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Interaction of cholesterol and lysophosphatidylcholine in determining red cell shape

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Abstract The effect of lysolecithin on the shape of human erythrocytes of varied cholesterol content was examined by scanning electron microscopy. Under the conditions of these experiments, all of the [^{14}C]lysolecithin incubated with cells was shown to be located in the external membrane leaflet. The membrane lysolecithin required to induce echinocytosis (spiculation) in normal cells (0.8 mol cholesterol/mol phospholipid) was approximately 0.08–0.10 μmol/10^{10} cells, which contributed 1.6–2.0 μm^2 or 1% of the cell surface area. This value is consistent with the premise that echinocytosis was caused by a slight differential expansion of the outer surface of the bilayer. The lysolecithin required for echinocytosis decreased as the membrane cholesterol content increased; from 0.14 μmol/10^{10} cells at 0.5 mol cholesterol/mol phospholipid to 0.03 μmol/10^{10} cells at 1.4 mol cholesterol/mol phospholipid. These data were interpreted in terms of a bilayer couple mechanism. Assuming that the two amphipaths acted additively, the amount of lysolecithin required to induce echinocytosis was used to estimate the partition of cholesterol between the two leaflets of the red cell membrane. A value of about 51:49% in favor of the outer leaflet was found at all cholesterol levels.—**Lange, Y., and J. M. Slayton.** Interaction of cholesterol and lysophosphatidylcholine in determining red cell shape. *J. Lipid Res.* 1982. **23:** 1121–1127.

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The dramatic effect of intercalated amphipathic compounds on red cell shape (1) was rationalized by Sheetz and Singer (2) in terms of the asymmetric distribution of anionic phospholipids between the two sides of the bilayer (3, 4). They proposed in their bilayer couple hypothesis that charged amphipaths will partition so as to minimize electrostatic free energy and that the preferential intercalation of a compound into one side of the bilayer will expand its area relative to that of the other side, altering membrane curvature and hence cell shape. The hypothesis suggests that the anion, 2,4-dinitrophenolate causes red cells to become echinocytic (spiculated) by expanding the outer (nearly neutral) leaflet, whereas chlorpromazine, a cation, induces stomatocytosis (invagination) by expanding the inner, anionic leaflet. However, Conrad and Singer (5) recently reported that these compounds are not incorporated into red cell membranes in measurable amounts, raising doubts as to the mechanism of their effect on cell shape.

Because of the uncertainty associated with the application of charged, exogenous amphipaths, we felt it important to evaluate the bilayer couple hypothesis in terms of two neutral, naturally occurring membrane lipids, cholesterol and lysolecithin, which alter red cell shape and are readily taken up into bilayers in measurable amounts. Mohandas, Greenquist, and Shohet (6) showed that lysolecithin (LPC) crossed the membrane slowly and therefore could be introduced preferentially into either leaflet of the bilayer so as to induce echinocytosis or stomatocytosis. Our own recent studies (7) and those of Chailley, Giraud, and Claret (8) suggested that cholesterol biases red cell membranes toward evagination or outward curvature. We report here that these two physiological compounds, lysolecithin and cholesterol, modulate red cell shape in an interdependent fashion consistent with a bilayer couple mechanism. Furthermore, we present a quantitative analysis demonstrating that cholesterol equilibrates so as to favor the outer leaflet by a small margin.

MATERIALS

Lyso-[palmitoyl-1-^{14}C]phosphatidylcholine (sp act 51.0 mCi/mmol) and egg lysophosphatidylcholine were purchased from New England Nuclear (Boston, MA) and Sigma (St. Louis, MO), respectively. Defatted bovine serum albumin was obtained from Sigma.

METHODS

Fresh blood from healthy human donors was collected in 10 mM EDTA. All manipulations were at 0–

Abbreviations: LPC, lysophosphatidylcholine; RBC, red blood cells; C/P, mol cholesterol/mol phospholipid.

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5°C unless noted. Plasma and buffy coat were separated from the cells after a 5-min centrifugation at 3,000 *g*. The cells were washed three times in 10 vol of 150 mM NaCl–5 mM Na phosphate (pH 7.5). Enrichment and depletion of erythrocyte cholesterol using sonicated liposomes were as described by Lange, Cutler, and Steck (7), except that 0.1% albumin was present in the buffer and after incubation with the liposomes the red cells were given an additional wash in 20 vol of buffer containing 1% albumin to remove any lysophospholipid that may have accumulated in the membrane during the incubation. The cells then were washed twice more in 20 vols of 150 mM NaCl–5 mM Na phosphate (pH 7.5) to remove the albumin. Membrane cholesterol content was determined in lipids extracted from ghosts as the molar ratio of cholesterol to phospholipid (7). Red cell concentration was determined in a Coulter Counter.

