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^{31}P NMR Visibility and Characterization of Rat Liver Mitochondrial Matrix Adenine Nucleotides†

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ABSTRACT: Compartmentation and NMR visibility of mitochondrial adenine nucleotides were quantitated in isolated rat liver mitochondria respiring on succinate and glutamate in vitro at 8 and 25 °C. Intra- and extramitochondrial nucleotides were discriminated by adding the chelator *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA). T_1 values of about 0.2–0.3 s for magnesium-bound matrix nucleotides were determined. Adenine nucleotide T_1 values were influenced by the ionic environment; only magnesium-free ATP T_1's were affected by temperature. Intra- and extramitochondrial adenine nucleotide ratios were varied in ATP-loaded mitochondria with added ATP and phosphate using the mitochondrial inhibitors oligomycin and carboxyatractyloside, and adenine nucleotides were quantitated by using NMR and enzymatic analysis. There was good agreement between matrix ATP concentrations (magnesium-bound ATP) calculated by using NMR and standard biochemical techniques. Although matrix ADP could be detected by NMR, it was difficult to quantitate accurately by NMR. The data indicate that mitochondrial ATP is NMR-visible in isolated mitochondria in vitro.

Discrepancies between estimates of adenine nucleotide metabolites determined by phosphorus-31 nuclear magnetic resonance (^{31}P NMR)[1] spectroscopy and estimates determined by enzymatic analysis have suggested the existence of NMR-invisible pools within the cell (Freeman et al., 1983; Iles et al., 1985; Stubbs et al., 1984; Murphy et al., 1988). The largest inconsistencies have been observed with ADP. In muscle, this has been attributed to the binding of ADP to actin, which results in a broadening of the ADP resonances. On the other hand, in the liver and kidney, which do not contain significant amounts of actin, it has been suggested that it is the mitochondrial pool of ADP which is not observable (Iles et al., 1985; Freeman et al., 1983). Good agreement has been found between ATP measured by ^{31}P NMR in vivo or in perfused livers and ATP determined in acid extracts of liver tissue (Iles et al., 1985; Stubbs et al., 1984; Cunningham et al., 1986; Desmoulin et al., 1987). In fact, the liver β-ATP signal was used as a calibration standard by Iles et al. (1985). However, differences between ATP levels measured by ^{31}P NMR and enzymatic assays of perchloric acid extracts of freeze-clamped livers subjected to ischemia have been reported recently (Murphy et al., 1988). On the basis of published values (Aw et al., 1987) for ATP compartmentation in isolated hepatocytes subjected to hypoxia, Murphy et al. (1988) concluded the differences could be attributed to invisibility of ATP in the mitochondrial pool. NMR-silent ATP has also been reported in the perfused heart (Takami et al., 1988; Neely et al., 1988).

In the original NMR studies of isolated mitochondria, Ogawa et al. (1978), Shen et al. (1980), and Ogawa and Lee (1982) concluded that mitochondrial adenine nucleotides were

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[1] Abbreviations: ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; T_1, spin–lattice relaxation time; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone.

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NMR-visible although there was some suggestion that in anaerobic mitochondria there was NMR-invisible ATP (Ogawa et al., 1978). However, direct comparison of adenine nucleotide concentrations determined by ^{31}P NMR and by enzymatic assay was not made. Whether or not a fraction of the cellular adenine nucleotides is invisible will have a significant impact on the interpretation of metabolite concentrations determined in vivo and in perfused organ systems using ^{31}P NMR. Since it has been suggested that the discrepancies between enzymatic and ^{31}P NMR estimates of ADP and ATP are due to NMR invisibility of the mitochondrial pool (Iles et al., 1985; Freeman et al., 1983; Murphy et al., 1988), in this study matrix and extramitochondrial adenine nucleotide concentrations have been determined by using both ^{31}P NMR and enzymatic analysis in isolated liver mitochondria. Over a wide range of matrix and extramitochondrial adenine nucleotide concentrations, matrix ATP was NMR-visible.

Experimental Procedures

Preparation and Incubation of Mitochondria. Male Sprague-Dawley rats were used. Livers were perfused with ice-cold isolation medium containing the following: 0.225 M mannitol; 0.075 M sucrose; 0.1 mM EDTA; 5 mM Mops, pH 7.0. The liver was removed and homogenized in isolation medium, and mitochondria were prepared by differential centrifugation (Schneider & Hogeboom, 1950). Protein was determined by the biuret reaction in the presence of 0.125% deoxycholate using bovine albumin standards. The respiratory control ratio of each mitochondrial preparation was determined separately in the presence of magnesium by measuring the ratio of the respiratory rate in the presence of ADP (0.5 mM) to the rate measured after the cessation of ADP phosphorylation in medium containing the following: 0.15 M KCl; 50 μM EDTA; 5 mM KH_2PO_4; 5 mM $MgCl_2$; 20 mM Mops, pH 7.2, at 37 °C; 20 mM glutamate; 1 mM malate. No mitochondrial preparation with a ratio less than 6.0 was used.

For NMR measurements, the mitochondria (30 mg/mL) were incubated at 8 °C in the standard medium containing 140 mM KCl, 15 mM succinate, 10 mM glutamate, 20 mM Mops or Pipes, and 1 mM CDTA, pH 6.3–7.0. At 8 °C, oxygenation was maintained by bubbling the sample for 5 min with 100% oxygen which was followed by a 7- or 8-min period without bubbling for obtaining high-resolution NMR signal accumulation. Thus, each bubbling cycle lasted 12–13 min. At 25 °C, 30% fluorocarbons (Fluoronert Electronic liquid FC433M, Cordova, IL) and 3% Pluronic F108 Prill (BASF Corp., Parsippany, NJ) were included in the incubation medium, and oxygenation was maintained by continuous bubbling with oxygen during signal accumulation. The oxygenation sequences were established in pilot experiments where oxygen consumption was measured with a Microelectrodes oxygen meter (Model OM-1) immersed directly in the NMR tube. At the end of each bubbling cycle (8 °C), an aliquot of the mitochondrial suspension was removed and the respiratory control ratio determined. The respiratory control ratio remained above 3 even after four bubbling cycles. After the second and fourth cycles, aliquots were taken for analysis of adenine nucleotides. In the NMR experiments, the mitochondrial sample was taken out of the magnet, and aliquots were removed for measurement of adenine nucleotides.

