Black Lipid Membranes at Bifaces

Formation characteristics, optical and some thermodynamic properties

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ABSTRACT Black lipid membranes (BLM) less than 90 A thick have been shown to be the most realistic approach to biological membrane models. This paper describes the formation characteristics, optical properties, and thermodynamics of BLM at water/oil/water bifaces. In particular, the nature of the Plateau-Gibbs border which supports the black membrane is analyzed in some detail. The formation of BLM at the biface involves a spontaneous reduction of the free energy of the system. As long as the integrity of the membrane is maintained, the limiting structure of the BLM represents the lowest free energy configuration.

Although at present the molecular organization of natural membranes has not been definitely settled, the general picture of membrane structure, widely accepted today, is that based upon the bimolecular lipid layer with adsorbed protein monolayers (for a current review, see reference 1). This model of the cell membrane rests, in part, on the foundation of the bimolecular lipid leaflet concept of Gorter and Grendel (2), which was deduced from the classical monolayer experiments. The postulated adsorbed protein layers had their origin in experiments initiated by Harvey and Shapiro (3) and by Cole (4) in the 1930s. Indirect experimental evidence in support of the bimolecular leaflet model (with adsorbed protein layers) has been provided by numerous studies such as X-ray diffraction, electron microscopy, permeability, electrical resistance and capacitance, and chemical analyses (5). Therefore, it has been evident from earlier years that, if the bimolecular lipid layer were indeed the major structural component of cellular membranes, knowledge concerning the properties and the formation of such a structure in vitro would be of considerable significance, both experimentally and theoretically. It was readily apparent also that a detailed physical chemical description of natural membranes would be best approached by studies of simpler, well-defined models, owing to the great complexity of living systems.

Among the various membrane models investigated, it is generally recog-
nized that bimolecular lipid membranes (or BLM) represent the closest approach to the problem of natural membrane models. A review of the studies on BLM is available. In the present paper, optical and interfacial properties of BLM at the biface are discussed together with the results of further investigations summarized in the following paragraphs.

**FORMATION CHARACTERISTICS OF BLM**

Experimentally, a BLM is formed as follows. A small amount of suitable lipid solution (see Table I) is introduced onto an opening in the wall of a hydrophobic support (such as Teflon or polyethylene) immersed in an aqueous solution. The formation characteristics of lipid membranes are quite similar to those of soap films in air. When first formed, the lipid membrane at the biface is usually thick, and interference colors may not be visible as viewed through a low-power microscope (10–40X) in reflected white light. Under suitable conditions, the thick membrane begins to thin spontaneously. When the membrane is still sufficiently thick (0.1–1 μ), the structure of the membrane is pictured as being similar to that of a sandwich consisting of an organic phase between two adsorbed monolayers of lipid molecules at the biface. At a distance more than 100 Å, the attraction of the lipid monolayers across

<table>
<thead>
<tr>
<th>Compound</th>
<th>γ (dyn/cm)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hexadecyltrimethylammonium bromide (HDTAB)*</td>
<td>0.15</td>
<td>Dissolved in the aqueous phase, cationic</td>
</tr>
<tr>
<td>2. Dioctadecyl phosphite (DODP)*</td>
<td>5.7</td>
<td>Nonionic</td>
</tr>
<tr>
<td>3. Dodecyl acid phosphate (DAP)*</td>
<td>1.1</td>
<td>Anionic</td>
</tr>
<tr>
<td>4. Glycerol distearate (GDS)</td>
<td>1.3</td>
<td>Nonionic</td>
</tr>
<tr>
<td>5. Glycerol dioleate (GDO)</td>
<td>1.5</td>
<td>Nonionic</td>
</tr>
<tr>
<td>6. Sodium dodecyl sulfate (Na-DS)*</td>
<td>1.5</td>
<td>Anionic</td>
</tr>
<tr>
<td>7. N-Lauryl,myristyl-β-aminopropionic acid (Deriphat-170C)*</td>
<td>0.9</td>
<td>Amphoteric</td>
</tr>
<tr>
<td>8. Lecithin (egg)</td>
<td>1.9</td>
<td>?</td>
</tr>
</tbody>
</table>

* Used in combination with freshly recrystallized cholesterol.

1 Since the thickness does not greatly differ from the length of two lipid molecules used, and owing to the optically "black" appearance, these membranes are called bimolecular, bilayer, or black lipid membranes, abbreviated as BLM.

2 The word "biface" is introduced to describe the two coexisting water/oil or solution/membrane interfaces.
the biface is small. The chief cause of thinning therefore takes place because of the presence of a Plateau-Gibbs border, i.e. the edge where the membrane is terminated. At the Plateau-Gibbs border, the pressure is less than in the interior of the thick membrane owing to the concave interfaces. The Plateau-Gibbs (or P-G) border at the biface therefore exerts a strong suction upon the interior of the thick membrane, causing a rapid reduction in its thickness. The rate of thinning prior to the appearance of "black spots" follows that of \( T^2 \approx \text{constant relation} \), where \( T \) is the time and \( t \) is the membrane thickness. However, the appearance of black spots (bimolecular or multiples of bilayer thickness) results in a greatly increased rate of thinning. It has been suggested that a "zipper-like" action, which is apparently also important in producing changes in a thin membrane, is an additional cause for the rapid growth of black area. It should be mentioned that the interference colors and black formation are highly variable using the brush technique. Fig. 1 shows an atypical picture of BLM during its formation. 

