

Single Cell Protein Production on C₁ Compounds. The Bioefficiency

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Pertaining to the optimal SCP production from one-carbon compounds, a theory is presented to support that the reasons for the unusual behavior of biomass yields in methylotrophs are kinetic rather than biosynthetic. The theory stresses the branching of the carbon flow toward either carbon dioxide or biomass and explains easily why yields change with dilution rate, temperature, pH, and other culture conditions, although the actual efficiency remains unchanged. The various available methods for assessing the bioenergetics of a process are discussed, with a view to derive useful conclusions from existing data and to design optimally future experiments. Thus, comparisons of computed to experimental yields suggest that the P/O ratio for methanol oxidation is 1, that for formaldehyde oxidation to formate is 1 or 3, and the one for formate oxidation possibly 3. For the serine pathway, the bioenergetics of either the icl^- or the icl^+ variant appear to explain adequately the experimental yields under the assumptions of the proposed theory.

1. Introduction

1.1. Significance and Scope. The production of single cell protein (SCP) from reduced one-carbon (C₁) compounds is most likely to become the predominant process of production of single-cell protein in the near and distant future, for a number of reasons that have been detailed in the past (Cooney, 1975) and may be summarized in the following. Reduced C₁ compounds (methanol, formaldehyde, and formate) are completely soluble in water and thus, unlike any gaseous or liquid hydrocarbons, additional mass-transfer limitations are avoided. Moreover, C₁ compounds may be obtained in an absolutely pure form, thus avoiding any toxicological questions on the final product, which have arisen for products deriving from liquid hydrocarbons, for example. Substrate availability and cost provide additional advantages to reduced C₁ compounds, as they can be readily derived from coal, natural gas, but also from renewable resources (should that become necessary), while they are produced in large quantities, and it is expected that their production volume will double within the present decade. There are also a number of more specific advantages that will become clear from the discussion that follows in this paper.

The biomass yield ($Y_{X/S}$) is perhaps the single most important parameter that determines the economic feasibility of any SCP process on reduced C₁ compounds (Cooney, 1975; Papoutsakis et al., 1978a), not only because of the lower substrate cost per weight unit of product, but, perhaps even more importantly, because of the lower specific oxygen demand ($Y_{O_2/X}$) and lower heat removal needs that accompany higher biomass yields. The relation between the specific oxygen demand and the biomass yield is adequately represented by (Papoutsakis et al., 1978a)

$$Y_{O_2/X} = \frac{1.5}{Y_{X/S}} - 1.33 \quad (1)$$

The specific heat of fermentation of the process, being directly proportional to the specific oxygen demand, has the same functional dependence on the biomass yield.

Indeed, improvements of the biomass yield by only a few percentage points would have a major impact on the economics of the process. It is in recognition of this fact that a systematic effort to improve the biomass yield has been recently reported for the well-known ICI (Imperial Chemical Industries) process (Walgate, 1980; Windass et al., 1980). A 3-7% improvement of the biomass yield has been reported for this process which utilizes the bacterium *Methylophilus methylotrophus* (AS1) growing on methanol. Using a genetic-engineering technique, that effort concentrated on improving the efficiency of nitrogen (ammonia) assimilation by inserting the *E. coli* gene of glutamate dehydrogenase in AS1 while thermally rendering inoperative, by the 37 °C process, the "inefficient" glutamate synthase. The present theoretical work represents an effort in a different direction toward improving the biomass yield, but also the productivity of the SCP process, over and above any improvements that would derive from an improved efficiency of nitrogen utilization. The scope of the present work also includes an effort to critically assess the available or possible routes and methods for achieving the aforementioned goal, to look at the available experimental information in perspective, and to derive conclusions pertinent to a better understanding of the process bioenergetics.

1.2. Past Work. Biomass and other associated ($Y_{O_2/X}$, Y_{ATP}) yields are determined by the efficiency with which aerobic microorganisms conserve energy via substrate-level and oxidative phosphorylation and subsequently use this energy for growth (Stouthamer, 1977). This fundamental idea has been the basis of a number of calculations for predicting biomass and other yields of C₁-compound utilizers (methylotrophs) (Harrison et al., 1972; van Dijken and Harder, 1975; Harder and van Dijken, 1976; Anthony, 1978). Theoretical computations like the above always assume that there exists an ideal coupling between energy (in the generalized sense, i.e., in the form of NAD(P)H and/or ATP) yielding and energy requiring processes and thus the predicted values merely represent upper bounds for the quantities under consideration. It is apparently

possible that more energy and/or NAD(P)H may be produced than it is required and that such an "uncoupling" will result in lower than theoretically predicted biomass yields; this point will be pursued in detail in the present paper. In this light, although theoretical predictions have been used to determine values for P/O ratios based on experimental biomass yields (e.g., Goldberg et al., 1976), such P/O values should be viewed and used with due care.

It is yet in principle possible to measure directly the P/O ratios that correspond to the oxidation of the various substrates and their derivatives (e.g., Tonge et al., 1977; O'Keefe and Anthony, 1978; Drozd and Wren, 1980) on the basis of the chemiosmotic theory, but this method is not without its pitfalls either. First, it is difficult to assess the effect of further oxidation of the substrate, and second if a second oxidation route is possible, as we shall see shortly, further uncertainties do arise. Other difficulties may also be encountered and atypical findings have resulted (Tonge et al., 1976). Thus, confusing conclusions have been and may be produced. Still, even if accurate P/O information may be guaranteed, NAD(P)H limited biomass yields (Anthony, 1978) or multiple oxidation schemes render such P/O information insufficient for definite biomass-yield predictions.

In short, none of the three methods discussed above, that is, the direct measurement of the biomass yield, the direct measurement of the various P/O ratios, or the theoretical computation of the biomass yield, is able to safely assess the biological efficiency of the particular microorganism and thus to serve as the sole criterion toward selecting microorganisms for SCP production. We have of course assumed that pertinent biochemical information will be available for the last two methods. It is yet not to be implied that the above three methods are exactly equivalent for the selection process; in fact, they are complementary in a sense.

