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An isothermal microcalorimetric titration/perfusion vessel equipped with electrodes and spectrophotometer

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Abstract

A new microcalorimetric titration/perfusion vessel has been developed with special reference to needs in thermodynamic and analytical investigations of complex processes. The vessel, which is part of a twin heat conduction microcalorimeter, is equipped with electrodes for determination of pH and oxygen concentration and with light guides forming part of a diod array spectrophotometer. The dynamic properties of the instrument has been investigated. The calorimeter and its analytical sensors has been tested in titration experiments and in bacterial growth experiments. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Microcalorimetry; pH electrode; Oxygen electrode; Spectrophotometer; Bacterial growth

1. Introduction

The term 'isothermal microcalorimeter' is commonly used for calorimeters designed for work in the microwatt range under essentially isothermal conditions. The design of isothermal microcalorimeters and of experimental procedures for such instruments have been much improved during the past decades [1]. Current important application areas include, for example, ligand binding studies using titration techniques, estimation of stabilities for compounds and products of technical importance and in the monitoring of living cellular processes.

Practically all processes give rise to heat effects. Calorimetry is therefore a very powerful technique for the detection and quantification of unknown or unexpected events being parts of complex systems. However, the non-specific nature of heat measurements will often make it difficult to interpret results from calorimetric investigations of complex reaction systems. It is therefore often necessary to support such measurements by specific measurements, preferably conducted in parallel, on the same reaction system. The calorimetric results will give an overall characterization of the process in terms of thermal power as a function of time, whereas the specific analytical results, where possible combined with known molar enthalpy data, will help to define the process and will facilitate a thermodynamic and kinetic discussion on a molecular level.

In flow or perfusion experiments it is often possible to connect analytical sensors on-line with the calorimetric reaction vessel, cf. [2,3]. Similarly, gaseous or liquid samples may be taken out from the flow-line of the calorimetric system, before or after the reaction vessel [3], or a gas may be allowed to diffuse from a calorimetric reaction vessel to an analytical instrument positioned outside the calorimeter [4].

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It is specially attractive to position the analytical sensor(s) in the sample compartment of the calorimetric reaction vessel. We have earlier reported experiments where oxygen and pH electrodes were placed in the reaction vessel of a microcalorimeter used for measurements of living cells [5]. The electrodes could be used without causing any significant disturbances of calorimetric experiments conducted on the microwatt level. Schaarschmidt and Lamprecht have reported the measurement of light absorption carried out in microcalorimetric vessels in order to monitor the concentration of growing yeast cells [6] and an oscillating chemical reaction [7], respectively.

In this report we describe the design and testing of a microcalorimetric titration/perfusion vessel equipped with pH and oxygen electrodes and with quartz light guides, forming part of a diod array spectrophotometer.

The dynamic properties of the instrument as reflected by its time constants under different experimental conditions, has been investigated. The simultaneous performance of the calorimeter, the electrodes and the spectrophotometer has been tested in titration experiments involving a neutralization reaction and in bacterial growth experiments.

2. Experimental

2.1. Instrument

The design of the calorimetric assembly is shown schematically in Fig. 1. The calorimeter is a twin thermopile heat conduction calorimeter which is inserted into a thermostated water bath, about $\pm 5 \times 10^{-4}$ K. The twin calorimeter and the reaction vessel is of the same type as reported earlier from this laboratory, see e.g. [5,8,9].

The reaction vessel is of the insertion type, meaning that it is normally taken out for inspection after each experiment and is cleaned and charged outside the calorimeter. Above the sample container (n) are two brass bolts (f) which are in thermal contact with the surrounding aluminium block (l) serving as the heat sink. An upper brass bolt (e) is in thermal contact with the thermostated water bath. The sample container was made from acid proof steel and has a volume of about 20 ml and an outer diameter of 27.5 mm (which makes the vessel compatible with the TAM microcalorimetric system (Thermometric, Järfälla, Sweden). Miniaturized electrodes for measurements of oxygen concentration (g) and pH (h), light guides (j, k), an injection device (s, t) and a steel tube which surrounds the stirrer shaft (q), are carried through the lid of the sample container and are sealed by O-rings. The reference vessel (p) has the same design as the sample container of the reaction vessel (n), but lacks stirrer, analytical sensors and injection device. The sample container and the reference vessel are each surrounded by five thermocouple plates (i), connected in series (CP 1, 4-71-06L; Melchor, Trenton, NJ, USA).

