

# Erythrocyte Morphology Reflects the Transbilayer Distribution of Incorporated Phospholipids

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**Abstract.** The transbilayer distribution of exogenous phospholipids incorporated into human erythrocytes is monitored through cell morphology changes and by the extraction of incorporated  $^{14}\text{C}$ -labeled lipids. Dilauroylphosphatidylserine (DLPS) and dilauroylphosphatidylcholine (DLPC) transfer spontaneously from sonicated unilamellar vesicles to erythrocytes, inducing a discocyte-to-echinocyte shape change within 5 min. DLPC-induced echinocytes revert slowly ( $t_{1/2} \sim 8$  h) to discocytes, but DLPS-treated cells revert rapidly (10–20 min) to discocytes and then become invaginate stomatocytes. The second phase of the phosphatidylserine (PS)-induced shape change, conversion of echinocytes to stomatocytes, can be inhibited by blocking cell protein sulfhydryl groups or by depleting intracellular ATP or magnesium (Daleke, D. L., and W. H. Huestis. 1985. *Biochemistry*. 24:5406–5416). These cell shape changes are consistent with incorporation of phosphatidylcholine (PC) and PS into the membrane outer monolayer followed by selective and energy-dependent translocation of PS to the membrane inner monolayer. This hypothesis is explored by correlating cell shape with the fraction of the exogenous

lipid accessible to extraction into phospholipid vesicles. Upon exposure to recipient vesicles, DLPC-induced echinocytes revert to discoid forms within 5 min, concomitant with the removal of most (88%) of the radiolabeled lipid. On further incubation, 97% of the foreign PC transfers to recipient vesicles. Treatment of DLPS-induced stomatocytes with acceptor vesicles extracts foreign PS only partially (22%) and does not affect cell shape significantly. Cells treated with inhibitors of aminophospholipid translocation (sulfhydryl blockers or intracellular magnesium depletion) and then incubated with either DLPS or DLPC become echinocytic and do not revert to discocytic or stomatocytic shape for many hours. On treatment with recipient vesicles, these echinocytes revert to discocytes in both cases, with concomitant extraction of 88–99% of radiolabeled PC and 86–97% of radiolabeled PS. The accessibility of exogenous lipids to extraction is uniformly consistent with the transbilayer lipid distribution inferred from cell shape changes, indicating that red cell morphology is an accurate and sensitive reporter of the transbilayer partitioning of incorporated exogenous phospholipids.

THE normal discoid shape of the human erythrocyte can be altered by the addition of certain amphipathic compounds (Deuticke, 1968; Mohandas and Feo, 1975; Fuji et al., 1979). The resulting cell shapes are dependent on the net charge of the amphipath. Neutral or negatively charged amphipaths cause discoid cells to become spiky, evaginate echinocytes, while positively charged amphipaths generate rounded, invaginated stomatocytes. Sheetz and Singer (1974) proposed that the red cell membrane behaves as a bilayer couple; selective intercalation of the agents into the outer or inner monolayer of the membrane results in expansion of one monolayer relative to the other, inducing the observed shape changes. Amphipaths that produce echinocytes associate preferentially with the outer monolayer, probably as a result of their inability to cross the bilayer, or because

of charge repulsion by negatively charged inner monolayer lipids. Conversely, those compounds that induce stomatocytosis do so by partitioning selectively into the cell inner monolayer, perhaps by association with inner monolayer lipids or proteins.

Phospholipids having short ( $<C_{15}$ ) acyl chains exchange rapidly between lipid bilayers, and can be introduced into cells by incubation with vesicles of appropriate compositions (Fuji and Tamura, 1983; Fuji et al., 1985; Ferrell et al., 1985*b*; Daleke and Huestis, 1985). Upon incorporation into erythrocytes, exogenous phospholipids effect shape changes that depend on their head group composition (Ferrell et al., 1985*a*; Daleke and Huestis, 1985). Phosphatidylcholine (PC)<sup>1</sup> induces echinocytosis, concurrent with uptake of the

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1. *Abbreviations used in this paper:* CDTA, trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; DLPC, dilauroylphosphatidylcholine;  $^{14}\text{C}$ -DLPC, 1-lauroyl-2-[1- $^{14}\text{C}$ ]-lauroylphosphatidylcholine;  $^{14}\text{C}$ -DLPS, 1-lauroyl-2-[1- $^{14}\text{C}$ ]-lauroylphosphatidylserine;  $^{14}\text{C}$ -DOPC, 1,2-di-[1- $^{14}\text{C}$ ]-oleo-

exogenous lipid. This crenated shape is stable for many hours and is followed by a slow reversion to discoid forms. This shape reversion, generally attributed to transbilayer equilibration of the lipid, occurs at rates that depend on the lipid acyl chain length (Fuji and Tamura, 1983; Fuji et al., 1985; Tamura et al., 1986; Middelkoop et al., 1986). In contrast, cells incubated with phosphatidylserine (PS) vesicles crenate only transiently, converting rapidly to discocytic and then to indented, stomatocytic shapes (Daleke and Huestis, 1985). This shape reversion is independent of PS acyl chain length (Daleke and Huestis, 1985). The bilayer couple model for cell morphology would interpret these observations as evidence that, in the short term, exogenously introduced PCs remain in the cell membrane outer monolayer, while aminophospholipids accumulate in the membrane inner monolayer.

If this inference from cell morphology is correct, the transbilayer distribution of exogenous phospholipids parallels that of endogenous phospholipids. In the human erythrocyte membrane, the choline phospholipids, PC and sphingomyelin, are found predominantly in the outer phospholipid leaflet, while the aminophospholipids, phosphatidylethanolamine and PS, reside largely or exclusively in the inner leaflet (Bretscher, 1972*a,b*; Gordesky and Marinetti, 1973; Verkleij et al., 1973; Zwaal et al., 1975). Direct and indirect techniques have demonstrated that asymmetric lipid distribution is maintained in part in erythrocytes (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985; Zachowski et al., 1986; Tilley et al., 1986), platelets (Daleke and Huestis, 1985; Sune et al., 1987; Bevers et al., 1982; also, our unpublished observations), pig lymphocytes (Zachowski et al., 1987), and hamster fibroblasts (Martin and Pagano, 1987) by a  $Mg^{+2}$ - and ATP-dependent, head group-specific protein that translocates aminophospholipids from the outer to the inner membrane monolayer. Support for this mechanism has been inferred from the accessibility of incorporated spin-labeled analogues to reduction (Seigneuret and Devaux, 1984), radiolabeled lipids to phospholipase hydrolysis (Tilley et al., 1986), fluorescent lipids to extraction (Martin and Pagano, 1987), and from the morphological response of human erythrocytes and platelets to spontaneously incorporated exogenous phospholipids (Daleke and Huestis, 1985).

If the interpretation of the morphological effects of exogenous phospholipids is correct, foreign PS is a substrate for the aminophospholipid translocator, and cell shape changes in response to its incorporation provide a convenient, continuous monitor of transport activity and asymmetry maintenance. However, the approach is indirect and model dependent, and it cannot yield quantitative information about transport events until a correlation between lipid distribution and cell morphology is demonstrated.

This investigation was undertaken to examine the validity of morphology-based assays of transbilayer lipid distribution and to quantify the amount of lipid required to produce stomatocytic morphology in erythrocytes. Cell morphology changes induced by dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylserine (DLPS) are correlated with

their transbilayer distribution by extracting  $^{14}C$ -labeled lipids from the cell outer monolayer into exogenous vesicles. Lipid distributions inferred from cell morphology are compared with lipid accessibility to phospholipase treatments. The results indicate that the effects that short, saturated acyl chain phospholipids exert on cell morphology accurately reflect the transbilayer distribution of the incorporated lipids.

