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Effect of concentration of substrates and products on the growth of *Klebsiella pneumoniae* in chemostat cultures

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Non-equilibrium thermodynamics (NET) can be used to describe microbial growth. In this description, the concentrations of products contribute to the driving forces of the metabolic processes (anabolism and catabolism). Thus, in contrast to the model of bacterial growth of Monod (Recherches sur la Croissance les Cultures Bactériennes (1942) Herman et Cie, Paris.), it is predicted that the growth rate of a bacterial chemostat culture is, in principle, dependent on the concentration of the catabolic product (for instance HCO_3^-) during catabolite limitation and on the concentration of the anabolic product (for instance biomass) during anabolite limitation. In order to test this prediction, *Klebsiella pneumoniae* was grown in aerobic citrate-limited, glucose-limited or ammonia-limited chemostat cultures. Ammonia-limited cultures were considered to be essentially anabolite-limited, whereas citrate limitation was used as a representative for catabolite limitation. In ammonia-limited or in glucose-limited cultures it was found that the growth rate was independent of the biomass concentration present. In the NET description this means that the 'back' reaction (i.e., in the direction from biomass to substrates) is saturated with respect to biomass. On the other hand, in citrate-limited cultures, the steady-state concentration of citrate increased with the concentration of the catabolic product HCO_3^-. At relatively low concentrations of HCO_3^-, 'thermodynamic back-pressure' of growth (i.e., increase in product concentration was compensated by an increase in substrate concentration so that the driving force for growth remained almost constant) was demonstrated as predicted by the NET model. At concentrations above 40 mM, a kinetic (allosteric) effect of HCO_3^- was detected. This was concluded from a reduced growth yield on citrate, and from a significant decrease in the maximal growth rate and the maximal oxygen consumption rate after relief of the citrate limitation.

Table of symbols and abbreviations used in this paper

Symbol	Unit	Definition
J	h^{-1}	flow or flux
L		phenomenological constant
ΔG	$kJ \cdot mol^{-1}$	Gibb's free-energy difference
$\Delta G°$	$kJ \cdot mol^{-1}$	standard Gibb's free-energy difference
$\Delta G^{\#}$	$kJ \cdot mol^{-1}$	a constant fixing the range of ΔG where J varies
$\tilde{\mu}$	$kJ \cdot mol^{-1}$	free energy or chemical potential
$\tilde{\mu}^{\#}$	$kJ \cdot mol^{-1}$	a constant fixing the range of $\tilde{\mu}$ where J varies
R	$8.3\ J \cdot mol^{-1} \cdot K^{-1}$	gas constant
T	K	absolute temperature
μ	h^{-1}	specific growth rate (in the steady-state chemostat $\mu = D$)
μ_{max}	h^{-1}	maximal growth rate
K_s	M	Michaelis-Menten constant
$\bar{s}$	M	steady-state concentration of limiting substrate in chemostat culture
$\bar{p}$	M	steady-state concentration of product in chemostat culture
S_r	M	concentration of limiting substrate in reservoir medium
D	h^{-1}	dilution rate
dw	$g \cdot l^{-1}$	dry weight
A_{540}		absorbance at 540 nm
NET		non-equilibrium thermodynamics
MNET		mosaic non-equilibrium thermodynamics
subscript a		anabolism
subscript c		catabolism
subscript s		substrate
subscript p		product
subscript i		process i, compound i

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Introduction

Mathematical modelling of bacterial growth and product formation has attained much attention in recent years (for a review see Ref. 1). These descriptions have been developed in order to try to combine our knowledge of the biochemistry of a microbial cell with microbial growth kinetics. Unfortunately, some of these new approaches are very complicated and require sophisticated computer technology to calculate predictions of growth and product formation (see, e.g., Ref. 2).

The simple classical model of Monod [3] has been quite successful and has been used as the basis for more refined models [4–7]. In this model, the bacterium is considered to be a catalytic unit, able to convert materials into products together with progeny with the same catalytic properties. The process can be described by simple Michaelis-Menten kinetics. Thus, during nutrient-limited growth, as is the case in chemostat cultures, the growth rate of the bacterium is solely dependent on the concentration of the limiting substrate (and on the μ_{max} and the K_s, both properties of the organism)

$$\mu = \mu_{max} \cdot \frac{\bar{s}}{K_s + \bar{s}} \tag{1}$$

In this equation, μ is the specific growth rate, μ_{max} the maximal growth rate, $\bar{s}$ the steady-state concentration of the growth-limiting substrate in the culture, and K_s the half-saturation constant for growth.

This kinetic model is phenomenological in nature, i.e., it describes growth without detailed references to the underlying metabolism. It implies that the relationship between μ and $\bar{s}$ is unique under a defined set of environmental conditions.

