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High-resolution NMR studies of transmembrane cation transport: Use of an aqueous shift reagent for ^{23}Na

(lanthanide complex/hyperfine shift/unilamellar vesicles/egg lecithin/gramicidin)

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ABSTRACT **^{23}Na NMR studies of large unilamellar vesicles of egg lecithin in salt solutions are reported. A shift reagent, the dysprosium nitrilotriacetate ion $Dy[N(CH_2CO_2)_3]_2^{3-}$ has been used to distinguish between $^{23}Na^+$ inside and outside the vesicles. When both are present and the shift reagent is present on only one side, two clearly distinct resonances are observed. Creation of a Na^+ concentration gradient and subsequent catalysis of passive transport induced by the introduction of gramicidin can be monitored easily by using the relative intensities of the two resonances. We report the observation of transport both out of and into vesicles.**

The transport of metal cations across biological membranes is a very important process (1) usually facilitated by integral membrane proteins (2). Unfortunately, the major cations transported (Na^+, K^+, Mg^{2+}, and Ca^{2+}) are among the hardest of all chemical species to monitor, having little direct spectroscopic accessibility. We have analyzed in some detail an increasingly popular vesicle high-resolution NMR technique that uses paramagnetic metal cations (usually lanthanides) as surrogates for the physiological cations (3). Under certain conditions, the transport of these ions has been induced by various substances, including ionophores, detergents, and membrane proteins (see ref. 3 for references). The essence of this NMR technique lies in the rendering of resonances of species on opposite sides of the membrane anisochronous (i.e., having different resonance frequencies). An attractive feature of this quality is the possibility of distinguishing between the two general modes of metal cation transport—i.e., whether the ions cross the membrane in bursts or one at a time. However, there are two major drawbacks with the method as it now stands. First, it is limited to use with small vesicles (up to *ca.* 50 nm in diameter) because it uses resonances of the membrane phospholipid molecules themselves, and the corresponding resonances of larger vesicles, organelles, cells, or organs are much too broad for this high-resolution technique. (For similar reasons, it is limited to studies above the lipid phase-transition temperature). Second, the method does involve the study of surrogate cations; although many substances have been found to transport these, biological systems are notoriously selective. One can be sure that many (if not most) membrane transporting proteins are quite specific for a particular metal cation. Toward overcoming these drawbacks, we report here some preliminary studies of passive Na^+ transport out of and into large vesicles that used an aqueous shift reagent for high-resolution $^{23}Na^+$ NMR (4). A separate communication on similar studies with living cells will be published elsewhere (5).

MATERIALS AND METHODS

Large unilamellar vesicles (LUV) of egg lecithin (Sigma) were prepared essentially by the dialytic detergent-removal technique of Reynolds and coworkers (6), which produces a reasonably monodisperse population of vesicles averaging 240 nm in diameter. In a typical preparation, 2 ml containing 10 mM egg lecithin, 150 mM octyl β-D-glucopyranoside (Calbiochem-Behring), and 60 mM NaCl or 40 mM LiCl/5 mM $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2$ (triethanol-ammonium dysprosium nitrilotriacetate) was dialyzed (Spectrapor no. 3 tubing) two or three times (an average of *ca.* 13 hr each) against a large quantity (an average of *ca.* 3 liters) of 60 mM NaCl or 40 mM LiCl/5 mM $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2$. This removed the detergent and produced the LUV. The NaCl-containing LUV were further dialyzed against 2 liters of 60 mM LiCl for 13 hr to remove external Na^+. External NaCl was added to the LiCl-containing LUV. Passive transport was induced by injecting a small amount (microliters) of a concentrated methanol solution of gramicidin D (Calbiochem–Behring), a mixture of gramicidins. Conditions for the NMR spectra are given in the text and figure legends.

