Biochimica et Biophysica Acta, 1153 (1993) 277–282

BBAMEM 76143

Heteroacid phosphatidylcholines with different amounts of unsaturation respond differently to cholesterol

J. Hernandez-Borrell [a,1] and K.M.W. Keough [a,b,*]

[a] *Department of Biochemistry and* [b] *Discipline of Pediatrics, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3X9 (Canada)*

(Received 7 April 1993)
(Revised manuscript received 10 August 1993)

Key words: Cholesterol; Phosphatidylcholine; Phase transition; DSC; Unsaturation; Lipid–cholesterol interaction

The effectiveness of cholesterol in removing the gel to liquid crystal phase transition of dispersions of pure molecular species of phosphatidylcholines (PC) that is detectable by differential scanning calorimetry (DSC) has been explored. The effect of cholesterol on 16:0-18:0 PC, 16:0-18:1 PC, 16:0-18:2 PC, 16:0-20:4 PC and 16:0-22:6 PC has been determined. Cholesterol caused a concentration-dependent removal of the detectable phase transitions in all cases. It required very little cholesterol to remove the phase transition 16:0-18:2 PC (< 17 mol% of cholesterol in PC). It required ≥ 35 mol% cholesterol to remove ΔH for 16:0-18:0 PC and 16:0-22:6 PC. About 20–25 mol% cholesterol caused disappearance of the transitional endotherm of 16:0-18:1 PC and 16:0-20:4 PC. The findings indicate that the magnitude of the influence of cholesterol on phospholipid is dependent on the degree of unsaturation in the lipid.

Introduction

Cholesterol, an important component of cellular membranes, occurs in varying amounts in different cells and different subcellular membranes of the same cell (review in Ref. 1). Early work with monolayers and bilayers showed that cholesterol interacted with phospholipids in such a way as to condense packing in monolayers or to reduce acyl chain motion in lipids which were above their rigid to fluid thermotropic phase transition temperature, and to allow for increased motion and reduced order in acyl chains of lipids at temperatures below their respective phase transitions (e.g., Refs. 2–4). Subsequently, a large number of investigations have provided information about the nature and magnitude of effects of cholesterol on phospholipids in model bilayer systems. From these studies, some features of phospholipid–cholesterol interactions which appear to be common to all systems have been found. These investigations have, however, been limited in respect to the variety of phospholipid types that have been studied.

Some studies (e.g., Refs. 5–12) have provided evidence that not all phospholipids interact in the same way, or at least to the same extent, with cholesterol. In light of these findings, the diversity of lipid species, and different amounts of cholesterol in various membranes, requires that details of lipid–cholesterol interactions be known for specific lipids or groups of lipids in order to properly appreciate the effect of cholesterol in diverse biological membranes.

In previous studies we have observed that the position and nature of acyl chains of phosphatidylcholines have a bearing on the differential scanning calorimetric behavior of membranes made with individual species of PC and cholesterol [9–12]. A finding of previous studies was that it took less cholesterol to remove the detectable phase transition of a heteroacid PC containing a saturated chain and a dienoic chain than the amount necessary to remove the transition of a heteroacid PC with the same saturated chain and a monoenoic chain, or of a disaturated PC [12]. The previous studies, however, did not include two very important classes of biological heteroacid PC, those containing a tetraenoic or a hexaenoic chain, that appear in

* Corresponding author. Fax: +1 (709) 7372552.
[1] Permanent address: Departament de Farmacia, Unitat de Fisicoquimica, Universitat de Barcelona, 08028 Barcelona, Spain.

Abbreviations: 16:0-16:0 PC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 16:0-18:0 PC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine; 16:0-18:1 PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 16:0-18:2 PC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine; 16:0-20:4 PC, 1-palmitoyl-2-archidonoyl-*sn*-glycero-3-phosphocholine; 16:0-22:6PC, 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

substantial portions in some membranes. In this study, therefore, we have investigated by differential scanning calorimetry how cholesterol affects the gel to liquid-crystalline phase transitions of a series of heteroacid PC which have palmitate (16:0) in the *sn*-1 position and one of a series of chains having zero (stearate, 18:0), one (oleate, 18:1 Δ9), two (linoleate, 18:2 Δ9,12), four (arachidonate, 20:4 Δ5,8,11,14) or six (docosahexaenoate, 22:6 Δ3,6,9,12,15,18) *cis* double bonds. The results indicate that these lipids fall into at least three groups whose transitions are removed by different amounts of cholesterol.

