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Oestradiol changes the dielectric structure of bilayer membranes

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Abstract. The addition of the hormone Oestradiol to Phosphatidylcholine-Cholesterol membrane changes the frequency dependence of the membrane impedance. It increases severalfold the electrical admittance of the polar regions and consequently provides a conducting shunt from the hydrocarbon region to the aqueous phase.

Key words: Bilayer lipid membranes, Oestradiol, impedance, dielectric structure

Introduction

Liposomes and vesicles have been proposed as drug delivery systems because of their ability to protect encapsulated drugs from enzymes and soluble reagents (Gregoriadis 1976, 1979). While some soluble drugs are encapsulated in the aqueous interior of liposomes, in many cases the drugs are amphiphilic and are therefore expected to partition into, or at the surface of, the liposome membrane. The location of a hydrophobic drug in a liposome membrane is potentially important for clinical applications. The location may determine its accessibility for interaction with soluble reagents. Further, the introduced drug may change the liposome membrane structure and thus alter the interaction between the liposome and tissue cells.

One drug that has been studied for encapsulation in liposomes is 17 β Oestradiol (Lundberg 1979; Salin-Drouin 1985) which is used in hormone therapy (Basdevant and de Lignieres 1980). This substance has lower toxicity than some other oestrogens. When it is administered orally, however, it does not appear in tissues in large concentration. It is hoped that encapsulation in liposomes will allow it to pass biological barriers. 17 β Oestradiol has low solubility in water and partitions principally into the liposome membrane.

Attempts have been made to determine the location of Oestradiol in phospholipid bilayers using the electron spin resonance of labelled stearic acid. It was shown that the introduction of 17 β Oestradiol affected the motion of the label attached to the fifth carbon in the alkyl chain of stearic acid, but not to that attached to the sixteenth carbon (Salin-Drouin 1985).

A technique which has been used to determine the location of added amphiphiles in bilayer membranes and to determine the structural changes induced in the membranes thereby is the analysis of the electrical structure of membranes using impedance spectroscopy (Coster and Smith 1974; Smith 1977; Ashcroft et al. 1977, 1981, 1980; Laver et al. 1984). This technique has the advantage that it may be conducted on a single membrane without perturbing it by the introduction of probes or by fixation and that it has a possible resolution in the direction of the membrane normal of rather better than 1 nm. It has the further important advantage that the only extant instruments are all located within a few km of some excellent beaches, restaurants, theatres and pubs.

In this study, we have used this technique to investigate the location of 17 β Oestradiol in bilayer lipid membranes of phosphatidylcholine and cholesterol, and to observe its effects on the dielectric substructure of these membranes. Phosphatidylcholine and cholesterol were chosen to allow direct comparison with the biochemical studies of Lundberg (1979) and Salin-Drouin (1985).

Materials and methods

Bilayer lipid membranes

Bilayer lipid membranes were produced by extruding a sonicated solution of lipid in *n*-hexadecane mixture

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from a syringe while passing the syringe tip past a 2.8 mm diameter hole in a polycarbonate chamber separating two volumes of aqueous salt solution (100 mol m^{-3} KCl). The thick film of lipid solution became thinner as the hydrophobic solution floated up to the top of the chamber (bringing closer together the monolayers which face the aqueous solution). Subsequently, patches of very low reflectance (called black) appear, (possibly as a result of film instabilities) and these expand quickly. The membrane quickly gains an area-specific capacitance which is consistent with a thickness of a few nm. Membranes were formed at 40 °C and then slowly cooled to 35 °C and equilibrated over ten to fifteen minutes. Measurements were made at 35°, 30° and 25 °C, with slow cooling between temperatures. At each temperature, three to five impedance spectra from 0.01 Hz to 10 kHz were measured. The membranes formed spontaneously at 40 °C to became "black" (as observed by reflected white light) over most of the aperture. After 1 h, the membrane capacitance was stable (drift less than 1% per h).

