Transmembrane Migration ('Flip-Flop') of Cholesterol in Erythrocyte Membranes

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After exchange with [^{14}C]cholesterol-labelled plasma lipoproteins for 0.5–4 h, erythrocytes were extracted with bile-salt solutions. The extracted cholesterol (mainly from the outside of the erythrocyte membrane) had the same specific radioactivity as the residual sterol. Thus cholesterol equilibrates rapidly (half-time less than 1 h) between the two sides of the membrane.

The first measurement of the rate at which sterol molecules transfer from one side of a lipid bilayer to the other (usually called 'flip-flop') was made by Smith & Green (1974). They used the phenolic sterol sterophenol [1-methyl-10-norcholesta-1,3,5(10)-trien-3-ol] in phosphatidylcholine vesicles and found that the half-time for the process was about 70 min, making it very slow compared with lateral diffusion, but appreciably faster than the transmembrane movement of phospholipids. On the other hand, experiments with cholesterol in similar vesicles and with virus membranes indicated that cholesterol movement across the bilayers occurred at a negligible rate, the half-times being at least several days (Poznansky & Lange, 1976; Lenard & Rothman, 1976). Since the present experiments were completed, however, Lange *et al.* (1977) have reported that cholesterol equilibrates rapidly (half-time less than 50 min) across the human erythrocyte membrane.

The above experiments have all used the ability of cholesterol in natural and model membranes to exchange with that of plasma lipoproteins or liposomes (Bruckdorfer & Graham, 1976) as a criterion of whether cholesterol is in the inner or outer half of the bilayer under study. Coleman and his colleagues (Coleman & Holdsworth, 1976; Billington *et al.*, 1977) have provided a direct method for studying components of the outer surface of cell plasma membranes. They have shown that at low concentrations the bile salts taurocholate and glycocholate can selectively remove lipids and proteins from the outer surface without any gross damage to the membrane. We have now used this method to compare the specific radioactivity of the cholesterol in bile-salt extracts of erythrocytes with that of the residual cholesterol after labelling the cells by exchange with [^{14}C]cholesterol-labelled plasma lipoproteins.

Materials and Methods

Sodium taurocholate and sodium glycocholate were obtained from Calbiochem, Hereford, U.K.

Rat erythrocytes labelled *in vivo* were obtained from animals 3 h after intraperitoneal injection of

10 μCi of [^{14}C]mevalonic acid (potassium salt). They were washed three times with buffered saline (0.154 M-NaCl, 1.5 mM-Hepes,* pH 7.4) (Coleman & Holdsworth, 1976).

For labelling rat erythrocytes *in vitro*, they were incubated with plasma obtained from the injected animals. For human and sheep erythrocytes, the plasma used was labelled with [^{14}C]cholesterol by the method of Werb & Cohn (1971). After 2 h at 37°C it was filtered through a membrane filter (0.45 μm pore size) before incubation with the cells.

[^{14}C]Cholesterol was incorporated into the erythrocytes by incubating 10 ml of packed cells with 40 ml of the labelled plasma at 37°C for the required length of time. The cells were separated by centrifugation (1000g for 10 min), rinsed briefly with unlabelled plasma diluted with 3 vol. of buffered saline, and then washed three more times with 10 vol. of buffered saline.

The cells were extracted with the bile-salt solutions as described by Coleman & Holdsworth (1976) and centrifuged at 1000g for 3 min. Tests were carried out on the cells to find the concentration that gave a good extraction of membrane lipids with minimal haemolysis. For the human and rat erythrocytes, 0.25–0.3% taurocholate was used, whereas for the sheep cells, 1.35% glycocholate proved optimal.

Lipid extraction and determination of phospholipid and cholesterol were as previously described (Graham & Green, 1970). Radioactivity was measured in an Intertechnique SL30 liquid-scintillation counter, with 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole in toluene (8 g/litre) as scintillant.

Results and Discussion

Although erythrocytes do become more fragile after treatment with bile salts, haemolysis in the experiments shown in Table 1 was only 2.4–8.3%, whereas 10–25% of the cholesterol (and 12–26% of

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Table 1. *Extraction of cholesterol from erythrocytes after exchange with [^{14}C]cholesterol-labelled plasma lipoproteins*
 Labelling of the cells with [^{14}C]cholesterol is described in the Materials and Methods section. In Expt. 1 the cells were labelled *in vivo* and in Expt. 2 they were labelled *in vitro* by incubation with the plasma obtained from the animals used in Expt. 1. In the remaining experiments the cells were labelled by incubating them with autologous plasma into which [^{14}C]cholesterol had been incorporated *in vitro*.

