The ^{31}P NMR Visibility of ATP in Perfused Rat Liver Remains about 90%, Unaffected by Changes of Metabolic State†

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ABSTRACT: The ^{31}P NMR visibility of ATP of the perfused rat liver was tested over a wide range of metabolic conditions, including normoxic and hypoxic perfusions, fructose loads, and various intervals of normothermic ischemia, for both ad libitum fed and 24-h fasted rats. The ^{31}P NMR signal of ATP was compared to the concentration of ATP determined by enzymatic assays on liver biopsies performed at the end of NMR acquisition. In a first series of experiments, the NMR resonance of intracellular ATP was quantitated in absolute terms by applying the ^{1}H NMR water signal as internal reference: during normoxic and hypoxic perfusions, a constant amount of ATP (0.43 ± 0.19 mM, mean ± SD), approximately 12% of the cellular ATP, is not detected by NMR. Nevertheless, there is a high correlation (slope = 0.96 ± 0.09; $r^2 = 0.93$) between the measurements of ATP by ^{31}P NMR spectroscopy and by biochemical analysis. In a second series of experiments, there was a highly significant correlation between the NMR and analytical biochemical measurements of ATP for whole range of metabolic states, i.e., fructose loads (1.0–10 mM) and various intervals of normothermic ischemia (ranging from 2 to 12 min), indicating unchanged ATP visibility. Thus, as opposed to the studies of Murphy et al. [Murphy, E., et al. (1988) *Biochemistry 27,* 526–528], it is concluded that ATP at 37 °C remains almost entirely visible in the perfused rat liver, also during ischemia.

Although ^{31}P NMR spectroscopy is now widely used for experimental studies (Jaroszewski et al., 1988; Lundberg et al., 1990) as well as clinical diagnosis (Cohen, 1987), the NMR visibility of phosphorylated metabolites (mainly ATP, ADP, and P_i) has not been unequivocally defined. These metabolites might be NMR invisible when located in intracellular environments with high viscosity or in the presence of a high concentration of paramagnetic ions or when tightly bound to macromolecules or cellular structures. It is possible that NMR visibility in vivo of a given compound not only will differ between tissues (Stubbs et al., 1984; Zahler et al., 1987; Painter et al., 1989; Unitt et al., 1992) but also may depend on the metabolic state, like during ischemic stress (Takami et al., 1988; Jeffrey et al., 1989; Humphrey & Garlick, 1991) or substrate deprivation (Pianet et al., 1991).

In the present study, we have focused on the NMR visibility of ATP in perfused rat liver. The assessment of ATP visibility is important as this compound is often taken as an internal concentration reference in many experimental and clinical reports, where relative metabolic alterations are expressed as changes in the ratio of intensity or area of the resonances of ATP and other relevant metabolites. Since there are conflicting reports on ATP visibility, this procedure might be misleading. Indeed, only some 45% of ATP was reported NMR observable in perfused rat liver submitted to brief intervals of ischemia, and it was suggested that the NMR-invisible ATP was mitochondrial ATP (Murphy et al., 1988). Likewise, a constant fraction of approximately 16% of cellular ATP remains invisible by ^{31}P NMR spectroscopy in freeze-trapped livers, either during normoxia or during a short ischemic period (Ikai et al., 1991). In human erythrocytes, Petersen et al. (1989) found 80–90% of ATP visibility. On the other hand, ATP has been reported fully visible in the rat liver during a normoxic perfusion (Cohen, 1983; Desmoulin et al., 1987), during a 4-h period of cold ischemia (Gallis et al., 1991), or in situ (Iles et al., 1985). Moreover, using isolated liver mitochondria, Hutson et al. (1989) showed full visibility of matrix ATP over a wide range of intra- and extramitochondrial nucleotide concentrations.

The rationale of this study was to compare quantification of ATP performed by ^{31}P NMR spectroscopy and biochemical analysis (on the same organ) over a wide range of metabolic conditions by subjecting livers from fed or fasted rats to hypoxia, varying loads of fructose, or periods of ischaemia. We have shown a highly significant linear relationship, with a slope not significantly different from 1, between the concentration of ATP measured by biochemical analysis and by NMR, during hypoxia. Nevertheless, a constant amount, equivalent to ca. 12% of cellular ATP in normoxia, was not NMR visible. Finally, the amount of invisible ATP remained unchanged during fructose loads or intervals of ischemia.

