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Evidence for bidirectional transverse diffusion of spin-labeled phospholipids in the plasma membrane of guinea pig blood cells

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The distribution and transverse diffusion kinetics of four spin-labeled phospholipid analogues (two with choline heads: phosphatidylcholine (PC) and sphingomyelin (SM); two with amino heads: phosphatidylserine (PS) and phosphatidylethanolamine (PE)) were studied in the plasma membrane of guinea pig blood cells: erythrocytes, reticulocytes, and leukemic lymphocytes. Nitroxide reduction by the internal content of the cells was used as an indicator to determine the phospholipids that penetrated the cells. The reduction rates were in the order, PS > PE > PC > SM in all cells. Reoxidation of phospholipids extracted by serum albumin revealed the distribution of the phospholipids at a given time. In all cells, the distribution equilibrium was reached in less than 2 h and the amounts left in the external leaflet were in the following proportional order: PS < PE < PC < SM. In the erythrocytes and especially in the reticulocytes, the shape change induced by adding phospholipids relaxed partially or completely at a lower speed but kept the same proportional order as at equilibrium. All the results were analyzed quantitatively with a simple kinetic model including the rates of transverse diffusion (flip and flop), the exchange between plasma membrane and internal membranes, and the reduction rate of free radicals (determined in either the internal or external membrane leaflet). The calculated rate constants of transverse diffusion varied from $2 \cdot 10^{-3}$ to $1.2 \cdot 10^{-1}$ min^{-1} for the flip and from $4 \cdot 10^{-3}$ to $1.2 \cdot 10^{-1}$ for the flop, depending on the polar head and the cell type. Possible interpretations of the external phospholipid reduction mechanism and cell deformation are discussed.

Introduction

In the classical textbooks on cell biology, the asymmetric distribution of the different phospholipids in the eukaryote cell plasma membrane is considered to be fully elucidated. The analytical methods to determine this distribution are based on the common principle that external phospholipids react with non-penetrating reagents such as phospholipases, phospholipid exchange proteins, or chemical substances [1–4]. However, none of these methods is entirely rigorous. The main problem encountered is the varying or incomplete reactivity of external phospholipids [2,5], as well as the difficulty of estimating the fraction of phospholipids present in the plasma membrane in relation to total phospholipids. Although the measure-

Abbreviations: CMC, critical micellar concentration; ESR, electronic spin resonance; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine; LDL, low-density lipoproteins.

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ment of equilibrium distribution is not very precise, it appears that the aminophospholipids (PE and PS) are found principally on the inner leaflet, and the choline derivatives on the outer leaflet [6,7].

The origin of this transverse phospholipid asymmetry has been explained by two conflicting hypotheses. The first is based on the assumption that the transport, which is probably active, reestablishes plasma or other membrane distributions by acting against the slow passive transverse diffusion of phospholipids. The second hypothesis relies on the specific interactions between some phospholipids (PE and PS) and the cytoskeleton. The diffusion equilibrium (involving transport or not) is putatively displaced by this interaction. Evidence of a selective ATP-dependent transport of aminophospholipids from the outer to the inner leaflet (flip) of erythrocyte membrane appears to confirm the hypothesis of a facilitated diffusion [8–10]. Other data about fibroblasts [11], platelets [12], lymphocytes [13] and different types of pathological erythrocytes [14–16] show that there are rapid transverse flip movements in the plasma membrane, at least in the cases of PS and PE. There are still no precise data in the literature on the inverse movement (flop), which must be present to assure that a stable distribution equilibrium is reached independently of any added exogenous phospholipids. Only theoretical considerations lead certain authors to assume that the transverse diffusion is reversible, either by taking into account [17], or not [18], specific interactions between certain phospholipids (PS) and the cytoskeleton.

The objective of the present work was to provide data on the kinetics of transverse diffusion (flip and flop) in three types of blood cells of the same organism (guinea pig), i.e., erythrocytes, reticulocytes, and B lymphocytes (obtained by leukemic development) [19]. The movements of spin-labeled phospholipid analogues in the cells were monitored by ESR according to the methods initially introduced by Devaux et al. for studying erythrocytes [8,20] and later extended to platelets [12,21]. In the present paper, the transverse diffusion movement (flip and flop) of phospholipids is demonstrated. The kinetic rates were found to depend on the polar head and the cell type. The cell shape was modified, at least in the erythrocytes and the reticulocytes, by the non-equilibrated phospholipid distribution, and the initial shape was restored at a rate corresponding to the rate of phospholipid entry into the cells.

Materials and Methods

Cells

Lymphocyte preparation. The leukemia affecting the cells used in this study arose spontaneously in a strain 2 guinea pig [19] and was serially passaged in syngenic animals. L_2C lymphocytes were harvested and purified by Lymphoprep gradient centrifugation, as previously described [22]. Cells were washed in Hanks' balanced salt solution, and finally suspended in the same buffer at $2 \cdot 10^8$ cells/ml. Cell viability was routinely determined in each lymphocyte preparation and was always greater than 96% both before and after cell experimentation.

Erythrocyte preparation. Guinea pig erythrocytes from fresh blood collected in citrate-phosphate-dextrose and heparin were washed five times with Hanks' solution, pH 7.4.

Preparation of reticulocytes. Reticulocytosis was induced in guinea pigs by intraperitoneal injection of phenylhydrazine hydrochloride (Fluka AG) (500 µl at 20 mg/ml) in saline on four successive days [23]. Two days after the end of treatment, animals were anesthetized and approx. 15 ml of blood was collected by cardiac puncture in syringes containing heparin and citrate-phosphate-dextrose. The blood was then washed four times in 150 mM NaCl, Tris-HCl buffer, pH 7.4, after which 3 ml of the washed cell suspension (hematrocrit 33%) was mixed with 31 ml of an isotonic Percoll (Pharmacia) solution [24]. After centrifugation at 15000 rpm for 20 min at 10 °C, the red blood cells were separated into two major bands in the density gradient. The remaining leukocytes had accumulated at the top of the gradient and were discarded. The first band, containing mostly reticulocytes, was collected, and the density gradient solution was removed by washing the cells four times. Reticulocytes stained with Brilliant Cresyl Blue (Serva) were counted by light microscopy (the proportion was always greater than 80%). Sterile bood samples in appropriate nutritive

medium, containing more than 80% reticulocytes, were incubated without shaking in closed tubes for 3 days at 37°C. At specified times: 4, 12, 24, 36, 48 and 72 h, blood samples were removed by a sterile technique for determining reticulocyte number, morphology, hemolysis, ATP content, and the transferrin receptor number, to show that the reticulocytes obtained were capable of maturation.

