# APPLICATION OF PULSED-GRADIENT $^{31}P$ NMR ON FROG MUSCLE TO MEASURE THE DIFFUSION RATES OF PHOSPHORUS COMPOUNDS IN CELLS

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ABSTRACT Pulsed-gradient $^{31}P$ NMR was used to measure the diffusion rates of phosphorus compounds in aqueous solution and in living muscles. The diffusion rates of creatine phosphate and inorganic phosphate in intact frog muscle cells were reduced by a factor of ~2 from those in aqueous solution, which suggests that the apparent intracellular viscosity is ~2 times larger than in aqueous solution.

Recently, magnetic field gradient NMR has been used to measure the diffusion coefficient of muscular water (1–3). This method is also applicable to Fourier transform NMR spectroscopy (4, 5). In this paper, application of pulsed-gradient $^{31}P$ Fourier transform NMR to measure the diffusion coefficients of phosphorus metabolites in frog muscle is reported together with the diffusion coefficient of muscular water measured by $^1H$ NMR.

The femoral biceps muscles of bullfrog, *Rana catesbeiana*, were used. A pair of excised muscles (wet weight ~1.6 g) was inserted into an NMR tube (10 mm diameter) with a 2-mm capillary tube containing $^2H_2O$ for NMR lock. The details of the method have been reported previously (6).

NMR studies were carried out at room temperature, 24°C, by a PFT-100 (Japan Electron Optics Laboratory, Akishima, Tokyo, Japan) with Fourier transform operating at 40.29 MHz ($^{31}P$) and 99.54 MHz ($^1H$). The spinning of the sample tube was not performed to avoid intensity modulation artifact associated with sample spinning. In $^{31}P$ NMR spectra, chemical shifts were represented in relation to a standard of 85% $H_3PO_4$, and the power spectra were used for analysis.

To produce the magnetic field gradient, the modulation coil built in the cross-coil probe of the NMR spectrometer was used. The connections of the coils were selected to produce appropriate polarity resulting in a $z$-field gradient, and a pulsed-current source designed to generate the gradient was used (5). The absolute magnitude of the gradient was calibrated against the value of the diffusion coefficient of pure water at 24°C, $2.25 \times 10^{-5}$ cm$^2$/s, calculated by the interpolation from Mills's data (7).

In the pulsed-gradient method, the spin-echo measurements are performed by the Hahn's pulse sequence (8) which consists of a 90° pulse followed by a 180° pulse, and the pulsed gradient is turned on before and after the 180° pulse (2–5). The paired gradient pulses each have intensity $G$, duration $\delta$, and interval $\Delta$ (time between the two pulses of the pair). The time of diffusion is defined by the spacing of the gradient pulses and is given by $\Delta - \delta/3$. The diffusional attenuation of the signal, measured at the time of the echo, is given by $\ln [M(G)/M(0)] = -\gamma^2 G^2 D^2 \times \delta^2 \cdot (\Delta - \delta/3)$, where $M(G)/M(0)$ is the ratio of echo amplitudes in the presence and absence of the gradient, $\gamma$ is the nuclear gyromagnetic ratio ($^1H$: $2.675 \times 10^4$ gauss$^{-1}$ s$^{-1}$, $^{31}P$: $1.083 \times 10^4$ gauss$^{-1}$ s$^{-1}$). $D$ is the diffusion coefficient, and is determined from the slope of a plot of $\ln [M(G)/M(0)]$ vs. $\delta^2 (\Delta - \delta/3)$. In our experiments, the intensity of magnetic field gradient pulses $G$ was 39.0 gauss (G)/cm and the gradient pulse interval $\Delta$ was kept constant at 20 ms. The duration of gradient pulse $\delta$ was varied from 1 to 2 ms ($^1H$) and from 2 to 10 ms ($^{31}P$). The diffusion time was ~20 ms. The interval between 90° and 180° pulse was kept constant at 20 ms. The pulse sequence was applied at 30 s intervals.

The spin echoes of water proton in muscle were accumulated 10 times. The echo attenuation $M(G)/M(0)$ is demonstrated together with that of water proton in model solution containing 30 mM creatine phosphate, 10 mM inorganic phosphate, 5 mM ATP, 100 mM KCl, and 5 mM $MgCl_2$ in $H_2O$ (pH 7.4) (as shown later in Fig. 2). The slope is smaller in muscle than that in solution, indicating a reduced diffusion rate of water in muscle.

