NUCLEAR MAGNETIC RESONANCE STUDIES OF SODIUM AND POTASSIUM IN ETIOLATED PEA STEM

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ABSTRACT Based on nuclear magnetic resonance (NMR) spectroscopy and other evidence, it has been argued that tissues accumulate, and retain, ions in a binding process by a highly structured water-protoplasm system; thus active membrane transport need not be involved. Recent evidence has accounted for the loss of resonance intensity usually found when investigating quadrupolar ions in animal tissue. Using continuous wave NMR spectroscopy, we have examined two quadrupolar ions, Na^+ and K^+, in pea stem cells where about 90% of the ion content is in the largely aqueous vacuoles having a membrane barrier. The NMR resonances from these ions correspond to almost 100% of that expected from independent measurements of total ion content. This indicates that the ions are retained as free ions after accumulation. The small fraction which is NMR invisible may represent ions in an ordered, anisotropic environment, such as that in the wall or cytoplasm.

INTRODUCTION

Most studies on salt relations with living cells appear to be based on the premise that the membranes act as a major barrier to ionic diffusion and serve as the site of active transport. This interpretation, however, is not universally held. From activity measurements with microelectrodes in epithelial cells of bullfrog small intestine, Lee and Armstrong (1972) have concluded that a substantial fraction of cellular Na^+ is osmotically inactive. Neville (1972) has recently suggested that amino acid accumulation should be considered in terms of G. N. Ling's (1962) hypothesis that ions are bound in highly structured protoplasm. Damadian (1971) reported that disrupted cell fractions of *Escherichia coli* bind K^+ in amounts equal to that of living cells with intact membranes. He has suggested that the role of intermediate metabolism in active transport is to increase the ion exchange capacity of the cell polyelectrolytes.

Conflicting data and ideas are easy to find in the literature, and there is much

evidence favoring membrane theory. Kushmerick and Podolsky (1969) have demonstrated that the diffusion coefficients in muscle cells of ions like K^+, Na^+, SO_4^{-2}, ATP^{-4} are similar to those of sucrose and sorbitol. Hinke (1970) has estimated that only 68% of the water in barnacle muscle fibers is osmotically active, while maintaining that the ions are predominantly free. One of the most potent arguments in favor of membrane theory lies in the demonstration of ion transport and accumulation by isolated membrane systems such as red cell ghosts and membrane vesicles which do not enclose any protoplasm (see reviews by Kaback, 1970, and Henderson, 1971).

One of the strongest cases for bound ions has been based on NMR spectroscopy. Ling and Cope (1969) used ^{23}Na resonances to examine the state of Na^+ in animal tissue. From the intensity of the observed signal, they concluded that a large portion of the intracellular Na^+ is in a bound state. Czeisler and co-workers (1970) reexamined muscle tissue and attempted to locate the NMR invisible sodium signal. More recently several groups of investigators have explained the missing or bound Na^+ signal as a phenomenon associated with quadrupolar ions in some kind of an ordered matrix (Shporer and Civan, 1972; Berendsen and Edzes, 1972; Edzes et al., 1972; Chen and Reeves, 1972). When dispersed in an ordered system, such as muscle tissue or DNA fibers, quadrupolar ions such as Na^+ ($I = 3/2$) will exhibit three resonance lines. Only 40% of the total intensity is visible in the central line. This 40% signal corresponds to the NMR visible signal observed by most workers. The absence of 60% of the expected signal from total sodium cannot be interpreted as evidence for binding. Recently Civan and Shporer (1972) have examined the oxygen-17 spectra of water in frog muscles. They have used the resonance of this quadrupolar nucleus, in the light of these most recent developments, to demonstrate that two types of water may be present. These would be either as a mobile and immobile state or in two compartments, one of which is characterized by an anisotropic medium.

