Biochimica et Biophysica Acta, 403 (1975) 256—265
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

BBA 67586

DETERMINATION OF HEAT CHANGES IN THE PROXIMITY OF IMMOBILISED ENZYMES WITH AN ENZYME THERMISTOR AND ITS USE FOR THE ASSAY OF METABOLITES

KLAUS MOSBACH, BENGT DANIELSSON, ANDERS BORGERUD and MARGARETHA SCOTT

Biochemical Division, Chemical Center, University of Lund, P.O. Box 740, S-220 07 Lund 7 (Sweden)

(Received April 18th, 1975)

Summary

A device, the enzyme thermistor, is described which is capable of measuring changes in heat due to enzymic reactions. The sensor, a thermistor, is in direct contact with the site of reaction through its placement in a microcolumn filled with an immobilised enzyme preparation. The substrate solution flows past the thermistor tip, and as much as approx. one half of the total heat evolved can be registered as temperature change, Δt.

Glass-bound glucose oxidase (EC 1.1.3.4), penicillinase (EC 3.5.2.6), trypsin (EC 3.4.21.4) and urease (EC 3.5.1.5) were used for the determination of glucose, penicillin G, benzoyl-L-arginine ethyl ester and urea respectively.

Linear relationships between the Δt recorded and the concentration of substrate were obtained in all cases.

Introduction

Microcalorimetry for the study of biological systems such as enzymic reactions, and as an analytical tool, has received increasing attention during the last few years [1]. Excellent microcalorimeters are now commercially available although wider application of these instruments may be hampered by their relatively high cost and the long periods of measurement necessary. In the present paper an alternative and simple approach is offered to the thermal analysis of biological systems, using what may be described as an "enzyme-thermistor". Here the sensor is a thermistor which is placed in close contact to the site of reaction. This is accomplished by placing the thermistor in the bed of a microcolumn filled with immobilised enzyme and permitting the substrate solution to flow over the thermistor tip. It is this same utilisation of proximity, i.e. of sensor to enzymic reaction, which is also the underlying principle of

enzyme electrodes and which in the latter case has already found wide application [2,3].

Immobilised enzymes have already been used in connection with microcalorimetry, when the enzyme preparation has been packed in a flow-through cell of a LKB thermopile conduction calorimeter [4]. The inherent advantages of using immobilised enzymes, such as the possibility of repeated use and the often observed increased stability, can be utilised in this manner.

With the device discussed in the present paper, under the conditions given as much as approx. one half of the total heat evolved could be registered as temperature change, Δt. This high efficiency can be ascribed to (a) the above-mentioned proximity of sensor to site of reaction, (b) to the fact that the heat-sensing device is placed in the path of the heat flow and thus will also register the heat formed in the lower regions of the column and (c) to the low heat capacity of the unit. The enzyme-thermistor was applied to the determination, in particular, of D-glucose, penicillin G and urea using glass-bound glucose oxidase, penicillinase and urease respectively. Linear relationships of Δt measured and concentration of substrate present were observed. Electrical calibration also showed linearity between the applied power (W) and the temperature differences recorded.

Materials and Methods

Materials

Urease (jack bean, type III, 28 units/mg), trypsin (twice crystallised, 36 units/mg) and glucose oxidase (*Aspergillus niger*, type V, 1250 units/6.25 mg) were obtained from Sigma (St. Louis, Mo., U.S.A.). Penicillinase (B grade 1360 units/vial, total wt 25 mg) was purchased from Cal Biochem. (San Diego, Calif.). Urea was obtained from Merck (Darmstadt, G.F.R.) benzoyl-L-arginine ethylester and penicillin G (benzyl-penicillin, sodium salt) were purchased from Sigma (St. Louis, Mo., U.S.A.). G-Aminopropyltriethoxysilane was obtained from Pierce Chemical Company (Rockford, Ill. U.S.A.) and CPG-10 controlled porous glass beads (72.9 nm, mesh 120/200) were purchased from BDH Chemicals (Poole, England). Control serum (lyophilised) was purchased from DADE (Division of American Hospital Supply Corporation, Miami, Fla. U.S.A.).

