Correlation NMR Spectroscopy with a Water Signal Subtracting System

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A modified correlation NMR method which includes the baseline correction systems in both hard- and softwares has been developed. By applying this method the proton signals of cytochrome b_5 and adenosine 5′-monophosphate at low concentration in H_2O solutions were obtained.

In the investigation of biological systems by proton magnetic resonance very strong signal of the solvent H_2O or HOD is troublesome. It is especially serious in the measurements of the imino and amino proton signals of proteins and nucleic acids. They are slowly exchangeable with water protons and their signals can only be detected in H_2O solutions. If a normal pulse Fourier transform (FT) NMR is applied for H_2O solutions, the signals of the sample at low concentration are hardly detected because the ratio of the sample signal to the solvent signal is too small that the finite dynamic ranges of the receiver and analog-digital (A/D) converter overlook the sample signal. Some groups have tried to eliminate or reduce the effect of the strong solvent signal by modifying the pulse FT NMR.[1-6] Another important device to solve the problem is correlation NMR, which has been introduced by Dadok and Sprecher,[7] and developed by other groups.[8-10] In this method the spectrum can be scanned only in the necessary frequency region and optimum conditions for observing the spectra are easily adjustable. We tried to develope the method taking its advantage into account. At the present work we will show a system of correlation NMR spectroscopy containing baseline correction systems in a sampling process, which is useful to eliminate the tail of the solvent signal. And also some applications to the biological substances in H_2O solutions will be presented. We are successful in getting the spectra at low concentrations.

Instrumentation

The principle for obtaining a correlation spectrum is based on the report of Gupta *et al.*[8] The method with a reference signal is employed instead of a theoretical function method to eliminate wiggles.

Figure 1 shows the block diagram of our system of the correlation NMR spectroscopy. A JEOL PS-100 NMR spectrometer was used together with a DP-1A pulse programmer and a EC-100 computer. The computer has 20 K words of memory (16 bits/word). We constructed a sweep generator where sawtooth waves are digitalized by the use of a D/A converter (12 bits). The digitalization of the sawtooth wave is essential to determine the chemical shift because we can know the exact sweep range from the frequency counter by holding the

