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PROPERTIES OF *SACCHAROMYCES CEREVISIAE* MITOCHONDRIA PREPARED BY A MECHANICAL METHOD

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SUMMARY

1. Mitochondria exhibiting respiratory control were prepared from bakers' yeast and from *Saccharomyces cerevisiae* strain D-261 after disrupting the cells in a colloid mill at low speeds with glass beads. Optimal values of the variables involved were determined. Yields of 13–25 mg mitochondrial protein per 100 g packed cells were routinely obtained. Mitochondrial quality was similar to that of particles prepared by a method employing snail gut enzymes to digest cell walls.

2. The functions of mitochondria prepared under the mildest homogenization conditions were only slightly affected by large alterations in pH or Mg^{2+} concentration of the reaction medium.

3. Oligomycin and atractyloside were potent inhibitors of phosphorylating respiration in yeast mitochondria.

4. Respiration was specifically stimulated by ADP, with an apparent $K_m =$ 26.5 μM. Phosphate also stimulated malate-supported respiration, with an apparent K_m of 1.5 mM. Varying the phosphate concentration with succinate as substrate led to a transition from ADP-controlled respiration at high phosphate to uncontrolled respiration at low phosphate.

5. P:O or ADP:O values for this preparation were about 1.8 during succinate oxidation, 1.8 during ethanol oxidation, 2.4 during α-ketoglutarate oxidation and 1.7 during oxidation of malate *plus* pyruvate.

INTRODUCTION

Facultative anaerobic yeasts are uniquely suited for the genetic[1,2] and environmental[3–7] manipulation of respiratory processes. Since all the energy required for growth may be derived from fermentation, yeast mitochondria may be subjected to drastic genetic modifications which would be lethal to obligately aerobic organisms[2]. Moreover, the cytochromes of yeast are inducible enzymes whose biosynthesis is dependent upon oxygen supply, energy source, and the stage of cell growth[3–7]. Thus both mitochondrial function and development are especially amenable to analysis in yeast.

The most severe obstacle to the study of intact yeast mitochondria has been the lack of rapid, reproducible preparatory methods which are readily applicable to

cells in any stage of culture growth. Among the various methods reported[9–15], OHNISHI AND HAGIHARA[15] described briefly a mechanical method for releasing mitochondria from *Saccharomyces carlsbergensis*. They have apparently abandoned this method in favor of a longer method employing digestion of yeast cell walls with snail gut juice (helicase) to form protoplasts, which are then ruptured by a brief blendor treatment[16].

This method is most successfully applied to a particular strain of *S. carlsbergensis* which displays a high sensitivity to snail gut digestive enzymes when cells are isolated during logarithmic growth. The resulting mitochondria exhibit ADP-mediated respiratory control but lack both the rotenone-sensitive component located at the NADH-cytochrome *b* segment of the respiratory chain and the associated capacity to phosphorylate ADP (phosphorylation site I)[30,31].

This report describes a rapid method employing mechanical rupture of yeast cells with a commercially available colloid mill. By carefully studying the variables associated with this procedure, a successful, reproducible method was devised allowing the isolation of small yields of relatively intact mitochondria from *Saccharomyces cerevisiae* cells in any growth stage. This method allows the use of a standard set of conditions, and avoids the need, in the enzymic method, for repeated restandardization of digestion conditions with change in physiological state or yeast strain. The mechanical method also avoids the potentially toxic effects of snail gut juice on the isolated mitochondria[16]. In the present paper it is shown that the intactness of yeast mitochondria may be estimated from their ability to retain respiratory control in the presence of Mg^{2+} and at high pH.

The affinities of the mitochondria for ADP and inorganic phosphate and their sensitivity to inhibitors of phosphorylation have also been determined.

