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CALORIMETRIC STUDIES ON THE USABILITY OF ENERGY-BALANCES OF MICROBIAL POPULATIONS FOR AN ECOTOXICOLOGICAL TESTING STRATEGY

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ABSTRACT

Direct calorimetry was used to measure the metabolic heat production of Escherichia coli growing on a synthetic culture medium. The principle of the chemostat culture allowed to change the growth conditions of the population easily and well reproducible. The steady-state growth rate of the cultures altered significantly the response of heat-flow rates and energy balances when toxic chemicals were added to the cultures. So we found a more pronounced increase of the heat flow after the addition of low concentrations of uncoupling agents at lower dilution-rates than at higher ones. The performance of our investigations was based almost on the properties of a specially designed heat-conduction-twin-calorimeter which containes the growing cultures directly inside its measuring cells.

INTRODUCTION

Recently toxicological methods for assessing toxic properties of environmental chemicals became more and more important (ref.1), in particular methods using microbial populations as biological targets (ref.2). From an ecological point of view the main goal is focused on the analysis of toxic effects at the level of biocoenoses (ref.3) or on mass- and energy flows in ecosystems (ref.4).

Microbial batch tests almost provide conditions of extreme physiological states, when microbial populations are allowed either to respire or to grow with the maximum speed (ref.2/5/6). Compared to this a chemostat culture facilitates to simulate the limitation of energy recources or to study competing mixed microbial populations as has been demonstrated by Mayfield et al (ref.7). The authors investigate effects of mercury on chemostat cultures among other things.

Fundamental work on the operation of chemostats has been published (ref.8/9). Changes of the physiological state of the cultures, concerning steady-state growth rates are easily made possible by changing the dilution-rate. Biological calorimetry on chemostats is carried out mostly with a combination of a flow-microcalorimeter and a fermenter (ref.10). This concept requires mathematical correction terms to recalculate the heat production rate of the chemostat culture from calorimetrically obtained data. The technique is used to investigate steady-state conditions of chemostats but not to analyse transient disturbances of it.

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(ref. 11). Recently we published results on toxic effects of chemicals on chemostat cultures of the aerobic bacterium Acinetobacter calcoaceticus (ref.12) which demonstrate the applicability of direct calorimetry for toxicity testing and a simple method to estimate toxicity data from heat-flow-measurements.

The problems concerning the flow-calorimetry on chemostats are avoided by a specially designed calorimeter of the heat-conduction principle, which has been developed on the basis of the calorimeter published by Marti et al (ref.13). The twin principle of the calorimeter allowed a good long term stability since disturbances from the surroundings were canceled out nearly completely. The calorimeter can be brought into operation with various dilution rates without recalibration since the effect of the medium flow through the measuring cells is a constructive compound of the heat conductivity between measuring cells and their surroundings. Some details of our construction are discussed below.

Experiments on aerobic chemostat-cultures of Escherichia coli were performed related to the question, wheather the heat production rate of a microbial culture is representative of disturbances being induced by toxic chemicals and how far the reaction of a chemostat culture on toxic chemicals depends on the physiological state of the microbial population.

MATERIALS AND METHODS

Escherichia coli (Enterobacteriaceae) was isolated from raw wastewater of a small sewage plant in 1981 and was maintained as stock culture on nutrient agar slants. In our chemostat experiments we used a synthetic culture medium containing 2.0 mM glucose, 6.55 mM NaH_2PO_4, 3.78 mM KH_2PO_4, 3.78 mM $(NH_4)_2SO_4$, 0.068 mM $CaCl_2$, 0.406 mM $MgSO_4$ and 1 ml of each trace element solution and Fe-EDTA-Complex according to Rippka et al (ref.14). The medium had a pH of 7.1.

Monitoring of the cultures

The cultures were monitored for glucose concentrations using the GOD-Perid-Method commercially available from Boehringer Mannheim. The concentration of bacterial dry matter was measured photometrically at 578 nm using an empirically determined calibration coefficient.

Growth rate and biomass-productivity were calculated from biomass data following Park et al (ref.15). To calculate energy-conversion-rates we used the standard value of the heat of combustion according Prochazka et al (ref.16) being 22.54 kJ / g dry bacterial matter.

