Mitochondrial Transmembrane pH and Electrical Gradients: Evaluation of Their Energy Relationships with Respiratory Rate and Adenosine 5′-Triphosphate Synthesis†

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ABSTRACT: Mitochondria from rat liver were suspended in ionic media, and parallel measurements were made of the respiratory rate, intramitochondrial volumes, and the phosphorylation state [[ATP]/([ADP][P_i])], as well as transmembrane pH gradients (from the distribution of labeled weak acids and bases) and electrical gradients (from the distribution of labeled lipid-soluble ions and of K^+ in the presence of valinomycin). A decrease in the pH gradient was observed with additions of sodium propionate, acetate, or lactate. In the case of propionate, the pH gradient decreased from −0.94 ± 0.10 (control) to −0.09 ± 0.08 unit (with 3 mM propionate) without change in any of the other measured parameters. Variation of the intramitochondrial volumes was achieved by changing [K^+] (+valinomycin) and by diluting the choline chloride media. The transmembrane electrical potential decreased from −150 mV under control conditions to approximately −75 mV in a 70 mosM medium or when 8 mM K^+ (+valinomycin) was present. The changes in [ATP]/([ADP][P_i]) were as follows: from 1.8×10^4 (control) to 3.9×10^2 M^{-1} with 8 mM K^+ (+valinomycin) or 1.0×10^4 M^{-1} when the osmolarity was decreased from 300 to 70 mosM. This corresponds to a decrease in the energy utilized for ATP synthesis from 14.1 to 11.9 kcal/mol (from 58.9 to 49.7 kJ/mol) with added K^+ and to 13.8 kcal/mol (57.7 kJ/mol) with decreased osmolarity. No direct correlation was found between the changes in transmembrane electrical potential, pH gradient, or total proton electrochemical gradient and either the respiratory rate or [ATP]/([ADP][P_i]).

Mitochondria, as part of their normal function, utilize the energy available from substrate oxidation for both ATP synthesis and solute transport. Interest in the two processes derives not only from their individual importance in mitochondrial function but also from the proposal that the electrogenic transport of one particular ion, H^+, serves as an essential intermediate in oxidative phosphorylation (Mitchell, 1966, 1977). In a metabolic process with a directional net flow, in this case oxidative phosphorylation, any intermediate must fulfill the inequality

$$a\Delta G_{o-r} \geq b\Delta G_i \geq c\Delta G_{ATP} \text{ or } d\Delta G_T \quad (1)$$

where ΔG_{o-r}, ΔG_i, ΔG_{ATP}, and ΔG_T are the respective negative free energies for the transfer of reducing equivalents across an energy coupling site, breakdown of the metabolic intermediate, hydrolysis of ATP, and dissipation of the electrochemical gradient of the transported solute. In each case, the calculations must include the appropriate relative stoichiometries, expressed here as *a*, *b*, *c*, and *d*. This is a particularly useful relationship in oxidative phosphorylation because electron transport from intramitochondrial NADH to cytochrome *c* has been shown to be near equilibrium both for suspensions of isolated mitochondria (Erecinska et al., 1974) and for mitochondria in situ (Wilson et al., 1974a,b; Hassinen & Hiltunen, 1975). As equilibrium is approached, the ΔG values in eq 1 must approach equality.

Considerable effort has been devoted to analyzing the free-energy relationships in oxidative phosphorylation (Slater et al., 1973; Erecinska et al., 1974; Wilson et al., 1974a,b; Holian et al., 1977) and in mitochondrial ion transport (Cockrell et al., 1966; Rottenberg, 1975; Azzone et al., 1978b, 1979; Fiskum et al., 1979; Holian & Wilson, 1980). Since the transport of any charged species across a membrane is dependent upon both its activity (concentration) gradient and the transmembrane electrical potential (*E*), i.e., on its electrochemical potential gradient, much effort has been directed toward developing suitable analytical methods for measurement of these gradients. As the methodology has improved and the number of reported measurements has increased, the values for the proton electrochemical gradient have consistently been lower than that predicted by Mitchell (1966, 1977). In addition, when differences in experimental conditions have been reported to give changes in the proton electrochemical gradient, they have not correlated well with the changes in measured ΔG_{ATP} or ΔG_{o-r}.

Consideration of a possible cause and effect relationship between parameters of a metabolic system requires examination of the most extensive range of experimental conditions still consistent with reasonably normal function of the system. In the present work, a large number of experimental conditions were studied in an attempt to manipulate the various parameters of mitochondrial function. The sets of conditions described in this paper were selected to best illustrate cases of almost independent variation of the mitochondrial transmembrane pH and electrical gradients. The effects of systematically decreasing these gradients on both the rate of ATP synthesis (respiratory rate) and the energy utilized for ATP synthesis (ΔG_{ATP}) are quantitated. The results are discussed in the context of possible energy-coupling and transport mechanisms.

Materials and Methods

Rat liver mitochondria were isolated according to the method of Schneider (1948) and suspended at a protein concentration of 45–65 mg/mL (biuret method; Gornall et al., 1949) in mannitol–sucrose medium [0.225 M mannitol, 0.075 M sucrose, 2 mM 3-(*N*-morpholino)propanesulfonate (Mops), and 0.4 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), pH 7.1]. Respiratory control values were 6–15 when glutamate and malate were used as substrates. Assay media consisted of 120 mM choline chloride, 15 mM Mops, and 0.2 mM EGTA at pH 7.1, or with variations in osmolarity as noted

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0006-2960/82/0421-1438$01.25/0

for the individual experiments. Oxygen consumption was measured with a Yellow Springs Instrument Co. (Yellow Springs, OH) oxygen electrode in a 1.2-mL closed glass chamber (Gilson Medical Electronics, Middleton, WI).

