The energetics of flux through carbamyl phosphate synthetase and of citrulline formation from added ammonia, bicarbonate, and ornithine have been investigated in liver mitochondria from rats fed a high protein diet. In the presence of an oxidizable substrate, but in the absence of ornithine, carbamyl phosphate accumulated as a function of the medium phosphate concentration (Km~ 1.5 mm) up to values of 30 nmol/mg of protein. Upon addition of ornithine, citrulline was produced at the rate of 70 nmol/mg/min, and the carbamyl phosphate content fell to below 1 nmol/mg. The intramitochondrial ATP/ADP ratio decreased after ornithine addition, indicating that release of inhibition of carbamyl phosphate synthetase by carbamyl phosphate predominated over the expected inhibition due to the fall of the ATP/ADP ratio. Under partially uncoupled conditions in the presence of ornithine, citrulline formation decreased linearly with a fall of the calculated intramitochondrial MgATP/MgADP ratio.

Changes of the thermodynamic parameters of mitochondrial phosphorylation potential, ΔGp(m), proton electrochemical gradient, ΔpH+, and oxidation-reduction potential difference between NAD+ and cytochrome c, ΔEH, were measured under conditions of enhanced respiration induced by citrulline synthesis and compared with ADP-stimulated respiration. Under both conditions, ΔGp(m) decreased and ΔEH also decreased due to a net oxidation of NADH and reduction of cytochrome c. However, ΔpH+ showed no change after citrulline addition although it decreased during ADP-stimulated respiration. The average H+/2e stoichiometry over the first two phosphorylation sites calculated from the ΔEH/ΔpH+ ratio ranged from 3.0 to 3.5, while the H+/ATP stoichiometry calculated from the ΔGp(m)/ΔpH+ ratio ranged from 2.0 to 2.5. The calculated ratios of H+/2e and H+/ATP both increased as ΔpH+ was lowered by addition of an uncoupling agent. The overall data are apparently not in accordance with the commonly held view that ΔpH+ is an obligatory intermediate between the oxidation-reduction pumps of the respiratory chain and ATP synthase.

The first step in the disposal of ammonia via the urea cycle is the intramitochondrial production of carbamyl phosphate from NH₄⁺, HCO₃⁻, and 2 mol of MgATP²⁻. This energy-dependent reaction, mediated by carbamyl phosphate synthetase, also has an absolute requirement for Mg²⁺ and N-acetylglutamate and is considered the primary site for regulation of the urea cycle in vivo (1–3). The second reaction of the urea cycle, also intramitochondrial, is the carbamylation of ornithine to citrulline by ornithine transcarbamylase. Since this reaction has a much higher capacity than carbamyl phosphate synthetase (4), flux is readily monitored in isolated mitochondria by measuring the rate of citrulline synthesis in media supplemented with ammonia, bicarbonate, ornithine, and a respiratory substrate. Citrulline synthesis by coupled mitochondria is associated with a 2- to 3-fold stimulation of respiration in accordance with enhanced rates of formation and utilization of intramitochondrial ATP (5–7). Under these conditions citrulline synthesis is independent of the presence of atracyloside, an inhibitor of ATP and ADP transport across the mitochondrial membrane.

When an extramitochondrial ATP-trapping system such as glucose and hexokinase is added to mitochondria to induce flux through the adenine nucleotide translocator, the rates of respiration and glucose 6-phosphate formation are inversely related to the extramitochondrial ATP/ADP ratio in the range from 5 to 100 (8–11). A high extramitochondrial ATP/ADP ratio corresponds to the resting state 4 condition and near thermodynamic equilibrium is thought to be achieved between the extramitochondrial phosphorylation potential, the proton electrochemical gradient across the mitochondrial membrane and the oxidation-reduction potentials of the electron transport carriers of the respiratory chain (12–14).

Respiratory stimulation associated with ATP synthesis may thus be induced either by a fall of the extramitochondrial ATP/ADP ratio, where its effects are exerted on the intramitochondrial ATP/ADP ratio through the adenine nucleotide translocator, or directly on the intramitochondrial ATP/ADP ratio through a stimulation of citrulline synthesis. Both effects are expected to induce alterations in the thermodynamic relationships between the energy-transducing parameters of oxidation-reduction potential free energy, proton electrochemical gradient, and phosphorylation potentials (15). When both glucose 6-phosphate and citrulline synthesis are allowed to occur simultaneously, there is a competition for ATP produced by oxidative phosphorylation between ATP export on the adenine nucleotide translocator and ATP utilization by carbamyl phosphate synthetase such that the rate of citrulline synthesis decreases as the extramitochondrial ATP/ADP ratio falls (6, 11, 16–18). However, the nature of this competition in relation to the matrix concentrations of ATP, ADP, and Mg²⁺ is not yet fully understood (18, 19).

The primary purpose of the present experiments was to investigate the steady state relationships between the intramitochondrial ATP/ADP ratio, the phosphorylation potential, the mitochondrial proton electrochemical gradient, and the oxidation-reduction potentials of the pyridine nucleotides and cytochrome c in mitochondria before and after stimulation of citrulline synthesis by addition of ornithine. These
data are compared with results obtained after stimulation of respiration by addition of glucose and hexokinase as an extra-
mitochondrial ATP-trapping system. Rats were fed a high protein diet, which augments the intramitochondrial level of
N-acetylimidazole, maximizes rates of citrulline synthesis, and
increases respiration to about two-thirds of the rate achieved with glucose and optimal hexokinase (11, 20). In addition, the relationship between flux through carbamyl phosphate synthetase, as measured by citrulline formation, and the intramitochondrial ATP/ADP ratio has been inves-
tigated by varying the phosphate concentration and using submaximal concentrations of uncoupling agents to decrease the coupling between respiration and phosphorylation. These studies permitted calculation of the thermodynamic stoichiometries between the free energy in the oxidation-reduction
span from NADH to cytochrome c (ΔG°), the proton electrochemical gradient across the mitochondrial membrane (ΔpH), and the intramitochondrial phosphorylation potential (ΔGp(m)). The data provide information on steady state values for the H+/2e− ratio of the respiratory chain electrogenic proton pump, and the H+/ATP ratio of the mitochondrial ATP synthase in the mitochondria under different conditions of respiratory state and coupled oxidative phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Preparation of Rat Liver Mitochondria**—Male, Sprague-Dawley rats (approximately 200 g) were maintained on a high protein diet (80% casein) for 2 to 3 weeks prior to killing. Liver mitochondria were prepared by differential centrifugation essentially as described by Schneider and Hoogboom (21) except that the homogenizing medium consisted of 300 mM mannitol and 0.1 mM EDTA, pH 7.0. Sucrose was omitted from all experiments since it interferes with the colorimetric assay of citrulline (5). Respiratory control ratios were tested using 10 mM glutamate and 1 mM malate as substrate, and was 7 to 11 in normal medium containing 100 mM KCl, 5 mM KH2PO4, 20 mM MOPS, 2 mM MgCl2, and 1 mM EDTA, pH 7.2, and 3.5 to 5 in citrulline synthesis medium (see below).

