# Multiple Quantum Filtered $^{23}$Na NMR Spectroscopy of the Isolated, Perfused Rat Liver

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**Isolated, perfused rat livers were examined by single-quantum (SQ) and double-quantum-filtered (DQ-filtered) $^{23}$Na spectroscopy during prolonged global ischemia and during perfusion with ouabain, low-buffer potassium, or lithium-enriched buffer. Baseline separation of the intracellular ($Na_i^+$) and extracellular ($Na_e^+$) sodium resonances using $TmDOTP^{5-}$ allowed a direct comparison of temporal changes in SQ versus DQ-filtered $Na_i^+$. The SQ $Na_i^+$ signal increased ~150% during the first 15 min of global ischemia and then remained relatively constant over the next 45 min, while the DQ-filtered signal steadily increased ~400% over the same 60 min period. In similar experiments in which all perfusate sodium was replaced by lithium, the DQ-filtered $Na_i^+$ signal increased ~180% over a similar period of ischemia. Exposure of livers to ouabain also resulted in larger increases in DQ-filtered versus SQ signal of $Na_i^+$. The ~290% increase in DQ-filtered sodium observed during perfusion of livers with a hypokalemic buffer (1.2 mM $K^+$) could be completely reversed by continued perfusion with a buffer containing normal levels of $K^+$ (4.7 mM). These data suggest that the DQ-filtered $Na_i^+$ signal of liver does not simply report an increase in [$Na_i^+$], but may be exquisitely sensitive to other intracellular events initiated by altered physiology. Magn Reson Med 41:1127–1135, 1999.** © 1999 Wiley-Liss, Inc.

**Key words: $^{23}$Na MRS; perfused liver; multiple quantum filtered NMR; shift reagent**

Multiple quantum (MQ) filtered $^{23}$Na NMR spectroscopy may prove useful for monitoring intracellular sodium ($Na_i^+$) in isolated tissues (1–3). It is a particularly attractive technique for in vivo applications because MQ filters are based on differences in relaxation rates of intra- and extracellular $Na^+$ and do not require addition of an exogenous shift reagent (SR). A $^{23}$Na MQ-filtered signal is observed whenever sodium experiences biexponential relaxation due to interaction with electrostatic sites on ordered biopolymers (4,5). It has been demonstrated in the perfused rat heart and in the rat liver in situ that the small but measurable extracellular sodium ($Na_e^+$) signal that passes through a triple-quantum (TQ) filter is largely unchanged during ischemia and other physiological perturbations (2,3,6–8). Thus, changes in total TQ-filtered $^{23}$Na signal intensity in the absence of an SR may directly report changes in [$Na_i^+$]. However, changes in the TQ-filtered $Na_i^+$ signal of the in situ liver during global ischemia were shown not to parallel changes in the SQ $Na_i^+$ signal, using $TmDOTP^{5-}$ as an SR to resolve the signal of $Na_i^+$ from $Na_e^+$ (3). A similar disparity between SQ- and TQ-filtered signals has been seen in brain (9), but not in red blood cells (10) or hearts (11,12). This finding suggests that this phenomenon may be tissue dependent.

The reason(s) for the apparent differences in temporal changes in SQ versus MQ-filtered $Na_i^+$ signals in liver have not been delineated. One testable hypothesis, based on previous observations (3), is that structural alterations within hepatocytes occur rapidly after cessation of flow and those structural events expose more existing $Na_i^+$ to binding sites that were sequestered in normoxic tissue. Exposure of $Na_i^+$ to such sites could produce changes in the MQ-filtered signal without dramatic changes in total [$Na_i^+$] as measured by SQ spectroscopy. Alternatively, all new $Na_e^+$ that enters liver cells during ischemia may be exposed to a different cellular environment than the majority of preischemic $Na_i^+$. Both explanations require the existence of multiple $Na_i^+$ pools. Although small increases in the fast component of the transverse relaxation rate of $Na_i^+$ ($T_{2f}$) were detected in the in situ liver post ischemia (3), the magnitude of those changes could not differentiate between these two mechanisms. Lyon and McLaughlin (13), in the only other published DQ-filtered $^{23}$Na NMR study of perfused liver, have shown that a single-compartment model cannot adequately describe the relaxation characteristics of $Na_i^+$.

The present study was designed to investigate in more detail the apparent differences between the temporal changes in SQ versus MQ-filtered $Na_i^+$ signals in an isolated, perfused rat liver preparation and to test the hypothesis stated above. Another goal was to repeat the previously described in situ ischemia experiments (3) using a homogenous radio frequency (RF) coil to remove any possibility that the changes in MQ-filtered $^{23}$Na intensities arose because of the inhomogenous $B_1$ field generated by the surface coil in the reported in situ experiments. A third aim was to determine whether physiological perturbations other than ischemia produce a differential change in the SQ and MQ-filtered $Na_i^+$ and to examine the reversibility of such changes. Finally, the question of whether temporal changes in the MQ-filtered $^{23}$Na signal require entry of new $Na_e^+$ into cells during ischemia was tested in a model in which all extracellular $Na^+$ was replaced by $Li^+$ prior to ischemia.

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Abbreviations: MQ, multiple quantum; SQ, single quantum; DQ, double quantum; TQ, triple quantum; NMR, nuclear magnetic resonance; SR, shift reagent; $Na_i^+$, intracellular sodium; $Na_e^+$, extracellular sodium; $TmDOTP^{5-}$, thulium(III) complexed with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetra-(methylene phosphonate).

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## MATERIALS AND METHODS

### Liver Perfusions

Fed Sprague-Dawley male rats (~125 g) were anesthetized by intramuscular injection of 30 mg of ketamine HCl (Ketaset; Aveco, Fort Dodge, IA) and heparinized by intraperitoneal injection of 500 UI heparin (Elkins-Sinn, Cherry Hill, NJ). The livers were then isolated as previously described (14). Briefly, the portal vein was cannulated with a 20 G, 1 inch catheter (Demed, Canton, MA) and perfused with a phosphate-free Krebs-Henseleit buffer. The perfusate was kept at 37°C in a water-bath and continuously saturated with a gas mixture of 95% $O_2$ and 5% $CO_2$ to give a final pH of 7.4. The composition of the buffer was as follows: NaCl 118 mM, KCl 4.7 mM, $MgSO_4.7H_2O$ 1.2 mM, $CaCl_2.6H_2O$ 3.0 mM, $Na_2EDTA$ 0.5 mM, $NaHCO_3$ 25 mM, and glucose 5.5 mM. EDTA was added to chelate any adventurous paramagnetic ions that might affect the quality of the NMR spectrum. A 200 μl bulb containing methylene diphosphonic acid (0.1 M, pH 10), NaCl (4 M), and $TmDOTP^{5-}$ (0.1 M) was used as an external chemical shift and concentration reference (15). The livers were first perfused for 10 min with non-recirculating buffer to remove blood completely from all vessels and then either perfused with 250 mL of recirculating KH buffer (with SR) or perfused without recirculation (in experiments without SR).

