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An Analysis of the Growth of *Gluconobacter oxydans* in Chemostat Cultures

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Abstract. *Gluconobacter oxydans* was grown successively in glucose and nitrogen-limited chemostat cultures. Construction of mass balances of organisms growing at increasing dilution rates in glucose-limited cultures, at pH 5.5, revealed a major shift from extensive glucose metabolism via the pentose phosphate pathway to the direct pathway of glucose oxidation yielding gluconic acid. Thus, whereas carbon dioxide production from glucose accounted for 49.4% of the carbon input at a dilution rate (D) = 0.05 h^{-1}, it accounted for only 1.3% at D = 0.26 h^{-1}. This decline in pentose phosphate pathway activity resulted in decreasing molar growth yields on glucose. At dilution rates of 0.05 h^{-1} and 0.26 h^{-1} molar growth yields of 19.5 g/mol and 3.2 g/mol, respectively, were obtained. Increase of the steady state glucose concentration in nitrogen-limited chemostat cultures maintained at a constant dilution rate also resulted in a decreased flow of carbon through the pentose phosphate pathway. Above a threshold value of 15–20 mM glucose in the culture, pentose phosphate pathway activity almost completely inhibited. In *G. oxydans* the coupling between energy generation and growth was very inefficient; yield values obtained at various dilution rates varied between 0.8–3.4 g/cells synthesized per 0.5 mol of oxygen consumed.

Key words: *Gluconobacter oxydans* – Chemostat – Glucose oxidation – Gluconate oxidation – Growth yields.

Gluconobacter oxydans is known to oxidize glucose via a direct oxidative pathway to gluconic acid (King and Cheldelin, 1958) and ketogluconic acid (Stouthamer, 1960). However, glucose may also be oxidized, after phosphorylation (to glucose 6-phosphate) by the enzymes of the pentose phosphate pathway. Gluconate is similarly oxidized in this pathway, after phosphorylation to 6-phosphogluconate. An active tricarboxylic acid cycle is not present in this organism (Kitos et al., 1958). It was found during batch culture studies with *G. oxydans* growing in glucose-constaining media, that the carbon flow through the pentose phosphate pathway was dependent on both the pH of the culture and the precise concentration of glucose in the medium (Olijve and Kok, 1979). Thus, pH values below 3.5–4.0 inhibited phosphorylative glucose and gluconate oxidation completely, and resulted in quantitative accumulation of gluconate in the medium. When the pH value of the culture medium was automatically maintained in the optimal range for growth (4.0–6.0), a triphasic batch growth response was observed. Above a threshold concentration of 5–15 mM, glucose repressed pentose phosphate activity. As a consequence very low cell yields were obtained under these conditions; however, an accurate determination of the efficiency of growth was very difficult in batch culture, owing to the continuously changing chemical environment occurring throughout the growth cycle.

The chemostat mode of continuous culture opens us the possibility of studying the behaviour of microorganisms towards precise concentrations of different nutrients in the growth medium. In this report an analysis is given of the growth of *G. oxydans* in both a glucose-limited and a nitrogen-limited (glucose-sufficient) chemostat culture. Supporting evidence is presented for the regulatory role of the glucose concentration in the assimilation of glucose and gluconate by *G. oxydans*.

Materials and Methods

Microorganism and Cultivation. Gluconobacter oxydans, subspecies *suboxydans* (obtained from the American Type Culture Collection as

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Acetobacter suboxydans ATCC 621 H) was grown in chemostat cultures in both the complex and the chemically defined media described previously (Olijve and Kok, 1979). The defined and the complex media contained 10 g glucose per liter, when glucose-limited. When *G. oxydans* was grown at pH 2.5, the glucose concentration was raised to 50 g per liter in order to compensate for the diminished yield of organisms. The nitrogen-limited medium contained 185 mg per liter $(NH_4)_2SO_4$ and the L-glutamine was replaced by α-keto glutaric acid (100 mg per liter); the glucose concentration was raised to 20 g per liter (or as specified in the Results section). Cultivation was at 28 °C and the pH was controlled at either 2.5 or 5.5 by automatic adjustment with 2N NaOH. The equipment used has been described by Harder et al. (1974).

