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Energetic regulation of microbial growth

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1. Introduction

In today's talk I would like to limit myself to a discussion of energy-metabolism as it relates to microbial growth. Energetics of the formation of metabolic products, therefore, will not be emphasized. Figure 1 is a very qualitative picture and I want to try to make it a more quantitative one. For a more quantitative description of microbial growth, and for a description of both substrate flow and energy flow one needs to integrate knowledge from biochemistry, microbiology, biomathematics and statistics. The subject has been treated in several recent reviews [1, 2]. There are several possible approaches and I will start with a theoretical one which everybody can do at his desk.

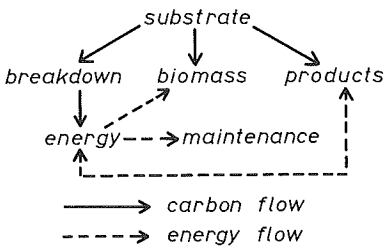

Fig. 1 Heterotrophic growth and product formation.

Table 1 summarizes the macromolecular composition of microbial cell material. This is taken from a publication of MOROWITZ [3] and his description is in fact much more detailed. The content is known of each aminoacid, each nucleic acid base and a number of lipids. From this we can calculate the amount of energy which is required to form biomass of this composition starting with various carbon sources (glucose, lactate, malate, acetate), ammonia and salts [4]. In order to do this we have to follow all metabolic pathways, for instance from glucose to each of the 20 aminoacids and determine the amount

of ATP which is needed to do this synthesis. The largest amount of energy is required for the formation of proteins, predominantly for the polymerisation of the aminoacids to build proteins. A great uncertainty of this kind of calculations is the estimate of the energy required for transport processes. I have assumed that for each molecule of an organic compound which is taken into the cell one molecule of ATP is required.

Wherever the proton motive force (pmf) represents the driving force for the uptake of solutes I have regarded pmf and ATP as equivalent. In this way one can calculate the total amount of ATP required for the formation of 1 gram of cells. With glucose, for example, one can calculate that 28.8 grams of cell weight is formed from 1 mole of ATP [4].

Table 1 ATP requirement for the formation of microbial cells

Macromolecule	Amount g/100 g cells	ATP required Glucose	Lactate	Malate	Acetate
Polysaccharide	16.6	0.21	0.71	0.51	0.92
Protein	52.4	2.04	3.39	2.85	4.27
Lipid	9.4	0.01	0.27	0.25	0.50
RNA	15.6	0.57	0.87	0.72	1.03
DNA	3.2	0.10	0.16	0.13	0.19
		2.93	5.40	4.46	6.91
Transport		0.54	2.02	2.02	3.08
		3.47	7.42	6.48	9.99
Y_{ATP}^{max}		28.8	13.4	15.4	10.0

2. The influence of the C-source

There are two main differences that might occur with various substrates. First the formation of the complete aminoacid mixture from acetate is much more energy expensive than the formation of the same aminoacid mixture with glucose as C-source [4]. These differences are due to the cost of monomer formation. Secondly, the amount of ATP required for transport processes is larger if the substrate consists of small molecules since many more of these molecules have to be transported into the cell. The amount of cell material that can be formed from 1 mole of ATP decreases therefore in the sequence glucose > lactate > malate > acetate. Experiments that illustrate this point are done in aerobic cell suspensions in which one measures growth yield per moles of oxygen consumed during growth (Y oxygen).

For complex substrates Y_o is large – for glucose a value of about 20 – and these values are low – about three to four – for growth on formate or oxalate. The calculations and the experimental observations are very consistent. How-

ever, the theoretical growth yields calculated in this way and the experimental growth yields differ by a factor of about 2, an observation that remains to be explained later. It is evident that the nature of the carbon source has a very profound influence on the ATP costs for the formation of microbial biomass. We can also see from Table 1 that the complexity of the medium has a great influence. In a glucose medium, where nearly all the ATP required for protein synthesis is needed for polymerisation, the addition of an aminoacid mixture will have a very small influence on the energetics whereas in the case with growth on acetate the addition of aminoacid mixture will have a very great influence.

3. The influence of the N-source

Also the nitrogen source is of great importance for the efficiency of energy expenditure. The amount of ATP which is required for the formation of one gram of cell material with glucose as C-source varies greatly whether one choses ammonia or nitrate as N-source. The amount of cell material formed with nitrate is smaller because nitrate assimilation which occurs via glutamine synthetase requires one ATP whereas ammonia is assimilated without the expenditure of ATP by the glutamate dehydrogenase. With N_2 as a nitrogen source the Y_{ATP} (the amount of cell material formed per mole of ATP) decreases drastically because nitrogen fixation requires a large amount of ATP. The amount is not accurately known because nitrogenase also reduces protons to form H_2. Various ATP to N ratios are listed in Table 2. A limitation of this approach becomes obvious. We calculate here only ATP requirements. This is acceptable for anaerobic growth. But for growth under aerobic conditions one would also have to account for reducing equivalents. Although there is a very large difference between Y_{ATP} for growth with nitrate and Y_{ATP} for growth with N_2, the molar growth yields ($Y_{SUBSTRATE}$) are about the same because nitrate reduction requires more reducing equivalents than N_2 fixation [5].

Table 2 Influence of N-source on theoretical Y_{ATP}^{max} values

Theoretical Y_{ATP}^{max}	
ammonia	nitrate
28.8	23.1
ATP requirement/atom N fixed	nitrogen
6	11.1
9	8.7
12	7.1
15	6.0

4. The influence of the biochemical capabilities of the cells

In addition to the carbon source the complexity of the medium and the nitrogen source there is also an influence of the pathway which is employed by the cells for the assimilation of substrates.

