Thermochimica Acta, 239 (1994) 17–32
Elsevier Science B.V., Amsterdam
SSDI 0040-6031(94)01678-A

Kinetic investigations of microbial metabolism by means of flow calorimeters

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(Received 17 December 1993; accepted 17 December 1993)

Abstract

A mathematical model for microbial growth is presented which combines a Michaelis–Menten kinetics for oxygen and a substrate inhibition kinetics of the Haldane-type of phenol. It is applied to flow calorimetric experiments of the growth of *Pseudomonas putida* on phenol and other aromatic compounds. The model describes features of the growth well and makes unexpected predictions which have been experimentally verified and compared with as yet unexplained observations from the literature. Possible applications to batch and flow calorimetric investigations of microbial growth are discussed with respect to the critical evaluation of the chosen instrumental set-up.

INTRODUCTION

After a few decades of stagnation, biological calorimetry is attracting increasing attention. From biochemistry with enzymatic reactions or protein investigations to microbiology with experiments on microbial growth or on pharmacological influences, from tissue cultures to studies of whole animals and to global evaluation of ecological systems, calorimetry has demonstrated its power as a universal, integral, non-destructive and highly sensitive tool for many biological questions. It has become a well-established method, with a broad spectrum of commercially available instruments containing vessels in the range of a few microlitres up to several hundred millilitres or even litres. Several books and some review articles offer comprehensive surveys on biological applications [1–3]. Nevertheless, biological calorimetry can still provide some unexpected surprises and unwanted pitfalls. The present paper deals with such an experience and offers some possible solutions.

Usually, the instruments applied in biological calorimetry are of the isoperibolic type and may be roughly divided into two classes: the batch and the flow systems. Both offer advantages and drawbacks and should be chosen carefully for investigations. While batch instruments are inevitable in

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experiments on whole animals or liquid systems with coarse particles, flow calorimeters have superior efficiency in investigations of microbial cultures, immobilized enzymes, and tissue cultures on glass beads or other carrier materials.

The advantage of batch experiments with microorganisms lies in the fact that the culture is enclosed within the calorimeter and that all the heat produced by metabolism during maintenance and growth is detected by the instrument. The important drawback of this method is connected with the thermal disturbances as a result of any mechanical interference with the system. Stirring to avoid sedimentation of cells, lack of homogeneity or decrease of oxygen pressure around the cells introduce heats of friction which are not completely compensated for by a similar stirring device in the twin chamber of the system. The addition of material or the removal of samples during the experiment in order to determine substrate or product concentrations or cell counts, disturbs the thermal signal; aeration introduces considerable heats of evaporation, and the simultaneous monitoring of pH value, optical density or oxygen pressure in the medium is difficult due to the limited space in the calorimetric vessel.

These problems are overcome by the application of a flow calorimeter connected to an external fermentor. None of the above-mentioned activities on the liquid culture in the fermentor have any influence on the calorimetric signal. However, the crucial disadvantage of this approach lies in the separation of fermentor and calorimeter and the necessary tube connection between the two. A minimum tube length is unavoidable due to geometrical reasons and the need for a highly effective thermal equilibration of the in-flowing liquid to the temperature of the calorimetric cell. This leads to a considerable change in the metabolic state of a liquid volume leaving the fermentor during the transport to the calorimeter, especially at high cell densities. A drastic increase in the pumping rate and, thus, a corresponding decrease in flow times is not possible because of the necessary thermal equilibration. Thus, in some situations the calorimetric signal is no longer a true picture of the metabolic situation in the fermentor and has to be evaluated with precaution [4–7]. "Simultaneous" determinations are not really simultaneous; the results may be "highlights of instrumental artifacts" as we will demonstrate in the present paper.

All the experimental power–time curves shown in this paper are from investigations on the microbial degradation of phenol by the bacterium *Pseudomonas putida*. However, the results of the mathematical modelling are also applicable to all other flow calorimetric investigations of microbial systems, as underlined by the examples cited from the literature. In our experiments, the gram negative, polar-flagellated, unicellular bacterium *Pseudomonas putida*, strain 548 (ATCC 17514, NCIB 10015; Deutsche Sammlung von Mikroorganismen DSM/Braunschweig) was applied. It not only tolerates phenol up to concentrations of more than 1 g l^{-1} but also uses

this compound as its sole energy and carbon source [8, 9]. It mineralizes phenol and related substances to carbon dioxide and water in a quantitative way. All batch experiments were run in a Calvet-type twin calorimeter (Setaram/Lyon) with mechanical stirring and vessels of 15 ml capacity; all flow experiments were run with an LKB flow calorimeter (type 10700, LKB/Bromma). More information about the system, the experimental approach and results may be found in the literature [10].

