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THE STOICHIOMETRY AND DYNAMICS OF LECITHIN–CHOLESTEROL CLUSTERS IN BILAYER MEMBRANES

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SUMMARY

NMR experiments comparing the immobilisation of lecithin chains by cholesterol and by 5α-androstan-3β-ol confirm the 1 : 1 stoichiometry of the lecithin–sterol complex and the molecular details of the interaction given by Darke et al. (Darke, A., Finer, E. G., Flook, A. G. and Phillips, M. C. (1972) J. Mol. Biol. 63, 265–279) (in particular, that the cholesterol OH lies next to the lecithin phosphate.) In bilayers containing less than equimolar amounts of cholesterol discrete regions of 1 : 1 complex separate out; this leaves (a) lecithin molecules at the boundaries of these regions, for which cooperative motions are not possible; and (b) clusters of free lecithin molecules in which the hydrocarbon chains undergo cooperative motions and which freeze at the usual gel-to-liquid-crystal transition temperature (T_c). There is a significant fraction of the latter type of lecithin molecule only when less than about 30 mole % cholesterol is present. When the level of cholesterol in lecithin bilayers is decreased, the increase in free lecithin cluster size causes the motions of the hydrocarbon chains in these regions to become more cooperative such that the transition enthalpy per molecule of Type (b) approaches that of pure lecithin. At room temperature ($T > T_c$), the exchange rate between complexed and uncomplexed lecithin probably lies between 10^{-1} s^{-1} and 10^2 s^{-1}.

INTRODUCTION

The extensive studies in the past twenty years of the interaction between phospholipids and cholesterol have been in broad agreement in showing that the presence of cholesterol affects the hydrocarbon chain packing in mixed monolayers and bilayers (for reviews, see refs 1 and 2). In particular, the restriction of hydrocarbon chain motions in the liquid-crystalline state and the perturbation of the gel-to-liquid-crystal transition have been well confirmed by a range of spectroscopic techniques (refs 3–11 and refs cited in refs 1 and 2). Another significant finding of the NMR studies [3, 4] is the fact that in mixed bilayers containing less than equimolar amounts of cholesterol, the sterol is not distributed evenly along the bilayer. This clustering of molecules or "lateral-phase separation" has since also been detected by wide angle

X-ray diffraction [12] and spin-label [13] experiments. These clustering effects, which can also occur in mixed phospholipid bilayers [14–16], are a consequence of the cooperative nature of the hydrocarbon chain motions and packing. In order to minimise the free energy of the phospholipid molecules in a single-component bilayer by maximising both the van der Waals interactions and the configurational entropy of the flexible molecules, the arrangement of the chains has to be close-packed and yet allow considerable segmental motion. This can only be achieved if these motions are cooperative. When rigid "solute" molecules (e.g. cholesterol, proteins) which cannot satisfy these requirements are introduced into a liquid–crystalline bilayer, the system minimises its free energy by retaining, to some extent, discrete regions of lecithin in which the cooperative chain packing can occur. This shows that the configurational entropy of the chains is more important than the entropy which would be gained by random mixing.

Given that such clustering occurs in lecithin–cholesterol bilayers, we need to know the composition of the cholesterol-rich regions. There is disagreement about the stoichiometry of the lecithin–cholesterol association (cf. refs 3, 4, 12, 13, 17) and the purpose of this communication is to present new data confirming the existence and molecular arrangement of an equimolar complex, and to discuss its lifetime. The cooperativity of the system leads to complicated behaviour at the gel-to-liquid-crystal transition, and failure to appreciate this fact has given rise to incorrect interpretations of various data; we show how the apparent inconsistencies in the literature can be rationalized if due account is taken of the cooperativity.

Molecular packing

When the lipid concentration (C) is less than 60 % (w/w) (i.e. excess water is present), the maximum amount of cholesterol which can be incorporated into lecithin bilayers is equimolar with the lecithin [18–20]. At this composition, the bilayer is comprised completely of 1 : 1 complexes and it is homogeneous along its plane. NMR determinations [3, 4] of the segmental motion of lecithin molecules in these bilayers as compared to pure lecithin bilayers have shown that in the apolar region the first ten methylene groups are rendered least mobile, leaving the terminal methyl groups relatively free; in the polar region the trimethylamino group is slightly restricted although the glycerol backbone has more motional freedom. The least mobile methylene groups have similar mobility to the cholesterol molecule; in the latter the alkyl chain at C-17 is not rotating freely* and contrary to the assumption by Rothman and Engelman [21] has roughly the same mobility as the steroid nucleus. These data are consistent with the lecithin and cholesterol molecules being juxtaposed with the cholesterol hydroxyl group hydrogen-bonded to the phosphate group of the lecithin and the remainder of the sterol molecule extending along the lecithin chains to C-10 (see photographs in refs 3 and 22).

