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The Thickness, Composition and Structure of Some Lipid Bilayers and Natural Membranes

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Summary. It has been shown that the capacitance, thickness and composition of black lipid films may depend strongly on the hydrocarbon solvent used in their formation. By the use of n-hexadecane, films have been formed which contain effectively no solvent and which are comparable to the leaflets of the mesomorphic phase of the pure lipid. These films have capacitances of ca. 0.6 $\mu F/cm^2$ and hydrocarbon thicknesses of ca. 31 Å. Thinner black films of higher capacitances are also described.

The capacitances of biological membranes are, in contrast, nearer to 1 $\mu F/cm^2$, and it is suggested that the hydrocarbon region in these membranes may often be thinner than in the lipid leaflets. This suggestion is consistent with some X-ray and lipid composition data. It is pointed out that if the membranes contain abnormally thin lipid leaflets, the area per polar head group of the phospholipid must be increased, and that hydrocarbon is thereby exposed to the aqueous phases. Non-polar protein residues could then interact with these hydrocarbon areas, thus tending to stabilize the expanded leaflet.

Optically black films of various lipids in non-polar solvents may be formed under aqueous solution. From thickness measurements, it may be inferred that the films are bimolecular leaflets, consisting of a non-polar region containing the lipid hydrocarbon chains and some solvent, sandwiched between the polar head groups of the lipid [17]. For certain purposes, therefore, the black film may be regarded as a model for a biological membrane, in accordance with the concepts which originated in the work of Gorter and Grendel, and Davson and Danielli. The value of the black film as a model for a biological membrane must depend on whether the two structures have comparable thicknesses, and also on whether the thickness of the black film may be varied in a controlled manner. The presence

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of hydrocarbon solvent in a black film also raises questions as to its value as a membrane model. One of the objects of the present paper is to demonstrate some of the factors which determine both the thickness and composition of black films. It will be shown that the thickness and composition may be closely interrelated, and that under certain conditions the distinction in terms of these characteristics between the black film and the pure lipid leaflet (of the smectic mesophase of lipid in aqueous solution) effectively vanishes.

The electrical capacitance values reported for biological membranes are generally higher than any obtained for black films. A possible reason for this discrepancy may be that the thickness of the hydrocarbon layer in artificial lipid leaflets is larger than in biological membranes. It will be shown, by invoking the principles now known to determine the capacitance of black films, that the thicknesses of the biological membrane obtained independently from electrical capacitance, lipid composition and certain X-ray diffraction observations are self-consistent if interpreted according to a predominantly bilayer model. The reason that the biological membrane might be the thinner is discussed. It is also pointed out that there is not necessarily an inconsistency between recent optical and spectroscopic data for biological membranes and a membrane model which consists of lipid arranged predominantly in a bimolecular leaflet form.

Methods and Materials

Methods

Films were formed across a hole in a polytetrafluorethylene (PTFE) pot by means of the brush technique. Several pots with hole diameters ranging from 1 to 2 mm were used.

The capacitance C of the system was measured at 550 Hz by methods described previously [17]. At this frequency, the capacitance was effectively that of the film itself, and from this value the thickness δ_e of the hydrocarbon core of the film may be calculated by means of the equation

$$C = \frac{\varepsilon A}{4\pi\delta_e} \tag{1}$$

where A is the area of the film and ε is the dielectric constant of the hydrocarbon in the core. This dielectric constant may be estimated from a knowledge of the composition of the core and the bulk dielectric constants [23] of the appropriate hydrocarbons. It has been shown elsewhere [12, 18] that the capacitance of the polar groups of the lipid and of the electrical double layers may be neglected. The justification for using bulk values of the dielectric constant of the hydrocarbon is that in the micelles of paraffin-chain surfactants in water the hydrocarbon has effectively the same density as in bulk [2, 11]; also, it has been pointed out [2] that, even if the chains in the bilayer were highly

oriented (which they clearly are not [2]), the birefringence would be almost negligible. Although it is not needed for present purposes, the total thickness of the films may be obtained from δ_e by appropriate allowance for the dimensions of the polar head groups. For the glyceride and glyceryl phosphoryl choline groups, the thicknesses are in the region of 4.5 and 6 Å, respectively. The total correction to the thickness is thereby twice these values. Thus for lecithin-decane films, the total thickness should be ca. 60 Å. Probably the most accurate data with which to compare this estimate are those of Cherry and Chapman [9] who found from optical studies ca. 62 ± 2 Å for ostensibly similar films.

Interfacial tensions of glyceryl mono-oleate and of phospholipid in hydrocarbon against aqueous solutions were determined by the drop-volume technique [3]. The activity of the glyceryl mono-oleate in the hydrocarbon was calculated from vapor pressure measurements. The vapor pressures were determined by means of a modified Hewlett Packard Type 302 Vapor Pressure Osmometer. Further details have been given elsewhere [1]. The phospholipid was insoluble in both hydrocarbon and aqueous phases, and the spreading pressure at the n-hexadecane-0.1 M NaCl interface was measured by means of a torsion balance and a PTFE Wilhelmy plate, on a trough equipped with movable barriers [7]. The phospholipid in ethanol solution was spread initially at a very low pressure by means of an Agla microsyringe. The phospholipid film was then compressed by adjustment of the barriers as in the conventional Langmuir trough technique.

Interfacial tension and vapor pressure measurements were carried out at 20 °C. The spreading and capacitance experiments were carried out at room temperature (21 °C) unless otherwise stated.

The dielectric constants of solutions of 5α-cholestane in n-decane were measured in a PTFE cell fitted with platinum electrodes. The cell constant was determined by use of the standards air, n-decane and n-hexadecyl bromide.

