Studies on the Hydrogen Belts of Membranes: I. Diester, Diether, and Dialkyl Phosphatidylcholines and Polyoxyethylene Glycerides in Monolayers with Cholesterol

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ABSTRACT

The hydrogen belts of membranes are defined as the regions consisting of hydrogen bond acceptors, i.e., the C=O groups of glycero- and sphingolipids, and hydrogen bond donors, i.e., cholesterol-OH, sphingolipid-OH, proteins, and water. Lipid-lipid hydrogen bonding in these belts has been suggested. The connection of such hypothetical bonding with the condensation effect, i.e., the apparent reduction of surface area occupied by phospholipids in mixed monolayers with cholesterol, has been tested with lipids possessing and lacking C=O groups: diester, diether, and dialkyl phosphatidylcholine, and analogous polyoxyethylene diglycerides. Condensation by cholesterol was observed for all lipids. Consequently, the hypothetical lipid-C=O-cholesterol hydrogen bonding is not a prerequisite for the condensation effect.

INTRODUCTION

The regions that separate the hydrophobid core from the outer, polar zones of a membrane have so far received little attention. These regions we call hydrogen belts (1) because they consist of hydrogen bond acceptors (C=O groups of glycero- and sphingolipids) and hydrogen bond donors (cholesterol-OH, sphingolipid-OH, water, perhaps proteins). The balance of these groups in the hydrogen belts is very probably of great biological importance. This is suggested by the difference in belt compositions between manufacturing and isolating membranes of mammals: membranes that manufacture protein (endoplasmic reticulum) or energy (mitochondria) are rich in lipid C=O and very poor in lipid OH groups; on the other hand, the hydrogen belts of the isolating plasma membrane, and especially the myelin membrane, are very rich in hydroxyls. We have suggested (1) that hydroxyl groups close membranes by lipid-lipid hydrogen bonding and that proteins and cholesterol compete for bonding to the ester C=O groups of phospholipids.

Cholesterol plasticizes as well as rigidifies phospholipid bilayers (2). Phospholipid monolayers are condensed by cholesterol, that is, the apparent surface area occupied by a phospholipid molecule is reduced when cholesterol is added (3,4). This effect reflects the intercalation of cholesterol and fatty acid chains; it is maximal with cholesterol and those other sterols that have a flat ring system, a side chain, and a β-oriented 3-hydroxyl (5). Cholesterol and such sterols also reduce the permeability of phospholipid bilayers for non-electrolytes (6) and cations (7). Steric and energetic arguments have led us to suggest (1) that the cholesterol-OH may be hydrogen-bonded to phospholipid C=O, and that such bonding might be a condition for the condensation effect. This hypothesis could be tested with polar lipids lacking the C=O groups, in which the possibility of C=O-cholesterol hydrogen bonding is eliminated. De Kruyff et al. (8) have already investigated monolayers of some relevant model lipids with cholesterol and reached the conclusion that neither the nature of the polar head group nor that of the hydrogen belt groups are responsible for the effect; however, a figure in this study (Ref. 8, Fig. 3) shows that the condensation effect was considerably smaller for a 1-ester-2-ether-phosphatidylcholine than for a diester phosphatidylcholine. To investigate the problem further, we have synthesized a phosphatidylcholine (diester-PC) and its diether and dialkyl analogs, and also some analogs in which the zwitterionic phosphorylcholine is replaced by a polyoxyethylene group. We have then determined the force-area curves, at room temperature, of monolayers of these lipids alone and together with cholesterol.

MATERIALS AND METHODS

All synthetic lipids have been listed with their full names in Table I.

