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Co^{2+} as a Shift Reagent for ^{35}Cl NMR of Chloride with Vesicles and Cells†

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ABSTRACT: Applications of high-resolution ^{35}Cl NMR to the study of chloride in vivo and in vesicles have hitherto been limited by problems of NMR detectability and of resolving internal from external signals. We have characterized the effects of Co^{2+} on the ^{35}Cl resonance of Cl^- in solution and have shown that when added to suspensions of lipid vesicles, Co^{2+} shifts the ^{35}Cl signal of the extravesicular Cl^-, allowing clear resolution and quantitation of two peaks. We have assigned these signals to chloride inside and outside the vesicles. The spectra do not change over a 90-min period, demonstrating the stability of the vesicles in the presence of Co^{2+}. This technique is shown to be applicable to red blood cell ghosts, where intravesicular and extravesicular chloride signals were separated and measured and chloride/sulfate exchange through the band 3 anion transport protein A was followed. In two plant species (an alga and a higher plant), an intracellular Cl^- signal can be observed and resolved from the extracellular signal. The intracellular transportable chloride was found to be fully NMR-visible (±5%) in the algal cells. The high steady-state levels of Cl^- seen in the alga were consistent with previous work using $^{36}Cl^-$ labeling on a related species [Doblinger, R., & Tromballa, H. W. (1982) *Planta 156*, 10-15]. Successive spectra acquired after adding Co^{2+} to *Chlorella* cells under deenergizing conditions allow us to follow the time course of movement of Cl^- out of the cells.

High-resolution ^{35}Cl NMR has been used in inorganic chemical studies of the interaction of Cl^- with metal ions and complexes in solution [reviewed by Lindman and Forsén (1976)] and in many investigations of chloride binding to proteins, both in solution [reviewed by Forsén and Lindman (1981)] and with integral membrane proteins (Baianu et al., 1984; Shami et al., 1977; Falke et al., 1984a,b; Shachar-Hill et al., 1989). However, the application of ^{35}Cl NMR to studying chloride in vivo has been restricted by two problems: the superposition of the signals from intra- and extracellular chloride and the fact that in all cells studied to date intracellular signals are too broad to quantify. This has been found in human red blood cells (Falke et al., 1984b; Brauer et al., 1985; Hoffman & Gupta, 1987), rat proximal tubules (Hoffman et al., 1987), and *Escherichia coli* (this study). This broadening of intracellular signals is due to the very rapid quadrupolar relaxation of the ^{35}Cl nucleus when its motion is restricted by binding to a protein or other slowly tumbling species where electric field gradients at the ^{35}Cl nucleus are

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not rapidly averaged to zero. For a fuller discussion of the NMR visibility of quadrupolar nuclei in vivo, see the review by Springer (1987).

Employing this NMR "invisibility" of intracellular chloride, ^{35}Cl NMR has been used to monitor Cl^- transport in erythrocytes where all the signal is extracellular (Brauer et al., 1985) and as a marker of extracellular volume in assessing intracellular volume in cell suspensions (Hoffman & Gupta, 1987; Hoffman et al., 1987). Here we report that intracellular ^{35}Cl in cells of two plant species is detectable and gives sharp signals. Further, these intracellular signals can be resolved from extracellular chloride signals by the addition of Co^{2+} to cell suspensions. We have used this approach to estimate intracellular chloride levels and to follow the time course of chloride efflux from *Chlorella* cells under deenergizing conditions. Our results are similar to those obtained with another *Chlorella* species (Doblinger & Tromballa, 1982) using $^{36}Cl^-$ labeling.

We have further assessed the potential of Co^{2+} as a shift reagent (SR)[1] for ^{35}Cl NMR in vesicle suspensions and show that when added to suspensions of lipid vesicles it shifts the ^{35}Cl signal of the extravesicular chloride, allowing clear resolution and quantitation of the signals from intravesicular and extravesicular chloride. The vesicles are stable in the presence of Co^{2+}. In lysed and resealed erythrocytes lacking hemoglobin (ghosts), chloride levels can be measured and transport followed, demonstrating the potential for studying chloride transport processes in vesicle suspensions by NMR.

Materials and Methods

^{35}Cl NMR. All spectra were taken on a Brücker Instruments 360-AM spectrometer operating at 35.3 MHz for the ^{35}Cl resonance. A 20-mm broad-band probe and a broad-band preamplifier were used. Ninety-degree pulses (60–80 ms) were used with acquisition times of 51–102 ms and a sweep width of 10000 Hz. Details for each spectrum are given in the figure legends. Processing of data was carried out both with spectrometer software and with the "NMR1" program (Syracuse University NMR center). Only exponential multiplication (1–5-Hz line broadening) was used before Fourier transformation and line-fitting, and integration of peaks was used for quantitation. T_1 and T_2 measurements were made using standard inversion recovery and spin-echo pulse sequences.

