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THE EFFECTS OF CHOLESTEROL INCLUSION ON THE MOLECULAR ORGANISATION OF BIMOLECULAR LIPID MEMBRANES

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Dielectric measurements on planar egg phosphatidylcholine bilayers formed from *n*-hexadecane solutions indicate that these bilayers contain very low equilibrium concentrations of alkane. In 100 mM KCl the capacitance of the hydrophobic region was found to be 7.0 ± 0.2 mF/m^2. The addition of cholesterol (at 2:1 mole ratio) was found to affect only marginally the capacitance of the hydrophobic region of such bilayers. Precise measurements of the frequency dependence of the bilayer impedance at very low frequencies now allow the resolution of several electrically distinct substructural regions within the bilayer. Examination of the effects of cholesterol inclusion upon the electrical parameters of these substructural regions indicate that cholesterol spans the acetyl region (i.e. the region containing the glycerol bridge of the phosphatidylcholine molecules in the bilayer) with the hydroxyl group of the cholesterol molecules located inbetween the phosphate group and the glycerol oxygens of the phosphatidylcholine molecules. The capacitance of the hydrophobic region of both phosphatidylcholine and phosphatidylcholine/cholesterol bilayers formed from *n*-hexadecane solutions was found to decrease slightly as the external KCl concentration was decreased.

Introduction

Cholesterol is a common constituent of cell membranes and is indeed, in some membranes, one of the major lipids present. A large number of studies have shown that cholesterol can play important and diverse roles in cell membrane function. Thus it has been shown that cholesterol can vary the temperature for the phase transition in the lipid bilayer region of cell membranes and can alter their fluidity and permeability [1,2]. Other studies indicate that cholesterol can also modify more specialised membrane functions such as the induction of anaesthesia [3] and active transport [4–6].

It has been shown previously that precise measurements of the impedance of egg phosphatidylcholine bilayers at very low frequencies (0.01–100 Hz) allow a characterization of electrically distinct substructural layers within the bilayer [7–9]. Consideration of the electrical time constants of the layers so characterised permitted their identification with the hydrophobic, acetyl and polar-head layers within the lipid bilayer.

Here the polar-head region refers to the layer containing the choline-phosphate dipoles of the phosphatidylcholine molecules at the bilayer/electrolyte interface, whereas the acetyl region refers to the layer containing the carboxyl-ester oxygens

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located between the polar-head groups and the central hydrophobic region [9,10]. Subsequent improvements to the resolution and frequency range of our four-terminal digital impedance measuring technique now permit resolution of additional substructural features.

Here we report the results of such dielectric measurements on the effects of the incorporation of cholesterol into single planar egg phosphatidylcholine bilayers at various external electrolyte (KCl) concentrations.

These studies on egg phosphatidylcholine bilayers with added cholesterol are of interest because their composition and structure are presumably closer to that of the lipid bilayer present in some plasma cell membranes than those of the pure egg phosphatidylcholine bilayers studied previously [7–9]. Furthermore these measurements allow investigation of whether the location of cholesterol in these single planar bilayers is similar to that determined previously for multilayers by diffraction techniques [11,12] and NMR studies [13–15].

Materials and Methods

Experimental aspects

Bilayers were generated as described previously [7–9] from solutions of egg phosphatidylcholine or egg phosphatidylcholine and cholesterol (2 : 1 mole ratio) dissolved in *n*-hexadecane (10 mM phosphatidylcholine). Measurements were made at 25–30°C with the electrolyte solution (KCl) adjusted to pH 7 by the addition of KOH. Bilayers were generated at higher temperatures (40°C) and the temperature was reduced only after the bilayer had become 'black'. Stable bilayers could not be generated at temperatures less than 30°C. Once the bilayer impedance had settled sufficiently, a series of scans of the variation of the bilayer impedance with frequency was commenced. Each scan involved 35–45 impedance measurements spanning the frequency range 0.001 to 10000 Hz.