Measurements were made of oxygen consumption, mitochondrial matrix volume, transmembrane pH, electrical and K^+ gradients, and [ATP]/([ADP][P_i]) in parallel incubations, all at room temperature. Aliquots of the concentrated mitochondrial suspensions were added to oxygen-saturated assay media to give a final protein concentration of 5–6 mg/mL, with additions of 3 mM NaP_i, 1 mM ATP, 5 mM glutamate, and 5 mM malate as substrates. Reactions were carried out in the O_2 electrode chamber or in glass vials, all equipped with magnetic stirring bars. Either sodium propionate, potassium acetate, KCl, and/or valinomycin (2.4×10^{-7} M) was also added at this time, depending upon the experimental conditions. Phosphorylation was initiated by the addition of ADP (0.7 mM) and was followed immediately by the addition of the probes.

Respiration was allowed to return to minimal rates (state 4), usually about 60 s, and then samples were taken. Duplicate aliquots (0.4 mL) of assay suspensions were transferred to 400-μL polypropylene centrifuge tubes (Eppendorf) containing 20 μL of silicone oil (specific gravity adjusted to stay below the medium in question; General Electric) and were subjected to a 1.5-min centrifugation in a Beckman Model B microfuge. Supernatant and pellet fractions were processed according to a previously described method (Deutsch et al., 1979b). Prepared samples were dissolved in ACS II aqueous scintillation fluid (Amersham, Arlington Heights, IL) and counted in a Searle Delta 300 scintillation counter.

Determination of Transmembrane Gradients. The intramitochondrial (matrix) water volume and the extramitochondrial water volume trapped in the mitochondrial pellet (trapped volume) were calculated from the total water content of the pellet as measured with ^3H_2O (0.63 μCi/mL) and the [^{14}C]sucrose (0.25 μCi/mL) content, assuming the latter to be impermeable to the membrane.

Transmembrane pH gradients were calculated primarily from the distribution of [^{14}C]acetate (25 μM) or [^{3}H]acetate (12.5 μM). This probe has nearly ideal properties for this purpose. (1) The uncharged acid has been shown to be very rapidly permeable to biological membranes and lipid bilayers [Klocke et al., 1972; Deutsch & Forster, 1977; see Deuticke (1973) for experiments with erythrocyte membranes]. (2) Salts of the anionic species (acetate) are very water soluble, and thus acetate would be expected to have a low affinity for most nonspecific anion binding sites. (3) The acetate ion is only very slowly permeable to lipid bilayers and, in general, is not transported across biological membranes except where low-specificity anion-exchange mechanisms are present. Thus, permeability of acetate is normally sufficiently less than that for acetic acid to allow the total distribution to accurately reflect an existing pH gradient [see Klocke et al. (1972) and Deutsch & Forster (1977)].

A series of experimental tests gave no evidence for metabolism of acetate or of high-affinity binding sites of such numbers that they would significantly contribute to the measured internal concentration. (1) The measured distribution of acetate was independent of concentration throughout the measured range of 10–800 μM. (2) The final distribution was attained in less than 30 s and remained constant for at least 5 min. (3) The final distribution was independent of the order of addition of labeled and unlabeled compounds; i.e., 100 μM unlabeled acetate added 30 s before 12.5 μM labeled acetate gave the same final isotope distribution as when the 12.5 μM labeled compound was used alone.

The weak base ethanolamine (83 μM) verified the existence of a pH gradient, alkaline inside, as its concentration was lower inside than outside. The other weak acids which were used, 5.4 μM 5,5-dimethyloxazolidine-2,4-dione [see Addanki et al. (1968)] and 15 μM trimethylacetic acid, gave patterns similar to that of acetate but with somewhat different absolute values. It has not proven possible to obtain quantitative agreement of a number of different probes for pH gradients over wide ranges of experimental conditions. This means that despite the apparent ideal behavior of acetate, some reservations remain concerning its absolute accuracy.

Calculation of ΔpH was made according to

$$\Delta pH_i = pK_a + \log [(T_i/T_e)(10^{pH_e - pK_a} + 1) - 1] \quad (2)$$

where T_i and T_e are the internal and external concentrations of a weak acid, pH_i and pH_e are the pHs of the intramitochondrial space and medium, respectively, and pK_a is that of the weak acid probe.

Transmembrane electrical gradients can be calculated according to the Nernst potential from the distribution of any passively permeable ion at equilibrium

$$E = -\frac{RT}{nF} \ln \frac{[C_i]}{[C_e]} \quad (3)$$

where $[C_i]$ and $[C_e]$ are the concentrations, internal and external, of a permeable ion. In this work, [^{3}H]triphenylmethylphosphonium ($TPMP^+$) [0.8–2.5 μM; results were found to be concentration independent in this range; see also Holian & Wilson (1980)] was used as the charged lipophilic probe (Skulachev, 1971). Distribution of potassium in the presence of valinomycin was determined by separate measurements of supernatant and pellet concentrations of the ion, using a Varian Series AA475 atomic absorption spectrophotometer. Preparation of samples was as follows: 50 μL of supernatant was diluted into 5 mL of buffer (0.1 N HNO_3 + 1 mg/mL cesium chloride). Pellets were first digested for 24 h with 50 μL of concentrated nitric acid and then diluted into 5–10 mL of buffer. The total proton electrochemical gradient, $\Delta\mu_{H^+}$, was calculated as the sum of ΔpH and the membrane potential as

$$\Delta\mu_{H^+} = E - \frac{RT}{F}(pH_e - pH_i) \quad (4)$$

and expressed in millivolts.