Cytosol preparation. Cell hemolysis was performed by brief sonication of a cellular pellet at 4°C using a titanium probe (Ultrasons, Annemase). Membrane was pelleted by centrifugation for 30 min (100000 × *g* at 4°C).

Lymphocyte treatment

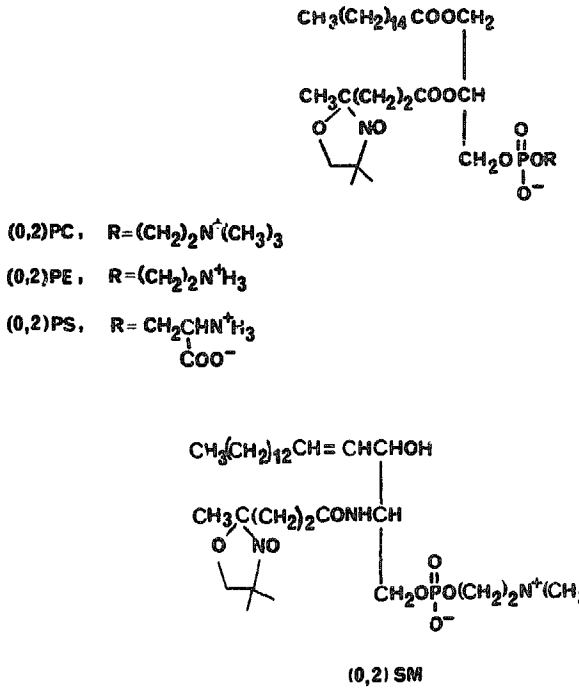
NEM treatment. A concentrated stock solution of NEM (Merck) in cell buffer was added to a cell suspension ($2 \cdot 10^8$ cells/ml) at 20°C (final concentration 2 mM), followed by gentle mixing for 10 min at 20°C before addition of spin-labels.

Ethylamine treatment. Cell suspensions were incubated with 1 mM ethylamine (Sigma) for 20 min at 37°C before addition of spin-probes.

Uptake and degradation assays of ^{125}I-labeled LDL in lymphocytes. All uptake (i.e. binding plus internalization) and degradation assays were carried out at 37°C as described previously [25], in the presence of absence of 1, 2 or 5 mM NEM.

Spin-labeling

The structural formulae of the spin-labeled phospholipids used are presented in Scheme I. They were synthesized as previously described [8,26]. For labeling, erythrocytes and reticulocytes were suspended to a final hematocrit of 75% in Hanks' buffer, pH 7.4, and L_2C lymphocytes were suspended to $2 \cdot 10^8$ cells/ml in the same buffer. The spin-label was added from a concentrated ethanol solution. The final amount of ethanol was less than 0.5% (v/v). The spin-label concentration (10–20 μM) corresponded, after incorporation, to less than 1% of the endogenous phospholipids. ESR experiments were carried out on a Brucker ER 200 D spectrometer, with a variable temperature accessory and connected to an Apple II + microcomputer. The concentration of nitroxides was determined by comparison with a standard, after subtraction of the base line and double integration of the signal.



Scheme I.

Determination of labeled lipids located in the outer leaflet of cell membrane lipid bilayer

The labeled phospholipid molecules located in the outer leaflet, and thus exposed on the outer surface of the cell membrane, were determined by a selective BSA (Sigma) extraction method. The labeled phospholipid was added to the cell suspension ($2 \cdot 10^8$ cells/ml and 75% hematocrit for lymphocytes and red cells, respectively) at a known concentration (determined by ESR) and incubated at 37°C. At specific times, aliquots of the cell suspension were mixed with 1% BSA and incubated for 5 min at 4°C. The mixture was then centrifuged for 30 s at 4°C. The quantity of signal present in the supernatant was determined before and after reoxidation of the probes with 10 mM $K_3Fe(CN)_6$, which represented the amount of labeled phospholipid localized in the outer leaflet.

The following conditions were verified:

- Incubation of cells with BSA for 5 min was sufficient to extract all external labeled phospholipids.
- Although the ferricyanide was paramagnetic, it did not change the ESR spectrum integral of the solubilized phospholipids in the presence of BSA.
- Ferricyanide completely reoxidized all the spin-label reduced by cell cytosol (cell disrupted by

sonication). In some cases, at the end of the 2 h incubation time, cells were disrupted (by vigorous shear stress in a Dounce homogenizer in the presence of hypotonic medium) and the label was reoxidized (ferricyanide) in order to check that ESR signal can be completely recovered by comparison with standard as described in the spin-labeling section.

A small hydrolysis (< 4% of the initial spin label amount) appears as measured according to Ref. 12.

Scanning electron microscopy

Spin-labeled phospholipid was added to the cell suspension and incubated at 37°C. At defined times, aliquots were transferred to vials containing an equal volume of 2.5% glutaraldehyde (Sigma), buffered at pH 7 with phosphate. Incubation with the fixative was continued for at least 30 min at 20°C, after which the fixed samples were treated with phosphate buffer containing 1% osmium tetroxide, dehydrated through a graded alcohol series, and dried with carbon dioxide by the critical point method. The dried samples were sputter-coated with gold and observed at 15 kV in a Jeol JSM 35 scanning microscope.

Optical microscopy

Labeled and fixed cell samples were viewed under a Zeiss WL phase contrast microscope using a magnification of ×630.

