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# Role of intramitochondrial pH in the energetics and regulation of mitochondrial oxidative phosphorylation

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**The dependence of ATP synthesis coupled to electron transfer from 3-hydroxy-butyrate (3-OH-B) to cytochrome *c* on the intramitochondrial pH ($pH_i$) was investigated. Suspensions of isolated rat liver mitochondria were incubated at constant extramitochondrial pH ($pH_e$) with ATP, ADP, $P_i$, 3-OH-B, and acetoacetate (acac) (the last two were varied to maintain [3-OH-B]/[acac] constant), with or without sodium propionate to change the intramitochondrial pH. Measurements were made of the steady-state water volume of the mitchondrial matrix, transmembrane pH difference, level of cytochrome *c* reduction, concentration of metabolites and rate of oxygen consumption. For each experiment, conditions were used for which transmembrane pH was near maximal and minimal values and the measured extramitochondrial [ATP], [ADP], and [$P_i$] were used to calculate log[ATP]/[ADP][$P_i$]. When [3-OH-B]/[acac] and [cyt $c^{2+}$]/[cyt $c^{3+}$] were constant, and $pH_i$ was decreased from approx. 7.7 to 7.2, log [ATP]/[ADP][$P_i$] at high $pH_i$ was significantly ($P < 0.02$) greater than at low $pH_i$. The mean slope ($\Delta$ log [ATP]/[ADP][$P_i$] divided by the change in $pH_i$) was $1.08 \pm 0.15$ (mean $\pm$ S.E.). This agrees with the slope of 1.0 predicted if the energy available for ATP synthesis is dependent upon the pH at which 3-hydroxybutyrate dehydrogenase operates, that is, on the pH of the matrix space. The steady-state respiratory rate and reduction of cytochrome *c* were measured at different $pH_i$ and $pH_e$ values. Plots of respiratory rate vs.% cytochrome *c* reduction at different intra- and extramitochondrial pH values indicated that the respiratory rate is dependent upon $pH_i$ and not on $pH_e$. This implies that the matrix space is the source of protons involved in the reduction of oxygen to water in coupled mitochondria.**

## Introduction

The electron transport and energy coupling reactions associated with the mitochondrial inner membrane involve many reactions. Relevant enzyme complexes are generally integral parts of the membrane; some extend fully across the permeability barrier and therefore contact both aqueous phases. Most of the reaction mechanisms examined to date, however, have been found to be highly selective, involving reactants from the aqueous phase on only one of the two sides of the membrane. Because the energetics and kinetics of many of the reactions are dependent upon the activities of such reactants, it is necessary to determine experimentally from which aqueous compartment a component is taken and/or into which compartment it is released. Protons, in particular, have been proposed to have a major role as both reactants and regulators in many of the reactions of oxidative phosphorylation. The studies presented in this report address questions concerning $H^+$ uptake and release by 3-hydroxybutyrate dehydrogenase and cytochrome *c* oxidase in isolated rat liver mitchondria.

In the study of the energy relationships of electron transfer between NADH and cytochrome *c* and ATP synthesis, the 3-hydroxybutyrate (3-OH-B) couple can serve as both a source of reducing equivalents and as an indicator of the oxidation-reduction state of the intramitochondrial $NAD^+$/NADH couple [1]. The half-reduction potential of the 3-OH-B/acetoacetate (acac) couple is pH-dependent, becoming 60 mV more positive for each pH unit more acid the environment [2]. As the dehydrogenation reaction occurs within the mitochondrial matrix (approx. 0.8 pH units more alkaline than

Abbreviation: 3-OH-B, 3-hydroxybutyrate.

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the suspending medium in isolated mitochondria), calculation of the energetics of oxidative phosphorylation from 3-OH-B have been based upon the intramitochondrial pH [3–5]. Several authors have alternatively suggested that these calculations should be based upon the extramitochondrial pH [6–8], because of an assumed coupling of the oxidation with proton extrusion to the cytoplasmic space [9]. Proper assignment of the pH dependence is an important issue in determination of the correct ATP/$2e^-$ stoichiometry, as the same experimental data based upon $pH_e$ would imply a stoichiometry of less than two ATP synthesized per two electron transferred, compared with the value of 2.0 when the intramitochondrial pH is used [4,5]. One question addressed in this paper is whether the energy available for ATP synthesis between 3-OH-B and cytochrome *c* is dependent upon the intramitochondrial or extramitochondrial pH.

Reduction of molecular oxygen to water at the third energy conservation site (cytochrome *c* oxidase) consumes four protons per dioxygen molecule reduced. The rate of this reaction is strongly pH-dependent, with an approximately second-order dependence upon the $[H^+]$ of the suspending medium [10–12]. When the pH of the suspending medium is experimentally varied, however, the intramitochondrial bulk-phase pH shifts in a similar manner (i.e., a relative constant transmembrane pH difference is maintained); therefore, it has not been determined whether the reaction was responding to changes in intra- or extramitochondrial pH. Evidence has been provided that protons are taken upon from the matrix space (see for examples Refs. 9, 13–19) and may be released into the extramitochondrial space [19].