We have compared the relative values for E as calculated by the distribution of K^+ and $TPMP^+$. The data obtained for all of the experimental conditions used in the present paper are plotted in Figure 1. The data fall along a straight line with a slope of 0.98 and correlation coefficient of 0.98. The y intercept of 27.7 mV is consistent with two-thirds of the phosphonium being bound to the mitochondrial membrane and matrix elements of the mitochondria which equilibrate with the internal free phosphonium. Similar results were reported for slightly different conditions by Holian & Wilson (1980). A correction for the binding of $TPMP^+$ has been included in all subsequent tables and plots. The reader may regenerate the original data (measured $[TPMP^+]_i/[TPMP^+]_e$) by reading from the abscissa (corrected value) and the ordinate (uncorrected value). Tritiated tetraphenylphosphonium (TPP^+) and tribenzylmethylammonium ($TBMA^+$) were also used as probes for the membrane potential. When the values for these probes were plotted against the K^+-derived values for the same experiment, relatively more binding was observed with TPP^+ (approximately 77%) and less for the $TBMA^+$ [approximately

Table I: Effect of Increasing Concentrations of Sodium Propionate on Mitochondrial Energetic Parameters[a]

sodium propionate (mM)	μL of matrix H_2O (mg of protein)$^{-1}$	ΔpH (acetate)	membrane potential (mV) ($TPMP^+$)	proton electrochemical gradient (mV)	log [[ATP]/([ADP][P_i])]	respiratory rate [nmol of O_2 min^{-1} (mg of protein)$^{-1}$]
0	1.23 ± 0.28 (5)	−0.92 ± 0.10 (4)	133 ± 6 (4)	188 ± 8 (4)	4.73 ± 0.16 (2)	11.7 ± 2.8 (4)
0.05	1.20 ± 0.17 (4)	−0.78 ± 0.18 (4)	132 ± 5 (4)	178 ± 8 (4)	4.78 ± 0.05 (2)	15.5 ± 2.1 (3)
0.1	1.15 ± 0.31 (5)	−0.29 ± 0.06 (4)	135 ± 4 (4)	152 ± 3 (4)	4.81 ± 0.04 (2)	13.5 ± 3.2 (3)
0.3	1.16 ± 0.21 (5)	−0.16 ± 0.09 (4)	127 ± 7 (4)	137 ± 11 (4)	4.78 ± 0.01 (2)	14.6 ± 2.7 (3)
1	1.14 ± 0.21 (5)	−0.14 ± 0.06 (4)	134 ± 5 (4)	142 ± 5 (4)	4.81 ± 0.03 (2)	13.4 ± 2.3 (3)
3	1.17 ± 0.35 (3)	−0.09 ± 0.08 (4)	127 ± 9 (4)	132 ± 12 (4)	4.75 ± 0.02 (2)	13.8 ± 0.2 (3)

[a] Concentrated mitochondrial suspension (45–65 mg of protein/mL in mannitol–sucrose medium) was added to the oxygen-saturated assay medium of 120 mM choline chloride, 15 mM Mops, and 0.2 mM EGTA to a final protein concentration of approximately 5 mg/mL. Substrates (5 mM glutamate and 5 mM malate), NaP_i (3 mM), ATP (1 mM), and propionate (as indicated) were added. Phosphorylation was initiated with 0.7 mM ADP, and the labeled probes were added approximately 15 s later. Samples were taken after respiration had returned to minimal rates (see Materials and Methods). All reactions were at pH 7.1 and were carried out at room temperature.

50%; see also Holian & Wilson (1980)] than for similar concentrations of $TPMP^+$. The slopes were similar to those generated by $TPMP^+$ and K^+.

Determination of $[ATP]/([ADP][P_i])$. After respiratory rates had returned to a minimum following addition of ADP, 1-mL samples were rapidly added to 1 mL of cold 4% perchloric acid. After 10 min at 0–4 °C, the samples were centrifuged to remove precipitated protein, and the supernatant fractions were neutralized to pH 6–6.5 with cold 2 M K_2CO_3–0.5 M triethanolamine solution. Adenine nucleotide concentrations were determined by high-performance liquid chromatography, using a Perkin-Elmer Series 2 liquid chromatograph. An isocratic elution was done with a 0.6 M ammonium phosphate buffer (pH 4.4) from a Du Pont 25 cm × 4.6 mm Zorbax SAX column, with the detector set at 260 nm. Standardized solutions at ATP and ADP were used for calibrations.

The energy required for synthesis of 1 mol of ATP, ΔG_{ATP}, was calculated with

$$\Delta G_{ATP} = \Delta G^{\circ\prime} + RT \ln \frac{[ATP]}{[ADP][P_i]} \qquad (5)$$

where the standard free energy of synthesis of ATP at 25 °C and at pH 7.0, for low $[Mg^{2+}]$, is 8.4 kcal/mol (35.1 kJ/mol) (Guynn & Veech, 1973).

$[^3H]TPMP^+I^-$ and $[^3H]TBMA^+I^-$ were synthesized and crystallized by one of us (D.F.W.) according to the methods of Lipsich and Lieberman as reported by Hong (1977) and of Birkofer (1942), respectively. Purity of synthesized $TMBA^+I^-$ was confirmed [see Holian & Wilson (1980)]. Other radioactive compounds were purchased from New England Nuclear, Boston, MA.

Carbonyl cyanide *p*-(trifluoromethyl)phenylhydrazone (FCCP) was the generous gift of Dr. P. G. Heytler, E. I. du Pont de Nemours and Co., Wilmington, DE. All substrates and reagents used were of the highest grade commercially available.

