Observation of a ^{1}H Double Quantum Filtered Signal of Water in Biological Tissues

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The observation of a ^{1}H double quantum filtered (DQF) NMR signal of water in bovine sciatic nerve, bovine articular cartilage, rat tail tendon, and rat brain is reported. The origin of this signal in rat tail tendon was found to be a result of residual dipolar interaction between water protons and macromolecular protons. The dependence of the width of the ^{1}H DQF spectra on the orientation indicated that in rat tail tendon the effective director of the residual dipolar interaction is parallel to the collagen fibers. ^{1}H DQF NMR may be applied in imaging where the contrast obtained is related to the degree of order in the tissue.

Key words: residual dipolar interaction; ^{1}H double quantum filtered NMR; tendon; sciatic nerve.

INTRODUCTION

It has recently been shown that quadrupolar nuclei can be used to detect anisotropy in biological tissues using multiple quantum techniques (1–10). Most of the quadrupolar nuclei suffer from low sensitivity due to a low gyromagnetic ratio or low concentration in biological tissues. Compared to quadrupolar nuclei, the advantages of the ^{1}H nucleus for *in vivo* NMR applications are apparent. Furthermore, measurements of ^{1}H allow simultaneous detection of water molecules and lipids.

Dipolar splitting of ^{1}H of water molecules has been directly observed by Berendsen and Migchelsen (11, 12) in collagen fibers equilibrated with water vapor. However, when the water content in the collagen fibers corresponded approximately to its amount in the native state, the splitting was not observable at room temperature. Recently, anisotropy-dependent dipolar splittings of ^{1}H resonances of creatine and phosphocreatine in human skeletal muscle has been detected by Kreis *et al.* (13–15).

In cases of anisotropic molecular tumbling, the averaging of the dipolar interaction is partial. The residual dipolar interaction allows even rank tensors to be formed, enabling the application of double quantum (DQ) techniques. The residual dipolar interaction may be of local or macroscopic nature. In both cases, DQF spectra will be detected, but only in the latter case will the spectra change when the sample is rotated.

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In this study, ^{1}H DQF spectra of several intact tissues were measured. We demonstrate that it is indeed possible to use DQ techniques for probing anisotropic motion of water molecules, thereby collecting information about local and macroscopic order in the system.

MATERIALS AND METHODS

Bovine sciatic nerve and bovine articular cartilage were obtained from the local slaughterhouse. Rat tail tendon and rat brain were isolated from rats anesthetized with sodium pentobarbital. The samples were immersed in a phosphate buffered saline containing 0.01% azide, and kept refrigerated at 4°C until measured. The medium contained 10% D_2O so we were able to perform ^{2}H measurements on the same samples. For NMR measurements, samples were wiped dry and immersed in Fluorinert (FC-77, obtained from 3M Co. Ltd.). Sciatic nerve and tendon were measured with their long axis parallel to the magnetic field. The tendon was also measured at an angle of 90° by rapping the tendon fibers on a capillary so that they were approximately perpendicular to the magnetic field. The main axis of the articular cartilage plug which is perpendicular to the cartilage surface was positioned parallel to the magnetic field. The two hemispheres of rat brain were separated and placed in a 10-mm NMR tube one on top the other, with their long axis parallel to the magnetic field.

Spectroscopic measurements were conducted on Bruker AMX 360-WB and Bruker ARX 500 spectrometers, using either a 10- or 5-mm probes.

The detection of DQ coherence is done using the following pulse sequence (16)

$$90^\circ_\phi - \frac{\tau}{2} - 180^\circ_{\phi_1} - \frac{\tau}{2} - 90^\circ_\phi - t_1 - 90^\circ_{\phi_2} - t_2 \text{ (acquisition)} \quad [1]$$

As a result of the residual dipolar interaction, double rank spherical tensor operators ($T_{2,1}$, $T_{2,-1}$) are created during τ, the creation time. In cases where the dipolar interaction predominantly couples two spins and the contribution of multispin coherences is negligible, higher rank tensors will not be created. The 180° pulse refocuses frequency shifts and magnetic field inhomogeneities. The second 90° pulse transforms the second rank tensors into double quantum coherence tensors ($T_{2,2}$, $T_{2,-2}$) that are selected using cycling of the phase, ϕ. The third 90° pulse transforms the coherence back to -1 so it may be detected during acquisition.

