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# Changes in pyridine nucleotide levels alter oxygen consumption and extra-mitochondrial phosphates in isolated mitochondria: a $^{31}$P-NMR and NAD(P)H fluorescence study

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**Isolated rat-liver mitochondria were used to study the relation between mitochondrial NADH levels, oxygen consumption ($QO_2$), and extra-mitochondrial phosphates. Alterations in NADH and $QO_2$ were accomplished by incubating mitochondria with different substrates or varying amounts of exogenous ATPase while monitoring $QO_2$ and NAD(P)H fluorescence. Two sets of conditions were studied: (1) in the presence of excess ADP and inorganic phosphate, an increase in NAD(P)H fluorescence was associated with a linear increase in $QO_2$; (2) when $QO_2$ was driven by the steady-state hydrolysis of ATP by exogenous ATPase, increases in $QO_2$ were associated with proportional decreases in NAD(P)H fluorescence. For all substrates tested this relation was linear; however, the slope was substrate dependent. Different substrates were able to maintain different NAD(P)H levels at the same $QO_2$. To investigate this further, effects of changing substrates at constant $QO_2$ on NAD(P)H and extra-mitochondrial phosphates were determined. Addition of glutamate + malate to mitochondria respiring on citrate caused a 50% increase in NAD(P)H fluorescence, a 41% decrease in ADP, and a 30% decrease in inorganic phosphate. Similar changes for the substrate jump, pyruvate + malate to glutamate + malate were found. Finally, it was determined that a linear relation holds between increases in NAD(P)H fluorescence and increases in $QO_2$ when substrates were varied at constant, physiologic levels of extra-mitochondrial ADP. These results indicate that $QO_2$ depends on NAD(P)H levels as well as on extra-mitochondrial phosphates over a wide range of respiratory rates.**

## Introduction

Recent studies on a number of different tissues have demonstrated that the control of mitochondrial ATP production in intact tissues when workload is varied is a complex process. The coupling between increases in work and increased oxygen consumption ($QO_2$) does not simply rely on the concentration of high-energy phosphates as early models predicted [1–3]. Studies using $^{31}$P-NMR have shown that levels of ADP, ATP and inorganic phosphate ($P_i$) remain constant over wide variations in work output [4–7]. These results suggest that other factors must be responsible for coupling increases in ATP utilization to increases in oxidative ATP production.

A primary candidate for an additional control

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Abbreviations: $QO_2$, oxygen consumption rate; $P_i$, inorganic phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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site is the mitochondrial pyridine nucleotide redox state. Previous investigators have suggested that the NADH/NAD redox state may be a controlling step when the $QO_2$ of a cell is increased during hormonal stimulation [4,8–10], with alterations in metabolic substrates [11–13], or upon work transitions [5–7]. Theories have been proposed relating the NADH/NAD redox state to $QO_2$ and the extra-mitochondrial phosphorylation potential [14–17]. However, no studies looking directly at how changes in the pyridine nucleotide redox state affect respiration or extra-mitochondrial phosphates over a range of conditions have been performed on isolated mitochondria.

In this study we have used isolated liver mitochondria incubated under a variety of conditions to see how alterations in the pyridine nucleotide redox state affect $QO_2$ and extra-mitochondrial phosphates. In order to lay the groundwork so that these studies can be extended to intact organs, fluorescence was used to monitor NAD(P)H levels and $^{31}$P-NMR was used to monitor extra-mitochondrial phosphates. The results obtained show that alterations in substrates which increase pyridine nucleotide levels can stimulate $QO_2$ at fixed levels of extra-mitochondrial ADP, ATP and $P_i$. In addition, at fixed levels of respiration increases in NAD(P)H levels were associated with decreases in ADP and $P_i$ levels. These studies should provide a basis for the interpretation of in vivo NAD(P)H fluorescence and $^{31}$P-NMR investigations of the regulation of oxidative ATP production. Preliminary results of this work were presented at the Biophysical Society Meeting, San Francisco, CA, U.S.A. (1986) Biophys. J. 49, 207a and the Society of Magnetic Resonance in Medicine Meeting, Montreal, Canada (1986).

## Materials and Methods

*Preparation of mitochondria.* Liver mitochondria from male Sprague-Dawley rats (200–400 g, Taconic Farms) were isolated following the procedure of Schnaitman and Greenawalt [18]. Briefly, excised liver was placed into a solution (solution A) containing 70 mM sucrose, 210 mM Mannitol, 2 mM Hepes and 0.5 g/l bovine serum albumin (Miles Lab.) at 4°C and pH 7.4. After mincing, the tissue was broken up with one or two passes in a loose-fitting homogenizer in a 1 : 3 (w/w) dilution of tissue with solution A. The resulting suspension was centrifuged at $480 \times g$ for 15 min; the supernatant was discarded and the pellet homogenized in a 1 : 10 (w/w) volume of solution A with five passes in a tight-fitting homogenizer. This suspension was spun at $480 \times g$ and the supernatant was saved and spun at $7710 \times g$ to obtain the mitochondrial pellet. This pellet was rinsed twice, resuspended in roughly 50 ml of solution A, then centrifuged at $12100 \times g$. The pellet was again rinsed twice, resuspended and centrifuged at $12100 \times g$. This final pellet was rinsed twice with the experimental solution (solution B: 137 mM KCl, 10 mM Hepes, 2.5 mM $MgCl_2$, 0.5 mM EDTA (pH 7.2)) and resuspended in a final volume of 3–4 ml of solution B to give a stock suspension of mitochondria. Typically 13 g of liver gave 3.9 ml of 38 mg protein/ml stock suspension.

