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Review

# P/O ratios of mitochondrial oxidative phosphorylation

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**Abstract**

Mitochondrial mechanistic P/O ratios are still in question. The major studies since 1937 are summarized and various systematic errors are discussed. Values of about 2.5 with NADH-linked substrates and 1.5 with succinate are consistent with most reports after apparent contradictions are explained. Variability of coupling may occur under some conditions but is generally not significant. The fractional values result from the coupling ratios of proton transport. An additional revision of P/O ratios may be required because of a report of the structure of ATP synthase (D. Stock, A.G.W. Leslie, J.E. Walker, Science 286 (1999) 1700–1705) which suggests that the H+/ATP ratio is 10/3, rather than 3, consistent with P/O ratios of 2.3 with NADH and 1.4 with succinate, values that are also possible.
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*Keywords:* Oxidation phosphorylation; P/O ratio; Proton leak; Slip; ATP synthase

## 1. Introduction

P/O ratios of oxidative phosphorylation (the ATP produced per oxygen atom reduced by the respiratory chain) were first studied in the 1940s and 1950s when the mechanistic values were assumed to be integers. Interest arose again in the 1980s because chemiosmotic theory allowed fractional values and studies of proton transport were not all consistent with integer P/O values. We proposed mechanistic P/O ratios of 2.5 and 1.5 with NADH-linked substrates or succinate, respectively, based on measurements by two methods [1–3] and compatible with $H^+$/O=10 or 6 with NADH or succinate, $H^+$/ATP=3 for ATP synthase and $H^+$/ATP=1 for ATP transport to the cytoplasm. Recent structural studies of ATP synthase [4] now suggest that even the $H^+$/ATP ratio may not be an integer, since it is the ratio of two molecular motors, and so P/O ratios should be reconsidered yet again. The current situation is that some textbooks give P/O's of 3 and 2, one saying they are "more established values" [5], others give 2.5 and 1.5, and a recent review said only that the values are variable [6].

The purpose of this review is to summarize old and new results relevant to P/O ratios and provide analyses of some results to help indicate what values are feasible.

## 2. Measurements of P/O ratios

A collection of P/O measurements is shown in Table 1. Most studies chosen were about P/O values rather than routine reports in papers about some other aspects of oxidative phosphorylation. Numbers have been rounded to two significant figures because, considering possible systematic errors, it is appropriate, and standard deviations are omitted because the degree of reproducibility does not reflect absolute accuracy. In some cases several numbers are reported in which case the range is listed. Most reports are with rat liver mitochondria and exceptions are noted in the text. Some comments on the values in Table 1 follow.

*Abbreviations:* BSA, bovine serum albumin; SMP, submitochondrial particles; $\Delta p$, the electrochemical proton gradient; state 3, respiration with ADP and $P_i$ present; state 4, respiration without ADP; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; RCR, respiratory control ratio

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Table 1
Uncorrected P/O and ADP/O measurements

| Authors | *Method* | NADH-$O_2$ | Succinate-$O_2$ | Site 2 | Site 3 |
|---|---|---|---|---|---|
| Ochoa 1943 [9] | A | 0.96–2.5 | | | |
| Lehninger and Smith 1949 [10] | A | 1.2–2.4 | | | |
| Cross et al. 1949 [11] | A | 0.7–3.3 | 0.88–1.6 | | |
| Copenhaver and Lardy 1952 [12] | A | 2.1–3.2 | 1.2–1.9 | 0.6 | |
| Chance and Williams 1955 [13] | B | 2.3–3.0 | 1.5 | | |
| Greengard et al. 1959 [14] | A | | 1.3–2.2 | | |
| Jacobs and Sanadi 1960 [15] | A | | | 0.45 | 0.95 |
| Estabrook 1967 [16] | B | | 1.7 | | |
| Lee et al. 1967 [17] | C | | | 0.99 | |
| Chamalaun and Tager 1969 [18] | A | | | | 0.94 |
| Klingenberg 1975 [19] | C | | 1.4 | | |
| Hinkle and Yu 1979 [1] | B, C | 2.2 | 1.4 | | |
| Pozzan et al. 1979 [20] | B, C | | | 0.44 | |
| Azzone et al. 1979 [21] | C | | 1.9 | | 1.3 |
| Lemasters 1984 [22] | B | 2.6 | 1.7 | | |
| Beavis and Lehninger 1986 [23] | D | 2.7–2.9 | 1.6–1.8 | | |
| Jensen et al. 1986 [24] | C | | 1.4–1.8 | | |
| Stoner 1987 [25] | C | 2.6 | 1.5 | 0.50 | 1.0 |
| Hafner and Brand 1988 [26] | B | | 1.4 | | |
| Luvisetto and Azzone 1989 [27] | C | | 1.3–1.5 | | |
| Toth et al. 1990 [28] | B | 3.3–3.5 | | | |
| Hinkle et al. 1991 [3] | B | 2.3 | 1.5 | 0.50 | 0.98 |
| Davis and Davis-von Thienen 1991 [29] | B | 2.5 | | | |
| Lee et al. 1996 [30] | B, C | 2.9 | 1.8 | | |
| Fontaine et al. 1997 [31] | C | | 1.3–1.5 | | |
| Devin et al. 1997 [32] | C | 2.5 | | | |
| Gnaiger et al. 2000 [33] | E | | 1.6 | | |