With the availability of methods to experimentally vary $\Delta$pH across the inner membrane, and hence of $pH_i$ independently of $pH_e$, it is possible to investigate these two questions. The approach used in the first set of experiments has been to quantitate the $[ATP]/[ADP][P_i]$ synthesized by mitochondria oxidizing 3-OH-B under conditions for which the $pH_e$ was held constant and $pH_i$ was experimentally varied. In the second set of investigations, the relationship between the respiratory rate and cytochrome *c* reduction has been assessed as a function of intra- and extramitochondrial pH. In both sets of experiments, evidence has been obtained that the reactions are dependent upon the intramitochondrial pH. A preliminary report of this work was presented in a symposium honoring Professor Tsoo E. King [20].

## Materials and Methods

Mitochondria were isolated from livers of male Sprague-Dawley rats in a medium containing 0.225 M mannitol, 0.075 M sucrose, and 0.4 mM EGTA, and were suspended to a concentration of 40–50 mg protein/ml in the same medium. Assays were carried out by diluting this suspension in either high ionic (120 mM choline chloride) or low ionic (0.2 M mannitol, 0.07 M sucrose) media, each containing 15 mM Mops and 0.4 mM EGTA, buffered to the pH indicated for each set of experiments. The media were bubbled with oxygen to an approximate concentration of 800 $\mu$M prior to addition of the mitochondria. Intramitochondrial pH was varied by addition of sodium propionate to the incubation media, or in the first set of experiments, by varying [3-OH-B] and [acac].

For steady-state metabolite samples, aliquots of assay suspension were quenched by rapidly mixing with an equal volume of ice-cold 4% perchloric acid, and the deproteinized supernatant fractions were neutralized to pH 6 with 0.04 vol. of 2 M $K_2CO_3$/0.57 M triethanolamine. 3-OH-B and acac were measured according to the methods of Williamson and Mellanby [21] and Mellanby and Williamson [22], respectively. ATP and ADP were determined by the enzymatic methods of Lamprecht and Trautschold [23], and inorganic phosphate was determined by the malachite green method [24]. Dependence upon the concentration of external adenine nucleotides has been shown [3,11]; therefore, the contributions of intramitochondrial and bound adenine nucleotides were subtracted from the total concentrations measured in the deproteinized supernatant fractions. Measurements of ATP and ADP from incubations without added adenine nucleotides were considered to be representative of endogenous (intramitochondrial + bound) values, and were subtracted from total measurements. This correction was in the range of 2 nmol ADP and 4 nmol ATP per mg mitochondrial protein.

For each set of experimental conditions, measurements were made of mitochondrial matrix volume and transmembrane pH difference ($\Delta$pH). The intra- and extramitochondrial ('trapped') volumes were calculated from the total contents of $^3H_2O$ and [$^{14}$C]sucrose, assuming sucrose to be impermeable to the inner membrane of the mitochondria. The difference between extramitochondrial and intramitochondrial pH was determined from the distribution of [$^{14}$C]- or [$^3$H]acetate or [$^{14}$C]5,5-dimethyloxazolidine-2,4-dione, and the transmembrane electrical difference was calculated according to the Nernst potential from the distribution of [$^3$H]triphenylmethylphosphonium after a correction for binding [25]. Samples were processed as follows (see Ref. 25 for detail). Duplicate aliquots (400 $\mu$l) of mitochondrial suspension were transferred to 400 $\mu$l centrifuge tubes containing approx. 20 $\mu$l silicone oil (specific gravities of 1.03 and 1.07 for the high ionic and low ionic media, respectively; General Electric) and centrifuged for 90 s in a Beckman Model B microfuge. Supernatant and pellet fractions were separately dissolved in aqueous scintillation fluid and counted in a Searle Delta 300 scintillation counter.

Oxygen consumption and reduction of cytochrome *c* were monitored in a specially constructed glass spectrophotometer cuvette in which a Clark-type oxygen electrode was mounted. The oxidation-reduction state of cytochrome *c* was measured by the absorbance at 550 minus 540 nm. A correction of 3% in the total absorbance change was made for the contribution of cytochrome *b* reduction at these wavelengths. This contribution was determined separately by addition of antimycin A and substrate to mitochondria in which the *b* cytochromes were fully oxidized by aerobic incubation with FCCP. Cytochrome *c* was considered to be fully reduced following anaerobiosis in the presence of FCCP plus substrate or after addition of dithionite.

Radiochemicals were obtained from New England Nuclear, Boston, MA. Hexokinase (for ATP assays) and reagents for $P_i$ determinations were purchased from Sigma, St. Louis, MO, and remaining enzymes for metabolite assays from Boehringer-Mannheim, Indianapolis, IN.