Results

Experimental Variation of the Transmembrane pH Gradient (*ΔpH*). In earlier experiments (Holian & Wilson, 1980), the pH gradients across the mitochondrial membrane under phosphorylating conditions but in different media were observed to vary from greater than 1 pH unit, alkaline inside, to near zero pH unit. In the present work, the conditions giving rise to these ΔpH values were systematically examined. The presence of acetate in the incubation medium has been reported (Holian & Wilson, 1980) to decrease the measured ΔpH, an observation confirmed during the current experiments. In extending these observations, we have found that propionate is much more effective in decreasing ΔpH than is acetate

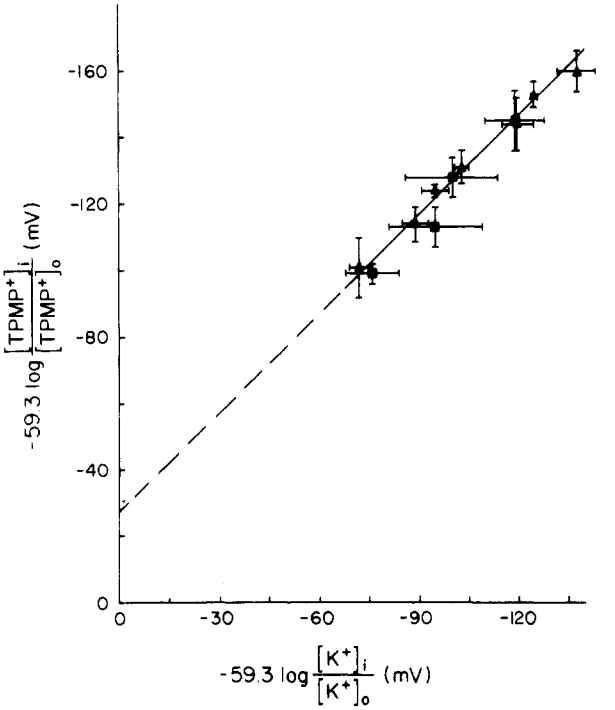

FIGURE 1: Comparison of $TPMP^+$ and K^+ concentrations in the presence of valinomycin as indicators of the transmembrane electrical potential. The membrane potentials, as calculated from the distribution of K^+ (abscissa) and $[^3H]TPMP^+$ (ordinate) for the various experimental conditions included in this study, are plotted in the figure. The data are from experiments in which the osmolarity (▲) and $[K^+]$ (■) were varied. A straight line is fitted to the data by the least-squares method. It has a slope of 0.98 and a *y* intercept of 27.7 mV. The correlation coefficient is 0.98.

(Table I). Additions of propionate (0–3 mM) in the incubation medium of mitochondria under phosphorylating conditons resulted in ΔpH decreasing from −0.94 ± 0.10 unit at 0 mM propionate to −0.09 ± 0.08 unit at 3 mM propionate. The respiratory rate remained unchanged at near 13.5 nmol of O_2 min^{-1} (mg of protein)$^{-1}$, and the generated phosphorylation state ratio, expressed as log [[ATP]/([ADP][P_i])], remained unchanged at near 4.8. The transmembrane electrical potential, as calculated from the distribution of $TPMP^+$, was unaffected by propionate concentration and remained constant at about −130 mV.

When the total proton electrochemical gradient was calculated, it decreased from −188 ± 8 to −132 ± 4 mV with increasing propionate concentrations. Similar effects were observed when acetate or lactate was added to the suspending medium except that much higher concentrations were required. For example, a 50% decrease in ΔpH was attained with 0.1 mM propionate, 3 mM acetate, or 30 mM lactate.

Table II: Effect of Varying Osmolarity of Suspending Medium on Mitochondrial Energetic Parameters[a]

tonicity (mosM)	μL of matrix H_2O (mg of protein)$^{-1}$	ΔpH (acetate)	membrane potential (mV)		av proton electrochemical gradient (mV)	log [[ATP]/([ADP][P_i])]	respiratory rate [nmol of O_2 min^{-1} (mg of protein)$^{-1}$]
			TPMP$^+$	K^+			
300	1.39 ± 0.18 (6)	−0.50 ± 0.05 (6)	117 ± 9 (4)	119 ± 9 (4)	148	4.28 ± 0.07 (3)	18.8 ± 3.9 (7)
160	2.04 ± 0.21 (6)	−0.38 ± 0.05 (6)	100 ± 6 (4)	100 ± 14 (4)	125	4.24 ± 0.07 (4)	26.4 ± 5.6 (6)
100	2.72 ± 0.14 (6)	−0.33 ± 0.05 (6)	85 ± 6 (4)	96 ± 14 (4)	110	4.09 ± 0.07 (4)	28.3 ± 8.1 (6)
70	3.72 ± 0.25 (4)	−0.24 ± 0.05 (4)	71 ± 3 (4)	76 ± 8 (2)	88	3.96 ± 0.15 (4)	34 ± 9.7 (6)

[a] Aliquots of concentrated mitochondrial suspensions (45–65 mg of protein/mL in mannitol–sucrose medium) were diluted with oxygen-saturated choline chloride assay media of decreasing osmolarities to give a final protein concentration of approximately 6 mg/mL. The media and the final osmolarities were as follows: 120 mM choline chloride, 15 mM Mops, and 0.4 mM EGTA (300 mosM); 60 mM choline chloride, 7.5 mM Mops, and 0.4 mM EGTA (160 mosM); 30 mM choline chloride, 5 mM Mops, and 0.4 mM EGTA (100 mosM); and 15 mM choline chloride, 5 mM Mops, and 0.4 mM EGTA (70 mosM). Substrates, NaP_i, and ATP were added as in Table I. Valinomycin (2.4×10^{-7} M) and potassium acetate (1 mM) were present in all assays to permit the K^+ distribution to be used as a measure of the transmembrane electrical potential. Phosphorylation was initiated by ADP (0.7 mM). Labeled probes were added, and samples were taken as described under Materials and Methods. In each case, the final pH was 7.1, and the incubations were carried out at room temperature.

