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Nuclear Magnetic Resonance Relaxation Times and Plasmalemma Water Exchange in Ivy Bark

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ABSTRACT

Measurement of nuclear magnetic resonance (NMR) relaxation times (transverse [T_2] and longitudinal [T_1]) for *Hedera helix* L. cv. Thorndale (ivy) bark water indicates the presence of at least two populations of water with different relaxation characteristics. One population of water with short T_2 and T_1 was found to be composed of both hydration water and extracellular free water. The second population of water with long T_2 and T_1 was identified as intracellular bulk water.

NMR relaxation of extracellular water protons is controlled by cell wall surface effects, possibly due to binding of paramagnetic cations by the cell walls. NMR relaxation of intracellular water protons is controlled by both water exchange to the extracellular environment and chemical exchange with a population of protons that is chemically shifted from that of the bulk water. The relaxation time of intracellular water is not measurably affected, either by intracellular paramagnetic ions or by increased viscosity of intracellular water. Manganese flux into the cells occurs at 1.7×10^{-15} moles cm^{-2} $seconds^{-1}$ and is independent of extracellular Mn^{2+} concentration in the range 5 to 20 mM.

The intracellular-extracellular water exchange time of ivy bark was found to be predominantly limited by membrane water permeability. A diffusional water permeability coefficient (P_d) of approximately 3×10^{-2} cm $seconds^{-1}$ was calculated for ivy cell membranes at 20 C.

The diffusional water permeability coefficient of multicellular plant tissue is difficult to measure because of extracellular unstirred layers of water (associated with cell walls) which offer resistance to diffusion of labeled water (5, 14). A NMR[3] technique of measuring diffusional water permeability that is free from the problem of extracellular unstirred layers has been applied to red blood cells (4). This NMR technique requires knowledge of a transverse relaxation time (T_{2a}) for the intracellular water protons that is controlled by intracellular dynamic magnetic interactions only. It also requires measurement of a transverse relaxation time (T'_{2a}) for the intracellular water protons that is controlled by both the intracellular dynamic magnetic interactions and by water exchange out of the cell. The water exchange time (τ_a) which is an exponential time constant for water diffusion out of the cell can then be calculated from T_{2a} and T'_{2a}. The value of τ_a depends upon water permeability and/or intracellular unstirred layers. A method to determine if P_d can be calculated from τ_a has been presented by Stout *et al.* (22). Conlon and Outhred (4) measured T_{2a} using packed red blood cells suspended in plasma and T'_{2a} using red blood cells suspending in Mn^{2+} solution. Paramagnetic cations such as Mn^{2+} cause water proton spins to relax in a short time. Thus, proton spins of water molecules diffusing out of the cells are relaxed in a short time that is characteristic of the extracellular Mn^{2+} solution. A valid measurement of T'_{2a} also required that the red blood cells were loosely packed. When the cells are too tightly packed or the spin relaxation rate of extracellular water is too slow, the probability that water molecules that have diffused out of the cells will diffuse back into the cells before their proton spins have been relaxed is high and results in a larger measured value of T'_{2a}. This effect is referred to as backflux (4). Another potential source of error to T'_{2a} that must be considered when using the Conlon-Outhred technique is whether or not the added Mn^{2+} is altering P_d and thus τ_a. The divalent cation Ca^{2+} alters water permeability of artificial lipid membranes (16). Conlon and Outhred (4) found no significant source of error in their measurements from effect of added Mn^{2+}.

The present study was performed to measure P_d of a complex multicellular plant system. Since water permeability may play an important role in drought (25) and cold (18) hardiness of plants, bark of *Hedera helix*, a plant tissue which can cold acclimate, was chosen. The NMR transverse relaxation properties of ivy bark were studied in some detail to determine if the principle of NMR used by Conlon and Outhred (4) could be used to measure P_d of ivy membranes. A study of NMR relaxation processes in ivy bark is also of interest since relaxation times may be interpreted to indicate the degree of molecular mobility of cellular water (2, 12).

MATERIALS AND METHODS

NMR Measurements. An NMR spectrometer similar to the design by Clark (3) operated at 18.5 MHz was used for the relaxation time measurements. The signal was averaged and recorded with a signal averager (Tracor Northern NS-575). For most measurements the sample was maintained at 20 C. To study hydration water, subzero temperatures were obtained by controlling the rate at which cold gaseous N_2 passed by the NMR sample tube. T_2 was measured using the Hahn spin-echo pulse sequence or the Carr-Purcell-Meiboom-Gill pulse sequence (8). T_1 was measured using the 180°, τ, 90° pulse sequence or the 90°, τ, 90° pulse sequence (8).

