A THEORY AND A MODEL FOR INTERPRETING THE PROTON NUCLEAR MAGNETIC RESONANCE SPECTRA OF WATER IN PLANT LEAVES

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ABSTRACT Some plant leaves display complex, orientation-dependent, proton nuclear magnetic resonance (^{1}H NMR) spectra. The spectral patterns vary as the angle between the leaf surface and the applied magnetic field is varied. They also vary with temperature and with the quantity of absorbed manganous ions, but they are independent of magnetic field strength. In this paper, we propose a theory to explain the origin of the spectra and a model from which the patterns can be calculated. The theory shows how heterogeneous magnetic susceptibilities and local dipolar magnetic fields in chloroplasts can shift the water-proton resonance field. The model describes a simplified leaf structure in which the chloroplasts are nonrandomly aligned with respect to the leaf surface. Model calculations are tested by comparison with experimental spectra from hawthorn leaves (*Crataegus* sp.).

INTRODUCTION

Plant leaves are mostly water. Therefore, we might expect the proton nuclear magnetic resonance (NMR) spectrum of a leaf to be essentially the spectrum of bulk water, i.e., a single peak. As anticipated, the spectrum of most plant leaves is a single peak; however, the leaves of some species display complex spectral patterns consisting of two or three peaks (1). Each species with a complex spectrum has its own characteristic pattern. The pattern of one species is distinguished from that of another by different peak splittings and different relative peak intensities (1). The patterns are orientation dependent; they vary as the angle between the leaf surface and the applied magnetic field is varied.

Before we can extract the information contained in a leaf spectrum, we require a theoretical foundation. Specifically, we need answers to such questions as: What causes the signals to split into a complex pattern? Why are the patterns orientation dependent? How are the patterns related to the internal structure or chemical composition of the leaf? And, why are the complex spectral patterns not seen in all plant species?

We propose a theory to answer the questions posed above, and a model from which a leaf spectrum can be simulated by calculation. We shall show how NMR spectra, with the aid of the model, can be used to measure the relative volumes of different water compartments in vivo. Model calculations also provide a method for measuring the orientations of thylakoid membranes with respect to the leaf surface.

MATERIALS AND METHODS

Leaf samples were field collected from ornamental hawthorn trees (*Crataegus* sp.). At the time the samples were collected, the trees had been in leaf for ~1 mo. The exact species used could not be determined with certainty because the hundreds of hawthorn species, varieties, and hybrids are difficult to classify, but all the trees appeared to be clones of the same cultivar, and all gave essentially identical results. Hawthorn leaves were chosen for this study because their NMR spectra are complex, unusually well resolved, and consistently reproducible (1).

Experimental proton NMR spectra were obtained as previously described (1). Disks 4 mm in diameter were excised from fresh leaves and placed in NMR sample-tube inserts designed to orient the sample so that a vector normal to its surface makes an angle of 0, 30, 45, 60, or 90° to the applied magnetic field. Spectra were recorded at 470 and 200 MHz. Sample temperatures were 293°K except as noted.

In almost all cases, spectra were recorded from each sample <10 min after the leaves had been harvested from the tree. For some experiments, short branches (25 cm) were removed from the tree and soaked with cut ends in 2.5 mM aqueous $MnSO_4$; leaf disks were prepared for study after the branches had soaked for time intervals from 1 h to 3 d.

EXPERIMENTAL RESULTS

Fig. 1 shows two hawthorn spectra obtained with leaf surfaces oriented perpendicular to the applied magnetic field (i.e., at $\theta = 0°$). The spectra were recorded using different field strengths; their similarity demonstrates that the spectral pattern is almost independent of magnetic field when plotted on a parts per million scale. Additional spectra from the same hawthorn cultivar may be found in reference 1.

To facilitate discussion, the peaks in Fig. 1 are labeled *A*, *B*, and *C*, starting from the low-field (left) side. *A* is a weak peak or shoulder (sometimes very weak), while peaks *B*