The details of this arrangement are in agreement with optical-rotatory-dispersion studies of a series of cholesterol-related steroids in lecithin bilayers [22] and an

* The relative lack of mobility of this group is probably due to the difficulty of rotating a branched chain. It has been clearly demonstrated [4] by (a) the fact that no high resolution spectrum was observed from any cholesterol protons in sonicated codispersions with denterated lecithin, and (b) a detailed analysis of the wide-line NMR spectrum of unsonicated codispersions.

infrared dichroism study of lecithin–cholesterol multibilayers [34]. The involvement of the hydroxyl group and the C-17 side chain in the interaction of cholesterol with lecithin has also been neatly demonstrated by comparison of the interactions of a series of related sterols [23, 24]. However, the idea (drawn from model-building studies [21]) of the cholesterol being more deeply immersed in the bilayer is incompatible with these findings. We have now obtained further evidence for the relative positions of the steroid nucleus and the lecithin molecule being as described above. High resolution NMR spectra (100 MHz, 26 °C) were obtained from sonicated dispersions of egg lecithin (0.5–1 % (w/v)) alone, and codispersed with either cholesterol or 5α-androstan-3β-ol (molar ratio 2 lecithin : 1 sterol). This isomer of androstanol differs from cholesterol only in that the eight-carbon side chain on C-17 is missing and that the ring system is saturated; removal of the double bond is not significant because the interactions of cholestanol and cholesterol with lecithin are the same [23]. The percentage of lecithin alkyl chain protons contributing to the high resolution integral [25], measured over 3 ppm and allowing for the sloping baseline caused by broad lines under the integrated signal, was 88 % with androstanol and 70 % with cholesterol. The figures are normalised to 100 % for lecithin alone; the actual figure observed for pure lecithin was only 92 %, probably because of incomplete sonication and some loss of signal in the wings of the peak [25].

The missing signal is from the protons on methylene groups whose mobility is decreased by contact with the sterol. These amount to only 12 % in the case of androstanol, but to 30 % with cholesterol. This difference is consistent with other reports [23, 26] which indicate that the condensation of lecithin by androstanol is less than that by cholesterol. The difference of a factor of 2.5 in the amount of broadened NMR signal can only be due to the effect of the branched cholesterol side chain, which is effectively six carbons long; it substantiates the earlier finding [4] that the side chain is rigid. If the length of the sterol nucleus in contact with the lecithin hydrocarbon chains is equivalent to x methylenes, and we assume that the stoichiometry of interaction is the same for each sterol and that every affected lecithin methylene gives a resonance too broad to be observed by our measurement [4], then* $x/(x+6) = 12/30$. Thus $x = 4$; this is in complete agreement with our original estimate [3, 4] for the relative positions of the two molecules, arrived at by completely different measurements.

Stoichiometry

The above NMR results with codispersions containing 2 : 1 mole ratios of lecithin–sterol cast light on the problem of the stoichiometry of the cholesterol-rich regions in non-equimolar mixtures of lecithin and cholesterol. Thus, the missing signal from protons on methylene groups whose mobility is decreased by contact with the sterol is a function of both (a) the relative positions of the molecules in the plane of their long axes (see above), and (b) the number of lecithin molecules affected by each cholesterol molecule (i.e. the stoichiometry of interaction). If each sterol molecule interacts with and immobilises n lecithin chains, then the fraction of lecithin chain NMR signal lost $= ny/$(total number of lecithin methylene groups per sterol

* This approximate calculation ignores the fact that 10 % of the chain carbons interacting with cholesterol are likely to be –CH= rather than $-CH_2-$.

