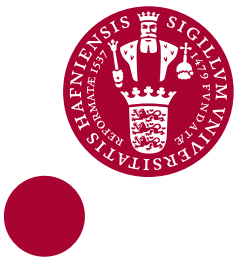

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A Possible Role of Inorganic Phosphate as a Regulator of Oxidative Phosphorylation in Combined Urea Synthesis and Gluconeogenesis in Perfused Rat Liver

A PHOSPHORUS MAGNETIC RESONANCE SPECTROSCOPY STUDY*

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Metabolic control of oxidative metabolism was studied in perfused rat liver by means of phosphorus magnetic resonance spectroscopy. Oxygen consumption, ATP, and P_i were measured with different rates of gluconeogenesis and urea synthesis by varying concentrations of the substrates in the perfusate. Five levels of oxygen consumption ($\dot{V}_{O_2}$) were obtained: an average control value of 1.94 ± 0.14 and 2.93 ± 0.25, 3.29 ± 0.46, 3.85 ± 0.26, and 4.18 ± 0.56 μmol/min/g liver (mean ± S.D., $n = 6$). The corresponding ATP concentrations were 2.51 ± 0.20, 2.39 ± 0.08, 2.24 ± 0.09, 2.13 ± 0.12, and 1.91 ± 0.13 mM. P_i increased stoichiometrically with the decrease in ATP. Free P_i (P_{i_f}) was calculated as NMR-visible P_i in control plus $-\Delta ATP$ (1.94 mM + ($-\Delta ATP$)). The kinetic relationship of oxidative phosphorylation as a function of P_{i_f} followed a Michaelis-Menten type of equation: $\dot{V}_{O_2} = 5.55/(1 + 0.24/([P_{i_f}] - 1.81))$. The observed K_m value for P_i of 0.24 mM approximates the reported K_m value in isolated mitochondria of 1 mM. The free P_i concentration of 1.94 mM is in the range of the K_m value, while the free ADP concentration of 200 μM exceeds the K_m value of 20 μM. Therefore, it is suggested that P_i play a major role in the regulation of mitochondrial oxidative phosphorylation in combined urea synthesis and gluconeogenesis.

Mitochondrial respiratory control is found to be determined by availability of ADP and P_i, adenylate translocation control, and thermodynamic equilibrium in isolated liver mitochondria or isolated hepatocytes (1–4). The ADP or P_i control of ATP synthesis in mitochondria has proved to be a fundamental principle of negative feedback control of oxidative metabolism (5). The K_m value of oxidative phosphorylation in isolated mitochondria was first found to be 20 μM for ADP and 1 mM for P_i (5, 6). The regulating chemicals must be in the range of their K_m values. The role of free ADP in the control of oxidative metabolism has been verified in skeletal muscle using NMR (7, 8). In liver analytical biochemistry shows that P_i concentration is 3–4-fold larger than the K_m value for P_i. Therefore, P_i has not appeared to be a likely candidate as a regulator of mitochondrial respiration *in vivo* with two exceptions of the phosphofructokinase-deficient limb (9) and the fructose-loaded liver (10). Comparisons of P_i value in liver as measured by NMR and biochemical analysis have shown that free P_i is approximately one-half of the total P_i (11, 12). Therefore, the role of free P_i in regulation of oxidative metabolism should be reconsidered. The free form of ADP is also differentiated from the bound form based on the difference of spin-lattice (T_1) relaxation time. However, *in vivo* studies on the role of free ADP and free P_i in the metabolic control of the liver has been performed.

A small decrease of ATP in perfused rat liver during the operation of a biosynthetic pathway such as gluconeogenesis has been reported by several investigators using analytical biochemistry (13, 14). Phosphorus magnetic resonance spectroscopy enables consecutive determination of small changes in ATP and P_i in the same liver. The β-ATP peak in phosphorus spectra in perfused rat liver stands independently and is easy to quantitate. However, the P_i peak is difficult to separate from those of phosphomonoester and phosphodiester. Liver as opposed to brain and muscle lacks the phosphocreatine/creatine energy buffering system. Therefore, the changes in P_i are attributable directly to ATP breakdown, if no sugar phosphate accumulates and no phosphate is lost from hepatocyte. ADP is also produced in an equal amount by ATP breakdown. Thus, the K_m value of the relation between oxygen consumption rate $(\Delta\dot{V})_{O_2}$ and changes in ATP concentration ($-\Delta ATP$) will provide a clue as to metabolic control based upon a 20 times difference between the K_m value in mitochondria for ADP and the value for P_i.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats, weighing 120–140 g, were fed laboratory chow *ad libitum*. Food was withdrawn 48 h before the start of the perfusion experiment. The animal had free access to water at all times. All chemicals were purchased from Sigma. Enzymes were obtained from Boehringer Mannheim.

