REFERENCES

Altona, C., & Sundaralingam, M. (1972) *J. Am. Chem. Soc. 94*, 8205-8212.

Brown, T., Hunter, W. N., Kneale, G., & Kennard, O. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2402-2406.

Calladine, C. R. (1982) *J. Mol. Biol. 161*, 343-352.

Chambers, J. L., & Stroud, R. M. (1977) *Acta Crystallogr. B33*, 1824-1837.

Dickerson, R. E. (1983) *J. Mol. Biol. 166*, 419-441.

Di Gabriele, A. D., Sanderson, M. R., & Steitz, T. A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1816-1820.

EMBO Workshop (1989) *EMBO J. 8*, 1-4.

Fairall, L., Martin, S., & Rhodes, D. (1989) *EMBO J. 8*, 1809-1817.

Haran, T. E., Shakked, Z., Wang, A. H.-J., & Rich, A. (1987) *J. Biomol. Struct. Dyn. 5*, 199-217.

Heinemann, U., Lauble, H., Frank, R., & Blocker, H. (1987) *Nucleic Acids Res. 22*, 9531-9550.

IUPAC-IUB Joint Commission on Biochemical Nomenclature (1983) *Eur. J. Biochem. 131*, 9-15.

Jain, S., & Sundaralingam, M. (1989) *J. Biol. Chem. 264*, 12780-12784.

Jain, S., Zon, G., & Sundaralingam, M. (1987) *J. Mol. Biol. 197*, 141-145.

Jain, S., Zon, G., & Sundaralingam, M. (1989) *Biochemistry 28*, 2360-2364.

Jones, T. A. (1985) *Methods Enzymol. 115*, 157-171.

Rubin, J. R., Brennan, T., & Sundaralingam, M. (1972) *Biochemistry 11*, 3112-3128.

Sundaralingam, M. (1969) *Biopolymers 7*, 821-860.

Sundaralingam, M. (1973) *Jerusalem Symp. Quantum Chem. Biochem. 5*, 417-456.

Sundaralingam, M. (1982) in *Conformation in Biology* (Srinivasan, R., & Sarma, R. H., Eds.) pp 191-225, Adenine Press, New York.

Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature 282*, 680-686.

Wang, A. H.-J., Fuji, S., van Boom, J. H., & Rich, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3968-3972.

Westhof, E., Dumas, P., & Moras, D. (1985) *J. Mol. Biol. 184*, 119-145.

Zon, G. (1990) in *HPLC in Biotechnology* (Hancock, W. S., Ed.) pp 301-397.

Mechanistic Stoichiometry of Mitochondrial Oxidative Phosphorylation†

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ABSTRACT: P/O ratios of rat liver mitochondria were measured with particular attention to systematic errors. Corrections for energy loss during oxidative phosphorylation were made by measurement of respiration as a function of mitochondrial membrane potential. The corrected values were close to 1, 0.5, and 1 at the three coupling sites, respectively. These values are consistent with recent measurements of mitochondrial proton transport.

Eleven years ago, we reported that measured stoichiometries of mitochondrial oxidative phosphorylation were significantly lower than the traditional values of 1 per coupling site (Hinkle & Yu, 1979). Early studies of P/O ratios usually yielded values less than 1 per site, but it was assumed that the value was an integer and the results were described as "approaching" the now classical integers of 2 with succinate and 3 with NADH-linked substrates. Our observations that the P/O ratio with succinate as substrate is close to 1.5 have been confirmed by four research groups (Van Dam et al., 1980; Stoner, 1987; Hafner & Brand, 1988; Luvisetto & Azzone, 1989). Other groups have found slightly higher values (Beavis & Lehninger, 1986; Lemasters, 1984). When individual coupling sites are measured, there is agreement that site 2 forms less than 1 ATP per electron pair (Brand et al., 1978; Pozzan et al., 1979, Stoner, 1987), the value probably being 0.5, but there is less agreement about sites 1 and 3 [for reviews, see Hinkle and Yu (1979), Stoner (1987), and Lemasters et al. (1984)].

Chemiosmotic theory (Mitchell, 1965) provides a rationale for fractional values of P/O ratios because the proton is the coupling currency and the number of protons transported by a coupling region (site) of the respiratory chain need not be an integral multiple of the protons needed to synthesize ATP from ADP and P_i. Since ATP synthesis is measured in the external medium, ATP efflux and ADP plus P_i influx across the inner membrane must also occur, and these have been shown to be coupled to the influx of one proton, electrically, as ADP^{3-}/ATP^{4-} antiport and, chemically, as P_i^-/OH^- antiport (Klingenberg & Rottenberg, 1977). The use of protons to drive ATP/ADP + P_i transport and the leakage of protons across the mitochondrial inner membrane would lower the observed P/O ratio, although the protons used in transport are considered a part of the ideal "mechanistic" stoichiometry, whereas the "leak" protons or other mechanisms of energy loss are not.

We report here measurements of P/O ratios made with particular attention to several common systematic errors, and other measurements to estimate energy losses during oxidative phosphorylation. It is concluded that ideal mechanistic stoichiometries are 1.0 at sites 1 and 3, and 0.5 at site 2, values suggested previously (Hinkle, 1981).