EXPERIMENTAL PROCEDURES

Animals. Male Wistar rats (250–300 g) fed ad libitum or fasted for 24 h were used. The animals had free access to tap water. Anesthesia was induced by an intraperitoneal injection of sodium pentobarbital (50 mg/kg).

Liver Perfusion. After a median laparotomy, two catheters were positioned in the portal vein and superior caval vein. The inferior caval vein was then ligated and the liver removed from the animal to a perfusion chamber (Figure 1). Krebs–Henseleit buffer (Krebs & Henseleit, 1932) was perfused in an anterograde direction in a nonrecirculating setup at a constant rate of 35 mL/min, in some experiments supplemented with 15 mM phosphocreatine. Oxygenation of the perfusate was achieved with a Silastic tubing gas exchanger, at 37 °C (Hamilton et al., 1974). A suction line fitted in the bottom of the probe containing the liver prevented accumu-

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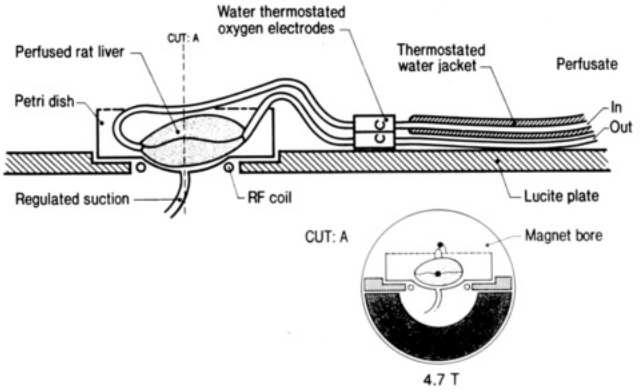

FIGURE 1: Setup of the rat liver perfusion in the horizontal magnet for NMR experiments.

lation of perfusate leaking from the liver. Hepatic oxygen consumption was measured with two flow-through oxygen electrodes (Yellow Spring Instrument Co.) placed upstream and downstream of the liver (Figure 1). Calculation of the consumption was based on the difference between the concentration of dissolved O_2 in the perfusate entering and leaving the organ, the flow rate, and the rat weight, assuming a 4% (w/w) liver weight (Sies, 1978).

NMR Experiments. Experiments were carried out on an Otsuka Vivospec spectrometer interfaced to a Magnex 4.7-T superconducting horizontal magnet (bore = 31 cm). ^{31}P NMR spectra were acquired at a frequency of 81.06 MHz, without proton decoupling. Perfused livers were positioned over a one-turn double-tuned (^{1}H/^{31}P) inductively driven surface coil of 35-mm diameter (see Figure 1). The magnetic field was shimmed on the water signal, and acquisition parameters were optimized on the β-NTP signal of liver. The pulse length was 50 μs, the interpulse delay 0.75 s, and the spectral width 10 kHz. Blocks of 160 scans were accumulated in 2K data points every 2 min. Saturation factors of the phosphorus metabolite signals were computed as the ratio of the area of signals acquired under the conditions given above and the area measured under fully-relaxed conditions, with a repetition time of either 7 s (experiments without PCr) or 25 s (experiments with added PCr).

Determination of the absolute ATP concentration from the NMR measurements was performed according to procedures derived from Wray and Tofts (1986) and De Bisschop et al. (1991), using the ^{1}H NMR signal from intraorgan water as a concentration reference. ^{31}P NMR spectra consisted in the accumulation of a block of 80 scans (4K data points) acquired with a pulse length corresponding to a 90° nutation angle at the center of the coil, and a repetition time of 7 s. Fully-relaxed ^{1}H NMR spectra were recorded before and after ^{31}P spectra acquisition and consisted of four scans acquired with a pulse corresponding to a 90° nutation angle at the center of the coil and a repetition time of 25 s. Experiments run daily, with a calibration solution consisting of 50 mM KH_2PO_4 in 150 mM NaCl, allowed measurement of the proportion between the concentration ratio and the NMR signal ratio for water and inorganic phosphate. The influence of saline concentrations on dielectric losses has been checked with concentrations ranging from 100 to 200 mM NaCl in the calibration solution (50 mM KH_2PO_4) and found insignificant, as previously reported by De Bisschop et al. (1991). The assumption of a constant content of intraorgan water during the course of perfusion experiments was validated by the acquisition of identical ^{1}H NMR signals before and after ^{31}P NMR acquisition (variation = $-1.08 \pm 3.13\%$, $n = 8$). A water content amounting to 76% w/w of the perfused liver was used for calculations. The areas from all the resonances were estimated by the cut-and-weigh procedure. In the calculation of the ATP concentration, it was assumed that ATP formed a constant 83% of the β-NTP signal (Floridi et al., 1977; Palombo et al., 1988). All NMR data were corrected for saturation by multiplication with the appropriate saturation factor as described above.