Table 3 Theoretical Y_{ATP}^{max} values for micro-organisms with different carbon assimilation pathways for growth with methane and methanol

Assimilation pathway	Theoretical Y_{ATP}^{max}
Ribulose monophosphate cycle, fructose diphosphate aldolase variant	27.3
Ribulose monophosphate cycle, 2-keto-3-deoxy--6-phosphogluconate aldolase variant	19.4
Serine pathway	12.5
Ribulose diphosphate cycle	6.5

Table 3 lists as an example the various assimilation pathways which are presently known for growth on C_1 compounds. Energy efficiency (Y_{ATP}) of these pathways varies greatly (Y_{ATP} = 27. 3 to 6.5).

The ribulosedisphosphate cycle (the CALVIN cycle) is the most energy expensive pathway for the assimilation of the C_1 compounds. It is used by *P. denitrificans* for growth on methanol which we will discuss later. These differences are also found in experiments where one measures yields of organisms growing with methanol and using these different assimilation pathways. So all these calculations come true with the one exception that the theoretical growth yields are much larger than the experimental growth yields.

5. Determinations of growth parameters

One could consider that the requirements for maintenance energy would be one of the factors which would explain the differences between experimental and theoretical growth yields. The reciprocal value of the actual molar growth yields $\left(\frac{1}{Y}\right)$, equals the reciprocal value of the apparent yield $\left(\frac{1}{Y}\,\text{max}\right)$, corrected for maintenance energy requirement plus the maintenance energy requirement divided by μ $\left(\frac{1}{Y} = \frac{1}{Y}\,\text{max} + \frac{m}{\mu}\text{, ref. 6}\right)$. This is normally determined in chemostat experiments in which one measures molar growth yields as functions of specific growth rates. One obtains a straight line in a plot of the reciprocal value of the molar growth yield against the reciprocal value of μ which allows the determination of the growth parameters Y_{Glu}^{max} and the maintenance coefficient [6]. In a number of cases this approach doesn't satisfy be-

cause one looks at substrate while one ought to look at ATP. Therefore, I have converted the equation above in $q_{ATP} = \frac{\mu}{Y_{ATP}^{max}} + m_e$. Thus the specific production q_{ATP} equals the specific growth rate divided by Y_{ATP}^{max} plus the maintenance coefficient [7]. Specific rate of ATP production becomes a linear function of the specific growth rate.

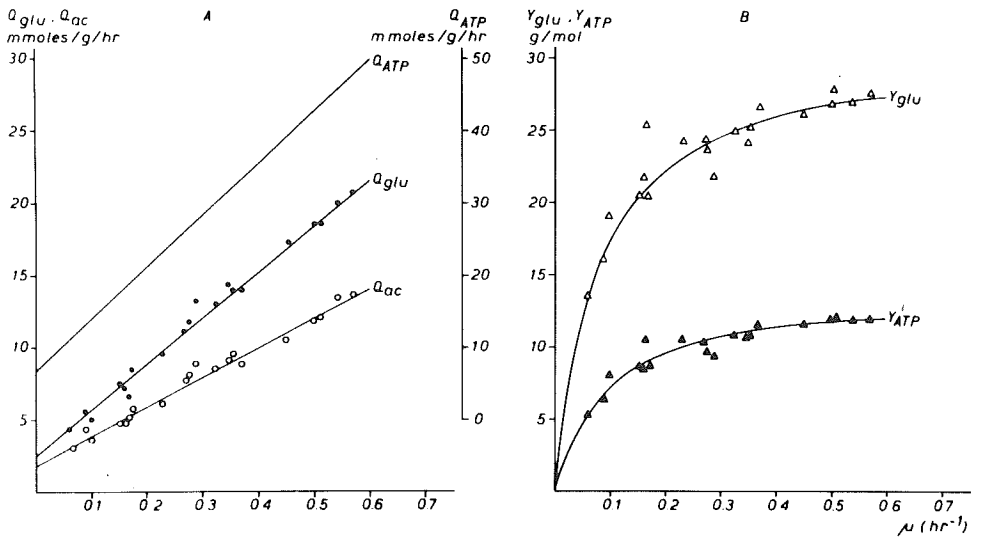

Fig. 2 Effect of the specific growth rate on the rates of glucose consumption and acetate and ATP production, and on the molar growth yields and Y_{ATP} by *Enterobacter aerogenes* during glucose-limited anaerobic growth. (A). Glucose consumption, •——•; acetate production, ○——○; ATP production, ——. (B). Molar growth yield for glucose, △——△; Y_{ATP}, ▲——▲. Data from ref. 2.

That this is indeed the case is shown in Figure 2 which shows anaerobic growth of *Enterobacter aerogenes* under glucose limitation in a chemostat. We have plotted specific rate of glucose consumption (qglu) against specific growth rate. It's a linear function of μ. The same applies to the specific rate of acetate production (qac). Because of the anaerobic growth conditions one can calculate exactly the rate of ATP production which is a linear function of the growth rate. The graph also allows to determine maintenance coefficient and Y_{ATP}^{max}. Y_{ATP} becomes a function of the specific growth rate (this was previously considered to be a constant). But from these experiments and from the previous theoretical calculations it is evident that Y_{ATP} is dependent on the medium composition and on the specific growth rate.

This approach enables one to determine growth parameters. Growth parameters are needed to understand energetic regulation of microbial growth.

