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Lateral diffusion of erythrocyte phospholipids in model membranes comparison between inner and outer leaflet components

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Abstract. The physical properties of lipid bilayers with a similar composition to the outer and inner leaflets of the human erythrocyte membrane have been examined in protein-free model systems. The outer leaflet (OL) was represented by a phospholipid mixture containing phosphatidylcholine and sphingomyelin extracted from human erythrocytes, while a mixture of phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine represented the inner leaflet (IL). The ratio of cholesterol to phospholipid was varied in both mixtures. The lateral diffusion coefficient of fluorescent phospholipids diluted in such lipid mixtures was determined by the modulated fringe pattern photobleaching technique. Contrast curves with a single exponential decay, indicative of homogeneous samples, were obtained only for temperatures above 15 °C and for a cholesterol to phospholipid molar ratio below 0.8. The rate of lateral diffusion was approximately five times faster in IL than in OL multilayers, in agreement with former results obtained in human erythrocytes (Morrot et al. 1986). Varying the cholesterol to phospholipid ratio from 0 to 0.8 (mol/mol) enabled us to decrease the diffusion constant by only a factor of approximately 2 for both IL and OL mixtures. The order parameter of a spin-labeled phospholipid was determined in the different systems and found to be systematically smaller in IL mixtures than in OL mixtures. The present study indicates that the difference in lipid diffusivity of the two erythrocyte leaflets may be accounted for solely by a difference in phospholipid composition, and may be independent of cholesterol and protein asymmetry.

Key words: Phospholipid asymmetry – Lateral diffusion – Photobleaching – Spin-labels – ESR – Cholesterol

Introduction

Several types of biophysical experiments have revealed that the erythrocyte inner and outer leaflets have different dynamic properties. These experiments include: ESR of spin-labeled probes specific for the inner or outer monolayer (Tanaka and Ohnishi 1976; Seigneuret et al. 1984; Morrot et al. 1989), photobleaching determination of the rates of lipid lateral diffusion (Henis et al. 1982; Morrot et al. 1986), fluorescent polarisation experiments (Schroeder 1981; Cogan and Schachter 1981; Hale and Schroeder 1982; Schachter et al. 1982), and membrane binding of a fluorescent dye (Williamson et al. 1982). The majority of the reports indicate a higher viscosity in the outer monolayer. However, there are a few reports at variance with this generalization (Schachter et al. 1982; Henis et al. 1982). Recently Schroeder and Nemecz have discussed possible pitfalls which might explain these few exceptions (Schroeder and Nemecz 1989). The difference in physical parameters between inner and outer monolayers can originate from several factors reflecting the asymmetry of the plasma membrane composition: *i*) asymmetry of protein composition (Heast 1982); *ii*) asymmetry of phospholipid composition (Verkleij et al. 1973); and, *iii*) asymmetry of cholesterol distribution (Schroeder and Nemecz 1989).

The role of cholesterol has been frequently emphasized to explain the difference in viscosity. However the asymmetric distribution of cholesterol in erythrocytes is still a controversial subject and depending on the authors, cholesterol in red blood cells (RBC) is reportedly found mainly in the outer monolayer, in the inner monolayer or is equally distributed (see the reviews by Schroeder and

Abbreviations: OL, outer leaflet; IL, inner leaflet; RBC, red blood cell; NBD-PC, 1-acyl-2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino] dodecanoyl phosphatidylcholine; NBD-PE: 1-acyl-2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] dodecanoyl phosphatidylethanolamine; NBD-PS: 1-acyl-2-[12-(7-nitrobenz-2-oxy-1,3-diazol-4-yl)amino] dodecanoyl phosphatidylserine; DMPC, 1,2 dimyristoyl-sn-glycero-3-phosphocholine; DMPS, 1,2 dimyristoyl-sn-glycero-3-phosphoserine; PC, phosphatidylcholine; C/P, cholesterol over phospholipid molar ratio; D, lateral diffusion coefficient; S, order parameter; ESR, electron spin resonance; NMR, nuclear magnetic resonance; EDTA, ethylene diamine tetraacetic acid; TRIS, tris-(hydroxymethyl)amino ethane

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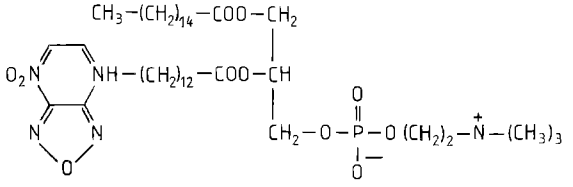
Nemecz 1988 and by Zachowski and Devaux 1989). Furthermore, the rates of lateral diffusion of phospholipids in the inner and outer monolayer of human RBC differ by a factor of 5, which seems to exceed the possible modulation of phospholipid lateral diffusion by cholesterol. For example, Thompson and Axelrod reported that a 30% cholesterol depletion of human erythrocyte membrane was not accompanied by a significant reduction of lipid lateral diffusion at 37 °C (Thompson and Axelrod 1980). In lipid bilayers comprising a single phospholipid species and cholesterol, no investigation has demonstrated that cholesterol enrichment is sufficient to account for, at a quantitative level, the difference in physical properties observed in RBC between the two leaflets; a factor of about 2 is the maximum effect reported (Devaux and McConnell 1972; Wu et al. 1977; Rubenstein et al. 1979; Kuo and Wade 1979; Lindbloom et al. 1981; Shin and Freed 1989).