MATHEMATICAL MODELLING

Growth is one of the important attributes of life and a target of many biological investigations. Organisms exhibit various time dependences of mass (or number) increase which are described by quite different mathematical equations [11]. Even the growth of microorganisms is rather diverse, demanding a variety of mathematical approaches [12]. But growth is often limited by some external constraints, including substrate or product concentrations, pH values, poisoning effects of metabolites and lack of oxygen, so that it may be described by just one common equation. While growth or mass increase are integral figures, the heat production or metabolic rates proportional to the change in mass are derivative figures. Therefore, in the following mathematical models of microbial metabolism, the first derivatives with respect to time of the growth equations are used.

Independently of the different kinetics applied later on, the following results can be drawn from a single calorimetric curve [13]. (i) The area between the power–time curve and the abscissa corresponds to the total heat Q_T evolved during the experiment. (ii) This value Q_T is proportional to the initial substrate concentration S_0. (iii) The amount of heat $Q(t)$ dissipated until a particular moment t corresponds to the amount of substrate $\Delta S(t)$ consumed during this period. (iv) The ratio $Q_T/S_0 = \Delta Q_S$ (in kJ mol^{-1}) allows $\Delta S(t)$ to be calculated from $Q(t)$ and, together with S_0, the momentary substrate concentration $S(t)$. (v) S_0 and Q_T are proportional to the total amount of biomass ΔX_0 produced during the experiment, rendering a mass yield $Y_{X/S}$ (in g dry matter per mol substrate) and a heat yield ΔQ_X (in kJ per g dry matter). (vi) The first derivatives with respect to time of $\Delta S(t)$ and $\Delta X(t)$ render rates of change in substrate and biomass concentrations that are proportional to the heat production rate $P(t)$. These figures provide all the necessary values to calculate the kinetic data from just two calorimetric results, $P(t)$ and $Q(t)$.

However, in the light of the mathematical model developed below for the calorimetric signal of the experimental results, it should be kept in mind that these statements are true for batch calorimeters [13], but for flow versions only if the calorimetric signal is a true picture of the metabolic events in the separated fermentor, i.e. under strong aeration and stirring and at high flow rates (see below).

Model after Verhulst–Pearl

In a fully unlimited microbial culture without any external constraints, growth follows an exponential law, at least for a certain time. It is represented by the equation of Malthus

$$N(t) = N_0 \exp(kt) \tag{1}$$

with $N(t)$ being the number of cells at time t, N_0 at time zero, k the constant of unlimited growth and t the experimental time. The heat production rate $P(t)$ during this time is then given by

$$P(t) = \epsilon \, \mathrm{d}N(t)/\mathrm{d}t = \epsilon k N_0 \exp(kt) = \epsilon k N(t) \tag{2}$$

with ϵ being the specific heat production per newly formed cell. Finally, the total heat produced from $t = 0$ to $t = t'$ is found by integration

$$Q(t') = \int_0^{t'} P(t) \, \mathrm{d}t = \int_0^{t'} \epsilon k N_0 \exp(kt) \, \mathrm{d}t = \epsilon N_0(\exp(kt') - 1) = \epsilon \, \Delta N(t') \tag{3}$$

and is thus directly proportional to the number of cells $\Delta N(t')$ formed during this period. This holds true only if there is no contribution from maintenance metabolism to the heat balance. Usually, this part may be neglected under the chosen conditions.

Unlimited growth of microbes is, fortunately, only possible for a short time. Soon, some of the constraints mentioned above gain influence, reduce the growth constant k and decelerate growth. This situation is described by the equation of Verhulst and Pearl ("logistic function") developed from the Malthus equation

$$\mathrm{d}N(t)/\mathrm{d}t = (k - mN(t))N(t) \tag{4}$$

$$P(t) = (k - mQ)Q \tag{5}$$

with m being an inhibition term. It reflects the decrease in substrate concentration, or increase in metabolic products, or some other external parameters. $P(t)$ is a parabolic function of $Q(t)$, but exhibits a nearly symmetrical, bell-shaped graph as a function of time (Fig. 1). This type of power–time curve is found in unstirred liquid batch cultures or in growth on a solid medium [14].