MATERIALS AND METHODS

Bakers' yeast was purchased from Federal Yeast Co., Baltimore, Md. Highly derepressed *S. cerevisiae*, strain D-261, was grown on a natural medium containing 0.1 % (w/v) glucose and 3 % (v/v) ethanol as previously described[17]. The Eppenbach MV–6–3 micromill (Gifford-Wood), and the centrifuge equipment previously used in preparing both loosely coupled[17,18], and tightly coupled yeast mitochondria[18] were employed. After washing, 400 g of cells were resuspended to a final volume of 600 ml, except as indicated below. The suspending medium contained 0.3 or 0.6 M mannitol, 10^{-4} M EDTA and 0.2 % fraction V bovine serum albumin (Calbiochem). Cell suspensions were placed in the precooled colloid mill operating with a maximum gap setting (0.076 inch) at a speed of 2500 rev./min (Powerstat setting 15). 500 ml of well-washed 0.2-mm glass beads (No. 4285–M–20, Arthur H. Thomas) were added to the mill. The resulting yeast-bead suspension was titrated to pH 6.8 with 1.0 M NaOH. The pH was continuously monitored with the aid of a combination pH electrode immersed in the recycling stream of the mill.

The pH was maintained at 6.8 by periodic additions of a few drops of 1.0 M NaOH. After 3 min the speed and gap settings were adjusted to the values which had been preselected for cell rupture, and cell breakage was carried out for 4–8 min. Optimum values for the variables, based on the quality of the isolated mitochondria, were as follows: mill speed, 7000 rev./min (Powerstat setting 25); gap setting, 0.040 inch; time, 8 min. In all cases the final temperature of the homogenate was

below 5°. About 10 ml of 1.0 M NaOH were required to maintain the pH at 6.8 during the grinding period. The homogenate was drained from the mill, decanted from the beads, and two 100-ml portions of homogenization medium were used to rinse the mill and the beads. The resulting fluid was added to the homogenate which was then centrifuged at 1200 × *g* for 10 min. The supernatant fluid, containing the mitochondria, was recentrifuged at 6000 × *g* for 15 min. The supernatant layer was discarded. The pellet material was gently resuspended in 40–80 ml of wash medium and recentrifuged at 750 × *g* for 7 min to remove any remaining large cellular debris. The wash medium consisted of 0.6 or 0.3 M mannitol containing 0.1 % fraction V bovine serum albumin; the pH was adjusted to 6.8 with 1.0 M NaOH.

The 750 × *g* supernatant layer was decanted and recentrifuged at 8000 × *g* for 15 min. The supernatant fluid and copious fluffy layers were removed by aspiration from the top of the darker mitochondrial pellets. Any remaining fluffy material was washed from the surface of the well-packed pellets with a small volume of wash medium. The pellets were then thoroughly drained and resuspended with a minimal amount of wash medium (usually 0.2 ml).

In more recent work it was found that by altering the centrifugation pattern, mitochondrial yield could be increased 2- to 3-fold with no observable alteration in functional properties. In the "modified procedure", the 750 × *g* supernatant layer described above was centrifuged at 20000 × *g* for 15 min. The turbid supernatant fluid was discarded, and the well-packed pellet material resuspended in 10 ml of wash medium per centrifuge tube and reisolated by centrifugation at 10000 × *g* for 15 min. The supernatant fluid and fluffy layers were removed and discarded. The well-packed mitochondria were resuspended with a minimal amount of wash medium (about 0.5 ml). With this method 13–25 mg of mitochondrial protein per 100 g packed cells is obtained. This represents a yield of about 1 % based on yeast cytochrome *c* content[19]. This yield is only 5 % of that obtained from the enzymic preparation[16], but is highly reproducible and the procedure is relatively simple, rapid and inexpensive.

Mitochondrial respiration was followed at 25° using a strip chart recorder and Clark oxygen electrode[20]. A semi-closed 3.0-ml chamber was used in which O_2 back-diffusion was only 0.25 mM per min at an O_2 concentration of 18.3 mM.

