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# ION PERMEATION ACROSS THE BILAYER OF ANNEALED PHOSPHATIDYLCHOLINE VESICLES AT ELEVATED TEMPERATURES

## CONCENTRATION DEPENDENCE AND THE MICELLE-BILAYER DYNAMIC EQUILIBRIUM

RÜDIGER LAWACZECK *, RONALD BLACKMAN and MASATSUNE KAINOSHO **

*Arthur Amos Noyes Laboratory of Chemical Physics* †, *California Institute of Technology, Pasadena, Calif. 91125 (U.S.A.)*

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## Summary

The relative stability of the lipid bilayer toward ions above the crystalline to liquid-crystalline phase transition temperature has been studied under isotonic conditions for small annealed vesicles of dilauroyl (DLPC), dimyristoyl (DMPC), dipalmitoyl (DPPC), and distearoyl (DSPC) phosphatidylcholine by using lanthanide ions as a probe. The bilayer stability increased as the chain length of the lipid fatty acid increased, and a rapid translocation of ions across the bilayer started at about 60, 70, and 80°C for DMPC, DPPC, and DSPC vesicles, respectively. The bilayer of DLPC vesicles is apparently permeable for the tested ions even at room temperature. Two other important phenomena concomitant with the observed translocation of ions were found. Firstly, the ion leakage occurred in an "all-or-none" fashion, i.e. as soon as the vesicles start to become permeable toward ions, the concentration of ions in the intra- and extravesicular media are equalized within a short time. Secondly, the rate of the relative number of inward facing lipid molecules which become exposed to extravesicularly added paramagnetic lanthanide is a function of the inverse phosphatidylcholine concentration. This feature explicitly excludes the possibilities that the observed ion leakage occurs through a diffusion, pore formation, or through the rupture of vesicle walls induced by vesicle-vesicle collisions. We instead propose as the most probable mechanism that a dynamic

* Present address: Institut für Physikalische Chemie der Universität, Marcusstr. 9/11, 8700 Würzburg, G.F.R.

** Present address: Central Research Laboratories, Ajinomoto Company Inc., Kawasaki, 210, Japan.

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Abbreviations: DPPC, L-α-dipalmitoyl phosphatidylcholine; DMPC, L-α-dimyristoyl phosphatidylcholine; DSPC, L-α-distearoyl phosphatidylcholine; DLPC, L-α-dilauroyl phosphatidylcholine.

equilibrium between the various states of the phosphatidylcholine molecules in water, such as monomers, micelles, vesicles, and multilamellar liposomes, is in fact responsible for the observed phenomena.

## Introduction

The problem of whether single-bilayer, phospholipid vesicles are appropriate models for biological membranes is still under discussion and is to some extent answered from the different viewpoint of each investigator. In any case, such model systems provide the advantage of a relatively well-defined system, and thus, it is possible to circumvent the complexities of biological membrane systems. Hence, we feel that detailed physicochemical studies of bilayer vesicles will give some insight into the functioning of biological membranes.

In two preceding contributions we discussed the formation and annealing of structural defects within the bilayer of phospholipid vesicles [1,2]. The conclusions which were drawn from those results can be summarized in the following way: phospholipid vesicles which were prepared below their respective crystalline to liquid-crystalline phase transition temperatures ($T_c$) show structural defects within their bilayer. These defects are responsible for a very fast ion permeation across these bilayers and for a high fusion rate of the vesicles. Raising the temperature above $T_c$ introduces an annealing of the structural defects, which therefore only exist below $T_c$. This annealing process is univesicular and seemingly irreversible. Once the vesicles have been annealed they are much more stable towards fusion and the bilayer forms a high diffusion barrier against the ions already investigated (i.e paramagnetic $Ln^{3+}$, $Mn^{2+}$). As an extension of these studies, experiments were performed to investigate the stability of phospholipid vesicles as a function of temperature. In this report, we would like to present data concerning vesicle stability and ion transport as manifested by the exposure of inward-facing phospholipid molecules to extravesicularly added paramagnetic shift reagents. We show that the processes which allow ions to permeate the membranes are not simply univesicular. It is further observed that the above-mentioned ion translocation depends on the chain length of the fatty acid residues of the respective phosphatidylcholines. This latter effect was previously observed at a single temperature by Hauser and Barratt [3]. Their explanation was based on an ion transport mediated by vesicle-vesicle collisions. The authors further argued that the vesicle systems consisting of shorter chain-length phosphatidylcholines were extremely unstable, if indeed, any vesicles were formed.