Carbon Balances. Glucose was assayed by the glucose oxidase method (Boehringer, Mannheim, Germany). Airflow rates, carbon dioxide production and oxygen consumption rates were determined as described previously (Olijve and Kok, 1979). For the determination of the dry weight of organisms in the culture, 100 ml samples were removed from the chemostat, centrifuged, washed twice with distilled water and dried to constant weight at 105° C. After weighing the carbon, hydrogen and nitrogen contents of the dried cells were determined by standard methods of organic analysis. Total acid formed was assayed by determination of the amount of alkali required to maintain the culture pH value constant. Since the primary product of direct glucose oxidation is glucono-δ-lactone, 5 ml culture supernatant was titrated to pH 10 (in order to allow complete hydrolysis of the lactone) using a titrator (Radiometer, Copenhagen, Denmark), equipped with an automatic burette (Dosimat E 535; Methrom Heritale, Switzerland); complete medium was used as a reference.

Paper Chromatography of Ketogluconates. For paper chromatography of ketogluconates, the method of Stouthamer (1960) was used. Preparation of samples was as described previously (Olijve and Kok, 1979). After elution, the paper was sprayed with a 2% (w/v) solution of o-phenylenediamine dihydrochloride in 80% aqueous ethanol (De Ley, 1955).

Measurement of the Potential Maximum Rate of Glucose and Gluconate Oxidation by whole Cells ($Q_{O_2}^{max}$). The $Q_{O_2}^{max}$ was determined using an oxygen electrode as described previously (Olijve and Kok, 1979).

Results

Growth of Gluconobacter oxydans in Glucose-Limited Chemostats at Controlled pH Values of 2.5 and 5.5. As reported previously (Olijve and Kok, 1979) *G. oxydans* is unable to grow in chemically defined media at pH values below 3.5 – 4.0. However, reasonable growth rates can be obtained in complex media, thus illustrating the increased nutritional requirements of *G. oxydans* when growing at these low pH values. In this connection, addition of several vitamins, amino acids and nucleotides to the defined medium did not result in growth at pH 2.5. The complex nutritional requirement was most probably caused by an almost complete inhibition of pentose phosphate pathway activity at pH 2.5, as suggested previously (Olijve and Kok, 1979). This is also shown by the results of an experiment in which a comparison was made of growth and glucose metabolism of *G. oxydans* in glucose-limited chemostat cultures at pH 2.5 and pH 5.5, respectively (Table 1).

Table 1. Growth and glucose oxidation in glucose-limited chemostat cultures of *Gluconobacter oxydans* at pH 2.5 and 5.5, (glucose-yeast extract media)

	D (h^{-1})	Y_{mol}[a]	Ratio %		Carbon-[c] recovery %
			gluconate/ glucose	ketogluconate/ glucose	
pH 2.5	0.05	1.73	98.0	3.4	101.4
	0.10	1.68	99.1	3.4	102.5
pH 5.5	0.05	27.4	4.9	16.6	21.5
	0.26	18.9	38.7	35.6	74.3

[a] G cells synthesized per mol glucose consumed
[b] Mol gluconate produced per mol glucose consumed × 100%; the ketogluconate concentration was calculated from the 2- and 5-ketogluconate concentrations in culture supernatants
[c] Carbon recovery was based on the gluconate and ketogluconate concentrations in the culture

Activity of the pentose phosphate pathway was completely absent when *G. oxydans* was grown at pH 2.5, since the glucose consumed could be quantitatively recovered as gluconates. These results show clearly that glucose is used under these conditions as an energy source and not as a source of carbon and here it is worth mentioning that yeast extract alone did not support growth. When the pH of the culture medium was controlled at pH 5.5, completely different oxidation kinetics were observed. A substantial amount of the added glucose was oxidized in the pentose phosphate pathway resulting in an increased molar growth yield on glucose.

