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Development of Microbodies in the Yeast *Kloeckera* Growing on Methanol

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A number of microbodies appear regularly in methanol-grown yeast cells, but rarely in ethanol- or glucose-grown cells. When one of representative methanol-utilizing yeasts, *Kloeckera* sp. no. 2201 (also known as *Candida bodinii*), was cultured on glucose and then transferred into a methanol medium, microbodies of small size could be observed in 2-h-old cells. The number of microbodies per sectioned cell reached five to six after 4 h of cultivation. Though the number of microbodies did not change during prolonged cultivation, their size became larger with the passage of cultivation time. The activities of catalase and alcohol oxidase were confirmed in the particulate fractions throughout the cultivation period, whereas the activities of formaldehyde dehydrogenase and formate dehydrogenase were not detected in the particles. The activity of isocitrate lyase was detected in the particulate fractions only at the early growth phase.

Occurrence of microbodies in yeast was first demonstrated by Avers and her co-workers (1, 2, 18) in various strains of *Saccharomyces cerevisiae*. They detected several enzymes including catalase in the microbody fraction from the yeast. However, the number of microbodies in *Saccharomyces* cells was too small to study their metabolic significance.

In the course of the studies on the physiology of hydrocarbon- and methanol-utilizing yeasts, we observed that numerous microbodies appeared regularly in the cells grown on hydrocarbon or methanol (6, 15). Localization of catalase activity in the organelle was also demonstrated (6, 20, 21). Similar phenomena in methanol-utilizing yeasts were also reported by van Dijken et al. (22) and Sahm et al. (17).

Isolation of microbodies was successfully achieved by means of sucrose density gradient centrifugation from methanol-grown cells of *Kloeckera* sp. no. 2201 (5) and from hydrocarbon-grown *Candida tropicalis* pK 233 (unpublished data). In the microbodies of methanol-grown *Kloeckera*, activities of catalase, flavin-dependent alcohol oxidase, and D-amino acid oxidase were confirmed. Localization of catalase, D-amino acid oxidase, and isocitrate lyase has been demonstrated in the microbodies from *C. tropicalis* so far studied.

The biogenesis and development of microbodies merits study inasmuch as these organelles are rarely found in cells grown on glucose or ethanol, but appear regularly in cells grown on hydrocarbon or methanol. It has been claimed that microbodies can be formed by division of preexisting microbodies in hydrocarbon-utilizing yeast cells, because the number of the organelles per one cell increases along with cell growth (14).

This paper deals with the development of microbodies in methanol-grown cells of *Kloeckera* sp. no. 2201. The number of the organelles in the cells seemed not to change, but their volume increased markedly during the utilization of methanol. The origin of the organelles, however, remained unclear.

MATERIALS AND METHODS

Microorganism. *Kloeckera* sp. no. 2201 was kindly supplied by K. Ogata, Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University. In the opinion of some microbiologists, this yeast should be considered to be a strain of *Candida boidinii*. The yeast, cultured on glucose, was transferred into a methanol medium and cultivated with shaking at 30 C as described previously (6).

Enzyme assay. Cells were harvested by centrifugation, washed twice with distilled water, and suspended in 0.05 M potassium phosphate buffer (pH 7.2). The cell suspension (2 mg of dry cell per ml) thus obtained was sonicated for 5 min at 20 kc/s below 4 C. The resulting homogenate was used as enzyme source. To obtain the particulate fraction, cells were digested with Zymolyase (Kirin Brewing Co., Tokyo, Japan) (11), as described previously (6). Protoplasts obtained were suspended into 0.01 M potassium phosphate buffer (pH 7.2) containing 0.65

M sorbitol and 0.5 mM ethylenediaminetetraacetic acid and disrupted for 5 min in a Teflon homogenizer under cooling. The resulting protoplast homogenate was centrifuged at 20,000 × *g* for 15 min, and the particulate fraction containing mitochondria and microbodies was obtained as sediment. The particles thus obtained were suspended into the same medium and used as enzyme source after dilution.

