Assimilation and Toxicity of Some Exogenous C₁ Compounds, Alcohols, Sugars and Acetate in the Methane-oxidizing Bacterium *Methylococcus capsulatus*

By M. ECCLESTON* AND D. P. KELLY

*Department of Microbiology, Queen Elizabeth College,
Campden Hill, London, W8 7AH*

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SUMMARY

Growth of *Methylococcus capsulatus* on methane was inhibited by methanol (0.1 %, v/v, and above), ethanol, *n*-propanol and *n*-butanol (0.01 %, v/v, and above), but was unaffected by galactose, glucose, fructose, maltose, sucrose (at 0.1 M) or lactose (0.05 M). About one organism in 7 million grew well on solid medium using methanol vapour as a sole source of carbon and energy, but [¹⁴C]methanol was readily metabolized and assimilated by cultures growing on methane. Labelling patterns from [¹⁴C]methane and [¹⁴C]methanol were similar, indicating their assimilation by a common pathway. Dissimilarities between the labelling patterns obtained with ¹⁴CH₄ and ¹⁴C-labelled formaldehyde, formate and carbonate indicated that the ribose phosphate cycle of formaldehyde assimilation may not account for all the carbon assimilated by *M. capsulatus*: significant incorporation of formate, carbon dioxide and possibly of intermediates of methane oxidation more reduced than formaldehyde may occur. ¹⁴C-Labelled ethanol and acetate showed restricted incorporation into lipid, leucine, glutamate, proline and arginine, indicating that *M. capsulatus* can produce acetyl coenzyme A from both compounds and introduce it into an incomplete biosynthetic tricarboxylic acid cycle. *Methylococcus capsulatus* was unable to assimilate more than trace amounts of [¹⁴C]glucose or sucrose.

INTRODUCTION

Methylococcus capsulatus represents one of the groups of obligately methylotrophic bacteria whose growth is supported only by the aerobic oxidation of methane, methanol and dimethyl ether (Foster & Davis, 1966; Whittenbury, Phillips & Wilkinson, 1970; Wilkinson, 1971; Quayle, 1972). Although intensive studies have been made of the kinetics and enzymology of the oxidation and incorporation of C₁ compounds by methylotrophs (Kemp & Quayle, 1966, 1967; Quayle, 1969, 1972; Higgins & Quayle, 1970; Lawrence, Kemp & Quayle, 1970; Ribbons, Harrison & Wadzinski, 1970; Ribbons & Michalover, 1970; Patel & Hoare, 1971; Davey, Whittenbury & Wilkinson, 1972), physiological studies have been largely concerned with high biomass production for use as food protein (Silverman, 1964; Hamer, Heden & Carenberg, 1967; Whittenbury, 1969; Wilkinson, 1971). Oxidation by methylotrophs of C₂ to C₅ primary alcohols as well as of formaldehyde and formate has been reported (Silverman, 1964; Patel & Hoare, 1971; Davey *et al.* 1972; Patel, Bose, Mandy & Hoare, 1972) but these compounds did not support growth. Quayle's group have proposed a ribose phosphate cycle for formaldehyde fixation which may be the principal

* Present address: Department of Biological Sciences, University of Warwick, Coventry, Warwickshire, CV4 7AL.

mechanism for entry of C₁-carbon into biosynthetic pathways in *M. capsulatus* (Kemp & Quayle, 1966, 1967; Lawrence *et al.* 1970; Quayle, 1972). Failure of the methylotrophs to grow on a wider range of organic substrates (Whittenbury *et al.* 1970) has not yet been satisfactorily explained, but the kinds of enzymatic 'lesions' and regulatory phenomena proposed to explain obligate lithotrophy (Kelly, 1971) may apply to the methylotrophs also (Whittenbury, 1971; Quayle, 1972). Amemiya (1972) failed to culture *Methanomonas* on glucose in the dialysis culture system that has allowed growth of some 'obligate' chemolithotrophs (Pan & Umbreit, 1972), and attributed failure to grow on glucose to an absence of hexokinase (Amemiya, 1972). Little work has been published on intermediary metabolism in methylotrophs, although preliminary reports indicate that acetate can be assimilated, and labelling patterns for this substrate indicate the presence of a tricarboxylic acid cycle lacking α -oxoglutarate dehydrogenase in *Methylococcus capsulatus* (Patel, Hoare & Taylor, 1969) and of a complete cycle in *Methanomonas methanooxidans* (Wadzinski & Ribbons, 1972). This is an example of the strikingly different biochemical properties of methylotrophs with Type I and Type II membrane systems (Davies & Whittenbury, 1970). For example, all Type I organisms so far examined lack the key enzyme of the serine pathway and have an incomplete Krebs's cycle, whereas Type II organisms use the serine pathway and have a complete Krebs's cycle (Lawrence & Quayle, 1970; Davey *et al.* 1972).

