MODELS OF ARTIFICIAL MEMBRANES

LECTURE

STUDIES ON THE RELEASE OF VESICLES FROM HUMAN RED BLOOD CELLS

Peter Ott

Institut für Biochemie und Molekularbiologie der Universität; Bühlstr. 28, CH-3012 Bern/Switzerland

ABSTRACT

Erythrocytes release membrane vesicles under various in vitro conditions such as ATP-depletion, Ca^{2+}-loading or upon incubation with suspensions of sonicated dimyristoylphosphatidylcholine (DMPC) or dilauroylphosphatidylcholine. All these pathways have in common, that an erythrocyte shape change is observed before vesicles are released.

DMPC-induced vesiculation starts after a incubation period of 60-90 minutes and is completed within 4 to 6 hours of total incubation time. After various preincubation protocols which result in a reduction of cellular ATP levels and a concomitant echinocyte formation, erythrocytes release vesicles much sooner upon addition of DMPC than do discocytes with normal ATP levels. Furthermore, the rate of vesicle release is accelerated. It appears that alterations which occur in metabolically depleted red cells make their membranes more susceptible to a further perturbation by the addition of exogenous DMPC.

On the other hand, vesicle release is abolished in deoxygenated sickle red blood cells. Only in oxygenated sickle cells - with depolymerized hemoglobin - DMPC-induced

Key words: ATP deplection, Ca^{2+}-loading, DMPC-induced vesiculation, echinocytes, vesicles release

vesiculation takes place. Obviously, the severe constraints that are imposed on the membrane skeleton of the cell by hemoglobin polymerization do no longer allow vesicle release.

Moreover, DMPC-induced vesiculation is dependent on a decrease of red cell membrane cholesterol levels. With unchanged membrane cholesterol content no vesiculation but merely a formation of echinocytes is observed when DMPC is introduced into the red cell membrane.

These observations and the fact, that the vesicles released from the cells are essentially free of skeletal proteins, suggest that the membrane skeleton has to be flexible enough to allow its local separation from the intrinsic domain before vesiculation can occur. This separation may be achieved during the formation of echinocytes that precedes vesicle release. Furthermore, only when two opposite regions of the lipid bilayer can approach each other very closely, a sufficiently destabilized lipid bilayer will allow the fusion event that has to be considered as a prerequisite of vesicle release from the echinocyte. This destabilization can be achieved by a modulation of the cholesterol level of the erythrocyte membrane.

INTRODUCTION

Shape changes of erythrocytes can be observed under a variety of conditions and have been related either to changes in the structural properties of individual protein components of the membrane skeleton (1-3) or to the action of certain membrane penetrating agents. Either stomatocytes or echinocytes can be formed (4). Echinocytes have further been shown to release membrane vesicles as a consequence of prolonged incubation in glucose free medium with concomitant ATP-depletion (5), or by increasing the intracellular calcium levels (6). Moreover, incubation of red cells with sonicated suspensions of dimyristoylphosphatidylcholine (DMPC) results in vesicle release, however without an ATP-depletion of the cells (7). Vesicle release has also been observed as a consequence of repeated deoxygenation and reoxygenation of sickle cells (8).

While the phospholipid composition of erythrocytes and vesicles appears to be comparable, clear differences are observed with regard to their respective protein composition. Integral membrane proteins, such as acetylcholinesterase, band 3 protein and glycophorin are present in vesicles but only traces of cytoskeletal components can be discovered (5, 6, 8, 9). This strongly indicates that interactions within the membrane and the membrane skeleton (2, 10) have to be changed or abolished prior to vesicle release. The mechanisms by which red cells can be stimulated to release vesicles are still

obscure. Both ATP-depletion of erythrocytes or incubation of cells with sonicated suspensions of DMPC first results in formation of echinocytes and then in release of vesicles. Under conditions of metabolic starvation more than 20 hours of incubation time are required to achieve ATP-depletion and initiate vesicle release (5). Upon incubation with DMPC, physiological ATP levels are maintained and only 4 hours are required for vesicle release (7).

Membrane penetrating agents, such as lysophospholipids and amphipathic drugs, are able to induce shape changes in human red blood cells as the result of a perturbation of the lipid bilayer balance but no vesicle release has been reported. Furthermore, a partial replacement of native erythrocyte phosphatidylcholine species by certain disaturated species results in the formation of echinocytes without membrane vesiculation (11). This suggests that echinocyte formation is not a sufficient prerequisite for the vesiculation to occur and indicates that an additional process is of importance in the mechanism of vesicle release from red blood cells.