The pH electrode, (h), is of the 'solid state type' and was obtained from Lazar Research Laboratories, Los Angeles, CA. It is fitted with a 0.5 m salt bridge enclosed by a teflon tube, connecting it with the reference electrode (r). The oxygen electrode (g), a Clark cell, was manufactured in our laboratory and has been described earlier [5]. The light guides (j, k) are single fibres made from quartz and were obtained from Thor Laboratories (Newton, NJ, USA). Light is passed from a deuterium–halogen light source, (a), DH-2000, manufactured by TOP Sensor Systems (Eerbeck, The Netherlands) into the calorimetric vessel by means of the light guide (j), diameter 0.6 mm. Light transmitted through the medium in the sample container is collected by the second light guide, (k), diameter 1 mm, and is passed to the spectrophotometer (c) consisting of a UV–VIS monochromator and a diod array detector, S 2000. Both units were obtained from Ocean Optics (Dunedin, FL, USA). The grating of the monochromator has 600 lines/mm and the diod array detector has 2048 elements.

The length of the gap between the light guides can be adjusted by means of a stainless steel holder (not indicated in Fig. 1). The stirrer (o) is of is the 'turbine' type [5], and is positioned unsymmetically in the vessel. Liquid passes into the upper and lower ends of the turbine cylinder and flows out through the holes in the mid part of the cylinder. Two small propellers were placed above and below the cylinder in order to improve the turbulence around the electrodes and near the bottom of the vessel. The turbine was made from PEEK and the propellers were made from 18 carat gold.

Medium can be perfused through the vessel as described earlier [5,10]. During a perfusion experiment medium is introduced into the sample compartment through the stirrer shaft which is made from a

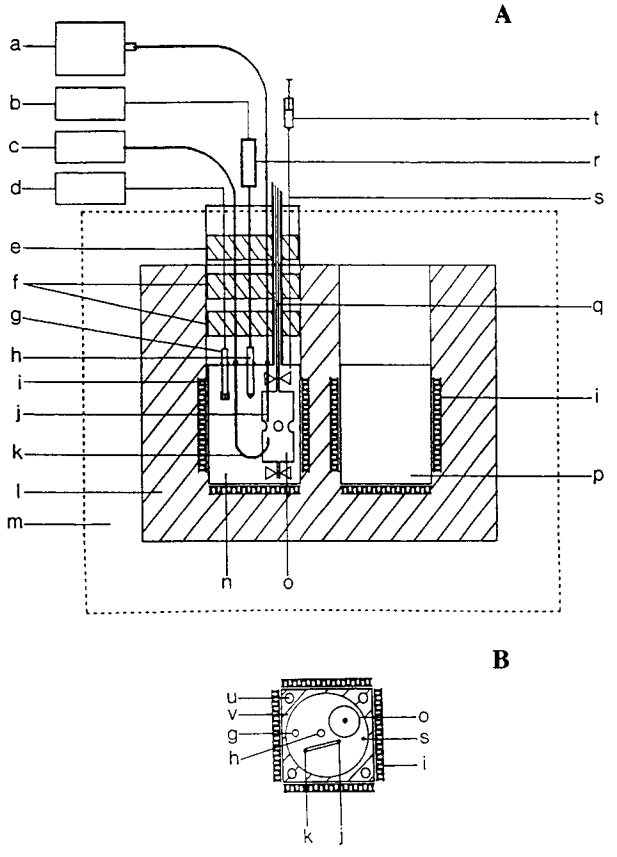

Fig. 1. (A) Schematic picture of the calorimetric assembly. (B) Horizontal section through the sample container. (a) Deuterium–halogen light source; (b) pH meter; (c) monochromator and diod array detector; (d) electronic unit of the polarografic oxygen sensor; (e) brass bolt; (f) brass bolts; (g) oxygen electrode; (h) pH electrode; (i) thermocouple plate; (j) light guide; (k) light guide; (l) heat sink; (m) thermostated water bath; (n) sample container; (o) 'turbine' stirrer; (p) reference vessel; (q) steel tube; (r) reference electrode; (s) injection needle; (t) Hamilton syringe and syringe drive; (u) hole with permanently installed calibration heater; (v) squared aluminium can.

thin-walled acid proof steel tube, inner diameter 1 mm. The medium will leave the vessel through the annular space between the stirrer shaft and the steel tube (q). With aqueous solutions a peristaltic pump is normally used.

Reagents or living cells are injected into the sample container by use of a titration device (s, t), similar to those we have used in vessels described earlier, cf. [5,8,9]. A Hamilton syringe fitted with a stainless steel injection needle (length 1 m, inner diameter 0.15 mm), is operated by use of a computer controlled syringe drive. A steel tube, inner diameter 0.5 mm, serves as a guide for the injection needle. The tip of the injection needle ends at the level of the upper propeller of the stirrer.

