Double-Quantum-Filtered ^{23}Na NMR Study of Intracellular Sodium in the Perfused Liver

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ABSTRACT We acquired double-quantum-filtered ^{23}Na NMR spectra from perfused liver, using a range of τ values from 0.2 to 24 ms, where τ is the separation between the first and second $\pi/2$ pulses in the radio-frequency pulse sequence. For each τ value we compared the amplitude of the double-quantum-filtered ^{23}Na NMR signal acquired from intracellular sodium ions when the liver was perfused with buffer containing the "shift reagent" $Dy(PPP)_2$ to the amplitude of the total double-quantum-filtered ^{23}Na NMR signal acquired when the liver was perfused with buffer containing no $Dy(PPP)_2$. For $\tau \leq 4$ ms, the average ratio of the two amplitudes was 0.98 ± 0.03 (mean ± SEM). For $\tau \geq 8$ ms, the average ratio was significantly less than 1. These results demonstrate that double-quantum-filtered ^{23}Na NMR signals acquired from perfused liver using short τ values arise almost exclusively from intracellular sodium ions, but double-quantum-filtered ^{23}Na NMR signals acquired from perfused liver using long τ values contain contributions from both intracellular and extracellular sodium ions. This conclusion suggests that multiple-quantum-filtered ^{23}Na NMR spectroscopy will be useful in studying intracellular sodium levels in the perfused liver, and possibly in the intact liver in vivo.

INTRODUCTION

Sodium ions are inhomogeneously distributed in tissues; in most cases, the intracellular sodium concentration is an order of magnitude lower than the extracellular sodium concentration. The resulting transmembrane sodium gradient is used to drive a number of physiological functions, e.g., transmission of the nervous impulse, maintenance of normal cell volume, and transport of substrates and ions into and out of the cell (Aidly, 1978; Ganong, 1987).

Conventional (single-pulse) ^{23}Na NMR spectroscopy has been used to monitor intracellular sodium concentrations in isolated cells and perfused organs (Gupta et al., 1984; Springer, 1987). These studies utilized "shift reagents" to separate the overlapping signals from intracellular and extracellular sodium ions. Shift reagents, however, can have toxic side effects (Endre et al., 1989) and may not be suitable for in vivo studies.

Multiple-quantum-filtered ^{23}Na NMR signals are sensitive to the relaxation rates of sodium ions (Pekar and Leigh, 1986; Jacard et al., 1986). If the relaxation properties of intracellular sodium ions were different from the relaxation properties of extracellular sodium ions, multiple-quantum-filtered ^{23}Na NMR spectroscopy might be able to distinguish the intracellular signal without the use of shift reagents. The double-quantum-filtered ^{23}Na NMR studies presented here were designed to test this approach with the perfused liver.

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MATERIALS AND METHODS

Liver perfusion

Complete Krebs-Henseleit buffer contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 26 mM $NaHCO_3$, 8 mM glucose, and 1.25 mM $CaCl_2$. In experiments with perfused livers the buffer was supplemented with 2% BSA. In many cases, inorganic phosphate was omitted from the buffer. The buffer was oxygenated with 95% O_2/5% CO_2 gas: the final pH was 7.4.

A clear stock solution of 50 mM dysprosium tripolyphosphate ($Dy(PPP)_2$) was prepared by adding an equal volume of 100 mM dysprosium chloride to 210 mM pentasodium tripolyphosphate (Wittenberg and Gupta, 1985). Stock 50 mM $Dy(PPP)_2$ was diluted with Krebs Henseleit buffer containing a reduced concentration of NaCl. The final sodium concentration in the buffer was 144 mM.

Male Fischer rats (Taconic Farms, Taconic, NY; 90–120 g), maintained on standard rat chow and water ad libitum, were anesthetized with sodium pentobarbitol (50 mg/kg). Surgery was performed as described by Exton (1975). The portal vein was cannulated, and the liver was rapidly perfused with oxygenated Krebs-Henseleit buffer maintained at 37°C. The liver was then removed from the body and placed in a 20 mm NMR tube. The flow rate was set at 3–4 ml/min/g liver weight, assuming the weight of the liver was 4% of the total body weight. A 20–30 μ porous Teflon (Zitex) filter (Cole-Parmer, Niles, IL) was placed in the perfusion line before the liver. The perfusate did not recirculate.

Aliquots of perfusate efflux were collected every 30 min to assay for lactate dehyrogenase (LDH). Activity is expressed in LDH units/ml (Wroblewski and LaDue, 1955).

NMR spectroscopy

A Bruker MSL-300 NMR spectrometer operating at 79.4 MHz for ^{23}Na and 121.5 MHz for ^{31}P was employed for these studies. The 20 mm double-tuned (^{23}Na/^{31}P) probe was maintained at 35°C. For ^{23}Na, the $\pi/2$ pulse was 30.5 μs, the sweep width was 4000 Hz, and free induction decays were acquired every 200 ms. For ^{31}P, the $\pi/2$ pulse was 23 μs, the sweep width was 6000 Hz, and free induction decays were acquired every 800 ms. For ^{23}Na NMR spectroscopy, the position of the signal from buffers containing no $Dy(PPP)_2$ was defined as 0 ppm. For ^{31}P NMR spectroscopy, the position of the signal

from the alpha phosphate of ATP was defined as −10.8 ppm: this definition is equivalent to defining the position of the signal from phosphoric acid as 0 ppm.