Materials

The glyceryl mono-oleate was obtained from Sigma and was >99% pure 1-isomer by thin layer chromatographic analysis. This was kindly carried out by Dr. H. van Zutphen. Pure egg yolk phosphatidyl choline was generously supplied by Mr. N. Miller of the Agricultural Research Council (Institute of Animal Physiology, Babraham, Eng.). The fatty acid content of this phospholipid was established by gas-liquid chromatography (Table 1). The glyceryl mono-palmitoleate was prepared from palmitoleic acid in the laboratory of Prof. R. G. Jensen (University of Connecticut) and was shown to be the pure 1-isomer by borate thin layer chromatography. The cholesterol was a Fluka

Table 1. *The chain composition and unsaturation of the egg yolk phosphatidyl choline*

Chain	% (w/w) of total fatty acid	Chain	% (w/w) of total fatty acid
16:0	26.2	22:6	4.4
16:1	2.0	18:4	
18:0	15.1	20:2	2.8
18:1	31.9	20:5	
18:2	12.2	22:5	
20:4	5.4		

puriss grade specimen. The hydrocarbons and the hexadecyl bromide were Koch-Light puriss grade, and were further purified by passage through an alumina column. The ethanol was analytic reagent spectroscopic grade. The 5α-cholestane was obtained from Sigma and was 99% pure by thin layer chromatography. The NaCl was analytic reagent grade and was roasted at 700 °C to remove organic impurities. Other electrolytes were also analytic reagent grade. The water was twice distilled, once from a commercial still and then from a Pyrex still fitted with a quartz column, condenser and receiver. The pH of the aqueous solutions was measured occasionally at the end of experiments and was always approximately 5.6.

Results

Glyceryl Mono-Oleate Systems

The capacitance of black films stabilized by glyceryl mono-oleate depends strongly on the hydrocarbon solvent in which the oleate is dissolved (Table 2). Further results are published elsewhere [1, 2]. The capacitance increases considerably for solvents of chain length greater than n-decane.

The thickness δ_e of the hydrocarbon part of the film, also shown in Table 2, was calculated from Eq. (A.6) of the Appendix. This equation takes into account the fact that both the oleyl chains and the hydrocarbon solvent contribute to the dielectric constant of the film. The proportions of these two components are estimated from adsorption data, as discussed below. The partial molar volume of the oleate chain in the film was assumed to be equal to the molar volume of 1-heptadecene in bulk. The partial molar volume of the hydrocarbon solvent in the film was taken as the molar volume in the pure liquid. The assumption of bulk liquid properties for the hydrocarbon in the film, which, as mentioned above, was made by analogy with the interior of surfactant micelles in aqueous solution [11], is consistent with the electrical conductances of the films.

The adsorption of glyceryl mono-oleate at hydrocarbon-0.1 M NaCl interfaces was determined by the measurement of interfacial tension as a function of oleate activity in the hydrocarbon, followed by the application

Table 2. *The thickness and composition of black films formed from glyceryl mono-oleate dissolved in various n-alkanes, in 0.1 N NaCl*

Solvent	C (μF/cm²)	ε	δ_e(Å)	A (Å² per molecule)	$\bar{\varphi}$
n-Heptane	0.389 ± 0.003	2.05	46.5	39.5	0.55
n-Decane	0.383 ± 0.008	2.08	48	39.5	0.53
n-Tetradecane	0.465 ± 0.004	2.11	40.1	36.5	0.70
n-Hexadecane	0.580 ± 0.004	2.13	32.4	38	0.83

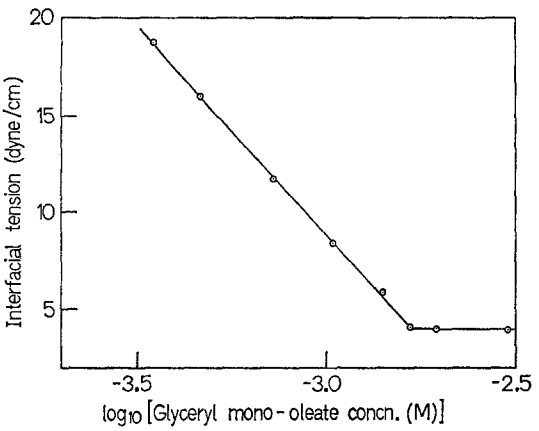

Fig. 1. The interfacial tension of glyceryl mono-oleate at the n-decane-0.1 N NaCl interface at 20 °C as a function of the glyceride concentration

of the Gibbs adsorption equation. As the oleate is very strongly adsorbed, and the two solvents are effectively insoluble in each other, the full Gibbs equation for this system reduces to [10]:

$$-d\gamma = \frac{kT}{A} d\ln a_2 \tag{2}$$

where γ is the interfacial tension, A is the area per molecule of the glyceryl mono-oleate, and a_2 is the activity of the oleate in the oil phase. The γ vs. $\log_{10}$ (oleate concentration) curves were very similar for each of the systems of Table 2. The curve for n-decane is shown in Fig. 1. The sharp levelling of the curve at higher concentrations is analogous to the results for many water-soluble surfactants and indicates micelle formation. Black films were stable only at concentrations greater than the critical micelle concentration, and hence the value of A required was that which corresponded to the maximum slope of the curve. Activity coefficients in n-heptane were determined by vapor pressure osmometry. The remaining solvents were not sufficiently volatile for this approach to be used, and the activity coefficients for n-heptane were employed. Although not ideal, this procedure is unlikely to produce any great error, especially as the activity correction in n-heptane has the effect of decreasing A by only 7%.

It has been shown theoretically [10] and for one system experimentally (D. A. Haydon & J. L. Taylor; *unpublished results*) that the adsorption of glyceryl mono-oleate in a black film differs only very slightly (<1%) from the adsorption at the bulk interface with which the film is in equilibrium.

The volume fraction $\bar{\varphi}$ of the oleate chains in a film was therefore calculated from δ_e and A.

From Table 2 and from other results [1, 2], it is found that for hydrocarbon solvents of chain length greater than n-decane, both the thickness of the black film and the volume fraction of solvent in the film decrease.

Lecithin Systems

The influence of the solvent on the capacitance of lecithin films is shown in Table 3. The increases of capacitance for longer chain length solvents are very similar to those for glyceryl mono-oleate. Film thicknesses were calculated as before from Eq. (A.6). Film compositions could not be determined as for glyceryl mono-oleate systems because lecithin is insoluble at a molecular level in hydrocarbon. Instead, an alternative procedure, based on the following arguments, has been used.