An alternative phenomenological method to describe bacterial growth and metabolism was developed by Westerhoff and others [1,8–11]. The foundations of the model are the thermodynamic laws with special emphasis on non-equilibrium thermodynamics (NET).

NET considers a bacterium to be an energy-transducing black box. Like the Monod model, its conclusions are not explicitly related to the internal biochemical characteristics (although the model can be refined so as to take these into consideration: resulting in the MNET model [1,8 – 10]). The black box couples an input flow to an output flow, or in microbial terms, catabolism (i.e., all energy-liberating processes) to anabolism (all biosynthetic reactions, e.g., the production of biomass). NET considers catabolism and anabolism to be driven by their conjugated forces as a consequence of the differences in free energy (ΔG) between substrates and products (including biomass).

This description leads to some testable predictions (see Theory section) of microbial growth which could not be derived from other models, including Monod's model. First, the relationship between growth rate (μ) and steady-state concentration of the growth-limiting substrate ($\bar{s}$) is not a hyperbole as predicted by the model of Monod (Michaelis-Menten kinetics) but a logarithmic curve.

Surprisingly, in the literature only a limited number of measurements of $\bar{s}$ in chemostat cultures could be found [6,7,12]. The data of Schulze and Lipe [13] and the results obtained previously by us [14,15] showed that a plot of μ versus $\bar{s}$ fits such a logarithmic function approximately equally well as the hyperbolic function proposed by Monod.

Second, the concentration of the limiting substrate, but also that of the products participate in the driving forces of the metabolic processes (ΔG), and both can, in principle, affect the growth rate. Concerning the concentration of the limiting substrate, it is universally agreed that this is a major parameter affecting microbial growth. This is immediately clear from the Monod model (Eqn. 1), but also from data reported in the literature [3–7,13,14]. However, a thermodynamic 'back pressure' exerted by the concentration of products (from the flow of limiting substrate) on rates of catabolism or anabolism is usually neglected in the microbiological literature. There are reports on the effects of end-products on yield and fermentation pattern in chemostat cultures, but $\bar{s}$ had not been determined in the experiments [16–18]. Consequently, from these experiments it is impossible to evaluate whether the observed effects are the result of thermodynamic (i.e., ΔG related) or (but more likely) from kinetic effects, or both. Thermodynamic 'back pressure' can only be demonstrated when growth rate and $\bar{s}$ are simultaneously monitored.

In the experiments described in this paper, *Klebsiella pneumoniae* was grown in ammonia-limited, glucose-limited or citrate-limited chemostat cultures at several dilution rates and with different concentrations of products (CO_2/HCO_3^- and biomass), to determine the relationship between the concentration of products and rates of anabolism and catabolism.

Theory

The non-equilibrium thermodynamic (NET) description of microbial growth considers catabolism (input of the black box system) and anabolism (output) to be driven by their two conjugated forces (ΔG_c and ΔG_a) as a consequence of differences in free energy between substrates and products

$$\Delta G_a = \tilde{\mu}_{ap} - \tilde{\mu}_{as} \tag{2}$$

$$\Delta G_a = \Delta G_a^\circ + RT \cdot \ln\left(\left[\frac{ap}{as}\right]\right) \tag{3}$$

$$\Delta G_c = \tilde{\mu}_{cs} - \tilde{\mu}_{cp} \tag{4}$$

$$\Delta G_c = \Delta G_c^\circ + RT \cdot \ln\left(\left[\frac{cs}{cp}\right]\right) \tag{5}$$

In these equations, ΔG is the Gibbs free-energy difference of a reaction, $\Delta G°$ the standard free-energy difference, $\tilde{\mu}$ the chemical potential of a compound (the $\tilde{\mu}$ is introduced to avoid confusion with the growth rate, μ), R is the gas constant and T the temperature. Subscripts a and c are abbreviations for anabolism and catabolism, respectively, with extensions s and p for substrate and product, respectively (ap, as, cp and cs).

In a first approximation, NET made use of linear relations of forces and fluxes of near-equilibrium systems

$$J_i = L_i \cdot \Delta G_i \tag{6}$$

Here J_i is the flux or flow through system i (rate of the process) and L_i the so-called phenomenological constant describing the conductance of the process. This constant is determined by system characteristics such as enzyme activities. Indeed, L_i can be affected by temperature, osmolarity, etc., but also by growth rate [19,20] or by allosteric effects of substrates or products. In the following these are referred to as 'kinetic effects'.

It appears that flow-force plots are, in general, S-shaped [21]: at low and at high free energies of a reaction, the reaction rate is independent of the force (a saturation or V_{max} effect), whereas at intermediate force values the flow-force relation is approximately linear. In general, this linear region does not extrapolate to the origin of the plot.