In this report, we describe similar experiments performed over a range of temperatures, and the results indicate that the postulated collision mechanism cannot adequately explain our findings. Hence, we suggest that the processes involved are far more complex.

## Experimental part

DLPC, DMPC, DPPC, and DSPC were purchased from Calbiochem and were used without further purifications, since thin-layer chromatography did not

reveal any detectable impurities. The lanthanide salts (nitrates), products of Research Organic/Inorganic Chemical Corp., were lyophilized from deuterium oxide (99.8%, Stohler Isotopic Chemicals) to reduce the residual $H^2HO$ peak in the proton NMR spectra.

The vesicles solutions were prepared as follows: the weighed lipids (in the range of 20—100 mg) were added to 1—2 ml of aqueous $La(NO_3)_3$ (2—20 mM) and the suspension heated to approx. 60—70°C. After Vortex mixing, the milky, homogeneous solution was placed in a 150 W MSE sonicator with a Ti-microtip and sonified at high power either: (i) continuously for 15 min at a temperature above $T_c$, or (ii) below $T_c$ in intervals of 30 s on and 30 s off for 30 min. The resultant, clear stock solution was centrifuged, filtered through a cotton layer, and then pipetted into a series of 5-mm NMR tubes. At this point, the solutions, which were sonified below $T_c$, were completely annealed [1,2]. The lipid was sonified in a $La(NO_3)_3$ solution so that it was possible to add shift reagents (at a desired concentration) without generating significant chemical potential differences across the bilayer. For the experiments designed to measure the dependence of the lanthanide salt concentration, a stock solution of unannealed vesicles in high salt was diluted with $^2H_2O$ to give the desired salt concentration. Under these conditions, the existence of the structural defects within the bilayer would allow a rapid equilibration of ions across the bilayer. The samples were then annealed to stop any further ion translocation, and the paramagnetic shift reagent was added at the respective concentration. Subsequently the samples were incubated either for the same time period at various temperatures or at various incubation times at fixed temperatures. 220 or 100 MHz, proton NMR spectra were recorded at about 5—10 degrees above the $T_c$ of the respective lipid. For each set of experiments, the spectrum of one sample was measured just after the last pipetting step (without any further incubation) to serve as a reference. Previous experiments had shown that, under these conditions, more than 90% of the expected intensities can be observed in the proton NMR spectrum [1].

In the case of an impermeable bilayer the extravesicularly added paramagnetic shift reagent will only induce chemical shift changes for those protons on the outward-facing lipid molecules [4,5] and then only if they are in close proximity to the binding site (the phosphate group [6]) of the trivalent paramagnetic cations (we used $Eu^{3+}$ or $Nd^{3+}$ for up- or downfield shifts, respectively). Thus the resonance due to the choline methyl protons is split into two well-separated signals, which reflect the choline moieties which are, respectively, exposed to or shielded from the extravesicularly added paramagentic lanthanide ions. As long as the bilayer of the phospholipid vesicles stays impermeable to the added ions, these two choline methyl signals provide an easy means to monitor the inward- or outward-facing lipid molecules.

The intensities of the shifted ($s$) and non-shifted ($n$) choline methyl resonance were used to calculate the intensity ratio $r = n/(n + s)$. This parameter was then plotted as a function of either the incubation temperature ($T$), the time ($t$) or the concentration ($c$). The sum, $(n + s)$, in the denomonator served as an internal intensity standard. The ratio, $r$, reflects the fraction of lipids which are not exposed to the shift reagent.

The corresponding ratio, $r_o$, observed for the standard sample in each set,

refers to the initial state in which all paramagnetic ions are extravesicular. Thus $r - r_o$ is a measure of the change in temperature, time, or concentration. For example, $-(r - r_o)/\Delta t$ is the relative rate at which the inward-facing lipid molecules become exposed to the shift reagent.

Thin-layer chromatography of the vesicles after the incubation period did not reveal measurable degradation products.

## Results

Fig. 1 illustrates the effects typically observed. Spectra A and B were obtained from the same sample and differ only by an additional incubation (0.5 h at 90°C) for sample B. While the signal intensities for the fatty acid protons ($-CH_2-$, $-CH_3$) are hardly affected by the second incubation period, there are intensity changes in the signals arising from the choline methyl protons. In going from A to B the intensity of the shifted high-field signal ($s$) increased while that of the non-shifted low-field signal ($n$) correspondingly decreased. During the additional incubation time, an increased number of the inward-facing phosphatidylcholine molecules were exposed to the $Eu^{3+}$.

