

## ENZYME THERMISTOR ANALYSIS IN CLINICAL CHEMISTRY AND BIOTECHNOLOGY

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**Abstract** - The combination of calorimetry and immobilized enzymes forms a pair with unique properties. Calorimetry is a general detection principle and specificity is introduced by the enzyme. We discuss here simple "semi-adiabatic" calorimeters and their application in clinical chemistry, enzyme immunoassay, process control and environmental control.

### INTRODUCTION

The enzyme thermistor is a simple analytical instrument that registers under flow conditions heat evolved in a small, well insulated flow chamber. Specificity is introduced by immobilized biocatalysts e.g. enzymes or whole cells placed in the chamber.

Calorimetry has long been recognized as a technique with great potential for studying biochemical systems (Refs. 1 & 2) because of its general detection principle. Almost every biochemical reaction is associated with heat evolution and this makes it possible in most cases to follow a primary reaction obviating the need for an additional auxiliary enzyme system (with its requirement for e.g. coenzymes). Furthermore, the calorimetric technique is independent of the optical properties of the sample studied.

Because of the generality of the technique all heat changes are registered. This lack of specificity is a limitation in determination of an individual kind of molecules which has to be compensated for by the inherent specificity of the biocatalyst utilized. On the other hand, when studying the overall reactions in complex biochemical systems, the generality is an advantage. In spite of its great potential, the applications of calorimetry to biochemical analysis have been hampered by the rather high instrument costs and also by the fact that calorimetry has been a field where conventional biochemists have had very little experience.

In recent years several simple, inexpensive devices for determination of e.g. urea and glucose, based on the combination of immobilized enzyme and the thermometric detection principle, have been introduced (Refs. 3-9). They are generally flow systems with less adiabatic properties than conventional calorimeters which makes them suitable for studies where no absolute thermodynamic information is required. The applicability of such simple calorimetric techniques is illustrated here by the variety of studies which have been carried out in the fields of clinical chemistry, enzyme immunoassay, process control and environmental control.

### EXPERIMENTAL

**Apparatus.** Although recent technical developments at our department include an enzyme thermistor with the columns housed in a thermostatted aluminium block, the major part of the work presented here has been performed with the simple apparatus originally described in Ref. 3 & 10 or with the split-flow device presented in Ref. 11. These are constructed mainly of plexiglass and are designed for use in a well thermostatted waterbath.

A simple single thermistor device is depicted in Fig. 1 A. A small plastic column (0.2-1 ml) contains the immobilized enzyme preparation. The column is mounted in the plexiglass housing surrounded by an airspace for thermal insulation in a holder designed for convenient change of columns. A small glass-encapsulated thermistor (e.g. Veco type 41A28, 1.5 x 6 mm, 10k $\Omega$  at 25 $^{\circ}$  C, temperature coefficient - 4.4 %; Victory Engineering Corporation, Springfield, N.J., U.S.A.) fixed at the tip of a stainless steel tube (2 mm outer diameter) constitutes the temperature probe, which is placed either directly above the Vyon (porous polyethylene) disc at the top of the column or inserted 2-3 mm into the column packing through a hole in the Vyon disc. The latter arrangement usually gives a more even temperature signal. All joints are O-ring sealed as indicated in the drawing. Buffer is continuously pumped through the enzyme thermistor using a peristaltic pump (Varioperpex, LKB-Produkter, Bromma, Sweden) first passing through a heat exchanger made of a piece of thin-walled

stainless steel tube (40-50 cm long, 0.8 mm inner diameter). The heat exchanger coil sits in a waterfilled plastic cup. The purpose of this arrangement is to minimize temperature fluctuations in the solution passing through the column. These temperature fluctuations are in the order of  $\pm 0.01^\circ\text{C}$  in the waterbaths normally used and less than  $\pm 10^{-4}^\circ\text{C}$  at the thermistor.

The temperature signal is registered with a potentiometric recorder coupled to a Wheatstone bridge. A suitable bridge is manufactured by Knauer Wissenschaftlicher Geratebau (W. Berlin, Germany). It delivers a 100 mV signal for a temperature change of  $0.02^\circ\text{C}$  at the most sensitive range. We have also used bridges of our own design with somewhat better resolution. Furthermore, it is often possible to increase the sensitivity by using a more sensitive recorder range. With the recorder span, e.g. at 10 mV, the temperature resolution will be  $10^{-3}^\circ\text{C}$  full scale.

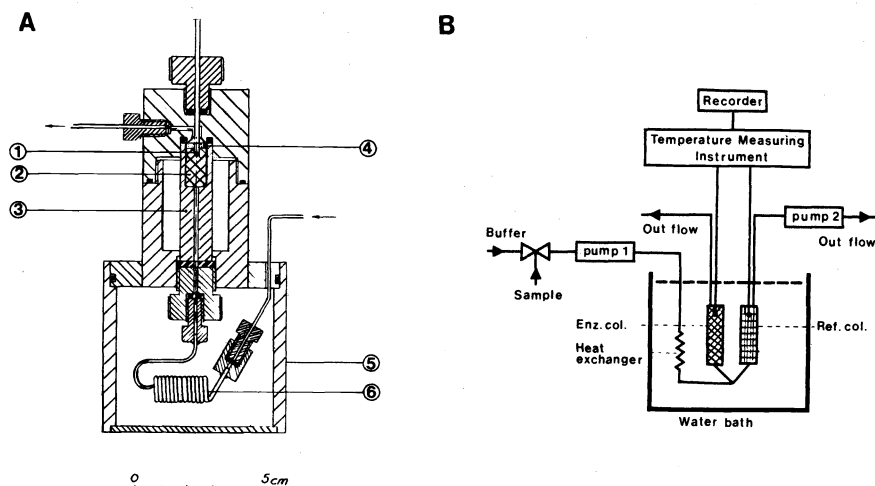


Fig. 1 A. A single thermistor device. 1. Thermistor. 2. Immobilized enzyme. 3. Plastic column. 4. Disc of porous polyethylene (Vyon). 5. Plexiglass container.

