Stoichiometry of Adenosine Triphosphate-driven Proton Translocation in Bovine Heart Submitochondrial Particles*

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SUMMARY

Inward-directed proton translocation driven by ATP hydrolysis was investigated in bovine heart submitochondrial particles under conditions where no net pH change occurred upon ATP hydrolysis. A biphasic time course of ATP hydrolysis, due to the presence of adenylate kinase activity, was observed by following the release of $\gamma$P, from $[\beta,\gamma^{-}\text{32P}]$ATP under comparable conditions. An equation was derived for calculation of the $H^+:ATP$ ratio from the time course of ATP hydrolysis and the transient pH change of the medium due to proton translocation. $H^+:ATP$ ratios as high as 1.7 were observed, although a seasonal variation was noted. However, oligomycin raised the $H^+:O$ ratios for respiration-driven proton translocation, suggesting that the experimentally observed $H^+:ATP$ ratios were suboptimal. The results indicate that the true stoichiometry of ATP-driven proton translocation, characteristic of the ATPase mechanism is $2H^+:ATP$. The same stoichiometry has been reported previously for outward-directed proton translocation in intact mitochondria.

Proton translocation across the mitochondrial inner membrane by a reversible ATPase is a fundamental requirement of the chemiosmotic hypothesis of oxidative phosphorylation (1, 2). The release of protons from mitochondria (3) and the uptake of protons by inverted submitochondrial particles (4, 5) have been observed during ATP hydrolysis. Since this process is thought to result from anisotropic, or vectorial, catalysis of the ATP hydrolysis reaction across the membrane, its stoichiometry is of importance in understanding the molecular mechanism of the membrane-bound ATPase complex. The stoichiometry of ATP-driven proton translocation has been investigated in rat liver mitochondria by Mitchell and Moyle (3), who calculated a ratio of 2$H^+$ translocated per ATP hydrolyzed. This finding was interpreted as indicating that the ATPase itself translocated 2 protons in catalysis of the hydrolytic reaction (3). However, independent studies characterizing the mitochondrial adenine nucleotide transport system suggested that this carrier was electronegative (6–9), and might thus have contributed to the stoichiometry of ATP-driven proton translocation observed with intact mitochondria (3, 10).

Since submitochondrial particles are inverted with respect to mitochondria, the ATPase is exposed to the external medium. In this case, ATP does not require a transport system in order to be hydrolyzed. Determination of the stoichiometry of ATP-driven proton translocation in submitochondrial particles can thus give a clearer description of the ATPase mechanism without ambiguities imposed by the adenine nucleotide transport system.

EXPERIMENTAL PROCEDURE

Materials

F1, the purified mitochondrial ATPase from bovine heart (11), was a gift from Dr. E. Racker. Adenylate kinase, alcohol dehydrogenase, ATP, oligomycin, carbonyl cyanide m-chlorophenyl hydrazine, and atractyloside were from Sigma. Valinomycin was from Calbiochem. $[\beta,\gamma^{-}\text{32P}]$ATP—$[\gamma^{-}\text{32P}]$ATP, prepared by the method of Avron (12), was a gift from Dr. E. Racker. It was converted into $[\beta,\gamma^{-}\text{32P}]$ATP by incubation at 25° for 20 hours in a reaction mixture containing 80 mM K+-morpholinopropane sulfonate, pH 7.0, 165 mM NaCl, 1 mM MgCl2, 0.2 mM $[\gamma^{-}\text{32P}]$ATP, 0.02 mM AMP, and 0.05 mg (70 units) of adenylate kinase in a final volume of 3.5 ml. The reaction mixture was then diluted 10-fold and the adenine nucleotides separated on a column (0.8 x 6 cm) of Dowex 1-Cl (Sigma) (13). Radiochemical purity of the $[\beta,\gamma^{-}\text{32P}]$ATP was 95% as determined by thin layer chromatography on polyethyleneimine cellulose plates (Machery-Nagel & Co.) developed with 1.3 M LiCl (14). Complete randomization of the label between the $\beta$ and $\gamma$ positions was verified by hydrolysis to ADP and $P_i$ with F1 ATPase. The assay contained 50 mM K+-morpholinopropane sulfonate, pH 7.0, 2 mM MgCl2, 0.1 mM $[\beta,\gamma^{-}\text{32P}]$ATP (specific radioactivity $4 \times 10^9$ cpm per pmole) and 0.02 mg (15 units) of F1 in a final volume of 1.0 ml. The reaction was terminated after 10 min at 25° by the addition

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1 The abbreviations used are: F1, coupling factor 1, the mitochondrial ATPase; ETPEI, electron transport particles prepared by sonicication of heavy layer bovine heart mitochondria.
of 5% trichloroacetic acid and the \( ^{32}P \) was determined as described under "Kinetics Experiments."

