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STUDIES ON YEAST MITOCHONDRIA

SOME PROPERTIES OF MITOCHONDRIA ISOLATED FROM *SACCHAROMYCES CARLSBERGENSIS* GROWN IN NORMAL GLUCOSE MEDIUM

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SUMMARY

1. Mitochondria isolated from *Saccharomyces Carlsbergensis* are found to have three phosphorylation sites in the respiratory chain for the oxidation of NADH and NAD^+-linked substrates and two for succinate oxidation. Freshly isolated mitochondria exist in an inhibited state with no respiratory control, but on ageing for 2–3 h a good coupled state is obtained. α-Ketoglutarate and α-glycerophosphate are poorly oxidized in these mitochondria.

2. Exogenous NADH is a very good substrate for yeast mitochondrial respiration and apparently has a very low K_m. However, one-third of the added NADH is not available for oxidation probably due to some form of compartmentation. Studies of both oxygen uptake and the redox changes of cytochrome *b* show complete oxidation of two-third of the added NADH.

3. Difference spectra of yeast mitochondria at liquid-nitrogen temperatures show all the characteristic peaks of cytochromes *a* (600 nm), *b* (558, 525 and 428 nm), c_1 (552 nm) and *c* (545 and 516 nm).

4. The reduction of cytochrome *b* by dicumarol in antimycin A inhibited mitochondria provides evidence for an energy conservation site on the substrate side of cytochrome *b*.

5. In the absence of added ADP, the oxidation of malate and pyruvate occurs in the yeast mitochondria in a new respiratory state (State X) where the oxygen uptake occurs at State 4 rate but the redox level of the flavins, cytochrome *b* and *c* are similar to State 3. State X respiration is believed to be due to depletion of the high energy intermediate C ~ I caused by the substrate anions accumulation.

6. The responses of yeast mitochondria to Ca^{2+} are qualitatively similar to those in rat liver mitochondria, particularly with respect to respiratory stimulation, membrane alkalinization and its accumulation in the mitochondria with succinate as the substrate in the presence and absence of acetate.

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INTRODUCTION

The conflicting reports concerning the oxidative phosphorylation efficiencies of yeast cells and mitochondria have caused considerable difficulty in the understanding of metabolic control phenomenon related to glycolysis and respiration in these organisms. LYNEN AND KOENIGSBERGER[1] obtained a P:O ratio of 1 for respiring bakers yeast whereas CHANCE[2] provided evidence for three phosphorylation sites in yeast cells from crossover data of different respiratory components and explained the low P:O value reported previously to high dephosphorylation reactions prevalent in yeast cells. In later work, however, LYNEN[3] obtained P:O ratios varying between 2 and 3 when yeast cells respired on endogenous substrate.

ROY[4] isolated particles containing normal respiratory pigments from bakers yeast broken by high pressure technique and made an important observation that during the oxidation of D-lactate in these particles electron transport occurs through a by-pass of the antimycin A site of the respiratory chain. This result was also observed in isolated mitochondria from *Saccharomyces Carlsbergensis*[5,6].

A rigorous study of the properties of isolated mitochondria from *S. Carlsbergensis* grown in lactate medium, was carried out by OHNISHI *et al.*[5,24]. They observed that these mitochondria could oxidise exogenous NADH and the respiratory chain had two phosphorylation sites for the oxidation of NAD^+-linked substrates, as well as for succinate. SCHUURMANS-STEKHOVEN[6], however, reported three phosphorylation sites for the oxidation of NAD^+-linked substrates and two for succinate in the mitochondria of *S. Carlsbergensis* grown in a medium containing both glucose and lactate as the carbon source. Non-existence of Site 1 in *Saccharomyces cerevisiae* was also reported by BALCAVAGE AND MATTOON[7], KOVAC *et al.*[8] and SCHATZ *et al.*[9].

It has been previously reported by us[10] that the presence or absence of inositol in the growth medium grossly alters the respiratory behavior of these yeast cells. Thus studies were undertaken to determine whether the presence or absence of inositol in the growth medium caused characteristic differences in the respiratory behavior of the yeast mitochondria. This paper elucidates the general properties of mitochondria isolated under our conditions from *S. Carlsbergensis* grown in normal medium containing both glucose and inositol. Only the properties that appeared different from those reported earlier together with some novel data on the stoichiometry of NADH oxidation, responses of these mitochondria to Ca^{2+} and the redox states of carriers such as cytochrome *b* with different substrates are reported here.

