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# Relationship of Transmembrane pH and Electrical Gradients with Respiration and Adenosine 5′-Triphosphate Synthesis in Mitochondria†

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ABSTRACT: The mechanism of mitochondrial oxidative phosphorylation and its regulation have been studied by using suspensions of isolated rat liver mitochondria. Parallel measurements were made of mitochondrial volume, respiration, transmembrane pH and electrical gradients, and adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and inorganic phosphate ($P_i$) concentrations under various experimental conditions. The transmembrane electrical gradients were calculated from the equilibrium distributions of [$^3$H]-triphenylmethylphosphonium ($TPMP^+$), [$^3$H]tribenzylmethylammonium ($TBMA^+$), and $K^+$ (plus valinomycin). The transmembrane distributions of labeled acetate, methylamine, and 5,5-dimethyloxazolidine-2,4-dione were used for the calculation of pH gradients. Evaluation of the data shows that the respiratory rate is strictly correlated with [ATP]/([ADP][$P_i$]) (free energy of ATP synthesis), whereas there is no consistent correlation between the transmembrane electrical potential, the pH gradient, or the total "protonmotive force" ($\Delta\mu_{H^+}$) and the respiratory rate. Thermodynamic analysis indicates that, in order for the proton electrochemical gradient to serve as an intermediate in ATP synthesis, from three to seven $H^+$ would have to be transported per each ATP synthesized, depending on the experimental conditions. These results suggest that the proton electrochemical gradient may not serve as a primary intermediate in oxidative phosphorylation.

The obligatory parameters of any intermediate(s) (I) between the oxidation–reduction reactions and ATP synthesis in mitochondrial oxidative phosphorylation can be determined from accurate kinetic and thermodynamic (free energy) measurements. Experimental evidence has been obtained that the first two sites of oxidative phosphorylation are in near equilibrium (Erecinska et al., 1974; Wilson et al., 1974a,b), and the overall rate of oxidative phosphorylation is regulated by extramitochondrial (Holian et al., 1977; Owen & Wilson, 1974; Wilson et al., 1977) or cytosolic (Wilson et al., 1974a,b; Erecinska et al., 1977) [ATP]/([ADP][$P_i$]). Therefore, intermediates in oxidative phosphorylation must fit the required relationship for the free energy change in the oxidation–reduction reactions to that utilized in ATP synthesis

$$\Delta G_{O\text{-}R} \geq \Delta G_I \geq \Delta G_{ATP} \quad (1)$$

where $\Delta G_{O\text{-}R}$, $\Delta G_I$, and $\Delta G_{ATP}$ are the free energy changes associated with the oxidation–reduction reactions, the inter-

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mediates (I), and ATP synthesis, respectively. Moreover in any process which dissipates $\Delta G_I$ without interferring with the ATP synthesis reactions, $\Delta G_{ATP}$ must decrease parallel to $\Delta G_I$.

Mitchell (1968) and Mitchell & Moyle (1969) have proposed that proton transport and its associated "protonmotive force" ($\Delta\mu_{H^+}$), which is the sum of the mitochondrial transmembrane pH and electrical ($E$) gradients, is the primary intermediate in oxidative phosphorylation. Direct measurements of the transmembrane electrical potential with microelectrodes are impractical but have been used in "giant" mitochondria where they indicate a low transmembrane potential (<60 mV) (Kinnally et al., 1978).

Measurements with fluorescent dyes have provided a variety of values for the electrical component of the protonmotive force in mitochondrial (Kinnally & Tedeschi, 1976; Kinnally et al., 1978; Laris et al., 1975; Maloff et al., 1978) and submitochondrial particles (Bashford & Thayer, 1977), which probably reflects their sensitivity to other membrane properties rather than strictly to the membrane potential (Hoffman & Laris, 1974). Inorganic ions have been used extensively to measure the transmembrane electrical potential in mitochondria (Azzone et al., 1978a,b; Nicholls, 1974; Padan & Rottenberg, 1973; Rottenberg, 1970, 1973; Rottenberg & Scarpa, 1974) but have come under criticism for lack of definitive knowledge on the mechanism(s) of transport (Diwan & Tedeschi, 1975; Tedeschi, 1975). Lipophilic ions were first used by Skulachev and co-workers (Bakeeva et al., 1970; Grinius et al., 1971; Skulachev, 1971) for the measurement of transmembrane electrical gradients. They have been used to measure electrical potentials in mitochondria (Azzone et al., 1976, 1978a,b), inverted inner mitochondrial membrane vesicles (Wehrle et al., 1978), submitochondrial particles (Azzone et al., 1978c; Grinius et al., 1970), and a variety of other systems (Deutsch et al., 1979; Deutsch & Kula, 1978; Lombardi et al., 1974; Schuldiner & Kaback, 1975). The use of lipophilic ions allows accurate measurement of the transmembrane electrical potential which is not subject to other membrane properties, including transport, as long as care is taken to correct for membrane binding of the probes (Deutsch et al., 1979; Levine et al., 1979). Weak acid and weak base distributions across membranes have been used widely for the measurement of transmembrane pH gradients since first introduced by Waddel & Butler (1959).

The $[H^+]/[ATP]$ ratios which would be required for the proton gradient to act as an intermediate in oxidative phosphorylation have been calculated from the reported values of $\Delta\mu_{H^+}$ and range from 2.0 (Laris et al., 1975; Mitchell, 1968; Nicholls, 1974) to >15 (Kinnally & Tedeschi, 1976). Independent measurements of proton movement in mitochondria have given $H^+$/site values from 2 (Moyle & Mitchell, 1973, 1978; Thayer & Hinkle, 1973) to 4 (Brand et al., 1976a,b; Reynafarje & Lehninger, 1978; Vercesi et al., 1978).

Unfortunately, this multiplicity of the reported values and the lack, in some cases, of essential measurements (such as intramitochondrial volume) require that comprehensive measurements be made in order to determine (1) whether $\Delta\mu_{H^+}$ fulfills the free energy requirements for an intermediate in oxidative phosphorylation (eq 1) and (2) the minimum value and reproducibility of the $[H^+]/[ATP]$ ratios.

## Materials and Methods

*Mitochondrial Preparation and Respiration Measurements.* Mitochondria were isolated from rat liver according to the method of Schneider (1948). Mitochondria were suspended at a protein concentration of 50–65 mg/mL in a medium of 0.25 M sucrose, 5 mM KCl, 5 mM morpholinopropanesulfonate (Mops), and 0.2 mM ethylenediaminetetraacetate (EDTA) at pH 7.0 (unless otherwise noted). Respiratory control values were 6–10 with glutamate plus malate as substrates. Protein was determined by the biuret method (Gornall et al., 1949) with crystalline bovine serum albumin as a standard.

Experiments were conducted after diluting the mitochondrial suspension to a protein concentration of 5–16 mg/mL in an oxygen-saturated medium consisting of 0.225 M mannitol, 0.075 M sucrose, 5.0 mM Mops, 1.0 mM acetate, and 0.2 mM EDTA at pH 7.0 with 10 mM glutamate and 10 mM malate as substrates or other media as described in the text. Oxygen consumption was measured by using a Yellow Springs Instrument Co. oxygen electrode in a 1.2-mL glass chamber from Gilson Medical Electronics. The electronics for the oxygen measurements were designed and built by the Johnson Research Foundation instrument shop.

