Properties of Lipid Bilayers at a Water-Water Interface

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Abstract

This paper is essentially a review of the work on black hydrocarbon films in aqueous media which has been carried out by the author and his colleagues during the last few years.

The theory of the formation and stability of the films is discussed in terms of the structure and physical properties of the constituent molecules. Particular consideration is given to the adsorption of the stabilizing molecule and the metastable equilibrium of the resultant thin film. The various systems which have been examined experimentally are described. The interrelation of the film capacitances, thicknesses, and compositions is discussed in the light of the theoretical expectations.

The films are permeable to water although the measurement of their permeability is complicated by the difficulty of stirring the boundary layers of the aqueous solutions. There is a discussion of the progress in this problem and the interpretation of the permeability measurements in terms of the structure and composition of the films.

Finally some conditions under which films become strongly conducting are described.

1. Introduction

IT IS NOW WELL KNOWN that solutions of certain lipids in some nonpolar solvents may be extended under aqueous solutions to give optically black thin films (1–5). The phospholipids have been the most widely used lipid solutes, and the solvents have ranged from complicated mixtures of solvents, some of which were very soluble in water (2,4), to pure aliphatic hydrocarbons (3,5), which were effectively insoluble. In the latter type of system, at least, it has become clear that, in general character, the thin films which are formed bear a close resemblance to aqueous soap films. Thus the films are formed from a two (or more)-component liquid mixture, strong adsorption of the solute occurs at the interfaces, the metastable thin film eventually formed is in thermodynamic equilibrium with its borders and the adjacent bulk phases. The film also contains two (or more) components although their mole ratio may well be quite different from that in the bulk lipid solution.

The electrical capacitance of these films is readily measured and can be used to give an apparently accurate estimate of the thickness of their hydrocarbon regions. The electrical resistance of the films is normally high although interaction with certain types of polypeptides can produce an enormous reduction of resistance. Regardless of their electrical resistance, the films are permeable to water to a readily measurable extent.

These topics are discussed in more detail in the following sections. Apart from reviewing published work, some as yet unpublished material is presented. In Section 5 on the water permeability the new results mentioned are those of W. R. Redwood, and Section 6 on polypeptides contains results obtained by J. L. Taylor and, more recently, by C. T. Everitt.

2. Film Stability

The factors which may be important in causing the thinning of liquid films have been described in some detail by Mysels, Shinoda, and Frankel (6). The three most generally encountered are gravity in association with capillary suction from the borders of the film, London-van der Waals forces, and the evaporation or dissolution of the film liquid in the sandwiching bulk phases. If these three types of influence are not opposed, one should not expect to obtain a stable, or metastable, thin film. As the two surfaces of the film approach each other however, the interaction of the two associated surface phases may produce a positive, free-energy change which tends to stabilize the film. This interaction can conveniently be examined by means of the general thermodynamic equation[1]

$$F-F^* = 2\Omega(\gamma-\gamma^*)_{n_i} + \Sigma\, n_i\,(\mu_i-\mu_i^*) \qquad [1]$$

where F is the Helmholtz free-energy of the system, Ω the area of one side of the film, γ the interfacial tension in the film, n_i the number of molecules of species "i" in the system, and μ_i their chemical potential. The symbols with an asterisk denote the value before, and those without, the value after the interaction. For most systems, and certainly for those of interest in the present paper, the second term on the right-hand side of Equation 1 is small and the equation reduces to

$$F-F^* = 2\Omega(\gamma-\gamma^*)_{\mu^*} \qquad [2]$$

The surface pressure of the molecules adsorbed at the film interfaces can be written as

$$\pi = \gamma_o-\gamma \qquad [3]$$

where γ_o is the tension of the solvent interface when there is no adsorption; γ_o will be assumed to be independent of film thickness. When ions are adsorbed, it is convenient to regard π as made up of two components,

$$\pi = \pi_u + \pi_e \qquad [4]$$

where π_e is that part of the surface pressure owing to the mean electrostatic surface potential, relative to zero in the bulk hydrocarbon phase, generated by the ion adsorption, and π_u is the surface pressure which the ions would exert if they were uncharged, together with an increment owing to image effects of the ions (8). This latter effect is fairly small and will be neglected. Equation 2 can now be rewritten as

$$F-F^* = 2\Omega\{(\pi_u^*-\pi_u) + (\pi_e^*-\pi_e)\}_{\mu_i^*} \qquad [5]$$

The second term in the bracket arises from the over-

[1] It must be emphasized that as F-F* in Equation 1 is the positive or "repulsive" part only of the free energy of interaction across the film, the interfacial tensions (δ) are not actual tensions; they are the tensions which would obtain if the tension of the pure solvent (δ_o) were independent of film thickness. In fact, owing to the existence of the attractive London-van der Waals forces the tension of the pure solvent is a function of film thickness, and in the present treatment this has been taken into account by Equation 9. The interfacial pressures (π) may, however, be regarded as actual pressures as they are differences between two tensions.

Hydrostatic pressure terms have been considered separately.

lapping of the diffuse ionic double layers of the two surfaces and can be estimated from the theory of Verwey and Overbeek (9). The first term is perhaps most readily pictured as arising from steric interference between the two surface phases. In attempts to estimate both terms it will be assumed that the thin film is in thermodynamic equilibrium with its surroundings. Experimental evidence in several different types of film supports this assumption.

The surface ion density because of adsorption will be assumed to be large compared with the diffuse charge on one side of the interface when ion adsorption is zero; following the assumption of equilibrium with adjacent bulk phases, the diffuse double layer overlap will be assumed to take place at a constant surface potential (of 252 mv) relative to the bulk of the film-forming phase. A typical value for the specific conductivity of a lipid-in-hydrocarbon solution from which a film may be formed is $\sim 10^{-10}\Omega^{-1}cm^{-1}$. If the whole of this conductivity is taken to be owing to uniunivalent ions (a doubtful but conservative assumption), one can estimate an electrolyte concentration of $\sim 10^{-9}$ mole/l. If the dielectric constant is then assumed to be 2.1, one can calculate the free-energy change for various film thicknesses from the tables given by Verwey and Overbeek.

The magnitude of the first, or nonionic, term is more difficult to assess as it is necessary to have a detailed knowledge of the adsorption of the lipid solute molecules at the interfaces. To a first approximation, adsorbed long-chain ions at hydrocarbon/water interfaces obey a two dimensional equation of state of the form (8,10):

$$(\pi - \pi_e)(A - A_o) = kT \qquad [6]$$

where A is the interfacial area per adsorbed molecule and A_o is the value of A which corresponds to $(\pi - \pi_e) = \infty$, k is the Boltzmann constant, and T is the absolute temperature. If the molecules have no net charge, $\pi_e = 0$, and Equation 6 reduces to

$$\pi_{(u)}(A - A_o) = kT \qquad [7]$$

By the use of statistical thermodynamics it can be shown that if an adsorbed monolayer obeys Equation 7, the adsorption of the molecules is determined by the Equation 11

$$\frac{A_o}{A - A_o} \exp \frac{A_o}{A - A_o} = a \exp\left(\frac{-\Delta\mu^\circ}{kT} + 1\right) \qquad [8]$$

where a is the activity of the solute in one of the bulk liquid phases, and $\Delta\mu^\circ$ is the standard chemical potential change for the transfer of one molecule of solute from the liquid phase to the interface. An estimation of $(\pi_u^* - \pi_u)$ for a system in which Equations 7 and 8 were applicable has been attempted along the following lines (12). The data on the adsorption of normal-chain lipid molecules at hydrocarbon/water interfaces suggest that in many systems the adsorbed molecules are oriented normally to the interface and that the chains can be regarded statistically as rigid rods.