Results

Reduction of the ESR signal by the internal content of the cells

The nitroxide radicals of spin-labeled phospholipids were rapidly reduced by the cell cytoplasm, which shows several features: (i) specialized enzymes that reduce free radicals [27] or intermediates such as peroxides [28], (ii) glutathione in its reduced form [29], or (iii) ascorbic acid (present in large amounts in animal feed). The reducing power was determined from the decrease in the ESR signal of spin-labeled acid (4-doxylpentanoic acid) in broken cells, corrected by the dilution factor of the cytoplasm in the extracellular medium (estimated by the proportion of tracer reoxidized by ferricyanide in the extracellular medium, after total reduction). The reducing power was found to be identical in all the cells ($t_{1/2}$ = 2–4 min), but was negligible in the external medium of washed cells ($t_{1/2}$ > 2 h) in the presence of (0,2) phospholipids. The latter result proved that the spin-labels did not provoke cellular lysis at the concentrations used. Cellular membranes, obtained by centrifugation, did not reduce the ESR signal of spin-labeled phospholipids (data not shown).

TABLE I

HALF-REDUCTION TIME AT 37°C OF SPIN-LABELED PHOSPHOLIPIDS IN DIFFERENT GUINEA PIG CELLS

The numbers represent the half-reduction times at 37°C of the four phospholipid analogues in different guinea pig blood cells and are followed by the standard deviation of the samples (n = 4).

	Erythrocytes (min)	Reticulocytes (min)	Lymphocytes (min)
SM	32±0.7	42±0.7	20±1.2
PC	32±0.4	32±0.8	16±0.9
PE	17±0.7	21±0.9	11±0.7
PS	7±0.2	12±2.0	6±0.5

In the presence of cells, the kinetics of nitroxide radical reduction depended on the particular head group of the phospholipid analogue and cell type (Table I), as previously described for erythrocytes [20] and platelets [12]. The ESR signal disappeared after 2 h of cell incubation at 37°C with any of the spin-labeled phospholipids (data not shown). The rates of reduction by the different cells were in the order, erythrocytes < reticulocytes < lymphocytes, for all phospholipids except PS, which was always reduced at the same rate ($t_{1/2}$ = 6–12 min). In all cells, the rates of phospholipid reduction were in the same order: PS > PE > PC > SM (Table I).

Endocytosis and nitroxide reduction

Phospholipid entry into cells could be obtained by (i) transverse diffusion across the plasma membrane, or (ii) membrane endocytosis. NEM, which reacts with the SH-group, abolishes aminophospholipid diffusion into erythrocytes, which is a specific proteic process [30]. Fig. 1 shows that PS and PE reduction was slowed down when lymphocytes were treated with 2 mM NEM,

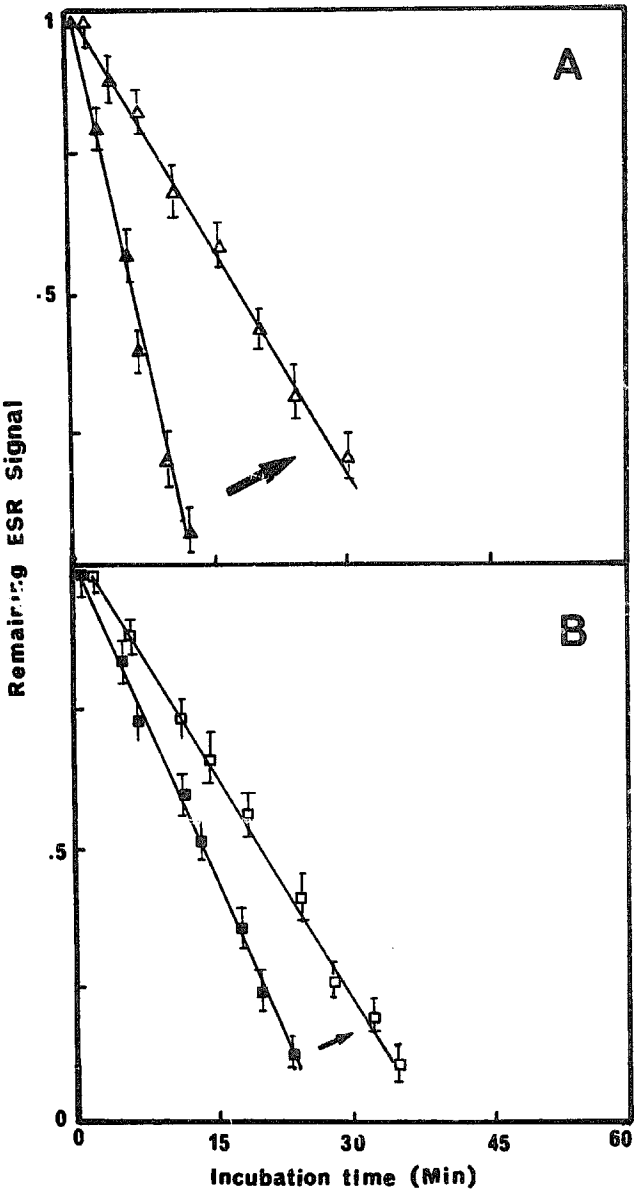


Fig. 1. Spontaneous reduction at 37 °C of (0,2)PS (panel A), and (0,2)PE (panel B) before (closed symbols) or after cell treatment with NEM (open symbols). Lymphocytes were incubated in the presence of 2 mM NEM for 10 min at 20 °C. The spontaneous reduction of (0,2)SM and (0,2)PC was not modified by this treatment.

whereas the reduction rates of the choline head derivatives (PC and SM) were not modified. The uptake and degradation of radioactive LDL (^{125}I-LDL) were reduced 20-fold by the same treatment, as previously described [31]. Ethylamine, another inhibitor of membrane endocytosis [32–34], did not modify the phospholipid reduction rate.

Localization of phospholipids in plasma membrane

The phospholipid proportions in the outer leaflet can be determined at any time by ferricyanide treatment of phospholipids extracted either in their oxidized or reduced forms) with serum albumin at 4 °C, as previously described in platelets [21]. Fig. 2 shows the results with all spin-labels and the three different cell types.