The surface pressures π^F and π^S of the insoluble molecules in the film and in the interface between the bulk phases at the meniscus, respectively, can be written

$$\pi^F = \gamma_0^F - \gamma^F \tag{3}$$

and

$$\pi^S = \gamma_0^S - \gamma^S \tag{4}$$

where γ^F is the (hypothetical) tension of one interface of the thin film and γ^S is the tension of the interface between the meniscus and the aqueous phase, and γ_0^F and γ_0^S are the corresponding values for the film and bulk interface in a system containing only the pure solvents. By subtraction of Eq. (3) from Eq. (4),

$$\pi^S - \pi^F = (\gamma^F - \gamma^S) - (\gamma_0^F - \gamma_0^S). \tag{5}$$

Table 3. *The thickness and composition of black films formed from lecithin dispersed in various n-alkanes, in 0.1* M *NaCl*

Solvent	C (μF/cm²)	ε	δ_e(Å)	A (Å² per molecule)	$\bar{\varphi}$
n-Decane	0.385 ± 0.013 [a]	2.09	48	61 [b]	0.69
n-Dodecane	0.443 ± 0.009	2.11	42.1	(61) [c]	0.75
n-Tetradecane	0.515 ± 0.010	2.13	36.6	(61) [c]	0.86
n-Hexadecane	0.603 ± 0.010	2.14	31.4	61	1.00

[a] Data of Ref. [17].
[b] Data of Ref. [10].
[c] Assumed values.

It has been shown for glyceryl mono-oleate systems by contact angle measurements that the first term is ca. -0.005 dyne/cm (D. F. Billet, *personal communication*). The second term is inaccessible to experiment because films cannot be formed from pure solvent, but should theoretically be very similar in order of magnitude to the first. Thus $(\pi^S - \pi^F)$ will be very small and, as the absolute values of π^S and π^F are in the region of 50 dyne/cm, these two surface pressures may, for present purposes, be taken as equal.

The magnitude of π^F or π^S may be found to a close approximation from measurements either of the tension of the thin film, or of the interface between the equilibrium bulk lipid and aqueous phases. The film tension (or $2\gamma^F$) may in principle be obtained by bulging the film under a known hydrostatic pressure. The present systems were too fragile for this approach to be used and it was necessary instead to measure the bulk phase interfacial tension, γ^S. π^S (and hence π^F) was then obtained from Eq. (4).

The number per unit area of lipid molecules which correspond to the pressure π^F or π^S may be found by spreading the insoluble lipid as a monolayer at the appropriate hydrocarbon-aqueous solution interface.

The above procedure was applied to the system lecithin + n-hexadecane. The interfacial tension for an 8 mM (stoichiometric) solution of lecithin in n-hexadecane against 0.1 M NaCl was less than 0.2 dyne/cm. The film interfacial tension γ^F was therefore between zero and this value. The interfacial tension, $\gamma_0^S (\approx \gamma_0^F)$, between pure n-hexadecane and 0.1 M NaCl is 53.9 dyne/cm [3] at 20 °C and thus $\pi^S (\approx \pi^F) \approx 53.7$ dyne/cm. In the Langmuir trough experiments, some collapse was found to occur at about 49 dyne/cm. However, the extrapolated area per molecule for a pressure of 53.7 dyne/cm is about 61 Å^2 (Fig. 2). This result for the lecithin + n-hexadecane system does not differ significantly from that reported previously for the lecithin + n-decane system [10]. The lecithin thus resembles the glyceryl mono-oleate in that the limiting area per molecule does not depend significantly on the chain length of the hydrocarbon solvent. There are ca. 1.6×10^{14} phosphatidyl choline molecules per cm^2 of black film interface in both the n-decane and n-hexadecane systems. This value has been used to calculate the composition of both the lecithin + decane and lecithin + hexadecane films and has also been assumed as an approximation to estimate the composition of the dodecane and tetradecane films.

In order to estimate the molar volume and dielectric properties of the lecithin chains, it has been assumed that there is, on the average, one octadecenoyl and one heptadecenoyl chain per molecule. This result was obtained by averaging the chain lengths and double-bond contents (Table 1). Thus a molar volume equal to one heptadecene and one hexadecene chain

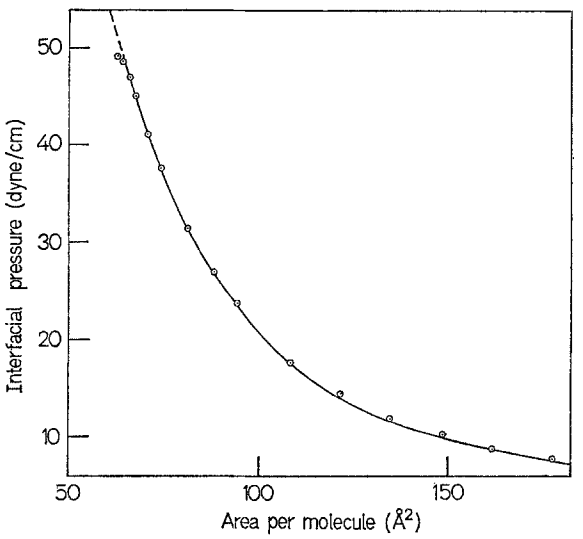

Fig. 2. The surface pressure versus area per molecule for lecithin at the n-hexadecane-0.1 N NaCl interface at 21 °C

has been used for each lecithin molecule, and the dielectric constant of 1-octadecene has been taken [17]. The estimated capacitances, thicknesses and compositions of the various types of lecithin film are given in Table 3. For comparison with the glyceryl mono-oleate systems of Table 2, it should be noted that the capacitance of films formed from lecithin + n-heptane is identical to that for films of lecithin + n-decane [17]. It follows, therefore, that the thickness and volume composition of the lecithin + heptane and lecithin + decane films are also identical.

Some Other Properties of Lecithin-Hexadecane Systems

The finding that films formed from hexadecane solutions contain little or no solvent is of particular interest in connection with their use as membrane models. Some other properties of these films will therefore be mentioned.