Catalase activity was assayed spectrophotometrically by following the decrease in absorbance of H_2O_2 at 240 nm according to the method of Roggenkamp et al. (16) except that 120 μmol of H_2O_2 was used. Alcohol oxidase activity was measured as follows. Enzymatic oxidation of methanol was carried out according to Tani et al. (19) with 100 μmol of methanol as the substrate; formaldehyde formed was assayed by the method of Nash (13). Formaldehyde dehydrogenase and formate dehydrogenase activities were measured by following the increase in absorbance of reduced nicotinamide adenine dinucleotide at 340 nm according to Kato et al. (9, 10) except that the concentrations of the substrates and the cofactors were three times those of the original ones. These assays were carried out at pH 7.2. Isocitrate lyase activity was measured by the modified method of G. H. Dixon and H. L. Kornberg (Biochem. J. 72:3P, 1959). The assay mixture contained 180 μmol of potassium phosphate buffer (pH 7.2), 12.5 μmol of trisodium DL-isocitrate, 5 μmol of phenylhydrazine hydrochloride, 7.5 μmol of $MgCl_2$, 3 μmol of cysteine hydrochloride, and enzyme in a final volume of 1.5 ml. The reaction was done at 30 C, and glyoxylate-phenylhydrazone formed was estimated by measuring the increase of absorbance at 324 nm. D-Amino acid oxidase was assayed essentially by the method of Hayashi et al. (8). D-Alanine was used as the substrate and 2,4-dinitrophenylhydrazine as the reagent to detect pyruvate formed (4). Protein was assayed by the method of Lowry et al. (12).

Electron microscopy. Cells were fixed with 5% glutaraldehyde and postfixed with potassium permanganate. In some cases, the cells fixed with glutaraldehyde were treated with Zymolyase and postfixed with osmium tetraoxide. These procedures were the same as described previously (6).

Estimation of the area of microbodies from electron micrographs. To estimate the area of microbodies, electron micrographs of cell sections cut along the long axis at their centers were selected. The estimation was carried out by means of the point-counting method. Although Hawley and Wagner (7) described a method to calculate the volume of mitochondria from electron micrographs by assuming constancy of shape, we could not confirm the constancy of yeast microbody shape. Therefore, the results in this paper were presented in terms of area instead of volume.

RESULTS

In glucose-grown cells of *Kloeckera* sp. no. 2201, microbodies were rarely detected. When the glucose-grown cells were transferred into a methanol medium and cultivated with shaking, microbodies of small size were sometimes observed in 1-h-old cells, although most electron micrographs showed the absence of the organelles (Fig. 1A). At the second hour of cultivation, two to three microbodies were observed clearly (Fig. 1B). The number of microbodies in a sectioned cell increased up to five to six, and their size became larger at 4 h (Fig. 1C). After this period of cultivation, the number of the organelles remained constant, whereas their size continued to increase (Fig. 1D–F). Sometimes the organelles were more than 1 μm in length along the long axis (Fig. 1F). The well-developed microbodies existed in a closely associated state. The relationship between cell growth and the development of microbodies is illustrated in Fig. 2. The average area fractions (the percentage of total area of the section occupied by the microbodies) and the numbers of microbodies were estimated by using 10 to 15 sections of cells. The number of microbodies reached the maximum at 4 h of cultivation, while their size increased gradually after this period. Crystalloids were observed clearly in microbodies as early as 4 h of cultivation, when the cell was postfixed with OsO_4 after treatment with Zymolyase (Fig. 3A). An enlarged photograph (Fig. 3B) shows a lattice structure of the crystalloid. This structure was also observed even in cells grown on methanol for 46 h (6).

In connection with the electron microscope studies, we investigated the time-course changes in the activities of several enzymes that were related to the methanol oxidation system (group A) and/or have been found in yeast microbodies (group B): flavin-dependent alcohol oxidase and catalase belonging to both groups A and B; formaldehyde dehydrogenase and formate dehydrogenase belonging to group A but not to group B; and D-amino acid oxidase and isocitrate lyase belonging to group B but not to group A. When the activities of these enzymes were measured using cell homogenates, all the enzymes investigated, except for D-amino acid oxidase, increased significantly during growth on methanol (Fig. 4). Only the activity of D-amino acid oxidase decreased slightly at the initial growth phase and was kept at a constant level during the incubation period (Fig. 4). Table 1 shows the relative enzyme activities recovered in the particulate fractions, which were obtained from the protoplast homogenates by centrifugation at 20,000 × *g* and found to be composed of a mixture of mitochondria and microbodies. It is of great interest that approximately one-half of the activities of catalase and alcohol oxidase, participating in the first step of methanol oxidation,

