BRIEF COMMUNICATION

[^{1}H]SPIN-ECHO NUCLEAR MAGNETIC RESONANCE IN PLANT TISSUE

I. The Effect of Mn(II) and Water Content in Wheat Leaves

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ABSTRACT The effect of age-dependent Mn(II)-gradients, as observed by electron paramagnetic resonance (EPR), on the [^{1}H]NMR spin-spin relaxation time (T_2) was studied in wheat leaves. A non-exponential T_2 spin-echo decay was always observed, revealing the presence of at least two different fractions of non- (or slowly) exchanging water in the leaves. No effect of the Mn(II)-concentration on T_2 of the separate water fractions (covering ~90% of the total water content) has been found. From these observations we conclude that Mn(II) is present in bound form. The dependence of T_2 on water content can be explained with a two-state model, demonstrating the occurrence of fast exchange within each of the two slowly exchanging water fractions.

INTRODUCTION

Proton-spin-lattice (T_1) and Proton-spin-spin (T_2) relaxation times have been widely used for the study of the physical properties of water in biological systems (1–4) and a relationship between T_1, T_2 and water content has been demonstrated (1, 5, 6, 7). The interpretation of the results is complicated by the complexity of the specimen. The main difficulties arise from the fact that to the measured parameters (T_1, T_2) contributions may be made by factors not directly related to the physical properties of water. It is well-known, that plants contain Mn(II) and other paramagnetic metalions as a constituent of the photosynthetic unit (8). These paramagnetic centers may shorten the relaxation time of water protons, obscuring the correlation between T_1, T_2 and water content.

Fedotov et al. (4) have shown that there is no, or only a small, effect of paramagnetic ions on T_1 of water in intact tissues of bean and crinum leaves, nor is there one in potato tuber. Samuilov et al. (9), using the Overhauser effect have found no effect of paramagnetic ions in seeds of Welsh onions, peas, broad beans and sunflower. According to Hazlewood et al. (2)

†H. van As and T. J. Schaafsma were deeply saddened by the death of W. P. A. van Vliet on July 5, 1980.

the relaxation mechanism in skeletal muscle is insignificantly affected by paramagnetic impurities. Stout et al. (10) suggest, however, that in ivy bark T_2 of extracellular water is shortened by paramagnetic ions in the cell walls. In aqueous suspensions of chloroplasts Wydrzynski et al. (19) concluded that T_1 and T_2 are largely determined by manganese, bound in the chloroplast membrane.

Here, we report the results of measurements on leaves of summer wheat, type SELTEP. Using EPR, we detected Mn(II)-gradients in the leaves, changing sign upon maturation. The effect of these gradients on the spin-spin relaxation time T_2 of tissue water was investigated as a check on the correlation between T_2 and water content. This research is part of a program aimed at the use of spin-echo NMR techniques to measure transport and exchange of water in plant stems (11, 18) and tissues.

MATERIALS AND METHODS

Plant Material

Wheat variety SELTEP, was grown under a 16-h light (23°)/8-h dark (20°C) cycle. No special culture was used. Proton spin-spin relaxation measurements were carried out using leaves in different stages of maturation and at different positions in the plants. Leaves were cut transversally in ~2 cm sections for NMR and EPR measurements. EPR signal amplitudes were not affected by waiting periods of ~2 h after segmentation.

EPR Measurements

Room temperature EPR spectra were obtained using a Varian E-3 spectrometer (Varian Associates, Instrument Div., Palo Alto, Calif.). Samples were contained in a quartz tube, 0.8 mm i.d., permitting reproducible sample positioning. Using standard precautions, the EPR signal amplitude of a 0.175 mM $MnCl_2$ solution was reproducible within 3%. Instrument settings: 100 kHz modulation amplitude, 10 G; microwave power, 40 mW; time constant, 0.3 s; scan rate, 250 G/min.

NMR Measurements

The experiments were carried out with a home-built 15-MHz spin-echo spectrometer, equipped with a Newport 7-in electromagnet (Newport Laboratories, Inc., Santa Ana, Calif.) (11), a home-made transmitter/receiver coil of the solenoid-type, with a length of 5 mm and a diameter of 11 mm. The spin-spin relaxation time T_2 was measured by the Carr-Purcell-Meiboom-Gill (CPMG) method (12). Because of the inhomogeneity and the size of the samples a time-dependent baseline ("baseline drift") must be expected (13, 14). A first order correction for this phonomena was made by the following pulse sequence:

$$90^\circ_x - \tau - (-180^\circ_y - 2\tau)_n - 5T_1 - 90^\circ_{-x} - \tau - (-180^\circ_y - 2\tau)_n - 5T_1.$$

By substracting the two CPMG-decays, the effect of missettings in the 180° pulses was diminished. The CPMG T_2 decay was measured by sampling the height of the echoes. The time 2τ was set on 1.6 ms. A graphical method as outlined by Hazlewood et al. (2) was used to analyze the data. The error in T_2 was evaluated at ±8%. All NMR experiments were made at probe temperature (29°C).