**Incubation of Mitochondria**—The medium found to provide optimal rates of citrulline formation (citrulline synthesis medium) was similar in essentials to that of Charles et al. (5) and consisted of 300 mM mannitol and 0.1 mM EDTA, pH 7.0. Sucrose was omitted from all experiments since it interferes with the colorimetric assay of citrulline (5). Respiratory control ratios were tested using 10 mM glutamate and 1 mM malate as substrate, and was 7 to 11 in normal medium containing 100 mM KCl, 5 mM KH2PO4, 20 mM MOPS, 2 mM MgCl2, and 1 mM EDTA, pH 7.2, and 3.5 to 5 in citrulline synthesis medium (see below).

**Assay of Metabolites**—Mitochondrial protein was deter-
mined by a modification of the biuret procedure using crystalline bovine serum albumin as standard (25). Citrulline was assayed colorimetrically according to the method of Guthohrlein and Knappe (26). Inorganic phosphate was assayed according to Treub and Derr (27), with the modification that the final sample volume was decreased to 1.2 ml and the optical densities were measured by a semiautomatic spectrophotometer. Ketone bodies and glucose-6-phosphate were determined by spectrophotometric enzyme assay procedures (28), while ATP and ADP were measured fluorometrically (29). The car-
bamyl phosphate content of the mitochondrial matrix was measured by a radioactive method according to Meijer.1 The normal mitochondrial incubation medium (with no added ornithine) was modified to contain 11.6 mM bicarbonate, and 3 ml of buffer equilibrated with 95% O2 plus 5% CO2 was added to 5 ml vials and sealed. The reaction was started by addition of mitochondria (12 mg of protein) and 5 mM [14C]bicarbonate and the incubation was continued with shaking at 25°C for the desired time interval (usually 8 min for steady state conditions). A similar vial with all ingredients except mitochondria was run in parallel for determination of the bicarbonate specific activity in the medium. At the end of the incubation, duplicate 1-ml aliquots of the mitochondrial suspension were rapidly centrifuged through silicone oil into 0.4 ml of buffer containing 20 mM MOPS, 0.5 mM mannitol, 10 mM EDTA, 2% Lubrol, and oligomycin (1 mg/ml of mitochondrial protein), pH 7.4. This buffer inactivated carbamyl phosphate synthetase and prevented further formation of carbamyl phosphate. Imme-
diately after the centrifugation, the supernatant was aspirated, and 10 ml of 5% trichloroacetic acid was added to each of the duplicate samples to allow conversion of [14C]carbamyl phosphate to
[14C]citrulline by action of ornithine transcarbamylase liberated from the lysed mitochondria. The second tube was used as a radioactive blank. After incubating the tubes for 15 min at 25°C, the reaction was stopped by addition of perchloric acid (final concentration 4%) and the tubes were placed in a boiling water bath for 3 min to complete removal of CO2. The residual counts in the blank tube were subtracted from the counts in the tubes containing ornithine for calculation of the carbamyl phosphate content of the original mito-
chondria. Recoveries by colorimetric citrulline assay showed that 92%

1 The abbreviations used are: MOPS, 3-(N-morpholino)pro-
panesulfonic acid; EGTA, ethyleneglycol bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; MES, 2-(N-morpholino)ethanesulfonic acid; TPMP+, triphenylmethyl phosphonium; DMO, 5,5-dimethyllox-
azolinedione-2,4-dione; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl-
yldrazone.

2 A. J. Meijer, personal communication.
to 96% of the carbamyl phosphate was converted to citrulline. This method has the advantage of high sensitivity, but a more direct assay based on the colorimetric determination of citrulline produced from carbamyl phosphate has been described recently (30).

**Measurement of Oxidation Reduction Potentials.**—The NAD⁺ oxidation-reduction state was estimated by three separate methods. The first was from measurements of the β-hydroxybutyrate/acetoacetate ratio, the matrix pH, and an assumed equilibrium of β-hydroxybutyrate dehydrogenase (Kₑₐ = 4.93 × 10⁻¹⁰). The second was from measurements of pyridine nucleotide fluorescence changes (396 nm excitation, 420 to 530 nm band pass emission) using a D. C. filter fluorometer manufactured by the Johnson Research Foundation (31, 32), and the third, spectrophotometrically by following absorption changes at 340 to 375 nm in a DW2a dual wavelength spectrophotometer (American Instrument Co.) fitted with a magnetic stirrer. In methods two and three, calibration was achieved by determining the fully reduced and fully oxidized state of the pyridine nucleotides. Complete oxidation was achieved by a careful titration with the uncoupler FCCP. After each addition of FCCP, oxidation of NADH occurred rapidly in a stepwise manner reaching a maximum at about 2 μM FCCP. Complete NADH reduction was obtained by addition of 0.5 μM of amcin A or 1 mM KCN. The NAD⁺ oxidation-reduction state, Eₙ(NAD⁺), under different experimental conditions was calculated according to the Nernst equation:

\[ E'_m = E'_n + \frac{2.3 \, R \, T \, n \, F \, \log (NAD^+/NADH)}{n \, F} \]  

where E'_m is the condition-dependent midpoint potential, is calculated from the standard NAD⁺ midpoint potential at pH 7.0 and 25 °C of -320 mV according to equation 2:

\[ E'_n = 220 + \frac{2.3 \, R \, T \, n \, F \, (7.00 - pH_n)}{n \, F} \]

pHₙ is defined as the mitochondrial matrix pH and the constant 2.3 RT/nF is 29.6 when n = 2 at 25 °C.

The cytochrome c oxidation-reduction state, E'_c(cyt c) was measured spectrophotometrically in the DW2a spectrophotometer using the wavelength pair 540 to 550 nm. Full oxidation was achieved by the addition of FCCP or 0.5 μg/ml of antimycin A and full reduction by N₃ anoxia or addition of 1 mM KCN. The cytochrome c oxidation-reduction state was calculated according to equation 3:

\[ E'_c(cyt c) = E'_m + \frac{2.3 \, R \, T \, F \, \log (cyt c^+/cyt c^-)}{F} \]

where Eₙ is the pH-independent midpoint potential of cytochrome c, is 235 mV (33). The oxidation-reduction potential span (ΔEₖ) from NADH to cytochrome c was calculated according to equation 4:

\[ \Delta Eₖ = E'_c(cyt c) - E'_n(NAD^+) \]

**Calculation of Mitochondrial Inorganic Phosphate.**—At relatively low extramitochondrial phosphate concentrations (up to 2 mM), the distribution of phosphate across the mitochondrial membrane is in approximate equilibrium with the ΔpH according to the equation 5 (34, 35):

\[ \log \left( \frac{P_m^-}{P_e^-} \right) = -n \, \Delta pH \]

where Pₐ and Pₑ are the internal and external mitochondrial phosphate concentrations. This equation, along with the Henderson-Hasselbach relationship for the ionization of phosphate, allows calculation of the intramitochondrial phosphate concentration from a knowledge of the extramitochondrial phosphate concentration and the pH of the extra- and intramitochondrial spaces according to equation 6:

\[ P_m^- = P_e^-[10^{\frac{pK}{2}}(1 - 1/1 + 10^{\frac{pK}{2}})] \times 10^{\frac{pH - 7.2}{2}} \]

where pK for H₂PO₄⁻/HPO₄²⁻ = 7.2, and pKₐ is the extramitochondrial pH.