Standard respiratory medium consisted of 0.01 M sodium phosphate buffer (pH 6.5), containing mannitol at either 0.3 or 0.6 M. The reaction chamber was filled with reaction medium, and additional reagents, substrates and mitochondria as required. The system was calibrated at zero oxygen by the addition of a rapidly respiring yeast cell suspension or several grains of dithionite. The oxygen concentration at 100 % air saturation was determined by the Winkler method[21]. Triple distilled, autoclaved water was used to prepare the reaction medium for O_2 determination. Standard 0.025 M sodium thiosulfate reagent was purchased from the Fisher Scientific Co.

Protein nitrogen was estimated by the method of JOHNSON[22]. P:O ratios were determined using ^{32}P and hexokinase–glucose as described by NIELSEN AND LEHNINGER[23] and modified by GREGG[24]. ADP:O ratios were calculated by the method of CHANCE AND WILLIAMS[25].

Oligomycin was dissolved in 100 % methanol. Sodium atractylate was obtained as a gift from Dr. A. L. Lehninger and was dissolved in triple distilled water. All other reagents were dissolved in triple distilled water and adjusted to pH 6.5 with NaOH or HCl.

For electron microscopy mitochondria were fixed in 6.25 % glutaraldehyde and 1 % OsO_4 in 0.1 M phosphate buffer (pH 7.2). Thin sections were stained with 1 % uranyl acetate and lead citrate[26].

RESULTS

Effect of varying homogenization conditions

In preliminary experiments the effectiveness of the colloid mill was evaluated with respect to the many variables involved in homogenization. Fig. 1 illustrates the effects on cell breakage obtained by altering homogenization conditions. From these results conditions were selected which were as mild as possible, but which yielded a high proportion of cell breakage. Mill speed (Powerstat setting), a primary determinant of shear forces, has little effect on the degree of cell rupture and can therefore be kept relatively low, thereby improving mitochondrial quality. Changes in gap, homogenization time, and glass bead concentration all altered the extent of cell rupture (Fig. 1). These variables have all been optimized for maximum cell breakage and minimal mitochondrial damage.

Similarly, cell content of the homogenization slurry was varied, using cell suspensions of 40, 50 and 60 % (w/v). With a gap of 0.040 inch, a Powerstat setting of 25, and a homogenization time of 8 min, a progressive increase in per cent cell rupture was observed up to 60–70 % with the 60 % cell suspension.

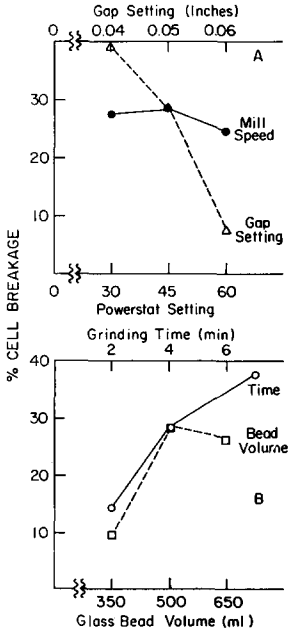

Fig. 1. Effects of alterations in homogenization conditions on the extent of cell breakage. 10 l of a bakers' yeast cell suspension (400 g wet weight/l) were prepared in 0.3 M mannitol homogenization medium. 1-l aliquots were removed and homogenized in the colloid mill. The basic homogenization conditions were: speed 45, gap 0.050 inch, time 4 min, glass beads 500 ml. These conditions were altered, one variable at a time, while retaining all other variables in the basic conditions. 3 samples were taken from each aliquot before and after homogenization and cells counted using a hemocytometer and standard cell counting techniques.

The above experiments were conducted with a constant weight of cells (400 g wet weight). By combining 100 ml of homogenization medium with 50, 100 or 200 g (wet weight) of cells and homogenizing in the presence of an equal volume of glass beads, under the same conditions, cell rupture of 50–65 % was obtained. The yield of intact mitochondria ranged from 13 to 25 mg protein per 100 g cells, a value comparable to that obtained with the 400-g preparation.