P/O or ADP/O ratios were measured by the following methods: (A) Warburg manometer and phosphate uptake, (B) Chance and Williams oxygen electrode and ADP pulse (see text), (C) oxygen electrode and phosphate or ATP assay, (D) oxygen electrode and pH change, (E) oxygen electrode and injection of ADP at a known rate.

### *2.1. Studies with succinate or NADH-linked substrates*

The earliest studies reported P/O ratios in minced tissue of about 2 [7,8]. A P/O ratio of 3 was first proposed by Ochoa in 1943, who corrected values of 0.96–2.5 (Table 1) to 2.4–4.0 (average 3.06) by measuring glycolysis in the same crude heart extract [9]. I discussed P/O ratios with Ochoa in 1987 and he was not surprised that they could be lower and was highly amused that they were still in question.

Lehninger and Smith [10] used a crude mitochondrial fraction with 3-hydroxybutyrate and AMP as substrates and reported P/O ratios that ranged from 1.5 to 2.44 at 4 min, and from 1.3 to 1.88 at 12 min. Studies with Warburg manometers usually give higher values at short times which have been shown to be overestimates (see later). They concluded that the ratio was "somewhat more than 2".

Cross et al. [11] used rabbit kidney and liver mitochondria (called "cyclophorase") and included hexokinase and glucose to trap the ATP synthesized, as have most groups since. The values show a wide range (Table 1). Assuming the variation was from random errors, the values are consistent with 2.5 or 1.5 with NADH or succinate, respectively.

Copenhaver and Lardy [12] reported a more narrow range of values (Table 1) that are also compatible with fractional ratios. The variation probably stemmed from the use of a Warburg manometer rather than the mitochondrial preparations because the preparations were essentially the same as were used later when there was high reproducibility using an oxygen electrode. However, people tended to attribute the lower values to bad mitochondria.

The first measurements of P/O ratios with an oxygen electrode were by Chance and Williams [13], who developed an excellent method that has been used in countless studies since. The amount of oxygen uptake stimulated by an addition of a known amount of ADP was used to calculate the P/O ratio, which is often called the ADP/O ratio when measured in this way. In that first study they used a vibrating oxygen electrode dipping into an optical cuvette, which gave much more diffusion of oxygen from the air than a modern closed cell does. They measured the diffusion into the cell as ". . .0.1 μM $O_2$ per second when half of the oxygen had been used up", but did not correct for it. With 3-hydroxybutyrate as substrate they gave three pulses of ADP and calculated ADP/O ratios of 2.3, 2.7, and 3.0, increasing as the experiment progressed, which they attributed to endogenous substrates lowering ratios of the first ADP pulses. The amounts of oxygen that diffused into the solution from the air can be calculated based on Fig. 2A of Ref. [13] and their estimate of the rate, which varies from zero at the top to 24 μM O/min at the bottom of the chart. Since the rate of diffusion from the air was less than 15% of the respiration rate, it is a reasonable approximation to use linear rates and calculate the amount of diffusion from the average rate during a state 3 burst and the length of time of

the burst. When the amount of oxygen that diffused in is added to the amount of apparent oxygen uptake, the corrected ADP/O values for the three ADP pulses are 2.2, 2.5, and 2.5. Thus, the major reason why the ratios increased with pulse number was diffusion of oxygen from the air. With succinate the rate of respiration was faster, the diffusion of oxygen less significant, and the reported value of 1.5 corrects down to 1.44. These corrected values are essentially the same as our proposed fractional values.

Greengard et al. [14] studied P/O ratios with succinate under various conditions. The highest values of 2.2 were obtained with low (6 mM) succinate concentration and no inhibitors. Lower values of 1.44–1.62 occurred at 60 mM succinate, and 1.28 or 1.36 were observed with amytal, which inhibits NADH-Q reductase, at 60 and 6 mM succinate, respectively. These results are compatible with a mechanistic ratio of 1.4. Slater had proposed that P/O ratios with NADH were lower than 3 [34] but, since there was no rationale, he dropped the subject when it was clear there were three coupling sites [14]. He later wrote that people who reported high values "either by selecting their 'best' results or by choosing uncritically the procedure giving the highest ratios can shelter behind the tacit assumption that their technique for the preparation of the mitochondria was superior" [35].