Enzyme-thermistor

All measurements were carried out with the apparatus shown in Fig. 1. The thin-walled glass column was filled with 0.8—1.0 ml of the enzyme-glass-bead preparation. The column was placed in a perspex cylinder with a lid equipped with outlet and a leaktight, adjustable holder for the thermistor-probe (thermistor type 44106, resistance at 25°C, 10 kΩ, temperature coefficient 4%, Yellow Spring Instruments, Ohio, U.S.A.). The thermistor (ITT type F23D) used for the experiment using glucose oxidase had a resistance at 25°C of 1.7 kΩ, temperature coefficient 4%. The thermistor could be placed at different heights inside the microcolumn depending on the range of substrate concentrations to be analysed. This arrangement in an intermediary position will permit measurements of Δt at low substrate concentrations, since in this case the enzymic reaction will be restricted to the lower part of the column. If

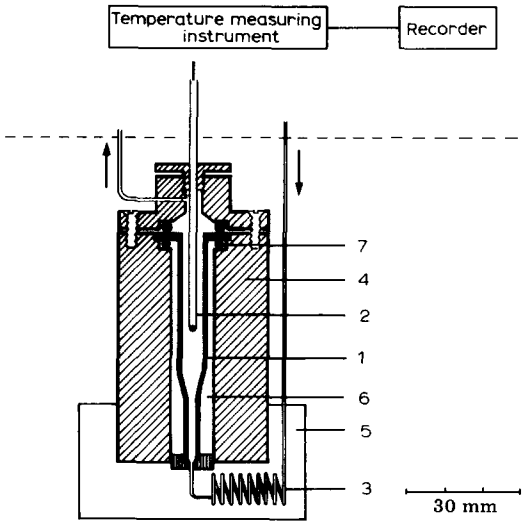

Fig. 1. The substrate solution is pumped through the heat exchanger (3) into the microcolumn (1) containing the immobilised enzyme preparation. The thermistor (2) is usually placed in the middle of the column. Around the lower part of the thermistor unit a water-jacket (5) made up of a plastic container is mounted. The space (6) between column (1) and the perspex cylinder (4) is airfilled. O-rings (7) are placed between the lid and the main unit. The entire measuring unit is immersed in a water bath, level as indicated by dotted line.

the sensor is placed at the top of the bed, heat losses to the surroundings are likely to be more pronounced. At the same time with the arrangement used also higher substrate concentrations can be analysed. All experiments reported here were performed with the thermistor bead placed in the centre of the enzyme bed and central in height. The substrate solutions were pumped from the bottom of the column after passing a coiled thin-walled stainless steel tube (250 mm long, 0.8 mm inner diameter) acting as heat exchanger. Constant ambient temperature was achieved by immersing the measuring cell in an ultra-stable water bath (Hetotherm, 05 PG 623 UO, Heto, Birkeroed, Denmark). As discussed below, far less sophisticated water baths can also be used. To minimise the effect of short-term temperature fluctuations due to convection in the water bath the heat exchanger was shielded by a plastic container. The water temperature was maintained at 27°C. Solutions were pumped through the system with a peristaltic pump (LKB-Beckman, Varioperpex), usually at 60 ml/h.

The variations in thermistor resistance were recorded with a high precision measuring bridge (Knauer, Wissenschaftlicher Geraetebau, W. Berlin, Germany) connected to a potentiometric recorder (Kontron, Switzerland). At the most sensitive range an output from the bridge of 100 mV corresponded to a temperature change of Δt = 0.023°C when using the YSI 44106 thermistor as calibrated with different water-bath temperatures. Buffer was constantly pumped through the cell and on turning a three way tap, the solution containing the substrate sample was pumped through the system at the same flow-rate as the buffer for either 60 s or 90 s. After each determination the system was allowed to equilibrate for 1—2 min. Substrate and buffer solutions were kept outside the water-bath.

Electrical calibration

Electrical calibration was carried out by placing an insulated, coiled manganin wire of known resistance (54.3 Ω/m) in the microcolumn. To cover as much as possible of the power range obtained in the enzymic reactions, two different lengths of resistance wires were used: 8.6 cm (4.7 Ω), 0—7 mW and 136 cm (73.7 Ω), 0—30 mW. The shorter wire was situated near the inlet, while the longer occupied approx. the lower one third of the column. The heating effect produced by the wire was varied by changing the current at constant voltage. A reference power supply (PT 2248, Philips, Netherlands) was used as constant voltage source and the voltage and current were measured with a digital multimeter from Keithley Instruments, Model 160, (Cleveland, Ohio, U.S.A.). The same flow-rate and buffer was used as for enzyme analysis (below).