A lecithin-hexadecane black film does not, at room temperature, seem to have a stable area but expands continuously at the expense of the meniscus. When the film reaches the edge of the hole, it may still continue to expand and its area, as indicated by its capacitance, may reach many times the value corresponding to the area of the hole (Fig. 3). The expansion beyond the area of the hole appears to occur partly through the movement of the perimeter of the film outwards across the surface of the PTFE, and partly

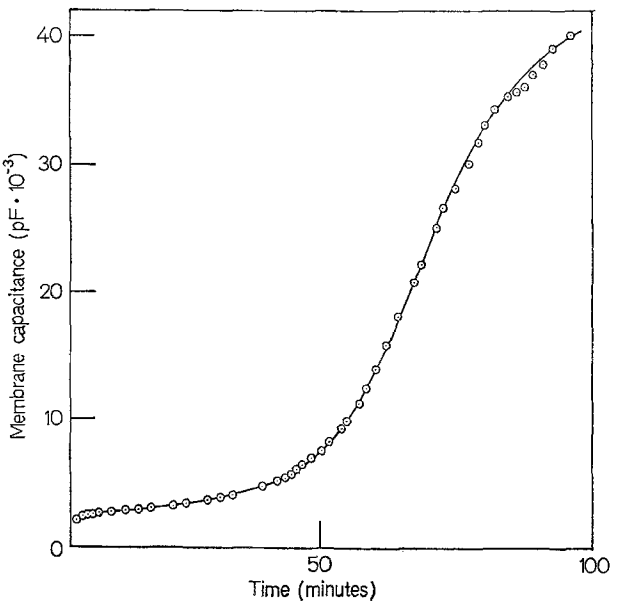

Fig. 3. The variation with time of the capacitance of a lecithin-hexadecane black film in 0.1 N NaCl. The film broke at the upper extremity of the curve

through bulging. A somewhat similar phenomenon was observed previously for lecithin-decane films, but only when their age exceeded ca. 3 hr [17]. The spontaneous generation of black film may be compared to the swelling and generation of lamellae which occurs when pure lecithin is placed in aqueous solution.

The expansion of the films makes the determination of specific capacitance rather difficult. This is particularly so at higher temperatures, where the expansion occurs much more rapidly. No significant effects of temperature on specific capacitance were noticed, however (Table 4).

When films were formed in $CaCl_2$ rather than in NaCl solutions, their behavior was quite different. The films were very fragile and showed no tendency to expand spontaneously at either room or higher temperatures. Accurate capacitance measurements were difficult to achieve, but the results indicated that the specific capacitance was in the region of 0.65 to 0.70 $\mu F/cm^2$ – somewhat higher than in NaCl.

Since several of the biological membranes used later for comparison are known to contain cholesterol, the effects of this steroid on lecithin + hexadecane films were investigated. Cholesterol is not very soluble in hexadecane, and the maximum concentration obtainable is only about 16 mM. The addition of cholesterol at this concentration in the bulk solution had no

Table 4. *The influence of cholesterol, Ca^{++} and temperature on black films formed from lecithin + n-hexadecane*

System	C (μF/cm²)	δ_e(Å)	Temp. (°C)
Lecithin + hexadecane in 0.1 M NaCl	0.613 ± 0.010	31	32
	0.607 ± 0.022		37
Lecithin + hexadecane + cholesterol [a] in 0.1 M NaCl	0.593 ± 0.007	32	24
	0.599 ± 0.008		40
Lecithin + hexadecane in 0.01 M $CaCl_2$	0.677 ± 0.015	28	39
	0.682 ± 0.014		21
Lecithin + hexadecane + cholesterol [a] in 0.01 M $CaCl_2$	0.612 ± 0.010	31	21

[a] Lecithin/cholesterol mole ratio in film nominally 2.5:1 (*see* Ref. [10]).

significant effect on the specific capacitance of the lecithin + hexadecane films (Table 4). As pointed out above, the adsorption of both lecithin and glyceryl mono-oleate appears to be independent of the chain length of the solvent. For a lecithin + decane system containing 14 mM cholesterol in the spreading solution, the film contains about two and a half moles of lecithin to one mole of cholesterol [10]. It is inferred that for the same adsorption, the hexadecane film will contain a somewhat similar ratio of lecithin to cholesterol.

The lack of knowledge of the dielectric contribution of cholesterol tends to hinder the interpretation of these results. As the hydroxyl group of the cholesterol should not affect the dielectric constant of the membrane, an attempt has been made to estimate this contribution by the use of 5α-cholestane (Fig. 4). The dielectric constants of various concentrations of 5α-cholestane in n-decane were measured, and these results were then extrapolated to unit mole fraction of the 5α-cholestane; this gave a dielectric constant for the pure compound of 2.22 ± 0.02. The additional contribution of a double bond at the 5–6 position was estimated by comparing the effects of the introduction of a double bond into cyclohexane and various straight-chain n-alkanes. The maximum possible fractional increase in the dielectric constant for a double bond in the '1' position in these hydrocarbons, multiplied by the number of carbons in the chain, was approximately constant, and between 0.5 and 0.6 for each of the hydrocarbons. Thus, the largest estimate of the effect of the double bond on cholestane would only make the dielectric constant 2.27 ± 0.02. Such a dielectric contribution for the cholesterol would have less than 3% effect on the overall dielectric

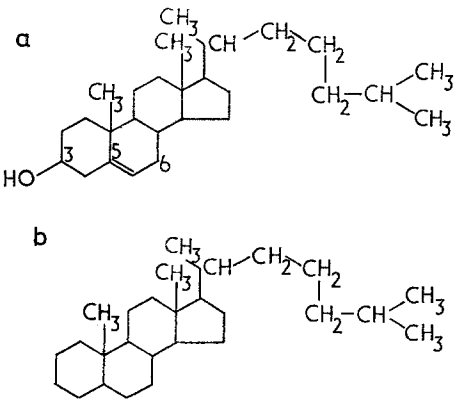

Fig. 4. (a) Cholesterol and (b) 5α-cholestane

constant of the films considered here. For the capacitance to remain the same, it would seem that the film thickens by less than 3%.

It is to be noted that, with the decane films, the maximum possible capacitance that has been observed on the addition of cholesterol [19] corresponds very closely to that obtained with pure lecithin-hexadecane films ($\sim$0.6 $\mu F/cm^2$). From the above argument, it seems unlikely that this cholesterol effect in the decane film is a result of a change of dielectric constant, but rather that the cholesterol has a similar effect to the hexadecane in that its introduction into the film results in a reduction in the average extension of the chains of the lecithin.

