MICROBIAL METABOLISM OF OXALATE AND ONE-CARBON COMPOUNDS

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ABSTRACT

Interest in one-carbon metabolizing micro-organisms is due to the possibility of utilizing them as a source of single cell protein. Furthermore investigations on the metabolism of these bacteria that assimilate oxalate in addition, would aid in elucidating the fundamental process of autotrophic-heterotrophic interconversions on these simple carbon compounds. The present review summarizes the recent developments in the latter field, while attempting to also point out the avenues for future investigations. It is divided into sections dealing with the metabolism of (A) Oxalate (B) Formate (C) Formamide (D) Methane, Methanol, Methylamine and CO_2 followed by comparative notes on (E) Autotrophy and Methylotrophy.

Key words: Oxalate, one-carbon compounds, Autotrophy, Heterotrophy, Methylotrophy.

INTRODUCTION

Ever since their discovery, the metabolic versatility displayed by microbes has awed and intrigued microbiologists, biochemists and molecular biologists. Most compounds on earth, either natural or man-made are metabolized, by microorganisms. The compounds range from inorganic atmospheric CO_2 to organic complex petrochemicals. The present review is concerned with the fascinating problems encountered in the microbial growth on the simple one- and two-carbon compounds oxalate, formate, formamide, methane, methanol, methylamine and CO_2 by autotrophic and heterotrophic pathways. Although at first sight the metabolism of these compounds may appear simple, it is only deceptively so. This is proved by the vast amount of work that has gone into their study-the last five years have brought forth a spate of published literature [1–6].

A. OXALATE METABOLISM

Oxalate which enjoys ubiquitous distribution in the animal and plant kingdoms is the most highly oxidized of organic compounds. In the animal

kingdom oxalate and its salts are present in urine and blood of mammals. Much of the oxalate in animals originates from the oxalate ingested with plant material, although minute amounts are synthesized by mammals through the oxidation of glyoxylate and ascorbate [7–8]. In the plant kingdom, enormous quantities of oxalate are often found in leaves of some plants and in fungi. Accumulation of oxalate by fungi, particularly in *Aspergillus, Penicillium* and *Mucor* species is of such an order that these fungi could be used for industrial fermentation for oxalate [9]. On the death and decay of plants containing oxalate, the oxalate can accumulate in the soil where its chelating properties will prove toxic and interfere with plant growth. The prevention of this accumulation is attributed to the activities of oxalate metabolizing soil microorganisms. [10]

The present status of knowledge, on the metabolism of oxalate is summarized below. Much of this matter has been taken from the many reviews and dissertations that have appeared on this subject [9–13, 22].

Oxalate formation

Two pathways of oxalate formation are well known (9).

(*a*) *Cleavage of a tricarboxylic acid cycle intermediate*

In *Aspergillus niger*, oxalate arises directly by the hydrolytic cleavage of oxaloacetate by oxaloacetate hydrolase (EC 3·7·1·1) but not by the oxidation of glyoxylate [4]. Further, the tricarboxylic acid cycle is involved in the degradation of citrate to oxalate.

(*b*) *Oxidation of glyoxylate*

Oxalate is formed by the oxidation of glyoxylate in plant tissues, especially in bulbs of *Oxalis pes-caprae* [14], and in the mold *Sclerotium rolfsii* [15].

Endogenous formation and high urinary excretion of oxalate which is unrelated to the dietary intake of oxalate occurs in pathological conditions in mammals as in " hyperoxaluria " and ' kidney stones ' or ' urocalculosis ' [7, 16[. This condition is thought to result due to a failure in the conversion of glyoxylate arising from the oxidative decarboxylation of glycine into formate, or in the reconversion of glyoxylate into glycine by glutamate-glycine transaminase. The excess of glyoxylate formed in these cases is oxidized to oxalate [7]. There is an interesting suggestion that treatment of kidney stones could probably be attempted with oxalate degrading enzymes

from microbes (Prof. J. V. Bhat and Dr. E. G. Afrikyan, personal communications).

(c) Conversion of oxalate to neurotoxins

Several species of *Lathyrus* plant seeds contain β-N-oxalyl L-α, β-diaminopropionic acid and γ-N-oxalyl-α, γ-diaminobutyric acid and α-N-oxalyl-α, γ-diaminobutyric acid. The biosynthesis of β-N-oxalyl L-α, β-diaminopropionic acid is of interest since this neurotoxin has been isolated from the seeds of *Lathyrus sativus*, the consumption of which causes the disease neurolathyrism in humans. The concentration of this non-protein amino acid in seeds increases on germination [87, 88]. Malati *et al.* [88] have found that β-N-oxalyl L-α, β-diaminopropionic acid is synthesized in the seeds of *L. sativus* from oxalic acid via oxalyl-CoA. Oxalyl-CoA is reported to be formed from oxalate by oxalyl-C_2oA synthetase [89] by the reaction:

$$\text{Oxalate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{++}} \text{Oxalyl-CoA} + \text{AMP} + \text{PPi}$$

Oxalate oxidation

There are three possible ways of oxalate degradation [9].

(a) Oxidation

The oxidation of oxalate is accomplished by a single enzyme oxalic acid oxidase in mosses and higher plants [9].

$$\text{COOH} \cdot \text{COOH} + O_2 \rightarrow 2CO_2 + H_2O_2$$

(b) Decarboxylation

The direct decarboxylation of oxalate is thought to be the system active in white rot, wood destroying fungi [9]. An enzyme specific for oxalate decarboxylation has in fact been purified from *Collybia velutipes* and requires catalytic quantities of oxygen [17].

$$\text{COOH} \cdot \text{COOH} \xrightarrow{O_2} \text{HCOOH} + CO_2$$

(c) Activation and decarboxylation

Oxalate degradation by this process is more complicated. Here oxalate is activated to oxalyl-CoA* and then decarboxylated to formyl-CoA (Fig. 1).

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* *Abbreviations*:

CoA, Coenzyme A; TPP, Thiamine pyrophosphate; NAD, Nicotinamide-adeninedinucleotide; NADH, Nicotinamide-adenine dinucleotide, reduced; NADP, Nicotinamide-adenine dinucleotide phosphate; NADPH, Nicotinamide-adenine dinucleotide phosphate, reduced; RuDP, Ribulose diphosphate; RuMP, Ribulose monophosphate; PGA, Phosphoglyceric acid; TSA, Tartronic semialdehyde; ATP, Adenosine 5′-triphosphate; ADP, Adenosine 5′-diphosphate; AMP, Adenosine 5′-monophosphate; DCPIP-2, 6-Dichlorophenolindophenol; C_1, One-carbon.

