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TEMPERATURE-DEPENDENT STRUCTURAL CHANGES IN PLANAR BILAYER MEMBRANES SOLVENT "FREEZE-OUT"

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SUMMARY

1 The structure of planar bilayer membranes formed from glycerylmonooleate in *n*-hexadecane has been investigated using precise measurements of specific geometric capacitance (C_g). Films were formed at 25–30 °C in 0.1 M NaCl and the temperature lowered at a rate of 0.1–0.2 °C/min. At about 16 °C the Plateau border (annulus) freezes but the film does not rupture. The freezing is accompanied by a large increase in C_g . $C_g = 0.625 \mu\text{F}/\text{cm}^2$ at 25 °C and $0.735 \mu\text{F}/\text{cm}^2$ at 10 °C.

2 Equations for C_g were derived which take into account the temperature-dependent changes in density and dielectric coefficient of the films above the melting point of the *n*-hexadecane. The density of *n*-hexadecane and 1-heptadecene (taken as equivalent to the glycerylmonooleate acyl chain) were measured as a function of temperature. Calculations using these equations and data indicate that the observed changes in C_g above the melting point cannot be accounted for unless there are significant changes in the composition of the bilayer.

3 The data are consistent with the following hypothesis. At 25 °C there are 7–8 *n*-hexadecane molecules per 100 glycerylmonooleate molecules in the true bilayer portions of the films. Additional solvent is trapped in microlenses. As the hexadecane freezes, the solvent in the true bilayer is disproportionated (i.e. it "freezes-out") into additional microlenses leaving extensive regions of bilayer which are largely free of solvent.

INTRODUCTION

The planar bilayer membrane first described by Mueller et al. [1] has proven to be a useful model system for biological membranes (see reviews, [2–5]). One of the major disadvantages of the system is the presence in the bilayer of the alkane solvent used for the formation of the film. White and Thompson [6] suggested that the solvent does not necessarily have a stoichiometric relation with the surface active lipids. They also demonstrated that disproportionation [7] of the solvent into mi-

microlenses can have significant effects on measurements of specific electrical capacitance. Montal and Mueller [8] have devised a method for forming solvent free bilayers from monolayers but the technique is rather difficult to use under some circumstances. During a series of experiments on thermal phase transitions in planar bilayers it was found that the annulus of the bilayer could be frozen without bilayer breakage by reducing the temperature below the freezing point of the solvent. The purpose of this paper is to report measurements of specific capacitance performed on frozen films composed of glycerylmonooleate and *n*-hexadecane. The data suggest that when the temperature is lowered beyond the freezing point of the solvent, the solvent disproportionates (i.e. "freezes-out") into microlenses leaving extensive regions of bilayer essentially free of solvent.

The effects of temperature on the structure of planar lipid bilayer membranes have been studied in several laboratories. Krasne et al [9] studied glyceride-decane membranes and found phase transitions to occur in the glycerides at about 40 °C. Phase transitions of lecithins with homogeneous acyl chain composition dispersed in *n*-decane were examined by Stark et al [10]. Both of these studies were concerned with the effects of phase transitions on antibiotic ion transport. Pagano et al [11] measured the reflectance of bilayers formed from glycerylmonosterate and *n*-hexadecane above and below the phase transition of the glycerylmonosterate (T_m of approx 60 °C). Their data suggested that the thickness of the bilayer increases below the phase transition. In none of these studies was the temperature lowered beyond the freezing point of the solvent.