A $\Delta G^{\#}$ term has been introduced to obtain a relatively simple mathematical description which is able to cover a wide range of actual flow-force relationships (for an explanation see Refs. 1, 8, 10)

$$J_i = L_i \cdot (\Delta G_i - \Delta G_i^{\#})$$

$\Delta G^{\#}$ is defined in the following way: when ΔG is so high (or low) that system i is at its maximum positive (or negative) reaction rate, then $\Delta G - \Delta G^{\#}$ is constant; when ΔG is in the range in which it influences the flow, then $\Delta G^{\#}$ is constant so that $\Delta G - \Delta G^{\#}$ varies linearly with ΔG.

The # here is assigned to ΔG. However, it is possible to make an extension to each component of the ΔG, the $\tilde{\mu}$ of substrates and products. Thus, in a generalised form

$$\Delta G_i - \Delta G_i^{\#} = \Sigma(\tilde{\mu} - \tilde{\mu}^{\#})_{ip} - \Sigma(\tilde{\mu} - \tilde{\mu}^{\#})_{is} \tag{8}$$

For a number of far-from-equilibrium systems the relations between flows and forces are linear, though not always proportional [8,1,22]. Thus, it seems attractive to describe the (catabolic and anabolic) flows and forces in the black-box system by one set of linear relations

$$J_c = L_{cc}(\Delta G_c - \Delta G_c^{\#}) + L_{ca}(\Delta G_a - \Delta G_a^{\#}) \tag{9}$$

$$J_a = L_{ac}(\Delta G_c - \Delta G_c^{\#}) + L_{aa}(\Delta G_a - \Delta G_a^{\#}) \tag{10}$$

In general, microbial growth is almost completely limited by the availability of one substrate; the other substrates may be considered to be present in 'saturating' concentrations. Thus, when the growth-limiting substrate is flowing into anabolism (e.g., NH_4^+) the catabolism may be saturated, resulting in an approximately constant $\Delta G_c - \Delta G_c^{\#}$ (or $[\tilde{\mu} - \tilde{\mu}^{\#}]_{cs\ or\ cp}$) in Eqns. 9 and 10. On the other hand, when a catabolite is growth-limiting, then $\Delta G_a - \Delta G_a^{\#}$ is expected to be constant.

Therefore, to predict effects of the concentration of products (or substrates) on rates of metabolism, one has to focus on ΔG_a during anabolite-limited growth or on ΔG_c during catabolite-limited growth. However, it is not a priori clear whether the concentration of products affects growth by way of thermodynamic 'back pressure' or is subjected to a saturation effect ($\tilde{\mu} - \tilde{\mu}^{\#}$ is constant) or/and modifies the conductance (L) of the cells.

Materials and Methods

Organism. Klebsiella pneumoniae was used throughout all experiments. Cells were maintained on nutrient agar (Gibco) and subcultured every 2 weeks.

Growth conditions in chemostat cultures. Cells were grown in glucose-limited, ammonia-limited or citrate-limited chemostat cultures (500 series L&H Engineering, U.K.) with a working volume of approx. 600 ml, in most experiments at a dilution rate of $0.38 \pm 0.02\ h^{-1}$ and a temperature of $36 \pm 1°C$. The pH was monitored constantly and maintained at 6.9 ± 0.1 by automatic addition of 4 M NaOH. The cultures were sparged with filtered air or mixtures of O_2/CO_2/air ($30 \pm 3\ l \cdot h^{-1}$, $pO_2 = 0.21 \pm 0.02$ atm) and stirred at 1000 rpm. Simple salts media were used as specified by Evans et al. [23] with the exception that 1 mM pyrophosphate was used instead of citrate as the chelating agent for the trace elements. In most experiments the concentration of the growth-limiting substrate in the reservoir medium (S_r) was 10 mM (glucose, ammonia or citrate). To adjust the biomass concentration, S_r was varied from 0.1 up to 20 mM. 100 mM glucose or 200 mM ammonia were present under non-limiting conditions.

Growth conditions in batch cultures. Batch experiments were performed in the same fermentors with a medium similar to that used for chemostat experiments. 10 mM citrate was added as carbon and energy source. The pH was monitored constantly and maintained at 6.9 ± 0.1 by automatic addition of 4 M NaOH or 1 M H_2SO_4. The cultures were sparged with filtered air or O_2/CO_2/air mixtures.

Sampling procedure. In order to determine $\bar{s}$, the sampling procedure was optimised and the input concentration of the growth-limiting substrate was lowered (with a concomitant decrease in biomass). Samples 1–2

ml were taken by removing culture fluid directly from the steady-state chemostat via a vacuum filtering technique (Whatman glass microfibre filter GF/F). The sampling procedure took always less than 2 s (mostly less than 1 s). Of this period, the cells flowed from culture to filter in a much shorter time. The samples were frozen directly after removal from the culture.

Despite these precautions, the determinations of $\bar{s}$ possibly show a systematic underestimation of the actual values. With an S_r of 10 mM, and a D of 0.38 h^{-1}, the rate of consumption is constant and about 1 $\mu M \cdot s^{-1}$. This means that all values are underestimated by a few tenths of a μM.

