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A ^{31}P NMR Study of Mitochondrial Inorganic Phosphate Visibility: Effects of Ca^{2+}, Mn^{2+}, and the pH Gradient†

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ABSTRACT: The effects of external pH, temperature, and Ca^{2+} and Mn^{2+} concentrations on the compartmentation and NMR visibility of inorganic phosphate (P_i) were studied in isolated rat liver mitochondria respiring on succinate and glutamate. Mitochondrial matrix P_i is totally visible by NMR at 8 °C and at low external concentrations of P_i. However, when the external P_i concentration is increased above 7 mM, the pH gradient decreases, the amount of matrix P_i increases, and the fraction not observed by NMR increases. Raising the temperature to 25 °C also decreases the pH gradient and the P_i fraction observed by NMR. At physiologically relevant concentrations, Ca^{2+} and Mn^{2+} do not seem to play a major role in matrix P_i NMR invisibility. For Ca^{2+} concentrations above 30 nmol/mg of protein, formation of insoluble complexes will cause loss of P_i signal intensity. For Mn^{2+} concentrations above 2 nmol/mg of protein, the P_i peak can be broadened sufficiently to preclude detection of a high-resolution signal. The results indicate that mitochondrial matrix P_i should be mostly observable up to 25 °C by high-resolution NMR. While the exact nature of the NMR-invisible phosphate in perfused or in vivo liver is yet to be determined, better success at detecting and resolving both P_i pools by NMR is indicated at high field, low temperature, and optimized pulsing conditions.

The phosphorylation potential [ATP]/[ADP][P_i] is frequently used as a measure of the available free energy of the cellular adenine nucleotide pool and thus is an important means of evaluating cell metabolism. In various tissues, a considerably higher phosphorylation potential is calculated using phosphorus-31 nuclear magnetic resonance (^{31}P NMR)[1] measurements (Iles et al., 1985; Cunningham et al., 1986; Desmoulin et al., 1987) than by using biochemical data (Siess et al., 1982; Soboll et al., 1978; Aw et al., 1987; Klingenberg

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[1] Abbreviations: ^{31}P NMR, phosphorus-31 nuclear magnetic resonance; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time; T_2^*, apparent spin-spin relaxation time including field inhomogeneity contribution; 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; DMO, 5,5′-dimethyloxazolidine-2,4-dione.

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& Heldt, 1982). The P_i signal from cellular compartments other than cytosol, and the degree of NMR invisibility of P_i in the different pools, could be a factor.

It appears that in intact liver a significant proportion of intracellular phosphate cannot be measured using ^{31}P NMR. Estimates of inorganic phosphate in isolated hepatocytes and liver range from 2 to 4 μmol/g wet weight liver (Siess et al., 1982; Aw et al., 1987; Cunningham et al., 1986; Desmoulin et al., 1987). However, estimates of ^{31}P NMR visible inorganic phosphate in liver in different metabolic states range from only 20% to 50% of chemically assayed inorganic phosphate (Iles et al., 1985; Cunningham et al., 1986; Desmoulin et al., 1987). It has been suggested that ^{31}P NMR detects only cytosolic P_i and thus measures only the cytosolic pH, while the mitochondrial phosphate signal appears to be NMR-invisible (Bailey et al., 1981). Adler et al. (1984) compared cellular pH in renal tubular cells using ^{31}P NMR and DMO distribution and concluded that the NMR-visible P_i was in the cytosol only. However, the validity of the traditional assignment of liver P_i to the cytosol has been questioned recently by Thoma and Ugurbil (1988). These authors found that the ^{19}F NMR chemical shift of difluoromethylalanine suggested an intracellular pH of 7.4 whereas the effects of uncoupler on the fructose 1-phosphate ^{31}P NMR chemical shift indicated a cytosolic pH of 7.0. In addition, we have recently verified (Hutson et al., 1989) the original observation (Ogawa et al., 1978) that the intramitochondrial phosphate ^{31}P NMR signal can be detected from isolated mitochondria incubated in vitro. A separate peak, tentatively assigned to mitochondrial matrix inorganic phosphate, has also been observed using isolated hepatocytes incubated at 4 °C (Cohen et al., 1983) and using perfused rat heart (Garlick et al., 1983). This peak, which was slightly downfield from the peak assigned to cytosolic inorganic phosphate, responded appropriately to alkalinization of the mitochondrial matrix by the K^+ ionophore valinomycin, and to acidification of the mitochondria by H^+ ionophores. Arruda et al. (1981) also observed two phosphate signals in turtle urinary bladder.

In a previous study from this laboratory, the NMR spectral characteristics of intramitochondrial adenine nucleotides were reported (Hutson et al., 1989). In this study, the mitochondrial inorganic phosphate signal has been characterized using isolated rat liver mitochondria. The effect of cellular divalent cations, temperature, and media composition on the NMR spectral characteristics of P_i have been investigated in order to evaluate factors which may be responsible for limited NMR visibility in the cell. Likewise, a careful assessment was made of the relationship of the pH gradient to the phosphate gradient and of the ability of matrix P_i to monitor intramitochondrial pH.

Experimental Procedures

Preparation and Incubation of Mitochondria. Male Sprague-Dawley rats were used. Livers were perfused with ice-cold isolation medium containing 0.225 M mannitol, 0.075 M sucrose, 0.1 mM EDTA, and 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 as described by Hutson et al. (1989). No mitochondrial preparation with a ratio less than 6.0 was used.

For NMR measurements, the mitochondria (30 mg/mL) were incubated at 8 or 25 °C in the standard medium containing 140 mM KCl, 15 mM succinate, 10 mM glutamate (15 mM at 25 °C), 20 mM MOPS or PIPES, and 1 mM CDTA, pH 6.3–7.0, as detailed in Hutson et al. (1989). At 8 °C, oxygenation was maintained by bubbling the sample for 5 min with 100% oxygen, followed by a 7- or 8-min period without bubbling in order to obtain high-resolution NMR data. 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. At intervals indicated in the table and figure legends, the mitochondrial sample was taken out of the magnet, and aliquots were removed for measurement of metabolites.

Analytical Methods. ATP, ADP, and AMP were assayed spectrophotometrically (Williamson & Corkey, 1969), and inorganic phosphate was assayed 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. Preparation of mitochondrial samples for extra- and intramitochondrial metabolite determinations was as described in Hutson et al. (1989). The pH gradient was determined biochemically on an aliquot of the mitochondrial suspension taken directly from the NMR tube using the distribution of ^{14}C-labeled DMO (Addanki et al., 1968) as detailed in Hutson (1987). Matrix water was determined in parallel samples using ^3H_2O and [U-^{14}C]sucrose.