### MRS Data Collection

Most NMR experiments were performed using a 4.7 T 15 cm vertical bore MSL-200 spectrometer (Bruker, Billerica, MA) fitted with a 30 mm broadband Helmholtz coil (Doty, Columbia, SC). The probe was tuned to the $^{23}Na$ frequency after each liver was placed in the center of the coil. The magnet was shimmed on the $^{23}Na$ FID to a linewidth of 18–23 Hz. SQ $^{23}Na$ spectra were collected using a pulse-acquire sequence with a 49 μsec 90° excitation pulse and a 10 μsec spectrometer dead time before acquisition of 4096 complex data points over a spectral width of 16 kHz. The MQ-filtered $^{23}Na$ spectra were acquired using the following filter (16):

$$\left[(\pi/2)_\phi - \frac{\tau}{2} - (\pi/2)_\phi - (\pi)_{\phi+\pi/2} - (\pi/2)_\phi - \frac{\tau}{2} - (\pi/2)_{\phi+\pi/2} - \delta - (\pi/2)_{\pi/2} - t_{acq}\right]_{N\pm}$$

where $\tau$ is the MQ preparation time, $\delta$ is the MQ evolution time, and $t_{acq}$ is the acquisition time. The signals associated with the DQ and TQ coherences were selected by using 32 and 48 phase cycling steps, respectively (17). Typically, optimal values of $\tau$ (2 msec) and $\delta$ (3 μsec) were used in all experiments, except for the transverse relaxation measurements, where $\tau$ or $\delta$ was varied. The optimal $\tau$ value was selected initially by collecting data using various $\tau$ delays in the DQ filter pulse sequence and choosing the value that gave maximum signal intensity. The evolution time was kept as short as possible to reduce signal loss due to DQ $T_2$ relaxation. As in the SQ experiments, a 10 μsec spectrometer dead time was followed by acquisition of 4096 complex data points over a spectral with of 16 kHz. SQ and DQ-filtered spectra were collected alternately; the number of acquisitions for SQ data was 128 and that for DQ-filtered data was 1024. The repetition time for both the SQ and the DQ-filtered pulse sequence was set to 260 msec to allow for complete relaxation of sodium.

In one experimental group, DQ-filtered and TQ-filtered $^{23}Na$ spectra of livers were recorded using a 4.7 T 40 cm horizontal bore CSI Omega spectrometer (Bruker, Fremont, CA) fitted with a 30 mm single-turn solenoid volume coil (90° $^{23}Na$ pulse of 30 μsec). Unlike the Bruker MSL system, both DQ- and TQ-filtered spectra could be obtained on this spectrometer so that the contribution of $Na_e^+$ in the two experiments could be directly compared using the same liver.

All $^{31}P$ measurements were recorded on the MSL-200 system using a 60 μsec, 90° pulse (100 μsec dead time) and 2048 data points acquired over a sweep width of 10 kHz. Six hundred free induction decays (FIDs) were averaged per spectrum using a 1 s delay between pulses. The chemical shift of the γ-phosphate of ATP was used as an internal reference at -2.54 ppm.

All NMR data were processed and analyzed using the PC-based NMR software NUTS (Acorn NMR, Fremont, CA). All FIDs were baseline corrected and multiplied by single exponentials corresponding to 10 Hz line broadening for $^{23}Na$ or 20 Hz line broadening for $^{31}P$ before Fourier transformation. A magnitude calculation was performed on the DQ-filtered spectrum to account for the opposite phases of two Lorentzians, and correction factors were applied to account for non-zero mean noise (3). SQ and DQ-filtered $^{23}Na$ peak areas were determined by integration between user-defined endpoints.

### Ischemia Experiments

The contribution of $Na_e^+$ to the DQ-filtered $^{23}Na$ signal and the temporal changes in the SQ and DQ-filtered signals during global ischemia were first measured in the presence of SR. After an initial 10 min stabilization period, a $^{31}P$ spectrum was acquired to get a qualitative measure of tissue viability. SQ and DQ-filtered $^{23}Na$ spectra were then recorded sequentially, and $TmDOTP^{5-}$ (5 mM) was added to the perfusate. After achieving a steady-state separation between the $Na_e^+$ and $Na_i^+$ resonances, a $^{31}P$ spectrum was once again collected to evaluate any possible effects of SR on high-energy phosphorus metabolism. Data collection was then switched back to $^{23}Na$ and, after collecting baseline SQ and DQ-filtered spectra in the presence of the SR, the liver was subjected to an extended period of zero-flow ischemia. In separate experiments, SQ and DQ-filtered $^{23}Na$ spectra were recorded alternatively during a normoxic perfusion period and during zero-flow ischemia in the absence of $TmDOTP^{5-}$.

Changes in SQ and DQ $Na_i^+$ transverse relaxation rates during controlled perfusion and zero-flow ischemia were measured using the following protocol. SQ $T_{2f}$ and $T_{2s}$ of $Na_i^+$ that passed a DQ-filter were evaluated from a series of 15 DQ-filtered spectra (NS = 1024) acquired using $\tau$ values ranging from 0.1 to 48 msec. Similarly, DQ transverse relaxation times ($T_{DQ}$) were determined from a series of 11 DQ-filtered spectra (NS = 1024) acquired using $\delta$ values ranging from 0.1 to 12.8 msec. ($T_{DQ}$ was measured using

two different $\tau$ values, 1 and 12 msec.) To correct for increases in $Na_i^+$ signal intensity after initiation of ischemia, the $\tau$ (or $\delta$) value order was randomized in all experiments, and the FIDs corresponding to each $\tau$ (or $\delta$) value were collected in blocks of 256 scans at various times over the ischemic period. Four such blocks were summed to yield 1024 scans for each $\tau$ (or $\delta$) value.

### Perfusions With Ouabain and Low Potassium Buffer

Two groups of livers were submitted to perturbations intended to alter the $Na_i^+$ content while SQ and DQ-filtered $^{23}Na$ spectra were alternatively recorded throughout each experiment. In the first group, livers were first perfused for 25 min with normal perfusate (with or without 5 mM $TmDOTP^{5-}$) and then for 85 min with the same medium supplemented with 0.1 mM ouabain, an inhibitor of the Na/K-ATPase. In the second group, livers were first perfused with recirculating normal buffer (without $TmDOTP^{5-}$) for 25 min, and then with a non-recirculating buffer containing only 1.2 mM potassium for 45 min. These livers were subsequently perfused with non-recirculating normal buffer for 35 min to evaluate the reversibility of any changes detected in the DQ-filtered $Na_i^+$ spectrum upon return of $[K^+]$ to normal levels.

