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PLASMA MEMBRANES FROM *CANDIDA TROPICALIS* GROWN ON GLUCOSE OR HEXADECANE

I. ISOLATION, IDENTIFICATION AND PURIFICATION

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Summary

Plasma membranes from *Candida tropicalis* grown on glucose or hexadecane were isolated using a method based on the difference in surface charge of mitochondria and plasma membranes.

After mechanical disruption of the cells, a fraction consisting of mitochondrial and plasma membrane vesicles was obtained by differential centrifugation.

Subsequently the mitochondria were separated from the plasma membrane vesicles by aggregation of the mitochondria at a pH corresponding to their isoelectric point. Additional purification of the isolated plasma membrane vesicles was achieved by osmolysis. Surface charge densities of mitochondria and plasma membranes were determined and showed substrate-dependent differences.

The isolated plasma membranes were morphologically characterized by electron microscopy and, as a marker enzyme, the activity of Mg^{2+}-dependant ATPase was determined.

By checking for three mitochondrial marker enzymes the plasma membrane fractions were estimated to be 94% pure with regard to mitochondrial contamination.

Introduction

Numerous authors have attempted to isolate plasma membranes from different yeast strains using a variety of methods. The most applicable techniques were mechanical disruption and subsequent fractionation by differential and density gradient centrifugation [1—6] and enzymatic removal of cell wall followed by osmotic lysis of protoplasts and differential and density gradient

centrifugation [7—10]. Principally, both techniques are based on the difference in densities of the various cell organelle membranes.

In contrast, Fuhrmann et al. [11] described an isolation method for yeast plasma membranes from *Saccharomyces cerevisiae* which is based on differences in the surface charge densities of mitochondrial and plasma membranes. In a vesicle suspension consisting of mitochondrial and plasma membrane vesicles, the mitochondria were aggregated by lowering the pH slightly below their isoelectric point. Aggregation of the mitochondria caused a higher sedimentation rate. The more negatively charged plasma membrane vesicles remained in the supernatant. After further purification by filtration, a pure plasma membrane fraction was obtained as judged biochemically and electron microscopically.

Slight modifications of this isolation technique allowed us to prepare plasma membranes from *C. tropicalis* cells, grown on glucose or hexadecane. The isolation and identification of these plasma membranes, according to morphological and enzymatical markers, will be discussed and compared with plasma membranes from *S. cerevisiae*.

Materials and Methods

Organisms. Two yeasts were used. *C. tropicalis* ATCC 32 113 was grown on glucose or hexadecane and, for comparison, *S. cerevisiae* LBG H 1022 was grown on glucose.

Experimental cultures. Cultivation (batch culture) was carried out in an aerated 30 l bioreactor with a working volume of 15 l. The temperature was kept constant at 30° C and the pH was maintained at 5.0 by automatic addition of 4 M NaOH. Oxygen uptake and carbon dioxide release were monitored continuously by gas analysis. Cells were harvested in early stationary phase, 1/2—1 h after oxygen uptake ceased, and were washed twice with distilled water and once with osmotic stabilizer solution (0.4 M KCl/20 mM triethanolamine, pH 7.0).

Growth medium. A synthetic, carbon-limited medium as described by Divjak and Mor [12] was used. The concentrations of carbon source were 1% for hexadecane and 2.3% for glucose.

Isolation of plasma membrane vesicles. Cells were mechanically disrupted in a cell homogenizer (Dyno Mill, W. Bachofen AG, Basel, Switzerland). 80—90 g of *S. cerevisiae* or 50—60 g of *C. tropicalis* cells (wet weight) were resuspended in 100 ml ice-cold osmotic stabilizer solution (0.4 M KCl/20 mM triethanolamine, adjusted to pH 7.0 with 1 M HCl) and homogenized with 250 ml of glass beads (diameter 0.25—0.5 mm) for 50 s at a stirrer speed of 15 m/s (periphery). During the breakage procedure, the temperature of the cell suspension was kept constant at 2° C by cooling the system with methanol at −15° C. The cell homogenate was separated from the glass beads by filtration through a glass filter (Sovirel G 1, France).

For isolation of plasma membrane vesicles from *S. cerevisiae* the procedure described by Fuhrmann et al. [11] was used. The scheme of plasma membrane preparation from *C. tropicalis* is shown in Fig. 1.