Determinations. Dry weight of cells in chemostat cultures was determined by the method of Herbert et al. [24].

Concentrations of possible excretion products in culture fluid were determined by HPLC chromatography using a Biorad Aminex ion-exclusion HPX 87 H column, a refractometer (Knauer, Berlin) and a Spectra physics SP 4270 integrator (CA. U.S.A.). Carbon dioxide and oxygen in the effluent gas flow were measured with an IR (Servomex PA404) and a paramagnetic (Servomex 580A) gas analyser, respectively. Gas flows were determined with Rota meters.

Glucose was analysed fluorimetrically with a hexokinase/glucose-6-phosphate dehydrogenase assay [25], ammonia was analysed according to a glutamate dehydrogenase method [26] and citrate was analysed by a citrate lyase/malate dehydrogenase system [27].

The μ_{max} was determined by recording the course of the absorbance at 540 nm (continuous flow system) of a batch culture and calculating the growth rate from the stage of logarithmic increase. The maximal rate of oxygen consumption (J_{O_2}) of cells was measured in chemostat medium (containing 10 mM citrate) with a Clark oxygen electrode (YSI 4004).

Results and Discussion

In probably all models of microbial growth, the steady-state concentration of the growth-limiting substrate in the culture ($\bar{s}$) is a major parameter affecting growth rate (μ). In comparison, NET also considers the concentration of products to be an integral part of the driving forces (ΔG) in metabolism affecting, in principle, μ. Therefore, we introduce here, analogous to $\bar{s}$, an associated parameter, $\bar{p}$, describing the steady-state concentration of product (the product formed from the growth-limiting substrate).

In the experiments described below, the relationship of $\bar{s}$ versus μ was checked first. Next, the relationship of $\bar{p}$ versus μ was investigated for two situations: ammonia-limited growth (anabolite limitation) and citrate-limited growth (catabolite limitation).

In our earlier study [14], the relationship of $\bar{s}$ versus μ of glucose-limited cultures of *K. pneumoniae* was investigated. We report here additional experiments for ammonia-limited and citrate-limited cultures of *K. pneumoniae* (Fig. 1). Also with these substrates, $\bar{s}$ is strongly dependent on D. For ammonia-limited cultures the reported $\bar{s}$ values ($K_s \cong 3$ μM) are in agreement with the known affinity of the ammonia-assimilation system [28]. Best fits, generated with non-linear regression analysis, showed convergence, random distribution of residuals (except for the lower $\bar{s}$), standard errors in parameters were less than 40% of the values and quotients of the mean sums of squares for the rival models of 1.7, 1.1 and 1.1 for the glucose data [14], the ammonia data, and the citrate data, respectively. Conse-

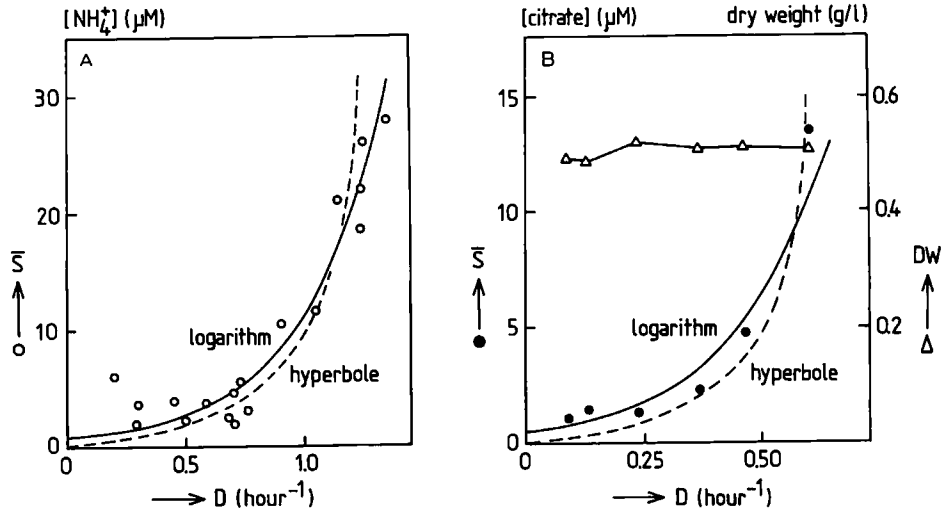

Fig. 1. Relationship between the dilution rate of ammonia- (A) or citrate-limited (B) chemostat cultures of *K. pneumoniae* and the steady-state concentration of ammonia or citrate ($\bar{s}$). In B dry weight is also indicated.