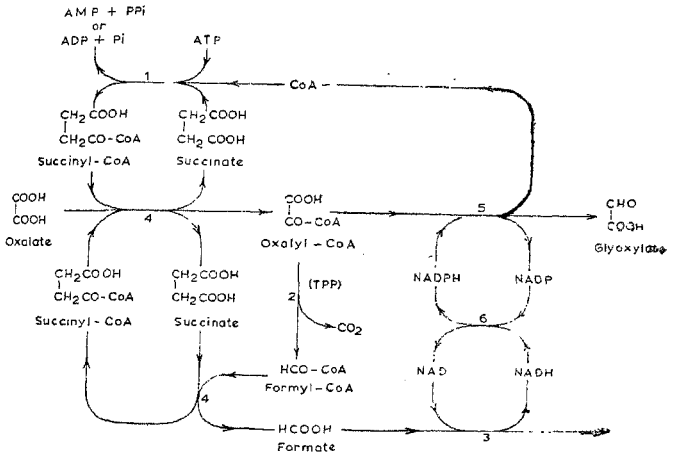

FIG. 1. The oxalyl-CoA cycle in *Pseudomonas oxalaticus* [19]. Reactions 1–6 catalysed by:

1. Succinyl-CoA synthetase
2. Oxalyl-CoA decarboxylase (EC 4.1.1.8)
3. Formate dehydrogenase (EC 1.2.1.2).
4. Transferase(s)
5. Oxalyl-CoA reductase (EC 1.2.1.17)
6. Transhydrogenase

This is the first step of degradation in all the oxalate utilizing bacteria studied todate [18–20]. All the enzymes of the oxalate decarboxylation cycle (Fig. 1) have been demonstrated in extracts of *Pseudomonas oxalaticus* OX1. Oxalyl-CoA decarboxylase has also been purified from *Ps. oxalaticus* and its presence demonstrated in *Ps.* OD1 [20]. Koch and Jaenicke [21] refer to the decarboxylation of oxalate by species of *Pseudomonas* in which phosphate, CoA and TPP were cofactors for the reaction. Formate which is formed as an intermediate during the oxidation is dehydrogenated by an NAD-linked formate dehydrogenase. Also this reaction is the sole energy yielding step in the complete oxidation of oxalate in the cycle. In the pink-pigmented organisms *Ps.* AM1, *Protaminobacter ruber* and *Ps. extorquens* and in the non-pigmented *Alcaligenes* LOx and *Thiobacillus novellus*, oxalate oxidation proceeds in a fashion similar to that in *Ps. oxalaticus* [1, 22].

Two distinctive features of the oxalyl-CoA cycle are:

(*a*) Oxalyl-CoA is stituated at the branch point of two reaction sequences (i) decarboxylation to formate which is the source of NADH and (ii) reduction by NADPH to glyoxylate which initiates the synthesis of cell constituents. A major factor controlling these two alternative pathways is probably the relative levels of reduced and oxidized pyridine nucleotides. These levels would enable a sensitive balance to be maintained [19].

(*b*) The oxalyl-CoA cycle is not a cycle in the same sense as are the tri- and dicarboxylic (glyoxylic) acid cycles. The essence of a true oxidation cycle (as exemplified by the latter two cycles) is (i) the condensation of the substrate by C–C bonding with an acceptor compound, (ii) expulsion of fully oxidized carbon atoms successively as CO_2 and (iii) regeneration of the acceptor molecule. Such condensation is not possible with a molecule at the oxidation level of oxalate. The explusion of CO_2 follows directly on activation of the molecule, the cyclic part of the process being concerned with the conservation of the energy required to synthesize an acyl-CoA bond rather than with oxidation *per se*. Thus oxalate marks the transition between the cyclic oxidation of C_2 compounds and the non-cyclic oxidation of C_1 compounds.

Oxalate assimilation

Utilization of oxalate as the sole carbon and energy source is the unique privilege of microorganisms. The high state of oxidation of oxalate requires the removal of only two electrons for CO_2 formation. This has led to the suggestion of the possible autotrophic growth of these organisms on oxalate [9, 23, 24]. However, all oxalate utilizers studied have turned out to be heterotrophic as far as oxalate assimilation is concerned. *Ps. oxalaticus* OX1 exhibits a unique phenomenon of autotrophic growth on the intermediate of oxalate oxidation, formate when presented as the sole source of carbon [25].

The assimilatory pathways of oxalate as in *Ps. oxalaticus* OX1 and *Ps.* AM1 (Fig. 2) have been extensively worked out by Quayle and others [1, 12, 19, 20, 26]. Oxalate assimilation proceeds with the reduction of the activated molecule to glyoxylate by oxalyl-CoA reductase in *Pseudomonas* species, *Alcaligenes* LOx and *Thiobacillus novellus* [27, 28]. Oxalyl-CoA reductase has been partially purified from extracts of oxalate-grown *Ps. oxalaticus* and shown to be present in *Ps.* OD1 [26]. Further reduction of glyoxylate to glycerate proceeds either by the 'glycerate' pathway or a variant of the 'serine' pathway (Fig. 2).

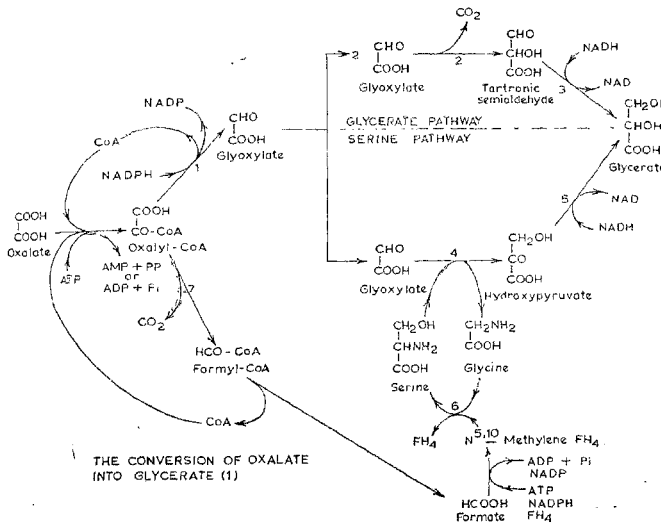

FIG. 2. The conversion of oxalate into glycerate [1]
Reactions 1–7 catalysed by:

1. Oxalyl-CoA reductase (EC 1.2.1.17)
2. Glyoxylate carboligase
3. Tartronic semialdehyde reductase (EC 1.1.1.60)
4. Serine-glyoxylate aminotransferase
5. Hydroxypyruvate reductase (EC 1.1.1.29)
6. Serine hydroxymethyltransferase (EC 2.1.2.1)
7. Oxalyl-CoA decarboxylase (EC 4.1.1.8).