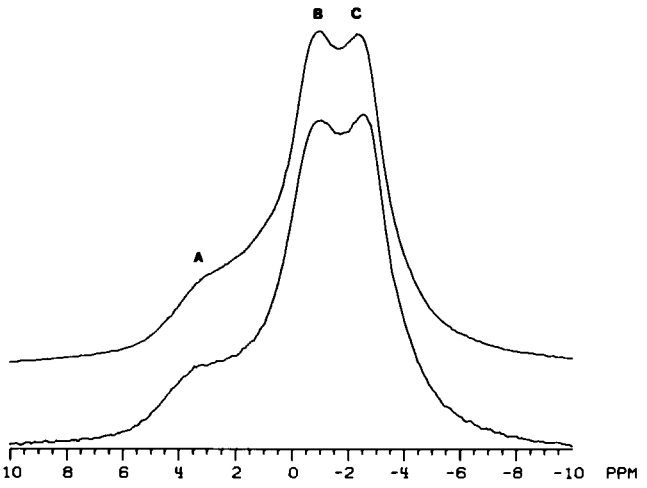

FIGURE 1 ^{1}H NMR spectra of disks (4 mm diam) cut from fresh hawthorn leaves and oriented in the NMR probe so that the leaf surface was perpendicular to the applied magnetic field. The upper trace was obtained at a proton resonance frequency of 470 MHz, while the lower trace was obtained at 200 MHz. Three peaks (labeled *A*, *B*, and *C*) can be discerned. The chemical shift scale can be used to measure peak splittings but not absolute chemical shift values because the origin (at 0 ppm) is arbitrary; no chemical shift reference was used.

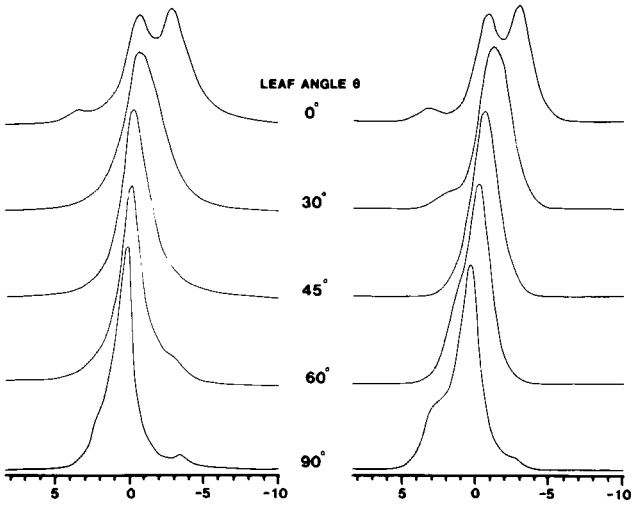

FIGURE 2 Comparison of experimental and computed spectra as a function of leaf angle, θ. Left side: experimental 470 MHz ^{1}H NMR spectra from a single hawthorn leaf disk. Right side: theoretical orientation-dependent leaf spectrum calculated using Eq. 7 with the input parameters discussed in the text. θ is defined as the angle between the applied field and a vector normal to the leaf surface. All spectra have been plotted on the same scale (in parts per million units) to allow direct comparison.

and *C* are stronger. The splitting (separation) between peaks *A* and *B* is greater than that between *B* and *C*. All fresh hawthorn samples produce an *ABC* pattern with peak splittings of about the same magnitude, but resolution varies from sample to sample. It is possible to change the relative peak intensities by partially drying the sample (1); peak *B* decreases in intensity more rapidly as the leaf dries than do peaks *A* and *C*. Peak positions and spectral resolution are relatively unaffected by dehydration.

The left side of Fig. 2 displays a series of experimental spectra obtained from another hawthorn sample at orientations from $\theta = 0°$ to 90°. These spectra represent about the best resolution that we have observed, although the resolution is not dramatically different from that of an average hawthorn sample.

Examining Fig. 2, we see that the spectrum at $\theta = 0°$ consists of three partially overlapping peaks. As the leaf is rotated in the field, peaks *A* and *C* converge on peak *B*. By 45° or 60° the three peaks have coalesced. Partial resolution of the component peaks returns at $\theta = 90°$; at this orientation, a weak, high-field peak or shoulder appears near the 0° position of peak *C*, and a stronger, low-field shoulder may be seen near the 0° position of peak *A*. Relaxation time measurements demonstrate (1) that peak *C* (the high-field peak at 0°) crosses through the center of the spectrum to become the low-field shoulder at 90°.

Fig. 3 shows the effect of temperature variation on the spectrum of a single leaf disk oriented at $\theta = 0°$. Peak *A* is very weak in this sample. The splitting between peaks *B* and *C* decreases by ~4 or 5% as the temperature increases from 11° to 30°C. The temperature effect is reversible. All three traces in Fig. 3 were recorded within a period of 20 min without disturbing the sample in the NMR probe. At constant temperature, a typical spectrum would not change perceptibly in a similar time interval.

