Channel-type Molecular Structures. Part 4. Transmembrane Transport of Alkali-metal Ions by 'Bouquet' Molecules

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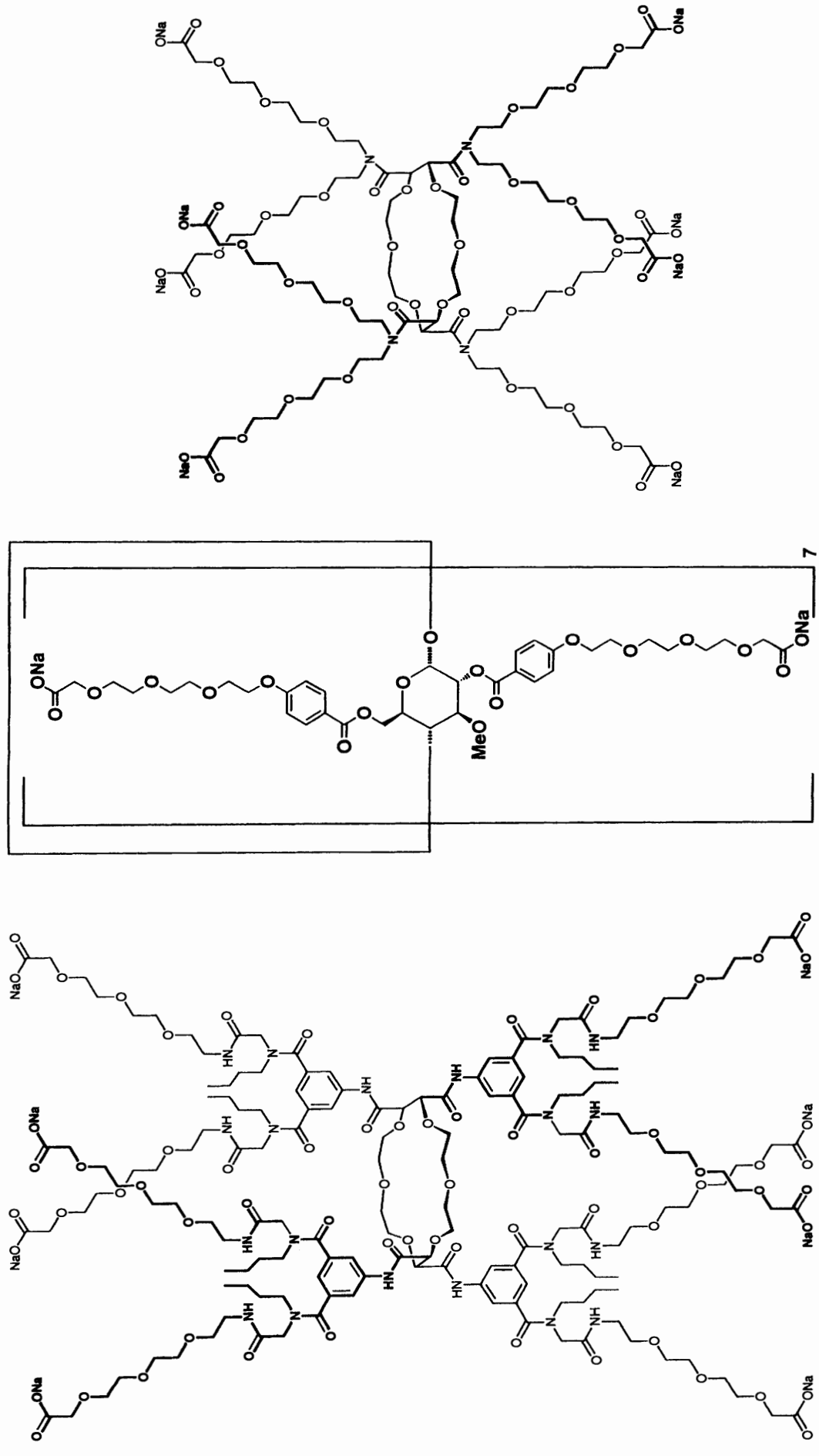
This report describes transport experiments with 'bouquet' molecules designed to act as artificial ion channels. The 'bouquets' are based on a central macrocycle which is either an 18-crown-6 (B_M) or a cyclodextrin derivative (B_{CD}) to which are attached poly(ethylene oxide) [poly(oxyethylene)] chains (B_M^o and B_{CD}^o) or polyalkyl chains (B_M^c and B_{CD}^c) tipped with carboxylate end-groups. The 'bouquets' were studied in liposomes prepared from egg phosphatidylcholine (egg PC), dipalmitoyl phosphatidylcholine (DPPC) and a mixture of egg PC, stearylamine and cholesterol. Opposing gradients in Li^+ and Na^+ concentration were created and the transport of alkali-metal ions down their concentration gradients was followed directly by ^7Li and ^{23}Na NMR spectroscopy. 'Bouquets' were found to cause a one-for-one exchange of Na^+ for Li^+ (antiport). In order to estimate transport rates, the extent of Na^+ entry into liposomes was followed as a function of time. All 'bouquets' transported ions at similar rates in fluid membranes. Comparison of transport rates in fluid- (egg PC) and gel-state membranes (DPPC) was used to distinguish carrier and channel mechanisms. Control experiments demonstrated that a known carrier (monensin A) gave significantly lower transport rates in gel-state membranes. Two 'bouquets', B_M^c and B_{CD}^o, were found to transport Na^+ at similar rates in fluid- and gel-state membranes; this suggests that ion passage occurs preferentially by the channel mechanism and not by the carrier mechanism. Variation of transport rate with 'bouquet' concentration was probed for B_M^o and B_M^c and the rates were found to increase with B_M^c concentration but not with B_M^o concentration. Since the transport rate is expected to be proportional to transporter concentration in both the carrier and channel mechanisms, this indicates that B_M^o uses neither a carrier nor a channel mechanism. The mechanism by which 'bouquet' molecules operate and the criteria which may be used to decide whether functioning channels have been created are discussed.

Eukaryotic cells are highly organised systems which are characterised by subdivision of their internal volume by membranes into organelles, which are specialised for different functions.[1] Bilayer membranes *per se* are relatively impermeable to many substances including charged species such as alkali-metal ions, permitting compartmentation of essential but mutually incompatible processes.[2] Specialised proteins catalyse the transport of selected species across membranes, allowing communication between compartments. This transfer may take place by a diffusive carrier mechanism or by a channel mechanism in which the protein spans the membrane as a conduit and the guest passes through the channel rather than through the membrane. Channels are ubiquitous in living systems and channels are often 'gated' to open only under specific conditions.[3]

Inspired by the biological role of ion channels, the construction of artificial ion channels has been an area of continuing interest in the fields of biomimetic and supramolecular chemistry. The design of artificial channels draws on the accumulated expertise in the field of supramolecular chemistry while their study offers the potential for revealing new facets of the physical chemistry of ion transport.

A number of different strategies have been used to construct artificial ion channels. Macrocyclic complexing agents may be stacked one upon another to form a tube long enough to span a bilayer.[4,5] This approach has recently resulted in the construction of functional artificial ion channels from self-assembling peptide nanotubes; these channels were shown to transport alkali-metal ions at three times the rate of a naturally occurring peptide channel, gramicidin A.[6] Monomeric species capable of traversing the membrane may self-associate to form an oligomeric pore in the manner of amphotericin.[7–10] Alternatively, several membrane-spanning monomers may be pre-organised by attachment to a central core.[5,11–18] The latter is the basis for the 'bouquet' approach to artificial ion channels.[11] Lastly, half-channel compounds may be constructed in this fashion and the half channels allowed to self-assemble in the membrane, mimicking the formation of ion channels by gramicidin dimerisation.[19] These approaches have led to compounds which have been demonstrated to be effective ion transporters.

