An Approach to Channel-type Molecular Structures. Part 3.† Incorporation Studies of the *Bouquet*-shaped B_M and B_{CD} in Phosphatidylcholine Vesicles‡

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Incorporation of *bouquet*-shaped molecules based either on a macrocyclic (B_M) or on a β-cyclodextrin (B_{CD}) core in phosphatidylcholine vesicular bilayers has been investigated. As can be shown by numerous techniques, these molecules are incorporated in moderate to high yields within vesicle membranes. Possible incorporation modes are then discussed.

In previous papers,[1,2] the synthesis and some properties in homogeneous media of two series of molecules expected to act as artificial ionic channels in lipid bilayers were reported. These molecules belong to a new family called *bouquet* in order to describe their symmetrical arborescent shape. The B_M series is based on the tetracarboxylic [18]-O_6 macrocycle[1] [Fig. 1(*a*)], whereas the B_{CD} series is built up on a modified β-cyclodextrin[2] [Fig. 1(*b*)].

In the present paper, the studies dealing with incorporation of these molecules in artificial bilayers of phosphatidylcholine vesicles are described. Our purpose was to define their mode of incorporation *via* the effects of the interactions between model bilayers and the *bouquets*. Evidence for an interaction between any molecule and membranes can be obtained by two different approaches: (*i*) the effect of incorporation in lipid media on some specific molecular features (effect of the lipid environment on the molecule); (*ii*) the observation of molecule-induced perturbations of the membrane system (effect of the molecule on the lipid environment).

(*i*) In order to detect the presence of the *bouquets* in membrane media, two types of intrinsic incorporation probe were introduced during the design of these molecules: (*a*) strongly absorbing UV chromophores give the opportunity to observe the core and to measure the number of *bouquets* present in the bilayer; furthermore, these chromophores might give valuable information concerning the nature of their environment; (*b*) at pH = 7 used in all the reported experiments, at least a part of the carboxylic groups borne on the *bouquets*' extremities is expected to be negatively charged; in a neutral or zwitterionic environment as is the case for the phosphatidylcholine bilayers, these *bouquets* display a local excess of negative charge which could be detected by a charge-sensitive probe like a cation.

(*ii*) Among possible membrane properties likely to indicate incorporation of foreign molecules, one finds fluidity, elasticity, etc. In the special case of lipids exhibiting a gel–liquid crystal transition, examination of phase-transition characteristics can often be equally informative.[3] Among the membrane systems in which one can observe such features, vesicles are probably the most appropriate.[4–6] The choice of the lipids used for these investigations was based, firstly on correspondence between the thickness of the bilayers they can form and the length of the *bouquets* in their extended-chain conformation (about 40–50 Å) and secondly on their ability to form vesicles in a wide range of conditions. Being already well documented, egg lecithin (EPC) was chosen for most of the studies. Dipalmitoylphosphatidylcholine (DPPC), giving approximately the same bilayer thickness as EPC while displaying a gel–liquid crystal transition was also occasionally used. At this stage, the importance of the lipid system in the *bouquet* concept must be emphasized. It is not just a matrix allowing the study of ionophore properties, but affecting the *bouquet* itself, it determines, at least partially, *bouquet* properties such as chain elongation and mobility, internal conformational energy potentials.

If the evidence of *bouquet* incorporation into the membrane constitutes an essential preliminary step to ion conduction studies, it does not allow one to draw *bouquet* structure–ion transport property relationships alone. Such a goal can only be reached after a careful analysis of the orientation, and possibly, the conformation of these molecules in the membranes. In the last part of this paper, we try to define the way in which the *bouquets* are incorporated in the lipid medium.

Experimental

All the reagents used in this study were of the highest grade. The lipids (EPC and DPPC Sigma) were kept at −20 °C in chloroform solutions. Unless specified, the molecules to be incorporated were added prior to vesicle formation, together with the lipids. The *bouquets* were kept at 4 °C in alcoholic solutions (methanol or ethanol). Except in the case of the titration experiments, the *bouquet* concentrations given in the text are the initial molar ratio *bouquet*: lipid of the vesicular preparations. In the following text, aqueous solution stands for Tris pH = 7 buffered aqueous solutions of the different salts used.

Vesicles were prepared according to classical procedures.[7] Preparation by sonication:[8] a chloroform solution containing 10 mg of DPPC was evaporated under vacuum at room temperature to produce a thin film on the walls of a small round-bottomed flask. After the addition of Tris pH = 7, 10 mmol dm^{-3} buffer (2 cm^3), the suspension was continuously sonicated (Branson B 50 equipped with a titane probe; 15 min; power 2; 55 °C). After being cooled to room temperature, the preparation was filtered on a short column of Sephadex G-25M (Pharmacia PD-10; elution Tris pH = 7, 10 mmol dm^{-3} buffer).

Preparation by injection[8] was performed for experiments using a mass of lipid injected in 2.5 cm^3 of aqueous solution of 100–150 mg for the concentrated and 10–25 mg for the diluted

† For Part 1 and Part 2 of this series, see references 1 and 2.

‡ Abbreviations used in the text: EPC, egg lecithin; DPPC, dipalmitoylphosphatidylcholine; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; B_M° or °COOR *bouquets* based on a [18]-O_6 crown ether (R = Et or Na)[1] and B_{CD}° or °COOR *bouquets* based on a β-cyclodextrin derivative (R = Et, Bn or H);[2] in both cases polyoxyethylenic (o) or alkyl (c) lateral amphiphilic chains. Tris: Trishydroxyethylaminomethane; Tris pH = 7: $TrisH^+$-Cl^-/Tris buffered at pH = 7; R = compartmentation ratio of the vesicular solution = internal volume/external volume.