The cell homogenate was centrifuged twice in a Sorvall RC 5-B centrifuge for 5 min at 2000 × *g* and the pellet, consisting of cell debris and unbroken cells,

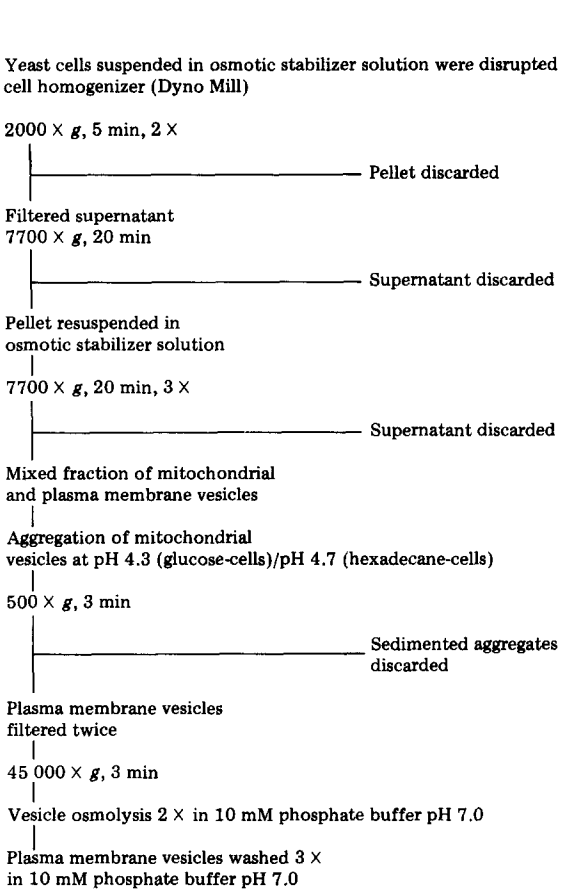

Fig. 1. Scheme of plasma membrane vesicle preparation from *C. tropicalis*.

discarded. The supernatant was filtered (glass fiber filter Sartorius 13'400) to remove remaining cell wall fragments and spun at 7700 × *g* for 20 min. After discarding the supernatant the pellet consisting of plasma membrane vesicles and mitochondria was washed three times in osmotic stabilizer solution always centrifuging as above. Thereafter the plasma membrane vesicles were separated from the mitochondrial vesicles by aggregation of the mitochondria. For this, the pH of the suspension was brought to pH 4.3 for glucose-grown cells or to pH 4.7 for hexadecane-grown cells. For that we used osmotic stabilizer solution, adjusted to pH 1.0 by 1 M HCl. After 20—30 min the clearly visible brownish mitochondrial aggregates were sedimented. The supernatant containing the white plasma membrane vesicles could easily be separated from the sedimented mitochondria by decanting after a centrifugation at 500 × *g* for 3 min. To remove small, unsedimented aggregates of mitochondria the supernatant was filtered twice (glass fiber filter Sartorius 13'400). Subsequently the plasma membrane vesicles were centrifuged at 45 000 × *g* for 5 min, lysed twice in 10 mM phosphate buffer and washed three times in the same buffer at 45 000 × *g* for 5 min.

Mitochondrial fraction. After aggregation the mitochondrial pellet was immediately resuspended in osmotic stabilizer solution at pH 7.0 and washed

three times in the same solution at 45 000 × *g* for 5 min.

Cell electrophoresis. Electrophoretic mobilities were determined in an apparatus described by Fuhrmann et al. [13] using a phase contrast optic. The operating conditions corresponded to those described by Fuhrmann et al. [11].

Electron microscopy. For freeze-cleaving all preparations were frozen in a propane jet according to Moor et al. [14]. Freeze-cleaving was carried out in a Balzers BA 360 apparatus by a slightly modified procedure of Moor [15].

For thin sectioning the plasma membrane vesicles were fixed, stained and embedded following the procedure of Kopp [16], except dehydration of the material was achieved by dimethoxypropane [17].

Biochemical assays. Protein was determined by the method of Lowry et al. [18].

Adenosine triphosphatase (EC 3.6.1.3) activity was measured according to Fuhrmann et al. [3] by following the release of inorganic phosphate from ATP.

Phosphate was determined by the method of Fiske and Subba Row [19].

Cytochrome oxidase (EC 1.9.3.1) activity was determined by the method of Polakis et al. [20], which measures the decrease of absorbance of ascorbic acid-reduced cytochrome *c* at 550 nm.

Malate dehydrogenase (EC 1.1.1.37) activity was measured as described by Flury et al. [21] with oxalacetate as the substrate.

Succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Caplan and Greenawalt [22].

Results

Morphological characteristics of plasma membrane and their isolated vesicles. Freeze-cleaved yeast plasma membranes exhibit characteristic invaginations and an asymmetric particle distribution of their fracture faces, as shown in Fig. 2. With all three cell types the plasmatic-fracture face (PF) is covered by a large number of particles, whereas on the exoplasmatic-fracture face remarkably less are located. For plasma membranes of *S. cerevisiae* some other specific structures such as hexagonal particle arrangements are described [3,23–25]. In Figs. 2a and 3b several of these hexagonal structures can be seen, but their appearance is dependent on the growth phase. In fast growing cells of a continuous culture the plasma membrane-intercalated particles are distributed randomly (Fig. 3a). In the stationary phase several areas on the plasmatic-fracture face exhibited hexagonal particle arrangements (Fig. 2a), which increased and became even more marked after starvation of cells in distilled water (Fig. 3a). In plasma membrane of *C. tropicalis* a random particle distribution was observed during all growth phases and the change of substrate also had no influence on the distribution pattern (Fig. 2, b and c).