Table III: Effect of Varying [K^+] (+Valinomycin) on Mitochondrial Energetic Parameters[a]

[KCl] (mM)	μL of matrix H_2O (mg of protein)$^{-1}$	ΔpH (acetate)	membrane potential (mV)		av proton electrochemical gradient (mV)	log [[ATP]/([ADP][P_i])]	respiratory rate [nmol of O_2 min^{-1} (mg of protein)$^{-1}$]
			TPMP$^+$	K^+			
0 (no val)	1.24 ± 0.13 (6)	−0.08 ± 0.20 (6)	144 ± 7 (6)		148	4.24 ± 0.19 (6)	12.1 ± 2.5 (5)
0 (+val)	1.39 ± 0.25 (5)	−0.17 ± 0.04 (5)	132 ± 6 (5)	138 ± 6 (5)	145	4.42 ± 0.17 (5)	13.2 ± 1.6 (3)
0.5 (+val)	1.71 ± 0.42 (2)	−0.15 ± 0.13 (2)	125 ± 4 (2)	125 ± 1 (2)	134	4.33 (1)	13.9 ± 1.6 (2)
1 (+val)	1.68 ± 0.34 (5)	−0.26 ± 0.04 (5)	116 ± 8 (5)	120 ± 5 (5)	132	4.01 ± 0.19 (4)	18.2 ± 4.2 (3)
2 (+val)	1.84 ± 0.35 (6)	−0.26 ± 0.04 (5)	103 ± 5 (6)	103 ± 2 (6)	119	3.66 ± 0.27 (5)	23.3 ± 3.7 (4)
3 (+val)	2.03 ± 0.59 (2)	−0.29 ± 0.01 (2)	96 ± 2 (2)	95 ± 4 (2)	113	3.95 ± 0.02 (2)	20.0 (1)
4 (+val)	2.06 ± 0.38 (4)	−0.33 ± 0.02 (4)	86 ± 5 (4)	89 ± 4 (4)	108	3.19 ± 0.41 (4)	38.0 ± 7.1 (2)
8 (+val)	2.24 ± 0.55 (3)	−0.35 ± 0.03 (3)	73 ± 9 (3)	72 ± 3 (3)	94	2.59 ± 0.11 (3)	45.1 ± 8.5 (2)

[a] Aliquots of concentrated mitochondrial suspensions (45–65 mg of protein/mL in mannitol–sucrose medium) were added to oxygen-saturated assay medium (120 mM choline chloride, 15 mM Mops, and 0.4 mM EGTA) to give a final concentration of approximately 5 mg/mL. Substrates, NaP_i, and ATP were added as given in the legend of Table I, along with KCl (as indicated) and valinomycin (2.4×10^{-7} M). Phosphorylation was initiated with 0.7 mM ADP. Labeled probes were added, and samples were taken as described under Materials and Methods. All incubations were at pH 7.1 and room temperature.

Effect of the Osmolarity of the Suspending Medium on Mitochondrial Energetics. Detailed measurements of the behavior of mitochondria isolated in a mannitol–sucrose medium and then diluted in a choline chloride assay media to give final milliosmolarities of 300, 160, 100, and 70 are presented in Table II. The respiratory rate of a suspension of mitochondria was higher for a 300 mosM choline chloride medium than for a 300 mosM mannitol–sucrose medium [approximately 19 nmol of O_2 min^{-1} (mg of protein)$^{-1}$ vs. approximately 13 nmol of O_2 min^{-1} (mg of protein)$^{-1}$] and increases with decreasing osmolarity of the choline chloride medium to 34 nmol of O_2 min^{-1} (mg of protein)$^{-1}$ in a 70 mosM medium. Even in the latter case, a respiratory control ratio of near 2 was observed. Maximal phosphorylation, expressed as log [[ATP]/([ADP][P_i])], decreased slightly from 4.28 ± 0.07 to 3.96 ± 0.18 with decreasing osmolarity as did ΔpH (from −0.50 ± 0.05 to −0.24 ± 0.05 unit). Valinomycin (2.4×10^{-7} M) and K^+ (1 mM) were present in these incubation media, allowing the transmembrane electrical potential to be calculated from the distribution of K^+ as well as from the distribution of TPMP$^+$. It decreased from approximately −118 mV at 300 mosM to −73.5 mV at 70 mosM. Thus, the calculated proton electrochemical gradient decreased from −147 mV in 300 mosM media to −87 mV in 70 mosM media.

Effect of Varying [K^+] (+Valinomycin) on Mitochondrial Energetics. When suspensions of mitochondria were treated with valinomycin and incubated in the presence of a permeant anion and varying concentrations of K^+, the intramitochondrial volume was determined by the maximal K^+ gradient formed and the osmolarity of the medium. As may be seen from Table III, when the permeant anions were propionate (3 mM) and phosphate (5 mM), increasing the added [K^+] from 0 to 8 mM was accompanied by an increasing respiratory rate until, at 8 mM, respiratory control was almost competely lost. Net ADP phosphorylation was observed at all concentrations of added K^+, but the energy available for phosphorylation, expressed as log [[ATP]/([ADP][P_i])], decreased from 4.42 ± 0.17 (0 added K^+) to 2.59 ± 0.11 with 8 mM K^+. The ΔpH increased slightly with increasing [K^+], from −0.08 ± 0.20 unit (no valinomycin) to −0.33 ± 0.02 unit (8 mM K^+ plus valinomycin), while the electrical gradient decreased from −144 to −73 mV. As a result, the calculated proton electrochemical gradient decreased from −148 to −94 mV.

