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Respiratory System of *Candida albicans*

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SUMMARY

Biochemical properties of mitochondria-like particles of *Candida albicans* FIA 1038 were examined and results were as follows:

1) Oxidation of members of the TCA cycle was found in the particles though pyruvate oxidase was not detectable.

2) Succinic dehydrogenase and oxidase activity in these particles was the strongest among the enzymatic activities of the TCA cycle. The dehydrogenase is firmly bound to the particles.

3) The presence of cytochrome oxidase and cytochromes in the particles was proved by experiments on oxidation of reduced cytochrome and PPDA, inhibitory experiments and by measurement of the optical density of cytochrome.

4) The mitochondria-like particles were rich in ribonucleic acid and phospholipids.

5) Observation of the particles was made with an electronmicroscope.

INTRODUCTION

The presence of mitochondria-like particles was reported in baker's yeast by Nossal (1954), Linnan and Still (1955), Agar and Douglas (1957) and Hebb, Montogomery and Slebodnik (1958). Their criteria for mitochondria-like particles can be summarized as follows:

(A) Morphological criteria are:

1) The size should be nearly or less than 1 μ and the shape round, ellipsoidal or rod-like.

2) There is a double-layered membrane.

3) The particles are osmotically sensitive.

4) They can be stained by Janus Green B.

(B) Biochemical criteria are:—

1) The particles contain enzymes of the TCA cycle.

2) Succinic dehydrogenase is firmly bound to the particles.

3) They have a terminal respiratory system.

Merkel and Nickerson (1953) have already reported the presence of "mitochondria" particles in *Candida albicans*. They stained the intracellular particles by TTC, Nile blue and Janus Green B in the presence of the disodium salt of EDTA and demonstrated stained particles released from crushed cells. They also assumed the presence of cytochrome oxidase in their "mitochondria" particles because there was no reduction of Janus Green B by the particles in the presence of CN^-. In 1958, Ward and Nickerson reported results proving the presence of cytochrome c oxidase in normal living cells and isolated mitochondria by differ-

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ential centrifugation. However they failed to prove it and regarded those particles as "mitochondria" deprived of cytochrome c oxidase. They further assumed that "the reduction of oxygen is mediated by flavoprotein oxidases." The reports of Nickerson *et al.* are somewhat confusing and this proof of the presence of mitochondria-like particles are not convincing.

Keeping these criteria in mind, we looked for mitochondria-like particle in *Candida albicans*. The present results are limited to investigations on the enzymatic nature of the subcellular fractions of *Candida albicans* FIA 1038 prepared by grinding cells in sucrose-controlled medium.

Fraction P_2 is mainly composed of particles, which are carrying cytochromes, cytochrome c oxidase and several enzymes of the TCA cycle. The size of these particles is about 0.3 to 1.0 μ and their shape ellipsoid. Experiments to demonstrate a double membrane, osmotic sensitivity and stainability with Janus Green B are still under way. By biochemical criteria, we have isolated mitochondria-like particles.

Materials and Methods

Fungi used: Candida albicans FIA 1038 (maintained in the Research Institute for Microbial Diseases, Osaka University) was grown up twice in CYG culture-medium (Casein acid-hydrolysate, 0.5%; Yeast extract, 0.5%; Glucose, 0.5%) for 24 hours at 35°C. The cells were inoculated into 100ml of fresh CYG medium in a shaking culture and incubated with shaking in air at 35°C.

Assay methods: Manometric experiments were performed at 35°C in air by the Warburg technique. Each flask contained 1.0 ml of the fraction (about 1 mg. protein), and sucrose KH_2PO_4-K_2HPO_4 buffer (pH 7.0) (K-K buffer) in the main chamber, 0.2 ml of neutralized 0.1M substrate in the side arm, and 0.2 ml of 20% KOH or KOH+KCN in the center well. The total volume was 2.2 ml and the final concentration of sucrose in the vessels was kept at 0.25 M. Enzymatic activity was determined during the period of linear oxygen uptake.

Thunberg-tubes were used for the estimation of dehydrogenases of the particulate fraction (P_2). After the particulate fraction had been dialyzed against 0.25 M sucrose K-K buffer for 42 hours at 5°C, it was recovered by centrifugation and resuspended in 0.25 M sucrose K-K buffer (pH 7.0).

