# NMR Observability of ATP: Preferential Depletion of Cytosolic ATP during Ischemia in Perfused Rat Liver

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ABSTRACT: The extent to which cellular metabolites are NMR observable is of fundamental importance in the interpretation of in vivo NMR studies. Analysis of ischemic rat liver shows that ATP resonances measured by $^{31}P$ NMR decrease considerably faster than total tissue ATP measured in extracts. This discrepancy demonstrates that, in liver, ATP is not 100% observable. Furthermore, the data are consistent with the supposition that in situ mitochondrial ATP resonances are not normally observable by in vivo NMR techniques. The specificity of the NMR measurement for cytosolic ATP indicates that $^{31}P$ NMR can be a valuable tool for the specific measurement of ATP in this compartment.

Although the application of phosphorus NMR to problems ranging from monitoring organ function (Ackerman et al., 1984; Balaban et al., 1986; Schmidt et al., 1986) to clinical diagnosis (Radda, 1986) and to the development of cell injury (Murphy & London, 1988) has proliferated rapidly in recent years, the fundamental question of whether all cell ATP is NMR observable has remained largely unanswered. ATP has generally been assumed to be 100% NMR observable, in contrast to the situation for ADP and $P_i$ (Iles et al., 1985; Stubbs et al., 1984; Cohen, 1983; Desmoulin et al., 1987). Intracellular metabolites may be unobservable by NMR techniques as a consequence of binding to macromolecular structures, localization to environments with a high viscosity, or association with paramagnetic ions. Probably the most well-characterized example of an "NMR invisible" metabolite arises in the case of ADP that, in nearly all cell types examined, is virtually undetectable by in vivo $^{31}P$ NMR but is present at relatively high levels in cell extracts. In muscle, this discrepancy has frequently been attributed to the binding of ADP to the protein actin, with the resulting $^{31}P$ resonances rendered too broad to permit detection. As discussed by Iles et al. (1985), however, this interpretation is not adequate for liver, in which the actin concentration is insufficient to account for the NMR invisible ADP pool. In this case, it has been suggested that the additional NMR invisible ADP may correspond to the mitochondrial pool (Iles et al., 1985; Stubbs et al., 1984; Desmoulin et al., 1987). Mitochondrial ADP could be NMR invisible due to high viscosity or the presence of paramagnetic ions or both (Balaban, 1984). Phosphorus NMR studies of isolated mitochondria have produced conflicting conclusions on the NMR visibility of mitochondrial high-energy phosphates (Wong, 1980; Ogawa et al., 1978). Optimal NMR observability of mitochondrial ATP has been achieved (Ogawa et al., 1978) when ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (EGTA) is present during the isolation of the mitochondria, as well as during accumulation of the NMR spectra, and this may be related to chelation of paramagnetic ions.

Two important questions will be addressed in this paper. First, is ATP in liver 100% NMR observable? This question is significant since ATP is frequently used as a concentration standard to which other phosphate-containing metabolites are referenced (Iles et al., 1985). Second, if ATP is not 100% observable, is NMR observability related to cellular compartmentation of the ATP pool? An experimental approach to these questions was suggested by the experiments of Aw et al. (1987), who reported that during anoxia there is a preferential decrease in cytosolic ATP, with mitochondrial ATP decreasing only slightly. The relatively high ratio of mitochondrial/cytosolic ATP, which is achievable during ischemia or anoxia, could provide an ideal situation for determination of the observability of mitochondrial ATP in the intact cell. Thus, the ATP resonances of the ischemic liver should exhibit a more precipitous decrease than the ATP concentration determined in cell extracts from freeze-clamped livers.

## Experimental Procedures

*Liver Perfusion.* Male Sprague-Dawley rats weighing 175–225 g were anesthetized with 50 $\mu$g of nembutal per gram of weight. The hepatic portal vein was cannulated, and perfusion was begun with Krebs–Ringer bicarbonate buffer. The superior vena cava was cannulated for the outflow of the perfusate. The buffer was circulated through an "oxygenator" that maintained the temperature at 37 °C and kept the buffer equilibrated with 95%/5% $O_2/CO_2$. The buffer flows immediately from the oxygenator into the hepatic portal vein.

*NMR Measurements.* $^{31}P$ NMR spectra were recorded by using a home-built 3.0 cm diameter probe operating at 146 MHz in a Nicolet NT-360 NMR spectrometer. The magnetic field was shimmed on $H_2O$ in the liver, and we routinely obtained nonspinning line widths at half-height of 0.3–0.5 ppm. We used a 40-$\mu$s pulse length, a 1-s delay, a 5K spectral width, and 4K data points.