Liver Perfusion—After anesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), the liver was perfused with phosphate-free Krebs-Henseleit solution with 0.5 mM EDTA equilibrated with 95% O_2 and 5% CO_2 following the method described by Sugano *et al.* (15). The excised liver was placed in a closed plastic cylinder with 30 mm diameter and 22 ml volume inside of a horizontally placed solenoid coil. A high perfusion flow rate of 27 ml/100 g body weight ensured sufficient oxygen supply with hemoglobin-free perfusion. The perfusate entered the liver at a constant temperature of 35 °C controlled by a water-jacketed line, and the pH of the perfusate was 7.35. The effluent from liver was collected by two suction lines. One was positioned at the bottom of the capsule to measure oxygen partial pressure without contamination of air. Oxygen partial pressure was recorded polarographically with a Clark-type

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oxygen electrode placed 50 cm distant from the NMR capsule. The oxygen consumption rate was calculated from the flow rate, and the difference in the oxygen partial pressure between the influent and the effluent using the Bunsen absorption coefficient in crystalloid perfusate was 3.25 ml O_2/ml/mm Hg O_2 (16). Urea and glucose production rates were determined with the sample collected from this line. The second suction line was placed at the top of the capsule at a flow rate of more than 50 ml/min and allowed the liver to bathe in the perfusion medium and prevented overflow outside of the NMR capsule.

Experimental Protocol—The liver was challenged by four levels of combined urea synthesis and gluconeogenesis by changing the perfusate containing these substrates. After recording two control phosphorus spectra without the addition of substrates, 5 mM lactate and 1 mM pyruvate were added to initiate gluconeogenesis. 20 minutes after the initiation of gluconeogenesis, the following four levels of substrates for urea production were further added for 20 min in the following four groups: 0.5 mM NH_4Cl in Groups 1, 2 mM NH_4Cl in Group 2, 2 mM NH_4Cl and 2 mM ornithine in Group 3, and 10 mM NH_4Cl and 2 mM ornithine in Group 4, respectively. The 20-min interval was allowed to reach steady state. At 5-min intervals, phosphorus spectrum, oxygen consumption rate, urea, and glucose production rates were measured. At the end of the experiment, the perfused liver was freeze-clamped with aluminum tongs precooled in liquid nitrogen for the biochemical measurement of adenine nucleotides and P_i. The first control spectrum was measured at 30 min, and within 80 min the entire experiment was finished. In the control group the liver was perfused without additional substrates, and phosphorus spectra and oxygen consumption were measured at three periods: 30–40, 50–60, 70–80 min. The liver sample freeze-clamped at 30 min after initiation of perfusion without substrates served as control for the biochemical analysis.

NMR Measurement—Phosphorus spectra were obtained at 5 tesla on a Brucker CXP 200 with a four-turn solenoid type probe tuned to 80.98 MHz. Spectra of perfused liver were obtained typically for 4.2 min by accumulating 512 free induction decays resulting from 47-μs radiofrequency pulses. The angle of the spin was 90°, and the delay between each pulse was 0.5 s. The repetition rate and the flip angle were selected to optimize the time resolution of β-ATP (17). Fourier transformation was performed with 20-Hz exponential line broadening. Dimethyl phosphonic acid in a capillary in the perfusion chamber was used as an external reference to standardize changes in signal intensity (11). Relative concentration of ATP, P_i, and phosphomonoester were calculated by triangulation (height *versus* width at half-height) from β-ATP, P_i, and phosphomonoester peak area. ADP concentration was estimated by measuring the difference in the area under the γ-ATP + β-ADP and the β-ATP peaks. The P_i/ATP and ADP/ATP ratios were corrected for saturation. In this study, the saturation parameters of P_i, β-ATP, and γ-ATP were 51.0 ± 2.0, 33.3 ± 1.2, and 31.0 ± 1.7% (n = 3, mean ± S.D.), respectively. The signal-to-noise ratio of β-ATP was around 20–30. Intracellular pH was calculated from the P_i chemical shift relative to α-ATP according to the equation described by Malloy *et al.* (18).

Biochemical Analysis—Biochemical analysis of ATP, ADP, AMP, and P_i concentration was performed in neutralized $HClO_4$ liver extracts (19–21). The concentrations expressed as micromoles per gram wet weight of liver were converted to millimolar using the value of 0.8 g of cellular H_2O/g of liver (22). Urea and glucose were measured by enzymatic spectrophotometric methods described in Refs. 23 and 24.

Statistical Analysis—The data were analyzed using an analysis of variance and unpaired Student's t test on the four groups *versus* control with the confidence limit at 95%. The values are expressed as mean ± S.D.

