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The monensin-mediated transport of sodium ions through phospholipid bilayers studied by ^{23}Na-NMR spectroscopy

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The monensin-mediated transport of sodium ions through the walls of large unilamellar vesicles of egg phosphatidylcholine was studied using ^{23}Na-NMR and aqueous shift reagents. The transport is dynamic on the NMR timescale and is strictly first order in monensin over the concentration ranges studied indicating that transport occurs by a 1:1 Na^+-ionophore complex. Transport appears to be inhibited by increasing concentrations of Na^+.

Introduction

The transport of materials through cell walls is a process of fundamental biochemical importance [1]. Materials that are transported are known to include amino acids, nucleotides, sugars and ions. In general it is believed that active transport involving enzymes is involved for these substances. For the ions, however, an alternative mode of transport can be established using ionophoric materials. With such transport processes it is believed that the metal ions are transported in the form of their complex with the ionophore [2]. Many such ionophores are active as antibiotics, which property presumably arises from their interference with cation transport.

Whilst complexation/decomplexation reactions of cations with ionophores have been widely studied, very little information is available concerning the transporting abilities of ionophores for ions through membranes. The recent availability of aqueous shift reagents for ^{23}Na- and ^{39}K-NMR based on lanthanide tripolyphosphate (PPP_i) complexes [3–7] and the relative ease with which phospholipid vesicles can now be grown by dialytic detergent removal [8] suggests a most convenient method for such studies. The method involves direct monitoring of the signals of the transported species on either side of the membrane (i.e., in and out of the vesicle). Several groups of workers have reported transport studies using such systems in which the time-course of a transport process has been followed [9,10]. We now report a dynamic NMR study of the transport of sodium ions through phospholipid bilayers mediated by the ionophore monensin.

Monensin is a monocarboxylic polyether antibiotic which shows a particularly high selectivity for sodium over other alkali metal cations [11]. The monensin molecule provides a polar interior for binding the cation and a hydrophobic exterior for solvating in the interior of the bilayer [12,13]. The overall 1 : 1 complex is electrically neutral.

Previous studies of monensin-mediated transport have been reported by several groups, notably by Degani and Elgavish [14] and by Hamilton et al. [15]. Degani and Elgavish used a technique similar to ours except that they used a relaxation agent outside the vesicles to relax the external sodium. An inversion recovery sequence was then used to remove the signal from the more rapidly relaxing Na(out) signal. The transport was shown

to be essentially first order in monensin. Hamilton et al. studied Na^+ uptake in membrane vesicles from SV3T3 (Simian virus 40) cells. They noted that with Na^+ concentrations above approx. 20 mM increasing amounts of monensin inhibited the transport of Na^+. Our results confirm the first but not the second of these reports. Our results do, however, show that increasing concentrations of Na^+ inhibit the ability of monensin to transport Na^+.

Experimental

Large unilamellar vesicles (LUV) were prepared from egg yolk phosphatidylcholine (PC) by the dialytic detergent removal technique introduced by Reynolds and co-workers [8]. In a typical preparation 60 μmol of PC together with 15 equivalents of *n*-octyl-β-glucopyranoside (0.9 mmol) were dissolved in 3 cm^3 200 mM NaCl solution. This solution was then dialysed against 5 litres of 200 mM NaCl solution in a cold room (approx. 277 K). The external solution was changed twice at 10–12 h intervals. This sequence formed the vesicles. A final dialysis was then performed against 5 litres (20 mM Na_5PPP_i/100 mM NaCl). The resulting vesicles thus had 200 mM Na inside and outside, the internal anion was chloride and the external anions were chloride and tripolyphosphate (PPP_i). Since the preparation of the vesicles resulted in equal concentrations of Na^+ on both sides of the membrane the transport experiments involve no net movement of sodium. A slight dilution of the preparation occurred during dialysis and the average concentration of PC per cm^3 of LUV prepared was 18.8 μmol.

1.5 cm^3 of this suspension of vesicles was taken and sufficient of a 1 M solution of $DyCl_3$ to create a 10–12 ppm shift difference was added (approx. 6 μl). The vesicle preparations consistently gave approx. 2.6% of the sodium trapped inside the vesicles measured by integration of the signals. The integrations accounted for the total amount of sodium used thus there was no evidence of 'invisible' sodium. Electron microscopy revealed a uniform spread of vesicle diameters between about 200 and 600 nm.

