# Shape Changes in Human Erythrocytes Induced by Replacement of the Native Phosphatidylcholine with Species Containing Various Fatty Acids

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ABSTRACT Phosphatidylcholine-specific transfer protein from beef liver has been used to replace native phosphatidylcholine (PC) molecules from intact human erythrocytes by a variety of PC species differing in fatty acid composition. These replacements changed neither the total phospholipid content of the membrane, nor the composition of this fraction in terms of the various phospholipid classes. The morphology of the erythrocyte was not modified when native PC was replaced by 1-palmitoyl,2-oleoyl PC, 1-palmitoyl,2-linoleoyl PC, egg PC, or PC isolated from rat liver microsomes. Replacement with the disaturated species 1,2-dimyristoyl PC, 1,2-dipalmitoyl PC, and 1,2-distearoyl PC resulted in the formation of echinocytes and, at higher levels of replacement, in spheroechinocytes. Echinocyte-like erythrocytes were also observed after replacement with 1-palmitoyl,2-arachidonoyl PC, whereas stomatocytes were formed upon replacement with PC species containing two unsaturated fatty acids, e.g., 1,2-dioleoyl PC and 1,2-dilinoleoyl PC. The observations show that the erythrocyte membrane structure and the overall discoid cell shape of the human erythrocyte are optimally stabilized by PC species that contain one saturated and one mono- or diunsaturated fatty acid, and that the cell tolerates only limited variations in the species composition of its PC.

The shape of the erythrocyte is thought to be maintained by the membrane skeleton in close interaction with the plasma membrane. Numerous studies have brought evidence that both elements have their specific function in this respect, although the major contribution to shape maintenance has been ascribed to the spectrin–actin network underlying the membrane (for recent reviews, see references 1–4).

The role of the membrane has been investigated mainly by modification of its composition or the structure of its individual constituents, and the consequences that such modifications might have for the shape of the cell have been assessed. Some typical examples of such studies are the following. When the cholesterol content of the membrane is modified, the shape of the cell changes; cholesterol depletion yields stomatocytes whereas an increase in cholesterol content results in a discocyte-to-echinocyte transformation (5–8). A similar transformation results from the insertion of lysophospholipids and free fatty acids in the membrane (9, 10), whereas selective removal of phospholipids from the outer membrane leaflet results in stomatocytosis (11). Lipid-soluble compounds, in particular, charged amphipathic agents, which partition randomly or specifically in one of both leaflets, also give shape changes, the nature of which depends on the localization of the compound (9, 12–14). In addition, the conversion of phospholipids by phospholipase treatment of the intact cells results in echinocyte formation (after phospholipase $A_2$ treatment) or stomatocytic cells (after phospholipase C or D treatment( (15–17).

Recently, a phosphatidylcholine-specific exchange protein was used to replace phosphatidylcholine (PC)[1] from intact rat erythrocytes by dipalmitoyl PC, and even this modest change in composition of the bilayer resulted in the formation of echinocytes (18). The latter approach has been applied now on human erythrocytes using a variety of well-defined PC species. The characteristic feature of this modification procedure is that the alteration that occurs upon exchange of one

[1] *Abbreviation used in this paper:* PC, phosphatidylcholine.



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PC molecule against another is limited to the apolar region of the outer monolayer, leaving constant the number and nature of polar headgroups of phospholipids in the outer monolayer. It will be argued that, in order to prevent, in the apolar region of the outer monolayer, packing defects that may arise upon replacement, the outer monolayer has to bend, either inwards or outwards, depending on the PC species introduced. This change in overall structure of the outer monolayer is followed by the inner monolayer and the membrane skeleton.

A model is proposed as a refinement of the bilayer couple hypothesis by Sheetz and Singer (19), to account for the observed shape changes. It will be argued that, in addition to the coupling of the two monolayers, a coupling of the inner monolayer to the cytoskeleton is an essential prerequisite for the formation of abnormal erythrocyte shapes.

## MATERIALS AND METHODS

### Materials

Human erythrocytes were obtained from healthy volunteers by venipuncture; acid citrate-dextrose was used as anticoagulant (20). All biochemical reagents were of analytical grade and obtained from Calbiochem-Behring Corp. (San Diego, CA), Sigma Chemical Co. (St. Louis, MO), Merck (Darmstadt, Federal Republic of Germany), or Pierce Chemical Co. (Rockford, IL). 1,2-Dimyristoyl PC, 1,2-dipalmitoyl PC, and 1,2-dilinoleoyl PC, synthesized following standard procedures (21), were gifts from colleagues of the Department of Biochemistry (Utrecht, The Netherlands). 1-Palmitoyl,2-oleoyl PC and egg PC were obtained from Sigma Chemical Co.; 1,2-distearoyl PC and 1,2-dioleoyl PC were purchased from Larodan (Malmö, Sweden); 1-palmitoyl,2-linoleoyl PC was bought from P. L. Biochemicals (Milwaukee, WI); and 1-palmitoyl,2-arachidonoyl PC was bought from Avanti Polar Lipids (Birmingham, AL).