*Mitochondrial Volume Measurements.* Intramitochondrial water was measured for conditions identical with those used in measurements of transmembrane electrical potential, pH gradient, and $[ATP]/([ADP][P_i])$. After mitochondria and medium were added to the oxygen electrode chamber, substrates were added along with 1.5 mM $P_i$, 3.0 mM ATP, catalase, and enough $H_2O_2$ to bring the oxygen concentration to $\sim$1 mM. A "modifier" of mitochondrial function was then added along with $2 \times 10^{-7}$ M valinomycin and finally 1.5 mM ADP. When the extramitochondrial pH was varied, the mitochondria were first diluted to 10 mg/mL, then 10 mM glutamate, 10 mM malate, 3.0 mM ATP, and 1.5 mM $P_i$ were added, and aliquots were adjusted to pH 6.5 or 7.6. When the osmolarity of the extramitochondrial medium was varied, in either ionic medium (117 mM KCl, 33 mM NaCl, 5 mM Mops, 0.2 mM EDTA, and 1 mM sodium acetate, pH 7.0) or sucrose–mannitol medium, the mitochondria were isolated in sucrose medium (see Mitochondrial Preparations and Respiration Measurements) and then after 30 min they were diluted 10-fold in the indicated medium, recentrifuged, and resuspended in the same medium (all at 0–4 °C). The osmolarity was varied by adding $H_2O$ to the medium. For all the conditions, respiration after ADP addition was allowed to return to minimal rates (state 4), and then samples for the various assays were taken during steady-state conditions. Aliquots (0.25 mL) of mitochondrial suspensions were quenched by a 1-min centrifugation in an Eppendorf microfuge through silicone oil (versilube F-50, or a combination of silicone oils giving a specific gravity of 1.015 when ionic media were used, General Electric) in 400-$\mu$L polyethylene tubes and treated as described previously (Deutsch et al., 1979). Initial conditions were chosen (3.0 mM ATP, 1.5 mM $P_i$, 1.5 mM ADP, and low mitochondrial protein concentration) such that in the final steady-state conditions the intramitochondrial adenine nucleotides did not contribute significantly to the total amounts present in the reaction mixture. All experiments consisted of triplicate sets of measurements.

Matrix water was determined from the distribution of $[^3H]H_2O$ by using [$^{14}$C]sucrose as the extramatrix marker. Radioactive counting was done with Aqueous Counting Solution from Amersham in a Searle Delta 300 scintillation counter.

*Measurements of Transmembrane Electrical and pH Gradients.* The transmembrane electrical gradient was calculated from the distribution of $K^+$ (plus valinomycin) or triphenylmethylphosphonium ($TPMP^+$) or tribenzylmethylammonium ($TBMA^+$) ions between the supernatant and pellet fractions.

The effect of varying external $[K^+]$ was determined by incubating mitochondrial suspensions at 5 mg/mL in 120, 45, 10, and 1 mM KCl solutions, while adjusting the mannitol and sucrose concentrations to keep osmotic strength constant, plus 5.0 mM Mops, 0.2 mM EDTA, and 1.0 mM acetate, pH 7.0. The suspensions at various $[K^+]$ were incubated without added substrate in the presence of 15 $\mu$M rotenone plus $2 \times 10^{-7}$ M valinomycin for 3 min and then centrifuged and treated as described previously for the measurement of intramitochondrial water spaces.

For $K^+$ measurements, 100-$\mu$L aliquots of supernatant were placed in 2.5–5.0 mL of 0.1 N $HNO_3$. The pellets were digested with 50 $\mu$L of concentrated $HNO_3$, incubated for 2 h at 60 °C, and then diluted with 5–10 mL of $H_2O$. Measurements were made with a Varian 1200 atomic absorption spectrophotometer. Standard curves were established by using $K^+$ solutions of 0.01–0.15 mM KCl.

Transmembrane pH gradients were determined from the measured radioactive distributions of $[^3H]$- or $[^{14}C]$acetate, $[^{14}C]$methylamine, and $[^{14}C]$-5,5-dimethyloxazolidine-2,4-dione (DMO). The supernatants and pellets from the electrical and pH probes were treated in a similar fashion as described for volume measurements. The pH gradients were calculated from eq 2 (Waddell & Butler, 1959):

$$\Delta pH = \log ([H^+]_i/[H^+]_0) \quad (2)$$

The total proton electrochemical gradient ($\Delta\mu_{H^+}$) was calculated from the sum of the $\Delta$pH and $E$:

$$\Delta\mu_{H^+} = RT/F \ln ([H^+]_i/[H^+]_0) + E \quad (3)$$

or

$$\Delta\mu_{H^+} = -n(RT \ln ([H^+]_i/[H^+]_0) + FE) \quad (4)$$

Detailed explanation of the theory of these techniques can be found in a number of publications (Deutsch et al., 1979; Skulachev 1971; Waddell & Butler, 1959).

*Measurements of* $[ATP]/([ADP][P_i])$. [ATP]/([ADP][$P_i$]) ratios were calculated from measurements of total ATP, ADP, and $P_i$ from deproteinized samples. The free energy of ATP hydrolysis was calculated on the basis of a $\Delta G°'$ of −8.4 kcal/mol for very low $[Mg^{2+}]$ (Guynn & Veech, 1973). Aliquots (1 mL) of mitochondrial suspensions were quenched in cold (4 °C) perchloric acid (0.15 mL of 40%), centrifuged to remove precipitated protein, neutralized with $K_2CO_3$ and triethanolamine, and then assayed for ATP, ADP, and $P_i$. Inorganic phosphate was assayed by the method of Martin & Doty (1949). ATP and ADP were assayed according to the procedures of Lamprecht & Trautschold (1963) and Adam (1963), respectively. At the time of centrifugation the oxygen concentration was usually between 300 and 500 $\mu$M, an amount sufficient for at least 3 min of respiration. This permitted ample time for penetration of the mitochondria through the silicone oil layer before the oxygen could be depleted. All assays were carried out with a Shimadzu Spectronic 210 digital double-beam spectrophotometer.

*Reagents.* $[^3H]H_2O$, $[^{14}C]$sucrose, $[^{14}C]$acetate, $[^{14}C]$-methylamine, and $[^{14}C]$DMO were all obtained from New England Nuclear. The substrates glutamic acid and malic acid were obtained from Sigma Chemical Co. (St. Louis), as were valinomycin, ATP, ADP, Mops, mannitol, and trizma base. The enzymes and reagents for the ATP and ADP assays were also obtained from Sigma Chemical Co. $[^3H]TPMP^+$ was kindly donated by Dr. H. R. Kaback, Roche Institute of Molecular Biology, Nutley, NJ. Nigericin was generously supplied by Dr. P. Hammill, Eli Lily Co., Indianapolis, IN. $[^3H]TBMA^+I^-$ was synthesized by one of us (D.F.W.) by a method similar to that of Birkofer (1942). Parallel synthesis of unlabeled $TBMA^+I^-$ gave a crystalline solid which melts at 186 °C in agreement with the literature values. The uncoupler 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) was the generous gift of Dr. P. Hamm of Monsanto Chemical Co.