The impedance and phase angle measurements were made with an improved version of a four-terminal computer-controlled digital impedance spectrometer described previously [7–9,16]. All the results presented are for bilayers in which the 'dissolved' alkane solvent had reached its equilibrium value (as evidenced by no detectable variation in the bilayer capacitance over an extended period). This was achieved typically 30–60 min after the bilayer had become 'black'. Bilayers were observed under a stereo microscope and care was taken to ensure that the membranes were flat by adjusting the hydrostatic pressure on each side of the bilayer. This could be verified by a minimum in the measured capacitance. The bilayer area was determined to an accuracy of $\pm 1\%$ using a calibrated graticule.

Determination of bilayer substructure

A bilayer composed of a series of layers with different electrical time constants will exhibit a frequency dependent total capacitance and conductance due to electrical polarization at the interfaces of adjoining layers (a Maxwell-Wagner dispersion).

All the experimental data for the lipid bilayers showed such a frequency dependent capacitance and conductance which could be accurately fitted to the theoretical Maxwell-Wagner dispersion expected from a multilayer sandwich of substructural layers with different electrical time constants. This procedure yielded values for the individual capacitance and conductance of each layer in the bilayer.

In principle a dielectric model of the structure of lipid bilayers should allow for continuously varying dielectric properties. The finite accuracy of the experimental data, however, restricted us to a coarser subdivision of the bilayer structure. In the analysis of the data, additional structural subdivisions were included in the theoretical multilayer model only when this significantly improved the fit to the data (see Ref. 9 for further details). Thus continuously varying dielectric properties were modelled by a series of dielectrically distinct layers. Previously we reported the results of only three distinct regions (acetyl, hydrophobic and polar-head). The higher precision data presented here allowed us to resolve further subdivisions within each of the three regions (e.g., hydrophobic-1 and hydrophobic-2, etc.).

It should be noted here that any conductance not intrinsic to the bilayer (e.g. that of the torus) was determined by the technique of Hanai et al. [17], whereby the area of the bilayer is altered by

hydrostatic bowing. If the bilayer conductance is then plotted against the bilayer capacitance, the 'leak' conductance can be estimated from extrapolation to zero capacitance (i.e., zero area). The data presented in the tables, but not that in the figures, have been corrected for this 'leak' conductance.

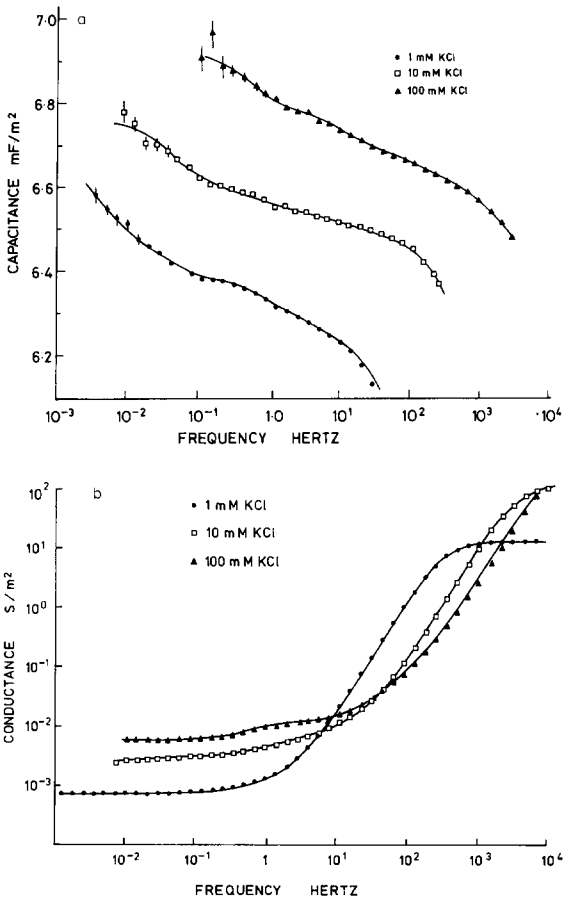

Fig. 1. The equivalent parallel capacitance (a) and conductance (b) of representative egg phosphatidylcholine bilayer/electrolyte systems formed from *n*-hexadecane solutions of the lipid. The error bars (generally too small to be visible) give the standard error of three to five frequency scans upon a single bilayer. The solid curves show the Maxwell-Wagner theoretical dispersion of the capacitance and conductance of a bilayer/electrolyte system in which five distinct types of substructural regions are present in the bilayer. In the theoretical curves, the independently measured 'leak conductance' was included as a separate parameter in the fitting routine.

