Energy Production and Growth of *Pseudomonas oxalatus* OX1 on Oxalate and Formate

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Abstract. The efficiency of oxidative phosphorylation in *Pseudomonas oxalatus* during growth on oxalate and formate was estimated by two methods. In the first method the amount of ATP required to synthesize cell material of standard composition was calculated during growth of the organism on either of the two substrates. The $[\text{Y}_\text{ATP}^\text{max}]$ values thus obtained were 12.5 and 6.5 for oxalate and formate respectively, if the assumption were made that no energy is required for transport of oxalate or carbon dioxide. When active transport of oxalate requiring an energy input equivalent to 1 mole of ATP per mole of oxalate was taken into account, $[\text{Y}_\text{ATP}^\text{max}]$ for oxalate was 9.4. True Y$_\text{ATP}$ values were derived from these data on the assumption that the energy produced in the catabolism of *Pseudomonas oxalatus* is used with approximately the same efficiency as in a range of other chemoorganotrophs. P/O ratios were calculated using the equation $\text{P/O} = \text{Y}_\text{O}/\text{Y}_\text{ATP}$. The data for Y$_0$ and m$_e$ required for these calculations were obtained from cultures of *Pseudomonas oxalatus* growing on oxalate or formate in carbon-limited continuous cultures. The P/O ratios calculated by this method were, for oxalate, 1.3 (or 1.0 if active transport were ignored), and for formate, 1.7.

In the second method the stoichiometries of the respiration-linked proton translocations with oxalate and formate were measured in washed suspensions of cells grown on the two substrates. The $\text{H}^+/\text{O}$ ratios obtained were 4.3 with oxalate and 3.9 with formate. These data indicate the presence of two functional phosphorylation sites in the electron transport chain of *Pseudomonas oxalatus* during growth on both substrates. A comparison of the P/O ratio on oxalate obtained with the two methods indicated that the energy requirement for active transport of oxalate has a major effect on the energy budget of the cell; about 50% of the potentially available energy in oxalate is required for its active transport across the cell membrane. Translocation of formate requires approximately 25% of the energy potentially available in the substrate. These results offer an explanation for the fact that molar growth yields of *Pseudomonas oxalatus* on oxalate and formate are not very different.

Key words: P/O ratio – $\text{Y}_\text{ATP}^\text{max}$ – $\text{H}^+/\text{O}$ quotient – Oxalate – Formate – *Pseudomonas oxalatus* OX1.

The synthesis of cell constituents during growth of *Pseudomonas oxalatus* on oxalate has been shown (see Blackmore et al., 1968) to involve reduction of oxalate to glyoxylate and subsequent conversion of glyoxylate by the glycerate pathway to 3-phosphoglycerate (see Fig. 1).

$$2\text{oxalate} + 3\text{ATP} + 2\text{NADPH} + \text{NADH} + 3\text{H}^+ \rightarrow 3\text{phosphoglycerate} + 2\text{P}_i + 3\text{ADP} + 2\text{NADP}^+ + \text{NAD}^+.$$ (1)

The necessary energy is derived from the oxidation of oxalate after its conversion to formate as follows:

$$\text{oxalate} + \text{NAD}^+ \rightarrow 2\text{CO}_2 + \text{NADH}.$$ (2)

In the catabolism of oxalate only NADH is produced whereas its assimilation requires NADPH for the reduction of oxalyl CoA to glyoxylate (see Fig. 1). In order to transfer reducing power from NADH to NADP a transhydrogenase must be present. The available evidence (Quayle et al., 1961) seems to indicate that in *Pseudomonas oxalatus* this enzyme is not energy-requiring; Eq. (1) gives therefore the...
overall energy requirement for the conversion of oxalate to 3-phosphoglycerate.

