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# Reversible uncoupling of oxidative phosphorylation at low oxygen tension

(mitochondrial efficiency/hypoxia/adenine nucleotide assay/electron transport)

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**ABSTRACT** **The stoichiometry of oxidative phosphorylation at low oxygen tension (< 3 torr; $O_2$ < 5 μM) has been measured in rat liver mitochondria. In a steady-state model in which respiration rate was experimentally controlled by either oxygen or substrate (succinate) limitation, flux-dependent variation in the phosphorylation efficiency (P/O ratio) of stimulated mitochondrial respiration was evaluated. P/O ratio remained constant over a wide range of respiration rates in mitochondria limited only by substrate availability. In contrast, oxygen-limited mitochondria demonstrated a continuous decline in P/O ratio as respiration was increasingly restricted. Significant differences in the two test conditions were demonstrated throughout the range of analysis. The effect of oxygen limitation on phosphorylation efficiency was shown to be completely reversed by restoring zero-order kinetics associated with high oxygen tension. These findings are discussed in regard to a proposed uncoupling of mitochondrial coupling site II at low oxygen tension arising as a consequence of energy-dissipating electron flux through the ubiquinone-cytochrome *b*-$c_1$ region of the respiratory chain (complex III).**

Living cells utilize and conserve energy in various forms. In this regard, the cell shares with other mixed-energy-domain systems the ability to convert energy internally from one physical form to another. For aerobic organisms, the existence of one such biological energy transduction has been extensively documented: the coupling of electron transport in the mitochondrial respiratory chain (cellular respiration) to the synthesis of ATP. Indeed, so effectively does oxidative phosphorylation support the many energy-requiring reactions of aerobic cells that there is a tendency to assume that mitochondrial ATP synthesis proceeds with maximum stoichiometric efficiency under nearly all physiologic conditions.

However, it is well established that mitochondrial respiration is not obligatorily linked to the formation of high-energy phosphate bonds (1–4). The phenomenon of uncoupling has been demonstrated routinely in the laboratory by using chemical agents that direct the energy released by electron transport into mitochondrial energetic activities other than phosphorylation. The possibility of intrinsic mitochondrial uncoupling has been considered by several investigators and is predicated on the hypothetical existence of energy-dissipating pathways of electron flux through the respiratory chain. In this connection, Trumpower (5) commented on a potential uncoupling of Mitchell's proposed "Q-cycle" (6) related to the disposition of the unpaired electron of ubisemiquinone interacting with the Rieske iron-sulfur protein at coupling site II. An explicit separation of energy-transducing and energy-dissipating electron transport pathways in submitochondrial particles is described in the dual respiratory chain model of Norling *et al.* (7). Chance (8) proposed energy-dissipating electron flux between quasi-equipotential carrier groups to explain the initial transient response of the cytochromes *b* in oxygen pulse studies, an experimental situation that is particularly interesting to us because of our primary focus on the regulation of energetics of cellular respiration at low oxygen tension.

Much of our present understanding of cellular respiration is derived from experiments conducted under the qualifying condition of high ambient oxygen tension, in which respiration demonstrates zero-order kinetics with respect to oxygen concentration. This experimental setting is not necessarily analogous to the physiologic situation in which oxygen is continuously supplied into a tissue micro-environment that may be poised at a very low $P_{O_2}$ (9, 10). The role of oxygen in the control of mitochondrial behavior has been explored from a number of perspectives; for example, the kinetics of cellular respiration (11), the redox state of respiratory chain carriers (12), the preservation of energetic function (13), and the thermodynamic relationship between the mitochondrial oxidation-reduction reactions and the extramitochondrial phosphorylation state (14). Many of these observations have been incorporated into the "near-equilibrium" model of oxidative phosphorylation (15, 16), which remains a convenient conceptual device for the assessment of mitochondrial behavior, in spite of compelling theoretical objections (17). An important prediction of the near-equilibrium model is the maintenance of efficient ATP synthesis by mitochondrial electron transport at low oxygen tension (14), thereby rejecting the physiologic significance of energy-dissipating electron flux through the respiratory chain. However, support for this prediction heretofore has been circumstantial.