Estimation of the cytochrome c oxidase activity and the observation of cytochrome components were made in a Beckman spectrophotometer. Reduced cytochrome c was prepared by the addition of a very small amount of hydrosulfite. Excess hydrosulfite was oxidized by bubbling air through the solution.

Protein was determined with the Folin phenol reagent.

Fractionation of RNA, DNA and phospholipid was performed by the method of Schmidt-Thannhauser. The estimations of RNA, DNA and phospholipid-phosphorus were made by reaction of Orcinol, diphenilamine and Fiske-Subarow reagents respectively.

Electron microscopy of the particles: Samples were fixed with 1% OsO_4-Veronal acetate buffer (pH 7.2) for 30 minutes at room temperature, washed with physiological saline, resuspended in distilled water and shadowed (tan angle $\frac{1}{4}$) with

germanium on a grid. The specimens were photographed under a Hitachi electron microscope (Model HU 9D).

RESULTS

Growth of Candida albicans FIA 1038 :

Cells of *Candida albicans* are usually very hard to destroy. We intended to compare the fragility of *C. albicans* at various growth phase, so it was necessary to study the growth curve of this organism. As there was no report in the literature we studied the growth curve in CYG medium, Figure 1.

After a lag phase for about 3 hours, there was a linear growth phase for about 17 hours, which is followed by a stationary phase.

The preparation of subcellular fractions :

During the logarithmic growth phase, the cells are most fragile when ground. Considering the yield of cells, cultures were used after 15—16 hours growth for preparation of subcellular fractions. The cells were harvested and washed twice with 0.05 M KH_2PO_4-K_2HPO_4 buffer (pH7.0). The pellet was ground in a pestle and mortar with glass powder in the cold and resuspended in cold 0.25 M sucrose K-K buffer (pH 7.0). The disrupted material was centrifuged at 3,000 r.p.m. for 5 minutes to remove the glass powder and a crude extract (CE) was separated. The glass powder was washed with fresh sucrose K-K buffer and centrifuged at the same speed. The resulting supernatants were added to the crude extracts. Precipitates and supernatants obtained from the CE by centrifugation at 6,000 r.p.m. for 20 minutes were named P_1 and S_1 respectively. S_1 was centrifuged at 12,000 r.p.m. (14,500 × g) for 25 minutes and the precipitate P_2 and supernatant S_2 were obtained. Precipitate P_3 and supernatant S_3 were obtained by the centrifugation of S_2 at 30,000 r.p.m. (68,000 × g) for 60 minutes. The precipitates P_1, P_2 and P_3 were resuspended in cold 0.25 M sucrose K-K buffer (pH 7.0). All procedures were performed at 5°C.

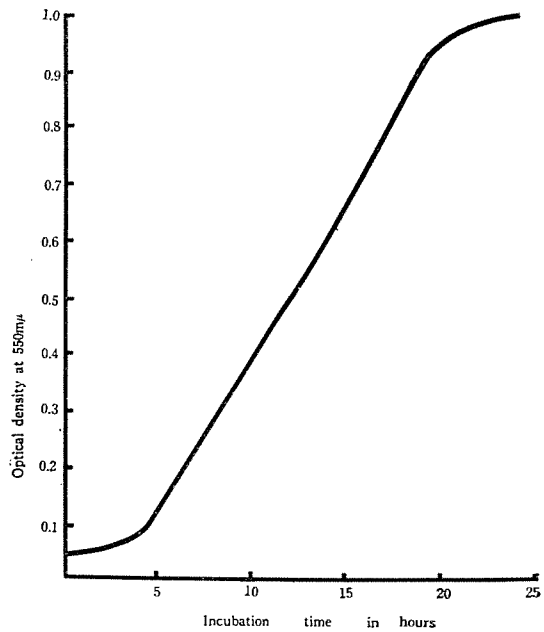

Fig. 1. Growth of *Candida albicans* FIA 1038, at 35°C.

Chemical constitution of each fraction :

The concentration of protein, RNA, DNA and phospholipid-phosphorus were estimated in each fraction and the ratio of RNA/protein in each fraction was compared. The results obtained are shown in Table 1.

Table 1. Chemical constituents of subcellular fractions.