*Oxygen-consumption measurements.* $QO_2$ measurements were made using a Clarke-type $O_2$ electrode (Yellow Springs Instruments, OH) placed in a 1.0 ml stirred chamber which was water jacketed to maintain a constant temperature of 25°C. The electrode was calibrated with air-saturated solution B, which was assumed to contain 470 μM oxygen atom [19].

To standardize each experiment the amount of mitochondrial protein added to the chamber was adjusted to $1.94 \pm 0.07$ mg protein/ml ($n = 17$). This represented 50–80 μl from the stock solutions and gave a $QO_2$ of $187 \pm 5.2$ natoms O/mg protein per min ($n = 51$) when incubated with 5 mM inorganic phosphate ($P_i$), 5 mM glutamate (glut), 5 mM malate (mal), and 700 μM ADP (State 3 conditions) * in solution B. For each particular mitochondrial preparation the State 3 rate was taken as 100% and all other $QO_2$ values were expressed as a percent of this control value. This rate varied less than 4% over the course of a day of experiments (10–12 h).

As a test of the integrity of each mitochondrial preparation, the acceptor control ratio, defined as the State 3 rate divided by the residual $QO_2$ measured after all the ADP was phosphorylated to

* Respiratory states are named following Chance and Williams [2].

ATP (State 4), was determined. Mitochondria with acceptor control ratios of less than 6 were discarded. The average ratio for all preparations was $10.6 \pm 0.3$ at the start of each days experiments which fell to a value of $9.7 \pm 0.4$ at the end of each days experiments.

*Fluorescence measurements.* When excited by light in the near ultraviolet mitochondria fluoresce blue light. It has been appreciated since the earliest fluorescence studies of yeast [20] and isolated mitochondria [21,22] that NADH, NADPH, and flavins can all contribute to the fluorescence signal. A comparison between changes in absorption, fluorescence and chemical analysis of NADH and NADPH concluded that changes in fluorescence from isolated mitochondria correlated with changes in NADH + NADPH levels [23]. Furthermore, it was determined that due to the effects of binding to proteins NADH fluorescence is enhanced 2- to 3-fold that of NADPH in mitochondria [22,23]. Therefore it was estimated that the majority of the fluorescence from State 4 mitochondria is due to NADH [23]. Studies examining changes in NADH and NADPH for State 4-to-State 3 transitions in isolated liver mitochondria indicated that both NADH and NADPH levels changed significantly [24]. Therefore the fluorescence signal represents a complex sum of NADH and NADPH and was used in this study as a qualitative measure of pyridine nucleotide redox state. Further justification for using the fluorescence signal comes from data which indicates that the NADH/NAD couple is in equilibrium with the NADPH/NADP couple in liver mitochondria [25].

Fluorescence measurements were made simultaneously with $QO_2$ measurements. The chamber used was designed so that a portion of the sample compartment was not water jacketed to allow the efficient transfer of excitation and fluorescent light. Mitochondrial fluorescence was excited with a $N_2$ laser at 337 nm (Laser Science Inc.) and detected with a silicon-intensified target multichannel analyser (PAR 1216, Princeton Applied Research) after spectral dispersion of the emitted light in a monochromator (Instruments SA). In this way an approx. 300 nm bandwidth could be sampled at the video frame rate of 33 ms. The laser pulse was triggered at the beginning of each video frame using logic lines from the PAR 1216 controller. The output of the PAR 1216 controller was interfaced to a PDP 11/23 computer (Digital Equipment Corp.). To enhance signal to noise, 30 video frames were averaged. The temporal resolution of the fluorescence measurements was varied as necessary by introducing a delay (15–60 s) between spectral acquisitions. Data were analyzed by taking the ratio of the fluorescence intensities at appropriate wavelengths.

*NMR measurements.* $^{31}$P-NMR measurements were made on an NT-360 (Nicolet) widebore spectrometer operating at a phosphorus frequency of 146.15 MHz. A homebuilt probe [26] incorporating a chamber (5 ml sample volume) which continuously stirred and oxygenated the mitochondrial suspension was used. Oxygen tensions of approx. 1200 μM oxygen atom were maintained by simply blowing 100% $O_2$ over the surface of the stirred suspension. Temperature was maintained at 25°C by blowing temperature regulated air over the probe.

For the NMR experiments, the experimental volumes were scaled up from 1 ml to 5 ml and a portion of the 5 ml volume was used to check $QO_2$ values.

$^{31}$P-spectra were obtained in 5 min blocks using a 30 μs pulse (90°) and 3 s recycle time. At the end of an NMR experiment the suspension was removed from the probe and $QO_2$ measurements were made. Mitochondria were stable for at least 1.5 h, as determined from the $^{31}$P-NMR spectra as well as $QO_2$ measurements made at the end of NMR experiment. Because a 3 s recycle time was not sufficient to allow the nuclei to relax fully, at the end of each experiment a fully relaxed spectrum (30 s recycle time) was obtained along with a 3 s delay spectrum and the ratio between the peak areas obtained from these two spectra were used to correct all prior spectra. Peak areas were determined using the Nicolet integration routine. Phosphate concentrations were quantitated by dividing the total amount of added phosphate by the determined peak areas.