In Fig. 2, this effect is further illustrated for various incubation temperatures and phosphatidylcholines. Here, we have summarized several sets of experiments for DMPC, DPPC, and DSPC (all at the same concentration) and have plotted the ratio, $r$, versus the incubation temperature. Fig. 2 indicates that $r$ stays constant up to a specific, chain-length-dependent temperature, $T_s$, at which point $r$ suddenly drops and within a narrow temperature range approa-

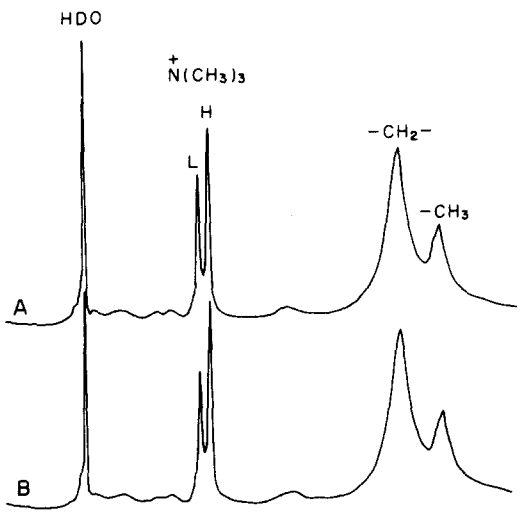


**Fig. 1. 100 MHz proton NMR spectra of small, annealed DPPC vesicles (2.5 w/v%) in an aqueous solution of 5 mM $La(NO_3)_3$ where 5 mM $Eu(NO_3)_3$ was added extravesicularly. The spectra were taken at 60°C. (A) Spectrum of the sample just after adding the $Eu(NO_3)_3$ shift reagent. (B) Spectrum of the same sample after an additional incubation period of 0.5 h at 90°C. The spectral assignments indicated correspond to the choline methyl protons ($-\overset{+}{N}(CH_3)_3$) and the methylene and methyl protons of the hydrocarbon chain ($-CH_2-$, $-CH_3$), HDO refers to the residual water signal. H and L correspond to the high-field, shifted (H) and low-field, non-shifted (L) parts of the signal of the choline methyl protons. The plotted spectral width is 500 Hz.**

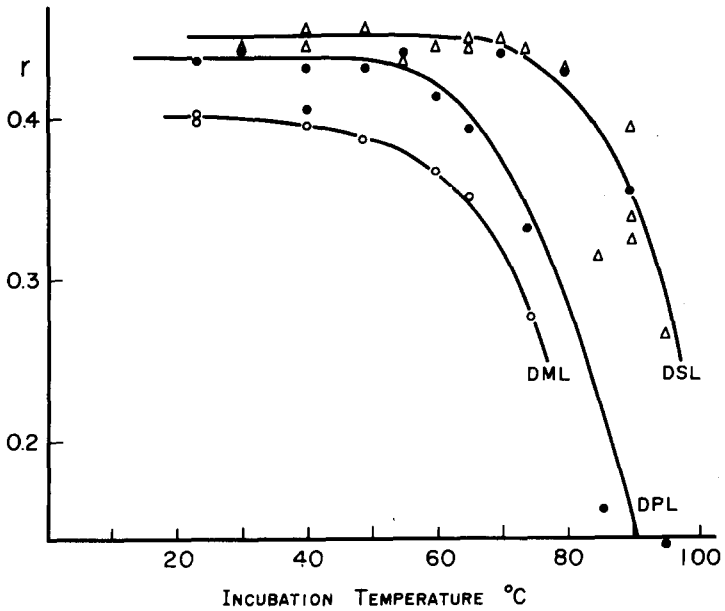


Fig. 2. Ratio $r$ (the relative number of impermeable vesicles) versus the incubation temperature at a fixed incubation time (1.5 h) for DML (dimyristoyl phosphatidylcholine) (○), DPL (dipalmitoyl phosphatidylcholine) (●), and DSL (distearoyl phosphatidylcholine) (△) vesicles. The phosphatidycholine (1.25 w/v%) and the 10 mM $La(NO_3)_3$, $Nd(NO_3)_3$ concentrations were the same throughout the experiments.

ches zero. Concomitant with this drop in $r$ there is a small but pronounced loss in the signal intensities of the methylene protons of the fatty acid residues. This loss in signal intensity (under high resolution conditions, and especially for the methylene protons) must be interpreted either in terms of a direct vesicle-vesicle fusion, a disruption of vesicles leading to the formation of multilamellar structures [1,2,7,8], or more indirectly in terms of a growing of the vesicle sizes via intermediate lipid states (see below). In either instance the number of small vesicles which contribute predominantly to the observed signal intensities is diminished.