MATERIALS AND METHODS

Culture conditions and strain of yeast

S. Carlsbergensis, Hillman Hospital Strain 4228 was obtained from the American Type Culture Collection and grown in 1.5 % glucose medium as described previously[11]. The growth temperature was 25° and the medium was continuously aerated. Cells were harvested after 17 h growth, centrifuged and washed with distilled water three times, then suspended in double the volume of 0.1 M KH_2PO_4 and aerated for 3 h. The cells were then centrifuged and washed twice with distilled water and once with 1.3 M sorbitol. These cells were then used for making protoplasts and mitochondria as described below.

Preparation of protoplasts and mitochondria

Protoplasts of *S. Carlsbergensis* were made, using snail gut juice extracts, by slight modifications of the methods described by OHNISHI *et al.*[5] and SCHUURMANS-STEKHOVEN[6]. Lyophilized snail gut juice (L'Industrie Biologique Française S.A.) was dissolved in 0.1 M Tris–maleate buffer (pH 5.8) containing 1 mM EDTA and dialysed against the same buffer, with three changes (2 l each time), for 7–8 h. The dialysed enzyme, containing 30–40 mg protein/ml, was centrifuged at 10000 × *g* for 10 min. The yellow fatty layer was removed and the clear supernatant fraction was frozen for later use. The washed cells were suspended in double the volume of 1.3 M sorbitol solution containing 0.1 mM EDTA and 0.1 M Tris–maleate buffer (pH 5.8) and incubated with the snail enzyme preparation (2 mg protein/ml) at 28° for 60–75 min. The incubated cells, containing 75–85 % protoplasts, were centrifuged at 5000 × *g* for 8 min. The residue was twice washed with 1.3 M sorbitol and centrifuged at the same speed. All the operations after enzyme incubation were performed at 0°.

The protoplasts were suspended in 0.45 M mannitol (1:5), containing 0.1 mM EDTA and purified bovine serum albumin (0.5 mg/ml), and homogenized in a Waring blender at low speed for 30 sec. The homogenized suspension was centrifuged at 2000 × *g* for 8 min. The supernatant fraction was recentrifuged under the same conditions. The supernatant fraction from this spin was then centrifuged at 8500 × *g* for 10 min. The supernatant fraction from this spin was discarded and the fatty layer adhering to the sides of centrifuge tubes removed with cheese cloth. The residues in each tube were suspended in the mannitol–EDTA–bovine serum albumin medium, collected in one tube and centrifuged at 10000 × *g* for 10 min. The pellet was washed once more in the same way and the final pellet suspended in a small volume of the same medium and kept in ice for 2–3 h before use. For Ca^{2+} uptake studies, however, the mitochondria were washed twice with 0.45 M mannitol and resuspended in the same medium.

Oxygen uptake

Oxygen uptake studies were performed polarographically at room temperature (22°) using a Clark oxygen electrode connected through an amplifier to a recorder. ADP:O ratios were calculated as described by ESTABROOK[12]. The oxygen concentration of the reaction medium at 22° was determined by both polarographic and enzymic[28] methods using air saturated distilled water as the standard ($[O_2]$ 275 μM at 22°)[27].

Low-temperature cytochrome spectra

Cytochrome spectra of yeast mitochondria were obtained at liquid-nitrogen temperatures according to the method of ESTABROOK[13], using a splitbeam spectrophotometer.

Changes in cytochrome b reduction

The changes in reduced cytochrome *b* of yeast mitochondria in different respiratory states were measured with a dual wavelength spectrophotometer using 430 nm and 410 nm as the measuring and reference wavelength, respectively.

Dual wavelength spectrophotometric methods were also used for measuring the redox changes of flavin (465–495 nm) and cytochrome *c* (550–540 nm).

The effects of Ca^{2+} on intramitochondrial pH changes

These effects were studied by the method of CHANCE AND MELA[15] using bromothymol blue as the pH indicator.

Ca^{2+} uptake by mitochondria was determined by centrifuging the Ca^{2+}-treated mitochondrial suspension at 10000 × *g* for 10 min and extracting the Ca^{2+} from the pellets by boilling with 0.1 M HNO_3 for 10 min. The extracts were centrifuged and the supernatant fraction used directly for measuring the Ca^{2+} content by atomic absorption methods, using 422.7 nm as the measuring wavelength.

