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# Discrimination between the Different Compartments in Sciatic Nerve by $^2$H Double-Quantum-Filtered NMR[1]

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**The $^2$H double-quantum-filtered (DQF) NMR spectrum of isolated rat sciatic nerve, equilibrated with deuterated saline, is composed of three quadrupolar-split water signals. On the basis of the time course of their shift by Co-EDTA$^{2-}$ and $CoCl_2$, the signals with quadrupolar splittings of about 120, 470, and 9 Hz were assigned to water in the epineurium, endoneurium, and intra-axonal compartments, respectively. The signal of the bulk water, which experiences isotropic motion, was eliminated by the DQF pulse sequence. As the maximum intensities of the water signals in the three anisotropic compartments occur at different creation times, in the DQF pulse sequence, it is possible to resolve the signals and measure their properties, such as relaxation times, independently, without perturbing the system with shift reagents.**


*Key Words:* **$^2$H double-quantum-filtered (DQF) NMR; sciatic nerve; quadrupolar splitting.**

$^2$H single-pulse and double-quantum-filtered (DQF) spectra of rat sciatic nerve equilibrated in deuterated saline have revealed three water populations with distinct quadrupolar splittings. The one with the largest quadrupolar splitting was assigned to the water in the endoneurium, the intermediate splitting to the water in the epineurium, and the very small splitting to the axonal water. This last signal could be observed only by DQF spectroscopy since the large signal of isotropic water was efficiently eliminated by this technique.

Previous attempts to separate and assign water populations in different compartments in nerves were based on the resolution of $^1$H NMR multiexponential relaxation decay curves (*1–5*). $^2$H NMR in ordered biological tissues provides an additional parameter for characterizing the water environments, i.e., the residual quadrupolar splitting. The introduction of multiple-quantum-filtered pulse sequences has greatly extended the scope of NMR studies of quadrupolar nuclei in biological tissues (*6–8*). In particular, these techniques enable observation of small residual quadrupolar interactions (*9*), which allows the detection of order in these tissues (*10*).

A graphic representation of the general structure and dimensions of the rat sciatic nerve is given in Fig. 1. The nerve comprises bundles of nerve fibers surrounded and held together by a collagen network—the endoneurium (*11*). The nerve fibers consist of axons surrounded by a phospholipid bilayer. Most of the axons are tightly wrapped by many layers of the myelin sheath, and hundreds of myelinated axons are enclosed in the flat squamous cell layer—the perineurium (*12*). The perineurium, whose structure is similar to that of the endothelium of blood vessels, serves as the blood nerve barrier and is permeable to water and ions (*13, 14*). Several perineurial bundles are wrapped together and separated from the rest of the tissue by an outer layer of collagen fibers and fibrocytes—the epineurium.

Wistar Hamamatsu rats (250–350 g, 7–13 weeks) were anesthetized with sodium pentobarbital (50 mg/kg body wt ip). The sciatic and the vagus nerve were isolated and the outer coat of adipose and connective tissue was carefully removed in saline solution (155 m*M* NaCl). For measurements of $^2$H spectra, the nerves were equilibrated in deuterated saline. Each nerve was placed in a 100-$\mu$L capillary tube and positioned with its long axis parallel to the magnetic field. For some of the measurements, the external saline was replaced by fluorinated oil (Fluorinert, FC-77, 3M), which has a low water solubility and the same magnetic susceptibility as water (H. Yamada, T. Koyano, and A. Tasaki, private communication). Collagenase I was obtained from Sigma.

NMR measurements were carried out on ARX500 and AMX300 WB Bruker NMR spectrometers, operating at a $^2$H frequency of 76.8 and 46.0 MHz, respectively. No significant effect of the field strength was found on the spectral lineshapes and relaxation times. All experiments were conducted at room temperature (about 22°C).

DQF spectra of $I = 1$ nuclei are conventionally measured using the multiple-quantum-filtering pulse sequence

$$90°-\tau/2-180°-\tau/2-90°-t_1-90°-\text{Acq.} \qquad [\text{PS1}]$$

[1] This work was presented at the Fourth Annual Meeting of the Society of Magnetic Resonance, New York, 1996.