In the present work we have purified RBC phospholipids and produced bilayers with a composition of either the inner or outer monolayer in an aqueous suspension. The rate of lateral diffusion was measured by the modulated fringe pattern photobleaching technique (Davoust et al. 1982; Morrot et al. 1986). In addition, the order parameter of a nitroxide spin-labeled phospholipid was determined by ESR. Samples with and without cholesterol were compared using both techniques. Finally, earlier data obtained on the native membrane enabled us to evaluate the overall effect of the proteins on lipid fluidity. The conclusion of the present investigation is that the asymmetric fluidity of the erythrocyte is primarily due to the difference in phospholipid head group composition between inner and outer monolayers rather than to the influence of cholesterol or protein.

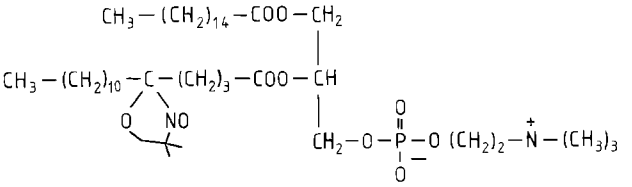
Materials and methods

Lipids

Erythrocyte phospholipids were extracted by the method of Rose and Oklander (1965) and purified by chromatography on CM-cellulose according to Comfurius and Zwaal (1977). Egg phosphatidylcholine was extracted from fresh yolks according to Singleton et al. (1965). Lipids were stored at −25 °C under argon to avoid peroxidation. Cholesterol (95% pure) was purchased from Fluka AG (Switzerland). 12-(7-nitrobenz-2-oxo-1,3-diazo-4-yl) aminododecanoic acid (NBD-FA) was synthesized from 12-aminododecanoic acid and NBD chloride. 1-acyl-2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) aminododecanoyl] phosphatidylcholine (NBD-PC):



was synthesized by the method of Samuel et al. (1985). The corresponding phosphatidylethanolamine (NBD-PE) and phosphatidylserine (NBD-PS) were synthesized from NBD-PC by the method of Comfurius and Zwaal (1977). The following spin-labeled lipid was synthesized according to Hubbell and McConnell (1971):



Oriented multilayers

2 mg of the desired lipid mixture, containing 0.2 mol% of fluorescent phospholipid, were dissolved in 100 µl of organic solvent (chloroform/methanol: 1:1; v/v) and applied as drops on a cleaned glass microscope slide at 40 °C. The solvent was evaporated under reduced pressure (16 mbars; 1 h). Two different methods were then used to hydrate the samples: (*i*) The slide was placed in a glass chamber which contained a reservoir of distilled water. The chamber was flushed with argon gas, to avoid oxidation, and maintained at 40 °C, for 24 h. The sample was then rapidly covered with a glass coverslip and the slide-lipid-coverslip assembly pressed between two metal blocks for 15 s. The pressed sample was sealed with glue, as rapidly as possible to minimize water evaporation. (*ii*) The dried lipids were exposed to water vapor at a temperature of approximately 45 °C for 30 s and immediately pressed and sealed. The sealed sample was then allowed to stand for 24 h at 40 °C before use. The samples were inspected optically using Nomarski interference-contrast to evaluate the extent of defects in the multilayers. The sealed samples appeared to be stable for several days. Storage at 40 °C improved the lipid ordering.

Lateral diffusion measurements

An argon laser (Spectra Physics model 164-08) tuned to 488 nm was used as the excitation source for a fluorescence inverted microscope (Zeiss IM 35). An oil immersion objective lens with a magnification of ×25 was employed. The modulated fringe pattern photobleaching technique produces a bleaching pattern composed of interference fringes with the decay of the fluorescence contrast between bleached and non bleached regions plotted either directly or after semi-logarithmic transformation (Davoust et al. 1982 and Morrot et al. 1986). The time constant (τ) of the decay curve for a single diffusion constant (D) is:

$$\tau = i^2/4\pi^2 D$$

where i is the interfringe distance which was varied from 1.4 to 8 µm so that τ was typically of the order of 500 ms to give diffusion coefficients in the range $1\text{–}40 \cdot 10^{-9}$ $cm^2\ s^{-1}$).