[*Methyl*-$^{14}$C]egg PC, synthesized according to Stoffel et al. (22), was donated by G. van Meer. [*Methyl*-$^{14}$C]dipalmitoyl PC and 1-palmitoyl[2-$^{14}$C]oleoyl PC and [$^3$H]glycerol-trioleate were obtained from Amersham (Cardiff, U.K.).

### Methods

PREPARATION OF VESICLES: The various PC species that were used to prepare vesicles were mixed with 6 mol % of phosphatidate (obtained by phospholipase D treatment of egg PC), trace amounts of [$^{14}$C]PC and [$^3$H]-glycerol-trioleate (0.1% of the total PC present), and cholesterol in an equimolar amount to the total PC. The lipid mixture was dried from solution and dispersed in a buffer containing 150 mM NaCl, 25 mM glucose, 1 mM EDTA, 0.2% Na-azide, and 10 mM Tris-HCl, pH 7.4 (referred to as buffer throughout). The dispersion was sonicated under nitrogen at 70 W using a Branson sonifier (Branson Sonic Power Co., Danbury, CT) for 3–6 min at a constant temperature which was above the transition temperature of the PC species used, and centrifuged at 100,000 *g* for 45 min. The recovery varied from 50 to 85% depending on the type of PC used.

INCUBATION CONDITIONS: Erythrocytes were washed three times with a fourfold excess of buffer. PC-specific transfer protein purified from beef liver (23, 24) was used in concentrated solution as described before (25, 26). Cells were incubated at 37°C, using a clinical blood rotator at 4 rpm. The 30–40% erythrocyte suspension contained 2–3 $\mu$M transfer protein and a two- to fourfold excess (based on PC present in the erythrocytes) of vesicle PC. Aliquots were taken at timed intervals and added to 5 ml of buffer at 37°C. Cells were isolated by centrifugation for 15 min at 2,500 *g* and the resulting supernatant was used to determine hemolysis by absorbance measurement at 408 nm. Intact cells were washed twice in a 30-fold volume of buffer which was kept at a temperature of 25–35°C.

LIPID ANALYSIS: Cells were extracted according to Rose and Oklander (27) and lipid extracts were separated by two-dimensional thin-layer chromatography (28). The specific radioactivity of PC was determined by radioactivity measurement and phosphate analysis (29) of the PC isolated from the TLC plate. From these data the extent of PC replacement was calculated as described before (26). Corrections for contamination of erythrocyte PC with vesicle PC that was not due to exchange, were carried out on the basis of the amount of [$^3$H]glycerol-trioleate in the erythrocyte lipid extract.

ASSAY OF MOLECULAR SPECIES COMPOSITION: The total lipid extract of 1 ml of packed cells was separated by thin-layer chromatography on Silicagel (Merck 60 DC-Fertig platten) using as solvent chloroform-methanol-acetic acid-$H_2O$ (75:45:2:6 by vol). The PC was isolated by elution from the silicagel with chloroform/methanol (2:1, by vol), dried and dissolved in 0.5 ml of methanol. Diglycerides were prepared from the PC by addition of 1 ml of buffer containing 100 mM Tris and 5 mM $CaCl_2$, pH 7.4; 2 ml of diethylether and 100 $\mu$l (1.75 IU) phospholipase C from *C. welchii*. After 1 h of incubation at 37°C with vigorous mixing, the diacylglycerols were extracted with ether and dried over $Na_2SO_4$. Diglycerides were converted to their trimethylchlorosilane derivatives according to Myher and Kuksis (30) and the various molecular species were separated on a 3% Silar 5 CP column (Applied Science Laboratories, Inc., State College, PA) in a Packard gas chromatograph model 805 (Packard Instrument Co., Inc., Downers Grover, IL) equipped with a dual glass U tube (180 × 0.3 cm) at 270°C.

SCANNING ELECTRON MICROSCOPY: Samples of 10 $\mu$l of packed erythrocytes were washed three times in 150 mM NaCl, 10 mM Tris, pH 7.4, and fixed for 1 h at 20°C in 1 ml buffer containing 10 mM NaCl, 40 mM Na citrate, and 0.5% formaldehyde. After washing, a fixation with 1% $OsO_4$ for 30 min was applied, followed by dehydration of the samples in a graded series of ethanol, transfer in isopropanol, and drying at the air. Cells were covered with a thin gold layer by the Sputter process. Microscopy was performed with a Cambridge Stereoscan 600 M (Cambridge Technology Inc., Cambridge, MA).

## RESULTS

### Replacement of Native PC by Various PC Species

The phosphatidylcholine-specific exchange protein can be applied to exchange PC between various membranes and to modify in this way, systematically and specifically, the fatty acid composition of PC in a membrane. Three types of PC have been used in the present studies: disaturated PC molecules, the concentration of which is low in the human erythrocyte; 1-saturated-2-unsaturated PC molecules, the major type occurring in this membrane, and finally, diunsaturated PC molecules, some of which do occur, but only as minor constituents. The relative concentration of these three types in the native human erythrocyte membrane are 13, 67, and 15% of the total PC, respectively, and the species are distributed randomly over the inner and outer layer of the membrane (31–33).