Materials and Methods

Materials

The phospholipids 16:0-16:0 PC, 16:0-18:0 PC, 16:0-18:2 PC, 16:0-20:4 PC and 16:0-22:6 PC, were obtained from Avanti Polar Lipids, Pelham, AL, and 16:0-18:1 PC and cholesterol were purchased from Sigma Chemical Company, St. Louis, MO. Before use, all lipids were analyzed by TLC and gas liquid chromatography [14]. All were pure by thin layer analysis and gave expected molar ratios of constituent fatty acids on gas chromatographic analysis. Water was deionized and doubly-distilled, the second distillation being from dilute potassium permanganate solution. Solvents were of reagent grade or higher quality and were from Fisher Scientific Company, Dartmouth, Nova Scotia. Water and solvents were purged thoroughly with argon before use.

Phospholipids and cholesterol were mixed in chloroform, dried under N_2 and evacuated in the presence of P_2O_5 overnight. Lipid dispersions were made at 25–30% (w/w) by vigorously vortexing the dried film in water at temperatures above their respective transition temperatures. For saturated species additional brief bath sonication was employed.

Methods

Differential scanning calorimetry was performed on a Perkin-Elmer DSC-2 calibrated with pure indium (Perkin-Elmer, Norwalk, CN) and hexadecane (Aldrich Chemical Company, Milwaukee, WI). All calorimetric scans and standardizations were carried out at 5 degrees/min and at an instrument sensitivity of 1 mcal/s. Transitional endotherms for 18:0-18:1 PC-cholesterol mixtures in excess water were obtained by supercooling the water in the sample to −12° to −16°C and then heating through the transition. These conditions were employed so as to avoid masking of the lipid endotherms by the large ice-water endotherms. Endotherms of 16:0-18:2 PC, 16:0-20:4 PC and 16:0-22:6 PC were obtained in ice. Additives such as ethylene glycol or glycerol which could depress the freezing point of the water were avoided in most cases, since these compounds might themselves influence the transitions to some extent (e.g., Refs. 15,16).

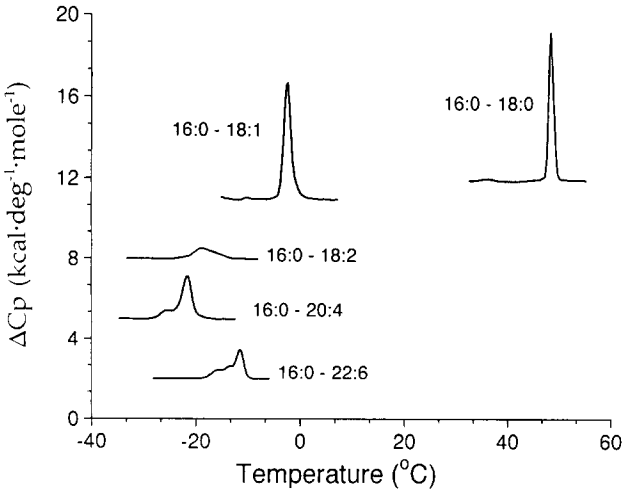

Fig. 1. Normalized endotherms for transition of dispersion of 16:0-18:0 PC, 16:0-18:1 PC, 16:0-18:2 PC, 16:0-20:4 PC and 16:0-22:6 PC. Traces are shifted on the specific heat axis for clarity of presentation.

After DSC scans were completed, samples were extracted and analyzed for phosphorus as described previously [14,17]. Oxidation was monitored by ultraviolet spectroscopy after redissolution of dried aliquots of the post-DSC extracts in doubly-distilled ethanol [16,18,19]. Some post-DSC samples were also assessed by TLC and fatty acid analysis [16,18,19]. No appreciable degradation was observed in any samples except for some samples of 16:0-22:6 PC. The effects of oxidation in these samples will be discussed below.