EXPERIMENTAL PROCEDURES

Rat liver mitochondria were prepared in 250 mM sucrose with two washes (Pederson et al., 1978). Many variations in the preparation were tried, and while respiratory control and respiration rates depended on the method, the P/O values were very insensitive to the details of the procedure. The standard medium for measurements reported was 250 mM sucrose, 10 mM K-MOPS (pH 7.2), 5 mM KP_i (pH 7.2), 5 mM $MgCl_2$,

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Table I: Summary of ADP/O Ratios[a]

	substrate	RCR	respiration (nmol min^{-1} mg^{-1})	ADP/O ratio	ADP/ΔO ratio
site 2	succinate–ferricyanide	1.9	165	0.49 ± 0.02	1.10 ± 0.05
site 3	ascorbate–O_2	2.6	86	0.98 ± 0.09	1.60 ± 0.12
sites 2 + 3	succinate–O_2	7.6	163	1.48 ± 0.04	1.72 ± 0.05
sites 1 + 2 + 3	3-hydroxybutyrate–O_2	5.0	58	2.27 ± 0.08	2.85 ± 0.15

[a] ADP/O and ADP/ΔO ratios were measured as described in the text in the following additions to standard medium: site 2, 5 mM succinate, 1 mM KCN, 2 mM ferricyanide, and 3 μM rotenone; site 3, 20 mM ascorbate, 4 mM ferrocyanide, and 1.4 μM myxathiazol; sites 2 + 3, 5 mM succinate and 3 μM rotenone; sites 1 + 2 + 3, 20 mM 3-hydroxybutyrate. RCR is the respiratory control ratio.

and 1 mg/mL fatty acid free bovine serum albumin.

Oxygen uptake was measured with a Clark electrode (Model LN5320, Yellow Springs Instrument Co.), in a glass cell (Gilson) of 1.7-mL volume, thermostated at 25 °C and stirred with a glass-covered magnetic stir bar cut from a large paper clip. The oxygen electrode was calibrated with NADH and submitochondrial particles during each experiment as described by Chappell (1964) and previously (Hinkle & Yu, 1979).

The reducing agent ferrocyanide/ascorbate and the oxidizing agent ferricyanide react at cytochrome *c*, allowing sites 3 and 2 to be measured independently. Ferricyanide reduction during the site 2 assay was measured in a Perkin-Elmer 356 dual-wavelength spectrophotometer at 440–490 nm using an extinction coefficient of 0.59 mM^{-1} cm^{-1}.

NADH concentrations were determined with a Cary 219 spectrophotometer with correction for nonoxidizable forms essentially as described (Lemasters, 1984). The purity of ADP was measured by enzymatic assay (Lamprecht & Trautschold, 1974) and by HPLC. An LKB Model 2152 HPLC was used with a Partisil Sax (10 μm) anion-exchange column eluted by a linear gradient of 0.007 M sodium phosphate, pH 4.0, and 1.0 M sodium phosphate, pH 4.5, at 1.5 mL/min. Peaks were quantitated with a Spectra-Physics 4270 chromatography integrator. The total nucleotide concentration was also confirmed by the absorbance at 259 nm (E_{mM} = 15.4). The AMP content of ADP used was from 2 to 4%, and the effective ADP concentration for calculation of P/O ratios was taken as [ADP] + (2 × [AMP]). When nucleotides were analyzed following incubation with mitochondria, the reaction was stopped with ice-cold 7% TCA and the TCA removed by extraction into 0.5 M tri-*n*-octylamine in Freon 113 (Khym, 1975).

Mitochondrial membrane potential was measured with a tetraphenylphosphonium (TPP^+) electrode (Kamo et al., 1979). The potential was calculated from the Nernst equation, and the internal TPP^+ concentration was calculated from the uptake above a base line with 0.2 μM of the uncoupler SF 6847 and an internal volume of 1.0 μL/mg. The potentials are overestimates because no correction was made for binding of TPP^+ inside the mitochondria, but this is satisfactory for the present work because the values are used to compare different conditions rather than for energetic calculations.

Results

We have used the method of Chance and Williams (1955) to measure P/O ratios because of its simplicity and directness. A known amount of ADP is added to mitochondria respiring in an oxygen electrode cell in a medium that contains phosphate. Respiration is stimulated while the ADP is being phosphorylated to ATP, and the coupling ratio can be calculated from the amount of ADP added and the amount of oxygen taken up. When measured in this way, the P/O ratio is called the ADP/O ratio. For accurate measurements, the oxygen electrode must be calibrated and concentrations of ADP and contaminating AMP determined. The accuracy of the spectrophotometer should be confirmed, and the absorbance values measured should be in the optimal range around 0.3 ODU. The volumes of ADP or NADH added were at least 5 μL from a 10-μL Hamilton syringe.

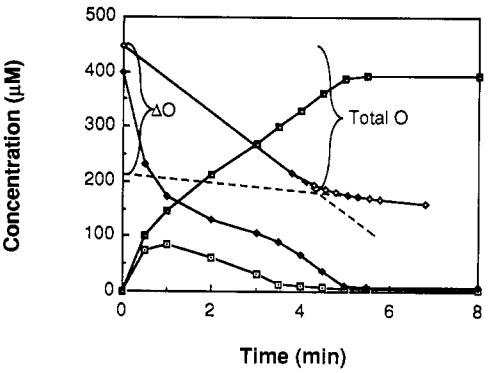

FIGURE 1: ADP pulse time course. Mitochondria (0.3 mg/mL) were incubated in standard medium with 5 mM succinate and 5 μM rotenone. At zero time, 390 μM ADP was added, and samples were withdrawn for nucleotide analysis by HPLC. A fraction of the same reaction mixture was added to the oxygen electrode cell for measurement of oxygen uptake. Concentrations of ADP (♦), ATP (■), AMP (□), and oxygen (◇) are shown. The lines extrapolating the oxygen uptake curve are included, and the amounts of oxygen corresponding to "total O" and "extra O" (ΔO) are indicated.