### Perfusions With $Li^+$-Enriched Buffer

Another group of livers was perfused with $Li^+$-enriched buffer in which all sodium was replaced by an equivalent amount of lithium. (LiCl and $Li_2CO_3$ replaced NaCl and $NaHCO_3$.) After livers were perfused with standard K-H buffer ($Na^+$ form) for 10 min, the perfusate was switched to $Li^+$-enriched buffer, and then the livers were perfused for an additional 60 min while $^{31}P$ NMR spectra were collected. Subsequently, perfusate flow was stopped, and SQ and DQ-filtered $^{23}Na$ spectra were alternately recorded for an additional 60 min.

## RESULTS

Figure 1 shows SQ and DQ-filtered $^{23}Na$ spectra of an isolated, perfused liver before addition of SR (first 2 spectra in each stack), after addition of 5 mM $TmDOTP^{5-}$ to the perfusate (next 4 spectra in each stack) and during zero-flow ischemia (remaining 18 spectra in each stack). The SQ and DQ-filtered spectra were collected sequentially every 5 min (~30 sec for each SQ spectrum and ~4.5 min for each DQ-filtered spectrum). The chemical shift of tissue $^{23}Na$ in the SQ spectrum before addition of SR was set to 0 ppm, while the signal from $Na^+$ in the reference bulb was shifted to 21 ppm by excess $TmDOTP^{5-}$. After addition of SR to the perfusion medium, the SQ resonance representing $Na_e^+$ moved downfield and stabilized by the third spectrum. This indicated that $TmDOTP^{5-}$ was uniformly distributed into all the extracellular space of the liver after about 10 min of perfusion. We previously quantified $[Na_i^+]$ in perfused mouse liver both by $^{23}Na$ NMR and by atomic absorption spectroscopy (AAS) and found it to be 29 ± 5 mM (15). Assuming that $[Na_i^+]$ is similar in rat liver, the large $Na_e^+$ resonance seen in these spectra clearly had a large contribution from $Na^+$ in the KH buffer bathing the liver in the NMR tube. The SQ $Na_i^+$ resonance remained unshifted throughout the experiment, showing that $TmDOTP^{5-}$ remained in the extracellular compartment.

The bottom panel of Fig. 1 shows DQ-filtered $^{23}Na$ spectra collected on the same liver over the same period of time. Clearly, the entire DQ-filtered $^{23}Na$ signal arises from $Na_i^+$ at 0 ppm. $Na^+$ in the external reference bulb, and $Na_e^+$ were not detected in the DQ experiment, at least using this number of spectral acquisitions. A slight decrease in intensity and broadening of the DQ-filtered $Na_i^+$ signal was evident upon addition of SR to the perfusate, but the area of the DQ-filtered signal was unaffected by SR. These minor perturbations probably reflect a change in bulk magnetic susceptibility of the sample introduced by the paramagnetic SR.

Figure 2 shows plots of normalized SQ $Na_i^+$ and DQ-filtered $^{23}Na$ signal intensities from livers before and after onset of zero-flow ischemia ($n = 3$). The normalized SQ $Na_i^+$ signal increased by ~1.5-fold during the first 15 min of zero-flow ischemia, and then reached a plateau before gradually declined over the remaining 45 min. In comparison, the normalized DQ-filtered signal increased continuously throughout the ischemic period, reaching a maximum of nearly fourfold after 60 min. Another DQ-filtered experiment was performed without SR, and the increases in signal intensity during zero-flow ischemia were identical to those observed with SR ($n = 3$). This confirms that $Na_e^+$ does not contribute to the total DQ-filtered $^{23}Na$ signal from perfused liver. Since others have reported that $Na_e^+$ can contribute to a MQ-filtered $^{23}Na$ spectrum (3,6–8), we also compared DQ-filtered and TQ-filtered $^{23}Na$ spectra of rat livers perfused with SR. Figure 3 shows that a contribution from $Na_e^+$ is indeed seen in the TQ-filtered $^{23}Na$ spectrum of liver but not in the DQ-filtered $^{23}Na$ spectrum (both using $\tau = 2$ msec). The signal from the reference bulb positioned near the liver within the coil and containing aqueous $Na^+$ and excess $TmDOTP^{5-}$ (similar to that shown in the SQ spectra of Fig. 1) was effectively filtered by both the DQ and TQ sequences.

### $^{23}Na$ Relaxation Times of Perfused Rat Liver

The DQ-filtered $^{23}Na$ signal from livers without SR was measured as a function of preparation time, $\tau$, both during normoxic perfusion and during zero-flow ischemia (data not shown). The DQ-filtered $^{23}Na$ signal reached an optimum near $\tau \approx 2$ msec in both cases. A fit of data collected during normoxic perfusion to a double exponential function gave $T_{2f}$ and $T_{2s}$ values of 0.61 ± 0.03 msec and 10.66 ± 0.53 msec, respectively ($n = 4$). During zero-flow ischemia, four successive series of data points were collected. Table 1 summarizes the values of $T_{2f}$ and $T_{2s}$ evaluated separately from each data series. On average, over the entire ischemic period, $T_{2f}$ and $T_{2s}$ was 0.74 ± 0.07 msec and 8.41 ± 0.72 msec, respectively.

The DQ-filtered $^{23}Na$ signal of livers without SR was also measured as a function of evolution time, $\delta$, at two different preparation times (1 and 12 msec) during normoxic perfusion and during zero-flow ischemia. The transverse relaxation time, $T_{DQ}$, was evaluated by fitting the resulting decay curves (not shown) to a monoexponential function. During normoxic perfusion, $T_{DQ}$ of liver was 0.24 ± 0.03 msec and 7.33 ± 0.05 msec for $\tau = 1$ and 12 msec,