*Incubation conditions.* Three different classes of experiments were performed. In these experiments either the $QO_2$ or the NADH levels were varied. In order to control $QO_2$ an ATPase was added. To vary NADH levels mitochondria were incubated

with different substrates. The incubation conditions for these different experiments were the following.

(1) State-3 measurements: mitochondria were incubated with various substrates (figure captions) in solution B with 5 mM $P_i$ and no ADP (pseudo-State 4). After steady-state fluorescence and $QO_2$ values were obtained, 700 μM ADP was injected into the chamber-initiating State 3. Fluorescence and $QO_2$ were continuously monitored until all the ADP was phosphorylated and a new steady state was reached. Typically two experiments were performed per mitochondrial preparation per substrate and repeated on three different mitochondrial preparations.

(2) Steady-state measurements. To maintain steady-state levels of $QO_2$ between State 4 and State 3 varying amounts of an ATPase [27,28] (potatoe apyrase, Sigma Grade IV) were added to the mitochondrial suspension containing 5 mM $P_i$, 5 mM MgATP, and various substrates. NAD(P)H fluorescence and $QO_2$ were continuously monitored. Apyrase was added from a Stock solution containing 100 units/ml.

(3) Substrate-jump experiments: to monitor the effects of changing substrates on NADH levels and extra-mitochondrial phosphates a transition from one substrate regime to another was made. Mitochondrial NAD(P)H fluorescence, $QO_2$, and NMR measurements of extra-mitochondrial phosphates were made. The incubation medium in these studies consisted of 8.3 mM phosphocreatine, 8.3 mM creatine, 8.3 mM $P_i$, 8.3 mM MgATP in solution B which was modified by decreasing the KCl content so that an osmolarity of 290 + 10 mosm was maintained. In these studies a fixed amount of apyrase (1.5 μl of the stock solution) was added. In addition, creatine kinase (Sigma type 1 from rabbit muscle) was added to allow for the determination of ADP concentrations [28,29]. 7.5 μl of a 200 units/ml stock solution of creatine kinase, or approx. 10 × more creatine kinase activity than apyrase activity was used. After steady-state measurements were obtained with a starting substrate a second substrate was added and the effects on NAD(P)H fluorescence and extra-mitochondrial phosphates were monitored.

Mitochondrial protein was measured on stock suspensions after freeze thawing and dissolving in equal volumes of 1 M NaOH using a protein assay employing Coomassie brilliant blue (Bio-Rad) with -globulin standards. The osmolalities of final incubation solutions (solution B + substrate and phosphates) were measured using a Wescore model 5100 C vapor pressure osmometer and adjusted to 290 ± 10 mosM by varying the KCl content of solution B. All compounds used were obtained from Sigma and used without further purification unless otherwise noted. Results are expressed as the average ± standard error.

## Results

*Quantitative fluorescence*

An advantage of using an array detector to measure fluorescence is that spectral information is rapidly obtained. The maximum of the fluorescence occurs between 440 and 450 nm with a width at half height of 90–95 nm in agreement with earlier reports [21,22]. One of the main purposes of the present study was to compare the effects of variations in mitochondrial NAD(P)H levels on $QO_2$ of isolated mitochondria. To do this it was important to find a suitable reference which would allow quantitative comparison between different protocols. The scattered light from the laser (i.e., 337 nm line) has been used to quantitate in vivo surface fluorescence [30] but was found to be very sensitive to chamber position and substrate regime (pyruvate absorbs 337 nm light). Adding a fixed amount of an exogenous fluorescent probe to standardize each experiment circumvented all of the above problems because alterations in excitation light intensity or positioning of the chamber with respect to the slit would affect both the intrinsic as well as the exogenous fluorescence. Following a suggestion by Kramer and Pearlstein [31], Rhodamine B (2 μM) was added to the mitochondrial suspension. Rhodamine B fluoresces at approx. 586 nm and can be excited by 337 nm light. In addition, changes in membrane potential have been reported not to affect the fluorescence [32]. For most of the substrates used, Rhodamine B had no significant effect on $QO_2$. The one exception was the hexanoyl-carnitine derivate; Rhod B was found to be a potent inhibitor of hexanoyl-carnitine supported $QO_2$. Thus, for most experiments Rhod B was included in the

incubation mixture for the entire time-course of an experiment. However, with the hexanoyl-carnitine derivate, Rhod B was only added at the end of an experiment to calibrate the system.

Besides having no effect on $QO_2$, Rhodamine B, at the levels used, had no effect on the fluorescence intensity of NAD(P)H or the relative fluorescence changes found for a State 4-to-State 3 transition with glutamate + malate as substrates. Similarly, changes in NAD(P)H levels had no effect on Rhodamine B fluorescence as illustrated in Fig. 1 for the pseudo-State 4-to-State 3-to-State 4 transition with mitochondria respiring on 5 mM pyruvate. From these results it was concluded that Rhodamine B was a suitable reference for quantitatively comparing NAD(P)H fluorescence from different mitochondrial suspensions.