Biochemical Analyses. At the end of NMR experiments, the probe with perfused liver was withdrawn from the magnet, and the left lateral lobe of the liver was rapidly excised, blotted on filter paper, and freeze-clamped between aluminum tongs precooled at liquid nitrogen temperature (Quistorff & Poulsen, 1980). The time from circulation interruption to freeze-clamping amounted to less than 5 s. The time between the end of NMR acquisition and freeze-clamping was routinely $\approx$45 s. The frozen liver biopsies were then stored at –80 °C. Hepatic metabolites were extracted with 2 M perchloric acid and the extracts neutralized with 4 M KOH/0.2 M triethanolamine. Standard enzymatic assays were performed to measure the hepatic concentrations of ATP and PCr (Lowry & Passonneau, 1972) and ADP and AMP (Jaworek et al., 1970). The specific activity of yeast hexokinase, the enzyme used for ATP assay, is much higher for ATP than for other nucleoside 5′-triphosphates, amounting to only 0.75% and 3.75% of its activity toward ATP, for GTP and UTP, respectively (Darrow & Colowick, 1962). Inorganic phosphate was assayed by the method of Penney (1976).

Calculations and Statistics. The kinetics of ATP concentration followed by ^{31}P NMR spectroscopy during ischemia are displayed with a time scale accounting for the nonlinear decrease of this nucleotide. The time points have therefore been corrected according to an assumed first-order decay of ATP as fitted from the time course observed during biochemical analyses. Data are presented as means ± SD. Unpaired Student's t test was used for statistical analyses between groups of data. A p value less than 0.05 was considered significant. The linear regression procedures were performed with the C-Stat software package (Cherwell Scientific Publishing, Oxford, U.K.); slopes are expressed with a 95% confidence interval.

RESULTS

Hepatic ATP Visibility during Hypoxia. Figure 2 displays typical ^{31}P NMR spectra of the isolated liver from fed (Figure 2a) and 24-h fasted rats (Figure 2b). Peak assignments are reported in the figure legend, according to Desmoulin et al. (1987) and Quistorff et al. (1988). A characteristic difference between the fed and the fasted liver is the higher ATP/P_i concentration ratio of the former. The signal to noise ratio for the β-NTP resonance during the present conditions of 2-min data acquisition is of the order of 25 in the fed and 15 in the fasted state. Consequently, the detection limit for this peak is reached when the concentration has decreased 6–7-fold, corresponding to approximately 0.4 mM.

In a first series of experiments, the hepatic ATP concentration has been decreased by perfusing livers from either fed or 24-h fasted rats with buffer equilibrated with different oxygen tensions, from 95% (normoxia) down to 20% (hypoxia). Alternate ^{1}H and ^{31}P fully-relaxed NMR spectra have been acquired as described under Experimental Procedures, and the absolute concentration of ATP has been derived from the ratio of the areas from the resonances of intraorgan water and β-NTP. Intracellular ATP was then assayed on perchloric acid extract from livers freeze-clamped at the end