After 2 h of incubation, a near-equilibrium situation was reached in all cases. Whatever the cell type, SM was always the phospholipid that entered the cells most weakly (less than 5, 20, or 50% in erythrocytes, reticulocytes, or lymphocytes respectively). The level increased slightly for PC (15, 30 and 55%), markedly for PE (55, 70 and 70%) and maximally for PS, which completely entered the cells. Half-entry times are given in Table II for all spin-labels and cell types. It must be emphasized that in every case after the 2 h incubation, the ESR signal was completely recovered by ferricyanide oxidation of disrupted cells.

TABLE II

QUANTITATIVE ANALYSIS OF TRANSVERSE DIFFUSION RATES [a]

	$t_{1/2}$ reduction calc. [b] (min)	S_{eq} [c]	k_1	$k_{-1}/(1+K)$	k_3
Erythroctyes					
SM	32	0.91	0.2	2	2
PC	31	0.77	0.7	2.4	1.5
PE	20	0.33	1.2	0.6	2.5
PS	11	0.04	3.1	0.05–0.2	4
Reticuloctyes					
SM	40	0.80	1.3	15	1
PC	32	0.66	0.8	1.7	0.7
PE	20	0.29	2.1	0.9	1.7
PS	13	0.02	8.5	0.17	1.2
Lymphocytes					
SM	21	0.43	1.5	1.2	2.5
PC	16	0.45	1.8	1.5	2.5
PE	16	0.25	2.7	0.9	2.5
PS	7	0.005	9.3	0.05	2.5

[a] Rate constants ($\times 10^2$) are expressed in min^{-1}.

[b] Data calculated with the k_1, $k_{-1}/(1+K)$ and k_3 reported in the table (to compare with results in Table I).

[c] S_{eq} is the equilibrium fraction of phospholipids at the external side of the plasma membrane.

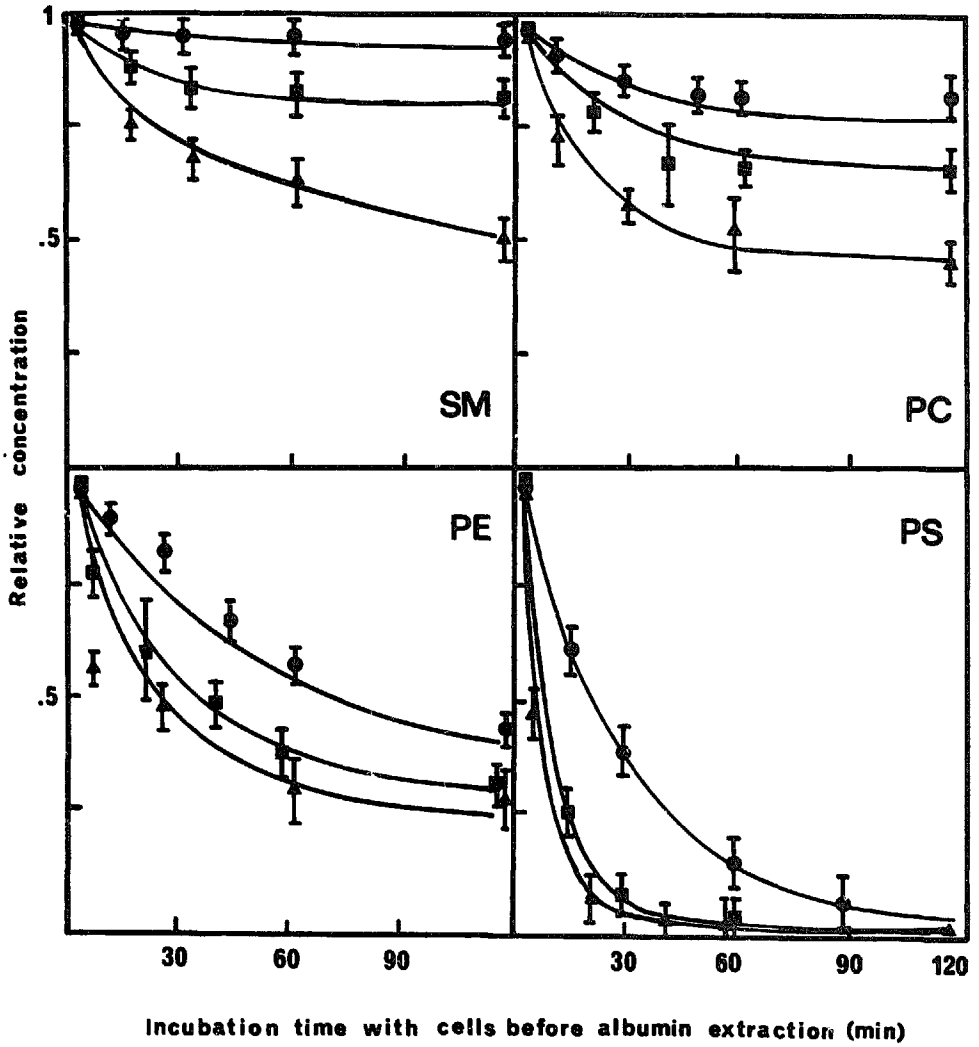


Fig. 2. Time course of the relative ESR signal of phospholipids associated with BSA in ferricyanide-oxidized supernatant after addition of spin-labeled phospholipids to erythrocytes (●), reticulocytes (■) and L_2C lymphocytes (▲). Error bars correspond to the maximum deviation in three different experiments. The lines correspond to the theoretical values calculated with the model (see text) using the rate constant values in Table II.

Relationship between transverse diffusion and cell shape

The addition of an excess of exogenous phospholipids on the outer plasma monolayer induces a clear shape change in the case of erythrocytes. In guinea pig cells, as observed with human erythrocytes [8,35], an echinocytic shape was obtained (not shown). The reticulocyte form, which was initially less regular than the erythrocyte form (Fig. 3A, B and C), became crenated after addition of all the markers used in this study (Fig. 3D, E and F), thus resembling the different echinocytic states previously defined by Bessis [36] for erythrocytes. Finally, lymphocytes, whose plasma membranes have numerous microvilli and invaginations (Fig. 4), showed no shape variation after addition of phospholipids, as previously observed by Sheetz et al. [37].

