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Chapter 1

THE PHYSICAL NATURE OF PLANAR BILAYER MEMBRANES

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1. Introduction

A major part of the general strategy for defining the structure–function relationships of ion channels is to reconstitute isolated channel proteins into planar lipid bilayer membranes separating two aqueous compartments. In principle, this part of the strategy allows one to manipulate the lipid and aqueous environments of the protein to elucidate their roles in channel assembly and function *in vivo*. Its success depends on how well one can use the natural physicochemical behavior of the bilayer system to control the reconstitution process and the composition and properties of the lipid bilayer itself. Consequently, the serious student of reconstitution must come to terms with the physical chemistry of these bilayers variously referred to as planar lipid bilayers, black lipid membranes, or thin lipid films.

My aim in writing this chapter is to give the reader a basic understanding of planar bilayers and how to control them. My approach will be largely qualitative because I suspect that most readers are primarily interested in ion channels and only secondarily in the bilayers themselves. Planar bilayers can be incredibly frustrating. All of us who have worked with them have, at one time or another, been reduced to irrational (but satisfying) acts of anger. The frustration usually arises because one is, unknowingly, trying to do something that is thermodynamically unlikely. The number of beakers I sent crashing against the laboratory wall decreased as my understanding of the physical chemistry of the system increased. Here, then, are the essentials of black lipid membranes as I see them.

1.1. Theme of the Chapter

The basic theme of this chapter is that the planar bilayer itself is only one part of a highly heterogeneous physicochemical system. To understand the bilayer, one must understand it in the context of the whole system, which consists

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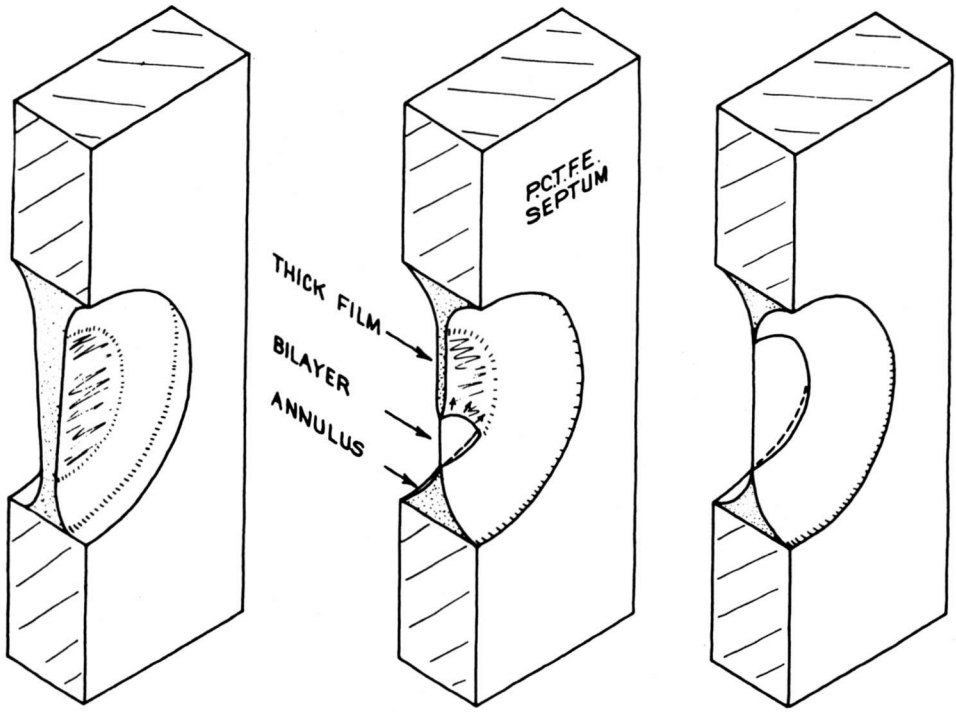


Figure 1. Summary of the process of formation of planar lipid bilayers from lipids dispersed in alkane solvents by the method of Mueller *et al.* (1962). The lipid solution is applied beneath water across an aperture in a nonpolar material such as polychlorotrifluoroethylene (PCTFE). Note that the lipid solution "wets" the septum and forms an annulus (Plateau–Gibbs border) around the bilayer. The initially thick film thins spontaneously because of the Plateau–Gibbs border suction. (From White *et al.*, 1976.)

of several different phases. Consider, for example, the classic black-film system first described by Mueller *et al.* (1962). A bilayer forms spontaneously as in Fig. 1 after a "forming solution" is spread across a small (1–2 mm) aperture in a hydrophobic partition separating two aqueous phases. The forming solution consists of a surface-active lipid "dissolved" in a nonpolar liquid such as *n*-decane. The resulting structure consists of the bilayer film surrounded by an annulus of the bulk forming solution. When viewed under reflected light, the very thin (25–50 Å) bilayer reflects little light relative to its surroundings and thus appears "black"; hence the term black lipid membrane.

The bilayer itself consumes most of our attention, and we thus largely ignore the annular border. Such neglect is not wise if one wishes to control the bilayer. These two parts or phases of the system tend toward chemical equilibrium with one another. Because the annulus has a mass at least a million times that of the bilayer, the chemical potentials of the components of the bilayer are constrained to be the same as those of the annulus. The secret to the control of bilayer behavior

and composition is the control of the annulus or any other more massive phases it contacts.

The annulus, then, must receive much of our attention as we seek to use the bilayer as a controlled environment for ion channels. It is not a simple solution of lipid in organic solvent. The lipid is present in the solvent as hydrated aggregates (micelles), which tend toward equilibrium with the bulk aqueous phase. The annulus is a multicomponent system and can exhibit a complex phase behavior. We are most experienced as biologists with the phase behavior of pure lipid dispersed in water. The behavior of hydrated lipid in a nonpolar phase can be quite different.

The planar bilayer system thus consists of several phases that tend to equilibrate with one another. To understand it, one must think about it in terms of the general principles of equilibrium in heterogeneous systems. This can be a confusing business. I have found it to be less so if I organize my thinking around Gibbs's phase rule. This chapter is written, therefore, with the phase rule as its central focus.

1.2. Organization of the Chapter

I proceed as follows. I first discuss the most common strategies for reconstituting channels in planar bilayers to set the context for the material to follow. The discussion is brief because most of this book is devoted to the specific details of reconstitution. It gives me an opportunity, however, to identify topics I believe to be central to the reconstitution problem and to reference key reviews for those topics not reviewed in detail.

I next discuss the general principles of planar bilayer formation and stability. Of particular importance are the differences between those formed by spreading films from bulk solutions by the method of Mueller *et al.* (1962) and those formed by the folding up of monolayers by the method of Montal and Mueller (1972). These principles reveal the complex heterogeneous nature of the planar bilayer. I then discuss the physical chemistry of heterogeneous systems in the context of the bilayer system. This sets the stage for a discussion of the control of bilayer behavior. I close by explaining some of the vagaries of planar bilayers, which have frustrated more than a few biophysicists.

2. Overview of Reconstitution

2.1. Strategies for Reconstitution

Of the many different approaches to reconstitution that have been tried [see reviews by Miller (1983a,b, 1984) and by Montal and his colleagues (1981)], two have emerged as the principal ones (Fig. 2). In the first, planar bilayers are formed either from bulk solutions (Mueller *et al.*, 1962) or from monolayers (Montal and Mueller, 1972). The channels are transferred to the bilayer from vesicles by Ca^{2+}-

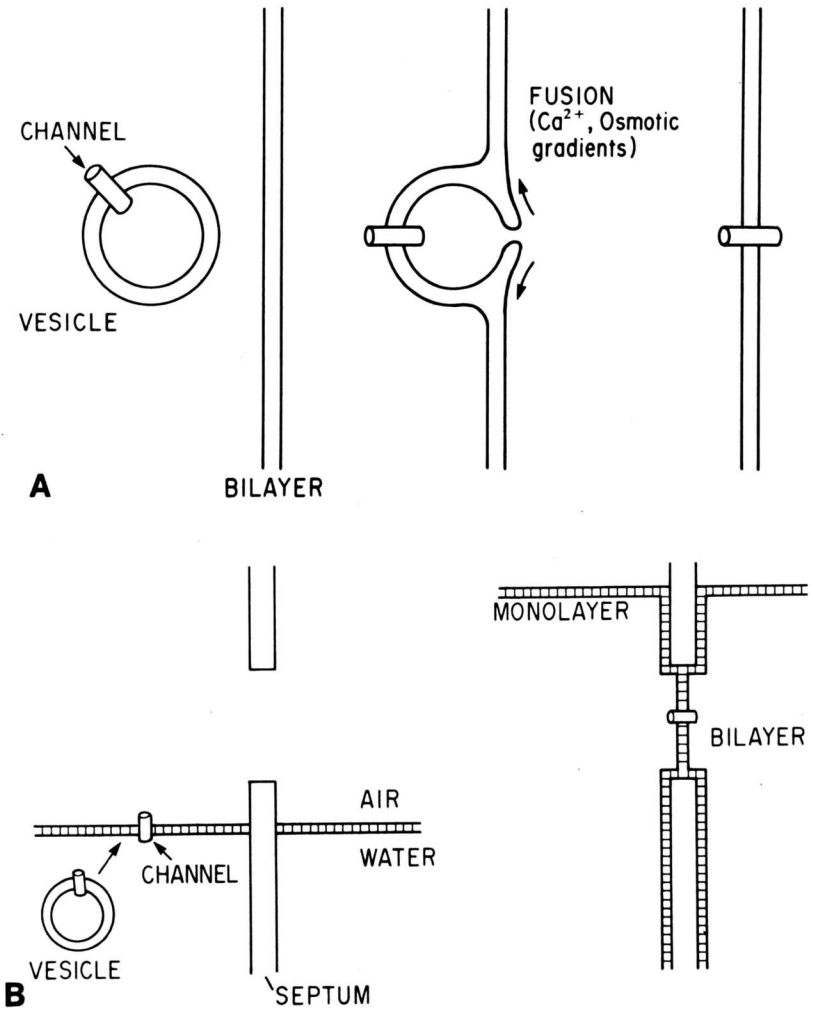


Figure 2. Summary of the two principal methods for incorporating ion channels into planar bilayers. Both methods utilize vesicles with the protein incorporated into their bilayers. (A) The method of Miller and Racker (1976) incorporates the protein by fusing vesicles made with charged lipids to preformed bilayers. The fusion is controlled with Ca^{2+} and the creation of osmotic gradients across the vesicles and planar bilayer. It is assumed that the fusion is complete so that the contents of the vesicles are released *trans* to the side the vesicles are added to. (B) The method of Schindler (1980) involves forming bilayers by folding up monolayers across an aperture in the manner of Montal and Mueller (1972). The monolayers are formed by allowing the protein-containing vesicles to spread spontaneously at the air–water interface. It is assumed that the protein is incorporated into the monolayers.

induced fusion controlled by osmotic gradients across the vesicles and the planar bilayer, which contain negatively charged lipids (Miller and Racker, 1976). The channels are incorporated into the vesicles by dialysis of solutions containing the protein and excess lipid solubilized in detergent. The osmotic stress is viewed as causing the vesicles to swell and rupture in such a way that fusion occurs.

