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Observation of Triplet Traces Obtained with Inversion Recovery Method in Both Residual Water- and H_2O/D_2O-Albumin Mixture by Using 400 MHz Proton NMR

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NMR studies involving H_2O/D_2O mixtures are mainly based on a single spectrum of water. However, some recent papers have revealed the presence of an HDO triplet in H_2O/D_2O, which is caused by the splittings of H_2O signal by deuterium (D). Observation of inversion recovery (IR) traces of this triplet should be very useful for analyzing relaxation data in such mixtures. In this work, in order to obtain the traces, inversion recovery signal intensities of residual water and a mixture of 0.10 H_2O and 0.90 D_2O were acquired *versus* a set of short delay times in the presence or absense of 0.2 g albumin. T_1 and T_2 curves of the same samples were also obtained by using different sets of much longer times for checking the effect of radiation damping on the traces. Experiments were carried out with a 400 MHz proton NMR spectrometer equipped with a topspin programme for relaxation measurements. Inversion recovery data of the traces and T_1 curves were obtained with the inversion recovery method, while T_2 curves were obtained with the Carr-Purcell-Meiboom-Gill method. Data was processed through an analysis programme of the topspin. Data showed that radiation damping is effectively reduced upon addition of albumin. Furthermore, the data of the residual water- and the mixture with albumin demonstrated the traces of the triplet at the initial part of a single inversion recovery line. Our results suggest that the splitting of water proton signal by deuterium can be detectable by the inversion recovery method for D_2O- and the mixture containing albumin.

Key Words: NMR, ^1H, H_2O/D_2O mixture, Albumin, H-D splittings, Triplet.

INTRODUCTION

H_2O/D_2O mixtures are being used as solvent for proteins and some other chemical compounds[1-7]. In the mixture of H_2O and D_2O, there are three molecular species H_2O, D_2O and HDO in a dynamic equilibrium[8,9]. However, NMR spectrum of the mixture shows a single line at all static magnetic fields. Accordingly, the studies involving such solvents are mainly based on this single spectrum of water. The interpretation of the spin-lattice (T_1) and spin-spin (T_2) relaxation data in H_2O/D_2O mixture is known to be difficult[8,9]. The analysis of the equilibrium data among H_2O, D_2O and HDO was also reported to be difficult[10]. On the other hand, the small spin-spin splittings of H_2O signal by deuterium (D) in H_2O/D_2O were studied by proton NMR for deuteriated water[10]. In addition, a triplet of ^1H NMR signals, which is the splitting of H_2O signal by deuterium, was recently presented through a special free induction decay (FID) processing programme[11]. The splittings may be demonstrated further by the relaxation effects of the triplet components. The demonstration may be achieved by acquiring inversion recovery (IR) data of the triplet. It should contribute to the interpretation of NMR relaxation mechanisms in H_2O/D_2O medium. However, a strong interaction between high proton magnetization and FID current induced in RF coils causes radiation damping (RD)[12-16]. Radiation damping interferes with the relaxation times. For this reason, a particular attention must be paid to the effect of radiation damping when such inversion recovery data is investigated at higher fields.

In the present work, one group of experiments was designed to observe the traces of the triplet. In this type of experiments; inversion recovery signal intensities of residual water and a mixture of 0.10 H_2O and 0.90 D_2O, in the presence or absence of albumin, were obtained *versus* a set of short delay times. The relationship between signal intensities and delay times was then displayed in the relaxation window. Second group of experiments was also carried out to determine the effect of radiation damping on the traces. In this case, inversion recovery and Spin Echo (SE) signal intensities of the same samples were obtained *versus* a set of much longer times. The experimental data was fitted by using the magnetization recovery and echo decay formulas. T_1 and T_2 curves of the samples with and without protein were then compared to evaluate the effect of radiation damping.

EXPERIMENTAL

Preparation of samples: D_2O with 99.9 % purity (0.1 mL residual water per 100 mL of solution) was purchased from Merck (Merck KGaA, Darmstadt/Germany). The mixtures of H_2O/D_2O were prepared by adding 0.10 mL H_2O to 0.90 mL D_2O so that total volume is 1 mL. The samples containing the protein were prepared by adding 0.2 g of albumin to 1 mL of each of D_2O and the mixture. Probe temperature was kept at 25 ºC by using an automatic temperature controller unit. Prior to NMR measurements, all samples were deoxygenated with a flowing nitrogen stream for one hour to remove oxygen by a method similar to those given in previous works[4,5].