Mitochondrial and extramitochondrial Ca^{2+} and Mn^{2+} were measured at 432 and 280 nm, respectively, by atomic absorption analysis using a Perkin-Elmer 360 spectrophotometer. One milliliter of the mitochondrial suspension was diluted with 1 mL of ice-cold 120 mM KCl and 20 mM MOPS, pH 6.8, and centrifuged immediately at 14000*g* for 1 min at 4 °C. The supernatant was removed, diluted, and filtered (0.45-μm Nalge cellulose acetate filter) before assaying for Ca^{2+} or Mn^{2+}. The mitochondrial pellet was extracted overnight with 2 N nitric acid and then centrifuged, and the supernatant was assayed for Ca^{2+}. For measurement of Mn^{2+}, two mitochondrial pellets were pooled, ashed overnight at 500 °C, diluted with 1 N nitric acid, and assayed for Mn^{2+}. Standard curves for Ca^{2+} and Mn^{2+} were prepared using the same solutions and procedures.

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 as described in Hutson et al. (1989). The acquisition time was 84 ms, the sweep width was 12 kHz, and 4K data points were used. Zero-filling to 8K points preceded application of an exponential filter and Fourier transformation. The pulse flip angle and repetition (interpulse delay) times were selected to optimize the signal-to-noise ratio for matrix metabolites. Pulse repetition times and the number of accumulations are shown in the figure legends. Unless indicated otherwise, a Lorentzian line broadening of 20 Hz was applied prior to Fourier transformation. Overlap of P_i (in with out) was minimal in most spectra. Nevertheless, a cutting/weighing procedure or a standard spectral simulation program was used to further reduce the error. Before cutting, a line was first drawn at the base of the high-resolution components to reduce contributions from overlapping PME and broad underlying phospholipids or unknown slow tumbling macromolecular complexes (cf. Figure 1). When the simulation program was used, a broad 250-Hz component and two narrow components for PME were assumed in addition to the narrow P_i signals. The broad

Table I: Effect of Environment on T_1 Values for Inorganic Phosphate[a]

additions	n	temp (°C)	T_1 (s) matrix (+Mg^{2+})	T_1 (s) external (+CDTA)
incubation buffer	1	8	3.10 ± 0.20	7.58 ± 0.30
	1	8		6.75 ± 0.30[b]
	1	25	4.85 ± 0.20	11.67 ± 0.30
mitochondria				
not loaded	4	8	1.09 ± 0.06	3.38 ± 0.34
ATP-loaded	9	8	0.54 ± 0.05	2.98 ± 0.28
	4	25	0.44 ± 0.05	
ATP-loaded + 15 μM FCCP	7	8		4.89 ± 0.32
	2	25		9.15, 9.90

[a] T_1 values were determined for inorganic phosphate as described under Experimental Procedures using the standard incubation medium with and without mitochondria. For T_1 determinations in the incubation buffer, 5 mM P_i, 2 mM ATP, and 5 mM $MgCl_2$ were added, and the pH was adjusted to 7.7 in order to mimic the matrix environment (Hutson et al., 1989). The external environment was mimicked by adding 2 mM P_i, 2 mM ATP, and 5 mM CDTA and adjusting the pH to 6.8. Mitochondria were incubated at pH 6.8 in the standard buffer with 1 mM P_i and 0.5 mM ATP added. In some cases, mitochondria were loaded with ATP, as described by Austin and Aprille (1984). Means and standard errors of the means are presented. [b] No added CDTA.

component comprised about 16% of the total signal in the P_i region. The areas were similar to the values obtained using a deconvolution function to eliminate the broad spectral contribution (Gordon et al., 1982). Similar values were obtained after a computer-generated polynomial correction of the base line.

Spin–lattice relaxation times (T_1) were computed as detailed in Hutson et al. (1989). A modified progressive saturation experiment was used with respiring mitochondria, and the data were analyzed using an equation derived from Becker et al. (1979). This equation assumes exponential relaxation behavior. Using reference solutions, the intensity ratios gave similar T_1's to the inversion–recovery method. The reported T_1 represents an average of numerous values calculated for each bubbling cycle and between separate bubbling cycles. For the incubation buffer measurements and with added FCCP, an inversion–recovery sequence (Freeman & Hill, 1969) was also used and analyzed by a three-parameter fit to the equation $S(t) = A + Be^{-t/\tau_1}$ (Levy & Peat, 1975; Kowaleski et al., 1977) using a nonlinear curvefit (Johnson & Frasier, 1985). Saturation factors were calculated from the T_1 values, and corrected areas were determined.

At the end of each experiment, FCCP was added to the mitochondrial sample to uncouple oxidation and phosphorylation, and spectra of the inorganic phosphate were obtained with the same delay times as in the prior experiment, as well as under fully relaxed conditions. Thus, an internal area standard was obtained for each mitochondrial sample. In some experiments, a capillary containing methylenediphosphonic acid inserted into the center of the sample was used as a chemical shift reference. The chemical shift scale is presented relative to phosphocreatine.

Results

Determination of Matrix P_i by NMR Spectroscopy. As a first step in the estimation of NMR-visible P_i in the mitochondrial matrix, the apparent relaxation times ($T_{1,app}$) of medium and matrix phosphate were measured. Since it is not always practical to acquire fully relaxed spectra of P_i with isolated mitochondria, T_1 values are necessary to correct the estimates acquired at short pulse delays. The data are summarized in Table I. Values were first determined without the addition of mitochondria in the standard incubation medium and also under conditions which have been shown to mimic the mitochondrial matrix environment (Hutson et al., 1989). At both 8 and 25 °C, addition of magnesium to the buffer resulted in about a 50% decrease in the T_1. T_1 values for P_i in solution increased when the temperature was raised from 8 to 25 °C. Significantly shorter apparent T_1 values for both intra- and extramitochondrial P_i were measured in the presence of mitochondria oxidizing glutamate and succinate in state 4. As with adenine nucleotide T_1 values (Hutson et al., 1989), the T_1 for matrix P_i was short (<1 s) and did not vary significantly with temperature. This insensitivity to temperature may result from offsetting temperature dependences of the inherent spin–lattice relaxation in the absence of exchange (vide supra) and of the contributions from rate constants for exchange with ATP, and with external phosphate, which should increase at higher temperatures. This interpretation is strengthened by the observation that the T_1 for extramitochondrial P_i appeared to be sensitive to the metabolic state of the mitochondria (as expected, longer T_1 values were found for P_i with added uncoupler than without). T_1 values of <1 s for matrix P_i have been reported by Ogawa et al. (1978, 1981). The T_1 of P_i in intact liver has been reported to be 0.6 s (Malloy et al., 1986) or slightly shorter (Vanstapel et al., 1990), which is similar to that which we observed in the mitochondrial matrix space.

