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Methane-Oxidizing Microorganisms

I. J. HIGGINS,[1]* D. J. BEST,[1] R. C. HAMMOND,[2] AND D. SCOTT[1]

Biotechnology Unit, Cranfield Institute of Technology, Cranfield, Bedford, MK43 0AL,[1] and Unilever Research, Colworth Laboratory, Sharnbrook, Bedford, MK44 1L9,[2] United Kingdom

INTRODUCTION

Despite studies of methane-oxidizing microorganisms (methanotrophs) spanning some 75 years, their importance in global elemental cycles is neither widely acknowledged nor fully understood. Nevertheless, particularly in the last 15 years, many groups have accepted the challenge of methanotrophic microbiology, in some cases spurred on by the prospects of commercial exploitation of these microorganisms, initially for single-cell protein production and more recently as biocatalysts or sources of useful polymers. There is now a substantial body of knowledge of methanotrophy, but it is not our intention here to offer an extensive historical review; rather, we wish to highlight recent discoveries and to assess the environmental importance and possibilities for commercial exploitation of this fascinating group. For complementary information on methanotrophs, the recent reviews by Colby et al. (20), Hanson (50), Wolfe and Higgins (195), and Higgins (59) should be consulted. The microbial transformation of C_1 compounds in general is discussed by Higgins and Hammond (63), and two excellent articles on the microbial assimilation of C_1 compounds were recently published by Quayle (131, 132).

The magnitude of turnover in the biosphere of the major carbon source for methanotrophs, methane, is not widely recognized. Approximately 50% of total organic carbon degraded by anaerobic microflora is converted to methane. Sources of atmospheric methane are summa-

rized in Table 1, which was compiled from the data of Ehhalt (41). Biogenic methane is the major source of atmospheric methane and is quantitatively similar to the output from natural gas wells. It is equivalent to about 0.5% of the annual production of dry organic matter, but recent environmental studies (discussed in Biology) indicate this to be only a small fraction of total biogenic methane, most of which is oxidized microbiologically to CO_2 before the carbon reaches the atmosphere. Methane is, therefore, one of the most abundant organic compounds on this planet, and this is reflected by the abundance of methanotrophs in the environment. They can represent up to 8% of the total "heterotrophic" population, and in some sediments methanotroph counts of 5×10^7/ml have been reported (184). Thus, methanotrophs constitute one of the major groups of free-living microorganisms, and their metabolic activities play a major role in determining the nature of the environment. Although it is self-evident that methanotrophs may play a role in maintaining low levels of methane in the atmosphere (1.4 ppm, vol/vol), other consequences of their activities are less well understood or quantifiable at the time of writing. There is, nevertheless, accumulating evidence that this group of microorganisms plays important roles in the nitrogen cycle and in the degradation of complex, particularly hydrophobic, organic compounds (60).

In the last few years, several concepts about the nature of methanotrophy have been questioned by a number of studies. The most interesting aspects concern the prevalence and nature of obligate methanotrophy versus facultative methanotrophy, anaerobic methane oxidation, ancillary metabolism of complex compounds, the multiplicity of C_1 incorporation pathways in some species, the nature of methane monooxygenases (MMOs), and the role of intracytoplasmic membranes. These aspects are examined in this review, and an attempt is made to give a contemporary view of the environmental significance of these microorganisms and to assess the potential for their industrial exploitation.

TABLE 1. *Sources of atmospheric methane*

Source	Global methane production (Mt/yr)
Biogenic[a]	
Enteric fermentation in animals	101–220
Paddy fields	280
Swamps, marshes	130–260
Freshwater lakes	1.3–25
Upland fields	10
Forests	0.4
Tundra	0.8–8
Oceans	
Open	4–6.7
Continental shelf	0.07–1.4
Total biogenic	528–812
Other sources	
Coal mining	6.3–22
Lignite mining	1.6–5.7
Industrial losses	7–21
Automobile exhaust	0.5
Volcanic emissions	0.2
Total other sources	15.6–49.4
TOTAL ALL SOURCES	544–862
Other relevant figures for comparison	
Output of natural gas wells for consumption (1965)	520
Dry organic matter	165,000
Atmospheric methane (about 1.4 ppm, vol/vol)	4,000

[a] These figures indicate only methane released into the atmosphere and do not include biogenic methane oxidized by methanotrophs.

BIOLOGY

Taxonomy

Most methanotrophic bacteria that have been isolated are obligate for reduced C_1 substrates and gram-negative, obligately aerobic rods, vibrios, or cocci. The most extensive amount of published data on isolation of these organisms is due to Whittenbury and his colleagues (188), who classified the isolates into type I (*Methylococcus*, *Methylomonas*, and *Methylobacter*) and type II (*Methylosinus* and *Methylocystis*). The major division between types I and II was based upon differences in intracytoplasmic membrane arrangement and carbon incorporation pathways. Most workers accept this as a basis for development of a formal classification, and most organisms isolated since this work fall more or less into one of these groups. As more detailed knowledge has accumulated, the need for two subgroups (A and B) above the genus level has arisen for classification of type I organisms (188), and the new genus of facultative methanotrophs, *Methylobacterium* (117, 122), has been accommodated as a subgroup within type II. It shares with type II species, which use only reduced C_1 substrates, the characteristics of paired peripheral membranes when growing on methane and C_1 incorporation via the serine pathway. Recent deoxyribonucleic acid (DNA) homology studies (50) suggest, however, that this classification is inappropriate, for there is little homology between the DNAs of *Methylobacterium organophilum* XX and a representative type II species,

Methylosinus trichosporium OB3b. Type I species have membranes that are bundles of vesicular disks, and they use the ribulose monophosphate (RMP) cycle. Subgroup B species also possess the ribulose diphosphate pathway of CO_2 fixation. Attempts are now being made to devise a formal taxonomy and nomenclature of methanotrophs (140, 142), although there is a continuing need to accommodate the unexpected, e.g., *Methylococcus capsulatus* (Bath), which has three pathways for incorporation of C_1 units: ribulose diphosphate, RMP, and serine pathways (166). This may represent a third major group, type X (185).

Recently, yeasts have been isolated which are facultative methanotrophs (192) and have been identified as strains of *Sporobolomyces roseus*, *Sporobolomyces gracilis*, *Rhodotorula glutinis*, and *Rhodotorula rubra* (193).

Some bacterial species can oxidize methane anaerobically by coupling the oxidation to sulfate reduction (109), but these remain to be identified. The recent finding that well-characterized methanogenic bacteria can also oxidize methane, albeit slowly, is fascinating (198). Although the rates involved are only 0.001 to 0.3% of the rate of methane formation, the mechanism appears to be distinct and not a simple reversal of the methanogenic system. There is inconclusive evidence that some algae and fungi can oxidize methane (42, 197).

Ecology

The vast majority of methane-utilizing species isolated from a wide range of natural environments are aerobic, obligate, methanotrophic bacteria. Whether this is a true reflection of relative abundance or an artifact due to isolation procedures remains to be established.

Methane is continually generated in anaerobic environments, and there is good evidence for its anaerobic oxidation linked to sulfate reduction by uncharacterized microorganisms present in sediments (50, 109). In most situations where methane diffuses into aerobic environments, large populations of aerobic methanotrophs can be found, e.g., soils, surface layers of sediments, and natural waters. As mentioned in Introduction, their numbers can be extremely high in sediments, and counts of 2.5×10^5/ml have been measured in lake water (77). The greatest numbers in water columns are found in the thermocline zone of meromictic lakes. The indigenous population is often highly effective in oxidizing methane. For example, in experiments with columns of sediments, in some cases none of the methane generated in the lower, anaerobic regions is released into the atmosphere above the sediment (184). Similarly, gas bubbles released from a lake bottom showed a 75% decrease in methane content over a vertical distance of 10 m (41). It is clear, even from the relatively few determinations of numbers of methanotrophs in the environment, that they are ubiquitous and represent a significant proportion of the natural microflora. There is no indication that particular species are habitat specific; rather, a variety of species are found in all habitats (184). Thermophilic and thermotolerant strains have been isolated (96, 184, 188).

With a fluorescent antibody staining technique, Reed and Dugan (134) surveyed the distribution of *Methylomonas methanica* and *M. trichosporium* in Cleveland Harbor (Cleveland, Ohio). Concentrations of the two methanotrophs were inversely proportional to sampling depth, suggesting that they grew primarily in or about 1 m above the sediment. *M. methanica* was observed at every sampling station and *M. trichosporium* was observed at only two, but a seasonal winter variation in the former, but not in the latter, correlated with the laboratory observation that *M. methanica* was cold sensitive. This study supports the view that methane oxidizers grow primarily just above the anaerobic environments, in areas of low oxygen tensions.

It may be that some methanotrophs, although having an obligate requirement for reduced C_1 compounds, commonly co-utilize ancillary carbon and energy sources. This is discussed further in Carbon Metabolism. It is even possible that some species have an obligate requirement for methane plus another organic substrate. To our knowledge there have been no attempts to isolate such strains. Ecological aspects are not discussed in detail here, since a highly authoritative review of methylotrophic ecology by Hanson has recently been published (50).

Morphology and Physiology

An outstanding morphological feature of methanotrophs is the ability to develop extensive intracytoplasmic membrane structures. These have been described by several groups of workers in many species (195) and, as discussed above, are used as a taxonomic criterion. Similar membranes are found in photosynthetic bacteria, ammonia and nitrite oxidizers, blue-green algae (cyanobacteria), and some higher hydrocarbon utilizers, and they contain lipids that are unusual in bacteria (195).

Accepting that these membranes need not fulfil the same role in these different groups, in the current context, what is their role in methanotrophs? They are clearly not necessary for growth on methanol, since facultative methylo-

trophs are devoid of them. It has therefore been assumed that the membranes are associated specifically with the initial metabolism of methane, and this contention would appear to be supported by the fact that facultative methanotrophs only contain such membranes when grown on methane (94, 123, 195).

Fine structure of type I methane-utilizing bacteria. Type I methanotrophs possess uniform arrays of membranes, distributed evenly throughout the cytoplasm, consisting of a stacked series of flattened discoidal vesicles (28, 30, 57, 76, 126, 150). Early studies suggested a uniformity of morphology in these arrays, each saccule being limited by a unit membrane 7.5 to 8.0 nm in width and adjacent saccules being separated by a 2.0-nm gap. The saccule width varied between 15.0 and 20.0 nm (150). Continuity between these internal membrane arrays and the cytoplasmic membrane has been noted (28, 30, 46). In species subject to detailed examination (*M. capsulatus*, *Methylomonas* sp.), the internal membrane system is extensive, occupying most of the cell volume (30, 76, 150). Branching of these internal membranes has also been noted (30).

Recently, however, it has been demonstrated that the internal morphology of these type I methane utilizers is radically affected by culture age and condition (76). Exponentially growing organisms exhibit the classical orderly parallel stacks of membrane sacs described above. However, as the culture enters the early stationary phase, these membrane arrays become less numerous and less orderly, and many organisms contain electron-lucent droplets of unknown composition. In the late stationary phase this disordered state becomes even more pronounced, the membrane sacs being widely distended, if present at all, and many spheroidal inclusions are found. In addition to this degeneration of the regular membrane arrays in older cultures, other morphological pecularities have been reported. In a *Methylomonas* sp. two types of polar organelle have been described, both apparently contiguous with the cytoplasmic membrane. One is 20.0 nm wide, from the cytoplasmic to the limiting membrane, the interspace being filled with "spikes" perpendicular to the bordering layer. The second possesses two bordering layers, each 7.0 nm thick and 1.5 nm apart, parallel to and 45.0 nm from the cytoplasmic membrane. The intervening space is filled with spikes 30.0 nm in length. Older cells also contain myelin-like structures, consisting of a variable number of electron-dense and electron-transparent striated layers with a periodicity of 3.2 nm. It has been suggested that lipid material from degenerating membranes is transformed into these structures (30).

Later stationary-phase cultures of *M. capsulatus* often possess crystalloid inclusions, presumably protein, with regular 10-nm spacing (76). In addition to the uniform membrane stacks observed in *Methylococcus mobilis* sp. nov. (57), regular patterns of tubular structures 22 nm in diameter have been observed when this species is grown with peptone as the organic nitrogen source. The significance of these various structures and their prevalence are unknown.

All detailed studies of type I methanotrophs have involved organisms from batch cultures in shake flasks. Little is known of their morphology under carefully defined growth conditions, i.e., in a fermentor, although De Boer and Hazeu (30) noted that the membrane stacks were less extensive under these conditions, more areas of low electron density being scattered throughout the cytoplasm, with the outer double layer of the cell wall forming unusual projections.

The presence of membranes in methanol-grown type I species has also been reported (30, 46, 76). However, organisms are largely filled with electron-lucent droplets, but transfer back to growth on methane induces membrane formation, concomitant with a reduction in the number of electron-lucent droplets (76). It has thus been concluded that intracytoplasmic membranes are induced by methane per se. Internal membranes have, however, been observed during the growth of *Methylococcus* strain NCIB 11083 on methanol (91), although the possibility that they have been induced by ammonia, a methane analog, could not be completely ruled out.

The behavior of the stacks of intracytoplasmic membranes during binary fission has been described as both an arbitrary division and redistribution of the membranes between daughter cells (30, 46, 98) and invagination of the cytoplasmic membrane. Monosov (98) concluded that the processes involved in membrane genesis are connected with the accumulation of phospholipid micelles in growing organisms. The centers of micelle formation are closely associated with the cytoplasmic membrane and may be precursors of the intracytoplasmic bilayers.

Fine structure of type II methane-utilizing bacteria. Davies and Whittenbury (28) first described the two types of membrane organization in methane-oxidizing bacteria. They reported that membranes from methane- and methanol-grown type II methanotrophs were similar in appearance to the cytoplasmic membrane, but were less ordered than type I mem-

brane arrays. Membranes in type II species were always paired and enclosed variable-sized lumens. Although they were usually distributed throughout the cytoplasm, in some sections they appeared to be mainly peripheral. Extensive peripheral "stacks" of membranes have also been observed in various type II methanotrophs, e.g., *Methanomonas methanooxidans* (*Methylomonas methanooxidans*) (149) and *M. trichosporium* OB3b (181); flattened vesicles have been seen in the latter strain (180, 181), and large vesicles have been seen in *Methanomonas margaritae* (106, 162).

M. trichosporium OB3b also shows marked changes in intracytoplasmic membrane content and internal morphology which are dependent on growth conditions (10, 13, 62, 146). During growth in shake flasks, the membrane content appeared to be related to the amount of oxygen in the gas phase (146). Organisms grown with little air or oxygen showed extensive paired membranes (Fig. 1A), whereas correspondingly less membranes were observed in those grown with more air or oxygen (Fig. 1B) Growth in continuous culture at 30°C under a variety of conditions, for example, methane, oxygen, or nitrogen limitation or at maximum growth rate, yielded organisms lacking parallel paired membranes and containing only membrane-bound vesicles (Fig. 1C) Interestingly, the appearance of membranes in shake flask-grown organisms correlated with the particulate location of MMO (146) (see Energy Metabolism). Organisms with extensive membrane arrays were obtained recently by growth in continuous culture at low cell density with excess nitrate and low methane and oxygen partial pressures (D. Scott, unpublished data).