The 'Bouquet' Approach to Artificial Ion Channels.—The 'bouquet' approach to artificial ion channels consists in grafting poly(ethylene oxide) chains onto a central macrocycle, which is either an 18-crown-6 (B_M^o) or a β-cyclodextrin derivative (B_{CD}^o). The crown ether forms labile complexes with alkali-metal ions while the cyclodextrin makes a rigid 'hole' wide enough to allow the passage of hydrated alkali-metal ions. The chains are modified to bear lipophilic groups to position the central part of the 'bouquet' near the midpoint of the membrane and they are tipped with charged carboxylate groups to anchor the ends of the chains at the membrane/water interfaces (see Scheme 1). For both the crown ether- and cyclodextrin-based compounds an analogue having polyalkyl chains in place of poly(ethylene oxide) chains was also synthesized (B_M^c, B_{CD}^c). Thus, four compounds with two types of central annulus and two types of pendant chains were in hand, permitting structure–activity correlations for ion transport to be made. We also prepared another set of crown ether-based compounds bearing shorter polyoxy or polyalkyl chains with carboxylate endgroups (C_M^o and C_M^c) for comparison (see Scheme 1). These compounds



Scheme 1 Structures of 'bouquet' molecules B_M^o (left), based on an 18-crown-6 derivative, and B_{CD}^o (middle), based on cyclodextrin. As a control, a shorter analogue based on an 18-crown-6 derivative, denoted C_M^o (left) was prepared. For each of the compounds shown above, a polyalkyl analogue having polyalkyl chains in place of poly(ethylene oxide) chains was also synthesized. These are denoted B_M^c, B_{CD}^c and C_M^c, respectively.

are presumably too short to span the membrane in the proper orientation to form ion channels and are used as controls.

The present report describes the investigation of ion transport by the 'bouquet' molecules (for a preliminary description, see ref. 20). Previous publications have described the synthesis of the 'bouquet' molecules,[11-13] their properties in homogeneous media[12,13] and studies of their incorporation into bilayer membranes.[14]

Study of 'Bouquets' in Liposomes; Monitoring Transport by NMR Spectroscopy.—We chose to study transport by 'bouquet' molecules in liposomes, which are bilayer membrane structures that enclose a volume of solution. They are easily prepared, stable, and may be characterised by electron microscopy or dynamic light scattering measurements. 'Bouquet' molecules have previously been shown to be well incorporated into the bilayer membranes of liposomes made from egg PC and DPPC.[14]

Transport of alkali-metal ions in liposomes may be followed by a variety of techniques, the more common of which include the pH-stat method,[16b,c] fluorescence and UV–VIS spectroscopy,[9,16a,19] and conductimetry in planar bilayers.[18b,21] In many cases, alkali-metal ion transport is coupled to another process such as proton transport and the rate of this second process is followed. We chose to follow the transport of alkali-metal ions across the membrane by alkali-metal NMR using a shift reagent to distinguish metal ions inside and outside the liposomes.[22] This method had the advantage that the transported species are observed directly, making interpretation of the results straightforward. Using a multinuclear probe, a number of different alkali-metal ions may be observed concurrently, giving insight into the mechanism of transport.

Transport Mechanism: Synport or Antiport, Carrier or Channel?—We sought to understand the mechanism of transport by 'bouquet' molecules in a number of different ways. Comparison of rate and extent of transport of Na^+ and Li^+ in our experiments revealed the detailed mechanism of transport (synport or antiport). Comparison of transport rates in membranes above and below their liquid crystal-to-gel phase transition temperature (T_c) was used to distinguish carrier and channel transport mechanisms and we also studied the order of the reaction with respect to the transporter with this goal in mind. The results shed light on the mechanism by which 'bouquet' molecules operate as transporters.

Results

Characterisation of Liposomes by Electron Microscopy.—Freeze-fracture electron microscopy was used to characterise the form and size distribution of the liposomes used in transport studies, which were made from egg phosphatidylcholine (egg PC or EPC), dipalmitoyl phosphatidylcholine (DPPC) or a mixture of egg PC, stearylamine and cholesterol (7:2:1 molar ratio). For egg PC, liposomes having incorporated 'bouquet' molecules were also studied.

The size distributions of liposomes with different membrane compositions in the absence of 'bouquets' are compared in Fig. 1 and Table 1. The liposomes were predominantly large with mean diameters of 100–600 nm and each was composed of a single bilayer membrane. Such liposomes may be termed large unilammelar vesicles or LUVs. With the exception of the mixture of egg PC, stearylamine and cholesterol, the proportion of multilammelar vesicles was negligible.

It may be seen that DPPC gave the smallest vesicles, egg PC gave vesicles of moderate size, and the mixture egg PC–stearylamine–cholesterol gave significantly larger vesicles. More significantly, incorporation of 'bouquets' into liposomes during preparation by dialytic detergent removal does not result in morphological changes: large unilammelar vesicles were seen with and without incorporated 'bouquet'.

Table 1 Size distribution of vesicles with and without incorporated 'bouquet' molecules prepared by the dialytic detergent removal technique[a]

Preparation	Size/nm	MLV (%)
EPC	200 ± 300	0
DPPC	90 ± 120	0
EPC–stearylamine–cholesterol	600 ± 500	12
EPC + B_M^o	200 ± 120	0
EPC + B_M^c	150 ± 120	0
EPC + B_{CD}^o	380 ± 180	0
EPC + B_{CD}^c	150 ± 120	0

[a] The size is given as the position of the peak in the profile of population *vs.* size ± the width at half height of the peak. The proportion of multilammelar vesicles in each preparation is also given.

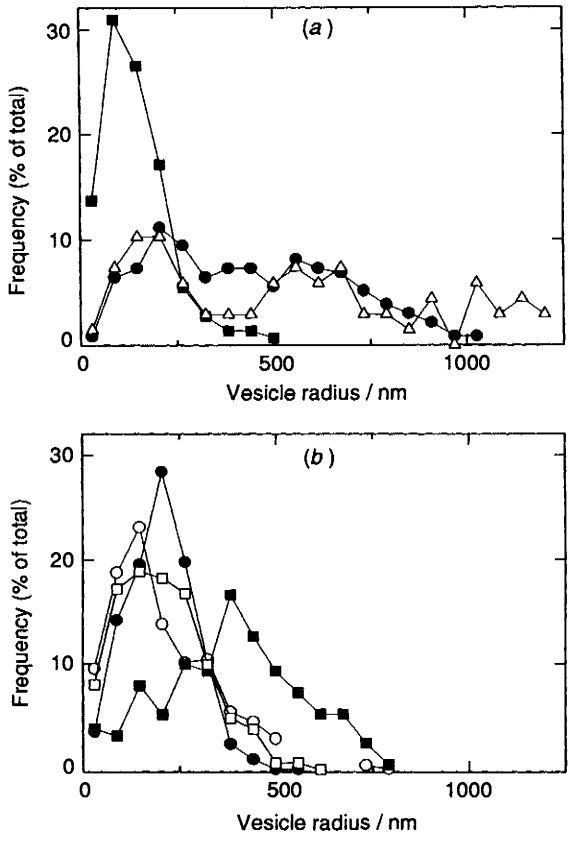


Fig. 1 Size distributions of LUV preparations. (*a*) Comparison of size distributions of liposomes prepared from egg phosphatidylcholine (●), dipalmitoylphosphatidylcholine (■) and a mixture of egg phosphatidylcholine, stearylamine and cholesterol (7:2:1 molar ratio) (△). (*b*) Comparison of size distributions of liposomes prepared from egg phosphatidylcholine containing incorporated 'bouquet' molecules B_M^o (●), B_M^c (○), B_{CD}^o (■) and B_{CD}^c (□).