## Results

*Coupling of ATP synthesis and electron transport across the first two sites of ATP synthesis*

The relationship between the energy available from electron transfer from NADH to cytochrome *c* and maximal steady-state ATP synthesis was measured in order to verify the integrity of the mitochondrial preparations. Mitochondria were suspended in oxygen-enriched ionic medium (pH 7.0), incubated with ADP and $P_i$ in order to deplete endogenous substrates, and then supplemented with ATP, 3-OH-B, and acac. Oxygen consumption increased with addition of oxidizable substrate, and returned to minimal rates after approx. 1 min. Two min further incubation was allowed for equilibration of the 3-OH-B/acac and $NAD^+$/NADH couples, after which samples were quenched and processed as described in Materials and Methods.

The approach to equilibration of electron transport between 3-OH-B and cytochrome *c* and ATP synthesis can be quantitated from the measured concentration ratios of the reactants and products. The reaction is:

$$\text{3-OH-B} + 2\ \text{cyt}\ c^{3+} + 2\ \text{ADP} + 2\ P_i \rightleftharpoons 2\ \text{ATP} + \text{acac} + 2\ \text{cyt}\ c^{2+} \quad (1)$$

where the ADP, ATP and $P_i$ are in the extramitochondrial space. The mass action ratio for this reaction can be calculated from the measured concentration of reactions by the formula:

$$K_{MA} = \left(\frac{[\text{ATP}]_e}{[\text{ADP}]_e[\text{Pi}]_e}\right)^2 \left(\frac{[\text{acac}]}{[\text{3-OH-B}]}\right)\left(\frac{[\text{cyt}\ c^{2+}]}{[\text{cyt}\ c^{3+}]}\right)^2 \quad (2)$$

The subscript e is used to indicate the extramitochondrial compartment. Steady-state measurements from several experiments in which the starting substrate concentrations were varied are presented below:

| $pH_i$ | log $K_{eq}$ | $\frac{[ATP]}{[ADP][P_i]}$ | $\frac{[acac]}{[3\text{-}OH\text{-}B]}$ | $\frac{\%\ cyt\ c^{2+}}{\%\ cyt\ c^{3+}}$ | log $K_{MA}$ |
|---|---|---|---|---|---|
| 7.52 | 6.46 | $\frac{(1.48)}{(0.028)(1.70)}$ | $\frac{0.11}{0.31}$ | $\frac{8.5}{91.5}$ | 6.47 |
| 7.57 | 6.53 | $\frac{(1.29)}{(0.043)(1.07)}$ | $\frac{0.26}{0.85}$ | $\frac{9.8}{90.2}$ | 6.45 |
| 7.37 | 6.12 | $\frac{(1.25)}{(0.061)(1.20)}$ | $\frac{1.20}{4.45}$ | $\frac{11.2}{88.8}$ | 6.10 |

All concentrations are in mM, and $pH_i$ was calculated as $pH_e$ plus the measured transmembrane pH difference. Since the extramitochondrial pH was held constant, changes in $pH_i$ are equivalent to changes in $\Delta$pH. The near equality of the equilibrium constant ($K_{eq}$), calculated according to Ref. 4, and the mass action ratio ($K_{MA}$) is consistent with previous reports [3,4].

*Effects of varying intramitochondrial pH on the maximal steady-state level of synthesis of ATP by mitochondria oxidizing 3-OH-B*

When isolated rat liver mitochondria are incubated with increasing concentrations of some weak acids, the measured intramitochondrial pH becomes more acidic, approaching $pH_e$ [25–27]. In the present experiments, $pH_i$ was decreased by additions of sodium propionate or by increasing the concentrations of 3-OH-B and acac while maintaining the concentration ratio of the latter constant. Using this method and a $pH_e$ of 7.0, $pH_i$ could be decreased from about 7.7 to about 7.2 (i.e., $\Delta$pH decreased from 0.7 to 0.2, alkaline inside). For each experiment, samples were taken under conditions selected for both high and low values of $pH_i$. Each experimental point consisted of the mean of four trials $\pm$S.E. The transmembrane electrical gradient was approx. $-120$ mV for all conditions, in good agreement with previously determined values [4,5,25], and the mitochondrial matrix volumes remained unchanged with changes in $pH_i$.

The concentrations of ATP and $P_i$ are much higher (approx. 1.5 mM and 1.1 mM, respectively) than that of ADP ($\mu$M). Thus, any change in the [ATP]/[ADP][$P_i$] must be due primarily to changes in [ADP]. In experiments in which the intramitochondrial pH was decreased by increasing concentrations of weak acids, [ADP] increased an average of 37% for each 0.1 pH unit decrease in $pH_i$. This increase in [ADP] indicates a decrease in the steady-state phosphorylation state ratio attained by the mitochondria; thus, the ability to synthesize ATP decreased with decreasing $pH_i$. The possibility that this increase in [ADP] was due to an increased degree of uncoupling at lower $pH_i$ was excluded (see ahead). In every experiment, the value for log [ATP]/[ADP][$P_i$] was significantly lower at the lower

value of $pH_i$ ($P < 0.02$; paired $t$-test). Data from seven experiments are plotted in Fig. 1A (scale on left-hand side) as log [ATP]/[ADP][$P_i$] vs. $pH_i$. The average value of $\Delta$log [ATP]/[ADP][$P_i$] divided by the difference in $pH_i$ ($\Delta pH_i$) was $1.08 \pm 0.15$ (mean $\pm$ S.E.).