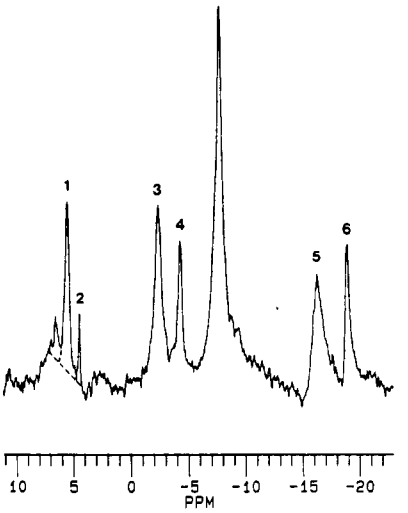

FIGURE 1: ^{31}P NMR spectrum of ATP-loaded mitochondria oxidizing succinate and glutamate at 8 °C. A total of 1352 acquisitions were accumulated with 68° radio-frequency pulses and a 0.31-s repetition time. Peak assignments are (1) matrix P_i, (2) external P_i, (3) matrix γ-ATP + β-ADP, (4) external γ-ATP + β-ADP, (5) matrix β-ATP, and (6) external β-ATP. The external adenine nucleotide peaks are more upfield due to the removal of Mg^{2+} by CDTA chelation. The dashed line under the narrow P_i components defines the base line of the area which was integrated, as described under NMR Measurements.

Figure 1 shows a representative spectrum of ATP-loaded mitochondria oxidizing glutamate and succinate in the presence of added ATP and P_i at 8 °C. P_i in the more alkaline mitochondrial matrix (peak 1) can be resolved from extramitochondrial P_i (peak 2). The quantitation of matrix and extramitochondrial P_i by biochemical assay and by NMR was then examined over a range of P_i concentrations at 8 °C; the data are presented in Figure 2. There was excellent agreement between the two methods, with a correlation coefficient of 0.99 for extramitochondrial P_i and 0.95 for matrix P_i at low concentration. However, when total external P_i concentration was greater than 7–8 mM, it appeared that some of the matrix P_i

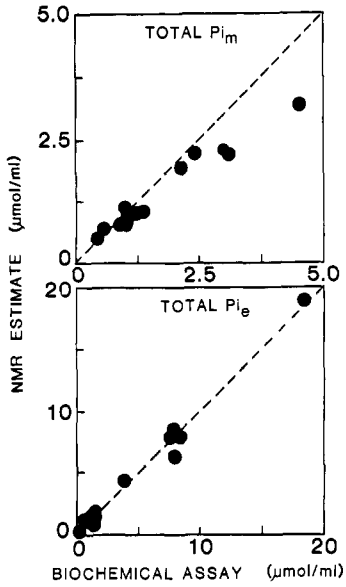

FIGURE 2: Comparison of P_i concentrations determined by biochemical assay and ^{31}P NMR at 8 °C. Mitochondria were incubated as described under Experimental Procedures and in Hutson et al. (1989). Both matrix P_i and extramitochondrial P_i are presented as micromoles per milliliter of mitochondrial suspension. The dashed line represents a slope of 1.

was not detected by NMR. The regression line for all matrix P_i data points below 2.5 μmol/mL mitochondrial suspension (about 80 nmol/mg of mitochondrial protein) was $y = 0.86x \pm 0.088$, where y is the concentration determined by NMR and x is the concentration measured biochemically. Therefore, at physiological concentrations of phosphate, the matrix P_i appeared to be NMR-visible at 8 °C.

Effect of Metal Ions on Mitochondrial ^{31}P NMR Metabolites. The NMR visibility of inorganic phosphate and adenine nucleotides could be affected by complexation with biological divalent cations such as Ca^{2+} and Mn^{2+}. Therefore, trials were carried out to quantitate the effects of these cations on the spectral characteristics of mitochondrial P_i and ATP. The initial level of Ca^{2+} in the mitochondria, incubated in nominally Ca^{2+}-free media (no added EDTA or CDTA), was 1.87 nmol/mg of mitochondrial protein. Freshly isolated mitochondria incubated in medium with 1 mM CDTA had a Ca^{2+} content of 1.00 nmol/mg of mitochondrial protein. Representative ^{31}P spectra of mitochondria oxidizing glutamate and succinate with 1 mM P_i at 8 °C after addition of graded amounts of Ca^{2+} are shown in Figure 3. Mitochondrial Ca^{2+} concentrations were determined in parallel experiments; all of the added Ca^{2+} was found in the mitochondria. When matrix calcium exceeded 5 nmol/mg of protein, calcium appeared to have a significant effect on spectral intensities of matrix P_i and adenine nucleotide peaks. As expected, at concentrations of matrix Ca^{2+} which can result in precipitation of calcium salts within the matrix [for a review, see Gunter and Pfeiffer (1990)], P_i and adenine nucleotides were almost completely NMR-invisible (see Figure 3, 100 nmol/mg of added Ca^{2+}). Separate control studies showed that the T_1 of P_i was not changing with additional calcium. Also the line widths ($1/\pi T_2^*$) do not increase under the conditions of the experiment.

