Methane-oxidizing Activity and Membrane Morphology in a Methanol-grown Obligate Methanotroph, *Methylosinus trichosporium* OB3b

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The obligate methylotroph *Methylosinus trichosporium* OB3b was capable of growth on methanol as sole carbon source at concentrations as high as 4% (v/v), and viability was maintained over many successive transfers. Methane mono-oxygenase, detected by epoxidizing and hydroxylating activity, was retained. The gross morphology of the organism on this substrate was dependent on culture conditions. It varied from organisms containing extensive peripheral membrane systems to those with extensive inclusions, the latter representing a poorly developed membrane system which predominated under most growth conditions.

INTRODUCTION

Methylosinus trichosporium OB3b is classified as a Type II methane-utilizer on the basis of its gross structural morphology, showing extensive peripheral intracytoplasmic membranes (Weaver & Dugan, 1975), and its intermediary metabolism, whereby formaldehyde and carbon dioxide are incorporated into cell material by the serine pathway (Lawrence & Quayle, 1970).

Methanol is toxic to many methane-utilizing methylotrophs at concentrations as low as 0.01% (v/v), and only poor growth of methane-utilizing organisms has been reported with methanol as sole carbon source (Leadbetter & Foster, 1958; Stocks & McCleskey, 1964; Whittenbury *et al.*, 1970*a*). Viability of *Methylococcus* NCIB 11083, a Type I methylotroph, was lost after successive transfers in shake flask culture with methanol at a concentration of 0.1% (v/v), due to the accumulation of formaldehyde in the culture supernatant (Linton & Vokes, 1978). Growth of the same organism under conditions of methanol limitation resulted in the retention of viability in chemostat cultures. A method for the adaptation of the methylotroph *Methylocystis parvus* OBBP to methanol at concentrations of up to 4% (w/v) has been described (Hou *et al.*, 1979*a*).

The enzyme responsible for the oxidation of methane in *Methylosinus trichosporium* OB3b has been described in detail (Tonge *et al.*, 1977; Stirling & Dalton, 1979). Methane mono-oxygenase (MMO) is an enzyme of broad specificity, oxidizing n-alkanes, n-alkenes, aromatic and alicyclic compounds (Colby *et al.*, 1977; Higgins *et al.*, 1979, 1980, 1981). Methanol has been reported to be a substrate (Colby *et al.*, 1977) and a competitive inhibitor of the enzyme (Colby *et al.*, 1975). The loss of this epoxidizing and hydroxylating activity by cultures of *M. trichosporium* OB3b grown on methanol has led to the suggestion that the appropriate enzyme system is inducible (Hou *et al.*, 1979*b*).

The ultrastructure of methanol-grown methanotrophs has been described by several groups of workers. Some have reported that the organisms possess intracytoplasmic membranes (De Boer & Hazeu, 1972; Linton & Vokes, 1978); others describe variable membrane content or

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vesicles (Whittenbury *et al.*, 1970*a, b*; Ohtomo *et al.*, 1977; Takeda & Tanaka, 1980) or, in some cases, the absence of intracytoplasmic membranes (Hyder *et al.*, 1979; Patt & Hanson, 1978).

This paper describes the growth of *M. trichosporium* OB3b on methanol, concomitant retention of epoxidizing and hydroxylating activities characteristic of methane mono-oxygenase, and changes in morphological characteristics, dependent on factors other than the growth substrate.

METHODS

Organism. The source and maintenance of *Methylosinus trichosporium* OB3b are described in the preceding paper (Scott *et al.*, 1981).

