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Registry No. C, 7440-44-0; $KNi[Fe(CN)_6]$, 53295-14-0; $K_2Ni[Fe(CN)_6]$, 13601-16-6; $In[Fe(CN)_6]$, 15418-33-4; $KIn[Fe(CN)_6]$, 55742-38-6; $K_3[Fe(CN)_6]$, 13746-66-2; $K_4[Fe(CN)_6]$, 13943-58-3; $NiCl_2$, 7718-54-9; $InCl_3$, 10025-82-8; KCl, 7447-40-7; K, 7440-09-7.

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Nigericin-Mediated Transport of Cesium Ions through Phospholipid Bilayers Studied by a ^{133}Cs Magnetization-Transfer NMR Technique

The use of ^7Li, ^{23}Na, and ^{39}K NMR spectroscopy to study the transport of alkali-metal ions through the limiting membranes of cells[1-6] or through model phospholipid bilayers[7-11] is becoming an established technique. We now report that ^{133}Cs NMR spectroscopy may be used to study the nigericin-mediated transport of Cs^+ ions through phosphatidylcholine bilayers by use of a magnetization-transfer technique in a process shown to be first order in nigericin. The long relaxation time of the ^{133}Cs nucleus allows extremely slow exchange rates (relative to those measurable by other dynamic NMR techniques) to be measured.

The NMR methods for the study of membrane transport of alkali-metal ions through model biological membranes that have been developed involve a compartmentalized system of cells (e.g. erythrocytes) or of vesicles and employ a shift reagent[12] or a relaxation agent[13] to contrast the signals from the intracompartmental and extracompartmental metal ions. For slow exchange rates the time course of signal intensities can be followed. For rapid exchange dynamic NMR line-broadening effects are observed. Magnetization transfer offers a third option for intermediate exchange rates that has so far not been fully utilized.[11]

The magnetization-transfer technique for a two-site case involves placing a magnetic label at one site by inverting the spin populations (inverted signal) and then following the signal intensity at the other site as the inverted signal relaxes back. If there is chemical exchange on the time scale of the relaxation process, a reduction in intensity of the monitored signal will be observed.[11,14,15]

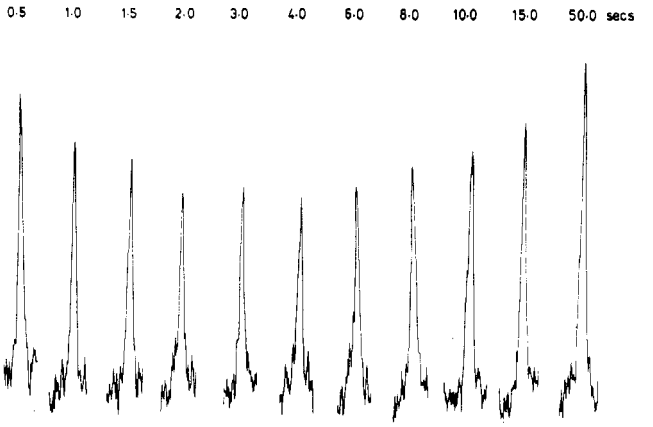

Figure 1. Typical variation of the intensity of the Cs^+(in) peak vs delay time (τ) for 25 mM Cs^+ and nigericin. Conditions are as in Table I with rate $k(\text{in} \rightarrow \text{out}) = 0.13\ s^{-1}$. The reduction in signal intensity arises from transport of inverted magnetization from the Cs^+(out) signal. The recovery arises from normal relaxation processes. The transport rate constant is obtained from the variation in signal intensity with time.

The magnetization-transfer technique is most useful in the region where the transfer rates are similar to or somewhat slower than the relaxation rates, i.e. in the slow exchange limit just before measurable line broadening begins. We have previously used ^7Li magnetization transfer to study the membrane transport of lithium mediated by the ionophores monensin[9,11] and M139603,[10] and it has also been used by Shungu and Briggs for ^{23}Na transport studies.[15] We now report that a similar technique can be applied to ^{133}Cs transport. To demonstrate this, we chose to examine transport mediated by nigericin, an ionophore known to favor the medium to large alkali-metal ions, whose maximum selectivity is for K^+ and which should therefore transport Cs^+ but with a much reduced efficiency.

^{133}Cs NMR spectroscopy has been used by Davis, Murphy, and London to study the uptake of Cs^+ ions into human erythrocytes and into perfused rat hearts.[16] These workers observed a chemical shift difference between the intra- and extracellular $^{133}Cs^+$ resonances without the addition of shift reagent. In our vesicular systems there was no naturally occurring shift difference. It is, however, known that ^{133}Cs chemical shifts are much more anion dependent than those of the other alkali metals, and we found that the resonance of $^{133}Cs^+$ is shifted appreciably by the presence of linear tripolyphosphate (PPPi) ions. The differences observed in cellular systems may well, therefore, arise from different anionic compositions between the intra- and extracellular fluids.

