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The transport of Li^+, Na^+ and K^+ ions through phospholipid bilayers mediated by the antibiotic M139603 studied by ^{7}Li-, ^{23}Na- and ^{39}K-NMR

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Addition of the ionophoric antibiotic M139603 to preparations of large unilamellar vesicles made from egg-yolk phosphatidylcholine in lithium, sodium or potassium chloride solutions gives rise to dynamic effects in the ^{7}Li-, ^{23}Na- and ^{39}K-NMR spectra. The dynamic spectra arise from M139603-mediated transport of the metal ions through the membrane. The kinetics of the transport are followed as a function of the concentrations of M139603 and the metal ion and are compatible in all cases with a model in which one M139603 molecule transports one metal ion. The transport of metal ions mediated by M139603 is appreciably faster than that of monensin and nigericin. The results show that diffusion of the complex through the membrane cannot be (wholly) rate-limiting for Na^+ and K^+ transport. For Li^+ transport dissociation is the rate-limiting step. The stability constants for complex formation are evaluated. M139603 shows some selectivity in its complexes with the alkali metal ions with the Na^+ complex being the most stable.

Introduction

The ionophoric antibiotics have widespread biological action [1]. In particular, they are efficient mediators of the transport of metal ions and H^+ through the limiting membranes of cells. This property is presumed to be responsible for their biological activity through the dissipation of trans-membrane ion gradients. It is believed that in most cases the transport occurs by the ionophore transporting the metal ion through the membrane in the form of a 1 : 1 metal-ionophore complex.

The discovery of M139603 (Fig. 1), an ionophoric antibiotic from the aerobic fermentation of *Streptomyces longisporoflavus* NCIB 11426, was announced by ICI in 1980 [2]. M139603 is therefore a comparative latecomer amongst this class of compound. Its structure is different from that of other commonly used acidic ionophoric antibiotics in that it possesses a biosynthetically rare acid group i.e. the form of an acyl tetronic acid moiety. Another feature of difference is the lack of an internal hydrogen bond to assist in the coiling of the ionophore round the metal ion. M139603 and several of its derivatives are useful as coccidiostats and as growth promoters in ruminant animals, reducing methane production and increasing the propionate/acetate ratio in the rumen [2,3].

Compared with the extensive literature available on other commercially available ionophoric antibiotics, remarkably little has been published on this material. The structure of M139603 has been determined crystallographically from the 4-bromo-3,5-dinitrobenzoyl derivative of the sodium salt [4]. The sodium ion is six-coordinated through five oxygen atoms from the molecule, two of which come from the acyl tetronic acid, and a water molecule which occupies the sixth position. The sodium atom is at the centre of a very distorted octahedron.

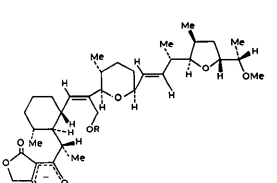

Fig. 1. Structure of the ionophore M139603.

Abbreviation: PC, phosphatidylcholine.

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NMR spectra of the acid and its sodium salt show that the solution conformation is similar to that determined crystallographically in the solid [5]. The same workers measured the binding constants of M139603 to various metal ions finding that the most stable complex with an alkali metal ion was with Na^+, the next most stable with K^+ and that the binding constant to Li^+ was too small to be measured. No information is available, however, on the relative efficacy of M139603 compared to other ionophores in transporting metal ions through biological membranes.

We have previously demonstrated that dynamic NMR techniques can be used to study the ionophore-mediated transport of alkali metal ions through phospholipid bilayers [6–9]. Briefly, the experiments involve the preparation of PC vesicles with equal concentrations of metal ions inside and outside, the establishment of a chemical shift difference by use of an aqueous shift reagent for the metal ions and a dynamic line broadening [7,8] or magnetisation transfer [6,9] experiment to obtain transport rates as the ionophore is added. The dynamic NMR results allow the rates of transport to be measured for various concentrations of ionophore and metal ion. We have also demonstrated [7] that the classical mobile carrier system presented by Painter and Pressman [10] satisfactorily accounts for the observed kinetics. This model is presented in Fig. 2 and has been discussed by us before [7–9].

The rate equation derived from this model [7] (Eqn. 1) predicts that the reciprocal of the rate constant for transport should vary linearly with metal ion concentration.

$$\frac{1}{\tau_{M^+,in}} = \frac{A\,k_{diff}\cdot k_d\cdot[L]_T}{V_{in}(k_d+2k_{diff})([M^+]+k_d/k_f)} \qquad (1)$$

(This equation describes the lifetime, τ, of a metal ion, M^+, inside a vesicle of volume, V_{in}, and surface area, A, with a total ionophore concentration (complexed + uncomplexed) $[L]_T$.)