Liposomes for ESR and ^{31}P-NMR experiments

Magnetic resonance experiments were performed with hand shaken liposomes. 20 mg of phospholipids in chloroform: methanol (1:1; v/v) were added to a suitable amount of cholesterol and dried under vacuum for several hours before addition of buffer (TRIS 20 m*M*, NaCl 150 m*M*, EDTA 1 m*M*, pH 7.4). The concentration of lipids was of the order of 50 mg/ml. The lipid suspension was vortexed in the presence of glass beads for at least two minutes. Five cycles of freeze-thawing were then applied to the sample before use. For NMR experiments, the buffer contained a mixture of H_2O-D_2O (1:1). For ESR, 1% of the spin-labeled phospholipid was mixed with the unlabeled phospholipids before evaporating the organic solvent.

ESR experiments

50 µl sealed capillaries were used for ESR. The spectra were recorded with a VARIAN E109 century line spectrometer equipped with a temperature control accessory and connected to a Tektronix 4052 calculator. Order parameters were calculated by the following formula (Gaffney 1976):

$$S_{\text{eff}} = \frac{T'_{/\!/} - T'_{\perp}}{T_{zz} - 1/2(T_{xx} + T_{yy})} \frac{a_0}{a'_0}$$

with $a'_0 = (T'_{/\!/} + 2T'_{\perp})/3$; $a_0 = 15.2$ G; $T_{xx} = 6.3$ G; $T_{yy} = 5.8$ G and $T_{zz} = 33.6$ G.

NMR experiments

5 mm diameter tubes were used for NMR. ^{31}P-NMR experiments were run on a BRUKER MSL-300 at 121 MHz with broad proton decoupler, functioning at two levels (high decoupling during echo sequence) to avoid sample heating. A 90-τ-180-τ sequence was used, with $\tau = 20$ µs. The 90° pulse was obtained in 4 µs.

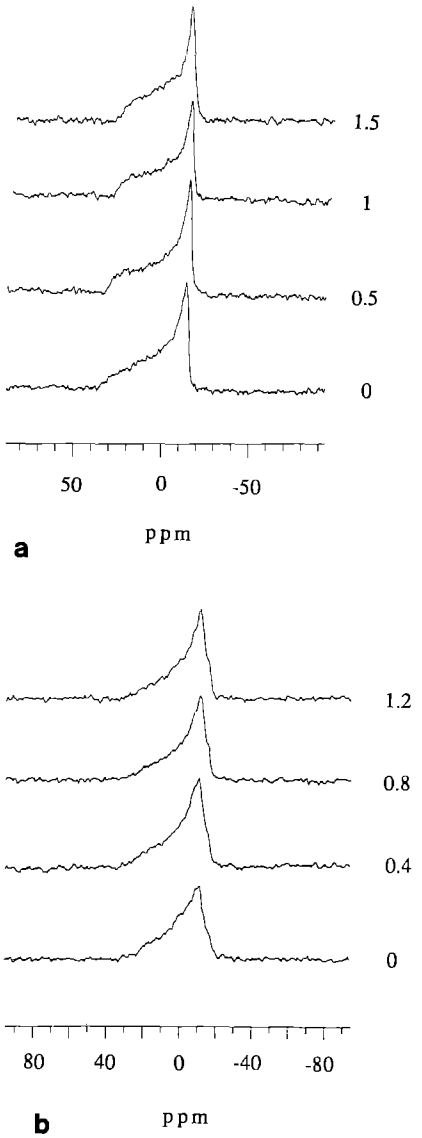


Fig. 1 a, b. ^{31}P-NMR spectra of liposomes made with lipids extracted from erythrocytes: **a** outer leaflet mixture (OL); **b** inner leaflet mixture (IL). The cholesterol to phospholipid molar ratio is indicated for each spectrum. Temperature 37 °C

Results

Throughout this investigation OL mixtures consisted of 50 mol% sphingomyelin, 40 mol% phosphatidylcholine and 10 mol% phosphatidylethanolamine, whereas IL mixtures contained 5 mol% sphingomyelin, 15 mol% phosphatidylcholine, 50 mol% phosphatidylethanolamine and 30 mol% phosphatidylserine. The ratio of phospholipid to cholesterol (mol/mol) varied from *0* to 2 for the magnetic resonance experiments (ESR and NMR) and from *0* to 0.8 for the photobleaching experiments.

NMR results

Figure 1 shows the ^{31}P-NMR spectra of OL and IL mixtures at 20 °C with various phospholipid to cholesterol ratios. All specta have a line shape characteristic of a lipid bilayer. The low field shoulder in some of the spectra is undetectable, suggesting partial liposome orientation in the magnetic field (Seelig et al. 1985; Speyer et al. 1987; Roux et al. 1988). The same characteristic spectra were obtained for temperatures between 5 °C and 37 °C (data not shown).