molecule) where the length of the sterol molecule in contact with the lecithin hydrocarbon chains is equivalent to y methylenes. The total number of lecithin methylene groups present per sterol molecule is about 62 (for NMR purposes, $CH_3 = 1.5\,CH_2$), plus four –CH= groups which are not included in the integrated signal. For androstanol, $y = 4$ as found above; for cholesterol, $y = 10-$ (average number of –CH= groups per chain) $= 9$. Thus for androstanol, $4n/62 = 0.12$: $n = 1.9$. For cholesterol, $9n/62 = 0.3$: $n = 2.1$. Thus $n = 2.0 \pm 0.1$. Since each lecithin molecule has two chains, the stoichiometry of the interaction is $n/2 : 1$, i.e. equimolar. This stoichiometry is entirely consistent with the lecithin–cholesterol–water phase diagram because when $C \leqq 60\,\%$ (w/w), crystalline cholesterol separates if the cholesterol/lecithin ratio is increased above 1 : 1 (refs 18 and 19). This fact has been overlooked by Shimshick and McConnell [13] who detected clustering by spin-label studies, but claimed that there was no evidence for the clustering being due to separation of stoichiometric complexes.

Measurements such as the above, which look mainly at the properties of individual molecules, show unambiguously that the interaction stoichiometry is 1:1. However, measurements which rely on the cooperative properties of assemblies of uncomplexed lecithin molecules have given results which have sometimes been interpreted in terms of a 2 : 1 stoichiometry [12, 17]. This discrepancy originates from the facts that lecithin–cholesterol bilayers contain two phases* (one being the free lecithin clusters and the other the regions of complex), and that the cooperativity of lecithin molecular motions varies with complex concentration (see below). This will now be discussed further.

The term "cooperativity" is normally applied to a transition (e.g. melting, helix → coil [35]), and is generally described by the number of molecules (n) acting in concert, i.e. by the size of the cooperative unit. We wish to describe the cooperativity not only of the lecithin gel-to-liquid-crystal transition, but also of the segmental motions of neighbouring lecithin molecules above and below the transition. In this case the transition enthalpy (ΔH) also provides useful information, because it measures how efficiently packing is maintained in the two phases. As an example, the average measured ΔH would be reduced by the presence of a number of uncomplexed molecules which were badly packed, and which could only undergo non-cooperative motions and therefore not undergo the transition.

The temperature range over which the calorimetric transition occurs, ΔT, is related to both n and ΔH, since it is inversely dependent [17] on the van 't Hoff heat, which itself is the product of n and ΔH. Thus

$$(\Delta T)^{-1} \propto n \cdot \Delta H \tag{1}$$

The derivation of Eqn 1 assumes that the transition behaves like normal melting transitions, in that it is a cooperative two-state phenomenon. Then $(\Delta T)^{-1}$ is a convenient measure of the cooperativity for our purpose. It is important to note that the interpretation of Eqn 1 is different for one- and two-phase systems. Thus for pure lecithin bilayers where there is no clustering or lateral phase separation, $\Delta H = \Delta H'$, the transition enthalpy per mole of total lecithin. On this basis, for pure lecithin in dilute dispersions $n \approx 10^2$ (refs 27 and 28). In an ideal lattice n would be infinite

* A small amount of miscibility of the two phases is not ruled out by the experimental data.

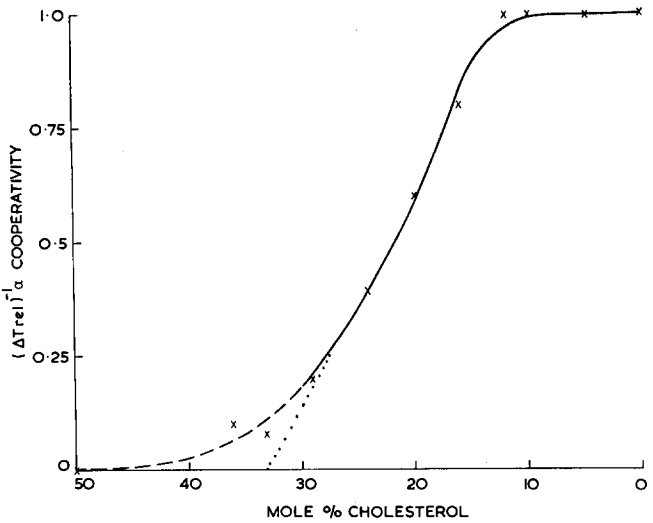

Fig. 1. The variation with composition of the cooperativity of the gel-to-liquid-crystal transition in mixed dipalmitoyllecithin–cholesterol bilayers. The relative widths of the transition (ΔT_{rel}) were obtained by smoothing published data [17, 18] and from some unpublished measurements of ours by normalising with respect to ΔT for pure dipalmitoyllecithin bilayers. $(\Delta T)^{-1}$ is a measure of the cooperativity of the transition, as explained in the text. The exact form of the curve in the dashed region is dependent on sample history.

