ASPECTS OF THE REGULATION OF METHYLOTROPHIC METABOLISM

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1. Introduction

The ability of many organisms to synthesize all their cell constituents from C_1-compounds at levels of oxidation ranging from methane to carbon dioxide has long fascinated plant and microbial biochemists. Since autotrophic fixation of CO_2 by plants and bacteria has been intensively studied from the earliest times, it is understandable that the first suggestions as to the way in which microorganisms might assimilate reduced C_1-growth substrates tended to centre around an autotrophic mode, i.e. prior oxidation to CO_2 followed by CO_2 assimilation [1–3]. Researches over the last thirty years have shown that while this is true in some cases, there are now five other routes of net C_1-assimilation recognised in which four, at least, involve a major portion of carbon being incorporated at reduction levels higher than that of CO_2 [4]. This research activity has been regularly and comprehensively reviewed [4–12] and it would be both impossible and unnecessary to review all this ground in an article of the present size. Past work has mainly concentrated on elucidation of the basic biochemistry of the assimilation and dissimilation sequences, some of which are remarkably intricate; however, there has been much less attention paid to the regulation of these pathways and it is in this direction that we are now seeing, and can expect to see in the future, more research effort. In this regard the techniques of continuous culture and molecular genetics will play key roles.

It is the purpose of this article to highlight some features which, in my view, present particularly interesting problems of control and it is in the nature of the present state of the art that problems rather than solutions will be presented. My selection of topics and literature throughout is selective rather than comprehensive.

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

2. Energy generation from reduced C_1-compounds

2.1. *Anaerobic*

All methanogenic bacteria catalyse the following reaction:

$$4\ H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3\ H_2O;$$

$$\Delta G'_o = -32.4\ \text{kcal/reaction} \qquad (1)$$

from whence they derive their energy. The mechanism of this reaction, let alone its regulation, has not been fully elucidated [11]. In addition, some methanogens can convert methanol, formate or carbon monoxide to methane by processes that are again not fully understood. Ability to grow anaerobically in the light on methanol or formate is found in representatives of seven species of Rhodospirillaceae [13,14]. The dehydrogenation of the carbon substrate follows a linear sequence similar to that encountered in many aerobic organisms, via formaldehyde and formate to carbon dioxide [15].

Non-photosynthetic organisms which are able to grow on a C_1-substrate anaerobically by using an inorganic electron acceptor, e.g., *Paracoccus denitrificans* on methanol/nitrate or *Hyphomicrobium* X on methanol or methylated amines/nitrate, do so by a similar linear sequence involving formaldehyde and formate as intermediary metabolites [16–18].

2.2. *Aerobic*

2.2.1. Oxidation of methane to methanol

The biochemistry of this transformation which is carried out by aerobic methanotrophs has recently

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been penetrated to the enzyme level principally by research groups at Canterbury and Warwick [11,12]. The enzyme systems responsible in *Methylosinus trichosporium* 0b3b and *Methylococcus capsulatus* (Bath) are monooxygenases catalysing the reaction:

$$CH_4 + O_2 + NAD(P)H_2 \rightarrow$$

$$CH_3OH + NAD(P) + H_2O \quad (2)$$

Both enzyme systems consist of three component proteins which apparently differ in some respects between the two microbial species. Initially it was thought that the two systems further differed in that the complex from *M. trichosporium* was particulate, and its reducing agent was a CO-binding cytochrome *c* [11]. This compared with the enzyme complex from *M. capsulatus* which was soluble and whose reducing agent was $NAD(P)H_2$ [12]. A detailed comparison of the two enzyme systems by Stirling et al. [19] has now shown that these differences may not be fundamental since the complex purified from *M. trichosporium* in the Warwick laboratories, in contrast to that initially purified in the Canterbury laboratories, was soluble and reduced by $NAD(P)H_2$; furthermore, cross-reactivity occurred between two of the components of the enzyme complex from *M. capsulatus* and one of the components from that of *M. trichosporium*, indicating close functional similarity. It now seems likely that different growth and storage conditions of *M. trichosporium* may affect the characteristics of the enzyme complex and hence explain the apparently different results from the two laboratories.

One of the most remarkable properties of methane oxygenases is their quite astonishing versatility with respect to substrate: alkanes, alkenes, ethers, carbon monoxide, ammonia, alicyclic, aromatic and heterocyclic compounds have been reported to be oxygenated [20,21]. It is not known whether this lack of specificity is a fortuitous result of the active site chemistry of the enzyme and/or whether it has some physiological significance for methanotrophs in their natural habitat.

Most of the well-characterised methanotrophs are obligate methane- or methanol-utilizers and hence study of the regulation of the methane oxygenase system in such organisms has necessarily been limited. However, two facultative methanotrophs have now been isolated, viz., *Methylobacterium organophilum* XX [22,23] and *Methylobacterium* R6 [24]. Study of *M. organophilum* by Hanson and his group has shown that formation of a complex intracytoplasmic membrane system, probably involved in the oxidative process, is repressed by substrates other than methane [22], is high during growth under O_2-limiting conditions but decreases at higher O_2 tensions [25]. Apart from these preliminary findings little is known as to how methanotrophs regulate the flow of carbon from methane to methanol.