The hydrophobic solvent in the torus partitions to differing degrees into the bilayer. Large solvent molecules partition into the membrane to a lesser extent, and all solvents studied are progressively excluded from the membrane at low temperature. *n*-hexadecane was chosen as the hydrophobic solvent for the membrane-forming solution because it partitions sparingly into bilayers. Laver (1983) and Coster and Laver (1986) measured the membrane capacitance as a function of temperature for phosphatidylcholine membranes formed from solution in a range of alkanes from dodecane to *n*-hexadecane (the aqueous solution was 1 mol m^{-3} KCl). They found that the capacitance reached the same limiting value at low temperature for all alkanes, and interpreted this value as the capacitance of a solvent-free bilayer. For hexadecane, this limiting value was reached at 30 °C. These authors also determined the partitioning of dodecane into Phosphatidylcholine/Cholesterol membranes formed from solutions with a range of Phosphatidylcholine : Cholesterol ratios. The fraction of solvent decreased with increasing cholesterol fraction.

Each membrane was photographed several times during its life and the area determined using a planimeter. This measurement introduces the largest error into the absolute measurements of area-specific parameters. Because of the difficulty of discerning the microscopic geometry of the torus and membrane boundaries, the error is of the order of one percent.

Over the hours (or days) taken for each experiment, differential evaporation from the two sides of the chamber tended to bow the membrane. To keep the membrane planar, a micrometer-syringe driven by a stepping motor was attached to a tube of solution leading to one side of the chamber. The flatness was observed by the reflection of white light and maintained by adjusting the speed of the stepping motor. Errors in membrane area due to curvature of the membrane were an order of magnitude smaller than those mentioned above.

Materials

Egg Lecithin (Phosphatidylcholine) and Cholesterol were obtained from Sigma and used as supplied. Oestradiol was kindly supplied by Laboratory Besins Iscovesco. The lipid solutions were prepared by mixing the lipids (with or without oestradiol) in solution in chloroform or chloroform-methanol 4:1. Chloroform and methanol were redistilled from Analar grade solvents. Phosphatidylcholine/Cholesterol solutions contained the two lipids in mole ratio 2:1. Phosphatidylcholine/Cholesterol/Oestradiol membranes contained the three lipids in mole ratio 2:1:1. The mixture was dried at 40 °C for 3 h. Subsequently, *n*-hexadecane was added and the preparation was sonicated for ten minutes in a nitrogen atmosphere. Samples of the lipid solutions of each type were analysed using gas chromatography and mass spectrometry to determine if there were any chemical changes (such as oxidation) to the lipids during the preparation and storage of samples. No such changes were observed in samples treated in the manner described above.

Aqueous solutions were made from distilled water driven through an Organex MiliQ (Milipore) system and subsequently degassed under vacuum. KCl was Analar BDH 99.8% used without further purification.

A model for the frequency dependence of membrane impedance

In several studies of the impedance of bilayer membranes (e.g. Coster and Smith 1974; Ashcroft et al. 1977, 1981, 1983: Smith 1977), the membrane has been modelled as 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 (see Fig. 1). Dispersions of impedance with frequency arise from such admittances in series when the time constants C/G of different layers are unequal. Such dispersions with frequency are called Maxwell-Wagner dispersions.

For a circuit comprising n elements, each of which is the parallel combination of a conductor G_i and a capacitor C_i, the admittance is

$$Y = \left[\sum_{i=1}^{n} (G_i + j\omega C)^{-1} \right]^{-1}, \quad (1)$$

where $j^2 = -1$ and $\omega = 2\pi f$ is the angular frequency. We define the conductance $G(f)$ as the real part of Y