Analytical Methods. ATP, ADP, and AMP were assayed spectrophotometrically (Williamson & Corkey, 1969) and inorganic phosphate by the method of Baginski et al. (1967). In some experiments, the mitochondria were loaded with ATP as described by Austin and Aprille (1984). This procedure included a wash step which removed ATP that had not been transported into the mitochondria. For determination of the total metabolite concentrations, a 0.5-mL aliquot of the mitochondrial suspension was diluted with 0.5 mL of ice-cold 40 mM EDTA in KCl medium and precipitated with perchloric acid (3.5% final concentration). The supernatant was neutralized with 3 M KOH containing 0.5 M Mops and centrifuged and the supernatant assayed for adenine nucleotides and inorganic phosphate. In preliminary experiments, the perchloric acid pellet was extracted twice with 3.5% perchloric acid and 20 mM EDTA. The three supernatants were then combined and neutralized before assaying for metabolites. Differences between the two procedures were found to be ≤5%, so the extraction was discontinued. For determination of extramitochondrial metabolites, the mitochondrial suspension was first diluted in ice-cold KCl–EDTA medium and then centrifuged immediately at 14000g for 1 min at 4 °C. An aliquot of the supernatant was precipitated with perchloric acid, and the supernatant was neutralized before assay. Matrix nucleotides were calculated from the difference between the total and extramitochondrial values.

NMR Measurements. ^{31}P NMR spectra were acquired at 162.0 MHz using a Bruker AM-400 wide-bore spectrometer equipped with a 20-mm $^{13}C/^{31}P$ double-tuned probe. A 15-mm NMR tube which contained the mitochondrial sample was fitted inside a 20-mm NMR tube containing D_2O. The D_2O was used to maintain a frequency lock. Field homogeneity was optimized on the water proton signal in the sample such that the external inorganic phosphorus line width was 8–15 Hz. ^{31}P NMR spectra were recorded by using 68–70° flip angles. The acquisition time was 840 ms with a sweep width of 12 kHz and 4K data points. One zero fill was used to yield a final number of 8K data points. The pulse flip angle and repetition (interpulse delay) times were selected to optimize the signal-to-noise ratio for matrix ATP. Pulse repetition times and the number of accumulation are shown in the figure legends. A Lorentzian line broadening of 20 Hz was applied prior to Fourier transformation except in Figure 2, where it was 10 Hz.

Spin–lattice relaxation times (T_1) were computed by using two different procedures. For respiring mitochondrial samples, a modified progressive saturation experiment was used. Pairs of signal intensity ratios were obtained for the corresponding repetition times (total time, T, was kept constant), and a 68° flip angle was used. The following equation which was derived from Becker et al. (1979) was used:

$$\left(\frac{S_A}{S_B}\right)_T = \left(\frac{t_B}{t_A}\right)\left[\frac{1 - e^{-t_A/T_1}}{1 - (e^{-t_A/T_1})\cos\theta}\right]\left[\frac{1 - (e^{-t_B/T_1})\cos\theta}{1 - e^{-t_B/T_1}}\right]$$

where S_A, t_A and S_B, t_B are the signal intensities and interpulse delay times for two acquisitions (A and B) with the total time for each experiment kept constant. The reported T_1 represents an average of numerous values calculated for each bubbling cycle and between separate bubbling cycles. With added uncoupler, which collapsed the inorganic phosphate peaks into a single peak, an inversion–recovery sequence (Freeman & Hill, 1969) was also used and analyzed by a three-parameter fit to the equation $S(t) = A + B^{-t/T_1}$ (Levy & Peat, 1975; Kowaleski et al., 1977). The two methods gave comparable T_1 values. Saturation factors were calculated from the T_1 values, and corrected areas were determined. The chemical shift scale is presented relative to phosphocreatine. At the end of each experiment, uncoupler was added to the mitochondrial

Table I: Intra- and Extramitochondrial Adenine Nucleotides Determined by Biochemical Assay[a]

additions	nmol/mg of mitochondrial protein						
	ATP		ADP		AMP		total ATP + ADP + AMP
	matrix	external	matrix	external	matrix	external	
mitochondria							
none	5.66 ± 0.46	2.72 ± 0.39	4.39 ± 0.64	0.46 ± 0.05	1.77 ± 0.26	0.08 ± 0.05	15.1 ± 0.9
+P_i	8.18 ± 0.47	3.97 ± 0.45	2.11 ± 0.24	0.50 ± 0.10	0.57 ± 0.17	0.08 ± 0.05	15.4 ± 0.7
ATP-loaded mitochondria							
+P_i	20.5 ± 1.8	6.6 ± 0.55	2.16 ± 0.13	0.31 ± 0.06	0.24 ± 0.09	ND[b]	29.9 ± 1.9
+P_i + ATP	18.4 ± 1.4	26.6 ± 1.15	1.60 ± 0.30	0.72 ± 0.11	0.41 ± 0.07	ND	47.7 ± 1.9

[a] Mitochondria were incubated under the same conditions as in the NMR experiments. Samples were removed for analysis of adenine nucleotides after two or four bubbling cycles. Additions were 1 mM phosphate (P_i) and 0.5 mM ATP. Means and standard errors of the means are given for three to five separate experiments. [b] ND, not detectable.