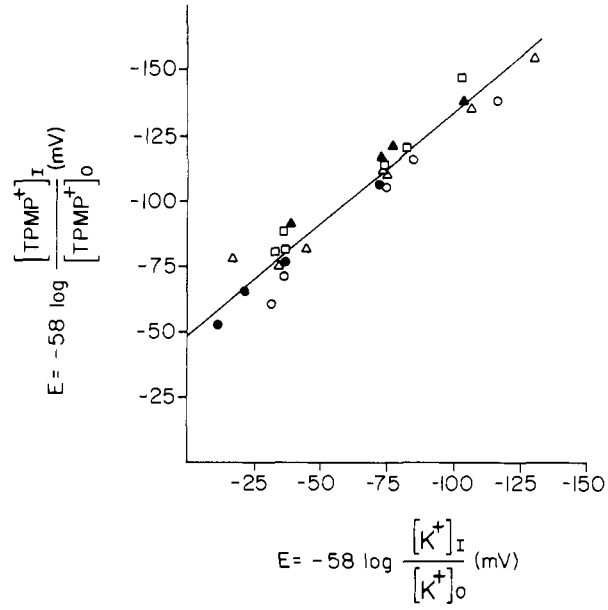


FIGURE 1: $TPMP^+$ electrical potential vs. $K^+$ diffusion potential. Mitochondrial suspensions (5 mg/mL) were incubated at various $[K^+]$ for 3 min in the presence of 15 $\mu$M rotenone (no added substrate) and $2 \times 10^{-7}$ M valinomycin for measurement of volumes, $TPMP^+$ electrical potential, and $[K^+]$. The osmotic strength of the various media was kept constant by adjusting the sucrose and mannitol concentrations while still using 5.0 mM Mops, 1.0 mM acetate, and 0.2 mM EDTA (pH 7.0). Each point represents one set of determinations of volume, $TPMP^+$ distribution, and $[K^+]$.

## Results

*The Transmembrane Electrical Gradient from* $TPMP^+$ *Distributions at Varying* $[K^+]$ *Gradients (Plus Valinomycin).* The data obtained from parallel measurements of mitochondrial volume and $TPMP^+$ and $K^+$ distributions at varying $[K^+]$ are presented in Figure 1 as the calculated transmembrane electrical potentials from the $TPMP^+$ vs. $K^+$ distribution. These determinations were conducted without added substrate and in the presence of rotenone in order to limit the available energy supply (there was no measureable oxygen consumption). However, the remaining endogenous energy supply was sufficient so that neither the mitochondrial water volume nor internal $[K^+]$ remained constant but each increased over twofold with increasing extramitochondrial $[K^+]$. At a measured external $[K^+]$ of 1.6 mM the internal $[K^+]$ was 97 mM, while at 42 mM external $[K^+]$ the internal $[K^+]$ was 200 mM. When the calculated Nearnst potentials for $K^+$ and $TPMP^+$ distribution are plotted against each other (Figure 1), the points fall along a line with a slope of ~0.85 and a $y$ axis intercept of >0.

In the remainder of the results, values of the calculated $E$ from the $TPMP^+$ distribution are both given without correction and corrected for binding based on Figure 1. In Tables I–IV the uncorrected values are given in parentheses next to the corresponding corrected values.

*Effects of an Uncoupler (S-13), Valinomycin and Increasing* $[K^+]$, *Nigericin, Sodium Nitrate, and Sodium Acetate on Thermodynamic Parameters of Isolated Mitochondria. Uncoupler.* Measurements (state 4) of mitochondrial volumes,

Table I: Effect of S-13, Valinomycin and Increasing [$K^+$], Nigericin, Sodium Nitrate, and Sodium Acetate on Thermodynamic Parameters[a]

| condition | μL of matrix $H_2O$/mg | ΔpH | transmembrane electrical and $H^+$ electrochem gradients (mV) TPMP+ $E$ | TPMP+ | TPMP+ $\Delta\mu H^+$ | $K^+$ $E$ | $K^+$ $\Delta\mu_{H^+}$ | nmol of $O_2$ $min^{-1}$ $mg^{-1}$ | log [ATP] / [ADP][$P_i$] |
|---|---|---|---|---|---|---|---|---|---|
| (A) control | 0.87 ± 0.16 | −0.70 ± 0.09 | −156 ± 3 | (−183 ± 3) | −197 ± 8 | −138 ± 2 | −179 ± 5 | 7.6 ± 0.5 | 5.35 ± 0.24 |
| 0.45 μM S-13 | 0.95 ± 0.12 | −0.62 ± 0.06 | −136 ± 6 | (−165 ± 6) | −172 ± 9 | −133 ± 4 | −169 ± 1 | 18.4 ± 0.5 | 4.93 ± 0.25 |
| 0.75 μM S-13 | 0.89 ± 0.23 | −0.56 ± 0.08 | −120 ± 6 | (−152 ± 6) | −152 ± 10 | −125 ± 6 | −157 ± 5 | 25.7 ± 1.8 | 4.17 ± 0.19 |
| 1.13 μM S-13 | 0.75 ± 0.17 | −0.45 ± 0.04 | −104 ± 3 | (−138 ± 3) | −130 ± 6 | −120 ± 3 | −146 ± 3 | 32.9 ± 2.3 | 3.52 ± 0.21 |
| (B) control | 0.57 ± 0.07 | −0.71 ± 0.03 | −173 ± 5 | (−197 ± 5) | −214 ± 6 | | | 3.7 ± 0.6 | 5.54 ± 0.22 |
| $2 \times 10^{-7}$ M valinomycin | 0.96 ± 0.07 | −0.68 ± 0.05 | −150 ± 3 | (−177 ± 3) | −189 ± 5 | −140 ± 4 | −180 ± 7 | 6.2 ± 1.7 | 5.39 ± 0.10 |
| $2 \times 10^{-7}$ M valinomycin + 2.5 mM $K^+$ | 1.14 ± 0.02 | −0.68 ± 0.09 | −122 ± 2 | (−153 ± 2) | −161 ± 4 | −113 ± 4 | −151 ± 2 | 10.4 ± 0.9 | 5.37 ± 0.08 |
| $2 \times 10^{-7}$ M valinomycin + 5.0 mM $K^+$ | 1.26 ± 0.02 | −0.67 ± 0.01 | −102 ± 4 | (−136 ± 4) | −140 ± 5 | −98 ± 4 | −137 ± 4 | 14.8 ± 0.8 | 4.50 ± 0.56 |
| (C) control | 0.65 ± 0.14 | −0.83 ± 0.12 | −159 ± 6 | (−185 ± 6) | −207 ± 13 | −152 ± 11 | −200 ± 18 | 8.3 ± 0.5 | 5.30 ± 0.28 |
| $2 \times 10^{-8}$ M nigericin | 0.58 ± 0.12 | −0.79 ± 0.17 | −160 ± 6 | (−186 ± 6) | −206 ± 9 | −134 ± 5 | −180 ± 13 | 10.3 ± 1.2 | 5.40 ± 0.26 |
| $5 \times 10^{-8}$ M nigericin | 0.41 ± 0.09 | −0.70 ± 0.11 | −174 ± 6 | (−198 ± 7) | −214 ± 9 | −126 ± 6 | −167 ± 12 | 12.4 ± 1.6 | 5.11 ± 0.30 |
| $1 \times 10^{-7}$ M nigericin | 0.31 ± 0.09 | −0.84 ± 0.17 | −184 ± 7 | (−206 ± 7) | −233 ± 16 | −124 ± 8 | −173 ± 17 | 13.8 ± 2.0 | 5.20 ± 0.31 |
| $2 \times 10^{-7}$ M nigericin | 0.20 ± 0.02 | −0.35 ± 0.23 | −193 ± 2 | (−214 ± 2) | −213 ± 12 | −108 ± 5 | −128 ± 17 | 16.6 ± 2.5 | 4.02 ± 0.22 |
| (D) 15 mM sodium nitrate | 0.53 ± 0.18 | −1.05 ± 0.06 | −156 ± 7 | (−182 ± 7) | −217 ± 9 | −149 ± 8 | −209 ± 11 | 7.2 ± 0.8 | 5.35 ± 0.04 |
| 0.075 mM sodium acetate | 0.67 ± 0.10 | −0.84 ± 0.08 | −149 ± 4 | (−176 ± 4) | −198 ± 8 | −145 ± 4 | −193 ± 8 | 6.1 ± 0.8 | 5.39 ± 0.09 |
| 0.75 mM sodium acetate | 0.69 ± 0.09 | −0.69 ± 0.06 | −150 ± 2 | (−177 ± 2) | −190 ± 5 | −146 ± 3 | −186 ± 5 | 6.4 ± 0.9 | 5.37 ± 0.06 |
| 60 mM sodium acetate | 0.80 ± 0.12 | −0.42 ± 0.03 | −147 ± 1 | (−174 ± 1) | −171 ± 1 | −145 ± 1 | −170 ± 4 | 9.0 ± 1.0 | 5.07 ± 0.07 |