Fig. 4 shows that the splitting between peaks *B* and *C* increases as the sample absorbs manganous ions. The peak-to-peak splitting in the lower curve, from a branch soaked 72 h in 2.5 mM $MnSO_4$, is 1.92 ppm, while in the upper curve, representing a sample from the same branch

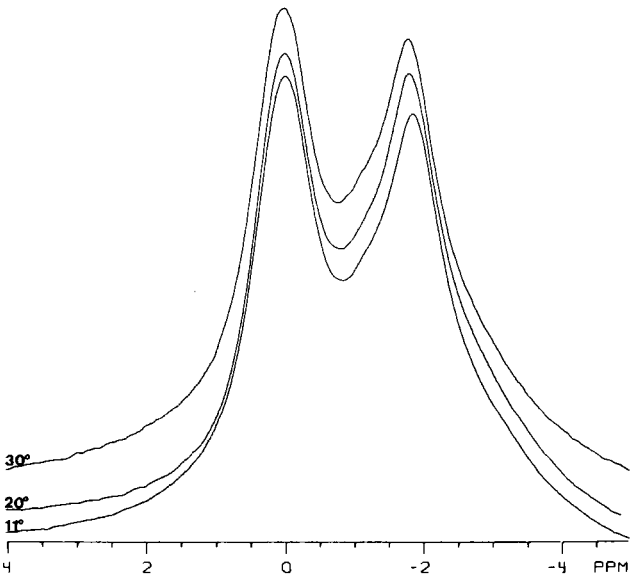

FIGURE 3 470 MHz ^{1}H NMR spectra from a single hawthorn leaf disk at three different temperatures (11, 20, and 30°C). The splitting between peak *B* (near 0 ppm) and peak *C* (near −2 ppm) increases progressively as temperature decreases.

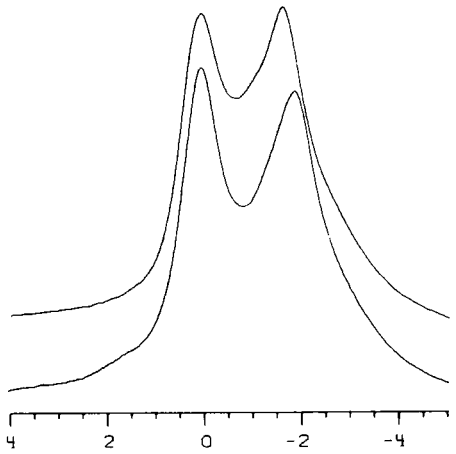

FIGURE 4 470 MHz ^{1}H NMR spectra from two different leaf disks. Both samples were taken from a hawthorn branch soaked with its cut end in 2.5×10^{-3} molar aqueous $MnSO_4$ solution. Upper trace: soaked for 1 h. Lower trace: soaked 72 h. The splitting between peaks *B* and *C* increases with soaking time. The horizontal scale registers field displacement in parts per million units from an arbitrary origin.

soaked only 1 h, the splitting is 1.68 ppm. The standard deviation of experimental scatter for repeated measurements of peak splitting is ±0.02 ppm.

DISCUSSION OF EXPERIMENTAL RESULTS

To explain the proton NMR spectrum of a leaf, a successful theory must be consistent with the following experimental facts:

(*a*) The major features in the spectrum are intense and relatively narrow, with typical peak widths between 1 and 3 ppm; therefore, the spectrum must be predominantly that of liquid water. No other fluid component in a leaf has comparable proton concentrations, and signals from protons in membranes or in solid particles are expected to have peak widths >3 ppm.

(*b*) Different peaks in the spectrum have different proton spin-lattice relaxation times (1, 2); this fact demonstrates that the various peaks are signals from protons in different water compartments.

(*c*) Different peaks decrease in amplitude at different rates as a leaf dries; they also freeze at different rates (1). This is additional evidence that the peaks represent signals from different water compartments.

(*d*) The peak from one water compartment can be distinguished from the peaks of other compartments by its unique spin-lattice relaxation time. Using this method, peak shifts can be followed as leaf orientation is changed. Results show that peaks *A* and *C* cross through the position of peak *B* as the sample is rotated (1); i.e., peaks *A* and *C* trade places as θ changes from 0° to 90°. This suggests that peaks *A* and *C* originate from water in magnetically anisotropic compartments that are preferentially oriented with respect to the leaf surface, and that peak *B* is the signal from water in one or more isotropic compartments.

(*e*) Peak splittings increase as temperature decreases, or as the concentration of maganous ions increases. The splittings are independent of field when measured on a ppm scale; i.e., they are proportional to field on an absolute scale. These observations provide important clues concerning the origin of the splittings.