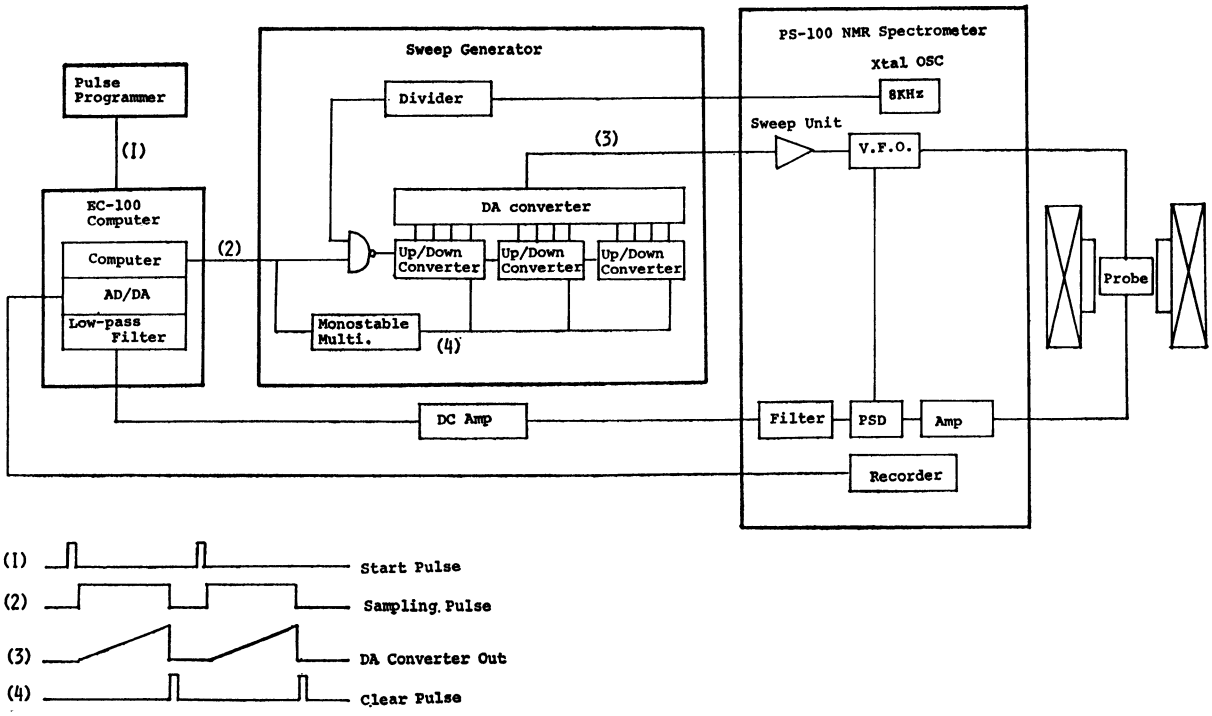

Fig. 1. A block diagram of a correlation spectrometer and a timing diagram for a computer control of a digitalized frequency sweep.

voltage of the sawtooth at the beginning and the end of the scan. The shapes of the control pulses and their relations with each other are also shown in Fig. 1. The transient response to the frequency sweep is passed through a low-pass filter and A/D converter (12 bits), then collected in the computer. The computer program for the system was written down by modifying the JEOL PFT-100 computer program. When we collect a transient response of a sample in a 4K words memory area and that of a reference signal in another 4K words memory area, we can get a final spectrum in a 8K words memory area (a 4K area for a real part and the other 4K area for an imaginary part). We confirmed the linearity of the frequency sweep and the accuracy of the chemical shift by measuring the mixture of chloroform, dichloromethane, dioxane, 1,1,1-trichloroethane, acetone, cyclohexane, and tetramethylsilane. The linearity was good enough so that the error of the chemical shift of every signal was less than 0.1%.

A hardware system for the baseline correction in the process of collecting the data was added to this correlation NMR spectrometer in order to reduce the effect of the tail of the H_2O signal. The flow chart of this system was illustrated in Fig. 2. Two kinds of baseline correction treatments were introduced into the hard and softwares. The process to eliminate the effect of a large tail of the solvent signal is as follows. At the first step, a function (a quadratic or exponential function) is subtracted from the transient response in an electronic circuit just before the phase sensitive detector (hardware subtraction). Beforehand the same treatment was done for the signal of pure solvent under the same conditions and stored in a certain memory area of the computer. This residual baseline of the solvent is further subtracted from the hardware treated sample spectrum in the computer (software subtraction). The transient response can be accumulated for certain times before this subtraction. The resultant spectrum was further accumulated in another memory area. It should be noted that the first baseline correction took away to some extent the saturation of the phase sensitive detector (PSD) by the strong solvent signal and improved the signal condition to pass through the dynamic range restriction of the A/D converter. The second step enchanced the efficiency of accumulation.

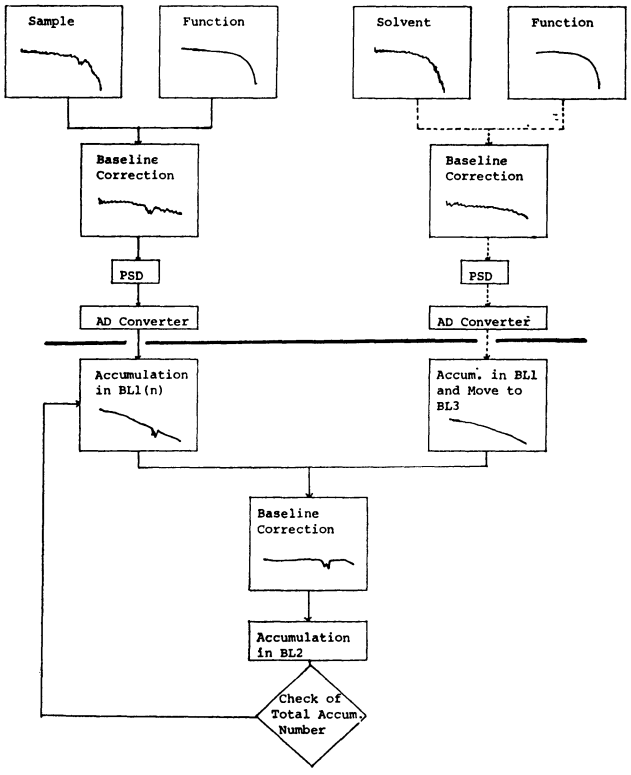

Fig. 2. A flow chart of a baseline correction system. Upper: in a hardware, lower: in a software. The part shown in broken lines indicates the one which should be operated before the treatment of the sample signal. BL1 (2 or 3): Block 1 (2 or 3) in the memory area.