Table II: Effect of Mitochondrial Inhibitors on Intra- and Extramitochondrial Adenine Nucleotides Determined by Biochemical Assay[a]

additions	bubbling cycle	nmol/mg of mitochondrial protein						
		ATP		ADP		AMP		
		matrix	external	matrix	external	matrix	external	total
none	1	25.8 ± 0.6	26.0 ± 1.4	3.5 ± 0.1	0.76 ± 0.07	0.8 ± 0.1	0.18 ± 0.09	57.1 ± 1.6
oligomycin	2	8.19	26.62	9.93	1.01	2.83	0.08	48.7
	4	1.51 ± 0.65	18.8 ± 1.1	12.8 ± 1.0	10.4 ± 1.3	3.5 ± 1.2	3.15 ± 0.9	50.1 ± 2.3
oligomycin + CAT	2	15.72	15.26	4.58	9.63	0.84	3.31	49.3
	4	8.5 ± 2.7	9.3 ± 0.7	6.7 ± 1.8	13.2 ± 0.6	3.9 ± 1.7	10.0 ± 1.7	51.6 ± 4.1

[a] Mitochondria were loaded with ATP and incubated in the presence of 1 mM phosphate and 0.5 mM ATP as described under Experimental Procedures. Mitochondria were incubated for one bubbling cycle before addition of mitochondrial inhibitors. Samples were removed for determination of adenine nucleotides at the end of the first (before inhibitor), second, and fourth bubbling cycles. Oligomycin was 40 μg/mg of mitochondrial protein, and carboxyatractyloside (CAT) concentration was 30 μM. Means and standard errors of the means are presented for three separate experiments. Values without standard errors are from duplicate measurements which did not differ more than 7%.

Table III: Relationships between Intra- and Extramitochondrial Adenine Nucleotides in the Presence and Absence of Mitochondrial Inhibitors[a]

additions	bubbling cycle	ATP_m/ADP_m ratio	ATP_e/ADP_e ratio	matrix ATP + ADP + AMP (nmol/mg of mitochondrial protein)
mitochondria				
none	2 and 4	1.3 ± 0.2	5.9 ± 0.8	11.8 ± 0.8
P_i	2 and 4	3.9 ± 0.4	7.9 ± 1.6	10.9 ± 0.6
ATP-loaded mitochondria				
P_i	2 and 4	10.5 ± 0.9	21.3 ± 4.1	22.9 ± 1.8
P_i + ATP	1	7.4 ± 0.2	34.2 ± 3.2	30.1 ± 0.6
	2 and 4	11.3 ± 2.0	36.9 ± 5.6	20.4 ± 1.5
+oligomycin	2	0.8	26.4	21.0
	4	0.12 ± 0.05	1.8 ± 0.2	17.8 ± 1.7
+oligomycin + CAT	2	3.4	1.6	21.1
	4	1.3 ± 0.4	0.70 ± 0.05	19.1 ± 3.6

[a] Ratios were determined from mean values presented in Tables I and II. Matrix nucleotides are designated by ATP_m, ADP_m, while external nucleotides are designated by ATP_e, ADP_e. Standard errors are shown.

sample, and spectra of the inorganic phosphate were obtained with the same delay times as in the experiment as well as under fully relaxed conditions. Inorganic phosphate was assayed as described above. Thus, an internal area standard was obtained for each mitochondrial sample. An aqueous solution of methylenediphosphonic acid (Burt et al., 1976) contained in a capillary and inserted into the center of the 15-mm tube was also used as an area and shift standard in most experiments.

Results

Liver mitochondria oxidizing succinate plus glutamate were incubated under a variety of conditions which were designed to vary both matrix and external ATP and ADP concentrations over a wide range. The data for mitochondria incubated in the absence and presence of mitochondrial inhibitors are shown in Tables I and II. As shown in Table I, total nucleotides were about 15 nmol/mg of mitochondrial protein (<500 μM) without ATP loading and about 30 nmol/mg of mitochondrial protein (≤1 mM) after loading. The net increase in concentration of matrix nucleotides can be maintained at 8 °C, because the ATP–Mg/P_i carrier is not active at low temperature (Aprille, 1988). Without inhibitors, no differences were seen in adenine nucleotide concentrations between bubbling cycles 2 and 4, so the data from both cycles were combined.

Mitochondrial and extramitochondrial ATP/ADP ratios calculated from the data presented in Tables I and II are summarized in Table III. ATP/ADP ratios were maximized by addition of inorganic phosphate (Table III). In the presence of excess phosphate, matrix nucleotides represented about 70% of total ATP and 80–90% of total ADP. When mitochondrial inhibitors were added, there were significant changes in ATP, ADP, and AMP concentrations between bubbling cycle 2 (immediately after adding inhibitor) and cycle 4 (see Table II).

Consistent with previous observations (Klingenberg & Heldt, 1982), the ATP/ADP ratio in the mitochondria was lower than in the extramitochondrial compartment. Extramitochondrial ATP/ADP ratios greater than 30 were observed routinely with loaded mitochondria regardless of the extramitochondrial ATP concentration. The stability and reproducibility of the preparation were also indicated by the fact that after cycle 1, where some loss of nucleotides occurred,

Table IV: T_1 Values for Intra- and Extramitochondrial Adenine Nucleotides[a]

		T_1 (s)			
		ATP β-phosphate		ATP γ- + ADP β-phosphate	
additions	temp (°C)	matrix (+Mg^{2+})	external (+CDTA)	matrix (+Mg^{2+})	external (+CDTA)
incubation buffer	8	0.20	1.51	0.25	2.32
	25	0.23	2.75	0.24	5.34
mitochondria					
not loaded	8	0.22		0.27 ± 0.04	
ATP-loaded	8	0.22 ± 0.02	0.80 ± 0.06	0.20 ± 0.02	1.12 ± 0.15
	25	0.22 ± 0.00		0.23 ± 0.02	

[a] T_1 values were determined for adenine nucleotides as described under Experimental Procedures using the standard incubation buffer at 8 and 25 °C with and without mitochondria. For T_1 determinations in incubation buffer, a solution which mimicked the mitochondrial environment and contained 5 mM ATP ± 2 mM ADP and 5 mM $MgCl_2$ at pH 7.7 was placed in the 15-mm NMR tube. The 15-mm tube was placed inside a 20-mm tube which contained 2.5 mM concentrations of adenine nucleotides plus 5 mM CDTA without $MgCl_2$ at pH 7.0. Up to 30% fluorocarbons had no effect on adenine nucleotide T_1 values. In the mitochondrial experiments, the medium pH ranged from 6.3 to 7.0. Means and standard errors of the means are presented from three to nine separate experiments. Values without standard errors are means from single experiments.

