STUDIES ON THE OXIDATIVE METABOLISM OF *SACCHAROMYCES CEREVISIAE*

II. Morphology and Oxidative Phosphorylation Capacity of Mitochondria and Derived Particles from Baker's Yeast

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ABSTRACT

A specially designed high-speed blendor and glass beads have been used to disintegrate yeast cells. The method enables large quantities of cells to be fragmented quickly at low temperature, and cell-free mitochondrial particles to be prepared in high yield. The particles are isolated in a sucrose-Tris-EDTA medium and extensively refractionated in the same medium. The success of the fractionation is dependent upon the presence of the Tris buffer, as the latter prevents the aggregation of the particulate material. Two morphologically and enzymatically different particle types have been obtained: a heavy fraction corresponding to mitochondria in size and internal organization, and a light fraction consisting of vesicular, single-membrane particles of a smaller size. The light particles oxidize DPNH and succinate, but do not oxidize pyruvate-malate, and lack the capacity for phosphorylation. The heavy particles oxidize pyruvate-malate as well as the citric acid cycle intermediates, although their α-ketoglutaric dehydrogenase activity is low. Oxidation by the heavy particles is coupled to phosphorylation, and P/O ratios of about 1.5 have been obtained. Lactic acid dehydrogenase is also present in the heavy fraction, and lactate is oxidized with a P/O ratio of about 0.7.

INTRODUCTION

Yeast is one of the simplest organisms in which the existence of mitochondria has been demonstrated (1, 24). Attempts have been made in the past to isolate yeast mitochondria, and cell-free particles capable of terminal oxidation and some phosphorylation have been described (18, 22). The isolation of mitochondrial fractions catalyzing the oxidation of the citric acid cycle substrates has also been reported (15, 23), but the rates of the enzymatic activity indicated that those fractions probably contained some particulate material other than mitochondria.

A serious limitation imposed on previous studies of yeast particulate fractions has been the difficulty of preparing the material in sufficient quantity for further fractionation. The development of a simple cell-breaking device capable of rupturing 100 ml. of yeast suspension every 4 minutes has now overcome this limitation.

The present communication is an extension of the earlier studies (15, 16) and describes the subfractionation of cell-free particulate material. The morphology of the isolated mitochondria and derived particles, as seen in the electron microscope, is correlated with their ability to catalyze the citric acid cycle and terminal oxidations

together with oxidative phosphorylation. The morphology of the mitochondria *in situ* is described in the preceding paper (24).

METHODS

Cell Disintegration

A stainless steel high-speed blendor especially designed for the rupture of large quantities of yeast with glass beads was used. The machine is similar to the well known Waring blendor with modifications to prevent glass beads from entering the motor and to permit temperature regulation. An overhead direct drive motor is situated on top of a serrated stainless steel bowl of about 350 ml. capacity. The temperature is controlled throughout the operation by immersion of the bowl in an alcohol–dry ice bath. Details of this apparatus will be described in a separate publication currently in preparation.

Freshly grown baker's yeast was obtained from Mauri Bros. & Thomson Ltd., Sydney, every day. The cells were thoroughly washed in water and then cooled in ice. All subsequent steps were carried out at 0–2°C.

The packed cells were suspended in an equal volume of medium containing 1.0 M sucrose, 0.02 M tris-(hydroxy-methyl)-amino methane (Tris)[1] buffer, and 0.001 M sodium ethylenediamine tetraacetate (EDTA),[1] pH 7.4. The pH of the suspension was adjusted to about 7.2–7.4 with 1 N KOH, using bromthymol blue as an external indicator, and maintained at this level throughout the preparative procedure.

Aliquots of the cell suspension (100 ml.), together with 130 ml. of glass beads (average diameter 0.15 mm.), were placed in the steel blendor bowl and homogenized at top speed (about 15,000 r.p.m.) for 3 to 4 minutes. The contents of the blendor were washed into a measuring cylinder with a solution containing 0.5 M sucrose, 0.01 M Tris, and 0.0005 M EDTA, pH 7.4, and made up to 250 ml. The glass beads were allowed to settle, the supernatant was decanted, and the beads were washed with a further 100 ml. of the above medium. Four disintegrations were carried out successively and the supernatants and washings pooled. The combined material was then centrifuged at 2400 *g* for 20 minutes and the sediment containing unbroken cells and debris was discarded. Particulate material was isolated from the supernatant by centrifuging at 20,000 *g* for 15 minutes in the Spinco Model L preparative ultracentrifuge. The sediment was collected, washed in the sucrose-Tris-EDTA medium, and used for experiments or fractionated further as described in the text. The yield of protein in the sediment at this stage was about 1200 mg.