Various reasons have been proposed to account for the discrepancies between experimental and theoretical yields, which among others include an energy requiring transport of the substrate (CH₃OH) across the cell membrane, a varying ATP yield from the reduced coenzymes (van Dijken and Harder, 1975), and repressive effects of carbon sources upon the efficiency of oxidative phosphorylation (Goldberg et al., 1976). It has been also theorized (Goldberg et al., 1976) that a major difficulty in understanding and predicting biomass yields lies in the fact that the natural electron acceptor of the oxidation step from methanol to formaldehyde is yet to be identified. All of the so far proposed explanations for the lower (than the theoretical) experimental yields have essentially ignored the effect of the growth conditions on the biomass yield, and as such they are not specific to any growth parameters.

One of the most peculiar growth macro-characteristics of methylotrophs is the strong dependence of their biomass yield on the specific growth rate (μ), both in continuous cultures (Battat et al., 1974; Ballerini et al., 1977; Papoutsakis et al., 1978a; 1981) but also in batch cultures (Papoutsakis et al., 1978a; Reuss et al., 1974). Thus the biomass yield, in steady-state continuous (chemostatic) culture, is a bell-shaped function of the dilution rate ($D = \mu$), which increases initially reaching a maximum, and then decreases gradually as the washout dilution rate is approached. In batch cultures, the (average) biomass yield generally decreases with decreasing specific growth rates or increasing initial substrate concentrations. Even more peculiarly, the biomass yield changes significantly also during the course of a batch (Krug, 1977) or extended batch culture (Reuss et al., 1974). Unusually strong de-

pendences of the biomass yield on temperature (Ballerini et al., 1977; Papoutsakis et al., 1978a) and pH (Papoutsakis et al., 1981) have also been reported. Finally, we have recently reported (Papoutsakis et al., 1981) that despite the fact that formate provides NADH, and thus ATP, through its oxidation, it appears to have no effect on improving the yield on its cosubstrates in mixed-substrate cultures of an RMP-cycle microorganism (see also section 2.1).

Several attempts have been made (Reuss et al., 1974; Ballerini et al., 1977; Rock et al., 1978) to explain the biomass yield variation with the specific growth rate based on the familiar equation

$$-r_s = \frac{r_x}{Y_{\max}} + mX \quad (2)$$

or its equivalent form

$$\frac{1}{Y_{X/S}} = \frac{1}{Y_{\max}} + \frac{m}{\mu} \quad (3)$$

but such an explanation does not appear well justified. According to eq 3, $Y_{X/S}^{-1}$ is a linear function of μ^{-1} such that in the limit where the specific growth rate becomes infinitely large, the biomass yield approaches the maximum yield, Y_{\max} . Clearly then, eq 3 predicts a monotonically increasing $Y_{X/S}$ with μ (or D , for the steady-state continuous cultures) in the form of saturation-type kinetics. However, as we have discussed above, this is not true for the C₁-compound utilizers. Besides, eq 2 cannot explain why the yield changes during the exponential phase of batch cultures where μ is constant.

1.3. Specific Objectives. The following three questions will be entertained in the present paper. (i) What could be the possible reason(s) for the unusual biomass yield behavior of methylotrophs? (ii) What are the values of the P/O ratios for the various oxidative reactions that affect the biomass yield of methylotrophs? (iii) What is the best strategy for obtaining the answers to (i) and (ii) and what are the practical implications of these answers?

2. Methods

We shall concern ourselves only with the two major pathways of one-carbon-compound utilization and their variants: the Ribulose Monophosphate (RMP) cycle (Strøm et al., 1974) and the serine pathway (SP) (Quayle, 1972; 1975; Harder et al., 1973). The other pathways (Quayle, 1972; Strøm et al., 1974; Anthony, 1978) are of no practical importance for our present objectives. A number of experimental findings may be crystallized into a series of, presently so-called, assumptions. Some of these assumptions have been well established and amply discussed in the past; for these we provide only the appropriate references. The rest are discussed as necessary following their statement. These assumptions are necessary and/or enlightening for and furnish rigor and system to the methods and discussion that follow. The discussion and any conclusions related to objective (i) apply perhaps also to methylotrophic yeasts, although it is rather clear (van Dijken et al., 1978) than yeasts do not utilize the RMP cycle. Thus, experimental evidence from yeasts is used only for those assumptions that are necessary in the discussion of objective (i), i.e., assumptions 4, 5, 6, 9, and 12.

2.1. Assumptions. (1) The formula used for cell material is C₄H₈O₂N (MacLennan et al., 1971; van Dijken and Harder, 1975; Goldberg et al., 1976; Anthony, 1978).

(2) The ATP yield (Y_{ATP}) for assimilation of phosphoglycerate (PGA) is 10.5 g/mol of ATP; the reductant for biosynthesis from PGA is NAD(P)H; the nitrogen source

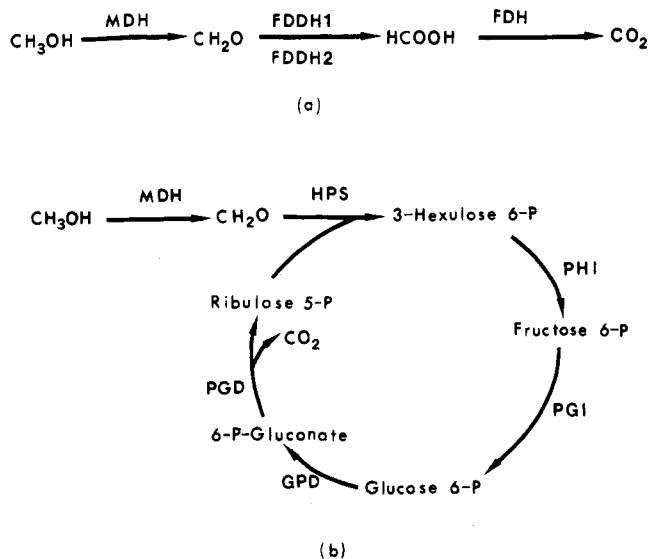
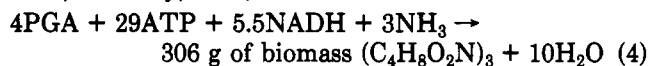


Figure 1. The two possible complete oxidation schemes for C_1 compounds: (a) oxidation through formate and (b) cyclic oxidation scheme (Strøm et al., 1974).

is ammonia. Thus, the following equation for assimilation of PGA into cell material is used (van Dijken and Harder, 1975; Anthony, 1978)



(3) The transport of the growth substrates into the cells does not require any energy (ATP) expenditure (Anthony, 1978).