Phospholipids

Phosphatidylcholine (diester-PC), Table I, 1,

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TABLE I

Abbreviations and Structures of Lipids Synthesized

		Structure
1	Diester-PC	1-0-hexadecanoyl-2-0-(*cis*-9'-octadecenoyl)-*sn*-glycero-3-phosphocholine[a]
2	Diether-PC	1-0-hexadecyl-2-0-(*cis*-9'-octadecenyl)-*sn*-glycero-3-phosphocholine[b]
3	Dialkyl-PC A	2,2-di-(9,10-methyleneoctadecyl)ethoxy-1-phosphocholine
4	Dialkyl-PC B	2,2-di(*cis*-9-octadecenyl)ethoxy-1-phosphocholine
5	Diester glyceride A	1,2-0-di(9,10-methyleneoctadecanoyl)-3-0-(2-(2-methoxyethoxy)ethyl)-*rac*-glycerol
6	Diether glyceride A	1,2-0-di(9,10-methyleneoctadecyl)-3-0-(2-(2-methoxyethoxy)ethyl)-*rac*-glycerol
7	Diester glyceride B	1,2-0-di(*cis*-9-octadecenoyl)-3-0-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-*rac*-glycerol

[a]Position 1 contained 32% stearic acid, 2% other homologs.

[b]Position 1 contained 18% stearyl residue, 12% other homologs.

was prepared by modification of egg lecithin rather than by total synthesis in order to make its fatty acid chain composition similar to that of the available diether-PC. Egg lecithin (9) was deacylated in position 2 with phospholipase 2 (10) and reacylated with oleic acid anhydride (11). The lipid contained 48 mole % oleic acid (18:1), 35 mole % palmitic (16:0), 16 mole % stearic (18:0), and 2 mole % other fatty acids (14:0, 15:0, 16:1, 17:0, 18:2). It was, therefore, mainly 1-palmitoyl-2-oleoyl-PC (nearly 70%) with an admixture of 1-stearoyl-2-oleoyl-PC (about 30%).

Diether-PC (Table I, 2) was prepared from commercial chimyl alcohol (Sigma, St. Louis, MO) which contained 70 mole % 1-palmityl (16:0)-*sn*-glycerol, 18 mole% stearyl analog, and 15% other homologs. It was tritylated in position 3 (12), an oleyl group was introduced in 2 with oleyl bromide and KOH in toluene (12); the product was purified by chromatography and detritylated with 90% acetic acid (13), followed by alkaline hydrolysis of the diether-acetate, to yield 1-alkyl-2-oleyl-*sn*-glycerol. This was converted to diether-PC with 2-bromoethyl dichlorophosphate and trimethylamine (14). Mol wt assuming 75% C_{16}, 25% C_{18} chains in position 1: 757.1, phosphorus calculated, 4.1%, found 4.1%. This lipid contained, according to synthesis, 70 mole % 1-0-palmityl-2-0-oleyl-*sn*-glycerol-3-phosphorylcholine, 18 mole % 1-0-stearyl-, and the rest closely related homologs. The product was pure as judged by thin layer chromatography (TLC), as were all other lipids used in our experiments.

The synthesis of dialkyl-PC B, (Table I, 4) started with a repeated malonic ester condensation of oleyl (18:1) bromide (15). The disubstituted malonic ester was saponified, decarboxylated by heating in vacuum at 180 C, esterified (methanol-H_2SO_4), and reduced with $LiAlH_4$ to the alcohol which was converted as above to dialkyl-PC B, 2,2-dioleylethoxy-1-phosphorylcholine. Mol wt, $C_{43}H_{88}O_4PN \cdot H_2O$, 730.1; P, 4.2%, found 4.2%.

Dialkyl-PC A (Table I, 3) contained two aliphatic chains with a cyclopropane ring. The starting material was 9,10-methyleneoctadecanoic methyl ester [from methyl oleate with diiodomethane and zinc (16), which was reduced with $LiAlH_4$ to the alcohol, which was converted to the bromide with CBr_4 and triphenylphosphine (17)].