The specific aim of the following study was to evaluate directly the stoichiometry of oxidative phosphorylation at low $P_{O_2}$. Because of the unique demands of this particular experimental setting, a steady-state model of mitochondrial respiration has been developed with electron flux rate as the control parameter. Flux-dependent phosphorylation efficiencies were quantitated for both oxygen-limited and substrate-limited respiration.

## EXPERIMENTAL

**General.** Rat liver mitochondria were prepared by a modification of the method of Schneider and Hogeboom (18) using a sucrose/Tris·HCl/EDTA isolation medium (19). Incubation details are provided in the legends. All studies were performed at room temperature (23–24°C). Mitochondrial integrity was assessed by standard polarographic parameters with succinate

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Abbreviations: RCR, respiratory control ratio; $Ap_5A$, $P^1$,$P^5$-di(5′-adenosyl) pentaphosphate.

as substrate. Minimal performance characteristics were strictly enforced: ADP/O > 1.5 and respiratory control ratio (RCR) > 5.5; ADP/O ratios (succinate) rarely exceed 1.65 when measured at room temperature (20). Oxygen consumption was measured with a Clark electrode. Respiration was terminated with $HClO_4$ (final concentration, 3%). Quenched samples were maintained in ice-cold acidic medium for 1 hr before neutralization with a KOH/ammonium phosphate buffer. Adenine nucleotide concentrations were measured by chromatographic separation as described below. Low-oxygen P/O determinations were performed in an anaerobic glove box purged for 24 hr with nitrogen followed by argon (research grade: $O_2 < 1$ ppm). All media used in conjunction with the low-oxygen determinations, including the quenching medium, were deoxygenated with argon. Mitochondrial protein was estimated by the biuret method. An inhibitor of adenylate kinase, $P^1,P^5$-di(5'-adenosyl) pentaphosphate ($Ap_5A$; Sigma) was used in the P/O evaluations (21). All studies were completed within 10 min after the mitochondria were suspended in the reaction medium to minimize "aging" effects (22).

**P/O Determinations with HPLC.** In the analysis of the phosphorylation efficiency of both oxygen-limited and substrate-limited respiration, HPLC was used to monitor the synthesis of ATP. Although not commonly used for this purpose, the technique proved to be an ideal choice for processing the large number of samples required in this study. HPLC provides a complete profile of the concentration of all three adenine nucleotide species (23). From this information, phosphorylation activity is readily corrected for the effects of adenylate kinase (EC 2.7.4.3.). An additional feature of chromatographic analysis is its ability to detect free adenosine, accumulation of which can compromise estimates of phosphorylation efficiency. No adenosine accumulation was detected in any of the analyses presented in this report.

Mitochondrial suspensions (1–10 mg of mitochondrial protein per 5 ml of reaction medium) forced to respire at selected rates by either substrate- or oxygen-deprivation were stimulated with 200 μM ADP. After a 30-sec delay to ensure steady-state conditions, the suspension was repetitively sampled at regular intervals. Six samples were collected for each P/O determination; only 100 μl was required for each sample, representing slightly less than 2% of the total volume of the mitochondrial suspension. The quenched samples were centrifuged to separate precipitated protein, neutralized as described, filtered, and applied directly to the HPLC column with no additional preparation. Complete separation of adenine nucleotides for a single sample was performed in less than 6 min on a $C_{18}$ μBondapack column (Waters) with 50 mM ammonium phosphate at pH 5.5 as solvent. On-line quantitation of adenine nucleotide concentration was provided by a 254-nm photometer linked to a digital integrator (Waters M730 data module).

