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MICROCALORIMETRIC METHODS FOR SUBSTRATE DETERMINATION IN FLOW SYSTEMS WITH IMMOBILIZED ENZYMES

H.-L. SCHMIDT, G. KRISAM * and G. GRENNER

Lehrstuhl für Allgemeine Chemie und Biochemie, Technische Universität München, D 8050 Freising-Weihenstephan (G.F.R.)

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Summary

The enthalpy of processes catalyzed by immobilized enzymes in the reaction cell of a LKB-flow calorimeter is used for determination of urea (0.5—5 μmol) and glucose (0.03—0.5 μmol). Accuracy is 2—5% and the time needed for one analysis is 20 min.

A sensitive "enzyme thermistor" consisting of a flow through cell with an immobilized enzyme and two thermistors is described, which permits glucose determinations (0.05—1 μmol ± 0.03 μmol) by means of temperature difference caused by reaction heat.

Coupling of enzyme reactions for increasing reaction heat and consequently sensitivity in calorimetric determinations is demonstrated.

Introduction

As a result of the development of efficient and sensitive instrumentation, calorimetry [1] can be introduced into biochemical analysis. Because of the specificity of enzymatic catalysts, measurements of defined reactions and determinations of substrates have become possible using flow systems: the kinetic behaviour of hydrolases [2—4] and determination of inhibitors of these enzymes [5,6] have been studied.

Nevertheless, most of these experiments have been performed in homogeneous solutions of enzymes and substrates. Homogeneous flow systems are not very suitable for the determination of a substrate because large amounts of enzymes and coenzymes are needed. Further progress towards routine assay in

* Part of thesis G. Krisam.

biochemical analysis using microcalorimetry only became possible after introduction of immobilized enzymes [7,8].

Commercially available microcalorimeters were originally developed for precise determination of ΔH values and high sensitivity; however, because of their relatively high time constant they are not always suitable for measurements of fast reactions [9], and their application in flow-through systems for analytical routine assay is limited.

On the other hand thermistor devices with rather low time constants have been used in chromatographic flow systems for detection and determination of dissolved substances from adsorption enthalpy [10—12], and combination of thermistors with immobilized enzymes led to sensors for thermometric detection of substrates in flow systems [13,14]. The intention of the following investigation has been to test the energy yield of suitable enzyme catalized reactions by means of a heat flow calorimeter and to use these reactions for testing a new enzyme thermistor detector.

Materials and Methods

Immobilized enzymes were obtained from Boehringer Mannheim Co., Mannheim (Enzygel®-samples), from Miles-Seravac, Lausanne (Enzite®-samples) and from our own laboratory. Their specific activities were determined in suspension according to the conditions for the corresponding soluble enzymes [15,16]; an Aminco DW-2 spectrophotometer was used in all assays. The enzyme gels (30—60 mg dry weight depending on the specific activity) were suspended in a suitable buffer (see Results) and put into the reaction cell of the devices.

Buffer flow was effected by an Ismatec mp-Pg peristaltic pump, and substrate samples dissolved in 30—130 µl buffer were added to the flowing buffer (5 and 20 ml/h) by means of a Chromatronix SV 8031 valve. The heat flow measuring device consisted of an LKB microcalorimeter system 10700-1 with a prototype of a flow cell, and a Kontron recorder with integrator. It was kept in a thermostated room of 22 ± 1°C while the reaction temperature was 25 or 27°C. Details of the enzyme thermistor system are given under Results.

Results

(I) Urea determination in the LKB heat flow calorimeter

The enthalpy for the enzymatic hydrolysis of urea at pH 7.0 has been determined by Brown et al. [17] in a batch system; the reaction has been studied in a flow calorimeter in a homogeneous phase [4]. Therefore, this reaction was chosen for initial studies of heat yield in a system of heterogeneous enzymatic catalysis.

