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Influence of the phospholipid structure on the stability of liposomes in serum *

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The effect of serum on the structural integrity of liposomes consisting of ether and/or carbamyl analogs of 1,2-diester phosphatidylcholine (PC) has been evaluated by measuring both the efflux of the entrapped 6-carboxyfluorescein and the lipid transfer to serum proteins, and the results have been compared with the egg PC liposomes. Replacement of the C-1 ester bond in PC by an ether linkage did not significantly enhance the liposome stability, but it was markedly increased upon introducing further structural changes in the C-2 ester region of the resulting 1-ether-2-ester PC. However, the stability was not influenced by altering the steric configuration of the latter phospholipid. These results strongly suggest that lysis of liposomes in serum can be prevented by structurally modifying the ester bond(s) in the phospholipid component of liposomes.

Liposomes are lysed upon their incubation with blood, plasma or serum due to their interaction with high-density lipoprotein (HDL) [1,2]. This lytic effect of HDL is known to be mediated through transfer of lipids from liposomes to the protein, which in turn results in an enhanced leakage of the entrapped solutes in the medium [3,4]. Our previous studies [5,6] have, however, shown that both the lipid transfer and the enhanced leakage are considerably reduced by introducing an appropriate structural modification in the phospholipid component of liposomes. To further explore the role of phospholipid structure in determining the stability of liposomes in blood (or serum), we have now examined the effect of monkey serum on the stability of liposomes consisting of carbamyl and ether analogs of phosphatidylcholine (PC) (1–4, Fig. 1). Similar studies, using the PC analogs 2–4 described here, have earlier been reported by Hermetter and Paltauf [7].

Egg PC and egg [*methyl*-^{14}C]PC (35 μCi/μmol) were prepared as described earlier [8]. 1-*O*-Hexadecyl-2-*O*-*N*-(heptadec-8-*cis*-enyl)carbamyl-*sn*-glycero-3-phosphocholine (1), 1-*O*-hexadecyl-2-*O*-oleyl-*sn*-glycero-3-phosphocholine (2), 1-*O*-hexadecyl-2-oleoyl-*sn*-glycero-3-phosphocholine (3) and 3-*O*-hexadecyl-2-oleoyl-*sn*-glycero-1-phosphocholine (4) were synthesized as in Ref. 9. The ^{14}C-labeled 1 (25 μCi/μmol), 2 (23 μCi/μmol), 3 (40 μCi/μmol) and 4 (22 μCi/μmol) were prepared from 1, 2, 3 and 4, respectively, using the published procedure [8]. All the phospholipids exhibited single spots on Silica gel G-60 TLC plates. HDL was isolated from monkey serum using the known method [10]. The purity (greater than 90%) of the protein was established by polyacrylamide gel electrophoresis. 6-Carboxyfluorescein (Eastman Kodak) was used after its purification as in Ref. 11.

Unilamellar liposomes were prepared from PC (20 μmol), cholesterol (10 or 20 μmol), traces of the corresponding ^{14}C-labeled PC (about 5 μCi)

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Abbreviations: PC, phosphatidylcholine; HDL, high-density lipoprotein.

and self-quenched concentrations (0.2 M) of 6-carboxyfluorescein [12] in 0.8 ml Tris-buffered saline (10 mM containing 150 mM NaCl, pH 7.4) by probe sonication [5], and fractionated by centrifugation in a Beckman L5-65B ultracentrifuge at $100000 \times g$ for 1 h at 5°C. Only the liposomes found in the top 3/4 of the supernatant were used. Free and liposomal 6-carboxyfluorescein were separated by gel filtration over Sephadex G-50 [5]. The liposome size was determined by analytical molecular sieve chromatography over Bio-Gel A-50 m using Southern bean mosaic virus and γ-globulin as the column standards [13]. The average Stokes radii of the vesicles were between 15 and 17.5 nm.