*Analytical Methods.* The liver was freeze-clamped with liquid nitrogen cooled aluminum tongs. A sample was weighed and homogenized with a mortor and pestle in 8% perchloric acid (PCA) in the presence of dry ice. The homogenate was centrifuged at 39200*g* at 4 °C for 15 min, and the supernatant was then removed and neutralized with potassium carbonate. Removal of the extract from the precipitated protein prior to neutralization assures that we do not have adenylate kinase contamination in our neutralized extracts (Williamson & Corkey, 1969). ATP was assayed spectrophotometrically via NADPH-linked reactions.

## Results

Figure 1 shows $^{31}P$ NMR spectra of a perfused rat liver. A preischemic spectrum (a) is shown in Figure 1, and successive 4-min spectra of the liver, made ischemic by turning

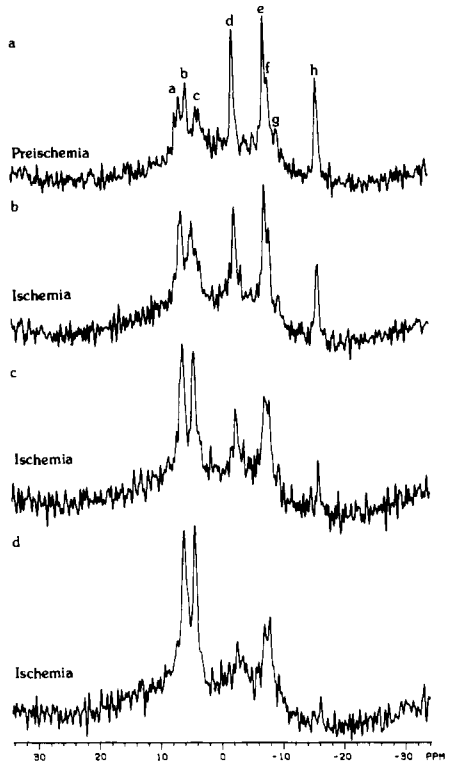


FIGURE 1: Phosphorus NMR spectra of ischemic rat liver. Spectrum a was accumulated prior to ischemia, and spectra b–d were acquired during ischemia. Each spectrum took 4 min. Ischemia lasted a total of 12 min. Resonance identification is as follows: a = phosphate monoesters; b = inorganic phosphate (intra- and extracellular); c = glycerophosphocholine and glycerophosphoethanolamine; d = $\gamma$-phosphate of ATP; e = $\alpha$-phosphate of ATP; f = NAD(H) and NADP(H); g = UPD sugars; h = $\beta$-phosphate of ATP.

Table I: Hepatic ATP Levels during Ischemia

| condition | ATP[a] ($\mu$mol/g wet wt) | % control |
|---|---|---|
| control | 2.60 ± 0.15 ($n$ = 3) | |
| ischemia (12 min) | 0.79 ± 0.13 ($n$ = 3) | 30.4 |

[a] Values are mean ± SEM; $n$ = number of experiments. The (blotted) wet weight/dry weight, which did not change between control and ischemic livers, averaged 3.2 ± 0.1. ATP was measured as described under Experimental Procedures. The control ATP was obtained from nonischemic livers, frozen within 5 min after perfusion was started.

off the perfusion pump, were acquired over the next 12 min (b–d). By the 8–12-min accumulation period (d), in three livers the ATP level, as judged by the change in intensity of the $\beta$-phosphate resonance, is only 13.5 ± 2.5% of its control value. At the end of the accumulation of spectrum d, the liver was removed from the magnet and rapidly frozen. The results of enzymatic assay of ATP in an extract from this liver are summarized in Table I. Enzymatic assay of ATP in three livers, ischemic for 12 min, yielded a value 30.4 ± 4.8% of control levels, a value statistically different ($p < 0.05$) than that measured by NMR (13.5%). It is noted that all potential errors in this study would further increase the discrepancy between the NMR and extract data. The NMR spectra were acquired over the period from 8 to 12 min of ischemia, while the extracts were prepared after 12 min. Additionally, any hydrolysis that occurred during extraction would further decrease the ATP level in the extracts.

The discrepancy in ATP levels between the NMR measurements and the enzymatic assays of PCA extracts of freeze-clamped livers suggests that during ischemia as much as 60–70% of liver ATP is not NMR observable. Interestingly, under similar conditions of anoxia, mitochondrial ATP has been shown by Aw et al. (1987) to comprise 60–70% of the cell ATP. Aw et al. (1987) reported that, after 30 min of anoxia, total ATP had decreased by 84%, with cytosolic and mitochondrial pools reduced by 92% and 43%, respectively. Assuming that the $^{31}$P NMR observation is sensitive to the cytosolic pool, whereas the extraction procedure measures the total ATP pool, the present study shows that, after 12 min of ischemia, total and cytosolic ATP decreases by 70% and 87%, respectively.

