31P NMR quantitation of phosphorus metabolites in rat heart and skeletal muscle in vivo

SAM HITCHINS, JULIE M. CIESLAR, AND GEOFFREY P. DOBSON
Department of Physiology and Pharmacology, School of Biomolecular and Molecular Sciences, James Cook University, Townsville QLD 4811, Australia

Received 13 November 2000; accepted in final form 29 March 2001

APPLICATION OF 31P NMR SPECTROSCOPY has gained increasing popularity in recent years in providing a window into metabolism and cellular bioenergetics, but a number of concerns remain (2, 6, 14, 19, 20). Notwithstanding the problem of NMR sensitivity (>0.5 mM), a longstanding difficulty has been how to convert the number of “spins” into an absolute tissue concentration (12). Two popular methods for quantifying the absolute phosphorus concentration in tissues are using an external reference standard (9, 28) and using tissue ATP measured independently after the experiment and equating the content to the area under the βATP peak (8, 13, 14, 20, 29). Other methods of quantitation include internal [1H]H2O calibration (27) and the use of a synthesized NMR reference produced by the transmittance of signal from an electronic device received at the same time as the sample signal (2). The assumptions of all these methods are not trivial and involve knowledge of the radiofrequency and coil loading properties, inhomogeneity in the B1 coil, volume sensitivities, the effect of motion, and the choice of spectral analysis program (28, 29).

Using differences between an external standard and measured ATP has led some investigators to propose an up to 40% invisibility of ATP in the heart (26, 30). Invisibility is a complex term but largely reflects line broadening from the highly decreased mobility of a phosphorus compound, e.g., from compartmentation. One of the greater difficulties, however, with invoking invisibility is that one has to be confident that all the technical assumptions mentioned above are not a contributing factor. The aim of this study is to take these factors into account and compare the two most common methods of an external standard and comparing the phosphorus concentrations with freeze-clamp ATP values measured from the same tissues in rat heart and gastrocnemius muscle in vivo. Our study showed that by positioning the external standard above the coil and providing symmetrical volume sensitivities, both methods agreed to within 5–10% at 7 T. The heart showed the greatest discrepancy due to the lower signal-to-noise ratio, primarily from motion effects.

EXPERIMENTAL PROCEDURES

Surgical Protocol

Heart. Seven male Sprague-Dawley rats weighing 300–350 g were obtained from the James Cook University (JCU) breeding colony and housed in the animal facility. Rats were supplied with unrestricted access to food and water. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg rat weight). The left femoral artery and vein were cannulated with polyethylene (PE)-50 tubing. The venous line was used for maintenance of anesthesia (15 mg/ml solution of pentobarbital sodium in 0.9% saline), whereas the arterial line was used for blood collection and continuous monitoring of blood pressure. The arterial
line was flushed periodically with a 100 U/ml solution of heparin in 0.9% saline (David Bull Laboratories; Melbourne, Australia). Blood pressure was measured using a pressure transducer interfaced to a Maclab 200 (AD Instruments; Castle Hill, Australia) and Apple Macintosh computer. After cannulation, animals were artificially ventilated on room air with a Harvard Small Animal Ventilator (Harvard Apparatus; South Natick, MA) to give a blood PO$_2$ of ~90 mmHg, a PCO$_2$ of ~40 mmHg, and a pH of ~7.4. Blood (0.2 ml) was drawn from the arterial line and analyzed with the Ciba-Corning 865 blood gas analyzer (Ciba Corning Diagnostic; Medfield, MA) to ensure that blood gases were within the correct range.

A thoracotomy was performed, and a custom-built flexible arm surface coil (9-mm outer diameter) tunable to $^{31}$P was placed on the left ventricle. The coil was made of Teflon-coated copper wire (1.25 mm thick) and was sufficiently flexible to follow the movement of the heart and maintain contact without excessive pressure against the heart. A thin-walled latex balloon containing 10 mM phenylphosphonic acid (PPA) in D$_2$O (Aldrich Chemical; Milwaukee, WI) was placed on top of the surface coil. The configuration of coil, heart, and standard is illustrated in Fig. 1. No significant alteration in blood pressure or heart rate was seen on placement of the coil.

The sampling depth of the surface coil was determined on a multilayer phantom. Four plastic disks enclosed on both sides by a thin latex membrane were assembled and filled with 100 mM Na$_2$HPO$_4$, phosphocreatine (PCr), PPA, and glucose-6-phosphate standards, respectively. The disks were then layered to form four successive layers, each 2 mm thick (Fig. 2). A fully relaxed $^{31}$P spectrum was obtained using the same surface coil described above to determine the relative signal contribution from each layer.