According to the current model vesicle release from red blood cells can be divided into three steps. First, a cell shape change from discocyte to echinocyte takes place which then is followed (or even paralleled) by a (partial) dissociation of the intrinsic domain (lipids and transmembrane proteins) from the membrane skeleton. Finally, a membrane fusion event has to occur to allow the vesicle release.

The present contribution describes three approaches that have been used to elucidate the mechanism of vesicle release from red blood cells. First, ATP-depletion was applied in combination with DMPC to probe the influence of cellular metabolism on the vesiculation process (12). Then, sickle red cells, which are know to have several acquired membrane defects, were subjected to the treatment with DMPC (13) and finally, the influence of membrane cholesterol levels on red cell vesiculation was investigated (14).
In all experiments membrane bound erythrocyte acetylcholinesterase was used as a marker enzyme to characterize the velocity and the extent of vesicle release.

THE ROLE OF ATP

Incubation of red cells with sonicated DMPC suspensions results in vesicle release which starts after a lag period of approx. 60 to 90 minutes without significant decrease in the ATP level of the cells, and approximately 70 % of the total acetylcholinesterase activity is recovered in the supernatant within 4 hours after initiation of vesiculation. On the other hand, incubation of red blood cells in glucose free medium for more than 22 hours results in ATP-depletion and spontaneous vesicle release. The release rate, however, is very slow and 40-70 % of the total acetylcholinesterase activity can be recovered in the supernatant after 50 hours. Addi-

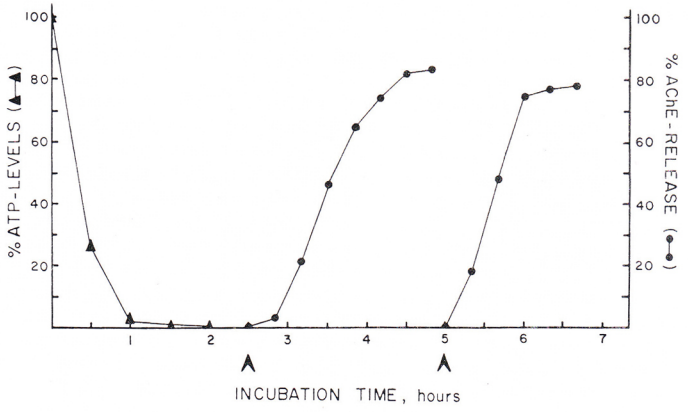

FIGURE 1

DMPC-induced vesicle release after rapid ATP depletion of red blood cells. Vesicle release was monitored by measuring acetylcholinesterase activity in the cell free supernatant. Filled triangles represent ATP content of erythrocytes and filled circles represent the acetylcholinesterase activity measured in the cell free supernatant. DMPC (0.5 mg/ml, final concentration) was added to red blood cells after ATP-depletion with iodoacetate for 2 1/2 hours (left arrow) or five hours (right arrow).

tion of iodoacetamide to red blood cell suspensions results in rapid ATP depletion within 2 1/2 hours of incubation, when ATP levels are less than 2 % of the initial values, but over 70 % of the cells are still discocytic (15). Incubation of these ATP-depleted cells results in vesicle release that starts shortly after the addition of DMPC and acetylcholinesterase activity in the supernatant reaches 70 % within 2 hours (Fig. 1). After 5 hours of preincubation under the same conditions all cells are transformed to echinocytes (15). Release of vesicles starts immediately after adding DMPC and the release rate is accelerated to such an extent that acetylcholinesterase activity in the supernatant reaches 70 % of the total amount in less than 1 hour. These results show that after preincubation protocols which induce echinocyte formation or reduce cellular ATP levels, erythrocytes release vesicles much sooner after the addition of DMPC than do discocytes with normal ATP levels. In addition, the rate of vesicle release is accelerated. Apparently, alterations that occur in metabolically depleted red cells make their membranes more susceptible to a further perturbation by addition of exogenous DMPC.

Although some controversy exists in the literature concerning the influence of ATP on the red cell membrane and its cytoskeletal network several effects of ATP-depletion have been described. Besides a dephosphorylation of spectrin (16) metabolic depletion also results in a decreased protection against the oxidative damage that promotes cross-linking of spectrin (17) and possibly a change in membrane lipid asymmetry (18, 19). Furthermore, red cell shape recovery mechanisms appear to be energy dependent (20) and metabolic depletion has been shown to promote cell shape changes (15). Likewise it was reported that diacylglycerol, a fusogenic lipid component, was formed in ATP-depleted red cells (21) and promoted to some extent membrane vesiculation processes. Furthermore, Huestis and coworkers have shown that phosphoinositide metabolism is affect by ATP-depletion which again does affect cell shape (22).