The resistance of the lecithin-hexadecane films is not significantly different from those reported previously for films formed with other solvents. In the absence of cholesterol, the films sometimes tend to be leaky, but even then the resistances are in the region of 10^7 to 10^8 $\Omega\,cm^2$.

Other Systems

The thickness of the hydrocarbon region may be an important parameter in understanding the interaction of molecules across a membrane. For this reason, an attempt has been made to obtain thinner films than those so far described.

One obvious and relatively easy method of achieving this is by the use of shorter chain lipids. Thus, it was found that glyceryl monopalmitoleate + hexadecane films had a specific capacitance that was to be expected from the decrease in length of the lipid chain by two carbon atoms (Table 5).

A different approach is to examine the effects of other types of solvent. Films formed from glyceryl mono-oleate and alkyl bromides have specific

Table 5. *Some exceptionally thin black films*

System	Aqueous phase	C (μF/cm²)	δ_e(Å)[a]
Glyceryl mono-palmitoleate + n-hexadecane	0.1 M NaCl	0.674 ± 0.004	28.1
Glyceryl mono-palmitoleate + n-hexadecyl bromide	0.1 M NaCl	0.852 ± 0.009	22.2
Glyceryl mono-oleate + n-hexadecyl bromide	0.1 M NaCl	0.761 ± 0.004	24.9
	satd. NaCl	0.808 ± 0.010	23.4
Glyceryl mono-oleate + n-hexadecyl bromide + cholesterol[b]	0.1 M NaCl	0.684 ± 0.004	28

[a] Dielectric constant of 2.14 assumed throughout.
[b] Glyceryl mono-oleate: cholesterol mole ratio 1:2 in film-forming solution.

capacitances considerably higher than those for the corresponding alkanes [1]. The interpretation of these capacitances in terms of thickness is not simple, however, as the dielectric constants of the alkyl bromides (ca. 3.8 to 5) are considerably different from those for the alkanes (ca. 2.0). This difficulty could be largely overcome if the composition of the films were known. However, the activity of the glyceryl mono-oleate in the alkyl bromides is difficult to determine by vapor pressure osmometry; for this and other reasons, the composition is not readily estimated. A maximum value for the area per molecule of glyceryl mono-oleate at the hexadecyl bromide-saturated NaCl interface of 43 Å² has, however, been calculated [1]. From this value it can be deduced from Eq. (A.6) that the maximum possible thickness of the hydrocarbon region of the black film is 23 Å and that there must be less than 1% by volume of hexadecyl bromide in the film. The reduced thickness of films when hexadecyl bromide is used is attributable to a smaller adsorption together with a tendency for the oleate chains to pack tightly into the region immediately adjacent to the interfaces of the film. The results for various films formed from hexadecyl bromide solution are shown in Table 5. A few films were formed from lecithin in this solvent and, although they also gave high specific capacitances, they were so unstable as to be of little use.

It should be pointed out that, although films formed in this type of solvent may be useful as models of thinner membranes, they are not strictly comparable with 'natural' systems. The interaction of the chains of a lipid monolayer with a layer of n-alkane is directly analogous to the interaction

of these chains with the chains of an opposing monolayer in a pure lipid bilayer. For the interaction of a monolayer of lipid with a layer of alkyl bromide, this analogy is no longer valid. This is reflected in the fact that the area per lipid molecule in a film formed in an n-alkane solvent (but not in an alkyl bromide) is comparable with the area per molecule in the lamellae of a smectic mesophase containing that lipid.

Discussion

Comparison of the Black Film with the Lamellae of the Smectic Mesophase

It has been shown that when hexadecane solutions are used, black films stabilized by a single polar lipid may be formed which contain practically no hydrocarbon solvent. The way in which the nature of the solvent influences the structure and composition of the black film has been described elsewhere [2]. It was found that the variation of the hydrocarbon solvent does not significantly affect the area per molecule of the polar lipid in the film, but it greatly affects the conformation of the chains. The longer the chain length of the solvent, the smaller the average extension of the chains of the polar lipid. The film thus becomes thinner and the volume fraction of solvent in the film becomes smaller. For hexadecane, the chains of the polar lipid are coiled down into practically their own volume, and solvent is almost completely excluded. These films are obviously very similar to the lamellae of the smectic mesophase of the pure polar lipid in aqueous solution.

The thickness of the black film formed from lecithin + hexadecane is very similar to that deduced from X-ray diffraction data on the lamellar phase of lecithin in water (Table 6). The value for the hydrocarbon thickness in the latter system was obtained by subtracting an appropriate length from the thickness of the lamellae deduced from the X-ray data, in order to allow for the fact that the glyceride group had been included with the hydrocarbon. It is not entirely clear if the differences between the thicknesses and areas per molecule in the two systems are significant. Different molar volumes for the lipid chains were assumed in the two instances (590 ml/mole for the black film as against ca. 570 ml/mole for the smectic mesophase), and this helps to account for the discrepancy. The series of assumptions concerning the densities of the lipid and aqueous regions in the smectic mesophase could introduce some error into the derived values for thickness and area per molecule of the lecithin in the lipid leaflet. A smaller area per molecule corresponds to a thicker leaflet. Finally, there must, for thermody-

Table 6. *A comparison of lipid membranes*

System	Hydrocarbon thickness (Å)	Partial molar area of phospholipid molecule (Å^2)	Ref.
Black films of lecithin + hexadecane	31	61	—
Lamellar phase of lecithin in water	~26	67–72	[24, 32, 35]
Lamellar phase of lecithin + cholesterol (1:1 mole ratio)	~29	54	[24]
Mammalian erythrocyte	22[a]	87.5[b]	

[a] Value deduced from area per molecule assuming that the core of the membrane is predominantly hydrocarbon.

[b] From lipid analysis of mammalian erythrocyte (Ref. [4]).

namic reasons, be some hexadecane in the lecithin black film, although this should not contribute appreciably to the discrepancy in the thickness.