Acquisition parameters and data processing: All measurements were made in the absence or presence of 0.2 g albumin. The experiments were carried out on a BRUKER AVANCE-400 MHZ Proton NMR spectrometer equipped with the QNP probe. The probe was adjusted to the tuned position for all cases. The spectrometer was also equipped with the Topspin Programme (Topspin 1.3) for relaxation measurements. The inversion recovery and Spin Echo curves of residual water and the mixture were obtained by the inversion recovery and CPMG (Carr-Purcell-Meiboom-Gill) methods. Pulse repetition time of each experiment was set to a value above $5T_1$ of the every corresponding sample. The T_1 of every sample was determined by pioneer experiments. Puls repetition times of pure residual water, residual water with albumin, pure mixture and the mixture with albumin were 100, 25, 55 and 15 s, respectively. This set of the pulse repetition times were used for whole of the experiments (the traces and also T_1 and T_2 curves). Delay times in inversion recovery experiments and also echo times in CPMG experiments were altered by equal steps. Observation frequency, number of scans and 90º pulse were 400.132 MHz, 1 scan and 9.75 m, respectively. The other experimental conditions were the same for all cases.

Furthermore, an analysis programme of the topspin was used to process the acquired data in the relaxation mode. Following standard procedures (FT of FID signals, phase correction and integration), inversion recovery or Spin Echo curves were obtained in the relaxation window.

Experiments designed for observing the inversion recovery traces of the triplet and the characteristics of spectra related to the traces

First step: Successive FID signals of residual water and the mixture were acquired *versus* a set of delay times altering from 20-2000 m. The incremental increase between the successive delay times was selected to be 20 m. The data was acquired and processed as explained in the part of "acquisition parameters and data processing". The traces of the triplet were then displayed in relaxation mode by a plot of signal intensities *versus* delay times.

Second step: The FT of each FID signal relevant to the traces was made for the mixture with and without albumin. The shape of spectra with albumin was compared with that of spectra without albumin. A similar comparison was also made for the residual water.

Third step: The chemical shift of each water spectrum was calculated for 27 successive points creating the traces. The chemical shift of the following 27 points on the single inversion recovery line was also calculated for evaluating reading error.

Experiments designed for explaining the effect of radiation damping on the traces of the triplet: The successive FID signals were acquired *versus* one hundreds of delay times altering up to a value around $5T_1$ for every sample. Fixed steps of pure residual water, residual water with albumin, pure mixture and the mixture with albumin were 0.8, 0.2, 0.5 and 0.1 s, respectively. In addition, the successive echo decays of the same samples were obtained by using fifty of echo times. In this case, fixed steps of pure residual water, residual water with albumin, pure mixture and the mixture with albumin were 100, 8, 160 and 12 m, respectively. Each fixed step was used to obtain a set of delay times or echo times. The data was acquired and processed in accordance with the explanation given in "acquisition parameters and data processing" section. The data obtained with inversion recovery was fitted by using the magnetization recovery formula describing the return of the z-component of magnetization to the equilibrium value, while that with CPMG was fitted by the formula describing dephasing of transfer magnetization. The former fit gives T_1 relaxation curve, while later gives T_2 relaxation curve.

RESULTS AND DISCUSSION

Results related to the traces and the characteristics of the relevant signals: The data of the residual water giving the traces are shown in Fig. 1, while that of the mixture are shown in Fig. 2. In this figure and the following figures, (a) represents pure samples, while (b) represents the samples doped with albumin. In the absence of albumin, no triplet is detectable for residual water. However, the triplet is clearly detectable in the presence of 0.2 g albumin. In addition, the triplet can be chosen for the mixture without and with albumin, too.