(*a*) *The glycerate pathway*

This pathway similar to the one found in glycollate-grown *Escherichia coli* and *Pseudomonas* sp. [29], operates in *Ps. oxalaticus*, *A.* LOx and *T. novellus* [12, 22, 26, 28]. The reactions here are catalysed by three enzymes (Fig. 2). The key enzyme glyoxylate carboligase forms TSA and CO_2 from two molecules of glyoxylate. Glyoxylate carboligase has been purified to homogeneity from *Ps. oxalaticus* and shown to be a flavin requiring enzyme [30]. TSA is reduced by TSA reductase to glycerate which is then phosphorylated by glycerate kinase. The glyocerate pathwa y is also utilized

frequently in the assimilaton of many C_2 compounds like glycollate, glyoxylate, glycine, and others like uric acid, allantoin and glutamate [31, 32].

The overall equations for the dissimilation and assimilation of oxalate by the glycerate pathway are:

$$\text{Oxalate} \rightarrow 2CO_2 + 2\ NADH_2$$

$$2\ \text{Oxalate} + 3\ ATP + 2\ NADPH_2 + NADH_2 \rightarrow PGA + CO_2.$$

To quote from Harder [12]: "From these equations it appears that the energy yield for the oxidation of oxalate is very small. In addition, large amounts of ATP and reducing power are required to reduce the molecule to PGA. It is therefore not surprising that the molar growth yield of an organism growing on oxalate is very low".

(*b*) *The serine pathway*:

Oxalate-grown *Ps.* AM1, *Ps.* AM2, *Pr. ruber*, *Ps. extorquens*, *Ps.* MOx and *Ps.* AM1 (var 470) do not contain glyoxylate carboligase [1, 28] and synthesize cell constituents from oxalate by a variant of the serine pathway (Fig. 2). Here glyoxylate is converted into hydroxypyruvate by an aminotransferase reaction. The latter is reduced to glycerate by the key enzyme hydroxypyruvate reductase. The serine pathway is not only involved in the assimilation of oxalate but is also a common pathway of C_1 assimilation (discussed in a later section). Simultaneous occurrence of the glycerate and serine pathways is not likely [1].

B. FORMATE METABOLISM

Formate is the simplest of organic C_1 compounds. It occurs in plant and animal tissues and is also an end product frequently encountered in the fermentation of carbohydrates. The features relevant to the discussion here are (*a*) its formation as an intermediate during the oxidation of oxalate and several C_1 compounds (CH_4, CH_3OH, CH_3NH_2, etc.) and (*b*) its ability to support the autotrophic or heterotrophic growth of some bacteria.

Formate oxidation

The enzymes catalyzing the dehydrogenation of formate are widespread in animals, plants and microbes. All aerobic C_1 utilizing microorganisms possess an NAD-linked formate dehydrogenase [33]. Among the chemolithotrophs *Nitrobacter agilis* has a particulate nitrite-formate oxidase [28] and formate-grown *Thiobacillus* A2 an NAD-linked formate

dehydrogenase [34]. *Alcaligenes* LOx has both a particulate formate oxidase and an NAD-linked formate dehydrogenase [28]. *T. novellus* also appears to have two different formate oxidizing mechanisms as our investigations have revealed an NAD-linked formate dehydrogenase in oxalate- and formate-grown cells and its absence in thiosulphate-grown cells [22] while Aleem [35] has reported a soluble formate-cytochrome C reductase in thiosulphate-grown cells. Particulate formate oxidizing activities have also been reported in *E. coli*, *Ps. denitrificans*, *N. agilis*, *N. winogradskyi* and *Ps.* MS [28]. Among anaerobes, strains of *Rhodopseudomonas palustris* have a DCPIP or NAD-linked formate dehydrogenase [33], and *R. acidophila* strain 10050 an NAD-linked formate dehydrogeanse [36], while *Clostridium thermoaceticum* has an NADP-linked formate dehydrogenase which has been recently purified [37]. Reactions occurring during the anaerobic dismutation of formate and C_1 compounds by methanogenic bacteria are not clearly understood, and the path of carbon assimilation during these processes remains unexplored [33, 38].

Formate assimilation

Utilisation of formate as sole carbon source is not as widespread a phenomenon as is the oxidation of formate. Aerobic bacteria like *Ps. oxalaticus*, *Ps.* AM1 (1), *Bacterium formoxidans* [39], *Hydrogenomonas eutropha* Z–I [3], *N. winogradskyie* [40], *Thiobacillus* A2 [34], *Alcaligenes* FOR_1 [41] and facultative methylotrophs are known to utilize formate as sole carbon source. The ability of photosynthetic bacteria to grow on C_1 compounds is not well documented, but there is definite evidence that *R. palustris* grows on formate in a manner paralleling *Ps. oxalaticus* [33]. Anaerobic fermentation of formate by methanogenic bacteria is well established [33, 38].

Till 1958 very little was known of the mode of biosynthesis of cell constituents from reduced C_1 growth substrates. Bhat and Barker [23] and Van Niel [42] had pointed out the possibility that an organism growing on a highly oxidized C_1 substrate such as formate, might utilize an autotrophic type of metabolism in which the energy of oxidation of the substrate is coupled to the assimilation of CO_2. In fact, several authors have assumed that this takes place without there being any proof that it does [43].

(*a*) *Autotrophic assimilation of formate*

Of all the organisms tested so far, evidence for autotrophic growth on formate has been obtained in only five aerobic bacteria, *viz.*, *Ps. oxalaticus*

OX1 [33]. *H. eutropha* Z–1 [3], *B. formoxidans* [39], *T. novellus* [22] and *Alcaligenes* FOR$_1$ [41]. The two organisms in which autotrophic growth on formate is not conclusively demonstrated are *N. winogradskyi* [40] and *T.* A2 [34] although they are reported to utilize formate. It is likely that *Micrococcus denitrificans* utilizes formate autotrophically as it utilizes this mode growth on methanol, but enzymic or other evidence for it is still lacking [44].