Determination of lysophosphatidylcholine uptake by red cells

Red cells were incubated for 15 min at 37°C in 19 vol of 150 mM NaCl–5 mM Na phosphate (pH 7.5) containing different amounts of lysolecithin and albumin and 0.025–0.05 μCi of [^{14}C]LPC/ml. Aliquots of the mixture were taken for the determination of total radioactivity and for microscopy. Additional aliquots were centrifuged in an Eppendorf microfuge to pellet the cells. The cells were washed three times in 15 vol of 150 mM NaCl–5 mM Na phosphate (pH 7.5) and resuspended in 19 vol of the same buffer. Aliquots of this mixture were taken for the determination of cell number and radioactivity incorporated into the red cells. Aquasol (New England Nuclear) was used as counting fluid. Identical volumes of red cells (3–4 μl) were counted for the determination of red cell radioactivity and total radioactivity so that quench corrections were not necessary. Furthermore, this small volume of cells did not give significant quenching as demonstrated in experiments using Protosol (New England Nuclear) and H_2O_2 to decolorize the cells and by internal standardization. Data were expressed as uptake of radiolabeled lysolecithin, ignoring the contribution of endogenous lysolecithin which was minimal due to prior washing of the cells with albumin.

The transbilayer distribution of lysolecithin was determined by washing the erythrocytes with buffer containing 1% albumin, as described by Mohandas et al. (6). At the end of the experimental manipulations, the red cell lipids were extracted by the procedure of Folch, Lees, and Sloane Stanley (9). One-dimensional thin-layer chromatography of the extracted lipids was performed using chloroform–methanol–acetic acid–water 25:15:4:2 (v/v) and the spots were visualized by iodine vapor. The zones corresponding to lysolecithin and phosphatidylcholine standards were collected by scraping the plates, and the radioactivity was determined by liquid scintillation counting.

Red cell morphology

Red cells were fixed in 1% glutaraldehyde, washed twice in deionized water, spread on clean glass slides, and air dried at room temperature. The smears were coated in an evaporator with gold–palladium 60:40 and photographed at a magnification of 2000 with a JEOLCO TSM-U3 scanning electron microscope, operated at 25 kv with the stage at a 20° angle.

RESULTS

Uptake of lysolecithin by red cells

Preliminary to our study of the interplay between two blood lipids in affecting red cell shape, we examined the influence of red cell cholesterol on the uptake of added [^{14}C]lysolecithin. Red cells rapidly took up the lysolecithin from incubation buffers; uptake was completed within 5 min at 37°C. The amount of lysolecithin taken up was varied either by changing its concentration in the buffer or by adding different amounts of albumin to the buffer as a sink. At constant buffer lysolecithin concentration, the amount of lysolecithin taken up by the cells decreased 30-fold as the buffer albumin concentration increased from 0 to 0.5% (**Fig. 1A**).

It has been shown that lysophosphatidylcholine introduced at the outer surface of intact erythrocytes is translocated slowly across the bilayer to the inner leaflet where it becomes acylated to phosphatidylcholine (6, 10). However, during the brief duration of our experiments, more than 95% of the lysolecithin was still located at the outer membrane surface and more than 95% of the radiolabel comigrated with lysolecithin in thin-layer chromatograms. Therefore, our findings refer to red cells enriched in lysolecithin exclusively at the outer surface.

Red cells depleted of cholesterol took up the same amount of lysolecithin as did control cells (Fig. 1A). In marked contrast, cholesterol-enriched cells took up significantly more lysolecithin at all albumin concentrations (Fig. 1B). A similar result was obtained when lysolecithin was varied at constant albumin concentration (not shown). Enriched cells typically took up 1.4–1.6 times more lysolecithin than control cells.

