BBA 75749

# PHYSICAL PROPERTIES OF LECITHIN–CEREBROSIDE BILAYERS

A. W. CLOWES*, R. J. CHERRY AND D. CHAPMAN
*Department of Chemistry, Sheffield University, Sheffield S3 7HF (Great Britain)*
(Received May 7th, 1971)

SUMMARY

The incorporation of ox brain cerebroside into egg-lecithin model membranes (bilayers) typically increases the electrical resistance and breakdown voltage and decreases the a.c. capacitance. At 1:1 mole ratio the capacitance measured at 1000 Hz is 0.24 ± 0.02 $\mu$F/cm$^2$ as compared with 0.38 ± 0.02 $\mu$F/cm$^2$ for egg lecithin alone. By comparison with the effects of including sphingomyelin and dibehenoyl lecithin in bilayers, it is concluded that the reduced capacitance is in part due to a thicker hydrocarbon region resulting from the relatively longer hydrocarbon chains of the cerebroside. Combined measurements of a.c. and d.c. capacitance indicate the presence of a low frequency dispersion from which it is inferred that the remaining reduction in a.c. capacitance is due to a contribution from the polar groups. An analysis of the bilayer equivalent circuit shows that such a contribution can be expected only if the polar region has a low conductance and a low dielectric constant.

Calorimetric studies demonstrate that hydrated ox brain cerebroside has a gel–liquid crystalline phase transition at about 55°. The transition temperature of hydrated cerebroside–egg lecithin samples falls as the proportion of lecithin is increased, reaching 20° at about 1:1 mole ratio. There is circumstantial evidence that this is close to the maximum amount of cerebroside that can be incorporated into the bilayer at this temperature.

## INTRODUCTION

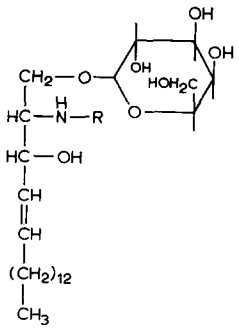
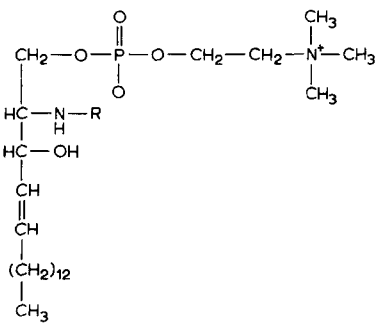
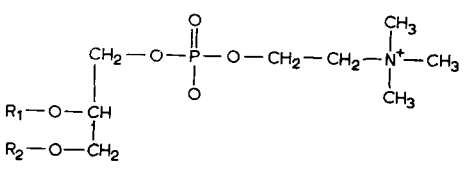
Sugar lipids have been identified as important membrane components with a specific tissue distribution[1] and a frequently defined antigenic role[2]; they have, however, been the subject of only a few biophysical studies. The particular function of the sugar group in membrane interactions is unknown. HOWARD AND BURTON[3] reported that they were able to form black films from cerebroside in *n*-decane but only at elevated temperatures (35–45°). Recently HOPFER *et al.*[4], found that bilayers formed from diglucosyldiglyceride (extracted from *Staphylococus aureus*) in *n*-decane had a resistance of $1.10^8$–$2 \cdot 10^9$ $\Omega \cdot$cm$^2$, a d.c. capacitance of 0.35–0.46 $\mu$F/cm$^2$, and a slight anionic selectivity. Other studies on crude brain extracts containing sugar lipids[5] have been made but the contribution of the various sugar lipids is not clear.

In this paper we report the properties of mixed ox brain cerebroside–egg yolk

* Present address: Harvard Medical School, 25 Shattuck Street, Boston, Mass. 02030, U.S.A.

TABLE I

STRUCTURE AND FATTY ACID DISTRIBUTION

| *Structure* | | *Principal fatty acids* | | *Mol. wt. (approx.)* |
|---|---|---|---|---|
| | | *(R)* | (%) | |
| (1) *Cerebroside* | * | 24:0 | 7 | 811 |
|  | | 24:1 | 16 | |
| | | (OH) 18:0 | 9 | |
| | | (OH) 24:0 | 17 | |
| | | (OH) 24:1 | 8 | |
| (2) *Sphingomyelin* | * | 18:0 | 40 | 800 |
|  | | 24:0 | 11 | |
| | | 24:1 | 25 | |
| (3) *Egg yolk lecithin* | ** | 16:0 | 38 | 750 |
|  | | 18:1 | 33 | |
| | | 18:2 | 17 | |
| (4) *Dibehenoyl lecithin* (same base as egg yolk lecithin) | | 22:0 | 100 | 902 |

* Adapted from O'BRIEN AND ROUSER[8].
** From SINGLETON *et al.*[34].

lecithin–*n*-decane bilayers. For comparison a limited number of observations on synthetic dibehenoyl $C_{22}$ lecithin and ox brain sphingomyelin in egg yolk lecithin–*n*-decane bilayers are included.

Cerebroside has been established as predominantly 1-galactosyl-2-acylsphin-

gosine (1-*O*-β-D-galacto-pyranosyl-2*S*:3*R*-2-tetracosanoylamido-*trans*-octadecene-1,3-diol)[6] with a variable fatty acid composition. In human brain[7] and ox brain[8] the fatty acids are mostly of the $C_{24}$ type as is shown in Table I. A β-linkage between the galactose anomeric carbon and the first carbon of the sphingosine base has been demonstrated by several techniques[9,10].

## MATERIALS AND METHODS

Black film studies were performed with bulk ox brain cerebroside generously provided by Drs. W. E. Van Heyningen and J. Mellanby. Koch–Light samples were used for the calorimetric work. Individual cerebroside fractions from human brain (nervone, cerasine, cerebrone, and sulfatide) were provided by Professor E. KLENK. The bulk ox brain cerebroside was chromatographed on Silica gel G (Merck) and developed in chloroform–methanol–7 M aq. ammonia (230:90:15, v/v/v). Under iodine vapor the bulk fractions were clearly seen to be a mixture of individual components with the cerasine fraction in greatest aboundance. Egg yolk lecithin was prepared by the method of DAWSON[11] and was pure by thin-layer chromatograpy. It was stored as a 1% solution in chloroform under nitrogen in sealed ampoules at −20°. *n*-Decane and ox brain sphingomyelin were obtained from Koch–Light. Synthetic dibehenoyl lecithin was a gift from Unilever. Tris (hydroxymethyl)methylamine was obtained from British Drug Houses. Distilled water and Analar grade KCl, $CaCl_2$ and sucrose were used for the preparation of the various solutions.

Mixtures of egg yolk lecithin and ox-brain cerebroside were prepared in chloroform–methanol (1:1, v/v), evaporated to dryness under nitrogen, and redissolved in *n*-decane at 1% concentration. Using the brush technique, films of 1–2 mm diameter were formed on teflon supports in an apparatus similar to that of MUELLER AND RUDIN[12].