Vesicle Preparation. Lipid vesicle suspensions with large trapped volumes were prepared by a modification of the ether injection method (Deamer & Bangham, 1976; Alger & Prestegard, 1979); 150 mg of L-α-phosphatidylcholine (type V-EA from Sigma) was dissolved in 50 mL of USP-grade diethyl ether (Malinckroft Inc.) and injected at a rate of 1.5 mL/h into 5 mL of 40 mM KCl kept at 65 °C. The suspension was then dialyzed at room temperature for an additional 2 days to eliminate residual ether.

Ghost Preparation. Human erythrocyte ghosts with very low hemoglobin content were prepared by osmotic lysis, washing, and resealing according to the method of Bodeman and Passow (1972). The method was modified by increasing the volume in which the red cells were lysed to 140 times the volume of cells used; also, the membranes were washed once in the resealing solution at 0 °C before resealing at 37 °C. This procedure produces "white" ghosts of very low hemoglobin content. For NMR measurements of sulfate/chloride exchange, the ghosts were washed and resealed in a solution containing 100 mM NaCl, 5 mM $MgCl_2$, 10 mM MOPS, and 0.1 mM TRIS–EDTA at pH 7.5 and were pelleted and resuspended in a solution containing 5 mM $MgSO_4$, 10 mM MOPS, 40 mM Na_2SO_4, and 10 mM $CoCl_2$ just before NMR measurement was begun. For inhibition of anion exchange, 50 μM DIDS was included in the resealing solution.

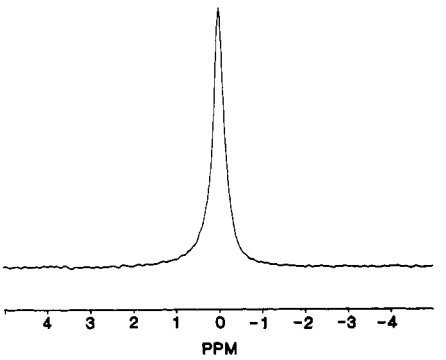

FIGURE 1: ^{35}Cl spectrum of 0.3 mL of 100 mM NaCl. The line is Lorentzian with a width at half-height of 10 Hz (=0.28 ppm).

Escherichia coli Samples. E. coli (strain MRE 600) were grown, harvested, and prepared for NMR as described by Castle et al. (1986). The cells were resuspended to high density in growth medium and aerated by bubbling in the magnet.

Chlorella Samples. Chlorella pyrenoidosa [strain 252 from the algal collection at the University of Texas at Austin (Starr & Zeikus, 1987)] was grown at 23 °C under cool white fluorescent lighting with aeration in Gorham's culture medium 11 (Hughes et al., 1958) with the omission of the A4 salts (paramagnetics) and with half the concentration of phosphate. This medium contains 2 mM chloride when fresh. These modifications of growth medium did not affect growth rates. Stock cultures were maintained on plates (growth medium solidified with 1.5% agar), and each growth was started by inoculation from plates into 150 mL of medium in 250-mL Erlenmeyer flasks and grown to saturation (3–4 days) before transfer to 4 L of medium in 8-L flasks. Growth to mid- or late-log phase (where the cells were still vacuolated) took a further 3–4 days. For harvest, 3 L of culture was cooled on ice to 5 °C with aeration and then centrifuged for 15 min to a soft pellet which was then resuspended to 12 mL for NMR experiments. To induce chloride efflux, the sample was kept at ~40 °C without oxygenation in the magnet.

Higher Plant Cells. Cells of tobacco (*Nicotiana tobbaccum*) grown from isolated protoplasts were the kind gift of Dr. Neal McHale of the Connecticut Agricultural Experiment Station. The cells were grown heterotrophically in liquid culture on a defined medium in petri dishes for a total of 12 days after protoplast isolation at which point they had regenerated cell walls and undergone 2–3 divisions to produce clumps having several cells each. For sample preparation, the cells were centrifuged and resuspended in growth medium. The sample was aerated throughout acquisition of NMR spectra. Cell size and count measurements were made by light microscopy for estimates of intracellular volumes.

Results and Discussion

The $^{35}Cl^-$ Signal. ^{35}Cl is a spin $^3/_2$ nucleus with a gyromagnetic ratio (γ) of 2.621 and a quadrupole moment of −0.08; it has a natural abundance of 75.77%. Figure 1 is a spectrum of 0.3 mL of 100 mM NaCl. The spectrum can be fitted to a Lorentzian line shape with a width at half-height of 10 Hz (=0.28 ppm). The measured spin–lattice relaxation time (T_1) of the same sample of 41 ms is consistent with values previously reported, reviewed by Lindman and Forsén (1976).

