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Differential effects of cholesterol and oxidised-cholesterol in egg lecithin bilayers

Christopher Karolis [a], Hans G.L. Coster [a,*], Terry C. Chilcott [a], Kevin D. Barrow [b]

[a] *UNESCO Centre for Membrane Science and Technology and Department of Biophysics, School of Physics, University of New South Wales, Sydney 2052, Australia*

[b] *School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney 2052, Australia*

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Abstract

Low frequency impedance measurements of pure egg lecithin (phosphatidylcholine) bilayers have revealed the presence of four layers which can be attributed to the acyl chain, carbonyl, glycerol bridge and phosphatidylcholine regions of the lecithin molecule. Measurements on bilayers formed in the presence of unoxidised-cholesterol revealed that cholesterol molecules were located in the hydrocarbon region of the bilayer with its hydroxyl groups aligned with the carbonyl region of the lecithin molecules. Measurements of oxidised-cholesterol lecithin bilayers revealed that these molecules protruded less into the hydrocarbon region and their polar hydroxyl group aligned with the glycerol bridge region of the lecithin molecule. © 1998 Elsevier Science B.V.

Keywords: Cholesterol; Lecithin; Bilayer

1. Introduction

X-ray diffraction studies [1,2] and ESR studies [3] of aqueous mixtures of lecithin and cholesterol suggest that the presence of cholesterol leads to an increase in thickness of the hydrocarbon region of lipid bilayer membranes and an increase in the number of lecithin molecules per unit area of membrane. This condensing effect was first observed by Leathes [4] in 1925 and has since been confirmed by NMR and ESR studies [5–7]. The change in molecular area is associated with an ordering of the lipid acyl chains due to the strategic positioning of the rigid sterol moiety. In bilayers, this ordering manifests in changes in the viscosity and permeability properties [8].

To explain the condensing effect of cholesterol, Weiner and Felmeister [9] proposed that the effective area of the cholesterol molecule is the same as in a monolayer of pure cholesterol and the interaction of cholesterol with the lecithin hydrocarbon chains reduces the effective area. Their ''interaction'' model is supported by the measurements of Oldfield and Chapman [10], Hsia et al. [11] and Butler et al. [12] as well as by studies of the behaviour of cholesterol in mono-layers [13].

The change in fluidity of lecithin–cholesterol membranes is related to the degree to which fatty acids in the lipids are saturated and the homogeneity of the lipid component of the membrane. Experiments performed with different phospholipids containing various proportions of cholesterol, [7,10,14] show widely varying effects. For example, increasing

* Corresponding address: Fax: +61 2 9385 5981.

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the cholesterol content of egg lecithin bilayers results in an extension of the fatty acid chains and a decreased amplitude of motion of the long axis. Cholesterol increases the chain order and this also reduces the solubility of short-chain hydrocarbons in the lipid bilayer [15,16]. The latter may explain the conflicting reports in the literature on the effects of the cholesterol on the thickness of artificial lipid bilayers [1]. Increasing the cholesterol content of dipalmitoyllecithin bilayers, on the other hand, results in an increase in mobility and amplitude of motion of the fatty acid side chains.

The osmotic permeability of phospholipid membranes appears to be influenced by the presence of cholesterol [8,17,18].

The autoxidation of cholesterol dispersed in aqueous solution has been thoroughly investigated [19]. The initial products are mainly 7a- and 7b-hydroperoxides which decompose into the common impurities in commercial cholesterol, 3b-hydroxycholest-5-ene-7-one and cholesta-3,5-diene-7-one. Radical autoxidation can occur in the solid state and similar products are formed. Cholesterol can be oxidised in air and hence a highly purified sample of cholesterol must be stored under an inert atmosphere in the dark and preferably at low temperature. The oxidisation products of cholesterol, such as 7-dehydrocholesterol, are known to play a vital role in some membrane functions [20]. Planar bilayers have been produced from oxidised-cholesterol alone [20], and in association with phospholipids [22].

The position of the cholesterol hydroxyl group in the bilayer and the influence, if any, of hydrogen bonding is largely unresolved [5,21,22].