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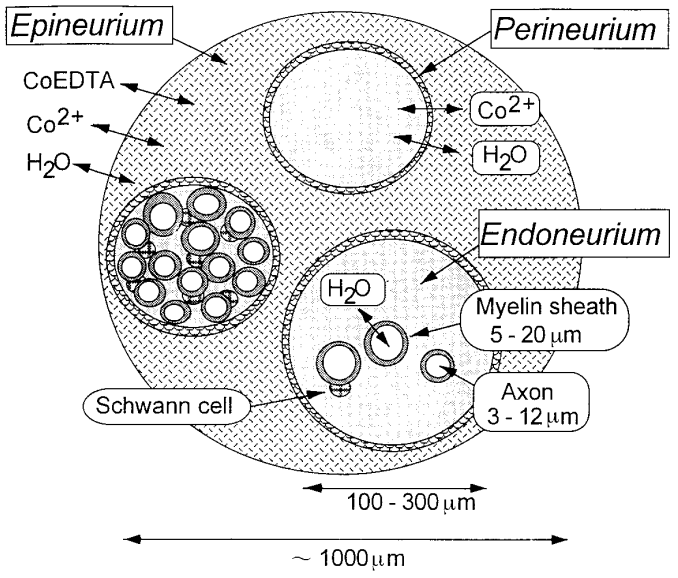


**FIG. 1.** Graphic representation of the different structures of rat sciatic nerve. Approximate dimensions are given in the figure.

For systems in which either macroscopic or local order exists, the first-rank single-quantum (SQ) coherences formed by the first 90° pulse, $T_{1,1}$ and $T_{1,-1}$, evolve under the nonzero quadrupolar interaction, $\nu_Q$, into the second-rank SQ coherences, $T_{2,1}$ and $T_{2,-1}$. The second 90° pulse converts these SQ coherences into double-quantum coherences, $T_{2,2}$ and $T_{2,-2}$. The 180° pulse inserted between the two 90° pulses serves to remove field inhomogeneities and off-resonance effects. The double-quantum coherences are transformed back to SQ coherences, $T_{2,1}$ and $T_{2,-1}$, by the last 90° pulse, and these evolve into the observable coherence, $T_{1,-1}$, during the acquisition. The DQ evolution time, $t_1$, is kept short (10–20 $\mu$s) in order to eliminate any evolution during that time. The quadrupolar-split satellites are obtained in antiphase by the DQF technique.

Since the second-rank tensor is formed only when the quadrupolar interaction is not averaged to zero, the detection of a DQF signal for $I = 1$ nuclei indicates an order in the system. The method has the advantage of not detecting water molecules in isotropic phases, such as the bulk water, which means that small quadrupolar splittings can be resolved. The detected water molecules are inside anisotropic compartments which contain water molecules bound at anisotropic sites and water molecules in fast exchange with them.

The relaxation time of the DQ coherence, $T_{DQ}$, was measured by a modification of [PS1]. This was done by using a constant value of $\tau$ corresponding to the maximum signal, $\tau_{max}$, and varying the DQ evolution time, $t_1$:

$$90°-\tau_{max}/2-180°-\tau_{max}/2-90°-t_1/2-180°-t_1/2-90°-\text{Acq.} \quad [PS2]$$

The relaxation time of the DQ coherence is given by $1/T_{DQ} = 1/4\chi^2(J_1 + 2J_2)$, where $\chi$ is the quadrupolar coupling constant and $J_n$ is the spectral density at a frequency of $n\omega_0$.

In a system where the quadrupolar interaction does not average to zero, the transverse relaxation time of the SQ coherence is modulated by this interaction. To obtain the relaxation time, the quadrupolar interaction must be refocused. This is accomplished with the DQF–SQE pulse sequence (*15, 16*):

$$90°-\tau/2-90°-\tau/2-\tau_{max}-60°-t_1-60°-\text{Acq.} \quad [PS3]$$

The second 90° is a refocusing pulse for both the first- and the second-rank tensors formed during $\tau/2$. At the end of the preparation time, $T_{2,-1}$ goes through a null and is recreated from $T_{1,-1}$ during $\tau_{max}$. The 60° pulse transfers the $T_{2,-1}$ into a double-quantum coherence. If $\tau_{max}$ is set to zero, no double-quantum coherences will be formed. The SQ transverse relaxation rate obtained from a plot of the peak height as a function of $\tau$ is given by $1/T_2 = 1/4\chi^2(3J_0 + 3J_1 + 2J_2)$. Since this relaxation rate has a contribution from $J_0$, it is proportional to $\tau_c$ when $\omega_0\tau_c \gg 1$ and therefore most sensitive to binding to macromolecules.