[1] Abbreviations: S/N, signal-to-noise ratio; SR, shift reagent; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

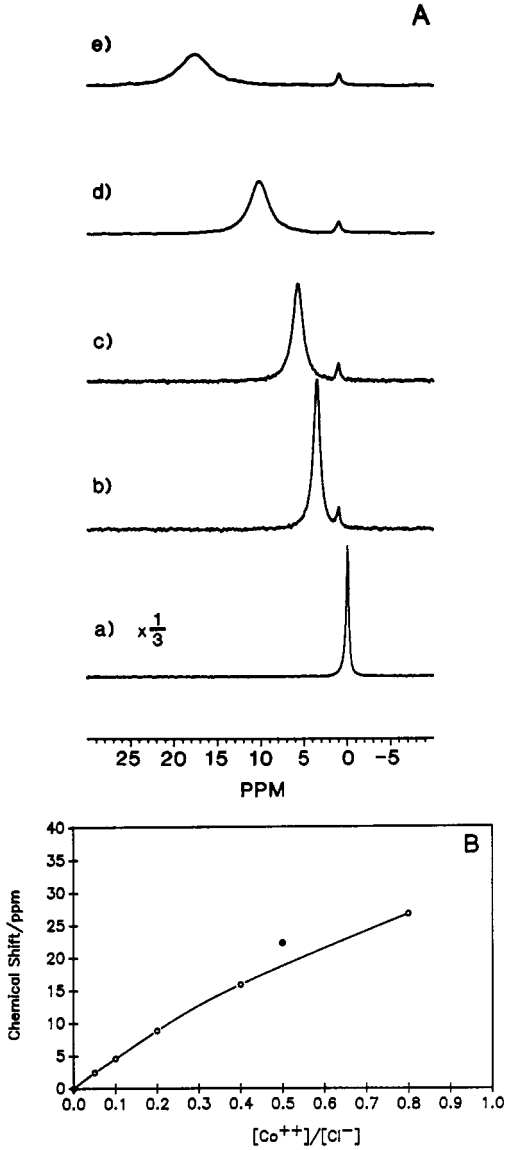

FIGURE 2: (A) ^{35}Cl spectra taken at 37 °C of a two-compartment sample containing 25 mM chloride with increasing amounts of Co^{2+} added to the larger compartment. Concentrations of Co^{2+} added: (a) 0 mM; (b) 1.25 mM; (c) 2.5 mM; (d) 5 mM; (e) 10 mM. (B) Plot of the shifts induced by different concentrations of Co^{2+} added to the phantom of (A). Open symbols are for Co^{2+} added as $Co(NO_3)_2$. The closed symbol is for Co^{2+} added as $CoCl_2$. The temperature was 37 °C and the chloride concentration 25 mM throughout. The line is to guide the eye only.

The spin–spin relaxation is monoexponential with a T_2 of 30 ms (consistent with the 10-Hz line width and Lorentzian line shape seen for well-shimmed samples).

The short T_1 makes rapid pulsing possible, and together with moderate line widths, this means that good signal-to-noise ratios (S/N) are obtainable in moderate acquisition times from samples of low chloride concentrations despite the low γ. For example, a spectrum with an S/N value of 7 was obtained from a 12-mL sample of 1 mM NaCl in 20 s. This represents an S/N of 12 mM^{-1} which could be improved by the use of a dedicated chlorine preamplifier and/or probe (see Materials and Methods).

Shifting the $^{35}Cl^-$ Resonance. In attempting to shift the $^{35}Cl^-$ resonance at this field, we found Co^{2+} to be effective—in agreement with previous findings at lower fields (Barabalat-Rey, 1969)—and also found several trivalent lanthanide metal cations (Pr^{3+}, Dy^{3+}, and Gd^{3+}) to be ineffective at moderate concentrations. We also found Mn^{2+} (broadens very effectively), Cu^{2+} (which broadens and shifts), and Fe^{2+} (which shifts less than Co^{2+}) to be inappropriate. In order to apply Co^{2+} as a SR in vesicle or cell preparations, the shift and broadening induced by the addition of varying amounts of Co^{2+} to chloride in inorganic solutions were examined. For this purpose, a two-compartment sample, consisting of two concentric coaxial NMR tubes, was used. The smaller internal compartment contained a 25 mM NaCl solution to act as a shift reference, and the larger one contained 25 mM NaCl to which increasing amounts of Co^{2+} were added as $Co(NO_3)_2$ from a 2 M stock solution. Figure 2A shows spectra of the sample with increasing amounts of Co^{2+} added to one compartment. The Co^{2+} induces a downfield shift and also a broadening of the $^{35}Cl^-$ resonance. Lorentzian line shapes are observed here and elswhere for Cl^- in the presence of Co^{2+}. Upon warming the sample of Figure 2, there is an increase in the downfield shift and a decrease in the line width of the shifted peak (data not shown).