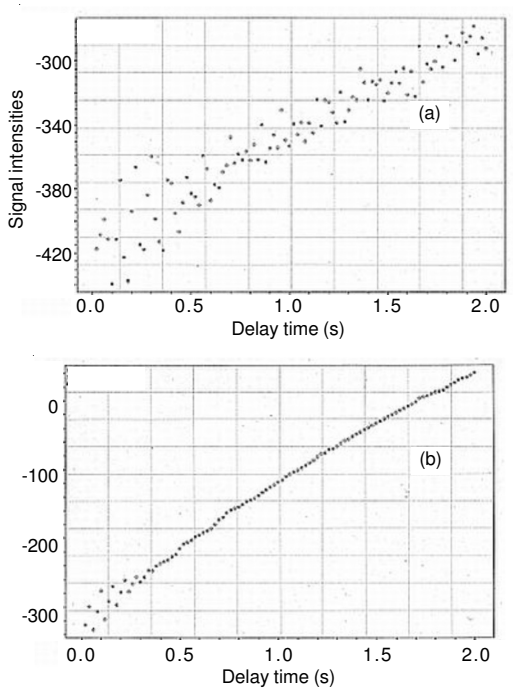


Fig. 1. Inversion recovery curves of residual water in the absence (a) or presence (b) of albumin

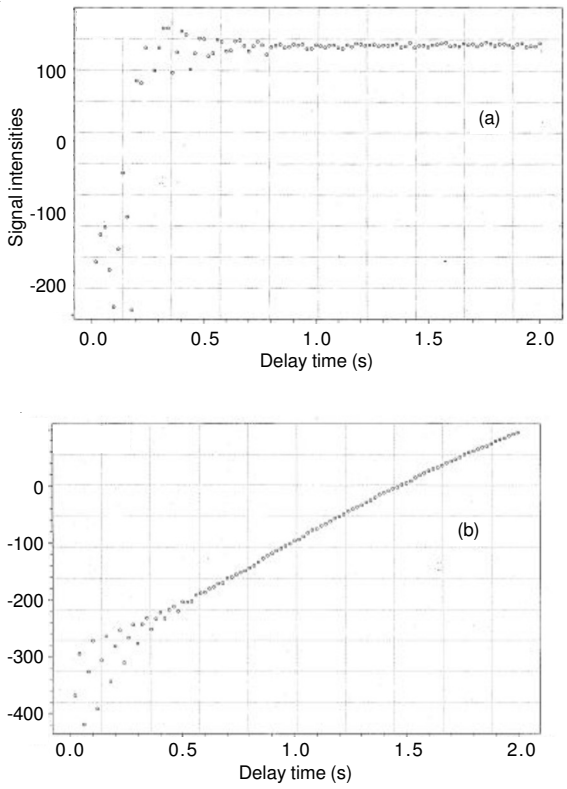


Fig. 2. Inversion recovery curves of the mixture of 0.10 H_2O and 0.90 D_2O in the absence (a) or presence (b) of albumin

Fig. 3a shows a representative spectrum belong to the traces given in Fig. 2a for pure mixture, while Fig. 3b shows that belong to the traces given in Fig. 2b for the mixture with 0.2 g albumin. In case of pure mixture, almost all spectra have phase distortions. However, all spectra of the samples with albumin have a Lorentzian shape similar to that given in Fig. 3b. In the presence of albumin, the signals creating the inversion recovery curves of the triplet were inverted along (-z)-axis and they decrease as the delay times increase. The traces merge

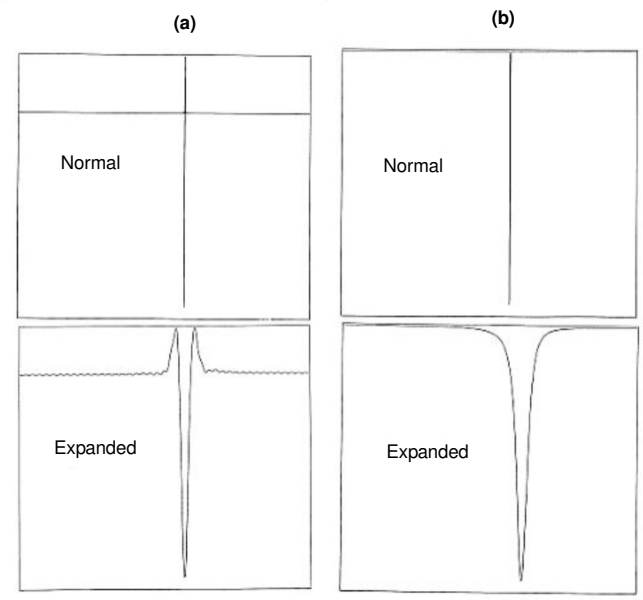


Fig. 3. NMR spectrum of water in the mixture of 0.10 H_2O and 0.90 D_2O in the absence (a) or presence (b) of albumin

at delay times around 500 m. On the other hands, the points of each curve of the triplet were selected in serial way. If the short delay times of 20 m, 40 m, 60 m, 80 m, … are denoted by 1, 2, 3, 4, 5, …; the points of one curve are selected as 1, 4, 7, 10 …., those of second curve are 2, 5, 8, 11… and the numbers of third one are 3, 6, 9, 12… . That is, inversion recovery data of the traces is alternately recorded.