During growth of the organism on formate as sole carbon source, the synthesis of cell constituents involves the Calvin cycle of carbon dioxide fixation. The overall conversion of carbon dioxide into the primary output molecule of the cycle, 3-phosphoglycerate, can be summarized in the following equation:

$$3 \text{CO}_2 + 8 \text{ATP} + 5 \text{NADH} + 5 \text{H}^+ \rightarrow 3\text{-phosphoglycerate} + 8\text{ADP} + 7\text{P}_i + 5\text{NAD}^+. \quad (3)$$

Energy for growth on formate is generated by a NAD-linked formate dehydrogenase:

$$\text{formate} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{NADH} + \text{H}^+. \quad (4)$$

During growth of Pseudomonas oxalaticus on both oxalate and formate the oxidation of formate according to reaction (4) is the only energy yielding reaction: the complete oxidation of either substrate yields 1 mole of NADH.

Whereas the energy yield of the oxidation of oxalate and formate is the same, there appears to be a large difference in the energy- and reducing power requirement for the synthesis of 3-phosphoglycerate from each of the carbon sources [Eqs. (1) and (3)]. In fact the heterotrophic pathway is more economical in terms of energy requirement to the extent of 2 NADH + 5 ATP/mole of 3-phosphoglycerate formed. Consequently a large difference may be expected between the energy- and reducing power requirement for the synthesis of cell material from oxalate and formate 

(Fig. 1. Metabolism of oxalate
in Pseudomonas oxalaticusOX1

there may be a difference in the efficiency of oxidative phosphorylation between autotrophic and heterotrophic cultures as has been observed in Paracoccus denitrificans (Knobloch et al., 1971; van Verseveld and Stouthamer, 1976) and Pseudomonas saccharophila (Ishaque et al., 1971). Another possibility is that one or more energy-requiring processes in the metabolism of oxalate or formate, which have not been accounted for above, have a larger effect on the energy budget of the cell in the case of growth on oxalate. Translocation of the substrates from the medium into the cell is one of the possibilities. This question is discussed in detail in the accompanying paper (Dijkhuizen et al., 1977).

We decided, therefore, to study the relation between energy production and growth in Pseudomonas oxalaticus by trying to estimate P/O ratios during growth of the organism on oxalate and formate. One of the methods available to calculate P/O ratios involves the use of the equation $P/O = Y_{O}/Y_{\text{ATP}}$. In facultative anaerobes $Y_{O}$ is generally measured in aerobic continuous cultures and $Y_{\text{ATP}}$ in anaerobic cultures at the same growth rate (Stouthamer and Bettenhausen, 1975). Since Pseudomonas oxalaticus is an obligate aerobe, we had to estimate $Y_{\text{ATP}}$ values in a different way. The method employed involves the calculation of the $[Y_{\text{ATP}}^\text{max}]$ values (Stouthamer, 1973) for oxalate and formate. The true $Y_{\text{ATP}}^\text{max}$ values were derived from these data on the assumption that a similar coupling between energy generation and growth exists in Pseudomonas oxalaticus as in a range of other chemoorganotrophs (Forrest and Walker, 1971; Harder and van Dijken, 1976). The P/O ratios obtained using this partly theoretical approach have been compared with the $P/O$ ratios measured with the oxygen pulse method (Mitchell and Moyle, 1967) in cells grown on oxalate and formate. According to Mitchell's chemiosmotic hypothesis of oxidative phosphorylation two protons are translocated from inside to outside per energy conservation site when
two electrons are transferred along the respiratory chain (Mitchell, 1972). A measurement of the \( \text{H}^+/\text{O} \) ratios will therefore permit an estimation to be made of in vivo P/O ratios. The implications of the results of these studies for the overall energy budget of \( \text{Pseudomonas oxalaticus} \) during growth on oxalate and formate is discussed.

A preliminary report of this work has been presented elsewhere (Dijkhuizen and Harder, 1977).