Resistance measurements were carried out by applying a d.c. voltage to the film and measuring the current with a Keighley 417 Picoammeter. A.c. capacitance measurements from 15 to 50000 Hz were carried out using an Advance A.E. Generator Type J Model 2, and a Wayne-Kerr Universal Bridge B221-Mk. III with an external null indicator (Brookdeal 450 low noise amplifier). D.c. capacitance measurements were made using a circuit similar to the one described by HANAI, HAYDON AND TAYLOR[13] coupled with the bridge as shown in Fig. 1 so that sequential d.c. and a.c. measurements could be made on each film. The d.c. capacitance was calculated in

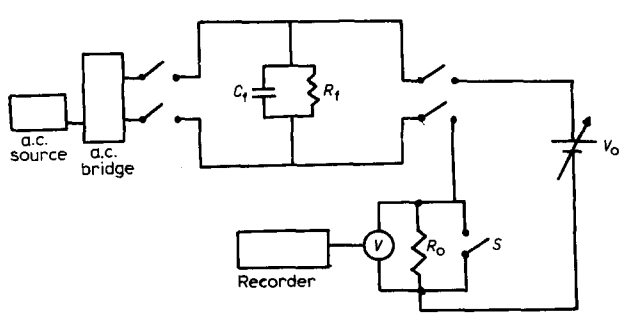



Fig. 1. Diagram of circuit for a.c. and d.c. capacitance measurements. Bilayer is represented by a resistance ($R_f$) and a capacitance ($C_f$) in parallel.

the following manner; $\tau$, the circuit time constant, was determined from a semi-log plot of the voltage rise across the resistor $R_0$ following the opening of the switch S. $R_f$, the film resistance was determined from the ratio of the applied voltage, $V_0$, to the final steady voltage $V$ across $R_0$.
Then

$$C_{d.c.} = \frac{\tau(R_0 + R_f)}{R_0 R_f} \quad (1)$$

where

$$R_0 = 0.986 \cdot 10^8$$

and

$$R_f = \frac{V_0 - V}{V} \cdot R_0$$

For both a.c. and d.c. capacitance measurement the applied potentials were always less than 50 mV.

The area of each film was determined by measuring the diameter with a travelling microscope. In the d.c. experiments the measurement of area was avoided by determining the ratio of the d.c. capacitance to the a.c. capacitance at 1000 Hz ($C_{d.c.}/C_{a.c.}$) for each film. This method is advantageous because the area is the least accurate of the measurements.

The bilayer thickness was determined optically using a modification of a previously described arrangement[14]. Reflectivity measurements were made from black films formed in aqueous solutions of $CaCl_2$ or sucrose in 50 mM Tris (pH 7.2) at various values of refractive index; these refractive indices were measured on an Abbé refractometer. A Hewlett Packard 9100 calculator was used to fit the experimental points to linear plots using the method of least squares.

The films were illuminated at near-normal incidence by light from a helium–neon laser ($\lambda$ = 6328 Å) and, to avoid birefringence errors, the beam was polarized perpendicular to the plane of incidence. Reflectances were calibrated by substituting a quartz plate of known reflectivity in place of the membrane. To simplify the experimental technique the reflectivity of the quartz plate reference was measured immediately after the bilayer in the same aqueous solution; a correction factor was then applied to account for the attenuation of the absolute reflectivity of the quartz plate with increasing refractive index of the aqueous phase. The absolute reflectivity of the film, $R_m$, is then given by

$$R_m = \frac{I_m}{I_Q / G(n_0)} \cdot R_Q$$

where $R_Q$ is the reflectivity of the quartz plate in distilled water ($=1.86 \cdot 10^{-3}$); $I_m$, the intensity reflected by the film; $I_Q$, the intensity reflected by the quartz plate and $G(n_0)$ the attenuation factor for the quartz plate which is dependent upon the refractive index $n_0$ of the aqueous phase. Although the optical rotation of the most concentrated solution (55%) of sucrose was large, the path length between the cuvette wall and the film was small so that the amount of beam rotation was small and produced an insignificant error in the thickness calculations.

Thermal measurements were carried out using a polarizing microscope with a hot stage and a Perkin–Elmer differential scanning calorimeter Model 1b (*cf.* LADBROOKE AND CHAPMAN[15] review) using cold-welded aluminium pans. Samples were mixed in chloroform–methanol, dried thoroughly under nitrogen, and mixed with excess water (lipid/water = 1:1) at 55° producing a homogeneous dispersion which gave a lipid and a water transition. It was found necessary to freeze the samples in liquid nitrogen for a day in order to get reproducible peaks. The samples, after extensive cooling, were rapidly transferred to the machine and heating runs begun at −100°.

## RESULTS

### *Membrane formation*

Stable films were formed from solutions of cerebroside–egg yolk lecithin (1 % in *n*-decane) with mole fractions of cerebroside varying from 0 to 0.7. Stable bilayers could not be formed at greater cerebroside concentrations. With increasing amounts of cerebroside the films showed greater reluctance to thin and at the higher mole ratios it was found expedient to increase the thinning rate by application of a potential. Cerebroside, dibehenoyl lecithin and sphingomyelin mixed with egg yolk lecithin all formed larger area films (on the same 2-mm aperture) with a less pronounced Gibbs ring than egg yolk lecithin alone.

### *Electrical measurements*

Cerebroside–lecithin films made from various mole ratios characteristically lasted longer, had a higher breakdown resistance, a higher breakdown potential and

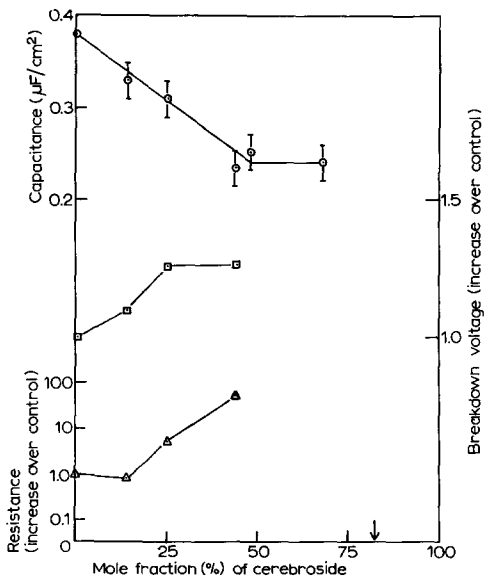



Fig. 2. Graph of bilayer capacitance (⊙), breakdown voltage (⊡), and resistance (△) as a function of the mole fraction of cerebroside in egg yolk lecithin (1 % total lipid in *n*-decane). Breakdown voltage and resistance are plotted as the fraction of the lecithin control value; the control lecithin was taken from the same batch and the films made on the same teflon supports as the cerebroside–lecithin mixtures. Arrow: films did not form at this mole ratio.

a lower a.c. capacitance than the lecithin controls (Fig. 2.) For cerebroside–lecithin (1:1) the resistance was typically in the range $10^7 - 10^8\ \Omega \cdot cm^2$, the breakdown voltage approximately 200 mV, and the capacitance 0.24 $\mu F/cm^2$.