Table 4 Growth parameters for some organisms growing anaerobically in a chemostat under energy-limited conditions

Organism	Substrate	Y_{ATP}^{max}	m_e
Lactobacillus casei	glucose	24.3	1.5
Enterobacter aerogenes	glucose	14.0	6.8
	citrate	9.0	2.3
Escherichia coli	glucose	10.3	18.9
	glucose	8.5	6.9
Saccharomyces cerevisiae	glucose	11.0	0.5
	glucose	13.0	0.25

The experimental Y_{ATP}^{max} value approaches the theoretical value only for *Lactobacillus casei* of all the organisms listed in Table 4. In all other cases the experimental values are much lower than the theoretical values. The values are higher for glucose than for citrate, as has been predicted by the calculations. Maintenance energy is not responsible for the difference between theoretical and experimental growth yields.

6. Anaerobic versus aerobic growth

Experiments under anaerobic conditions are easy because one knows the degradation pathways and one can calculate the Y_{ATP} values. Therefore, anaerobic cultures are ideal tools to study the influence of environmental and experimental factors on the energetics of microbial growth. Under aerobic conditions one finds, just as in anaerobic experiments, that the rate of glucose consumption measured as the rate of oxygen reduction is a linear function of the specific growth rate. But now we have to deal with an additional unknown parameter: the efficiency of oxidative phosphorylation. Efficiency of oxidative phosphorylation in microbial systems is very difficult to establish due to a certain variability in the number of phosphorylation sites coupled to the respiratory chains which are often branched as it was mentioned by WHATLEY for *P. denitrificans*. This is further illustrated for aerobic growth of *P. denitrificans* with gluconate as a carbon source and nitrate as a nitrogen source (Table 5). One can define an assimilation equation using the elemental composition of microbial cell material [5]. This way one can assign a molecular weight to microbial cell material. For this study a molecular weight of 152 has been assumed. As outlined previously for anaerobic growth one can also determine Y_{Gluc}^{max}, the amount of gluconate assimilated and the amount of gluconate dissimilated. With this elemental balance method one can then calculate oxygen uptake, CO_2 evolution and $Y_{O_2}^{max}$. Direct measurements of these values agree well with the theoretical ones. In the literature one may find a large number of values for these parameters which unfortunately do not agree with theoretical calculations. The investigators have either overlooked the excretion of other

products, in that case the balance will never fit, or they have made inaccurate determinations. Determinations of Y_{ATP}^{max} rest on an assumption about a certain number of phosphorylation sites. *P. denitrificans* may express a respiratory chain with two or three sites which gives different values for Y_{ATP}^{max}. The difficulty of aerobic experiments is that either one must know Y_{ATP}^{max} to determine the number of phosphorylation sites or one must know the number of sites for a determination of Y_{ATP}^{max}. There are various ways to circumvent the problem: 1. deviation from linearity in the rates of substrate consumption and specific growth rate; 2. comparison of substrate and anaerobic growth; 3. comparison of substrate and sulfate-limited growth. (During sulfate-limited growth phosphorylation site one is often lost.); 4. independent determination of one growth parameter e.g. the P/O ratio and 5. use of mixed substrates. In the following section each possibility is treated separately. All these possibilities have a number of uncertainties. It is therefore safe to apply as many of these possibilities as possible.

Table 5 Gluconate-limited growth of P. denitrificans with Nitrate as N-source

Equation: $C_6H_{12}O_7 + 1.4\ HNO_3 + 6.85''\ H_2'' \rightarrow C_6H_{10.84}N_{1.4}O_{3.07} + 8.13H_2O$

$Y_{GLUC}^{max} = 69.2$. Dissimilated 54.4% Assimilated 45.6%

	Calculated	Observed
O_2 uptake (mol/mol gluconate)	1.43	1.52
CO_2 evolution (mol/mol gluconate)	3.26	3.03
$Y_{O_2}^{max}$	48.4	45.5
ATP Formation	2 sites	3 sites
1.77 NADH/mol	3.54	5.31
1.09 $FADH_2$/mol	1.09	2.18
substrate phosphorylation	1.63	1.63
Total	6.26	9.12
Y_{ATP}^{max}	11.0	7.6

6.1. Deviation from linearity

Fig. 3 shows glucose-limited aerobic growth of *Enterobacter aerogenes*. At a μ value at about 0.5 the organisms start to excrete acetate [1]. At the same time the rate of oxygen uptake decreases and the rate of glucose consumption increases. By comparing the parts of the curves before and after $\mu = 0.5\ h^{-1}$ one has enough information for an independent resolution of all the growth parameters. One finds a value of Y_{ATP}^{max} of 12.4 and a P/O ratio of 1.4. The Y_{ATP}^{max} compares favorably with the anaerobic value which was about 14. One can therefore use the first part of the curve in Fig. 3 (below $\mu = 0.5\ h^{-1}$) with the results of anaerobic growth under glucose-limited conditions (Fig. 2). The same values are then obtained for the P/O ratio. The deviation from linearity is found very rarely.