B. Schematic representation of a split-flow enzyme thermistor system. (This figure is reprinted from Ref. 9 with permission from the publisher).

Increased baseline stability and insensitivity to non-specific heat production caused by dilution or interactions with the matrix etc. has been achieved by using a split-flow enzyme thermistor (Ref. 11). Fig. 1 B is a schematic illustration of such an apparatus and ancillary equipment. Here two columns are used, one containing the immobilized enzyme and the other the reference column, which contains support material only and is inactive. Non-specific effects are assumed to be equal in the two columns so that the differential temperature signal recorded by the two thermistors at the outlets of the columns thus represents the true heat signal from the enzymic reaction. In this apparatus thermistors can also be mounted at the inlet of the column and this is valuable when working with continuous or large volume sample introduction since the reference column can then be omitted. The reason for this omission is that unspecific effects are generally noticeable only at the very ends of a sample plug. Since it is important that the flow through each column is identical, two pumps are used in an arrangement shown in Fig. 1 B.

**Procedure.** The system was equilibrated while buffer was pumped through the column(s) with a flow rate of 0.5-1 ml/min. The waterbath temperature was usually  $27.0^\circ\text{C}$ . Samples were introduced into the continuous flow either via a three-way valve before the pump or with different kinds of sample-loop injection valves placed after the pump. An advantage of the first arrangement is that sample volume can be easily changed, while the latter system gives highly reproducible sample volumes. We have also employed a septum injection valve for volumes of the order of  $10\ \mu\text{l}$  for concentrated samples. During its passage through the enzyme bed the substrate is converted into product and heat is liberated. The increased temperature of the fluid is registered by means of the thermistor at the top of the enzyme bed.

The samples may be introduced as short pulses resulting in temperature peaks as shown in Fig. 2 A. When the pulse length is increased a plateau is obtained. In this case, when the system operates under thermally steady state conditions, a higher amplitude is obtained. The sensitivity can thus be increased by using a long sample pulse, but at the expense of rapidity. We have generally used sample volumes in the range of 0.25-1 ml giving a sample handling capacity of 15-30 samples per hour. The steady state procedure is especially

useful at very low concentrations or in cases of non-specific heat effects due to differences in composition between perfusing buffer and sample solution. As already mentioned such effects are usually significant only at the ends of a sample pulse. Regardless of pulse length the temperature peak has been found to be a suitable measure of reaction heat.

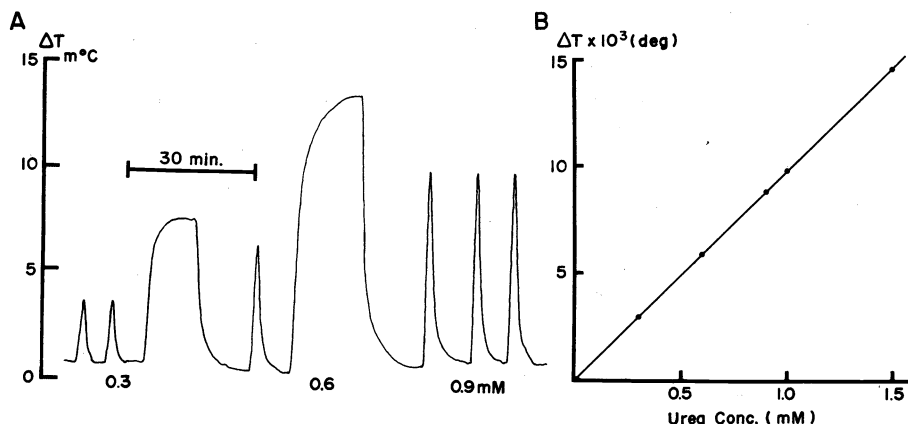


Fig. 2 A. Recorder tracing obtained at the determination of urea with a single column enzyme thermistor containing CPG-bound urease. Urea samples were introduced for 1 min. or for 12 min. in 0.1 M sodium phosphate buffer, pH 7.0, containing 2 mM glutathione and 1 mM EDTA at a flow rate of 0.74 ml/min. B. Standard curve for urea obtained with the same system using 1 min. urea pulses.

Linear correlation between the height of the temperature peaks and substrate concentration is obtained generally over large substrate ranges as illustrated by Fig. 2 B, which shows a standard curve for urea. The integral of the temperature peak is also linearly related to substrate concentration (Ref. 5) and may even give more accurate data than peak height but is more difficult to obtain. In addition, the slope of the ascending temperature peak is a useful measure of substrate concentration and may be of particular value in automated evaluation systems.

