Growth of Pseudomonas C on C₁ Compounds:
Continuous Culture

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Pseudomonas C was grown in continuous culture on methanol, formaldehyde, or formate as sole carbon source. On methanol $\mu_{max} = 0.49/h$ and yield constant (Y) = 0.54; on formaldehyde and on unsupplemented media, $\mu_{max}$ was about 0.2/h and Y was 0.15, whereas addition of p-aminobenzoic acid, folic acid, serine, or glycine to the medium raised Y to about 0.26 to 0.29, and addition of p-aminobenzoic acid, folic acid, serine, nicotinamide adenine dinucleotide, and Tween 80 raised the yield to 0.35. On formate and on unsupplemented media, $\mu_{max} = 0.2/h$ and Y = 0.02, whereas addition of 0.1 mM p-aminobenzoic acid increased $\mu_{max}$ to about 0.47 and Y to about 0.23. At low cell concentrations or growth rates a beneficial effect of CO₂ was observed. Formaldehyde or formate, when added together with methanol, were utilized simultaneously with the methanol.

Continuous culture provides a convenient means for studying the physiology of microorganisms in that it permits growth in a constant environment (see ref. 4). Furthermore, by choosing the carbon source as limiting nutrient, relatively toxic materials can be used as substrates since their steady-state residual concentration in the growth vessel may be sufficiently low so that the toxicity is not exhibited (6).

Although some information is available on the properties (yield, growth rates, substrates, etc.) of microorganisms grown on methanol in batch culture (3), little has been published (1, 12) on their properties in continuous culture. Because of the usefulness of continuous culture as a tool for physiological studies, because of its application for production of single-cell protein (14), and because of its potential for exploring the utilization of inhibitory substrates, the present investigation was undertaken.

MATERIALS AND METHODS

Microorganisms, media, and continuous culture techniques. The bacterium, media, and continuous culture techniques used in this study have been described previously (2, 13). Any modifications introduced are noted.

Formaldehyde (36 to 37%) containing 10% methanol as a stabilizer was obtained from Palestine Frutaron Ltd., Haifa, and was used for those experiments in which the presence of methanol was not objectionable. When methanol-free formaldehyde was used as a sole carbon source, it was prepared by heating paraformaldehyde solution (11).

All other chemicals were of the usual analytical grades.

Assays. Cell concentration was determined by measurement of the optical density (OD) of the cell suspension at 650 nm with a 1-cm cell in a Gilford model 240 spectrophotometer. The curve relating OD to dry weight was linear up to 0.8 OD, and 1 OD was equal to 0.73 mg (dry wt)/ml.

Methanol was measured by gas chromatography as previously described (2).

Formic acid was measured by noting the change in OD at 450 nm when 1 ml of potassium permanganate solution (0.5 g/liter) was added to 1 ml of medium and incubated for 15 min at room temperature. This method was usable only at formic acid concentrations above 3 mg/liter. Under the conditions of growth used in the investigation there was no interference by the medium or by metabolites excreted during growth, and 1 OD unit equalled 150 $\mu$g of formic acid in the reaction mixture.

Formaldehyde was measured by the Nash reagent (9).

Notation. The notation used for continuous culture is that of Herbert et al. (8). When multiple substrates were used, their concentration in the medium reservoir (inflowing medium) is denoted by $S_i—CH_2OH$ for methanol and similarly for other substrates, whereas their concentration in the growth vessel is denoted by $S—CH_2OH$, etc.

The yield constant, Y, has units of grams of cell (dry weight) per gram of substrate used. Since the ratio between the molecular weights of formic acid and methanol is approximately 1.5, the yield based on formate (Y — HCOOH) was multiplied by this factor for comparison with the molar yield based on methanol and is denoted by $Y_m—HCOOH$.

The cell concentration, X, is in grams of cell (dry weight) per liter.

RESULTS AND DISCUSSION

Methanol as a sole carbon source. Preliminary studies (13) led to an improved medium which supported cell densities of up to about 12
g (dry weight)/liter and gave yield constants of about 0.5 to 0.55, with methanol as the limiting nutrient. When the concentration of methanol in the inflowing medium was fixed at 1.0 g/liter, an anomalous behavior of the cell culture was observed at low dilution rates (D) (Fig. 1). Not only is the yield constant reduced at dilution rates below about 0.25/h, but the residual methanol rises from about 45 mg/liter at D = 0.2/h to 90 mg/liter at D = 0.1/h.