In the second method, lipid monolayers are produced at the air–water interface from the channel-containing vesicles (Schindler, 1980). These monolayers presumably contain the channels, which become incorporated into the bilayer when the monolayers are raised across the aperture by the method of Montal and Mueller (1972).

Both methods can lead to the incorporation of channels into bilayers, but it is not proven that the methods work precisely according to the cartoons shown. Regardless of the accuracy of the cartoons, however, they do illustrate the topics that should attract the interest of the serious student of reconstitution.

2.2. Important Topics for Reconstitution

The two principal methods of forming planar bilayers must be understood thoroughly. These are the subject of the present chapter. In addition, however, the general nature of monolayers at the air–water interface must be appreciated. I highly recommend the reviews of Gershfeld (1974, 1976) and the superb monograph by DeFay and Prigogine (1966). The particularly relevant major issues addressed by Gershfeld concern the phase behavior of monolayers, the equilibrium between bulk lipid phases and adsorbed layers at the air–water interface, and the heterogeneity of surface films. Particular attention should be paid to the question of whether surface films are necessarily monomolecular in thickness and to film penetration by chemical components (e.g., proteins) in the subphase.

Another obvious topic for study is that of the physical chemistry of liposomes and vesicles. Bangham, who is the originator of these systems, has written several reviews that are essential reading (1974, 1980). Israelachvili *et al.* (1980) and Tanford (1980) have summarized the physical principles of liposome and micelle formation. A recent volume edited by Ostro (1983) contains a number of useful papers.

Because the joining together of vesicles with bilayers is central to reconstitution, knowledge of the energetics and mechanics of bilayers in the context of adhesion and deformation is essential. Thorough reviews of these and related topics have been written by Evans and Skalak (1979a,b) and by Parsegian and his colleagues (Parsegian and Rand, 1983; Evans and Parsegian, 1983; Parsegian *et al.*, 1984). The role of osmotic stress in fusion is likely to be more subtle and complicated than simple vesicle swelling. In this regard, the effect of water activity on bilayer mechanics (Parsegian *et al.*, 1984) and the organization of water near the membrane surface (Gruen and Marcelja, 1984) should receive special attention. The molecular packing density of water and the headgroups of phospholipid bilayers are likely to be important also (White and King, 1985).

Finally, the planar bilayer system is best described by the laws of the physical

chemistry of surfaces. A familiarity with surface chemistry can be gained from textbooks by DeFay and Prigogine (1966), Adamson (1967), and Aveyard and Haydon (1973).

3. Formation and Stability of Planar Bilayers

Several books and reviews have been devoted to this general subject (Tien and Diana, 1968; Henn and Thompson, 1969; Haydon, 1970; Jain, 1972; Tien, 1974; Finkelstein, 1974; Fettiplace *et al.*, 1975), and I do not intend to be encyclopedic in my approach. Rather, I wish to highlight central ideas that are useful for the later discussion. The most succinct review of the physical chemistry is that of Fettiplace *et al.* (1975).

Planar bilayers formed from either bulk solutions or monolayers are largely indistinguishable from the physicochemical point of view. The major difference is the nature of the bulk phases from which they are formed. Because the first method reveals the general principles better, I begin with it.

3.1. Formation by Spreading from Bulk Solutions

3.1.1. Forming Solution

The first step in the formation of black films by the original method of Mueller *et al.* (1962) is to dissolve—more properly, disperse—a surface-active lipid in a nonpolar solvent. The lipids most commonly used are the monoglycerides (MG) or lipids commonly found in cell membranes such as phosphatidylcholines (PC), phosphatidylethanolamines (PE), or phosphatidylserines (PS), which may be mixed with varying amounts of cholesterol. Phosphatidylserine is particularly useful for conferring fixed charge at the surface, but phosphatidylglycerol (PG) and phosphatidic acid (PA) are also used for this purpose. Films were originally formed from whole lipid extracts of membranes, and these are still used for some purposes. The most common nonpolar solvents these days are alkanes or other long-chain hydrocarbons such as squalene. In the early days, standard lipid extraction solvents (e.g., chloroform:methanol) augmented with alkanes were used, but these are more difficult to use, probably because of time-dependent changes in composition and phase behavior in the presence of excess water.

The lipid in the solvent tends very strongly to form large aggregates (micelles) organized to shield the polar regions of the lipids from the nonpolar solvent (Fig. 3). The simplest micelles are those of the monoglycerides, which tend to form clusters of 25 or so molecules with the acyl chains facing outward (Andrews *et al.*, 1970). Black films cannot be formed unless the aggregates are present; i.e., the concentration of lipid must be above the critical micelle concentration (cmc).

The forming solution remains in contact with the bilayer as the annulus for the bilayer's entire life. In addition, the bilayer and annulus reside in an excess aqueous phase. Because the annulus is so massive compared to the bilayer, the

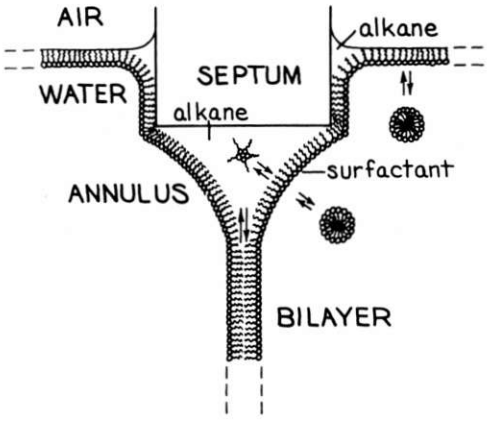


Figure 3. A schematic representation of the black lipid membrane system and the various locations and states of the surface-active lipid. The lipid in the annulus is represented as simple inverted micelles, but other much larger and complex aggregates are possible. The arrows indicate the equilibria between the various phases of the system. The drawing is not to scale. For bilayers formed by spreading from bulk nonpolar solutions, the mass of the annulus is a million times that of the bilayer. Therefore, the chemical potentials of the lipid and solvent (alkane) in the annulus determine the chemical potentials in the bilayer.

chemical potential of the lipid in the aggregates determines the state of the lipid in the bilayer itself. Consequently, the phase behavior of the forming solution in excess water is important to understand. This is not trivial, because one ends up dealing with microemulsions, which are the subject of another whole complex area of physical chemistry (e.g., Ekwall, 1969; Dominquez *et al.*, 1979).

Microemulsions are generally formed from three-component systems consisting of a surfactant, a nonpolar solvent, and a polar solvent. The bulk forming solution has these ingredients when one considers that water, even if not specifically added, must eventually be present in the annulus. I will not not describe the complex behavior of microemulsions; the interested reader can gain entry to the literature from a recent symposium volume edited by Luisi and Straub (1984).

3.1.2. Spreading the Film

A droplet of the bulk forming solution is placed around the rim of an aperture in a Teflon®, Kel-F®, or polyethylene partition submerged in the aqueous phase. Bilayers will not form across the aperture spontaneously; they must be spread with a small brush or, preferably, an air bubble protruding from a fine glass capillary. I make this obvious point because it means that the black film is inherently not an equilibrium system—work must be done to create it. There is always a lower free energy state for the system, which is the bulk solution with no black film; that is, the films tend toward the broken state. The membranes can, however, exist for many hours if other aspects of the complex equilibrium of the system are tended to. It is reasonable to consider them as metastable equilibrium systems (Guggenheim, 1977).

A crucial feature of the black-film system is the interface between the bulk solution of the annulus and the aqueous phase. The lipid adsorbs at the interface from the micelles to a potential energy well resulting from the insolubility of the acyl chains in the aqueous phase and the insolubility of the polar groups in the

nonpolar phase. The lipid lowers the interfacial tension γ of the interface (at equilibrium) according to the Gibbs absorption equation

$$d\gamma = -\Gamma \, d\mu \tag{1}$$

where

$$\mu = \mu_0 + RT \ln a \tag{2}$$

In these equations, μ is the chemical potential of the lipid with activity a in the annulus, and Γ is the interfacial concentration (mol/cm^2). μ_0 is the chemical potential in the standard state or a suitable reference state. The activity (a) may be written as ξc_L where c_L is the lipid concentration and ξ the activity coefficient. Equation 1 is the foundation of surface chemistry. It simply states that the decrease in interfacial tension is directly proportional to the change in the chemical potential of the lipid in the annulus. It allows one to determine the interfacial concentration (Γ) of the lipid, which leads in turn to the area/molecule of the lipid in the bilayer (Andrews *et al.*, 1970).

3.1.3. Thinning of the Film

The film formed by spreading the solution across the aperture is initially several micrometers thick but thins spontaneously to the planar bilayer state (Fig. 1). It thins under the influence of several driving forces. The major one in the early stages is the Plateau–Gibbs border suction, which arises from the curvature of the annulus (Plateau–Gibbs border). The curvature is necessary to satisfy simultaneously the contact angles the border must make with the aperture and the film. [These angles are related to the interfacial tensions of the various substances in contact. See Adamson (1967).] Wherever there is a curved interface at mechanical equilibrium, there must be a hydrostatic pressure difference ΔP across it described by the law of LaPlace

$$\Delta P = 2\gamma/R \tag{3}$$

where γ is the interfacial tension and R the radius of curvature. The pressure is always greater on the concave side of the surface so that the pressure in the border must be less than that in the flat region (Fig. 3). Therefore, bulk solution will flow from the flat film into the border, causing the film to thin.