**Calculation of Phosphorylation Potential.**—The phosphorylation potential of the adenine nucleotides in the mitochondrial matrix (ΔGₚ) in millivolts is calculated according to equation 7:

\[ \Delta Gₚ = \Delta G'_c + 2.3 \, R \, T \, F \, \log \left( \frac{ATP}{ADP \cdot P} \right) \]

where Pₑ is the inorganic phosphate concentration in moles per liter and ΔGₚ is the standard phosphorylation potential, which at Mg²⁺ = 1 mM and pH 7.8 was taken to be 330 mV (12, 36). The standard phosphorylation potential was not corrected for the small changes of matrix pH observed after ornithine addition, which would raise the value by no more than 0.5%.

**Calculation of Free and Mg²⁺-chelated Adenine Nucleotides.**—The free Mg²⁺ concentration of the mitochondrial matrix with mito-

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**RESULTS**

**Optimization of Citrulline Synthesis.**—The effect of variation of medium components on the stimulation of respiration induced by ornithine was measured polarographically. An optimal NH₄Cl concentration of 10 mM was determined, in agreement with Charles et al. (5). The ornithine-induced stimulation of respiration increased by 26% after addition of 2 mM MgCl₂, and was further increased by 1 mM EGTA. These findings are in accordance with recent observations that carbamyl phosphate synthetase is inhibited by matrix Ca²⁺ and that this inhibition is counteracted by Mg²⁺ (38, 39). Respiratory stimulation upon ornithine addition was independent of pH over the range 7.0 to 7.8 and of toxicity from 150 to 300 mosmol. In contrast with the data reported by Charles et al. (5), we found that the rate of citrulline production was 38% lower with 10 mM pyruvate than with 10 mM glutamate as substrate (cf. 30). Succinate also led to lower rates of citrulline synthesis.

Different medium components altered the state 4 rate of respiration of the mitochondria. Addition of bicarbonate and NH₄Cl separately decreased the respiratory control ratio at concentrations as low as 1.6 mM NH₄Cl or 5 mM bicarbonate, while the state 5 rate remained unchanged. The effect of NH₄⁺ is in accordance with an uncoupling like effect due to the transhydrogenase activity of glutamate dehydrogenase combined with a stimulation of the energy-linked transhydrogenase (34). Hydrolysis of carbamyl phosphate may also account for a lowering of the respiratory control ratio.

**Effects of Ornithine on Mitochondrial Energy State.**—In order to investigate the effect of ornithine addition on changes of the intramitochondrial adenine nucleotides and other parameters of the mitochondrial energy state, liver mitochondria from rats fed a high protein diet were incubated for 32 min with the standard medium required for citrulline synthesis in the presence of 10 mM glutamate as respiratory substrate and 3 μg/mg of protein of carboxyatractlyoside to inhibit the adenine nucleotide translocator. In the experiment shown in

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**Energetics of Citrulline Synthesis**

**Fig. 1. Time course of citrulline formation.** Liver mitochondria (4 mg/ml) from rats fed a high protein diet were incubated with citrulline synthesis medium with 10 mM glutamate as substrate (see "Experimental Procedures") for 16 min prior to addition of 10 mM ornithine.

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**3** J. R. Williamson and B. E. Corkey, unpublished experiments.
Energetics of Citrulline Synthesis

Fig. 1, ornithine (10 mM) was added after 16 min of incubation, and samples were removed for metabolic analyses at 4-min intervals prior to and after ornithine addition. Fig. 1 shows that after an initial lag period, the rate of citrulline formation became linear at a rate of 70 nmol/mg/min compared with rates of about 20 nmol/mg/min observed with normal rat liver mitochondria incubated with glutamate as substrate (41). This lag is typical for glutamate as respiratory substrate, and is also seen in the stimulation of respiration produced after ornithine addition. It is probably caused by an increase of N-acetylglutamate synthesis from acetyl-CoA and glutamate during the experiment (41). This cofactor is an obligatory allosteric activator of carbamyl phosphate synthetase (42). In parallel experiments where oxygen consumption was also measured, the calculated P/O ratio was 3.10 ± 0.14 (22 observations) based on 2 mol of ATP utilized/mol of citrulline formed. This compares with a mean value of 1.86 ± 0.13 (18 observations) for the P/O ratio for glucose 6-phosphate formation in the same medium with glutamate as substrate but in the absence of ornithine and carboxyatractyloside (7). The higher P/O ratio for citrulline synthesis compared with glucose 6-phosphate formation relates to the fact that under the latter conditions ATP must be transported out of the mitochondria via the energy consuming adenine nucleotide translocator.

Fig. 2A illustrates that ornithine addition to mitochondria incubated in the citrulline synthesis medium caused a prompt fall of intramitochondrial ATP and rise of ADP. This was reflected by about a 50% fall of the intramitochondrial ATP/ADP ratio from initial values of 1.15 (Fig. 2B). Increased flux through the electron transport chain induced by the stimulation of ATP utilization is thus associated with a fall of the phosphorylation state of the intramitochondrial adenine nucleotides.

Assay of the total inorganic phosphate in the mitochondrial extracts showed that there was an apparent large fall instead of the expected small increase on the basis of the 1.5 nmol/mg drop of the ATP content. The spurious values for assayed inorganic phosphate were caused by the rapid hydrolysis of carbamyl phosphate, which accumulated prior to ornithine addition. The amount of carbamyl phosphate accumulation in the mitochondria depended on the concentration of inorganic phosphate in the incubation medium, as illustrated in Fig. 3. In the absence of added phosphate, the carbamyl phosphate content was about 2 nmol/mg of protein, and reached a maximum of almost 30 nmol/mg of protein with 5 mM phosphate. In each case measurements were made after 8 min of incubation. After ornithine addition, the carbamyl phosphate levels decreased to about 0.5 nmol/mg of protein.