**Immobilization Techniques.** When using immobilized enzymes in flow systems some basic criteria must be fulfilled: a) the support must possess mechanical stability high enough to withstand physical stress (Ref. 12), b) the enzyme preparation must be stable from an operational point of view, i.e. contain an excess of catalytic power capable of compensating for any denaturation that may take place (Ref. 13) or, alternatively, the enzyme should be easily renewable on the support, preferably without taking the support out of the flow stream (i.e. reversible immobilization) (Ref. 14 & 15), c) the immobilized enzyme preparation must have good and constant flow properties and d) the support per se must not have any catalytic activity or tendency to interact with the sample that would disturb the measurement of the enzymic reaction.

It is evident therefore that the choice of support material and immobilization technique needs careful consideration. We have generally used controlled pore glass (CPG) as a support since it is well characterized, and has high enzyme-binding capacity, simple immobilization chemistry (Ref. 12), good stability towards pressure and it is not attacked by microorganisms. However, its structure is easily fragmented on grinding and some non-specific adsorption may occur. Thus, when no specific effects concerning flow properties or adsorption phenomena could be predicted, the enzyme was immobilized on CPG (40-80 mesh, pore diameter 55 nm; Corning Glass Works, Corning, N.Y., U.S.A.) derivatized with  $\gamma$ -aminopropyltriethoxysilane and glutaraldehyde prior to coupling (Ref. 12).

In immunological applications any unspecific adsorption of antigen or enzyme-labelled antigen to the immunosorbent would effectively prevent meaningful measurements. In these cases Sepharose CL 4B was used as support. As the mechanical stability of Sepharose is not as high as that of CPG, a lower flow rate was used in the enzyme thermistor. The antibodies were immobilized to the Sepharose beads using the BrCN-technique (Ref. 16).

When applying crude solutions to the enzyme thermistor system, as for example in the analysis of lipemic sera in clinical samples, in the analysis of samples from microbiological fermentations or of waste water samples in environmental control, a flow system with a low tendency to clog should be used. In these cases it is advantageous to use nylon tubing. The enzyme was covalently coupled to the inner surface of the nylon tubing and the

lumen of the tubing was free for transport of particles present in the sample (Ref. 17). Coupling of the enzyme was performed using glutaraldehyde after treatment of the nylon tubing with dimethylsulphate followed by derivatization with polyethyleneimine (Ref. 18).

Cells have been immobilized using two different procedures: a) microbial cells were entrapped within a three-dimensional lattice of 15 % polyacrylamide (T/C = 95/5) and b) animal cells (e.g. red blood cells) were immobilized utilizing the biospecific interaction between lectins and glycoproteins on the cell membrane (Ref. 19).

**Amplification.** Although most enzymic reactions are accompanied by heat production of at least 20 kJ/mol, permitting enzyme thermistor determinations with a sensitivity of  $10^{-6}$  -  $10^{-5}$  M, some reactions produce very little heat. For example, on hydrolysis of an ester by trypsin (E.C. 3.4.4.4) almost no heat is evolved. In spite of this the reaction can be followed thermometrically because the hydrolytic step produces a proton which protonates the buffer and the protonization heat can be registered. As the heat of protonization varies markedly between different buffers, it is important to use buffers with high values of heat of protonization (Ref. 20). It was thus shown using a flow microcalorimeter that the heat signals were approximately 10 times higher when the reaction was carried out in TRis-HCl buffer as compared with phosphate buffer (Ref. 21).

Another method of amplifying the heat signal is to use co-immobilized sequentially operating enzymes. This means that when the substrate is converted in the primary enzyme-catalyzed reaction, a second sequentially acting enzyme in close proximity to the first continues the modification of the former substrate molecule. The heat signals from the enzyme reactions are superimposed and registered as a measure of the first enzyme's substrate concentration, which makes the analysis more sensitive. Furthermore, the co-immobilized sequentially acting multi-step enzyme systems offer other advantages (Ref. 22), for example a faster conversion as compared with the situation where the enzymes are immobilized on separate polymer beads and better efficiency at low substrate concentrations. These facts have been utilized in some analytical applications, with the enzyme thermistor (Ref. 23) as well as with enzyme electrodes (Ref. 24). Finally, specific arrangements to amplify single reactions may be possible. It was demonstrated that all enzymes using oxygen as "cosubstrate" are limited at higher substrate concentrations due to oxygen deficiency. The concentration range for analysis was substantially increased by co-immobilizing an artificial oxygen supplier, e.g. red blood cells together with the enzyme (Ref. 19). A similar effect is obtained by co-immobilizing catalase (E.C. 1.11.1.6.), which restores half of the oxygen consumed by the oxidase thereby extending the linear range and simultaneously increasing heat production. The sensitivity of the system was increased due to the extra heat from the catalase reaction (Ref. 9).

#### ENZYME THERMISTOR APPLICATIONS IN CLINICAL CHEMISTRY

The applicability and properties of the enzyme thermistor were first investigated in the field of clinical chemistry and it is within this area that most work has been done. Practically useful methods have been designed for the determination of urea (Ref. 5) and glucose (Ref. 9) in serum samples. Other metabolites studied are cholesterol and cholesterol esters, oxalic acid, creatinine, various antibiotics, sugars, phenol and hydrogen peroxide.