The chemical shifts of the 27 signals constituting the triplet in Fig. 2b were changed from 4.7161-4.7183 ppm, while those of the following 27 signals of the single line were changed from 4.7175-4.7181 ppm. The standard deviation for the former case is 0.0006 ppm, while that for the later case is 0.0002 ppm.

Results giving a clue for the effect of radiation damping on the curves of the triplet: The data of residual water giving the T_1 curve are shown in Fig. 4, while that of the mixture are shown in Fig. 5. The T_2 curves of the same samples are shown in Figs. 6 and 7. It can be clearly seen that both T_1 and T_2 curves of the pure mixture suffer from radiation damping. On the other hand, T_1 curves of residual water- and the mixture with albumin obey $M_z = M_0(1\text{-}2\ \exp - t/T_1)$, while T_2 curves of the same samples obey $M_{xy} = M_0\ \exp - t/T_2$. This indicates that the radiation damping is effectively reduced after addition of albumin. In the other words, there is a negligible effect of the radiation damping on the relaxation of the samples with albumin.

Molecular species in H_2O/D_2O mixture: In the residual water, there are mainly three molecular species such as H_2O, D_2O and HDO in the solution[7]. The dynamic reaction among H_2O, HDO and D_2O can be expressed by the following equation[8-11].

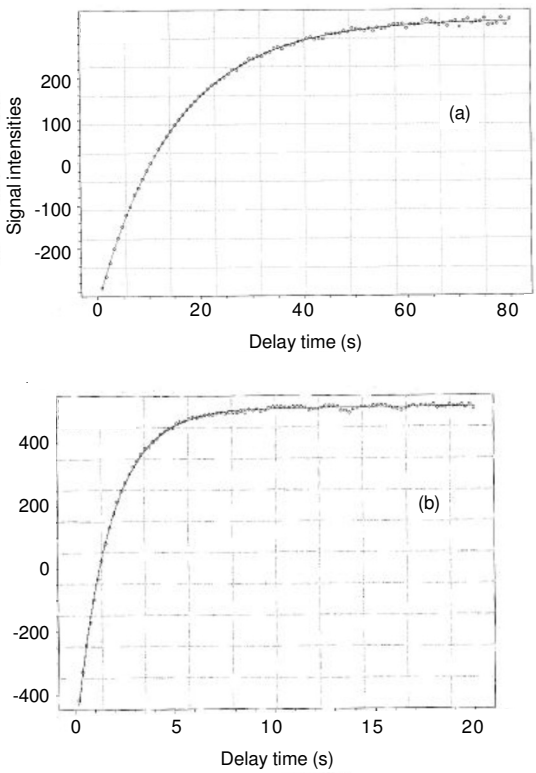


Fig. 4. T_1 curves of residual water in the absence (a) or presence (b) of albumin

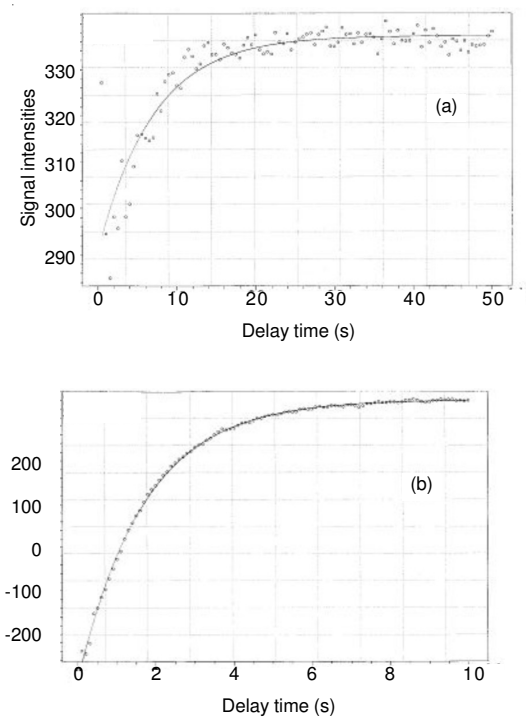


Fig. 5. T_1 curves of the mixture of 0.10 H_2O and 0.90 D_2O in the absence (a) or presence (b) of albumin