In the case of phosphorus metabolites, the latter half of the echo signal was accumulated 100 times and Fourier transformed spectra are shown in Fig. 1. The spectra show, from left to right, inorganic phosphate, creatine phosphate, $\gamma$, $\alpha$, and $\beta$ phosphate groups of ATP in the model solution, in which the concentration of ATP and $MgCl_2$ was increased to 20 mM to show the ATP signals clearly. The

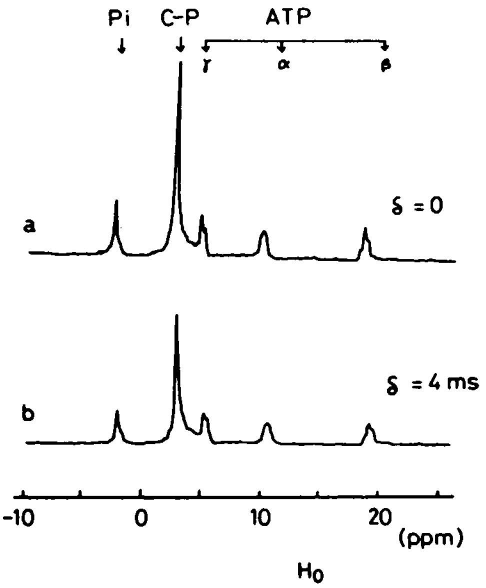

FIGURE 1 Pulsed-gradient $^{31}P$ NMR spectra of phosphorus compounds in model solution containing 30 mM creatine phosphate, 10 mM inorganic phosphate, 20 mM ATP, 100 mM KCl, and 20 mM $MgCl_2$ (pH 7.4). (*a*) The power spectra of spin-echo obtained without pulsed-gradient. The spectrum shows, from left to right, the signals of inorganic phosphate (Pi), creatine phosphate (C ~ P), γ, α, and β phosphate groups of ATP. The $M(0)$ values for each compound were obtained from the peak heights of the corresponding signals. (*b*) The spectra obtained by the application of pulsed-gradient. The gradient pulse length applied ($\delta$) was 4 ms and each signal was reduced in height depending on the diffusion rate of each compound. The $M(G)$ values were obtained from the peak heights of the corresponding signals.

upper spectrum (*a*) was obtained in the absence of the gradient pulses, and $M(0)$ values were obtained from the peak heights of the individual signals. The lower spectrum (*b*) was obtained in the presence of the gradient pulses (pulse duration $\delta = 4$ ms), from which $M(G)$ values were obtained. $M(G)/M(0)$ ratios in different pulse durations were obtained and plotted in Fig. 2.

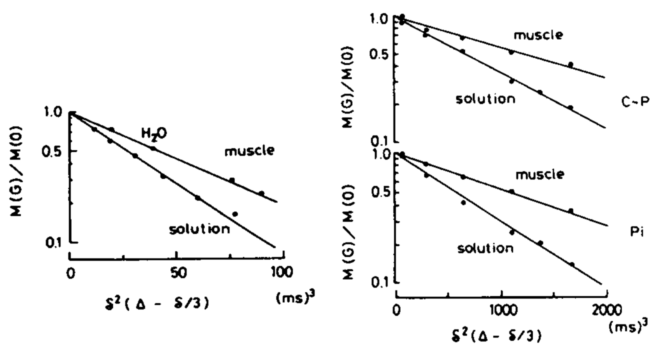

FIGURE 2 The determination of diffusion coefficients of water ($H_2O$), creatine phosphate (C ~ P), and inorganic phosphate (Pi) in frog muscle and in model solution. The latter contains 30 mM creatine phosphate, 10 mM inorganic phosphate, 5 mM ATP, 100 mM KCl, and 5 mM $MgCl_2$ in $H_2O$ (pH 7.4). The slopes of the lines are smaller in muscle than those in solution indicating the reduced diffusion rates in muscle. The diffusion coefficients calculated from the slopes, are summarized in Table I.

Similarly, the spin-echo spectra of phosphorus metabolites in frog muscle were measured. However, the content of creatine phosphate decreased gradually with the concomitant increase in inorganic phosphate in frog muscle under anaerobic conditions, as reported previously (6). To exclude the effect of change in contents, the spectra obtained in the absence of the gradient pulses and those obtained in the presence were alternately measured throughout the experiments. The peak heights of the individual signals in the spectrum obtained in the presence of gradient pulses were divided by the average values of the corresponding peak heights in the two adjacent (before and after) spectra obtained in the absence of gradient pulses. The calculated values were used as $M(G)/M(0)$, and were plotted in Fig. 2. The slopes for both creatine phosphate and inorganic phosphate were smaller in muscle than those in solution, indicating reduced diffusion rate of these compounds in muscle.