The relationship between NAD(P)H fluorescence and mitochondrial $QO_2$ was investigated under two different conditions. The first involved the effect of incubating mitochondria with different substrates on NAD(P)H levels and on $QO_2$ when ADP and $P_i$ were in excess (State 3). The second involved monitoring the variation in NAD(P)H fluorescence and $QO_2$ with increasing rates of extra-mitochondrial ATP hydrolysis caused by addition of apyrase (steady-state measurements). Under this condition it is generally believed that ADP [2,33], ATP/ADP [34], or the phosphorylation potential [35,36] controls oxidative phosphorylation. Finally, the effects of varying substrate in a jump like fashion on NAD(P)H fluorescence, $QO_2$, and extra-mitochondrial phosphates was studied while maintaining the ATPase rate constant (substrate jump measurements).

*State-3 measurements*

To correlate State 3 $QO_2$ with NAD(P)H fluorescence, mitochondria were incubated with a variety of NADH-linked substrates. Simultaneous $QO_2$ and fluorescence recordings were obtained with suspensions undergoing a State 4-to-State 3 transition. Fig. 1 illustrates typical fluorescence spectra obtained for these transitions for mitochondria incubated with 5 mM pyruvate. A drop in fluorescence occurs on transition from State 4 to State 3 due to the stimulation of electron transport by ADP. The fluorescence intensity only partially recovers on return to State 4. Fig. 2

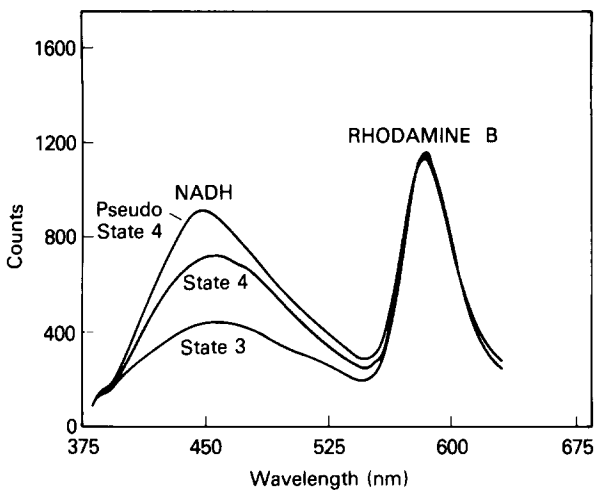


Fig. 1. A fluorescence spectrum obtained from a mitochondrial suspension incubated with 5 mM pyruvate. The two peaks seen are from mitochondrial NAD(P)H (440 nm) and rhodamine B (586 nm, 2 μM). The three spectra correspond to mitochondria respiring in (a) pseudo-State 4, (b) State 3, which was initiated by injection of 700 μM ADP, and (c) State 4. The spectra are overlayed to illustrate that changes in NAD(P)H fluorescence occurred without any change in rhodamine B fluorescence.

shows a time-course of the changes in $QO_2$ and fluorescence levels with mitochondria incubated with 5 mM glutamate + 5 mM malate before and after addition of 700 μM ADP (arrow). It can be readily seen that addition of ADP leads to a new

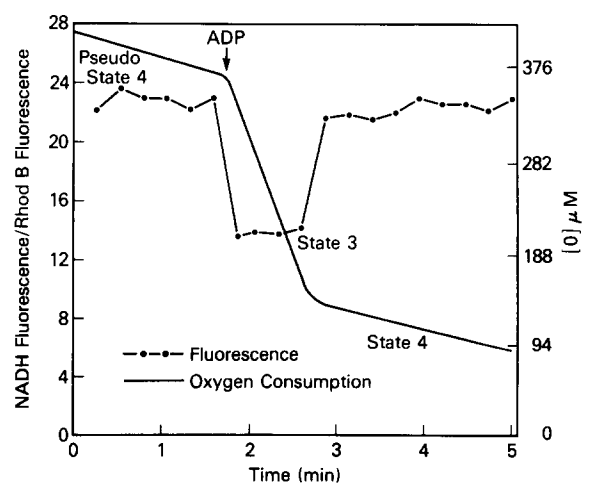


Fig. 2. Simultaneous recordings of $QO_2$ (solid line) and NAD(P)H fluorescence levels (dotted line) from a suspension of mitochondria incubated with 5 mM glutamate + 5 mM malate at 25 °C and pH 7.2. 700 μM ADP was added to the chamber at the arrow. Rhod B, rhodamine B.

steady-state level of fluorescence which recovers after the ADP is phosphorylated to ATP. Using data of this kind the State 3 $QO_2$ was compared to the State-3 level of fluorescence for a series of substrates. Fig. 3 shows a plot of $QO_2$ vs. NAD(P)H fluorescence with the State-3 values of these parameters obtained for 5 mM glutamate + 5 mM malate assigned the value 100%. A steep linear relation with a slope of 2.1 ($r = 0.92$) was found.

*Steady-state measurements*

In these studies the steady-state levels of mitochondrial NAD(P)H and $QO_2$ were compared under conditions where $QO_2$ was driven by ATP hydrolysis. The steady-state $QO_2$ was varied by addition of exogenous apyrase to the suspension. Stimulation of respiration by increasing ADP levels due to added ATPase has been shown to lead to a decrease in mitochondrial NADH levels [2]. Fig. 4 shows an example of the increases in $QO_2$ and decreases in fluorescence intensity measured during sequential additions of apyrase (arrows) to a suspension respiring on 5 mM glutamate + 5 mM malate. Fig. 5 shows a plot of the variation in $QO_2$ and fluorescence as respiration was stimulated with apyrase for four different substrates. There was a linear relation found for changes in $QO_2$ and NAD(P)H fluorescence for each substrate. For any given substrate, higher levels of NAD(P)H were associated with lower $QO_2$ in mitochondria respiring between States 3 and 4 (Fig. 5) than for mitochondria respiring in State 3 (Fig. 3), in the presence of excess ADP. This comparison demonstrates that NAD(P)H levels alone do not control $QO_2$ but there is control by ADP as well [2].