As the film approaches thicknesses of a few hundred Angstroms, a second driving force becomes apparent. It is the London–van der Waals attraction between the aqueous phases separated by the thin film. This force is moderately long ranged and has been described in detail (e.g., Parsegian, 1975; Brooks *et al.*, 1975). Because of it, the free energy change of the film (ΔF) relative to infinite separation is

$$\Delta F = -H/12\pi d^2 \tag{4}$$

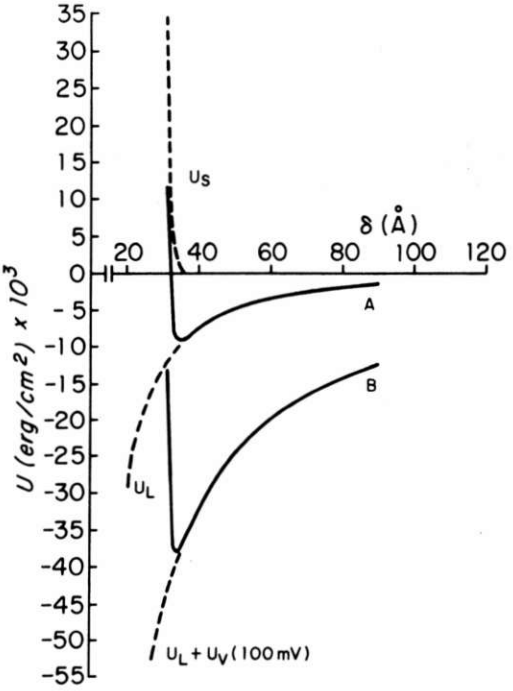


Figure 4. Hypothetical potential energy-versus-thickness curves for planar bilayer membranes. Two opposing forces stabilize the membrane: a London–van der Waals attractive potential (U_L) between the separated aqueous phases (equation 4; $\Delta F = U_L$) and a steric repulsive force (U_S) arising from the interactions of the apposed lipid acyl chains of the bilayer. When an electric field is present, an additional attractive potential (U_V) exists given by equation 6 ($\Delta F_V = U_V$). (From White, 1970.)

where H is the Hamaker coefficient and d the thickness of the film. It is this force that leads to the final thinning and is the major force attempting to thin the membrane at the equilibrium bilayer thickness. The black film (bilayer) usually appears first at the lower margin of the film and propagates rapidly over the surface (Fig. 1). The film is thinner at the lower margin because of the buoyancy of the forming solution.

3.1.4. Stability and Composition of the Film

The bilayer would not be stable unless the van der Waals force were opposed by another set of forces. When the opposing surfaces of the thicker film meet, there is a tendency for the apposed acyl chains on the lipid to interdigitate and to exclude the solvent molecules trapped in the film (Taylor and Haydon, 1966). However, this tendency to "demix" tends to lower the entropy of the bilayer and consequently to raise the free energy of the system, giving rise to an opposing force called the steric repulsive force. The bilayer thus resides in a local free energy well and is stable (Fig. 4).

Another force resisting the thinning of the film, albeit a temporary one, is viscous drag of the solvent flowing out from between the advancing interfaces. When the surfaces are very close, the viscosity of the solvent resists the thinning. As a result, the films are initially somewhat thicker. The excess solvent separates within 15–30 min into microlenses about 1 μm in diameter scattered over the

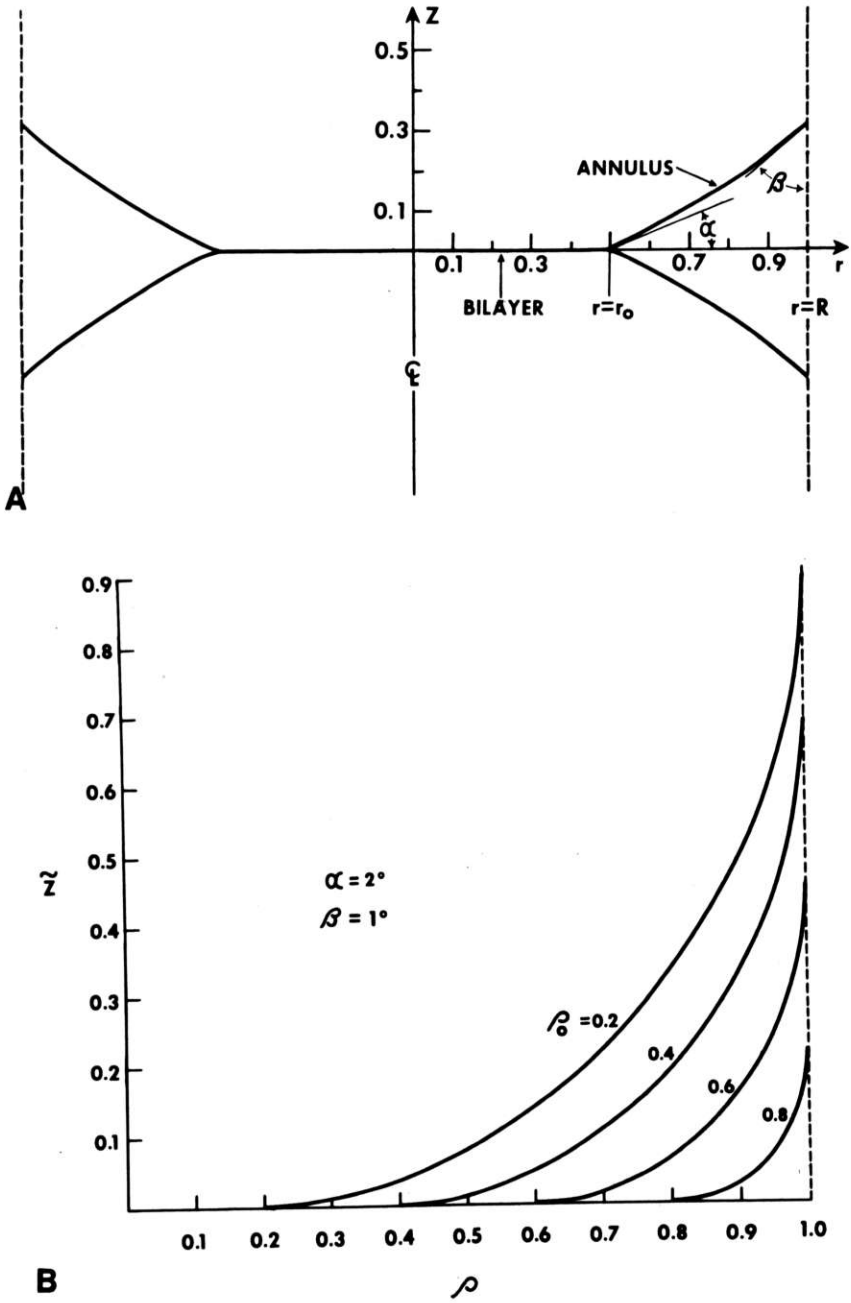
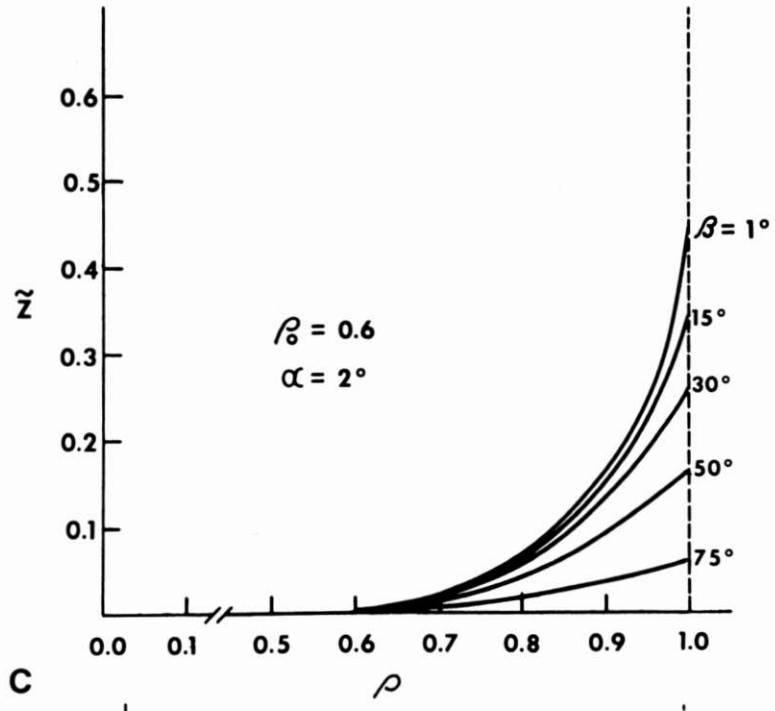


Figure 5. The shape of the annulus surrounding the planar bilayer membrane showing how changes in annulus volume (related to R), the contact angle between the annulus and film (α), and the contact angle between annulus and aperture (β) affect the shape. (A) Overview of system. In B, C, and D, the figures represent the shape in the first quadrant only. Normalized coordinates are used: $\rho = r/R$ and $\tilde{Z} = Z/R$. (B) Variation in shape with changes in ρ. (C) Variation in shape with changes in α. (D) Changes in shape with β. (From White, 1972.)



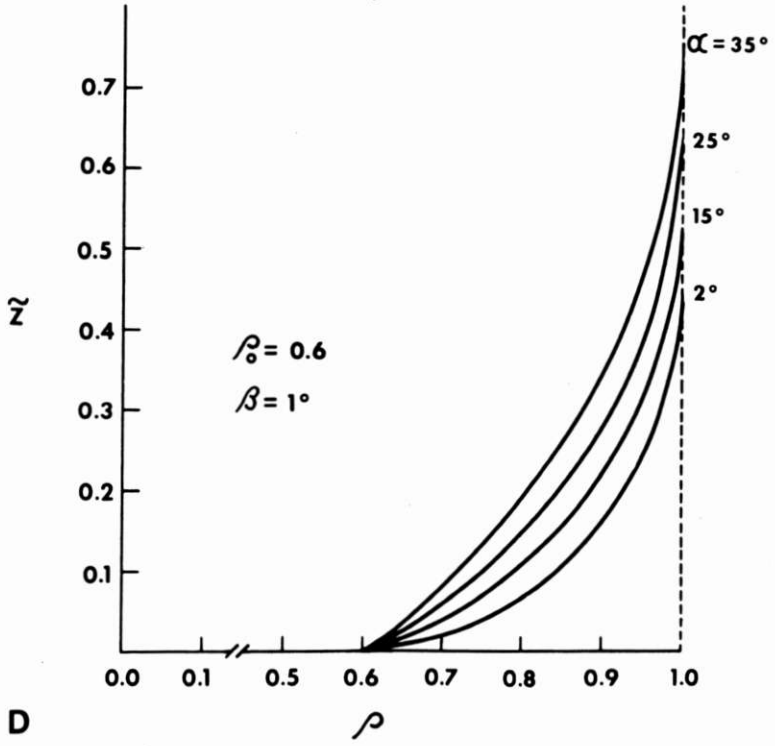


Figure 5. (*continued*)

surface, which are easily seen in reflected light. They do not usually interfere with most electrical measurements and eventually coalesce with the annulus.