Most of the transport studies were carried out on a Bruker WP80 FT NMR spectrometer in Stirling operating at 21.19 MHz. The spectrometer was field/frequency locked on the ^2H resonance of ^2H_2O in the inner compartment of a coaxial tube. The spectra were obtained at 303 K and were normally line broadened by 2 Hz to improve the signal-to-noise ratio. Typically 4000 free induction decays were collected into 512 data points with a sweep width of 1500–2000 Hz. The FID was zero filled and transformed in 4k data points. Some spectra were obtained on the WH360 spectrometer in Edinburgh to study the transport by a magnetisation transfer technique. We thank the SERC for an allocation of time on this instrument.

Monensin was used as its sodium salt supplied by Sigma and was recrystallised before being made into a standard solution in methanol. Microlitre amounts of these approx. $2.0 \cdot 10^{-2}$ M solutions were added and the line broadening of the peak due to internal sodium was measured. A typical set of spectra is shown in Fig. 1.

Results and Discussion

As small aliquots of monensin are added to the vesicle preparation both lines in the spectrum broaden. The relative increase in linewidth of the inner resonance is much greater than that for the outer for increasing monensin concentration. The line broadenings are consistent with a dynamic exchange process between the Na(in) and the Na(out) resonances with the site of lower population broadening more rapidly. Mean lifetimes τ for the Na^+ inside the vesicles were obtained both by approximation from the line broadening (Eqn. 1) and by calculation of line shapes [16]. Both methods gave excellent agreement. The rate constants $(1/\tau)$ for the exchange in the direction Na(in) → Na(out) are recorded in Table I.

$$k = \frac{1}{\tau} = \pi \text{ (line broadening)} \quad (1)$$

For every concentration of Na^+ studied (100–250 mM) the rate varies linearly with monensin concentration. This variation indicates a first-order relationship between monensin concentration and transport rate which is consistent with a 1 : 1 complex between the transporting and the transported species. The variation of rate constant with sodium concentration is more puzzling

