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STUDY OF THE TRANSVERSE DIFFUSION OF SPIN LABELED PHOSPHOLIPIDS IN BIOLOGICAL MEMBRANES

I. HUMAN RED BLOOD CELLS

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SUMMARY

Spin labeled analogs of phosphatidylcholine were used to study the transverse diffusion (flip-flop) of phospholipids in the erythrocyte membrane. The nitroxide spin label was placed either on the β acyl chain or on the choline group. These labeled phosphatidylcholine molecules were incorporated into the membrane by incubation of the red cells at 22 °C with sonicated spin-labeled phosphatidylcholine vesicles from which all traces of free fatty acids and lyso derivatives were carefully removed by bovine serum albumin treatment. This incorporation did not provide any change in the morphology of the cell as indicated by scanning electron microscopy. When spin-labeled phosphatidylcholine, having a nitroxide on the β chain but near the polar head-group, was incorporated into the erythrocyte membrane, ascorbate treatment at 0 °C allows selective reduction of the signal coming from the outer layer of the membrane. When the label was on the polar head-group, the inner content of the erythrocyte rapidly reduced the label facing the cytoplasm, thus creating a spontaneous anisotropy of the labeling. The anisotropic distribution of spin-labeled phosphatidylcholine in the erythrocyte membrane was found to be stable at 22 and 37 °C for more than 4 h. It is therefore concluded that the rate of outside-inside and inside-outside transition is so slow that the anisotropic distribution of the phospholipids in the erythrocyte membrane can be maintained during cell life.

INTRODUCTION

There is now increasing evidence that many biological membranes show an asymmetric distribution of proteins [1–3] and lipids [1, 4, 5] between the inner and the outer membrane surface. The erythrocyte membrane is so far the most thoroughly investigated membrane with respect to its composition and the asymmetrical distribu-

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

tion of its phospholipids [1, 4, 5]. This inside-outside lipid asymmetry is of interest as regard to both the biological function and the biosynthesis of the membrane [6].

A particularly pertinent physicochemical parameter of such an asymmetry is the rate constant of the lipid transfer from one surface to the other, known as "flip-flop". Kornberg and McConnell [7] found that the transverse diffusion of phosphatidylcholine in sonicated egg lecithin bilayers had a half-time of 6.5 h at 30 °C. More recently, Johnson et al. [8] measured a half-time of 40 h at 20 °C in the same model system, while Sherwood and Montal [9] estimated the half-time for the flip-flop of the oleyl acid phosphate to be between 15 and 19 h at 22 °C. However, values of the order of a few minutes were obtained by McNamee and McConnell [10] in vesicles of excitable membranes. Furthermore, Grant and McConnell [11] suggested that, in *Acholeplasma laidlawii*, the characteristic time for the flip-flop of spin labeled phosphatidylcholine was 15 s or less. The former results would indicate that there is a highly restricted rate of flip-flop in artificial bilayers but the latter suggest that phospholipid flip-flop is a non-negligible event in the life-time of a biological membrane. If the flip-flop rate constant is very small, then newly synthesized asymmetric membranes would be expected to maintain this asymmetry for a relatively long time. On the other hand, if the rate constant is large, there must be a biosynthetic mechanism which restores this asymmetry.

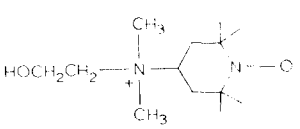
To determine the flip-flop rate of phospholipids in human red blood cells, where anisotropic distribution of phosphatidylcholine and phosphatidylethanolamine exists [4], we used two different spin-labeled analogs of phosphatidylcholine.

A morphological study of the labeled erythrocytes was also undertaken using scanning electron microscopy to determine whether any local perturbations were caused by the lipid nitroxide analogs.

MATERIALS AND METHODS

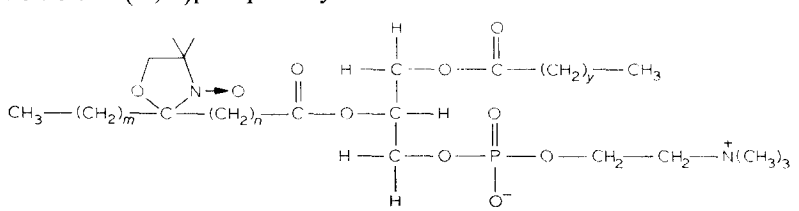
Spin-labeled molecules

TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) was a gift from Dr. Rassat (C.E.N. Grenoble). TEMPO-choline chloride:

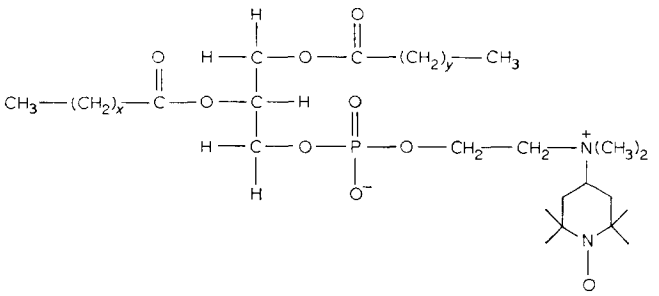


was prepared according to Kornberg and McConnell [7].

Various types of phosphatidylcholine molecule were prepared; (10,3)phosphatidylcholine and (7,6)phosphatidylcholine labeled on the β chain by an oxyoxazolidine ring according to Hubbell and McConnell [12]. Their general formula can be written as (m, n)phosphatidylcholine:



Another spin label analog, TEMPO-phosphatidylcholine, containing a probe on the choline group was synthesized from hydrogenated phosphatidylcholine according to Kornberg and McConnell [7].