Because of very small difference in specific gravity between the lipid solution and the bathing medium, the lipid membranes formed in the usual manner (i.e. with vertical orientation) behave essentially like horizontal soap films. As can be seen in Fig. 1, the boundary between the black and thicker region is circular instead of a horizontal line. It has been noted by earlier workers (7) that BLM are most prone to rupture during their formation. This premature demise of BLM may be explained by the fact that the boundary between the two areas is the seat of high concentration stress and turbulence.

**Figure 1.** The growth of a black (bimolecular) lipid membrane in aqueous solution. This photograph is reproduced from a frame of a motion picture first shown at the Symposium on the Plasma Membrane held by the New York Heart Association, New York, December 1961. Magnification about \( \times \) 50.
owing to the rapid drainage of the lipid solution. Therefore, the rupture would most likely occur somewhere along this boundary. It is of some interest here that the transition from a black area to that of a thicker region does not appear to be gradual, but rather is very sharply delineated. The reason for this apparent sharp boundary may be made clear by the following analysis, which is the explanation used in the case of soap films (9). We may consider that the black area of a lipid membrane is, to a first approximation, an interface between two aqueous solutions, I and II (Fig. 2). At point N, where three interfaces meet—thick film/solution I, the interface between the two solutions, and solution II/thick film—the angles are set at 120° apart. The distance B, the transition region from the black to the thicker part of the membrane, is given by

\[ B = \frac{S}{2} \cot \theta \]

For a silvery-golden membrane (i.e. before the transition to the black state), S is about 1000 A. The transition region B is therefore calculated to be only about 290 A. Hence a very sharp demarcation is to be expected when the boundary is observed under low magnification.

It should be pointed out that a black or grayish-black lipid membrane may not necessarily be bimolecular or bilayer in thickness. In fact, we have observed at least three shades of black for BLM formed from Escherichia coli lipids. Under appropriate conditions, these BLM can be made to go from one stage of "blackness" to the next, with a very long time interval between the stages. However, removal of lipid solution around the membrane or otherwise disturbing the thick membrane by mechanical means would generally speed up the growth of the secondary (limiting) black structure.
THE PLATEAU-GIBBS BORDER AND BLM STABILITY

There are at least two major problems associated with BLM investigations. First, we do not know precisely what molecular species are present in the membrane. The second problem is the fragility of the structure, which presents considerable experimental difficulties in investigating its mechanical and other properties. In many ways, therefore, our description of the BLM will necessarily be qualitative. In our consideration of the relationship between the BLM and its Plateau-Gibbs border, we shall follow the analysis put forth by investigators in the field of soap films (10-12). As mentioned earlier, the edge where the BLM is terminated is called the Plateau-Gibbs border and is believed to be essential for the integrity of BLM structure. It has been frequently observed that the area of BLM does not usually extend over to the entire aperture in the membrane support. That means that the BLM may occupy anywhere from nearly none to almost 100% of the total available area at the biface. Before offering an explanation of why this is so, let us consider qualitatively the factors which are likely to be involved in the BLM stability.

During the formation stages of BLM, when a drop of lipid solution is introduced onto the aperture in the membrane support, thereby creating two coexisting interfaces or a biface, three events are likely to take place: (a) the lipid solution will thin down spontaneously to give a BLM in equilibrium with its P-G border or break; (b) the lipid solution will drain sufficiently to give a colored lipid film but will remain in this state for a long period of time if not disturbed; (c) the lipid solution will remain as a globule. We shall limit our consideration to case a. It is evident that the existence of a BLM at the biface must be due to a balance of forces. At equilibrium the attractive forces must be equal to repulsive forces. In terms of the free energy $F$ of the system, $\frac{dF}{dt} = 0$, where $t$ is the thickness of BLM. The attractive forces most likely would include van der Waals' attraction and P-G border suction, whereas the counterforces consist of Born repulsion and electric double layers situated at the biface. The Born repulsion or steric effect arises at a very short distance (at the bimolecular length of the lipid used) and increases rapidly when the limiting thickness of BLM is passed. At equilibrium, Born repulsion is perhaps much more important than the double-layer repulsion in counteracting the van der Waals forces and P-G border pressure. As has been mentioned earlier, a number of "black" states are possible for BLM. This means that in the free energy vs. BLM thickness plot a number of minima are present, corresponding to each "black" state. If the final black state (secondary black) represents the limiting structure of the BLM consisting of a bimolecular leaflet of lipid molecules, the other black states must be multiples of this basic unit. Although
quantitative calculations along these lines are as yet unavailable, the interplay of these forces must be responsible for the existence of these BLM (8).

In going from the "colored" state to the final "black" state, the excess lipid solution used usually ends up in the P-G border. A small fraction of it could either form microlenses adhering to the BLM or dissolve in the ambient solution. We now attempt an explanation for case a, where a BLM is formed spontaneously but the observable BLM area varies when formed under different experimental conditions.