Plant Material. *Hedera helix* L. cv. Thorndale (ivy) bark was chosen because it provides a relatively uniform cell size. Both epidermal and cortical cells are parenchyma cells of about the

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[3] Abbreviations: NMR: nuclear magnetic resonance; T_1: longitudinal relaxation time; T_2: transverse relaxation time; P_d: diffusional water permeability coefficient; PMR: proton magnetic resonance; R_c: rate change; HSE: Hahn spin-echo pulse sequence; CPMG: Carr-Purcell-Meiboom-Gill pulse sequence.

same size. We assumed that the cells were spherical. The average cell diameter determined from 160 cells was 36 μm.

Bark was peeled from ivy stems and cut into small pieces (size range of 1–4 mm) with a razor blade. The bark was then put into NMR sample tubes (i.d., 6 mm) and gently packed to the bottom. If measurements were to be made on bark exposed to solution, vacuum infiltration (pressure, 252 mm Hg) was used to remove air from the intercellular spaces. Following vacuum infiltration excess solution was poured out of the sample tube. Further removal of excess extracellular solution was done by blotting with Kimwipes until only a film of Mn^{2+} solution covered the bark surfaces. The excess extracellular solution was removed so that the extracellular signal from the Mn^{2+} solution would not interfere with measurement of the intracellular water relaxation time. Twenty cycles of vacuum infiltration were given where one cycle represents the time for the vacuum to reach minimum pressure plus 2 min at minimum pressure before the vacuum was released. Since vacuum infiltration took 1 hr, the first NMR measurement was made 1 hr after the bark was exposed to solution.

The relaxation properties of live and dead ivy bark were studied to determine membrane effects. An ivy bark sample was placed into a NMR sample tube and T_1 and T_2 were measured for living bark. To kill the ivy bark cells the stoppered sample tube containing the bark was plunged into liquid N_2, left there for 2 min, and then thawed at room temperature. Following thawing, T_1 and T_2 of the dead bark were measured.

Sample water content was measured gravimetrically by drying in a vacuum oven at 80 C for 19 hr. Sample water content was also measured by NMR. At fixed gain settings of the receiver, the height of the free induction decay 250 μsec after the 90° pulse of radio frequency energy was determined for quantities of water (1 mM $MnCl_2$ solution) ranging between 0 and 100 mg. A standard curve of signal height *versus* quantity of water was then plotted. The signal height from an ivy bark sample was then measured and the quantity of water estimated from the standard curve (19).

NMR Relaxation in Isolated Cell Walls. To determine if ivy bark cell walls cause water in their vicinity to have a small T_2 structurally intact cell wall material was isolated and then rehydrated so that the T_2 of water in close proximity to cell walls could be measured. To remove lipid materials the bark was soaked in a large volume of 95% ethanol for 2 hr. Next, to remove water-soluble materials the ethanol-washed bark was put into "tea bags" (7) and the tea bags were put into a large beaker. Tap water was continuously run into the beaker for 24 hr. The bark following washing in ethanol and water represents the "cell wall" material used for the NMR measurements. The cell wall material was then dried for 2 hr at 85 C. To rehydrate the bark it was dipped into distilled H_2O for various lengths of time and then blotted with Kimwipe tissue to remove water adhering to the outer surface. The amount of rehydration was measured gravimetrically following a measurement of T_2.

Hydration Water in Ivy Bark. To measure unfrozen water at subzero temperatures the amplitude of the free induction decay 250 μsec after the 90° pulse was measured. The 250-μsec time delay allowed for complete decay of nonmobile proton signals, such as from ice. The temperature was decreased in steps of 3 to 6 C and at least 20 min were allowed for temperature equilibration at each temperature. The proton magnetic resonance signal height was determined as a per cent of the signal height in the sample before it was frozen. Following NMR measurements the water content of the bark was determined gravimetrically. The tissue water content at subfreezing temperatures was calculated from the tissue water content before freezing and the per cent reduction in the PMR signal at freezing temperatures.

Chemical Exchange in NMR Relaxation. The effect of $CaCl_2$ and $MnCl_2$ on chemical exchange in ivy bark was studied. Bark was vacuum-infiltrated in solutions containing $CaCl_2$ and $MnCl_2$ or in solutions containing only $MnCl_2$. T_2 was then measured, by both the HSE and CPMG techniques, as a function of the time bark was in solution. At each time the rate change (R_c) was calculated as the difference in values of ($1/T_2$) measured by HSE and CPMG. A relaxation rate is simply expressed as the reciprocal of the appropriate relaxation time. If this rate change were due to chemical exchange, as believed, it would equal $P_\alpha P_\beta (S\omega)^2/k$, where P_α is the proportion of protons at α sites, P_β is the proportion of protons at β sites, $S\omega$ is the chemical shift between site α and site β, and k is the rate constant for exchange between site α and site β (8). Populations α and β referred to here need not be the same populations *a* and *b* referred to when discussing the Conlon-Outhred technique of measuring water exchange.