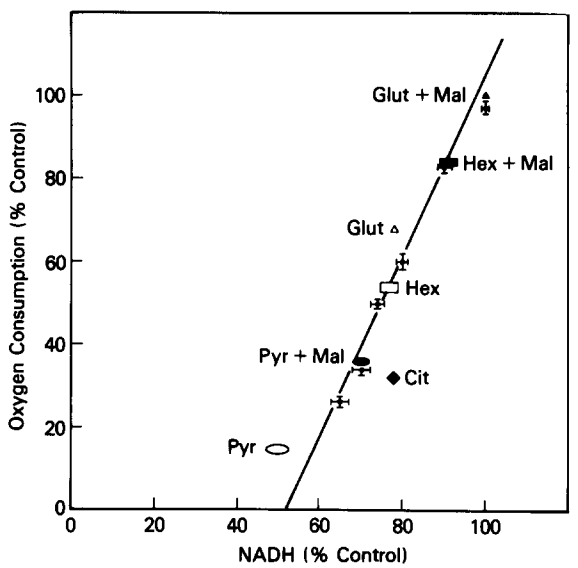


Fig. 3. A plot of $QO_2$ vs. NAD(P)H fluorescence for mitochondria respiring in State 3 with different substrates. 100% values were taken to be the State 3 rates of $QO_2$ and fluorescence measured with mitochondria incubated with 5 mM glutamate (Glut) + 5 mM malate (Mal). Substrates used were: ▲, 5 mM glutamate + 5 mM malate; ■, 0.4 mM D,L-hexanoyl-carnitine (Hex) + 5 mM malate; △, 5 mM glutamate; □, 0.4 mM D,L-Hex; ●, 5 mM pyruvate (Pyr) + 5 mM malate; ◆, 5 mM citrate (Cit); ○, 5 mM pyruvate; and + represents varying amounts of glutamate (0.13, 0.25, 0.5, 1.0, 1.5, 2.5 and 5 mM) + 5 mM malate. The size of each symbol represents ± standard errors. The solid line is the best least squares fit to the expeirmental points and represents a slope of 2.1 ($r = 0.92$).

*Substrate-jump measurements*

An interesting result illustrated in Fig. 5 is that at the same $QO_2$, different levels of NAD(P)H can be achieved with different substrates. For example, at a $QO_2$ of 30%, mitochondria incubated with citrate have a NAD(P)H fluorescence of 80%, while mitochondria incubated with glutamate + malate have an NAD(P)H level of 148%. To de-

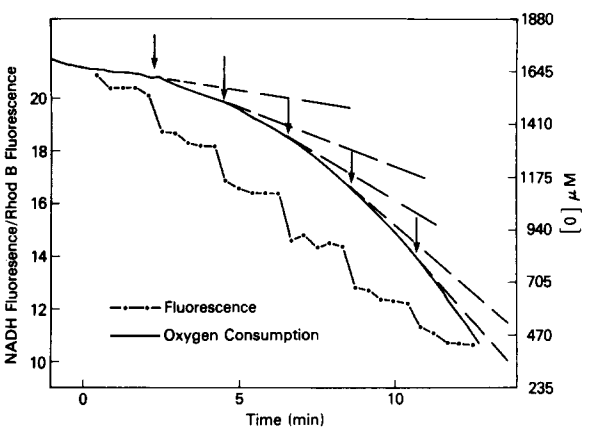


Fig. 4. The effects of increasing additions of apyrase on $QO_2$ (solid line) and NAD(P)H fluorescence levels (dotted line) from a mitochondrial suspension incubated with 5 mM glutamate + 5 mM malate and 5 mM MgATP. The arrows indicate when 3 μl of a 100 units/ml solution of apyrase was added except the last arrow when 5 μl was added. The dashed lines extending from the $QO_2$ curve are to illustrate the linear portions of the line and were used to measure $QO_2$. Rhod B, rhodamine B.

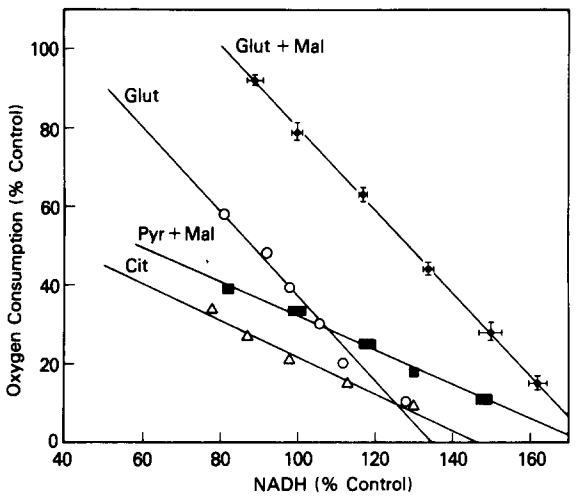


Fig. 5. A plot of $QO_2$ vs. NAD(P)H fluorescence for additions of apyrase to: ●, 5 mM glutamate (Glut) + 5 mM malate (Mal); ○, 5 mM glutamate; ■, 5 mM pyruvate (Pyr) + 5 mM malate; △, 5 mM citrate (Cit). The size of the symbols represent ± standard errors. Apyrase was added in aliquots from a 100 units/ml stock. Solid lines through the points represent best least-squares fits with slopes of −1.1 ($r = 0.99$) for glutamate + malate and glutamate, −0.47 ($r = 0.99$) for citrate, and −0.44 ($r = 0.99$) for pyruvate + malate.