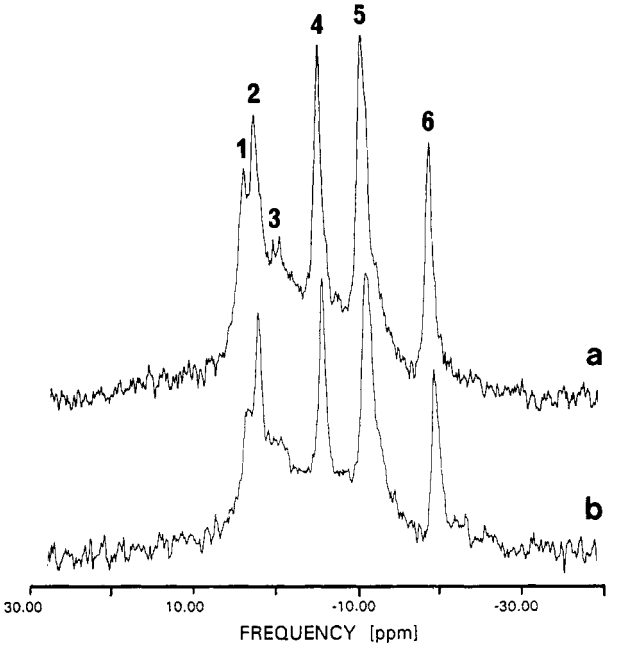

FIGURE 2: ^{31}P NMR spectra from perfused rat livers. The livers from rats fed ad libitum (a) or 24-h fasted (b) were perfused with a Krebs–Henseleit buffer for 30 min. Spectra were then recorded at 81.06 MHz without proton decoupling and with the following parameters: angle of nutation at the center of the coil, 55°; spectral width, 10 kHz; 2K data points; interpulse delay, 0.75 s; 160 scans (total time of acquisition, 2 min). They were processed with an exponential filter corresponding to a 15-Hz line broadening. Assignments: 1 = phosphomonoesters (mainly adenosine 5′-monophosphate and phosphorylcholine); 2 = inorganic phosphate (P_i); 3 = phosphodiesters (glycerophosphorylethanolamine and glycerophosphorylcholine); 4 = γ-phosphorus of NTP and β-phosphorus of NDP; 5 = α-phosphorus of NTP and NDP, diphosphodiesters (mainly NAD^+ and uridine 5′-diphosphoglucose); 6 = β-phosphorus of NTP.

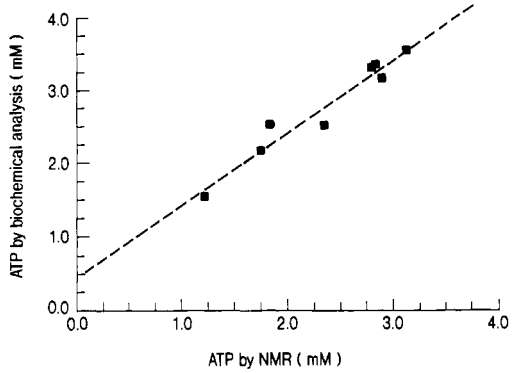

FIGURE 3: Relationship between the hepatic concentration of ATP determined by biochemical assay and by ^{31}P and ^1H NMR spectroscopy. Livers from fed or 24-h fasted rats were perfused with a Krebs–Henseleit buffer and submitted to various levels of hypoxia. Hepatic ATP concentrations obtained from biochemical assays (ordinate) are plotted vs absolute hepatic ATP concentrations determined by ^{31}P and ^1H NMR spectroscopy (abscissa). The water content of the perfused rat liver has been assumed to be 76% of the liver weight and ATP to make up 83% of total NTP. The regression line follows the equation $y = 0.96x + 0.43$, with a coefficient of correlation $r^2 = 0.93$.

of NMR acquisition. The relationship between the concentration of ATP determined by NMR spectroscopy and by biochemical assays on the same liver is presented in Figure 3. There was a high correlation between both measurements during normoxia and hypoxia. Indeed, linear regression analysis of the data followed the equation: $[ATP]_{Biochemistry} = (0.96 \pm 0.09)[ATP]_{NMR} + 0.43$ ($r^2 = 0.93$). This strongly suggests that ATP remained visible by ^{31}P NMR spectroscopy during hypoxia in the perfused rat liver, except for a constant

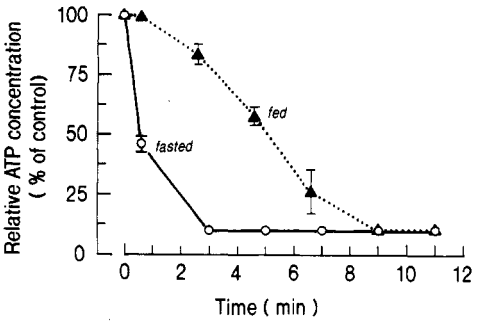

FIGURE 4: Hepatic ATP content during normothermic ischemia. Livers from fed (▲) or 24-h fasted (○) rats were perfused for 30 min with a Krebs–Henseleit buffer. The perfusion was then stopped at the zero time point and the organ superfused with a warm P_i-free Krebs–Henseleit buffer to maintain a temperature of 37 °C. Kinetics of ATP content were recorded by ^{31}P NMR spectroscopy every 2 min. Data are calculated as a percentage of the preischemic level and presented as the mean ±SD for a minimum of three experiments for each data point.

amount of 0.43 ± 0.19 mM ATP (ca. 12% of normoxic cellular ATP in fed liver).