2.2.2. Oxidation of methanol to formaldehyde

As far as is known, all methylotrophic bacteria accomplish the oxidation of methanol to formaldehyde by way of a methanol dehydrogenase first discovered by Anthony and Zatman [26]. Many such enzymes have now been purified and in general are characterised by low substrate specificity towards primary alcohols, requirement for high pH, coupling to phenazine methosulphate in the presence of ammonia or a primary amine as activator. All the enzymes possess a prosthetic group which Anthony and Zatman [27] suggested might be a pteridine derivative. The properties of this enzyme have been extensively described but it is only recently that decisive steps have been taken in its detailed biochemistry. It has now been shown [28–30] that the prosthetic group is an entirely novel coenzyme form of a nitrogen-containing orthoquinone of structure shown in fig.1. and given the trivial name of methoxatin. The coenzyme functions as an electron carrier and a functional coupling to cytochrome *c* has been demonstrated with the enzyme from *Hyphomicrobium* [31].

All methanol dehydrogenases tested so far are able to oxidize formaldehyde to formate, probably due to the fact that formaldehyde exists in aqueous solution

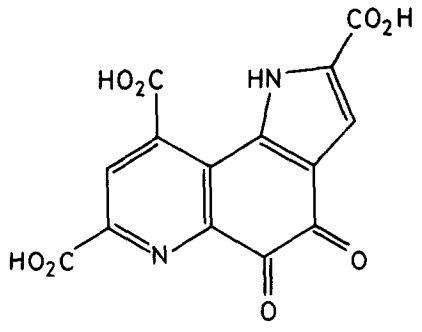


Fig.1. Structure of methoxatin, the coenzyme of methanol dehydrogenase.

largely as its hydrated form, $CH_2(OH)_2$, and hence similar to the structure of methanol [32]. The question of the possible physiological significance of this dual substrate specificity will be returned to later.

The methanol dehydrogenase described above is confined to bacteria. In methylotrophic yeasts, oxidation of methanol is effected within peroxisomes by methanol oxidase, the formation of which is induced during growth on methanol [33–35]:

$$CH_3OH + O_2 \rightarrow HCHO + H_2O_2 \quad (3)$$

The bulk of the formaldehyde is exported to the cytoplasm where it is oxidised to CO_2 by NAD-linked dehydrogenases (see later) although under some conditions some formaldehyde may be oxidised to formate in the peroxisomes either by the methanol oxidase itself, which possesses dual substrate specificity [36], or by the reaction of hydrogen peroxide and catalase, also present in the peroxisomes [37]. The regulation of the synthesis of C_1-catabolic enzymes in yeast will be returned to later.

2.2.3. Oxidation of methyl amines to formaldehyde

A wide variety of enzyme systems has been found in different organisms which oxidize *N*-methyl groups to formaldehyde [4–12]. Dehydrogenases, oxygenases and oxidases are encountered but in very few cases are pyridine nucleotide-linked dehydrogenases encountered. Many yeasts are able to utilize methylamine as nitrogen source by oxidising it to formaldehyde and ammonia with a methylamine oxidase synthesized within the peroxisomes [38].

2.2.4. Oxidation of formaldehyde to formate

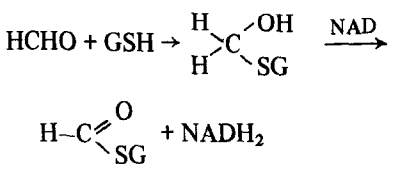
The further oxidation of formaldehyde to formate may take place in several different ways (see [39–41] for current reviews):

(i) Further dehydrogenation or oxidation by enzyme systems already producing formaldehyde from methanol, e.g., methanol dehydrogenase, methanol oxidase

(ii) Dehydrogenation by NAD(P)-independent aldehyde dehydrogenases, often at apparently low specific activity when measured using artificial electron acceptors e.g. the dye-linked aldehyde dehydrogenase of *Pseudomonas* AMI [42]

(iii) Dehydrogenation by NAD(P)-dependent dehydrogenases, e.g., from *Bacterium* 4B6 [43]

(iv) Dehydrogenation by NAD(P)-,glutathione-dependent dehydrogenases. In such cases the reaction probably proceeds via a thiohemiacetal derivative as in the dehydrogenation of *S*-hydroxymethylglutathione to *S*-formylglutathione in methylotrophic yeasts [44,45]:



(4)

(v) Total oxidation of formaldehyde to CO_2 by a cyclic mechanism in organisms using the ribulose monophosphate (RuMP) cycle of formaldehyde fixation [4].

In an intact cell, metabolic pathways are split into component reactions each of which is normally catalysed by a discrete enzyme. It is most uncommon to find two successive reactions catalysed by the same enzyme, particularly if the intermediary product is itself an important metabolite with other metabolic connections. The regulatory difficulties posed in such a situation are obvious. It is thus interesting to find that this possibility exists in the oxidation of methanol to formaldehyde and thence to formate in the case of bacterial methanol dehydrogenase and yeast methanol oxidase. Such a two-step oxidation can readily be demonstrated in cell-free extracts of appropriate organisms. To what extent does this reaction occur in vivo? In organisms which grow autotrophically on reduced C_1-compounds such a question may seem almost academic because formaldehyde is but an intermediary catabolite during the complete oxidation of the growth substrate to CO_2; however, it is interesting that all species of bacteria which are presently known to utilize reduced C_1-compounds autotrophically also possess NAD(P)-linked formaldehyde dehydrogenases [46]. A small amount of formaldehyde may be abstracted in the form of C_1-folate for use as an 'active C_1-unit' in biosynthesis; this need only be a small proportion of the total C_1-flux. However, in organisms which grow non-autotrophically on reduced C_1-compounds, formaldehyde stands at a major cross-road of metabolism. A major part of it must be assimilated into cell constituents at the level of formaldehyde and only the remainder further oxidised to provide additional energy (and some formate for biosynthetic use as formyl tetrahydrofolate). In such cases the question

of the dual substrate specificity of the methanol dehydrogenase/oxidase is far from academic.