Earlier work by Feo and Mohandas (15) has shown that after 2 1/2 hours of fast ATP-depletion erythrocytes still maintain their discocytic shape while after 5 hours a complete transformation from discocytes to echinocytes takes place. These observations together with the results shown in Fig. 1 strongly suggest that it is primarily the increased number of echinocytes observed after ATP-depletion which accelerates the rate of vesicle release in these cells after addition of DMPC. On the other hand, the mechanism by which this shape change is promoted in energy depleted cells is not yet fully elucidated and may even have different causes which complement each other.

THE ROLE OF SICKLING

In sickle red blood cell several acquired defects have been reported, while the major congenital defect is an amino acid substitution which leads to a polymerization of the hemoglobin in the deoxygenated condition. Under oxygenated conditions such sickle cells show the same vesiculation behavior as normal cells (Fig. 2). However in a nitrogen atmosphere i. e. under deoxygenated conditions, the vesiculation process is significantly reduced. The inhibition of vesiculation by deoxygenation is fully reversible immediately upon reoxygenation of the cells (Fig. 3). The uptake of DMPC by sickle cells is comparable to normal cells and not decreased in the deoxygenated state. On the other hand vesiculation experiments under hypertonic conditions demonstrate that not the sickle morphology but rather the formation of polymerized hemoglobin is responsible for the reduced vesiculation in sickle erythrocytes. All these observations indicate that sickle hemoglobin polymers have a major influence on membrane rigidity which in turn affects the capability of the deoxygenated cells to release membrane vesicles when incubated with DMPC.

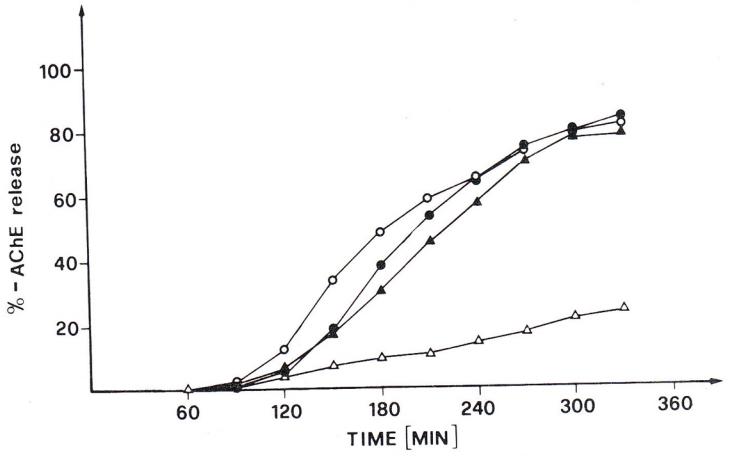

FIGURE 2

DMPC induced vesicle release of normal and sickle red blood cells. Vesicle release was monitored by measuring acetylcholinesterase activity in the cell free supernatant. The cells were incubated under oxygenated conditions (room air; filled symbols) or deoxygenated conditions (nitrogen; open symbols) with DMPC (0.5 mg/ml final concentration). Circles represent normal cells and triangles represent sickle red cells. Please note that oxygenated sickle cells show the same vesiculation behavior as normal cells.

THE ROLE OF CHOLESTEROL

As mentioned above a first step observed during vesicle release is the formation of echinocytes. However, such echinocytosis is also observed as a consequence of treatments with other membrane penetrating agents that do not induce vesicle release. Hence, the question arises what makes a cell progress to a point where it sheds vesicles. Up to now this second process, initiating membrane vesiculation of previously formed echinocytes, is not fully understood. It is well known that incubation of red blood cells with sonicated phosphatidylcholine vesicles decreases the cholesterol level in their membranes (23). Therefore the possibility has to be considered that, besides a formation of echinocytes, cholesterol depletion may be an important prerequisite of DMPC-induced