In this study, we have used electrical admittance dispersion spectra to determine the effects of cholesterol on the dielectric structure of egg lecithin bilayers and the differential effects and location of oxidised and unoxidised-cholesterol in the bilayer structure (Fig. 1). We do not, however, attempt to separate out the effects of the various oxidation products of cholesterol.

Diagrams and Captions

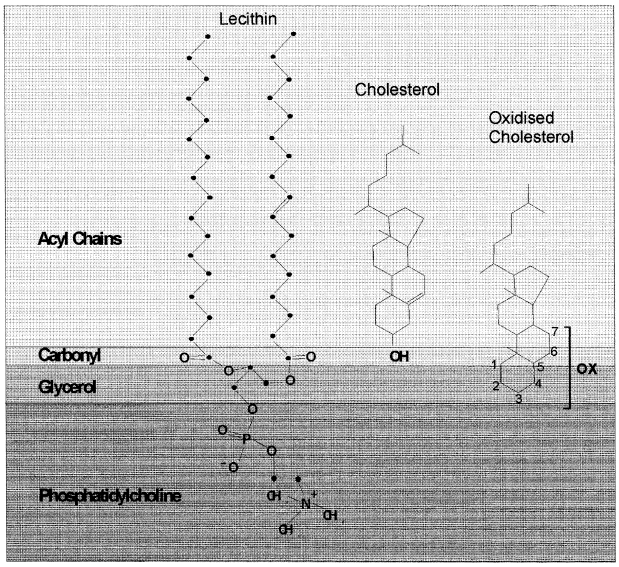

Fig. 1. Hypothetical alignment of cholesterol and oxidised cholesterol molecules within lecithin bilayers. Impedance measurements of egg lecithin bilayers have identified four electrically distinct layers that correspond to the acyl chain, carbonyl, glycerol bridge and phosphatidylcholine regions of the lecithin molecule [22]. The hypothesis is that the cholesterol molecule is located in the hydrocarbon region of the lecithin bilayer with its hydroxyl groups aligned with the carbonyl region whereas the oxidised cholesterol molecule protrudes less into the hydrocarbon region with its polar (oxidised) hydroxyl group (OX) aligned with the glycerol bridge region. (Note that a simplified representation of the molecular structure for oxidised cholesterol is shown since autoxidation of cholesterol initially produces mainly 7a- and 7b-hydroperoxides which later decomposes to 3b-hydroxycholest-5-ene-7-one and cholesta-3,5-diene-7-one [19]).

2. Materials and methods

2.1. Purification of cholesterol

Cholesterol was purified by converting it into the crystalline dibromide/acetic acid complex and then reaction in ether solution with zinc dust as detailed by Fieser [23]. The cholesterol was then recrystallised several times from methanol and obtained as large

[1] For artificial lipid bilayers swollen with alkane ''solvents'' (from which these bilayers are generated) a decrease in the alkane solubility would lead to a decrease in the thickness. Thus, for BLM generated from *n*-hexadecane dispersions of the lipid, the effect of cholesterol on the membrane thickness would be less than for BLMs generated from *n*-decane solutions of the lipids, since, the solubility of the alkanes in BLMs increases sharply as the alkane chain length decreases.

colourless crystals; m.p. 149–150° (corr.) $[\alpha]_D^{25}$ −40° (C = 2, $CHCl_3$).

^{1}H NMR, ^{13}C NMR and infra-red spectra were consistent with a very high degree of purity. The sample was stored under nitrogen in glass tubes wrapped in foil and kept at −20°C. Examination by TLC confirmed that hydroperoxides were absent [19]. The purified sample of 0.25 g was dissolved in a 10 ml solution of a 3:1 chloroform:methanol mixture (v/v) and stored at −4°C in a light free container. Oxidised-cholesterol was produced by bubbling oxygen through a cholesterol/chloroform solution for 4 h.

2.2. Formation of bilayers

Egg lecithin (phosphatidylcholine) was obtained from Sigma and used as supplied. Bilayers were produced from lipid mixtures of either egg lecithin alone; egg lecithin–cholesterol (mole ratio 2:1) or egg lecithin–oxidised-cholesterol (mole ratio 2:1). The mixture was dried over N_2 at 40°C for 3 h. Subsequently, *n*-hexadecane was added and a film of the preparation was painted with a syringe over a small hole in a polycarbonate septum separating chambers containing 100 mol m^{-3} KCl at pH 7.2.