In the mixed fraction (7700 × *g* pellet) of mitochondria and plasma membrane vesicles the latter could easily be identified by their characteristic invaginations (Fig. 4a). The vesicles contain some kind of unidentified inclusions (Fig. 4, b and c) which disappear after osmolytic treatment. After aggregation of the mitochondria and release of cytoplasmic content by cytolysis, these invaginations disappear almost completely (Fig. 5, a and b), but the asymmetric particle distribution between EF and PF was equal as in situ. Thin sections

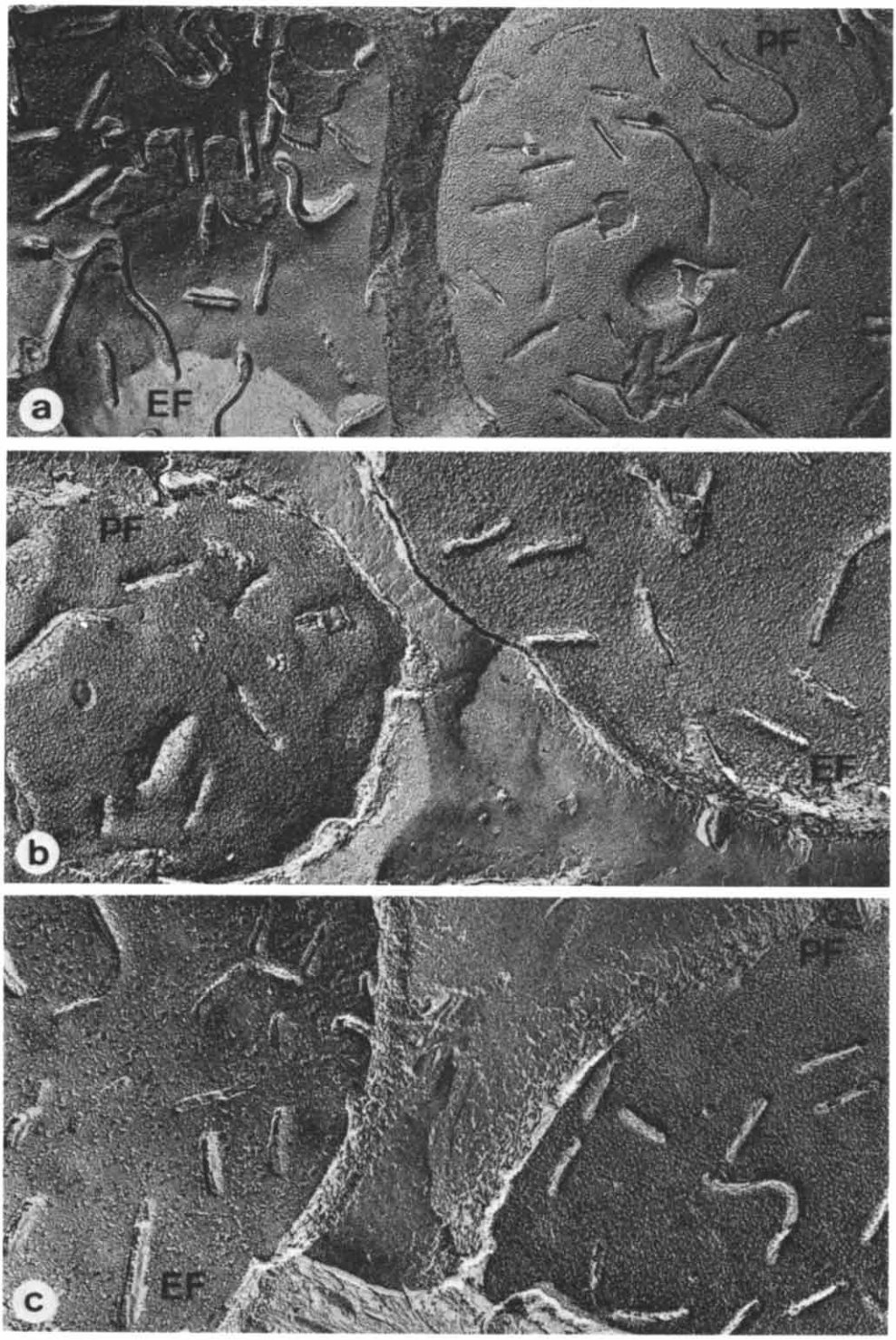

Fig. 2. Freeze-cleaved cells in early stationary phase from (a) *S. cerevisiae* grown on glucose (magnification X 45 000); (b) *C. tropicalis* grown on glucose (magnification X 57 000); (c) *C. tropicalis* grown on hexadecane (magnification X 57 000) PF, plasmatic-fracture face; EF, exoplasmatic-fracture face.