Spectral Measurement

The above described correlation NMR spectroscopic method was applied to the aqueous solutions of some biological substances. Figure 3 is the 100 MHz proton magnetic resonance spectrum of the aromatic region of the oxidized cytochrome b_5 (solubilized with trypsin from rabbit liver microsomes) in D_2O solution (0.1 M phosphate buffer pD 7.0). The protein concentration is 7 mM (*MW=ca.* 12000). The chemical shift is expressed relative to the internal standard of sodium (2,2,3, 3-tetradeuterio-3-trimethylsilyl)propionate (TMSP-d_4). The conditions of the measurement are given in the figure legend. All spectra hereafter were measured under the internal lock on H_2O (or HOD) signal. And their correlation was taken with a transient response

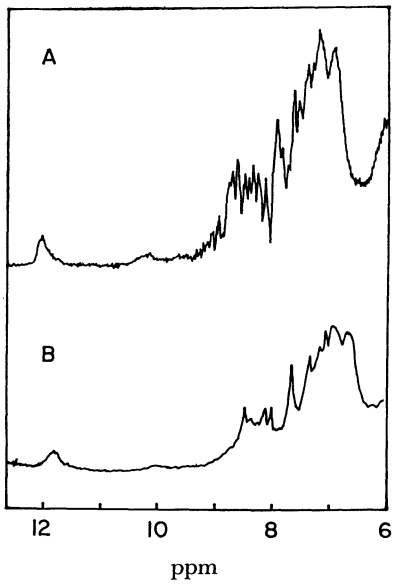

Fig. 3. (A) Transient response and (B) correlation spectrum of oxidized cytochrome b_5 from rabbit liver microsome in D_2O (0.1 M phosphate buffer pD 7.0). The concentration is 7 mM (*MW=ca.* 12000). The reference is TMS signal. Sweep direction: from higher to lower field, sampling point: 4096, sweep rate: 381.5 Hz/s, sampling time: 2.03 s, sweep repetition time: 2.10 s, filter: 250 Hz, number of accumulation: 3000. Chemical shifts are given relative to the signal of TMSP-d_4.

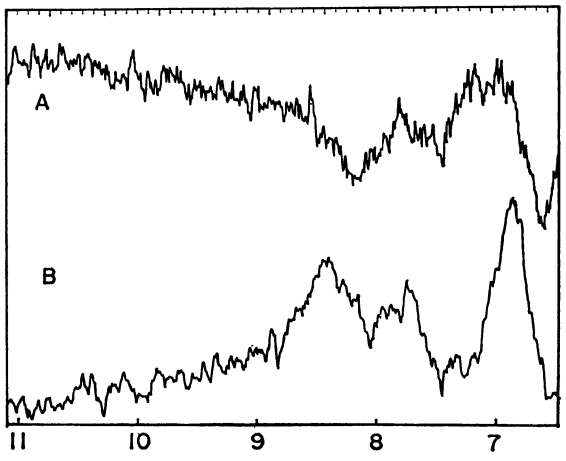

Fig. 4. (A) Transient response and (B) correlation spectrum of oxidized cytochrome b_5 in H_2O(0.1 M phosphate buffer pH 7.0). Concentration: 1 mM, reference: TMS, sweep direction: from lower to higher field, sampling point: 4096, sweep rate: 122.3 Hz/s, sampling time: 4.08 s, sweep repetition time: 5.00 s, filter: 20 Hz (sample), 125 Hz (reference), number of accumulation: 3000. Chemical shifts are given relative to the signal of TMSP-d_4.