After incubation of erythrocytes (not shown) and reticulocytes (Fig. 5) with spin-labeled phospholipids at 37°C, the spikes disappeared at very different rates, depending on the spin-label and the cell type. Guinea pig erythrocytes (Fig. 6A), like human erythrocytes, showed considerable form relaxation in the presence of PE and especially PS, while the shape change induced by adding PC and SM was irreversible. With reticulocytes (Fig. 6B), the shape relaxation was

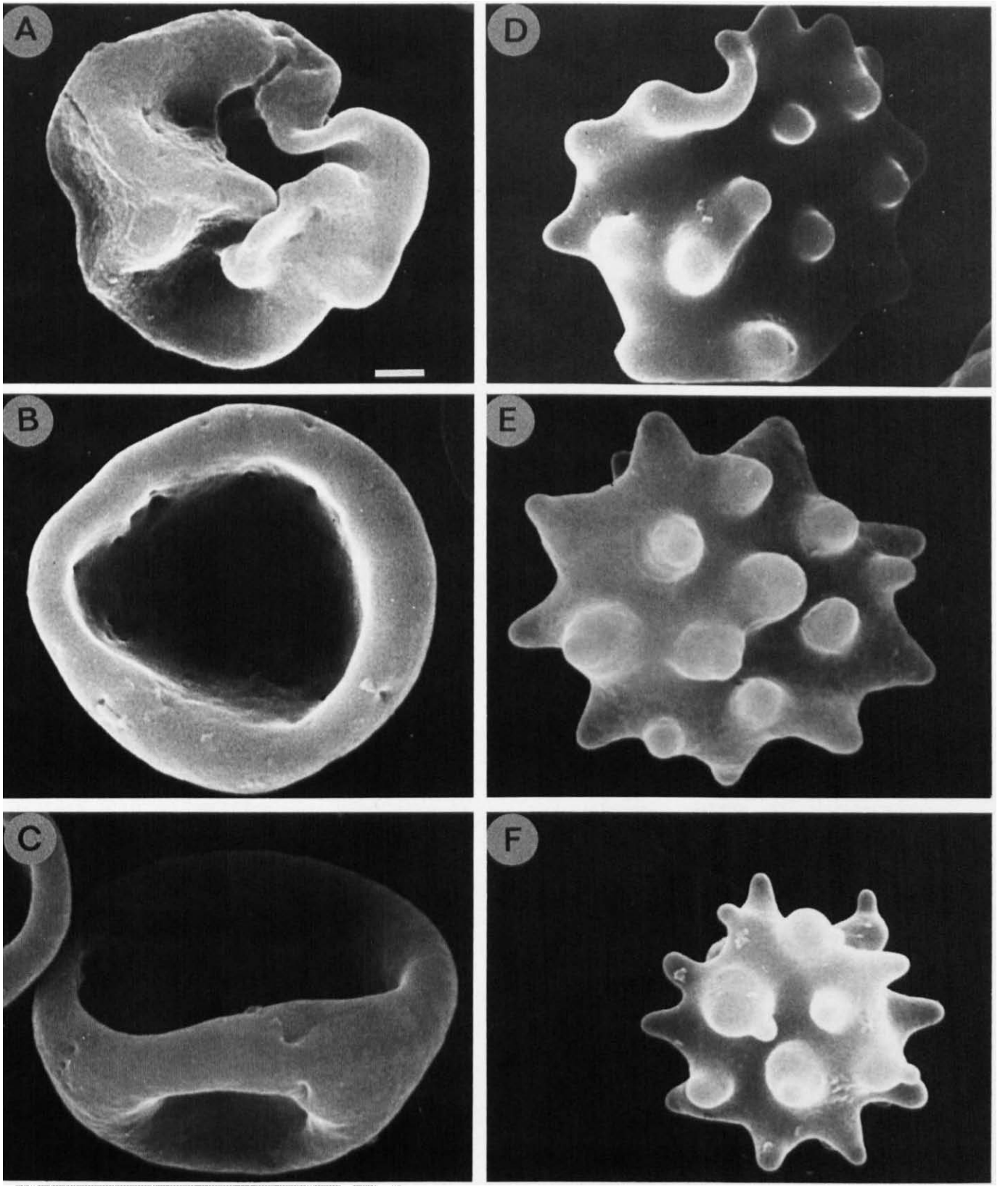


Fig. 3. Scanning electron micrographs of reticulocytes. Control reticulocytes before addition of spin-labels, had irregular (panel A), monoconcave (panel B) or triconcave shapes (panel C). Five minutes later adding (0,2)PC at 37 °C, the reticulocytes became crenated and had different echinocytic shapes: disco (panel D), II (panel E) and III echinocytes (panel F). Bar = 1 μm.

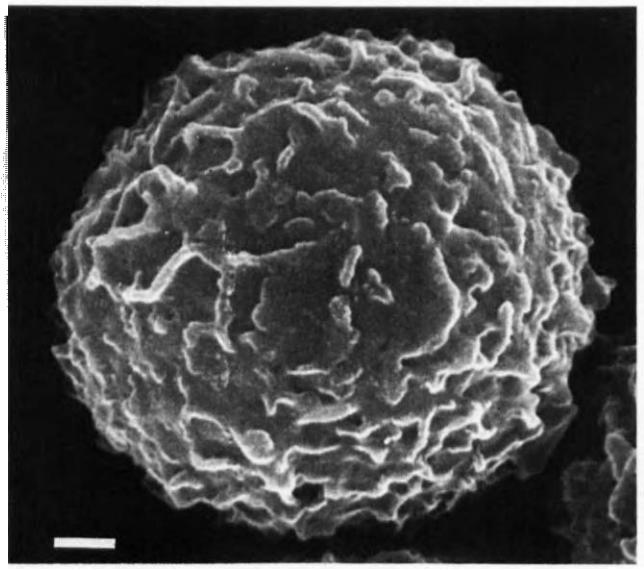
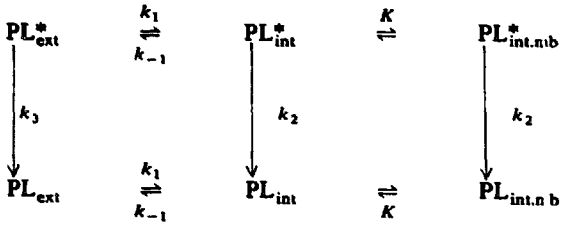


Fig. 4. Scanning electron micrograph of a typical L_2C lymphocyte at 37°C. The abundance of microvilli that are normally present in an irregular array on the lymphocyte surface made it impossible to determine whether any significant change in cell surface morphology was produced by adding spin-labeled phospholipids. Bar = 1 μm.

perceptible for both PC and SM (30% relaxation after 120 min), but it was quicker in the case of PS and PE than for the other phospholipids.