(*f*) The NMR spectral patterns are more or less reproducible within a species, but vary widely from one plant species to another (1). This fact suggests that we shall find an explanation for the complex spectral patterns in some feature of leaf structure or leaf chemistry that varies dramatically with species.

PREVIOUS THEORIES TO EXPLAIN COMPLEX, WATER-PROTON SPECTRA

Complex, water-proton NMR spectra often have been seen in biological samples. Several explanations have been advanced to explain the phenomena. These explanations may be correct for the systems to which they were applied, but we shall show that they are inconsistent with our experimental results, and that a new theory is required to explain leaf spectra.

A single, water-proton NMR peak can be distorted to produce a complex pattern if the sample or the sample-holder distorts the magnetic field. For example, Burke et al. (3) obtained complex, orientation-dependent water proton spectra from dogwood stems, and they determined that the pattern was produced by effects related to the cylindrical sample shape. However, the complex patterns we obtain from leaves are not caused by sample shape effects. The sample-tube inserts used in our experiments were designed to minimize field inhomogeneities (1). If shape effects produced the complex patterns, then similar spectra should have been obtained from all disk-shaped samples but we recorded single peaks from most leaves as well as from disks of moist filter paper (1).

Orientation-dependent doublet patterns from structured water have been identified in NMR spectra from clay, DNA fibers, and a number of other systems (4, 5, 6). Theory shows that the doublets result from nuclear dipole-dipole interactions, and that to produce the effect structured water must exist in a hydration layer adjacent to oriented fibers, membranes, or surfaces (6). The doublet peaks are seen only in relatively dry samples. When protons from the structured water can exchange with those of free water molecules in the bulk liquid, the doublet spectrum is replaced by a single peak (5). The nuclear dipole-dipole mechanism requires peak splittings to be inversely proportional to the applied magnetic field strength when measured on a parts per million scale (5). Our results show conclusively that nuclear dipole-dipole interactions do not contribute significantly to either peak widths or peak shifts in the NMR spectra of hawthorn leaves (or leaves of any other species we have examined) because the spectra are independent of field (Fig. 1). In

addition, our spin-lattice relaxation times are inconsistent with the nuclear dipole-dipole mechanism, which requires both peaks to have identical relaxation times.

Lenk et al. (4) obtained doublet spectra from spinach leaves, and they attribute the patterns to nuclear dipole-dipole interactions in structured water. Our results show a single peak from spinach (1). We suspect that Lenk's doublet pattern may have been caused by sample shape effects.

Chloroplasts are known to have anisotropic magnetic susceptibility tensors (7). This property allows isolated, resuspended chloroplasts to be oriented by the force of a magnetic field (8, 9). The anisotropic susceptibility of oriented chloroplasts would be a sufficient explanation for anisotropic NMR spectra, except that the tensor components are orders of magnitude too small to account for the observed peak shifts.

A NEW THEORY TO EXPLAIN THE NMR SPECTRA OF LEAVES

The experimental results suggest that peaks *A* and *C* originate in anisotropic water compartments that are preferentially oriented with respect to the leaf surface. We contend that these compartments must be chloroplasts, since chloroplasts are the only structures in a leaf that are both anisotropic and oriented. Chloroplasts contain a significant fraction of the total leaf water, often >25% of it. Our theory, outlined below, explains how the NMR signal from chloroplast water protons can be orientation dependent, and why the shifts are much larger in some species than in others.

Manganese is an essential micronutrient for plants. Most of it is found in several distinct environments or pools (10) within the chloroplasts. One pool is at the active site for water splitting in photosystem II; this pool has roughly constant concentration in all plants (~1 Mn atom/100 chlorophyll molecules) (10). Another pool is the reserve or weakly bound manganese; these are Mn^{2+} ions bound near the outer surface of the thylakoid (11) in such a way that their crystal field axes are oriented preferentially with respect to the plane of the membrane (1, 12).

The function of reserve manganese has not been established definitely; it may act as a superoxide dismutase. In any case, the quantity of Mn^{2+} in the reserve pool is extremely variable (10). Some species (e.g., spinach) have very little, while other species have much higher concentrations (1, 13). From one species to another, the concentration can differ by several orders of magnitude. We have no information concerning Mn^{2+} concentrations in the majority of the species that we have tested; however, the few species that we know to contain large amounts of reserve manganese show complex NMR spectra, while those that we know to have small amounts give single peaks (1). These facts lead us to suggest that variation in reserve Mn^{2+} concentration is the dramatic, species-dependent difference that causes some leaves to show a complex NMR spectrum, while others give only a single peak.

