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A ^{13}C NMR METHOD FOR DETERMINATION OF THE TRANSBILAYER DISTRIBUTION OF PHOSPHATIDYLCHOLINE IN LARGE, UNILAMELLAR, PROTEIN-FREE AND PROTEIN-CONTAINING VESICLES

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Summary

(1) Large unilamellar vesicles have been prepared from *N*-[Me_3-^{13}C]-18 : 1_c/18 : 1_c-phosphatidylcholine, both with and without the major intrinsic proteins from the human erythrocyte membrane incorporated in the bilayer.

(2) It is shown that the inside-outside distribution of the lipid molecules in these large unilamellar structures can be determined using ^{13}C NMR.

(3) Large vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine containing glycophorin show an enhanced permeability to Dy^{3+}. It is shown that the permeability barrier of these vesicles can be restored by addition of 10 mol% 18 : 1_c/18 : 1_c-phosphatidylethanolamine or 1-18 : 1_c-lysophosphatidylcholine.

Introduction

An asymmetrical distribution of phospholipids has been reported for a number of biological membranes [1] and in some cases evidence has been presented for the occurrence of transbilayer movement of lipid molecules [2,3]. Sonicated aqueous lipid dispersions (vesicles) have commonly been used as model systems for studying these phenomena [4—14]. However, these vesicles have a strongly curved bilayer and this makes it questionable whether the curvature itself does not influence the transbilayer distribution and movement of lipids. Handshaken liposomes lack this strong curvature, but they are not very suitable for this type of study because of their multilamellar structure. Recently methods have been described for the preparation of large unilamellar vesicles, either

with or without proteins from the human erythrocyte membrane incorporated in the bilayer [15—19]. As model systems, these vesicles have a better resemblance to biological membranes than sonicated vesicles or handshaken liposomes.

The distribution of phospholipid molecules across the bilayer of sonicated vesicles has been studied by ^{1}H, ^{31}P and ^{13}C NMR, using paramagnetic metal ions to differentiate between the inner and outer surfaces [20—23]. Whereas ^{1}H and ^{31}P NMR do not yield high resolution spectra of non-sonicated lipid dispersions, ^{13}C NMR has the advantage of limited resonance broadening in structures with restricted motion [24]. This is particularly the case for the carbon atoms of the *N*-methylcholine group of phosphatidylcholine molecules, which undergo a considerable, almost isotropic, internal motion.

In this paper it is shown that ^{13}C NMR of *N*-[Me_3-^{13}C]phosphatidylcholine offers the possibility of studying the distribution of these lipid molecules across the bilayer of large unilamellar vesicles. This is a necessary step for future studies on transbilayer movements in these systems.

Materials and Methods

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18 : 1_c/18 : 1_c-phosphatidylethanolamine) were synthesized as described before [25,26]. 1-Oleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c-lysophosphatidylcholine) was obtained by hydrolysis of 18 : 1_c/18 : 1_c-phosphatidylcholine by pancreatic phospholipase A_2. *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine was synthesized according to Stockton et al. [27]: 34.5 mmol [^{13}C]methyliodide and 6.7 mmol phosphatidyl ethanolamine, isolated from soya beans, were incubated in 200 ml chloroform/methanol (1 : 1, v/v) with 10 g $NaHCO_3$ at room temperature, with stirring, for 10 days. The phosphatidylcholine formed was purified by silica gel column chromatography and subsequently deacylated [25]. The *N*-[Me_3-^{13}C]-labelled glycerylphosphatidylcholine was reacylated with oleic acid according to Warner and Benson [28]. *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine was isolated from the incubation mixture by silica gel column chromatography.

Ghosts were prepared from fresh human blood according to Dodge et al. [29] and lyophilized (when used for the purification of glycophorin) or stored at −20°C (when used to prepare a Triton X-100 extract). Glycophorin was purified according to Marchesi and Andrews [30] and delipidated as described before [19]. A crude Triton X-100 extract was obtained from ghosts as described previously [16], with minor modifications. Band 6 * protein was solubilized first by mixing 1 vol. of ghost suspension with 20 vols. 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0. The membranes were sedimented and washed once with 20 vols. 50 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0. Intrinsic proteins, including band 3, were extracted with 2 vols. 0.5% Triton X-100 in 50 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0.