Results

Fig. 1 shows the equivalent parallel capacitance and conductance of a representative egg phosphatidylcholine bilayer/electrolyte system (formed with *n*-hexadecane solvent) as a function of frequency for three concentrations of external KCl electrolyte. The capacitance data for 1 mM KCl shows the presence of three prominent frequency dispersions. The two dispersions at lower frequencies are a consequence of the three main types of electrically distinct substructural regions in the bilayer viz; (hydrophobic, acetyl, and polar-head regions). The large dispersions at the highest frequencies occurs because of the presence of the electrolyte external to the bilayer surface. The 'leak' conductance (i.e. that extrinsic to the bilayer) was always less than 60% that of the conductance measured at 1 Hz.

Effect of cholesterol

Bilayers were found to form more easily and had enhanced stability when cholesterol was present. The dispersion with frequency of the capacitance of representative egg phosphatidylcholine bilayer/electrolyte systems with and without cholesterol in 1 mM KCl is shown in Fig. 2. For these two systems it is possible to resolve further dielectric substructures within each of the three main regions characterised previously.

Determination of the equivalent electrical substructural parameters (see Table I) * revealed that the primary effects of the inclusion of cholesterol in egg phosphatidylcholine bilayers formed in 1 mM KCl were:

(a) a small (4%) increase in the total capacitance of the hydrophobic region.

(b) a 40–50% reduction in the capacitance of the acetyl region.

These alterations in the substructural parameters show that cholesterol is indeed present in the bilayer phase, and that the previously observed absence of an effect of cholesterol on the total

* The values listed in Tables I and II refer to the series combination of electrically identical regions on either side of the bilayer. The bilayer is assumed symmetric about its midplane.

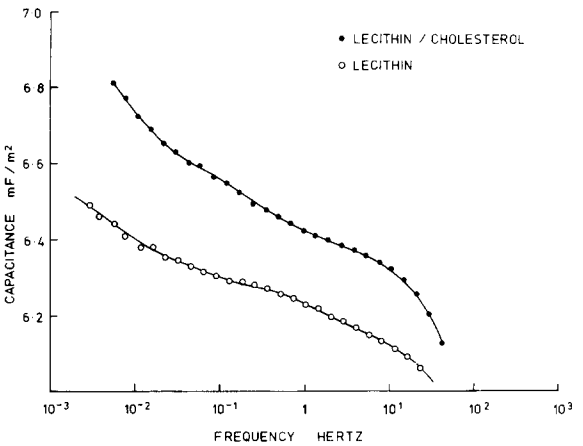

Fig. 2. The frequency dependence of the equivalent parallel capacitance of representative egg phosphatidylcholine bilayer/electrolyte systems formed with and without cholesterol from *n*-hexadecane solutions in 1 mM KCl electrolyte. Note the increased dispersion in the capacitance at low frequencies for the bilayer containing (2:1 mole ratio) phosphatidylcholine/cholesterol. Cholesterol, however, was found to only marginally affect the frequency dependence of the equivalent parallel conductance of such bilayers.

measured capacitance of such bilayers [18] could not simply have been due to its exclusion from the bilayer phase when bilayers are generated from phosphatidylcholine/cholesterol mixtures.

Effect of varying the external electrolyte concentration

The capacitance of the hydrophobic region was found to increase with increasing external KCl concentration for bilayers formed with and without cholesterol. The effect of cholesterol on the impedance dispersion of egg phosphatidylcholine bilayers was reduced at higher KCl concentrations. A summary of the dielectric parameters of bilayers with and without cholesterol at various KCl concentrations is given in Table II.

