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BIOSPECIFIC THERMAL ANALYZER COUPLED WITH A FLOWTHROUGH IMMOBILIZED ENZYME COLUMN

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A thermal analyzer consisting of a pair of glass thermistors was coupled with a flowthrough immobilized enzyme reactor for the determination of ATP concentration. Hexokinase was covalently bound to poly(chloromethyl-styrene-divinyl benzene) beads and packed in the reactor. The thermal analyzer was designed so as to respond to heat generation in the reactor. A maximum temperature change was exhibited within 1.5 min after the injection of a sample solution containing ATP together with glucose. Thermal decay occurred within 1 min. The thermal analyzer responded reproducibly to ATP at a constant concentration. Assays could be repeated at 3-min intervals. The effects of sample volume and flow rate on the response were examined with regard to an empirical equation. Concentration of ATP was determined with the thermal analyzer in the range of 0–10 mM. The thermal analyzer may be a simple device for the rapid determination of ATP concentration.

INTRODUCTION

Over the past decade immobilized enzymes and other biologically active molecules have found application in clinical and environmental analysis (1–4). These recently developed analytical systems are characterized by the combination of solid/liquid biochemical reactions and physicochemical detection methods including colorimetric, electrochemical, thermometric, and radioisotope techniques (5–12). In a series of publications, we have reported the development of various novel sensors employing a solid-phase biochemical reaction to an electrochemical detector (13–17). The purpose of the present work is to develop a solid-phase analytical system for monitoring a specific substance of biochemical importance that is normally difficult to measure electrochemically.

The combination of an immobilized enzyme and a thermometric detector has been investigated by several groups. The "enzyme thermistor" has been applied to a variety of analyses, including trypsin (18–20), glucose (20–22), lactose (21), uric acid (21), cholesterol (21), penicillin G (21), and

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urea (20–22). These analyzers, however have all suffered from low sample throughput and large volume of sample required, partly because of slow thermal response characteristics and the compressibility of gel type enzyme supports. To overcome these problems, the present immobilized enzyme thermal analyzer was assembled by making use of a flow through reactor incorporating an enzyme immobilized to polystyrene derivatized beads.

MATHEMATICAL EXPRESSION OF THE THERMAL RESPONSE

The performance of the immobilized enzyme thermal analyzer is ultimately limited by the capabilities of the immobilized enzyme reactor used. A packed-bed type of immobilized enzyme reactor has been modeled in terms of reaction plates rather than separation by the same mathematics that apply to chromatographic systems (23). In the following model, we have assumed that the sample species is instantly converted to product, that there is no heat loss due to poor adiabaticity, that the sample zone is thermally homogeneous in the radial direction, and that the detector responds very rapidly to the temperature change.

The configuration of the immobilized enzyme reactor is schematically illustrated in Fig. 1. Reference and detecting thermistors are located at the inlet and the outlet of the reactor. Volume V_s (ml) of the sample flows through the reactor at a constant flow rate of F (ml/min). The generated heat dispersed in the space of V_r transfers at the same rate as F. The maximum temperature change caused by the enzymatic reaction of the sample at a concentration of C_s (mol/l) will be expressed by ΔT_{max} (°C). The sample and the heat phases require time $\theta = V_s/F$ and $\theta' = V_R/F$ to pass through the reactor, respectively. Temperature difference between the detecting and the reference thermistors, $\Delta T(t)$, will be a function of t, θ, and θ':

$$\Delta T = \Delta T_{max} \cdot f(t, \theta, \theta') \tag{1}$$

The function of $f(t, \theta, \theta')$ determines the shape of the response curves. We applied an error function derived by Sternberg (23) to express the response curve. The error function of $\mathrm{erf}(x)$ is defined as $(2/\sqrt{2})\int_0^x \exp((-Z^2)dz$, where $\mathrm{erf}(0) = 0$ and $\mathrm{erf}(\infty) = 1$. The resulting function can be evaluated numerically using tabulated values of the error function, $\mathrm{erf}(x)$:

$$\Delta T(t) = \frac{\Delta T_{max}}{2}\left[\mathrm{erf}\left(\frac{t - t_{max}}{\sqrt{2}(\theta/\theta')}\right) - \mathrm{erf}\left(\frac{t - t_{max} - \theta}{\sqrt{2}(\theta/\theta')}\right)\right] \tag{2}$$