Effect of Mg^{2+} on respiration

In early experiments mitochondria isolated in 0.3 M mannitol, using high mill speeds, exhibited respiratory control only in the absence of Mg^{2+}, thus indicating a considerable loss of structural integrity. It was possible to decrease the apparent mitochondrial damage by the combined use of milder homogenization conditions and media of high osmolarity. With 0.6 M mannitol in preparatory and assay media the specific respiratory rates were depressed as expected[27], but respiratory control ratios of 2.0 or greater were retained even in the presence of 10 mM Mg^{2+}.

Effect of pH on respiration

Respiratory control of damaged mitochondria may be improved by lowering the pH of the reaction medium. OHNISHI, KAWAGUCHI AND HAGIHARA[16] have indicated that the optimum pH for respiratory control in yeast mitochondria lies well below pH 7.0. Our findings indicate that the pH optimum for respiratory control is a function of the quality of the mitochondrial preparation. Thus the less intact the mitochondria, the lower will be the observed pH optimum for respiratory control. Conversely, with a highly intact preparation, respiratory control is essentially independent of pH between 6.0 and 7.5. From the preceding results it may be concluded that a valid estimation of mitochondrial intactness must take into account the effect of those variables which govern the assay of respiratory control.

Effect of mannitol on O_2 solubility

Since in mammalian mitochondrial assays relatively low osmolarities are employed, the effects of solute concentration on oxygen solubility and response of the oxygen cathode are often ignored[28]. However, at high osmolarities the oxygen content will be greatly depressed[29]. Hence, Winkler assays[21] of the oxygen content in reaction media of varying mannitol concentration were performed. The results, as presented in Table I, were used in all P:O and Q_{O_2} calculations.

TABLE I

OXYGEN CONTENT OF MANNITOL SOLUTIONS

In addition to mannitol, all solutions except the distilled water contained 10 mM sodium phosphate buffer, pH 6.5. Autoclaved water was used in all solutions and each solution was equilibrated with air for 2 h at 25°.

Sample	*Temperature*	*Oxygen content* (μM)
Distilled water	25	245 ± 3
0.2 M mannitol	25	232 ± 2
0.4 M mannitol	25	213 ± 1
0.6 M mannitol	25	196 ± 2

The polarographic tracings presented in Fig. 2 indicate the high quality of yeast mitochondria which we now routinely prepare. Respiratory control ratios greater than 3.0 may be obtained with succinate as substrate, while values of 4.0 to 6.0 may be obtained with α-ketoglutarate as substrate. Like other investigators[16,30,31] we find an apparent absence of site I phosphorylation in Saccharomyces mitochondria. Thus, as shown in Table II, P:O or ADP:O ratios of 2 are obtained with either pyridine nucleotide-linked substrate or with succinate.

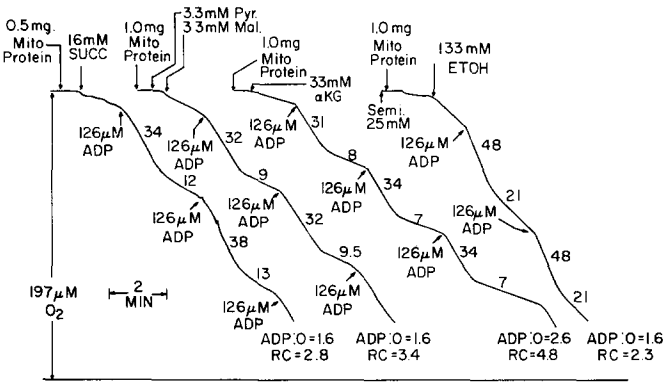

Fig. 2. Polarographic tracings illustrating the respiratory capacity of Saccharomyces mitochondria with 4 representative substrates. SUCC, succinate; αKG, α-ketoglutarate; Pyr., pyruvate; Mal., malate; ETOH, ethanol; Semi., semicarbazide. Substrates and ADP are indicated as final concentrations. Numbers accompanying the tracings indicate the respiratory rates in μM O_2/min. ADP:O and respiratory control ratios (RC) for each substrate are presented below their respective tracings. Standard reaction medium at 25 ± 0.5° was employed in the 3.0-ml system. Mitochondria were isolated according to the "modified method" described in the text, after homogenizing 400 g cells for 8 min at speed 25, gap 40.