Water Content

Water content was determined by weighing freshly excised samples, drying until constant weight in an oven at 90°C, followed by reweighing.

Manganese Determination

The total manganese content in the wheat leaf segments was determined by atomic absorption spectroscopy. Measurements were made with a Perkin-Elmer HGA-74 type 460 atomic absorption

spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Dried leaf segments were digested with 100 μl concentrated $HClO_4$ at ≃150°C. Digested samples were evaporated and dissolved in 100 μl concentrated HCl. The final volume was brought to 100 ml by adding distilled water. Standards at 0.02, 0.06, and 0.10 ppm Mn were prepared in an acidified water medium.

RESULTS AND DISCUSSION

EPR signals are easily detected in wheat leaves. Two types of spectra can be discerned: (*a*) a sextuplet hyperfine structure, typical for Mn(II), and (*b*) a weak peak close to the center of the manganese multiplet.

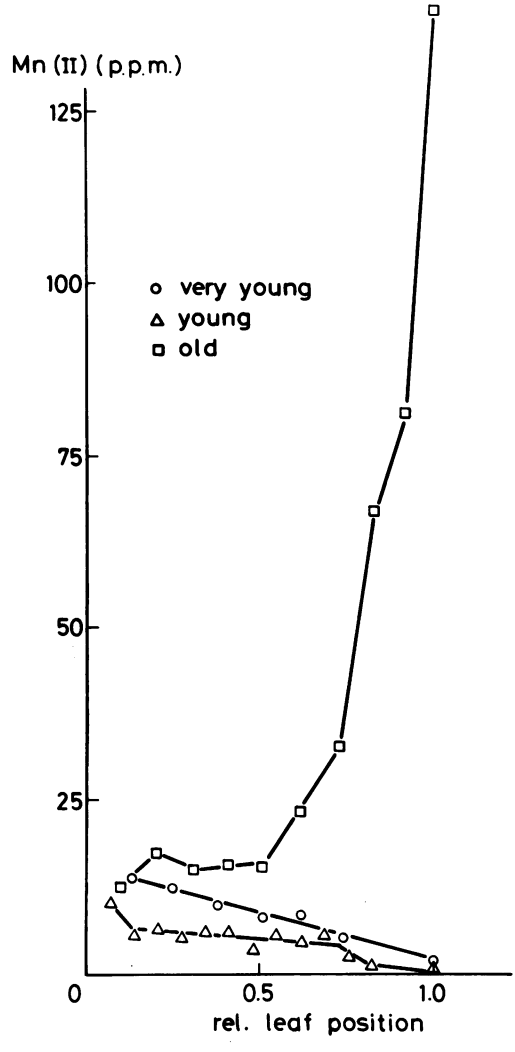

FIGURE 1 Mn(II) in ppm of dry matter vs. the position in the leaf. The relative position is defined as the ratio of the distance from the base of the leaf and the total leaf length. The results are given for three wheat leaves in different stages of maturation: (○) very young, not full-grown (eighth leaf of a total of eight, growth stage of the wheat, after the decimal code of Zadoks et al. (20, 17); (△) young, nearly full-grown (ninth of a total of ten, growth stage 19); (□) old, full-grown (sixth of a total of ten, growth stage 19). Mn(II) is measured by EPR.

Fig. 1 represents the results of Mn(II) EPR measurements for three wheat leaves in various stages of growth. For very young leaves the Mn(II) signal decreases from base to top; upon maturation this Mn(II) gradient reverses sign.

We measured T_2, Mn(II), and water content of wheat leaves at various positions between the base and the top of the leaf, using spin-echo NMR, EPR, and drying to constant weight, respectively (Fig. 2). Invariably, a nonexponential T_2 decay was observed, indicating that there are several fractions of tissue water with distinguishable T_2-values. Because of the limited signal-to-noise ratio we have used two water fractions, constituting 90% of the total tissue water for data fitting. The resulting T_2-values of the two fractions differ by a factor of 3. The ratio between the amplitudes of the slowest and the fastest relaxing water fractions varies from 1 to 2 for young vs. old leaves. Considerable scatter in the T_2-values of the two separate fractions occurs due to a strong correlation between the fitting parameters (amplitudes and relaxation times). A mean effective relaxation rate $\overline{R}_2$ is defined by:

$$\overline{R}_2 = P_a T_{2a}^{-1} + P_b T_{2b}^{-1}. \tag{1}$$

Here P_a and P_b designate the two fractions of tissue water. In solution, R_2 is linearly dependent on the Mn(II)-concentration, but such linear relationship does not obtain in either type of leaf (Fig. 2) for R_2 of the separate fractions.