Since accurate values for the intramitochondrial phosphate concentration were required for calculation of the adenine nucleotide phosphorylation potential, estimates of the mitochondrial phosphate concentration in the absence of ornithine were made, (a) by subtracting the separately assayed carbamyl phosphate from the assayed inorganic phosphate, and (b) by calculating the matrix phosphate from the medium phosphate concentration and the pH gradient across the mitochondrial membrane. These data are summarized in Table I for incubation conditions with 1 mM phosphate and either glutamate alone or glutamate plus malate as substrate. Rates of citrulline formation were 69 nmol/mg/min, with no effect of malate addition (cf. 41). In the absence of ornithine, about

![Figure 2A](image1.png)  Effect of ornithine on intramitochondrial ATP and ADP contents (A) and ATP/ADP ratio (B) in liver mitochondria from rats fed a high protein diet. The incubation conditions were the same as Fig. 1.

![Figure 2B](image2.png)

![Figure 3](image3.png)  Effect of inorganic phosphate on accumulation of carbamyl phosphate in liver mitochondria from rats fed a high protein diet. Mitochondria were incubated in the absence of ornithine in citrulline synthesis medium with 10 mM glutamate as substrate.

**Table 1**

Comparison of calculated and assayed inorganic phosphate contents of liver mitochondria in the presence and absence of ornithine

Liver mitochondria (4 mg/ml) from rats fed a high protein diet were incubated in citrulline synthesis buffer, pH 7.2, containing 1 mM phosphate for 22 min, with 10 mM ornithine added after 10 min. Substrate concentrations were: glutamate, 10 mM; malate, 1 mM. Values shown are means ± S.E. of four to six determinations. The volume of the mitochondrial matrix space was 0.86 ± 0.02 μl/mg of protein.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ornithine</th>
<th>ΔpH</th>
<th>Carbamyl phosphate</th>
<th>P, measured</th>
<th>P, from ΔpH</th>
<th>P, corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>P, mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Glutamate</td>
<td>~</td>
<td>0.51 ± 0.01</td>
<td>12.6 ± 0.6</td>
<td>18.6 ± 0.6</td>
<td>6.3 ± 0.4</td>
<td>6.0 ± 0.4</td>
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<tr>
<td></td>
<td>+</td>
<td>0.56 ± 0.01</td>
<td>12.6 ± 0.6</td>
<td>7.8 ± 0.4</td>
<td>8.4 ± 0.1</td>
<td>6.8 ± 0.1</td>
</tr>
<tr>
<td>Glutamate plus malate</td>
<td>~</td>
<td>0.49 ± 0.02</td>
<td>13.6 ± 0.4</td>
<td>20.4 ± 1.3</td>
<td>7.4 ± 0.4</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.55 ± 0.01</td>
<td>13.6 ± 0.4</td>
<td>20.4 ± 1.3</td>
<td>7.4 ± 0.4</td>
<td>7.8 ± 0.2</td>
</tr>
</tbody>
</table>

* The intramitochondrial P, was calculated from the measured pH of the medium and matrix spaces using a pKₐ of 7.31 for H₄PO₄⁻/H₂PO₄⁻.

* The measured P, concentration in the matrix was corrected for the carbamyl phosphate concentration.
12 nmol/mg of protein of carbamyl phosphate was formed and the measured phosphate after correction for carbamyl phosphate agreed closely with values calculated from the ΔpH. In the presence of ornithine, the directly measured phosphate values agreed with those calculated from the ΔpH. Consequently, in further work with 1 mM phosphate, matrix phosphate concentrations both in the absence and presence of ornithine were calculated from measurements of the extra-mitochondrial phosphate concentration and the ΔpH.

Fig. 4A shows that the intramitochondrial adenine nucleotide phosphorylation potential, ΔGp(m), fell by about 20 mV after ornithine addition. This was associated with a fall of the membrane potential, ΔΨ, of about 3.5 mV (Fig. 4B) and an approximately equivalent rise of ΔpH (Fig. 4C). Consequently, the proton electrochemical gradient, ΔµH⁺ (defined here as ΔΨ + 59.2 ΔpH), showed no statistically significant change after ornithine addition. The effect of ornithine to diminish ΔΨ and increase ΔpH, thereby producing no statistically significant change of ΔµH⁺, was confirmed in a separate series of experiments with 10 mM glutamate and 1 mM malate as respiratory substrate (Fig. 5) rather than 10 mM glutamate alone (Fig. 4). The increase of ΔpH may be accounted for by the fact that ornithine is transported across the mitochondrial membrane electroneutrally by exchange with a proton (43), which would tend to increase the alkalinity of the matrix space (see also 44).

The change of the mitochondrial phosphorylation potential after ornithine addition also caused perturbations of the oxidation-reduction state of the components of the electron transport chain. Fig. 6 illustrates the results of a typical experiment with glutamate as substrate in which the pyridine nucleotide oxidation-reduction state was measured by changes of pyridine nucleotide fluorescence, while changes of the cytochrome c oxidation-reduction state were measured spectrophotometrically. As seen from Fig. 6, ornithine caused a relatively slow oxidation of the pyridine nucleotides and a rapid reduction cytochrome c. However, the simultaneous changes of the β-hydroxybutyrate/acetoacetate ratio as an alternative monitor of the NAD⁺ oxidation-reduction state were very small. Analyses of NAD⁺ and NADP⁺ and their reduced forms in mitochondria under similar conditions incubated with glutamate alone as substrate showed that NADH decreased from 0.53 ± 0.07 to 0.46 ± 0.03 nmol/mg while NADPH decreased from 3.33 ± 0.06 to 2.83 ± 0.10 nmol/mg (six determinations). Likewise, the change of the NAD⁺/NADH ratio was small (from 7.8 to 9.4), while the change of the NADP⁺/NADPH ratio was much greater (from 0.14 to 0.39). Consequently, the decrease of the pyridine nucleotide fluorescence in Fig. 6 can be attributed primarily to an oxidation of the mitochondrial NAD⁺ system. This effect on NADPH oxidation, however, was not observed when glutamate plus malate was used as substrate.

The effects of ornithine on the NAD⁺ oxidation-reduction state calculated from the β-hydroxybutyrate/acetoacetate ratio and on the cytochrome c oxidation-reduction state for glutamate alone and glutamate plus malate conditions are summarized in Table II. Similar effects of ornithine were observed in the absence and presence of malate, namely a small oxidation of NADH and a larger reduction of cytochrome c, despite the fact that malate addition caused a 31 mV reduction of the NAD⁺ system. Consequently, the stimulation of electron transport flux by ornithine addition caused a contraction in the oxidation-reduction span from NADH to cytochrome c (ΔEₐ) of 12 to 13 mV.