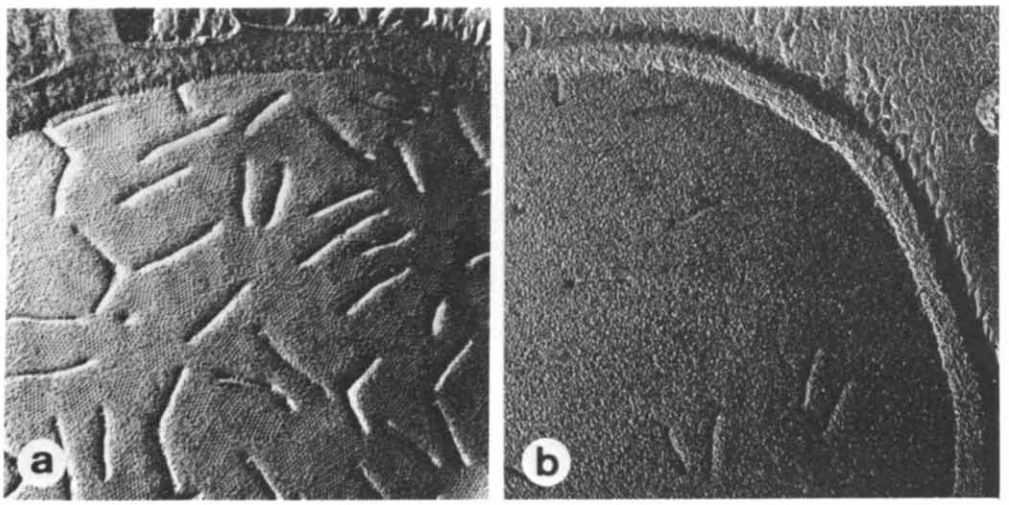

Fig. 3. Plasmatic-fracture face (PF) of the plasma membrane from *S. cerevisiae* in (a) starved cells and (b) cells of a continuous cultivation. Magnification × 48 000.

showed closed and empty vesicles with a typical unit membrane structure (Fig. 5, c and d).

Surface charge density. As previously stated, the isolation method utilizes the difference in surface charge density to separate the mitochondria from the plasma membranes. To determine the optimal pH for aggregation, the surface charge density was measured for plasma membranes and mitochondria from all three cultures. The results (Fig. 6, a—c) show that the surface charge densities were not the same and varied with both cell type and carbon source.

The isoelectric points of the mitochondria are in the same range, varying from pH 4.3 to 4.7. In contrast, the isoelectric points of the plasma membranes differ considerably, ranging from below pH 3 for *S. cerevisiae* up to pH 4 for hexadecane-grown *C. tropicalis.* This means that the difference between the isoelectric points of the mitochondria and the plasma membranes also varies considerably, but is sufficient in all cases to permit aggregation of the mitochondria without concomitant aggregation of plasma membranes.

Activity of Mg^{2+}-dependent ATPase. Fig. 7, a and b shows the pH dependence of Mg^{2+}-ATPase activity from *C. tropicalis.* With plasma membranes from glucose-grown cells in accordance with *S. cerevisiae* [3] an optimal activity at pH 7.0 is found. However, pH range is extremely narrow as no siginificant activity can be measured at pH 6.5 and 7.5. On the other hand plasma membranes from cells grown on hexadecane show a broad pH spectrum over a pH range 6.0—9.0 with an optimum around pH 8.0. Similar maximal turnover rates (approx. 150 μmol PO_4^{3-}/g protein per 10 min) were determined in both fractions in agreement with results of Fuhrmann et al. [3], measured with plasma membranes of *S. cerevisiae.*

Assessment of membrane purity. As already descibed above, the 7700 × *g* pellet is a mixed fraction of mitochondria and plasma membrane vesicles. For this reason the purified plasma membrane preparation was checked especially