Although the concept that a large fraction of Na^+ is bound, as observed by magnetic resonance, is not reasonable in light of recent explanations, we have attempted to study the resonances of $^{39}K^+$ and $^{23}Na^+$ in the vacuolated cells of higher plants, particularly pea epicotyl tissue. This system is composed of many cells, each having a large central vacuole which constitutes the bulk of the tissue volume. The vacuole is considered to be largely aqueous and ordinarily to contain little protein. The antagonists of membrane theory have not considered the case of vacuolated cells, although this system is probably a good model for study of ion uptake. In pea epicotyl tissue it is estimated that about 84% of the volume of the tissue is occupied by the largely aqueous vacuole, while the cytoplasm constitutes about 3%, with the remainder being wall and intercellular space (Macklon and Higinbotham, 1970). It is difficult to conceive of any agent or mechanism which could bind the K^+ (54–79 μmol/ml); the same could be said of Na^+. NMR spectroscopy, however, could provide a way for studying the nature of ions in the total tissue. If only 40% of the

expected signal were observed, one would have the same result as found with other tissue. The only interpretation possible would be that the ions experienced a field gradient as a result of anisotropy within the tissue. However, with the large vacuoles in epicotyl tissue, where ions might be considered free in solution, one might expect a large fraction of the ions' signal to be visible. Indeed, a signal representing 100% of the total ion concentration could only be explained by assuming that the ions were totally free in a nonordered system. Obviously, under conditions where the signal is totally visible, no uptake resulting from binding by proteins or metabolites need be hypothesized.

Previous attempts have been made to determine the ionic activities in the cytoplasm and vacuole. Etherton (1968) used an ion selective microelectrode to measure K^+ activities in the cytoplasm and vacuole of pea root cells; quite high activities were found, particularly in the vacuole, but in the absence of direct measures of concentrations no conclusions about binding can be made. Cell electropotentials, however, exceeded those predicted from K^+ diffusion. Vorobiev (1968) and Khitrov and Vorobiev (1971) also used ion selective microelectrodes to measure K^+ and Cl^- activities in the cytoplasm and vacuoles of *Nitella* and *Chara*. They found that the activities corresponded to the concentrations in each case, and, further, that the Nernst potential for K^+ corresponded to the membrane electropotential although Cl^- was actively accumulated (i.e., transported against the electrochemical potential gradient).

We have examined both the $^{23}Na^+$ and $^{39}K^+$ spectra of these ions absorbed by etiolated epicotyl tissue of *Pisum sativum*. Continuous wave NMR spectroscopy was used with time averaging. The ^{23}Na signal is quite strong, but the ^{39}K signal is extremely weak. One study of ^{39}K in halophilic organisms has been reported (Cope and Damadian, 1970). From measurements of ^{39}K relaxation times, the authors believe that the K^+ is in a highly ordered state in the halophiles. With our techniques we have been able to study the line width and the signal intensity very carefully. Audio modulation of the resonance is at a much higher frequency than the line width. Low radio-frequency power levels could be used because the signal-to-noise ratio could be increased by spectral accumulation. In general, most of the problems discussed by Czeisler and co-workers (1970) can be accounted for, except for the inhomogeneity of the biological tissue itself. The stem tissue, however, may be an exceptionally good system because of the structural properties discussed above.

METHODS AND MATERIALS

NMR spectra were obtained with a modified Varian DP-60 NMR spectrometer (Varian Associates, Instruments Div., Palo Alto, Calif.). The field was locked to an external water capillary with resonance frequency of 60 MHz. Spectra of sodium-23 (15.872 MHz) and potassium-39 (2.797 MHz) were then obtained by direct sweeping of the radio frequency. Audio modulation of 2,000 Hz was used in conjunction with a Princeton Applied Research model 121 lock-in amplifier (Princeton Applied Research Corp., Princeton, N. J.) for baseline stabilization. A Fabri-Tek 1072 signal averager (Nicolet Instrument Corp., Madison,

Wis.) was used for time averaging and integration of the signal. For concentration determinations of the NMR visible signals, the integrated signal was compared against standards of sodium chloride containing Carbowax 1500 (Union Carbide Corp., Carbon Products Div., New York). The Carbowax broadened the signal by increasing the viscosity and allows one to prepare concentrations of any given line width. Within the limits of the NMR experiment, the potassium-39 signals measured for total intensity had line widths corresponding to potassium chloride dissolved in water. Total ion content was determined with a Perkin-Elmer 303 atomic absorption spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). The tissue samples were completely digested in nitric acid for elemental analysis by atomic absorption.