Changes in the concentrations of the respective adenine nucleotides were evaluated over the set of six samples. Typically, a linear increase in ATP and a corresponding decrease in ADP was encountered. Changes in AMP generally were small ($Ap_5A$ was present in the reaction medium to inhibit adenylate kinase). This pattern is shown in Fig. 1. Linear regression analysis was used to quantitate the changes in ATP and ADP concentrations independently; all coefficients of correlation for the regression analyses exceeded 0.995 for ATP and −0.995 for ADP. The computed slopes were combined to derive an estimate for the rate of phosphorylation [nmol of high-energy phosphate bonds (~P) per min]. The estimated rate of phosphorylation was adjusted for oligomycin-insensitive ATP hydrolysis, which was measured independently for each preparation of isolated mitochondria (by the above technique with rotenone-inhibited,

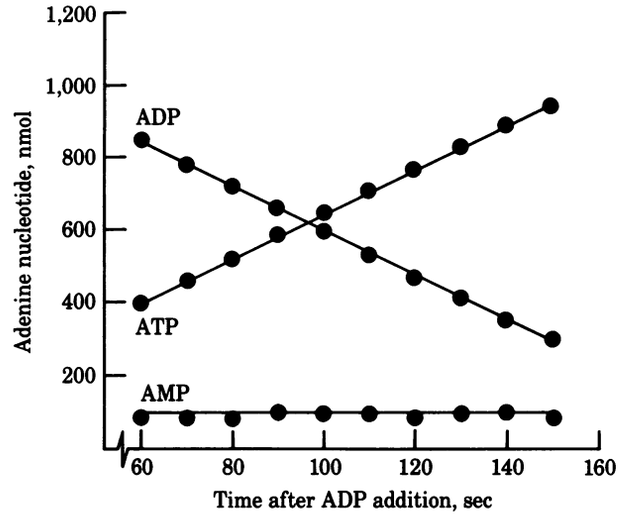


FIG. 1. Changes in adenine nucleotide concentrations during an interval of ADP-stimulated respiration. A mitochondrial suspension (1 mg of mitochondrial protein per ml) utilizing 10 mM succinate in the presence of excess oxygen was sampled at 10-sec intervals after stimulation with 260 μM ADP. The rate of phosphorylation was estimated from the linear changes in ATP and ADP concentrations, by using regression analysis. HPLC was used to assess adenine nucleotide concentrations. Incubation medium (used in all experiments) was 75 mM KCl/50 mM Tris·HCl/12.5 mM $K_2HPO_4$/5 mM $MgCl_2$/1 mM EDTA/200 μM $Ap_5A$/10 μM rotenone, pH 7.4; incubation was at 23–24°C.

substrate-free mitochondria suspended in a medium containing 20 nmol of oligomycin per mg of mitochondrial protein and 100 μM ATP). The adjustment was small in all cases and was negligible at the higher rates of respiration (<3% of the uncorrected estimate). Respiration rates (nmol of $O_2$ per min) during the assay period were measured directly with a Clark electrode in the substrate-limited studies. For the oxygen-limited determinations, the rate of oxygen consumption was controlled as described below. P/O values were computed from the respective rate measurements in the conventional way.

**Controlled Oxygen Consumption at Low $O_2$.** In a typical experiment, the assay vessel was purged with argon and filled with 5 ml of partially deoxygenated reaction medium containing succinate as substrate. Oxygen tension was continuously monitored polarographically (Fig. 2). Mitochondria were added to the assay vessel and allowed to consume residual dissolved oxygen. The anaerobic condition was maintained for a maximum of 30 sec. The suspension then was continuously reoxygenated with air-saturated infusate delivered at a fixed flow rate by a syringe pump. After approximately 30 sec the mitochondria were stimulated with 200 μM ADP. The system spontaneously attained a steady-state oxygen tension which supported a rate of respiration equal to the rate of oxygen infusion, as indicated by a plateau in the polarographic record. All measurements of ATP synthesis were made during this steady-state interval. Total mitochondrial protein in the assay vessel was maintained essentially constant (within 2%) throughout the study. The infusion pump was stopped at the completion of the analysis, allowing the suspension to return to the anaerobic state for calibration of the oxygen electrode.