If it is accepted from the above discussion that the lecithin + hexadecane black film is not significantly different from the lamellae of the smectic mesophase, it would be expected that the addition of cholesterol to each would have a similar effect. The addition of cholesterol to the lecithin + hexadecane film produces an increase in thickness of approximately 1.0 Å (Table 4), whereas a comparable amount of cholesterol in the lipid lamellae produces an increase in thickness of ca. 2.5 Å [24]. Again, it is not clear if this difference is significant.

The Black Film or Lipid Leaflet in Relation to the Biological Membrane

The capacitances reported so far for well-characterized black films have been low compared to those for biological membranes, the latter being in the region of 1 μF/cm^2 (Table 7). This could, to some extent, be attributed to the fact that the capacitances and particularly the membrane areas in the biological systems have been less accurately determined than for the black films. For the mammalian erythrocyte, however, this explanation seems unlikely as, in this instance, the dimensions of the cell are fairly well known and, furthermore, the specific capacitances for various species of erythrocytes are very similar [14]. It has also been suggested that in the biological membrane (in contrast to the black film) there are polar pores which increase the dielectric constant of the membrane and are thus responsible for the

Table 7. *Thickness and capacitance data on various biological membranes*

Membrane	C ($\mu F/cm^2$)	Hydrocarbon thickness (Å)		Ref.
		from capacitance[a]	from X-ray diffraction	
Mammalian erythrocyte	0.8	24	—	[14]
Osmotically lysed mammalian erythrocyte	0.9	21	—	[34]
Nerve myelin	0.6	32	—	[21]
	1.2	16	—	[36]
	—	—	35	(D. L. D. Caspar; *pers. comm.*)
Rod outer segments				
(surface membrane)	0.8–1.5	24–13	—	[13]
(disc membrane)	—	—	18	[5]
Squid giant axon	1.0–1.1	18	—	[20, 31]
	0.8	24	—	[37]

[a] A dielectric constant of 2.14 has been assumed.

high capacitance. The assumption that such polar regions are present in a membrane is made, however, chiefly in order to explain membrane conductance. But calculations based on a simple pore model show that the fraction of the membrane which need consist of pores in order to account for the conductance is much too small to influence the capacitance. Thus less than 10^{-6} of the membrane area need be composed of conducting units or 'pores' filled with 0.1 M KCl to give a membrane conductance of $10^{-2}\ \Omega^{-1}\ cm^{-2}$. The estimate of less than 13 sodium channels per μ^2 for the lobster axon also illustrates this point [28]. A further possibility is that a certain amount of the protein is inserted between the hydrocarbon chains of the lipid, and that this affects the dielectric constant but not the resistance of the membrane. It is difficult at present to assess the probable magnitude of this effect.

Yet another, and very simple explanation for the relatively high specific capacitances of the biological membranes is indicated by the results of Tables 2–6. Thus, it might be that the core of the membrane *is* predominantly hydrocarbon, but that this layer is merely somewhat thinner than in most of the artificial membranes. A recent X-ray diffraction study of the outer segments of retinal photoreceptors gave an electron density profile which was interpreted, by the use of derived values for the lipid and protein density, as indicating a hydrocarbon thickness of ca. 18 Å [5]. If the hydro-

carbon chains alone contributed to the dielectric constant, this thickness would correspond to a specific capacitance of 1 $\mu F/cm^2$. Unfortunately the available capacitance for rod outer segments is somewhat uncertain (Table 7).

Nerve myelin appears to have a thicker lipid bilayer ($\sim$35 Å) than the outer segment membrane [5; D. L. D. Caspar, *personal communication*]. The available capacitances for nerve myelin are as uncertain as those for the rod outer segment (Table 7), but the lowest value corresponds to about 32 Å. The discrepancy between the hydrocarbon thickness of the pure lecithin leaflet and the myelin may be accounted for solely by the fact that the latter contains a considerable amount of sphingolipid and cerebroside with an average chain length of 22 carbon atoms, and also about 25% by weight of cholesterol [29, 30]. The difference in thickness between the pure lipid leaflet and the rod outer segment membrane may not be accounted for by this type of argument because the outer segment membrane, although appearing to be considerably thinner than a pure 18-carbon lipid leaflet, contains lipids with a mean chain length of 19 to 20 carbon atoms [6].

The problem of hydrocarbon thickness may also be approached from data on the total lipid composition of the membrane. The red blood cell contains about equimolar amounts of phospholipid and cholesterol, the phospholipid having an average chain length of 18 carbon atoms [28]. For the membrane lipid to surround completely the red blood cell in the form of a bilayer, the partial molar area of a phospholipid molecule in the bilayer must be ca. 87.5 $Å^2$, each phospholipid plus cholesterol occupying ca. 125.5 $Å^2$ [4]. This may be compared with the smectic mesophase of lecithin and cholesterol (1:1 mole ratio) where the partial molar area of the phospholipid is ca. 54 $Å^2$, each phospholipid plus cholesterol occupying ca. 92 $Å^2$ [24]. If it is assumed that the hydrocarbon density is approximately the same in the natural and artificial systems, the increase in area per molecule must correspond to a decrease in thickness of the hydrocarbon region from 29 Å to ca. 22 Å (Table 6). The latter value is in excellent agreement with the thickness calculated from the specific capacitance, assuming that only the hydrocarbon chains contribute to the dielectric constant.

From the foregoing discussion, it appears that if the data obtained independently from electrical capacitance, lipid composition and certain X-ray diffraction observations are interpreted in terms of a predominantly bilayer model, they all suggest that the thickness of the hydrocarbon region of the bilayer is either similar to or thinner than in comparable artificial membranes. For the hydrocarbon chains to pack into a smaller thickness, while at the same time retaining a normal hydrocarbon density, the area per