* The major polypeptides of the human erythrocyte membrane are numerated according to Steck [31].

^{3}H-labelled Triton X-100 (0.22 mCi/g) was a gift from Dr. A.M. Rothman of Rohm and Haas Company. Triton X-100 was obtained from Rohm and Haas, Bio Beads SM-2 from Bio-Rad, $^{13}CH_3I$ from Prochem and $^{2}H_2O$ from Merck, Sharp and Dohme. Dy_2O_3 was purchased from British Drug House and converted to its chloride by HCl. All other chemicals were analytical grade.

Methods. Protein-free vesicles of *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine were prepared by the ethanol-injection method of Batzri and Korn [32], as modified by Kremer et al. [15]. 150 μl of *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine solution in absolute ethanol (50 mg/ml) were injected during a period of 5 min by a moter-driven syringe at room temperature with stirring into 5 ml 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0 buffer. Ethanol was removed by dialysis for 1 h at room temperature against 1 l of the same buffer. The size of these vesicles was measured by angular-dependent light scattering, using a FICA light-scattering photometer and techniques described by Kremer et al. [15]. Alternatively, protein-free vesicles of *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine were obtained by hydrating a lipid film in a Triton X-100 containing buffer, followed by removal of the detergent by SM-2 beads [16]. Lipid dispersions (5 mM) were made in 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0, containing 0.5% Triton X-100. The mixture was slowly rotated overnight at 4°C with 0.3 g of wet SM-2 beads/ml. The beads were removed by filtration through glass wool. Where indicated the vesicles were sedimented at 150 000 × *g* for 90 min or concentrated by vacuum dialysis.

Glycophorin-containing lipid vesicles were prepared according to MacDonald and MacDonald [17]. A mixture of glycophorin and lipids (in a 1 : 400 molar ratio) was dissolved in chloroform/methanol/water (150/75/1, v/v) and dried by evaporation. The lipid-protein film was hydrated in 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 M EDTA, pH 7.0 buffer.

Band 3 protein-containing vesicles were prepared using a crude Triton X-100 extract from human erythrocyte ghosts, as described previously [16]. Lipids were dried from chloroform and dispersed in fresh, ice-cold Triton X-100 extract (50 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, 0.5% Triton X-100, pH 7.0) to a concentration of 5 mM. The NaCl concentration was adjusted to 150 mM by addition of 1.05 M NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0. The mixture was slowly rotated overnight at 4°C with 0.3 g of wet SM-2 beads/ml. After removal of the beads by filtration through glasswool, the vesicles were sedimented at 150 000 × *g* for 90 min.

The internal volume of the vesicles was determined from the amount of trapped K^+, according to van Zoelen et al. [33]. The vesicles were prepared in 150 mM KCl, 10 mM Tris/acetic acid, 0.2 M EDTA, pH 7.0, and the non-trapped K^+ was removed by gel filtration at 0°C over Sephadex G-50 coarse, eluting with 150 mM choline chloride, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0 buffer. The K^+ content of the vesicles, released by the addition of 2% Triton X-100, was measured with a K^+-specific glass electrode as described before [34].

Freeze-fracture electron microscopy was performed as described previously [35]. Glycerol was added to prevent freeze damage.

^{13}C NMR measurements were performed on a Bruker 360 WS spectrometer

at a frequency of 90.5 MHz, as described in detail previously [12,13]. Prior to the NMR experiments 10—20% of ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0, was added to the samples.

Protein was measured by the method of Lowry et al. [36], with 3% dodecyl sulphate in the alkaline copper reagent, in case Triton X-100 was present in the samples. Dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to Steck and Yu [37]. Triton X-100 was measured by its absorbance at 275 nm or by using ^3H-labelled Triton X-100. Lipid extractions were carried out as described by Renkonen et al. [38]. Phospholipids were separated by two-dimensional thin-layer chromatography using the procedure of Broekhuyse [39] and determined as phosphorus by a modification [40] of the procedure of Fiske and Subbarow.

Results

Large protein-free and protein-containing vesicles

The following structures have been investigated: protein-free vesicles, prepared by the ethanol-injection method [32] and by treatment of a lipid/Triton-X-100 mixture with SM-2 beads [16], glycophorin-containing vesicles, prepared by hydration of a dried lipid-glycophorin film [17] and band 3 protein-containing vesicles, prepared by SM-2 beads treatment of a mixture of lipids and a crude Triton X-100 extract from human erythrocyte ghosts [16].