but this is reduced in real systems by the presence of dislocations, caused by impurities and lattice vacancies. However because of clustering, the lecithin–cholesterol mixed bilayer is a two phase system; in this case there is an added complication in the application of Eqn 1. Complexed lecithin molecules cannot undergo the gel-to-liquid-crystal transition and therefore ΔH has to be per mole of uncomplexed lecithin (the mid-point of the transition of the uncomplexed lecithin occurs at the usual temperature, T_c); i.e. $\Delta H \neq \Delta H'$. ΔH cannot be calculated without knowledge of the stoichiometry of the lecithin–cholesterol association.

Fig. 1 shows a plot of $(\Delta T)^{-1}$ as a function of composition for mixed dipalmitoyl lecithin–cholesterol bilayers. From 0–15 mole % cholesterol, n and ΔH are constant and the only effect of cholesterol is to remove lecithin to form regions of 1 : 1 complex. The reduction in cooperativity when the cholesterol concentration is greater than approximately 15 mole % indicates a steady change, with composition, of the properties of the lecithin clusters and thus a reduction of either n or ΔH, or both. From Eqn 1 and using $\Delta H'$ to calculate the ΔH expected on the basis of a 1 : 1 interaction, it can be shown that both n and ΔH decrease from the values expected for pure lecithin. The reduction in n with increasing cholesterol suggests that eventually the cluster size becomes less than that of the original cooperative units. The reduction in ΔH occurs because the interaction energy between neighbouring lecithin molecules in the gel phase is reduced if the motions cannot be completely cooperative, as is the case when the clusters become smaller. A further cause of the reduction in average ΔH per molecule of uncomplexed lecithin is that the clusters are surrounded by a border of lecithin molecules (Fig. 2), which cannot undergo the same cooperative motions as molecules inside the

clusters. This is because they are juxtaposed with rigid lecithin–cholesterol complexes. These boundary molecules are fairly mobile, since they are not directly associated with cholesterol molecules; however they will have a much smaller (possibly zero) gel-to-liquid-crystal transition enthalpy, and will in any case melt over a wide temperature range. There may also be a few uncomplexed lecithin molecules within the complex regions, behaving in the same way as those at the borders. Another paper (Finer, E. G. and Phillips, M. C., unpublished) will discuss the relative importance of the effects of the boundary molecules, and of changes in the cooperativity of molecules inside the clusters, on the reduction in average ΔH.

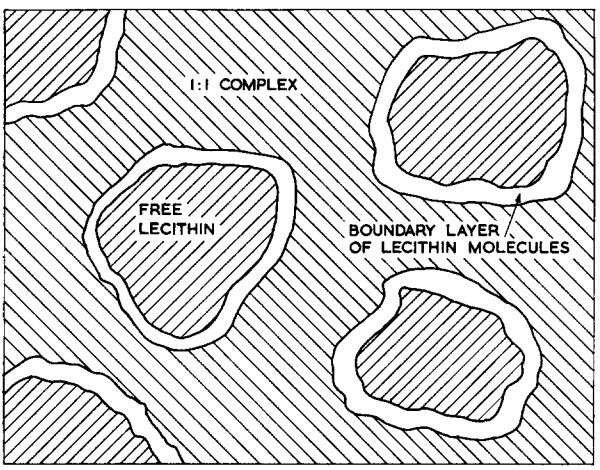

Fig. 2. Schematic representation of the structure of a mixed lecithin–cholesterol bilayer which contains less than equimolar amounts of cholesterol. The three different environments of lecithin molecules are shown. The clusters of free lecithin are of the order of 10^2 Å in diameter at high cholesterol concentrations.

The number of clusters and distribution of cluster sizes in a bilayer such as is shown in Fig. 2 will depend on the history of the sample under examination, especially when the sample is stored at $T < T_c$. Even pure phospholipid systems undergo slow structural reorganizations under these conditions [37]. This explains why irreproducible results have been obtained even with the most careful measurements [17, 18] on chemically well-defined systems.