From the enthalpy of hydrolysis of urea by different samples of immobilized urease at pH 7.0 a single calibration curve was obtained (Fig. 1). The molar reaction enthalpy as determined from the slope of this curve was −1.7 ± 0.2 kcal/mol, which is in good agreement with the value found by Brown et al. [17] in batch experiments (−1.57 kcal/mol). The result indicates total hydrolysis of the samples in the flow system. Within a given range it was independent of the

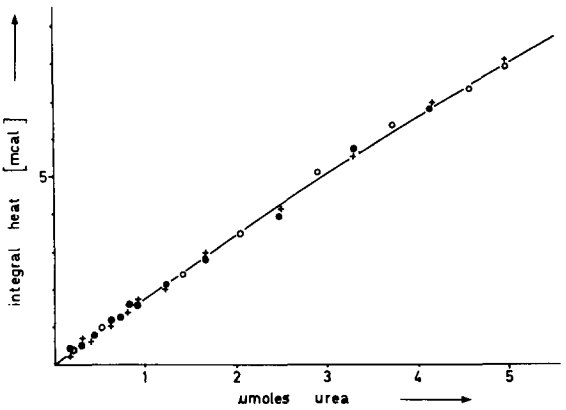

Fig. 1. Heat production from the hydrolysis of urea catalized by immobilized urease (EC 3.5.1.5). 0.05 M Tris · HCl buffer, pH 7.0; flow rate, 5 and 20 ml/h; reaction temperature, 27°C; ○———○, 6 units Enzygel®-urease (0.08 units/mg dry gel); +———+, same sample after 3 weeks at 27°C,; ●———●, 9.5 units Enzite®-urease (0.2 units/mg dry gel).

sample volume (30—130 μl) and buffer flow rate (5—20 ml/h). 0.5—5 μmol urea can be determined in this way with an error of 5%. For analysis of urea in urine 10 μl were needed; deviations from results obtained with other methods were not larger than 5%. As expected from the equation

$$O{=}C\begin{matrix}NH_2\\ \diagdown\\ \diagup\\ NH_2\end{matrix} + H_2O \xrightarrow{\text{urease}} NH_4^+ + NH_3 + HCO_3^- \qquad (1)$$

the reaction enthalpy was dependant on the pH ($\Delta H = -2.0 \pm 0.2$ kcal/mol at pH 8.0); Tris being the buffer with the highest protonation enthalpy [2,8] had been used in all experiments. Yet the small increase compared to the reaction enthalpy at pH 7.0 would not justify running the reaction at this pH rather than the pH optimum of urease.

(II) Investigations with the coupled enzyme system glucose oxidase-catalase for the calorimetric assay of glucose

Commercially available preparations of glucose oxidase are contaminated by catalase. Therefore, glucose dehydrogenation is normally accompanied by at least partial decomposition of the H_2O_2 formed. In the presence of sufficient catalase activity any H_2O_2 produced should be decomposed, and the reaction enthalpy measured should correspond to the oxidation of glucose by molecular oxygen (Reaction 4).

$$C_6H_{12}O_6 + O_2 \xrightarrow{\text{glucose oxidase}} C_6H_{10}O_6 + H_2O_2 \qquad (2)$$