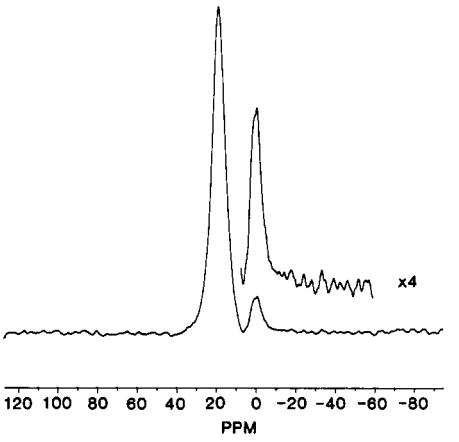

FIGURE 3: Spectrum of a suspension of lipid vesicles in 40 mM KCl at 37 °C. Lipid concentration was 30 mg/mL. The [Co^{2+}] [added as $Co(NO_3)_2$] was 16 mM. The spectrum is the sum of 3000 scans and took 5 min to acquire.

Figure 2B is a plot of the shifts induced by different concentrations of Co^{2+} with the chloride concentration constant at 25 mM. The shifts were referenced to the unshifted peak and were corrected for bulk susceptibility efffects by using the ^1H spectrum of the same sample (in all cases, this correction was less than 5%). This correction does not take into account any specific shift in the ^1H resonance of water induced by the Co^{2+}, but this specific shift is known to be very small at the low Co^{2+} concentrations used here (Luz & Shulman, 1965). The solid point in Figure 2B is from a spectrum taken under the same conditions as those in Figures 2A with the exception that 12.5 mM $CoCl_2$ in H_2O was used in the outer compartment; thus, the concentration of chloride was 25 mM as for the other points, but NO_3^- (and also Na^+) was absent. This shows that the presence of NO_3^- significantly affects the shift induced by the Co^{2+}. NO_3^- (and also SO_4^{2-}) complexes with Co^{2+} and reduces the concentration of free Co^{2+} available to interact with chloride. In the presence of phosphate or carbonate, precipitation of Co^{2+} occurs at neutral and alkaline pHs.

Vesicle Suspensions. Figure 3 shows a spectrum of a suspension of lipid vesicles in 40 mM KCl at 37 °C taken after the addition of Co^{2+} [as $Co(NO_3)_2$] to a concentration of 16 mM. The spectrum consists of a small unshifted Cl^- peak and a larger peak that is shifted and broadened though still Lorentzian. Three observations (not shown) support the assignment of the small unshifted peak to chloride within the vesicles and the large peak to extravesicular chloride: first, the relative

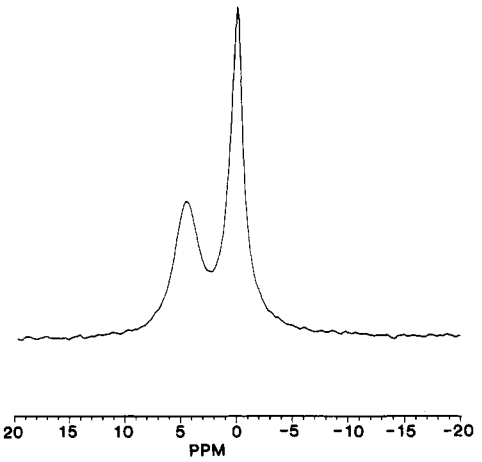

FIGURE 4: Spectrum of a suspension of red blood cell ghosts at 37 °C. The ghosts contained 100 mM chloride and no sulfate (see Materials and Methods for preparation) and were suspended in a solution containing 20 mM chloride, 45 mM sulfate, and 10 mM Co^{2+}. This spectrum is the sum of 1000 scans and was acquired in 80 s.

integral of the signals (10:1) is consistent with about 10% trapped volume, reasonable for large unilamellar vesicles (Alger & Prestegard 1979); second, addition of NaCl to the suspension increases the size of the large signal only; third, addition of detergent (Triton X-100) to permeabilize the membrane removes the unshifted signal, so that all the $^{35}Cl^-$ intensity is under the downfield-shifted resonance.

The size of the shifts and broadening of the extravesicular signal seen in the presence of vesicles at various temperatures and concentrations of Co^{2+} are very similar to those seen in the absence of vesicles. Also there is no split water peak in the ^1H spectrum of such preparations. These two observations indicate that the separation of the two peaks and the broadening of the shifted peak in Figure 3 are due to the same specific interaction of the Co^{2+} and Cl^- seen in analytical solutions. The integrated areas of spectra taken before and after adding Co^{2+} are the same, and this shows that to within experimental accuracy (typically 5% on the broadened peaks) the addition of Co^{2+} does not affect the NMR visibility of chloride in solution. This was observed for analytical samples, for vesicle suspensions, with ghosts, and for the plant cell suspensions described below.

A series of 5-min spectra such as that shown in Figure 3 were acquired over a 1.5-h period and showed no changes. This was found only for vesicles which had been dialyzed (48 h) after formation to remove residual ether and to allow stabilization of vesicle size. More permeable vesicles showed variable decreases in intravesicular signal size in successive spectra and showed shifting and broadening of the internal peak consistent with Co^{2+} leakage into the vesicles.