The instrument is normally calibrated by use of an electrical heater inserted into the sample compartment of the reaction vessel (not shown in Fig. 1). The heater, made from lacquered 0.15 mm manganine wire, was wound to form a horizontal ring, diameter about 10 mm. The ring is positioned around the stirrer shaft, between the turbine cylinder and the upper gold propeller. Lacquered copper leads, diameter 0.15 mm, are in direct contact with the medium before leaving the sample compartment. The insertion calibration heater was normally not inserted into the sample compartment during chemical or biological experiments. Four permanently installed resistors connected in series (u, Fig. 1B) were also tested as a calibration heater. They were positioned in holes

drilled in the corners of a squared aluminium can (v), serving as a receiver for the vessel.

2.2. Test experiments

Unless otherwise is noted, test experiments were conducted with reaction and reference vessels charged with 20 ml of water. The reaction vessel was stirred at 120 rpm. Uncertainties are given as ±SD. The instrument was normally calibrated electrically using the insertion heater. Calibration experiments were also conducted by use of the permanently installed electrical heater and by dilution of 1-propan-1-ol [11].

In order to employ thermopile heat conduction calorimeters in kinetic work and in 'fast' titration experiments [12] it is necessary to know the dynamic properties of the instrument. For a calorimetric vessel where the content has a high thermal conductivity, for example a well stirred liquid, the dynamic properties can normally be expressed accurately by the Tian equation

$$P = \varepsilon\left(U + \tau\frac{\mathrm{d}U}{\mathrm{d}t}\right), \tag{1}$$

where P is the thermal power, ϵ the calibration constant, U the thermopile potential, τ the time constant of the instrument and t is the time. Values for the time constant are usually derived from the shape of the cooling curve following switching off an electrical heating current [12,13].

In cases where the sample has a low thermal conductance, for example many solid samples and non-stirred liquids, significant temperature gradients may arise. It can then be necessary to use Tian type of equations extended to include more than one time constant, Eq. (2) [12,13].

$$P = \varepsilon\left[U + (\tau_1 + \tau_2)\frac{\mathrm{d}U}{\mathrm{d}t} + \tau_1\tau_2\frac{\mathrm{d}^2U}{\mathrm{d}t^2}\right]. \tag{2}$$

The dynamic properties of the instrument has been investigated by several series of electrical calibration experiments using the insertion heater, with or without efficient stirring of the liquid sample.

In a series of titration experiments 20 ml of 0.1 M 'tris' solution (tris-(hydroxymethyl)-methylamine, 99.9%, Aristar, BDH) was titrated stepwise with 0.1 nitric acid solution (Titrisol, Merck, Darmstadt, Germany). Hydrochloride acid solution is normally used in this test reaction [10] but here nitric acid solution was chosen as it is less corrosive towards stainless steel. Carbon dioxide free water was used and care was taken to avoid contamination of the tris solution with carbon dioxide during its preparation and when the calorimetric vessel was charged. A small amount of *m*-cresol red (Merck) was added to the tris solution in order to make it possible to monitor the experiment by the spectrophotometer. The gap between the light guides (j, k) was adjusted to 3 mm. The change in pH was followed by means of the pH electrode. About 12 μl of acid solution was injected in each step. The experiments were conducted at 25.00°C and 37.00°C.

Bacterial growth experiments were conducted with a strain of *E. coli* (C 600) in a medium prepared from 2 g glucose, 10 g trypton, 5 g yeast extract, 10 g NaCl and 1000 g distilled water. Cells and medium were kindly provided by Division of Applied Microbiology, Chemical Center (B. Hahn-Hägerdal). In the growth experiments reported here, the sample container of the calorimetric reaction vessel was completely filled with medium (i.e. there was no gasphase in the vessel). The sample was stirred but no perfusion of medium took place. The experimental temperature was 25.00°C and the gap between the light guides was adjusted to 10 mm. Growth experiments were initiated by injection of about 10^4 cells which were suspended in 0.3 ml of growth medium. The cells were in a phase of exponential growth.

3. Results and discussion

3.1. Baselines, dynamic properties and calibration experiments

Several long term baseline experiments were conducted. Typically, the deviation from a mean value was less then ± 0.7 μW during a 24 h period. This value is significantly larger than those we normally have observed for similar twin microcalorimeters [8]. However, it may be noted that the present instrument is quite far from an ideal twin system as the reference vessel lacks stirrer and the analytical devices.

No interference with the calorimetric measurements could be seen from the use of the electrodes. However, during spectrophometric measurements there can be a significant release of thermal power due to light which

is not collected by the receiving light guide (k). Typical values observed as baseline displacements, which can be corrected for, were in the range 1–10 μW.