[a] Effects on mitochondrial matrix volume, transmembrane electrical and pH gradients, respiration, and [ATP]/([ADP][$P_i$]). Measurements were carried out on suspensions of mitochondria treated with (A) an uncoupler (S-13), (B) valinomycin and increasing external [$K^+$], (C) nigericin, and (D) sodium nitrate and increasing concentrations of sodium acetate. The mitochondria were suspended in an oxygen-saturated medium of 0.225 M mannitol, 0.075 M sucrose, 5.0 mM Mops, 1.0 mM acetate, and 0.2 mM EDTA (pH 7.0) to a protein concentration of 16 mg/mL. Glutamate and malate, 10 mM each, were added along with 1.5 mM $P_i$, 3.0 mM ATP, catalase, and enough $H_2O_2$ to reach ~1 mM oxygen concentration. Then $2 \times 10^{-7}$ M valinomycin [except for the control in (B)] was added along with the S-13, $K^+$, nigericin, sodium nitrate or sodium acetate, and then 1.5 mM ADP. After respiration returned to minimal rates (state 4), three 0.25-mL aliquots were taken for the desired type of assay and treated as described under Materials and Methods. The data represent the mean ± SD of three separate experiments except for part C where five experiments were done.

respiration, [ATP]/([ADP][$P_i$]), and transmembrane electrical and pH gradients were made under various experimental conditions. Table I (A) summarizes the data obtained by using S-13, a potent uncoupler. The relatively high concentrations of S-13 used in these experiments are required by the high protein concentrations. The uncoupler S-13 is essentially water insoluble, and the titer is dependent on the mitochondrial protein concentration (Wilson, 1969). The matrix water volume initially increases at 0.45 μM S-13 and then decreases by 12% at 1.13 μM S-13. Levels of S-13 which gave inhibition of respiration (data not presented here) result in a 90% decrease of the control matrix water volume.

Control mitochondria, at an external pH of 7.0, gave a measured internal pH of 7.70 which shifted to pH 7.45 with 1.13 μM S-13. The transmembrane electrical gradient decreased 52 and 18 mV (more positive) as measured with $TPMP^+$ and $K^+$, respectively, upon addition of 1.13 μM S-13 compared to the control. Respiration steadily increased and the [ATP]/([ADP][$P_i$]) ratio steadily decreased as the uncoupler concentration was increased.

*Valinomycin Plus $K^+$.* The addition of valinomycin to mitochondria [Table I (B)] resulted in a significant increase of matrix volume and decrease in electrical potential as measured by $TPMP^+$. There was a minimal increase in respiration, a decrease in [ATP]/([ADP][$P_i$]), and no change in the transmembrane pH gradient. These trends continued as the external [$K^+$] was increased by adding 2.5 and 5.0 mM $K^+$ to the 1 mM $K^+$ already present in the external medium. At the highest $[K^+]_0$ the mitochondrial volume had increased 2.2-fold over that of the control, and the calculated transmembrane electrical potential had become over 60 mV more positive as measured with either $TPMP^+$ or $K^+$ distributions. There was no change in the transmembrane pH gradient and minimal change in [ATP]/([ADP][$P_i$]) as the external $K^+$ was increased, the latter consistent with the observed stimulation of respiration.

*Nigericin.* Nigericin, in the presence of $2 \times 10^{-7}$ M valinomycin, produced markedly different and more complex changes than addition of valinomycin by itself [Table I (C)]. As the nigericin concentration was increased, the matrix water volume decreased almost 70% (these volume changes limit the usefulness of experiments using higher concentrations of nigericin), the transmembrane electrical potential became 29 mV more negative with $TPMP^+$ and 44 mV more positive with $K^+$, respiration increased slightly, and [ATP]/([ADP][$P_i$]) declined slightly. The pH gradient remained constant up to $1 \times 10^{-7}$ M nigericin and then shifted 0.49 pH unit more acidic with $2 \times 10^{-7}$ M nigericin.

*Nitrate and Acetate.* Experiments with sodium nitrate and sodium acetate [Table I (D)] resulted in changes only in the

Table II: Comparison of Three pH Probes for Gradient Measurements[a]

| condition | μL of matrix $H_2O$/mg | ΔpH acetate | ΔpH methylamine | ΔpH DMO | nmol of $O_2$ $min^{-1}$ $mg^{-1}$ |
|---|---|---|---|---|---|
| 15 mM sodium nitrate | 0.48 | −1.05 | −0.74 | −1.10 | 5.5 |
| 0.075 mM sodium acetate | 0.59 | −0.84 | −0.53 | −0.80 | 4.8 |
| 0.75 mM sodium acetate | 0.69 | −0.69 | −0.46 | −0.71 | 4.7 |
| 60 mM sodium acetate | 0.81 | −0.42 | −0.38 | −0.52 | 7.1 |

[a] Evaluation of three different probes of transmembrane pH gradient measurements. Mitochondria were suspended in an oxygen-saturated medium of 0.225 M mannitol, 0.075 M sucrose, 5.0 mM Mops, 1.0 mM acetate, and 0.2 mM EDTA (pH 7.0) to a protein concentration of 16 mg/mL. Glutamate and malate, 10 mM each, were added along with 1.5 mM $P_i$, 3.0 mM ATP, catalase, and enough $H_2O_2$ to reach ~1 mM oxygen concentration. Then $2 \times 10^{-7}$ M valinomycin was added along with the sodium nitrate or sodium acetate and then 1.5 mM ADP. Respiration was allowed to return to minimal rates (state 4), and then three 0.25-mL aliquots were taken for volume and pH gradient assays using [$^{14}$C] methylamine and [$^{14}$C] dimethyloxazolidine-2,4-dione and treated as described under Materials and Methods. The data represent the average of two experiments. The [$^{14}$C] acetate data were taken from Table I (D).

matrix water volume and the transmembrane pH gradient. In changing from a medium containing 15 mM sodium nitrate to media containing up to 60 mM sodium acetate, the volume increased (maximum change 50%) and the internal pH became more acidic (by 0.63 pH unit).