Discussion

'Solvent-free' lipid bilayers made from hexadecane solutions

The specific capacitance of the hydrophobic region of egg phosphatidylcholine bilayers formed in 100 mM KCl with *n*-hexadecane solvent was found to be 7.0 ± 0.2 mF/m^2. Bilayers formed by monolayer apposition, believed to be essentially solvent free [19], have comparable values for the total capacitance (e.g. egg phosphatidylcholine 7.6 ± 0.4 mF/m^2 [20]; dioleoylphosphatidylcholine 7.28 ± 0.19 mF/m^2 [21], 7.21 ± 0.21 mF/m^2 [19]). It should be emphasized here that the values of capacitance we present in this paper are for bilayers that were 'black' for typically one hour. Capacitances determined soon after a film had become entirely black were around 6.5 mF/m^2,

TABLE I

THE SUBSTRUCTURAL PARAMETERS OF EACH ELECTRICALLY DISTINCT REGION WITHIN EGG PHOSPHATIDYLCHOLINE BILAYERS WITH AND WITHOUT CHOLESTEROL

The figures present means ± S.D. for a number of different bilayers (*n*). PC, phosphatidylcholine; Chol, cholesterol.

Region	Capacitance (mF/m^2)		Conductance (mS/m^2)	
	PC $n = 10$	PC/Chol $n = 20$	PC $n = 10$	PC/Chol $n = 20$
Hydrophobic 1	12.7 ± 0.2	13.2 ± 0.2	0.3 ± 0.2	0.5 ± 0.3
Hydrophobic 2	12.7 ± 0.2	13.2 ± 0.2	0.5 ± 0.3	1.0 ± 0.3
Acetyl 1	330 ± 50	200 ± 25	20 ± 10	20 ± 10
Acetyl 2	600 ± 100	300 ± 75	250 ± 100	250 ± 50
Polar-head 1	600 ± 100	500 ± 100	$(3.5 \pm 1.3) \cdot 10^3$	$(5.5 \pm 1.5) \cdot 10^3$
Polar-head 2	550 ± 100	450 ± 75	$(25 \pm 2.5) \cdot 10^3$	$(45 \pm 15) \cdot 10^3$
Polar-head 3	350 ± 50	250 ± 50	$(2.5 \pm 0.5) \cdot 10^5$	$(2.5 \pm 0.15) \cdot 10^5$

TABLE II

THE TOTAL PARALLEL CAPACITANCE AND CONDUCTANCE OF THE THREE MAIN REGIONS OF BOTH EGG PHOSPHATIDYLCHOLINE AND EGG PHOSPHATIDYLCHOLINE/CHOLESTEROL BILAYERS AT DIFFERENT ELECTROLYTE (KCl) CONCENTRATIONS

The parameters for each main region are the series combination of the electrical parameters of several subregions. The * refers to parameters that could not be reliably determined from our experimental data. The conductances referring to the polar heads give the total range of conductances of the resolvable regions in the polar heads. Note how the conductance of different parts of the polar-head region can differ by several orders of magnitude. The figures present means ± S.D. for a number of different bilayers (l for phosphatidylcholine (PC) bilayers; m for phosphatidylcholine/cholesterol (PC/Chol) bilayers).

		Capacitance (mF/m^2)			Conductance (mS/m^2)		
		1 mM KCl $l = 10$ $m = 20$	10 mM KCl $l = 5$ $m = 3$	100 mM KCl $l = 4$ $m = 7$	1 mM KCl $l = 10$ $m = 20$	10 mM KCl $l = 5$ $m = 3$	100 mM KCl $l = 4$ $m = 7$
Hydrophobic	PC	6.35 ± 0.1	6.7 ± 0.2	7 ± 0.2	0.2 ± 0.1	1.5 ± 0.5	2.5 ± 0.5
	PC/Chol	6.6 ± 0.1	6.8 ± 0.2	7 ± 0.2	0.35 ± 0.15	1.5 ± 0.5	2.5 ± 0.5
Acetyl	PC	600 ± 100	400 ± 50	*	250 ± 100	250 ± 25	*
	PC/Chol	300 ± 50	200 ± 50	*	250 ± 50	300 ± 30	*
Polar-head	PC	160 ± 30	260 ± 40	130 ± 20	$(3–250)\cdot10^3$	$(2.5–300)\cdot10^3$	$(1.2–2500)\cdot10^3$
	PC/Chol	120 ± 20	180 ± 20	200 ± 50	$(5–250)\cdot10^3$	$(1–300)\cdot10^3$	$(1–7000)\cdot10^3$

which are similar to values reported in the literature previously for such bilayers [18,21].

