*Biochimica et Biophysica Acta*, 772 (1984) 1–9
Elsevier



BBA 72023

# THE THICKNESS OF THE HYDROPHOBIC AND POLAR REGIONS OF GLYCEROL MONOOLEATE BILAYERS DETERMINED FROM THE FREQUENCY-DEPENDENCE OF BILAYER CAPACITANCE

D.R. LAVER *, J.R. SMITH ** and H.G.L. COSTER ***

*Biophysics Laboratory, School of Physics, University of New South Wales, Sydney, NSW 2033 (Australia)*

(Received July 28th, 1983)
(Revised manuscript received December 5th, 1983)

*Key words: Capacitance; Bilayer thickness; Dielectric structure; Glycerol monooleate bilayer; Frequency dependence*

**The frequency dependence of the equivalent parallel capacitance and conductance of glycerol monooleate bilayers formed with *n*-hexadecane and squalene solvents has been measured. The capacitance of such bilayers was always found to decrease by 3–5% with increasing frequency over the range 0.1 Hz to 5000 Hz. For KCl concentrations equal to or above 0.1 M it was found that the capacitance of the hydrophobic region was in the range 6.5–7.0 mF/m$^2$ for bilayers formed with *n*-hexadecane and 7.6–7.9 mF/m$^2$ for squalene. By fitting a model of a multilaminar dielectric sandwich to the experimental data it was possible to determine the equivalent parallel capacitance and conductance of 4 or 5 distinct layers within the bilayer. Using their electrical time constants as a guide, the dielectric constant within each polar region was estimated and consequently the thickness of each substructural region could be calculated from its measured capacitance. In this fashion the overall thickness of each polar region was estimated to be in the range 0.5–0.75 nm. We compare our results with those calculated previously from a combination of optical and high-frequency capacitance measurements, and a discussion is given of the magnitude of the systematic errors that will ensue as a consequence of the neglect of the frequency dependence of the bilayer capacitance and how these errors can be reduced.**

## Introduction

Although not a lipid typically present in cell membranes, planar bilayers formed from glycerol monooleate have in recent years been frequently studied. In part this is related to the relative ease with which bilayers containing low solvent concentrations may be generated (e.g., if *n*-hexadecane [1] or squalene [2] is used as the solvent). Furthermore it is possible to achieve good reproducibility in such experiments. For comparison with various theoretical models it is of considerable interest to determine the relative dimensions of the polar and hydrophobic regions within such single planar glycerol monooleate bilayers. Towards this end two experimental approaches have been previously used for planar bilayers.

One approach utilises measurements of the optical reflectance of the bilayer surface to determine the thickness of the bilayer. Unless the refractive index of the bilayer is independently known, e.g. by measurement of the Brewster angle, the reflectance must usually be measured as a function of either the angle of incidence or the

* Present address: School of Chemistry, University of Sydney, N.S.W., Australia 2006.
** Present address: Biophysics Laboratory, School of Biological Sciences A12, University of Sydney, N.S.W., Australia 2006.
*** To whom reprint requests should be addressed.

refractive index of the aqueous medium. Assuming that the overall properties of the bilayer remain unaltered by this latter procedure, the overall thickness, $\delta$, (i.e., hydrophobic plus polar regions) of the bilayer can then be calculated (see, for example, Ref. 3). Theoretical studies suggest that the interpretation of such results should be essentially independent of the nature of any bilayer substructure [4]. The results of these optical measurements are then combined with electrical measurements in the following fashion. If the dielectric constant $\varepsilon_H$ of the hydrophobic region can be estimated, the thickness $\delta_H$ of this region can then be calculated from its capacitance $C_H$ via the relation

$$\delta_H = \varepsilon_0 \varepsilon_H / C_H \tag{1}$$

where $\varepsilon_0$ is the permittivity of free space.

It is generally assumed that the capacitance, $C^*$, of the bilayer measured at high frequencies, where its capacitance is essentially frequency independent, is equal to that of the hydrophobic region $C_H$. If this assumption were correct then

$$\delta_H = \varepsilon_0 \varepsilon_H / C^* \tag{2}$$

The thickness $\delta_P$ of each polar region (assuming symmetry) is simply given by

$$2\delta_P = \delta - \delta_H \tag{3}$$

This combination of optical and electrical measurements has been used to determine $\delta_P$ for egg phosphatidylcholine [3] and glycerol monooleate [5] bilayers.

A difficulty with the above approach is that the presence of the dielectric substructure associated with the hydrophobic and polar regions requires, as a necessary consequence, that the capacitance and conductance of the bilayer itself must be frequency dependent because of interfacial polarisation (see, for example Refs. 6 and 7). If, as a first approximation, the bilayer substructure is assumed to consist only of hydrophobic and polar regions, it can be shown that for frequencies much higher than that of the consequent Maxwell-Wagner dispersion

$$C^* = C_H / (1 + (2C_H / C_P)) \tag{4}$$

where $C_P$ is the capacitance of each polar region.