M. trichosporium OB3b, grown on methanol, also undergoes morphological changes with varying growth conditions (10). Cells grown in batch culture display many electron-lucent vesicles throughout the early logarithmic and midlogarithmic phase. Towards stationary phase, vesicles no longer prevail and irregular membrane arrays are seen. The vesicular morphology is retained during continuous culture at 30°C, but at 22°C well-ordered paired membranes are present.

There are reports of surface structures similar to those of some type I species in two *Methylocystis* strains. Spiral, tubular appendages perpendicular to and distributed over the outer surface have been observed in *Methylocystis echinoides* (sp nov.) (160) and *Methylocystis* sp. strain IC493S/5 (55).

Three facultative methanotrophs have been examined for intracytoplasmic membranes after growth on different substrates. *M. organophilum* exhibits typical type II membranes only when grown on methane, not when methanol or glucose are carbon sources (123). Similarly, both *Methylobacterium ethanolicum* and *Methylobacterium hypolimneticum* have membranes during methane growth, but not when heterotrophically grown (94).

Effect of growth conditions on intracytoplasmic membranes. It is now clear that the internal morphology of methanotrophs is dependent upon growth conditions. As previously mentioned, *M. organophilum* contains intracytoplasmic membranes only during growth on methane (123), and the membrane content is dependent on dissolved oxygen tension. At very low dissolved oxygen tension, three to four layers of paired membranes are present, whereas at higher dissolved oxygen tension, typically only one peripheral paired membrane is observed. Similar low dissolved oxygen tension during methanol, glucose, or succinate growth does not induce membranes. Synthesis of membranes is also encouraged by low temperature and low growth rates. Nevertheless, the nature of regulation of membrane synthesis remains unclear. In several methanotrophs it appears that methane per se does not induce membrane formation, for methanol-grown organisms under certain conditions possess arrays of intracytoplasmic membranes (10, 28, 30, 91). It is conceivable that under these latter circumstances, membranes are synthesized in response to a methane analog, ammonia or methanol (10, 91).

Recent studies indicate that such membrane arrays are not static assemblies, but are in a dynamic state of flux, and changes in the arrays occur in response to environmental changes (10, 76, 106, 146, 162). Degradation of intracytoplasmic membranes occurs in stationary-phase batch cultures, yielding distended membrane lumens. Paired membranes are seldom present in organisms grown in continuous culture under a variety of conditions. The predominant morphology is represented by either distended membrane stacks or vesicles in type I or type II methanotrophs, respectively. These structures probably arise from lipid accumulation in the organism during unbalanced growth. Over-utilization of carbon source occurs in many methanotrophs, with the concomitant accumulation of intracellular storage polymers (90). Thus, in the first instance, morphology may be determined by the relative levels of carbon (methane or methanol) and nitrogen sources. The subsequent extent of membrane synthesis may then be controlled by the dissolved oxygen tension (123, 146), which may reflect the specific localization of electron transfer proteins (146).

Phospholipid and fatty acid compositions

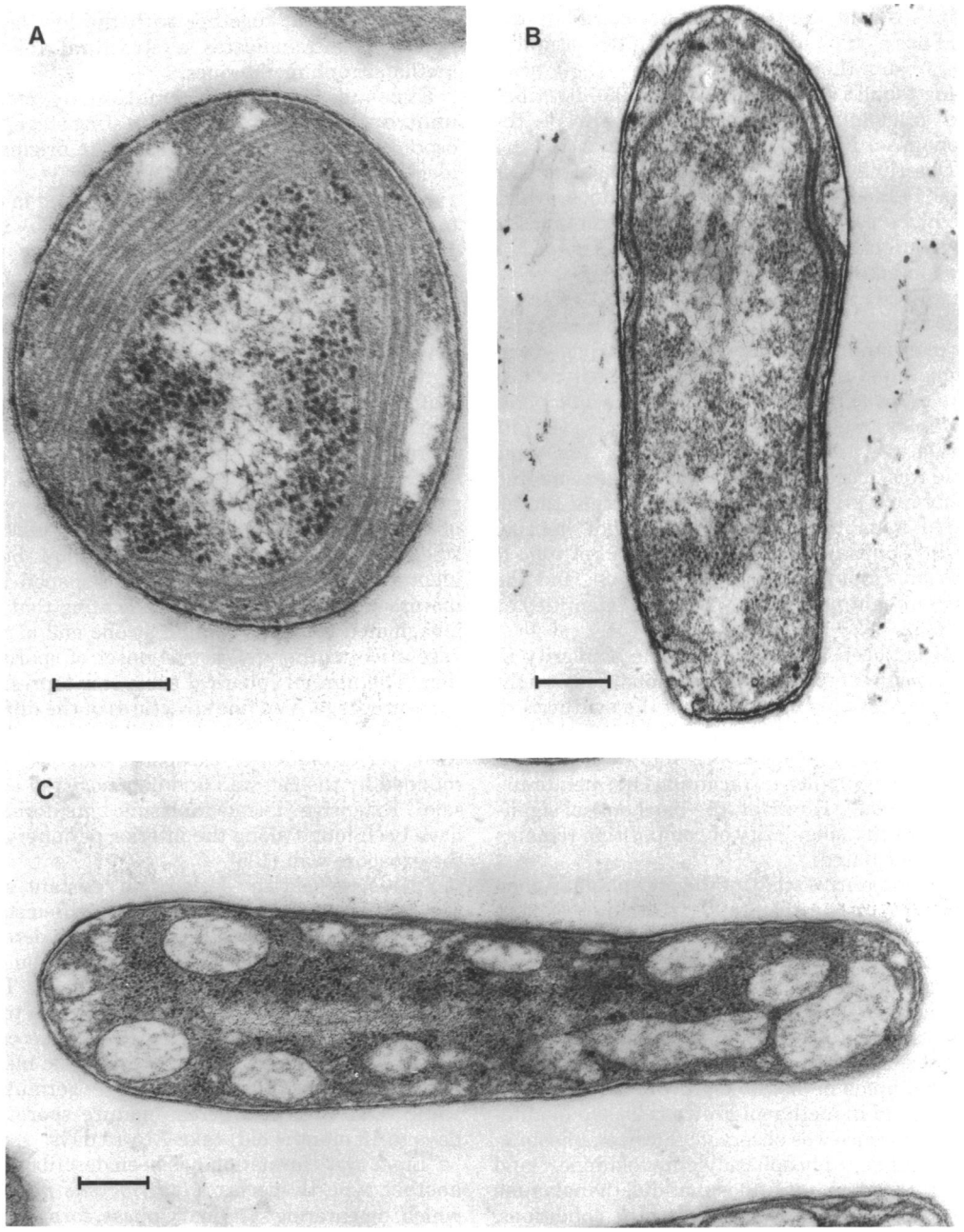


FIG. 1. *Effect of growth conditions on internal morphology of M. trichosporium OB3b (146): transmission electron micrographs of thin sections of M. trichosporium OB3b prepared after growth of the bacterium under different conditions, i.e., (A) oxygen-limited shake flask culture, (B) shake flask culture with high oxygen levels, (C) chemostat cultivation (specific growth rate, 0.02/h; cell density, 0.2 g/liter). Bar = 0.2 μm.*

of methanotrophs. It has been suggested that the fatty acid composition of methane-oxidizing bacteria is probably correlated with the structure of the membrane system (46). Type I methanotrophs, as exemplified by *M. capsulatus*, exhibit a typical gram-negative bacterial phospholipid pattern (95), the major component is phosphatidylethanolamine with, in addition, phosphatidylglycerol, phosphatidylcholine, and cardiolipin. A characteristic of type I methano-

trophs is that the predominant esterified fatty acid has a chain length of 16, both the saturated ($C_{16:0}$) and the monounsaturated ($C_{16:1}$) acids being found (1, 95, 150). Makula (95) described the predominant positional isomers of the monounsaturated acid as Δ^9, Δ^{10}, and Δ^{11}.

Descriptions of the phospholipid patterns in type II methanotrophs are at variance, which might be ascribed to differences in methods of cultivation (95, 182). For *M. trichosporium* the predominant phospholipid was reported as phosphatidylglycerol (182) or the methyl esters of phosphatidylethanolamine (95), small amounts of cardiolipin and lysophosphatidylglycerol being found in the latter case. One striking feature is the similarity of the ratio of acidic phospholipids to phosphatidylethanolamine and its derivatives (about 1:4) in all strains. The same ratio was observed in both whole organisms and membrane preparations, indicating that the internal membrane composition is not unusual (182). The major esterified fatty acid of type II methanotrophs is $C_{18:1}$ (95, 149, 182), and the predominant positional isomer was identified as Δ^{10} (95).

It has been suggested that the similarity in fatty acid composition of phospholipids might be advantageous in facilitating the synthesis of various phosphatides via a common precursor, and this might be especially important to organisms with complex intracytoplasmic membrane systems (182). However, the biochemical significance of this singularity of composition remains to be elucidated.

A comparative study of the phospholipid compositions of the facultative methanotroph *M. organophilum* grown under different conditions was performed in an attempt to identify lipids specifically associated with intracytoplasmic membranes (123). The amount of total lipid correlated with the amount of membrane material observed in thin sections, and the amount of neutral lipids in methane-grown cells was twice that found in methanol-grown cells. No qualitative difference was observed; however, phosphatidylcholine, phosphatidylethanolamine, and methyl esters of phosphatidylethanolamine were present under all growth conditions. Growth on methane resulted in a decrease in the ratio of phosphatidylcholine to phosphatidylethanolamine. Several sterols, differing from those identified in *M. capsulatus* (11), were found, most notably in organisms grown on methane at low dissolved oxygen tension, conditions favoring membrane formation. Sterols are believed to play an important structural role in membrane formation, but in this study the sterols were not confined to the intracytoplasmic membrane material. This fact, together with the low levels present, contraindicates a structural role in methanotroph membranes.

Exposure and cyst formation by methanotrophs. Three varieties of resting stage, exospores and two types of cysts, were originally identified in methanotrophs (187).

Exposure formation has been described in detail in strains of *M. trichosporium* (135, 136, 187). Spore formation is initiated during the early stationary phase. The onset is characterized by the appearance at the polar tip, distal from the holdfast, of a second capsule. The development of this second capsule may be monitored by fluorescent antibody staining techniques (135) by determining the increase in the intensity of fluorescence relative to the stage of sporulation. Antigens that are different from vegetative cell antigens are thus present on the surfaces of the exospores and exospore regions of vegetative cells. Both immature and mature stages of spore development have now been identified (136). Cup- and crescent-shaped immature exospores are found, indicating that an invaginated sphere is formed at one end of the vegetative mother cell at the onset of sporulation. The normal spherical exospores represent a mature stage. The fine structures of the different types of spores are essentially similar. The thick, electron-dense exospore wall is surrounded by the cell wall and its associated capsule. Extensive intracytoplasmic membranes have been found along the interior periphery of the exospore wall (136).

Spores are heat and desiccation resistant, surviving at least up to 18 months in the dried state in the absence of methane, and have no detectable respiratory activity (187). Germination occurs after aerobic exposure to methane. The thick exospore wall disappears, and a germ tube develops, which lacks the extensive fibrillar capsule and into which the intracytoplasmic membrane extends. Freshly formed spores germinate within 2 to 3 days, whereas mature spores (7 days to 18 months old) take 7 to 15 days.

"Lipid cyst" formation has been described for another type II species, *Methylocystis parvus*, which, on entering stationary phase, forms large lipid inclusions, mainly poly-β-hydroxybutyrate (PHB), and loses its internal membrane system (187).

Type I methanotrophs, in general, form *Azotobacter*-like cysts that are resistant to desiccation, but not heat. Encystment appears to be promoted by conditions of low oxygen tension. Strains of *Methylomonas* and *Methylococcus* species form immature cysts, with structures intermediate between vegetative cells and ma-

ture cysts, that are not desiccation resistant but that will survive the absence of methane for longer periods than will vegetative cells (187).

Ultrastructure of methane-utilizing yeasts. The ultrastructure of two yeast strains capable of growth on methane was studied by Wolf and Hanson (194). Both *S. roseus* strain Y and *R. glutinis* strain Y when grown on methane contained microbodies which were sites of catalase activity. Their rare occurrence in glucose-, ethanol-, or acetate-grown cells suggests that microbodies play a role in methane metabolism in these strains. Similar organelles were also found in *R. glutinis* C7 cells grown on hexadecane (194).

CARBON METABOLISM

Obligate methanotrophs that use the serine pathway to incorporate carbon at the oxidation levels of formaldehyde and carbon dioxide into cell constituents have been isolated less frequently than those that employ the RMP pathway. This may reflect the greater efficiency of the latter cycle (159) in respect to growth rates on methane alone, although it does not necessarily reflect the abundance of either type of organism in the environment. The possibility that type II species with the serine pathway and an intact tricarboxylic acid (TCA) cycle may grow better on mixtures of C_1 substrates and more complex carbon sources is discussed below.

The Serine Pathway

Details of the serine pathway have been elucidated in facultative methylotrophs and have been authoritatively reviewed (20, 128, 195). The carbon assimilation pathways in type II methanotrophs, classified according to morphological characteristics, were first studied by Quayle and co-workers, who demonstrated that such bacteria possess high activities of the soluble enzyme hydroxypyruvate reductase (87, 88). High activities of this key enzyme have subsequently been taken to indicate the pathway's operation in a variety of type II methanotrophs, but little effort has been devoted to confirming the details of the pathway in most of these species. The following are species for which there is evidence for operation of the pathway: *M. trichosporium* strains PG and OB3b (88), *Methylosinus sporium* (88), *M. parvus* OBBP (88), *M. methanooxidans* (88), *Methylovibrio soehngenii* strains A and B (58), strains 1 and NIG 3.1 (56), *Methylosinus* sp. strains, CRL15 and CRL16 (119), *Methylocystis* sp. strain CRL18 (119), strain M102 (99) *M. trichosporium* subsp. *methanolicum* (171), *M. parvus* subsp. *fuscus* (171), *M. organophilum* (122), isolate R6 (118), *M. ethanolicum* (94), and *M. hypolimneticum*.