Such data as exist on the adsorption of lipids which can stabilize black hydrocarbon films suggest that in the film these molecules are not close-packed. Hence, when the film thickness becomes less than twice the length of the lipid solute molecules, there is a tendency for interspersion of the chains of the opposing monolayers to take place. Such an interspersion takes up sites which would otherwise be available for the chains of the molecules originally present. This loss of adsorption sites produces, through Equations 7 and 8, a simultaneous partial desorption of the lipid molecules and fall in surface pressure. The free energy of the interaction is thus positive (from Equations 1 or 5), and the two opposing monolayers tend to repel each other. A serious difficulty arises in trying to relate the extent of overlap of the two monolayers to the loss of adsorption sites. However, in practice, according to a model based on Equations 7 and 8, the dependence of repulsive free-energy on chain overlap is so strong that it is only for very weak adsorption that the repulsion is not effectively infinite as soon as the monolayers touch. In other words, the finer details of the model are not of great importance, provided one is dealing with solute molecules which have more than about 12 carbon atoms in their chains and form monolayers from dilute solutions that are more than about half close-packed.

Potential energy curves for hydrocarbon films as a function of their thickness are shown in Fig. 1. The London-van der Waals energy was calculated from the expression [9]

$$V_A = \frac{A_H}{48\pi d^2} \times \text{retardation correction} \qquad [9]$$

where d is half the thickness of the film and A_H is the Hamaker constant for hydrocarbon and water. This was taken to be 10^{-13} erg (13). The calculation of the ionic repulsion has already been mentioned. For the steric repulsion it has been arbitrarily assumed that the monolayers make contact with each other at a film thickness of 50 Å. Finally a correction for the influence of gravity on the film material has been included although this is significant only in thick films. In the range of thicknesses where the films would be optically black, it can be seen that there is only one minimum, which is formed by the abrupt onset of the chain interaction. According to the above theory, this minimum is likely to occur when the thickness of the hydrocarbon part of the film is approximately twice the length of the solute chains. The electrical free-energy as it has been calculated, is not insignificant but nevertheless changes so slowly with film thickness that it should not affect the behavior of the very thin films. It is interesting to note however that the electrical repulsion is of such long range that in combination with the gravitational term a shallow minimum is produced at a thickness of about 0.5 μ (Fig. 1, inset).[2]

Some of the requirements for stable black film formation can now be summarized in the light of Fig. 1 and the background theory. The solute molecules in the hydrocarbon must be strongly adsorbed, that is, the value of $\Delta\mu^\circ$ must be sufficiently large and negative for A to be of the same order of magnitude as A_o at an accessible value of the activity a. For long-chain molecules which are adsorbing from hydrocarbon to water the value of $\Delta\mu^\circ$ is determined chiefly

[2] An inconsistency in the above arguments arises because, on the one hand, it has been assumed that the lipid molecules ionize and form electrical double layers and, on the other, that $(\pi_u^* - \pi_u)$ can be calculated from Equations 7 and 8, which are for nonionized molecules. This inconsistency has been disregarded for simplicity and for lack of concern with any particular system.

A further point arises from the assumption that the film is in equilibrium with the adjacent bulk phases. If the film-forming lipid solution contains substances which are appreciably soluble in water and with which the adjacent bulk aqueous phases are not in equilibrium, diffusion out of the film will occur. Under these conditions the films may well become thinner.

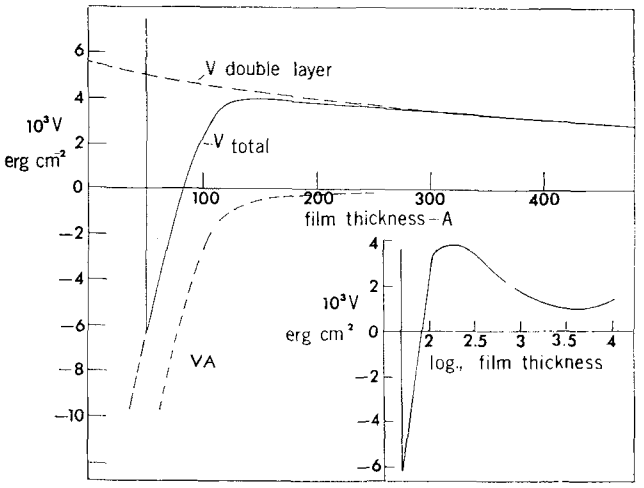

FIG. 1. The energetics of lipid bilayer formation. Estimates of the electric double-layer, London–van der Waals, steric repulsion and gravitational free energies have been combined to give an over-all curve of free energy as a function of thickness.

by the size and polarity of the head group as this is the only part of the molecule which actually transfers from one phase to the other. Lipids with large polar head groups therefore promote stable film formation. Yet such groups tend to diminish the solubility of the solute in the hydrocarbon and so lower the maximum value of a.

It is also probable, although few data are available, that characteristics which favor strong adsorption also favor association and micelle formation in the hydrocarbon. In this way also, the maximum activity of the single molecules may be limited. The nature of the solvent is likely to be of relatively minor importance. Aside from the question of dissolution or evaporation from the film (which can be an important reason for instability) it is noted that, in Equations 7 and 8, which apply to strongly adsorbed solutes, there is no term explicitly for the solvent. Furthermore the replacement of a nonpolar solvent, such as an aliphatic hydrocarbon, by a more polar one, such as chloroform, may increase the solubility of the lipid solute and perhaps raise the maximum value of a, but at the same time it is almost certain that $\Delta\mu^\circ$ will become less negative. The adsorption may therefore not change a great deal. Whether or not the solute molecules ionize at the interface is (Fig. 1) evidently irrelevant to the stability, provided that the ion concentration in the hydrocarbon is low.[1]

From the estimation of the steric repulsion generated by the chain interaction (12) it might seem that the black films should be indefinitely stable. It is well known that this is not so. In fact, films of hydrocarbon in water seem somewhat less stable than many types of soap film in air. The reason for this is not yet clear. It may be that the model which has been used is seriously inapplicable and the overlap free-energy, a large over-estimate. Or it may be that other factors are involved. It is clear that if the surface tension in the film is finite, complete collapse will occur if a hole of greater than a critical size is formed. De Vries has calculated, on the basis of a simple model, that the activation energy for formation of the critical sized hole is (14)

$$E_{act.} = 11.7d^2\gamma \qquad [10]$$

For a film of thickness 50 Å and tension 1 dyne/cm, $E_{act.} = 7.3 \times 10^{-13}$ erg or about 18kT. The structure is therefore not obviously unstable from this point of view. A further possible source of instability may lie in a tendency for the film liquid to de-wet, and become detached from, the supporting material, usually polyethylene (2,4) or, better, polytetrafluorethylene (3,5). There are indications however that this latter source of instability is not the major one. Thus films can be formed in such a way that they do not have supporting frames, by allowing droplets of the aqueous phase under the lipid solution to rest gently against an interface with a second aqueous phase (Fig. 2). Black films formed in this way, even though they may be considerably smaller than those made on solid supports, are not obviously more stable. In conclusion, then, the precise reasons why black hydrocarbon films in aqueous solutions are relatively unstable, especially when they are composed of two pure components, are not at present very clear.

3. Electrical Capacitance

The accurate measurement of film capacitance is probably best achieved by a.c. methods, which have been described previously (5). In order to interpret the capacitance so as to give further information about the film, a physical model is required for the film and its surroundings.