Relationship of the Proton Electrochemical Gradient to Respiratory Rate and ΔG_{ATP}. When propionate concentration in the suspending medium was the experimental variable, the proton electrochemical gradient decreased with increasing propionate concentration, exclusively through decreasing ΔpH, while neither respiratory rate nor ΔG_{ATP} changed. With osmolarity of the choline chloride suspending medium or [K^+] (+valinomycin) as experimental variables, a decrease in ΔG_{ATP} was observed as the proton electrochemical gradient decreased (Figure 2). In each case, an approximately linear relationship is observed, but the best-fit lines for each set of data are different. For example, plotted data from Table II give a y intercept of 13.0 kcal/mol with a correlation coefficient of 0.98 while those from Table II (K^+ + valinomycin) give a y intercept of 7.7 kcal/mol with a correlation coefficient of 0.97.

The respiratory rate showed similar disparity in its response to the conditions which modified the proton electrochemical

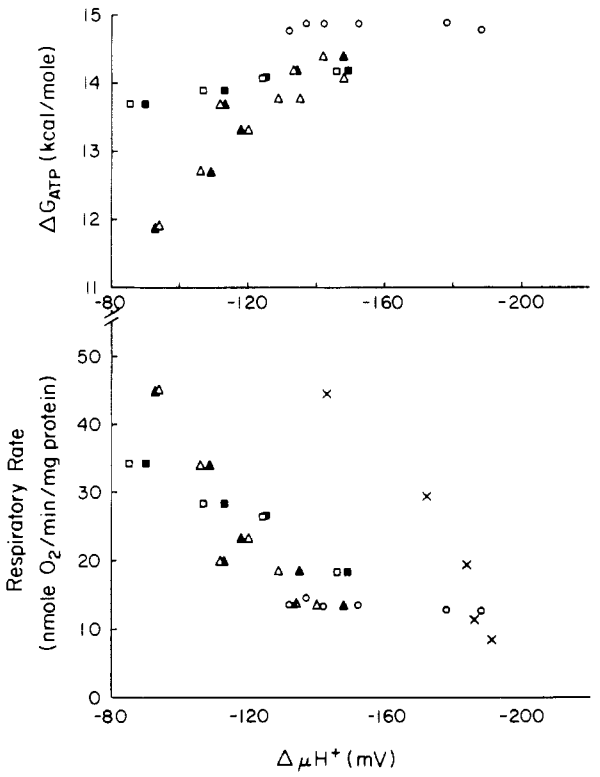

FIGURE 2: Relationship of the mitochondrial respiratory rate and the free energy utilized for the synthesis of ATP (ΔG_{ATP}) to the proton electrochemical gradient. The data are taken from Tables I–IV and presented graphically to permit visualization of the effect of varying the experimental conditions. Data are presented for experiments in which the concentration of propionate (○), the concentration of K^+ in the presence of valinomycin (△, ▲), the osmolarity of the suspending medium (□, ■), and the concentration of FCCP (×) were varied. In the experiments for which the membrane potential was measured both by [$TPMP^+$] and by [K^+] (plus valinomycin), the former data are represented with open symbols and the latter with closed symbols.

gradient. Titration of mitochondria with uncoupler (FCCP) is included not only because it represents another method for varying both transmembrane gradients and respiratory rate but also because other workers have reported similar measurements, and this permits direct comparison with their work (Holian & Wilson, 1980; Rottenberg, 1975; Nicholls, 1974; Küster et al., 1981).

Discussion

As expressed in eq 1, the free-energy change associated with the primary "high-energy" intermediate of oxidative phosphorylation (ΔG_i) should have a well-defined relationship with the free energy of ATP synthesis (ΔG_{ATP}). It follows that the decrease of ΔG_{ATP} by any method excepting inhibition of ATP synthetase should result in a parallel decrease in ΔG_i. The stoichiometry of the coupling reaction can then be directly determined by the stoichiometry required to give equal changes in ΔG_i. In the special case in which proton transport is the intermediate, the minimum number of protons which must be utilized to synthesize ATP is $\Delta G_{ATP}/\Delta G_{H^+}$. The experimental conditions of the studies reported in this paper were carefully selected to minimize damage to the mitochondria (respiratory control was observed in all cases) and to not inhibit the rate of ATP synthesis. Although the experimental conditions used resulted in large changes in the volume of the mitochondrial matrix, only in the case of 100 and 70 mosM choline chloride medium was the physiologic volume of near 2.0 μL/mg of protein (Holian & Wilson, 1980; Deutsch et al., 1979a) exceeded.[1] The data show, however, that the capacity for oxidative phosphorylation was not compromised.

