Biochimica et Biophysica Acta, 769 (1984) 519–522
Elsevier

BBA Report

BBA 70140

COMPARISON OF CHOLESTEROL-PHOSPHOLIPID INTERACTION IN BILAYERS OF LIPOSOMES AND THE RED CELL MEMBRANE

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(Received September 8th, 1983)

Key words: Cholesterol-phospholipid interaction; Cholesterol distribution; (Liposome, Erythrocyte membrane)

The energetics of interactions of cholesterol with phospholipid in simple liposome bilayers were compared with those in the bilayer of the human erythrocyte membrane, by measuring cholesterol distribution between erythrocytes and liposomes prepared from their whole phospholipid extract. With liposomes of a range of initial cholesterol contents, the equilibrium value for *r*, the ratio of cholesterol/phospholipid in the liposomes to that in the cells, is in the range 1.1–1.2. The closeness of this value to 1.0 indicates that overall cholesterol-phospholipid interaction in the cell membrane is similar to that in liposomes. However, while the deviation from 1.0 is small, and could arise from average cholesterol-phospholipid interactions in the membrane being only 0.06 to 0.1 kcal · mol^{-1} weaker than in liposomes, it could also result from 10 to 20% of the cell membrane phospholipid being unavailable to mix with cholesterol.

The fact that cholesterol decreases the fluidities of phospholipid bilayers in cell membranes and artificial liposomes [1], indicates that the general nature of the interactions between these lipids in the two systems is similar. This present work examines the extent to which the energetics of the cholesterol-phospholipid interactions in the erythrocyte membrane are affected by factors peculiar to membrane structure and not present in liposomes. For example, some fraction of the cell membrane phospholipid may not mix freely with cholesterol as a result of binding to protein [2], or may be subject to conformational constraints associated with restriction to a particular leaflet [3] or domain [4] of the bilayer. The comparison of interactions in the cell membrane and liposomes was made on the basis of the equilibration of cholesterol between intact human erythrocytes and liposomes. The latter were prepared from the whole phospholipid extract of erythrocytes, to avoid specificity based on species of phospholipid polar group and characteristics of the hydrocarbon chains, as found by Lange et al. [5] for cholesterol distribution between erythrocyte membranes and liposomes prepared from various individual phospholipids.

The experiments reported here were with whole erythrocytes instead of the cell ghosts which were used by Lange et al. [5]. This modification was made after preliminary experiments indicated (a) excessive sedimentation of liposomes at the centrifuge speeds required to sediment ghosts, and (b) that erythrocytes could be incubation for prolonged periods without hemolysis provided that agitation was only intermittent (see below). The use of whole cells also has the obvious advantage of avoiding alterations in the cell membrane accompanying the conversion of cells to ghosts.

Distribution of cholesterol between erythrocytes and liposomes was determined by incubation of whole cells with liposomes for up to 93 h at 37°C. The incubation mixtures contained typically about 35% (v/v) erythrocytes, and had liposome phospholipid contents 30%–70% of that of the

erythrocytes. Incubations were in screwcap glass culture tubes; intermittent slow rotation of the tubes, on a 1 min on-15 min off cycle, was sufficient to prevent sedimentation of the cells, while keeping hemolysis (as measured by the 550 nm absorbance of the liposome phase) to less than 1% in 93 h. The incubation media was 150 mM NaCl, 5 mM Tris (pH = 7.4), 0.5% glucose, 0.25 mM Na EDTA (to minimize binding of liposomes to cells [6]), 0.25 mM NaN_3, and 0.25 vol.% gentamicin antibiotic (Upjohn). To terminate the incubation, cells and liposomes were separated by centrifuging at $2500 \times g$. Cells were then washed three times with 10–20 volumes of 150 mM NaCl, and cells and liposomes extracted with isopropanol/chloroform [7]. Light microscope examination indicated that cell shapes were similar after incubations in the presence and absence of liposomes; after 93 h, about half the cells were echinocytes and half retained the discoidal shape.