Growth of the organism. Adaptation of *M. trichosporium* OB3b to growth on methanol was achieved essentially as described for *Methylocystis parvus* OBBP (Hou *et al.*, 1979*a*) except that methanol was supplied in the vapour phase from a centre-well in a 250 ml conical flask. Flasks were inoculated with methane-grown colonies on agar plates and incubated with shaking at 30 °C. Growth occurred within 7–10 d and subsequent adaptation to aqueous methanol was achieved by the sequential transfer of cultures into media with progressively higher concentrations of methanol (0.1, 0.5, 1.0, 2.0, 4.0%, v/v). Growth was followed by determining turbidity in an EEL colorimeter and relating this to cell dry weight using a previously constructed calibration curve.

Stock cultures were routinely maintained in liquid culture with 1.0% (v/v) methanol, subcultured every 4–5 d.

Continuous cultivation was carried out in a 5 litre LKB 1601 Ultraform fermenter operating at 30 °C (impeller speed, 700 rev. min⁻¹; pH, 6.8; air flow rate, 2 l min⁻¹). At the methanol feedstock concentration of 0.25% (v/v) routinely used, the culture was under carbon limitation. The growth yield (Y_{Methanol}) was determined from measurements of the difference in methanol concentration in the feed and overflow, estimated gas chromatographically as described for propene oxide (see below), and of dry weights of culture samples, corrected for inorganic salt content.

Preparation of samples for electron microscopy. Samples of methanol-grown organisms were fixed using the standard procedure of Kellenberger *et al.* (1958). Where indicated, appropriate amounts of sucrose were added to the osmium tetroxide fixative and the Kellenberger washing buffer. Fixed samples were embedded in agar, diced, stained for 2 h in 0.5% (w/v) uranyl acetate in Kellenberger buffer, and sequentially dehydrated in a graded ethanol/water series, before embedding in Spurr's low viscosity resin (Spurr, 1969). Polymerization occurred overnight at 60 °C. Sections cut on an LKB Ultratome III were post-stained according to Reynolds (1963) and examined in an AEI EM801 electron microscope.

Determination of epoxidizing and hydroxylating activity. (a) *Whole organism biotransformation.* Organisms were harvested by centrifugation (10000 g, 10 min, 4 °C) and resuspended in 20 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-MgCl₂, to a cell density of 10 mg dry wt ml⁻¹. Samples of this suspension (5 ml) were incubated at 30 °C in 50 ml conical flasks capped with Suba-seals and the substrate (propene, 5 ml; ethylbenzene, 10–20 µl) was added by direct injection. Rates of oxidation were determined by monitoring product formation gas chromatographically using a Pye Unicam 204 instrument fitted with a flame-ionization detector, under the following conditions. (i) Conversion of propene to propene oxide (and methanol determinations). Cell suspension (2 µl) was directly injected on to a column (2.1 m × 4 mm i.d.) of Chromosorb 101 (nitrogen flow rate, 40 ml min⁻¹; injector temperature, 150 °C; column oven temperature, 110 °C; detector oven temperature, 200 °C). Retention times under these conditions were 7.1 min for propene oxide and 2.4 min for methanol. (ii) Conversion of ethylbenzene to 4-hydroxyethylbenzene (Higgins *et al.*, 1979, 1980, 1981). Cell suspension (100 µl) was extracted with an equal volume of ethyl acetate and the layers were separated by centrifugation. Samples of the organic layer (2 µl) were injected on to a column (2.1 m × 4 mm i.d.) containing 10% (w/w) SP2100 on Chromosorb W (nitrogen flow rate, 40 ml min⁻¹; injector temperature, 150 °C; column oven temperature, 120 °C; detector oven temperature, 200 °C). The retention time of the major product, 4-hydroxyethylbenzene, was 4.2 min.