Methylotrophic yeasts present a particularly interesting problem because of the presence of both methanol oxidase and catalase within peroxisomes. Thus methanol may be oxidized to formaldehyde by methanol oxidase by reaction (3), or by catalase itself making use of the peroxide generated in reaction (3) [37]:

$$CH_3OH + H_2O_2 \rightarrow HCHO + 2\ H_2O \qquad (5)$$

Furthermore, formaldehyde can be oxidised to formate both by methanol oxidase [36] and catalase [37] in an exactly analogous pair of reactions to (3) and (5), respectively. From considerations of K_m-values of the enzymes concerned, the molar growth yields of yeasts grown on methanol and the behaviour of mutants, it has been concluded [35,36] that little formaldehyde is oxidized in vivo in the peroxisomes by reactions analogous to (3) and (5). Instead, most of the formaldehyde generated in the peroxisome is excreted into the cytoplasm, converted into *S*-hydroxymethylglutathione and dehydrogenated to *S*-formylglutathione, as in reaction (4). In this respect glutathione serves as a trap for the formaldehyde and renders it inert to further oxidation by methanol oxidase or catalase. The implications of this with respect to assimilation of formaldehyde will be discussed later. Kinetic studies of formaldehyde dehydrogenase from methanol-grown *C. boidinii* have indicated that the regulation of the activity of the enzyme may be effected in vivo by NADH and ATP, both of which are inhibitors of the enzyme [41].

In methylotrophic bacteria the situation is far from clear and in view of the variety of formaldehyde-oxidising enzymes each organism has to be examined individually in the light of its individual enzyme profile. In bacteria which possess defined formaldehyde dehydrogenases at high specific activity there would seem to be no need for the methanol dehydrogenase to carry out oxidation of formaldehyde to formate (to what extent it actually does do so in vivo is difficult to determine). There are however, some bacteria, e.g., *Pseudomonas* AMI, *Pseudomonas methylica* and *Hyphomicrobium* X where the only direct formaldehyde-oxidising enzyme which has been detected is a non-specific dye-linked aldehyde dehydrogenase at low specific activity [42,47]. It has been assumed previously that this dehydrogenase is probably involved in formaldehyde oxidation by *Pseudomonas* AMI [7,42]. The fact that a mutant (M15A) of *Pseudomonas* AMI which lacked methanol dehydrogenase was still able to grow unimpaired on methylamine and to oxidize formaldehyde proved that methanol dehydrogenase was not necessary for oxidation of formaldehyde by this organism [48]. The dye-linked formaldehyde dehydrogenase was present in extracts of the methylamine-grown mutant; bearing in mind that methylamine is oxidised via formaldehyde in *Pseudomonas* AMI by a methylamine dehydrogenase [49], the simplest conclusion was to ascribe a formaldehyde-oxidising function to the dye-linked formaldehyde dehydrogenase. However, Marison and Attwood [47] have recently surveyed the occurrence of this enzyme in a range of methylotrophic bacteria grown on a number of different substrates and have purified the enzyme from methanol- and ethanol-grown *Hyphomicrobium* X; their conclusion from this survey is that the dye-linked formaldehyde dehydrogenase is unlikely to play a major role in the oxidation of formaldehyde during the dissimilation of C_1 compounds. If this is so the route of formaldehyde oxidation in organisms such as *Pseudomonas* AMI remains an open question. A further possibility suggested by Johnson and Quayle [42] is that the formaldehyde is oxidised by a sequence of tetrahydrofolate (THF)-linked enzymes:

Formaldehyde + THF $\rightarrow$ $N^{5,10}$-methylene THF (6)

$N^{5,10}$-Methylene THF + NADP $\rightarrow$ $N^{5,10}$-methenyl THF + $NADPH_2$ (7)

$N^{5,10}$-Methenyl THF + $H_2O \rightarrow N^{10}$-formyl THF (8)

N^{10}-Formyl THF + ADP + $P_i \rightarrow$ THF + formate + ATP (9)

Sum: Formaldehyde + NADP + ADP + $P_i \rightarrow$ formate + $NADPH_2$ + ATP (10)

Reaction (6) can occur non-enzymatically and the presence of enzymes catalysing reactions (7–9) in extracts of methanol-grown *Pseudomonas* AMI have been demonstrated by Large and Quayle [50]. To what extent this scheme operates in vivo in organisms such as *Pseudomonas* AMI as a means of oxidising formaldehyde is not known. If it does operate, then methylene THF would stand at a metabolic cross-road between assimilation via the serine pathway and dissimilation. This point will be referred to later.

