

CARBON OXIDATION AND INCORPORATION PATTERNS

IN BATCH CULTURES OF *METHYLOMONAS* L3

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Summary A recently developed methodology of directly measuring the oxidation and incorporation patterns of carbon substrate in continuous cultures of RuMP-type methylotrophs is extended to batch cultures of the obligate methylotroph *Methylomonas* L3. The ratio of cyclic to total substrate oxidation varies with the initial methanol concentration from 0 to 68%. Formaldehyde, as a methanol cosubstrate, enhances the net substrate oxidation. The substrate oxidation and incorporation pattern is also affected by the state of the culture inoculum.

INTRODUCTION

In Ribulose Monophosphate(RuMP)-type methylotrophic bacteria, methanol or formaldehyde oxidation can be accomplished via either a linear (via formate) or a cyclic oxidation scheme (Anthony, 1982). In view of the different enzymes involved in each oxidation scheme, and since methylotrophs derive their biosynthetic energy through substrate oxidation, the degree of involvement of each oxidation scheme would affect the growth bioenergetics and in particular the biomass yield (Anthony, 1982; Papoutsakis and Lim, 1981). The extent of utilization of each oxidation scheme among the various RuMP-type methylotrophs, its cellular regulation, its possible dependence on the culture conditions and its correlation to *in vitro* enzyme activities are of considerable fundamental and practical importance. They have also been the subject of significant research effort and debate in the last few years (Anthony, 1982; Hirt et al, 1978; Ben-Bassat et al, 1980; Häggström and Dostálek, 1981; Samuelov and Goldberg, 1982; Zatman, 1981). A direct measurement of the oxidation pattern would be valuable in elucidating the bioenergetic aspects of methylotrophic growth and, in particular, in addressing the question on the degree of coupling between energy production and utilization.

Previous efforts to measure directly the substrate oxidation pattern in *Pseudomonas* C (Ben-Bassat et al, 1980; Samuelov and Goldberg, 1982) have been criticized (Anthony, 1982) because of the use of uniformly labeled ^{14}C -glucose as a tracer. This tracer overestimates, in a complex to measure quantitatively way, the extent of utilization of the linear oxidation scheme at the expense of the cyclic one (Anthony, 1982; Chu et al, 1984a; 1984b).

We have recently developed a procedure to measure directly the substrate oxidation and incorporation patterns in RuMP-type methylotrophs, under steady-state, chemostatic conditions, (Chu et al, 1984a; 1984b). The method employs ^{14}C -methanol, ^{14}C -formate and 1- ^{14}C -

glucose as tracers. Here, the procedure is applied to the exponential growth of batch cultures of *Methylobionas* L3 (Hirt et al, 1978), to measure directly the carbon oxidation and incorporation patterns under various growth conditions. Based on the low *in vitro* activities of the linear-oxidation enzymes (formate and formaldehyde dehydrogenases), and the high activities of cyclic-oxidation enzymes, Zatman (1981) has concluded that RuMP-type methylotrophs predominantly employ the cyclic scheme of substrate oxidation. The continuous-culture experiments (Chu et al, 1984a; 1984b) and the present batch experiments will demonstrate that in *Methylobionas* L3, the cyclic oxidation scheme is not predominantly used and that, in fact, the extent of utilization of each oxidation scheme varies dramatically with culture conditions.

MATERIALS AND METHODS

The growth media and conditions for the batch cultures of *Methylobionas* L3 on methanol at 30°C have been described previously (Hirt et al, 1978). The microorganism was grown on various methanol and formaldehyde concentrations. The analyses of methanol and formaldehyde, the dry-weight determination, and the measurement of biomass yields have been described earlier (Hirt et al, 1978). All chemicals employed were of reagent grade. Radiochemicals were purchased either from ICN Pharmaceuticals (Irvine, CA) or Amersham (Arlington Heights, IL).

M.L3 cells were grown in batch in 2 l flasks with 400 ml media at various initial methanol and formaldehyde concentrations. When the cultures reached mid-exponential phase, three portions of 50 ml culture suspension were withdrawn aseptically and put into three 250 ml flasks. Each of the three labeled tracers was then injected to one of the three 250 ml flasks. After one hour, growth was terminated by injecting 3 ml 100% w/v TCA. Sterile air was passed through each culture, via ports in the stopper of the 250 ml flasks, during the entire run. The procedures for CO₂ trapping, biomass harvesting and subsequent treatments, and liquid-scintillation counting were similar to those employed for the chemostatic experiments (Chu et al, 1984a; 1984b).