Enzyme thermistor determination of serum urea has been carried out using urease (E.C. 3.5.1.5) immobilized on controlled pore glass. A standard curve obtained for aqueous urea standards is shown in Fig. 2 B. Linearity has been observed for remarkably wide ranges of

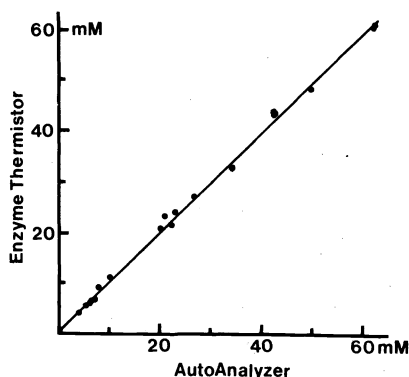


Fig. 3. Urea determination in human serum. Comparison between results obtained with a single-thermistor enzyme thermistor and with an AutoAnalyzer using the diacetyl monoxime procedure.

urea concentration - typically 0.01-200 mM. Serum samples were usually diluted 10-fold, so the working range was approx. 0.3-10 mM. The high catalytic excess of the enzyme column gives the system high operational stability. The precision is high - the coefficient of variation for samples within a run is less than 1% - being largely governed by the precision in the sample introduction. Because of the rapid and simple starting up procedure of an equilibrated enzyme thermistor as well as the operational stability, the enzyme thermistor is especially useful for urgent urea determination. Fig. 3 shows the correlation between serum urea concentrations determined with the enzyme thermistor and a common colorimetric method (Ref. 5). Urea analysis with an enthalpimetric technique similar to ours is described in Ref. 25.

For glucose determination we have employed glucose oxidase (E.C. 1.1.3.4), generally in combination with catalase (Ref. 9). These enzymes have also been used in another enzyme thermistor-like technique (Ref. 7). Glucose determinations have furthermore been successfully carried out by flow enthalpimetry using immobilized hexokinase (E.C. 2.7.1.1) (Ref. 4). We have preferred the use of glucose oxidase because of its high stability and because no cofactors are required. A disadvantage is, however, that linearity is obtained only up to 0.45 mM glucose (or 0.7 mM if catalase is also employed) as is illustrated in Fig. 4 A. This means that for glucose determination in human serum, samples have to be diluted 50- to 100-fold. However, it is also possible to inject small volumes (5-20  $\mu$ l) of serum directly into the buffer stream entering the enzyme thermistor. In these determinations both single thermistor and split-flow apparatus have been used. The precision in the determinations in both cases was high: coefficient of variation less than 0.6% for within-day samples. We encountered some difficulties in the analysis of serum samples containing sodium fluoride with single thermistor devices. The sodium fluoride, which is added to the blood sample as a preservative, has a considerable heat of dilution resulting in a high and irreproducible background heat signal. This problem was, however, easily overcome by including at least 0.1 M sodium fluoride in the assay buffer. Glucose levels determined by the enzyme thermistor in serum samples correlated well with the values obtained with a conventional, spectrophotometric, enzymic technique used at a hospital's routine laboratory (Fig. 4 B).

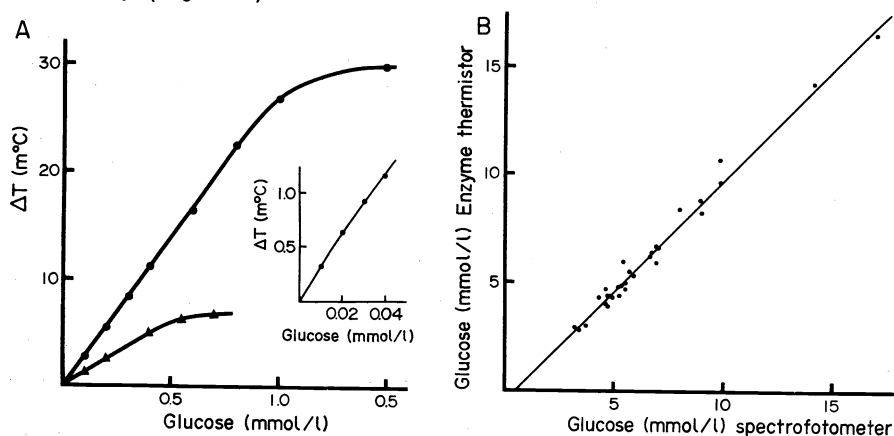


Fig. 4 A. Temperature response as function of glucose concentration in 1 ml samples at a flow rate of 1 ml/min. A single column enzyme thermistor was used and the enzyme preparation was either glucose oxidase + catalase ( $\bullet$ ) or glucose oxidase only ( $\blacktriangle$ ).

B. Serum glucose as measured with the enzyme thermistor (split-flow) versus a spectrophotometric hexokinase/glucose-6-phosphate dehydrogenase technique. (The figures are reprinted from Ref. 9 with permission).

Cholesterol has been measured using cholesterol oxidase (from *Nocardia erythropolis*, E.C. 1.1.3.6) (Ref. 23). The assays were run in phosphate buffer containing 12% ethanol and 8% Triton X-100. The temperature response was linear only in the concentration range 0.03-0.2 mM cholesterol due to low enzyme load. Cholesterol in serum could be determined after organic solvent extraction, evaporation of the organic phase and dissolving the residue in the above mentioned buffer. A feasible system capable of discriminating between cholesterol and cholesterol esters could be readily accomplished by using a precolumn of immobilized cholesterol esterase (E.C. 3.1.1.13), which is commercially available, arranged so that it could be switched in and out of the flow to the enzyme thermistor.