During autotrophic growth, formate is oxidised to CO_2 which is then then fixed by the RuDP cycle (Fig. 3) similar to that found in plants and

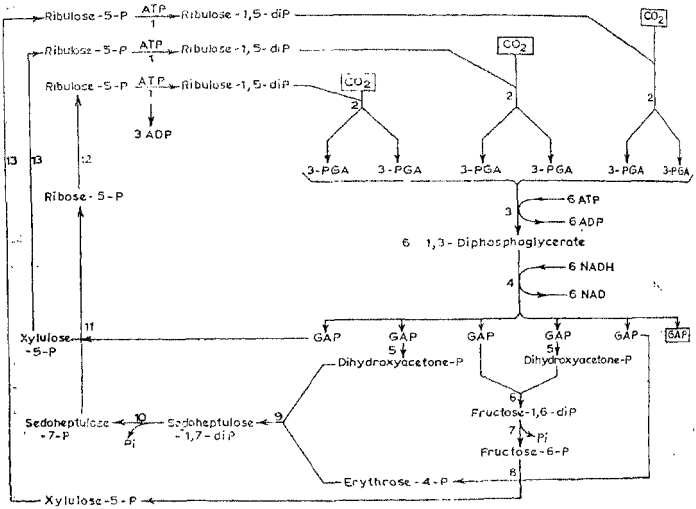

FIG. 3. The ribulose diphosphate cycle of CO_2 fixation [84].
Reactions 1–13 catalysed by:

1. Phosphoribulokinase
2. Carboxydismutase/Ribulose-1, 5-diphosphate carboxylase (EC 4.1.1.39)
3. Phosphoglycerate kinase (EC 2.7.2.3)
4. Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12)
5. Triosephosphate isomerase (EC 5.3.1.1)
6. Aldolase (EC 4.1.2.7)
7. Fructose-1, 6-diphosphatase
8. Transketolase
9. Aldolase
10. Phosphatase
11. Transketolase (EC 2.2.1.1)
12. Phosphoriboisomerase (EC 5.3.1.6)
13. Ribulose-5-phosphate-3-epimerase (EC 5.1.3.1)

chemolithotrophic and photoautotrophic bacteria [33, 45]. The necessary energy for these reactions is derived by the oxidation of formate by NAD-linked formate dehydrogenase.

$$HCOOH \rightarrow CO_2 + H_2O$$

$$CO_2 + RuDP \rightarrow 2\ PGA.$$

The energy requirements for the synthesis of a triose phosphate in this cycle are expressed as:

$$3CO_2 + 9ATP + 6NADH_2 + 5H_2O \rightarrow \text{Triose phosphate} + 9ADP + 8Pi + 6NAD.$$

Blackmore and Quayla [46] have shown that when *Ps. oxalaticus* is grown on an equimolar mixture of formate and oxalate, it adopts a metabolism in which the autotrophic metabolism of formate predominates. This was unexpected as it can be seen that heterotrophic growth on oxalate is more economical in terms of energy requirements. The growth rate of *Ps. oxalaticus* on formate alone is 38% faster as compared to growth on oxalate [46]. Here the behaviour of *Ps. oxalaticus* provides a good example of Pardee's generalization that in bacterial metabolism maximal growth rate is the guiding principle rather than maximal efficiency [46]. However, we have observed that *T. novellus* grows faster heterotrophically on oxalate (11 hr. generation time) than autotrophically on formate (32 hr.) [22].

The finding of autotrophic growth on formate by *Ps. oxalaticus* led Peck [47] to speculate that "growth on formate represents an intermediate stage in the evolution of autotrophy". Peck [47] also considers *Ps. oxalaticus* as a facultative chemolithoautotroph. More rightly using the terminologies of Rittenberg [48] this organism should perhaps be called a 'Chemo-organotrophic autotroph'.

(*b*) *Heterotrophic assimilation of formate*:

The heterotrophic assimilation of formate where its reduction level is conserved is widespread in aerobic bacteria and occurs *via* the serine pathway. The reactions of this cycle are shown in figure 4 and discussed in a later section.

From a survey of the literature regarding oxalate and formate metablism presented in the sections above, the salient features and avenues for future investigation that can be pointed out are:

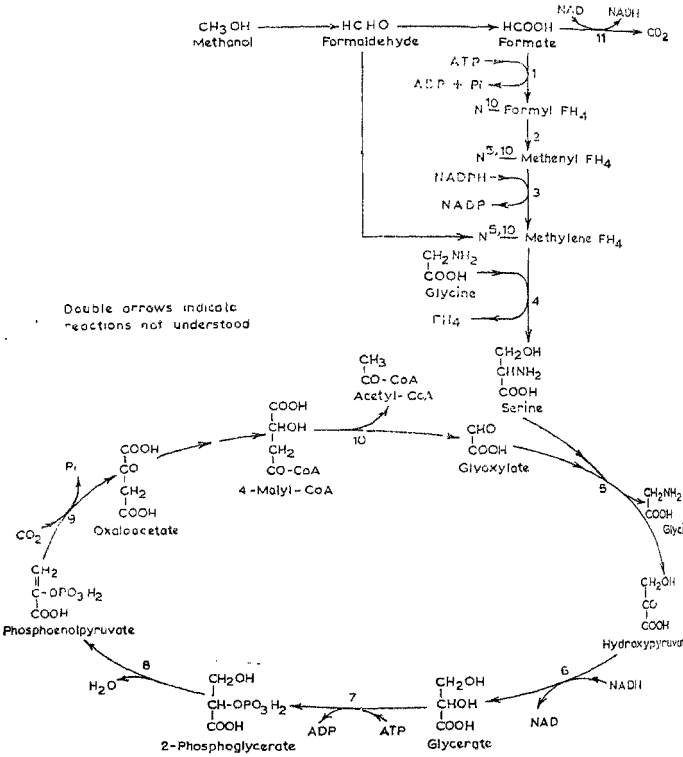

FIG. 4. The serine pathway in *Pseudomonas* AM1 [62, 65, 85, 86]
Reactions 1–11 catalysed by:

1. Formyltetrahydrofolate synthetase (EC 6.3.4.3)
2. Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9).
3. Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5)
4. Serine hydroxymethyltransferase (EC 2.1.2.1).
5. Serine-glyoxylate aminotransferase
6. Hydroxypyruvate reductase (EC 1.1.1.29)
7. Glycerate kinase (EC 2.7.1.31)
8. Enolase (EC 4.2.1.11)
9. Phosphoenolpyruvate carboxylase (EC 4.1.1.31)
10. Malyl-CoA lyase
11. Formate dehydrogenase (EC 1.2.1.2)

(*a*) *Ps. oxalaticus* OXI is a special member among the different classes of chemo- and photoautotrophs as judged from its ability to utilize formate autotrophically and oxalate heterotrophically. *T. novellus* is the only bacterium shown recently to utilize oxalate and formate by metabolic patterns similar to *Ps. oxalaticus*. It is also the first facultatively autotrophic chemolithotroph and member of the thiobacilli to utilize oxalate.