Fraction	RNA/mg. Protein	DNA γ/mg. protein	Phospholipid-phosporus γ/mg. protein
CE	0.17	2.9	1.2
P_1	0.20	1.8	2.7
P_2	0.32	5.4	2.8
P_3	0.30	2.0	2.0
S_3	0.10	0.25	0.45

The three particulate fractions P_1, P_2 and P_3 are rich in RNA, DNA and phospholipid in contrast to those of the supernatant fraction S_3. There is no significant difference in the constitution of these particulate fractions, and the ratio of RNA/protein in fraction P_1 was no larger than that of fractions P_2 and P_3. The chemical constitution of fraction P_2 was very similar to that of fraction P_3. Fraction S_3 was relatively poor in these chemical components.

The low but significant content of DNA per mg. protein in the P_2 particles is quite analogous to that of ghost of *Pseudomonas fluorescens* A3-12 (Nozu *et al.*, in press), though there was a very large ratio of DNA/protein in bacterial cytoplasm.

Oxidative activities in each fraction:

To prove the presence of the TCA cycle in the particulate fraction, the oxidative activity of each fraction for glucose, lactate, succinate, malate and fumarate was estimated and compared with that in intact cells. The results obtained are shown in Table 2.

Table 2. Oxidative activity of intact cells and subcellular fractions

Fraction	Enzymatic activity Q_{O_2} (protein)							
	Glucose	Lactate	Sodium pyruvate	Sodium citrate	Sodium α-keto-glutarate	Sodium succinate	Sodium malate	Sodium fumarate
Intact cell*	210	93	0	0	0	0	0	0
Crude extract	3	2	0	4	5	16	1	5
P_1	15	—	0	9	18	20	7	7
S_1	5	—	0	3	1	11	4	8
P_2	12	4	0	3	4	81	33	30
S_2	3	2	0	2	3	11	4	5
P_3	0	—	0	0	1	12	0	1
S_3	1	—	0	1	1	1	1	1

*Values in intact cells are expressed per mg. dry weight.

Glucose and lactate were oxidized by the intact cells (IC) of *C. albicans* but other TCA-cycle members were not oxidized.

Fraction P_2 contains all the enzymes of the TCA cycle and there is high succinoxidase activity in this fraction. Succinoxidase is the only enzyme detectable

in fraction P_3.

Though TCA cycle enzymes were not detectable in IC, they were found in fraction P_2. The absence of enzymatic activity in IC is perhaps due to low penetration through the thick cell wall of *C. albicans*. Pyruvic oxidase could not be found in any fraction, though Linnan and Still (1955) by the addition of other TCA cycle members to pyruvate demonstrated pyruvic oxidase in isolated mitochondria of *Saccharomyces cerevisiae*.

Dehydrogenase activities:

From the above experiments, it is obvious that P_2 particles contain enzymes of the TCA cycle. In the following experiment, the second biochemical criterion was examined. The succinic dehydrogenase activity was estimated on dialyzed fraction P_2 in the absence and presence of DPN. The results are shown in Table 3.

Table 3. Dehydrogenase activities in fraction P_2

Substrate	Before dialysis	After dialysis	
		DPN (+)	DPN (—)
Succinate	1.0	0.5	0.43
Lactate	0.6	0.06	0.05
Alcohol	0.3	0.01	0.006
Glucose	0.25	0.03	0.00
No substrate	0.03	0.03	0.00
No enzyme no substrate	—	0.00	0.00
No enzyme succinate	—	0.00	0.00

System: fraction P_2, 1.0 ml (0.77 mg protein); 0.1% DPN, 0.5ml; Methylene blue (in 10,000), 0.7ml; and substrate 1.0 ml (0.1M succinate, 0.1M lactate, 0.1M glucose, 1M ethanol); and 0.25M sucrose K.-K buffer (pH 7.0).
The Final volume was 5.0 ml and final concentration of reaction system was adjusted to 0.25M. When alcohol was used as substrate, hydrosulfite was added. Temperature 30°C.

The effect of DPN can not be seen with succinic dehydrogenase, but it is significant that enzyme activity is still bound to P_2 fraction after dialysis.

The lactic, glucose and alcohol dehydrogenases activities were estimated in dialyzed preparations in the presence and absence of DPN. Table 3 shows that these dehydrogenases are less active after dialysis. It is interesting that lactic dehydrogenase in *C. albicans* does not seem to be strictly DPN-dependent.