**Preparations**

*Mitochondria*—Heavy layer bovine heart mitochondria prepared on a large scale (15) were generously provided by Dr. E. Racker.

\( ETP_m \) \( (\text{Mg}^{2+}, \text{Mn}^{2+}) \) submitochondrial particles (16) were prepared from heavy layer bovine heart mitochondria as described by Bever (17), except that sonication was conducted at 0° for 20 s, rather than at −10° for 20 s. The \( ETP_m \) were suspended in 0.25 M sucrose and stored on ice. All studies reported in this paper were done with freshly prepared \( ETP_m \) used within a maximum of 10 hours after preparation, during which time no decrease in proton translocation activity was observed. However, freezing resulted in a 25 to 50% decrease in proton translocation activity.

**Analytical Procedures**

Measurement of \( \text{pH} \)—\( \text{pH} \) was measured during proton translocation experiments in a 1-ml glass cell constructed by fusing two 7/25 outer ground glass joints at an 80° angle. One joint, approximately horizontal, held a Beckman model 39045 \( \text{pH} \) electrode, while the vertical joint served as an addition port. A 7/25 inner ground glass joint, heated at the small end to close the opening to a 0.5 mm diameter, served as a top for the addition port. Additions were made through this top with Hamilton syringes. This arrangement minimized \( \text{pH} \) drift due to atmospheric CO\(_2\) and allowed work under anaerobic conditions. A glass capillary tube was connected to the side of the cell to make contact with the reference electrode. The contents of the cell were stirred by a 7-mm Teflon "flea" driven magnetically.

A glas.5 capillary tube was connected to the side of the cell to make contact with the reference electrode. The contents of the test tube were stirred magnetically throughout the experiment. The reaction was terminated after the desired time by the addition of 0.05 ml of 50% trichloroacetic acid with a Clark oxygen electrode. This aliquot of the supernatant was then analyzed by a modification of the procedure of Lindberg and Fronter (21) in a manner analogous to that described above in anaerobic conditions. Also, for \( \text{pH} \) measurement, \( [\beta, \gamma-\text{32P}] \text{ATP} \) was added to give the desired final concentration of ATP, and \( \text{pH} \) was measured with a Clark oxygen electrode exactly as described by Schatz and Racker (20).

Kinetics Experiments—For determination of the kinetics of hydrolysis of \([\beta, \gamma-\text{32P}]\text{ATP} \), \( ETP_m \) and valinomycin were added to an assay containing a buffer mixture in a final volume of 0.30 ml. The buffer mixture contained 40 \( \mu \)moles of KCl, 0.3 \( \mu \)mole of glycyglycine, 1 \( \mu \)mole of MgCl\(_2\), and 10 \( \mu \)moles of 2-[\text{N-morpholinoethane sulfonic acid, final pH 6.1}]. After 1 min of equilibration, \( [\beta, \gamma-\text{32P}]\text{ATP} \) (approximately 10\(^4\) cpm), containing unlabeled MgATP to give the desired final concentration of ATP, was added in 0.20 ml with a syringe. The contents of the test tube were stirred magnetically throughout the experiment. The reaction was terminated after the desired time by the addition of 0.05 ml of 50% trichloroacetic acid with a Clark oxygen electrode. The reaction conditions which best satisfied this requirement were reaction conditions under which \( j \) would equal 0. The reaction conditions which best satisfied this requirement were reaction conditions under which \( j \) would equal 0. The reaction conditions which best satisfied this requirement were reaction conditions under which \( j \) would equal 0.

Protein Concentration-Protein concentration was determined by the biuret method in the presence of 0.33% deoxycholate with bovine serum albumin as standard (22).

**ATP Concentrations**—ATP concentrations were determined spectrophotometrically based on a molar absorption coefficient of 1.54 \( \times 10^4 \text{M}^{-1} \text{cm}^{-1} \) at 260 nm (23).