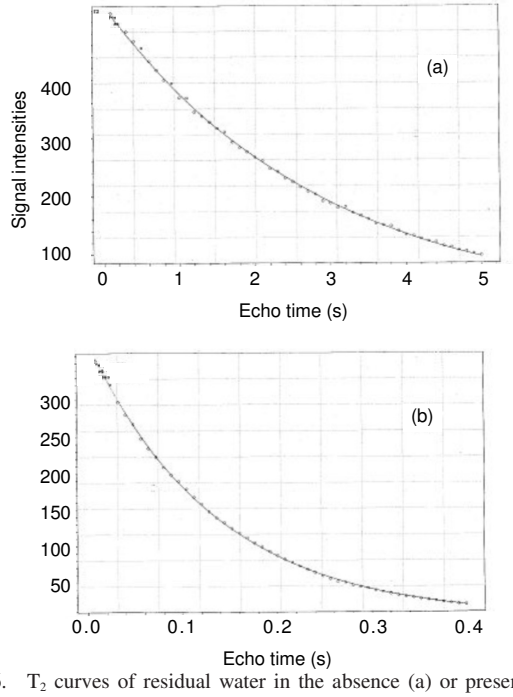
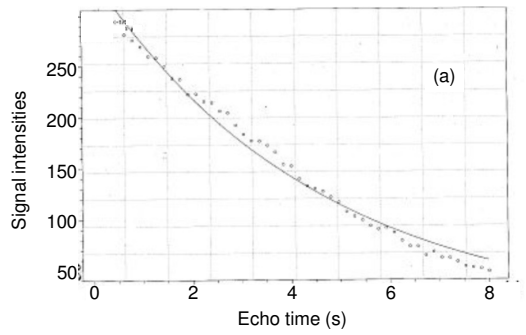


Fig. 6. T_2 curves of residual water in the absence (a) or presence (b) of albumin



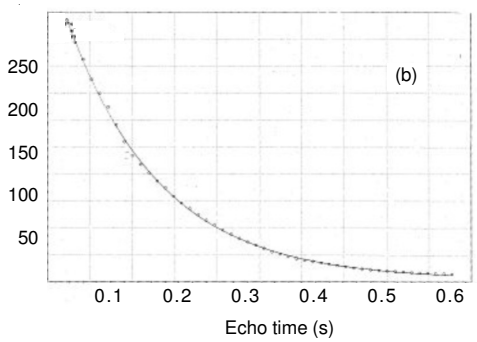


Fig. 7. T_2 curves of the mixture of 0.10 H_2O and 0.90 D_2O in the absence (a) or presence (b) of albumin

$$H_2O + D_2O \leftrightarrow 2HOD \quad (1)$$

In the case of H_2O/D_2O mixture, H_2O is added to D_2O solution containing residual water. As a result, there is a formation of fresh HDO molecules through the interaction of H_2O and D_2O in accordance with eqn. 1. In the presence of albumin, the some molecules of residual water are bound to albumin and the others stay to be free[4,5]. A fast chemical exchange of protons takes place between both states. On the other hand, spin quantum number of deuterium is known to be 1. Accordingly, deuterium can split an H signal into 3 through different orientations denoted as -1, 0 and 1. The traces and the following single line are consistent with the spin quantum number of deuterium and the molecular diversity given by eqn. 1. However, the relevant spectra of the splitting are not detectable since the chemical shifts of the splitting are very small[11].

Effect of radiation damping on the inversion recovery and Spin Echo curves: When the current in the RF coils induced by the processing magnetization is highly strong, it produces a torque on the magnetization and drive the magnetization back to z-axis[12-16]. The rotation of magnetization towards z-direction by such a torque is called radiation damping[12-16]. The radiation damping causes distortions in the shape of spectra[16]. In the presence of radiation damping, inversion recovery or Spin Echo data can not be fitted to the relevant relaxation formulas well.