Acetic acid and formic acid were analysed by gas-chromatography after derivatising them to methylesters. 2.0 ml raw sample, 1.0 ml Methanol and 1.0 ml 25% H_2SO_4 were sealed in 10 ml reaction vials, held at 60°C for two hours and then analysed on a column of two meters length packed with Porapak Q (80/100mesh)

The gas chromatograph was brought into operation with a temperature programme of 5 minutes at 100°C increasing to 160°C at a rate of 10°C / minute. Injector and detector temperature was 180°C. For analysis the samples were kept at 45°C in a water circulating bath and 1.0 ml of the gasphase were injected into the GC using a gas-tight syringe. We used the GC-Mini-2 (Shimadzu) with FID-detectors.

Ethanol was analysed using the system alcohol-dehydrogenase-NAD. The final processing of the data was carried out on a Hewlett-Packard series 86 personal computer. Thermogram data were transfered to the calculator from chart-recorder prints by hand.

Design and performance of the calorimeter

Figure 1 showes a scheme of a cross section of the calorimeter. In contrast to several flow-calorimeters ist did not operate with countercurrent heat exchangers (ref. 17). The culture medium arrived in the measuring cells with the temperature of the electronically regulated thermostat and left them with a temperature being changed for ΔT. The expensive equilibration and thermostatisation of the air-supply was neccessary to minimize disturbances resulting from the evaporation process inside the measuring cells (ref.13). Nevertheless the aeration of the calorimetric vessels introduced serious noise and instability on account of mechanical work and endothermic gas-expansion.

FIG 1 The complete Calorimeter

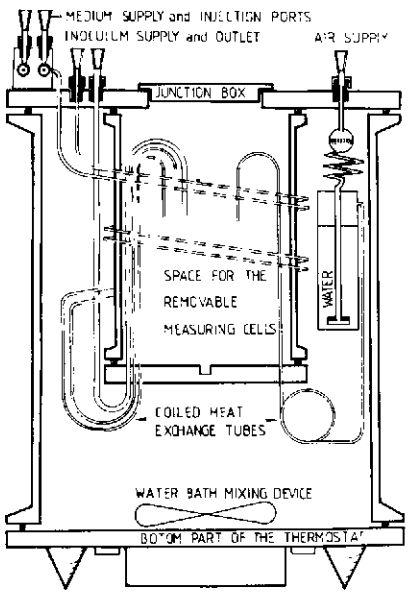


Figure 1: The design of the calorimeter (schematically)

A glas vessel of 50 cm diameter served for the thermostat and contained the neccessary supporting elements of the construction, such as the heat exchangers, gas washing bottles a mixing device, and a large inner space for mounting the measuring cells.
The top nad bottom parts of the calorimeter were made of aluminium. The upper lid supported connectors for the peripheral Instruments and the bottom lid supported the heating elements of the electronically regulated thermostat. The use of glas cylinders allowed a visual inspection of the calorimeter during operation.

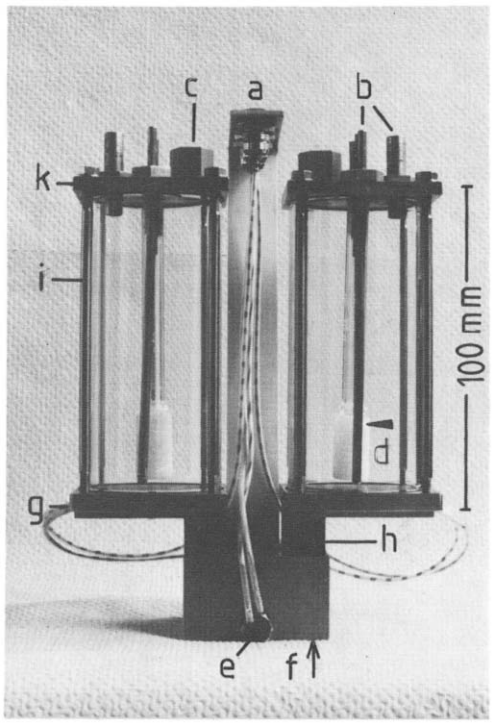


Figure 2: Photograph of the measuring cells of the calorimeter.