*Comparison of Different Probes for Measurement of pH Gradients.* Since [$^{14}$C]acetate was used to measure the pH gradient even when the external acetate concentration increased up to 60 mM, it was important to use alternative probes to validate the measured pH gradients. For this purpose [$^{14}$C]methylamine (a weak base) and [$^{14}$C]DMO (a weak acid) were used under conditions identical with those that generated Table I (D), and the results are summarized in Table II.

Since methylamine is a weak base, it is excluded when the intramitochondrial pH is more alkaline than the external medium, making the intramitochondrial pH values calculated from its distribution susceptible to error at higher pH gradients. The calculated values indicate a pH gradient, alkaline inside, of 0.74 pH unit for 15 mM $NaNO_3$ and 0.38 pH unit for 60 mM acetate. This is in reasonable agreement with the values of 1.1 and 0.52 pH units calculated from the distribution of DMO and 1.05 and 0.42 pH units calculated from the distribution of acetate. The latter (DMO and acetate) are weak acids and are included with this pH gradient, their distributions giving the more reliable calculated internal pH values.

*Effects of Varying External pH on Mitochondrial Volume, Respiration, [ATP]/([ADP][$P_i$]), Transmembrane Electrical Potential, and Transmembrane pH Gradients.* The mitochondria had a 21% smaller matrix water volume at pH 7.6 compared to pH 6.5, with intermediate values at pH 7.0 (Table III). The pH gradient decreased as the external pH was made more alkaline, resulting in intramatrix pH values of 7.31, 7.68, and 8.07, respectively, for medium pH values of 6.5, 7.0, and 7.6. The transmembrane electrical gradient as calculated from the $TPMP^+$ distribution did not appreciably change, while that calculated from the $K^+$ gradient (plus valinomycin) shifted 24–28 mV more positive at pH 7.6 compared to pH 6.5. There were only small changes in respiration (slightly elevated at pH 7.6) and [ATP]/([ADP][$P_i$]) at the three values tested.

*Effect of Changing Composition and Osmolarity of Mitochondrial Medium on Matrix Water Volume, Respiration, [ATP]/([ADP][$P_i$]), and Transmembrane Electrical and pH Gradients.* The osmolarities of the media were altered by varying the amount of water added to the original media, i.e., to 0.350, 0.195, and 0.110 osmolar. The calculated osmolarities include contributions from the mitochondrial suspension, substrates, and ATP. Experiments with the ionic medium (117 mM KCl, 33 mM NaCl, 5 mM Mops, 0.2 mM EDTA, and 1 mM sodium acetate, pH 7.0) were evaluated without valinomycin, while $1 \times 10^{-7}$ M valinomycin was added to the sucrose–mannitol medium. The data are presented in Table IV.

There were no appreciable differences in respiration and [ATP]/([ADP][$P_i$]) between the media or when the osmolarity in either medium was varied. In each case there was swelling of matrix space when the osmolarity was lowered, with the changes being more pronounced in the sucrose–mannitol medium. The transmembrane pH gradients were higher (more alkaline inside) by ~0.2 pH unit in the sucrose–mannitol medium, and in both media the pH gradient became slightly smaller (0.07–0.17 pH unit) as the osmolarity was decreased. Values of the transmembrane electrical gradients, calculated from the $TPMP^+$ distribution, were comparable for the two media and became less negative by 14–19 mV as the osmolarity was decreased. The electrical gradients measured from $K^+$ distribution became 38 mV more positive as the osmolarity was decreased.

*Comparison of $TPMP^+$, $TBMA^+$, and $K^+$ (Plus Valinomycin) for Measurement of the Electrical Potential in a Choline Chloride Medium.* The data in Table IV (C) show the comparison of the three different probes for evaluating *E* in choline chloride medium while varying the external pH and adding 20 mM sodium acetate. None of the three probes

Table III: Effects of Varying External pH on Mitochondria[a]

| external pH | μL of matrix $H_2O$/mg | ΔpH | transmembrane electrical and $H^+$ electrochem gradients (mV) $TPMP^+$ *E* | $TPMP^+$ | $TPMP^+$ $\Delta\mu_{H^+}$ | $K^+$ *E* | $K^+$ $\Delta\mu_{H^+}$ | nmol of $O_2$ $min^{-1}$ $mg^{-1}$ | log [ATP]/([ADP][$P_i$]) |
|---|---|---|---|---|---|---|---|---|---|
| 6.5 | 1.18 ± 0.27 | −0.81 ± 0.11 | −137 ± 8 | (−166 ± 8) | −184 ± 12 | −136 ± 9 | −184 ± 13 | 9.0 ± 0.6 | 5.95 ± 0.19 |
| 7.0 | 0.96 ± 0.07 | −0.68 ± 0.05 | −150 ± 3 | (−177 ± 3) | −189 ± 5 | −140 ± 4 | −180 ± 7 | 6.3 ± 1.8 | 5.39 ± 0.10 |
| 7.6 | 0.90 ± 0.15 | −0.47 ± 0.14 | −135 ± 8 | (−164 ± 8) | −162 ± 16 | −112 ± 28 | −139 ± 36 | 18.1 ± 1.8 | 5.43 ± 0.10 |

[a] Effect of varying external pH on mitochondrial matrix volume, transmembrane electrical and pH gradients, respiration, and [ATP]/([ADP][$P_i$]). Mitochondria were suspended to a protein concentration of 10 mg/mL in an oxygen-saturated medium of 0.225 M mannitol, 0.075 M sucrose, 5.0 mM Mops, 10 mM acetate, 0.2 mM EDTA, 3.0 mM ATP, 10 mM glutamate, 10 mM malate, and 1.5 mM $P_i$. Then aliquots were adjusted to pH 6.5 and 7.6. Catalase and enough $H_2O_2$ were added to reach ~1 mM oxygen concentration along with $2 \times 10^{-7}$ M valinomycin and 1.5 mM ADP. After respiration returned to minimal rates (state 4), three 0.25-mL aliquots were taken for the desired type of assay and treated as described under Materials and Methods. The data represent the mean ± SD of three experiments. The data for pH 7.0 were taken from Table I (B).