*Free energy changes*

The data can also be plotted as the free energy of hydrolysis of ATP in the extramitochondrial space ($\Delta G_{ATPe} = \Delta G^{0\prime} + RT \ln$ [ATP]/[ADP][$P_i$] vs. $pH_i$ (Fig. 1A, scale on right-hand side). The straight line with the least-squares best fit to the data extrapolated to the $y$-axis, where its intercept was 13.42 kcal/mol. The dotted line represents the linear relationship predicted from calculation of the free energy of electron transport between 3-OH-B and cytochrome $c$ using the intramitochondrial pH. The value for the $y$-intercept of the theoretical curve, 13.34 kcal/mol, was calculated from the equilibrium constant of $2.58 \cdot 10^5$ $M^{-1}$ for the free energy of hydrolysis of ATP at pH 7.0 [28–30] and measured values of substrate ratios and cytochrome $c$ reduction from 17 determinations, assuming an ATP/$2e^-$ stoichiometry of 2.0 for the first two sites. The experimental values agree, within error, with the values of $\Delta G_{ATPe}$ predicted by these calculations.

There were systematic variations in the phosphorylation state ratio from one experiment to the next due to experimental variations in the final [3-OH-B]/[acac] and possibly to differences in the properties of the mitochondria. In order to visualize the relationship between [ATP]/[ADP][$P_i$] and $pH_i$, independent of experiment-to-experiment variability, the data were normalized and replotted in Fig. 1B. Normalization involved calculation of the average value of log [ATP]/[ADP][$P_i$] and of $pH_i$ for each experiment. The correction required to have the average value of log [ATP]/[ADP][$P_i$] coincide with the theoretical curve at a value equal to the average $pH_i$ was added or subtracted from the data at both high and low $pH_i$. This normalization factor for log [ATP]/[ADP][$P_i$] had a mean value of $0.14 \pm 0.04$ (mean $\pm$ S.E. for seven experiments). Linear regression analysis of the replotted data yielded a best fit line with a slope of 0.97 and a correlation coefficient of 0.96. This, and the aforementioned analysis of the unnormalized data, indicate a good fit to the pH-dependence predicted by the theoretical curve.

*Analysis of the energetics of oxidative phosphorylation for the first two sites as a function of $pH_i$*

The pH dependence of the overall reaction of oxidative phosphorylation for the first two sites is also readily observed by plotting the log of the mass action ratio, $K_{MA}$, against $pH_i$ when $pH_e$ is constant. For the purposes of this analysis, we have assumed an ATP/$2e^-$ stoichiometry of 2.0 for the first two sites [4,5]. There