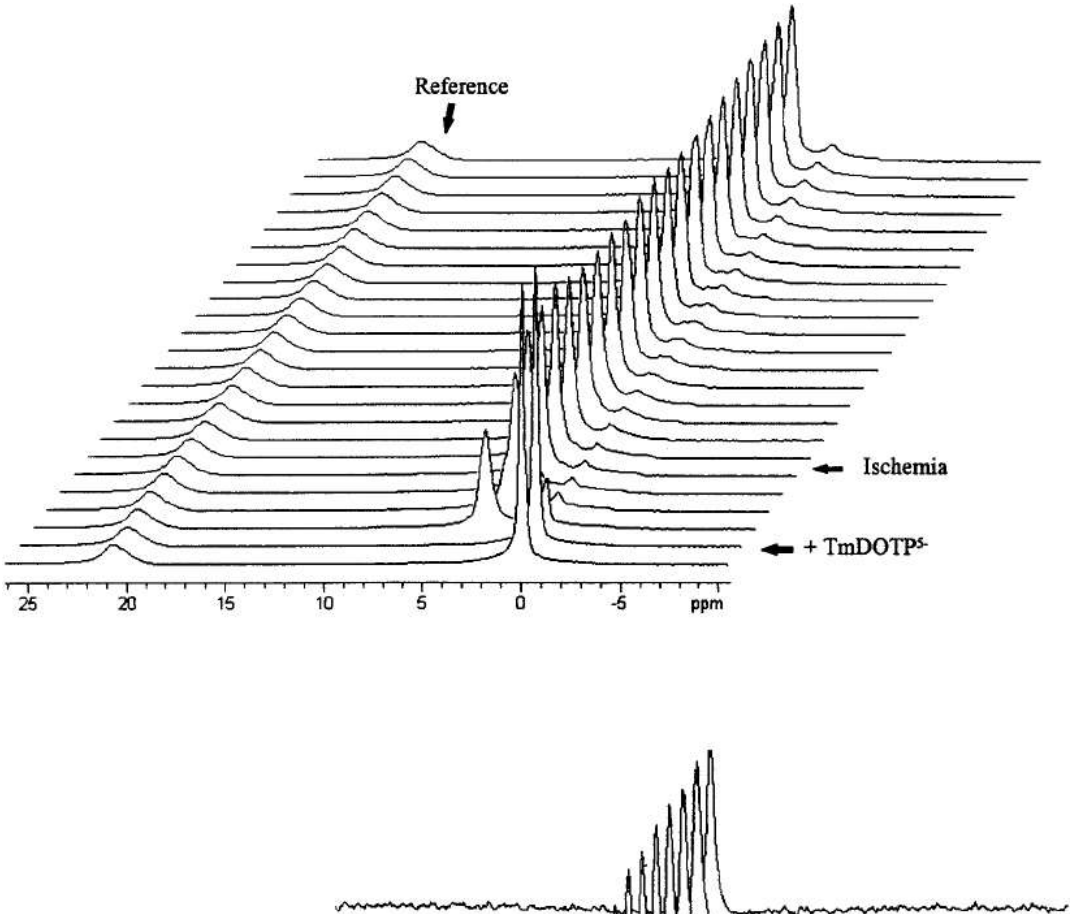



FIG. 1. Evolution of SQ $^{23}$Na spectra (top) and DQ-filtered $^{23}$Na spectra (bottom) of an isolated rat liver perfused prior to addition of TmDOTP$^{5-}$ to the perfusate, after addition of 5 mM TmDOTP$^{5-}$, and during subsequent zero-flow ischemia.

respectively. During ischemia, $T_{DQ}$ averaged 0.44 ± 0.02 msec and 10.57 ± 0.03 msec for $\tau$ = 1 and 12 msec, respectively.

### Perfusions With Ouabain and Low Potassium Buffer

Perfused rat livers were also submitted to perturbations designed to alter $Na_i^+$. First, one group of livers ($n$ = 4) was perfused with 0.1 mM ouabain, an inhibitor of Na/K-ATPase. Figure 4 (top) compares the evolution of the DQ-filtered $^{23}$Na signal of livers perfused with ouabain, in the presence and absence of TmDOTP$^{5-}$ ($n$ = 4 in each experiment). In both cases, the DQ-filtered $^{23}$Na signal intensity increased early after addition of ouabain and continued to increase over 120 min. After 90 min, the

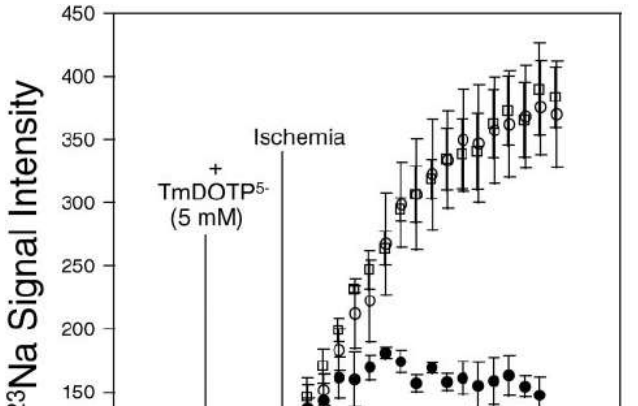



FIG. 2. Comparison of SQ (filled circles) and DQ-filtered (open symbols) signal intensities of $Na_i^+$ in isolated rat livers perfused with (squares) or without (circles) TmDOTP$^{5-}$ during zero-flow ischemia.

Table 1
$^{23}$Na Transverse Relaxation Times ($T_{2s}$ and $T_{2f}$) of $Na_i^+$ in the Isolated Rat Liver During Normoxia and Zero-Flow Ischemia (Mean ± SEM)

| Perfusion condition | $T_{2s}$ (msec) | $T_{2f}$ (msec) |
|---|---|---|
| Normoxic | 10.66 ± 0.53 | 0.61 ± 0.03 |
| Zero-flow ischemia | | |
| 0–15 min | 7.61 ± 1.97 | 0.75 ± 0.21 |
| 15–30 min | 8.69 ± 0.78 | 0.74 ± 0.08 |
| 30–45 min | 8.58 ± 0.52 | 0.73 ± 0.05 |
| 45–60 min | 8.49 ± 0.17 | 0.75 ± 0.02 |
| Average of four ischemic periods | 8.41 ± 0.72 | 0.74 ± 0.07 |

signal intensity had increased about 3½ times its initial value. On average, no significant differences were found between the DQ-filtered $^{23}$Na signals collected in the presence or absence of SR. This demonstrates that the DQ-filtered $^{23}$Na signal does not have a contribution from $Na_e^+$ when ouabain is present and that TmDOTP$^{5-}$ does not alter ouabain activity to any significant extent. The SQ signal intensity also increased during ouabain treatment but to a lesser extent, increasing by about 1.7–1.8 times its original intensity.