Expt. no.	Source of erythrocytes	Time exposed to labelled plasma (h)	Bile salt used (sodium salts)	Cholesterol extracted (%)	Cholesterol specific radioactivity (c.p.s./mg)		
					Whole cells	Bile salt extract	Extracted cells
1	Rat	3.5	Taurocholate	25.0	2660	2750	2680
2	Rat	4.0	Taurocholate	23.0	372	282	300
3	Human	0.5	Taurocholate	22.5	3500	3460	3690
4	Human	4.0	Taurocholate	16.0	10800	10700	10900
5	Sheep	0.5	Glycocholate	19.3	2420	2250	2450
6	Sheep	3.5	Glycocholate	9.6	8880	8880	8280

the phospholipid) was extracted. Qualitative t.l.c. of the phospholipids present in the extracts of human and sheep cells confirmed the quantitative results of Coleman & Holdsworth (1976) and Billington *et al.* (1977) in showing that the phospholipids extracted are enriched in those found mainly in the outer half of the membrane bilayer.

Table 1 shows that, after a short period of exchange with plasma lipoproteins containing [^{14}C]cholesterol, the labelled sterol in the membrane is uniformly distributed between the fraction that can be extracted by bile salts and that which remains behind. Thus the two fractions behave as if they are derived from a common pool of membrane cholesterol. This could be explained in several ways.

Firstly, all (or almost all) of the erythrocyte cholesterol could be present in the outer leaflet of the membrane bilayer. The distribution of cholesterol between the two surfaces of the membrane has not been firmly established. There is evidence that the distribution can vary with the conditions and that there may be more sterol in the outer leaflet than in the inner one (Fisher, 1976), but the latter always contains a substantial share and may contain one-half of the total (Fisher, 1976; McCabe & Green, 1977).

Secondly, it is possible that, during the exchange process, cholesterol is simultaneously inserted into both leaflets of the bilayer rather than only the outer one. This may be true if liposomes are used as the exchanging species instead of plasma lipoproteins, since fusion of the two bilayers does occur (Papahadjopoulos *et al.*, 1974). However, similar fusion of erythrocytes and plasma lipoproteins, which have their non-esterified cholesterol present in a monolayer at the surface, does not occur (Bruckdorfer & Graham, 1976). (The previous estimates of the trans-membrane migration of cholesterol have also contained the assumption that cholesterol exchange only

takes place between the exposed surface of the bilayer under study and the other component.)

A third possibility is that the bile salts perturb the membrane, thus causing a rapid mixing of the sterol in the two halves of the bilayer during extraction. This would involve some specific and novel effect, since it does not involve the membrane phospholipids. Because protein and phospholipid are preferentially removed from the outer surface of the membrane and there is no obvious disruption of the basic structure, it is reasonable to conclude that cholesterol is similarly treated.

The remaining interpretation is that cholesterol transfers from one side of the erythrocyte membrane to the other at a rate rapid enough to allow equilibration between the two to occur within the time of the experiments. Because of the time involved in washing the cells *etc.*, during which equilibration would still proceed, the shortest time interval between exposure of the cells to labelled plasma and the end of extraction was over 2 h. Thus it may be concluded that the half-time for sterol migration across the membrane is less than 1 h. This is consistent with the value originally found in a model system (Smith & Green, 1974) and with the results of Lange *et al.* (1977) for human erythrocytes. The results of Haran & Shporer (1977) also suggest that trans-bilayer migration is rapid. As would be expected (Smith & Green, 1974), the rate is faster than that for phospholipids, which give a half-time of 4.5 h in intact rat erythrocytes (Renooij *et al.*, 1976) and 2.3 h in resealed 'ghosts' (Bloj & Zilversmit, 1976).

Comparison of the findings of previous studies of cholesterol exchange between erythrocytes and plasma (Green, 1977) reveals that although experiments performed *in vivo* do not provide evidence for the existence of more than one pool of erythrocyte sterol, some experiments performed *in vitro* do so. It is therefore noteworthy that the experiments

performed *in vivo* and *in vitro* in the present investigation give very similar results. Some factors that might affect cholesterol movement within and between membranes and lipoproteins can be eliminated from the similarity of the results obtained when different species are used to provide the cells and plasma and when different methods of labelling the plasma and different bile salts are used. But if the rate of transmembrane transfer could vary in different conditions to give half-times of from just under 1 h to about 3 h, then the different results obtained with the erythrocyte/plasma lipoprotein system could be explained. However, the present experiments, like those of Lange *et al.* (1977), do not allow a minimum half-time to be estimated. It could be very much less than 1 h. Even if two pools of erythrocyte cholesterol, exchanging at different rates, do exist, they need not necessarily be in different leaflets of the membrane bilayer.

The extremely low rates of cholesterol transfer reported for phospholipid vesicles by Poznansky & Lange (1976) could be a function of the small size of the structures. The molecular packing and the distribution of cholesterol between the two halves of the bilayer in such structures can vary markedly with the radius of curvature (de Kruijff *et al.*, 1976). Although the influenza viruses studied by Lenard & Rothman (1976) are larger than the vesicles, they are much smaller than erythrocytes and their membrane contains many glycoprotein molecules, which could restrict the ability of lipid molecules to transfer across the bilayer.

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