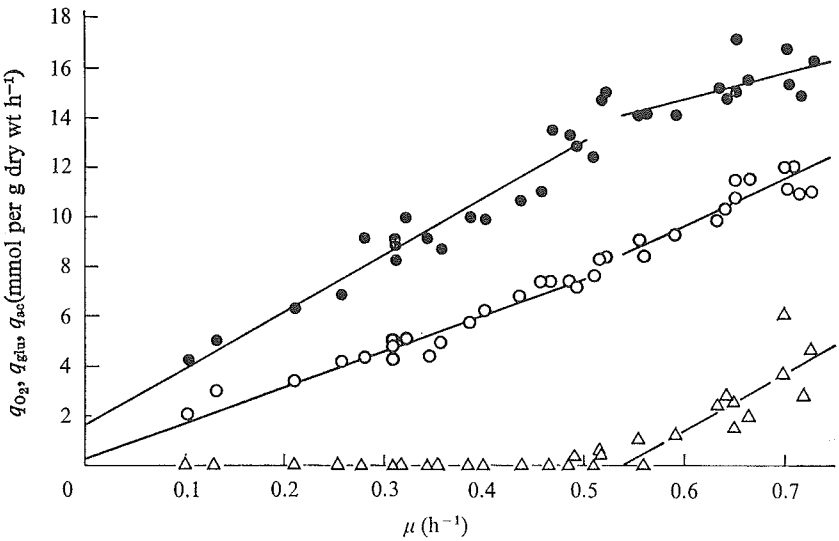

Fig. 3 Effect of specific growth rate on the rates of glucose and oxygen consumption and acetate production by *Enterobacter aerogenes* during glucose-limited aerobic growth with nitrate as nitrogen source. ●——● specific rate of oxygen consumption (q_{O_2}); ○——○ specific rate of glucose consumption (q_{glu}); △——△ specific rate of acetate production (q_{ac}). For those parameters where two lines are given, the first is for $\mu < 0.51\ h^{-1}$ and the second for $\mu > 0.54\ h^{-1}$. From ref. 1

6.2. Aerobic and anaerobic growth

A comparison of aerobic and anaerobic growth is shown in Table 6 [1]. The confidence limits indicate the inaccuracy of this kind of experiments. Y_{ATP}^{max} for anaerobic growth with citrate is between 8.3 and 10.6. The confidence limits for the maintenance coefficients are even larger. We have calculated growth parameters for chosen values of the P/O ratio and then we have determined Y_{ATP}^{max}. We have discussed that Y_{ATP}^{max} has the same value for aerobic and anaerobic growth and we look for the P/O ratio for which this applies. A P/O ratio

Table 6 Anaerobic and aerobic growth of E. aerogenes with citrate

P/O	Y_{ATP}^{max}	m_e	Y_{ATP} at $\mu = 0.2$
	8.3–10.6	0.4– 3.9 anaerobic	8.1– 9.0
		aerobic	
1.0	12.4–17.2	0.6– 7.7	10.4–12.3
1.2	10.5–14.6	0.6– 9.5	8.3– 9.8
1.4	9.2–12.7	0.7–10.1	7.8– 9.2
1.6	8.1–11.2	0.7–11.3	6.9– 8.2
1.8	7.3–10.0	0.7–12.5	6.2– 7.3
2.0	6.6– 9.1	0.8–13.7	5.6– 6.6

of about 1.6 fits best. A value of between 1.4 and 1.6 for the P/O ratio in *Enterobacter aerogenes* is very reasonable. It indicates two sites in an organism which lacks cytochrome c. This approach is applicable only to facultative organisms; one cannot use it for strict aerobes.

6.3. Sulphate-limited growth

For studies of the aerobic metabolism of *P. denitrificans* we have compared succinate- and sulphate-limited growth [8] (Table 7). In all cases the carbon source is succinate. When there are two sites the experimental P/O ratio for the complete oxidation of succinate is 1.7. When sulphate is limiting the first site is lost, and one gets a P/O ratio of 1. If there are three functional phosphorylation sites these values are 2.7 and 2.0 respectively. Calculations of Y_{ATP}^{max} for two and three sites are given in Table 7. Only in the case of two phosphorylation sites the same value of about 9 is found for Y_{ATP}^{max} for succinate- and sulphate-limited cells. From these data we concluded that during heterotrophic growth of *P. denitrificans* two phosphorylation sites are active [7]. This was confirmed by anaerobic experiments (with nitrate as electron acceptor) in which the third site cannot function. We conclude, therefore, that at least under our growth conditions only site one and two function in *P. denitrificans*. H^+/O ratios decrease from 7.5 to 3.4 when the first site is lost. And we interpret this as an excretion of about three to four protons per phosphorylation site.

Table 7 Y_{ATP}^{max} from growth of *Paracoccus denitrificans* with succinate

Limiting factor	Hydrogen acceptor	Theoretical P/2e⁻-ratio		Y_{ATP}^{max}		H^+/O
		2 sites	3 sites	2 sites	3 sites	
succinate	oxygen	1.7	2.7	9.1	5.9	7.5
sulfate	oxygen	1.0	2.0	9.3	4.9	3.4

Fig. 4 is an EPR spectrum of membrane particles of *P. denitrificans* showing succinate-limited cells and sulphate-limited cells [9]. Iron sulfur centers one to four are present at the same g positions in the spectrum as has been observed for mitochondria. The sulphate-limited cells show no signal at g-values of 2.05 and 1.93 which are characteristic for the iron sulfur center 2. Under sulphate-limited growth conditions the cells do not form iron sulfur center 2 [9]. Concomitantly they loose sensitivity towards rotenone. We have experienced that sensitivity to rotenone, the presence of iron sulfur center 2 and phosphorylation at site one always go together. In summary, we have found for *P. denitrificans* that under succinate-limited conditions site one and two function, while under sulphate-limited condition only site two is operative.