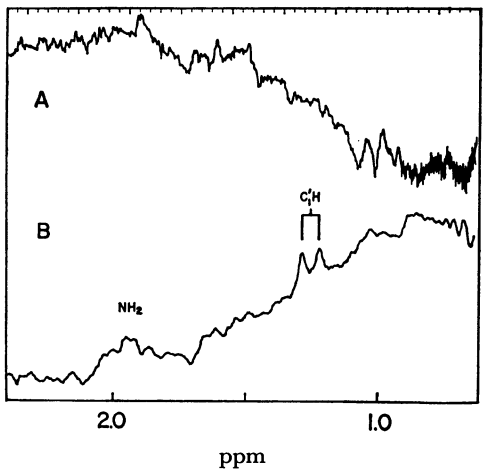

Fig. 5. (A) Transient response and (B) correlation spectrum of adenosine 5′-monophosphate sodium salt in H_2O.
Concentration: 2.5 mM, reference: TMS, sweep direction: from lower to higher field, sampling point: 4096, sweep rate: 51.3 Hz/s, sampling time: 4.09 s, sweep repetition time: 6.00 s, filter: 20 Hz, number of accumulation: for the second baseline correction(in BL 1) 10, and total 1000. A quadratic function was subtracted in the first baseline correction. Chemical shifts are given relative to the signal of H_2O.

of tetramethylsilane (TMS). Figure 3(A) is the transient spectrum and (B) is the obtained final spectrum. The spectrum shows good quality. As can be seen in Fig. 3(B) the effect from the linewidth of the reference signal (TMS) is negligible in the case of such macromolecules as proteins.

At the next step we tried to get the spectrum of the same sample in a dilute H_2O solution in the same frequency region. The spectra in Fig. 4 were obtained from the 1 mM H_2O solution of the oxidized cytochrome b_5 (0.1 M phosphate buffer at pH 7.0). Because of its severe conditions the spectrum in the higher field region than 7 ppm is not reliable. Comparing it with Fig. 3 we can see the contribution from NH_2 or NH group of this protein at 8.5 ppm. It is often required to take the spectra of proteins in H_2O at such a low concentration because of the low solubility and limitted amount of the sample.

Finally we tried to get a weak signal located closely to the solvent H_2O signal with the aid of our baseline correction system. Figure 5 shows the 100 MHz proton spectrum of the H_2O solution of adenosine 5′-monophosphate sodium salt (5′-AMP) at 2.5 mM. The observed doublet signal at about 1.2 ppm lower field from water signal comes from the C-1 proton of the ribose moiety and the broad signal at about 2 ppm is due to the amino protons of the adenine base. The long range noises arise from the subtracted baseline in a software. They obscure the broad signal to some extent. The important point of this system is to use a highly smoothed baseline at the second step of baseline correction. Otherwise, the noises are accumulated and disturb the spectrum. It should be noted, however, that a weak signal at about 1 ppm from water (6 ppm from TMSP-d_4) could be obtained, while it was impossible without the baseline correction system as shown in Fig. 4.

Discussion

Although a modified WETT method,[3] the water signal saturation method,[4] and Redfield pulse method[5] in FT NMR have shown their usefulness for aqueous solution systems, they have some disadvantages respectively. Those methods usually can eliminate only one strong signal. And it is necessary for the first method that the relaxation time of nuclei which give the signal to be eliminated is much longer than those of the other nuclei. The second method can not be applied to the exchangeable protons such as NH_2 and NH protons because of the saturation transfer from the irradiated solvent protons. The Redfield method is hard in setting the pulse condition and troublesome in baseline correction. The correlation method can avoid such disadvantages and Ozawa and Arata introduced an elegant wing processing method in correlation NMR to correct a titled baseline.[11] However correlation NMR had not yet been so far successful in getting weak signals within 1 ppm from water resonance, which was performed by the modified WEFT method.[3] It can be said that our method with a baseline correction system is promising in getting the proton signals of biological substances very close to the water resonance of solution. The system can also be applied to biomembranes or proteins in detergents where not only the solvent signal but the strong signals due to the CH_2 group of phospholipids and detergents also disturb the observation of the aimed resonance signals.

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