RESULTS

Four levels of oxygen consumption rate were obtained by substrate loading for urea synthesis and gluconeogenesis, as shown in Fig. 1*A* and Table I. Combined urea synthesis and gluconeogenesis caused a decrease in ATP concentration in response to the four substrate combinations, as shown in Fig. 1*B* and Table II. Within 15 min after the addition of substrates, oxygen consumption, urea and glucose production rate, and ATP level reached steady state. In the control group without substrate loading, ATP and P_i concentration remained at the initial level at least for 80 min of perfusion. Typical spectra before and during combined gluconeogenesis and urea synthesis and the difference spectrum are shown in Fig. 2.

By analytical biochemistry ATP and P_i concentration in

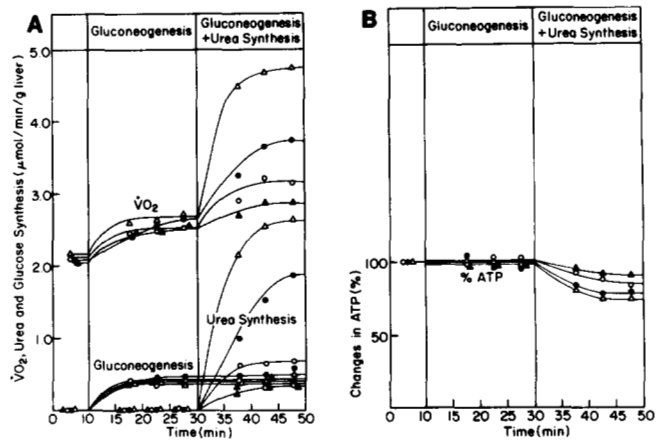

FIG. 1. **The changes in oxygen consumption, urea production, glucose synthesis (*A*), and ATP (*B*) in the four groups.** Representative cases are shown. *Closed triangles, open circles, closed circles,* and *open triangles* show the changes in Group 1, Group 2, Group 3, and Group 4, respectively. Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine.

TABLE I

Oxygen consumption, urea synthesis, and gluconeogenesis during gluconeogenesis and combined urea synthesis and gluconeogenesis in perfused rat liver

Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine; A = control; B = gluconeogenesis; C = combined urea synthesis and gluconeogenesis. Values are expressed as mean ± S.D. The number of rats used is shown in parentheses.

		Control (n = 6)	Group 1 (n = 6)	Group 2 (n = 6)	Group 3 (n = 6)	Group 4 (n = 6)
Oxygen consumption (μmol/min/g)	A	2.01 ± 0.14	1.89 ± 0.08	1.90 ± 0.13	1.96 ± 0.17	1.95 ± 0.17
	B	2.00 ± 0.15	2.39 ± 0.14[a]	2.30 ± 0.18[a]	2.49 ± 0.19[a]	2.43 ± 0.28[a]
	C	2.08 ± 0.21	2.93 ± 0.25[a]	3.29 ± 0.46[a]	3.85 ± 0.26[a]	4.18 ± 0.56[a]
Difference (μmol/min/g)	B − A		0.47 ± 0.15	0.41 ± 0.06	0.49 ± 0.12	0.49 ± 0.12
	C − A		0.96 ± 0.27	1.44 ± 0.38	1.85 ± 0.24	2.23 ± 0.44
Urea synthesis			0.60 ± 0.14	0.90 ± 0.29	1.99 ± 0.38	2.41 ± 0.42
Gluconeogenesis (μmol/min/g)			0.44 ± 0.06	0.44 ± 0.08	0.43 ± 0.15	0.53 ± 0.13

[a] Significantly different from the control group without substrates load ($p < 0.05$).

TABLE II

The changes in ATP, P_i, phosphomonoester, P_i/ATP ratio, ADP/ATP ratio, and intracellular pH as measured by NMR during gluconeogenesis and combined urea synthesis and gluconeogenesis in perfused rat liver

Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine; A = control; B = gluconeogenesis; C = combined urea synthesis and gluconeogenesis; PME = phosphomonoester. Values are expressed as means ± S.D. The number of rats used is shown in parentheses.