CALCULATIONS

The density [12] and, consequently, the dielectric coefficient [13] of alkanes and related compounds depend upon temperature. These variations will affect the specific capacitance of bilayers and must therefore be accounted for. Fettplace et al [23] treat the two component planar bilayer membrane as a thin liquid film whose dielectric coefficient ϵ_B will be given by

$$\epsilon_B = F\epsilon_{AC} + (1-F)\epsilon_s \quad (1)$$

where F is the volume fraction of the film occupied by surfactant acyl chains, ϵ_{AC} the dielectric coefficient of the acyl chains, and ϵ_s the dielectric coefficient of the solvent. They assume the specific volumes of the acyl chains and solvent molecules in the bilayer to be the same as in bulk solution. The acyl chains and solvent molecules are also assumed to form ideal mixtures in the bilayer. Given the number of acyl chains and solvent molecules per unit area of film, it can be shown that the thickness of the bilayer δ_B is given by

$$\delta_B = \frac{N_{AC}}{N_A} \left[\left(\frac{1}{X_{AC}} - 1 \right) \left(\frac{M_s}{\rho_s} \right) + \frac{M_{AC}}{\rho_{AC}} \right] \quad (2)$$

where

$$X_{AC} = \frac{N_{AC}}{N_{AC} + N_s} \quad (3)$$

N_{AC} is the number per unit area, M the molecular weight, ρ the absolute density (g/cm^3),

and N_A Avogadro's number. The subscripts AC and S refer to the acyl chains and solvent respectively. The volume fraction (F) of the acyl chains is given by

$$F = \left[1 + \left(\frac{1}{X_{AC}} - 1 \right) \frac{M_S \rho_{AC}}{M_{AC} \rho_S} \right]^{-1} \quad (4)$$

The specific geometric capacitance (C_g) of the hydrocarbon layer of the bilayer can be calculated from equations 1 and 2 using

$$C_g = \frac{\epsilon_0 \epsilon_B}{\delta_B} \quad (5)$$

where $\epsilon_0 = 8.854 \times 10^{-14}$ F/cm, δ_B and ϵ_B will be temperature dependent because of the temperature dependence of density. The density of most alkane solvents depends linearly upon temperature [12] and consequently

$$\rho_i(T) = g_i + h_i T \quad (6)$$

g_i and h_i are constants and i is either AC or S. The dielectric coefficient of the solvent and acyl chains can be calculated from [13]

$$\epsilon_i(T) = \frac{1 + 2(\rho_i^2 m + \rho_i b_i)/M_i}{1 - (\rho_i^2 m + \rho_i b_i)/M_i} \quad (7)$$

where

$$b_i = \frac{\epsilon_i(20) - 1}{\epsilon_i(20) + 2} \frac{M_i}{\rho_i(20)} - m \rho_i(20) \quad (8)$$

and (20) means the values at 20 °C. The constant m is nearly independent of alkyl structure and has a value of about $-3 \text{ cm}^6/\text{g} \cdot \text{mol}$ (Mopsik, F. I., personal communication).

These equations can be used to calculate specific geometric capacitance (C_g) as a function of temperature given bilayer composition or, conversely, bilayer composition from measurements of C_g . The equations are probably reliable only when the bilayer is in a liquid state. The equations are used in this paper only when this condition exists.

MATERIALS AND METHODS

The method of White [14, 15] and White and Thompson [6] was used to determine the specific geometric capacitance of the bilayers with a precision of $\pm 0.3\%$ and accuracy of $\pm 1\%$. In essence, the total capacitance is measured using an AC bridge and the area using the photographic weight-area method [15]. The membrane-electrolyte system consists essentially of the membrane capacitance in series with the electrolyte resistance. The bridge measures the parallel equivalent capacitance and resistance. Consequently there is a dispersion which must be accounted for to find the actual total capacitance of the bilayer [6]. In addition, the total capacitance contains a contribution from the electrolyte double layers at the surfaces of the bilayer. White [14] has shown that these double layer capacitances in series with the geometric

capacitance of the hydrocarbon layer can be accurately calculated using well known equations [21, 22] All measurements of specific capacitance reported in this paper represent the geometric specific capacitance of the hydrocarbon layer obtained as described elsewhere [14] All measurements were made at a frequency of 100 Hz The a c voltage across the bilayer from the bridge was kept constant at 7 mV (r m s).