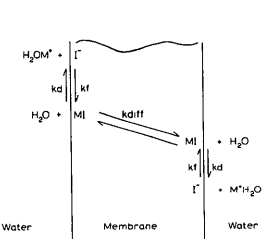

Fig. 2. Mechanism for the ionophore-mediated transport of metal ions through phospholipid membranes. (Formation rate for ionophore metal complex = k_f; dissociation rate for ionophore metal complex = k_d; diffusion rate = k_{diff}.)

The slope and intercept of such a graph are functions of k_f, k_d and k_{diff}. Two limiting cases can be distinguished. If diffusion is not rate-limiting, the slope is proportional to k_d^{-1} and the intercept proportional to k_f^{-1}, allowing these rate constants to be evaluated. Alternatively, if diffusion is the slow rate-limiting step, the slope is proportional to k_{diff}^{-1} and the intercept is proportional to $k_d\cdot k_{diff}^{-1}\cdot k_f^{-1}$.

We now present our results for M139603 transporting lithium, sodium and potassium ions through PC bilayer membranes which are in accord with the model in Fig. 2 and Eqn. 1. The results show that M139603 transports these ions through PC bilayers at rates faster than those of any other ionophore that we have so far reported.

Experimental procedure

Large unilamellar vesicles were prepared from egg-yolk PC by a modification of the dialytic detergent removal technique introduced by Reynolds and co-workers [11] described in our previous papers [7–9]. A typical preparation would have a total of approx. 30 μmol of lipid in 1.5 cm^3 of salt solution. Three dialyses (> 2 litres each) at 40 °C against the chloride of the appropriate metal produced large detergent-free unilamellar vesicles with the same concentrations of metal ion inside and out. Two final dialyses introduced tripolyphosphate into the external medium as described previously [7–9]. Sufficient $DyCl_3$ ($^7Li^+$ and $^{23}Na^+$) or $Tb(NO_3)_3$ ($^{39}K^+$), typically a few microlitres of a 1 M solution (Na^+, K^+) or an 80 mM solution (Li^+) was then added to generate a shift difference of approx. 10 ppm (Na^+, K^+) or 25–45 Hz (Li^+). The sole difference between techniques previously adopted and those described here was the use of 2 cm^3 of vesicle suspension containing 40 μmol of PC for NMR work on ^{39}K transport.

Sodium and potassium transport was studied by dynamic line broadening [7,8]. Lithium transport was studied by magnetisation transfer [6,9]. Transport studies were performed on a Bruker WP80 FT NMR spectrometer in Stirling University at 21.19 MHz (^{23}Na) and 31.14 MHz (^7Li) and on a Bruker AM300 spectrometer in St. Andrews at 14.01 MHz (^{39}K). In all cases the spectrometer was field-frequency locked on the ^2H resonance of ^2H_2O in the inner compartment of a coaxial tube and all spectra were obtained at 303 K. Spectra were line broadened typically by 1 Hz (Li), 2 Hz (Na) or 5 Hz (K) to improve the signal-to-noise ratio. For ^{23}Na and ^{39}K, the spectra recycle times were at least 3 T_1. For the lithium magnetisation transfer experiments, the recycle times were at least 5 T_1.

Addition of small aliquots (microlitre amounts) of a standard solution of M139603 in methanol (0.005–0.01 M) gave rise to dynamic line broadening and magnetisation transfer effects. The results, obtained as described previously, are given in Tables I–III. The ratios of PC to M139603 used in this work vary from approx. 1 : 300 to 1 : 19000. This corresponds to a concentration range of M139603 in the membrane of approx. $7 \cdot 10^{-5}$ to $4 \cdot 10^{-3}$ M.

All lipids were purchased from Lipid Products. We thank Dr. N. Elmore of ICI Pharmaceuticals for a generous gift of M139603.

Results and Discussion

As we have observed previously for both monensin and nigericin, adding small aliquots of M139603 to the vesicle preparation broadened the Na^+ and K^+ lines and gave rise to magnetisation transfer effects for Li^+ consistent with a dynamic exchange process between the M^+_{in} and the M^+_{out} populations. The rates of transport measured for Li^+, Na^+ and K^+ are shown in Tables I–III, where the derived k' values incorporate terms in area and volume of the vesicles and in ionophore-to-lipid ratio. For all three metals at every concentration studied, the transport rate in the direction in → out varies linearly with the M139603 concentration, indicating a first-order relationship between M139603 concentration and the transport rate. The linear relationship between k^{-1} and metal ion concentration (Figs. 3 and 4) is also in accord with our model. Taken together, these results present strong evidence that the transporting species is a 1 : 1 complex between ionophore and M^+.