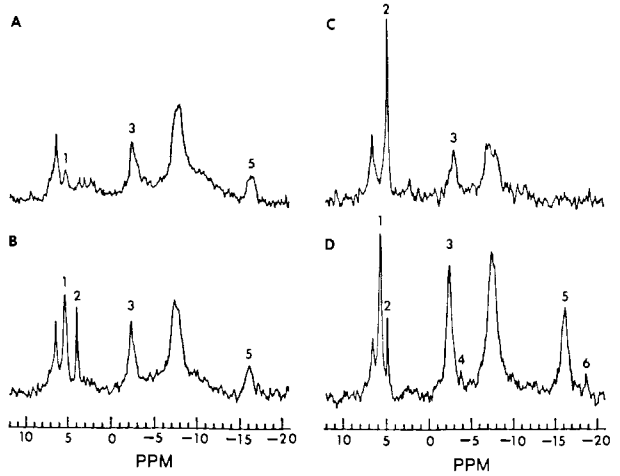

FIGURE 1: ^{31}P spectra of unloaded and ATP-loaded liver mitochondria oxidizing succinate and glutamate. Signals were accumulated with 68° radio-frequency pulses at a 0.31-s repetition time. (A) Signals from mitochondria (28.6 mg/mL) respiring on 25 mM succinate and 10 mM glutamate. The spectrum is the sum from three bubbling cycles, 1560 accumulations each. (B) Addition of 1 mM P_i to the mitochondria (28 mg/mL). The spectrum is the sum of two bubbling cycles, 1560 accumulations each. (C and D) Spectra from ATP-loaded mitochondria (24.8 mg/mL) with 1 mM added phosphate (C) after and (D) before addition of 15 μM FCCP, 1330 accumulations. Peak assignments are (1) matrix P_i, (2) external P_i, (3) matrix γ-ATP + β-ADP; (4) external γ-ATP + β-ADP, (5) matrix β-ATP, and (6) external β-ATP. The peaks marked external are more upfield due to the chemical shifts caused by removal of Mg^{2+} due to chelation. According to the procedure developed by Ogawa et al. (1978), we assumed that only external ATP was affected by the chelator CDTA.

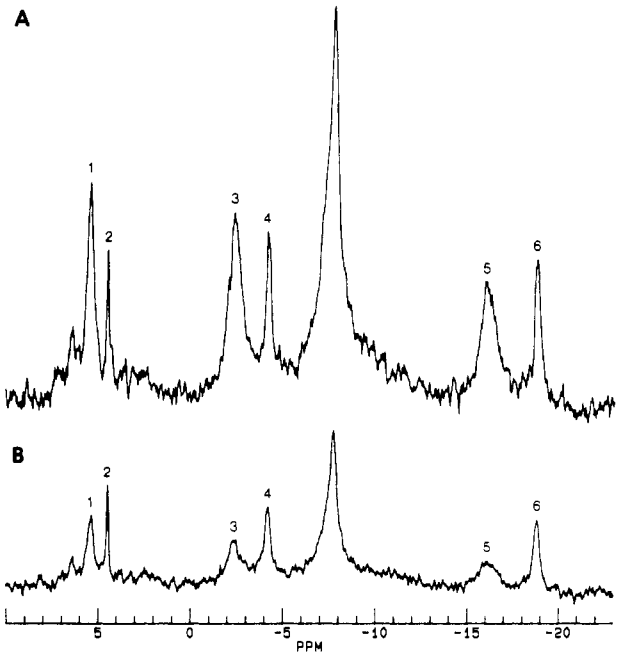

FIGURE 2: Spectra from ATP-loaded mitochondrial oxidizing succinate and glutamate with 1 mM added P_i and 0.5 mM added ATP. The mitochondrial concentration was 37.9 mg/mL. Signals were accumulated by using 68° radio-frequency pulses and a 0.3-s repetition time with 2400 accumulations (upper spectrum) or a 1.2-s repetition time with 600 accumulations (lower spectrum). Line broadening was 10 Hz. Peak assignments are defined in the legend to Figure 1.

the sum of all adenine nucleotides remained constant (Tables I and II). There was also no significant change in matrix nucleotide content (see Table III).

As shown in Figure 1A,B, in mitochondria not loaded with ATP, matrix ATP was detectable by NMR, and matrix inorganic phosphate (3–4 nmol/mg of mitochondrial protein) was just visible (Figure 1A). With the addition of phosphate, both matrix and external phosphate were readily visible (Figure 1B). When uncoupler was added to collapse the pH gradient across the inner mitochondrial membrane, the matrix inorganic phosphate peak merged with the external inorganic phosphate peak, and the loss of ATP was indicated by the disappearance of the β-phosphate of ATP (Figure 1C). As determined by enzymatic assay, in the presence of uncoupler matrix ATP was 0.9 ± 0.5 nmol/mg of mitochondrial protein (n = 3) or a concentration of <50 μM ATP, which was below the detection limit of this NMR experiment. Figure 1D shows a spectrum from ATP-loaded mitochondria incubated in the presence of phosphate. The matrix ATP (magnesium-bound ATP) resonances are at the same chemical shift as endogenous ATP in mitochondria that were not loaded (compare Figure 1B and Figure 1D).

Figure 2A,B shows representative spectra from ATP-loaded mitochondria (Austin & Aprille, 1984) oxidizing glutamate and succinate in the presence of added ATP and phosphate. Since the ^{31}P chemical shifts of ATP are dependent upon metal ion complexation, and because the matrix ATP is 95% magnesium bound (Corkey et al., 1986) (see Figure 1), extramitochondrial nucleotides could be discriminated from matrix nucleotides by adding CDTA to the medium. The spectra in Figure 2 were obtained by using repetition times of 0.3 and 1.2 s. It is clear that T_1 values are considerably shorter for matrix metabolites than for extramitochondrial metabolites, and that extramitochondrial resonances are significantly more saturated with the short repetition times used for most of the experiments. Measured T_1 values for adenine nucleotides are summarized in Table IV. T_1 values for matrix nucleotides at 8 and 25 °C were on the order of 0.2–0.3 s, which are similar to values obtained in liver at 37 °C (Iles et al., 1985; Malloy et al., 1986; Desmoulin et al., 1987). No significant

differences were found in T_1 values determined in the presence or absence of the mitochondrial inhibitors oligomycin and carboxyatractyloside, so these data were included in the mean values shown in Table IV. With CDTA in the extramitochondrial compartment, any Mg^{2+} and paramagnetic divalent ions such as Mn^{2+} are tightly bound to the chelator. T_1 values for extramitochondrial ATP and ADP were 4–5 times longer than for magnesium-bound matrix nucleotides. Differences in T_1 values for magnesium-bound and free nucleotides were greater in the absence of mitochondria (Table IV). Comparing T_1 values of ATP and ADP obtained in respiring mitochondria to those of the nucleotides in incubation buffer alone, it is apparent that matrix adenine nucleotide T_1 values were influenced primarily by the ionic environment.