Enzyme immobilisation

Preparation of alkylamino glass with G-aminopropyltriethoxysilane was carried out following a procedure developed by Weetall and described in the literature [7].

Coupling of the enzymes to alkylamino glass was carried out after activation with 2.5% glutaraldehyde and followed essentially a procedure given in the literature [7]. To 1 g (dry wt) of alkylamino glass were added: 3 ml of trypsin solution (125 mg) in 0.1 M sodium phosphate buffer (pH 7.0), or 4 ml of glucose oxidase solution (25 mg) in 0.1 M sodium phosphate buffer (pH 7.5), or 3 ml of urease solution (100 mg) in 0.1 M sodium phosphate buffer (pH 6.0) and one vial of penicillinase (25 mg) dissolved in 3 ml of 0.1 M sodium phosphate buffer (pH 7.0).

Assays

Enzymic determinations of the various compounds with the thermistor were carried out as follows: Penicillin G was pumped through the column in 0.1 M sodium phosphate buffer (pH 7.0), containing 0.006% sodium azide; urea in 0.1 M sodium phosphate buffer (pH 7.0), 1 mM in both EDTA and reduced glutathione; benzoyl-L-arginine ethylester in 0.1 M Tris buffer (pH 8.0), 1 mM in $CaCl_2$; D-glucose in 0.1 M sodium phosphate buffer (pH 7.0).

Determinations of the effluents obtained after passage through the column, serving as a check on the extent of the enzymic reactions, were carried out spectrophotometrically. Penicillin G was determined according to the described procedure [5] and urea also according to the literature [6]. An alternative approach, i.e. recycling of the effluent through the thermistor to ensure completeness of reaction was carried out in the glucose oxidase experiment.

Results and Discussion

Enzyme thermistor

The device shown in Fig. 1 has been applied throughout for the various measurements. In our opinion the arrangement of the sensor directly in the flow-path as utilised here represents a major advance over the previously described device in which the thermistor was kept separate and merely encircled

by a coil of tubing packed with the immobilised enzyme preparation [8]. The advantages are that the system is far simpler to handle, that higher flow-rates can be obtained and that the sensor and the site of the enzymic reaction are closer to each other. It is also far more efficient in heat response than recently reported devices in which trypsin was either directly immobilised onto a thermistor by crosslinking with glutaraldehyde [9] or by entrapping trypsin within a dialysis bag around a thermistor [10]. For instance, as will be seen below under similar conditions, addition of a solution of benzoyl-L-arginine ethylester containing 10 μmol to immobilised trypsin resulted in Δt changes of 0.25 m°C [9] and 3 m°C [10] respectively whereas with the unit described here a Δt of 46 m°C was recorded. We feel that the far lower Δt found with the two first mentioned devices can be ascribed to great losses of heat to the surrounding medium. Also problems of immobilising sufficient enzyme activity (particularly with the first approach) as well as diffusional hindrance of substrate/product through the membrane in the second device will occur. The enzyme-thermistor described here combines two advantages over the devices discussed above: (a) the microcolumns can be filled with, in most cases, sufficient enzyme preparation to ensure complete reaction and (b) through insulation of the column, and, more important, through the actual transport of heat to the thermistor by the flow, losses of heat to the surroundings are minimised; in addition the flow of substrate reduces diffusional hindrances. The situation prevailing can thus be described as semi-adiabatic. It should be added that filling of the column is easily accomplished on removing the lid and that no reference thermistor was found necessary for the various enzymic experiments described below.

The enzymes used, penicillinase, urease, glucose oxidase and trypsin were immobilised to alkylamino glass following glutaraldehyde activation. Glass was used as support because of its mechanical stability. Other immobilised enzyme preparations such as those based on acrylic polymers [4,11,12] as matrix, should also be applicable for the enzyme thermistor.

Amino acid analysis of one of the glass-bound enzymes, urease was carried out. 44 mg of protein was found bound to 1 g of dry glass support.

