The Effect of Growth Conditions on Intracytoplasmic Membranes and Methane Mono-oxygenase Activities in *Methylosinus trichosporium* OB3b

By D. SCOTT, J. BRANNAN AND I. J. HIGGINS*†

Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

(Received 31 October 1980; revised 2 January 1981)

The distribution of methane mono-oxygenase (MMO) activities between particulate and soluble fractions of cell-free extracts of *Methylosinus trichosporium* OB3b was dependent upon growth conditions. Particulate activity was associated with the presence of intracytoplasmic membranes observed only under oxygen-limiting conditions in shake flask cultures. Particulate and soluble activities showed substantially different sensitivities to a range of potential inhibitors. The particulate enzyme was inhibited by metal-chelating agents, thiol reagents and amytal, whereas the soluble MMO was not inhibited by these compounds; both activities were sensitive to KCN, ethyne and 8-hydroxyquinoline. NAD(P)H was the only suitable electron donor. The activities were unstable at 0 °C but the soluble enzyme could be partially stabilized by several compounds. The particulate and soluble MMO activities are compared with previously reported particulate and soluble MMO enzymes from this species and other methane oxidizers.

INTRODUCTION

There have been many reports of intracytoplasmic membranes in methane-utilizing bacteria (Wolfe & Higgins, 1979). The assumption that these membranes are involved in the enzymic process of methane oxidation is supported by the fact that the facultative methanotroph *Methylobacterium organophilum* contains intracytoplasmic membranes when grown on methane, but not when grown on 'heterotrophic' substrates, e.g. glucose (Patt & Hanson, 1978). The membrane content of this organism, however, can be changed by altering the oxygen tension. Similarly, the intracytoplasmic membrane content of *Methylosinus trichosporium* OB3b, a Type II obligate methanotroph, can be varied by altering the proportion of air and methane in gassing mixtures of shake flasks (Brannan & Higgins, 1978). This organism shows very few intracytoplasmic membranes when grown in a chemostat, an effect also seen in a *Methylomonas* sp. (De Boer & Hazeu, 1972). Degeneration of intracytoplasmic membranes with ageing of cultures has been described for *Methylococcus capsulatus* (Texas), a Type I methanotroph (Hyder *et al.*, 1979).

Recently, there has been considerable controversy over the nature of cell-free methane mono-oxygenase (MMO) activity in *M. trichosporium* OB3b. MMO in this organism was originally described by Tonge *et al.* (1975, 1977) and Higgins *et al.* (1977) who reported that the activity was associated with particulate fractions of cell-free extracts, was sensitive to a wide range of inhibitors and utilized ascorbate as well as NAD(P)H as electron donor. Stirling & Dalton (1979) subsequently reported cell-free MMO in this organism to be entirely soluble and similar to the soluble MMO from *Methylococcus capsulatus* (Bath). However,

† Present address: CIT/Compiègne Unit in Biotechnology, Cranfield Institute of Technology, Cranfield, Bedford MK43 0AL, U.K.

Patel *et al.* (1979) found MMO in *M. trichosporium* OB3b to be particulate and similar to that originally described (Tonge *et al.*, 1975).

This paper describes the effect of growth conditions on intracytoplasmic membranes and MMO in *M. trichosporium* OB3b and findings are presented which account, in part, for inconsistencies in the recent literature concerning the nature of the MMO in this organism (Tonge *et al.*, 1975; Stirling & Dalton, 1979; Patel *et al.*, 1979).

METHODS

Materials. Most chemicals were the finest grade available and were obtained from sources previously reported (Tonge *et al.*, 1977). In addition, propene and propene oxide were obtained from BDH, and phenyl-methylsulphonyl fluoride was purchased from Sigma.

