APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 7, 127–134 (1982)

The Enzyme Thermistor

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Accepted February 18, 1981

Abstract

This is a brief review of some applications and a description of the enzyme thermistor, which in effect is a flow-calorimeter designed for routine analysis in clinical chemistry, biochemistry, environmental control, and biotechnology. The heat produced in a small column filled with immobilized enzyme is measured with a thermistor, hence the name enzyme thermistor (*1*, *2*).

Index Entries: Enzyme thermistor; thermistor, enzyme; calorimetry, with an enzyme thermistor; immobilized enzyme thermistor; glucose, enzyme thermistor for; lactose, enzyme thermistor for; cellobiose, enzyme thermistor for; ethanol, enzyme thermistor for; immunoassay, enzyme thermistor for.

Introduction

Calorimetry is a universal analytical method and is particularly attractive in enzymic analysis since most enzymic reactions are associated with a heat evolution of at least 20 kJ/mol. An important advantage is that the process can be followed irrespective of the optical properties of the sample, *viz.* color and turbidity. In fact, a greater flexibility is offered in the choice of reactions to be followed since there is no demand for reactions giving rise to a color change or a pH-shift as in spectrophotometry or titrimetry. It may also be possible to avoid reaction sequences involving cofactors or other expensive reactants. Generally, a single enzymic step produces sufficient heat for reliable determinations and this allows simple analytical methods to be used. Wider application of calorimetry has, however, been hampered by the relatively high cost and complexity of existing calorimeters. In recent years several inexpensive, less complicated devices for biochemical analysis have been developed combining the universality of calorimeters with the specificity of enzymic reactions (*2*).

0273–2289/82/0912–0127$02.00

The currently used version of the enzyme thermistor developed in our laboratory has a maximal temperature resolution of 10^{-5} degrees. This enables concentration determinations down to $10^{-5}M$ for reactions of moderate enthalpy. Since the efficiency of heat detection is at least 50–75%, concentrations around 1 mM can be determined with an error of less than 1%, assuming a temperature resolution of 10^{-4} °C and an enthalpy change of 80 kJ/mol.

Apparatus

Figure 1 shows the design of the calorimeter part of an enzyme thermistor. This apparatus comprises two aluminum cylinders. The outer cylinder is provided with electrical heaters for thermostatting. The inner cylinder is isolated from the outer one by an airspace. This arrangement creates a very stable temperature inside the cavities into which the enzyme columns (volume 0.2–1 mL) are inserted. Buffer is continuously pumped through the system at a flow rate of 0.5–2 mL/min with a peristaltic pump. Samples are generally introduced through a chromatography injection valve with a 0.5 or 1 mL loop. Before entering the enzyme column the solution passed through a heat exchanger. The temperature at the outlet of the column is registered continuously by a thermistor connected to a Wheatstone bridge (*1*). The apparatus has two column ports that can be used independently, or with one loaded with an inactive enzyme preparation as a reference column. The sample flow in this case is split into two equal parts and the temperature difference between the two columns is measured by connecting both thermistors to the bridge.

This split-flow arrangement (*3*) is useful for elimination of unspecific heat signals arising from dilution effects, viscosity or pH changes, and so on. Differential temperature determination also improves the baseline stability.

Enzymes are usually immobilized on CPG (Controlled Pore Glass), which is a mechanically stable support with high binding capacity, but other supports have been used as well (*1*). A large excess of catalytic capacity (10–1,000 units) is normally used, ensuring very good operational stability and a substantial range of linearity.