Injection of an ethanolic solution of lipid into an aqueous solution has been shown to result in the formation of vesicles [15,32]. In analogy with the results of Kremer et al. [15] on dimyristoylphosphatidylcholine, the diameter of 18 : 1_c/18 : 1_c-phosphatidylcholine vesicles can be varied by the lipid concentration in the ethanol. Using light-scattering techniques [15] diameters of 415, 490, 690 and 800 Å were found with 18 : 1_c/18 : 1_c-phosphatidylcholine concentrations in ethanol of 12.5, 25, 50 and 72.5 mg/ml, respectively. Electron microscopy of vesicles prepared by injection of a 50 mg/ml ethanolic solution of *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine in buffer shows unilamellar vesicles with an average diamter of 580 ± 40 Å (Fig. 1A). For the trapped volume of these vesicles 3.5 μl/μmol lipid was found, which is considerably more than the value of 0.8 μl/μmol reported for the 250 Å diameter sonicated vesicles [41].

Hydration of a dried lipid-glycophorin film according to MacDonald and MacDonald [17] results in the formation of large vesicles with the protein incorporated in the bilayer [14]. Small intramembraneous particles (40 Å diameter) have been observed on the fracture faces of these structures [14]. The size of these vesicles ranges from 1000 to 5000 Å (Fig. 1B) and for the trapped volume a value of 2.9 μl/μmol lipid was found.

Removal of Triton X-100 with SM-2 beads from a mixture of lipids and Triton X-100 extract from human erythrocyte ghosts produces vesicles, which show particles on the fracture faces which are very similar to those of the erythrocyte membrane [16]. The crude Triton X-100 extract used in this study contained 15% of the total ghost protein and 45% of the phospholipids. Dodecyl sulfphate-polyacrylamide gel electrophoresis showed that band 3, 4.2, 4.5, 7 and PAS1 were the major protein components (not shown). The pres-