The end point of oxygen uptake has traditionally been calculated in two ways (see Figure 1): For "total oxygen" (O or 2e), the transition from rapid to slow respiration when ADP is exhausted ("state 3" to "state 4" transition) is taken as the crossing point of two lines extending the two rates, and for "extra oxygen" (ΔO or Δ2e), the state 4 rate is extended back to the point of ADP addition. Thus, "total oxygen" is the entire amount of oxygen consumed during the rapid respiration following ADP addition, and "extra oxygen" has had the basal rate subtracted, presuming that the basal rate continues during ADP phosphorylation. Figure 1 also shows an analysis of ATP, ADP, and AMP levels following the addition of ADP to mitochondria oxidizing succinate. About one-quarter of the ADP is rapidly converted to AMP and ATP by adenylate kinase present in the mitochondrial intermembrane space. As the phosphorylation of ADP to ATP by oxidative phosphorylation proceeds, the levels of nucleotides pass the equilibrium position of the adenylate kinase reaction, and the AMP is phosphorylated by adenylate kinase. An analysis of ADP and AMP remaining at the end point of oxygen uptake is presented below. A summary of ADP/O and ADP/ΔO values with different substrates is shown in Table I. The values of ADP/O are close to 0.8, 0.5, and 1 for sites 1, 2, and 3, respectively. The values of ADP/ΔO are significantly higher and do not fit with simple summations of contributions from the three sites. In considering how to interpret these results, we have examined many possible systematic errors of the method and have taken two experimental approaches to determine how the measured values should be corrected to give the ideal mechanistic stoichiometry of coupling at each site.

Possible Systematic Errors. Beginning with possible systematic errors, we first consider the measurement of oxygen.

This measurement is probably prone to the most error in calibration because of the large temperature coefficient of its solubility and slow equilibration between air and solutions. We have calibrated the oxygen electrode during each experiment with NADH as described under Experimental Procedures. A glass cell is used, and glass magnetic stir bars were made to avoid the oxygen dissolved in Teflon bars. By equilibrating the bars with 100% O_2 and then placing them in the oxygen electrode, we determined that air-equilibrated 8 × 1 mm Teflon bars contain about 30 nmol of O and 10 × 2 mm bars contain 65 nmol of O. This oxygen dissolved in the Teflon equilibrated with the medium with a half-time of 30 s for small bars and 70 s for the larger bar. Thus, the Teflon stir bars usually used or other Teflon parts should be avoided because they serve as an oxygen reservoir and make calculation of oxygen uptake less accurate.

Diffusion of O_2 into the cell through the syringe port is also a possible source of error which is quite variable because it depends on the diffusion through the unstirred water in the port. Lemasters estimated back-diffusion in the same model cell to be 1.7 nmol/min (Lemasters, 1984), which, at the low mitochondrial concentration used in Figure 1 to prolong the transitions, would cause a 3% overestimation of the P/O ratio. At the usual protein concentration of 1 mg/mL, the error would be only 1%.

The ADP solution is kept at 0 °C where the solubility of oxygen in water is about twice that at 25 °C. Thus, a small error is introduced from the extra oxygen added to the cell with the ADP. We use a high ADP concentration of about 100 mM so that this error is only 0.5–1%. If less concentrated ADP solutions were used or the ADP addition were made when the O_2 concentration in the cell had reached low values, the error would be higher.

The rather slow response time of a Yellow Springs oxygen electrode causes the pen trace to lag a few seconds behind the actual oxygen concentration. The extrapolation of the end point for total O is at a delayed time, so a similar extrapolation must be made at the onset of fast respiration so that the electrode response is not included in the time of state 3 [as described by Lemasters (1984)]. This was done for the measurements reported here but not in our previous measurements (Hinkle & Yu, 1979). The data in Figure 1 were corrected for the oxygen electrode response.

Another error occurs because not all the added ADP is phosphorylated at the end point. In Figure 1, the concentrations of ADP and AMP at the time when the oxygen concentration reaches the extrapolated end point for total oxyen uptake were 15 and 5.5 μM, respectively, causing a 6.5% overestimation of the P/O ratio. The effect of remaining AMP could be more significant in other measurements made in the absence of added Mg^{2+} where adenylate kinase activity is low and the reversal of AMP formation is slower.

Possible side reactions of the added ADP were investigated by analysis of nucleotides by HPLC following incubation of ADP or AMP with mitochondria. We found that 0.33 nmol min^{-1} (mg of protein)$^{-1}$ was formed, presumably by 5′-nucleotidase. This low rate is not significant.

If we add the systematic errors discussed above for a typical ADP/O determination, the factors leading to an overestimation of the true value are about 7% under optimal conditions. These errors have not been corrected for in the results reported in Table I.

Corrections for Energy Loss (Proton Leaks). To begin the analysis of energy "leaks" to interpret the data in Table I, we partially inhibited respiration with malonate, a method first described by Tsou and Van Dam (1969). As shown in Figure 2, the ADP/O for sites 2 plus 3 and the ADP/2e ratio for site 2 are approximately constant at about 1.5 and 0.5, respectively, as respiration is inhibited. P/ΔO and P/Δ2e values rose to unreasonably high values, however, indicating that use of ΔO is an overcorrection for energy leaks. The site 2 assay gave a value of 0.48 for the P/2e ratio which fell to only 0.45 at 50% inhibition of respiration, whereas the P/Δ2e ratio was 1.2 and rose to 2.3 at 50% inhibition. This is particularly significant in view of the agreement that $H^+/2e = 2$ (matrix side) at this coupling site, consistent with a mechanistic P/2e = 0.5 if $H^+/ATP = 4$ (see below). This indicates that the uncorrected values of P/2e are very close to the mechanistic values.