The uptake of lysolecithin by red cells enriched to different degrees was determined (**Fig. 2**). These data showed that enrichment to C/P ~ 1.0 led to an increase in the amount of lysolecithin taken up by the cells. Further enrichment to C/P = 1.24 did not cause any ad-

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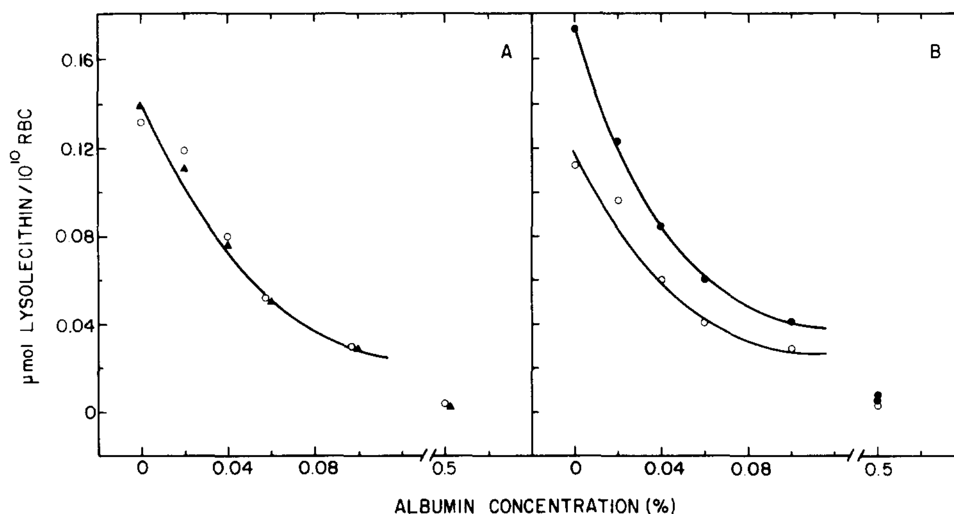

Fig. 1. Uptake of lysolecithin by red cells of modified cholesterol content at different buffer albumin concentrations. Red cells were incubated for 15 min at 37° in 19 vol 150 mM NaCl–5 mM Na phosphate (pH 7.5) containing [^{14}C]lysolecithin with 8 μM unlabeled compound and 0–0.5% albumin. Aliquots were taken for the determination of uptake as described in Methods. Errors in uptake measurement as assessed by the range of duplicate determinations were within 5%. Panel A: Comparison of uptake in control (○) and depleted (▲) cells with cholesterol contents of 0.86 and 0.54 mol cholesterol/mol phospholipid, respectively. Panel B: Comparison of uptake in control (○) and enriched (●) cells with cholesterol contents of 0.86 and 1.24 mol cholesterol/mol phospholipid, respectively.

ditional increment in uptake. These data, together with those illustrated in Fig. 1, show that the capacity of the red cell membrane for lysolecithin abruptly shifts as its cholesterol content reaches C/P ~ 1.0.

Effect of cholesterol and lysolecithin on cell shape

The morphology of erythrocytes of different cholesterol and lysolecithin content was examined. Incubation of normal erythrocytes with lysolecithin converted the discocytes to echinocytes (**Fig. 3,** panels A and B). Depletion of red cell cholesterol rendered normal cells stomatocytic (pitted and cupped) (Fig. 3, panel D). Cells that had been both depleted of cholesterol and treated with lysolecithin were discocytes (Fig. 3, panel E).

A quantitative analysis of the impact of these two lipids on cell shape was performed. Cells with C/P ~ 0.6 were cup-shaped (not shown, but cf. ref. 8), and cells with C/P ~ 0.5 exhibited pits or indentations as well as cupping (Fig. 3, panel D). When cholesterol-depleted cells were made to a concentration of 0.076 μmol LPC/10^{10} RBC, they were not echinocytic (Fig. 3, panel E), but control red cells at the same lysolecithin content were (Fig. 3, panel B). The amount of lysolecithin uptake that induced 50% echinocytosis was 0.14 μmol/10^{10} RBC in depleted cells and 0.080 μmol/10^{10} RBC in control cells (**Table 1,** Experiment 2).

Red cells enriched in cholesterol to C/P ~ 1.2 were somewhat enlarged and flattened in the absence of lysolecithin (not shown but see refs. 11 and 12). These cells became echinocytic at a lysolecithin content of 0.085 μmol/10^{10} RBC (Fig. 3, panel F) whereas control cells still were discocytes with 0.099 μmol/10^{10} RBC (Fig. 3, panel C).