Erythrocyte Ghosts. The $^{35}Cl^-$ NMR signal from intracellular chloride has been found to be too broad to quantitate in erythrocytes (Falke et al., 1984b; Brauer et al., 1985). In the presence of erythrocyte membranes alone, however, the $^{35}Cl^-$ NMR signal can be measured easily (Shami et al., 1977; Falke et al., 1984a,b). These results made erythrocyte ghosts a promising system for testing the present approach to monitoring chloride levels. In addition, much data exist on anion transport in red blood cells and red cell ghosts from previous work with radioisotopes, and this provides an important standard for assessing the validity of this method.

Figure 4 shows a spectrum of a suspension of ghosts at pH 7.5 taken at 37 °C. The sample consisted of 4 mL of pelleted sealed ghosts containing 100 mM chloride and no sulfate resuspended to a total volume of 7 mL in a solution containing

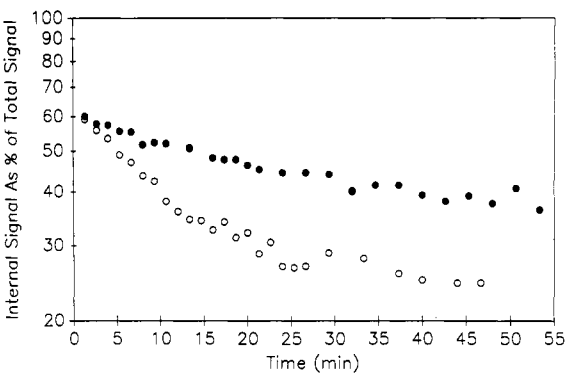

FIGURE 5: Plot of internal chloride levels with time for suspensions of ghosts prepared as for Figure 4. One suspension was prepared with 50 μM DIDS (closed symbols) and the other suspension (open symbols) without. Data points correspond to one 80-s spectrum each except for the open symbols after 27 min where two or three free induction decays (FID's) were added together for each data point. Exponential multiplication of 3 Hz was used before Fourier transformation of FID's. The integrated internal resonance was expressed as a fraction of the total integrated intensity.

10 mM chloride and 45 mM sulfate. Thus, there is an outwardly directed chloride gradient and an inwardly directed sulfate gradient. The unshifted signal in Figure 4 was assigned as arising from chloride inside the ghosts by similar means to those used for the spectra of lipid vesicles (decribed above), and its shift was set to 0 ppm. The large size of the internal resonance in Figure 4 is due to the higher concentration of chloride initially inside the ghosts. Subsequent decreases in the internal peak and increases in the external peak were followed by acquiring successive spectra over the next 45–100 min. Figure 5 shows plots of the percentage of chloride remaining inside ghosts as a function of time for two samples of ghosts from the same preparation; the ghosts in one sample were resealed in the presence of 50 μM DIDS, and those in the other sample were not. There is a rapid decrease in the internal chloride signal (half-time 15 min) which is inhibited by DIDS and also a slow component of decrease (half-time) >100 min). This behavior is reproducible ($n = 3$) and expected from the known ability of band 3 protein to exchange chloride for sulfate. The rate of this process has been previously measured in red cells at 37 °C under various similar but not identical conditions using radioisotope flux methods (Lepke & Passow, 1971). Such work shows that chloride/sulfate exchange takes place on a time scale of minutes, consistent with the rate observed here.

The slow component of loss of the internal chloride signal which is not affected by DIDS was seen in all ghost preparations, whether or not concentration gradients of anions were present (not shown). This slow component was temperature-dependent (inhibited by placing the suspensions on ice) and may have been due to lysis of the ghosts.

In Vivo Applications. Despite reports that intracellular chloride is invisible in mammalian cells (Falke et al., 1984b; Brauer et al., 1985; Hoffman & Gupta, 1987; Hoffman et al., 1987), we have tried to use Co^{2+} to resolve signals from intracellular chloride in vivo in cells of *Escherichia coli*, *Chlorella pyrenoidosa* (a unicellular green alga), and *Nicotiana tobbaccum*. We were able to obtain good shifts in the position of the chloride signal in the presence of *E. coli* cells but did not see an unshifted peak in any of our spectra. This we believe to be due to NMR invisibility of Cl^- in these cells because of two observations: (i) the addition of Mn^{2+} to the *E. coli* suspensions broadened *all* the chloride signal, contrary to observations with vesicles and *Chlorella* cells (see below); (ii) upon addition of NaCl to a suspension of *E. coli* cells (in the