As the values of the hydrophobic capacitance reported here are similar to those reported for 'solvent-free' bilayers, it is hard to escape the conclusion that these bilayers formed from *n*-hexadecane must contain correspondingly low solvent concentrations. Further support for this view is derived from measurements of the capacitance of the hydrophobic region as a function of temperature (Coster, H.G.L. and Laver, D.R., unpublished data) which show that below 30°C the capacitance is essentially independent of temperature. However above 30°C variations in the capacitance with temperature are related to alterations of the alkane 'solubility' in the bilayer [22].

Dielectric structure of phosphatidylcholine bilayers

To investigate the location of cholesterol within these bilayers it is first necessary to consider the dielectric substructure of egg phosphatidylcholine bilayers in more detail. Interpretation of the spatial dielectric structure from the data presented in Tables I and II requires estimates of the dielectric constant of each region in the bilayer. While little is directly known about their values, the constraints imposed by their possible dimensions within a bilayer (estimated from molecular models), and their known chemical nature, narrow the range of possible values. Additionally it appears reasonable to assume that both the dielectric constant and conductivity decrease towards the interior of the bilayer.

The parameters listed in Table I, indicate that the hydrophobic region is composed of two types of substructural region with electrical time constants differing by a factor of approximately 2. That these two time constants are present is clearly evident from the impedance dispersion in the frequency range 0.003 Hz–0.03 Hz. It is difficult to successfully determine the relative thicknesses of the two electrically distinct regions. However the geometrical division determined from the dielectric measurements is similar to that obtained in NMR studies using deuterated lipids, which show that bilayers have a constant order parameter extending from the glycerol group to about half way into the hydrophobic region [13,14]; the bilayer midplane having a much lower order parameter.

The parameters of the acetyl region are consistent with an inner region being associated with the first few carbon atoms of the acyl chains and an outer region being localised in the oxygen-dense region containing the carbonyl-ester linkages.

Our high frequency dielectric data, which contains information concerning the structure of the headgroup dipoles, is partially masked by the impedance of the thin layer of external electrolyte in series with the bilayer [9] (between the bilayer and the plane containing the tips of the potential measuring electrodes). Therefore the assignment of substructural regions within the polar heads is less certain. Studies on multilayers indicate that in the absence of external ions the choline-phosphate dipole is aligned parallel to the interface [12,23–25] although there is some evidence that this dipole can be extended normal to the interface for bilayers [26].

Fig. 3 shows the putative identification (relative to the location of phosphatidylcholine molecules) of the electrically distinct regions in an idealized schematic of an egg phosphatidylcholine molecule in a bilayer. The estimated dielectric constant for each is given.

