Biochimica et Biophysica Acta 863 (1986) 110–114
Elsevier

BBA 70357

BBA Report

Biophysical properties of unusual phospholipids and sterols from marine invertebrates *

Eser Ayanoğlu [a], Nejat Düzgüneş [b], W.M. Donald Wijekoon [a,**] and Carl Djerassi [a]

[a] *Department of Chemistry, Stanford University, Stanford, CA 94305 (U.S.A.) and* [b] *Cancer Research Institute and Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143 (U.S.A.)*

(Received 28 May 1986)
(Revised manuscript received 22 September 1986)

Key words: Phospholipid; Sterol; Phase transition; (Marine invertebrate)

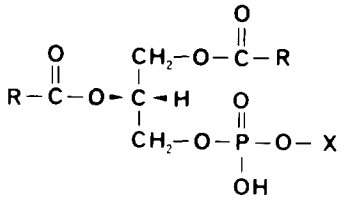
Liposomes composed of 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine underwent an endothermic phase transition at 42°C. Cholesterol or the marine sterols studied did not affect this transition to an appreciable extent, but rather were excluded from the phospholipid bilayers. Membranes composed of 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphoethanolamine displayed very similar phase properties. Effects of the marine sterols on dipalmitoylphosphatidylcholine bilayers were also investigated.

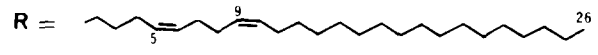
In recent years, nearly 200 sterols which have no counterparts among terrestrial organisms have been isolated from marine invertebrates, especially demosponges, gorgonians and soft corals. 19-Nor- or A-norsterols as well as very unusual side chains which contain unprecedented cyclopropane, cyclopropene, allene and acetylene functions as well as unusual branching [1–3] have been encountered. Furthermore, during the last decade, over fifty fatty acids with no counterparts in terrestrial sources have also been discovered [4–6] in the phospholipids of sponges. These are characterized by very long chain lengths (C_{24}–C_{30}) and unexpected locations of double bonds (Δ^5 and/or $\Delta^{5,9}$ positions) in comparison with the fatty acids (C_{14}–C_{20}) of phospholipids found in other animals. Some very long chain fatty acids were encountered in certain procaryotic organisms, such as mycoplasma. In the sponges we have studied so far, in addition to unusual double bond patterns, large quantities of very long chain branched, methoxy, acetoxy, cyclopropane, brominated mono- and di-olefinic, or totally saturated structures have also been found. Furthermore, unexpectedly high levels of PE, PG and PS instead of the 'animal phospholipid' PC were encountered in sponges [5,7]. Normally, the presence of such unusually long-chain acids with different unsaturation patterns and substitutions would demand molecular interactions distinct from those associated with more conventional fatty acids. The physical and physiological properties of such membranes would be different from those of eucaryotic or most procaryotic membrane systems. While some researchers concluded [8] that some sponge membranes have distinctive bilayer structures not found in other living organisms, other investigators [9] challenged this idea and considered a unique constitution unlikely.

* Part 13 in the series 'Phospholipid studies of Marine Organisms'.

** Present address: Department of Chemistry, University of Nevada, Reno, NV 89507, U.S.A.

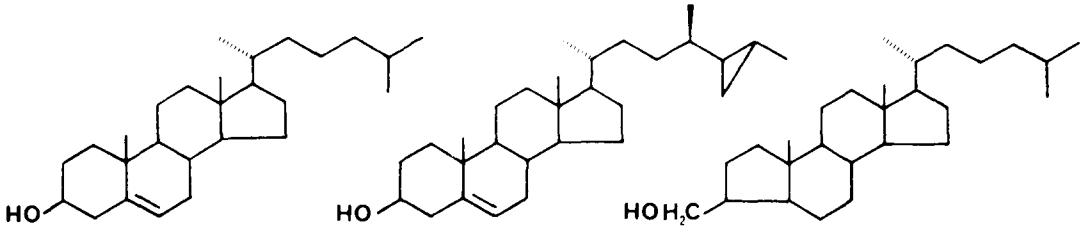
Correspondence: Dr. C. Djerassi, Department of Chemistry, Stanford University, Stanford, CA 94305, U.S.A.





1: X= CHOLINE

2: X= ETHANOLAMINE



CHOLESTEROL PETROSTEROL A-NORCHOLESTEROL

Recently, we have started investigating some of the biophysical properties of these unusual phospholipids and sterols, in comparison with their more conventional counterparts. For our initial studies we chose 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine (PC (26 : 2, 26 : 2)) (1) and 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphoethanolamine (PE (26 : 2, 26 : 2)) (2) (Fig. 1) which were recently synthesized in our laboratory [10,11]. As typical sterols, we investigated petrosterol which contains a cyclopropyl group in the side chain [12–14], and A-norcholesterol [15] which has a unique sterol nucleus (Fig. 1). We now wish to report some unusual membrane properties of these molecules, especially of the phospholipid 1.