Influence of Dilution Rate on the Growth and Rate of Oxidation of Glucose by G. oxydans in Glucose-Limited Chemostat Cultures. Gluconobacter oxydans was grown in a glucose-limited chemostat culture in a chemically defined medium with the pH value controlled at 5.5. Culture contents of organisms, substrate and products were determined at several dilution rates. The relationship found between molar growth yield and the dilution rate is shown in Fig. 1. These data reveal that a remarkable decrease in growth yield with increasing dilution rate, although the steady state glucose concentrations remained very low up to dilution rates of 0.15 h^{-1}. This observation prompted us to construct carbon mass balances over the whole range of dilution rates. Complete carbon recovery was obtained at D = 0.05 h^{-1}, 0.075 h^{-1}, 0.19 h^{-1} and 0.26 h^{-1} from determinations of cell material, carbon dioxide, gluconate and 2-keto- and 5-ketogluconate. At D = 0.16 h^{-1}, however, these compounds accounted for only 87% of the carbon input. Examination of the culture supernatant for keto acids by paper chromatography revealed a third component (besides, of 2-keto- and 5-ketogluconate) which had a yellow-brownish

colour in daylight after spraying the chromatograph with the o-phenylenediamine reagent. The R_f value of this component was about the same as that reported for 2,5-diketogluconate by Stouthamer (1960). Moreover, calculations based on the total amount of acid gave a complete carbon recovery when the amount of acid found was taken to be C_6-monocarboxylic acids. It therefore seems probable that this compound is indeed 2,5-diketogluconic acid. The 2,5-diketogluconate concentration in the culture medium was calculated by subtracting the gluconate and ketogluconate concentrations from the total amount of acid. The elementary cell composition of *G. oxydans* was found to be $C_5H_{8.9}NO_{1.9}$; and this elementary cell composition was constant over the whole range of dilution rates studied (0.05 h^{-1} to 0.26 h^{-1}). The overall stoicheiometry of growth of the organism, as a function of the dilution rate, is given in Table 2. These data clearly show that the decrease in cell yield coincides with decreasing pentose phosphate pathway activity. The carbon flow through the direct pathway of glucose oxidation leading to gluconic acid and ketogluconic acids, and through the pentose phosphate pathway, is thus dependent on the dilution rate (Fig. 2). At D

Table 2. Overall stoicheiometry of growth of *Gluconobacter oxydans* on glucose in glucose-limited chemostat cultures as a function of the dilution rate

D (h^{-1})	Molar Ratios										Carbon recovery %
	Substrates				Products						
	Glucose	$+O_2$	$+ NH_3$	→	Cell material ($C_5H_{8.9}NO_{1.9}$)	$+ CO_2$	+ Gluconic acid	+ 2- and 5-ketogluconic acid	+ 2,5-diketo-[a] gluconic acid	$+ H_2O$	
0.05	10	35.3	1.7	→	1.7	29.6	0.5	3.0	–	43.6	97.0
0.075	10	26.4	1.4	→	1.4	22.5	0.8	4.2	–	30.4	97.2
0.16	10	13.2	0.8	→	0.8	7.7	6.0	0.9	1.4	11.9	101.0
0.19	10	7.8	0.4	→	0.4	2.3	8.3	0.8	–	6.8	95.4
0.26	10	5.9	0.3	→	0.3	0.8	9.1	0.7	–	1.3	101.0