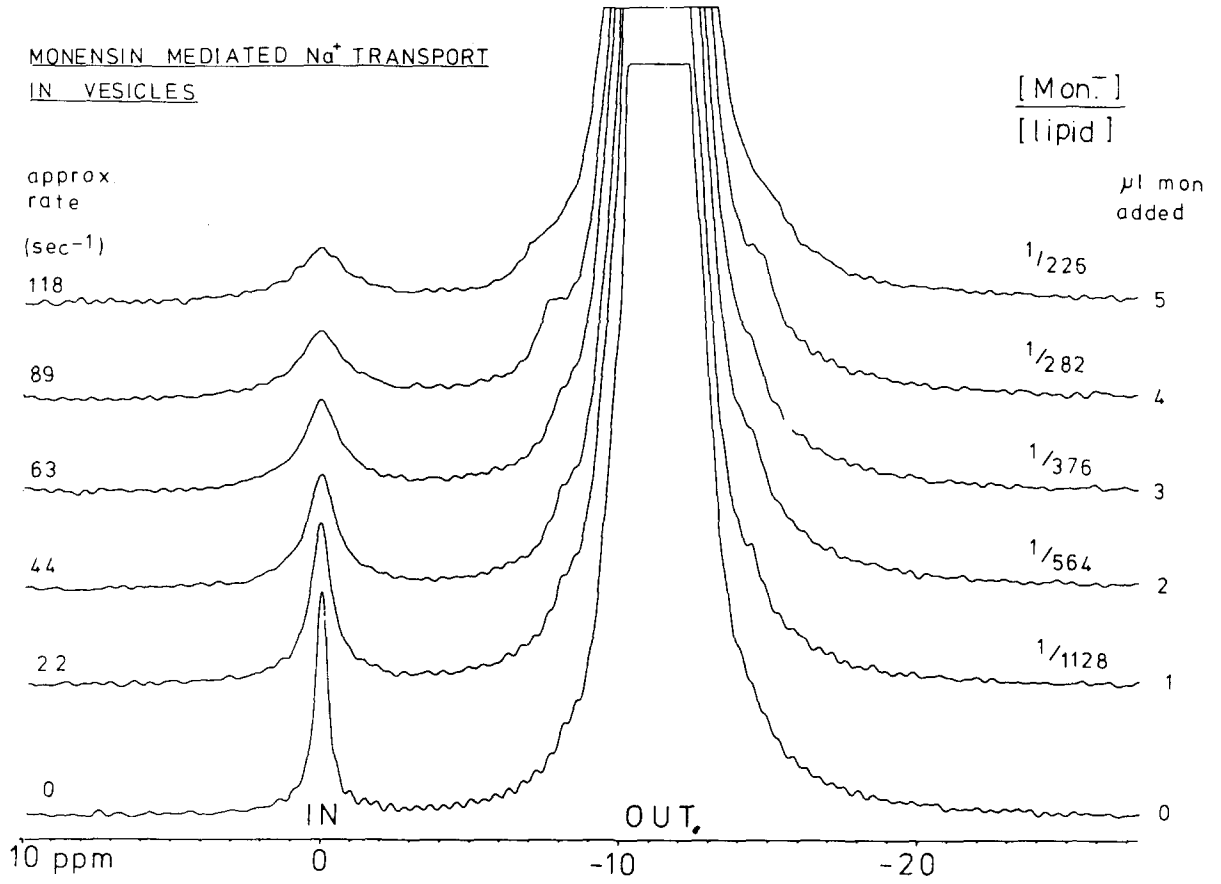

Fig. 1. Observed changes in ^{23}Na-NMR spectra of LUV containing 120 mM NaCl on addition of increasing microlitre amounts of $2.446 \cdot 10^{-2}$M sodium monensin in methanol solution at 303 K. The surrounding medium contains 10 mM Na_5PPP_i, 70 mM NaCl and 4.0 mM $DyCl_3$. PPP_i, tripolyphosphate.

because the rate constants decrease with increasing sodium concentration but appear to level off beyond approx. 200 mM (Fig. 2 and Table II). We currently do not have an explanation for this behaviour.

The exchange that we are studying is in reality a three-site exchange problem with the sites being Na(in), Na(out) and Na(mon) which presumably occupies a site in the membrane. Relaxation from the more rapidly relaxing Na(mon) causing the line broadening has to be ruled out as a cause of the observations. In a typical experiment in which this effect might be expected to be seen we might have approx. $6 \cdot 10^{-8}$ mol of monensin and $4 \cdot 10^{-6}$ mol Na^+(in), i.e. at least 100-times more Na(in) than Na(mon) in the membrane. The linewidth of Na(mon) in methanol solution at the temperature used in this experiment is approx. 300 Hz thus a maximum contribution to the line broadening from this source can be estimated as 3 Hz which is negligible in comparison with observations under the above experimental conditions.

In order to prove further that the line broadening arises in reality from a chemical exchange of nuclear spins across the membrane a magnetisation transfer experiment was set up. In this experiment, conducted on a WP360 spectrometer, the spins of the Na(out) were inverted by a 90°-D_1-90°-τ-90°-FID sequence in which D_1 was selected to lead to a 180° separation of the two signals in the $X'Y'$ plane. In accordance with our expectations selective inversion of the Na(out) peak caused a time-dependent reduction in the signal of the Na(in) peak during the recovery of the Na(out)

TABLE I

MONENSIN-MEDIATED TRANSPORT OF Na^+ IN LARGE UNILAMELLAR VESICLES OF EGG PHOSPHATIDYLCHOLINE

[NaCl] (mM)	μmol Na(mon) per cm^3 of LUV	k (s^{-1})
100	0.0000	0.0
100	0.0086	18.5
100	0.0215	43.0
100	0.0301	69.1
120	0.0000	0.0
120	0.0122	22.0
120	0.0245	44.0
120	0.0367	63.0
120	0.0489	87.0
120	0.0612	118.0
150	0.0000	0.0
150	0.0253	29.0
150	0.0378	43.2
150	0.0504	63.6
150	0.0630	79.3
175	0.0000	0.0
175	0.0131	18.4
175	0.0263	27.7
175	0.0394	43.8
175	0.0525	50.7
175	0.0630	69.1
200	0.0000	0.0
200	0.0126	12.3
200	0.0252	27.4
200	0.0378	39.9
200	0.0504	49.1
200	0.0630	64.5
250	0.0000	0.0
250	0.0118	9.6
250	0.0236	23.0
250	0.0354	32.3
250	0.0473	43.8
250	0.0591	51.5

magnetisation. Rate constants obtained from this experiment are comparable with those obtained from the line broadening. We believe that this experiment proves unambiguously that Na^+ is being transported through the phospholipid membrane as we implied from the line broadening experiments.