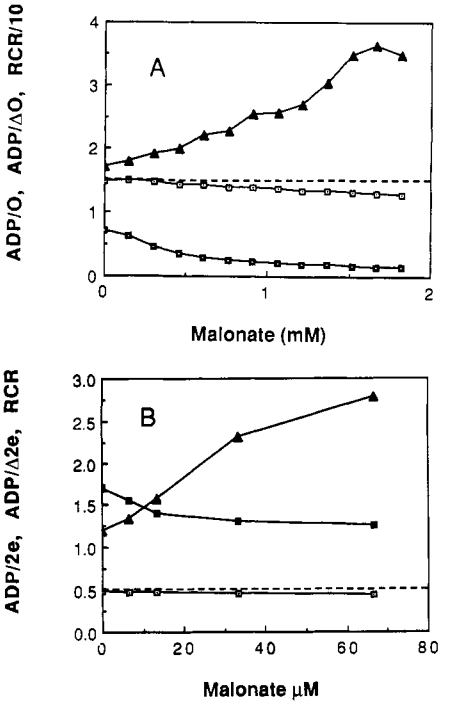

FIGURE 2: Malonate inhibition of oxidative phosphorylation. (A) Sites 2 plus 3. Mitochondria (1.25 mg/mL) were suspended in standard medium with 9 mM succinate, 1.2 μM rotenone, and malonate, at various concentrations as shown, in the oxygen electrode cell. ADP/O (□) and ADP/ΔO (▲) ratios and the respiratory control ratio (RCR) (■) were measured following a pulse of 640 nmol of ADP. Each malonate concentration is a separate experiment. (B) Site 2 as (A) except that 1 mM KCN and 2.1 mM ferricyanide were also added as electron acceptor.

To analyze energy losses in more detail, we have used the method of Nicholls (1974). Figure 3 shows measurements of the respiration rate ("current") as a function of membrane potential ("voltage") varied with an inhibitor or by changing the substrate concentration. The curves without ADP can be interpreted as either a current–voltage curve for proton permeability or the slippage of proton pumps leading to a variable stoichiometry of proton transport coupling to respiration, or a combination of the two. The curves with ADP present (solid symbols) represent the current–voltage curve of ATP synthesis. The highest point on these curves corresponds to normal oxidative phosphorylation. The energy loss which occurs during oxidative phosphorylation can be obtained from the current–voltage curve without ADP at the same value of membrane potential which occurs during normal oxidative phosphorylation. This rate of energy loss, expressed as the fraction of respiration, is 3.7% at sites 2 plus 3 (Figure 3A) and 7.1% at site 3 (Figure 3B). These corrections can also be expressed as the fraction of basal respiration measured

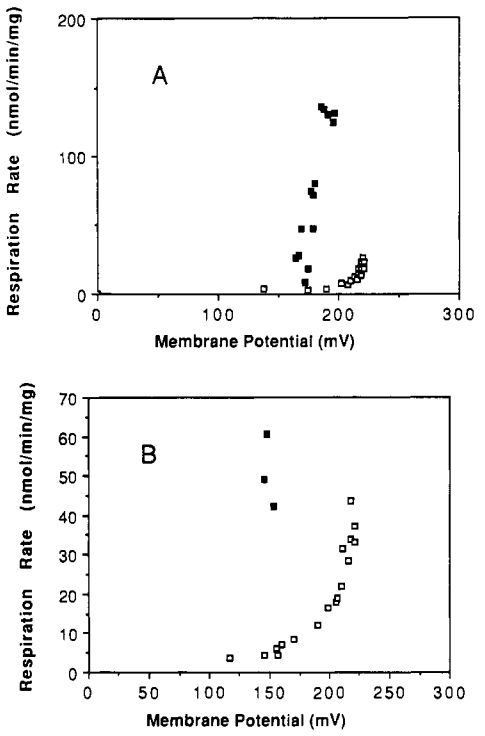

FIGURE 3: Current–voltage curves. In each case, the rate of electron flow was varied by titration with substrate or inhibitor, and the resulting membrane potential was measured with the TPP^+ electrode in the presence (■) and absence (□) of 0.3 mM ADP. Mitochondria (0.5 mg/mL) were suspended in standard medium with 5 μM TPP. (A) Sites 2 plus 3: the medium also contained 3 μM rotenone and 5 mM succinate, and respiration was inhibited with malonate from 0 to 6 mM. (B) Site 3: the medium also contained 1.4 μM myxathiazol and 20 mM ascorbate, and respiration was varied by different levels of the mediator sodium ferrocyanide from 0 to 1.8 mM.

without ADP. The results from Figure 3 are 17% at sites 2 plus 3 and 11% at site 3. In other words, about one-tenth to one-sixth of the basal (state 4) respiration is associated with energy loss during oxidative phosphorylation, consistent with the results shown in Figure 2 which indicate that subtraction of the entire basal rate from the total rate of respiration, i.e., the use of ΔO, causes an overcorrection for energy loss. A small part of the energy loss is due to ATPase activity or proton conduction by F_0, because we observed that oligomycin inhibited state 4 respiration by about 15%. Calcium ion transport was not a significant factor in energy loss because EGTA did not inhibit state 4 respiration. Application of the corrections for energy loss from Figure 3 to the data in Table I yields corrected ADP/O values of 1.05 for site 3 and 1.54 for sites 2 plus 3. These upward corrections must then be corrected downward for the systematic errors of about 7% from oxygen in the ADP solution and ADP plus AMP remaining at the extrapolated end point of the stimulated respiration discussed previously. Thus, within a small error which is hard to evaluate because it involves so many factors and calibrations, the measured values of P/O are the mechanistic ratios.