**RESULTS**

Simplified Conditions for Measurement of ATP-driven Proton Translocation—The hydrolysis of ATP results in a net \( \text{pH} \) change of the medium at most values of \( \text{pH} \) and Mg\(^{2+} \) concentration. The net \( \text{pH} \) change is useful as the basis for an assay for ATP hydrolysis (3, 24), but complicates the measurement of proton translocation. The observation of ATP-driven proton translocation can thus be simplified by choosing reaction conditions which yield no net \( \text{pH} \) change upon ATP hydrolysis.

Because of adenylate kinase activity, both Reactions 1 and 2 must be considered in submitochondrial particles.

\[
\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{P}_1 + j\text{H}^+ \tag{1}
\]

\[
\text{ATP} + 2\text{H}_2\text{O} \rightarrow \text{AMP} + 2\text{P}_1 + n\text{H}^+ \tag{2}
\]

Theoretical calculations of Altman (25, 26) were used to select reaction conditions under which these reactions would go to completion. The reaction conditions which best satisfied this requirement were those of \( j = n = 0 \). The reaction conditions which best satisfied this requirement were those of \( j = n = 0 \). The reaction conditions which best satisfied this requirement were those of \( j = n = 0 \).

The specific reactivity of the medium described under "Experimental Procedure," the hydrolysis of ATP under these conditions was verified experimentally with soluble enzymes.
Under these conditions, no pH change was observed upon ATP hydrolysis by F₁, the soluble mitochondrial ATPase, indicating that \( j = 0 \) in Reaction 1. In addition, no pH change was observed upon ATP hydrolysis by a mixture of F₁ and adenylate kinase, indicating that \( n = 0 \) in Reaction 2.

The advantage of employing reaction conditions giving no net pH change upon ATP hydrolysis was a significant simplification in the calculation of the \( \text{H}^+ : \text{ATP} \) ratio. In addition, this pH was also within the pH range reported to be technically optimal for measurement of respira- tion-driven proton translocation in submitochondrial particles deficient in coupling factors (19). However, use of these conditions required the development of an additional procedure for measuring the hydrolysis of ATP.

**Typical ATP-driven Proton Translocation Experiment**—Fig. 1A shows a tracing of the pH recording obtained during a typical proton translocation experiment. Upon addition of ATP, the pH of the medium rose rapidly, reaching a maximum in about 20 s, and slowly returned to approximately the original pH. Fig. 1B shows the pH recording obtained during an identical experiment in the presence of the uncoupler, carbonyl cyanide \( m \)-chlorophenyl hydrazone (1 \( \mu \)M). In this case, little proton translocation was observed. Addition of the uncoupler any time after the initiation of proton translocation with ATP caused a rapid return of the pH to the base-line (not shown). The prevention and reversal by an uncoupler, which increases the proton permeability of the membrane (see References 10, 27, and 28), of the pH change occurring upon ATP addition confirmed that the pH change reflected the generation and decay of a pH gradient across the membrane. The cause of the small, rapid decrease (approximately 0.03 ng ions of H⁺ per nmole of ATP) of the pH base-line during the course of the experiment was not determined. However, as mentioned previously, this was not associated with a net pH change accompanying the ATP hydrolysis reaction. In all cases, the final, rather than initial, pH was used as the reference pH for analysis of the proton translocation experiments.

Fig. 1C shows the pH recording obtained upon ATP addition in the presence of the inhibitor, oligomycin (2.1 \( \mu \)g per mg of protein). As expected, no pH change characteristic of proton translocation was observed under these conditions, although a transient acidification of the medium was noted. This transient acidification was not uncoupler sensitive, as a similar pH change occurred in the presence of both oligomycin and carbonyl cyanide \( m \)-chlorophenyl hydrazone. The cause of this transient acid formation remains unknown, but may reflect interaction of ATP with the inhibited ATPase or a secondary reaction of ATP in the absence of hydrolysis.

**Kinetics of ATP hydrolysis by ETP₈**—The kinetic data for ATP hydrolysis by ETP₈ were measured by following the release of \( ^{32} \text{P} \) from [\( \beta,\gamma^{-32} \text{P} \)]ATP as described under “Experimental Procedure.” The conditions were identical to those used in the proton translocation experiments, except for a higher buffer concentration to insure accuracy of the pH. The results of the kinetics experiment corresponding to the proton translocation experiment of Fig. 1A are shown in Fig. 2A. The results are expressed as percentage of hydrolysis of the added ATP to facilitate recognition of the biphasic nature of the kinetics. As shown, the added ATP was first hydrolyzed in a rapid burst, corresponding roughly to the time required for the pH change associated with proton translocation to reach a maximum (Fig. 1A). The second phase of the reaction reflected the lower, but still significant, adenylate kinase activity which generated further ATP. Since the over-all reaction occurring in this system was effectively Reaction 2, used of doubly labeled ATP was required for complete monitoring of the reaction.