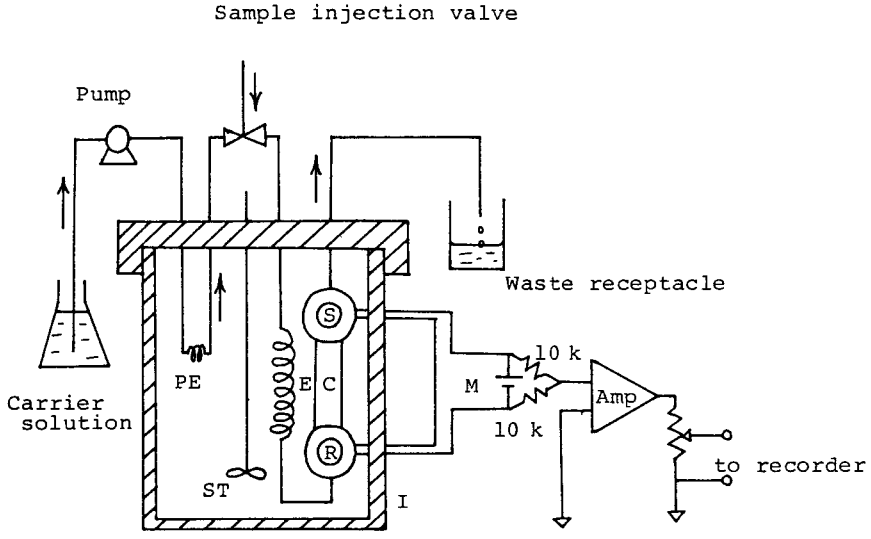

FIG. 1. Schematic representation of the thermal analyzer coupled with a flowthrough immobilized enzyme column. PE: pre-equilibration coil; E: equilibration coil; R: reference thermistor; C: immobilized hexokinase column, S: sensing thermistor; M: mercury cell (1.3 V); Amp: amplifier; ST: stirrer; I: water bath controlled within 0.005°C.

where t_{max} is the time at T_{max}. The function of $\Delta T(t)$ gives the peak height (ΔT_{peak}) at $t_p = t_{max} + \theta/2$.

$$\Delta T_{peak} = \Delta T_{max} \cdot \mathrm{erf}(\theta'/2\sqrt{2}) \tag{3}$$

EXPERIMENTAL

Reagents

Hexokinase (EC 2.7.1.1), 0.37 U/mg in lyophilized form, was obtained from Oriental Yeast Co., Ltd. (Tokyo). Adenosine triphosphate was purchased from Kyowa Hakko Co., Ltd. (Tokyo). All other chemicals used were of reagent grade.

Preparation of Immobilized Hexokinase

Poly(chloromethyl-styrene-co-divinyl benzene) beads (20/50 mesh) were reacted with hexamethylenediamine at 50°C for 60 min and thoroughly washed with water. Twenty milligrams of the aminated polymer beads were packed in a column for a reactor. Into the packed column, 0.25%

glutaraldehyde solution (0.1 M phosphate buffer at pH 7.0) was fed for 15 min. After sufficient washing with 0.1 M phosphate buffer (pH 7.0), 1000 U of hexokinase, dissolved in 2 ml phosphate buffer (pH 7.0), was circulated through the column at 20°C for 60 min. The immobilized enzyme was exhaustively washed with 0.1 M phosphate buffer at pH 7.0.