Procedure

Samples are generally introduced as rather short pulses, 0.25–1 mL, in a continuous buffer stream at a flow rate of 0.5–2 mL/min. Thermal steady state will not be obtained for such short sample pulses, but the enzymic reaction will generate a temperature peak the height of which is normally taken as a measure of the substrate concentration. The linear range of the relationship between temperature peak height and substrate concentration is usually at least 0.01 –100 mM when not limited by reactant concentrations (as oxidases are by the supply of oxygen) or other factors. The area under the temperature peak or the ascending slope are also useful measures of substrate concentration, as has been discussed previously (*4*). The maximum number or samples that can be analyzed by the present system is 15–30

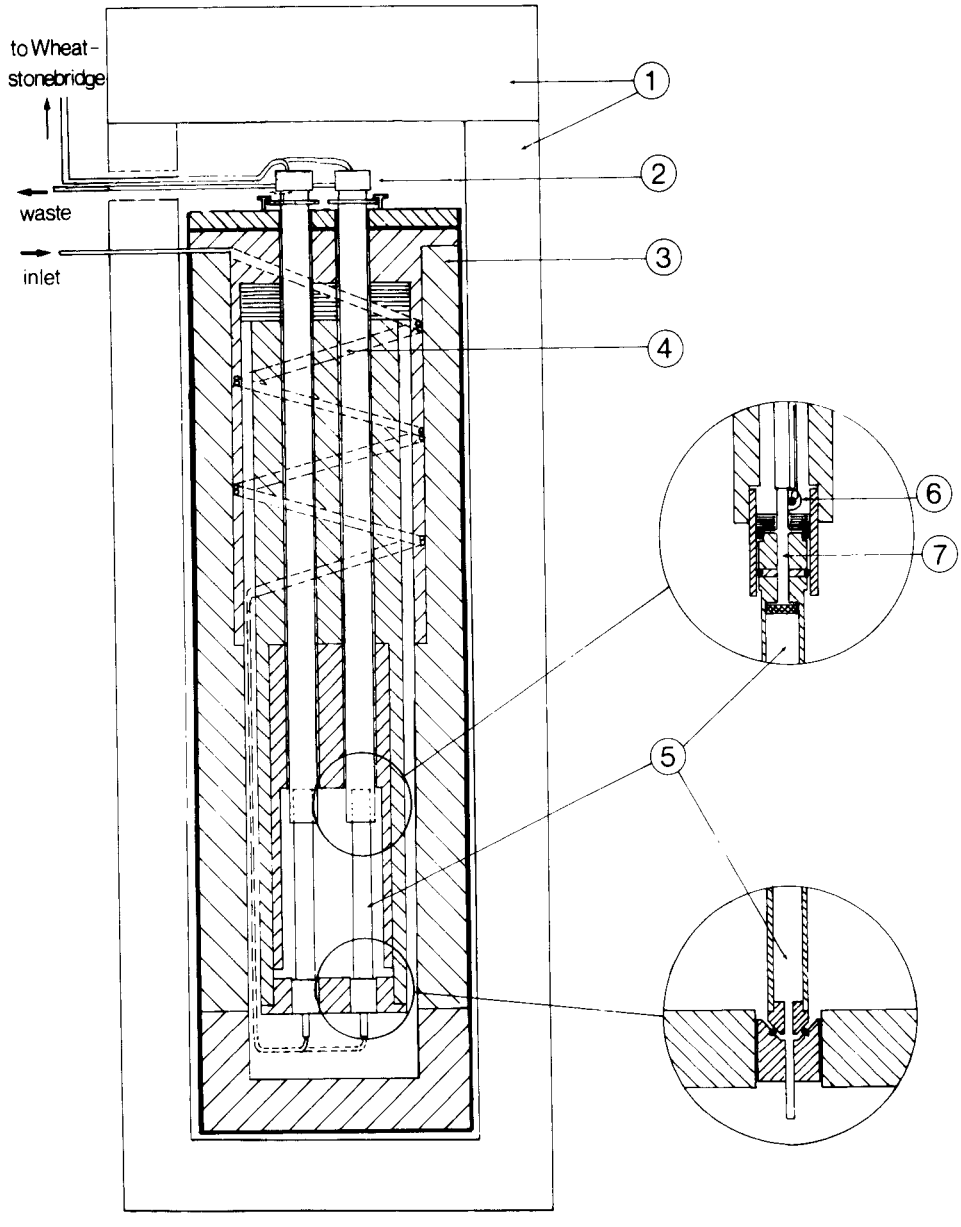

Fig. 1. Schematic drawing of the calorimeter part of an enzyme thermistor. 1. Polyurethane insulation. 2. Plexiglass holders for the columns. 3. Outer, thermostatted aluminum cylinder. 4. A pair of heat exchangers made of 0.8 mm id acid-proof steel tube. 5. Plastic column. 6. Thermistor attached to a short length of gold tube. 7. Column outlet.

samples/h with the above mentioned sample volumes. Increasing the sample volume eventually gives a steady-state temperature. This is higher than the peak value and also gives a linear relationship to substrate concentration. The enzyme thermistor can be used for continuous measurements and the signal is stable enough to be used for continuous control of a process (5). An advantage of continu-

ous sample introduction is that nonspecific effects owing to dilution or solvation heat are automatically eliminated since they are present only at the ends of a sample plug.