Our model predicts that if diffusion is the rate-limiting step, the slope in Fig. 3 would be proportional to k^{-1}_{diff}. Since it would be expected that the Na^+ and K^+ complexes would have almost identical molecular volumes and shapes, the diffusion coefficients should be almost identical for both metals. Unlike the cases of monensin and nigericin, the slopes of the Na^+ and K^+ lines for M139603 are identical within experimental error. In addition, the slopes are smaller than those obtained for monensin and nigericin. This suggests either faster dissociation rates for M139603 that are almost identical for both metals, or that diffusion is the rate-limiting step for M139603 transporting Na^+ and K^+. We note that for most studies of ionophores in non-aqueous solvents, dissociation rates almost invariably differ as the metal ion is changed [12], which suggests that diffusion is the rate-limiting step in the current case. However, our recent results indicate that

TABLE I

Rate constants for Li^+ transport in egg PC vesicles

From these results, $k'_f = 5488 \pm 525\ s^{-1}$, $k'_d = 479 \pm 33\ M \cdot s^{-1}$, $K_s = 11.5 \pm 1.9\ M^{-1}$.

$[Li^+]$ (M)	k (mol lipid/mol M139606 per s)
0.025	4279 ± 211
0.050	4111 ± 107
0.075	2728 ± 117
0.100	2402 ± 53
0.150	1964 ± 51
0.200	1730 ± 27

TABLE II

Rate constants for Na^+ transport in egg PC vesicles

From these results, $k'_f = (20.5 \pm 5.0) \cdot 10^4\ s^{-1}$ [a], $k'_d = (0.855 \pm 0.098) \cdot 10^4\ M \cdot s^{-1}$ [a], $K_s = 24.0 \pm 8.2\ M^{-1}$.

$[Na^+]$ (M)	k (10^5 mol lipid/mol M139603 per s)
0.025	1.343 ± 0.153
0.050	0.853 ± 0.030
0.100	0.642 ± 0.176
0.150	0.438 ± 0.089

[a] Calculated on the basis that diffusion is not the rate-limiting step.

TABLE III

Rate constants for K^+ transport in egg PC vesicles

From these results, $k'_f = (5.65 \pm 0.76) \cdot 10^4\ s^{-1}$ [a], $k'_d = (0.804 \pm 0.155) \cdot 10^4\ M \cdot s^{-1}$ [a], $K_s = 7.02 \pm 2.03\ M^{-1}$.

$[K^+]$ (M)	k (10^5 mol lipid/mol M139603 per s)
0.050	0.406 ± 0.046
0.075	0.383 ± 0.014
0.100	0.314 ± 0.025
0.125	0.335 ± 0.019
0.150	0.261 ± 0.025

[a] Calculated on the basis that diffusion is not the rate-limiting step.

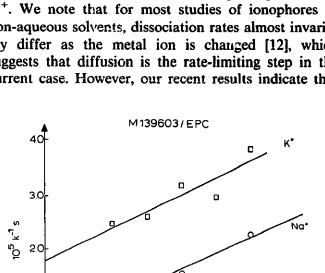

Fig. 3. Variation of the reciprocal of the transport rate with metal ion concentration for the M139603-mediated transport of Na^+ and K^+ ions. EPC, egg PC.

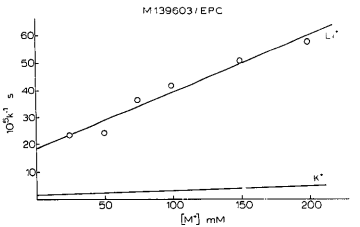

Fig. 4. Variation of the reciprocal of the transport rate with metal ion concentration for the M139603-mediated transport of Li^+ ions. EPC, egg PC.

K^+ is transported faster by the ionophore, salinomycin, than by M139603, with a lower value for the slope of the k^{-1} vs. $[M^+]$ curve ($k'_d = 1.39 \cdot 10^1\ M \cdot s^{-1}$). This indicates that M139603 transport cannot be (wholly) diffusion-controlled.

The values in Tables II–IV for k'_d and k'_f are calculated on the basis that diffusion is not the slow rate-limiting step. If diffusion is rate-limiting, the intercepts on our graphs are given by $k'_d \cdot k'_{diff}/k'_f$ (i.e., $k'_{diff} \cdot K_s^{-1}$) and we are not able to extract k'_f, although the ratio of slope to intercept still gives K_s.