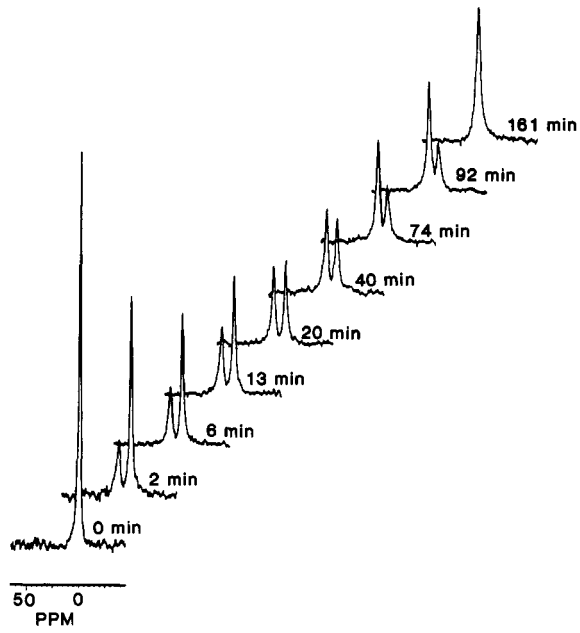

FIGURE 6: Spectra of a suspension of *Chlorella pyrenoidosa* cells in growth medium taken before and at increasing times after the addition of 8 mM Co^{2+} as $Co(NO_3)_2$. Total time for each spectrum was 5.1 min. Times after addition of cobalt are indicated for each spectrum.

absence of Co^{2+}), the single $^{35}Cl^-$ signal increases; when, following this, the cell membranes are partially permeabilized with toluene, the $^{35}Cl^-$ signal *decreases*. This is consistent with movement of chloride from a compartment of higher visibility to one of low visibility.

Several observations indicate that the NMR invisibility of Cl^- in these cells is due largely to interaction with proteins. First, proteins are well-known to broaden the $^{35}Cl^-$ resonance (Forsén & Lindman, 1981). We found that addition of 120 mg/mL BSA to a solution of 25 mM NaCl causes sufficient broadening of the signal to make the resonance too broad to be observed (not shown). With 24 mg/mL BSA added, the $^{35}Cl^-$ peak showed two coresonant components and had a line width at half-height of >400 Hz. The concentration of soluble proteins in the cytoplasm of *E. coli* is 135 mg/mL (Ingraham et al., 1980). Second, increasing the viscosity of an aqueous solution of NaCl by adding glycerol to 50% by volume increased the line width only to 120 Hz, indicating that viscosity alone does not account for intracellular invisibility.

We have been able to observe sharp intracellular chloride signals in the unicellular green alga *Chlorella pyrenoidosa* and in higher plant cells as described below. Figure 6 shows spectra taken before and at various times after the addition of Co^{2+} to a suspension of *Chlorella pyrenoidosa* cells in growth medium; 8 mM $Co(NO_3)_2$ was added, although the resulting concentration of Co^{2+} in solution was less due to some precipitation with phosphate. The initially large unshifted peak is assigned to intracellular chloride and the downfield shifted peak to extracellular, as follows: (i) addition of 5 mM KCl to the suspension increases the shifted peak only; (ii) adding 10 mM EDTA eliminates the shifted peak, all the intensity becoming coresonant and unshifted (EDTA removes the shift induced in the ^{35}Cl signal of analytical samples by chelating the Co^{2+}); (iii) alternatively, adding Mn^{2+} causes the shifted peak to be greatly broadened, leaving the unshifted peak unchanged. Thus, the Co^{2+} is acting to shift the same chloride pool as is rapidly accessible by EDTA and Mn^{2+}.

The intracellular volume in this case was approximately 5% of the total sample volume (estimated by cell counts and microscopy), and thus the unshifted signals in Figure 6 correspond to intracellular chloride concentrations that are higher than the extracellular (initially 200 times higher). This is consistent with previous work on chloride levels in another *Chlorella* species using $^{36}Cl^-$ labeling (Doblinger & Tromballa, 1982). In order to induce chloride efflux, the cells in this experiment were kept warm and in the dark without oxygenation (otherwise little Cl^- movement occurs). The reduction in the intracellular peak size and the increase in the extracellular signal allow one to quantitate the NMR visibility of chloride in these cells. This is done by comparing the decrease in the intracellular signal over time to the increase in the extracellular signal over the same time period. These being the same, one concludes that intracellular visibility is not significantly different from 100% ($n = 3$). We suggest that the intracellular signal arises largely or entirely from the large vacuolar compartment in these cells (>80% of cell volume).