The method of WERKHEIM AND BARTLEY[29] for the separation of mitochondria form suspensions was also employed, using silicone as the middle layer and 3% $HClO_4$ as the bottom layer during centrifugation. The bottom layer was heated at 80° for 5 min, cooled, centrifuged and the supernatant fractions neutralised with K_2CO_3 and recentrifuged. The supernatant fraction from this spin was used for Ca^{2+} estimation by atomic absorption.

Reaction medium A

Reaction medium A, used for the studies of the respiratory activity of yeast mitochondria, was composed of 0.45 M mannitol containing 5 mM KCl, 10 mM potassium phosphate buffer (pH 6.5), 10 mM triethanolamine buffer (pH 6.5) and 0.1 mM EDTA.

Reaction medium B

Reaction medium B, used for the studies on Ca^{2+}-induced bromothymol blue color changes in yeast mitochondria, contained 0.45 M mannitol, 10 mM Tris–HCl buffer (pH 6.5), 400 μM Tris–succinate and 6 μM bromothymol blue.

RESULTS

Concentration of oxygen in the reaction medium at 22°

The oxygen concentration in the reaction medium was found to be 240 ± 5 μM by both the polarographic and enzymic methods. This value was used throughout this work because all the reactions were carried out at 22°.

Changes in the coupling characteristics of yeast mitochondria

Freshly prepared yeast mitochondria show relatively high oxygen uptake rates with succinate, citrate and a mixture of malate and pyruvate, but do not show any respiratory control, *i.e.* the mitochondria do not show State 4–3–4 transitions upon ADP addition. This is shown in Fig. 1A. It is interesting to note that oligomycin causes an inhibition of this respiration from 13.2 to 7.2 μM O_2/min.

On ageing for 40–60 min at 0°, the rate of respiration of these mitochondria on any of the substrates mentioned previously, shows stimulation on addition of ADP. This is shown in Fig. 1B. It may be observed that at this state the mitochondria do not show a State 3–4 transition. On the contrary, it becomes further uncoupled with ADP.

After 1.5–2 h of ageing, the mitochondria show the usual State 4–3–4 transitions shown in Fig. 1C. The ADP:O ratios at this stage, however, are quite low. The ADP:O ratio with malate and pyruvate as substrate was found to be 1.9. After 3 h of ageing the mitochondria showed maximum respiratory efficiency as judged by the ADP:O ratio.

Respiratory control and ADP:O ratios of yeast mitochondria with different substrates

Mitochondria aged over 3 h were used in all subsequent studies. Typical traces of oxygen utilization by these mitochondria with succinate or a mixture of malate and pyruvate as substrate are given in Figs. 2 and 3. The traces were obtained from the same preparation of mitochondria and the data with these two substrates are com-

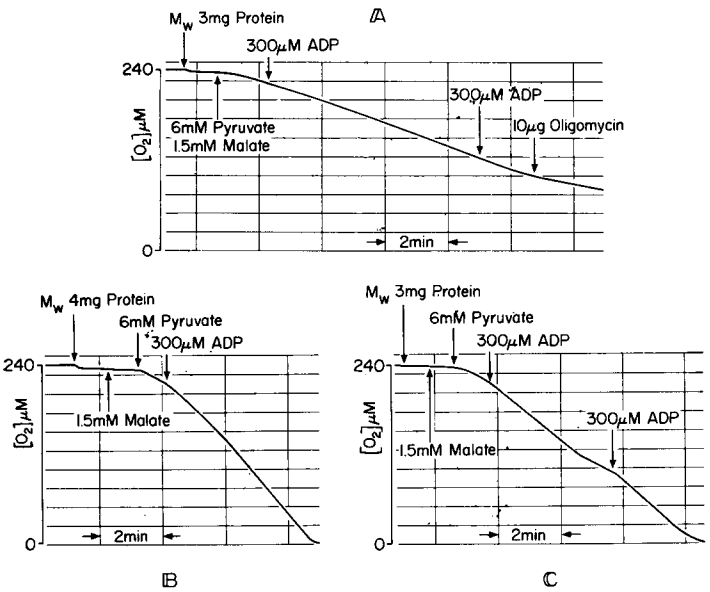

Fig. 1. Coupling behavior of *S. carlsbergensis* mitochondria (M_w) on ageing: 0.1 ml mitochondria (3–4 mg protein) added to 3 ml 0.45 M mannitol containing 5 mM KCl, 10 mM potassium phosphate buffer (pH 6.5), 10 mM triethanolamine buffer (pH 6.5). The oxygen uptake was monitored with a Clark oxygen electrode. The additions of substrate, ADP, *etc.*, are noted on the figure. The respiratory behavior of fresh, 1 h aged and 2 h aged mitochondria are shown in A, B and C, respectively.