MATERIALS AND METHODS

Organism

The organism used, \( \text{Pseudomonas oxalaticus} \) OxI, was obtained from Professor J.R. Quayle, University of Sheffield, England and still has all its originally described properties (Quayle, 1961), including the ability to grow autotrophically on formate as the sole source of carbon and energy. The organism was maintained as described by Dijkhuizen and Harder (1975).

Continuous Culture Experiments

Growth yields of \( \text{Pseudomonas oxalaticus} \) on oxalate or formate were determined in mineral medium in carbon- and energy-limited continuous cultures, using a 1.5 l fermenter of the type described by Harder et al. (1974a). Air flow rates were determined with a precision gas meter, type 1 (Meterfabrick Dordrecht, The Netherlands). Oxygen consumption was measured with a paramagnetic oxygen analyzer (Type OA 180, Servomex Controls Ltd., Crowborough, Sussex, U.K.) and the carbon dioxide production with an infrared gas analyzer (Model SR2, Grubb Parsons Ltd., Newcastle-upon-Tyne, U.K.). The medium composition was (g/l): K2HPO4, 1.0; NaH2PO4, 0.75; (NH4)2SO4, 1.75; MgSO4 · 7 H2O, 0.35. Per liter of medium 0.75 ml of a trace elements solution with a composition as described by Dijkhuizen and Harder (1975) was added. The concentration of oxalate or formate was 0.2 M, in both cases supplied as a mixture of the acid (0.15 M) and the salt, dipotassium oxalate and sodium formate, respectively (0.05 M). This composition was chosen in order to minimize the effects on dilution rate of the addition of acid necessary for pH control, because of the rapid increase of the pH during growth on oxalate or formate. The pH, set at 7.5, was controlled by the automatic addition of 1 M sulphuric acid, also containing CaCl2 · 2 H2O (1 g/l) and FeCl3 · 4 H2O (1 g/l). The medium was sterilized by filtration through a Seitz EKS filter (Seitz GmbH, Bad Kreuznach, W.-Germany). The culture was inoculated with cells grown on 0.8% yeast extract and the temperature maintained at 28°C. During growth of the organism on oxalate or formate in the chemostat, a steady state was assumed to be established after not less than 5 volume changes of the culture fluid. For dry weight determination samples of 40 ml were centrifuged, washed twice with distilled water and dried to constant weight at 110°C. After weighing, in some samples the carbon, hydrogen and nitrogen content was then determined by standard methods of organic analysis.

Measurement of \( \text{H}^+/\text{O} \) Ratios

\( \text{Pseudomonas oxalaticus} \) was grown in batch culture in 2 l of medium as described by Dijkhuizen and Harder (1975). The concentrations of oxalate and formate used were 15 and 20 mM, respectively. The pH was kept constant at 7.5 by the automatic addition of 1.5 N oxalic acid or 5 N formic acid. Cells were grown on sodium succinate (20 mM) without pH control in conical flasks containing 200 ml of medium. The organisms were harvested in the late logarithmic growth phase, washed twice with 1 mM Tris-HCl buffer containing 140 mM KCl, pH 7.0 and resuspended in the same solution to a final density of 10 mg dry weight per ml. The \( \text{H}^+/\text{O} \) ratios were measured as described by Mitchell and Moyle (1967). This involved the measurement of the acidification of the lightly buffered (1 mM Tris-HCl + 140 mM KCl) cell suspension when a pulse of air saturated 140 mM KCl was added. The reaction mixture also contained potassium thiocyanate (final concentration 100 mM) to neutralize any electrical charges across the membrane and to maximize the pH difference, and carbonic anhydrase (30 µg/ml) was included to catalyze the CO2/H2CO3/HCO3 equilibrium (West and Michell, 1974). A Radiometer (Copenhagen) model 26 pH meter was used with a glass semi-micro combination electrode (GK 2321C), and a Telsec type X chart recorder with offset control. The scale was calibrated by the addition of nitrogen sparged 0.01 M HCl. The cell suspension was kept at a constant temperature of 30°C in a Rank oxygen electrode chamber (Rank Bros., Bottisham, Cambridge, U.K.).