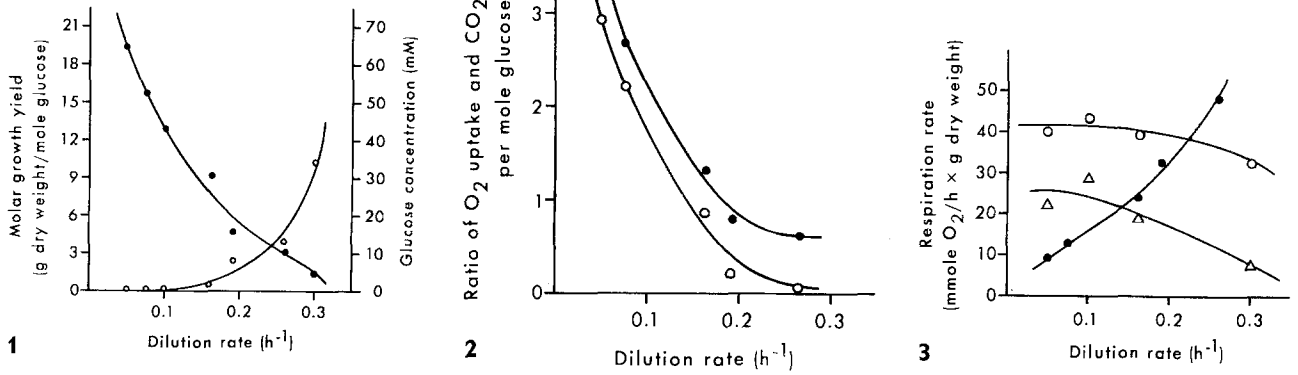


Fig. 1. Growth of *Gluconobacter oxydans* in a glucose-limited chemostat culture as a function of the dilution rate. Growth is expressed as molar growth yield (●), the glucose concentration (○) is given in mM. Growth conditions: defined medium, pH 5.5, temperature 28 °C

Fig. 2. Extent of glucose oxidation in a glucose-limited chemostat culture of *Gluconobacter oxydans* as a function of the dilution rate. The plotted values were calculated from the stoicheiometry of growth as given in Table 2. Symbols: (●) mol oxygen consumed per mol glucose, (○) mol carbon dioxde produced per mol glucose

Fig. 3. Q_{O_2} (●) and Q^{max} for glucose (○) and gluconate (▲) of *Gluconobacter oxydans* grown in a glucose-limited chemostat as a function of the dilution rate. $Q_{O_2}^{max}$ was determined by measuring the oxygen uptake of cell suspensions in the presence of 120 mM substrate. Q_{O_2} was calculated from carbon balances

$= 0.05\ h^{-1}$ nearly 50% of the glucose carbon was recovered as carbon dioxide, while at $D = 0.26\ h^{-1}$ only about 1.5% of the glucose carbon appeared as carbon dioxide.

Small amounts of keto acids were formed at dilution rates above $0.20\ h^{-1}$, corresponding with glucose concentrations of 10 mM and higher, while at dilution rates below $0.20\ h^{-1}$ the gluconate produced from glucose was almost quantitatively oxidized to ketogluconates. This is a similar situation to that observed in batch culture (Olijve and Kok, 1979) where, in the first growth phase no accumulation of ketogluconates was found, while in the second phase small amounts of keto acids were produced. Although the ratio between 2- and 5-ketogluconate concentrations changed with the dilution rate (Table 3) 2-ketogluconate was principally produced by this strain.

In the glucose-limited cultures the respiration rate was limited by the rate of supply of glucose to the organisms. Cells were thus unable to express their full respiratory potential. However, the full respiratory potential could be measured with washed cell suspensions, and the observed respiration rate (Q_{O_2}) and the respiratory potential ($Q_{O_2}^{max}$) of organisms growing at different dilution rates were compared (Fig. 3). A large overcapacity of the cells to oxidize glucose existed at low dilution rates. This over-capacity decreased slightly at increasing dilution rates until at dilution rates above $0.22\ h^{-1}$ the observed $Q_{O_2}^{max}$ could no longer account for the calculated oxygen uptake rate of the culture. Although several explanations may be put forward to explain this discrepancy, the precise reasons for it remain obscure.

Growth of Gluconobacter oxydans and Glucose Oxidation in Nitrogen-Limited Chemostat Cultures. The relationship between dilution rate and growth of *G. oxydans* in a nitrogen-limited chemostat culture is shown in Fig. 4. Bacterial concentration clearly was independent of the dilution rate, except at growth rates close to the critical dilution rate (D_c). In these carbon-sufficient cultures the organisms were able to express their full respiratory potential. When the Q_{O_2} values in situ are compared with the corresponding $Q_{O_2}^{max}$ values for glucose and gluconate, at each dilution rate (Table 4), similar values were found, but with increasing dilution rates these organisms showed an increased capacity to oxidize glucose and a decreased capacity to oxidize gluconate. These results suggest that changes in the cells' content of glucose- and gluconate dissimilating enzymes or of transport capacity had occurred, that were dependent on the dilution rate. The $Q_{O_2}^{max}$ value obtained at $D = 0.05\ h^{-1}$ with cells grown in a glucose-limited chemostat culture was higher than that of the nitrogen-limited cells by a factor of about 2.5.