In these high salt concentrations, it was necessary to warm the electrolyte to 35°C in order to facilitate thinning of the film. Initially, coloured interference fringes were visible across the hole indicating that the film thickness was of the order of the wavelength of light. As the buoyant hexadecane diffused into the electrolyte, black spots appeared near the lower perimeter of the hole indicating that the film thickness in these regions was less than the wavelength of light and hence, of the order of that for a lipid bilayer. Measurements of capacitance at low frequencies (see Section 2.4) over this period were found to increase, confirming that the film was thinning. The black regions grew and merged to occupy the centre of the hole. Even when most of the hole aperture was black, the capacitance continued to increase, indicating that diffusion of excess hexadecane out of the bilayer was producing further thinning. When the capacitance had stabilised it was assumed that the diffusion process was complete, although some hexadecane molecules would remain ''dissolved'' in the interior. Then the temperature was slowly adjusted to 25°C. Measurements were begun only after the low frequency capacitance (at a frequency of 1 Hz) was stable; increasing by less than 1% per hour.

2.3. The frequency dependence of bilayer impedance

The multi-layered dielectric model of the lipid bilayer [24] consists of a series of electrically distinct layers to each of which is attributed an area specific conductance G and capacitance C. The equivalent circuit for such a model is a series combination of capacitors, each shunted by a conductor. Dispersions of impedance with frequency (Maxwell–Wagner dispersions) arise from such admittances in series when the time constants C/G of different layers are unequal [25]. For a circuit comprised of n elements, each of which is the parallel combination of a conductor G_i and a capacitor C_i the transfer function is:

$$TF(s) = \sum_{i=1}^{n} \frac{1}{G_i + sC_i} \quad (1)$$

where s is the Laplace complex frequency and

$$\omega_i = \frac{G_i}{C_i} = \frac{1}{t_i} \quad (2)$$

define the ''natural'' frequencies (or reciprocal time constants t_i) of the system. When $s = j\omega$ ($j^2 = -1$) the transfer function gives the impedance, that is

$$Z = TF(j\omega)$$

where ω is the angular frequency of the *ac* signals used to measure the impedance.

We define $G(\omega)$ as the real part of the admittance $(1/Z)$ and the capacitance $C(\omega)$ as $-j/\omega$ times the imaginary part. In the low frequency limit of a two element circuit (i.e. for $\omega < \omega_1$ $(= 1/t_1)$ where $t_1 < t_2$), the total capacitance is

$$\frac{C_1G_2^2 + C_2G_1^2}{(G_1 + G_2)^2}$$

which reduces to

$$\frac{C_1C_2}{C_1 + C_2}$$

at the high frequency limit (i.e. for $\omega >> \omega_2$). The effects of additional series elements with time constants t_i (where $t_1 < t_i < t_2$) are to introduce addi-

tional dispersions near their respective ''natural'' frequencies ω_i and to further reduce the total capacitance at the high frequency limit (refer to Coster et al. [26] for further details). Now it is reasonable to assume that the conductivity and dielectric permittivity both decrease towards the bilayer centre [22,27] and therefore by careful selection of the C_i and G_i values it is possible to generate a set of parameters that describes the frequency dispersion in capacitance as well as that in conductance.

2.4. Impedance measurements

The difficulties associated with the detection of dispersions in the capacitance and conductance have been described in detail by Coster and Smith [25] and account for the lack of success of early work based on high frequency 2-terminal measuring systems [24,28]. The present experiments were made with a computer based, 4-terminal, high resolution, ultra low frequency impedance spectrometer [29] which is an improved version of that described earlier [30]. The accuracy and resolution of this device was tested with a hard-wired circuit, hypothetically representing a lipid bilayer, consisting of a series of RC elements with time constants differing one from the other by approximately a factor of 10. The resistance and capacitance values were chosen to be similar to those that could be expected for a real bilayer spanning a septum [31–33,22,27] with a hole area of 3.87×10^{-6} m^2. A more complete description of the impedance spectrometer is given by Coster et al. [26].