Michaelis–Menten kinetics

Many enzymatic reactions are governed by the so-called Michaelis–Menten kinetics

$$v = v_{\max} S/(K_m + S) \tag{6}$$

where v is the variable enzymatic rate, $v_{\max}$ the maximum rate, S the substrate concentration and K_m the Michaelis constant. It corresponds to

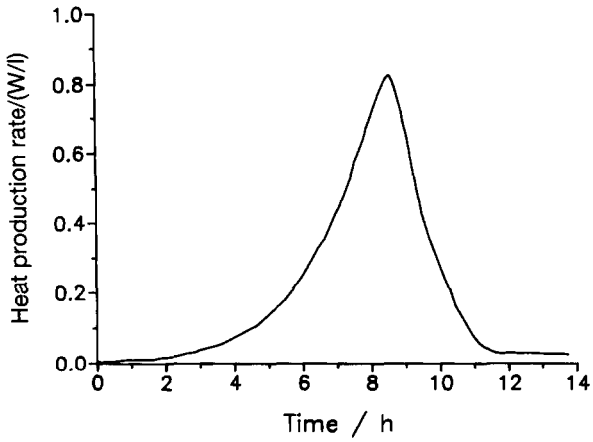

Fig. 1. Experimental power–time curve of microbial growth following the Verhulst–Pearl equation presented in eqn. (5).

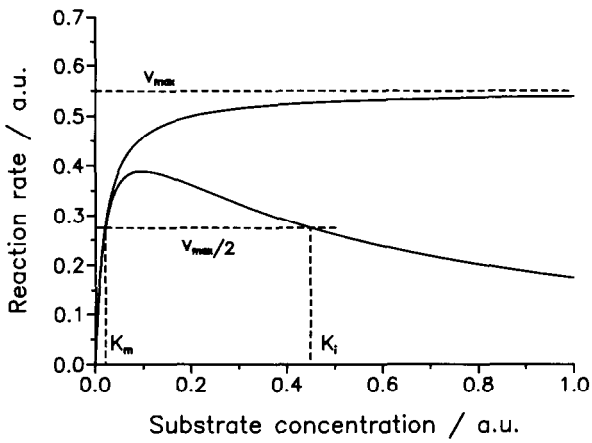

Fig. 2. Enzymatic reaction rate as a function of substrate concentration (both in arbitrary units a.u.) following a Michaelis–Menten kinetics (upper curve, eqn. (6)) and following a Haldane kinetics (lower curve, eqn. (8)). The maximum reaction rate v_{max}, the Michaelis constant K_m and the inhibition constant K_i are indicated.

that substrate concentration where $v = v_{max}/2$. This kinetics is depicted in the upper curve of Fig. 2. To obtain an understanding of the kinetic situation in the culture, the curve $v = f(S)$ in Fig. 2 should be read in the negative direction: time increases with decreasing substrate concentration, the culture "slides" along the graph from right to left, with minor variations at the beginning and very major ones at the end, in a manner that is directly proportional to the substrate concentration. The Michaelis–Menten kinetics is apparent only at the end of the power–time curve at the sharp drop from the maximum to the zero line (Fig. 3). It is this part of the calorimetric

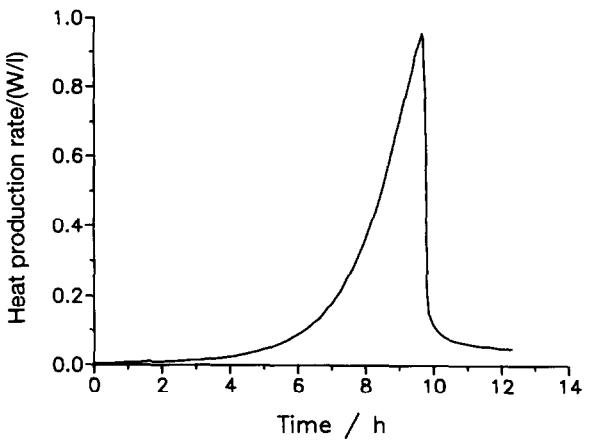

Fig. 3. Experimental power–time curve of the growth of *Pseudomonas putida* at low phenol concentrations following the Michaelis–Menten kinetics of eqn. (7).

graph in which the characteristic data K_m and v_{max} are determined. A double reciprocal plot of $1/v$ against $1/S$ (Lineweaver–Burk plot) renders a straight line whose intercepts with the axes give $1/v_{max}$ and $-1/K_m$. Thus, v_{max} and K_m are easily obtained from the experimental curve.