Discussion

An evaluation of the many measurements of P/O ratios over the years must take into account experimental design, which can lead to systematic errors, and the theory of proton-coupled systems for correction of measured values to give the ideal mechanistic value. In addition, there is another factor which is not usually discussed; the theoretical milieu in which the measurements were made, which can lead to unconscious selection of data by the experimenter. This last factor has probably been significant in some cases, since after it became clear that there were three "coupling sites" but before there was a rationale for fractional values, we are told that it was difficult to publish values that were not "approaching" an integer for each coupling site. Considering reports over the past 10 years when fractional values were considered possible, almost all of the measured values are close to those reported here. Furthermore, a variety of methods were used, indicating that the lower values are not the result of a particular method. Phosphorylation was measured by ADP determination (Lemasters, 1984; Hafner & Brand, 1988; Luvisetto & Azzone, 1989), chemical determination of phosphate (Stoner 1987), and measurement of proton uptake (Beavis & Lehninger, 1986). One recent study (Toth et al., 1990) reported higher values of 1.9 with succinate and 3.2 with 3-hydroxybutyrate but found that the values decreased as the concentration of mitochondria was increased. The P/O values and respiratory control ratio also increased in successive ADP pulses as the oxygen concentration decreased, suggesting to us that diffusion of oxygen into the medium occurred, probably from the large Teflon stirring bar used. Diffusion of oxygen into the cell from the stirring bar or the addition port has probably caused systematic errors which increased the P/O ratio in many studies.

The measured P/O ratio we observed are close to 1, 0.5, and 1 for sites 1, 2, and 3, respectively, and the question is whether these are the mechanistic stoichiometries or whether they should be corrected to higher values. We have not included a detailed study of site 1 in this report, but earlier studies strongly support the conclusion that the measured P/O ratio for site 1 is 1.0 (Pozzan et al., 1979; Stoner, 1987). We have considered various corrections for experimental artifacts, the largest of which is an overestimation by the ADP pulse method because of ADP and AMP remaining at the end point. The end point determined in the traditional way involves a partial subtraction of basal respiration even for "total oxygen". We have also estimated corrections for proton leakage or possible slip of respiratory proton transport which leads to underestimation of the mechanistic stoichiometry. There is evidence both for (Zoratti et al., 1986) and against (Brown, 1989) slip of the proton transport mechanisms. However, our method to correct for energy loss, measuring membrane potential as a function of respiration rate with and without ADP, would correct for either slip or proton leak, assuming that the rate of slip or proton leak is controlled by the back-pressure of the electrochemical proton gradient (Westerhoff & Van-Dam, 1987). The correction upward for energy (proton) leaks and the correction downward for incomplete phosphorylation of ADP and AMP were both in the range of 5–10%, and under most conditions cancel each other, leaving the uncorrected P/O ratio calculated by using total oxygen consumption very close to the true mechanistic stoichiometry.

Others have concluded that a larger correction for proton leakage is necessary. Lemasters (1984) used equations of nonequilibrium thermodynamics to conclude that measured values of ADP/ΔO close to 1.25, 0.5, and 1.5 at the three sites, respectively, were the mechanistic values. This conclusion resulted from the linear equations (forced through zero) used, which are not consistent with the highly nonlinear relation between basal respiration and membrane potential shown by the method of Nicholls (1974) in Figure 3. In another study, Beavis and Lehninger (1986) concluded that the mechanistic values for P/O ratios were 1, 0.5, and 1.25 at the three sites, respectively, based on estimates of the proton leak from uncoupler titrations. The natural proton permeability has different membrane potential dependence from that induced by

uncouplers, however [see Krishnamoorthy and Hinkle (1984)].

We should also consider whether mechanistic P/O ratios of 1, 0.5, and 1 for the three coupling sites, respectively, are consistent with measured H/O ratios of the respiratory chain and the H/ATP ratio for ATP synthesis. Ideally, the P/O ratio for a given substrate is equal to the H/O ratio divided by the H/ATP ratio. The H/ATP ratio of ATP synthesis in whole mitochondria is the H/ATP ratio for synthesis of ATP by the F_0F_1 ATP synthase plus the H/ATP ratio for transport of ATP from the mitochondrial matrix to the cytoplasm in exchange for ADP and P_i. This latter ATP transport is generally agreed to have H/ATP = 1, with one electric charge transported by the ATP/ADP antiporter (Klingenberg & Rottenberg, 1977) and one proton transported chemically inward by the P_i/OH antiporter (Coty & Pedersen, 1974). The H/ATP ratio of mitochondrial F_0F_1 ATP synthase has been evaluated in inverted submitochondrial particles by measurement of ΔG_{ATP} and the electrochemical proton gradient, Δp, during ATP synthesis and ATP hydrolysis, the equilibrium value being bracketed by these two conditions (Berry & Hinkle, 1983). The value after correction for probe binding in the measurement of Δp was close to 3, indicating the mitochondrial H/ATP = 4. However, measurement of ΔG_{ATP} and Δp in mitochondria during oxidative phosphorylation (Woelders et al., 1985) indicates H/ATP = 3 for synthesis and transport, although the data points ranged as high as 3.6. This discrepancy with the results in submitochondrial particles is probably caused by a lack of true equilibrium in the mitochondrial system caused by slow ATP hydrolysis in the matrix and external medium which prevents the true high value of ΔG_{ATP} from being achieved. In addition, the experimental design with mitochondria did not bracket the equilibrium value by comparison of results during ATP synthesis and hydrolysis. There may also have been an overestimation of Δp which would cause an underestimation of the H/ATP ratio.