The chemical potentials (equation 2) of the solvent and lipid in the bilayer must be equal to those of the annulus when the film reaches its local equilibrium. Because the annulus is much more massive than the bilayer, the solvent in the bilayer tends to come into equilibrium with the annulus. This is equivalent to the situation a single biological cell faces when suspended in a large volume of buffer. We can thus describe an osmotic pressure on the solvent in the annulus trying to drive solvent into the bilayer, which is opposed by the van der Waals force squeezing the bilayer (Andrews, 1970; White, 1980). Most properly then, we must describe the black film's "equilibrium" not as a regular equilibrium (pressure everywhere uniform) but as an osmotic equilibrium (Guggenheim, 1977).

3.1.5. Film Boundary Conditions

An annulus surrounds all black films regardless of method of formation and is essential for the existence of the bilayer (White *et al.*, 1976). It connects the bilayer to the aperture, so its mechanical properties have an effect on one's ability to form and manipulate the bilayer. The influence of the annulus on mechanical stability can be appreciated from a mathematical analysis of the shape of the annulus using the methods of variational calculus (White, 1972). The shape is determined by the contact angle between the forming solution and the aperture (β), the contact angle between the bilayer and the annulus (α), and the volume of solution placed on the aperture (Fig. 5). If one forms the bilayer on an aperture that is the shape of a right circular cylinder, the aspect ratio of the aperture (its length divided by diameter) is likely to be important in achieving mechanical stability because there is a maximum annulus width that can be accommodated by a particular aperture.

One problem with films is that they tend to drift along the length of the cylindrical aperture. Another is that they exchange bulk solution with the surface of the partition. If all surfaces of the forming chamber are thoroughly coated with the bulk forming solution, then bilayer diameter will tend to shrink because solution flows from the septum surface to the annulus as a result of the border suction that led to film thinning in the first place. Both of these problems can be controlled reasonably well by using a countersunk aperture (Fig. 6). The film becomes trapped at the point where the aperture changes diameter. Further, the shelf of the countersink helps to define the volume of the annulus and therefore the bilayer's diameter.

The contact angle (α) between the bilayer and annulus is determined by the difference in free energy (ΔF of equation 4) between a thick film (e.g., the annulus) and a bilayer (Haydon and Taylor, 1968). The relationship between the two is

$$\Delta F = 2\gamma(\cos \alpha - 1) \tag{5}$$

where γ is the annulus–water interfacial tension. Haydon and his colleagues (Haydon and Taylor, 1968; Requena and Haydon, 1975b; Brooks *et al.*, 1975) have

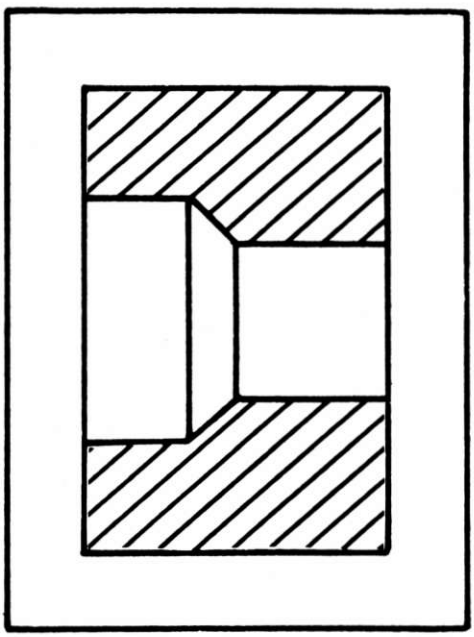


Figure 6. Cross-sectional view of a convenient aperture for forming black lipid films. The countersink keeps the film from drifting along the length of the aperture and helps stabilize the volume of lipid in the annulus to minimize changes in diameter with time.

measured ΔF (the depth of the free energy minimum in Fig. 4) by measuring the contact angle optically.

The application of an electric field to the bilayer adds a compressive force in addition to that of the van der Waals attraction (equation 4) and leads to further thinning of the film (Fig. 4). The change in free energy ΔF_V caused by an electric potential V is

$$\Delta F_V = -\frac{\epsilon_0 \epsilon}{2d} V^2 \tag{6}$$

where ϵ_0 is the permitivity of free space ($8.85 \cdot 10^{-14}$ F/cm^2), ϵ the dielectric coefficient of bilayer, and d the thickness. As implied by equation 5, a change in the contact angle must accompany the voltage-induced change in free energy, as Requena and Haydon (1975a) have verified. This causes the shape of the annulus to change in such a way that the diameter of the bilayer increases (White and Chang, 1981).

3.2. Formation of Black Films from Monolayers

The lipid and some of the solvent from the bulk forming solution will inevitably adsorb at the air–water interface and form either monolayers or possibly

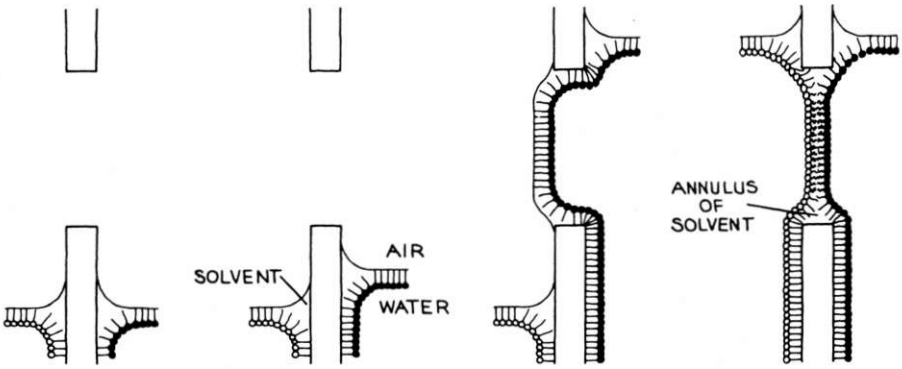


Figure 7. A sequence of sketches showing how a bilayer is produced from monolayers when alkane is present. The alkane causes the acyl chains of the monolayer to have a favorable orientation for bilayer formation. The septum is shown for artistic convenience as being thin; it is orders of magnitude thicker in reality. (From White *et al.*, 1976.)

so-called duplex films (Langmuir, 1933). Another part, then, of the now clearly complex black film sysktem is the lipid and solvent at this interface. Another way of forming black films is to float droplets of the bulk forming solution on the surfaces of the aqueous compartments, which are initially below the level of the aperture. A bilayer can be formed by raising first one and then the other aqueous phase above the aperture, as shown in Fig. 7. There is no fundamental difference between bilayers formed in this way and those formed by painting.

3.2.1. Solvent-Free Bilayers

The basic strategy for forming bilayers by folding up monolayers was introduced by Takagi *et al.* (1965) and perfected by Montal and Mueller (1972). The original rationale was to produce bilayers free of solvent because solvent was nonbiological. Further, because volatile alkanes are anesthetics, the fear arose that solvent-containing bilayers might be anesthetized and thus create a poor environment for ion channels. The main issue is whether or not the bilayer of nerve membranes mediates the anesthetic action. There is strong evidence (Franks and Lieb, 1978, 1981) that it does not, in which case the fear is unfounded.

In any case, Montal and Mueller's goal was to form bilayers with as little solvent as possible. In the original form, lipid was spread from a volatile solvent at the air–water interfaces so that solvent-free monolayers remained after the solvent evaporated. This is the classic method of forming monolayers for study using the Langmuir surface balance (see Gaines, 1966). Prior to the spreading of the monolayers, the initially dry septum was "conditioned" with petroleum jelly applied from a dilute hexane solution. The petroleum jelly provides a small amount of nonpolar lipid for the annulus. Without this material, bilayers cannot be formed as is illustrated in Fig. 8. An annulus of some sort is absolutely required (White *et al.*, 1976). This does not mean that the bilayers necessarily contain very much

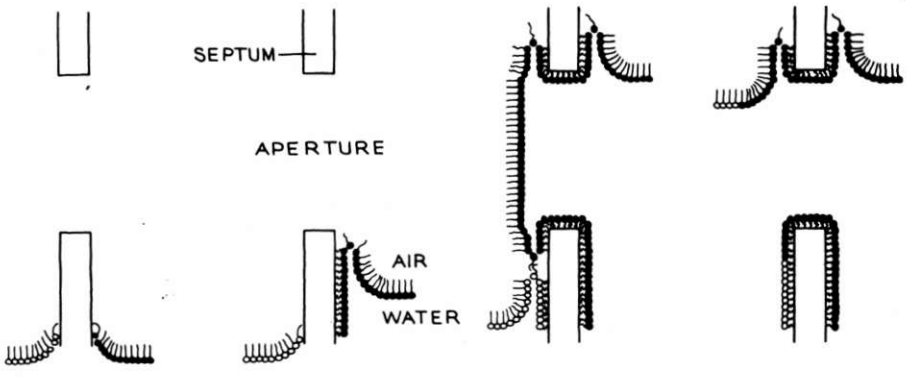


Figure 8. A sequence of sketches showing what happens if one attempts to form bilayers from monolayers in the absence of alkane or other nonpolar solvent. The raising of the left-hand water level "peels off" the monolayer on the right aqueous phase, shown here protruding through the aperture (third and fourth frames). (From White *et al.*, 1976).

solvent. The constituents of the petroleum jelly are probably not very soluble in bilayers.