## Materials and Methods

### Materials

DLCP, dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylglycerol (DPPG), 1-lauroyl-lysophosphatidylcholine, bee venom phospholipase  $A_2$ , cabbage phospholipase D (type V), and *Staphylococcus aureus* sphingomyelinase C were obtained from Sigma Chemical Co. (St. Louis, MO). Dimyristoylphosphatidylserine (DMPS) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). [ $^{14}C$ ]-Lauric acid (56 Ci/mol) and 1,2-di- $[^{14}C]$ -oleoylphosphatidylcholine ( $^{14}C$ -DOPC, 112 Ci/mol) were purchased from Amersham Corp. (Arlington Heights, IL). The ionophore A23187 was purchased from Calbiochem-Behring Corp. (San Diego, CA). The scintillation cocktail Aquasol was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade.

1-Lauroyl-2- $[^{14}C]$ -lauroylphosphatidylcholine ( $^{14}C$ -DLPC, 16.3 Ci/mol) was synthesized from 1-lauroyl-lysophosphatidylcholine and  $^{14}C$ -lauric acid either by the method of Warner and Benson (1977) or Montecucco and Schiavo (1986). 1-Lauroyl-2- $[^{14}C]$ -lauroylphosphatidylserine ( $^{14}C$ -DLPS, 7.2 Ci/mol) was synthesized by phospholipase D-catalyzed head group exchange of  $^{14}C$ -DLPC with L-serine (Comfurius and Zwaal, 1977). Synthesized lipids were purified by CM-52 cellulose (Whatman Inc., Clifton, NJ) chromatography (as described in Comfurius and Zwaal, 1977).

### Cells

Human erythrocytes were obtained from adult volunteers by venipuncture and collected into EDTA or citrate. Erythrocytes were pelleted by centrifugation (5,000 g, 5 min), washed three times with 4 vol of 150 mM NaCl, and washed once with 4 vol of 138 mM NaCl, 5 mM KCl, 6.1 mM  $Na_2HPO_4$ , 1.4 mM  $NaH_2PO_4$ , 5 mM glucose, pH 7.4 (NaCl/P<sub>i</sub>). Cells were used within 6 h of isolation.

### Vesicle Preparation

Unilamellar vesicles were prepared by sonication under argon, using a bath sonicator heated to  $>10^\circ$  above the gel-to-liquid crystalline phase transition temperature of the lipid. Lipid suspensions in NaCl/P<sub>i</sub> (concentrations as noted in the text) were sonicated until clear and used immediately. Lipid mixtures were prepared in chloroform solution; solvent was evaporated under a nitrogen stream and lipids were resuspended and sonicated in NaCl/P<sub>i</sub>.

### Cell-Vesicle Incubations

Sonicated vesicles were incubated with cells at 50% hematocrit,  $37^\circ C$ , for the time intervals specified in the figure legends. Aliquots of cells were separated from supernatant vesicles by centrifugation (8,800 g, 2.5 min) and washed twice by resuspension in NaCl/P<sub>i</sub> and centrifugation.

Sonicated DLPS or DLPC vesicles labeled with  $^{14}C$ -DLPS or  $^{14}C$ -DLPC, respectively, were incubated with cells as described above. Radio-label in aliquots of the supernatant and the washed cell pellet was assessed by liquid scintillation counting.

### Inhibition of Aminophospholipid Translocation

Modification of protein sulfhydryl groups or cytosolic magnesium depletion inhibits aminophospholipid translocation (Daleke and Huestis, 1985). The effects of these treatments on cell shape changes and phospholipid distribution were examined. Sulfhydryl group modification was accomplished by treating cells with 5 mM diamide or 2 mM *N*-ethylmaleimide (NEM) (oxidizing and alkylating agents, respectively) in NaCl/P<sub>i</sub> at 20% hematocrit (HCT) for 1 h. In some experiments, as indicated in figure legends, cells were treated with 1 mM NEM in NaCl/P<sub>i</sub> at 10% HCT for 15 min. To ac-

phosphatidylcholine; DLPS, dilauroylphosphatidylserine; DMPS, dimyristoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; HCT, hematocrit; MI, morphological index; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine; PS, phosphatidylserine; SA, surface area.

comply magnesium depletion, cells were suspended at 20% HCT in 140 mM KCl, 5 mM NaCl, 6.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM glucose, pH 7.4 (KCl/P<sub>i</sub>) containing 1 mM trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) or 1 mM EDTA, warmed to 37°C, and treated with 5 μM A23187 for 15 min. Cells were pelleted by centrifugation (8,800 g, 2.5 min), washed three times, and then treated with vesicles as above. Magnesium-depleted cells were treated with vesicle suspensions in KCl/P<sub>i</sub> supplemented with 1 mM EDTA or 1 mM CDTA.

### Extraction of Incorporated DLPS or DLPC

The extent of extraction of transferrable lipid from cells by exogenous vesicles depends on the relative surface areas (SAs) of cells and vesicles in a given suspension. The total surface area of a suspension of red cells (SA<sub>cell</sub>) at a particular HCT can be calculated from the volume and surface area of a discocyte (94 fl and 135 μm<sup>2</sup>, respectively; Wintrobe, 1981):

$$SA_{\text{cell}} L^{-1} = (1.35 \times 10^{13} \mu\text{m}^2 L^{-1}) \times \text{HCT}\%. \quad (1)$$

The total surface area of a sonicated vesicle suspension (SA<sub>ves</sub>) can be calculated from the vesicle lipid cross-sectional area (0.6 nm<sup>2</sup>/PC molecule; Tanford, 1980), the fraction of outer monolayer lipid per vesicle (0.735, calculated for a 25-nm vesicle with a 5-nm bilayer thickness; Huang, 1969), and the vesicle lipid concentration ([lipid]):

$$SA_{\text{ves}} L^{-1} = (3.61 \times 10^{14} \mu\text{m}^2 \text{mmol}^{-1}) \times [\text{lipid}] \text{ mM}. \quad (2)$$

The fractional SAs are given by:

$$SA_{\text{cell}}/SA_{\text{total}} = \text{HCT}\% / (\text{HCT}\% + [26.7 \times \{\text{lipid}\} \text{ mM}]) \quad (3)$$

and

$$SA_{\text{ves}}/SA_{\text{total}} = [\text{lipid}] \text{ mM} / ([\text{lipid}] \text{ mM} + [0.0374 \times \text{HCT}\%]). \quad (4)$$

Equations 3 and 4 can be used to determine conditions for maximal extraction of transferable lipid from cells by vesicles. Assuming the transferable lipid present in the cell membrane outer monolayer is capable of equilibrating with the outer monolayer of the vesicle fraction and that the on and off rates for lipid transfer are not significantly different for the two membranes, the relative surface areas represent the maximal amount of extraction of cell outer monolayer transferable lipid into recipient vesicles. A high vesicle-to-cell surface area ratio can be attained by using low cell concentrations or high vesicle lipid concentrations. Sonicated vesicle concentrations of 20 mM DOPC are easily achieved, and require a cell concentration of 5% HCT to achieve >99% of the total suspension SA in the vesicle fraction.

Cells containing DLPC or DLPS incorporated as described above were incubated with 20 mM DOPC/DPPG (97:3) vesicles at 5% HCT, 37°C. (DOPC does not transfer at appreciable rates and therefore is a passive recipient for shorter chain phospholipids. Inclusion of small amounts of DPPG in the DOPC vesicles impedes red cell-vesicle and vesicle-vesicle aggregation and aids in the separation of vesicles from cells [Newton, 1986].) In parallel experiments, control cells were incubated with 20 mM DOPC/DPPG (97:3) vesicles containing trace <sup>14</sup>C-DOPC. After the time intervals indicated in the figure legends, aliquots of suspension were separated by centrifugation. Supernatants were assayed for radioactivity, and aliquots of cells were fixed for morphological analysis.