Study of Alkali-metal Ion Transport using the Gradient Technique.—When using NMR spectroscopy to follow transport in liposomes, experiments may be divided into two general classes. In the first, the system is at thermodynamic equilibrium, with solutions of identical composition inside and outside liposomes. When separate resonances corresponding to species inside and outside liposomes can be observed, dynamic NMR methods such as inversion-recovery experiments are a simple and elegant means to study the transport process since the

degenerate exchange reaction interconverting M^+(in) and M^+(out) may be followed.[23,24] However, the relaxation rate of the observed nucleus limits the experiment to the observation of relatively high transport rates. Preliminary work showed that transport by 'bouquets' was too slow to be observed by dynamic NMR experiments under equilibrium conditions.[23]

We therefore chose to follow the transport of ions down a concentration gradient by means of a second class of methods that involves non-equilibrium conditions: a gradient in metal ion concentration is created and the return of the system to equilibrium is monitored. In the present case, the collapse of opposing gradients in the concentrations of two different alkali-metal ions was followed.[22]

Liposomes were made in an aqueous solution of LiCl (100 mmol dm^{-3}), which was then present at equal concentrations in the internal and external volumes. Addition of a shift reagent for alkali-metal ions to the external volume allowed separate resonances for Li^+(in) (unshifted) and Li^+(out) (shifted) to be observed and quantified. The shift reagent used was a mixture of tetramethylammonium tripolyphosphate [$(Me_4N)_5PPP$] and dysprosium chloride in a 3:1 molar ratio which caused upfield shifts in the resonances of both Li^+ and Na^+. Addition of an iso-osmolar NaCl solution to the liposome preparation caused the creation of gradients in sodium ion (present outside, $[Na^+]_{ext}$ *ca.* 50 mmol dm^{-3}, but not inside vesicles, $[Na^+]_{int} = 0$) and lithium ion (concentration in the external volume reduced by dilution, $[Li^+]_{ext}$ *ca.* 50 mmol dm^{-3}, internal concentration unchanged, $[Li^+]_{int}$ = 100 mmol dm^{-3}). Furthermore, these gradients were in opposing senses: Na^+ was present in greater concentration outside the vesicles than inside, while for Li^+ the reverse was true. The salt solutions inside and outside liposomes were unbuffered, both with pH *ca.* 6.5; the external pH remained constant throughout the transport experiment. Since chloride was the counterion in both salts, it was present in almost equal concentrations in the internal and external volumes. Therefore, in these experiments, significant gradients in metal ion concentration were created, while proton and chloride ion concentration gradients were small.

Monitoring of Na^+ *entry into liposomes.* The use of a multinuclear NMR probe allowed both Na^+ and Li^+ to be observed for as long as the liposomes remained stable (a timescale of days). While both entry and exit of ions may be observed almost simultaneously, we routinely followed only the entry of sodium ions by integrating the peak corresponding to internal Na^+ as a function of time. Initially, Na^+ was present only outside the liposomes, so any entry was due to transport. Some vesicle breakage was apparent during the longer transport experiments: observing entry rather than exit meant that vesicle breakage would not be counted as transport.

Measurement of the trapped volume allowed comparison of the results of different experiments. In practice, ^7Li spectra were recorded before the addition of NaCl and the internal and external peaks integrated. This allowed the calculation of the total volume trapped inside liposomes. The trapped volume varied between liposome preparations: greater trapped volume meant a greater capacity for Na^+ entry. Therefore, the internal sodium integral (measuring transport) must be scaled with respect to the trapped volume to allow comparison of transport results from different liposome preparations.

The measured trapped volume, together with the known volumes and concentrations of all solutions, allowed the prediction of the integral of the internal sodium peak when the system has returned to equilibrium: $(I_{int}^{Na})_{eq}$ (at equilibrium, $[Na^+]_{ext} = [Na^+]_{int}$). This prediction assumed that no vesicle breakage takes place. We defined a parameter called the extent of entry which has a value of zero when no sodium has entered liposomes and a value of 100 at equilibrium. At any given time t the extent of entry of sodium (E_{Na}) is given by $E_{Na} = (I_{int}^{Na})_t/(I_{int}^{Na})_{eq} \times 100$. (See the Experimental section for details on the calculation of the extent of entry.) The fact that extents of entry greater than 100 were not observed (within experimental error) supports the validity of this approach.

Transport Experiments in Fluid-state Membranes.—Preparation of liposomes containing incorporated 'bouquets' by dialytic detergent removal. 'Bouquet' molecules were incorporated into the membrane phase of liposomes made from egg phosphatidylcholine (egg PC). Egg PC was chosen as a typical phospholipid whose properties are well documented and which had a membrane thickness similar to the length of the extended 'bouquet' molecules. For experiments in egg PC, the 'bouquet' molecules were mixed with phospholipid and octyl glucoside detergent in an organic solvent, the solvent was removed under vacuum, the residue dissolved in aq. LiCl solution, and the detergent was removed by dialysis, creating liposomes containing 'bouquet'. This method of incorporation amounts to a 'co-preparation' since 'bouquets' are introduced into the vesicles as they are formed.

Acceleration of Na^+ *entry by 'bouquets'.* When opposing trans-membrane gradients of Na^+ and Li^+ concentrations were created, Na^+ entered liposomes having incorporated 'bouquets' faster and to a greater extent than in control preparations without added 'bouquets'. Fig. 2(*a*) shows plots of the extent of entry of Na^+ as a function of time for liposomes with and without 'bouquets'; the corresponding data are available in the Supplementary Material.† Entry was relatively slow and rates were similar for all 'bouquet' molecules.

The extent of entry as a function of time for the shorter-chain analogues of the 'bouquets', C_M^o and C_M^c, in liposomes made from a mixture of egg PC, DPPC, stearylamine and cholesterol (fluid state) is shown in Fig. 2(*b*). Since these molecules did not contain chromophores, their final concentration in the transport experiment could not be assayed. However, C_M^o is appreciably more water soluble than its polyalkyl analogue, so the lower rate of transport observed for C_M^o probably reflects a lower final concentration due to greater losses to the external solution during preparation of liposomes by dialysis.

One-for-one exchange of Na^+ *for* Li^+ *by bouquet molecules.* Use of the dysprosium chloride–tetramethylammonium tripolyphosphate shift reagent allowed the internal and external ions to be differentiated for both Na^+ and Li^+. By switching between ^{23}Na and ^7Li detection during the course of a transport experiment, the evolution of both Na^+ and Li^+ signals could be monitored. Since the total amounts of Na^+ and Li^+ are known, the amount of Na^+ or Li^+ having entered or exited the liposomes could be calculated at the time corresponding to any spectrum. It was found that as Na^+ entered liposomes, Li^+ exited. The amounts of Na^+ ions having entered and Li^+ ions having exited liposomes at various times are compared for two transport experiments in Fig. 3(*a*) and (*b*). The relatively good correspondence between the two sets of values is consistent with a mechanism in which there is a one-for-one exchange of sodium ions for lithium ions across the membrane.