As the rate of substrate consumption v is proportional to the heat production rate $P(t)$, eqn. (6) can be transformed to

$$P = P_{max} S/(K_m + S) \tag{7}$$

It has been shown in the literature that growth is often limited by an enzymatic master reaction so that eqn. (7) can be chosen to describe microbial growth in liquid cultures [13]. In many enzymatic reactions, the maximum reaction rate v_{max} (and correspondingly here P_{max}) is linearly dependent on the enzyme concentration. As the latter increases with proceeding growth of the culture, P_{max} is no longer a constant but increases proportionally to the cell number or biomass which can be calculated from the calorimetric curves as demonstrated above.

Haldane kinetics

Some substrates block their own enzymatic degradation at higher concentrations although they obey the Michaelis–Menten kinetics at low values. This behaviour can be described by a Haldane kinetics adding a quadratic inhibition term to the denominator of eqn. (6)

$$v = v_{max} S/(K_m + S + S^2/K_i) \tag{8}$$

with the inhibition constant K_i. The geometric form of this kinetics is presented in the lower graph of Fig. 2. K_i is defined as that concentration where $v = v_{max}/2$ (similar to K_m). The observed maximum reaction rate v' is

given by the geometric mean of K_m and K_i. At high substrate concentrations, K_m may be neglected against S^2/K_i, leading to a hyperbolic dependence of v on S. At low substrate concentrations, the last term is negligible and the Haldane kinetics changes to a usual Michaelis–Menten kinetics. Thus, the first part of a power–time curve of microbial growth at higher substrate concentrations follows eqn. (8), the final part follows eqn. (6).

Corresponding to the Lineweaver–Burk plot of a Michaelis–Menten kinetics, an easy way to evaluate v_{max} and K_i for a Haldane kinetics is to plot $1/v$ against S for high substrate concentrations. The slope of the linear functions renders $1/(v_{max}K_i)$, the intercept with the abcissa $-K_i$, while K_m is found at low substrate concentrations.

Double kinetics

At first glance it seems that phenol is the only limiting substrate in the growth medium of *P. putida* [10]. However, with an oxygen solubility of 0.237 mmol l^{-1} at 30°C and a stoichiometric ratio of 1 mol phenol per 3.3 mol oxygen at intensive growth or 7 mol at maintenance, it becomes clear that oxygen appears as the main limiting substrate, even at medium phenol concentrations. Typical initial phenol concentrations of 0.333 g l^{-1} correspond to 3.5 mmol l^{-1} or 15 times the amount of oxygen dissolved in water. Combined with the stoichiometry mentioned above, the oxygen concentration is just one fiftieth of that needed for complete oxidation of phenol.

During growth in the fermentor, phenol is consumed while oxygen is kept approximately constant due to intensive aeration and stirring. But at the moment when a small suspension volume enters the tube to flow to the calorimeter, further oxygen supply is cut off and oxygen concentration decreases in parallel with phenol, although much more quickly. Under appropriate rate conditions, all oxygen is consumed before the suspension volume enters the calorimetric cell and the aerobic phenol degradation ceases: the calorimeter signal returns to the base line. Thus, no growth is taking place although it proceeds with unretarded speed in the fermentor.

A rough estimation yields the critical values. At the peak of a typical growth curve (Fig. 3) the heat flow rate of 1.0 W l^{-1} corresponds to a phenol consumption rate of 2.0 mmol l^{-1} h^{-1} and an oxygen consumption rate of 6.7 mmol l^{-1} h^{-1}. With a maximum oxygen solubility of 0.237 mmol l^{-1}, it takes just 2 min until the oxygen concentration reaches zero. And with a typical flow rate of 30 ml h^{-1}, a suspension volume reaches the calorimeter after 6 min, i.e. at a time when the oxygen concentration is already zero.

Taking this into account, the Haldane-type expression of eqn. (8) has to be further modified with respect to oxygen by a Michaelis–Menten kinetics

$$v = v_{max}S/(K_m + S + S^2/K_i)(S'/(K'_m + S')) \quad (9)$$

where S' represents the oxygen concentration in the calorimetric vessel and K'_m the corresponding Michaelis–Menten constant. Depending upon the growth phase, either the first or second term dominates and exhibits the limiting factor of the calorimetric signal, though not, of course, of growth in the fermentor. This means that under such conditions of higher initial substrate concentrations and low flow rates, the calorimeter no longer gives a true picture of the metabolic events and growth in the fermentor. This will be explained below in more detail when discussing the results of the mathematical model.