Digitization of the DSC scans was performed using a Hewlett-Packard 7470A Plotter and Graphpad InPlot software to read the curves into films on a personal computer. The data were normalized per mole of phospholipid phosphorus as described previously [9] for presentation in Fig. 1. Values of ΔH were calculated directly from the raw endotherms using the phosphorus contents of the samples determined after DSC [17].

Results

Fig. 1 shows normalized endotherms for dispersions of samples of 16:0-18:0 PC, 16:0-18:1 PC, 16:0-18:2 PC, 16:0-20:4 PC and 16:0-22:6 PC. Fig. 2 shows examples of normalized endotherms for dispersions of those lipids in the presence of various amounts of cholesterol. While all samples were heated and cooled at least twice, and exotherms obtained on cooling were consistent with the corresponding endotherms, only some examples are shown in Figs. 1 and 2 for the sake of clarity of presentation and economy of space. Thermodynamic data for the transitions of the dispersions of pure phosphatidylcholines are given in Table I.

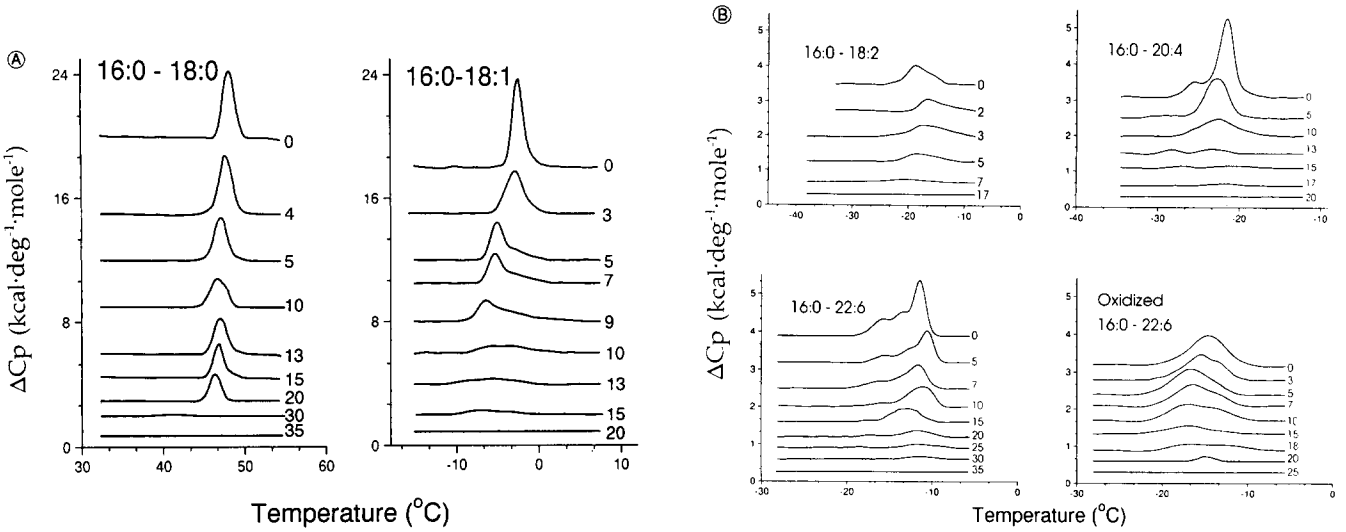

Fig. 2 (A) Normalized endotherms for transitions of 16:0-18:0 PC, 16:0-18:1 PC, and 16:0-18:2 PC in the presence of different amounts (mol%) of cholesterol. (B) Normalized endotherms for transitions of 16:0-20:4 PC, 16:0-22:6 PC, and an oxidized sample of 16:0-22:6 PC in the presence of different amounts (mol%) of cholesterol.

Where there were overlapping multicomponent endotherms, all were included in the calculations of ΔH and ΔS and the temperature of the transitions was taken at the temperature of the maximum rate of heat flow into the sample.