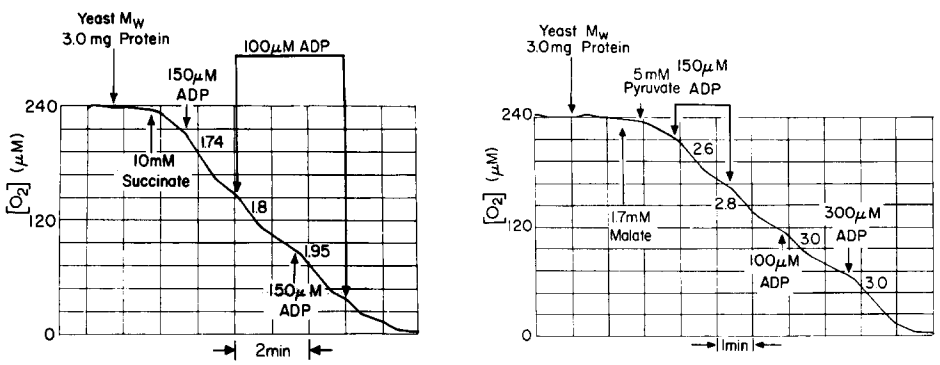

Fig. 2. Respiratory control and ADP:O ratios of yeast mitochondria (M_w) using succinate as the substrate. The reaction conditions are the same as in Fig. 1 and the numbers on the trace show the ADP:O ratios after each addition of ADP.

Fig. 3. Respiratory control and ADP:O ratio of yeast mitochondria respiring on malate *plus* pyruvate as the substrate. The reaction conditions are the same as in Fig. 1. Numbers on the trace show the ADP:O ratios after each addition of ADP.

TABLE I

RESPIRATORY CONTROL AND ADP:O RATIOS IN *S. Carlsbergensis* MITOCHONDRIA WITH DIFFERENT SUBSTRATES

Substrate	*Number of experiments*	*Respiratory control ratio*	*ADP:O ratio*
Succinate (10 mM)	10	1.5 — 2.0	1.6 — 2.0
NADH (0.8 mM)	8	2.0 — 2.7	2.2 — 2.4
Ethanol (20 mM)	10	1.7 — 2.2	2.4 — 2.6
Citrate (7 mM)	10	1.8 — 2.8	2.6 — 3.0
Malate (1.7 mM) *plus* pyruvate (5 mM)	10	2.0 — 2.8	2.5 — 3.0

parable. It may be seen from the figures that the ADP:O ratios with succinate, and malate and pyruvate as substrates are nearly 2 and 3, respectively. The respiratory control and ADP:O ratios with different substrates are given in Table I. It is quite evident that the ADP:O ratio for NAD^+-linked substrate oxidation is more than 2 and for succinate it is 2. Rotenone, however, does not inhibit oxidation of NAD^+-linked substrates. The rate of oxidation of α-ketoglutarate and also α-glycerophosphate is very low in these mitochondria. As ADP:O and P:O ratios do not differ significantly, only the data on ADP:O ratios are included here.

Stoichiometry of NADH to oxygen

An interesting result was obtained from oxygen uptake studies when NADH was used as the substrate, as shown in Fig. 4. It would appear from the trace that the added NADH was fully oxidized and the ratio of NADH to oxygen consumed was 1.5–1.6. The stoichiometry of NADH to oxygen did not change when the substrate was oxidized in either State 3 or State 4. Addition of 400 μM ADP after the exhaustion of NADH in State 4 produced a small burst of respiration. Higher values of NADH:O ratio (1.8–1.9) were also obtained on other occasions, but the oxidative activity of these mitochondria was found to be much less, especially with substrates other than NADH. It was also observed that the NADH:O ratio decreased with the ageing of the mitochondria (15–18 h). The concentration of the NADH solution used was measured enzymically as described by WILLIAMSON AND HERCZEG[26].