(b) *Cell-free activity.* Organisms harvested by centrifugation (10000 g, 10 min, 4 °C) were washed once in ice-cold 20 mM-sodium phosphate buffer, pH 7.0, containing 5 mM-MgCl₂, and resuspended in the same buffer. Organisms were broken by sonication (2 × 45 s), and cell debris was removed by centrifugation (5000 g, 10 min, 4 °C). This extract was further centrifuged (38000 g, 30 min, 4 °C) yielding supernatant (S38) and particulate (P38) fractions. Methane mono-oxygenase activity was assayed as previously described (Colby & Dalton, 1978) by following the conversion of propene to propene oxide. Assays were carried out in sealed 10 ml conical flasks at 30 °C in a reciprocating water bath shaker. Reaction mixtures contained (in 1 ml): sodium phosphate buffer, pH 7.0 (20 µmol), MgCl₂ (5 µmol), NADH (ethanol-free; Colby *et al.*, 1977) (2.5 µmol), propene gas (84 µmol) and test protein (0.5–5.0 mg). Propene oxide was assayed as described above.

Polarographic measurements. Substrate-stimulated oxygen consumption by whole organism suspensions was measured at 30 °C using a jacketed Rank oxygen electrode (working volume 3 ml; Rank Bros, Bottisham, Cambs) containing: 2.5 ml (or 2.65 ml, with methanol as substrate) sodium phosphate buffer, pH 7.0, 250 µl cell suspension (1–3 mg dry wt organisms), and 250 µl methane- or propene-saturated phosphate buffer or 100 µl methanol (100 mM). At 30 °C, the solubilities of the gases in water are: methane, 1.23 µmol ml⁻¹; propene, 19.9 µmol ml⁻¹; oxygen, 0.23 µmol ml⁻¹ (International Critical Tables of Numerical Data).

Poly-β-hydroxybutyrate determinations. The poly-β-hydroxybutyrate content of organisms was determined according to Law & Slepecky (1961).

RESULTS

Batch growth of M. trichosporium OB3b on methanol

This organism would not grow on methanol (even at concentrations as low as 0.01 %, v/v) by direct transfer from cultures growing on methane. However, it could be adapted from plate growth on methane to growth on methanol vapour in liquid culture over a period of 7–10 d (see Methods). The organism grew well on methanol at initial concentrations of up to 4 % (v/v) after lag periods of 48–72 h. Specific growth rates of 0.063–0.086 h⁻¹, compared with a maximum specific growth rate of about 0.05 h⁻¹ on methane, were routinely observed. The organism grew well with either nitrate or ammonium as nitrogen source.

Stock cultures of methanol-adapted *M. trichosporium* were routinely maintained in liquid culture on 1 % (v/v) methanol; no loss of viability was observed, even after numerous transfers over many months. It was difficult, however, to maintain stocks on nitrate mineral salts agar (Whittenbury *et al.*, 1970a) to which methanol was supplied either directly or as vapour. Although growth to single colonies occurred on initial transfer from liquid to solid culture, viability was lost on subsequent transfer to fresh plates. Viability was maintained with the use of highly purified agar.

Continuous cultivation of the organism with methanol as sole carbon source

Growth of *M. trichosporium* in a fermenter is described in Fig. 1. A methanol feedstock concentration of 0.25 % (v/v) was routinely used and at this concentration the culture was carbon-limited, as evidenced by an increase in steady-state cell density on increasing the methanol feedstock concentration to 0.5 and 1.0 % (v/v). Under these conditions the exponential growth rate prior to establishing continuous cultivation ranged between 0.043 and 0.055 h⁻¹. Y_{Methanol} was 0.40 g dry wt (g methanol)⁻¹, a typical value for a methanol utilizer (Goldberg *et al.*, 1976). Once again, nitrate or ammonium served equally as well as nitrogen source. Under conditions of carbon limitation, levels of the endogenous reserve poly-β-hydroxybutyrate were very low (approximately 0.5 % of dry weight), compared with values of 25 % of dry weight when grown on methane under conditions of carbon excess.