Instrumentation

The immobilized enzyme was packed in the column-type reactor (2.1 cm, 0.1 cm ID) facilitated with the reference and detecting thermistors at the bottom and the top. The geometry is represented in Fig. 2. The carrier solution of 0.1 M phosphate buffer (pH 7.0) is delivered from the reservoir at a constant flow rate by a peristaltic pump. The solution passes through a pulse suppressor and a preequilibration coil (25 cm, 0.1 cm ID stainless steel tubing), reaching the sample injection valve, where the sample is introduced into the carrier solution. The carrier solution and sample plug then pass through an equilibration coil (25 cm, 0.1 cm ID stainless steel tubing). During its residence in the coil the sample is thermally equilibrated with the water in the bath. The biochemical reaction of the sample is performed in the immobilized enzyme reactor. The heat generated is detected by a detecting

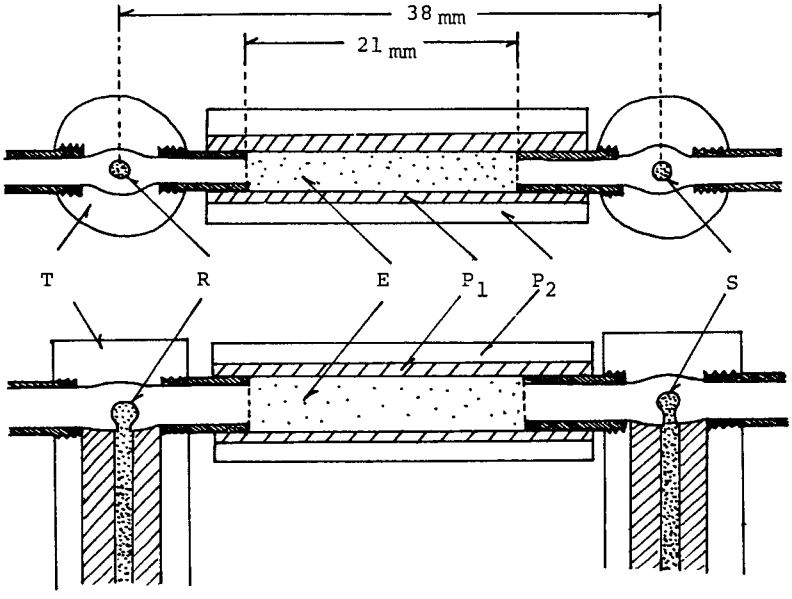

FIG. 2. Configuration of the immobilized enzyme column. R,S: reference and sensing thermistors; T: Teflon holder for the thermistor; E: immobilized enzyme; P_1, P_2: insulator.

thermistor in comparison to the reference thermistor. The solution is then discarded to a waste receptacle. The temperature difference measured by the thermistors is converted to a disbalance voltage by a DC bridge attached to an electrometer (Model PM-18N, Toa Electronics Co., Ltd., Tokyo). The configuration of the immobilized enzyme reactor and the thermistors is shown in Fig. 2.

Measurements

Assay of the immobilized hexokinase was based on the following reaction:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+ \quad (4)$$

About 10 mg of immobilized hexokinase was contacted with 5 ml of 0.1 M phosphate buffer (pH = 7.5) containing 0.02 M ATP (0.35 ml), 10% glucose (3.6 ml), 0.013 M $NADP^+$ (0.5 ml), 0.1 M $MgCl_2$ (0.20 ml), and 30 U/ml G-6-PDH (0.15 ml), and was allowed to stand at 25°C with shaking. Absorbance at 340 nm due to NADPH was recorded. The activity of hexokinase was calculated as 0.3 U/mg in the matrix according to the following equation:

$$\text{Activity (U/mg)} = \frac{\Delta A_{340\,\text{nm}}/\text{min}}{6.2 \times \text{mg immobilized enzyme/ml reaction mixture}}$$

RESULTS AND DISCUSSION

Response Curves of the Thermal Analyzer to ATP

A solution of ATP and glucose was injected at various concentrations into the thermal analyzer through the injection value. Sample volume was maintained at 200 μl. A carrier solution of 0.1 M phosphate buffer (pH 7.0) was delivered at a constant flow rate of 1.02 ml/min. Since the immobilized hexokinase is packed in the reactor, ATP was converted to ADP with resulting heat generation.