Double-quantum-filtered ^{23}Na NMR spectra were acquired using the pulse sequence $\theta - \tau/2 - 2\theta - \tau/2 - \theta - \delta - \theta - t$, with a 128-step phase cycling procedure (Bax et al., 1980). Assuming that $\theta = \pi/2$, the resulting double-quantum-filtered ^{23}Na NMR signal, $s(\tau, \delta, t)$, is given by the expression (Pekar and Leigh, 1986; Jaccard et al., 1986)

$$s(\tau, \delta, t) = -(3/20)\, A_0 N[e^{-R_1^{(1)}\tau} - e^{-R_2^{(1)}\tau}]e^{-\mathscr{R}_1^{(2)}\delta}[e^{-\mathscr{R}_1^{(1)}t} - e^{-\mathscr{R}_2^{(1)}t}], \quad (1)$$

where τ is the preparation time and δ is the double-quantum evolution time. $R_1^{(1)}$ and $R_2^{(1)}$ are the single-quantum transverse relaxation rates for the "outer" and "central" transitions, respectively. We assume that inhomogeneous broadening can be included in Eq. 1 by the addition of a second term to the relaxation rates. $\mathscr{R}_1^{(1)}$ is defined as the sum of the single-quantum transverse relaxation rate for the outer transition plus a term due to inhomogeneous broadening. $\mathscr{R}_2^{(1)}$ is defined in a similar manner. $\mathscr{R}_2^{(2)}$ is defined as the sum of the double-quantum transverse relaxation rate plus a term due to inhomogeneous broadening. N is the number of sodium ions contributing to the ^{23}Na NMR signal, and A_0 is defined by the equation

$$s(t) = A_0 N\left[\frac{3}{5}e^{-\mathscr{R}_1^{(1)}t} + \frac{2}{5}e^{-\mathscr{R}_2^{(1)}t}\right], \quad (2)$$

where $s(t)$ is the free induction decay observed after a $\pi/2$ pulse. Fourier transformation of Eq. 1 gives the double-quantum-filtered ^{23}Na NMR spectrum. Assuming that $\mathscr{R}_2^{(2)}\delta \ll 1$, the amplitude at the center of the signal, $S(\tau)$, is given by the expression

$$S(\tau) = -(3/20)A_0 N[e^{-R_1^{(1)}\tau} - e^{-R_2^{(1)}\tau}][1/\mathscr{R}_1^{(1)} - 1/\mathscr{R}_2^{(1)}]. \quad (3)$$

The amplitude of the signal observed from Krebs-Henseleit buffer lacking inorganic phosphate using the double-quantum-filtered pulse sequence (see Fig. 3 *A*) was subtracted from the amplitude of the double-quantum-filtered ^{23}Na NMR signal from perfused liver. For this correction, which was performed separately for each value of τ, it was assumed that the liver occupied half of the sensitive volume of the sample and that the extracellular space occupied approximately 30% of the liver.

The apparent linewidth of the narrow component of the double-quantum-filtered ^{23}Na NMR signals from perfused liver was calculated by drawing a "baseline" between the two minimum values of the spectrum, and measuring the full-width at half-height of the narrow component from this baseline. All double-quantum-filtered ^{23}Na NMR spectra were acquired using $\delta = 20$ μs, except for the experiments illustrated in Fig. 6.

Statistical analysis

The τ dependence of the double-quantum-filtered ^{23}Na NMR signal from perfused liver was fit to Eq. 7 using the KaleidaGraph (Synergy Software) program, which also estimated the SD of the calculated parameters.

RESULTS

Effects of Dy(PPP)$_2$ on conventional ^{23}Na NMR spectra from perfused liver

Fig. 1 *A* shows a conventional ^{23}Na NMR spectrum of rat liver perfused with complete Krebs-Henseleit buffer. The spectrum was collected 15 min after the start of perfusion with buffer containing 3 mM Dy(PPP)$_2$. Reproducible spectra were obtained within 10 min, indicating that the shift reagent had equilibrated throughout the extracellular space. The signal at −3.6 ppm was assigned to extracellular sodium ions (Gupta et al., 1984), whereas the signal at +0.2 ppm was assigned to intracellular sodium ions. The downfield shift of the signal from intracellular sodium ions is presumably due to bulk magnetic susceptibility effects (Chu et al., 1990). For liver perfused with buffer containing no Dy(PPP)$_2$, the linewidth of the ^{23}Na NMR signal was 16 Hz (after correction for 10 Hz line-broadening). For liver perfused with buffer containing 3 mM Dy(PPP)$_2$, the linewidths of the signals from extracellular and intracellular sodium ions were approximately 82 and 95 Hz, respectively (after correction for line-broadening).

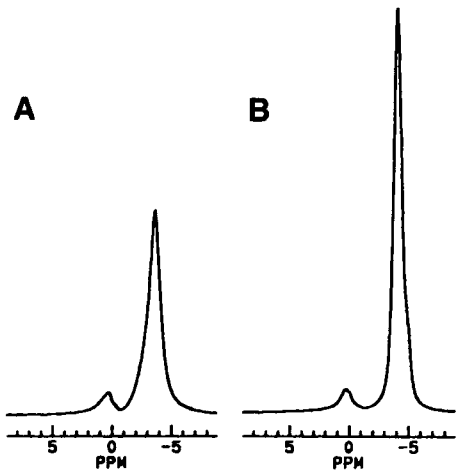

FIGURE 1 Conventional ^{23}Na NMR spectra of perfused rat liver. Livers were perfused with complete Krebs Henseleit buffer (*A*) or Krebs Henseleit buffer lacking inorganic phosphate (*B*). In both cases, the perfusion buffer contained 3 mM Dy(PPP)$_2$.

Fig. 1 *B* shows a conventional ^{23}Na NMR spectrum of rat liver perfused with Krebs Henseleit buffer lacking inorganic phosphate. The spectrum was collected 25 min after the start of perfusion with buffer containing 3 mM Dy(PPP)$_2$. The signal at −4.0 ppm was assigned to extracellular sodium ions, whereas the signal at +0.3 ppm was assigned to intracellular sodium ions. The linewidths of the signals from extracellular and intracellular sodium ions were approximately 60 and 94 Hz, respectively (after correction for line-broadening).