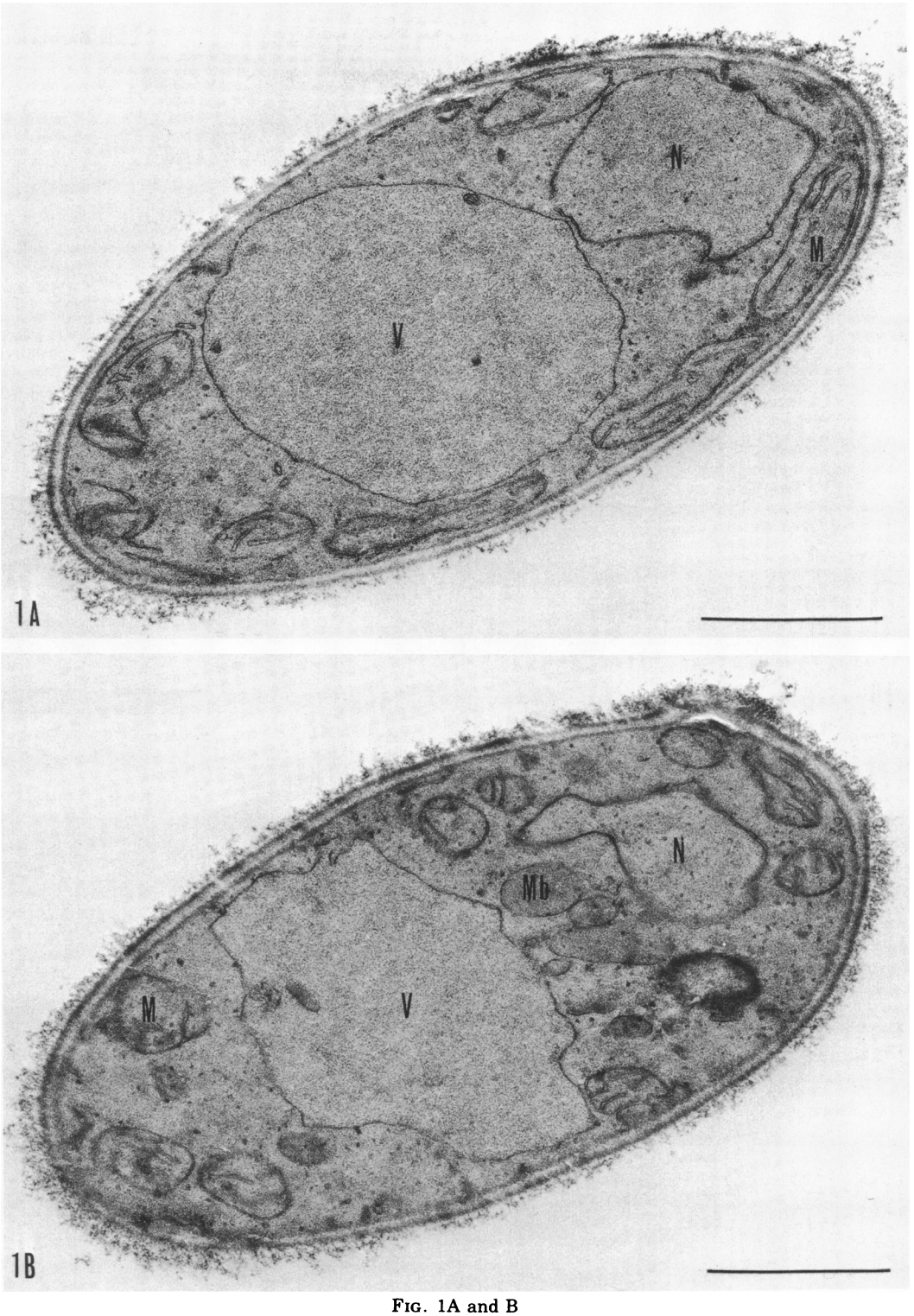

FIG. 1A and B

FIG. 1. *Ultrastructure of Kloeckera cells cultivated on methanol. The yeast cultured on glucose was transferred into a methanol medium and cultivated with shaking at 30 C. (A) 1 h; (B) 2 h; (C) 4 h; (D) 8 h; (E) 16 h; and (F) 32 h. Glutaraldehyde-potassium permanganate fixed. Bar, 1 μm.*