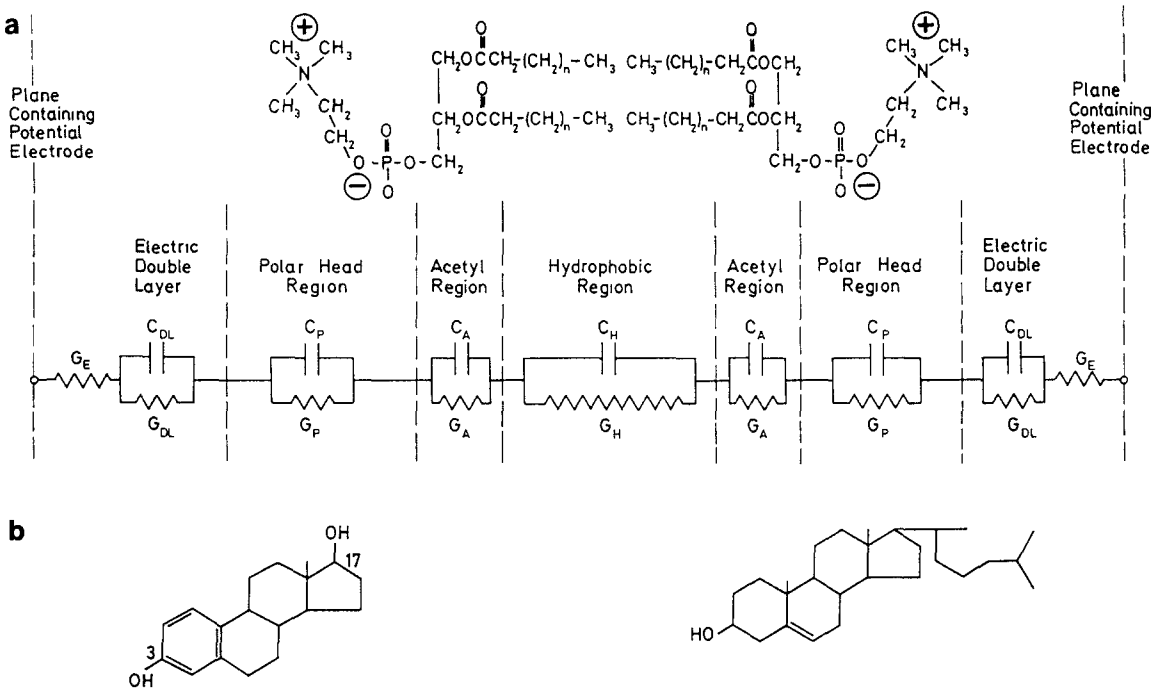

Fig. 1. A representation of a bilayer-electrolyte system and an approximate equivalent circuit for that system, after Ashcroft et al. (1977). Each chemically distinct region of the membrane is represented by a layer with a finite conductivity and dielectric constant, as is the electric double layer. The charge supplied by the current electrodes and which causes the potential difference across the membrane is predominantly found in an electric double layer near the membrane surface. The structures of 17β Oestradiol (*top*) and cholesterol (*bottom*) are shown in **b**

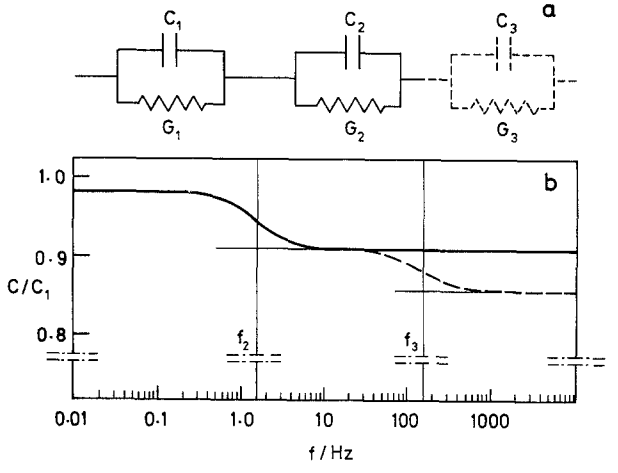

Fig. 2. A simple series circuit of two admittances ($C_1 \parallel G_1$ and $C_2 \parallel G_2$) is shown (*solid lines*). If their time constants C/G are different, the total capacitance (defined as the imaginary part of the admittance divided by $j\omega$) is frequency dependent and disperses at the higher frequency f_2. The high frequency limit is the series combination of C_1 and C_2 (*fine line*). If a further series admittance ($C_3 \parallel G_3$) is added, a further dispersion appears at the frequency f_3 (*dotted line*). The high frequency limit is the series combination of C_1, C_2 and C_3. For this plot the following values were used: $C_1/G_1 = 1$ s; $C_2 = 10\,C_1$, $G_2 = 100\,G_1$; $C_3 = 15\,C_1$, $G_3 = 15{,}000\,G_1$. The values were chosen to exaggerate the dispersions. In membranes, the dispersions are several, closer together in frequency, and smaller in amplitude

and the capacitance $C(f)$ as $(-j/\omega)$ times the imaginary part.

