Substrate inhibition in *Pseudomonas oxalaticus* OX1: a kinetic study of growth inhibition by oxalate and formate using extended cultures

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Pseudomonas oxalaticus OX1 has been grown in a mineral salts medium with oxalate or formate as the sole source of carbon and energy. At concentrations of these substrates above 50 mM inhibition of growth was indicated by a long and variable lag phase in batch culture. This inhibition was further studied by estimating maximum specific growth rates at different substrate concentrations using the extended culture technique for control of the substrate concentration. With formate, inhibition became apparent at substrate concentrations above 20 mM, whereas oxalate inhibited growth at concentrations above 15 mM. Complete inhibition was not observed even at concentrations of 100 mM. A number of inhibition functions were fitted with the experimental data using computer analysis. The results indicated that the Haldane equation was the simplest function to describe quantitatively the kinetics of the observed substrate inhibition. Studies on the rate of oxygen uptake at different concentrations of oxalate indicated that respiration was much more sensitive to inhibition than growth. However with formate, inhibition of respiration was not observed up to concentrations of 50 mM, indicating that different mechanisms may underlie the observed growth inhibition by the two substrates.

INTRODUCTION

Pseudomonas oxalaticus OX1 is able to grow in a mineral salts medium with dipotassium oxalate or sodium formate as the sole source of carbon and energy. The pathways of oxalate and formate metabolism in this organism have been studied by Quayle and coworkers (Quayle, 1961). It was shown that during growth on oxalate the glycerate pathway is the main route for carbon assimilation (Quayle, Keech and Taylor, 1961). When the organism is grown on formate

on the other hand, all the carbon is assimilated from CO_2 which is fixed via the Calvin cycle (Quayle and Keech, 1959). During studies on the regulation of the choice between autotrophic and heterotrophic metabolism in *P. oxalaticus* when the organism is grown on mixtures of oxalate and formate in batch and continuous cultures, we have been troubled by a long and variable lag of growth. In some cases growth did not start at all. Since a possible explanation for the observed behaviour was that at the concentrations used (50 mM) these substrates inhibit growth, we decided to investigate the occurrence of substrate inhibition during growth of *P. oxalaticus* on each of the two compounds.

A detailed analysis of substrate-inhibited growth can be made by estimating specific growth rates at different substrate concentrations. Such a study of the kinetics of substrate inhibition has only been reported for a limited number of inhibitory substrates (Edwards, 1970; Pawlowsky and Howell, 1973). Kinetic functions have been proposed to model substrate inhibition of enzyme activity or growth of microorganisms on the basis of different assumptions on inhibition mechanisms at the molecular level (Webb, 1963; Edwards, 1970). It was expected that a detailed analysis of substrate inhibition by oxalate and formate in *P. oxalaticus* would allow a choice to be made of a particular inhibition function. This in turn would throw light on the molecular mechanism of inhibition of growth by these compounds. This paper records studies of the kinetics of substrate inhibition in *P. oxalaticus* in which a number of inhibition functions have been fitted with experimental data using computer analysis.

MATERIALS AND METHODS

Organism. Pseudomonas oxalaticus OX1 was obtained from Professor J. R. Quayle, Department of Microbiology, University of Sheffield, Sheffield, England. The organism was reisolated from a continuous culture with oxalate as the limiting nutrient after 3 weeks at a dilution rate of 0.05 hr^{-1}. It was maintained on 0.8% yeast extract slopes (Difco Laboratories, Detroit, Michigan, USA). Stock cultures were grown at 30 C, stored at 2 C and subcultured every 3 weeks. Purity was checked by streaking out for single colonies on yeast extract agar. The organism differs from that described by Höpner and Trautwein (1971) in that it can grow on formate as the sole source of carbon and energy.