RESULTS

Fig. 1 depicts the ^{23}Na NMR spectrum (132.3 MHz, Bruker WM-500) of a dispersion of LUV prepared from egg lecithin so that, in the NMR tube, there was 60 mM NaCl inside the vesicles, and the aqueous space outside the vesicles was 35 mM in LiCl and <0.5 mM in NaCl. Fig. 1*a* shows the spectrum when no shift reagent was added. The single sharp resonance represents the Na^+ both inside and outside the vesicles, referred to as Na^+_{in} and Na^+_{out}. Fig. 1*b* shows the spectrum after the outside aqueous space was made 6.3 mM in $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2$. The single resonance in Fig. 1*a* is split into two peaks in Fig. 1*b*; the smaller one is shifted upfield by 190.4 Hz, and the larger one, downfield by 9.8 Hz. Because we found $Dy[N(CH_2CO_2)_3]_2^{3-}$ to induce upfield isotropic hyperfine shifts (Δ) in the $^{23}Na^+$ resonance, the assignment of the upfield peak to Na^+_{out} is clear. That the splitting increased with shift reagent concentration (not shown) and that the magnitude of the splitting is not inconsistent with observed shifts (4) help to confirm this. The small downfield shift of the Na^+_{in} resonance is interesting. It may be related to similar observations of shifts of inside lipid headgroup ^1H resonances induced by paramagnetic lanthanide aquo ions outside of small vesicles (7). However, its observation here may tend to contradict the explanation offered.

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Abbreviations: LUV, large unilamellar vesicles; FID, free-induction decay(s).

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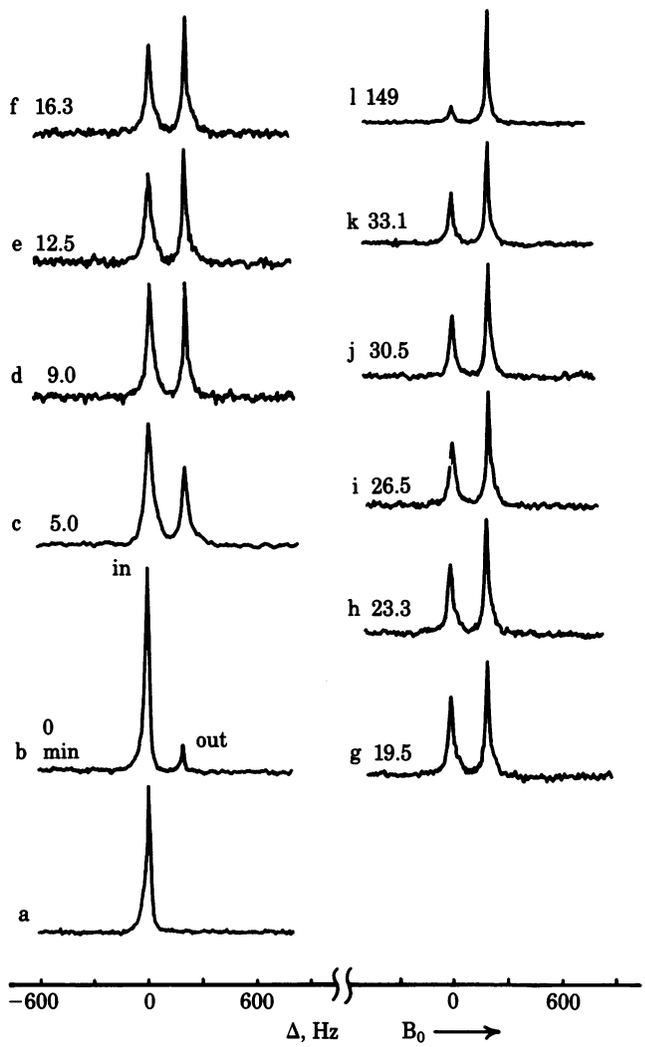

FIG. 1. ^{23}Na NMR spectra (132.3 MHz, 11.74 T) of a dispersion of LUV in ^2H_2O. (*a*) In the NMR tube, the final concentrations were: egg lecithin, 6.0 mM; $NaCl_{in}$, 60 mM; $NaCl_{out}$, 0.27 mM; and $LiCl_{out}$, 35 mM. (*b*) Concentrations were as in *a* except that the outside aqueous space was made 6.3 mM in $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2$. For *a* and *b*, 512 free-induction decays (FID) were accumulated in 208 sec. (*c–l*) Spectra are labeled with the minutes elapsed after the solution was made 0.16 μM in gramicidin. The times recorded are those of the midpoints of the data accumulation periods, which were 156 FID in 64 sec (*c*), 128 FID in 52 sec (*d–j*), and 512 FID in 208 sec (*k* and *l*). The temperature was *ca.* 297 K.