Intra- and extramitochondrial adenine nucleotide ratios were varied in ATP-loaded mitochondria with added ATP and phosphate using the mitochondrial inhibitors oligomycin and carboxyatractyloside (see Table II). Since oligomycin blocks mitochondrial ATP synthesis, intramitochondrial ATP concentration fell drastically in its presence, because under energized conditions matrix ATP is released via the adenine nucleotide translocase in exchange for extramitochondrial ADP. The extramitochondrial ATP/ADP ratio fell slower than the matrix ratio (see Table III). This slow fall in ATP concentration may be due to ATPase in broken fragments of mitochondria. The atractyloside derivative, carboxyatractyloside, inhibits the adenine nucleotide translocase [reviewed by Klingenberg and Heldt (1982)]. In the presence of oligomycin and carboxyatractyloside, oxidative phosphorylation is blocked, but matrix nucleotides are trapped inside the mitochondria. Because the translocase is blocked, there is a dramatic rise in extramitochondrial ADP concentration (see Table II). Under these conditions, the matrix ATP/ADP ratio fell less than the extramitochondrial ratio, and was considerably higher than with oligomycin alone (Table III). In fact, about 50% of the total ATP and >30% of the total ADP present were in the mitochondrial compartment in the presence of carboxyatractyloside plus oligomycin (Table II). This distribution mimics the compartmentation of nucleotides between mitochondria and cytosol reported by Aw et al. (1987) in hepatocytes subjected to anoxia. Representative spectra are shown in Figure 3. After addition of oligomycin, the decrease in matrix ATP concentration which was accompanied by an increase in matrix ADP concentration could be seen as a decrease in the matrix β- and γ-ATP peaks and a rise in β-ADP. Changes in matrix nucleotides were accompanied by an increase in matrix inorganic phosphate concentration with no significant change in intramitochondrial pH (compare Figure 3A and Figure 3B). As shown in Figure 3C, with oligomycin plus carboxyatractyloside the fall in extramitochondrial ATP concentration proceeded faster than changes in matrix ATP concentration with a concomitant increase in inorganic phosphate concentration in both compartments.

Matrix and extramitochondrial nucleotide concentrations were measured by using ^{31}P NMR and enzymatic assay under the experimental conditions described in Tables I and II. These data are shown in Figure 4. Although there was some scatter in these data, there was reasonably good agreement between the two methods for measuring extramitochondrial ATP concentration (correlation coefficient of 0.83). Under all conditions tested, there was an excellent agreement between matrix ATP concentrations calculated by using NMR and determined by standard biochemical techniques, with an overall correlation coefficient of 0.92. On the other hand, ADP concentrations were difficult to estimate quantitatively by using

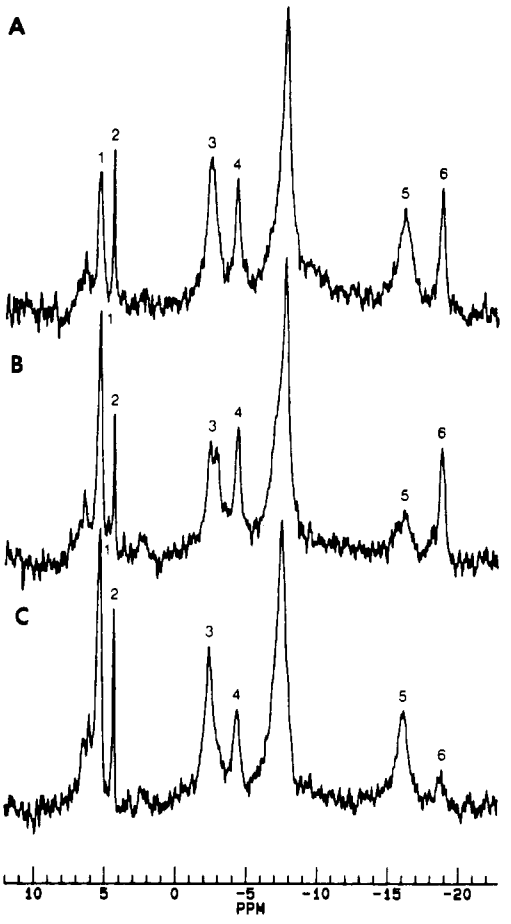

FIGURE 3: Effect of oligomycin and carboxyatractyloside on ^{31}P spectra from ATP-loaded mitochondria. Additions were 0.5 mM ATP and 1 mM P_i. Signals were acquired by using 68° radio-frequency pulses and a 0.3-s repetition time with 1332 accumulations. (A) Spectrum from mitochondria (33.6 mg/mL) before addition of mitochondrial inhibitors. (B) After addition of oligomycin (40 μg/mL) to the mitochondria (29.9 mg of protein/mL). (C) After addition of oligomycin (40 μg/mL) plus carboxyatractyloside (30 μM) to the mitochondria (33.6 mg of protein/mL). Peak assignments are defined in the legend to Figure 1.

^{31}P NMR, particularly when the ATP/ADP ratio was high. When the ADP concentration was $\leq$0.2 mM (near the detection limit of these ^{31}P NMR experiments), there was poor agreement between NMR and enzymatic estimates, and the differences were in opposite directions for matrix and extramitochondrial ADP. Linear regression equations and correlation coefficients for matrix and external ADP are given in the legend to Figure 4.