Peas, *P. sativum* cv. Alaska, were grown in darkness for 7 days in vermiculite moistened with a standard nutrient solution having the following composition in moles per liter: KCl, 1.0; $Ca(NO_3)_2 \cdot 4H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.25; $NaH_2PO_4 \cdot H_2O$, 0.904; Na_2HPO_4, 0.048 (Higinbotham et al., 1967). Excised epicotyl segments, 3 cm long, were then placed in the above solution and gently agitated for varying periods of time at 20°C. Each sample consisted of 20–25 segments, each from a separate plant, with a total weight of 2.2–2.6 g. Each point in Figs. 3–5 represents a separate sample, so that Fig. 4 represents epicotyl segments from over 200 individual plants. For NMR measurements the stem segments were placed in a test tube of 15 mm diameter. They were kept moist with deionized water but were well aerated. The absolute values of the ion accumulation would vary from one series of uptake experiments to another, but in all series of experiments the general pattern is that observed in Figs. 3 and 4.

To improve uptake for the potassium experiments KCl was added (to the foregoing solution) to give a final concentration of 30 mM K^+. For sodium uptake KCl was omitted and NaCl added to give 30 mM Na^+.

RESULTS AND DISCUSSION

Segments of plant tissues accumulate ions when bathed in aqueous solutions of salts. Pea epicotyl segments are known to accumulate K^+ when exposed to a nutrient solution (Higinbotham et al., 1967). The content of Na^+, Mg^{++}, and Ca^{++} remain at very low levels. Only K^+ approaches an electrochemical equilibrium. With plant cells two membranes, the plasmalemma separating the cytoplasm from the cell wall and the tonoplast separating the cytoplasm from the vacuole, must be considered. From electrochemical measurements, Macklon and Higinbotham (1970) have concluded that K^+ is actively transported into the cytoplasm and then diffuses passively into the vacuole. The plasmalemma then is the site of initial accumulation. It has also been suggested that with higher plant cells Na^+ is pumped out at the plasmalemma and into the vacuole at the tonoplast (Pierce and Higinbotham, 1970). Etiolated stem tissue will accumulate Na^+ when subjected to nutrient solutions with high concentrations of Na^+ providing K^+ is absent.

The NMR spectrum of sodium-23 is easily detected. A spectrum of the sodium ion in stem segments is shown in Fig. 1. Spectra were taken after different periods of Na^+ accumulation. The line width of the resonance was constant at 27 Hz. The resonance of $^{39}K^+$ obtained from tissue allowed to accumulate K^+ is shown in Fig. 2. Because of the lower sensitivity, the spectra were much more difficult to obtain

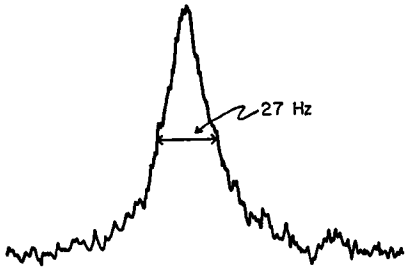

FIGURE 1

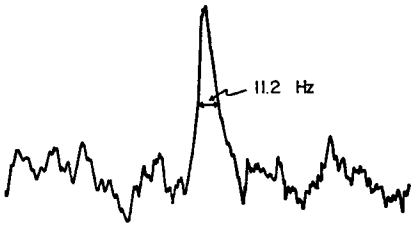

FIGURE 2

FIGURE 1 ^{23}Na spectrum at 15.872 MHz, 80 scans, of pea epicotyl segments in high Na nutrient solution for 36 h.