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP} \quad (5)$$

The generated heat was detected with a pair of thermistors. Typical response curves are presented in Fig. 3. Within 30 sec after the sample injection, a slight endothermic change occurred. The exothermic change reached a peak

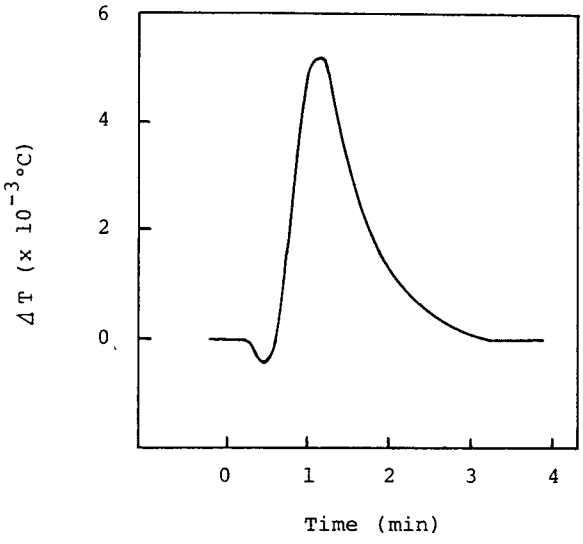

FIG. 3. Typical response curve of the thermal analyzer to ATP. Sample volume: 0.2 ml; flow rate: 1.02 ml/min; ATP concentration: 10 mM.

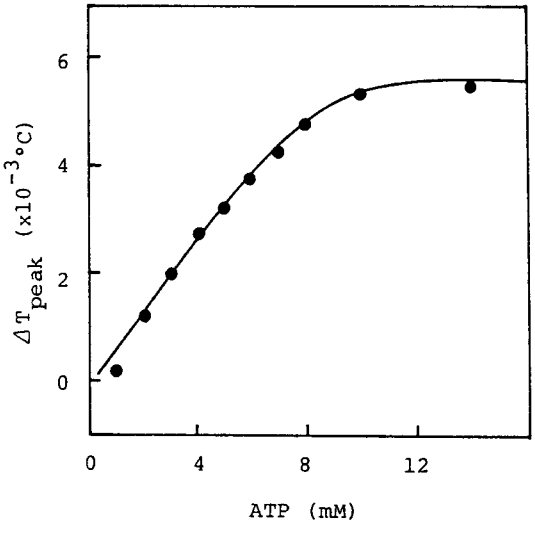

FIG. 4. Temperature change at peak as a function of ATP concentration. Sample volume: 0.2 ml; flow rate: 1.02 ml/min.

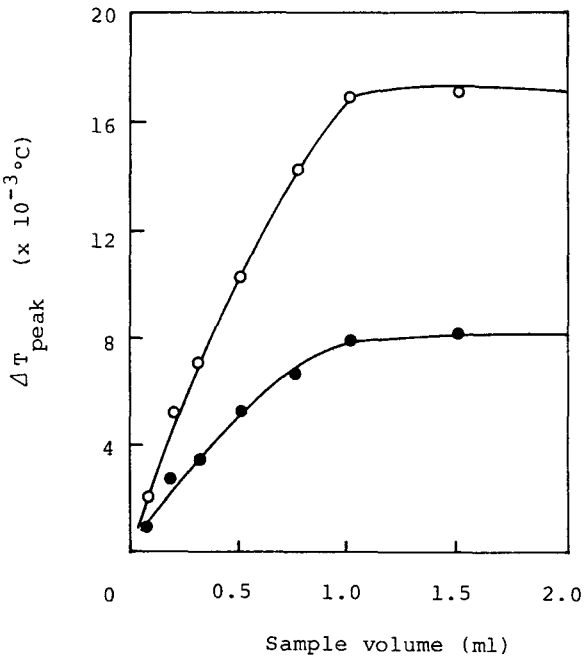

FIG. 5. Effect of sample volume on T_{peak}. Flow rate: 1.02 ml/min; open circles: 10 mM ATP; solid circles: 5 mM ATP.

in 1 min. Another minute was required to recover the initial equilibration. The response curve was almost symmetrical in shape. In the lower concentration range below 0.1 mM ATP, however, symmetry of the response curve was not observed. The response was disturbed by noise, partly due to heat generation by the thermistors.