If the lipid bilayer is impermeable to ions, then $r$ will truly reflect the fraction of inward-facing lipids. This holds for annealed vesicles but, as we have just shown, only when they are kept at temperatures below $T_s$. Under these conditions, $r$ can be calibrated by electron microscopic methods and offers an easy means to estimate the average size of the vesicles. The fact that the initial values of $r$, in Fig. 2 increase with longer chain-length probably reflects an increasing vesicle size due to the additional methylene groups and was already observed by de Kruijff et al. [9] using similar techniques.

For incubation temperatures above $T_s$, an increasing fraction of the inner bilayer becomes exposed to the extravesicular shift reagent possibly due to an increasing permeability toward these ions. The temperature ranges for $T_s$ are: DMPC 40—60°C, DPPC, 50—65°C, and DSPC 75—85°C.

The experimental data for DLPC vesicles could not be included in Fig. 2, since the shift reagent leaks through the DLPC bilayers even at room temperatures. This effect, and a time-dependent loss of signal intensity and line

braodening was observed by Hauser and Barratt [3]. They interpretet this effect being due to a lack of single-walled vesicles and a predominance of larger multilamellar structures thus implying a pronounced instability of DLPC vesicles. However, we find that at 220 MHz, and in the absence of paramagnetic ions, the choline methyl signal can be resolved into the two resonances from inside and outside lipids. As will be considered in a separate communication, proton NMR relaxation rates for the choline methyl, the fatty acid methylenes, and methyl protons for DLPC vesicles are in accord with their homologs containing longer fatty acid chains and show no abrupt deviation with respect to vesicles of other phosphatidylcholines in their temperature profile of the relaxation rates. It was always found that the $T_1$ of the inward-facing choline methyl protons is slightly shorter than the corresponding $T_1$ from the outward-facing lipids (the same holds for $T_2^*$). This supports the idea that the inward-facing lipid molecules are motionally more restricted than the outward-facing ones [2]. These results allow us to suggest that DLPC phosphatidylcholine does, in fact, form single-walled bilayer vesicles, but that the $T_s$ for the ion permeation (or the exposure of the inside molecules to extravesicular shift reagent) is now below room temperature for these vesicles ($T_c$ = 11°C, 0°C according to refs. 10 and 11, respectively, $T_s < RT$).

The experiments leading to Fig. 2 are difficult to quantitate because, at $T_s$, the relative rate of the observed increase in the ion permeation (or the exposure of inside molecules to the extravesicular shift reagent) is a function of the lipid (or vesicle) concentration. The results of concentration-dependent studies are summarized in Figs. 3 and 4. In Fig. 3a the difference $r_o - r$ for DPPC vesicles (incubated at 85°C in the presence of extravesicular $Eu^{3+}$) is plotted versus the phosphatidylcholine concentration and, in the insert, versus the inverse of the phosphatidylcholine concentration. Fig. 3b shows the relative chemical shift of the two choline resonances before and after the incubation at 85°C.

Another interesting feature of the observed phenomenon is that the signal due to the inward-facing choline protons did not shift significantly up- or downfield but showed only a decrease in its intensity as a result of the incubation at temperatures above $T_s$. The comparison of the choline methyl chemical shifts with that of the methylene protons of the fatty acid residues, which for our purpose can serve as a chemical shift standard, revealed that it is the outside signal which moves toward the inside one as consequence of the 1 h incubation at 85°C. This might be due to a decreasing number of paramagnetic ions in the extravesicular medium, because some of these ions become translocated and are thus lost from the extravesicular compartment. We did not observe intermediate states where only a few paramagnetic ions were translocated which would result in a range of spectral positions for the signals of the inner choline protons. These facts imply that the ion leakage or translocation occurs through an "all-or-none" process. This further suggest that once the vesicles allow ions to permeate an approximate equilibrium between the concentrations of ions in the inside and the outside media is rapidly established, so that a spectral discrimination between the inner and outer moieties is lost.