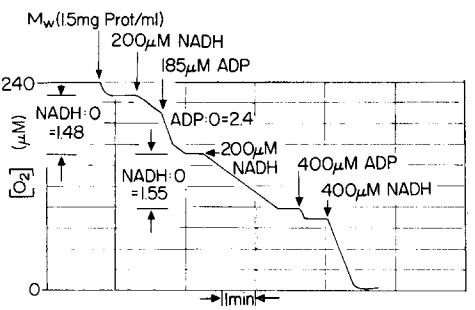

Fig. 4. ADP:O and NADH:O ratios of yeast mitochondria (M_w) using NADH as the substrate. The reaction conditions are the same as in Fig. 1.

Low-temperature difference spectrum

The low-temperature difference spectrum of yeast mitochondria (not shown) has all the characteristic cytochrome peaks, such as *a*-α (600 nm), *b*-α, *b*-β and *b*-γ, (558, 525 and 428 nm) and c_1-α (552 nm) and *c*-α, *c*-β (545 and 516 nm).

Changes in the redox state of cytochrome b

Changes in the redox state of cytochrome *b* were studied under two conditions: (A) when stirring of the mitochondrial suspension was fairly rapid to ensure some diffusion of air, but without affecting spectroscopic measurements, and (B) when stirring was much milder and little or no diffusion of air took place.

Studies were made under condition A for substrates such as citrate, succinate, and malate *plus* pyruvate and it is evident from Fig. 5 that a large number of ADP-

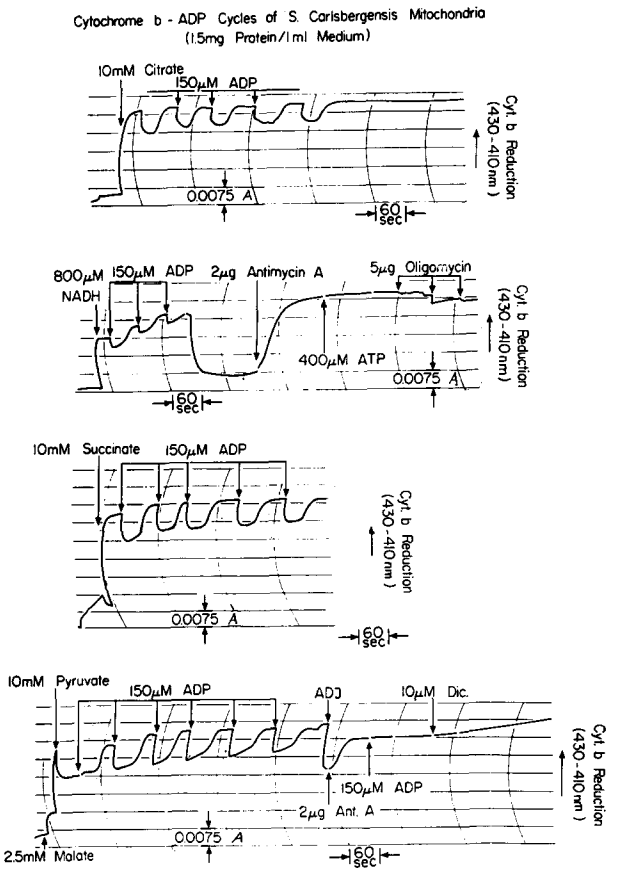

Fig. 5. Changes in the redox states of cytochrome *b* of yeast mitochondria under different experimental conditions: Mitochondria (1.5 mg protein/ml) were suspended in reaction medium as in Fig. 1 and the changes in the redox state of cytochrome *b* due to additions of different substances, as noted on the figures, were monitored with a Chance dual-wavelength spectrophotometer using 430 nm as the measuring and 410 nm as the reference wavelength. With substrates, citrate, succinate and malate *plus* pyruvate, experiments were performed in Condition A (rapid stirring) and for NADH in Condition B (slow stirring). All traces show the oxidation–reduction cycle of cytochrome *b* upon addition of ADP. The effects of oligomycin, and dicumarol (Dic.) are shown in the 2nd and 4th trace from the top, respectively.

induced cycles of cytochrome *b* oxidation–reduction were obtained with each of the above substrates.