As a simple example, consider the two element circuit of $(C_1 \parallel G_1)$ in series with $(C_2 \parallel G_2)$ (Fig. 2). The admittance of this circuit is readily calculated from (1) by expanding and rationalising the denominator and yields:

$$C = \frac{\omega^2 C_1 C_2 (C_1 + C_2) + C_2 G_1^2 + C_1 G_2^2}{(G_1 + G_2)^2 + \omega^2 (C_1 + C_2)^2},$$

$$G = \frac{G_1 G_2 (G_1 + G_2) + \omega^2 (G_1 C_2^2 + G_2 C_1^2)}{(G_1 + G_2)^2 + \omega^2 (C_1 + C_2)^2}. \qquad (2)$$

Let us set the first time constant $\tau = C_1/G_1$ larger than the second. When $f \ll 1/\tau_1$, (the low frequency limit) the capacitance is $(C_2 G_1^2 + C_1 G_2^2)/(G_1 + G_2)^2$. When $f \gg 1/\tau_2$, (the high frequency limit) the capacitance is just the series capacitance $C_1 C_2/(C_1 + C_2)$. $C(f)$ for this simple circuit is shown in Fig. 2 (solid line).

There is little point in writing explicitly the expanded expression for $G(f)$ and $C(f)$ in circuits containing many elements. First, the length of such expressions increase very rapidly with n (Smith 1977) so they are not instructive; further, the algorithm used for comparing them is derived from (1) rather than from the expanded form. Briefly, the effect of adding an

additional series element with smaller time constant τ is to introduce an additional dispersion near the frequency $1/\tau$ which reduces the capacitance at high frequency to that of the series combination of all the capacitors (Fig. 2b, dashed line).

For membranes, the time constant of successive layers is thought to increase towards the centre of the membrane (Ashcroft et al. 1983; Laver et al. 1984) i.e. the conductivity and dielectric constant both decrease towards the centre. At frequencies well below the reciprocal of the time constant of the external solution, for example, ($f \ll G_{ES}/C_{ES}$), the admittance of the solution approaches its conductance and so the total capacitance of the system is increased. At successively lower frequencies, a further increase in C is observed near the characteristic frequency (G/C) of each layer.

A general one-dimensional model of a membrane would have a local conductivity and dielectric constant which would be continuous functions of position along the membrane normal, and would thus require an infinite number of series elements to represent it precisely. The finite precision of impedance measurements limits analysis to a small number of discrete layers, identified for instance with the hydrophobic interior, the polar head region, the acetyl region between the two, and (with maximum instrumental resolution) various subdivisions of these.

Measurements of bilayer impedance

Measurements of membrane impedance at ultra-low frequency are commonly subject to two difficulties. First, bridges are inaccurate and impractical at such frequencies. Second, two-terminal measurements necessarily include the potential across the solution-electrode interface in the measurement of transmembrane potential. The electrical measurements in this study were made using a four terminal system developed in this laboratory which has been described elsewhere (Bell et al. 1975; Ashcroft et al. 1983; Smith 1977). Briefly, sinusoidal potentials are generated digitally and applied via buffer amplifiers to the current electrodes on either side of the membrane and a reference impedance in series. Ultra-high impedance electrometer amplifiers are connected to voltage electrodes on either side of the membrane and to the reference impedance. Thus no appreciable current passes through the voltage electrodes and so there is no a.c. potential across these electrode-solution interfaces. The potential differences across the membrane and the series impedance are digitized and sinusoids of the input frequency are fitted by least squares to these data. The amplitude of the impedance is determined to within $\pm 0.1\%$ and the phase angle to within $0.01°$. Conductance and capacitance parameters of the model were determined by fitting the finite element model [Eq. (1)] to the measured data of the impedance (magnitude and phase angle at each frequency). We used a (non-linear), standard error weighted least squares fit obtained by the method of steepest descent.

Results

Bilayers containing Oestradiol formed more rapidly and were more stable than were the Phosphatidylcholine/Cholesterol bilayers. Membranes of both types could be maintained over at least several hours.