Intramitochondrial and external phosphate and matrix ATP concentrations shown in Figure 4 were quantitated either by biochemical assay or by ^{31}P NMR. Both the biochemical and NMR measurements showed increased uptake of medium P_i by the mitochondria with increased calcium loading (Figure 4, external P_i). At the highest calcium loading (100 nmol of

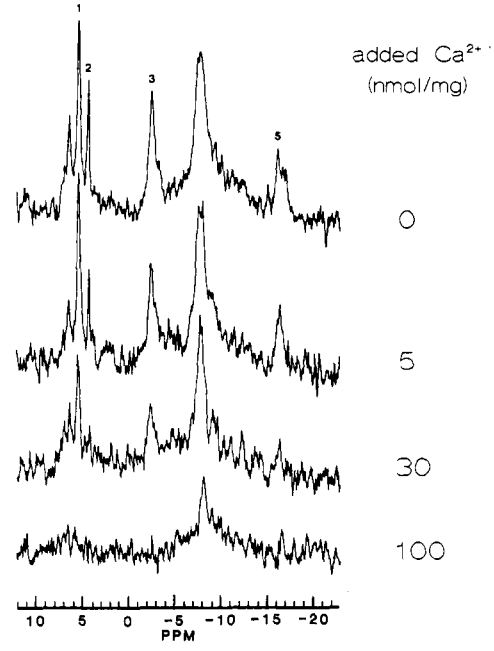

FIGURE 3: Representative spectra showing the effect of graded amounts of Ca^{2+} on ^{31}P spectra of mitochondria oxidizing succinate and glutamate at 8 °C. Mitochondria (28.7 mg/mL) were incubated in the presence of 1.0 mM P_i without added CDTA or ATP. Spectra were obtained before and after addition of Ca^{2+}. The repetition time was 0.31 s with 1352 accumulations, using a 68° flip angle. Peak assignments are defined in the legend to Figure 1.

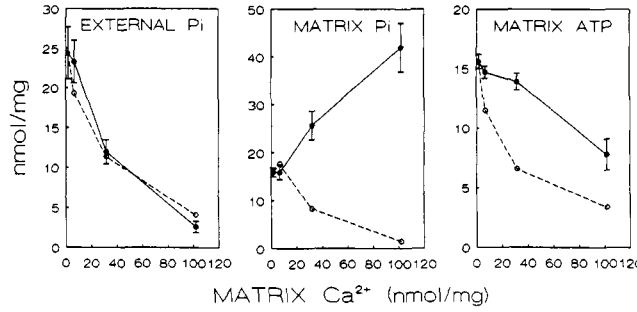

FIGURE 4: External and matrix P_i and matrix ATP determined by ^{31}P NMR and biochemical assay as a function of increasing matrix Ca^{2+} concentration. Experimental conditions were as described in the legend to Figure 3. P_i and ATP concentrations were determined by ^{31}P NMR (○) or by biochemical assay (●) as described under Experimental Procedures. For metabolites determined by biochemical assay, means and standard errors of the means from six experiments are presented. The NMR values shown were determined in a single experiment. Essentially identical data were obtained in a duplicate experiment performed on a separate day. The uncertainty in the NMR estimates as judged by signal-to-noise limitations and the disagreement between the duplicate experiments was ±3 nmol/mg.

Ca^{2+}/mg of mitochondrial protein), essentially all of the medium P_i had been accumulated by the mitochondria. During calcium titration, the sum of matrix and medium P_i measured biochemically remained constant at 36.4 ± 0.8 nmol/mg of mitochondrial protein. However, when measured by NMR, matrix P_i showed a small initial increase at 5 nmol of Ca^{2+}/mg of mitochondrial protein followed by a progressive decrease at higher Ca^{2+} levels (see Figure 4, matrix P_i). At very high Ca^{2+} loading (100 nmol of Ca^{2+}/mg of mitochondrial protein), 96% of the assayable matrix P_i was NMR-invisible. After uncoupler-induced release of Ca^{2+} and P_i from the matrix space into the larger volume of the medium, subsequent removal of Ca^{2+} from the medium by EDTA addition induced a 30% increase in the P_i standard area. At all concentrations of added mitochondrial Ca^{2+}, a fraction of the

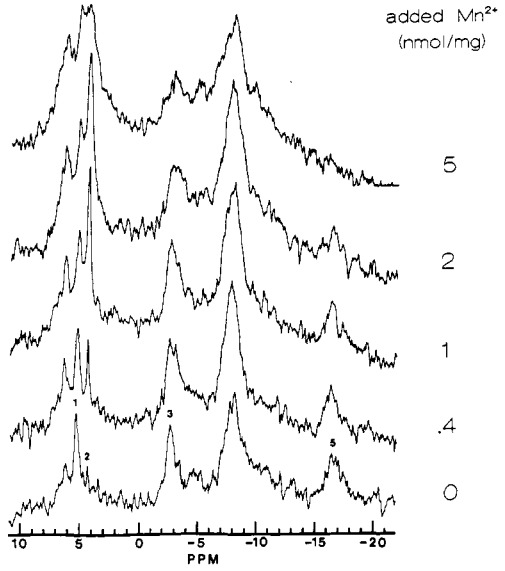

FIGURE 5: Representative spectra showing the effect of Mn^{2+} concentration on the ^{31}P spectra of mitochondria oxidizing succinate and glutamate at 8 °C. Mitochondria (33.7 mg of protein/mL) were incubated with 1.0 mM phosphate without added CDTA. Spectra were obtained before and after addition of Mn^{2+}. The repetition time was 0.15 s with 2000 accumulations using a 68° flip angle. Peak assignments are defined in the legend to Figure 1.

matrix ATP was NMR-invisible. Both NMR and biochemical assay showed a decrease in matrix ATP with added Ca^{2+}.

Separate experiments were carried out to measure the effect of Mn^{2+} on the mitochondrial spectra. The protocol was similar to the one described above for Ca^{2+}, because mitochondria will accumulate Mn^{2+} in an energy-dependent process via the Ca^{2+} transporter [for a review, see Gunter and Pfeiffer (1990)]. However, lower levels of Mn^{2+} than of Ca^{2+} were added to the mitochondria, since the paramagnetic Mn^{2+} ion can be expected to have a more profound effect on the ^{31}P spectra than the diamagnetic Ca^{2+} ion, due to enhanced T_1 and T_2 relaxation. Figure 5 shows ^{31}P NMR spectra of mitochondria obtained before and after additions of Mn^{2+} equivalent to 0.4, 1, 2, and 5 nmol/mg of mitochondrial protein. Another spectrum was taken with 0.1 nmol of added Mn^{2+}/mg of protein, but is not shown because it was virtually identical to the control spectrum. At each level of added Mn^{2+}, total matrix Mn^{2+} was assayed by atomic absorption spectrophotometry. In contrast to the results with Ca^{2+}, which was totally incorporated by the mitochondria, only about 37% of the added Mn^{2+} was actually taken up by the mitochondria. The initial level of matrix Mn^{2+} was 0.30 ± 0.01 nmol/mg, in excellent agreement with previous estimates (Thiers & Vallee, 1957) of the Mn^{2+} content of isolated rat liver mitochondria. Increasing Mn^{2+} to levels above 0.5 nmol/mg of mitochondrial protein decreased NMR-visible ATP only slightly, while increases to 1 nmol/mg caused a 50% decline in NMR-observable mitochondrial matrix ATP. Very little (<20%) matrix ATP could be detected at matrix Mn^{2+} levels above 2 nmol/mg. No effect of Mn^{2+} on biochemically assayed matrix ATP was found, however. In contrast, Mn^{2+} increased the spectral peak area of extramitochondrial P_i. Since most of the added Mn^{2+} (63%) remained in the medium, the apparent increase in extramitochondrial P_i magnetization is due to a large decrease in the T_1 of the medium P_i in the presence of Mn^{2+}. Separate control studies (Figure 6) showed that the T_1 of P_i decreases in the presence of Mn^{2+}, which is especially apparent during these rapid pulsing experiments. This conclusion is also supported by the biochemical data, which showed that both matrix and extramitochondrial P_i