FIGURE 2 ^{39}K spectrum at 2.797 MHz, 937 scans, of pea epicotyl segments in normal K nutrient solution for 72 h.

than for Na^+. The line widths obtained for K^+ were constant for different times of K^+ accumulation. The consistency of these line widths throughout the experiment indicates little binding of the ions to any membranes or protoplasmic constituents. Under the simplifying conditions of rapid exchange, one can expect to observe a line width which is an average of that from bound and free ions (Stengle and Baldeschwieler, 1967; Ward, 1969; Magnuson and Bothner-By, 1971), and is represented in the following equation:

$$\Delta\nu_{obs} = \Delta\nu_f X_f + \sum_i \Delta\nu_{bi} X_{bi}.$$

$\Delta\nu_{obs}$ is the observed line width, and $\Delta\nu_f$ and $\Delta\nu_b$ represent the line width of free and bound ions, respectively. The X's are the respective fractions, and the i's allow for summation over a variety of binding sites. If the relative fraction of binding sites stays constant, then $\Delta\nu_{obs}$ would be expected to remain constant. Alternatively, if the ions are all predominantly free, then the line width would be expected to remain constant and narrow. It has been demonstrated that, ion interaction with membranes (Magnuson et al., 1970) and cells (Magnuson and Magnuson, 1972) can be detected by line width changes. Because of the consistency observed in the line widths of the resonance for either K^+ or Na^+ in pea stem tissue, it can be concluded that little interaction with the cell components is occurring. It should be noted that the simplifying conditions of rapid exchange may not hold in this case, and other factors may contribute to the observed line width. The constant line width, however, indicated that if other factors are contributing, they are not changing within an experiment. The invariant line width, slightly broader than that of free Na^+ in water, may reflect invariance in the local magnetic field inhomogeneities caused by a heterogeneous sample.

To further investigate the state of ions, intensities of the signals were obtained by integration. Fig. 3 presents the results for the total Na^+ accumulation, obtained by atomic absorption spectrophotometry, and the NMR signal intensity for stem tissue subjected to nutrient solutions high in Na^+. The NMR signal is always close to