Crude sterol mixtures were obtained from the acetone-soluble lipid extracts of *Axinella verrucosa* and *Petrosia ficiformis*. The extracts were subjected to saponification and open column chromatography followed by GLC and HPLC as outlined in previous reports [12,13]. A preparative HPLC system (comprised of a Waters M6000 pump, a UK injector and a R403 differential refractometer) and two Altex Ultrasphere ODS columns (10 mm i.d. × 25 cm) in series with absolute methanol was employed as the mobile phase for the final purifications. The phospholipid and fatty acid detection was carried out as described earlier [5]. For the gas chromatography-mass spectrometry measurements, a Ribermag GC-MS-DS system, combining a Ribermag R 10-10 quadrupole mass spectrometer with a Carlo Erba series 4160 Fractovap chromatograph, containing an SE-54 silica column was utilized. The synthesis of the phospholipids was achieved as reported earlier [10,11]. For the preparation of the liposomes, lipids were dried by rotoevaporation under vacuum, hydrated above their phase transition temperature (55–60 °C) and centrifuged to concentrate the samples. They were transferred to aluminum calorimeter pans and sealed hermetically under

pressure and then scanned at a rate of 5 Cdeg/min at a sensitivity setting of 0.5 or 1 mcal/s in a Perkin Elmer DSC-2 differential scanning calorimeter. The entalpies of transition were calculated by determining the area under the curve, by cutting and weighing. The transition temperature was taken as the peak of the heating curve. The release of carboxyfluorescein from the lipid vesicles was determined by the method employed for dipalmitoylphosphatidylcholine (DPPC) vesicles [16]. The lipids were hydrated in 50 mM carboxyfluorescein (obtained from Eastman Organic Chemicals and purified by Sephadex LH-20 chromatography) as multilamellar vesicles and extruded successively through polycarbonate membrane filters of 0.4, 0.2 and 0.1 μm pore diameter in order to achieve a uniform size distribution, and chromatographed on Sephadex G-75, using 100 mM NaCl, 5 mM Tes at pH 7.4 as the elution buffer. Light scattering (90°) was observed simultaneously with carboxyfluorescein release by setting the emission monochromator at 430 nm. Carboxyfluorescein fluorescence (F) was determined by exciting at 430 nm and detecting the emission above 530 nm using a 3-68 Corning cut-off filter in the second emission channel of an SLM 4000 fluorometer. F_{max} was determined by lysing the vesicles with 0.1% w/v Triton X-100 at 25°C. The temperature was controlled by circulating water around the fluorometer cell, and detected continuously by a Bailey Instruments probe thermometer. In the first set of experiments, dipalmitoylphosphatidylcholine (DPPC) (99% pure, Sigma Chemical Company) was selected as a standard phospholipid and its interaction with different sterols (cholesterol, petrosterol, A-norcholesterol and *A. verrucosa* total sterols) studied. For each sterol, three different molecular ratios (5, 9, 20 mole%) were examined. Differential scanning calorimetric studies indicated that all of these sterols had a very similar effect on the transition temperature (41°C) of DPPC; furthermore, these sterols did not differ from cholesterol in broadening the transition peak of DPPC with increasing mole ratios.

Liposomes composed of the synthetic marine phospholipid 1,2-di-(5Z,9Z)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine (1) displayed a transition endotherm with a peak at 42°C (Fig. 2A)

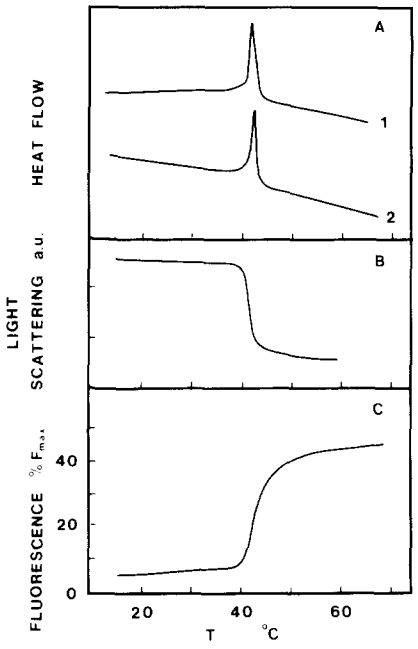


Fig. 2. Thermotropic behavior of 1,2-di-(5Z,9Z)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine in 100 mM NaCl, 5 mM Tes (pH 7.4). (A) Differential scanning calorimetry: curve 1, heating scan; curve 2, cooling scan. (B) 90°C light scattering at 430 nm. (C) Release of liposome-encapsulated carboxyfluorescein. The heating rate for A was 5 Cdeg/min, the heating rate for B and C was 1.4 Cdeg/min.

with an ethalpy of 51.1 ± 3.8 kJ/mol (12.2 ± 0.9 kcal/mol). The width of the endotherm at half peak height was 3 Cdeg.