Phosphatidylcholine was extracted from egg yolk according to the method of Singleton et al. [13]. Fatty acid-free bovine serum albumin was purchased from Pentex (Miles Laboratories, USA). Fresh human blood and lyophilized human serum albumin were provided by the Centre National de Transfusion Sanguine, Paris. Ascorbic acid was purchased from Merck.

Treatment of erythrocytes

Freshly collected human red cells from citrate/phosphate/dextrose-treated blood were washed three times by centrifugation (5 min, 2500 $\times g$) with the following isotonic buffer, referred to as buffer A: 150 mM NaCl, 8 mM KCl, 2 mM $MgSO_4$, 10 mM Tris/maleate (pH 7.4), 10 mM glucose, 1 % human serum albumin. Washed cells were ready for incubation with spin-labeled phosphatidylcholine analogs.

Erythrocyte ghosts were prepared according to Bodemann and Passow [14]. Only tested resealed ghosts (Type II) were used in our experiments.

Incorporation of the labeled phosphatidylcholine into erythrocyte membranes

(*a*) *Preparation of vesicles.* 3.6 μmol of the dry labeled phospholipid were suspended by Vortex mixing in 1 ml of the following buffer, referred to as buffer B: 150 mM NaCl, 8 mM KCl, 2 mM $MgSO_4$, 10 mM Tris/maleate (pH 8.2), 10 mM glucose. The suspension was then sonicated for 45 min at room temperature in an ultrasonic disintegrator (Ultrasons Anemasse) under argon. After sonication the suspension was treated with fatty acid-free bovine serum albumin polymerized by glutaraldehyde according to Avrameas and Ternink [15, 16] to remove lyso derivatives and fatty acids that appeared during sonication. This suspension was centrifugated at 30 000 $\times g$ for 35 min at 22 °C in a Beckmann model J 21 B centrifuge to remove the largest vesicles and titanium particles from the suspension. Pure (10,3) phosphatidylcholine and (7,6)phosphatidylcholine were used in preparing these vesicles. TEMPO-phosphatidylcholine was mixed with 50 % egg lecithin to obtain small vesicles.

(*b*) *Incorporation of labeled phosphatidylcholine molecules into erythrocyte membranes by fusion.* 1 ml of labeled phosphatidylcholine vesicle suspension (3.6 μmol/ml) was mixed with 1 ml of packed red blood cells (10 μmol membrane phospholipids/ml) and left to incubate for 1 h at room temperature. The red cells were then washed three times with the isotonic buffer A and collected by centrifugation. The molar ratio of

incorporation after incubation was about 2 % with (10,3)phosphatidylcholine and almost ten times lower with TEMPO-phosphatidylcholine.

Electron paramagnetic resonance spectra

Packed spin-labeled red cells were transferred to a 50 μl quartz sample cell and mounted vertically in a variable temperature accessory of a Varian E9 spectrometer. For most experiments the peak to peak amplitude of the midfield line of the derivative spectrum was used as a measure of the relative spin label concentration.

Measurement of the reduction of the nitroxide radical by ascorbate

1 ml of ice-cold 5 mM sodium ascorbate solution (pH 7.4) in buffer A was added at 0 °C to 1 ml of packed spin labeled erythrocytes. The mixture was immediately transferred to an EPR sample cell and the spectrum was recorded at 0 °C. The reduction by ascorbate was followed for 1–4 h in the case of (10,3)phosphatidylcholine labeling and for 15 min in the case of TEMPO-phosphatidylcholine. The intensity of a given peak divided by its initial amplitude at time 0 (h/h_0) was plotted versus time to determine unambiguously the level of maximum reduction. In all our experiments, there was at least a ten-fold excess of sodium ascorbate over spin label concentration in the treated sample.

Scanning electron microscopy

(a) The packed labeled erythrocytes were diluted a thousand fold with 2 % glutaraldehyde in Hanks medium at pH 7.4. After 20 min at room temperature, the cells were washed four times with Hanks medium and the pellet was fixed by adding a solution of 2 % osmic acid in distilled water. The preparation was left for 30 min at room temperature and the fixed cells were washed four times with double-distilled water. The pellet was submitted to several dehydrations with 50, 60, 90, 96 and 100 % ethanol and then diluted in propylene oxide to 5 ml. The suspension was then spread on a chemically clean glass slide and air dried at room temperature. The spread cells were shadowed with gold-palladium (60/40) and viewed at 4000–12 500 magnification with a Cameca MB-07 scanning electron microscope operated at 25 kV and 30° tilt angle. For differential counts several fields were selected at random and photographed at 4000 magnification. Cells incubated for 1 h in isotonic buffer A were used as control.

(b) The vesicle suspension was spread on a freshly cleaved mica slide and left 10 min for adsorption at room temperature. The preparation was fixed by immersing the slide rapidly into a solution of 2 % osmic acid, and left for 30 min at room temperature. After several washings and dehydrations with ethanol and liquid freon, the preparation was submitted to a last and complete dehydration using the freon critical point drying technique. The sample was then shadowed and observed as previously described.

RESULTS

(I) Morphological study

Scanning electron microscopy enables us to control the morphological aspect of red blood cells when incubated with spin-labeled vesicles.