[1] The following abbreviations are used in the text: ATP, adenosine triphosphate; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; EDTA, ethylenediamine tetraacetate; GTP, guanosine triphosphate; Tris, tris-(hydroxymethyl)-amino methane.

Reagents

All chemicals used were commercial products, except yeast hexokinase, which was prepared to stage 3*a* by the method of Berger *et al.* (4).

Assay Methods

Inorganic phosphate was determined by the method of Allen (2), and protein was estimated by the biuret method of Gornall *et al.* (7) using crystalline bovine serum albumin as a standard. α-Keto acids were determined by the method of Koepsell and Sharpe (11), and α-ketoglutaric dehydrogenase activity was measured by the method of Sanadi *et al.* (19).

Manometric measurements were made in air at 30°C. by the conventional Warburg technique. The values for oxygen uptake given in the text have been corrected for "blank" oxidation. The duration of experiments, excluding equilibration period (6 minutes), was 15 to 30 minutes. Under standard conditions for manometric assay of oxidative phosphorylation each manometer contained: ATP, 5 μmoles; $MgSO_4$, 10 μmoles; potassium phosphate, pH 7.4, 30 μmoles; glucose, 100 μmoles; yeast hexokinase, an amount capable of esterifying 3 μmoles of glucose per minute; sucrose, 1.5 mmoles; NaF, 60 μmoles; bovine serum albumin, 10 mg.; thiamine pyrophosphate, 0.3 μmoles; DPN, 0.5 μmoles; Tris buffer, 10 μmoles (added with the particle suspension); EDTA, 0.5 μmoles (added with particle suspension); and 1.0 ml. of particle suspension containing 2 to 8 mg. of protein. The amounts of substrate added to the manometers, except where otherwise indicated, were 40 μmoles of succinate, pyruvate, citrate, α-ketoglutarate, lactate, or ethanol, 5 μmoles of malate, and 10 μmoles of DPNH. The total volume added to each flask was 3.0 ml., excluding alkali in the centre well (0.2 ml.).

Phosphorylation was determined by measuring the decrease of inorganic phosphate in the reaction medium.

Electron Microscopy

For electron microscope studies the particle suspensions were fixed for 30 to 60 minutes with 1 per cent osmium tetroxide, buffered at pH 7.2 with 0.02 M veronal acetate. The fixed material was washed in water, dehydrated, embedded in methacrylate, and sectioned as described in the preceding paper (24).

RESULTS

Subfractionation of Particulate Material

The sediment isolated from disintegrated yeast contains about 10 to 12 per cent of the total protein of the cell-free homogenate. It oxidizes various substrates and is capable of oxidative phosphorylation, but the low P/O ratios obtained in the presence of succinate as well as very low rates of pyruvate-malate oxidation (Table I) suggested that the material might be a mixture of metabolically different particle types. Subfractionation of the material was therefore attempted along the lines developed by Hatefi and Lester (9) for beef heart mitochondria. The procedure finally adopted is set out in Fig. 1.

For this fractionation the particulate material was suspended in the sucrose-Tris-EDTA medium to contain 7 to 12 mg. of protein per ml., and the thick suspension centrifuged at 40,000 *g* (average) for 20 minutes in the No. 40 rotor of the Spinco Model L ultracentrifuge. The resulting pellet was distinctly stratified, containing a dark-brown bottom layer (Heavy I), an intermediate one, and a reddish-brown layer on the top (Light I). The clear supernatant was discarded and the upper layer (Light I) resuspended in fresh sucrose medium and removed. The intermediate layer was similarly resuspended and discarded. The remaining bottom layer (Heavy I) was resuspended in fresh medium and the centrifugation procedure repeated twice in exactly the same manner. Each time the two upper layers were discarded to leave, in turn, Heavy II and Heavy III. Light I was also recentrifuged under the same conditions, but on this occasion the top layer was collected (Light II) and the two bottom layers discarded. Before use in the assays each fraction was washed and finally resuspended in the sucrose medium. Most of the initial sediment was made up of the light fraction, the heavy particles (Heavy III) constituting only 5 to 10 per cent of the total protein.