Medium. The medium had the following percentage (w/v) composition: $(NH_4)_2SO_4$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.02; K_2HPO_4, 0.17; NaH_2PO_4, 0.14; $FeCl_2 \cdot 4H_2O$, 0.35×10^{-3}; $CaCl_2 \cdot 2H_2O$, 0.2×10^{-5}. Per liter of this medium 0.5 ml of a trace element mixture of the following composition was added

(mg/liter): $ZnSO_4 \cdot 7H_2O$, 50; $MnCl_2 \cdot 4H_2O$, 400; $CoCl_2 \cdot 6H_2O$, 1; $CuSO_4 \cdot 5H_2O$, 0.4; H_3BO_3, 2000; $Na_2MoO_4 \cdot 2H_2O$, 500. The concentration of dipotassium oxalate and sodium formate varied between 1 and 90 mM. The media were heat-sterilized for 15 min at 120 C; phosphates and the carbon sources were sterilized seperately and added to the mineral salts solution after cooling.

Growth conditions. The organism was grown in 500 ml conical flasks containing 200 ml medium. Incubation was on a rotory shaker (Gyrotory incubator-shaker G25, New Brunswick, New Jersey, USA) at 28 C. For the determination of the specific growth rate $\mu = \frac{1}{x} \cdot \frac{dx}{dt}$, where x is a parameter of culture density, the organism was grown in an all-glass fermenter described by Veldkamp and Kuenen (1973). Growth was followed turbidometrically using a Vitatron UC 200 colorimeter (Vitatron, Dieren, The Netherlands) equipped with a 433 nm filter. The pH of the culture was kept at pH 7.5 with the aid of a modified pH controller (Kuenen, Cuperus and Harder, 1973) using either 0.5 M oxalic acid or 1.0 M formic acid as neutralizing agents. The temperature was maintained at 28 C.

Assay procedures. Formate was estimated according to the method of Lang and Lang (1972). Oxalate was estimated using the volumetric method described by Vogel (1955).

Computer analysis. The inhibition functions were fitted to the experimental data by varying the fittable parameters in any given function to minimize the sum of the squares of the differences between the observed data and the function values at each substrate concentration. The nonlinear least squares fits were obtained with a Syber computer (Control Data Corp., Minnesota, USA) using program number 1720 of the Computer Centre, University of Groningen. This program is available from the authors on request.

Respiration studies. Cells grown at different substrate concentrations were harvested by centrifugation (6500 *g*, 20 C, 15 min), washed twice with 50 mM sodium phosphate buffer pH 7.5 and the cell density adjusted to 0.5 mg dry wt/ml. 0.5 ml of this suspension was added to the compartment of an oxygen electrode (Biological Oxygen Monitor, Model YSI B53, Yellow Spring Instruments Co., Yellow Springs, Ohio, USA). 4.5 ml of an air-saturated 50 mM sodium phosphate buffer was added to the suspension and the increase of the rate of oxygen uptake, after addition of various concentrations of oxalate and formate contained in 0.2 ml, was corrected for the endogenous rate of oxygen uptake. Oxygen uptake rates were calculated as μl O_2/mg dry wt of cells/hr. The temperature was maintained at 28 C.

RESULTS AND DISCUSSION

Observation of a pronounced and variable lag phase in batch cultures. In a study of mathematical models for growth of microorganisms with inhibitory substrates, Andrews (1968) demonstrated the occurrence of a long lag phase of growth when microorganisms are inoculated in media containing growth-inhibitory substrates. The model, which was based on the Haldane equation (Table 4, function 1), also predicted a significant decrease of the lag phase when a larger inoculum is used. Since a study of the effect of substrate concentration and inoculum size on the length of the lag phase is comparatively easy to perform we decided to investigate the possible occurrence of substrate inhibition in *P. oxalaticus* using this criterion. In the actual experiments, cells grown in media with 10 mM oxalate or 10 mM formate – which concentrations were kept constant using the extended culture technique (see below) – were harvested from the late exponential growth phase, washed twice with 50 mM sodium phosphate buffer pH 7.5 and resuspended in this buffer. Conical flasks containing mineral medium with different concentrations of oxalate or formate were inoculated with this suspension to give initial optical densities at 433 nm of 0.04, and in another series, of 0.08. In each case the elapsed time before growth started was recorded. The results (Tables 1 and 2) indicate increased lag phases of growth with increasing concentrations of the inhibitory substrate. The lag phase was longest when oxalate was present as the substrate. With formate a lag of growth of 2 hr was only observed at concentrations above 150 mM. When the inoculum size was increased twofold the lag period decreased by 1–4 hr both with oxalate and formate. These results indicate that growth inhibition of *P. oxalaticus* by