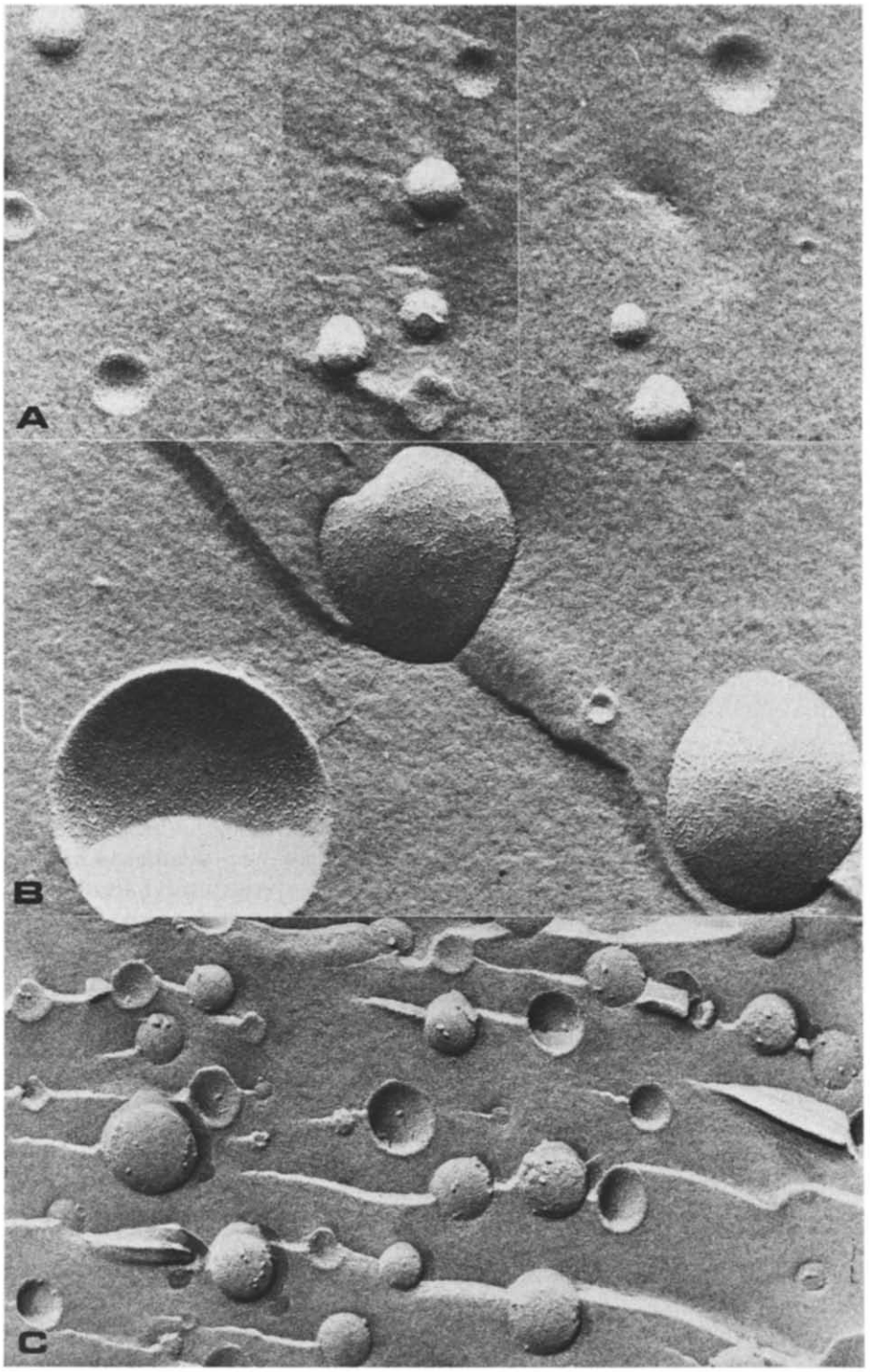

Fig. 1. Freeze-fracture electron micrographs of 18 : 1_c/18 : 1_c-phosphatidylcholine vesicles in 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, pH 7.0. (A) Vesicles prepared by the ethanol-injection method and concentrated by vacuum dialysis. (B) Vesicles prepared with purified glycophorin by hydrating a dried lipid-glycophorin film (lipid/protein molar ratio, 400 : 1) and sedimented at 125 000 × *g* for 60 min. (C) Vesicles prepared with a crude Triton X-100 extract from human erythrocyte ghosts by SM-2 beads treatment (lipid/protein weight ratio, 18 : 1) and sedimented at 150 000 × *g* for 90 min. Magnification, ×110 000.

ence of 80-Å particles on the fracture faces of the reconstituted vesicles (Fig. 1C) indicates the incorporation of band 3 protein into the bilayer [16,18,42]. Thin-layer chromatography of the lipids in the Triton X-100 extract showed the presence of phosphatidylethanolamine (37%), phosphatidylcholine (30%), phosphatidylserine (25%) and sphingomyelin (7%). In the vesicles obtained from 18 : 1_c/18 : 1_c-phosphatidylcholine and the crude Triton X-100 extract approximately 10% of the total lipid has originated from the Triton X-100 extract. Removal of Triton X-100 with SM-2 beads [43], as indicated in the methods section, results in a Triton X-100 concentration of 0.005—0.01% (determined with ^{3}H-labelled Triton X-100), which means a molar ratio Triton X-100 to phospholipid in the vesicles of 2—3 to 100. Repeated treatment with SM-2 beads was found to lower this ratio to about 1—100. Vesicles formed in this way have a diameter of 500—1500 Å (Fig. 1C). For the trapped volume a value of 2.2 μl/μmol lipid was found. Protein-free vesicles were made in the same way from a dispersion of lipid in a Triton X-100 containing buffer [16]. NMR measurements, discussed below, indicated that the final centrifugation step had to be omitted, as it caused the formation of multilamellar structures.