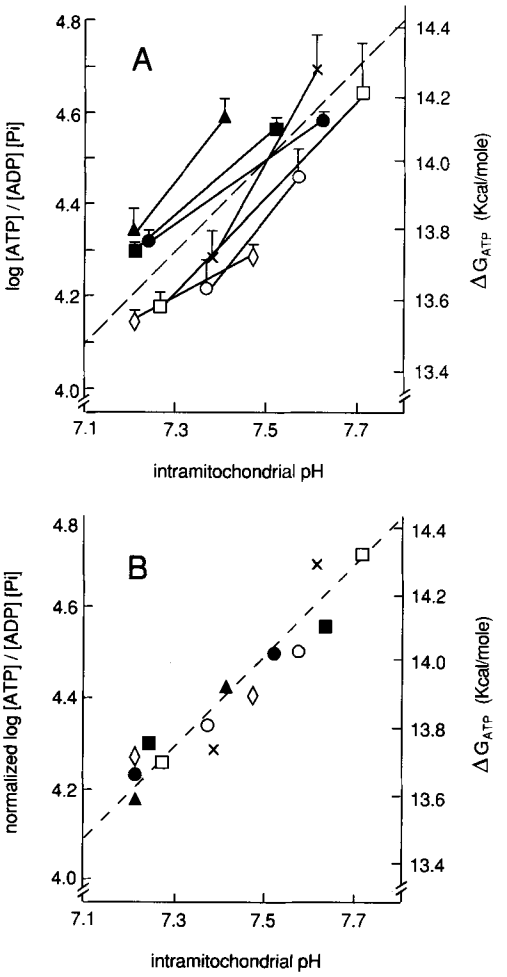


Fig. 1. Effect of decreasing $pH_i$ on the maximal steady-state extramitochondrial [ATP]/[ADP][$P_i$] and $\Delta G_{ATP}$. Concentrated suspensions of rat liver mitochondria were diluted in 120 mM choline chloride, 15 mM Mops, 0.4 mM EGTA (pH 7.0) to a final protein concentration of approx. 4 mg/ml. Intramitochondrial pH was varied by addition of sodium propionate or increasing [3-OH-B + acac] (keeping the final ratio constant). Experiments were carried out as described in the text. Measurements were made of the steady state $\Delta$pH, ATP, ADP, $P_i$, 3-OH-B, acac, oxygen consumption and cytochrome $c$ reduction. (A) Final values of [ATP]/[ADP][$P_i$] (mean of four trials $\pm$ S.E.) for high and low values of $pH_i$ from seven experiments were corrected for endogenous concentrations of adenine nucleotides and plotted as either the log [ATP]/[ADP][$P_i$] (scale on left side) or the free energy of hydrolysis of ATP (scale on right side) against the measured $pH_i$. Individual experiments are represented by different symbols. The mean value of the difference in log [ATP]/[ADP][$P_i$] divided by $pH_i$ is $1.08 \pm 0.15$ (S.E.). (B) The data from (A) were normalized according to the procedure described in the text. The dotted line represents the theoretically predicted relationship between $pH_i$ and log [ATP]/[ADP][$P_i$] for the case in which ATP synthesis between 3-OH-B and cytochrome $c$ is dependent upon the intramitochondrial pH. They $y$-intercept of $4.00 \pm 0.02$ was calculated from the equilibrium constant of electron transport to cytochrome $c$ at pH 7.0 ($2.58 \cdot 10^5$ $M^{-1}$; see Ref. 28) and the measured cytochrome $c$ reduction state and the average [3-OH-B]/[acac] values from 17 determinations. A straight line fitted to the data by the least-squares method has a slope of 0.97 and a correlation coefficient of 0.96.

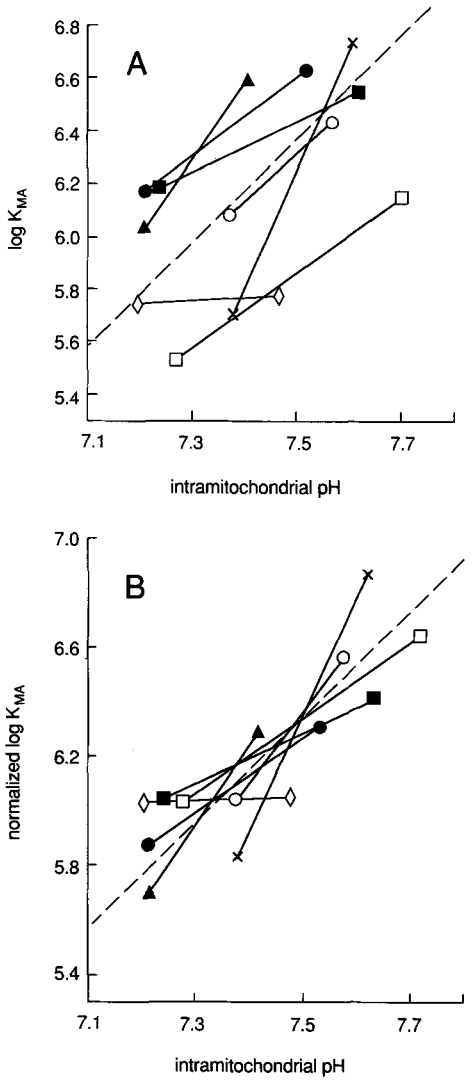


Fig. 2. Effect of decreasing $pH_i$ on the energetics of oxidative phosphorylation for the first two sites. (A) The log of the calculated mass action ratios ($K_{MA}$) for each of the reported experiments was plotted against the measured intramitochondrial pH, using the same symbols for individual experiments as in Fig. 1. The dotted line represents the theoretical value for dependence of the energetics of oxidative phosphorylation for the first two sites on the intramitochondrial pH, assuming an ATP/$2e^-$ stoichiometry of 2.0 (B) The log $K_{MA}$ were normalized by the same procedure as used in Fig. 1B and replotted.

was a small increase in cytochrome *c* reduction associated with addition of propionate or increased substrate concentration (from $7.8 \pm 1.7\%$ reduced to $9.2 \pm 1.1\%$, mean $\pm$ S.D., statistically significant at the $P < 0.05$ level), concomitant with a 10% to 20% increase in the respiratory rate. Final concentrations of acetoacetate and 3-OH-B ranged from 0.10 to 1.28 mM and 0.25 to 4.06 mM, respectively, but the ratio of the two remained relatively constant at $0.29 \pm 0.03$ (S.E.) for 14 trials.

Mass action ratios were compared with the respective intramitochondrial pH for each experiment (Fig. 2A). The average difference in the log of the mass action ratio divided by the difference in $pH_i$ for high and low values of the latter was $1.84 \pm 0.5$ for seven experiments. This is consistent with the theoretically predicted value of 2.0 if oxidative phosphorylation were to be dependent upon the intramitochondrial pH and substantially different from the value of 0 if it were dependent upon $pH_e$.

In order to better visualize the relationship between the mass action ratio and $pH_i$ for each experiment, data were normalized as described for log [ATP]/[ADP][$P_i$] and replotted in Fig. 2B. The theoretically predicted line has a *y*-intercept which corresponds to the equilibrium constant for the overall reaction at $pH_e$ (see Ref. 4).

