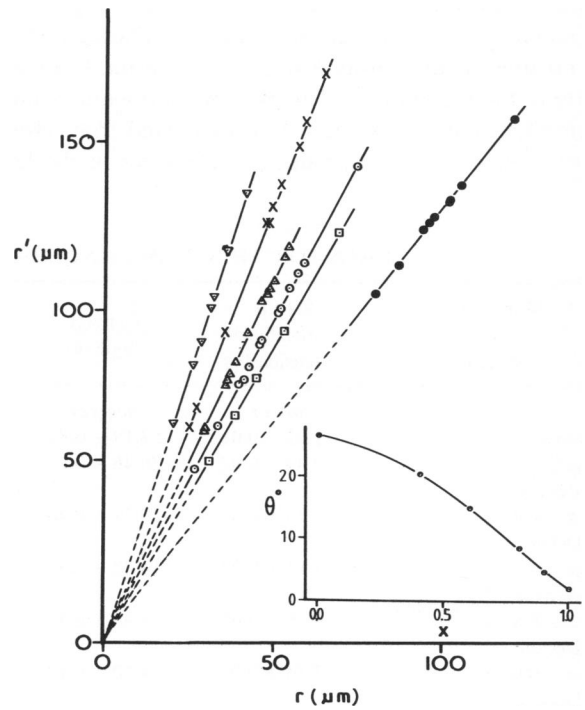

FIGURE 3 Lens radius (r') versus droplet radius (r) for various monoolein-solvent systems in 0.1 M NaCl. ●, triolein; □, squalene; ○, *n*-decane (x = 0.4)-squalene; △, *n*-decane (x = 0.6)-squalene; x, *n*-decane (x = 0.8)-squalene; ▽, *n*-decane (x = 0.9)-squalene. The linear regression lines are shown. *Inset:* The variation of the contact angle (θ) with the mole fraction (x) of *n*-decane in the monoolein-*n*-decane-squalene film-forming solutions. T = 25°.

The membrane tension, σ, is then given by

$$\sigma = 2\gamma\cos\theta, \quad (3)$$

where γ is the interfacial tension of the equilibrium film-forming solution in the Plateau-Gibbs border. The hydrostatic pressure contribution to the balance of forces has been disregarded as negligible in the present systems. The change in Helmholtz free energy, ΔA, during formation of the black film from thick lipid is (17, 22)

$$\Delta A = 2\gamma\,\mathcal{A}(\cos\theta - 1), \quad (4)$$

where $\mathcal{A}$ is the area of the film.

RESULTS

The systems examined together with the results are presented in Table I. Monoolein was the film stabilizer in all instances and the solvent consisted of squalene-*n*-decane mixtures, the mole fractions (x) of which are shown. Results were obtained for monoolein-*n*-hexadecane so as to check the technique against the interference method. For completeness, the result for pure *n*-decane measured by the interference method is included. One further system, that of monoolein ($x = 0.1$) plus triolein (24), was examined.

The measured contact angle was not a function of droplet-lens volume in any of the systems. To check that the lens volume tended to zero with the droplet volume, the lens-base radius was plotted against the droplet radius (Fig. 3). The plots are linear, as required theoretically and, in each instance, appear to stem from the origin, suggesting that no change in drop size occurred after photographing and no lipid solution was lost in the transfer from the pipette to the membrane. (The maximum amount of lipid solution that could have been lost, estimated from visual observation, would have produced <2% error in the lens radius.) The validity of the present technique was also confirmed by the close agreement between the contact angles obtained for monoolein-*n*-hexadecane by this method and by the interference method (Table I).