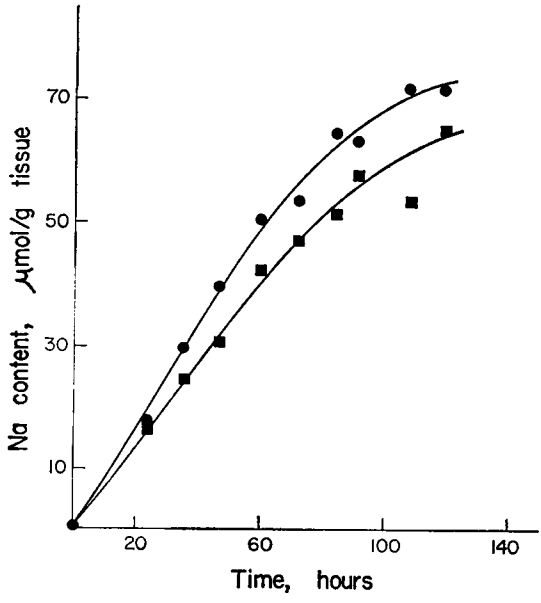

FIGURE 3

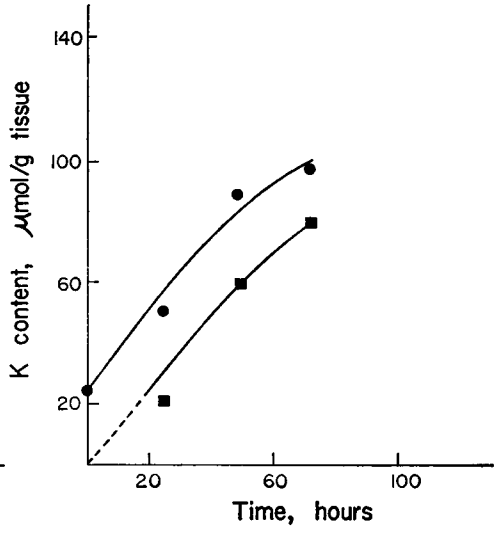

FIGURE 4

FIGURE 3 Na^+ uptake results for pea epicotyl segments in high Na^+ nutrient solution. Total Na^+ from atomic absorption, ●; integrated NMR intensity, ■. Each point represents a separate sample of about 2 g of tissue from approximately 20 plants.

FIGURE 4 K^+ uptake results for pea epicotyl segments in normal nutrient solution. Total K^+ from atomic absorption, ●; integrated NMR intensity, ■. Each point represents a separate sample of about 2 g of tissue from approximately 20 plants.

100% of the total Na^+ visible. Within the limits of the NMR experiment, it appears that the NMR signal observed could account for all the Na^+. The NMR intensity is consistently lower than that determined by atomic absorption and this may account for a small amount of Na^+ within an ordered state. This high integrated NMR intensity is in marked contrast to the 40% usually found in animal tissue, and which has been explained by quadrupolar ions in an environment where an electric field which does not average to zero is experienced by the ion. Because the value for the pea stems is so close to 100%, the ions are presumably in a free aqueous environment which, most certainly, is predominantly in the cell vacuole. It should be noted again that although there was variability in ion uptake between different series of samples, the comparisons of the atomic absorption with NMR measurements within a given series were always similar. Approximately 10 series were examined for Na^+ accumulation and 5 for K^+ accumulation.

Changes in the Q of the sample coil, when comparing pea stems with sodium chloride solutions, must be considered. To examine this, signals from sodium chloride solutions were compared with sodium chloride solutions surrounding stem segments. When the spaces between 20 or more segments of dead tissue in a 15 mm NMR tube were filled with sodium chloride solution, intensities of the Na resonances

corresponded exactly to values expected from the volume of solution. After equilibrating throughout the dead tissue for 120 h, the intensity remained unchanged. The deviation between the expected signal, based on that predicted from the standard, was never more than 2%. This clearly indicates that variations in Q are not important and strongly implies that inhomogeneity of the tissue, as discussed above, is not influencing the experimental data considerably.

The results of a K^+ accumulation experiment in which ^{39}K resonances were obtained are shown in Fig. 4. At zero time when the stem tissue contains about 20 μeq K/g, no ^{39}K resonance could be detected. This may be entirely due to the low sensitivity of ^{39}K. Again with ^{39}K, most of the K^+ is observed in the NMR signal, in direct contrast to a 40% intensity which might be observed if the K^+ were in an ordered environment. The ^{39}K data agree, therefore, with the ^{23}Na experiments. It should be noted that the experimental error in the ^{39}K measurements are larger than with ^{23}Na because of lower signal intensity. An estimate of error for the ^{23}Na experiments would be $\pm$5–10%. For the ^{39}K experiments, one can estimate 10–20%.

To investigate the nature of ion balance between K^+ and Na^+, pea stem segments were first exposed to Na^+. After a period for Na^+ accumulation, the stem tissue was placed in a normal potassium nutrient. The results after a 120 h exposure to high Na^+ are shown in Fig. 5. This experiment is similar to that conducted by Ling and Cope (1969) who replaced intracellular K^+ with Na^+. In this experiment a small