Table I summarizes the diffusion coefficients of creatine phosphate, inorganic phosphate, and water in muscle and in the model solution, and the ratios of the diffusivities in muscle to those in the solution. The diffusivities of both creatine phosphate and inorganic phosphate in muscle cells were reduced by 0.4 times and that of water by 0.6 times from those in aqueous solution.

The diffusion rate of molecules in muscle cells has been measured by isotope-tracer method. Bunch and Kallsen (9) reported that the diffusion coefficients of radioactively labeled water, urea and glycerol in muscle fiber of giant barnacle were not different from those values reported for aqueous solution. On the other hand, Kushmerick and Podolsky (10) reported that diffusion coefficients of potassium, sodium, sulfate, sorbitol, sucrose, and ATP in skinned fibers of frog muscle were reduced by 0.41–0.56 times from those in aqueous solution. Our results obtained in intact frog muscle were also reduced by ~50% from those in aqueous solution. The consistency of these findings suggests that a single factor, most likely the viscosity, underlies the reduction of diffusivities of molecules in muscle cells.

TABLE I

THE DIFFUSION COEFFICIENTS MEASURED BY PULSED-GRADIENT NMR

| | $D$ | $D_0$ | $D/D_0$ |
|---|---|---|---|
| | *(× $10^{-6}$ $cm^2/s$)* | *(× $10^{-6}$ $cm^2/s$)* | |
| C~P | 2.6 ± 0.4(5) | 6.2 ± 0.8(4) | 0.4 |
| Pi | 3.3 ± 0.4(5) | 7.8 ± 1.1(4) | 0.4 |
| $H_2O$ | 14.1 ± 0.9(3) | 22.5* | 0.6 |

*The diffusion coefficient of water reported by Mills (7) was used to calculate the other values of diffusion coefficients.

The diffusion coefficients of creatine phosphate (C~P), inorganic phosphate (Pi), and water ($H_2O$) in frog muscle ($D$) and in model solution ($D_0$), and the ratios of diffusivities ($D/D_0$) measured by pulsed-gradient NMR. The model solution contains 30 mM creatine phosphate, 10 mM inorganic phosphate, 5 mM ATP, 100 mM KCl and 5 mM $MgCl_2$ in $H_2O$ (pH 7.4). The number of samples is shown in parenthesis.

The diffusion coefficient of water in muscle measured by $^1$H NMR has been reported to be a function of diffusion time, which is determined by experimental conditions, and the obtained values are the weight-averaged values during the diffusion time (2, 3). The value of reduction rate was in general agreement with those reported by Tanner (2). Meanwhile, the ratio of diffusivity (muscle/aqueous solution) of water was slightly larger than the ratios of diffusivities of creatine phosphate and inorganic phosphate. The difference suggests that intracellular viscosity might be slightly higher than extracellular viscosity, because the diffusivities of creatine phosphate and inorganic phosphate reflect only the intracellular viscosity and that of water might be due to both intracellular and extracellular viscosity.

The diffusion coefficients have been measured by $^1$H NMR using either the steady-gradient method (1) or the pulsed-gradient method (2, 3). However, the pulsed-gradient method has certain advantages over the steady-gradient method. As already reported (2, 3), the pulsed-gradient method provides a definite diffusion time, $\Delta - \delta/3$, which may be varied independently with the radiofrequency pulses, and the magnitude of the gradient used in the pulse can be increased more than in the steady-gradient method. Thus, with the pulsed-gradient method, the diffusion time can be reduced to a considerably smaller value, which makes it possible to observe restricted water diffusion effects as reported by Tanner (2). Furthermore, the diffusion coefficients of various compounds can be obtained simultaneously with the pulsed-gradient method, because the high resolution NMR spectra can be obtained when the gradient pulse is turned off, which is impossible for the steady-gradient method.

Thus, the pulsed-gradient method of $^{31}$P NMR was successfully applied to measure the diffusion coefficients of phosphorus compounds in muscle cells and in aqueous solution. This technique can be applied to other types of living cells and organs without destructive results.

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