Morphological observations of the multilayers

Observation of the hydrated lipid multibilayers in the polarizing microscope enables the optical anisotropy or birefringence of lamellar lipid phases to be visualized. For the fluid phase lamellar, large domains without defects or with arrays of linear defects typical of bilayer stacking edges (Wu et al. 1977), can be selected for the photo-

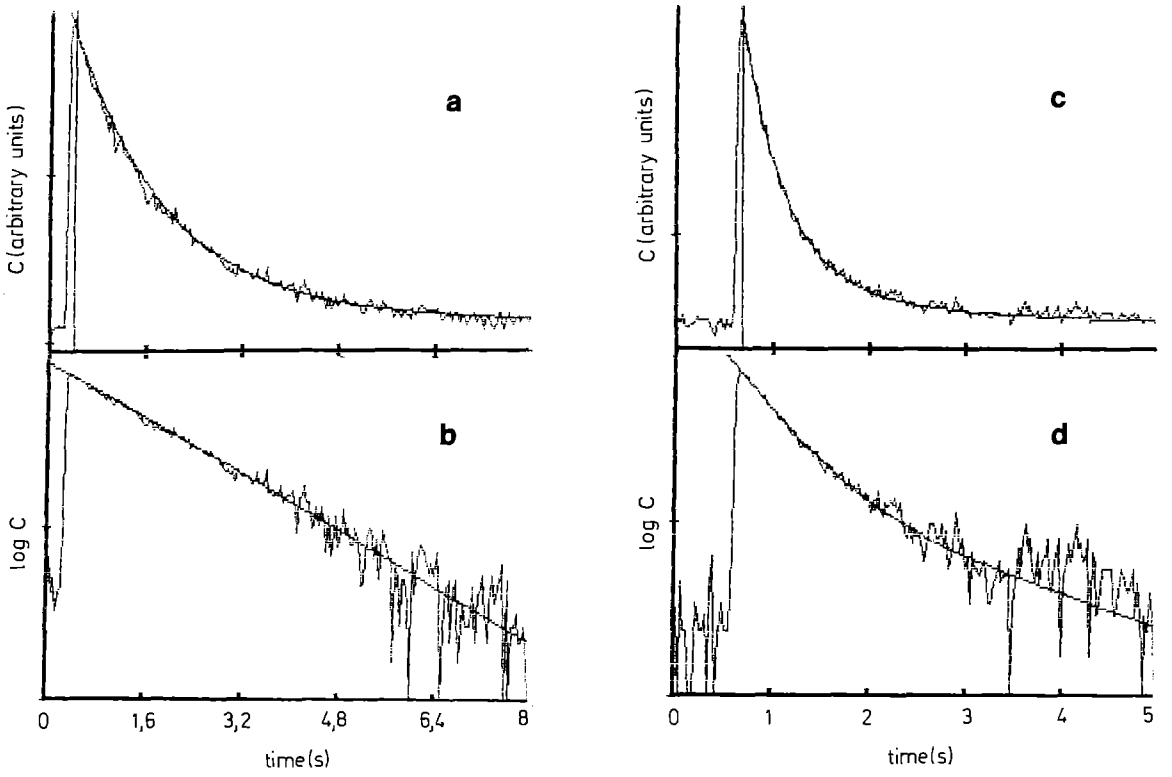


Fig. 2a–d. Decays of the fluorescence contrast after photobleaching (second harmonic of the modulation) for NBD-PC. **a, b** OL lipids at 37 °C, no cholesterol, bleaching time 50 ms, interfringe spacing 4.69 μm. $D = 3.5\ 10^{-9}\ cm^2\ s^{-1}$. **c, d** OL lipids at 5 °C, no cholesterol, bleaching time 100 ms, interfringe spacing 1,86 μm, $D_1 = 4.8\ 10^{-10}\ cm^2$ (11%) and $D_2 = 2.0\ 10^{-10}\ cm^2\ s^{-1}$ (89%). Note that the semi-logarithmic transformation reveals clearly whether the decay is mono-or multi-exponential. The curves superimposed on the experimental recording correspond to the best fit with either one or two exponentials