We can now see how the complexity of this system can lead to difficulties in the interpretation of plots of the composition dependence of parameters such as the gel-to-liquid-crystal transition enthalpy [17, 18] and the intensity of wide-angle X-ray-diffraction patterns [12]. Such plots depend not only on the amount of uncomplexed material but on the cooperativity of its motions and packing. Fig. 1 shows that the cooperativity extrapolates to zero for bilayers containing about a 2 : 1 molar ratio (i.e. 33 mole % cholesterol, see dotted line in Fig. 1). Therefore any plot dependent on cooperativity will be the same and extrapolations of such data do not provide evidence for a 2 : 1 complex (cf. ref. 13).

Dynamics

The cholesterol-rich regions in Fig. 2 consist of 1 : 1 complex packed in the same fashion as that described above for homogeneous equimolar lecithin–cholesterol bilayers. These regions have a state of segmental fluidity which is, taking the average along the lecithin chain, intermediate between lecithin gel and liquid–crystalline phases [4]. The rate of lateral diffusion of lecithin molecules in these regions is decreased by a factor of 1.5–2.0 as compared to pure, liquid–crystalline lecithin bilayers [29]. In the latter, effective pairwise exchange of neighbouring molecules occurs with a frequency of the order of 10^7 s^{-1}. Since diffusion coefficients are inversely proportional to molecular size, assuming constant lateral viscosity [36], the diffusing unit in the mixed bilayer is probably the 1 : 1 lecithin–cholesterol complex. The lifetime of the 1 : 1 complex is particularly important and we need to know the frequency of exchange of lecithin molecules from the regions of complex into the boundary layers. A lower limit to the residence time of lecithin in a region of complex was deduced [4] from NMR data by assuming that the slight broadening of the methylene resonance from the free lecithin in a 2 : 1 codispersion was caused only by exchange with the complexed lecithin; this analysis indicated that the lifetime of lecithin in the regions of complex in egg yolk lecithin–cholesterol bilayers at 20 °C is at least 30 ms (refs 3 and 4). This means that a complexed lecithin molecule changes position at least 10^5 times before it passes across a boundary into the region of free lecithin. Although we do not have any direct evidence, it is probable that dissociation of the complex and exchange of lecithin molecules between regions of complex and free lecithin coincide.

Although the NMR experiments indicate a complex which is long-lived in terms of the timescale of molecular motions (10^{-9} s), the relative rates of exchange of lecithin and cholesterol between bilayers indicate that the association is short-lived on the timescale of laboratory experiments (min–h). Thus cholesterol molecules can exchange readily between lecithin- and cholesterol-containing bilayers [30], whereas an exchange protein [31, 32] is required for rapid exchange of lecithin molecules. Since the exchange rates in the absence of protein are different, the lifetime of the complex ($\tau_{complex}$) must be less than the time required for cholesterol exchange. This time is of the order of tens of minutes [33] which at first sight appears inconsistent with the idea of a complex. However the following approximate calculation shows that $\tau_{complex} < 10$ min is consistent with the energetics likely to be involved in this type of system.

A rough value of the upper limit to the lifetime of the 1 : 1 lecithin–cholesterol complex can be derived from the possible differences between the interaction enthalpies of lecithin–cholesterol in the complex, and lecithin–lecithin in a pure lecithin bilayer. We assume that the dissociation of the complex and of pairs of lecithin nearest neighbours follow a reaction pathway which contains no energy maximum corresponding to an activated complex, i.e. that the activation energy equals the enthalpy difference between "reactants" and "products". We further assume that the entropies of dissociation of a complex, and of a pair of lecithin neighbours, are roughly equal. Then

$$\tau_{complex}/\tau_{lecithin} \cong \exp(\Delta E/kT) \qquad (2)$$

where the lifetime $\tau_{lecithin} = 10^{-7}$ s for bilayers [29] at a temperature $T > T_c$,

and ΔE is the enthalpy difference between the complex and a pair of lecithin neighbours (> 0 if the complex is more stable). A reasonable upper limit for ΔE is 10 kcal/mole; then $\tau_{complex} <$ about 2 s. The lower limit of 30 ms for $\tau_{complex}$, found from NMR studies [4], if it is assumed that no rapid exchange of partners occurs within regions of complex, corresponds to $\Delta E = 8$ kcal/mole, i.e. a figure greater than the approx. $10\,kT$ needed to define a reasonably stable complex. Overall, we can set the limits 10^{-2} s $< \tau_{complex} < 10$ s.