Estabrook [16] reported that ADP/O=1.7 with 3.3 mM succinate and no rotenone. The reaction time for ADP/O measurements is shorter than studies with a Warburg manometer, but it is likely that these conditions contributed to the value higher than 1.4, as did the use of plastic in the apparatus (see later).

Pfaff and Klingenberg [36] discovered that ATP/ADP exchange across the mitochondrial inner membrane was driven by membrane potential, which implied that synthesis and export of ATP use more protons than synthesis alone. Klingenberg [19] then studied P/O ratios and found that the value depended on the gradient of ATP/ADP across the membrane, decreasing as the gradient increased, proposed to result from variable charge transport. This was later changed to a strict ratio of one negative charge per ATP [37,38]. However, even in the earlier work [19], the maximum P/O ratio observed was 1.4 with succinate, compatible with one charge per ATP and with the low P/O being the mechanistic value.

In 1979 we reported P/O ratios of 1.4 with succinate and 2.2 with 3-hydroxybutyrate by both the Chance and Williams method and by $^{32}$Pi esterification [1] and suggested they were close to mechanistic. The mitochondria had respiratory control ratios (RCR) as high as 12.5.

Lemasters [22] used the Chance and Williams method with an additional refinement of measuring the slope of ATP vs. oxygen at different ADP pulse sizes, which gave values of 1.7 and 2.6 with Succinate or 3-hydroxybutyrate, respectively. Ogata et al. [39] earlier suggested that there is oxygen uptake inherently associated with the ADP pulse transition, but that was because they did not use the Chance and Williams method for extrapolation of the end point. Lemasters then used a simple non-equilibrium thermodynamic analysis to indicate that basal respiration should be subtracted and reported corrected values of 2.03 and 3.23 with succinate or 3-hydroxybutyrate (see later).

Beavis and Lehninger [23] measured P/O ratios with oxygen and pH electrodes and found a lower limit by malonate titration of 1.63 and 2.66 with succinate and 3-hydroxybutyrate, respectively, from which they suggested mechanistic ratios of 1.75 and 2.75. This was a valid experimental approach but it was not established that the measurement of ATP synthesis from the change of pH was accurate.

Jensen et al. [24] measured P/O ratios of 1.38 to 1.75 with succinate by $^{32}$Pi uptake and corrected the value to 1.91 by a formalized version of the Nicholls [40] method (see later). The correction for proton leak seems about right but the raw P/O values seem high, possibly because of the elaborate procedure used where five samples were withdrawn to measure the rate and from the temperature of 10 °C, which can make calibration of the oxygen electrode more difficult. Not enough details were given to offer a better explanation.

Stoner [25] reported a detailed study of P/O ratios in heart mitochondria, measured by chemical disappearance of Pi and an oxygen electrode. The results were compatible with fractional values (Table 1). He also carried out titrations with four inhibitors and the P/O values were not sensitive to inhibition in the first half of the titration, indicating they were close to mechanistic.

Toth et al. [28] reported, with heart mitochondria from young chickens, that the ADP/O increased on subsequent ADP additions from 3.3 to 3.5 with pyruvate plus malate, and the respiratory control ratio increased from 18 to 93. However, they used an unusually large (9×3 mm.) Teflon stirring bar. We studied smaller bars [3] and by extrapolation estimate the oxygen in their air-equilibrated bar as about 150 nmol, or 18% of the oxygen in the cell. This error of experimental design invalidates the high values.

Davis and Davis-van Thienen [29] reported an ADP/O of 2.5 with pyruvate plus malate and concluded from studies with added ATP that the uncorrected value was probably the best estimate of the mechanistic value.

Lee et al. [30] reported measurements by the Chance and Williams method, but without $Mg^{2+}$ ions, which gave ADP/O=2.90 with 3-hydroxybutyrate and 1.84 with succinate. We [1] reported results with and without $Mg^{2+}$ and found no difference in the ADP/O except that without $Mg^{2+}$ the state 3–4 transition was slower, making the endpoint hard to draw. This is explained by the fact that free ATP is a competitive inhibitor of ADP transport but Mg-ATP is not [36]. Lee et al. [30] state that the constancy of the ADP/O with pulse number showed ". . .there was no diffusion of air and/or outgassing of the Teflon stirring bar. . .". Whenever a Teflon stirring bar is used, there must be outgassing of the oxygen which does not necessarily cause the ADP/O to depend on pulse number because the Teflon equilibrates

quite rapidly and simply acts as additional volume not included in the calculation. In criticizing our measurements as having error from the presence of $Mg^{2+}$ ions, Lee et al. [30] misunderstood how we calculate ADP/O ratios. We stated [1,3] that we used [ADP]+2×[AMP] as the effective ADP concentration, as do others [23,27], because AMP is also phosphorylated by adenylate kinase in the presence of magnesium ions. They used the ADP concentration, ignoring the 5% AMP contamination, which gives obviously erroneous results. Thus, they did not reproduce our results, because they made the calculation differently, and probably overestimated the P/O ratios because of the use of Teflon and other possible errors.