(*b*) The metabolic pattern of *Ps. oxalaticus* and *T. novellus* raises several questions about the factors that regulate their mode of growth. As formate is the key compound involved during both autotrophic and heterotrophic growth, these oxalate utilizing organisms form ideal probes for investigating the biochemical basis of heterotrophic-autotrophic interconversions. *T. novellus* shoud prove particularly useful in such investigations as it is a chemolithotroph. More permutations and combinations of carbon source for studies with mixed substrates (oxalate + thiosulphate, formate + oxalate, etc.) and mixotrophic growth is possible with *T. novellus*.

(*c*) Autotrophy on formate, an energetically unfavourable mode of growth, has been well established in only 5 species as mentioned. An investigation into the prevalence of this type of growth in more bacteria appears desirable.

(*d*) In studies of the growth patterns of *Ps. oxalaticus* on mixed substrates (formate + oxalate, formate + glycollate etc.), Blackmore and Quayle [46] comment that "it is not known what metabolites actually function as inducers or co-repressors of synthesis of formate dehydrogenase and RuDP carboxylase, although the apparent simplicity of the substrates and products of the former enzyme invites further study of this question". Blackmore and Quayle [46] have also pointed out the existence of an unknown formate oxidizing enzyme system different from the NAD-linked formate dehydrogenase in *Ps. oxalaticus* grown on substrates like succinate or succinate-plus-formate. The presence of this system makes it clear that the repression of RuDP carboxylase under the above growth conditions is not due to the exclusion of formate from the cell [46]. These facts emphasize the need for study on formate oxidizing enzyme mechanisms other than the NAD-dependent dehydrogenase in oxalate utilizing bacteria.

It is of interest here that our investigations have revealed two different formate oxidizing enzyme systems in *A.* LOx, *viz.*, (i) the constitutive particulate formate oxidase in oxalate- and succinate-plus-formate grown cells and (ii) the inducible NAD-linked formate dehydrogenase in soluble fraction of oxalate-grown cells alone, and the simultaneous occurrence of both

the systems in oxalate-grown cells [28]. In the light of these findings in *A.* LOx, supported by those of Quayle and Keech [25], it seems likely that in *Ps. oxalaticus* particulate fractions were involved in the oxidation formate in mixed substrate grown cells. Furthermore these findings are likely to influence the calculations of Y_{ATP} and growth yields of *Ps. oxalaticus* and other organisms on oxalate and formate. Hence further investigations in this important area of formate metabolism deserves much attention.

(*e*) It was mentioned earlier that formate oxidation by the NAD-dependent dehydrogenase is the only energy yielding reaction during growth on oxalate and formate, although the energy requirements for biosynthesis differ significantly. Harder *et al* [5] and Knight *et al.* [2] in recent investigations of these problems have shown (*a*) that even the transport of oxalate and formate into the cell is energy dependent and (*b*) that there is a difference in the NADH level of the cells during an oxalate-formate change. This brings us to the question—Is there a difference in the properties of the NAD-linked formate dehydrogenase in oxalate- and formate grown cells? It may be pointed out that this NAD-dependent enzyme has been purified and studied only from formate-grown cells [49].

(*f*) Comparison of oxalate and formate metabolism in a large number of bacteria is obviously necessary before any hypothesis can be put forth to explain these intriguing phenomena of heterotrophic-autotrophic interconversions.

C. FORMAMIDE METABOLISM

The microbial metabolism of formamide closely resembles that of formate. Several species of *Pseudomonas* utilize amides for growth. Recently Thatcher and Weaver [50] have suggested an important role for formamide utilizing organisms in a novel carbon-nitrogen cycle involving cyanogenic plants parasitized by certain fungi. In these plants cyanides are converted to formamide which in turn is utilized as carbon and energy source by a *Pseudomonas* species.

Formamide oxidation

The first step is the hydrolysis of the amide by formamide amido-hydrolase (EC 3.5.1.4) releasing ammonia and formate. This is known to occur in *Ps.* SL–4 [50] and suggested to occur in *T. novellus* also [22]. Formate is further oxidized by NAD-linked formate dehydrogenase in both the organisms [22].

Formamide assimilation

Whether formamide will be utilized as sole carbon source or not will depend on the presence or absence of the relevant anabolic pathway enzymes for the assimilation of formate produced on hydrolysis of formamide. *T. novellus* utilizes formamide as sole carbon source and formamide carbon is assimilated autotrophically just as during growth on formate [22]. A few other one-carbon metabolizing organisms also utilize it as carbon source [51], while some just hydrolyse it and others utilize it as a nitrogen source [52].

D. Methane, Methanol, Methylamine and CO_2 Metabolism

Studies on the mode of carbon assimilation from oxalate and formate by Quayle and coworkers at Sheffield University from 1958 onwards, created an impetus in the study of microbial growth on various other C_1 compounds* like CH_4, CH_3NH_2, CH_3OH etc. The biosynthetic and degradative pathways of these C_1 compounds share a number of features in common with those of oxalate and formate. In the following sections, some of the latest developments in the field of C_1 metabolism will be highlighted briefly as several reviews on the subject have appeared in close succession in the past decade [12, 43, 53–55]. The excellent reviews by J. R. Quayle [33] and C. Anthony [56] are quite exhaustive.

One carbon compounds occur in abundance throughout nature, *e.g.*, methane is found in coal and oil deposits and is evolved during ruminant digestion and sewage decomposition. Methanol is formed by the breakdown of methylated natural products like pectin. Methylated amines occur widely in tissues of plants, animals, fishes and in the excreta of fish. CO_2 is present in the atmsophere, in water, and in the earth as solid carbonates. With such a natural abundance of C_1 compounds it is not surprising that a wide variety of microorganisms utilize them. A representative list of such organisms has been indicated by Quayle [54].