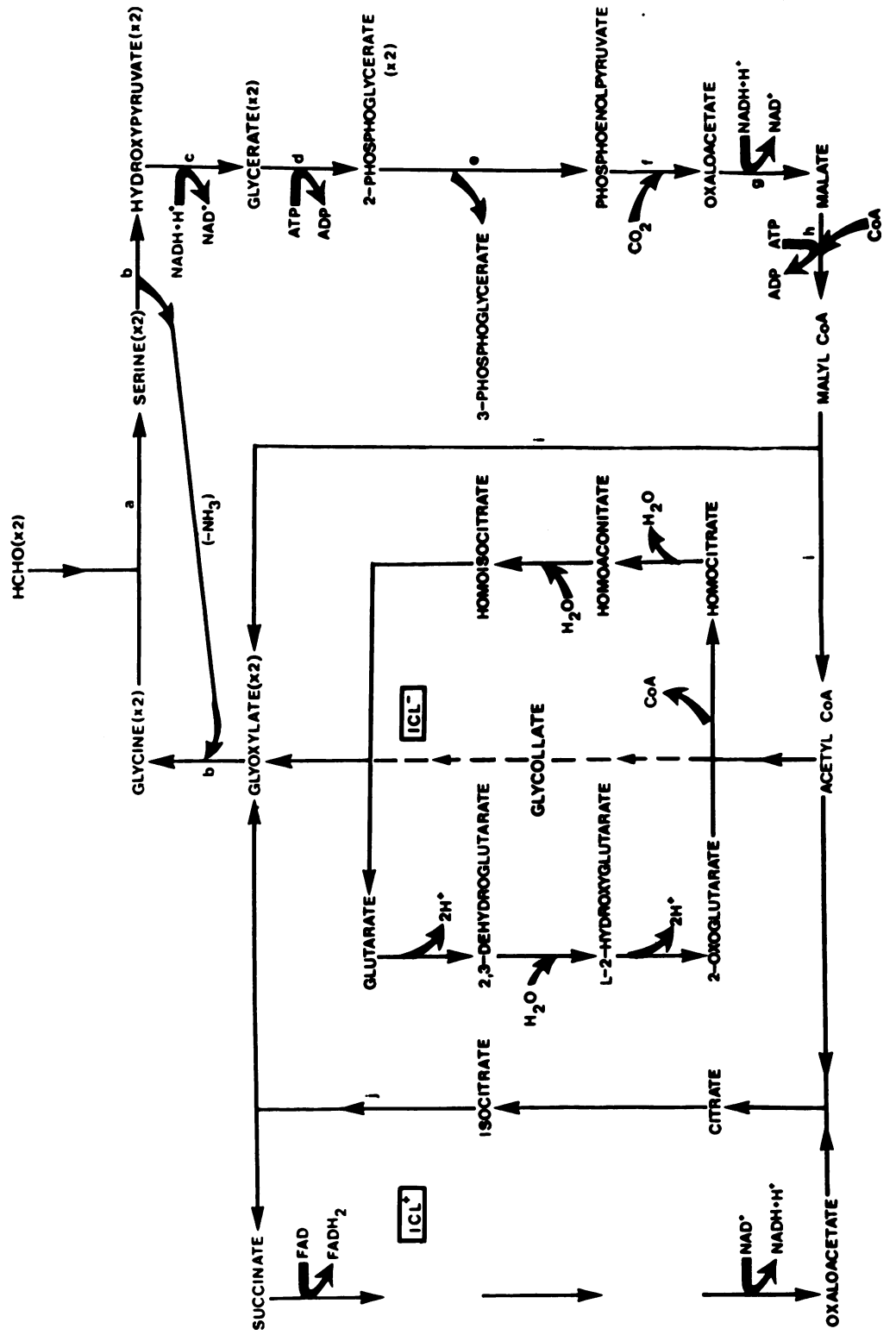
The original studies of Lawrence et al. (87) on methane-grown *M. methanooxidans* described early labeling of C_4 carboxylic acids, serine, glycine and "unknown compounds" when the cells were incorporating [^{14}C]methanol at 30°C. The unknown compounds were tentatively identified as folate derivatives. At lower temperatures, the early label resided predominantly in serine and glycine, and the labeling pattern suggested that the assimilation pathway was similar to that described for *Pseudomonas* strain AM1 (86).

Strøm et al. (159) could not detect the key enzymes of the RMP pathway in *M. trichosporium*. Other enzymes associated with pentose sugar rearrangement had low activities compared with those in type I methane utilizers.

All facultative methane-utilizing bacteria possess high levels of hydroxypyruvate reductase and are assumed to be serine pathway species (26, 117, 128). Formaldehyde, a highly reactive molecule generated by oxidation of methane via methanol, enters the pathway by reacting chemically with tetrahydrofolate to produce N^5,N^{10}-methylenetetrahydrofolate (Fig. 2). The first enzymatic step, catalyzed by serine transhydroxymethylase (serine hydroxymethyltransferase, EC 2.1.2.1), involves the condensation of the methylene tetrahydrofolate with glycine to form serine. This enzyme is present in most bacteria, being involved in the synthesis of serine and purines, but in methylotrophic bacteria using the serine pathway, it assumes a more central role in metabolism.

O'Connor and Hanson (102) purified two serine transhydroxymethylase activities from the facultative methane utilizer *M. organophilum* XX. One activity predominated during growth on methanol, and the other predominated during growth on succinate. These two isoenzymes were purified, and their properties differed substantially. The methanol-induced enzyme had twice the molecular weight of the succinate-induced enzyme, possessed different electrophoretic and sedimentation properties, and, unlike the other isoenzyme, was not stimulated by the metal ions Mg^{2+} and Zn^{2+}. Both activities were competitively inhibited by glycine, but only the methanol-induced enzyme was stimulated by glyoxylate. This result is not surprising in view of the fact that glyoxylate is the precursor of glycine via the action of serine-glyoxylate aminotransferase.

There is little information concerning regulation of the channeling of formaldehyde into either oxidative or assimilative metabolism (132). Harder and Attwood (51) proposed that


HCHO(x2)
SERINE(x2)
HYDROXYPYRUVATE(x2)
GLYCERATE(x2)
2-PHOSPHOGLYCERATE (x2)
PHOSPHOENOLPYRUVATE
OXALOACETATE
MALATE
MALYL CoA
3-PHOSPHOGLYCERATE
GLYCINE(x2)
GLYOXYLATE(x2)
HOMOISOCITRATE
HOMOACONITATE
HOMOCITRATE
ACETYL CoA
GLYCOLLATE
GLUTARATE
2,3-DEHYDROGLUTARATE
L-2-HYDROXYGLUTARATE
2-OXOGLUTARATE
ISOCITRATE
CITRATE
SUCCINATE
OXALOACETATE
ICL
NADH+H+
NAD+
ATP
ADP
CO2
H2O
CoA
FAD
FADH2
2H+
(-NH3)

the reactivity of free formaldehyde with tetrahydrofolate is such that it is only when all the coenzyme is derivatized that formaldehyde is available for oxidative energy generation. Control would hence be effected through the relative levels of reduced nicotinamide adenine dinucleotide (NADH), adenosine 5'-triphosphate (ATP), and tetrahydrofolate. Quayle (132) pointed out, however, that a tetrahydrofolate-mediated mechanism can participate in the oxidation of formaldehyde to formate and bypass such control.

In facultative methanotrophs, the following key serine pathway enzymes are inducible by growth on methanol: serine-glyoxylate aminotransferase, glycerate kinase, and glyoxylate-stimulated serine transhydroxymethylase (102). These activities are not repressed during growth on methanol if succinate is added to the medium.

Half of the 2-phosphoglycerate generated by formaldehyde fixation is converted to the net product of the pathway, 3-phosphoglycerate. The remainder is converted to phosphoenolpyruvate, the acceptor molecule for the second important carbon fixation step in this pathway (Fig. 2). Phosphoenolpyruvate is carboxylated to form oxaloacetate, which is then reduced to malate (85). Carbon dioxide is derived mainly from the oxidation of reduced C_1 substrates. It has been calculated that approximately 50% of the carbon derived from methanol is assimilated as carbon dioxide by the facultative methylotroph *Pseudomonas* AM1 (84). The original short-term labeling experiments implicated the carboxylation of phosphoenolpyruvate, which was found to be irreversible (85). Strøm et al. (159) predicted a higher carbon dioxide requirement for serine pathway organisms than for RMP pathway organisms, and the fact that carboxylase activities in the former are an order of magnitude higher than those in the latter has reinforced this view (92, 171). However, the exact nature of the carboxylase systems of the serine pathway methane utilizers is unclear.

Phosphoenolpyruvate carboxylase plays a key role in methylotrophic metabolism. The studies of Newaz and Hersh (100) suggested that this enzyme was at the crossroads between carbon assimilation and energy generation. The phosphoenolpyruvate carboxylases of methylotrophic strains examined to date are predominantly of the class 3 type, subject neither to activation nor to inhibition. Studies of methylamine-grown *Pseudomonas* strain MA (ATCC 23319) (100) indicated, however, that the carboxylase is activated by NADH and, in this organism at least, thus becomes a focal point for regulation of metabolism.

In contrast, O'Connor (101) described two isoenzymes of phosphoenolpyruvate carboxylase in *M. organophilum*. The isozyme of methanol-grown cells is insensitive to both acetyl coenzyme A (acetyl-CoA) and NADH, but succinate-grown organisms possess an acetyl-CoA-stimulated activity. The two activities fractionate at different ammonium sulfate concentrations, indicating two different forms of the enzyme.

Loginova and Trotsenko (92) found high activities of phosphoenolpyruvate carboxylase in two strains of *M. trichosporium*. Activity was greater in the presence of Mg^{2+} than in the presence of Mn^{2+}. A similar activity has been identified in a type II methanotroph, strain M102 (99). It had previously been suggested that carbon dioxide fixation by this strain depended upon the presence of methane to furnish intracellular acceptor molecules (108). Radiolabeling studies in both whole organisms and extracts of this strain have led to the postulation of the induction of several carboxylating systems, namely, phosphoenolpyruvate carboxylase, NADH(-phosphate) [NAD(P)H]-dependent "malic enzyme," phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxytransphosphorylase, and pyruvate carboxylase (99).

There is only one other report of the presence of phosphoenolpyruvate carboxykinase in a methylotroph (82), and other workers have found the complete absence of other carboxylating systems in serine pathway strains (92, 171, 172). Naguib (99) also described variable locations of carboxylating enzymes according to the physiological condition of the organism, a situation reminiscent of that reported for the methane monooxygenase in *M. trichosporium* OB3b and discussed in Comparison of methane monooxygenases from different species (14, 146). However, Naguib (99) ascribed this variation to stripping of the activities from the membranes during cell breakage, implying that they are predominantly membrane bound in vivo. He contends that the operative carboxylating systems depend on both the physiological status and the mode of metabolic control in these organisms. On the basis of the published data, it appears to be difficult to assess these enzyme

FIG. 2. *Serine pathway. a, Serine transhydroxymethylase; b, serine-glyoxylate aminotransferase; c, hydroxypyruvate reductase; d, glycerate kinase; e, enolase; f, phosphoenolpyruvate carboxylase; g, malate dehydrogenase; h, malate thiokinase; i, malyl-CoA lyase; j, isocitrate lyase. ICL^+, Isocitrate lyase pathway; ICL^- possible alternatives to ICL^+. FAD, Flavin adenine dinucleotide; $FADH_2$, reduced FAD; ADP, adenosine 5'-diphosphate.*

activities in methylotrophs, and therefore this postulate awaits confirmation.

The various carboxylating activities found in serine pathway organisms can be summarized as follows:

(i) "malic enzyme"

pyruvate + NAD(P)H + H^+ + CO_2 $\rightleftarrows$ malate + oxidized NADP ($NADP^+$)

(ii) pyruvate carboxylase

pyruvate + CO_2 + ATP $\rightleftarrows$ oxaloacetate + adenosine 5'-diphosphate + inorganic phosphate

(iii) phosphoenolpyruvate carboxylase

phosphoenolpyruvate + CO_2 $\rightarrow$ oxaloacetate + inorganic phosphate

(iv) phosphoenolpyruvate carboxykinase

phosphoenolpyruvate + CO_2 + adenosine 5'-diphosphate $\rightleftarrows$ oxaloacetate + ATP

(v) phosphoenolpyruvate carboxytransphosphorylase

phosphoenolpyruvate + CO_2 + inorganic phosphate $\rightleftarrows$ oxaloacetate + pyrophosphate

In the present context it is relevant to examine the role of pyruvate in the metabolism of type II methanotrophs. Quayle (129) presented a generalized scheme for the serine pathway which included pyruvate carboxylase. The reports of pyruvate-carboxylating systems are, as described above, at variance, and Trotsenko and Shishkina (172) concluded that known pathways for using pyruvate for gluconeogenesis are not physiologically functional in *M. trichosporium* strains and *Methylocystis minimus*. Furthermore, they suggested that the formation of acetyl-CoA from pyruvate is of minor importance in these species, since the activity of the E_1 component of the pyruvate dehydrogenase complex is very low. In contrast, early reports suggested the pyruvate dehydrogenase activity in type II methane utilizers was easily detected in cell-free extracts (27). A definitive comparative survey would help to clarify the situation. The facultative methylotroph *Hyphomicrobium* strain X possesses limited ability to utilize pyruvate (52), lacking most enzymes associated with pyruvate metabolism. Mutants of *Pseudomonas* AM1 lacking the E_2 component of pyruvate dehydrogenase have been described (12). Because of the loss of this enzyme, the mutants had the properties of restricted facultative methylotrophy, similar to the hyphomicrobia.

Malate dehydrogenase has been reported in both obligate and facultative methanotrophs (117, 122). Reported specific activities are in the ranges 108 to 480 and 14 to 1,410 nmol/min per mg of protein in obligate and facultative methylotrophs, respectively.

The generation and cleavage of malyl-CoA is another important step in the serine pathway (Fig. 2). Extracts of methane-grown *M. trichosporium* OB3b contain no detectable ATP- and CoA-dependent malate lyase, but do catalyze Mg^{2+}-dependent cleavage of CoA (144). This suggests that malate thiokinase, the enzyme responsible for malyl-CoA generation, might be limiting in the first case, and hence any malyl-CoA generated would be rapidly hydrolyzed by the lyase. Alternatively, an acyl-CoA transferase might be involved. Malyl-CoA has been detected at high activities in methanol-grown *M. organophilum* (101).

The action of malyl-CoA lyase regenerates one acceptor molecule of glyoxylate and one molecule of acetyl-CoA. The problem encountered by all serine pathway organisms is the regeneration of a second glyoxylate acceptor molecule from acetyl-CoA. Three biochemical solutions have been proposed, but only two can be applied to type II methanotrophs, for they lack the key enzyme involved in the first solution (below), namely, isocitrate lyase (117, 171, 172).

(i) The isocitrate lyase pathway, reviewed elsewhere (20, 128, 195), uses enzymes of the TCA cycle in conjunction with isocitrate lyase to cleave isocitrate to succinate and glyoxylate.