*Dependence of the relationship between cytochrome c reduction and respiratory rate on intra- and extramitochondrial pH*

Rat liver mitochondria were suspended to approx. 4 mg/ml protein in low ionic medium (see Materials and Methods). Rotenone (1 nmol/mg protein) was added in order to prevent oxidation of NADH-linked endogenous substrates, and then the mitochondria were supplemented with 8 mM ascorbate, followed by 3 mM ATP. Continuous measurements were made of oxygen consumption rate and cytochrome *c* reduction state with the oxygen concentration always above 400 $\mu$M. Additions of TMPD (a mediator or electron flow between ascorbate and cytochrome *c*) from 10 $\mu$M to 200 $\mu$M, either sequentially or in independent trials, caused cytochrome *c* to become increasingly reduced, concomitant with an increase in respiratory rate. When oxidative phosphorylation was uncoupled from electron transport by adding 0.7 $\mu$M FCCP, the plot of cytochrome *c* reduction vs. respiratory rate (Fig. 3) was linear and independent of pH of the suspending medium or presence of ATP. However, when suspensions of coupled mitochondria at high [ATP]/[ADP][$P_i$] were used, the respiratory rate was inhibited by increasing alkalinity, and the degree of inhibition was greater when cytochrome *c* was more oxidized (see also Ref. 10).

The relationship between cytochrome *c* reduction and respiratory rate at high [ATP]/[ADP][$P_i$] (approx. $10^5$ $M^{-1}$) was examined as a function of intra- and extramitochondrial pH. Plots of cytochrome *c* reduction vs. oxygen consumption for $pH_e$ values of 7.0 and 7.6 (Fig. 3) were nonlinear and markedly pH-dependent: for each value of % cytochrome $c^{2+}$, the respiratory rate was greater for more acid $pH_e$ values.

Mitochondrial matrix water spaces and $\Delta$pH were measured for each set of conditions in separate but parallel assays. The $\Delta$pH values were $0.7 \pm 0.1$ and $0.6 \pm 0.1$ (alkaline inside) for $pH_e$ of 7.0 and 7.6, respectively, equivalent to mean intramitochondrial bulk phase pH values ($n = 3$) of 7.7 and 8.2. Transmembrane electrical potentials remained approximately constant at 120 mV (negative inside).

In order to determine whether the reaction was dependent upon the intra- or extramitochondrial pH, similar titrations were then carried out in which 10–20 mM sodium propionate was added to the medium to decrease $pH_i$ (Fig. 3). There was no change in the mitochondrial matrix water volume with these additions, but $\Delta pH$ decreased to $0.3 \pm 0.1$ pH units for mitochondria suspended at either pH 7.0 or 7.6, resulting in mean intramitochondrial bulk phase pH values of 7.3 and 7.9, respectively. Measurements of cytochrome *c* reduction and respiratory rate demonstrated an increased respiratory rate for each level of reduction of cytochrome *c*. This increase in respiratory rate occurred whether propionate was added before the first addition of TMPD or at any point in the titration.

Measurements of the metabolites in perchloric acid-quenched aliquots of the mitochondrial suspensions from titrations at $pH_e$ 7.6 indicated that, in the presence of high concentrations of propionate, there was a slightly increased final [ADP] ($0.075 \pm 0.02$ mM ADP, as compared with $0.067 \pm 0.01$ mM ADP without propionate). In order to exclude the possibility that the observed increase in respiration was solely due to the resultant small decrease in [ATP]/[ADP][$P_i$] (from $1 \cdot 10^5$ $M^{-1}$ without propionate to $9 \cdot 10^4$ $M^{-1}$ with propionate), dependence of the respiratory rate on intramitochondrial pH at [ATP]/[ADP][$P_i$] of $10^3$ $M^{-1}$ was also examined, as a similar small increase in [ADP] under these conditions would result in less than a 2% change in the final phosphorylation state ratio. Initial respiratory rates following addition of varied [TMPD] had a similar dependence upon the intramitochondrial pH as in the experiments with addition of ATP only (approx. 15–25% increase in respiratory rate for a given % of cytochrome *c* reduction). These data indicate that the decrease in the phosphorylation state due to the inclusion of propionate did not contribute significantly to the increased respiratory rate calculated at constant cytochrome *c* reduction levels in these experiments or in those assessing the dependence of ATP synthesis of the intramitochondrial pH. In addition, the decrease in phosphorylation state ratio in the experiments measuring the pH-dependence of electron transfer between 3-OH-B and cytochrome *c* was not due to an uncoupling effect of sodium propionate.