Currently much attention has been focussed on obtaining methane and methanol utilizing yeasts and bacteria to produce single cell protein, organic acids and amino acids. Methanol is an attractive raw material for such fermentations, with advantages like low cost, high purity (>99·8%), complete

* 'One-carbon compounds' have one or more carbon atoms without C—C bonds, *e.g.*, CH_3OH, $(CH_3)_3NH^+$, $(CH_3)_2NH_2^+$ $CH_3NH_3^+$ etc., according to Colby and Zatman [59]. Other organic compounds have more than one carbon atom with C—C bonds, *e.g.*, CH_3COOH, COOH. COOH, CH_3CH_2OH, etc.

water miscibility and restricted use by certain microorganisms [57, 58]. The advantages with methane are its natural abundance and gaseous nature. These properties enable both the easier processing of the cells grown in the gas atmsophere and the overcoming of problems of substrate limitations affecting maximal growth yields.

Methane, methanol and methylamine (mono-, di- and tri-) oxidation

Many enzymes that catalyse the oxidation of one-carbon compounds at a va iety of oxidation levels have now been purified and studied [33]. The dissimilation of these compounds is generally thought to proceed as follows:

$$CH_4 \xrightarrow{1} CH_3OH \xrightarrow{2} HCHO \xrightarrow{3} HCOOH \xrightarrow{7} CO_2$$

$$(CH_3)_3\,NH^+ \xrightarrow{4} (CH_3)_2NH_2{}^+ \xrightarrow{5} CH_3NH_3{}^+$$

Reactions 1–6 are not fully understood in all cases, although detailed investigations on particular steps have been done in many bacteria [33]. In the majority of organisms studied reaction 7 is catalysed by an NAD-dependent dehydrogenase [12]. Study of reactions 1–3 has assumed a lot of importance at the moment. Van Dijken and Harder [60] carried out a theoretical study on the growth yields of microorganisms on methane and methanol. They point out that different amounts of ATP formed by these reactions when energy yielding, significantly influence Y_{ATP} values, maximal growth yields and hence athe choice of microorgansims for single cell protein.

CO_2 assimilation and methane, methanol and methylamine assimilation

The biochemical singularity of C_1 utilizing organisms is their ability to synthesise in net fashion a C_3 carbon skeleton from a C_1 unit. From a C_3 compound, established pathways of intermediary metabolism leads to the synthesis of cellular constituents. There is evidence that there are lesions even in the intermediary metabolism of some C_1 utilizers, *e.g.*, lack of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and succinyl-CoA synthetase [33, 59]. In fact, it has recently been suggested that lack of 2-oxoglutarate dehydrogenase may well be the basis of obligate methylotrophy* in some bacteria [61]. Such lesions have also been observed in some chemolithotrophs [45, 47, 48]. These aspects, how-

* 'Obligate methylotrophy' utilizes only 'one carbon compounds', 'Facultative methylotroph' utilizes 'one-carbon compounds' and several organic compounds [59].

ever, are not considered here. Only the progress made in elucidating the mechanism of building up a C_3 compound is discussed. C_1 assimilation can proceed from the most highly oxidized carbon level as in CO_2 or from a more reduced level like that in formate or formaldehyde. The pathways accomplishing this assimilation and a representative list of the micro-organisms and carbon sources in which these are found are show in Figs.3–5 and Table I.

TABLE I

Distribution of oxalate and one-carbon assimilation pathways

Organism	Oxalate	Formate	Metha-nol	Methyl-amine	Tri methyl amine	Methane	Refe-rence
Hydrogeno-monas eutro-pha Z–1	—	RuDP	—	—	—	—	[3]
Bacterium formoxidans	—	RuDP	—	—	—	—	[39]
Micrococcus denitrificans	—	—	RuDP	—	—	—	[44]
Alcaligenes FOR_1	—	RuDP	—	—	—	—	[41]
Thiobacillus novellus	Glycerate	RuDP	—	—	—	—	[22]
Alcaligenes LOx	Glycerate	—	—	—	—	—	[28]
Pseudomonas KOx	Glycerate	—	—	—	—	—	[28]
Ps. MOx	Serine	Serine	—	—	—	—	[28]
Ps. AM1 (var. 470)	Serine	Serine	—	—	—	—	[28]
Protamino-bacter FOR_2	..	Serine	—	—	—	—	[41]
Ps. oxalaticus OXI	Glycerate	RuDP	—	—	—	—	[46]

TABLE I—(*Contd.*)

Organism	Oxalate	Formate	Methanol	Methylamine	Trimethyl amine	Methane	Reference
Ps. AM1	Serine	Serine	Serine	Serine	—	—	[1, 62, 73]
Ps. AM2	Serine	Serine	Serine	—	—	—	[1]
Ps. extorquens	Serine	—	—	—	—	—	[1]
Protaminobacter ruber	Serine	—	—	—	—	—	[1]
Ps. PRL-W4	—	—	Serine	..	—	—	[33]
Hyphomicrobium vulgare	—	—	Serine	—	—	—	[33]
Diplococcus PAR	—	—	—	Serine	—	—	[33]
Hyphomicrobium X	—	—	Serine	—	—	—	[74]
Ps. MS	—	—	—	Serine	—	—	[75]
Ps. MA	—	—	—	Serine	—	—	[64]
Isolate XX	—	—	—	—	—	Serine	[76]
Gliocladium deliquescens[1]	—	—	Serine	—	—	—	[77]
Paecilomyces varioti[1]	—	—	Serine	—	—	—	[77]
Organism W1	—	—	RuMP	RuMP	—	—	[78]
Ps. C[2]	—	—	RuMP	—	—	—	[79]
Bacterium W6	—	—	RuMP	—	—	—	[80]
Bacterium 4B6	—	—	—	—	RuMP	—	[59]
Ps. 3A2	—	—	—	—	Serine	—	[59]
Bacterium 5B1	—	—	—	—	Serine	—	[59]

TABLE I—(*Contd.*)

Organism	Oxalate	Formate	Methanol	Methylamine	Trimethylamine	Methane	Reference
Organism C2A1	—	—	—	—	RuMP	—	[81]
Organism W3A1	—	—	—	—	RuMP	—	[81]
Organism W6A	—	—	—	—	RuMP	—	[81]
Bacillus S2A1	—	—	—	—	RuMP	—	[81]
Bacillus PM6	—	—	—	—	RuMP	—	[81]
Bacterium 2B2	—	—	—	—	RuMP	—	[68]
Methylomonas sp.	—	—	—	—	—	RuMP	[82]
Methylobacter sp.	—	—	—	—	—	RuMP	[82]
Methanomonas methanooxidans	—	—	—	—	—	Serine	[82]
Methylococcus capsulatus	—	—	—	—	—	RuMP	[66]
Ps. methanica	—	—	—	—	—	RuMP	[66]
Methylosinus trichosporium OB3B	—	—	—	—	—	Serine	[66]
Kloeckera sp. 2201	—	—	RuMP	—	—	—	[83]
Pichia pastoris M3	—	—	RuMP	—	—	—	[83]
Candida boidinii No. 0302	—	—	RuMP	—	—	—	[83]
Candida N-16	—	—	RuMP	—	—	—	[67]

— = Unreported in the reference mentioned.