Table 3. Ketogluconate formation by *Gluconobacter oxydans* grown in glucose-limited chemostats at pH 5.5

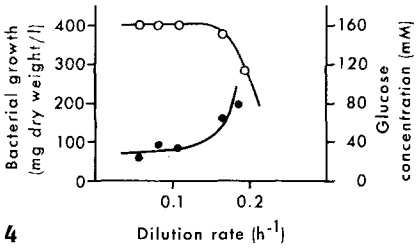
Dilution rate (h^{-1})	Steady state concentrations (mM)		
	Glucose	2-keto-gluconate	5-keto-gluconate
0.05	0.03	10.2	5.8
0.075	0.04	20.8	3.0
0.16	1.4	17.2	4.0
0.19	9.0	2.9	0.8
0.26	13.8	1.9	1.0

Table 4. Maximal respiration rates ($Q_{O_2}^{max}$) and respiration in situ (Q_{O_2}, Q_{CO_2}) as a function of the dilution rate in nitrogen-limited chemostat cultures of *Gluconobacter oxydans*

Dilution rate (h^{-1})	Respiration rate (mmol/h × g dry weight)			
	Q_{O_2} in situ	Q_{CO_2} in situ	$Q_{O_2}^{max}$-glucose	$Q_{O_2}^{max}$-gluconate
0.054	20.4	8.3	17.4	11.8
0.11	26.9	8.5	32.7	15.9
0.16	46.9	8.2	57.7	9.5
0.18	60.6	6.6	52.2	4.7

One must conclude, therefore, that the inherent low affinity of whole cells for glucose ($K_s = 13$ mM; Olijve and Kok, 1979) can be compensated for by the synthesis of large amounts of those enzymes responsible for glucose oxidation, under conditions where the extracellular glucose concentration is low. In nitrogen-limited cultures, the rate of oxidation of glucose by enzymes of the pentose phosphate pathway was seemingly constant over the range of dilution rates studied, since the Q_{O_2} did not vary significantly with the dilution rate. Thus, as the growth rate was increased more glucose was oxidized via the direct pathway.

The coupling between respiration and growth in nitrogen-limited cultures is different from that extant in glucose-limited cultures (Table 5). The yield values for oxygen, obtained with cultures of *G. oxydans* growing in both glucose and nitrogen-limited chemostats were very low, an observation in agreement with the results of Whittaker and Elsden (1963), who reported a Y_o of 4.0 g organisms synthesized per 0.5 mol oxygen consumed. In glucose-sufficient cultures energy conservation from substrate oxidation was even less efficient than in glucose-limited cultures. The influence of an increasing glucose input on the relationship between growth and respiration was studied with a nitrogen-limited chemostat culture growing at a fixed dilution



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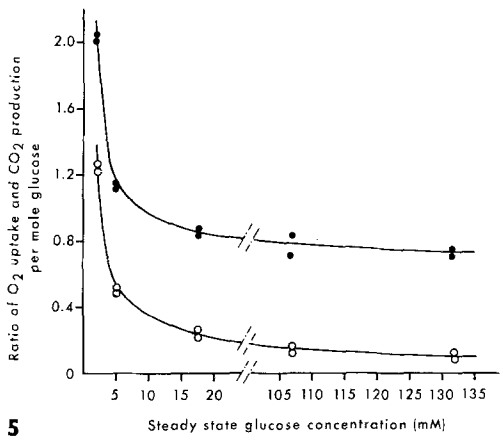

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Fig. 4. Bacterial dry weight (O) and glucose concentration (●) in nitrogen-limited chemostat cultures of *Gluconobacter oxydans*, as a function of the dilution rate. Glucose concentration in feed: 230 mM