Demonstrations of inhibition of growth of *Methylococcus capsulatus* by various organic compounds (Eroshin, Harwood & Pirt, 1968; Eccleston & Kelly, 1972*a*) indicate that such compounds can probably enter metabolic sequences in this organism. Further experiments showed that the common pathways for biosynthesis of the aspartic and glutamic acid families of amino acids occur in this organism which also possesses a transport system for accumulating amino acids into its cells (Eccleston & Kelly, 1972*a, b*).

We now present our observations on (i) the response of *Methylococcus capsulatus* to primary alcohols, including the response of methane-grown organisms to methanol, formaldehyde and formate as alternative substrates; (ii) metabolic implications from long-term labelling patterns from ¹⁴C-labelled C₁ compounds, acetate and ethanol; (iii) growth response and assimilation of sugars by organisms growing on methane.

METHODS

Organism and culture conditions. There were described previously (Eccleston & Kelly, 1972*a, b*).

Growth experiments in liquid culture. These were conducted as described previously (Eccleston & Kelly, 1972*a, b*). Growth of wild-type *Methylococcus capsulatus* in liquid culture using methanol in place of methane as the carbon and energy source was tested by inoculating 1 ml of a 24 h culture into 9 ml of medium supplemented with various methanol concentrations. Flasks were sealed with sterile rubber bungs, and incubated, without methane, under standard conditions (Eccleston & Kelly, 1972*a, b*) for periods of up to 4 weeks. Control flasks were treated similarly, but were gassed with methane prior to incubation.

Growth experiments in plate culture. Toxicity of organic compounds to growth of *Methylococcus capsulatus* in Petri dish culture was normally tested by adding the sterile organic compound to minimal agar medium (Eccleston & Kelly, 1972*b*) to give the appropriate final concentration. Petri dishes were inoculated by spreading 0.1 ml of a 24 h liquid culture. Separate control plates were always set up under standard incubation conditions under methane + air to check the viability of inoculum cultures.

Methanol was tested as a carbon and energy source by the above method, and by diffusion

from methanol-impregnated, uninoculated agar plates (see below). Formaldehyde was tested as a potential carbon and energy source by distributing a total of 0.1 g or 1.0 g paraformaldehyde powder over the surface of three uninoculated agar plates placed at the bottom of desiccators. Water vapour in contact with the paraformaldehyde powder generated formaldehyde within the desiccators. Desiccators were set up with or without methane to check for inhibition effects.

All Petri dishes were examined for growth after 5 days, and at weekly intervals thereafter for up to 6 weeks at 37 °C.

Isolation of methanol mutants. Mutants of *Methylococcus capsulatus* capable of growth in plate culture on methanol rather than methane were obtained by spreading 0.1 ml samples of 24 h wild-type cultures (1 to 5×10^9 colony-forming units/ml) on to dishes of minimal medium (Eccleston & Kelly, 1972*b*). Dishes were incubated in air-filled sealed desiccators at 37 °C. Methanol was supplied by diffusion from three to four dishes placed in the bottom of each desiccator and containing a total of 1 ml methanol absorbed into uninoculated minimal medium. Inspection after 10 days of incubation showed many tiny mutant colonies of various sizes, and several larger colonies. After 3 weeks of incubation each plate supported the growth of 30 to 40 large colonies (0.3 to 1.0 mm diam.), occurring with a frequency of about 1 in 7 million. Several hundred smaller colonies (< 0.3 mm diameter) were also visible. Individual large mutant colonies were picked off and separated from smaller colonies by single colony isolation through several weekly transfers, until several strains of uniform colony size, capable of good growth on methanol, were obtained. Colonies were identical in size, shape, colour and microscopic appearance to those of the wild-type grown on methane.