In Fig. 3 A, a front view of BLM is shown with different observable areas labeled 1 and 2. At equilibrium, there are three forces intersecting along a line of contact (represented by point N), which is shown in a cross-sectional view in Fig. 3 B. Here BLM separates aqueous phases I and II. The third liquid phase, designated by III, is the lipid solution in the P-G border. The law of Neumann's triangle states that, at equilibrium,

\[ \gamma_{BLM} = \gamma_{I-III} \cos \alpha + \gamma_{II-III} \cos \beta \]  

(2)
where the subscripts to the $\gamma$'s refer to the various liquid phases. At equilibrium, the theorem of Neumann's triangle states (13) that the magnitude of each of three interfacial tensions is proportional to the sine of the angle between the other two interfaces,

$$\frac{\gamma_{\text{BLM}}}{\sin (\alpha + \beta)} = \frac{\gamma_{\text{I-III}}}{\sin (\pi - \beta)} = \frac{\gamma_{\text{II-III}}}{\sin (\pi - \alpha)}$$

(3)

Normally, the two aqueous phases I and II are identical. Therefore, $\gamma_{\text{I-II}}$ equals $\gamma_{\text{II-III}}$, and $\alpha = \beta = \theta$. From elementary trigonometry, it can be shown that

$$\gamma_{\text{BLM}} = 2\gamma_{\text{ML}} \cos \theta$$

(4)

where $\gamma_{\text{ML}}$ denotes the interfacial tension between two bulk phases in the presence of a lipid monolayer. At the P-G border, the interfaces are circular according to the Young-Laplace equation, with radius equal to $R$. At equilibrium, the downward tension ($\gamma_{\text{BLM}}$) must be balanced by the upward tension $\gamma_s$, which may be split into two terms, and given by

$$\gamma_s = 2\gamma_{\text{ML}} \cos \alpha + \frac{S\gamma_{\text{ML}}}{R}$$

(5)

where on the right-hand side the first term is the vertical component of the interfacial tension and the second represents the P-G border suction pressure. The horizontal distance, $S$, is given by

$$S = 2R (1 - \cos \alpha)$$

(6)

The observable circular area of BLM is governed by $\gamma_{\text{BLM}}$, $\gamma_{\text{ML}}$, and the angle $\theta$. If $\theta$ is zero, then, upon substitution of $S$ into equation 5, we have

$$\gamma_{\text{BLM}} = 2\gamma_{\text{ML}}$$

(7)

In that case no BLM is obtainable. If $\theta$ is greater than zero, the distance $S$ is

$$S = 2R (\cos \theta - \cos \alpha)$$

(8)

which means that $\gamma_{\text{BLM}}$ must be less than $2\gamma_{\text{ML}}$. In order for this to be true, the P-G border must move toward the rim of the aperture as dictated by equation 4. The latter situation is shown by the dashed line in Fig. 3 B. The ideas discussed here may be further developed to provide an alternative means of evaluating the tension of the BLM.
As mentioned in the preceding paragraphs, BLM are formed in aqueous solution by a process similar to the generation of black soap films in air. The optics of BLM are essentially those of very thin films. (According to the definitions used in thin film optics, a thin film is a layer with parallel faces whose thickness, \( t \), is of the order of the wavelength, \( \lambda \), of light used. A very thin film has \( t \) less than \( 0.01\lambda \).) The common optical measurement thus far made on BLM is the reflectance at a small angle of incidence using visible electromagnetic radiation. The results are interpreted in terms of either a homogeneous structure (single-layer model) or a triple-layer model. In the latter model, the polar (or ionic) portions of the lipid molecules situated at the biface form the two outer layers with a liquid hydrocarbon core in the center. To evaluate the thickness of BLM from reflectance measurements, the following equations are used:

\[
R_s = \frac{4r^2 \sin^2 \delta}{(1 - r^2)^2 + (2r \sin \delta)^2}
\]  
(9)

for the single-layer model, and

\[
R_t = \frac{r_1^2 + r_2^2 + 2r_1r_2 \cos (\theta_1 - \delta_h)}{1 + r_1^2 r_2^2 + 2r_1r_2 \cos (\theta_1 - \delta_h)}
\]  
(10)

for the triple-layer model. In equations 9 and 10, \( r \)'s are the various Fresnel reflection coefficients, \( \delta = (2\pi n_\delta \cos \theta)/\lambda \), in which \( t \) is the thickness, \( \theta \) (or

### Table II

<table>
<thead>
<tr>
<th>BLM from</th>
<th>Solvent</th>
<th>Aqueous phase</th>
<th>Thickness*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin</td>
<td>( n )-Decane</td>
<td>0.1 n NaCl</td>
<td>69±10</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77±10†</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Lecithin</td>
<td>( CHCl_3 + CH_3OH + n )-tetra-decane</td>
<td>0.1 n NaCl</td>
<td>72±10</td>
<td>17</td>
</tr>
<tr>
<td>Phospholipids (?)</td>
<td>?</td>
<td>?</td>
<td>74±15</td>
<td>18</td>
</tr>
<tr>
<td>Glycerol distearate</td>
<td>( n )-Hexane</td>
<td>Various</td>
<td>50±5</td>
<td>8</td>
</tr>
<tr>
<td>Oxidized cholesterol</td>
<td>( n )-Octane</td>
<td>0.1 n NaCl</td>
<td>40±10</td>
<td>8</td>
</tr>
<tr>
<td>7-Dehydrocholesterol</td>
<td>( C_7-C_{14} )</td>
<td>0.1 n NaCl</td>
<td>40±10</td>
<td>8</td>
</tr>
</tbody>
</table>