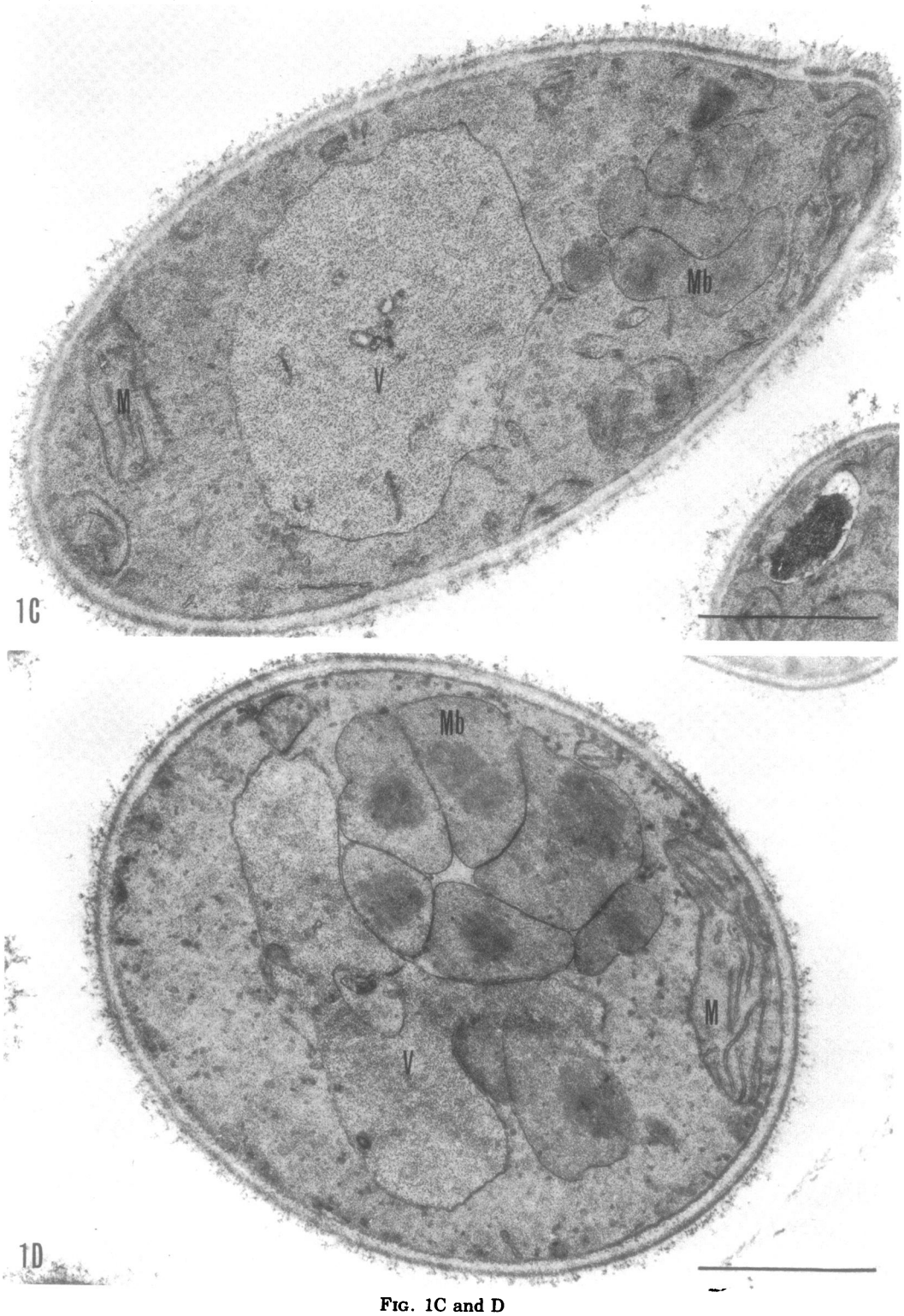

FIG. 1C and D

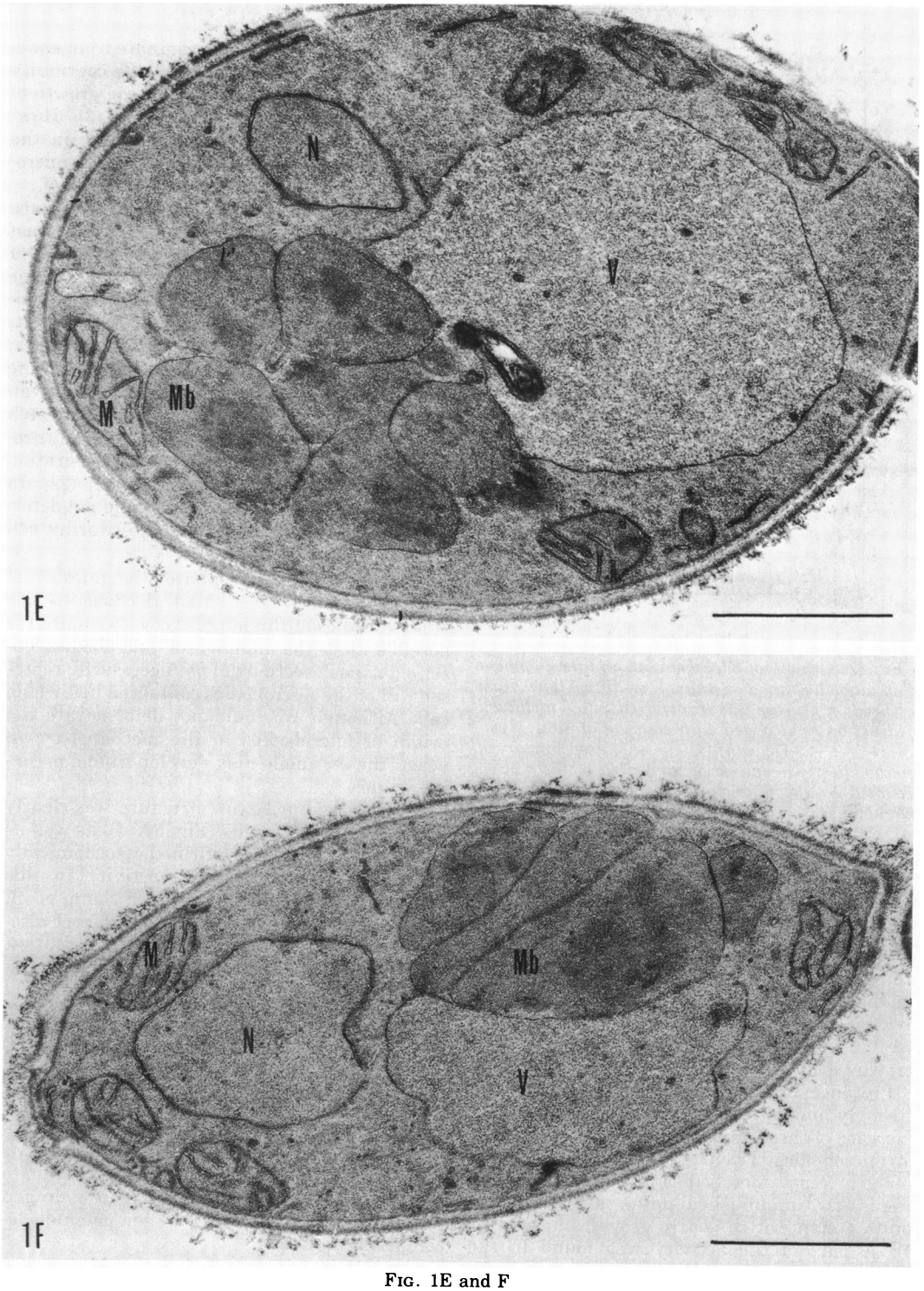

FIG. 1E and F

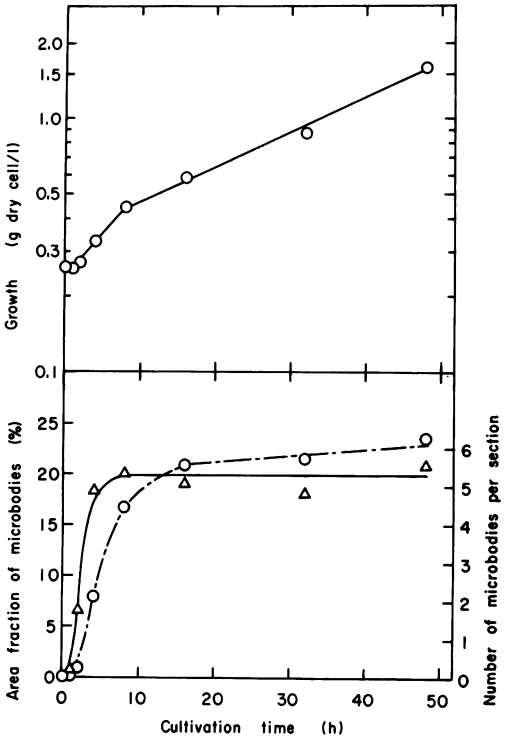

FIG. 2. *Growth of Kloeckera sp. and development of microbodies in a methanol medium. The yeast cultured on glucose was transferred into a methanol medium and cultivated with shaking at 30 C. Symbols: (○——○) Growth; (○- - -○) area fraction of microbodies (percentage of total area of the section occupied by the microbodies); and (△) number of microbodies.*

were found in the particulate fractions, whereas those of formaldehyde and formate dehydrogenases, the enzymes participating in the later steps of methanol oxidation, were hardly detected in these fractions. About 60% of D-amino acid oxidase activity was also observed in the particulate fractions. Catalase, alcohol oxidase, and D-amino acid oxidase were demonstrated to be microbody enzymes of methanol-grown yeast (5).

Presence of these enzymes in microbodies strongly indicates a correlation between the increase of these enzymes and the development of microbodies. Isocitrate lyase, whose occurrence in yeast microbodies was confirmed in *C. tropicalis* (unpublished data), showed maximum activity at the early growth phase (Fig. 4). A part of the activity was found in the particulate fraction at this phase, but no activity was detected in the particles at the later growth phases (Table 1).