The inset to Fig. 3 shows how the contact angle varies with mole fraction of *n*-decane in the *n*-decane-squalene system. Bulk interface and half bilayer tensions for this system are compared in Fig. 4. The thickness of the hydrocarbon region of the membrane is also shown. As can be seen, the tension rises with increasing *n*-decane concentration. The thickness changes in these systems reflect, to a good approximation, the changes in volume of the hydrocarbon in the membrane because the adsorption of the monoolein is not a strong function of the nature of the hydrocarbon (25). It follows that relatively large tension increases occur for small volume adsorptions of *n*-decane. In the monoolein-triolein system, where no hydrocarbon was present, the bilayer thickness is only slightly smaller than for the monoolein-squalene system, but the tension is lower by more than a factor of two and the contact angle is much greater.

DISCUSSION

Two salient features of the present results are evident from Figs. 4 and 5. Fig. 4 shows that, at low values of x, the membrane tension rises more rapidly with increasing *n*-decane mole fraction than does the membrane thickness. From Fig. 5 one can see that, for membrane thicknesses $\lesssim$ 46Å, for a given thickness, the systems containing *n*-decane have a considerably larger tension than the highest attainable in those containing longer homologues. Also, the points for *n*-heptane- and *n*-octane-saturated systems exhi-

TABLE I
DATA FOR THIN FILMS AND THEIR EQUILIBRIUM BULK LIPID SOLUTIONS

Monoolein/solvent system	Bulk interfacial tension (γ)	Contact angle (θ)	Half bilayer tension ($\sigma/2$)	Free energy of thinning(ΔA^*)	Capacitance per unit area (C_f)	Hydrocarbon region thickness (h)
	mN m^{-1}	*degrees*	*mN m^{-1}*	*μJ m^{-2}*	*μF cm^{-2}*	*Å*
Triolein	1.82 ± 0.01	57.19 ± 0.81	0.98 ± 0.02	−1673.2 ± 44.4	—	22.7§
Squalene	2.44 ± 0.02	26.46 ± 0.34	2.18 ± 0.02	−511.2 ± 13.6	0.751 ± 0.003	25.79 ± 0.11
n-Decane $x = 0.4$ in Squalene	2.79 ± 0.02	20.31 ± 0.46	2.62 ± 0.02	−347.3 ± 15.8	0.712 ± 0.005	27.10 ± 0.18
n-Decane $x = 0.6$ in Squalene	3.11 ± 0.02	14.72 ± 0.32	3.01 ± 0.02	−204.2 ± 8.9	0.663 ± 0.004	28.93 ± 0.16
n-Decane $x = 0.8$ in Squalene	3.50 ± 0.02	8.46 ± 0.30	3.46 ± 0.02	−76.2 ± 5.4	0.588 ± 0.008	32.35 ± 0.41
n-Decane $x = 0.9$ in Squalene	3.74 ± 0.01	4.60 ± 0.12	3.73 ± 0.01	−24.1 ± 1.3	0.511 ± 0.006	36.91 ± 0.43
n-Decane $x = 0.95$ in Squalene	—	—	—	—	0.443 ± 0.006	42.23 ± 0.60
n-Decane‡	3.84 ± 0.02	1.97 ± 0.02	3.84 ± 0.02	−4.5 ± 0.1	0.378 ± 0.004	49.15 ± 0.48
n-Hexadecane	—	4.63 ± 0.09	—	—	—	—
n-Hexadecane‡	—	4.82 ± 0.12	—	—	—	—

The aqueous phase was 0.1 M NaCL. $T = 25°$.
‡Measured by interference technique.
§From Ref. 24.