The longitudinal relaxation time, $T_1$, of the ordered molecules and those in fast exchange with them can be measured by adding a DQ filter after the inversion recovery pulse sequence (*17*):

$$180°-\tau-90°-\tau_{max}/2-180°-\tau_{max}/2-90°-t_1-90°-\text{Acq.} \quad [PS4]$$

The longitudinal relaxation rate is given by $1/T_1 = 1/4\chi^2(J_1 + 4J_2)$.

The single-pulse $^2$H spectrum of rat sciatic nerve equilibrated in deuterated saline is composed of five peaks: a central one and two pairs of satellite transitions (Fig. 2). The satellite splittings are 170 and 500 Hz and, to within a few hertz, are centered on the central peak. These satellites are due to quadrupolar splitting originating from water molecules in two nonexchanging anisotropic compartments. Since the nerve is immersed in a $D_2O$ solution, the central transition is due mostly to the free $D_2O$ molecules.

The $^2$H DQF spectra of rat sciatic nerve at different creation times are given in Fig. 3. The spectra are composed of a superposition of three pairs of satellite transitions evolving at different rates. It is evident from the spectra that the large central water signal has been eliminated by the DQ filter. On the other hand, a new, very narrow signal, which evolves at longer creation times, is revealed. Thus, we are detecting ordered water in three different compartments. The observation of distinct signals means that relative to their quadrupolar splitting, the exchange between these compartments is slow. Figure 4 illustrates the dependence

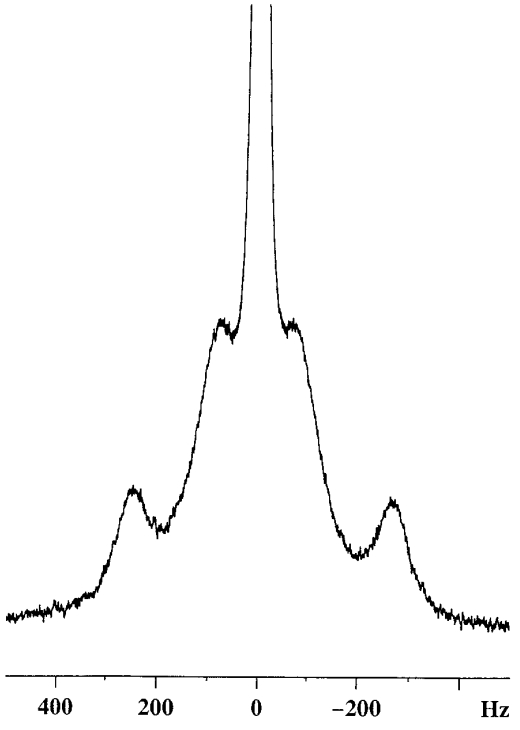


**FIG. 2.** Single-pulse $^2$H NMR spectra at 76.8 MHz of rat sciatic nerve (wet weight 50 mg) equilibrated in deuterated saline.