Inhibition of respiration by KCN and NaN_3:

From the above results it is concluded that the TCA cycle is present in fraction P_2, though it is not certain whether it is present in intact cells. In accordance with the third biochemical criterion described above, the terminal respiratory enzyme system should also be found in fraction P_2. To investigate this, the inhibition of KCN and NaN_3 on respiration was studied in intact cell

and fraction P_2 using glucose and succinate as substrates. The final concentration of each inhibitor was 10^{-2}, 10^{-3} and 10^{-4} M. The results obtained are shown in Table 4.

Table 4. Inhibition of respiration by KCN and NaN_3

Fraction	Substrate	Qo_2 (protein)						
		No inhibitor	KCN (M)			NaN_3 (M)		
			10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
Intact cells*	Glucose	71	160	108	100	0	37	77
P_2	Succinate	7.6	0	1.7	3.1			
		30				0	0	16.6

*Values for intact cells expressed per mg dry weight.

The respiration of the intact cell with glucose as substrate was increased in proportion to the concentration of KCN. NaN_3 inhibited the respiration of IC at a concentration of 10^{-2} and 10^{-3} M, but increased it slightly at a concentration of 10^{-4} M. Both KCN and NaN_3 inhibited the succinate oxidation in fraction P_2.

The stimulation of respiration by the inhibitors observed in IC are similar to results of Ward *et al* (1958). Stimulation by KCN may be due to the stimulation of catalase activity. Inhibition of respiration fraction P_2 indicates the presence of cytochrome oxidase in this fraction.

Cytochrome c oxidase:

Since the presence of cytochrome oxidase is indicated by the inhihibitory experiments with KCN and NaN_3, it is necessary to prove the presence of cytochrome c oxidase. Its activity in fraction P_2 was measured by the rate of decrease in the optical density at 550 mμ. The results obtained are shown in Fgiure 2.

Though the activity is not as high as was expected, it is significant. Its presence was also demonstrated by the oxidation of *p*-phenylenediamine (PPDA) and of succinate in the presence of cytochrome c, Table 5. It can therefore be stated that PPDA oxidase and the succinoxidase exist in fraction P_2. Succinoxidase activity was increased by addition of oxidized cytochrome c (yeast).

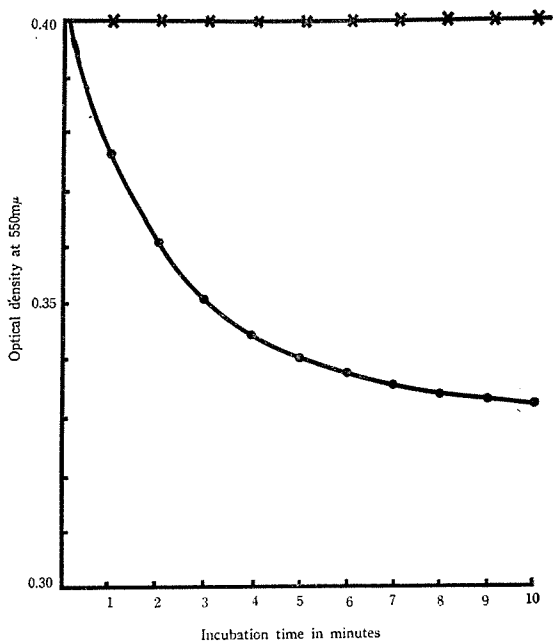

Fig. 2. Oxidation of reduced cytochrom c (yeast) by the cytochrom c oxidase in fraction P_2 of *Candida albicans* FIA 1038:
—•— Experimental : —×— Control (autoxidation)

Table 5. p-Phenylenediamine (PPDA) oxidases and effect of cytochrome c on the activity of succinic oxidase in fraction P_2

Qo_2 (protein)		
PPDA	Succinate	Succinate+cytochrome c
5.1	6.4	
7.6	4.7	
6.1	7.6	10.4
2.1	3.7	5.1
	3.1	6.9

During oxidation of these substrates, electrons are transmitted to oxygen by cytochrome oxidase, so that particles P_2 contain cytochrome oxidase.

The absorption of cytochromes:

From the results of oxidation of PPDA and succinate, cytochromes in addition to cytochrome oxidase are present in particles P_2. To demonstrate their further presence, the absorption bands of cytochromes were examined in the oxidized and reduced state in fraction P_2. The differential spectrum of cytochromes is shown in Figure 3.