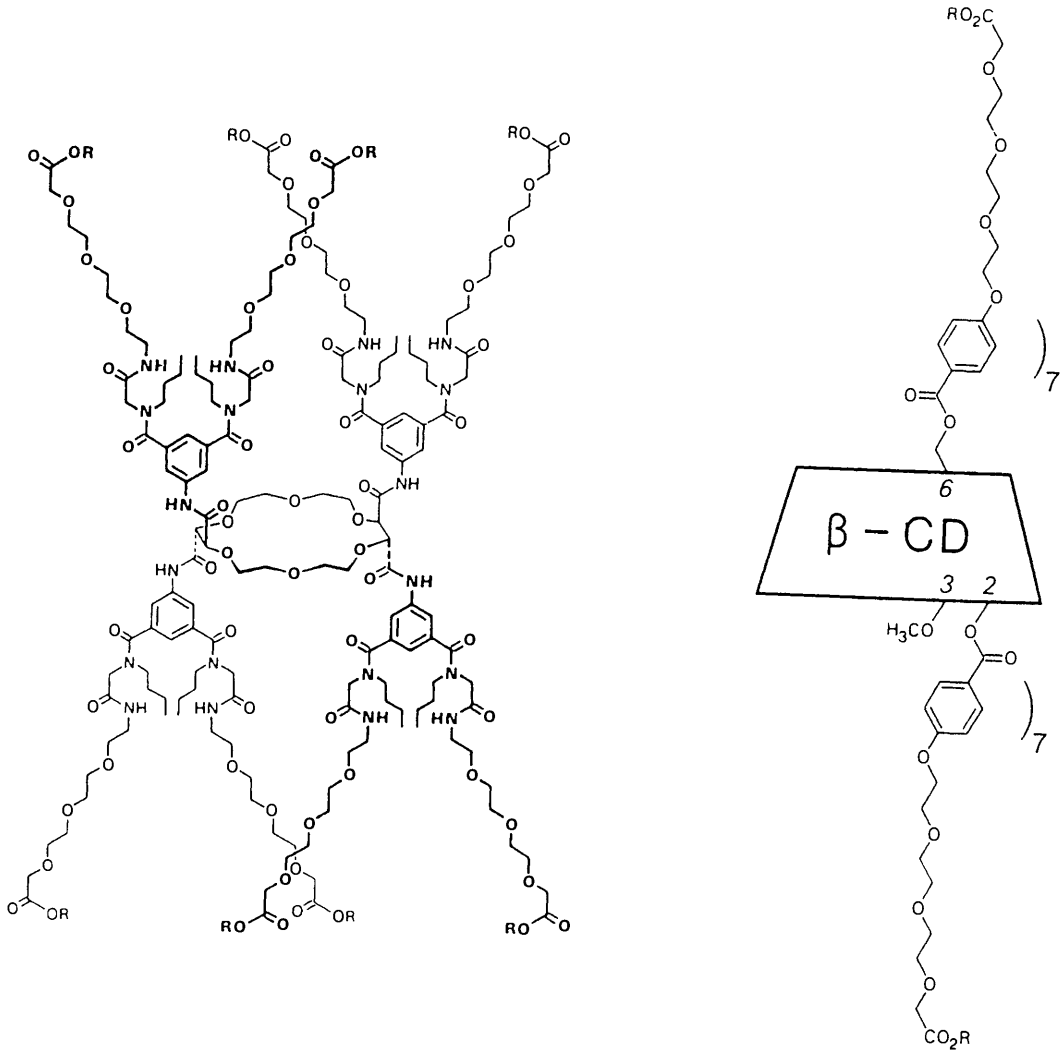

Fig. 1 Structure of the *bouquet* molecules (*a*) $B_M°$, (R = Na); (*b*) $B_{CD}°$, (R = H)

systems. A syringe was filled with a solution of the lipid in a mixture of absolute ethanol (0.5 cm^3) and either diethyl ether, methylene chloride or chloroform (2 cm^3). The solution was injected under argon (13 cm^3 h^{-1}) into 2–2.5 cm^3 of aqueous solution previously degassed with argon bubbling for 10 min at 60 °C (Et_2O or CH_2Cl_2) or 70 °C ($CHCl_3$; in this case a weak aspiration was maintained above the injection system). After the end of the organic solution introduction, the vesicular preparation stayed for 15 min at injection temperature and was then cooled to room temperature. The preparation was finally dialysed against the injection aqueous solution (0.5–1 l) and filtered over Sephadex G-25M (Pharmacia PD-10; elution with the same aqueous solution).

Preparation by dialysis. [9] A mixture of EPC (30 mg), β-octyl D-glucopyranoside (15 equivalents) in methylene chloride (5 cm^3) was evaporated under vacuum at room temperature. The resulting film was dissolved in aqueous solution (2 cm^3) and the mixture was then poured into a dialysis bag (Viskase; MWCO: 6000–8000). The dialysis was run in two or three steps against 1 dm^3 of aqueous solution (one night, then 24 h, and finally a few hours). Finally, the preparation was filtered over Sephadex G-25M (Pharmacia PD-10; elution with the same aqueous solution).

Incubation experiment. A solution of EPC vesicles (4 cm^3) was prepared by dialysis according to the procedure described above. This preparation was first diluted with the dialysis solution (NaCl 100 mmol dm^{-3} Tris pH = 7 10 mmol dm^{-3}) and then divided into three 2 cm^3 samples. 100 μl of concentrated methanolic solution of $B_M°$ (respectively, 60 μl of concentrated ethanolic solution of B_M^c) were then poured into the first (respectively, the second) vesicular sample to reach a concentration of 0.5 mol of *bouquet* for every 100 mol of lipid. After 36 h of incubation at room temperature, a first series of UV spectra was recorded. After addition of 1 cm^3 of NaCl 100 mM Tris pH = 7 in each sample, gel permeation chromatography (Sephadex G-25M; Pharmacia PD-10) was carried out and the first 2 cm^3 of turbid vesicular solution were collected. A second series of UV spectra was recorded at this stage.

UV spectra were recorded on a Perkin–Elmer 554 spectrometer with a slit width of 1 or 2 nm. All spectra were collected at room temperature unless otherwise indicated. In the latter case, thermostatted cells were used. For each experiment, two solutions of vesicles were prepared under the same conditions. The first one, without *bouquet*, was put into the reference cell, the second one containing the vesicles prepared in the presence of the molecules to be incorporated was put into the working cell. It was checked that errors due to poor

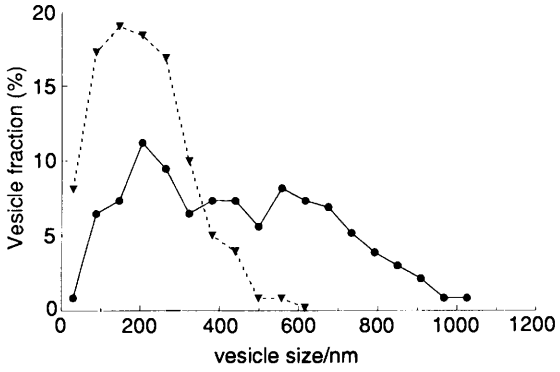

Fig. 2 Distribution of size of EPC vesicles prepared by dialysis either with (- - - -) or without (——) B_{CD}^{c}

compensations of light diffusion were less than 10% of the recorded absorbances.

CD spectra were recorded at room temperature on a Mark V Jobin-Yvon spectrometer. In every case, absorbance was less than 1.4. As for the UV spectroscopy, the recorded spectra were obtained by subtraction of the spectrum without *bouquet* from the spectrum with it.

^{7}Li, ^{23}Na and ^{133}Cs NMR spectra were recorded at room temperature (22 ± 2 °C) on a Bruker AM 200SY spectrometer equipped with a broad band probe at 77.7, 52.9 and 26.25 MHz respectively (90° pulse width: 27, 16.5 and 27.5 μs; acquisition time (AQ + RD): 5.3 (s), 483 (ms), 3.9 (s); resolution: 0.095, 0.52, 0.128 Hz/point; 8, 16, 8 scans; line broadening factor: 0.1, 1.2, 0.3 Hz). Longitudinal relaxation times T_1 were measured by the inversion recovery technique. The partial inversion recovery technique was also used to access the zero passage time τ.[10] It was checked under all conditions and for all the investigated ions: $T_1 \approx 1.5$–1.7τ. The measurements were recorded on 2 cm^3 vesicular samples containing at least 10% D_2O. The separation of the ion NMR signals arising, respectively, from the internal and the external vesicular compartment was achieved using methods previously described.[11] These methods were extended to the cesium ion using the couple (Tm^{3+}, tripolyphosphate ion: $P_3O_{10}^{5-}$) as a shift reagent. A vesicular solution was prepared in presence of MCl (M = Li, Na or Cs) and an M NMR spectrum is recorded (^{7}Li, ^{23}Na or ^{133}Cs NMR). The vesicular wall effects being weak, one observed initially one peak corresponding to M^+ in its aqueous environment. A shift reagent was then added to the external pool, so as to shift the ion signal, giving therefore a two-peak spectrum (one for the internal compartment, one for the external one).[11] Under appropriate accumulation conditions, the compartmentation ratio R = internal vesicular volume/external vesicular volume is deduced from the respective areas of the two peaks.

Calorimetry measurements were made with a Perkin-Elmer DSC 2 Differential Scanning Calorimeter on one-drop samples of vesicular preparations.