Lee et al. [30] also measured P/O ratios by stopping the reaction with perchloric acid and determining ATP, ADP and AMP by HPLC. The results were 2.78 with pyruvate plus malate. Their version of this method, however, underestimates the oxygen uptake because the reaction was stopped before the oxygen concentration was zero and the slow response of the oxygen electrode makes the trace indicate less oxygen uptake than the true value. Others have avoided this problem by allowing the trace to hit bottom before stopping the reaction (e.g., Refs. [1,26]) but Lee et al. [17] specifically recommend against this.

Devin et al. [32] reported that average P/O ratios ranged from 2.37 to 2.46 with glutamate plus malate as substrate in six different media: sucrose, KCl, NaCl, K glucuronate, Na glucuronate, and choline Cl. I do not recommend using glutamate as substrate because of unknown amounts of substrate-level phosphorylation, but in liver it is apparently not a problem. In heart mitochondria glutamate P/O ratios can be over 3 [41].

Gnaiger et al. [33] reported a P/O of 1.58±0.02 with succinate, measured by a new method of injecting ADP at a known rate while measuring oxygen uptake. They concluded that 1.58 is significantly higher than 1.5 but did not suggest an explanation.

Saturation transfer $^{31}P$ NMR has been used to measure P/O ratios in whole tissues. A value of 2.36±0.15 was found with pyruvate as substrate in perfused rat heart [42]. This method tends to overestimate the mechanistic value because it measures the unidirectional flux of Pi into ATP, which can include glycolysis and Pi-ATP exchange by ATP synthase. Both of these problems were minimized in the study. A P/O of 2.46±0.20 was also reported in perfused rat kidney using the NMR method [43]. These were physiological measurements and yet seem close to the mechanistic values, indicating that in these tissues the physiological state is close to state 3. In resting hepatocytes the effective P/O ratio was only 50% of the maximum based on 2.5 and 1.5 values [44].

### 2.2. Studies of individual sites

Jacobs and Sanadi [15] reported a study of the second site, using succinate to ferricyanide, which gave P/2e values of 0.30 to 0.45, and the third site, using silicomolybdate or ferrocyanide as a mediator to cytochrome *c* with ascorbate as the reductant. P/O values with silicomolybdate without inhibitors ranged from 1.33 to 1.65, but with antimycin A the value was 0.95 (Table 1). With ferrocyanide the value was 0.92 with or without antimycin A.

Howland [45] reported a third-site assay with TMPD-ascorbate gave P/O values greater than 1.4, which were inhibited 50% by antimycin A, but Chamalaun and Tager [18] found a value of 0.94 with rotenone, indicating that, as with succinate, an inhibitor of the first site is necessary to avoid additional ATP synthesis by endogenous substrates. Azzone et al. [21] reported 1.34 with ascorbate–ferrocyanide and 1.9 with succinate plus rotenone (Table 1), but ATP was measured enzymatically which can include synthesis by adenylate kinase. Later Luvisetto and Azzone [27] reported ADP/O ratios of 1.3–1.5 with succinate. The values of a third-site assay are very significant because the only possible scenario for integer P/O rates is that site 3 has a P/O of 1.5, to make up for the value of 0.5 at site 2. It seems that even the early work ruled that out.

In 1979, Pozzan et al. [20] reported a site 2 ADP/2e of 0.44±0.03 and pointed out that the value was consistent with the electrogenic transport of 2 $H^+$/2e for the Q-cycle and $H^+$/ATP=4. Other reports of P/2e's at site 2 are all compatible with a mechanistic value of 0.5 except that of Lee et al. [17] which was 0.99 (Table 1). The later work of Lee et al. [30] giving high P/O values was presented as consistent with P/2e=1 at site 2.

Stoner [25] seems to have made the only study of P/2e at site 1 in whole mitochondria, with $CoQ_1$ plus myxothiazol as the oxidant. With pyruvate plus malate the result was 1.09 and with 3-hydroxybutyrate it was 1.0. The difference was attributed to substrate level phosphorylation or proton efflux from transhydrogenase using NADPH generated from isocitrate dehydrogenase, and evidence presented for the latter.

### 2.3. Possible errors in P/O determinations

Accurate measurement of P/O ratios requires skills that are passed on in laboratories and sometimes lost. An example is a recent report of ADP/O=3.00±0.01 with isolated muscle mitochondria and pyruvate as substrate [46]. The group had previously reported a value of 2.47 [47]. One could argue that they had learned to make the measurement better in the intervening years, but the 3.00 value was determined with a very small (5 μM) ADP pulse which would have given a deflection of the pen on the chart recorder of about a millimeter, making the value not credible. When people are not aware that the measurement they are making is in question, they can be surprisingly uncritical.