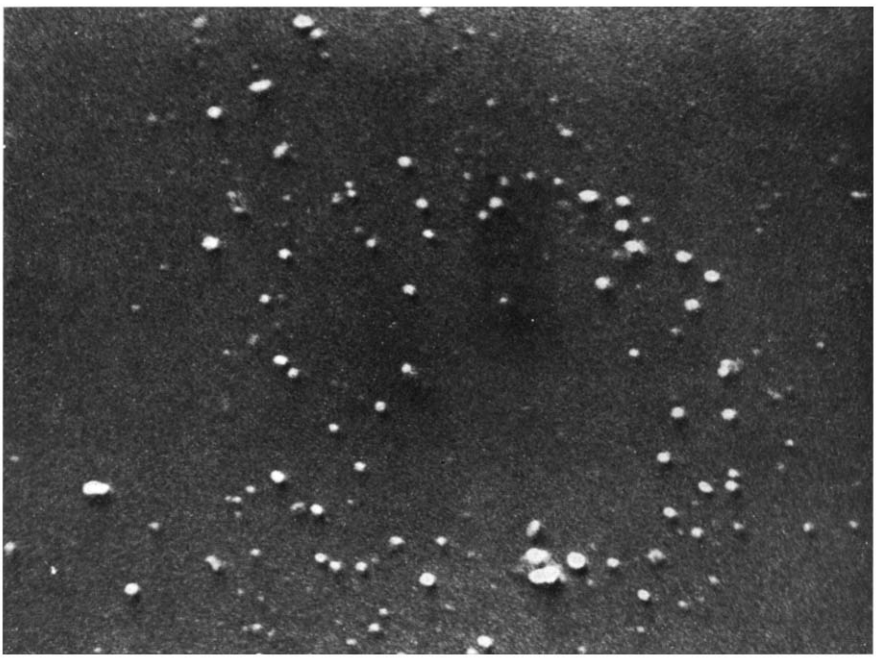


Fig. 1. Scanning electron micrograph of pure (10,3)phosphatidylcholine vesicles sonicated for 45 min in buffer B at 22 °C. The average diameter of the vesicles is 700 Å. Magnification × 12 500.

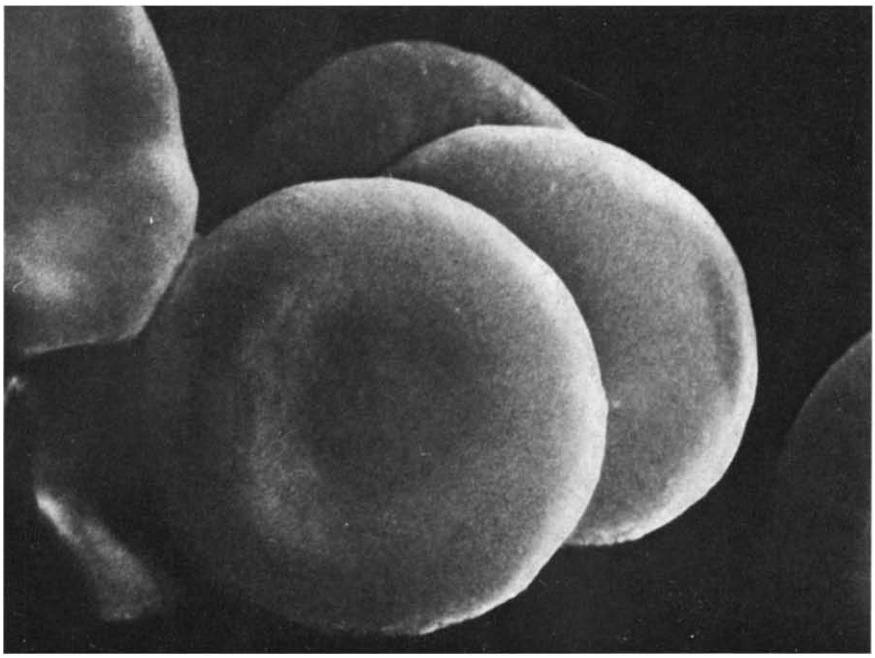
(*a*) *Vesicles*. Fig. 1 shows a representative population of spin-labeled phosphatidylcholine vesicles with an average diameter of 700 Å. These small vesicles are likely to be formed by single bilayers [7].

(*b*) *Erythrocytes*. Fig. 2 shows representative micrographs of untreated cells (2a, 2b) and cells exposed to sonicated vesicles for 1 h at 22 °C (2c, 2d). Most of the cells (in both preparations) exhibit a biconcave morphology. Less than 2 % of these cells (treated or untreated) present knob-like perturbances. No "scars" from fusion appear on treated erythrocyte membranes. Fig. 3 exhibits red blood cells incubated in the presence of liposomes (unsonicated vesicles) for 1 h at 22 °C. The size of the liposomes stuck onto the red blood cell membrane is about seven times larger than the size of the vesicles presented in Fig. 1. No fusion seems to occur in this case, as confirmed by the corresponding EPR spectrum representative of unincorporated labeled phosphatidylcholine. Fig. 4 is representative of a population of erythrocytes treated for 1 h at 22 °C by a suspension of sonicated vesicles which had not been submitted to the treatment with bovine serum albumin polymer. The appearance of echinocyte cells must certainly be due to the free fatty acids and lyso derivatives formed by degradation of the phospholipids during sonication.