Retention of epoxidizing and hydroxylating activity in methanol-grown organisms

Polarographic studies on washed cell suspensions derived from batch cultures grown on methanol at high concentrations revealed that the cells required copious washing to remove the residual methanol, which was largely responsible for apparently high endogenous respiration rates. As methanol is a substrate for MMO and therefore competitively inhibits oxidation of other substrates (Colby *et al.*, 1975), cell suspensions were preincubated in an open-topped oxygen electrode for 30 min to allow residual methanol to be metabolized. Table 1 summarizes the results obtained for various batch cultures. The rate of methane oxidation, as monitored by methane-stimulated oxygen uptake, was approximately 40 % of that measured for methane-grown organisms. Rates of methanol-stimulated oxygen uptake were similar to those measured in methane-grown organisms. Initial rates of propene oxidation were extremely variable, probably due to the different residual methanol concentrations present in the samples tested, but they were similar to rates of methane oxidation. The oxidation of ethylbenzene to its 4-hydroxy derivative was used as an indication of the

Fig. 1. Continuous cultivation of *M. trichosporium* OB3b on methanol. The organism was grown as described in Methods under the following conditions: methanol feedstock concentration, 0.25% (v/v); air flow rate, 2 l min⁻¹; pH, 6.8; temperature, 30 °C; dilution rate, 0.05 h⁻¹. Increases in steady-state organism concentrations are shown on increasing the methanol feedstock concentration to 0.5 and 1.0% (v/v) at the times indicated by the arrows.

Table 1. *Methane mono-oxygenase activities in methanol-grown M. trichosporium* OB3b

The organism was grown in batch culture on methanol. The abilities of washed suspensions to oxidize methane and methanol and to convert propene to propene oxide were measured as described in Methods.

Methanol concn in batch culture (% v/v)	Methane-stimulated oxygen uptake [nmol O ₂ utilized min ⁻¹ (mg dry wt) ⁻¹]	Methanol-stimulated oxygen uptake [nmol O ₂ utilized min ⁻¹ (mg dry wt) ⁻¹]	Conversion of propene to propene oxide [nmol propene oxide formed min ⁻¹ (mg dry wt) ⁻¹]
0.1	ND	ND	13.6
0.5	ND	ND	56.0
1.0	13.8	ND	12.2
2.0	12.5	83.2	21.0
4.0	14.4	93.0	14.0

ND, Not determined.

presence of a broad-specificity hydroxylating activity. In all methanol-grown samples tested this activity was present and was inhibited by inclusion in the incubation mixture of ethyne, a potent inhibitor of MMO (Dalton & Whittenbury, 1976). All cultures when spread on agar plates and incubated under methane/air (1:1, v/v) were morphologically homogeneous after 14 d growth.

Cultures of *M. trichosporium* maintained solely on methanol for 9 months retained both epoxidizing and hydroxylating activity.

Organisms harvested from steady-state continuous culture possessed epoxidizing and hydroxylating activities similar to those described above. The enzyme responsible for these activities was located in the soluble fraction of disrupted organisms and could be linked only to NAD(P)H. Its specific activity was similar to that of the soluble enzyme in extracts of methane-grown organisms [20–30 nmol min⁻¹ (mg protein)⁻¹]. The location of the enzyme in methane-grown organisms is dependent on the intracytoplasmic membrane content (Scott *et al.*, 1981) and this may well be so for methanol-grown organisms.

Fig. 2. Transmission electron micrographs of thin sections of *M. trichosporium* OB3b grown on methanol under different conditions: (a, b) batch culture, early-exponential phase; (c) batch culture, mid-exponential phase; (d, e, f) batch culture, deceleration phase; (g, h, i) continuous culture, dilution rate 0.057 h^{-1} . The bar markers represent $0.25 \mu\text{m}$. PHB, Poly- β -hydroxybutyrate; V, vesicle; IM, intracytoplasmic membranes; IN, invagination of the cytoplasmic membrane.