### Cell-to-Cell Transfer of DLPS

Erythrocytes were incubated in NaCl/P<sub>i</sub> with or without 5 mM diamide (an inhibitor of aminophospholipid translocation; Daleke and Huestis, 1985) for 1 h, washed by centrifugation and resuspension, and incubated with 0.2 mM DLPS for 45 min. The cells were washed, resuspended at 50% HCT, and added to an equal volume of untreated discocytes (also at 50% HCT). The cell mixture was incubated at 37°C, and, after the times indicated in the figure legends, aliquots were removed and fixed for morphological analysis.

### Phospholipase Treatments

Cells were treated with 1 mM NEM (10% HCT for 15 min at 37°C), or with 1 mM CDTA + 5 μM A23187 in KCl/P<sub>i</sub> (20% HCT for 5 min at 37°C), as described above. Cells were washed and treated with 50 μM <sup>14</sup>C-DLPS at 50% HCT for 45 min (normal cells) or 15 min (NEM or CDTA/A23187-treated cells) at 37°C. (This concentration of <sup>14</sup>C-DLPS does not induce shape changes and was chosen to avoid shape-dependent

alterations in phospholipid accessibility [Daleke, D. L., and W. H. Huestis, unpublished observations].) Cells were washed and suspended at 20% HCT in 10 mM Hepes, 140 mM KCl, 5 mM NaCl, pH 7.4 (Hepes/KCl) containing 0.25 mM CaCl<sub>2</sub>. Phospholipase A<sub>2</sub> (bee venom; 25 IU ml<sup>-1</sup>) was added and the sample was incubated for 1 h at 37°C. Sphingomyelinase C (*S. aureus*; 0.1 IU ml<sup>-1</sup>) was then added, and the incubation continued for another hour. Aliquots were added to 10 vol of 1 mM EDTA, and the cells were pelleted by centrifugation, washed once, and frozen. Frozen cells (15 μl) were lysed in 0.5–1.0 ml of 10 mM Tris, 2 mM EDTA, pH 7.4 (Tris/EDTA), pelleted by centrifugation (8,800 g, 10 min), and washed once. Cell lipids were extracted by vortexing with 0.3 ml (18 vol) of 2:1 methanol/chloroform. Water (0.1 ml, 6 vol) and chloroform (0.1 ml, 6 vol) were added, the organic and aqueous layers were separated by centrifugation, and the isolated organic layer was dried with a nitrogen stream. Lipids were reconstituted in 10 μl chloroform/methanol 4:1 and separated by one-dimensional TLC on silica gel hard layer plates (Analtech Inc., Newark, DE) with 65:25:5 chloroform/methanol/95–97% formic acid (Daleke and Huestis, 1985). Lipid spots were visualized with iodine vapor, and those corresponding to DLPS were scraped from the plates and analyzed for radioactivity. In the TLC system used, short acyl chain, saturated phospholipids migrated at R<sub>f</sub> values different from cell phospholipids. Typical R<sub>f</sub> values for cell phospholipids were as follows: sphingomyelin, 0.18; PC, 0.30; PS, 0.37; phosphatidylethanolamine, 0.46; and fatty acids, 0.82. At the same time, R<sub>f</sub> values for DLPS and DMPS were 0.30 and 0.32, respectively. The percent of <sup>14</sup>C-labeled phospholipid hydrolyzed was determined by comparison with cells treated with <sup>14</sup>C-labeled lipid but not exposed to phospholipases.

### Cell Morphology

Erythrocyte suspensions were prepared for morphological analysis by fixing 5 μl aliquots of 5–50% HCT suspensions in 50 μl of 0.5% glutaraldehyde in NaCl for at least 10 min at room temperature. Samples were analyzed by light microscopy. Echinocytes were assigned scores of +1 to +5 (increasing value denoting more severe crenation, Fig. 1, *a–e*); discocytes were scored 0 (Fig. 1 *f*); and stomatocytes were given scores of –1 to –4 (Fig. 1, *g–j*, Bessis, 1973; Fuji et al., 1979; Ferrell et al., 1985a; Daleke and Huestis, 1985). The average score of a field of 100 cells is defined as the morphological index (MI).

Fixed cells were prepared for scanning EM by washing once with 100 vol of distilled water and freeze- or air-drying 2 μl of a 1% HCT suspension on a glass coverslip. Samples were sputter coated with 15–18-nm gold-palladium before viewing.

### Scintillation Counting

Samples were prepared for liquid scintillation counting by bleaching aliquots for 4–12 h at 60°C in 10 vol of 30% hydrogen peroxide + 0.1 vol of Aquasol. After cooling, samples were suspended in Aquasol and counted with a scintillation counter (model LS-380; Beckman Instruments, Inc., Palo Alto, CA). Counts were corrected to disintegrations per minute with a standard quench curve. In some experiments, the bleaching step was omitted and similar results were obtained after quench correction.

## Results

### DLPS- and DLPC-induced Erythrocyte Morphology Changes

Within 1–2 min of exposure to DLPC or DLPS vesicles, normal discoid erythrocytes (Fig. 2, *a* and *e*) became echinocytic (Fig. 2, *b* and *f*). DLPC-treated cells retained this crenate shape for several hours before slowly (*t*<sub>1/2</sub> ~ 8 h) reverting to discocytes (not shown). DLPS-treated cells spiculated only transiently, reverting rapidly to discocytic (Fig. 2, *c* and *g*) and then stomatocytic shapes (*t*<sub>1/2</sub> ~ 15 min; Fig. 2, *d* and *h*). These cell shape changes can be represented graphically by scoring representative samples on an MI scale (Fig. 1; Bessis, 1973; Fuji et al., 1979; Ferrell et al., 1985a; Daleke and Huestis, 1985).

The time course of erythrocyte shape changes induced by DLPS and DLPC, expressed as the MI, is shown in Fig. 3

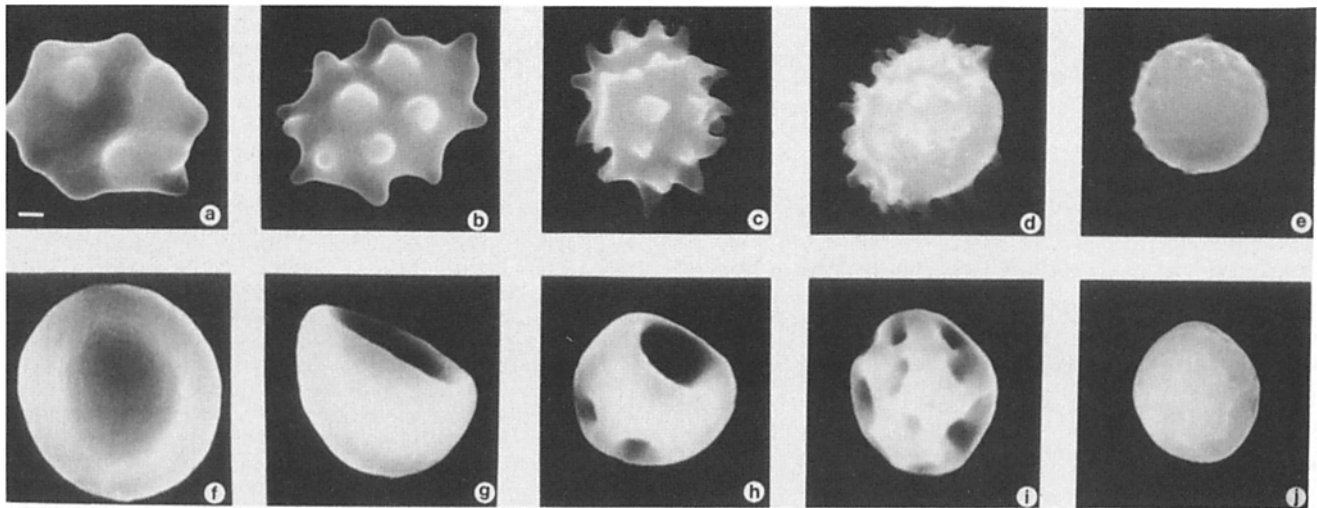


Figure 1. Scanning electron micrographs of a discocyte (f), echinocytes (stage +1 [a], stage +2 [b], stage +3 [c], stage +4 [d], stage +5 [e]), and stomatocytes (stage -1 [g], stage -2 [h], stage -3 [i] and stage -4 [j]). Bar, 1  $\mu\text{m}$ .