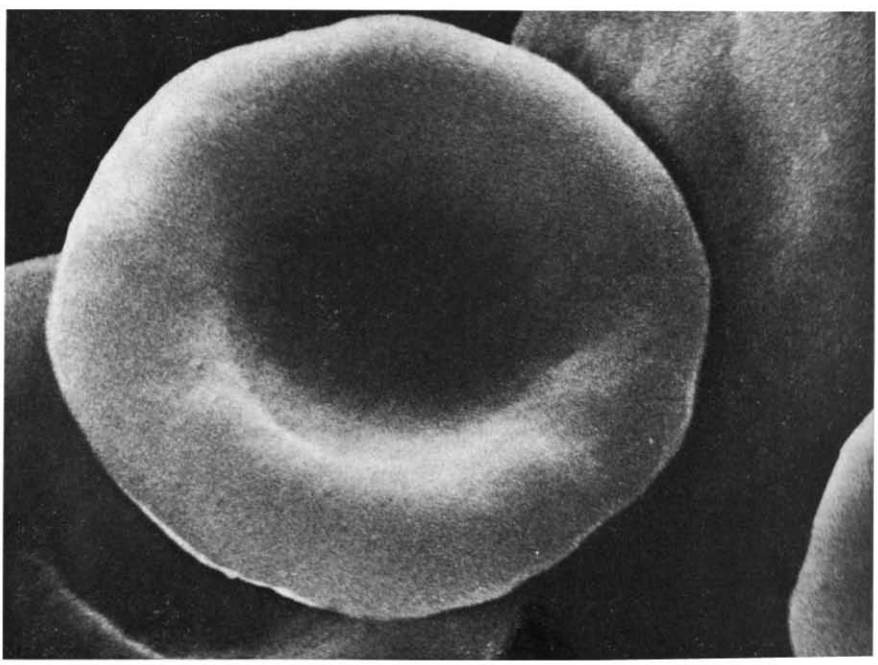
(II) Labeled phospholipids contained in the sonicated vesicles are incorporated into the erythrocyte membrane and diluted in the lipid matrix

As shown in Figs $5a_1$ and $5b_1$, the unpaired electrons in vesicles of pure (10,3)phosphatidylcholine or of 50 % pure TEMPO-phosphatidylcholine interact

(a)



(b)



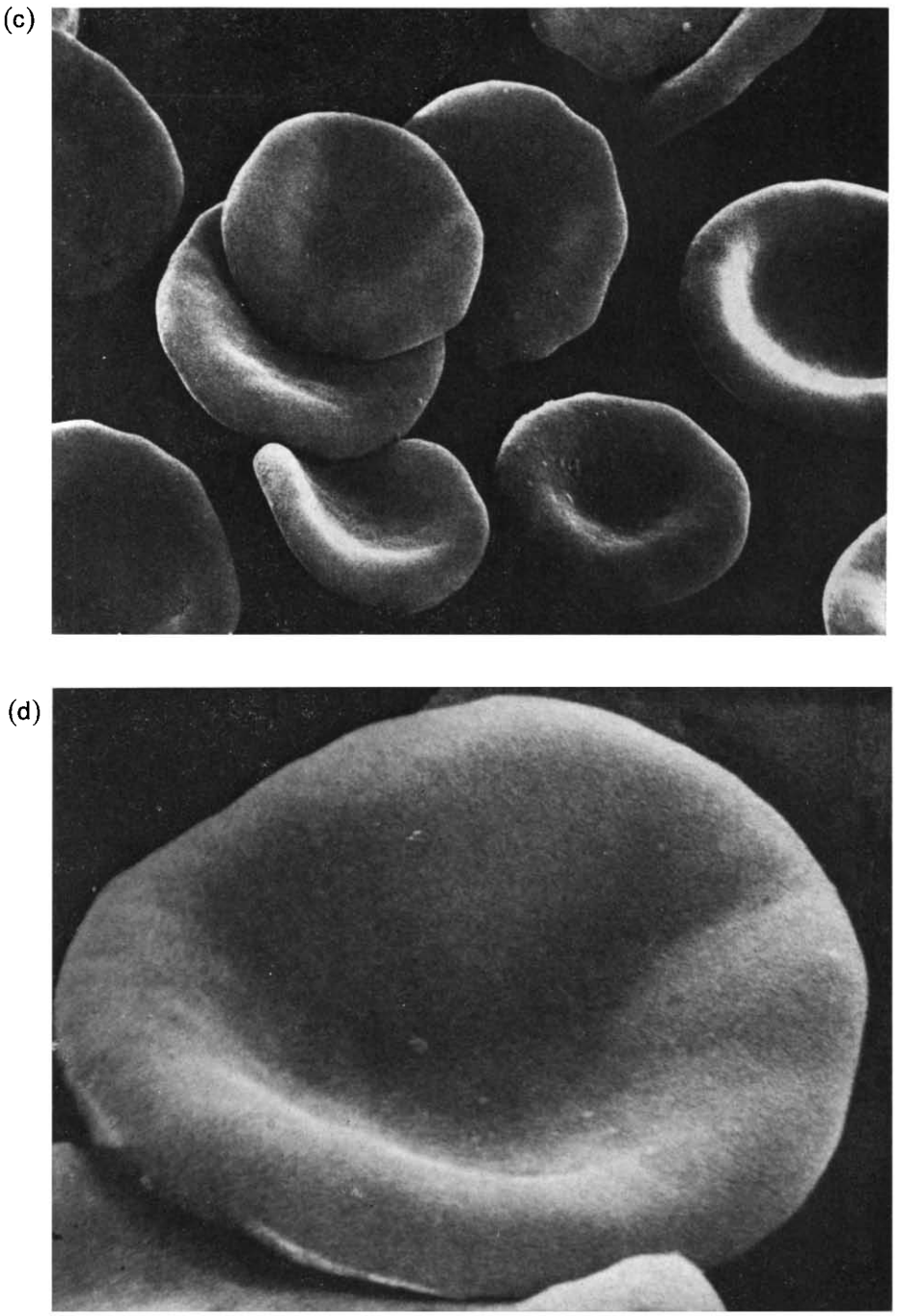


Fig. 2. Scanning electron micrographs of erythrocytes. (a–b) Untreated red blood cells, (c–d) (10,3) phosphatidylcholine labeled red blood cells. 1 ml of packed erythrocytes was incubated with 1 ml of (10,3) phosphatidylcholine vesicle suspension (3.6 μM/ml) for 1 h at 22 °C. 98 % of the erythrocytes in both preparations exhibit a biconcave morphology. Magnification: a, c × 2000; b, d × 6250.