As seen previously [16,20], the progressive introduction of double bonds did not lead to a continuous decrease in the transition temperature or in ΔH for the transition in dispersions of the pure PC (Fig. 1 and Table I). The transition temperature was lowest when there were two or four double bonds in the *sn*-2 chains; the lowest ΔH was seen for the PC with two double bonds. The T_{max} of 16:0-22:6 PC was higher than that of either 16:0-18:2 PC or 16:0-20:4 PC, consistent with previous studies [16,20–24]. The entropy change was smallest for 16:0-18:2 PC, the next lowest being that of 16:0-22:6 PC.

Fig. 2 shows normalized endotherms for the various PC in the presence of increasing amounts of cholesterol. Cholesterol caused a broadening, and a diminution in the magnitude, of the transitions without substantially altering their temperatures. For all the PC, progressive removal of the measurable enthalpy change was observed as the cholesterol concentrations increased. The relative rate of disappearance of ΔH varied with the individual lipid interacting with cholesterol (see also Fig. 3). The relative effect of cholesterol on the original ΔH is shown in Fig. 3 for each of the phospholipids. For the calculations the whole areas under the curves from 18:0-20:4 PC and 16:0-22:6 PC were included. The ultraviolet spectra of the sam-

TABLE I

Thermodynamic parameters for pure lipids

Values are means ±95% confidence limits for two or three samples.

Phosphatidylcholine	T_{max} (K)	ΔH (kcal mol^{-1})	ΔS (kcal mol^{-1} K^{-1})
16:0-16:0 [a]	314.1	8.03	0.025
16:0-18:0	321.4±0.4	8.53±0.06	0.026
16:0-18:1	270.7±0.2	7.86±0.22	0.029
16:0-18:2*	254.5	2.62	0.010
16:0-20:4	252.6±0.2	5.40±0.41	0.021
16:0-22:6	261.9±0.0	4.11±0.38	0.016

[a] Only one sample studied. Values are consistent with those found for these lipids in previous work [4,12,16,20].

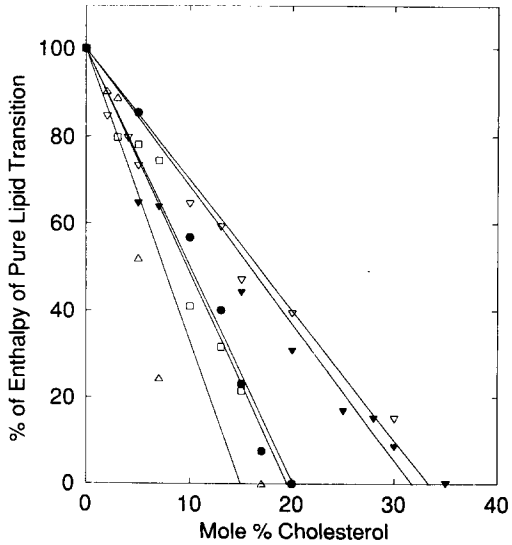

Fig. 3. The proportion of detectable enthalpy change as a function of cholesterol concentration for various lipids plus correlation coefficients (R) for the fitted lines. 16:0-18:0 PC, ▽ ——— ▽, $R = 0.99$; 16:0-18:1 PC, □ ——— □, $R = 0.99$; 16:0-18:2 PC, △ ——— △, $R = 0.92$; 16:0-20:4 PC, ● ——— ●, $R = 0.99$; and 16:0-22:6 PC, ▼ ——— ▼, $R = 0.98$.

ples obtained after the calorimetric experiments indicated that little oxidation had occurred [16,18,19]. This was confirmed in some samples by TLC [18,19].

Fig. 2b also includes a series of endotherms from 16:0-22:6 PC which had undergone some oxidation as evidenced by TLC and ultraviolet analysis. This group of DSC samples had molar extinctions at 233 nm ranging from 1560 to 4230 and at 270 nm of 1000 to 1500. With one exception, equivalent samples of the essentially unoxidized 16:0-22:6 PC had extinctions in the range of 530 to 820 at 233 nm and 230 to 550 at 270 nm. All samples of 16:0-20:4 PC had molar extinctions of less than 550 at 233 nm and less than 330 at 270 nm. The oxidized samples 16:0-22:6 PC appeared to be slightly more effected by cholesterol than were the unoxidized ones. This is probably due to the disruptive effects of the oxidation products themselves, not because of an increased impact of cholesterol only.