RESULTS AND DISCUSSION

Source of PMR Signal from Ivy Bark and the PMR Relaxation Times. The water content estimated by NMR was 104 ± 5% ($\bar{X}$ ± SE, N = 9) of the water content measured gravimetrically indicating that the PMR signal is predominantly from bark water. A slight overestimate of the water content by NMR is expected since soluble solids (*e.g.* sugars) have long relaxation times and will therefore contribute to the signal being observed 250 μsec after the 90° pulse (19). However, since the value from the NMR technique was not statistically significantly higher than that measured gravimetrically, it can be assumed that the ivy bark PMR signal arises only from bark water.

HSE measurements revealed the presence of at least two populations of water, each with different PMR relaxation characteristics (Table I). One population, representing 32 ± 5% of the total water signal, had a T_2 of 10 ± 1 msec. The second population, representing the remaining 68 ± 5% of the water signal, had a T_2 of 64 ± 2 msec. Equations presented by Hazlewood *et al.* (12) demonstrated that the measured population size ($P'_{a,b}$) need not equal the actual population size ($P_{a,b}$). The presence of two populations of water was also observed from T_1 measurements. One population representing 40 ± 1% of the total water signal had a T_1 of 28 ± 4 msec and a second population representing 60 ± 1% had a T_1 of 182 ± 8 msec (Table I). The population fraction with the shorter T_2 and T_1 was labeled population *b* and the population fraction with the longer T_2 and T_1 was labeled population *a* (Table I).

The value of the larger T_2 depends upon the method of measurement (Table I). A larger value for T_2 was measured, with the CPMG technique, suggesting that either chemical exchange or diffusion in a field gradient is shortening the value of the T_2 measured by the HSE technique (8).

The applied magnetic field used for NMR has a gradient of about 30 mgauss cm^{-1} across the sample which is much too weak (8) to account for the difference in transverse relaxation rates $R_c = (T_2)^{-1}_{HSE} - (T_2)^{-1}_{CPMG}$. Heterogeneity of the sample can cause local changes in sample magnetization over distances of the order of a cell radius. It can be calculated from plausible estimates of the magnetic susceptibility of cell material that local field gradients associated with heterogeneous magnetization could possibly account for the value of R_c = 3.7 sec^{-1} (1/64 − 1/84) measured (Table I). Experiments with $CaCl_2$ and $MnCl_2$ added to the extracellular water, rule out diffusion in local field gradients as the mechanism responsible for R_c.

$MnCl_2$ increased R_c and $CaCl_2$ decreased R_c (Table II). These results with $CaCl_2$ and $MnCl_2$ indicate that the rate change R_c is due to an extracellular event since the effect of the cations occurs within 1 hr, a time period when little intracellular penetration of the cations has occurred.

The larger population of water which has the larger T_2 is believed to be intracellular water. The presence of $MnCl_2$ or $CaCl_2$ in the extracellular water could not alter diffusion of intracellular water. It has been suggested (11) that HSE T_2 values of animal cells are shortened by diffusion in local magnetic field gradients. More recent studies indicate that animal cell T_2 values are not shortened by diffusion in local magnetic field gradients (2, 12).

Table I. NMR relaxation times of ivy bark water.

Population[1]	Measured relaxation time[2] (msec) T_2' CPMG	T_2' HSE	T_1'	Measured size of the[3] population fraction from HSE T_2	from T_1
a	84 ± 3	64 ± 2	182 ± 8	0.68 ± 0.05	0.60 ± 0.01
b	9(n=1)	10 ± 1	28 ± 4	0.32 ± 0.05	0.40 ± 0.01

[1] The water fraction with a short T_2 was labelled b and the water fraction with a long T_2 was labelled a.

[2] The values are $\bar{x} \pm$ SE for n = 3.

[3] The population fractions measured from T_1 and T_2 measurements were not significantly different at P = 0.05.

Table II. The influence of $MnCl_2$ or $CaCl_2$ on the rate change R_c $((1/T_2)_{HSE} - (1/T_2)_{CPMG})$.

Treatment	Time exposed to soln (hrs)	T_2 (msec)[1] CPMG (t_{cp} = 5 msec)	HSE	R_c (sec^{-1})	Rate change as a percent of the rate change at zero time
25 mM $MnCl_2$ plus 1 mM $CaCl_2$	0	89	58	6	100
	1	68	41	10	170
	4	51	34	10	170
30 mM $MnCl_2$	0	100	76	3	100
	4.5	52	41	5	170
100 mM $CaCl_2$ plus 5 mM $MnSO_4$	0	90	64	4	100
	4	98	97	0	0
	6	132	114	1	25
	8	134	124	1	25

[1] The T_2 values are for popn a.