A.c. capacitance measurements of lecithin–cerebroside (1:1) films over the range 15 to $5 \cdot 10^4$ Hz showed only one dispersion which shifted to lower frequency when the salt concentration was decreased (Fig. 3). D.c. capacitance measurements gave time constants, $\tau$, between 0.41 and 0.79 sec corresponding to a frequency range of 0.20–0.39 Hz. As shown in Table II the calculated d.c. capacitances were 13 % greater than the a.c. capacitances measured at approximately the same time at 1000 Hz. Lecithin alone showed no significant difference between a.c. and d.c. measurements.

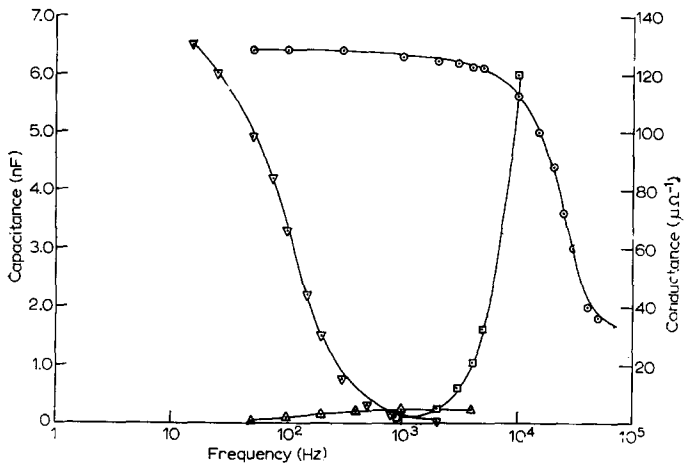



Fig. 3. A.c. capacitance ($C_{a.c.}$) and conductance ($G$) as a function of frequency for two cerebroside–egg yolk lecithin (1:1) films; 100 mM KCl: $C_{a.c.}$ (⊙) and $G$ (⊡); 0.01 mM KCl: $C_{a.c.}$ (▽) and $G$ (△). Measurements made on films formed across a 2-mm diameter hole.

TABLE II

(a) a.c. CAPACITANCE MEASUREMENTS (AT 1000 cycles/sec)

| *Lipid mixture (1 % in n-decane)* | $C_{a.c.}$ ($\mu F/cm^2$) |
|---|---|
| (1) Egg yolk lecithin | 0.38 ± 0.02 |
| (2) Egg yolk lecithin–cerebroside (1:1) | 0.24 ± 0.02 |
| (3) Egg yolk lecithin–sphingomyelin (1:1) | 0.31 ± 0.02 |
| (4) Egg yolk lecithin–dibehenoyl lecithin | |
| (i) 4:1 | 0.31 ± 0.02 |
| (ii) 25:1 | 0.31 ± 0.02 |

(b) d.c. CAPACITANCE MEASUREMENTS

| *Lipid mixture (1 % in n-decane)* | $\tau$ (*sec*) | $C_{d.c.}/C_{a.c.}$ (%) |
|---|---|---|
| (1) Egg yolk lecithin–cerebroside (1:1) | 0.41–0.79 | 113 ± 5 |
| (2) Egg yolk lecithin | 0.22–0.59 | 103 ± 5 |
| (3) Egg yolk lecithin–sphingomyelin (1:1) | 0.60–0.74 | 102 ± 5 |

Over the range of applied potential (20–40 mV) used in the d.c. capacitance measurements, no variation of capacitance with potential was observed.

Both sphingomyelin–egg yolk lecithin and dibehenoyl lecithin–egg yolk lecithin films were found to have an a.c. capacitance of 0.31 $\mu$F/cm$^2$ (Table II). The sphingomyelin films showed only a single dispersion (at 20 kHz in 0.1 M KCl) in the frequency range $2 \cdot 10^{-1}$–$5 \cdot 10^4$ Hz. Dibehenoyl lecithin still yielded capacitances of 0.31 $\mu$F/cm$^2$ even when the mole ratio of dibehenoyl lecithin to egg yolk lecithin was decreased to 1:25.

*Optical measurements*

Reflectivity measurements were made on lecithin–*n*-decane and lecithin– cerebroside–*n*-decane films and are plotted in Fig. 4a as the square root of the reflectivity

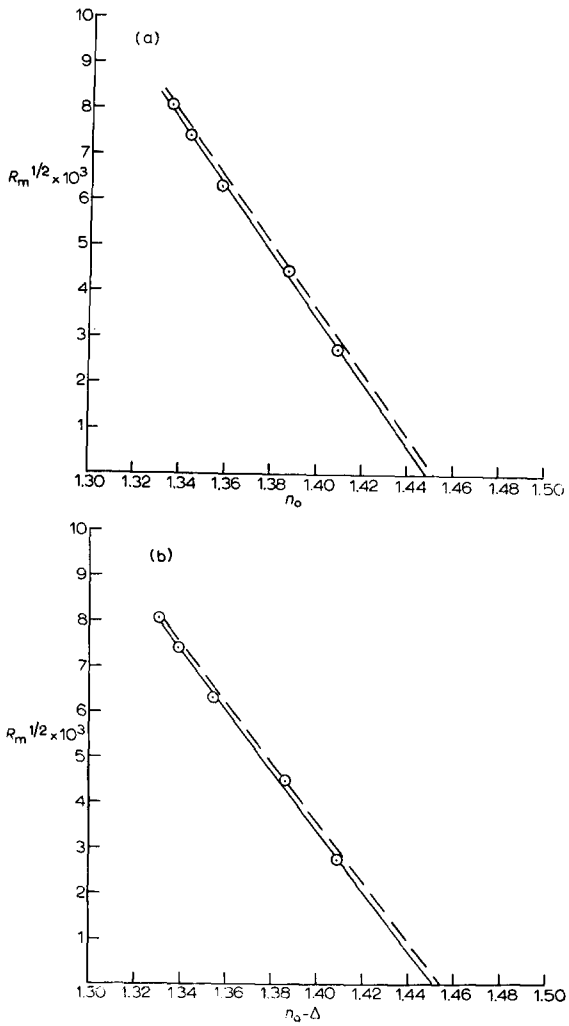



Fig. 4. a. Square root of the reflectivity plotted against the refractive index of the aqueous solution ($n_0$). – – –, egg yolk lecithin; ⊙—⊙, egg yolk lecithin–cerebroside (2:1). b. Square root of the reflectivity plotted against ($n_0 - \Delta$). – – –, egg yolk lecithin; ⊙—⊙, egg yolk lecithin–cerebroside (2:1).