In older leaves, the sign of the Mn(II) gradient is reversed compared with younger leaves (Fig. 1), but the dependence of the $\overline{R}_2$ curve on the position in the leaf does not change. Evidently, the effect of the Mn(II) gradient measured by EPR is not reflected in the R_2 values of tissue water determined by NMR. This indicates that Mn(II) is present in bound form inaccessible to water on the timescale of our experiments. This is not in conflict with the fact Mn(II) has been detected by EPR by Meirovitch and Poupko (15) and by other authors. Siderer et al. (16) have observed Mn(II) EPR signals in lettuce chloroplasts similar to our experimental results on intact tissue. Mn(II)-concentrations (Figs. 1 and 2) are calculated from the EPR spectra using aqueous $MnCl_2$ solutions for calibration. Whether these Mn(II) signals in wheat leaves have the full possible intensity or only 9/35 of the total intensity ($M_s = \pm 1/2$ transitions) (15, 16) cannot be decided from the observed spectra.

When all manganese is present as Mn(II) in a bound form, allowing the observation of only the $-1/2 \rightleftarrows 1/2$ EPR transitions, the amount of manganese deduced from atomic absorption and EPR measurements should be in a ratio of ~4. We observed a ratio of ~8. However, manganese is not necessarily present as Mn(II). In dark-adapted chloroplasts Wydrzynski et al. (19) have found that only part of the bound manganese is present as Mn(II).

Because Mn(II) had no effect on the spin-spin relaxation time of >90% of the total water, we will now consider the relationship between T_2 of the water protons and the total water content in the two fractions. Separate R_2-values for the two fractions indicate that 90% of the total water is present in two slowly exchanging fractions. For a model with two states a and b, slow exchange is defined by the condition:

$$\tau_a^{-1} + \tau_b^{-1} \ll (T_{2a}^{\circ})^{-1} - (T_{2b}^{\circ})^{-1}, \tag{2}$$

neglecting the chemical shift difference between the two states; τ_a, τ_b are the lifetimes of water molecules in states a and b, and T_{2a}°, T_{2b}° are the spin-spin relaxation times of water in the

