THE RATE OF CHOLESTEROL 'FLIP-FLOP' IN LIPID BILAYERS AND ITS RELATION TO MEMBRANE STEROL POOLS

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1. Introduction

The movement of lipid molecules from one side of a lipid bilayer to the other (the so-called 'flip-flop') was first studied in films of stearate [1]. At 25°C stearate molecules transferred with a halftime of 25 min in the absence of Ca^{2+} and 50 min in its presence. Of more relevance to cellular membranes is the 'flip-flop' of the major structural lipids, phospholipids and unesterified sterols. Phospholipid transfer has been investigated by a spin-labelling technique in liposomes of egg lecithin [2]. The half-time at 30°C was 6.5 hr and the process is at least eight orders of magnitude slower than translational migration within the plane of the bilayer [3].

We have now utilized the ability of iodide ions added to one side of a lipid bilayer to quench the fluorescence of the sterol sterophenol (1-methyl-19-nor-cholesta-1,3,5(10)trien-3-ol; fig. 1) on that side of the bilayer, to measure sterol 'flip-flop' in liposomes. The process is much faster than that of phospholipids, with a half-time at 30°C of 72 min. This finding is of relevance to studies of cholesterol exchange between plasma lipoproteins and cell membranes and to the finding in such studies [4,5] of more than one pool of membrane sterol.

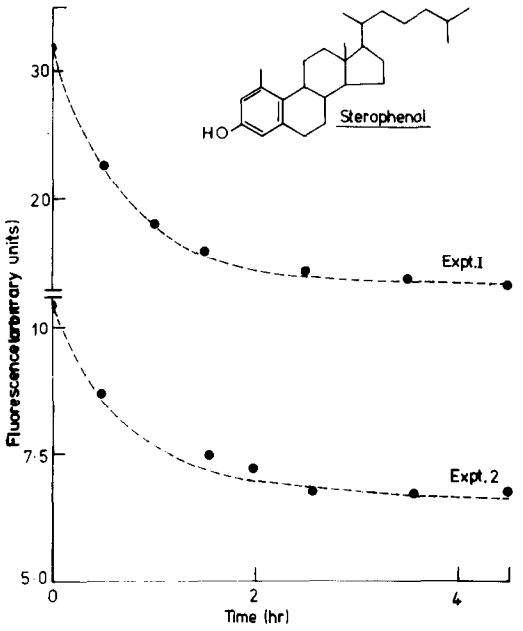

Fig. 1. Time course of the quenching by externally added KI of the fluorescence of sterophenol initially mainly on the inner surface of liposomes. (----) Calculated time course; (•): experimentally determined values.

2. Materials and methods

Egg lecithin preparation, lipid extraction, estimation of phospholipid and cholesterol were as described previously [6,7]. Low density lipoproteins were obtained from human plasma by ultracentrifugation [8] and dialysed against 0.15 M phosphate buffer, pH 7.4. Sterophenol was prepared from cholesta-1,4,6-trien-3-one [9] and estimated spectrophotometrically in ethanol solution (λ_{max} 285 nm, ϵ 1995).

Liposomes were prepared as before [7] except that only three one minute periods of sonication interrupted by 30 second cooling periods were used. After

centrifugation at 105 000*g* for 20 min, the supernatant liposome dispersion consisted almost entirely of single-shelled vesicles [10]. Low density lipoproteins were separated from liposomes by precipitation with dextran sulphate [11]. Complete separation, confirmed by the absence of fluorescence from lipoprotein tryptophan, was obtained by increasing the concentration of the dextran sulphate used to 2.5%.

Fluorescence measurements were made with a Hitachi Perkin–Elmer MPF 2A fluorescence spectrophotometer, using the conditions previously described [12]. Excitation was at 290 nm and fluorescence was measured over the range 300–380 nm.

2.1. *Experimental procedure*

The principle of the method was to prepare liposomes containing the fluorescent sterol in both halves of the bilayer and then, by incubating in the presence of a large excess of low density lipoproteins, to exchange the sterophenol on the outside of the liposomes for cholesterol. Iodide ions penetrate liposomes relatively slowly [13] but quench the fluorescence of sterophenol very efficiently. If they are added to the liposomes, they quench none of the fluorescence at first, but as sterophenol molecules exchange with cholesterol via the 'flip-flop' their fluorescence will be quenched by the added I^-. Hence the increase in the degree of quenching of the sterophenol fluorescence provides a measure of the rate at which the sterol transfers across the bilayer.

Two experiments were performed using liposomes with molar ratios of sterophenol to lecithin of 0.71 and 0.56. To 3 ml of each preparation (1 mg of lecithin/ml) was added enough low density lipoprotein solution to provide a 15-fold excess of cholesterol over sterophenol in a final volume of 6 ml. The mixtures were incubated at 30°C for about 90 min then an equal volume of 0.15 M NaCl followed by 0.1 ml of 2.5% dextran sulphate were added. After 15 min the precipitated lipoproteins were sedimented at 20 000 *g* for 15 min. Two samples (0.8 ml) of each were removed. To one sample (control) 0.2 ml of 1.5 M KCl and to the other, 0.2 ml of 1.5 M KI were added and their fluorescence spectra recorded. The remainder of the liposomes were incubated at 30°C and further samples removed at intervals for measurement of fluorescence in the presence of KI.