Fig. 5. Extent of glucose oxidation in a nitrogen-limited chemostat culture of *Gluconobacter oxydans* ($D = 0.13\,h^{-1}$), as a function of the steady state glucose concentration. Symbols: (●) mol O_2 consumed per mol glucose consumed, (O) mol CO_2 produced per mol glucose consumed

Table 5. Yield values for oxygen obtained with glucose- and nitrogen-limited chemostat cultures of *Gluconobacter oxydans* (pH 5.5; 28°C; Y_o = g organism synthesized/0.5 mol oxygen consumed)

	Glucose limitation	Nitrogen limitation
$D = 0.05\,h^{-1}$	3.0	1.1
$D = 0.16\,h^{-1}$	3.4	1.5

Table 6. Yield values for oxygen of *Gluconobacter oxydans* grown in a nitrogen-limited chemostat at $D = 0.13\,h^{-1}$ at increasing glucose input

Steady state glucose concentration (mM)	Y_o (g cells synthesized per 0.5 mol oxygen consumed
2	1.8
5	1.9
18	1.2
105	0.8
132	0.8

rate. The results (Table 6) show that increasing the glucose input concentration effected a decrease in the coupling between respiration and growth. In the same experiment the carbon flow through either the direct pathway of glucose oxidation or the pentose phosphate pathway was determined as a function of the residual glucose (Fig. 5). These data show that the extent of glucose oxidation was dependent on the steady state glucose concentration. Below a glucose concentration of 15 – 20 mM, oxidation of glucose (and of gluconate) via the pentose phosphate pathway was increased.

It is noteworthy that considerable amounts of glucono-δ-lactone accumulated in the culture medium. Lien (1959) determined the half-time of glucono-δ-lactone hydrolysis as a function of the pH value. Determination of the difference between the amount of alkali added to maintain the culture pH constant and the total amount of acid produced, revealed that the amount glucono-δ-lactone thus obtained at the various dilution rates, was closely comparable with the theoretical value calculated from the data of Lien. This result indicates that glucono-δ-lactone (as the primary product of direct glucose oxidation) rapidly accumulated in the culture.

Discussion

The experiments described above show that *Gluconobacter oxydans* was able to modulate the flow of carbon through either the direct pathway of glucose oxidation or through the pentose phosphate pathway. This modulation was clearly apparent when the dilution rate of a glucose-limited chemostat culture was varied. At low rates, where the pentose phosphate pathway was most active nearly 50% of the added carbon was recovered as carbon dioxide, whilst at high growth rates only a very small part of the glucose added was oxidized via the pentose phosphate pathway. Thus, gluconate and ketogluconate accumulated in the culture medium. Since the decrease in growth yield coincided with the decrease in pentose phosphate pathway activity, it seemed reasonable to suppose that the decrease in cell yield was caused by inhibition of the pentose phosphate pathway enzymes. It was also shown that increased extracellular glucose concen-

trations resulted in a decreased flow of carbon through the pentose phosphate pathway. Assimilation of glucose via this pathway was completely absent when *G. oxydans* was grown at pH values of 2.5, thereby causing the organisms to have increased nutritional requirements. These results are similar to those in batch culture (Olijve and Kok, 1979). Very low growth yields were obtained with *G. oxydans* when grown in glucose-containing media. These low cell yields correlated with low Y_o values thus indicating very poor coupling between respiration and growth (Tables 5 and 6) extant under the various growth conditions. These Y_o values are much lower than the values found with cultures of other organisms, growing on glucose [e. g. 20.2 for *Escherichia coli* (Whittaker and Elsden, 1963), 16.0 for *Pseudomonas fluorescens* (Whittaker and Elsden, 1963) and 31.9 for *Aerobacter aerogenes* (Hadjipetrou et al., 1964)]. Phosphorylation efficiency apparently was low in *G. oxydans* (Klungsöyr et al., 1957), most probably due to the organism's possession of a restricted electron transport system (Smith, 1961; Yamada et al., 1969). *Gluconobacter oxydans* possesses membrane-bound dehydrogenases which are claimed to be responsible for the rapid single step oxidation carried out by this organism (Widmer et al., 1956; Kersters et al., 1965). Apart from these particulate enzymes, which most probably are linked to the cytochrome chain, cytoplasmic dehydrogenases effecting direct substrate oxidation often are found, which use soluble cofactors such as NAD or NADP (Cummins et al., 1957). In *G. oxydans* both a membrane associated and an NADP-linked glucose dehydrogenase has been detected (King and Cheldelin, 1958; Stouthamer, 1960), and detailed enzymatic analysis of glucose grown *G. oxydans* has indicated that the NADP-linked dehydrogenase is the more important enzyme for glucose oxidation (Olijve, unpublished data).