Total oxidation of formaldehyde to CO_2 by the cyclic sequence shown in fig.2 has been demonstrated in several bacteria operating the RuMP cycle [4,51–56]; the first two enzymes of the oxidation cycle, viz., hexulose phosphate (HuMP) synthase and phospho-hexuloisomerase, are common to the assimilatory RuMP cycle and hence such a mode of formaldehyde dissimilation is necessarily limited to organisms which possess that assimilatory cycle. The occurrence of this dissimilatory cycle is often accompanied by low or undetectable activities of formaldehyde and formate dehydrogenases. No striking modulation by small molecule effectors of the activity of purified HuMP synthase or phosphohexuloisomerase from several organisms has yet been reported. However, complex, non-Michaelis–Menten kinetics of HuMP synthase in crude extracts and permeabilized cells of *Pseudomonas oleovorans* and *Bacterium* MB 58, together with evi-

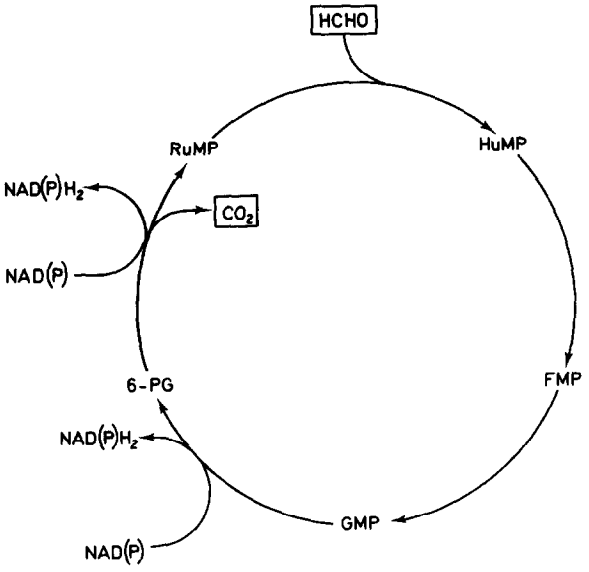


Fig.2. Cyclic sequence for oxidation of formaldehyde. *Abbreviations:* in text

dence for multiple, interconvertible forms of the purified enzyme from these organisms, suggest that it may be subject to complex regulation by, as yet undefined, sub-unit interactions [57–59]. It may be recalled that Ferenci et al. [60] noted that HuMP synthase from *M. capsulatus* dissociated under mild changes of pH and ionic strength from a species of mol. wt 310 000 to a species of mol. wt 49 000.

Reduced pyridine nucleotides and ATP have been shown to inhibit the activity of the pyridine nucleotide-linked glucose 6-phosphate (GMP) dehydrogenases of *Pseudomonas* W6 [61], *Methylomonas* M15 [62], *Methylophilus methylotrophus* [63] and *Pseudomonas* C [64]. Reduced pyridine nucleotides and ATP also inhibit the NADP- and NAD-linked 6-phospho-gluconate (6-PG) dehydrogenases of *M. methylotrophus* [4,63] and the single NADP/NAD-linked 6-PG dehydrogenase in *Pseudomonas* C [64].

In general, therefore, indicators of high energy status, viz., reduced pyridine nucleotides and ATP, inhibit the operation of the dissimilatory cycle of formaldehyde fixation, and this seems a logical control. A further control may be exerted at 6-PG in the case of organisms operating the Entner–Doudoroff cleavage variant of the RuMP cycle (see section 3.1.).

It may be noted that oxidation of methanol via the cyclic scheme shown in fig.2, in organisms such as *M. methylotrophus* which possess no detectable independent formaldehyde dehydrogenase and only low levels of formate dehydrogenase, means that the methanol is dehydrogenated only as far as formaldehyde and, despite the dual substrate specificity of methanol dehydrogenase with respect to both methanol and formaldehyde, the majority of the formaldehyde is transferred intact to HuMP synthase. The means whereby the transfer of this highly reactive molecule is effected within the milieu of an enzyme which can readily oxidize it further in vitro, is not known. It may depend upon juxtaposition of the two enzymes, perhaps in association with a membrane, and the shielding of formaldehyde from water to prevent its transformation from CH_2O into $CH_2(OH)_2$, the latter probably being the second substrate for the methanol dehydrogenase.

2.2.5. Oxidation of formate to carbon dioxide

Formate is oxidised to CO_2 in both methylotrophic yeasts and bacteria by NAD-linked formate dehydrogenases, there are, however, important differences between the eucaryotic and procaryotic systems.

In three species of methylotrophic yeast, viz., *H. polymorpha, C. boidinii* and *Pichia pinus* it has been found that the formate dehydrogenase catalyses the NAD-linked dehydrogenation of both formate and *S*-formylglutathione, but the K_m for the latter substrate was at least an order of magnitude lower than that for the former [44]. This suggested that *S*-formylglutathione is the natural substrate, this would of course be consistent with the properties of yeast formaldehyde dehydrogenase which is GSH-dependent and thus produces *S*-formylglutathione as product. Van Dijken et al. [44] made the further interesting observation that the 'formate dehydrogenase' appeared to catalyse the hydrolysis of the *S*-formylglutathione but only in the presence of NAD. This suggested that the dehydrogenation/hydrolysis of *S*-formylglutathione to CO_2 and glutathione occurred in two stages, with hydrolysis proceeding on the enzyme before dehydrogenation of the resulting enzyme-bound formate, the whole process being necessarily dependent on the presence of enzyme-bound NAD. In contrast to this, Kato et al. [65] have found an *S*-formylglutathione hydrolase in methanol-grown *Kloeckera* sp 2201 which was quite separate from the 'formate dehydrogenase' of the same organism.

The synthesis of four dissimilatory enzymes involved in methanol metabolism by yeasts, viz., methanol oxidase, catalase, formaldehyde and formate dehydrogenases, appears to be controlled by derepression rather than induction, as is the formation of peroxisomes [66].

Despite the apparent simplicity of the dehydrogenation of formate to CO_2 by bacteria, its enzymology is highly complex. In the case of methylotrophic bacteria, the formate dehydrogenases of two species have so far been studied in detail, viz., *P. oxalaticus* and a 'methylotrophic bacterium strain 1'.