From general surface chemical considerations there seems little doubt that the black film consists of a thin sheet of lipoidal material at the surfaces of which the stabilizing polar molecules are adsorbed so that their polar groups are in the adjacent aqueous solutions and their chains extend into the lipid phase. One can envisage the films therefore as consisting essentially of a layer of hydrocarbon sandwiched between regions consisting of a mixture of polar head groups and aqueous solution. If the polar head groups are ionized, then it is probable that ionic double layers will be present at the film surfaces. Even if the polar heads are not ionized, adsorption of ions from the aqueous solution may produce double layers. The

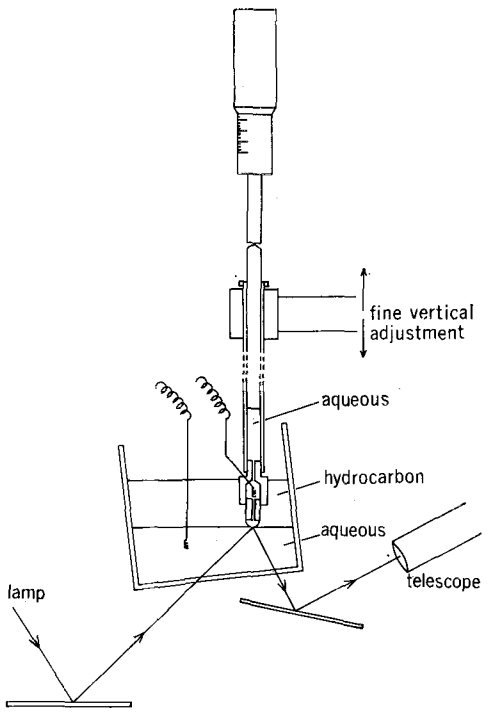

FIG. 2. The formation of "unsupported" black lipid films under conditions where area and electrical properties may be examined.

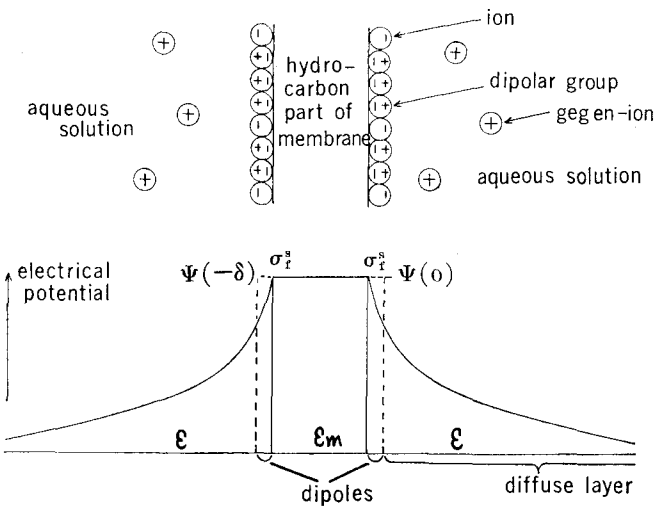

FIG. 3. The model for the capacitance calculations.

model is illustrated in Fig. 3. The behavior of the system in an alternating field can be analyzed in terms of the equations for a three-layer dielectric consisting of hydrocarbon and polar regions and the aqueous phase (15). It has been shown that, apart from their role in originating double layers, the polar group region has too high a capacitance and too low a resistance compared with that of the hydrocarbon layer (taking optical data to give a rough indication of the thickness of the latter) to contribute significantly to the over-all electrical properties (15). In an earlier treatment the electrical double-layer capacitance was ignored because rough calculations showed that, under normal conditions of electrolyte concentration, this was very large compared with that of the hydrocarbon. However the electrical double layers cannot, in general, be neglected. For an infinite insulating plane sheet carrying an equal fixed surface-charge density at each of its surfaces, immersed in an aqueous solution, the capacitance of the sheet in a normally oriented field can be shown to be given by the equations (the derivation will be given elsewhere):

$$CV = \frac{-\epsilon\kappa kT}{\Psi\pi ze}\left\{2\left(\cosh\frac{ze}{kT}\left[\Psi(o)-\frac{V}{2}\right]-1\right)\right\}^{1/2} + \sigma_f^s \qquad [11]$$

$$\frac{\epsilon_m}{\delta}\left[\Psi(o)-\Psi(-\delta)\right] = \frac{-\epsilon\kappa kT}{ze}\left\{2\left(\cosh\frac{ze}{kT}\left[\Psi(o)-\frac{V}{2}\right]-1\right)\right\}^{1/2} + \Psi\pi\sigma_f^s \qquad [12]$$

$$\frac{\epsilon_m}{\delta}\left[\Psi(o)-\Psi(-\delta)\right] = \frac{\epsilon\kappa kT}{ze}\left\{2\left(\cosh\frac{ze}{kT}\left[\Psi(-\delta)+\frac{V}{2}\right]-1\right)\right\}^{1/2} - \Psi\pi\sigma_f^s \qquad [13]$$

C is the capacitance of the sheet per unit area
V is the applied potential difference
ϵ is the dielectric constant of the aqueous solution, which is assumed to remain constant up to the phase boundary.
ϵ_m is the dielectric constant of the insulating sheet
σ_f^s is the density of the fixed surface charge
δ is the thickness of the insulating sheet.

The $\Psi(o)$ and $\Psi(\delta-)$ are the potentials at the two surfaces of the sheet, and κ is the Debye-Hückel reciprocal length parameter for the aqueous solution $\kappa = \sqrt{\frac{8\pi z^2e^2n_o}{\epsilon kT}}$ where n_o is the number of ions of one species per cm^3 of solution far from the membrane surface).

In deriving Equations 11 to 13, it has been assumed that the fixed charge of σ_f^s is not a function of V. It has been assumed that the Poisson-Boltzmann equation for point ions is applicable and that the electrolyte is symmetrical with ions of valence z. Image effects have been neglected. In this form the equations hold only when $(\partial\Psi/\partial x)$ at $x = 0$ is negative.

For practical purposes the most important aspect of the equations is that, for electrolyte concentrations of greater than about 10^{-3} mole/1, they reduce effectively to the expression,

$$C = \frac{\epsilon_m}{\Psi\pi\delta} \qquad [14]$$

This is because under these circumstances the electrical double-layer capacitances become so large that, when added in series with the geometrical capacitance of the insulating sheet, they make an insignificant contribution. At lower concentrations however, the diffuse double-layer capacitances are lower and cannot be neglected. Their effect is to lower the over-all capacitance. The special case of zero fixed surface-charge has been examined by Läuger et al. (16). Although this particular system is almost unrealizable in practice, owing to ion adsorption, it is simpler to analyze. In fact, under these conditions, it turns out that the geometrical and double-layer capacitances are reciprocally additive. When the fixed charge density is not zero, these capacitances are reciprocally additive only when the quantities $[\Psi(o) - \frac{V}{2}]$ and $[\Psi(-\delta) + \frac{V}{2}]$ are sufficiently smaller than kT/ze for the exponential factors to be approximated to the first two terms. It is only under these conditions also that the membrane capacitance is independent of the fixed charge density.