Table 1. Lag phases of growth of *Pseudomonas oxalaticus* OX1 in batch culture with different initial concentrations of dipotassium oxalate

Oxalate concentration (mM)	Initial turbidity $OD_{433}^{1\,cm} = 0.04$ Lag phase (hr)					Initial turbidity $OD_{433}^{1\,cm} = 0.08$ Lag phase (hr)				
	2	4	6	10	14	2	4	6	10	14
10	+	+	+	+	+	+	+	+	+	+
50	−	+	+	+	+	+	+	+	+	+
90	−	−	+	+	+	+	+	+	+	+
120	−	−	+	+	+	−	+	+	+	+
150	−	−	−	+	+	−	−	+	+	+
180	−	−	−	−	+	−	−	−	−	+
200	−	−	−	−	−	−	−	−	−	−

$+ = \Delta E_{433}^{1\,cm} \geqslant 0.01$ $\quad - = \Delta E_{433}^{1\,cm} < 0.01$

Table 2. Lag phases of growth of *Pseudomonas oxalaticus* OX1 in batch culture with different initial concentrations of sodium formate

Formate concentration (mM)	Initial turbidity $OD_{433}^{1\,cm} = 0.04$ Lag phase (hr)				Initial turbidity $OD_{433}^{1\,cm} = 0.08$ Lag phase (hr)			
	1	2	3	4	1	2	3	4
10	+	+	+	+	+	+	+	+
50	−	+	+	+	+	+	+	+
90	−	+	+	+	+	+	+	+
120	−	+	+	+	−	+	+	+
150	−	+	+	+	−	+	+	+
180	−	−	+	+	−	+	+	+
200	−	−	−	+	−	+	+	+

$+ = \Delta E_{433}^{1\,cm} \geqslant 0.01$ $\quad - = \Delta E_{433}^{1\,cm} < 0.01$

oxalate and formate already occurs at the relatively large inoculum sizes used. It was decided to study the kinetics of this inhibition in more detail.

Growth at controlled substrate concentrations. When studying the kinetics of the inhibition of growth of a microorganism by inhibitory substrates, specific growth rates have to be determined at different substrate concentrations, which should be kept constant over a prolonged period of time. Such a situation is not obtained in a classical batch culture where during growth the substrate concentration decreases with time. In the case of inhibitory substrates this will lead to decreasing inhibition and non-logarithmic growth will be the result. Logarithmic growth in the presence of a constant concentration of a growth-inhibitory substrate can be achieved using a semicontinuous culture technique for which the name extended culture has been proposed (Edwards et al., 1970). In the case of *P. oxalaticus* growing on oxalate or formate such a culture can be set up without elaborate systems such as an exponential gradient mixer (Martin and Felsenfeld, 1964) or other devices (Edwards et al., 1970). This is due to the molar growth yield of *P. oxalaticus* on the two substrates being very low (Harder, Wiersma and Groen, 1974). The rate of substrate disappearance from the medium is therefore relatively high and since this process is accompanied by a rise of the pH, control of the substrate concentration in the growing culture can be achieved by means of pH control through titration with either oxalic or formic acid of suitable concentration. Thus the oxalate concentration could be maintained at any value between 1.2 and 90 mM for at least 8 hr and in the case of formate, concentrations between 2 and 90 mM were kept constant for at least 6 hr. This is illustrated in Figs. 1 and 2. These examples are representative for the results obtained at various concentrations. The small decrease of the substrate concentration after 6–8 hr may be due to slight increase in the volume of