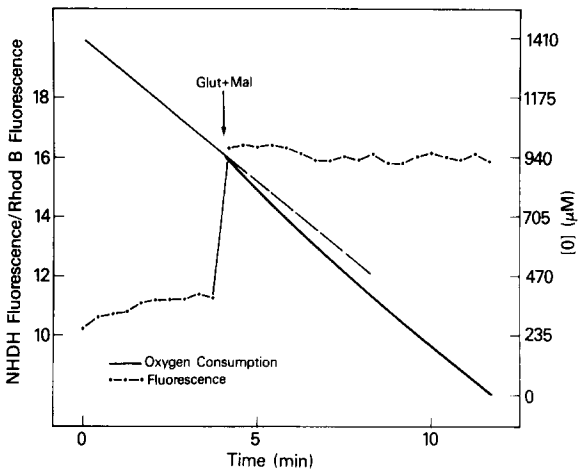


Fig. 6. Simultaneous recordings of $QO_2$ (solid line) and fluorescence (dashed line) for the substrate jump, 5 mM citrate to 5 mM glutamate (Glut) + 5 mM malate (Mal). Mitochondria were incubated at 25 °C (pH 7.2) in the presence of 8.3 mM phosphocreatine, 8.3 mM creatine, 8.3 mM $P_i$, 8.3 mM MgATP, 0.15 units apyrase, and 1.5 units of creatine kinase. The arrow indicates the time when glutamate + malate were added to the suspension. Rhod B, rhodamine B.

termine whether an increase in NAD(P)H at constant $QO_2$ can affect extra-mitochondrial phosphates, jump experiments were performed. Fig. 6 shows the $QO_2$ tracing and fluorescence levels obtained from a suspension of mitochondria respiring on 5 mM citrate with added apyrase to maintain a steady state. In these experiments creatine kinase, phosphocreatine and creatine were added to allow determination of ADP levels. After addition of 5 mM glutamate + 5 mM malate (arrow) there was a rapid increase in fluorescence as predicted by the results illustrated in Fig. 5. Immediately after addition of glutamate + malate there is a transitory stimulation of $QO_2$, which after 5–10 min returns to the rate measured before addition of glutamate + malate. If it is assumed that the external ATPase rate remains constant, a transitory stimulation of $QO_2$ implies an alteration in levels of extra-mitochondrial phosphates if respiration remains coupled to ATP synthesis.

To determine whether extra-mitochondrial phosphates were altered after the substrate jump, $^{31}$P-NMR spectra were obtained on mitochondrial suspensions which duplicated the conditions of Fig. 6. Fig. 7a shows a typical NMR spectrum obtained from a mitochondrial suspension respiring on 5 mM citrate. Because a dilute suspension of mitochondria was used, only extra-mitochondrial phosphates could be detected. Peaks from $P_i$, phosphocreatine and the three phosphates of ATP were detected. The ADP level was too low to be detected in the NMR spectrum. The peak intensities were stable for at least 1.5 h. Addition of 5 mM glutamate + 5 mM malate to the suspension leads to a decrease in the $P_i$ peak and an increase in the phosphocreatine peak (Fig. 7b). This is most clearly seen in the difference spectrum (Fig. 7c). These changes took approx. 10 min to reach the new steady state, in agreement with the time it took for the $QO_2$ to slow down to the control level after the initial stimulation seen upon addition of glutamate + malate (Fig. 6).

Using the NMR-derived concentrations of phosphocreatine and ATP (Table I), knowledge of the total phosphocreatine + creatine added to the solution, and the appropriate equilibrium constant for the reaction catalysed by creatine kinase (see footnote to Table I), the levels of ADP were calculated [28,29]. Data summarizing the effects of addition of glutamate + malate to citrate are

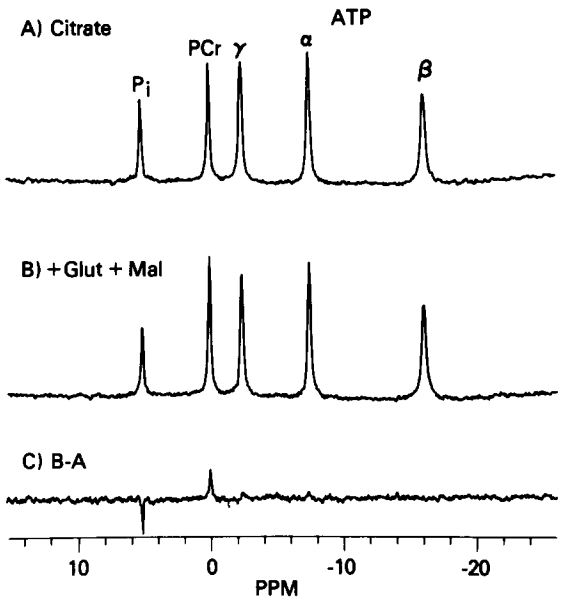


Fig. 7. $^{31}$P-NMR spectra obtained from a mitochondrial suspension. Conditions were as in Fig. 6. (a) spectrum from mitochondria respiring on citrate, (b) spectrum from mitochondria at steady state after addition of 5 mM glutamate (Glut) + 5 mM malate (Mal), (c) the difference; (b) − (a). Spectra were acquired in blocks of 100 scans using a 30 μs 90° pulse and 3 s delay. Each spectrum is the addition of five blocks or 500 scans. A 10 Hz exponential filter was applied prior to Fourier transformation. Peaks are as labeled. PCr, phosphocreatine.