In theory there are two situations where ^{1}H DQ coherences can be formed in the presence of isotropic motion. One is the presence of homonuclear J coupling, as is in

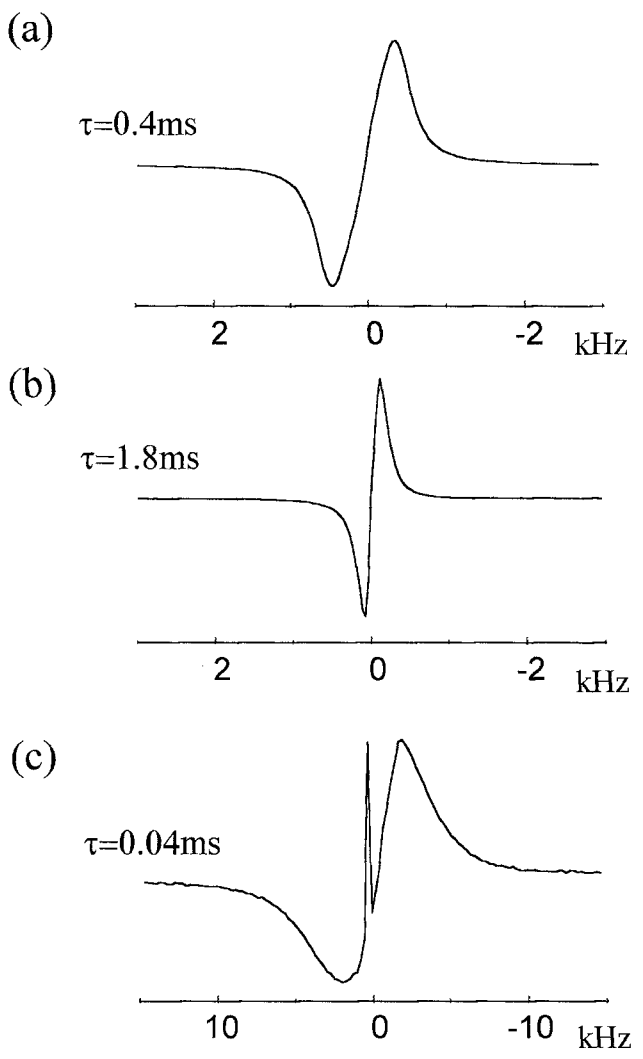

FIG. 1. ^{1}H DQF NMR of: (a) rat tail tendon; (b) bovine articular cartilage; (c) rat brain. The number of accumulations was 32 for a and b and 128 for c. The spectra were taken with the τ values which give the maximum signal. The values of τ are indicated in the figure.

the INADEQUATE experiment. However, for water protons, no homonuclear J coupling is expected from either intra- or intermolecular interaction. The second case is dipolar interaction between three protons in the slow motion condition. In this case a third rank tensor $T_{3,2}$ can be formed giving rise to DQF signal. This possibility can be tested by searching for $T_{3,3}$ using triple quantum-filtered NMR techniques. Such a test, with negative results is described in the Results section. Thus, the detected DQ signal of water results exclusively from anisotropic molecular motion.

In order to verify that the signal obtained by the pulse sequence given in Eq.[1] originates from DQ coherences and not from a leakage of single quantum (SQ) coherences through the DQ filter, the evolution of the DQ coherences was measured at an off-resonance frequency. The amplitude of the resulting signal as a function of t_1 is expected to be modulated by a frequency of $p(\omega_{r.f.}-\omega_o)$, where $\omega_{r.f.}$ is the carrier frequency, ω_0 is the Larmour frequency, and p is the coherence which is 2 in our case. This experiment is denoted as an off-resonance DQ relaxation (DQR) experiment. Another way to verify that the signal obtained by Eq. [1] originates from DQ coherences is to sum up the signals obtained from Eq. [1] with the phases ϕ and ϕ_1 alternating between ϕ, ϕ_1 and $\phi+90°$, $\phi_1+90°$, respectively. Due to the phase evolution of the DQ coherences, the addition of 90° to ϕ and ϕ_1 effectively adds 180° to the acquired signal. As a consequence, summation of the signals gives zero signal if it originates from DQ coherences (U. Eliav, personal communication). This experiment is called a DQF-zero experiment. Since this method does not require an offset of the carrier frequency, it is useful for systems with very broad spectral lineshapes.