In the cell, >90% of the ATP is present as the magnesium complex (Siess et al., 1982; Corkey et al., 1986). Our spectra are consistent with the idea that a similar percentage of mitochondrial ATP is magnesium bound. Therefore, mitochondrial and cytosolic adenine nucleotides cannot be resolved by ^{31}P NMR on the basis of their chemical shifts. In a case where the equilibrium of two species greatly favors one over the other, as here for the magnesium-complexed versus free ATP, it is difficult to tell if Mg^{2+} is in fast exchange with ATP. In this case, one motionally averaged resonance should occur very near that of fully magnesium-complexed ATP. In the case of slow exchange of Mg^{2+} with ATP, one large peak at the fully complexed position and one small one (possibly barely above noise level) at the free position should occur. It has been observed recently (Sontheimer et al., 1986) that at field strengths above 8.5 T and temperatures below 15 °C, the slow-exchange regime prevails for Mg^{2+} and ATP. This may

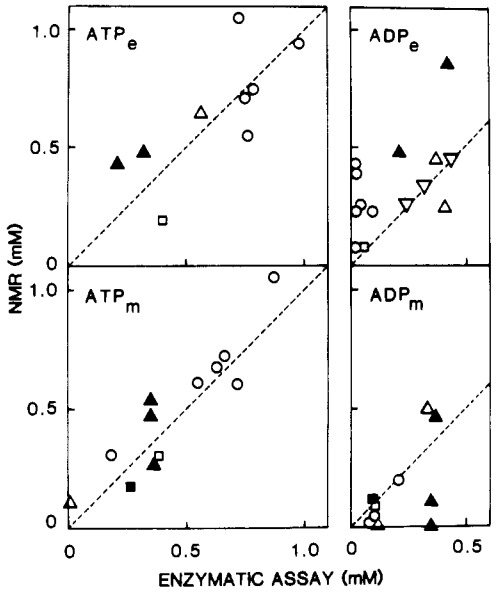

FIGURE 4: Comparison of adenine nucleotide concentrations determined by enzymatic assay and ^{31}P NMR at 8 °C. Mitochondria were incubated as described under Experimental Procedures. The following symbols are used to differentiate additions to ATP-loaded mitochondria: (□) 1 mM P_i; (○) 1 mM P_i and 0.5 mM ATP; (▲) 1 mM P_i and 0.5 mM ATP with 40 μg/mg oligomycin or (△) oligomycin plus 30 μM carboxyatractyloside; (▽) 20 μM FCCP. For mitochondria not loaded with ATP, (●) represents addition of 1 mM P_i. Both matrix (ATP_m, ADP_m) and extramitochondrial (ATP_e, ADP_e) nucleotide concentrations are presented as micromoles per milliliter of mitochondrial suspension. The dashed line represents a slope of 1.0. Best fits were to the following equations: $Y = 0.82X + 0.13$ ($r = 0.75$) for ATP_e, $Y = 1.00X + 0.04$ ($r = 0.92$) for ATP_m, $Y = 0.69X + 0.20$ ($r = 0.58$) for ADP_e, $Y = 0.90X + 0.05$ ($r = 0.60$) for ADP_m.

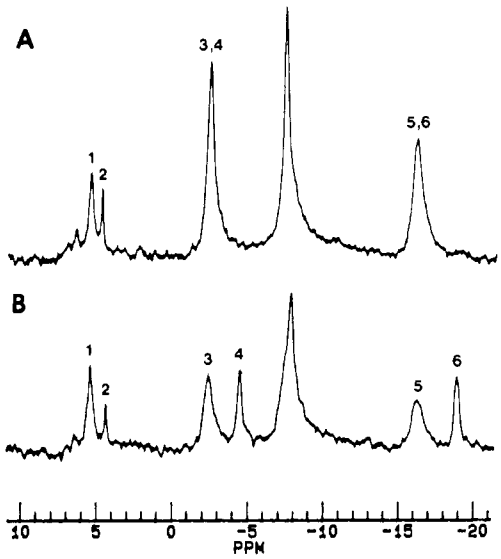

FIGURE 5: Effect of magnesium on the spectrum of ATP-loaded mitochondria oxidizing succinate and glutamate. (A) Mitochondria (32.3 mg/mL) oxidizing succinate and glutamate without added CDTA were incubated with 0.5 mM ATP, 1 mM P_i, and 2 mM $MgCl_2$. (B) The addition of 5 mM CDTA permits resolution of intramitochondrial and extramitochondrial ATP β- and γ-phosphate resonances. The repetition time was 0.3 s with 1200 accumulations. Peak assignments are defined in the legend to Figure 1. The α-phosphate region of (B) is presumably smaller in area than for (A) because of the expected lengthening of T_1 and thus the increase in saturation with added CDTA to the external medium (Table IV). Likewise, peaks 4 and 6 will be saturated more in (B) than in (A) due to the longer T_1's in the presence of CDTA (Table IV).

also be the case in our mitochondrial samples, as indicated by the hint of a broad resonance just above noise level at about −19 ppm (Figure 5A). Figure 5 shows ^{31}P spectra from

Table V: ^{31}P NMR and Enzymatic Estimates of Adenine Nucleotides in the Presence of Magnesium[a]

	adenine nucleotide estimate (μmol/mL)[b]			
	ATP		ADP	
additions	enzymatic	NMR	enzymatic	NMR
none	1.49	1.48	0.13	0.11
oligomycin + CAT	0.67	0.61	0.51	0.44
	0.54	0.47	0.54	0.32

[a] Mitochondria were incubated at 8 °C in the standard incubation buffer without CDTA with the following additions: 2 mM $MgCl_2$; 1 mM phosphate; 0.5 mM ATP. Oligomycin and carboxyatractyloside (CAT) concentrations were 40 μg/mg of mitochondrial protein and 30 μM, respectively. [b] Nucleotide concentrations are presented as micromoles per milliliter of mitochondrial suspension. Individual values from three separate experiments are shown.