(4) The oxidation level of carbon incorporation into biomass is formaldehyde for yeasts and the RMP-cycle bacteria (Strøm et al., 1974; Krug et al., 1979a) and formaldehyde and/or formate (and CO_2) for the SP bacteria (Large and Quayle, 1963; Quayle, 1972). For the latter, the incorporation extents at the formaldehyde or formate levels are not known and may be expected to vary with growth conditions and among various bacteria.

(5) Formate is oxidized to CO_2 by an NAD-linked formate dehydrogenase (FDH) (Quayle, 1972; Anthony, 1978; Krug et al., 1979a). In yeasts and RMP-cycle bacteria, formate can thus only be oxidized to CO_2 and cannot be incorporated into biomass; i.e., reduction of formate to formaldehyde does not occur (Hirt et al., 1978; Papoutsakis et al., 1981; Krug et al., 1979a).

(6) Serine-pathway bacteria grow on any single or any combination of methanol, formaldehyde, and formate (Quayle, 1972; Goldberg et al., 1976). RMP cycle bacteria and methylotrophic yeasts grow (continuously, at least) only on methanol although they may readily utilize (but not grow alone on) formaldehyde and/or formate (Reuss et al., 1974; Hirt et al., 1978; Papoutsakis et al., 1978a; Krug et al., 1979a).

(7) Both of the RMP-cycle variants, the fructose diphosphate (FDP) variant and the Entner-Doudoroff (ED) variant, operate in RMP bacteria (Strøm et al., 1974; Anthony, 1978), albeit in unknown extents each.

(8) In the serine pathway, the formation of malyl-CoA from malate uses 1 molecule of ATP regardless of mechanism (Anthony, 1978; Krug et al., 1979a). In the icl^+ variant, the oxidation of acetyl-coA to glyoxylate yields NADH and the reduced flavoprotein of succinate dehydrogenase (FPH_2) (Quayle, 1972; Krug et al., 1979a). The P/O ratio of the oxidation of FPH_2 is taken to be 2 regardless of the P/O ratio for NADH oxidation. For the icl^- variant only the extreme possibility is examined, where no reducing power is produced from the oxidation of

acetate to glyoxylate (Anthony, 1978; Krug et al., 1979a).

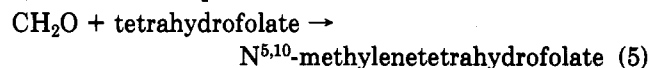
(9) In RMP cycle bacteria, both the oxidation schemes (a) and (b) of Figure 1 operate for methanol and/or formaldehyde oxidation, in unknown extents each (Strøm et al., 1974; Anthony, 1978). In serine pathway bacteria, only the oxidation scheme of Figure 1a operates. Yeasts possess only the scheme of Figure 1a (Schlanderer et al., 1978).

(10) Methanol oxidation, in either RMP cycle or SP bacteria, is mediated by a methanol dehydrogenase (MDH) whose function is independent of NAD^+ . Although its natural electron acceptor is not known, the enzyme is being assayed using the (artificial electron carrier) system PMS-DCPIP. The enzyme is possibly a pteridoprotein and interacts perhaps at the level of cytochrome c with the electron transport system (Anthony, 1978). As such, the P/O ratio for methanol oxidation is most likely not smaller than 1 and not larger than 2. The enzyme also oxidizes formaldehyde to formate at a relative rate apparently varying among bacteria.

(11) Formaldehyde is oxidized by the above MDH and/or another PMS-DCPIP formaldehyde dehydrogenase (FDDH1) which energetically will be indistinguishable from MDH. An NAD-linked formaldehyde dehydrogenase (FDDH2) operates in all SP and many RMP-cycle bacteria (Krug et al., 1979a). The extent of the involvement of each of FDDH1 and FDDH2 in formaldehyde oxidation to CO_2 via formate is unknown and may be expected to vary among various bacteria.

(12) The key enzymes of C_1 -compound oxidation (MDH, FDDH1, FDDH2, FDH, PGD) and incorporation (HPS, THF, SHT) are inducible and many of them repressible (Papoutsakis et al., 1978b; Beinor, 1978; Krug et al., 1979a; Large and Quayle, 1963; Ben-Bassat and Goldberg, 1977; Krug, 1979; Schlanderer et al., 1978; Sahn, 1977).

(13) For the serine pathway, and for the case of direct formaldehyde incorporation into biomass (see assumption 4) the first incorporation reaction



which may or may not be enzymatically catalyzed (Large and Quayle, 1963), is assumed to have no energy requirements (Anthony, 1978).

In regard to assumption 2, the 10.5 figure for Y_{ATP} is an experimental one. Yet, if a higher Y_{ATP} is chosen, its effect on the biomass yield is disproportionately low (Anthony, 1978). Assumption 3 appears well accepted among most researchers although there is some recent evidence that formaldehyde may be actively transported in the RMP-cycle *Methylomonas* L3 (Krug et al., 1979b). This and formate transport will be subsequently discussed in more detail. In clarifying assumption 4, although carbon from either CH_3OH or CH_2O can enter into cell mass through reaction 5 for serine pathway organisms, we cannot exclude the possibility that carbon may also be incorporated into cell mass through HCOOH. Indeed, Large and Quayle (1963) demonstrated that when the serine pathway operates, the enzymes responsible for the incorporation of HCOOH into cell mass were present when the cells were grown on either HCOOH or CH_3OH . Regardless of whether these enzymes are coordinately induced or derepressed along with other enzymes of C_1 metabolism or whether they are induced by intracellular HCOOH, the fact remains that they may be responsible for a fraction of the total amount of C_1 units fixed into cell mass. As-