Evaluation of rate-constants of transverse diffusion

The kinetic model given below was used to represent all the phenomena occurring between the addition of spin-labeled phospholipids to the cells and the total reduction of the ESR signal. The asterisk denotes the spin-labeled analogues, and the absence of an asterisk means that the nitroxide was reduced. The subscripts 'ext' and 'int' mean that the phospholipids were localized in the plasma membrane and 'int.mb' corresponds to phospholipids unable to diffuse to the outer leaflet, since they were situated in intracellular membranes (other than plasma membrane).



The model is based on the following hypotheses: (i) for a given polar head, a phospholipid can move from one monolayer to the other at the same rate, whether the nitroxide radical is reduced or not; (ii) the passage from the inner plasma membrane leaflet to intracellular membranes is fast compared to transverse diffusion (equilibrium always attained); (iii) some nitroxide reduction can take place without phospholipid penetration into the cells (k_3) whatever the reduction mechanism (see Discussion).

According to this model, the sum of the native and reduced label concentrations in the outer leaflet ($PL^*_{ext} + PL_{ext}$) changes in a two-step process with the second step being much faster than the first one. Equilibrium concentration is reached with an experimental rate k_{exp} given by

$$k_{exp} = k_1 + k_{-1}/(1+K) = k_{-1}/[(1+K)\times S_{eq}]$$

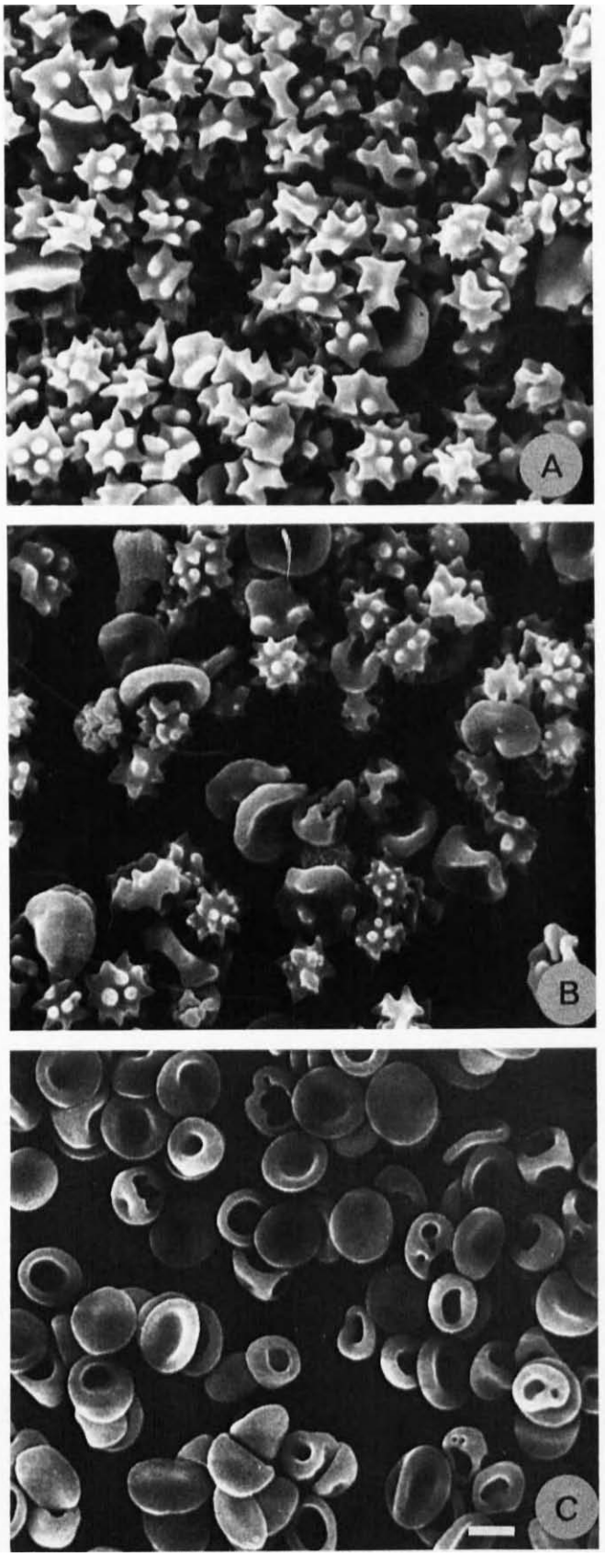
(Where S_{eq} is the final proportion of marker localized in the outer cell monolayer) allowing the calculation of k_1 and $k_{-1}/(1+K)$ from experiments. It is easy to verify that $1/S_{eq} = 1 + K_1(1+K)$, where $K_1 = k_1/k_{-1}$. The theoretical variation of the marker concentrations in the outer leaflet was calculated with the best values reported in Table II. There is an excellent agreement between the experimental data and the simulation curve (Fig. 2).

The amplitudes of the ESR signal (all phospholipids marked with an asterisk) are related by a series of differential equations that were solved by the Runge Kutta-Verner method. This was done for a group of equilibrium and rate-constant values with: (i) $k_2 = 0.2$–0.5 min^{-1} being the reduction rate of cytosol which was found to be the same in all cell types; (ii) k_3 being adjusted (Table II) to fit the calculated half-reduction time to experimental data (Table I).