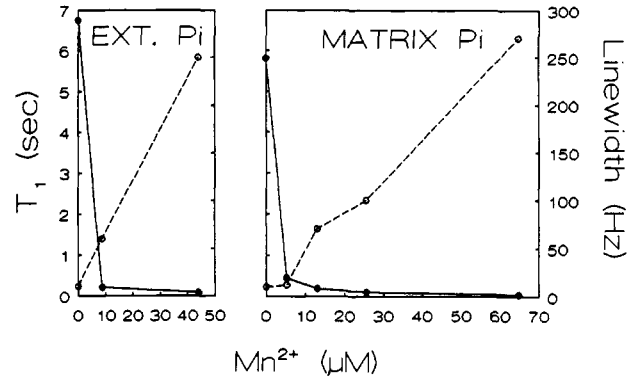

FIGURE 6: Results of solutions modeling the T_1 and line-width effects on matrix and external phosphate as a function of increasing Mn^{2+}. The solution which mimicked the matrix environment contained 140 mM KCl, 20 mM MOPS, 15 mM succinate, 10 mM glutamate, 2 mM $MgCl_2$, 3.7 mM P_i, and 0.4 mM ATP, pH 7.7. Mn^{2+} was added to approximate the amounts represented in the spectra of Figure 5, taking into account the fact that only 37% of that added is taken up by the mitochondria (0.4 nmol/mg ≃ 5 μM; 1 nmol/mg ≃ 13 μM; 2 nmol/mg ≃ 26 μM; 5 nmol/mg ≃ 65 μM). The solution which mimicked the external environment was the same except $MgCl_2$ was omitted and the pH was 6.8 (0.4 nmol/mg ≃ 9 μM; 2 nmol/mg ≃ 44 μM). Open circles represent line-width data, and closed circles are T_1 data. The uncertainty in the line widths is no more than ±7 Hz in all cases. The error in the T_1 values was within the symbol except for the initial points which were ±0.3 s.

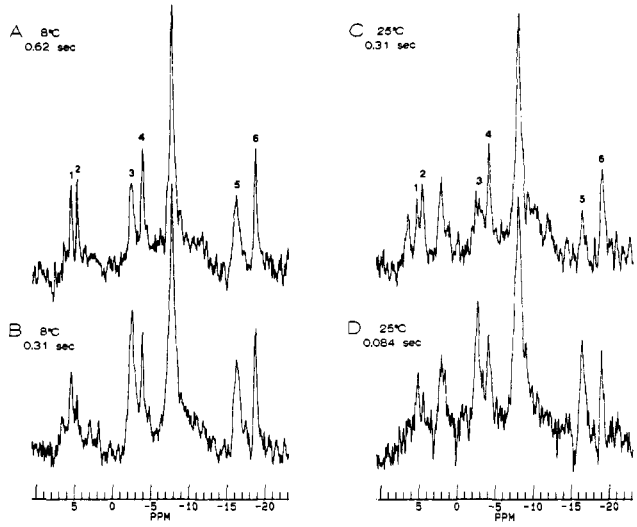

FIGURE 7: Effect of repetition time and temperature on ^{31}P NMR spectra of ATP-loaded mitochondria oxidizing glutamate and succinate. Mitochondria (26 mg/mL) were incubated as described under Experimental Procedures in the presence of 0.5 mM ATP and 1 mM P_i (0.75 mM P_i at 25 °C). The temperatures and repetition times are given next to the corresponding spectra, and the number of accumulations were 360, 720, 1000, and 3572 for spectra A–D, respectively.

remained constant during Mn^{2+} titration at 16.0 and 25.0 nmol of P_i/mg of mitochondrial protein, respectively. It was difficult to assess the matrix P_i quantitatively by ^{31}P NMR at an added Mn^{2+} concentration of 5 nmol of Mn^{2+}/mg of mitochondrial protein, because the spectrum is clearly broadened by T_2 effects, as was shown in separate control studies (Figure 6). The matrix P_i peak is little changed by added Mn^{2+} at levels equal to or lower than 2 nmol of added Mn^{2+}/mg of mitochondrial protein (~42 μM external Mn^{2+}).

Effects of Temperature. Next, the effect of temperature on visibility and quantitation of matrix P_i was investigated. Spectra, at two different repetition times, of mitochondria oxidizing succinate and glutamate in the presence of added ATP and P_i at 8 and 25 °C are shown in Figure 7. At 25

Table II: Comparison of the Mitochondrial pH Gradient (ΔpH) Determined at 8 °C by ^{31}P NMR and the Distribution of DMO[a]

conditions	medium pH	n	matrix pH		ΔpH	
			NMR	DMO	NMR	DMO
			Control Mitochondria			
no additions	6.95 ± 0.05	6	>7.90	8.10 ± 0.06	>0.95	1.15
+P_i	6.85 ± 0.15	9	7.63 ± 0.02	7.71 ± 0.02	0.78	0.86
			ATP-Loaded Mitochondria			
ATP + P_i	6.90 ± 0.10	6	7.69 ± 0.02	7.66 ± 0.02	0.79	0.76

[a] The ΔpH is defined as the difference between the matrix and medium pHs. The NMR values for matrix and medium pHs were calculated from the chemical shift of P_i using an experimentally determined pK_a for phosphate of 6.86 at 8 °C. Alternatively, the matrix pH was calculated from the distribution of ^{14}C-labeled DMO as described under Experimental Procedures in mitochondrial samples taken directly from the NMR tube. When added, ATP and phosphate concentrations were 2 mM. Means and standard errors of the means are presented.