Table I. Computed Biomass Yields on Methanol with Both the RMP-Cycle Variants

X	Y	Z	$Y_{\text{CH}_3\text{OH}}$	
			ED variant	FDP variant
1	1	2	0.53	0.56
1	1	3	0.56	0.59
2	2	3	0.70	0.71
1	2	2	0.57	0.60
1	3	3	0.61	0.63
2	3	3	0.71	0.73

assumption 7 is based on the experimental fact that the enzymes for both variants exist in RMP-cycle bacteria (Strøm et al., 1974). The hypothesis (in assumption 8) that the P/O ratio for FPH₂ is 2 is perhaps more justified (Netrusov et al., 1977) than Anthony's one (1978) that the P/O ratio is one less than that of NADH. This, however, will have no effect on our conclusions, since as we shall see, most probably Z = 3 and the two hypotheses become equivalent. Also, in assumptions 8, the icl⁻ variant is, perhaps, more efficient than assumed. Thus, biomass yield computations with both icl⁺ and icl⁻ variants will cover all the actual possibilities of biomass yields for SP bacteria. In reference to assumption 9, SP bacteria do not possess the enzymes of the cyclic oxidation scheme of Figure 1b (Strøm et al., 1974; Ben-Bassat and Goldberg, 1977). Finally, in regard to assumption 12, the following more detailed reference information is in order. Beinor (1978) studied and showed experimentally that the enzymes HPS, MDH, and FDDH1 are inducible and repressible in *Methylomonas* L3 (Hirt et al., 1978). Extensive experimental results (Krug, 1979) have shown that PGD is also an induction-repression regulated enzyme in strain L3.

2.2. Assimilation Pathways. Ribulose Monophosphate (RMP) Cycle. In view of assumptions 4 and 7, the following equations describe the formaldehyde conversion to PGA when the RMP cycle operates (Strøm et al., 1974) (in simplified form)

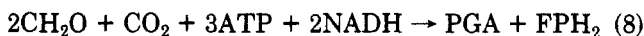


for the FDP variant, and

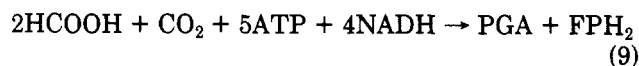


for the ED variant.

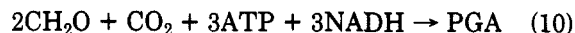
Serine Pathway (SP). If assumptions 4, 6, 8, 9, 11, and 13 are taken into account, the following four equations may be written for the production of PGA from either formaldehyde or formate, as appropriately, and CO₂



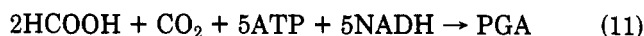
for the icl⁺ variant and incorporation at the formaldehyde level



for the icl⁺ variant and incorporation at the formate level



for the icl⁻ variant and incorporation at the formaldehyde level, and



for the icl⁻ variant and incorporation at the formate level.

2.3. Computation of Biomass Yields. Biomass yields on any C₁ substrate, given the assimilation pathway (section 2.2) and oxidation scheme (Figure 1), are computed by eliminating PGA from eq 4 and the appropriate assimilation equation (eq 6–11). Parts of or the complete oxidation schemes of Figure 1 are used, first, to bring if necessary the C₁ compound to the oxidation level (see eq 6–11) at which incorporation into biomass occurs, and second, to meet the needs of the resulting equation for NADH and ATP. The latter requires that the P/O ratios for the oxidation of the reduced coenzymes, involved in the oxidation schemes, be known or specified. It is clear in view of assumptions 4, 7, 9, and 11 that for those cases where both the oxidation schemes and/or two assimilation schemes, (e.g., eq 6 and 7) and/or two energetically different enzymes are involved (see assumption 11), the actual biomass yields will range between the values computed with each oxidation scheme or enzyme separately, for the extent of involvement of each scheme or enzyme is not known and is most probably varying as we elaborate subsequently. Note also that when YH₂ (the reduced coenzyme of formaldehyde dehydrogenase) is NADH₂, the two oxidation schemes of Figure 1 are energetically indistinguishable. In those entries of Tables I and II where the P/O ratio assigned to YH₂ is the same to that of NADH₂ (which we will take to be only 2 or 3) it will always be that YH₂ ≡ NADH₂. Finally, the actual theoretical yields may be higher by 2.5% (to account for ash (van Dijken and Harder, 1975)) than the computed yields.

2.4. Three Axioms. The following three statements, redundant though they may look, will provide further economy and clarity in the development of the arguments and conclusions that follow.

Axiom 1. Enzyme repression or derepression (induction) mechanisms are continuous-process functions of their repressor or inducer concentrations, following (negative or positive) saturation kinetics.

Table II. Computed Biomass Yields on Methanol, Formaldehyde, and Formate with the Serine Pathway^a

X	Y	Z	C ₁ -unit incorporation into cell mass at the CH ₂ O level				C ₁ -unit incorporation into cell mass at the HCOOH level					
			$Y_{\text{CH}_3\text{OH}}$		$Y_{\text{CH}_2\text{O}}$		$Y_{\text{CH}_3\text{OH}}$		$Y_{\text{CH}_2\text{O}}$		Y_{HCOOH}	
			icl ⁻	icl ⁺	icl ⁻	icl ⁺	icl ⁻	icl ⁺	icl ⁻	icl ⁺	icl ⁻	icl ⁺
1	1	2	0.38*	0.45*	0.31	0.36	0.29*	0.32*	0.26	0.31		
1	2	2	0.44	0.52	0.38	0.44	0.41	0.48	0.35	0.41	0.11	0.13
1	1	3	0.38*	0.45*	0.33	0.41	0.29*	0.32*	0.27	0.32		
					(0.24)	(0.29)			(0.19)	(0.28)		
1	3	3	0.47	0.55	0.43	0.50	0.45	0.52	0.41	0.47	0.13	0.15
					(0.36)	(0.42)			(0.34)	(0.39)		

^a The $Y_{\text{CH}_2\text{O}}$ values in parentheses have been computed with the additional assumption that formaldehyde is actively transported upon the expenditure of 1 mol of ATP/mol CH₂O. For biomass-yield values marked by an asterisk, the computation does not depend upon the exact values of X and Y as long as X ≠ 0, Y ≠ 0, and YH₂ ≠ NADH₂; for example, both combinations (2, 2, 3) and (1, 1, 3) give the same methanol-yield value for an icl⁻ bacterium.