Analytical treatment of a two reaction system such as this is quite complex (29). In addition, analysis of the present system was limited experimentally by an inability to measure true initial velocities and by an unknown endogenous adenosine nucleotide content. In view of these limitations, the simplest mathematical function describing the observed results was employed. The observed product-time curve could be well approximated as the sum of two first order exponentials, regarding the endpoint of the reaction as 100% hydrolysis of the added ATP, as in Equation 3.

\[
P_t = S_0 \left( e^{-\alpha_1 t} - e^{-\alpha_2 t} \right)
\]

In this equation, \( P_t \) represents phosphate released at time \( t \); \( S_0 \) is the amount of ATP added initially; and \( \alpha_1 \) and \( \alpha_2 \) are kinetic parameters similar to conventional rate constants describing the
time course. Such a calculated curve, fit to the hydrolysis data, is shown as a solid line (---) in Fig. 2A. For this experiment, \( \alpha_1 = 0.25 \text{ s}^{-1} \) and \( \alpha_2 = 0.011 \text{ s}^{-1} \). The greatest deviations from the calculated curve occurred at early reaction times. However, calculation of the \( \text{H}^+ : \text{ATP} \) ratio was not greatly affected by variations of \( \alpha_2 \).

Fig. 2B shows the time course of ATP hydrolysis in the presence of a maximally inhibitory amount of oligomycin (2.1 \( \mu \)g per mg of protein). In this case, the release of \( ^{32} \text{P} \) from \([\beta, \gamma, ^{32} \text{P}]\)-ATP followed a linear time course for at least the initial 2 min of the reaction. Oligomycin sensitivity of the ATPase, determined from the data of Fig. 2 by comparison of the initial rates of hydrolysis in the presence and absence of oligomycin, was greater than 95%, which was in good agreement with measurements obtained by a more conventional assay (30). In view of this observation, we decided to treat all of the observed ATP hydrolysis as contributing to proton translocation for calculation of the \( \text{H}^+ : \text{ATP} \) ratio. Since only the oligomycin-sensitive portion of the observed ATP hydrolysis was expected to be coupled to proton translocation (3), this simplification leads to a small underestimation of the true \( \text{H}^+ : \text{ATP} \) ratio.

**Calculation of \( \text{H}^+ : \text{ATP} \) Ratio.** Having measured both proton translocation and the kinetics of hydrolysis of the added ATP, the \( \text{H}^+ : \text{ATP} \) ratio can be calculated by the following procedure. This procedure is an extension of theoretical considerations described by Mitchell (3, 31) applied to the present experimental system.

The general relationship describing the generation and decay of the spatial pH gradient (\( \Delta \text{pH} \)) across the inner mitochondrial membrane (M phase) due to ATP-driven proton translocation as a function of time (t) is given by Equation 4 (31).

\[
B \frac{d \Delta \text{pH}}{dt} = h \frac{dP}{dt} - ZC_M \Delta \text{pH}
\]

(Equation 4)

The nomenclature is that used by Mitchell (3), i.e. B is the differential buffering power (micromoles of \( \text{H}^+ \) per \( \text{pH} \) unit); \( C_M \) is the effective proton conductance of the M phase (micromoles per volt per s); \( Z \) is an electrical unit conversion factor equal to 2.303 \( RT/F \) (in which \( R \) is the gas constant, \( F \) the Faraday, \( T \) the absolute temperature) with a value of 59 mV at 25°C; \( h \) is the \( \text{H}^+ : \text{ATP} \) stoichiometry, and \( B \) is the phosphate released in the ATPase reaction. The expression for \( B \) was given earlier (Equation 3), and thus

\[
\frac{dP}{dt} = \alpha_1 s e^{-\alpha_1 t} + \alpha_2 s e^{-\alpha_2 t}
\]

(Equation 5)

Equation 4 can be solved by a standard technique of differential equations to give Equation 6.