The fraction of the total peak area due to the inside resonance was measured (by planimetric integration) to be 0.92 in the Fig. 1*b* spectrum. (The spectra were obtained with NMR conditions such that there should be no differential T_1 effects.) The value for the fraction of the total aqueous volume inside the LUV was interpolated for 6 mM lipid to be 0.05 (6). Combining these numbers with 60 mM Na^+_{in} yields a Na^+_{out} concentration of 0.27 mM. This corresponds to removal of >99% of the Na^+_{out} during the final dialysis. Essentially complete removal can be attained in a subsequent chromatographic step (6) not used in this study. Here, there also could be some error in the calculated volume ratio because of inaccuracy in our knowledge of the final lipid concentration. In other studies, where we had prepared LUV and the gradients chromatographically by the method of Enoch and Strittmatter (8), and where we had been able to conduct phosphate analyses on the final solutions, our measured area ratios were in good agreement with expected values (9). Also, of course, any leakiness of the vesicles would affect the observed ratio. The spectrum in Fig. 1*b* was obtained *ca.* 1.5 hr after the LUV were removed from the LiCl dialysate.

Immediately after the Fig. 1*b* spectrum was obtained, the solution was made 0.16 μM in the ionophore gramicidin (10–12). This amounts to *ca.* 13 gramicidin molecules per vesicle (6) and induces a rapid efflux of Na^+ down its concentration gradient, as can be seen in spectra *c–l* in Fig. 1, which depict some of the spectra obtained and show the time evolution of the spectrum measured in minutes from the time of addition of gramicidin. The inner peak decreased in area at the expense of the outer peak. This happened quite quickly at first (the fractional area inside dropping to 0.5 in the first 10 min) and then more slowly. The fractional area obtained at 149 min, 0.16 (inner/total), had still not reached the equilibrium value—0.05, the inner-to-total volume ratio assumed above. A plot of the logarithm of the ratio R of the fractional area (inner/total) at any time to the fractional area at time zero (0.92) against time is shown in Fig. 2. It is reasonably fitted with two exponential decay terms [$R = 0.41\exp(-0.18t) + 0.53\exp(-0.0096t) + 0.05$, with t shown in minutes]. The faster first-order rate constant is $3.0 \pm 0.3 \times 10^{-3}$ sec $^{-1}$, whereas the slower one is $1.6 \pm 0.2 \times 10^{-4}$ sec $^{-1}$. These can be converted to permeability coefficients (P) through the relationship: $P \simeq$ (rate constant)(vesicular inner aqueous volume)(vesicular surface area)$^{-1}$ (3, 6). Using the average values for the volume (6.54×10^{-15} ml) and area [3.5×10^5 (nm)^2] given by Mimms *et al.* (6), we obtained P of 5.6×10^{-9} cm/sec and 2.9×10^{-10} cm/sec.

Transport of Na^+ into LUV is shown in Fig. 3. The spectrum in Fig. 3*a* is that of a sample of LUV prepared similarly to that of Fig. 1 except that $[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2$ was present both inside (5.0 mM) and outside (3.7 mM) the vesicles, as was LiCl (40 mM inside, 29 mM outside). The single