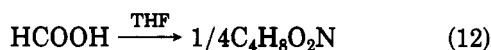
Axiom 2. If a methylotroph growing on a C_1 compound displays a growth efficiency equivalent to that of an appropriate assimilation equation (eq 6–11), at least the same growth efficiency is expected for growth of the microorganism on another C_1 compound, and thus the indicated assimilation equation may be safely used to predict *obtainable* biomass yields on this other C_1 compound.

Axiom 3. If a P/O-ratio value for an oxidation reaction in a methylotroph is safely derived from growth data on a C_1 compound, at least that P/O-ratio value should be expected for the same reaction and growth on an other C_1 compound.

Axiom 2 would allow, for example, the derivation of safe low bounds for biomass yields of a methylotroph from the development of, perhaps empirical, an assimilation equation from reliable experimental data, despite the possible lack of exact biochemical information (e.g., for the icl⁻ variant of the SP). On the basis of this axiom, only the high experimental values for biomass yields should be retained, the lower values being rejected for reasons to be subsequently elaborated. Similarly, we may treat P/O-ratio values on the basis of axiom 3.

3. Results and Discussion

3.1. The Kinetic Concepts: Branching of the C_1 -Unit Flow. As we saw already, in methylotrophs there exist two distinct carbon-flow processes; one is the assimilation process (see eq 6–11) and the other is the C_1 -compound oxidation process (Figure 1) which produces NADH and energy required for assimilation. In computing biomass yields in the previous section, we implicitly assumed that the two processes are finely tuned, so that only so much NADH and/or ATP is produced through oxidation as is required for assimilation. This fine tuning of the two processes produces the maximal possible biomass yields. Thus, achieving maximal yields with methylotrophs involves critical control of the branching of C_1 unit flow toward the two final products, CO_2 and cell mass. The branching between catabolic and anabolic pathways is determined by both the induction of enzymes (assumption 12) and the regulation of their activity once induced, while the ratio of the two branch-flows determines the biomass yield. Consider, for example, the case of exclusive carbon incorporation at the formate level (serine pathway). Since the overall assimilation reaction is



the biomass yield on HCOOH may be represented by

$$Y_{HCOOH} = (\text{mol of HCOOH converted to } C_4H_8O_2N) / (\text{mol of HCOOH totally consumed}) \quad (46) = (0.554) \frac{r_{thf}}{r_{thf} + r_{fdh}} = \frac{0.544}{1 + r_{fdh}/r_{thf}} \quad (13)$$

where r_{fdh} is the rate of HCOOH conversion to CO_2 catalyzed by FDH, and r_{thf} is the rate of incorporation of HCOOH into cell mass catalyzed by THF. We may obviously also write

$$r_{thf} = r_{thf}((THF), (HCOOH), T, pH) \quad (14)$$

$$r_{fdh} = r_{fdh}((FDH), (HCOOH), (NAD), T, pH) \quad (15)$$

where the concentrations in the above two expressions are understood to be intracellular. Thus, if a formate yield of 0.15 (see Table II) can be achieved, we may obtain from (13) that

$$r_{fdh}/r_{thf} = 2.63 \quad (16)$$

Also, it may be readily shown (Papoutsakis et al., 1978a) that under steady-state conditions

$$\mu = D = Ar_{thf} \quad (17)$$

for growth on formate, where A is a physical constant of the system.

Consider now, for example, chemostatic, steady-state growth under formate limiting conditions. Since the residual extracellular (and thus the intracellular) formate concentration changes with dilution rate and so do the concentrations of FDH and THF (which are obviously induced by formate or a product derived from formate, assumption 12), according to the molecular theory of enzyme induction (axiom 1), eq 16 will possibly hold only for a particular value (or a narrow region of values) of D , according to eq 14 and 15. This will become possible only if the culture conditions such as temperature, pH, and other nutrients will permit the establishment of concentrations of cofactors, HCOOH, FDH, and THF such that eq 16 is satisfied for a particular value of D . Under any other conditions, more NADH or energy will be produced through formate oxidation than required for formate incorporation into biomass. Clearly, to any submaximal Y_{HCOOH} will correspond a unique r_{fdh}/r_{thf} value obtained from eq 13. It is, of course, difficult to find the exact conditions for the maximal yield, and extensive experimentation will be required for each microorganism. It should also be emphasized that comparison of theoretical yields to continuous steady-state (chemostatic) yields is useful only when a variety of growth conditions and dilution rates have been examined. Finally, there is no guarantee that every microorganism is capable of achieving the maximal yield suggested by its biochemical microcharacteristics.

From a theoretical point of view, it is interesting to note that according to eq 13, the yield will change by only changing either r_{fdh} or r_{thf} , which according to eq 14 and 15 can happen by changing any of the concentrations, (HCOOH), (THF), (FDH), (NAD) or any of the culture conditions, T , pH, or other-nutrient concentrations. In other words, once the carbon-flow-branching concept is recognized, the possibility of a highly variable biomass yield is subject to only trivial assumptions which seem to be always satisfied.