Solvent-free bilayers can be formed by the painting method simply by using squalene, which does not dissolve in the bilayer but forms a good annulus (White, 1978). Di- or triglycerides (Waldbillig and Szabo, 1979) can be substituted for the squalene. A big advantage of the Montal–Mueller method is that it is possible to form asymmetric bilayers. Another is that one can minimize the total overall effect of the nonpolar solvent so that the properties of the lipid in an aqueous phase rather than a nonpolar phase dominate the system. For example, Pattus *et al.* (1978) and Schindler (1979) showed that one can spread the monolayers from lipsomes rather than from lipid dissolved in solvent. This sort of procedure has actually been used for years by surface chemists studying equilibrium of spreading of monolayers at the air–water interface from bulk lipid (see, e.g., Eriksson, 1971; Phillips and Hauser, 1974; Tajima and Gershfeld, 1978).

3.2.2. The Bulk Phase Distinguishes the Two Methods

The most important difference between the two methods of forming bilayers is the nature of the bulk phase with which the bilayer is attempting to equilibrate. In the first method, one begins with a solution of hydrated lipid in hydrocarbon solvent so that the phase behavior of the lipid–water–hydrocarbon bulk phase determines the state of the bilayer. In the second method, minimum amounts of solvent are present, so that the phase behavior of a predominantly lipid–water bulk phase determines the state of the bilayer.

Figure 9 shows a phase diagram for glycerol monooleate–triolein mixtures in the presence of excess water. The left-hand side represents the situation in which one forms bilayers from bulk nonpolar solutions. At room temperature, the bulk phase is mostly triolein with small amounts of GMO dispersed as micelles.

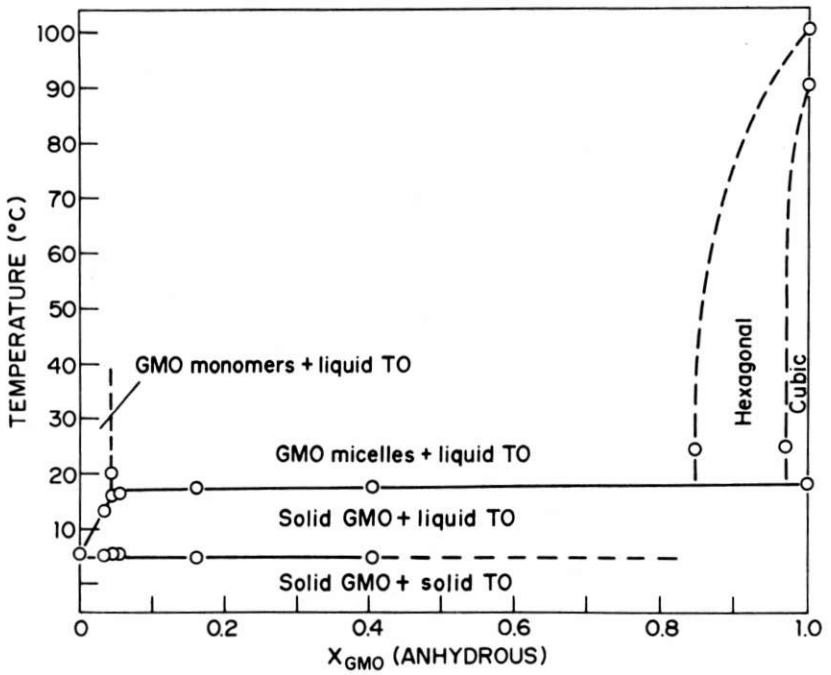


Figure 9. Phase diagram for a mixture of glycerol monooleate (GMO) and triolein (TO) in excess water. This is an example of the type of phase behavior one can expect of the black-film "forming solution" constituting the annulus of the black film. The left-hand side corresponds to the situation for bilayers formed by the spreading method of Mueller *et al.* (1962). The right-hand side corresponds to the situation for bilayers formed by the folding up of monolayers by the method of Montal and Mueller (1972). (Based on unpublished data by S. H. White and T. J. McIntosh, Duke University, Durham, North Carolina.)

On the right is the situation in which one uses minimal amounts of solvent (triolein) as in the formation from monolayers under the conditions of Montal and Mueller. When nearly pure GMO is present, the bulk phase is either a hexagonal or a cubic structure. Thus, if one wishes to understand the differences between bilayers formed by the two methods, one must look to the phase diagram for the system. Because the bulk phases from which the bilayers arise in the two cases are different, the resulting bilayers can be different.

4. Black Films as Hetergeneous Systems

It should be clear by now that the planar bilayer is the smallest part of a complex heterogeneous system. We cannot manipulate it for reconstiution studies unless the combined behavior of the several phases of the whole system is taken into account. To do a proper job, the physical chemistry of heterogeneous systems must be appreciated. Gibbs set down in 1876 the basic rules governing such sys-

tems (see Gibbs, 1961). Among the many contributions of his monumental work, his wonderfully elegant phase rule is perhaps the most important.

4.1. Equilibrium in Heterogeneous Systems: The Phase Rule

A heterogeneous system is one comprised of several different phases. If there are no curved interfaces between the various phases and the system is at equilibrium, the phase rule describes the relationship among the number of experimental variables (f, also called degrees of freedom or variance) that can be independently varied, the number of chemical components (c), the number of phases (p), and the number of intensive variables (i) such as temperature, pressure, and electric field. The great utility of the phase rule is that it forces one to think through the physical chemistry of a system in a systematic fashion. The phase rule is

$$f = i + c - p \tag{7}$$

This equation is a consequence of the condition for the equilibrium of a heterogeneous system, which is that the chemical potential (equation 2) of each component be the same in all phases. At equilibrium, in other words, there is no net difference in the partial molar free energy of a chemical species between any of the phases. The major result of this is that the concentrations of the components in the various phases are not independent of one another; the phase rule is the statement of this fact.

The simplest example of an application of the phase rule is the melting of ice at constant pressure. Here, $i = 1$ and $c = 1$, so that if liquid water and solid water coexist, $p = 2$, leading to $f = 0$. This means that the melting of ice is an isothermal event.

4.2. The Applicability of the Phase Rule to Black Films

Because the phase rule is true only at equilibrium, the first question that must be addressed is that of the equilibrium of the system. I noted earlier that the black film system is not inherently an equilibrium system because the free energy of the system is lowered if the membrane breaks. On the other hand, black films can exist for many hours without noticeable changes in properties because they reside in a local potential energy minimum at the higher energy state. We are thus at liberty to treat a black film as a system in a metastable or frozen equilibrium in the sense described by Guggenheim (1977). There are situations, however, in which the system is definitely not at equilibrium. These situations express themselves in two ways. One is that some measured parameter such as specific electrical capacitance may vary strongly with time or be nonreproducible. The other is that films may be difficult to form or may break prematurely. The causes of these frustrating events are discussed in the last section of this chapter.

Another aspect of equilibrium must also be considered because the hydro-

static pressure in the annulus is different from that in the film (section 3.1.3). The phase rule must be modified in such systems to include mechanical equilibrium as described by the Law of LaPlace (equation 3). However, one can show that for black films generated from bulk organic phases using reasonable aperture geometries, the curvature of annulus can be ignored.

Yet another issue concerns the surface phases of the system. They should be included in some way. The phase rule as applied to interfacial systems in general has been discussed elegantly by DeFay and Prigogine (1966). The interfaces of a system appear explicitly in the phase rule, it turns out, only if it is possible to have two or more different states of matter present in a given surface simultaneously. The classic example is the compression of a monolayer from the gaseous state to the liquid expanded state. Over some region of the compression, both phases are present at the air–water interface.

One can show that the complete phase rule for the black film system is

$$f = i + c - p + (s - n) \tag{8}$$

providing the curvature of the annulus can be ignored. In this equation, n is the number of types of surface, and s the total number of surface phases (e.g., liquid expanded, gaseous). The types of surface present in the black film system are the air–water interface, the annulus–water interface, and the bilayer, so that $n = 3$. If each surface type contains only one surface phase, then equation 8 reduces to equation 7. I consider next the application of the phase rules to the films formed by spreading a bulk solution across an aperture.

4.3. Phase Rule Analysis of the Black Lipid Membrane

Specifically, consider a black film formed from lipid dispersed in alkane spread across an aperture immersed in a NaCl solution. Assume that there is only one surface phase per surface so that equation 7 can be used. We may consider first the number of phases present. There are at least a bulk aqueous phase, a bulk air phase (we wish to include the air–water interface), and a bulk organic phase. The bulk organic phase may itself consist of more than one phase depending on how the lipid aggregates in it.

If one uses glycerol monooleate, which forms relatively small aggregates (Andrews *et al.*, 1970), then the activity of the alkane solvent depends rather strongly on the concentration of GMO (Waldbillig and Szabo, 1978) because the aggregates behave as a molecular species. It is generally agreed that under these circumstances, the organic phase should be considered as a single phase (Tolman, 1913). If one uses a phospholipid, on the other hand, very large aggregates result, which have little effect on the activity of the alkane (Fettiplace *et al.*, 1971). In this case, the organic phase should be considered as a two-phase system: bulk alkane plus bulk lipid aggregates. Thus, the bulk forming solution is counted as either one or two phases depending on the behavior of the hydrated lipid in the nonpolar solvent.

There are five components, which include water, NaCl, lipid, alkane, and "air." (Including air is somewhat arbitrary because for most purposes we can ignore it as it appears once in both c and p and thus cancels its presence. I include it for completeness.) The number of intensive variables will be three if one varies temperature, pressure, and the electric field across the system. In general, however, the pressure is constant, and I take $i = 2$. We thus find that either $f = 2 + 5 - 3 = 4$ (GMO) or $2 + 5 - 4 = 3$ (phospholipid). The experimental variables that must be controlled in the case of GMO ($f = 4$) are temperature, electric field, salt concentration, and GMO concentration. If the lipid forms a separate phase, as the phospholipids tend to, then the only variables ($f = 3$) are temperature, electric field, and NaCl concentration. Should the lipid undergo a thermal phase transition at some temperature so that two lipid phases are present (solidus and fluidus), then the number of degrees of freedom would be reduced by one as long as both phases are present. The reduction in f would most likely be revealed by the thermal behavior of the system.

In speaking of the concentrations of salt and lipid as independent variables, it should be kept in mind these statements are equivalent to saying that water and alkane activities are independent variables. Recall that one of the major methods of reconstitution involves osmotic gradients across vesicles and bilayers, suggesting that for some purposes it is more convenient to view the system from the point of view of water activity and its effect on the physical state of the lipid.