TABLE II

P:O AND ADP:O VALUES FOR TWO SUBSTRATES

Mitochondria were prepared and ADP:O values determined in 0.6 M mannitol solutions under the conditions described in Fig. 2. ^{32}P:O values were determined for the same mitochondrial preparation using the methods described in the text.

Substrate	*Rotenone (μM)*	*2,4-Dinitrophenol (μM)*	*ΔO (mμatoms)*	*Esterified ^{32}P (mμatoms)*	*^{32}P:O*	*ADP:O*
Succinate	—	—	803	1424	1.8	1.7
Ethanol	—	—	803	1412	1.8	1.6
Ethanol	—	—	770	1402	1.8	1.6
Ethanol	20.8	—	341	690	2.0	—
Ethanol	8.3	—	440	837	1.9	—
Ethanol		166	660	266	0.4	—

Affinities for ADP and phosphate

Analysis of the yeast mitochondrial affinity for ADP yielded an apparent K_m of 26.5 μM which is in good agreement with values reported for mammalian mitochondria[32].

Like mammalian mitochondria[33], yeast mitochondria exhibit a specific requirement for ADP as a phosphate acceptor in State 3 respiration. State 3 respiration

was not initiated by CDP, UDP, GDP or IDP when tested at the same concentration as ADP or at higher concentrations.

During malate *plus* pyruvate oxidation, a value of 1.6 mM phosphate was obtained for half-maximal stimulation of State 3 respiration (Fig. 3). This value is similar to that obtained for rat liver mitochondria oxidizing β-hydroxybutyrate[32], but dissimilar to values observed with rat liver or pigeon heart mitochondria during succinate *plus* glutamate oxidation[32]. With yeast mitochondria, oxidizing succinate, phosphate acts as a regulator of State 4 respiration, or as a "coupling" factor (Fig. 4).

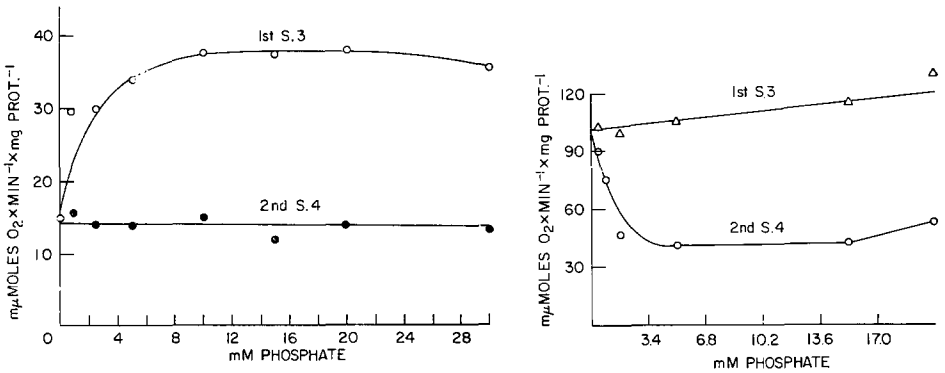

Fig. 3. The effect of phosphate concentration on malate-supported respiration. Mitochondria were added to reaction medium in which the phosphate concentrations were varied to give the indicated final concentrations at pH 6.5. Reactions were initiated by addition of 30 mM malate and 3.0 mM pyruvate. After 2 min 125 μM ADP was added to initiate State 3 (S.3). The State 4 (S.4) rate is that obtained after ADP or phosphate exhaustion. Other conditions were as described in Fig. 2.