The maximum pulse height was calculated by extrapolation of the semilogarithmic plot of proton decay back to zero time (Scholes and Mitchell, 1970). In some experiments cells were starved for 2 h or overnight in medium without carbon source, then washed twice with 1 mM Tris-HCl buffer containing 140 mM KCl, pH 7.0, before respiration-driven proton translocation was measured.

RESULTS

The relation between bacterial dry weight and dilution rate during growth of \( \text{Pseudomonas oxalaticus} \) on oxalate or formate is shown in Figure 2. Elemental analysis of the cell material revealed a cell composition corresponding to C4H2O2N. This composition was independent of the dilution rate over the range 0.03 to 0.15 h⁻¹. Material balances for carbon, hydrogen, oxygen and nitrogen at a dilution rate of 0.1 h⁻¹ are shown in Table 1. \( Y_0 \) values and \( Q_{CO2} \) data calculated from these equations were in very good agreement with the experimentally observed values. The molar growth yields on oxalate and formate found in these experiments, 3.8 and 3.4 respectively, were unexpectedly close (see "Introduction"). The low growth yields on these simple and highly oxidized substrates reflect their poor capacity to serve as an energy source, as already indicated by Whitaker and Elsden (1963) in their study on the relation between growth and oxygen consumption in microorganisms.

The maintenance requirement was determined by reploting the data of Figure 2 in Figure 3 according to the Pirt equation (Pirt, 1965):

\[
\frac{1}{Y} = \frac{m_w}{D} + \frac{1}{Y_0}
\]  \quad (5)

The values of \( m_w \) calculated from these plots (2.3 mMole of oxalate/g dry weight · h and 3.3 mMole of formate/g dry weight · h) are quite low indicating that the effect of maintenance requirement of the organism on the growth yields at a dilution rate of 0.1 h⁻¹ is very small.

In order to estimate P/O ratios during growth on oxalate and formate the theoretical \( Y_{ATP} \) values were
Fig. 2. Relationship between bacterial dry weight and dilution rate of *Pseudomonas oxalaticus* OXI during growth on 0.2 M oxalate (A) and 0.2 M formate (B).

Table 1. Material balances for C, H, O and N during growth of *Pseudomonas oxalaticus* OXI on oxalate or formate in continuous culture at a dilution rate of \( D = 0.10 \text{ h}^{-1} \)

<table>
<thead>
<tr>
<th>Material</th>
<th>Oxalate</th>
<th>Formate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1000 C(_2)H(_4)O(_2) + 343 O(_2) + 37 NH(_3)</td>
<td>1000 CH(_2)O(_2) + 360 O(_2) + 33 NH(_3)</td>
</tr>
<tr>
<td>Oxalate</td>
<td>( \rightarrow 37 \text{ C}_6\text{H}_5\text{O}_2\text{N} + 1852 \text{ CO}_2 + 908 \text{ H}_2\text{O} )</td>
<td>( \rightarrow 33 \text{ C}_6\text{H}_7\text{O}_2\text{N} + 868 \text{ CO}_2 + 918 \text{ H}_2\text{O} )</td>
</tr>
<tr>
<td>Y(_0)</td>
<td>5.5</td>
<td>4.7</td>
</tr>
<tr>
<td>RQ</td>
<td>5.4</td>
<td>2.4</td>
</tr>
<tr>
<td>Y(_\text{mol})</td>
<td>3.8</td>
<td>3.4</td>
</tr>
</tbody>
</table>

Fig. 3. Relationship between the reciprocal Y\(_s\) value and the reciprocal dilution rate of *Pseudomonas oxalaticus* OXI during growth on 0.2 M oxalate (A) and 0.2 M formate (B).