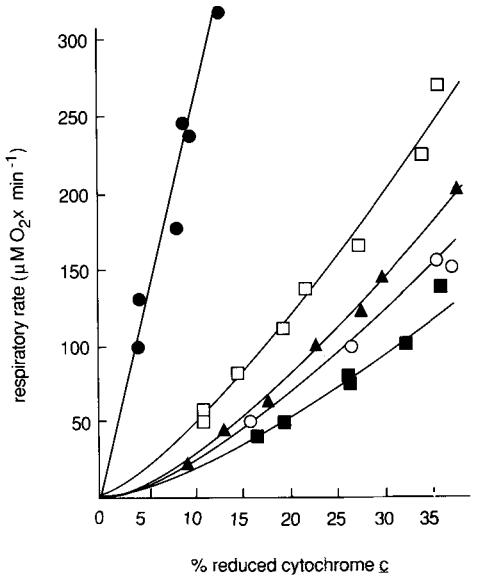


Fig. 3. The relationship between cytochrome *c* reduction and respiratory rate at different intra- and extramitochondrial pH values. Isolated mitochondria were suspended at a protein concentration of approx. 4 mg/ml in a medium of 0.2 M mannitol, 0.7 M sucrose, 15 mM Mops, and 0.4 mM EGTA, at either $pH_e$ 7.0 (□, ▲) or $pH_e$ 7.6 (○, ■). Open symbols represent incubations to which 20 mM sodium propionate had been added. Titrations in which oxidative phosphorylation was uncoupled from electron transport by inclusion of 0.7 μM FCCP in the assay medium are symbolized by ●. The points were obtained from a representative experiment as described in the text. Curves drawn through the points are included in order to better visualize the relationships and are approximate fits to the data.

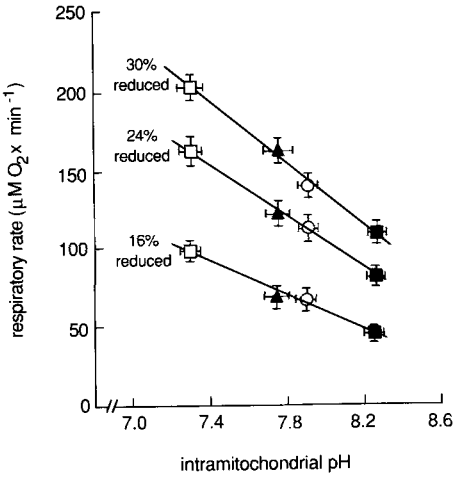


Fig. 4. Dependence of the respiratory rate on intramitochondrial pH. Mean values (±S.E.) of the respiratory rate corresponding to 16%, 24%, and 30% reduced cytochrome *c* from titration curves of six experiments (see Fig. 3 as an example) were plotted against the intramitochondrial pH (mean ± S.E. for three determinations). Symbols for trials at $pH_3e$ of 7.0 and 7.6, with and without added sodium propionate, are the same as those used in Fig. 3.

In order to evaluate the degree of dependence of the respiratory rate upon intramitochondrial pH, the respiratory rates from curves corresponding to selected levels of cytochrome *c* reduction (16%, 24%, and 30% reduced) were plotted vs. the calculated intramitochondrial pH value in Fig. 4. For each level of reduction of cytochrome *c*, the plotted points describe a curve which appears linear in this experimental range, indicating a direct correlation between respiratory rate and intramitochondrial pH. Measurements made at more alkaline pH values indicate a greater dependence on $pH_i$. This dependence approaches the second power dependence reported earlier for the dependence on $pH_e$ [10]. Mitochondrial preparations become somewhat unstable

at $pH_e$ values greater than about 7.6, and quantitative evaluation was not pursued beyond this value.

## Discussion

*The role of pH in the energetics of oxidative phosphorylation*

The overall reaction for the oxidation of 3-OH-butyrate by molecular oxygen,

$$\text{3-hydroxybutyrate} + 1/2\ O_2 \rightarrow \text{acetoacetate} + H_2O \quad (3)$$

is composed of two half-reactions:

$$\text{acetoacetate} + 2H^+ + 2e^- \rightarrow \text{3-hydroxybutyrate} \quad (4)$$
$$E_{m7} = -0.266\ \text{V}$$

$$1/2\ O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad (5)$$
$$E_{m7} = +0.820\ \text{V}$$

The half-reduction potentials of both the 3-hydroxybutyrate/acetoacetate couple and the $H_2O/O_2$ couple are pH-dependent in the physiological pH range. Moreover, the pH dependencies are equivalent in that the $E_m$ values become 60 mV more negative with each unit the pH becomes more alkaline. Thus, in a single compartment system, the standard free energy change of the overall reaction is independent of the pH, and the value of $\Delta E_m$ remains constant at $-1.086$ V. Mitochondria, however, represent a two-compartment system, and it is possible that the relevant hydrogen ions of the two half-reactions are taken from, or released into, different compartments (either the matrix or cytoplasmic spaces).