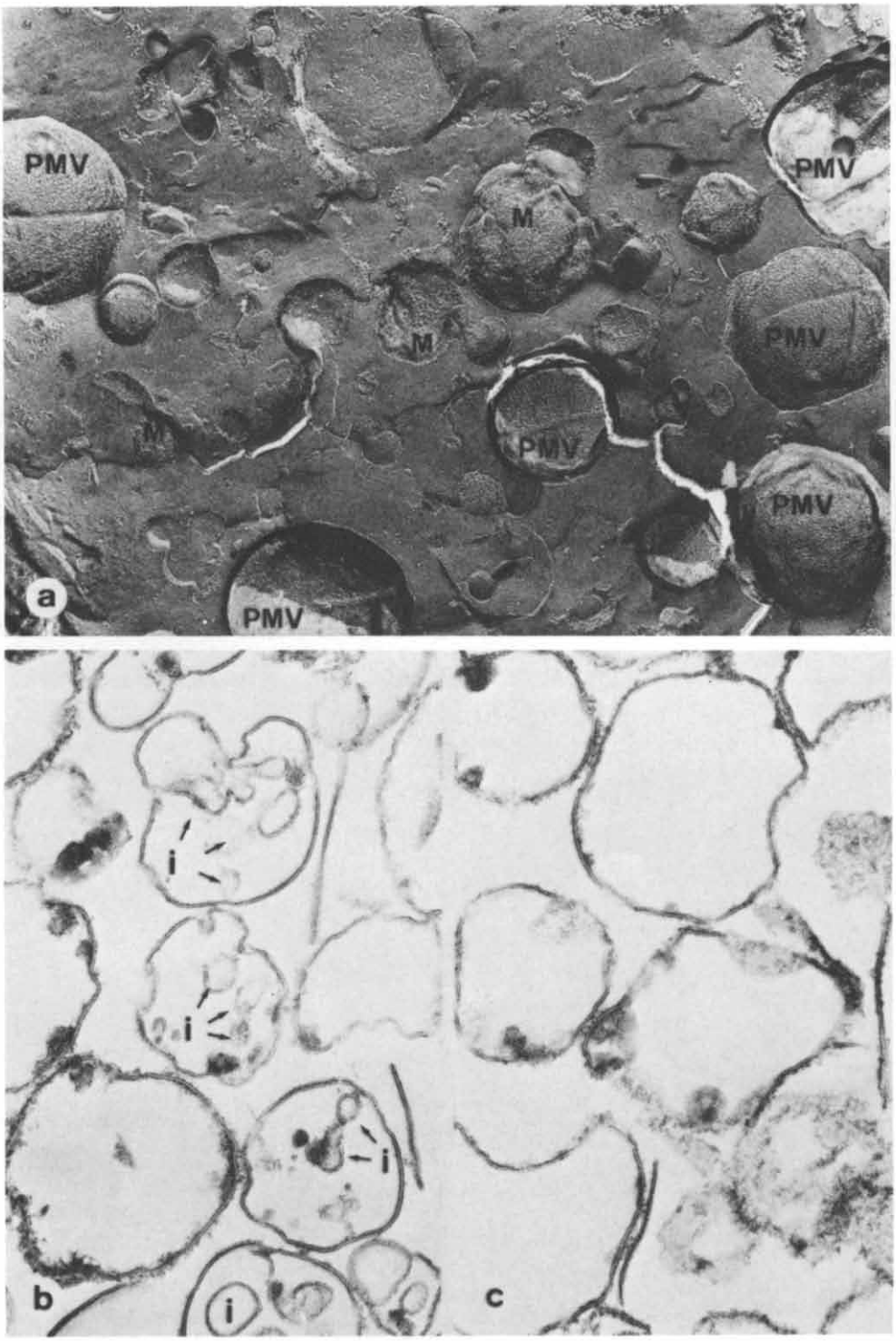

Fig. 4. (a) Freeze-cleaved 7700 $\times g$ pellet of mixed fraction of mitochondrial (M) and plasma membrane vesicles (PMV). Fracture faces of plasma membrane vesicles show typical invaginations. Magnification × 57 000. (b and c) Thin sections of plasma membrane vesicles from *S. cerevisiae* before (b) and after (c) osmolysis. Magnification × 84 000. i, inclusions.