Note that in one experiment (Fig. 3, panel B) control cells were echinocytic at a lysolecithin content that left control cells in a different experiment discocytic (Fig. 3, panel C). The sensitivity of the cells to echinocytosis

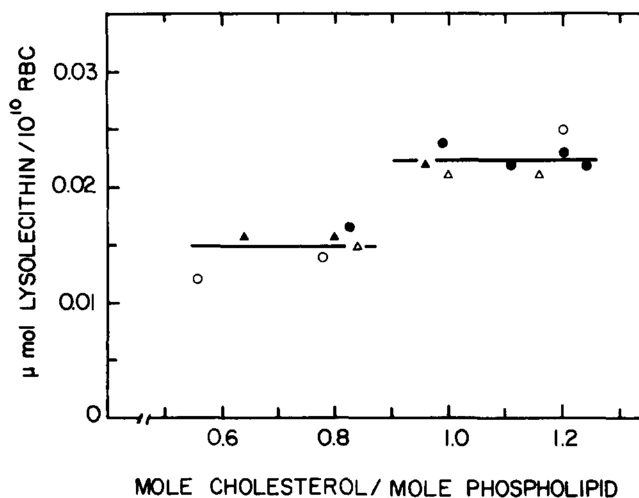

Fig. 2. Uptake of lysolecithin by red cells as a function of membrane cholesterol content. Red cells were enriched or depleted of cholesterol by incubation with sonicated liposomes for 5–16 hr (see Methods). Lysolecithin uptake was measured as described in the legend to Fig. 1, except that the buffer contained 0.05% albumin and 3 μM lysolecithin. Data from four experiments are given, each symbol referring to an experiment.

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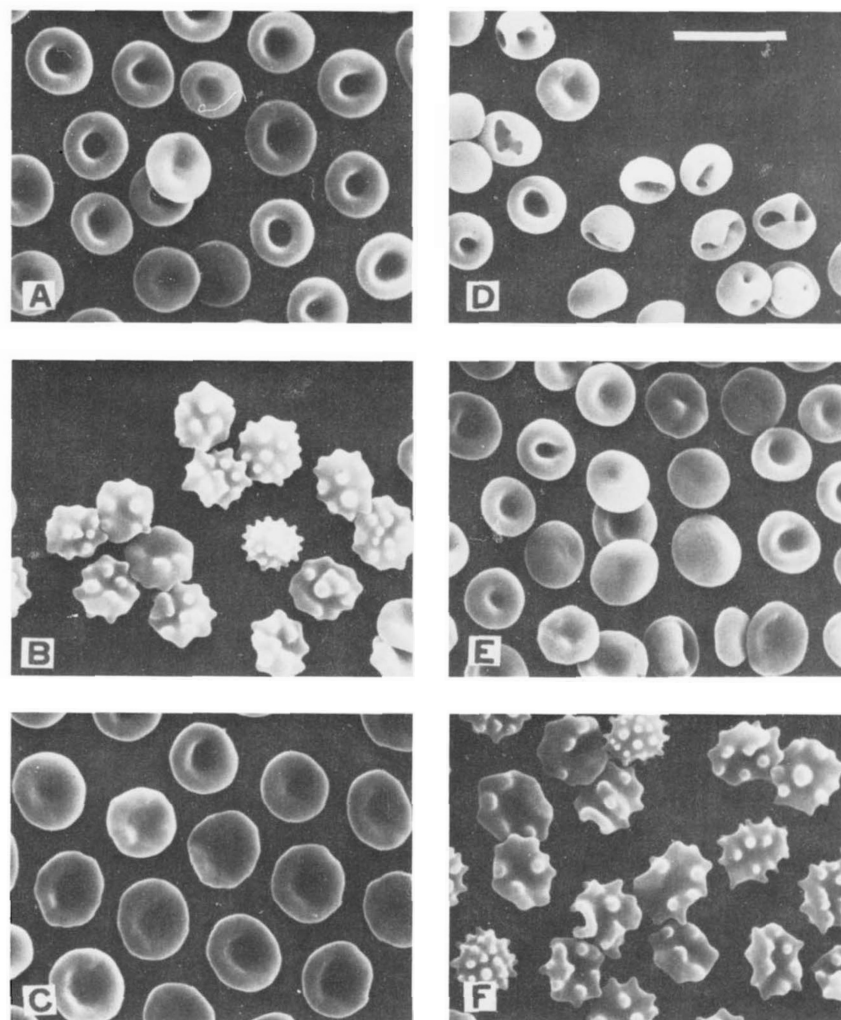