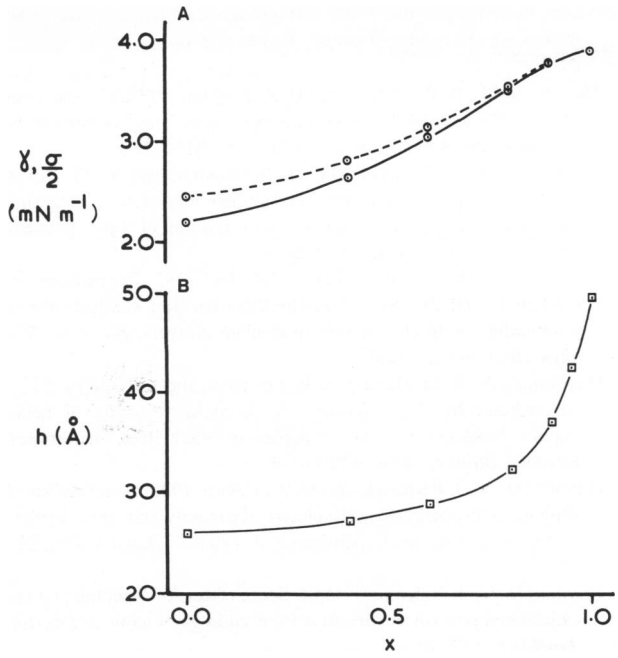

FIGURE 4 The dependence of (*A*) bulk (γ) and membrane ($\sigma/2$) interfacial tensions, and (*B*) of hydrocarbon thickness (h) of the membrane on the mole fraction (x) of *n*-decane in monoolein-*n*-decane-squalene/0.1 M NaCl systems. (*A*) - - -, bulk lipid solution; ___, membrane. $T = 25°$.

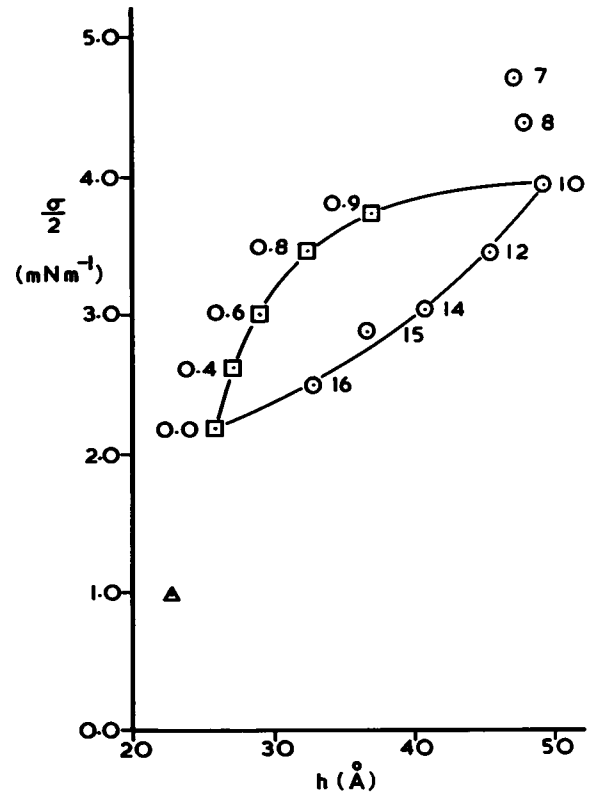

FIGURE 5 The half-membrane tension ($\sigma/2$) versus the membrane hydrocarbon thickness (h). □, monoolein-*n*-decane-squalene/0.1 M NaCl systems, where the mole fractions of *n*-decane are indicated; ○, monoolein-*n*-alkane/0.1 M NaCl systems, where the chain length of the *n*-alkane is indicated; △, monoolein ($x = 0.1$) in triolein/0.1 M NaCl.

bit higher tensions than for *n*-decane at comparable thicknesses. In general, therefore, it appears that the smaller the hydrocarbon the more effective it is in raising the membrane tension. A qualitative explanation for this observation can be found in a recently developed statistical mechanical model for alkane adsorption into bilayers (18, 19).

This model predicts that the longer the chain of a particular *n*-alkane, the more it will distribute into the center rather than the outer parts of the chain region of a bilayer. This is essentially because the insertion of additional hydrocarbon chains into the outer part of the bilayer, where the segmental order parameter is relatively high (26), tends both to constrain the hydrocarbon solvent to lie roughly parallel to the lipid chains and also to straighten the lipid chains (in order to produce holes for the solvent molecules). These processes are unfavorable in free energy terms, the more so the longer the chain length of the alkane. The smaller alkanes, therefore, have a greater tendency to reside in the surface of the bilayer and hence to increase the area per molecule of the lipid. This in turn leads to the exposure of more hydrocarbon to water, which could contribute to the larger tensions. (Changes in the state of the lipid in solution in the Plateau-Gibbs border cannot, however, be ruled out [27].) An extrapolation of this trend suggests that very small hydrocarbons such as cyclopropane should produce the largest tension changes for a given thickness increase.