(ii) Studies of mutants of the facultative methylotroph *Pseudomonas* AM1 (2) suggested that in this organism, at least, enzymes may be present that cause acetyl-CoA to be directly oxidized to glyoxylate, with glycolate as a possible intermediate (Fig. 2). Bellion (6) eliminated any other known biochemical routes by which this might occur by failing to demonstrate any of the key enzymes of either the glycerate (tartronic semialdehyde) pathway or the β-hydroxyaspartate pathway in *Pseudomonas* strain PAR, a non-isocitrate lyase serine pathway organism.

(iii) There is little direct biochemical evidence to support the direct oxidation proposal (ii), and work by Kortstee (83) was based on the supposition that acetyl-CoA might be oxidized subsequent to its condensation with an unknown compound which was then cleaved to yield glyoxylate. It was not possible to demonstrate the direct oxidation of acetate or acetyl-CoA in ex-

tracts of the facultative methylotroph *Pseudomonas* strain 80 or the presence of glycolate during conversion of acetyl-CoA to glyoxylate. Kortstee discovered that only one of a large number of compounds would condense with acetyl-CoA and produce glyoxylate as an end product when incubated with cell-free extracts. This compound was 2-oxoglutarate, and its addition to extracts effected an almost complete conversion of acetyl-CoA to glyoxylate.

Labeling studies demonstrated that the two carbon atoms of glyoxylate were derived from acetyl-CoA. Incubation of extracts with both homocitrate and homoisocitrate resulted in the formation of products whose 2,4-dinitrophenylhydrazones were indistinguishable from authentic glyoxylate and glycerate derivatives. On the basis of these experimental data, Kortstee proposed the homoisocitrate pathway in which acetyl-CoA condensed with 2-oxoglutarate and, with subsequent rearrangement, produced homoisocitrate (Fig. 2). Cleavage of this C_7 molecule regenerated one molecule of glyoxylate and one of glutarate. The mechanism of regeneration of 2-oxoglutarate from glutarate is tentatively proposed to parallel the oxidation of succinate to oxaloacetate in the TCA cycle. At the time of writing, however, this pathway has not been described in any serine pathway methanotroph, and it awaits unambiguous confirmation.

The Ribulose Monophosphate (Quayle) Cycle and Other Aspects of Carbon Assimilation by Type I Methanotrophs

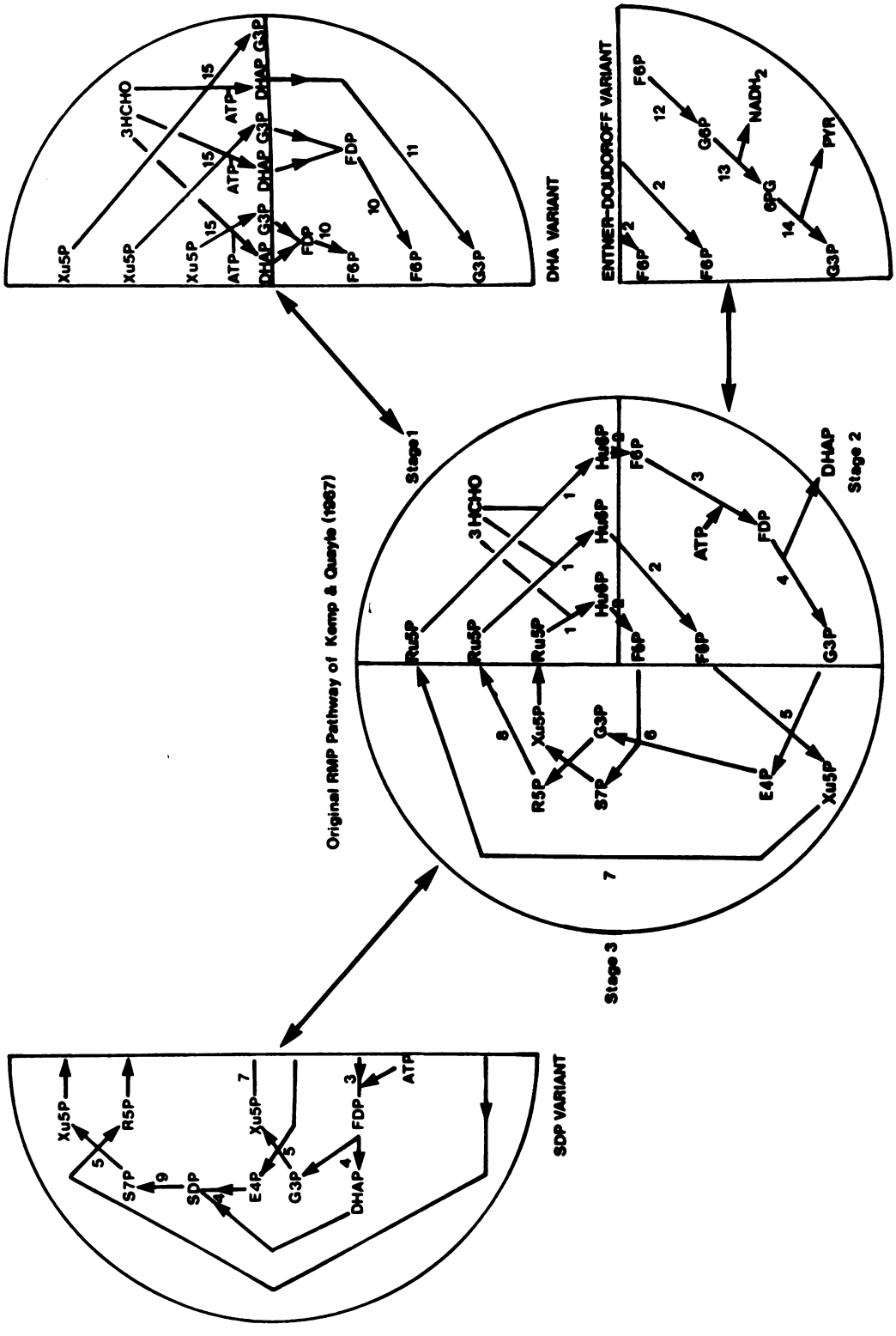
The biochemistry of formaldehyde assimilation by type I methylotrophs (*M. capsulatus* [Texas] [89], *M. capsulatus* [Bath] [89], *Methylococcus ucrainicus* [96], *Methylococcus thermophilus* [96], *Methylococcus albus* [96], *Methylococcus minimus* [96], *M. mobilis* [57], *M. methanica* [89], *Methylomonas rubrum* [96], *Methylomonas agile* [88], *Methylomonas rosaceus* [88], *Methylomonas* sp. [30], *Methylobacter capsulatus* [88], *Methylobacter vineclandii* [88], and *Methylobacter bovis* [96]) has been the subject of much more intense study that that of their serine pathway counterparts. Since the early 1960s, workers, notably at Sheffield and Warwick, United Kingdom, have meticulously documented the details of the RMP pathway of formaldehyde assimilation (Fig. 3), and this area has been adequately reviewed elsewhere (20, 128, 133, 195). The details of the cycle differ somewhat between species, and the three variants are shown in Fig. 3. It was suggested that this pathway may have been the progenitor of the closely related ribulose diphosphate cycle (133). Quayle (132) recently discussed aspects of regulation in the RMP pathway.

For some years it was thought that the classification of methylotrophs as type I or type II based upon morphological criteria rigorously corresponded to their modes of carbon assimilation (128). In every methanotroph examined, the dual presence of key enzymes of both assimilation pathways could not be demonstrated (88). Recently, however, it has become increasingly clear that some methanotrophs which assimilate carbon via the RMP pathway contain significant activities of enzymes involved in other assimilation pathways. This has been most persuasively elucidated in the case of *M. capsulatus* (Bath).

The low activities of hydroxypuruvate reductase (159) and malyl-CoA lyase (144) originally found in both *M. capsulatus* (Texas) and *M. methanica* were attributed to involvement in protein and prophyrin synthesis. Significantly higher incorporation of label from formaldehyde, as compared with that from methane or methanol, into serine and glycine in *M. capsulatus* (Texas) suggested that its total carbon assimilation might not be due entirely to the RMP pathway (40). Entry of formaldehyde into cellular carbon via purine synthesis would not account for the observed variation in labeling pattern; similar results were obtained with formate. At the time, assimilation via CO_2 was thought to be negligible (40).

In the light of quantitative differences in growth yields of the type I strains *Methylomonas albus* and *M. capsulatus* (Bath), which presumably possessed the same single carbon assimilation pathway, the key enzymes of both assimilation pathways of both strains were reexamined (186). *M. albus*, with the greater growth yield, contained only enzymes of the RMP pathway, whereas *M. capsulatus* (Bath) possessed, in addition, hydroxypyruvate reductase. Hydroxypyruvate reductase activity was detected at 45°C, but not at 30°C, and a similar high temperature optimum for this enzyme in other type I methanotrophs was noted by Malashenko (96), who also demonstrated that his isolates contained serine-glyoxylate aminotransferase. Pulse-labeling studies in continuous cultures of *M. capsulatus* (Bath), using [^{14}C]methanol as a carbon source, demonstrated the incorporation of label into sugar phosphates of the RMP pathway at both 30 and 45°C, but label was found to reside also in serine and glycine at 45°C. Label was also incorporated from formate at 45°C, formate acting as a source of carbon dioxide (184).

Among the 12 type I isolates examined by Trotsenko (171), only 1 was found to contain


Original RMP Pathway of Kemp & Quayle (1967)
Stage 1
Stage 2
Stage 3
DHA VARIANT
ENTNER-DOUDOROFF VARIANT
SDP VARIANT
3HCHO
Xu5P
Ru5P
Hu6P
F6P
G3P
DHAP
FDP
ATP
R5P
S7P
E4P
SDP
6PG
G6P
PYR
NADH2

solely the RMP pathway. Low activities of hydroxypyruvate reductase and serine-glyoxylate aminotransferase were found in the others. Supplementing the growth medium with formate caused a substantial increase in the levels of hydroxypyruvate reductase. Formate was thus considered to be an inducer for the serine pathway in these isolates.

Malashenko (96) and Colby et al. (20) referred to the caution required to assess the roles of certain enzymes of the serine pathway that were detected in these organisms and stressed that the enzymes may not be indicative of the complete cycle itself, but may merely be involved in a serine exchange reaction sequence. The evidence in the case of *M. capsulatus* (Bath) appears to be rather more substantial in this regard, although the exact contribution made by this minor pathway has not been elucidated. However, Colby et al. (20) suggested that it may be "significant under certain environmental conditions" and, indeed, "of crucial importance to survival."

In addition to the two carbon assimilation pathways discussed above, the presence of the key enzymes of the Calvin (ribulose diphosphate) cycle, ribulose diphosphate carboxylase and phosphoribulokinase, was demonstrated in cell-free extracts of *M. capsulatus* (Bath) (164, 165). The rate of CO_2 fixation by intact organisms was low, however, and contributed only about 2.5% (wt/wt) of the total cell carbon. Fixation depended upon the presence of methane, indicating an energy requirement for incorporation (165). Radiotracer studies demonstrated early incorporation of label into 3-phosphoglycerate and also aspartate, citrate, and malate, the latter three arising from carboxylation of C_3 metabolites. Ribulose diphosphate carboxylase thus functions in vivo in this methane utilizer, generating labeling patterns similar to those observed for autotrophic bacteria grown heterotrophically. Nevertheless, despite the presence of a complete Calvin cycle, *M. capsulatus* (Bath) could not be grown autotrophically on CO_2.

Ribulose diphosphate carboxylase from this organism requires a divalent cation for activity, has an alkaline pH optimum, is inhibited by 6-phosphogluconate, and possesses an oxygenase activity (165). This secondary activity of the enzyme generates phosphoglycolate, which may be subsequently cleaved by a specific phosphoglycolate phosphatase, recently discovered in extracts of *M. capsulatus* (Bath) (166). The metabolic fate of the glycolate may possibly involve incorporation via glyoxylate, using the serine pathway, thus providing a role for this pathway in a type I strain, which incorporates label from [^{14}C]glycolate into serine and glycine (166). The presence of ribulose diphosphate carboxylase was demonstrated in only two strains of *M. capsulatus* (Bath).

Fixation of CO_2 by type I methanotrophs can occur by a variety of different mechanisms (92, 171). Certain type I methanotrophic strains (*Methylobacter chrococcum* 90, *M. methanica* 12, *M. bovis* 90) do not possess pyruvate carboxylase, and activities of phosphoenolpyruvate carboxylase, found only with Mn^{2+} ions in this case, are an order of magnitude lower than those found in serine pathway organisms. Phosphoenolpyruvate carboxykinase and "malic enzyme" activities have also been detected. The variation in activities of carboxylases between type I methanotrophs is thought to reflect differences in the contribution made to the overall carbon balance by the serine pathway (171). For instance, carboxylases are present in *M. bovis* 90, which possesses key enzymes of the serine pathway which are not found in *M. methanica* 12, possessing solely the RMP pathway. Thus, one might place methanotrophs in groups based on CO_2 assimilation which directly correlates with their primary assimilation pathways: serine $\rightarrow$ RMP + serine $\rightarrow$ RMP. No account is taken here of the contribution made by the recently discovered presence of the Calvin cycle in some methanotrophs.

The Dissimilatory Ribulose Monophosphate Pathway

Gluconate 6-phosphate dehydrogenase is present in methane-utilizing bacteria that pos-

FIG. 3. *RMP pathway (adapted from Colby et al. [20]). Ru5P, Ribulose-5-phosphate; Hu6P, D-erythro-L-glycero-3-hexulose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; G3P, glyceraldehyde 3-phosphate; DHA, dihydroxyacetone; DHAP, DHA-phosphate; E4P, erythrose 4-phosphate; Xu5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; SDP, sedoheptulose-1,7-diphosphate; R5P, ribose-5-phosphate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; PYR, pyruvate. 1, 3-Hexulose phosphate synthase; 2, phospho-3-hexuloisomerase; 3, 6-phosphofructokinase; 4, fructosediphosphate aldolase; 5, transketolase; 6, transaldolase; 7, ribulosephosphate epimerase; 8, ribulosephosphate isomerase; 9, sedoheptulose diphosphatase; 10, fructose diphosphatase; 11, triosephosphate isomerase; 12, glucosephosphate isomerase; 13, glucose-6-phosphate dehydrogenase; 14, 6-phosphogluconate dehydratase + phospho-2-keto-3-deoxygluconate aldolase; 15, transketolase + triokinase. (Reproduced with permission from the Annual Review of Microbiology.)*

sess the assimilatory RMP pathway with the Entner-Doudoroff variant of glyceraldehyde 3-phosphate production (20, 27, 131, 159). The presence of this enzyme permits cyclic oxidation of formaldehyde to CO_2 by the RMP pathway without the intermediacy of formate. The pathway furnishes NADPH, which is not otherwise generated by the oxidation of methane to CO_2 (195), although an $NADP^+$-linked formaldehyde dehydrogenase was recently described in *M. capsulatus* (Bath) (155). The occurrence of this cycle is often accompanied by low or undetectable activities of formaldehyde dehydrogenase and formate dehydrogenase (131). The operation of this cyclic oxidative pathway could not be detected in *M. methanica*, which possesses a highly active formaldehyde dehydrogenase (173).