Fedotov *et al.* (9) concluded that the HSE T_2 of plant cells was shortened by chemical exchange between water protons and cell wall hydroxyl protons.

Identification of Populations of Water That Give Rise to Two Relaxation Times. Water associated with bacterial cell walls has a short T_2 (17). To test if the T_2 of 10 ± 1 msec could be due to cell wall relaxation, a crude cell wall preparation was made and the T_2 for cell walls of varying water content was measured (Table III). It was found that water associated with ivy cell walls does have a short T_2. At the higher water contents (1.2–3.01 g/g dry weight) the value of the smaller T_2 was near that of the short T_2 (10 ± 1 msec) found for intact ivy bark. This experiment suggests that the 10 ± 1 msec T_2 of intact ivy bark is at least partly from extracellular water.

Hazlewood *et al.* (12) recently measured a T_2 of 5 msec in skeletal muscle which was attributed to hydration water of macromolecules. If hydration water of ivy bark also has a T_2 near 5 msec it would probably not be resolved as a separate signal, but would be included in the water population (P'_b) found to have the T_2 of 10 ± 1 msec. Since the water population, P'_b, represents 32 ± 5% of the cellular water it is unlikely that it is all from extracellular water because estimates of plant free space are in the 5 to 10% range (26). Therefore, the water population, P'_b, may actually contain more than one type of water. Bulk water freezes at a higher temperature than hydration water (1). If water population P'_b contains hydration water and population P'_a contains only bulk water, there should be a smaller decrease in population P'_b than in population P'_a when bark is exposed to subzero temperatures. The presence of hydration water in water population P'_b was tested for in this way by freezing cold-acclimated ivy bark. This type of experiment was difficult to do with nonacclimated bark, because of the lower amount of unfreezable water in nonacclimated bark (21). P'_a decreases and P'_b increases (by definition $P'_a + P'_b = 1$) at subzero temperatures (Fig. 1) indicating that P'_b contains hydration water. This hydration water would be both intracellular and extracellular.

Table III. The transverse relaxation time of hydrated "cell wall" material.

Degree of hydration measured gravimetrically (gH_2O/g dry weight)	HSE T_2 (msec) for Popn b	for Popn a	P'_a
0.21	≈0.8	none	
0.47	≤2.0	none	
1.21	6.5	none	
1.84	4.5	18	----
3.01	7.0	32	0.41

Evidence that the long T_2 is from intracellular bulk water was acquired by shortening the extracellular T_2 by adding Mn^{2+} to the extracellular water. The larger T_2 of ivy bark after 1 hr in a 25 mM

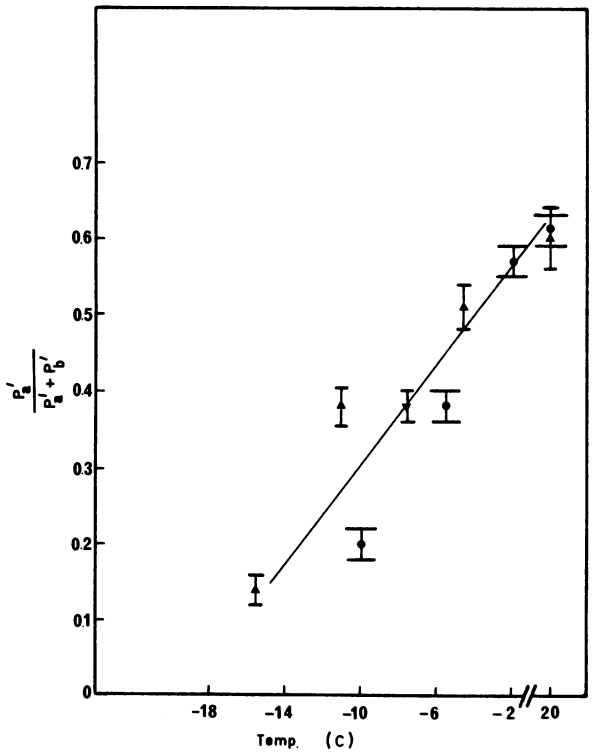

FIG. 1. Relative size of water population with long T_2 (population *a*) for cold-acclimated ivy bark at subzero temperatures. Error bars indicate error of each measurement for plant 1 (▼) and plant 2 (●).