°C, both matrix and extramitochondrial P_i peaks were broader than at 8 °C, and the phosphodiester (PDE) peaks between 1.7 and 2.1 ppm were significantly more intense than at 8 °C. At 25 °C, the intensity of the PDE peaks in our proton-coupled high-field spectra is often variable. Under these conditions, the phosphodiester peaks are likely to be due to contributions from more mobile membrane phospholipid structures as well as glycerophosphocholine and glycerophosphoethanolamine which are on the pathways of phospholipid catabolism (Murphy et al., 1989; Bates et al., 1989; Burt, 1985). The increased P_i line widths at 25 °C could be due to faster spin–spin relaxation and/or chemical exchange, but are probably in large part due to susceptibility-induced inhomogeneous broadening resulting from bubbling required for adequate oxygenation during data collection. (At 8 °C, the bubbling could be halted during data collection; intermittent oxygenation was sufficient to maintain mitochondrial viability at 8 °C but not 25 °C.) Visibility of matrix P_i was improved by increasing the number of acquisitions using very short repetition times (see Figure 7) which resulted in higher signal/noise ratios and greater saturation of the extramitochondrial P_i due to its longer T_1. Greater saturation of the external P_i signal reduced the overlap between the internal and external P_i spectral peaks. Because the fluorocarbons interfered with the P_i assay, biochemical determinations of P_i at 25 °C were only possible in samples incubated without fluorocarbons. Under these circumstances, mitochondria could not be maintained for more than 20 min in the spectrometer. Although a wide range of P_i concentrations could not be tested at 25 °C, in a limited number of samples (total matrix $[P_i]$ ≤0.4 μmol/mL medium) NMR observability of matrix P_i was apparent, since the ratio of matrix P_i determined by NMR to that determined by biochemical methods was 1.21 ± 0.20 (n = 6).

Sensitivity of the Chemical Shift of Matrix P_i to pH. Initially the pH dependence of the chemical shift of P_i in the incubation media was measured. An experimentally observed pK_a of 6.86 ($K_d = 1.36 \times 10^{-7}$), with limiting chemical shifts of δ_A = 3.42 ppm and δ_B = 5.66 ppm, was determined at 8 °C by titrating P_i in the incubation medium in the absence of mitochondria. This value is similar to that determined by Ogawa et al. (1978) (pK_a = 6.7) at 0 °C using uncoupled mitochondria. The validity of this pK_a for matrix P_i was examined by comparing the pH determined by the distribution of DMO and the pH determined by ^{31}P NMR. These data are summarized in Table II. In the absence of added P_i, the intramitochondrial pH was greater than 1 pH unit higher than the pK_a for P_i, and thus could not be precisely determined using ^{31}P NMR. Results from the two methods were in qualitative agreement for matrix pH >7.8 and in excellent agreement for matrix pH <7.8, e.g., after addition of medium P_i. The data in Table II also show that within a medium pH range of 6.7–7.0 the mitochondria maintained a constant matrix pH.

Table III: Relationship between the Inorganic Phosphate (P_i) Distribution and ΔpH in Respiring Liver Mitochondria at 8 °C[a]

medium pH	calculated n value	
	NMR estimate	biochemical estimate
6.85 ± 0.18	2.05 ± 0.17	2.08 ± 0.28

[a] The following equation described by Greenbaum and Wilson (1985) was used: $\log([P_i]_m/[P_i]_e) = \log[([H^+]_m + K_d)/([H^+]_e + K_d)] + n\Delta pH$. Concentrations of matrix P_i and extramitochondrial P_i were determined in samples taken from the NMR experiments where external P_i concentration was between 0 and 2 mM and used to calculate the biochemical estimate of n. A K_d for P_i of 1.36×10^{-7} (see Results) was used in the calculations. Mean matrix water volume was 1.12 ± 0.05 μL/mg of mitochondrial protein (n = 25). Means and standard deviations of the means from 17–20 separate determinations are presented.

At room temperature, a pK_a of 6.77 (Elliot & Dawson, 1988), δ_A = 3.21 ppm, and δ_B = 5.70 ppm were used to calculate matrix pH. Mean matrix pH was lower at 25 °C than at 8 °C. Over the medium pH range of 6.59–6.84, the mean ΔpH in ATP-loaded mitochondria was 0.47 ± 0.03 (n = 9) at 25 °C in the presence of 1 mM added P_i.

Using both ^{31}P NMR and biochemical assay measurements, the relationship between the P_i distribution and ΔpH was examined in mitochondria respiring at 8 °C (Table III). When the external P_i concentration was ≤2.0 mM, which is near the K_m for P_i transport (Coty & Pedersen, 1974; Fonyo, 1978; Ligeti et al., 1985), the data fit the equation described by Greenbaum and Wilson (1985) for stoichometrically coupled exchange of $H_2PO_4^-$ with OH^-; i.e., a calculated value of n = 2 was observed (see Table III). The relationship between ΔpH and the predicted and observed P_i gradients ($[P_i]_m/[P_i]_e$) was investigated over a wide range of medium P_i concentrations (see Figure 8). Both NMR and biochemical determinations of the P_i gradient, as well as matrix pH determined by ^{31}P NMR, are shown. As the medium phosphate concentration increased, the increased accumulation of P_i in the mitochondrial matrix resulted in a decrease in the pH gradient across the inner mitochondrial membrane (see Figure 8). As suggested by data presented in Table III, with medium P_i concentrations at or below the K_m for P_i transport, the measured P_i gradient for both NMR and biochemical determinations of P_i fit the predicted value. However, the relationship between the P_i gradient and ΔpH did not hold at higher medium P_i concentrations regardless of the method used to measure matrix P_i. At saturating concentrations of P_i, NMR gave lower values for matrix P_i than biochemical determinations (see Figure 8). Thus, as is also suggested by the data in Figure 2, the NMR visibility of matrix P_i appeared to be a function of P_i concentration. The validity of the NMR determination of matrix P_i was checked by comparing T_1 values for matrix and external P_i at 2.0 and 10.0 mM added P_i. Neither matrix nor external P_i T_1 values were affected by increasing P_i concentrations (data not shown).