* Unless otherwise noted, the thickness values were calculated using equation (9).
† Calculated using equation 10.
$x_1 - \delta_h$) is the reflected angle in the membrane, $n$ is the membrane refractive index, and $\lambda$ is the wavelength of the visible radiation used (see, for example, reference 14). In applying equation 9, one has to assume or know the value of the membrane refractive index, $n_b$. For a homogeneous and isotropic structure of BLM, Brewster’s law ($\tan \phi_p = n_b/n_w$) may be used to estimate the index of the membrane. It is based upon the property that for light polarized with the electric vector in the plane of incidence, the reflectance of a transparent film of refractive index $n_b$ immersed in a medium of index $n_w$ is minimum at the polarizing angle $\phi_p$. The thickness values obtained using equation 9 are shown in Table II, and lend support to the contention that BLM consists of a bimolecular layer of lipid held together by van der Waals’ forces. The large error (approximately 10%) in the results is due mainly to the uncertainty in

The Brewster’s angle determination. Furthermore, there is some question concerning the applicability of Brewster’s law to the determination of the refractive indices of very thin films such as BLM. This difficulty in assessing refractive index of the membrane using Brewster’s law may be avoided, however, if we assume that the index of refraction of the BLM varies little with wavelength of light used. We are currently experimenting with this approach, and the steps are outlined below.

It is evident from equation 9 that two equations are required to determine the two quantities $n_b$ and $t_b$. Experimentally, one needs to measure $R_t$ as a function of wavelength $\lambda$, as has been suggested by Abelas (14). The quantities dependent on the wavelengths $\lambda_1$ and $\lambda_2$ are designated by the subscripts 1 and 2 and

$$A_1 = \frac{1 + R_{1t}}{1 - R_{1t}}$$

(11)

<table>
<thead>
<tr>
<th>$t_b$</th>
<th>$n_b = 1.42$</th>
<th>$n_b = 1.44$</th>
<th>$n_b = 1.46$</th>
<th>$n_b = 1.55$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_1$</td>
<td>$\lambda_2$</td>
<td>$\lambda_1$</td>
<td>$\lambda_2$</td>
</tr>
<tr>
<td>1</td>
<td>5.129</td>
<td>7.477</td>
<td>10.309</td>
<td>32.325</td>
</tr>
<tr>
<td>2</td>
<td>6.208</td>
<td>8.769</td>
<td>11.816</td>
<td>34.949</td>
</tr>
<tr>
<td>3</td>
<td>7.388</td>
<td>10.162</td>
<td>13.425</td>
<td>37.675</td>
</tr>
<tr>
<td>4</td>
<td>8.671</td>
<td>11.658</td>
<td>15.136</td>
<td>40.500</td>
</tr>
<tr>
<td>5</td>
<td>10.056</td>
<td>13.255</td>
<td>16.948</td>
<td>43.426</td>
</tr>
</tbody>
</table>
The optical thickness of the membrane is given by the equation

\[ A_2 = \frac{1 + R_{12}}{1 - R_{12}} \] (12)

since \( \delta_2 = \left( \frac{\lambda_1}{\lambda_2} \right) \delta_1 \), the only unknown in equation 13 is \( \delta_1 \), which can be readily evaluated from experimental data. The refractive index \( n_b \) of the membrane may be found using the following equation:

\[ n_b^4 \left[ \frac{(2n_w^2)(1 + \cos \delta_1) - 4n_w^2 A_1}{1 - \cos \delta_1} \right] n_w^2 + n_w^4 = 0 \] (14)

TABLE IV

<table>
<thead>
<tr>
<th>( t )</th>
<th>( A )</th>
<th>( A )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.959</td>
<td>4.660</td>
</tr>
<tr>
<td>6</td>
<td>5.623</td>
<td>6.452</td>
</tr>
<tr>
<td>8</td>
<td>6.561</td>
<td>7.454</td>
</tr>
<tr>
<td>10</td>
<td>7.571</td>
<td>8.527</td>
</tr>
<tr>
<td>14</td>
<td>9.801</td>
<td>10.883</td>
</tr>
</tbody>
</table>

It remains to be seen whether this method, due to Abeles (14), can provide us with more information concerning the detailed structure of BLM.

The considerations presented above are based upon the supposition that the structure of BLM is homogeneous and isotropic. However, it has been proposed that a BLM is not likely to be a homogeneous and an isotropic structure. This stems from the fact that the compounds so far established for BLM formation are all amphipathic in nature. Hence, a more realistic model consisting of three layers is proposed for the BLM (15). The details will not be repeated here. The following paragraph is concerned with the results of further computation using equation 10, and points out their significance in relation to the structure of BLM.