The inclusion of a small amount of antioxidant (recrystallized hydroquinone) in preparations of 16:0-22:6 was done in a few cases without substantial effect on the transition temperature or shape. It was observed that if oxidation had begun in the original 'pure' lipid sample as evidenced by its behavior on chromatography and ultraviolet adsorption, adding antioxidant did not change the overall properties of the endotherms. If oxidation had not begun prior to dispersion of the lipid and cholesterol, it was found that they did not become oxidized over the time of the experiment, even in the absence of antioxidant. For this reason, antioxidants generally were not used in the study.

Discussion

The shapes of the transitional endotherms of the dispersions of the pure lipids were different from one another. 16:0-18:0 PC showed a fairly symmetric transitional profile whereas 16:0-18:1 PC showed a unimodal peak with a small degree of high temperature asymmetry. 16:0-18:2 PC displayed a more highly skewed peak than 16:0-18:1 PC. The temperatures and shapes of these endotherms were similar to those seen previously in other scanning calorimetric investigations (e.g., Refs. 9,15,17,20). Thus, adding double bonds up to two per chain reduces cooperativity of the gel to liquid crystal phase transition. The transition of 16:0-20:4 PC, was bimodal, with a low temperature minor component, and a relatively narrow higher temperature major component. The transition of pure 16:0-22:6 PC was trimodal, displaying two low temperature minor components, and a relatively narrow major high temperature component. This property was seen in the samples showing very little evidence of oxidation either by TLC or by UV analysis. On the other hand, when some oxidation had taken place, as in the sample shown in the last panel in Fig. 2, the transition of 16:0-22:6 PC was less cooperative and apparently unimodal. The presence of bi- or tri-modal endotherms suggests that there might be complex rearrangements taking place, but it is not possible to ascribe the endotherm shoulders to any specific physical reorganization. On the other hand, the broad endotherms may just arise from a non-cooperative melting of the acyl chains. In keeping with this view, Litman et al. [24], using Raman spectroscopy, found transition widths of 6, 15 and 20°C for the lipids 16:0-18:1 PC, 16:0-20:4 PC, and 16:0-22:6 PC, respectively. The transition temperature of the 16:0-22:6 PC was similar to that found by others for cooling scans (e.g., Refs. 21,25), but was below that seen using other techniques for heating scans [21,24,25]. We have no simple explanation for this difference except that the techniques measure different aspects of the lipid melting. We have had a second totally separate lot of 16:0-22:6 PC synthesized and we found it had the same calorimetric properties. Use of ethylene glycol in the dispersing medium to avoid ice formation did not change the properties other than those which could be ascribed to previously seen influences of ethylene glycol (e.g., Refs. 15,16).

Fig. 3 shows the percentage of the ΔH of the pure lipid transition which was detectable as a function of cholesterol content for the whole series of lipids. As the number of double bonds in the *sn*-2 chains increased from zero to two, the lipids became progressively more 'susceptible' to cholesterol, the observable ΔH being removed by progressively less cholesterol, an influence seen in previous work [10–12,20]. This trend was reversed, however, on further addition of double bonds in the *sn*-2 chain. More cholesterol was required to remove the transition of 16:0-20:4 PC than that of 16:0-18:2 PC, and an even larger amount to remove that of 16:0-22:6 PC. The loss of detectability of ΔH was not related to the absolute magnitude of the transition of the pure lipid nor to lack of instrument sensitivity. The ΔH for pure 16:0-22:6 PC is less than that of either 16:0-18:1 PC or 16:0-20:4 PC, yet the transition of 16:0-22:6 PC was detectable in the presence of more cholesterol than either of those other two lipids. Likewise, the detectable transitions of 16:0-18:1 PC and 16:0-20:4 PC were removed by about the same amount of cholesterol, but the former had a greater ΔH for the pure lipid transition than did the latter. Finally, previous work with lipids with highly unsaturated chains in both *sn*-1 and *sn*-2 positions showed detectable enthalpy from transitions even in the presence of 50 mol% cholesterol [13], although the ΔH from transitions of the pure lipids was extremely low [14]. Thus the differences seen here in amounts of cholesterol leading to lack of detectable enthalpy

change were dependent on the type of phospholipid involved.