Applications

Clinical Chemistry

Our first enzyme thermistor application studies were in the field of clinical chemistry and we have now studied quite a few metabolites, some of which are found in Table 1. Detailed studies aiming to evaluate the practicality of the calorimetric method in comparison with methods currently used have been performed in several cases, e.g., for glucose (*6*), urea (*7*), and triglycerides (*8*). For these metabolites the enzyme thermistor methods compare favorably with existing methods. They also appear to offer definite advantages for some other, less well-studied metabolites, such as oxalate and lactate. A simple and specific assay for oxalate is presently under investigation using the enzyme oxalic acid oxidase (E.C. 1.2.3.4) as

TABLE 1
Substances Analyzed with the Enzyme Thermistor

Substance	Enzyme(s)	Concentration range,[a] mmol/l
Ascorbic acid	Ascorbic acid oxidase	0.05–0.6
ATP	Apyrase or hexokinase	1–8
Cephalosporin	Cephalosporinase	0.005–10
Cholesterol	Cholesterol oxidase	0.03–0.15
Creatinine	Creatinine iminohydrolase	0.01–10
Glucose	Glucose oxidase/catalase	0.002–0.8
Hydrogen peroxide	Catalase	0.005–10
Lactate	Lactate 2-monooxygenase	0.01–1
Lactose	Lactase and glucose oxidase/ catalase	0.05—10
Oxalic acid	Oxalate oxidase	0.005–0.5
Penicillin G	Penicillinase	0.01–500
Sucrose	Invertase	0.05–100
Triglycerides	Lipase, lipoprotein	0.1–5
Urea	Urease	0.01–500
Cellobiose	β-glucosidase + glucose oxidase/ catalase	0.05–5
Ethanol	Alcohol oxidase	0.01–1
Galactose	Galactose oxidase	0.01–1

[a]These values have been obtained from a number of different studies and do not necessarily represent the limits of detection possible with the enzyme thermistor.