Fig. 4. The effect of phosphate on succinate-supported respiration. Except for the utilization of succinate (16.6 mM) as substrate, all other conditions were similar to those described in Fig. 3.

Thus in the absence of ADP, and at low phosphate concentration, the respiratory rate approximates the maximum rate observed under conditions of high phosphate and non-limiting ADP (*i.e.*, State 3 conditions). In the absence of phosphate and with succinate as substrate, *S. cerevisiae* mitochondria thus appear to be loosely coupled or uncoupled.

Effects of oligomycin and atractyloside

In order to further investigate the phosphorylation mechanism of mitochondria, oligomycin and atractyloside were characterized with respect to their ability to inhibit coupled respiration. In agreement with OHNISHI, KAWAGUCHI AND HAGIHARA[16] we find yeast mitochondria to be considerably less sensitive to oligomycin than mammalian mitochondria, with 100 % inhibition of State 3 respiration occurring at 8.5 μg oligomycin per mg protein, and 50 % inhibition at 3.4 μg per mg. On the other hand, atractyloside, in the presence of 200 μM ADP, produced a 50 % inhibition of State 3 respiration at 4.4 μM, a value comparable to that reported for mammalian mitochondria by VIGNAIS *et al.*[34]. Complete inhibition was obtained at 30 μM. Like the latter investigators, we find that atractyloside inhibition may be completely reversed by high concentrations of ADP.