Table IV: Comparison of Media of Different Composition and Osmolarity[a]

| condition | μL of matrix $H_2O$/mg | ΔpH | transmembrane electrical and $H^+$ electrochem gradients (mV) TPMP+ $E$ | | TPMP+ $\Delta\mu_{H^+}$ | $K^+$ $E$ | $K^+$ $\Delta\mu_{H^+}$ | nmol of $O_2$ $min^{-1}$ $mg^{-1}$ | log [ATP] / [ADP][$P_i$] |
|---|---|---|---|---|---|---|---|---|---|
| (A) ionic medium | 0.92 | −0.50 | −119 | (−150) | −148 | | | 7.1 | 5.63 |
| dil ionic medium | 1.19 | −0.52 | −110 | (−143) | −140 | | | 6.7 | 5.61 |
| $H_2O$ | 1.45 | −0.43 | −102 | (−136) | −127 | | | 7.6 | 5.58 |
| (B) sucrose–mannitol medium | 0.53 | −0.79 | −119 | (−151) | −165 | −138 | −184 | 6.6 | 5.64 |
| dil sucrose–mannitol | 1.09 | −0.62 | −109 | (−142) | −145 | −120 | −156 | 7.7 | 5.82 |
| $H_2O$ | 1.70 | −0.62 | −98 | (−132) | −134 | −100 | −137 | 11.5 | 5.45 |

| condition C | μL of matrix $H_2O$/mg | ΔpH | $E$ TPMP+ | $E$ TBMA+ | $E$ $K^+$ | $\Delta\mu_{H^+}$ TPMP+ | $\Delta\mu_{H^+}$ TBMA+ | $\Delta\mu_{H^+}$ $K^+$ | nmol of $O_2$ $min^{-1}$ $mg^{-1}$ | log [ATP] / [ADP][$P_i$] |
|---|---|---|---|---|---|---|---|---|---|---|
| choline chloride, pH 7.0 | 1.60 | −0.35 | −84 | (−121) | −82 | −80 | −105 | −103 | −100 | 14.2 | 3.90 |
| choline chloride, pH 7.6 | 1.19 | −0.32 | −89 | (−125) | −87 | −75 | −108 | −106 | −94 | 19.5 | 3.68 |
| choline chloride + 20 mM sodium acetate, pH 7.6 | 1.08 | −0.19 | −93 | (−128) | −94 | −78 | −105 | −106 | −89 | 19.8 | 3.46 |

[a] Dependence of the mitochondrial transmembrane pH and electrical gradients, matrix volume, respiration and [ATP]/([ADP][$P_i$]) on the composition and osmolarity of the suspending medium. (A) Mitochondria were isolated in sucrose medium and then after 30 min were suspended in 117 mM KCl, 33 mM NaCl, 5 mM Mops, 0.2 mM EDTA, and 1 mM sodium acetate at pH 7.0 (ionic medium) and recentrifuged; the resulting pellet was resuspended in the ionic medium to 56 mg/mL. Experiments were carried out as usual for three different conditions: (1) 0.75 mL of ionic medium; (2) 0.25 mL of ionic medium and 0.50 mL of $H_2O$; (3) 0.75 mL of $H_2O$. In each case oxygen-saturated medium, 10 mM glutamate, 10 mM malate, 3 mM ATP, and 1.5 mM ADP were added. (B) Experiments were conducted similarly to (A) but with mitochondria that were washed and suspended in 75 mM sucrose, 225 mM mannitol, 5 mM Mops, and 1 mM acetate at pH 7.0 (sucrose–mannitol medium). Additionally, $1 \times 10^{-7}$ M valinomycin was added in (B) to allow $K^+$ gradient measurements. (C) Mitochondria were isolated and suspended (50 mg/mL) in sucrose–mannitol medium. Aliquots (0.1 mL) of mitochondria were then suspended in 0.9 mL of oxygen-saturated medium comprised of 150 mM choline chloride, 5 mM Mops, 0.2 mM EDTA, 1 mM sodium acetate, 4 mM $P_i$, 10 mM glutamate, and 10 mM malate at either (1) pH 7.0, (2) pH 7.6, or (3) pH 7.6 with 20 mM sodium acetate. Experiments were carried out as usual containing $5 \times 10^{-8}$ M valinomycin.

indicated major changes in the values of the transmembrane electrical potential among the experimental conditions tested. The values calculated for the $TBMA^+$ distributions (without corrections) are in good agreement with those calculated from the $K^+$ distribution. The measured equilibrium time for $TBMA^+$ distribution under these experimental conditions was <2 min.

*Mitochondrial Volume.* Volume measurements were also conducted with $^{14}C$-labeled poly(ethylene glycol) ($M_r$ 4000) in place of [$^{14}C$]sucrose. Poly(ethylene glycol) is impermeable to the outer mitochondrial membrane, thereby giving values of the total intramitochondrial space of 1.96 ± 0.22 μL/mg (nine measurements).

## Discussion

In experiments in the present paper when the matrix space was swollen, e.g., by decreasing osmolarity or by adding valinomycin and $K^+$, the matrix space approached but did not exceed the outer membrane volume. Electron micrographs of mitochondria in intact cells typically show the inner membrane extended to the outer membrane (Fawcett, 1966). This suggests that the volume mitochondria occupy in intact cells can be estimated by assuming 2.0 μL/mg of mitochondrial protein present in the cell. Thus, isolated mitochondria suspended in the typical sucrose and/or mannitol medium have a highly condensed matrix with the enzymes in an aqueous volume less than one-third of that present in whole cells.

The observed matrix volume changes can be interpreted on the basis of ion movements. The volume increase produced by valinomycin and $K^+$ is due to the accumulation of $K^+$ and acetate with consequent osmotic swelling: the higher the external [$K^+$], the greater the swelling. The acetate-induced volume increase may be for a similar reason.

A reverse process would be responsible for the volume decrease observed with nigericin. Nigericin increases the permeability of $K^+$ [primarily by $K^+$ for $H^+$ exchange; Pressman (1968)], thereby depleting mitochondria of $K^+$. Uncoupler (plus valinomycin) also decreased mitochondrial matrix volume, indicating an increased permeability of the mitochondrial membrane. The observed volume decrease which accompanies increasing extramitochondrial pH also must reflect increases in ion permeabilities which occur as the pH is increased (Brierly & Jurkowitz, 1976).

*Transmembrane pH and Electrical Gradients.* The transmembrane pH gradients were reproducible among the experiments, and the agreement of the measured gradients using three different probes (acetate, DMO, and methylamine) indicated good reliability for the calculated pH gradient.