Determination of penicillin G was first described in one of the earlier enzyme thermistor studies (Ref. 10). With penicillinase (beta lactamase, E.C. 3.5.2.6) bound to CPG-glass it has been possible to measure benzylpenicillin concentrations down to 0.01 mM with linear response up to 200-300 mM.

Current studies on antibiotics include measurement of cephalosporins utilizing cephalosporinase (E.C. 3.5.2.8). Fig. 5 A shows a response curve for aqueous cephalosporin standards obtained with the enzyme thermistor containing cephalosporinase bound to CPG (Ref. 26). Similar results have been obtained with the enzyme coupled to agarose (Sea Sep., Marine, U.S.A.). The sensitivity of the direct enzyme thermistor methods is in most cases not sufficient for determination of antibiotics in therapeutic doses in blood. For such purposes a special enzyme immunoassay (TELISA) has been designed, where the enzyme thermistor is used to follow the activity of the marker enzyme (see below and in Ref. 27).

Besides determination of glucose we also have described a procedure for the determination of lactose (Ref. 23). The sample solution was passed through a column containing CPG-bound lactase (E.C. 3.2.1.23) before entering the glucose oxidase/catalase thermistor. Based on the glucose standard curve, linear response can be expected in the range 0.01-0.7 mM lactose assuming complete conversion in the precolumn. In a similar way other disaccharides such as sucrose, maltose (Ref. 28) and cellobiose (Ref. 29) can be measured (see Table 1). In addition, sucrose has been measured directly with the enzyme invertase (E.C. 3.2.1.26) placed in the enzyme thermistor. The measuring range was 0.05 to 100 mM and this example demonstrates one of the advantages associated with the enzyme thermistor since this reaction can be followed directly, which is not possible with other techniques as photometry. Ascorbic acid is another substance in this category and using ascorbic acid oxidase (E.C. 1.10.3.3) linearity was obtained between 0.05 and 0.6 mM (Ref. 28).

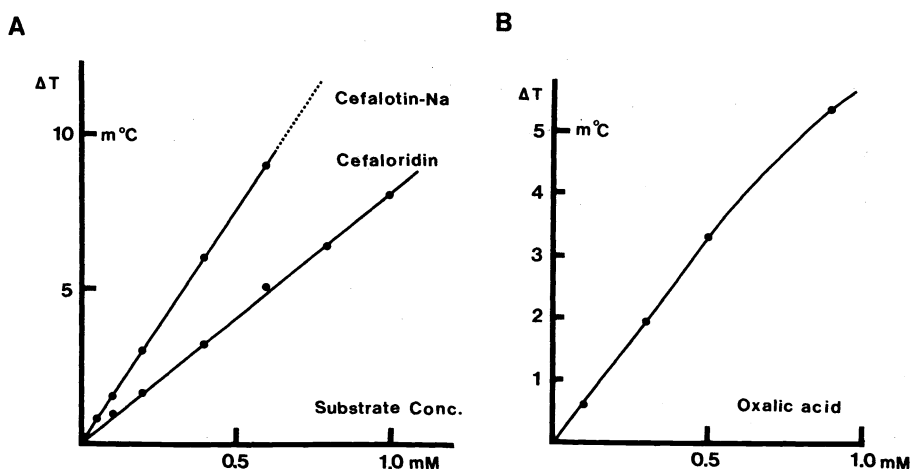


Fig. 5 A. Temperature response curves for two different commercial cephalosporin preparations: cephaloridine and the sodium salt of cephalothin. Sample pulses (1 ml) were introduced with a flow rate of 0.8 ml/min. into a simple column enzyme thermistor containing 100 units of CPG-bound cephalosporinase. The buffer used was 0.1 M Tris-HCl, pH 8.3.

B. Response curve for oxalic acid dissolved in 0.1 M citric acid - 0.05 M sodium phosphate buffer, pH 3.4, containing 2 mM EDTA obtained with a simple thermistor enzyme thermistor, operated at a flow rate of 0.7 ml/min. Sample pulse length was 1 min.

Promising results have been obtained in the determination of oxalic acid and creatinine. An assay procedure for oxalic acid based on oxalic acid decarboxylase (E.C. 4.1.1.2) was described earlier (Ref. 30); this enzyme had rather limited stability and gave only moderate heat. Preliminary results with oxalic acid oxidase (E.C. 1.2.3.4), prepared from barley roots (Ref. 31) have been very encouraging since this enzyme is more stable and the reaction yields considerable heat. The linear range obtained so far is 0.01-0.5 mM (Fig. 5B). Tests with urine samples revealed problems with non-specific heat production, which however can be avoided if an ion exchange step is included. After passing the urine through a weakly basic ion exchanger, the oxalic acid can be readily eluted and applied to the enzyme thermistor.

For the determination of creatinine there are also two different enzymes available: creatinine amidohydrolase (E.C. 3.5.2.) which produces creatine and creatinine iminohydrolase (E.C. 3.5.4.2.1) which cleaves off an imino group producing N-methylhydantoin. The former enzyme is used in a spectrophotometric assay as the first enzyme in a long sequence (Ref. 32) but has poor stability and limited heat production in the enzyme thermistor. The latter enzyme, the deiminase, on the other hand, seems to be more stable and produces heat enough for a lowest measurable concentration of at present  $10^{-5}$  M creatinine in buffer solution (Ref. 33). This sensitivity may be sufficient for the determination of serum creatinine,

but more work remains to be done before a working, useful method is developed.