The results presented in Table I show typical oxidation rates and P/O ratios observed with the fractions at successive stages of the purification. The light and heavy fractions were distinctly different in metabolic activity. With succinate the oxidation rate remained more or less constant in all fractions, but the P/O ratio in the heavy fraction increased about tenfold (to 1.4) as compared with the initial sediment. There was no phosphate esterification in the light fraction. On the other hand, the oxidation rate of pyruvate-malate increased very considerably with the purification of the heavy fraction to a rate similar to that for succinate oxidation, whereas the light fraction did not oxidize pyruvate-malate. The P/O ratios associated with pyruvate-malate oxidation were largely the same wherever this substrate was oxidized.

TABLE I

Oxidative and Phosphorylative Activity of Particles at Various Stages of Fractionation

Fraction*	Succinate		Pyruvate-malate	
	Oxidation rate‡	P/O	Oxidation rate‡	P/O
Initial sediment	0.14	0.1	0.02	1.2
Light I	0.11	0.0	0.01	1.2
Light II	0.14	0.0	0.00	—
Heavy I	0.13	0.8	0.05	1.2
Heavy II	0.12	1.0	0.12	1.3
Heavy III	0.13	1.4	0.15	1.4

Standard assay conditions.
* Fractions designated as indicated in Fig. 1.
‡ μatoms of oxygen/min./mg. protein.

The light fraction has proved to be an excellent source material for the preparation of a yeast particle capable of very rapid aerobic oxidation of succinate and DPNH. Further enzymatic studies on this fraction are in progress and will be the subject of another communication.

Oxidation of Substrates Coupled to Phosphorylation

The oxidation rates and P/O ratios obtained with fraction Heavy III in the presence of various substrates are given in Table II. As indicated, the results show some variation over a large number of experiments. This is mainly due to the efficiency of separation of the various layers throughout the procedure of fractionation. The boundary between the heavy and intermediate layers is not always distinct, and it is difficult on occasions to separate them completely. The intermediate layer appears to be a mixture of the heavy and the light particles.

All members of the citric acid cycle were oxidized at a rapid rate with the exception of α-ketoglutarate, which was oxidized very slowly. For the oxidation of pyruvate a source of oxalacetate was

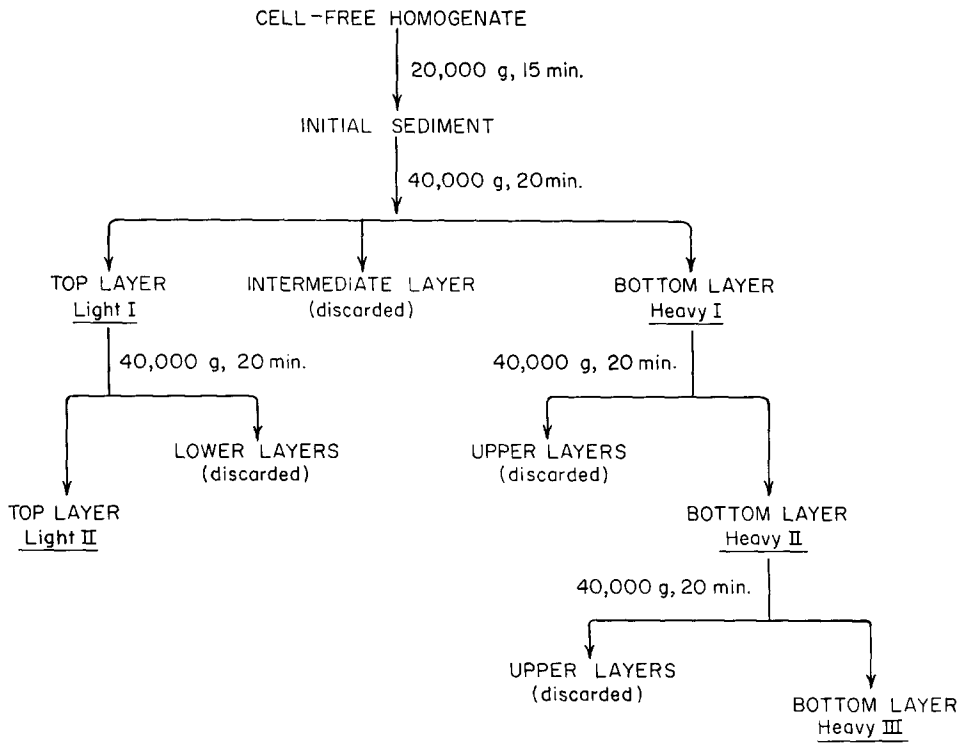


FIGURE 1

Scheme for the subfractionation of cell-free particles

required which could be supplied by malate, fumarate, or succinate. Because of the low oxidation rate of α-ketoglutarate this intermediate as well as citrate failed to "spark" pyruvate oxidation.