Then $C^*$ must under-estimate $C_H$ and consequently $\delta_H$ calculated by Eqn. 2 must, to some extent, be in error. This error will then be magnified significantly when $\delta_P$ is calculated via Eqn. 3. To correct for this source of error a knowledge of the frequency dependence of the bilayer capacitance due to its intrinsic substructure is required so that a correct value can be calculated for $C_H$ and hence $\delta_H$ via Eqn. 1.

An alternative approach to this whole problem is based solely upon electrical measurements. Although Hanai et al. [8] using measurements on egg phosphatidylcholine bilayers concluded "that no dielectric dispersion resulting from the presence of the polar groups is either predicted or detected", subsequent higher precision measurements performed in this laboratory [6,7] have shown that their measured capacitance is indeed frequency dependent. By fitting the theoretical behaviour of a multilaminar dielectric sandwich to such experimental data it is then possible to calculate the equivalent dielectric parameters, not only for the polar-head and hydrophobic regions, but also under some conditions for finer substructural divisions [9–11]. Once the capacitance $C_i$ and dielectric constant $\varepsilon_i$ of a substructural region within a bilayer are estimated, then the thickness $\delta_i$ of this region can be calculated via

$$\delta_i = \varepsilon_0 \varepsilon_i / C_i \tag{5}$$

The main difficulty in this latter approach lies in estimating the dielectric constants within the polar regions.

In this paper we report the results of measurements of the frequency dependence of the capacitance and conductance of glycerol monooleate bilayers formed with squalene and *n*-hexadecane solvents. Analysis of this dielectric data then provides an independent estimate of the dimensions of the polar and hydrophobic regions within these bilayers. Comparison is made with values of $\delta_H$ and $\delta_P$ determined previously by Dilger [5] via a mixture of optical and high-frequency capacitance measurements and it is shown how errors associated with this latter technique can be reduced.

## Materials and Methods

Bilayers were generated from films of glycerol monooleate with either squalene or *n*-hexadecane

solvent. The films were placed across a hole (diameter approx. 1.5 mm) in a polycarbonate septum which divided a chamber containing the aqueous solutions of KCl at 20–25°C. When the membrane capacitance had settled sufficiently (varying by less than 0.1% in 300 s), a series of measurements (scans) of the membrane impedance over a range of frequencies was commenced. Each scan involved measuring the membrane impedance at 20–30 different frequencies spanning the range from 0.1 Hz up to 5000 Hz. Each such scan of frequencies lasted approximately 1 ks. Full details of the low-frequency, digital impedance spectrometer in four terminal operation (precision in measurement of $C$ better than 0.1%) are given elsewhere in conjunction with the details of fitting a model of a multilaminar dielectric sandwich to the experimental data [10–12]. The r.m.s. amplitude of the sinusoidal voltage across the bilayer was always less than 10 mV. At this voltage level the electrical behaviour of the bilayer was found to be extremely linear, and the correlation index between the measured sinusoidal voltage responses and the sinusoidal least-squares fit typically exceeded 0.9999.

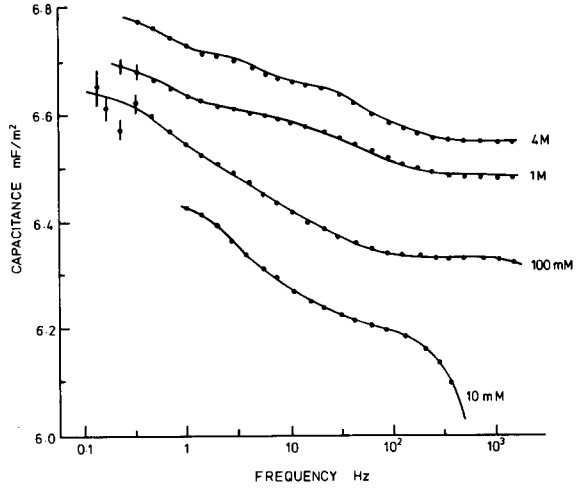


Fig. 1. The equivalent parallel capacitance as a function of frequency for glycerol monooleate/*n*-hexadecane bilayers formed in 0.01 M, 0.1 M, 1 M and 4 M KCl electrolyte. The capacitance of bilayers formed in 1 mM KCl was too small to appear on this figure. The data shown for bilayers formed in 0.1 M and 1 M KCl are each the average for two successive frequency scans. The error bars, which are smaller than the datum points at all but very low frequencies, illustrate the experimental scatter. The solid curves show the theoretical behaviour of a multilaminar dielectric sandwich with parameter values that produced a best fit to the experimental data.

## Results

Bilayers formed from glycerol monooleate were found to thin rapidly and to typically attain a steady capacitance within 300–500 s. However, the lifetimes of glycerol monooleate bilayers formed with squalene were found to be quite short and rarely exceeded 1 ks. Consequently, the frequency dependence of their impedance could not be satisfactorily measured at an adequate number of frequencies over a sufficiently large frequency range to yield detailed information on their dielectric substructure. For a KCl concentration of 0.1 M, the capacitance at 1 Hz of glycerol monooleate/squalene bilayers was found to be in the range 7.5–7.8 mF/m$^2$ (10 bilayers). To obtain an estimate of the frequency dependence of the capacitance of each glycerol monooleate/squalene bilayer, its capacitance was alternately measured at 1 Hz and 1000 Hz. The capacitance measured at 1000 Hz was found to be less than that measured at 1 Hz by 0.18–0.22 mF/m$^2$, this corresponded to a dispersion in capacitance of 2.5–3.1%.