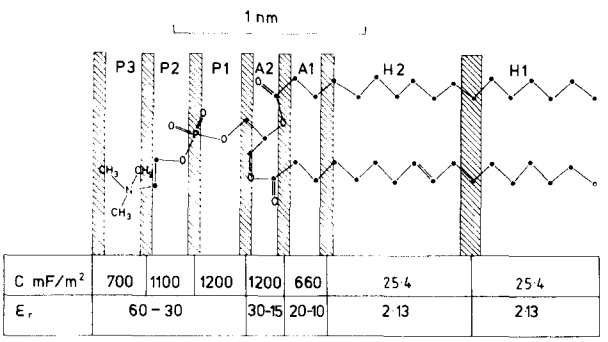

Fig. 3. A schematic model of an egg phosphatidylcholine molecule showing the putative location of each distinct dielectric region. The estimated range of dielectric constant of each distinct layer is shown at 1 mM KCl concentration. The polar head of the phosphatidylcholine molecule is shown here at approx. 45° to the bilayer normal, however, other orientations are equally likely. The data are representative of an average of 10 egg phosphatidylcholine bilayers. The dielectric constant ϵ_r, capacitance C, and the thickness d of any region are related by $C = \epsilon_r \epsilon_0 / d$ where ϵ_0 is the permittivity of free space. H1 and H2 refer to the hydrophobic region, A1 and A2 to the acetyl regions and P1–P3 to the polar head regions. It must be stressed however, that these dielectric regions need not correspond to specific parts of the phosphatidylcholine molecules, but because of thermal motions will represent both time and spatially average values over different parts of the phosphatidylcholine molecules.

The location of cholesterol

The location of cholesterol within the bilayer can now be determined from examination of its effects upon the individual structural parameters. Alteration of the effective dielectric constant of any region should produce a corresponding change in the equivalent parallel capacitance of that region. The dielectric constants of the cholesterol molecule (with the exception of the hydroxyl group) is thought to be 2.27 whereas that of the phosphatidylcholine acyl chains is thought to be 2.13 [18]. Therefore the presence of cholesterol should not significantly alter the average dielectric constant of the hydrophobic region. However, the dielectric constant of the acetyl region of the egg phosphatidylcholine bilayers appears to be significantly higher (10–30). Thus if the ring structure of cholesterol, with its low dielectric constant, bridges the acetyl region, an approx. 30% reduction in the average dielectric constant of that region would result for a 2 : 1 phosphatidylcholine/cholesterol mole ratio. If the cholesterol ring structure extended into the polar-head region a similar decrease in capacitance of the polar-head region should be expected. The results in Tables I and II show a reduction of at least 30% in the capacitance of the acetyl region and only a small alteration of the polar-head capacitance. This indicates that the cholesterol ring structure certainly extends outwards past the glycerol bridge but not as far as the inner portion of the polar heads of the phosphatidylcholine molecules.

On the other hand the hydroxyl group on the cholesterol molecule should be reasonably polar, presumably having a significantly higher effective dielectric constant than the rest of the molecule. Thus if the hydroxyl group of the cholesterol were located within the acetyl region, the dramatic decrease always seen in the acetyl region capacitance upon the inclusion of cholesterol would not be expected. On the other hand, the dielectric constants of the hydroxyl group of cholesterol and that of the phosphatidylcholine polar-head regions are likely to be similar and so no gross change of the average dielectric constant (and hence capacitance) of the inner polar-head region would be expected if the hydroxyl group were located there.

The dielectric data are thus consistent with the cholesterol hydroxyl group being located inbe-

tween the phosphate group and glycerol oxygens of the phosphatidylcholine molecules. Diffraction studies on multilayers [11,12] indicate that the hydroxyl group of cholesterol is near the glycerol bridge. Some NMR studies on vesicles, however, have found interactions between the hydroxyl group of cholesterol and the phosphatidylcholine carbonyl group [27,28] whereas others found interactions with the phosphatidylcholine phosphate group [29,30]. Our dielectric data suggest a location intermediate to these two models, and perhaps reflects the fact that our data refer to a different lipid system, i.e., a single planar bilayer in the presence of abundant water.

The presence of cholesterol in egg phosphatidylcholine bilayers would increase the average separation of the head-group dipoles of the adjacent phosphatidylcholine molecules thus decreasing their attractive interaction and allowing them to extend slightly. Such a change in the head-group tilt would result in an effective thickening of the polar head region which is consistent with the small but consistently observed change in the polar head capacitances (see Tables I and II).

Effect of cholesterol on bilayer thickness

The absence of an effect of cholesterol on the thickness of phosphatidylcholine/*n*-hexadecane bilayers formed in 100 mM KCl, which have a hydrophobic region 2.7 nm in thickness (from our capacitance data), suggests that the hydrophobic region of the cholesterol molecules extends to approx. 1.35 nm from the interface. Thus for bilayers thicker than 2.7 nm, the effect of cholesterol should be to reduce the thickness by enhanced kinking of the phosphatidylcholine acyl chains as they fill the 'void' spaces that would otherwise exist at the bilayer center adjacent to the end of cholesterol molecules.