The case of carbon incorporation exclusively at the formate level, which we analyzed above as an illustrative example, is certainly the simplest possible. In contrast, growth on methanol through the RMP cycle, with both the oxidation schemes of Figure 1 and with both FDDH1 and FDDH2 operating simultaneously, is the most complicated but yet the most important practically case. This situation presents a larger number of factors that may influence the biomass yield as is indicated by the equations

$$Y_{CH_3OH} = (\text{mol of } CH_3OH \text{ converted to } C_4H_8O_2N) / (\text{mol of } CH_3OH \text{ totally consumed}) \quad (32) =$$

$$(0.797) \frac{r_{hps} - r_{pgd}}{r_{hps} + r_{fd1} + r_{fd2}} = (0.797) [1 - (r_{pgd}/r_{hps})] / [1 + ((r_{fd1} + r_{fd2})/r_{hps})] \quad (18)$$

where r_{hps} is the rate of the formaldehyde-condensation reaction catalyzed by HPS

$$r_{hps} = r_{hps}((CH_2O), (\text{ribulose 5-P}), (HPS), T, pH) \quad (19)$$

r_{pgd} is the rate of CO_2 production from 6-P-gluconate catalyzed by PGD (Figure 1b) and for which we can write

$$r_{pgd} = r_{pgd}((6\text{-P-gluconate}), (PGD), (NADP), T, pH) \quad (20)$$

and r_{fd1} , r_{fd2} are the rates of formaldehyde oxidation to formate catalyzed by FDDH1 and FDDH2, respectively, and thus

$$r_{fd1} = r_{fd1}(\text{FDDH1}, (\text{CH}_2\text{O}), (Y_{fd1}), T, \text{pH}) \quad (21)$$

$$r_{fd2} = r_{fd2}(\text{FDDH2}, (\text{CH}_2\text{O}), (\text{NAD}), T, \text{pH}) \quad (22)$$

with Y_{fd1} being the oxidized form of the FDDH1 coenzyme. Note also that the rate $r_{hps} - r_{pgd}$ is also affected indirectly by the further branching of the carbon flow which results from the two RMP-cycle variants (assumption 7). The yield expressions for any other cases may be readily derived from or similarly to eq 18.

A number of experimental findings pertain to the above discussion and provide more specific information: (a) the biomass yield has only one inflection point (the maximum point) (Papoutsakis et al., 1978a; Battat et al., 1974; Ballerini et al., 1977; Papoutsakis et al., 1981); (b) many submaximal and some maximal yields can be obtained under two different culture conditions (Papoutsakis et al., 1978a; Papoutsakis et al., 1981); (c) maximal yields are obtained always under steady-state continuous (chemostatic) growth, while batch or unstable-steady-state yields are considerably lower than continuous chemostatic yields (Papoutsakis et al., 1978a; DiBiasio et al., 1979; Krug et al., 1979a); (d) high yields are associated with very low residual substrate concentrations while low yields are accompanied by high residual-substrate concentrations, either in continuous cultures (towards washout) or in batch cultures (Papoutsakis et al., 1978a; Battat et al., 1974; Ballerini et al., 1977; Held et al., 1978; DiBiasio et al., 1979; Papoutsakis et al., 1981).

A qualitative explanation for the above discussed behavior of the biomass yield can be provided by the model proposed by Papoutsakis et al. (1978b) (see also Krug et al., 1979b; Papoutsakis et al., 1981; Held et al., 1978) which is based on the role that formaldehyde plays in regulating growth. The discussion and ideas of this section pertain to both methylotropic bacteria and yeasts.

3.2. Computed and Experimental Yields and P/O Ratios. Table I presents computed biomass yields as discussed in section 2.3, for both variants of the RMP cycle with assumptions 3, 4, 5, 7, 9, 10, and 11 taken into account, while Table II presents computed biomass yields for the serine pathway with the additional assumptions 6 and 8. Some of these values have been presented before (van Dijken and Harder, 1975; Anthony, 1978), but they are presented again here for completeness and to allow easy comparison with experimental values as follows.

Computed biomass yields serve basically two purposes. First, they allow us to estimate upper bounds for experimental yields, given the pertinent biochemical information. Secondly, together with proper experimental data and pertinent biochemical information, they enable us to conclude about P/O ratios, preferred pathways, or oxidation schemes and to decide whether we must accept or reject experimental data. The latter purpose is rather risky and difficult to accomplish. Conclusions deriving, for example, from experimental information of a single microorganism can be misleading. On the contrary, conclusions based on trends and synergetic information derived from several microorganisms (possessing similar enzymes and enzymatic reactions) are less prone to falsehood. What follows represents an extensive attempt to accomplish the second purpose primarily, as stated above, in the light of the foregoing discussion.

3.2.a. The RMP Cycle. For *Methylomonas* EP-1 (an RMP-cycle bacterium) we have reported (Papoutsakis et al., 1978a) the existence of the enzymes MDH, FDDH1, FDDH2, and FDH and a maximum biomass yield of 0.65. The RMP-cycle *Methylomonas* L3 possesses the enzymes MDH, FDDH1, and FDH and although capable of oxidizing formaldehyde through the cyclic oxidation scheme

of Figure 1b (Krug, 1979), oxidation through formate appears to be the in vivo preferred scheme (Hirt et al., 1978); a maximum biomass yield of 0.57 has been reported (Hirt et al., 1978; Papoutsakis et al., 1981) for this microorganism. Finally, *Pseudomonas* C possesses the enzymes MDH, FDDH1, FDDH2, and FDH but appears to oxidize formaldehyde primarily through the scheme of Figure 1b (Ben-Bassat and Goldberg, 1977); a maximum yield of 0.56 has been reported (Battat et al., 1974) for strain C.

The combination $X = 1, Y = 3, Z = 3$ (briefly denoted as (1, 3, 3)) appears to suit best the experimental findings for *Methylomonas* EP-1. One could perhaps say that the FDP variant is the preferred pathway, while FDDH1 does not appear to be responsible, in an appreciable extent, for formaldehyde oxidation. It seems most unlikely for two microorganisms that have so similar C_1 -compound oxidation and incorporation enzymes to have different electron transport systems. Thus, that the combination (1, 1, 3) is the most suitable for the experimental information concerning strain L3, was not unexpected. Further, it may be suggested from the figures of Table I, that the cyclic scheme of formaldehyde oxidation (Figure 1b), combination (1, 3, 3), does not appear indeed responsible for much of formaldehyde's oxidation. A few (2-4) percentage points differences between theoretical and maximal experimental yields may be attributed to uncertainties related to the ash content of dry biomass or to assumption 1. In this light, experimental yields for *Pseudomonas* C do not contradict the computed yields of Table I. Both the most likely combinations for strain C, (1, 2, 2) and (1, 3, 3) give values that overestimate somewhat the maximal experimental yield. As it was discussed earlier (sections 2.4 and 3.1), however, lower than expected yields should not be used to derive conclusions from comparisons with computed yields. For, either the microorganism is not capable to achieve the maximal yield for kinetic reasons (section 3.1) or the proper conditions to do so have not been attained.