Intracellular and Extracellular Polymers Produced by Methane-Utilizing Bacteria

Early studies revealed sudanophilic granules, identified as PHB, deposited in large quantities (25% of the dry weight of 4-day-old cultures) in *M. methanica* (53, 81). Many of the methane-utilizing strains identified by Whittenbury et al. (188) were packed with lipid inclusions, mainly PHB. In addition, *M. parvus* forms large PHB inclusions on entering stationary phase (187). Two strains of the methane-using vibrio *M. soehngenii* (58) and cysts of thermophilic methanotrophs (96) were found to possess various amounts of PHB. Lipid inclusions identified as PHB have also been noted in the type II methanotroph *M. trichosporium* OB3b (10, 167, 182) at levels from 2.4% of total dry weight under unspecified growth conditions (87) to 30% in methane excess continuous culture (10). An enzyme involved in PHB mobilization, β-hydroxybutyrate dehydrogenase, was found in extracts of methylotrophs, and acetone accumulation concomitant with PHB degradation was demonstrated (167). Acetone production, probably catalyzed by acetoacetate decarboxylase, occurred during the cooxidation of ethane by this strain. It is unlikely that the production was related to acetate formation, since acetone also accumulates during other cooxidative reactions, such as epoxidation, catalyzed by this strain (D. J. Best, unpublished data). The precise reasons for this mobilization of PHB reserves and the seemingly wasteful excretion of acetone under these conditions are not clear. Degradation of PHB to acetoacetic acid would provide reducing equivalents via the action of the oxidized nicotinamide adenine dinucleotide (NAD^+)-linked β-hydroxybutyrate dehydrogenase. Subsequent metabolism of acetoacetic acid might prove energetically expensive under conditions where the organism is challenged with an assimilable substrate, and thus acetone may be excreted to maintain NADH levels. Clearly, a more extensive survey of PHB metabolism is called for in order to clarify its role as a storage polymer in methanotrophs and the regulation of its synthesis and subsequent motilization.

Accumulation and excretion of polysaccharides has been noted in a number of methanotrophs (38, 73, 90, 151). *Methylococcus* NCIB 11083, grown in continuous culture on methane under ammonia limitation, accumulates a branched polysaccharide, composed almost entirely of glucose residues, with α-1 → 4 and α-1 → 6 linkages and with an average molecular weight of 2×10^5 to 4.5×10^5. Accumulation, which reaches 32.5% of the dry weight at low dilution rates in continuous culture, is inversely proportional to the dilution rate. Polyglucose is rapidly metabolized at 42°C in the absence of CH_4 without any change in culture viability. This catabolism is accompanied by the uptake of ^{35}S, indicative of protein synthesis, and the yield of organism has been estimated to be 0.26 g/g of polyglucose metabolized. Chemical characterization of the polymer showed that it possessed an absorption spectrum similar to that of amylopectin when complexed with iodine, but the extent of attack by α- and β-amylases suggested it was similar in chain branch length to glycogen (90). Accumulation of this polymer, which performs an energy storage function, is not confined to conditions of low growth rate under nitrogen limitation, but may also occur when a rapidly growing culture is limited by some other medium constituent. Linton and Cripps (90) suggested that the polymer accumulates due to "over-utilization" of methane to deal with the problem of nonregulated generation of excess carbon and reducing equivalents. Similarly, the type II methanotroph *M. trichosporium* OB3b accumulates PHB in the early logarithmic phase of growth on methanol (10). Whether such over-utilization of carbon source will result in excretion or accumulation of storage materials seems to depend upon the particular species.

Excretion of polysaccharide by a type II methanotroph, *N. parvus* OBBP (67), is surprising in view of the fact that type II species possess few enzymes of carbohydrate metabolism (27, 159, 171). After growth on methanol, this strain contains 62% polysaccharide, consisting of the monomers D-glucose (82%) and L-rhamnose (14%) together with minor quantities of L-glucose, D-rhamnose, and D-galactose. Further investiga-

tion of such polysaccharide synthesis in type II methanotrophs is of vital importance with regard to establishing the overall carbon balance of these strains. A recent report on polysaccharide production in a putative *M. methanica* strain described differences in polymer structure dependent on whether the enrichment culture was methane or methanol grown; the polymer contained glucose, galactose, mannose, fucose, and rhamnose in the proportion 6:2:1:2:1 or 6:4:0:0, respectively (73). This led to the tantalizing suggestion that the isolate was a symbiotic association of a methane utilizer and a methanol utilizer and that, when grown on methane, the methane utilizer deoxygenated the galactose and mannose residues of the polysaccharide produced by the methanol utilizer, as some form of oxygen conservation mechanism. The situation, however, is far from clear. Methanotrophs are also known to excrete a variety of colored pigments, largely uncharacterized, but presumed to be carotenoids.

Intermediary Metabolism, the Tricarboxylic Acid Cycle, and Utilization of Ancillary Carbon Sources

Serine pathway methanotrophs do not possess some of the enzymes of carbohydrate metabolism, i.e., glucose-6-phosphate dehydrogenase or gluconate 6-phosphate dehydrogenase (27, 159, 171). This is also true for methane-grown facultative methane utilizers. Glucose-6-phosphate dehydrogenase is induced only when the organisms are grown on glucose (117, 122), and the activity is either NAD or NADP linked. In contrast, type I methanotrophs possess hexokinase (171), NADP-linked glucose-6-phosphate dehydrogenase, and gluconate 6-phosphate dehydrogenase (27, 159, 171). Nevertheless, despite early reports of a stimulation of growth by glucose in *M. methanica* (39), most workers have concluded that type I methanotrophs do not assimilate more than trace amounts of glucose (40). The enzymatic activities associated with pyruvate metabolism in these strains were discussed in previous sections.

One of the most significant biochemical differences between type I and type II methanotrophs is that the TCA cycle is incomplete in the former, but complete in the latter. The crucial enzymatic lesion is the absence of 2-oxoglutarate dehydrogenase (27, 171). All other enzymatic activities associated with the cycle have been demonstrated, albeit at relatively low levels. Isocitrate dehydrogenase in *M. capsulatus* (Texas) is strictly NAD dependent (27).

Complete TCA cycles have been reported in facultative methanotrophs (26, 94, 117, 122). Specific activities of the enzymes of the cycle were severalfold higher in succinate-grown than in methane-grown organisms (117). Two TCA cycle enzymes, citrate synthase and 2-oxoglutarate dehydrogenase, were repressed in methanol-grown *M. organophilum*, suggesting that the TCA cycle is not as important to this species during growth on C_1 compounds as during growth on succinate (103).

The classical experiments to further elucidate the nature and role of the TCA cycle in methanotrophs involved measurements of the incorporation of radioactive acetate into cellular constituents. In the representative type I strain, *M. capsulatus* (Texas), both 2-oxoglutarate dehydrogenase and aconitate hydratase are undetectable and the endogenous rate of acetate uptake is stimulated by methane, methanol, or formate (114, 115). Label is incorporated mainly into lipids, but 25% resides in only four amino acids, glutamate, proline, arginine, and leucine. The incorporation of label into glutamate indicates that aconitate hydratase is, in fact, present. In contrast, similar experiments with the type II methane utilizer *M. methanooxidans* demonstrated not only that the addition of acetate to the medium enhanced the growth yield but also that both acetate carbons were incorporated into amino acids of the glutamate and aspartate families, as well as those derived from pyruvate. Radioactive CO_2 was also released, which is entirely consistent with a functional TCA cycle in vivo in a type II methanotroph (177).

Oxidation of C_1 compounds and primary alcohols supports the assimilation of acetate by cell suspensions of *M. methanica* (Texas) and *M. trichosporium* Pa, with similar patterns and rates of incorporation in both strains (113). Assimilation of acetate coupled with primary alcohol oxidation suggests that this oxidation furnishes the energy necessary for acetate uptake.

The role of the TCA cycle in methanotrophs is not fully elucidated or understood, but it was suggested that type II organisms, with their high demand for NAD(P)H for MMO and the serine pathway, could not exist without a fully functional TCA cycle (184). Lack of detailed knowledge in this area may be responsible for the conclusion that the TCA cycle is not particularly important in methylotrophic metabolism (132), which is probably true of type I species, but the presence of a catabolic TCA cycle in type II organisms requires an explanation.

Many specialist bacteria lack a complete TCA cycle (148). The absence of 2-oxoglutarate dehydrogenase confers a purely biosynthetic role on the other enzymes of the cycle rather than

the dual roles of biosynthesis and catabolism. In general, the mode of regulation of citrate synthase is a good indication of the presence or absence of a complete cycle. NADH inhibits the enzyme when the cycle is primarily catabolic, whereas 2-oxoglutarate will inhibit the enzyme in strains that use the incomplete cycle in a purely biosynthetic capacity. Although this corollary appears to hold for specialist strains, such as phototrophs and lithotrophs, it does not hold for methanotrophs examined to date. The citrate synthases of both *M. trichosporium* and *M. albus* are insensitive to NADH and 2-oxoglutarate (22). The absence of feedback control is consistent with a catabolic function of the TCA cycle in these species. Inhibition of citrate synthase by high levels of ATP occurs, but is regarded as physiologically unimportant (22). Further studies of TCA cycle regulation are required to clarify its role in methanotrophs.

A second approach to the evaluation of the cycle in specialist strains, as suggested by Smith and Hoare (148), now seems especially applicable to type II methanotrophs, where comparisons can be made between versatile species identical to specialist strains in all respects other than the capacity for growth on organic compounds. The identification and rigorous characterization of facultative type II methane utilizers now makes possible such an approach to the problem.

A third approach also asks important questions concerning the role of the TCA cycle, with special reference to type II methanotrophs with a complete cycle. There are many reports of broad-specificity oxidoreductases present in methanotrophic bacteria, and these are discussed in Energy Metabolism. Of particular interest is the oxidation of alkanes to alkanoic acids, which may be β-oxidized to acetate, and the implications that such activity raises concerning the utilization of ancillary carbon sources and the nature of obligate methanotrophy itself. The precise contribution to the overall carbon assimilation by methanotrophs still remains to be elucidated, but it has been pointed out elsewhere that "co-oxidation is not some aberrant and useless activity but can be directly beneficial to methanotrophs provided methane is also available" (184). The simplest example is the oxidation of ethane to acetate, which might provide the organism with reducing power, ATP, and acetate, that may be used as a carbon (and energy) source (via the TCA cycle) (184). This concept of metabolically useful co-oxidized substrates was expanded upon by Higgins and coworkers (60, 61, 64), who observed the oxidation and subsequent degradation of radioactive hexadecane to radioactive CO_2 and certain phenylalkanes by *M. trichosporium* OB3b. Such oxidative processes would most probably yield acetate, which could be assimilated under conditions where the primary growth substrate, methane, is limiting. Certainly the role of acetate as a central metabolite in such a type II strain should not be doubted, for it stands at a metabolic crossroads between the serine pathway, the TCA cycle, and carbon and energy storage in the form of PHB, a polymer that can only be mobilized via acetyl-CoA (Fig. 4). Thus, it has been argued that the ability to use co-oxidizable substrates via conventional acetate metabolism confers an advantage on methanotrophic organisms that possess a complete TCA cycle (60–61, 64). Further evidence in support of this hypothesis is found from observations that, under carbon limited conditions, hexadecane will significantly increase the cell yield of *M. trichosporium* OB3b (F. Taylor, personal communication). It was suggested that in their natural environments, methylotrophs are unlikely to have access to a continuous supply of their specific energy source; their survival will then depend on an ability to obtain metabolically useful energy from energy sources by means other than specialized oxidation reactions (148). It is likely that methane is usually present in the natural habitats of methanotrophs, but it may often be growth limiting, thereby offering an advantage to species that can co-utilize ancillary carbon and energy sources.

Such approaches as these do, however, once again beg the question of the biochemical basis of obligate methanotrophy. Although it was demonstrated that the mutational loss of 2-oxoglutarate dehydrogenase does confer obligacy upon an otherwise facultative methylotroph (163), the absence of this enzyme does not offer a rational explanation for methylotrophic fastidiousness. Many strains with the same enzymic lesion are capable of heterotrophic growth (148). It certainly does not offer any explanation for the obligacy of type II methane utilizers, and, to date, only hypotheses are available to explain this dietary particularity. These include (i) substrate toxicity; (ii) substrate impermeability or an energetic requirement for substrate uptake as demonstrated for acetate; (iii) coarse metabolic control by enzyme induction or repression, enzymes being strictly regulated at levels that allow efficient growth on one substrate only; (iv) unusual or inadequately coupled electron transport mechanisms and energy transduction; (v) specific biosynthetic deficiencies; (vi) absolute dependence on a metabolite available only during growth on the characteristic carbon source;

(vii) failure to oxidize NADH, although this is not tenable in the case of methanotrophs, since they possess NADH oxidase activities (27); and (viii) multiple enzymic lesions (172). For a much more detailed discussion of this problem, the reader is once again referred to the excellent review of Smith and Hoare (148).

Methane-Utilizing Yeasts

Methanol-utilizing yeasts incorporate formaldehyde via a xylulose-5-phosphate cycle, partially reminiscent of the RMP pathway (132). However, due to the difficulty in obtaining sufficient biomass, very little is known of the pathways of carbon incorporation of the recently isolated methane-utilizing yeasts. Large, yeast-like microorganisms were observed in methanotrophic enrichment cultures from shield lakes (143), but the isolation of pure methane-utilizing yeast strains was first reported by Wolf and Hanson (192). Every isolate oxidized methane to CO_2, but such intermediates as methanol, formaldehyde, and formate would not support growth. The greatest cell yields were found in cultures supplied with an atmosphere containing 10% (vol/vol) CO_2. In this case no radioactive label from methane was incorporated into TCA-precipitable material. If the CO_2 level was lowered, however, label was found in cellular components, suggesting that these yeasts assimilate carbon as CO_2 and not as one of the intermediates of methane oxidation. These isolated strains have a wide nutritional capacity. They use methylamine, butane, octane, some higher hydrocarbons, ethanol and other alcohols, acetate, and some sugars. Further elucidation of the pathways involved in these eucaryotic organisms is keenly awaited.

ENERGY METABOLISM

The pathway of methane oxidation to carbon dioxide via methanol, formaldehyde, and formate in methanotrophic bacteria has been well established for many years. Although considerable attention has been focused on the enzymes of this oxidative sequence, until recently little was known of the mechanisms involved. Significant advances have now been made towards elucidating the enzymic mechanisms of methane and methanol oxidation at the molecular level.