Values of the H^+/O ratio at the three coupling sites have also been controversial, but there has been an increasing consensus in recent years. Part of the problem has been one of terminology because at each coupling site there is proton absorption or formation by the overall reactions of the respiratory chain in addition to proton transport, so that at each site the number of protons appearing on the cytoplasmic side of the membrane is not equal to the number disappearing from the matrix side (see Figure 4). For example, at site 2, the overall reaction is succinate + 2 cyt c_{ox} → fumarate + 2 cyt c_{red} + $2H^+$, and these two scalar protons appear on the cytoplasmic side. There is general agreement that two protons disappear from the matrix, two positive charges cross the membrane in an outward direction, and four protons appear in the cytoplasm per electron pair going from succinate to cytochrome *c*, consistent with a "Q-cycle" mechanism (Mitchell, 1976; Trumpower, 1990). Because the synthesis and transport of ATP utilize protons electrogenically, site 2 is thus properly referred to as transporting two protons per electron pair, even though four protons appear on the cytoplasmic site. Since site 3 has the opposite imbalance with two fewer protons appearing in the cytoplasm than disappearing in the matrix, there is no ambiguity when both sites are considered together. Taking this complexity into account, the transport of two protons per electron pair at site 2 is firmly established in mitochondria (Mitchell & Moyle, 1967; Alexandre & Lehninger, 1979) and reconstituted ubiquinone–cytochrome *c* reductase (Leung & Hinkle, 1975). The transport of four protons per electron pair at site 1 is indicated by direct

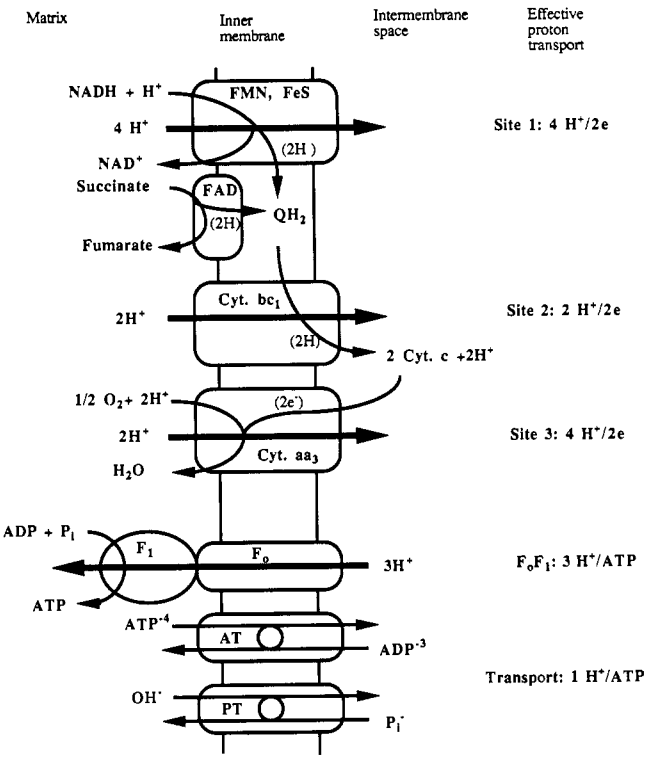

FIGURE 4: Mitochondrial proton transport. The flow of hydrogen (2H, electrons and protons) down the respiratory chain from NADH to H_2O is shown as curved arrows. The resulting proton transport is shown as thick horizontal arrows. The effective proton transport per electron pair is four at site 1, two at site 2 (although two additional protons are formed when cytochrome *c* is reduced in the intermembrane space), and four at site 3 (two by direct transport and two from the movement of electrons across the membrane and uptake of protons to form H_2O). Three protons are required for synthesis of ATP by ATP synthase (F_0F_1), and one proton is used in the uptake of ADP and P_i by the adenine nucleotide transporter (AT) and phosphate transporter (PT).

measurement (Pozzan et al., 1979; Villalobo et al., 1984) and by comparison of site 1 with site 2 (Wikström, 1984a) or with ATP hydrolysis (Scholes & Hinkle, 1984) although values of 3 (DeJonge & Westerhoff, 1982) and 5 (Freedman & Lemasters, 1984) have been suggested. Site 3 has been the most controversial, partly because it is not reversible and there is an excess of free energy in the respiratory chain from cytochrome *c* to oxygen. The value of four charges transported per electron pair has been found by direct measurement with reconstituted cytochrome *c* oxidase (Wikström, 1977; Coin & Hinkle, 1979; Sigel & Carafoli, 1980; Proteau et al., 1983; Thelen et al., 1985) and by comparison with site 2 (Wikström, 1984b), but values of six (Azzone et al., 1979; Costa et al., 1984) and five (Beavis & Lehninger, 1986) have also been reported. Thus, it is reasonable to conclude that the H/O ratios of electrogenic proton translocation at the three sites are 4, 2, and 4, respectively. A scheme showing these ratios is shown in Figure 4. These values of the H/O ratios and H/ATP ratio are compatible with P/O ratios of 1, 0.5, and 1 at the three sites, respectively, and this compatibility contributes to the view that the proton transport ratios shown are correct.