As mentioned above, the quantity most accessible to direct experimental measurement is the reflectance of the membrane. Calculations are therefore carried out for the reflectance, \( R_t \), using equation 10. The calculations are made for varying thicknesses, \( t_p \), of the polar (or ionic) layer and refractive indices. The wavelength of the incident light is 4350 Å. The thickness of the
central hydrocarbon region is assumed to be either 48 or 50 Å. The results for two typical cases are shown in Tables III and IV. In both tables, columns 2–4 give the reflectances of BLM according to the triple-layer model. Columns 5 show the reflectances of membranes having a homogeneous single-layer structure. The last column of each table presents the over-all thicknesses of the BLM using \( t = 2t_p + t_h \). It is evident from the results of calculations that the reflectances of BLM are very sensitive to the polar group thickness and membrane refractive index used, with the latter having a much greater effect than the former. The results presented in these tables are useful in that they may be employed to check the experimentally measured reflectance values.

The relationship between the single-layer and triple-layer models has been discussed in an earlier publication (15). In general, the thickness is about 10% lower as calculated according to Rayleigh's equation.

**METHOD OF MEASURING BLM TENSION BY BULGING**

Since the classical techniques for measuring the tension of films at interfaces (oil/water or air/water) are not easily extended to BLM, a novel method has been developed using highly sensitive pressure transducers which have become...
recently available (Sanborn model-270 and Pace model P-90D have been used). The theory on which the "bulging" method of measuring BLM tension is based is very simple. It is essentially a modification of the so-called maximum bubble pressure method (16). In applying the method to BLM systems, it is assumed that the conventional definition of interfacial tension is valid. The other assumptions are that equilibrium conditions exist and the BLM support is completely wetted by the lipid solution. Experimentally, a BLM is first formed at the biface. The hydrostatic pressure is then applied by slowly infusing a solution to one side of the membrane. The pressure difference, $P$, across the BLM is continuously recorded and reaches a maximum when the BLM is bulged into a hemisphere. The tension of the BLM is calculated at this point using the relation $\gamma = Pd/8$, where $a$ is the diameter of the opening. Fig. 4 illustrates diagrammatically one type of experimental arrangement used in the determination of interfacial tension of the BLM at the biface. The calculated results, together with the composition of BLM forming solutions are given in Table I.

It should be pointed out that the bulging method is not only useful for BLM systems at W/O/W bifaces but suitable also for other situations where two bulk phases are being separated by a thin layer (soap films, monolayers, multilayers, or immiscible liquids). Certain advantages of this method may be stated: (a) only a small quantity of test liquid is necessary; (b) temperature control is easy; (c) interfaces (or biface) can be easily renewed, thus reducing contamination. It seems that at the biface the only "mechanical" property which is directly measurable is the interfacial tension. Therefore, the method should also be of value in studying the interactions of drugs, proteins, and other agents with BLM.

**pH AND SALT EFFECTS ON $\gamma_i$**

Although no systematic studies of pH and salt effects on the interfacial tension of BLM have been carried out, the following tentative conclusions may be drawn from a few scattered experimental observations. (a) For BLM generated from lipids bearing ionizable groups, the $\gamma_i$ values are lower than for BLM containing anionic lipids at higher pH's. The opposite may be said to hold for BLM with cationic lipids. (b) For BLM formed from neutral lipids, pH has only secondary effects on $\gamma_i$. This is also true for changing salt concentrations. However, a much more noticeable salt effect is observed for BLM produced from lipids with charged groups. For example, the interfacial tensions of BLM formed from cholesterol-HDTAB-dodecane in water and in 0.1 N NaCl are, respectively, 0.15 and 0.65 dyne/cm. Similarly, the dioctadecyl phosphite membrane in water has a $\gamma_i$ value of 5.7 dynes/cm as compared with 3.9 dynes/cm when measured in 0.1 N NaCl.

To sum up the observations on the $\gamma$'s of the BLM generated from a num-
ber of compounds, the results strongly suggest that a low $\gamma$ value is a pre-
requisite for stable BLM formation; the lower limit is slightly above zero.
The upper limit of the $\gamma$ value, if it exists, cannot exceed about 9 dynes/cm.

THE THERMODYNAMICS OF BLM
AT THE BIFACE

In this section the classical thermodynamic equations of the interface between
two bulk phases developed by Gibbs will be applied to the BLM system (19).
We shall assume that each extensive property of the system, such as the free
energy $F$, the entropy $S$, or the number of moles of each component, is the
sum of three parts: (a) the contribution of aqueous phase I, (b) the similar
contribution of aqueous phase II, and (c) the BLM, which has a small but
definite uniform thickness. This is consistent with the usual picture of two
homogeneous aqueous solutions between which a BLM is interposed. Since
BLM has a “well-defined” structure, we may speak of its temperature $T$,
free energy, etc., just as for the two aqueous phases I and II. The exceptions
to these quantities are the pressure and interfacial tension. In the case of
pressure, the force per unit area exerted perpendicularly on the BLM is

$$p = P_L - \frac{\gamma}{t}$$

(15)

Therefore, the $pV$ work for the interfacial layer is equal to

$$pV_{BLM} - \gamma A$$

(16)

where $V_{BLM} = t \cdot A$, and $A$ is the area of the membrane. For the BLM, the
general variation of the Helmholtz free energy is given by

$$dF_{BLM} = -S'_{BLM} dT - p dV_{BLM} + \gamma dA + \sum_i \mu_i dn$$

(17)

where $\mu$ and $n$ are the chemical potential and the number of moles in each
phase, respectively. By manipulating these equations in the customary man-
ner, we can obtain the Gibbs-Duhem equation for BLM, i.e.