The stability of liposomes in the presence of monkey serum was ascertained by measuring both the serum-induced leakage of the entrapped 6-carboxyfluorescein and the phospholipid transfer to serum proteins. The serum-induced leakage was determined by measuring the efflux of the entrapped dye in the presence of serum as well as buffer. The leakage rates of this dye from egg PC/cholesterol liposomes, in the presence of buffer, were almost identical to those observed for liposomes consisting of one of the PC analogs 1–4 and cholesterol. Fig. 2 shows that the 6-carboxyfluorescein efflux from the egg PC/cholesterol liposomes is considerably enhanced by including serum in the incubation mixture. This enhanced leakage was not significantly reduced upon replacing egg PC in liposomes by one of the PC analogs 2–4. However, the 6-carboxyfluorescein leakage remained virtually unaffected by incubating 1/cholesterol liposomes with serum. These observations clearly indicate that interactions of the egg PC/cholesterol liposomes with the serum constituents markedly alter the permeability behavior of these liposomes, and that this effect of serum is prevented by a complete replacement of egg PC in the liposomes by the PC analog 1.

In order to confirm that the enhanced leakage of the entrapped dye in serum is due to lipid transfer to serum proteins [3,4], we measured the phospholipid transfer to these proteins (Fig. 3). Table I shows that considerably larger amounts of phospholipids are transferred to serum proteins from the liposomes consisting of egg PC and cholesterol or one of the PC analogs 2–4 and cholesterol, as compared to that from the 1/cholesterol liposomes. Furthermore, the extent of the phospholipid transfer from egg PC/cholesterol liposomes was reduced upon replacing egg PC in the liposomes by 2, 3 or 4. At 50 mol% cholesterol concentration, the reduction was appreciably greater using the PC analog 2, as compared to 3 or 4. To further examine whether HDL is the major serum protein participating in the lipid transfer [3,4], we also measured the phospholipid transfer to HDL, after interacting lipo-

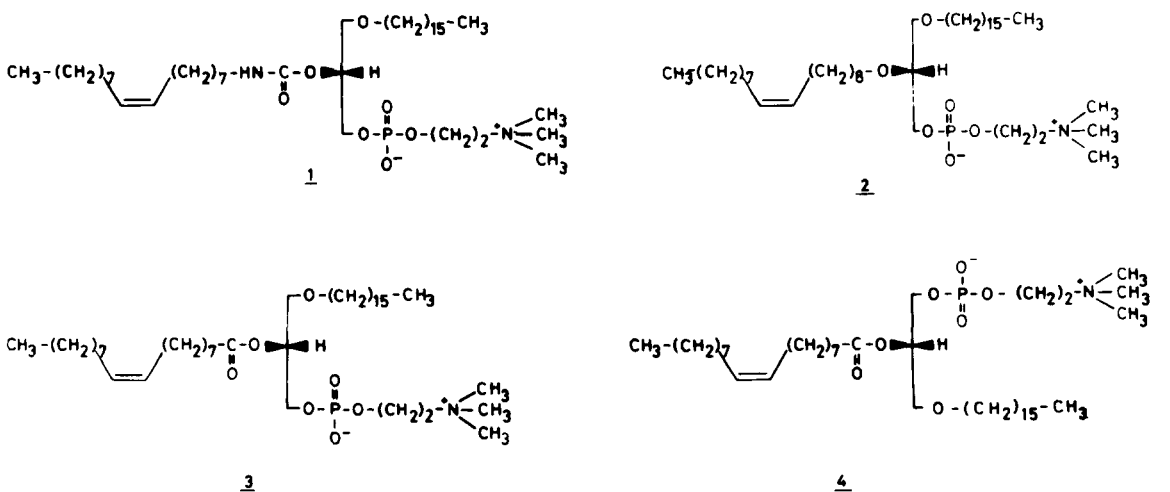

Fig. 1. Carbamyl and ether analogs of phosphatidylcholines (1–4).

somes with the pure protein. Results of these experiments revealed that from egg PC/cholesterol liposomes about 31% of the total phospholipid was transferred to HDL in 3 h at 37°C, whereas under identical conditions, the extents of phospholipid transfer from 1/cholesterol, 2/cholesterol, 3/cholesterol and 4/cholesterol liposomes were about 4, 14, 22 and 23%, respectively. Also, the elution profiles of HDL-associated phospholipids from the Sepharose 6B column were almost identical to those shown for serum protein-associated phospholipids in Fig. 3. These results indicate that the various structural modifications introduced in the PC component inhibit the lipid transfer to HDL (or serum proteins), which in turn leads to greater stability of the liposomes in serum.