a) electrical connectors
b) inlet and outlet for air, medium and culture waste
c) space for mounting a calibrating heater for example a Pt 100 resistor
d) air sparger
e) temperature sensor of the electronically regulated thermostat
f) thermal contact area to the thermostat
g) transistors of the calorimetric measuring bridge mounted in the bottom part of the cells
h) rod-shaped heat-conducting parts of the bottom part of the twin measuring cell configuration (precisely manufactured stainless steel component)
i) mounting screws of the calorimetric vessels.
k) lids of the measuring cells (stainless steel)

Figure 2 shows the measuring twin cells which were made of stainless steel and easily removable from the calorimeter after removing a central mounting screw and the feeding tubes.

The calorimetric measurement of the difference temperature between the two measuring cells worked with a pair of transistors operating like diodes at a constant current source (100µA). The temperature coefficient of this bridge was found to be -2.28 mV / K being constant within a temperature range from 0°C to 60°C.

The heat conductivity between measuring cells and surroundings was analysed to be 0.262 W/K without medium flow and increased for 0.0121 W/K with an increase of the medium flow rate for 1 ml/h. The heat capacity was determined using the Newtonian cooling function to be 998 J/K at 30°C and the time constant of the instrument was 3800 s without medium flow. It decreased with increasing medium flow. The large time constant required a method of deconvolution since growth dynamics of microbes may show very fast responses of the heat flow rate. The Tian equation (1) offers a simple method of transformation and was applied according to equation (2) to reconstruct the heat flow rate using discrete thermogram data (ref. 18).

$$\dot{Q}_{(t)} = K \cdot \Delta T + \frac{d\,\Delta T}{dt} \cdot C_p \qquad (1)$$

$$\dot{Q}_{(t_1 t_2)} = K \cdot \frac{\Delta T_1 + \Delta T_2}{2} + \frac{\Delta T_2 - \Delta T_1}{t_2 - t_1} \cdot C_p \qquad (2)$$

The constant K in these equations means the heat conductivity between the cell and the surrounding and depended on the medium flow rate (see above). C_p of our calorimeter did not depend on the medium flow or aeration rate of our instrument.

Noise and long term stability

Our calorimeter showed a resolution of better than 1 mW short term noise related to an operating volume of 140 ml when equation (2) was used for deconvolution and the time t_2-t_1 was 180 s. The long term stability depended on the stability of the aerationoperating conditions as well as on the medium flow rate and did not exceed 1-2 mW during a chemostat experiment of several days duration (ref. 12).

Operation of the chemostat cultures

The chemostats were brought into operation as it had been published recently (ref. 12) but using Escherichia coli instead of Acinetobacter calcoaceticus. The growth of the cultures was limited by the carbon and energy source of the culture medium. The maximum specific growth rate of E. coli was determined to 0.77 /h using batch cultures and 0.82 /h to 0.84 /h using the wash-out technique on the chemostat. Determinations of μ_{max} during wash-out being based on biomass data resulted in the same values as calculations from heat production rates or substrate consumption.

EXPERIMENTAL RESULTS

Reaction after application of additional substrate amounts

Figure 3 shows the heat flow rate of E. coli growing in the calorimeter when 200 mg Glucose were added to the culture at various dilution rates. The extra substrate was consumed by the culture resulting in a simultaneous increase of the heat flow. The reaction rate significantly decreased with increasing dilution rates and nearly disappeared at D=0.75/h. The monitoring of formic acid, acetic acid and ethanol during these experiments showed neither formic acid nor ethanol but large amounts of acetic acid being formed during the first response. The reaction of the cultures started up with an instantaneous heat production maximum followed by an exponential increase up to the peak maximum. The smaller