Polyoxyethylene Diglycerides

Diester glyceride A, Table I, 5, was prepared by acylation (acid chloride, pyridine) of the substituted glycerol *rac*-3-0-(2-(2-methoxyethoxy)ethyl)-glycerol. This had been prepared from methoxyethoxyethanol which was converted, with PBr_3 (ether, room temperature, 20 hr) to methoxyethoxyethyl bromide, bp 51 C/3 mm, which was condensed with 1,2-isopropylideneglycerol (KOH, toluene) (product bp 112 C/3 mm). The isopropylidene group was then removed with 10% aqueous acetic acid, 2 hr at 100 C. The diester glyceride A was purified by high performance liquid chromatography on Porasil® (Waters Associates, Milford, MA) with hexane-1% isopropanol. On silicic acid thin layer chromatography in hexane: ether, 6:4, its retention was similar to that of a diglyceride. Analysis by gas liquid chromatography after transesterifiecation, with methyl oleate as internal standard: 9,10-methylenestearate, calculated as acid, for the monoester 62.75%; for the diester: 79.9%; found: 79.3%.

Diether glyceride A, Table I, 6, the diether analog of diester glyceride A, was synthesized from *rac*-3-0-(2-(2-methoxyethoxy)ethyl) glycerol and 9,10-methylenestearyl bromide (KOH, toluene). Separation from a slower running product (monoether) was achieved by chromatography on a silicic acid column with hexane:ether, 6:4. The lipid, of which no further analysis is available, was homogeneous by TLC and moved close to diglyceride.

Diester glyceride B, *rac*-3-0-(2-(2-(2-meth-

TABLE II

Area of Phospholipid or Glyceride Molecule in Monolayer at 20 dyn·cm^{-1} and 22 C

		Without cholesterol	With cholesterol, 1:1	% Condensation
1	Diester-PC	72.6 ± 1.5	51.9 ± 1.4	29
2	Diether-PC	75.0 ± 1.0	51.7 ± 0.2	31
3	Dialkyl-PC A[a]	74.4 ± 1.0	59.9 ± 0.4	19.5
4	Dialkyl-PC B[b]	81.3 ± 1.1	61.3 ± 0.6	25
5	Diester glyceride A	70.5 ± 0.6	64.1 ± 2.0	9
6	Diether glyceride A	74.5 ± 1.3	65.5 ± 1.2	12
7	Diester glyceride B	77.9 ± 0.6	74.9 ± 0.2	4

[a]A, di-9,10-methylenestearyl lipids.

[b]B, dioleyl lipids.

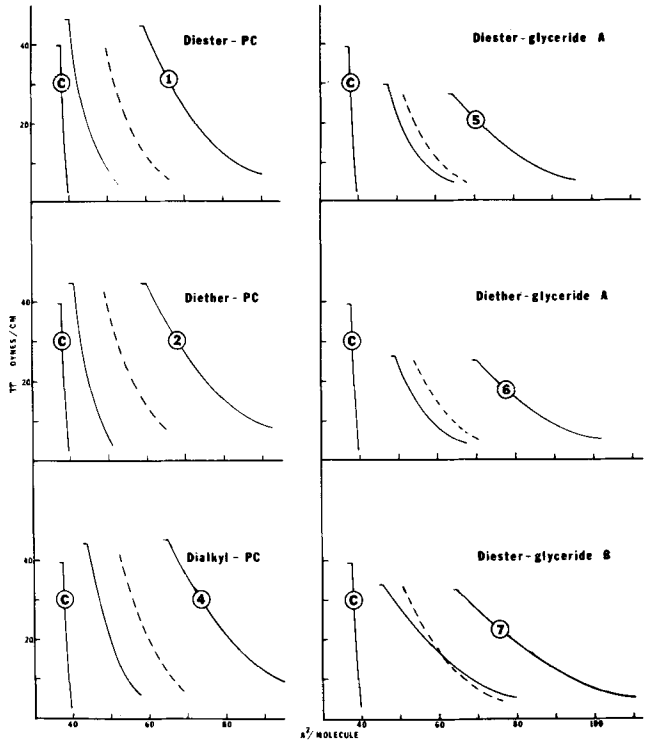

FIG. 1. Force-area curves of lipid monolayers at 22 C. To the right in each graph, with numbers referring to the compounds of Table I, the polar lipid. C is cholesterol. The dashed curve would be expected for an equimolar mixture without condensation. The solid unmarked line is the actually measured force-area curve for an equimolar mixture of polar lipid and cholesterol.