It is difficult to compare our results with those of Degani and Elgavish [14]. Firstly, they used '10%' egg PC which presumably means that they had much more phospholipid present than in our experiments. Secondly, they used a different

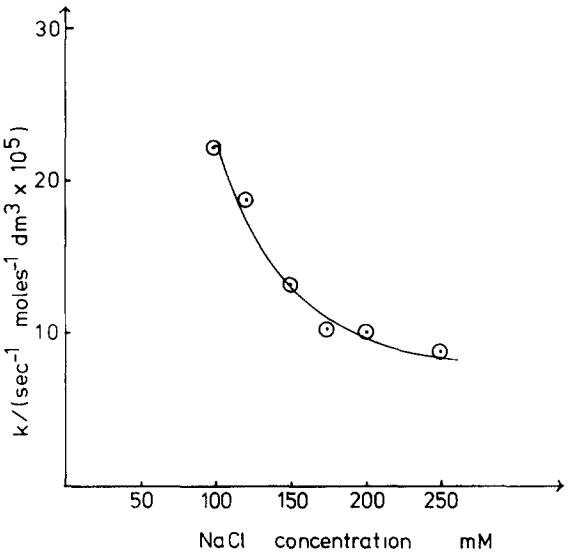

Fig. 2. First-order rate constants for Na^+ transport plotted against concentration of internal NaCl.

method to prepare their vesicles, with the consequence of a great difference in size (radius of approx. 60 Å (6 nm) versus 200 nm). Degani and Elgavish were able to conclude from their results that the "... transport rate increases fairly linearly with ionophore concentration, indicating that the dominant transporting species is a 1 : 1 complex of the sodium ionophore." Our results put this conclusion on much firmer basis.

The results of Hamilton et al. [15] in which they found increasing amounts of monesin to inhibit Na^+ transport above 20 mM Na^+ are more difficult to reconcile with ours. It should be noted, however, that the vesicle preparations they employed were from whole biological membranes

TABLE II

FIRST-ORDER RATE CONSTANTS FOR TRANSPORT AT DIFFERENT CONCENTRATIONS OF NaCl

[NaCl] (mM)	k(s^{-1}·mol^{-1}·dm^3)(×10^5)	Correlation coefficient
100	22.3±1.5	0.995
120	18.9±0.6	0.998
150	12.7±0.5	0.998
175	10.3±0.6	0.992
200	10.1±0.3	0.998
250	8.9±0.3	0.998

incorporating proteins and their vesicles appear to have been set up to be leaky in the first place. Their experiments and results cannot be sensibly compared with ours.

In principle the monesin-mediated transport of sodium through membranes could be studied by following the time dependence of signal intensities if the system were set up with different initial concentrations of Na^+ on either side of the membrane as has been done by other workers [9,10]. In practice this is more difficult than appears at first sight. The rates that we are observing are at least 10^6-times greater than those observable by such experiments. Were an experiment to be set up using concentrations of monesin 10^6 lower than in our experiments it would require the presence of a counterion to equalise the ionic concentrations in order to avoid swelling or even worse bursting of vesicles. As sodium ions were transported from the region of high $[Na^+]$ the counterion would have to be transported in the opposite direction. This counterion transport would then probably become the rate determining step for sodium transport.

Our results, reported above, and the recent successful use of shift reagents and vesicles to study the time course of other transport processes [5] shows the tremendous promise that ^{23}Na- and ^{39}K-NMR hold for enhancing our understanding of this important field. Other ionophoric materials have been investigated, and there is clearly a potential for the use of this technique to investigate the mode of action of membrane transport enzyme systems.

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