Table 2. ATP requirement for the formation of microbial cells from glucose, oxalate and carbon dioxide as the carbon source in a mineral salt medium

<table>
<thead>
<tr>
<th>Synthesis of</th>
<th>ATP requirement (mol x 10(^{-4}) per g cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>20.5</td>
</tr>
<tr>
<td>Protein</td>
<td>203.0</td>
</tr>
<tr>
<td>Lipid</td>
<td>1.5</td>
</tr>
<tr>
<td>RNA and DNA</td>
<td>55.1</td>
</tr>
<tr>
<td>Turnover m-RNA</td>
<td>14</td>
</tr>
<tr>
<td>Transport of a) carbon source</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>52</td>
</tr>
<tr>
<td>Total</td>
<td>348</td>
</tr>
<tr>
<td>(Y_{\text{ATP}})(_\text{theor.})</td>
<td>28.7</td>
</tr>
</tbody>
</table>

\* The composition of the cells was taken as: polysaccharide 16.6\%, protein 52.4\%, lipid 9.4\%, RNA 15.7\% and DNA 3.2\% of the dry weight (Stouthamer, 1973).

\( \text{Data of Stouthamer (1973)} \)

calculated (Table 2). The input of energy to synthesize a \( \text{C}_3 \) compound via the glycerate pathway or via the Calvin cycle is shown in Eqs. (1) and (3) respectively. Using these data the energy requirement for the synthesis of the various monomers from 3-phosphoglycerate was then calculated. It was assumed that 3-phosphoglycerate was converted into key intermediates of the glycolytic pathway via the glycolytic reactions and that \( \text{C}_4 \)-dicarboxylic acids were synthesized via PEP-carboxylase. The composition of cell material and values for the energy requirement for polymerization of monomers, for turnover of m-RNA and for transport of ions were those used by Stouthamer (1973). In the case of biosynthesis of cell material from oxalate two calculations have been made: In column 1 of Table 2 the energy requirement for the synthesis of cells from oxalate was calculated on the basis of the assumption that oxalate is transported across the cell membrane of *Pseudomonas oxalaticus* by a process that does not require energy; column 2 shows the total energy required in the case where energy (1 mole of ATP/mole of oxalate, see Dijkhuizen et al., 1977) is consumed. A comparison of the resulting \( Y_{\text{ATP}}\)\(_{\text{theor.}} \) values obtained, shows that such an active transport process has a significant effect (ca. 20\%) on the total energy requirement of biomass formation. From the \( Y_{\text{ATP}}\)\(_{\text{theor.}} \) values (Table 2) it can be seen that biosynthesis of cell material from carbon dioxide is far more expensive than from oxalate. This is completely due to the difference in energy demand between the Calvin cycle and the glycerate pathway for the production of the primary output molecule.
Experimentally determined \( Y_{\text{ATP}} \) values are always very much smaller than theoretical \( Y_{\text{ATP}}^\text{max} \) values (Stouthamer and Bettenhaussen, 1975; Harder and van Dijken, 1976); this difference is only partly caused by energy required for so-called maintenance processes (Pirt, 1965, 1975; van Gent-Ruyters et al., 1975). The small correction for the effect of maintenance on the \( Y_{\text{ATP}} \) values can be made by applying the modified (de Vries et al., 1970) formula of Pirt (1965):

\[
1/Y_{\text{ATP}} = m_e \mu + 1/Y_{\text{ATP}}^\text{max}.
\]

(6)

Stouthamer and Bettenhaussen (1975), working with Enterobacter aerogenes grown in glucose-limited chemostat culture, corrected the generally reported value of \( Y_{\text{ATP}} \) of 10.5 (Stouthamer, 1969; Forrest and Walker, 1971) to a \( Y_{\text{ATP}}^\text{max} \) value of about 14. There is still a large discrepancy between this value and the theoretical \( Y_{\text{ATP}}^\text{max} \) value of 28.8 for growth on glucose in a mineral medium (Stouthamer, 1973). To account for this discrepancy Harder and Veldkamp (unpublished) suggested the use of a coupling factor \( k \) as follows:

\[
y_{\text{ATP}}^\text{max} = k \cdot [Y_{\text{ATP}}^\text{max}]_{\text{theor}}.
\]