($R_m^{\frac{1}{2}}$) against the refractive index of the aqueous phase ($n_0$). The thickness of the bilayer is calculated using equations previously derived[14,16].

$$\frac{n_1 R_m^{\frac{1}{2}}}{\sin \beta} = n_1 - n_0 + \Delta \tag{3}$$

where

$$\Delta = \frac{(n_1 - n_0)^2}{n_1 + n_0}$$

and

$$\beta = \frac{2\pi n_1 d}{\lambda}$$

$n_1$ is the mean refractive index in the plane of the bilayer, $\lambda$ is the wavelength of the incident light, and $d$ is the total bilayer thickness. By extrapolation of the line to zero reflectivity an approximate value for the refractive index of the film is obtained. Using this value of $n_1$ the data is replotted as $R_m^{\frac{1}{2}}$ against $n_0 - \Delta$ (Fig. 4b). From this graph the thickness and the mean refractive index of the film are obtained.

To eliminate any possibility of ion binding effects[17] optical measurements were carried out in sucrose solutions in addition to calcium chloride. These measurements gave a thickness of 66 ± 3 Å and a mean refractive index of 1.454 ± 0.002 for egg yolk lecithin which is in good agreement with previous determinations[14,18]. Measurements on films from lecithin–cerebroside mixtures (2:1, w/w) in sucrose solutions gave a thickness of 67 ± 2 Å and a mean refractive index of 1.450 ± 0.001. A 2:1 (instead of a 1:1) mixture was used to increase the thinning rate and to keep the experiments from being prohibitively long.

To check the prediction that the reflected light intensity goes to zero as the refractive index of the aqueous solution approaches the refractive index of the film, visual observations were made on films in a series of highly concentrated $CaCl_2$ solutions. At $n_0$ = 1.430 the photo-multiplier could no longer distinguish the reflected beam from the background scatter. As $n_0$ was further increased the intensity of the reflected beam continued to fall until at 1.450 (saturated $CaCl_2$ solution) the reflected beam was barely visible by eye.

*Calorimetry*

The change of hydrated cerebroside from the gel to the liquid crystal phase, observed visually and in the differential scanning colorimeter, occurred at approximately 50–55° and gave a broad endothermic peak as is to be expected with mixed hydrocarbon chains[19]. The relationship of transition temperature ($T_c$) of hydrated mixtures of cerebroside and egg yolk lecithin ($T_c$ approx. −15°) to mole fraction of cerebroside is illustrated in Fig. 5. The liquid crystal-hydrated transitions were dependent on the mole fraction of the components, although it was difficult to assign precise transition temperatures because of the broadness of the peaks.

## DISCUSSION

*Film composition and calorimetry*

Attempts have been made previously to determine bilayer composition by interfacial tension measurements[20] and radioactive techniques[21,22]. The present

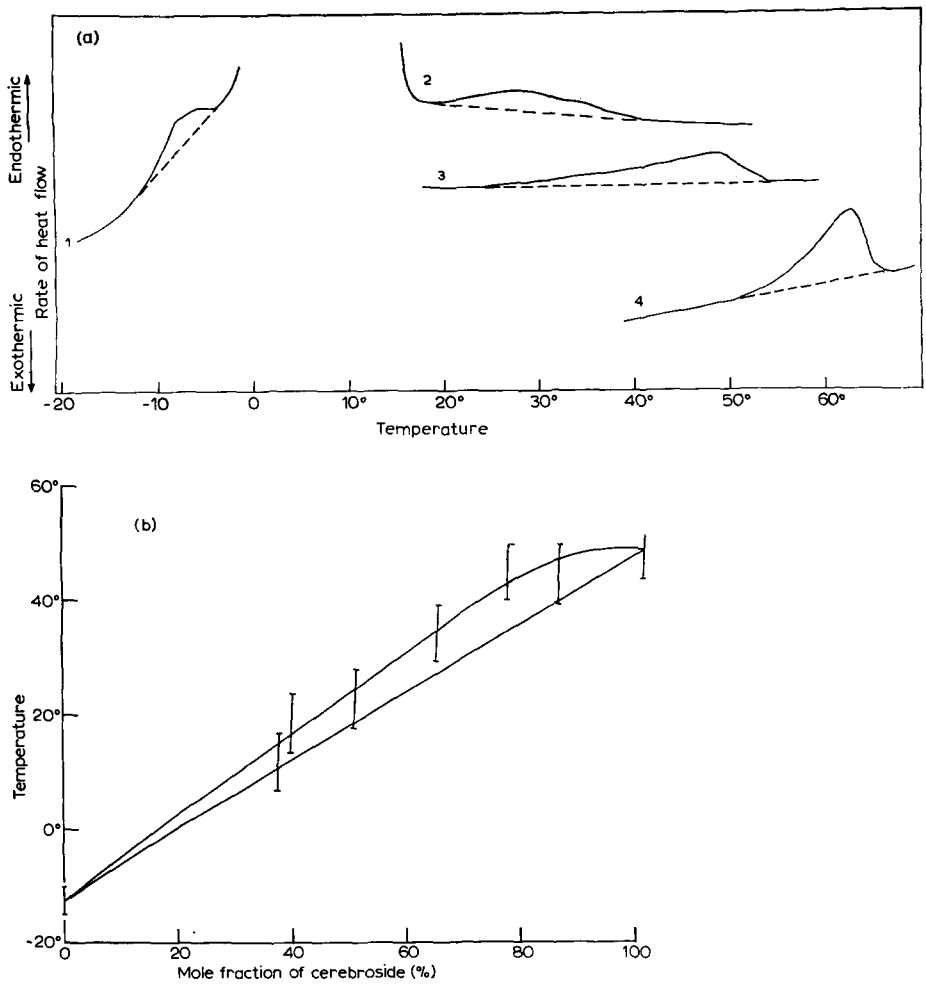



Fig. 5. a. Transition peaks of various water–lipid mixtures (1:1, w/w) in the differential scanning colorimeter (range, 4; scan, 9 degrees/min). 1, egg yolk lecithin 100%; 2, egg yolk lecithin–cerebroside, 61:39%; 3, egg yolk lecithin–cerebroside, 50:50%; 4, cerebroside 100%. Dashed line indicates assumed base line. b. Graph of onset temperature of transition peak *versus* mole fraction of cerebroside in egg yolk lecithin. Values from 0 to 10° were obscured by the water peak. Straight line between pure egg yolk lecithin and cerebroside transitions indicates values expected in the event of ideal mixing.

capacitance data shows a point of inflection at approx. 1:1 cerebroside–lecithin in *n*-decane (bulk solution) (Fig. 2), suggesting that there is a maximum amount of cerebroside which can be incorporated into the bilayer. Although the relationship between the composition of the film and the bulk solution is not clear, a plausible value for the cerebroside content of the film may be deduced from the available calorimetric data.