DISCUSSION

Microbody is thought to originate from endoplasmic reticulum. In this case, the continuity to endoplasmic reticulum during the growth of microbody has been demonstrated (3). However, we could not observe continuity in the hydrocarbon-grown yeast cells, in which microbodies are presumed to grow by division (14).

The results described in this paper revealed that the microbodies of methanol-grown yeast cells grew in size in spite of the constancy of number (Fig. 2). No continuity to endoplasmic reticulum was observed. The number of organelles per sectioned cell was almost constant, five to six, although many more microbodies, up to 10 to 12, were found in rare cases. The constancy of number was also reported by van Dijken et al. (22), that is, 6 per section in batch cultures and up to 12 in a chemostat culture. Furthermore, they demonstrated the migration of microbodies into the daughter cell (22). In the present study, however, we could not determine the behavior of microbodies during cell division.

At the early growth phase, the number of microbodies was rather small. Because the size of microbodies at this stage was very small, it is possible that the number of microbodies observed in each section did not necessarily represent all of the microbodies contained in a whole cell. Although we could not demonstrate the origin of microbodies in the methanol-grown yeast, the organelle may develop from a preexisting one.

A crystalloid or lattice structure was clearly observed in 4-h-old cells (Fig. 3A, B) as well as 46-h-old cells (6). We confirmed cytochemically the localization of catalase activity in this structure (6). On the other hand, Sahm et al. (17) demonstrated a close relationship of alcohol oxidase to the crystalloid by using a mutant lacking alcohol oxidase. These two enzymes, participating in the initial step of methanol oxidation, may be associated in this structure. Approximately one-half of catalase and alcohol oxidase activities was found in the particulate fractions, whereas formaldehyde and formate dehydrogenases, which were also involved in the yeast methanol oxidation system, were observed exclusively in the supernatant fractions (Table 1). These results indicate an intimate relationship in microbodies between hydrogen peroxide-generating and hydrogen peroxide-decomposing systems.