Incorporation of labelled compounds. Incorporation of labelled glucose and sucrose was measured using the methods described previously (Eccleston & Kelly, 1972*a*). Incorporation of labelled ethanol, methanol and formaldehyde was determined after mixing 0.2 ml samples from cultures with 0.8 ml of 5% (v/v) acetic acid in ethanol, and drying samples of the suspensions on Whatman GF/A glass-fibre discs (2.1 cm diameter) prior to scintillation counting. Dried discs and Millipore membranes were assayed for ^{14}C by immersing in 5 ml 0.5% (w/v) butyl PBD (2-(4'-*t*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole) in toluene and counting at 10 °C in a Philips liquid scintillation analyser, programmed to compute d.p.m. values from a predetermined channels ratio equation.

Fractionation of ^{14}C -labelled organisms. The procedures previously described were used (Eccleston & Kelly, 1972*a*). Carbon-14 in the fractions was assayed by scintillation counting using 0.5% (w/v) butyl PBD in 10 ml of toluene containing sufficient ethanol to produce single phase systems.

Leucine, isoleucine and phenylalanine were isolated from the other amino acids in protein hydrolysates by 2-dimensional chromatography (Eccleston & Kelly, 1972*a*) and resolved from each other by descending chromatography (21 °C, 42 h) on Whatman no. 1 paper (prewashed by chromatography in *n*-butanol+acetic acid+water; 12:3:5, by vol) using methyl ethyl ketone+*n*-butanol+0.880 ammonia solution +water (20:20:1:10, by vol). Nucleic acid hydrolysates were fractionated by 2-dimensional ascending chromatography on Whatman no. 1 papers using 70% (v/v) *tert*-butyl alcohol in 0.8 N-HCl followed by *sec*-butyl alcohol +99% formic acid+water (70:10:20, by vol). ^{14}C -Regions on chromatograms were detected by autoradiography and ^{14}C counted after immersing the paper segments in 20 ml of 0.5% (w/v) butyl PBD in toluene.

^{14}C -Labelled chemicals. These were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. [^{14}C]Paraformaldehyde (0.446 mg, 100 μCi) was converted to formaldehyde by adding 7.45 ml water and heating in a sealed tube at 80 to 90 °C for 2 h.

Fig. 1. Effect of several concentrations (% v/v) of different primary alcohols on the exponential growth of *Methylococcus capsulatus* (wild-type). Control cultures received only water in each case. (a) Methanol; (b) ethanol; (c) *n*-propanol; (d) *n*-butanol.

Fig. 2. Incorporation of $[2\text{-}^{14}\text{C}]\text{ethanol}$, $[^{14}\text{C}]\text{methanol}$ and $[^{14}\text{C}]\text{formaldehyde}$ by growing cultures of *Methylococcus capsulatus*.

RESULTS

Effect of methanol on growth. Exponentially growing wild-type cultures of *Methylococcus capsulatus* were inhibited by methanol at concentrations of 0.1 % (v/v) and above (Fig. 1*a*). High concentrations (5 %, 10 %, v/v) had an immediate toxic effect. Lower concentrations (0.25 % to 1.0 %, v/v) did not exert any noticeable toxic effect during the first 2 h of exposure, after which growth became very irregular (Fig. 1*a*). In some cases (e.g. 0.5 %, 1.0 %, v/v) growth temporarily ceased, resuming at slower rates for short periods, but bacterial yields were never greater than 0.6 mg dry wt/ml even after incubation for a further 16 h.

Methanol was also toxic to growth of wild-type organisms in Petri dish culture. Although minimal agar medium supplemented with 0.1 % (v/v) methanol supported a lawn of growth, sparser growth was observed on a 0.5 % (v/v) methanol after the incubation of spread-plate cultures under standard conditions in a methane+air atmosphere. Higher concentrations (1 %, 1.5 %, v/v) resulted in extremely poor growth.