Growth and maintenance of organism. *Methylosinus trichosporium* OB3b, originally obtained from Professor R. Whittenbury (Department of Biological Sciences, University of Warwick), was maintained on ammonia mineral salts agar plates (Whittenbury *et al.*, 1970) under methane/air (1:1, v/v), subcultured every 3–4 weeks. It was grown at 30 °C on ammonia or nitrate mineral salts medium (Whittenbury *et al.*, 1970) supplemented with $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ($2.5 \mu\text{g l}^{-1}$), either in continuous culture at dilution rates of $0.02\text{--}0.05 \text{ h}^{-1}$ with methane (routinely 20–50%, v/v, in air) as carbon source, or in shaken 2 litre Buchner flasks ($120 \text{ rev. min}^{-1}$; amplitude, 5 cm) each containing 500 ml medium with various methane/air or methane/oxygen gas mixtures (see Results). Organisms for electron microscopic studies were grown as above and also in shaken 250 ml Buchner flasks containing 80 ml medium and various gas mixtures.

Preparation of cell-free extracts. Organisms from steady-state continuous cultures or from 2 litre shake flask cultures after 2–5 d growth ($A_{600} 0.5\text{--}0.7$) were harvested by centrifugation (10 000 g, 10 min, 4 °C), washed once in ice-cold 20 mM-sodium phosphate buffer, pH 7.0, containing 5 mM- MgCl_2 , and resuspended in the same buffer. Organisms were broken by sonication (MSE 150 W, $2 \times 45 \text{ s}$, 12–16 μm amplitude, 1 min cooling on ice between pulses), and cell debris and unbroken organisms were removed by centrifugation (5000 g, 10 min, 4 °C). The resulting cell-free extract was further centrifuged (150 000 g, 20 min, 4 °C) yielding supernatant (S150) and particulate (P150) fractions. The S150 fraction was frozen immediately by dropwise addition to liquid nitrogen and stored under liquid nitrogen. The P150 fraction was resuspended in ice-cold 20 mM-sodium phosphate buffer, pH 7.0, containing 5 mM- MgCl_2 , homogenized and treated similarly.

Methane mono-oxygenase (MMO) assays. MMO was assayed essentially as described by Colby & Dalton (1976) by following propene oxidation 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), MgCl_2 (5 μmol), NADH (ethanol-free; Colby *et al.*, 1977) (2.5 μmol) and test protein (0.5–5 mg); propene gas (2 ml) was injected through Suba-seals. The product, propene oxide, was estimated by gas chromatography using a glass column (2.1 m $\times$ 4 mm i.d.) of Chromosorb 101 (80–100 mesh) in a Pye Unicam 204 instrument fitted with a flame-ionization detector (nitrogen flow rate, 40 ml min^{-1} ; oven temperature, 110 °C).

Purification of CO-binding cytochrome c. CO-binding cytochrome c was purified using a modification of the procedure described by Tonge *et al.* (1977). Larger gel filtration columns were used containing Sephadex G-150 (5 $\times$ 80 cm), G-75 (5 $\times$ 80 cm) and were eluted in an upwards direction. Cytochrome-containing fractions were pooled, extensively dialysed against 5 mM-sodium phosphate buffer, pH 7.0, and applied to the top of a CM52 cation exchange column (2.2 $\times$ 33 cm). The cytochrome was eluted with a linear gradient of KCl (0–0.3 M) in 5 mM-sodium phosphate buffer, pH 7.0, at 4 °C. Finally, cytochrome-containing fractions were pooled and dialysed against 5 mM-sodium phosphate buffer, pH 7.0.

Determination of protein. Protein was measured either by a modified biuret method (Gornall *et al.*, 1949) or by a modified Lowry method (Kennedy & Fewson, 1968) with bovine serum albumin (Fraction V) as standard.

Electron microscopy. Organisms were fixed immediately after harvesting either (i) in glutaraldehyde (2.5%, w/v) in cacodylate buffer, pH 7.0, for 1 h, washed with cacodylate buffer, and then in osmium tetroxide (1%, w/v) in veronal acetate for 1 h or (ii) using the standard procedure described by Kellenberger *et al.* (1958). Fixed samples were embedded in agar (2%, w/v), diced, stained for 2 h in uranyl acetate (0.5%, w/v) 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.

Assay of poly- β -hydroxybutyrate. Poly- β -hydroxybutyrate from whole organism suspensions was extracted and quantified by the method of Law & Slepceky (1961).