From these observations, it would appear that the interactions between HDL and liposomes are not significantly affected upon replacing the C-1 ester bond in PC by an ether linkage or by changing the steric configuration of the resulting phospholipid (3), which is consistent with the earlier findings [7]. These interactions were, however, markedly influenced by introducing an appropriate structural change in the C-2 ester region of 3. This effect was maximum when the C-2 ester bond in 3 was replaced by a carbamyl linkage.

The observed differences in interactions of HDL with the various types of liposomes could not be a result of tighter phospholipid packing in the liposome bilayer [14,15], as the thermal phase-transition temperatures of the PC analogs 1–4 as well as the interactions of cholesterol with at least 2–4 are expected to be similar to that of the corresponding diacyl PC [5,16–19]. Neither can it be attributed to a difference in the size of liposomes [20], since the liposome size was found not to be influenced by the structure of the phospholipid component. It may, therefore, be envisaged that replacement of the ester linkage(s) in PC by an ether and/or carbamyl bond(s) probably reduces the affinity of HDL for liposome phospholipids, which in turn affects the lipid transfer to this protein. This is quite in accordance with the earlier studies [5,7].

Replacement of the ester linkage in PC by a carbamyl bond should apparently confer hydrogen bond-forming ability on the resulting phospholipid molecule. The existence of intermolecular hydrogen bonds in carbamyl PC bilayers has already