An energetic analysis of the three coupling sites of the respiratory chain is shown in Table II. We have calculated the maximum free energy that could be conserved from each coupling site if the P/O ratios are 1, 0.5, and 1, respectively. The pH values used to calculate midpoint potentials of the hydrogen carriers are an estimate of the matrix pH of 8.0 for site 1 and the external pH of 7.4 for sites 2 and 3 used in the

Table II: Energetic Analysis of the Respiratory Chain[a]

	$E_{m7.4}$ (mV)	$E_{m8.0}$ (mV)	E_h (mV)	ΔE_h (mV)	maximum values	
					ΔG_{ATP} (kcal/mol)	Δp (mV)
site 1 NADH		−350	−361	359	−16.5	180
Q		+5	−2			
site 2 Q	+41		+34	213	−19.6	213
cyt c+c_1	+225		+247			
site 3 cyt c+c_1	+225		+247	~503	−23.2	252
O_2	+796		~+750			

[a] The components defining the reactants and products of each coupling region are shown. NADH, Q, and cytochrome c+c_1 are approximately 70%, 63%, and 30% reduced, respectively, during state 4 respiration with 3-hydroxybutyrate as substrate (Muraoka & Slater, 1969), from which the actual redox potentials (E_h) are calculated. The standard midpoint potentials for NADH (Burton & Wilson, 1953), Q (Urban & Klingenberg, 1969), cyt c+c_1 (Ohnishi et al., 1972), and O_2 were calculated by using the matrix pH of 8.0 for site 1 and the external pH of 7.4 for sites 2 and 3 (see text). The O_2 concentration varies and can be very low, so the E_h value is only approximate. The maximum values of ΔG_{ATP} consistent with the redox potentials were calculated by using P/O ratios of 1, 0.5, and 1 at the three sites, respectively, the conversion factor 23.062 kcal/(V·mol), and the fact that two electrons reduce one oxygen atom. The maximum electrochemical proton gradient (Δp) is calculated from ΔE_h and the proton/electron ratio at each site.

original study (Muroaka & Slater, 1969). This is because the scalar protons formed or consumed in the reactions are in the matrix for NADH + H^+ but in the intermembrane space for QH_2 and O_2. This is not obvious for oxygen because the protons which form water come from the matrix. When the proton flux from the synthesis of ATP is included, however, the net proton loss from the overall reaction occurs in the intermembrane space. The maximum values of ΔG_{ATP} and Δp are calculated from ΔE_h assuming 100% efficiency (i.e., equilibrium) and the new values for P/O ratios. The measured maximum value of ΔG_{ATP} formed by oxidative phosphorylation is about −16 kcal/mol (Slater et al., 1973; Wanders et al., 1984), which is less than the maximum values calculated in Table II, particularly for site 3 where the assumption that the system is at equilibrium leads to a large overestimate of ΔG_{ATP} and Δp. The maximum values of Δp predicted from the redox potentials are 180 and 213 mV for the first two sites, respectively. These values are reasonably close to the measured value of Δp during state 4 respiration of about 190–200 mV [e.g., see Brown and Brand (1988)], considering possible errors in E_h. The chemically determined E_{m7} for ubiquinone in ethanolic solution is +112 mV [see Brown and Brand (1988)]. If that value is used in place of +65 mV determined by succinate/fumarate titration of mitochondria (Urban & Klingenberg, 1969), the maximum value of Δp at sites 1 and 2 would be 198 and 178 mV, respectively, although the biochemically determined value is more appropriate. If the matrix pH value used were 7.4 instead of 8.0, the maximum Δp at site 1 would be 189 mV, but the other sites would be unchanged.

Finally, the traditional calculation of the number of moles of ATP synthesized during the oxidation of 1 mol of glucose should be reconsidered. The complete oxidation of 1 mol of glucose yields 8 mol of matrix NADH which on oxidation would yield 20 mol of ATP, 2 mol of succinate yielding 3 mol of ATP, 2 mol of cytoplasmic NADH yielding 3 mol of ATP via the glycerol phosphate shuttle, 2 mol of cytoplasmic ATP from substrate level phosphorylation, and 2 mol of matrix GTP from succinyl-CoA synthetase. The matrix GTP forms ATP by nucleoside diphosphokinase. However, since the ATP must still be transported to the cytoplasm transporting 1 proton for each ATP, the amount of ATP that can be synthesized by oxidative phosphorylation is decreased by 2 protons or 0.5 ATP. Thus, the overall yield of ATP from glucose oxidation is 29.5 ATP per glucose, rather than the traditional value of 36 ATP per glucose based on integer values of the P/O ratios. If cytoplasmic NADH is oxidized via the malate–aspartate shuttle, then 4.5 ATP would be synthesized during oxidation of the 2 mol of cytoplasmic NADH, because glutamate/aspartate exchange is coupled to the influx of 1 proton per glutamate (LaNoue & Schoolworth, 1979), and the overall yield would be 31 ATP per glucose.

Registry No. H^+, 12408-02-5; ATP, 56-65-5; NADH, 58-68-4; glucose, 50-99-7.

References

Alexandre, A., & Lehninger, A. L. (1979) *J. Biol. Chem. 254*, 11555–11560.

Azzone, G. F., Pozzan, T., & DiVirgilio, F. (1979) *J. Biol. Chem. 254*, 10206–10212.

Beavis, A. D., & Lehninger, A. L. (1986) *Eur. J. Biochem. 158*, 315–322.

Berry, E. A., & Hinkle, P. C. (1983) *J. Biol. Chem. 258*, 1474–1486.

Brand, M. D., Harper, W. G., Nicholls, D. G., & Ingledew, W. J. (1978) *FEBS Lett. 95*, 125–129.

Brown, G. C. (1989) *J. Biol. Chem. 264*, 14704–14709.

Brown, G. C., & Brand, M. D. (1988) *Biochem. J. 252*, 473–479.

Burton, K., & Wilson, T. H. (1953) *Biochem. J. 54*, 86–94.

Chance, B., & Williams, G. R. (1955) *J. Biol. Chem. 217*, 383–393.

Chappell, J. B. (1964) *Biochem. J. 90*, 225–237.

Coin, J. T., & Hinkle, P. C. (1979) in *Membrane Bioenergetics* (Lee, C. P., Schatz, G., & Ernster, L., Eds.) pp 405–412, Addison-Wesley, Reading, MA.