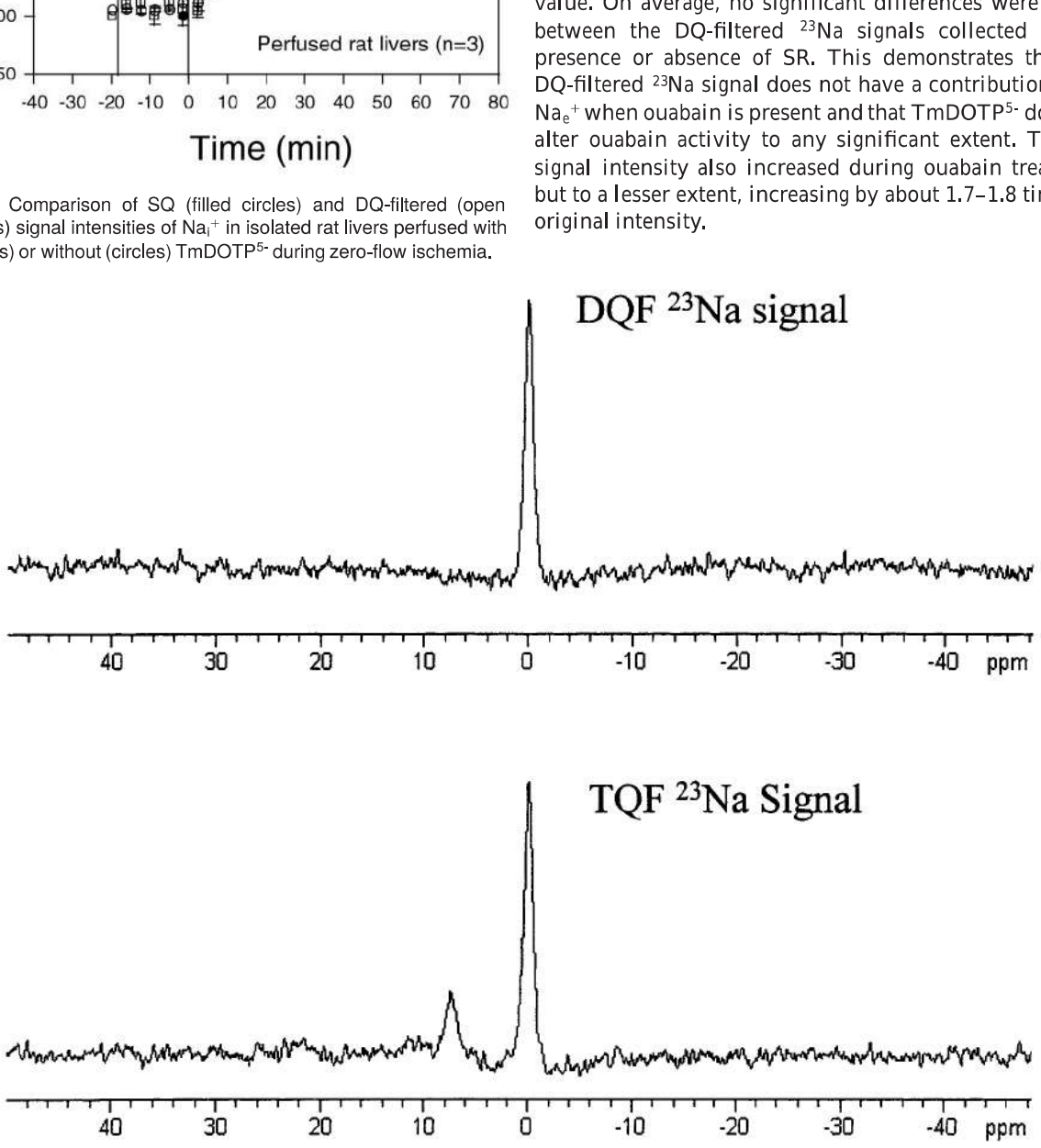
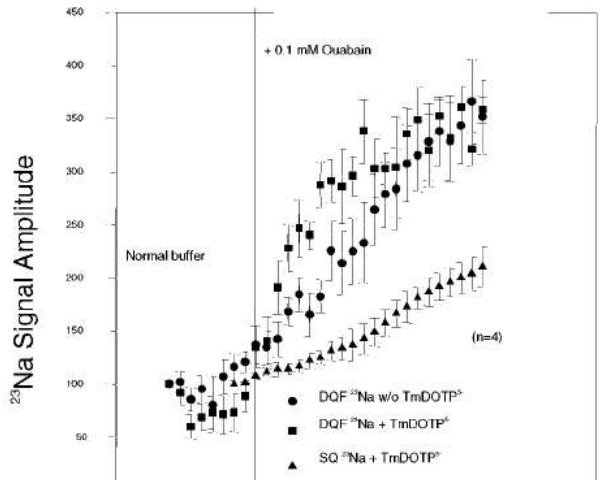



FIG. 3. Comparison of DQ-filtered and TQ-filtered $^{23}$Na spectra of an isolated rat liver perfused with 5 mM TmDOTP$^{5-}$ in a phosphate-free Krebs-Henseleit buffer. Note that the a signal from the shifted extracellular sodium ($Na_e^+$) resonance passes the TQ-filter but not the DQ-filter. These spectra were collected using a horizontal bore 4.7 T magnet and a Helmholtz coil.



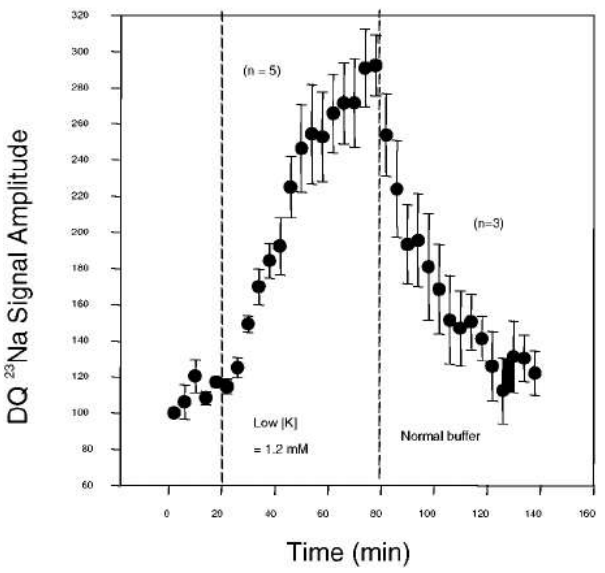




FIG. 4. Influence of 0.1 mM ouabain on the SQ and DQ-filtered $^{23}$Na signals of perfused rat livers (top). Evolution of the DQ-filtered $^{23}$Na signal of the isolated rat liver during the perfusion with an hypokalemic solution (1.5 mM $K^+$) and after switching the perfusate back to normal $K^+$ (3.7 mM).

A second group of livers ($n$ = 5) were perfused with a Krebs-Henseleit solution containing low potassium (1.2 mM). This perturbation resulted in an increase in DQ-filtered $^{23}$Na signal intensity (Fig. 4, bottom) that was reversed upon reperfusion with normal levels of potassium (4.7 mM). The DQ-filtered $^{23}$Na signal returned to pre-hypokalemic levels ($n$ = 3) after about 60 min of perfusion with 4.7 mM $K^+$.

### Perfusions With $Li^+$-Enriched Buffer

A final group of livers was examined to test the hypothesis that the increase in the DQ-filtered $Na_i^+$ signal during the perturbations described above (ischemia, ouabain, low potassium) reflects an alteration in the chemical environment of existing $Na_i^+$ and not influx of new $Na_e^+$ into cells. To limit $Na_e^+$ from entering liver cells during ischemia, a perfusate was prepared in which LiCl and $Li_2CO_3$ replaced NaCl and $NaHCO_3$, respectively (the buffer contained no sodium). Livers ($n$ = 3) were perfused with standard K-H buffer ($Na^+$ form) for 10 min and then switched to $Li^+$-buffer (no $Na^+$) and perfused for an additional 60 min. $^{31}$P NMR spectra (not shown) collected over this time interval indicated that total ATP and intracellular pH were unchanged during perfusion with $Li^+$-enriched buffer.