A manganous ion is paramagnetic, and its five, unpaired electrons make a substantial contribution to the local magnetic field. The magnetic field experienced by a proton in the vicinity of a Mn^{2+} ion constantly fluctuates as the ion relaxes and as the proton moves, but at any instant of time the net field is the sum of three terms: the external applied field, the sum of dipolar fields from nearby ions, and a susceptibility correction that depends in part on the local concentration of manganous ions. Peak positions in the NMR spectrum are determined by the average field to which the protons are exposed.

The time-averaged dipolar field, H_d, at any point in space near a manganous ion is given by the strength of the instantaneous dipolar field (14) multiplied by a Boltzmann factor. At the high temperature limit (where $g\beta H \ll kT$):

$$H_d/H = g^2 \beta^2 S(S+1)(1 - 3\cos^2\theta)(r^3 kT)^{-1}, \quad (1)$$

where $g = 2.0$, $S = 5/2$, β is the Bohr magneton, k is Boltzmann's constant, r is the ion-to-proton distance, T is the temperature, H is the strength of the applied field, and θ is angle between the ion-proton vector and the applied field. H_d is angle dependent and temperature dependent in a manner consistent with experimental leaf spectra. H_d/H is a measure of the fractional shift in field (or in peak position) caused by the dipolar field from a single ion. At $T = 293°K$, and at a point in space defined by $\theta = 0°$ and $r = 1$ nm, Eq. 1 gives $H_d/H = 1.5 \times 10^{-4}$ (i.e., at that location, the dipolar field is 150 ppm of the applied field).

As chloroplast water protons diffuse, they experience a net average dipolar field, $\langle H_d \rangle$, which is the average field over all the volume accessible to water. For protons occupying a spherical volume around the manganous ions (e.g., for aqueous Mn^{2+} solutions), $\langle H_d \rangle$ is zero. This is also true for protons diffusing through a random structure containing many ions. But for an ordered system of ions embedded in membranes that are oriented with respect to the external field, $\langle H_d \rangle$ can be different from zero. If the geometry at every membrane binding site is identical, the effect of each embedded ion is additive and the net average dipolar field experienced by the protons is proportional to the number of ions and inversely proportional to chloroplast volume.

The detailed structure of a thylakoid membrane is unknown. Therefore, we cannot describe the actual geometry around reserve Mn^{2+} ions. The calculation presented below features a purely speculative geometry and is intended only to demonstrate that manganese can produce NMR shifts of the magnitude observed in leaves.

Suppose that Mn^{2+} ions are embedded in ordered thylakoid membranes so that water molecules can approach the ions only through a cone that penetrates the membrane. The cone's apex is the Mn nucleus while its axis is directed

perpendicular to the plane of the membrane and parallel to the applied field (i.e., at $\theta = 0°$). Assume, also, that due to membrane geometry, the average field produced by an ion is zero outside the membrane and the penetrating cone. For this model, the NMR peak shift is given by the integral:

$$\langle H_d \rangle / H = N/V \int_{r_a}^{r_b} (H_d/H)\,(1 - \cos\psi)\, 2\pi r^2 dr, \qquad (2)$$

where ψ is an angle that defines the width of the cone, and H_d/H is the same expression as in Eq. 1 but with the term $(1 - 3\cos^2\theta)$ replaced by its average value over the spread of the cone (e.g., in this example from $\theta = 0°$ to $\theta = \psi$). N/V is the number of ions per unit volume. Integration is performed from r_a, the distance of closest Mn-proton approach, to r_b, the penetration depth of the cone.

A typical chloroplast has a volume of $\sim 3 \times 10^{-17}$ m^3, and may contain $\sim 3 \times 10^8$ chlorophyll molecules (7). Chloroplasts with a very large concentration of reserve manganese might have as many as one Mn^{2+} ion per chlorophyll molecule, and so it is conceivable that concentrations as high as $N/V = 1 \times 10^{25}$ m^{-3} could be found in the chloroplasts of some species. Using these speculative numbers in Eq. 2 with $r_a = 0.2$ nm, $r_b = 1.0$ nm, and $\psi = 55°$ gives $\langle H_d \rangle / H = 4$ ppm. This calculation demonstrates that the dipolar field from reserve manganese is potentially sufficient to explain the magnitude of the *ABC* splittings. If the calculation were repeated at different membrane orientations, it would show that the splitting varies as $(1 - 3\cos^2\theta)$. Note that $\langle H_d \rangle / H$ is not equivalent to an anisotropic magnetic susceptibility.