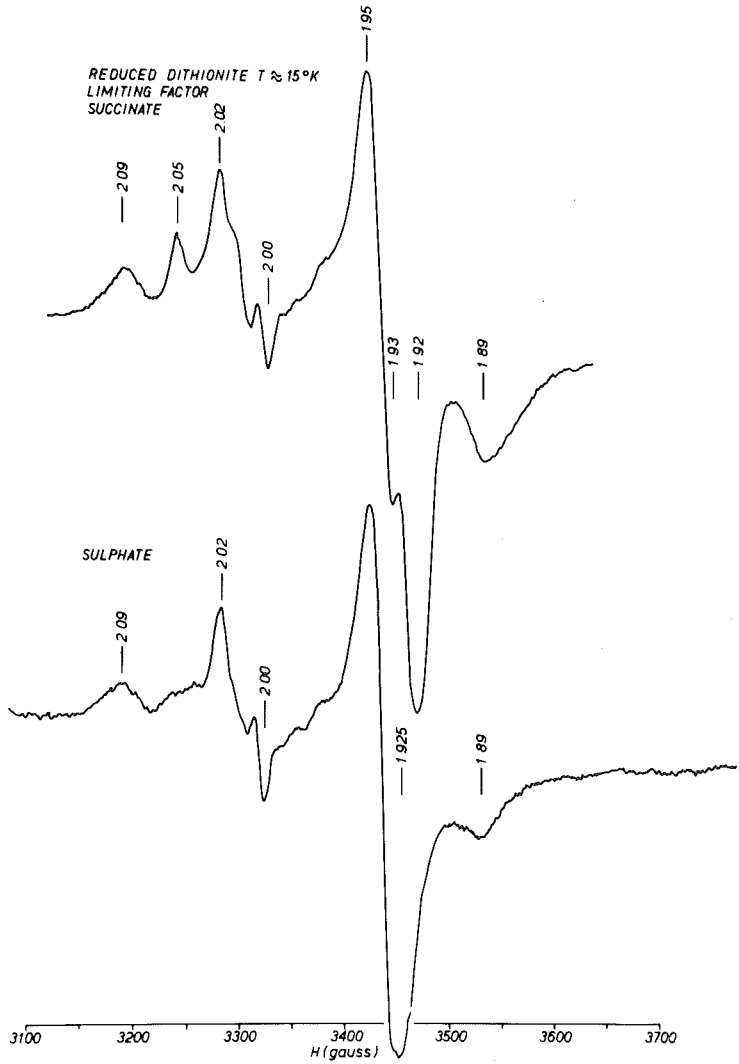

Fig. 4 Comparison of the EPR spectra of membranes of succinate- and sulphate-limited cells of *P. denitrificans*. Data from ref. 9.

6.4. Growth of *P. denitrificans* with methanol

Methanol is first oxidized to formaldehyde with cytochrome c as electron acceptor. This cytochrome reacts with carbonmonoxyde. Formaldehyde and formate are oxidized to carbon dioxyde with NAD^+ and the CO_2 is assimilated by the ribulosediphosphate cycle (the CALVIN cycle).

The growth equation $6\ CO_2 + 1.5\ NH_3 + 12.3\ NADH + 12.3\ H^+ \rightarrow C_6H_{10.8}N_{1.5}O_{2.9} + 9.1\ H_2O$ starts with CO_2 which requires a lot of NADH to be reduced to cell material [10]. The experimental values of $Y_{O_2}^{max}$ and the theoretical values based on $Y_{methanol}^{max}$ give a very good balance. The same applies to Y_{ATP}^{max} (Table 8). Therefore, no other products are being formed under these conditions. For the calculations of Y_{ATP}^{max} we assume that phosphorylation sites 1, 2 and 3 are active (Table 9). An overall P/O ratio of 2.05 gives a Y_{ATP}^{max} of 3.8. We take into consideration that the theoretical Y_{ATP}^{max} for assimilation of CO_2 by the ribulosediphosphate cycle is only 6.5 (Table 3) and that we always find experimental values of about halve of the theoretical value. Therefore, we can ex-

Table 8 Growth parameters of *P. denitrificans* for methanol-limited growth in the chemostat

g/mol	mmoles $g^{-1} h^{-1}$
$Y_{meth}^{max} = 13.4\ (12.1-15.0)$	$m_{meth} = 1.7\ (0.7-2.7)$
$Y_{O_2}^{max} = 15.5\ (13.5-18.2)$	$m_{O_2} = 2.7\ (1.5-3.8)$
$Y_{CO_2}^{max} = 24.8\ (22.3-28.0)$	$m_{CO_2} = 0.3\ (-0.2-0.8)$

Growth equation: $6CO_2 + 1.49\ NH_3 + 12.28\ NADH_2 \rightarrow C_6H_{10,83}N_{1.49}O_{2.9}$

On the basis of the growth equation and Y_{meth}^{max}:

Expected $Y_{O_2}^{max} = 14.0$ and $Y_{CO_2}^{max} = 25.0$

Table 9 Growth parameters for *P. denitrificans* growing with methanol and formate

Carbon source (Substrate)	Y_{Sub}^{max}	$Y_{O_2}^{max}$	Theoretical P/2e	Sites	Y_{ATP}^{max}
Methanol	13.4	15.5	2.05	1, 2, 3	3.8
			1.50	*	5.1
			1.05	1, 2	7.4
Formate	2.9	10.5	3.00	1, 2, 3	1.8
			2.00	1, 2	2.6
			1.70**	1, 2	3.1
			1.50**	1, 2	3.5

* NADH passes sites 1 and 2, only methanol passes also site 3.

** Formate is transported with $1H^+$.

Theoretical $^P/_{2e^-}$ ratios of 1.7 and 1.5 are obtained for $^{H^+}/_{ATP}$ ratios of 3 and 2 respectively.

clude that only site 1 and 2 function, since the corresponding Y_{ATP}^{max} value is higher than the theoretical value. If we assume that site one and two function in the oxidation of NADH and that site three functions in the oxidation of methanol we get $Y_{ATP}^{max} = 5.1$, which is too close to 6.5 (experimental values are only about 50% of the theoretical) to take this seriously. So we reject all other possibilities and think that sites 1, 2 and 3 are operative under these conditions. Formate, which is assimilated in the same way, leads to a completely different situation (Table 9). We conclude that during growth on formate only site 1 and 2 are operative. It is, therefore, not the switch from heterotrophic to autotrophic growth which turns on the third site but it is specifically the growth on methanol.