From measurements performed on such hard-wired circuits [34], it was found that the capacitance and conductance of individual elements could be determined within 0.1%, even when the circuit contained seven elements. Other factors such as determination of the bilayer area increase the uncertainty to about 2% for real bilayers.

Bilayer formation was observed with a low powered microscope in conjunction with electrical measurements of capacitance at a frequency of 1 Hz. Impedance measurements at other frequencies commenced only when the capacitance and conductance had stabilised (less than 0.1% change per cycle). A micro-syringe was used to inject or withdraw electrolyte into or from one side of the bilayer and thereby compensate for small differences in hydrostatic pressure that might develop across the bilayer during the experiment and which cause the bilayer to bow. The planar area of the bilayer was determined from photographs taken through a low powered microscope [34]. Impedance measurements were made in the frequency range 10^{-3}–10^4 Hz at multiples of 1.412 in frequency. To obtain a complete spectrum required 15 min (largely determined by the period of the ac signals of the first few, lowest, frequencies).

3. Results

Bilayers with cholesterol generally had longer lifetime than those of pure lecithin. In this study, the longest lifetime in membranes containing cholesterol was 50.4 ks compared to 21.6 ks for membranes containing only lecithin. The lifetimes were sufficiently long (average lifetime $\sim$10 ks) to permit at least three impedance spectra to be obtained for each bilayer. The frequency dependence of the capacitance and conductance of a lecithin bilayer is shown in Fig. 2. There is a noticeable decrease in the capacitance with frequency particularly at frequencies greater than 10^3 Hz. If the bilayer behaved like a pure capacitor, spanned by small conducting pathways, i.e. a homogeneous dielectric structure, its total capacitance would be independent of frequency. However, between the lowest frequency and 10^3 Hz the capacitance decreased by about 9%, consistent with capacitance dispersions reported previously by Ashcroft et al. [31–33].

To facilitate comparison between the different bilayers, the dispersion described by a Maxwell–Wagner model was fitted to the averaged impedance spectra obtained from lecithin (Fig. 2), lecithin–cholesterol (Fig. 3 and Fig. 4) and lecithin–oxidised-cholesterol bilayers (not shown). A four-layer Maxwell–Wagner model was used for the bilayers in addition to elements representing the bulk solution [27].

The layers in the Maxwell–Wagner model could be identified directly from the transfer function Eq. (1), when the latter was plotted along a negative frequency axis as shown in Fig. 5. The peaks corre-

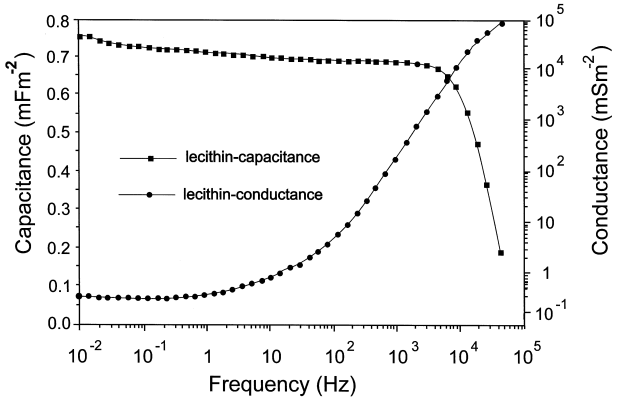

Fig. 2. Capacitance and conductance dispersions for an egg lecithin bilayer formed in 100 mM KCl at 25°C. In order to observe the characteristic features of the impedance dispersions we express the impedance Z in terms of admittance $(1/Z = G(\omega) + j\omega C(\omega))$ which is comprised of parallel conductance $G(\omega)$ and capacitance $C(\omega)$ components. If the bilayer were homogeneous it could be represented by a single layer with capacitance and conductance properties that are independent of frequency. In contrast, the conductance and capacitance of the bilayer were found to be strong functions of frequency over several decades of frequency. In fact, the theoretical curves fitted to the data correspond to a four-layer model of the type defined by Eqs. (1) and (2). See Fig. 7 for average conductance and capacitance values for the layers comprising such a model. Note that the symbols used to represent the data are larger than the experimental errors.