Skeletal muscle. Six male Sprague-Dawley rats weighing 350–400 g were obtained from the Animal Resources Centre (Canning Vale, Australia) and housed in the animal facility at JCU. Rats were supplied with unrestricted access to food and water. Animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg rat weight), tracheotomized, and ventilated using a Harvard Small Animal Ventilator to give a blood PO$_2$ of ~90 mmHg, a PCO$_2$ of ~40 mmHg, and a pH of ~7.4. Blood gases were analyzed with the Ciba-Corning 865 blood gas analyzer. Anesthesia was maintained by delivering 1% isoflurane (Abbott Australasia; Kurnell, Australia) in compressed air via the ventilator at a rate of 0.5 l/min. The right carotid artery was cannulated with PE-50 tubing and utilized for blood collection and continuous monitoring of blood pressure. The arterial line was flushed periodically with a 100 U/ml solution of heparin in 0.9% saline. Blood pressure was measured using a Statham P23 XL pressure transducer interfaced to a Maclab 200 and Apple Macintosh computer.

The animal was transferred to a custom-built Perspex cradle. The cradle was prefitted with a 37°C water-heated pad. The gastrocnemius was exposed, and the muscle was carefully denuded of overlying tissue and covered with a plastic film to prevent drying of exposed tissue. A three-turn surface coil (14-mm outer diameter) tunable to $^{31}$P was placed on the center of the gastrocnemius muscle. A thin-walled latex balloon containing 10 mM PPA was placed on top of the surface coil. The sampling depth of the surface coil was determined in the same manner as described for the heart.

**NMR Spectroscopy**

Heart. $^{31}$P NMR experiments were performed at 121.47 MHz in a 110-mm horizontal bore Oxford 7.05-T superconducting magnet. The magnet is the property of the School of Molecular and Biomedical Sciences (JCU). $^{31}$P spectra were obtained using a Varian Inova NMR spectrometer. Magnetic field homogeneity was maximized on the $^1$H free induction decay (FID) measured off resonance using an Oxford Instruments 15-channel shim supply. Radiofrequency pulses of 8-μs duration at an ~40° flip angle were applied with a 1-s interpulse delay. FIDs were acquired over 0.4 s with a total of 1,024 FIDs averaged. An spectral width of 8,000 Hz was used, and 6,400 data points were obtained. An exponential line broadening factor of 10 Hz was applied to the $^{31}$P NMR spectra, which were then fitted using the Varian FitSpec software. After integration, all peaks were multiplied by a saturation correction factor specific for each peak. These factors were determined experimentally by comparing the peak integrals of partially relaxed spectra, obtained using the acquisition parameters described above, to the peak integrals of fully relaxed spectra (20-s interpulse delay). Mean values were used in NMR calculations of individual phosphorus compounds in the heart (and skeletal muscle) for a 1-s acquisition delay.
In the heart, the mean correction factors for the 10 mM PPA external standard, Pi, PCr, and βATP were 1.12 ± 0.03, 0.97 ± 0.08, 1.24 ± 0.04, and 0.87 ± 0.03 (± SE, n = 7), respectively. On completion of spectral acquisition, the heart was freeze-clamped in vivo at the temperature of liquid nitrogen. Tissue was ground to a powder in liquid nitrogen and stored at −80°C for later enzymatic analysis of tissue ATP concentration (described below).

**Skeletal muscle.** 31P NMR experiments in muscle were performed using the same instrumentation as for the heart experiments. Radiofrequency pulses of 8-μs duration at an −90° flip angle were applied with a 1-s interpulse delay. FIDs were acquired over 0.8 s with a total of 256 FIDs averaged. A spectral width of 6,000 Hz was used, and 9,600 data points were obtained.

Spectral intensities of phosphorus compounds were determined by computer integration using the Varian (VNMRX) software. Opposed to heart spectra, a line-fitting program was not required for muscle because of the superior signal-to-noise ratio. The partially relaxed 31P spectra were calibrated by comparison with fully relaxed spectra using the same process described for the heart (see above). In muscle, the mean correction factors for the 10 mM PPA external standard, Pi, PCr, and βATP were 1.29 ± 0.08, 0.98 ± 0.10, 1.03 ± 0.01, and 0.88 ± 0.09 (± SE, n = 6), respectively. On completion of spectral acquisition, the muscle was freeze-clamped in vivo, and the tissue was powdered in liquid nitrogen for enzymatic determination of ATP concentration.