The lower protein content of the vacuole in plant cells would account for the narrow line and apparent 100% visibility of intracellular chloride in these cells. The question arises as to whether the changes seen in successive spectra of Figure 6 are caused or accelerated by the addition of Co^{2+}. This might occur either by cobalt entering the cells and shifting the $^{35}Cl^-$ signal of intracellular chloride [as in the case of Mn^{2+} entry into maize root tip cells (Pfeffer et al., 1986) which affects the ^{31}P NMR spectrum even after extracellular Mn^{2+} is removed] or by cobalt toxicity causing leakage of chloride from the cells. This question is addressed by the following observations: (i) Addition of EDTA to such samples, even after 2 h, removes the shift, immediately making all the signal coresonant (if slow entry of cobalt were shifting the intracellular chloride signal, then chelating extracellular Co^{2+} would be expected to leave some of the signal from intracellular chloride still shifted). (ii) Samples kept warm and without oxygen (as in Figure 6 but without Co^{2+}) for some time reveal, upon the addition of Co^{2+} and acquiring a spectrum, that chloride leakage has taken place to a comparable extent to samples where Co^{2+} was present throughout. Thus, the presence of Co^{2+} does not accelerate chloride loss under these conditions. Results from a different *Chlorella* species (Doblinger & Tromballa, 1982) shows that $^{36}Cl^-$ leaks in the dark over several hours even under oxygenated conditions. Neither the presence of Co^{2+} nor the conditions used to induce chloride efflux (see Materials and Methods) caused significant cell lysis. This was examined in the *Chlorella* samples after NMR experiments by (i) light microscopy and (ii) spinning down the cells and looking for chlorophyll release.

In the application of any SR to living systems, a key issue is the possible toxicity of the reagent to the cells in question. As discussed above, adding Co^{2+} to *Chlorella* cells did not significantly affect the rate of chloride leakage under the conditions above, and there is no sign of Co^{2+} entry or cell integrity loss on a large scale. However, we found that *Chlorella pyrenoidosa* does not grow at all in the presence of 10 mM Co^{2+} and so Co^{2+} clearly has undesirable effects. Indeed, Co^{2+} is known to be toxic to various living cells. Thus, having once demonstrated that intracellular chloride is visible in at least two plant cells, one can seek other, nontoxic SR's.

The spectrum in Figure 7 is of a suspension of cells of *N. tobbaccum* grown in liquid culture (see Materials and Methods). The cells were in growth medium at 27 °C and were oxygenated by bubbling O_2 during the acquisition, conditions similar to those of growth except for the addition of Co^{2+} to 20 mM. Two successive 15-min spectra showed no significant changes. The intracellular chloride signal is much less than in *Chlorella* despite the presence of sizable vacuoles in these

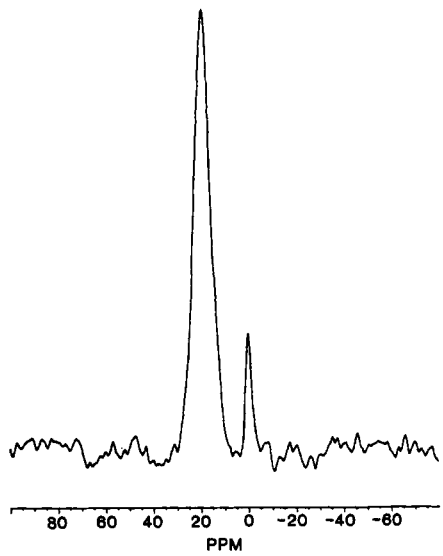

FIGURE 7: Spectrum of tobacco cells grown in liquid culture from isolated protoplasts. Cells were being oxygenated in growth medium at 27 °C throughout acquisition. [Co^{2+}] was 20 mM. Total time for acquisition was 15 min.

cells, thus indicating much lower intracellular chloride concentrations for these cells.

CONCLUSIONS

The results of this study show that ^{35}Cl NMR can be used to monitor chloride levels in vesicles using Co^{2+} as a SR to resolve internal from external signals. In red cell ghost preparations, this method allows accurate continuous measurement of chloride transport under physiological conditions and yields results consistent with established radioisotope methods. In two plant species, we have shown that intracellular chloride signals are visible and can be resolved in vivo, in contrast with results from mammalian and bacterial cells. The transportable chloride in *Chlorella* cells is fully NMR-visible, and both the intracellular levels and the rate of efflux measured by this method are consistent with values for total cell chloride previously measured by radioisotopic methods. It is proposed that the intracellular signals arise from vacuolar chloride.

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APPENDIX

Theory of Exchange Applied to the Interaction of Cl^- with Co^{2+}. The effect on the ^{35}Cl spectrum of adding Co^{2+} to chloride in solution may be understood in terms of the exchange of chloride between two chemically different environments: hydrated in free solution and complexed to a Co^{2+} ion. The predominant chloride complex of cobalt under conditions of moderate temperature and concentration is the octahedral $(CoCl{\cdot}5H_2O)^+$ (Barabalat-Rey 1969):