Finally, with an enzyme thermistor technique based on specific reversible immobilization using antigen-antibody interaction (Ref. 34) phenol, tyrosine and hydrogen peroxide among others could be conveniently measured. Measuring ranges are given in Table 1, which summarizes current results obtained with various substances.

#### THERMOMETRIC ENZYME-LINKED IMMUNOSORBENT ASSAY (TELISA)

The use of immunosorption for assay of endogenous and exogenous compounds in biological fluids has received enormous attention during the last decade. Initially radio immunoassay was developed (Ref. 35) and has later been followed by methods based on the use of other markers e.g. fluorescent probes (Ref. 36), phages (Ref. 37) and enzymes (Ref. 38). Enzyme immunoassay (EIA) is based on the interaction between an antigen and a specific antibody with one of the moieties involved in the interaction being labelled with an enzyme.

The enzyme thermistor has mainly been used in a competitive enzyme-linked immunosorbent assay (ELISA) procedure. The column in the thermistor unit is filled with an immunosorbent consisting of antibodies immobilized on Sepharose CL 4B. The assay scheme is shown in Fig. 6. Here it is seen that the time of contact between the immunosorbent and the sample to be analyzed is very short - of the order of only 1-2 minutes. This means that the binding reaction between antigens and antibodies is far from equilibrium. Furthermore, it can be seen from the same figure that after analysis of the amount of bound enzyme, a dissociation step is inserted and because of this the immunosorbent can be reused over prolonged periods of time (Ref. 39). The dissociation step is critical since any remaining enzyme on the column would severely influence the results of subsequent analyses. Glycine-HCl, pH 2.2, fulfilled the demands set on this step in the system studied.

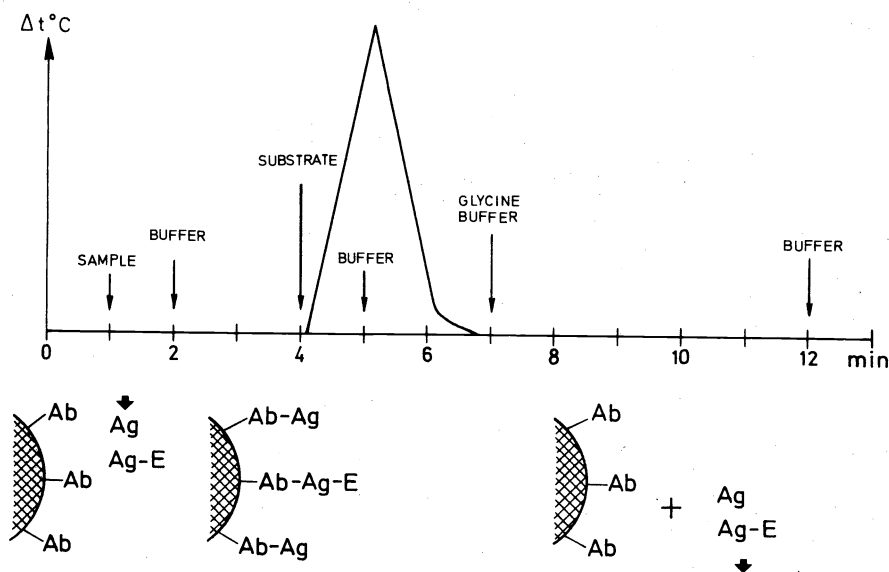


Fig. 6. Schematic presentation of a reaction cycle in the TELISA procedure. The arrows indicate changes in the perfusing medium (flow rate 0.8 ml/min.). The cycle starts with potassium phosphate buffer pH 7.0 (0.2 M). At this time the thermistor column contains only immobilized antibodies. At the arrow "sample" a mixture of antigen and catalase-bound antigen is introduced. The system is then washed with potassium phosphate buffer for two minutes. The sites on the antibodies of the column are now occupied by antigen as well as by catalase-labelled antigen. The amount of catalase bound is measured by registering the heat produced during a one-minute pulse of the substrate 1 mM  $H_2O_2$ . After the heat pulse is registered, the system is washed with 0.2 M glycine/HCl, pH 2.2, to split the complex. After five minutes of washing, phosphate buffer is introduced, and the system is ready for an other assay. (Reprinted from Ref. 27 with permission from the publisher).

The enzyme antigen conjugates (usually involving catalase or peroxidase as marker enzymes) were prepared following conventional methods and they were purified using gel chromatography.

In one study, gentamicin was analyzed in standard solutions as well as in serum samples (Ref. 40). First a standard curve was set up (Fig. 7 A); 100 % was set equal to the heat signal obtained with no free antigen in the assay mixture.

It is thus seen that on increasing the amount of free antigen less and less enzyme labelled antigen is bound to the column. Serum samples were then assayed and the results were compared with the values obtained from conventional microbiological assays; as seen in Fig. 7 B, the two methods correlate well ( $y = 0.98x$ ). Owing to the few pipetting steps, the omission of separate washing steps and the isokinetic character of the system (requiring well defined, constant reaction condition with respect to time, temperature, pH, flow rate etc.) a very high reproducibility was obtained.