The oxidation of α-ketoglutarate was not stimulated by additions of coenzyme A or GTP. When citrate was rapidly oxidized by the particles in experiments of short duration, almost equimolar amounts of α-ketoglutarate accumulated in the reaction mixture (Table III). If, however, a small amount of citrate (5 μmoles) was used and the experiment continued over a long period, a smaller proportion of α-ketoglutarate was recovered. The same was observed when pyruvate-malate was used as substrate (Table III). Direct assay of the α-ketoglutaric dehydrogenase complex showed that the slow oxidation of α-ketoglutarate was due to the low concentration of this enzyme in the cell. It was not found to occur in significant amounts in any of the cell fractions other than the heavy particles.

The standard assay system contained added DPN, but the cycle substrates were also oxidized without the addition of this compound. This indicates that significant quantities of bound DPN are present in the heavy particles. However, the oxidation rates of citrate and of pyruvate-malate were usually increased in the presence of added DPN by about 50 and 100 per cent, respectively. On the other hand, the P/O ratios observed with different substrates were not greatly influenced by external DPN concentration. Added DPNH in substrate amounts was very rapidly oxidized and the oxidation was coupled to phosphorylation with a P/O ratio of about 1.5 (Table II). This value is the same as that obtained in the oxidation of substrates by enzymes requiring DPN as a cofactor, so that these preparations do not distinguish between free DPNH and bound DPNH.

Yeast lactic dehydrogenase has been shown by Appleby and Morton (3) to be a cytochrome b_2 hemoflavoprotein, and it was of interest to determine whether it is localized in the mitochondria together with the other cytochromes. Lactic acid was found to be oxidized by the heavy fraction

with an observed P/O ratio of around 0.7. In the presence of small amounts of malate, the oxidation rate was doubled and the P/O ratio increased to about 1.0 (Table II).

Ethanol alone was found to be very slowly oxidized by the heavy fraction, with a P/O ratio exceeding 1.0. The presence of an aldehyde trapping reagent such as semicarbazide, to remove acetaldehyde, considerably increased the rate of ethanol oxidation without affecting the P/O ratio.

Phosphorylation in presence of any substrate was completely uncoupled by 2:4-dinitrophenol (10^{-3} M).

Effect of EDTA and Sucrose

Under standard conditions the heavy particles were isolated in the presence of 0.0005 M EDTA, and this reagent was essential for maximal activities. Heavy particles isolated in sucrose-Tris medium, which did not contain EDTA, barely oxidized pyruvate-malate, while succinate and citrate oxidation rates were largely unchanged (Table IV). Phosphorylation associated with succinate oxidation was about halved, while with citrate the P/O ratio remained more or less constant. The concentration of EDTA was not critical over a range of 0.005 M to 0.0002 M.

Sucrose concentration was varied in the isolation medium from 0.25 M to 0.8 M without striking effect on the activity of the heavy particles. However, variations in the concentration of sucrose in the assay system did influence the activity of the particles. The standard procedure was to assay the preparations in 0.5 M sucrose. Sucrose concentrations greater than 0.5 M (up to 0.8 M) were largely without effect on both the succinate and

TABLE II

Oxidative and Phosphorylative Activity of Heavy Particles

Substrate	Oxidation rate*	P/O
Succinate	0.10–0.18	1.0–1.6
Pyruvate	0.005	—
Malate	0.005	—
Pyruvate-malate	0.10–0.18	1.0–1.6
Pyruvate-malate (2 μmoles)	0.10	1.4
Pyruvate-fumarate (2 μmoles)	0.10	1.3
Pyruvate-succinate (2 μmoles)	0.08	1.4
Pyruvate-α-ketoglutarate (2 μmoles)	0.005	—
Pyruvate-citrate (2 μmoles)	0.005	—
Citrate	0.10–0.21	1.0–1.5
α-Ketoglutarate	0.01–0.02	1.0–1.5
Lactate	0.07–0.10	0.6–0.7
Lactate-malate	0.15–0.20	0.8–1.1
Ethanol + semicarbazide (10 μmoles)	0.04	1.2
DPNH	0.19–0.23	1.1–1.6

Standard assay conditions.

* μatoms of oxygen/min./mg. protein. These rates have been corrected for the oxidation of the "sparker" compound.