All water molecules within a single chloroplast experience the same average magnetic field because diffusion thoroughly mixes them during a proton's spin-spin relaxation time, T_2. T_2 represents the averaging time over which a nucleus responds to its local environment (15). Experimental T_2 values in leaves range from 3 to 30 ms (1); during this time interval, water molecules diffuse a distance of from 2 to 6 μm, a range comparable to the dimensions of a chloroplast. Furthermore, chloroplast water is isolated for times considerably longer than T_2 from water in other compartments. Saturation-transfer experiments have shown that chloroplast water exchanges relatively slowly with water in the cytoplasm (2). Therefore, the NMR spectrum of an individual chloroplast is a single peak at a position that represents the average spectral position of all its water protons. NMR signals from other water compartments are averaged also, but the averaging process may not be quite as effective because other compartments are larger than chloroplasts.

Manganous ions in aqueous solution are effective spin-lattice relaxation agents, but Mn^{2+} in leaves apparently is not. Leaves showing *ABC* patterns have spin-lattice relaxation times from 0.3 to 1.2 s (1); this may indicate that the ions are fully chelated so that water molecules do not enter the first coordination sphere.

THE MODEL

This section describes a leaf model in sufficient detail that its internal magnetic field distribution (i.e., its NMR spectrum) can be calculated. The model has been simplified and idealized until it contains the minimum number of adjustable parameters necessary to reproduce the general features of experimental leaf spectra. Therefore, it should not be regarded as a complete or accurate description of the internal structure of real leaves.

Leaf water is contained in relatively isolated compartments. Each cell is a compartment, and within it there are subcompartments: the chloroplasts, vacuole, and cytoplasm. Water may be present also in extracellular compartments.

A leaf is divided into parallel layers of cells; these are the palisade, spongy mesophil, and epidermal layers. The palisade cells are a layer of narrow, cylindrical cells, with axes directed perpendicular to the leaf surface. The spongy layer consists of irregularly shaped cells and a considerable amount of air space. Epidermal cells occupy the leaf surfaces; they contribute little to the NMR spectrum because they contain only a small fraction of the total leaf water.

The magnetic field distribution inside a layered structure depends on magnetic susceptibility differences between the layers, and on the orientation of the layers with respect to an external field (1). A susceptibility difference will cause the relative positions of signals from different layers to be orientation dependent. The effect also causes the entire NMR spectrum of a leaf to be offset if there are susceptibility differences between the leaf and the sample holder; however, we shall neglect the offset because it is irrelevant when no chemical shift reference is used.

The average magnetic susceptibility of the spongy mesophil is different from that of the palisade layer because the proportions of air and water are different, and because air and water have different magnetic susceptibilities; susceptibility differences also may be caused by differing concentrations of paramagnetic ions. Therefore, we may expect the relative positions of NMR peaks from water in these two layers to be orientation dependent.

Leaf layers are most conveniently modeled by choosing a reference layer from which all other susceptibility differences are measured. The palisade layer is a convenient reference because it contains the chloroplasts. In such a model, the external magnetic field that must be applied in order to observe resonance signals from protons in the spongy layer, H_b, is given by (1, 15):

$$H_b = H_p\,(1 + \delta\cos^2\theta), \qquad (3)$$

where δ is the susceptibility difference between the spongy mesophil and palisade layers, θ is the angle between the applied field and a vector normal to the leaf surface, and

H_p is the field required to resonate protons in the palisade layer at the fixed transmitter frequency used in the NMR spectrometer. We define H_b and H_p in terms of fields required for resonance because our object is to calculate the positions of the spectral peaks, not the internal field strengths per se.

It is possible to estimate limits for the range of values that δ may exhibit. One limit is determined by the susceptibility difference between air and water (equivalent to a shift of 0.7 ppm); the other by the effect of paramagnetic ions concentrated in the chloroplasts. The manganous ions inside a chloroplast change its isotropic volume magnetic susceptibility by an amount δ' given by (16):

$$\delta' = \mu\, Ng^2\beta^2 S(S+1)\,(3kTV)^{-1}, \qquad (4)$$

where $\mu = 4 \times 10^{-7}$. Using $N/V = 1 \times 10^{25}$ m^{-3}, $g = 2.0$, $S = 5/2$, and $T = 293$°K, Eq. 4 gives $\delta' = 3.1$ ppm. This should be regarded as an upper limit for δ'; in a typical leaf N/V would be $<10^{25}$. The value of δ depends on δ', and on the volume fraction of air and chloroplasts in each layer. For example, by varying the proportions of air and water and the relative numbers of chloroplasts, δ can be made to vary between a low extreme value of -0.7 ppm (if the spongy layer is all air and no chloroplasts are present) to an upper limit of δ' (if chloroplasts make up all of the palisade layer and none of the spongy mesophil).