RESULTS

Effect of growth conditions on intracytoplasmic membranes in M. trichosporium OB3b

Methylosinus trichosporium OB3b showed marked changes in intracytoplasmic membrane content and internal morphology depending on growth conditions. Thin sections of organisms grown in 250 ml shake flasks gassed with methane/air (5:1, v/v) or methane/oxygen (5:1, v/v) showed extensive peripheral membranes (Fig. 1*a, b*). Organisms from shake flasks with increased air or oxygen in gassing mixtures, e.g. methane/air (1:5, v/v) or methane/oxygen (1:5, v/v), showed correspondingly fewer intracytoplasmic membranes and some vesicles appeared (Fig. 1*c, d*). Degeneration of membranes to vesicles in late-exponential and early-stationary phase organisms occurred, as observed in *Methylomonas* sp. (De Boer & Hazeu, 1972) and *Methylococcus capsulatus* (Texas) (Hyder *et al.*, 1979). In contrast, organisms grown in continuous cultures at 30 °C under a variety of conditions, e.g. methane, oxygen or nitrogen limitation, never contained peripheral intracytoplasmic membranes but showed large vesicles within the cytoplasm (Fig. 2*a, b*). Those cultures grown under nitrogen-limiting or carbon-excess conditions also contained large granules of poly- β -hydroxybutyrate (Fig. 2*c*). Poly- β -hydroxybutyrate constituted 20–25% of dry weight under these conditions. Vesicles appeared to be formed by invagination of the cytoplasmic membrane (Fig. 2*d*). When organisms were stored aseptically in collector vessels at 15 °C for 48 h, a reduction in vesicle size was noted, and continuous cultures grown at 22 °C were predominantly vesicular, although a small percentage (5–10%) had poorly developed membrane systems (Fig. 2*e*). Vesicles are not artefacts caused by fixation procedures (Best & Higgins, 1981).

Distribution of MMO activity in cell-free extracts of M. trichosporium OB3b grown under different conditions: correlation with intracytoplasmic membrane content

Table 1 summarizes the effect of growth conditions on the distribution of MMO activity in cell-free extracts of *M. trichosporium* OB3b. When organisms were grown in 2 litre shake flasks under oxygen-limiting conditions (3–6%, v/v, oxygen in methane) a substantial, but variable, proportion (30–66%, 10 determinations) of total MMO activity was located in the 150 000 g particulate fraction. These cultures exhibited an extensive peripheral intracytoplasmic membrane system. However, organisms grown in continuous culture at various dilution rates (0.02–0.05 h⁻¹) and with different gassing mixtures (5–50%, v/v, methane in air; 50–95%, v/v, methane in oxygen) always had soluble MMO activity (12 determinations). No MMO activity was detected in any particulate preparations from these cultures. In

Table 1. *Effect of growth conditions on the distribution of MMO activity in cell-free extracts of M. trichosporium OB3b*

Growth, preparation of fractions of cell-free extracts and assay of MMO activities are described in Methods.

Growth condition (gas mixture, v/v)	MMO activity (% of total)	
	Particulate	Soluble
Shake flask (CH ₄ /O ₂ , 15:1)	30–66	34–70
Shake flask (CH ₄ /air, 5:1)	0–50	50–100
Shake flask (CH ₄ /air, 1:5)	0	100
Shake flask (CH ₄ /O ₂ , 1:5)	0	100
Chemostat, 30 °C (5–50% CH ₄ in air; 50–95% CH ₄ in O ₂)	0	100
Collector vessel, 15 °C, 48 h	7	93
Chemostat, 22 °C (20% CH ₄ in air)	8	92

Fig. 1. Electron micrographs of ultra-thin sections of *Methylosinus trichosporium* OB3b grown in shake flasks under different conditions. (a, b) Organisms grown under methane/oxygen (5:1, v/v) showed extensive peripheral intracytoplasmic membranes (M). (c, d) Organisms grown under methane/air (1:5, v/v) showed fewer membranes and some vesicles (V). The bar markers represent 0.2 μm.