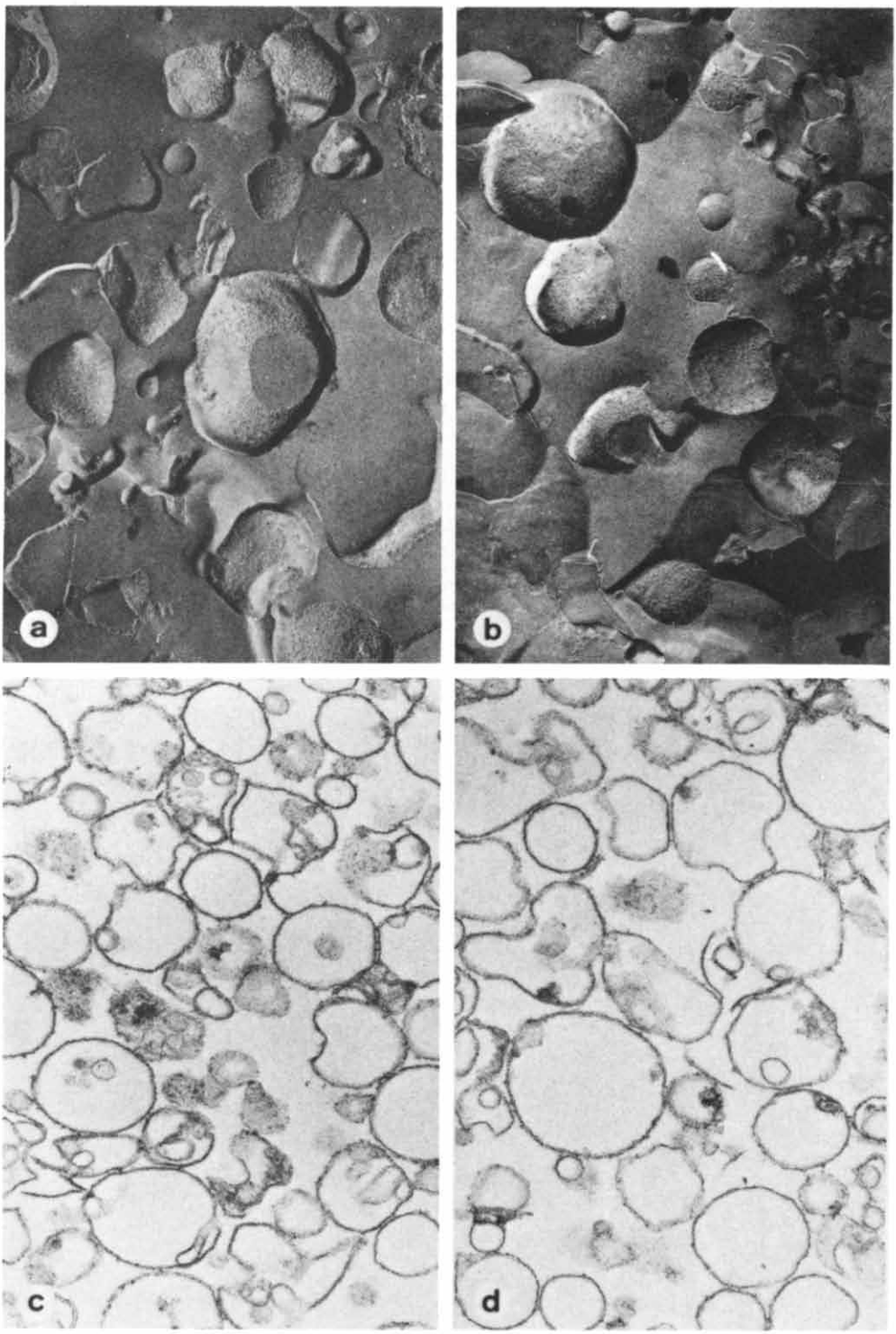

Fig. 5. (a and b) Purified plasma membrane vesicles from *C. tropicalis* grown on glucose (a) or hexadecane (b) after freeze-cleaving. Magnification × 48 000. (c and d) Thin sections of the same preparations (c) glucose- (d) hexadecane-grown cells. Magnification × 45 000.