Growth on methanol as the carbon and energy source. Attempts to grow our wild-type strain of *Methylococcus capsulatus* in liquid culture using methanol (0.05, 0.1, 0.5, 1.0 %, v/v) in place of methane as the carbon and energy source were unsuccessful, and always resulted in lysis of the cells when methane was not supplied. Mutant strains could, however, be isolated in Petri dish culture when methanol was used in place of methane as the carbon and energy source. Mutant strains survived at least seven weekly transfers on solid medium, but did not grow when transferred to liquid medium containing methanol (0.01, 0.05, 0.1, 0.5 or 1 %, v/v).

Table 1. *Fractionation of Methylococcus capsulatus after growth with ¹⁴C-labelled methane, methanol, formaldehyde, formate, carbonate, ethanol or acetate*

On reaching a cell density of 0.31 mg dry wt/ml, cultures received one of the following: [¹⁴C]methane (7 μ Ci); [¹⁴C]methanol (0.1 mM; 30 215 d.p.m./nmol); [¹⁴C]formaldehyde (0.1 mM; 13 672 d.p.m./nmol); [¹⁴C]formate (0.018 mM; 1 μ Ci/ml); Na₂ ¹⁴CO₃ (0.036 mM; 2 μ Ci/ml); [2-¹⁴C]ethanol (0.124 mM; 14 467 d.p.m./nmol); [U-¹⁴C]acetate (0.009 mM, 0.5 μ Ci/ml). Cultures receiving ¹⁴C-carbonate, formate or acetate were harvested after 1 h. The other cultures were harvested for fractionation on reaching about 1 mg dry wt/ml (late exponential phase).

Fraction	¹⁴ C content (%) in fraction						
	¹⁴ C-labelled substrate						
	Methane	Methanol	Formaldehyde	Formate	Carbonate	Ethanol	Acetate
Cold 5 % TCA	10.4	9.5	9.5	24.5	19.9	4.8	10.3
70 % ethanol	30.4	37.5	24.8	14.6	18.1	69.4	59.2
Ethanol + ether	1.1	1.6	1.7	0.4	0.1	2.9	2.1
Hot 5 % TCA	22.9	20.8	42.0	25.2	24.7	3.5	3.8
Residue (protein)	35.2	30.6	22.0	35.3	37.2	19.4	24.6
% recovery of ¹⁴ C in bacteria	92.3	99.5	107.6	107.5	108.0	104.0	103.0

Table 2. *Distribution of ¹⁴C in Methylococcus capsulatus protein after incorporation of ¹⁴C-labelled compounds (see Table 1)*

Identity of compound	¹⁴ C content of compounds* (d.p.m./1000 d.p.m. in glutamate)						
	¹⁴ C-labelled substrate						
	Methane	Methanol	Formaldehyde	Formate	Carbonate	Ethanol	Acetate
Glutamate	1000	1000	1000	1000	1000	1000	1000
Proline	549	561	581	644	542	515	475
Arginine	701	746	921	{ 2565	960	564	322
Lysine	808	967	876		466	—	29
Leucine	926	1068	887	{ 2586	{ 1008	801	662
Isoleucine	706	507	420			28	35
Phenylalanine	644	610	638			15	38
Aspartate	743	677	697	1218	1123	23	42
Serine + glycine	651	671	1138	5745	804	46	38
Threonine	432	402	392	390	395	13	10
Alanine	825	771	574	778	218	13	15
Tyrosine	536	528	537	1166	365	15	12
Methionine + valine	752	666	502	2048	235	12	17
Unknowns	103	109	85	—	—	—	—

* Mean of duplicate determinations.

Effect of methanol, n-propanol and n-butanol on growth. Ethanol was very inhibitory when added to exponentially growing liquid cultures of *Methylococcus capsulatus*, exhibiting slight but noticeable toxicity at concentrations as low as 0.005 % (v/v) (Fig. 1 b). *n*-Propanol (Fig. 1 c) and *n*-butanol (Fig. 1 d) were also potent growth inhibitors, which at moderate concentrations (0.025 to 0.1 %, v/v) did not show an effect until 2 to 3 h after administration, when the growth rate fell abruptly (cf. methanol inhibition).