$$Cl^- + (Co{\cdot}6H_2O)^{2+} = (CoCl{\cdot}5H_2O)^+ + H_2O$$

$$K_{eq} = [CoCl{\cdot}5H_2O]/[Co^{2+}][Cl^-] \quad \text{(A1)}$$

The Lorentzian line shape observed for chloride in the presence of Co^{2+} in solution is due to rapid tumbling of the complex in solution; for more slowly tumbling species such as chloride bound to BSA, quadrupolar effects cause a non-Lorentzian line shape (Lindman & Forsén, 1976). The NMR behavior of the ^{35}Cl nucleus is very different in terms of chemical shift and relaxation in the two environments, but the exchange rate between bound and free states is rapid enough that only one resonance is observed. The situation can be analyzed in terms of the Bloch equations as modified by McConnell to include exchange (McConnell, 1958). These equations have been solved by Swift and Connick (Swift & Connick, 1962; Swift, 1973) for situations where at any instant there are many more nuclei in one environment than in the other. K_{eq} of A1 is small [0.17 according to Barabalat-Rey (1969)] so that, for the concentration ranges in which we are interested, the large majority of chloride ions are not complexed to Co^{2+} and thus Swift and Connick's treatment applies. The shift induced by Co^{2+} in the resonant frequency of $^{35}Cl^-$ relative to the frequency observed without Co^{2+} is given by

$$\omega_{obs} = \omega_F - \frac{\Delta\omega_B}{\tau_F\tau_B[(1/T_{2B} + 1/\tau_B)^2 + \Delta\omega_B^2]} \quad \text{(A2)}$$

and the observed transverse relaxation time by

$$\frac{1}{T_{2obs}} = \frac{1}{T_{2F}} + \frac{1}{\tau_F}\frac{(1/T_{2B})^2 + 1/T_{2B}\tau_B + \Delta\omega_B^2}{(1/T_{2B} + 1/\tau_B)^2 + \Delta\omega_B^2} \quad \text{(A3)}$$

where the subscripts B and F refer to the two environments sampled by the chloride ions, bound and free, respectively, and where τ_F and τ_B and the lifetimes of an ion in the two environments, T_{2F} and T_{2B} are the transverse relaxation times in the two environments, and $\Delta\omega_B = \omega_F - \omega_B$ is the difference between the ^{35}Cl resonant frequencies of Cl^- in the two environments.

In order to select appropriate SR(s) for use under different conditions and in order to interpret and quantify the observed spectra, it is important to know the relative contributions of the different terms in eq A2 and A3. It is often possible to simplify these equations greatly with such information. Work by Barabalat-Rey at low fields on this system yielded values for $\Delta\omega_B$, τ_B, and $1/T_{2B}$ which (after scaling $\Delta\omega_B$ up to our field strength) allowed us to predict the shifts and line widths to be expected. Unfortunately, these predictions were far from what we observed, necessitating further investigation of the relative sizes of the different terms in eq A2 and A3. By a comparison of the shifts and line widths observed for the two stable isotopes of chlorine in combination with field strength comparisons (unpublished results), it was possible to simplify eq A2 and A3 to

$$\Delta\omega = P_B\Delta\omega_B \quad \text{(A4)}$$

and

$$1/T_2 = 1/T_{2F} + P_B\tau_B\Delta\omega_B^2 \quad \text{(A5)}$$

where $P_B = [CoCl{\cdot}5H_2O]/[Cl]_{tot} = [CoCl{\cdot}5H_2O]/([Cl] + [CoCl{\cdot}5H_2O])$ is the fraction of total chloride that is bound. These correspond to one of the limiting cases treated by Swift and Connick.

The assignment of this SR/ligand system a limiting exchange regime allows one to interpret several observations using eq A4 and A5: (i) The finding that the shift is increased by temperature must be due to an increased P_B; this is consistent with the previous finding of a positive free energy of formation for the $CoCl{\cdot}5H_2O$ complex (Barabalat-Rey, 1969). (ii) The observation that the line width of the shifted resonance is *decreased* with increased temperature is explained by the

fact that τ_B decreases more with temperature than P_B increases.

Because K_{eq} in A1 is small, $[Cl^-] \simeq [Cl^-]_{tot}$ and $[Co^{2+}] \simeq [Co^{2+}]_{tot}$ for low concentrations of Cl^- and Co^{2+}, so that P_B is given to a good approximation by $K_{eq}[Co^{2+}]_{total}$. Thus, as P_B is a factor in both eq A4 and A5, one predicts that the total concentration of Co^{2+} should determine the shift and broadening much more than the ratio of SR to shifted species. This explains whey in the experiments described above, similar concentrations of Co^{2+} were required in samples which differed considerably in the concentration of chloride present. It also explains why the trivalent lanthanides are ineffective at low concentrations—Gd^{3+}, for instance, gives ~30 ppm shift at 1 M $GdCl_3$ (Barabalat-Rey, 1969), but we found very little shift in the millimolar concentration range despite the fact that $[Gd^{3+}]/[Cl^-]$ was identical. Such conditions (rapid exchange and small K_{eq}) apply to many SR systems, and in these cases, the abolute concentration of SR added determines the shift observed as much or more than does the ratio of added SR to shifted species.

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