## RESULTS

A common reference state for measuring the rate of oxygen consumption ($Q_{O_2}$) in well-coupled mitochondrial suspensions is the "static head" condition (17), state 4, in which the rate of respiration is limited by the availability of phosphate acceptor.

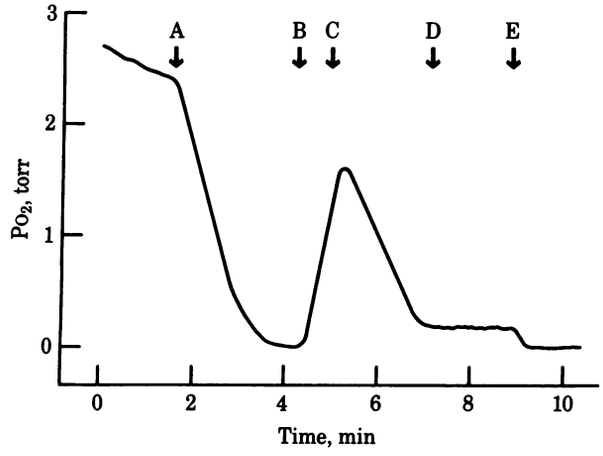


FIG. 2. Steady-state respiration at low oxygen tension. Oxygen tension was continuously monitored with a Clark electrode during an infusion study of mitochondrial phosphorylation efficiency at low $P_{O_2}$, conducted within the zero-oxygen environment of an anaerobic glove box. At A, mitochondria were added to the reaction vessel and allowed to consume the residual oxygen. The infusion of air-saturated reaction medium was begun at B; 200 μM ADP was added at C. The suspension spontaneously attains a steady-state oxygen tension at which the rate of respiration is equal to the rate of infusion (points D and E). Samples routinely were collected during this interval. Infusion was terminated at E. Reaction vessel contained 1 mg of mitochondrial protein, 5 ml of incubation medium, and 10 mM succinate. Infusate was 1 mg of mitochondrial protein in 5 ml of incubation medium. (One torr = 133 Pa.)

For convenience, we adopted the state 4 respiration rate as a reference for assessing the effect of substrate limitation and oxygen limitation on the efficiency of oxidative phosphorylation. Previous experience in our laboratory has indicated that state 4 $Q_{O_2}$ (nmol of $O_2$ consumed per min per mg of mitochondrial protein) is relatively constant from one preparation of isolated liver mitochondria to another, as long as certain performance standards are satisfied.

Stimulation of respiration with 200 μM ADP under conditions of excess substrate (succinate, 10 mM) and oxygen ($O_2$ > 50 μM) increases the rate of electron flux through the respiratory chain to a value >550% that of the static head rate. Direct examination of P/O ratio under these conditions was performed on five different preparations of mitochondria. A mean (±SD) of 1.62 ± 0.11 was found. In each case, the directly estimated P/O ratio compared favorably to the polarographic ADP/O ratio.

In subsequent studies, the rate of ADP-stimulated respiration was experimentally limited by controlling the availability of either substrate or oxygen. In both test populations, flux-dependent variation in phosphorylation efficiency was evaluated over a 25-fold range of stimulated respirations rates, from 20% to 500% of the state 4 $Q_{O_2}$ (Fig. 3).

For the assessment of substrate-limited respiration, malonate, a competitive inhibitor of succinic dehydrogenase (complex II), was used. Desired rates of respiration were attained by adjusting the malonate concentration (0.1–25 mM) in suspensions of rotenone-inhibited mitochondria utilizing 10 mM succinate. Nineteen evaluations were performed (Fig. 3 *Upper*). As expected, the P/O ratio remained constant over a broad range of respiration rates. Only when respiration was constrained to a rate below that permitted under state 4 conditions did a significant decrease in phosphorylation efficiency occur. The dramatic decline in P/O ratio at extremely low rates of electron flux through the respiratory chain is consistent with previous observations of inhibited respiration (24). This behavior may relate to internal processing of the free energy released by electron transport, in which the energetic requirements of the mitochondrion itself take precedence over the process of phosphorylation.