All quantitative determinations of the *bouquet* concentrations in membranes were performed after gel permeation chromatography (Sephadex G-25M; Pharmacia PD-10). During these experiments, the initial concentration in *bouquet* was 1 mol for every 100 mol of lipid. The lipid concentrations were measured after mineralization. The amount of phosphate groups in a vesicular aliquot was estimated through phosphomolybdic complex formation and colorimetric determination.[12] Typically, for a vesicular preparation initially containing 20 mg of DPPC or EPC in 2.5 cm^3 of aqueous solution, aliquots in the range 100–200 μl were mineralized. The *bouquet* concentrations were measured by UV spectroscopy after lyophilization of the vesicle solution, taking up in an appropriate amount of ethanol 95% and filtration of residual salts (*bouquets* B_{CD}: $\varepsilon_{210} = 1.3\ 10^5$ cm^{-1} dm^3 mol^{-1};[1] *bouquets* B_{CD}, $\varepsilon_{258} = 2.0\ 10^5$ cm^{-1} dm^3 mol^{-1})[2]. For an initial molar ratio *bouquet:* lipid of about 1–2%, 0.8–1.3 cm^3 aliquots were lyophilized.

Results

Characterization of the Vesicular Systems.—Vesicular sonication preparations were characterized by light-scattering measurements. Injection or dialysis vesicular preparations were analysed by electron microscopy (freeze fracture technique). In the case of sonication, the results are not consistent with the existence of freely moving small vesicles: the observed 'object' mean diameter of 500–800 nm and the polydispersity suggest that the small unilamellar vesicles (SUV) produced under such conditions are probably aggregated. The population of vesicles prepared by injection was rather heterogeneous, containing multilamellar vesicles (MLV) and large unilamellar vesicles (LUV), even at low lipid concentrations. As already described,[7,9,13] vesicles prepared by dialysis were essentially LUV. No influence of *bouquet* on the vesicle type was ever observed by these techniques. The vesicular preparations were also routinely characterized by ^{7}Li NMR, ^{23}Na NMR and ^{133}Cs NMR to show the aqueous solution compartmentation and to measure the internal volume of the vesicular system. Typical results are presented in Table 1. For a given amount of lipid material, one observes that:

—the SUV prepared by sonication have the smallest internal volume; this is in agreement with expectations based on their small vesicular radius

—the LUV prepared by dialysis have the largest internal volume; our values are in agreement with values previously described[13]

—the values observed for the injection preparations are in accordance with electron microscopy observations; the internal volume is clearly less than in the case of the dialysis LUV; this can be explained by the presence of MLV among vesicles thus prepared.

As far as can be estimated from the technique of investigation and except for the case of vesicular preparations obtained by dialysis, the *bouquets* did not alter the membrane systems. On the other hand, the *bouquets* exert a significant and reproducible effect on vesicles prepared by dialysis: the compartmentation ratio R decreases significantly in the order: no *bouquet* > B_M° > B_M^{c} = B_{CD}° > B_{CD}^{c}. This effect was confirmed by the measurement of vesicle sizes from the electron microscopy replicas. Introduction of the *bouquets* during the vesicle preparation decreases significantly the mean diameter of the vesicle population. All profiles of the fractured lipid shells were circular, and it could therefore be assumed that they were derived from spherical vesicles. Histograms of the measured diameters of the lipid shells were subjected to stereological analysis to correct for the effect of non-equatorial fractures on size distribution. Each diameter range of the freeze-fractured shells was thus multiplied by a factor of $4/\pi$ to obtain roughly the diameter range of the corresponding vesicles.[14] As an example, Fig. 2 gives the distribution of the size of vesicles prepared by dialysis at 25 mg EPC/cm^3 of 100 mmol dm^{-3} LiCl solution without or with B_{CD}^{c} at a concentration equal to 0.4 mol of B_{CD}^{c} for 100 mol of lipid.

As estimated by NMR techniques, the reproducibility of all the vesicle preparations obtained under the same conditions was fairly good. The standard deviation of the internal vesicular compartmented volume was less than 20%, that of the longitudinal relaxation time T_1 or the width at half-height $W_{1/2}$ of the internal NMR signal of the elements investigated, ^{7}Li, ^{23}Na and ^{133}Cs being less than 10%. The vesicle stability at room temperature, examined by the same methods, was good enough to perform all our experiments; no significant change, either of

Table 1 Measurements of the compartmented ratio R = internal vesicular compartmented volume/external vesicular compartmented volume by M NMR of alkali-metal ions M: $^{7}Li^{+}$, $^{23}Na^{+}$ and $^{133}Cs^{+}$

Method of vesicle preparation[a]	C_{lipid}[b]	R(%)
Sonication	10 mg/2 cm^3	< 1
Injection	10–25 mg/ 2.5 cm^3 (CH_2Cl_2–$CHCl_3$)[c]	≈ 1[d]
	10–25 mg/2.5 cm^3 (Et_2O)[c]	≈ 5[d]
	100–150 mg/2.5 cm^3 (Et_2O)[c]	≈ 20–30[d]
Dialysis	30 mg/2 cm^3	
EPC		13.5[e]
EPC + $B_M{}^o$ 5.10^{-3} mol/mol lipid		11.5[e]
EPC + $B_M{}^c$ 5.10^{-3} mol/mol lipid		9.5[e]
EPC + $B_{CD}{}^o$ 5.10^{-3} mol/mol lipid		9.5[e]
EPC + $B_{CD}{}^c$ 5.10^{-3} mol/mol lipid		6.5[e]

[a] See the Experimental part. [b] Lipid concentration: m mg of EPC in x cm^3 of aqueous solution. The vesicles were prepared as described in the Experimental part at the initial lipid concentration presented in the table. The results displayed by DPPC and EPC for sonication and injection vesicular preparation were similar and only the latter are given. During the preparations by sonication and injection, the presence of the *bouquets* exerted no influence on R and only one value is reported. [c] Solvent used during the injection of EPC. [d] Mean value obtained under several conditions of salt concentrations in the aqueous phase (range 100–200 mmol dm^{-3}). [e] Mean value based on three experiments with three different aqueous solutions: 100 mmol/dm^{-3} MCl (M = Li, Na, Cs), Tris pH = 7 10 mmol dm^{-3}.

the chemical shift difference between the signals corresponding to internal and external vesicular compartments, of the longitudinal relaxation times T_1 or of the widths at half-height $W_{1/2}$ was observed after 48 h.

Detection of the Bouquets *in Membranes by UV Spectroscopy.*—In bilayer incorporation studies, the UV spectra may sometimes provide important information.[15] Firstly, the presence of a signal in agreement with the expected spectrum of the molecule to be incorporated and an absorbance value greater than the theoretical value resulting only from the molecules dissolved in the internal pool of the vesicles, indicate membrane incorporation.* Secondly, supposing that no lipid is lost during the vesicle preparation and that the molecular extinction in the lipid medium may be estimated from a systematic study of the solvent influence on the UV absorption of the molecules, the absorbance roughly gives the incorporation yield. Finally, the changes in the UV spectra arising from the perturbations exerted on the bilayer by temperature modifications, lipid nature, vesicular structure, etc., might give useful insights into the orientation and conformation analysis in the membrane medium.[15] During these studies, special attention was paid to the maximum absorption wavelengths and the half-height width of the corresponding absorptions.

To analyse the mode of incorporation of the *bouquets* in the membrane media, UV spectra were collected in several vesicular systems essentially produced in three different ways. Sonication treatment of an aqueous lipid suspension yielded SUV.[8] A dialysis method gave LUV.[9] In addition to these methods, vesicular preparations were also performed by injection[8] to yield a mixture of vesicles containing a great amount of MLV. Thus, a collection of methods giving vesicles of various curvature radii was available. In the two *bouquet* series [Fig. 1(*a*) R = Na and (*b*) R = H], UV spectra were recorded for all these types of vesicle. Furthermore, in the B_M series and in the case of vesicles prepared by dialysis, UV spectra were measured whereas the *bouquets* were added either before or after vesicle formation.†

The influence of two parameters was qualitatively investigated: the curvature of the lipid system and the temperature. The first one can be approached by recording UV spectra of MLV, LUV and SUV. It is also possible, for a given preparation, to examine the effect of gel permeation chromatography. Such an operation effectively modifies the size distribution of a vesicle preparation so that comparing spectra of the initial vesicular solution and of the first drops collected after chromatography allows the study of the influence of this parameter. Indeed after chromatography, the vesicle mean-diameter is expected to be shifted towards larger values whereas the size distribution width is narrowed.