When the replacement is carried out using various types of PC molecules, large differences in rate and extent of exchange are observed. Fig. 1 shows some typical examples of replacement with various PC molecules. It is obvious that unsaturated molecules are incorporated faster in the erythrocyte membrane than disaturated species. To obtain sufficient replacement of erythrocyte PC by such disaturated molecules,

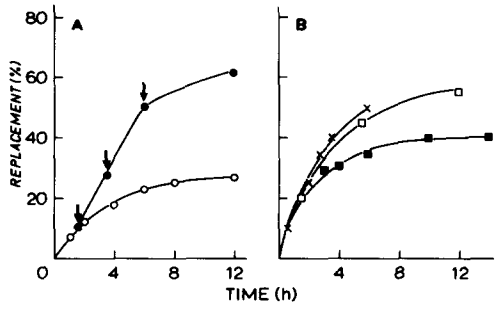


FIGURE 1 Replacement of native erythrocyte PC by various PC species. Erythrocytes were incubated for various time periods with a variety of PC molecular species. Lipid analysis as described in Materials and Methods was carried out after the incubation and the data obtained are expressed as the amount of erythrocyte PC (in percent) that has been replaced by the donor PC species. (*A*) Replacement with 1,2-dipalmitoyl PC (○). At the time points indicated by the arrows the erythrocytes were isolated and reincubated with fresh donor PC vesicles and exchange protein to improve the extent of replacement (●). (*B*) Replacement with 1-palmitoyl, 2-oleoyl PC (□), 1-palmitoyl, 2-linoleoyl PC (■), and 1,2-dilinoleoyl PC (×).

the donor vesicles have to be replaced repeatedly by fresh material as indicated in the figure. In this way up to 60% of the native PC can be replaced by dipalmitoyl PC. Similar replacements can be obtained using species such as 1-palmitoyl,2-oleoyl PC, 1-palmitoyl,2-linoleoyl PC, and 1-palmitoyl,2-arachidonoyl PC. In these cases, it was not necessary to replace the vesicle system. Also exchange with 1,2-dioleoyl PC and 1,2-dilinoleoyl PC proceeds relatively fast (results not shown). Extensive replacement (up to 60%) can be obtained with dioleoyl PC, but with dilinoleoyl PC hemolysis starts to occur when 40% of the erythrocyte PC has been replaced. The extent of replacement depends on the excess of donor PC present in the incubation mixture: in general, a threefold excess of donor PC is sufficient to replace up to 60% of the native PC. The extent of replacement has been determined in all cases, using radioactive PC tracer molecules that had been added to the donor vesicles as described in Materials and Methods. Alternatively, the extent of PC replacement can be determined by analysis of the fatty acid composition of the erythrocyte PC before and after replacement or, more directly, to analyze the molecular species composition by gas chromatography as described by Myher and Kuksis (30). Table I summarizes data obtained with the latter technique on erythrocytes before and after replacement of PC by 1-palmitoyl, 2-arachidonoyl PC and 1,2-dioleoyl PC, respectively. The two major species present in the native cell are 1-palmitoyl,2-oleoyl PC and 1-palmitoyl,2-linoleoyl PC, and upon replacement both species are decreased in favor of the newly introduced PC. The data indicate that replacement of the 1-palmitoyl,2-linoleoyl PC is more pronounced than replacement of the 1-palmitoyl,2-oleoyl PC, in particular when dioleoyl PC is introduced.

TABLE I

*Molecular Species of PC from Human Erythrocytes before and after PC Replacement*

| PC species | Control | 16:0/20:4 enriched | 18:1/18:1 enriched |
|---|---|---|---|
| | | % | |
| 16:0/16:0 | 20 | 19 | 15 |
| 16:0/18:1 | 83 | 63 | 59 |
| 16:0/18:2 | 100 | 70 | 52 |
| 18:0/18:1 | 18 | 15 | + |
| 18:1/18:1 | + | + | 124 |
| 18:0/18:2 | 21 | 28 | 7 |
| 16:0/20:4 | 15 | 63 | + |

The PC from human erythrocytes was isolated and analyzed for its fatty acid species composition as described in Materials and Methods. The data are expressed as percentage of total amount of 1-palmitoyl, 2-linoleoyl PC (16:0/18:2 PC) present as major species in the native (control) cells, and arbitrarily set at 100%. The replacement with 1-palmitoyl, 2-arachidonoyl PC (16:0/20:4 PC) and 1,2-dioleoyl PC (18:1/18:1 PC) amounted to 20 and 48% of the native PC, respectively. Only the major PC species are listed.

Control experiments were performed to demonstrate that the exchange process only involved PC and that none of the other membrane lipid constituents were modified to an appreciable extent (Table II). The precautions that were taken to prevent such modifications include the addition of cholesterol to the donor PC vesicle system in concentrations similar to that found in the erythrocytes. A nonexchangeable marker molecule, [$^3$H]glycerol-trioleate, was used to measure the extent of contamination of the erythrocyte suspension, after the incubation and subsequent washings, with residual vesicle material. The amount of vesicle PC found in the erythrocyte pellet due to this contamination, was usually <4% of the erythrocyte PC.