oxyethoxy)ethoxy)ethyl)-glycerol-1,2-dioleate, Table I, 7, is similar to diester glyceride A but contains oleic acid and one additional oxyethylene link. 2-(2-(2-Methoxyethoxy)ethoxy)-ethanol (Olin Chemicals, Brandenburg, KY) was converted with PBr_3 to the bromide; bp 76 C/1 mm; condensated with 1,2-isopropylidene glycerol, product bp 128 C/1 mm; removal of the isopropylidene group with 10% acetic acid; acylation with oleoyl chloride in benzene-pyridine. "Diester glyceride B", mol wt 767.2, calc., C:72.02%; H:11.30%; found, C:72.16%; H:11.07%. In TLC, the lipid behaved like a diglyceride.

Cholesterol was of specially purified grade (Supelco, Inc., Bellefonte, PA) or analytic reagent recrystallized three times from ethanol; no difference was noted between the preparations.

Monolayer Experiments

Lipid monolayers on water were prepared in

a Teflon trough, 30 x 15 x 0.5 cm, with a Teflon slide bar connected to a controllable mechanical drive. Surface tension of the monolayer was continuously monitored with a platinum blade suspended from a No. FTA-10-1 Transducer connected to a No. 8805-P Preamplifier (Hewlett Packard Inc., Paramus, NJ). The signal was continuously recorded. Monolayers were made by gently placing drops of the lipid in hexane (1 mM) on the water surface from a microsyringe. One hundred nmoles of cholesterol, 50 nmoles of lipid, or an equimolar mixture of cholesterol and lipid, 100 or 50 nmoles total, was used for each run, which lasted 12 min. The force-area curves shown have been averaged from four to five measurements taken at room temperature, 22 C. The standard errors for the areas were around ± 1.0 Å^2 (Table II).

RESULTS

Force-Area Curves of the Phospholipids and Condensation by Cholesterol

The force-area characteristics of monolayers of diester-PC, alone and with cholesterol (Fig. 1, 1), agree with those determined by de Kruyff et al. (8) for the same compound. The strong condensation effect is indicated by the distance between the experimental curve (in the middle) and the theoretical (dashed) curve for the mixed lipids. Table II gives surface areas per molecule for diester-PC, as well as the other lipids, at a surface pressure of 20 dyn·cm^{-1}, the apparent area after condensation, calculated assuming cholesterol to be incompressible, and the apparent condensation in percent.

The force-area curve for diether-PC B (Fig. 1, 2) is indistinguishable from that of the diester-PC within the limits of accuracy. Paltauf et al. (18) obtained a similar result with dipalmityl-PC. The condensation effect with cholesterol is of exactly the same magnitude as for diester-PC (Table II, 2).

The dialkyl-PC B monolayer, with the lipid having two oleyl chains, is slightly more expanded (Fig. 1, 4; Table II, 4). Cholesterol again causes condensation, although the area of the condensed phospholipid remains somewhat larger than for diester- and diether-PC. Dialkyl-PC A (Table II, 3; no figure shown), with cyclopropane fatty alkyl chains, has the same surface area as diester- and diether-PC and is also condensed but to a somewhat lesser extent.

Polyoxyethylene Glycerides

The glycerides A containing a methoxyethoxyethoxy polar group (Fig. 1, Table II; 5 and 6) yielded force-area curves similar to those of the phospholipids, but the monolayer collapsed at lower surface pressure. Both lipids were condensed by cholesterol, though only moderately.

The diester glyceride B substituted with a methoxyethoxyethoxyethoxy group (Fig. 1, Table II; 7) formed a somewhat more stable monolayer, with the expected force-area characteristics. Cholesterol caused considerable condensation at high pressure only; at 17 dyn·cm^{-1}, condensation was no longer noted; and at lower pressure the film with cholesterol appeared actually to be expanded.