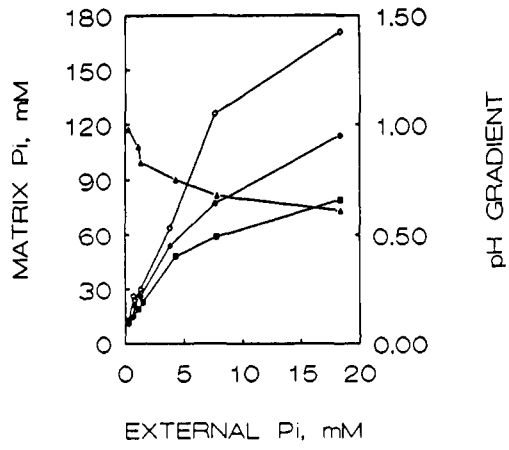

FIGURE 8: Comparison of predicted and observed matrix P_i concentration as a function of increasing extramitochondrial P_i concentration. Mitochondria oxidizing succinate and glutamate were incubated with 0.5 mM ATP and varied concentrations of P_i. Predicted matrix P_i concentrations (○) were calculated using the equation described by Greenbaum and Wilson (1985). Extramitochondrial P_i concentrations used in the calculations were determined either by biochemical assay or by ^{31}P NMR, and ΔpH was determined using ^{31}P NMR (▲). The uncertainty in ΔpH is estimated to be ±0.025 pH unit. The experimental observed matrix P_i concentrations were determined either by biochemical assay (●) or by ^{31}P NMR (■). The NMR determinations were carried out in duplicate, and different values determined with different external P_i concentrations were carried out with separate mitochondrial preparations on different days. The uncertainty in the NMR estimate as judged from signal-to-noise limitation is ±3 μmol/mL. Biochemical assays were performed on a portion of the NMR sample and on a parallel experiment which mimicked the NMR conditions. The standard error for the two trials was within the symbol dimensions.

Discussion

The ability to observe mitochondrial P_i by ^{31}P NMR depends upon the amount of matrix P_i present, its chemical shift, and its line width. Enough must be present to be detectable in a reasonable amount of time, and the peak must not be excessively broadened by interaction with paramagnetic species or relatively immobile cellular constituents. The line width must be narrow enough, and the chemical shift must be sufficiently distinct, to permit resolution from and to prevent overlap with the resonances of extramitochondrial P_i and other species with similar chemical shifts. Also, the external P_i–mitochondrial P_i exchange rate must be slow compared to the frequency separation of the two resonances in the ^{31}P NMR spectrum.

Since phosphate enters the mitochondria in concert with exit of hydroxyl ion and is therefore accumulated as a function of the pH gradient (Coty & Pedersen, 1974; Fonyo, 1978; Ligeti al., 1985), the phosphate inside the mitochondria in liver should represent a significant proportion of the intracellular pool. Indeed, about half of the total hepatic inorganic phosphate of 2–4 μmol/g wet weight is in the mitochondria (Siess et al., 1982), an amount quite adequate for NMR detection.

The ^{31}P chemical shift of P_i is sensitive to the pH of its environment, which allows pH to be measured by ^{31}P NMR (Gadian et al., 1979). In each cellular compartment, the P_i spectral line is the fast-exchanging, population-weighted average of the mono- and dibasic forms of the phosphate ion. Since the pH inside the mitochondrial matrix is believed to be close to 1 pH unit more alkaline than the cytosol (Hoek et al., 1980; Strzelecki et al., 1984), the mitochondrial P_i resonance should be shifted about 1 ppm downfield from the cytosolic peak. The relatively slow exchange of P_i between the mitochondrial and cytosolic spaces [V_{max} = 200 nmol/(min·mg of mitochondrial protein), K_m = 1 mM, 0 °C] (Coty & Pedersen, 1974) indicates that the two P_i peaks should not be coalesced and that exchange broadening should be minimal at physiological temperatures and available field strengths. Indeed our measurements indicate that the internal and external P_i resonances are resolvable at concentrations of mitochondria approaching those found in liver cells. The effects on line width of paramagnetic metal ions and complexation of phosphate with slowly tumbling macromolecules are less well documented.

Although most of the available data suggest that a significant proportion of the intracellular phosphate in perfused or in vivo liver is NMR-invisible (Iles et al., 1985; Cunningham et al., 1986; Desmoulin et al., 1987), the exact location of this NMR-invisible intracellular pool has not been determined. An attractive hypothesis is that this pool is located inside the mitochondria (Cunningham et al., 1986); however, not all the data are consistent with this notion (Thoma & Ugurbil, 1988). Several reports have appeared in the literature aimed at assessing the cytosolic and mitochondrial compartmentation of hepatic metabolites including phosphate (Siess et al., 1982; Aw et al., 1987). These studies have employed isolated rat hepatocytes, which apparently contain 7–9 μmol of P_i/g dry weight. Rapid separation techniques place 30–50% of this within the mitochondrial matrix. Since the mitochondrial matrix space comprises about 15% of the total cell volume (LaNoue et al., 1984), the cytosolic concentration would be about 3 mM and the mitochondrial concentration greater than or equal to 8 mM. Careful estimates of chemically measurable phosphate and NMR-visible inorganic phosphate in two recent publications (Cunningham et al., 1986; Malloy et al., 1986) in in vivo rat livers suggest slightly higher levels of total inorganic phosphate (about 10 μmol/g dry weight), about half of which is NMR-visible. Thus, the NMR-invisible pool is the same order of magnitude as the mitochondrial pool. However, when the uncoupler dinitrophenol was infused into the livers studied by Cunningham et al. (1986), total phosphate doubled, ATP decreased, and presumably the ΔpH across the mitochondrial membrane decreased. Under these conditions, one would expect that the fraction of the total tissue P_i compartmented within the mitochondria would decrease. However, the percent visibility of the total pool remained constant at 50%. This places some doubt on whether the invisible fraction is exclusively mitochondrial.

The present data indicate that the mitochondrial phosphate pool is not inherently NMR-invisible. In fact, at low temperatures and low concentrations of P_i, all of the matrix pool is NMR-visible. Using the cryo-NMR method in which respiration is completely uncoupled, P_i concentrations measured by NMR and biochemical analysis were generally in agreement in perfused rat liver before and following ischemia (I. Ikai, University of Pennsylvania, personal communication). It was suggested that partial invisibility of ATP to NMR was not due to high concentrations of paramagnetic ions or high microviscosity in the mitochondria but rather due to binding to macromolecular sites (Ikai et al., 1991).