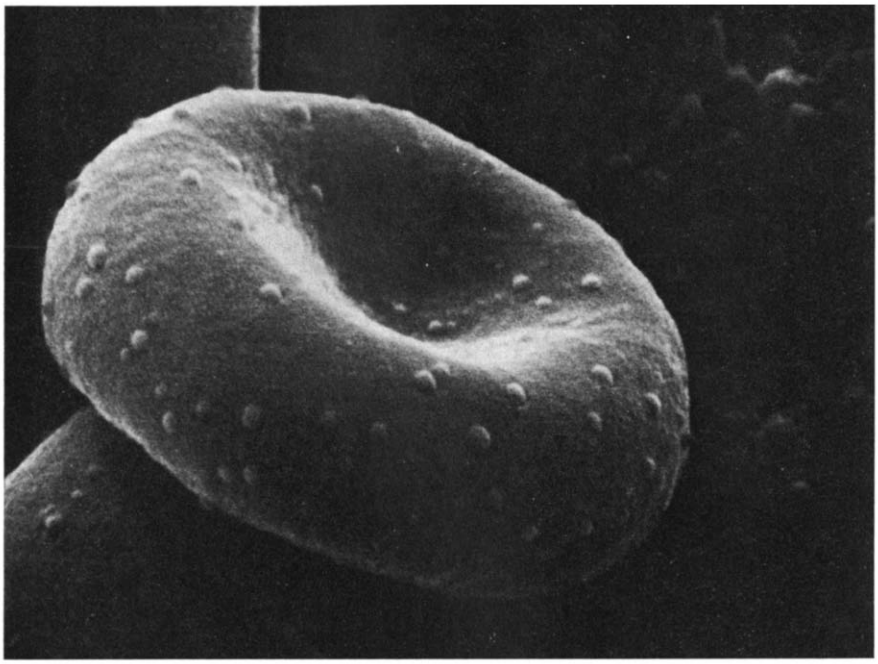


Fig. 3. Scanning electron micrograph of red blood cells incubated in the presence of (10,3)phosphatidylcholine liposomes (non-sonicated) for 1 h at 22 °C. Note that the liposomes are stuck on the cells membrane. No fusion seems to appear. The average size of the liposomes is about 5000 Å. Magnification × 6250.

strongly, giving rise to a broad EPR resonance line. After mixing the red blood cells with the spin-labeled vesicles (1 h at 22 °C) a new resonance pattern appears (Figs $5a_2$ and $5b_2$) very different from the broad resonance spectrum of the vesicles. This new pattern is attributed to spin labels that are incorporated into the membranes, and diluted into the membrane phospholipids [17].

(III) Incorporation of spin labeled phosphatidylcholine by fusion creates a symmetrical distribution of labeled phospholipids between both sides of the membrane

The method used to differentiate between the inner and the outer layer of the membrane containing spin-labeled phospholipids consists in a selective reduction of the outwardly exposed nitroxide by sodium ascorbate, which does not pass the phospholipid bilayer at 0 °C [7]. If the nitroxide is on one of the fatty acid chains of a phospholipid but not too far from the polar head-group (e.g. (10,3)phosphatidylcholine), it is still possible to reach the paramagnetic group with ascorbate, although the rate of reduction of the signal is much slower than with the TEMPO-phosphatidylcholine.

The EPR signal of (10,3)phosphatidylcholine-labeled erythrocytes, submitted to ascorbate treatment for 45 min, is reduced to 50 % of its initial value (Fig. 6). A plateau is reached and is maintained for 2 h. Fig. 7 shows the striking difference in the kinetics of reduction of the signal arising from (10,3)phosphatidylcholine and

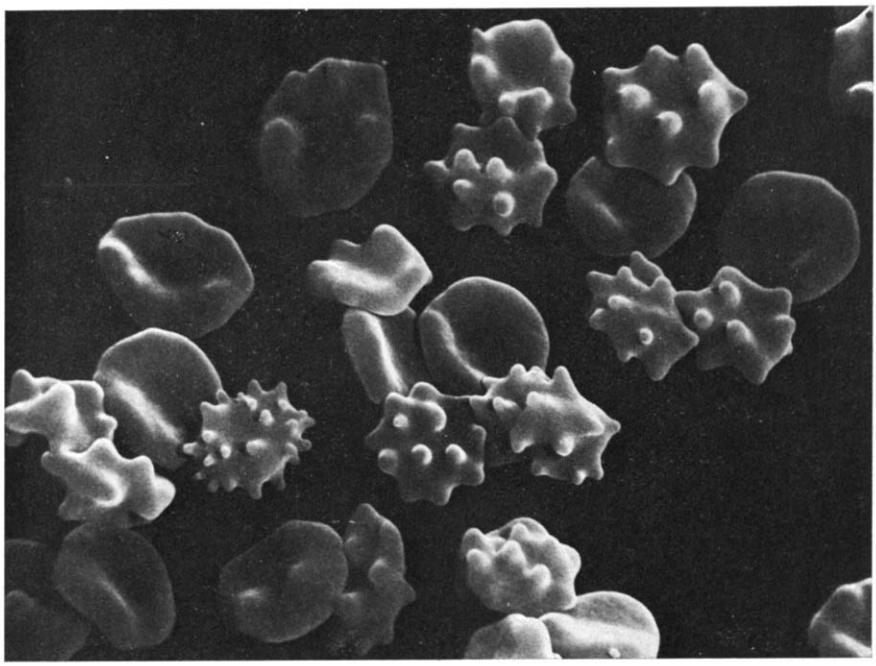


Fig. 4. Scanning electron micrograph of red blood cells incubated for 1 h at 22 °C in the presence of (10,3)phosphatidylcholine vesicles non-treated with bovine serum albumin polymer. Most of the cells exhibit an echinocyte morphology. Magnification × 1500.