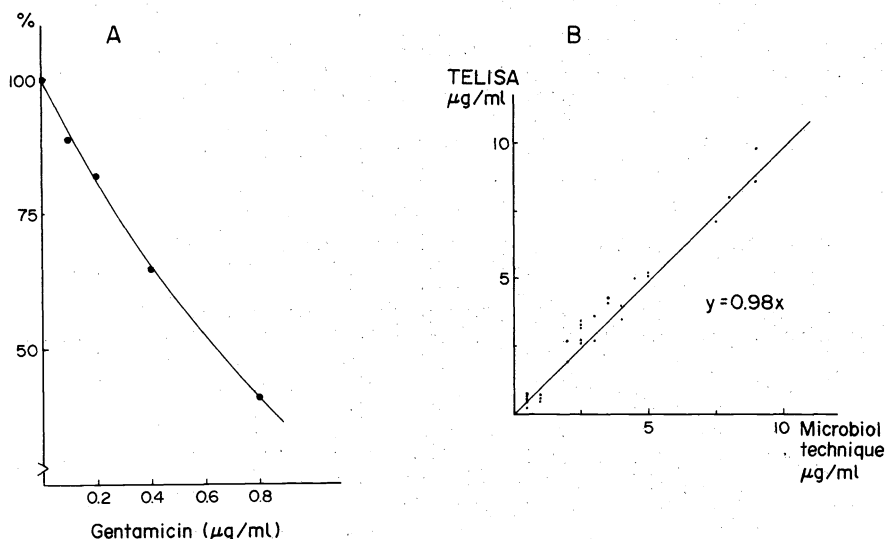


Fig. 7 A. Standard curve for gentamicin showing the decrease in temperature response obtained on introducing 1 mM  $H_2O_2$  in 1 minute long pulses to the antibody-thermistor column after exposure of the bound antibody to a mixture of a given amount of aggregate and varying concentrations of free gentamicin.

B. Correlation of results on gentamicin determination obtained with the TELISA-technique and a microbiologic technique. (The figures are taken from Ref. 40 with permission).

Using the TELISA-technique analysis down to a concentration of  $10^{-13}$  M can be carried out. Even if some sensitivity is lost in a non-equilibrium assay other advantages are gained, e.g. speed of analysis (total time for one assay including regeneration step is 12 min.) and reproducibility. Furthermore, by reusing the immunosorbent it is necessary to set up a standard curve only when a new immunosorbent preparation is used. A 100 % standard needs to be run each day as an internal calibration (Ref. 39).

Applying the TELISA-technique macromolecules e.g. human serum albumin as well as smaller molecules such as insulin and gentamicin have been analyzed. In recent studies a versatile immunosorbent has been used. Protein A from *Staphylococcus aureus* was bound to Sepharose and the appropriate amount of antibody was introduced for each assay (Ref. 41).

#### PROCESS CONTROL

Most analytical processes used in process control except for pH,  $pO_2$  and  $pCO_2$  determinations are discontinuous. Often the goal in development of analytical processes must be to make the procedures continuous because this reduces costs of sample handling, personnel costs and gives much more information per unit time. In addition, direct determinations of components formed or consumed in a process are preferable to indirect estimates based on changes in e.g. pH or  $pO_2$ . From our current studies it can be judged that the enzyme thermistor will be very useful also in this field.

In an attempt to follow the events in a penicillin fermentation, samples were taken out from the fermentor and analyzed (Ref. 42). As the sample probably contained particulate matter, a device with the enzyme bound to nylon tubing was used. Following a procedure



which was identical to conventional sample treatment, analysis results fitted very well with those from conventional spectrophotometric assays (Ref. 43),  $y = 0.998 X + 3.8$ . The intercept was shown to be due to microbial growth in the thermistor unit and could in later studies be eliminated totally by using buffers containing 0.02 %  $\text{NaN}_3$ .

A similar analytical approach was used to follow the enzymic or microbiological degradation of cellulose. In this process two products, cellobiose and glucose were of interest and were determined. To do this a three enzyme system consisting of  $\beta$ -glucosidase (hydrolysing cellobiose into two glucose units), glucose oxidase and catalase was used. Since  $\beta$ -glucosidase is inhibited by the product from the glucose oxidase catalyzed reaction  $\delta$ -gluconolactone, co-immobilization was disadvantageous (Ref. 44). Therefore, a precolumn of  $\beta$ -glucosidase was used in combination with co-immobilized glucose oxidase and catalase placed in the enzyme thermistor unit. By using a by-pass coil over the  $\beta$ -glucosidase column, glucose already present in the sample could also be analyzed (Ref. 29).

The next step was to apply the enzyme thermistor in continuous process control (Ref. 45). In order to investigate whether the thermistor unit was suitable for such an application a continuous concentration gradient of substrate was pumped through the enzyme thermistor column. The results obtained were in good agreement with those from conventional spectrophotometer analysis.

After showing that the thermistor unit was suitable for continuous analysis it was adapted to the effluent from an enzyme reactor containing  $\beta$ -galactosidase. In the reactor lactose was hydrolysed to glucose and galactose and the glucose levels in the effluent were measured using a glucose oxidase/catalase thermistor. The heat signal registered by the thermistor was used, via a control unit, to regulate the flow of substrate through the enzyme reactor. Thus, it was possible to keep the product composition in the effluent constant despite clogging phenomena etc. This is shown in Fig. 8.