There is good evidence that black films, like myelin figures, only form when the transition temperature of the hydrated lipids is below the environmental temperature. Thus it was previously found for synthetic dipalmitoyl lecithin ($T_c$, 42°) that bilayers

would not form at room temperature[23]; cerebroside and sphingomyelin ($T_c$ about 40°) bilayers formed only after the surrounding bath was heated to a higher temperature (35–45°)[3]. However, the relationship between black film formation and the $T_c$ of the lipids is complicated by the presence of *n*-decane in the films. With hydrated dipalmitoyl lecithin–*n*-decane mixtures we have observed a typical solvent effect on the thermal transition at 42°. This peak becomes broadened and is shifted by about 10° to lower temperature when *n*-decane is present.

It was found that cerebroside–egg yolk lecithin mixtures containing between 40 and 50% cerebroside give a transition at about room temperature. This mole ratio is close to that found at the inflexion point of the capacitance data. Thus there is circumstantial evidence that the cerebroside–egg yolk lecithin ratio in the bilayer is similar to that of the bulk solution.

*Electrical and optical measurements*

It has been shown that optical measurements determine the total thickness of the film[16] while the capacitance measurements, at least in the case of egg yolk lecithin–*n*-decane, are determined only by the hydrocarbon thickness[13,24]. The finding that the thickness of lecithin and lecithin–cerebroside films are approximately the same by optical methods, although the capacitance of the latter is greatly diminished, suggests three possibilities:

(1) The hydrocarbon thickness is the same and a change in dielectric constant produces the capacitance change.

(2) The drop in capacitance is due to an increase in hydrocarbon thickness resulting from the longer cerebroside fatty acid chains (Table I). In this case there must be a compensating decrease in polar thickness.

(3) The hydrocarbon thickness is the same in both cases and therefore a contribution from the polar region must account for the drop in capacitance.

The first possibility is unlikely since the relevant long chain hydrocarbons have dielectric constants that do not vary by more than ± 5%. (In subsequent calculations we arbitrarily use the same hydrocarbon dielectric constant (2.05) as was previously deduced for egg yolk lecithin–*n*-decane films[24].)

Evidence relevant to the second possibility is obtained from the measurements with ox brain sphingomyelin and dibehenoyl lecithin. Since sphingomyelin has a sphingosine base and a large amount of $C_{24}$ fatty acid, it differs from cerebroside only in the polar head group. Its capacitance was found to be constant over all frequencies examined and hence can be related directly to the hydrocarbon thickness[13]. Using the formula for a parallel plate capacitor,

$$C = \frac{\varepsilon_m A_F}{4\pi d_H}$$

where $A_F$ is the area of the film, $\varepsilon_m$ the dielectric constant and $d_H$ the hydrocarbon thickness, we arrive at a value of 59 ± 4 Å. The results with dibehenoyl lecithin where the capacitance directly relates to the hydrocarbon thickness, indicate that even in low dilution in egg yolk lecithin the longest fatty acid chains dominate the capacitance. Thus the incorporation of $C_{22}$ and $C_{24}$ chains does increase the hydrocarbon thickness and decrease the capacitance to 0.31 $\mu$F/cm$^2$ but this alone is not sufficient to account for the full drop in capacitance observed with cerebroside. By analogy the drop in

cerebroside capacitance to 0.31 $\mu$F/cm$^2$ would be due to a thicker hydrocarbon region, and the drop from 0.31 to 0.25 $\mu$F/cm$^2$ must then be accounted for by a contribution from the polar groups (*i.e.* possibility (3)).

A contribution from the polar groups may arise either from their geometrical capacitance or from a charge double layer effect. Using a simplified model, EVERITT AND HAYDON[25] derived an expression for the membrane capacitance (for low applied potentials relative to the surface potential $\psi_0$):

$$\frac{1}{C_{\text{total}}} = \frac{2}{\frac{\varepsilon_0 K}{4\pi}\,\text{Cosh}\left(\frac{ze\psi_0}{2kT}\right)} + \frac{1}{\varepsilon_{m/4\pi d}} \tag{4}$$

where $K$ is the Debye–Hückel reciprocal length parameter =

$$\left(\frac{8\pi z^2 e^2 c_0}{\varepsilon_0 kT}\right)^{1/2}$$

($c_0$ = electrolyte concentration of valence $z$, $\varepsilon_0$ = dielectric constant of bulk aqueous solution, and $k$ = Boltzmann's constant). The first term is the expression for the contribution of the electrical double layer and the second the contribution of the geometrical capacitance of the membrane. Examination of the EVERITT–HAYDON graph of capacitance *versus* electrolyte concentration for various ion surface densities suggests that no drop of capacitance due to a double layer effect should be observed for any film in 0.1 M KCl, no matter how low the surface density of charged groups becomes. Therefore we are forced to account for the cerebroside–egg yolk lecithin results in terms of the geometrical capacitance.

In order to discuss the effect of the polar groups on the membrane capacitance, we make use of the circuit model of HANAI *et al.*[13]. The hydrocarbon region, the polar region and the aqueous phase are represented as three parallel combinations of capacitance and resistance in series (Fig. 6a). It was shown that the equivalent complex capacitance $C^*$ is given by

$$C^* - C_{\text{stray}} = C_h + \frac{C_l - C_i}{1 + jw\tau_p} + \frac{C_i - C_h}{1 + jw\tau_q} + \frac{1}{jw} G_l \tag{5}$$

where

$$G_l = \frac{G_a G_b G_c}{D} \tag{6}$$

$$C_h = \frac{C_a C_b C_c}{A} \tag{7}$$

$$C_l = \frac{C_a(G_b G_c)^2 + C_b(G_c G_a)^2 + C_c(G_a G_b)^2}{D^2} \tag{8}$$

$$C_l - C_i = \frac{1}{D(\tau_p - \tau_q)}\left[-E + F\tau_p + \frac{1}{\tau_p} C_a C_b C_c - \tau_p^2 G_a G_b G_c\right] \tag{9}$$