Using Warburg manometers involved adjusting and reading a shaking manometer (or 10 being run simultaneously) while reading the clock and writing down the results. They could give accurate rates of oxygen uptake

after the temperature and $CO_2$ levels equilibrated. Most errors, however, led to underestimates of oxygen uptake [48,49].

Oxygen electrodes are easy to use but there are some possible errors. The solubility of oxygen is very temperature-dependent, being almost twice as great at 0 °C as at 25 °C [16]. People usually kept media in the refrigerator and sometimes did not appreciate how long it took to equilibrate at a higher temperature (1 h with bubbling). This was only a problem if the calibration was made by using established solubilities but if not done correctly led to an overestimation of ADP/O ratios. Some groups calibrated by using submitochondrial particles (SMP) and NADH additions, in which case the accuracy of the NADH solution is important. The calibration of the ADP solution is also not trivial.

Plastics contain $O_2$ gas, and early oxygen electrode cells were made of plastic. This allows $O_2$ to diffuse out of the plastic into the solution during the experiment and thus the $O_2$ uptake is underestimated. Teflon has a high $O_2$ solubility [3] and the ubiquitous Teflon stirring bars can cause problems, as discussed earlier. In the 1960s the oxygen electrode I used was a copy of Estabrook's [16], had a Teflon entry port and stirring bar and was made of Plexiglass. The error from plastic depends on how the instrument is calibrated, but it is best to avoid it altogether.

The systematic errors in a typical ADP/O measurement with succinate plus rotenone in a glass apparatus with no Teflon are as follows: a 3.7% underestimate from the proton leak and/or slip [3], a 6.5% overestimation from AMP and ADP remaining at the endpoint [3], a 1% overestimation from diffusion of oxygen from the entry port [22], and a 0.5–1% overestimation from oxygen in the cold ADP solution used for the ADP pulse. Thus, the measured value is about 4% too low and about 8% too high. The largest error was from AMP and ADP remaining. There is very little ADP and AMP remaining when the respiration burst is completely over, but at the endpoint, the intersection of extrapolations of the state 3 and 4 traces, there is significant AMP and ADP [3]. This correction is not related to the small AMP contamination of the ADP used for the pulse, but to AMP formed by adenylate kinase. The 2–5% AMP contamination in added ADP was corrected for in all our measurements [1,3] by using an effective ADP concentration of [ADP]+2×[AMP] in the calculation of ADP/O.

Finally, there is the question of the quality of the mitochondria. Different labs or individuals have slightly different degrees of contamination of their mitochondria with microsomes and other possible differences even if they use the same method, and mitochondria degrade with time. It is generally recognized that the quality of a mitochondrial preparation is indicated by the degree of stimulation of respiration by ADP, or RCR, and that the P/O ratio does not vary even when the RCR has decreased from the usual maximum of about 5 to half that. For example, inhibition of succinate respiration by 1 mM malonate lowered the RCR from 7.1 to 2.4 but only lowered the ADP/O ratio from 1.5 to 1.42 (Fig. 2 of Ref. [3]). I doubt that preps have been a factor in P/O values since about 1950.

## 3. Thermodynamic analysis

The distribution of redox energy between the three sites is far from equal, with the largest (470 mV) at site 3, the next (360 mV) at site 1, and the smallest (250 mV) at site 2 [3,50,51]. (There was an error in the $\Delta E_h$ calculation for site 2 in Ref. [3] because the level of reduction of cytochrome *c* was mistakenly given as 30% instead of 10%.) Slater et al. [51] proposed that site 2 extends from cytochrome *b* to cytochrome $a_3$ to make it have more energy, but it is clear it begins at CoQ and ends at cytochrome *c*. These calculations are compatible with the fractional P/O ratios where the coupling at site 2 is half that of the other sites.

Forman and Wilson [52] and Greenbaum and Wilson [53] have measured the energetics of ATP synthesis from 3-hydroxybutyrate to cytochrome *c* in mitochondria and concluded that P/2e=2. The logic seems compelling; the equilibrium position of the reaction was bracketed by measurements where ATP synthesis was driven by respiration and where reverse electron flow was driven by ATP hydrolysis. The $\Delta G_{redox}$ in each case was about 30 kcal/2e and the $\Delta G_{ATP}$ was about 15 kcal/mol giving a P/2e of $1.97 \pm 0.06$.