P. oxalaticus, whose methylotrophic ability is limited to autotrophic growth on formate, possesses two formate oxidising systems: a particulate formate oxidase and a soluble NAD-linked formate dehydrogenase [67–70]. The function of the former enzyme is thought to be mainly respiratory in which electrons are fed into an electron transport chain, whereas the physiological function of the latter enzyme is thought to lie in the provision of NADH for biosynthetic purposes [70]. Little is known, as yet, about the formate oxidase system but the formate dehydrogenase has been characterized in a most elegant and incisive manner by a group at Heidelberg [70]. It is a complex multimeric flavoprotein of mol. wt 315 000 containing 2 FMN, 18–25 non-heme iron atoms and 15–20 acid-labile sulphide groups. The enzyme is extremely labile and sensitive towards oxygen. Many questions need to be answered as to the role of the flavin and acid-labile sulphide groups in the mechanism of action and regulation of activity of this highly interesting enzyme.

An NAD-linked formate dehydrogenase has been purified from a 'methylotrophic bacterium, strain 1' by a group in Moscow [71]. The enzyme shows many different properties from that of *P. oxalaticus*. It is a dimer of mol. wt 80 000, although it is sensitive to oxygen it can be almost completely stabilised in the presence of EDTA or mercaptoethanol, it shows no reaction with artificial electron acceptors, the presence within it of a flavin group has not been reported.

There is no indication as to the mode of metabolism of 'bacterium, strain 1' and hence it is not yet possible to speculate as to whether autotrophic growth on formate will in general involve a very different formate-oxidising system from that operating in an organism which incorporates C_1-carbon at the level of formaldehyde; the profound difference in the energy demands of the two processes does however invite this kind of speculation.

3. Assimilation of C_1-compounds

The following discussion will be confined to aerobic and facultatively aerobic organisms which assimilate a major portion of their carbon at a level more reduced than CO_2. Space does not permit treatment of those strict anaerobes which are able to grow on C_1-compounds as sole source of carbon; in many cases the biochemistry of their assimilatory pathways is not yet at a stage where detailed study of regulation is appropriate [11,72]. Neither does space permit discussion of those methylotrophic bacteria which grow autotrophically on reduced C_1 compounds by first oxidising them to CO_2 and then assimilating the bulk of their carbon by the ribulose bisphosphate (RuBP) cycle of CO_2 fixation. The regulation of this cycle has been long and intensively studied and has been reviewed recently [10]. The reader is also referred to interesting work currently being carried out on the regulation of the autotrophic metabolism of formate by *P. oxalaticus* [73–75] and *Thiobacillus* A2 [76].

3.1. *RuMP cycle of HCHO fixation*

Several recent reviews contain detailed presentations of this cycle and its four possible variants [4,11,12,77]; these will not be repeated here.

Entry of carbon into the RuMP cycle is effected via HuMP synthase catalysing:

$$HCHO + RuMP \rightarrow HuMP \quad (11)$$

As mentioned previously there is a complete absence of knowledge as to how the formaldehyde is apportioned between the HuMP synthase, the further oxidative action of methanol dehydrogenase, and NAD(P)-linked formaldehyde dehydrogenase(s) in those organisms possessing such enzyme(s). Furthermore, formaldehyde is such a highly reactive compound, as any investigator who has radioautographed the products of reaction of [^{14}C]formaldehyde with any crude cell-free extract knows to his cost, that the enzymatic machinery necessary to handle the enormous flux of this reactive and potentially lethal compound which is generated within an actively growing methylotroph must be very precise and foolproof. The nature and control of this machinery presents a fascinating unsolved problem.

It has already been noted that appreciable small-molecule modulation of the activity of HuMP synthase or the following enzyme, phosphohexuloisomerase, has not yet been encountered, although subunit interactions may be involved.

Cleavage of the skeleton of a C_6 sugar phosphate to two C_3 fragments is an integral part of a RuMP cycle. Two such cleavage variants are known, each of which will be dealt with now in turn.

3.1.1. Cleavage via fructose bisphosphate aldolase

The first variant of the cycle uses the familiar glycolytic enzymes:

$$\text{Fructose 6-P(FMP)} + ATP \rightarrow \text{fructose 1,6-bis P(FBP)} + ADP \quad (12)$$

$$FBP \rightarrow \text{glyceraldehyde 3-P(GAP)} + \text{dihydroxyacetone-P(DHAP)} \quad (13)$$

One molecule of triose phosphate has then to be recycled into the rearrangement reactions necessary for regeneration of the acceptor molecule for formaldehyde, i.e., RuMP, and emergence of the resultant essential stoicheiometry:

$$3\ HCHO + ATP \rightarrow \text{triose-P} + ADP \quad (14)$$

It is well known [78] that the enzyme which catalyses reaction (12) during glycolysis by non-methylotrophic bacteria is subject to allosteric regulation and it would be of interest to see if this enzyme is subject to like regulation when involved in the RuMP cycle.

Two patterns are known for the rearrangement to three molecules of RuMP of the 15 carbon atoms contained in the combination of FMP/GAP/DHAP emerging from the fixation and cleavage sequence. One pattern involves transaldolase, transketolase and pentose phosphate isomerase and epimerase; all these enzymes are freely reversible and hence unlikely to be targets for control. The second pattern involves sedoheptulose 1,7-bisphosphatase (SBPase) in place of transaldolase and, as in the RuBP cycle of CO_2 fixation, would be an obvious target for control. To my knowledge, no such information of this enzyme has been published yet.