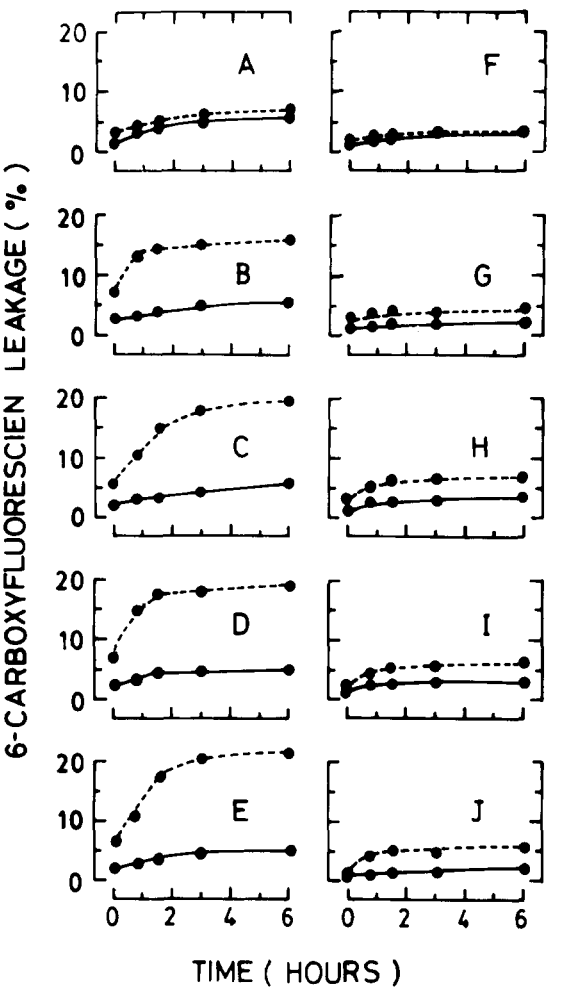

Fig. 2. Kinetics of efflux of the entrapped 6-carboxyfluorescein upon incubating liposomes with buffer (●———●) or serum (● — — — ●). Liposomes (0.5 ml, 3–4 μmol lipid P) were incubated with 1.0 ml monkey serum or buffer at 37°C. Measured aliquots from the incubation mixture were withdrawn at different periods of time and diluted to 1.0 ml with Tris-buffered saline. Free 6-carboxyfluorescein in the mixture was measured on an Aminco SPF-500 fluorimeter using the excitation and emission wavelengths of 490 and 520 nm, respectively. Total amount of 6-carboxyfluorescein was determined after lysing the liposomes with Triton X-100 (1% final concentration). Percent 6-carboxyfluorescein release was calculated from $100 \times Dye_f/Dye_t$, where f and t denote free and total dye, respectively. Values shown are mean of three independent experiments. Maximum variation was ±2%. Molar ratios: (A) 1/cholesterol (2:1); (B) 2/cholesterol (2:1); (C) 3/cholesterol (2:1); (D) 4/cholesterol (2:1); (E) egg PC/cholesterol (2:1); F, 1/cholesterol (1:1); G, 2/cholesterol (1:1); H, 3/cholesterol (1:1); I, 4/cholesterol (1:1); J, egg PC/cholesterol (1:1) liposomes.