Since, similarly to residual quadrupolar interaction, the residual dipolar interaction is not a dissipative process but a coherent one, it can be refocused using multiple quantum filtered echo techniques (17, 18). The following DQF-SQE pulse sequence was used

$$90^\circ_{\phi_1} - \frac{\tau}{2} - 90^\circ_{\phi_2} - \frac{\tau}{2} - \tau_1 - 60^\circ_{\phi_3} - t_1 - 60^\circ_{\phi_4} - t_2 \text{ (acquisition)} \qquad [2]$$

The phase cycling selects the pathway: $P = 1 \rightarrow -1 \rightarrow -2 \rightarrow -1$. Notice that the refocusing pulse is the second 90° and not a 180° pulse.

RESULTS

Observation of ^{1}H DQF Signals for Various Tissues

^{1}H DQF spectra of bovine articular cartilage, rat tail tendon, bovine sciatic nerve, and rat brain are shown in Figs. 1 and 2. The spectra of the different tissues exhibited different splitting and different dependencies on τ (see Table 1).

Off-resonance DQR measurements for tendon and cartilage were performed in order to verify that the signals obtained by Eq. [1] originate from DQ coherences and are not leakage of SQ. A frequency shift of 1500 Hz was used. From the fit of the peak intensities to a sinusoidal function, multiplied by a decaying exponent, we have found that the intensity oscillates with a frequency of 3009 and 2975 Hz for the tendon and the cartilage, respectively. These frequencies are in very good agreement with twice the frequency offset, indicating that we are indeed observing DQ coherences.

For brain we were unable to detect the rise of the DQF as a function of the creation time τ, and observed only its decay. This may be due to very fast formation of the DQF signal that occurred during the pulses. A sharp signal 90° out of phase from the broad signal with random intensity was always observed in this case (Fig. 1). Due to the large linewidth of about 3600 Hz and the short relaxation times of the DQF signal of the brain tissue, off-resonance DQR measurement was not feasible. However, in the DQF-zero experiment the broad signal was completely eliminated while the narrow component persisted, indicating the DQ origin of the broad component and the SQ origin of the narrow one.

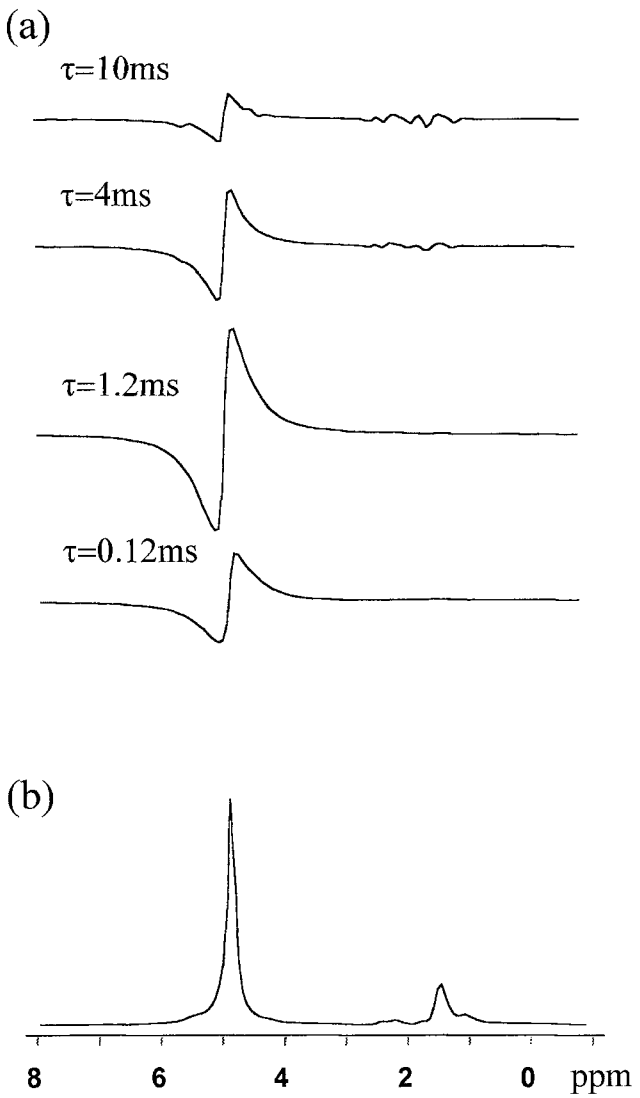

FIG. 2. (a) ^1H DQF spectra of bovine sciatic nerve at various creation times. The values of τ are indicated in the figure. (b) ^1H SQ spectrum of the same sciatic nerve.