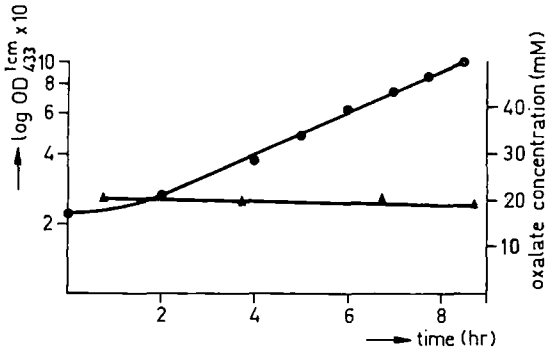

Fig. 1. Growth of *Pseudomonas oxalaticus* OX1 in extended culture at a constant oxalate concentration of 20 mM.
●—● optical density; ▲—▲ oxalate concentration.

the culture due to the incoming titrant and to consumption of NH_4^+ ions from the culture fluid. This results in a slightly falling pH of the culture and as a consequence less oxalic or formic acid is titrated than would be required to maintain the substrate concentration exactly constant. These results demonstrate that specific growth rates of *P. oxalaticus* at various substrate concentrations of oxalate and formate can be estimated using the extended culture technique.

Specific growth rates at various substrate concentrations. The determination of the specific growth rate μ of *P. oxalaticus* at various substrate concentrations of oxalate and formate were performed in an all-glass culture vessel with a working volume of 200 ml (Veldkamp and Kuenen, 1973). A log phase culture of the

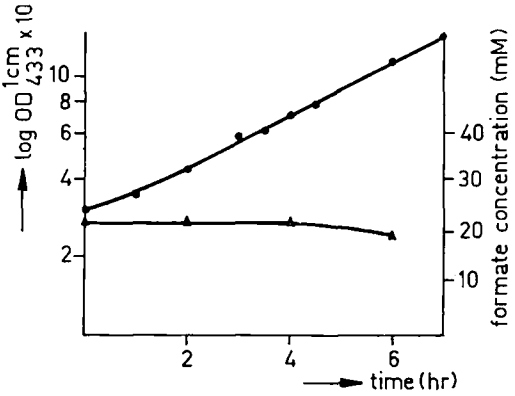

Fig. 2. Growth of *Pseudomonas oxalaticus* OX1 in extended culture at a constant formate concentration of 21 mM.
●—● optical density; ▲—▲ formate concentration.

organism, which was grown at the substrate concentration to be tested, was partly removed from the growth vessel. The remaining volume of the culture (ca. 15 ml) was used to inoculate fresh sterile medium, which was introduced into the vessel. The concentration of oxalate or formate in this medium was chosen in such a way that the final concentration in the culture was between 1 and 90 mM. The culture was stirred and aerated and immediately after mixing the pH was adjusted to pH = 7.6 with 0.5 N H_2SO_4. Any following change in the pH of the culture was then automatically corrected by addition of 0.5 M oxalic acid or 1.0 M formic acid with the aid of a pH controller. Growth at 28 C was followed by reading the optical density of the culture at suitable time intervals. At each substrate concentration at least three runs were conducted, both with oxalate and formate, and in each case a clear logarithmic growth phase was observed (Figs. 1 and 2). Specific growth rates of *P. oxalaticus* were calculated from the linear parts of the plots obtained when the logarithm of the optical density at 433 mM log ($OD_{433}^{1\,cm}$) was plotted against time and the average value at each substrate concentration is given in Table 3. The results indicate that maximum growth rates were obtained at 15 mM in the case of oxalate and 21 mM in the case of formate. At higher concentrations of these substrates the specific growth rate decreased, which indicates growth inhibition by these compounds. However, complete inhibition was not observed even at concentrations of 100 mM. On the basis of these results substrate concentrations of oxalate and formate employed in media used for batch cultivation and during the initial stages of continuous cultivation are now kept at 15–20 mM. Lag of growth of the organism has since not been observed.