Some flow-calorimetric reflections

In the Introduction, the advantages and drawbacks of batch and flow calorimeters were discussed briefly. Due to their mechanical construction, the classical LKB flow calorimeters (type 10700) dissipate the heat produced in the flow-through cell via the two Peltier elements (heat flux sensors) on both sides, the air gap and the liquid leaving the measuring cell [7]. Thus, the effective sensitivity of the instrument significantly changes with the flow rate [4, 6, 7]. Figure 4 shows the calibrating responses at five different flow rates. Figure 5 shows the sensitivity as a function of the pumping rate. The sensitivity decreases to 67% when the flow changes from 0 to 200 ml h^{-1}. At the same time the baseline noise and fluctuations increase considerably so that such high flow rates are not recommended under normal experimental conditions (but see refs. 6, 15 and 16). Rates of 30–50 ml h^{-1} are usually found in the literature.

The tubing from the fermentor to the calorimeter is composed of the necessary external connections and the various internal heat exchangers,

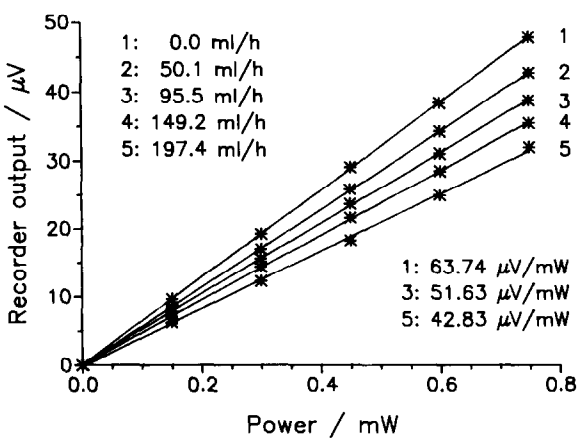

Fig. 4. Electrical calibration of the calorimeter at 5 different flow rates. The sensitivities at flow rates 1, 3, and 5 are presented in the lower inset.

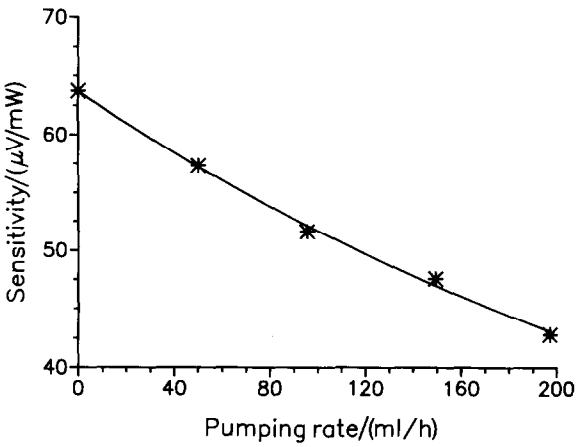

Fig. 5. Sensitivity of the calorimeter as a function of the flow rate.

with a total length of 250 cm or a corresponding volume of 3.2 ml suspension. At the flow rates of 30 ml h^{-1} usually applied in these investigations, a flow time of 6 min is observed. This has to be compared with the consumption rates and times calculated above.

Computer simulation

The ideas and equations explained above were used for the development of a simulation program working on a personal computer. The aim was to describe experimental calorimetric power–time curves, to predict extreme situations and to gain more insight into the metabolic events in the combination fermentor/flow calorimeter. It had to be kept in mind that the model should be applicable to two partly independent systems: to both the fermentor and the flow calorimeter with its tube connections.

Two assumptions were incorporated into the model. (i) It is assumed for the fermentor that aeration and stirring are so effective that the suspension is homogeneous and that the oxygen concentration remains constant near saturation even at higher metabolic rates. Under such conditions, eqn. (8) suffices to describe the rates of heat production and metabolism. (ii) The connecting tubes between the fermentor and the calorimeter shall be impermeable to oxygen so that its concentration diminishes countinuously due to metabolic consumption. When a volume element leaves the fermentor it is assumed to develop its own metabolic rate: eqn. (9) holds good in this situation. Moreover, this tube system has its own characteristic time, always starting at zero for each volume element entering the tube, while the fermentor time proceeds from the very beginning of the calorimetric experiment.