		Control (n = 6)	Group 1 (n = 6)	Group 2 (n = 6)	Group 3 (n = 6)	Group 4 (n = 6)
% changes in						
ATP	B/A	99.2 ± 1.8	98.2 ± 2.1	100.7 ± 2.6	99.0 ± 1.5	101.5 ± 2.6
P_i		103.5 ± 4.1	95.2 ± 8.4	98.5 ± 4.9	95.3 ± 4.5	94.8 ± 9.0
PME		100.0 ± 5.3	100.4 ± 4.2	99.5 ± 6.2	102.8 ± 6.1	101.2 ± 10.2
% changes in						
ATP	C/A	99.5 ± 1.9	95.4 ± 3.3[a]	89.2 ± 3.5[a]	84.7 ± 4.7[a]	76.2 ± 5.3[a]
P_i		103.2 ± 2.5	101.4 ± 6.2	107.9 ± 10.2	115.8 ± 11.7[a]	126.9 ± 11.2[a]
PME		100.2 ± 7.1	104.1 ± 7.0	99.0 ± 7.9	105.8 ± 8.7	103.2 ± 7.2
P_i/ATP ratio	A	0.81 ± 0.20	0.85 ± 0.09	0.84 ± 0.21	0.84 ± 0.25	0.84 ± 0.22
	B	0.87 ± 0.18	0.87 ± 0.26	0.83 ± 0.18	0.83 ± 0.26	0.79 ± 0.23
	C	0.86 ± 0.21	0.91 ± 0.15	1.08 ± 0.30	1.19 ± 0.37	1.43 ± 0.41
ADP/ATP ratio	A	0.08 ± 0.05	0.08 ± 0.05	0.08 ± 0.04	0.07 ± 0.03	0.09 ± 0.04
	B	0.09 ± 0.04	0.06 ± 0.03	0.08 ± 0.04	0.06 ± 0.03	0.09 ± 0.04
	C	0.07 ± 0.05	0.08 ± 0.04	0.10 ± 0.06	0.10 ± 0.03	0.12 ± 0.06
Intracellular pH	A	7.32 ± 0.04	7.30 ± 0.03	7.24 ± 0.09	7.29 ± 0.07	7.31 ± 0.07
	B	7.28 ± 0.05	7.21 ± 0.06	7.20 ± 0.05[a]	7.20 ± 0.09	7.21 ± 0.09
	C	7.30 ± 0.04	7.21 ± 0.03[a]	7.13 ± 0.05[a]	7.13 ± 0.06[a]	7.09 ± 0.06[a]

[a] Significantly different from the control group without substrate load ($p < 0.05$).

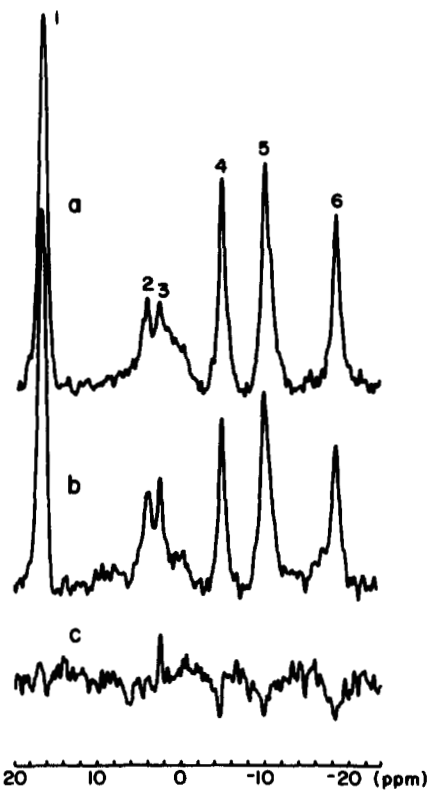

FIG. 2. **Phosphorus spectra before and during combined urea synthesis and gluconeogenesis with 10 mM NH_4Cl, 2 mM ornithine, 5 mM lactate, and 1 mM pyruvate and the difference spectrum between them.** *Trace a* shows the control spectrum, *Trace b* shows the spectrum during combined urea synthesis and gluconeogenesis, and *Trace c* shows the difference spectrum between *a* and *b*. A typical case is shown. Assignment: *1*, reference; *2*, phosphomonoester; *3*, P_i; *4*, γ-ATP + β-ADP; *5*, α-ATP + α-ADP; *6*, β-ATP.

the control was 2.51 ± 0.20 and 3.24 ± 0.26 mM as shown in Table III. The P_i/ATP ratio measured by analytical biochemistry in the control was 1.34 ± 0.22, while the ratio by NMR was 0.81 ± 0.20. Assuming the 100% visibility of ATP, comparison of these values revealed that NMR-visible P_i is approximately 60% of P_i determined by biochemical analysis, that is 1.94 mM, as summarized in Table IV. The comparison of ADP/ATP ratio by NMR and biochemical analysis in control showed that NMR-visible ADP is approximately 20% of ADP measured by enzymatic analysis, that is 0.20 mM. Combination of urea synthesis and gluconeogenesis evoked significant decrease in ATP by 4.6, 10.8, 15.3, and 23.8% (0.12, 0.27, 0.38, and 0.60 mM) relative to each control in the four groups, respectively, while NMR-visible P_i concentration was significantly increased in Group 3 and Group 4 by 15.8 and 26.9% (0.31 and 0.52 mM), as shown in Table II. Gluconeogenesis alone did not change ATP or P_i concentration.