$MnCl_2$ solution had a value near 30 to 45 msec (Fig. 2). The T_2 of 30 to 45 msec must be from intracellular water since extracellular water would have a small T_2 value near that of the $MnCl_2$ solution (1.1 msec for 25 mM $MnCl_2$). It can be concluded that most of the 10 ± 1 msec population is extracellular water since Mn^{2+} can only have the effect of shortening T_2. If intracellular water of untreated ivy bark has a T'_{2a} of 58 to 68 msec (Fig. 2) it could easily have its T_2 decreased to 30 to 45 msec by the addition of extracellular Mn^{2+} owing to the Mn^{2+} decreasing the contribution of the backflux term (23).

The T_2 time dependence for ivy bark exposed to Mn^{2+} (Fig. 2) is similar to that observed for *Elodea* leaves (22). For this experiment vacuum infiltration was done in $CaCl_2$ solution before exposing the bark to $MnCl_2$ so that the effect of Mn^{2+} penetration into the bark could be observed. Evidence was obtained using *Elodea* leaves and radioactive Mn^{2+} that the initial rapid decrease of T_2 (Fig. 2, region I) is related to time being required for Mn^{2+} to diffuse throughout the extracellular space and reach the cell membrane where the Mn^{2+} would become effective in relaxing protons on water molecules diffusing out of the cells. The slow gradual change in the value of T_2 over several hr (Fig. 2, region II) is believed due to Mn^{2+} penetration into the cell where it would alter the dynamic magnetic interactions occurring inside the cell (23). The Mn^{2+} influx rate across the ivy bark plasmalemma was calculated by the method proposed by Stout *et al.* (22) to be 1.7 × 10^{-15} mol cm^{-2} sec^{-1} when extracellular $MnSO_4$ was present at concentrations of 5, 10, or 20 mM. This influx rate is typical for divalent cations. Spanswick and Williams (20) measured an efflux rate for $^{45}Ca^{2+}$ from *Nitella* cells of 4.6 × 10^{-14} mol cm^{-2} sec^{-1}. Mn^{2+} penetration into ivy bark cells is therefore not rapid. The independence of flux rate on Mn^{2+} concentration implies that uptake sites were saturated by the lowest Mn^{2+} concentration used.

Measurement of Water Exchange Time. Because transverse relaxation in ivy cell walls is not slow, the value of the intracellular water T_2 is at least partly determined by the mechanism of water exchange across the membrane to extracellular regions where transverse relaxation is fast. Therefore the T_2 of intracellular water, measured in the absence of extracellular Mn^{2+}, is a measure of T'_{2a} and not of T_{2a}. Since T_{2a} of ivy bark cannot be measured directly, P_d cannot be calculated using the Conlon-Outhred technique in its simplest form. In a similar experiment it was possible to measure directly T_{2a} of *Elodea* leaves (22). It is not known why *Elodea* cell walls do not cause the T_2 of its extracellular water to be short. It may be that ivy cell walls, but not *Elodea* cell walls, contain paramagnetic ions which act as PMR relaxation centers. It is known that *Chlorella* cell walls accumulate Mn^{2+} when cells are exposed to $MnCl_2$ solution due to their cation exchange capacity (23).

When the actual value of the extracellular T_2 (T_{2b}) is small compared to the value of τ_a and T_{2a} then to good approximation the equation:

$$\frac{1}{T'_{2a}} = \frac{1}{T_{2a}} + \frac{1}{\tau_a} - \frac{P_a T_{2b}}{P_b \tau_a^2} \qquad (1)$$

describes transverse relaxation (23). The third term on the right side of equation 1 is equivalent to the backflux term of Conlon and Outhred (4). A Conlon-Outhred experiment involves making the backflux term negligible by adding extracellular Mn^{2+} to make T_{2b} small. It can be seen from equation 1 that a plot of $(1/T'_{2a})$ *versus* T_{2b} should be a straight line with slope equal to $P_a/(P_b\tau_a^2)$ and intercept equal to

$$\left(\frac{1}{T_{2a}} + \frac{1}{\tau_a}\right).$$

To determine τ_a for ivy bark cells it was necessary to measure T'_{2a} as a function of T_{2b} so that τ_a could be calculated from the slope. A range of T_{2b} values was acquired using different concentrations of extracellular Mn^{2+} and assuming that T_{2b} equaled the T_2 of the Mn^{2+} solutions. It was recognized that this assumption was not strictly valid since the real T_{2b} would depend upon the cell wall relaxation effect as well as on the added extracellular Mn^{2+} effect.