There are several reasons why these calculations overestimated the P/2e ratio. First, as pointed out by two groups [54,55], the $E_h$ of 3-hydroxybutyrate was incorrectly based on the matrix pH, the rationale being that the dehydrogenase is located in the matrix. The problem is that in the overall equation for the reaction, the scalar protons formed from the reduction of cytochrome *c* by 3-hydroxybutyrate appear outside as part of the Q-cycle, so the medium pH should be used or else an additional term added, which amounts to the same thing. When the medium pH is used the calculated P/2e is about 1.8 instead of 1.97. Greenbaum and Wilson [53] addressed this criticism, showing that when matrix pH is varied with added propionate the calculated P/2e remained constant only when the matrix pH was used. However, addition of propionate stimulated respiration twofold and lowered $\Delta p$, indicating that it was a weak uncoupler and the constancy of P/2e fortuitous. In addition, the measurements of $\Delta G_{ATP}$ were too low because of ATP hydrolysis during the stopping by perchloric acid, as was shown later [56]. Measurements with a phenol stop [56,51] and by NMR [57] gave ATP/ADPxPi ratios that were 20 to 100 times higher. Also, neither the forward nor the reverse reactions were actually at equilibrium. The forward reaction from external 3-hydroxybutyrate to cytochrome *c* was in steady state with a small amount of ATPase activity and proton leaks and it is hard to say how close to equilibrium it was. The reverse reaction was run after a period of oxidation

without added substrate and with an uncoupler present to deplete endogenous substrates. There were still endogenous substrates present, however, as shown by the results where the reduction of acetoacetate greatly exceeded the oxidation of cytochrome *c*. The combination of these errors could easily bring the calculated P/2e ratio down to 1.5 or lower.

Analyses of $\Delta G_{redox}/\Delta G_{ATP}$ in SMP have shown values of 1.3 [58] and 1.4 [54] at the first site, compatible with a P/2e=1 in mitochondria, and 0.89 at the second site, compatible with P/2e=0.5 in mitochondria when the roles of $\Delta\Psi$ and ΔpH are taken into account [54].

Non-equilibrium thermodynamics (NET) has been used by several groups to analyze oxidative phosphorylation. In this theory the "Phenomenological Stoichiometry", *Z*, is calculated in two ways. When *Z* was based on $\Delta G_{redox}/\Delta G_{ATP}$, it was found that *Z*=2.1 (succinate), 3.0 (NADH) [59] and 1.22, 0.53, and 1.53 at the three sites, respectively [60]. When the original method of calculating *Z* from the coefficients of the linear energy–rate relationships was used, the result was *Z*=3.0 with succinate [61]. This was considered "unsatisfactory" and so a modified method, called mosaic NET, was used which subtracted a fraction of $\Delta G_{redox}$ before calculating the coefficients, giving *Z*=1.996 with succinate [61]. As pointed out by Wilson [62], the application of NET to enzymatic systems is problematic. Walz [63] ends his discussion of the relevance of *Z* by saying that the linearity of the energy-rate equations "...does not justify the assumption that these systems behave like 'near equilibrium' while in fact being far from it." Respiration slows down as ADP runs out and $\Delta p$ increases not because the system is approaching equilibrium but because of kinetic limitations, based on the fact that the measured P/O ratios are lower than predicted from the energy available. Estimations of P/O ratios by NET are not theoretically sound and should be ignored.

## 4. Proton transport stoichiometries

Proton transport stoichiometries have had a turbulent history, which has probably caused many to discount the subject. However, there is essentially complete agreement today.

### 4.1. $H^+/O$ ratios

Rossi and Azzone [64] reported high charge/O ratios (after Cockrell et al. [65]) measured as uptake of potassium with valinomycin, which gave $K^+/O=6$ with succinate. Costa et al. [66], using rapidly responding oxygen and pH electrodes and extrapolating to zero time, claimed $H^+/O=8$ with succinate but the method was convincingly criticized by Krab and Wikstrom [67] and the result shown to be compatible with a value of 6. Values of 6 and 10 for succinate and NADH, respectively, are generally accepted, largely from the work of Wikstrom and coworkers. The arguments seem to be over although not all groups have accepted the consensus explicitly.

The third site was most in question because it does have an excess of redox energy, but a third generation study of cytochrome oxidase vesicles by Antonini et al. [68] has nailed down the value of pumped protons per 2e (the total coupling being two more charges per 2e) as 2.02±0.20, "independent of the number of turnovers, proton back-leak rate, or type of experiment (oxidant or reductant pulse)." They used a rapid scanning spectrophotometer, stopped flow apparatus and computer analysis to separate the spectral changes of phenol red and cytochrome redox changes. Most reports using a pH electrode had shown variable results with these parameters because of the slow response time.

The second site $H^+/2e$ has not been controversial, although the terminology has often been misunderstood. Four protons are formed outside and two protons absorbed inside per 2e transferred from $CoQH_2$ or succinate to cytochrome *c* [69]. Two charges cross the membrane, as electrons on cytochrome *b*'s, per 2e. Thus, two protons are transported electrogenically from the matrix to the cytoplasm, although many call the value 4 because four protons are formed in the cytoplasm. The extra two protons come from the substrate and are not transported. Exactly the opposite imbalance between the protons formed and absorbed on the two sides occurs at site 3 so there is no problem defining the values for physiological substrates.

The first site value, $H^+/2e=4$, was indicated by many studies of multiple sites and recently by direct measurement in SMP from NADH to $CoQ_1$ [70].