Unbuffered NaCl solutions (10^{-1} M) were prepared as described earlier [14] The pH of the solutions was about 6 Bilayers were formed from glycerylmonooleate (Sigma Chemical Co., St. Louis, Mo.) and 99.5% pure *n*-hexadecane (LaChat Chemicals, Chicago Heights, Ill.). The glycerylmonooleate migrated as a single spot on thin-layer plates using diethyl ether-benzene-ethanol-acetic acid (40:50:20:2, by vol.) as a developer [16] The *n*-hexadecane was purified prior to use by passage through alumina The glycerylmonooleate was dispersed in the hexadecane (20 mg/ml) after lyophilization from benzene

TABLE I

TEMPERATURE DEPENDENCE OF THE DENSITY OF *n*-HEXADECANE AND 1-HEPTADECENE

Density was measured using 0.25 ml pycnometers [17] as a function of temperature The data were fitted to the equation $\rho(T) = g + hT$ The regression coefficient in both cases was 0.998 The values of $\rho(20)$ calculated from the equation are compared in the last two columns with values published in the Chemical Rubber Company's Handbook of Chemistry and Physics, 46th Edn (C R C)

Hydrocarbon	Temperature range (°C)	$g \pm S D$ (g/cm ³)	$h \pm S D$ (10^{-4} g/cm ³ /C)	Calculated $\rho(20)$	C R C $\rho(20)$
<i>n</i> -Hexadecane	16.5–28.0	0.7864 ± 0.0003	-6.402 ± 0.133	0.7736	0.7733
1-Heptadecene	10.5–28.0	0.8015 ± 0.0003	-6.431 ± 0.139	0.7886	0.7892

The acyl chain of the glycerylmonooleate was assumed to be equivalent to 1-heptadecene for purposes of calculation The densities of 1-heptadecene and *n*-hexadecane were measured as a function of temperature using the pycnometric technique of Lipkin et al. [17] The results of these measurements are shown in Table I Measurements of the densities of various mixtures of the two compounds indicated that mixtures were nearly ideal The dielectric coefficients at 20 °C were calculated from $\epsilon = n_D^2$ where n_D is the tabulated (C R C Handbook of Chemistry and Physics, 46th Edn) index of refraction at 20 °C at the wavelength of the sodium D line $\epsilon_s(20) = 2.056$ and $\epsilon_{AC}(20) = 2.083$

Temperature was measured using a small (1 mm diameter) glass encased thermistor probe attached to a d c bridge constructed in the laboratory The temperature was controlled by circulating water through a water jacket surrounding the experimental chamber The warming or cooling rate during controlled temperature variations was about 0.1–0.2 °C/min

All calculations were performed on a Wang Corp. (Tewksbury, Mass.) 600-6TP programmable calculator

RESULTS

The bulk lipid solution was applied to the aperture at a temperature of 25–30 °C. No measurements were made for 30–60 min after the black film was formed to assure that equilibrium was achieved and that solvent disproportionation [7] was complete. Microlenses generally appeared about 3 min after full black and became well developed and evenly distributed after about 10 min. The effects of microlenses on measurements of specific capacitance are discussed in detail elsewhere [6].