The most characteristic change in cytochrome *b* was obtained when malate *plus* pyruvate was used as the substrate. Addition of malate caused a small reduction of cytochrome *b* but addition of pyruvate caused at first a large reduction and then an immediate oxidation of the cytochrome *b*. In State 4 respiration, in the absence of added ADP, the cytochrome was reduced only an anaerobiosis (not shown in the figure). The first addition of ADP in State 4 did not show any significant change in the redox state of the cytochrome, but it was reduced after the exhaustion of ADP. Normal ADP induced cycles were obtained thereafter. The same type of redox behavior of cytochrome *c* (550–540 nm) and flavin (465–495 nm) was observed with this substrate mixture.

It is also evident from the malate and pyruvate trace that after antimycin A treatment, addition of 10 μM dicumarol caused a slow but quite significant reduction of cytochrome *b*. In a separate experiment, addition of 1.5 mM ATP after antimycin A treatment produced a small but significant oxidation of cytochrome *b*.

The redox change of cytochrome *b* was studied under condition B when NADH was used as the substrate. This was done to calculate the NADH:O ratio, if and when the substrate is fully oxidized and no further electron transport occurs through the respiratory chain. It is evident from the NADH trace (Fig. 5) that after a few ADP cycles, cytochrome *b* was fully oxidized. Addition of antimycin A reduced the cytochrome *b* probably due to the flow of electrons from coenzyme Q or compartmentized NADH. A small oxidation–reduction cycle was observed in this system after the addition of 10 μg oligomycin. However, 400 μM ATP did not produce any change. The NADH:O ratio was found to be 1.67 in this mitochondrial preparation.

Effect of Ca^{2+} on yeast mitochondria

Effects of added Ca^{2+} on the oxygen uptake characteristics of yeast mitochondria are shown in Fig. 6. With succinate as substrate (Fig. 6A) Ca^{2+} stimulates the rate of respiration from 28 to 53 μM O_2 per min. ADP does not change this rate showing that respiration is uncoupled in the mitochondria. The effect of Ca^{2+}-addition when a mixture of malate and pyruvate is the substrate, is quite interesting (Fig. 6B). It stimulates the rate of respiration from 19 to 28 μM O_2 per min but addition of ADP

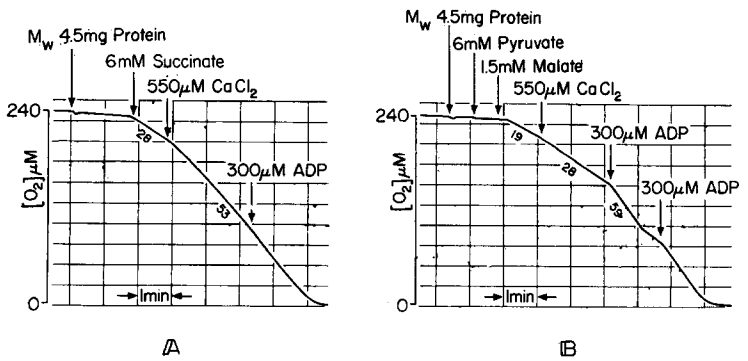

Fig. 6. Effect of Ca^{2+} on the respiratory behavior of yeast mitochondria (M_w) with succinate (A) and malate *plus* pyruvate (B) as the substrates. Reaction conditions were the same as in Fig. 1.

increases the rate still further to 59 μM O_2 per min and shows characteristic State 4–3–4 transitions and the ADP:O ratio is found to be 2.4.

The effect of Ca^{2+} on mitochondrial alkalinity was studied using reaction medium B containing low succinate to avoid its possible effect as a permeant anion. The changes in the bromothymol blue absorbance (618–702 nm) due to Ca^{2+} and phosphate is shown in Fig. 7. It is quite evident that Ca^{2+} causes membrane alkalinization and phosphate reverses this effect. The K_m for the reversal reaction by phosphate is 1.5 mM. Citrate is very efficient in reversing the Ca^{2+}-induced alkalinity (not shown in the figure) and has a similar effect with a K_m of 2.5 mM.