Bilayers formed with *n*-hexadecane were more stable (lifetimes up to 4 ks). Thus measurements of their impedance dispersion with frequency could be made in detail over a range sufficiently large to yield detailed information of their substructure. For many bilayers, however, only one complete frequency scan spanning the range 0.1 to 1000 Hz was feasible before the bilayer 'broke'. For the occasional bilayer with an unusually long lifetime, it was possible to repeat the scan of frequencies and it was found that the dispersion in membrane capacitance obtained from consecutive scans was reproducible to better than 0.2% for frequencies above 1 Hz. Some representative results for the measured frequency dependence of the capacitance of glycerol monooleate bilayers formed with *n*-hexadecane in aqueous solutions of KCl ranging in concentration from 10 mM to 4 M are shown in Fig. 1. A representative variation of the measured bilayer conductance with frequency is shown in Fig. 2. The measured capacitance was always found to decrease with increasing frequency. It is apparent from Fig. 1 that for bilayers with KCl

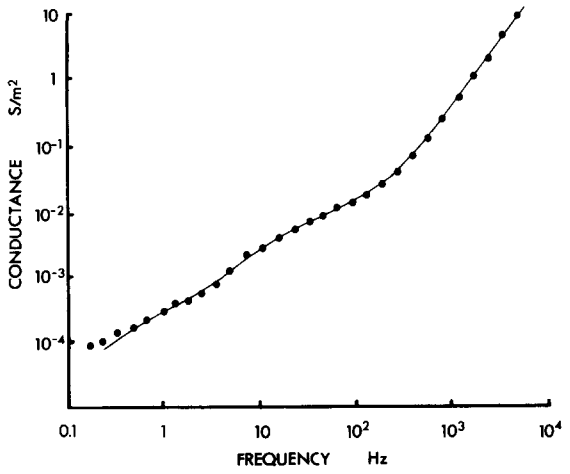


Fig. 2. The measured frequency dependence of the equivalent parallel conductance of a glycerol monooleate/*n*-hexadecane bilayer formed in 1 M KCl. The frequency dependence of the capacitance (measured simultaneously) of this particular bilayer is given in Fig. 1. The solid line is the theoretical behaviour of a multilaminar dielectric sandwich with the same parameter values as were used for Fig. 1.

concentration equal to or above 0.1 M, the capacitance measured at approx. 1000 Hz will typically be smaller than the low-frequency value by 3–5%. When the external concentration of KCl decreases, the conductance of the external electrolyte located in-between the bilayer surface and the voltage-measuring electrodes will also decrease. This will then increase the magnitude of the resultant dispersion between bilayer and electrolyte at frequencies above 100 Hz [7], and is responsible for the greater frequency dependence of the measured capacitance of the bilayer/electrolyte system when the concentration of KCl is below 0.1 M.

*Dielectric substructure*

Examination of the data shown in Fig. 1 for bilayers formed with KCl concentrations equal to or above 0.1 M, where the contribution of the external electrolyte to the frequency dispersion will be very small, reveals that more than one frequency dispersion is present in the bilayer capacitance. Thus the dielectric substructure of glycerol monooleate bilayers is more complicated than that of a simple homogeneous hydrophobic region bounded by uniform polar regions, and in this regard is similar to phosphatidylcholine and phosphatidylcholine/cholesterol bilayers [10,11]. However, bilayers formed with glycerol monooleate differed from those formed with phosphatidylcholine [11] in that the capacitance of glycerol monooleate bilayers above 300 Hz was essentially independent of frequency up to 5 kHz. (Because 5 kHz was the upper limit of measurement with our present apparatus, we were unable to determine whether additional dispersions in capacitance at still higher frequencies were present, as reported by Sargent [13] for oxidised cholesterol bilayers and by Stark and Gisin [14] for glycerol monooleate and dioleoylphosphatidylcholine bilayers. However, these authors did not think that these additional high-frequency dispersions were associated with the dielectric substructure. Thus it appears that our equivalent circuit is adequate to describe the dielectric substructure, but extra elements might be necessary to describe any additional dispersion (if present) above 5 kHz.)

The continuous lines in Fig. 1 are the theoretical fits to the experimental data using a model for the bilayer composed of either four or five dielectrically distinct layers (i.e., layers with different electrical time constants). The parameters associated with the dielectric substructure of glycerol monooleate/*n*-hexadecane bilayers formed in 1 mM, 0.1 M and 4 M KCl are given in Table I. We have ascribed the layer with the largest electrical time constant to the hydrophobic region, and have followed other authors [2,15], in assuming that $\varepsilon_H = 2.16$ for glycerol monooleate/*n*-hexadecane and $\varepsilon_H = 2.2$ for glycerol monooleate/squalene. The dielectric constant associated with each individual polar region was estimated using its electrical time constant $\tau (= C/G)$ as a guide (see Appendix) and its thickness $\delta_i$ then calculated using Eqn. 5. The overall thickness $\delta_P$ $(= \Sigma\delta_i)$ and capacitance $C_P$ $(= 1/(\Sigma(1/C_i)))$ of the polar regions were then calculated by appropriate summation of the individual substructural values.