The design of the present instrument was governed by the need to combine heat measurements with specific analytical determinations. As a consequence, the reaction vessel become quite non-ideal (from a calorimetric point of view) as the analytical sensors, the stirrer shaft and mechanical support form significant thermal conductors bypassing the thermopile. This may possibly lead to systematic errors in electrical calibration experiments and it is therefore important to compare results from different calibration methods. Calibration constants were determined at 25.00°C and the vessels charged with 20 ml of water. Mean values obtained with the permanently installed calibration heater and by chemical calibration using dilution of 1-propan-1-ol [11] were both 0.2% higher than value determined by use of the insertion heater. The difference is not significant. If the water in the sample container was not stirred the calibration constant, determined with the insertion heater, increased by 1.4%.

When the vessels were charged with 20 ml of water and the reaction vessel was efficiently stirred, (120 rpm) the time constant was found to be $\tau = 268 \pm 2$ s. Fig. 2 shows results of an experiment where electrical energy (0.133 J) was released during 5 s, using the insertion calibration heater. The continuous curve shows the experimental calorimetric record, i.e. values proportional to the thermopile potential (ϵU) versus time, following switching off the electrical current. The dashed curve, calculated from Eq. (3), cf. [12,13], shows the calculated heat flow, $\phi_{\text{calc.}}$, from the vessel to the heat sink versus time (taking the time constant into account),

$$\phi_{\text{calc.}} = U_0 e^{-t/\tau}, \quad (3)$$

U_0 is the differential thermopile potential at the time when the electrical power was switched off. It is seen that the two curves will overlap about 2 min after the switch-off time.

Fig. 3 shows results from the same type of experiment as shown in Fig. 2, except that the liquid was not stirred. From Fig. 3A, showing curves corresponding to those in Fig. 2, it is the seen that a significant difference remains between the two curves until about 6 min after the switch-off time. In Fig. 3B the cooling curve was derived using two time constants, Eq. (3) [13]. In that case recorded and corrected curves were in agreement after about 2 min following the switch-off time.

3.2. Acid base titration experiments

Tris solutions containing small amount of *m*-cresol red were titrated with nitric acid solution at 25.00°C

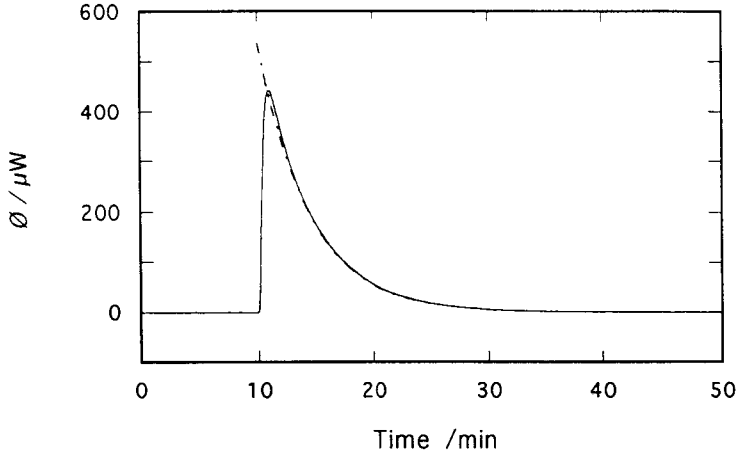

Fig. 2. Heat flow curves following the release of a pulse of electrical energy using the insertion heater. The water in the sample compartment was efficiently stirred. The continous curve shows the experimental calorimetric record. The dashed curve is dynamically corrected using one time constant, cf. the text.