The thermodynamic parameters of adenine nucleotide phosphorylation potential (ΔGp(m)), proton electrochemical...
gradient ($\Delta \tilde{\mu}H^+$) and oxidation-reduction span for the first two phosphorylation sites ($\Delta E_i$) in two separate series of experiments with either glutamate alone or glutamate plus malate as respiratory substrate, are summarized in Table III. Ornithine addition produced qualitatively similar changes in both experiments, namely a fall of $\Delta Gp(m)$ and $\Delta E_i$ and no change of $\Delta \tilde{\mu}H^+$. Malate addition caused a significant reduction of the pyridine nucleotides without a corresponding change of the cytochrome c oxidation-reduction state, so that $\Delta E_i$ increased (cf. Tables II and III). The decrease of $\Delta E_i$ with ornithine was the same in the absence and presence of malate, but the decrease of $\Delta Gp(m)$ was smaller. Table III shows that the ratio $\Delta Gp/m/\Delta \tilde{\mu}H^+$, which represents the proton stoichiometry for intramitochondrial ATP synthesis ($H^+/ATP$) was in the range of 2.1 to 2.5. Similarly, values for the ratio of $\Delta E_i/\Delta \tilde{\mu}H^+$, which represents the average $H^+/2e^-$ stoichiometry over the first two phosphorylation sites, was in the range of 3.1 to 3.5. On the other hand, the ratio of $\Delta E_i/\Delta Gp(m)$ was remarkably constant under all conditions (range 1.38 to 1.44).

Effects of Extramitochondrial Glucose-Hexokinase Trap on Mitochondrial Energy State—In another series of experiments, mitochondria were incubated in the citrulline synthesis buffer with 10 mM glutamate and 1 mM malate as respiratory substrate in the absence of ornithine and respiratory stimulation was induced by addition of 10 mM glucose and 3 units/ml of hexokinase. Fig. 7 illustrates that the transition from state 4 to state 3 respiratory activity was associated with an oxidation of the pyridine nucleotides and a reduction of cytochrome c. Data from five paired experiments showed that the NAD$^+$ oxidation-reduction state changed by 10.3 ± 2.4 mV while the cytochrome c oxidation-reduction state changed by 5.4 ± 2.1 mV.

The changes of the intramitochondrial ATP/ADP ratio, phosphorylation potential, and oxidation-reduction span from NADH to cytochrome c were similar to those produced by the addition of ornithine to stimulate intramitochondrial ATP utilization (Table IV). In contrast to the lack of effect of $\Delta \tilde{\mu}H^+$ when respiration was stimulated by addition of ornithine, addition of ADP to induce state 3 respiration caused a significant fall of $\Delta \tilde{\mu}H^+$. Values for the ratios of $\Delta Gp(m)/\Delta \tilde{\mu}H^+$ and $\Delta E_i/Gp(m)$ were similar to those in Table III and showed no change between state 4 and 3 conditions. When ornithine was added to mitochondria under conditions of nonmaximal state 3 respiration (produced by addition of limiting hexokinase concentrations), there was a further fall of the intramitochondrial ATP/ADP ratio, but the effects on $E_a(NAD^+)$, $E_a$ (cytochrome c), and $\Delta \tilde{\mu}H^+$ were negligible (data not shown).

Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NAD$^+$ oxidation-reduction state</th>
<th>Change after ornithine</th>
<th>Cytochrome c oxidation-reduction state</th>
<th>Change after ornithine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>$-332 \pm 0.3$</td>
<td>$2.4 \pm 1.1^b$</td>
<td>$287 \pm 4.0$</td>
<td>$-9.5 \pm 1.5'$</td>
</tr>
<tr>
<td>Glutamate + malate</td>
<td>$-363 \pm 0.8$</td>
<td>$3.8 \pm 0.8^b$</td>
<td>$290 \pm 4.5$</td>
<td>$-9.2 \pm 2.2'$</td>
</tr>
</tbody>
</table>

$^a$ A positive change denotes an oxidation while a negative change denotes a reduction.

$^b$ p < 0.05

$^c$ p < 0.01

Table II

Effect of ornithine on NAD$^+$ and cytochrome c oxidation-reduction potentials

Liver mitochondria (4 mg/ml) from rats fed a high protein diet were incubated for 32 min in citrulline synthesis buffer, pH 7.2, containing 1 mM phosphate, with 10 mM ornithine added after 15 min. The NAD$^+$ oxidation-reduction potential was calculated from the β-hydroxybutyrate/acetocetate ratios between 12 and 16 min (control) and between 28 and 32 min (plus ornithine). Substrate concentrations were: glutamate, 10 mM; malate, 1 mM. Values shown are mean ± S.E. of eight determinations.

Liver mitochondria (4 mg/ml) from rats fed a high protein diet were incubated without ornithine in citrulline synthesis medium with 10 mM glutamate and 1 mM malate as substrates. Stoichiometric relationships between $\Delta Gp(m)$, $\Delta \tilde{\mu}H^+$, and $\Delta E_i$ in rat liver mitochondria

Liver mitochondria (4 mg/ml) from rats fed a high protein diet were incubated in citrulline synthesis buffer, pH 7.2, containing 1 mM phosphate for 32 min. Ornithine (10 mM) was added after 15 min. Substrate concentrations were: glutamate, 10 mM; malate, 1 mM. Samples were removed for analyses at 2-min intervals. Values shown for $\Delta Gp(m)$ and $\Delta \tilde{\mu}H^+$ are means ± S.E. for averages of the determinations before and after ornithine addition for four separate experiments. Values for $\Delta E_i$ represent the mean ± S.E. for seven determinations.

Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Ornithine</th>
<th>$\Delta Gp(m)$</th>
<th>$\Delta \tilde{\mu}H^+$</th>
<th>$\Delta E_i$</th>
<th>$\Delta Gp(m)/\Delta \tilde{\mu}H^+$</th>
<th>$\Delta E_i/\Delta Gp(m)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>-</td>
<td>$450 \pm 0.4$</td>
<td>$177 \pm 0.6$</td>
<td>$619 \pm 4$</td>
<td>$2.54$</td>
<td>$3.50$</td>
</tr>
<tr>
<td>Change</td>
<td>$-193 \pm 0.9^*$</td>
<td>$0 \pm 0.7$</td>
<td>$-12 \pm 2^*$</td>
<td>$2.18$</td>
<td>$3.15$</td>
<td>$1.44$</td>
</tr>
<tr>
<td>Glutamate + malate</td>
<td>+</td>
<td>$452 \pm 0.8$</td>
<td>$207 \pm 1.9$</td>
<td>$653 \pm 5$</td>
<td>$2.14$</td>
<td>$3.08$</td>
</tr>
<tr>
<td>Change</td>
<td>$-7 \pm 1.1^*$</td>
<td>$1 \pm 2.1$</td>
<td>$-13 \pm 2^*$</td>
<td>$2.14$</td>
<td>$3.08$</td>
<td>$1.44$</td>
</tr>
</tbody>
</table>