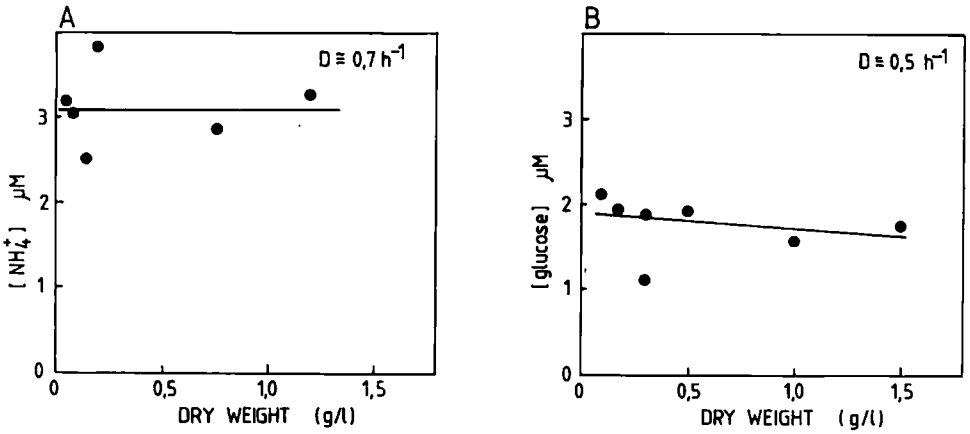

Fig. 2. Relationship between the concentration of biomass (dry weight) of ammonia (A) or glucose-limited (B) chemostat cultures of *K. pneumoniae* at $D \approx 0.70\ h^{-1}$ or $0.50\ h^{-1}$, respectively, and the steady-state concentration of ammonia or glucose ($\bar{s}$).

quently, there are no strong arguments [29] to destinguish between the hyperbolic description (according to the model of Monod) or logarithmic relationship (NET).

The chemostat system is designed to achieve a steady-state situation by fixing the dilution rate and thus the mean growth rate, at a constant value. However, it would be nicer to control $\bar{s}$ in a so-called 'substratostat', because $\bar{s}$ is usually the parameter directly controlling growth rate. For glucose limitation, ammonia limitation, or citrate limitation, such a sophisticated apparatus is not yet feasible. Thus, to draw conclusions about particular effects on the growth rates of microbial cultures during substrate-limited growth, one has to fix the D value and determine the $\bar{s}$ value. This has been done to study the effect of $\bar{p}$ and μ.

Ammonia-limited cultures of *K. pneumoniae* were considered to be essentially anabolite limited, because ammonia is only assimilated into cell material (anabolic product) and not involved in catabolism [28]. The steady-state biomass concentration ($\bar{p}$) in ammonia-limited cultures ($D \cong 0.7\ h^{-1}$) was adjusted by changing the medium reservoir composition (S_r). The results of determinations of $\bar{s}$ are presented in Fig. 2A and are simply described with a linear function. It is clear that $\bar{s}$ did not vary significantly with increasing $\bar{p}$ in ammonia-limited cultures of *K. pneumoniae*. The same result was obtained with glucose-limited (partly anabolite-limited) cultures ($D \cong 0.5\ h^{-1}$) as is depicted in Fig. 2B. In other words, during anabolite-limited growth, μ is independent of $\bar{p}$ (concentration of biomass). This is not unexpected; for a cell, the concentration of other cells is not relevant.

The situation for purely catabolite-limited growth is more complicated. The reason for this is that, in general, the substrate for catabolism contains also the carbon required for biosynthesis. However, it is reasonable to assume that with highly oxidised substrates (such as citrate) the growth is essentially limited by the amount of free energy, while for substrates with a higher degree of reduction, the availability of carbon becomes the limiting factor, so that the latter cultures are (partly) anabolite limited [11,30,31]. This is probably the most quantitative argument available as long as no thorough analysis exists of the extent of the control exercised by each reaction of the intricate network of total metabolism on anabolism and catabolism. It would be interesting to apply the direct analysis of control of anabolism and catabolism (and leak) on growth rate, described by Kell [32], on the growth-limiting conditions in chemostat cultures. In this study, citrate limitation was used as a representative for a catabolite limitation.

The time course of $\bar{s}$ in citrate-limited cultures of *K. pneumoniae* at a fixed dilution rate ($D \cong 0.4\ h^{-1}$) is depicted in Fig. 3. Surprisingly, the results show clearly that for these cultures a long-term adaptation pattern seems to exist, i.e., the concentration of citrate continued to decrease for a prolonged period (about 100