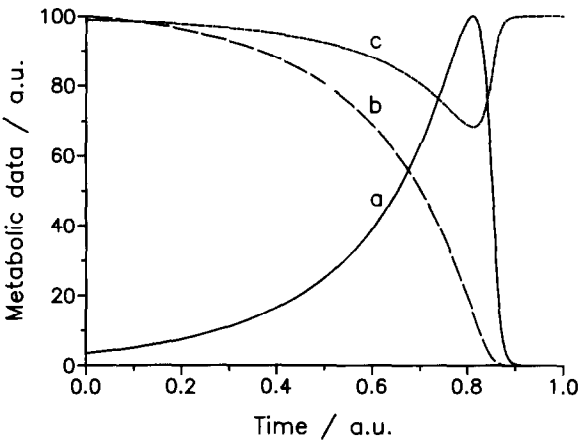

Fig. 6. Calculated power–time curve (a) for growth at medium phenol concentration and high flow rate, together with the corresponding phenol (b) and oxygen concentrations (c). All traces are shown in arbitrary units taking their maxima as 100%.

The mathematical model calculates the following values as a function of time: biological mass, substrate concentration, metabolic and heat production rates, and total heat dissipation both in the fermentor and in the calorimeter. Some of these functions are displayed on the screen and compared with experimental power–time curves. Figure 6 shows a typical, calculated power–time curve of growth at medium phenol concentrations together with substrate and oxygen concentrations in the calorimetric cell. The residence time, i.e. the flow time from the fermentor to the calorimeter, was chosen as in most experiments. Figure 6 exhibits a good approximation to the experimental curves when the characteristic parameters found in the literature are slightly changed. Moreover, it demonstrates that the steep decline beyond the maximum follows the phenol concentration which becomes rate-limiting while the oxygen concentration changes only slightly.

Changing the model, a second peak appeared (Fig. 7) which has already been observed in experimental curves but was assumed to be due to a diauxic metabolic behaviour. As such a situation could not be described by our simple mathematical model of eqn. (8), this structure must be inherent in the system. Comparing heat production rate and oxygen concentration revealed that the latter was rate-limiting until the phenol concentration reached such low values that the limitation was lifted, the oxygen concentration increased and the heat production rate could increase again. The steep increase equals the corresponding decrease beyond this maximum and can be extrapolated backwards to show the complete Michaelis–Menten kinetics of phenol degradation at low concentrations. Thus, this second peak is not the expression of a diauxic metabolic behaviour, as often stated in the literature, e.g. refs. 17 and 18, but merely an instrumental artifact introduced by oxygen depletion. The correctness of the calculated figures could

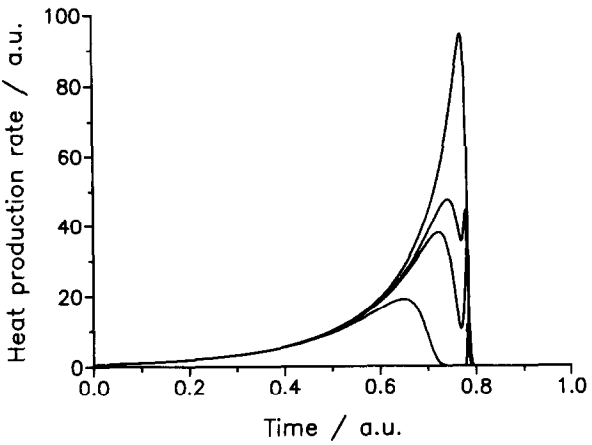

Fig. 7. Calculated power–time curves at various flow rates, i.e. residential times, but identical initial phenol concentration. The flow rates differ at 20:10:8:1 a.u. from top to bottom.

be proved by means of a polarographic oxygen sensor incorporated into the flow line behind the calorimetric cell.

Prolonging the residence time should lead to an earlier appearance and a smaller amplitude of the first maximum, a deeper minimum and a more distinct separation of the two maxima. Such a behaviour is clearly demonstrated in Fig. 7 for four residence times. After these mathematical predictions, we lowered the experimental flow rate to exceptional values, confirming the theoretical results. Figure 8 shows an experimental power–time curve at high phenol concentration and a prolonged residence time. Such curve shapes have appeared in the literature [17–20] and have been

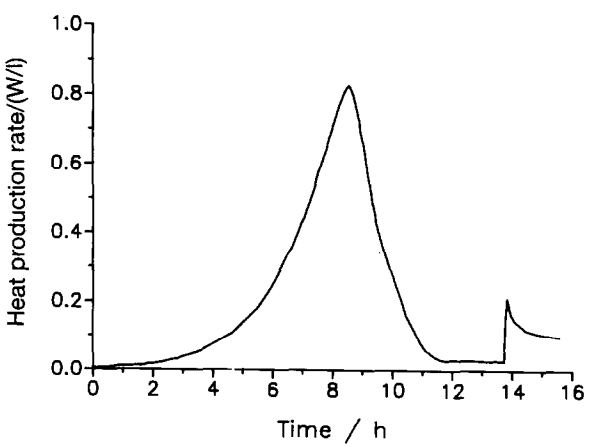

Fig. 8. Experimental power–time curve of growth at high initial phenol concentration and strongly reduced flow rate. Compare with the lowest trace in Fig. 7.

discussed in metabolic terms but with no sound explanation. Here they follow as a necessary consequence of the chosen experimental conditions.