The observed shape changes have to be ascribed to phospholipid replacement only because it was shown, confirming earlier results (25, 26, 34) that the protein composition and content of the erythrocyte membranes were not affected by the PC exchange, and furthermore, it was found that incubations of erythrocytes with the various vesicle systems but without exchange protein did not change the erythrocyte shape. The property of the PC-specific exchange protein to enhance a one-for-one exchange only has been documented in detail (35) and could be confirmed recently by the following type of experiment. Donor vesicles of various composition were incubated with erythrocytes under identical conditions as described here. The transfer of radioactive PC from vesicles to erythrocytes was determined by measuring specific radioactivity of the erythrocyte PC after the incubation. Transfer of PC from the erythrocyte to the vesicles was analyzed by determining the fatty acid and PC species composition of the vesicles. The data obtained from these experiments matched precisely—that is, the amount of radioactive PC that moved into the erythrocyte membrane was found to be identical to the amount of erythrocyte PC, characterized by its fatty acid composition, which moved into the vesicles. A full account of these observations, describing in detail the kinetic param-

TABLE II

*Lipid Composition of Erythrocytes after PC Replacement*

| PC species introduced | Extent of replacement* | Phospholipid composition‡ | | | | Cholesterol content§ |
|---|---|---|---|---|---|---|
| | | PC | PE | SPH | LPC | |
| | % | | % | | | *mol* |
| — | — | 28 ± 2 | 27 ± 1 | 26 ± 2 | 2 ± 1 | 80 ± 10 |
| 16:0/16:0 | 30 | 29 | 29 | 28 | 2 | 100 |
| 16:0/18:0 | 30 | 28 | 27 | 28 | 0.5 | 90 |
| 16:0/18:1 | 60 | 29 | 28 | 28 | 3 | 80 |
| 16:0/18:2 | 60 | 30 | 29 | 30 | 2 | 80 |
| 16:0/20:4 | 60 | 30 | 27 | 27 | 3 | 100 |
| 18:1/18:1 | 60 | 27 | 25 | 26 | 2 | 90 |
| 18:2/18:2 | 30 | 30 | 28 | 28 | 2 | 95 |

The phosphatidylserine and phosphatidylinositol contents remained constant and are not included here.

* The extent of replacement is expressed as the percentage of original erythrocyte PC that has been replaced.

‡ The phospholipid composition is given as percentage of total phospholipid present.

§ Cholesterol content is presented as moles of cholesterol per 100 moles of phospholipid present.

eters of the exchange, will be published elsewhere (Child, P., J. J. Myher, F. A. Kuypers, J. A. F. Op den Kamp, A. Kuksis, and L. L. van Deenen, manuscript submitted for publication).

## Shape Changes Induced by PC Replacement

Before discussing the experiments in which shape changes of the modified erythrocytes were observed, it is useful to summarize those experiments in which modification of the erythrocyte PC does not result in altered morphology. Fig. 2 shows the normal discoid appearance (*A*) as well as the shape of the cells (*B*) after a 20-h incubation during which 50% of the native PC had been replaced by 1-palmitoyl,2-oleoyl PC. A small number of echinocytes is observed, but a sample of control cells, incubated for the same time period in the absence of donor PC and exchange protein, appeared to contain a similar number of echinocytes (not shown). When incubations were carried out with 1-palmitoyl,2-linoleoyl PC, egg PC, or PC from rat liver microsomes as the donor system, the resulting erythrocytes, in which >50% of the native PC had been replaced, similarly maintained their discoid shape.

Dramatic changes in erythrocyte shape are observed when the unsaturation index of PC in the membrane, which is defined as the total number of double bonds relative to that of the fatty acids, becomes lower than 0.5 or higher than 1.0. The first situation is obtained when the erythrocyte PC is replaced by dimyristoyl PC, dipalmitoyl PC, or distearoyl PC. Fig. 2*C* shows that echinocytes are formed when ~25% of the native PC has been replaced by dipalmitoyl PC. Further increase in the content of disaturated species leads to the formation of spheroechinocytes (Fig. 2*D*) and finally, hemolysis starts when ~40% of the total PC present in the erythrocyte membrane is composed of dipalmitoyl PC. The same observations have been made with distearoyl PC (data not shown). A more precise and quantitative correlation between the extent of PC replacement and changes in erythrocyte shape is difficult to make. Replacement can only be measured as an average value of the total erythrocyte population, whereas the individual cells show various extents of echinocyte formation (Fig. 2, *C* and *D*).