Fig. 3(*a*) and Fig. 3(*b*) report the UV spectra of *bouquets* $B_{CD}{}^oCOOH$ and $B_{CD}{}^cCOOH$ in homogeneous solution or in membrane media. Tables 2(*a*) and 2(*b*) summarise the results corresponding to all *bouquets*. Except for the spectra recorded in vesicles prepared by injection, all the spectra recorded in membrane media display significant changes compared with homogeneous solutions, which may be roughly described as an overall red-shift of the absorption maximum λ_{max} and a broadening of the corresponding absorption. This latter phenomenon is more pronounced with the *bouquets* B_{CD}. The alkyl B^c and the polyoxyethylenic B^o *bouquets* exhibit parallel behaviour. Besides these general observations, one notices that: (*i*) the influence of the chemical nature of the lipid used to produce vesicles only weakly affects the UV spectra; in the B_M series, similar results were observed for the vesicles prepared by injection with EPC or DPPC [Table 2(*a*)]. (*ii*) For a given *bouquet*, the UV spectrum is strongly dependent on the mode of incorporation; as an example, while the vesicles used were in both cases prepared by dialysis, UV spectra of $B_M{}^o$ and $B_M{}^c$ appeared overall to be red-shifted in the incubation experiment (addition of the *bouquets* after vesicle formation) compared with those recorded when the *bouquets* are co-added with the lipids before the formation of the vesicles. (*iii*) While exhibiting only one UV absorption maximum in most of the conditions, one notices that in vesicles prepared by dialysis, the absorption of $B_{CD}{}^o$ presents a shoulder, the position of which corresponds to the absorption in octanol.

The effect of gel permeation chromatography is shown in Table 3. This investigation was done while studying *bouquet* incorporation by incubation. In every case, one observes an overall blue-shift in the course of the chromatography.‡

The influence of temperature was examined on a DPPC vesicular preparation obtained by sonication. While increasing the temperature and crossing the gel to liquid-crystal transition

* An illustration of such a calculation is given below (see the Discussion).

† No experiments were performed with the *bouquets* B_{CD} in view of their poor solubility in hydrophilic solvents, thus preventing the B_M incubation procedure to be applied.

‡ In addition to the reported experiments, similar behaviour was observed for preparations by sonication, the blue-shift obtained in this case being 5 nm.

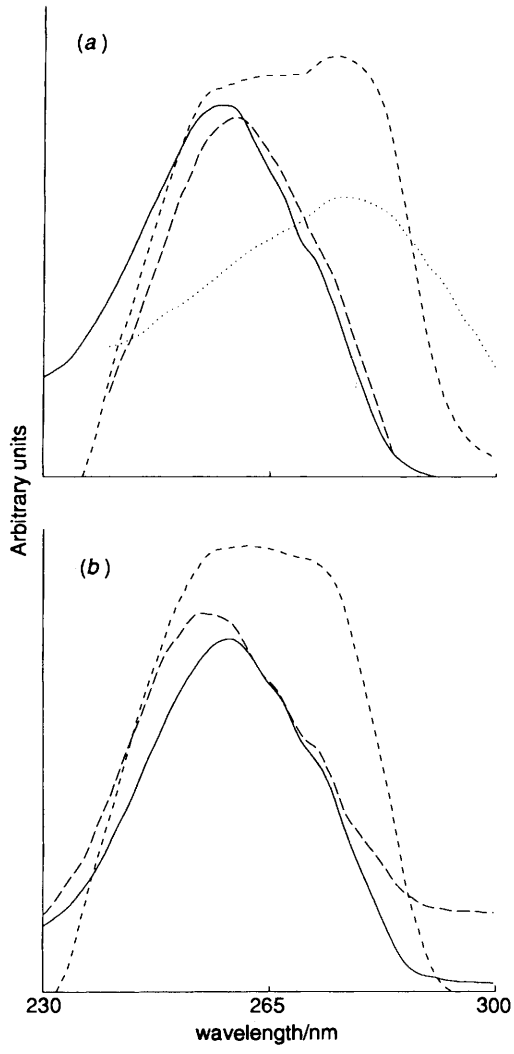

Fig. 3 UV absorption spectra of the *bouquet* molecules under several conditions of homogeneous solution and of lipid media: (*a*) $B_{CD}{}^{c}CO_2H$: ethanol 95% (——); vesicles EPC (dialysis) (- - - -); vesicles EPC (injection) (– – –); octanol (· · · ·); (*b*) $B_{CD}{}^{c}CO_2H$: ethanol 95% (——) vesicles EPC (dialysis) (- - - -); vesicles DPPC (injection). (– – –)

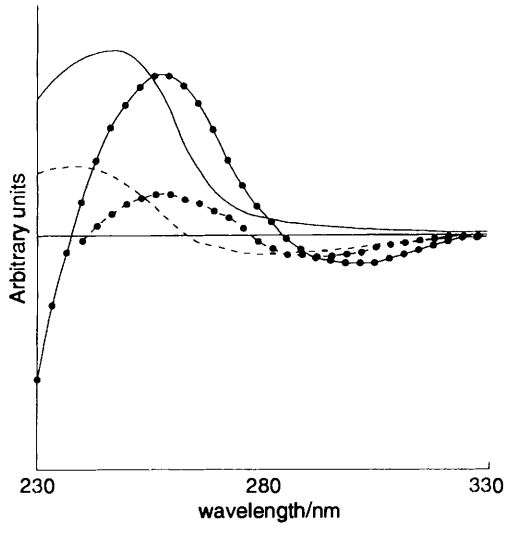

Fig. 4 Circular dichroism spectra of $B_M{}^{o}CO_2Na$ and $B_M{}^{c}CO_2Na$ in DPPC membranes prepared under several conditions: $B_M{}^{o}CO_2Na$, sonication (——); $B_M{}^{c}CO_2Na$, sonication (– – –); $B_M{}^{o}CO_2Na$, injection (– – –); $B_M{}^{c}CO_2Na$, injection (– · – · –)

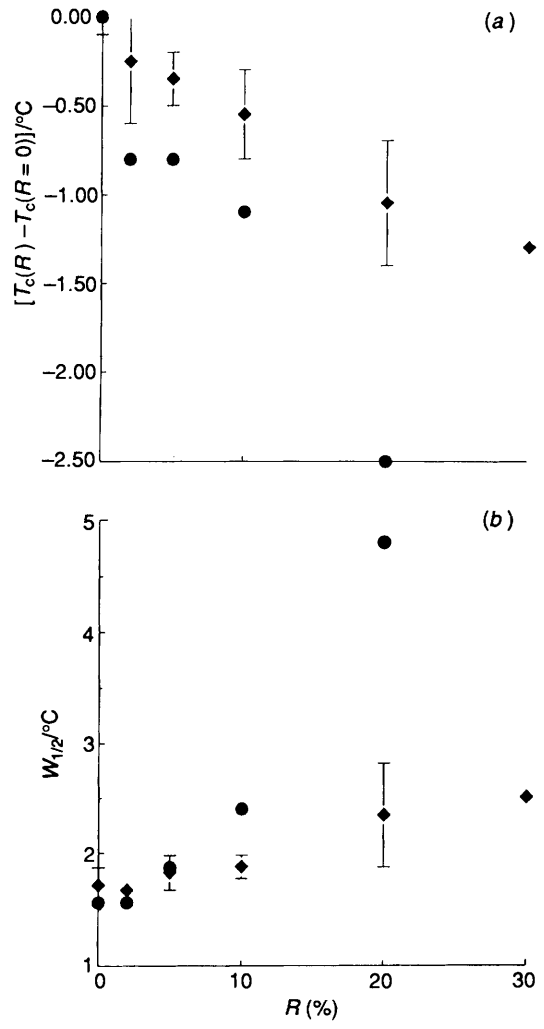

Fig. 5 (*a*) B_M *bouquet*-induced relative decrease of the transition temperature $T_c(R) - T_c(R = 0)$ of DPPC and (*b*) increase of the width at half-height $W_{1/2}$ of the transition peak, *versus* the theoretical molar ratio *R bouquet*: lipid [$B_M{}^{o}CO_2Na$(●), $B_M{}^{c}CO_2Na$(◆)]. In the case of the $B_M{}^{c}$ series, the measurements were reproduced twice; the error bars indicate the dispersion of the results.

temperature for DPPC, the absorption maximum overall is red-shifted (10 nm).