The diffusion rate for the Li^+ complex would be expected to be similar to those of Na^+ and K^+. The 18-fold difference in slope between Na^+/K^+ and Li^+ means that diffusion cannot be rate-limiting for Li^+ transport. Therefore, the graph of k^{-1} vs. $[Li^+]$ allows extraction of the values for the association and dissociation rates of the M139603-Li^+ complex in the membrane surface.

The recognition processes for ionophores are complex with as many as six oxygen atoms in the inner coordination sphere of the metal needing to be exchanged to form or to break down the metal-ionophore complex. During the complexation process for monensin and nigericin (k_f) six water molecules are exchanged for six oxygens of the ionophore and during the release step (k_d) the six oxygens of the ionophore are replaced by six water molecules. These are almost certainly sequential reactions, i.e., the steps designated by k_f and k_d have probably six or more stages each. These steps will not all occur with the same rate. For the dissociation process it seems likely that replacement of one of the ionophore oxygens with a water molecule would be the actual slow step. Unlike monensin or nigericin, M139603 carries one molecule of water with it through the membrane and therefore has fewer stages to go through in the formation and dissociation reactions.

A comparison of the derived rates for molecular recognition and apparent stability constants at the membrane surfaces for M139603 (assuming that diffusion is not rate-limiting), monensin and nigericin is given in Table IV. For all three alkali metals the transport rates of M139603 are greater, with the recognition rates typically being at least 3-times larger (with the exception of k_f for K^+/nigericin), despite the stability constants being lower than those for monensin. Whatever the nature of the rate-limiting step, our results show that the dissociation rate for M139603 is greater than that for monensin and nigericin. Two possible explanations can be advanced for this faster rate for M139603.

For both monensin and nigericin the known structures of the metal complexes include a hydrogen bond from the carboxylic acid group to a hydroxyl group at the other end of the ligand. There is no such hydrogen bond in M139603. In addition, the sodium complex of M139603 and, by inference, also the lithium and potassium complexes possess a water molecule bound to the central metal ion [4]. The lack of a hydrogen bond and the presence throughout of one molecule of water bound to the metal ion will lead to fewer steps in the proceses defined by k_f and k_d. The lower number of steps could in turn lower the activation energies for the recognition processes thus accelerating them.

The second and more likely explanation relates to the severe distortion of the octahedral ligand distribution around the central sodium ion in the structure of the M139603 sodium complex. [4] This distortion will lead to strain in the complex, which will be reflected in a higher dissociation rate and lower stability constant.

Irrespective of which process is rate-limiting, the ratio of slope-to-intercept gives the stability constant for the metal-ionophore complex in the membrane

TABLE IV

Comparison of rate and stability constants for monensin, nigericicin and M139603 in egg PC vesicles

n.d., not determined (values were too slow to be measured). Units: k'_f, s^{-1}; k'_d, $M \cdot s^{-1}$; K_s, M^{-1}.

	Li^+			Na^+			K^+		
	$10^{-4}\,k'_f$	$10^{-4}\,k'_d$	K_s	$10^{-4}\,k'_f$	$10^{-4}\,k'_d$	K_s	$10^{-4}\,k'_f$	$10^{-4}\,k'_d$	K_s
M139603	0.549	0.0479	11.5	20.52	0.855	24.0	5.65	0.804	7.03
Monensin	0.103	0.00558	18.5	4.878	0.150	32.6	2.30	0.433	5.3
Nigericin	n.d.	n.d.	n.d.	7.638	0.352	22	9.61	0.0997	96

surface. The stability constants derived from our work show that the Na^+ complex of M139603 is more stable than those of Li^+ and K^+ but, interestingly, we find the Li^+ complex to be more stable than the K^+ complex. This is in contrast to the results obtained by Grandjean and Laszlo, using methanol water as solvent, who reported that the stability constant of the Li^+ complex was too small to be detected [5]. This discrepancy points to the problems in inferring behaviour in biological membranes from results in other solvent systems.

The stability constant for the K^+ complex of M139603 ($7.02 \pm 2.03\ M^{-1}$) is amongst the lowest we have yet measured using our NMR techniques. Using this stability constant, one can calculate that at 50 mM K^+, only 26% of the added ionophore is bound in its K^+ complex. Despite this low binding, the efficient transport of K^+ still occurs, because of the rapid association, dissociation and diffusion reactions, However, in competition with Na^+ or Li^+, one would expect K^+ transport to be greatly reduced by the greater stability of the other metal ion complexes which would preferentially utilise the ionophore.

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