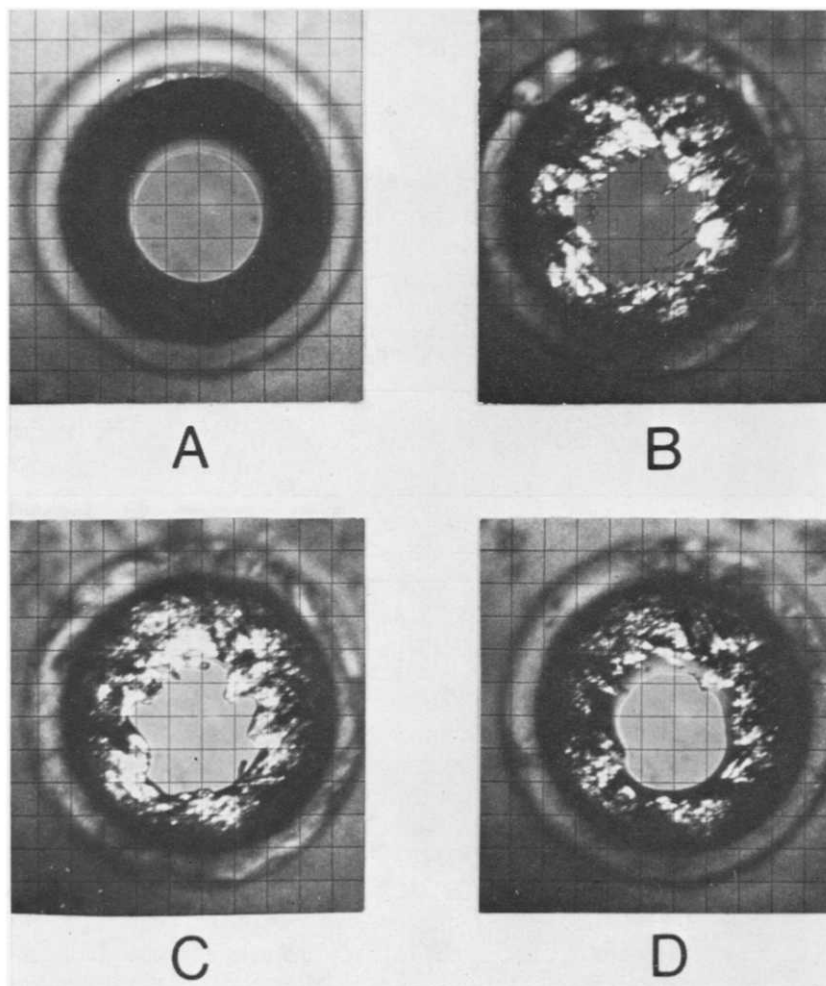


Fig. 1. Photographs illustrating the freezing and melting of the annulus-bilayer system. The diameter of the aperture is about 2 mm. The photographs were made using transmitted light. A shows the membrane and annulus at 22 °C. The system froze at about 17 °C and appeared as in B. Note the crystals extending from the annulus into the film. The early stages of melting are shown in C (18.3 °C) and D (18.37 °C). The membrane appeared as in A after melting but had a smaller area. Magnification of all photographs $\times 23$. The film was formed from glycerylmonooleate in *n*-hexadecane.

The temperature was slowly lowered after the waiting period to approx 10°C and then returned slowly to the starting temperature. The annulus freezes at approx 16°C (the approximate melting point of *n*-hexadecane) and casual observation suggests that the film breaks. However, the capacitance measurement belies this conclusion. Closer examination reveals that the film has a highly irregular border and a somewhat warped surface. These conditions complicate the area determinations. The area of bilayer was assumed to remain constant below the freezing point and five photographic determinations of area were made. The area was taken as the mean of the five measurements. The standard error of the mean was only about $\pm 0.4\%$ which compares favorably with the estimated precision ($\pm 0.3\%$) for single area determinations on liquid films above the melting point [6].

Fig 1 shows a series of photographs of a film freezing and melting. The photographs were made using transmitted light as described earlier [6, 15]. The annulus in this case froze suddenly and rapidly at about 17°C and assumed the configuration shown in the upper right hand photograph of Fig 1. Crystals (presumably solvent) extend into the bilayer but these are not seen in most cases. As the temperature was raised, the annulus slowly melted as shown in the remaining photographs of Fig 1. No film has yet been formed which ruptured upon freezing. Below the freezing point the films are stable for hours and relatively insensitive to mechanical disturbances. The films were most likely to rupture during the warming process as the annulus began to melt but this occurred in no more than about 20% of the films.