Ultrastructure of methanol-grown *M. trichosporium*

The standard Kellenberger fixation procedure (Kellenberger *et al.*, 1958) was preferred to other fixation methods as the integrity and shape of the bacteria was preserved to a greater extent. There were no peripheral intracytoplasmic membranes in organisms grown in shake flasks (1.0 and 0.1%, v/v, initial methanol concentrations) but there were extensive areas of

Fig. 3. Transmission electron micrographs of thin sections of *M. trichosporium* OB3b grown on methanol in continuous culture at a dilution rate of 0.025 h^{-1} and fixed under a variety of osmotic conditions (see Results). The bar markers represent $0.25 \mu\text{m}$. V, Vesicle; Va, vacuole.

electron-lucent material around the periphery. Chemostat batch cultures grown on methanol at an initial concentration of 0.25% (v/v) had a similar morphology in early-exponential phase. Large reserves of lipid, presumably poly- β -hydroxybutyrate, seen as light grey, smooth-surfaced granules, were scattered throughout the cytoplasm, surrounded by extended vesicles (Fig. 2*a, b*). Mid- and late-exponential phase organisms adopted a more vesicular appearance, the membrane-bound vesicles being predominantly peripheral and containing an amorphous material, less electron-dense than the surrounding cytoplasm. The membranes bounding the vesicles were not obviously contiguous with the cytoplasmic membrane and, as shown below, these structures did not represent osmotically 'blown' membrane sacs. This type of morphology predominated at this stage of growth (Fig. 2*c*).

In the deceleration phase the organisms had a morphology resembling the classical arrangement of Type II peripheral membranes, although the assembly was much less extensive than originally described for methane-grown organisms (Weaver & Dugan, 1975). The membranes were arranged somewhat irregularly around part of or the entire periphery and the intramembranous areas were devoid of ribosomes, found evenly scattered throughout the cytoplasm. Organisms possessing such poorly developed membrane assemblies were devoid of the membrane-bound vesicles characteristic of earlier phases of growth and, in general, contained little poly- β -hydroxybutyrate (Fig. 2*d, e, f*).

In steady-state continuous culture at a dilution rate equal to the batch culture exponential growth rate few of the peripheral membranes described above were seen. A large proportion

Fig. 4. Transmission electron micrographs of thin sections of *M. trichosporium* OB3b grown on methanol in continuous culture: (a, b, c) dilution rate 0.0125 h^{-1} ; (d, e, f) dilution rate 0.057 h^{-1} (before starvation); (g, h, i) dilution rate 0.057 h^{-1} , subsequent to starvation for 24 h. The bar markers represent $0.25 \text{ } \mu\text{m}$. V, Vesicle; FV, flattened vesicle; IM, intracytoplasmic membranes.

of the cell volume was occupied by vesicles formed by invagination of the cytoplasmic membrane (Fig. 2g, h, i). In some cases the degree of invagination was extraordinary, leaving very large areas between the cell wall and the cytoplasmic membrane. The occurrence of such large invaginations prompted an examination of the effect of the osmotic strength of the Kellenberger fixative on morphology.

Fig. 5. Transmission electron micrographs of thin sections of *M. trichosporium* OB3b grown on methanol at 22 °C: (a, b, c) batch culture, deceleration phase; (d, e, f) continuous culture, dilution rate 0.025 h⁻¹. The bar markers represent 0.25 μm. FV, Flattened vesicle; IM, intracytoplasmic membranes; OIM, obliquely cut intracytoplasmic membranes.

Organisms from continuous culture were fixed in six fixatives covering a wide osmotic range: (a) osmium tetroxide in distilled water; (b) osmium tetroxide in nitrate mineral salts medium; (c) standard Kellenberger fixative; (d) Kellenberger fixative plus 0.5% (w/v) sucrose; (e) Kellenberger fixative plus 1.0% (w/v) sucrose; (f) Kellenberger fixative plus 2.0% (w/v) sucrose. Post-fixation procedures were identical in all cases. Representative micrographs of sections from organisms fixed under the six different conditions are shown in Fig. 3 (a–f). The appearance of the organisms did not alter over the range of conditions (a)–(e). However, osmotic stabilization of the Kellenberger buffer with 2.0% (w/v) sucrose resulted in osmotic shock of the cells, the cytoplasmic membrane pulling away from the cell wall to leave large empty vacuoles. These were completely different in character from the cytoplasmic invaginations observed earlier, as they did not contain any amorphous material and were not localized intrusions into the cytoplasm. The occurrence of cytoplasmic invaginations was therefore unlikely to be an artefact of the fixation procedure. This was further supported by the fact that the electron density of the cytoplasm of osmotically shocked organisms was far greater than that of organisms possessing large vesicles or invaginations.