\[
B \Delta \text{pH} = h s
\]

(Equation 6)

where

\[
Y = \left( \frac{\alpha_1}{\alpha_1 - ZC_M/B} + \frac{\alpha_2}{\alpha_2 - ZC_M/B} \right) e^{-\left( \frac{ZC_M/B}{\alpha_2 - ZC_M/B} \right) t}
\]

\[
- \left( \frac{\alpha_1}{\alpha_1 - ZC_M/B} \right) e^{-\alpha_1 t} - \left( \frac{\alpha_2}{\alpha_2 - ZC_M/B} \right) e^{-\alpha_2 t}
\]

(Equation 7)

As an important consequence of the use of experimental conditions giving no net pH change upon ATP hydrolysis, the following relationship is valid. Namely

\[
R \Delta \text{pH} = R_0 \Delta \text{pH}_0
\]

in which \( \Delta \text{pH}_0 \) represents the pH change of the outer phase and \( R_0 \) is the outer phase buffering power. Combining Equation 8 and Equation 6, one obtains Equation 9.

\[
h = \frac{B_0 \Delta \text{pH}_0 e^{\left( ZC_M/B \right) t}}{S_0 X}
\]

(Equation 10)

where

\[
X = \left[ \left( \frac{\alpha_1}{\alpha_1 - ZC_M/B} \right) e^{-\left( \frac{ZC_M/B}{\alpha_1 - ZC_M/B} \right) t} + \left( \frac{\alpha_2}{\alpha_2 - ZC_M/B} \right) e^{-\left( \frac{ZC_M/B}{\alpha_2 - ZC_M/B} \right) t} \right]
\]

(Equation 11)

By taking the logarithm of Equation 10 and rearranging, one obtains Equation 12.

\[
\ln \left( \frac{B_0 \Delta \text{pH}_0/S_0 X}{} \right) = \ln h - \frac{ZC_M/B}{t}
\]

(Equation 12)

Therefore, a semilogarithmic plot of \((B_0 \Delta \text{pH}_0/S_0 X)\) versus time will give a line with a slope \(-ZC_M/B\) with an intercept on the ln axis at \( t = 0 \) which is equal to \( h \), the \( \text{H}^+ : \text{ATP} \) stoichiometry.

Equation 12 was useful for analyzing the experimental data. The quantity \( B_0 \Delta \text{pH}_0 \) was obtained from the pH recording (Fig. 1). The parameters \( \alpha_1 \) and \( \alpha_2 \) were derived from the parallel kinetics experiment (Fig. 2A). As shown by Equation 12, it was not necessary to determine \( C_M \) and \( B \) individually since they occurred only as a ratio. In fact, the term, \( ZC_M/B \), did not have to be measured experimentally at all. Instead, an approximate value of \( ZC_M/B \) could be used for calculating \( X \), and the slope of the graph of Equation 12 determined. Since the slope of the graph of Equation 12 was equal to \( ZC_M/B \), the new value could be used to recalculate Equation 12 and the procedure repeated until agreement was obtained. Such an iterative method for obtaining \( ZC_M/B \) has been previously described (3).

Figure 3 shows the application of Equation 12 to the data presented in Figs. 1A and 2A. For this experiment, the value of \( ZC_M/B \) was 0.018 s\(^{-1}\), which corresponds to a \( t_{1/2} \) of \( \Delta \text{pH} \) decay of 30 s, while the intercept on the ln axis was 1.77 - the \( \text{H}^+ : \text{ATP} \) ratio. Only a single decay phase of the pH gradient was observed, in contrast to the biphasic decay observed with intact mitochondria (3).

The complexity of the calculation procedure described here compared to that used for the analysis of respiration-driven proton translocation (18, 19) is a consequence of the more complex kinetics of the enzymes, both ATPase and adenylyl kinase, involved in ATP hydrolysis by ATPase.

The effect of this analysis is to mathematically transform the generation of the pH gradient from a time-dependent to an instantaneous phenomenon. The pH gradient then decays with a \( t_{1/2} \) characteristic of the intrinsic proton permeability of the membrane. The latter situation is similar to that occurring in oxygen-pulse measurements of respiration-driven proton translocation. In this case, because of the low \( K_m \) for oxygen, respiration proceeds at a constant rate for only a short time (about 2 s) giving rapid generation of the pH gradient due to respiration-driven proton translocation (18, 19). The term \( ZC_M/B \) can be
for, since AMP will favor the adenylate kinase reaction initially, causing a lowering of $\Delta \text{H}^+$. However, it should be noted that by using preparations. For example, high concentrations of endogenous concentration were obtained. This was most likely due to a variation of the parameters $a_1$ and $a_2$ with protein or ATP concentration. No significant differences in the kinetic parameters or the term $ZC_M/B$ were observed between seasons.