DISCUSSION

In this study, we set out to determine if the possibility of hydrogen bonding between the carboxyl C=O groups of polar lipids and the hydroxyl of cholesterol was a prerequisite of the monolayer condensation effect. To this purpose, a phosphatidylcholine was compared with analogs lacking its hydrogen bond accepting C=O groups: a diether and a dialkyl phosphatidylcholine. Both compounds displayed the area-condensation effect with cholesterol, the diether-PC to exactly the same degree as diester-PC, the dialkyl-PC somewhat less. It should appear, then, that phospholipid-cholesterol hydrogen bonding, if it exists, is not responsible for the condensation effect. Strictly, however, the results only show that phospholipid-C=O-cholesterol bonding is not required, since the ether oxygen of the diether lipid might also be a potential hydrogen bond acceptor. [The possibility of phosphate=O-cholesterol bonding in the case of the dialkyl-PC will be discussed in the following paper (19)]. However, the ether oxygen is a weaker hydrogen bond acceptor than the C=O group, and molecular models show that it would be less freely accessible to the cholesterol-OH proton. Since the condensation effect for the ether lipid is, nevertheless, exactly equal to that of the diester-PC, it is unlikely that the hypothetical lipid-lipid hydrogen bonding is of critical importance for the effect. The experiment by de Kruyff et al. (8) showing a reduced condensation effect for a 1-ester-2-ether-PC, as compared to diester-PC, does not quite conform to our results; perhaps an ester-ether lipid may behave differently from both diester and diether lipid, though this does not seem likely. In any case, the authors concluded from the condensation effect that did exist, although reduced, that this result provided no evidence for cholesterol-phospho-

lipid bonding in the hydrogen belt. From our results, we arrive at the same conclusion.

The fact that a modification of the hydrogen belt, as performed in our experiments, does not influence the condensation of phospholipid monolayers by cholesterol does not, of course, prove that such a modification will be insignificant in all other respects; nor does it disprove the existence of phospholipid-cholesterol hydrogen bonding. In the following paper (19), we report that an effect of such a modification appears as a change of the activation energy of bilayer permeation.

The polyoxyethylene glycerides were synthesized to serve as membrane lipid analogs containing hydrophilic head groups which, in contrast to those of natural lipids, contained neither electrical charges nor hydrogen bond donors, and only weak hydrogen bond acceptors (the ether oxygens). These lipids formed monolayers somewhat less stable than those of phospholipids (Fig. 1: 5, 6, 7), and we have not been able to prepare liposomes from them. The cyclopropane, long chain lipids were employed in order to eliminate the danger of oxidation and to allow a catalytic hydrogenation step during the synthesis, though this procedure was, eventually, not used. Van Deenen had reported (20) that cyclopropane groups, just as double bonds, conferred fluidity on membranes. As shown by the force-area curves, all lipids were, in fact, in a liquid-expanded state at room temperature.

Both diester and diether glyceride A are condensed by cholesterol (Fig. 1: 5 and 6); again, it is clear that glyceride-C=O-cholesterol hydrogen bonding, if it exists, cannot be indispensible for condensation. The condensation effect is appreciably smaller for the glycerides than for the phospholipids. This might be interpreted as evidence for cholesterol-OH-phosphate=O interaction in the case of phospholipids; however, there are strong theoretical arguments against this possibility (1), as well as counterevidence from nuclear magnetic resonance (NMR) experiments (21), and de Kruyff et al. (8) found that condensation by cholesterol of a glycolipid (which contains neither phosphate nor double-bonded oxygen in the head group) equalled that of a phospholipid. The reason for the smallness of the effect in the polyoxyethylene lipids is probably the low polarity of these compounds, which might at best bind three water molecules to their head group as opposed to the 10-11 tightly bound by phosphorylcholine (22). The polyoxyethylene head groups may, perhaps, be partially inserted into the hydrophobic region of the monolayer and fill the interstices that would receive the cholesterol. Diester glyceride B, with a longer hydrophilic head group, and greater monolayer stability, shows appreciable condensation by cholesterol at high surface pressure, perhaps because the head group is now squeezed out into the aqueous phase. At low surface pressure, addition of cholesterol paradoxically appears to extend the area occupied by the glyceride. It appears that the cholesterol molecules must occupy more than their usual 38.5 $Å^2$ as a result, perhaps, of tilting from their usual vertical position because the aqueous subphase is shielded by polyoxyethylene residues and the monolayer becomes depolarized.

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