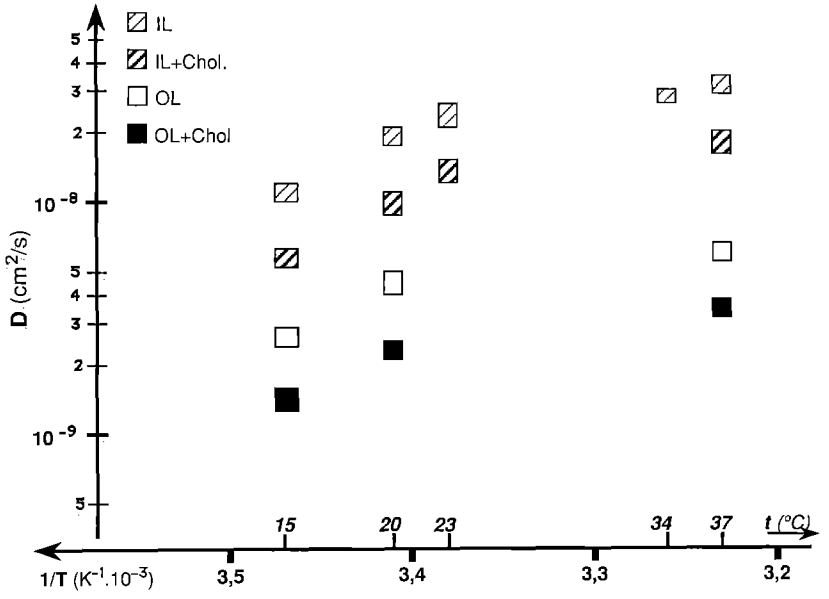


Fig. 3. Lateral diffusion of the NBD-PC probe in oriented multibilayers as a function of temperature. (▨) IL without cholesterol; (▨) IL with cholesterol, C/P = 0.8; (□) OL without cholesterol; (■) OL with cholesterol, C/P = 0.8. Error bars are explained in the text. Approximate activation energies of 18 kcal $mol^{-1}\ K^{-1}$ and 5 kcal $mol^{-1}\ K^{-1}$ can be calculated in the low and high temperature domains

bleaching experiments. In the presence of low cholesterol concentration, local defects are frequently seen at the junctions of the linear defects. If the cholesterol concentration exceeds a molar ratio of C/P ~ 0.8, large non-fluorescent domains with an apparent crystalline structural reveal an inhomogeneous lipid organization. This effect is accentuated at temperatures below 15 °C. The crystalline domains then appear even at low C/P. The temperature dependence of the domains suggests that they are not simply formed by cholesterol precipitation from the solvent during evaporation. In practice, the samples with a high C/P (i.e. above 0.8) were not suitable for photobleaching, probably because even the areas out of the cholesterol domains were inhomogeneus and not flat. Similarly lateral diffusion could not be determined reliably below 15 °C.

Lateral diffusion of fluorescent lipids

In preliminary experiments, we measured the rate of lateral diffusion of NBD-PC, NBD-PE, NBD-PS and NBD-FA in egg-PC multilayers. The four lipid probes gave fluorescent contrast curves corresponding to a single exponential associated with a lateral diffusion coefficient, at 20 °C, equal to $1.6 \pm 0.1\ 10^{-8}$ cm^2/s (data not shown). Thus, in agreement with previously published data, the diffusion of phospholipids in the liquid crystalline state determined using fluorescent labelled phospholipids, is essentially independent of the detailed structure of the lipid probe (Derzko and Jacobson 1980).

The results of Fig. 2 were obtained with NBD-PC in an OL mixture, at 37 °C (a, b) and 5 °C (c, d). The temperature dependence of the lateral diffusion coefficient D for this probe in OL and IL mixtures with and without cholesterol (0.8 mol/mol) is shown in Fig. 3 for the temperature range covering 15 to 37 °C. Intermediate concentrations of cholesterol gave intermediate results, however the uncertainty of each value was such that it was impossible to determine any relationship between diffusion coefficient and cholesterol concentration. Very similar results were obtained with fluorescent lipids having a different head group. In Fig. 3, the results for samples giving the higher diffusion coefficient for each temperature are shown. The uncertainty of the ordinate is associated with the determination of D on a single decay curve. However, repeating the same experiment on a new sample with similar composition, often gave a significantly different result, although associated again with a single exponential with low noise. The spread of the diffusion coefficient was greater for IL than OL, and greater for bilayers containing high cholesterol than for those containing no cholesterol. Figure 4 shows the actual distribution of the diffusion coefficients at 15 °C and 37 °C. Visual control of the samples by polarizing microscopy, suggested that the lower D values are in fact associated with incomplete hydration of the samples. Overnight incubation generally decreased the scatter of the diffusion data and favored the higher values.

At temperatures below 15 °C, or at high temperature for high C/P, all samples gave rise to multi-exponential decay curves (Fig. 2c). D values varied considerably from one sample to another. The decomposition into two diffusion coefficients indicated in the legend to Fig. 3 is just one example. Other samples gave more than two diffusion coefficients and no reliable values could be gathered from such samples.

Order parameter determinations with spin-labeled phospholipids

Figure 5 shows a comparison at 20 °C of the ESR spectrum of spin-labeled PC recorded after dilution in OL or in IL. As expected, the spectrum associated with the OL mixture indicates more immobilization than the IL mixture (larger hyperfine splitting). Figure 6 shows the effective order parameter calculated from the ESR spectra for different cholesterol to phospholipid ratios and for different temperatures.