Effects of Dy(PPP)$_2$ on double-quantum-filtered ^{23}Na NMR spectra from perfused liver

Fig. 2 *A* shows double-quantum-filtered ^{23}Na NMR spectra from rat liver perfused with Krebs-Henseleit buffer lacking inorganic phosphate. Spectra shown were acquired with $\tau = 0.5$ ms (*left*) and $\tau = 8.0$ ms (*right*). The amplitude of the double-quantum-filtered ^{23}Na NMR signal acquired with $\tau = 8$ ms was approximately 0.3% of the amplitude of the conventional ^{23}Na NMR signal. Double-quantum-filtered ^{23}Na NMR spectra from liver perfused with complete Krebs-Henseleit buffer were similar to the spectra shown in Fig. 2 *A*. Fig. 2 *B* shows double-quantum-filtered ^{23}Na NMR spectra from liver perfused with complete Krebs Henseleit buffer including 3 mM Dy(PPP)$_2$. The large double-quantum-filtered ^{23}Na NMR signal at −3.6 ppm in both spectra was assigned to extracellular sodium

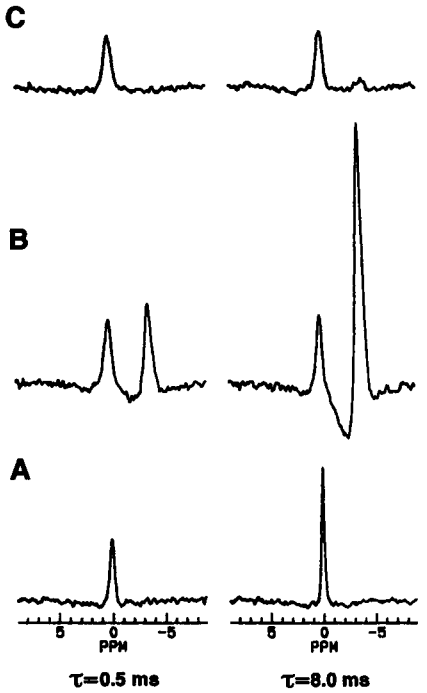

FIGURE 2 Double-quantum-filtered ^{23}Na NMR spectra of perfused rat liver. Livers were perfused with Krebs Henseleit buffer lacking inorganic phosphate (*A*, *C*) or complete Krebs-Henseleit buffer (*B*). Spectra were acquired under control conditions (*A*) or 25 min after the addition of 3 mM $Dy(PPP)_2$ to the perfusion buffer (*B*, *C*). The preparation time (τ) is given below each set of spectra.

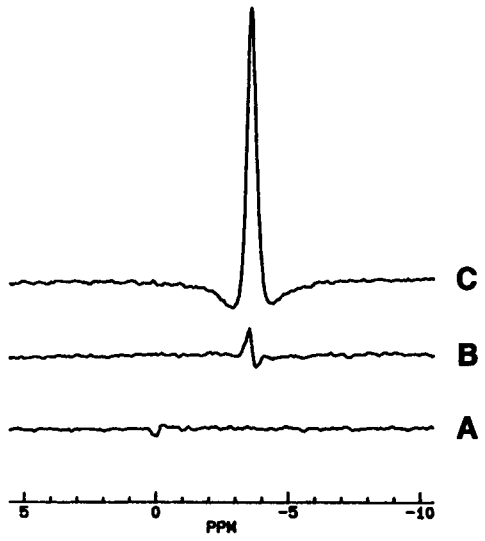

FIGURE 3 Double-quantum-filtered ^{23}Na NMR spectra of Krebs-Henseleit buffers. (*A*) Complete Krebs Henseleit buffer; (*B*) Krebs Henseleit buffer lacking inorganic phosphate, plus 3 mM $Dy(PPP)_2$; (*C*) complete Krebs Henseleit buffer plus 3 mM $Dy(PPP)_2$. The spectra shown in traces *B* and *C* were acquired approximately 1 h after the addition of $Dy(PPP)_2$. τ = 8 ms for all spectra. T = 35°C. The vertical scale and acquisition parameters were identical for all spectra.

ions. Fig. 2 *C* shows double-quantum-filtered ^{23}Na NMR spectra acquired from rat liver perfused with Krebs Henseleit buffer including 3 mM $Dy(PPP)_2$, but lacking inorganic phosphate. The small double-quantum-filtered ^{23}Na NMR signal occuring at −3.8 ppm in the spectrum acquired with τ = 8 ms was assigned to extracellular sodium ions. No double-quantum-filtered ^{23}Na NMR signal was observed at −3.8 ppm for the spectrum acquired with τ = 0.5 ms.

A shifted double-quantum-filtered ^{23}Na NMR signal was also observed from a sample consisting only of Krebs Henseleit buffer after the addition of $Dy(PPP)_2$ (Fig. 3 *C*). The amplitude of the signal shown in Fig. 3 *C* was approximately 1% of the amplitude of the conventional ^{23}Na NMR signal and was not reduced by filtration of the solution through a 0.2-μ filter (data not shown). The shifted double-quantum-filtered ^{23}Na NMR signal was substantially reduced if inorganic phosphate was omitted from the buffer (Fig. 3 *B*). All of the double-quantum-filtered ^{23}Na NMR spectra used for the data analysis given below were acquired from livers perfused with Krebs-Henseleit buffer lacking inorganic phosphate. The ^{31}P NMR results and the enzymatic assays (see below) suggest that the omission of inorganic phosphate from the perfusion buffer did not compromise the intracellular ATP level or the cellular integrity of the liver.

The amplitude of the double-quantum-filtered ^{23}Na NMR signal was measured as a function of τ while the liver was perfused with buffer containing no $Dy(PPP)_2$. The perfusion buffer was then changed to a similar buffer containing $Dy(PPP)_2$, and the amplitude of the double-quantum-filtered ^{23}Na NMR signal assigned to intracellular sodium ions was measured as a function of τ. The amplitudes of the signals from individual livers were normalized to the amplitude of the control double-quantum-filtered ^{23}Na NMR signal acquired with τ = 8 ms, which was assigned a value of 10.