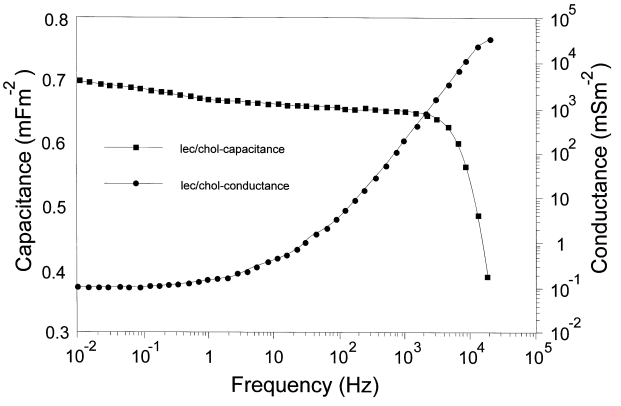

Fig. 3. Capacitance and conductance dispersions for an egg lecithin cholesterol bilayer formed in 100 mM KCl at 25°C. Although the general character of the dispersions are very similar to those for lecithin bilayers (Fig. 2), the presence of cholesterol can be seen to have lowered the overall capacitance and conductance of the bilayer.

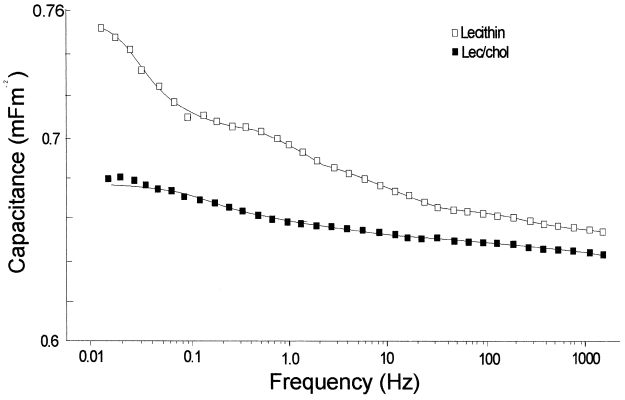

Fig. 4. Effects of cholesterol revealed by a closer examination of the capacitance dispersions from Figs. 2 and 3. The direct comparison of the capacitance dispersions on an enlarged scale, reveals the more subtle effects of cholesterol on the bilayer. These effects are more strikingly revealed in the transfer function of the model (see Fig. 5) and in the integral of the transfer function (see Fig. 6).

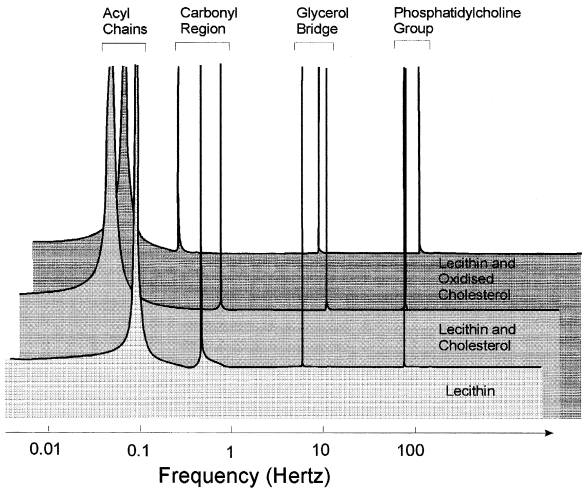

Fig. 5. Impedance spectra showing how cholesterol and oxidised cholesterol affect the ''natural'' frequencies of the lecithin bilayer. The spectra are plots of the transfer function magnitude Eq. (1) along the negative real frequency axis (i.e. for when the Laplace transform variable $s = -\omega$). The peaks in each spectrum occur at frequencies given by $\omega = |-G_i/C_i|$ and therefore identify the ''natural'' frequencies (i.e. reciprocal time constants t_i) of the four layers that comprise each of the models for the three types of bilayer. Each of the peaks corresponds to the indicated regions of the lecithin molecule (see Fig. 1). The cholesterol and oxidised cholesterol can be seen to cause a displacement in each of the regional peaks indicating a structural rearrangement of the lecithin bilayer at the sub-molecular level.

spond to the ''natural'' frequencies (or reciprocal time constants) of the different layers Eq. (2) and the integral of the transfer function along these axes gives the reciprocal capacitances of these layers (see Fig. 6). Specifically, the steps in the integral at the peaks gives the reciprocal capacitances of the layers. The transfer function therefore identifies the conductances and capacitances of the layers, the values for which are presented in the bar charts of Fig. 7.