$$S'_{BLM} dT - V_{BLM} dp + A d\gamma + \sum_i n d\mu = 0$$

(18)

If equation 18 is divided throughout by $A$, it becomes

$$S'_{BLM} dT - t dp + d\gamma + \sum_i \Gamma du = 0$$

(19)

where $S_{BLM}$ is the entropy per unit area of BLM, and $\Gamma$ is the so-called inter-
facial excess. It is evident that equation 19 is a form of the Gibbs adsorption
equation, which will be considered further in connection with the BLM structure. We can also write the change of \( \gamma \) with temperature, which is given by

\[
- \frac{d\gamma}{dT} = S_{BLM} - \sum \Gamma \frac{d\mu}{dT}
\]  

(20)

We shall now attempt to apply these equations to the BLM system. Before doing so, it seems appropriate to recall the behavior of molecules at oil/water interfaces and their relation to the bifaces. Generally speaking, relatively high tensions are observed at the bulk oil/water interfaces (about 50 dynes/cm for long-chain hydrocarbon vs. \( H_2O \)). By introducing polar (or ionic) groups into the hydrocarbon molecules, the \( \gamma \) can be greatly lowered. When a BLM is extended, the external work done on the membrane is \( \gamma dA \). If the process is carried out isothermally and at constant pressure and composition, we can define, in the conventional manner, the entropy of BLM formation as \(-\frac{d\gamma}{dT}\). Recalling that \( dF \) is a perfect differential of \( F \) (the Helmholtz free energy) with respect to the two variables, \( T \) and \( A \), we obtain

\[
\left( \frac{\partial \gamma}{\partial T} \right)_A = - \left( \frac{\partial S}{\partial A} \right)_T
\]  

(21)

Since \( dq = Tds \), where \( q \) is the quantity of heat involved, it follows also that

\[
\left( \frac{\partial q}{\partial A} \right)_T = -T \left( \frac{\partial \gamma}{\partial T} \right)_A
\]  

(22)

according to the laws of thermodynamics. The total internal energy of the BLM for a unit area (1 cm\(^2\)) increase is then the sum of two quantities; i.e.

\[
E_{BLM} = \gamma_{BLM} - T \left( \frac{\partial \gamma}{\partial T} \right)_A
\]  

(23)

The first term on the right side of equation 23 is the so-called free energy of the membrane. The quantity \( T(\partial \gamma/\partial T)_A \) is the amount of energy which must be supplied from the bathing solution. Rigorously speaking, the second term on the right-hand side of equation 23 is not to be neglected. However, in using the \( \gamma = Pd/8 \) relation, it has been assumed that the process is carried out isothermally with that portion of the energy provided by the surrounding medium.

**THERMODYNAMICS OF BLM FORMATION**

The formation of a BLM at a biface may be imagined to take place in three experimentally distinguishable steps. These are (a) the creation of a water/oil/water (or W/O/W) biface, (b) the adsorption or migration of interfacially
active molecules to the biface, and (c) the formation of the BLM from the monolayers situated at the biface. These individual steps, together with the accompanying free energy changes, are given by the following equations:

\[
\text{Liquid hydrocarbon (O) + H}_2\text{O (W) } \rightarrow \text{W/O/W + } \Delta F_1 \quad (24)
\]

\[
\text{W/O/W + lipid } \rightarrow \text{monolayers (ML) + } \Delta F_2 \quad (25)
\]

\[
\text{ML } \rightarrow \text{bipolar lipid membrane (BLM) + } \Delta F_3 \quad (26)
\]

It is evident that the overall reaction is

\[
\text{Liquid hydrocarbon + H}_2\text{O + lipid } \rightarrow \text{BLM + } \Delta F_i \quad (27)
\]

where \(\Delta F_i\) is the sum of three free energies. In equations 25 and 26, it is understood that both the monolayers and the BLM are in equilibrium with the Plateau-Gibbs border. From surface film studies, it is generally known that the tendency for a liquid hydrocarbon (e.g. \(n\)-dodecane) to spread on a clean water surface is nil, owing to the negative spreading coefficient. Therefore, \(\Delta F_1\) is approximately equal to zero. Expressing other \(\Delta F_i\)'s in terms of interfacial tension (free energy), we have

\[
\Delta F_2 = \gamma_{\text{ML}} - \gamma_{\text{O/W}} \quad (28)
\]

\[
\Delta F_3 = \gamma_{\text{BLM}} - \gamma_{\text{ML}} \quad (29)
\]

\[
\Delta F_i = \gamma_{\text{BLM}} - \gamma_{\text{O/W}} \quad (30)
\]

where \(\gamma_{\text{O/W}}\) is the interfacial tension of the oil/water interface, and \(\gamma_{\text{ML}}\) and \(\gamma_{\text{BLM}}\) are, respectively, the interfacial tension of the monolayer and the BLM.