**Metabolic Analysis**

All chemicals used in the metabolic assays were purchased from either Sigma or Boehringer-Mannheim and were of the highest grade. Frozen powdered heart or muscle tissue (100 mg) was weighed at the temperature of liquid nitrogen in a mini-bead beater (Biospec, Australia) using the enzymatic method of Lowry and Passonnau (21).

**Phosphorus Quantification**

Two independent methods of peak standardization were utilized to determine the concentration of Pi, PCr, and ATP. First, the βATP peak was used as an internal standard. In this method, the enzymatically determined ATP concentration in freeze-clamped tissues was equated with the integral of the βATP peak. The saturation-corrected phosphorus metabolite integrals were then standardized from the saturation-corrected βATP peak as follows

\[
[P_i]\text{[μmol/g wet wt]} = \frac{\text{SCF}_{P_i} \times \text{integral}_{P_i}}{\text{SCF}_{\beta\text{ATP}} \times \text{integral}_{\beta\text{ATP}}} \times [\text{ATP}][\text{μmol/g wet wt}]
\]

\[
[\text{PCr}][\text{μmol/g wet wt}] = \frac{\text{SCF}_{\text{PCr}} \times \text{integral}_{\text{PCr}}}{\text{SCF}_{\beta\text{ATP}} \times \text{integral}_{\beta\text{ATP}}} \times [\text{ATP}][\text{μmol/g wet wt}]
\]

where SCF is the saturation correction factor for each metabolite in heart and skeletal muscle (see above).

The second method employed PPA as an external standard. Notwithstanding issues of signal-to-noise ratios and peak integration methods, the following equations (Eq. 4–10) present the theory of using an external reference positioned symmetrically above the coil and heart tissue as follows.

The observed signal (S) (or integral of the phosphorus metabolite in heart) is given by

\[
S = \int_{\text{allspace}} \rho(x, y, z) \times B_1\text{ coil}(x, y, z) dV
\]

where \(\rho\) is the spin density distribution of the source signal, \(V\) is volume where signal is measured and \(B_1\) coil is the sensitivity of the coil system, which is generally the transverse field at a point in space normalized to the amplitude per mole of phosphorus metabolite. For the reference signal, \(\rho = [\text{PPA}] \times f_{\text{PPA}}(x, y, z)\), where \(f_{\text{PPA}}(x, y, z)\) is the spatial distribution. If we take \(f = 1\) inside the sphere containing the standard PPA and \(f = 0\) outside, therefore

\[
S_{\text{PPA}} = [\text{PPA}] \int_{\text{sphere}} B_1\text{ coil} dV
\]

The tissue phosphorus signal (e.g., PCr) from Eq. 4 above will therefore be

\[
S_{\text{PCr}} = \int_{\text{allspace}} [\text{PCr}](x, y, z) \times B_1\text{ coil}(x, y, z) dV
\]

which leads to

\[
[\text{PCr}] = \frac{S_{\text{PCr}}}{\int_{\text{allspace}} f_{\text{PCr}}(x, y, z) \times B_1\text{ coil} dV}
\]

and assumes that PCr is uniformly distributed. If \(f = 1\) inside the heart and \(f = 0\) outside, as for PPA in the sphere (Eq. 5), the volume integral is limited to the convoluted volume of the heart. Therefore, given these conditions

\[
[\text{PCr}] = \frac{S_{\text{PCr}}}{\int_{\text{heart}} B_1\text{ coil} dV}
\]

From these equations, PPA can be used as the external standard (Eq. 5) to determine tissue PCr (Eq. 8). The two basic assumptions of the method are that 1) [PCr] is uniformly distributed in the heart, and 2) the sensitivity of the coil system yields identical signal amplitude per mole of 31P between PPA and phosphorus compounds in the heart. Therefore

\[
\int_{\text{heart}} B_1\text{ coil} dV = \int_{\text{sphere}} B_1\text{ coil} dV
\]

If these assumptions are correct, then

\[
[\text{PCr}] = \frac{S_{\text{PCr}} \times [\text{PPA}]}{S_{\text{PPA}}}
\]
The concentration of PCr then becomes the equivalent of micromoles of metabolite per gram wet weight in equal volumes (taking into account that 1 ml of water is equivalent to 1 g, and 1 ml tissue is equivalent to 1.1 g).