A distinctly different pattern was seen in the oxygen-limited model—phosphorylation efficiency was compromised throughout the range of evaluation (Fig. 3 *Lower*). In none of the 20 determinations did the measured P/O ratio exceed 1.5. Although a precipitous decline in P/O ratio was evident at the lower range of electron flux in both experimental settings, significant differences between substrate-limited and oxygen-limited respiration were found at all measured rates. In comparing the approximated phosphorylation efficiency curves for the respective conditions, the greatest discrepancy is noted at intermediate respiration rates: the P/O ratio for the oxygen-limited mitochondrial suspension falls to nearly 1.0 before any detectable decrease occurs in the substrate-limited preparation—a net difference of 0.6 high-energy phosphate bond per atom of oxygen consumed. As electron flux rates approach zero, the curves tend to converge. Similarly, convergence is suggested as respiration approaches maximally stimulated rates (state 3 $Q_{O_2}$).

Restoration of phosphorylation efficiency at higher ambient oxygen tension is suggested by the apparent monotonic increase in P/O ratio with respect to respiration rate. The limitations of the controlled infusion technique have made direct confirmation impossible. However, complete restoration of phosphorylation efficiency at high oxygen tension (90 torr, zero-order kinetics with respect to $O_2$) was uniformly documented polarographically by using mitochondrial suspensions maintained anaerobically for several minutes (both with and without

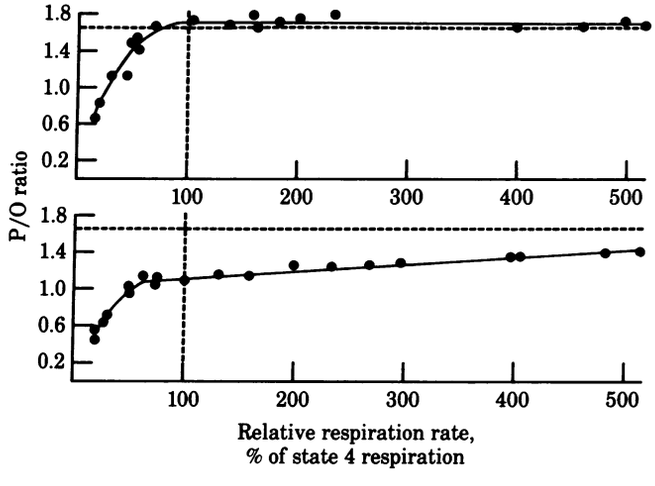


FIG. 3. Phosphorylation efficiency (P/O ratio) under substrate-limited and oxygen-limited conditions. (*Upper*) Substrate-limited. Malonate (0.1–25 mM) was used to limit the rate of ADP-stimulated respiration by mitochondria metabolizing 10 mM succinate at atmospheric oxygen tension. (*Lower*) Oxygen-limited. Oxygen availability was regulated by the controlled infusion of oxygenated medium (see Fig. 2 legend for details) into an uninhibited mitochondrial suspension metabolizing excess succinate (10 mM). The infusate was identical in all respects to the mitochondrial suspension except that it was air-saturated ($O_2$ ≡ 240 μM) and contained neither substrate nor ADP. Continuous oxygen monitoring and repetitive sampling of the suspension were conducted within an anaerobic glove box. In both traces, each point represents an individual determination of P/O ratio referenced for convenience to the suspension's measured state 4 rate of respiration. P/O ratios were estimated as described in the text, with linear regression analysis. In all cases, the coefficient of correlation for the statistic exceeded 0.995. The horizontal broken lines (----) show, for reference, the average P/O ratio determined by direct assay in five uninhibited preparations metabolizing 10 mM succinate at atmospheric oxygen tension (ADP/O = 1.62; SD ± 0.11). The solid lines are fitted visually.

added ADP). Neither ADP/O ratio nor RCR was significantly changed as a consequence of exposure to anaerobic or low-oxygen-tension conditions.