3.1.2. Cleavage via Entner–Doudoroff enzymes

This variant of the cycle uses the Entner–Doudoroff sequence of reactions for cleavage of hexose phosphate:

$$\text{6-PG} \rightarrow \text{2-keto-3-deoxy-6-phosphogluconate (KDPG)} + H_2O \quad (15)$$

$$KDPG \rightarrow \text{pyruvate} + GAP \quad (16)$$

In those organisms such as *M. methylotrophus* [4,63] and *Pseudomonas* C [53] which use a cyclic mechanism for oxidation of formaldehyde (fig.2), 6-PG thus stands at a branch point between dissimilation and assimilation. It has already been mentioned that reduced pyridine nucleotides and ATP inhibit the NADP- and NAD-linked 6-PG dehydrogenases of *M. methylotrophus* [4,63] and the single NADP/NAD-linked 6-PG dehydrogenase of *Pseudomonas* C [64], hence under conditions of high energy status, formaldehyde is diverted from dissimilation to assimilation via pyruvate plus GAP. It will be interesting to see if the enzymes catalysing reactions (15) and (16) are subject to regulatory control but there are no such reports of this as yet.

It may be noted that the overall stoicheiometry of a RuMP cycle employing Entner–Doudoroff cleavage and transaldolase rearrangement is:

$$3\ HCHO + NAD(P) \rightarrow pyruvate + NAD(P)H_2 \quad (17)$$

The GAP produced by the Entner-Doudoroff cleavage is, of course, recycled into the RuMP cycle for the necessary regeneration of the RuMP acceptor molecules. When any intermediates of the RuMP cycle are withdrawn from the cycle for biosynthetic purposes, net quantities of pyruvate will need to be converted into triose phosphate to top up the cycle. The nature of this gluconeogenic transformation and its possible control is presently under study in the Sheffield laboratories.

As with the FBP aldolase cleavage variant described in section 3.1.1., control of the fully reversible enzymes involved in the transaldolase-catalysed rearrangement sequence is not to be expected. It is most unlikely that an SBPase-catalysed rearrangement sequence operates in conjunction with Entner-Doudoroff cleavage owing to the very unfavourable energetics which result [4,77] and no organisms have yet been reported which possess this particular enzyme profile.

3.1.3. Future problems

It can be seen that much remains to be learnt about control of the RuMP cycle and its different variants: there is a complete lack of knowledge concerning the coupling of methanol dehydrogenase to HuMP synthase; also, since much of the basic biochemistry of the RuMP cycle has been learnt from obligate methylotrophs, there is little information on regulatory phenomena involved in switching between heterotrophic and methylotrophic metabolism and growth on mixed substrates. There are however, some facultative RuMP cycle methylotrophs already isolated which should make such studies possible [79–82].

3.2. *Xylulose monophosphate cycle of HCHO fixation*

Recent work has indicated that methylotrophic yeasts incorporate carbon from methanol via a xylulose 5-phosphate (XuMP) cycle of HCHO fixation [83–89]. The key steps of this cycle are a special transketolase [85,87,89] catalysing the transfer of a glycolaldehyde equivalent to formaldehyde:

$$XuMP + HCHO \rightarrow GAP + \text{dihydroxyacetone (DHA)} \quad (18)$$

followed by phosphorylation of the DHA by a triokinase:

$$DHA + ATP \rightarrow DHAP + ADP \quad (19)$$

These steps can then be incorporated into a cycle in which net synthesis of triose phosphate can be effected from three molecules of formaldehyde (fig.3). Little is yet known about the regulation of this cycle. It may be recalled that methylotrophic yeast dehydrogenate methanol to formaldehyde by way of an oxidase contained within peroxisomes. The formaldehyde is exported into the cytoplasm where it is further oxidised in the form of *S*-hydroxymethylglutathione by reaction (4). There is no evidence as yet that the transketolase catalysing reaction (18) is other than soluble and further study is needed to clarify how the formaldehyde flux is divided effectively between assimilation (in the form of formaldehyde) and dissimilation (in the form of *S*-hydroxymethylglutathione). Two obvious targets for regulatory control at other loci in the XuMP cycle are the triokinase and the FBPase and these enzymes are presently under investigation.

3.3. *Serine pathways of HCHO/CO_2 fixation*

The serine pathways comprise the isocitrate lyase$^+$ (icl$^+$) and icl$^-$ variants [4,7,9–12]. The icl$^+$ variant may be represented in the form of three phases (fig.4). This figure indicates by means of broken arrows that it is still not known how malate is activated to malyl-CoA or how acetyl-CoA is oxidised to glyoxylate in organisms such as *Pseudomonas* AMI which possess neither malate thiokinase nor isocitrate lyase. Thus the basic biochemistry which is a prerequisite for the understanding of control of the icl$^-$-serine pathway remains incomplete.