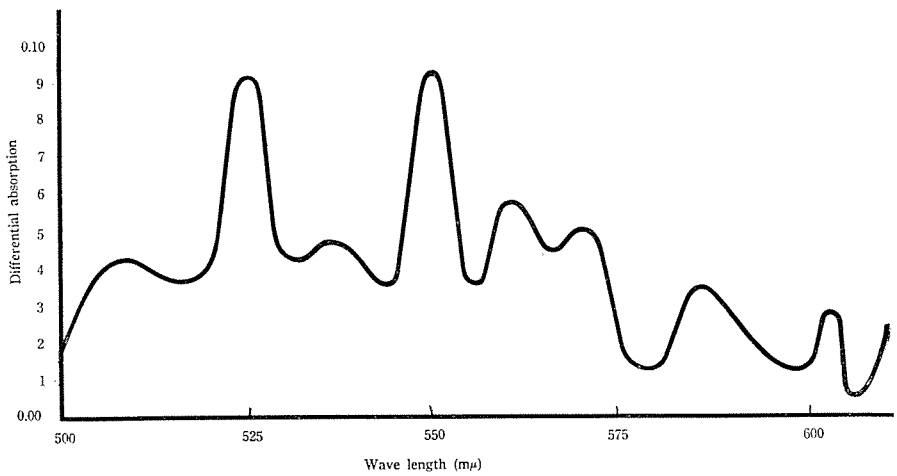

Fig. 3. Differential spectrum of cytochroms in fraction P_2 of *Candida albicans* FIA 1038.

Not a great change in absorption was found, but significant absorption bands were seen at 526 mμ and 550 mμ. An absorption near 603 mμ was not so definite.

From this experiment, it can be stated that the cytochrome system is present in particles P_2.

Electronmicroscopic observations of the particulate fractions:

From the analysis of the electronmicrographs, it was judged that fraction P_1 was not particulate, but that fractions P_2 and P_3 were. P_2 particles are ellipsoidal and their size varies from 0.3 to 1.0 μ. P_3 particles seem to be granular with a diameter of about 50 mμ. It is not clear whether particles P_3 are fragments of the

P_2 particles, or whether the two kinds of particles exist in the cell of *Candida albicans* as independent entities.

It is not yet clear which part of the cell these particles are derived from. The authors have found, by thin section electronmicroscopy of the same size as those of P_2 inside and along the cytoplasmic membrane of cells which have been cultured in Sabouraud's glucose medium for 7 days, Fig. 4. However we have no proof of their identity to P_2 particles. One should be cautious in such cases, because the protoplasmic membrane of bacteria contains cytochromes, cytochrome oxidase and enzymes of the TCA cycle. If one could exclude the possibility extrapolated from bacteria, it would be more easy to say that P_2 particles are mitochondria-like particles. However, from purely biochemical criteria, P_2 particles are mitochondria-like.

DISCUSSION

The present work was designed to study the question of whether mitochondria are present in a non-sporeforming yeast, *Candida albicans*. Ward and Nickerson (1958) consider that respiration in *C. albicans* is not mediated by cytochrome oxidase but by flavoprotein oxidases. Nevertheless, they claim the presence of mitochondria in this organism (Merkel and Nickerson, 1953; Ward and Nickerson, 1958). We cannot accept their concepts, because we think mitochondria should have a terminal respiratory system.

The cell wall of *C. albicans* is very thick and rigid for enzymatic studies on subcellular unit. The problem of cell disruption is not easy. We decided that grinding was the most effective method for disruption of cells and preparation of active enzyme systems. Thus in our experiments we avoided the problem of impermiability to substrates and inhibitors.

The results obtained that intact cells could oxidize glucose and lactate rapidly but not members of the TCA cycle, may be explained by differences in the rate of penetration through cell wall and cytoplasmic membrane.

The results that oxidase activity of several TCA cycle members are found in the isolated P_2 fraction, satisfy the criterion required as evidence for mitochondria-like particles, but pyruvate oxidase activity was not found in any subcellular fraction. Linnan and Still (1955) have studied the oxidation of pyruvate in *Saccharomyces cerevisiae* and concluded that "pyruvate oxidases are at least in part firmly attached to or inside the mitochondria" because they found strong activation of pyruvate oxidase on addition of the other members of TCA cycle. Though analogous experiments have not been performed with *C. albicans*, we assume that pyruvate oxidase of the similar nature may exist in fraction P_2.