As Fig. 3a clearly indicates, the rate of exposure of the inner lipid molecules decreases inversely with the concentration of lipids. We have measured the effects of variations in the $Nd^{3+}$ concentration, and have plotted the ratio $r$

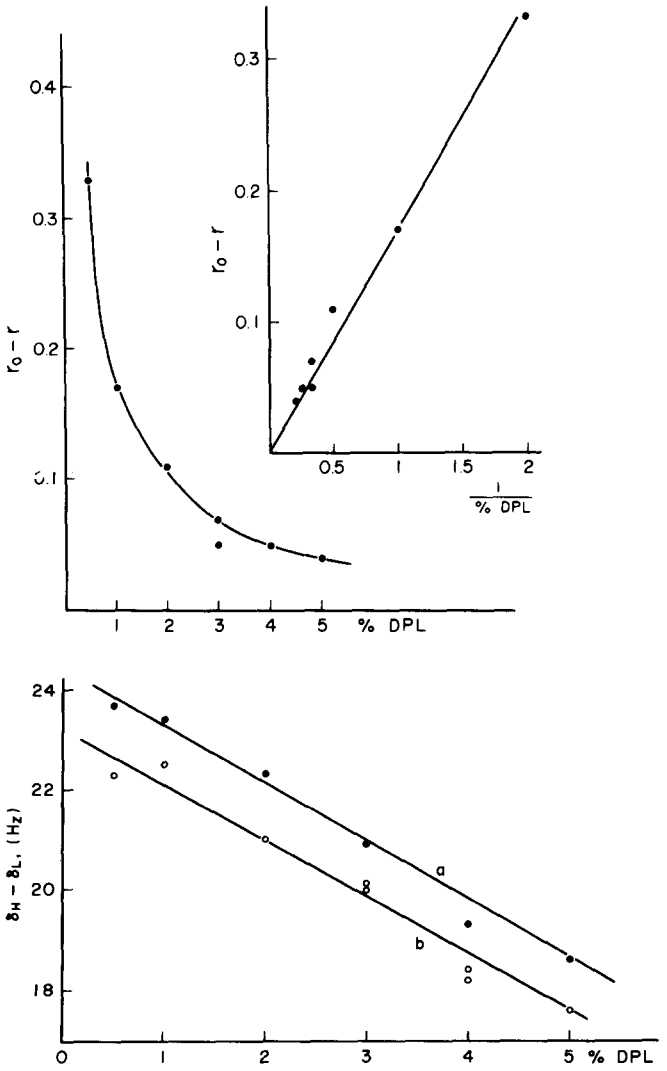


Fig. 3. Ion exposure of the inward-facing choline molecules in DPL (dipalmitoyl phosphatidylcholine) vesicles toward extravesicularly added $Eu^{3+}$ as function of the phosphatidylcholine concentration (w/v%) at a fixed 5 mM $La(NO_3)_3$, $Eu(NO_3)_3$ concentration. The spectra from which $r$ was calculated were taken after an incubation period of 1 h at 90°C, while $r_o$ was calculated from spectra without this additional incubation period. (a) The difference $r_o - r$ as function of the phosphatidylcholine concentration. $r_o - r$ corresponds to the relative number of inward-facing lipid molecules being exposed to the extravesicular added $Eu^{3+}$ during the additional incubation period. The insert shows the same difference $r_o - r$ as function of the inverse of the phosphatidylcholine concentration. (b) The chemical shift differences $\delta_H - \delta_L$ between the shifted (outward) and non-shifted (inward) signals of the choline methyl protons before (•, a) and after (○, b) the additional incubation period of 1 h at 90°C. Same samples as in A. The comparison with the methylene proton signals of the fatty acid chain revealed that the peak which corresponds to the outward and exposed choline methyl molecules moves toward the inward peak which remains at fixed spectral position.

(Fig. 4c) and the intensity of the high-field peak relative to that of the methylene signal (Fig. 4b) versus the $Nd^{3+}$ concentration at a fixed DPPC concentration. In Fig. 4a, the relative chemical shift changes are shown. It is clear from Figs. 4b and 4c that the ratio $r$ or a similarly defined quantity are not influ-