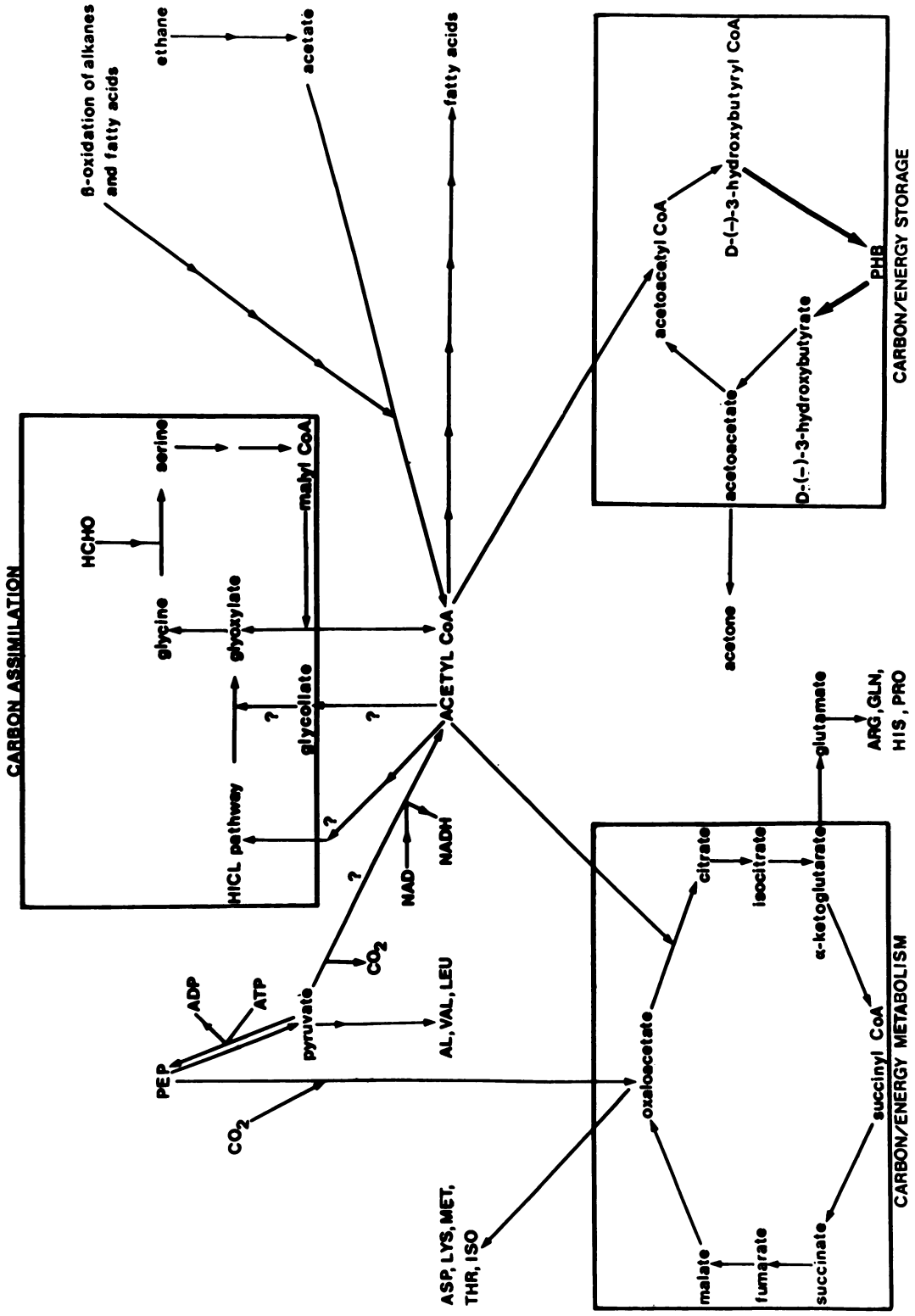
Methane Monooxygenase

Early reports of cell-free methane- and NADH-dependent oxygen consumption in particulate preparations of *M. capsulatus* (Texas) (137, 138) and *M. methanica* (43) were followed by descriptions of the properties of cell-free MMO from *M. methanica* (19), *M. trichosporium* OB3b (170) and *M. capsulatus* (Bath) (16). Methane monooxygenase activity was purified and studied in detail from the latter two strains and, at present, is best characterized in *M. capsulatus* (Bath).

A three-component MMO from *M. capsulatus* (Bath) was purified by Dalton and co-workers (24) by conventional chromatographic techniques (Fig. 5). Component A has a molecular weight of about 220,000, with two nonheme iron atoms and two acid-labile sulfur atoms per molecule. This oxygen-labile protein is purified under anaerobic conditions and is thought to be the hydroxylase component of the enzyme. Component B, a colorless protein with a molecular weight of about 15,000, is stable in the presence of phenylmethylsulfonyl fluoride. Although its role in the oxygenase reaction is uncertain, it is essential for activity. Component C has a molecular weight of 44,000, and each molecule contains one molecule of flavin adenine dinucleotide, two atoms of nonheme iron, and two atoms of acid-labile sulfur. The ability to be reduced by NADH (17) and the ability to catalyze the reduction by NADH of other acceptors (18) indicate that component C is the reductase component of the monooxygenase complex. Component A is not directly reduced by NADH, but gives an electron paramagnetic resonance signal on reduction with sodium dithionite. This signal is enhanced in the presence of a substrate, ethylene, suggesting that component A binds substrate when in a reduced form (20). Thus, component C transfers electrons from NADH to component A, which then binds the substrate, allowing oxidation (Fig. 6) (24).

The methane-oxidizing enzyme from *M. trichosporium* OB3b was purified (169), although recently this purification procedure has not yielded active enzyme in our laboratory. The three-component enzyme comprised a soluble CO-binding cytochrome *c*, a copper-containing protein (protein 1), and a small protein, with molecular weights of 13,000, 47,000, and 9,400, respectively (169). The soluble CO-binding cytochrome *c* has an oxidase function (170), and although evidence suggests that it binds MMO substrates, such as methane and ethylene, (49) as well as oxygen, it was also thought to be the reductase component (169). The roles of the other two components remain unclear, although protein 1 may be involved in oxygen binding (169).

More recent work by Dalton's group and in our own laboratory revealed MMO activities in cell-free extracts of *M. trichosporium* OB3b of a substantially different nature from those published earlier for both the cell-free enzyme and


β-oxidation of alkanes and fatty acids
ethane
acetate
fatty acids
CARBON ASSIMILATION
HCHO
serine
malyl CoA
glycine
glyoxylate
glycollate
HICL pathway
ACETYL CoA
acetoacetyl CoA
D-(-)-3-hydroxybutyryl CoA
acetoacetate
D-(-)-3-hydroxybutyrate
PHB
CARBON/ENERGY STORAGE
acetone
glutamate
ARG, GLN, HIS, PRO
NAD
NADH
ADP
ATP
PEP
pyruvate
CO2
AL, VAL, LEU
citrate
isocitrate
α-ketoglutarate
oxaloacetate
succinyl CoA
malate
fumarate
succinate
CARBON/ENERGY METABOLISM
ASP, LYS, MET, THR, ISO

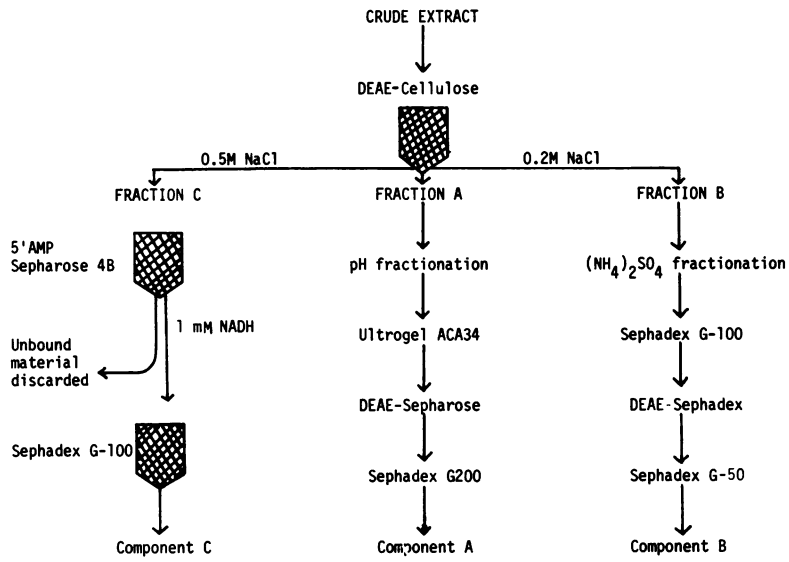


FIG. 5. *Purification scheme for the three components of MMO from M. capsulatus (Bath) (from Dalton [24]).*

the purified enzyme (62, 146, 151). Stirling and Dalton (157) resolved the MMO of this species into two fractions by ion-exchange chromatography. Although reduced activity was observed when the two fractions were reconstituted, full activity was restored to fraction 1 by addition of purified components B and C from *M. capsulatus* (Bath). No complementation of activity was observed with fraction 2 of *M. trichosporium* OB3b extracts, but these results suggest that fraction 1 contains the hydroxylase component of MMO and that there may be some homogeneity between the two enzyme systems (157).

Discrepancies in the nature of the *M. trichosporium* MMOs found by different workers may be due partly to different growth and preparation procedures (see Comparison of methane monooxygenases from different species).

Comparison of methane monooxygenases from different species. The ability of MMO to oxygenate a wide range of substrates in vitro was demonstrated with cell-free extracts of *M. capsulatus* (Bath) (21), *M. trichosporium* OB3b, and *M. methanica* (153). Substrates included *n*-alkanes, *n*-alkenes, ethers, and alicyclic, aromatic, and heterocyclic hydrocarbons for *M. capsulatus* (Bath) and *M. trichosporium* OB3b. *M. methanica* had a more limited substrate specificity: *n*-hexane and *n*-heptane and all cyclic compounds were not oxygenated. The facultative methane-oxidizing bacteria appear to have a similar varied substrate range, although its extent is unclear, since oxidation of aromatic, alicyclic, and other complex hydrocarbons have not been reported. *M. organophilum* CRL26, for instance, oxidizes C_2 to C_4 *n*-alkanes and *n*-alkenes at rates similar to those of obligate methanotrophs (68, 69), and both *M. ethanolicum* and *M. hypolimneticum* yield propene oxide and ethanol from propylene and ethane, respectively (94). Although some reactions catalyzed by MMO are analogous to the oxidation of its natural substrate (e.g., formation of primary alcohols from *n*-alkanes and 1-phenylalkane side chains) many of the substrates attacked do not resemble methane. Hydroxylation of aromatic, heterocyclic, and multi-ring compounds may, therefore, indicate a free radical reaction (74) or some other reactive oxygen mechanism.

It is particularly interesting that broad substrate oxygenation also occurs in whole organism suspensions of *M. trichosporium* OB3b (60, 62, 64) and *M. capsulatus* (Bath) (24), although some products of MMO activity are subject to further attack by other nonspecific oxidoreductases (see below).

MMOs from most sources appear to be obligatorily NAD(P)H linked. The enzymes from *M. methanica* (19, 43), *M. capsulatus* (Texas)

FIG. 4. *Central position of acetyl-CoA in the metabolism of type II methanotrophs. HICL, Homoisocitrate lyase; PEP, phosphoenol-pyruvate; ADP, adenosine 5'-diphosphate; AL, alanine; VAL, valine; LEU, leucine; ASP, aspartic acid; LYS, lysine; MET, methionine; THR, threonine; ISO, isoleucine; ARG, arginine; GLN, glutamine; HIS, histidine; PRO, proline.*

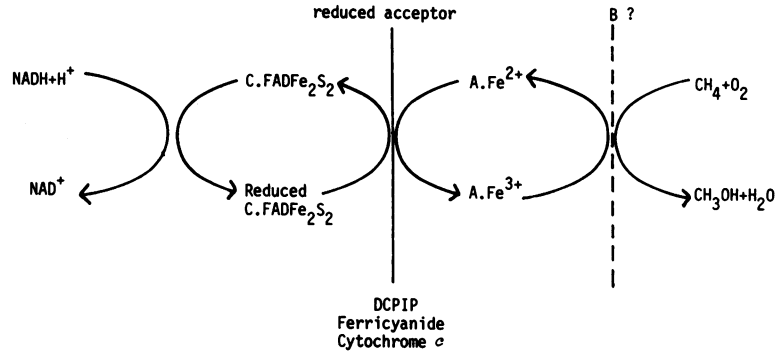


FIG. 6. *Pathway of electron transfer between the MMO components in the oxidation of methane. The prosthetic groups of components A and C are indicated as Fe and Flavin adenine dinucleotide (FAD)-Fe_2S_2. The role of B is uncertain. DCPIP, dichlorophenol indophenol. (From Dalton [24].)*

(138), *M. capsulatus* (Bath) (16), *M. trichosporium* OB3b (62, 146, 153, 157), and *M. organophilum* CRL26 (118) all require reduced pyridine nucleotides for in vitro activity. Tonge et al. (170) originally reported cell-free MMO from *M. trichosporium* OB3b to utilize ascorbate and methanol, as well as NAD(P)H as an electron donor. Later, ascorbate-mediated cell-free MMO was also described in *M. capsulatus* grown at 45°C (125). It is unclear whether NAD(P)H is the direct electron donor in vivo in some methane-oxidizing bacteria, for there is evidence that intermediate electron transfer protein(s) may be involved in extracts of *M. trichosporium* OB3b grown under oxygen-limiting conditions (146) (see below).

A study of the location of MMO activity in cell-free extracts of *M. trichosporium* OB3b revealed an interesting correlation with the enzyme's sensitivity to inhibitors (146). During oxygen-limiting growth conditions in shake flasks, particulate MMO activity sensitive to chelating agents, thiol reagents, amytal, and KCN is present. Similar inhibitors were reported for particulate MMOs of *M. capsulatus* (Texas) (137), *M. methanica* (19, 44), *Methylosinus* sp. CRL15 (118), and, originally, for *M. trichosporium* OB3b (169). Recently, it was found that growth of *M. trichosporium* OB3b in a chemostat under low methane and oxygen partial pressures with excess dissolved nitrate yields organisms that are packed with intracytoplasmic membranes and that show only particulate MMO activity (146).

In extracts prepared from *M. trichosporium* OB3b grown under all conditions other than oxygen limitation, MMO was present only in the soluble fraction. Activity was insensitive to chelating and thiol reagents and amobarbital, as shown earlier for soluble activities in this strain (157) and *M. capsulatus* (Bath) (16, 154).