Transport experiments in egg PC–stearylamine–cholesterol membranes. Further experiments on transport by 'bouquet' molecules made use of vesicles made from a mixture of egg PC, stearylamine and cholesterol. As seen by electron microscopy, the introduction of the positively-charged stearylammonium ion into the membrane results in larger vesicles and a greater proportion of multilamellar vesicles than for egg PC alone [Fig.

† Supplementary material (extent of entry as a function of time for the experiments corresponding to Figs. 2–6) is available. Supplementary material has been deposited under the Supplementary Publications Scheme. For details, see 'Instructions for Authors (1995)', *J. Chem. Soc., Perkin Trans. 2*, 1995, issue 1 [Supp. Pub. No. 57063, 5 pp.].

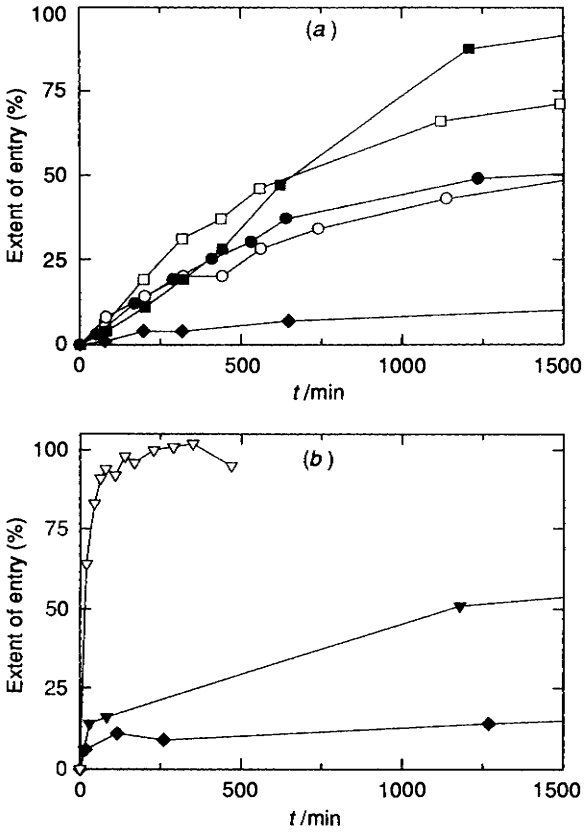


Fig. 2 (*a*) Extent of entry of Na^+ into liposomes as a function of time in the presence of 'bouquet' molecules B_M^o (●), B_M^c (○), B_{CD}^o (■) and B_{CD}^c (□) and in their absence (◆). The lines are simply a guide for the eye. See text for an explanation of the calculation of extent of entry. 'Bouquet' concentrations were 62 (B_M^o), 27 (B_M^c), 56 (B_{CD}^o) and 51 μmol dm^{-3} (B_{CD}^c). Liposomes were prepared from egg phosphatidylcholine and lipid concentrations ranged from 8.4 to 11.2 mg cm^{-3}. (*b*) Extent of entry of Na^+ into liposomes as a function of time in the presence of C_M^o (▼) and C_M^c (▽), and in their absence (◆). The lines are simply a guide for the eye. Liposomes were prepared from a mixture of egg PC, DPPC, stearylamine and cholesterol. The concentrations used (estimated from quantities added before dialysis) were egg PC: 4.5, DPPC: 7.5, stearylamine: 0.5, cholesterol: 0.39 mg cm^{-3}; C_M^o: 0.14 and C_M^c: 0.14 mmol dm^{-3}.

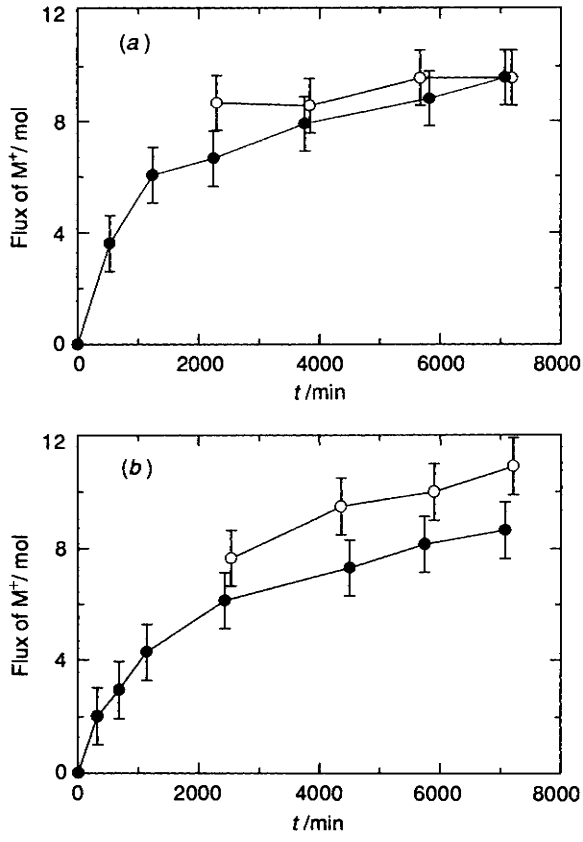


Fig. 3 Comparison of the quantity (M) of Na^+ having entered liposomes (●) with the quantity of Li^+ having exited (○) as a function of time during a transport experiment. The lines are simply a guide for the eye. The data refer to LUV made from egg phosphatidylcholine with incorporated (*a*) B_M^o and (*b*) B_M^c. Error bars are estimates of relative errors.

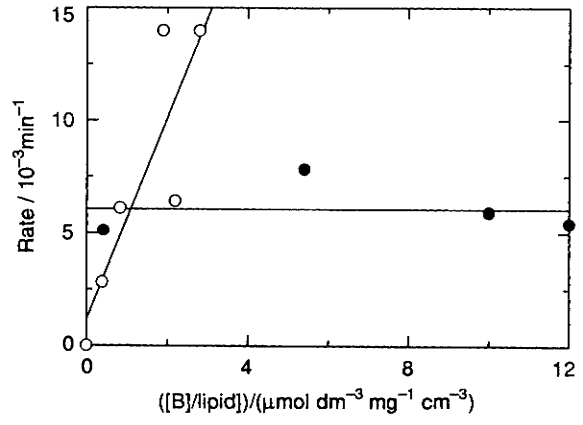


Fig. 4 Variation of the rate of entry of Na^+ into LUV (egg PC–stearylamine–cholesterol) as a function of the ratio of 'bouquet' to lipid for B_M^o (●) and B_M^c (○). The lines are from linear regression analysis of the data.

1(*a*)]. More importantly, the net trapped volume is significantly increased, resulting in larger observed changes in I_{int}^{Na} and therefore greater sensitivity in transport experiments. Note that incorporation of charged molecules into membranes is often facilitated by including species having the opposite charge into the membrane matrix.[25,26] In these experiments, 'bouquets' were introduced into the membrane using the pre-incubation method (see below).

We used this system to study the relationship between 'bouquet' concentration and transport rate for the two compounds based on the macrocyclic crown ether, B_M^o and B_M^c. The amount of 'bouquet' present was measured by UV absorption and the amount of lipid was assayed by phosphate analysis after mineralisation (see Experimental section).[14] We assumed that all 'bouquet' remaining in the LUV preparations was in the membrane phase and was active only there. Previous experiments demonstrated that 'bouquets' introduced into liposomes by incubation were found predominantly in the membrane phase.[15] Thus, transport results should be judged with respect to the ratio of 'bouquet' to lipid. Since the lipid concentration varied slightly between preparations, we used this ratio to compare the transport results. Losses of lipid during vesicle formation were found to be small in all cases.