Our results show that cholesterol can alter the width of bilayers containing low concentrations of alkane solvent. Whether the widely varying physiological and biochemical effects of cholesterol in cell membranes are to some degree a consequence of altered stresses in the intrinsic membrane proteins, which could arise from variations in the thickness (due to cholesterol) of the surrounding lipid matrix, remains to be resolved. If the lipids surrounding such proteins had, for example, fully extended acyl chains, then cholesterol could produce large effects in the lipids adjacent to these proteins without significantly altering the rest of the bilayer.

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References

1 Pang, K.Y. and Miller, K.W. (1978) Biochim. Biophys. Acta 511, 1–9
2 Deuticke, B. and Ruska, C. (1976) Biochim. Biophys. Acta 433, 638–653
3 Stephens, C.L. and Shinitsky, M. (1977) Nature 270, 267–268
4 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603–615
5 Warren, G.B., Houslay, M.D., Metcalfe, J.C. and Birdsall, N.J.M. (1975) Nature 255, 684–687
6 Madden, T.D., Chapman, D. and Quinn, P.J. (1979) Nature 279, 538–541
7 Coster, H.G.L. and Smith, J.R. (1974) Biochim. Biophys. Acta 373, 151–164
8 Ashcroft, R.G., Coster, H.G.L. and Smith, J.R. (1977) Biochim. Biophys. Acta 469, 13–22
9 Ashcroft, R.G., Coster, H.G.L. and Smith, J.R. (1981) Biochim. Biophys. Acta 643, 191–204
10 Ashcroft, R.G., Thulborn, K.R., Smith, J.R., Coster, H.G.L. and Sawyer, W.H. (1980) Biochim. Biophys. Acta 602, 299–308
11 Franks, N.P. (1976) J. Mol. Biol. 100, 345–358
12 Worcester, D.L. and Franks, N.P. (1976) J. Mol. Biol. 100, 359–378
13 Seelig, J. and Seelig, A. (1974) Biochemistry 13, 4839–4845
14 Stockton, G.W. and Smith, I.C.P. (1976) Chem. Phys. Lipids 17, 251–263
15 Turner, G.L. and Oldfield, E. (1979) Nature 277, 669–670
16 Bell, D.J., Coster, H.G.L. and Smith, J.R. (1975) J. Physics E. 8, 66–70
17 Hanai, T., Haydon, D.A. and Taylor, J. (1965) J. Theoret. Biol. 9, 433–443
18 Benz, R., Fröhlich, O., Läuger, P. and Montal, M. (1975) Biochim. Biophys. Acta 394, 323–334
19 Fettiplace, R. (1978) Biochim. Biophys. Acta 513, 1–10
20 Benz, R. and Janko, K. (1976) Biochim. Biophys. Acta 455, 721–738
21 Fettiplace, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membrane Biol. 5, 277–296
22 White, S.H. (1978) Biophys. J. 23, 337–347

23 Büldt, G., Gally, H.U., Seelig, A., Seelig, J. and Zaccai, G. (1978) Nature 271, 182–184
24 McIntosh, T.J. (1978) Biochim. Biophys. Acta 513, 43–58
25 Büldt, G., Gally, H.U., Seelig, A., Seelig, J. and Zaccai, G. (1979) J. Mol. Biol. 134, 673–691
26 Hauser, H., Philips, M.C., Levine, B.A. and Williams, R.J.P. (1976) Nature 261, 390–394
27 Huang, C.H. (1976) Nature 259, 242–244
28 Chatterjie, N. and Brockerhoff, H. (1978) Biochim. Biophys. Acta 511, 116–119
29 Phillips, M.C. and Finer, E.G. (1974) Biochim. Biophys. Acta 356, 199–206
30 Darke, A., Finer, E.G., Flook, A.G. and Phillips, M.C. (1972) J. Mol. Biol. 63, 265–279