^{13}C NMR measurements on large vesicles

The transbilayer distribution of *N*-[*Me*$_3$-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine in sonicated vesicles can be measured by ^{13}C NMR using Dy^{3+}, which causes a downfield shift and broadening of the resonance originating from the labelled carbon atoms of the lipid molecules in the outer monolayer of the vesicle [12—14]. Furthermore, the intensity of the shifted resonance is strongly decreased by the loss of the Nuclear Overhauser Enhancement [12,14]. The 90.5 MHz ^{13}C NMR spectrum of *N*-[*Me*$_3$-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine vesicles, prepared by the ethanol-injection method, is shown in Fig. 2A. Besides the resonance from the external dioxane reference (0 ppm), and Tris (8 ppm) only the resonance of the *N*-methyl carbon atoms (13 ppm) is observed. The addition of Dy^{3+} to the vesicles causes a decrease in the intensity of this signal, without the appearance of a shifted resonance (Fig. 2B). Similar

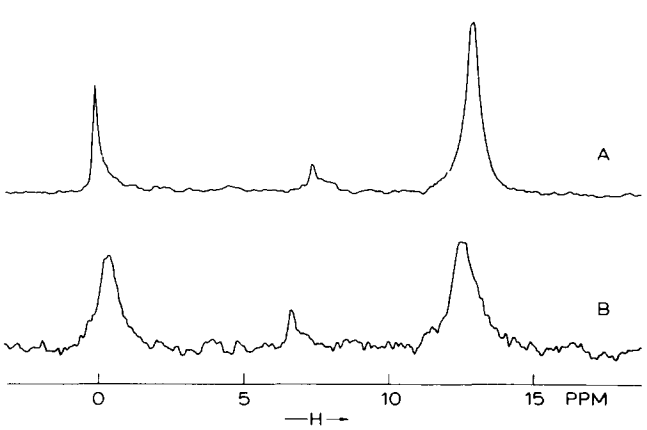

Fig. 2. 90.5 MHz ^{13}C spectrum of a 3 mM *N*-[*Me*$_3$-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine vesicle suspension in a ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, p^{2}H 7.0 buffer, prepared by the ethanol-injection method, (A) in the absence and (B) in the presence of 5 mM $DyCl_3$. Chemical shifts are upfield from external 1,4-dioxane.

spectra were obtained with N-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine vesicles, prepared by the Triton X-100/SM-2 beads procedure, both using a Triton X-100 containing buffer or a crude Triton X-100 extract from human erythrocyte ghosts. As shown in Fig. 3 the intensity of the N-[Me_3-^{13}C] resonance decreases with increasing Dy^{3+} concentration, until a value near to 50% has been reached. Higher Dy^{3+} concentrations do not affect the intensity of the remaining signal, not even after a 4 h incubation at 25° C. This strongly suggests that the resonance from the molecules in the outside layer of the vesicles is broadened by Dy^{3+} beyond detection, whereas the resonance from the molecules in the inside layer is not affected.

Fig. 4 shows the 90.5 MHz ^{13}C spectra of glycophorin-containing vesicles of N-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine, in the absence and presence of 5 mM $DyCl_3$. It is clear that upon addition of Dy^{3+} nearly all intensity of the N-[Me_3-^{13}C] resonance is lost, which can only mean that these vesicles are permeable to the trivalent cation and that molecules in the inner layer are accessible to the Dy^{3+}. Considering the possibility that this high permeability could be due to the 18 : 1_c/18 : 1_c-phosphatidylcholine being unable to provide a proper packing around the glycophorin molecules, small amounts of other lipids were introduced into the system, which could possibly restore the permeability barrier of the vesicles. Fig. 5 shows the ^{13}C spectra of such a system, in which the lipid part consisted of N-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine and 18 : 1_c/18 : 1_c-phosphatidylethanolamine in a 9 : 1 molar ratio.