$^*$ By the Student t test, p < 0.01.
Table IV
Stoichiometric relationships between ΔGp(m), ΔμH⁺, and ΔEₗ in rat liver mitochondria during state 4 and state 3 conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>ATP (ADP)</th>
<th>ΔGp(m)</th>
<th>ΔμH⁺</th>
<th>ΔEₗ</th>
<th>ΔGp(m)</th>
<th>ΔμH⁺</th>
<th>ΔEₗ</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 4</td>
<td>1.83 ± 0.10</td>
<td>442 ± 2.2</td>
<td>196 ± 1.9</td>
<td>628 ± 2.2</td>
<td>2.26</td>
<td>3.20</td>
<td>1.42</td>
</tr>
<tr>
<td>State 3</td>
<td>1.47 ± 0.14</td>
<td>437 ± 1.7</td>
<td>188 ± 4.2</td>
<td>612 ± 1.5</td>
<td>2.32</td>
<td>3.25</td>
<td>1.40</td>
</tr>
<tr>
<td>Change</td>
<td>−0.36 ± 0.17</td>
<td>−5 ± 2.4</td>
<td>−8 ± 4.6</td>
<td>−16 ± 3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05.

**Fig. 8. Relationship between the rate of citrulline synthesis and the mitochondrial ATP/ADP ratio.** Liver mitochondria (3 mg/ml) from rats fed a high protein diet were incubated in citrulline synthesis medium containing 0 to 6 mm inorganic phosphate and 10 mM glutamate.

Table V
Effect of varying external phosphate on mitochondrial energy state

<table>
<thead>
<tr>
<th>Phosphate ADP</th>
<th>ATP (ADP)</th>
<th>ΔGp(m)</th>
<th>ΔμH⁺</th>
<th>Δψ</th>
<th>Δψ/Eₗ(NAD⁺)</th>
<th>Δψ/(c)</th>
<th>ΔEₗ</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
<td>mV</td>
</tr>
<tr>
<td>No ornithine</td>
<td>0.33</td>
<td>456</td>
<td>0.67</td>
<td>157</td>
<td>176</td>
<td>−336</td>
<td>255</td>
</tr>
<tr>
<td></td>
<td>1.07</td>
<td>456</td>
<td>0.56</td>
<td>140</td>
<td>173</td>
<td>−331</td>
<td>251</td>
</tr>
<tr>
<td></td>
<td>1.51</td>
<td>451</td>
<td>0.51</td>
<td>142</td>
<td>172</td>
<td>−321</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>1.78</td>
<td>444</td>
<td>0.44</td>
<td>140</td>
<td>166</td>
<td>−317</td>
<td>262</td>
</tr>
<tr>
<td>Plus ornithine</td>
<td>0.24</td>
<td>433</td>
<td>0.66</td>
<td>138</td>
<td>178</td>
<td>−335</td>
<td>255</td>
</tr>
<tr>
<td></td>
<td>0.68</td>
<td>440</td>
<td>0.60</td>
<td>138</td>
<td>174</td>
<td>−327</td>
<td>257</td>
</tr>
<tr>
<td></td>
<td>1.11</td>
<td>456</td>
<td>0.58</td>
<td>141</td>
<td>176</td>
<td>−320</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
<td>427</td>
<td>0.52</td>
<td>138</td>
<td>169</td>
<td>−314</td>
<td>259</td>
</tr>
</tbody>
</table>

* p < 0.01.

In order to investigate the relationship between rates of citrulline synthesis and the mitochondrial ATP/ADP ratio in greater detail, various concentrations of the uncoupler FCCP from 0.006 to 0.12 nmol/mg were added to mitochondria incubated in the citrulline synthesis medium in the presence of ornithine. Fig. 9 shows that the relationship between the total matrix ATP/ADP ratio and the ΔμH⁺ was nonlinear, such that at high levels of ΔμH⁺ a small change caused a large change of the ATP/ADP ratio, while at low levels of ΔμH⁺ a large change caused only a small change of the ATP/ADP ratio. Under control conditions, the matrix free Mg²⁺ concentration was 1.3 mM. This is well above the reported Km of 0.2 mM for free Mg²⁺ activation of isolated carbamyl phosphate synthetase (45). Using this value, and from a knowledge of the matrix concentrations of total ATP, ADP, and inorganic phosphate, the matrix pH and the Mg²⁺ dissociation constants of the different ligand-binding species, the bound Mg²⁺, and the concentration of Mg-bound nucleotides were calculated. The amount of Mg²⁺ bound to nucleotides and phosphate was 12.5 nmol/mg compared with a total assayed magnesium content of 38.4 nmol/mg, which did not change after addition of uncoupler. The amounts of ATP, ADP, and Pi in the free forms were about 1%, 14%, and 90%, respectively. For the purposes of the calculations for the uncoupler-treated mitochondria, the free plus nucleotide-bound Mg²⁺ was maintained constant, and as the ATP/ADP ratio fell, the calculated free...
MgADP ratio. Under control conditions, the matrix MgATP concentration is about 7 mM while the MgADP concentration of citrulline synthesis was linearly related to the MgATP/MgADP ratio. Inhibition by MgADP would be expected to exert a strong regulatory influence on flux through carbamyl phosphate synthetase.

The degree of coupling between electron transport and the phosphorylation of proteins is assumed to remain at the normal concentration of HC03- in the mitochondria, while the Kt for phosphate is very high. In addition, the enzyme requires free Mg2+ as cofactor, which must bind before MgATP. Also, N-acetylglutamate is an obligatory allosteric activator (45, 50). The concentrations of cofactors as well as the other substrates and products in mitochondria are normally within the regulatory range of their kinetic constants, thus making it more difficult to evaluate the regulatory significance of a particular parameter when several are varying simultaneously.

In the present experiments, NH4+, HCO3-, and Mg2+ are maintained well above their Kt values, while use of high protein-fed rats and glutamate as respiratory substrate assures a high intramitochondrial concentration of N-acetylglutamate (20, 52). Consequently, the main experimental variables to account for flux changes through carbamyl phosphate synthetase are MgATP2-, MgADP2-, and carbamyl phosphate. With ornithine present, so that flux through carbamyl phosphate synthetase is coupled with ornithine transcarbamylase, carbamyl phosphate is decreased to a low, constant level. Under these defined experimental conditions, the rate of citrulline synthesis should be a function of MgATP/MgADP. In the absence of ornithine, it is apparent from the data in Fig. 5 and respiratory studies (not shown) that flux becomes limited due to the accumulation of carbamyl phosphate to 3 times greater than its Kt value of 10 mM (45). Hence, the reported stimulatory effect of ornithine on carbamyl phosphate synthetase (53) can probably be accounted for largely on the basis of removal of carbamyl phosphate as the inhibitory reaction product. This effect may also be responsible for the ornithine-induced stimulation of urea formation in isolated hepatocytes incubated with ammonia (54, 55). In a recent paper, somewhat similar conclusions have been reached by Cohen et al. (56), although their experimental data differed from ours in several important respects. Notably, they observed a marked fall of mitochondrial carbamyl phosphate after an initial increase to about 16 mM at 2 min in the absence of ornithine, without a stimulation of flux. In our hands, the mitochondrial carbamyl phosphate content was stable at least over 20 min. Since Cohen et al. (56) observed that only 14% of the total carbamyl phosphate in their mitochondrial suspensions was in the mitochondrial matrix after 10 min, differences of membrane
permeability to carbamyl phosphate may account for the different results.