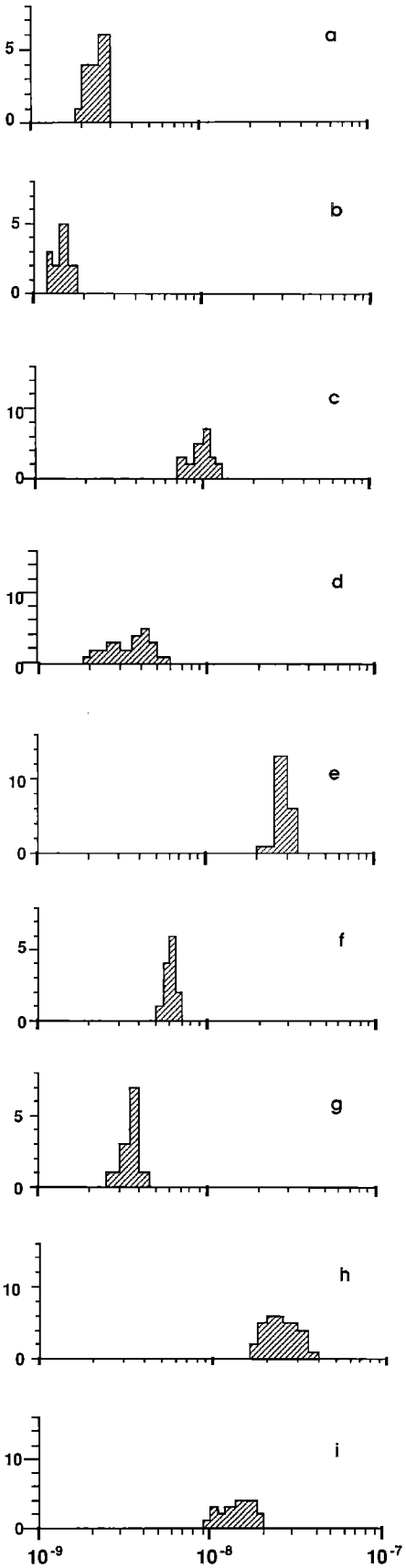


Fig. 4a–i. Distribution of measured D values of number n, in cm^2 s^{-1} as actually measured at 15 °C, in: **a** OL without cholesterol; **b** OL with cholesterol; **c** IL without cholesterol; **d** IL with cholesterol and, at 37 °C, in: **e** egg PC; **f** OL without cholesterol; **g** OL with cholesterol; **h** IL without cholesterol; **i** IL with cholesterol

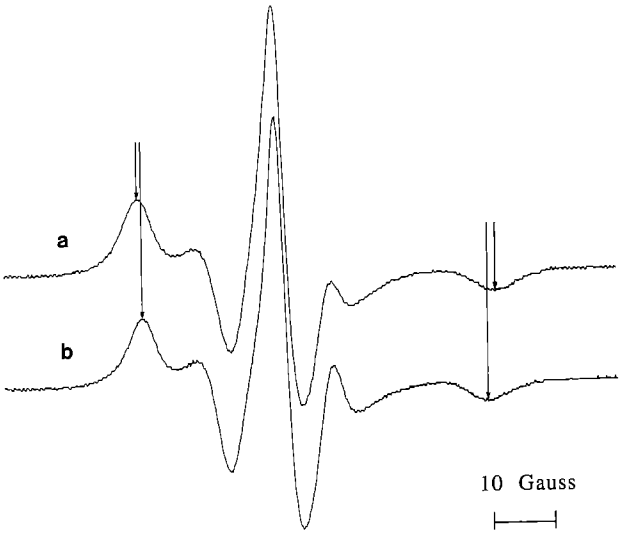


Fig. 5. ESR spectra of the C5-spin-labeled phospholipid probing: *a* OL; *b* IL. Temperature 15 °C. The outer extreme splittings are indicated by arrows

Table 1. Phospholipid lateral diffusion coefficients at 37 °C, as determined using NBD-PC for oriented lamellae of IL and OL lipids prepared as described in the text, $\times 10^9$ cm^2/s

	RCB[a]	Extracted phospholipids + cholesterol ($C/P \sim 0.8$)[b]	Extracted phospholipids[b]	White ghosts[c]	Total extracted lipids[c]
IL	8 ±1	18 ±2	32 ±3	2.0±0.3	7.8±0.7
OL	1.4±0.3	3.5±0.35	6.1±0.6		

[a] from Morrot et al. (1986), actual temperature 35 °C
[b] this work
[c] from Golan et al. (1984)