Increase in transport rates with B_M^c *concentration, but not* B_M^o *concentration.* Fig. 4 and Table 2 show the relationship between transport rate and 'bouquet' concentration for B_M^o and B_M^c. Transport rates were calculated as slopes of ln [ext(∞) − ext(*t*)] *vs. t*, assuming first-order behaviour (see Experimental section for details). The results show significant scatter, but it may be seen nonetheless that transport rates increase with increasing concentrations of B_M^c, but are unchanged despite wide variations in B_M^o concentration. In both carrier and channel mechanisms, transport rate is expected to increase with the concentration of transporter, as for B_M^c. The fact that transport rates do not increase with B_M^o concentration implies

Table 2 Rates of entry of Na^+ into liposomes containing incorporated B_M^o or B_M^c [a]

'Bouquet'	[Lipid]/mg cm^{-3}	[Bouquet]/10^{-5} mol dm^{-3}	[Bouquet]/[Lipid]/ (μmol dm^{-3} mg^{-1} cm^3)	Rate constant/ 10^{-3} min^{-1}
B_M^o	12.0	0.50	0.42	5.1
		6.5	5.4	7.8
		12	10	5.9
		14	12	5.4
B_M^c	9.5	0.37	0.39	2.8
	9.5	0.79	0.83	6.1
	9.4	1.8	1.9	14
	9.4	2.1	2.2	6.4
	9.4	2.6	2.8	14

[a] Liposomes were prepared from a mixture of egg PC, stearylamine and cholesterol; 'bouquets' were introduced by the pre-incubation method. See Experimental section for details on the calculation of rate constants.

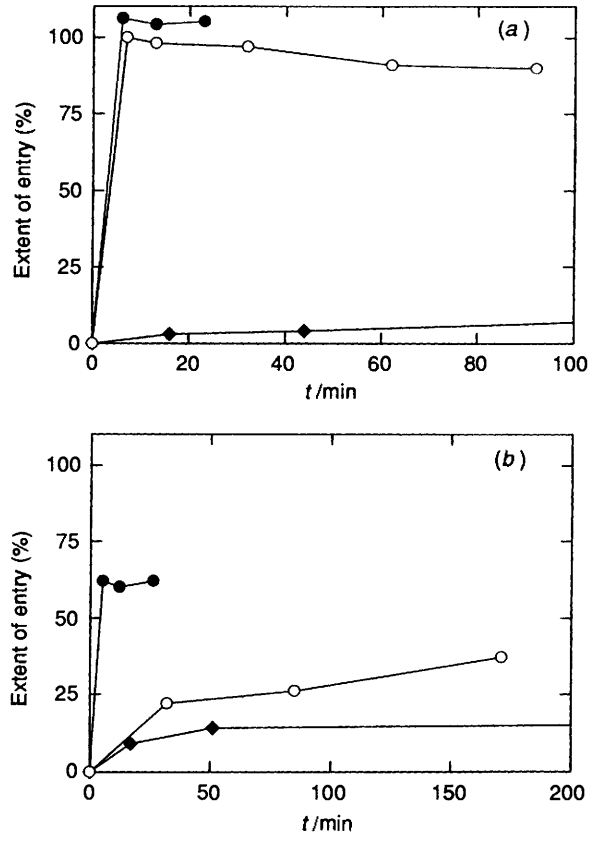


Fig. 5 (*a*) Variation of the extent of entry of Na^+ into LUV (egg PC) as a function of time in the presence of 48 μmol dm^{-3} monensin (○) and 57 μmol dm^{-3} gramicidin (●) introduced by the pre-incubation method and in the absence of added transporter (◆). The lines are simply a guide for the eye. (*b*) Variation of the extent of entry of Na^+ into LUV(DPPC) as a function of time in the presence of 96 μmol dm^{-3} monensin (○) and 29 μmol dm^{-3} gramicidin (●) introduced by the pre-incubation method and in the absence of added transporter (◆). Incomplete entry in the case of gramicidin may be due to a small amount of vesicle breakage.

that some other mechanism is responsible for transport in this case.

Transport Experiments in Gel-state Membranes.—Comparison of transport rates in fluid and 'frozen' membranes to distinguish carrier and channel mechanisms. In order to gain further insight into the mechanism of transport by 'bouquet' molecules, we made use of the liquid crystal-to-gel phase transition of phospholipid bilayers. It is known that the rate of transport by carrier mechanisms is markedly reduced below the phase transition temperature (T_c) of bilayers, while transport by the channel mechanism is relatively unaffected.[27] The carrier mechanism, in which the carrier–metal ion complex must shuttle back and forth through the membrane, is sensitive to membrane fluidity: the rate of transport is reduced significantly on passing from the fluid state to the gel state. In the channel mechanism, ions pass through the immobile channel molecule rather than through the membrane, so changes in membrane fluidity do not affect the rate of transport. Thus, comparison of transport rates in fluid membranes (above T_c) and gel-state membranes (below T_c) would allow a distinction to be made between the two mechanisms (*vide infra*).

Behaviour of known carrier and channel compounds in fluid and 'frozen' membranes. In order to test this hypothesis, rates of transport of Na^+ across liposomal membranes in the gradient experiment were measured for monensin A, an ionophore antibiotic which is known to transport Na^+ and Li^+ by the carrier mechanism,[28] and gramicidin A, a channel-forming peptide. This was done at 25 °C for liposomes made from egg phosphatidylcholine (EPC, $T_c = -15$ to -7 °C, fluid at 25 °C)[29] and for liposomes made from dipalmitoyl phosphatidylcholine (DPPC, $T_c = 41$ °C, gel at 25 °C).[29] Egg PC is a mixture of saturated and unsaturated phospholipids extracted from egg yolk having an average acyl chain length of 14–16 carbons. DPPC is a saturated phosphatidylcholine having 16-carbon acyl chains. Thus egg PC and DPPC have approximately the same bilayer thickness while showing widely different gel-to-liquid phase transition temperatures.

Decrease in the rate of transport by the carrier monensin in 'frozen' membranes. In egg phosphatidylcholine vesicles, transport was rapid and complete in less than 10 min with both 48 μmol dm^{-3} monensin and 57 μmol dm^{-3} gramicidin. In DPPC liposomes, transport was slow and less than halfway to completion after 100 min with 96 μmol dm^{-3} monensin, but transport with 29 μmol dm^{-3} gramicidin remained rapid, as shown in Fig. 5(*a*) and (*b*). Therefore, the rate of transport by a diffusive carrier was markedly reduced in a gel-state bilayer relative to a fluid-state bilayer, as expected. This demonstrates that the comparison of transport rates in LUV(EPC) and LUV(DPPC) will help to determine whether a transporter operates by a carrier mechanism: a transporter operates by the carrier mechanism only if the transport rate in LUV(DPPC) is lower than in LUV(EPC) at 25 °C. Thus, observation of similar transport rates in LUV(EPC) and LUV(DPPC) implies that the carrier mechanism is not operating.