Fig. 3. Scanning electron micrographs of lysolecithin-treated red cells of modified cholesterol content. Enriched, depleted, and control red cells from the experiments illustrated in Fig. 1 were photographed. Control red cells (cholesterol/phospholipid mole ratio = 0.86) in the absence of lysolecithin (LPC) (Panel A) and with 0.08 μmol LPC/10^{10} RBC (Panel B) are compared with depleted cells (cholesterol/phospholipid mole ratio = 0.54) in the absence of added lysolecithin (Panel D) and with 0.076 μmol LPC/10^{10} RBC (Panel E). In a separate experiment, control red cells (cholesterol/phospholipid mole ratio = 0.86) with 0.099 μmol LPC/10^{10} RBC (Panel C) are compared with enriched red cells (cholesterol/phospholipid mole ratio = 1.24) with 0.084 μmol LPC/10^{10} RBC (Panel F). Calibration bar = 10 μm.

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by lysolecithin thus varied among experiments. Nevertheless, whenever two preparations were compared in the same experiment, cells that had more cholesterol invariably required less lysolecithin for echinocytosis and vice versa (Table 1).

The lysolecithin content of red cell membranes has been estimated as 1–2% of total phospholipids (13). Insofar as it affects cell shape, this endogenous lysolecithin can be considered to be in the same pool as other membrane phospholipids and therefore need not be accounted for in the interpretation of our data. To prevent possible lysolecithin incorporation during treatment of red cells with sonicated liposomes, serum albumin was included in the buffer. Furthermore, prior to shape studies, the cells were washed with buffer containing albumin, a treatment which has been shown to remove lysolecithin from red cell membranes (6).

DISCUSSION

This study makes two points. The first is that the level of membrane cholesterol has a distinctive influence on the incorporation of lysolecithin: an abrupt transition is observed at C/P ~ 1.0. This effect is unlikely to reflect a specific interaction between cholesterol and the lysophospholipid since uptake is independent of cholesterol content both below C/P = 0.9 and above unity

TABLE 1. Lysolecithin incorporation required to induce echinocytosis in red cells of modified cholesterol content

Expt	C/P	Cholesterol (Molecules per Cell × 10^{-6})	LPC μmol/10^{10} RBC	LPC (Molecules per cell × 10^{-6})	Cholesterol Equivalents (Molecules per cell × 10^{-6})	Calculated Cholesterol Distribution Outer/Inner
1	0.86	189	0.08	5	3	1.03
	0.54	118	0.14	8		
2	0.83	182	0.05	3	4	1.04
	0.62	136	0.12	7		
3	0.82	180	0.09	5		
	1.18	259	0.05	3	2	1.02
4	0.86	188	0.11	7		
	1.24	272	0.04	2.5	4	1.04
5	0.84	184	0.10	6		
	1.38	303	0.03	2	4	1.04

Red cells were enriched or depleted of cholesterol and the lysolecithin uptake that resulted in 50% echinocytosis was determined. From the differential in the number of lysolecithin molecules taken up by control and modified cells, the equivalent number of cholesterol molecules was calculated as described in the text. Red cell cholesterol, C/P (mol/mol phosholipid), was expressed as number of cholesterol molecules per cell, calculated from the standard value of 3.66 mol phospholipid/10^{10} RBC.

(Fig. 2). The sharp change in lysolecithin uptake occurring at a membrane cholesterol content of C/P = 0.9–1.0 is reminiscent of other abrupt changes in membrane properties in this region. The susceptibility of red cell cholesterol to oxidation by cholesterol oxidase and the orientation (sidedness) of vesicles from isolated red cell membranes both exhibited sharp transitions at C/P = 0.9–1.0 (7). The data presented here provide further evidence for a change in membrane organization at this cholesterol content.

The major point of this study deals with cell shape. The determinants of the characteristic biconcave shape of the mature human erythrocyte are unknown but both the lipid bilayer and a submembrane reticulum of filamentous proteins have been implicated (14–16). Since the orientation of vesicles derived from isolated cell membranes depends strongly on their cholesterol content, we have proposed that cholesterol biases membrane contour in the outward direction (7). The observation that cholesterol depletion induces a discocyte to stomatocyte transition (Fig. 3, panel D and ref. 8) adds support to our hypothesis that cholesterol constrains the membrane against invagination.

It has been suggested that the effect of cholesterol depletion on red cell shape is mediated through the action of a membrane-bound kinase (8). However, cholesterol enrichment biases membrane curvature even in ghosts where the kinase is not operative (7). Moreover, the effect of lysolecithin on cell shape is essentially instantaneous and hence is unlikely to be a metabolic effect. Therefore, we prefer a structural hypothesis and interpret the effects of lysolecithin and cholesterol on erythrocyte morphology in terms of a bilayer couple mechanism.