Chloroplasts are irregular in shape, but they approximately correspond to ellipsoids with three unequal dimensions. As such, they may be described in terms of three perpendicular axes, with two of the axes defined by the longest and the shortest distances through the center of the chloroplast. Chloroplasts contain thylakoid membranes, which are planar structures arranged in parallel stacks that are aligned perpendicular to the shortest axis (9).

Most of the chloroplasts are located inside the palisade cells where they are nonrandomly oriented with respect to the leaf surface (1). The chloroplasts are not attached to other structures, but are generally found firmly pressed against cell walls. The mechanical effect of pressing an ellipsoidal chloroplast against the cylindrical inner wall of a palisade cell aligns the shortest chloroplast axis along a radius vector perpendicular to the axis of the cell, and the longest chloroplast axis parallel to the cell's axis.

Isolated, resuspended chloroplasts can be oriented by the force of a magnetic field (8), but the force is very weak and is barely sufficient to overcome thermal forces that tend to randomize the orientations of suspended chloroplasts. To be capable of orienting chloroplasts effectively, mechanical forces inside a cell must be much stronger than the thermal forces. Therefore, the mechanical forces must be strong enough to dominate magnetic forces, and we may confidently assume that net chloroplast orientation with respect to the surface of a leaf is independent of the leaf's orientation in a magnetic field.

We shall define f_c as the fraction of leaf water present inside chloroplasts that are pressed against the cylindrical walls of palisade cells (i.e., with long axes perpendicular to the leaf surface). Other chloroplasts may be pressed against the ends of the palisade cells or against the tops or bottoms of other cells; we define f_a as the fraction of leaf water in chloroplasts aligned in this way, i.e., with short axes perpendicular to the leaf surface. Both sets of chloroplasts (represented by f_a and f_c) are aligned with one of their axes perpendicular to the leaf surface, while the other two axes are rotated by an arbitrary angle ϕ with respect to the laboratory axis system. The angle ϕ is not fixed by sample geometry and is different for each chloroplast.

The net magnetic field experienced by water protons inside a chloroplast is the sum of the applied field (modified by appropriate susceptibility corrections) and the average dipolar field from reserve Mn^{2+} ions; it may be described by a tensor function aligned with respect to the thylakoid membranes, i.e., along the same axis system as defined by the ellipsoidal shape. We shall define the dipolar field tensor components as α, β, and γ along the short, intermediate, and long chloroplasts axes, respectively. A dipolar field averages to zero over all orientations; therefore: $\alpha + \beta + \gamma = 0$. At this time we cannot predict the values of α, β and γ, but according to the theory outlined above, we can predict that their magnitudes are proportional to the concentration of manganous ions in the chloroplasts.

The NMR peak for chloroplasts aligned with long axes perpendicular to the leaf surface (i.e., of water fraction f_c) is a function of the leaf angle θ and the internal rotation angle ϕ. The peak (peak C) appears in the spectrum at a field H_c, given by:

$$H_c = H_p\,(1 + \gamma\cos^2\theta + (\alpha\sin^2\phi + \beta\cos^2\phi)\sin^2\theta) \qquad (5)$$

and for parallel chloroplasts (peak A or water fraction f_a) the field is:

$$H_a = H_p\,(1 + \alpha\cos^2\theta + (\gamma\sin^2\phi + \beta\cos^2\phi)\sin^2\theta). \qquad (6)$$

Eqs. 3, 5, and 6 predict the positions of three peaks in the spectrum of a model leaf. These are: peak A from water fraction f_a (chloroplasts with long axes parallel to the leaf surface), peak B from fraction f_b (water in the spongy mesophil), and peak C from fraction f_c (chloroplasts with long axes perpendicular to the surface). Of course, in a real leaf there are chloroplasts with intermediate orientations, and there are a number of nonchloroplast water compartments (including the epidermal cells as well as vacuolar and cytoplasmic water in the palisade cells) but we assume (for simplicity) that these water fractions are either so small relative to f_a, f_b, and f_c that they may be neglected, or that their NMR signals are located so close to H_a, H_b and H_c that the peaks are unresolved.

Assuming Lorentzian shape functions, the NMR spectrum can be calculated from:

$$I(H,\theta) = \sum_{\phi=0^\circ}^{\phi=90^\circ} f_a[1 + (H - H_a)^2W^2]^{-1} + f_b[1 + (H - H_b)^2W^2]^{-1} + f_c[1 + (H - H_c)^2W^2]^{-1}, \quad (7)$$

where I, the spectral intensity, is a function of H, the applied magnetic field strength, and θ, the leaf angle, and where W is a peak width parameter.