The findings imply that the magnitude of the effect of cholesterol on the phospholipid is dependent upon the specific molecular species of PC involved. The greatest effect of cholesterol was seen on the PC with two double bonds in the *sn*-2 chain, with progressively more cholesterol being required for an equal effect on ΔH as the number of double bonds progressed from two to zero or from two to six. The addition of double bonds beyond two does not lead to continued decreases in transition temperatures (Table I) [16,20–23]. Statistical mechanical modelling suggests that the melting properties are due to a balance between packing density in the gel and available rotational states in the liquid crystal [16]. In keeping with that idea, the unsaturated chain of 16:0-22:6 PC is fairly condensed and restricted in potential conformational states [26,27]. It would appear that the 'magnitude' of the lipid-cholesterol effect follows a similar pattern to the transition temperatures and entropy changes of the pure lipids, and perhaps it is governed by the same factors that govern the latter parameters.

Previous work with monolayers indicated that the magnitude of the condensing effect of cholesterol is influenced by the degree of unsaturation of the chain in lipids comparable to those studied here [5–8]. Ghosh and Tinoco [6] and Demel et al. [8] found that the condensing effect of cholesterol was greater on 16:0-18:2 PC than on 16:0-20:4 PC, and Demel et al. [8] found that 16:0-22:6 PC was condensed least of these three polyunsaturated PCs. The magnitude of the condensing effect in monolayers was correlated with the amount of cholesterol required to remove the transitions seen in this work. Cholesterol also had different influences on the permeability of liposomes containing a series of heteroacid PC with different degrees of unsaturation [8]. The findings all suggest that cholesterol influences PC with 20:4 and, especially 22:6, chains less than PC containing an 18:2 chain in the *sn*-2 position.

The amount of cholesterol required to abolish the transition of 16:0-20:4 PC and 16:0-22:6 PC approaches that required for 16:-18:1 PC and 16:0-18:0 PC, respectively. This may not mean, however, that the modes of interaction are exactly the same in these systems. The two PC containing 20:4 and 22:6 chains may not mix very well with cholesterol, whereas there is no reason to believe that PC containing 18:0 or 18:1 chains do not mix well with the sterol. Evidence for lack of mixing in such polyunsaturated systems comes from the reduced condensing effect on 16:0-20:4 and 16:0-22:6 PC [5–7] and from other considerations. Demel et al. [8] found that PC containing two polyunsaturated chains were not condensed at all by cholesterol. Similarly, we observed that the calorimetrically detectable transitions of PC with two polyunsaturated chains, especially those containing two 20:4 or 22:6 chains, was hardly affected by up to 50 mol% cholesterol. A potential explanation for those findings and of the effect of cholesterol on the heteroacid lipids studied here is that cholesterol does not mix as well with PC containing 20:4 and 22:6 chains as it does with other PC. In those systems with highly unsaturated chains there could be lateral separation into cholesterol-rich and cholesterol-depleted regions. In more saturated systems, more random distribution of cholesterol could occur. In these systems the magnitude of the cholesterol effect (ability to remove the detectable phase transition) would increase with progressive unsaturation (zero to two double bonds). In PC-cholesterol mixtures where the PC have more than two double bonds, the magnitude of the cholesterol effect would decrease with increasing numbers of double bonds because of decreasing 'solubility' of cholesterol in those systems. The work of Van Blitterswijk et al. [28] supports the interpretation of a difference in the nature of the lipid–cholesterol interaction for systems with 20:4 or 22:6 chains. Other recent work supports the idea that the degree of unsaturation of acyl chains influences the extent or magnitude of the phospholipid–cholesterol interactions [29,30].