TABLE III

Accumulation of α-Ketoglutarate during the Oxidation of Citrate and Pyruvate-Malate by Heavy Particles

Substrate	Duration of expt.	Oxygen uptake	Accumulated α-ketoglutarate
	min.	*μatoms*	*μmoles*
Citrate (40 μmoles)	20	19.3	18.0
Citrate (5 μmoles)	45	8.8	2.7
Pyruvate (40 μmoles) + malate (5 μmoles)	15	15.4	4.6
Pyruvate (5 μmoles) + malate (5 μmoles)	45	17.6	3.7

TABLE IV

The Effect of EDTA on Oxidation and Phosphorylation by Heavy Particles

Fraction Heavy III used for all assays. The EDTA concentrations cited refer to the isolation medium, which was composed of 0.5 M sucrose, 0.01 M Tris, pH 7.4, and EDTA where indicated. Other conditions of assay as described in the section on Methods.

	EDTA (μmoles/ml.)			
	0		0.2 to 5.0	
Substrate	Oxidation rate*	P/O	Oxidation rate*	P/O
Pyruvate-malate	0.01	—	0.12	1.4
Citrate	0.16	1.1	0.14	1.3
Succinate	0.12	0.6	0.13	1.5

* μatoms of oxygen/min./mg. protein.

the pyruvate-malate systems. However, at lower sucrose concentrations (0.08 M and 0.17 M) succinate oxidation rate decreased by some 50 per cent, while the P/O ratio tended to increase up to 1.8. On the other hand, pyruvate-malate at these sucrose concentrations was oxidized by the heavy particles at a greatly increased rate (100 per cent increase), while the P/O ratio decreased by about 20 per cent.

Morphology of the Isolated Particles

Yeast mitochondria as described in the preceding paper (24) are morphologically similar to those present in the cells of higher organisms, except for their smaller size (0.2 to 0.6 μ). The morphology of particles in the isolated fractions—initial sediment, Heavy III, and Light II—are shown, respectively, in Figs. 2, 3, and 4.

The unfractionated sediment consists of a wide variety of particle types ranging from those that can be recognized as partially degraded mitochondrial forms to empty vesicles and some structureless material.

The fraction Heavy III is made up of the more intact mitochondrial forms originally present in the unfractionated sediment. Compared with yeast mitochondria *in situ*, these particles have undergone some structural change, but many still show the presence of cristae and internal organization while others are more swollen and show little internal structure. On the whole, this fraction is similar in morphology to isolated mitochondria prepared from other cells.

The fraction Light II is composed of small vesicles and some amorphous material. Studies now in progress (to be published) have shown that the vesicular material is of mitochondrial origin and that it is similar to the electron transport particles derived from heart mitochondria as reported by Ziegler *et al.* (25).

DISCUSSION

Previously the most satisfactory way to fragment yeast cells for the study of particulate enzyme systems has been the shaking of a mixture of glass beads and cells at high speed in a Nossal shaker (17, 15, 22). However, the small amount of material which could be processed by this machine was a serious limitation to further study of the mitochondrial systems. The high-speed blendor used in this study now enables the convenient handling of large quantities of cells and permits the isolation of cell-free particles in sufficient quantity for further fractionation and study.

The procedure described for the subfractionation of the initial particle sediment is similar to that developed for the study of heart mitochondria (9). The important feature of the method is the use of Tris buffer in the fractionation medium. In the absence of buffer, or when it is replaced by phosphate or β-glycerophosphate, the particles aggregate and are not separable.

The morphology and biochemical properties of the yeast fractions also resemble those of the heart particles (25). The light fraction is similar in biochemical properties to the electron transport particle described by Green and associates (8). It catalyzes the rapid aerobic oxidation of DPNH and succinate, but is unable to oxidize the substrates of the citric acid cycle and has no capacity for phosphorylation. The ability to oxidize pyruvate-malate and the capacity for oxidative phosphorylation are confined to the heavy or more intact mitochondrial fraction. The oxidation rate of pyruvate-malate by isolated yeast mitochondria is of about the same order as that by liver and