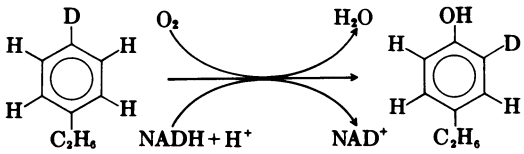
It is possible, therefore, that particulate MMOs retain a close association with electron transfer proteins and that NAD(P)H may be an indirect electron donor in vivo. Soluble MMO, however, shows no sensitivity to electron transport inhibitors or chelating agents, suggesting that NAD(P)H interacts directly with the enzyme. Further evidence for the latter case is provided by the properties of component C of the soluble *M. capsulatus* (Bath) enzyme (17, 18) (see above).

Hou and co-workers have reported the absence of MMO activity in whole organism suspensions of methanol-grown obligate and facultative methanotrophs (119, 168) and suggest, therefore, that MMO is only induced during growth on methane. Although this certainly appears to be the case for the facultative methane utilizers *M. ethanolicum* and *M. hypolimneticum* (94), MMO activity was demonstrated in the following methanol-grown obligate methanotrophs: *Methylococcus* NCIB 11083 (91), *M. capsulatus* (Texas) (76), and *M. trichosporium* OB3b (10). In these strains, at least, MMO is apparently constitutive.

Mechanisms of oxidation by methane monooxygenase. In view of the substrate specificity of the MMOs studied so far, the involvement of free radicals appears likely. Hutchinson et al. (74) proposed a free radical mechanism for methane oxidation involving generation of a methyl radical, stabilized by coordination to an iron atom, which reacts with molecular oxygen to produce a methylperoxy-iron derivative. Addition of two methyl peroxide radicals with subsequent loss of an oxygen molecule and further proton loss or addition generates methanol or formaldehyde. Direct experimental evidence for radical involvement is, however, lacking. Attempts to detect radicals by using spin-trapping agents have proved unsuccessful (D. J. Best and

D. Scott, unpublished data). Although the theoretical calculations of barrier heights for methane oxidation by a P450 hydroxylase favor a concerted "attachment-rearrangement" mechanism involving ^{1}s oxygen atoms (barrier height $\simeq$ 2 kcal/mol) (127), the experimental activation energy of methane oxidation by cell-free MMO from *M. capsulatus* at temperatures above 20°C was determined to be about 18 kcal/mol (125). This is, however, close to the theoretical barrier height of about 14 kcal/mol for a triplet hydroxylation with ^{3}p oxygen atoms involving a two-step "abstraction-recombination" mechanism with the formation of radical intermediates.

Nuclear magnetic resonance studies of MMO in *M. capsulatus* (Bath) showed the operation of an "NIH shift" mechanism during the ring hydroxylation of 1-phenylethane (25), thus:



This is analogous to the classical NIH shift mechanisms found for aromatic hydroxylases (78, 168).

Methanol Dehydrogenase

The broad-specificity NAD(P)$^+$-independent methanol dehydrogenase of the type first described by Anthony and Zatman (5) is common to all methane- and methanol-utilizing bacteria studied so far. Methanol dehydrogenases have been characterized from several methane oxidizers and, in general, appear to be closely similar (110–112, 176). In vitro, methanol dehydrogenase can be coupled to phenazine (m)ethosulfate in the presence of ammonia or a primary amine as activator and will oxidize many primary alcohols.

The enzyme is thought to be coupled to the electron transport chain at the level of cytochrome *c* in vivo. Recently, Duine et al. (35) reported the isolation of methanol dehydrogenase from *Hyphomicrobium* X with a functional coupling to cytochrome *c*. Potassium ferricyanide-oxidized cytochrome *c* shows a characteristic reduced spectrum on addition of methanol to the complex. Furthermore, this coupling is only functional in anaerobically prepared extracts and is irreversibly destroyed on exposure to oxygen, yielding the conventional, NH_4^+-activated, dye-linked dehydrogenase.

A major contribution to our understanding of the biochemistry of all methane- and methanol-utilizing bacteria is the elucidation of the structure of the prosthetic group of methanol dehydrogenase. Evidence had accumulated that the coenzyme is a novel, nitrogen-containing *o*-quinone (29, 34, 37, 183).

Salisbury et al. (145) used X-ray crystallography to determine the structure of a purified degradation product of the cofactor and proposed the trivial name "methoxatin" for the cofactor (I in Fig. 7). Confirmation of the structure came from Duine et al. (36), and they proposed the name "pyrrolo-quinoline quinone," since the *o*-quinone structure is essential for activity. Although the prosthetic group of methanol dehydrogenase cannot, at present, be reconstituted with its own apoenzyme, a functional holoenzyme can be reconstituted with glucose dehydrogenase, indicating that this group of "quinoproteins" have similar cofactors (36).

Forrest et al. (45) have proposed a mechanism for the oxidation of methanol involving methoxatin (I) (Fig. 7), which forms an addition compound with the amino group of a lysine residue (or with ammonia or a primary amine) at the C_4 position (II). 1,4-Elimination of water produces the quinone analog (III) in the active enzyme. 1,4-Addition of primary alcohol allows a cyclic rearrangement with release of the oxidized product.

Oxidation of primary alcohols by the facultative methane utilizer *Methylobacterium* CRL26 is also catalyzed by an ammonium ion-requiring, phenazine (m)ethosulfate-dependent methanol dehydrogenase (117). Similar activity was also reported for *M. organophilum* XX (191); additionally, this enzyme oxidized secondary and tertiary alcohols at low activities. Ammonium ions were not required, but were stimulating for newly purified enzyme, although ammonium ions were essential for activity after storage. The newly isolated species *M. ethanolicum* also has an ammonium ion-requiring, phenazine methosulfate-linked methanol dehydrogenase when grown on methane, but this activity is about sevenfold lower in extracts of ethanol-grown organisms (94). The presence of an alternative ethanol-oxidizing enzyme was not reported, but this may be similar to the situation during growth of two facultative methylotrophs on methanol and ethanol (51). During methanol growth of strain PAR, only a typical methanol dehydrogenase with a molecular weight of 112,000 was detected, which oxidized primary and secondary alcohols. In ethanol-grown organisms, however, this enzyme was absent and was replaced by an NAD-dependent alcohol dehydrogenase with a molecular weight of 150,000. The latter enzyme was not found during growth on methanol, and although it, too, showed broad substrate specificity, it did not oxidize methanol (7).

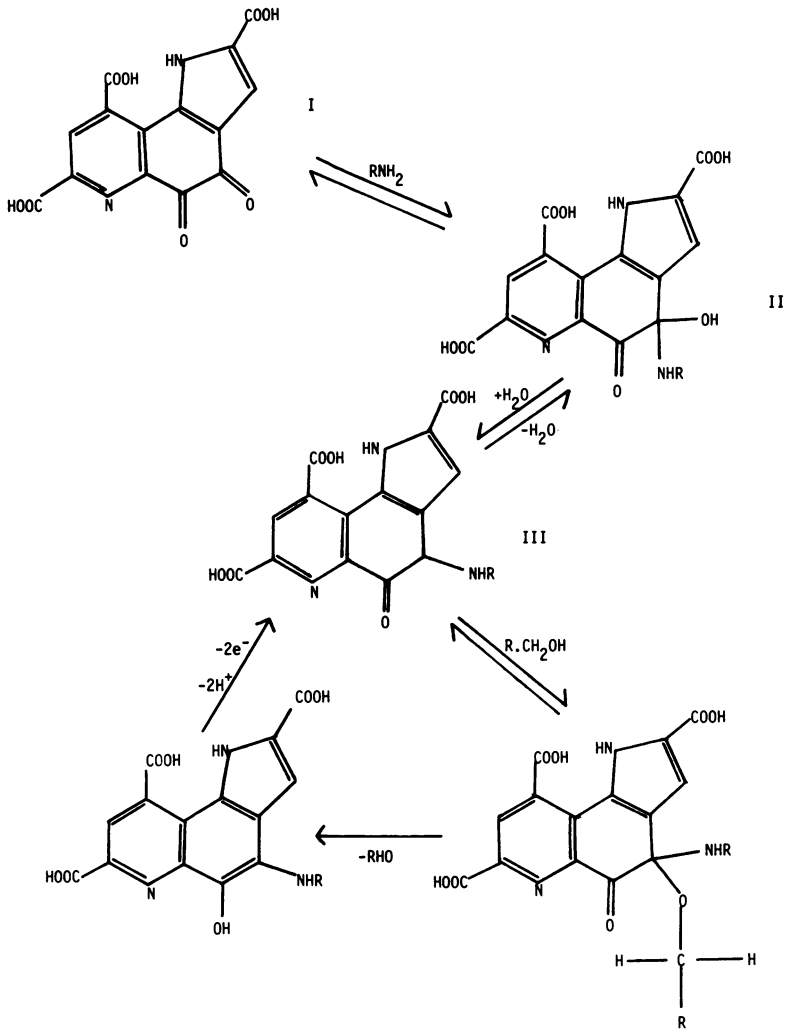


FIG. 7. *Proposed mechanism for the role of methoxatin in methanol oxidation by methanol dehydrogenase (adapted from Forrest et al. [45]).*

Formaldehyde Dehydrogenase

C_1-utilizing bacteria contain several different types of formaldehyde dehydrogenase activity (2, 155). These can be classified into two groups: (i) $NAD(P)^+$-linked enzymes and (ii) $NAD(P)^+$-independent enzymes that require an artificial electron acceptor (e.g., phenazine methosulfate or dichlorophenol indophenol) for in vitro activity. Both groups encompass enzymes which are specific for formaldehyde and nonspecific aldehyde dehydrogenases.

Stirling and Dalton (155) purified a $NAD(P)^+$-linked formaldehyde dehydrogenase from *M. capsulatus* (Bath) and subsequently reported preliminary evidence for an NAD^+-linked enzyme in cell-free extracts of *M. trichosporium* OB3b (157). In addition, a heme-containing aldehyde dehydrogenase was purified from *M. trichosporium* PG which required phenazine methosulfate for in vitro activity (116). This activity was demonstrated in cell-free extracts of several type I, type II, and facultative methane-utilizing strains, although extracts of *M. capsulatus* (Texas and CRL24) and *Methylosinus sporium* 5 showed very low activity (6 to 7 nmol/min per mg of protein). Cyclic oxidation of formaldehyde of the type discussed in Carbon Metabolism, which would yield two NAD(P)H molecules per C-1 unit, has yet to be demonstrated in methanotrophs.

The ability to derive readily available reducing equivalents in the form of NAD(P)H from formaldehyde dehydrogenases may be impor-

tant in influencing the yield during growth on methane (3, 4) and the usefulness of supplementary carbon sources (see Carbon Metabolism).

Formate Dehydrogenase

A typical NAD-linked formate dehydrogenase has been found in several methylotrophs and three methane oxidizers (80, 112, 139).

Secondary Alcohol Dehydrogenase

Phenazine methosulfate-dependent methanol dehydrogenase from methane-utilizing bacteria has a broad substrate specificity for C_1 to C_{10} primary alcohols, but shows either little or no activity towards secondary alcohols (see above). Recently, however, a secondary alcohol-specific dehydrogenase activity was observed in cell-free extracts and whole organism suspensions of several types of obligate and facultative methane and methanol utilizers (71). Both primary and secondary alcohols are the products of short-chain *n*-alkane oxidation by MMO (21, 119). Hou and co-workers described the accumulation of methylketones from secondary alcohols (reflecting secondary alcohol dehydrogenase activity) (71) and from *n*-alkanes (reflecting MMO and secondary alcohol dehydrogenase activities) (72, 120) by intact methanotrophs. The activity could not be demonstrated in extracts of either of the facultative methanotrophs *M. ethanolicum* or *M. hypolimneticum*, although whole organisms oxidized 2-butanol (94). Although it has been known for some time that *n*-alkanes are co-oxidized to methylketones by *M. methanica* (89, 93), the Exxon group showed that this is due to NAD^+-dependent secondary alcohol dehydrogenase present in cell-free extracts, in addition to MMO.

Secondary alcohol dehydrogenase was purified from methanol-grown *Pseudomonas* sp. ATCC 21439 (70) and has a molecular weight of about 95,000, comprising two identical subunits. Although the dehydrogenase was specific for secondary alcohols, it did not oxidize C_7 to C_{10} alcohols and showed maximum activity towards 2-butanol. Hou et al. (70) do not ascribe a physiological function to secondary alcohol dehydrogenase activity in C_1 metabolism, but do suggest that this NAD^+-linked activity would be advantageous for methanotrophic organisms, which may be NAD(P)H limited (4). However, the fact that the oxidation of *n*-alkanes via secondary alcohols to methylketones yields no net increase in NADH or other reducing agent seems to have been overlooked. Although some secondary alcohols are present in the environment, the relatively specific secondary alcohol dehydrogenase shows no activity towards secondary alcohols above 2-hexanol, i.e., it is unlikely to attack secondary alcohols that might be derived from substances common in the environment.

Oxidation of Multicarbon Compounds

The capacity of methane-oxidizing bacteria to metabolize an extensive and varied range of organic compounds has prompted considerable research effort and much speculation over the metabolic significance of these processes (60, 61, 158) to methanotrophs. Although MMO has been at the forefront of this attention and is almost always responsible for the initial oxidative attack on these compounds, many transformation products are the result of further oxidation by other oxidoreductases. Whole organism suspensions of *M. trichosporium* OB3b and *M. capsulatus* (Bath) oxidize *n*-alkanes, *n*-alkenes, aromatic and alicyclic hydrocarbons, phenols, long-chain and alicyclic alcohols, and heterocyclic, multi-ring, and halogenated hydrocarbons (24, 60). The rate of these oxidations can be similar to the methane oxidation rate (e.g., ethane, propane, propene) or, in the case of more complex compounds, an order of magnitude less. An important factor in determining the rate and extent of whole organism biotransformations is the level of endogenous energy reserves. Organisms harvested from carbon excess, nitrogen-limited cultures of *M. trichosporium* OB3b have high levels (20 to 30%) of PHB (146) and show concomitantly faster propene oxidation and greater product accumulation than those organisms with low levels of PHB from carbon-limited, nitrogen excess growth conditions (Best and Scott, unpublished data). A similar situation arises in *M. capsulatus* (Bath) (24), with accumulation of storage polymers in low specific-growth-rate cultures (0.03/h). This fact may have been overlooked in some cases and should be carefully considered when comparing the in vivo oxidation abilities of methane oxidizers.

Stirling and Dalton (156, 158) defined the oxidation of nongrowth substrates catalyzed by MMO and subsequent enzymes as "fortuitous," suggesting that these activities are a consequence of the mechanistic requirement for methane, methanol, and formaldehyde oxidation. Higgins and co-authors (60, 61), in contrast, argued that as carbon (acetate) and energy (NADH and ATP) can be derived from oxidation of some environmentally important hydrocarbons (e.g., *n*-alkanes) a selection pressure may have existed to retain or develop nonspecific enzymes, and that this metabolism should be termed "supplementary" (60). (This is discussed further in Evolution of Methanotrophs.)