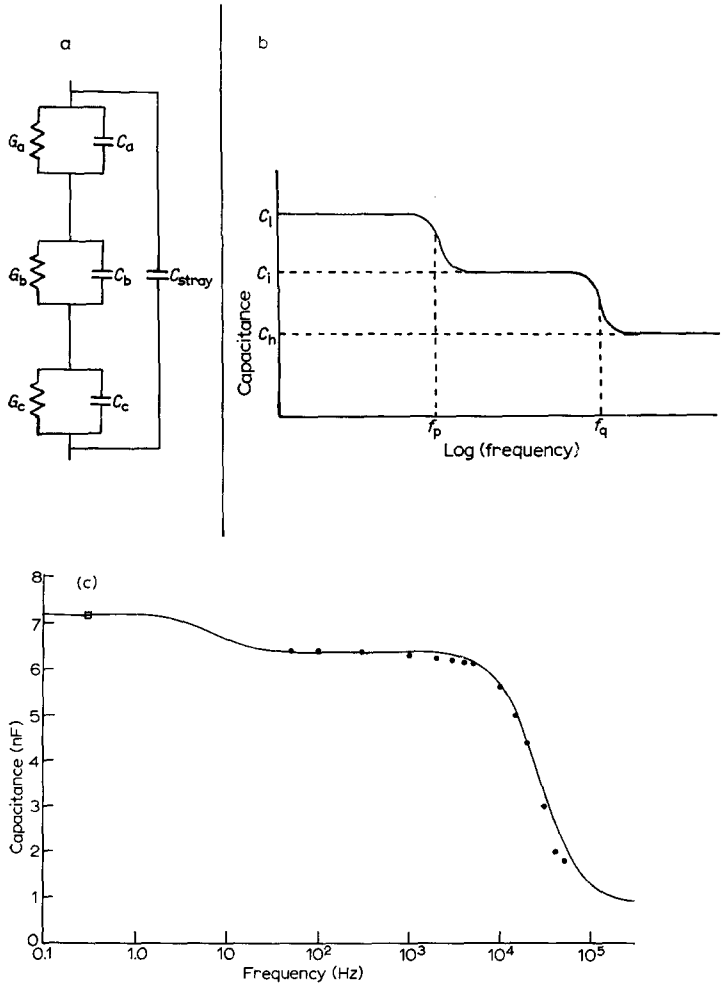



Fig. 6. a. Equivalent circuit of bilayer and aqueous phase. $C_a$,$G_a$ and $C_b$,$G_b$ and $C_c$,$G_c$ are the capacitances and conductances of the polar region, hydrocarbon region and aqueous phase respectively. b. Form of dispersion curve for circuit shown in a. c. Solid line: theoretical dispersion curve calculated for the parameters $C_a = 55$ nF, $C_b = 7.2$ nF, $C_c = 1$ nF, $G_a = 2.54 \cdot 10^{-6}\,\Omega^{-1}$, $G_b = 2.54 \cdot 10^{-10}\,\Omega^{-1}$, $G_c = 1.25 \cdot 10^{-3}\,\Omega^{-1}$. The values for $C_c$ and $G_c$ correspond to measured values for 0.1 M KCl. ●, experimental points (a.c.) for lecithin–cerebroside bilayer of area $2.54 \cdot 10^{-2}$ cm² in 0.1 M KCl. □, 'd.c.' capacitance (calculated from the $C_{d.c}/C_{a.c.}$ ratio given in Table II). (a and b are from HANAI *et al.*[13].)

$$Ci - C_h = \frac{1}{D(\tau_p - \tau_q)}\left[E - F\tau_q - \frac{1}{\tau_q} C_aC_bC_c + \tau_q^2 G_aG_bG_c\right] \quad (10)$$

$$A = C_aC_b + C_bC_c + C_aC_c \quad (11)$$

$$B = C_a(G_b + G_c) + C_b(G_c + G_a) + C_c(G_a + G_b) \quad (12)$$

$$D = G_aG_b + G_bG_c + G_cG_a \quad (13)$$

$$E = C_aC_bG_c + C_bC_cG_a + C_cC_aG_b \tag{14}$$

$$F = C_aG_bG_c + C_bG_cG_a + C_cG_aG_b \tag{15}$$

$$\tau_p = \frac{B + (B^2 - 4AD)^{1/2}}{2D} \tag{16}$$

$$\tau_q = \frac{2A}{B + (B^2 - 4AD)^{1/2}} \tag{17}$$

and $C_a$, $G_a$ and $C_b$, $G_b$ and $C_c$, $G_c$ are the capacitances and conductances of the polar region, the hydrocarbon region and the aqueous phase respectively.

The above equations enable a dispersion curve to be calculated for any set of parameters for the membrane and the aqueous phase. In general such a curve will show two dispersions corresponding to the relaxation times $\tau_p$ and $\tau_q$ (Fig. 6b) However, the actual form of the dispersion curve depends on the relative values of $G_a$, $G_b$ and $G_c$. Since the d.c. conductance of the circuit is considerably reduced when a membrane is present, we can be sure that $G_c$ is not the lowest of the three conductances. Thus the cases to be considered are (a) $G_b \ll G_c \ll G_a$. (b) $G_a \ll G_c \ll G_b$ (c) $G_a, G_b \ll G_c$. HANAI *et al.*[13] have demonstrated that in case (a) only one dispersion is observable and that at low frequency the capacitance is frequently independent and determined only by $C_b$. Case (b) is physically unreasonable since it is most unlikely that the membrane hydrocarbon region would be the most conducting part of the circuit. Thus we are left with case (c) to account for the present results with egg yolk lecithin–cerebroside bilayers, where a second dispersion is indicated by the finding that the d.c. capacitance is 13% higher than the a.c. value.

As shown in the following analysis a second dispersion should be observed when $G_a, G_b \ll G_c$. Under these conditions the equations given above simplify to give

$$C_l = \frac{C_aG_b^2 + C_bG_a^2}{(G_a + G_b)^2} \tag{18}$$

$$C_l - C_i = \frac{1}{C_a + C_b}\left(\frac{C_aG_b - C_bG_a}{G_b + G_a}\right)^2 \tag{19}$$

$$\tau_p = \frac{C_a + C_b}{G_a + G_b} \tag{20}$$

$$\tau_q = \frac{C_aC_b + C_bC_c + C_aC_c}{G_c(C_a + C_b)} \tag{21}$$

Further, if $G_a \gg G_b$ we obtain

$$C_l = C_b \tag{22}$$

$$C_l - C_i = \frac{C_b^2}{C_a + C_b} \tag{23}$$

Estimates of the parameters which are consistent with the experimental data may be made as follows. For simplicity we consider a membrane of unit area. Firstly,

suppose that the low frequency dispersion lies between the frequency of the lowest a.c. measurement (50 Hz) and the equivalent frequency of the 'd.c.' measurement (0.3 Hz), then the 'd.c.' capacitance is $C_1$ and the a.c. capacitance is $C_1$. Using the measured values adjusted to unit area of $C_1 = 0.25\ \mu F$ and $C_1/C_1 = 1.13$ we calculate $C_b = 0.29\ \mu F$ and $C_a = 1.8\ \mu F$ from Eqns. 22 and 23. (The condition $G_a \gg G_b$ is likely to be satisfied since hydrocarbons have extremely low conductances relative to polar molecules.) Using these values of $C_a$ and $C_b$, we deduce from Eqn. 20 that $5 \cdot 10^{-6} < (G_a + G_b) < 5 \cdot 10^{-4}\ \Omega^{-1}$ in order that $f_p\ (= 1/2\pi\tau_p)$ be less than 50 Hz. Since the measured d.c. conductance of the bilayer (determined mainly by $G_b$) is $10^{-7} - 10^{-8}\ \Omega^{-1}$ we may conclude that $5 \cdot 10^{-6} < G_a < 5 \cdot 10^{-4}\ \Omega^{-1}$.