Such behavior is not in accordance with that predicted by the standard theoretical development of continuous culture (8), and an explanation was sought. The fact that this behavior was observed only at low cell concentrations and low growth rates suggested that a requirement for carbon dioxide might exist. This was also suggested by data showing a significant positive effect of carbon dioxide on the incorporation of \[^{14}C\]formate into methanol-grown cells (19). Figure 2 shows the results of an experiment in which carbon dioxide was added to the inlet air stream and indicates clearly that the presence of carbon dioxide increases the yield value significantly.

The concentration of carbon dioxide necessary to avoid the above-mentioned anomaly can be calculated, based upon S\(_{\text{CH}_3\text{OH}}\) = CH\(_2\)OH = 1.0 g/liter, \(Y = 0.5\), \(D = 0.2\) h, and the assumption that the cells contain about 50% carbon. Thus, under these conditions, carbon dioxide is being produced at a rate of 32 mg/h, which at the air flow rate for these experiments (100 ml/min) results in a partial pressure of 0.003 atm \(CO_2\) in the growth vessel, about 10-fold higher than the normal concentration of \(CO_2\) in the atmosphere. It may be assumed, with essentially no error, that the gas and liquid phases are in equilibrium in regard to the partial pressure of carbon dioxide.

**Utilization of formaldehyde.** It has been observed that *Pseudomonas C* growing on methanol in batch culture was capable of incorporating \[^{14}C\]formaldehyde into trichloroacetic acid-precipitable compounds (19). A series of continuous culture experiments were conducted in which the carbon source(s) was chosen as the growth-limiting nutrient, and methanol and formaldehyde were used together in the nutrient medium. Table 1 presents the results, indicating that methanol and formaldehyde can be utilized simultaneously. Since the carbon sources (methanol and formaldehyde) were the limiting nutrients, it would be expected that the yield of cells would be additive. This was not the case, as is shown by the decrease in yield calculated for formaldehyde (Y—HCHO) as the concentration of formaldehyde was increased. This suggests metabolic interactions between these substrates, and this problem is under investigation to clarify their nature.

To determine whether formaldehyde could be used as a sole source of carbon and energy, a
continuous culture experiment was carried out in which the concentration of methanol in the inflowing medium was held constant and the concentration of formaldehyde was slowly raised. This was done so as to avoid a concentration of formaldehyde in the growth vessel high enough to inhibit growth and cause "wash-out" of the culture. When the desired final concentration of formaldehyde was reached, the medium reservoir was replaced by a new batch of medium containing the same final concentration of formaldehyde but no methanol. The details of the experiment and the results are given in Table 2.

The cells obtained from growth on formaldehyde alone had a tendency to flocculate in the growth vessel, and together with the low yield constant of 0.15 suggested that improved conditions might lead to better results. Table 2 shows the effect obtained by adding various growth factors to the medium. Whereas the addition of Tween 80 did not increase the yield of cells, it reduced the propensity of the cells to form aggregates.

Whereas wash-out occurred at dilution rates above about 0.2/h when unsupplemented M-3 medium was used, the use of the supplements permitted steady states to be attained at dilution rates up to about 0.28/h, with significantly better yields than those obtained at 0.2/h (Fig. 3). The level of residual formaldehyde was found to be approximately 2 mg/liter (Fig. 3).

When formaldehyde was included in the inflowing nutrient feed, but ammonium sulfate was added separately, normal growth and yields were obtained (results not shown). Thus, it does not appear that the requirement for addition of ammonia and formaldehyde in a single stream as claimed by Hitzman and Alquist (U.S. patent 3,642,578, 1972) is relevant for Pseudomonas C.