Table V shows that total values of (ATP + NMR-visible P_i) of 4.36, 4.33, 4.38, and 4.37 mM in the four groups were maintained almost at the same level as the control of 4.45 mM. Analytical biochemistry showed that P_i concentration in the effluent was under the detectable level both in the presence and absence of substrates at least for 80 min. Phosphomonoester peak area, which contains phosphorylcholine, sugar phosphate, AMP, and 3-phosphoglycerate (25), did not significantly change during this substrate loading, as shown in Table II. ATP and P_i concentration in control remained at the initial level at least for 80 min. These results suggest that there is a stoichiometric relationship between an increase in NMR-visible P_i and the decrease in ATP.

Intracellular pH was significantly decreased both during gluconeogenesis alone and during combined urea synthesis and gluconeogenesis as shown in Table II. However, the values of oxygen partial pressure in the effluent during combined urea and glucose synthesis were 287 ± 54, 272 ± 63, 228 ± 63, 175 ± 45 mm Hg (n = 6) in the four groups which strongly suggests that sufficient oxygen was supplied to liver.

Cytosolic free ADP may be calculated from the difference

TABLE III

The changes in adenine nucleotides and inorganic phosphate as measured by biochemical assay during combined urea synthesis and gluconeogenesis

Control, 30 min after initiation of perfusion without substrates; Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine. Values are expressed as means ± S.D. The number of rats used is shown in parentheses.

	Control ($n = 6$)	Group 1 ($n = 5$)	Group 2 ($n = 5$)	Group 3 ($n = 5$)	Group 4 ($n = 5$)
ATP (mM)	2.51 ± 0.20	2.38 ± 0.42	2.16 ± 0.19[a]	2.06 ± 0.32[a]	1.85 ± 0.32[a]
ADP (mM)	0.98 ± 0.11	0.98 ± 0.09	1.02 ± 0.08	1.02 ± 0.17	1.14 ± 0.15
AMP (mM)	0.26 ± 0.04	0.29 ± 0.06	0.32 ± 0.07	0.33 ± 0.15	0.45 ± 0.18[a]
P_i (mM)	3.24 ± 0.26	3.24 ± 0.38	3.32 ± 0.30	3.37 ± 0.71	4.18 ± 1.30
P_i/ATP	1.34 ± 0.22	1.41 ± 0.36	1.53 ± 0.25	1.87 ± 0.43	2.51 ± 1.11

[a] Significantly different from the control ($p < 0.05$).

TABLE IV

The comparison between NMR measurement and biochemical analysis of adenine nucleotides and P_i in the control liver

Biochemical analysis	
ATP	2.51 (mM)
ADP	0.98 (mM)
P_i	3.24 (mM)
P_i/ATP	1.34
ADP/ATP	0.39
NMR measurement	
P_i/ATP	0.81
ADP/ATP	0.08
Visibility of P_i	0.81/1.34 = 0.60
Visibility of ADP	0.08/0.39 = 0.20
NMR-visible P_i	3.24 × 0.60 = 1.94 (mM)
NMR-visible ADP	0.98 × 0.20 = 0.20 (mM)

TABLE V

The stoichiometric relationship between decrease in ATP and increase in free P_i during combined urea synthesis and gluconeogenesis

Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine; P_{i_f}, free P_i. ATP and free P_i concentration were calculated according to the following equation.

ATP = ATP in control (2.51 mM)

× % change as measured by ^{31}P NMR

$P_{i_f} = P_{i_f}$ in control (1.94 mM) × % change as measured by ^{31}P NMR

	Calculated		
	ATP	P_{i_f}	ATP + P_{i_f}
		mM	
Control	2.51	1.94	4.45
Group 1	2.39	1.97	4.36
Group 2	2.24	2.09	4.33
Group 3	2.13	2.25	4.38
Group 4	1.93	2.46	4.37

between the (γ-ATP + β-ADP) and β-ATP area in the ^{31}P NMR spectrum. The NMR-measured relative ratio of ((γ-ATP + β-ADP) − β-ATP) to β-ATP has been reported to be in the wide range of 0.06–0.14 (11, 12, 25). In the present study, the NMR-measured ADP/ATP ratio in control was 0.08 ± 0.04 ($n = 30$) with large standard deviation. Table VI shows the changes in calculated NMR-visible ADP concentration during combined urea synthesis and gluconeogenesis. The calculated NMR-visible ADP concentration was not significantly changed from the control value of 200 μM in response to the four levels of substrate loading. However, the

TABLE VI

The changes in calculated free ADP concentration during combined urea synthesis and gluconeogenesis

Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine. ATP was calculated according to the following equation:

ATP = ATP in control (2.51 mM)

× % change as measured by ^{31}P NMR

	Measured ADP/ATP	Calculated	
		ATP	Free ADP
		mM	
Control	0.08	2.51	0.20
Group 1	0.08	2.39	0.19
Group 2	0.10	2.24	0.22
Group 3	0.10	2.13	0.21
Group 4	0.12	1.93	0.23

rather low sensitivity of ADP measurement by ^{31}P NMR should be considered.