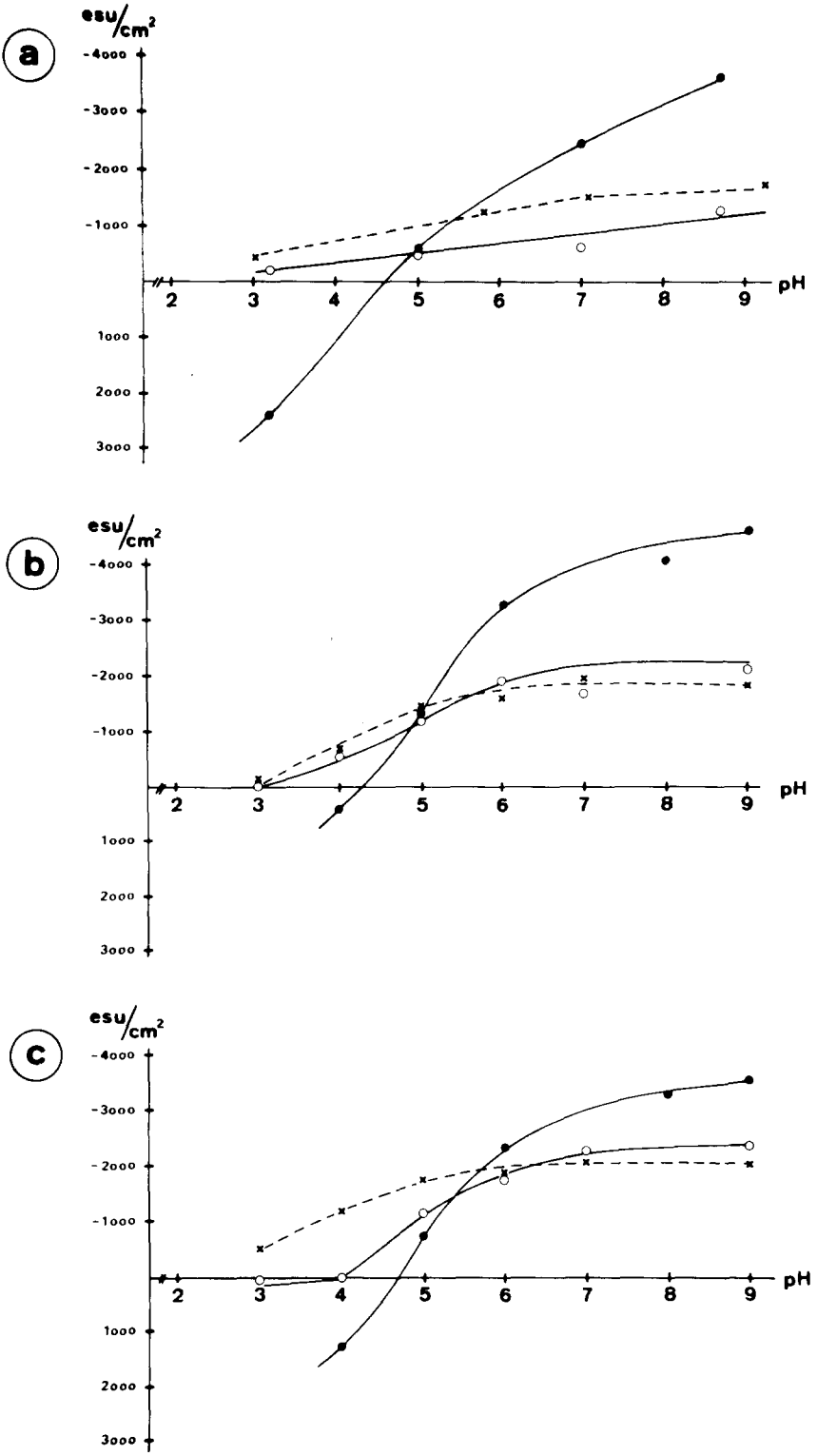

Fig. 6. Surface charge density of whole cells (x- - - - -x), mitochondria (●———●) and plasma membrane vesicles (○———○) from (a) *S. cerevisiae* grown on glucose, (b) *C. tropicalis* grown on glucose and (c) *C. tropicalis* grown on hexadecane in relation to pH. Results of 10 measurements expressed as mean ± S.E. (within the points). esu, electrostatic unit.

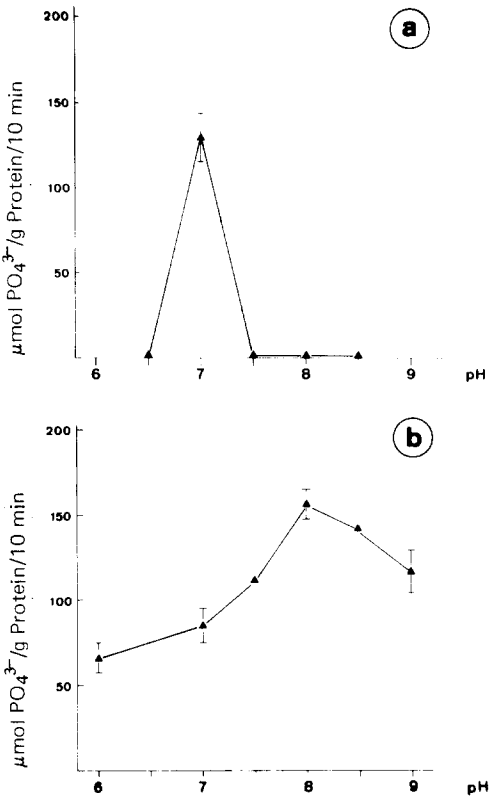

Fig. 7. pH-dependence of Mg^{2+}-activated ATPase of plasma membranes from *C. tropicalis* grown on glucose (a) and grown on hexadecane (b). Results are expressed as mean ± S.D. from 5—7 experiments. The number of experiments was 2 for pH 7.5 and 8.5 for plasma membranes from hexadecane-grown cells.

for mitochondrial contamination.

The mitochondrial fraction obtained by aggregation shows a low oxygen uptake rate with NADH as substrate and, with the addition of ADP no increase in oxygen uptake is observed. It seems that even a short incubation at low pH may disturb the functions of mitochondria irreversibly. Due to these facts it was not possible to estimate the degree of mitochondrial contamination by respiration measurements.