In Fig. 2a the response measured as Δt is depicted after letting a pulse (1.5 ml) of 10 mM penicillin G pass over the glass-bound penicillinase. As is seen, a Δt as high as 0.08°C is obtained. The base-line in the figure is somewhat idealized; however, the peaks of the slightly wavy baseline only reached at most 2 per cent of the substrate peak found. Simple water-baths have recently been employed yielding baselines sufficiently good for a number of substrate determinations in the concentration range of 1 mM.

In Fig. 2b the response to the same concentration continuously flowing through the system is depicted. The fact that the recorded Δt is higher when continuous flow is applied is due to the fact that the amount of substrate present in the pulse is not sufficient to give heat equilibrium. This is in agreement with previous findings using conventional flow-microcalorimeters [4].

In fig. 3a, b the recorded Δt values are plotted against the amount (μmol) of penicillin G present in 1.5 ml pulses. As is seen from the figures linear relationships were found in the concentration range from 1 μmol of penicillin and higher. The slight curvature found below 1 μmol in the sample might be due to interference from some non-specific heat whose contribution will be

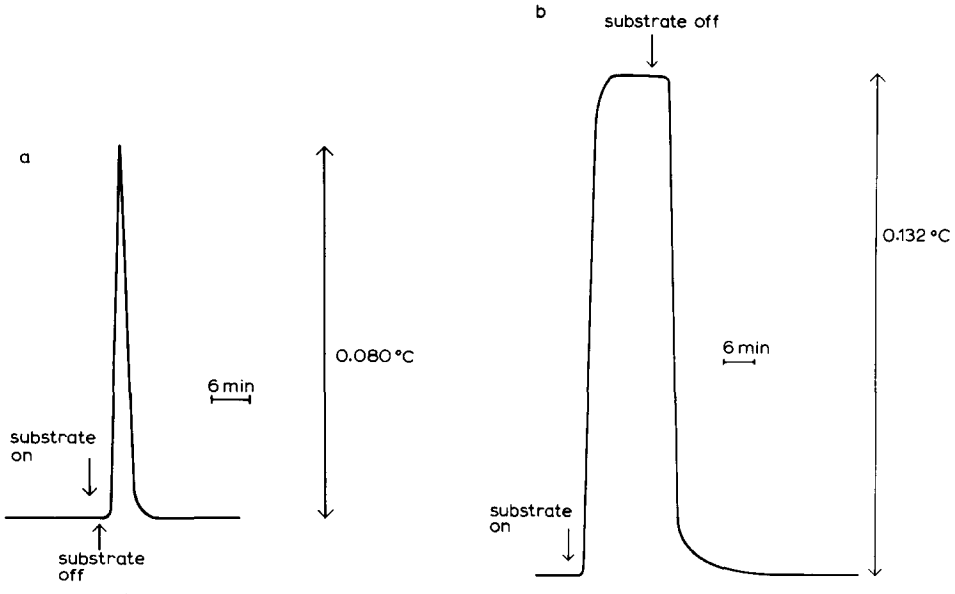

Fig. 2a. Experimental curve obtained after injection of a pulse of 1.5 ml of 10 mM penicillin G solution at a flow-rate of 60 ml/h through the enzyme thermistor containing glass-bound penicillinase. (b). Experimental curve obtained by continuously flowing substrate, 10 mM penicillin, through the enzyme thermistor at the same flow-rate.

more pronounced at lower substrate concentrations. A Δt of as high as 1 °C was obtained for 150 μmol of penicillin G. On the other hand as little as 0.38 μmol (=0.25 mM) which gave a Δt of 0.004 °C can be safely determined. Reproducibility of the Δt recorded was excellent varying usually be less than ±2 per cent on successive runs or when tested at 1-day's interval. The storage stability of the enzyme preparation was also good. Thus, after storage at room temperature for about 3 weeks the Δt response to a 10 mM solution of penicillin G showed no reduction. However it should be pointed out that since excess enzyme activity was probably present in the column some decrease in total enzyme activity due to denaturation would escape detection and therefore no general statement on increased stability of the immobilised penicillinase can be made.