Reliability of the Measured Values for the Transmembrane Electrical Gradients. Transmembrane pH and electrical gradients are difficult to measure for vesicular systems which are too small for the use of microelectrodes. The methods for measurement of pH gradients are discussed under Materials and Methods. Several methods have been used for measurements of electrical gradients including distribution of K^+ and Rb^+ in the presence of valinomycin, distribution of lipid-soluble cations or anions, and fluorescence changes in certain organic dyes. The first method has been most generally used because in model systems valinomycin has been shown to be highly selective in cation binding and to catalyze electrogenic movement of these cations across lipid bilayer membranes (Pressman, 1976; Mueller & Rudin, 1969). Similar data have been obtained for the lipophilic ions, but in this case, binding of the ions to the mitochondrial membrane and contents of the matrix space appears to be more significant than in the case of K^+ and Rb^+ (see Materials and Methods). The fluorescent indicators are not primary standards but must be empirically calibrated, usually by using K^+ plus valinomycin, and, because they are sensitive to many experimentally variable aspects of their environments (Walsh Kinnally et al., 1978; Sims et al., 1974), are qualitative and not quantitative indicators of the electrical potential. Microelectrodes have been used to measure the electrical potential in giant mitochondria (Tupper & Tedeschi, 1969; Walsh Kinnally et al., 1978; Maloff et al., 1978). These authors report that in such mitochondria under phosphorylating conditions the transmembrane electrical potential is +19 mV. These data must be kept in mind, but the difference in experimental material and conditions prevents direct comparisons. For these reasons, we will restrict our discussion of normal mitochondria to data for which the first two methods were used.

Role of the Transmembrane Gradients. The present data are not consistent with the transmembrane pH gradient playing a significant role in regulation of mitochondrial respiration or provision of an energy source for ATP synthesis. The ΔpH does not appear to be energetically coupled to the electrical potential; i.e., a decrease in ΔpH at constant respiratory rate and [ATP]/([ADP][P_i]) is not accompanied by an energetically equivalent increase in transmembrane electrical potential. It has been proposed (Mitchell, 1966, 1977) that such a coupling should occur if the chemiosmotic mechanism is operative.

Experimental conditions, however, can be created in which ion transport processes not otherwise seen in mitochondria can be induced, giving rise to a coupling of ΔpH and E. Some antibiotics, for example, nigericin, catalyze an exchange, in this case H^+ for K^+, which results in such a coupling. Another

[1] Electron microscopy of mitochondria in situ shows the inner and outer membranes to be closely apposed with no observable intermembrane space. For isolated mitochondria, particularly those isolated in low ionic strength media, electron micrographs show a fully extended outer membrane with the inner membrane and matrix space occupying only part of the volume within the outer membrane. The ^{14}C-labeled poly(ethylene glycol) used in these studies is impermeable to the outer mitochondrial membrane and allows the water space within the outer membrane to be measured. This space of 1.8–2.0 μL/mg of protein (this paper; Holian & Wilson, 1980) may be a slight underestimate as it is not possible to be sure that 100% of the mitochondria have intact outer membranes. A similar value of 2 μL/mg of protein has been reported for mitochondria in intact neuroblastoma cells by using electron micrographs to estimate the fraction of cell volume and the measured cellular and mitochondrial cytochrome c contents per milligram of protein to estimate the fraction of the cellular protein which is mitochondrial (Deutsch et al., 1979a).

Table IV: Effect of FCCP Concentration on Mitochondrial Matrix Volumes, Transmembrane pH and Electrical Gradients, and Respiratory Rate[a]

FCCP (μM)	μL of matrix H_2O (mg of protein)$^{-1}$	ΔpH (acetate)	membrane potential (mV) (TPMP$^+$)	proton electrochemical gradient (mV)	respiratory rate [nmol of O_2 min^{-1} (mg of protein)$^{-1}$]
0 (2)	1.39	−0.71	149	191	8.7
0.05 (2)	1.32	−0.65	148	186	11.8
0.10 (2)	1.24	−0.59	149	184	19.5
0.15 (2)	1.17	−0.49	143	172	29.6
0.30 (2)	1.15	−0.36	122	143	44.5

[a] Concentrated mitochondrial suspensions in mannitol–sucrose medium (45–65 mg of protein/mL) were added to oxygen-saturated 120 mM choline chloride, 15 mM Mops, and 0.4 mM EGTA to a final protein concentration of approximately 5 mg/mL. Substrates, ATP, and NaP_i were added as in the legend of Table I. Phosphorylation was initiated by addition of ADP (0.7 mM). Labeled probes were added, and samples were taken as described under Materials and Methods. All incubations were carried out at room temperature and at pH 7.1.

such set of conditions is that of active transport of a cation, such as K^+, in the presence of valinomycin. Net transport of cations must be accompanied by anions, in effect providing the energy for a reverse gradient of the total weak acid with an accompanying ΔpH. A slight effect of this type is seen in Table III where the measured ΔpH increased with increasing $[K^+]$. This effect can be eliminated by increasing the concentrations of the weak acid. For most of the experimental conditions reported in this paper, there was significant net uptake of K^+ (in the presence of valinomycin). The calculated energy requirement for K^+ transport (ΔG_T) ranged from a high of 3.18 kcal/mol to a low of near 1.66 kcal/mol; this would correspond to $[K^+]/[ATP]$ ratios of 4.5–7.2. It is interesting to note that in their pioneering studies, Cockrell et al. (1966) reported K^+ accumulation with $[K^+]/[\sim P]$ of 6.5 under optimal steady-state conditions. Although these authors used assumed intramitochondrial volumes and thereby intramitochondrial $[K^+]$ values which were probably too low, their calculated ΔG_T value was 1.64 kcal/mol, similar to the minimal value reported in the present studies. Many other workers have measured $[K^+]/[\sim P]$ values [see also Coin & Hinkle (1979), Reynafarje & Lehninger (1978), and Azzone et al. (1979)], but the reported values vary, making it impossible at this stage to determine if a unique stoichiometry exists for the transport process or if the stoichiometry is dependent upon the experimental conditions.