This analysis serves to illustrate the major factors affecting the properties of the black-film system. I have not, however, shown explicitly that the degrees of freedom of the system are reflected in the properties and organization of the bilayer. I illustrate this in the next section by considering the control of bilayer properties.

5. Controlling Bilayer Properties

The best method for assessing the state of a bilayer is to measure its specific electrical capacitance (Hanai *et al.*, 1964; White, 1970, 1977; Fettiplace *et al.*, 1971; Requena and Haydon, 1975b), and I thus use this parameter to describe how the bilayer structure changes under various circumstances. The measured specific capacitance of a bilayer, $C_m = C_T/A_m$, is obtained in my laboratory by measuring the total capacitance C_T by standard electrical methods (White and Blessum, 1975) and the membrane area A_m photographically (White, 1970). C_m is determined primarily by the thickness and dielectric coefficient of the hydrocarbon core. I refer to this as the geometric capacitance C_g given by

$$C_g = \epsilon_0 \epsilon_{hc}/d_{hc} \tag{9}$$

where ϵ_{hc} and d_{hc} are the dielectric coefficient and thickness of the hydrocarbon core of the bilayer. The thickness is proportional to the number of lipid molecules n_L per unit area of film and the number of solvent molecules n_S per unit area. Thus,

$$d_{hc} = n_L V_{ac} + n_S V_S \tag{10}$$

where V_{ac} and V_S are the combined molecular volumes of the two acyl chains of the lipid and the molecular volumes of the solvent molecules, respectively (Fettiplace *et al.*, 1971, 1975; White, 1977).

There can also be a contribution C_{dl} to the specific capacitance because of the electric double layers at the bilayer surface (Everitt and Haydon, 1968) and one contributed by the geometric capacitance of the polar headgroups (Coster and Smith, 1974). The latter is not observed, however, without heroic efforts, and I do not discuss this contribution here. The contribution of C_{dl} to C_m can be significant and is accounted for by the equation

$$1/C_m = (1/C_g) + (2/C_{dl}) \tag{11}$$

because the capacitances are in series. C_{dl} has been described in detail by Everitt and Haydon (1968). It is equivalent in form to equation 9 using the dielectric coefficient and Debye length of the double layer. The latter is a strong function of ionic strength and thus electrolyte concentration.

The following sections address in turn how changes in the state of the bulk phases and the intensive variables affect the structure of the bilayer. They focus primarily on black films formed from the monoglycerides dispersed in alkanes because these are the best-studied systems and the ones that are clearly capable of being in a frozen equilibrium.

5.1. The Aqueous Phase

Figure 10 shows the variation in C_m as a function of NaCl concentration for GMO–*n*-decane membranes (White, 1973). The capacitance depends on electrolyte concentration and, except for the 1 M point, shows the expected behavior based on equation 11 (solid curve). The more interesting question, and the one more relevant to the reconstitution issue, is that of the effect of water activity on bilayer structure. The 1 M point in Fig. 10 deviates significantly from the expected curve, and this is probably because of a water activity effect. Andrews *et al.* (1970) made a similar observation and showed the cmc of GMO in alkane solvents to be greatly affected by the salt concentration. This finding makes the important point that aqueous phase hydration affects the phase behavior of the bulk forming solution, which in turn affects the bilayer. It is important to keep in mind that the bilayer will always tend to come into equilibrium with the monolayers adsorbed at the annulus–water interface. If this adsorption changes, the concentration n_L (equation 10) of the lipid in the bilayer changes. Very few measurements of effects of water activity have been reported in the literature. This is unfortunate in face of reconstitution methods that utilize osmotic stress.

The monoglycerides are slightly soluble in the aqueous phase, and it is important that the aqueous phase be in saturation equilibrium with the bulk forming solutions. No rigorous attempt was made to do this (to my embarrassment!) in the data of Fig. 10. As a result, C_g is slightly high (0.41 $\mu F/cm^2$) compared to the

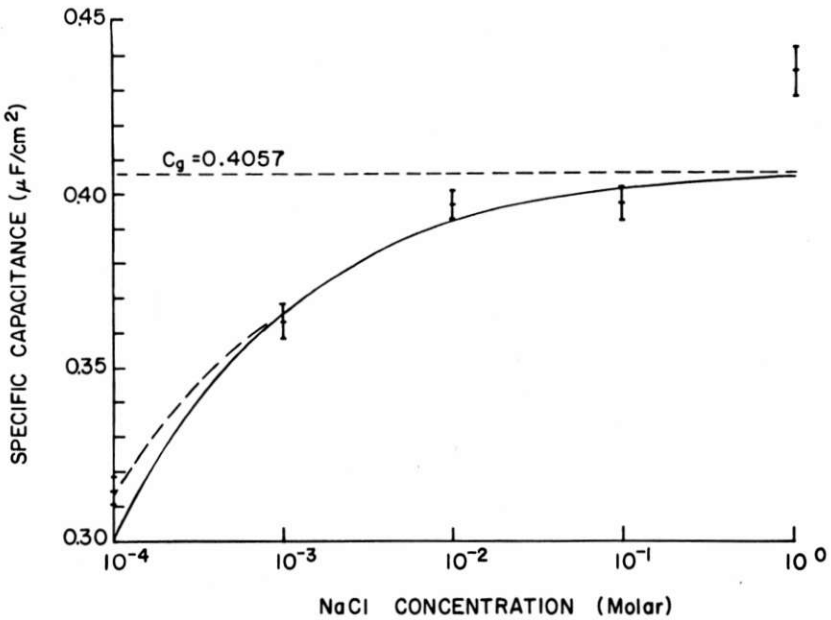


Figure 10. The specific capacitance (C_m) of bilayers formed from glycerol monooleate in NaCl solutions. the solid curve represents the theoretically expected curve (Everitt and Haydon, 1968) caused by the capacitance (C_{dl}) of the double layers at the surface of the membrane assuming the value of geometric capacitance (C_g) shown. The point in 1 M salt is high because of the effect of water activity on the state of the GMO in the annulus (Andrews *et al.*, 1970). (From White, 1973.)

correct value, 0.38 μF/cm^2, obtained when the aqueous phase has been properly equilibrated.

5.2. The Annulus Phase: Alkane Activity

Waldbillig and Szabo (1978) showed that the capacitance of films formed from GMO in *n*-decane depends strongly on GMO concentration, just as expected from the phase rule analysis described in Section 4.3. This observation confirms that the annulus in this case should be considered a single phase.

In addition to its dependence on the activity of the alkane in the annulus, the amount of alkane in the bilayer also depends on the structure of the alkane. Figure 11 shows the solubility of different alkanes in GMO bilayers as reflected by the thickness of GMO membranes (White, 1977). In all cases, the concentration of GMO was 10 mg/ml in the bulk solution so that the differences in solubility of the alkanes reflect primarily differences in molecular interactions with the bilayer itself. The solubility (thickness) depends on the size of the alkane, plotted here as the molecular volume. The figure makes another point that is important: the size parameter that determines solubility is the length of the alkane. This is il-

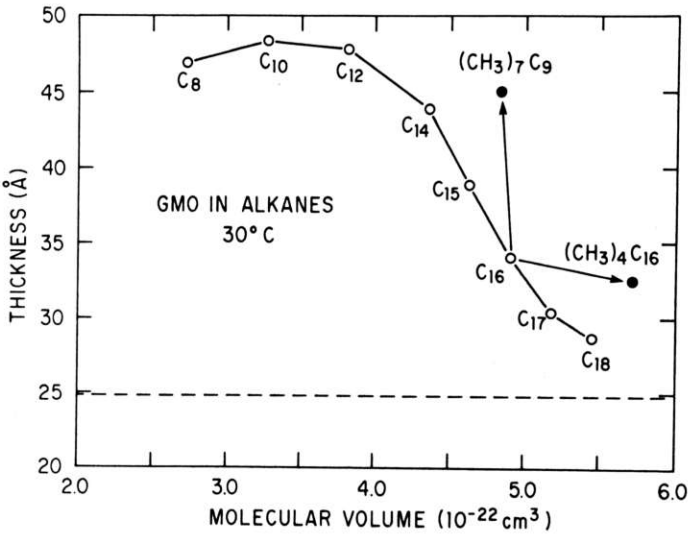


Figure 11. The thickness of planar bilayer membranes formed from glycerol monooleate (10 mg/ml) dispersed in various alkanes at 30°C as a function of alkane molecular volume. The shaded points for heptamethylnonane [labeled $(CH_3)_7C_9$] and tetramethylhexadecane [labeled $(CH_3)_4C_{16}$] demonstrate that the variable that determines alkane solubility in the bilayer is length. The dashed line represents the hypothetical solvent-free bilayer thickness. (From White, 1977.)

lustrated by the hexadecane analogues heptamethylnonane [labeled $(CH_3)_7C_9$] (which has the volume of hexadecane but the length of about decane) and tetramethylhexadecane [labeled $(CH_3)_4C_{16}$] {which has the volume of eicosane $[(CH_3)_2(CH_2)_{18}]$ but the length of hexadecane}.

An important implication of Fig. 11 is that with an alkane of sufficient length, one should be able to create a bilayer that contains little solvent by spreading from bulk solution. This possibility was borne out by forming membranes using squalene, which is about 34 carbons long. Solvent-free membranes with a thickness of about 25 Å (Fig. 11) should have a capacitance of about 0.78 $\mu F/cm^2$. Figure 12 shows the capacitance of GMO–squalene membranes as function of temperature (White, 1978). The expected solvent-free capacitance is observed, suggesting that little solvent is present. Waldbillig and Szabo (1979) extended this idea to the use of triglycerides, which also seem to be insoluble in the bilayer.

Also shown in Fig. 12 are measurements reported by Petersen (1983) for GMO–triolein. These membranes are somewhat thinner, suggesting that there may be very small amounts of squalene remaining in the squalene-formed membranes. This point is not entirely clear, however, because the interfacial adsorption from triolein and squalene solutions might be different, leading to different areas/molecule in the bilayer.