The amplitude of the double-quantum-filtered ^{23}Na NMR signal acquired from intracellular sodium ions when the liver was perfused with buffer containing 2 mM $Dy(PPP)_2$ (Fig. 4, *filled triangles*) is smaller than the amplitude of the total double-quantum-filtered ^{23}Na NMR signal acquired when the liver was perfused with buffer containing no $Dy(PPP)_2$ (Fig. 4, *filled circles*). Part of this difference is due to the effect of $Dy(PPP)_2$ on the apparent linewidth of the narrow component of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions. We assume that extracellular $Dy(PPP)_2$ cannot affect the homogeneous relaxation rates of intracellular sodium ions, i.e., $R_1^{(1)}$ and $R_2^{(1)}$. The effects of $Dy(PPP)_2$ on the apparent linewidth of the narrow component of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions are thus due to an effect of $Dy(PPP)_2$ on the inhomogeneous relaxation rates of intracellular sodium ions, i.e., $\mathscr{R}_1^{(1)}$ and $\mathscr{R}_2^{(1)}$. This conclusion is consistent with the observation that extracellular paramagnetic shift reagents increase the inhomogeneous relaxation rates of intracellular water (Rosen et al., 1990). Both effects could be mediated through magnetic fields generated inside the cell by the gradient in magnetic susceptibility across the cell membrane (Rosen et al., 1990).

Equation 3 shows that, if $Dy(PPP)_2$ in the perfusion buffer increases the relaxation rates $\mathscr{R}_1^{(1)}$ and $\mathscr{R}_2^{(1)}$ of intra-

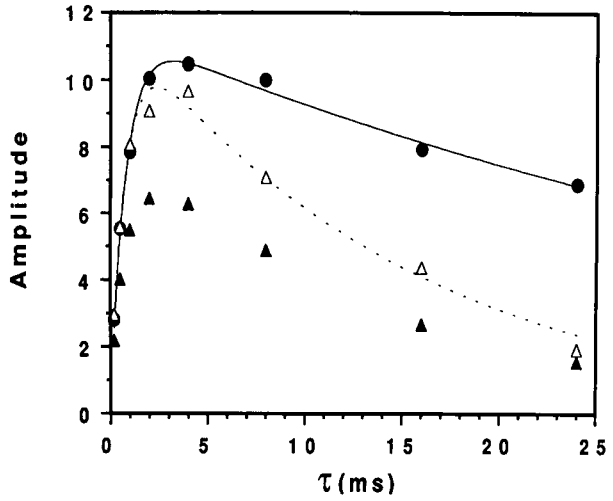

FIGURE 4 τ-Dependence of the amplitude of the double-quantum-filtered ^{23}Na NMR signal from perfused liver. Livers were perfused with Krebs Henseleit buffer lacking inorganic phosphate. For the control data (●), the points denote the amplitude of the total double-quantum-filtered ^{23}Na NMR signal. For data acquired from livers perfused with buffer containing 2 mM $Dy(PPP)_2$ (▲, △), the points denoted the amplitude of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions. (▲) Uncorrected data; (△) corrected data (see Results). The solid curves are the best fits of the data to Eq. 7.

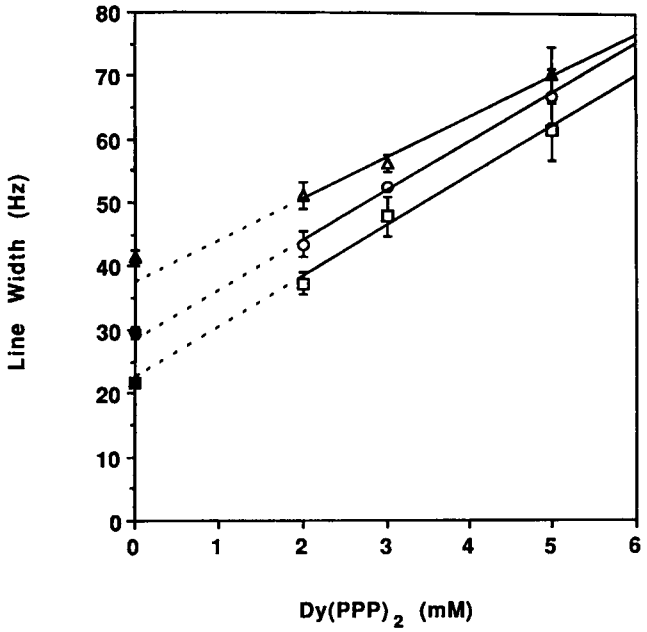

FIGURE 5 Effect of $Dy(PPP)_2$ on the linewidth of the double-quantum-filtered ^{23}Na NMR signal. The ordinate denotes the linewidth of the double-quantum-filtered ^{23}Na NMR signal acquired from intracellular sodium ions in livers perfused with Krebs Henseleit buffer lacking inorganic phosphate. The abscissa denotes the $Dy(PPP)_2$ concentration in the perfusion buffer. Data were acquired with τ = 0.2 ms (□), τ = 4 ms (○), and τ = 16 ms (△). Data points are mean values ± SEM (N = 4). The solid lines are the best linear fit to each data set. Solid symbols denote the line width of the total double-quantum-filtered ^{23}Na NMR signal acquired from liver perfused with buffer containing no $Dy(PPP)_2$. These points were not included in the fitting procedure.

cellular sodium ions, the amplitude of the observed double-quantum-filtered ^{23}Na NMR signal will decrease by a factor α, where

$$\alpha = \frac{1/\mathscr{R}_{1sr}^{(1)} - 1/\mathscr{R}_{2sr}^{(1)}}{1/\mathscr{R}_{1}^{(1)} - 1/\mathscr{R}_{2}^{(1)}}. \quad (4)$$

$\mathscr{R}_{1sr}^{(1)}$ and $\mathscr{R}_{2sr}^{(1)}$ are the values of $\mathscr{R}_1^{(1)}$ and $\mathscr{R}_2^{(1)}$, respectively, when the perfusion buffer contains $Dy(PPP)_2$. Assuming that $\mathscr{R}_1^{(1)} \gg \mathscr{R}_2^{(1)}$ and $\mathscr{R}_{1sr}^{(1)} \gg \mathscr{R}_{2sr}^{(1)}$, Eq. 4 simplifies to

$$\alpha = \frac{1/\mathscr{R}_{2sr}^{(1)}}{1/\mathscr{R}_{2}^{(1)}}. \quad (5)$$

We assume that $1/\mathscr{R}_{2sr}^{(1)}$ for intracellular sodium ions can be approximated by $\pi\Delta\nu_{sr}$, where $\Delta\nu_{sr}$ is the apparent linewidth of the narrow component of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions in liver perfused with buffer containing $Dy(PPP)_2$ (see Materials and Methods). We also assume that $1/\mathscr{R}_2^{(1)}$ can be approximated by $\pi\Delta\nu_0$, where $\Delta\nu_0$ is the value of $\Delta\nu_{sr}$ extrapolated to zero $Dy(PPP)_2$ concentration. With these assumptions, Eq. 5 simplifies to

$$\alpha = \frac{\Delta\nu_0}{\Delta\nu_{sr}}. \quad (6)$$

Fig. 5 shows the values of $\Delta\nu_{sr}$, and the linear fits to the data, for three values of τ. The measured values of $\Delta\nu_{sr}$ and the calculated values of $\Delta\nu_0$ were used to calculate α for each τ value, using Eq. 6. The values of α were then used to calculate the corrected amplitude of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions (see Fig. 4, *open triangles*, and Table 1).