Discussion

Choline head phospholipids can diffuse rapidly across the plasma membrane of certain cells

The amino head phospholipids (PE and especially PS) were more rapidly reduced by the intracellular content (Table I) than the choline head phospholipids (PC and SM). This confirms the high transverse diffusion speed of these phospholipids, which has previously been demon-



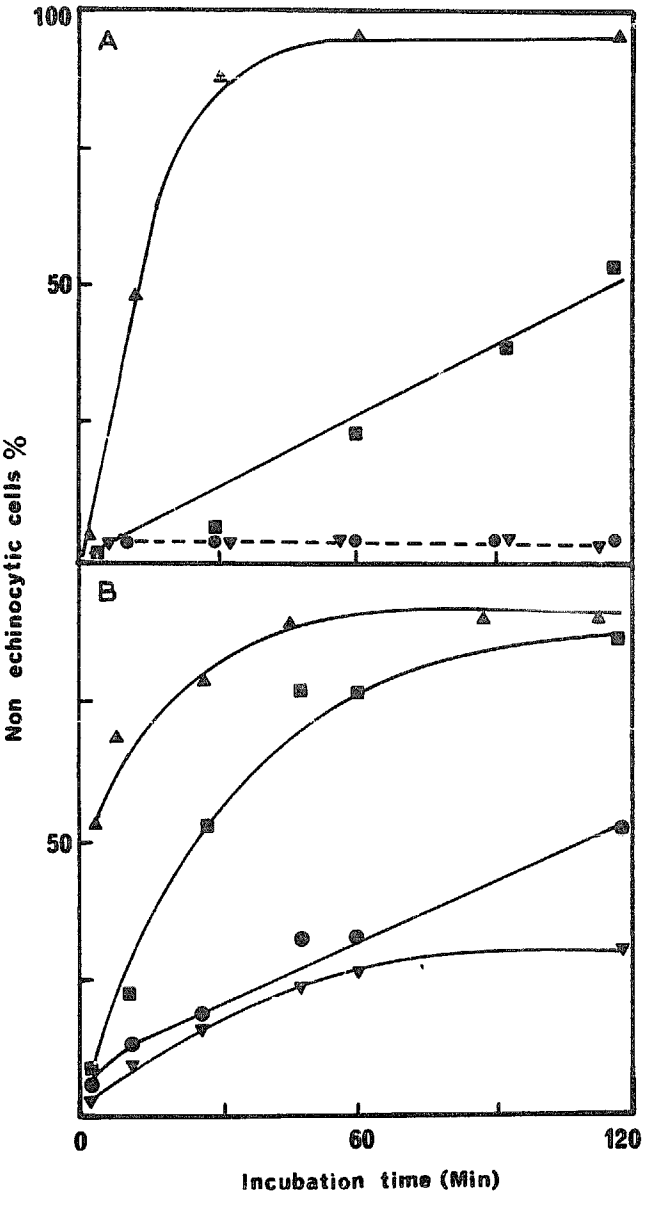


Fig. 6. Percentage of non-echinocytic cells (erythrocyte – panel A; and reticulocyte – panel B) versus incubation time at 37 °C for cells labeled with (0,2)PC (●), (0,2)SM (▽), (0,2)PE (■) or (0,2)PS (▲). Each data point represents at least 300 cells distributed in five fields selected at random and counted using a phase contrast microscope.

strated by different experimental methods using other cells, i.e. human erythrocytes [8,10,35], pig lymphocytes [13] and fibroblasts [11]. They are

Fig. 5. Scanning electron micrographs of reticulocytes incubated for 2 (panel A) or 120 min (panels B and C) with (0,2)PC (panels A and B) or (0,2)PE (panel C), respectively. Bar = 5 μm.

probably transported by a specific protein, since NEM decreased their reduction to the value observed for PC reduction (as in the case of erythrocytes [9]). It should be noted, however, that even the choline head phospholipids were reduced rapidly ($t_{1/2} = 20–40$ min) in the three types of cells, in contrast with what has been observed in human erythrocytes [20], but in agreement with results obtained in malaria-infected monkey erythrocytes [14]. To demonstrate that this reduction occurred essentially in the inner leaflet of the plasma membrane, it was necessary to exclude two other possible hypotheses:

(i) Endocytosis. We confirmed that NEM, which completely inhibits the uptake and degradation of LDL by lymphocytes possibly by endosome proton ATPase inhibition [38], was unable to prevent the reduction of choline head phospholipids. It has been demonstrated in pig lymphocytes that cytochalasin B and colchicine have no inhibitory effects on spontaneous reduction of spin-labeled phospholipids at 37°C [13]. In reticulocytes, transferrin receptor endocytosis is a very well known and effective process [39]. Nevertheless, it implies a very quick recycling of endosomes to the plasma membrane, which is incompatible with contact between labeled phospholipids situated in the inner endosome monolayer (identical to the external leaflet of plasma membrane) and cytoplasm [40]. Endocytosis cannot explain the differences between the phospholipid reduction rates since the phospholipidic composition of endosomes is analogues to that of the plasma membrane [41].

(ii) Spin label reduction on the outer leaflet. The extracellular medium had a very low reducing power in the case of erythrocytes and reticulocytes (less than 1% of cytosol reducing power) and was completely unable to reduce nitroxide groups in the case of lymphocytes. If nitroxide reduction occurs without phospholipid translocation to the inner leaflet of the plasma membrane, another mechanism is needed to explain the phenomenon (see below). Whatever the case, the possible mechanism cannot explain the difference in the reduction rates of PC and SM, which was observed in all three cell types (Table I), if there is no flip diffusion of PC and SM, at least in the case of reticulocytes and lymphocytes.

In conclusion, all the phospholipids, regardless of their polar head, were able to diffuse rapidly across the plasma membranes of guinea pig cells. The rate of inside-outside translocation of amino head phospholipids was faster than that of choline head phospholipids, as previously described in other organisms.