The phase rule analysis of Section 4.3 and the findings of Waldbillig and Szabo (1978) noted above show that the activity of the alkane in the annulus determines the concentration in the bilayer. Because Squalene is insoluble in the bilayer but

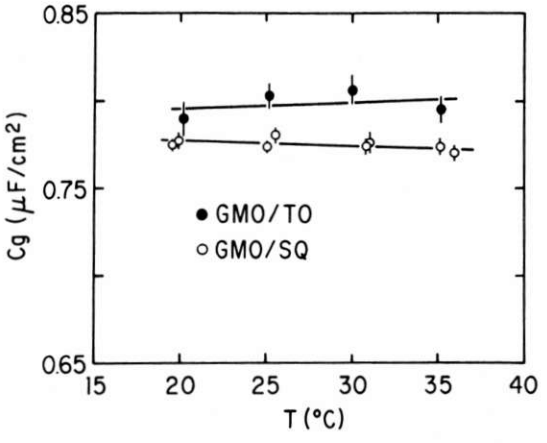


Figure 12. The specific capacitance of "solvent-free" bilayers formed from glycerol monooleate dispersed in squalene (SQ) or triolein (TO). The capacitances correspond closely to those expected of a membrane containing no solvent and having a thickness of 25 Å (Fig. 11). The TO curve is higher than the SQ curve because either some SQ remains in the bilayer or the adsorption of GMO at the SQ–water and TO–water interfaces of the annuli are different. The SQ data are from White (1978), and the TO data from Petersen (1983).

completely miscilbe with alkanes, it is possible to control arbitrarily the amount of alkane in the bilayer by mixing various mole fractions of squalene into the bulk forming solution (White, 1979; Needham and Haydon, 1983). Figure 13 shows the mole fraction of decane in bilayers formed from decane–squalene–GMO solutions with various mole fractions of decane (White, 1979). Here I have plotted mole

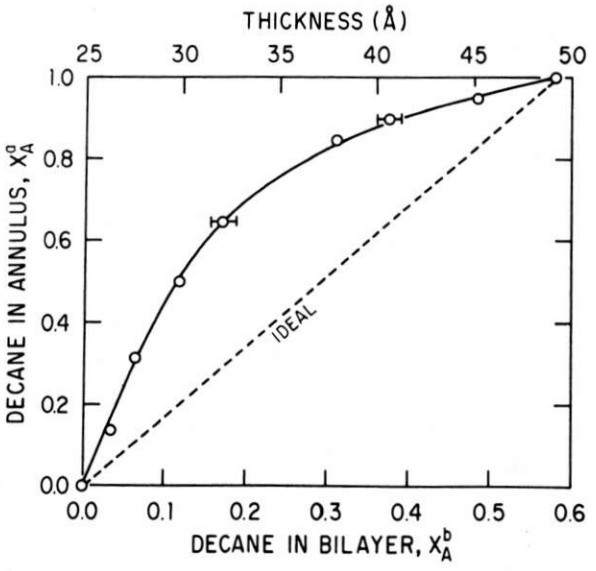


Figure 13. The amount of *n*-decane in GMO bilayers is determined by the activity of *n*-decane in the annulus. $X_A{}^b$ is the mole fraction of *n*-decane in the bilayer, and $X_A{}^a$ is the mole fraction in the annulus resulting from the mixing of squalene with the *n*-decane. The data are plotted in this way because $X_A{}^a$ will be proportional to the vapor pressure of the squalene–*n*-decane mixture; the nonideality of the interaction of the decane with bilayer is thus demonstrated. The deviation of the curve from the ideal case (dashed curve) is positive, indicating a tendency toward phase separation. Data based on those of White (1979).

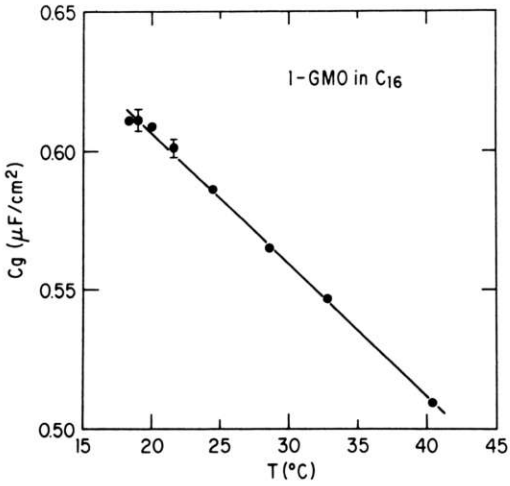


Figure 14. The specific geometric capacitance (C_g) of planar bilayers formed from GMO–*n*-hexadecane as a function of temperature. The solubility of *n*-hexadecane in the bilayer increases with temperature, causing the thickness of the bilayer to increase. Data taken from White (1976).

fraction X_A^a of alkane in annulus (proportional to the vapor pressure of the decane solution) against mole fraction X_A^b of alkane in bilayer to examine directly the ideality of the decane–bilayer solution. If the decane and bilayer mix ideally, then a Raoult's Law straight line relationship would result (dashed curve).

Figure 13 shows two things. First, the amount of decane in the film clearly depends on the activity of the decane in the annulus. Second, it forms a very nonideal solution in the bilayer, showing a large positive deviation from ideality. This nonideal behavior causes the thickness of the bilayer to be strongly dependent on electric field strength (*vide infra*).

The solubility of alkanes in black films can be strongly temperature dependent (White, 1970, 1976). Figure 14 shows the specific capacitance of GMO–hexadecane membranes as a function of temperature under equilibrium conditions. The amount of hexadecane in the bilayer depends very strongly on temperature, with solubility increasing as temperature increases. Because the bilayer is in saturation equilibrium with the annulus, one can determine the enthalpy and entropy of solution of the hexadecane in the bilayer from such measurements (White, 1976). The enthalpy of solution in this case is 3.8 ± 0.1 kcal/mol for the transfer of hexadecane from annulus to bilayer. This means that the binding energy of the hexadecane in the bulk phase of the annulus is considerably larger than in the bilayer. This is strong evidence that the interior of the bilayer is not equivalent to a bulk alkyl liquid. The main point is, however, that temperature can be an important experimental parameter as expected from the phase rule. Only if the enthalpy of solution of the alkane in the bilayer were zero or the alkane were insoluble in the bilayer would the capacitance change with temperature be small, as in Fig. 12.

A surprising result is that the annulus may be frozen without the bilayer breaking (White, 1974, 1975). Figure 15 shows measurements of C_g for GMO–hexadecane films as the temperature is decreased to less than the melting point (T_m = 16°C) of *n*-hexadecane. There is an abrupt increase in C_g at the T_m of

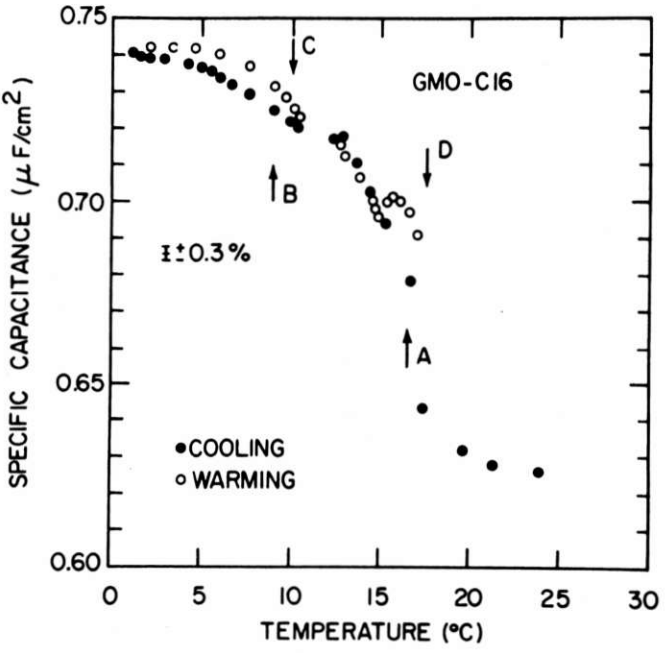


Figure 15. The temperature dependence of the specific geometric capacitance (C_g) of GMO–*n*-hexadecane membranes as the temperature is lowered through the melting point of *n*-hexadecane (16°C). The annulus freezes, but the membrane does not break. There is an abrupt increase in C_g at the freezing point because solid hexadecane is soluble in the bilayer. (From White, 1975.)

hexadecane, apparently because of the freezing out of the hexadecane from the bilayer. This means that hexadecane in the solid state is much less soluble in the bilayer than in the liquid state. It is of interest to apply the phase rule (equation 7) to this system. Assuming no electric field and constant pressure, $i = 1$. There are five components (air, water, NaCl, hexadecane, lipid) and, above T_m, three phases, so that $f = 3$ (water activity, alkane activity, and temperature). Below T_m, the annulus has solidified. If it remains a single phase (a GMO–hexadecane mixture), there are still $f = 3$ degrees of freedom, and the capacitance will depend on the composition of the annulus. If the annulus forms two phases (solid hexadecane and GMO do not mix), then $f = 2$, and C_g will be independent of alkane activity. No experiments have been done to test this issue.

5.3. The Annulus Phase: Lipid Activity

Little work on the effect of lipid-phase behavior on planar bilayer properties has been reported in the literature. The first that I am aware of is that of Krasne *et al.* (1971), but they worked with a complex solution of uncertain composition that was not characterized well physicochemically. Pagano *et al.* (1973) used

optical measurements of thickness to study membranes formed from hexadecane solutions containing GMO, glycerol monostearate (GMS), or 1:1 mixtures of GMO–GMS. The GMO and GMS in hexadecane in the presence of excess water have melting isotherms at T_m = 16°C and T_m = 55°C, respectively. When $T < T_m$ for GMS-formed membranes, there was a reversible increase in thickness that was consistent with the formation of a gel-state lipid in the bilayer, possibly accompanied by a phase separation of the hexadecane into microlenses. Interestingly, when the GMS–GMO mixture was used and $T < T_m$ for GMS, the bilayer seemed to consist of two phases, judging by the appearance of very bright spots on the surface of the membrane. These were interpreted as GMS crystalline bilayer regions dispersed in liquid–state GMO. The phase rule allows us to decide if this is reasonable.