Lipid determinations of the cell and liposome extracts were carried out in parallel. Cholesterol was determined by the phthalaldehyde method [8], and phospholipid by the procedure of Rouser et al. [9]. Determinations were done in triplicate, with an average deviation from the mean typically of less than 1%; in instances in which this quantity exceeded 2%, determination of both lipids, in both cell and liposome extracts, was repeated.

Erythrocytes were obtained from blood drawn from normal human donors into acid/citrate/dextrose. Following separation of cells from plasma by $2500 \times g$ sedimentation, the buffy coat was removed and the cells washed three times with 150 mM NaCl. Incubations were started within 4 h of drawing the blood.

Phospholipids for the liposomes were extracted from cells several days before the planned incubations, also using the isopropanol-chloroform procedure, and stored 1:1 chloroform/methanol at −20°C. Cholesterol was removed from this lipid extract by chloroform elution of a silica gel (Unisil, Clarkson Chemical Co., Williamsport, PA) column, followed by methanol elution of the phospholipids. Tracer amounts of L-α-1-palmitoyl-2-[1-^{14}C]oleoylphosphatidylcholine (New England Nuclear, Boston, MA) were added to the extracted phospholipids. Appropriate cholesterol/phospholipid ratios (C/P) in the liposomes were obtained by reconstituting the phospholipid with this sterol.

Liposomes were prepared a day before the start of incubation by standard ultrasonication technique [10] using a Model W-350 Heat Systems (Plainview, NY) instrument, equipped with the standard tip. Sonication was carried out in 150 mM NaCl, under nitrogen, in an ice water bath for 1 h (setting 9, 70% duty cycle). The liposome suspension was centrifuged at $100\,000 \times g$ for 1 h following sonication.

Thin-layer chromatography (Applied Science Prekotes, chloroform/methanol/water/30% ammonia (90:40:4:4, v/v)) indicated similar contents of the major erythrocyte phospholipids (phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, and phosphatidylserine) for the original extracted lipid, for that subjected to silica gel chromatography, and for sonicated liposomes. The other, minor, phospholipids [11] were not detected. No effect of incubation on the phospholipid composition of either cells or liposomes was found.

The experimentally measured distribution ratio, r, is defined by:

$$r = \frac{(C/P)_l}{(C/P)_m} \tag{1}$$

Where C/P is the molar ratio of cholesterol to phospholipid, and the subscripts l and m refer to liposomes and cell membranes.

Errors in the determination of r, which would bring the experimentally measured distribution coefficient closer to unity, may arise from incomplete separation of cells from liposomes. First, hemolysis results in the formation of cell ghosts, which would not sediment at the low centrifugation speeds used. This does not introduce a significant error, since hemolysis was less than 1%. Second, as reported by Ott et al. [12], on prolonged incubation with phospholipid liposomes, erythrocytes may shed membrane particles which would not sediment at low centrifugation speed. However, no evidence for such particles was indicated from the phospholipid content of the supernatant; this is consistent with the findings of Eytan et al. [13], that only liposomes made with saturated phospholipids produce such shedding. The remain-

ing, and major factor interfering with complete separation, was the co-sedimentation of liposomes with cells. Control experiments showed that about 5% of the liposomes sediment on low speed centrifugation after 93 h incubation, while radioactivity determination, on the erythrocyte membrane extract, indicated sedimentation of another 5% due to attachment to erythrocytes. Since, as will be shown below, the measured distribution coefficients are close to 1.0, sedimentation of 10% of the liposomes along with erythrocytes does not produce a major error. Exchange of phospholipid between cells and liposomes during incubation need not be considered, since no change in liposome specific radioactivity was found.

Lange et al. [7] found that equilibration of cholesterol between erythrocyte membranes and liposomes is a slow process, with a half-time of about 22 h at 46°C. In the present work, the attainment of equilibrium was evaluated by noting the effect of extending the incubation time, and by approaching the equilibrium distribution from both directions.