REFERENCES

1 Phillips, M. C. (1972) Prog. Surf. Membrane Sci. 5, 139–221
2 Chapman, D. (1973) Biological Membranes, Vol. 2, Academic Press, New York, 91–144
3 Darke, A., Finer, E. G., Flook, A. G. and Phillips, M. C. (1971) FEBS Lett. 18, 326–330
4 Darke, A., Finer, E. G., Flook, A. G. and Phillips, M. C. (1972) J. Mol. Biol. 63, 265–279
5 Boggs, J. M. and Hsia, J. C. (1972) Biochim. Biophys. Acta 290, 32–42
6 Mendelsohn, R. (1972) Biochim. Biophys. Acta 290, 15–21
7 Badley, R. A., Schneider, H. and Martin, W. G. (1971) Biochem. Biophys. Res. Commun. 45, 174–183
8 Cogan, U., Shinitzky, M., Weber, G. and Nishida, T. (1973) Biochemistry 12, 521–528
9 Hemminga, M. A. and Berendsen, H. J. C. (1972) J. Magn. Res. 8, 133–143
10 Marsh, D. and Smith, I. C. P. (1973) Biochim. Biophys. Acta 298, 133–144
11 Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I. C. P. (1973) Chem. Phys. Lipids 10, 11–27
12 Engelman, D. M. and Rothman, J. E. (1972) J. Biol. Chem. 247, 3694–3697
13 Shimshick, E. J. and McConnell, H. M. (1973) Biochem. Biophys. Res. Commun. 53, 446–451
14 Phillips, M. C., Ladbrooke, B. D. and Chapman, D. (1970) Biochim. Biophys. Acta 196, 35–44
15 Phillips, M. C., Hauser, H. and Paltauf, F. (1972) Chem. Phys. Lipids 8, 127–133
16 Shimshick, E. J. and McConnell, H. M. (1973) Biochem. 12, 2351–2360
17 Hinz, H.-J. and Sturtevant, J. M. (1972) J. Biol. Chem. 247, 3697–3700
18 Ladbrooke, B. D., Williams, R. M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340
19 Lecuyer, H. and Dervichian, D. G. (1969) J. Mol. Biol. 45, 39–57
20 Green, J. R. and Green, C. (1973) Biochem. Soc. Trans. 1, 365–368
21 Rothman, J. E. and Engelman, D. M. (1972) Nat. New Biol. 237, 42–44
22 Green, J. R., Edwards, P. A. and Green, C. (1973) Biochem. J. 135, 63–71
23 Demel, R. A., Bruckdorfer, K. R. and van Deenen, L. L. M. (1972) Biochim. Biophys. Acta 255, 311–320
24 de Kruyff, B., Demel, R. A., Slotboom, A. J., van Deenen, L. L. M. and Rosenthal, A. F. (1973) Biochim. Biophys. Acta 307, 1–19
25 Finer, E. G., Flook, A. G. and Hauser, H. (1972) Biochim. Biophys. Acta 260, 59–69
26 Ladbrooke, B. D. and Chapman, D. (1969) Chem. Phys. Lipids 3, 304–356
27 Hinz, H.-J. and Sturtevant, J. M. (1972) J. Biol. Chem. 247, 6071–6075
28 Träuble, H. (1971) Naturwissenschaften 58, 277–284
29 Devaux, P. and McConnell, H. M. (1972) J. Am. Chem. Soc. 94, 4475–4481
30 Bruckdorfer, K. R., Edwards, P. A. and Green, C. (1968) Eur. J. Biochem. 4, 506–511
31 Kamp, H. H., Wirtz, K. W. A. and Van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 318, 313–325
32 Demel, R. A., Wirtz, K. W. A., Kamp, H. H., Geurts van Kessel, W. S. M. and van Deenen, L. L. M. (1973) Nat. New Biol. 246, 102–105
33 Ehnholm, C. and Zilversmit, D. B. (1973) J. Biol. Chem. 248, 1719–1724
34 Verma, S. P. and Wallach, D. F. H. (1973) Biochim. Biophys. Acta 330, 122–131
35 Zimm, B. H. and Bragg, J. K. (1959) J. Chem. Phys. 31, 526–535
36 Joos, P. (1970) Chem. Phys. Lipids 4, 162–168
37 Finer, E. G. and Darke, A. (1974) Chem. Phys. Lipids 12, 1–16