As a specific example, the experimental data are available for BLM formed from lecithin-dodecane system: \(\gamma_W \approx 72, \gamma_O \approx 22 (n\text{-dodecane}), \gamma_{\text{O/W}} \approx 50, \gamma_{\text{ML}} \approx 6.5,\) and \(\gamma_{\text{BLM}} \approx 0.9\). Thus, the free energy change in the formation of BLM from lecithin at a clean W/O/W biface is about 50 ergs/cm². Assuming the area occupied per lecithin molecule in the BLM to be 50 Å², the molar free energy change is calculated to be 3.6 kcal.

It is seen that the formation of BLM at bifaces is akin to the process of spreading monolayers at air/water or oil/water interfaces. With suitable lipid solutions, such as oxidized cholesterol in octane, a BLM is formed spontaneously. That is, when a drop of lipid solution is introduced into the aperture, it never forms a stable thick membrane, but the drop thins down rapidly to the P-G border in equilibrium with a BLM. Since \(\gamma_{\text{BLM}}\) for the membrane is very small (usually less than 6 dynes/cm²), the molecules in the membrane must be under very high compression. The resulting structure (BLM) has a close-packed arrangement which is thought to be not unlike
that of liquid crystals in two dimensions. Further discussion of the BLM structure will be given in the section on the Gibbs adsorption isotherm.

Using BLM generated from dodecyl acid phosphate–cholesterol–dodecane in 0.1 n NaCl as illustrated, the temperature variations of the $\gamma$'s have been determined. From equations presented above and the experimental data, the entropy, free energy, and enthalpy of BLM formation may be calculated. The results show that the free energy of formation of BLM decreases slightly with increasing temperature for the temperature range investigated (25–44.5 C). This slight decrease is consistent with the idea that the major portion of the work involved is the formation of a duplex film (using Harkins' terminology) at the biface. At higher temperatures, the concentration of adsorbed lipid molecules will be less. Hence a lower free energy should result. The entropies of formation are all negative, ranging from 0.008 at 25°C to 0.264 erg/cm²-deg at 44°C. This means that the latent heats of formation are also negative. Therefore, in the process of formation of BLM, an evolution of heat takes place, which is also accompanied by a decrease in entropy. One obvious explanation is the changes in the orderliness of constituent molecules at the biface. As would be expected, the lipid molecules possess a higher degree of order in the BLM state than in the lipid solution. The larger decrease of entropy at a higher temperature seems to imply that a change has taken place in the monolayer structure, whereas the organization of the BLM is not much affected by a moderate rise in temperature. Similarly, much the same argument may be used to explain the negative enthalpy of formation data.

**APPLICATION OF GIBBS' ADSORPTION EQUATION TO BLM SYSTEM**

As mentioned in the section on the P-G border and BLM stability, one of the major problems is that we do not have any precise knowledge concerning the chemical composition of the membrane. The need for such knowledge is evident, since any quantitative considerations of BLM would have proceeded more rapidly had we possessed such information. At present, the use of direct chemical analysis for BLM composition is not feasible owing to the very small areas and thickness of the membrane (8). One way to approach this problem would be to resort to the use of Gibbs' adsorption isotherm given earlier (equation 19). The use of equation 19 might be possible if the quantity $\gamma$ is identified with the $\gamma_{BLM}$. In order to calculate the excess concentration of lipid molecules in the BLM, the usual extrathermodynamic assumption would also have to be made in that the only species in the membrane would be the surface-active lipid molecules. Since the Gibbs equation relates the $\gamma_{BLM}$ and the concentration of the dissolved material, therefore, a knowledge of the interfacial concentration of adsorbed molecules would permit an evaluation of the area occupied by BLM-forming molecules. From this information
certain deductions concerning the structural aspects of the membrane might be made. We now present results of an investigation of BLM generated from the cholesterol-dodecane-HDTAB (hexadecyltrimethylammonium bromide) system as a specific example.

With the aforementioned assumptions in mind, equation 19 may be written as

\[-d\gamma_{\text{BLM}} = RT \gamma_c d \ln [C] + RT \Gamma_h d \ln [\text{HDTAB}] \quad (31)\]

under constant temperature and pressure conditions, where \(\gamma_c\) and \(\Gamma_h\) refer to the interfacial excess concentrations of cholesterol and HDTAB, respectively. The respective interfacial excess may be obtained from the slope of \(\gamma\) vs. concentration plots in the usual manner. In the present study, all \(\gamma\) measurements were made at the biface using the bulging method (Fig. 4). The calculated results are given in Table V. It should be stated that ideally one would like to obtain the \(\gamma\) vs. concentration plot for one component while holding the concentration of the other constant. However, we have not been able to produce BLM from either cholesterol or HDTAB alone, and so the data even for this simple BLM system are not complete. Therefore, the discussion which follows is presented to show that structural information may be obtained in spite of the limited data. It should be mentioned that the values for the area occupied per molecule given in Table V are expressed in terms of minimum area because of the lack of activity coefficient data and drastic simplifications used in the application of Gibbs' equation. In the case of HDTAB in the presence of cholesterol, the area is found to be about 200 Å². This value is calculated from the slope of \(\gamma_{\text{BLM}}\) vs. \([\text{HDTAB}]\) in the aqueous phase at a constant concentration of cholesterol. If we assume that the area occupied by the respective species remains unchanged in the membrane, this

\[\text{TABLE V}\]