The sciatic nerve DQF spectra exhibited, in addition to the water signal, multiple peaks originating predominantly from lipids (Fig. 2). The dependence of the lipid DQF signal on the creation time, τ, was different from that of the water signal. The maximum height of the water DQF peak was observed at $\tau = 0.89 \pm 0.09$ ms, while the lipids gave a maximum DQF signal at $\tau = 10 \pm 2$ ms. In the off-resonance DQR experiments with a carrier frequency shifted by 1500 Hz from the water peak and 2715 Hz from the main lipid signal, the modulation frequencies of water and lipid signals as a function of t_1 were 3100 and 5400 Hz, respectively, as expected for DQF coherences (Fig. 3).

We would like to comment here that the DQF signals obtained in these experiments are not a result of the dipolar field effect (19, 20). Unlike signals due to the dipolar field effect, all the DQF signals (except from the brain tissue) exhibited a biexponential dependence on τ, with exponents of opposite signs. In fact, the dipolar field effect is not expected in our experiment since the DQ coherences were selected using phase cycling and not using gradients. Moreover, the proton content was relatively low due to the presence of Fluorinert.

Table 1
Water ^1H Dipolar Splitting at τ_{max} for the Different Tissues

Tissue	Splitting (Hz)	τ_{max} (ms)[a]
Tendon (n = 4)[b]	660 ± 150[c]	0.46 ± 0.05
Articular cartilage (n = 3)	210 ± 52	1.5 ± 0.3
Sciatic nerve (n = 4)	120 ± 10	0.89 ± 0.09
Brain (n = 4)	3900 ± 110	<0.04

[a] The values of τ_{max} were obtained by fitting the DQF signal intensities to a biexponential function.
[b] n represents the number of samples taken from different animals.
[c] The given errors are standard deviations.

In order to obtain a better understanding of the interactions responsible for the ^1H DQF spectra, a series of measurements were performed on rat tail tendon which has a simple structure, being composed mainly of collagen fibers.

Dependence of the DQF Signal on H_2O/D_2O Ratios

^1H SQ and DQF spectra were measured for rat tail tendon immersed in saline containing 10% D_2O and in saline containing 90% D_2O (Fig. 4). Analysis of the spectra indicated the presence of three ^1H signals with different widths. The narrowest peak seen in the SQ spectrum of the solution of 10% D_2O (Fig. 4c) disappears in the DQF spectrum (Fig. 4a) and hence is due to free, isotropically rotating water molecules. In the SQ spectrum of the 90% D_2O solution the narrowest peak mostly disappeared revealing an ^1H peak with intermediate width. At the same time, the intensity of the broad component decreased as well. The peak with the intermediate width passes the DQ filter but with lower efficiency as compared with the broad component. The ^2H spectra measured on the same samples (Fig. 5) allow a better insight as for the nature of the ^1H resonances. The ^2H SQ spectra consist only of a broad doublet and a narrow signal which completely disappears in the DQF spectra. The ratio between the narrow and the broad components in the SQ spectra is independent of the H/D ratio. We can conclude that in the ^2H spectra both narrow and broad components are due to water deuterons, where the narrow peak stems from free, isotropically rotating water molecules. The peak with the intermediate width appearing in the ^1H spectra is missing from the ^2H spectra and is more prominent in the ^1H spectra at 90% D_2O. Hence it must stem from nonexchangeable protons.