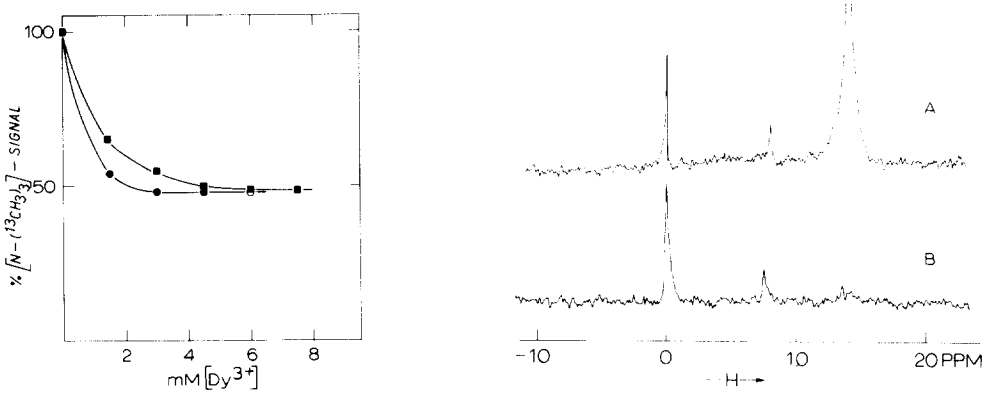

Fig. 3. Effects of Dy^{3+} on the intensity of the N-[Me_3-^{13}C] (N-($^{13}CH_3$)$_3$) resonance of N-[Me_3-^{13}C]-18 : 1_c/18 : 1_c-phosphatidylcholine vesicles in a ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, p^{2}H 7.0 buffer at 90.5 MHz. To a 3 mM N-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine vesicle suspension increasing amounts of $DyCl_3$ were added and the intensity of the remaining N-[Me_3-^{13}C] signal was determined. ■, vesicles prepared by the ethanol-injection method; ●, vesicles prepared with a crude Triton X-100 extract from human erythrocyte ghosts, by removal of Triton X-100 with SM-2 beads; ○, idem, after 4 h incubation with 6 mM Dy^{3+} at 25° C.

Fig. 4. 90.5 MHz ^{13}C spectrum of a glycophorin-containing vesicle suspension of N-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine (2 mM) prepared according to MacDonald and MacDonald [17], in ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, p^{2}H 7.0 buffer, (A) in the absence and (B) in the presence of 5 mM $DyCl_3$.

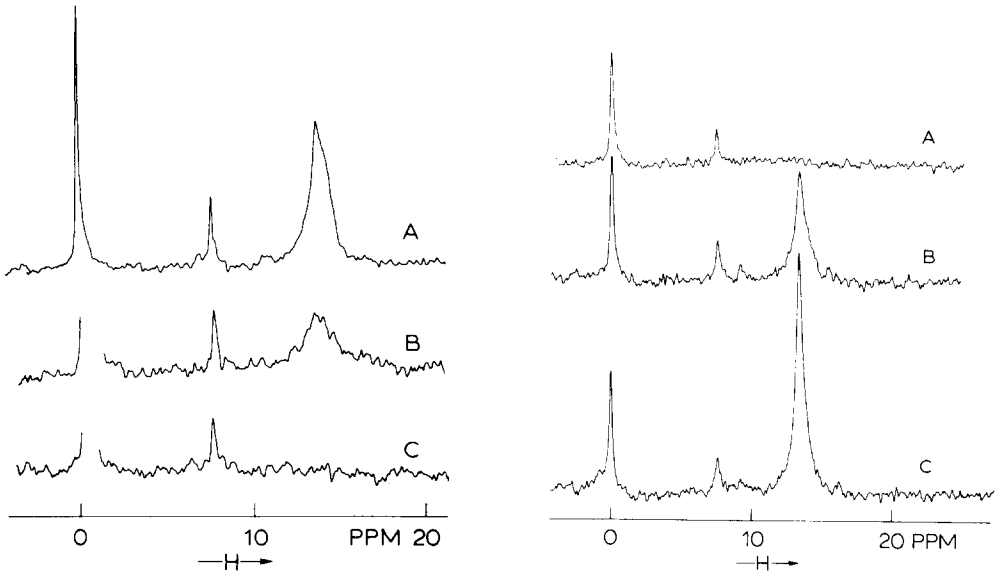

Fig. 5. 90.5 MHz ^{13}C spectrum of a glycophorin-containing vesicles suspension of 90 mol% *N*-[Me_3-^{13}C]-18 : 1_c/18 : 1_c-phosphatidylcholine (2 mM) and 10 mol% 18 : 1_c/18 : 1_c-phosphatidylethanolamine, prepared according to MacDonald and MacDonald [17], in a ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, p^{2}H 7.0 buffer, (A) in the absence, (B) in the presence of 5 mM $DyCl_3$, and (C) after freezing-thawing in the presence of 5 mM $DyCl_3$.

Fig. 6. 90.5 MHz ^{13}C spectrum of a glycophorin-containing vesicles suspension of 90 mol% *N*-[Me_3-^{13}C]-18 : 1_c/18 : 1_c-phosphatidylcholine (2 mM) and 10 mol% 18 : 1_c/18 : 1_c-phosphatidylethanolamine, prepared in the presence of 5 mM $DyCl_3$ according to MacDonald and MacDonald [17], in ^2H_2O-containing 150 mM NaCl, 10 mM Tris/acetic acid, 0.2 mM EDTA, p^{2}H 7.0, (A) in the absence, (B) in the presence of 7 mM EDTA, and (C) after freezing-thawing in the presence of 7 mM EDTA.