Alternatively, $f_p$ may be in the region of the 'd.c.' capacitance measurement. In this case $G_a \approx 5 \cdot 10^{-6}\ \Omega^{-1}$. Taking the sphingomyelin value of $0.31\ \mu F/cm^2$ for $C_b$ (since in this case $C_1/C_i$ is not known), Eqns. 18 and 19 yield values of $C_a$ in the range 1.12–1.32 $\mu F$ corresponding to the range of $10^{-7}$–$10^{-8}\ \Omega^{-1}$ for $G_b$. (It is preferable to use Eqns. 18 and 19 in this case since $G_a$ is now not completely negligible compared with $G_b$.) These calculations cover all possibilities since $f_p$ cannot lie at much lower frequency than 0.5 Hz without the d.c. and a.c. capacitances becoming identical. As an example, we have calculated a theoretical dispersion curve using a set of parameters consistent with the above arguments (Fig. 6c).

The important feature which emerges from the above calculations is that the polar groups must form a region of low conductance and low dielectric constant. The value of $C_a = 1.8\ \mu F$ corresponds to a dielectric constant of 2.2 for a 10-Å layer while the maximum conductance of $5 \cdot 10^{-4}\ \Omega^{-1}$ corresponds to a specific conductance of $5 \cdot 10^{-11}\ \Omega^{-1} \cdot cm^{-1}$. These are unexpectedly low values considering the polar nature of the lipid head groups. Even if the maximum experimental uncertainty is taken into account, the dielectric constant of the polar region cannot be much greater than 4, and the conclusion concerning $G_a$ is unaltered.

*Model of the membrane*

Inspection of Fig. 7a shows that $C_{24}$ cerebroside has two fatty acid chains which are effectively 24 and 14 carbons long when all the polar groups on the ceramide base are placed at the hydrocarbon–polar region interface. Therefore the maximum and minimum theoretical thicknesses of the hydrocarbon region (with fully extended chains) are 61 Å and 51 Å according to whether or not the chains interdigitate (Fig. 7b). These dimensions are also applicable to $C_{24}$ sphingomyelin for which we have determined a hydrocarbon thickness of $59 \pm 4$ Å. We have also argued that the hydrocarbon thickness of the cerebroside bilayer is likely to be similar to that of sphingomyelin. Thus it appears that for both cerebroside and sphingomyelin there is little interdigitation of the hydrocarbon chains in the bilayer. (Although the films contain substantial amounts of *n*-decane, it was shown previously that the hydrocarbon solvent does not have an effect on the hydrocarbon thickness as deduced from the capacitance[26].).

Subtraction of the determined hydrocarbon thickness from the cerebroside–lecithin optical thickness gives a value which lies between zero and sixteen angstroms for the sum of the two polar regions. Since each polar region is less than 8 Å thick, we conclude that the galactose and choline phosphate groups are not extended and the most probable thickness is consistent with the polar groups lying parallel to the plane

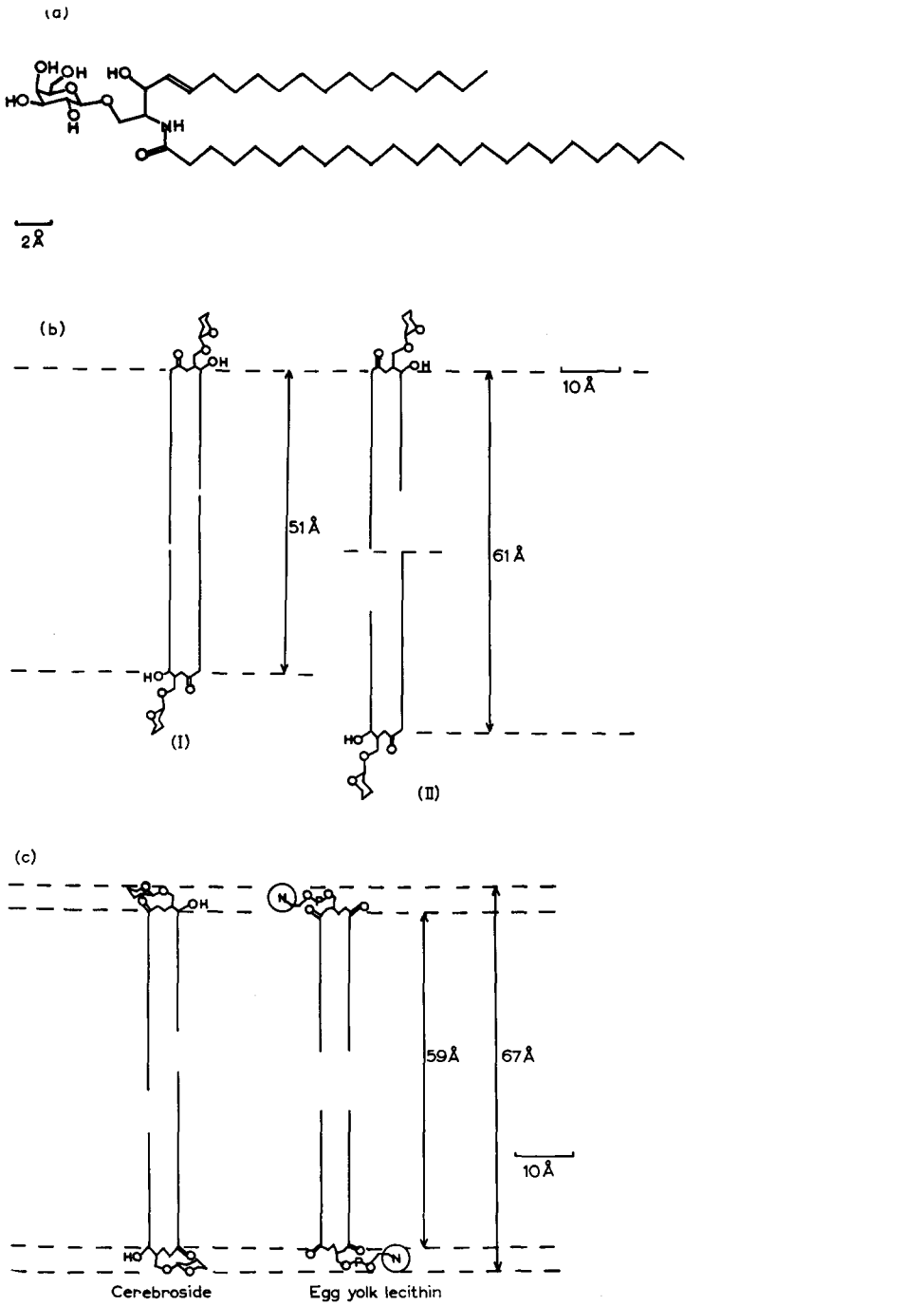



Fig. 7. a. Cerebroside molecule (with $C_{24}$ fatty acid). b. Schematic diagram indicating maximum and minimum hydrocarbon thickness. (i) Interdigitation; (ii) no interdigitation. c. Cerebroside–lecithin bilayer model based on optical and capacitive thicknesses.

of the film. (The polar groups would occupy 10–12 Å if fully extended.) In this state the surface potential should be zero. From the above considerations we conclude that the most probable organisation of the bilayer is that shown schematically in Fig. 7c.