Explanation of Figures

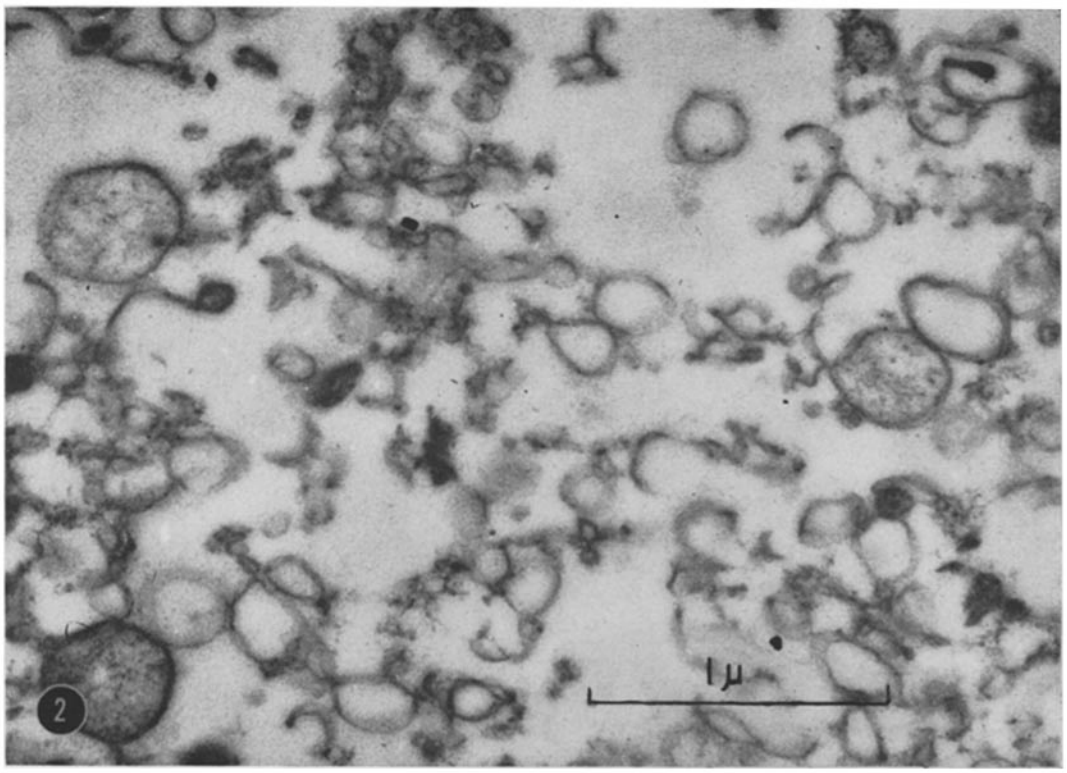
Electron micrographs of sections through different yeast particle fractions, fixed in osmium tetroxide. × 40,000.

FIGURE 2

Initial sediment. Several forms suggestive of mitochondria are evident, together with considerable amounts of vesicular and structureless material.