*Variation of C with KCl concentration*

The measured dependence of $C_H$ upon KCl concentration for glycerol monooleate/*n*-hexadecane bilayers is shown in Fig. 3. The overall decrease in $C_H$ observed with decreasing KCl concentration is similar to that reported previously by White [16] for glycerol monooleate/*n*-decane bi-

TABLE I

THE PARAMETERS OF DIELECTRICALLY-DISTINCT SUBSTRUCTURAL REGIONS WITHIN GLYCEROL MONOOLEATE/*n*-HEXADECANE BILAYERS

| Region | $C_i$ (mF/m$^2$) | $G_i$ (mS/m$^2$) | $\tau_i$ (ms) | $\varepsilon_i$ | $\delta_i$ (nm) |
|---|---|---|---|---|---|
| 1 mM KCl, three bilayers | | | | | |
| Hydrophobic | 5.7 ± 0.2 | 0.1–0.3 | $(19–57)\cdot10^3$ | 2.16 | 3.24–3.48 |
| Polar 1 | 500 ±100 | $(2–4)\cdot10^2$ | $(1–3)\cdot10^3$ | 4–5 | 0.06–0.07 |
| Polar 2 | 860 ±200 | $(1–3)\cdot10^3$ | 220–1000 | 5–14 | 0.05–0.19 |
| Polar 3 | 1100 ±200 | $(1–2)\cdot10^4$ | 45–130 | 17–22 | 0.12–0.22 |
| Polar 4 | 1000 ±200 | $(8–10)\cdot10^4$ | 8–15 | 28–33 | 0.21–0.37 |
| Total polar | 200 ± 80 | | | | 0.60 ± 0.12 |
| 100 mM KCl, three bilayers | | | | | |
| Hydrophobic | 6.7 ± 0.2 | 0.1–1.0 | $(6.7–67)\cdot10^3$ | 2.16 | 2.77–2.94 |
| Polar 1 | 700 ±100 | $(1.5–3)\cdot10^3$ | 200–530 | 10–15 | 0.11–0.22 |
| Polar 2 | 850 ±100 | $(1–1.5)\cdot10^4$ | 50–95 | 19–22 | 0.18–0.26 |
| Polar 3 | 1200 ±100 | $(1–2)\cdot10^5$ | 5.5–13 | 30–37 | 0.20–0.30 |
| Total polar | 300 ± 60 | | | | 0.64 ± 0.08 |
| 4 M KCl, six bilayers | | | | | |
| Hydrophobic | 6.8 ± 0.2 | 1–3 | $(2.2–6.9)\cdot10^3$ | 2.16 | 2.73–2.90 |
| Polar 1 | 1300 ±200 | $(1–2)\cdot10^3$ | 550–1500 | 5–9.5 | 0.03–0.08 |
| Polar 2 | 1500 ±300 | $(3–5)\cdot10^4$ | 24–60 | 21–25 | 0.1–0.18 |
| Polar 3 | 1500 ±300 | $(2–4)\cdot10^5$ | 3–4.5 | 38–42 | 0.19–0.31 |
| Polar 4 | 1700 ±300 | $(1–2)\cdot10^6$ | 0.7–2 | 46–56 | 0.2–0.36 |
| Total polar | 370 ±120 | | | | 0.73 ± 0.10 |

layers. To investigate whether this decrease in $C_H$ reflects an actual increase in $\delta_H$ or is a consequence of the presence of electrical double layers external to the bilayer [16,17], we have indicated on Fig. 3 the theoretical relationship that would be expected if $\delta_H$ was independent of KCl concentration and if electrical double layers were present adjacent to the surface of an uncharged, unstructured bilayer. The agreement with the experimental data suggests that, at least for these bilayers, $\delta_H$ itself might be relatively independent of KCl concentration. For KCl concentrations greater than 0.1 M the dependence of $C_H$ upon KCl concentration for glycerol monooleate/*n*-hexadecane bilayers was within the experimental error, and the 10% difference in capacitance between 0.1 M and saturated electrolyte reported previously [1] for glycerol monooleate/*n*-hexadecane bilayers in NaCl was not evident.

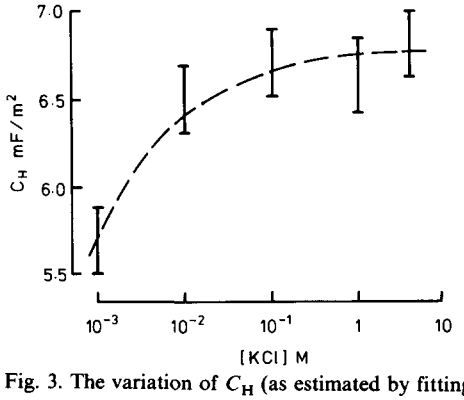


Fig. 3. The variation of $C_H$ (as estimated by fitting the experimental data) as a function of the external KCl concentration for glycerol monooleate/*n*-hexadecane bilayers. Error bars indicate the experimental scatter. The dashed line shows the theoretical dependence which would occur if $C_H$ was independent of the KCl concentration (and $= 6.8$ mF/m$^2$), and if electrical double layers were present in the external electrolyte adjacent to the (uncharged) bilayer surface [17].

