Chloride Transport through Model Biological Membranes studied by ^{35}Cl NMR

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A method is presented by which chloride transport through the walls of phosphatidylcholine vesicles can be measured using ^{35}Cl NMR and it is used to show that tetrabutylammonium ions mediate chloride transport *via* an ion pair but that potassium valinomycin mediated chloride transport as an ion pair is negligible.

The use of alkali metal NMR 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 a well established technique. There is an equally important field of anion transport through biological membranes[12] to which NMR techniques should be able to make a significant contribution but where virtually no work has been reported other than on erythrocyte suspensions.[5,13] The importance of chloride transport processes has been emphasised recently by the sequencing of the gene responsible for cystic fibrosis which is believed to lead to abnormalities in chloride transport.[14] We now report a general method by which ^{35}Cl NMR can be used to study chloride ion transport through biological membranes. Its utility is demonstrated in work on chloride transport through phosphatidylcholine bilayers *via* ion pairing with the tetrabutylammonium ion. In contrast, valinomycin, which has been suggested to co-transport chloride as an ion pair when potassium is transported, has a negligible transport rate for chloride under our conditions.

The NMR methods that have been developed for the study of the membrane transport of alkali metal ions involve a compartmentalised system of cells (*e.g.* erythrocytes) or of vesicles and employ a shift reagent[15] or a relaxation agent[16] to contrast the signals from the intra- and extra-compartmental metal ions. Rather than attempt to develop a shift reagent for ^{35}Cl we decided to attempt to develop a relaxation agent to differentiate the 'in' and the 'out' signals.

Chloride is very efficiently relaxed by Mn^{2+} ions. However, as with other divalent metal ions, Mn^{2+} is also very efficient at promoting bursting and fusion of phospholipid vesicles.[17–20] The half life of vesicles made by a dialytic detergent removal technique,[7] subject to sufficient Mn^{2+} to broaden the ^{35}Cl(out) line to *ca.* 1000 Hz, was a few minutes at 303 K. We reasoned that the line broadening was due to transient binding between the Mn^{2+} and the Cl^- ions causing paramagnetic relaxation of ^{35}Cl, whilst at the same time interaction of co-ordination sites on the Mn^{2+} with the bilayer surface caused vesicle destruction. We therefore searched for ligands

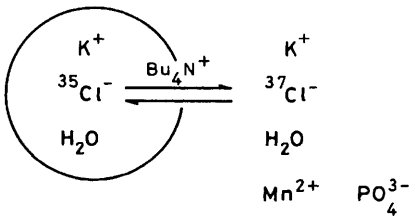

Figure 1. Experimental set-up to demonstrate mediated chloride transport.

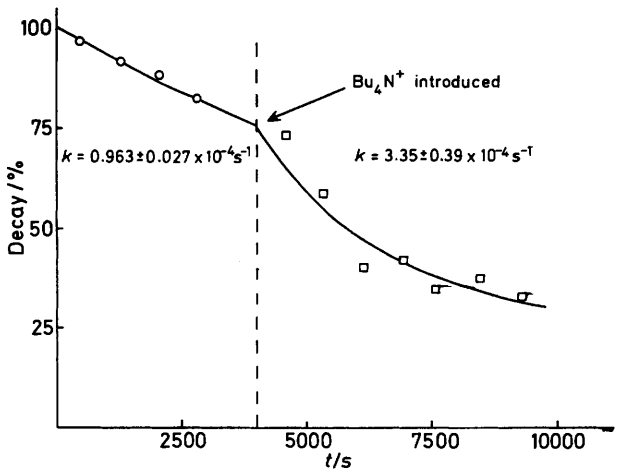

Figure 2. Decay of the $^{35}Cl^-$(in) signal as a function of time at 303 K before and after the introduction of tetrabutylammonium ions. Concentrations (mM) immediately after addition of Bu_4N^+ are: PC 18.72; K^+ 50(in), 50 (out); Na^+ 56.2 (out); Cl^- 50(in), 52.5 (out); PO_4^{3-} 20 (out); Mn^{2-} 2.5 (out); Bu_4N^+ 1.25; Br^- 1.25.