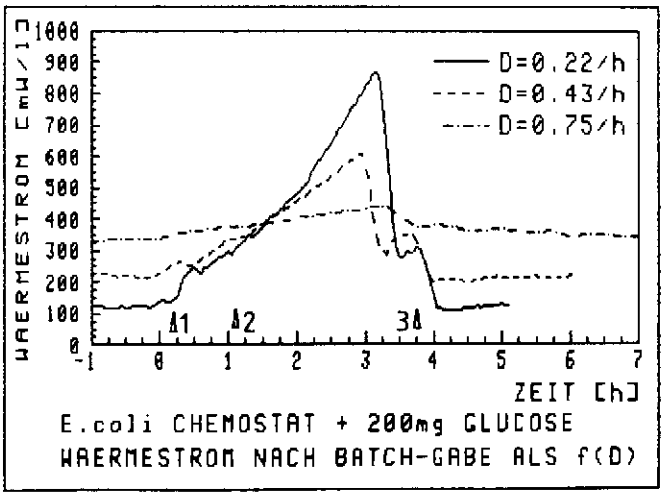


Figure 3: Reaction of the heat production rate of E. coli growing in the calorimeter following the addition of extra substrate at time zero. Arrow 1 indicates an instantaneous enhancement of the heat production, arrow 2 points on the exponential phase and arrow 3 marks the second heat flow peak resulting from acetic acid turnover.

second heat flow maximum resulted from a rapid turnover of acetic acid and began after the exhaustion of the excess glucose to concentrations near zero.

Reactions after applications of toxic chemicals

Figure 4 shows the heat flow from E. coli cultures when 2-nitrophenol was added. With increasing initial concentrations of the toxic chemical the chemostats showed increasing inhibitory effects regarding the heat production. The lowest concentration which resulted in inhibitory effects decreased with increasing dilution rates. For example doses of 22 mg 2-nitrophenol/l resulted in small inhibitory effects at a dilution rate of 0.75/h but at D=0.158 ist resulted in a significant increase of the heat flow.

The available increase of the heat flow after intoxications was much larger at low dilution rates than at higher ones.

Figure 5 shows the energy-conversion pattern of E. coli growing at a dilution rate of 0.428/h. 2-Nitrophenol did not reduce the turnover of glucose but decreased the heat flow drastically and induced the formation of acetic acid but no synthesis of ethanol or formic acid. On the whole the diagram shows serious deviations of the energy bilance from completeness.

Figure 6 shows a comparison of the energy conversion when pentachlorophenol was added to E. coli cultures at various dilution rates. The heat of combustion of the glucose being consumed was larger than the output of catabolic heat and

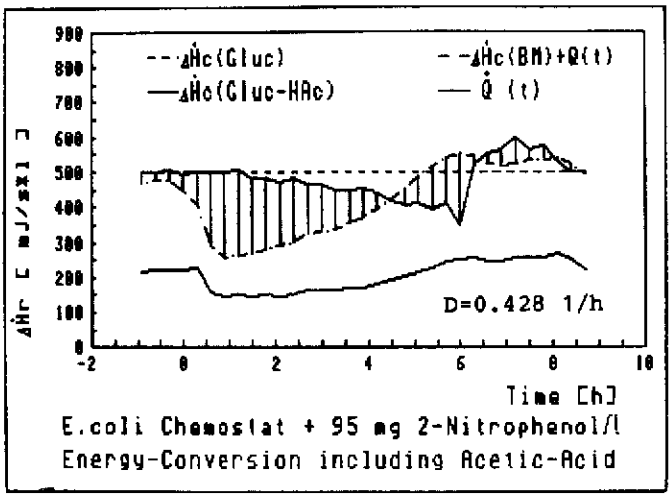


Figure 5: Energy bilance of E. coli after batch application of nitrophenol. The hatched area indicates deficites of the energy balance that cannot be accounted for by the monitoring data.

the heat of combustion of the biomass being formed. Differences between the input and output may be due to the formation of intermediary substances or bacterial slimes. The smallest sensitivity to toxic effects regarding pentachlorophenol was found with the culture growing at half maximum speed. The slowest recovery from disturbances showed the data at D=0.75 and a transient biomass productivity of zero was found in the culture growing at D=0.158.