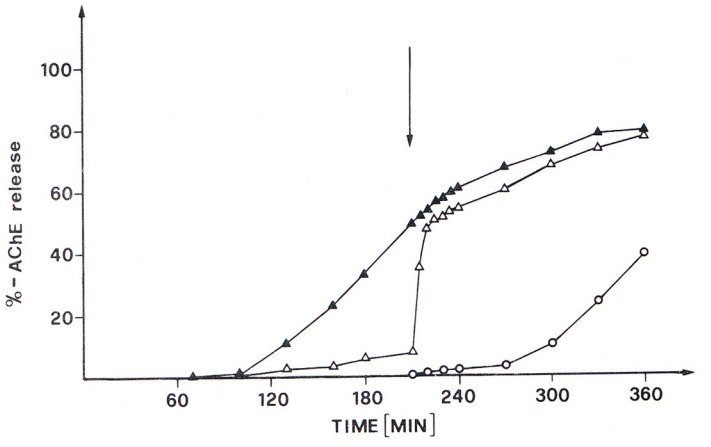

FIGURE 3

Effect of reoxygenation on vesiculation behavior of deoxygenated sickle cells. Vesicle release was monitored by measuring acetylcholinesterase activity in the cell free supernatant. Oxygenated (filled triangles) and deoxygenated (open triangles) sickle cells were incubated with DMPC as described in Fig. 2. After 3.5 hours of incubation the cells were reoxygenated by exposure to a stream of oxygen for 5 minutes (indicated by an arrow). To exclude an influence of the sickling-uncycling cycle on vesiculation in a control experiment (open circles) sickle cells were incubated under deoxygenated conditions in absence of DMPC for 3.5 hours. DMPC was then added to the specimen and after 2 minutes reoxygenation was started.

vesiculation. This notion also arises from the observation that vesicle release is suppressed in all incubations where the cholesterol level of the membrane is not altered although the uptake of DMPC into the membrane is the same under all conditions observed.

To study these questions cells were first loaded with DMPC and subsequently depleted of cholesterol or vice versa. Incubations were carried out in presence of a phosphatidylcholine specific phospholipid transfer protein, to ensure reproducible phospholipid transfer rates. With sonicated DMPC/cholesterol donor vesicles (1:1, mole/mole), in the presence of transfer protein no significant membrane vesicle release was observed (Fig. 4), not even when incubations were carried out for up to five hours. The extent of replacement of red cell phosphatidylcholine by DMPC was slightly over 20 % (Fig. 4). Only when these DMPC-loaded cells were subsequently

incubated with sonicated egg phosphatidylcholine vesicles, to reduce the cholesterol content in their membranes, an instantaneous release of vesicles was observed (Fig. 4). On the other hand with sonicated egg phosphatidylcholine/cholesterol vesicles (1:1, mole/mole) - which do not alter red cell membrane cholesterol levels - only about

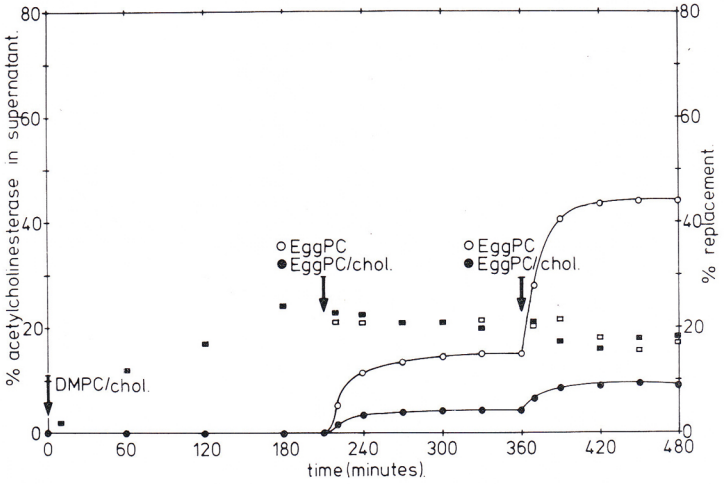

FIGURE 4

Incubation of human erythrocytes with sonicated DMPC-cholesterol vesicles in presence of phospholipid transfer protein, followed by cholesterol depletion. Vesicle release was monitored by measuring acetylcholinesterase activity in the cell free supernatant (circles). During the first three hours erythrocytes were incubated with DMPC/cholesterol vesicles (1:1, mole/mole) in presence of the phosphatidylcholine-specific transfer protein. After removal of those vesicles, cells were incubated with sonicated vesicles composed of either egg-phosphatidylcholine (open circles), or egg-phosphatidylcholine : cholesterol (filled circles), (1:1, mole/mole); these vesicles being replaced by new batches after another two hours of incubation. The extent of replacement of erythrocyte phosphatidylcholine by DMPC is expressed as % of total red cell phosphatidylcholine (squares). Open symbols represent incubation with DMPC/cholesterol vesicles, followed by egg phosphatidylcholine vesicles and filled symbols the incubation with DMPC/cholesterol vesicles, followed by egg phosphatidylcholine/cholesterol vesicles.