Thus, $H^+_{in}/O$ and charge/O ratios of 4, 2 and 4 for the three sites are well supported and do not vary except possibly under unusual conditions [6].

### 4.2. $H^+/ATP$ of ATP synthase

There is evidence from thermodynamic measurements with SMP that the $H^+/ATP$ ratio of ATP synthase is about 3 [71,72] but for technical reasons that measurement is the least accurate of all the ratios discussed, and so it should be deduced from the $H^+/O$ and P/O, or from structure. Partial structures of ATP synthase from X-ray crystallography [4] and studies of ATP-driven rotation of the γ subunit [73] have led to a mechanism of coupling between proton transport and ATP synthesis that involves two motors: $F_1$, an ATP-driven motor, and $F_O$, a $\Delta p$-driven motor [74]. This structure implies that the $H^+/ATP$ ratio should be determined by the number of c subunits in $F_O$ divided by the number of αβ subunits in $F_1$. Stock et al. [4] reported that yeast mitochondrial $F_OF_1$ has 10 c subunits in $F_O$ which together with the three αβ pairs in $F_1$ gives an $H^+/ATP$ of 10/3. If this structure is the same in mammalian ATP synthase then the mechanistic P/O for NADH oxidation should be 10/(10/3+1)=2.3, and for succinate oxidation 6/(10/3+1)=1.4.

One study has concluded that the number of c subunits does not determine the $H^+$/ATP ratio [75]. Chloroplast ATP synthase, which has 14 c subunits [76], was reconstituted in liposomes, a $\Delta p$ imposed and ATP synthesis measured with luciferase. The resulting $H^+$/ATP was 4.0±0.3 but the predicted ratio was 4.7. The use of luciferase limited the concentrations of ATP to 0.1–0.5 μM, much lower than physiological, which makes the conclusion questionable. The $H^+$/ATP ratio of 4.7 fits well with the chloroplast $H^+$/2e of 6 and the measured P/2e of 1.3 [77]. To this reviewer the number of c subunits currently seems the best indicator of the ratio.

For other ATP synthases the $H^+$/ATP ratio can be different, with the numbers of c-type subunits or domains reported so far in homologous enzymes (including a V-ATPase [78]) being 7 [78], 10 [4,79], 11 [80], 13 [81], and 14 [76]. Strikingly, none of these values are the previously expected 9 or 12, which would have given $H^+$/ATP=3 or 4. For the second time, a new mechanism has surprised the field by allowing fractional coupling ratios, and it is now even suggested that there is a principle of symmetry mismatch that forbids 9 or 12 for the number of c subunits [4,74].

## 5. Corrections for uncoupling activities are small

When it was assumed that mechanistic P/O ratios were integers, there seemed to be significant uncoupling activity and NET and slip were brought in to try to explain it. However, when fractional mechanistic ratios are considered, the need for such explanations vanishes and we are left with the proton leak, possibly some slip, and a rate of total energy wastage that is less than basal respiration.

### 5.1. Proton leaks

Proton permeability of mitochondria has been reviewed [82–85]. A seminal observation was made by Nicholls [40] who measured the $\Delta p$ and respiration during a titration of succinate oxidation with malonate under state 4 conditions, and interpreted the results as a current–voltage curve for proton permeability of the mitochondrial inner membrane. The curve was not linear (Ohmic) but showed an increase in current at higher voltage. Several groups (see Ref. [83]) measured proton permeability directly and observed such non-Ohmic behavior, although not necessarily explaining all of the Nicholls-type measurements. The relevance of these measurements for correcting P/O ratios is that the proton leak in state 4 at high $\Delta p$ is much higher than the rate when the $\Delta p$ is the same value as in state 3, so only 10–17% of the basal respiration should be subtracted from the total [3].

### 5.2. Slip

In 1981 Pietrobon et al. [86] proposed that part of basal respiration is caused by "slip" of proton pumps and numerous studies of such slip followed. The definition of slip was basal respiration that is not accounted for by proton leak and the early studies compared the current–voltage curves at different sites and pointed out that the differences must be caused by slip. In the first paper, however, the greatest discrepancy between sites was caused by the incorrect use of 4 $H^+$/2e for the proton transport by site 2. When the correct value of 2 $H^+$/2e is used the *I–V* curve is similar to the other sites. The evidence for and against slip has been reviewed [82,84] and no clear conclusion reached. Azzone, a major proponent of slip, most recently concluded that basal respiration is largely due to slip at 15 °C and to leak at 37 °C [87].

Slip is a mixture of two mechanisms and, in contrast to the uncertain nature of slip, there are several well-established cases that fit the definition but are not called slip. Plant mitochondria have an alternative oxidase that oxidizes CoQ without driving proton transport and which has a higher $K_M$ for $QH_2$ than the coupled pathway, making coupling ratios decrease at higher $QH_2$ levels [88]. Bacteria have mixtures of coupled and uncoupled respiratory enzymes which are made depending of growth conditions [89,90]. Another well-established slip occurs in chloroplast ATP synthase [91,92], which conducts protons when the ATP and ADP concentrations are below 20 μM, although this would not occur physiologically.