Detection of Bouquets *in Membranes by Circular Dichroism.*—CD spectroscopy showed the presence of the chiral *bouquets* B_M [sodium carboxylate; Fig. 1(*a*); R = Na] in membrane media. The spectra were recorded in vesicles of DPPC prepared either by sonication or by injection (Fig. 4). They present a shape in agreement with the spectra recorded in homogeneous media.[1,2]

Detection of Bouquets *in Membranes by Differential Scanning Calorimetry.*—Among the lipids used during incorporation studies, DPPC displays at T_c = 41 °C, a transition between a gel-like phase under T_c, and a liquid-crystal phase above T_c.[5] In the case of perfect thermodynamic behaviour, one expects, simultaneously with the increase in the amount of an incorporated molecule, a modification of the thermodynamic parameters of the lipid–molecule mixture relative to the pure lipid (especially a decrease of T_c)[3] and a broadening of the transition peak due to the local perturbation in the neighbourhood of the incorporated molecule.

A study of the incorporation effects of the *bouquets* B_MCOONa was performed in vesicles of DPPC prepared by

Table 2 (*a*) UV absorption maxima of the *bouquet* molecules B_MCOONa in membrane systems (concentration: 1 mol for 100 mol of lipid)

Method of vesicle preparation	(B_M°COONa)/nm λ_{max}		(B_M°COONa)/nm λ_{max}	
	DPPC	EPC	DPPC	EPC
Sonication	242		244	
Injection[a]	214	216		
Dialysis[b]		228		220

[a] Lipid concentration: 15 mg injected in 2.5 cm^3 of NaCl 200 mmol dm^{-3}, Tris pH = 7, 10 mmol dm^{-3}. [b] Aqueous solution: NaCl 100 mmol dm^{-3}, Tris pH = 7, 10 mmol dm^{-3}.

(*b*) UV absorption maxima of the *bouquet* molecules B_{CD}COOH in membrane systems (concentration: 1 mol for 100 mol of lipid)

Method of vesicle preparation	(B_{CD}°COOH)/nm λ_{max}		(B_{CD}°COOH)/nm λ_{max}	
	DPPC	EPC	DPPC	EPC
Injection[a]	258		258	
Dialysis[b]		268		256–262

[a] Lipid concentration: 15 mg injected in 2.5 cm^3 of CsCl 200 mmol dm^{-3}, Tris pH = 7, 100 mmol dm^{-3}. [b] Aqueous solution: NaCl 100 mmol dm^{-3}, Tris pH = 7, 10 mmol dm^{-3}.

Table 3 Effect of gel permeation chromatography on UV absorption maxima of B_MCOONa in membrane media (init., before chromatography; fin., after chromatography)

Method of vesicle preparation	*Bouquet* (concentration[a]) Lipid	λ_{max}(init.)/ nm	λ_{max}(fin.)/ nm
Dialysis incubation	B_M°COONa (0.5) EPC	248[b]	243[b]
Dialysis incubation	B_M°COONa (0.5) EPC	255[b]	254[b]

[a] In mol for 100 mol of lipid. [b] Absorbances at λ_{max}(init.): B_M° 0.65; B_M° 0.75; absorbances at λ_{max}(fin): B_M 0.30; B_M° 0.50. Experimental details are given in the Experimental section.

sonication [Fig. 5(*a*) and Fig. 5(*b*)]. One observes a decrease in T_c and a broadening of the transition width $W_{1/2}$ when the amount of the *bouquets* B_MCOONa is increased.

Detection of Bouquets *in Membranes by NMR Experiments.*—In an aqueous environment in the 0.1 mol dm^{-3} concentration range, interactions between ions are far from negligible. Interaction of the sodium cation with partially neutralized polyacrylic acid as a counter-ion in aqueous solution and the influence of the degree of neutralization on ^{23}Na NMR characteristics were studied.[16] Although showing non-linear behaviour, it was observed that an increase in the degree of neutralization (here anionic charge) induced a decrease in the longitudinal relaxation time T_1 and a simultaneous increase of the half-height width $W_{1/2}$ of the ^{23}Na NMR signal. All things being equal, it therefore seems reasonable to suspect an increase in the anionic charge density when T_1 decreases. Being negatively charged at pH = 7, the *bouquets* would induce such a phenomenon.

In principle, it would thus be possible to use either T_1 or $W_{1/2}$ and an M NMR spectrum (M = ^{7}Li, ^{23}Na or ^{133}Cs) either before or after splitting by a shift reagent to show the appearance of negative surface charge density. Because the charge-induced perturbation is in relation to the ratio of the surface of the membrane system to the volume of aqueous solution, the properties of the internal vesicular pool are the most sensitive probes and investigations have to be performed after splitting of the initial signal. Furthermore, it was noticed during this work that $W_{1/2}$(int) is too sensitive to other parameters such as the structure of the vesicular population to be a valuable surface charge probe. Thus, T_1(int) appeared to be the best indicator for the detection of incorporation of *bouquets* by NMR spectroscopy. Table 4 reports the results on vesicles prepared by dialysis with or without *bouquets*. These experiments were made with several cations (Li^+, Na^+, Cs^+) in order to investigate the sensitivity of this method. For lithium and cesium cations, one observes effectively in the presence of the *bouquets* a decrease of the longitudinal relaxation time T_1(int). This effect is more pronounced in the B_{CD} series, displaying corresponding increases in $W_{1/2}$(int).

Quantitative Determination of the Bouquet *Concentration in Membranes.*—Among all of the methods used to prove the presence of the *bouquets* in the membrane medium, only titration experiments provide a means to determine the exact amount incorporated. This method at once gives: (*i*) the concentration of the *bouquets* in moles of *bouquets* for one mole of matrix lipid; (*ii*) the incorporation yield of the *bouquets* which is obtained by dividing the final *bouquet* concentration by the initial one. The final concentration is important for the calculation of the molar efficiency of the *bouquets* in eventual ion transport activity. The incorporation yield gives an approximate criterion of *bouquet* design for membrane incorporation.

Table 5 shows that the final *bouquet* incorporation can be rather high (10^{-3}–10^{-2} mol/mol lipid range), the yields being in fair agreement with the rough estimations obtained from UV spectra, extending from 20–25% to 80%.

As the quantitative determination of the lipid concentration in the vesicle sample is required to measure the *bouquet* incorporation yield, this study also allows us to determine whether any lipid loss occurred during the course of the dialysis process. Indeed, one has only to compare the amount of lipid used to prepare the vesicles with the effective amount measured after vesicle preparation. All measurements performed for dialysis preparations showed that the yield of vesicle preparation from EPC was in the 100% range.