Fig. 2. Electron micrographs of ultra-thin sections of *Methylosinus trichosporium* OB3b grown in continuous culture under different conditions. (a, b) Organisms grown at 30 °C under a variety of gas mixtures (see text) showed vesicles (V), often membrane-bound, and lacked a peripheral membrane system. (c) Organisms grown at 30 °C under nitrogen-limiting and methane-excess conditions had large granules of poly- β -hydroxybutyrate (PHB) in addition to vesicles (V). (d) Vesicles appeared to be formed by invagination of the cytoplasmic membrane (arrows). (e) A small percentage (5–10%) of organisms grown at 22 °C showed a poorly developed membrane system. The bar markers represent 0.2 μ m.

Table 2. *Effect of various compounds on the stability of MMO in soluble preparations stored at 0 °C for 24 h*

Samples (1 ml) of soluble extract were kept at 0 °C in sealed polythene tubes in the presence of the test compound for 24 h. The following compounds had no stabilizing effect: bovine serum albumin (1 mg ml⁻¹), 2-mercaptoethanol (10 mM), NAD⁺ (2.5 mM), propene (20%, v/v), CuCl₂ (5 mM), MnCl₂ (5 mM), MgCl₂ (50 mM). The original MMO activity was 21 nmol propene oxide produced min⁻¹ (mg protein)⁻¹.

Test compound	Concn (mM)	MMO activity (% of original)
None	—	0–5
Phenylmethylsulphonyl fluoride	1	44
	5	32
	10	0
Dithiothreitol	1	35
	5	11
	10	12
Sodium thioglycollate	1	16
	5	12
	10	13
NADH	1	24
	5	0
Anaerobic conditions	—	41

organisms stored in collector vessels at 15 °C for 48 h or harvested directly from continuous cultures at 22 °C, 7 and 8%, respectively, of MMO activity was in the particulate fraction.

Thus, organisms which possess vesicles and no intracytoplasmic membranes have entirely soluble MMO activity. The appearance of a poorly developed membrane system in some of the population is coincident with the sedimentation of 7–8% of MMO activity in the particulate fraction.

Electron donor specificity of soluble and particulate MMO

Both soluble and particulate MMO activities could only be linked to NAD(P)H; ascorbate or methanol could not be used as electron donors, although occasional low soluble activity was observed with ascorbate/tetramethyl-*p*-phenylenediamine. Addition of highly purified CO-binding cytochrome *c* to particulate fractions showing NADH-linked activity did not result in ascorbate-linked MMO activity. The involvement of this cytochrome in MMO activity is discussed later.

Stability of soluble and particulate MMO

MMO activity in soluble and particulate fractions was lost after storage at 0 °C for 24 h, although both preparations retained full activity when frozen and stored in liquid nitrogen for 24 h. Soluble activity could be partially stabilized for 24 h at 0 °C by several compounds (Table 2).

Effect of inhibitors on soluble and particulate MMO activities

Table 3 summarizes the effects of a variety of potential inhibitors on the two MMO activities. The inhibitor sensitivity of the soluble MMO was essentially the same as reported earlier for *M. trichosporium* OB3b (Stirling & Dalton, 1979) and similar to the *Methylococcus capsulatus* (Bath) soluble MMO (Colby & Dalton, 1976; Stirling & Dalton, 1977). The enzyme was, in our hands, more sensitive to KCN, particularly at low protein concentrations. Figure 3 shows the inhibition of MMO by KCN at various protein concentrations of soluble extract in the reaction mixture.

Table 3. *Effect of potential inhibitors on soluble and particulate MMO activities*

Assays were carried out as described in Methods. Extracts were incubated with the test inhibitor for 3 min before initiating the reaction with NADH. Values for KCN inhibition are for the same soluble or particulate protein concentration. The uninhibited (control) activities of the soluble and particulate fractions were 26 and 18 nmol propene oxide produced $\text{min}^{-1} (\text{mg protein})^{-1}$, respectively.