$$H_2O_2 \xrightarrow{\text{catalase}} 1/2\ O_2 + H_2O \qquad (3)$$

$$C_6H_{12}O_6 + 1/2\ O_2 \rightarrow C_6H_{10}O_6 + H_2O \qquad (4)$$

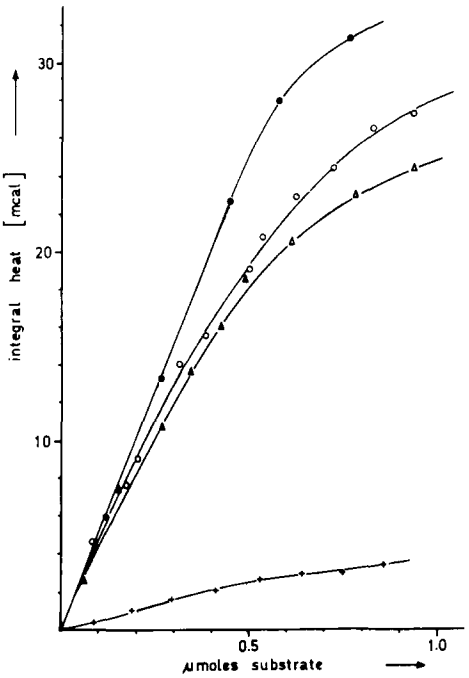

Fig. 2. Heat production from glucose oxidation catalized by immobilized glucose oxidase-catalase (EC 1.1.3.4 and EC 1.11.1.6). 0.066 M phosphate buffer, pH 7.0; flow rate, 5 and 20 ml/h; reaction temperature, 27° C. +———+, 6 units Enzygel®-glucose oxidase (0.1 units/mg dry gel), H_2O_2 as substrate; △———△, same sample with glucose as substrate; ○———○, special enzyme sample, containing 12 units glucose oxidase + 150 units catalase; ●———●, same, buffer oxygen saturated, all with glucose as substrate.

The oxidation of glucose by immobilized glucose oxidase has already been investigated by Johansson [7] in the LKB microcalorimeter system. Since in Johansson's paper the dependence of heat production on substrate amount was not linear, even for low substrate amounts, we supposed one of the reactions to be incomplete and have now attempted to control them under different conditions. The calibration curve for glucose obtained with immobilized glucose oxidase (Boehringer Enzygel®-glucose oxidase) was similar to that of Johansson [7]. Catalase activity in the enzyme preparation is demonstrated (Fig. 2). After addition of an excess of immobilized catalase a small prolongation of the linear part of the calibration curve could be obtained. Similar results were found with a special sample of highly active glucose oxidase. Saturation of the buffer with oxygen resulted in a remarkable extension of the linear part of the calibration curve. It can be calculated that, under these experimental conditions, oxygen is present in sufficient amounts to oxidize about 90 μg of glucose in the reaction volume. Therefore, the reaction must proceed quantitatively up to this amount of substrate.

From the slope of the calibration curve a total reaction enthalpy of 49.5 kcal/mol is calculated. Since the enthalpy for H_2O_2 decomposition is approx. 30 kcal/mol [18], 20 kcal/mol remain for the formation of gluconolactone or gluconate. No data for comparison are available, but this seems to be a reasonable value for this reaction.

(III) Experiments on microcalorimetric assay of NAD^+-dependent dehydrogenations

NAD^+-dependent dehydrogenations are reversible and their equilibrium is almost exclusively in favour of the oxidized coenzyme. Therefore heat production permitting calorimetric substrate determination can only be expected from a reaction consuming NADH. An example tested in the calorimetric flow system was the reduction of pyruvate catalyzed by immobilized lactate dehydrogenase (EC 1.1.1.27, from pig heart, immobilized by reaction with Affi-gel 10, Bio Rad Laboratories, Richmond, Calif., following the manufacturer's instruction; specific activity 0.01 unit/mg dry weight of gel) according to the reaction:

$$H_3C\text{-}CO\text{-}COOH + NADH + H^+ \xrightarrow{\text{lactate dehydrogenase}} H_3C\text{-}CHOH\text{-}COOH + NAD^+ \quad (5)$$

The reaction was carried out in 0.07 M phosphate buffer, pH 7.0, containing 10 mg NADH/ml. Heat production corresponded to the amount of substrate added in the range of 0.15—2 μmol of pyruvate. The reaction enthalpy (—10 ± 0.5 kcal/mol) calculated was in good agreement with the value determined by Katz [19] using a batch calorimeter (10.7 kcal/mol). The relatively high error is due to the low specific activity of the enzyme; the sample was also very unstable, making further experiments impossible.

Quantitative dehydrogenation of a substrate by NAD^+ is only possible when the product is eliminated by a coupled reaction. Therefore, microcalorimetric determination of ethanol by the alcohol dehydrogenase reaction was tried in the presence of semicarbazide. Heat effects found dit not correlate to the amounts of ethanol used, and the immobilized alcohol dehydrogenase was very instable under the experimental conditions.