As an alternative to the use of the water ^1H NMR signal for absolute quantification, an external (vascular) standard compound may be selected. Phosphocreatine (PCr) fulfills the requirements for a suitable standard. Thus, livers from 24-h fasted or fed rats were perfused with Krebs–Henseleit medium supplemented with 15 mM PCr. The NMR-derived ATP/PCr ratio, suitably corrected for saturation, was expressed as a function of the ATP/PCr ratio determined by biochemical assays on biopsies from the same livers for nine experiments. Linear regression of the two data sets gave a slope very different from unity ($\alpha = 0.31$, data not shown). It was observed that perfusate was unavoidably lost from the excised biopsy prior to freeze-clamping, probably explaining the much higher ATP/PCr ratio in the biopsy. Consequently, this procedure was abandoned as a means of absolute quantification.

Time Course of Changes during Ischemia. In order to assess the NMR visibility of ATP under metabolic conditions where the distribution of ATP between the cytosol and the mitochondria is known to change, two other methods have been used to perturb the hepatic ATP concentration: either various intervals of ischemia representing non-steady-state conditions or different fructose loads creating metabolic steady-state at different ATP concentrations. The time required to perform an absolute measurement of ATP applying the water calibration technique described above was not suitable with the fast kinetics of ATP decrease during ischemia (see below). Therefore, in the following experiments, the area of the resonance arising from β-NTP is expressed in arbitrary units.

Figure 4 displays the time course of ATP concentration during ischemia at 37 °C in the fed and in the fasted state at a time resolution of 2 min as measured by ^{31}P NMR spectroscopy. In these non-steady-state experiments (various intervals of ischemia), it is important to realize that while the biopsy measurements reflect the ATP concentration at the well-defined time of freeze-clamping, the spectroscopic data integrate a 2-min measuring interval. Consequently, the time points representing the NMR data in Figure 4 have been drawn assuming a weighted signal average (e.g., the time point of the 0–2-min spectrum is indicated at time 0.6 min; see Experimental Procedures). Similarly, Table I shows the levels of adenine nucleotides and P_i reached in livers from either fed or 24-h fasted livers after various intervals of normothermic ischemia (2, 4, 6, or 12 min) as measured in the

Table I: Hepatic Adenine Nucleotide and P_i Levels during Normothermic Ischemia[a]

	[ATP] (mM)		[ADP] (mM)		[AMP] (mM)		[P_i] (mM)	
condition	fed	fasted	fed	fasted	fed	fasted	fed	fasted
control	3.57 ± 0.30	2.95 ± 0.30	0.98 ± 0.07	1.35 ± 0.14[b]	0.19 ± 0.05	0.30 ± 0.07	2.85 ± 0.48	4.60 ± 0.94[b]
ischemia (2 min)	3.28 ± 0.61	0.45 ± 0.04[b]	1.64 ± 0.32	1.32 ± 0.07[b]	0.82 ± 0.28	2.17 ± 0.23[b]	4.52 ± 0.71	11.31 ± 0.96[b]
ischemia (4 min)	1.67 ± 0.31	0.38 ± 0.07[b]	1.61 ± 0.14	0.95 ± 0.01[b]	0.67 ± 0.34	2.65 ± 0.34[b]	6.05 ± 0.57	13.05 ± 0.74[b]
ischemia (6 min)	0.97 ± 0.27	0.16 ± 0.04[b]	1.47 ± 0.06	0.59 ± 0.07[b]	1.20 ± 0.21	2.52 ± 0.11[b]	8.19 ± 0.56	13.52 ± 0.14[b]
ischemia (12 min)	0.21 ± 0.04	0.15 ± 0.07	1.01 ± 0.12	0.52 ± 0.03[b]	1.82 ± 0.23	2.29 ± 0.19	11.46 ± 0.88	14.47 ± 0.52[b]