Another typical example of changes in cell shape after replacement with disaturated PC species is shown in Fig. 3*A*. In these cells close to 40% of the native PC has been replaced by 1,2-dimyristoyl PC during a 12-h incubation. The cells are spheroechinocytic and it was observed that additional replacement resulted in hemolysis. Spheroechinocyte-like erythrocytes are also found to be formed when the original PC from the erythrocyte is replaced by 1-palmitoyl,2-arachidonoyl PC (Fig. 3*B*). This figure shows the situation after replacement of 20% of the total erythrocyte PC by this species, but similar shapes have been observed after replacement of up to 60% of the PC, which results in an increase of the double-bond index

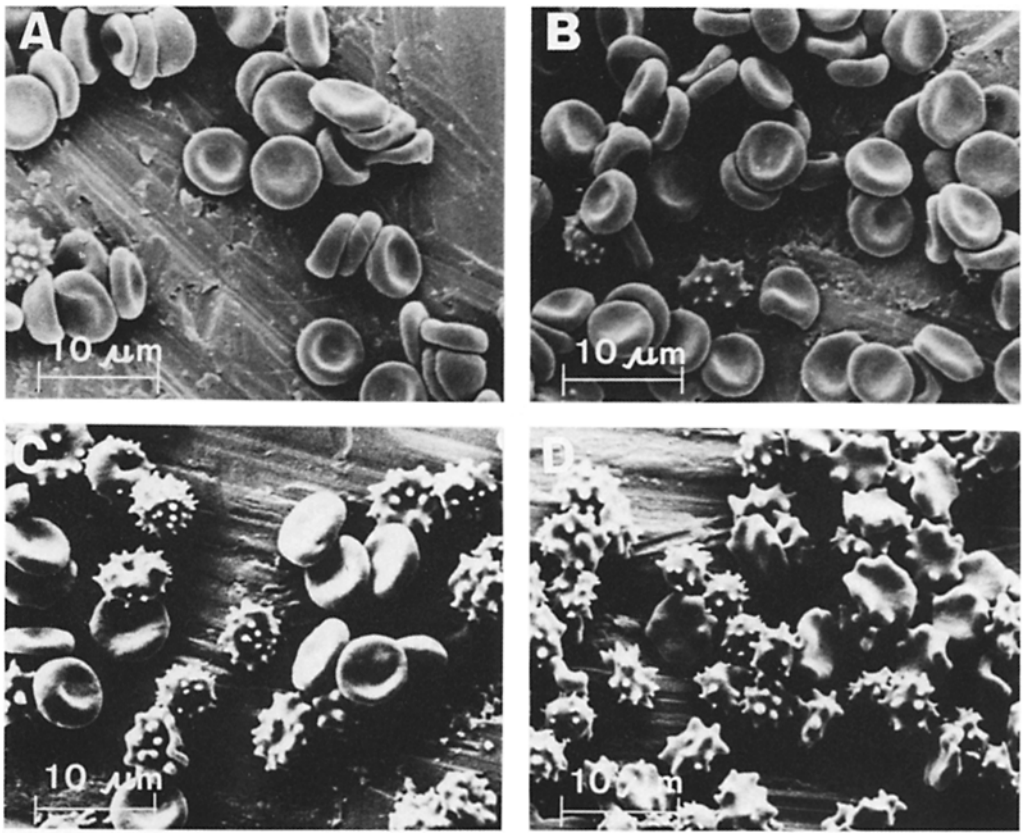


FIGURE 2 Scanning electron micrographs of human erythrocytes before and after PC replacement. The replacement procedure and preparation technique are described in Materials and Methods. Bars, 10 μm. (*A*) Control erythrocytes at the start of the experiments. (*B*) Cells in which 50% of the native PC has been replaced by 1-palmitoyl, 2-oleoyl PC during a 20-h incubation. Similar pictures (not shown) were obtained by incubating control cells for a long period and by replacement incubations using, as donor PC, 1-palmitoyl, 2-linoleoyl PC, egg PC or, PC from rat liver microsomes. (C) Erythrocytes in which ~30% of the PC had been replaced by 1,2-dipalmitoyl PC in a 15-h incubation. (*D*) Erythrocytes in which 40% of the PC had been replaced by 1,2-dipalmitoyl PC in a 20-h incubation. Similar erythrocytes were obtained by replacement experiments with 1,2-distearoyl PC.