### Table 1. Effect of different concentrations of formaldehyde on the steady-state concentration and yield of Pseudomonas C cells grown on methanol and formaldehyde

| S—HCHO (g/liter) | S—CH₂OH (g/liter) | X (g of cell/liter) | S—CH₂OH (mg/liter) | Y* | Y—HCHOc |
|------------------|-------------------|---------------------|---------------------|----|---------|
| 0.0              | 1.00              | 0.54                | 40                  | 0.56| 0.56    |
| 0.1              | 1.02              | 0.59                | 42                  | 3.2 | 0.55    |
| 0.5              | 1.12              | 0.72                | 45                  | 4.0 | 0.46    |
| 1.0              | 1.25              | 0.88                | 43                  | 4.0 | 0.40    |
| 2.0              | 1.50              | 0.98                | 46                  | 3.8 | 0.29    |
| 4.0              | 2.00              | 1.21                | 42                  | 4.2 | 0.22    |

* Cells were grown in M-3 medium (13), containing (NH₄)₂SO₄ (2.5 g/liter) as a nitrogen source, at 35 C. The stirring was 600 rpm with aeration of 0.5 volumes of air/volume of growth medium per min and dilution rate of 0.35/h. X, cell concentration; Y, yield constant.

** Based on utilization of methanol plus formaldehyde.

* Calculated on the assumption that the methanol is utilized at the same yield as in the absence of formaldehyde (Y = 0.56).

### Table 2. Effect of different substances on the yield of Pseudomonas C growing in a chemostat culture with formaldehyde as a sole carbon source

| Additions to the growth medium | Y—HCHO |
|-------------------------------|--------|
| None                          | 0.15   |
| PABA                          | 0.26   |
| FA                            | 0.29   |
| SER                           | 0.26   |
| GLY                           | 0.26   |
| TW-80                         | 0.15   |
| NAD                           | 0.19   |
| PABA + FA                     | 0.29   |
| PABA + FA + NAD               | 0.31   |
| PABA + FA + SER               | 0.33   |
| PABA + FA + SER + GLY         | 0.24   |
| PABA + FA + NAD + TW-80       | 0.31   |
| PABA + FA + NAD + TW-80 + CO₂| 0.31   |
| PABA + FA + SER + NAD + TW-80 | 0.35   |
| PABA + FA + SER + NAD + TW-80 | 0.43   |

(D = 0.53/h)

* Cells were grown with methanol (3 g/liter) as described in Table 1. Heated paraformaldehyde was added stepwise to the medium reservoir to a final concentration of 2 g/liter. After a steady state was established the medium was changed to fresh M-3 medium (13) containing 1 g of formaldehyde per liter, and the dilution rate was changed to 0.1/h. Y, yield constant.

* The concentrations of the different substances added to the medium were: PABA, 0.1 mM; folic acid (FA), 0.1 mM; glycine (GLY) 0.1 mM; nicotinamide adenine dinucleotide (NAD), 10 mg/liter; Tween 80 (TW-80), 0.1 ml/liter, and L-serine (SER), 0.1 mM. CO₂ was added by aeration of the culture with a gas mixture consisting of 95% air and 5% CO₂.

The fact that Pseudomonas C can be grown quite well in continuous culture on formaldehyde as a sole source of carbon and energy suggests that the earlier failure to observe growth in batch culture (2) was due simply to formaldehyde having been present at a toxic
concentration. If concentrations of formaldehyde of 30 to 50 mg/liter inhibit growth, it would be very difficult, if not impossible, to detect growth in batch cultures using noninhibitory levels of formaldehyde. Continuous culture permits growth at high cell concentrations together with very low concentrations of formaldehyde in the growth vessel, provided great care is taken during start up and operation to avoid transients giving rise to formaldehyde concentrations high enough to be inhibitory. Reexamination of the organisms *Pseudomonas methanica* (5), *Pseudomonas AM-1* (16), *Pseudomonas PRL-W4* (10), *Pseudomonas oxalaticus* (17), *Methyllococcus capsulatus* (15), and *Pseudomonas methanica* (7), which are known to utilize methanol but were reported unable to utilize formaldehyde, might reveal that they indeed are capable of formaldehyde utilization provided the concentration is kept low. A possible simplification in such a reexamination might be to check for ability to use formaldehyde while growing on methanol in continuous culture, as described above, before going on to the more arduous work of using formaldehyde as sole carbon source.

**Utilization of formate.** As was noted in the case of formaldehyde, cultures growing on methanol were able simultaneously to utilize formate. Table 3 presents the results of experiments showing simultaneous utilization of methanol and formate.