(IV) Glucose determination by an enzyme thermistor detector

Measuring device. Any instrument for precise temperature difference determination in small volumes may be used for the construction of a thermometric sensor; hence Mosbach's enzyme thermistor [13] is based on the Universal Temperatur-Messgerät of the Knauer Co., Berlin. The device developed by us (Fig. 3) consists of a flow-through cell with two thermistors (Siemens K 19, 12 kΩ), which form part of a Wheatstone bridge. Continuous current (1—2 V) is supplied to the bridge from an integrated circuit (LM 109 K). The voltage of the bridge is amplified by an Analogic® MP 221 chopper amplifier and transmitted to a compensation recorder and integrator. The whole assembly is kept in an electronic thermostat. The flow-through cell (Fig. 4) is made of plexiglass connected to gold capillaries. Because of their low time constant (0.3 s), non-insulated thermistors were used which were mounted outside the cell in good thermal contact to the catalyst area.

The electronic stability of the assembly (shift of the zero line) is smaller than 2% of full scale per day. The recorder scale corresponds to 10^{-2}°C, while differences of $5 \cdot 10^{-4}$°C may be detected; this corresponds approximately to detection of ≈200—500 μcal/min at a buffer flow rate of 17 ml/min. The response time of the whole assembly is about 2 s.

Determination of glucose. Because of its high reaction enthalpy the glucose oxidation reaction was chosen to test the thermistor system. 30 mg Enzygel®-

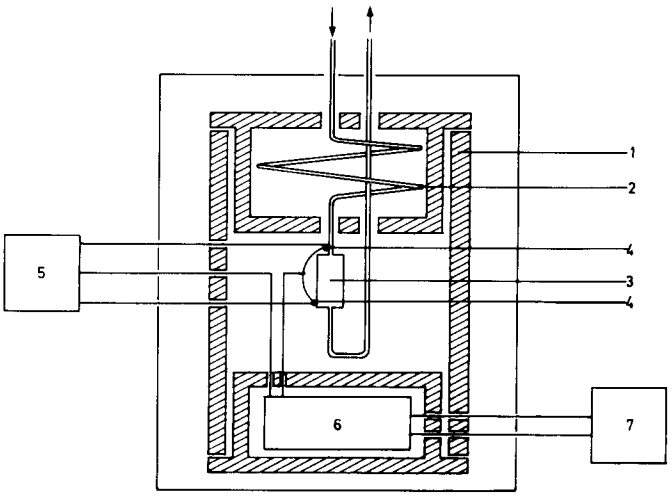

Fig. 3. Thermistor detector. 1, Aluminium box in thermostat; 2, gold capillary; 3, reaction cell (see Fig. 4); 4, sample and reference thermistors; 5, Wheatstone bridge; 6, amplifier; 7, recorder and integrator.

glucose oxidase (special sample corresponding to 12 units glucose oxidase and 150 units catalase) were applied in the reaction cell.