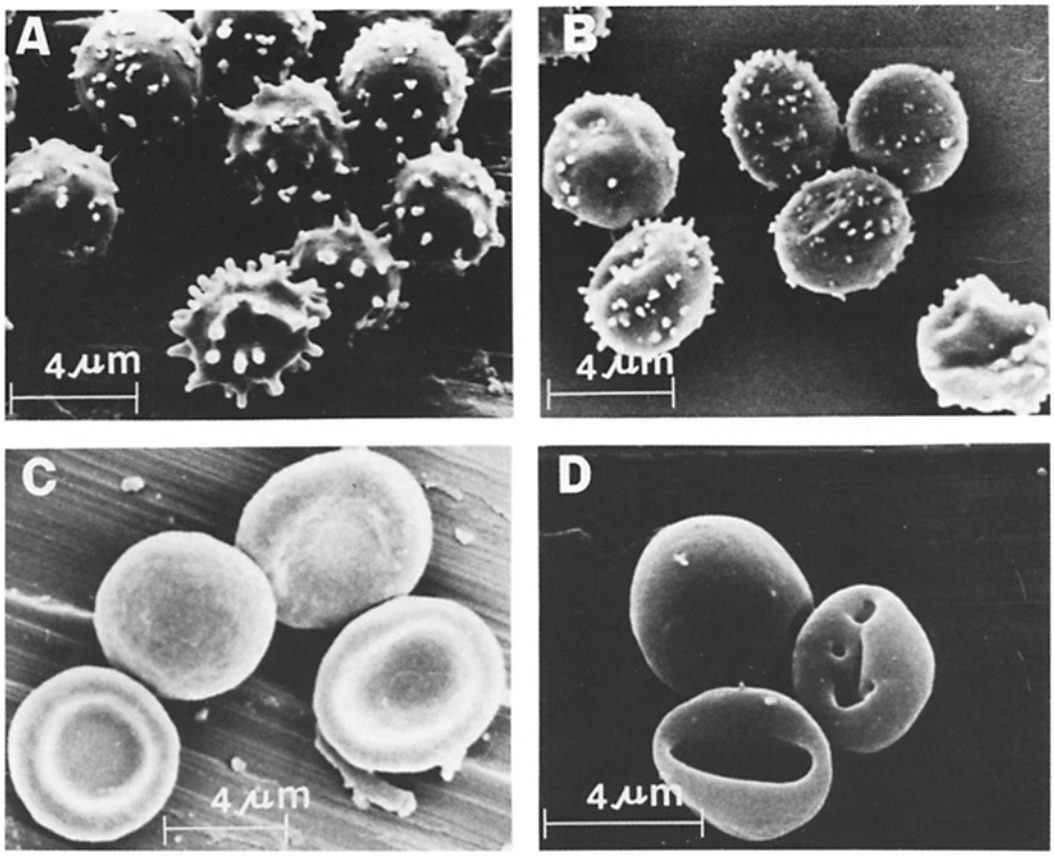


FIGURE 3 Scanning electron micrographs of human erythrocytes after PC replacement. The replacement procedure and preparation technique are described in the Materials and Methods section. Bars, 4 μm. (*A*) Spheroechinocytes obtained by replacing 40% of the erythrocyte PC by 1,2-dimyristoyl PC in a 12-h incubation. (*B*) Erythrocytes after replacement of 20% of the native PC by 1-palmitoyl, 2-arachidonoyl PC in a 15-h incubation. (*C*) Erythrocytes in which 48% of the native PC had been replaced by 1,2-dioleoyl PC in a 15-h incubation. (*D*) Erythrocytes after replacing 30% of the native PC by 1,2-dilinoleoyl PC in a 6-h incubation.

to a value higher than 1.0. The cells are spheroechinocytic at first appearance, but, in addition, show typical dimples. It is possible that these are due to the loss of internal osmotic pressure, since it was observed that replacement with 1-palmitoyl, 2-arachidonoyl PC made the cells leaky for ions (34).

Replacement of native PC by species that contained two unsaturated fatty acids results in the formation of stomatocytes. After replacement of >40% of the PC with 1,2-dioleoyl PC, the cells show a clear tendency to adopt a stomatocytic morphology (Fig. 3*C*). Typical stomatocytes are observed when 20–30% of the PC had been replaced by 1,2-dilinoleoyl PC in a 6-h incubation (Fig. 3*D*). A further increase in the concentration of the latter species in the membrane causes the cells to lyse.

## DISCUSSION

The mixed acid PC molecules, having one saturated and one mono- or diunsaturated fatty acid, are the most abundant PC species in the human erythrocyte (see Table I), and the exchange experiments by which these species are even more enriched in the membrane show that this type of PC molecule has the most optimal structure for maintaining the cellular shape. On the other hand, when the amount of these species is reduced in the membrane by replacement with disaturated, di-unsaturated, or mixed acid species with a highly unsaturated fatty acid, drastic shape changes are noted. Control experiments have shown that the exchange protein does not modify the overall phospholipid content, the amount of cholesterol, and the protein composition of the membrane (see also references 25, 26, and 34). The protein has been shown to give a one-for-one exchange of PC molecules (35), and therefore we have to ascribe the observed effects exclusively to modifications in fatty acid composition of the PC in the membrane.