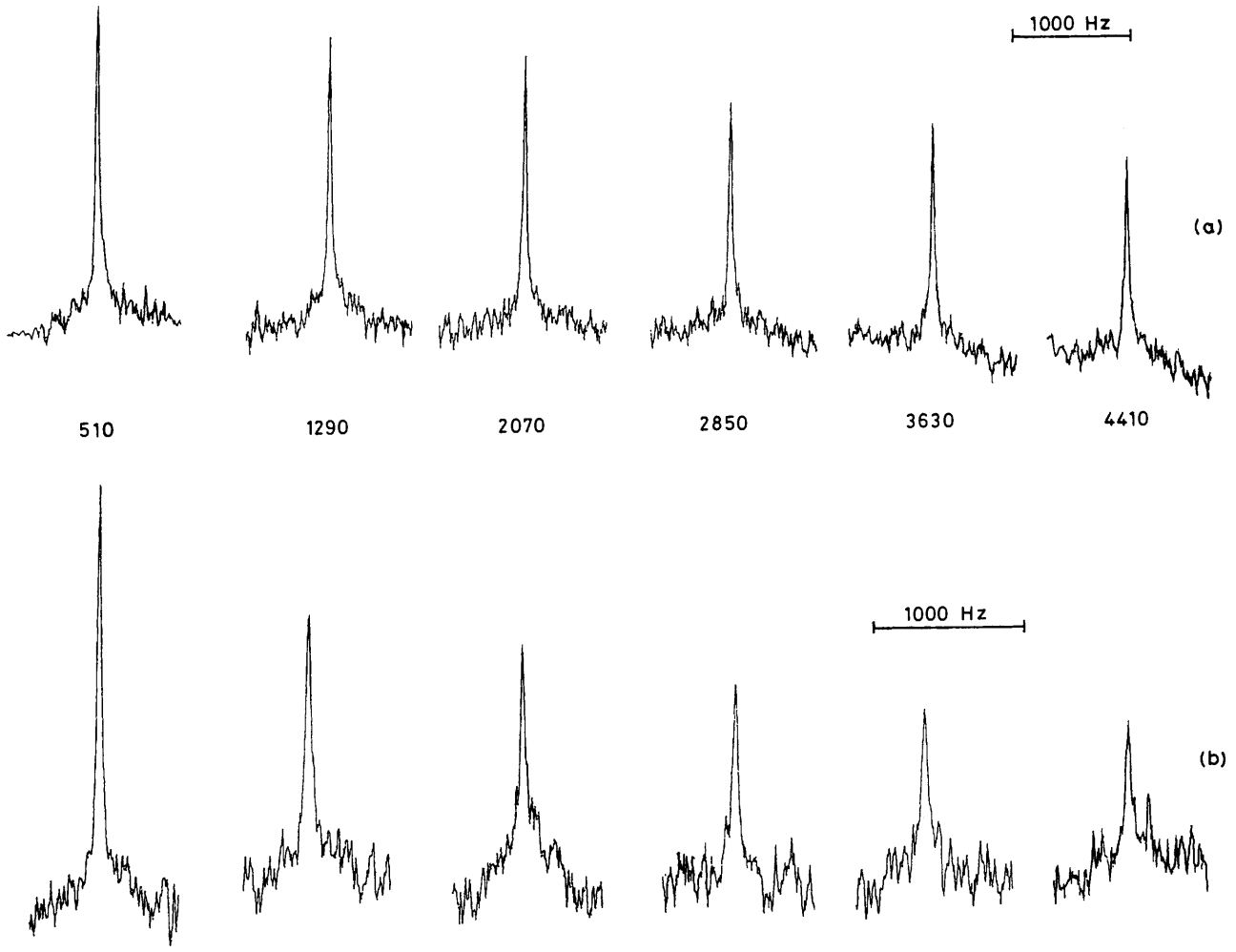

Figure 3. Experimental ^{35}Cl spectra at 29.41 MHz showing the more rapid decay of the $^{35}Cl^-$(in) signal in the presence of tetrabutylammonium ions using NaCl as the electrolyte, otherwise conditions are as in Figure 2: (a) no Bu_4N^+ present, (b) Bu_4N^+ present. Each spectrum is from 6000 free induction decays.

that might be strongly bound to some of the co-ordination sites on Mn^{2+} leaving some of the others still open to interaction with chloride but not with the membrane. If such a ligand could be found it should allow line broadening without causing destruction of the vesicles. Polydentate ligands known to form relatively strong complexes with Mn^{2+} in aqueous solution that we examined, but that proved ineffective for our purposes included iminodiacetic acid, nitrilotriacetic acid, and kojic acid.