THEORY

The reactions for substrate oxidation and incorporation into biomass, as well as the general decarboxylation and carboxylation reactions associated with biosynthesis (Anthony, 1982) can be condensed into a diagram as in Fig. 1 (Ben-Bassat et al, 1980; Chu et al, 1984a; 1984b). The r_i 's in Fig. 1 represent the reaction rates of the various carbon flows in the metabolism and have units of C-mole per unit time and either per unit dry-biomass weight or per unit reactor volume. From steady-state mass balances then we obtain

$$r_1 = r_2 + r_3 = r_7 + r_8 \quad , \quad r_3 = r_4 + r_5 \quad (1),(2)$$

$$r_7 = r_5 + r_6 \quad , \quad r_2 + r_4 = r_6 + r_8 \quad (3),(4).$$

Since r_1 (the rate of CH₃OH uptake) can be directly measured, three more equations are needed to determine all the r_i 's. Because of the three branch points in the metabolic pathway (at formaldehyde, CO₂/bicarbonate and 6-phosphogluconate (GPG)), three radioactively labeled compounds should be used as tracers to provide the needed information. As we elaborate elsewhere (Chu et al, 1984a; 1984b) these tracers are ¹⁴CH₃OH,

¹⁴C-formate (or ¹⁴C-bicarbonate) and an ideally labeled ¹⁴C-glucose. Labeled glucose is used to trace the incorporation of methanol or formaldehyde into biomass and their oxidation to CO₂ via the cyclic scheme and the general decarboxylation reactions. Because of the complex rearrangement reactions of the metabolic schemes of the RuMP-type methylotrophs, the ideally labeled glucose must have a certain distribution of ¹⁴C-atoms among its first, second and third carbons, which, although can be precisely determined, is not practically possible (Chu et al, 1984a; 1984b). If U-¹⁴C-glucose is used, the usual interpretation of the results leads to an overestimation of the extent of utilization of the linear oxidation scheme at the expense of the cyclic scheme (Anthony, 1982; Chu et al, 1984a; 1984b). A quantitative interpretation of the results employing U-¹⁴C-glucose is not possible. On the other hand, if 1-¹⁴C-glucose is employed as a tracer, the results obtained are amenable to accurate quantitative treatment (Chu et al, 1984a; 1984b). Let A, B, C and β be the ratios (called the *Q ratios*) of radioactivity incorporated into biomass over the total radioactivity in biomass and CO₂/bicarbonates when ¹⁴C-methanol, ideally labeled ¹⁴C-glucose, ¹⁴C-formate and 1-¹⁴C-glucose are used as tracers, respectively. Then, the rates (or ratios of rates) of Fig. 1 are obtained from the following system of equations (Chu et al, 1984a; 1984b)

$$r_2/r_1 = (B-A)(B-C)^{-1} \quad , \quad r_3/r_1 = (A-C)(B-C)^{-1} \quad (5),(6)$$

$$r_4/r_1 = (A-C)(1-B)(B-C)^{-1}(1-C)^{-1} \quad (7)$$

$$r_5/r_1 = (A-C)(1-C)^{-1} \quad , \quad r_6/r_1 = C(1-A)(1-C)^{-1} \quad (8),(9)$$

$$r_7/r_1 = A \quad , \quad r_8/r_1 = 1-A \quad (10),(11)$$

$$r_9/r_1 = r_4/r_1 - 0.2 r_5/r_1 \quad , \quad B = B' + (1-B')C \quad (12),(13)$$

$$\beta = \beta' + (1-\beta')C \quad , \quad B' = 4.17 \beta'(1+3.33\beta')^{-1} \quad (14),(15).$$