[a] Livers from fed or 24-h fasted rats were perfused outside the NMR magnet for 30 min with Krebs–Henseleit buffer. The perfusion was then stopped at the zero time point and the organ superfused with a warm P_i-free Krebs–Henseleit buffer to maintain a temperature of 37 °C. The livers were submitted to 2-, 4-, 6-, or 12-min normothermic ischemic intervals. ATP, ADP, AMP, and P_i were measured enzymatically as described under Experimental Procedures. Data are presented as means ± SD for three experiments. [b] $p \leq 0.05$ comparing fed vs fasted by means of the unpaired Student's t test.

biopsies. The results from Figure 4 and Table I demonstrate a very distinct difference in the time course of ATP decrease in the fed and fasted animals. Thus, in the fasted state, ATP decreased by more than 85% from a control value of 2.95 ± 0.30 to 0.45 ± 0.04 mM after 2 min of ischemia, while in the fed liver, ATP fell only slightly from 3.57 ± 0.30 to 3.28 ± 0.61 mM, amounting to a decline of less than 10% (Table I). Only after 8–10 min of ischemia did the ATP concentration in the fed livers reach the NMR detection limit (see Figure 4). Since the oxygen consumption in the fed state under these conditions, with endogenous substrates only, is similar to or even larger than that in the fasted state [1.77 ± 0.07 (n = 5) and 1.63 ± 0.08 μmol min^{-1} g^{-1} wet weight (n = 4), respectively], it is clear that glycolysis in the fed state must be supplying a major part of the ATP consumption as was indeed shown by Thurman and Scholz (1977). Thus, the rate of ATP decline in the fasted state is of the order of 2 μmol min^{-1} (g of liver)$^{-1}$ wet weight during the first minute of ischemia (see Figure 4). Assuming a P/O ratio of 2.5 and 30% cyanide-insensitive oxygen consumption (Younes & Strubelt, 1988), the preischemic oxygen consumption, however, indicates an ATPase activity some 2–3-fold higher. Hence, at the time resolution of the present studies, the initial ΔATP appears to underestimate the ATPase activity in the fasted state of the liver prior to ischemia. In the fed state, the situation is complicated by the presence of anaerobic glycolysis, and ΔATP during the initial interval of ischemia is only some 0.3 μmol min^{-1} g^{-1} wet weight. It is clear, therefore, that the value obtained for ΔATP during the initial 1–2 min of ischemia is not a useful measure of the ATP turnover of the organ, in the fed state, while in the fasted state without glycogen improved time resolution in the estimate of the initial ΔATP during ischemia might provide a more realistic measure of the preischemic oxygen consumption of the organ (Lowry et al., 1964), although it should be noted that Ikai et al. (1990) found a similar, surprisingly low ΔATP, even with a much better time resolution of 15–30 s.

Comparison of ATP Visibility during Ischemia and Fructose Loads. The intracellular concentration of ATP has also been manipulated by submitting livers from fed rats to fructose administration at concentrations of 1, 2, 3.5, or 10 mM. After 10–12 min of fructose phosphorylation, a new steady-state ATP concentration was reached for all fructose concentrations. The left lateral lobe of the organ was then rapidly freeze-clamped, and metabolites were extracted to allow comparison between the concentration of ATP and the area of the β-NTP resonance in the last spectrum acquired. The relationship between biochemical and ^{31}P NMR quantification for ATP concentration during fructose administration is presented in Figure 5a. A similar relationship established during intervals of normothermic ischemia is displayed in Figure 5b. The slopes of the lines fitted by