3. Results and discussion

To establish that sterophenol transfer was a true measure of cholesterol exchange, liposomes containing roughly equal amounts of [^{14}C]cholesterol and sterophenol were incubated as described above with low density lipoproteins. Samples of the lipoprotein were taken over 5 hr and analysed for ^{14}C and fluorescence. The two labels entered the lipoproteins at the same rate, showing there was no preferential exchange of either sterol with lipoprotein cholesterol.

The optimum concentration of KI was established in preliminary experiments. Its non-penetration of liposomes in the few minutes needed to measure fluorescence was confirmed by comparing the fluorescence of liposomes containing sterophenol (a) after sonication in the presence of KCl (b) as for (a) but with KI added afterwards (I^- on outside only) and (c) after sonication in the presence of KI (I^- on both sides of bilayer). The fluorescence of (a) was roughly double that of (b) whereas (c) gave no detectable fluorescence.

In the two experiments described above, comparison of the fluorescence in the presence and absence of I^- of the liposome samples taken immediately after incubation with the lipoproteins, gives the initial proportion of sterophenol in each half of the bilayer. In expt. 1 only 8% of the fluorescence was quenched by added KI, indicating that 92% of the sterophenol was on the inner surface. In expt. 2, 68% was on the inner surface. On incubation, quenching of fluorescence by the external I^- increases in both experiments (fig. 1) showing that sterophenol is transferring from the inner to the outer surface of the bilayer. The process appears to be essentially complete in 4–5 hr. The final degree of quenching of sterophenol fluorescence is a measure of the distribution of total sterol molecules between the two surfaces of the bilayer if random exchange of cholesterol and sterophenol occurs. In both experiments, 54% appears to be on the outside, and 46% on the inside. Thus the liposomes are not so asymmetric as those used by Kornberg and McConnell [2] in their study of phospholipid exchange. However their vesicles contained no sterol and were sonicated for a much longer period of time and so were probably slightly smaller than those used here. Since the plasma membranes of animal cells have a much larger radius of

curvature than these liposomes, it seems probable that cholesterol is fairly uniformly distributed between the two sides of their bilayers.

The rate at which fluorescence quenching increases (fig. 1) is not a simple exponential process as in the studies of phospholipid exchange [2]. In the latter, spin-labelled molecules were chemically reduced as they transferred across the bilayer, whereas in the present study, the probe is unchanged and as it builds up in the outer half of the bilayer, it begins to exchange back with the sterophenol remaining on the inside. Hence the apparent rate of movement slows with time. A similar phenomenon was observed in studies of the exchange of [^{14}C]cholesterol between plasma lipoproteins and erythrocyte membranes [5] and ascribed to possible changes in their structure with time. However, this could also be only an apparent slowing of the exchange rate caused by recycling of the isotope.

At the start of expt. 1 when almost all of the sterophenol was on the inside of the bilayer, the measured rate of transfer is not affected by this counter-transfer and represents the true rate. If this rate remains constant, then from the known distribution of total sterol between the two sides of the bilayer, and assuming a completely random exchange of sterophenol and cholesterol molecules, the theoretical distribution of sterophenol on each side of the bilayer at various time intervals can be calculated. The distribution of sterophenol with time can also be calculated for expt. 2, where initially an appreciable amount of sterophenol is on the outside of the bilayer, assuming that the rate of transfer is the same as in expt. 1. It can be seen (fig. 1) that the measured quenching of sterophenol fluorescence fits very closely the calculated curves. After correction for counter-transfer, semilog plots of sterophenol transfer against time give straight lines. The average half-time for sterol 'flip-flop' is 72 min, movement from inside to outside being slightly quicker (half-time 62 min) and movement from outside to inside, slightly slower (half-time 82 min). As the curves in fig. 1 were calculated assuming that an equal number of sterol molecules transfer each way across the bilayer in a given time interval, 'flip-flop' probably occurs via the coupled movement of molecules from each side as proposed by Kornberg and McConnell [2].

The finding that sterol 'flip-flop' is much quicker than that of phospholipids was expected. Sterol molecules have only about half the mass of phospholipids and have a much smaller hydrophilic group so much less energy would be needed for their transfer across the hydrophobic region of a lipid bilayer.

The results could explain the finding of two separate pools of membrane sterol exchanging with plasma lipoproteins [4,5]. The unesterified cholesterol of plasma lipoproteins is probably in a monolayer at the surface [12]. If red blood cells are obtained from an animal soon after injecting radioactive cholesterol or mevalonic acid, the cholesterol on the outside of the membrane bilayer could be more heavily labelled than that on the inside. On mixing with unlabelled plasma or lipoprotein solutions exchange will proceed simultaneously between the two halves of the lipid bilayer and between the outside of the bilayer and the external lipoproteins. The specific activity of the unesterified sterol in the latter could then rise above the average specific activity of the red cell cholesterol, as has been found [4,5]. This effect would not be seen if the isotopic label were initially in the lipoproteins or if ghosts, permeable to lipoproteins were used [6] where exchange could occur at both surfaces of the cell membrane. The rate of lipid 'flip-flop' in natural membranes will probably be slower than in liposomes because of the presence of protein which inhibits the molecular motion of the lipids [14].

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