The capacitance of the hydrocarbon (acyl chains) region of the lecithin bilayers, $C_h = 7.7\,mF\,m^{-2}$, is in excellent agreement with mono-layer studies [35–37]. The addition of either cholesterol or oxidised-cholesterol reduced this value slightly to $7.2\,mF\,m^{-2}$.

In contrast, the conductance of the acyl chain region in lecithin bilayers was found to be $4.8 \pm 0.9\,mS\,m^{-2}$, more than twice the value of $2.3 \pm 1.0\,mS\,m^{-2}$ obtained for cholesterol bilayers but less than twice the value for bilayers with oxidised-cholesterol. It is these differences in conductance which account largely for the observed shift in the peak of the transfer function which corresponds to the acyl chain region (see Fig. 5).

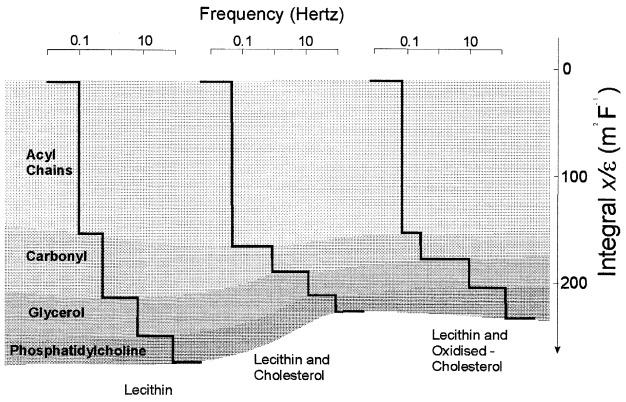

Fig. 6. Integral of the impedance spectra showing dielectric/geometrical effects of cholesterol and oxidised cholesterol on the lecithin bilayer. Characteristic features of the integral are steps which occur at the ''natural'' frequencies (i.e. at $\omega_i = |-G_i / C_i|$) of the transfer function Eq. (1). The magnitudes of the steps give the reciprocal capacitances (i.e. $1/C_i$) and hence the ratio of thickness to dielectric permittivity (i.e. x/ε) for the layers in the impedance models. These layers are shown to derive from the indicated regions of the lecithin molecule. The $x{:}\varepsilon$ ratios for these regions are shown to decrease in the presence of cholesterol and oxidised cholesterol, with cholesterol producing the most significant decrease.

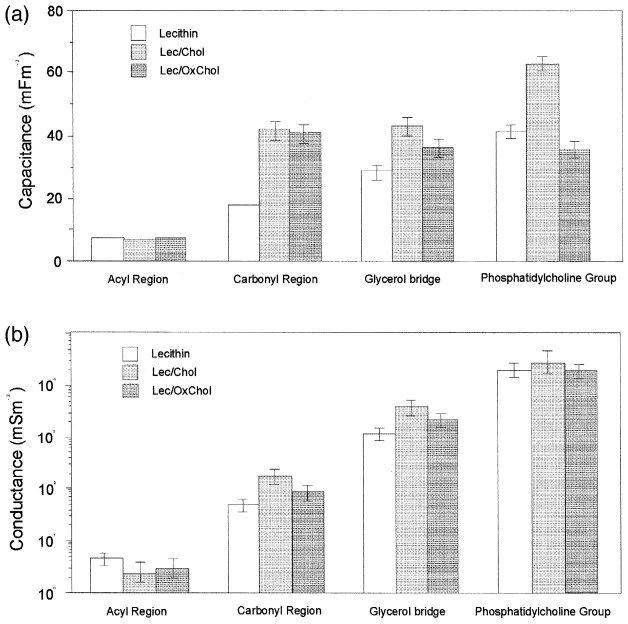

Fig. 7. Averaged conductance and capacitance values for submolecular regions of bilayers formed from lecithin, lecithin–cholesterol and lecithin–oxidised cholesterol. The capacitances for the acyl chain regions were 7.75, 7.15 and $7.25\,mF\,m^{-2}$ for the respective types of bilayers. Note that the standard errors for these estimates ($\sim 1.5\%$, $n >= 9$) were too small to be shown as is the that for the capacitance of the acetyl region for lecithin bilayers.