Thus, there may be qualitatively and quantitatively different types of interactions between cholesterol and biological lipids of different degrees of unsaturation. This possibility should be kept in mind when considering potential effects of cholesterol in biological membranes. Further work to consider this possibility is desirable, especially to elucidate the specific molecular attractions, or lack of them, that may occur in the PC-cholesterol systems.

Acknowledgements

This work was supported by the Medical Research Council of Canada. We thank Lorne Taylor for help with digitization of endotherms and preparation of figures, and E. Davidson for technical assistance.

References

1 Green, C. (1977) Int. Rev. Biochem. 14, 101–152.
2 Van Deenen, L.L.M., Houtsmuller, U.M.T., De Haas, G.H. and Malder, E. (1982) J. Pharm. Pharmacol. 14, 429–444.
3 Chapman, D., Owens, N.F. and Walter, D.A. (1966) Biochim. Biophys. Acta 120, 148–155.
4 Ladbrooke, B.D., Williams, R.M. and Chapman, D. (1968) Biochim. Biophys. Acta 150, 333–340.
5 Ghosh, D., Lyman, R.L. and Tinoco, J. (1971) Chem. Phys. Lipids 7, 173–184.
6 Ghosh, D. and Tinoco, J. (1972) Biochim. Biophys. Acta 266, 41–49.
7 Ghosh, D., Williams, M.A. and Tinoco, J. (1973) Biochim. Biophys. Acta 291, 351–362.

8 Demel, R.A., Geurts van Kessel, W.S.M. and Van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 266, 26–40.
9 Davis, P.J. and Keough, K.M.W. (1983) Biochemistry 22, 6334–6340.
10 Davis, P.J. and Keough, K.M.W. (1984) Biochim. Biophys. Acta 778, 305–310.
11 Davis, P.J., Kariel, N. and Keough, K.M.W. (1986) Biochim. Biophys. Acta 856, 395–398.
12 Keough, K.M.W., Giffin, B. and Matthews, P.L. (1989) Biochim. Biophys. Acta 983, 51–55.
13 Kariel, N., Davidson, E. and Keough, K.M.W. (1991) Biochim. Biophys. Acta 1062, 70–76.
14 Keough, K.M.W. and Kariel, N. (1987) Biochim. Biophys. Acta 902, 11–18.
15 Davis, P.J., Fleming, B.D., Coolbear, K.P. and Keough, K.M.W. (1981) Biochemistry 20, 3633–3636.
16 Coolbear, K.P., Berde, C.B. and Keough, K.M.W. (1983) Biochemistry 22, 1466–1473.
17 Keough, K.M.W. and Davis, P.J. (1979) Biochemistry 18, 1453–1459.
18 Coolbear, K.P. and Keough, K.M.W. (1983) Biochim. Biophys. Acta 732, 531–540.
19 Keough, K.M.W. and Parsons, C.S. (1990) Biochem. Cell Biol. 68, 300–307.
20 Keough, K.M.W., Giffin, B. and Kariel, N. (1987) Biochim. Biophys. Acta 902, 1–10.
21 Deese, A.J., Dratz, E.A., Dahlquist, F.W. and Paddy, M.R. (1981) Biochemistry 20, 6420 6427.
22 Keough, K.M.W. (1986) Biochem. Cell Biol. 64, 44–49.
23 Keough, K.M.W. (1990) Biochem. Soc. Trans. 18, 835–837.
24 Litman, B.J. Lewis, E.N. and Levin, I.W. (1991) Biochemistry 30, 313–319.
25 Barry, J.A., Trouard, T.P., Salmon, A. and Brown, M.F. (1991) Biochemistry 30, 8386–8394.
26 Stubbs, C.D. and Smith, A.D. (1984) Biochim. Biophys. Acta 779, 89–137.
27 Applegate, K.R. and Glomsett, J.A. (1986) J. Lipid Res. 27, 658–680.
28 Van Blitterswijk, W.J., Van der Meer, B. and Hilkmann, H. (1987) Biochemistry 26, 1746–1756.
29 Cohen, E.D. and Carey, M.C. (1991) J. Lipid Res. 32, 1291–1302.
30 Slotte, J.P. (1992) Biochemistry 31, 5472–5477.