Transport of Na^+ *in 'frozen' membranes by two 'bouquets'.* For experiments in DPPC membranes a different method of incorporation (pre-incubation) was used for 'bouquets'. LUV(DPPC) were prepared and added to a dried film of 'bouquet' compound in a small test-tube, then incubated at 50 °C for at least 1 h. Incubation accelerated incorporation of

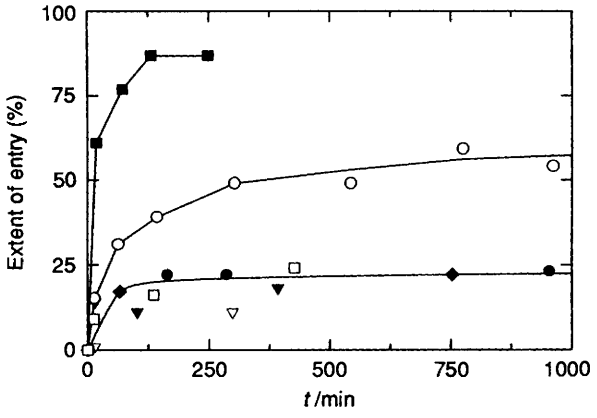


Fig. 6 Extent of entry of Na^+ into LUV(DPPC) as a function of time in the presence of 'bouquet' molecules B_M^o (●), B_M^c (○), B_{CD}^o (■), B_{CD}^c (□), C_M^o (▼) and C_M^c (▽) and in their absence (◆). The lines are simply a guide for the eye. See text for an explanation of the calculation of extent of entry. 'Bouquets' were introduced by pre-incubation and 'bouquet' concentrations were 63 (B_M^o), 31 (B_M^c), 28 (B_{CD}^o), 29 (B_{CD}^c), 29 (C_M^o) and 28 μmol dm^{-3} (C_M^c). The lipid concentration was approx. 12 mg cm^{-3}.

the 'bouquet' into the membrane and resulted in a stable liposome preparation. For experiments with monensin A and gramicidin A, a similar method was used except that the transporters were added from an alcoholic stock solution. Control experiments showed that added alcohol did not affect vesicle permeability. The pre-incubation method was used instead of co-preparation for LUV(DPPC) to reduce the length of time 'bouquets' are exposed to elevated temperatures. LUV(DPPC) were prepared by dialysis at 50 °C over 2 days; dialysis must be done above the phase transition temperature.

Thus, 'bouquets' were incorporated into the membranes of liposomes made from DPPC and Na^+ entry was followed in gradient experiments (Fig. 6). Entry of sodium ions in excess of the amount seen for a control without 'bouquet' was seen for only two 'bouquets': B_M^c and B_{CD}^o. Transport of Na^+ across DPPC membranes was not seen for B_M^o, B_{CD}^c, C_M^o and C_M^c.

Discussion

All 'Bouquets' Transport Ions at Similar Rates in Fluid Membranes.—All 'bouquets' transported Na^+ and Li^+ rather slowly in fluid membranes made up of egg phosphatidylcholine and compounds having different central cores (18-crown-6 or β-cyclodextrin) and different types of 'arms' [poly(oxyethylene) or polyalkyl] transported ions at roughly similar rates. One might have expected the compounds with polyalkyl chains to be less effective transporters since they have fewer sites available for alkali-metal ion complexation between the end-groups and the central core. However, as discussed below, complexation is only one factor involved in the functioning of an artificial ion channel.

Transport of Na^+ *and* Li^+ *by 'Bouquets' Proceeds by Antiport.*—Various mechanisms may be envisaged for alkali-metal ion transport across membranes. To preserve electroneutrality, passage of an alkali-metal cation across the membrane in one direction may be balanced by passage of another cation in the opposite sense (antiport).[30] In the present system, this cation may be either another alkali-metal ion or a proton. Alternatively, the alkali-metal ion and an anion, such as chloride, may cross the membrane in the same direction (synport).[30] During the gradient experiments, no significant change in pH was observed during the transport experiment, making cation-proton antiport unlikely. NMR experiments in which ^7Li and ^{23}Na resonances were followed simultaneously during a transport experiment showed that the amount of Na^+ having entered liposomes was roughly equal to the amount of Li^+ having exited liposomes. This confirmed that transport by the 'bouquets' proceeded by a Na^+–Li^+ antiport mechanism. Since transport of Na^+ and Li^+ is coupled, it is reasonable to assume that the more slowly transported ion determines the overall rate of transport.

B_M^c *and* B_{CD}^o *Do Not Use a Carrier Mechanism.*—Experiments comparing transport rates in fluid egg PC membranes and 'frozen' (gel-state) DPPC membranes showed that the rate of transport was drastically reduced for a diffusive carrier like monensin on going from fluid to 'frozen' membranes. Since two 'bouquets', B_M^c and B_{CD}^o, were found to transport Na^+ and Li^+ at similar rates in egg PC and DPPC membranes, the carrier mechanism may be excluded for these compounds. Therefore, these compounds may function by a channel mechanism, among others. The fact that these two 'bouquets' were, respectively, a crown ether-based compound with polyalkyl chains and a cyclodextrin-based compound with polyoxy chains led us to consider the factors involved in the functioning of an artificial channel of the 'bouquet' type.

C_M^o *and* C_M^c *Probably Use a Carrier Mechanism.*—The results for C_M^o and C_M^c provide a useful control for the results in the B_M and B_{CD} series. Consideration of the structures of C_M^o and C_M^c using molecular models shows that they are too short to span a bilayer membrane composed of phospholipids having 16-carbon acyl chains in a conformation with the crown ether moiety perpendicular to the acyl chains. These compounds cannot act as channels and yet are effective transporters in fluid membranes, probably using a carrier mechanism.

If C_M^o and C_M^c act as carriers, by analogy it is reasonable to suppose that B_M^o and B_M^c, which have similar structures, could also use the carrier mechanism. In 'frozen' DPPC membranes, C_M^o and C_M^c are inactive, consistent with a carrier mechanism provided that these compounds remain in the membrane upon 'freezing', a reasonable assumption for lipophilic C_M^c, at least. In contrast, B_M^c and B_{CD}^o are active in 'frozen' DPPC membranes, showing that they are not carriers. It may be inferred that the channel mechanism (or some alternative mechanism) may be more efficient than the carrier mechanism for B_M^c and B_{CD}^o, but the balance between the two mechanisms may be a fine one in this class of compounds since not all are able to use the non-carrier pathway. These comparisons underline the importance of experiments with membranes in both the fluid and gel states which may serve to exclude a carrier mechanism for those compounds which transport in both the fluid and gel states.

Other Artificial Ion Channel Mimics Resembling 'Bouquets' Transport Alkali-metal Ions.—Ion-channel mimics which resemble 'bouquet' molecules have been investigated.[17] Transport of alkali-metal ions across the fluid membranes of liposomes was coupled to proton transport using a proton carrier. Opposing gradients of proton and metal-ion concentration were created and the return to equilibrium was followed by the pH-stat technique.[17]

Several compounds were classified as channel-like. Two of these, denoted $(A8_2P)_4Tet$ and $(A8TrgP)_4Tet$, resemble 'bouquet' molecules, having a central 18-crown-6 annulus, polyalkyl and/or polyoxygenated side chains and carboxylate end-groups. The two compounds transport K^+ at similar rates despite the fact that they differ in the number of ligand oxygens present in the side chains, all other structural features being the same. This is reminiscent of our results for B_M^o and B_M^c in fluid membranes.

In a very recent study, sterol-based ionophores were found to

give transport rates similar to those of 'bouquet' molecules. A sterol–polyether conjugate, composed of a rigid hydrophobic androstenediol core to which were attached two hexaethylene-glycol chains, was incorporated into LUV(EPC) and alkali-metal transport was assayed by following Na^+ entry as in the present study.[31] Transport rates were similar to those found with 'bouquet' molecules under very similar conditions. Transport activity was comparable in fluid-state egg PC membranes and gel-state DPPC membranes, suggesting the formation of pores formed by the association of a number of sterol monomers.