3.3.1. Modulation of enzyme activity

One of the main ports of entry of carbon into either of the serine pathways is via reactions (6) and (20):

$$\text{Methylene THF} + \text{glycine} \rightarrow \text{serine} + \text{THF} \quad (20)$$

There is no evidence that reaction (6) is catalysed by an enzyme in bacterial systems despite an early, but unconfirmed, report that such an enzyme existed in extracts of pigeon liver [90]. Once again, there is no knowledge as to how the formaldehyde flux is divided out between assimilation via reactions (6) and (20) and further oxidation by methanol dehydrogenase or other formaldehyde-oxidising systems. On the assump-

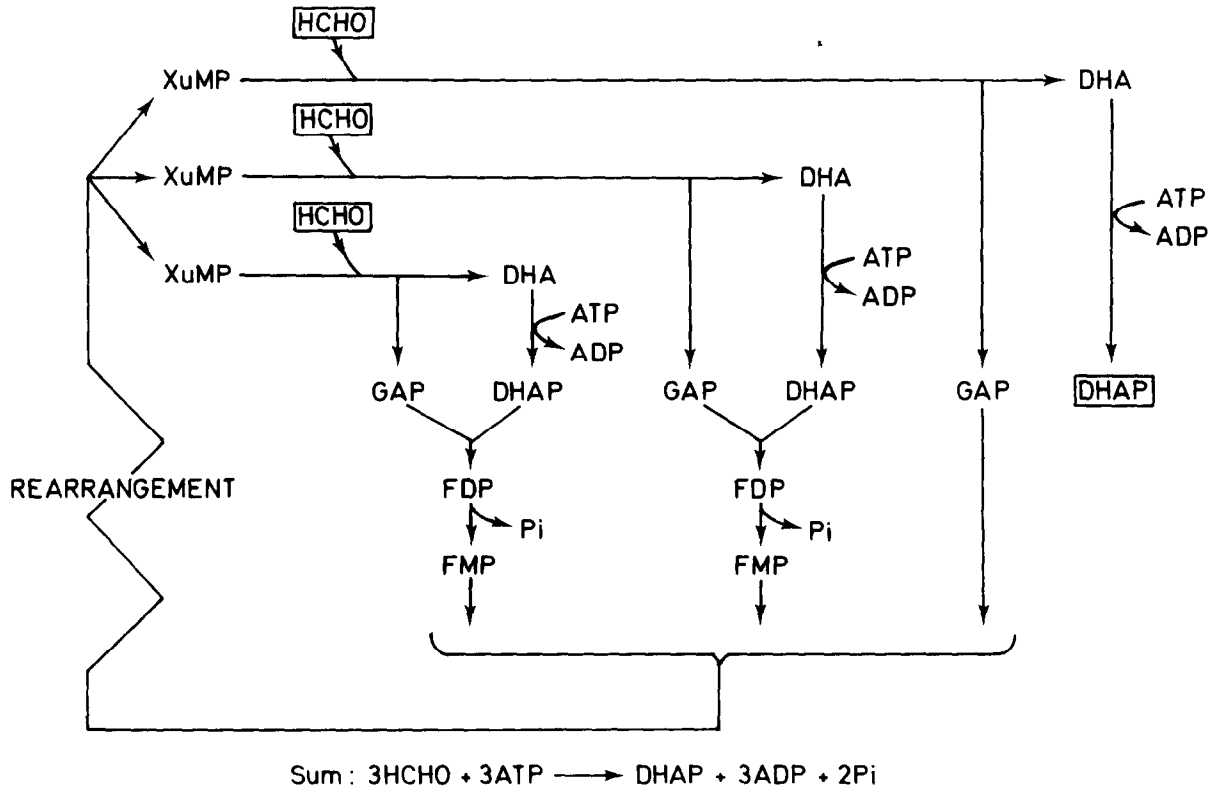


Fig.3. Xylulose monophosphate cycle of formaldehyde fixation. (Reproduced from Transactions of the Biochemical Society with permission.) *Abbreviations:* in text

tion that it is free formaldehyde which is further oxidised to formate, and bearing in mind that formaldehyde reacts rapidly with THF via reaction (6), Harder and Attwood [91] have suggested that only when all the THF in the cell is in the form of C_1 derivatives would formaldehyde be available for conversion into formate and consequent energy generation. Such energy generation in the form of NADH and ATP would activate phase I of the serine pathway, thus releasing THF again. The control of assimilation/dissimilation would therefore be effected through the relative levels of NADH and ATP on the one hand and THF on the other. It has been noted earlier that the mechanism of formaldehyde oxidation in many organisms using a serine pathway is still unclear. While this remains unclear so must the validity of the suggested control via THF, particularly as one of the possibilities for formaldehyde oxidation is by way of reactions [6–10]. An alternative cyclic pathway for formaldehyde oxidation in *Pseudomonas* MA, which grows on methylamine by the icl^+-serine pathway [92], has been proposed by Newaz and Hersh [93]. In this scheme, formaldehyde is inserted into phase I of fig.4 and is oxidised to CO_2 by a rather complex combination of serine pathway, glyoxylate and tricarboxylic acid cycles. PEP is at a branch point of metabolism; carboxylation to oxaloacetate commits the flow of carbon into assimilation whereas dephosphorylation to pyruvate commits the flow into dissimilation via the glyoxylate and tricarboxylic acid cycles. Newaz and Hersh found the PEP carboxylase of *Pseudomonas* MA which was synthesized during growth on methylamine to be activated by $NADH_2$ which would be consistent with a signal of high energy status switching on the assimilatory pathway.

Three instances of isofunctional enzymes have been reported in bacteria using serine pathways. O'Connor and Hanson [94] found two serine transhydroxymethylases in the facultative methylotroph *M. organophilum* (organism XX). One enzyme is predominant during growth on succinate, while the other is formed during growth on methane or methanol.