(7)

The equation indicates that not all the energy that is generated from the oxidation of a substrate is used efficiently for biosynthetic purposes, but that, in addition to energy required for maintenance, a further part or the energy is lost due to a certain amount of uncoupling of energy generation and growth (see Harder and van Dijken, 1976).

The value of \( k \) can be approximated from a mean value for \( Y_{\text{ATP}}^\text{max} \) of 14 of cells growing on glucose as limiting substrate in a chemostat and is found to be approximately 0.5 (Harder and van Dijken, 1976; see also Farmer and Jones, 1976). If it is assumed that Pseudomonas oxalaticus uses energy produced in the dissimilation of oxalate or formate with about the same efficiency for biosynthetic purposes as in a range of other chemooorganotrophs (Forrest and Walker, 1971), \( Y_{\text{ATP}}^\text{max} \) values for growth on oxalate and formate can be calculated (Table 3).

The \( Y_{\text{ATP}} \) values at a dilution rate of 0.1 \( h^{-1} \) were calculated from the \( Y_{\text{ATP}}^\text{max} \) values using the modified Pirt equation [Eq. (6)]. Since, for this purpose the maintenance energy requirement has to be expressed in terms of ATP, the \( m_e \) values were calculated, assuming a \( P/O \) ratio of 1 on oxalate and 1.5 on formate (see "Discussion"). These calculations resulted in \( Y_{\text{ATP}} \) values for oxalate of 5.5 (without active transport of oxalate) or 4.2 (assuming that active transport of oxalate requires an amount of energy equivalent to 1 mole of ATP/mole of oxalate transported) and for formate of 2.8. In the case of growth of Pseudomonas oxalaticus on formate, cell material is synthesized from carbon dioxide. Since this carbon dioxide needs not necessarily be generated from formate, energy cost of transport of the carbon source has not been accounted for in the present calculations. [When it is assumed that all carbon dioxide is assimilated from intracellular formate, which uptake requires 0.5 mole of ATP/mole of formate (Dijkhuizen et al., 1977), then the \( P/O \) ratio for formate becomes 1.9.] From these \( Y_{\text{ATP}} \) values \( P/O \) values were calculated using the equation \( P/O = Y_o/Y_{\text{ATP}} \). Values from 1.0 and 1.3 for oxalate and 1.7 for formate respectively (see Table 3). These ratios indicate the net synthesis of approximately 1.0 to 1.3 and 1.7 moles of ATP per mole of oxalate or formate oxidized respectively.

To compare these results, obtained in a partly theoretical approach, with values from a more direct approach, proton translocations associated with the oxidation of oxalate and formate by whole cells were measured. Immediately after the addition of a small volume of air-saturated KCl (140 mM) to an anaerobic cell suspension of Pseudomonas oxalaticus a rapid phase of acidification was observed, followed by a decay back to the original value with a half time of about 60 s. No proton extrusion was observed in the absence of potassium thiocyanate and the proton pulses were maximal at a thiocyanate concentration of 100 mM or higher. The addition of the uncoupler carbonylcyanide m-chlorophenylhydrazone (8 \( \mu \)M) caused a rapid decay of the proton pulse, due to its effect in increasing the proton conductance across the membrane phase, as predicted by the chemiosmotic hypothesis. The results are summarized in Table 4. With oxalate and formate as electron donors to \( H^+/O \) ratios close to 4 were measured which indicates the presence of two energy-conservation sites in the respiratory chain of Pseudomonas oxalaticus during growth on both substrates (Mitchell and Moyle, 1967).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Without Active Transport</th>
<th>With Active Transport</th>
<th>Formate</th>
<th>Oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Y_{\text{ATP}} )</td>
<td>( Y_{\text{ATP}}^\text{max} )</td>
<td>( Y_{\text{ATP}} )</td>
<td>( Y_o )</td>
<td>( P/O )</td>
</tr>
<tr>
<td>Oxalate</td>
<td>5.5</td>
<td>2.8</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Formate</td>
<td>6.5</td>
<td>3.3</td>
<td>2.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Table 3. Calculation of \( P/O \) ratios in Pseudomonas oxalaticus OX1 from data of growth in oxalate- and formate-limited continuous culture at a dilution rate of \( D = 0.10^{-1} \)
Table 4. $\Delta H^+/O$ ratios measured in whole cells of Pseudomonas oxalaticus OX1 respiring different added substrates