The saturation-corrected phosphorus metabolite integrals were standardized from the saturation-corrected 10 mM PPA integral as follows:

\[
[P_i] (\mu \text{mol/g wet wt}) = \frac{\text{SCF}_{\text{PPA}} \times \text{integral}_{\text{PPA}}}{\text{SCF}_{\text{PPA}} \times \text{integral}_{\text{PPA}}} \times 10 \text{ (mM)} \tag{11}
\]

\[
[\text{PCR}] (\mu \text{mol/g wet wt}) = \frac{\text{SCF}_{\text{PCR}} \times \text{integral}_{\text{PCR}}}{\text{SCF}_{\text{PCR}} \times \text{integral}_{\text{PPA}}} \times 10 \text{ (mM)} \tag{12}
\]

\[
[\text{ATP}] (\mu \text{mol/g wet wt}) = \frac{\text{SCF}_{\text{ATP}} \times \text{integral}_{\text{ATP}}}{\text{SCF}_{\text{PPA}} \times \text{integral}_{\text{PPA}}} \times 10 \text{ (mM)} \tag{13}
\]

**Intracellular pH**

Intracellular pH (pH\textsubscript{i}) was calculated from the chemical shift (δ, in parts per million (ppm)) of P\textsubscript{i} relative to PCR in the \textsuperscript{31}P spectra using the NMR version of the Henderson-Hasselbalch equation (1):

\[
pH_{i} = 6.75 + \log \left( \frac{\delta - 3.25}{5.69 - \delta} \right) \tag{14}
\]

**Free Magnesium**

Intracellular free Mg\textsuperscript{2+} concentration ([Mg\textsuperscript{2+}]i) was calculated from the observed chemical shift difference (δ\textsubscript{dab}; in ppm) between βP and αP resonances of ATP in the \textsuperscript{31}P spectra using a modified form of the London equation (11):

\[
[Mg^{2+}]_{i} = K_D \left[ \delta_{\text{dab}} + \alpha \delta_{\text{dab}} - \delta_{\text{dab}}(1 + \beta) \right] \tag{15}
\]

where α = [H\textsuperscript{+}]/K\textsubscript{H} and β = K\textsubscript{H}/K\textsubscript{d}. K\textsubscript{H} is the dissociation constant for the H\textsuperscript{+}/ATP\textsuperscript{4-} equilibrium, K\textsubscript{d} is the dissociation constant for the ATP\textsuperscript{4-}/Mg\textsuperscript{2+} equilibrium, and K\textsubscript{D} is the dissociation constant for the ATPH\textsuperscript{2+}/Mg\textsuperscript{2+} equilibrium. The parameters δ\textsubscript{1}, δ\textsubscript{2}, δ\textsubscript{d}, and δ\textsubscript{dab} were assigned published values of 10.600, 11.660, 8.165, and 8.52 ppm, respectively; K\textsubscript{D} was 9.0 × 10\textsuperscript{-5} M, K\textsubscript{H} was 3.4 × 10\textsuperscript{-7} M, and K\textsubscript{D} was 7.2 × 10\textsuperscript{-4} M (11).

**Statistical Analysis**

All values are means ± SE. Student’s t-tests were applied for statistical comparisons, with the α-level of significance set at P < 0.05. P values are also reported for comparisons between the two methods of quantification.

**RESULTS**

The sampling depths of the surface coils used for in vivo determination of phosphorus metabolite concentrations in heart and skeletal muscle are shown in Fig. 2. In the heart, ~70% of the signal came from the first 2 mm of tissue below the coil and ~90% came from within the first 4 mm. With the larger coil for skeletal muscle, 50% came from the first 2 mm and a total of 85% came from the first 6 mm below the coil. The thickness of the ventricle wall directly beneath the coil was measured in our study and found to be ~4 mm in thickness and 8 mm in rat gastrocnemius muscle below the site of coil placement. Given these distances, we conclude that over 90% of signal was received from both ventricular and skeletal muscle.

Table 1 summarizes the data obtained from the two methods of quantitation of ATP, PCr, and P\textsubscript{i} in heart and skeletal muscle in vivo. With the use of the external standard (10 mM PPA), the mean concentrations of ATP, PCr, and P\textsubscript{i} in the heart were 4.48, 9.21, and 2.25 μmol/g wet wt, respectively. With the use of the internal ATP standard measured enzymatically on the same tissue, the mean contents were 4.78, 9.83, and 2.51 μmol/g wet wt, respectively. There were no significant differences for each metabolite and the two methods of quantification in the heart. The P values for ATP, PCr, and P\textsubscript{i} for the two methods are 0.41, 0.34, and 0.46, respectively (Table 1).