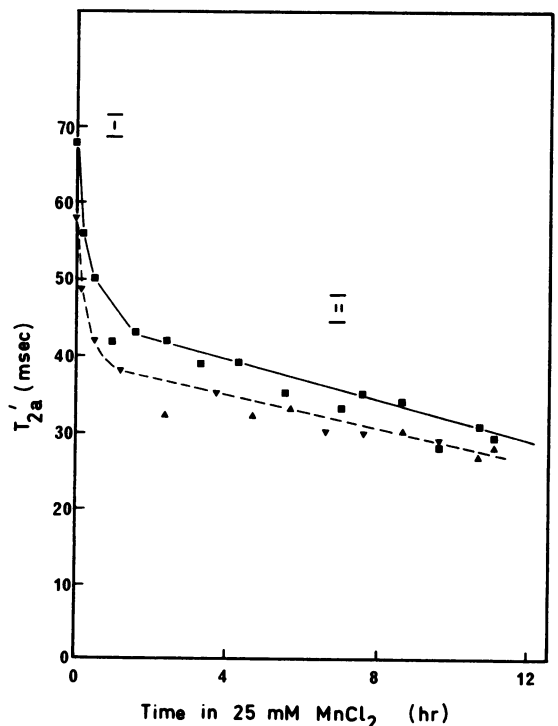

FIG. 2. T_2 for intracellular water protons of ivy bark, in the presence of extracellular 25 mM $MnCl_2$, as a function of time. The bark had been vacuum-infiltrated in 26 mM $CaCl_2$ before being put into $MnCl_2$ solution. The Mn^{2+} solution also contained 1 mM $CaCl_2$. T_2 was measured by the HSE technique. Data shown for plant 1 (■) and plant 2 (▼).

Ivy cell walls might also be expected to accumulate Mn^{2+} due to their cation exchange capacity.

To calculate τ_a from a plot of $(T'_{2a})^{-1}$ *versus* T_{2b} it is necessary to know the values of P_a and P_b. No experimental evidence was acquired to estimate the free space of ivy bark. If it is assumed that the extracellular volume (free space plus intercellular air space) is equal to 20% of the bark volume then P_a equals 0.8. From the measured slope (Fig. 3) and the value 0.8 for P_a, τ_a was estimated to be approximately 40 msec. If a value of 0.7 had been chosen for P_a, τ_a would be 30 msec and if a value of 0.9 had been chosen for P_a, τ_a would be 60 msec. Thus assuming P_a equal to 0.8 can only result in τ_a being in error by less than a factor of 2.

Once τ_a was estimated from the slope of the line (Fig. 3) the rate $(T_{2a})^{-1}$ was estimated from the intercept ($T_{2b} = 0$) of the line to be 4 sec^{-1} for ivy bark. This rate of 4 sec^{-1} would be caused by all of the relaxation mechanisms other than intra-extracellular water exchange. One mechanism that contributes to $(T_{2a})^{-1}$ is chemical exchange. The total chemical exchange rate was estimated earlier to be 3.7 sec^{-1} (Table I). Within experimental error chemical exchange can explain the value of 4.0 sec^{-1} estimated for $(T_{2a})^{-1}$ and so other relaxation mechanisms have little influence on $(T_{2a})^{-1}$. The usual nuclear dipole-dipole interaction between water molecules and interaction with paramagnetic impurities within the cell are other mechanisms that might be expected to affect $(T_{2a})^{-1}$. The dipole-dipole mechanism is weak in pure water and would account for about 0.1 sec^{-1}. With air dissolved in the water the rate is typically 0.5 to 1 sec^{-1}. However, the dipole-dipole mechanism is affected by viscosity of the cell water, and it has been suggested that cell water viscosity exceeds that of pure water and would thus increase the contribution to relaxation from this mechanism (9, 12). The present results indicate that this mechanism is not strong in ivy bark.

Mineral analysis revealed that ivy bark contained 30 ± 3 μg/g (based on dry weight) Mn and 6 ± 1 μg/g Fe. Knowing these cation concentrations and the bark per cent water it was calculated that if the cations were freely dissolved in the bark water the transverse relaxation rate due to these paramagnetic ions would be 8.3 sec^{-1}. Since interaction with paramagnetic impurities does not contribute significantly to $(T_{2a})^{-1}$ it can be concluded that paramagnetic cations are associated with cell constituents which either physically isolate the cations from cell water or alter the number of free electrons, thus decreasing the effectiveness of the paramagnetic cations on NMR relaxation (9).