Effects of compartmentation on the double-quantum-filtered ^{23}Na NMR signals

If all the sodium ions that contributed to the observed double-quantum-filtered ^{23}Na NMR signal had the same $R_1^{(1)}$ and $R_2^{(1)}$ values, Eq. 3 could be used to analyze the data shown in Fig. 4. However, two observations suggest that the single-compartment model is not appropriate for the data from liver perfused with buffer containing no $Dy(PPP)_2$. First, the solid points in Fig. 5 show that the linewidth of the narrow component of the double-quantum-filtered ^{23}Na NMR signal, which is approximately equal to $\pi/\mathscr{R}_2^{(1)}$, is a function of τ. Second, Fig. 6 (see below) shows that the dependence of $S(\tau, \delta)$ on δ is a function of τ (Lyon et al., 1991). The data, however, can be analyzed using a modified form of Eq. 3, i.e.,

$$S(\tau) = B_0[e^{-\langle R_1^{(1)}\rangle\tau} - e^{-\langle R_2^{(1)}\rangle\tau}] \quad (7)$$

where $\langle R_1^{(1)}\rangle$ and $\langle R_2^{(1)}\rangle$ are "average" values of the transverse relaxation rates from different intracellular and extracellular compartments, and B_0 is independent of τ (see Appendix 1). The first row in Table 2 shows the values of B_0, $\langle R_2^{(1)}\rangle$ and $\langle R_1^{(1)}\rangle$ calculated from a fit of the "control" data to Eq. 7 (see Fig. 4).

Fig. 5 also shows that both $\Delta\nu_{sr}$ and $\Delta\nu_0$ for intracellular sodium ions are a function of τ. This observation implies that the single-compartment model is not valid even for intra-

TABLE 1 Effect of 2 mM Dy(PPP)$_2$ on the amplitude of double-quantum-filtered ^{23}Na NMR signals from perfused liver

τ (ms)	A_{con}*	A_{Dy}§	A_{Dy}/A_{con}	p‖
0.2	2.8 ± 0.2 (N = 12)	3.0 ± 0.3 (N = 4)	1.06	0.70
0.5	5.5 ± 0.2 (N = 11)	5.6 ± 0.7 (N = 4)	1.01	0.92
1.0	7.9 ± 0.3 (N = 12)	8.1 ± 1.1 (N = 4)	1.03	0.80
2.0	10.0 ± 0.4 (N = 12)	9.1 ± 1.1 (N = 4)	0.90	0.31
4.0	10.5 ± 0.3 (N = 11)	9.7 ± 1.2 (N = 4)	0.92	0.37
8.0	10.0‡	7.1 ± 1.1 (N = 4)	0.71	<0.001
16	8.0 ± 0.2 (N = 12)	4.4 ± 0.8 (N = 4)	0.55	<0.001
24	6.9 ± 0.1 (N = 8)	2.0 ± 0.4 (N = 4)	0.28	<0.001

Livers were perfused with Krebs Henseleit buffer lacking inorganic phosphate. A_{con} and A_{Dy} are mean values ± SEM.

* Amplitude of the double-quantum-filtered ^{23}Na NMR signal acquired from liver perfused with buffer containing no Dy(PPP)$_2$. Data from all control experiments were combined.

‡ Normalized to 10.0 for individual livers (see Results).

§ Corrected amplitude of the double-quantum-filtered ^{23}Na NMR signal acquired from intracellular sodium ions in liver perfused with buffer containing 2 mM Dy(PPP)$_2$.

‖ p value (Students t test) for comparison of A_{Dy} and A_{con} at specific τ values.

cellular sodium ions. The data acquired from intracellular sodium ions were analyzed using Eq. 7 (see Table 2 and Fig. 4).

δ Dependence of double-quantum-filtered ^{23}Na NMR spectra from perfused liver

Fig. 6 shows the δ dependence (see Materials and Methods) of the amplitude of the double-quantum-filtered ^{23}Na NMR signal from liver perfused with Krebs-Henseleit buffer lacking inorganic phosphate. The data were acquired from the same liver, using two values of τ (1 and 16 ms, respectively). The dashed curves show the best-fit of the data to a single exponential decay. The rate constants derived from the fits are 3519 s^{-1} (τ = 1 ms) and 678 s^{-1} (τ = 16 ms).

Effects of Dy(PPP)$_2$ on ^{31}P NMR spectra from perfused liver

Fig. 7 shows ^{31}P NMR spectra of rat liver perfused with Krebs-Henseleit buffer in the absence and presence of 3 mM Dy(PPP)$_2$. Livers perfused with complete Krebs-Henseleit buffer (Fig. 7 *A*) have a higher phosphomonester signal than livers perfused with Krebs-Henseleit buffer lacking inorganic phosphate (Fig. 7 *B*), although the ATP signals are similar. Livers perfused with buffer containing 3 mM Dy(PPP)$_2$ show a small broadening of all signals, but no substantial change in the area of the ATP signals.

Enzymatic determination of liver damage

LDH assays of the perfusate at the start of the experiment showed an average activity of ~10 units/ml (Exton, 1975).