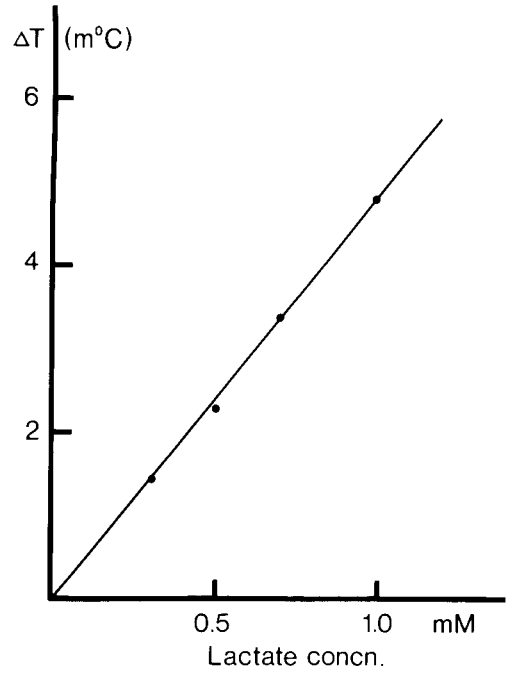

Fig. 2. Calibration curve for determination of lactate in 0.2*M* sodium phosphate buffer, pH 7.0. A 0.5 mL CPG-column with 50 IU (applied) of lactate oxidase was used at a flow rate of 1 mL/min. Sample volume: 1 mL.

sole enzyme and has given promising results (*1*). A very simple procedure has been designed for lactate determination. Here also only a single enzyme, lactate oxidase (lactate 2-monooxygenase, E.C. 1.13.12.4), is required. Figure 2 shows a calibration curve for this system. It has been found to perform well with crude samples such as fermentation broths and serum. the clinical applicability is now being investigated.

Biotechnology

A major part of our efforts to develop calorimetric techniques is now directed towards this area. Compounds of primary concern studied here include carbohydrates such as sucrose, lactose, cellobiose, antibiotic such as penicillins and cephalosporins, and others such as ethanol and lactate (*4*). Sucrose can be determined in the range of 0.05–100 m*M* in a very simple and reliable fashion using the enzyme invertase (E.C. 3.2.1.26). Other glucose-containing disaccharides such as lactose and cellobiose can be determined by a glucose oxidase/catalase thermistor after enzymic hydrolysis of the disaccharide. This is preferably done with the hydrolytic enzyme in a precolumn, as was done with β-glucosidase in a recent study on cellobiose (*9*). As shown in another report in this issue, enzyme thermistor assays for carbohydrates are sufficiently accurate to be used for control of biotechnological processes (*5*).

Very good results have been obtained in studies on antibiotics. Penicillin can be measured in discrete samples or continuously with the aid of immobilized

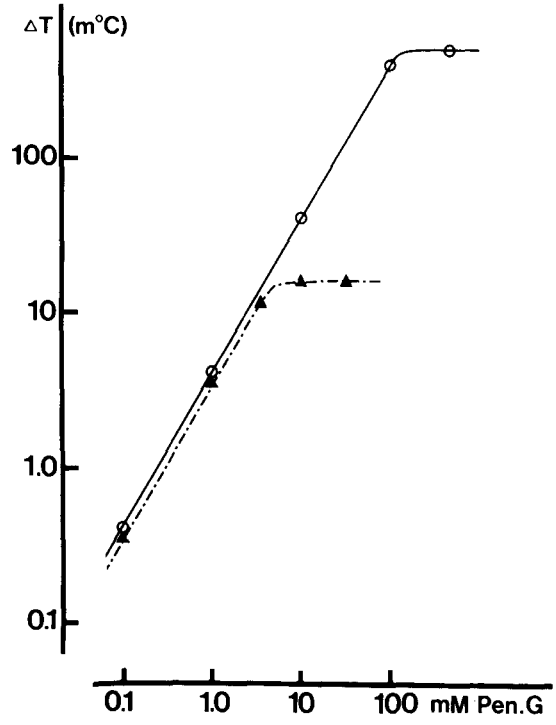

Fig. 3. Calibration curves (log–log scale) for penicillin G with β-lactamase as the enzyme. Comparison of the linear ranges obtained for CPG-bound (o) and for nylon tubing-coupled (▲) enzyme.

penicillinase (β-lactamase, E.C. 3.5.2.6). The enzyme has been covalently bound either to CPG or to the inside of nylon tubing. Both systems work well. CPG gives a somewhat higher sensitivity, but the nylon tubing is less susceptible to fouling (Fig. 3). Enzyme thermistor assay of penicillin in fermentation broths competes favorably with existing methods (*10*). Another promising enzyme thermistor application is the assay of cephalosporins using cephalosporinase (*1*) as mentioned above. It has the same advantages as the penicillin assay. Figure 4 shows calibration curves for two different commercial preparations of cephalosporin.