To determine the status of $Na_i^+$ in livers perfused with $Li^+$ prior to and during ischemia, $TmDOTP^{5-}$ was added to $Li^+$-enriched buffer to resolve the signals of $Na_i^+$ and $Na_e^+$ (Fig. 5, top). Although there was no $Na^+$ in the perfusate before addition of SR, addition of $Na_4HTmDOTP$ did add about 20 mM $Na^+$ to the perfusate. The inset to Fig. 5 shows the resolved $Na_e^+$ and $Na_i^+$ resonances from a liver perfused with buffer containing 143 mM [$Li^+$] and ~20 mM [$Na_e^+$] (entirely from SR), while the data (top panel) show the SQ intensity of $Na_i^+$ as a function of time after cessation of flow ($n$ = 3). A slight decrease in $Na_i^+$ was detected during the first 10 min of ischemia (presumably as some $Li_e^+$ exchanged with $Na_i^+$), but those changes were relatively minor. No further changes were detected throughout the 75 min ischemic period. This verified that $Na_i^+$ did not increase during the duration of this experiment.

In subsequent experiments, SR was not added to perfusate so that *all* $Na_e^+$ could be excluded. Livers were perfused for 8 min with standard K H buffer, then perfused with $Li^+$-enriched buffer for 8 min to wash all $Na_e^+$ from the NMR tube and all extracellular space, and finally submitted to global ischemia. After switching to $Li^+$-enriched buffer but prior to ischemia, the intensity of the DQ-filtered $Na_i^+$ signal decreased to ~65% of its control value prior to initiation of ischemia, presumably reflecting even more exchange of $Na_i^+$ with $Li_e^+$. Upon initiation of ischemia, the intensity of the DQ-filtered $Na_i^+$ signal increased progressively over 75 min (Fig. 5, bottom panel), eventually reaching an 1.8-fold increase in intensity ($n$ = 3). This intensity increase is not due to movement of new $Na_e^+$ into cells during the ischemic period. Also shown in Fig. 5 for comparison are data from livers perfused with normal KH-buffer without SR (similar to the data shown in Fig. 2). Although the DQ-filtered sodium signal intensity increases were larger in livers perfused with KH versus $Li^+$-enriched buffer (by a factor of ~1.6), the data show that some of this change must reflect an alteration in the chemical environment of existing $Na_i^+$.

## DISCUSSION

MQ-filtered $^{23}$Na NMR spectroscopy can potentially discriminate between tissue $Na_i^+$ and $Na_e^+$ without addition of an exogenous hyperfine shift reagent (1). However, the ultimate utility of this technique may be limited by two factors. The first is the extent to which $Na_e^+$ contributes to the MQ-filtered $^{23}$Na signal and whether this contribution, if any, changes during a physiological intervention. The second relates to whether any change detected in a MQ-filtered spectrum reflects a change in [$Na_i^+$] or some other less well-defined factor such as exposure of existing $Na_i^+$ to newly exposed binding sites.

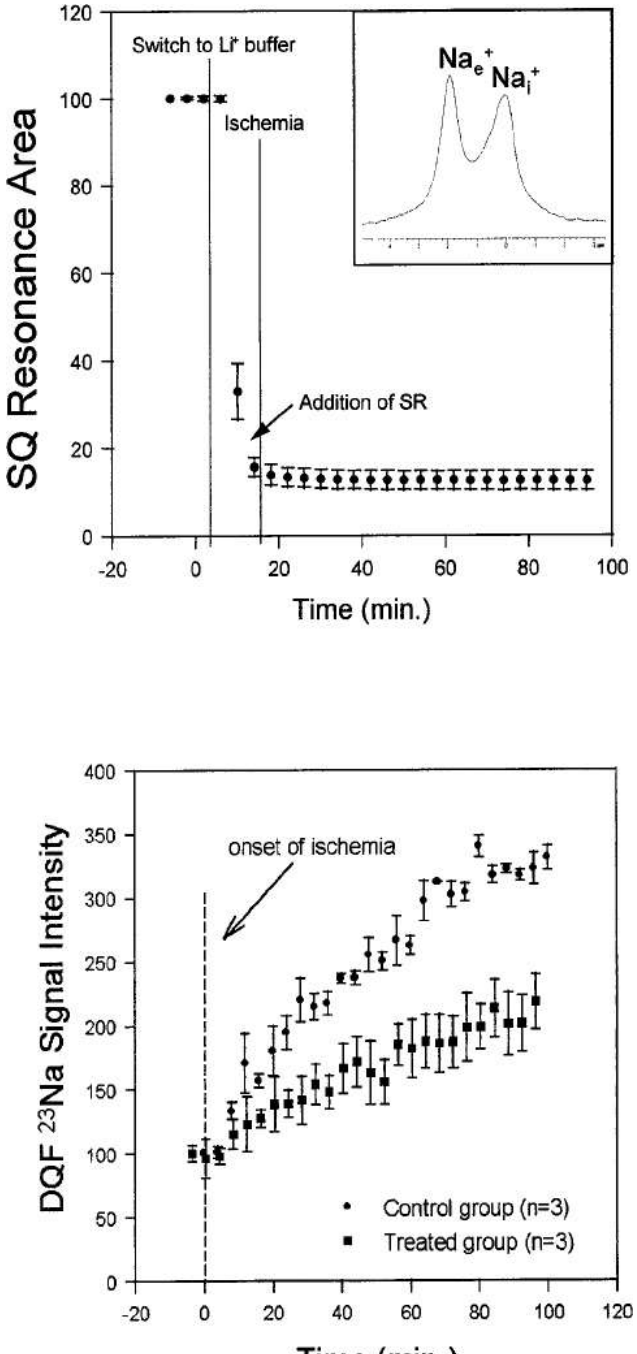



FIG. 5. SQ $^{23}$Na NMR spectra of a liver before and after switching from normal Krebs-Henseleit buffer to one containing $Li^+$ instead of $Na^+$ (see Materials and Methods for details). The sequence of events for this experiment were as follows: Livers were first perfused with normal KH buffer, and the resonance area of the resonance corresponding to total sodium was set equal to 100. After switching to $Na^+$-free, $Li^+$-enriched buffer, the total sodium resonance area dropped to about 30 units. At this point, no sodium was present in the perfusate. $Na_4$ HTmDOTP (5 mM) was then added to the perfusate to resolve the $Na_e^+$ and $Na_i^+$ resonances (see insert). The top panel shows the area of the $Na_i^+$ resonance as a function of time after initiate of zero-flow ischemia. Note that the initial resonance areas reflect total $Na^+$, while the resonance areas to the right of the line labeled "ischemia" reflect only $Na_i^+$. The bottom panel compares the changes in DQ-filtered $Na_i^+$ in livers perfused with $Li^+$-enriched buffer (solid squares) versus normal KH buffer (solid circles, data from Fig. 2).