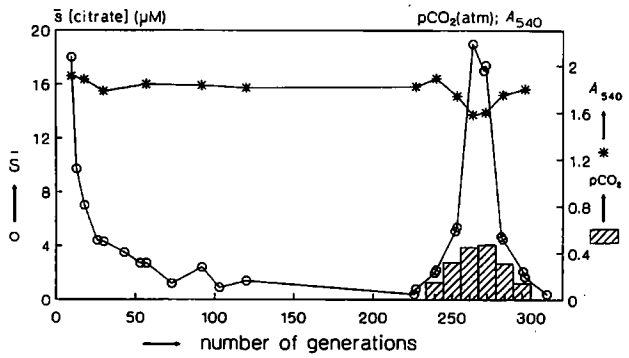

Fig. 3. Complete course of the steady-state concentration of citrate ($\bar{s}$) in a citrate-limited cultures of *K. pneumoniae* at $D = 0.38\ h^{-1}$. After about 200 generations (±2 weeks), the effect of CO_2/HCO_3^- on $\bar{s}$ was investigated by increasing the pCO_2 stepwise to 0.45 atm. (225–265 generations). Next, hysteresis was examined by the reverse experiment (265–310 generations). Also the course of the A_{540} is depicted.

generations) while the yield (as derived from A_{540}) remained constant.

This phenomenon is very similar to the observations, with glucose-limited cultures, of *K. pneumoniae* [14], in which the glucose concentration continued to decrease for about 60 generations. The underlying mechanisms of these adaptations (genotypic/phenotypic) are intriguing, but beyond the scope of this paper. The most important conclusion from this observation is that great care has to be taken when defining a steady state. Therefore, samples for determinations of $\bar{s}$ in glucose-limited, citrate-limited or ammonia-limited cultures were only taken after at least 100 generations in the chemostat (for an example, see Fig. 3).

Citrate-limited cultures of *K. pneumoniae* were used to study the influence of the concentration of the catabolic product carbon dioxide and/or bicarbonate ($\bar{p}$) on $\bar{s}$.

The complete course of this experiment is depicted in Fig. 3. After a long-term adaptation period (about 200 generations), the carbon dioxide pressure (pCO_2) in the influent gas flow was increased stepwise (0–0.45 atm). Subsequently, samples were withdrawn from the culture at each steady state. To check the influence of possible (long-term) adaptation and hysteresis effects, the pCO_2 after having been raised to 0.45 atm was lowered again stepwise down to 0.003 atm. The differences in $\bar{s}$ in the steady state upon increasing or decreasing the influent pCO_2 do not seem significant (see Fig. 3).

In order to examine the effect of $\bar{p}$ more precisely, the results are recapitulated in Fig. 4. Clearly, there is a strong influence of $\bar{p}$ (pCO_2) on $\bar{s}$ of citrate-limited cultures of *K. pneumoniae*. Also, the absorbance at 540 nm decreased significantly at pCO_2 above 0.3 atm.

It appeared to be impossible to construct suitable carbon balances, because much of the carbon was recovered in CO_2. However, with HPLC analysis of culture supernatants, no acetate, ethanol, succinate or other excretion products could be detected. Thus, the only products during aerobic citrate-limited growth of cultures of *K. pneumoniae* were assumed to be biomass and CO_2.

We determined whether the observed phenomenon was indeed the result of increasing $\bar{p}$ (CO_2/HCO_3^-) and not the result of the concomitant increase in Na^+ concentration used as neutralising ion. The concentration HCO_3^- in the cultures at pCO_2 values ranging from 0.01 to 0.45 atm was calculated according to the procedure described by Dixon et al. [33]. In our experimental set-up, a pCO_2 of 0.45 atm was equivalent to about 60 mM HCO_3^-.

In a control experiment there was no effect of NaCl up to 60 mM on $\bar{s}$ in citrate-limited cultures of *K. pneumoniae*. Thus, the increase in $\bar{s}$ was a direct result of the increase in the concentration of aqueous CO_2 and/or HCO_3^-.

One of the first approximations in the NET description is the implicit assumption that the cell composition (conductance) is independent of μ, $\bar{s}$ and $\bar{p}$ (Eqns. 9 and 10). In other words: the phenomenological constants (the L values) were considered to be constant (as is implicit in most growth models, including Monod's [1,3,9]). However, it is well known that L changes with μ [19,20] and, quite likely, L will also change with $\bar{p}$ (and $\bar{s}$).

For instance, HCO_3^- affects reactions of the Krebs cycle (e.g., succinate dehydrogenase) and (de)carboxylation reactions (e.g., phosphoenol pyruvate carboxylase) (for a review see Ref. 34). In anaerobic chemostat cultures, futile cycles involving CO_2 have been proposed [16,17]. Thus, in other words, it may be anticipated that HCO_3^- modifies L.

Fig. 4 shows that $\bar{s}$ increased with $\bar{p}$; this increase ($\pm$ 20 μM) is too small (0.2% of the S_r value) to influence biomass concentration in the culture significantly. Thus, the A_{540} of the culture (see Fig. 4) reflects the yield value directly. Besides thermodynamic 'back pressure', possibly some metabolic uncoupling with high pCO_2 (> 0.3 atm) occurred, resulting in a decreased yield on citrate.