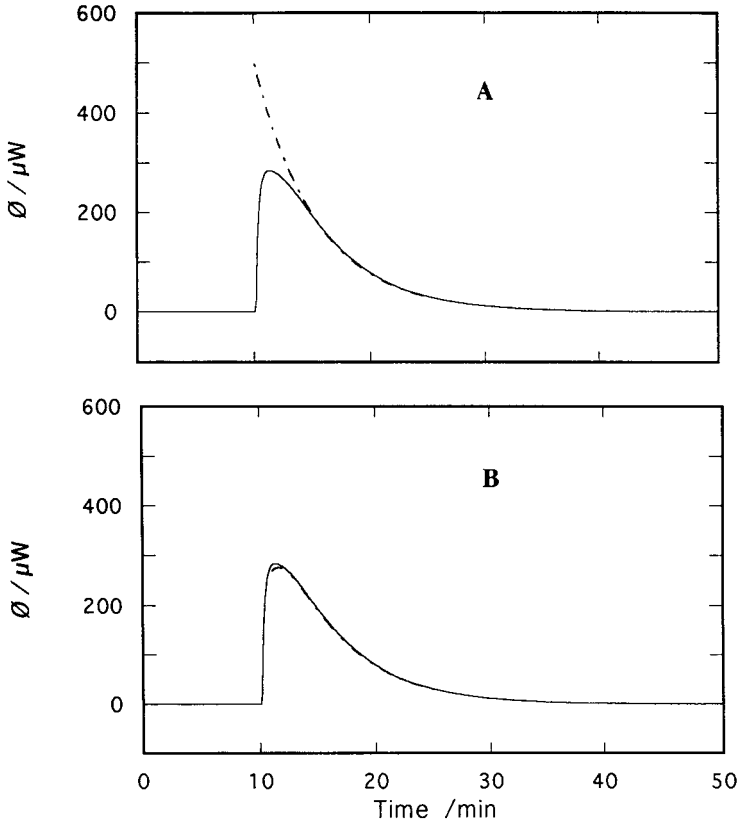

Fig. 3. Heat flow curves following the release of electrical energy using the insertion heater. The water in the sample compartment was not stirred. (A) Results corresponding to those given in Fig. 2. The dashed curve in (B) was derived using two time constants, Eq. (2).

and 37.00°C and the processes were monitored simultaneously by the calorimeter, the pH meter and the spectrophotometer. Typical results from measurements conducted at 37°C are illustrated in Fig. 4A–D. The calorimetric titration curve is shown in Fig. 4A. As expected, the heat quantities for each titration step are essentially constant until pH decreased to about 7.1 (pK_a for tris at 37°C is 7.73 [14,15]). The value derived for the enthalpy of protonation of tris at 37°C is $\Delta H = -46.97 \pm 0.15$ kJ/mol (mean value for five series; corrections were applied for dilution of the acid). The value is in fair agreement with van't Hoff values reported in the literature [14,15], $\Delta H = -46.74 \pm 0.10$ kJ/mol (the uncertainty is an estimate). The value determined at 25°C was -47.50 ± 0.29 kJ/mol (mean value for six series) in agreement with out value determined using an accurate macro calorimeter, $\Delta H = -47.48 \pm 0.03$ kJ/mol [16].

The spectrophotometric record in Fig. 4A shows the change in optical density at 572 nm for the added indicator substance. It is seen that the optical density value will decrease also between the fast stepwise changes caused by the change in pH. As the spectrophotometer is a single beam instrument we interprete this effect as due to a gradual decrease in the intensity of the light beam.

In measurements of a chemical reaction system in solution it is usually possible to find a wavelength in the UV–VIS spectrum where the absorption is zero for the measured spieces. Further, one may assume that the ratio of the light intensity between different wavelengths is constant. Some part(s) of the measured spectrum, where the absorption of the reaction system does not change with the progress of the reaction and where the light absorption of the solvent is zero, can then be used to determine the change in light intensity

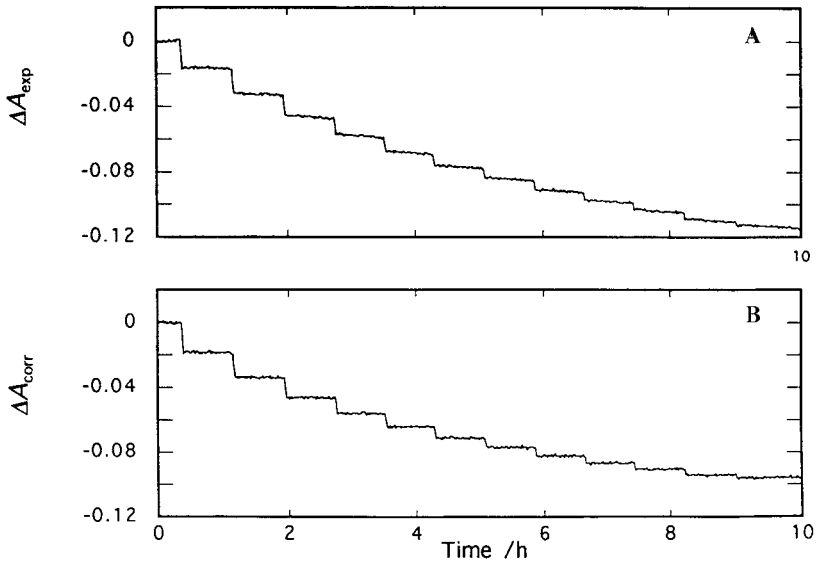

Fig. 4. Typical results from a stepwise 'slow' titration of an aqueous solution of tris with nitric acid solution (indicator added). (A) Recorded optical density–time curve. (B) Optical density–time curve corrected for drift in the light intensity, cf. the text.

with time [17]. Fig. 4B shows the spectrophotometric results corrected according to this method.