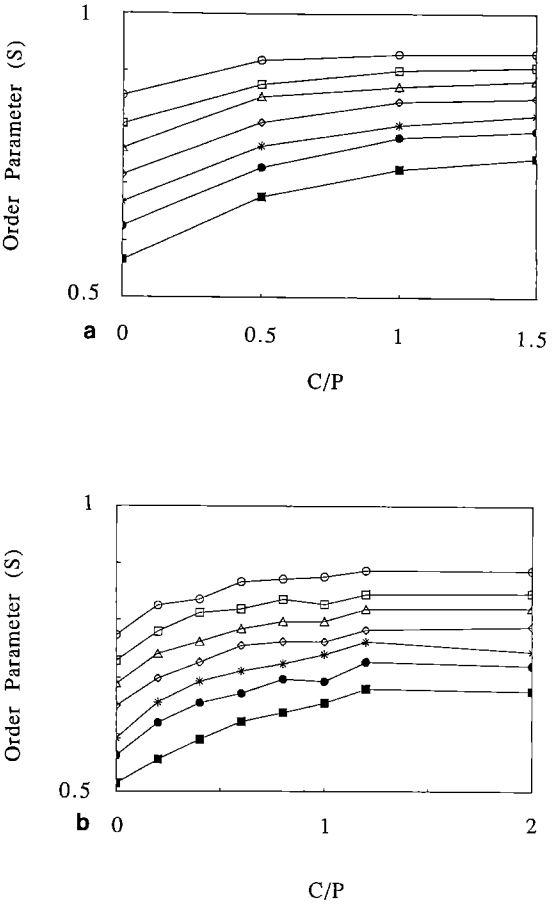


Fig. 6a, b. Order parameter of the spin-labeled phospholipid as a function of C/P ratio and for different temperatures, in: **a** OL; and **b** IL. For C/P values above ~1. *S* is barely sensitive to the cholesterol level, which suggests inhomogeneous lipid mixing above a certain cholesterol threshold. (○), 5°; (□), 10°; (△), 15°; (◇), 20°; (*), 25°; (●), 30°; (■), 37°

Discussion

Phospholipids in the erythrocyte membrane form a bilayer (Finean 1969; Cullis and Grathwohl 1977). Furthermore, Hope and Cullis showed by ^{31}P-NMR that lipid mixtures, analogous to the lipid composition of each erythrocye leaflet, separately form bilayers at 20 °C, at least in the absence of Ca^{2+} ions (Hope and Cullis 1979). We have confirmed this behaviour with the IL and OL mixtures. From 4 to 37 °C, the ^{31}P-NMR spectra for both IL and OL mixtures, with or without cholesterol, were characteristic of lipid bilayers. However, NMR experiments were carried out with an excess of water, conditions under which photobleaching cannot be performed since oriented multilayers with a minimum of water are required for the latter measurements. In addition, ^{31}P-NMR is a rather inappropriate technique to determine whether a bilayer is composed of a homogeneous fluid phase or a mixture of fluid and gel phase. In practice, NMR reflects the average phospholipid structure. Thus, there are certain difficulties inherent to the model systems used in the present investigation that should be discussed before relating our data to the red blood cell membrane.

As indicated previously by McCown et al. (1981), for a given phospholipid mixture and a given temperature, the rate of lateral diffusion can be taken as an indication on the degree of hydration. Prolonged incubation in the presence of water vapor generally resulted in a progressive increase of the diffusion and a diminished scatter of the data recorded with different samples. Following this criteria, we have found that the hydration of phospholipids extracted from RBC, particularly IL lipids, was more difficult and less reproducible than in the case of egg-PC. As a general rule, we have considered the highest diffusion coefficient to be the more significant, i.e. to correspond to optimum hydration (see Fig. 5).

In the present investigation, each sample mimicing one layer of the erythrocyte membrane contained at least three phospholipid species, plus, in some instances, cholesterol. Thus, it might be anticipated that complicated phases exist with mixed lipid dispersions at low temperature, as indeed was suspected from visual inspection and from the diffusion data. Microscopic inspection with polarized light revealed large domains of defect-free stacked bilayers only at temperatures, above ~15 °C and preferentially with samples containing a low amount of

cholesterol. The visual heterogeneity was generally, but not always, accompanied by multi-exponential decay curves. There is a vast literature concerning the miscibility of cholesterol in a single phospholipid species (see for example the review by Vaz et al. (1982) and references cited therein). The general conclusion seems to be that above a certain threshold, close to 20% cholesterol, heterogeneous domains are formed, with cholesterol being concentrated in certain areas of low lipid diffusivity. In practice, we have restricted our diffusion measurements to a maximum of 0.8 mol of cholesterol per mol of phospholipid. Above this ratio, no homogeneous looking sample could be formed. Such a molar ratio is close to that corresponding to an average distribution of cholesterol between both erythrocyte monolayers. This means that we have not been able to measure the influence of a putative asymmetric cholesterol distribution, which in fact may be physically impossible to generate. At any rate, as discussed below, the mere difference in phospholipid composition of the two erythrocyte leaflets can explain the difference in rates of phospholipid lateral diffusion, independantly of a putative cholesterol modulation. The discussion is therefore restricted to results obtained at 37 °C which is the RBC physiological temperature and where single exponential fluorescent decay results were obtained. The data which have been presented in the "Results" correspond essentially to NBD-PC. A few experiments with other headgroup lipids gave the same results.