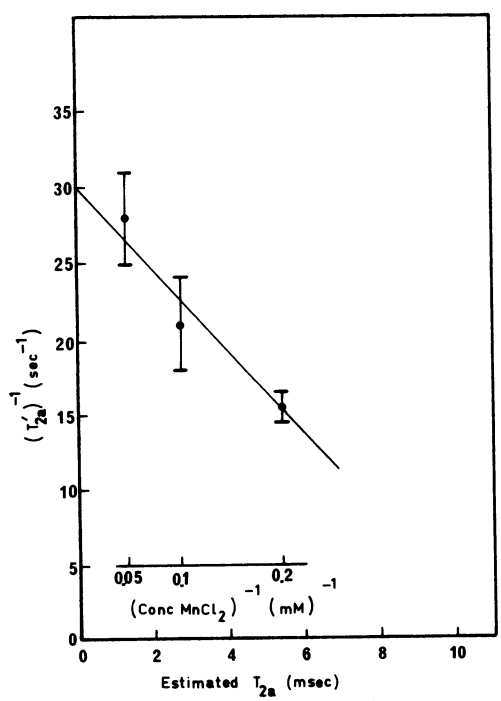

FIG. 3. Transverse relaxation rate of intracellular water protons of ivy bark, as a function of T_{2b}. Values are $\bar{X}$ ± SD. Sample size was four for 5 mM, six for 10 mM, and three for 20 mM. Transverse relaxation rates were determined from T_2 measured by the HSE technique.

Our model for relaxation in ivy bark is that there is fast relaxation in extracellular regions due to concentration of paramagnetic ions in cell walls during growth, and chemical exchange. Intracellular relaxation is due to water exchange through the membrane to the fast relaxing extracellular region. Both extracellular mechanisms affect intracellular water relaxation through the permeability of the membrane. The relative importance of these mechanisms cannot be assessed from our data. Dipole-dipole relaxation and intracellular paramagnetic cations do not contribute significantly to intracellular relaxation.

Evidence that extracellular regions provide relaxation centers for intracellular water (through water exchange across the membrane) is contained in an experiment measuring T_1 and T_2 in dead ivy bark. Both T_1 and T_2 were decreased (Table IV) in value after ivy bark was killed by freezing in liquid N_2. The decreased relaxation times can be explained by disrupted cell membranes which decrease τ_a. In the live ivy bark, the cell size and permeability of the intact membrane control the access of intracellular water to the extracellular regions where relaxation is fast.

Determination of Water Exchange from CPMG Measurements of T_2. It has been concluded that the rate $(1/T'_{2a})$ in a HSE experiment contains a significant contribution from chemical exchange. Since the CPMG technique eliminates the effect of chemical exchange on T_2, and the rate from other mechanisms in intracellular water is small ($\ll$ 4 sec^{-1}), the $(1/T_{2a})$ term of equation 1 can be ignored when the CPMG technique is used. τ_a can then be calculated from the intercept of a plot of $(T'_{2a})^{-1}$ *versus* the reciprocal of Mn^{2+} concentration since T_{2b} is proportional to the reciprocal of Mn^{2+} concentration. Using concentrations of $MnCl_2$ up to 200 mM, it was found that the rate $(T'_{2a})^{-1}$ did not change linearly with the reciprocal of Mn^{2+} concentration (Fig. 4). A linear change would be due to the backflux term being decreased by increasing concentrations of Mn^{2+}. The nonlinearity indicates that the Mn^{2+} might be affecting τ_a. A decrease of P_a by $MnCl_2$ would also result in nonlinearity of the plot, but this is not believed to occur appreciably in ivy bark. From the measured osmotic potential of ivy bark ($\psi_{os} = -17 \pm 1$ bar) cell plasmolysis would not occur until the $MnCl_2$ reached a concentration of 238 mM assuming complete dissociation, yet the nonlinearity of the plot begins to occur at a lower concentration. Also high concentrations of extracellular sucrose did not cause this type of nonlinearity (21). Thus, P_a is not being decreased as a result of cell shrinkage. The nonlinearity of the plot does not arise from increased Mn^{2+} flux into the cells since the change of T_2 with time in Mn^{2+} solution was similar for bark exposed to high Mn^{2+} concentration and for bark exposed to low Mn^{2+} concentration (21). Since diffusion of water through the intracellular water would not be affected by extracellular Mn^{2+}, τ_a is being decreased by high Mn^{2+} concentration due to P_d being increased and not due to high Mn^{2+} concentration affecting intracellular diffusion.

Table IV. NMR relaxation times for water of living and dead ivy bark.