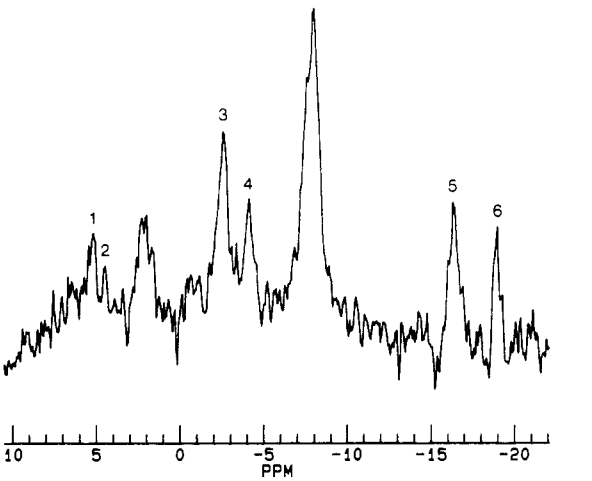

FIGURE 6: Spectrum of ATP-loaded mitochondria (26.14 mg/mL) oxidizing succinate plus glutamate at 25 °C with addition of 0.75 mM P_i and 0.5 mM ATP. The repetition time was 0.084 s with 3572 accumulations. Peak assignments are defined in the legend to Figure 1.

mitochondria respiring on glutamate plus succinate in the presence of added ATP and phosphate and 2 mM $MgCl_2$ (no CDTA), followed by addition of excess CDTA (Figure 5B) which then allowed separation of the resonances for matrix and extramitochondrial nucleotides (Figures 5A,B). After addition of CDTA, the ratio of the T_1-corrected areas for magnesium-bound versus free ATP was 0.65, while the ratio of matrix to external ATP determined by biochemical assay was 0.60. This result is consistent with our assumption that the magnesium-bound ATP corresponds to matrix ATP. Comparisons of ATP and ADP concentrations determined by ^{31}P NMR and enzymatic analysis in the presence of magnesium are shown in Table V. There was good agreement between ^{31}P NMR and enzymatic estimates of ATP and ADP in the presence of magnesium.

The effect of temperature on the visibility of adenine nucleotides was also examined. At 8 °C, it was relatively easy to oxygenate the dense mitochondrial sample adequately. To maintain adequate oxygenation of the mitochondria at 25 °C, it was necessary to bubble continuously with oxygen and include 30% fluorocarbons to increase the oxygen capacity of the medium. T_1 values for matrix nucleotides determined at 25 °C were similar to values obtained at 8 °C (see Table IV). Matrix ATP was visible at 25 °C, and a typical spectrum obtained at 25 °C is shown in Figure 6. Matrix ATP concentrations determined by ^{31}P NMR and enzymatic assay are compared in Figure 7. There was good agreement between matrix ATP concentrations calculated by using NMR and those determined by standard biochemical techniques, with

an overall correlation coefficient of 0.90. Thus, temperature did not affect visibility of matrix ATP in liver mitochondria in vitro. At 25 °C, there was more scatter between NMR and enzymatic estimates of mitochondrial ADP concentrations, but some matrix ADP was detectable (slope of 0.79, $r = 0.68$) (data not shown).

Discussion

Quantitation of adenine nucleotides in complex systems is difficult in part because NMR is relatively insensitive and errors of 10% in the measurements are not unusual. Accurate quantitation of peaks in phosphorus NMR spectra is usually done without proton decoupling under "fully-relaxed" conditions, in which the interpulse delay time is at least 5 times longer than the longest T_1 in the spectrum. Only then are all peak areas equally proportional to the concentration or amount of each chemical species. If the signal-to-noise ratio per unit time is important, it is more advantageous to pulse rapidly, even though the peak intensities then are also a function of T_1 values. In isolated mitochondria, where steady-state physiological conditions cannot be maintained indefinitely, maximizing the signal-to-noise ratio for the metabolite of interest is often preferable. Accurate quantitation is still possible, if one of two corrective procedures is used: (a) multiplication of the areas by a saturation factor, the reciprocal of the peak attenuation due to rapid pulsing compared to the fully relaxed areas; (b) calculation of saturation factors from a knowledge of the T_1 values, pulse flip angle, and interpulse delay time. We chose the flip angle and pulse delay to maximize the signal-to-noise ratio for matrix nucleotides. The metabolite T_1 values which were determined were representative of values reported for cellular nucleotides (Malloy et al., 1986; Iles et al., 1985; Desmoulin et al., 1987).

In isolated hepatocytes, mitochondrial ATP concentration has been estimated at about 15–25% of total cellular ATP concentration (Siess & Wieland, 1976; Aw et al., 1987). In our study, matrix ATP concentration was varied from 7 to 74% of the total ATP concentration in the NMR tube. Analysis of mitochondrial and extramitochondrial nucleotides in respiring mitochondria at both 8 and 25 °C confirms that mitochondrial matrix ATP concentration can be quantitated by using ^{31}P NMR. Hence, our data are in agreement with Desmoulin et al. (1987) where a good correlation was found between ATP measured by NMR in the perfused liver and ATP measured in acid extracts. It is possible to argue that the agreement between NMR and freeze–clamp data for normoxic liver total ATP may result from the fact that the low percentage (15–25% of total tissue ATP) of mitochondrial ATP in this condition is not much greater than the experimental uncertainties of the measurements. Determination of matrix ATP concentration by enzymatic assay and by NMR analysis gave no indication that even a fraction of the mitochondria is not observable by ^{31}P NMR spectroscopy.

Murphy et al. (1988) observed that 60–70% of the enzymatically assayable ATP in perfused livers made ischemic for 30 min was not observable by NMR spectroscopy. These workers assigned this NMR-"invisible" pool to the mitochondrial matrix compartment of the cell largely on the basis of the data of Aw et al. (1987), who found that 58% of the total ATP in anoxic hepatocytes is in the mitochondria. Possible explanations for NMR invisibility in mitochondria include microviscosity and binding to proteins, rapid exchange across a memebrane separating two compartments of different magnetic environment, and broadening due to association with paramagnetic ions. The observability of matrix ATP in isolated mitochondria (Ogawa et al., 1978; this study), and total ATP in livers perfused under a variety of different conditions including brief hypoxia (Desmoulin et al., 1987), indicates that microviscosity and binding to proteins are probably not responsible for the apparent invisibility of ATP during total ischemia. Since Aw et al. (1987) have proposed that during anoxia mitochondrial ATP and ADP movements may be inhibited, this makes rapid exchange an unlikely explanation. Also, we saw no effect of carboxyatractyloside, which inhibits the adenine nucleotide translocase, on visibility of matrix ATP. During the first few minutes of ischemia, total ATP reduction observed by Murphy et al. (1988) could be estimated to be about 25% [from Murphy et al. (1988), Figure 1] which is consistent with the observations of Desmoulin and co-workers for a similar time period of anoxia.