[1]These fungi also utilize formaldehyde by the serine pathway [77.]

CO_2 assimilation

Assimilation occurs by the RuDP cycle. In some cases, the acetyl-CoA cycle of CO_2 assimilation operates in addition to the RuDP cycle,

(*a*) *The ribulose diphosphate pathway*

The RuDP cycle or the Calvin cycle of CO_2 fixation has been well established in photosynthetic plants, photosynthetic bacteria and chemoautotrophs [33, 45]. The scheme of reactions is shown in Fig. 3. Phosphoribulokinase and RuDP carboxylase (carboxydismutase), are the key enzymes of this cycle and catalyse the formation of RuDP from ribulose-5-phosphate and PGA from RuDP and CO_2 respectively. The enzymic reactions that lead to the conversion of PGA to the cell constituents and the regeneration of the acceptor pentose phosphate molecule are well known [33]. The RuDP cycle, as mentioned earlier, also functions in the assimilation of CO_2 formed from formate in *Ps. oxalaticus, T. novellus* etc. An interesting recent finding is the demonstration of this cycle in methanol-grown *Micrococcus denitrificans*, a H_2 oxidizer [44]. This is the first record of an autotrophic growth on a reduced organic compound other than formate. *M. denitrificans* is also reported to utilize methylamine and formate although the pathway of their assimilation is not established [44]. We believe that *M. denitrificans* represents the first case of a 'facultative autotroph' also being a 'facultative methylotroph'. The only other example of such a type of organisms is *T. novellus* which we found to utilize methanol (in presence of yeast extract), formate, and formamide just as facultative methylotrophs [22]. However, the mode of methanol metabolism in *T. novellus* is uncertain in view of the low activities of RuDP carboxylase and hydroxypyruvate reductase that we detected in methanol-grown cells [22]. The RuDP cycle also operates in *Rhodopseudomonas acidophila* strain 10050 grown anaerobically on methanol in the presence of light [36].

(*b*) *The acetyl-CoA cycle* (*reductive carboxylic acid pathway*)

The acetyl-CoA cycle of CO_2 fixation was found to operate in anaerobic photosynthetic bacteria like *chlorobium thiosulfatophilum* and *Rhodospirillum rubrum* [54]. It involves two carboxylation reactions:

$$\text{Acetyl-CoA} + CO_2 + 2\text{FdH} \longrightarrow \text{Pyruvate} + \text{CoA} + 2\text{Fd}$$

catalyzed by pyruvate synthease and,

$$\text{Succinyl-CoA} + CO_2 + 2\text{FdH} \longrightarrow \alpha\text{-Oxoglutarate} + \text{CoA} + 2\text{Fd}$$

(FdH = reduced ferredoxin)

catalysed by α-oxoglutarate synthase. The overall energy input for the cycle may be given by:

$$4CO_2 + 3ATP + NADH_2 + NADPH_2 + \text{Flavin } H_2 + 4FdH \longrightarrow \text{Oxaloacetate} + AMP + 2ADP + 4Pi + NAD + NADP + \text{Flavin} + 4Fd.$$

There is evidence that the acetyl-CoA and RuDP cycle function simultaneously in photosynthetic bacteria [54]. There is no reference to the operation of this cycle in C_1 utilizing organisms and aerobic chemoautotrophs [33, 54].

Methane, methanol and methylamine assimilation

Assimilation generally occurs by the serine pathway or the ribulose monophosphate pathway. It is worth recalling here that *M. denitrificans*, however, assimilates methanol autotrophically.

(a) The serine pathway.

The serine pathway operates during growth on a variety of C_1 compounds varying in reduction level between methane and formate. The majority of bacteria utilizing this pathway are facultative methylotrophs. Quayle [33] therefore, speculates that "the serine pathway may turn out to be as widely distributed amongst this type of organism as is the RuDP cycle amongst autotrophs". This pathway has been extensively investigated using isotopes, mutant organisms and folate antagonists of growth [33]. The occurrence of the pathway, the scheme of reactions and the enzymes involved are shown in Table I and Fig. 4. Formate enters the cycle by the formation of N^{10}-formyl FH_4. Methane, methanol and methyl-amine enter by the formation of HCHO [33, 62]. The presence of isoenzymes of serine hydroxymethyltransferase was recently demonstrated in a facultative methylotroph grown on methane, methanol and succinate [63]. Serine is converted to glycerate by the activities of serine-glyoxylate amino-transferase and hydroxypyruvate reductase, the key enzymes of this pathway. A possibility that in *Ps.* MA, serine is converted by serine dehydratase into pyruvate which is further converted by malic enzyme into malate was suggested [64]. However, the detection of low and variable level of serine dehydratase and high level of hydroxypyruvate reductase and phosphoenol pyruvate carboxylase has now indicated that the serine pathway is the major metabolic route of C_1 assimilation in *Ps.* MA also [64].

The operation of the serine pathway requires the synthesis of one molecule of glycine (in reaction 4, Fig. 4) for each molecule of PGA formed. There

is evidence that the immediate precursor of glycine is glyoxylate [33, 56]. The mechanism of the net synthesis of glyoxylate from one-carbon units has been intensively investigated [33, 56]. Glyoxylate is suggested to be formed from malate, 4-malyl-CoA or isocitrate by the activitivies of malate lyase, malyl-CoA lyase or isocitrate lyase [65, 56].