The experimentally measurable quantities are A, C, β and r₁. As we detail elsewhere (Chu et al, 1984a; 1984b), the essential assumptions necessary for the above quantitative interpretation of the results have been amply tested for *M.L3* and are expected to be valid for most RuMP-type methylotrophs. These assumptions have as follows. First, we assume that formate is only oxidized to CO₂ and is not reduced to formaldehyde. Second, we assume that glucose-6-P (which results from the phosphorylation of glucose as the first step of its utilization by the microorganisms) is assimilated by the KDPG/TA (2-keto-3-deoxy-6-phosphogluconate/transaldolase) variant of the RuMP cycle. Thirdly, we assume that glucose-6-P is oxidized to CO₂ via glucose-6-P dehydrogenase and 6PG dehydrogenase, as well as the general decarboxylation enzymes. Finally, we assume that approximately (0.8 ± 0.2) moles of CO₂ are produced from general decarboxylation reactions per 4 moles CH₃OH incorporated into biomass and that (80 ± 20)% of the carbon of these decarboxylation reactions originates at position 1 of the glucose-6-P molecule. The overall (experimental and computational) accuracy of the results obtained via this procedure is ± 5%.

The above analysis can be applied, in an average sense, to investigate the substrate oxidation and carbon-incorporation patterns of cultures of non-steady-state growth, as well. This would require that no

severe transients occur, that the different residence times of the gas and liquid phases are taken into account in continuous reactors, and that radioactivity is collected over a reasonably short time for a meaningful averaging. So long that no severe transients occur, the pseudo-steady-state hypothesis for the key intermediates can be argued to be valid, such that eqs. (1) through (4), and those derived there from are valid. The pseudo-steady-state hypothesis for the various intermediates of interest (formaldehyde, 6PG, etc.) states that the rate of accumulation of each intermediate is very small compared to the various rates of reactions by which it is produced and consumed. Simple calculations (see, e.g., Attwood and Quayle (1984)) can readily demonstrate the validity of the hypothesis, even when small amounts of the intermediate accumulate in the reactor. Application of the procedure during mid-exponential batch growth as described in Materials and Methods does satisfy the necessary conditions, and should give, therefore, meaningful results. These results must be interpreted in an average, over the experiment time, sense, despite the widely accepted belief that mid-exponential growth is *unrestricted* growth, whereby the key metabolic reactions operate at constant rates.

RESULTS AND DISCUSSION

A number of batch experiments were performed to investigate the effect of initial methanol and formaldehyde concentrations on the oxidation and metabolic patterns of carbon substrate in *M.L3*. The Q ratios obtained with the three tracers (A for ^{14}C -methanol, C for ^{14}C -formate and β for $1\text{-}^{14}\text{C}$ -glucose), the rates of Fig. 1 as calculated from the Q ratios according to eqs. (5)-(15), the initial methanol ($(\text{MeOH})_0$) and formaldehyde ($(\text{CH}_2\text{O})_0$) concentrations, the specific growth rate (μ) and the biomass yield (Y_s , g biomass per g substrate(s) utilized) for five experiments are shown in Table 1. The biomass yields shown in Table 1 are the average yields calculated over the 1-hour period of the tracer experiments. Biomass yields over the entire batch runs were typically lower.

$2\text{-}^{14}\text{C}$ -glucose was used as a tracer in a similar batch experiment to check the validity of the assumption that $80 \pm 20\%$ of general decarboxylation involves carbons originating from the 1-C position of pyruvate. Less than 300 dpm radioactivity appears in the carbon dioxide fraction per 1 million dpm radioactivity of the tracer employed. A proper blank experiment without the cells is needed to eliminate radioactivity coming from the impurities in the labeled compound in this case. After the correction made by the blank experiment, the Q ratio for $2\text{-}^{14}\text{C}$ glucose was found to fall between 0.92 and 1.0. Assuming that the 3-C is decarboxylated to the same extent as the 2-C, it can be calculated that 70 to 100% of the general decarboxylation involves 1-C. Therefore, our assumption (which was based on a detailed accounting of all the general decarboxylation reactions (Chu et al., 1984b)) is valid.