## Discussion

The values of $C_H$ derived herein from the measured frequency dependence of the capacitance of

glycerol monooleate bilayers formed with *n*-hexadecane (see Table I) are consistently higher than those reported previously for electrolyte concentrations equal to or above 0.1 M (e.g., $5.80 \pm 0.04$ mF/m$^2$ at 550 Hz [18]; 6.1–6.25 mF/m$^2$ at 100 Hz [19,20]; $5.84 \pm 0.03$ mF/m$^2$ at 550 Hz [21]). A reason for some of this difference is apparent in Fig. 1 which shows that the capacitance measured at frequencies above 100 Hz will certainly underestimate the true low frequency value (denoted by $C_L$) which is a reasonable approximation to $C_H$ (see, for example, Ref. 7). (The discrepancy between $C_H$ and $C_L$, the latter measured at frequencies below that of the Maxwell-Wagner dispersion produced by the hydrophobic and polar regions, can be shown to be approximately given by $C_L - C_H = 2C_P(G_H/G_P)^2$. Our measurements revealed that the ratio $G_H/G_P$ was typically $10^{-4}$ and in this situation the discrepancy could be less than one part per million.)

Our four-terminal measurements of $C$ at 1 kHz for glycerol monooleate/squalene bilayers (7.3–7.6 mF/m$^2$) are comparable with those reported previously for two-terminal measurements at high frequencies (e.g. $7.771 \pm 0.041$ mF/m$^2$ at 100 Hz [2]; $7.94 \pm 0.13$ mF/m$^2$ at 1 kHz [22]; $7.58 \pm 0.07$ mF/m$^2$ at 500 Hz [23]; 7.44–7.51 mF/m$^2$ at 500 Hz [5]). They are sufficiently close to $C$ reported for bilayers made by monolayer apposition (e.g., $7.45 \pm 0.24$ mF/m$^2$ at 2 kHz [24]; $7.5 \pm 0.3$ mF/m$^2$ at 5 kHz [25]; $7.9 \pm 0.1$ mF/m$^2$ at 5 kHz [26]) to suggest that solvent concentrations in the bilayers reported herein were also low.

The magnitude of the measured decrease in $C$ between 1 Hz and 1 kHz was similar for bilayers formed with squalene or *n*-hexadecane (see Fig. 1). Thus we will assume, as a first approximation, that the dielectric substructures of glycerol monooleate bilayers formed with squalene and *n*-hexadecane differ only in their values of $C_H$.

*Determination of $\delta_H$ and $\delta_P$*

Depending upon the nature of the available data, there are several methods, of varying accuracy, which could be employed to calculate $\delta_H$ and $\delta_P$. Five different methods using either electrical measurements alone (methods 1, 2) or a combination of optical and electrical measurements (methods 3, 4, 5) will now be discussed and values calculated for $\delta_H$ and $\delta_P$.

*Method 1: From detailed dielectric measurements.* From our detailed measurements of $C$ and $G$ as a function of frequency it was possible to estimate $C_i$ and $G_i$ for each substructural region.

TABLE II

ESTIMATED THICKNESS OF HYDROPHOBIC AND POLAR REGIONS OF GLYCEROL MONOOLEATE BILAYERS

Values of $C_L$ and $C^*$ are from our measurements, except the two bracketed values of $C^*$ measured by Dilger [5] used in method 4. The values of $\delta$ used in methods 3–5 were also measured optically by Dilger. The solvents used were *n*-hexadecane (C16) and squalene (SQL) and it was assumed that $\varepsilon_H = 2.16$ and 2.2 respectively. For methods 2 and 5 it was assumed that $\varepsilon_P = 25$.

| | Solvent | [KCl] (M) | $C_L$ (mF/m$^2$) | $C^*$ (mF/m$^2$) | $\delta_H$ (nm) | $\delta_P$ (nm) | $\delta$ (nm) |
|---|---|---|---|---|---|---|---|
| Method 1 | C16 | 4 | 6.80 | – | 2.81 | 0.73 | 4.27 |
| Detailed dielectric measurements | C16 | 0.1 | 6.70 | – | 2.85 | 0.64 | 4.13 |
| Method 2 | C16 | 0.1 | 6.70 | 6.42 | 2.85 | 0.72 | 4.29 |
| From $C_L$ and $C^*$ | SQL | 0.1 | 7.80 | 7.52 | 2.50 | 0.53 | 3.56 |
| Method 3 | C16 | 0.1 | 6.70 | – | 2.85 | 0.59 | 4.03 |
| From $\delta$ and $C_L$ | SQL | 0.1 | 7.80 | – | 2.50 | 0.58 | 3.66 |
| Method 4 | C16 | 0.1 | – | (5.85) | 3.27 | 0.38 | 4.03 |
| From $\delta$ and $C^*$ | SQL | 0.1 | – | (7.44) | 2.62 | 0.52 | 3.66 |
| | C16 | 0.1 | – | 6.42 | 2.98 | 0.53 | 4.03 |
| | SQL | 0.1 | – | 7.52 | 2.59 | 0.54 | 3.66 |
| Method 5 | C16 | 0.1 | – | 6.42 | 2.88 | 0.58 | 4.03 |
| From $\delta$, $C^*$ and $\varepsilon_P$ | SQL | 0.1 | – | 7.52 | 2.49 | 0.59 | 3.66 |