A. Cells treated with 0.2 mM DLPC exhibited an MI of +3.2 within 5 min, and retained this morphology for 2 h. Cells treated with 0.25 mM DLPS crenated on the same time scale as DLPC-treated cells but did not reach the same extent of echinocytosis, reverting to an MI of -2.8 within 30 min.

#### Incorporation of $^{14}\text{C}$ -DLPS and $^{14}\text{C}$ -DLPC

The incorporation of DLPS and DLPC into cells was monitored using the corresponding  $^{14}\text{C}$ -labeled lipids. As shown in Fig. 3 B, DLPC and DLPS were incorporated into erythrocytes at similar rates, on a time scale similar to the observed echinocytosis. For DLPC, the time courses of radiolabeled lipid incorporation and shape change were identical, while only 75% of maximal DLPS incorporation had occurred in cells at the peak of the crenation phase (2 min). Incorporation of  $^{14}\text{C}$ -DLPS continued as the cells reverted to discocytes and stomatocytes, reaching completion at 15 min, just before completion of the shape change. In both cases, >97% of the  $^{14}\text{C}$ -lipid became associated with the cells within 15 min.

The extent of DLPS- and DLPC-induced cell shape changes increased with increasing amount of incorporated lipid (Fuji and Tamura, 1983; Fuji et al., 1985; Ferrell et al., 1985a; Daleke and Huestis, 1985). Cells were incubated for 45 min with vesicles at various lipid concentrations (0.01–0.5 mM), and the amount of  $^{14}\text{C}$ -labeled DLPS or DLPC incorporated into cells and the resultant MI were measured. The MI varied approximately linearly with incorporated foreign lipid in the range 10–250  $\mu\text{mol L}^{-1}$  cells (MI = +0.5 to +3 and -0.5 to -2.5; Fig. 4). This range of lipid incorporation corresponds to 0.25–6.25% of the total cell phospholipid. DLPS-treated cells with an average morphology of -1 had incorporated  $\sim 80 \mu\text{mol lipid L}^{-1}$  cells, while those with an average morphology of -2.5 had incorporated  $\sim 250 \mu\text{mol lipid L}^{-1}$  cells (Fig. 4, ■). After incubation with DLPC, stage +1 cells had incorporated 50  $\mu\text{mol lipid L}^{-1}$  cells, and those crenated to stage +3 had incorporated 150  $\mu\text{mol lipid L}^{-1}$  cells (Fig. 4, ▲). The values found for DLPC are similar to those reported previously (Fuji and Tamura, 1983; Ferrell et al., 1985a). The extremes of the MI scale are less sen-

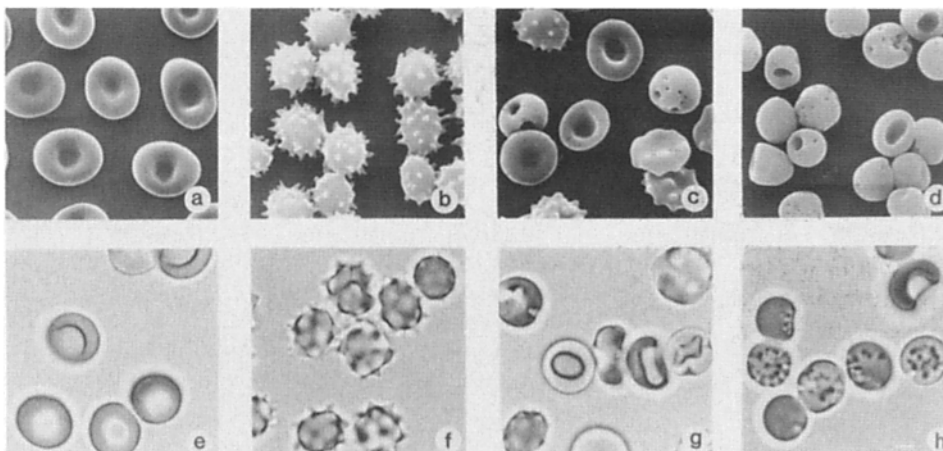
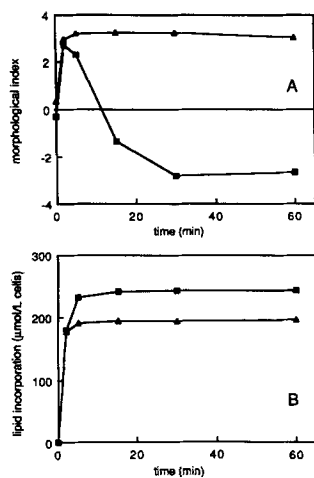
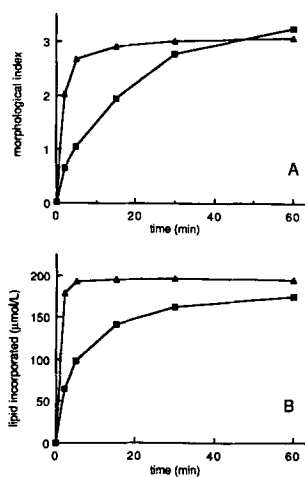


Figure 2. Scanning electron micrographs (a–d) and light micrographs (e–h) of normal erythrocytes (a and e) and erythrocytes treated with DLPS for 5 (b and f), 10 (c and g), or 30 (d and h) min. Bar, 2  $\mu\text{m}$ .



**Figure 3.** Time course of erythrocyte shape change and  $^{14}\text{C}$ -lipid incorporation upon treatment with DLPS or DLPC vesicles. Cells were incubated with 0.25 mM DLPS (■) or 0.2 mM DLPC (▲) containing trace amounts of the corresponding radiolabeled lipid. At the times indicated, aliquots were removed and fixed for morphological indexing or washed and analyzed for cell-bound radioactivity. (A) Time course of erythrocyte shape change (MI). (B) Incorporation of  $^{14}\text{C}$ -DLPS or  $^{14}\text{C}$ -DLPC.



**Figure 5.** Time course of shape change and  $^{14}\text{C}$ -DLPS or  $^{14}\text{C}$ -DLPC incorporation into erythrocytes after inhibition of aminophospholipid transport. Cells were treated with 1 mM EDTA + 5  $\mu\text{M}$  A23187 before incubation with radiolabeled DLPS (0.2 mM, ■) or DLPC (0.2 mM, ▲). At the times indicated, aliquots were removed and fixed for morphological indexing or washed and analyzed for cell-bound radioactivity. (A) Erythrocyte shape change (MI). (B) Incorporation of  $^{14}\text{C}$ -DLPS or  $^{14}\text{C}$ -DLPC.

sitive to lipid incorporation; incorporation of  $<15 \mu\text{mol}$  of either PC or PS per liter of cells had no significant morphological effect, and additional incorporation in excess of  $200 \mu\text{mol/liter}$  had diminished effect.