In order to find out whether the interaction responsible for the DQF signal of the water protons originates from intra- or intermolecular proton-proton coupling, the dependence of the intensity of the broad component in the ^1H DQF signal on the H/D ratio was quantified. The ratio of the intensities of the ^2H satellites observed at 10% and 90% D_2O is a measure of the final ratio of the concentrations of ^2H in the two samples. Comparison of the ^2H DQF peak intensities for samples equilibrated with 90% and 10% D_2O gave a ratio of 6.7 ± 0.5. As expected, exactly the same ratio was obtained when comparing the intensity of the satellites in the SQ spectra.

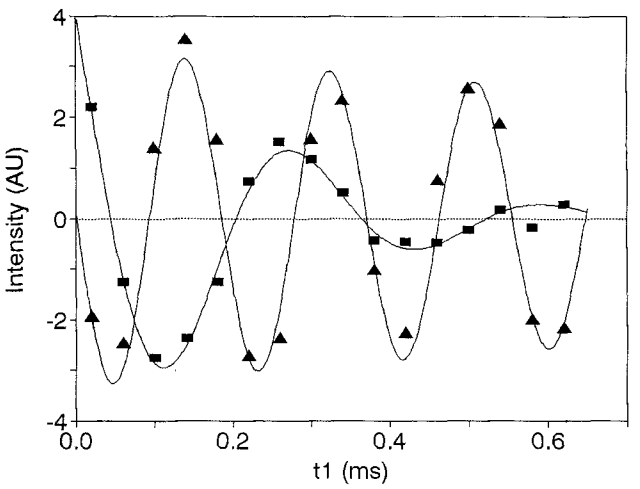

FIG. 3. The signal intensities of the off-resonance DQR experiments of the water peak (squares) and of the lipid peak (triangles) of sciatic nerve. The symbols indicate the experimental results and the lines indicate the curve that was fitted to the function $I = I_0\cos(2\pi\nu t_1 + \phi)\exp(-t_1/T_{DQ})$.

When the fractions of H and D are p and (1-p), the water species distribution is of p^2, 2p(1-p), and $(1-p)^2$ of H_2O, HDO, and D_2O, respectively. If intramolecular dipolar interactions between the two water protons give the predominant contribution to the DQF signal, this signal is expected to be proportional to the concentration of the H_2O species, i.e., p^2. From the ^2H spectra the ratio of the fractions between the two samples is 6.7. Thus the expected ratio of the intensities of the broad component of the ^1H spectra is $6.7^2 = 45$. The ratio of the intensities of the broad components for the two solutions can be obtained for the ^1H DQF spectra (Fig. 4a and 4b). The ratio obtained is 7 ± 1, i.e., within the experimental error proportional to p and not to p^2.

Hence, it can be concluded that the main interaction contributing to the DQF signal is an intermolecular interaction between the water protons and species whose protons do not exchange with water. These species can be tentatively assigned to the oriented macromolecular structure, which, in the case of the rat tail tendon, is mainly the collagen fibers. This result is in accordance with several works that demonstrate that there is a significant interaction between water molecules and macromolecules that affect relaxation times such as T_1 and T_2 (21, 22).

Dependence of the ^1H DQF Linewidth on the Orientation

An important characteristic of the dipolar interaction is its dependence on orientation. DQF experiments were conducted on rat tail tendon in two orientations, θ', of the long axis of the tendon relative to the magnetic field (Figs. 6a and 6b). At short creation times, a ratio of 1.7 between the spectral widths at $\theta' = 0°$ and $\theta' = 90°$ is obtained (see Table 2). In the presence of macroscopic order, the width of the DQF spectra should follow the factor $(3\cos^2\theta - 1)$, where θ is the angle between the director of the average residual dipolar interaction over the whole sample and the magnetic field. If the direction of the dipolar interaction is aligned parallel to the collagen fibers, changing the fiber orientation from 0° to 90° relative to the magnetic field should result in a change by a factor of two in the splitting of the spectra. The precision of the determination of the splitting in each of the spectra was 5%. A larger error occurred in the determination of θ', especially for the perpendicular arrangement which was obtained by wrapping the tendon on a capillary. The difference between the experimental ratio of 1.7 and the theoretical one is within the experimental error of the determination of θ'.

Due to the heterogeneity of the system, the lineshape varies as a function of the creation time, an effect which is most pronounced at long creation times (5). As a consequence, the width of the lineshape does not follow the factor $(3\cos^2\theta' - 1)$ at long creation times as is evident from Table 1.