Table I summarizes the kinetic parameters used to fit the data obtained from the hydrolytic time course experiments. No correlations of the parameters $a_1$ and $a_2$ with protein or ATP concentration were obtained. This was most likely due to a variable endogenous adenine nucleotide content between ETP$_H$ preparations. For example, high concentrations of endogenous AMP will favor the adenylate kinase reaction initially, causing a lowering of $a_1$. However, it should be noted that by using $[\beta, \gamma, \Delta^2 \text{P}]$AMP all such complications are automatically accounted for, since a $\Delta^2 \text{P}$ will always be released by the ATPase regardless of interconversion of the label by the adenylate kinase activity. Errors due to variation in the kinetic parameters were minimized by performing the kinetic experiments parallel to the proton translocation experiments, i.e., with the same ETP$_H$ preparation and ATP concentration. No significant differences in the kinetic parameters or the term $ZC_M/B$ were observed between seasons.

The standard deviations of the parameters $a_1$ and $a_2$ (Table I) were relatively large. However, it was found that variation of these parameters independently or together over the range of their standard deviations changed the calculated $\text{H}^+:\text{ATP}$ ratio by less than 0.15. Calculation of the $\text{H}^+:\text{ATP}$ ratio was thus relatively insensitive to such variations. For this reason, the average kinetic parameters were used to analyze other proton translocation experiments for which detailed time courses of ATP hydrolysis were unavailable, including some from the previous winter (January 1972). Some representative $\text{H}^+:\text{ATP}$ ratios calculated with average kinetic parameters are also shown in Fig. 4. Values obtained by this less accurate calculation procedure all fell within the same range of $\text{H}^+:\text{ATP}$ ratios obtained as described in the text, for determination of the $\text{H}^+:\text{ATP}$ ratio from the data presented in Figs. 1A and 2A.

These were calculated as described in the text for $\text{H}^+:\text{ATP}$ ratio determinations. The standard deviations of the parameters $a_1$ and $a_2$ for ATP hydrolysis by ETP$_H$ preparations, calculated as described in the text, for determination of the $\text{H}^+:\text{ATP}$ ratio from the data presented in Figs. 1A and 2A.

Table I: Average kinetic parameters for ATP hydrolysis by ETP$_H$ under conditions of ATP-driven proton translocation.

<table>
<thead>
<tr>
<th></th>
<th>$a_1$</th>
<th>$a_2$</th>
<th>$ZC_M/B$</th>
<th>$\text{H}^+:\text{ATP}$</th>
<th>Number of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter ETP$_H$</td>
<td>±0.06</td>
<td>±0.03</td>
<td>±0.001</td>
<td>±0.001</td>
<td>8</td>
</tr>
<tr>
<td>Summer ETP$_H$</td>
<td>±0.07</td>
<td>±0.002</td>
<td>±0.001</td>
<td>±0.001</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 3. Determination of the $\text{H}^+:\text{ATP}$ ratio. Semilogarithmic plot of the quantity $(B\Delta \text{pH}/S_0 \Delta X)$ versus time calculated by Equation 12 as described in the text, for determination of the $\text{H}^+:\text{ATP}$ ratio from the data presented in Figs. 1A and 2A.

FIG. 4. Summary of $\text{H}^+:\text{ATP}$ ratio determinations. $\text{H}^+:\text{ATP}$ ratios, calculated as described in the text, for determination of the $\text{H}^+:\text{ATP}$ ratio from the data presented in Figs. 1A and 2A.
mycin requirement was probably due to the presence of sufficient divalent cations, such as Mg$^{2+}$ in the medium or Mn$^{2+}$ introduced during sonication, to act as permeable co-ions in this system.

The addition of atracyloside (23 $\mu$M), a specific inhibitor of the mitochondrial adenine nucleotide transport system, had no effect on the observed proton translocation or H$^+:ATP$ ratio. This result was expected, since the external orientation of the ATPase in submitochondrial particles eliminated the need for an ATP transport system.