10 % of the acetylcholinesterase activity was found in the supernatant.

Apparently, erythrocytes containing appreciable amounts of DMPC will release vesicles as soon as their cholesterol level is decreased to about 80 % of its original value. Similarly, the lag period observed between the addition of DMPC and the onset of vesiculation is clearly reduced when membrane cholesterol levels are previously decreased to approximately 85 % of the normal value before the exogenous phospholipid is added. It appears therefore, that crenation without additional cholesterol depletion, is not sufficient to initiate membrane vesiculation.

It is most obvious that release of membrane vesicles from the intact red cell involves a fusion process between regions of the inner monolayer that approach each other very closely. This may occur during formation of echinocytes which is induced in our studies by the incorporation of DMPC into the membrane. Another and equally important prerequisite that should be fulfilled is a destabilization of the lipid bilayer to trigger the fusion process. Such processes have been reported to be critically dependent on an appropriate cholesterol/phospholipid ratio. In the present studies this ratio is apparently established by a decrease in the cholesterol content of the membrane.

In a recent report, Chabanel et al., (24) suggested that a decrease in red cell membrane cholesterol primarily increases the fluidity of the inner leaflet of the membrane which is highly enriched in the aminophospholipids, phosphatidylserine and phosphatidylethanolamine (25). Phosphatidylethanolamine is known to readily adopt a non bilayer configuration, such as the hexagonal H II phase, unless it is sufficiently stabilized in a bilayer configuration by other components. Hence, it may be suggested that a moderate cholesterol depletion of the erythrocyte results in an increased tendency of the red cell membrane phosphatidylethanolamine to adopt a non bilayer configuration. Such configurations have indeed been considered as an intermediate step in membrane fusion processes (26). So far, phosphatidylcholine induced vesiculation of erythrocytes has only been demonstrated upon incubation of the cells with sonicated DMPC or dilauroylphosphatidylcholine vesicles, but does not take place when dipalmitoyl- phosphatidylcholine is used although the latter lipid also induces the formation of echinocytes (11). Furthermore, also the amount of DMPC incorporated into the red cell membrane appears to be critical, since only limited vesiculation is observed when no more than 10 % of the red cell membrane phosphatidylcholine has been replaced by DMPC (Fig. 3). This indicates that the properties of the fatty acid moieties of the added lipid is equally important.

In conclusion, the studies summarized in this overview show that the stability and flexibility of the entire membrane as well as the fluidity of the lipid phase are important in vesicle release. Factors which influence these parameters do also influence or even modulate this fusion related process. Since membrane fusion is a

prerequisite of vesicle release, the studies presented here are of general interest in the investigation of processes where fusion-related phenomena - such as endocytosis or exocytosis - do occur.