4. Discussion

The division of the capacitance frequency spectrum into regions characterised by different time constants suggests that the bilayer consists of dielectrically resolvable sections. Figs. 2 and 3 show that the dominant feature of the capacitance dispersion was that occurring at low frequencies and which can be largely identified with the hydrocarbon moiety of the interior. Such an assignment can be made readily since the capacitance of this region derives from a dielectric constant that should be similar to that of straight chain alkanes which, for those with 12 or more carbon atoms, has a value of ~ 2.2, [38–40] and the approximate thickness of the bilayers which must have a value somewhat less than the twice the alkyl chain length (see [15,27,31,32]). This capaci-

tance is of a similar order to that measured at low frequencies.

Fig. 4 shows the detail of the effects of cholesterol inclusion on the low frequency capacitance of the bilayer and that the thickness of the hydrocarbon region increased in the presence of cholesterol. It has been argued [41,34] that because of the capacitance of lecithin bilayers formed from *n*-hexadecane is similar to bilayers formed from mono-layer apposition, there was almost no solvent retained in the bilayer. A dielectric constant of ~ 2.2 for this region would therefore seem to be appropriate [40]. This value yields a thickness of 2.51 nm for the hydrocarbon region in lecithin-only bilayers and 2.73 nm in lecithin–cholesterol bilayers.

This $\sim 9\%$ increase in thickness does not simply arise as a consequence of an increase in the partitioning of hexadecane into the membranes. Indeed, cholesterol has been shown [15] to lead to a decrease in the alkane partitioning into lecithin bilayers. The increase in membrane thickness on addition of cholesterol therefore reflects the direct effect on the lipid organisation and not a modulation of the alkane retention of the membranes. Such is most likely due to decrease in the number of possible conformations of the acyl chains of the lecithin (and hexadecane) in the vicinity of the larger ring structure of cholesterol [15]. The increased chain order (decrease in micro viscosity) is correlated to the electrical conductance which was reduced by 50% with the inclusion of cholesterol (see Fig. 7(B)). Substitution of cholesterol with oxidised-cholesterol reduced the conductance by only about 25%.

Bilayer stability may be related to the formation of pores of a critical size [42–44]. The effects reported here of the inclusion of cholesterol into lipid bilayer membranes plus the observation that lecithin bilayers become more stable physically with the inclusion of cholesterol, can be expected from the energy required to form pores of the critical size [44].

Basically, the energy to form a pore of radius R is given by:

$$E_p = 2\pi W - \pi R^2\left(\gamma + \varepsilon_0 \varepsilon_b \frac{V^2}{2}\right) \quad (4)$$

where W is the perimeter energy per unit perimeter length, γ is the interfacial free energy of the bilayer, ε_0 is the dielectric constant of free space, ε_b the dielectric constant of the bilayer and V is the transmembrane potential. The first term represents the cost of making the new ''edge'' where entropic and energy items are unfavourable for the packing of lipid molecules into a highly curved surface. The second term represents the energy of the area of bilayer now occupied by the pore (i.e. energy saved). For small pores the energy cost to form the perimeter is larger than the energy saved by a reduction in area of the planar bilayer. Eventually, the differential increase in the perimeter energy required equals the differential decrease in surface energy. At that point, the bilayer would be unstable; a pore of this radius would grow uncontrollably leading to bilayer rupture. The distribution of the pore sizes can now be obtained via the Boltzmann distribution function. A molecule such as cholesterol, which has a small polar head and a large hydrophobic region will not pack well into the highly (negatively) curved pore region. The presence of such a molecule in the bilayer would therefore tend to increase the perimeter energy and shift the distribution of pore radii towards smaller radii [44]. This would reduce the bilayer conductance. The critical pore radius (at which the bilayer would rupture) is also increased (statistically a rarer event) and this would be reflected in greater bilayer stability. Further statistically, the distribution of pore sizes with cholesterol present, would shift to smaller pore sizes. This would reduce the conductance of the bilayer.