In order to compare the anisotropy obtained for water protons with that measured by the quadrupolar splitting of deuterons, we measured ^2H SQ spectra of the same sample. Two satellites were clearly observed equidistant from the central peak of the free water, with a separation of 1800 Hz and 1060 Hz for $\theta' = 0°$ and $\theta' = 90°$, respectively (Figs. 6c and 6d). The ratio of the splittings between the two orientations, 1.7, is exactly the same as that for the protons. This means that the orientation of the effective directors of the ^2H quadrupolar interaction and the ^1H dipolar interaction are the same, and coincide with the orientation of the collagen fibers. As was mentioned above the main contribution to the error in the splitting ratio between the two orientations stems from the inaccuracy of θ' in the perpendicular orientation. Thus, the fact that the same deviation of this ratio from the theoretical value of 2.0 was obtained for the two nuclei is reasonable as they were measured on the same sample.

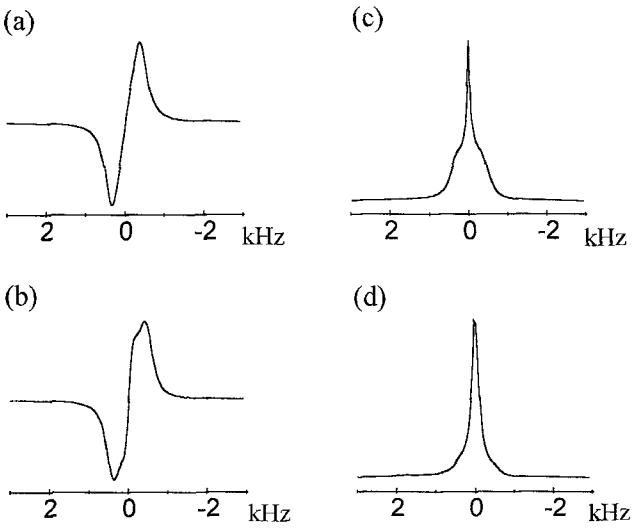

FIG. 4. ^1H DQF (a, b) and SQ (c, d) spectra of rat tail tendon immersed in saline containing 10% (a, c) and 90% (b, d) D_2O. The creation time of the DQF spectra was 0.4 ms in order to obtain maximum intensity.

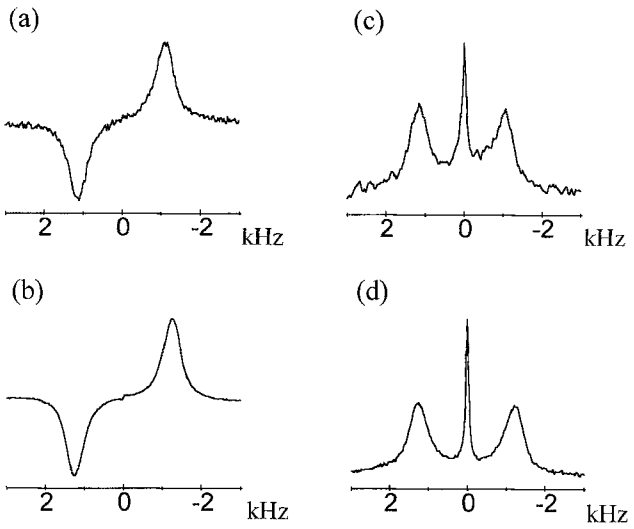

FIG. 5. ^2H DQF (a, b) and SQ (c, d) spectra of rat tail tendon immersed in saline containing 10% (a, c) and 90% (b, d) D_2O. The creation time of the DQF spectra was 0.12 ms in order to obtain maximum intensity.

Dipolar Echo

Residual dipolar interaction is a coherent interaction and, as a result, can be refocused using the DQF-SQE pulse sequence Eq. [2] (17, 18). This pulse sequence refocuses any first order interaction including the Zeeman interaction and the residual dipolar interaction. No refocusing is expected if the spectral linewidth is determined by relaxation processes only. DQF-SQE experiments were run with $\tau_1 = 0$. At the end of the creation time, τ, the second rank tensor, $T_{2,-1}$, is zero due to the refocusing effect of the second 90° pulse. Thus, when $\tau_1 = 0$, second rank tensors will not be formed and no DQ coherence will be observed.