Here the addition of the Dy^{3+} clearly causes the disappearance of about 50% of the signal. A similar result was obtained by introducing 10 mol% 1-18 : 1_c-lysophosphatidylcholine instead of 18 : 1_c/18 : 1_c-phosphatidylethanolamine. Fig. 5C shows that freezing-thawing in the presence of Dy^{3+} causes the disappearance of all signal intensity. This indicates firstly that indeed on interaction with Dy^{3+} in these conditions the resonance of *N*-[Me_3-^{13}C] carbon atoms are broadened beyond detection and secondly, that all lipid molecules are accessible to shift reagent. Another way to establish this has been shown in Fig. 6, again using glycophorin-containing vesicles of *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine and 18 : 1_c/18 : 1_c-phosphatidylethanolamine in a molar ratio of 9 : 1. When prepared in the presence of 5 mM $DyCl_3$ no signal at all is observed from the *N*-[Me_3-^{13}C] carbon atoms (Fig. 6A). Complexation of the external Dy^{3+} with 7 mM EDTA causes the appearance of the resonance of the molecules in the outer layer (Fig. 6B), whereas freezing-thawing in the presence of 7 mM EDTA causes the complexation of all Dy^{3+}, and the appearance of the resonance of all molecules (Fig. 6C). These experiments show that it is possible to calculate the percentage of molecules in the inner layer from the ratio of intensities of the *N*-[Me_3-^{13}C] signals in the absence and presence of sufficient Dy^{3+} to cause the broadening of the signal of all molecules in the outer layer beyond detection. This Dy^{3+} concentration is easily found by a titration exper-

TABLE I

TRANSBILAYER DISTRIBUTION OF *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-PHOSPHATIDYLCHOLINE IN LARGE UNILAMELLAR VESICLES

	% Molecules in inner monolayer
Protein-free vesicles	
Prepared by the ethanol-injection method	48.5
Prepared by the Triton X-100/SM-2 beads procedure	49.5
Glycophorin-containing vesicles	
Prepared according to MacDonald and MacDonald [17]	indeterminant *
Prepared according to MacDonald and MacDonald [17], containing in addition 10 mol% 18 : 1_c/18 : 1_c-phosphatidylethanolamine	49.5
Prepared according to MacDonald and MacDonald [17], containing in addition 10 mol% 1-18 : 1_c-lysophosphatidylcholine	48.6
Band 3 protein-containing vesicles	
Prepared by the Triton X-100/SM-2 beads procedure with a crude Triton X-100 extract from human erythrocyte ghosts	49.5

* These vesicles are permeable to Dy^{3+}, and a reduction of the signal intensity was observed to about 10%.

iment as in Fig. 3. Results obtained in this way have been summarized in Table I. Of the *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine molecules 48.5 and 49.5% were detected in the inner layer of the protein-free vesicles, prepared by ethanol-injection method and Triton X-100/SM-2 beads procedure, respectively *. Similar values were found for the glycophorin-containing vesicles with 10 mol% 18 : 1_c/18 : 1_c-phosphatidylethanolamine (49.5%) or 10 mol% 1-18 : 1_c-lysophosphatidylcholine (48.6%) and for the band 3 protein-containing vesicles (49.5%). With glycophorin-containing vesicles of only *N*-[Me_3-^{13}C]-18 : 1_c/18 : 1_c-phosphatidylcholine a reduction to about 10% of the signal intensity was observed upon addition of Dy^{3+}, due to the leakiness of these vesicles. With *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine vesicles, prepared by the Triton X-100/SM-2 beads procedure, about 85% of the signal did not disappear upon addition of Dy^{3+}, if these vesicles had been sedimented by high speed centrifugation. Obviously, the centrifugation caused fusion of the vesicles to multilamellar structures. Multilamellar structures were also formed after concentration of these vesicles by vacuum dialysis to a lipid concentration of 50 mM, this accounts of 65% of the *N*-[Me_3-^{13}C] signal not disappearing upon addition of excess Dy^{3+}.