## DISCUSSION

In recent years, nonequilibrium thermodynamics has been increasingly applied to the description of biological systems, particularly in regard to free energy transductions involving incompletely coupled biochemical processes. Using this formalism, Rottenberg (25) confirmed that, in tightly coupled mitochondria respiring in the presence of excess oxygen, the degree of coupling between electron transport and phosphorylation is <1.0. It has been shown that, as a consequence of incomplete coupling, the stoichiometry of oxidative phosphorylation (P/O ratio) demonstrates two distinct dependencies: the first on the degree of coupling and the second on the relative affinities of the input (electron transport) and output (phosphorylation) reactions (26).

We used P/O ratio in assessing the coupling status of mitochondria respiring at low ambient oxygen tension. Two considerations influenced this decision. (*i*) The simplifying assumptions normally used in directly estimating the degree of coupling (linearity and the Onsager relation) have not been confirmed for respiration restricted by oxygen availability and may not apply (27). (*ii*) At the cellular level, where the efficient management of both material and energy reserves is a prerequisite to optimal performance, stoichiometry, as an index of both material and energy conservation, is perhaps the most important measure of the physiologically relevant efficiency of oxidative phosphorylation.

We report here the reversible uncoupling of oxidative phosphorylation at low oxygen tension. ADP-stimulated mitochondria metabolizing succinate and constrained by oxygen availability demonstrated a marked decrease in phosphorylation efficiency, significantly greater than that occurring as a consequence of electron flux limitation *per se*. The character of the dependency of phosphorylation efficiency on the degree of oxygen limitation, in particular the distinct inflection occurring at a P/O ratio of approximately 1.0 (Fig. 3), is not inconsistent with the proposal that one of the relevant coupling sites (site II or site III) may be selectively bypassed or disengaged at low oxygen tension. Earlier speculations have focused upon the ubiquinone:cytochrome $b$-$c_1$ region (complex III, coupling site II) of the respiratory chain as the possible site of an alternative energy dissipating pathway of electron flux (5, 7, 8). Preliminary unpublished studies of our own which reveal that the kinetic properties of complex III are sensitive to oxygen concentration may prove relevant to the possibility of selective uncoupling of site II. Our experiments to date do not distinguish, mechanistically, whether the effect of low oxygen tension is mediated indirectly as a passive consequence of increased mitochondrial electron density (8) or in fact reflects a direct interaction between oxygen and one or more unspecified mitochondrial components (28, 29).

We are inclined to view the uncoupling of oxidative phosphorylation at low oxygen tension as a general property of mitochondria, relevant, for example, to our particular interest in the pathophysiology of cerebral hypoxia and ischemia. Measurements of regional $P_{O_2}$ in rat brain revealed that mitochondria may routinely respire at intracellular $O_2$ $\phi\pm'' = \mu M$ (9). It therefore is not surprising that several lines of evidence suggest that reversible uncoupling occurs *in vivo*. For example, the phenomenon may be discernible in studies, performed in Siesjö's laboratory, in which sustained (and even increased) rates of rat cerebral oxygen consumption (30) were observed at levels of arterial hypoxemia previously characterized by a significant loss of brain phosphocreatine stores (31), levels at which it has also been demonstrated that cerebral energy utilization is, if anything, slightly decreased (32).

The apparently universal distribution, among aerobic cells, of the enzyme superoxide dismutase implies that oxygen-dependent organisms are continuously threatened by free radical oxygen (33, 34). Coupled mitochondria are significant generators of oxygen free radicals, producing $O_2^-$ (and $H_2O_2$) in direct proportion to ambient oxygen tension (35). Cells that are continuously exposed to high oxygen concentrations are particularly vulnerable to free radical reactions which degrade structural and enzyme proteins, nucleic acids, and membrane phospholipids (36). On the other hand, the unique energetic efficiency that characterizes aerobic oxidative phosphorylation is significantly compromised when oxygen availability restricts the rate of mitochondrial respiration. These conflicting considerations dramatize the predicament common to all aerobic life forms and support the proposition that cellular respiration is optimally conducted when oxygen tension is maintained at the lowest level compatible with cellular energy requirements.

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