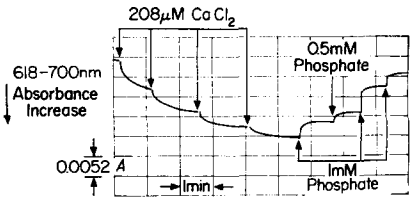

Fig. 7. Effect of Ca^{2+} on the membrane alkalinization of yeast mitochondria. Mitochondria (1.5 mg protein/ml) were suspended in reaction medium B containing 0.45 M mannitol, 10 mM Tris–HCl (pH 6.5), 400 μM Tris–succinate and 6 μM bromothymol blue and changes in the bromothymol blue absorbance after addition of Ca^{2+} and phosphate were monitored with a dual wavelength spectrophotometer using 618 nm and 700 nm as the measuring and reference wavelength, respectively.

Accumulation of Ca^{2+} inside yeast mitochondria with succinate and succinate *plus* acetate as the substrates is shown in Table II. It may be seen from the table that yeast mitochondria accumulate 40–44 nmoles of Ca^{2+} per mg protein when succinate is used as the substrate. Addition of acetate (7 mM) to a mitochondrial suspension containing succinate and Ca^{2+}, increases the Ca^{2+}-accumulation to 60 nmoles per mg protein. Also, it may be seen that rat liver mitochondria accumulate much more Ca^{2+} even when its concentration in the suspending medium is less than that used for yeast mitochondria. Ca^{2+} content in the mitochondrial pellet after the separation of suspending medium by the method of WERKHEIM AND BARTLEY[29] was also determined. No significant differences in the results from those noted above were obtained.

TABLE II

COMPARISON OF Ca^{2+} ACCUMULATION IN *S. Carlsbergensis* MITOCHONDRIA AND RAT LIVER MITOCHONDRIA

Data obtained from five experiments.

Substrate	*Amount of Ca^{2+} (mM) added to medium*		*Amount of Ca^{2+} (nmoles/mg protein) accumulated*	
	yeast mitochondria	*rat liver mitochondria*	*yeast mitochondria*	*rat liver mitochondria*
Succinate (7 mM)	1.5	0.58	35–40	60–70
Succinate (7 mM) *plus* acetate (7 mM)	1.5	0.58	55–60	110–120

DISCUSSION

Respiratory control and phosphorylation efficiency

Studies on the respiratory behavior of mitochondria isolated from *S. Carlsbergensis* clearly show that freshly isolated mitochondria stay in an inhibited state (with no respiratory control) without completely losing the mechanism of coupling, as shown by the effect of oligomycin on these respiring mitochondria. It is unlikely that mitochondria exist in an inhibited or uncoupled state in yeast cells and so the observed effect is probably created during the isolation process.

The appearance of coupling activity on ageing is probably due to a gradual inactivation of the ATPase activity which is somehow activated during the isolation procedure, or it may be due to the gradual destruction of an inhibitor of phosphorylation which somehow accumulated in the mitochondria during the isolation process. It is not possible at this time to characterize which one of the above possibilities is responsible for the generation of respiratory control in the ageing yeast mitochondria.

The ADP:O ratios, which reflect the phosphorylation efficiencies of mitochondria[12,14] were more than 2 for NADH or NAD^+-linked substrates suggesting three phosphorylation sites in the respiratory chain. These observations on ADP:O ratios confirm those reported by SCHUURMANS-STEKHOVEN[6] who grew the yeasts in a medium containing glucose and lactate. These results were communicated to Dr. T. Ohnishi who obtained similar ADP:O ratios in yeast mitochondria[16] isolated in the manner described in this paper. These results show some similarity with plant mitochondria as reported by IKUMA AND BONNER[25].

The peculiarity of yeast mitochondria is that the respiratory control ratios obtained with different substrates are quite low (1.8–3.0) as compared to those of rat liver mitochondria where one can obtain ratios of more than 10 with malate *plus* glutamate as the substrate. Whether the low respiratory control is an inherent characteristic of yeast mitochondria or is caused by the methods used in their isolation, is yet to be determined.

The low respiratory control in yeast mitochondria which possibly reflects a partial reversible uncoupling do not interfere with the phosphorylation efficiencies. The reversible uncoupled state is defined here as the uncoupled state where the ADP:O ratio is not low[14] and the irreversible uncoupled state is the one where ADP:O ratio decreases[17] or approaches zero. The low ADP:O ratios with NAD^+-linked substrates reported earlier by OHNISHI *et al.*[5,24] and others[5,7,8], are probably due to an irreversible uncoupling of one phosphorylation site in yeast mitochondria because of growth conditions or isolation procedures.