For skeletal muscle, using the external standard, the concentrations of ATP, PCr, and P\textsubscript{i} were 6.09, 23.44, and 1.81 μmol/g wet wt, and, for the internal standard, the concentrations were 6.03, 23.30, and 1.82 μmol/g wet wt, respectively. In skeletal muscle, as in the heart, no significant differences were found between the two methods and each phosphorus compound. The P values for ATP, PCr, and P\textsubscript{i} for the two methods are 0.83, 0.93, and 0.97, respectively (Table 1). Free [Mg\textsuperscript{2+}]i was assessed by the relative chemical shift of the αATP to βATP peaks in \textsuperscript{31}P NMR spectra and found to be 0.46 ± 0.04 mM (average chemical shift of 8.64 ± 0.03) in heart and 0.57 ± 0.04 mM (average chemical shift of 8.58 ± 0.01) in skeletal muscle. pH\textsubscript{i}, determined from the chemical shift between P\textsubscript{i} and PCr, was 7.32 ± 0.04 (average chemical shift of 5.17 ± 0.04) in heart and 7.18 ± 0.02 (average chemical shift of 5.03 ± 0.02) in skeletal muscle.

Table 1. \textsuperscript{31}P NMR-determined ATP, PCr, and P\textsubscript{i} in rat heart and gastrocnemius muscle in vivo derived from an external PPA standard and an internal ATP standard measured enzymatically on tissue extracts from the same hearts

<table>
<thead>
<tr>
<th>Concentration From External Standard, μmol/g wet wt</th>
<th>Concentration From Internal Standard, μmol/g wet wt</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>4.48 ± 0.33</td>
<td>4.78 ± 0.19†</td>
</tr>
<tr>
<td>PCr</td>
<td>9.21 ± 0.65</td>
<td>9.82 ± 0.18†</td>
</tr>
<tr>
<td>P\textsubscript{i}</td>
<td>2.25 ± 0.16</td>
<td>2.51 ± 0.33†</td>
</tr>
<tr>
<td><strong>Muscle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>6.09 ± 0.19</td>
<td>6.03 ± 0.19†</td>
</tr>
<tr>
<td>PCr</td>
<td>23.44 ± 0.88</td>
<td>23.30 ± 1.30†</td>
</tr>
<tr>
<td>P\textsubscript{i}</td>
<td>1.81 ± 0.18</td>
<td>1.82 ± 0.19†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7 animals for heart studies and 6 animals for muscle studies. Free intracellular Mg\textsuperscript{2+} concentration was calculated from the chemical shift of αATP relative to βATP using NMR and was 0.46 ± 0.04 mM for the heart and 0.57 ± 0.02 mM for muscle. Intracellular pH was calculated from the NMR chemical shift of P\textsubscript{i}, relative to phosphatidylcholine (PCr) and was 7.32 ± 0.04 for the heart and 7.18 ± 0.02 for muscle. PPA, phenylphosphonic acid. *ATP content measured enzymatically (see text for equations used to calculate PCr and P\textsubscript{i}). P values reflect differences between the two methods. †Not significant between the two methods for each phosphorus metabolite using a Student’s t-test.
DISCUSSION

Our study shows that $^{31}$P NMR determination of absolute concentrations of ATP, PCr, and Pi in heart and skeletal muscle can be accurately measured using either external references or an internal ATP standard. No significant differences were found between the two methods in determining each phosphorus compound in heart or skeletal muscle. The higher $P$ values in skeletal muscle demonstrate a tighter agreement (100–101% difference in means of metabolite concentrations between the two methods) compared with heart (89–94%).

External and Internal Standardization of NMR Spectra

The major assumptions of the external PPA method are outlined in EXPERIMENTAL PROCEDURES. The method is only valid to the extent that the sensitivity profile and homogeneity of the PPA reference is identical (or similar) to heart tissue. The excellent agreement between the two methods suggests that both of these assumptions were adequately satisfied. The major advantage of the external PPA method is that continuous spectra can be acquired over the time course of the experiment and precludes use of more invasive techniques to estimate internal concentrations of ATP.