Sample	Relaxation times for Popn a	Popn b	P'a
	HSE T_2 (msec)		
Living bark	77	10	0.48
Dead bark	21	5	0.55
	T_1 (msec)		
Living bark	163	15	0.64
Dead bark	22	13	0.51

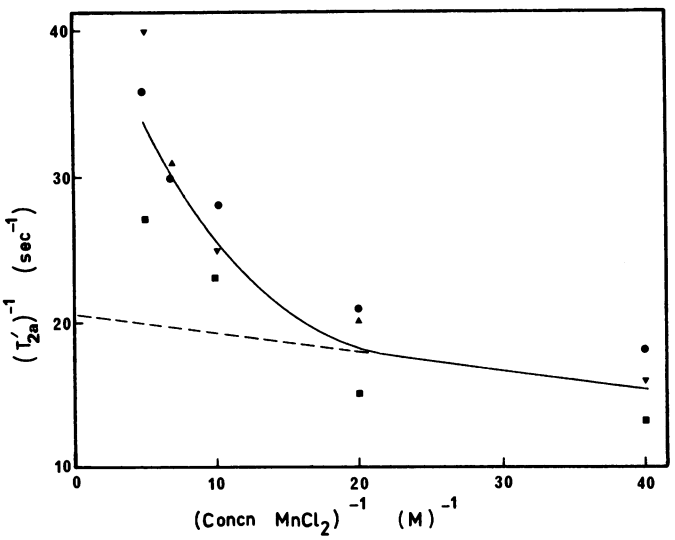

FIG. 4. Transverse relaxation rate of intracellular water protons of ivy bark as a function of $1/Mn^{2+}$. Transverse relaxation rates were determined from T_2 measured by the CPMG technique (t_{cp} = 5 msec). Data for plant 1 (■), plant 2 (▼), and plant 3 (●).

If the water exchange time is estimated using only the results for 25 mM and 50 mM $MnCl_2$ (Fig. 4), a τ_a in the range 40 to 60 msec is estimated. This estimate of τ_a agrees well with the value of 40 msec estimated for τ_a in the HSE experiment where the highest concentration of Mn^{2+} used was 20 mM. Thus τ_a is not altered significantly by Mn^{2+} concentrations less than 50 mM. If the lowest concentration of Mn^{2+} used, 5 mM, does not alter membrane water permeability significantly, it can be concluded that the water exchange time is approximately 40 ± 20 msec.

Calculation of the Diffusional Water Permeability Coefficient. When the water exchange time is controlled only by membrane permeability, P_d can be calculated using $P_d = V/(A\ \tau_a)$. The test that τ_a is being controlled only by membrane water permeability and not by intracellular unstirred layers is that the quantity ($3D\ \tau_a/r^2$) is greater than one for spherical cells (22). For ivy bark, r = 18 μm, $\tau_a \approx 40$ msec, and $D = 1.43 \times 10^{-5}$ cm^2 sec^{-1} (21). The value 0.53 for ($3D\ \tau_a/r^2$) is less than 1, indicating that τ_a of ivy bark is not solely limited by membrane water permeability. However, the value 0.53 is in the range 0.5 to 1.0, indicating that a theoretical correction factor can be applied to estimate P_d. A value for P_d of approximately 3×10^{-2} cm sec^{-1} at 20 C is calculated for ivy bark cells after correcting for internal unstirred layers by the method described by Stout *et al.* (22). This P_d is only accurate by a factor of 1 or 2 due to the uncertainty in the estimate of τ_a. It should also be pointed out that it is not known what effect the internal cell structure will have on this calculation. It is expected that the internal structure of the cell might have the effect of decreasing the effective cell radius. If this is true and a correction could be made for the reduced cell radius, the correction would be in a direction that would reduce the uncertainty in the determination of P_d.

Existing water permeability measurements for plants indicate a wide range of values. For example, P_w (permeability coefficient of water) values near 10^{-2} cm sec^{-1} have been measured for giant algal cells (6) while a P_d of 4.5×10^{-6} cm sec^{-1} was measured for maize roots (13). According to Dainty (5) the "only good" estimate of a plant cell P_d gave a value of 2.4×10^{-4} cm sec^{-1} for *Valonia* cells (10). It is not known if the wide range of estimates for P_d is due to the different experimental techniques by which they were measured or if they represent a species difference. The value of P_d estimated for ivy bark indicates a highly permeable membrane. Our determination of P_d for ivy bark along with the estimate of P_d for *Valonia* cells (10) suggest that species differences result in P_d values differing by a factor of 100. P_d values for plant cells differing by a factor of 30 or more have been measured by NMR. *Elodea* leaves were found to have a $P_d \gtrsim 4.7 \times 10^{-2}$ cm sec^{-1} (22) and *Chlorella* cells were found to have a $P_d = 2.1 \times 10^{-3}$ cm sec^{-1} (23). If the estimate of P_d for ivy bark is typical of multicellular plant membranes, the membrane water permeability of multicellular plants is high and within the range found for liposomes (15) and single cells (6, 23) rather than extremely low as reported by Jarvis and House (13). Using our estimate of P_d for ivy membranes it was argued that it would not be necessary for P_d to increase during cold acclimation of ivy plants to prevent intracellular ice formation during natural freezing (24).

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