ATP concentrations obtained by enzymatic analysis in the liver extract were significantly decreased from the control value of 2.51 ± 0.20 to 2.16 ± 0.19, 2.06 ± 0.32, and 1.85 ± 0.32 mM in Groups 2–4, as shown in Table III. The changes in the P_i concentration were not significant. With biochemical assay total ADP concentration was increased from the control value of 0.98 ± 0.11 to 1.14 ± 0.15 mM in Group 4.

DISCUSSION

Decrease in ATP—ATP synthesis should be equal to ATP consumption in the steady state metabolism. Initial depletion of ATP was caused by combination of urea synthesis and gluconeogenesis due to lack of energy buffering systems such as phosphocreatine/creatine which exist in brain and muscle but not in liver. The decrease in ATP is induced by fructose infusion or massive hepatectomy (26, 27). Fructose is rapidly phosphorylated to fructose 1-phosphate by ketohexokinase at the expense of ATP, resulting in depletion of ATP and sequestration of P_i. Since liver regeneration requires increased ATP synthesis due to protein and nucleic acid synthesis, the concentration of ATP in the remnant rabbit liver 24 h after 70% hepatectomy is decreased by 40%.

Gluconeogenesis alone did not significantly affect the ATP level in the present study. Lactate and pyruvate act both as substrates for gluconeogenesis and as more effective energy-yielding substrates than endogenous substrates (14). Conversely, ammonium chloride is solely a metabolic load to liver from the viewpoint of energy balance. Ammonia arises almost exclusively from protein breakdown, and the concentration of

ammonia in the portal vein reaches 1.0 mM in rats fed with high protein food (28). Hyperammonemia is known to deteriorate liver function and is considered as one of the causes of hepatic failure. Although the rapid perturbation by combination of gluconeogenesis and urea synthesis with 10 mM NH_4Cl and 2 mM ornithine plus 5 mM lactate and 1 mM pyruvate is far from a physiological condition, a depletion of adenine dinucleotides was not observed. The decrease in ATP during the perturbation could not be ascribed to deterioration of the liver judging from time course changes in the control group without substrate loading.

Urea synthesis from ammonium chloride and gluconeogenesis from lactate and pyruvate require four ATP per urea, six ATP per glucose, respectively. Urea and glucose production need one oxygen per glucose and two-third oxygen per urea, assuming the P/O ratio to be 3. The balance between oxygen consumption and glucose and urea synthesis may be estimated by the following equation: $\Delta\dot{V}_{O_2}/(\Delta G + 2\,\Delta U/3)$, where $\Delta\dot{V}_{O_2}$ is the difference of oxygen consumption rate between the accelerated and the control value, ΔU and ΔG are net production of urea and glucose. The calculated values are 1.14, 1.36, 1.05, and 1.04 in Groups 1, 2, 3, and 4, as shown in Table VII. The values over 1 suggest that the small decrease in ATP is not due to hypoxia. The values of oxygen partial pressure in the effluent showed that relative hypoxia in the pericentral area could be avoided by the high perfusion flow rate. The observed slight decrease in intracellular pH might be due to urea synthesis itself which partially involves carbonic anhydrase reaction and produces protons (29).

$$2\ NH_4^+ + 2\ HCO_3^- \rightarrow \text{urea} + CO_2 + 3\ H_2O$$

Carbonic anhydrase converts CO_2 and H_2O to HCO_3^- and H^+ and provides HCO_3^- for carbamylphosphate synthetase.

P_i Control and ADP Control—NMR-visible P_i and ADP are "free" and are likely to participate in mitochondrial oxidative phosphorylation. Invisible P_i and ADP are "bound." Based on both the present NMR study and enzymatic analysis, the concentrations of ATP and free cytosolic P_i (P_{i_f}) in the control were estimated to be 2.51 and 1.94 mM. The controlling chemical of oxidative metabolism must be in the range of the K_m value. Since free P_i concentration in hepatocyte is close to the reported K_m value for respiration of 1 mM, P_i is considered to be one of the regulators.

Fig. 3 shows the kinetic relationship between $\Delta\dot{V}_{O_2}$ ($\Delta\dot{V}_{O_2}$ = $\dot{V}_{O_2}$ accelerated − $\dot{V}_{O_2}$ at rest) and decrease in ATP (−ΔATP). Oxygen consumption ($\dot{V}_{O_2}$) includes all ATP-consuming processes and cyanide-insensitive respiration. Even at the resting state ATP is utilized continuously for maintenance of cell integrity and function using endogenous substrates. Therefore, $\Delta\dot{V}_{O_2}$ and ΔATP are net oxygen consumption by mitochondria and net changes in ATP associated with urea synthesis and gluconeogenesis. The relationship fits a Michaelis-Menten type of equation with the parameters shown.