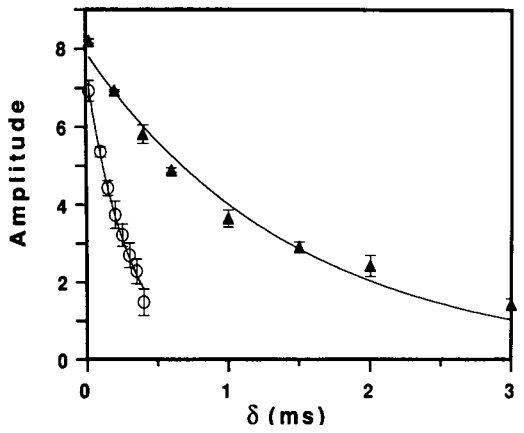

FIGURE 6 δ-Dependence of the amplitude of the double-quantum-filtered ^{23}Na NMR signal from perfused liver. Data were acquired with τ = 1 ms (○) and τ = 16 ms (▲). Points are mean values ± SEM (N = 4). Solid curves are the best fits of the data at each τ value to single exponential curves (see Results). Livers were perfused with Krebs Henseleit buffer lacking inorganic phosphate.

TABLE 2 Effect of Dy(PPP)$_2$ on the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions in perfused liver

	B_o (arbitrary units*)	$\langle R_2^{(1)} \rangle$ ms^{-1}	$\langle R_1^{(1)} \rangle$ ms^{-1}
Control	12.0 ± 0.1	0.023 ± .001	1.24 ± 0.06
2 mM Dy(PPP)$_2$‡	12.5 ± 0.8	0.073 ± .005	1.26 ± 0.15
3 mM Dy(PPP)$_2$	14.5 ± 1.2	0.081 ± .006	1.31 ± 0.2
5 mM Dy(PPP)$_2$	17.6 ± 1.4	0.077 ± .006	1.70 ± 0.2

B_o, $\langle R_2^{(1)} \rangle$ and $\langle R_1^{(1)} \rangle$ were derived from a fit to Eq. 7 of the τ dependence of the amplitude of the double-quantum-filtered ^{23}Na NMR signal from perfused liver (see Materials and Methods). For livers perfused with no Dy(PPP)$_2$ in the perfusion buffer (control), the total signal was used for the analysis. For livers perfused with buffer containing Dy(PPP)$_2$, the intracellular signal was used for the analysis. Livers were perfused with Krebs-Henseleit buffer lacking inorganic phosphate. For perfusion buffers containing Dy(PPP)$_2$, each data set was the average of four separate experiments. Control values for the three data sets were combined.

* The amplitude of the control double-quantum ^{23}Na NMR signal acquired with τ = 8 ms was normalized to 10.0 (see Results).

‡ Concentration in the perfusion buffer.

LDH activities at the end of the experiment were always below 130 units/ml. In the absence of inorganic phosphate in the perfusion buffer, they were below 50 units/ml.

DISCUSSION

Effects of Dy(PPP)$_2$ on double-quantum-filtered ^{23}Na NMR signals from intracellular sodium ions in perfused liver

Fig. 4 shows a comparison between the corrected amplitude of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions in liver perfused with buffer containing 2 mM Dy(PPP)$_2$ (*open triangles*) and the amplitude of the total double-quantum-filtered ^{23}Na NMR signal from liver perfused with buffer containing no Dy(PPP)$_2$ (*circles*). For short τ values the amplitudes are not significantly different (see Table 1). For example, for $\tau \leq 4$ ms, the average

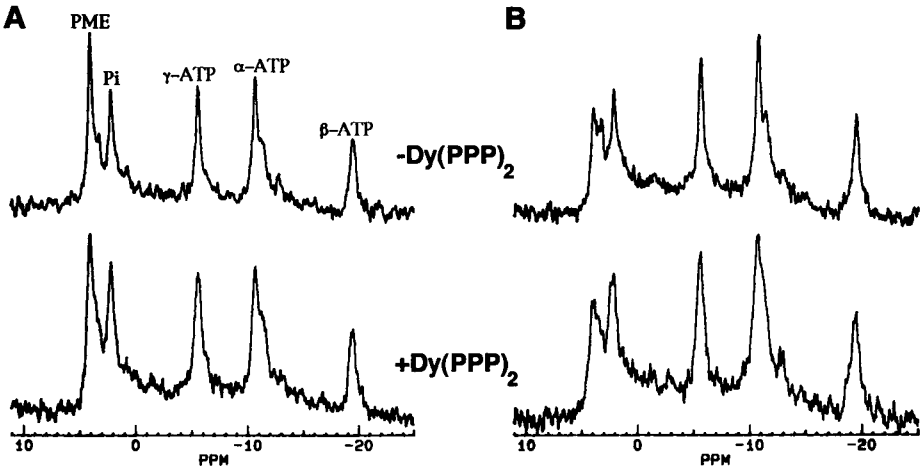

FIGURE 7 ^{31}P NMR spectra of perfused rat liver. Livers were perfused with complete Krebs Henseleit buffer (*A*) or Krebs Henseleit buffer lacking inorganic phosphate (*B*). Spectra were acquired with no shift reagent ($-Dy(PPP)_2$) or 3 mM shift reagent ($+Dy(PPP)_2$) in the perfusion buffer. The vertical scale and acquisition parameters were identical for all spectra.

ratio of the amplitudes is 0.98 ± 0.03 (SEM). The close correspondence between the two sets of data at short τ values demonstrates that, under these conditions, the double-quantum-filtered ^{23}Na NMR signal from perfused liver arises almost entirely from intracellular sodium ions.

At long τ values (i.e., $\tau \geq 8$ ms), the corrected amplitude of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions in liver perfused with buffer containing 2 mM $Dy(PPP)_2$ is significantly less that the amplitude of the double-quantum-filtered ^{23}Na NMR signal from liver perfused with buffer containing no $Dy(PPP)_2$ (see Fig. 4 and Table 1). Because extracellular $Dy(PPP)_2$ cannot affect the homogeneous relaxation rates of intracellular sodium ions, the simplest explanation for this observation is that the double-quantum-filtered ^{23}Na NMR signal collected from perfused liver using long τ values is the sum of two components, one arising from intracellular sodium ions and the other arising from extracellular sodium ions.