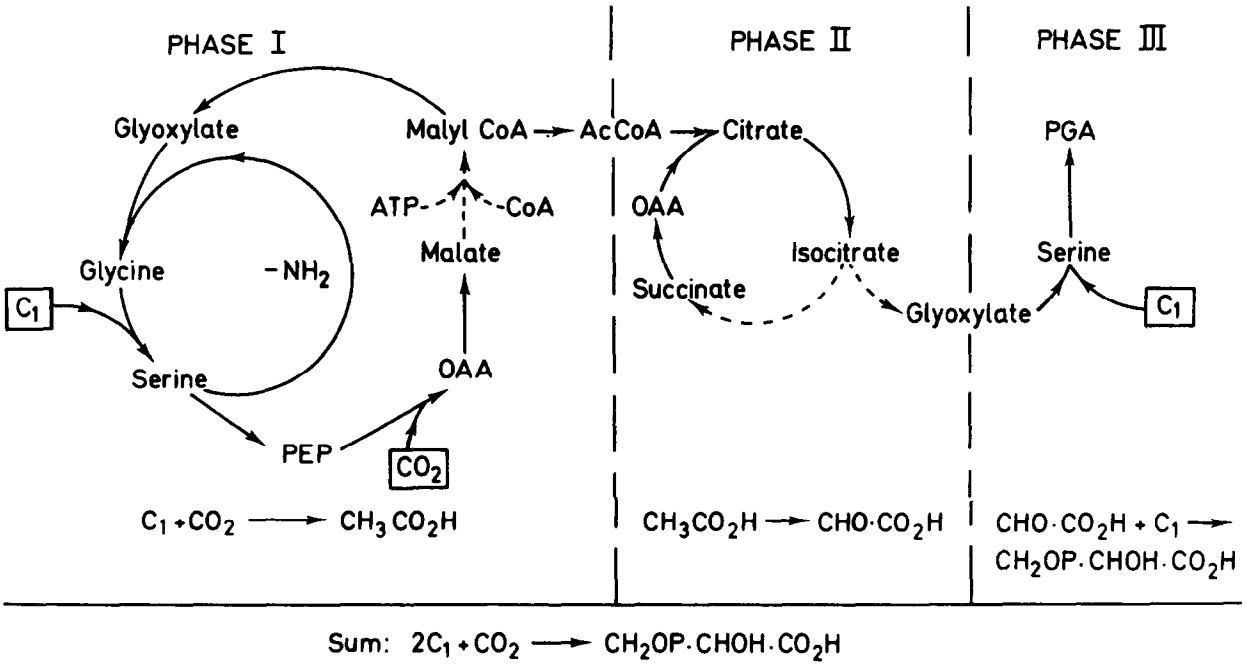


Fig.4. Serine pathways of formaldehyde/carbon dioxide fixation. (Reproduced from Transactions of the Biochemical Society with permission.)
Abbreviations: PEP, phosphoenolpyruvate; OAA, oxaloacetate; AcCoA, acetyl coenzyme A; PGA, phosphoglycerate

The latter enzyme, but not the former, is activated by glyoxylate. Since *M. organophilum* uses the icl^--serine pathway, the activation of reaction (20) by glyoxylate may have regulatory significance as a positive feed-forward effect. The other instances of isofunctional enzymes occur in *Pseudomonas* MA which elaborates two different isocitrate lyases, one during growth on acetate and presumably functioning in the glyoxylate cycle and the second during growth on methylamine and functioning in the icl^+ serine pathway [95]. No suggestive modulation effects on these two enzymes have been reported. This organism also synthesizes two PEP carboxylases, one during growth on methylamine and the other during growth on succinate. The former enzyme is activated by $NADH_2$ and the latter by acetyl-CoA.

3.3.2. Genetic control of enzyme synthesis

Many bacteria which use a serine pathway are facultative methylotrophs and a good deal is therefore known about variation of enzyme levels during growth of a given organism on different substrates. Dunstan et al. [96] observed that in *Pseudomonas* AMI three enzymes of phase I (fig.4) (serine–glyoxylate aminotransferase, hydroxypyruvate reductase and glycerate kinase) appeared to be regulated coordinately, being repressed when succinate was added to methanol growth medium. This contrasted with either methanol or methylamine dehydrogenases which continued to be synthesized when succinate was added to the respective C_1 growth media. A somewhat different picture for *M. organophilum* has been obtained by O'Connor and Hanson [97] using a DNA transformation system for genetic analysis. Their analysis indicates that in this organism synthesis of four enzymes of phase I and methanol dehydrogenase is linked coordinately under the control of a single regulatory agent.

4. Concluding remarks

This article is dedicated to Professor Sir Hans Krebs on the occasion of his 80th birthday. In view of the impact of his discovery of the tricarboxylic acid cycle on so many areas of intermediary metabolism it would have been particularly appropriate if I could have linked methylotrophic metabolism more closely with

the tricarboxylic acid cycle. Yet, by the nature of things, this is not possible because, in general, methylotrophs (with the possible exception of organisms such as *Pseudmonas* MA) do not use the tricarboxylic acid cycle for energy generation. Their energetic systems are, so to speak, sub-tricarboxylic cycle and unique to themselves. Indeed, obligate methylotrophy may largely result from lack of α-oxoglutarate dehydrogenase and hence of a functional tricarboxylic acid cycle. The obligate methylotroph may therefore be only one step away from richer pastures of intermediary metabolism and no better demonstration of this exists than the banishment of *Pseudomonas* AMI from such pastures to the leaner ones of obligate methylotrophy merely by mutational loss of α-oxoglutarate dehydrogenase [98].

Despite this, a large part of research into methylotrophic metabolism consists of a study of cycles and the inspiration as to what constitutes a metabolic cycle and what criteria must be satisfied before a sequence can be considered as a metabolic rather than a paper cycle comes from Sir Hans' own work, past, present and no doubt future.

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