Discussion

Influence of the Bouquets *on the Vesicular System Morphology.*—During the course of this work, no qualitative influence of the *bouquets* on vesicle types has been observed. It was only for vesicle preparations by dialysis, that variations of the internal vesicular pool volume were obtained. All things being equal, the decrease in the compartmentation ratio *R* in the presence of the *bouquets* in dialysis experiments might be due to several cases: (*i*) induced fragility of vesicles finally giving fewer vesicles; (*ii*) induced loss of lipid material during the formation of vesicles, which, being fewer, enclose less aqueous solution; (*iii*) morphological changes introducing either a *bouquet*-induced decrease in the yield of vesicle formation or a decrease in the mean vesicle size value (modification of the surface: volume ratio), both these cases giving for a same amount of lipid material a smaller value for *R*. The first explanation can be easily eliminated: for any vesicular preparation, either in the presence of *bouquets* or not, *R* remains constant for more than 48 h. Loss of lipids during vesicle preparation can also be rejected as a possible explanation. It is already known that phosphatidylcholines do not produce micelles which could cross the dialysis bag walls during the dialysis process.[7] During quantitative lipid determinations, it was checked that no lipid loss was *bouquet*-induced during the dialysis process. Consequently, the *bouquet*-induced morphological changes remain as the most probable explanation. Assuming that the vesicle population consists mainly of spherical LUV (which is strongly

Table 4 NMR characteristics of several alkali-metal cations in vesicular systems obtained by dialysis

System[a]	x (M')	τ_{int}[c]	$W_{\frac{1}{2}}$(int)/Hz[c]	τ_{ext}[c]	$W_{\frac{1}{2}}$(ext)/Hz[c]
^{7}Li	5 (Dy)[b]	±0.05 (s)	±0.25	±0.01 (s)	±0.25
A		7.00	1.25	0.47	0.75
B		6.60	1.00	0.44	1.50
C		6.70	1.00	0.43	1.50
D		5.80	1.25	0.50	0.50
E		4.5–5.0	1.25	0.43	1.00
^{23}Na	20 (Dy)[b]	±0.5 (ms)	±0.5	±0.25 (ms)	±0.5
A		29.5	8.0	17.0	14.0
B		28.0	10.0	17.0	14.0
C		30.0	9.0	17.0	14.0
D		28.0	10.0	17.0	14.0
E		27.0	10.0	17.5	14.0
^{133}Cs	10 (Tm)[b]	(s)		±0.1 (s)	±0.1
A		6.3 ± 0.1	4.0 ± 0.2	3.3	0.8
B		5.0 ± 0.2	4.4 ± 0.2	3.3	0.8
C		6.1 ± 0.3	1.6 ± 0.2	3.5	1.1
D		5.0 ± 0.6	7.0 ± 0.5	3.2	1.1
E		6.1 ± 1	9.0 ± 1	3.3	1.6

[a] Aqueous solution MCl 100 mmol dm^{-3} (M = Li, Na, Cs), Tris pH = 7, 10 mmol dm^{-3}; conditions of NMR spectrum recording: 1.8 cm^3 of vesicular preparation +0.200 cm^3 of solution of Na_5TPP 50 mmol dm^{-3} in D_2O + x μl of aqueous solution of $M'Cl_3$ 100 mmol dm^{-3} (M' = Dy ou Tm). A, EPC; B, A + 0.5% B_M°; C, A + 0.5% B_M^{c}; D, A + 0.5% B_M°; E, A + 0.5% B_M^{c} (all the concentrations are given in mol of *bouquet* per mol of lipid.) [b] Mean chemical shift differences between the signal corresponding to internal and external compartments Δδ: ^{7}Li 0.87 ± 0.08; ^{23}Na 4.83 ± 0.13; ^{133}Cs 0.81 ± 0.09. [c] τ (respectively, $W_{\frac{1}{2}}$) designates the zero time passage (respectively the width at half-height) of the NMR signal of the investigated elements in the corresponding internal or external vesicular compartment.

Table 5 Final concentration C (in mol for 100 mol of lipid) and incorporation yields ρ of the *bouquet* molecules in vesicles prepared by injection

Bouquet	Lipid	C ± 0.1	ρ ± 10 (%)
B_M°	DPPC[a]	0.4	40
	EPC[b]	0.8	80
B_M^{c}	DPPC[a]	0.6	60
B_{CD}°	DPPC[c]	0.2	20
B_{CD}^{c}	DPPC[c]	0.6	60

[a] An ether solution containing DPPC (20 mg) and B_M (1 mol for 100 mol of DPPC) was injected in 2.5 cm^3 of NaCl 200 mmol dm^{-3} Tris pH = 7, 10 mmol dm^{-3} at 333 K. [b] A chloroform solution containing EPC (20 mg) and B_M° (1 mol for 100 mol of DPPC) was injected in 2.5 cm^3 of NaCl 200 mmol dm^{-3} Tris pH = 7, 10 mmol dm^{-3} at 343 K. [c] An ether solution containing DPPC (10 mg) and B_{CD} (1 mol for 100 mol of DPPC) was injected in 2.5 cm^3 of CsCl 200 mmol dm^{-3} Tris pH = 7, 10 mmol dm^{-3} at 333 K.

suggested by the freeze-fracture pictures), a modification of the entrapped volume by a factor 13.5/6.5 (comparison of R with or without B_{CD}^{c}COOH; Table 1) should be accompanied by a reduction of the vesicle mean diameter of about 28%. Such a decrease is qualitatively in line with our electron microscopy observations (Fig. 2). It was already noticed that incorporation of some charged species in the lipid material is often accompanied by a change in the vesicle size.[7] That the compartmentation ratio R decreases in the order no *bouquet* > B_M° > B_M^{c} = B_{CD}° > B_{CD}^{c} would therefore appear convincing as this order corresponds to an increase in the surface charge density expected from a larger number of charged terminal groups for B_{CD} than for B_M and a better yield of membrane incorporation for the alkyl *bouquets* B^c than polyoxyethylenic *bouquets* B°.

Moreover, the absence of variation of the chemical shift difference between the signals corresponding to the internal and the external vesicular compartments during the NMR characterization experiments, constitutes an essential observation. This means that the membrane does not contain holes compatible with lanthanide-ion passage. Therefore, if the *bouquets* are incorporated into these membranes, their presence does not create defects in the lipid structure which would explain eventual ionophore properties. It is therefore reasonable to relate any ion transport to the *bouquet* species itself and not only to local perturbations of the lipid matrix induced by the *bouquet* molecules.

Evidence for Incorporation of the Bouquet *Molecules into the Membrane Medium.*—The observation of a suitable signal in UV spectra does not imply incorporation of the *bouquets*. Even after extensive washing of the external vesicular pool, the resulting system still contains, in the internal pool, the initial aqueous solution in which the *bouquets* might have been solubilized at the beginning of the experiment. Consequently, a signal might be observed even without membrane incorporation. In the latter case (no membrane incorporation; *bouquet* dissolution in the internal vesicle pool), if one assumes that the UV absorbance of the *bouquet* initially present is equal to 1, the remaining *bouquet* UV absorbance after vesicle preparation and extensive washing at constant volume of the external vesicular pool would be equal to the compartmentation ratio of the vesicular solution R = internal volume/external volume. Taking as an example the experiments done on vesicles prepared by dialysis, the absorbances of vesicular solutions at λ_{max} in 2 mm wide cuvettes were always greater than 1 whereas the initial concentrations of *bouquets* were 5 × 10^{-3} mol/mol of lipid (corresponding to an absorbance in the range 1–2 for B_M and 3–4 for B_{CD} under the same record conditions). Since observations made in homogeneous media showed that solvent influence on UV spectra was weak,[1,2] incorporation should not significantly increase the molecular extinction coefficient. Taking into account the dilution factor arising from gel permeation chromatography, one finds that about 50% (respectively 25%) of *bouquets* B_M (respectively B_{CD}) absorption remains in the vesicular preparation at the end of the experiments. The mean value of the compartmentation ratio R for all vesicle preparations given in Table 1 is always less than 13%. Comparison between R values and observed absorbances strongly suggests that an important part of the *bouquets* are incorporated *into* or *onto* the vesicle walls. Such an argument also applies during titration experiments. In the latter case, the compartmentation ratio R of a few