The present titration experiments, intended to demonstrate the use of the calorimeter in combination with the pH electrode and the spectrophotometer, can be looked upon as models for ligand binding experiments. Such measurements may lead to the simultaneous determination of the enthalpy change and the equlibrium constant, and thus the entropy change, for the binding process [1]. Many ligand binding processes, for example binding reactions involving proteins, are accompanied by proton release and it can therefore be important to monitor calorimetric titrations of such systems by pH determinations. Sometimes ligand binding processes can be followed by spectrophotometry, in which case the present type of instrument can be used for simultaneous, but independent, determination of the equilibrium constant(s). In particular for processes more complex than 1 : 1 binding reactions, that technique could lead to more safe thermodynamic determinations than microcalorimetric titrations alone [1].

The titration series with 12 injections reported here required 10 h to perform, Fig. 4. It should be noted, however, that stepwise titrations can be conducted in a much shorter time, even with a heat conduction calorimeter with relatively large time constant (as in the present case), provided that the investigated process is fast. Using a 'fast' titration technique [12] injections can be made before the baseline has been reached following a previous injection. Both the pH and the spectrophotometric measurements are fast and may directly be combined with such 'fast' calorimetric titrations. Further, for single beam spectrophotometers the effect of possible drift in the light intensity will be much reduced. Typical results from a 'fast' titration of tris solution with nitric acid solution at 37°C is shown in Fig. 5. The lower curve with rounded peaks is the experimental record. The curve with sharp peaks shows the corrected titration curve. Results were within uncertainty limits the same as those obtained with the 'slow' technique.

3.3. Microbial growth experiments

Four series of growth experiments with *E. coli* were conducted with consistent results. After an equilibration period of about 1 h the measurements started by observing the baselines during a short time, for the calorimeter, the electrodes and the spectrophotometer. The growth experiments were initiated by injection of cell suspension into the sample container which was completely filled with medium. The experiments were interrupted after about 6 h. Records from one of the

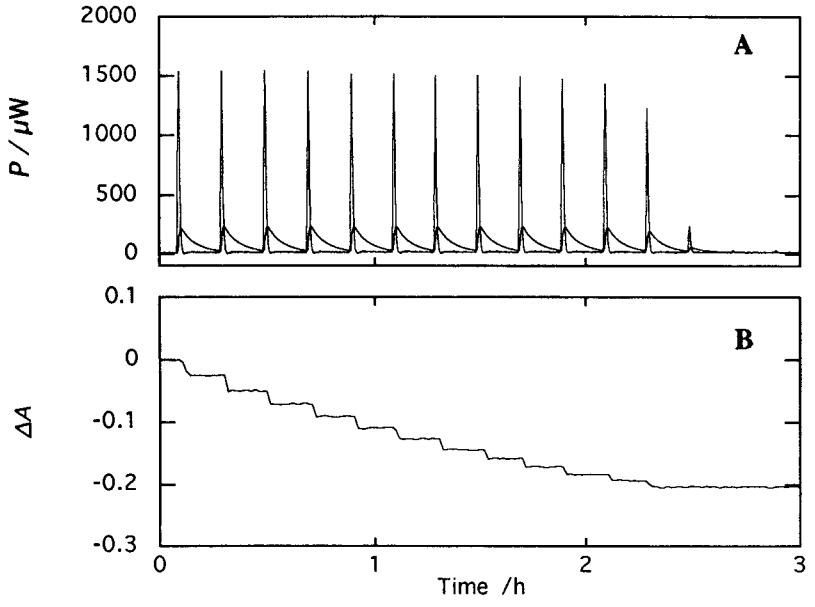

Fig. 5. Typical results from titrated of tris solution with nitric acid solution (indicator added) using the 'fast' titration technique. Fig. 5A shows results of the calorimetric measurements and in Fig. 5B corresponding spectrophotometric record is shown.

experiments are shown in Fig. 6. The thermal power–time curve, A, was dynamically corrected using Eq. (1). Following the initial disturbance caused by the injection of the cell suspension, the curve shows two distinct peaks during the experimental period. For the four experiments the time period between these peaks varied up to 0.5 h. As seen from curve B, the oxygen concentration decreases exponentially with time and reaches zero at the same time as curve A starts to decline after its first peak. The oxygen concentration of the injected cell suspension was low, which caused the initial small drop in curve B. After the oxygen concentration had reached the zero level, a small increase, presumably due to a drift in the electrode baseline value, was observed. The pH–time curve, C, increases slowly during a period of about 2.5 h after which a rapid increase takes place which ends at the time when the oxygen level reached zero. Thereafter, the pH decreases slowly during the rest of the experiment. The shape of the pH curves recorded in the different experiments and the final pH values varied somewhat. However, in all experiments a fast increase in pH was observed immediately before the oxygen level reached zero.