In order to estimate the extent of the signal reduction upon setting $\tau_1 = 0$, we conducted the same experiment setting the tilt angle of the second pulse to 180° (18). In this case the residual dipolar interaction is not refocused, and second rank tensors are present at the end of the creation time, τ. The signal intensity with a tilt angle of 90° was only 5% of that at 180°, indicating that the DQF signal stems predominantly from the residual dipolar interaction.

The linewidth of the signal obtained by the DQF pulse sequence is a sum of the contributions of the relaxation rate, the residual dipolar interaction, and the field inhomogeneity. The contribution of the residual dipolar interaction to the linewidth was checked by comparing the ^1H transverse relaxation rate measured by Eq. [2] with the linewidth. In these experiments a value of $\tau_1 = 0.4$ ms, which gives the maximum DQF signal, was chosen. The transverse relaxation time of the SQ coherence as obtained by the DQF-SQE sequence is 1.2 ms, which corresponds to a linewidth of 265 Hz. The observed linewidth of 560 Hz is significantly larger. This means that there is a significant contribution of the residual dipolar interaction to the linewidth. The contribution of the inhomogeneity of the magnetic field to the linewidth could be neglected since it was smaller than 60 Hz as was estimated from the single pulse spectrum.

Triple Quantum Filter Measurements

In order to check whether the contribution to the DQF spectra originates from more than two interacting protons, a TQF experiment was conducted on the rat tail tendon sample. The TQF experiment gave zero signal. This confirms our assumption that the contribution to the residual dipolar interaction originates predominantly from couples of interacting protons.

DISCUSSION

The work presented here demonstrates that it is possible to detect ^1H DQF signal of water in biological tissues resulting from residual dipolar interaction. The lineshape of the detected ^1H DQF signal was found to be sensitive to the type of tissue. As is evident from Table 1, more than an order of magnitude differences were found for the splitting and τ_{max} in the different tissues. One may note that such sensitivity is much higher than that obtained by other NMR methods like magnetization transfer, diffusion measurements, or inversion recovery experiments (23) for the same tissues.

One of the differences between ^1H and ^2H NMR spectra is that while the ^2H spectrum consists only of the water signals, that of the ^1H exhibits in addition a signal of nonexchangeable protons. For rat tail tendon we found that the ^1H signal with the intermediate width cannot be assigned to the water protons. This result is supported by the studies of Dehl and Hoeve (24) and Migchelsen and Berendsen (12) that obtained SQ spectrum of oriented collagen fibers equilibrated with water vapors. The spectrum contained two satellites originating from water molecules with anisotropic motion and a central line with intermediate width which was assigned to mobile side chains of the proteins. Due to the limited amount of H_2O in their experiments, they did not observe the narrowest peak of free water.

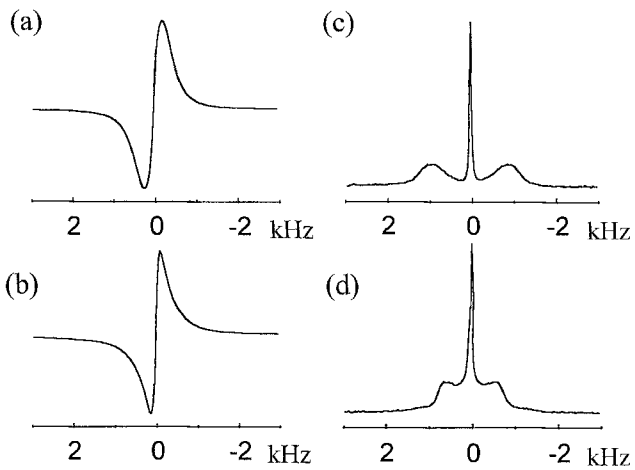

FIG. 6. ^1H DQF lineshapes (a, b) and ^2H SQ lineshapes (c, d) of rat tail tendon positioned at an angle of 0° (a, c) and 90° (b, d) relative to the magnetic field. The creation time of the DQF spectra was 0.12 ms.