Others have reported that $Na_e^+$ contributes to a MQ-filtered $^{23}$Na signal (3,6–13,18–22). Jelicks and Gupta, using $Dy(PPP)_2^{7-}$ as SR, reported that $Na_e^+$ contributes to the DQ-filtered $^{23}$Na spectrum in both red blood cells (18) and perfused rat hearts (19,20). They demonstrated that this contribution could be quenched by $Gd(PPP)_2^{7-}$, an efficient relaxation reagent of $Na_e^+$ (18,20). Using a different approach, Navon (22) demonstrated that the TQ-filtered $Na_e^+$ signal from isolated perfused hearts could be eliminated by using appropriate time delays and off-resonance effects, again using an SR. Hutchison et al (11), using $Dy(PPP)_2^{7-}$ as an SR, reported little contribution from $Na_e^+$ to DQ-filtered $^{23}$Na spectra of perfused hearts, while Dizon et al. (12), using $TmDOTP^{5-}$ as an SR, reported a significant contribution from $Na_e^+$ to the TQ-filtered signal of perfused hearts. However, the latter group also demonstrated that the contribution from $Na_e^+$ did not change during hypoxia, global ischemia, or ouabain treatment. Lyon and McLaughlin (13) reported that $Na_e^+$ contributes to the DQ-filtered $^{23}$Na spectrum (using $\tau \leq 4$ msec) of livers perfused with KH-buffer that included $P_i$, but not when perfused with KH-buffer lacking $P_i$. They also reported that $Dy(PPP)_2^{7-}$ alone induced a DQ-filtered sodium signal, the intensity of which was again sensitive to $P_i$. [We attempted these same experiments using $TmDOTP^{5-}$ as an SR, but found no effect of SR on either the TQ- or DQ-filtered $^{23}$Na signals (N. Bansal, unpublished results).] We recently reported that $Na_e^+$ contributes to the TQ-filtered $^{23}$Na spectrum of in situ rat liver (3), but also showed that the intensity of this signal did not change during ischemia. This was confirmed in the present study using an isolated, perfused rat liver preparation and a solenoid volume coil, thereby removing any doubt about this observation being an artifact due to $B_1$ inhomogeneity of the surface coil used in the in situ experiments. Using $TmDOTP^{5-}$ as an SR, we have now shown that $Na_e^+$ makes no contribution to a DQ-filtered $^{23}$Na spectrum of perfused liver. Our observation that DQ-filtered $^{23}$Na signal of livers subjected to zero-flow ischemia or perfusion with ouabain without SR exactly paralleled the increases seen in livers perfused with SR verified that the $Na_e^+$ signal is not simply quenched by the SR. Hence, we conclude that DQ-filtered $^{23}$Na NMR can be used to monitor $Na_i^+$ in livers without SR. The fact that we do detect a small signal from $Na_e^+$ in TQ-filtered spectra of liver [both perfused and in situ (3)] probably arises because a TQ-filtered signal contains contributions only from the third-rank tensors ($T_{3,-3}$ and $T_{3,+3}$) whereas the DQ-filtered signal contains contributions from both the third-rank ($T_{3,-2}$ and $T_{3,+2}$) and the second rank ($T_{2,-2}$ and $T_{2,+2}$) tensors. Since second and third rank tensors have opposite phase, these could in principle cancel in the DQ experiment but not in the TQ experiment (23).

Zero-flow ischemia resulted in increases in both the SQ and the DQ-filtered signals of $Na_i^+$ in perfused liver. The SQ $^{23}$Na signal has been shown to be 100% visible in liver (15,24), so the 1.5-fold increase in the SQ signal during ischemia must reflect the actual increase in $[Na_i^+]$ due to inhibition of the Na/K-ATPase and passive diffusion of $Na^+$ into cells. However, as we reported previously for the in situ rat liver (3), the time-dependent increase in the MQ-filtered $Na_i^+$ signal (TQ-filter for the in situ experiments and DQ-filter for the perfused livers reported here)

was more dramatic than the corresponding change in SQ signal. After 60 min of zero-flow ischemia, the DQ-filtered $^{23}Na$ signal increased nearly fourfold compared with control, while the SQ $^{23}Na$ signal increased by a factor of ~1.5 over this same time period. This phenomenon may be even more accentuated in ischemic brain, in which the DQ-filtered $^{23}Na$ signal (total $Na^+$) increased 800% while the SQ signal decreased by 10–15% (8). Heart tissue, however, seems to respond differently to ischemia than liver and brain. Navon et al. (25) observed a 1.6-fold increase in TQ-filtered $Na_i^+$ over 15 min of global ischemia that was completely reversed after ~5 min of reperfusion. Although concurrent SQ $Na_i^+$ was not measured in those experiments, the TQ-filtered $^{23}Na$ signal were assumed to reflect changes in [$Na_i^+$] directly (25). Hutchison et al. (11) monitored $Na_i^+$ in ischemic hearts by both SQ and DQ-filtered $^{23}Na$ NMR and found that although the SQ signal increased continuously over 60 min of ischemia, the DQ-filtered signal increased very quickly over the first few minutes, but then remained relatively constant. Their observation that the DQ/SQ intensity ratio decreased with time during ischemia and reperfusion suggested that qualitative changes were occurring in intracellular $Na^+$ binding sites during these perturbations. More recently, Dizon et al. (12) demonstrated that the TQ-filtered signal of $Na_i^+$ in perfused hearts increases with time during periods of hypoxia-aglycemia or zero-flow ischemia (~2.5-fold over 50 min). Again, SQ spectra were not reported so the relative temporal increases in SQ and TQ-filtered $^{23}Na$ NMR signal intensities could not be compared. However, Tauskela et al. (6), using $TmDOTP^{5-}$ and isolated perfused hearts, showed that the contribution of $Na_e^+$ to the DQ- and TQ-filtered $^{23}Na$ signals decreased during an ischemic episode. They also reported that the rate of increase in TQ-filtered $Na_i^+$ signal, when corrected for changes in transverse relaxation times ($T_{2f}$ and $T_{2s}$), paralleled the rate of increase in SQ $Na_i^+$ intensities during global ischemia. Similarly, Schepkin et al. (8) reported that the rise in TQ-filtered $^{23}Na$ signal during a variety of physiological perturbations (zero flow, 1 mM ouabain, low $K^+$, zero $Ca^{2+}$ and $Mg^{2+}$) parallels increases in $Na_i^+$ as measured analytically using $CoEDTA^-$ as a marker of extracellular space. Collectively, these reports indicate that changes in MQ-filtered $^{23}Na$ signal intensities parallel actual changes in [$Na_i^+$] in heart tissue. In liver and brain, however, changes in an MQ-filtered signal may in part reflect changes in cell structure that either expose or mask electrostatic sites or ordered macromolecular structures to existing $Na_i^+$.