Since vesicles of a number of lipid species with saturated acyl chains release their contents at the phase transition [16,17], it was of interest to determine if 1,2-di-(5Z,9Z)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine liposomes would display a similar behavior. Encapsulated carboxyfluorescein was released at the same transition temperature (42°C) (Fig. 2B) as measured by differential scanning calorimetry. In addition, light scattering measurements were performed out using the same type of liposomes, since light scattering is also a sensitive indicator of the phase transition of vesicles [16]. Again the same transition temperature was observed (Fig. 2C).

The phase transition temperature of 1 is suprisingly similar to that of DPPC although its hydrocarbon chain is larger by 10 carbon atoms. It appears that the presence of two *cis* double bonds close to the glycerol backbone on each C_{26} fatty

acid chain introduces substantial kinks in the membrane, thus reducing the phase transition from that expected from a C_{26} hydrocarbon chain.

We also investigated the interaction of cholesterol with 1 and found that the phase transition of the phospholipid was not significantly altered in the presence of the sterol. Moreover, there appeared to be a physical separation, an exclusion of cholesterol from the lipid phase during the formation of the liposomes, despite the fact that temperatures 20 Cdeg above the T_c were utilized for hydration. Each attempt to prepare liposomes together with cholesterol gave a turbid liquid and an accumulation of aggregates at the air/water interface. A transition temperature at 42°C was observed for each sample. The width at half peak height broadened slightly with these samples but not completely, even at 20% mole ratio of cholesterol. A reverse phase evaporation technique was also attempted with and without carboxyfluorescein. The dye was encapsulated in the liposomes, but the same aggregation was observed even at elevated temperatures (65°C) for hydration and long periods of ultrasonication. Attempts to form a well-dispersed liposome phase from the aggregates using polycarbonate membrane filters at high pressures and temperatures were not successful.

The aggregates were separated from the liposomes by the use of a glass filter. The chemical analysis of the filtered material showed that cholesterol was basically excluded from the marine phospholipid vesicles. Our lipid analysis on different preparations showed that the ratio of cholesterol in the liposomes always remained below 5%. On the other hand, the aggregates separated by filtration were composed of practically pure sterol. The effect of the various molar ratios of the marine sterols petrosterol, A-norcholesterol and *A. verrucosa* total sterols were also examined. Again, these sterols were excluded from the liposomes.

These findings suggest that the attractive energy of interaction between hydrocarbon chains of the lipids prevents significant phospholipid-sterol interactions and forces the cholesterol to be excluded from the bilayer, to form its own phase which then leads to precipitation. The long hydrocarbon chains are likely to generate an unusual bilayer thickness, which may cause the exclusion of cholesterol. It is also possible that, because of the kinking of the chains, the sterols do no not fit comfortably into the intermolecular spaces that are produced by the lipid. However, it appears that the lipid bilayer itself is very much intact from the fact that we could encapsulate carboxyfluorescein and did not observe any leakage until the phase transition was reached. Above the phase transition, carboxyfluorescein did not leak completely. This leads us to believe that even in the fluid state the permeability barrier is maintained. Therefore, even though the acyl chains might be kinked, the packing between the hydrocarbon chains is strong enough to maintain a high permeability barrier, except at the phase transition temperature. This suggests that the reorganization process at T_c is extensive.

We also measured the transition temperature of one of the synthetic double bond stereoisomers of 1, 1,2-di-(5*Z*,9*E*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine. The transition temperature was raised to 49°C (Fig. 3) ($H = 30.6$ kJ/mol or 7.3 kcal/mol), because of the presence of a *trans* double bond at C-9 instead of a *cis* one. On the other hand, the transition temperature of 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphoethanolamine (2) was very similar to that of the 5*Z*,9*Z*-phosphocholine isomer 1 (Fig. 3) while the enthalpy of transition was 45.2 kJ/mol (10.8 kcal/mol). This species of PE also excluded cholesterol from the bilayer. In general, the transition temperatures of more conventional phosphatidylethanolamines are higher than those of