Frequency dependence of the capacitance

Figure 3 shows the frequency dependence of the capacitance of BLMs made from phosphatidylcholine and cholesterol (PC/Ch) and those made from phosphatidylcholine, cholesterol and oestradiol (PC/Ch/Oes). Each plot is the average of 15 spectra on different membranes and the bars show the standard error. The variation is largely due to differences between membranes: measurements made on any one membrane at intervals of 50 min (the time taken to measure one complete spectrum) yielded error bars which were several times smaller. At all frequencies the variation is larger than the instrumental precision (about 0.1% in middle frequencies). The measurements shown were made at 30 °C; similar impedance measurements were made on all samples at 25° and 35 °C (not shown). For PC/Ch bilayers, there was little discernible variation with temperature over this range. The results for PC/Ch/Oes at different temperatures were very similar in form but displaced vertically; at any frequency, the

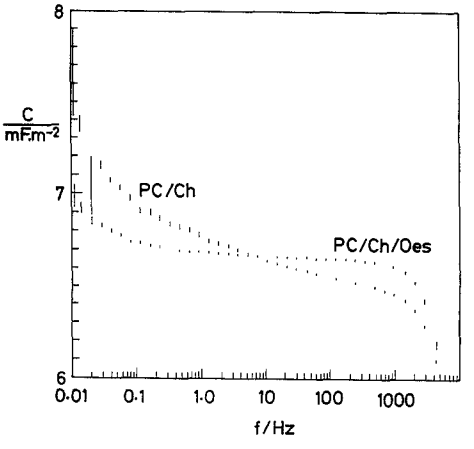

Fig. 3. The mean capacitance per unit area as a function of frequency is plotted for membranes at 30 °C made from phosphatidylcholine and cholesterol; and from phosphatidylcholine, cholesterol and oestradiol. Here and in Fig. 4, the *bars* show standard error

capacitance was about 0.8% higher at 25 °C and about 1.6% lower at 35 °C.

The capacitance of PC/Ch membranes is about 6.9 mF m^{-2} at 0.1 Hz and decreases with frequency by about 0.12 mF m^{-2} per decade between 0.1 Hz and 1 kHz. As noted in the Method section, the absolute value of area-specific parameters includes a possible error of 1%; variations in these parameters are known with greater precision, however, as a whole spectrum is measured on each membrane. Below 0.1 Hz, the capacitance increases more strongly with frequency and varies both among membranes, and over time (hours to days) for the same membrane. Above 1 kHz the capacitance decreases very rapidly with frequency. These results are very similar to those reported by Ashcroft et al. (1983) for the same temperature and solution, although these authors made no measurements below 0.1 Hz under these conditions.

The capacitance of PC/Ch/Oes membranes is about 6.7 mF m^{-2} at 0.1 Hz. It is only weakly dependent on frequency between 0.1 Hz and 1 kHz: it decreases by about 0.03 mF m^{-2} per decade over this range. As with PC/Ch membranes, the capacitance increases more strongly with frequency both below 0.1 Hz and above 1 kHz.

The frequency dependence of the conductance

Figure 4 plots the conductance as a function of frequency. The low frequency conductance of the BLM and torus in parallel varied both among membranes and for the same membrane as a function of time (over many hours). During the life of a membrane, including periods of maximum building vibration[1], the variation was as greater as a factor of 100. The average value of the low frequency limit of the conductance was 27 mS m^{-2} for PC/Ch and 7.0 mS m^{-2} for PC/Ch/Oes membrane-torus combinations. We do not attach much importance to these data. Electrical leaks may be associated with the boundary of such phases, and we believe this conductance to be a strong function of the macroscopic geometry of the membrane-torus system[1] and possibly much larger than the intrinsic conductance of the membranes. While the low frequency limit or parallel conductance may be due in large part to leaks in parallel with the membrane, increases in conductance with frequency demonstrate the impedance dispersion due to the internal dielectric structure of the membrane. Among membranes, the variation with frequency of the magnitude of these changes in conductance was of order 1%.

Discussion

Membrane impedance as a function of frequency

Dispersions of impedance with frequency arise from admittances in series (as shown in Fig. 2) because the time constants G/C are in general unequal. In a series of papers, Coster and others have calculated structures for various membranes, usually in low concentrations of salt (Ashcroft et al. 1977, 1981, 1983; Laver et al. 1984). The hydrocarbon, acetyl and head group regions been further divided into sub-regions in order to explain in detail the observed dispersion.