The fall of carbamyl phosphate also accounts for the fact that addition of ornithine to mitochondria incubated in the presence of HCO₃⁻, NH₄⁺, and an oxidizable substrate causes a stimulation of citrulline formation but a fall of ATP content and the ATP/ADP ratio (Fig. 3). Similar results have been obtained by Letko and Kuster (11) and Wanders et al. (18). It remains to be determined whether increased mitochondrial concentrations of N-acetylglutamate also contribute to the increased flux through carbamyl phosphate synthetase after addition of ornithine since this activator can counteract a fall of the ATP/ADP ratio (18). On the other hand, citrulline synthesis decreases with a fall of the mitochondrial ATP content or the ATP/ADP ratio when phosphate is depleted (Fig. 8), after addition of uncoupling agents (Fig. 9) or by stimulation of ATP efflux from the mitochondria on the adenine nucleotide translocator (18), and increases with an increased ATP/ADP ratio by stimulation of substrate level phosphorylation, or by addition of ATP to uncoupled mitochondria in the presence of oligomycin (18, 19). At high ATP/ADP ratios, saturation characteristics are observed (e.g. Fig. 8). Thus, at low carbamyl phosphate concentrations, it is clear that citrulline synthesis increases as a function of the intramitochondrial ATP/ADP, or more accurately, the MgATP/MgADP ratio (cf. 17).

When citrulline synthesis is allowed to occur in the presence of an extramitochondrial ATP-trapping system, there is a competition for ATP between carbamyl phosphate synthetase and the adenine nucleotide translocator. Stimulation of flux through either process independently causes a fall of the intramitochondrial ATP/ADP ratio, and when both occur simultaneously there is a further fall of this ratio (18). However, the competition is unequal since increasing concentrations of hexokinase with a glucose-hexokinase ATP-trapping system causes a large inhibition of citrulline synthesis whereas ornithine addition in the presence of hexokinase causes only a relatively small inhibition of glucose 6-phosphate formation (18). This difference in sensitivity probably reflects differences in the kinetic regulation between the adenine nucleotide translocator and carbamyl phosphate synthetase. The adenine nucleotide translocator is regulated by the free ATP/ADP ratio on both sides of the mitochondrial membrane in a manner which is not yet completely defined kinetically (57-59). The translocator binds extramitochondrial ATP with a low Michaelis constant and is inhibited by extramitochondrial ATP (59). Likewise, intramitochondrial ATP serves as a substrate for the translocator while intramitochondrial ATP is probably inhibitory. However, the fact that ATP₃⁻ is exported in exchange with ADP₃⁻ by an electroneutral mechanism results in an unequal ratio of ATP/ADP between the extra- and intramitochondrial spaces. When conditions of equilibrium are established, intra- and extramitochondrial ATP and ADP are distributed in accordance with the membrane potential ∆ψ, such that:

$$\Delta \psi = 2.3 \frac{RT}{F} \log \left[ \frac{[\text{ATP}/\text{ADP}]_{\text{Mr}}}{[\text{ATP}/\text{ADP}]_{\text{Mr}}} \right]$$

where ([ATP]/[ADP]ₘᵣ and [ATP]/[ADP]ₘᵣ refer to the extra- and intramitochondrial spaces, respectively (60). With addition of glucose-hexokinase as an extramitochondrial ATP-utilizing system, the extramitochondrial ATP/ADP ratio falls, respiration is stimulated, and the right-hand side term of equation 8 falls to a greater extent that the fall of ∆ψ (61). In experiments under conditions similar to those of the present investigation, Letko and Kuster (11) found that with the combined glucose-hexokinase citrulline-synthesizing system, different rates of respiration were obtained at the same extramitochondrial ATP/ADP ratio, depending on the rate of citrulline synthesis. Since it is now known that the intramitochondrial ATP/ADP ratio falls when citrulline synthesis is stimulated under these conditions, it may be concluded from the present data and studies by Letko and Kuster (11), Kunz et al. (62), and Wanders et al. (18) that during competition for intramitochondrial ATP between the adenine nucleotide translocator and carbamyl phosphate synthetase, the translocator flux is regulated by both the extramitochondrial and intramitochondrial ATP/ADP ratio. From presently available data there is no need to postulate a compartmentation of intramitochondrial adenine nucleotides (19) or a special kinetic association between the adenine nucleotide translocator and either the ATP synthase or carbamyl phosphate synthetase (17).

While the adenine nucleotide translocator is regulated by the free ATP/ADP ratio, carbamyl phosphate synthetase is regulated by the intramitochondrial MgATP/MgADP ratio. From computer calculations with the present data, the mitochondrial free ATP/ADP ratio was linearly related to the MgATP/MgADP ratio in the proportion of 0.076:1. Changes of free Mg⁺ in the range from 1 to 3 mM exert little effect on the relationship between free ATP/ADP and MgATP/MgADP. Hence, it may be concluded that the relative flux through the adenine nucleotide translocator and carbamyl phosphate synthetase is under purely kinetic regulation.

**Control of Respiration with Intramitochondrial ATP-consuming Process—H⁺/2e⁻ and H⁺/ATP Stoichiometries—** It is clear from the present data that stimulation of mitochondrial citrulline synthesis and flux through the adenine nucleotide translocator both cause a fall of the intramitochondrial phosphorylation potential (∆Gₚ(m)). The question arises how these effects are coupled to a stimulation of electron transport flux and increased rate of ATP synthesis. According to the chemiosmotic theory of oxidative phosphorylation, electrogenic efflux of protons from the mitochondria generated by transfer of electrons through the respiratory chain from NADH to O₂ establishes a proton electrochemical gradient (∆A⁺H⁺) across the mitochondrial inner membrane (14). Synthesis of ATP from intramitochondrial ADP and P, occurs via the Mg²⁺-dependent ATP synthase spanning the mitochondrial membrane, and is driven by an electrogenic influx of protons. The proton electrochemical gradient, ∆A⁺H⁺, is considered an intermediate of oxidative phosphorylation, such that influx of protons via the ATP synthetase decreases the ∆A⁺H⁺, which is maintained by oxidation-reduction-linked proton efflux. Under conditions of true thermodynamic equilibrium, i.e. when net flux is zero, the respiratory carrier oxidation-reduction potential driving force for proton efflux should be equal to ∆A⁺H⁺ with a fixed stoichiometry determined by the number of protons per phosphorylation site (the H⁺/2e⁻ ratio). In turn, ∆A⁺H⁺ should equal to the back pressure exerted by the intramitochondrial adenine nucleotide phosphorylation potential, also with a fixed stoichiometry determined by the number of protons required for ATP synthesis (the H⁺/ATP ratio). For further discussion see (15, 63, 64).