Curve D in Fig. 6 shows the apparent optical density (or turbidity), A′, versus time measured at the wavelength band 650–670 nm. The A′ value for a bacterial suspension is largely a measure of the light scattered by the bacteria and is often taken as a measure for the cell concentration [18]. In all experiments the turbidity curves increased until a breakpoint was reached when the growth conditions changed to anaerobic.

The mean value derived for the heat evolution during the aerobic phase was $\Delta H = -442 \pm 15$ kJ/mol of oxygen. This value approaches that for oxidation of glucose in aqueous solution to carbon dioxide and water (no buffer present), $\Delta H = -487$ kJ/mol [19].

The thermal power–time curve decreases rapidly when all oxygen was been consumed, but increased again and a second peak is seen after about 1.5 h under the anaerobic condition. Following a small decline the calorimetric curve increases once more before the experiment was terminated. Profiled calorimetric curves like curve A are typical for bacteria growing in rich media, see e.g. [20], and are believed to reflect different metabolic phases caused by the ongoing change in the composition of the growth medium.

The aerobic part of the increase and the decrease of curves A and B, respectively, are logarithmic. Corresponding part of curve D also appears to be logarithmic, except for the first hour where the turbidity is

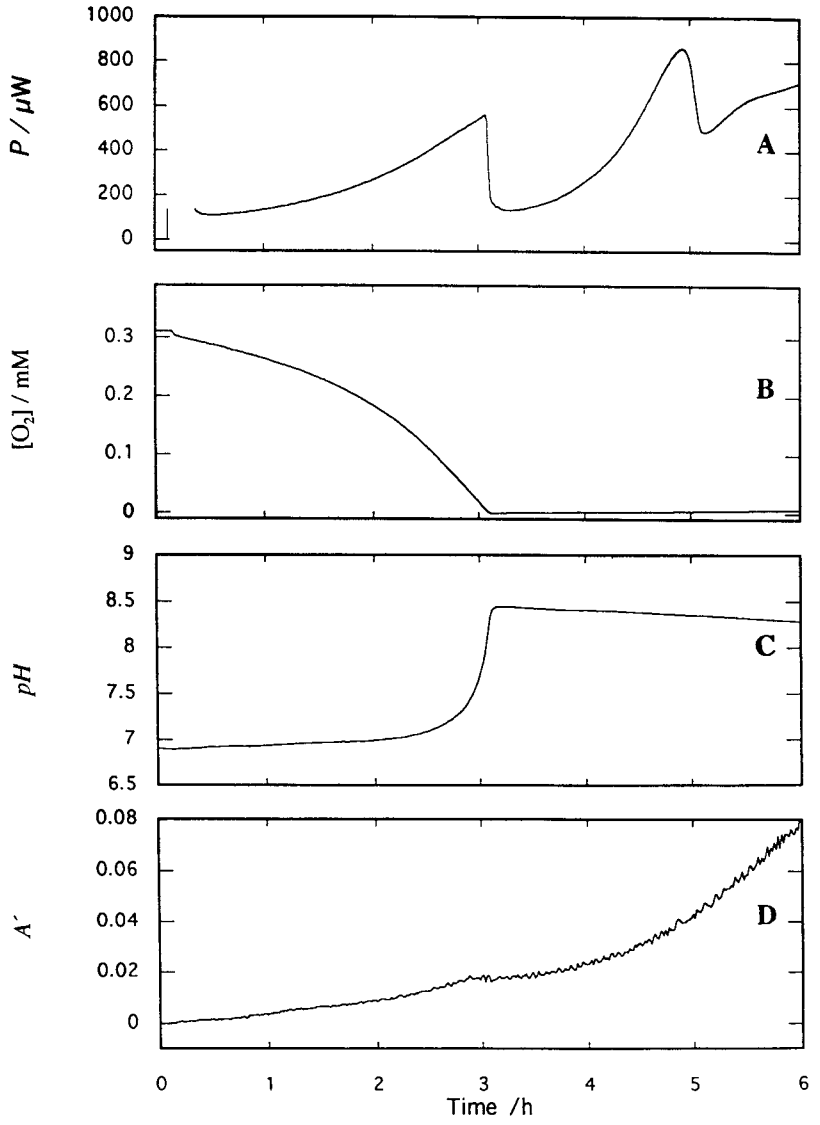

Fig. 6. Records from a growth experiment with *E. coli*. The sample compartment was completely filled with medium. A, thermal power–time curve. The experimental curve was dynamicaly corrected using Eq. (1). (B) oxygen concentration versus time. (C) pH–time curve. (D) Apparent optical density (turbidity) versus time.