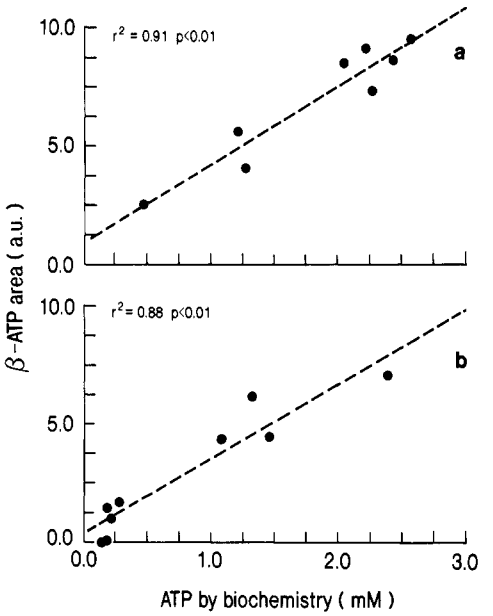

FIGURE 5: Correlation between hepatic ATP content determined by NMR and by biochemical assay during fructose administration or ischemia. Perfused livers were submitted either to fructose administration at various concentrations (a) or to intervals of ischemia (b). ATP content (arbitrary units of area) determined by ^{31}P NMR from the last spectrum before freeze-clamping is plotted vs values of ATP content obtained in freeze-clamped biopsies. The regression lines gave correlation coefficients of r^2 = 0.91 ($p < 0.01$) and r^2 = 0.88 ($p < 0.01$) for the fructose and ischemia experiments, respectively.

regression analysis for the fructose administration and ischemic stress experiments were not significantly different (α = 3.675 ± 0.484 and α = 3.732 ± 0.483 for fructose and ischemia experiments, respectively). Moreover, when all the points from both types of experiments were treated as a single set of data, there was a strong correlation (r^2 = 0.93, $p <$ 0.01), suggesting a similar extent of NMR visibility for ATP during periods of normothermic ischemia or fructose metabolism as was observed in the studies of hypoxia described first.

DISCUSSION

In the present study, the extent of ^{31}P NMR visibility of ATP in perfused rat liver has been shown to remain unchanged and about 90% during various metabolic states such as hypoxia, ischemic stress, or fructose administration, perturbations which cause ATP to vary from the control value of 3.0–3.5 mM to about 0.3 mM.

Literature reports dealing with NMR visibility of phosphorylated metabolites in vivo suggest that there may be a tissue-specific and possibly metabolic state related visibility change of ATP. Particularly, transitions involving brief intervals of ischemia (Murphy et al., 1988; Takani et al., 1988;

Humphrey & Garlick, 1991) or glucose deprivation (Pianet et al., 1991) could decrease NMR visibility of ATP. This effect has been attributed to a redistribution of nucleotides between intracellular compartments, especially from the cytosol to the mitochondria where the matrix is thought to be sufficiently different from the cytosol in terms of concentrations of paramagnetic ions, protein, and water to induce a decreased NMR visibility. Other reports, however, have indicated that ischemia did not change ATP visibility in the perfused rat liver (Desmoulin et al., 1987; Ikai et al., 1991; Gallis et al., 1991) or in the perfused rat heart (Jeffrey et al., 1989). These latter results are consistent with the fact that experiments with isolated mitochondria show 100% visibility of ATP (Hutson et al., 1989), although this view is challenged by some early observations by Ogawa et al. (1978). Morever, it has been recently reported that matrix mitochondrial P_i was mostly visible in isolated rat liver mitochondria under ionic and metabolic concentrations intended to mimic physiological environment, at temperatures ranging from 8 to 25 °C (Hutson et al., 1992).

ATP Visibility for Various Metabolic States. We have submitted perfused rat livers to metabolic stress purporting to induce (i) graded decreases in the cellular amounts of ATP and (ii) redistribution of ATP between the cytosolic and mitochondrial compartments. Indeed, fructose administration leads to a decrease in total hepatic adenine nucleotide concentration (Woods et al., 1970) and a preferential depletion of nonmitochondrial ATP, which makes up only 70% of cellular ATP after incubation of hepatocytes with 12 mM fructose (Grivell et al., 1991). Moreover, during short periods of ischemia, it has been observed that cytosolic ATP represents only 33% (Murphy et al., 1988) to 42% (Aw et al., 1987) of cellular ATP, compared to 84% in normoxia (Soboll et al., 1978; Aw et al., 1987). Despite this wide range of intracellular ATP distribution invoked in the present study by ischemia and fructose loads, we found that ATP remained about 90% visible in the perfused liver (Figure 3). This value is in good agreement with the amount of invisible ATP (ca. 16% of total cellular ATP) reported in freeze-trapped livers using ^{31}P cryo-NMR (Ikai et al., 1991). Moreover, in agreement with others (Desmoulin et al., 1987; Gallis et al., 1991), we have demonstrated that the extent of visible ATP remained unchanged during various metabolic perturbations. On the other hand, Murphy et al. (1988) have reported as much as 56% invisible ATP in perfused rat liver subjected to ischemia, suggesting that mitochondrial ATP was not observable by in vivo NMR spectroscopy. This apparent discrepancy might arise partly from the limited accuracy of the quantitation of the β-NTP resonance in ^{31}P NMR spectra, especially during ischemia with low levels of nucleotides (Blum et al., 1991). In fact, taking the data from Murphy et al. (1988), it would appear from their Figure 1 that the NTP signal after 12 min of ischemia amounts to approximately 25% of the control value, which is about equivalent to the concentration of 0.79 μmol/g wet weight actually measured (Murphy et al., 1988, Table I, p 527). The fact that Murphy et al. found a higher ATP concentration after 12 min of ischemia than in the present study could possibly be ascribed to a lack of control of temperature in their study. We observed that without superfusing the liver with warm perfusate during the ischemic interval, the temperature of the organ decreases quickly to room temperature. During a 12-min experiment, this would cause a much slower ATP hydrolysis. For instance, we have found that omitting the warm superfusion of the organ during 12 min of ischemia led to an NMR-detected ATP level of 30.5 ± 5.6% (n = 3) of the control value in fed rat livers, which is some 3-fold higher that what is found when the temperature is kept at 37 °C. This figure is in good agreement with the value reported by Murphy et al. (1988). Finally, it should be noted that the NMR-invisible ATP (16% of cellular ATP) found by Ikai et al. (1991) also did not change from normoxia to ischemia and since their method of detection involved disruption of cells the intracellular distribution of ATP could not have been the cause of ATP invisibility.