We also observed a substantially larger increase in DQ-filtered versus SQ $Na_i^+$ signal intensities in the presence of ouabain, an inhibitor of Na/K-ATPase. Inhibition of the ATP-dependent $Na^+$ pumps caused a gradual increase in the SQ $Na_i^+$ signal to about twofold after a 90 min exposure. During this same period, the DQ-filtered $Na_i^+$ signal increased by a factor of 3.5, in both the presence and absence of SR. Ouabain has been used extensively in MQ-filtered $^{23}Na$ studies of the heart. Dizon et al. (11), using $TmDOTP^{5-}$ as an SR, reported an ~3.3-fold increase in the TQ-filtered $Na_i^+$ signal after exposure of hearts to 0.2 mM ouabain for 25 min. Schepkin et al. (7), using TQ-filtered $^{23}Na$ spectroscopy without an SR, reported an ~2.1-fold increase in the total TQ-filtered $^{23}Na$ signal intensity after exposure of hearts to 1 mM ouabain for 30 min. Neither study reported concurrent SQ measurements of $Na_i^+$. Most recently, Tauskela et al. (6) compared the intensity changes of SQ (using $TmDOTP^{5-}$ to resolve $Na_i^+$) and TQ-filtered $Na_i^+$ in hearts exposed to either 0.5 or 0.75 mM ouabain. They found that the SQ and TQ-filtered $Na_i^+$ intensities were proportional during the first approximately twofold increase, and then began deviating, with the TQ-filtered signal either growing faster ($\tau$ = 14 msec) or slower ($\tau$ = 0.8 msec) than the SQ signal. We did not measure SQ spectra of livers perfused with 1.2 mM $K^+$ so do not know whether the presumed increase SQ intensity deviated from the increase in DQ-filtered $Na_i^+$ signal. Nevertheless, this experiment demonstrates that the rise in DQ-filtered $^{23}Na$ signal upon exposure of livers to hypokalemic buffer is readily reversible, making the method potentially useful for evaluating tissue recovery.

The 400% increase in DQ-filtered signal of liver $Na_i^+$ during ischemia could reflect a combination of 1) an increase in [$Na_i^+$]; 2) altered relaxation behavior of $Na_i^+$; and/or 3) an increase in the fraction of $Na_i^+$ exhibiting MQ coherence. The actual increase in [$Na_i^+$] as reported by the SQ spectrum accounts for about 38% of the total observed increase in DQ-filtered signal. As the DQ-filtered $^{23}Na$ signal depends on the relative values of slow ($T_{2s}$) and fast ($T_{2f}$) SQ relaxation components, changes in either or both of these values could also contribute to the changes detected in the DQ signal. The experimental relaxation data (Table 1) show that $T_{2s}$ decreases while $T_{2f}$ increases during the ischemia. Given that the DQ-filtered signal intensity depends on the difference between two biexponential relaxation terms, ($e^{-\tau/T2f} - e^{-\tau/T2s}$), the measured changes in $T_{2f}$ and $T_{2s}$ between normoxic and ischemic periods predict an ~9% decrease in signal intensity during ischemia, not a 400% increase as measured experimentally. These estimates (38% from an increase in [$Na_i^+$] minus 9% from alterations in relaxation behavior) suggest that the largest contribution to the 400% increase in DQ-filtered signal during ischemia arises from an increase in the fraction of existing $Na_i^+$ that exhibits MQ coherence. This was directly demonstrated by experiments in which all extracellular $Na^+$ was replaced by $Li^+$. The similar increase in DQ-filtered $^{23}Na$ signal during ischemia in $Li^+$ perfused livers proves that the DQ-filtered $^{23}Na$ signal does indeed reflect exposure of existing $Na_i^+$ to new binding sites.

The DQ-transverse relaxation time ($T_{DQ}$) of $Na_i^+$ is also sensitive to sodium-macromolecular interactions. Since $T_{DQ}$ should equal $T_{2f}$ for a uniform sample, our observation that $T_{DQ}$ differs from $T_{2f}$ provides further evidence for multiple $Na_i^+$ environments. During normoxic perfusion, $T_{DQ}$ was 30 times longer for a preparation time of 12 msec ($T_{DQ}$ = 7.33 ± 0.05 msec) than for 1 msec ($T_{DQ}$ = 0.24 ± 0.03 msec). For a uniform sample, $T_{DQ}$ is essentially independent of preparation time (9), so the observed dependence of $T_{DQ}$ on preparation time suggests the liver has multiple $Na_i^+$ binding environments with different relaxation properties. A short preparation time preferentially excites DQ coherence in strongly relaxed spins, reflected by shorter $T_{DQ}$ values, while a long preparation time preferentially excites DQ coherence in less strongly relaxed spins, reflected by longer $T_{DQ}$ values. $T_{DQ}$ increased

during ischemia for both preparation periods, consistent with reduced quadrupolar interactions. However, the increase in $T_{DQ}$ during ischemia was somewhat larger for a preparation time of 1 msec (1.8×) compared with 12 msec (1.4×), and this indicates that strongly relaxed spins are altered most during ischemia. The observation that the DQ-filtered $^{23}Na$ signal amplitude increases fourfold during ischemia while $T_{DQ}$ increases 1.5–1.8-fold also suggests that alterations within the cell during ischemia increases the fraction of $Na_i^+$ exhibiting MQ coherence, but that the average quadrupolar interaction decreases.

The exact origin of the structural changes that occur within the cell during ischemia or during perfusion with ouabain or with low levels of $K^+$ has not been delineated in these experiments. Numerous studies indicate that ischemic cell injury and ATP depletion in cell culture models result in a marked enhancement of actin polymerization, disruption of cortical and microvillar actin cytoskeleton, and redistribution and aggregation of filamentous actin throughout the cytoplasm (26). Shinohara et al. (27) have also shown that the cytoskeleton of liver is quickly disrupted during ischemia and that this process can be reversed if the liver is reperfused within 60 min. Treatment of liver with either colchicine, a known toxin of microtubule formation, or ouabain also disrupts liver cytoskeleton structure (28). Thus, one unifying explanation for our consistent observation of a larger increase in DQ-filtered versus SQ $^{23}Na$ signal in ischemic liver and in livers exposed to ouabain is that a greater fraction of $Na_i^+$ (both preexisting and new $Na_i^+$) interacts with strong electrostatic binding sites created by disruption of the liver cytoskeleton. If this hypothesis proves correct, then DQ-filtered $^{23}Na$ spectroscopy or imaging may be exquisitely sensitive for detection of liver dysfunction.

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