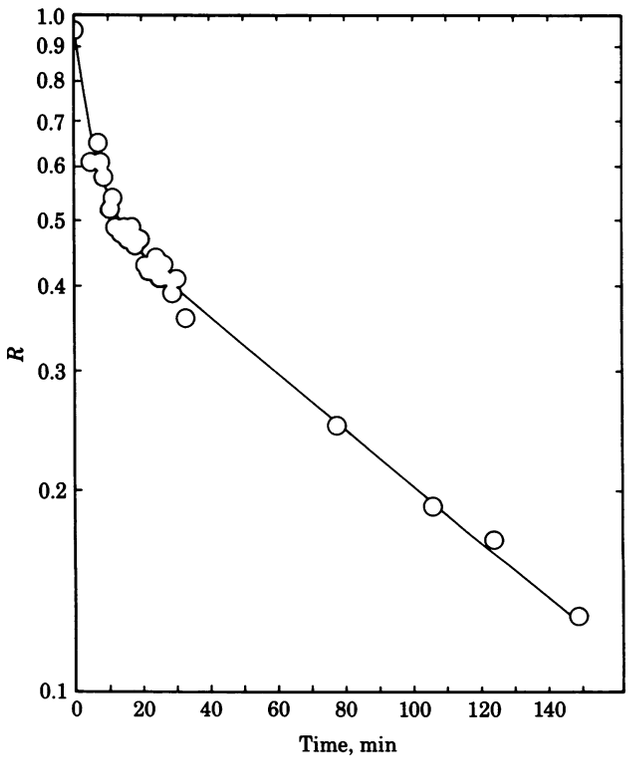

FIG. 2. The time dependence of the relative intensities of the two peaks of Fig. 1. The data are plotted as log R [≡ (fractional area inside)/(fractional area inside at time zero; spectrum in Fig. 1*b*)] versus time. The solid curve is the result of computer fitting.

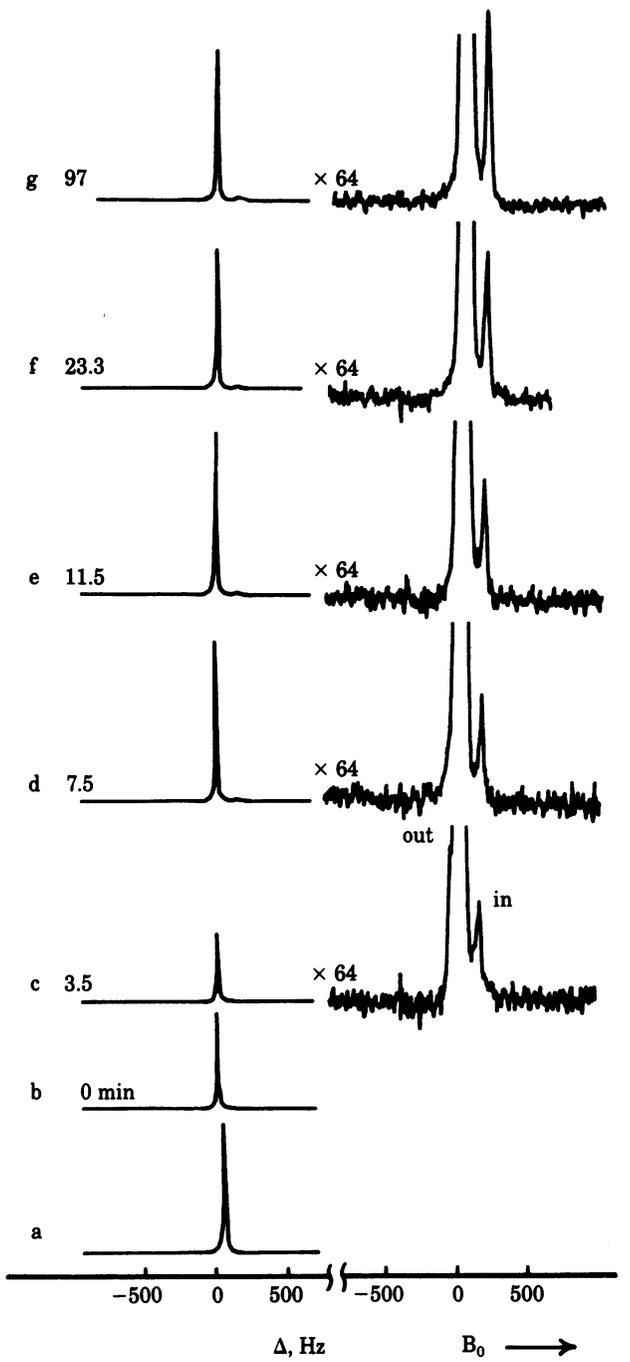

FIG. 3. ^{23}Na NMR spectra (132.3 MHz, 11.74 *T*) of a dispersion of LUV in ^2H_2O. (*a*) The final concentrations in the NMR tube were: egg lecithin, 7.5 mM; $LiCl_{in}$, 40 mM; $LiCl_{out}$, 29 mM; $NaCl_{out}$, 43 mM;$\{[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2\}_{in}$, 5.0 mM; and $\{[HN(CH_2CH_2OH)_3]_3Dy[N(CH_2CO_2)_3]_2\}_{out}$, 3.7 mM. (*b*) Concentrations were as in *a* except that the outside aqueous space has been made 5.3 mM in $LuCl_3$. (*c–g*) Spectra are labeled with the times elapsed after the solution was made 0.16 μM in gramicidin. The times recorded are those of the mid-points of the data accumulation periods, which were 128 FID in 52 sec (*c–e*) and 256 FID in 105 sec (*f* and *g*). On the right-hand side of each of the spectra in *c–g*, a plot is recorded where the vertical scale has been expanded. The temperature was *ca.* 297 K.