Linear regression analysis of the relationship between respiratory rate and membrane potential shows that fit to a straight-line relationship is attained with correlation coefficients of −0.357, −0.994, −0.915, and −0.913 for the data in Tables I–IV, respectively (Table V). The slopes of the lines represent the change in respiratory rate per millivolt of membrane potential and range from −0.13 (−0.354 if Table I is excluded) to −1.15 nmol of O_2 min^{-1} mg^{-1} mV^{-1}. Such large differences in slope are inconsistent with the membrane potential being the primary determinant of the mitochondrial respiratory rate [see also Padan & Rottenberg (1973) and Azzone et al. (1978a)]. Some authors (Nicholls, 1974; Küster et al., 1981) have reported good correlations between these two parameters, but this is the result of having used only a limited set of experimental conditions, such as titration with uncouplers, and is not a general observation. It should be emphasized that measurements for comparable conditions have yielded similar results.

Essential to mechanisms in which proton transport is the high-energy intermediate in the pathway to ATP synthesis is (1) a correlation between the proton electrochemical gradient and ΔG_{ATP} and (2) provision of adequate free energy by ΔG_{H^+} for the synthesis of ATP for all steady-state conditions (for which net ATP synthesis is occurring). Our data do not support the first requirement; as the propionate concentration

Table V: Linear Regression Analysis[a]

(A) Membrane Potential and Respiratory Rate

y intercept (nmol of O_2 min^{-1} mg^{-1})	slope (nmol of O_2 min^{-1} mg^{-1} mV^{-1})	correlation coefficient	data source
30.9	−0.130	−0.357[b]	Table I
61.5	−0.354	−0.994	Table II
73.5	−0.462	−0.915	Table III
186.0	−1.15	−0.913	Table IV
192.0	−1.32	−0.986[c]	Holian & Wilson (1980)

(B) Proton Electrochemical Gradient and log [[ATP]/([ADP][P_i])]

y intercept log [[ATP]/([ADP][P_i])]	slope (log units/mV)	correlation coefficient	data source
4.861	-5.45×10^{-4}	−0.391[b]	Table I
3.484	5.59×10^{-3}	0.964	Table II
0.0158	3.05×10^{-2}	0.911	Table III
−4.694	5.64×10^{-2}	0.997[c]	Holian & Wilson (1980)
−6.18	5.80×10^{-2}	[d]	theoretical

[a] The mean values for the indicated parameters were subjected to linear regression analysis according to the equation $y = A + Bx$. The intercept (A) and the slope (B) of the best-fit straight line are presented in addition to the correlation coefficient. The y intercept is the respiratory rate when the line is extrapolated to a membrane potential of zero (part A) and the value of log [[ATP]/([ADP][P_i])] when the line is extrapolated to a proton electrochemical gradient of zero. [b] The range over which the variables were changed is too small for reasonable analysis. This set of values should not be given much emphasis. [c] Data for titration with uncoupler taken from Table I of Holian & Wilson (1980) for comparison. [d] This line was calculated by assuming that, starting at the point −188 mV, log [[ATP]/([ADP][P_i])] = 4.73, as the proton electrochemical gradient decreased to zero log [[ATP]/([ADP][P_i])] would decrease to its equilibrium value of −6.18.

was raised, $-\Delta G_{H^+}$ decreased by 31% with no change in ΔG_{ATP}. Similarly, when the osmolarity of the choline chloride medium was decreased (Table II), $-\Delta G_{H^+}$ fell by 37% while ΔG_{ATP} decreased only 5%. The overall decrease in $-\Delta G_{H^+}$ was 54% while ΔG_{ATP} decreased only 8%. The lines fitted to the experimental data by linear regression analysis all extrapolate to values of log [[ATP]/([ADP][P_i])] at zero proton electrochemical gradient which are much greater than the expected equilibrium value of −6.2. This suggests that there is no direct coupling of the two processes.

Required Stoichiometry for Coupling of the Proton Electrochemical Gradient to ATP Synthesis. The data allow calculation of the minimum number of moles of protons which would have to pass down their electrochemical gradient to provide the energy for synthesis of 1 mol of ATP. The

$[H^+]/[ATP]$ calculated from $\Delta G_{ATP}/\Delta G_{H^+}$ is a minimum value and is consistent with any mechanism utilizing at least this many protons per ATP. In the present work, $\Delta G_{ATP}/\Delta G_{H^+}$ is not constant, as would be expected for straightforward stoichiometric coupling, but ranges from 2.8 to 7.0, depending upon the experimental conditions.

General agreement is observed (Mitchell & Moyle, 1969; Rottenberg, 1975; Azzone et al., 1978a; Nicholls, 1974; Holian & Wilson, 1980) that for conditions which give maximal values for the proton electrochemical gradient ΔG_{H^+} is near −4.8 kcal/mol, for which the calculated $[H^+]/[ATP]$ is approximately 3. There is also good agreement that when the measurements are made for mitochondria suspended under other conditions the proton electrochemical gradient can be much less than the maximal value with little or no decrease in ΔG_{ATP}. In several laboratories, ΔG_{H^+} values of near −2.0 kcal/mol have been obtained under conditions for which the required $[H^+]/[ATP]$ would be at least 7 (Azzone et al., 1978a,b; Walsh Kinnally et al., 1978; Holian & Wilson, 1980; this paper).

Summary. The transmembrane pH and electrical gradients have been measured for suspensions of isolated rat liver mitochondria. The pH gradient can be decreased to near-zero without affecting the respiratory rate, ATP synthesis, or the membrane potential. This indicates that the pH gradient does not play a significant role in either regulation of respiration or ATP synthesis. The total proton electrochemical gradient can be decreased by 58% with only a slight (8%) change in the energy utilized for ATP synthesis. These data make it unlikely that the transport of H^+ is equilibrated with the high-energy intermediate(s) of oxidative phosphorylation.

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