CALCULATIONS

To simulate a spectrum, $I(H)$, at a given θ, Eq. 7 requires nine input parameters ($f_a, f_b, f_c, \alpha, \beta, \gamma, \delta, W$ and H_p), but only six of these are independent variables. H_p establishes an absolute field scale, but this is irrelevant because no chemical shift reference was used; H_p may be eliminated by defining it arbitrarily as the origin of the chemical shift scale and by defining α, β, γ, and δ as chemical shifts (in parts per million units) from H_p. Furthermore, since α, β, and γ are a set of purely anisotropic shifts, only two of them are independent parameters, i.e., $\beta = -(\alpha + \gamma)$. The water fractions also represent two independent parameters since $f_a + f_b + f_c = 1$.

Of the six independent variables, five can be measured directly from an experimental NMR spectrum. W is the peak width (in parts per million) at half height. At $\theta = 0°$ the spacing between peaks A and C is equal to $\gamma - \alpha$ while the spacing from peak B to peak C is $\gamma - \delta$. The measured heights of peaks A, B, and C are proportional to f_a, f_b, and f_c, respectively.

We have written a computer program that calculates the spectral shape from six input parameters. In principle, the entire set of orientation-dependent spectra can be simulated using five fixed input parameters measured, as described above, from the $\theta = 0°$ spectrum and one variable parameter that is adjusted for an optimum fit to the experimental data. In practice, we find that the model with one adjustable parameter reproduces the spectrum and its orientation dependence rather well, but that somewhat better fits at all the angles can be obtained if W is allowed to be a function of θ.

Fig. 2 compares our model calculations with experimental spectra. Input parameters to Eq. 7 were $f_a = 0.07, f_b = 0.45, f_c = 0.48, \alpha = -3.1$ ppm, $\beta = 0, \gamma = 3.1$ ppm, and $\delta = 0.7$ ppm. W ranged in uniform steps from 2.2 ppm at $\theta = 0°$ to 1.5 ppm at 90°.

Note that the model reproduces the general features of the hawthorn spectrum at all angles. The calculations suggest that a very large fraction of the water resides in chloroplasts (i.e., f_a and f_c add to 55% of the total water). Hawthorn leaves were chosen for these model calculations in part because their chloroplasts are well oriented and they contain an unusually large water fraction; these factors improve the spectral resolution. We have used the model to simulate spectra from a number of other plant species; typically we find good fits to the spectra. The spectrum of each species is characterized by a different set of input parameters; most of the species have lower chloroplast water fractions than hawthorn. Results from other species will be published elsewhere.

A large fraction of the total peak width (1.5 to 2.2 ppm) may be caused by local variations in magnetic susceptibility within the sample. Field inhomogeneities up to 0.7 ppm can result from the susceptibility difference between air and bulk water (15), and comparable or even larger susceptibility differences could be caused by local concentrations of paramagnetic ions.

An exact fit to the data cannot be achieved within our simple model. In particular, the Lorentzian shape function gives a poor fit at the extreme wings of the spectrum, far from the peak centers. Perhaps this is due in part to the presence of broad background signals superimposed on the relatively narrow major peaks. It also may be due to the fact that the Lorentzian shape function is incorrect for the case of broadening caused by magnetic heterogeneity, but simulation using a Gaussian shape function, in a form equivalent to Eq. 7, reproduced the experimental spectra even less accurately.

CONCLUSION

Using a simple model, we have been able to simulate the major features of the orientation-dependent proton NMR spectra of plant leaves. The model provides a spectral assignment and a method for studying the net alignment of thylakoid membranes.

The theory is consistent with all our experimental data. For example, according to Eqs. 1 and 2, the splitting should be independent of field (on a parts per million scale), it should be proportional to manganous ion concentration, and it should vary with temperature approximately as T^{-1}; i.e., it should vary by 6% over the range from 11° to 30°C. This is approximately the experimental value.

Our success with model calculations by no means constitutes a proof of the theory. A number of crucial experimental tests remain to be tried. For example, an independent measurement of chloroplast water fractions and chloroplast orientations is needed. We have not found the relevant data in the literature; it is not easy to make such measurements using microscopy, and the experiments would have to be done on species that display complex NMR spectra. Another important test is a quantitative correlation of Mn^{+2} concentration with splitting; we have made qualitative correlations, but more work is needed. Other experiments include a comparative study of sun leaves and shade leaves from the same plant, and a study of leaves from plants grown in soil with various levels of manganese deficiency.

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