The above suggested value $X = 1$ that corresponds to MDH agrees with the fact that the enzyme is possibly a pteridoprotein that interacts with the electron transport system at the level of cytochrome c (assumption 10) and thus only 1 ATP can be produced. Similarly for FDDH1 (see assumption 10). A number of reports on direct measurement of the P/O ratio (Tonge et al., 1977; van Verseveld and Stouthamer, 1978; Hammond and Higgins, 1978; Drozd and Wren, 1980) suggested also an $X = 1$ value for methanol oxidation.

Production of 3 ATP's from NADH ($Y = Z = 3$) in methylotrophs appears to be less accepted in the literature of direct P/O-ratio measurements. In fact, there exist only two reports (Hammond and Higgins, 1978; van Verseveld and Stouthamer, 1978) with a suggested P/O ratio for NADH oxidation higher than 2. However, if the observation reported by Papoutsakis et al. (1981), that formate transport is associated with a process that removes protons from the growth medium, is generally true, it would be understandable that lower than the actual P/O ratios would be measured. In any case, a P/O ratio of at least 2 for NADH oxidation appears rather well established (Netrusov et al., 1977; Dijkhuizen et al., 1977). Finally it should be noted that most of the work of direct P/O-ratio measurement has been performed with SP bacteria, and thus the presently derived conclusions should apply to the discussion of SP-bacteria yields which follows.

3.2.b. The Serine Pathway. Serine pathway bacteria in pure cultures are of less practical importance for SCP production (Papoutsakis et al., 1978a) since, as a comparison of Tables I and II would show, their biomass yields

Table III. Experimentally Determined Yields and Residual Substrate Concentrations of C_1 -Compound Utilizers Following the Serine Pathway. Data of Goldberg et al. (1976)

bacterium	S_{CH_3OH} , mg/L	Y_{CH_3OH}	S_{CH_2O} , mg/L	Y_{CH_2O}	S_{HCOOH} , mg/L	Y_{HCOOH}
<i>Pseudomonas</i> 1	<5	0.38	<5	0.32	<5	0.14
<i>Pseudomonas</i> 135	<5	0.38	<5	0.27	<5	0.15
<i>Pseudomonas</i> AM-1	<5	0.30	<5	0.24	<5	0.104
<i>Pseudomonas</i> M-27	<5	0.41	<5	0.28	150	0.097
<i>P. rosea</i>	<5	0.41	<5	0.24	166	0.073

are considerably lower than the corresponding RMP-cycle yields. They are however of considerable theoretical interest for comparative studies, as they can grow on formaldehyde and formate as sole substrates and they have a practical potential for SCP production in mixed cultures with RMP-cycle bacteria.

The biochemical characteristics that affect the biomass yield in SP bacteria are very uniform and well described within assumptions 4, 5, 6, 8, 10, 11, and 13. Thus, and in view of the discussion in the previous subsection 3.2.a, Table II presents computations only for combinations of X or $Y = 1$ and $Y = Z = 2$ or 3. Table III presents data of Goldberg et al. (1976), with chemostatic yields and residual-substrate concentrations for five SP bacteria, for comparison purposes.

First, we will concentrate on formate yields in order to infer information about the validity of eq 9 or eq 11, as the yield computation for this case involves only the Z -value uncertainty.

In view of the discussion in section 3.1 (see in particular item (d) in the third paragraph from the end) regarding the kinetic concepts and the yield expression, the S_{HCOOH} values of the last two entries in Table III suggest that the corresponding formate yields are far from the maximal obtainable. Because of the high residual formate concentrations (150 and 166 mg/L), we infer that either steady states were not achieved, or that the dilution rates (D) were close to wash out, or that a nutrient other than formate was growth limiting (Mateles and Battat, 1974). Moreover, the microorganisms which gave the lowest yield on formate gave the highest yield on methanol, which does not support a possibility that the low yields could be due to inherently low metabolic efficiency. Similarly, we tend to believe that higher yields on all substrates are obtainable for *Pseudomonas* AM-1. Indeed, MacLennan et al. (1971) reported a higher maximum μ value for strain AM-1 than the one reported by Goldberg et al. (1976), a maximum $Y_{CH_3OH} = 0.33$, and an 11% loss of carbon material, part of which at least was due to lysed cells. Thus, the actual yield on methanol could be as high as 0.41. For these reasons we are inclined to believe that yields on formate as high as 0.15 can be obtained for all SP bacteria and that culture conditions are responsible for the lower reported yields.

Comparing now the reported experimental yields on formate for *Ps.* 1 and 135 with the theoretical yields of Table II, one concludes that either one of the eq 9 and 11 is valid and $Z = 3$, or that the actual serine pathway is more energetic than either of the icl^+ and icl^- variants and $Z = 2$. In any case, since Z cannot be any higher than 3 and in view of axioms 2 and 3, efficiency at least equivalent to that of icl^+ or icl^- should be expected for growth of these microorganisms on methanol or formaldehyde. Finally, even if formate transport requires 0.5 mol of ATP/mol of HCOOH (Dijkhuizen et al., 1977) (which is not supported, however, by our observations as discussed in our recent paper (Papoutsakis et al., 1981)), the yield is calculated to be 0.147 with $Z = 3$, which changes none of our foregoing arguments.

In the light of the discussions in the previous paragraphs

and in subsection 3.2.a, the most likely combinations for computing yields on methanol and formaldehyde are (1, 1, 3) and (1, 3, 3) (see also assumptions 10 and 11). Comparing thus experimental to computed methanol yields, and assuming that for all SP bacteria values at least as high as 0.41 can be obtained, it appears that carbon incorporation through formate and through the enzymes of the combination (1, 1, 3) are favored, but no discrimination between icl^- and icl^+ would be possible. Similar conclusions would be derived from comparing calculated to experimental yields on formaldehyde (assuming again that yields at least as high as 0.32 can be achieved for all SP bacteria). It appears however that experimental formaldehyde yields are somehow lower than the yields suggested by Table II; this may be perhaps due to the possible energy required for transport as was discussed in section 2.1 regarding assumption 3. Thus, formaldehyde yields have been computed with the additional requirement of 1 mol ATP/mol of CH_2O transported, and these yields are shown in parentheses in Table II. These yields appear to predict experimental values better.