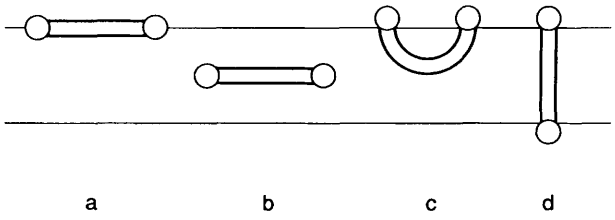

Fig. 6 Different possible modes of incorporation: (*a*) adsorption on the bilayer; (*b*) incorporation within the core of the bilayer; (*c*) hemimembrane incorporation; (*d*) transmembrane incorporation

per cent, is clearly inferior to incorporation yields, thus allowing the elimination of the hypothesis that a simple compartmentation of the aqueous solution by the vesicular walls might account for the observed results. Moreover, in the case of a simple compartmentation of the aqueous medium, the influence of parameters such as vesicle type, convexity, and perhaps even temperature, on the *bouquet* characteristics would not be easily explained. Indeed the *bouquets* would be in an identical aqueous environment in each case. Although overall they are similar in each series, the UV spectra present small variations concerning the wavelength of maximal absorption, as well as the half-height width of the corresponding bands. This leads us to suppose that the chromophore environments are different in the cases examined.

The other experiments strongly favour incorporation into the membrane system. The CD signals recorded in membrane media are in agreement with those in organic solvents (such as methanol) strongly suggesting the presence of the chiral *bouquets* B_M in the vesicles. The DSC experiments display clearly the membrane perturbation induced by the *bouquets* and give evidence of their interaction with the DPPC membrane. The decrease in T_c and the corresponding increase in $W_{1/2}$ while increasing the membrane concentration of *bouquets* correspond to the expected qualitative behaviour of an ideal mixture. Without proving that the mixture *bouquet*–DPPC is really ideal, the decrease in the transition temperature suggests that the B_M *bouquets* do not aggregate on the DPPC membrane. If this were the case, during demixing, one would expect the transition temperature of DPPC to remain constant, independently of the *bouquet* concentration. The greater perturbation induced by B_M^o compared with B_M^c is convincing because the polyoxyethylenic chains experience more conformational variations in the lipid environment than do the alkyl chains. Finally, NMR observations are also satisfactory: the ion characteristics are significantly perturbed by the surrounding charged *bouquets* in vesicles. Since B_{CD} *bouquets* carry more anionic carboxylate groups than do B_M *bouquets* one would expect that, at a given concentration, the increase in T_1 would be greater for the former series.

It seems therefore highly probable that *bouquets* are incorporated *within* the membrane.

Significance of Bouquet *Incorporation Yields.*—The membrane incorporation phenomenon results from the more favourable energetic accommodation of the *bouquet* molecules in the hydrophobic cores of bilayers than in an aqueous phase. This is related not only to the hydrophobic character of the species to be incorporated, but also to its geometrical fit with the strongly anisotropic lipid medium. In the case of vesicles, this accommodation occurs at each step of vesicle formation and, among other things, depends naturally on factors such as the vesicle preparation method, the intensity of the energy supplied during vesicle formation, *etc.* The evolution of R which was observed during *bouquet* incorporation in vesicles prepared by dialysis gives evidence of such a mutual *bouquet*–lipid interaction during vesicle formation.

The concentration of *bouquets* was quantitatively measured in vesicles prepared by injection. In other cases, similar trends based on UV absorbance measurements were observed. The results indicate that: (*i*) the incorporation yields are higher for the alkyl *bouquets* than for the polyoxyethylenic ones; (*ii*) the incorporation yields are in the same range for B_M^c and B_{CD}^c whereas there is a significant difference between B_M^o and B_{CD}^o, the former being incorporated in higher yields; (*iii*) lastly, although measured for only one *bouquet*, the incorporation yield is higher in EPC than in DPPC. The first observation (*i*) confirms intuitive assumptions. The alkyl and the polyoxy *bouquet* analogues are of the same shape as already shown.[2] The partition coefficient into the hydrophobic membrane phase is probably greater for *bouquets* containing alkyl chains than for those which contain polyoxyethylenic chains. Moreover, as DSC experiments have already shown, polyoxyethylenic B_M^o *bouquet* molecules introduce a larger perturbation than do alkyl B_M^c *bouquets*. This agrees with the expected membrane behaviour resulting from the greatest conformational disorder of the polyoxyethylenic lateral chains compared with alkyl chains.[17] It suggests that *bouquet* incorporation takes place with a more favourable energetic accommodation in the case of alkyl *bouquets*. Point (*ii*) stresses the significance of the butyl residue in the B_M^o *bouquet*. The relative weight of the central macrocyclic core is weak compared with that of the lateral chains. Therefore, one can analyse their structural features mostly in terms of lateral chains. The two *bouquet* series B_M and B_{CD} share a similar type of lateral chain: a benzene unit, a central triethyleneglycol part, a carboxylate end. However, the B_M *bouquet* lateral chains bear, in addition, a butyl branch. Although introducing a slight deviation from the cylindrical geometry which should promote membrane incorporation, the butyl groups increase the hydrophobicity and therefore should increase incorporation yields. On the other hand, the butyl units should contribute only weakly to the hydrophobicity of the B_M^c molecules containing alkyl chains. Finally, although injection of the organic lipid solutions occurred at two different temperatures, comparison between EPC and DPPC incorporation yields for B_M^o [observation (*iii*)] would be in agreement with the greater molecular disorder exhibited by the EPC lipids at room temperature.[5] Indeed this lipid preparation is a mixture, containing a *cis* double bond in one of the hydrophobic tails, and displays a gel liquid-crystal transition only at low temperature. This disorder is considerably reduced in the case of DPPC which is a pure compound, containing saturated alkyl chains and with a gel liquid crystal transition at T_c = 41 °C.

This study emphasizes the significance of several structural parameters of the molecules to be incorporated. As expected, a favourable hydrophobic–hydrophilic balance, minimal molecule-induced local disorder of the membrane matrix, the choice of an unsaturated lipid have a positive effect for achieving membrane incorporations in high yields.

Orientation and Conformation of the Bouquet *Molecules in Membrane Media.*—Numerous data are consistent with the incorporation of the *bouquets* in all the lipid bilayers used in the present experiments. Nevertheless, incorporation alone is not sufficient for establishing structure–property relationships in ion-transport experiments. This required a detailed study of the orientation and, if possible, of the conformation of the *bouquet* molecules in the bilayers.

Among the possible orientations of *bouquets*, only four types were considered: (*a*) adsorption on the bilayer surfaces; (*b*) incorporation in the core of the bilayer; (*c*) hemimembrane incorporation; (*d*) transmembrane incorporation (Fig. 6). It was assumed that the orientation of the polyoxy and alkyl analogues in the lipid bilayer for each set of conditions of vesicle preparation were identical. Former studies of *bouquet*

shapes in homogeneous solution strongly support this hypothesis.[2]

Adsorption of *bouquets* on bilayer surfaces (*a*) does not appear convincing. Such surface interaction is too weak to explain features like stability towards dialysis and gel permeation chromatography, the different behaviour of alkyl and polyoxyethylenic analogues (DSC), UV spectral perturbations associated with the changes of vesicular systems. Likewise, incorporation of *bouquets* into the membrane core (*b*) does not readily account for the monovalent alkali-metal cations' ability to interact with the *bouquets* in the bilayers as suggested by the ^{7}Li, ^{23}Na and ^{133}Cs NMR experiments. Indeed, lipid membranes are almost impermeable to alkali-metal cations and would prevent ion penetration into the bilayer core. Moreover, the strongly hydrophobic core of the membranes would be incompatible with the polar terminal carboxylic groups and the polyoxyethylenic chains of the *bouquet* species.