As the data of Table 1 indicate, the extent of cyclic substrate oxidation varies dramatically with the growth conditions from 0 to 68% of the total substrate oxidized. Thus, the ratio of cyclic to linear oxidation (r_9/r_2) is the lowest at the lowest $(\text{CH}_3\text{OH})_0$, and highest (1.32) at an intermediate $(\text{CH}_3\text{OH})_0$ value, for growth on methanol alone. A comparison of experiments 2 and 3 shows that the oxidation and incorporation pattern depends not only on the initial substrate concentration but also on the specific growth rate and Y_s values. The latter two are considerably affected by the state of the inoculum. Thus,

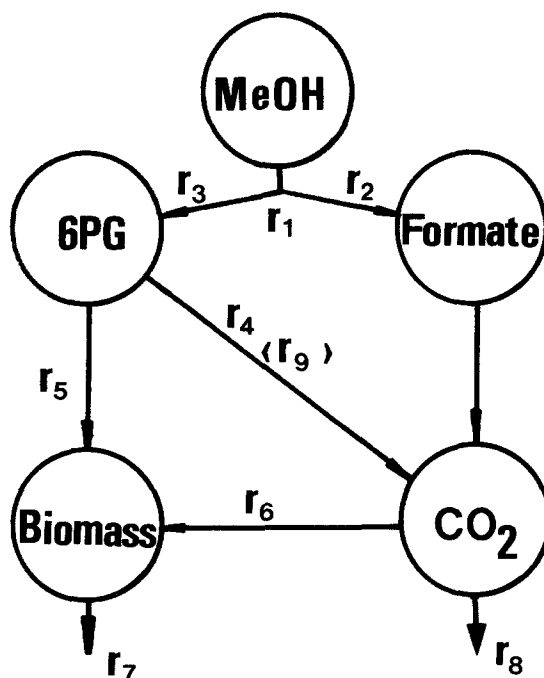


Figure 1

Carbon flow diagram; for legend see overleaf.

TABLE 1: Oxidation and incorporation patterns of carbon substrate in batch cultures of *M.L3*. For symbol definitions see text and Fig. 1. The numbers for the normalized rates (r_i/r_1) have been rounded to the second decimal figure.

Experiment	1	2	3	4	5
(MeOH) ₀ , g/l	0.63	2.38	2.38	14.45	2.80
(CH ₂ O) ₀ , g/l	0	0	0	0	0.07
μ , h ⁻¹	0.19	0.29	0.50	0.17	0.18
Y_S (g/g)	0.44	0.45	0.50	0.15	0.35
A	0.506	0.575	0.681	0.167	0.375
C	0.026	0.095	0.158	0.054	0.084
β	0.678	0.370	0.505	0.423	0.204
r_2/r_1	0.43	0.16	0.14	0.83	0.20
r_3/r_1	0.57	0.84	0.86	0.17	0.80
r_4/r_1	0.08	0.31	0.24	0.05	0.48
r_5/r_1	0.49	0.53	0.62	0.12	0.33
r_6/r_1	0.01	0.05	0.06	0.05	0.05
r_7/r_1	0.51	0.58	0.68	0.17	0.38
r_8/r_1	0.49	0.42	0.32	0.83	0.63
r_9/r_1	ca. 0.0	0.21	0.11	0.03	0.41
r_9/r_2	ca. 0.0	1.32	0.80	0.03	2.10

cells exercised prior to inoculation by a few consecutive fast transfers usually result in higher biomass yields and specific growth rates. At a higher $(\text{CH}_3\text{OH})_0$, the ratio r_9/r_2 drops again (exper. 4). The primary effect of the initial CH_2O presence in the medium is to slow the growth, enhance the total and cyclic substrate oxidation, and thus reduce the biomass yield (exper. 5). The results of Table 1 indicate that the extent of utilization of the cyclic oxidation scheme does not correlate with either μ or Y_S independently of the initial substrate concentration. The oxidation and incorporation pattern of carbon substrate for the lowest $(\text{CH}_3\text{OH})_0$ (exper. 1) is quite similar to the patterns obtained in continuous cultures (Chu et al, 1984a; 1984b), whereby cyclic oxidation plays an insignificant role in substrate oxidation. Continuous cultures are characterized by very low (0 - 20 mg/l) residual methanol concentrations.

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Figure 1. Carbon flow diagram in ML3. r_1 , rate of methanol oxidation; r_2 , rate of formaldehyde oxidation to formate; r_3 , rate of formaldehyde condensation via the hexulose phosphate synthase; r_4 , combined rate of CO_2 production via 6PG dehydrogenase and via other, general decarboxylation enzymes; r_5 , rate of carbon incorporation into biomass excluding r_6 ; r_6 , sum of rates of general carboxylation; r_7 , rate of appearance of methanol carbon in collected biomass; r_8 , rate of accumulation of trapped CO_2 ; r_9 , rate of cyclic formaldehyde oxidation.