A characteristic feature of the exchange process is that it occurs exclusively at the outer layer of the plasma membrane (26). Due to the slow transbilayer movement of PC in the intact cell (26, 36), the effect of PC replacement is therefore first of all restricted to the outer layer of the membrane. Even in case a complete equilibration of newly introduced PC over the two membrane layers should occur, the outer layer will be affected more, relative to the inner one, because it contains three times as much PC. The replacement of native PC by other PC molecules can result therefore in a disturbance of the preexisting equilibrium (in structure and properties) between the two layers of the membrane and, as summarized already at the opening of this article, unilateral modifications in the composition of one of the two layers of the erythrocyte membrane may cause shape changes. It is obvious that in our experiments alterations in the apolar part of the PC molecules are directly responsible for the change in shape because the amount of polar headgroups as well as their chemical com-

position does not change. If one considers what effect the change in apolar side chains can have on the overall structure of a lipid bilayer, two parameters in particular—the shape of the PC molecule and its interaction with cholesterol—seem to be relevant in this respect. The importance of the geometry of lipid molecules has been emphasized recently, following the theoretical consideration of Israelachvili (37), by studies on model membrane systems carried out by de Kruijff and co-workers (reviewed in reference 38), and on the membrane of *Acholeplasma laidlawii* by Wieslander et al. (39, 40). Information on PC–cholesterol interactions has been obtained mainly by monolayer studies (for a review, see reference 41), and, although this information can be helpful for understanding the physicochemical properties of the individual PC species, a straightforward extrapolation of monolayer data to the erythrocyte membrane has to be handled carefully as will be evidenced below.

The most common PC species in the erythrocyte membrane contains one saturated and one unsaturated fatty acid. If one replaces this type of molecule by 1,2-diunsaturated species, a crenation is to be expected, rather than a cup formation, because monolayer studies show that the area occupied by dilinoleoyl PC (90Å$^2$ at 21 dynes/cm) will be larger than that of the 1-saturated,2-unsaturated species (65–80Å$^2$ for palmitoyl-oleoyl- and palmitoyl-linoleoyl PC, respectively). However, the areas that are measured by this technique represent an average value of the hydrophobic part of the molecule and do not account for the geometry of the PC molecule which will contribute considerably to the effects observed. According to Wieslander et al. (40), each lipid molecule in a membrane can be visualized by a certain optimal surface area, hydrocarbon chain length and volume. The organization of a membrane can be visualized then as in the working model, presented in Fig. 4.

The 1-saturated,2-unsaturated PC species have a moderate cone shape, whereas that of the 1,2-diunsaturated species is more pronounced. When the latter type of species replaces a native PC molecule in the outer leaflet, it will occupy more space in this part of the membrane, in particular at the very end of the fatty acyl chains, deeply buried in the hydrophobic core of the membrane. In terms of the model depicted above, the average geometrical index of the outer leaflet will relatively increase and, when no other compensation is possible, a proper interaction between individual constituents can now be achieved only by bending of the outer layer of the membrane inwards. The inner monolayer and the membrane skeleton will easily follow, and the cell adopts a stomatocytic morphology.

In a similar way, crenation can be explained when dipalmitoyl PC is introduced into the outer monolayer. Again, a contradiction with monolayer data is evident, because the area occupied by the dipalmitoyl PC (41Å$^2$ at 21 dynes/cm), is much smaller than that of the native PC molecules (65–80Å$^2$). Even the presence of cholesterol, which has an expanding effect on packing of 1,2-disaturated species (and a condensing effect on that of 1-saturated,2-unsaturated molecules) cannot give a satisfactory explanation because the area occupied by the cholesterol–dipalmitoyl PC complex (83Å$^2$ at 21 dynes/cm) is still smaller than that of the cholesterol–palmitoyl-oleoyl PC combination (91Å$^2$). As long as no data are available on the properties of PC molecules in the complex mixtures (as in the outer monolayer of the erythrocyte) and at higher surface pressures, the extrapolation of data from

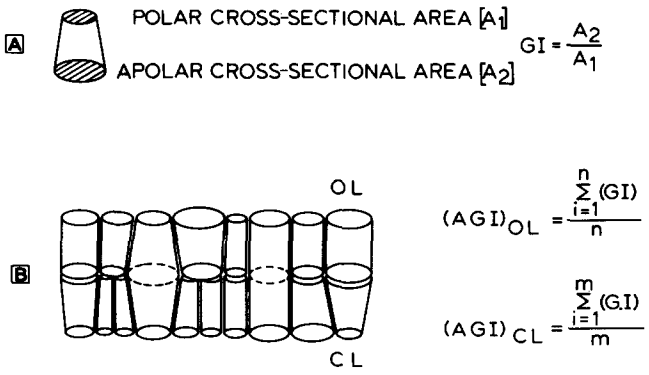


FIGURE 4 Working model to illustrate the effect of lipid geometry on overall organization of a membrane. (*A*) The geometrical index (*GI*) of a molecule is defined as the ratio between the space occupied by that part of the molecule deeply buried in the apolar core of the bilayer, and the optimal surface area of the polar part of this molecule. (*B*) A biological membrane of a complex mixture of constituents with different geometric parameters. The average geometrical index (*AGI*) of the outer layer (*OL*) and the cytoplasmic layer (*CL*) are defined as described. *n* and *m* represent the number of molecules in outer and cytoplasmic layer, respectively. It is possible to describe the curvature of a membrane with the ratio between the average geometrical indices of outer and inner layer, being 1.0 in a completely flat membrane. It can be assumed that under normal conditions the relation between the various parameters is optimal and energetically most favorable. Disturbances will result in an increase in the free energy content of the system and compensation for this has to be sought. This may lead to a rearrangement in the membrane by lateral and/or transbilayer movements of molecules and it can also lead to bending of the system in order to maintain proper interactions between individual membrane constituents. Of course, it is realized that the validity of such a static model is limited when applied to a dynamic system as most biological membranes are.