The main features of the model and its analysis have been confirmed by experiment although so far only results in relatively high electrolyte solutions have been reported. The film capacitances are proportional to area and above 10^{-3} mole/1 are independent of electrolyte concentration, at least up to 0.1 M (5,22). As will be seen below, Equation 14 gives reasonable interpretations of the measured capacitances. The fact that the polar groups and double layers can be disregarded in relatively high electrolyte concentrations means that the system of aqueous solution and membrane should behave as a two-layer dielectric and should therefore exhibit only one relaxation time. This is, in fact, what is found. The frequency dependence of a system consisting of an egg phosphatidyl choline and n-decane membrane in various sodium chloride solutions is shown in Fig. 4. The relaxation frequency increases with the concentration of NaCl accurately in agreement with theory (5). Each curve can be shown to be consistent, to within experimental error, with only one relaxation time. This is illustrated, for instance, by the semicircular Cole-Cole plots (Fig. 5). The capacitance of the membrane itself is thus independent of frequency. By the use of transient in addition to sine wave techniques, this has been established for the range $\sim 5 \times 10^{-3}$ to 10^7 c/s (15).

Under conditions in which Equation 14 is valid, the measurement of a black film capacitance and a

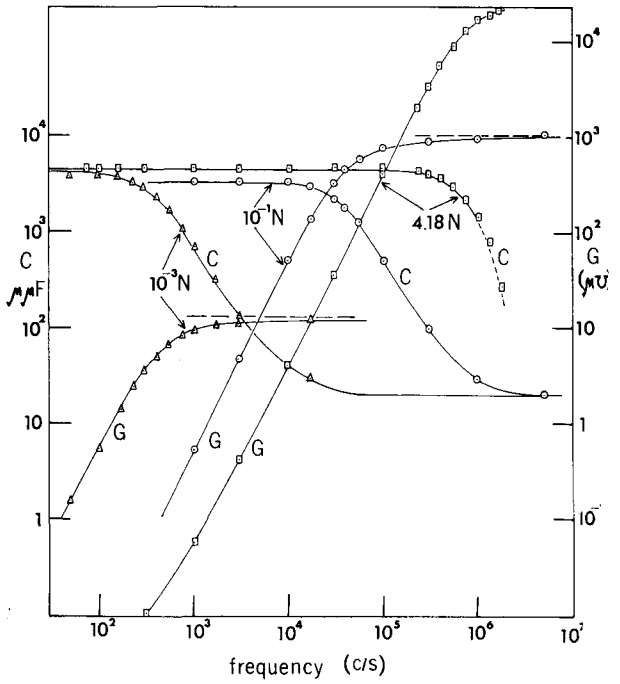

FIG. 4. Capacitances and conductances of the film and Teflon pot in NaCl solutions as a function of frequency. △ 10^{-3}N; ⊙; 10^{-1}N, ⊡ 4.18N (5).

knowledge of the dielectric constant of the insulating region enable one to determine the thickness of this region. Consideration of the model leads to the conclusion that only the hydrocarbon region is likely to be insulating and that this region is likely to begin at the point where the chains of lipid molecules join the polar head group. As has been seen in Section 2, this region is evidently composed of the chains of the stabilizing lipid molecules and of the hydrocarbon solvent. Data on the electrical resistance of the films (Section 4) suggest that this region contains little polar material. The comparison with soap micelles in which the partial molar volume of the methylene group is close to that for liquid hydrocarbons supports this conclusion (22,31). The appropriate dielectric constant to assume therefore seems to be that for the appropriate liquid hydrocarbon or hydrocarbon mixture. For linear aliphatic hydrocarbons this must be within a few per cent of 2.0. If cyclic material, such as in cholesterol, is present, the position is less clear and the appropriate value may well be rather higher.

The thickness of the hydrocarbon region of black films composed of normal-chain hydrocarbon and stabilized by lipids of different chain-lengths has been estimated by the above procedure. The resulting film thicknesses were a linear function of the number of carbon atoms in the stabilizer chain and the line passed through the origin (Fig. 6). The actual thickness values were within approximately an angstrom unit of twice the chain length of the fully extended stabilizer chains, thus producing a picture satisfactorily in agreement with the theoretical considerations of Section 2.

From a knowledge of the thickness of the hydrocarbon region of the film, an estimate of the composition of this region can be made by the use of Equations 6 or 7. For a nonionizing stabilizer molecule the interfacial area per molecule for a given surface pressure should be equal to, or less than, the value calculable from Equation 7, according to whether or not cohesion between the stabilizer molecules is significant. At hydrocarbon-water interfaces this factor is often small and will be disregarded for present purposes. An initial attempt to use this approach (5) was based on a surface pressure of 25.7 dyne/cm for egg phosphatidyl choline in n-decane against

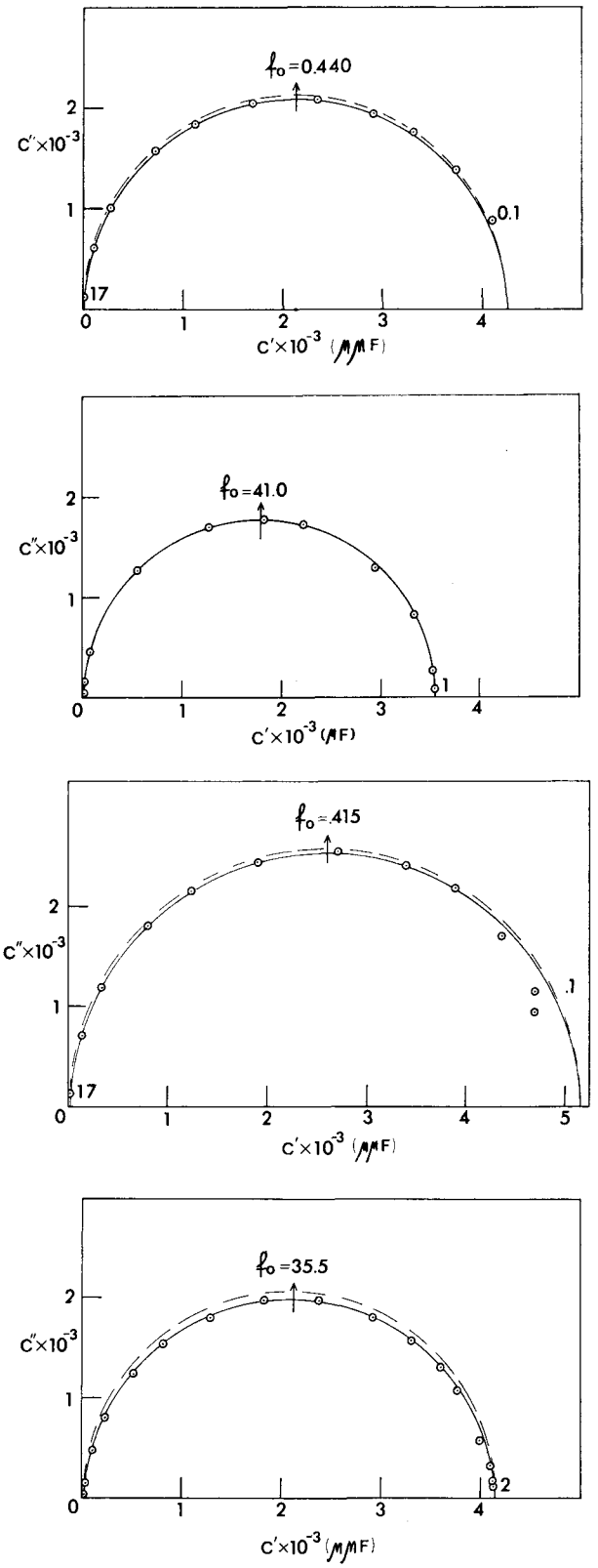

FIG. 5. The Cole-Cole plots for the film and Teflon pot in electrolyte for constant (but different) black film area in each case. (a) 10^{-3} N–NaCl; (b) 10^{-1} N–NaCl; (c) 10^{-3} N–$CaCl_2$; (d) 10^{-1} N–$CaCl_2$. The corresponding frequencies are shown on the graphs. The dashed line indicates the true semicircle (5).