Two series of experiments were carried out, each involving incubation of erythrocytes with liposomes of several initial C/P values. In the first series, where incubations were carried out for 72 h, the distribution coefficients continued to change, by about 10%, during the last 24 h. In the second series, in which incubations lasted for 93 h (the maximum feasible because of increasing hemolysis), the changes during the final 24 h were smaller, but nevertheless significant. The time course of the latter series is shown in Fig. 1, and Table I lists the distribution coefficients found at 72 h in the first series and at 93 h in the second series. While these distribution coefficients are not true equilibrium values, some considerations indicate that the systems are not far from equilibrium. The approach to equilibrium from both directions brackets the equilibrium value to between 1.1 and 1.2. Also, cholesterol movement between erythrocyte membranes and liposomes is known to follow a simple exponential time course after the initial stages [7]. On the basis of half-times of about 25 h indicated (after 24 h) by the data of Fig. 1 and the corresponding data for the 72 h series, values of r would be within 10% of the equilibrium value after 72 h, and within 5% after 93 h.

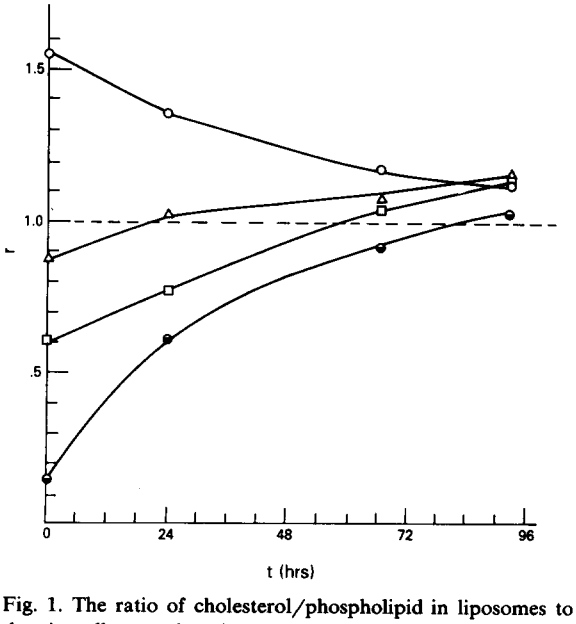

Fig. 1. The ratio of cholesterol/phospholipid in liposomes to that in cells as a function of incubation time. Original C/P mole ratio: ○, 1.42; △, 0.80; □, 0.54; ◒, 0.11.

TABLE I

INITIAL AND FINAL LIPOSOME C/P, AND FINAL r, VALUES

Original liposome C/P	Final liposome C/P	Final r
A. Incubation time 72 h		
1.32	1.20	1.19
0.76	0.91	1.09
0.56	0.78	1.03
B. Incubation time 93 h		
1.42	1.11	1.12
0.80	0.97	1.15
0.54	0.81	1.16
0.12	0.54	1.04

The preferential uptake of cholesterol by liposomes indicated by an equilibrium distribution coefficient in the range 1.1–1.2, though small, appears to be real since (a) although the values of r are computed from four experimental quantities (i.e., amounts of cholesterol and phospholipid in liposomes and cells), the uncertainties in these quantities are only 1–2%, and (b) the deviation from unity is opposite to that which would arise from incomplete separation of cells and liposomes.

An equilibrium distribution coefficient of 1.1–1.2 could result from average free energy of cholesterol-phospholipid interactions in the cell membrane being only 0.06–0.1 kcal · mol^{-1} weaker than in liposomes (from $\Delta G° = -RT \ln r$, where $\Delta G°$ is the standard free energy change for transfer of cholesterol between cells and liposomes, and R and T have their usual meaning. The deviation of r from unity could also arise from 20% of the phospholipid molecules being unavailable to mix with cholesterol, with the remainder behaving similarly to that in liposomes. While detailed speculation is obviously not warranted, it is suggestive that this fraction of phospholipid is similar to that which has been ascribed to boundary phospholipid in membranes of other cells [2], and is also approximately the fraction of the major individual phospholipid species in the erythrocyte membrane [11]. However, the main conclusion from this work is that the equilibrium distribution coefficient is close to unity, and that the overall phospholipid-cholesterol interaction in the cell membrane is not very different from that in liposomes.

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