Table 1 shows a comparison between the diffusion coefficients, at 37 °C of NBD-PC in each monolayer of intact RBC, in ghosts, in total lipid extract from RBC and in IL or OL mixtures, with (molar ratio 0.8) or without cholesterol. The diffusion constant for extracted lipids either with or without cholesterol, shows a 5 fold increase for the IL lipid mixture when compared with the OL mixture. The addition of cholesterol modifies D by only a factor of 2, in accordance with previous investigations on the diffusion of DMPC or DOPC with or without cholesterol (Vaz et al. 1982). Thus the difference in the chemical nature of the phospholipids normally present respectively in the inner and outer leaflets imposes a large difference in phospholipid diffusivity and in fact precludes a strong cholesterol asymmetric distribution.

Phospholipids of human erythrocyte inner and outer leaflets differ not only in the nature of their head groups (choline versus serine and ethanolamine) but also in the degree of chain unsaturation, the lipids of the inner leaflet (IL) being more unsaturated than those of the outer leaflet (OL) (Ways and Hanahan 1964). Published data on lipid lateral diffusion indicates that, at the same temperature, the diffusion constant is higher in unsaturated lipids than in saturated lipids, in the liquid crystalline phase (Vaz et al. 1985). However the differences are quite small between, for example, a series of phosphatidylcholine molecules. The differences are probably too small to account for the differences seen between the two erythrocyte leaflets, this is probably mainly due to head group differences.

As for the proteins, their overall effect on the phospholipid diffusion rate in red blood cells can be estimated by comparing the results obtained with extracted lipids and with native membranes. The average effect (compare columns 1 and 2 in Table 1) is a 2 fold diffusion coefficient change, which, most importantly, is the same for both layers. Thus, the interaction of the cytoskeleton with the lipids present in the RBC inner monolayer has no major influence on the phospholipid lateral diffusion constant, as already suggested by previous studies (Chang et al. 1981; Maksymiw et al. 1987; Calvez et al. 1988; Bitbol et al. 1989). In addition, PS, PE and PC diffuse at approximately the same rate when in the inner leaflets of intact RBC (Morrot et al. 1986), this is also an indication of the limited influence of the proteins on lipid diffusivity.

While the diffusion coefficient varies by a factor ~ 5 between IL and OL mixtures, PC order parameter S differs only by approximately 10% between the two lipid mixtures. Cholesterol addition can induce comparable or even larger S changes. Similarly proteins can increase S by more than 10%. However there is no direct relation between order parameters and lipid lateral diffusion. Shin and Freed (1989) have found an empirical logarithmic relationship between D and S measured with a spin labeled phospholipid, which at least suggests that D is a more sensitive parameter than S. Therefore, the ESR data alone do not enable one to discriminate between the respective influences of proteins, cholesterol and phospholipid composition on the local fluidity. However the ESR data are consistent with the photobleaching data, in the sense that the difference in S values could be due to the difference in phospholipid composition between inner and outer leaflets.

Attempts were made to relate the rates of lateral diffusion to membrane fluidity, in particular within the framework of the "hydrodynamic theory" of Saffman and Delbruck (1975). However, it appears that lipid translational diffusion may be more appropriately described by the "free volume theory", first introduced in the area of membranes by Galla and coworkers (Galla et al. 1979) than by the hydrodynamic theory. See the review by Clegg and Vaz (1985). Thus, rather than emphasizing a difference in viscosity beween the two red cell monolayers, it is perhaps more appropriate to speak of a packing difference. Schlegel and Williamson have emphasized for many years this asymmetric packing of the erythrocyte membrane (Schlegel et al. 1980). The tight packing on the outer monolayer would protect the cell from fusion; a loosening of the lipids makes the cell fusion competent (Schlegel and Williamson 1988). It is also very probable that this asymmetric packing influences the red cell shape.

Our general conclusion is that the dominant phenomenon which explains the asymmetric lateral diffusion in RBC seems to be the head-group segregation of the phospholipids between inner and outer leaflets rather than cholesterol or protein asymmetry. One of the consequences of this finding is that the asymmetric fluidity of red cells cannot be used as an argument to demonstrate cholesterol asymmetry, which remains a controversial subject. As for the phospholipid asymmetry, it is mainly due to the aminophospholipid translocase activity (Seigneuret and Devaux 1984; Devaux 1988). Thus, one of the functions of the aminophospholipid translocase in red

cells would be to preserve the membrane asymmetric viscosity and, thereby, to play a role in the control of cell morphology.

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