The temperature difference between the two thermistors registrated as a

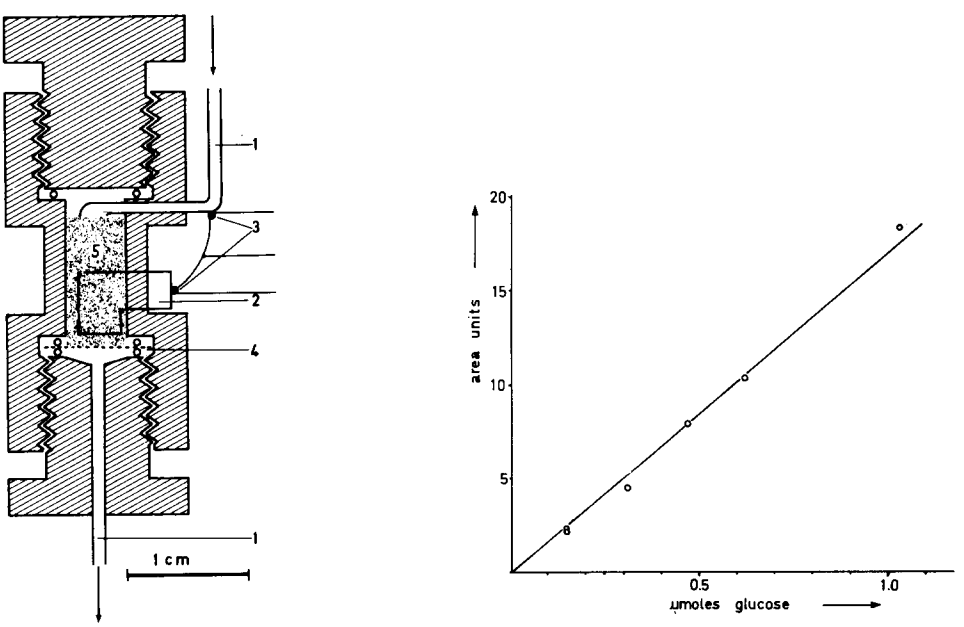

Fig. 4. Reaction cell (plexiglass, total volume 0.3 ml). 1, gold capillary (inner diameter 0.5 mm); 2, gold sheet, 3, thermistors; 4, stainless steel sieve between O-rings (pore size 40 µm); 5, immobilized enzyme.

Fig. 5. Calibration curve for glucose determination in the "enzyme thermistor". Integral of temperature course at buffer flow rate 17 ml/h, reaction cell containing 30 mg Enzygel®-glucose oxidase (12 units glucose oxidase + 150 units catalase); 0.066 M phosphate buffer, pH 7.0; temperature, 27°C.

function of time was, for a given amount of substrate strongly dependent on the buffer speed. At a low speed, response and sensitivity increased, but so also did time constant and error. Nevertheless for a constant buffer speed the area under the temperature curve corresponded to the amount of glucose added.

For comparison to the heat flow calorimeter, the buffer speed was adjusted so that a glucose sample could be determined every 17 min. The maximum temperature difference for the oxidation of 0.5 μmol glucose under these conditions was $2.5 \cdot 10^{-3}$°C. According to the calibration curve (Fig. 5) glucose could be determined between 0.05 and 1 μmol with an absolute accuracy of 0.03 μmol.

Discussion

Substrates in flow systems can be determined from the reaction enthalpy evolved at a reaction catalized by an immobilized enzyme. Heat flow calorimetry allows precise and absolute determination of this enthalpy, because the instrument can be electrically calibrated. As expected on the other hand, a marked disadvantage of flow calorimeters in enzymatic substrate determination is the high time constant. Although the enzymatic reaction occurs within 1—3 min, heat flux is registrated for about 20 min. This time is implied by the method itself and cannot be reduced.

For the same time of analysis the sensitivity and accuracy of the enzyme thermistor as detector are lower. Moreover, absolute heat determination by this device is not possible. Nevertheless the thermistor system is by far less expensive, and its sensitivity can be increased by better thermostatisation of the cell. Further improvements would be possible by use of immobilized enzymes of higher specific activity because cell volume and consequently heat capacity would be reduced.

Hence in enzymatic flow systems, heat flow calorimetry should be applied for precise determination of reaction enthalpies while thermistor detectors should be of interest in systems for analytic routine assay. For further improvement of both methods, immobilized enzymes of higher specific activity and better stability are needed. Mechanical qualities of the enzyme gels should be improved, especially their flow resistance should be diminished. Enzite®-gels (enzymes bound to cellulose) were found to be of relative high specific activity, but to have considerably higher flow resistance than Enzygel®-gels (enzymes in polyacrylamide gel).

A final aspect is the increase of energy yield in enzymatic catalyzed reactions. Enzyme catalyzed reactions which proceed quantitatively, such as hydrolyses or oxidation reactions, are suitable for microcalorimetric substrate analysis in flow systems. A reaction enthalpy of 5—10 kcal/mol is sufficient to determine a substrate between 0.25 and 0.5 μmol with an accuracy of 5%. Increased heat production and hence sensitivity can be achieved in many cases by coupling reactions. For example, any H_2O_2-producing dehydrogenation (as in flavine enzyme-catalysed reactions) may be coupled to a catalase-catalized H_2O_2 decomposition. Our own intentions are directed towards finding coupled reactions for NAD^+-dependent dehydrogenations.

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