6.5. Growth on mixed substrates

When one adds two substrates together to a batch culture one normally gets catabolite repression. I would like to consider catabolite repression as a batch culture artefact. During growth in a chemostat under carbon-limited conditions both substrates are always used simultaneously. So if one introduces two substrates, e.g., mannitol and methanol, then mannitol will be used as a carbon source and methanol will be used as an energy source [11]. The growth yield for mannitol in this case, is a linear function of the ratio of the concentrations of substrate 2 to substrate 1. This holds true down to a critical value of this ratio, where the total amount of substrate 1 has been assimilated. One can determine the P/O ratio directly because when growth with mannitol alone is considered energy generation differs from the case with mannitol + methanol [12]. During the complete oxidation of mannitol one gets 11 $NADH^+$, 2 FADH and either 4 or 3 molecules of ATP by substrate level phosphorylation via the glycolytic or the ENTNER-DOUDOROFF pathway respectively. Methanol yields 2 NADH and 1 XH_2. From the different ways of energy generation one can determine the P/O ratio. Growth with mannitol alone gives a $Y_{O_2}^{max}$ of about 35. Lines for $Y_{O_2}^{max}$ in Fig. 5 represent the values for three sites and for two sites respectively with various ratios of methanol/mannitol. When there is too much of the first substrate (methanol) it will be used as a carbon source too. The energetic efficiency for growth on methanol is lower than that for growth on mannitol as sole C-source. The experimental values fit best if one assumes three phosphorylation sites. This observation is important for the production of single cell protein from methanol (Table 8). Fig. 5 shows that when one adds a small amount of mannitol or glucose to the medium that the yield of cell material from methanol is increased but also the amount of oxygen required to form this cell mass is much smaller ($Y_{O_2}^{max}$ is larger, therefore, the amount of oxygen required for the formation of one gram cell material is lower than during growth on methanol alone). This indicates that one needs less energy for stirring and thereby less energy for cooling, if one uses mixed substrates for the formation of single cell protein. Similar calculations

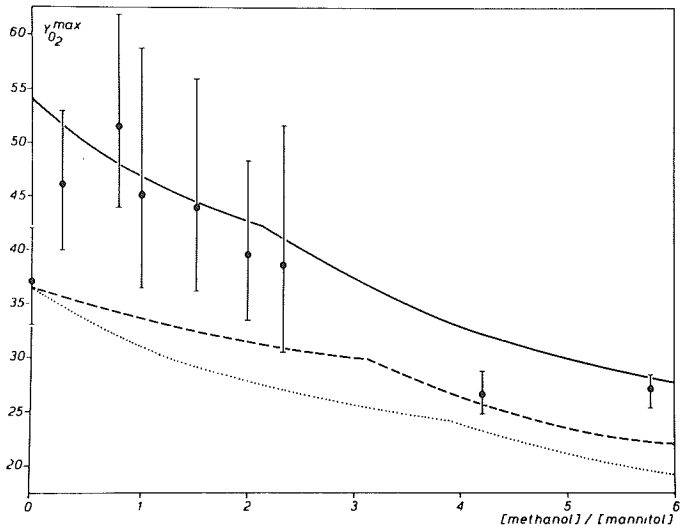

Fig. 5 Theoretical curves of $Y_{O_2}^{max}$ against the ratio [methanol]/[mannitol] of *Paracoccus denitrificans*. The curves were calculated for 3 sites (——), 3 sites but only electrons from XH_2 via the third site (– – –) and 2 sites (.....) present in the electron-transport chain. Experimental values of $Y_{O_2}^{max}$ including their 95% confidence intervals are indicated in the figure.

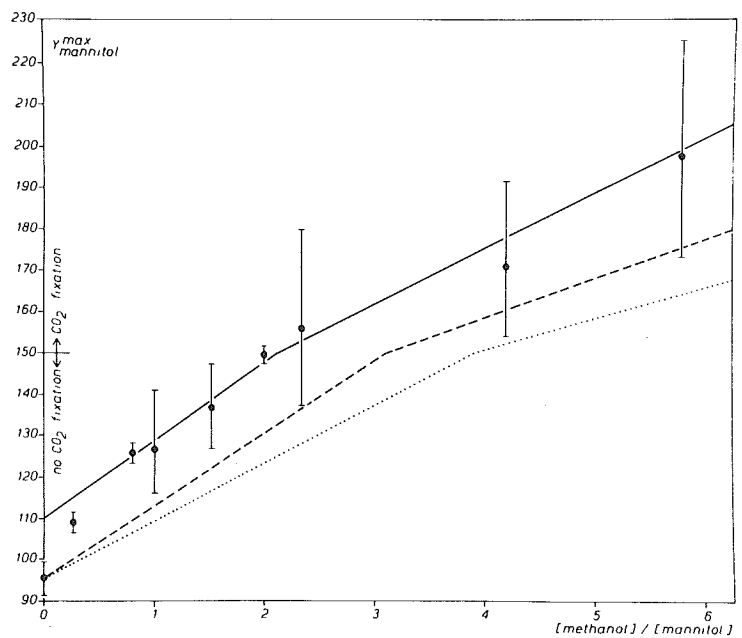

Fig. 6 Theoretical lines of $Y_{mannitol}^{max}$ against the ratio [methanol]/[mannitol] of *Paracoccus denitrificans*. The lines were calculated for 3 sites (——), 3 sites but only electrons from XH_2 via the third site (– – –) and 2 sites (.....) present in the electron-transport chain. Experimental values of $Y_{mannitol}^{max}$, including their 95% confidence intervals are indicated in the figure.

can be made for $Y^{max}_{mannitol}$ (Fig. 6) and here the value for mannitol alone is on the line for two sites. Already at low methanol/mannitol ratios there is a strong increase in $Y^{max}_{mannitol}$ and the line follows that one which is characteristic for three sites. In this region the points are on a straight line. This obscured a bit because one goes from two to three sites. The same value for Y^{max}_{ATP} of about 9 is found if we take 2 sites for growth with mannitol alone and 3 sites for growth with mannitol + methanol. At high methanol/mannitol ratios (Fig. 7) Y^{max}_{ATP} decreases because under these conditions methanol is used as a carbon source and growth on methanol is energy expensive. The explanation lies in the respiratory chain of *P. denitrificans* (Fig. 8, 13).