Using the time constant, $\tau_i$, for each region as a guide it was possible to estimate $\varepsilon_i$ (see Appendix) and hence the width $\delta_i$ of each region was calculated (see Table I). Addition of the appropriate widths then gave $\delta_H$ and $\delta_P$ for glycerol monooleate/*n*-hexadecane bilayers (see Table II).

*Method 2: From $C_L$ and $C^*$.* If detailed dielectric measurements are unavailable and consequently method 1 is unsuitable, the following simplified method can be used. Because $C_L$ is approximately equal to $C_H$ (see note above), $\delta_H$ can be calculated from $C_L$ via Eqn. 1. To evaluate $\delta_P$ an estimate of the average dielectric constant $\varepsilon_P$ within the polar regions is necessary. The following equation can then be used (derived from Eqns. 1, 3 and 4)

$$\delta_P = \varepsilon_0\varepsilon_P(C_L - C^*)/2C_LC^* \quad (6)$$

This equation assumes that only homogeneous polar and hydrophobic regions are present. The results based upon detailed dielectric measurements presented herein for glycerol monooleate/*n*-hexadecane bilayers, and also those for egg phosphatidylcholine bilayers [11], suggest that the effective average $\varepsilon_P = 25 \pm 5$. The results for glycerol monooleate/squalene and glycerol monooleate/*n*-hexadecane bilayers presented in Table II were calculated in this fashion using Eqn. 6. The data shown in Fig. 1 suggest that the capacitance measured at 1 Hz will under-estimate $C_L$ by approx. 1% and this was corrected for in the calculations for glycerol monooleate/squalene bilayers.

*Method 3: From $\delta$ measured optically and $C_L$.* Calculation of $\delta_P$ from methods 1 and 2 requires some estimate of $\varepsilon_P$ which is unnecessary if optical measurements of $\delta$ are available. Because $C_H \sim C_L$, $\delta_H$ can be calculated from Eqn. 1 and $\delta_P$ from Eqn. 3. The data given in Table II combine the optical measurements of $\delta$ by Dilger [5] with the values of $C_L$ reported in this paper.

*Method 4: From $\delta$ measured optically and $C^*$.* In this method, which has been used previously [3,5], the presence of any frequency dependence in the bilayer capacitance is neglected. Thus $C_H$ is assumed equal to $C^*$ and consequently $\delta_H$ is calculated via Eqn. 2 and $\delta_P$ via Eqn. 3. However our results clearly demonstrate that neglect of the frequency dependence of the bilayer capacitance associated with its intrinsic substructure will produce significant systematic errors in determining this same substructure. Therefore calculation of $\delta_H$ from $C^*$ (rather than $C_L$) using Eqn. 2 must overestimate the true value. The associated error can be estimated by two methods. Firstly the measurements of the frequency dependence of the bilayer capacitance shown in Fig. 1 indicate that this error might be typically 3–5%. Alternatively the error can be estimated by calculating the difference between $C^*$ and $C_H$ using Eqns. 4, 5 and values for $C_H$ and $\delta_P$ from other experiments (this is a more direct approach than that used by Dilger, Fisher and Haydon [15]). As an example if $C_H = 7$ mF/m$^2$, $\delta_P = 0.5$ nm [5], and $\varepsilon_P$ varied from 10 to 30, the discrepancy between $C^*$ and $C_H$ could vary between 7 and 2.5%. $\delta_H$ calculated from Eqn. 2 would then vary by the same extent. Because Eqn. 3 involves the difference between parameters of similar magnitude, any error in $\delta_H$ will be considerably magnified when $\delta_P$ is calculated. This method has been applied to the optical and capacitance measurements of Dilger [5], and also our measurements of $C^*$, in Table II.

*Method 5: From $\delta$ measured optically. $C^*$ and $\varepsilon_P$.* This method (like Method 4) is applicable where sufficiently detailed measurements of the frequency dependence of $C$ are not available, perhaps due to problems associated with bilayer instability similar to those we report here for glycerol monooleate/squalene bilayers, or perhaps because appropriate apparatus to measure the very low frequency capacitance is unavailable. Rearrangement of Eqns. 1, 3, 5 and 6 gives the following relationships

$$\delta_P = [\varepsilon_P/(\varepsilon_P - \varepsilon_H)][\delta - (\varepsilon_0\varepsilon_H/C^*)]/2 \quad (7)$$

$$\delta_H = [\varepsilon_H/(\varepsilon_P - \varepsilon_H)][(\varepsilon_0\varepsilon_P/C^*) - \delta] \quad (8)$$

Thus an estimate of $\varepsilon_P$ will permit more accurate evaluations of $\delta_H$ and $\delta_P$ using Eqns. 7 and 8 than will occur if Eqns. 2 and 3 are used as in method 4. For example if $\delta = 4$ nm and $C^* = 6.5$ mF/m$^2$, neglect of the dielectric substructure (method 4) would yield values of $\delta_H = 3.00$ nm and $\delta_P = 0.50$ nm (assuming $\varepsilon_H = 2.2$). Once the dielectric substructure is considered, however, more accurate values would be $\delta_H = 2.72$ nm and $\delta_P =$

0.64 nm if $\varepsilon_P = 10$, and $\delta_H = 2.92$ nm and $\delta_P = 0.54$ nm if $\varepsilon_P = 30$. We have used this method in conjunction with our measurements of $C^\star$ and those of $\delta$ measured optically by Dilger [5] (see Table II).