As expected form Eq. (3), ΔT_{peak} depended linearly on ATP concentration in the appropriate concentration range when the sample volume and flow rate were fixed. A ΔT_{peak} versus ATP concentration curve is presented in Fig. 4. A straight line was obtained in the ATP concentration range of 0–8 mM. However, ΔT_{peak} was almost constant above 10 mM ATP. Michaelis–Menten's equation indicates that an enzyme reaction proceeds in the zero order in the case where the substrate concentration far exceeds the Michaelis constant K_m. As hexokinase has a $K_m = 2 \times 10^{-3}$ M for ATP, the present enzyme reaction could not depend on ATP concentration in the range above 10 mM.

Effects of Sample Volume and Flow Rate on Response

The important factors involved in Eq. (3) are sample volume V_s and flow rate F. The effects of these factors on the response were investigated at a constant ATP concentration.

The sample volume dependency is presented in Fig. 5. The measurements were made at ATP concentrations of 5 and 10 mM while maintaining a flow rate of 1.02 ml/min. Sample volume was varied in the range 0–2 ml. ΔT_{peak} increased linearly with sample volume in the range 0–1 ml. However, no appreciable effects of sample volume were observed when more than 10 ml was applied to the column.

Equation (3) suggests that $\Delta T_{peak}/\Delta T_{max}$ is independent of ATP concentration when a flow rate is constant. ΔT_{max} was tentatively assumed to be the maximum ΔT_{peak} in each series. Data presented in Fig. 5 were replotted in Fig. 6 so as to represent a relationship between $\Delta T_{peak}/\Delta T_{max}$

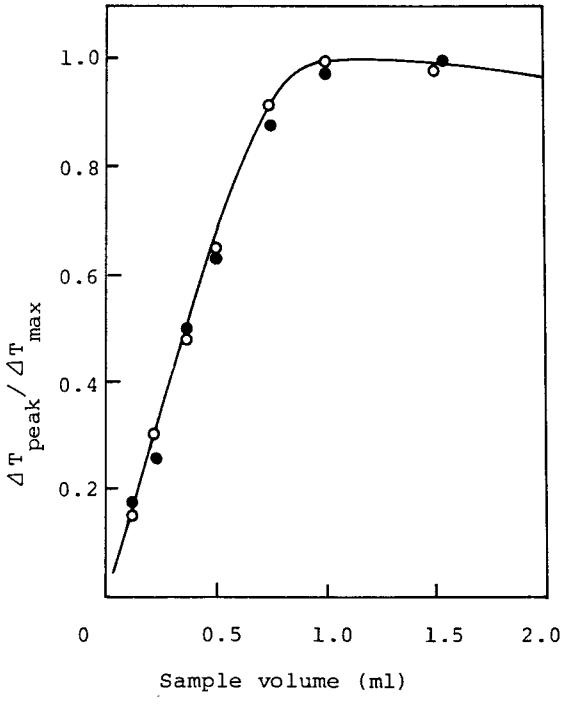

FIG. 6. Correlation of $\Delta T_{peak}/\Delta T_{max}$ and sample volume (V_s). Flow rate: 1.02 ml/min. Solid Circles: 5 mM ATP; open circles: 10 mM ATP. The curve was calculated from $\Delta T_{peak}/\Delta T_{max} = \mathrm{erf}(V_s/2\sqrt{2}V(\theta'/\theta))$ on the assumption of $\theta'/\theta = 0.15$.

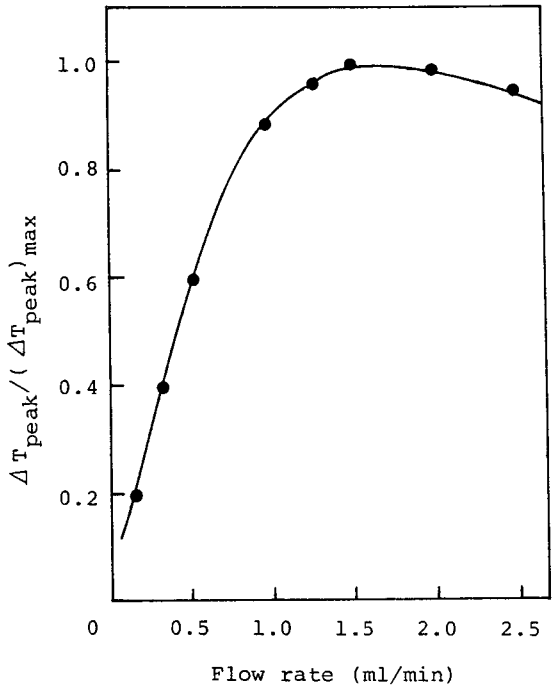

FIG. 7. Effect of flow rate on ΔT_{peak}. ATP concentration: 10 mM; sample volume: 0.2 ml.