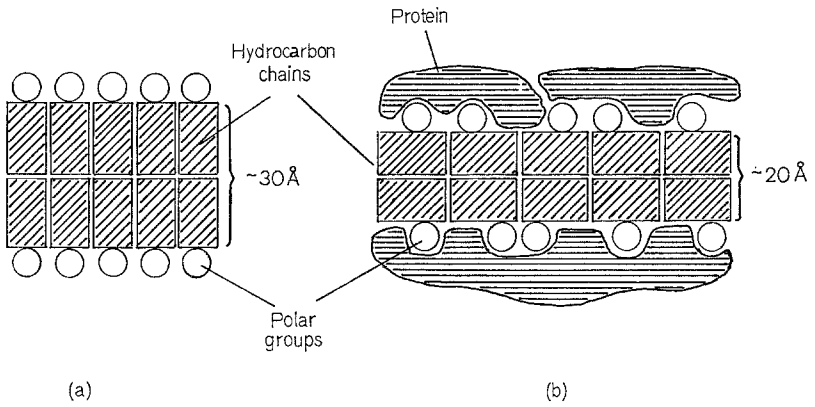

Fig. 5a and b. An illustration of the means by which non-polar residues of protein may interact with hydrocarbon chains of lipid molecules in a stretched bilayer. (a) Normal lipid bilayer. (b) The interaction of protein with exposed hydrocarbon in the stretched leaflet

molecule would have to be larger than in the artificial membranes. In fact, the area per molecule would be considerably larger than the area of a polar head group, and consequently significant expanses of hydrocarbon would be exposed to the aqueous phases at the surfaces of the leaflet. These hydrocarbon-water interfaces would be available to interact with the non-polar parts of proteins. The expansion of a pure lipid leaflet beyond its equilibrium area is energetically unfavorable, and it would be reasonable to regard the membrane protein as stabilizing the expanded leaflet (Fig. 5). As an increase in the area per lipid molecule corresponds to a decrease in the hydrocarbon thickness, the stronger the protein-hydrocarbon interaction (per unit area), the thinner would be the membrane. (Nerve myelin may be atypical in having a thicker hydrocarbon region on account of its containing about four times as much by weight of lipid as protein [29, 30], whereas most other membranes have at least as much protein as lipid.) These ideas have experimental support from the X-ray diffraction evidence that the addition of certain protein causes lipid lamellae in bulk phases to become as much as 20% thinner [16].

Interaction between protein and lipid chains in biological membranes has also been postulated on the basis of optical [25, 38] and nuclear magnetic resonance studies [8]. The above ideas are consistent with this but do not require that the protein be located in the interior of the leaflet.

Furthermore, a location of the protein at the interface rather than within the hydrocarbon does not necessarily rule out a partial α-helical conformation for this protein. From monolayer studies, it is known that

both synthetic polypeptides and natural proteins may retain considerable α-helical structure when spread at the air-water interface [22, 26, 27].

It is important to emphasize that in Fig. 5b a uniform thickness of the hydrocarbon region is indicated more for convenience than because it is demanded by the data. Provided the average thickness remained the same, considerable fairly short-range (say 50 Å) irregularities could be present. If such irregularities became very pronounced, the model would obviously tend towards the protein-lipid mosaic model. Moreover, if the rotation of the protein molecules were largely restricted, their dielectric constant could be low (5–10) [15], and the capacitance data could allow a substantial fraction of the membrane to have a very thin hydrocarbon region. However, although a severely distorted bilayer is not necessarily inconsistent with the data, it seems very doubtful if a true mosaic, in which the protein completely penetrates the membrane over an appreciable fraction of its area, could be consistent with the electrical resistances.

Appendix

Calculation of the Hydrocarbon Thickness δ_e from Adsorption and Capacitance Data

Provided that the film may be treated as a parallel plate capacitor,

$$\delta_e = \frac{\bar{\varepsilon}}{4\pi C} \tag{A.1}$$

where C is the capacitance per unit area. The film consists essentially of two hydrocarbon components, 1 and 2, and it is assumed that the mean dielectric constant $\bar{\varepsilon}$ may be obtained by summation of the dielectric constants of the component hydrocarbons on a volume fraction basis; i.e.

$$\bar{\varepsilon} = x\varepsilon_2 + (1-x)\varepsilon_1 \tag{A.2}$$

where x is the volume fraction of component 2.

If A is the area per polar lipid molecule (Å^2), N is the number of molecules per cm^2 of film interface and v is the molecular volume (cm^3)

$$\frac{N_1 v_1}{\delta_e} = 1-x; \quad \frac{N_2 v_2}{\delta_e} = \frac{2\times 10^{16} v_2}{\delta_e A} = \kappa; \quad 0 \leqq \kappa \leqq 1. \tag{A.3}$$

From (A.2) and (A.3),

$$\begin{aligned} \bar{\varepsilon} &= \frac{2\times 10^{16} v_2 \varepsilon_2}{\delta_e A} + \left(1 - \frac{2\times 10^{16} v_2}{\delta_e A}\right)\varepsilon_1 \\ &= \varepsilon_1 - \frac{2\times 10^{16} v_2}{\delta_e A}(\varepsilon_1 - \varepsilon_2). \end{aligned} \tag{A.4}$$

From (A.1) and (A.4),

$$\delta_e = \frac{1}{4\pi C}\left\{\varepsilon_1 - \frac{2\times 10^{16}\upsilon_2}{\delta_e A}(\varepsilon_1-\varepsilon_2)\right\}.$$

Rearranging,

$$\delta_e^2 - \frac{\varepsilon_1}{4\pi C}\delta_e + \frac{10^{16}\upsilon_2}{2\pi CA}(\varepsilon_1-\varepsilon_2)=0. \tag{A.5}$$

Solving the quadratic for δ_e,

$$\delta_e = \frac{1}{2}\left\{\frac{\varepsilon_1}{4\pi C} \pm \left[\frac{\varepsilon_1^2}{16\pi^2 C^2} - \frac{2\times 10^{16}\upsilon^2(\varepsilon_1-\varepsilon_2)}{\pi CA}\right]^{\frac{1}{2}}\right\}. \tag{A.6}$$