It now remains to discuss the relative merits of each of these five methods. The most accurate and unequivocal is probably method 3, but at present this combination of optical and low-frequency electrical measurements has not been performed upon the same bilayer. For a mixture of optical and high-frequency electrical measurements, method 5 should always be more accurate than method 4, despite the uncertainty in $\varepsilon_P$. The methods based solely on impedance measurements (methods 1 and 2) require an estimation of the local $\varepsilon$ within the bilayer. However the agreement between $\delta$ calculated by methods 1 and 2 and $\delta$ measured optically by Dilger [5] (see Table II) suggests that the assumptions involved in estimating $\varepsilon$ are not unreasonable. Method 2 will be generally less accurate than method 1 because it assumes homogeneous polar regions, whereas method 1 is more consistent with the experimental data in that this restriction is unnecessary.

Both our dielectric determination of $\delta$ and the optical determination of $\delta$ of Dilger [5], are significantly smaller than some other optical determinations of $\delta$ (e.g., 4.9 nm [27] for glycerol monooleate/*n*-hexadecane; 5.7 nm [28] for glycerol monooleate/squalene). It is also interesting that if the optical data of Dilger is re-analysed in conjunction with our capacitance measurements (see method 4 in Table II), the difference between $\delta_P$ for glycerol monooleate/*n*-hexadecane and glycerol monooleate/squalene bilayers originally reported by Dilger is significantly reduced.

In conclusion we have demonstrated that the capacitance of glycerol monooleate bilayers is frequency dependent in a similar fashion to that reported previously for egg phosphatidylcholine and egg phosphatidylcholine/cholesterol bilayers [11]. By estimating the behaviour of $\varepsilon$ within the bilayer it has been possible to calculate $\delta_H$ and $\delta_P$ and these were found to be in agreement with those obtained by optical measurements. It has also been shown how to correct for the small systematic errors that will occur in the estimation of $\delta_P$ if only high-frequency measurements of the capacitance are used without regard to its frequency dependence.

## Appendix

*Estimation of dielectric constant from electrical time constant*

Evaluation of the width of each substructural region within the bilayer from its individual measured capacitance requires an estimate of the effective dielectric constant $\varepsilon$ within each region. Because our measurements yield both the equivalent parallel capacitance $C$ and conductance $G$ of each region, one possible approach is to utilise the electrical time constant $\tau$ ($= C/G$) of each region as an indicator of the value of $\varepsilon$. This approach is feasible because, although the dielectric constant $\varepsilon$ only varies by a factor of approx. 35 from the external electrolyte ($\varepsilon \sim 78.5$) to the bilayer interior ($\varepsilon \sim 2.2$), the conductivity $g$ varies by a very large amount (for example in 1 M KCl electrolyte $g \sim 1$ S/m whereas in the bilayer interior $g \sim 10^{-14}$ S/m). The electrical time constant $\tau = C/G = \varepsilon_0 \varepsilon / g$ will thus vary enormously from the polar/external electrolyte interface to the polar/hydrophobic interface, and should primarily depend upon the local value of $g$ rather than $\varepsilon$. Thus if the relationship between $\tau$ and $\varepsilon$ within the

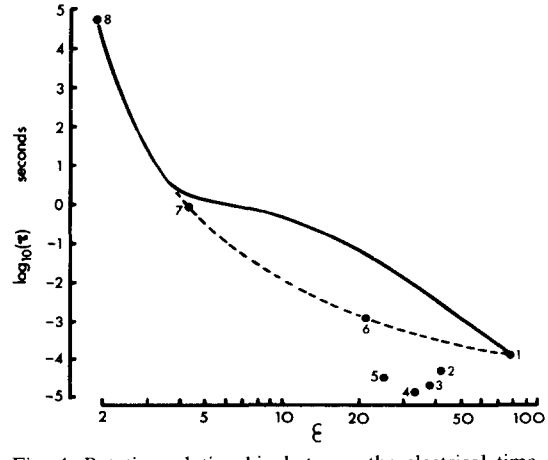


Fig. 4. Putative relationship between the electrical time constant $\tau$ and the dielectric constant $\varepsilon$ of substructural regions within a glycerol monooleate bilayer. The numbered data points refer to the following compounds; (1) water; (2) glycerol; (3) glycol; (4) methanol; (5) ethanol; (6) acetone; (7) diethyl ether; (8) hexane.

bilayer was known, $\varepsilon$ for each region could be estimated from $\tau$ and then $\delta$ could be calculated from $C$.