In Fig. 4 the Δt response found for urea using immobilised urease is plotted. Again strict linearity is found except at the highest urea concentration tested which is probably due to insufficient amounts of active urease present in the column. However within the range 2.5 mM (4 μmol)–7 mM (11 μmol), which represents the concentration of urea normally found in human blood serum, strict linearity is obtained. In a preliminary test to check the potential of the method for clinical analysis the urea content of freeze-dried commercially available standard serum (DADE) was determined and found to be in good agreement with the values given by the manufacturer (= determined with an autoanalyser). More detailed studies on this aspect are in progress.

Finally in Fig. 5 the response on addition of glucose to a column containing glucose oxidase was measured. Again linearity was found which was rather abruptly lost at higher concentrations of glucose which we ascribe to a limiting amount of glucose oxidase activity present in the column and/or limiting

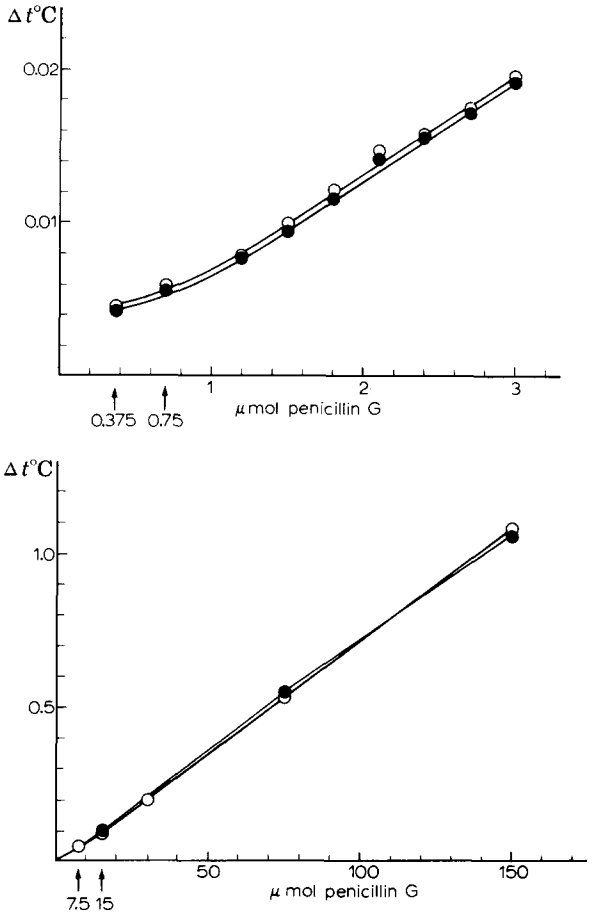

Fig. 3a,b. Measured peak heights (Δt) in °C as a function of the amount of penicillin G present in 1.5 ml and pumped through the enzyme thermistor containing glass-bound penicillinase at a flow-rate of 60 ml/h. The values obtained are from two successive runs at 1-day's interval. ●, first day; ○, second day.

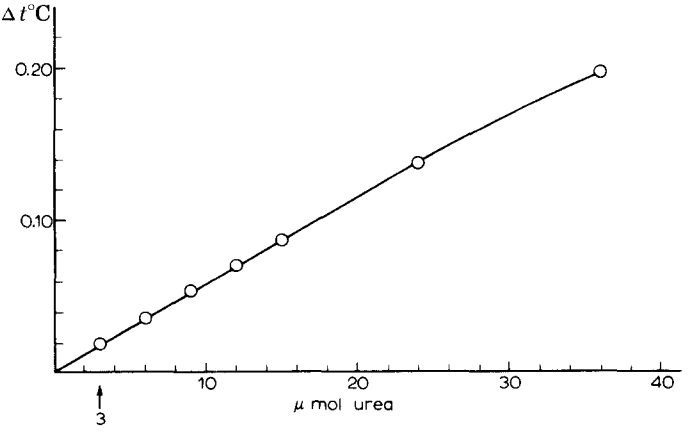

Fig. 4. Measured peak height (Δt) in °C as function of the amount of urea present in 1.5 ml and pumped through the enzyme thermistor containing glass-bound urease at a flow-rate of 60 ml/h.