(b) The ribulose monophosphate cycle

The RuMP cycle of HCHO fixation has been clearly elucidated ([56, 66] ; Fig. 5). The cycle effects a net conversion of HCHO units derived from methane or methanol into fructose-6-phosphate by the key enzyme hexulose phosphate synthase and phospho-3-hexulose isomerase. Regeneration of ribulose-5-phosphate is accomplished by the fructose diphosphate aldolase variant of the cycle as in the RuDP cycle or by the participation of enzymes of the Entner-Doudoroff pathway [66]. The RuMP cycle is viewed as a variant of the RuDP cycle with the reductive steps stripped out. The RuMP cycle operates in obligate methylotrophs with Type I membrane system, while the serine pathway occurs in those with Type II membranes [56]. It has lately been found to occur in facultative methylotrophs as well [67, 68].

An interesting situation where the RuMP cycle also constitutes a cyclic mechanism for the complete oxidation of HCHO to CO_2 by glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase and without involving NAD or DCPIP-linked HCHO and formate dehydrogenase, is reported in two methylotrophs [81].

Energy requirements of the RuDP, RuMP and serine pathway

Strom *et al.* [66] have calculated the energy requirements of each cycle for the synthesis of the common output molecule, pyruvate:

(1) Ribulose diphosphate cycle:

$$3CO_2 + 7\ ATP + 5\ NAD\ (P)\ H + 5H^+ + 5\ H_2O \longrightarrow Pyruvate + 7\ ADP + 5\ NAD\ (P)^+ + 7\ Pi$$

(2) Serine pathway:

$$2\ HCHO + CO_2 + 2\ ATP + 2\ NADH + 2H^+ + FP \longrightarrow Pyruvate + 2\ ADP + 2\ Pi + 2\ NAD^+ + FPH_2$$

(FP = flavoprotein of succinate dehydrogenase).

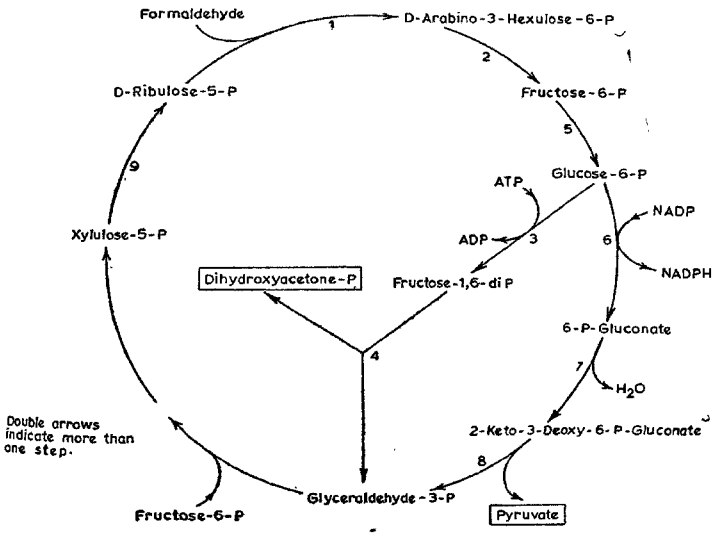

FIG. 5. The ribulose monophosphate cycle of formaldehyde fixation [66]
Reactions 1–9 catalysed by:

1. 3-Hexulose phosphate synthase
2. Phospho-3-hexulose isomerase
3. Phosphofructokinase
4. Fructose diphosphate aldolase
5. Phosphoglucoisomerase
6. Glucose-6-phosphate dehydrogenase
7. Phosphogluconate dehydratase
8. Phospho-2-keto-3-deoxygluconate aldolase
9. Ribulose 5-phosphate 3-epimerase.

If the organisms can oxidize HCHO to CO_2 *via* pyridine nucleotide linked dehydrogenase and thus gain 2 molecules of NADH, the above equation can be modified to

$$3\ HCHO + 2\ ATP + FP \longrightarrow Pyruvate + 2ADP + 2Pi + FPH_2$$

(3) Ribulose monophosphate cycle:

(*a*) Entner-Doudoroff variant

$$3\ HCHO + NADP^+ \longrightarrow Pyruvate + NADPH + H^+$$

(*b*) Fructose diphosphate aldolase variant

$$3HCHO + ADP + Pi + NAD^+ \longrightarrow Pyruvate + ATP + NADH + H^+$$

Comparison of the equations shows that the energy requirements for the different pathways decrease in the order $1 > 2 > 3a > 3b$. It may therefore be predicted that the most efficient C_1 utilizing organisms will be found to utilize the RuMP cycle [66]. In contrast, autotrophs on formate and methanol should be considered as the least efficient. This fact stimulates much interest in a study of the occurrence of bacteria of these typpe

E. AUTOTROPHY AND METHYLOTROPHY

In recent years a study of the well known 'facultative' and 'obligate' autotrophs with reference to their organic carbon metabolism has become essential in an understanding of their physiological roles in the environment. This has been emphasized in the reviews by Rittenberg [48] and Kelly [45]. Similarities between the 'obligate methylotrophs' and 'obligate chemolithotrophs' and their evolutionary sequence have been analysed by several authors [33, 45, 47]. It is surprising, however, that there is no reference to a systematic screening of the methylotrophic potential of facultative and obligate autotrophs, except for a few reports mainly dealing with formate and methanol in some species [3, 34, 44]. A re-evaluation of the ability of chemo- and photoautotrophs to utilize C_1 compounds in media with different growth factors and pH values [69] and in continuous culture [70] has been called for. The problems in positioning of chemolithotrophs among evolving autotrophs has also been discussed by McFadden [71]. He points out that there is a paucity of data on the structure, function and regulation of RuDP carboxylase in the various organisms and that such knowledge will yield much information about the evolution of autotrophy. There is also a dearth of knowledge on the different energy coupling mechanisms in sulphur and nitrite-oxidizing bacteria [47, 72]. Lastly there is an interesting suggestion that methane utilizers may have evolved from an ammonia oxidizing autotroph [33, 56].

CONCLUDING REMARKS

It is obvious that an explosive accumulation of valuable information has occurred since the discovery of oxalate and one-carbon metabolizing

bacteria, The sophistication achieved in detecting and evaluating the different one-carbon metabolic pathways has been very useful in determining the mode of growth of these microorganisms, their role in nature and their possible use as source of single cell protein. The mechanism of autotrophic heterotrophic conversions on one-carbon compounds and their *in vivo* regulation, however, remains obscure. We believe that a clearer understanding of such dynamic processes on these simple carbon compounds would give interesting insights into the evolution of autotrophy and heterotrophy.

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