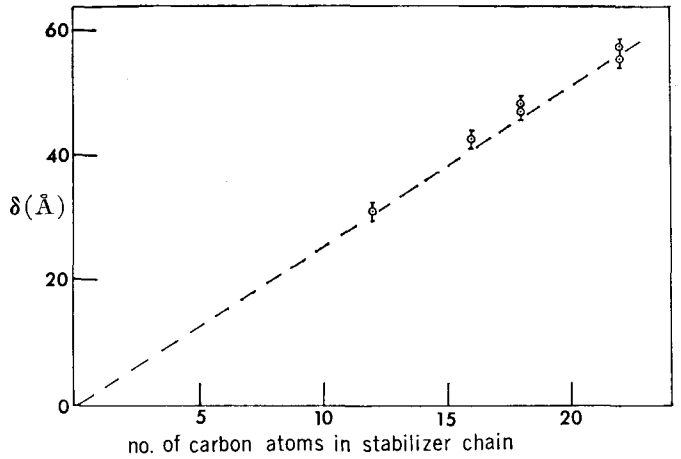

FIG. 6. The thickness, δ, of the hydrocarbon region of the black films as a function of stabilizer chain-length. The dashed line corresponds to the thickness of twice the chain length of the stabilizer (12).

N/10 NaCl. Subsequent measurements (unpublished data) suggest that the true value is nearer 50 dyne/cm, corresponding to an interfacial tension of 1–2 dyne/cm. Taking A_0 to be 58 Å^2 per molecule, the former pressure gives A = 73.7 and the latter A = 66.1 Å^2 per molecule. The volume fraction of n-decane in the film can then be estimated as 0.50 and 0.46 respectively. The neglect of the intermolecular attraction between the lecithin molecules makes these estimates upper limits; nevertheless one sees that there could be 3 to 3.5 n-decane molecules for every lecithin molecule.

The capacitance of a black film of n-decane stabilized by oleyl chain molecules is 0.39 μF/cm^2. This value is independent of temperature between 20C and 37C and, provided the aqueous phase is saturated with the lipid solution, is independent of the chain length of the hydrocarbon solvent from n-pentane to n-tetradecane (12). Phospholipids extracted from natural sources usually contain a mixture of chain lengths which can give somewhat different black film capacitances, depending on their composition. The purified egg phosphatidyl choline, used in the author's laboratory and supplied by R. M. C. Dawson, usually gave ~0.38 μF/cm^2. On the addition of cholesterol to the phospholipid solution, the films had capacitances which increased with cholesterol concentration up to a value of ~0.6 μF/cm^2. The precise result was somewhat dependent on the specimen of phospholipid which was used. This high capacitance was interpreted as partly owing to the thinning of the films on incorporation of cholesterol and partly to the probable higher dielectric constant of the cyclic hydrocarbon in the cholesterol (17). The adsorption of several different proteins on the surfaces of the films did not affect the capacitance at any frequency (17). The physical interpretation of this result is probably that the protein merely forms, in effect, an extension of the highly conducting polar group region.

A tacit assumption in the use of Equation 14 to give film thicknesses is that the field which must be applied in order to measure the capacitance does not itself perturb the film. In principle this assumption is invalid, as a field normal to the film surface aids the London-van der Waals forces and tends to compress the films to less than their normal equilibrium thickness (18,19). This effect seems to be detectable experimentally but is fairly small, and different types of film respond to different extents. The effect tends to be larger in high electrolyte concentrations. This can be seen from the results of Hanai et al. (5), where, under an applied a.c. potential of ~40mV, the capacitance per unit area of an egg phosphatidyl choline and n-decane black film in 4.18 N NaCl is approximately 5% higher than in 0.1 N or 0.001 N. In the latter concentrations the effect seems immeasurably small. Other workers have drawn attention to this phenomenon (16), but no data have been published. It is important to distinguish between a genuine compression and the increase in area in a field noted by Babakov et al. (18), which also yields an increased capacitance.

The comparison of thickness measurements by electrical (5,12) and optical (20,21) means can only be achieved if an estimated thickness of the polar group regions is added to the former values. According to the type of molecules involved, this can usually be done to an accuracy of two or three angstrom units. Thus if one assumes 10 Å for the thickness of the glyceryl phosphatidyl choline group and adds 20 Å to the thickness of the hydrocarbon region of egg lecithin films given by Hanai et al. (5) (~48 Å), one obtains a value close to the optical result (20). The two methods are thus to a large extent complementary in that one gives the thickness of the hydrocarbon region and the other gives (in the first instance, at least) the combined thickness of hydrocarbon and polar regions. The electrical capacitance is a stable, reproducible, and easily measured quantity, provided that the films are in equilibrium with their surroundings and that no diffusion of solvent or solute is occurring. A particularly valuable use of the capacitance is in accurate film area estimation; once a capacitance per-unit-area for a particular type of film is known, a capacitance measurement immediately gives the area of black film present. Visual inspection is often difficult unless the films have a symmetrical form and even then involves considerable error unless many measurements are made.

4. Electrical Conductance

The measurement of the conductance of black film is probably still best carried out by forming them over a small hole in a cylinder of polytetrafluorethylene (3,5). Although a conductance is easy to measure under these conditions, a value truly representative of the black part of the film is extraordinarily difficult to achieve. It is obvious that, owing to the nature of the system (a poor conductor in a good one), imperfections in the films or their supports are likely to yield high, rather than low, over-all conductances. Thus, in a sequence of films, which will usually give a succession of somewhat different apparent specific conductances, it is probable that only the lowest value has any chance of being a correct result. It is still necessary to demonstrate that the low conductance in question is proportional to the area of the black film. Without this test the conductance must be regarded with suspicion. By applying these considerations, it has been shown that, for black films composed of egg phosphatidyl choline, cholesterol, and n-decane,

TABLE I

The Specific Conductance of Black Films of Egg Phosphatidyl Choline, Cholesterol, and n-Decane (22)

NaCl (Concentration)	Conductance (Ω^{-1} cm^{-2})
0.01 N	0.5×10^{-9}
0.1 N	1.3×10^{-9}
1.0 N	2.5×10^{-9}

the specific conductance is approximately 10^{-9} Ω^{-1} cm^{-2}. The precise value is dependent on the concentration of electrolyte in the aqueous solution (22) (Table I).

The mechanism of the conduction is by no means clear although it is interesting to note that the mechanism of conduction in the black films is evidently quite different from that in the bulk solution of lecithin and cholesterol in the n-decane. The specific conductance of this solution was in the region of 10^{-11} Ω^{-1} cm^{-1}. The conductance of 1 cm^2 of black film of thickness 50 Å should, in proportion, be about 2×10^{-5} Ω^{-1} cm^{-2}, some 10^4 times more than actually observed. The explanation may be that micelles, which are unlikely to be present in the black film, are responsible for most of the bulk conduction whereas the black film behaves more like a sheet of pure aliphatic hydrocarbon. Anomalously high values of film conductance can evidently arise from leakage at the edges of the films. Apart from the possibility of microscopic water channels running along the surface of the polytetrafluorethylene (5), conduction through the thick meniscus of lipid solution bordering the black films can also be important. For the egg phosphatidyl choline cholesterol n-decane system mentioned above, a meniscus of average thickness 0.005 cm and width 0.01 cm, bordering a black film of 0.01-cm diameter, should have a conductance of approximately 0.6×10^{-11} Ω^{-1}. This value is, according to Table I, similar to that which the black film itself would have. Some lipid solutions, e.g., purified egg phosphatidyl choline in aliphatic hydrocarbon, may have considerably lower specific conductances; thus the problem in these systems is less serious. Addition of polar solvents, such as methanol or chloroform, may however reverse this situation.