The specific geometric capacitance of a single film as a function of temperature is shown in Fig 2. These values rarely varied more than 1% from film to film. The important result shown in this figure is the large increase in specific capacitance

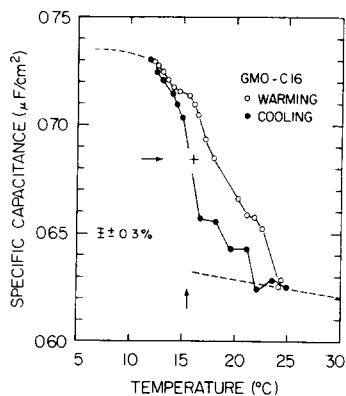


Fig 2 The specific geometric capacitance (C_g) of a single bilayer as a function of temperature. The approximate precision of each measurement is $\pm 0.3\%$. The vertical arrow indicates the temperature (15.5°C) at which the annulus of this particular membrane was observed to freeze upon cooling. The lower dashed curve shows how C_g should have varied with temperature if the temperature dependence were due to the temperature dependent density changes shown in Table I assuming there were $5.4 \cdot 10^{14}$ glycerylmonooleate (GMO) molecules per cm^2 with a mole fraction of 0.923. The cross (indicated by the horizontal arrow) shows the capacitance expected if the hexadecane solvent disproportionates during freezing to give an acyl chain mole fraction of 1.0. The conclusion suggested by these data is that solvent is "frozen-out" leaving extensive regions of bilayer essentially free of solvent.

which accompanies the freezing of the annulus. The reverse occurs during the melting of the annulus. There is a slight hysteresis which is always observed but otherwise the warming and cooling curves are quite similar. The capacitance tends to be somewhat erratic during the cooling phase but quite regular during the warming phase. This is observed in all films so far examined. There are several inflections in the curves which may be related to the thermal phase transitions in glycerylmonooleate-hexadecane-water reported by Pagano et al [11]. Andrews et al [18] have found that the specific capacitance of glycerylmonooleate films depends upon the length of solvent molecules used to form the films. In general, as the length increases, the solvent volume fraction of the film decreases with a concomitant increase in capacitance. It seems likely that the increase in capacitance observed here during cooling is partially due to the disproportionation of the *n*-hexadecane solvent. Data to be published elsewhere will show that the increase is also due to the thermal phase transition of the glycerylmonooleate which occurs at about the freezing point of the *n*-hexadecane [11].

DISCUSSION

The first point to establish is that the increase in capacitance observed upon cooling is not due to the temperature dependence of density reported in Table I. Eqns 1-8 were programmed on the Wang 600 calculator. The value of N_{AC} (0.1 M NaCl aqueous phase) was estimated from the data of Andrews et al [18] as $5.4 \cdot 10^{14} \text{ cm}^{-2}$. N_{AC} was assumed to remain constant from 16 to 30 °C. Measurements of the interfacial tension of glycerylmonooleate in *n*-hexadecane against 0.1 M NaCl (White, S. H., unpublished data) suggest this assumption is nearly correct. The mole fraction (X_{AC}) of acyl chains in the film was varied in the equations until a value of $C_g = 0.625 \mu\text{F}/\text{cm}^2$ was obtained for $T = 25 \text{ }^\circ\text{C}$. X_{AC} was found to be 0.923 while F (the volume fraction occupied by acyl chains) was found to be 0.925. The calculations were made assuming no microlenses were present. Having established a value for X_{AC} , C_g was calculated as a function of temperature holding X_{AC} constant. The results of the calculation are shown as the lower dashed curve in Fig. 2. It is apparent that the hydrocarbon density variations with temperature shown in Table I cannot explain the observations. Theory and experiment have been compared only over the region where the film-annulus system is liquid. The experimental freezing point of the system is indicated on Fig. 2 by the vertical arrow.