Only the root is taken for which $0 \leqq x \leqq 1$, i.e., for which

$$\delta_e \geqq \frac{2\times 10^{16}\upsilon_2}{A}.$$

References

1. Andrews, D. M. 1970. Ph. D. Thesis, University of Cambridge.
2. — Haydon, D. A., Manev, E. D. 1970. The composition and energy relationships for some thin lipid films, and the chain conformation in monolayers at liquid-liquid interfaces. *Special Disc. Faraday Soc.* No. 1, p. 46.
3. Aveyard, R., Haydon. D. A. 1965. Thermodynamic properties of aliphatic hydrocarbon/water interfaces. *Trans. Faraday Soc.* **61**:2255.
4. Bar, R. S., Deamer, D. W., Cornwell, D. G. 1966. Surface area of human erythrocyte lipids: Re-investigation of experiments on plasma membrane. *Science* **153**:1010.
5. Blaurock, A. E., Wilkins, M. H. F. 1969. Structure of frog photoreceptor membranes. *Nature* **223**:906.
6. Borggreven, J. M. P. M., Daemen, F. J. M., Bonting, S. L. 1970. Biochemical aspects of the visual process. VI. The lipid composition of native and hexane-extracted cattle rod outer segments. *Biochim. Biophys. Acta* **202**:374.
7. Brooks, J. H., Pethica, B. A. 1964. Properties of ionized monolayers. Part 6. Film pressures for ionized spread monolayers at the heptane/water interface. *Trans. Faraday Soc.* **60**:208.
8. Chapman, D., Kamat, V. B., De Gier, J., Penkett, S. A. 1968. Nuclear magnetic resonance studies of erythrocyte membranes. *J. Mol. Biol.* **31**:101.
9. Cherry, R. J., Chapman, D. 1969. Optical properties of black lecithin films. *J. Mol. Biol.* **40**:19.
10. Cook, G. M. W., Redwood, W. R., Taylor, A. R., Haydon, D. A. 1968. The molecular composition of black hydrocarbon films in aqueous solutions. *Kolloid Zeit.* **227**:28.
11. Corkill, J. M., Goodman, J. F., Walker, T. 1967. Partial molar volumes of surface-active agents in aqueous solution. *Trans. Faraday Soc.* **63**:768.
12. Everitt, C. T., Haydon, D. A. 1968. Electrical capacitance of a lipid membrane separating two aqueous phases. *J. Theoret. Biol.* **18**:371.
13. Falk, G., Fatt, P. 1968. Passive electrical properties of rod outer segments. *J. Physiol.* **198**:627.
14. Fricke, H. 1931. The electric conductivity and capacity of disperse systems. *Physics* **1**:106.
15. Grant, E. H. 1966. Dielectric dispersion in bovine serum albumen. *J. Mol. Biol.* **19**:133.

16. Gulik-Krzywicki, T., Sctecter, E., Luzzati, V., Faure, M. 1969. Interactions of proteins and lipids: Structure and polymorphism of protein-lipid-water phases. *Nature* **223**:1116.
17. Hanai, T., Haydon, D. A., Taylor, J. 1964. An investigation by electrical methods of lecithin-in-hydrocarbon films in aqueous solutions. *Proc. Roy. Soc. (Lond.) A* **281**:377.
18. — — — 1965*a*. Polar group orientation and the electrical properties of lecithin bimolecular leaflets. *J. Theoret. Biol.* **9**:278.
19. — — — 1965*b*. The influence of lipid composition and of some adsorbed proteins on the capacitance of black hydrocarbon membranes. *J. Theoret. Biol.* **9**:422.
20. Hodgkin, A. L., Huxley, A. F., Katz, B. 1952. Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**:424.
21. Huxley, A. F., Stämpfli, R. 1949. Evidence for saltatory conduction in peripheral myelinated nerve fibres. *J. Physiol.* **108**:315.
22. Isemura, T., Yamashita, T. 1959. Surface chemistry of synthetic protein analogues. VII. Polytyrosine and its related polypeptides. *Bull. Chem. Soc. Japan* **32**:1.
23. Landholt, H. H., Börnstein, R. 1959. Zahlenwerte und Funktionen, vol. II, p. 6. Springer-Verlag, Berlin.
24. Lecuyer, H., Dervichian, D. G. 1969. Structure of aqueous mixtures of lecithin and cholesterol. *J. Mol. Biol.* **45**:39.
25. Lenard, J., Singer, S. J. 1966. Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. *Proc. Nat. Acad. Sci.* **56**:1828.
26. Llopis, J. 1968. The polypeptide chain at liquid interfaces. Proc. 5th Int. Congress on Surface Activity (Barcelona). Vol. III, pp. 1–18. Ediciones Unidas, Barcelona.
27. Malcolm, B. R. 1968. Molecular structure and deuterium exchange in monolayers of synthetic polypeptides. *Proc. Roy. Soc. (Lond.) A* **305**:363.
28. Moore, J. W., Narahashi, T., Shaw, T. I. 1967. An upper limit to the number of sodium channels in nerve membrane. *J. Physiol.* **188**:99.
29. O'Brien, J. S., Sampson, F. L. 1965. Lipid composition of the normal human brain: gray matter, white matter and myelin. *J. Lipid Res.* **6**:537.
30. — — 1965*b*. Fatty acid and fatty aldehyde composition of the major brain lipids in normal human gray matter, white matter and myelin. *J. Lipid Res.* **6**:545.
31. Palti, Y., Adelman, J. W. 1969. Measurement of axonal membrane conductances and capacity by means of a varying potential control voltage clamp. *J. Membrane Biol.* **1**:431.
32. Reiss-Husson, F. 1967. Structure des phases liquide-cristallines de différents phospholipides, monoglycérides, sphingolipides, anhydres on en présence d'eau. *J. Mol. Biol.* **25**:363.
33. Rouser, G., Nelson, G. J., Fleischer, S., Simon, G. 1968. Lipid composition of animal cell membranes, organelles and organs. *In*: Biological Membranes, Physical Fact and Function. D. Chapman, editor. p. 1. Academic Press, London, New York.
34. Schwan, H. P., Carstensen, E. L. 1957. Dielectric properties of the membrane of lysed erythrocytes. *Science* **125**:985.
35. Small, D. M. 1967. Phase equilibria and structure of dry and hydrated egg lecithin. *J. Lipid Res.* **8**:551.
36. Tasaki, I. 1955. New measurements of the capacity and the resistance of the myelin sheath and the nodal membrane of the isolated frog nerve fiber. *Amer. J. Physiol.* **181**:639.
37. Taylor, R. E. 1965. Impedance of the squid axon membrane. *J. Cell. Comp. Physiol.* **66**:21.
38. Wallach, D. F. H., Zahler, P. H. 1966. Protein conformation in cellular membranes. *Proc. Nat. Acad. Sci.* **56**:1552.