The fact, that firmly bound succinic dehydrogenase is present in fraction P_2 indicates their mitochondria-like character.

Cytochrome oxidase in particles P_2 could be demonstrated by oxidation of PPDA, reduced cytochrome C and by the characteristic absorption spectra of cytochromes in these particles P_2. These results also support the mitochondrial properties of the P_2 particles.

However the absorption bands of cytochromes in fraction P_2 were very weak.

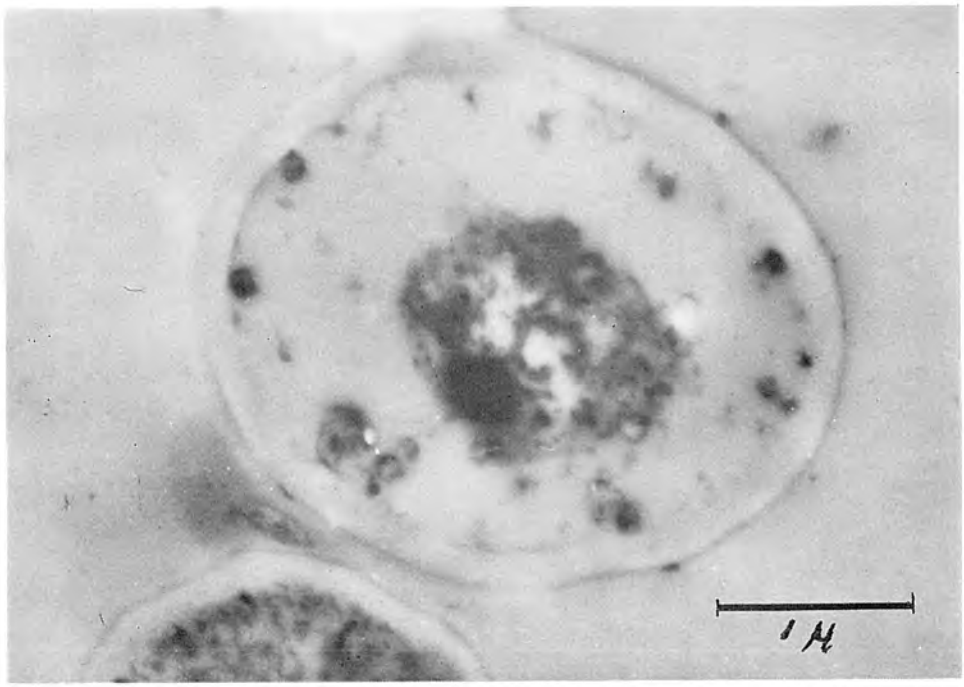

Fig. 4. The electronmicrograph of ultrathin section of *Candida aibicans* FIA 1038 cultured in Sabouraud's glucose medium for 7 days.
Similar sized particle to the P_2 particle are present inside and along the cytoplasmic membrane of the cell.

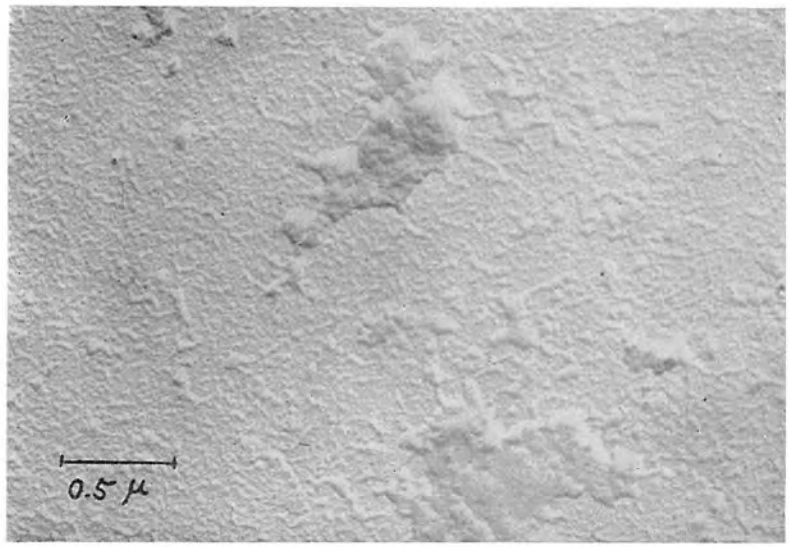

Fig. 5. The electronmicrograph of fraction P_1 of C. *albicans* FIA 1038 cultured in CYG medium for 15 hours. The fraction was prepared from organisms disrupted by grinding and centrifugation at 4,000 x g for 20 minutes.