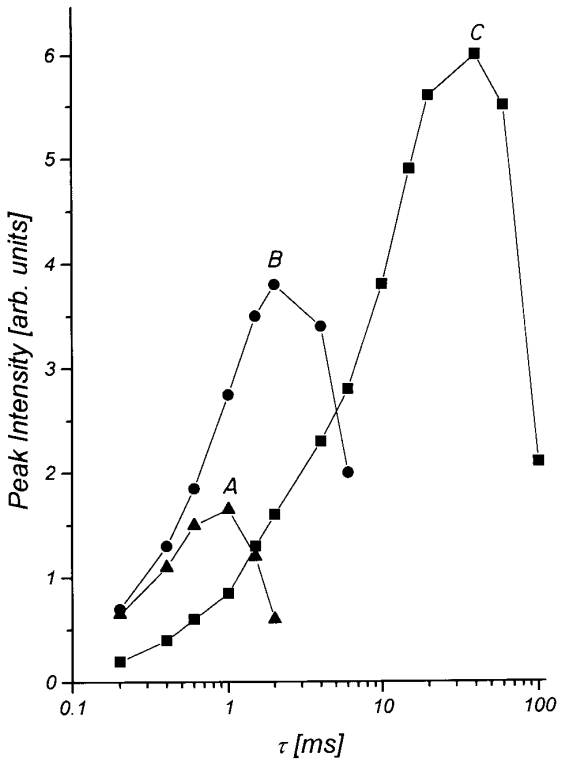


**FIG. 4.** The peak to peak intensity of the three different signals shown in Fig. 3, as a function of the creation time, $\tau$. The signals A, B, and C are those with the spectral splitting of 489, 117, and 8.5 Hz, respectively.

of the signal intensity on the creation time for the three signals. The fact that the maximum DQF signal for the three components is obtained at different $\tau$ values improves the ability to resolve the signals and measure their relaxation times. This property also allows independent measurement of the diffusion coefficients in each of the three compartments (*18*).

In the case of imperfect phase cycling, a residual signal from the free water may interfere with the narrow signal observed at long $\tau$ values. In order to determine whether the narrow signal originates from a DQ coherence or is a leakage of SQ coherences, we performed an off-resonance DQ evolution experiment by varying the time interval $t_1$ in

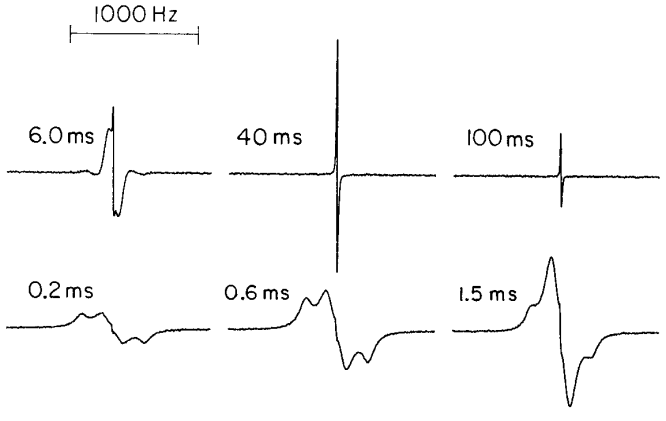


**FIG. 3.** $^2$H DQF NMR spectra at 46.0 MHz measured by [PS1] of rat sciatic nerve equilibrated in deuterated saline. Spectra are given as a function of the creation time, $\tau$. Number of accumulations, 128.

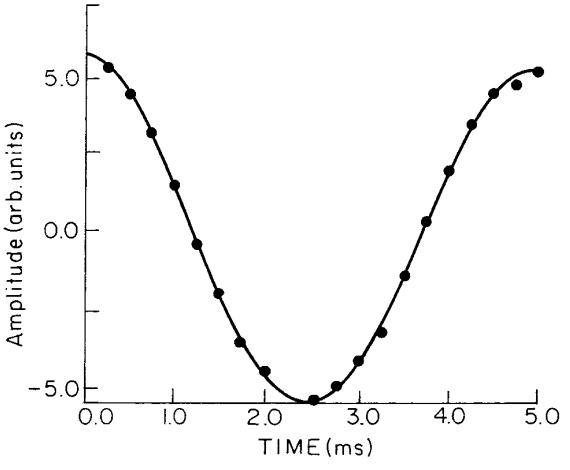


**FIG. 5.** The signal intensity of the narrow signal in Fig. 3, at a creation time of 30 ms and an offset frequency $\Delta\nu_0 = 100$ Hz, as a function of the DQ evolution time, $t_1$. The points are experimental. The line is fitted to the function $I = I_0\cos(4\pi\Delta\nu_0 t_1)\exp(-t_1/T_{DQ})$.