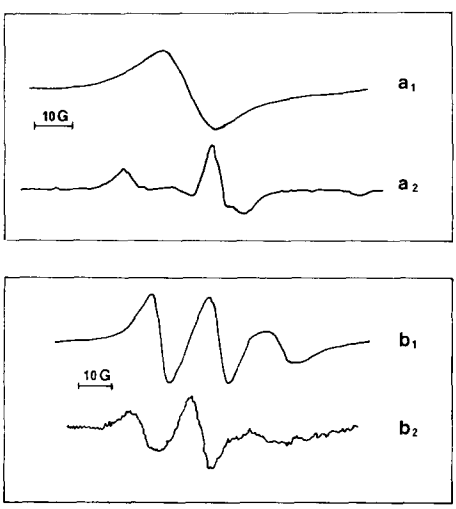


Fig. 5. EPR spectra of vesicles (a_1, b_1): a_1, pure (10,3)phosphatidylcholine vesicles; b_1, 50 % pure TEMPO-phosphatidylcholine vesicles. These spectra indicate a strong spin-spin interaction between the unpaired electrons of the nitroxide groups. EPR spectra of the spin-labeled erythrocytes (a_2, b_2): a_2, (10,3) phosphatidylcholine-labeled erythrocytes; b_2, TEMPO-phosphatidylcholine-labeled erythrocytes. These spectra indicate that the spin labels have been diluted (very likely) in the phospholipid bilayer.

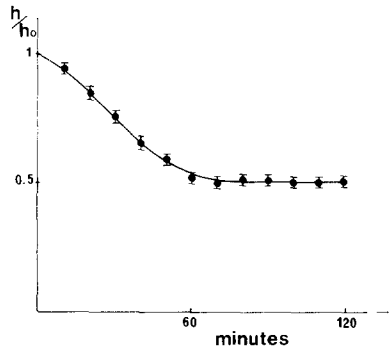


Fig. 6. Kinetics of reduction (10,3) phosphatidylcholine incorporated into erythrocyte membranes by sodium ascorbate ($2.5\ 10^{-3}$ M) at 0 °C. After 60 min 50 % of the nitroxides are reduced. This value is maintained for 2 h.

(7,6)phosphatidylcholine-labeled erythrocytes. In (10,3)phosphatidylcholine the nitroxide is near the polar head-group of the phospholipid, whereas in (7,6)phosphatidylcholine it is in the middle of the hydrocarbon chain. This provides a good control of the nonpenetration of sodium ascorbate in the membrane bilayer during the time of the treatment. We also showed that in erythrocyte resealed ghosts [14] containing 10^{-4} M tempocholine, no diminution of the EPR signal occurred in the presence of $2 \cdot 10^{-3}$ M ascorbate in the external medium for 2 h at 0 °C. These results indicate that, with the above conditions, sodium ascorbate does not cross the membrane and that fusion produces a symmetric labeling of the membrane with (10,3)phosphatidylcholine.

After reduction by ascorbate, the labeled phosphatidylcholine is only on the inner face of the membrane. It is interesting to note that no change in the shape of the signal accompanies the decrease of intensity. Thus the degree of motion, as detected by (10,3)phosphatidylcholine, is the same on both sides of the membrane in spite of a

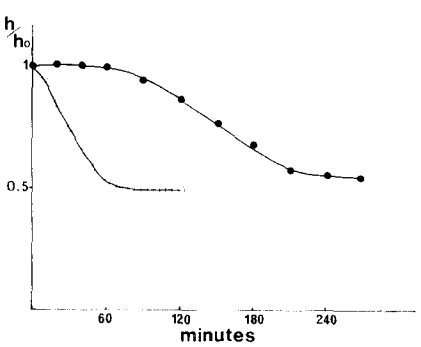


Fig. 7. Reduction by sodium ascorbate of two different spin analogs of phosphatidylcholine incorporated into erythrocytes. ($2.5 \cdot 10^{-3}$ M, 0 °C). ●, (7,6) phosphatidylcholine; ○, (10,3) phosphatidylcholine. The kinetics of reduction is more rapid in the case of (10,3) phosphatidylcholine-labeled cells (50 % after 45 min) than (7,6) phosphatidylcholine-labeled cells (40 % after 4 h). In (10,3)phosphatidylcholine the probe is near the polar head-group, in (7,6) phosphatidylcholine in the middle of the hydrocarbon chain.