The contribution of substrate level phosphorylation towards high ADP:O ratios with NAD^+-linked substrates, as reported in this paper, can be safely ruled out from the fact that these mitochondria do not oxidize exogenous α-ketoglutarate at a reasonable rate.

Stoichiometry of NADH oxidation

The absence of any significant oxygen uptake rate when the added NADH was exhausted and the NADH:O ratio of 1.5–1.6 suggests that this substrate is in some way compartmentized whereby only part of it (nearly two-thirds) is available for oxidation in the mitochondria. Presumably, this compartmentation plays an important

role in the regulation of metabolism in yeast cells as there is no potential barrier in the mitochondria for the oxidation of cytoplasmic NADH. The functional role of this NADH compartmentation in metabolic control is now being investigated. The small burst of respiration caused by 400 μM ADP after the exhaustion of NADH in State 4, is not clearly understood.

Responses of cytochrome b to substrates and other reagents

The large number of ADP induced oxidation–reduction cycles of cytochrome *b* obtained under experimental condition A clearly shows that the mitochondria isolated from *S. Carlsbergensis* were in good condition and that the results reported in this paper are not due to any form of degeneration of the mitochondria. These studies also show that in an antimycin A blocked system oligomycin (NADH trace Fig. 5) or high amounts of ATP (1.5–2.0 mM) causes oxidation of cytochrome *b*, which suggests an energy conservation site (Site 1) on the substrate side of the cytochrome.

The most significant evidence for Site 1 comes from the effect of dicumarol on antimycin A blocked systems (malate *plus* pyruvate trace Fig. 5) where quite a significant reduction of cytochrome *b* was observed. The absence of a very sharp or large change in the redox state of cytochrome *b*, due to oligomycin or dicumarol addition, is believed to be due to the reversible uncoupled state (low respiratory-control ratio) of these mitochondria.

When NADH was used as the substrate, the oxidation of cytochrome *b*, after a few ADP-induced cycles, shows that the substrate was completely oxidized. This observation and the calculated NADH:O ratio of 1.67 give additional support to our speculation that NADH is compartmentized in the mitochondria such that nearly one-third of the added NADH is unavailable for oxidation through the respiratory chain.

The interesting observations of the redox states of cytochrome *b*, flavin and cytochrome *c*, when malate and pyruvate were added in State 2, are very difficult to explain. This appears to be a new respiratory state of mitochondria (State X) where the rate of oxygen uptake is the same as the State 4 rate (obtained after the exhaustion of added ADP) but the redox state of the respiratory carriers is the same as in State 3. This respiration is not due to any by-pass of the electron transport chain (from flavin to cytochrome *c*) because it is inhibited by antimycin A (5 μg/ml), NaN_3 (50 μM) and KCN (0.5 mM).

Responses of yeast mitochondria to Ca^{2+} addition

With succinate as the substrate, addition of small amounts of Ca^{2+} greatly enhance the rate of respiration but the absence of a State 3–4-transition shows an uncoupling effect of Ca^{2+}. With malate *plus* pyruvate as the substrate, the enhanced rate of respiration is small and addition of ADP shows regular State 4–3–4-transitions. The relatively small enhancement of the rate is believed to be due to lower permeability of Ca^{2+} in the presence of this substrate. Ca^{2+} in this case produces a partial reversible uncoupling which does not interfere with the ADP reaction or ADP:O ratio.

The bromothymol blue experiments show some qualitative similarity between yeast mitochondria and mammalian mitochondria in producing membrane alkalinization and its reversal with phosphate (Fig. 7), and acetate or citrate (not shown). But the K_m for phosphate (1.5 mM) and acetate (2.5 mM) for the reversal reaction suggests

a much lower permeability of these anions in yeast mitochondria under this condition[23].

Ca^{2+} accumulation by yeast mitochondria with succinate as the substrate (40 nmoles/mg protein), and the effect of acetate in increasing the amount accumulated (60 nmoles/mg protein) shows again a qualitative similarity between these mitochondria and mammalian mitochondria, but quantitatively the effects are much less than in rat liver mitochondria. CARAFOLI *et al.*[18,19] reported similar data concerning Ca^{2+} accumulation in the mitochondria of *S. cerevisiae* but did not observe any stimulation of respiration on addition of this cation.

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