Eventually we found that the monodentate ligand phosphate increased the life of our vesicle systems whilst still allowing effective relaxation of the ^{35}Cl(out). The lifetime of our vesicles increased as the PO_4 : Mn ratio was increased. To effect relaxation of the ^{35}Cl(out) we then added a solution of $MnCl_2$, containing eight phosphates per Mn^{2+}, to our vesicle suspensions. This gives half lives for egg yolk phosphatidylcholine vesicles of several hours, long enough to allow chemical kinetics to be measured whilst still allowing the 'out' line to be broadened to *ca.* 1000 Hz. Subtraction of a reference spectrum containing only the broadened line from the spectrum of our vesicles allows the 'in' line to be examined and integrated. The amount of ^{35}Cl(in) can thus be followed as a function of time.†

The following experimental procedure was adopted in preliminary experiments on chloride transport. Vesicles were grown in aqueous KCl solution (50 mM) as previously described. Prior to the transport measurements, two dialyses (*ca.* 0.75 h each) were performed against labelled $K^{37}Cl$ (50 mM). After these dialyses the vesicles were substantially all $^{35}Cl^-$(in) and $^{37}Cl^-$(out). Sufficient of our Mn/PO_4 reagent was added to relax any external ^{35}Cl to a line width of *ca.* 1000 Hz. The ^{35}Cl spectrum at this point consisted of a relatively sharp peak ($w_{1/2}$ *ca.* 20 Hz) for the ^{35}Cl(in) superimposed on a very broad peak for the residual ^{35}Cl(out). The vesicles were

† *Note added in proof*: the previous two paragraphs describe the reasoning that led us to the method we employ. We have since found (F. G. Riddell and S. J. Tompsett, unpublished results) that if the vesicle suspension is adjusted to pH 4 (the pH after addition of Mn/PO_4) without phosphate being present, similar stability of vesicles is observed. The stabilisation of the vesicles is, therefore, an effect due to pH not to the presence of phosphate.

then monitored for a period of *ca.* 1 h whilst the decay of the ^{35}Cl(in) signal, due to passive chloride exchange, vesicles bursting or vesicles fusing, was followed. A small quantity of an aqueous solution of tetrabutylammonium bromide was then added and the intensity of the ^{35}Cl(in) peak was followed. The chemical situation at this point is summarised in Figure 1 and chemical compositions are as in the legend to Figure 2. Under these conditions the ^{35}Cl(in) peak decayed at *ca.* 3.5 times the rate before (Figure 2). Similar experiments using NaCl (50 mM) in which both processes were followed with separate sets of vesicles produced similar transport rates, although passive exchange appeared to be more rapid than with KCl (Figure 3).

The loss of the 'in' signal on addition of tetrabutylammonium bromide cannot be due to vesicles opening and closing since there is no substantial broadening of the ^{35}Cl(in) line that would be expected should this process occur and allow ingress of Mn^{2+}. Neither can the decay be due substantially to destruction of the vesicles since the decay is exponential to an expected infinity value of *ca.* 25% of the original intensity. Even more conclusively, when the experiment was repeated and followed by ^{37}Cl NMR (at 24.50 MHz), the ^{37}Cl(in) peak increased in intensity after the addition of tetrabutylammonium ions at a rate comparable to the decrease of the ^{35}Cl(in) peak in the former experiment. We conclude, therefore, that tetrabutylammonium ions are acting here in their known role as phase transfer agents[21] and carrying chloride into and across the membrane.

Similar experiments were performed with valinomycin as carrier since it has been postulated that when valinomycin transports a potassium ion across a membrane, it carries a chloride with it as an ion pair.[22] The rate of loss of the 'in' signal was the same in the presence of valinomycin as in its absence within experimental error. In a separate experiment with the same concentration of valinomycin we confirmed that K^+ transport was occurring at a rate many times that observed for loss of ^{35}Cl(in). With an identical vesicle preparation and ionic concentrations, the total exchange of K^+(in) with Rb^+(out) was complete in less than 7 min (the time resolution in our experiment).

We conclude that ^{35}Cl NMR can be employed as a means of following chloride transport processes in biological membranes using the protocols that we have developed and are developing, that tetrabutylammonium ions mediate the transport of chloride ions through biological membranes as ion pairs, but that under our conditions chloride transport mediated by the K^+ valinomycin complex as an ion pair is negligible and much slower than K^+ transport.

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