A reasonable hypothesis is that the abrupt increase in capacitance is caused by solvent disproportionation acting in the following way. During freezing the solvent in the bilayer per se (i.e., the regions of film containing no microlenses) condenses into frozen microdroplets trapped in the bilayer leaving extensive regions of bilayer essentially free of solvent. Frozen microdroplets are expected to coexist with the bilayer because the annulus-bilayer system is stable upon freezing. The effects of microlenses on capacitance can be calculated [6] given the average contact angle and radius of the lenses. Without resorting to this calculation, however, an upper limit for the specific capacitance C_g can be established by ignoring the lenses and taking $X_{AC} = 1$ for $N_{AC} = 5.4 \cdot 10^{14} \text{ cm}^{-2}$. It is an upper limit because the relatively thick lenses act to decrease specific capacitance. The value of C_g obtained under these conditions at 16 °C is $0.684 \mu\text{F}/\text{cm}^2$ and is indicated by a cross (opposite horizontal

arrow) on Fig 2. Assuming N_{AC} has the correct value, the calculation suggests that the bilayer is solvent free about midway through the capacitance transition. The remainder of the capacitance increase is likely due to the phase transition of the glycerylmonooleate. This however is the subject of a separate paper and will not be discussed here.

The above calculations were performed ignoring the existence of the microlenses. Pagano et al [19] have made direct measurements of the composition of glycerylmonooleate-hexadecane films. Their data can be used to calculate the effects of microlenses on the capacitance. They report that at 25 °C $N_{AC} = 4.7(\pm 0.4) \cdot 10^{14} \text{ cm}^{-2}$ and $N_S = 2.8(\pm 0.7) \cdot 10^{14} \text{ cm}^{-2}$. This value of N_{AC} is smaller than that estimated from the data of Andrews et al [18]. Eqn 2 shows that bilayer thickness (δ_B) is directly proportional to N_{AC} . Consequently, the smaller value of N_{AC} will lead to larger values of specific capacitance. The values of N_{AC} and N_S given by Pagano et al. [11] give an F value of 0.63. If all of the solvent is distributed uniformly in the bilayer, C_g is calculated to be $0.490 \mu\text{F}/\text{cm}^2$ at 25 °C which is much smaller than observed. The conclusion must be that much of the solvent is distributed as microlenses. The effects of microlenses were estimated using the equations of White and Thompson [6] assuming a lens diameter of approx $1 \mu\text{m}$ (10^{-4} cm) [7] and a lens-bilayer contact angle of 2° [20]. For $T = 25^\circ\text{C}$, the calculated value of C_g is $0.624 \mu\text{F}/\text{cm}^2$ assuming $N_{AC} = 4.7 \cdot 10^{14} \text{ cm}^{-2}$ and $N_S = 3.5 \cdot 10^{13} \text{ cm}^{-2}$ with $2.45 \cdot 10^{14} \text{ cm}^{-2}$ solvent molecules distributed in about $2 \cdot 10^7$ microlenses/ cm^2 . This number of microlenses would occupy about 14 % of the apparent bilayer area. If, upon freezing, the solvent in the bilayer also disproportionates into lenses, then at 16 °C the specific geometric capacitance would be about $0.662 \mu\text{F}/\text{cm}^2$. This value is smaller than both the observed value and the value calculated earlier on the basis of the data of Andrews et al [18]. An upper limit for the capacitance for $N_{AC} = 4.7 \cdot 10^{14} \text{ cm}^{-2}$ can be obtained by assuming $X_{AC} = 1.0$ and that there are no lenses. The value of C_g in this case is $0.735 \mu\text{F}/\text{cm}^2$ which is about the value observed at 10 °C.

The above calculations support the hypothesis that upon freezing the solvent molecules of the bilayer disproportionate into microlenses leaving large areas (86 % or more) of bilayer which are largely free of solvent.

Another observation of importance is the close similarity of the warming and cooling specific capacitance curves. If the hypothesis of solvent "freeze-out" is correct, then upon warming the solvent must return to the bilayer in the same mole fraction as prior to freezing. The obvious conclusion is that there is a stoichiometric relation between solvent molecules and acyl chains in the bilayer portions of the film. X_{AC} at 25 °C calculated from the data of Andrews et al [18] assuming no lenses was 0.923 while the data of Pagano et al [19] assuming lenses gives $X_{AC} = 0.931$. It appears that there are only 7 or 8 *n*-hexadecane molecules for every 100 glycerylmonooleate molecules.

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