Confidence in the accuracy of the transmembrane electrical gradient measurements generally poses more of a problem. In experiments where large gradients, negative inside, are encountered, the use of negatively charged probes such as tetraphenylboron and thiocyanate is unreliable due to extent of exclusion of these probes by the electrical gradient. Since binding of the positive lipophilic ions such as $TPMP^+$ can be a problem, the $TPMP^+$ distribution was compared with the $K^+$ distribution in the presence of valinomycin (plus rotenone without substrates) which allows free diffusion of $K^+$ in the absence of a significant energy supply. On the basis of the assumption that $K^+$ is not bound or transported, the results from the graph of the two sets of calculated diffusion potentials suggest a significant level of binding of $TPMP^+$ to mitochondria. The distribution of the lipophilic ion $TBMA^+$ is very similar to that of $K^+$, without any correction for $TBMA^+$

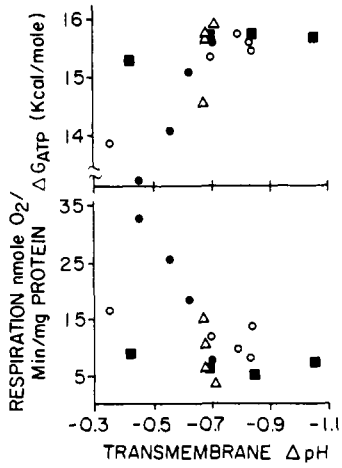


FIGURE 2: Free energy of ATP hydrolysis and rate of oxygen consumption plotted against the transmembrane pH gradient. The data were taken from Table I and are for titrations with S-13 (●), extramitochondrial $K^+$ (Δ), nigericin (O), and $NaNO_3$ or sodium acetate (■). The correlation coefficients to linear relationships are 0.68 for the free energy of ATP hydrolysis vs. transmembrane pH gradient (upper figure) and 0.53 for the rate of oxygen consumption vs. transmembrane pH gradient (lower figure).

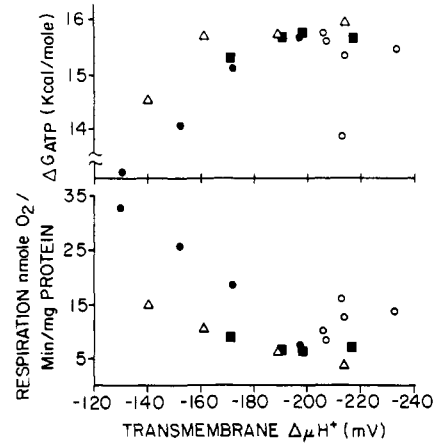


FIGURE 3: Free energy of ATP hydrolysis and rate of oxygen consumption plotted against the proton electrochemical gradient. The data were taken from Table I and are for titrations with S-13 (●), extramitochondrial $K^+$ (Δ), nigericin (O), and $NaNO_3$ or sodium acetate (■). The correlation coefficients to linear relationships are 0.58 for the free energy of ATP hydrolysis vs. proton electrochemical gradient (upper figure) and 0.61 for the rate of oxygen consumption vs. proton electrochemical gradient (lower figure).

binding, suggesting $TBMA^+$ is not significantly bound. The corrected $TPMP^+$ values and direct $TBMA^+$ values are in excellent agreement.

All these results indicate that the $TPMP^+$ values are an accurate measure of the true transmembrane electrical gradient after correction for binding. The valinomycin plus $K^+$ values, although probably reliable in many cases, are suspect when the possibility of active transport occurs (Diwan & Tedeschi, 1975) and when alternate routes of $K^+$ movement are present (such as nigericin).

The data can be analyzed with respect to three important questions. (1) Are the pH, electrical potential, and the total proton electrochemical gradient ($\Delta\mu_{H^+}$) possible determinants of the respiratory rate? (2) Is the behavior of the proton electrochemical gradient consistent with its proposed function as an intermediate in oxidative phosphorylation? (3) What is the minimum $[H^+]/[ATP]$ ratio consistent with the proton electrochemical gradient being an intermediate in oxidative phosphorylation?

*Is the Proton Electrochemical Gradient Correlated with Respiratory Rate and ATP Synthesis*? Two parameters are correlated only if the correlation plots have the same slope independent of the method used to perturb the system, as long as the reaction pathway between the two is not modified. The slope need not be linear but should be independent of the method of perturbation. The degree of correlation can be quantitatively evaluated through calculation of correlation coefficients which can range from 1.0 (perfect correlation) to 0.0 for random data points. When the transmembrane pH gradients are plotted (Figure 2) against the rate of respiration or $\Delta G_{ATP}$, there was no common trend for the points in either case, and correlation coefficients were 0.53 and 0.68, respectively. For example, when the pH gradient was changed by increasing the acetate and nitrate concentrations in the suspending medium, it gave a totally different behavior than did nigericin addition. Similar results are seen in Figure 3 with plots of $\Delta\mu_{H^+}$ vs. the respiratory rate or $\Delta G_{ATP}$; correlation coefficients were 0.61 and 0.58, respectively. Corrected values of $TPMP^+$ transmembrane electrical potentials were used for the plots of $\Delta\mu_{H^+}$ instead of valinomycin–$K^+$ distribution for the reasons described earlier dealing with possible active $K^+$ transport.

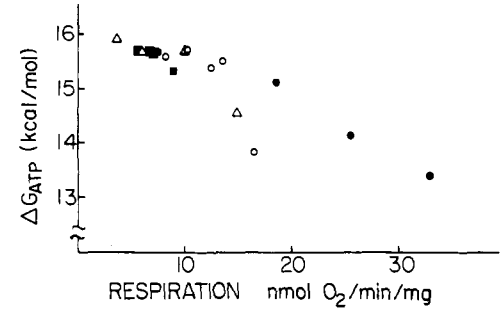


FIGURE 4: Rate of oxygen consumption plotted against the free energy of hydrolysis of ATP. The data were taken from Table I and are for titrations with S-13 (●), extramitochondrial $K^+$ (Δ), nigericin (O), and $NaNO_3$ or sodium acetate (■). The correlation coefficient to a linear relationship is 0.91.

The respiratory rate is plotted against $\Delta G_{ATP}$ in Figure 4. There is a correlation between these parameters because the majority of points, whatever the method of perturbation, fall along the same general curve and give a high correlation coefficient, 0.91. The observed relationship agrees well with that previously reported (Holian et al., 1977).

The apparent lack of correlation of $\Delta\mu_{H^+}$ with respiration and $\Delta G_{ATP}$ can also be noted in the experiments varying external pH [$\Delta G_{ATP}$ corrected at different pH values according to Rosing & Slater (1972)] and external osmolarity. In these experiments both the respiration and $\Delta G_{ATP}$ remained fairly constant, while $\Delta\mu_{H^+}$ changed by up to 47 mV.