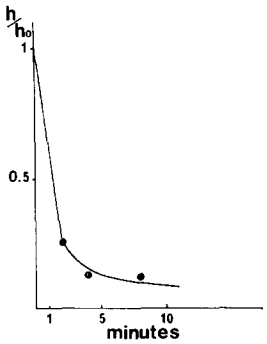


Fig. 8. Kinetics of reduction of TEMPO-phosphatidylcholine-labeled erythrocytes by sodium ascorbate ($2.5 \cdot 10^{-3}$ M, 0 °C). In this case the nitroxide group is on the polar head-group. 90 % of the signal has disappeared in 10 min.

possible difference in composition (see discussion). When TEMPO-phosphatidylcholine-labeled erythrocytes were submitted to the same ascorbate treatment, the EPR signal was rapidly abolished, indicating that this signal came from the outer layer of the membrane only. (Fig. 8). On the other hand, when ghosts labeled with TEMPO-phosphatidylcholine were submitted to the ascorbate treatment, the EPR signal rapidly decreases to a plateau at 50 % of the initial signal amplitude. Therefore, TEMPO-phosphatidylcholine incorporated by fusion is distributed on both sides of the membrane, but the nitroxide groups facing the inside of the red blood cell are reduced by the intracellular contents. Several controls were carried out to justify this hypothesis. When TEMPO-phosphatidylcholine-labeled erythrocytes were incubated with the supernatant of a sonicated preparation of red blood cells, a complete reduction of the signal occurred in less than 15 min at room temperature. On the other hand, after incubation of unlabeled erythrocytes with 10^{-4} M TEMPO, a penetrant molecule at 20 °C [10], the EPR signal amplitude of this small molecule was rapidly reduced (90 % in 15 min) indicating that the penetration of this molecule into the erythrocyte was accompanied by the reduction of the nitroxide group. These facts are in good agreement with the presence of a reducing agent in the inner content of the cell.

(IV) Study of the randomisation of an anisotropic labeling in erythrocyte membrane

(a) Use of (10,3)phosphatidylcholine labeling. An asymmetric labeling of erythrocyte, incubated in the presence of (10,3)phosphatidylcholine vesicles, was created by treatment of the cells with $2.5 \cdot 10^{-3}$ M sodium ascorbate for 50–60 min at 0 °C (Fig. 9). Erythrocytes were then submitted to several washings in buffer A at 0 °C to remove ascorbate from the medium. The packed cells were resuspended in an equal volume of isotonic buffer and left at room temperature for 0, 1, 2, 3 and 4 h before being submitted to a second ascorbate treatment. A sample was kept for control after the first ascorbate treatment to show that no further reduction or oxidation occurred. The reduction curves obtained after the double ascorbate treatment are shown in Fig. 9. It is clear that the initial slopes of the curve as well as the level of the plateau vary with the time between the two ascorbate treatments ("diffusion time"). This is indicative of a change in the spin label distribution after different incubation periods.

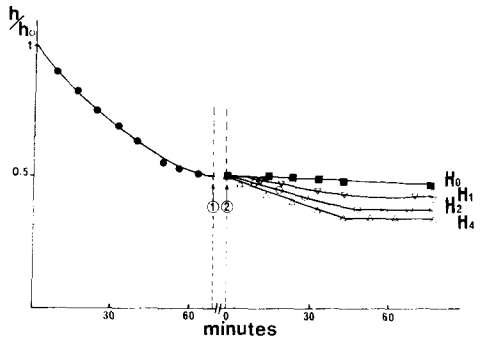


Fig. 9. Study of the flip-flop of (10,3)phosphatidylcholine in erythrocyte membranes. ●, Kinetics of reduction of the label by sodium ascorbate. 1, end of the first sodium ascorbate treatment; 2, beginning of the second ascorbate treatment. Ascorbate was in both treatments $2.5 \cdot 10^{-3}$ M. ■, diffusion time = 0 min (control); ▽, diffusion time = 1 h; ○, diffusion time = 2 h; △, diffusion time = 4 h. The level of the plateau reached after 45 min depends on diffusion time, indicating a slow flip-flop process.

We may describe the translocation of the spin-labeled molecules from the inner layer to the outer layer of the membrane, related to diffusion period t, by the following equation:

$$H = H_0 \exp(-t/\tau)$$

where τ represents the time after which $H/H_0 = 1/e$. H is the level of the plateau at time t and H_0 at time 0. Fig. 10 represents the variation of log H/H_0 versus diffusion time t; $1/\tau$ is the slope of that curve. The time necessary to transfer 50 % of the labeled phospholipid molecules from the inner to the outer layer of the membrane is about 7 h. This time represents the lower limit for the translocation.

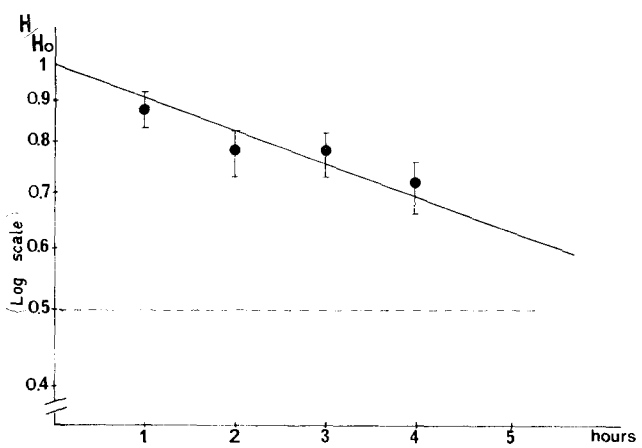


Fig. 10. (10,3)phosphatidylcholine flip-flop rate in erythrocyte membranes. The signal amplitude corresponding to a diffusion time t is measured from the value of the plateau reached after the second ascorbate treatment (see Fig. 9). The time necesary to transfer 50 % of the labeled phosphatidylcholine molecules from the inner to the outer layer of the membrane is about 7 h.