The two observations discussed above show that although the contribution from intracellular sodium ions dominates the observed double-quantum-filtered ^{23}Na NMR signal at short τ values, the contribution from extracellular sodium ions becomes significant at long τ values. This conclusion suggests that, in the perfused liver, sodium ions in intracellular and extracellular compartments have different relaxation properties. The difference in relaxation properties could be due to differences in binding constants or differences in correlation times of bound and free sodium ions in intracellular and extracellular compartments (Rooney and Springer, 1991a, b).

In principle, the amplitude of the double-quantum-filtered ^{23}Na NMR signal acquired with short τ values can be used to calculate the intracellular sodium concentration, using Eq. 3. This calculation requires a knowledge of the relaxation rates $R_1^{(1)}$, $R_2^{(1)}$, $\mathscr{R}_1^{(1)}$, and $\mathscr{R}_2^{(1)}$, and the intracellular volume of the liver. The relaxation rates could be obtained from a fit of the corrected data shown in Fig. 4 (*open triangles*) to Eq. 3, and a knowledge of the observed line-widths. However, the use of Eq. 3 assumes that all intracellular sodium ions have the same relaxation rates, an assumption that is not supported by the data (see Results). Under these conditions, a more complete analysis that includes compartmentation is required (see Appendix 1).

The data summarized in Table 2 show that although the calculated value of $R_2^{(1)}$ for intracellular sodium ions is essentially independent of the $Dy(PPP)_2$ concentration in the perfusion buffer, the calculated value of B_0 increased at the higher $Dy(PPP)_2$ concentrations. The simplest explanation for the increased values of B_0 is that high concentrations of $Dy(PPP)_2$ increased the intracellular sodium concentration. The explanation for the apparent increase in the calculated value of $R_1^{(1)}$ for intracellular sodium ions at the higher $Dy(PPP)_2$ concentrations is unclear.

These observations emphasize the importance of working at low $Dy(PPP)_2$ concentrations. For example, the data shown in Fig. 4 and Table 1 were obtained with 2 mM $Dy(PPP)_2$, which caused no effect on B_0 (see Table 2).

Effects of residual quadrupolar splitting on the double-quantum-filtered ^{23}Na NMR spectrum from intracellular sodium ions

The double-quantum-filtered ^{23}Na NMR signals from brain in vivo (Lyon et al., 1991), erythrocytes (Shinar et al., 1993), and bovine nasal cartilage (Eliav et al., 1992) show a marked change in line-shape at intermediate τ values. At long τ values, the line-shape is characteristic of a " typical" double-quantum-filtered signal from sodium ions in an isotropic envoronment (Pekar and Leigh, 1986; Jaccard et al., 1986), but at short τ values the signals contain components with more complicated line shapes. Eliav et al. (1992) and Shinar et al. (1993) showed that the more complicated line-shapes observed at short τ values could be explained by unresolved residual quadrupolar splitting.

A comparison of the observed τ dependence of the double-quantum-filtered ^{23}Na NMR signal from perfused liver (see

Fig. 2) to the τ dependence of the double-quantum-filtered ^{23}Na NMR signal observed from brain (Lyon et al., 1991), erythrocytes (Shinar et al., 1993), and bovine nasal cartilage (Eliav et al., 1992), and the τ dependence of the theoretical line-shapes expected in the presence of unresolved residual quadrupolar splitting (Eliav et al., 1992; Shinar et al., 1993), shows that the effects of unresolved residual quadrupolar splitting are very small in the liver. The data thus suggest that the observed τ dependence of the apparent line-width of the narrow component of the double-quantum-filtered ^{23}Na NMR signal is due to compartmentation, and not due to the presence of an unresolved residual quadrupolar splitting. This conclusion could be confirmed with experiments using triple-quantum-filtered ^{23}Na NMR approaches, which are not sensitive to unresolved residual quadrupolar splitting (Shinar et al., 1993).

In any event, the presence of unresolved residual quadrupolar splitting would not affect the analysis of the data or the major conclusion of the studies, i.e., that at short τ values essentially all of the double-quantum-filtered ^{23}Na NMR signal from the perfused liver arises from intracellular sodium ions.

Effects of Dy(PPP)$_2$ on double-quantum-filtered ^{23}Na NMR signals from extracellular sodium ions in perfused liver

Liver perfused with complete Krebs-Henseleit buffer containing Dy(PPP)$_2$ shows a large upfield-shifted double-quantum-filtered ^{23}Na NMR signal (see Fig. 2). An upfield-shifted signal of similar intensity is also observed on the addition of Dy(PPP)$_2$ to Krebs-Henseleit buffer (see Fig. 3). For the Krebs-Henseleit buffer sample, this signal is presumably due to aggregation of some of the components of the buffer on the addition of Dy(PPP)$_2$ (Chu et al., 1984). At least part of the double-quantum ^{23}Na NMR signal accquired from liver perfused with Krebs-Henseleit buffer containing Dy(PPP)$_2$ thus arises from sodium ions in the perfusate, i.e., not in the tissue. This observation complicates the interpretation of the double-quantum-filtered ^{23}Na NMR signal from "extracellular" sodium ions in liver perfused with buffer containing Dy(PPP)$_2$.

This double-quantum-filtered ^{23}Na NMR signal observed from Krebs-Henseleit buffer on the addition of Dy(PPP)$_2$ is not removed by filtration of the buffer after the addition of Dy(PPP)$_2$ (see Results). The signal is also present if 2% BSA is added to the Krebs Henseleit buffer or if bicarbonate is deleted from the buffer (data not shown). However, the signal can be reduced substantially if inorganic phosphate is removed from the Krebs-Henseleit buffer, and most of the perfused liver experiments were performed with this buffer.