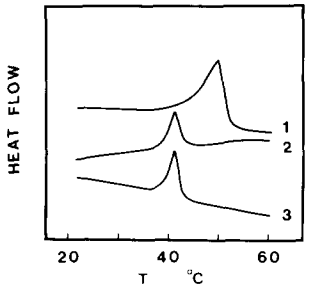


Fig. 3. Differential scanning calorimetry of: 1. 1.2-di-(5*Z*,9*E*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine; 2, 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphoethanolamine 3, 1,2-di-(5*Z*,9*Z*)-5,9-hexacosadienoyl-*sn*-glycero-3-phosphocholine.

the corresponding phosphatidylcholines [18]. The higher transition temperature of the PE species is usually attributed to hydrogen bonding between the amino group on PE with a neighboring phosphate [19,20]. One possible explanation for our result is that the pressure of very long acyl chains and/or the location of the *cis* double bonds overshadow the effect of the head group on the phase transition. Studies with various fatty acyl chains connected to different head groups are currently in progress.

Exclusion of sterols from natural phospholipids is an uncommon observation and might have significant implications in terms of lipid-lipid interactions in membranes. In the case of the two unusual phospholipid species investigated here, it appears that cholesterol does not fit in an energetically favorable configuration near the lipid/water interface, where the 3β-hydroxyl group is thought to partition. It will be of interest to investigate the miscibility of 1,2-di-(5*Z*-9*Z*)-5,9-hexacosadienoyl-*sn*-glycerol-3-phosphocholine and similar unusual marine phospholipids with shorter chain phospholipids, to examine the behavior of cholesterol as well as marine sterols in such mixtures, and to determine with which lipid(s) the sterols do associate in the membranes of marine invertebrates. The intracellular distribution and biological role of the phospholipids and sterols are also being investigated in our laboratories.

We thank Professor Robert Simoni of (Stanford University, Biology Department) and Professor Demetrios Papahadjopoulos (University of California, San Francisco, Department of Pharmacology and Cancer Research Institute) for valuable discussions. Financial support from the National Institutes of Health (Grant GM-06840, GM-28117) and American Heart Association (Grant in Aid 83-636) is gratefully acknowledged. We are also grateful to Dr. G Sodano and Professor D. Sica (Istituto di Chimica Organica dell'Universita di Napoli, Italy) for their generosity in supplying us with sponge material from the Mediterranean.

References

1 Ikekawa, N. (1985) in Sterols and Bile Acids (Danielsson, H. and Sjovan, J., eds.), pp. 199–230 (and leading references cited therein for other reviews), Elsevier Science Publishers, Amsterdam
2 Djerassi, C. (1981) Pure Appl. Chem. 53, 873–890
3 Djerassi, C. (1984) in Proceedings of the Alfred Benzon Symposium, Vol. 20 (Krosgsgaard-Larsen, P., Christensen, S.B. and Kofod, H., eds.), pp. 164–176, Munksgaard, Copenhagen
4 Litchfield, C., Tyszkiewicz, J. and Dato, V. (1980) Lipids 15, 200–202
5 Ayanoglu, E., Walkup, R.D., Sica, D. and Djerassi, C. (1982) Lipids 17, 617–625.
6 Ayanoglu, E., Kurtz, K., Kornprobst, J.M. and Djerassi, C. (1985) Lipids 20, 141–144
7 Kostetski, E.Y. and Shchipunov, Y.A. (1983) Zh. Evol. Biochim. Fiziol. 19, 8–15
8 Litchfield, C. and Morales, R.W. (1976) in Aspects of Sponge Biology (Harrison, W. and Cowden, C., eds.), pp. 183–200, Academic Press, New York
9 Lethias, C., Garrone, R. and Mazzorana, M. (1983) Tissue Cell 15, 523–535
10 Mena, P., Pilet, O. and Djerassi, C. (1984) J. Org. Chem. 49, 3260–3264
11 Mena, P. and Djerassi, C. (1985) Chem. Phys. Lipids 37, 257–270
12 Sica, D. and Zollo, F. (1978) Tetrahedron Lett. 837–840
13 Ravi, B.N. Kokke, W.C.M.C., Delseth, C. and Djerassi, C. (1978) Tetrahedron Lett. 5379–5382
14 Khalil, M.W., Durham, L.J., Djerassi, C. and Sica, D. (1980) J. Am. Chem. Soc. 102, 2133–2134
15 Minale, L. and Sodano, G. (1974) J. Chem. Soc. Perkin I, 2380–2384
16 Düzgüneş, N., Wilschut, J., Hong, K., Fraley, R., Perry, C., Friend, D.S., James, T.L. and Papahadjopoulos, D. (1983) Biochim. Biophys. Acta 732, 289–299
17 Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330–348
18 Keough, K.M.W. and Davis, P.J. (1984) Biomembranes 12, 55–97
19 Chapman, D. (1975) Q. Rev. Biophys. 8, 185–235
20 Boggs, J.M. (1980) Can. J. Biochem. 58, 755–770