Arguments for an inside-outside translocation (flop)

Nitroxide reduction did not completely eliminate phospholipids from the outer leaflet, as shown in Fig. 2. The phospholipids reached an equilibrium situation, in which SM and PC were essentially localized on the outer leaflet, while PE and especially PS were situated on the inner leaflet. This result is qualitatively in agreement with those obtained with erythrocytes [6] and platelets [42] for endogenous cell phospholipids. It appears very likely that in the different plasma membranes studied here, spin-labeled analogues of phospholipids have an equilibrium behavior identical to that of endogenous phospholipids, as previously described in human erythrocytes [8].

The simultaneous occurrence of total phospholipid reduction and transverse diffusion equilibrium implies that after the phospholipids are translocated into the cells, they are able to reemerge after reduction by the cytoplasm. The kinetic model proposed (see Results) allows a direct determination of flipping rate (k_1) and of the ratio $k_{-1}/(1 + K)$ (Table II), depending only on the speed at which equilibrium is reached, as shown in Fig. 2. The k_1 rate-constant was markedly higher in guinea pig erythrocytes than those that have been measured in human erythrocytes with the same spin-labeled phospholipids (2–3-fold higher for PS and PE, and 50-fold higher for PC) [17,43], thus indicating that transverse diffusion is a very species-dependent phenomenon. In an identical animal species (the guinea pig), the flipping rate was found to be notably lower in erythrocytes than in the other cells studied. This result seems to be general, since other cell types, such as platelets [12,21] and malaria-infected monkey erythrocytes [14], showed faster transverse diffusion rates than in the corresponding normal erythrocytes [20]. By contrast with purely phospholipidic model systems where the transverse diffusion rate is very slow, it ap-

pears that phospholipids can diffuse rapidly between the two monolayers of numerous plasma membranes, perhaps in connection with lipid metabolism.

Unlike the endogenous phospholipids, the spin-labels used here have some hydrophilic properties that allow rapid exchange between membranes. The rate of transfer between vesicles by an aqueous diffusion mechanism seems directly related to the CMC of lipids [44]. With a CMC value of $1 \cdot 10^{-6}$ M for (0,2) phospholipids [45,46], a halftime of 5 min can be estimated for the transfer between membranes, in agreement with the hypothesis of this paper. Consequently, the experimental equilibrium obtained after 120 min depends on the distribution equilibrium in the plasma membrane and of the exchange between plasma membrane and intracellular membranes. However, the constant K characterizing this transfer cannot be evaluated safely for three reasons: experimental results are not very accurate; contradictory values were obtained with the two limit assumptions which could be made regarding the partition between membranes (a constant partition of all spin-labeled phospholipids between internal and plasma membranes or a similar distribution of a given phospholipid in all cell types); some additional mechanisms should occur, such as flip-flop transfer in internal membranes [47,48] or interactions between some phospholipids and the cytoskeleton [49,50].

Nitroxide reduction on the external leaflet of the membrane

The reduction rates observed could only be simulated if the external reduction constant (k_3) was not equal to zero but not exceeding a value 10-fold lower than the internal reducing power. This k_3 rate was remarkably constant in a given cell type (Table II). Since neither the external medium or isolated membranes were able to reduce nitroxide, this reduction can only be explained by a combined effect of cytosol and plasma membrane. It is known that the reduction of free radicals is essential for cell life [48], especially for maintaining normal membrane fluidity [51] and avoiding malondialdehyde formation from unsaturated phospholipids, since this component is a very effective protein-coupling agent [52]. The reduction of peroxides obtained after oxidation of the double bonds of phospholipids may occur in the cytosol as a result of hydrolysis of the modified chains by intracellular phospholipase A_2. This mechanism cannot occur with the oxidized phospholipids situated on the external leaflet of plasma membranes. It is thus possible that the nitroxide reduction in this leaflet takes place by electron transfer between the cytosol and free radicals, via an electron carrier passing from one face of the plasma membrane to the other. The most efficient carrier currently known is α-tocopherol (present in guinea pig feed) which can easily exchange electrons with ascorbic acid [53].

Relationship between transverse diffusion and cell shape relaxation

The relatively fast entry of all the phospholipids was confirmed by the kinetics of spike disappearance induced in the erythrocytes and reticulocytes after addition of spin-labeled phospholipids (Figs. 5 and 6). The initial spiculated form of these cells (Fig. 3) appears to be due only to the phospholipid excess on the external leaflet of the plasma membrane [54]. The relaxation of cell shape could thus be a result of the equalization process, i.e. of phospholipid internalization. The phospholipids that entered the cells more rapidly were those that induced a reversible cell deformation (Table II and Fig. 6). However, the relationship between these two phenomena is not clearcut since the distribution equilibrium of PC, for example, which occurred after 2 h (Fig. 2), was not sufficient to reverse the erythrocyte shape. Consequently, it must be assumed that PC reaches its equilibrium distribution before the complete resorption of the excess phospholipid on the outer leaflet. The last event must be very slow since it has to be preceded by a change in the transverse distribution of the other phospholipids, such as PE and PS, which have a lower flopping rate in erythrocytes than in reticulocytes (Table II). This may explain the partially reversible deformation of reticulocytes by the choline head phospholipids (Fig. 5 and 6).

In conclusion, the kinetic model proposed here can explain all the results obtained (reduction, transverse diffusion equilibrium, and cell shape relaxation) on the basis of some reasonable and

coherent hypotheses. Although the experimental results can be represented by a mechanism excluding facilitated transport, the model is not in contradiction with the existence of such a mechanism [9]. In the cases studied here, however, a first-order kinetics of transverse diffusion is sufficient to explain the events. This order can be obtained in mechanisms involving an enzyme or a carrier only if the substrate concentration is smaller than the K_m, which seems to be the case in our experiments [9,55].

The results of this investigation, apart from the exchange of phospholipids between plasma membrane and intracellular membranes, can be extended without difficulty to endogenous phospholipids. The flip and flop diffusion processes may be rapid in some cell types, even for the choline head phospholipids, due to easier diffusion in these membranes. There are other examples of nonspecific diffusion increases in parasite cells [14] and in erythrocytes treated with large amounts of various components [56,57], probably because of a perturbation of the membrane structure. The compatibility between a fast diffusion process and preserved phospholipid distribution remains to be clarified.

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