We have indeed found that inclusion of cholesterol increases the maximum lifetime of lecithin bilayers and reduces the electrical conductance.

The first dispersion in capacitance which took place at about 0.05 Hz can be described in terms of a theoretical 2-layer model. The region under consideration is the interface defined by the C-1 and C-2 carbon atoms of the acyl chains. Huang [21] has suggested that hydrogen bonding is a dominant factor in positioning of the 3β-OH group [2] of cholesterol. The time constant of this region was noticeably different for the three types of bilayers, ranging from approximately 1 for lecithin-only bilayers to more than 4 for lecithin–oxidised-cholesterol bilayers. The

[2] This is the OH group of cholesterol shown in Fig. 1 and which is depicted as an OX group on oxidised cholesterol.

capacitance of this region was nearly three times less in lecithin layers than in those containing cholesterol. There was, therefore, a marked difference in the thickness of this region or the dielectric constant was three times larger with cholesterol in the bilayer, or perhaps a combination of these.

We believe that the dielectric constant of this region increased due to the presence of the 3β-OH group of cholesterol. The slightly higher conductance (25–50%), observed in lecithin/cholesterol bilayers supports this view. When the dielectric constant increases the concentration of charge carriers would also increase (since the Born energy for partitioning of ions decreases with increasing dielectric constant) and this would result in an increase in the conductance.

Observations made on a very long lived membrane (>10 h) made from lecithin and cholesterol revealed a lowering of the conductance in this region with time. We believe that the cholesterol molecule in these long lived membranes is oxidised in situ and moves out of the bilayer interior, albeit only slightly. If the oxidised portions of the cholesterol are sufficiently polar one might expect them to be located at the hydrophobic–hydrophillic interface; i.e. in the glycerol bridge region. The cholesterol, on oxidation would thus be drawn further out of the bilayer interior. If part of the ring structure now occupies the carbonyl region the dielectric constant will be reduced which would account for the drop in capacitance and conductance of that region.

The second dispersion occurred around 0.1 Hz and appeared to be associated with the glycerol bridge. The change in the packing density resulting from the inclusion of cholesterol would allow a greater number of water molecules to enter into this region. This was evidenced by the relatively large increase in conductance for this region observed in going from lecithin to lecithin–cholesterol bilayers.

If our notion of oxidised-cholesterol is correct it would place the 3β-OH group in the region of the glycerol bridge and therefore, restrict the number of water molecules. This would have the effect of lowering the conductance of the region, as indeed observed.

The third dispersion occurred at 1 Hz, and was much less pronounced in all the bilayers. There were also much smaller differences between bilayers. The conductance of this region was fairly high but it was still lower than the external solution. We are tempted to suggest that this dispersion was associated with the phosphatydylcholine group, and the next dispersion, not shown in Fig. 4, could thus be due to the choline group.

5. Conclusions

Low frequency impedance spectrometry was found capable of resolving as many as 7 separate elements in lipid bilayer bilayer/electrolyte systems. Four of these were identified with regions in the bilayer. Using this technique, the structure of lecithin, lecithin–cholesterol and lecithin–oxidised-cholesterol was characterised.

Lecithin bilayers appear to have a low dielectric constant region at the hydrophobic–hydrophillic interface. The addition of cholesterol to lecithin bilayers resulted in an increase in the polar nature of the hydrophobic–hydrophilic interface possibly due to an increase in water penetration into this region on addition of cholesterol. The effect was greatest for unoxidised-cholesterol.

The cholesterol molecules were located in the hydrocarbon interior of the bilayer with their hydroxyl groups spanning the carbonyl-interface. Oxidised-cholesterol molecules were located just slightly out of the hydrocarbon interior, with their polar regions spanning the glycerol bridge; this could be inferred from the change in capacitance and conductance of the carbonyl and glycerol regions of the bilayer on substituting oxidised for unoxidised cholesterol, and during in situ oxidation of cholesterol in bilayer membranes.

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