Thayer & Hinkle (1975) have reported that in submitochondrial particles the initial rate of ATP synthesis is slightly faster when driven by artificially imposed pH and $K^+$ (plus valinomycin) gradients than when driven by substrate oxidation. The authors suggest that these measurements support the idea that the $\Delta\mu_{H^+}$ and ATP synthesis are in near equilibrium. Two considerations make this questionable. (1) The experimental conditions for initial rate measurements (highly irreversible reaction) are very different from those of near equilibrium (fully reversible). In regulated enzyme systems there is rarely the same enzymatic activity for the two conditions. (2) Little ATP synthesis was observed in submitochondrial particles until the imposed $\Delta\mu_{H^+}$ exceeded 165 mV, and the reported high rates of ATP synthesis are for values near 265 mV. In the current paper, by use of intact mito-

Table V: Possible Stoichiometry for Coupling of $H^+$ Electrochemical Gradient with the Free Energy of ATP Synthesis[a]

| condition | $\Delta G_{ATP}$ [kcal/mol (kJ/mol)] | $\Delta G_{H^+}$ (C) $TPMP^+$ [kcal (kJ)] | $\Delta G_{H^+}$ (D) $K^+$ [kcal (kJ)] | $\Delta G_{ATP}/\Delta G_{H^+}$ $TPMP^+$ | $\Delta G_{ATP}/\Delta G_{H^+}$ $K^+$ |
|---|---|---|---|---|---|
| control | 15.9 (66.5) | 4.9 (20.5) | | 3.2 | |
| Alterations Principally in $E$ | | | | | |
| $2 \times 10^{-7}$ M valinomycin | 15.7 (65.7) | 4.4 (18.4) | 4.2 (17.6) | 3.6 | 3.8 |
| $2 \times 10^{-7}$ M valinomycin + 2.5 mM $K^+$ | 15.7 (65.7) | 3.7 (15.5) | 3.5 (14.6) | 4.2 | 4.5 |
| $2 \times 10^{-7}$ M valinomycin + 1.13 M S-13 | 13.4 (56.1) | 3.0 (12.6) | 3.4 (14.2) | 4.5 | 4.0 |
| $2 \times 10^{-7}$ M valinomycin + $2 \times 10^{-7}$ M nigericin | 13.7 (57.3) | 4.9 (20.5) | 2.9 (12.1) | 2.8 | 4.6 |
| osmotically swollen, in $K^+$ and $Na^+$ medium | 16.0 (66.9) | 2.9 (12.1) | | 5.5 | |
| $1 \times 10^{-7}$ M valinomycin, osmotically swollen, in sucrose–mannitol medium | 15.8 (66.1) | 3.1 (13.0) | 3.2 (13.4) | 5.1 | 5.0 |
| Alterations Principally in $\Delta$pH | | | | | |
| $2 \times 10^{-7}$ M valinomycin + 15 mM sodium nitrate | 15.7 (65.7) | 5.0 (20.9) | 4.8 (20.1) | 3.1 | 3.3 |
| $2 \times 10^{-7}$ M valinomycin + 60 mM sodium acetate | 15.4 (64.4) | 3.9 (16.3) | 3.9 (16.3) | 4.0 | 3.9 |
| $2 \times 10^{-7}$ M valinomycin, pH 6.5 | 16.1 (67.4) | 4.2 (17.6) | 4.2 (17.6) | 3.8 | 3.8 |
| $2 \times 10^{-7}$ M valinomycin, pH 7.6 | 16.5 (69.0) | 3.7 (15.5) | 3.2 (13.4) | 4.5 | 5.1 |
| $5 \times 10^{-8}$ M valinomycin, 150 mM choline chloride medium, pH 7.0 | 13.7 (57.3) | 2.4 (10.0) | 2.3 (9.6) | 5.7 | 6.0 |
| $5 \times 10^{-8}$ M valinomycin, 150 mM choline chloride, 20 mM sodium acetate medium, pH 7.6 | 13.1 (54.8) | 2.4 (10.0) | 2.0 (8.4) | 5.5 | 6.6 |

[a] Listed are various conditions taken from Tables I, III, and IV, in which the ratio of $\Delta G_{ATP}/\Delta G_{H^+}$ is calculated, as determined from both $TPMP^+$ and $K^+$ values of $\Delta\mu_{H^+}$. In each case the total proton electrochemical gradient (eq 4) was used to calculate the free energy change for proton translocation.

chondria, maximal [ATP]/([ADP][$P_i$]) values were achieved when the $\Delta\mu_{H^+}$ was less than −100 mV, and maximal rates of ATP synthesis occurred for even lower values of $\Delta\mu_{H^+}$.

*Thermodynamically Required [$H^+$]/[ATP] Ratios for Chemiosmotic Mechanisms.* If the assumption is made that the proton electrochemical gradient is an intermediate in oxidative phosphorylation, the minimum number of moles of $H^+$ that would be required to be transported per ATP synthesized can be calculated from $\Delta\mu_{H^+}$ and $\Delta G_{ATP}$. These calculations are shown in Table V as $\Delta G_{ATP}/\Delta G_{H^+}$ which is equivalent to the moles of $H^+$ that need to be translocated for an energy balance between $\Delta\mu_{H^+}$ and $\Delta G_{ATP}$. The free energy change for proton translocation was calculated from eq 4. Values of $\Delta G_{ATP}/\Delta G_{H^+}$ ranging from 2.8 to 5.7 ($TPMP^+$) and 3.3 to 6.6 ($K^+$ and $TBMA^+$) can be calculated, depending on the experimental conditions.

As indicated in the introduction, measurements of $\Delta G_{ATP}$ and $\Delta\mu_{H^+}$ under phosphorylating conditions have been carried out in several laboratories (Azzone et al., 1976, 1978b; Hoffman & Laris, 1974; Laris et al., 1975; Mitchell, 1968) with variable results. The reported $\Delta G_{ATP}$ values range from −13 to −16.5 kcal/mol, but many of the lower values may be attributed to the use of kinetically limiting concentrations of ADP or $P_i$ (Azzone et al., 1978b; Nicholls, 1974). With this consideration there appears to be reasonable agreement that $\Delta G_{ATP}$ is near −16 kcal/mol for maximum ATP synthesis (state 4). Values of $\Delta\mu_{H^+}$ are much more variable, in part due to the use of widely different methods. The methods with a firm theoretical basis for measuring transmembrane electrical potentials include microelectrode measurements and distribution of lipophilic ions (such as $TPMP^+$ and $TBMA^+$) and $K^+$ with valinomycin, assuming no active-transport system for $K^+$. Distributions of lipophilic ions usually give higher values for the calculated electrical potential than do the distributions of $K^+$ and $Rb^+$. The discrepancy is tentatively assumed to result from greater binding of these ions to the membrane than $K^+$ or $Rb^+$. Binding corrections for the lipophilic ions bring the two methods into good agreement. Using these methods, several laboratories have reported $\Delta\mu_{H^+}$ values from −0.14 to −0.23 V for similar experimental conditions (Azzone et al., 1976, 1978b; Hoffman & Laris, 1974; Laris et al., 1975; Mitchell & Moyle, 1969). Our values are in good agreement with Padan & Rottenberg (1973) and Azzone et al. (1978a,b) but clearly lower than the values reported by Nicholls (1974), Mitchell (1968), and Mitchell & Moyle (1969). An important point, in which we are in agreement with Azzone et al. (1978a,b), is that, when other media or reaction conditions are used, (1) good phosphorylation ($\Delta G_{ATP} > -13$ kcal/mol) can be observed when $\Delta\mu_{H^+}$ is −100 mV or less and (2) changes in $\Delta G_{ATP}$ and $\Delta\mu_{H^+}$ are not strictly correlated.

These results establish that, first, phosphorylation can occur under conditions where transmembrane movement of six or more protons would have to be coupled to the synthesis of each ATP, if the proton electrochemical gradient was an intermediate in mitochondrial oxidative phosphorylation, and, second, the coupling mechanism between proton translocation and ATP synthesis must incorporate variable stoichiometry in order for the $H^+$ electrochemical gradient to be a required intermediate in ATP synthesis from respiration.

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