Particulate and soluble enzyme systems for gluconate oxidation also are present in *G. oxydans* (De Ley and Stouthamer, 1959), the former yielding 2-ketogluconate, and the latter leading to the formation of both 2- and 5-ketogluconate. Shinagawa et al. (1976) reported that the activity of the particulate enzyme in cell-free extracts of several *Acetobacter* and *Gluconobacter* strains was about 100 times that of the soluble NADP-linked enzymes. It was postulated that the membrane-bound enzyme was mainly responsible for the formation of 2-ketogluconate whereas the NADP-linked enzymes were thought mainly to contribute to the reduction of ketogluconates to gluconate. This is in agreement with the conclusion of Stouthamer (1960) that ketogluconates were only further metabolized by *Acetobacter* after reduction to gluconate. Our results show that ketogluconates were only produced at low dilution rates, when *G. oxydans* was grown under glucose-limitation. Although, at high dilution rates, reasonable $Q_{O_2}^{max}$ values were found with gluconate, only small amounts of keto acids accumulated in the medium. This could be partly explained by the observation that, especially at these high dilution rates, glucono-δ-lactone was present in the culture medium. It is, however, more likely that under these conditions sufficient $NADPH_2$ is produced from glucose by the action of NADP-linked glucose dehydrogenase to reduce the ketogluconate (produced by the particulate gluconate dehydrogenase) to gluconate. This simultaneous operation of a particulate (cytochrome chain-linked) gluconate dehydrogenase and an NADPH-linked ketogluconate reductase (which effects a regeneration of oxidized NADP) represents a "futile cycle" but nevertheless allows the organisms to maintain a dynamic balance between the levels of its oxidized and reduced pyridine nucleotides. This is a very important consideration in *G. oxydans* since this organism supposedly lacks both pyridine nucleotide transhydrogenase and NADPH-oxidase (Eagon, 1963). A similar system has been described by Mowshowitz et al. (1974) for *Gluconobacter cerinus*. This strain excreted 5-ketofructose when grown on fructose, but only after cell growth ceased. It was, however, found that during the exponential growth phase 5-ketofructose was produced by a particulate dehydrogenase, though accumulation of this compound did not occur owing to the rapid rate at which it could be reduced back to fructose by a soluble NADPH-linked 5-ketofructose reductase. In *G. oxydans*, oxidation of $NADPH_2$ is vital for functioning of both the pentose phosphate pathway and for the activity of NADP-linked glucose dehydrogenase. Moreover, in the regulation of the flow of glucose over the direct pathway and the pentose phosphate pathway an important role may be played by the first products of direct glucose oxidation (namely $NADPH_2$ and ATP). Many authors, (Olive and Levy, 1967; Lee and Lessie, 1974; Lessie and Vander Wijk, 1972; Westwood and Doelle, 1974) have suggested that the cellular concentrations of $NADPH_2$ or (NADP/$NADPH_2$) ratio, or both, exert a significant control over the activity of the pentose phosphate pathway. Changes in the level of glucose- and gluconate dehydrogenases, and of ketogluconate reductases, will then result in changes in the (NADP)/($NADPH_2$) ratio, thus modulating the pentose phosphate pathway activity. The role of both the particulate glucose dehydrogenase and the NADP-linked soluble glucose dehydrogenase in direct glucose oxidation is not known and will require further investigation.

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