Samples derived from continuous culture at 30 °C at different growth rates all possessed a vesicular morphology (Fig. 2g, 3c, 4a), although with decreasing growth rate the amorphous vesicular material became more electron-dense, the vesicles occupied less of the cell volume

and, in some cases, the membranes of the flattened vesicles paired together and the cells adopted a more classic morphology (Fig. 4*b, c*). Similar changes were observed when organisms derived from continuous cultures at a specific growth rate of 0.057 h^{-1} were starved of carbon source for 24 h (Fig. 4*d-f, g-i*).

The batch culture exponential growth rate of organisms at $22\text{ }^{\circ}\text{C}$ was 0.025 h^{-1} and late-exponential phase organisms clearly possessed a more well-defined membrane assembly, with the paired membranes regularly stacked around the periphery (Fig. 5*a, b*). In obliquely cut sections of such organisms the membrane assembly appeared as a greyish, unresolved peripheral area, but in complete contrast to the extended membrane-bound vesicular structures described earlier (Fig. 5*c*). Continuous culture at $22\text{ }^{\circ}\text{C}$ at dilution rates of 0.025 or 0.013 h^{-1} produced organisms with restricted membrane arrays, as found in the $30\text{ }^{\circ}\text{C}$ deceleration phase cultures, and these paired membranous structures appeared to arise from flattening of the vesicles (Fig. 5*d, e, f*).

DISCUSSION

Despite the widely reported toxicity of methanol to obligate methylotrophs (Leadbetter & Foster, 1958; Stocks & McCleskey, 1964; Whittenbury *et al.*, 1970*a*), growth on methanol at high concentrations is clearly possible for *Methylocystis parvus* OBBP (up to 4%, w/v; Hou *et al.*, 1979*a*), *Methylococcus* NCIB 11083 (up to 0.2%, v/v; Linton & Vokes, 1978), and *Methylosinus trichosporium* OB3b (up to 4%, v/v; this paper). The mechanism by which these organisms adapt to growth on methanol at high concentrations is unknown but may reflect physiological adaptation of the population to the substrate or the selection of a mutant resistant to either methanol or formaldehyde.

Loss of ability of methanol-grown organisms to oxidize methane or substrate analogues has been reported for Bacterium B6 (Patel *et al.*, 1978) and for three other methylotrophs (Hou *et al.*, 1979*b*). In contrast, other workers have demonstrated the retention of methane-oxidizing activity in Type I methylotrophs (Linton & Vokes, 1978; Hyder *et al.*, 1979). Our results contrast with those obtained by Hou *et al.* (1979*b*) for the same strain of *M. trichosporium*. Retention of such activity would suggest that the enzyme is either constitutive or that it is induced by methanol. As methanol is a competitive inhibitor of methane mono-oxygenase (Colby *et al.*, 1975), one might postulate that failure to detect MMO activity in previous work may have arisen from the presence of residual methanol in the incubation mixtures. In the case of the fortuitous oxidation of propene, as opposed to supplementary oxidative metabolism (Higgins *et al.*, 1980), lack of epoxidizing activity might reflect the use of organisms from carbon-limited cultures for the following reason. In the absence of methane, the reducing power required for the oxidation of non-growth substrates is derived from endogenous reserves, in particular poly- β -hydroxybutyrate. Thomson *et al.* (1976) have demonstrated the concomitant loss of poly- β -hydroxybutyrate with the oxidation of non-growth substrates in *M. trichosporium* OB3b. Organisms from carbon-limited culture have low contents of this endogenous reserve and this may be reflected in reduced activity towards non-growth substrates in whole organism suspensions. Supplementing the incubation medium with formaldehyde or potassium formate as an exogenous energy source restores whole cell MMO activity (Dalton, 1980). Measurements of cell-free activities, where the reducing power is supplied in the form of exogenous NADH, of similar values to those for methane-grown cells would indicate that this may well be the case.