The phase rule (equation 8) for the situation in which there is more than one surface phase on a given surface can be applied to the experiment. No electric field is present, and pressure is fixed, so $i = 1$. The components are air, water, GMO, GMS, hexadecane, and salt, making $c = 6$. The bulk phases are water, air, and the annulus phases. Above T_m for GMS, the annulus may be counted as a single phase, whereas below T_m it may be counted as two phases because of the precipitated GMS or possibly a GMS–GMO complex. Thus p is taken as 3 for $T > T_m$ and 4 for $T < T_m$. The types of surfaces are the air–water interface, the annulus–water interface, and the bilayer, so that $n = 3$. The major question concerns the number of surface phases. Can the bilayer have liquid GMO and gel GMS on the surface simultaneously?

Consider first the situation above T_m, where I assume $n = s$. There are four degrees of freedom ($f = 1 + 6 - 3 = 4$) which include temperature, NaCl concentration, and the GMS and GMO concentrations. Below T_m, if there are two surface phases on each surface, then $s = 6$ and $f = 1 + 6 - 4 - (6 - 3) = 0$. This could occur only if hexadecane activity ceased depending on GMO concentration in the still-liquid hexadecane and if water activity no longer had an effect on the GMO adsorption. These situations seem unlikely, and I conclude that there are not two surface phases on each surface. If, on the other hand, each surface has only one surface phase, and we attribute the bright spots to GMS crystallized into microlenses, then $s = 3$ and $f = 1 + 6 - 4 - (3 - 3) = 3$ degrees of freedom, which presumably would be temperature, NaCl concentration, and GMO concentration, the GMS having precipitated to form a bulk lipid phase of fixed activity. The point of this exercise has been to indicate that phase rule analysis allows one to think through a complex situation systematically. It further suggests the types of experiments that would have to be done to test various possibilities for the state of the bilayer.

Boheim *et al.* (1980) have done an experiment similar to that of Pagano *et al.* (1973) except they used a mixed-chain PC and formed the bilayers by the method of Montal and Mueller (1972). Hexadecane was used to pretreat the septum. They observed the thickness of the black film to increase 12% above the T_m of the lipid, indicating a structural change in the bilayer accompanying the change in the state of the lipid in the aqueous phase. The phase rule analysis: if T is not equal to T_m, $i = 1$, $c = 5$, $p = 4$ (bulk water, bulk lipid, air, annulus), and $n =$

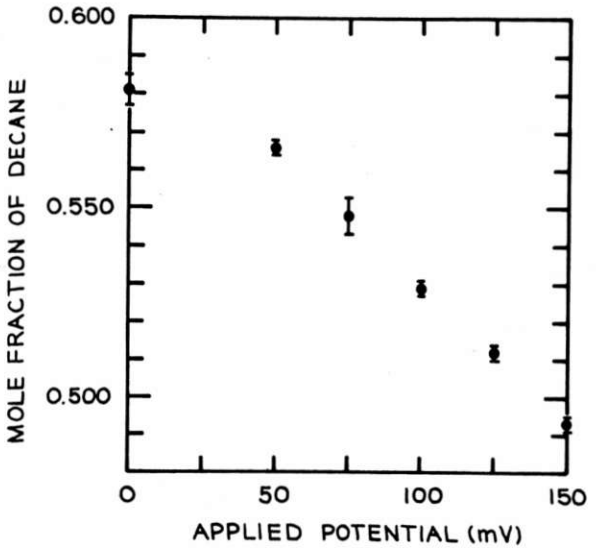


Figure 16. The amount of *n*-decane in a GMO bilayer as a function of the voltage across the membrane. The voltage creates a small pressure (~0.1 atm for 100 mV) in the bilayer, which raises the chemical potential of the decane relative to the annulus. Because the chemical potential of the alkane in the bilayer must be the same as in the annulus, there is a compensatory decrease in amount of decane in the membrane. A pressure of the size induced by the field could not have such a large effect unless the decane formed a very nonideal mixture with the bilayer. Figure 13 shows the nonideality. (From White, 1980.)

s, then $f = 1 + 5 - 4 = 2$, with the variables being water activity and temperature. During the melt there will be two lipid phases present simultaneously so that $p = 5$; the system is isothermal, and $f = 1$ (water activity). It is very interesting that Boheim *et al.* observed significant current fluctuations at the transition temperature as might be expected if two surface phases (gel and liquid crystalline) were present in the bilayer at T_m.

5.4. Electric Field Effects

It was observed very early in their history that electric fields affect C_T of black films (Rosen and Sutton, 1968). White (1970) demonstrated conclusively that this is a result of an increase in C_g resulting from a decrease in thickness. Andrews *et al.* (1970) showed that the extent of the effect depended on the alkane used, decane-formed films being much more sensitive than hexadecane formed films. Figure 16 shows the amount of decane in a GMO bilayer as a function of applied voltage V (White, 1980). This very strong effect is a result of the nonideal behavior of the decane in the bilayer (Fig. 13) (Andrews, 1970; White, 1979, 1980). The electric field causes an increase in pressure in both the annulus and the bilayer through electrostriction (equation 6), which is proportional to V^2. Because the

bilayer is very thin compared to the annulus, the pressure in the bilayer is orders of magnitude greater than that in the annulus. As a result, a 100 mV potential will increase the pressure in the bilayer by about 0.1 atm relative to the annulus, which therefore raises the chemical potential relative to the annulus. Because the annulus is so massive compared to the bilayer, the chemical potential of the bilayer must be reduced in some way in the face of the electric field. The reduction comes about by a decrease in the amount of decane in the film, since activity is proportional to concentration. The electric field can have this effect only if the alkane and the bilayer form a highly nonideal solution. If the decane formed an ideal mixture with the GMO of the bilayer, the effect shown in Fig. 16 would be an order of magnitude smaller. Note in Fig. 13 that bilayer thickness is extraordinarily sensitive to the mole fraction (activity) of the decane in the annulus.

The fact that hexadecane-formed bilayers are not very sensitive to voltage can be attributed to two factors. First, there is a much smaller amount of hexadecane in the membrane (Fig. 11). Second, hexadecane forms a more nearly ideal solution with the bilayer, i.e., it does not have the large positive deviation from ideality seen for decane (Fig. 13).

6. The Vagaries of Black Films: Nonequilibrium

The time dependence of the specific capacitance of black films provides a convenient criterion for equilibrium (metastable) because C_g depends strongly on composition. C_g can be measured with a precision of $\pm 0.3\%$ (White and Blessum, 1975); for films formed from monoglycerides and alkanes, C_g does not change within this limit for many hours at a time. Therefore, these films appear to be in equilibrium (metastable). That is not necessarily true for films formed from oxidized cholesterol (White, 1970) or diacylphospholipids (White and Thompson, 1973; Benz and Janko, 1976), whose specific capacitances can change drastically with time and be very nonreproducible.

The changes must occur because the alkane and/or lipid concentrations in the film change. The loss of alkane to the annulus and microlenses is partly responsible for the changes in oxidized cholesterol membranes because the application of a potential for a few minutes leads quickly to membranes with time-independent capacitances (White, 1970). The electric field hastens the movement of the solvent into the annulus and microlenses. Applied voltages have little effect, however, on the time dependence of diacylphospholipid capacitances (White and Thompson, 1973), and for these systems it is probably the adsorption of the lipid at the annulus–water interface and, consequently, the bilayer that is changing.

There are at least four circumstances under which adsorption is likely to change. First, the chemical structure of the lipid might change as a result of oxidation (Huang *et al.*, 1964). Second, the adsorption of the lipid at the interfaces is slow so that a long time is required for equilibrium. Johnson and Saunders (1973) have demonstrated that the interfacial tension at the interface between water and a cyclohexane–lecithin mixture changes from 40 dyne/cm to 2 dyne/cm over a 5-hr period. Third, the aqueous phase is not in equilibrium with the

bulk forming solution, so that the lipid desorbs into the aqueous phase, causing the interfacial concentration to be low compared to equilibrium. I indicated earlier that the capacitance of GMO–decane membrane is lowered from about 0.41 to 0.38 $\mu F/cm^2$ when care is taken to equilibrate the two phases. Solubility in the aqueous phase is not likely to be a problem with lecithin. However, one must consider the possibility that lecithin dispersed in excess water has a lower free energy than lecithin dispersed in alkane, leading to a steady loss of lipid to the creation of another phase. Fourth, the aggregated state of the lipid in the annulus may be changing with time because of changes in lipid hydration. Most of the changes observed for the diacylphospholipids (White and Thompson, 1973) are probably caused by this effect. Many of the frustrations of black films can be traced to these nonequilibrium conditions as discussed below.

A common scenario for the formation of black films from lecithins is as follows. A certain amount of lecithin is prepared by lyophilization from a choloform solution. Then alkane is added to make a 1% solution. However, unless the lipid is allowed to absorb some water from the atmosphere or a small amount of methanol is added, it will not disperse and remains as a lump in the bottom of the flask. Immediately after dispersal, the forming solution has the appearance and gross properties of pure alkane. However, if the "solution" is allowed to stand for several hours exposed to moist air (as during summer in Virginia, where I first observed this effect), it assumes the characteristics of a gel. If a small glass rod is dipped into the solution and withdrawn, long strands of it can be pulled from the surface several centimeters. The lecithin apparently continues to take on water and ultimately forms a microemulsion.

If one forms bilayers with the freshly formed solutions, their properties are likely to change drastically with time. Further, one's ability to form the bilayers is time dependent. Often when one attempts to form lecithin films from fresh solutions it is difficult to get them to span the aperture. Even if this step is accomplished, the films are likely to break before reaching the fully black state. If one waits for an hour, the bilayers form without problems, but the specific capacitance is likely to be highly variable. If several additional hours pass, the now well-hydrated forming solution becomes very gellike, and the membranes thin slowly and, once formed, have a stringy appearance with long strands of the annulus stretching into the bilayer region. At the same time the optical reflectance may be highly irregular as though there were patches of multilayers: end of experiment.

7. Epilogue

Finkelstein (1974) has related ". . . some of the folklore surrounding the technique of making lipid membranes, as revealed in both the written literature and the oral tradition." He further comments, ". . . when everything is working properly, bilayers that are stable indefinitely can be made at will. Like any technique, it has its own mysticism." I hope this chapter helps to remove black films from the realms of folklore and and mysticism. There is nothing unusual about

them as long as they are accepted as complex heterogeneous systems that operate according to the standard rules of physical chemistry. Biological systems must also obey these rules. In the process of learning how to reconstitute channels into black films whose composition we can control, I suspect we will also learn a good deal about how biological systems use the rules of physical chemistry to accomplish the same goal.

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