On the other hand, the major advantage of the internal ATP enzymatic method is that the molar sensitivities and coil-sensitive volumes are identical. However, two potential sources of error require comment. First, it is known that a small fraction of the total tissue ATP resides in the mitochondria (5 and 10% in muscle and the heart, respectively), and this would lead to higher levels of ATP, PCr, and Pi than reported in Table 1. Second, the internal method assumes that 100% of enzymatically measured ATP is equal to the total nucleoside triphosphate (NTP) pool under the ATP peak. We (8) have shown previously that ATP represents ~93% of the total NTP (ATP + UTP + CTP + ITP + GTP) in the rat heart. The net effect of underestimation of ATP from mitochondrial sequestration and overestimation from using the total NTP area appears to be of similar magnitude in opposing directions. Therefore, we conclude that equating tissue measurements of ATP with the area under the ATP peak is valid for heart and skeletal muscle given the excellent agreement between both methods.

Differences Between Heart and Skeletal Muscle

The lower $P$ values found in heart compared with skeletal muscle (Table 1) may relate to the assumption that the phosphorus compounds are not homogenous in the myocardium (see EXPERIMENTAL PROCEDURES). The ventricular chamber is one obvious difference between heart and skeletal muscle, which may have led to the lower ATP and PCr measurements found in the heart but not in skeletal muscle using the PPA method. A second reason for the lower $P$ values in the heart may be lower signal-to-noise ratios from movement itself, the rat heart beats at 350 beats/min. Another small but significant factor in the heart may also be contaminating phosphorus compounds from ventricular blood. The possibility is supported in part by the high Pi value (2.25 ± 0.16 μmol/g wet wt from the external standard) compared with literature values of ~1 mM (18). The higher values in our study are probably due to high concentration of 2,3-diphosphoglycerate (~4 mM) in rat blood (3). With the use of a heart wall thickness of 4 mm and the data in Fig. 2, a chamber blood contamination of ~10% would not be out of the question. With a 10% chamber blood space and a 10% tissue whole blood space (8), the Pi would decrease by ~50%, to a value of ~1.3 μmol/g. The Pi in rat gastrocnemius skeletal muscle (1.81 μmol/g wet wt or 2.85 mM intracellular water; Table 1) agrees well with the NMR published values of 1.9 μmol/g wet wt of Madhu et al. (22), 2.7 mM of Shoubridge et al. (25), and 4.9 mM of Mizuno et al. (24).

NMR Visibility of ATP

Over the past few decades, there has been much controversy as to whether ATP is 100% $^{31}$P NMR visible in the heart. Within the errors of the methods, we conclude that ATP is entirely visible in both normoxic rat heart and skeletal muscle. “Invisibility” is a complex term and generally refers to extreme line broadening due to the relative immobility of phosphorus nuclei from macromolecular binding, containment of nuclei within highly viscous compartments, or association with paramagnetic ions (16).

Our data agree with most other findings in the perfused rat heart (1, 7, 10, 13, 30) but are in contrast to those studies that have identified an invisible fraction of ATP ranging from 30 to 40% of total ATP (26, 30). Differences of this magnitude are difficult to reconcile given all the checks and balances required to quantify the concentrations as outlined earlier. ATP invisibility in the heart, but not skeletal muscle (4, 23), may also relate to differences in lower signal-to-noise ratios and incomplete myocardial occupancy of the coil sensitive volume through cardiac motion and/or chamber contamination. As discussed, these factors can introduce errors that significantly underestimate the ATP concentration.

While our study indicates that there is little or no NMR invisibility of ATP in rat heart and skeletal muscle, it does not exclude the possibility of intracellular compartmentalization within the NMR “visible” volume. The possibility of microcompartmentalization has been explored within the context of high-energy phosphate shuttling between the sites of ATP production and utilization (15, 17).

In conclusion, the present study demonstrates the use of a symmetrical external standard configuration positioned above the coil, which can provide accurate concentrations of ATP, PCr, and Pi in both heart and skeletal muscle in vivo. The method is valid to the extent that the sensitivity profile and homogeneity of the reference is identical (or similar) to the tissue. Our
estimates of ATP, PCr, and P_i were not significantly different from the internal method of employing ATP measured on tissue extracts prepared from the same tissue. The main disadvantage of using enzymatic ATP as an internal standard is that larger numbers of animals are required at different times during an experiment compared with the external method. Our study further concludes that PCr, ATP, and P_i are nearly 100% visible in the normoxic heart and non-working skeletal muscle given the errors of measurement.

This study was supported by National Heart Foundation Grant G00B 0547 (to G. P. Dobson).

REFERENCES