The activity of isocitrate lyase was rather low in the cells of methanol-grown *Kloeckera*, and its participation in methanol assimilation by

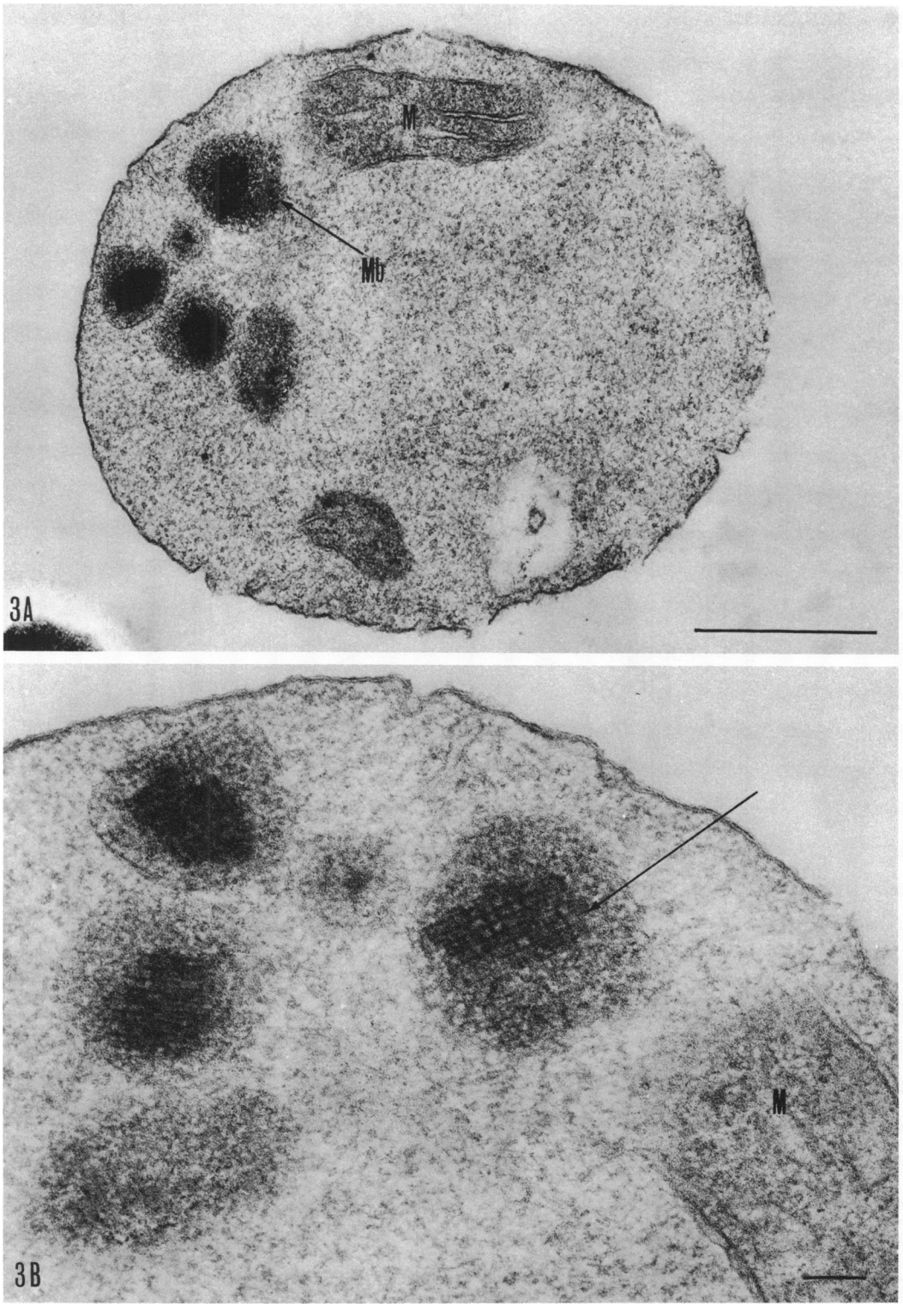

FIG. 3. *Appearance of crystalloids in the microbodies of Kloeckera sp. The yeast was cultivated for 4 h on methanol. (A) Crystalloids can be seen as an electron-dense area in microbodies. Bar, 1 μm. (B) Lattice structure is clearly observed in the microbodies. Bar, 0.1 μm. Cells were fixed with glutaraldehyde, treated with Zymolyase, and postfixed with* OsO_4.