### Incorporation of $^{14}\text{C}$ -DLPS and $^{14}\text{C}$ -DLPC into Inhibited Cells

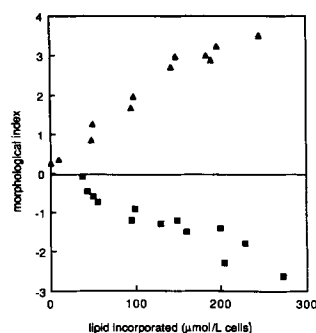
Cells treated with A23187 plus EDTA (or CDTA), resulting in  $\text{Mg}^{2+}$  depletion, are incapable of aminophospholipid translocation (Daleke and Huestis, 1985). Magnesium-depleted cells crenated when exposed to either 0.2 mM DLPS or 0.2 mM DLPC, and neither showed significant reversion to discocytes or stomatocytes (Fig. 5 A; Daleke and Huestis, 1985). Erythrocytes subjected to this treatment and then exposed to PC vesicles incorporated foreign lipid normally (compare Figs. 3 B and 5 B, ▲), and crenated in response to PC to an extent indistinguishable from controls (compare Figs. 3 A and 5 A, ▲). Incorporation of radiolabeled lipids was concurrent with shape change for DLPC and DLPS (Fig. 5 B), though both events were slower for DLPS than for DLPC. Magnesium depletion inhibited the second phase of PS-induced shape change completely; the cells crenated but did not revert to stomatocytes (Fig. 5 A, ■). The rate of incorporation of  $^{14}\text{C}$ -DLPS (Fig. 5 B, ■) and corresponding shape change (Fig. 5 A, ■) were slow compared with nor-

mal cells (Fig. 3, ■). This difference in rate may reflect an effect of EDTA on DLPS transfer into cells; NEM pretreatment also inhibited PS translocation (Daleke and Huestis, 1985) but the rate of transfer of DLPS from vesicles to NEM-treated cells was identical to untreated cells (not shown).

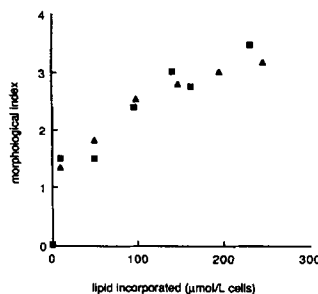
Cells that had been  $\text{Mg}^{2+}$  depleted, oxidized with diamide, or treated with NEM responded similarly to DLPC and DLPS treatment; both lipids crenated the cells in degrees proportional to lipid uptake. Approximately  $25 \mu\text{mol lipid L}^{-1}$  cells were required to produce stage +1 morphology, and  $200 \mu\text{mol lipid L}^{-1}$  cells were required to produce stage +3 morphology in inhibited cells (Fig. 6).

### Lipid Transbilayer Distribution by Extraction

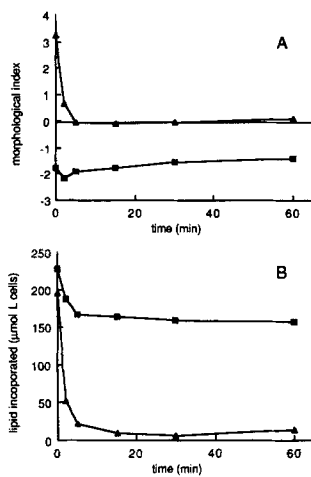
Lipids incorporated into the outer monolayer of erythrocytes and other cells can be extracted into exogenous vesicles (Struck and Pagano, 1980; Mashino et al., 1983). The rate of lipid transfer from cells to vesicles is a sensitive function of the water solubility of the lipids concerned: diacyl phospholipids having  $<15$  carbon atoms/acyl chain are extracted relatively rapidly ( $t_{1/2} < 1$  h), while more hydrophobic lipids (e.g., most naturally occurring phospholipids and many sterols) are not extracted appreciably in many hours of exposure to vesicles. The extent of extraction even of hydro-



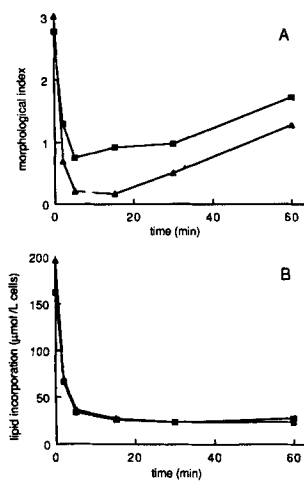
**Figure 4.** Morphological index and  $^{14}\text{C}$ -DLPS or  $^{14}\text{C}$ -DLPC incorporation. Cells were treated with various concentrations (0.01–0.25 mM) of DLPS (■) or DLPC (▲) labeled with the corresponding  $^{14}\text{C}$ -lipid for 45 min. Aliquots were removed and fixed for morphological indexing or washed and analyzed for cell-bound radioactivity.



**Figure 6.** Morphological index and  $^{14}\text{C}$ -DLPS or  $^{14}\text{C}$ -DLPC incorporation into inhibited cells. Cells were treated with 5  $\mu\text{M}$  A23187 + 1 mM EDTA and then incubated with varying concentrations (0.01–0.25 mM) of DLPS (■) or DLPC (▲) containing trace amounts of the corresponding  $^{14}\text{C}$ -lipid for 45 min. Aliquots were removed and fixed for morphological indexing or were washed and analyzed for cell-bound radioactivity.



**Figure 7.** Timecourse of erythrocyte shape change (A) and  $^{14}\text{C}$ -lipid content (B) during extraction of cells pretreated with 0.2 mM  $^{14}\text{C}$ -DLPS (■) or 0.2 mM  $^{14}\text{C}$ -DLPC (▲) and treated with DOPC/DPPG vesicles.



**Figure 8.** Morphological index and lipid content during extraction of A23187/EDTA-inhibited cells pretreated with 0.2 mM  $^{14}\text{C}$ -DLPS (■) or 0.2 mM  $^{14}\text{C}$ -DLPC (▲) and treated with DOPC/DPPG vesicles.

philic lipids is a function of the relative surface areas of the cells and vesicles in the suspension. If conditions are chosen such that the total surface area of the vesicles is much greater than the surface area of the cells, most of the transferable lipid in the cell outer monolayer will be extracted into the acceptor vesicle membrane.

Cells containing incorporated  $^{14}\text{C}$ -DLPS or  $^{14}\text{C}$ -DLPC (by prior incubation with 0.2 mM DLPC or DLPS containing radiolabeled tracers) were washed and resuspended at 5% HCT in a suspension of recipient vesicles (19.4 mM DOPC + 0.6 mM DPPG) and incubated at 37°C. DLPC-induced echinocytes reverted rapidly (5 min) to discocytes, concomitant with the appearance of >95% of  $^{14}\text{C}$ -DLPC in the vesicle fraction (Fig. 7). In contrast, DLPS-treated cells became slightly more stomatocytic, and 75% of radiolabeled DLPS could not be extracted from the cells. With continued incubation, DLPS-treated cells became slightly less stomatocytic concomitant with progressive removal of  $^{14}\text{C}$ -DLPS from the cells. Similar experiments using pure DOPC acceptor vesicles yielded similar results, indicating that the negatively charged DPPG normally present in the acceptor vesicles is not responsible for differential extraction of PC and PS. In parallel experiments, untreated cells were incubated with DOPC/DPPG vesicles containing trace amounts of  $^{14}\text{C}$ -DOPC. Less than 0.1% of the  $^{14}\text{C}$ -DOPC became associated with the cells during a 1-h incubation.

Ionophore plus EDTA-treated cells were crenated by incorporation of either 0.2 mM DLPS or 0.2 mM DLPC, and then extracted with recipient vesicles. The cells reverted rapidly to discocytes, coincident with removal of 86% of both radiolabeled lipids (Fig. 8). Cells restored to discoid morphology eventually crenated again (Fig. 8), a likely result of A23187/EDTA-induced ATP depletion (Daleke, D. L., and W. H. Huestis, unpublished observations). In similar experiments employing NEM instead of A23187/EDTA pretreatment, >95% of the radiolabeled lipid of both types could be extracted (not shown).