In an attempt to determine what this relationship might be like within the polar regions of a bilayer we have plotted the electrical time constant of simple compounds with chemical structures that might be appropriate to the glycerol monooleate polar regions as a function of their bulk dielectric constant (see Fig. 4). The time constants were calculated from experimental conductivity values given in the International Critical Tables and the dielectric constants, $\varepsilon$, are listed in the CRC Handbook of Physics and Chemistry. Because the glycerol monooleate bilayer capacitance appeared to be essentially frequency independent from 300 Hz to 5 kHz (see Fig. 1) the range of time constants given in Table I presumably reflects those varying from the bilayer/electrolyte interface up to the hydrophobic interior. As these measured values of $\tau$ all exceed that of water, the possible relationship for bulk regions appropriate to the bilayer (indicated by the dashed line in Fig. 4) has been drawn through the data points corresponding to compounds with $\tau$ greater than that of water. Obviously within the bilayer itself the situation will be more complicated. One major difference is that there will be water penetration into the polar regions [29]. This would presumably raise their dielectric constant and probably not increase their conductivity appreciably provided that the individual water molecules remain reasonably isolated. Because the time constants within the bilayer (see Table I) are greater than that of pure water it appears unlikely that there is significant electrolyte penetration into the polar regions and so its contribution can be neglected. In the absence of more definite information for glycerol monooleate bilayers we have assumed that for dielectric constant values of $\varepsilon = 50$, 30, 20 and 10 the polar regions would contain the following volume fractions of water; 50%, 30%, 20% and 10% respectively. The resultant relationship used in interpreting our experimental data is shown by the full line on Fig. 4. Although it has been necessary to make several assumptions in determining this relationship, a check upon the validity of these assumptions can be made by comparing $\delta$ obtained via our dielectric measurements with those measured optically.

## Acknowledgement

The authors wish to thank the Australian Research Grants Scheme which funded and made this research possible.

## References

1 Andrews, D.M., Manev, E.D. and Haydon, D.A. (1970) Special Disc. Far. Soc. 1, 46–56
2 White, S.H. (1978) Biophys. J. 23, 337–347
3 Cherry, R.J. and Chapman, D. (1969) J. Mol. Biol. 40, 19–32
4 Cherry, R.J. and Chapman, D. (1969) J. Theoret. Biol. 24, 137–146
5 Dilger, J.P. (1981) Biochim. Biophys. Acta 645, 357–363
6 Coster, H.G.L. and Smith, J.R. (1974) Biochim. Biophys. Acta 373, 151–164
7 Ashcroft, R.G., Coster, H.G.L. and Smith, J.R. (1977) Biochim. Biophys. Acta 469, 13–22
8 Hanai, T., Haydon, D.A. and Taylor, J. (1965) J. Theoret. Biol. 9, 278–296
9 Ashcroft, R.G., Thulborn, K.R., Smith, J.R., Coster, H.G.L. and Sawyer, W.H. (1980) Biochim. Biophys. Acta 602, 299–308
10 Ashcroft, R.G., Coster, H.G.L. and Smith, J.R. (1981) Biochim. Biophys. Acta 643, 191–204
11 Ashcroft, R.G., Coster, H.G.L., Laver, D.R. and Smith, J.R. (1983) Biochim. Biophys. Acta 730, 231–238
12 Laver, D.R. (1983) Ph.D. Thesis, University of New South Wales
13 Sargent, D.F. (1975) J. Membrane Biol. 23, 227–247
14 Stark, G. and Gisin, B.F. (1979) Biophys. Struct. Mech. 6, 39–56
15 Dilger, J.P., Fisher, L.R. and Haydon, D.A. (1982) Chem. Phys. Lipids 30, 159–176
16 White, S.H. (1973) Biochim. Biophys. Acta 323, 343–350
17 Läuger, P., Lesslauer, W., Marti, E. and Richter, J. (1967) Biochim. Biophys. Acta 135, 20–32
18 Fettiplace, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membrane Biol. 5, 277–296
19 White, S.H. (1975) Biophys. J. 15, 95–117
20 White, S.H. (1976) Nature 262, 421–422
21 Fettiplace, R. (1978) Biochim. Biophys. Acta 513, 1–10
22 Waldbillig, R.C. and Szabo, G. (1979) Biochim. Biophys. Acta 557, 295–305
23 Elliot, J.R. and Haydon, D.A. (1979) Biochim. Biophys. Acta 557, 259–263
24 Benz, R., Frohlich, O., Läuger, P. and Montal, M. (1975) Biochim. Biophys. Acta 394, 223–234
25 Alvarez, O. and Latorre, R. (1978) Biophys. J. 21, 1–17
26 Reyes, J. and Latorre, R. (1979) Biophys. J. 28, 259–279
27 Pagano, R.E., Cherry, R.J. and Chapman, D. (1973) Science 181, 557–559
28 Bach, D. and Miller, I.R. (1980) Biophys. J. 29, 183–187
29 Griffith, O.H., Dehlinger, P.J. and Van, S.P. (1974) J. Membrane Biol. 15, 159–192