For several years now there has been speculation as to the role of intracytoplasmic membranes in methylotrophs, with particular reference to the oxidation of methane and to the physiological factors that promote their formation. The original reports describing intracytoplasmic membranes in Type II methylotrophs (Davies & Whittenbury, 1970) indicate that, compared with Type I methylotrophs, the membrane arrays are less well-ordered and bound lumens of varying dimensions, containing material less electron-dense

than the cytoplasm. This study would confirm that view but would go further in suggesting that particular cellular morphologies are determined by particular environmental situations. *Methylosinus trichosporium* OB3b grown on methanol possesses two characterized states – one containing vesicles, one possessing intracytoplasmic membranes – and several intervening morphologies. The type of morphology that predominates in any given culture is critically dependent on the growth conditions. Reports which state that methanol-grown methylophilic organisms do not possess intracytoplasmic membranes should therefore be regarded with caution as in many cases the organisms described have only been examined under one set of cultural conditions (Ohtomo *et al.*, 1977; Patt & Hanson, 1978; Hyder *et al.*, 1979; Takeda & Tanaka, 1980).

The vesicular morphology of *M. trichosporium* OB3b represents a poorly developed intracytoplasmic membrane array and is similar to that described for certain strains of hyphomicrobia (Conti & Hirsch, 1965) and, more recently, *Methanomonas margaritae* (Ohtomo *et al.*, 1977; Takeda & Tanaka, 1980). The morphological changes induced in the latter organism are ascribed to the disintegration of the regular lamellar membrane assembly in a medium devoid of copper. Clearly copper is not the cause of such structural changes in *M. trichosporium* OB3b as it was present at high concentrations (25 mg l⁻¹) under all conditions examined. To date, however, no single physiological parameter can unambiguously be associated with the induction of morphological changes in this organism and, indeed, it is possible that a combination of factors is responsible. Two factors have been implicated, apart from the presence or absence of copper; the first is the dissolved oxygen concentration, the second the carbon source (Patt & Hanson, 1978). The former may play some role in this organism (Brannan & Higgins, 1978; Higgins *et al.*, 1981), but the latter, the presence of methane or methanol as growth substrate, does not influence the formation of membranes. Our evidence would indicate that membranes are not induced by methane *per se* and neither are they obligatorily associated with the oxidation of methane, although they might play an important role in energy transduction. This study suggests that membranes are present in most circumstances to a very limited extent, bounding vesicles containing an amorphous material. These vesicles appear to arise from the invagination of the cytoplasmic membrane, and such a mechanism has already been suggested for the formation of membrane arrays (Davies & Whittenbury, 1970; De Boer & Hazeu, 1972). Subsequent to the formation of such large invaginations, in this case promoted by low growth rate and/or extreme carbon limitation, these vesicles tend to flatten out around the periphery of the organism, the membranes pairing up and the contained material becoming more electron-dense, but still devoid of ribosomes. Thus, the ultrastructure resembles that previously reported for this organism (Weaver & Dugan, 1975) when the membrane assembly was described as 'flattened, balloon-like vesicles, enclosing cavities within the cytoplasm'.

Clearly, elucidation of the factors which induce the formation of the intracytoplasmic membrane assembly and the role of these assemblies requires strict control of cultural conditions and current work is being directed to this end.

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