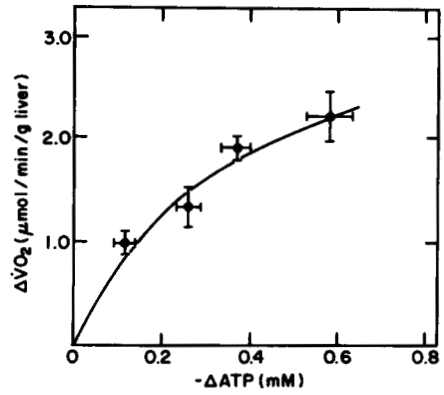

FIG. 3. **The relationship between $\Delta\dot{V}_{O_2}$ accelerated − $\dot{V}_{O_2}$ at rest) and (−ΔATP).** All points are mean ± S.E. (n = 6). The *line* represents the fitted equation: $\Delta\dot{V}_{O_2} = 3.50/(1 + 0.35/(-\Delta ATP))$.

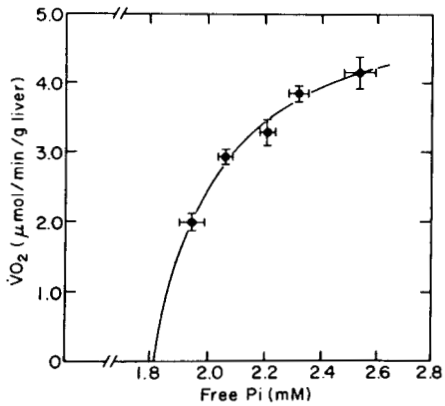

FIG. 4. **The relationship between oxygen consumption ($\dot{V}_{O_2}$) and calculated free P_i (P_{i_f}).** All points are mean ± S.D. (n = 6). The *line* represents the fitted equation: $\dot{V}_{O_2} = 5.55/(1 + 0.24/([P_{i_f}] - 1.81))$.

$$\Delta\dot{V}_{O_2} = 3.50 \times \frac{1}{1 + \dfrac{0.35}{-\Delta ATP}}$$

where $\Delta\dot{V}_{O_2}$ is μmol/min/g liver and −ΔATP is mM. This equation was fitted according to nonlinear least squares regression. This implies that K_m value of $\Delta\dot{V}_{O_2}$ for −ΔATP is 0.35 mM. P_i and ADP are theoretically produced in equal amounts by ATP breakdown. The K_m value for P_i was reported to be 1 mM, while the K_m value for ADP was reported to be 20 μM. The observed high K_m value of 0.35 mM favors that respiratory activity associated with combined urea synthesis and gluconeogenesis is regulated by changes in P_i, since 50-fold smaller changes of ATP would be expected if ADP were in control of liver metabolism.

The decrease in ATP can be substituted for the increase in P_i based on the stoichiometric relationship between them. Therefore, free P_i can be estimated as the following equation:

$$\text{Free } P_i\ (P_{i_f}) = 1.94 + (-\Delta ATP)\ (\text{mM})$$

Chemical reaction velocity does not respond to a differential change in substrate but to the molar concentration of substrate. Fig. 4 shows the kinetic relationship of oxygen con-

TABLE VII

The relative ratio of $\Delta\dot{V}_{O_2}$ to gluconeogenesis and urea synthesis

Group 1, 5 mM lactate + 1 mM pyruvate + 0.5 mM NH_4Cl; Group 2, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl; Group 3, 5 mM lactate + 1 mM pyruvate + 2 mM NH_4Cl + 2 mM ornithine; Group 4, 5 mM lactate + 1 mM pyruvate + 10 mM NH_4Cl + 2 mM ornithine.

	Group 1	Group 2	Group 3	Group 4
Measured O_2 consumption ($\Delta\dot{V}_{O_2}$) (μmol/min/g)	0.96	1.44	1.85	2.23
Calculated ATP consumption (6 ΔG + 4 ΔU) (μmol/min/g)	5.04	6.24	10.6	12.8
Calculated O_2 consumption (ΔG + 2 $\Delta U/3$) (μmol/min/g)	0.84	1.04	1.76	2.14
$\Delta\dot{V}_{O_2}/(\Delta G + 2\,\Delta U/3)$	1.14	1.36	1.05	1.04

sumption as a function of calculated free P_i. The relationship follows the equation in the range of P_{i_f} between 1.94 and 2.46 mM:

$$\dot{V}_{O_2} = 5.55 \times \frac{1}{1 + \frac{0.24}{[P_{i_f} - 1.81}}$$

where P_{i_f} is in mM. The result suggests that 0.24 mM increase of free P_i above a calculated base-line value of 1.81 mM causes half-maximal acceleration of oxidative metabolism and that P_i available for mitochondrial phosphorylation may be rather low. The reported K_m value of oxidative phosphorylation for P_i in isolated mitochondria varied with animal species and substrates. The value of rat liver mitochondria was reported as 1 mM in the presence of β-hydroxybutyrate (5), while the value of pigeon heart mitochondria was 0.13 mM in the presence of glutamate and succinate (30). The observed "K_m" value of 0.24 mM is thus close to the reported K_m value for P_i of isolated mitochondria.