[PS1]. The amplitude of the signal should oscillate with the product of the offset frequency and the coherence. Results obtained at a creation time of $\tau = 30$ ms, where the narrow signal has its maximum intensity, and an offset frequency of 100 Hz are given in Fig. 5. The amplitude of the signal oscillates as a function of the DQ evolution time, $t_1$, at a frequency of 201.5 Hz, which is in good agreement with $2 \times 100$ Hz, as expected from the evolution of the DQ coherence. Thus, the narrow signal originates from water molecules in an anisotropic compartment with small quadrupolar splitting, and is denoted compartment C.

The approximate quadrupolar splittings, $\nu_Q$, as well as the SQ and DQ relaxation rates for the three signals are given in Table 1. The transverse SQ relaxation times, obtained by the DQF–SQE pulse sequence [PS3], are very short for compartments A and B, and an order of magnitude longer for compartment C. When the nerve was immersed in Fluorinert, where the spectra are not contaminated by free water, we were able to measure the longitudinal relaxation times by the standard inversion recovery sequence. As shown in the table, the longitudinal relaxation times measured by the inversion recovery sequence and those measured by the DQF inversion are the same within the experimental error. This is an indication that there is no separate isotropic compartment in the nerve.

In order to assign the three $^2$H signals detected in the intact sciatic nerve (see Table 1) to the different structures in the nerve, we performed another series of experiments. When rat sciatic nerve was equilibrated in a solution of Co-EDTA$^{2-}$ in $D_2O$, the signal of compartment B immediately shifted, together with the signal of the bulk water (Fig. 6a), while the signal of compartment A remained unchanged. The shift of the bulk water signal revealed a narrow unshifted signal which, according to its DQF spectrum, was assigned to compartment C (Fig. 6b). The spectrum remained stable for more than 12 h after the addition of Co-EDTA$^{2-}$. The DQF sequence efficiently eliminated the signal of the bulk water and displayed the signals of the three compartments. These were most clearly seen at the following creation times: A at $\tau = 0.6$ ms, B at $\tau = 4$ ms, and C at $\tau = 30$ ms. A small leakage of the bulk water was also observed at a $\tau$ of 30 ms.

Adding $CoCl_2$ to the nerve caused an immediate shift of the signal from compartment B, and a slower shift of the signal from compartment A. The signal of compartment C did not shift throughout the experiment but its intensity diminished with a half life of a few hours. These results indicate that the compartment with the intermediate splitting (B in our nomenclature) is the outermost compartment of the nerve—the epineurium (see Fig. 1). The slow penetration of $CoCl_2$ into compartment A and its impermeability to Co-EDTA$^{2-}$ indicate that this is the endoneurium which is enclosed by a membranous sheath—the perineurium. The large quadrupolar splitting observed for this compartment is also in good agreement with this assignment, since electron microscopy has shown the endoneurium to contain thick well-aligned bundles of collagen fibers (*12*). Compartment C, with the small quadrupolar splitting of about 10 Hz, is unaffected by Co-EDTA$^{2-}$ and is affected by $CoCl_2$ very slowly. We assigned this signal to the intra-axonal water which is separated from the water in the endoneurium by both the myelin sheath and the phospholipid membrane, which are impermeable to electrolytes.

Our previous measurements on cartilage (*19*) and blood vessels (*20*) showed that the $^2$H quadrupolar splitting of the $^2H_2O$ water molecules stems from their interaction with the collagen fibers. The helical structure of the collagen molecules, which form covalently cross-linked fibers, can be disrupted by the action of collagenase which cleaves the peptide bonds located in the helical region, causing the ordered structure to collapse. When collagenase is applied to rat sciatic nerve at 37°C, a continuous change is observed in its $^2$H DQF

**TABLE 1**
**NMR Parameters Measured at 46.0 MHz of the Different Water Populations in Rat Sciatic Nerve Immersed in Saline and in Fluorinert**

| | Saline | | | Fluorinert | | |
|---|---|---|---|---|---|---|
| Compartment: | A | B | C | A | B | C |
| Splitting, Hz | 489 | 117 | 8.5 | 479 | 126 | 8.5 |
| $T_2$, ms | — | 8.9 ($\tau$ = 4 ms) | 65.2 ($\tau$ = 30 ms) | 3.6 ($\tau$ = 0.6 ms) | — | 72 ($\tau$ = 30 ms) |
| $T_{DQ}$, ms | 41 ($\tau$ = 0.6 ms) | 196 ($\tau$ = 4 ms) | 150 ($\tau$ = 30 ms) | 40.7 ($\tau$ = 0.6 ms) | 167 ($\tau$ = 4 ms) | 232 ($\tau$ = 30 ms) |
| $T_1$, ms[a] | | 250 ($\tau$ = 1.5 ms) | 283 ($\tau$ = 30 ms) | 126 ($\tau$ = 0.6 ms) | 234 ($\tau$ = 4 ms) | 291 ($\tau$ = 30 ms) |
| $T_1$, ms[b] | — | — | — | 120 | 230 | 285 |