Since the conclusions of this study depend heavily upon the studies of Aw et al. (1987), we undertook a study to confirm their results. A suspension of hepatocytes was subjected to 20 min of hypoxia; cells were then fractionated with digitonin (Zuurendonk et al., 1979) and spun through oil to obtain mitochondrial ATP. Unfractionated cells were also spun through oil to give total cell ATP; the cytosolic ATP level was then determined by subtraction. Enzymatic assay of ATP levels in these extracts indicated that after 20 min of hypoxia there was a drop in total cell ATP to 20–25% of control level, but 66% of the ATP was in the mitochondria, compared with 33% in the cytosol. In agreement with the results of Aw et al. (1987), these data demonstrate a greater than 90% fall in cytosolic ATP levels, with only a 35–40% drop in mitochondrial ATP.

## Discussion

Earlier attempts to determine whether in situ $^{31}$P NMR studies report total cellular ATP content have been limited by two factors: (1) the relatively small size of the mitochondrial ATP pool under control, aerobic conditions and (2) the difficulty of determining absolute metabolite concentrations in complex samples. Several groups using rapid fractionation studies have shown that, under control aerobic conditions, only 15–20% of the total liver cell ATP is localized in the mitochondria (Aw et al., 1987; Zuurendonk et al., 1979; Tischler et al., 1977; Siess & Wieland, 1976). Further, although NMR techniques have been demonstrated to provide accurate measurements of relative metabolite levels, as well as time-dependent changes in metabolite levels, the determination of absolute concentrations in tissues or perfused organs represents an extremely difficult problem. In general, the determination of absolute molecular concentrations by NMR spectroscopy requires the addition of an internal standard. However, in a perfused organ study it is not feasible to utilize an intracellular internal standard. Several approaches to this limitation have been proposed in the literature (Iles et al., 1982). For example, Wray and Tofts (1986) propose the utilization of the proton resonance of water as an internal standard. Calibration is achieved by carrying out a measurement on a standard with a known level of the metabolite in question and directly comparing the $^{1}$H and $^{31}$P resonance intensities. However, the existence of a significant pool of extracellular water clearly limits the accuracy of such an approach. Further, radio-frequency attenuation in the standard may differ from that in the sample, particularly for the case of a perfused organ.

As demonstrated here, studies of the response of hepatic ATP to anoxia or ischemia provide a solution to both of the limitations noted above. As a consequence of the differential depletion of cytosolic and mitochondrial pools, the fractional ATP content of the mitochondria increases dramatically. Further, the evaluation is based on observing changes in the metabolite levels, rather than relying on standards, so that the central problem of making absolute intensity determinations is obviated. The discrepancy between the in situ NMR determinations and the extract determinations is attributable to the invisibility of the mitochondrial ATP pool. The latter identification is supported by the analysis on isolated mito-

chondria carried out here and in studies reported by Aw et al. (1987). These results are consistent with conclusions regarding the invisibility of mitochondrial ADP resonances, and it is likely that both effects reflect the same combination of physical factors, e.g., relatively high levels of paramagnetic ions or high microviscosity. In this context, it is of interest to note that previous $^{13}C$ NMR investigations of yeast cells grown on media containing $^{13}C$-labeled nicotinate suggest an analogous invisibility of mitochondrial pyridine nucleotide resonances (Unkefer & London, 1984).

The compartmentation of cellular metabolites is a central aspect of cellular metabolism and a problem of long standing in the effort to utilize data obtained from cell extracts for the interpretation of metabolic regulation. The data in this study indicate that, in liver, ATP is not 100% NMR observable; only the cytosolic ATP appears to be NMR observable. This may have significant advantages for studies of metabolism, since it is cytosolic ATP that is important in regulating cytosolic enzymes and transporters; $^{31}P$ NMR measurements can provide a powerful tool for the selective measurement of this pool.

There has been considerable controversy concerning whether the fall in ATP concentration during ischemia is equal in all cellular compartments (Gudbjainason et al., 1970; Kubler & Katz, 1977; Farber et al., 1981). The data in this paper, in agreement with the report of Aw et al. (1987), show that cytosolic ATP falls preferentially during ischemia and anoxia. This preferential decrease in cytosolic ATP would explain observations such as the finding that, under conditions of ATP depletion (Soltoff & Mandel, 1984; Rau et al., 1977), Na rises and K falls as if the Na–K-ATPase were inhibited, despite the fact that enzymatic assay of ATP in extracts yields ATP values sufficient to maintain Na–K-ATPase activity, given the low $K_M$ for ATP of the enzyme. Thus, $^{31}P$ NMR measurements provide a powerful tool for analysis of cytosolic ATP and the effect of changes in cytosolic ATP on the regulation of cytosolic enzymes. There is little basis to anticipate that the present results are restricted to liver, and preliminary studies of other organs suggest similar NMR invisibility of the mitochondrial pool and a similar difference in the response to ischemia between the mitochondrial and cytosolic pools.

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