P_i content in rat liver has been reported to be in the wide range of 2.5–5.2 μmol/g wet liver. Hansen *et al.* (31) and Quistorff and Poulsen (32) claimed their low value of 2.4–2.9 μmol/g wet weight to be unbiased by sampling and contraction artifacts and explained the discrepancy due to the possibility of hydrolysis of labile organophosphate compounds (31, 32). Cunningham *et al.* (12) have reported that the NMR visibility of P_i is about 50% under various conditions (12). In the present study, P_i concentration in the control as measured by biochemical analysis was 2.59 μmol/g wet weight and NMR visible P_i represents about 60% of total P_i.

The present study revealed at the organ level that P_i may be a controller of liver mitochondrial oxidative metabolism based on the kinetic relationship. Chance and Hess (33) have found that transient activation of respiration by glucose addition in ascites tumor cell is characterized by a high ADP level and that the inhibition of respiration by glycolysis is related to the intracellular levels of phosphate. Wu and Racker (34) have reported in the study of the Pasteur effect that K_m value for P_i of glyceraldehyde-3-phosphate dehydrogenase of ascites tumor was 1.5 mM, while the intracellular concentration of P_i was 3–9 mM. The difference was interpreted by compartmentation and sequestration of P_i by organelles. Chance and Maitra (35) have found that only a small portion of the total phosphate in the cell is available to participate in the phosphate potential based on the difference between the spectroscopic method and chemical determination.

It should be noted that phosphate was omitted in the perfusion medium to evaluate the changes in intracellular P_i (25). Leakage of P_i from hepatocytes into the perfusate was negligible. Thus, NMR did not measure P_i in the perfusate in the capsule but P_i in hepatocytes. Both biochemical and NMR studies showed that P_i was increased by combined urea synthesis and gluconeogenesis. The urea synthesis rate was comparable to other reports using ordinary Krebs-Henseleit solution (14, 28, 29). These results eliminate the possibility that lack of phosphate in the medium requires P_i to be a regulator in oxidative phosphorylation.

ADP are largely bound as opposed to ATP and the free ADP has been proposed as a regulator of oxidative phosphorylation in muscle and brain, since the free ADP concentration calculated by the equilibrium constant of the near equilibrium reaction catalyzed by creatine kinase is in the range of K_m value (7, 8, 36). However, creatine kinase system is not expressed in the liver. The NMR measurement of ADP gives directly the free ADP concentration using the difference between (γ-ATP + β-ADP) and β-ATP, although the sensitivity of quantification of ADP by organ magnetic resonance spectroscopy should be considered (12). NMR-calculated free ADP concentration in the perfused rat liver was reported to be in the range of 176–300 μM (11, 12, 25). Veech *et al.* (22) calculated the cytosolic free ADP in hepatocytes as 46 μM from the measured components of glyceraldehyde-3-phosphate dehydrogenase:3-phosphoglycerate kinase/lactate dehydrogenase reactions. In the present study NMR measurement revealed that free ADP concentration in control was around 200 μM. Free ADP concentration exceeds the range of the K_m value. However, the NMR study showed that calculated free ADP concentration was increased in response to the perturbation by 10 μM with a large standard deviation. It might be suggested that a large part of ADP from ATP breakdown becomes the bound form. The reported 20 times difference between the K_m value for P_i and that for ADP might be explained by the difference of binding capability. The contribution of free ADP in the respiratory control mechanism remains unclear since the *in vivo* K_m value for free ADP could not be determined in this study.

Ylikahri *et al.* (10) reported that fructose infusion to rat liver induced transient inhibition of oxidative metabolism concomitant with decreases in P_i and ATP, as fructose is metabolized at a high rate via glycolysis. P_i was decreased by fructose infusion from 3.9 to 1.9 mM, which value is nearly equal to K_m value for P_i in oxidative phosphorylation. Moreover, they reported that mitochondrial oxygen uptake rates in mammals are related to plasma P_i concentration (37). However, systematic analysis for K_m value is necessary to verify the control mechanism of oxidative metabolism in liver. The present systematic analysis of oxidative metabolism during urea synthesis and gluconeogenesis has revealed that oxidative metabolism may be controlled by feedback of increased P_i.

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