Inhibitor	Concn (mM)	MMO activity (% of control)	
		Soluble	Particulate
None	—	100	100
Thiourea	0.1	112	49
Imidazole	0.1	106	52
8-Hydroxyquinoline	0.1	14	8
2-Mercaptoethanol	0.1	92	39
Dithiothreitol	0.1	104	21
KCN	0.01	78	54
	0.1	50	23
	1.0	29	ND
Amytal	2.0	130	68
Ethyne	4% (v/v)	0	0

ND, Not done.

Fig. 3. Effect of KCN on soluble MMO activity with different protein concentrations in the assay mixture: 8 mg protein ml^{-1} (○); 4 mg protein ml^{-1} (●); 1 mg protein ml^{-1} (□). The uninhibited (control) activities were 24 nmol propene oxide produced $\text{min}^{-1} (\text{mg protein})^{-1}$ at 8 and 4 mg protein ml^{-1} , and 11 nmol $\text{min}^{-1} (\text{mg protein})^{-1}$ at 1 mg protein ml^{-1} .

In contrast, the particulate MMO activity was sensitive to a wide range of inhibitors (Table 3) including chelating agents, thiol reagents, amytal and KCN, as originally reported for this organism (Tonge *et al.*, 1977). Similar inhibitions by some of these compounds have been reported for the particulate MMOs of *Methylosinus* sp. (CRL-15) (Patel *et al.*, 1979), *Pseudomonas methanica* (Ferenci *et al.*, 1975; Colby *et al.*, 1975) and *Methylococcus capsulatus* Texas (Ribbons, 1975).

DISCUSSION

The presence of extensive peripheral intracytoplasmic membranes in *Methylosinus trichosporium* OB3b was only observed in oxygen-limited shake flask cultures; these cultures exhibited substantial particulate MMO activity. Organisms from shake flasks with high

proportions of oxygen in the gas phase and from continuous cultures had entirely soluble MMO activity and peripheral intracytoplasmic membranes were limited or absent, respectively. Thus, localization of the MMO in the particulate fraction of cell-free extracts is coincident with the appearance of an extensive intracytoplasmic membrane system dependent on oxygen-limited shake flask culture growth conditions. This finding would account for differences in the observed cell-free location of MMO activity recently reported for *M. trichosporium*. Stirling & Dalton (1979) reported a soluble MMO from organisms grown in continuous culture with methane (20%, v/v, in air) as carbon source; Patel *et al.* (1979) described particulate MMO activity from organisms grown in shake flasks gassed with methane/air (1:1, v/v).

The MMO activity was originally reported to be particulate (Tonge *et al.*, 1975; Higgins *et al.*, 1977) from organisms grown in oxygen-limited continuous culture. Recently though, we have only found soluble MMO activity from chemostat-grown organisms, even from oxygen-limited cultures ($pO_2 < 0.1\%$). The exact growth conditions in continuous culture required to induce intracytoplasmic membranes and particulate MMO remain to be re-established.

In contrast to previous reports (Tonge *et al.*, 1975, 1977; Higgins *et al.*, 1977) particulate MMO from *M. trichosporium* OB3b was found to be unstable at 0–4 °C and ascorbate could not serve as an artificial electron donor. Both of these properties, however, may require the addition of purified CO-binding cytochrome *c* which has retained its electron-transfer activity. The involvement of this cytochrome in MMO activity, as originally suggested by Tonge *et al.* (1975), is, at present, in doubt (Stirling & Dalton, 1979). The cytochrome clearly is an unusual protein, as revealed by n.m.r. data and its ability to bind gaseous hydrocarbons, e.g. methane and ethane (Hammond *et al.*, 1979). In soluble preparations, however, both ascorbate and NADH reduce the cytochrome to the same degree, yet only the latter electron donor can provide reducing equivalents for the MMO, suggesting lack of involvement of the cytochrome in soluble activity (D. Scott, unpublished observations). Addition of highly purified cytochrome c_{CO} to particulate fractions, which showed NADH-linked MMO activity, did not result in ascorbate-linked activity. Although the purified cytochrome used retained 60–65% CO-binding ability, it had been frozen which may have led to an alteration of electron-transfer properties (Tonge *et al.*, 1975).