In earlier papers (17, 22) it was concluded that for many black lipid films the change in Helmholtz free energy per unit area on formation of the film from thick liquid could be accounted for almost entirely in terms of the work done by the van der Waals forces acting between the two aqueous phases across the hydrocarbon region of the film. It was also noted, however, that for certain types of film whose hydrocarbon thickness was <45 Å, the experimentally observed free energy of formation was significantly larger than that calculated from Lifshitz theory for a simple isotropic layer of hydrocarbon in water. The present results extend the previous finding to thinner, nearly solventless films of the same lipid and show that the discrepancy between the experimental and van der Waals free energies becomes enormous for these very thin films (Fig. 6). Discrepancies of this order of magnitude were predicted for somewhat similar systems by the mean-field statistical mechanical model referred to previously (19). Of particular interest is the prediction in Ref. 19 that for a dipalmitoylphosphatidylcholine membrane containing ~0.12 volume fraction of alkane, the free energy of formation would be ~740 μJ m^{-2}. This was compared with the largest experimental value then available (42.1 μJ m^{-2}) for a membrane containing 0.21 volume fraction. It was tentatively suggested that the different solvent contents might be the origin of the large discrepancy. Support for

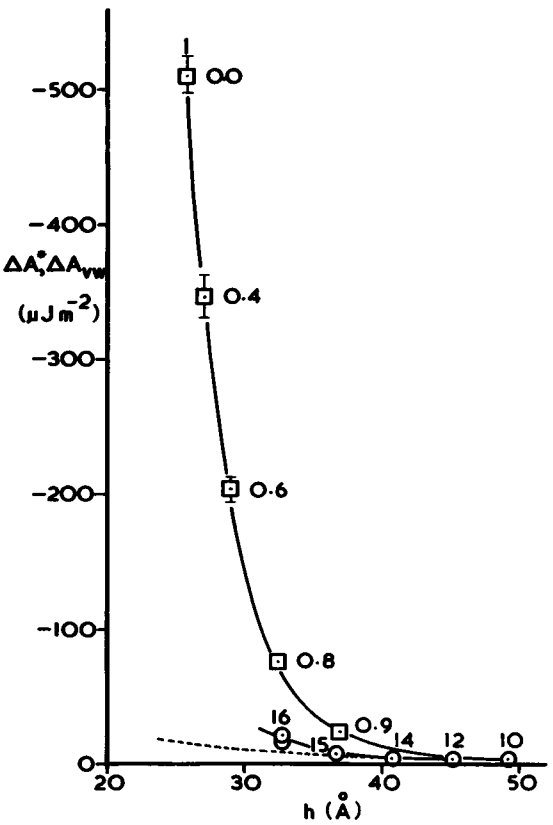

FIGURE 6 The free energy of thinning per unit area (ΔA^*) and the van der Waals free energy (ΔA_{vw}) calculated from the expression $\Delta A_{vw} = A_{Hamaker}/12\pi h^2$. $A_{Hamaker}$ has been set equal to 3.92×10^{-21} J, the value estimated from experiments with monoolein-*n*-decane systems (17) and which is very close to that calculated from Lifshitz theory (22). □, ΔA^* for monoolein-*n*-decane-squalene systems, where the mole fraction of *n*-decane is indicated; ○, monoolein-*n*-alkane systems, where the chain length of the *n*-alkane is indicated; - - -, ΔA_{vw}.

this suggestion lies in Fig. 6, where it can be seen that halving the solvent content at these solvent levels does yield order of magnitude increases in the free energy of formation. A more detailed discussion of the physical origins of the effect has been given in Ref. 19.

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