monolayer studies to the erythrocyte membrane can be misleading. Crenation of erythrocytes by dipalmitoyl PC can easily be explained, however, by considering the geometry of the molecule, which causes an effect opposite to that outlined above for 1,2-diunsaturated PC. When the native, cone shaped, PC is replaced by the cylindrical 1,2-disaturated species, the area occupied by the very end of the apolar tails (A2 in Fig. 4*A*) is decreased. Hence, the average geometrical index of the outer leaflet will decrease and, at a certain level of replacement, a bending outwards may be the way to maintain the most effective packing of the molecules in the membrane. Similar explanations, based on an increase or decrease of the geometrical index, can be given for the observed effects on shape after replacement with dimyristoyl PC, distearoyl PC, and dioleoyl PC.

This approach does not seem to provide a simple explanation for the complex effect found after replacement of the native PC by 1-palmitoyl,2-arachidonoyl PC. It has to be realized, however, that no data are available on the geometry and other physicochemical characteristics of this species and that replacement also leads to a strongly decreased osmotic fragility and increased leakiness of the cell (34).

The model depicted above means a refinement of the bilayer couple hypothesis of Sheetz and Singer (19), which considers shape changes of erythrocytes as a consequence of an increase in the total area occupied by the molecules in one monolayer of the membrane relative to the other layer. It is obvious that shape changes of the erythrocyte can also be

induced without any net increase in surface area of one of the monolayers. Physicochemical studies on the capability of phospholipid molecules to form either bilayer or non-bilayer structures, depending on both their fatty acyl constituents and nature of the polar headgroup, underline this approach. A recent publication (42, and the references cited therein) clearly illustrates this by showing that the ability for PC molecules to adopt hexagonal (non-bilayer) configurations is enhanced by the presence of two unsaturated fatty acids as for instance in the case of dilinoleoyl PC.

It has been estimated that only a very small (1%) increase or decrease in the surface area of the outer layer of the membrane is sufficient to induce changes in cell morphology (43). Also in the type of experiments described above, the change is very limited. Echinocytes are already formed when 20% of the native PC has been replaced by dipalmitoyl PC, and it can be estimated that this replacement accounts for ~2% of the total lipid complement.

A small modification in the conformation of the outer layer, resulting in crenation or cup formation, should be followed by the inner half of the bilayer, as well as by the membrane skeleton. Sheetz and Singer (19) provided the explanation for these phenomena by stating that the two monolayers are coupled. However, a major question remains unanswered by their hypothesis: Why are unilateral changes in surface area not instantaneously followed by transbilayer movement of one or more membrane constituents in order to regain equilibrium between the two monolayers? Such an event easily occurs in protein-free phospholipid vesicles, when one of the phospholipids in the outer layer is modified in its structure (44). In the erythrocyte, a proper candidate for such a compensatory process would be cholesterol, because it is present in large amounts and can undergo very rapid transbilayer movements (45). Nevertheless, compensation for unilateral modifications does apparently not occur this way, or only to a limited extent. Moreover, shape changes can even be induced by varying the amount of cholesterol (5–8).

We feel that the answer to the above question may be found in the assumption that the distribution of the lipid constituents over both halves of the bilayer is controlled by interaction with membrane proteins, and in particular, the phospholipids in the inner leaflet are fixed via interactions with the membrane skeletal network. Furthermore, recent findings indicate that the asymmetric distribution of sphingomyelin in the red cell membrane is highly static and that the individual molecules, unlike those of PC and phosphatidylethanolamine, may not experience transbilayer movements to any appreciable extent (46). Such a fixation may be expected to limit the freedom of the phospholipids to redistribute their total mass over both halves of the bilayer. Thus, a modification in the surface area organization of the outer monolayer can only to a limited extent be compensated for by a redistribution of lipid constituents over the two leaflets, and the compensation of the resulting tension between the two halves of the bilayer can only be found in bending of the whole membrane in either direction: outwards (echinocytes) or inwards (stomatocytes), depending on the nature of the change in the outer monolayer. Evidence is accumulating that the proposed interactions, at least with respect to the amino-phospholipids (phosphatidylethanolamine and phosphatidylserine) indeed exist, and that these are responsible for both maintaining the characteristic asymmetric distribution of the phospholipids over the two halves of the bilayer (3), as well as the relatively slow transbilayer movement of the individual phospholipid molecules (26, 36).

Finally, an additional observation that underlines this hypothesis has to be mentioned. Recovery from crenation has been reported recently by Alhanaty and Sheetz (47). Their observation that this recovery was a long-term, energy-requiring phenomenon indicates that structural rearrangements are necessary and that simple translocation of molecules is not possible. Our data on replacement with dipalmitoyl PC indicate the same: the potency for shape change recovery is limited and slow, and increased replacement with the (crenating) agent dipalmitoyl PC results in hemolysis.

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