sharp $^{23}Na^+$ resonance is due to NaCl added after vesicle formation and, thus, present only outside (43 mM). The spectrum in Fig. 3*b* was obtained after the outside aqueous solution of Fig. 3*a* was made 5.3 mM in Lu^{3+} (as the chloride). Because Lu^{3+} acts as an antishift reagent by inactivating $Dy[N(CH_2CO_2)_3]_2^{3-}$ (4), the Na^+ resonance is shifted back downfield by 58.6 Hz in Fig. 3*b*. However, the $Dy[N(CH_2CO_2)_3]_2^{3-}$ inside the vesicles (*ca.* 20×10^3 per vesicle) remained intact and able to shift the resonance frequency of any Na^+ transported in. This is seen in Fig. 3 *c–g* where the time-dependence of the spectrum, measured in minutes from the introduction of gramicidin (0.16 μM; *ca.* 11 gramicidin molecules per vesicle) is depicted. A small, sharp peak is seen to grow *ca.* 187 Hz upfield of the large resonance representing the Na^+ remaining outside. This also demonstrates that gramicidin does not transport Dy^{3+} across the vesicle membrane on the time scale of this experiment.

DISCUSSION

It seems reasonable that the Na^+ efflux in the experiment of Fig. 1 should be at least biphasic. After gramicidin is added and the transport of cations begins, one can visualize at least three distinct stages along the way to final equilibrium. The first stage to occur is likely a passive one-for-one Na^+-for-Li^+ exchange out of and into the vesicles, respectively, both ions moving down their concentration gradients. This stage ends when the Li^+ gradient is dissipated (33 mM§ both inside and outside). However, a Na^+ gradient still exists (27 mM inside, 2.0 mM outside, on the assumption that nonfacilitated Cl^- transport is slow enough to be ignored). At this point, the fraction of all Na^+ inside the vesicles would be 0.42 ($R = 0.45$).

A second stage would commence, whereby the Na^+ gradient would be partially dissipated at the expense of creating a new Li^+ gradient (inside > outside; Li^+ overshoot)—still through a one-for-one exchange. Such a stage would end when the ratio of inside concentration to outside concentration has the same value for both Na^+ and Li^+ (corresponding to the same diffusion potential). At this point, concentrations would be 55 mM Li^+_{in}, 32 mM Li^+_{out}, 5.1 mM Na^+_{in}, and 3.0 mM Na^+_{out} (still with Cl^- transport ignored; the diffusion potential would be 13 mV, outside positive). The fraction of total Na^+ inside the vesicles would be 0.08 ($R = 0.09$). Finally, the system will attain true equilibrium only after a third stage—passive nonfacilitated Cl^- transport out of the vesicles—is completed. At equilibrium Li^+_{in} and Li^+_{out} concentrations would be 33 mM, and those of Na^+_{in} and Na^+_{out} would be 3.0 mM. The fraction of total Na^+ that was inside the vesicles would be equal to the analogous volume fraction, 0.05. We are ignoring any Donnan effect caused by the impermeant $Dy[N(CH_2CO_2)_3]_2^{3-}$. The maximum effect (elaborated only if $HN(CH_2CH_2OH)_3^+$ were permeable) would only bring *R* down to 0.04. We are also ignoring any osmotic swelling effects (these would at most change the volume fraction from 0.05 to 0.06, and then only at the beginning of the transport experiment), and any small effects due to a pH gradient caused by permeation of $N(CH_2CH_2OH)_3$ (the pK_a of $HN(CH_2CH_2OH)_3^+$ is 9.5).