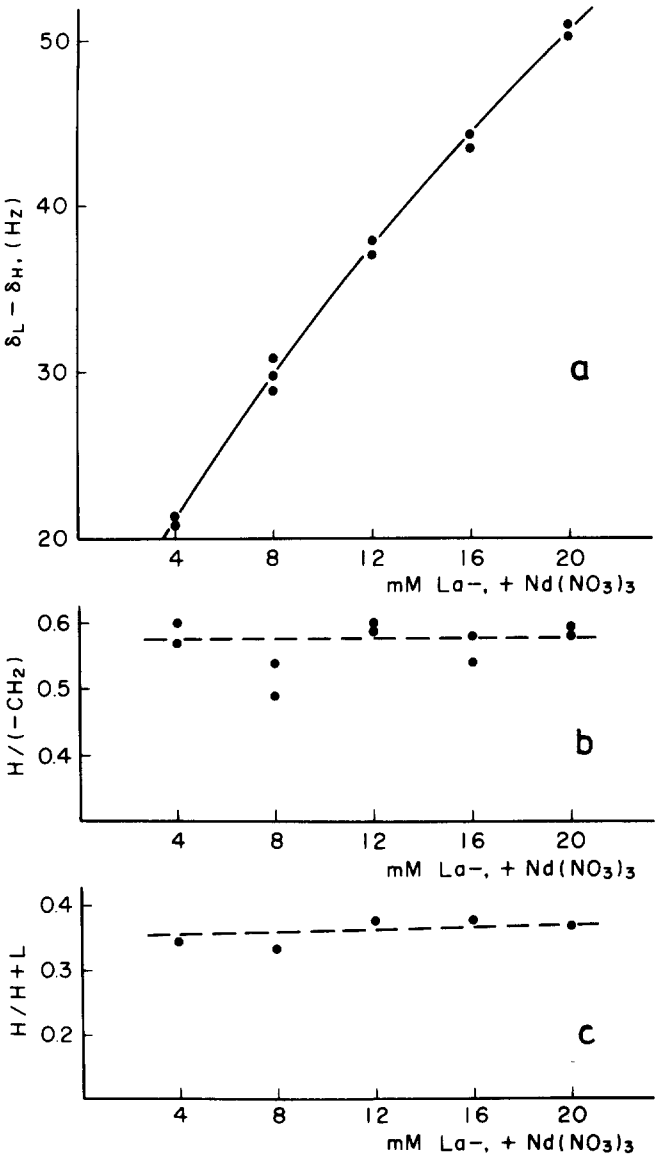


**Fig. 4. Exposure of the inward choline molecules in DPL (dipalmitoyl phosphatidylcholine) vesicles (1 w/v%) toward extravesicularly added $Nd^{3+}$ during an additional incubation period of 1 h at 85°C as function of the variable $La^{3+}$, $Nd^{3+}$ concentration. (a) Chemical shift differences between the shifted and non-shifted choline methyl signals ($\delta_L - \delta_H$). (b) Intensity of the non-shifted and inward choline methyl signals relative to the intensity of the signal of the methylene protons of the fatty acid chain. (c) ratio $r = (n/(n + s))$. Within this figure H equals $n$ and L equals $s$, respectively.**

enced by the changes in the lanthanide ion concentration. This conclusion is also supported by measurements on samples where the ratio of the lipid and lanthanide ion concentrations was kept constant while the total concentration was varied. In these experiments, the rate of exposure of the inward-facing lipid molecules followed an inverse concentration dependence with approximately the same slope as is indicated by the straight line in the insert of Fig. 3a. Final-

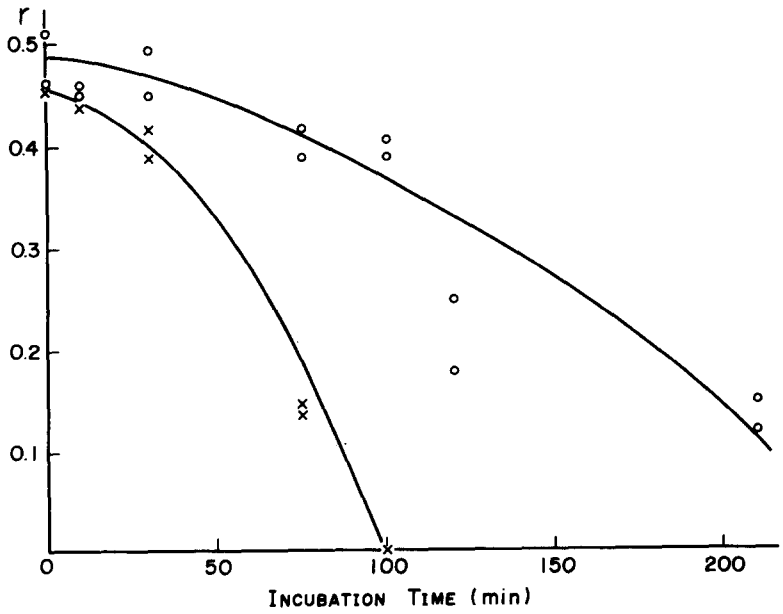


Fig. 5. Time dependence of the translocation of $Nd^{3+}$ across the bilayer of DPL (dipalmitoyl phosphatidylcholine) versicles at a fixed $La(NO_3)_3$, $Nd(NO_3)_3$ concentration of 20 mM. The ratio, $r$, at two phosphatidylcholine concentrations (○, 1.5 and X, 0.5 w/v%) in 20 mM $La(NO_3)_3$, $Nd(NO_3)_3$ is plotted versus the variable incubation time at an incubation temperature of 80°C.