Clearly, however, association of mitochondrial ATP with paramagentic ions could produce the observed "in vivo" invisibility. Tissues are homogenized in and mitochondria are invariably isolated in media containing divalent metal ion chelators such as EDTA in order to prevent uptake of divalent cations which could damage the mitochondria during the isolation procedure. Thus, livers were perfused with EDTA-containing media prior to the start of the mitochondrial isolation. With mitochondria isolated without EDTA and which were not loaded with extra ATP, addition of manganese had little effect on ATP line widths until added Mn^{2+} exceeded 1 nmol/mg of mitochondrial protein in excess of the endogenous levels (Hutson et al., unpublished results). It is possible that mitochondria in situ may have higher levels of paramagnetic ions such as manganese than do mitochondria isolated with EDTA. However, this explanation implies that the concentrations of free paramagnetic ions are higher in the mitochondria than in the cytosol, since mitochondrial ATP is more "invisible" than cytosolic. There is evidence that in isolated mitochondria the free Mn^{2+} concentration is lower inside than outside the mitochondria (Puskin et al., 1976). Since neither free Ca^{2+} nor Mg^{2+} levels are normally higher in the mitochondria than in the cytosol (Denton & McCormack, 1985; Corkey et al., 1986; Moreno-Sánchez & Hansford, 1988), this seems unlikely. However, it is clearly possible that during ischemia the intramitochondrial manganese levels, like Ca^{2+} levels, might rise, thus providing a basis for the data of Murphy et al. (1988). Clearly, estimates of free paramagnetic ion conent and compartmentation in the control and ischemic livers are required.

The ^{31}P chemical shifts of the α- and β-phosphates of ADP are so close to those of the α- and γ-phosphates of ATP that they cannot normally be well resolved except in tissue extracts. Hence, quantitation of ADP is extremely difficult, because the ADP concentration is calculated from the difference between two large numbers [(γ-ATP + β-ADP) − β-ATP]. Indeed, while enzymatic assays gave highly reproducible estimates of matrix and extramitochondrial ADP concentrations (Tables I and II), both were difficult to quantitate by using NMR. Under our experimental conditions, mitochondrial ADP concentration ranged from 32 to 90% of total; however, there was no consistent pattern which suggested a fixed percentage of matrix ADP was not being detected by NMR. ADP was NMR-observable in uncoupled mitochondria when ATP was below the limits of detection (Figure 1C). However, when matrix ADP within the receiver coil was <1 mmol (0.2 μmol/mL incubation medium), matrix ADP was consistently underestimated by NMR. In the presence of magnesium, mitochondrial ADP concentration was varied from 24 to 72% of total ADP concentration, and, except in one case, the NMR estimate was within 15% of the enzymatic estimate.

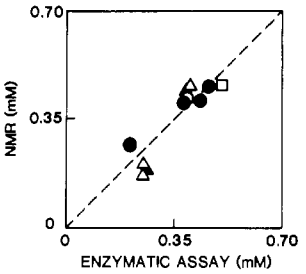

FIGURE 7: Comparison of matrix ATP concentrations determined by enzymatic assay and ^{31}P NMR at 25 °C. Mitochondria were incubated as described under Experimental Procedures. The following symbols are used to differentiate additions to ATP-loaded mitochondria: (□) none; (▲) 0.2 mM P_i; (○) 1 mM P_i, 0.5 mM ATP, and 2 mM $MgCl_2$; (△) 0.75 mM P_i and 0.5 mM ATP; (●) 0.2 mM P_i and 0.5 mM ATP. Matrix nucleotide concentrations are presented as micromolecules per milliliter of mitochondrial suspension. The dashed line represents a slope of 1.0. Best fit was to the following equation: $Y = 1.09X - 0.04$ ($r = 0.90$).

Estimates of NMR-visible ADP in liver and kidney range from 0 to 27% of the total ADP of about 1–1.4 μmol of ADP/g wet weight (Freeman et al., 1983; Iles et al., 1984; Stubbs et al., 1984; Cunningham et al., 1986). NMR estimates in intact liver are about 0.2–0.3 μmol of ADP/g wet weight (Cohen, 1983; Desmoulin et al., 1987; Cunningham et al., 1986). It has been suggested that the NMR-invisible ADP resides in the mitochondrial compartment (Stubbs et al., 1984; Iles et al., 1985; Cunningham et al., 1986). However, there are significant differences in ADP compartmentation in the intact liver and hepatocyte. Organic extraction of perfused liver gives estimates of at least 60% of total ADP in the mitochondria (Schwenke et al., 1981; Soboll et al., 1978), while in the isolated hepatocyte model, mitochondrial ADP represents 40–50% of total cellular ADP (Siess & Wieland, 1976; Aw et al., 1987). It is difficult to assign the NMR-invisible pool in the cell solely to the mitochondria on the basis of published estimates of ADP compartmentation, because these estimates differ depending on the methodology used to estimate the compartmentation (Schwenke et al., 1981; Soboll et al., 1978; Hummerich et al., 1988). Although there is some lack of consensus on the extent of ADP binding to liver proteins (Klingenberg & Heldt, 1982; Akerboom et al., 1978), it seems likely that the technical difficulty in measuring the low concentrations of ADP found in the cell, combined with binding of some ADP to cytosolic cellular proteins (either soluble or membrane proteins), contributes to the apparent invisibility of hepatic ADP. Binding to actin could account at most for 0.08 μmol/g wet weight (Gordon et al., 1977). The metabolic activity of the invisible pool of ADP also remains to be determined.

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