The present data show that under normal physiological conditions the divalent cations Ca^{2+} and Mn^{2+} are unlikely to play a role in the putative invisibility of matrix phosphate. When low levels of Ca^{2+} were added to mitochondria, it affected the NMR visibility of ATP more dramatically than that of phosphate. However, the effect of large unphysiological levels of Ca^{2+} on mitochondrial ATP visibility may explain the fact that after ischemia, a significant portion of total liver ATP becomes NMR-invisible (Murphy et al., 1988). The

invisibility of P_i at Ca^{2+} concentrations significantly greater than 5 nm/mg of mitochondrial protein is likely due to precipitation, as we have eliminated T_1 (intensity) and T_2 (line broadening) contributions. Nevertheless, in the normal physiological range (1–2 nmol/mg of mitochondrial protein, 0.1–0.2 μM free Ca^{2+}), Ca^{2+} appears to have relatively insignificant effects on the mitochondrial NMR spectra of phosphorus metabolites.

It would be difficult to assign the invisible pool solely to the mitochondria based on the supposition that divalent cations are concentrated in the mitochondria. To the contrary, recent studies from several laboratories (Gunter et al., 1988; Wan et al., 1989) indicate that cellular free Ca^{2+} levels are very similar inside and outside the mitochondrial membrane in nonpathological conditions. Studies of free Mn^{2+} distribution in mitochondria (Puskin et al., 1976; Gunter & Pfeiffer, 1990) indicate that the same is likely true of free concentrations of Mn^{2+} in the cell. Total rat liver Mn^{2+} has been measured as 32 nmol/g wet weight (Ash & Schramm, 1982; Bond et al., 1983). If all of this were inside the mitochondria, the mitochondrial content would be 0.43 nmol/mg of mitochondrial protein, but if it were distributed more evenly as measured by us, the content would be 0.16 nmol/mg. Thus, the physiological level in the mitochondria must be between 0.1 and 0.5 nmol/mg. In this physiological range, little or no evidence for an effect of Mn^{2+} on the NMR visibility of intra- or extramitochondrial P_i could be found. However, higher concentrations did affect both matrix ATP and P_i visibility due to T_2 line broadening effects (see Figures 5 and 6).

Our method of preparing liver mitochondria includes a preperfusion of the organ with the divalent cation chelator EDTA and inclusion of EDTA in isolation and resuspension media. Thus, the divalent cation content of this preparation of isolated liver mitochondria is quite low, and perhaps somewhat lower than that present in in situ mitochondria. However, without these precautions, isolated mitochondria contain high levels of Ca^{2+} ($\sim$10 nmol/mg) which are in excess of that present in the cell and which saturate the Ca^{2+} regulatory sites of mitochondrial enzymes known to be regulated by Ca^{2+} in situ (Sternickzuk et al., 1991). Thus, we have added back Ca^{2+} and Mn^{2+} through the physiological range (and beyond) in order to evaluate the significance of Ca^{2+} and Mn^{2+} as effectors of P_i visibility. The results suggest these particular cations are not effective in the physiological range. However, it may be that some other divalent cation present in the cell accumulates inside the mitochondria and broadens the P_i peak enough to render it invisible.

At low temperatures, ^{31}P NMR provides an accurate measure of mitochondrial phosphate as well as matrix pH. In fact, the data indicate that pK_a values for P_i determined in solution are the same as the pK_a in the intact mitochondria (see Table II). Using NMR, we were able to demonstrate under metabolizing conditions at 8 °C that there is a correlation between the distribution of P_i and the pH difference across the mitochondrial membrane. Palmieri et al. (1970) and later Greenbaum and Wilson (1985) had only been able to demonstrate this relationship in respiration-inhibited mitochondria. NMR could also be used to show the stoichiometry of P_i transport in exchange for OH^- under metabolizing conditions.

The present studies which demonstrate high NMR visibility of mitochondrial matrix P_i in isolated liver mitochondria make it appear unlikely that the mitochondrial matrix phosphate in liver cells is 100% invisible and the cytosolic pool 100% visible as suggested recently by Vanstapel et al. (1990) in an investigation of this problem in perfused rat livers. These elegantly performed studies showed that livers perfused with a phosphate-free medium lose about half the NMR-visible P_i pool in 30 min. Biochemical estimates of the phosphate washed out of the liver during this time indicate much more phosphate was lost than could be accounted for by the loss of NMR-visible phosphate. Therefore, they concluded that there was a large invisible pool which also contributed to the loss. Since they also found that the rate of (cytosolic) glycogenolysis was linearly related to the size of the NMR-visible pool, they concluded that the NMR-visible pool is the cytosolic P_i pool. This reasoning is somewhat flawed since their own data indicate that the visible and invisible pools are in close communication and are likely to change proportionally and in concert with each other.

The fact that phosphate is highly concentrated in the mitochondrial matrix space may allow a smaller fraction of the total to be NMR-observable compared to the cytosol. Also part of the cytosolic pool may be NMR-invisible due to the formation of slowly tumbling NMR-invisible complexes with Ca^{2+} or phospholipids. This would be consistent with the finding that the percent of the total cell phosphate which is NMR-visible remains constant when the liver is exposed to an uncoupling agent (Cunningham et al., 1986; I. Ikai, University of Pennsylvania, personal communication).

Part of the difficulty in observing the visible pool of mitochondrial matrix in liver cells may be the presence of nearby peaks (2-phosphoglycerate and possibly 3-phosphoglycerate, glucose-1-phosphate, and cytosolic P_i) (Gadian et al., 1979) which partially overlap the matrix P_i resonance. Indeed, Thoma and Ugurbil identified mitochondrial P_i as a shoulder on the cytosolic P_i peak in a perfused liver preparation. It should be possible to better resolve these peaks if ^{31}P NMR spectra are collected at (a) high field, (b) low temperatures (4–10 °C), and (c) with varied pulse delays to maximize intensity differences due to T_1. However, recent results of Thoma and Ugurbil (1988) illustrate the difficulty of sorting out P_i compartmentation. Clearly more work is required to define the role of compartmentation in NMR visibility of phosphorus metabolites in intact organs and tissues.

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