[a] Measured by DQF inversion recovery pulse sequence [PS4].
[b] Measured by inversion recovery.

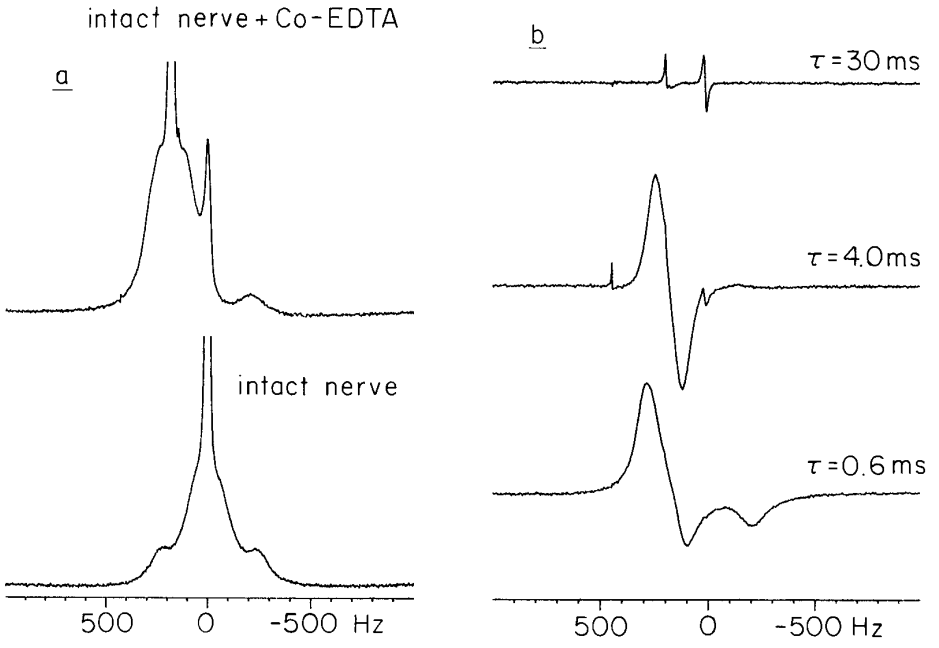


**FIG. 6.** (a) Single-pulse $^2$H NMR spectra of rat sciatic nerve and the same nerve equilibrated with a solution of 100 m*M* Co-EDTA$^{2-}$. (b) $^2$H DQF NMR spectra of the nerve equilibrated with a solution of 100 m*M* Co-EDTA$^{2-}$ at three creations times, 0.6, 4, and 30 ms, which are optimal for the selective observation of the three water populations. The creation times are indicated in the figure.

spectra (Fig. 7). Within an hour the signal of compartment B is lost, and only at longer incubation times does the signal of compartment A broaden and decay. This is another indication that the compartment most susceptible to modification is compartment B, i.e., the outermost compartment, the epineurium. The broadening and decreasing of the DQF signal of compartment A are followed by a decrease of the signal from compartment C as well (not shown). We propose that the DQF signal from compartment A originates from the interaction of water molecules with the collagen fibers of the endoneurium which fill the space between the axons and support them. With the loss of this orientational support, the DQF signal of the intra-axonal water which we assign to compartment C is expected to undergo changes as well.