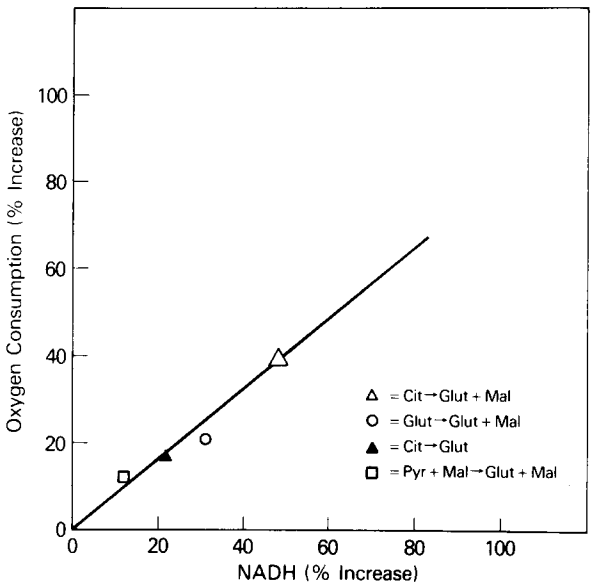


Fig. 8. A plot of the per cent increase in $QO_2$ vs. per cent increase in NAD(P)H fluorescence immediately following a substrate jump. Conditions were as in Fig. 6, except each point represents a different substrate jump with: □, 5 mM pyruvate (Pyr) + 5 mM malate (Mal) to 5 mM glutamate (Glut) + 5 mM malate; ▲, 5 mM citrate (Cit) to 5 mM glutamate; O, 5 mM glutamate to 5 mM glutamate + 5 mM malate; Δ, 5 mM citrate to 5 mM glutamate + 5 mM malate. The size of the symbol represents ± standard errors.

presented in Table I along with similar data obtained for the jump, 5 mM pyruvate + 5 mM malate to 5 mM glutamate + 5 mM malate + 5 mM pyruvate. Even though $QO_2$ remains constant in the steady state for the transition from citrate to glutamate + malate, a 50% increase in NAD(P)H is accompanied by a 41% decrease in ADP, a 30% decrease in $P_i$, and a 20% increase in phosphocreatine. For the jump pyruvate + malate to glutamate + malate + pyruvate a smaller in-

TABLE I

EFFECTS OF SUBSTRATE JUMPS ON $QO_2$, EXTRA-MITOCHONDRIAL PHOSPHATES AND NADH FLUORESCENCE

| Substrate | $QO_2$ [a] (%) | $P_i$ (mM) | PCr (mM) | ATP (mM) | Cr (mM) | ADP [b] (μM) | NADH [a] (%) |
|---|---|---|---|---|---|---|---|
| Citrate ($n = 6$) | 30 ± 3 | 6.6 ± 0.2 | 9.8 ± 0.4 | 8.5 ± 0.2 | 6.8 ± 0.4 | 44 ± 4.5 | 86 ± 0.6 |
| Glut + Mal | 31 ± 4 | 4.6 ± 0.4 | 11.8 ± 0.4 | 8.4 ± 0.1 | 4.8 ± 0.4 | 26 ± 3.3 | 129 ± 2.0 |
| Difference [c] | – | −2.0 ± 0.2 | +2.0 ± 0.2 | – | −2.0 ± 0.2 | −18 ± 2.0 | +43 ± 2.0 |
| Pyr + Mal ($n = 5$) | 28 ± 1 | 7.7 ± 0.3 | 8.7 ± 0.3 | 8.5 ± 0.1 | 7.9 ± 0.3 | 58 ± 3.7 | 106 ± 2.0 |
| Glut + Mal | 28 ± 1 | 6.8 ± 0.3 | 9.6 ± 0.2 | 8.5 ± 0.1 | 7.0 ± 0.2 | 46 ± 2.5 | 120 ± 2.9 |
| Difference [c] – | – | −0.9 ± 0.05 | +0.09 ± 0.05 | – | −0.9 ± 0.05 | −12 ± 1.2 | +14 ± 1.8 |

[a] The state 3 values for mitochondria respiring on 5 mM Glut + 5 mM Mal were taken as 100%.

[b] Calculated using the expression $[ADP] = [ATP][Cr]/[PCr]K_{eq}$, where $K_{eq} = 135$ at pH 7.2 and 2.5 mM free $Mg^{2+}$ [28].

[c] Differences were taken from paired determinations.

crease in NAD(P)H fluorescence (13%) leads to a 21% decrease in ADP, a 12% decrease in $P_i$, and an 11% increase in phosphocreatine.