Growth on formaldehyde and formate. Formaldehyde vapour or sodium formate (sterilized by filtration and tested at 0.1, 0.5, 1, 5, 10 and 100 mM) did not support the growth of *Methylococcus capsulatus* in Petri dish culture in the absence of methane. No mutant colonies capable of using these compounds as sources of carbon and energy in place of methane

Fig. 3. Incorporation of [U-¹⁴C]glucose and [U-¹⁴C]sucrose by growing cultures of *Methylococcus capsulatus*.

were detected after 6 weeks of incubation. Growth was not inhibited by formaldehyde vapour or by sodium formate (0.1 to 10 mM) when methane was supplied, but 100 mM-sodium formate prevented growth. Liquid cultures of *M. capsulatus* growing exponentially on methane were unaffected by the addition of 10 mM-sodium formate, but 50 mM-sodium formate completely prevented further growth.

Effect of some sugars on growth. The growth rate of exponentially growing liquid cultures of *Methylococcus capsulatus* was not significantly inhibited by the addition of any one of the following sugars (at 0.1 M): D(+)galactose, D(+)glucose, D(−)fructose, maltose, sucrose, or by 0.05 M-lactose. Glucose prolonged the lag when added to freshly inoculated cultures, but no growth occurred within 65 h when lactose (0.05 M), sucrose or galactose (0.01 M) were present in lag phase cultures (experiment performed by E. Y. T. Choi).

Assimilation of [¹⁴C]methanol. [¹⁴C]Methanol (0.1 mM) was rapidly metabolized by logarithmically growing cultures, about half of the added methanol-carbon being incorporated in the organisms (Fig. 2). Distribution of ¹⁴C among the fractions extracted from *Methylococcus capsulatus* grown with [¹⁴C]methanol + [¹²C]methane or with [¹⁴C]methane was essentially identical (Table 1), as was the labelling distribution among the protein amino acids (Table 2).

Assimilation of [¹⁴C]formaldehyde. About 30% of the [¹⁴C]formaldehyde (0.1 mM) added to growing cultures was rapidly incorporated into the organisms (Fig. 2). Formaldehyde-carbon appeared in all bacterial fractions, but its distribution was significantly different from that of [¹⁴C]methane or methanol (Table 1), with reduced labelling in lipid (70% ethanol) and protein fractions and raised labelling in nucleic acids (hot 5% TCA). Distribution of ¹⁴C among the protein amino acids was similar with [¹⁴C]formaldehyde, [¹⁴C]methane and [¹⁴C]methanol (Table 2), but labelling in serine + glycine relative to the other amino acids was about 41% higher after [¹⁴C]formaldehyde assimilation.

Assimilation of [2-¹⁴C]ethanol and [U-¹⁴C]acetate. [¹⁴C]Ethanol (0.124 mM) was incorporated by exponentially growing *Methylococcus capsulatus* at a rate of 325 nmol/mg dry wt of newly synthesized bacteria (i.e. about 1.6% of cell carbon supplied by ethanol). About 91% of the ethanol-carbon was assimilated (Fig. 2). [¹⁴C]Acetate (0.009 mM, 0.5 μ Ci/ml) was also incorporated by growing organisms; about 0.2 μ Ci/ml being assimilated in a 1 h incubation. Distribution within the organisms of ¹⁴C from acetate and ethanol was virtually identical, but differed markedly from that using [¹⁴C]methane (Table 1). In both cases most ¹⁴C was in the 70% ethanol-soluble lipid fraction with less in protein. Labelling in the protein was restricted to four amino acids: glutamate, proline, arginine and leucine (Table 2).

Assimilation of [¹⁴C]formate and [¹⁴C]carbonate. Growing cultures were exposed to small amounts of sodium [¹⁴C]formate (0.018 mM, 1 μ Ci/ml) or Na₂ ¹⁴CO₃ (0.036 mM, 2 μ Ci/ml) but no quantitative estimate of molar incorporation was possible because of the unknown and changing amounts of carbon dioxide and possibly formate within the culture vessels. Carbon from both materials was incorporated by *Methylococcus capsulatus*; total incorporation in 1 h was in each case about 0.015 μ Ci/ml. Relative to the labelling from [¹⁴C]methane, less ¹⁴C from formate and carbonate appeared in lipid but labelling in the nucleic acid and protein fractions was similar (Table 1). Formate-carbon in the protein fractions appeared to have been preferentially incorporated into glycine + serine, tyrosine and methionine + valine (Table 2). Carbonate-carbon appeared in all the amino acids, but except for proline and threonine tended to show a distribution pattern somewhat different from the normal carbon distribution obtained using [¹⁴C]methane (Table 2).