A partial inactivation by low pH is also observed for several specific enzymes of mitochondria. However, their remaining activity allows measuring the degree of contamination, as shown in Table I. Malate dehydrogenase, succinate dehydrogenase and cytochrome oxidase were chosen as marker enzymes. The latter two are known as exclusively mitochondrial bound, whereas for malate dehydrogenase two forms, a cytoplasmatic and a mitochondrial one, exist [21,26]. After washing the 7700 × *g* pellet three times no significant malate dehydrogenase activity is detectable in the supernatant. Therefore, it is assumed that the activity determined in this fraction is of mitochondrial origin.

Based on total activities, we estimated the degree of partial inactivation for the different marker enzymes between 30 and 80%. It was found that in *C.*

TABLE I

ASSESSMENT OF MITOCHONDRIAL CONTAMINATION

Estimation of mitochondrial contamination in the purified plasma membrane fractions from *S. cerevisiae* (1) and *C. tropicalis* on glucose (2) or hexadecane (3) according to the mitochondrial marker enzymes malate dehydrogenase, succinate dehydrogenase and cytochrome oxidase. Results are expressed as means of 2 experiments. I.U., international units (mmol/g protein).

		Mixed fraction of mitochondrial and plasma membrane vesicles			Aggregated mitochondria			Purified plasma membrane vesicles		
		1	2	3	1	2	3	1	2	3
Malate dehydrogenase	Total activity (I.U.)	1071	948	3930	728	357	1609	7	5	85
	Total activity (%)	100	100	100	68	37	41	0.7	0.5	2.2
	Relative activity (%)	—	—	—	100	100	100	1.0	1.4	5.3
Succinate dehydrogenase	Total activity (I.U.)	32	74	93	20	14	18	1.1	0.1	0.9
	Total activity (%)	100	100	100	67	19	20	3.4	0.1	1.0
	Relative activity (%)	—	—	—	100	100	100	5.5	0.7	5.0
Cytochrome oxidase	Total activity (I.U.)	31	46	74	19	12	43	0.9	0.4	2
	Total activity (%)	100	100	100	62	49	58	2.9	0.9	2.7
	Relative activity (%)	—	—	—	100	100	100	4.7	3.3	4.6

tropicalis all three enzymes were more inactivated than in *S. cerevisiae*. This can be explained by the longer aggregation time (incubation time at low pH) for *C. tropicalis* (30 min compared to 15 min with *S. cerevisiae*).

The total activities of mitochondrial fractions expressed as 100% relative activity resulted in a mitochondrial contamination of 6% at highest for purified plasma membrane fractions of all three cell types.

Discussion

The purity of membrane preparations is most important in view of biochemical examinations. Electron microscopical and enzymatical criteria were used to evaluate this purity qualitatively [1,3,5,11]. It was only Marriot [9] who gave quantitative data based on radioactive labeling methods. He estimated a 4% contamination level by mitochondrial fragments. In our preparations approx. 6% mitochondrial impurities were found based on assaying three mitochondrial marker enzymes. In addition, optical examination of the thin sections of the vesicles excluded any detectable presence of mitochondrial membrane fragments.

The membrane structure of the vesicle preparations reveals that the characteristic arrangement of particles of a given membrane [27] may vary in rather wide limits.

Drastic alterations are visible for example in Fig. 3, where the hexagonal

particle arrangements of stationary cells from *S. cerevisiae* disappear in chemostat cells. This and similar data by Takeo et al. [28] indicate that hexagonal arrangements are typical only for the final membrane structure. Whether or not this structure is due to spontaneous protein aggregation [29] is still open but it seems related to specific membrane functions including transport processes.

Unique membrane markers are represented by the invaginations, the high particle density and the asymmetric particle distribution between the two fracture faces (Fig. 2, b and c) of *C. tropicalis*. It is also noteworthy that, in contrast to *S. cerevisiae*, the pattern of particle arrangement seems to be independent of carbon source and rate of growth. The reduction of invaginations under certain conditions (Fig. 5) may be due to the preparation method applied (incubation at low pH). The principally different nature of the *Candida* membrane from *Saccharomyces* is also indicated by the fact that some modifications for the vesicle preparation procedure were necessary. Recognition of the *Candida* vesicles, however, was possible on the basis of their specific particle density.

Finally, ATPase activities were of great help for purity description. Yeast plasma membranes carry Mg^{2+}-dependent, moderately oligomycin-sensitive ATPase as a marker [1—3,5,6,9,31], whereas mitochondrial ATPase is strongly olygomycin sensitive [30]. Also, pH optima were very specific for plasma membrane ATPase of *S. cerevisiae* (pH 7.0—7.2) and *C. tropicalis* of glucose-grown cells and alkane-grown cells (pH 8.0). The data presented indicate that the modified Fuhrmann method for plasma membrane purification yields in reliable and characteristic vesicle preparation.

Acknowledgement

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