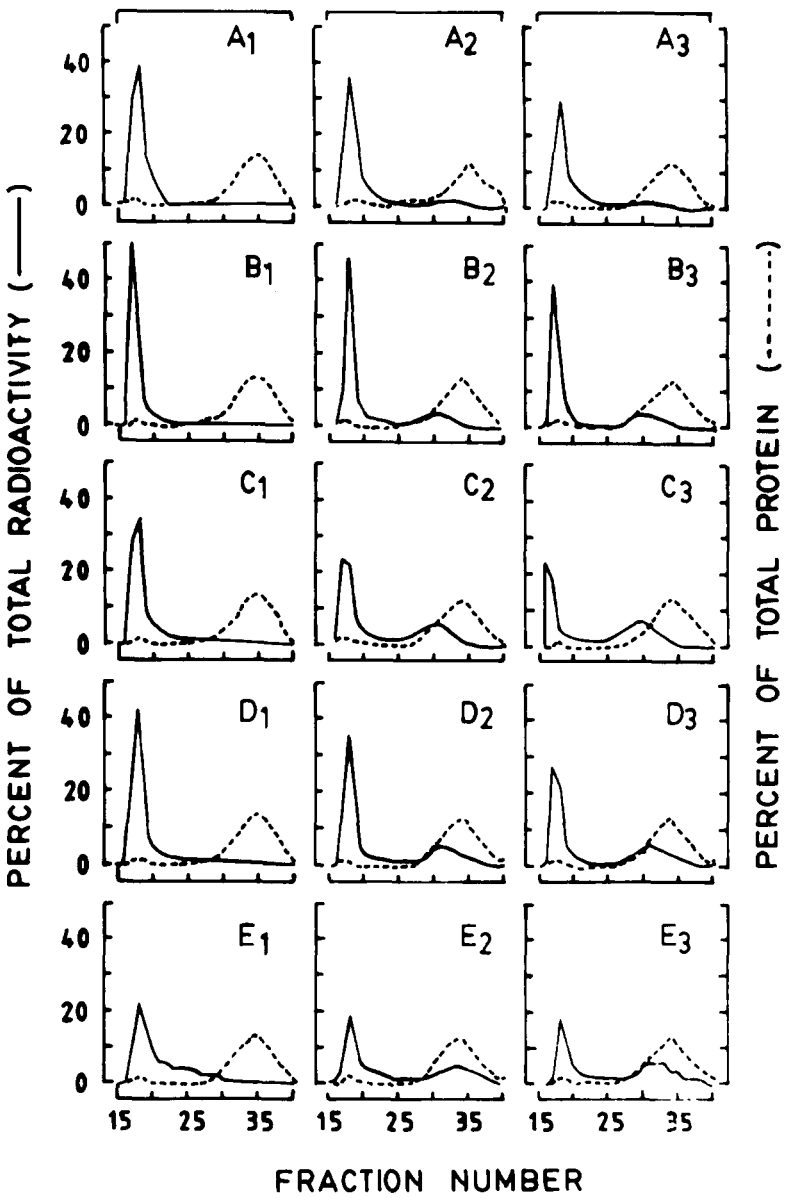

Fig. 3. Liposomes were incubated at 37°C with monkey serum, and a portion of the mixture was chromatographed over a Sepharose 6B column (34×1.4 cm) using Tris-buffered saline as the eluant. The elution patterns shown are for the incubation mixture consisting of PC/cholesterol (2:1, molar ratio) liposomes and serum. 1-ml fractions were collected. Each fraction was analysed by measuring absorbance at 280 nm, and by counting ^{14}C. Recoveries of lipids and protein from the column were over 90%. The percentage of lipid transfer to serum proteins was estimated as the increase in radioactivity associated to protein fractions at 3 and 6 h, as compared to the sample which was chromatographed immediately (0 h). A summary of data for phospholipid transfer is given in Table I. The liposomes, without incubating with serum, and pure HDL were also separately chromatographed on this column. The liposomes were recovered in fractions 16–20, whereas HDL eluted in fractions 27–33. (A) 1/cholesterol; (B) 2/cholesterol; (C) 3/cholesterol; (D) 4/cholesterol; (E) egg PC/cholesterol liposomes. Subscripts 1, 2 and 3 denote 0, 3 and 6 h incubation mixtures, respectively. For measuring the phospholipid transfer to HDL, PC liposomes containing 50 mol% cholesterol were mixed with HDL so that the ratio of phospholipid to HDL was about 2 [24]. The mixture was incubated at 37°C for 3 h. A measured aliquot of the incubation mixture was chromatographed on the Sepharose 6B column, and the phospholipid transfer was estimated essentially as above.

been suggested by an unusual thermal phase behavior of this phospholipid [21]. Since intermolecular hydrogen bonding between lipids is known to resist lysis of liposomes in serum [22], it may be inferred that lack of phospholipid transfer from 1/cholesterol liposomes to serum proteins (or HDL) is due to the hydrogen-bonding potential of the phospholipid component. This bonding probably does not exist in bilayers formed from 1 in the absence of cholesterol, as we observed no effect on the phospholipid transfer upon replacing egg PC in liposomes by 1. The absence of intermolecular hydrogen bonding in these bilayers is possibly due to lack of the C-1 ester group in 1, because replacement of egg PC in cholesterol-free liposomes by 1-ester-2-carbamyl PC has earlier been shown to inhibit the phospholipid transfer to serum proteins [5].

This study shows that the stability of liposomes in serum is greatly influenced by the structure of the phospholipid component. Liposomes consisting of 1 and cholesterol exhibited better stability

TABLE I

TRANSFER OF PHOSPHOLIPIDS FROM LIPOSOMES TO SERUM PROTEINS IN 3 AND 6 h AT 37°C

Values are expressed as mean of three determinations ± S.D. PL, phospholipid.

Liposomes	Molar ratio	PL transfer (%)	
		3 h	6 h
1/cholesterol	2	7.4 ± 1.4	10.8 ± 1.0
	1	5.2 ± 0.9	6.5 ± 1.4
3/cholesterol	2	18.7 ± 1.0	23.4 ± 1.7
	1	14.2 ± 0.8	17.6 ± 1.9
3/cholesterol	2	26.8 ± 3.5	33.8 ± 3.1
	1	19.4 ± 1.5	22.5 ± 1.5
4/cholesterol	2	24.2 ± 2.4	31.3 ± 1.7
	1	15.1 ± 0.9	18.2 ± 1.8
Egg PC/cholesterol	2	37.1 ± 4.6	43.1 ± 5.1
	1	28.6 ± 3.7	30.7 ± 3.4

than the 2/cholesterol liposomes, which in turn were more stable as compared to 3/cholesterol, 4/cholesterol or egg PC/cholesterol liposomes. As 1-ester-2-carbamyl PC has been shown to form liposomes that are considerably more stable and longer-living than the egg PC/cholesterol liposomes in circulation of rats [6], it may be expected that 1 liposomes would prove still better due to their phospholipase A_1- and A_2-resisting character [23]. Besides, the latter class of liposomes may not be toxic to the injected animals, since 1 is easily hydrolysed by phospholipase C [9]. Therefore, these liposomes should find some useful application as drug carriers in therapy [1].

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