The numerous technical difficulties involved in the accurate measurement of the true conductances of the black films makes the detailed investigation of the mechanism of conduction an unattractive proposition. It is doubtful, in the author's opinion, whether the conductances are ohmic. In early results, where border leakages may well have been significant, the voltage-current curves seemed approximately linear when other complications were apparently absent. In later work however, where great care was taken to eliminate border leakage, the results suggested slight curvature, with the concavity to the current axis. This was scarcely significant and could not be tested more thoroughly owing to the breakdown of the films at applied voltages of more than about 200–300 mV.

The results of Table I show that the conductance depends on sodium chloride concentration although not in a linear manner. The possible implications of this relationship have been discussed briefly (22), but it is difficult to say more from these results than that either the sodium or the chloride ion seems to cross the membrane. The effect of other inorganic ions on the conductance has not been fully investigated, but at present there is no strong evidence to suggest that any remarkable effects will be found. Laüger et al. have described a substantial increase of conductance in the presence of iodide ions (16). This was demonstrated clearly by changing over the solutions, surrounding a given membrane, from NaCl to NaI. However the initial conductance of the films in the KCl solutions was high compared with the data of Table I, thus suggesting that border leakage may have been present. In fact, the authors do not describe any quantitative tests which might cover this question.

5. Permeability to Water

Two approaches have so far been made to the determination of the water permeability of the lipid films. These are respectively the exchange diffusion method, as indicated by tritiated water transfer, and osmotic flow, produced either by electrolytes or nonelectrolytes, to which the films are effectively impermeable. The former technique, as originally used, gives results which, unless convection is carefully controlled, are somewhat irreproducible and are in the region of 2 μ/sec at 20C (23,24) and 4 μ/sec at 36C (4,25). The osmotic flow experiments yield considerably higher values for the permeability (23). At 20C the value for films formed from egg phosphatidyl choline cholesterol and n-decane in solutions of NaCl, $CaCl_2$, urea, and sucrose at various concentrations and concentration differences is approximately 19 μ/sec (24,26). It was confirmed that this permeability was independent of the area of the black film and that it held equally well for D_2O as for water (26). For films formed from different phospholipid preparations, Huang and Thompson, working at 36C, found osmotic permeabilities ranging from 17.3 μ/sec to 104 μ/sec. By the radiotracer technique however, the same systems showed only minor differences (25).

The most striking general feature of the results is the order of magnitude difference between the radiotracer and osmotic permeabilities. It was pointed out by Hanai et al. that the probable explanation for this discrepancy lay in the existence of unstirred (boundary) layers of aqueous solution adjacent to the film surfaces (24). This suggestion was made largely on the basis of the following experiments. When the radio-tracer diffusion experiment was combined with the osmotic flow experiment, the osmotic flow was not affected but the tracer diffusion increased by a factor of three independently of the direction of the osmosis. The reason for this was thought to lie in the fact that, during osmosis across a vertical membrane, concentration gradients of solute tend to be set up on one or both sides of the films and the resulting density gradients produce convection which stirs the boundary layers. Thus the presence of osmotic movement, which, owing to this convection, is normally influenced only to a minor extent by boundary layers, is able to enhance the tracer diffusion by partially removing such layers. It was also found that, when a highly permeable glass mesh was placed in a diffusion cell of similar geometry to that of the lipid membrane cell (i.e., the hydrodynamic conditions were similar in the two cases), an apparent permeability from tritiated water diffusion was obtained that was close to the lipid membrane value. It was therefore clear that, in the lipid membrane system, nearly all the resistance to transfer was in the boundary layers rather than in the membrane. In principle it should be possible from such data to calculate the effective thickness of the boundary layers and then to deduce the true lipid membrane permeability. For instance, the boundary layers of combined effective thickness l can be regarded as in series with the lipid membrane of true permeability P_t. The apparent permeability P is then given by

$$\frac{1}{P} = \frac{1}{P_t} + \frac{l}{D} \qquad [15]$$

where D is the diffusion coefficient of the water in the boundary layers. Unfortunately in order to carry out this procedure, high accuracy is required in P

and P_t for the "known" membrane, and this was not achieved in the above experiments.

Subsequently two approaches, which will be described in detail elsewhere, have been used to test quantitively the contribution of the boundary layers. The first of these has involved attempts to stir the lipid membrane system more efficiently. Mechanical methods, temperature, and density gradient methods, also combinations of the three, have been tried. The highest reproducible permeability obtained by tritiated water diffusion under these conditions was 12.3 μ/sec at 20C. This should be compared with the value of 19 μ/sec from osmotic measurements. A six-fold increase over the unstirred system was thus obtained, but with these techniques no closer agreement between osmotic and tracer experiments could be achieved.

The second method is based on the principle of the glass mesh experiment. An apparatus was constructed of polytetrafluorethylene, which was similar to that used in earlier lipid membrane work except that it could be split, and a cellophane membrane was inserted in the position normally occupied by the black film. With this apparatus, without any forced stirring, a value of 2.6 μ/sec was found for the lipid membranes at 20C. Under the same conditions the permeability of the cellophane membrane of single and double thickness was used to estimate the thickness of the boundary layers. These together were found to be 825 μ, a value, which in order of magnitude, is typical of that found in electrode reactions (27). By using this value for l in equation (15), the true permeability of the lipid film can be calculated as approximately 20 μ/sec. Similar experiments were carried out in which controlled mechanical stirring was incorporated. Under these conditions the apparent lipid membrane permeability rose to 6.2 μ/sec, the combined boundary layer thickness decreased to 275 μ, and the corrected lipid membrane value was again approximately 20 μ/sec.

It is concluded therefore that there is no real difference between the permeability of the lipid membranes to water under exchange or osmotic flow conditions.

Vreeman (28) has also presented an investigation by the tritiated water method of the permeability of black lipid films. The permeabilities obtained in an "unstirred" system decreased with time during any given experiment and ranged from 5.2 to 4.1 μ/sec at 20C. Vreeman considers his results in terms, first, of linear diffusion from one semi-infinite medium to another and then, in a qualitative manner, for circumstances more akin to his experimental conditions. He concludes from his analysis that the values must be considerably less than the true values for the membrane and furthermore that, owing to theoretical difficulties, a true permeability value cannot be obtained at present by the direct approach. Although Vreeman does not use the model involving convection and boundary layers, his conclusions, albeit qualitative, are essentially similar to ours in that he attributes the low apparent permeability under conditions of exchange diffusion to a change in tritiated water concentration in the aqueous phases adjacent to the black film.

Huang and Thompson (25) have reached a conclusion different from these. They consider that the discrepancy between osmotic and exchange diffusion permeabilities cannot be attributed to the presence of boundary layers. An alternative explanation which these authors consider is based on the assumption that the lipid membranes may have water-filled pores. Such an explanation is not obviously inconsistent with the d.c. conductances of $\sim 10^{-6}$ Ω^{-1} cm^{-2} which they found. For membranes such as our own, having conductances similar to those given in Table I however, this interpretation is untenable. As Vreeman points out (28), it is not necessary to assume the presence of pores in order to account for such conductances. Indeed, the presence of pores sufficient to account for the permeability discrepancy would be difficult to reconcile with these conductances.