**Effect of Oligomycin on H$^+:O$ Ratios**—Oligomycin is known to stimulate certain energy-linked reactions, such as the transhydrogenase (32), and is required in order to observe respiration-driven proton translocation (19) in submitochondrial particles which are deficient in coupling factors. It has been proposed that these effects of oligomycin result from the closing of a specific proton leak in the membrane at the F$_1$ attachment site (31).

Since ETP$_H$ are not regarded as deficient in coupling factors, they were not expected to show an oligomycin requirement for measurement of proton translocation. However, although ETP$_H$ did not require oligomycin, the addition of oligomycin raised the H$^+:O$ ratios for respiration-driven proton translocation to the maximum levels reported for mitochondria (18) and submitochondrial particles (19) as shown in Fig. 5. A seasonal variation was again noted, with “winter” ETP$_H$ showing approximately a 10% stimulation of the H$^+:O$ ratio compared with “summer” ETP$_H$, which exhibited about a 25% stimulation. As expected, less oligomycin was required for observing maximum H$^+:O$ ratios with “winter” ETP$_H$.

The pattern of stimulation of the H$^+:O$ ratios by oligomycin correlates qualitatively with the seasonal variation observed with the H$^+:ATP$ ratio. Winter ETP$_H$ exhibited both higher H$^+:ATP$ and H$^+:O$ ratios than summer ETP$_H$. In addition, the suboptimal nature of the H$^+:O$ ratios in the absence of oligomycin implies that the measured H$^+:ATP$ ratios were also suboptimal. Attempts to raise the H$^+:ATP$ ratio by the addition of small amounts of oligomycin were unsuccessful, presumably because of a lack of a significant difference in the affinity of oligomycin for the membrane “holes” as compared with complete ATPase complexes.

**Efficiency of Oxidative Phosphorylation**—Since a similar seasonal variation of P:O ratios has been reported (16), oxidative phosphorylation was measured as a further check on the degree of energy coupling exhibited by ETP$_H$. With NADH as substrate, P:O ratios of 1.7 to 1.9 were observed, whereas P:O ratios of 1.2 to 1.3 were found with succinate as substrate. Although the higher P:O ratios of the above ranges were observed with winter ETP$_H$, seasonal differences were probably not significant. Although these P:O ratios were not as high as originally described for ETP$_H$ (16), they were typical of those observed by other investigators (90). These suboptimal P:O ratios, compared with mitochondria, may further explain why an optimal H$^+:ATP$ ratio was not observed experimentally.

**DISCUSSION**

Proton translocation has been proposed as the primary means of energy conservation for the inner mitochondrial membrane and forms the basis of the chemiosmotic hypothesis of oxidative phosphorylation (1, 2). In this hypothesis, the energy derived from the oxidation of substrates by the respiratory chain is conserved as a proton motive force across the membrane, which can be utilized by a reversible proton translocating ATPase for synthesis of ATP. Because of the reversible nature of the coupling process, ATP hydrolysis can also generate a proton motive force. It is this latter phenomenon which was studied in this report.

There is substantial evidence that oxidation of respiratory substrates by mitochondria (18) and submitochondrial particles (19) is accompanied by proton translocation with an H$^+:O$ stoichiometry of 2 for each loop, or phosphorylation site. Proton translocation driven by ATP hydrolysis in rat liver mitochondria with a stoichiometry of 2H$^+:ATP$ has also been reported (3). In addition, a preliminary report of ATP-driven proton translocation in sonic particles of rat liver mitochondria at an efficiency of 0.5H$^+:ATP$, considered suboptimal by the authors, has been published (4). ATP-driven proton translocation has also been observed in certain other energy-conserving membrane systems, including chloroplasts (33), chromatophores (34), and reconstituted phospholipid vesicles containing mitochondrial ATPase (35).

As the results presented in this report show, H$^+:ATP$ ratios averaging as high as 1.7 have been found for ATP-driven proton translocation in submitochondrial particles. The methods and calculation procedure used have allowed full account of the influence of the ubiquitous adenylate kinase activity on ATP-driven proton translocation. Our results indicate that the true stoichiometry of ATP-driven proton translocation in submitochondrial particles is most likely 2H$^+:ATP$, in agreement with results obtained with intact mitochondria (3).