Table 2
^{1}H DQF Splitting for Rat Tail Tendon Measured at Orientations of $\theta' = 0°$ and $\theta' = 90°$ of the Long Axis of the Tendon Relative to the Magnetic Field

τ (ms)	Splitting at $\theta' = 0°$ (Hz)	Splitting at $\theta' = 90°$ (Hz)	Ratio
0.04	454	268	1.7
0.12	434	249	1.7
0.40	429	224	1.9
2.00	160	190	0.8

[a] Rat tail tendon was equilibrated with saline for 24 h.

In the ^{1}H spectra of the sciatic nerve a number of resonances other than water are observed. Most of them can be assigned to lipids. Although the lipid resonances are also observable in the SQ spectra (Fig. 2), the different τ dependencies of the water and the lipid DQF signals allow the enhancement of the lipid signal as compared to the water signal, and thus facilitate their study. As the source of the DQF signal must come from ordered structures it is most likely that the lipid signals originate from membranes.

The detection of ^{1}H DQF NMR indicates the presence of order in all biological tissues studied in the present work. Moreover, the spectral width of the ^{1}H DQF spectra of tendon exhibited dependence on the sample orientation, indicating an average macroscopic order in the tissue. The presence of macroscopic order in the other tissues mentioned in this work is presently under investigation.

The presence of macroscopic order of water in tendon is in accordance with earlier results of Berendsen *et al.* (11, 12), who found that in oriented collagen fibers equilibrated with water vapor, the width of the SQ spectrum depends on the orientation of the fibers relative to the field. Peto *et al.* (25) and Henkelman *et al.* (23) found that part of the transverse relaxation rate is sensitive to the orientation with a minimum value at the magic angle of 55°. However, as was discussed in the Introduction, macroscopic order is not a necessary condition for observing the DQF signal. Local order on a microscopic scale, without macroscopic order, will result in a DQF signal, but no orientation dependence is expected in this case. The ability to detect local order is unique to the DQF NMR method. Other NMR techniques, such as measurements of T_1, T_2, diffusion coefficients, and magnetization transfer, detect macroscopic order by measuring the dependence of these parameters on the orientation.

The quadrupolar splitting of the ^{2}H NMR spectra of rat tail tendon exhibited similar dependence on the sample orientation as the ^{1}H DQF spectra of the same sample (see Fig. 6 and Table 2). This is in spite of the fact that the orientation of their directors on the molecular scale is entirely different. The reason is that averaging by the molecular motion results in either residual quadrupolar or dipolar interactions whose directors coincide with the direction of the collagen fibers. This conclusion is independent of the detailed model of the molecular motion. Such models have been suggested by Berendsen (11) and Peto *et al.* (25). Their models which differ in their details, have the common property of implying that the bound water molecules rotate around the collagen fibers, which leads to effective directors parallel to the collagen fibers.

We have shown that for rat tail tendon the main interaction that contributes to the DQF signal originates from intermolecular dipolar coupling between protons of water and macromolecules. Berendsen (12) suggests the intramolecular water proton-proton interaction to be the main source of the residual dipolar splitting, Peto *et al.* (25) assigned the T_2 dependence on orientation to the interaction between the water protons and the collagen ones. Our results clearly support the latter interpretation. Comparison between the conclusions drawn on the basis of different NMR parameters, i.e., SQ NMR splitting (Berendsen (12)), dependence on orientation of the T_2 relaxation rate (Peto *et al.* (25), and Henkelman *et al.* (23)) and the DQF peak intensity measured in the present work, is allowed since the origin of all these measurements is the same, i.e., the residual dipolar interaction.

Due to the fact that the ^{1}H DQF NMR technique filters out contributions from free water molecules, it enables us to specifically measure NMR properties of water molecules in the vicinity of anisotropic sites. One application can be the direct measurement of the diffusion of water molecules that are bound to macromolecules or undergo exchange with bound water molecules, in nerves and other biological tissues. Such experiments using ^{2}H DQF NMR were already performed on a rat sciatic nerve (26) and rat brain (27). DQF techniques can also be utilized in imaging, thus enabling us to create "anisotropy-weighted" and "τ-weighted" contrast images similarly to ^{2}H DQF imaging (28), which is based on residual quadrupolar interaction. The use of ^{1}H has the obvious advantage of being applicable *in vivo*, having higher SNR, and being nondisruptive to the samples (in terms of adding D_2O).

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