Methylosinus trichosporium OB3b particulate MMO activity had the same overall inhibitor sensitivity as that originally described in this organism (Tonge *et al.*, 1977) and as that reported for other methanotrophs with particulate cell-free MMO (Ferenci *et al.*, 1975; Colby *et al.*, 1975; Ribbons, 1975; Patel *et al.*, 1979), while soluble MMO in *M. trichosporium* OB3b was similar to previously reported soluble MMO activities (Colby & Dalton, 1976; Colby *et al.*, 1977; Stirling & Dalton, 1979). On this evidence it would appear that the inhibitor sensitivities of the cell-free MMOs studied so far correlate with the location of the enzyme. Thus, particulate MMO activities are sensitive to chelating agents, thiol reagents and electron-transport inhibitors suggesting that the enzyme retains a close association with membrane-bound electron-transfer proteins and that either NAD(P)H is not the direct electron donor to the MMO or an alternative, inhibitor-sensitive, NAD(P)H acceptor-reductase component is synthesized. Soluble MMO activities are not inhibited by these compounds indicating that NAD(P)H is the direct electron donor.

The exact role of intracytoplasmic membranes in *M. trichosporium* OB3b has not yet been determined; clearly they are not obligatory for growth on methane. The presence of intracytoplasmic membranes and vesicles has been reported for growth of *M. trichosporium* OB3b on methanol (Higgins *et al.*, 1981). Synthesis of membranes in response to low dissolved oxygen tension in shake flask cultures may be to increase phosphorylating ability. During most growth conditions in the laboratory *M. trichosporium* OB3b does not have paired intracytoplasmic membranes.

Note added in proof. It is now possible to routinely obtain 100% particulate cell-free MMO activity in oxygen-limited chemostat cultures of *M. trichosporium* grown under the following conditions: methane/air/nitrogen, 1:1:4, by vol.; stirring rate, 500 rev. min⁻¹; dilution rate, 0.02 h⁻¹; biomass, 0.20–0.25 mg dry wt ml⁻¹. Under these conditions organisms have extensive paired intracytoplasmic membranes (see Fig. 1 a, b).

We thank Dr R. C. Hammond for assistance with the purification of cytochrome c, and the S.R.C. and I.C.I. Ltd for financial support.