Attempts to account for the "osmotic" permeability of the lipid films have so far been based on the assumption that they are effectively thin layers of bulk hydrocarbon. The solubility of water in a number of liquid aliphatic hydrocarbons is known (29). The diffusion coefficient of water in hydrocarbons has been measured (30) and can also be estimated approximately from empirical formulae (26). From these data and a knowledge of the thickness of the hydrocarbon region of the membrane (and from a neglect of the resistance of the polar groups), a permeability can be calculated. This model predicts the permeability of the egg phosphatidyl choline cholesterol n-decane membrane as closely as can be expected from the consideration that the membrane is not a slice of a bulk hydrocarbon but probably a highly organized mixture of saturated, unsaturated, and cyclic hydrocarbon in unknown proportions (26). A similar model has been considered by Vreeman, who reaches a similar conclusion (28).

The temperature variation for the water permeability of the egg phosphatidyl choline cholesterol *n*-decane members has been examined, and the plot of log P versus 1/T has been found to be linear between 20 and 40C. The slope of the line gives an "activation energy" of 14.6 ± 0.4 kcal/mole. Owing to the difficulty of keeping composition and also thickness constant during the temperature variation, a precise interpretation of this result is difficult. The isotropic sheet model however does again give rough agreement with the experimental data.

6. Highly Conducting Films

In the presence of certain types of macromolecules and polypeptides, black lipid films may become highly conducting. Perhaps the more interesting and certainly the most complicated behavior has been observed with poorly characterized materials (2,32). Polypeptides seem, so far, to exhibit rather simpler behavior, and concern will only be with such systems.

Of a large number of polypeptides examined, all those which produced an appreciable increase of conductance contained nine or more amino acids. Yet many polypeptides containing more than nine amino acids did not affect the conductance. The majority of the effective polypeptides were soluble either in the aqueous or lipid phases but not in both, and the conducting film could usually be produced merely by adding the polypeptide to the phase in which it was soluble. Exceptionally the polypeptide may be soluble in both phases. This is so for tyrocidine hydrochloride, for instance, and conducting films can be obtained regardless of which phase first contains the polypeptide.

The specific conductances of the films can become very high. Values of 0.1 Ω^{-1} cm^{-1} for glycerol monooleate gramicidin films have been obtained. For higher conductances, special techniques are required as the total film conductance becomes large compared

with that of the aqueous solution between the electrodes. The addition of the polypeptide to the bulk lipid phase does not usually affect significantly its bulk conductance. In these systems therefore, unlike those containing only lipids (Section 4), the "bubble apparatus" illustrated in Fig. 2 can be used to test whether or not the conduction in the presence of a polypeptide (at least, when this is high) is primarily attributable to border leakage. This has been done for glycerol mono-oleate n-decane membranes in the presence of gramicidin, with ohmic conductances of approximately 4×10^{-3} Ω^{-1} cm^{-2} and demonstration of the absence of border leakage. The approximate proportionality of conductance to area for egg phosphatidyl choline cholesterol n-decane isopropanol gramicidin membranes, as determined by bulging experiments (22), is shown in Fig. 7. These same membranes had capacitances per cm^2 insignificantly different from those of the membranes without gramicidin and which, together with the conductances, were independent of frequency. The Cole-Cole plots, as far as they were determined, are shown in Fig. 8.

Of great biological relevance is the ability of black films containing polypeptides to be selective in their ion permeabilities. In this connection the work of Lev et al. (33,34), in which this phenomenon has been demonstrated for valinomycin, is of particular interest.

The molecular mechanism by which conduction occurs in films is still largely a matter for speculation. The experimental evidence available so far suggests the following possibilities. The polypeptide molecules must in any case spend some time adsorbed in the surface of the membrane. Two essentially different mechanisms are then possible. The first may be termed the "polar pore" and the second, the "carrier" mechanism.

"Polar pore" formation requires either that the polypeptide extend so far into the lipid phase that it is able, by itself, to span the bilayer (thus being, in effect, adsorbed at both faces) or that, if the polypeptide is smaller, a certain proportion of the molecules associate across the film. In either case, the conducting channels would probably arise from the formation of continuous aqueous regions promoted by the hydration of the polar groups of the polypeptide. The adsorption would have to take place in such a way that the orientation of the polypeptide enabled it to extend at least half-way across the hydrocarbon region of the film. For a portion of the

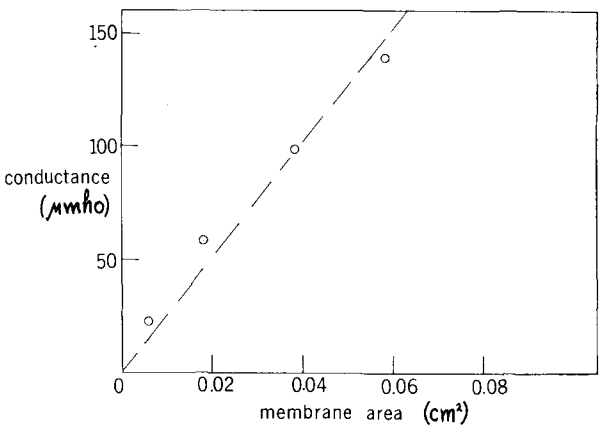

FIG. 7. The dependence of conductance on area for black films formed from solutions of egg phosphatidyl choline, cholesterol, and gramicidin A in *n*-decane and *n*-propanol in 0.1 N NaCl solution.

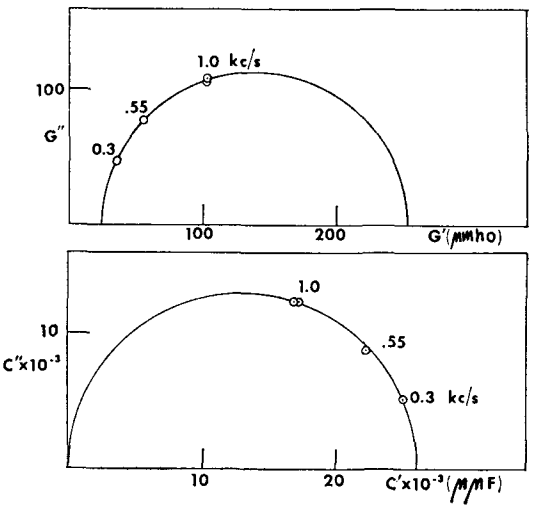

FIG. 8. The Cole-Cole plots (a) in the complex conductance and (b) in the complex capacitance planes for the system of Fig. 7.

polypeptide to be in the hydrocarbon phase at all it would have to be either very well endowed with hydrophobic groups or have groups which would interact with the polar groups of the film-stabilizing lipid, so enabling it to be "solubilized" in the hydrocarbon. If a molecule for which these various conditions seem impossible produces conduction in a film, it may be necessary to assume that aggregates of the polypeptide are adsorbed.

The introduction of this complication does not at present seem generally necessary although, for molecules as small as valinomycin, the formation of a pore by one molecule would involve severe distortion of the bilayer. The adsorption also must obviously be sufficient to account for the observed conductance. The relationship between specific membrane conductance and the number of adsorbed polypeptide molecules is problematical. However, from rough calculations, it seems possible to account for the conductance by assuming extremely low adsorption. This correlates well with the expectation that the adsorption would be very low, i.e., the concentration of the polypeptide in the bulk phase is usually small and the usual type of polypeptide structure, in which polar and nonpolar groups are not greatly separated in space, tends to yield a low $\Delta\mu^\circ$ (Equation 8). When these factors are considered, together with the fact that the polypeptide must compete with the strongly surface-active lipid molecules for space in the membrane surface, it becomes clear that there are likely to be quite rigorous structural requirements for a polypeptide to adsorb and orient itself in such a way as to be able to influence the film conductance.