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Cells grown on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxalate</td>
</tr>
<tr>
<td>None (endogenous)</td>
<td>4.16 ± 0.21 (5)</td>
</tr>
<tr>
<td>Oxalate</td>
<td>4.32 ± 0.04 (4)</td>
</tr>
<tr>
<td>Formate</td>
<td>4.25 ± 0.21 (4)</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
</tr>
<tr>
<td>Ascorbate-TMPD</td>
<td>2.20 ± 0.14 (2)</td>
</tr>
</tbody>
</table>

The values shown are presented as means ± S.D., with the number of observations in parentheses. No significant differences were observed in the $\Delta H^+/O$ ratios for the oxidation of endogenous substrates between samples measured directly or after starvation for 2 h or overnight. Final concentrations: K-ascorbate, pH 6.6, 5 mM; TMPD, 0.2 mM; oxalate, formate, succinate (potassium salts) and ethanol, 3 mM.

The magnitude of the proton pulses were independent of the pH over the range 6.5–7.5. With all substrates tested the respiratory activities were at least 5 times higher than the endogenous values.

DISCUSSION

The oxygen pulse method has been used to measure P/O ratios in a number of bacteria and evidence is available that the $\Delta H^+/O$ quotients obtained are indeed an indication of the efficiency of oxidative phosphorylation. For instance, in Escherichia coli, the respiration chain of which contains only energy-conservation sites 1 and 2 (Brice et al., 1974; Jones et al., 1975), Lawford and Haddock (1973) measured $\Delta H^+/O$ ratios of 4 with the NAD-linked substrate malate and of 2 with the flavin-linked substrates succinate and glycerol. The $\Delta H^+/O$ ratios measured in cells of Pseudomonas oxalaticus grown on oxalate and formate also point to the presence of two phosphorylation sites in the respiratory chain of this organism although the evidence presented here indicates the occurrence of energy-conservation sites 2 and 3.

It was therefore expected that an analysis of the growth yield data of the organism would lead to a P/O ratio of approximately 2. However, calculation of the P/O ratio using data derived from growing cultures gave a figure of about 1 for the number of phosphorylation sites in the case of oxalate. In the accompanying paper (Dijkhuizen et al., 1977) we demonstrate the presence of an active oxalate transport system, with an energy requirement probably equivalent to 1 mole of ATP per mole of oxalate transported. This would explain the discrepancy in the value of the P/O ratio during growth on oxalate obtained with the two methods used. Although the oxidation of oxalate is coupled to energy conservation at two sites, as indicated by the proton pulse experiments, the oxalate can only be oxidized after it has been transported into the cell, a process that probably consumes energy equivalent to 1 mole of ATP/mole of oxalate. Only half the energy potentially available in oxalate can therefore be used for biosynthesis as is made evident by the low value for the P/O ratio determined from growing cultures. No active transport system for formate was detectable in Pseudomonas oxalaticus (Dijkhuizen et al., 1977). Evidence is available which suggests that this substrate may cross the cytoplasmic membrane by diffusion in cotransport with one proton per molecule of formate (Garland et al., 1975). The energy requirement of formate transport is therefore half a mole of ATP/mole of formate which is compatible with the small difference observed in the P/O ratio on formate obtained in the two methods. Despite the presence of 2 energy-conservation sites in the electron transport chain of the organism, the net energy production from oxalate and formate is most likely 1 and 1.5 moles of ATP, respectively, per mole of substrate oxidized. These considerations explain why, in spite of the considerable difference in energy requirement of the carbon assimilation pathways operative during growth on the two substrates, the molar growth yields on oxalate and formate are quite close.