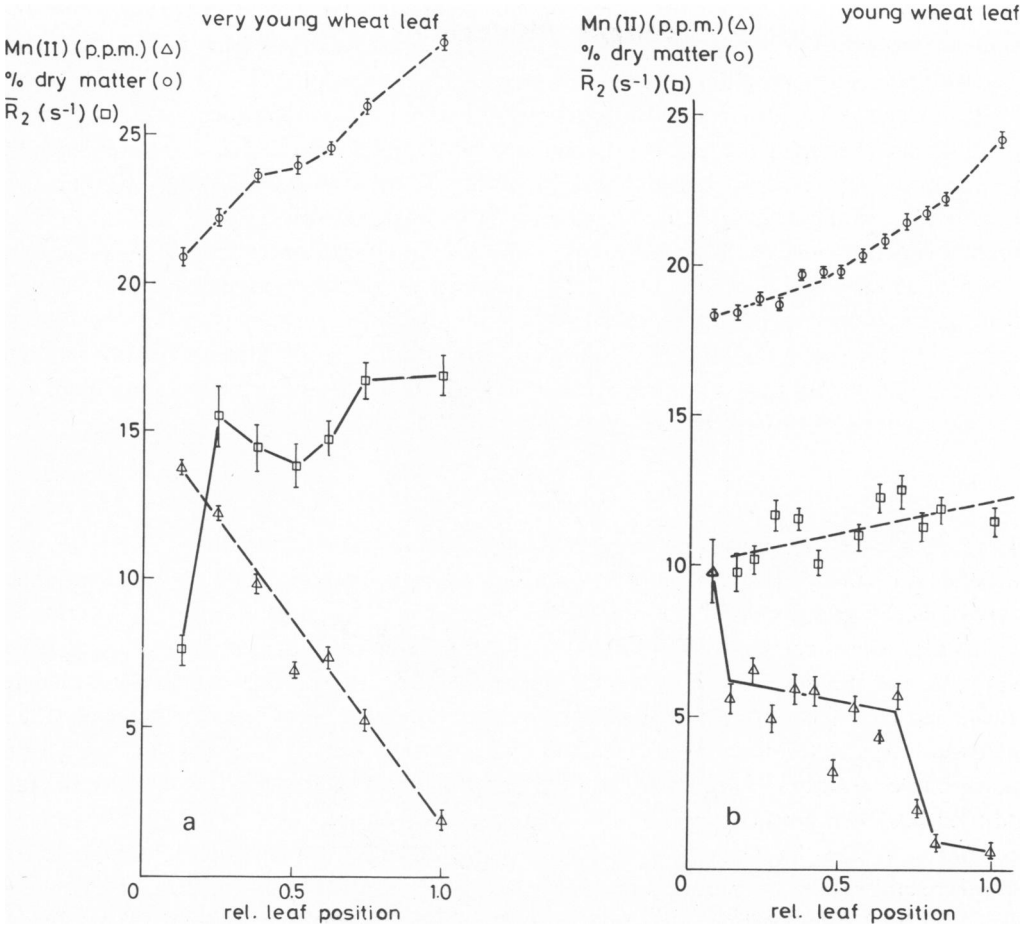

FIGURE 2 Mn(II), percent of dry matter, and spin-spin relaxation rate $\bar{R}_2$ as function of the leaf position for the very young (*a*) and the young (*b*) wheat leaf of Fig. 1. Mn(II) is measured by EPR. The spin-spin relaxation rate is given as the weighted average.

absence of exchange. The existence of fast exchange between two subfractions within each fraction is indicated by the dependence of R_2 on the dry matter/water ratio (Fig. 3).

For a two-state, fast-exchange model (1, 17), the observed single relaxation rate R_2 is the weighted average of the separate rates of two fractions consisting of "bound" and "free" water, yielding:

$$R_2 (\equiv T_2^{-1}) = T_{2f}^{-1} + P_b (T_{2b}^{-1} - T_{2f}^{-1}), \tag{3}$$

where subscripts *b* and *f* refer to "bound" and "free," respectively; P_b is the mole fraction of the "bound" fraction. The magnitude of P_b is proportional to the ratio between the tissue dry weight and the water weight, at least in the high water content region. Thus, in the two-state, fast-exchange model there is a linear dependence of R_2 on the ratio between tissue dry weight

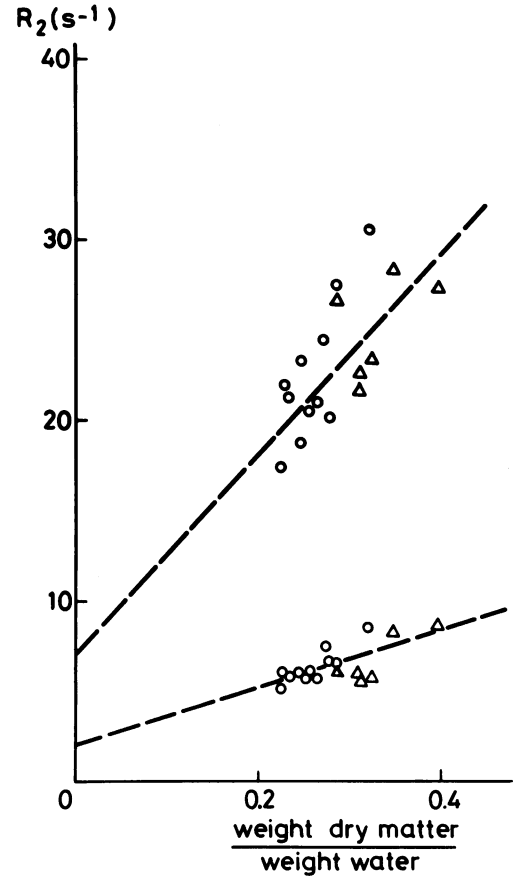

FIGURE 3 Proton relaxation rate R_2 in the wheat leaves of Fig. 2 *A* (Δ) and *B* (O) as a function of water content. A nonexponential T_2-decay was observed. The two fractions given here account for >90% of the tissue water.

and water weight in the high water content region as experimentally observed for both separate R_2s in wheat leaves (Fig. 3).

Therefore, we conclude that >90% of the total water is present in two slowly exchanging fractions within which there are fast-exchanging subfractions. Since the relaxation rate of the separate fractions varies inversely with water content, in this system the water content may be measured using T_2 values. This is not always possible (5) since variations of the exchange rate between different water fractions and of the dry matter structure may disturb the predicted linear dependence of R_2 on the ratio dry tissue weight and water weight.

CONCLUSIONS

(*a*) Wheat leaf contains age-dependent Mn(II) gradients. Part of the manganese is observable by EPR. (*b*) The Mn(II)-concentration does not affect the spin-spin relaxation time T_2 of water fractions representing >90% of the total water content of the leaf. (*c*) From *a* and *b* we conclude that the measured Mn(II) is present in bound form. (*d*) >90% of the total water is

present in two fractions exhibiting slow exchange on the T_2 timescale. Within these fractions there are fast-exchanging subfractions. (*e*) The relaxation rate of these two separate fractions varies inversely with water content, in agreement with the predictions from a simple two-state fast-exchange model.

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