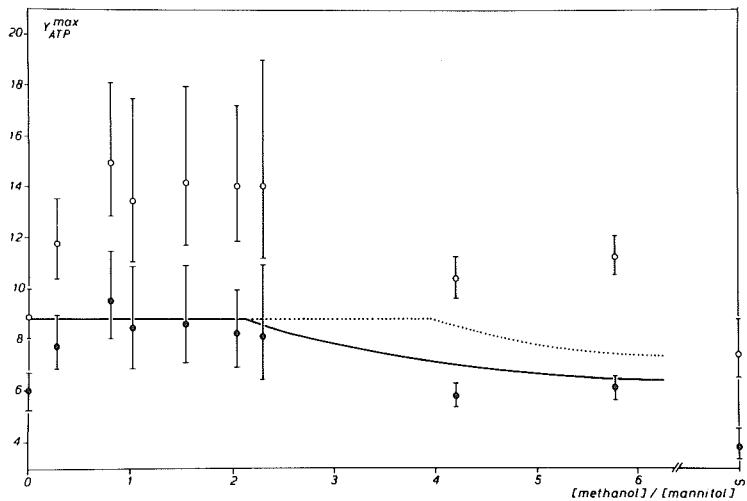

Fig. 7 Theoretical curves of Y^{max}_{ATP} against the ratio [methanol]/[mannitol] of *Paracoccus denitrificans*. The curves were calculated for 3 sites (——), and 2 sites (.....) present in the electron-transport chain. Experimental values of Y^{max}_{ATP} calculated for 3 sites (●) and 2 sites (○) 95% confidence intervals are indicated in the figure.

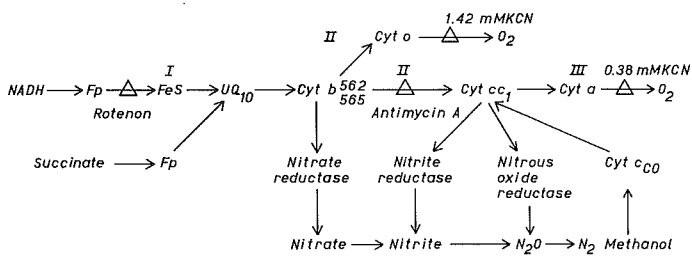

Fig. 8 Respiratory chain of *Paracoccus denitrificans*.

In cells grown with mannitol only, respiration is not sensitive towards Antimycin A. Electron flow is therefore towards cytochrome o. Secondly respiration is not sensitive towards low concentrations of cyanide which also indicates that the electron flow is towards cytochrome o. Electrons from methanol enter via cytochrome c (CO-sensitive) and pass to cytochrome aa_3 and then to oxygen. Cells grown on methanol contain much more cytochrome c. The increase in cytochrome c relative to cytochrome b enables the electrons to flow from cytochrome b towards cytochrome aa_3. Cells grown on methanol are therefore very sensitive towards Antimycin A and towards cyanide. In conclusion, the third site is switched on by an increased concentration of cytochrome c, which is characteristic for cells grown on methanol. In Fig. 8 we also see that electrons can flow towards nitrate, nitrite and nitrous oxyde. Nitrate accepts electrons from cytochrome b, nitrite and nitrous oxyde from cytochrom c. Molecular nitrogen is the product of denitrification.

7. The H^+/O-ratios

In Table 10 the H^+/O ratios of various cell suspensions are listed and I would like to draw your attention to methanol-limited cells. If we inhibit endogenous respiration with antimycine and rotenone and then add methanol we get very rapid respiration and about 3.5 protons are extruded per atom of oxygen reduced. I think this is direct proof that the cytochrome oxidase region of *P. denitrificans* is proton-translocating. For methanol-grown cells in the presence of succinate and rotenone the H^+/O value is much higher than for succinate-grown cells. This again indicates an extrusion of about 3 to 4 protons per site. For endogenous respiration the values fall into two classes: one type of cell suspensions has a mean value of about 7.16 whereas others have a value which is very close to 11. And we again estimate 3 to 4 protons extruded per site. Succinate-grown cells have two sites, site 1 and 2. The H^+/O ratio for endogenous respiration and succinate again indicate three to four protons per site. Sulphate-limited cells show the same H^+/O ratio for endogenous respiration and for succinate-driven respiration, again indicating one

Table 10 H^+/O ratios for starved *P. denitrificans* cells

Carbon source	H^+/O endogenous	succinate	succinate + rotenone	methanol + antimycine A. + rotenone
Succinate	7.50 ± 0.60(20)	3.71 ± 0.29(9)	3.03 ± 0.38(10)	–
Methanol	7.16 – 10.56(20)	6.16 ± 1.20(11)	5.12 ± 1.20(9)	3.49 ± 1.07(14)
Formate	6.31 ± 0.55(6)	3.92 ± 1.24(8)	3.47 ± 0.58(8)	–
Succinate (S-limited)	3.40 ± 0.45(32)	3.95 ± 0.40(15)	3.40 ± 0.31(19)	–

site. In our opinion respiration in *P. denitrificans* occurs with three to four protons which are excreted per phosphorylation site. This excludes the organization of the components of the respiratory chain of *P. denitrificans* in redox loops as proposed by MITCHELL. Redox loops are only consistent with 2 protons extruded per site.