This interpretation is supported by the finding that oligomycin stimulated respiration-driven proton translocation approximately 10% in comparable winter ETP$_H$ which consistently gave high experimental H$^+:ATP$ ratios. Lower H$^+:ATP$ ratios, about 1.2, were found with summer ETP$_H$. However, summer ETP$_H$ showed a greater stimulation of H$^+:O$ ratios by oligomycin, indicating that they were less coupled than winter ETP$_H$. Although the cause of this seasonal variation was not investigated, the variation probably reflects different stability of the proton translocation activity to the submitochondrial particle preparation procedure, rather than an intrinsic metabolic difference.
The finding of the same 2H\(^+:\)ATP stoichiometry in both mitochondria and submitochondrial particles is strong evidence in favor of the type II ATPase mechanism proposed by Mitchell (2). In this mechanism, translocation of two protons results directly from the hydro-dehydration reaction of the ATPase itself. Since the membrane is inverted in submitochondrial particles relative to intact mitochondria, no adenine nucleotide transport is required in order for ATP to gain access to the catalytic center of the ATPase. Our findings thus rule out an alternative mechanism, suggesting that the 2H\(^+:\)ATP stoichiometry observed with intact mitochondria might be due to an ATPase mechanism translocating 1 proton (type I) functioning concurrently with an electrogenic adenine nucleotide carrier translocating a second charge (10).

The possible electrogenic nature of the mitochondrial adenine nucleotide carrier was inferred from the observation that uncoupling stimulated the exchange of external ATP with internal ADP (6) and resulted in equilibration of the internal and external ATP:ADP ratios (7, 8). In addition, a transient stearoyl-CoA-sensitive proton uptake was observed upon the addition of ATP to rat liver mitochondria in the presence of an uncoupler and EDTA (3, 7). The magnitude of this proton uptake suggested that proton movement corresponding to about 0.5 H\(^+\) per ATP exchanged might be associated with the functioning of the adenine nucleotide carrier (7, 9) and led to the proposal that this carrier was "50% electrogenic." In contrast, our results indicate indirectly that the adenine nucleotide carrier operates electroneutrally, since the ATPase itself accounts fully for the 2H\(^+:\)ATP stoichiometry observed. However, complex interactions between transport systems in intact mitochondria, such as coupling between adenine nucleotide and phosphate transport (36), may explain some of these discrepancies.

It is relevant to note that nonintegral proton translocation stoichiometries might result if a catalytic residue of a protein involved in proton translocation had a pK\(_A\) near the pH of the medium. The observed proton translocation ratio would then reflect a statistical averaging of two different catalytic mechanisms and should be pH dependent. However, preliminary experiments have indicated that the H\(^+\):ATP ratio for ATP-driven proton translocation does not seem to be pH dependent, at least from pH 6 to 7.

The energy of ATP hydrolysis has also been shown to drive the transport of certain other ions in mitochondria, including Ca\(^{2+}\) (37, 38) and K\(^+\) in the presence of valinomycin (39). Higher ion uptake ratios than those predicted by the chemiosmotic hypothesis have been observed, indicating Ca\(^{2+}\):~ ratios of 2 (37) and K\(^+\):~ ratios as high as 7 (39) for the transport of these ions during ATP hydrolysis. In addition, a K\(^+\):ATP ratio of 4 during ATP synthesis driven by a potassium gradient in mitochondria has been reported (40).

Since under the conditions of our experiments other ions are also moving, it might be thought that the proton translocation observed is a secondary consequence of the primary transport of another ion. The evidence that proton translocation is primarily coupled to ATP hydrolysis rests mainly on the low proton permeability of the mitochondrial membrane. Such low proton permeability has been observed with intact mitochondria (41), submitochondrial particles (19), and is reflected in the low Z\(_{CM}\)/B values obtained in our experiments. Because of this low proton permeability, the rate of proton translocation observed is too high to be accounted for as ionic movement in response to the primary transport of another ion. Furthermore, since valinomycin creates passive K\(^+\) ion permeability (28, 42), if transport of an ion other than H\(^+\) were primary, then the addition of valinomycin plus K\(^+\) should decrease the observed proton translocation, in contrast to the increase which was observed. Also, the addition of an uncoupler, which increases the proton permeability of the membrane, should result in more, rather than less, H\(^+\) movement if transport of another ion were primary. For these reasons, we conclude that proton translocation at a stoichiometry of 2H\(^+:\)ATP is primarily coupled to ATP hydrolysis in submitochondrial particles.

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