Contribution of Non-Adenine Nucleoside Triphosphates to the β-NTP Signal. The resonance at ca. −18.65 ppm is composed of the signals from the β-phosphorus from several nucleosides 5′-triphosphates, and only some 80–85% is ATP (Floridi et al., 1977; Palombo et al., 1988). In the present study, as well as in several others (Murphy et al., 1988; Ikai et al., 1991; Blum et al., 1991), it is further assumed that the ATP fraction of the NTP signal remains constant during metabolic transitions. This may, however, not necessarily be correct. Thus, Gallis et al. (1991) reported a decrease in the contribution of ATP to NTP from a basal value of 80.3 ± 11.6% to 68.8 ± 13.5% in perfused rat liver after an extensive period of cold ischemia (4 h at 4 °C). GTP makes up 9–12% of total nucleoside 5′-triphosphates in the rat liver (Gallis et al., 1991). In the liver cells, this nucleotide is tightly associated with macromolecules during the processes of tubulin polymerization or the transduction of the hormonal signal by guanine nucleotide binding proteins (Spiegel, 1987), so the free concentration of GTP may be low, although, to our knowledge, not yet defined. Therefore, if cellular GTP is only partly NMR visible, the present data would indicate a higher, perhaps 100% visibility of ATP in the perfused rat liver.

Absolute Quantification of ATP by ^{31}P NMR Spectroscopy. Two different approaches were followed in this study in order to estimate the absolute intracellular ATP concentration. The first involved the addition of PCr to the perfusate as an external concentration reference, as previously applied by Thoma and Ugurbil (1987). However, due to an uncontrollable loss of PCr, leaking out of the vascular system of the liver during the process of freeze-clamping, this method was too inaccurate and was consequently abandoned. The second approach for absolute quantification has been recently implemented for the determination of ATP concentration in rat brain and muscle (Wray & Tofts, 1986; De Bisschop et al., 1991). It requires the acquisition of the ^1H NMR signal from water as an internal standard [assuming full visibility for water inside the liver, as was in fact observed for brain and muscle (Tofts, 1988)] and relies on the use of a double-tuned (^1H and ^{31}P) surface coil together with the same profile for spatial excitation of magnetization of both ^1H and ^{31}P nuclei (Thulborn & Ackerman, 1983; Tofts, 1988). A sample of KH_2PO_4 (50 mM in 150 mM NaCl) has been selected in order to determine the probe-dependent proportion between the concentration ratio and the NMR signal ratio for water and inorganic phosphate. This calibration has been executed before and after the experiments involving liver perfusions to minimize potential spectrometer instabilities (De Bisschop et al., 1991). Within the accuracy achieved in this work, we then conclude that ATP visibility was not affected by changes of metabolic state, remaining about 90%. Moreover, there was a constant amount of ATP not detected by ^{31}P NMR spectroscopy (0.43 ± 0.19 mM).

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Registry No. ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; fructose, 30237-26-4; phosphate, 14265-44-2.