### Cell-to-Cell Transfer of DLPS

Lipids sufficiently hydrophilic to be extracted from cells into vesicles should also exchange between cell membranes. Cell-to-cell transfer of DLPS was examined using the distribution of cell morphologies to indicate foreign lipid distribu-

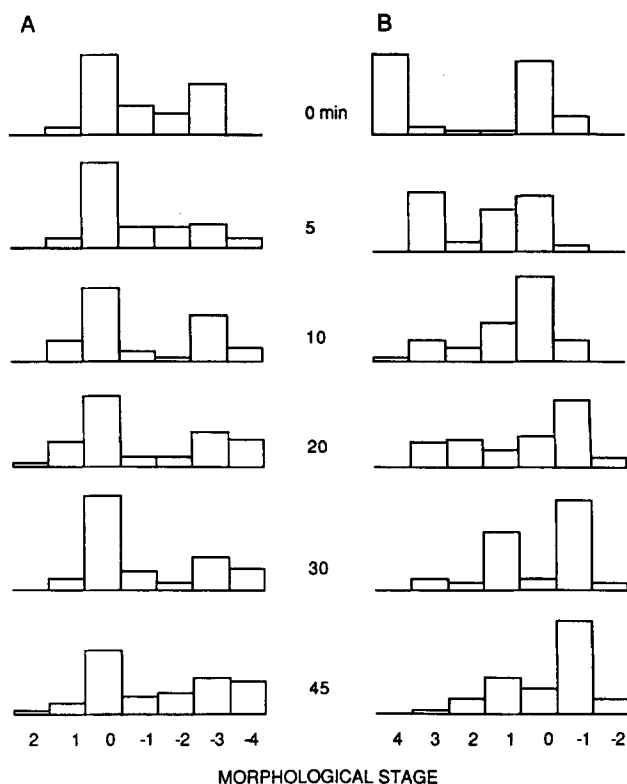
tion (Ferrell et al., 1985b). Control cells and cells inhibited with diamide were incubated with DLPS (0.2 mM) and, after the respective stomatocytosis or crenation was complete, washed free of exogenous lipid. When such cells were mixed with normal discocytes, the combined samples initially exhibited bimodal shape distributions: diamide-inhibited samples contained stage 4 echinocytes and discocytes but few intermediate forms, and the stomatocyte-discocyte mixture contained primarily discocytes and advanced stage stomatocytes (Fig. 9). Within 10 min of incubation, the two populations in the diamide-treated sample began to merge, generating intermediate morphologies (Fig. 9B). After 45 min of incubation, cells in this sample had reverted to discocytic and slightly stomatocytic morphologies. In contrast, normal discocytes mixed with PS-induced stomatocytes were largely unaltered after 45 min of incubation. The average morphology in this mixture shifted slightly toward stomatocytic shapes, but the bimodal shape distribution persisted (Fig. 9A).

### Phospholipase Experiments

Extraction of incorporated  $^{14}\text{C}$ -DLPS was compared to the accessibility of these lipids to phospholipase hydrolysis in intact cells. To avoid possible changes in phospholipid susceptibility due to cell morphology (Daleke, D. L., and W. H. Huestis, unpublished observations), cells were incubated with concentrations of  $^{14}\text{C}$ -DLPS that induce little or no shape change. Control cells or those treated with NEM or A23187/CDTA, as described above, were treated with 50  $\mu\text{M}$   $^{14}\text{C}$ -DLPS, washed, and exposed to phospholipase A<sub>2</sub> and sphingomyelinase C.  $^{14}\text{C}$ -DLPS accessibility to hydrolysis and DOPC/DPPG vesicle extraction are compared in Table I. The two approaches yield the same qualitative measure of accessible  $^{14}\text{C}$ -DLPS; less radiolabel was extracted or hydrolyzed in control cells than in NEM, diamide, or A23187/CDTA-treated cells, but the amount of lipid hydrolyzed by phospholipases was consistently less than that extracted by DOPC/DPPG vesicles.

### Discussion

The asymmetric distribution of phospholipids across certain biological membranes is well established. In the membrane



**Figure 9.** Cell-to-cell transfer of DLPS. Erythrocytes were treated with NaCl/Pi (A) or 5 mM diamide in NaCl/Pi (B), washed, incubated with 0.2 mM DLPS for 45 min, washed, and mixed with an equal volume of normal discocytes. At the timepoints indicated, aliquots were removed, fixed with glutaraldehyde, and the number of cells at each morphological stage was tabulated.

of the human erythrocyte, the choline phospholipids, PC and sphingomyelin, are located primarily in the outer leaflet of the bilayer (75 and 80%, respectively), while the aminophospholipids phosphatidylethanolamine and PS are sequestered in the inner leaflet (80 and 100%, respectively) (Bretscher, 1972a,b; Gordesky and Marinetti, 1973; Verkleij et al., 1973; Zwaal et al., 1975; Rothman and Lenard, 1977). An ATP-dependent aminophospholipid transporter that might maintain such asymmetry has been postulated, based on evidence from cell morphology changes (Daleke and Huestis, 1985), accessibility of spin-labeled aminophospholipid analogues to reduction (Seigneuret and Devaux, 1984; Zachowski et al., 1986), and the hydrolysis of radiolabeled lipids by phospholipases (Tilley et al., 1986). Evidence for a similar aminophospholipid transporter has been found in the plasma membrane of human platelets (Daleke and Huestis, 1985; Sune et al., 1987; also, our unpublished observations), pig lymphocytes (Zachowski et al., 1987), and Chinese hamster lung fibroblasts (Martin and Pagano, 1987). In addition, an outwardly directed phosphatidylcholine transporter has been reported in rat liver microsomes (Backer and Dawidowicz, 1987; Bishop and Bell, 1985; Kawashima and Bell, 1987). Such lipid transporting activities had been postulated from earlier studies of membrane asymmetry (Bretscher, 1973), but recent work has provided the first direct evidence for an active mechanism that generates and maintains phospholipid asymmetry in biological membranes.

**Table I.** Transbilayer Distribution of  $^{14}\text{C}$ -DLPS Incorporated into Normal, Diamide-treated, NEM-treated, or A23187/CDTA-treated Erythrocytes as Measured by Extraction or Phospholipase Treatment

Pretreatment	% of lipid extracted with DOPC vesicles ( $\pm$ SD)	% hydrolysis by phospholipases ( $\pm$ range)
None	16.0 $\pm$ 7.7	11.7 $\pm$ 5.8
Diamide	84.1 $\pm$ 4.1	—
A23187/CDTA	89.1 $\pm$ 2.4	48.8 $\pm$ 3.2*
NEM	66.9 $\pm$ 0.6 <sup>†</sup>	47.8 $\pm$ 5.9 <sup>‡</sup>
	96.9 $\pm$ 14.8 <sup>§</sup>	

\* Phospholipase A<sub>2</sub> only, 60 min.

<sup>†</sup> 1 mM NEM, 10% HCT for 15 min.

<sup>‡</sup> 2 mM NEM, 20% HCT for 1 h.

Examination of the mechanistic basis of membrane phospholipid asymmetry requires a highly sensitive assay for transbilayer phospholipid distribution. Such an assay should be specific for lipids on one side of the bilayer, nonperturbing to membrane structure, sensitive to small changes in lipid composition, and must sample the membrane on a time scale faster than transbilayer redistribution of the lipids. Two schemes have been used in such assays. In the first, chemical or enzymatic agents are used to alter lipids on the external face of the membrane, and the products of such reactions are isolated and quantified (Bretscher, 1972a,b; Gordesky and Marinetti, 1973; Roelofs and Zwaal, 1976; Whitely and Berg, 1974). Enzymatic or chemical modification of membrane lipids is usually slow, may perturb membrane structure leading to artefactual results (Franck et al., 1986), is insensitive to very small changes in lipid composition, and necessitates quantitative isolation and analysis of lipid degradation products or derivatives.