ly, we have measured the time dependence of the ion translocation. The results are summarized in Fig. 5 for two different phosphatidylcholine concentrations at a fixed incubation temperature. In agreement with Fig. 3a, we found that the exposure of the inner moieties to the extravesicularly added shift reagent is more rapid at the lower lipid concentration.

## Discussion

The results presented above concerning the chain-length and temperature-dependent transport of trivalent cations across the bilayer of vesicles demonstrate that a mechanism exists even for completely annealed vesicles, by which the previously protected inward-facing lipid molecules become exposed to the extravesicularly added shift reagent ions ($Eu^{3+}$, $Nd^{3+}$). Up to a temperature ($T_s$) about 20—35°C above the respective lipid phase transition temperature ($T_c$), the bilayer of the phospholipid vesicles forms a high diffusion barrier, indicated by the plateau in Fig. 2. At temperature above $T_s$, an increase in ion transport or, at least, an exposure of the inner molecules to the extravesicular cations begins, which is demonstrated by the increasing amount of lipid molecules interacting with $Eu^{3+}$ or $Nd^{3+}$. The concomitant, small but pronounced loss in signal intensity starting at $T_s$ together with the results of Figs. 3, 4 and 5 are not compatible with a purely diffusional process of the ions across the bilayer. At constant lipid concentration (Fig. 4) the measured ratio $r$ is hardly affected by an increase in the lanthanide ions, i.e. a higher surface cover does not per se stimulate the ion translocation. However, at variable lipid concentration this

ratio $r$ follows the inverse of the lipid concentration (Figs. 3 and 5). As the ratio $r$ was defined as a relative number, the absolute rate of the translocation of ions should almost be independent of the phosphatidylcholine and cation concentrations. In terms of a mechanistic interpretation we can thus rule out all processes which in one or the other way are functions of either the phosphatidylcholine or lanthanide ion concentrations, especially processes mediated by vesicle-vesicle collision (see refs. 3 and 12), the disruption of vesicles and the decomposition of the lipid material which should obey second- and first-order kinetics, respectively. It should be noted here that within each set of experiments all samples were derived from one stock solution, so that an equal size distribution of the vesicles, at least to start with, can be assumed.

In the following we would like to propose a mechanism by which ions can be transported across the vesicular bilayer, and which seems to be independent of the concentrations in question and thus could explain the observed results.

*Micelle formation*

As a starting point, it is informative to consider the various states of lipid molecules in an excess of water as presented schematically in Fig. 6. Here the CMC refers to the critical micelle concentration which means that above this phosphatidylcholine concentration free phosphatidylcholine molecules start to form micelles or similar aggregates. In exact analogy, we define the CBC as the concentration above which micelles tend to form bilayers, predominantly in the form of multilayers if no further treatment is involved. Fig. 6 differs from the representation of the phosphatidylcholine-water system of ref. 13 by the inclusion of the micelle state ordinarily found with phosphatidylcholines of shorter fatty acid chains [14] or in methanol/water mixtures [15].

Within this postulated scheme, the vesicles occupy a metastable state. They are usually generated by an ultrasonic irradiation of an aqueous lipid dispersion [16] or by dialysis procedures [17]. The vesicles are quite stable over a wide temperature range but they finally tend to fuse with a fusion rate constant, $k_f$,