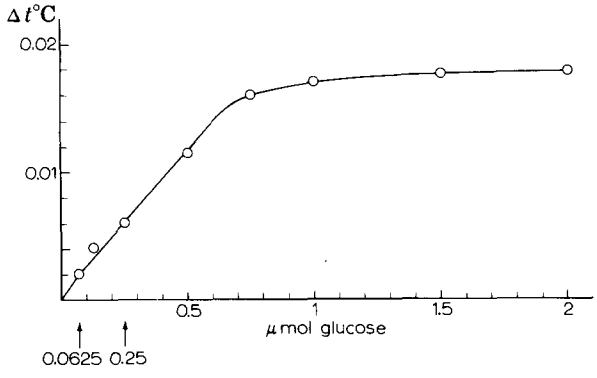

Fig. 5. Measured peak heights (Δt) in °C as a function of the amount of D-glucose in 1.0 ml and pumped through the enzyme thermistor containing glass-bound glucose oxidase at a flow-rate of 60 ml/h. In this experiment another type of thermistor (see Experimental section) was used giving an even more stable base-line.

amounts of oxygen dissolved in the medium. This was confirmed by the fact that at the higher concentrations tested, unreacted glucose was found in the effluent.

To further substantiate the observed linear relationships between Δt response and substrate concentration, electrical calibration was carried out. The short wire placed at the inlet of the column showed initial linearity between the Δt recorded and the power applied (Fig. 6) whereas the longer wire which was placed in the lower third of the column, thus representing a better parallel to the actual enzymic measurement, gave strict linearity over the whole range tested. (We are aware of, of course, that the heat-diffusion from a resistance-wire cannot wholly parallel the more homogenous heat-formation from the enzyme reaction of the column (as can be seen in Fig. 6). This linearity implies that within the range of our enzymic tests, there is no change in efficiency as higher temperature changes are measured. However, electrical calibration does not permit the determination of the efficiency of the enzyme-thermistor and thus does not give information as to the fraction of the total heat formed which is recorded as Δt. However, this can be estimated in the following indirect fashion. For example, using immobilised trypsin and applying a pulse of 1.5 ml of 5 mM benzoyl-L-arginine ethylester at a flow of 60 ml/h, a Δt of 0.034°C was obtained with the enzyme thermistor. From literature data [4] obtained with a microcalorimeter measuring the total heat of reaction, the ΔH of this reaction is 60 mJ/μmol. Thus 5 mW are formed (0.0833 μmol/s × 60 mJ/μmol). The liberated energy would theoretically give a Δt of 0.07°C in the sample volume (90 s × 5 mW/4.19 J/g, °C × 1.5 g). The dotted line in Fig. 6 represents this theoretical relationship between Δt and liberated energy in the sample. It can be seen that the Δt recorded is as high as approx. 50% of the theoretical value. To be able to obtain accurate determinations of the absolute heat of unknown reactions, however, more such comparative studies have to be carried out before an absolute efficiency coefficient of the simple device described here can be determined. It is obvious that both the flow rate and the

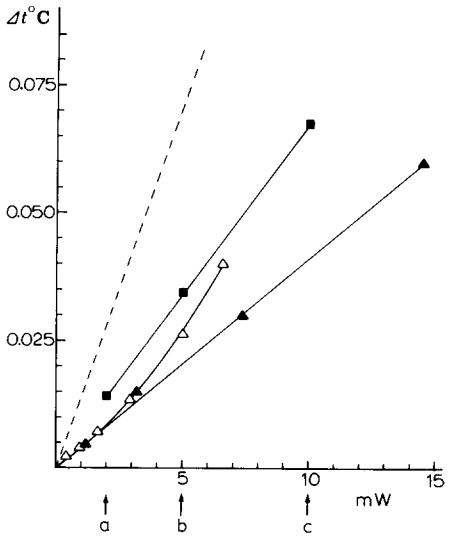

Fig. 6. Electrical calibration. Δt measured is plotted as a function of the power (mW) produced by two different resistance wires, 4.7 Ω (△) and 73.4 Ω (▲) respectively. In the same figure are also plotted the Δt values (■) recorded, obtained with immobilised trypsin at three different concentrations of benzoyl-arginine ethylester: (a) 2 mM, (b) 5 mM and (c) 10 mM applied as pulses of 1.5 ml of a flow rate of 60 ml/h. The corresponding power values (mW) on the abscissa were calculated from the known heat of reaction, ΔH = 60 mJ/μmol found under similar conditions [4] in 0.1 M Tris-buffer (pH 7.9), (μmol × 60 mJ/μmol × 1/90 s). The dotted line, which is said to represent 100% conversion of reaction heat to Δt recorded, shows the relationship between the power applied to 1.5 ml sample volumes and the resulting Δt, under ideal adiabatic condition and ignoring all other heat capacities than that of the sample volume itself.

position of the thermistor within the column have to be strictly defined. However such information is not required for analytical determinations, since calibration is relatively easily carried out. In order to construct a true calibration plot for samples of biological origin to be analysed, it is advisable to add known amounts of the substrate to be determined to the sample such as serum. In doing so Δt changes, which might be caused by components in the system other than substrate such as inhibitors are thus accounted for in the calibration plot.