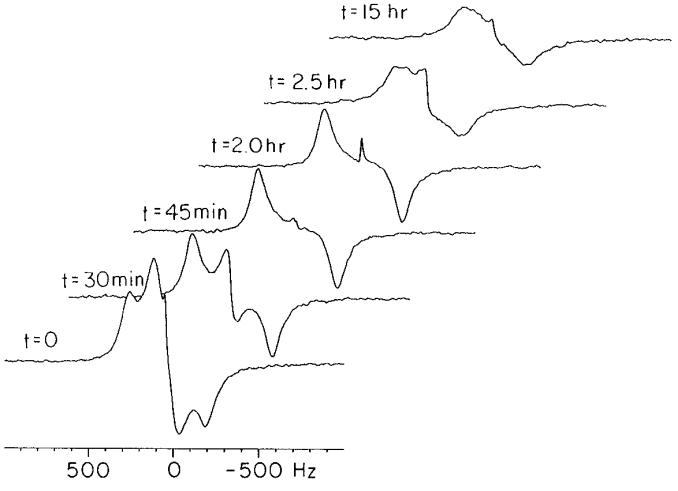


**FIG. 7.** $^2$H DQF NMR spectra of rat sciatic nerve incubated with 15 mg/ml collagenase, at 37°C. The spectra were measured at a creation time of 4 ms and are shown as a function of the incubation time.

We also checked the $^2$H SQ and DQF spectra of an unmyelinated rat nerve, the vagus nerve. Three distinct signals are observed in the $^2$H DQF spectra of the rat vagus nerve equilibrated in deuterated saline, similar to the case of the sciatic nerve (Fig. 8). However, their splitting is larger: 1560, 386, and 12 Hz for compartments A, B, and C respectively. These should be compared to the values of 467 ± 16 ($n$ = 8), 123 ± 23 ($n$ = 5), and 8.7 ± 0.9 Hz ($n$ = 7) for the same compartments in the sciatic nerve. In addition, the time of the appearance of these signals is much shorter. The DQ relaxation time of the water in the narrow compartment was found to be 162 ms, which is in good agreement with the 230 ms in the sciatic nerve. Thus the same behavior is observed for both the myelinated sciatic nerve and the unmyelinated vagus nerve.

Three different water compartments were identified and characterized in rat sciatic nerve equilibrated in deuterated saline by $^2$H SQ and DQF NMR techniques. To the best of our knowledge, no other method allows noninvasive separation and monitoring of these different water compartments. The three DQF $^2$H NMR signals were assigned to the water in the epineurium, endoneurium, and axon. Since the maxi-

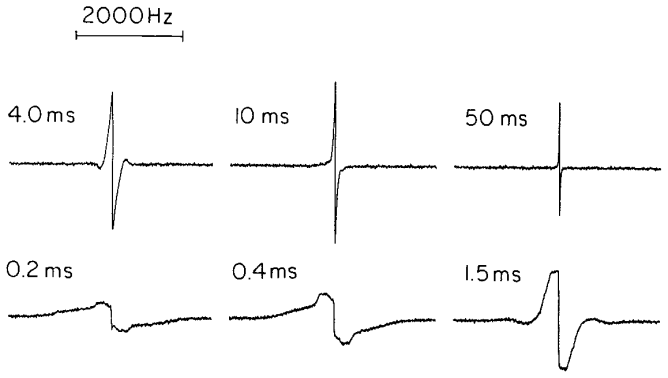


**FIG. 8.** $^2$H DQF NMR spectra at 46.0 MHz measured by [PS1] of rat vagus nerve equilibrated in deuterated saline. Spectra are given as a function of the creation time, $\tau$. Number of accumulations, 2048.

mum DQF signals for the three compartments occur at different $\tau$ values, it is possible to resolve the signals and measure the relaxation times independently. Moreover, this unique method completely eliminates the free water, enabling the monitoring of the intra-axonal compartment without perturbing the system with shift reagents.

According to recent reports, the differentiation of the water populations in the peripheral and central nervous systems is based on resolving the multiexponential transverse magnetization decay curves of the water. From this type of fitting procedure, both the relative fractions and the relaxation rates are obtained. Vasilescu *et al.* (*1*), Menon *et al.* (*2*), and Does and Snyder (*5*) reported three distinct transverse relaxation components of water in frog sciatic nerve, crayfish nerve cords, and sciatic nerve of *Xenopus laevis*, respectively. Henkelman *et al.* (*3*) reported two water populations in bovine optic nerve as well as in the brain white matter. In all these reports, the populations with short $T_2$ (10–50 ms) and intermediate $T_2$ (around 100 ms) were tentatively assigned to water in the myelin and axoplasm, respectively. These assignments were based on the assumption that the fast relaxing component must be associated with water interacting with macromolecules and phospholipids. Seo *et al.* (*4*) studied water compartmentation using relaxation times and diffusion coefficients of $^1H_2O$ in rat sciatic nerve, and found that the short $T_2$ components (ca. 30 ms) represent water in the axon and Schwann cells. A recent *in vivo* study on human brain (*21*) revealed two or three water populations whose relative weights were location-dependent.