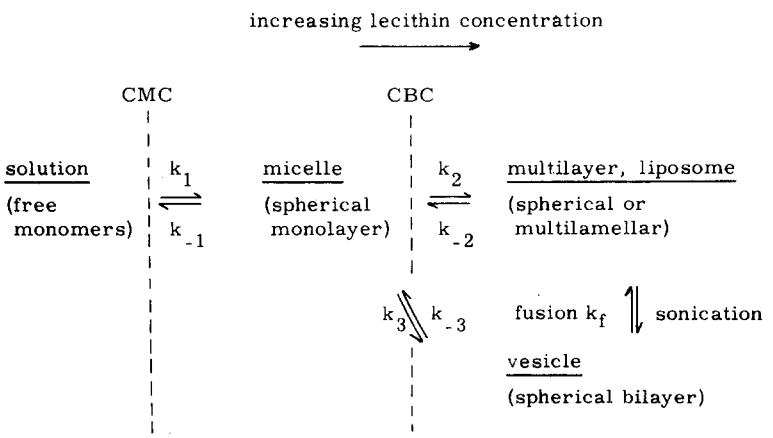


**Fig. 6. Diagram representing the dynamic equilibrium of the various states of phosphatidylcholine molecules in water. The states of the phosphatidylcholine molecules are shown in parentheses. CMC, critical micelle concentration; CBC, critical bilayer concentration.**

and ultimately they irreversibly form multilayers at concentrations above the critical bilayer concentration. We suggest that once the vesicles have been formed by some experimental preparation there should also exist, in analogy to the dynamic micelle-multilayer equilibrium, a similar equilibrium between micelles and vesicles. This equilibrium is governed by the rate constants $k_3$ and $k_{-3}$. The limiting concentrations, critical micelle concentration and critical bilayer concentration, as well as the respective rate constants are likely to be functions of the temperature, chain-length, ionic strength and other effects. In particular, it may be assumed that the dynamic equilibrium is shifted towards the free monomer and the micelle states with increasing temperature or decreasing chain-length of the fatty acyl residues. Consequently, there should be a critical temperature ($T_s$), characteristic of the lipid, above which vesicles eject micelles into the aqueous medium. For the present considerations we can assume an almost negligible multilayer formation and a slow direct fusion rate (i.e. $k_2/k_{-2} << k_3/k_{-3}$, and $k_f$ small). As our chosen phosphatidylcholine concentrations (in the range of 0.05—0.005 M) were always far above the critical micelle concentration (in the order of $10^{-10}$ M for DPPC, [15]) and presumably far above the critical bilayer concentration, these limiting concentrations become constant at a fixed temperature. Although the critical micelle concentration and critical bilayer concentration might be very low even at elevated temperatures, the dynamic equilibrium between vesicles and micelles could cause extravesicular ions to enter into the intravesicular compartment (or vice versa) and thus eventually lead to an equilibrium between the outside and inside media. It is this generation and turnover of micelles which become the rate-determining steps, and which make the whole process independent of the lanthanide ion concentration in the range studied.

At the lipid level one can rationalize the observed concentration dependence in the following manner: since the phosphatidylcholine concentration is higher than the critical micelle concentration and critical bilayer concentration the micelle concentration is assumed to be at a constant level, independent of the vesicle concentration. In a micelle-mediated exchange mechanism, therefore, the relative rate of exchange ($r$ is a dimensionless parameter which reflects the relative degree of exchange) will be higher at the lower vesicle concentrations. This micelle-mediated exchange is offered as an alternative to the recent discussion of the turnover of vesicles involving free monomer states as a means of mediating lipid exchange between DMPC and DPPC vesicles [13].

A detailed mechanistic consideration, i.e. whether during this micelle-generation pores are formed within the bilayer, or whether tightly bound lanthanide ions are translocated via the incorporation of micelles (or possibly monomeric lipid molecules) into the bilayer is beyond the scope of this discussion and must await further experiments. In a speculative manner this micelle-triggered translocation of ions can be compared to ion transport processes mediated by carrier molecules, one major difference being that the carrier is a specific and different molecule while the micelle shuttle is part of the lipid system.

It seems that the generation of micelles is not only a function of the chain length of the lipid molecules and the temperature but also a function of the vesicle size. Therefore the vesicle ensemble could participate in the micelle-triggered transport at different rates where small, and according to their high sur-

face curvature, less stable, vesicles might take part in the generation of micelles more efficiently than larger ones. Evidently it is this discrimination by the vesicle size which can account for the observed "all-or-none" mode of the ion leakage and for the increase of the vesicular size.

Other mechanisms such as pore formation [18,19], lipid exchange [13], vesicle-vesicle fusion [1,2,8,20], or flip-flop of lipid molecules between the two halves of the bilayer [21—23] can conceivably account for a transport or shuttle of ions across the bilayer. These mechanisms, however, all fail to explain the observed concentration dependence. These processes are likely to take place but in order that either of them be the sole origin of the observed ion translocation, additional arguments such as a protection or inhibition of the respective rates at higher phosphatidylcholine concentrations must be involved to account for the dependence of the observed and relative rate of ion translocation on the inverse of the lipid concentration.

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