REFERENCES

1. Mohandas, N., Chasis, J. A. and Shohet, S. B. The influence of membrane skeleton on red cell deformability, membrane material properties, and shape. (1983) Sem. Hematol. 20, 225-242.
2. Cohen, C. M. The molecular organization of the red cell membrane skeleton (1983) Sem. Hematol. 20, 141-158.
3. Goodman, S. R., Steven, R. and Shiffer, K. The spectrin membrane skeleton of normal and abnormal human erythrocytes. A review. (1983) Am. J. Physiol. 244, C121-C141.
4. Weed, R. I. and Chailley, B. In Red Cell Shape, Bessis, M., Weed, R. I. and Leblond, P. F., (editors), Springer Verlag, New York - Heidelberg - Berlin, 1973, pp. 55-68.
5. Lutz, H. U., Liu, S. C. and Palek, J. Release of spectrin free vesicles during ATP-depletion. (1977) J. Cell. Biol. 77, 548-560
6. Allan, D. and Thomas, P. Ca^{2+}-induced biochemical changes in human erythrocytes and their relation to microvesiculation (1981) Biochem. J. 198, 433-440.
7. Ott, P., Hope, M. J., Verkleij, A. J., Roelofsen, B., Brodbeck, U. and van Deenen, L. L. M. Effect of dimyristoyl phosphatidyl-choline on intact erythrocytes. Release of spectrin-free vesicles without ATP depletion. (1981) Biochim. Biophys. Acta 641, 79-87.
8. Allan, D., Limbrick, A. R., Thomas, P. and Westerman, M. P. Release of spectrin free spicules on reoxygenation of sickle cells. (1982) Nature 295, 612-613.
9. Weitz, M., Bjerrum, O. J., Ott, P. and Brodbeck, U. Quantitative Composition and Characterization of the Proteins in Membrane Vesicles Released from Erythrocytes by Dimyristoylphosphatidyl-choline. A Membrane System without Cytoskeleton (1982) J. Cell. Biochem. 19, 179-191.
10. Haest, C. W. M. Interactions between membrane skeleton proteins and the intrinsic domain of the erythrocyte membrane. Biochim. Biophys. Acta 694: 331-352, 1982.
11. Kuypers, F.A., Roelofsen, B., Berendsen, W., Op den Kamp, J.A.F. and Van Deenen, L.L.M. Shape changes in human erythrocytes induced by replacement of the native phosphatidylcholine with species containing various fatty acids. (1984) J. Cell Biol. 99, 2260-2267.
12. The influence of cellular ATP levels on dimyristoyl-phosphatidylcholine-induced release of vesicles from human erythrocytes". P. Bütikofer and P. Ott (1985) Biochim. Biophys. Acta 821, 91-96.

13. Effect of sickling on dimyristoylphosphatidylcholine-induced vesiculation of Human Red Blood Cells". P. Bütikofer, D. T.-Y. Chiu, B. Lubin and P. Ott, (1985) Biochim. Biophys. Acta 855, 286-292.
14. Effect of membrane cholesterol on dimyristoylphosphatidyl-choline-induced vesiculation of human red blood cells". E. J. Frenkel, F. A Kuypers, J. A. F. Op den Kamp, B. Roelofsen and P. Ott (1986) Biochim. Biophys. Acta 855, 293-301.
15. Feo, C. and Mohandas, N. Classification of role of ATP in red cell morphology and function (1977) Nature 265, 166-168.
16. Anderson, J. M. and Tyler, J. M. State of spectrin phosphoryla tion does not affect erythrocyte shape or spectrin binding to erythrocyte membranes. (1980) J. Biol. Chem. 255, 1259-1265.
17. Palek, J. and Lux, S. E. Red cell membrane skeletal defects in hereditary and acquired hemolytic anemias. (1983) Sem. Hematol. 20, 189-224.
18. Haest, C. W. M., Plasa, G., Kamp, D. and Deuticke, B. Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane (1978) Biochim. Biophys. Acta 509, 21-23.
19. Bergmann, W. L., Dressler, V., Haest, C. W. M. and Deuticke, B. Cross-linking of SH-groups in the erythrocyte membrane enhances transbilayer reorientation of phospholipids. (1984) Biochim. Biophys. Acta 769, 390-398.
20. Alhanaty, E. and Sheetz, M. P. Control of erythrocyte membrane. Shape recovery from the effect of crenating agents. (1981) J. Cell Biol. 91, 884-888.
21. Müller, H., Schmidt, U. and Lutz, H. U. On the mechanism of vesicle release from ATP-depleted human red blood cells (1981) Biochim. Biophys. Acta 649, 462-470.
22. J. E. Ferrell and W. H. Huestis. Phosphoinositide metabolism and the morphology of human erythrocytes. (1984) J. Cell Biol. 98, 1992-1998.
23. Giraud, F., Claret, M., Bruckdorfer, K.R. and Chailley, B. The effect of membrane lipid order and cholesterol on the internal and external cationic sites of the Na^+-K^+ pumps in erythrocytes (1981) Biochim. Biophys. Acta 647, 249-258.
24. Chabanel, A., Flamm, M., Sung, K.P.L., Lee, M.M., Schachter, D. and Chien, S. Influence of cholesterol content on red cell membrane viscoelasticity and fluidity (1983) Biophys. J. 44, 171-176.
25. Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. Organization of phospholipids in human red cell membranes as detected by the action of various purified phospholipases. (1975) Biochim. Biophys. Acta 406, 83-96.
26. de Kruijff, B., Cullis, P.R., Verkleij, A.J., Hope, M.J., van Echteld, C.J.A. and Taraschi, T.F. In The Enzymes of Biological Membranes, Martonosi, A.M., (editor) Plenum Press, New York, 1985, 2nd end., vol. 1, pp. 131-204.