and V_s. A solid curve was calculated from Eq. (3) on the assumption of $\theta'/\theta = 0.15$ (ml). The observed curve was simulated by Eq. (3). In addition, the effects of flow rate are presented in Fig. 7. The flow rate dependency is also simulated by Eq. (3).

Continuous Operation of the Thermal Analyzer

Reproducibility of the response was carefully checked during the continuous operation of the thermal analyzer. Typical performances are shown in Figs. 8 and 9. Sample volume and flow rate were fixed at 0.20 ml and 1.02 ml/min. When 10 mM ATP was injected every 3 min, the thermal analyzer responded to ATP as presented in Fig. 8. The extent $Xs\Delta T_{peak}$ was quite reproducible through the measurements. Figure 9 shows good reproducibility where ATP concentrations were varied. The standard deviation of 20 identical analyses was 0.005. It is reasonable to conclude that the thermal analyzer exhibited a very reproducible response.

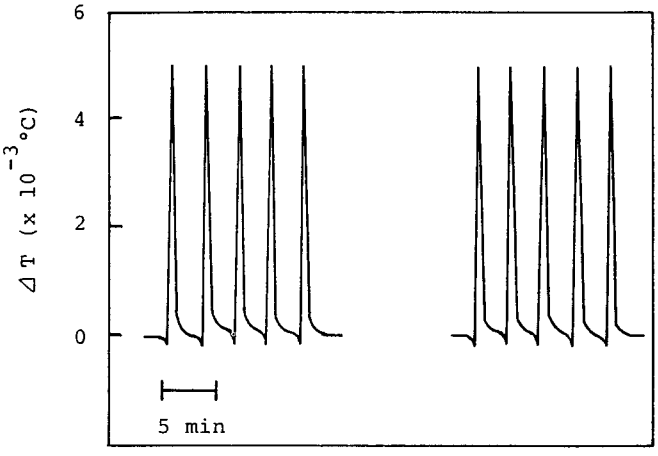

FIG. 8. Alternative responses of the thermal analyzer to 10 mM ATP. Flow rate: 1.02 ml/min; sample volume: 0.2 ml.

SUMMARY

In the present investigation immobilized hexokinase was used as a model system to evaluate the thermal analyzer coupled with a flowthrough immobilized enzyme column. The response of the thermal analyzer was successfully simulated by Eq. (3). The thermal analyzer offers a simple and rapid method for the determination of a biochemically important compound which could not be detected with bioelectrochemical sensors.

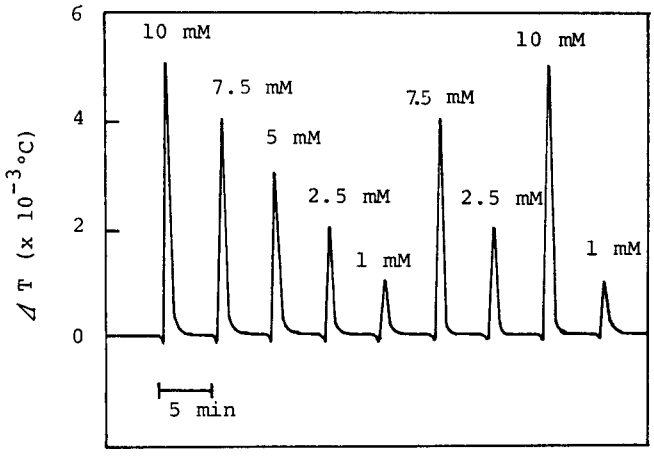

FIG. 9. Reproducibility of the responses to ATP. Flow rate: 1.02 ml/min.

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