The influence of pCO_2 on the maximal growth rate (μ_{max}) expressed after relief of the citrate limitation was also determined. In this experiment, citrate was added in saturating concentrations (10 mM). At relatively low pCO_2, there was no significant effect on μ_{max}, as is seen in Fig. 5. However, at higher pCO_2 a decrease in the μ_{max} was observed. This result supports the idea that high pCO_2 inhibits some part of the metabolism.

This was further corroborated by the finding that the maximal oxygen consumption rate (again with saturat-

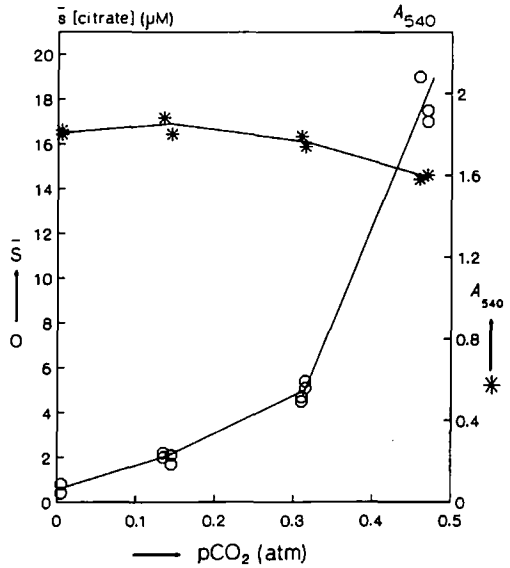

Fig. 4. Effect of CO_2/HCO_3^- on the steady-state concentration of citrate ($\bar{s}$) of a citrate-limited chemostat cultures of *K. pneumoniae* at $D = 0.38\ h^{-1}$. The concentration of biomass is indicated by A_{540}.

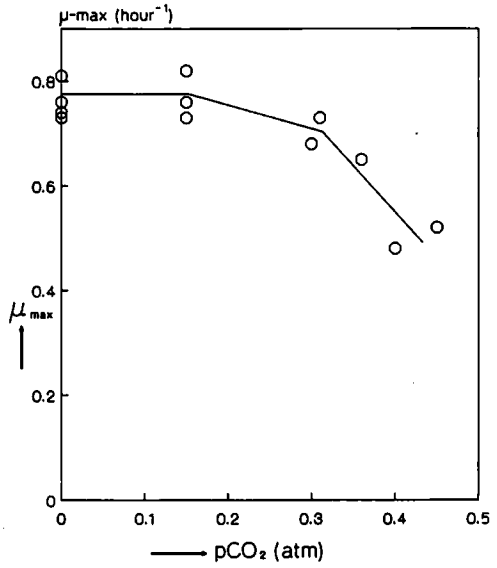

Fig. 5. Effect of CO_2/HCO_3^- on the μ_{max} of *K. pneumoniae* in minimal medium with citrate (10 mM) as carbon source.

ing citrate concentration) of cells harvested from a citrate-limited chemostat culture ($D = 0.38\ h^{-1}$) was initially not influenced by increasing HCO_3^- up to about 40 mM, corresponding to a pCO_2 of 0.30 atm (see Fig. 6). However, at high HCO_3^- concentrations, the maximum oxygen consumption rate was significantly decreased.

The decrease in μ_{max}, $J_{O_2}^{max}$ and yield on citrate with progressively increased pCO_2 can be explained by either

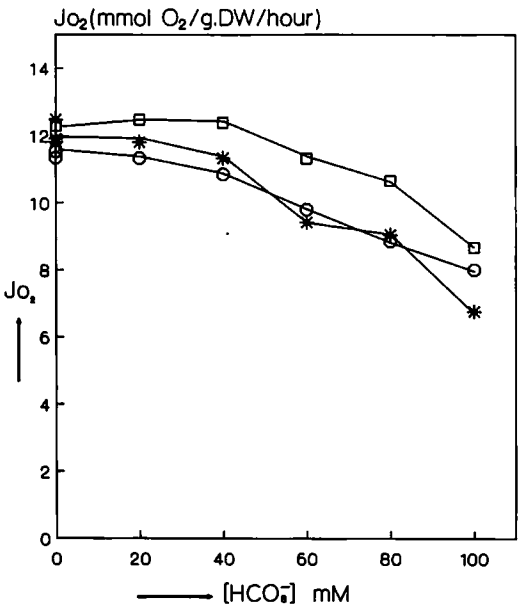

Fig. 6. Effect of CO_2/HCO_3^- on the J_{o_2} of cells in citrate-saturated (10 mM) minimal medium. Cells were harvested from a citrate-limited chemostat culture of *K. pneumoniae* at $D = 0.38\ h^{-1}$. □, titration with $NaHCO_3$ (with addition of NaOH to maintain pH at 7.0); *, incubation of cells in the appropriate medium directly after sampling; ○, cells were maintained at 0 °C before incubation.

inhibition of decarboxylation reactions (e.g., pyruvate decarboxylase) or by stimulation of futile cycles involving CO_2 or other processes of metabolic uncoupling [16,17,33,34]. The MNET model can be very useful to discriminate between the types of kinetic effect of high CO_2 (uncoupling, inhibition), but this is beyond the scope of this paper.