In Fig. 2, it is clear that the efflux slows down considerably when *R* reaches *ca.* 0.5. This would indicate that the fast process ($P = 5.6 \times 10^{-9}$ cm/sec) corresponds closely to the one-for-one exchange of Na^+ and Li^+ down their gradients. This is likely limited by the gramicidin-induced Li^+ transport which is *ca.* 1/6th as fast as that of Na^+ (13). Thus, the slow process ($P = 2.9 \times 10^{-10}$ cm/sec) would correspond to the essentially simultaneous occurrence of the second and third stages described above, implying that they both have very similar permeability coefficients. Support for this interpretation comes from the value of *P* reported for passive nonfacilitated transport of Cl^- (third stage) in these LUV to be 0.76×10^{-10} cm/sec at 24°C

§ This and all of the following concentrations for the experiment of Fig. 1 were calculated with the fractional inside aqueous volume, 0.05, assumed above.

(6). Our value is only *ca.* 4 times larger. There are a number of other possible tests for our interpretation. We also have used our technique to monitor nonfacilitated Na^+ leakage out of the LUV we prepare. Our results are in good agreement with those of Mimms *et al.*, who reported a value for $P = 9.5 \times 10^{-13}$ cm/sec at 24°C from $^{22}Na^+$ tracer studies (6).

An explanation of the biphasic nature of the Na^+ efflux involving intervesicular gramicidin exchange is probably not reasonable. This process has been reported to be slow compared to the time scale of our experiment (14).

The greatest mechanistic information content of the NMR study of ion transport occurs when the resonance frequency of an inside resonance depends on the number of ions that have entered the vesicle (3). This can be the case in the type of study reported here, when the only active shift reagent species is present inside the vesicles. A method for accomplishing this is demonstrated in Fig. 3.

By following the reasoning offered for the experiment depicted in Fig. 1, there should be a rapid influx of Na^+ and efflux of Li^+ until the LiCl gradient is dissipated. This stage should end when the LiCl both inside and outside is 30 mM,¶ the Na^+_{in} is 10 mM (39×10^3 ions per vesicle), and Na^+_{out} is 42 mM. Multiplying the volume ratio (V_{in}/V_{out}), 0.06, by the Na^+ concentration ratio gives a peak-area ratio (inner/outer) of 0.01. A second stage, involving formation of a Li^+ gradient (outside > inside; Li^+ overshoot) at the expense of dissipation of part of the Na^+ gradient should end when Li^+_{in} is 17 mM, Li^+_{out} is 30 mM, Na^+_{in} is 23 mM (91×10^3 ions/vesicle), and Na^+_{out} is 42 mM (Cl^- transport still ignored; the diffusion potential should be 15 mV, inside positive). The Na^+ peak-area ratio (inner/outer) should be 0.03 at this point. Final equilibrium should be reached only after completion of passive nonfacilitated transport of Cl^- into the vesicles. At this point the Li^+ concentration should be 30 mM both inside and out, the Na^+ concentration should be 40 mM both inside (16×10^4 ions per vesicle) and out, and the Na^+ peak-area ratio (inner/outer) should be equal to the volume ratio, 0.06. [Influx of the 16 mM Cl^-_{out} introduced with the Lu^{+3} can be discounted because the LUV are not significantly permeable to Lu^{+3} or Dy^{+3}. Also, we are again ignoring osmotic shrinking (in this case) and Donnan effects (which would be even smaller here).]

Although such small ratios are very hard to measure accurately, the experimental value of the Na^+ peak-area ratio (inner/outer) seems to climb rapidly to *ca.* 0.03 in the first 10 min. Then a much slower approach to the equilibrium ratio (0.06) is observed. The ratio at 97 min is found to be almost 0.05. The rate constant for the slower process is of the same order of magnitude as that of the slow process of Fig. 2, indicating that this represents the Cl^- diffusion stage. Thus, in this experiment, it seems that it is the first two stages that have similar permeability coefficients.