Vesicles were prepared by the dialytic detergent removal technique.[7-11] Typically egg yolk phosphatidylcholine (PC; 40 μmol) was dissolved in an aqueous 25 mM CsCl solution (2.0 mL) containing *n*-octyl glucopyranoside (ca. 600 μmol). Three dialyses against aqueous CsCl produced a suspension of large detergent-free unilamellar vesicles with the same concentration of Cs^+ inside and out. The internal volume was ca. 11.5% of the total volume. A final dialysis was performed against aqueous Cs_5PPPi ($[Cs^+]$ equimolar to earlier solution) with the ionic balance made up by choline chloride. This medium itself generated a chemical shift difference between the "in" and "out" ^{133}Cs peaks by moving the out signal to higher frequency. No paramagnetic lanthanide was added as in previously reported experiments with other alkali metals.

The pulse sequence $90°-t1-90°-\tau-90°$–FID was then employed with the transmitter set *d* Hz to low frequency of the Cs^+(in) signal

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0020-1669/90/1329-2397$02.50/0

Table I. Rate Constants (k) for Transport of Cs^+ as a Function of the Nigericin:PC Ratio in the Lipid Bilayer[a,b]

$10^3 \times$ nigericin:PC ratio	k(in → out)/s^{-1}
1.071	0.030
2.145	0.055
2.681	0.101
3.217	0.130
4.021	0.160

[a] These data give a first-order rate constant of 46.9 ± 3.3 mol of lipid·(mol of nigericin)$^{-1}$·s^{-1} (correlation coefficient 0.9807). [b] Conditions: 25 mM Cs^+; vesicles prepared from 40 × 10^{-6} mol egg yolk PC at 313 K; spectra run at 303 K on a Bruker AM300 spectrometer at 39.37 MHz. Nigericin:PC ratios vary between 1:934 and 1:249.

(where d = the chemical shift difference between the two sites and $t1 = 1/(2d)$. The first two pulses of this sequence specifically invert the Cs^+(out) magnetization. The variable delay then allows chemical exchange and relaxation to occur. This sequence is preferable to the DANTE sequence[15] when only two signals are involved, as it is over in a much shorter period with less loss of inverted magnetization. The relaxation times at each site were determined separately before the addition of any ionophore and were typically ca. 11.5 s (in) and 3.7 s (out). The Cs(out) relaxation time is ca. 5 times the Li(out) relaxation time observed in our $^7Li^+$ magnetization-transfer experiments[9,11] and allows rates that are slower than those for $^7Li^+$ transport to be measured.

The equations describing the behavior of two exchanging sites in a magnetization-transfer experiment have been derived by Morris and Freeman.[17] These equations were used in a least-squares program that allowed best fit values for the exchange rate to be calculated. Nigericin-mediated exchange rates for ^{133}Cs that we have measured by this technique vary between 0.02 and 0.3 s^{-1}. These values are about 1 order of magnitude less than the values we obtained for $^7Li^+$ transport and are probably the slowest rates yet measured by a dynamic NMR technique.

A typical set of intensity vs time measurements for the Cs^+(in) peak is shown in Figure 1, and a typical set of rate data is given in Table I. As in the case of Na^+ and K^+, the exchange is observed to be first order in nigericin, suggesting that one nigericin molecule transports one cesium ion. Confirmation of this depends upon a study of the dependence of rate on metal ion concentration.

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Investigation of Copper-Zinc Superoxide Dismutase Ser-137 and Ala-137 Mutants

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Ser- and Ala-137 mutants of human copper-zinc superoxide dismutase (Cu,ZnSOD) have been thoroughly characterized in an attempt to understand the subtle effect of the nature of the residue at position 137 on the structure of the copper site and on the activity profile. The results show that the nature of the residue at position 137 determines the presence of water in the active cavity as monitored through water ^1H nuclear magnetic relaxation dispersion. Also, the hyperfine shifts experienced by the protons of His-48 in the Cu_2Co_2 derivative are sensitive to the group at the 137 position. These effects are not detected through electronic and EPR spectroscopies. The activity profiles of Ser-137 and Ala-137 mutants are virtually identical and are very close to that of the Ile-137 mutant at pH < 10. The drop in activity above pH 10 closely parallels that observed in the wild type, at variance with the Ile-137 mutant that shows a marked decrease in activity already below pH 10. The activity profiles definitely show evidence of a pK_a between 6 and 7. The affinity of N_3^- for the Ser-137 and Ala-137 mutants is very similar to that for the wild type. This is again at variance with the Ile-137 derivative that shows an N_3^- affinity twice as large.

Introduction

Copper-zinc superoxide dismutase (SOD, hereafter) is a dimeric Cu,Zn system that is extraordinarily efficient as a catalyst for dismutation of superoxide.[1-8] This is accounted for by both the electrochemical potential of the pair Cu^{2+}/Cu^+, which is intermediate between the potentials of the pairs O_2/O_2^- and O_2^-/O_2^{2-},[8-10] and the residues at the entrance of the catalytic cavity that increase the affinity for the substrate.[8,11-14] Arg-143 (numbering of the human isoenzyme) is close to the copper ion[11-13] (Figure 1) and probably assists the substrate in entering the cavity;[8,11-13,15-18] such a role was proposed on the basis of chemical modification experiments[19,20] and recently demonstrated through site-directed mutagenesis.[21-23] Opposite Arg-143 is a Thr residue (Thr-137) (Figure 1) whose terminal OH is thought to form a

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