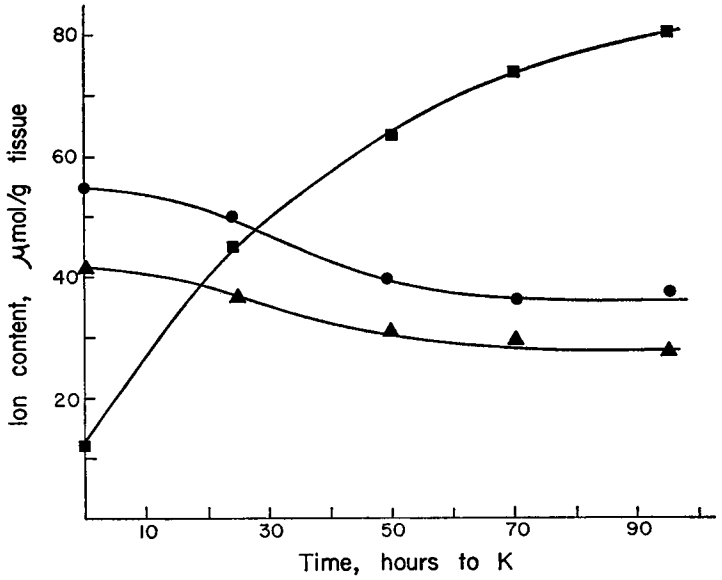

FIGURE 5 Ion content of epicotyl segments exposed for 120 h to high Na^+ nutrient and then exposed to normal nutrient. Time exposed to normal nutrient versus ion content. Total Na^+ from atomic absorption, ●; total K^+ from atomic absorption, ■; integrated NMR intensity for Na^+, ▲.

fraction of the Na^+ washes out. K^+ concentrations, however, reach quite high levels with no apparent exchange for accumulated Na^+. The best explanation for the initial decrease of Na^+ is loss from cell wall portions. It has been demonstrated by isotope efflux experiments that ions are exchanged very slowly from the vacuoles (Pierce and Higinbotham, 1970; Macklon and Higinbotham, 1970).

The NMR-integrated intensities are also presented in Fig. 5. In each case the NMR signal drops slightly with time, but the total Na^+ content drops more. This occurs within the first 30 h. The result of this is to bring the total Na^+ more into agreement with the NMR integrated intensity. Since this initial loss of total Na^+ is mostly from the cell wall portion, and possibly some from the cytoplasm, it appears that some of the difference between the total Na^+ value and NMR-integrated value may be from loss of Na^+ signal which is bound, for example, to the pectins. Perhaps, a portion of this difference is due to Na^+ which is experiencing partially averaged order in the cytoplasm or near the cell wall. It is apparent, nonetheless, that the major portion of the Na^+ is visible in an aqueous state free of order. The ions are free and relatively unaffected by any metabolites, proteins, or membranes.

One last comment should be made about the NMR technique. The NMR "invisible" signal has been successfully explained in terms of field gradients which do not average to zero. This has explained the presence of 40% signals in animal tissue such as muscle. Our results show clearly that the ions are in a free state just as in a solution. Of course, the model system is the plant cell which consists mostly of vacuole. It is known, however, that ions are accumulated in vacuoles, and now it seems clear that they must be retained as free ions in the concentration range of this study. As noted, with Fig. 3–5, the lower limits of detection are approximately 5 mM for Na^+ and 25 mM for K^+. Thus binding at these levels cannot be precluded. In pea epicotyl tissue the amounts of K^+ in the wall and cytoplasm have been estimated by tracer compartmental analysis as being about 5 μeq g^{-1} fresh weight (Macklon and Higinbotham, 1970). In oat coleoptile the corresponding values for both K^+ and Na^+ were found to be similar (Pierce and Higinbotham, 1970). Binding at these levels cannot be evaluated by the present methods. All the NMR results indicate, however, that the ions accumulated are predominantly, or almost entirely, free ions.

We thank Dr. H. J. C. Berendsen for allowing us to read a copy of his paper presented at the International Conference on the Physico-Chemical State of Ions and Water in Living Tissue and Model Systems, New York Academy of Sciences, January, 1972.

The authors wish to acknowledge the support of National Science Foundation grant no. GB 19201 to Noe Higinbotham and a Washington State University Research Committee grant to James A. Magnuson.

Received for publication 6 October 1972 and in revised form 26 March 1973.

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