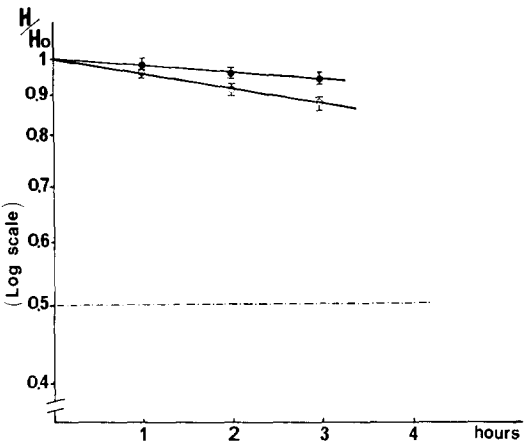


Fig. 11. Stability of the TEMPO-phosphatidylcholine signal at 22 °C and at 37 °C, after the inner content reduces the nitroxide. It is to be concluded that no translocation from outside to inside occurs for 3 h at 22 °C (○); at 37 °C (●) a slight reduction of the signal appears (15 % after 3 h).

(*b*) *Use of TEMPO-phosphatidylcholine labeling.* As mentioned previously, the asymmetry of labeling of erythrocytes incubated with TEMPO-phosphatidylcholine vesicles is created spontaneously by reduction of the inwardly exposed molecules by reducing agents inside the cell. In this case, the measure of the translocation rate of the labeled molecules from the outer to the inner layer is given by the decrease of the EPR signal amplitude as a function of diffusion time. Fig. 11 shows that practically no translocation occurs during 3 h at 22 °C. At 37 °C a slight difference appears with a decrease of 15 % of the initial signal after 3 h. In these experiments we consider that the process of flip-flop is too slow for determining the rate constant.

DISCUSSION

Our data provide evidence that fusion of sonicated labeled phosphatidylcholine vesicles with erythrocyte membranes allowed incorporation and dilution of the phosphatidylcholine analogs into the phospholipid bilayer. This incorporation was not perturbant for the cell morphology and occurred on both sides of the membrane, as demonstrated by a selective reduction method. Many controls were done showing that 2.5 mM ascorbate at 0 °C was a nonpenetrant agent and a good tool to reduce selectively the outwardly exposed labels. When we used TEMPO-phosphatidylcholine, a phosphatidylcholine analog with the label fixed on the polar head-group, the inner contents of the cell reduced the inwardly exposed labels, thus introducing a spontaneous asymmetry of the labeling. No treatment after incubation was necessary to study the redistribution of the label. Flip-flop in this case was measured by the evolution of the signal amplitude with "diffusion" time. This phenomenon was so slow that we could not evaluate the translocation rate. On the contrary, when (10,3)phosphatidylcholine was used to label the cell membrane, asymmetry had to be introduced by the ascorbate method, and redistribution of the inwardly exposed labels revealed by a second ascorbate treatment. The results obtained after these two ascorbate treatments of the cells gave a flip-flop value of 7 h.

The small difference in the flip-flop rate observed between (10,3)phosphatidylcholine and TEMPO-phosphatidylcholine labeled erythrocytes could be due to a possible perturbation of the membrane introduced by the ascorbate treatment in the case of (10,3)phosphatidylcholine-labeled erythrocytes. It is interesting to note that the preliminary experiments we have done with TEMPO-phosphatidylcholine-labeled erythrocyte ghosts indicate a flip-flop rate of 2 h at 22 °C. This more rapid flip-flop rate in red blood cell ghosts may be indicative of microscopic structural modifications of the bilayer induced by the "ghosting" procedure.

Again this suggests that perturbations of the membrane accelerate the flip-flop. Maybe introduction of spin labels into the erythrocyte membrane can create some perturbations in the structure of this membrane. Bieri et al. [18] observed that low concentrations (10^{-10}–10^{-5} M) of spin-labeled fatty acids can transform discocyte into echinocyte cells. Lysis of the cells occurred when labeled fatty acids were used at a concentration above 10^{-5} M. Deuticki [19] showed that after treatment by amphiphilic agents human red blood cells underwent morphologic transformations. Our studies show that the use of vesicles that contain degradation products (appearing during sonication) induced the formation of echinocyte cells (Fig. 4). The purification of the sonicated suspension using a bovine serum albumin polymer avoids such cell transformations by removing the lyso derivatives and the fatty acids from the suspension. Then measurements of the transverse diffusion of spin-labeled phosphatidylcholine in disc-shaped erythrocyte membranes gave a very long characteristic time (more than 24 h).

For all these experiments we have used labeled lipids. The two analogs of phosphatidylcholine are labeled by a different radical at different positions of the phospholipid structure. However, the results with these two spin labels are the same. In both cases, the effect of the perturbation induced on the lipids by the probe is probably to accelerate the flip-flop rather than to slow it down. Indeed the bulky oxy-oxazolidine of the (10,3)phosphatidylcholine certainly creates a local perturbation in the packing of the lipid chains. Since the tempo moiety of TEMPO-phosphatidylcholine is soluble in hydrophobic media as well as in hydrophilic solvents, the radical reduces the polarity of the head-group and should therefore facilitate flip-flop. The same conclusions were reached by Kornberg and McConnell [7].

It is reasonable to assume that our results with spin-labeled analogs of phosphatidylcholine can be generalized to ordinary phosphatidylcholine. The main conclusion is that the flip-flop of phosphatidylcholine (and therefore probably other phospholipids) is so slow that no exchange of phospholipids takes place between the two layers of the membrane by this process. The absence of transverse diffusion in erythrocyte membrane provides a good explanation for the maintenance of an asymmetrical composition of the membrane during the period of cell life. These facts strengthen the hypothesis of an asymmetrical biogenesis of the membrane.

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