Costa, L. E., Reynafarje, B., & Lehninger, A. L. (1984) *J. Biol. Chem. 259*, 4802–4811.

Coty, W. A., & Pedersen, P. L. (1974) *J. Biol. Chem. 249*, 2593–2598.

DeJonge, P. C., & Westerhoff, H. V. (1982) *Biochem. J. 204*, 515–523.

Freedman, J. A., & Lemasters, J. J. (1984) *Biochem. Biophys. Res. Commun. 125*, 8–13.

Hafner, R. P., & Brand, M. D. (1988) *Biochem. J. 250*, 477–484.

Hinkle, P. C. (1981) in *Chemiosmotic Proton Circuits in Biological Membranes* (Skulachev, V. P., & Hinkle, P. C., Eds.) pp 49–58, Addison-Wesley Publishing Co., Reading, MA.

Hinkle, P. C., & Yu, M. L. (1979) *J. Biol. Chem. 254*, 2450–2455.

Kamo, N., Muratsugu, M., Hongoh, R., & Kobatake, Y. (1979) *J. Membr. Biol. 49*, 105–121.

Khym, J. X. (1975) *Clin. Chem. (Winston-Salem, N.C.) 21*, 1245–1252.

Klingenberg, M., & Rottenberg, H. (1977) *Eur. J. Biochem. 73*, 125–130.

Krishnamoorthy, G., & Hinkle, P. C. (1984) *Biochemistry 23*, 1640–1645.
Lamprecht, W., & Trautschold, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 2101–2110, Academic Press, New York.
LaNoue, K. P., & Schoolworth, A. C. (1979) *Annu. Rev. Biochem. 48*, 871.
Lemasters, J. J. (1984) *J. Biol. Chem. 259*, 13123–13130.
Lemasters, J. J., Grunwald, R., & Emaus, R. K. (1984) *J. Biol. Chem. 259*, 3058–3063.
Leung, K. H., & Hinkle, P. C. (1975) *J. Biol. Chem. 250*, 8467–8471.
Luvisetto, S., & Azzone, G. F. (1989) *Biochemistry 28*, 1109–1116.
Mitchell, P. (1965) *Biol. Rev. 41*, 445–502.
Mitchell, P. (1976) *J. Theor. Biol. 62*, 327–367.
Mitchell, P., & Moyle, J. (1967) in *Biochemistry of Mitochondria* (Slater, C. C., Kaniuga, Z., & Wojtazak, L., Eds.) pp 53–74, Academic Press, London.
Muraoka, S., & Slater, E. C. (1969) *Biochim. Biophys. Acta 180*, 227–236.
Nicholls, D. G. (1974) *Eur. J. Biochem. 50*, 305–315.
Ohnishi, T., Wilson, D. F., Asakura, T., & Chance, B. (1972) *Biochem. Biophys. Res. Commun. 46*, 1631–1638.
Pederson, P. L., Greenawalt, J. W., Reynafarje, B., Hullihen, J., Decker, G. L., Soper, J., & Bustamente, E. (1978) *Methods Cell Biol. 20*, 411–481.
Pozzan, T., Miconi, V., DiVirgilio, F., & Azzone, G. F. (1979) *J. Biol. Chem. 254*, 10200–10205.
Proteau, G., Wrigglesworth, J. M., & Nicholls, P. (1983) *Biochem. J. 210*, 199–205.
Scholes, T. A., & Hinkle, P. C. (1984) *Biochemistry 23*, 3341–3345.
Sigel, E., & Carafoli, E. (1980) *Eur. J. Biochem. 111*, 299–306.
Slater, E. C., Rosing, J., & Mol, A. (1973) *Biochim. Biophys. Acta 292*, 534–553.
Stoner, C. D. (1987) *J. Biol. Chem. 262*, 10445–10453.
Thelen, M., O'Shea, D. S., Petrone, G., & Azzi, A. (1985) *J. Biol. Chem. 260*, 3626–3631.
Toth, P. P., Sumerix, K. J., Ferguson-Miller, S., & Suelter, C. H. (1990) *Arch. Biochem. Biophys. 276*, 199–211.
Trumpower, B. L. (1990) *J. Biol. Chem. 265*, 11409–11412.
Tsou, C. S., & Van Dam, K. (1969) *Biochim. Biophys. Acta 172*, 174–176.
Urban, P. F., & Klingenberg, M. (1969) *Eur. J. Biochem. 9*, 519–525.
Van Dam, K., Westerhoff, H. V., Krab, K., Van der Meer, R., & Arents, J. C. (1980) *Biochim. Biophys. Acta 591*, 240–250.
Villalobo, A., Alexandre, A., & Lehninger, A. L. (1984) *Arch. Biochem. Biophys. 233*, 417–427.
Wanders, R. J. A., Van den Berg, G. B., & Tager, J. M. (1984) *Biochim. Biophys. Acta 767*, 113–119.
Westerhoff, H. V., & Van Dam, K. (1987) *Thermodynamics and control of biological free energy transduction*, p 432, Elsevier, Amsterdam.
Wikström, M. (1977) *Nature 266*, 271–273.
Wikström, M. (1984a) *FEBS Lett. 169*, 300–304.
Wikström, M. (1984b) *Nature 308*, 558–560.
Woelders, H., van der Zande, W. J., Colen, A. M. A. F., Wanders, R. J. A., & Van Dam, K. (1985) *FEBS Lett. 179*, 278–282.
Zoratti, M., Favaron, M., Pietrobon, D., & Azzone, G. F. (1986) *Biochemistry 25*, 760–767.