Requirements for Functional Ion Channels.—In order for a 'bouquet' molecule to act as a true channel for alkali-metal ions, a number of conditions must be met: (*i*) the compound must be lipophilic enough to be incorporated into the membrane phase; (*ii*) the distribution of polar and apolar subunits of the 'bouquet' must be such that the molecule is incorporated into the membrane in a trans-membrane orientation; it must span the membrane, linking the internal and external aqueous phases;[11] (*iii*) the compound must associate with alkali-metal ions and the ions must be induced to pass through the 'bouquet' by complexation with the 'arms' and the central core.

We have attempted to determine whether the 'bouquet' molecules fulfill these three criteria. Previous reports have included evidence that the 'bouquets' are incorporated into the membrane phase of liposomes.[14] For example, incorporation of significant amounts of 'bouquets' into DPPC liposomes resulted in a lowering and broadening of the phase transition temperature. Based on these results, the 'bouquets' should not cause an observable shift in T_c at the low concentrations used in the transport experiments. We have shown that the crown ether and/or poly(ethylene oxide) chain subunits of the 'bouquets' interact with alkali-metal ions in the same manner as subunits alone.[12] Although there is a lack of adequate techniques to assess whether the 'bouquets' are incorporated in a trans-membrane orientation, the data obtained are consistent with such an orientation.

A compound may fail to act as a true channel for a variety of reasons. It may not be lipophilic to incorporate itself into the membrane or it may be incorporated into the membrane in an inactive non-trans-membrane orientation. It may be incorporated in a trans-membrane orientation, but may not interact strongly enough with alkali-metal ions to induce them to cross the membrane or may be deformed by lateral pressure from membrane phospholipids. Finally, an alternative mechanism, *e.g.* carrier mechanism or formation of a membrane defect, may provide a lower energy pathway for the compound to assist ions in crossing the membrane.

An inactive trans-membrane compound may cause local disruptions of membrane structure (defects) which allow an accelerated leakage of ions across the membrane, giving the appearance of transport in gradient experiments. However, it is difficult to imagine why only some 'bouquet' molecules would create defects in DPPC membranes, while others would not.

Channel and Defect Mechanisms for Transport by B_M^o, B_M^c *and* B_{CD}^o.—There are three possible explanations for the observation of transport with B_M^c and B_{CD}^o: (*i*) they act as true channels, albeit poor ones; (*ii*) they form a multi-'bouquet' aggregate creating a pore, in the same manner as alamethicin,[32] with ions passing not through the 'bouquet' interior but through the centre of the pore and (*iii*) they disrupt the regular packing of phospholipids in the bilayer, creating a defect in the membrane structure which allows the leakage of alkali-metal ions across the membrane.

Distinguishing the first possibility from the others amounts to determining whether ions pass through the 'bouquet' itself or not. If ions pass through the 'bouquet', the size of the central macrocycle (18-crown-6 or β-cyclodextrin) would be expected to confer selectivity on the transport process: ions small enough to pass through the macrocycle would be transported, while those that are too large to pass would not. Leakage of ions through a membrane defect or passage through a pore would not be expected to present the same selectivity pattern, if any, *e.g.* cation conductance by alamethicin, a pore-forming peptide, does not show any specificity.[32] Thus, a demonstration of selectivity in transport by 'bouquets' consistent with the known size of the central macrocycle would be evidence in favour of a true channel mechanism.

In this respect, liposomes containing incorporated 'bouquets' did not allow leakage of Dy^{3+} or the tripolyphosphate anion across the membrane. Indeed, leakage of the shift reagent into the internal volume would render the transport experiment impossible using the alkali-metal NMR technique. This indicates that at least the 'bouquets' do not induce major defects or pores allowing leakage of the more highly-charged ions of the shift reagent. Clearly, it would be of interest to investigate further the selectivity among alkali-metal ions of transport by 'bouquets'.

Lastly, experiments in liposomes made from egg PC, stearylamine and cholesterol showed that the rate of transport had a zero-order dependence on 'bouquet' concentration for B_M^o. This is inconsistent with carrier, channel or pore mechanisms, but could be explained if one admits a long-range formation of defects induced by B_M^o.

Conclusions

In experiments in which opposing gradients in Li^+ and Na^+ concentration were created and the transport of alkali-metal ions down their concentration gradients was followed, all 'bouquets' were found to transport ions at similar rates in fluid membranes made from egg PC. 'Bouquets' caused a one-for-one exchange of Na^+ for Li^+ (antiport). Comparison of transport rates in fluid- (egg PC) and gel-state membranes (DPPC) revealed that two 'bouquets' (B_M^c and B_{CD}^o) transport Na^+ at similar rates in the fluid and gel states. The carrier mechanism can be excluded for these compounds, based on complementary experiments with known carrier and channel compounds. Variation of transport rate with 'bouquet' concentration was probed for B_M^o and B_M^c and the rates were found to increase with B_M^c concentration, but not with B_M^o concentration. Thus, B_M^c and B_{CD}^o give results inconsistent with a carrier mechanism, but other alternatives to the channel mechanism, such as creation of a multi-'bouquet' pore or a membrane defect, cannot be ruled out definitely on the basis of our results.

This discussion illustrates the problems confronting attempts to construct artificial ion channels. Once a compound has been designed, synthesized, incorporated into membranes and shown to transport ions, there exist a number of possible mechanisms for transport (carrier, channel, pore and defect mechanisms). Transport must be shown to conform to criteria for the channel mechanism, assuming that a consensus is reached on what these criteria are. If several structurally-related compounds can be shown to use the channel mechanism, making structure–activity correlations is still difficult. Structural changes may affect partitioning of the compound into the membrane and its orientation in the membrane, as well as its intrinsic transport activity. Even if partitioning into the membrane can be measured, the orientation in the membrane is not readily characterised. A set of structural, physico-chemical and functional criteria must be devised and applied in order to determine whether a given compound creates an operational channel.

Experimental

Preparation of LUV.—Incorporation of bouquets by co-operation. Large unilamellar vesicles were prepared by the dialytic detergent removal technique[33] using L-α-phosphatidylcholine from egg yolk (egg PC or EPC, Sigma, *ca.* 99%) or L-α-dipalmitoyl phosphatidylcholine (DPPC, Sigma, 99$^+$%) or a mixture of egg phosphatidylcholine, stearylamine and cholesterol (7:2:1 molar ratio) [Sigma, Liposome Kit (Positive)], and octyl-β-D-glucopyranoside (Sigma, 98%, 15 equiv. with respect to lipid). Stock solutions of lipid and detergent in chloroform or dichloromethane were combined and concentrated to dryness. In cases when bouquet was incorporated by co-preparation, bouquet was also added at this point as a stock solution in methanol or chloroform–methanol.

The residue was dissolved in aq. LiCl solution (100 mmol dm^{-3}), giving a solution with 12.5 mg cm^{-3} lipid, then transferred to dialysis tubing (Viskase, M_w cut off 12 000–14 000). Detergent was removed by dialysis: typically, 7 cm^3 of detergent–lipid solution was dialysed against 3 dm^3 of 100 mmol dm^{-3} aq. LiCl and the dialysis solution changed three times over 2 days. For LUV(DPPC), all dialyses were carried out at 50 °C, *i.e.* above the phase transition temperature of the lipid, while for LUV(EPC), the first dialysis was carried out at 50 °C and subsequent dialyses were performed at room temperature. The final total volume of the LUV preparation was determined by weight and aliquots of the preparation were taken for phosphate analysis and assay of bouquet concentration. An aliquot of the final dialysis solution was also taken for osmolarity measurement.