The second general approach entails extraction or exchange of accessible lipids, either endogenous or exogenously introduced, by phospholipid exchange proteins (Wirtz, 1974; Van Meer et al., 1980), acceptor liposomes (Struck and Pagano, 1980; Mashino et al., 1983), or albumin (Mohandas et al., 1982; Tamura et al., 1985). Specific phospholipid exchange proteins are inconvenient to obtain, and incubations of many hours are required to exchange significant amounts of lipid. Alternately, the transbilayer distribution of exogenously incorporated lipids can be measured by extraction from the cell outer monolayer into acceptor liposomes. This approach is relatively nonperturbing, but it is limited by the rates at which phospholipids exchange between membranes, and thus is of little use in studying the distribution of most endogenous (long-chained) lipids.

A third approach to this problem takes advantage of the morphological effects that exogenous phospholipids exert on human erythrocytes and platelets (Daleke and Huestis, 1985; also, our unpublished observations). The basis for interpretation of these effects is the bilayer couple model (Sheetz and Singer, 1974), which proposed that exogenous amphipaths induce shape changes in erythrocytes by differential distribution between the two monolayers of the cell membrane. Such differential intercalation should expand one bilayer leaflet relative to the other, resulting in membrane evagination (echinocytosis) or invagination (stomatocytosis). Thus, positively charged amphipaths such as chlorpromazine, which should associate preferentially with the negatively charged

lipids of the inner monolayer, would be expected to have the stomatogenic effect that is in fact observed. Conversely, negatively charged amphipaths (e.g., dinitrophenol) or zwitterions that equilibrate very slowly across the bilayer (exogenous PC) are echinogenic, as the model predicts. Later work established the quantitative relationship between membrane monolayer expansion and cell shape, demonstrating that extremely small changes in stoichiometric bilayer balance have obvious morphological effects (Fuji and Tamura, 1983; Fuji et al., 1985; Ferrell et al., 1985a).

The morphological effects of exogenously introduced phospholipids suggest that they incorporate into erythrocyte and platelet plasma membranes in the same loci as the analogous native lipids. Erythrocytes incubated with PCs become echinocytic and remain so for many hours, consistent with accumulation of the foreign lipid in the membrane outer monolayer. Cells exposed to PSs also are transiently echinocytic, but convert rapidly to discocytic and eventually stomatocytic shapes (Fig. 2). If cells are first depleted of ATP or magnesium, or treated with sulfhydryl reagents, the conversion to discocytes and stomatocytes is prevented (Daleke and Huestis, 1985). This suggests that after initial incorporation into the membrane outer monolayer, exogenous PS is selectively transported by an energy and protein-dependent mechanism to the inner monolayer (Daleke and Huestis, 1985).

Provided exogenously added lipids cause no radical reorientation of other membrane components, such shape changes are reliable indicators of lipid distribution and can be used as a sensitive and continuous monitor of lipid uptake and transport. The experiments described in this contribution were undertaken to validate morphology-based assays by comparing lipid distributions inferred from cell shape changes with lipid accessibility to conventional enzymolysis or extraction.

### ***Temporal and Quantitative Correlation between Cell Shape Changes and Foreign Lipid Incorporation***

Cell morphology is an accurate measure of the time course and extent of lipid incorporation and transbilayer distribution. Erythrocytes exposed to foreign phospholipids change shape to extents proportional to, and at rates coincident with, lipid uptake. The rate of transfer of DLPC and DLPS from sonicated vesicles to cells is similar and coincides with the development of echinocytic shapes (Fig. 3). Cells treated with DLPC remain echinocytic for several hours, while cells treated with DLPS become stomatocytic, implying that PS, and not PC, translocates to the cell inner monolayer (Daleke and Huestis, 1985). In the range 10–250  $\mu\text{mol L}^{-1}$  the extent of shape change is linearly proportional to the amount of lipid incorporated; each unit increase in MI requires roughly 50  $\mu\text{mol lipid L}^{-1}$  cells for PC-induced echinocytes and 100  $\mu\text{mol lipid L}^{-1}$  cells for PS-induced stomatocytes (Fig. 4). Second, the morphology assay is reproducible and amenable to standardization. Erythrocyte morphology assays have been used in previous work to monitor the incorporation of foreign lipids into erythrocytes (Fuji et al., 1979; Daleke and Huestis, 1985; Ferrell et al., 1985a,b; Truong et al., 1986). Though the MI of samples is usually measured in a nonrandom fashion, when randomized, blind-coded samples are recounted by the same or different individuals using the MI scale shown in Fig. 1, identical results ( $\pm 0.1$  MI units) are

obtained (Daleke and Huestis, 1985). Thus, Figs. 4 and 6 provide a working standard curve for erythrocyte morphology as a function of DLPC or DLPS incorporation. For other lipids, differences in cross-sectional area and monolayer expansion require the establishment of similar standard curves. Third, phospholipid head group-dependent variations on monolayer expansion must be measured to compare morphological effects of different phospholipids. DLPS and DLPC have similar calculated head group cross-sectional areas and identical acyl chains. Thus, the amount of monolayer expansion for these lipids should be similar. However, the net charge of these phospholipids differs; PS is net negatively charged at physiological pH while PC is zwitterionic so their effective cross-sectional areas may be different. Under conditions in which PS is trapped in the outer monolayer (treatment with NEM or by magnesium depletion [Daleke and Huestis, 1985]), the morphological response of cells treated with PS or PC is essentially indistinguishable (Figs. 5 and 6), and PS, like PC, is accessible to extraction by exogenous acceptor vesicles (Fig. 8). Thus, PC and PS have similar effects on monolayer expansion per lipid incorporated (Fig. 6), and any charge or size differences do not contribute to their morphological effects. However, in normal cells, DLPS engenders slightly smaller absolute MI changes (stomatocytes) than the equivalent amount of DLPC (echinocytes, Fig. 4), indicating there may be differences in the sensitivity of each monolayer to expansion. This difference may result from the subjective choice of morphological stage indicators; the MI scale was determined arbitrarily and a stage 1 echinocyte does not necessarily correspond in monolayer expansion to a stage 1 stomatocyte. Thus, the incorporation of equivalent amounts of echinogenic and stomatogenic agents are not expected to produce equivalent absolute MIs. Alternately, the apparent lower potency of PS may result from incomplete translocation (see below) or from metabolism of the lipid in the cell inner monolayer. However, on the time scale of these experiments, TLC analysis indicates that erythrocytes do not convert incorporated  $^{14}\text{C}$ -PS into other forms (data not shown). Finally, this interpretation of cell shape changes and lipid distribution was verified by two independent methods: foreign lipid extraction by exogenous vesicles, and foreign lipid hydrolysis by exogenous phospholipases.

### ***Demonstration of Foreign Lipid Distribution by Extraction***

To measure lipid distribution by accessibility to extraction, erythrocytes loaded with radiolabeled DLPC or DLPS were treated with vesicles composed of DOPC, a relatively hydrophobic lipid that does not transfer readily between membranes. DLPC and DLPS, being significantly more water soluble, transfer rapidly between bilayers. Under the conditions chosen for extraction (a large excess of recipient vesicle membrane), the fraction of cell-bound lipids that is accessible to the extracellular medium transfers quantitatively to the DOPC vesicles within minutes.

DOPC vesicle treatment rapidly reverses DLPC-induced crenation, concomitant with removal of >99% of the incorporated radiolabel (Fig. 7 [▲]). In contrast, DOPC vesicles extract only 25% of DLPS from PS-induced stomatocytes, and the stomatocytic morphology of the cells persists (Fig.



7, ■). This accessibility to extraction is consistent with DLPC, and not DLPS, being localized to the cell outer monolayer.