DISCUSSION OF THE EXPERIMENTAL RESULTS

Dynamics of a microbial population regarding changes of environmental factors for example the addition of energy substrate or toxic substances cannot be predicted sufficiently using the MONOD equation (3).

$$\mu = \mu_{max} \cdot S / (k_S + S) \qquad (3)$$

The equation gives a description of microbial growth rate assuming steady-state (ref.8). Daigger and Grady (ref.19) deal with this subject extensively in a review and describe the phenomenon of dynamic responses of microbial populations with terms like 'ARP' (available reaction potential) and 'GRH' (growth rate hysteresis).

In our experiments the reaction of the cultures on batch doses of glucose demonstrated clearly several phenomena of transient state dynamics:

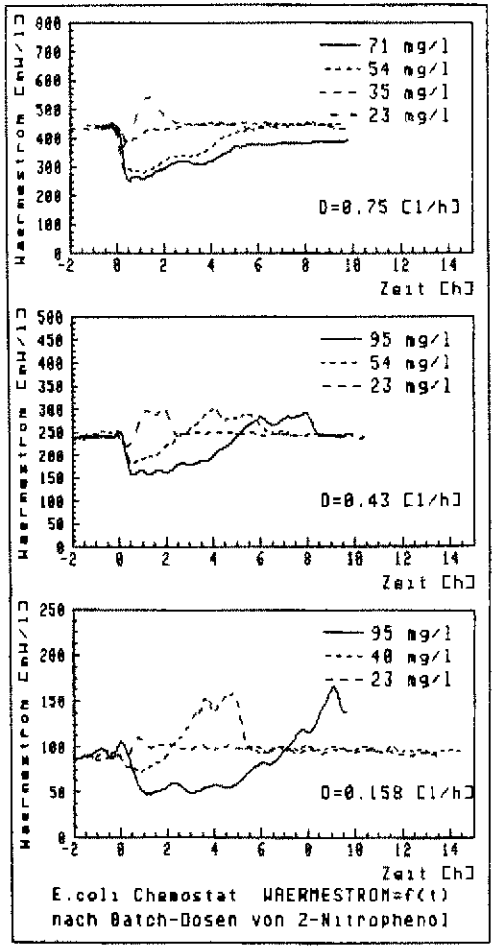
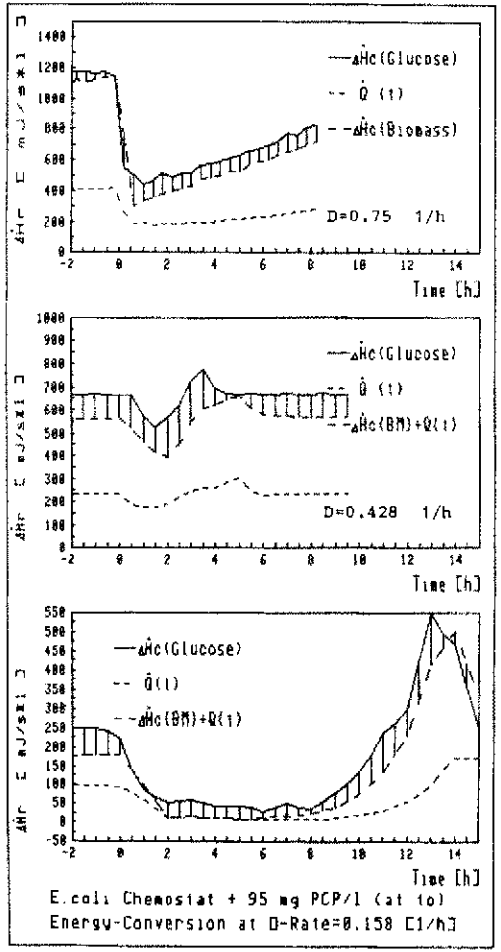


Figure 4 (left side): Response of the heat production rate of E.coli growing in a Chemostat-culture on batch doses of 2-nitrophenol. The reaction has been tested at three different dilution rates using various concentrations of the chemical.

The uncoupling effect of 2-nitrophenol on oxidative phosphorylation lead to high increasing rates of the heat production at low D-Rates. At high D-rates the heat production became decreased.

Figure 6 (right side): Energy-conversion by E. coli growing in chemostat cultures with various dilution rates. The Output of heat and chemical energy being bound in the biomass was lower than the combustion enthalpy of the glucose being consumed at the sam time.