4. Conclusions and Suggestions

In section 3.1 we presented arguments to theorize that the reasons behind the unusual behavior of the biomass yields of methylotrophs are kinetic rather than biosynthetic. This view explains why yields change with dilution rate, temperature, pH, and other culture conditions or even during the course of a batch culture, although the actual biosynthetic efficiency of the various microorganisms remains unchanged.

Comparison of computed to experimental yields strongly suggested that the P/O ratio corresponding to either MDH or FDDH1 is 1, a value that appears to agree with all the biochemical information that is available for these enzymes but also with direct P/O-ratio measurements. In addition, it appeared that in some microorganisms at least, NADH can yield 3 ATP's and in all microorganisms it can yield a minimum of 2 ATP's. The direct measurement of the P/O ratio for formate dehydrogenase fails perhaps to produce the (possible) 3 value probably because of the proton translocation that appears to accompany formate transport. RMP-cycle bacteria appeared to use both the ED and FDP variants and both C_1 -compound oxidation schemes in various and changing extents. SP bacteria appeared to incorporate carbon preferably through formate rather than formaldehyde. Experimental yields indicated that the actual bioenergetics of the SP may be equivalent to that of either the variants icl^+ and icl^- , although formate yields suggested bioenergetics closer to that of icl^+ . The lower than expected formaldehyde yields may be due to an energy-requiring transport of formaldehyde.

It is still necessary that all the above suggestions be further verified. The combination of preliminary suggestions from direct P/O-ratio measurements, theoretical computations and experimental-yield measurements could be used as in the present work for optimal experimental design and to derive final conclusions. Work according to this approach is presently in progress.

The fast growth rates that can be achieved in batch cultures, almost twice that of continuous cultures (Papoutsakis et al., 1978a), suggest that the enzyme quantities, under appropriate substrate concentrations, for greater growth rates do exist. It is a question then whether classical or other genetic techniques will allow to combine these high growth rates with the maximal possible yields, as suggested by the present analysis. It appears indeed that the bioenergetics of the RMP cycle would allow considerably higher yields than presently usually achieved, above perhaps the 0.7 mark. Improvements, for example, could result from removing or rendering inoperative the enzyme FDDH1, or perhaps removing the enzymes of C₁-compound oxidation through formate in favor of the cyclic oxidation scheme. Similarly, we could remove the enzymes of the ED variant in favor of the FBP variant. Perhaps another enzyme for methanol oxidation should be searched for, since methanol oxidation to formaldehyde would, energetically, permit the production of more than 1 ATP (Ribbons et al., 1970).

Nomenclature

A = a constant, eq 17
 ATP = adenosine 5'-triphosphate
 D = dilution rate (or space velocity)
 DCPIP = 2,6-dichlorophenolindophenol
 ED = Entner-Doudoroff (an RMP-cycle variant)
 FDDH1 = PMS-DCPIP formaldehyde dehydrogenase (see assumption 11 in section 2.1)
 FDDH2 = NAD-linked formaldehyde dehydrogenase (see assumption 11 in section 2.1)
 FDH = NAD-linked formate dehydrogenase
 FDP = fructose diphosphate (an RMP-cycle variant)
 FPH₂ = the reduced flavoprotein of succinate dehydrogenase
 GPD = glucose 6-phosphate dehydrogenase
 HPS = hexulose phosphate synthase
 icl⁺, icl⁻ = the SP variants with and without isocitrate lyase, respectively
 m = maintenance coefficient, eq 2 and 3
 MDH = methanol dehydrogenase
 NAD = nicotinamide adenine dinucleotide (oxidized form)
 NAD(P)H = nicotinamide adenine dinucleotide (phosphate) (reduced form)
 PGA = 3-phosphoglycerate
 PGD = phosphogluconate dehydrogenase
 PGI = phosphoglucoisomerase
 PHI = phospho-3-hexulose isomerase
 PMS = phenazine methosulfate
 P/O = moles of inorganic phosphate recovered in organic form (i.e., ATP) per atom of oxygen taken up
 RMP = ribulose monophosphate (cycle)
 r_s, r_x = rate of consumption, biomass production, respectively, eq 2 and 3
 r_{fdh} = rate of formate oxidation catalyzed by FDH
 r_{fd1}, r_{fd2} = rate of formaldehyde oxidation catalyzed by FDDH1 and FDDH₂, respectively
 r_{hps} = rate of the formaldehyde reaction catalyzed by HPS
 r_{pgd} = rate of the reaction catalyzed by PGD (Figure 1b)
 r_{thf} = rate of the formate reaction catalyzed by THF
 SCP = single cell protein
 SHT = serine hydroxymethyl transferase
 SP = serine pathway
 S_{CH_3OH} = residual methanol concentration in a chemostatic culture
 S_{CH_2O} = residual formaldehyde concentration in a chemostatic culture
 S_{HCOOH} = residual formate concentration in a chemostatic culture
 T = temperature
 THF = tetrahydrofolate formylase
 X = biomass concentration, eq 2 only
 X = the P/O ratio for methanol oxidation
 Y = the P/O ratio for formaldehyde oxidation to formate

Y_{ATP} = ATP yield, g of biomass synthesized per mole ATP generated by catabolic processes
 Y_{CH_3OH} = biomass yield on methanol substrate
 Y_{CH_2O} = biomass yield on formaldehyde substrate
 Y_{HCOOH} = biomass yield on formate substrate
 Y_{fd1} = the oxidized form of the FDH1 coenzyme
 Y_{max} = maximum yield, eq 2 and 3
 $Y_{O_2/X}$ = oxygen demand (or yield), g of O₂ consumed/g of biomass produced
 $Y_{X/S}$ = biomass yield, g of biomass produced/g of substrate consumed
 YH₂ = the reduced coenzyme of formaldehyde dehydrogenase
 Z = the P/O ratio for formate oxidation
 μ = specific growth rate

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