The echinocytic shape of lysolecithin-treated red cells has been attributed to an expansion of the external leaflet of the bilayer caused by preferential uptake of the compound in that membrane leaflet (6). The finding that the threshold lysolecithin concentration for echinocytosis depends on the cholesterol content of the cells suggests that its action depends on the other lipids present in the membrane, in keeping with a bilayer couple mechanism. In particular, the present data suggest that lysolecithin and cholesterol act additively in these experiments to determine shape, both differentially expanding the outer surface of the bilayer.

Our measurement of the incorporation of [^{14}C]lysolecithin into red cells permits a quantitative test of the bilayer couple mechanism. We have shown that all of the [^{14}C]lysolecithin remains confined to the outer surface of the membrane (see also ref. 6). The differential expansion of that surface may be simply estimated, provided no membrane molecules redistribute, as the product of the number of lysolecithin molecules incorporated times the cross-sectional area of that molecule. The cross-sectional area of a lysolecithin molecule in the red cell membrane is not known. However, it has been shown that the surface area available to one lysolecithin molecule and one cholesterol molecule in hydrated lamellar structures varies from 60–85 Å^2 (17). Assuming that the cholesterol molecule has a fixed area of about 39 Å^2 (18, 19), the area occupied by lysolecithin is between 21 and 46 Å^2. In the following calculations, the mean value of 34Å^2 will be assumed for the

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area of a lysolecithin molecule. At 0.08×10^{-10} μmol/cell (Table 1), this area amounts to 1.6 μm^2 which is 1.1% of the cell surface area of 140 μm^2. Beck (20) has calculated on geometric grounds that echinocytosis results from an excess of area at the outer leaflet of 0.52 μm^2. This close agreement provides strong support for the bilayer couple hypothesis.

Unlike lysolecithin (6), cholesterol equilibrates within seconds across the human erythrocyte membrane (21). If its effect on cell shape is through a bilayer couple mechanism, its equilibrium distribution across the membrane must be somewhat asymmetric. On the other hand, cholesterol content can be increased considerably without much effect on cell shape (11, 12), suggesting that the transmembrane partition could not be very far from uniform.

We have used the data obtained with [^{14}C]lysolecithin to estimate this cholesterol distribution as follows (Table 1). When the number of cholesterol molecules/cell was reduced from 189×10^6 to 118×10^6, an additional 3×10^6 lysolecithin molecules per cell were needed to cause the same degree of echinocytosis (Experiment 1). This amount of lysolecithin corresponds in area to 2.6 $\times 10^6$ molecules of cholesterol (1% of cell cholesterol), assuming the cross-sectional area of 39 Å^2 for the sterol. If the area of the additional lysolecithin needed for echinocytosis was exactly that lost by the reduction of cholesterol, it can be calculated that the outer leaflet in the normal cell contained approximately 3% more cholesterol than the inner leaflet, or contributed 51% of total membrane cholesterol. Experiment 2 similarly suggests that the outer leaflet in normal cells contributed 51% of total membrane cholesterol. Confirmation of this value comes from cholesterol enrichment studies, e.g., Experiment 5. In this case, the amount of lysolecithin required for a similar degree of echinocytosis was reduced by an equivalent of 4×10^6 molecules/cell by the extra cholesterol. If this effect were due to an asymmetric distribution of cholesterol, its partition between outer and inner leaflets in cholesterol-enriched cells would also be about 51:49%. The precise asymmetry estimated in this manner clearly depends on the values assumed for the surface areas of cholesterol and lysolecithin in the membrane. However, within a reasonable range of these parameters, the data indicate an excess of cholesterol of about 1–2% in the outer membrane leaflet. Of course, if cholesterol redistributes as a result of lysolecithin insertion in the outer leaflet, the asymmetry of cholesterol would be even less. In any case, our data indicate that there is a slight excess of cholesterol in the outer membrane leaflet of both normal and cholesterol-enriched red cells.

Finally, it is important to note that in these studies lysolecithin, like the other membrane phospholipids, is kinetically trapped at the surface of insertion and therefore the introduction of small amounts of this compound can have a major impact on cell shape which may however eventually be dissipated by transbilayer equilibration (6). In contrast, cholesterol moves rapidly between the two bilayer leaflets (21) and exerts its effect through its unequal equilibrium distribution. This feature may allow cholesterol to buffer cell shape (21).

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