DLPS-treated cells initially (<5 min) become more stomatocytic upon treatment with acceptor vesicles, consistent with contraction of the outer monolayer as a result of removal of residual outer monolayer DLPS (Fig. 7). After this initial contraction, cells revert slowly to more discoid forms and DLPS extraction continues at a slower rate. This shape reversion and lipid extraction likely result from transfer of inner monolayer DLPS to the outer monolayer and subsequent removal from the cells by acceptor vesicles. The half-time for this reversion is  $\sim 15$  h at  $37^\circ\text{C}$ . If the outward movement of DLPS is rate limiting and provided that cell-to-vesicle transfer of DLPS ( $t_{1/2} \sim 1$ -2 min; Fig. 3) is faster than inward transport ( $t_{1/2} \sim 9$  min), this is a measure of the inside-outside movement of DLPS. This value is 15-fold greater than that recently reported for the outward rate of movement of a spin-labeled analog of PS (1-palmitoyl-2-[4-doxy]pentanoyl]-phosphatidylserine)( $t_{1/2} = 0.96$  h; Bitbol and Devaux, 1988). In contrast, the inward rates reported for spin PS and spin PC movement ( $t_{1/2} = 0.06$  and  $7.7$  h, respectively) are similar to those measured using the morphological assay described in this work ( $t_{1/2} = 0.14$  and  $7.5$  h, respectively, for DLPS and DLPC; Daleke and Huestis, manuscript in preparation). The inward transport of PS requires ATP (Seigneuret and Devaux, 1984; Daleke and Huestis, 1985) and is independent of PS acyl chain composition (Daleke, D. L., and W. H. Huestis, 1985, manuscript in preparation). The differences in outward rate for DLPS and spin PS may indicate that this process is acyl chain dependent and occurs by a mechanism distinct from the inward transport, perhaps by flip-flop. Indeed, spontaneous transmembrane diffusion of phospholipids (flip-flop) is energy independent and strongly dependent on lipid acyl chain length (Van Meer and Op den Kamp, 1982; Middelkoop et al., 1986). Although recent calculations of the outward rate of PS transport from inward rate measurements indicate that outward movement of PS is also ATP dependent (Herrmann and Müller, 1986; Williamson et al., 1987), these calculations are indirect and model dependent.

Cells whose aminophospholipid transport is inhibited by magnesium depletion crenate upon treatment with either DLPS or DLPC. Extraction with DOPC vesicles temporarily restores all such cells to discocytic morphology, concurrent with removal of >90% of both DLPS and DLPC (Fig. 8). The availability of lipid to extraction is not dependent on cell shape; incubation with low concentrations of lipid (50  $\mu\text{M}$ ) induces no discernable change in cell shape, but the fraction of lipid accessible to extraction is similar to that observed at higher concentrations (Figs. 7 and 8; Table I). This accessibility to extraction, and the consequent morphological behavior of the cells, is consistent with the selective transbilayer lipid distribution inferred above.

#### **Assessment of Transbilayer Lipid Distribution by Phospholipase Treatment**

The accessibility of incorporated DLPS to exogenous agents was examined by phospholipase digestion, and results qualitatively similar to DOPC extraction were obtained (Table I). Inhibition of aminophospholipid transport increases the ac-

cessible fraction of subsequently incorporated DLPS four- to fivefold. Phospholipase digestion, however, yields values for the accessible population that are consistently less than (50-73%) the population accessible to DOPC extraction. This may be a result of incomplete hydrolysis of DLPS in the membrane outer monolayer, although under the conditions of this experiment all of the PC and PE in the outer monolayer are hydrolyzed. More extensive lipase digestion results in hemolysis, and is thus counterproductive as well as an apparently inescapable drawback to the lipase assay.

#### **Monolayer Expansion and Morphological Response**

As evinced by the partial accessibility of DLPS to extraction in normal cells (Fig. 7), sequestration of DLPS in the inner monolayer apparently is not complete. Since residual foreign PS in the outer monolayer would counter the stomatogenic effect of the translocated lipid to some extent, incomplete translocation may be partly responsible for the fact that PS appears less potent (less absolute MI) than PC in inducing shape changes in normal cells (Fig. 4).

Mathematical models of echinocyte shapes indicate that outer monolayer expansion due to PC intercalation is sufficient to account for its morphological effects (Ferrell et al., 1985a). Evidence from several sources indicates that red cell morphology may be more sensitive to inner monolayer than to outer monolayer expansion. Calculation of the monolayer surface areas yield excess outer monolayer surface areas of  $0.41 \mu\text{m}^2$  for discocytes,  $0.39 \mu\text{m}^2$  for stomatocytes, and  $0.93 \mu\text{m}^2$  for echinocytes (Beck, 1978). Thus, intercalation of equivalent amounts of similarly sized amphipaths should result in greater relative expansion of the inner monolayer. Indeed, erythrocytes incubated with 1-lauroyl-lysophosphatidylcholine are initially echinocytic and revert to discocytes within a few hours (due to flip-flop of the lipid to the inner monolayer), even though the equilibrium transbilayer distribution of the lipid favors the outer monolayer (Mohandas et al., 1978). Similarly, the concentration of anionic phenothiazines that produces spherocytic cells (Fig. 1 e) is 10-fold greater than that required of cationic phenothiazines to produce spherostomatocytes (Fig. 1 j) (Mohandas and Feo, 1975). As previously demonstrated (Daleke and Huestis, 1985), incubating cells with vesicles composed of an equimolar mixture of DLPS and DLPC (or DMPS and DMPC) results in no net shape change (MI = 0). Since DLPC and DLPS transfer into cells to the same extent and some (11-25%) of the DLPS remains in the outer monolayer, that leaflet may contain an excess of foreign lipid without evident morphological effect.

#### **Use of Morphology Changes to Monitor Intercell Lipid Transfer**

Cell morphology changes in mixed populations of discocytes and echinocytes have been used to demonstrate intercell transfer of hydrophilic PC (Ferrell et al., 1985b). When DLPS-induced stomatocytes are mixed with an equal volume of untreated discocytes, two populations of cells are apparent (Fig. 9 A). With extended incubation, the stomatocyte population becomes slightly more stomatocytic, and (except for a small, transient increase in echinocytes) the discocyte population shows little change. This behavior is consistent

with intercell equilibration of residual outer monolayer DLPS, while the bulk of the incorporated PS remains sequestered in the stomatocyte inner monolayer or in endocytotic vesicles. The persistence of two distinct morphological populations also indicates that the lipid does not move between sequestered and accessible pools on the time scale of this experiment.

In contrast, DLPS exchanges extensively from cells whose translocating capacity is inhibited. As with magnesium-depleted cells (Figs. 5 and 8), cells treated with diamide followed by DLPS crenate and remain echinocytic (Daleke and Huestis, 1985). When equal volumes of such echinocytes are mixed with untreated discocytes, two distinct cell populations are apparent initially (Fig. 9B). On a time scale consistent with intermembrane transfer of dilauroyl phospholipids, these two populations merge to yield a slightly stomatocytic MI. Thus, foreign PS in diamide-inhibited cells remains accessible to equilibration between cell populations.

### Summary

These experiments demonstrate a consistent correlation between erythrocyte shape and the accessibility of foreign lipid to exogenous agents. In echinocytic cells, such lipid is accessible to extraction into exogenous membranes and (to a lesser extent) to digestion by exogenous phospholipases. In stomatocytes generated by PS treatment of normal cells, <20% of incorporated PS is accessible to such agents. Erythrocytes whose aminophospholipid transport system is inhibited simply crenate when exposed to PS, and the incorporated PS is quantitatively accessible to extraction (85–97%) and partially accessible (50%) to phospholipase digestion. Thus, the transbilayer disposition of incorporated foreign lipid may be inferred from cell morphology, yielding results consistent with other assays. In contrast with assays involving lipid extraction or chemical and enzymatic modification, the morphology assay for lipid distribution is rapid and versatile, providing a continuous monitor of lipid uptake and transbilayer movement of time scales of minutes to hours. The method is convenient and conservative of materials, obviating separation and quantitation of extracted lipids or their derivatives. Finally, it is highly sensitive; very small changes in membrane bilayer balance effect readily measurable cell shape changes. These considerations make cell morphology a convenient and powerful way to study mechanisms of lipid transport and sequestration.

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