In conclusion the enzyme thermistor should be applicable for a wide range of substrates to be determined. It is noteworthy that a reference thermistor has been shown not to be required. However, when applying substrate concentrations in the 10^{-4} M range and below, the use of a differential arrangement with an additional thermistor mounted in the lower region of the microcolumn may be advantageous since this will give even better base-lines. It deserves mentioning that since in many enzymic reactions protons are formed, buffers of high exothermic enthalpy of protonisation to increase the Δt response can be used [4].

Another technique of thermal analysis, which is somewhat related to the enzyme thermistor in having the sensor placed in the reaction chamber, is that of direct injection enthalpimetry = thermal titrimetry [13,14]. This technique can be described as a sort of "instantaneous thermometric titration", i.e. a

temperature pulse is formed and recorded in an adiabatic cell on injection of a reagent to the enzyme solution in the vessel followed by rapid mixing. Whereas thermal titrimetry is a very sensitive method, the number of successive runs possible is limited because of accumulation of reaction product(s) in the vessel. Further it appears to be difficult to use immobilised enzymes with such a technique. In addition, whereas the enzyme thermistor can also be applied to continuous analysis of various processes, this will not be possible with thermal titrimetry.

Finally it deserves mentioning that the enzyme thermistor may also find use as a simple assay tool for the study of the influence of matrix binding on enzyme kinetics such as in the determination of the apparent K_m of immobilised enzyme preparations.

Acknowledgements

The authors are indebted to Professor Wadsö from the Thermochemistry Laboratory, Chemical Centre for valuable discussions and advice given in particular for the electrical calibration experiment. The linguistic help of Mr Richard Venn is acknowledged.

References

1 Spink, C. and Wadsö, I. Methods in Biochemical Analysis (D. Glick, ed.), Wiley-Interscience, N.Y. in press
2 Guilbault, G.G. (1971) Pure Appl. Chem. 25, 727—740
3 Nilsson, H., Akerlund, A. and Mosbach, K. (1973) Biochim. Biophys. Acta 320, 529—534
4 Johansson, Å., Lundberg, J., Mattiasson, B. and Mosbach, K. (1973) Biochim. Biophys. Acta 304, 217—221
5 Boxer, G.E. and Everett, P.M. (1949) Anal. Chem. 21, 670—673
6 Wriston, J.C. (1970) in Methods in Enzymology (Tabor, H. and Tabor, C.W., eds), Vol. 17A, pp. 732—742, Academic Press, N.Y.
7 Biomaterial Supports (1973) Corning Biological Products Department, Medfield, Mass., U.S.A.
8 Mosbach, K. and Danielsson, B. (1974) Biochim. Biophys. Acta 364, 140—145
9 Cooney, C.L., Weaver, J.C., Tannebaum, S.R., Faller, D.V., Shields, A. and Jahnke, M. (1974) in Enzyme Engineering, (Pye, E.K. and Wingard, Jr., L.B., eds), Vol. 2, pp. 411—417, Plenum Press, N.Y.
10 Mosbach, K., Mattiasson, B., Gestrelius, S. and Srere, P.A., Ibid, p. 151
11 Nilsson, H., Mosbach, R. and Mosbach, K. (1972) Biochim. Biophys. Acta 268, 253—256
12 Johansson, A. Chr. and Mosbach, K. (1974) Biochim. Biophys. Acta 370, 339—347
13 Wasilewski, J.C., Pei, P.T-S and Jordan, J. (1964) Anal. Chem. 36, 2131—2133
14 Marini, M.A. and Martin, C.J. (1973) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds), Vol. 27, pp. 590—616, Academic Press, N.Y.