FIGURE 3

Heavy particle fraction, "Heavy III." Variation in particle types is apparent, but the particles are mainly mitochondrial in form and show some retention of cristal structure.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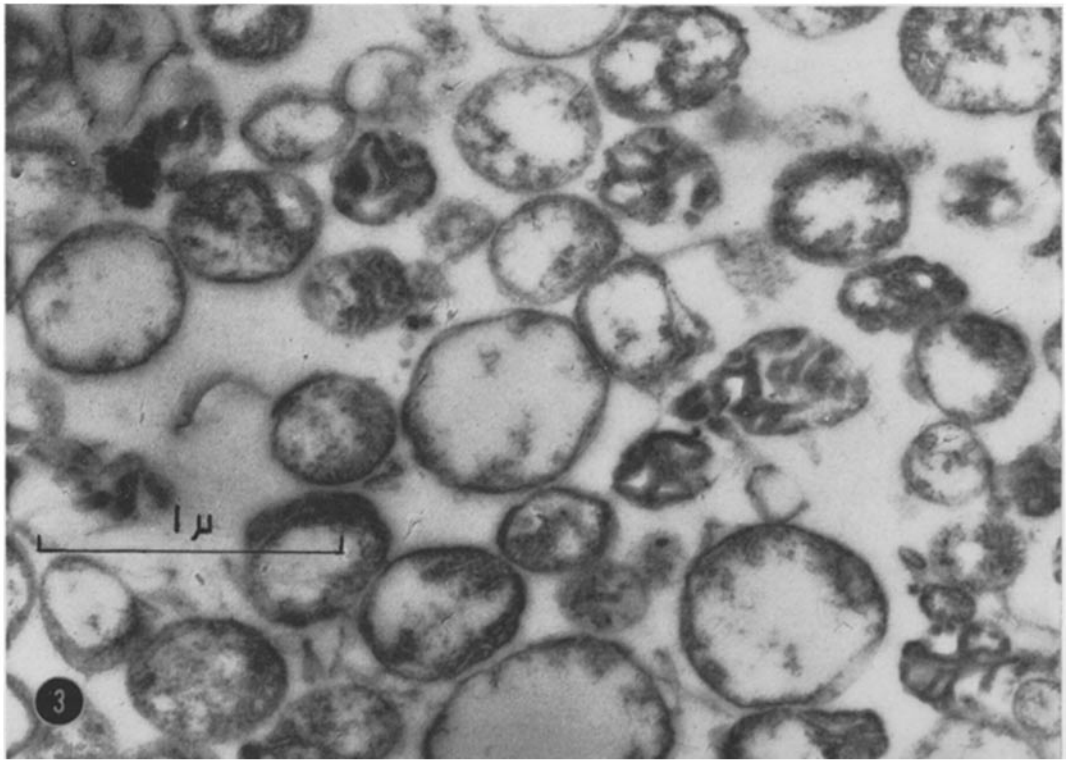
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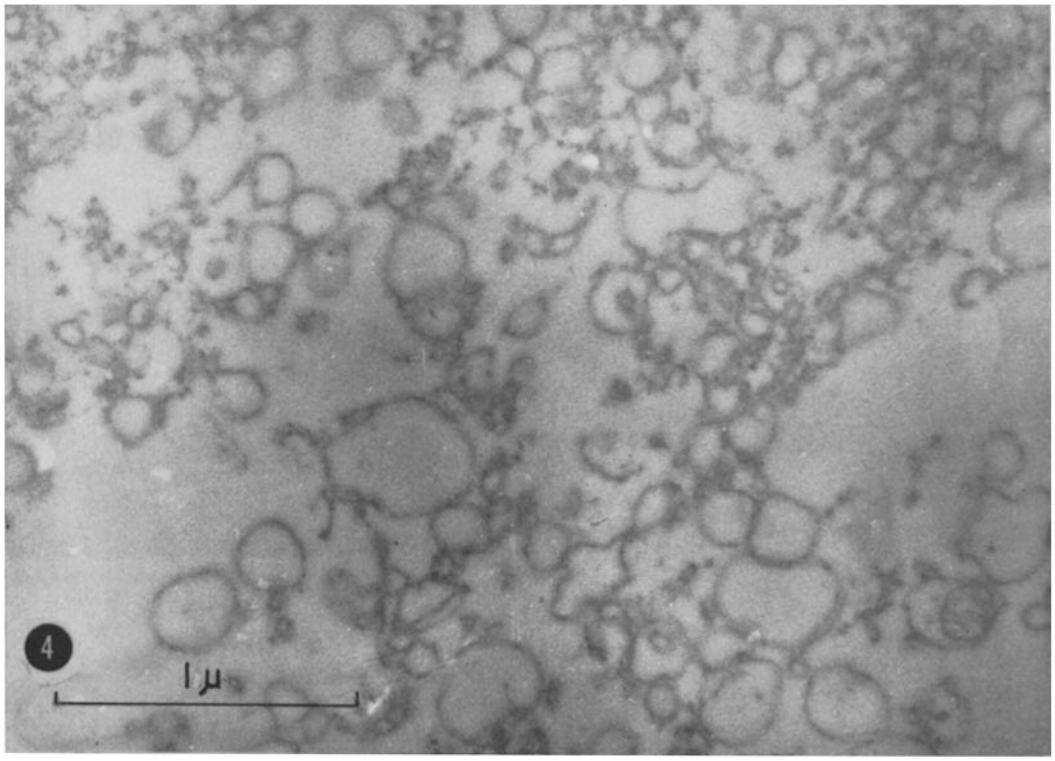

FIGURE 4

Electron micrograph of a section through the light particle fraction, "Light II." This contains mainly vesicles together with some amorphous material and small granules. × 40,000.

heart mitochondria, but the observed P/O ratios are lower and similar to those observed with succinate. The initial particle sediment contains considerable amounts of material capable of succinate oxidation, but unable to couple such oxidation to phosphorylation. During the preparation of the heavy fraction the non-phosphorylating particles are removed, and the final material oxidizes succinate with a P/O ratio close to the theoretical value of 2.0. Recently Utter *et al.* (22) have described a particle system from yeast which slowly oxidized succinate with a P/O ratio of 0.6. This system did not oxidize any of the other citric acid cycle substrates. These particles are therefore intermediate in activity between the heavy and light fractions described herein.