REFERENCES

- BEST, D. J. & HIGGINS, I. J. (1981). Methane-oxidizing activity and membrane morphology in a methanol-grown obligate methanotroph, *Methylosinus trichosporium* OB3b. *Journal of General Microbiology* **125**, 73–84.
- BRANNAN, J. & HIGGINS, I. J. (1978). Effect of growth conditions on the intracytoplasmic membranes of *Methylosinus trichosporium* OB3b. *Proceedings of the Society for General Microbiology* **5**, 69.
- COLBY, J. & DALTON, H. (1976). Some properties of a soluble methane mono-oxygenase from *Methylococcus capsulatus* strain Bath. *Biochemical Journal* **157**, 495–497.
- COLBY, J., DALTON, H. & WHITTENBURY, R. (1975). An improved assay for bacterial methane mono-oxygenase: some properties of the enzyme from *Methylobacterium methanica*. *Biochemical Journal* **151**, 459–462.
- COLBY, J., STIRLING, D. I. & DALTON, H. (1977). The soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath). Its ability to oxygenate *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic and heterocyclic compounds. *Biochemical Journal* **165**, 395–402.
- DE BOER, W. E. & HAZEU, W. (1972). Observations on the fine structure of a methane-oxidizing bacterium. *Antonie van Leeuwenhoek* **38**, 33–47.
- FERENCI, T., STRØM, T., & QUAYLE, J. R. (1975). Oxidation of carbon monoxide and methane by *Pseudomonas methanica*. *Journal of General Microbiology* **91**, 79–91.
- GORNALL, A. G., BARDAWILL, C. J. & DAVID, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *Journal of Biological Chemistry* **177**, 751–766.
- HAMMOND, R. C., TAYLOR, F. & HIGGINS, I. J. (1979). Studies on the mechanism of methane mono-oxygenase of *Methylosinus trichosporium* OB3b. *Society for General Microbiology Quarterly* **6**, 89.
- HIGGINS, I. J., KNOWLES, C. J. & TONGE, G. M. (1977). Enzymic mechanisms of methane and methanol oxidation in relation to electron transport systems in methylotrophs; purification and properties of methane oxygenase. In *Microbial Production and Utilization of Gases (H₂, CH₄, CO)*, pp. 389–402. Edited by H. G. Schlegel. Göttingen: Goltze Druck.
- HIGGINS, I. J., BEST, D. J. & SCOTT, D. (1981). Hydrocarbon oxidation by *Methylosinus trichosporium*: metabolic implications of the lack of specificity of methane mono-oxygenase. In *Proceedings of the 3rd International Symposium on Microbial Growth on C₁ Compounds*, pp. 11–20. Edited by H. Dalton. London: Heyden.
- HYDER, S. L., MEYERS, A. & CAYER, M. L. (1979). Membrane modulation in a methylotrophic bacterium *Methylococcus capsulatus* (Texas) as a function of growth substrate. *Tissue and Cell* **11**, 597–610.
- KELLENBERGER, E., RYTER, A. & SECHAUD, J. (1958). Electron microscope study of DNA-containing plasmids. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *Journal of Biophysical and Biochemical Cytology* **4**, 671–678.
- KENNEDY, S. I. T. & FEWSON, C. A. (1968). Enzymes of the mandelate pathway in bacterium N.C.I.B. 8250. *Biochemical Journal* **107**, 497–506.
- LAW, J. H. & SLEPECKY, R. A. (1961). Assay of poly-β-hydroxybutyric acid. *Journal of Bacteriology* **82**, 33–36.
- PATEL, R. N., HOU, C. T., LASKIN, A. I., FELIX, A. & DERELANKO, P. (1979). Microbial oxidation of gaseous hydrocarbons. II. Hydroxylation of alkanes and epoxidation of alkenes by cell-free particulate fractions of methane-utilizing bacteria. *Journal of Bacteriology* **139**, 675–679.
- PATT, T. E. & HANSON, R. S. (1978). Intracytoplasmic membrane, phospholipid, and sterol content of *Methylobacterium organophilum* cells grown under different conditions. *Journal of Bacteriology* **134**, 636–644.
- REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**, 208–212.
- RIBBONS, D. W. (1975). Oxidation of C₁ compounds by particulate fractions from *Methylococcus capsulatus*: distribution and properties of methane-dependent reduced nicotinamide adenine dinucleotide oxidase (methane hydroxylase). *Journal of Bacteriology* **122**, 1351–1363.
- SPURR, A. R. (1969). A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31–43.
- STIRLING, D. I. & DALTON, H. (1977). Effect of metal-binding agents and other compounds on methane oxidation by two strains of *Methylococcus capsulatus*. *Archives of Microbiology* **114**, 71–76.
- STIRLING, D. I. & DALTON, H. (1979). Properties of the methane mono-oxygenase from extracts of *Methylosinus trichosporium* OB3b and evidence for its similarity to the enzyme from *Methylococcus capsulatus* (Bath). *European Journal of Biochemistry* **96**, 205–212.

- TONGE, G. M., HARRISON, D. E. F., KNOWLES, C. J. & HIGGINS, I. J. (1975). Properties and partial purification of the methane-oxidizing enzyme system from *Methylosinus trichosporium*. *FEBS Letters* **58**, 293–299.
- TONGE, G. M., HARRISON, D. E. F. & HIGGINS, I. J. (1977). Purification and properties of the methane mono-oxygenase enzyme system from *Methylosinus trichosporium* OB3b. *Biochemical Journal* **161**, 333–344.
- WHITTENBURY, R., PHILLIPS, K. C. & WILKINSON, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *Journal of General Microbiology* **61**, 205–218.
- WOLFE, R. S. & HIGGINS, I. J. (1979). Microbial biochemistry of methane – a study in contrasts. In *International Review of Biochemistry*, vol. 21, pp. 267–353. Edited by J. R. Quayle. Baltimore: University Park Press.