8. What actually limits the rate of growth?

Only little experimental work has been done in this area. MAYNARD SMITH [14] is the only person who has speculated about it and according to him the growth rate is limited by the requirements of protein synthesis. We have looked at potassium-limitation because it is known that potassium transport, especially under potassium-limited conditions, requires a lot of energy. In experiments with three different concentrations of potassium in the inflowing medium we have noticed that μ_{max} is dependent on the input concentration of potassium. With increasing μ at one potassium concentration the amount of dry weight in the steady state decreases. This is due to the fact that the intracellular potassium concentration is a function of the specific growth rate. This is indicated in Table 11. The intracellular concentration increases from about

Table 11 Influence of dilution rate and of the input concentration of potassium on the steady state concentration of bacteria (x) and potassium (K_{ex}^+). From these data the intracellular potassium concentration (K_{in}^+) and the concentration gradient (K_{in}^+ / K_{ex}^+) were calculated.

D (h^{-1})	x (g dry weight. l^{-1})	% K	K_{ex}^+ (mM)	K_{in}^+ (M)	Concentration gradient × 1000
Potassium concentration: 0.21 mM					
0.034	0.66	1.0	0.036	0.13	3.6
0.050	0.61	1.2	0.026	0.15	5.8
0.090	0.46	1.6	0.023	0.20	8.7
0.093	0.45	1.5	0.040	0.19	4.8
0.107	0.50	1.4	0.026	0.18	6.9
Potassium concentration: 0.44 mM					
0.097	0.95	1.6	0.044	0.21	4.8
0.107	0.94	1.7	0.034	0.22	6.5
0.127	0.94	1.5	0.076	0.19	2.5
0.150	0.79	1.8	0.079	0.23	2.9
Potassium concentration: 0.66 mM					
0.117	1.32	1.6	0.127	0.20	1.6
0.157	1.11	1.7	0.169	0.22	1.3
0.179	1.04	1.8	0.169	0.24	1.4
0.197	0.95	2.1	0.146	0.27	1.9
Potassium concentration: 0.91 mM					
0.257	1.19	2.2	0.248	0.29	1.2

0.1 molar to 0.3 molar at the high specific growth rate. This results in high concentration gradients of potassium. The gradient between the extracellular concentration and the intracellular concentration in the steady state, amounts to 8700–1200. From these data we conclude that the maximum specific growth rate is not determined by the so-called MONOD equation which is derived from the MICHAELIS-MENTEN formalism, because the concentrations of potassium in the extracellular medium do not follow MONOD kinetics. During growth under potassium-limited conditions the cells have to do two things: to maintain the concentration gradient in the existing cells and to establish the gradient in the newly formed cells. Under increased specific growth rates it apparently gets too difficult to achieve both. At a higher input concentration of potassium a higher μ_{max} can be achieved, but the concentration gradient will be lower (Table 11). If one assumes that the same amount of ATP is required for the formation of biomass under succinate-, sulphate- and potassium-limited conditions, one has to allow for an additional amount of ATP for the establishment of the potassium gradients. This allows to calculate the amount of ATP which is required for potassium transport. From the experimental rate of potassium uptake and the amount of ATP required to maintain the potassium ion concentration inside the cell during one hour one can calculate the amount of ATP required for potassium transport. The value varies from 350 to 30 depending either on the concentration gradient or on the extracellular potassium concentration in the steady state. The large amounts of ATP required to establish and to maintain concentration gradients are an important factor, which explains the difference between theoretical and experimental growth yields [15, 16].

Maximum specific growth rate, therefore, does not depend on MONOD kinetics but rather on the rate at which the concentration gradient can be established. At even higher potassium concentrations, another factor will limit growth. If one starts with high concentrations of potassium one finds that the maximum specific growth rate is about $0.20\,h^{-1}$, with sulphate limitation about $0.21\,h^{-1}$ and with succinate limitation about $0.33\,h^{-1}$ (Table 12). A com-

Table 12 Influence of the nature of the growth-limiting nutrient on the maximum specific growth rate and the corresponding rates of succinate- and oxygen consumption in chemostat cultures of *Paracoccus denitrificans*. The data for sulphate- and succinate-limited cells are derived from MEIJER et al. (1977). * and **: Input potassium concentration = 0.66 resp. 0.91 mM.

Growth-limiting nutrient	Maximum specific growth rate (h^{-1})	Specific rate of production or consumption of (mmoles g dry weight^{-1} h^{-1})	
		Succinate	O_2
Potassium	0.20* – 0.26**	7.57 – 7.00	10.87 – 10.89
Sulphate	0.21	6.64	11.64
Succinate	0.33	8.82	11.16

parison of the specific rate of succinate consumption, the specific rate of oxygen uptake and the specific rate of ATP production under these circumstances shows that only the oxygen uptake is the same in all cases. Therefore, we have concluded that another factor which may limit growth of *P. denitrificans,* is the specific rate of oxygen uptake under coupled growth conditions. *P. denitrificans* can establish a higher respiration rate only under uncoupled conditions. I cannot explain this. I do not think it is the capacity of the respiratory chain which is limiting but it might be the rate of conversion of a pmf into ATP. But one thing became clear: the maximum specific growth rate is determined by the rate of energy production.

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