One point remains to be clarified: if transport of oxalate requires an amount of energy equivalent to 1 mole of ATP/mole of oxalate, the $\Delta H^+/O$ ratios...
with oxalate should be 2 instead of the reported 4. Oxalate transport was not, however, a factor in these experiments, because the cells were preloaded with oxalate, and the amount of oxygen added presumably did not exhaust intracellular oxalate.

The number of energy-conservation sites in the respiratory chain of bacteria seems to vary with species and growth conditions. Well-known examples are the absence of phosphorylation site 3 from heterotrophically-grown *Paracoccus denitrificans* (Knobloch et al., 1971; van Verseveld and Stouthamer, 1976; but see also Edwards et al., 1977), aerobically grown *Escherichia coli* (Lawford and Haddock, 1973) and *Enterobacter aerogenes* (Stouthamer and Bettenhausen, 1975). Moreover, in all three organisms mentioned phosphorylation site 1 is lost during sulphate-limited growth (Meijer et al., 1977; Poole and Haddock, 1974, 1975; Stouthamer and Bettenhausen, 1975).

At the moment it is not possible to decide conclusively which phosphorylation site is absent in *Pseudomonas oxalaticus*. Reduced minus oxidized cytochrome spectra reveal a normal cytochrome distribution including the presence of cytochromes of the a, b and c type. Since the functioning of site 3 phosphorylation appears to be predicated upon the presence of cytochrome (Scholes and Mitchell, 1970; Jones et al., 1975) the absence of site 1 or site 2 phosphorylation in *Pseudomonas oxalaticus* is more probable. The finding of →H+/O ratios of 4 in cell suspensions of *Pseudomonas oxalaticus* with substrates, the oxidation of which was either NAD-linked (ethanol) or FAD-linked (succinate), indicated that phosphorylation site 1 may be absent in this organism. Attempts to lose phosphorylation at site 1 by growing the organism on oxalate or formate in sulphate-limited continuous cultures did not result in a decrease in the →H+/O ratios, which were in both cases close to 4. Moreover, oxidation of NADH by membrane particles of *Pseudomonas oxalaticus* was not inhibited by the presence of amytal or rotenone. It is, therefore, tentatively concluded that site 1 phosphorylation is not functional in *Pseudomonas oxalaticus*. It is of interest to note that in addition to a NAD-linked formate dehydrogenase another formate-oxidizing enzyme has been detected in formate- and oxalate-grown *Pseudomonas oxalaticus* (Dijkhuizen et al., unpublished). This enzyme is a membrane-bound dehydrogenase which requires PMS-DCPIP for activity and in vivo may be associated with cytochrome b, similar to the formate dehydrogenase of *Escherichia coli* (Enoch and Lester, 1975). In the absence of a functional site 1 phosphorylation any role of this enzyme in the oxidation of formate will lead to the same P/O ratios as those discussed when a NAD-linked formate dehydrogenase is involved in either oxalate or formate oxidation.

The above results indicate that active transport of small molecules whose energy generation only yields a relatively small amount of biologically useful energy may require a substantial fraction of this energy for its translocation across the cell membrane. This is particularly manifested in *Pseudomonas oxalaticus* during growth on oxalate and formate, where 50% and 25% respectively, of the energy potentially available in the substrate is required for transport.

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