The observed P/O ratios with pyruvate-malate as substrate are lower than the expected theoretical value of 3.0. It was considered that the lower P/O ratio obtained with this substrate might be due to the uncoupled oxidation of DPNH which arose from the reduction of added, unbound DPN, as has been described by Lester *et al.* (14) with heart mitochondrial fragments. However, this does not appear to account for the observations. The same P/O ratio was obtained with substrate amounts of added DPNH as with pyruvate-malate. In addition, the P/O ratio with pyruvate-malate was the same irrespective of the presence of added DPN, although the rate of oxidation of this substrate was considerably increased by the presence of DPN. Thus DPNH oxidation in these preparations results in the same P/O ratio whether it arises from the reduction of DPN bound to the mitochondrial matrix, or from the reduction of added, unbound DPN. The study of "crossover" phenomena in intact yeast cells led Chance (5) to conclude that there are three phosphorylation sites in baker's yeast similar to those found in whole cells and isolated mitochondria of mammalian tissues. If Chance's interpretation may be accepted as definitive evidence, then the isolated yeast mitochondria have lost one of their phosphorylation sites. The two phosphorylation sites in the

electron transport chain common to, or similar for, both succinate and DPNH would be still active.

Yeast lactic dehydrogenase is a flavin-cytochrome b_2 enzyme (3). It might be considered in some respects as being similar to succinic dehydrogenase, except that the latter is linked to cytochrome *b*. The experiments reported herein suggest that the lactic dehydrogenase is localized in the mitochondria and a P/O ratio only about half of that observed with succinate is obtained, *viz.*, less than 1.0. Both substrates eventually donate electrons to cytochrome *c* (3), so that presumably a phosphorylation site common to both of them would be the one involving the oxidation of reduced cytochrome *c*. The reduction of cytochrome *c* via cytochrome b_2 evidently does not involve a site of phosphorylation. In contrast, succinate reduction of cytochrome *c* via cytochrome *b* is known to involve a phosphorylation site (12).

An interesting feature of the heavy fraction is its inability to oxidize α-ketoglutarate, except at very low rates. Determination of the α-ketoglutaric dehydrogenase activity in the soluble fraction and the various yeast particle preparations failed to detect significant concentrations of this enzyme in any fraction other than the mitochondria. The failure to detect the dehydrogenase in the soluble cell fraction precludes the possibility of the enzyme's being lost from the mitochondria during the fractionation procedure. The oxidation of citrate and pyruvate-malate by the isolated mitochondria was demonstrated to lead to α-ketoglutarate accumulation in the reaction medium, so that the low α-ketoglutarate dehydrogenase concentration limits the over-all rate of operation of the citric acid cycle. It can be concluded that in this strain of yeast only a sluggish citric acid cycle would be in operation.

EDTA was first used by Slater and Cleland (20) to stabilize the oxidative activity of heart mitochondria. Subsequently it was shown that EDTA functions to prevent swelling of mitochondria in the isolation medium (10) and the loss of bound DPN (13). No serious enzyme loss evidently occurs, as the addition of DPN restores the oxidation rate. In the present experiments the mitochondria prepared in the absence of EDTA were essentially without activity towards pyruvate-malate irrespective of the presence of added DPN. There appears to be no generalized loss of the soluble enzyme systems, as the oxidation of citrate was unaffected by the absence of EDTA. The results suggest that EDTA may have some specific effect on the pyruvate-malate enzyme system of yeast mitochondria.

It appears that the metabolic capacity of yeast mitochondria may vary a great deal. The low α-ketoglutarate dehydrogenase activity of the mitochondria described herein has not been suggested by earlier studies of such particles (15, 23). Linnane and Still (15) have described a crude yeast mitochondrial fraction which oxidized acetate only in the presence of α-ketoglutarate as a "sparker," presumably involving the conversion of acetate to acetylCoA by a transacetylation reaction between succinylCoA and acetate. On the other hand, crude mitochondrial fractions prepared by Vanderwinkel *et al.* (23) converted acetate directly to acetylCoA and oxidized it via the citric acid cycle. An extreme case of mitochondrial variation is seen with the mutant "petite" yeast described by Ephrussi and associates (6). Mitochondrial preparations from this organism contain the citric acid cycle enzymes (16), but are lacking in a number of the cytochrome enzymes, cytochromes *b*, *a*, and a_3, and therefore are unable to carry out aerobic oxidations. These variations in yeast mitochondrial activity may be ascribed to differences in strains and, in other cases, to different conditions of growth.

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