Incorporation of Bouquets by Pre-incubation.—Measured volumes of bouquet stock solution were dried to a film in a series of small test-tubes. LUV preparation was added to each and the septum-sealed tubes incubated in a water bath at 50 °C for at least 1 h. The tubes were mixed occasionally by inverting.

After incubation, the tubes were cooled slowly to room temperature, then filtered to remove undissolved bouquet. When the pre-incubation method was used lipid and 'bouquet' concentrations were not assayed as described below, but estimated from the amounts added.

Electron Microscopy.—Liposomes were analysed by freeze-fracture electron microscopy and measured diameters of liposomes were subjected to stereological analysis to correct for the effect of non-equatorial fractures on size distribution, as described previously.[34,35]

Preparation of Shift Reagent.—Penta(tetramethylammonium) tripolyphosphate [$(Me_4N)_5PPP$] was prepared from pentasodium tripolyphosphate (Na_5PPP) using a column of Dowex 50X-100 ion exchange resin (H^+ form, 100 g). The resin was treated with Me_4NOH to convert it to the Me_4N^+ form and washed to neutrality. Pentasodium tripolyphosphate (Na_5PPP, Sigma, practical grade, 0.5 g) was loaded and eluted with water. The basic eluent was collected, lyophilised and reapplied to the regenerated column (HCl, then Me_4NOH). Basic fractions were again collected and lyophilised. Residual water was removed azeotropically by repeated dissolution and evaporation from anhydrous ethanol. After drying under high vacuum, a concentrated solution of $(Me_4N)_5PPP$ was prepared and shown to contain Me_4N^+, but not Na^+ by ^1H and ^{23}Na NMR spectroscopy.

Shift reagent was prepared by mixing standard solutions of $(Me_4N)_5PPP$ and $DyCl_3$ (3:1 molar ratio), then adjusting to pH 7.0 with dilute HCl. A stock solution that was *ca.* 33 mmol dm^{-3} $(Me_4N)_5PPP$ and 11 mmol dm^{-3} $DyCl_3$ was typically used.

Osmolarity Measurements.—The osmolarity of all solutions were measured using a Roebling osmometer. The osmolarities of shift reagent and NaCl solutions were measured directly, while the osmolarity of the LiCl solution in the LUV preparation was taken as being identical to that of the final dialysis solution. After these measurements, stock solutions were diluted so that all solutions added to the LUV preparation had osmolarities which were equal to or slightly greater than that of the LUV preparation in order to prevent shrinkage or swelling of vesicles owing to imbalances in osmotic pressure.

Measurement of Lipid Concentration.—The lipid concentration of LUV preparations was measured by analysis for phosphate content after acid digestion, following the method of Chen *et al.* and Morrisson.[36]

Assay of 'Bouquet' Concentration.—The actual concentration of 'bouquets' in LUV preparations was measured using the UV absorption of the bouquets at 250 or 255 nm. The measured absorption coefficients were: $\varepsilon_{250}(B_M^c) = 5.97 \times 10^4$, $\varepsilon_{250}(B_M^c) = 7.75 \times 10^4$, $\varepsilon_{250}(B_{CD}^c) = 1.49 \times 10^5$ and $\varepsilon_{255}(B_{CD}^c) = 1.78 \times 10^5$ dm^3 mol^{-1} cm^{-1} (solvent: abs. ethanol). Since lipids also absorb at these wavelengths, the absorbance of the control preparation was subtracted from the absorbance of preparations containing bouquet, taking into account variations in lipid concentration between preparations.

NMR Experiments.—NMR experiments were performed on a Bruker 200 SY spectrometer thermostatted to 25 ± 1 °C. LUV and shift reagent solutions were placed in an 8 mm NMR tube which was in turn placed in a 10 mm tube containing D_2O for lock. Typically, 1000 μl (1 μl = 1 mm^3) of LUV in 100 mmol dm^{-3} aq. LiCl solution and 300 μl of shift reagent solution [30 mmol dm^{-3} $(Me_4N)_5PPP$ and 10 mmol dm^{-3} $DyCl_3$] were combined and ^7Li measurements were made. Subsequently, 800 μl of 100 mmol dm^{-3} aq. NaCl solution was added and transport was followed by ^{23}Na NMR spectroscopy.

For LUV in LiCl solution, the volume trapped inside liposomes was measured by ^7Li NMR. The 180° pulse width and the T_1 of the peak corresponding to entrapped Li^+ were measured for the sample of LUV plus shift reagent and this information was used to adjust the pulse width and recycle delay in order to obtain fully relaxed transitions and accurate integration. The trapped volume was calculated from the integrals of the peaks corresponding to internal and external Li^+ as well as the volumes of the solutions added (see below).

For some samples, particularly those containing LUV (egg PC–stearylamine–cholesterol), the LUV separated from solution and ^7Li spectra were obtained with spin rate = 0 and frequent pauses for mixing during acquisition. After addition of NaCl solution, vesicle separation was normally no longer a problem.

Typically, ^{23}Na spectra (1 K, 4096 scans) were zero-filled to 4 K and an exponential multiplication with a line broadening of 5 Hz was applied. Spectra were phased automatically, then corrected manually. A baseline correction was performed using a four-parameter polynomial fit. Finally, the peaks were integrated. In some cases, peaks corresponding to internal and external Na^+ overlapped slightly and deconvolution was carried out using the GLINFIT program (obtained from the Bruker program library).

Calculations: Internal Volume, Extent of Entry and Transport Rate.—Internal volume (V_{int}/μl), *i.e.* the volume inside LUV which is shielded from the shift reagent was calculated as follows. Known volumes of LUV and shift reagent were added to the NMR tube; the ^7Li spectrum yielded the integral of the internal peak as a percentage of the total integral [I_{int}^{Li}(%)].

Since $[Li]_{int}$ remains the same as [Li] in the LUV solution before addition of shift reagent, $V_{int}(\mu l) = V_{LUV} \times I_{int}^{Li}$.

In order to interpret the results of transport experiments, the internal volume as a percentage of the total volume after addition of NaCl solution $[V_{int}^{f}(\%)]$ was required. This parameter measures the capacity of a LUV preparation to allow entry of Na^+. It is important to note that the entrapped volume varied significantly among LUV preparation: measurement of V_{int} for each preparation was necessary. $V_{int}^{f}(\%) = V_{int}/V_{total}^{f}$.

The entry of Na^+ was measured as the integral of the internal peak as a percentage of the total integral $[I_{int}^{Na}(\%)]$. Initially, there was no Na^+ inside LUV and $I_{int}^{Na} = 0$. At equilibrium (when transport was complete), $I_{int}^{Na} = V_{int}^{f}(\%)$. The extent of entry may be normalised so that extent = 0 initially and at equilibrium extent = 100. Thus the normalised extent of entry at time t is $E_{Na}(t) = [I_{int}^{Na}(t)/V_{int}^{f}] \times 100$.

The increase of the extent of entry as a function of time in the presence of 'bouquet' molecules may be analysed to give an observed rate constant. First, a correction for 'passive' entry of Na^+ at each time was made by subtracting the extent of entry at the same time in the control experiment (no 'bouquet'). $E_{Na}^{corr}(t) = [E_{Na}(t)]_B - [E_{Na}(t)]_{control}$. Next, an infinity value for the corrected extent of entry in each experiment was identified. This was taken to be the largest observed value of $E_{Na}^{corr}(t)$. Finally, $\ln[E_{Na}^{corr}(\infty) - E_{Na}^{corr}(t)]$ was plotted against time, and the slope of the initial linear portion gave the observed rate constant (first-order behaviour is assumed).

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