The impedance dispersions of a wide range of membranes of different composition are qualitatively similar, and share the following features:

i) The low frequency ($\lesssim$ 0.1 Hz) capacitance is nearly equal to that of the hydrophobic interior. For most reconstituted membranes this is in the range 5–10 mF m^{-2} (Smith 1977).
ii) The outer regions of the membrane (acetyl and polar head regions of phospholipid membranes) contribute dispersions over the frequency range 0.1 to 1,000 Hz. The smaller the admittance of these regions, the larger the change in total capacitance over this range.
iii) The dispersion due to the electrolyte solution occurs at frequencies of about 1 kHz. This layer is relatively thick and therefore has a small capacitance. As a result, the total capacitance decreases substantially, and at high frequencies the capacitance of the system approaches that of the bulk solution and double layer.

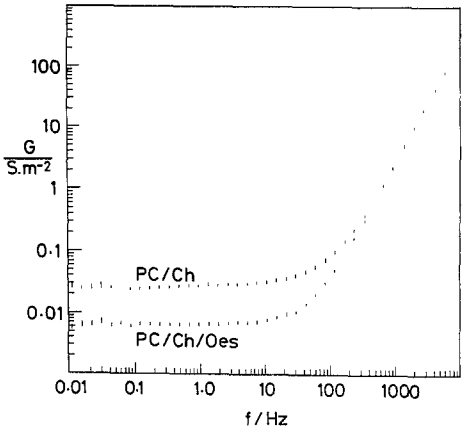

Fig. 4. The mean conductance per unit area as a function of frequency is plotted for the bilayer/torus system. The two data sets are for membranes of phosphatidylcholine and cholesterol; and of phosphatidylcholine, cholesterol and oestradiol

1 The conductance was lowest overnight (when the vibration in the building was least) and was greatest if the membrane was stretched by the hydrostatic pressure difference due to unequal evaporation from the two sides of the septum while the experiment was unattended. It was larger for membranes with very long irregular perimeters and for membranes which formed slowly, starting from several different black areas. It was larger for those which formed small multilayer phases (identified by bright interference colours) at the boundary between membrane and torus.

The results reported here for the PC/Ch membranes are very similar to those published by Ashcroft et al. (1983). Those authors, however, were unable to investigate frequencies below 0.1 Hz under comparable conditions and therefore did not report the substantial increase in capacitance at low frequency evident in Fig. 3. We observed a similar but smaller rise in capacitance at low frequencies in PC/Ch/Oes membranes. It is possible that this dispersion at very low frequency is due to a phenomenological impedance[2], rather than a Maxwell-Wagner dispersion as discussed above.

The small variation of specific capacitance with temperature suggests that there is very little hexadecane in the membrane (Laver 1983; Coster and Laver 1986).

Dielectric structure and the effect of Oestradiol

Figure 3 shows that the magnitudes of the capacitances of the two membranes are similar, but that the presence of Oestradiol has a dramatic effect on the dispersion of the capacitance.

The observed capacitance spectrum for PC/Ch membranes has been attributed to a hydrocarbon layer (whose dielectric constant and thickness give the low frequency capacitance at 0.1 Hz of 6.9 mF m^{-2}) in series with a layer containing the acetyl bonds and a layer containing the dipolar headgroups (Ashcroft et al. 1983). These outer layers disperse the impedance over the range 0.1 to 1,000 Hz and reduce the total capacitance by about 0.5 mF m^{-2}. The electrolyte solution is responsible for the dispersion above about 1 kHz.

To facilitate comparison with data published for other membranes, we have fitted a Maxwell-Wagner dispersion to the averaged impedance spectra. A five layer model for the membrane (not including the electric double layer and bulk solution) has been used (following Laver et al. 1984), and the ratio of the average residue squared to the variance was 0.0015 for PC/Ch and 0.0001 for PC/Ch/Oes. The layer are identified by these authors as the hydrophobic interior and four different exterior layers corresponding to the region containing the acetyl groups and the different head groups (see also Smith 1977 and the "Methods" section of this paper). Because of the likely contribution to the low frequency conductance by parallel leaks, the conductance of the hydrophobic region cannot be reliably estimated, although an upper estimate is evident in Fig. 4. The following parameters were obtained:

	C_1	C_2	C_3	C_4	C_5/mF m^{-2}
PC/Ch	6.9	330	480	460	500
PC/Ch/Oes	6.8	680	1,500	5,000	2,500
	G_1	G_2	G_3	G_4	G_5/S m^{-2}
PC/Ch	–	0.70	5.6	30	310
PC/Ch/Oes	–	0.65	35	1,300	7,500

For the purpose of comparison of the two types of membrane, however, an assignment of each of the layers to a specific chemical layer of the membrane is unnecessary: it is sufficient to regard the two capacitance spectra. For PC/Ch membranes, the capacitance of the hydrocarbon layer was $C_h \approx C(0.1\text{ Hz}) = 6.9$ mF m^{-2} while that of the whole membrane $C_m \approx C(1\text{ kHz})$ is smaller by about 0.5 mF m^{-2}. Using the equations for series admittance, and making the approximation that the conductance of the hydrocarbon layer is much less than that of the polar region, the series capacitance of the head groups and acetyl layer is approximately $C_h \cdot C_m/(C_h - C_m)$, i.e. 90 mF m^{-2}. For PC/Ch/Oes membranes, C_h is 6.8 mF m^{-2} and C_m(1 kHz) is less by about 0.12 mF m^{-2}. Consequently the series capacitance required is much larger: $C_h \cdot C_m/(C_h - C_m)$ is about 400 mF m^{-2}. The high frequency dispersion of the conductance (Fig. 4) gives less obvious information about the structure, however the information from the combined impedance data is represented in the table above. This table shows that, except for the first external layer, the effective conductance of the polar exterior layers of the bilayers is very much increased upon addition of Oestradiol.

The increase in admittance of the external layers of the PC/Ch/Oes membranes is an unexpectedly large effect: the susceptance is about five times larger than that of PC/Ch, and the conductance is also larger in the layers away from the hydrophobic core. Studies on the partitioning of Oestradiol into bilayer membranes (Heap et al. 1971; Lundberg 1979; Salin-Drouin 1985) indicate that Oestradiol partitions into Phosphatidylcholine and Phosphatidylcholine/Cholesterol membranes with a concentration of only about 4%. How is it possible that such a small concentration can cause

2 If the dispersion of capacitance at frequencies below 0.1 Hz were due to a Maxwell-Wagner dispersion, this would imply a larger limiting capacitance at low frequencies than has, to our knowledge, been reported. This in turn would suggest an unexpectedly high value for the capacitance of the hydrocarbon interior. One plausible candidate for a phenomenological capacitance is the polarisation at the membrane interface due to differential permeation of the membrane by the two ionic species (the "transport number" effect). Smith (1977) has calculated the capacitance as a function of frequency for membrane-solution systems with slightly different partition coefficient for the two ions. (Partitioning in this case may mean partitioning into membrane pores as well as into the membrane interior.) These calculations predict phenomenological capacitances which are rather larger than the geometric capacitance at or below 0.1 Hz.

such a large change in the admittance of the polar region?

That the dielectric constant and conductivity of the polar region are much lower than those of the external solution is probably a result of the lower concentration of ions in that region. The Born energy of an ion is low in the high dielectric medium of the solution, but it is much larger in the vicinity of lower dielectric media. To insert an ion among the closely packed head groups of a PC/Ch membrane presumably requires a large energy and the partitioning is therefore low.

The Oestradiol molecule is a four-ringed structure with a OH group at each end: these are the only hydrophilic groups of the molecule. It is therefore probable that it is located with the hydrocarbon rings in or at the edge of the hydrophobic region and the two OH groups pointing towards the solution. Cholesterol has also a four-ringed structure, but it has only one OH group (presumably anchored at the hydrophobic-hydrophilic interface) and the rest of the molecule (the rings and a branched hydrocarbon chain) are thought to extend into the bilayer interior. The proposed configuration of the Oestradiol molecule, in contrast, would create a "hole" among the polar head groups with an area of the same order as the cross-section of the Oestradiol molecule (in the plane of the rings). Such a water-filled hole would allow the partitioning of a small number of ions and thus provide a relatively low impedance shunt of the polar regions. This possible model suggests that Oestradiol, in concentration of a few percent, allows a rather larger number of ions to approach the hydrophobic interior of the membrane.

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