Table 3. Specific growth rates μ of *Pseudomonas oxalaticus* OX1 at different concentrations of oxalate or formate. Each value is a mean of at least 3 experiments; triplicates were always within 3% of the values given.

Growth substrate oxalate		Growth substrate formate	
Concentration (mM)	Specific growth rate μ (hr^{-1})	Concentration (mM)	Specific growth rate μ (hr^{-1})
1.2	0.173	2	0.220
5	0.189	5	0.232
10	0.207	10	0.237
15	0.211	13	0.243
20	0.193	21	0.246
30	0.167	30	0.223
50	0.151	55	0.208
70	0.131	70	0.178
75	0.124	90	0.157
80	0.118		
90	0.109		

Table 4. Kinetic models for substrate-inhibited growth

Function 1:	$\mu = \frac{\mu_{max}.S}{(K_s + S)(1 + S/K_i)}$
Function 2:	$\mu = \frac{\mu_{max}.S(1 + S/K)}{K_s + S + S^2/K_i}$
Function 3:	$\mu = \mu_{max}[\exp(-S/K_i) - \exp(-S/K_s)]$
Function 4:	$\mu = \frac{\mu_{max}.S\exp(-S/K_i)}{K_s + S}$

μ = specific growth rate at substrate concentration S; μ_{max} = maximum specific growth rate; S = substrate concentration; K_s = affinity constant; K_i and K = inhibition constants.

Determination of rate constants by computer analysis. An analysis of the kinetics of substrate inhibition in *P. oxalaticus* was made using a number of the functions tested by Edwards et al. (1970, Table 4). These functions have been selected arbitrarily from different mechanisms and models that have been proposed to kinetically describe substrate inhibition (Webb, 1963; Edwards et al., 1970). The first three functions result from various assumptions about organism–substrate interactions; the fourth was selected mainly to see whether an empirical relation for substrate inhibition derived from the data of Aiba, Shoda and Nagatani (1968) would provide a better fit with our experimental data. These four functions were fitted to the above substrate inhibition data by varying the fittable parameters (μ_{max}, K_s, K_i, K) in any given function to minimize the sum of squares of the errors calculated as the differences between the observed data and the values at each substrate concentration using the function. The results of these calculations have been summarized in Table 5. In the case of oxalate, the least squares fit was obtained with functions 1 and 3. Using the constants from Table 5, graphs have been drawn relating specific growth rate and substrate concentration of *P. oxalaticus* for functions 1 and 3 (Fig. 3). The experimental data have been included in the figure and it may be concluded that both functions give a satisfactory fit. A similar result was obtained for growth of *P. oxalaticus* on formate using the kinetic constants calculated for functions 1 and 4 (Fig. 4).

From the data (Table 5) it appears that function 2 gave the worst fit with both substrates. The calculated μ_{max} value is unrealistically high in both cases and in addition $K_s \gg K_i$. For these reasons function 2 has to be rejected. However, the functions 1, 3 and 4 give a comparable fit and it is difficult to favour any of these. Edwards (1970) and Pawlowsky and Howell (1973) arrived at similar conclu-

Table 5. Constants obtained by least squares fit of selected kinetic models to growth data of *Pseudomonas oxalaticus* OX1 growing on oxalate or formate