Returning to Fig. 7, it becomes clear that with increasing residence times the areas under the curves diminish and the total heat production determined calorimetrically decreases although the degraded amount of phenol remains constant. These predictions were also confirmed by direct measurements at different flow rates (not shown). Therefore, for exact quantitative flow-calorimetric determinations it is recommended that experiments be performed at several flow rates and the results extrapolated to a residence time of zero or to infinite rates. The differences between the calculated heat productions in the fermentor and the calorimetric cell increase with increasing initial substrate concentrations at otherwise constant conditions. Their ratios drops from 80% at 0.1 g l^{-1} phenol to less than 40% at 1 g l^{-1}, i.e. the calorimeter determines less than half of the correct metabolic heat.

A typical calorimetric curve with a second maximum exhibits a three-fold limiting character. In the first phase of slowly increasing heat output, the number of active microbial cells is limiting and heat dissipation grows proportionally to the cell count. Near the main maximum, the oxygen concentration becomes limiting and reduces the possible metabolic turnover (in the calorimetric vessel). Finally, oxygen concentration increases again in the third phase of the curve but phenol concentration tends towards zero and shapes the second maximum.

DISCUSSION

The model presented in this paper starts from the assumption that the combination fermentor/flow calorimeter is composed of two separate and partly independent units with their own experimental time courses. (i) The fermentor, with a large volume (as compared with the rest of the set up), sufficient stirring and perfect oxygen supply. Its time runs from the very beginning of the experiment. (ii) The tubing system with the flow-through heat sensor, and no further oxygen supply. Its time always starts at the moment when a volume element leaves the fermentor. In this way, the main volume in the fermentor has its own metabolic development that is different from that monitored by the calorimetric signal. As a consequence, the calorimeter presents an erroneous picture of the metabolic situation in the fermentor, as will be discussed below. Measuring only the oxygen concentration in the fermentor gives a misleading representation of the calorimetric state: the oxygen concentrations and, thus, the metabolic activities differ considerably in the two units. Placing a polarographic oxygen sensor in the fermentor is desirable; one directly behind the flow-through vessel is absolutely essential.

The model further assumes two terms, one describing a substrate inhibition kinetics of the Haldane type [8], here applied to phenol, the second one

a conventional Michaelis–Menten kinetics for oxygen. The first term holds in both the fermentor and the calorimeter, while the second is valid only in the tubings and the flow calorimeter. The various parameters used in the model are taken from the literature and are modified according to our own results.

The assumption of constant oxygen concentration in the fermentor is rather questionable, as the experiments show, but may be accepted as a first approximation to formulate a rather simple model of metabolic turnover. Polarographic determinations confirm that only in the case of very high stirring and aeration rates in the fermentor and with low microbial densities, is the oxygen demand of the culture fully met [15, 16]. A more precise model has to incorporate the oxygen concentration in the fermentor by calculating the metabolic consumption of oxygen and its transition from the gaseous into the liquid phase which is usually facilitated by appropriate stirring rates. The results of such an approach will be published elsewhere.

The model presented here is only suited for defined media with only one carbon and energy source. Many organisms need complex media for growth, composed of many, partly ill-defined, components which are metabolically degraded in succession. This results in polyauxic growth patterns and interference between the different substrates and their metabolites which are hard to describe in a mathematical model of heat production.

The model was developed for the demineralization of phenol, governed by a substrate inhibition kinetics. But it might be easily modified to describe calorimetric experiments with other substrates and other kinetics, e.g. competitive inhibition or Michaelis–Menten kinetics alone. The essential part is the contribution of the second (oxygen) term to the calorimetric response.