Discussion

The mechanisms which regulate transmembrane movements of lipids in biological membranes are still poorly understood. Recent studies have revealed that asymmetrical perturbations of the bilayer [13,44], phase transitions [45] and incorporation of glycophorin [14,46] greatly facilitate the transbilayer

* Recently the method was also applied to protein-free vesicles prepared by the ether-evaporation method described by Deamer and Bangham [41] and 49.6% of the molecules were detected in the inner monolayer (Noordam, P.C., unpublished).

movements of lipids in sonicated vesicles. The strong curvature of sonicated vesicles makes the significance of these observations for biological membranes uncertain, as the curvature itself might be one of the factors involved in the transbilayer movement of lipids. A prerequisite for studying transbilayer movements is a technique which can determine the distribution of molecules over inner and outer layers. NMR techniques like ^{1}H, ^{31}P and ^{13}C NMR have been successfully applied to sonicated vesicles to obtain this information [20—23]. With large structures both ^{1}H and ^{31}P NMR do not yield high resolution spectra, but ^{13}C NMR shows only a limited resonance broadening. In this paper it is shown that ^{13}C NMR of the *N*-[Me_3-^{13}C]-labelled lipids offers the possibility of measuring the distribution of the *N*-[Me_3-^{13}C] carbon atoms over the inner and outer layer of large unilamellar vesicles. On interaction with Dy^{3+} the resonance of the *N*-[Me_3-^{13}C] carbon atoms is broadened beyond detection, so the percentage of molecules in the inner layer can be calculated from the signal intensities in the absence and presence of Dy^{3+}. With this technique it should be possible to study transbilayer movements in large unilamellar vesicles along the lines followed previously with sonicated vesicles [12—14,46].

The finding that the large vesicles used in this study show an equal distribution of *N*-[Me_3-^{13}C] carbon atoms over inner and outer layers, indicates that these vesicles are virtually all large and unilamellar, and that they are impermeable to Dy^{3+}. In small sonicated vesicles of *N*-[Me_3-^{13}C]18 : 1_c/18 : 1_c-phosphatidylcholine only 37% of the molecules are located in the inner layer [12]. If multilamellar structures were present values of more than 50% would be found for the percentage of molecules in the inner layer, as was the case with vesicles prepared by the Triton X-100/SM-2 beads procedure after high speed centrifugation or concentration by vacuum dialysis. When vesicles are permeable to Dy^{3+} very low values are found for the percentage of molecules in the inner layer, as in the case of glycophorin-containing vesicles of *N*-[Me_3-^{13}C]-18 : 1_c/18 : 1_c-phosphatidylcholine.

Phospholipid molecules in the immediate environment of glycophorin molecules have been shown to have physico-chemical properties, which are different from the bulk of the phospholipids [33]. Discontinuities in the bilayer between protein-perturbed and free phospholipid molecules may be responsible for the observed high permeability of glycophorin-containing vesicles of 18 : 1_c/18 : 1_c-phosphatidylcholine. The observation that the barrier properties of glycophorin-containing bilayers of 18 : 1_c/18 : 1_c-phosphatidylcholine towards Dy^{3+} can be restored by the incorporation of 10 mol% 18 : 1_c/18 : 1_c-phosphatidylethanolamine or 1-18 : 1_c-lysophosphatidylcholine indicates that under these conditions the discontinuities in the lipid bilayer are less pronounced. From the model of Israelachvili [47] it appears that when the sizes of the hydrophobic regions of protein and lipid molecules do not exactly coincide, the lipid molecules will have to accomodate to the protein, which results in a curved meniscus of lipid molecules around the protein. It can be imagined that a tapered molecule like phosphatidylethanolamine, or a wedge-shaped molecule like lysophosphatidylcholine, will fit more easily into such a curved lipid meniscus than a more rectangular molecule like phosphatidylcholine. In this way a preferential interaction of certain lipid molecules with a protein will be observed, which originates from merely geometrical factors.

Band 3 protein-containing vesicles prepared from the crude Triton X-100 extract and 18 : 1_c/18 : 1_c-phosphatidylcholine are impermeable to Dy^{3+}. Whether this impermeability is also induced by phospholipids other than phosphatidylcholine, which are present in the Triton X-100 extract, is not yet known.

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