The complete oxidation of one molecule of citrate yields six molecules of CO_2. Applying this knowledge to Eqn. 5 suggests that ΔG_c would remain constant only if $[CO_2]^6/[\text{citrate}]$ is constant. However, in the citrate-limited chemostat system approximately two carbon atoms per molecule of citrate ($Y \approx 54$ g dw per mol citrate) are flowing into anabolism. Besides, some other reactions involving CO_2 may be in the (partly) saturated region. Thus, one should expect a less strong dependence ($< 6\times$) of the steady-state citrate concentration on pCO_2, and this fits well with the data of Fig. 4. The third alternative, some adaptation of the bacterial population to slightly changing concentrations of CO_2 cannot be excluded (change in L), not visible from yield, μ_{max} J_{o2}^{max}.

All observations together strongly suggest that concentration-dependent thermodynamic 'back pressure' is present with low CO_2/HCO_3^-, while at high values, kinetic (physiological) effects are also important.

One can speculate about the applicability of these experiments for discrimination between anabolite or catabolite limitation. With CO_2 as sole product of catabolism, one should expect thermodynamic 'back pressure' of CO_2 on the steady-state concentration of the carbon substrate only during (partly) catabolite-limited growth. Thus, the prediction is that during glucose-limited growth CO_2 affects the steady-state glucose concentration much less than with citrate-limited cultures.

Conclusions

The results of the experiments described in Figs. 1, 2 and 4 are summarised and interpreted in mathematical terms, of the NET description, in Table I. In this description, the concentration of substrates and products are an integral part of ΔG, the driving force for metabolic processes. However, in some cases a saturation effect may be observed so that the flow is independent of ΔG. For instance, the concentration of non-growth-limiting substrates and products does not affect growth. To describe this behavior mathematically, $\Delta G_i^{\#}$ and $\tilde{\mu}_i^{\#}$ were introducted as described above in the Theory section.

Thus, with steady-state chemostat cultures of *K. pneumoniae*, μ ($-J_a$) is strongly dependent on $\bar{s}$ in an approximately logarithmic fashion (Fig. 1 and Ref. 14), suggesting that $(\tilde{\mu} - \tilde{\mu}^{\#})_s$ are variables. Concerning the concentration of products ($\bar{p}$), two situations were not-

TABLE I

Presentation of $(\tilde{\mu} - \tilde{\mu}^{\#})_i$ as mathematical constants or variables involved in microbial metabolism

Subscripts a, c, s and p are abbreviations for anabolic, catabolic, substrate and product, respectively. Compound 'i' is in the unsaturated part of the flow versus force/chemical-potential relationship when $\tilde{\mu}_i^{\#}$ is constant. Compound 'i' is in the saturated part of the flow versus force relationship when $(\tilde{\mu} - \tilde{\mu}^{\#})_i$ is constant.

	Anabolite limitation (NH_4^+ limitation)	Catabolite limitation (citrate limitation)
$(\tilde{\mu} - \tilde{\mu}^{\#})_{cs}$	constant [a]	variable [b]
$(\tilde{\mu} - \tilde{\mu}^{\#})_{cp}$	constant [a]	variable [b]
$(\tilde{\mu} - \tilde{\mu}^{\#})_{as}$	variable [b]	constant [a]
$(\tilde{\mu} - \tilde{\mu}^{\#})_{ap}$	constant [b]	constant [a]

[a] Parameters derived from the Theory section.
[b] Parameters derived from the experiments.

iced: during NH_4^+ (anabolite)-limited growth (Fig. 2), μ was independent of $\bar{p}$ ([biomass]), whereas during citrate (catabolite)-limited growth (Fig. 4) μ was affected significantly by $\bar{p}$ ($[CO_2]$ or/and $[HCO_3^-]$) as a consequence of thermodynamic 'back pressure'. High CO_2/HCO_3^- also inhibits growth of citrate-limited cultures of *K. pneumoniae* kinetically.

In order to construct appropriate models of microbial growth, it is without doubt that any significant change in the physiology of the cells in the region of interest must be incorporated in these descriptions (for instance kinetic inhibition by end-products).

The driving force for processes of bacterial growth is dependent on the concentration of the growth-limited substrate. However, this paper has shown, in addition, the presence of thermodynamic 'back pressure' of the concentration of products during substrate-limited growth. Thus, it is important to account for this when designing and using microbial growth models.

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