Utilization of formate as a sole carbon source was achieved by starting a continuous culture on medium containing methanol and formate and switching to a media containing formate alone. Because of the relatively low toxicity of formate (2), the procedure was simpler than in the case of formaldehyde. Details are given in Table 4, which shows the yields obtained on

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**TABLE 4. Effect of different substances on the yield of *Pseudomonas C* growing in a chemostat culture with formic acid as a sole carbon source**

| Additions to the growth medium | \( Y_{m-HCOOH} \) |
|-------------------------------|---------------------|
| None                          | 0.02                |
| PABA                          | 0.22                |
| PABA + \( CO_2 \)             | 0.25                |
| PABA + NADH                   | 0.24                |
| PABA + SER                    | 0.24                |

*Cells were grown with methanol (3 g/liter) as described in Table 1. Formic acid was added to the medium reservoir to a final concentration of 3 g/liter. After a steady state was achieved, the medium was changed to fresh M-3 medium (13) containing 3 g of formic acid per liter, and the dilution rate was decreased to 0.1/h. When the different substances were added to the medium, the dilution rate was increased to 0.35/h and a steady state was reached.

*The concentrations of the different substances added to the medium were: PABA, 0.1 mM; dihydronicotinamide adenine dinucleotide (NADH), 1 mg/liter; \( L \)-serine (SER), 1 mg/liter. CO\(_2\) was added by aerating the culture with a gas mixture consisting of 95% air and 5% CO\(_2\).*

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**TABLE 3. Effect of different concentrations of formic acid on the steady-state concentration and yield of *Pseudomonas C* cells grown on methanol and formic acid**

| \( S_0-HCOOH \) (g/liter) | \( X \) (g of cell/liter) | \( S-CH_2OH \) (mg/liter) | \( S-HCOOH \) (mg/liter) | \( Y^* \) | \( Y_{m-HCOOH} \) |
|---------------------------|--------------------------|---------------------------|--------------------------|----------|-------------------|
| 0.0                       | 1.09                     | 45                        | 0                        | 0.56     | 0.07              |
| 0.3                       | 1.10                     | 47                        | 11                       | 0.51     | 0.12              |
| 1.0                       | 1.18                     | 43                        | 12                       | 0.45     | 0.12              |
| 2.0                       | 1.25                     | 45                        | 17                       | 0.39     | 0.12              |
| 3.0                       | 1.26                     | 46                        | 10                       | 0.31     | 0.08              |
| 4.0                       | 1.27                     | 45                        | 14                       | 0.28     | 0.06              |
| 5.0                       | 1.27                     | 49                        | 20                       | 0.23     | 0.05              |

*Same experimental conditions as in Table 1. Methanol was added to the medium to a final concentration of 2 g/liter. After a steady state was achieved, formic acid was added to the medium. The pH was constant in the range of 7.0 ± 0.1 by automatic titration with 5 N NaOH. X, cell concentration.

*Yield \((Y) = \text{grams of cell (dry weight) per gram of methanol plus formic acid utilized.}

*Calculated on the assumption that the methanol is utilized at the same yield as in the absence of formate \((Y = 0.56).
formate media together with various nutritional supplements.

Figures 4 and 5 summarize a series of experiments in which the organism was grown in continuous culture on formate as sole carbon source in medium supplemented with p-aminobenzoic acid (PABA).

Figure 5 indicates the effect of PABA on the yield constant of cells growing on formate. The optimal concentration of PABA was found to be in the range of 75 to 150 μM. The concentration of residual formate was not affected except at the highest PABA level, in which case it rose substantially, to about 150 mg/liter.

Effect of growth factors on yield. Tables 2 and 4 show clearly the effect of the addition of various growth factors on the yield constants of cells growing on formaldehyde and formate as sole carbon source. PABA and folic acid are known to be intermediates in the synthesis of tetrahydrofolic acid, which together with serine and glycine are believed to be involved in the assimilation of C₁ compounds via the so-called serine pathway (18). No effect of these growth factors was observed when the cells were growing on methanol, which suggests either differences in the metabolic pathways or in the requirements for these nutrients within the cells, when the cells grow on the different substrates.

As PABA had a very marked effect on yield, it would be expected that there would be a marked selective pressure during continuous culture in unsupplemented medium in favor of mutants that "overproduce" PABA and give a higher yield than the parent strain. In preliminary experiments carried out on unsupplemented medium at a low dilution rate (0.1/h), in fact, the yield increased from about 0.08 to 0.23 during a week of continuous culture. Experiments at dilution rates higher than 0.2/h were not successful in selecting for mutants.

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