The assignment of the different water populations in rat sciatic nerve in our study is based on results obtained by selective perturbation achieved by adding the shift reagents Co-EDTA$^{2-}$ and $CoCl_2$. We found that $CoCl_2$ penetrated the perineurial sheath, while Co-EDTA$^{2-}$ was hindered by this membranous structure. This result is in accordance with the impermeability to GdDTPA$^{2-}$ of the blood brain barrier, which has constituents similar to those of the perineurium. The assignment is also based on the action of collagenase which first affects compartment B, the epineurium, while compartment A is affected only at much longer incubation times.

In our previous studies on cartilage (*19*) and blood vessels (*20*), the $^2$H DQF NMR signal was seen to stem from the interaction of the deuterated water molecules with the oriented collagen fibers. The motional properties and residual quadrupolar splittings of the water in the three compartments, obtained by various relaxation measurements, are in accordance with the structure of the anatomical compartments of the nerve. Both the splitting and the transverse relaxation rates were the greatest for the endoneurium, which is known to have thick, well-oriented collagen fibers. For the intra-axonal compartment, the splitting was very small ($8.7 \pm 0.9$ Hz), and the $T_2$ relaxation time was an order of magnitude longer than that of the other compartments, pointing to a weaker interaction of water with ordered structures in the axon. Studies on the brain of rats fed heavy water also showed that the $^2$H splitting was of the order of 10 Hz (*22*). This is in good agreement with our data on the splitting of the intra-axonal water. The source of this interaction is not known but may be related to tubulin aggregates which are the major constituents of the cytoskeleton.

$^2$H DQF NMR is not suitable for estimating the water populations in the different compartments, since the DQF signal intensities depend on the relaxation times and the residual quadrupolar interactions. We therefore estimated the ratio of the water populations in compartments A and B and compartments A and C from the single-pulse experiments on the nerve without (Fig. 2) and with (Fig. 6a) the Co-EDTA$^{2-}$ shift reagent, respectively. The approximate result was 31, 44, and 25% for A, B, and C, respectively. Again, the results conform to the anatomical structure of the nerve and rule out the possibility that one of the signals arises from water in the myelin. Since our evaluation is based on the SQ spectra, the narrow signal assigned to the water in the axon might also include water from Schwann cells and fibrocytes; the fraction of these water populations is small and might not have been detected.

Our assignment of water compartments is strongly supported by the fact that the same type of $^2$H DQF NMR spectrum is obtained for the vagus nerve which, unlike the sciatic nerve, has only one layer of myelin. The similarity of the $^2$H DQF spectra for the myelinated and nonmyelinated nerves indicates that intramyelinic water is not observed as an independent signal in the spectrum.

Our preliminary diffusion measurements of $D_2O$ in sciatic nerve (*18*) indicated that the diffusion of water in compartment C, which we assigned here to the axon, is restricted in the perpendicular direction. The diameter of that compartment was found to be of the order of 7 $\mu$m. The diffusion in compartments A and B is not restricted, leading to the conclusion that the diffusion distance in these two compart-

ments is much larger than 7 $\mu$m. The average distance of 0.003 $\mu$m between two adjacent layers of the myelin sheath, and our failure to obtain such a diffusion distance further, confirms that we are not seeing intramyelinic water. This is, in fact, expected and may be due to a very small fraction of water plus a very short relaxation time.

This paper demonstrates that $^2$H DQF NMR enables resolving the water signals from different compartments in the nerve, without the addition of shift reagents. Moreover, the large signal of the bulk water is eliminated, further facilitating the assignment of the water signals to the different compartments and the measurement of their dynamic properties.

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