Comparison of results obtained with perfused liver to results obtained with other systems

Double-quantum ^{23}Na NMR techniques have been used recently to study rat brain in vivo (Lyon et al., 1991). These studies did not employ shift reagents and, thus, could not quantitate the relative contributions of intracellular and extracellular sodium ions to the observed double-quantum-filtered ^{23}Na NMR signal. However, in these studies the double-quantum-filtered ^{23}Na NMR signal acquired with short τ values (i.e., $\tau \sim 5$ ms) increased approximately threefold upon death, but the double-quantum-filtered ^{23}Na NMR signal acquired with long τ values (i.e., $\tau \geq 30$ ms) did not increase significantly upon death. The changes observed in brain are consistent with the conclusions reached in the present study with perfused liver—that double-quantum-filtered ^{23}Na NMR signals acquired with short τ values arise predominantly from intracellular sodium ions, whereas double-quantum-filtered ^{23}Na NMR signals acquired with long τ values arise from both intracellular and extracellular sodium ions.

A number of multiple-quantum-filtered ^{23}Na NMR studies of perfused heart have recently been reported (Jelicks and Gupta, 1989; Payne et al., 1990; Allis et al., 1991; Jelicks and Gupta, 1993). Some of these studies concluded that double-quantum-filtered ^{23}Na NMR approaches might not be useful for following intracellular sodium concentrations in the heart. These conclusions are different from the conclusions reached in the present study of perfused liver. However, the analysis of the double-quantum-filtered ^{23}Na NMR data from perfused heart was, in general, qualitative and did not take into account the effect of the shift reagent on the inhomogeneous relaxation rates of intracellular sodium ions.

CONCLUSIONS

The results of this study demonstrate that, although the double-quantum-filtered ^{23}Na NMR signal from perfused liver contains contributions from both intracellular and extracellular sodium ions, the double-quantum-filtered ^{23}Na NMR signal acquired with short τ values (i.e., $\tau \leq 4$ ms) arises almost exclusively from intracellular sodium ions. This conclusion suggests that multiple-quantum-filtered ^{23}Na NMR spectroscopy will be useful in studying intracellular sodium levels in the perfused liver, and possibly in the intact liver in vivo.

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APPENDIX 1

Effect of compartmentation on the double-quantum-filtered ^{23}Na NMR signal

Equation 3 gives the expression for the amplitude of the double-quantum-filtered ^{23}Na NMR signal for sodium ions in a single compartment. We define the relaxation rates in the kth compartment as $R_{1k}^{(1)}$, $R_{2k}^{(1)}$, $\mathscr{R}_{1k}^{(1)}$, and $\mathscr{R}_{2k}^{(1)}$ and define the number of sodium ions contributing to the ^{23}Na NMR signal from the kth compartment as N_k. Assuming that $\mathscr{R}_1^{(1)} \gg \mathscr{R}_2^{(1)}$, the

double-quantum-filtered ^{23}Na NMR signal from the kth compartment, $S_k(\tau)$, can be written as

$$S_k(\tau) = (3/20)\, A_o N_k [e^{-R_{1k}^{(1)}\tau} - e^{-R_{2k}^{(1)}\tau}][1/\mathscr{R}_{2k}^{(1)}]. \quad \text{(A1)}$$

The total double-quantum-filtered ^{23}Na NMR signal, $S_{tot}(t)$, is simply the sum of the signals from all the compartments, i.e.,

$$S_{tot}(\tau) = (3/20)\, A_o \sum_k N_k [e^{-R_{1k}^{(1)}\tau} - e^{-R_{2k}^{(1)}\tau}][1/\mathscr{R}_{2k}^{(1)}]. \quad \text{(A2)}$$

Equation A2 can be rearranged to give

$$S_{tot} = (3/20)\, A_o \left\{ \sum_k N_k/\mathscr{R}_{2k}^{(1)} [e^{-R_{1k}^{(1)}\tau}] - \sum_k N_k/\mathscr{R}_{2k}^{(1)} [e^{-R_{2k}^{(1)}\tau}] \right\}. \quad \text{(A3)}$$

Using the well known approximation of a sum of exponentials by a single exponential, Eq. A3 can be approximated by the expression

$$S_{tot}(\tau) \approx (3/20)\, A_o N_{tot} \langle 1/\mathscr{R}_2^{(1)} \rangle \{e^{-\langle R_1^{(1)} \rangle \tau} - e^{-\langle R_2^{(1)} \rangle \tau}\}, \quad \text{(A4)}$$

where

$$\langle R_1^{(1)} \rangle = \frac{\sum_k [N_k/\mathscr{R}_{2k}^{(1)}]\, R_{1k}^{(1)}}{\sum_k [N_k/\mathscr{R}_{2k}^{(1)}]}, \qquad \langle R_2^{(1)} \rangle = \frac{\sum_k [N_k/\mathscr{R}_{2k}^{(1)}]\, R_{2k}^{(1)}}{\sum_k [N_k/\mathscr{R}_{2k}^{(1)}]},$$

$$\langle 1/\mathscr{R}_2^{(1)} \rangle = \frac{\sum_k N_k/\mathscr{R}_{2k}^{(1)}}{\sum_k N_k}, \qquad \text{and} \qquad N_{tot} = \sum_k N_k.$$

Equation A4 has the same form as Eq. A1, except the parameters $R_{1k}^{(1)}$, $R_{2k}^{(1)}$, and $1/\mathscr{R}_{2k}^{(1)}$ are replaced by the "average" parameters $\langle R_1^{(1)} \rangle$, $\langle R_2^{(1)} \rangle$, and $\langle 1/\mathscr{R}_2^{(1)} \rangle$. Because $\langle R_1^{(1)} \rangle$, $\langle R_2^{(1)} \rangle$, and $\langle 1/\mathscr{R}_2^{(1)} \rangle$ are independent of τ, the τ dependence of the double-quantum-filtered ^{23}Na NMR signal from intracellular sodium ions can be used to calculate $\langle R_1^{(1)} \rangle$ and $\langle R_2^{(1)} \rangle$. The observation that the data in Fig. 7 *C* can be fit by Eq. A4 (see *dotted line* in Fig. 7) is consistent with the hypothesis that Eq. A4 is a reasonable approximation to Eq. A3.

Equation 7 has the same form as Eq. A4, which gives the relationship

$$B_o = (3/20)\, A_o N_{tot} \langle 1/\mathscr{R}_2^{(1)} \rangle.$$

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