Function fitted	μ_m (hr^{-1})	K_s (mM)	K_i (mM)	K (mM)	Minimal sum of squares of the differences ($\times 10^4$)
	Growth on oxalate				
1	0.288	1.93	58.06		2.34
2	18.750	406.50	7.85	25.1	5.09
3	0.231	2.54	118.41		2.35
4	0.250	1.22	107.43		3.68
	Growth on formate				
1	0.281	0.57	121.58		7.05
2	6.956	60.63	8.51	4.52	40.99
3	0.257	1.03	186.98		9.47
4	0.275	0.53	157.24		5.42

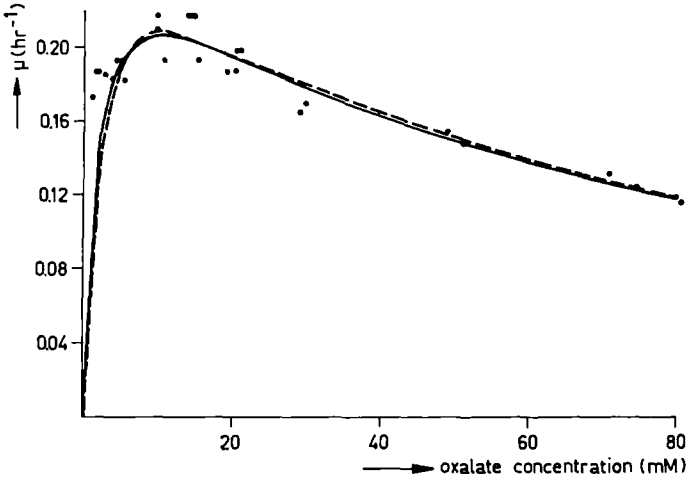

Fig. 3. Specific growth rate of *Pseudomonas oxalaticus* OX1 at different oxalate concentrations. The solid and dashed lines were calculated using functions 1 and 3 (Table 4), respectively. • • experimental data.

sions. Since functions 1, 3 and 4 mirror the trends in the experimental data with complete success either of the three functions may be chosen for a semiquantitative description of the growth inhibition of *P. oxalaticus* by oxalate and formate. Because the Haldane equation (function 1) is easier to manipulate than the other functions tested, it is concluded that this function is the equation of choice in describing the kinetics of substrate inhibition in *P. oxalaticus*.

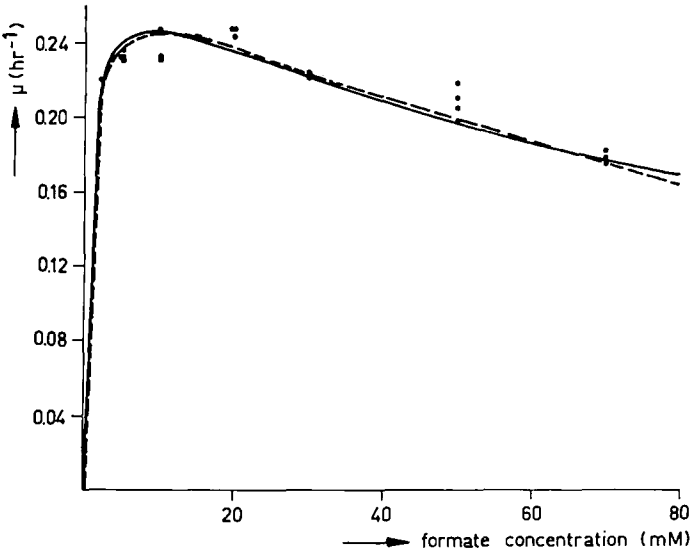

Fig. 4. Specific growth rate of *Pseudomonas oxalaticus* OX1 at different formate concentrations. The solid and dashed lines were calculated using functions 1 and 4, respectively.
• • experimental data.

Inhibition of substrate respiration by oxalate. Three of the inhibition functions tested above are based on different models about organism–substrate interactions. It was therefore expected that a computer analysis of the kinetics of substrate inhibition in *P. oxalaticus* would contribute towards an understanding of the mechanism of inhibition. Although the observed kinetics did enable us to reject function 2, it was impossible to favour either of the two other theoretical models tested.