Fig. 6. The electronmicrograph of fraction P_2 from disrupted cells of C. *albicans*. This fraction is precipitated at 14,500 x g for 25 minutes.

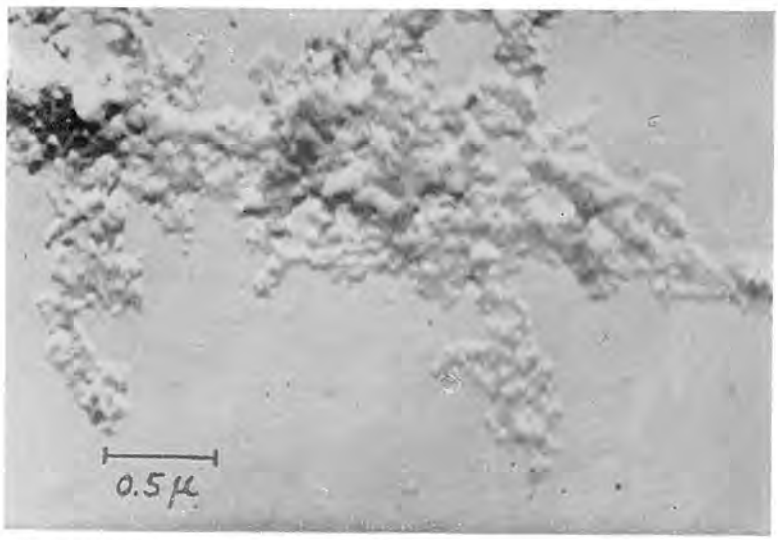

Fig. 7. The electronmicrograph of fraction P_3 centrifuged at 68,000 xg for 60 minutes.

We performed analogous experiments to those carried out by Nickerson et al., in which *C. albicans* was cultured in the 1000 ml of CYG medium in the presence of 50 mg of $FeCl_3$ and 10 mg of $ZnSO_4$. There was no differences in the enzymatic properties or cytochromes content of cells grown under normal and abnormal conditions. Though Ward and Nickerson found that cytochromes were induced in cells grown under the abnormal conditions of medium 2, we could not obtain such results under the abnormal conditions of CYG medium. The authors believe that the differences are due to differences in the culture medium.

Further studies should be carried out on oxidative phospholyration of high efficiency and on the osmotic sensitivity of particles P_2. However, from the above results mitochondria-like particles of *C. albicans* are rich in RNA and phospholipids.

Corwin, Schroeder and Mc-Cullough (1956) demonstrated in *Saccharomyces cerevisiae* that lipids can be synthesized by the incubation of the particulate fraction which is sedimentable at 11,500 × g with the supernatants and 1-C^{14}-acetate. The high concentration of RNA in the P_2 particles may support an assumption that similar results can be obtained with *C. albicans*.

It is unlikely that there are differences in the constitutents of P_2 and P_3 particles, though TCA cycle oxidases other than succinoxidase are not retained in fraction P_3. It is uncertain whether the particles P_3 are fragments of particles P_2, or whether they exist independently in the cells of *C. albicans*.

If the 80 S or 24-30 mμ particles exist in *C. albicans* as in *Saccharomyces cerevisiae* reported by Chao and Schachman (1956), Klein and Booher (1956), Agar and Douglas (1957) and Hebb, Montogomery and Slebodnik (1958), these should be present in fraction S_3 in our experiments. However, a study for such particles was not made during the present work. Further studies will be made to find small particles more rich in RNA in *C. albicans* than P_3 particles, with diameter of 50 mμ.

The intracellular position of the P_2-like particles was demonstrated by thin section electron micrographs of intact cells. However, we cannot say that the P_2 particles are the same as the particles which we found distributed along the cytoplasmic membrane, because we have no means of comparing the size of these particles.

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