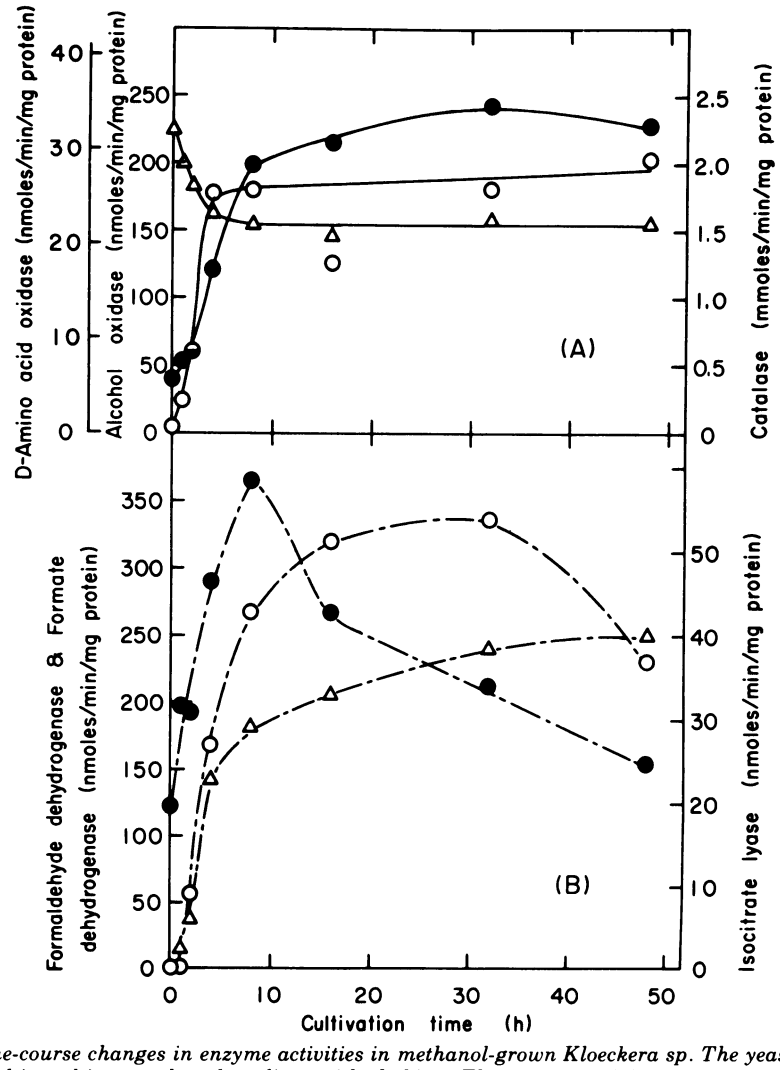

FIG. 4. *Time-course changes in enzyme activities in methanol-grown Kloeckera sp. The yeast cultured on glucose was cultivated in a methanol medium with shaking. The enzyme activities were measured by using cell homogenates. Symbols: (○——○) Alcohol oxidase; (●——●) catalase; (△——△) D-amino acid oxidase; (●– – –●) formaldehyde dehydrogenase; (△– – –△) formate dehydrogenase; and (●– – –●) isocitrate lyase.*

TABLE 1. *Particulate localization of enzymes*[a]

Enzyme	Relative activity in particulate fractions (%) cultivated for:				
	2 h	8 h	16 h	32 h	46 h
Protein	59	51	58	41	51
Catalase	57	39	61	59	62
Alcohol oxidase	63	37	53	45	51
Formaldehyde dehydrogenase	0	0	0	0	0
Formate dehydrogenase	0	0	0	0	0
Isocitrate lyase	45	64	20	0	0
D-Amino acid oxidase	62	43	55	57	65

[a] The particulate fractions were obtained from protoplast homogenates by centrifugation at 20,000 × *g* for 15 min. The total activity (particulate fraction + supernatant fraction) was expressed as 100%.

yeasts has not been demonstrated. However, the enzyme activity reached the maximum at early growth phase, and a part of the activity was found in the particulate fractions. The occurrence of this enzyme was demonstrated in the microbodies of hydrocarbon-grown *C. tropicalis* (unpublished data). The role of isocitrate lyase in the metabolism of yeasts growing on methanol remains to be elucidated.

As mentioned above, the microbodies of methanol-grown yeasts contained flavin-dependent alcohol oxidase and catalase participating in the initial oxidation step of methanol. The present study revealed that the development of microbodies is dependent on methanol

utilization. Although accumulated evidence emphasizes the distinct role of microbodies in methanol oxidation by yeasts, other roles of these organelles in methanol-grown yeast cells, e.g., their contribution to metabolism via the glyoxylate cycle, remains undetermined.

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