Assimilation of [U-¹⁴C]glucose and [U-¹⁴C]sucrose. Exponentially growing cultures were unable to incorporate large amounts of [¹⁴C]glucose (0.1 mM) or [¹⁴C]sucrose (0.1 mM) (Fig. 3). Less than 1% of the sugar added was assimilated.

DISCUSSION

Other workers have reported that *Methylococcus capsulatus* can grow on methanol as well as on methane (Foster & Davis, 1966; Whittenbury *et al.* 1970; Patel & Hoare, 1971) and can oxidize C₂ to C₅ chain length primary alcohols to aldehydes (Patel & Hoare, 1971; Davey *et al.* 1972) by means of a single primary alcohol dehydrogenase (Patel *et al.* 1972). Our results suggest that not all the members of a *M. capsulatus* population can grow on methanol and that longer chain alcohols, which do not support growth, may in fact be inhibitory. Whittenbury *et al.* (1970) found many methylotrophic isolates to be inhibited by methanol even at 0.01% (v/v), and were unable to grow *M. capsulatus* in liquid medium with 0.1% methanol. Our finding that only about 1 organism in 7×10^8 can grow readily on methanol as the sole carbon and energy source suggests that previous reports of growth on methanol have resulted from selection of variants within the inoculum population. Such variants might be better adapted to use exogenous methanol or might be organisms tolerating higher concentration of methanol than the majority of the population. Immediate inhibitory effects of methanol on cultures growing on methane became apparent at concentrations above 1% (v/v) and it should be noted that even heterotrophic organisms capable of growing on methanol may be inhibited by 1.5 to 5% (v/v) methanol (Shulgovskaya, Andreeva & Rabotnova, 1971).

Inhibition of growth on methane by C₂ to C₅ alcohols occurred at concentrations somewhat lower than those causing inhibition by methanol. As the inhibitory effects of ethanol, propanol and butanol became immediately apparent it seems possible that the alcohols themselves are toxic agents, although the rapid metabolic production of aldehydes from them might also account for their toxicity.