Therefore an attempt was made to investigate possible inhibition mechanisms of growth by studying the kinetics of the inhibition of substrate dissimilation in *P. oxalaticus*. This was done by following the rate of oxygen uptake of cell suspensions of *P. oxalaticus* at different concentrations of the substrate. Preliminary experiments showed that respiration of formate was not inhibited by concentrations up to 50 mM and therefore oxalate respiration was studied only. The organism was grown at different concentrations of dipotassium oxalate (between 5 and 90 mM) using the extended culture technique. Washed-cell suspensions were prepared from exponentially growing cultures and rates of oxygen uptake of these suspensions were measured at various oxalate concentrations ranging from 20 μM up to 50 mM. A typical result of such an experiment is shown in Fig. 5 in which the respiration rate is plotted as a function of the substrate concentration in the form of a Lineweaver-Burk plot. From the data obtained,

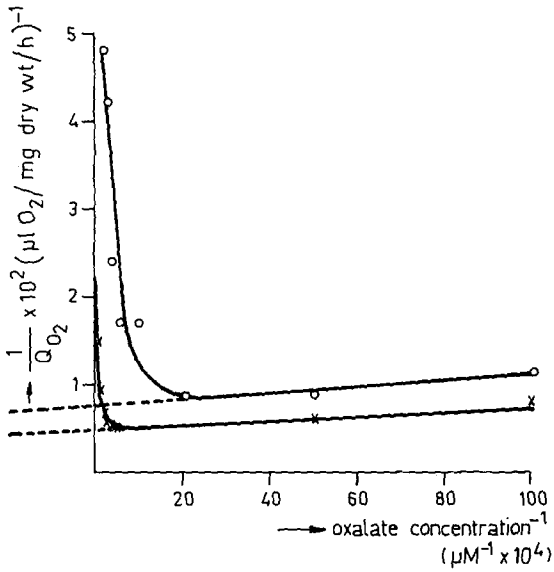

Fig. 5. Lineweaver-Burk plot of the rate of oxygen uptake of cell suspensions of *Pseudomonas oxalaticus* OX1 at different oxalate concentrations.
×—× organisms grown at a constant oxalate concentration of 15 mM;
○—○ organisms grown at a constant oxalate concentration of 70 mM.

the K_M value of the respiratory system for oxalate was estimated and found to be 40 ± 5 μM. This value is much lower than the K_s value calculated for growth (Table 5). The concentration of oxalate at which inhibition of the respiration became apparent was also much lower than that required for inhibition of growth, indicating that the respiratory system is more sensitive to inhibition by oxalate than is growth. Furthermore, the concentration of the substrate at which the cells had been grown had an effect on the concentration range in which inhibition of respiration became apparent. For example, cells grown at a constant oxalate concentration of 15 mM showed inhibition of respiration at concentrations above 2 mM, whereas cells grown at 70 mM oxalate showed a decreased rate of oxygen uptake above concentrations of 0.5 mM (Fig. 5). Another interesting phenomenon which appeared from these experiments was that the maximum rate of oxygen uptake of oxalate-grown cell suspensions calculated from the intercept with the vertical axis (Fig. 5), was dependent on the concentration of oxalate at which the cells had been grown. Although the full significance of these results is difficult to explain at present, the data show that the respiratory machinery required for the oxidation of oxalate has a high affinity for the substrate. In addition it is much more sensitive to inhibition by oxalate than is growth. This indicates that the primary process eventually leading to

growth inhibition may be related to respiration. In this respect it would be of interest to investigate the possible inhibition of active transport of oxalate in *P. oxalaticus* (Harder et al., 1974) at higher concentrations of this compound. Evidence for inhibition of the respiration of formate was not obtained even at concentrations of 50 mM. This indicates that the mechanism of growth inhibition by formate may be different.

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