It is known from earlier experimets that protein protons relaxes very fast[17,18]. Therefore, the magnetization of the present case is dominantly related to solvent protons. Decrease in the fraction of solvent water should be one reason for the reduction of radiation damping in the presence of albumin. Shortened T_2 values of samples containing albumin should be other reason for this reduction since it leads to smaller magnetization.

Possible causes of the observed traces showing a triplet: There are two main possibilities for the cause of the traces at the initial part of T_1 curve: (1) The traces may be caused by radiation damping. (2) The traces should be caused by the very small splittings of H signal by deuterium. The present data is in the favour of second choise. The clues for this claim can be as follows:

Imperfections in 180º pulse cause a small deviation of the inverted magnetization from -z-axis. This should lead to a poor contribution of radiation damping to inversion recovery data when proton magnetization is high. The solvent magnetization of residual water is higher than that of the same sample

with albumin. This is due to the decrease in the fraction of solvent (free) water in the later case. As a result, the poor contribution of radiation damping should be the source of distributive nature of data points on the upper part of the inversion recovery curve in Fig. 1a. However, when albumin is added, radiation damping is negligibly reduced for the same part of the curve.

The theory of radiation damping is explained in a very simple way in some works[16]. According to the theory, the magnetization keeps coherent when the recovery of magnetization is controlled by radiation damping[16]. It was also reported that the inverted magnetization grows before decaying[16]. However, the whole data of the traces are divided into three parts and the signal in each part decreases first. Therefore, the inversion recovery data of the traces obtained for samples with albumin is not consistent with the theory of radiation damping.

As mentioned above, radiation damping affects the shape of spectra. This can easily be seen in Fig. 3a, showing a twisted phase for the spectra of the pure mixture. Such a distortion was reported in the presence of radiation damping[16]. However, in the presence of albumin, the shape of spectrum given in Fig. 3b is Lorentzian as in the absence of radiation damping.

When inversion recovery magnetizations of components with different relaxation times are observed, the recovery of each component must follow a way related to its own relaxation. Accordingly, the inversion recovery data of the components are alternately recorded. Thus the alternate record observed for inversion recovery data of the traces is consistent with the presence of multiple relaxations.

T_1 curves obtained for the set of longer delay times are the extensions of inversion recovery curves obtained for the traces. That is, the traces can be seen as the initial part of T_1 curves. Therefore, such T_1 curves give an idea for the effect of radiation damping on the traces. The perfect fits between signal intensities and delay times (Figs. 4 and 5b) indicate that radiation damping is not effective for samples with albumin. Such an idea can be checked further by T_2 measurements. The perfect fits between echo intensities and echo times (Figs. 6 and 7b) confirm the negligibility of radiation damping in the presence of albumin.

The observations given through the items from (a) to (e) indicate that the traces in Figs. 1b and 2b are not caused by radiation damping. This eliminates the first choice to be the source of the traces. In addition, the traces are convenient to the study reporting small spin-spin splittings in H_2O/D_2O mixture[10]. They are also consistent with the previous one manifesting such a triplet by using a reconstitution programme[11].

The reconstitution programme permits the observation of the splittings of water signal. In the present case, the splittings were not observed. This should be due to very small chemical shift between the splittings. In fact, the small standard deviation (0.0002 ppm) obtained for the chemical shift of the points on the single line in Fig. 2b gives estimation for the range of the experimental error. The larger standard deviation (0.0006 ppm) obtained for the chemical shift of the points creating the traces should therefore be related to very small splittings of H signal by deuterium. It is clear that such a very small chemical shift prevents the observation of the splittings. Nevertheless, the presence of the splittings is being demonstrated through their relaxation effects.

Usefulness of the present results: In H_2O/D_2O mixture, the relaxation process involves inter- and intra-dipolar relaxation and chemical exchange between H-H and H-D[9]. The spin rotation mechanism was also considered[8,9]. There is also free and bound states of water molecules when protein is added to the mixture[4,5]. Such a variety makes the interpretation of relaxation to be difficult. The observation of the inversion recovery traces of the triplet should therefore contribute to the analysis of the relaxation in H_2O/D_2O. It may also be helpful for analyzing j-data of the mixture.

Conclusion

The present data suggest that the splittings of water proton signal can be detectable by the inversion recovery method for D_2O and the mixture of 0.10 H_2O and 0.90 D_2O containing albumin. Data also suggest that radiation damping is negligibly reduced after addition of albumin to the mixture.

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