Differentiating the two remaining incorporation modes, in hemimembrane (*c*) or transmembrane (*d*) fashion, constitutes a much more difficult task. Indeed, as observed from the aqueous solutions, transmembrane orientation can easily be confused with a random distribution of hemimembrane forms on both bilayer faces. Thus, it is probably the examination of the *bouquet* core features resulting from constraints on the rather rigid central cylindrical part of the *bouquets* which might yield some insight. Of all the techniques employed, only the UV spectra could be used to elucidate *bouquet* incorporation. Drawing convincing orientational and conformational conclusions from a detailed interpretation of UV spectra in several types of vesicle would have required the synthesis of compounds displaying characteristics suitable as valid UV models for hemimembrane incorporation and the preparation of samples for determining orientation in vesicles by UV linear dichroism techniques. In the absence of such data, it would be unrealistic at the present time to draw a definitive picture of *bouquet* orientation in membrane media. Nevertheless, the following points may be made.

—*The UV spectra of every* bouquet *in vesicles are not identical.* After incorporation, each vesicle contains numerous *bouquet* molecules and the collected UV spectra result from the superposition of the absorption of each of them. Obviously, the contribution of each individual *bouquet* molecule to the overall UV spectrum remains unknown. When, as observed during this work, a change occurs in UV spectra while performing gel permeation chromatography, one may conclude that a variety of individual contributions are made.

—*It is tempting to interpret the latter observation more in terms of orientation inhomogeneity of the* bouquets *in the membrane than in terms of aggregation phenomena.* Indeed (*i*) it has already been shown that solvent influence on B_M and B_{CD} *bouquet* chromophores was fairly weak except in the case of B_{CD}°COOH in rather long-chain alcohols;[2] (*ii*) DSC results suggested that no aggregation of the *bouquets* occurred in DPPC membranes; (*iii*) this observation is valid for both polyoxyethylenic and alkyl analogues and it would be rather surprising to observe the same aggregation behaviour for both types of derivative. UV spectral differences between B_M°COONa *bouquets* in the same lipid matrix (egg lecithin) prepared under the same condition (dialysis) but with different modes of incorporation (either codialysis lipid–*bouquets* or incubation) also support this explanation. That might mean that the systems examined were not necessarily under conditions of thermodynamic equilibrium.

—*Vesicle size is not necessarily the appropriate parameter determining the UV spectrum of* bouquets *in lipid media.* Spectral recordings before and after gel permeation chromatography performed on *bouquet*-containing vesicles emphasized the significance of vesicle size as a parameter affecting the shape of UV spectra. However, UV spectra may not be directly related to size but rather to parameters like the lipid pressure or more generally the lipid constraint exerted by the bilayer on the *bouquet* core.* The observation of a significant shift at T_c = 41 °C in B_M UV spectra recorded in DPPC SUV would support the relevance of these latter parameters. Indeed, the gel–liquid crystal transition in DPPC vesicles causes a variation in the molecular order in the alkyl tails, the bilayer being in a slightly expanded state above the transition temperature with a corresponding increase in the membrane internal pressure at constant membrane area. Such a constraint effect has already been involved to account for shifts in *bouquet* UV spectra in isotropic solutions.[1,2]

—*Is there any relationship between the* λ_{max} *of* bouquet *UV absorption and the* bouquet *incorporation mode?* As has already been shown, UV *bouquet* absorption is generally only slightly solvent dependent; nevertheless a significant red-shift was observed when poor solvents were used [see for instance on Fig. 3(*a*) the B_{CD}° UV absorption in ethanol and in octanol; the former is a good solvent for polyoxyethylenic chains whereas the latter is a poor solvent]. Thus if such a relationship existed, it might show up in spectra recorded for different incorporation preparations. Firstly, in homogeneous solutions in good solvents, it has already been argued that the B_{CD} *bouquet* shapes were more or less biconical, corresponding to a quasi-cylindrical environment of the central chromophore.[2] In terms of membrane media, this geometry corresponds to the transmembrane incorporation mode. In good solvents, *bouquet* λ_{max} values were the lowest recorded. In addition, during incubation experiments, given the slowness of flip–flop phenomena of charged lipids already investigated,[19] it makes sense to assume that *bouquets* B_MCOONa remained mainly incorporated in a hemimembrane fashion; in these experiments, *bouquet* λ_{max} values were among the highest recorded.

These two remarks led us to consider the hypothesis that a monotonous red-shift accompanies an increase of geometrical constraints exerted on the *bouquet* core. This would be in line with the trends expected to take place during *bouquet* incorporation in SUV prepared by sonication and in LUV prepared by dialysis. In the first case, the red-shift of B_M UV absorption observed was in the same range as that recorded during incubation experiments. This seems to indicate a large contribution of non-transmembrane incorporations corresponding mainly to B_M *bouquets* in a conical mean shape [Fig. 6(*c*)]; cones should accommodate the high curvature of the SUVs in a better way than cylinders which correspond to the transmembrane incorporation mode[20] [Fig. 6(*d*)]. In the case of LUVs prepared by dialysis, a moderate red-shift was observed but was, rather surprisingly, accompanied by a significant increase of the half-height width of the corresponding UV band; this would suggest very disperse incorporation modes extending from transmembrane to hemimembrane (Fig. 6). This would be in agreement with the changes occurring during the course of vesicle generation where the initial spherical EPC-β-octyl glucopyranoside micelle shape evolves to a planar one under conditions that do not allow flip–flop of the charged *bouquet* extremities to occur. Starting with the central region of the *bouquet* species located in the core of the spherical micelle and its side-chains dispersed all around the surface, evolution towards the planar shape would be almost independent of the number of *bouquet* lateral chains remaining on each planar face, eventually giving the *bouquet* either in a transmembrane or hemimembrane state or in any intermediate incorporation state.

In conclusion, the analysis of the available data suggests that

* Although its measurement remains largely speculative, the value of the membrane internal pressure is probably vesicle-curvature dependent.[18]

the incorporation of *bouquets* in bilayers yields a *collection of incorporation modes* dispersed continuously from a pure transmembrane to a pure hemimembrane mode, rather than a single type, either transmembrane or hemimembrane. Further studies may provide more insight into the mode of incorporation of *bouquet* type species in bilayers. Such information is not only essential for the interpretation of eventual ion conduction properties, but is also interesting for learning more, even indirectly, about the phenomenology of vesicle formation. Indeed, incorporation modes result from the accommodation between the respective energetic requirements of *bouquets* and of the lipid system. Thus, by considering the incorporation modes as fossils witnessing the formation of the vesicles, it might be possible to recreate the story behind the building of these molecular assemblies.

Conclusions

The available evidence permits us to conclude that the incorporation of *bouquets* takes place in good yields in several vesicular systems prepared from egg lecithin or dipalmitoylphosphatidylcholine. Furthermore, the observations support the idea that their orientation in the bilayer is not unique, but rather that they could be distributed between two limiting cases: transmembrane and hemimembrane incorporation modes (Fig. 6). Depending on factors such as the vesicular preparation method, vesicle radius and incorporation steps of the *bouquets*, different final states of orientation distribution are reached.

These results are essential for the investigation of the ion-transport properties *bouquet* molecules. Indeed, since they are apparently not incorporated in a way that would cause local disruptions of the membrane, ion transport properties will be determined directly by their structural features, themselves, even if the membrane exerts a determining influence on these features.

However, the lack of a precise knowledge of *bouquet* orientation in membrane media unfortunately limits the possibility of establishing direct structure–ion transport property correlations. Eventual ion transport properties are expected to result from a mixture of orientations of the *bouquet* species in the membrane media. One further complication arises from the fact that the cyclodextrin *bouquet* species could also present different shapes of the β-cyclodextrin core itself.[21] The present results stress the need for a detailed study of the modes of incorporation of channel-type molecules in general, if valid interpretation of ion-transfer properties is to be achieved.

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