If the above stoichiometric relationships remain invariant, a fall of ∆Gₚ(m) should induce a fall of ∆A⁺H⁺, which in turn should produce a fall of ∆A⁺, if near-thermodynamic equilibrium relationships are maintained when proton flux and ATP turnover increase. The extent of the flux stimulation should in principle be a function of the ratio of input and output forces and the degree of coupling of the system (63). Furthermore, under conditions when the turnover of ATP is entirely within the mitochondrial matrix, the P/O ratio should be higher than when ATP is transported out of the mitochondria.
by the electrogenic adenine nucleotide translocator, and should be equal to the total number of electrogenic protons exported for all three phosphorylation sites divided by the H+/ATP ratio.

The experimental results reported in this paper only partially support the above predictions of the chemiosmotic theory. Addition of ornithine to mitochondria incubated in citrulline synthesis medium in the presence of carboxyatractyslose caused a decrease of $\Delta G_p(m)$ and $\Delta E_a$, but no change of $\Delta H^\circ$ (Table III). On the other hand, when glucose-hexokinase was added to induce state 3 respiration, $\Delta H^\circ$ as well as $\Delta G_p(m)$ and $\Delta E_a$ decreased relative to the state 4 condition (Table IV), in accordance with other reports in the literature (see 15, 63, 64). However, although all the changes were statistically significant, the greatest decrease was no more than 4%, showing that these parameters deviated only slightly from thermodynamic equilibrium. Furthermore, the relative changes of $\Delta E_a$ and $\Delta G_p(m)$ with a flux increase tended to be opposite to those expected since the change of the back pressure, $\Delta \Delta G_p(m)$, would be expected to be greater or equal to the change of the driving force $\Delta \Delta E_a$.

There are several explanations that might account for these discrepancies. In the first instance, the coupling coefficient is lower in the citrulline synthesis medium than in normal mitochondrial incubation medium, as shown by the fall of the respiratory control ratio from 9 to about 3, and the lower than theoretical $P/O$ ratios when phosphorylation was measured by either glucose-6-phosphate or citrulline formation. Thus, not only does the control condition not represent a state of true thermodynamic equilibrium, but the degree of coupling may change after ornithine addition. Secondly, it is apparent that flux is regulated by only small differences between the thermodynamic driving force and back pressure, and that possible differences between $\Delta \Delta G_p(m)$ and $\Delta \Delta E_a$ are within experimental error. Thirdly, the $\Delta E_a/\Delta G_p(m)$ ratio is almost invariant and apparently independent of changes in $\Delta H^\circ$, irrespective of whether respiration is stimulated by ornithine addition in the presence of atracyloside (Table III), by an extramitochondrial ATP-trapping system (Table IV), or by uncoupling agents (data from Fig. 10). These findings suggest the possibility of a direct equilibration between $\Delta E_a$ and $\Delta G_p(m)$ without the mediation of $\Delta H^\circ$. Other studies (65–67) have shown that when mitochondrial respiration is stimulated by uncoupling agents or by K+ in the presence of valinomycin, the fall of $\Delta H^\circ$ for a given increase of respiration is greater than when respiration is increased to the same extent with an extramitochondrial ATP-trapping system. Our data are in agreement with these earlier observations. The findings reported in this paper concerning stimulation of respiration by a purely intramitochondrial ATP-utilizing system is the first illustration that respiration can be stimulated with no detectable fall of $\Delta H^\circ$. However, it should be stressed that under these conditions a fall of $\Delta V$ is observed which is compensated by a rise of $\Delta H$. Taken as a whole, the data indicate that $\Delta H^\circ$ may not be an obligatory intermediate of oxidative phosphorylation. The earlier data have in fact been interpreted as suggesting that phosphorylation, in addition to its indirect effect through $\Delta H^\circ$, interacts directly with the respiratory chain by a mechanism which does not require proton cycling through the bulk phases (63, 64). However, $\Delta V$ always falls when respiration increases, and it is possible that this component of $\Delta H^\circ$ interacts directly at the level of cytochrome $c$ oxidase. Unlike the reactions from NADH to cytochrome $c$, which are in near-equilibrium with the phosphorylation potential, the reaction from cytochrome $c$ to oxygen is far displaced from equilibrium, and may be kinetically controlled both by the level of reduced cytochrome $c$ (68) and by the membrane potential (69).

The measured values of $\Delta E_a/\Delta H^\circ$ and $\Delta G_p(m)/\Delta H^\circ$ are between 3.0 and 3.5, and between 2.0 and 2.5, respectively, depending on the conditions. Estimates of $H^+/2e^-$ and H+/ATP ratios for oxidative phosphorylation have been made by many workers using different approaches, and values for $H^+/2e^-$ vary from 2 to 4 and for H+/ATP from 2 to 3 (see Ref. 70 for further reference). Fig. 10 shows that when a proton leak across the mitochondrial membrane is induced by addition of small quantities of uncoupler, values of both $\Delta E_a/\Delta H^\circ$ and $\Delta G_p(m)/\Delta H^\circ$ increase hyperbolically with a fall of $\Delta H^\circ$, and extrapolate to integers of 3 and 2, respectively, at high values of $\Delta H^\circ$. According to the chemiosmotic hypothesis, the proton oxidation-reduction pumps provide the driving force for establishing $\Delta H^\circ$. Hence, incomplete coupling caused by proton leaks are expected to cause an increase of the $H^+/2e^-$ stoichiometry (63). On the other hand, if $\Delta H^\circ$ directly provides the energy for driving ATP synthesis, an increase of the $\Delta G_p(m)/\Delta H^\circ$ or $\Delta G_p(c)/\Delta H^\circ$ (66), ratio with a fall of $\Delta H^\circ$ is not readily explained by the chemiosmotic hypothesis (63). Phenomenological explanations advanced to account for this discrepancy include partial direct coupling between phosphorylation and the oxidation-reduction proton pump (63) or limited accessibility for protons around the respiratory chain and ATP synthetase complexes (64). However, it is obvious that the problem will not be solved without further knowledge at the molecular level.

Acknowledgments—We are indebted to Dr. Richard O. Viale for construction of a computer program to calculate the MgATP and MgADP concentrations.

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