A complete bilance of the mass- and energy turnover seemed to require the analysis of several products of the microbial metabolism.



i) The catabolic activity rose up instantly after the addition of substrate.
ii) Slowly the catabolic activity began to increase progressively until the glucose was eshausted.
iii) Transiently released acetic acid then became energy source for further catabolic activity after a short delay time.
iv) The culture reached steady state conditions again, when no acetic acid or glucose were present at important concentrations.

This cycle was found to be faster at low dilution rates than at high ones. Comparable variations of the reaction kinetics regarding toxic effects of chemicals were found. The transient formation of acetic acid clearly pointed out to non steady state conditions during the reaction cycle.

A simple modellisation of cellular energy transfer

An understanding of our observations on chemostat energetics during transient states should be discusses considering the circumstances that calorimetry only records catabolic reaction enthalpies (ref.20) in our experimental set up. Effects of chemicals acting on anabolic pathways may be monitored since anabolism and catabolism form a biochemical network inside the bacterial cell acting in combination.

Figure 7 shows a simple scheme of this network with particular compliance to catabolic processes and energy flows.

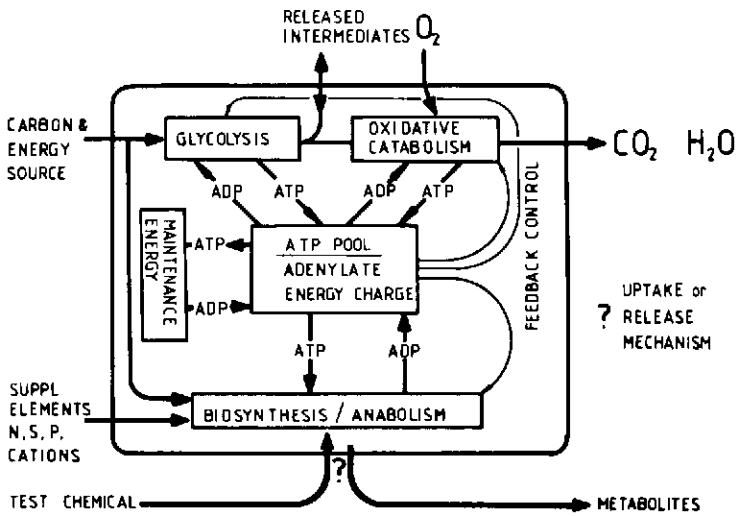


Figure 7: Scheme of the biochemical network of a bacterial cell. Regarding mass and energy flows the adenylate energy charge seemes to play a central part related to regulation and dynamic responses of catabolism.

The adenylate energy charge controles catabolic activities by feedback regulation and serves vor a unique form of biologically available chemically bound energy (ref.21). Toxic chemicals may affect the adenylate energy charge leading to a regulatory process of adaptation. Especially uncoupling agents which inhibit the formation of ATP from a proton-motive-force across the cell membrane affect the energy charge of aerobically growing organisms. This leads to a loss of feedback-control of the glycolysis. The chemical 2-nitrophenol being a typical uncoupling agent clearly showed the effect of increasing catabolic turnover rates in our experiments. It shiftet the mass and energy bilance towards higher catabolic activity and lower anabolic yields. The effect of uncouplers became more obvious with low dilution rates and we believe, that the maximum available glycolytic activity is determined by its value at the maximum specific growth rate.

Energy balances and assessment of toxicity

Calorimetry being a non specific method to quantify the enthalpy changes during metabolism allowed to monitor variations of metabolic activities without any specificy. In fact it is an andvantage if toxic effects have to be analysed regarding over-all reactions of microbial populations, as it has been allready shown in this paper but it is a disadvantage if details of the mode of action of a particular chemical have to be analyzed.

Moreover the catabolic heat production is only one aspect of the energy flow of growing microbial populations, another aspect is the anabolic energy conversion from low molecular weight substrates to the assembly of macromolecules which build up the living cell. Investigations on energy-conversion-stoichiometries requires analytical procedures to monitor metabolites, substrates and biomass. Such a complete assessment of microbial growth behavior during transient states becomes a complicated task as it has been shown with the example of 2-nitrophenol acting on E. coli cultures.

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