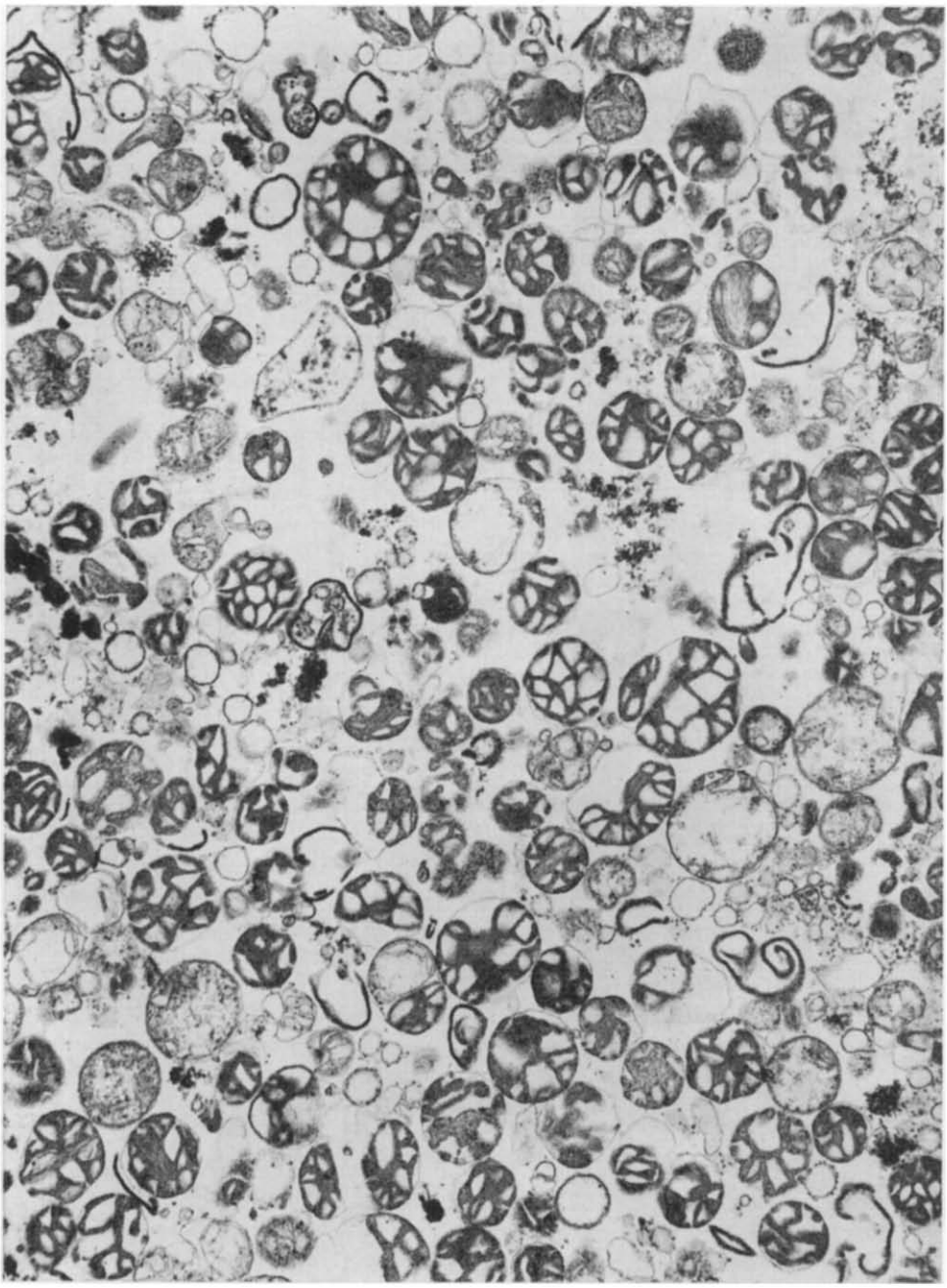

Fig. 5. Electron micrograph of freshly prepared *S. cerevisiae* mitochondria fixed with glutaraldehyde and OsO_4, and stained with uranyl acetate *plus* lead citrate as described in the text. 20000 ×.

Electron microscopy

Fig. 5 presents an electron micrograph of yeast mitochondria which were prepared as described in this paper. While contamination is present, the mitochondria themselves appear to be reasonably intact. In comparison with previously published electron micrographs of yeast mitochondrial preparations[16,35] these mitochondria appear to be as well, if not better, preserved.

DISCUSSION

Although the yield of mitochondria obtained from this procedure is relatively low, the method is simple, and the organelles appear to be equivalent or superior to those produced by the snail enzyme method[16,35]. This assertion is supported by the finding that the mechanically prepared mitochondria yield higher respiratory control ratios and apparently a more favorable response to alterations in assay conditions.

Damaged mitochondria might be expected to exhibit a distinct dependency on pH while intact mitochondria may react indifferently toward this variable[36,15]. The observations reported here would seem to support this idea.

The effects of Mg^{2+} on the enzymically prepared mitochondria have not been fully documented, although lowering of respiratory control has been reported[15]. EDTA has been routinely used in respiratory assays[15,16,35] and no Mg^{2+} was added in the system used by OHNISHI and coll.[15,16]. EDTA has no beneficial effect on the respiratory control of intact, mechanically prepared mitochondria, and added Mg^{2+} has only a minor effect on this parameter.

Since orthophosphate is an obligatory component of the energy conserving reactions of oxidative phosphorylation, it is significant that a differential effect of phosphate is observed between oxidation of succinate and malate, a pyridine nucleotide-linked substrate. While the mechanism of this effect remains to be elucidated, it may be that the phenomenon reflects a highly labile component of the coupling mechanism in yeast mitochondria. However, a labile, phosphate-dependent coupling factor has not been observed in mammalian mitochondria[32]. Alternatively, the phosphate effect described here may be related to a phosphate-dependent permeation system such as described by CHAPPELL AND HAARHOFF[37]. However, in their analysis, phosphate would appear to be required for any succinate-supported respiration, a situation which is the converse of that observed here.

Like other investigators[16,30,31] we are also unable to demonstrate the presence of Site I phosphorylation activity in Saccharomyces mitochondrial preparations. We are therefore unable to confirm the observations of STEKHOVEN[28,38] who reported P:O ratios of 3 for Saccharomyces mitochondria during pyridine nucleotide-linked substrate oxidation.

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