The "carrier" mechanism depends on the surface concentration of the polypeptide just as does the "polar pore" mechanism. However it is also dependent on the concentration of polypeptide and polypeptide-ion complexes within the membrane. At present there seems to be little evidence concerning these questions.

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Discussion

DR. ROBERT SCHEUPLEIN (Harvard Medical School, Boston, Mass.): I would like to ask Dr. Haydon about the assumption that the center of the membrane was liquid-like. You showed one slide which was a plot of the thickness versus the number of carbon atoms in the stabilizer, and the conclusion was that the molecules were arranged linearly and that they were oriented. In another part of your discussions, you showed that there was a very deep minimum in the various energy terms involved. How do you reconcile those two facts with the assumption that the inside of the membrane is liquid? It seems to me that these two observations may indicate that there is an abrupt phase change, or some kind of an abrupt change in state, and it might lead one to speculate on a different kind of membrane interior. Would you add to your comments the evidence that indicates that there is very little water in the center of the membrane? Is that true?

DR. HAYDON: To take the last point first, I think the conduction measurements are only consistent with a very small amount of water in the films. In fact the conductivity of the membrane is consistent with its being a thin sheet of water saturated aliphatic hydrocarbon. In other words the water concentration must be very small. Regarding the question of what the lipid is like inside the membrane, I think the evidence from the mechanical properties, particularly, for some kind of liquid state is much stronger than for a solid state. The reason that the films stop thinning at a thickness equal to twice the thickness of the two monolayers certainly depends on the physical state of the monolayers. If the monolayers are of the solid-condensed type then the reason for impenetrability of the two monolayers is self evident. However, I think that there are many types of black film where this is not the situation. In these systems the stability could be explained by the mobility of the adsorbed molecules in the plane of the film. Statistical calculations based on a completely mobile monolayer model do predict sufficient resistance to interpenetration to account for the film stability.

DR. ANDREOLI: We have carried out similar experiments on the bulk conductances of the lipid solutions from which the membranes were formed. The specific resistivity of the bulk lipid-decane solutions, measured by d.c. methods, is approximately 10^9 ohm-cm. If this value is applied to the membranes, their resistance should be 10^3 ohm-cm^2, rather than 10^8 ohm-cm^2, which is what we actually measure. We have rationalized this discrepancy by assuming that, in the membrane, there is relatively restricted motion of the polar heads of the phospholipid molecules into the dielectric medium.

DR. HAYDON: I do not really know how to reconcile the film specific conductances with the disproportionately high specific conductances of the bulk lipid phase. The likely explanation is that the bulk phase contains large micellar structures which in some way conduct the current and which are evidently not present in thin films.

The mechanism of conduction by the polypeptides is at present obscure. For large molecules like gramicidin the pore mechanism seems much more likely than a carrier mechanism. For valinomycin, on the other hand, the relatively small size of this molecule seems to favor a carrier mechanism.

DR. THOMPSON: I would like to make a comment about the water permeabilities. I was one of the original exponents of the views that the difference between these two values is due to something other than an unstirred layer. I would like to say that I think that the evidence which Dr. Haydon has produced this morning leaves very little doubt that the two values are, in point of fact, the same and that the explanation for its difference is the existence of an unstirred layer. In a recent paper Cass and Finkelstein (A. Cass and A. Finkelstein, "Water Permeability of Thin Lipid Membranes," J. Gen. Physiol. *50*, 1765–1784 (1967)) has come to the same conclusion based on different experimental evidence.

I wonder if I could ask you a question. What surface area do you calculate per phospholipid molecule, if you take the data which you showed us on the various kinds of films?

DR. HAYDON: From consideration of the surface tension in the film and from the application of a monolayer equation of state we reported in an earlier publication that in lecithin-decane films the decane occupied about 50% of the volume of the film. We still think that this is a reasonable estimate, although our recent data indicates nearer 40% for the decane volume.

DR. TIEN: Recently we have measured the interfacial tension of BLM quantitatively using a technique based on the maximum bubble pressure principle (J. Colloid and Interface Sci., *24*, 287, 1967). The interfacial pressure is the difference between the tension of the pure solvent and the membrane, which is about 50 dynes/cm or so. Therefore, the molecules in the BLM are under very high compression, and if I may venture to estimate the area occupied at the indicated interfacial pressure, we would expect the molecules to occupy their limiting area. For example, I have estimated the diameter of the polar group in lecithin molecules to be about eight Angstroms, that would give an area of about fifty Angstroms square.

DR. HAYDON: An examination of the influence of the solvent in the film on the water permeability is difficult largely because the film stability is adequate only for a very limited range of solvents. We have

made some attempts at this problem but the results are not particularly useful.

DR. GERALD EHRENSTEIN, (National Institutes of Health, Bethesda, Md.): The curves that you have shown demonstrated that the increase of the chain length continued to give you increased area and increased values for capacitance, indicating that two chains on both sides were just in contact. Now at the same time you indicated that the other material, decane presumably, just fits in some way. Do you have any idea as to what would happen if you changed the material, for example, if you changed the big neutral lipids, how would that go?

DR. HAYDON: We have varied the solvent from hexane to hexadecane, and noted that for the latter solvent the capacitance was significantly higher and hence the film thickness was evidently smaller. It is certainly interesting to consider what would happen if the solvent chain length were somewhat longer than that of the stabilizer or solute.

DR. GOLDUP: I would like to return to the question of water permeability. If one assumes the water is transported through pores the permeability coefficient for transport under an osmotic gradient and under isotope exchange would not be expected to be the same since in the former case the total flow is unidirectional and in the latter case bi-directional. Thus, it may be envisaged that under isotope exchange half the pores will be carrying water in one direction and in the other half the flow will be in the opposite direction. Dr. A. Finkelstein of Albert Einstein University in New York has recently been studying water permeability across phospholipid membranes and I believe under the best stirring conditions he still finds the isotope exchange permeability coefficient is still lower than the osmotic value by a factor of about two. If the unstirred layer has been effectively eliminated this would suggest the presence of pores. Is it not possible that the water molecules cross the membrane in a continuous hydrogen bonded chain which would be analogous to a pore?

DR. HAYDON: If pores are present in the membranes, then the permeability by the traces and osmotic methods may differ. In fact, I think there is little doubt that they do not differ. This does not mean that there are no pores in the membrane, but merely that if there are, there is an average of only one or less water molecule per pore. This situation is difficult to distinguish from an isotropic solution model, and the latter is at present the simplest way of interpreting the data. As one might expect, it is reasonably successful.

DR. ROBERT SCHEUPLEIN: Regarding the way your water molecules get through a bilayer that is only 50 Angstroms thick, I want to point out that fluctuations may turn out to play a very important role and that it is possible that local membrane regions might transiently open and close.

DR. HAYDON: It is, of course, difficult to be sure about the role of fluctuations in determining the mechanism of water permeability. Nevertheless, I think that the high energy, relative to kT, needed to make a hole through a membrane would make the frequency of such fluctuations very small, and the relatively low viscosity of the films would also make the half life of such fluctuations small.

I would like to point out that the Vrij paper is concerned primarily with very thick films, and the growth of fluctuations is extremely dependent on the thickness and extent of coupling between the two sides of the film. The thinner the film becomes the less easily do the fluctuations in thickness build up.