Slip, or inhibition of proton pumping, has also been induced by treatments such as DCCD [93], heat [94] or chloroform [95]. In the case of chloroform, the effect was shown to be caused by breakage of a fraction of the mitochondria, which then contributed to higher respiration but did not lower the estimate of $\Delta p$ [96].

The most recent example of specific slip is from Kadenbach [6] who studied the functions of the smaller subunits of eukaryotic cytochrome *c* oxidases. Some of these subunits are organ-specific and have phosphorylation sites and binding sites for ATP, ADP, palmitate or 3,5-diiodo-L-thyronine. In heart oxidase the proton pumping pathway is regulated by ATP levels in the matrix, decreasing from $2H^+$/2e to $1H^+$/2e when the ADP falls to about 2% of the ATP concentration. This is a 25% decrease in charge transport by the enzyme, and would not be observed in a typical ADP/O measurement because it would only occur at the end of the ADP pulse after the extrapolated endpoint. With the liver-type oxidase the same decrease in proton pumping was caused by palmitate, which was avoided in most P/O studies by the addition of bovine serum albumin (BSA).

### 5.3. ATPase

In some studies, ATPase activity would be concluded to be slip or proton leak. Masini et al. [97] reported that state 4 respiration was inhibited 75% by oligomycin, although we found only 15% inhibition [3], indicating that at least some of the basal rate is caused by ATP synthase and

ATPase activity. This ATPase activity, which is largely in the matrix, was inhibited by low concentrations of ADP and so does not occur during state 3 [97]. Most studies of slip are done in the presence of oligomycin but Nicholls' [40] and our [3] measurements were done without oligomycin and so include ATPase. This could account for at least part of the apparent non-Ohmic proton conductance or slip, because the rate of ATP synthesis is highly $\Delta p$-dependent.

### 5.4. Metabolite transport

Metabolite transport in most cases does not use energy because, although substrates enter mitochondria in exchanges which are equivalent to entering as the protonated acids, the protons are absorbed in the formation of $CO_2$ in subsequent metabolism and so do not contribute to use of the chemiosmotic gradient [98]. The only substrates that do use the $\Delta p$ during uptake are ADP plus phosphate (in exchange for ATP) and glutamate (in exchange for aspartate) [98]. The ATP/ADP and Pi transporters are included in the mechanistic P/O ratios of 2.5 and 1.5. They also lower the yield of ATP from succinate thiokinase from 1 to 0.75 because one proton is transported in which could have been used to make 0.25 ATP. The glutamate/aspartate transporter causes the malate–aspartate shuttle for oxidation of cytoplasmic NADH to have a lower P/O ratio than for matrix NADH by 0.25.

### 5.5. Calcium cycling

Calcium cycling is the uptake of calcium electrogenically and the efflux of calcium in exchange for protons or sodium ions, which amounts to uncoupling and would be included in proton leak measurement. It is estimated to be about 1% of the state 3 rate [99] and can be avoided by including EGTA.

### 5.6. Uncoupling proteins (UCPs)

UCPs are, most probably, transporters of anionic fatty acids which are present in mitochondria of specific tissues and are regulated [100]. Since the protonated forms of fatty acids are very permeant, UCP activity creates proton permeability. They are important for considerations of physiological energy metabolism but probably not for measurements of mechanistic P/O ratios, at least in liver and heart where, if present, they would be inactive because of the addition of BSA.

### 5.7. Transhydrogenase

The proton-transporting transhydrogenase is sometimes cited as a user of $\Delta p$ which would lower P/O ratios. The situation is very complicated, however, with several possible roles proposed for the enzyme [101–103]. NADP-linked isocitrate dehydrogenase generates matrix NADPH at a higher rate than the transhydrogenase [102] and it is not clear whether the transhydrogenase uses $\Delta p$ or contributes to it in state 3.

## 6. Conclusions

In a system as complex as oxidative phosphorylation, conclusions about the overall efficiency must be based on measurements with both whole mitochondria and isolated enzymes and involve measurements of proton transport as well as ATP synthesis. In addition, the mechanism of ATP transport to the cytoplasm is important. For most of the reports in Table 1 of uncorrected P/O values that appear to be higher than 2.5 with NADH or 1.5 with succinate, detailed explanations are given as to why they are overestimates. The ideal values could even be 10% lower, if the structural indications for $H^+$/ATP=3.3 for ATP synthase are correct. Studies of proton transport are completely consistent with, and explain, the fractional P/O values. There is still room for some fine tuning but the evidence from early and recent studies indicates that mechanistic (ideal, maximal) P/O ratios are close to 1, 0.5, and 1 at the three sites.

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