The previous data demonstrate that at a fixed $QO_2$, alterations in substrate associated with increases in NAD(P)H levels can lower ADP levels. As a measure of the effect that changes in substrates can have on $QO_2$ with the extra-mitochondrial ADP constant and at physiologic levels, the initial rate of $QO_2$ was measured immediately following a substrate jump. Because it takes approx. 10 min for the phosphates to reach a new steady-state in the large extra-mitochondrial space, the initial rate (first 60 s) of change in $QO_2$ can be assumed to occur at a constant level of extra-mitochondrial phosphates. Fig. 8 shows that an increase in NAD(P)H fluorescence is associated with a proportional increase in $QO_2$. This relation was linear with a slope of 0.81 ($r = 0.96$).

## Discussion

The prevalent models used to explain increased oxidative ATP production when a cell is stimulated have concentrated on the role of cytoplasmic phosphates such as ADP, $P_i$, and ATP [1–3]. Consequently, most work done with isolated mitochondria has been concerned with the effects of variations in extra-mitochondrial phosphates on $QO_2$ [33]. In general, these studies have been performed with a single substrate regime, such as glutamate + malate. However, metabolism of substrates by mitochondria in vivo is a complex affair and potentially an important regulatory site of oxidative phosphorylation. The effects of variations in NADH levels on $QO_2$ and extra-mitochondrial phosphates may be an important aspect of metabolic control when a tissue is stimulated.

It has been appreciated, since early studies with isolated mitochondria that substrates were needed to supply NADH to the electron-transport chain and that different substrates were able to support different rates of respiration [1,2,37–39]. Here we varied the amount of NAD(P)H in isolated liver mitochondria by utilization of different substrates. We have assumed that changes in substrate exert the observed effects on $QO_2$ and extra-mitochondrial phosphates due to their relative abilities to generate NADH alone. A linear relation between $QO_2$ and the NADH fluorescent signal obtained under conditions where neither ADP nor $P_i$, nor $O_2$ were rate limiting was found. These results are consistent with the notion that a production or delivery of NADH is rate limiting to oxidative phosphorylation under State 3 conditions. However, due to the nature of the NADH fluorescent signal from mitochondria, we suspect that the most significant NADH pool in this process is the bound NADH pool which has an enhanced fluorescence [23]. Indeed this bound NADH pool may represent a significant proportion of the NADH pool directly involved in the delivery of reducing equivalents to the cytochrome chain.

It is interesting that the linear relation found here between $QO_2$ and NAD(P)H fluorescence in State-3 mitochondria shows no sign of saturation over the range observed. The level of fluorescence obtained with 5 mM glutamate + 5 mM malate in State 3 is 60% of the maximal obtainable fluorescence achieved under anoxia or in the presence of cyanide. However, no substrate tested gave higher State 3 levels of fluorescence than glutamate + malate, thus, it is possible that at higher levels of State-3 fluorescence the linear correlation found will not hold.

As a measure of how much increases in mitochondrial NAD(P)H assoicated with changes in substrate can stimulate $QO_2$ at constant extra-mitochondrial phosphates, the initial change in $QO_2$ was measured immediately following a substrate jump. This was done at respiration rates of 30% maximal State-3 rates where extra-mitochondrial phosphates exert substantial control over respiration. A linear relation between per cent change in $QO_2$ and per cent change in NAD(P)H fluorescence was found (Fig. 8). The per cent change in $QO_2$ for a given change in NAD(P)H (slope = 0.8) was smaller under these conditions than found for State-3 mitochondria (slope = 2.1). This is most likely due to the sharing of control of oxidative phosphorylation by both the extra-mitochondrial phosphates and NADH levels in the region between State 4 and State 3. These studies bear directly on the extent of stimulation of $QO_2$ which can be expected if delivery of NADH is increased in an intact tissue at constant

levels of extra-mitochondrial phosphates.

That NADH levels are important in the control of $QO_2$ over the dynamic control region is best illustrated by the fact that at a fixed rate of $QO_2$ an increase in NAD(P)H fluorescence, due to a substrate jump, is associated with a substantial decrease in ADP as well as $P_i$ (Table I). This clearly shows that the magnitude of $QO_2$, even in the physiological zone between State 3 and 4, depends not only on extra-mitochondrial phosphates but on the NAD(P)H level different substrates can maintain as well.

Based on inhibitor titration studies it has been proposed that there is sharing of control among different enzymatic steps of oxidative phosphorylation [40]. The results presented here are consistent with these inhibitor titration studies where a portion of control of respiration was attributed to supply of NADH between States 3 and 4 [40]. Our results are also consistent with near equilibrium, thermodynamic models of oxidative phosphorylation [14–17,35,36]. A competing theory has been that the mitochondrial ATP/ADP translocase exerts kinetic control over respiration between States 4 and 3 [41]. The only way to reconcile the present data with this theory is to lead changes in NADH to changes in membrane potential which affect the kinetics of the translocase [42].

In summary, alterations in substrates which lead to increases in NAD(P)H fluorescence levels in isolated rat-liver mitochondria are associated with increases in $QO_2$ and alterations in extra-mitochondrial levels of phosphates. The alterations in NAD(P)H can exert effects on $QO_2$ over a wide range of respiratory rates. These results establish the connection between increases in reduced pyridine nucleotides and stimulation of $QO_2$, making it possible that it is increased in mitochondrial NADH which stimulate respiration in vivo when cellular work is increased [10].

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