The model makes several astonishing predictions which could be proved by varying the initial substrate concentration, the flow rate and the oxygen supply. The most unexpected one was the late appearance of the second maximum, even after a long thermo-neutral period. This effect has been described several times in the literature. In a paper by McIlvaine and Langerman [21], a thermo-neutral period of 150 min was observed in a growth curve of *Escherichia coli*, but was not discussed further in the text. Beezer and coworkers [17, 19] showed that the course of power–time curves is determined by "delicate oxygenation conditions" and concluded: "In the extreme situation the micro-organisms in the calorimeter inlet line and detection cell could be in an anaerobic environment even when the micro-organisms in the fermentor are shown to be in an aerobic environment." But they attributed the peak following the thermo-neutral period to the degradation of a limiting substrate formed during the first phase of aerobic metabolism. Bunker and James [18] changed the length of the thermo-neutral period at will by altering the experimental conditions such as aeration, stirring and glucose concentration. They stated: "There were

periods of growth without any increase in power output and *vice versa*. This is a phenomenon often observed during bacterial growth in rich media but which has never been satisfactorily explained"; and continue "The subsequent sharp decrease has been attributed to the exhaustion of a constituent necessary for growth, although this component has never been identified or named". Lamprecht et al. [20] investigated the "late peak" in calorimetric growth experiments with yeasts on glucose and found a thermo-neutral period which was not strictly zero but levelled at 17% of the maximum, presumably due to the anaerobic metabolism of the yeasts. In their experiments with yeasts on glucose, the second peak was much more pronounced than the present peak with *P. putida* on phenol, sometimes rendering the same heat production rate as the main maximum.

In addition to these direct presentations of the thermo-neutral period by other authors, the question of sufficient oxygenation in the calorimetric vessel has often been discussed in the literature and various proposals have been made for solving the problem. For example, Eriksson and Wadsö [22] developed (i) a special calorimetric cell for the simultaneous introduction of air and cell suspension and (ii) a mixed flow system of suspension and air bubbles; they obtained an oxygen pressure that never dropped below 10% of the saturation value. In these cases, the $p-t$ profiles are true pictures of the microbial metabolism and not calorimetric artifacts.

Moreover, the present investigations showed that the second peak is not bound to phenol but that it also appears with benzoic acid, catechol, or 3-oxoadipic acids, which are all closely connected to phenol metabolism. This proves that this peak is no separate metabolic event due to another energy source or to special metabolites but is a general phenomenon, i.e. an instrumental artifact. It can be produced at will and its position depends upon the flow rate chosen, the initial substrate concentration, the cell count and the degree of oxygenation. Thus, a calorimetric power–time curve with two maxima exhibits a sequence of different limitations: at the very beginning, the number of cells is limiting but steadily grows until oxygen becomes limiting at the first maximum. In the course of the subsequent minimum, both the substrate and oxygen concentrations determine the heat production rate, while beyond the second maximum the substrate concentration is the essential factor.

The present model can be applied not only to flow-calorimetric experiments but also to batch investigations. In this case, fermentor and calorimetric vessel are identical, their behaviour being described by one equation and one common time. Under "quasi-aerobic" or "micro-aerobic" conditions the second peak may also appear but not in such a distinct form as in flow experiments. The reason lies in the fact that in the latter case metabolism proceeds at high rates in the well-aerated fermentor, where the substrate concentration decreases enabling the recreation of oxygen supply in the calorimetric inlet line. In a batch experiment, metabolism is strongly

reduced in the calorimetric vessel, i.e. the fermentor, so that the interplay between the two concentrations is far less pronounced.

After finishing our calculations and their experimental verifications, and during preparation of the manuscript, we became aware of the papers by Livingston in which a similar approach was used for modelling the degradation of aromatic compounds in a fluidized bed [23, 24] and in membrane bioreactors [25]. The experimental conditions were chosen in such a way that phenol and oxygen were growth-limiting nutrients diffusing into and reacting within the biofilm established on solid support particles by natural adsorption. An equation similar to eqn. (9) was proposed which described growth of free and immobilized cells equally well. The necessary kinetic parameters for phenol degradation were determined in batch cultures of unsupported cells and showed sufficient agreement with literature data and those used in the present investigation.

Apart from these papers by Livingston for bioreactors [23–25], no publications are known to us that apply a double-kinetics model to evaluate microbial growth patterns in batch or flow calorimeters. Because it is not always possible to guarantee sufficient oxygenation of the medium at higher cell densities and with longer inlet lines to the calorimeter, such double-kinetics description may be useful for recalculating the "true" growth pattern of a microbial population or in avoiding pitfalls in establishing energetic balances. Moreover, these kinetic analyses provide a better understanding of the processes and limitations in calorimetric experiments and lead to more appropriate instrumental set-ups.

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