Long-term (1 to 7 h) incorporation of ^{14}C -labelled C_1 and C_2 compounds during growth has provided new information on intermediary metabolism in *Methylococcus capsulatus*. Previous work on short-term labelling with ^{14}C -labelled methane, methanol and formaldehyde and on cell-free extracts indicated the occurrence of a formaldehyde-fixing ribose phosphate cycle rather than the Calvin CO_2 -fixing cycle in *M. capsulatus* and *Pseudomonas* ('*Methylo-**monas*') *methanica* (Johnson & Quayle, 1965; Lawrence *et al.* 1970; Quayle, 1972). By this process the main flow of carbon into intermediary metabolism would proceed through formaldehyde generated from methane or methanol. The great similarity of gross and amino acid labelling patterns after assimilation of $[^{14}\text{C}]$ methane or $[^{14}\text{C}]$ methanol indicates that these are incorporated by a common pathway, each contributing to all the cellular constituents. Methanol was very rapidly metabolized (Fig. 2) and nearly half of that added to the culture was assimilated. The remainder was presumed to be oxidized to carbon dioxide, but little of any $^{14}\text{CO}_2$ produced was subsequently assimilated. Although $[^{14}\text{C}]$ formaldehyde was similarly rapidly metabolized and about one-third assimilated, the distribution of ^{14}C in the organisms was not exactly like that for methane or methanol (Tables 1 and 2). Less ^{14}C appeared in lipid and protein and more appeared in the hot TCA-soluble fraction. Further analysis of this fraction was inconclusive as more than half the ^{14}C in it was present as unidentified materials, probably derived from the polysaccharide capsule of *M. capsulatus*. The discrepancy between the observed labelling with $[^{14}\text{C}]$ formaldehyde and that expected (i.e. similar to $^{14}\text{CH}_4$) may perhaps be explained if additional mechanisms exist for incorporating formaldehyde when the organism is challenged by a sudden large supply of formaldehyde from the medium. The use of formaldehyde (and formate) specifically for purine synthesis is a possible point of entry, as occurs, for example, in *Escherichia coli* (Roberts, Abelson, Cowie, Bolton & Britten, 1955). Unless, however, there is discrimination in the organism between exogenous formaldehyde and that arising endogenously from methane, no significant change in labelling pattern should result. An alternative possibility is that *M. capsulatus* effects a significant proportion of its biosynthesis using carbon which leaves the methane oxidation pathway at a level more reduced than formaldehyde. Raised labelling of glycine + serine after $[^{14}\text{C}]$ formaldehyde assimilation (Table 2) is also consistent with a specific mechanism for incorporating C_1 -units from formaldehyde. Oxidation of $[^{14}\text{C}]$ formaldehyde to $[^{14}\text{C}]$ formate and subsequent incorporation of the formate explains this finding, as added $[^{14}\text{C}]$ formate contributed very heavily to serine + glycine in the protein (Table 2). Formate-carbon also contributed disproportionately to tyrosine and methionine + valine. Although ^{14}C from formate and carbonate appeared in all the cellular fractions and all the amino acids resolved, the quantitative importance of them as sources of carbon was not assessed because of the high and increasing level of carbon dioxide in the flasks. Raised labelling of aspartate from $[^{14}\text{C}]$ formate or carbonate relative to the other ^{14}C substrates suggests the operation of a $\text{C}_3 + \text{CO}_2$ carboxylation for a large part of their assimilation (Table 2). This contrasts with formate assimilation by the autotrophs *Thiobacillus neapolitanus* and *Chlorobium thiosulfatophilum* (Kelly, 1970, 1971) in which the bulk of ^{14}C from formate appeared in purines. From these observations and the failure of *M. capsulatus* to grow on formate it is clear that, unlike *Pseudomonas oxalaticus* (Quayle & Keech, 1959), *Hydrogenomonas* (Namsaraev, Nozhevnikova & Zavarzin, 1971) and *Rhodospseudomonas* (Stokes & Hoare, 1969), *M. capsulatus*, like other methylotrophs and C_1 -utilizing pseudomonads, is unable to develop a Calvin cycle or any means of assimilating compounds less reduced than formaldehyde as a sole source of carbon (Johnson & Quayle, 1965; Kelly, 1971; Kirikova & Romanova, 1972; Quayle, 1972).

The incorporation of acetate- ^{14}C only into leucine, glutamate, proline and arginine (Table 2)

confirms the presence of the incomplete 'biosynthetic' tricarboxylic acid cycle reactions in *Methylococcus capsulatus* (Patel *et al.* 1969). The essentially similar pattern for incorporation of [¹⁴C]ethanol (Tables 1 and 2) indicates that at least 91 % of the added 0.124 mM-ethanol (Fig. 2) must have undergone oxidation to acetate for passage into the leucine and glutamate pathways. Patel & Hoare (1971) previously reported quantitative oxidation of ethanol to aldehyde by *M. capsulatus* and its failure to oxidize acetaldehyde. Organisms growing on methane are clearly able to carry out a more extensive metabolism of ethanol than has previously been supposed. The presence of an incomplete Krebs's cycle in *M. capsulatus* and other Type I methylotrophs can be no more than a contributory cause of obligate methylotrophy, as *Methanomonas methanooxidans* and other Type II organisms possess a complete cycle (Davey *et al.* 1972; Wadzinski & Ribbons, 1972).

Methylococcus capsulatus was more tolerant in plate culture to high levels of sugars than of amino acids, although M/18 glucose completely inhibited radial growth of colonies and M/90 glucose was selective for glucose tolerant organisms (Eroshin *et al.* 1968). In contrast, exponential growth of liquid cultures was not usually significantly affected by M/10 glucose or any other of the sugars tested. The extremely low level of incorporation of ¹⁴C-labelled glucose and sucrose suggests that *M. capsulatus* may be impaired in ability to accumulate or phosphorylate these sugars. Glucose and lactose toxicity to plate cultures might thus result from altered permeability in lag phase cultures or from a change in the physical environment caused by the sugars. It is noteworthy that *Methanomonas* lacks hexokinase and is thus unable to assimilate glucose (Amemiya, 1972).

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