In oxidative phosphorylation all of the reducing equivalents transferred from 3-OH-butyrate to oxygen pass through cytochrome *c*. Evidence has been presented that this reaction is freely reversible [3–5,33,34] and approaches equilibrium from either the forward or reverse directions [3–5]. Since oxidation and reduction of cytochrome *c* does not involve stoichiometric uptake and release of $H^+$ [31,32], the oxidation-reduction reaction with 3-hydroxybutyrate may be written:

$$\text{3-OH-B} + 2\ \text{cyt}\ c^{3+} \rightleftharpoons \text{acac} + 2\ \text{cyt}\ c^{2+} + 2\ H^+ \quad (6)$$

for which the equilibrium constant is:

$$K_{eq} = \left(\frac{[\text{acac}]}{[\text{3-OH-B}]}\right)\left(\frac{[\text{cyt}\ c^{2+}]}{[\text{cyt}\ c^{3+}]}\right)^2 [H^+]^2 \quad (7)$$

In coupled mitochondria, the maximal free-energy change available for ATP synthesis is:

$$\Delta G = \Delta G^{0\prime} + RT\ \ln\left(\frac{[\text{acac}]}{[\text{3-OH-B}]}\right)\left(\frac{[\text{cyt}\ c^{2+}]}{[\text{cyt}\ c^{3+}]}\right) - 2RT\Delta\text{pH} \quad (8)$$

The system is very sensitive to the pH of the relevant compartment because a constant [acac]/[3-OH-B] and [cyt $c^{2+}$/cyt $c^{3+}$] the free energy of hydrolysis of ATP may decrease by approx. 1.36 kcal for each unit that the pH becomes more acidic. Otherwise stated, the extramitochondrial [ATP]/[ADP][$P_i$] must decrease 10-fold or the mass action ratio (Eqn. 2) must decrease by 100-fold for each pH unit acidification of the appropriate compartment. There is no dependence at all on the pH of the other compartment. Hence, the 'sidedness' of the energetics of this reaction can be determined. Figs. 1 and 2 show that both [ATP]/[ADP][$P_i$] and the calculated mass action ratios are fully dependent on $pH_i$, indicating that 3-hydroxybutyrate dehydrogenase equilibrates with protons from the matrix space and that the previous assignment of 2.0 ATP synthesized per 2 electrons transferred between 3-OH-B and cytochrome *c* [4,5] was correct. This conclusion is entirely consistent with the fact that 3-hydroxybutyrate dehydrogenase is inserted into the matrix side of the inner mitochondrial membrane and does not extend fully across the membrane [35,36].

In the present studies, the [3-OH-B]/[acetoacetate] ratio served to "clamp" the intramitochondrial [$NAD^+$]/[NADH] to the value for the 3-hydroxybutyrate dehydrogenase equilibrium value. The ratio of [3-OH-B]/[acetoacetate] in the blood is well recognized as being a good indicator of the intramitochondrial [NADH]/[$NAD^+$] ratio for liver mitochondria in situ. This is consistent with the fact that there is no net charge difference between 3-hydroxybutyrate and acetoacetate, there is no difference between their p*K* values and there are no known cotransported species. Thus changes in intracellular pH affect the phosphorylation state ratio through the pH-dependence of the equilibrium constant. Earlier measurements [25] in which an excess of reducing substrates, such as glutamate and malate, were added showed no correlation between changes in the transmembrane pH difference and the phosphorylation state ratio. In the earlier experiments the intramitochondrial [$NAD^+$]/[NADH] was kinetically determined through the pH-dependence of the several enzymes involved in substrate dehydrogenation. As such, there was no possibility of predicting the effect of intramitochondrial pH on the level of reduction of the NAD couple. It can be inferred from the current results that in the earlier experiments, kinetic changes compensated for the thermodynamic effect of pH, and that the redox potential of the NAD couple was not significantly altered by the pH changes.

*Origin of the $H^+$ ions for $O_2$ reduction and regulation of cytochrome c oxidase*

It is somewhat less direct to determine the source of the four protons used in reduction of each oxygen molecule to water by the analysis used in this study. However, in the present experiments, the electrical potential was unchanged, and changes in the rate of $O_2$

uptake were therefore indicative of dependence upon the concentration of $H^+$. The source of two of the four protons can be determined because they are taken up in reactions which kinetically modulate the enzymatic activity [10]. From results shown in Figs. 3 and 4, these appear to come specifically from the matrix space. The other two protons presumably also come from the matrix space, but their origin can only be inferred from these data. This result is consistent with determinations made of pH-dependence of electron flow between cytochrome *a* and cytochrome $a_3$ in reconstituted systems [39,40] and the vectorial accessibility of cytochrome $aa_3$ to the matrix side of mitochondria [41,42]. In addition, measurements of proton movements at site 3 are reported to show net appearance of protons in the extramitochondrial space (see, for example, Ref. 19) and this would require that protons, in addition to those used to form water, be transported from the matrix to the cytoplasmic side of the membrane. The roles of the individual components of the transmembrane electrochemical potential have been examined (Refs. 39–42, for example), and partial control of the cytochrome oxidase reaction by the electrical gradient has been reported.

The above considerations indicate that protons involved in both the 3-hydroxybutyrate dehydrogenase and cytochrome *c* oxidase reactions are derived from the matrix space. Thus the pH difference between the matrix and extramitochondrial spaces does not affect the overall free energy change associated with electron transfer from 3-hydroxybutyrate to oxygen.

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