<table>
<thead>
<tr>
<th>System</th>
<th>Concentration excess</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDTAB-C₁₂-H₂O</td>
<td>2.1×10⁻¹⁰ mole/cm²</td>
<td>80</td>
</tr>
<tr>
<td>HDTAB-cholesterol-C₁₂-H₂O*</td>
<td>7.8×10⁻¹¹ mole/cm²</td>
<td>200</td>
</tr>
<tr>
<td>Cholesterol-C₁₂-H₂O</td>
<td>5.6×10⁻¹⁰ mole/cm²</td>
<td>30</td>
</tr>
</tbody>
</table>

* Stable BLM obtained with this system only.

\[\text{For simplicity, we have used the concentrations instead of activities. Also, for the dissociable surfactant, HDTAB, a factor of } 2 \text{ should be taken into consideration.}\]
would mean that the molar ratio of the cholesterol to that of HDTAB is about 3:1 in the BLM. To account for this particular ratio, a most likely arrangement would be that of a hexagonal packing with 6 cholesterol molecules around each HDTAB. It is argued that the slenderness of the HDTAB hydrocarbon tail could easily fit into the space between the cholesterol molecules, with the head group of HDTAB sticking out at the membrane/solution interface. A probable structure of BLM produced from cholesterol-HDTAB as deduced from the above data is illustrated diagrammatically in Fig. 5. It seems likely that the insertion of HDTAB into the cholesterol "bilayer leaflet" may account for the BLM stability.

![Diagram of BLM structure](image)

**Figure 5.** BLM produced from the cholesterol-HDTAB system. A. A front view of a sheet of cholesterol molecules at the water/oil/water biface. B. Adsorption of hexadecyltrimethylammonium bromide (HDTAB) stabilized the system, leading to BLM formation. Open circles, cholesterol; larger circles with hatched centers, HDTAB.

**CONCLUSIONS**

BLM, a unique type of interfacial membrane, has been shown to be an important tool for the investigation of membrane phenomena in relation to biological membranes (8). Thus far, certain semblances have been demonstrated between natural membranes and BLM. However, we are still ignorant about the exact composition and structure of these membranes. The study of unmodified BLM, though important in its own right, cannot hope to elucidate the basic functions and structural aspects of natural membranes. Nevertheless, the fact that today we are able to manipulate a structure of molecular dimension, and, most of all, that such a delicate structure can be formed in vitro between two aqueous solutions, offers a wide variety of opportunities for its modification. These could then be used for studies such as membrane transport, conductivities, and membrane potentials. The important feature of the BLM lies in the possibility that BLM, when constituted from appropriate compounds, may be used as "model systems" in the understanding of visual process, and photosynthesis and related phenomena in which lamellar structures are known to be important. It is hoped that a detailed physical chemical description of the BLM system would be useful in providing a more rational basis for further development.

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REFERENCES

Discussion

*Dr. Mysels:* I wonder whether, Dr. Tien, in going to the surface area from the measurement of surface tension changes upon addition of quaternary ammonium salt, we don't have to make some assumption about the constancy of absorption of the cholesterol or something of that sort?

*Dr. Tien:* Yes. For a two-component system, the ideal situation is to measure interfacial tension change with respect to the concentration of one substance and holding the other one constant. You do the same thing for the other component. In this way you can obtain the sum of the two interfacial excesses.

Does that answer your question, Dr. Mysels?

*Dr. Mysels:* Yes.

*Dr. Miller:* I am not sure that I really understood every stage of the discussion because I couldn't see the tables very well, but I was wondering about the interpretation of the stability of the bilayer. If I did understand, it was based on consideration of the interfacial tensions. Am I correct?

*Dr. Tien:* Not quite. I think that a low value of interfacial tension is one of the prerequisites for the formation of a stable black lipid membrane.

*Dr. Miller:* What I really mean is the difference between the interfacial tension of the bilayer water interface and the oil/water interface, $\Delta F$. Is this $\Delta F$ related to some equilibrium phenomenon, or is it related to some transient process? That is, during the burst or destruction of the bilayer you create transiently an oil water interface by investment of the energy $\Delta F$, which in this case would become a component of an activation energy. Besides that, shouldn't some structural contributions and cohesion forces also be considered?

*Dr. Tien:* The interfacial free energy (or tension) to which I was referring is the free energy of formation measured at equilibrium conditions; that is, the energy required to form a black lipid membrane at the biface (i.e. the two coexisting water/oil or solution/membrane interfaces) from initially clean water/oil interfaces. With regard to the latter part of your comments, I can only say that the process leading to black lipid membrane formation is not very well understood at the present. Perhaps the question you posed and certain aspects I discussed in my paper will stimulate some interest in that direction.

*Dr. Mauro:* Would you care to speculate on your extensive investigations on lipid molecules? What would be the minimal length of the lipid chain that would give you a so-called secondary film?

*Dr. Tien:* In reply to your question, Dr. Mauro, I would like to say that a chain length of about 12 carbon atoms seems to be necessary (see Table I of my paper). This is based upon the fact that we have been able to produce black membranes stabilized by esters of lauryl alcohol. Perhaps $C_{10}$ or even lower hydrocarbon chain lengths are also possible, but I do not have any definite information.