The fact that the $^{23}Na^+_{in}$ resonance does not shift back downfield as it grows in intensity is consistent with the expected "all or nothing" aspect of the gramicidin transport mechanism (3). That is, the ions enter the vesicles only in bursts large enough so that one burst is sufficient to give a vesicle all the ions it will receive (91×10^3 at the end of the first two stages). However, the value of the resonance frequency of the inside resonance is somewhat puzzling. If Na^+_{in} is 10 mM (39×10^3 ions per vesicle) at the end of the first stage, and the inside shift reagent is 5.0 mM, one would expect an upfield shift of *ca.* 180 Hz by interpolation of measured titration curves (4). This is very close to the observed value. However, the peak-intensity ratio for this stage should be only 0.01, whereas the observed value rises quickly to 0.03 [consistent with Na^+_{in} being 23 mM (91×10^3 ions per vesicle) and an expected shift of *ca.* 155 Hz] and then more slowly to 0.05 [consistent with Na^+_{in} being 35 mM (14×10^4 ions per vesicle) and an expected shift of *ca.* 130 Hz]. The freed Dy^{3+} outside the vesicles will certainly be significantly bound to the outer vesicular surfaces (15), and this binding is known to produce an upfield shift of inside lipid headgroup resonances (7). Whether this would produce an extra upfield shift of *ca.* 30 Hz (187–155) in our experiment is not known. An alternative explanation of the nonshifting of the inside resonance is that the Na^+ ions are entering the LUV a few at a time, but as their concentration builds, the concentration of Li^+ (which compete for the shift reagent) decreases.

A much better investigation of these aspects can be made at higher lipid concentrations in which the inner aqueous volume is a more substantial fraction of the total. The quality of the current interpretation rests heavily on accurate measurements of very small peak-intensity ratios. Certainly, accurate determinations of the lipid concentrations in the final solutions are also required.

Thus, it seems clear that, with further developments, this method will allow quite sensitive study of passive and active metal cation transport in model membrane vesicles on reasonable time scales. In other experiments (9), we have monitored Na^+ efflux from LUV induced by the ionophore valinomycin (11). We have also prepared LUV by the reverse-phase evaporation technique (reverse-phase evaporation vesicles) (16). With these we have observed spectral splitting (with *ca.* 40% of the observable Na^+ inaccessible to the shift reagent at equilibrium). However, the lines are noticeably broader than those of Figs. 1 and 3 for reasons unknown to us.

The method reported here apparently avoids problems associated with severe quadrupolar relaxation (for ^{23}Na, nuclear spin $I = 3/2$) by observing the resonances of cations that are mostly freely solvated and unbound. The spectra observed in Figs. 1 and 3 are perhaps surprisingly sharp—certainly well within the "fast motional narrowing" condition (17). Also, by avoiding highly negatively charged bilayer membranes, one can eschew quadrupolar relaxation of the $^{23}Na^+$ resonance due to membrane-bound Na^+ (18).

Equilibrium spectra having anisochronous inside and outside resonances also will allow the study of fast, equilibrium transport through techniques available only with magnetic resonance. Thus, transport processes with lifetimes of the order of the magnitude of T_1 [57.0 msec for $^{23}Na^+$ at infinite dilution (19)] can be studied by the saturation transfer or selective population transfer approach (20). Processes, with equilibrium lifetimes of the order of milliseconds also will yield to total lineshape analysis (20). In fact, we have observed evidence of such phenomena (9, 21). We also have detected apparent equilibrium transport induced by the lithium salt of lasalocid A in the reverse-phase evaporation vesicle dispersions. In fact, the breadth of the $^{23}Na^+$ lines in such preparations, even before the addition of lasalocid A, might be due to equilibrium leakiness that is fast on the NMR time scale.

The major applications of this technique with regard to model membrane vesicles will probably be two. The first is the study of the inherent and stimulated leakiness of various LUV preparations. The second, and more important, is the study of the mechanisms of protein-mediated transport in reconstituted vesicle preparations. A number of Na^+-transporting proteins have been reconstituted with varying degrees of success: Na^+,K^+-ATPase (22), sodium channels (23), and the acetylcholine receptor (24). Also, important parameters such as the distribution

¶ This and all of the following concentrations were calculated assuming the volume ratio (V_{in}/V_{out}) to be 0.06, the value interpolated for 7.5 mM lipid (4).

of transport protein occupation numbers in vesicles will be easy to ascertain (3). In addition, it seems quite likely that this technique can be extended to the transport of the other physiological metal aquo cations—K^+, Mg^{2+}, and Ca^{2+} (unpublished data). In a separate communication, we shall show the application of this approach to the study of transport in living cells (5).

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