very low and the values are uncertain. From the shape of the aerobic parts of curves A, B and D the generation time for the bacteria can be calculated. Identical values were obtained from the calorimetric and the spectrophotometric curves, 1.3 ± 0.2 h, whereas the rate of oxygen consumption led to a slightly shorter value, 0.8 ± 0.1 h. From the anaerobic part of the turbidity curve the value for the generation time was derived to be 1.2 ± 0.2 h.

It is interesting to note that the second peak in curve A (at time 5 h), is not significantly reflected in the turbidity curve D. We may thus conclude that the anaerobic part of the calorimetric curve, in addition to a growth process, accounts for one or more processes

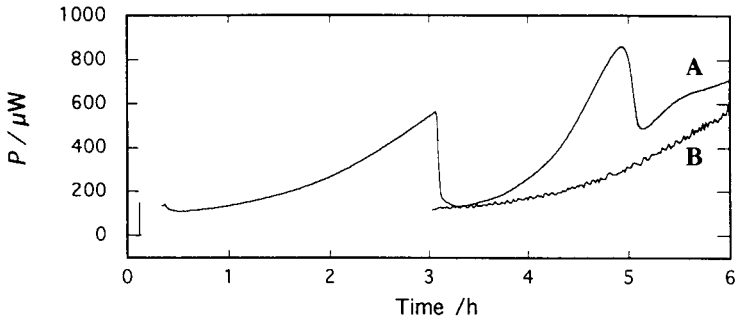

Fig. 7. Curve A is the same as curve A in Fig. 5. Curve B represents the maximum thermal power for the process causing the increase in the turbidity during the anaerobic phase (cf. the text).

which do not lead to any significant production of biomass.

We may tentatively assume that the thermal power for this 'non-productive' component in the anaerobic phase of curve A is zero at its first minimum (at about 3.3 h). A thermal power–time curve proportional to the turbidity curve may then be constructed assuming a generation time of 1.2 h. That hypothetical curve is shown in Fig. 7, together with the experimental calorimetric curve (A). The hypothetical curve (Fig. 7B) will represent the maximum rate of heat production for the process causing the increase of the anaerobic phase of the turbidity curve. The area between the curves will thus represent the enthalpy change from 'non-productive' process(es). The 'non-productive' part of the experimental thermal power–time curve, may not be zero at the first minimum of curve A, in which case that fraction of the released heat will be larger. The shape of any possible 'non-productive' thermal power–time curve, based on the shape of the turbidity curve, suggests that more than one 'non-productive' process is involved.

4. Conclusions

It has been demonstrated that the microcalorimetric vessel presented in this work can be useful for combined thermodynamic and specific analytical measurements. Further test experiments, with the emphasis on kinetic measurements, will be reported elsewhere [17].

As shown by the test experiments the calorimeter is, despite its non-ideal design, useful for accurate thermodynamic measurements and for long term monitoring of complex processes on the μW level. It is judged that the baseline stability, the limit of detection and thus the precision of the instrument would be significantly improved if the twin vessels were given a more identical design.

Results from the microbial experiments show the importance of combining specific and unspecific measurements in experiments conducted simultaneously on the same reaction system. Clearly, for processes as complex as a growth process, it would be desirable to equip the calorimetric vessel with additional specific sensors. In the present case, sensors for measurement of glucose, carbon dioxide and various simple metabolites formed and consumed during the course of the experiment would be particularly useful. A wide range of such sensors are now available and their performance in microcalorimetric vessels should be tested. With the modular design of the present vessel it would be possible to include several more sensors in the sample compartment.

The instrument can be used in experiments where medium is perfused through the reaction vessel and analytical instruments can then be connected on-line, see e.g. [3]. Cells can usually not be kept in a stirred suspension during perfusion experiments as they may partially be lost with the flow. However, many types of cells can be attached to microcarriers or to a solid support [21]. If measurements are conducted at a low sensitivity, as in the present microbial experiments, it is possible to suck out samples for analysis using the injection syringe, without disturbing the calorimetric measurement. Another general technique by which samples can be extracted for analysis from a micro-

calorimeter would be to install a microdialysis tube in the sample compartment, [22].

In conclusion, we believe that further developments of techniques where isothermal microcalorimetry and specific analytical methods are combined, are promising and should be given further attention.

Acknowledgements

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