Environmental Control

Because of its inherent specificity, the enzyme thermistor can be used in the determination of a specific component present in, for example, a waste water sample. With a more general biological approach, it can provide information on the integrated effects on living cells of all pollutants present in the sample. Two techniques have been utilized in the analysis of specific substances: determination as an inhibitor of a certain enzymic reaction and determination as a substrate of an enzyme. An example of the first one is the determination of heavy metals such Ag^{+}, Cu^{2+}, and Hg^{2+} by their inhibitory effect on urease (*11*). A simple and rapid regeneration procedure was applied and a sensitivity of 0.2 ppb of Hg^{2+} was found. Determination of cyanide using the enzyme rhodanese (E.C. 2.8.1.1) in the enzyme

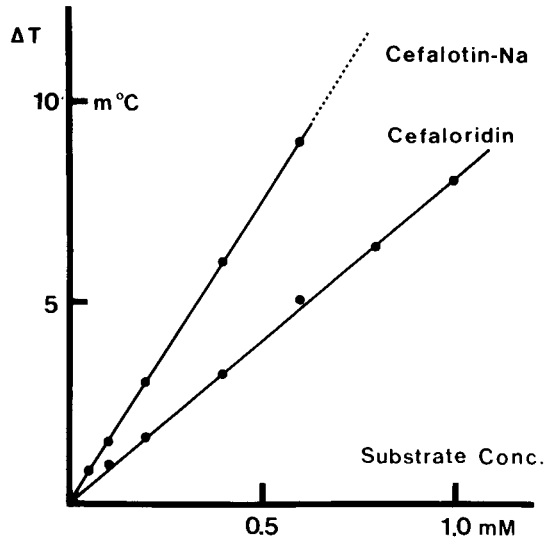

Fig. 4. Temperature response curves for two different commercial cephalosporins: cephaloridine and the sodium salt of cephalothin. Sample pulses (1 mL) were introduced with a flow rate of 0.8 mL/min. About 100 units of cephalosporinase were coupled to CPG. The buffer used was 0.1*M* Tris-HCl, pH 8.3.

thermistor column is an example of the second technique. Cyanide concentrations down to $10^{-5}M$ were determined (*12*).
The report on the "Microbe Thermistor" indicates the potential of a calorimetric instrument such as the enzyme thermistor in establishing the overall effects of pollutants on living organisms—in this case yeast cells entrapped in polyacrylamide (*13*).

TELISA

The enzyme thermistor has been used in a competitive ELISA procedure called TELISA (Thermometric Enzyme-Linked Immunosorbent Assay) (*1*). The column is then filled with an immunosorbent consisting of antibodies immobilized on Sepharose CL-4B. Very short contact times between immunosorbent and sample have been used, permitting a rapid assay procedure with a sensitivity down to $10^{-13}M$. The total time required for a sample cycle including separation is about 12 min.

Enzyme Activity Determination

Like other flow calorimeters, the enzyme thermistor unit can be employed for determinations of enzyme activities (*14*). The enzyme column was replaced with a piece of teflon tubing forming a 1 mL reaction coil in which the soluble enzyme to be analyzed reacted with excess of substrate present. The sample solution and an appropriate substrate solution were both passed through a heat exchanger and then mixed before entering the reaction coil. The sensitivity of the method appears to be 1 IU/mL or better for most enzymes. There are several variations on this theme.

These are now being investigated in order to develop useful monitoring techniques for analytical and preparative chromatography and for following enzyme purificaion procedures.

A related technique involving a modified enzyme thermistor unit can, as discussed below by Mandenius et al., be used for recording microbial power–time curves (thermograms). When combined with simultaneous enzyme thermistor determination of specific metabolites, this appears to be very powerful technique for following microbial metabolism.

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