Energy Transduction and Growth Yields

The electron transfer and energy transducing systems in methane-oxidizing bacteria are not fully understood, but our current knowledge was recently thoroughly reviewed by Higgins (59) and Wolfe and Higgins (195). Anthony (3, 4) discussed the prediction of growth yields in methylotrophic bacteria and identified several important considerations. First, yields for most methylotrophic bacteria are determined by NAD(P)H supply as well as ATP, and, in particular, methane-oxidizers may be NAD(P)H limited. The extent of this limitation depends primarily upon the mechanism of supply of the reducing source to the MMO. Bacteria that oxidize methane, utilizing NADH as the direct electron donor to the monooxygenase, have predicted yields in the range of 9.2 to 12.0 g (dry wt)/mol of substrate, depending on the type of carbon incorporation pathway, and are NADH limited. Alternatively, strains with a monooxygenase system coupled to reduced methanol dehydrogenase as the source of reducing equivalents, via CO-binding cytochrome *c* (65), have predicted yields as high as 17.4 g (dry wt)/mol of substrate and are predominantly ATP limited. If NADH is generated by reverse electron transport, yields would be between those for NADH-coupled and methanol dehydrogenase-coupled systems if ATP was required, and the same as the latter system if ATP was not required.

For organisms with an NADH-coupled monooxygenase system, the yield may be further affected by the type of reduced cofactor used in the formaldehyde dehydrogenase reaction. $NAD(P)^+$-linked formaldehyde dehydrogenase strains will have slightly higher yields than those which use an $NAD(P)^+$-independent enzyme. The experimental determination of growth yields on methane is difficult, but apparently reliable figures have been reported that are substantially greater than the theoretical predictions (3, 4). In addition, molar yields on methane and methanol are commonly found to be similar. This has been taken to indicate that MMO may be unusual among monooxygenases in being energetically conservative (65), but there are alternative explanations. Reconciliation of theory and practice awaits a more complete understanding of methanotrophic energetics.

Methanotrophic Yeasts and Anaerobic Methane Oxidation

The likelihood that methanotrophic yeasts obtain energy by oxidizing methane to CO_2 and assimilate carbon from CO_2 (192) is discussed in Carbon Metabolism. This apparently inefficient mechanism may be the reason for the very slow generation times (2 to 7 days). As yet, nothing is known of the enzymology of methane oxidation in these yeasts.

It was for many years widely assumed that methane is biologically inert in anaerobic environments, but recent geochemical evidence has indicated that methane does disappear in anaerobic environments (50). Zehnder and Brock (198), however, showed low levels of methane oxidation during anaerobic growth of some methanogenic bacteria. The ratio of methane oxidized to methane formed was between 0.001 and 0.3%, but preliminary evidence suggests that the route of methane oxidation is not simply reversal of the methane formation process. Anaerobic oxidation of methane also occurred in nonmethanogenic bacteria isolated from the sediment surface of Lake Mendota, Wis. (109). Sulfate, acetate, and methane were all required for anaerobic growth of the isolates, and it appears that acetate is directly assimilated and methane is oxidized to CO_2 without incorporation. Again, nothing is known of the nature of the enzymes involved.

NITROGEN METABOLISM

Methane-utilizing microorganisms play a role in the nitrogen cycle not only by virtue of the necessary incorporation of nitrogen into their biomass but also by transformation of inorganic nitrogen compounds and dinitrogen (195) and, perhaps, of complex, nitrogen-containing organics (21, 64). The ability of many methanotrophs to fix atmospheric nitrogen in addition to using nitrate or ammonia as a nitrogen source has been long established (128). Some information on pathways of ammonia assimilation by obligate methanotrophs is now available. Dalton and Whittenbury (26) detected glutamate dehydrogenase and glutamine synthetase activities in *M. capsulatus* (Bath). The activities of these two enzymes varied with different nitrogen sources, glutamate dehydrogenase having the highest activity in ammonia-grown organisms, whereas glutamine synthetase was the more active enzyme after growth under nitrogen-fixing conditions. Drozd et al. (33) found that an apparently different strain of *Methylococcus* did not possess glutamate dehydrogenase but was constitutive for enzymes of the glutamine synthetase–glutamine 2-oxoglutarate aminotransferase system (15).

In a more thorough study of seven obligate methanotrophs representing both type I and type II, Shishkina and Trotsenko (147) found that the type I strains tested, except *M. capsulatus* (Foster and Davis strain), had glutamate (or alanine) dehydrogenase and glutamine syn-

thetase activities. Type II methane utilizers had enzyme activities typical of the glutamine synthetase–glutamine 2-oxoglutarate aminotransferase system. *M. capsulatus* (Foster and Davis), a type I methanotroph, not only had alanine (but not glutamate) dehydrogenase and glutamine synthetase activities but in addition had glutamate synthase activity. Thus, this strain may possess two pathways for ammonia assimilation. In this study, enzyme activities varied according to the nitrogen source (NH_4^+, NO_3^-, or N_2).

Nitrogen fixation by *Methylosinus* strain 41 (32) is accompanied by hydrogenase activity which can support acetylene reduction (31). Nitrite is known to be reduced by methanotrophs (188), as it must be, prior to assimilation as ammonia.

Other transformations of nitrogen compounds reflect the activity of the broad-substrate-specificity MMO. Oxidation of ammonia to nitrite by methane utilizers was observed by Hutton and Zobell (75) and by Whittenbury and his colleagues (188). Initiation of this process is very probably due to MMO activity, for ammonia is a competitive inhibitor of methane oxidation (as methane is of ammonia oxidation in *Nitrosomonas* [107, 161]. Dalton (23) detected hydroxylamine as an oxidation product of ammonia by *M. capsulatus* (Bath), as did Higgins et al. (64) working with *M. trichosporium* OB3b.

Recently a specific hydroxylamine:cytochrome-*c* oxidoreductase, distinct from methanol dehydrogenase, has been described in *M. ethylococcus thermophilus* (152). Under some conditions, ammonia oxidation may well serve as a supplementary energy source (97), a concept discussed in Energy Metabolism. A number of groups, in monitoring the wide substrate specificity of MMO (Energy Metabolism), have shown that pyridine is a substrate, being oxidized to pyridine-*N*-oxide (21, 64). When such nitrogen compounds are available in the natural habitats of methanotrophs, it seems reasonable to suppose that they will be transformed by these organisms, which may thus play several roles in the nitrogen cycle (60).

GENETICS OF METHANOTROPHS

Progress in our understanding of the genetics of methane utilizers has been hampered by the difficulty of obtaining phenotypic mutants. Such mutants arose with a frequency of about 10^{-3} in *M. capsulatus* and *M. albus* treated with a chemical mutagen, such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, but generally reverted at high frequency (54, 190). Other chemical mutagens, such as alkylmethane-sulfonates, and irradiation with ultraviolet and γ sources were unsuccessful in mutating obligate methanotrophs, although both *N*-methyl-*N*-nitro-*N*-nitrosoguanidine and ultraviolet mutagenesis gave rise to auxotrophic mutants of the facultative methane utilizer *M. organophilum* (105). In this study, exogenous wild-type DNA transformed the mutants to prototrophy at a frequency of 5×10^{-3}, competence occurring at late logarithmic phase.

It seems likely that the inability to isolate mutants of obligate methanotrophs is not due to a fundamental difference in the genetics of these mutants compared with enterobacteria, for example, but is perhaps due to the use of inappropriate techniques. For example, at least some methanotrophs are probably not mutable by ultraviolet due to lack of error-prone "SOS" DNA repair mechanisms (189). Methanotrophs resemble other microorganisms in the possession of lytic bacteriophages (174, 175) and extrachromosomal DNA, at least in facultative methanotrophs, whose MMOs may be plasmid encoded (50, 196). A major aim of genetic studies is, of course, to understand the organization and regulation of the processes of metabolism, and it is not surprising that studies of C_1-oxidative and C_1-assimilative pathways are furthest advanced in facultative methylotrophs. In the facultative methanotroph *M. organophilum*, O'Connor (101) and O'Connor and Hanson (103, 104) showed by DNA transformation experiments that many of the key enzymes of C_1 oxidation and assimilation (with the notable exception of formate dehydrogenase) are coordinately regulated and appear to be located in a single operon.

The use of promiscuous plasmids to both generate mutants by transposon mutagenesis (9) and map mutants by chromosome mobilization (66, 124) in methanotrophs offers the best hope for rapid elucidation of genetic systems in these species. Transfer of the antibiotic resistance markers carried by such plasmids to facultative methylotrophs has been reported by several groups (8, 47, 79, 178). In addition, Warner et al. (179) reported transfer of the plasmid R68.45 to the methanotroph *M. trichosporium* OB3b.

EVOLUTION OF METHANOTROPHS

The possibility that the present-day aerobic methane utilizers might represent those species which evolved the capacity to oxidize and grow on methane when the earth's atmosphere became sufficiently oxidized is set in context by Quayle and Ferenci (133). They discuss the current relationships between autotrophy and methylotrophy, of which methanotrophy is a specific case, and suggest evolutionary routes for

the development of similar carbon assimilation pathways in these two groups of species.

There are, for instance, considerable morphological similarities between methane utilizers and nitrifying bacteria in their internal membrane arrays; the two groups initiate the oxidation of their respective energy sources, CH_4 and NH_3, by a monooxygenase, and both nitrifiers and type I methanotrophs lack a complete TCA cycle. In addition, some methanotrophs appear to be capable of partial lithotrophic metabolism, deriving energy from ammonia oxidation. Carbon assimilation pathways are not the same, however, though Quayle and Ferenci (133) have suggested possible evolutionary sequences by which the type I methanotrophic RMP pathway of carbon assimilation might be linked with the closely related ribulose diphosphate cycle of the nitrifying bacteria.

Shishkina and Trotsenko (147) found that *M. capsulatus*, used in their studies of nitrogen assimilation (see above), possessed metabolic activities typical of both type I and type II methanotrophs and suggested that the species might represent an intermediate between the two types. Further evidence to this effect is cited by Taylor et al. (166), who detected the enzymes of the autotrophic Calvin cycle in *M. capsulatus* (Bath) as well as [1-^{14}C]glycolate incorporation by a pathway probably involving enzymes found in the type II serine pathway for carbon assimilation. Our present state of knowledge of methanotrophs is far from complete, and the alternative suggestions of reclassifying *M. capsulatus* (Bath) as a type X methylotroph (185) or the division of *Methylococcus* into two genera (141) highlight our lack of understanding of those relationships within this group of methylotrophs which is necessary for detailed comparison with other microbial groups.

Of particular interest in the present context is the evolution of MMO. Although there is considerable controversy as to the protein chemistry of MMO (132), the remarkable scope of MMOs from different sources to effect oxidations of many organic compounds (see above) is worthy of further mention. This phenomenon has been variously termed "cometabolism" and "fortuitous metabolism" (156) and Quayle (130) has proposed that the term "cometabolism" be confined to the oxidation of substrates, such as CO, for which reducing power must be provided by the addition of a suitable cosubstrate. It should not be overlooked, however, that the cosubstrate need not be exogenously supplied, and reducing power can be generated from endogenous storage polymers. "Fortuitous metabolism" would then describe oxidation of such a substrate as ethane. In this case, the reducing power for the initial oxidative attack by MMO is replenished by further oxidation of the product of the MMO reaction.

It has been suggested (60) that there may have been selection pressures for evolution of broad-specificity MMOs and other oxidoreductases or for the retention of such enzymes, since they may confer some physiological advantages on methanotrophs. Although either case defies conclusive proof, some possible products of MMO activity, such as ethanol derived from ethane oxidation or alcohols derived from long-chain alkanes, can be incorporated by methanotrophs after further oxidation by nonspecific oxidoreductases (see above and reference 60) and appear to increase cell yield (177) (Taylor, personal communication). In this context the term "supplementary metabolism" seems more appropriate.

It is possible to speculate on the development of MMO. As discussed by Quayle and Ferenci (133), oxygen appeared in the atmosphere after a considerable period of biotic evolution. One might consider a "primitive" anaerobe, fermenting formaldehyde, confronted with a developing aerobic environment. The ability to supplement dwindling supplies of formaldehyde by oxidizing (though perhaps also dwindling) methane and methanol would have conferred considerable advantage. A relatively inefficient MMO with poor substrate specificity would have been the sufficient outcome of such selection pressure at the original event, especially since other previously unavailable reduced organic compounds might also then be oxidized, yielding perhaps energy and carbon for incorporation. Subsequent specialization of the methane utilizers to their present habitats must have been accompanied by selection pressure for development of a highly active MMO, not subject to competitive inhibition by other commonly occurring compounds, unless the ability to oxidize those other compounds conferred an advantage slightly outweighing that of improved catalysis of methane oxidation. That the latter case seems to exist today, at least in those few MMOs so far examined, suggests that there is indeed some advantage to maintaining a broad-substrate-specificity MMO. It would appear, however, that facultative methane utilizers could be at an advantage over obligate strains in situations where carbon sources other than methane are available.

Perhaps facultative methane utilizers find little advantage in an MMO with catholic specificity because they can avail themselves of other carbon sources. Whole methane-grown cells of *Methylobacterium* strain R6, the facultative methane utilizer studied by the Exxon group, can oxidize carbon monoxide, ethane, propane,

ethylene, and propylene (117), and the two facultative isolates of Lynch et al. (94) can oxidize ethylene and propylene if grown on methane. More detailed studies of the substrate specificities of the MMOs of facultative methane utilizers are unfortunately not available.

A particularly fascinating question is whether the isolation of facultative methanotrophs, which has led only to species similar to type II obligate organisms, is fortuitous or truly representative. This can only be resolved by the isolation of more species, but it would not be surprising if it proved invariably to be the latter case. The ability of type II obligate strains to metabolize non-C_1 substrates makes the basis of their obligacy and their relationship to facultative strains more difficult to define. It does, superficially, suggest a close evolutionary link, which is not, however, supported by DNA hybridization studies (50).

BIOTECHNOLOGICAL APPLICATIONS OF METHANOTROPHS

For many years, methanotrophs have been considered for single-cell protein processes, and development in this area continues (48). The discovery of the extensive range of chemical transformations catalyzed by methanotrophs has opened up the possibility of their exploitation for biocatalysis, and several patents have been filed on this subject (60). It seems likely that such processes will be developed within the next few years. They may also prove useful as sources of biopolymers.

To capitalize fully on the biotransformational capacities of these bacteria, it may well be necessary to develop reliable techniques for their genetic manipulation. Successful development of such techniques would also facilitate expression of foreign DNA coding for products of industrial or medical importance. There may be significant advantages in using methanotrophs for synthesis of foreign proteins, as they appear to have low protease activities, as exemplified by long-term stability of enzyme activities in nongrowing organisms. The fact that many enzymes of C_1 metabolism are apparently constitutive in obligate methanotrophs suggests that they possess operons suitable for cloning and the subsequent expression of foreign DNA in these bacteria.

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