The conclusion that the dielectric constant and the the conductance of the polar region are low leads us to believe that water and ions are excluded from the polar region. If appreciable water were present then a high value of $\varepsilon$ would be expected ($\varepsilon_{H_2O} = 80$). (A value of 36 for the Stern layer of long chain pyridinium iodides was measured by MUKERJEE AND RAY[27] using charge transfer techniques.) This conclusion is unexpected in view of the four hydroxyl groups on the galactose head group of cerebroside. However, if the galactose is parallel to the plane of the membrane, it is possible for the hydroxyl groups to orient at the outermost surface of the membrane. In this position they can hydrogen bond with the surrounding aqueous phase without any appreciable penetration of water into the polar region.

*Comparison with other physical techniques*

The hypothesis that the lecithin–cerebroside or lecithin–sphingomyelin bilayers have a thicker hydrocarbon region than lecithin is supported by X-ray data showing that hydrated ox brain cerebroside and sphingomyelin bilayers in the liquid crystal state are 49 and 48 Å thick, respectively, as opposed to 38 Å for egg yolk lecithin[28]. However, these thickness calculations are based on a lipid density which does not take into account the true distribution of water in the lipid polar region. Further, the effect of *n*-decane on lipid thickness is not known, and therefore one must be careful about drawing a comparison between thicknesses measured by X-ray and capacitance.

Monolayer[29–31] and X-ray[28] experiments indicate that cerebroside and sphingomyelin both have lower molecular areas than lecithin, which suggests that bilayers formed from *n*-decane–lecithin–cerebroside or *n*-decane–lecithin–sphingomyelin may be more compact than lecithin–*n*-decane films. Taken with the finding that the effective dielectric thickness is increased, these related observations may account for the increased resistance and breakdown voltage of these mixed lipid films relative to lecithin alone.

*Cerebrosides and myelin*

In older experiments and more recent detailed studies[1], cerebrosides have consistently been shown to be a major component of myelin[1]. BRANTE[32] found in general that myelin was composed of cholesterol, cerebroside and phospholipids in the proportion 2:1:2. For peripheral nerve JOHNSON, *et al.*[33] suggested a proportion of 1:1:2. We have shown that cerebrosides in similar proportions increase the stability and resistance of artificial membranes. These properties may be of advantage in myelin whose principal function is that of an electrical insulator.

ACKNOWLEDGEMENTS

We gratefully acknowledge the samples from Drs. Van Heyningen, Mellanby, Professor Klenk, and Unilever Ltd., and the loan of electrical equipment from Dr. D. Dunmur. We also thank the Science Research Council for financial support.

REFERENCES


1 E. G. LAPETINA, E. F. SOTO AND E. DE ROBERTIS, *J. Neurochem.*, 15 (1968) 437.
2 M. M. RAPPORT AND L. GRAF, *Prog. Allergy*, 13 (1969) 273.
3 R. E. HOWARD AND R. M. BURTON, *J. Am. Oil Chemists' Soc.*, 45 (1968) 202.
4 U. HOPFER, A. L. LEHNINGER AND W. J. LENNARZ, *J. Membrane Biol.*, 2 (1970) 41.
5 F. P. WIRTH, H. E. MORGAN AND C. R. PARK, *Fed. Proc.*, 24 (1965) Abstr. 2520, 588.
6 H. E. CARTER AND F. L. GREENWOOD, *J. Biol. Chem.*, 199 (1952) 283.
7 E. KLENK, *Prog. Chem. Fats Lipids*, 10 (1969) 409.
8 J. S. O'BRIEN AND G. ROUSER, *J. Lipid Res.*, 5 (1964) 339.
9 M. MARTIN-LOMAS AND D. CHAPMAN, *J. Biol. Chem.*, to be published.
10 Y. FUJINO AND T. NEGUISKI, *Bull. Agric. Chem. Soc. Japan*, 20 (1956) 183.
11 R. M. C. DAWSON, *Biochem. J.*, 88 (1963) 414.
12 P. MUELLER AND D. O. RUDIN, in H. PASSOW AND R. STAMPFLI, *Laboratory Techniques in Membrane Biophysics*, Springer-Verlag, Berlin, 1969, p. 141.
13 T. HANAI, D. A. HAYDON AND J. TAYLOR, *J. Theor. Biol.*, 9 (1965) 278.
14 R. J. CHERRY AND D. CHAPMAN, *J. Mol. Biol.*, 40 (1969) 19.
15 B. D. LADBROOKE AND D. CHAPMAN, *Chem. Phys. Lipids*, 3 (1969) 304.
16 R. J. CHERRY AND D. CHAPMAN, *J. Theor. Biol.*, (1969) 137.
17 R. J. CHERRY, D. E. GRAHAM AND D. CHAPMAN, *Chem. Phys. Lipids*, 6 (1971) 125.
18 C. LUSSAN, A. HOU AND P. BOTHEREL, *C. R. Acad. Sci. Paris*, 269 (1969) 639.
19 M. C. PHILLIPS, B. D. LADBROOKE AND D. CHAPMAN, *Biochim. Biophys. Acta*, 196 (1970) 35.
20 G. M. W. COOK, W. R. REDWOOD, A. R. TAYLOR AND D. A. HAYDON, *Kolloid-Z. Z. Polym.*, 227 (1968) 28.
21 F. A. HENN AND T. E. THOMPSON, *J. Mol. Biol.*, 31 (1968) 227.
22 D. M. ANDREWS AND D. A. HAYDON, *J. Mol. Biol.*, 32 (1968) 149.
23 C. HUANG, L. WHEELDON AND T. E. THOMPSON, *J. Mol. Biol.*, 8 (1964) 148.
24 T. HANAI, D. A. HAYDON AND J. TAYLOR, *Proc. R. Soc. London, Ser. A*, 281 (1964) 377.
25 C. T. EVERITT AND D. A. HAYDON, *J. Theor. Biol.*, 18 (1968) 371.
26 J. TAYLOR AND D. A. HAYDON, *Disc. Faraday Soc.*, 42 (1966) 51.
27 P. MUKERJEE AND A. RAY, *J. Phys. Chem.*, 70 (1966) 2144.
28 F. REISS-HUSSON, *J. Mol. Biol.*, 25 (1967) 363.
29 H. HAUSER AND R. M. C. DAWSON, *Eur. J. Biochem.*, 1 (1967) 61.
30 D. O. SHAH AND J. H. SCHULMAN, *Lipids*, 2 (1967) 21.
31 J. H. RAPER, D. B. GAMMACH AND G. H. SLOANE-STANLEY, *Biochem. J.*, 98 (1965) 21p.
32 G. BRANTE, *Acta Physiol. Scand.*, 18 (1949) Suppl. 63.
33 A. C. JOHNSON, A. R. MCNABB AND R. J. ROSSITER, *Biochem. J.*, 43 (1948) 578.
34 W. S. SINGLETON, M. S. GRAY, M. L. BROWN AND J. L. WHITE, *J. Am. Oil Chemists' Soc.*, 42 (1963) 53.