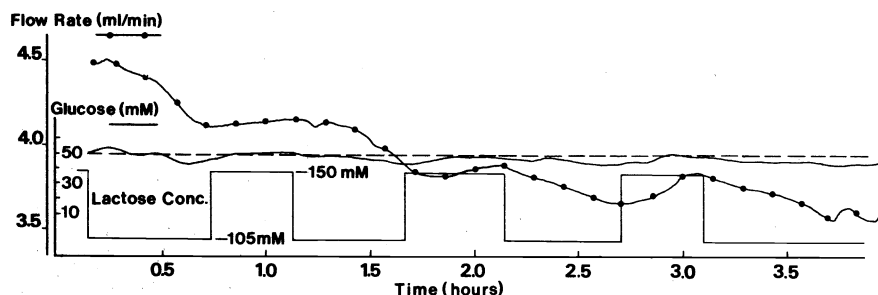


Fig. 8. Glucose concentration (—) recorded upon pumping 100 % whey (150 mM in lactose) and alternating with 70 % whey (105 mM lactose) through a 50 ml enzyme reactor containing Sepharose-bound lactase. The desired glucose concentration (-----) was set at 50 mM. The pump speed curve (-●-) expresses the number of revolutions of the pump and is proportional to flow only at moderate back pressures. The sample solution continuously fed to the enzyme thermistor was diluted 100-fold and the reactor was operated at 55° C.

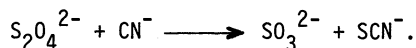
#### ENVIRONMENTAL CONTROL

A general analytical system such as the enzyme thermistor can be applied to analyze a very broad spectrum of media because of its independence from the physical properties of the sample to be analyzed. Such is the case in environmental control analysis. Environmental control analysis with the enzyme thermistor can either be applied to the determination of a specific component present in for example water, or alternatively, using a more general biological approach, information on the integrated effects on living cells of all the different pollutants present in the waste water can be obtained.

Two different methods may be used in the specific analysis of substances - either one can take advantage of the inhibitory effect of the substance on a certain enzyme reaction and from the degree of inhibition deduce the concentration of the substance or, alternatively, an enzyme that can use the substance to be analyzed as a substrate may be used.

The inhibitory effect of heavy metal ions on urease activity was used for assaying  $\text{Cu}^{2+}$ ,  $\text{Hg}^{2+}$  and  $\text{Ag}^+$  in buffer solutions (Ref. 46). Using a step-wise washing and regenerating procedure it may also be possible to discriminate between more than one species of heavy metal ions that may be present in the sample. A sensitivity of 0.2 ppb for  $\text{Hg}^{2+}$  was found with this system.

In the analysis of cyanide present in waste-water from blast furnace, the enzyme rhodanese, which catalyses the reaction shown below, was used and it was possible to measure  $\text{CN}^-$  concentrations down to  $10^{-9}$  M (Ref. 47).



$\text{CN}^-$  could from a theoretical point of view have also been analyzed by its inhibitory effect on many enzyme reactions, but by using the system above, where  $\text{CN}^-$  is a substrate, no regeneration of the enzyme is necessary and consequently continuous analysis is possible.

Microcalorimeter analysis of insecticides has been performed utilizing their inhibitory effect on acetylcholine esterase (Ref. 48). In addition, recently enzyme systems acting on this group of substances have been isolated allowing also a direct approach for the analysis of pesticide molecules can be envisaged (Ref. 49).

TABLE 1. Substances analyzed with the enzyme thermistor.

Substance	Enzyme(s)	Conc. range mmol/l	Ref.
Ascorbic acid	Ascorbic acid oxidase	0.05 - 0.6	28
Cellobiose	$\beta$ -glucosidase + glucose oxidase/catalase	0.05 - 5	29
Cholesterol	Cholesterol oxidase	0.03 - 0.15	23
Cholesterol esters	Cholesterol oxidase + cholesterol esterase	0.03 - 0.15	23
Cephalosporins	Cephalosporinase	0.005 - 10	26
Creatinine	Creatinine imino- hydrolase	0.01 - 10	33
Cyanide	Rhodanese	0.02 - 1	47
Glucose	Glucose oxidase + catalase	0.002 - 0.7	9
Hydrogen peroxide	Catalase	0.005 - 10	34
Lactose	Lactase + glucose oxidase/catalase	0.05 - 10	23
Oxalic acid	Oxalate oxidase	0.01 - 0.5	
Oxalic acid	Oxalate decarboxylase	0.1 - 3	30
Penicillin G	Penicillinase	0.01 - 500	10
Phenol	Tyrosinase	0.1 - 1	34
Sucrose	Invertase	0.05 - 100	28
Urea	Urease	0.01 - 500	5

#### WHOLE CELLS

Because of their metabolic versatility immobilized living cells possess a great potential in analysis. The advantage lies in their ability to act on a broad spectrum of substrates. However, when a single substance in a crude solution is to be measured a purified enzyme preparation is to be preferred since no side reactions can take place, whereas when an estimate on the total content of a group of substances is desired such broad specificity is advantageous.

In a model study yeast cells were entrapped in polyacrylamide and packed in the thermistor column (Ref. 50). From Fig. 9A it is seen that the system responded to changes in glucose concentration in the medium, but it can also be seen that the system was much slower to respond to changes in the medium than a conventional system based on immobilized enzymes. This may in part be explained by the severe diffusion restrictions caused by the large polyacrylamide beads used, but also by transport processes through the cell membrane and by the sluggishness of the catalytic processes within the cell.

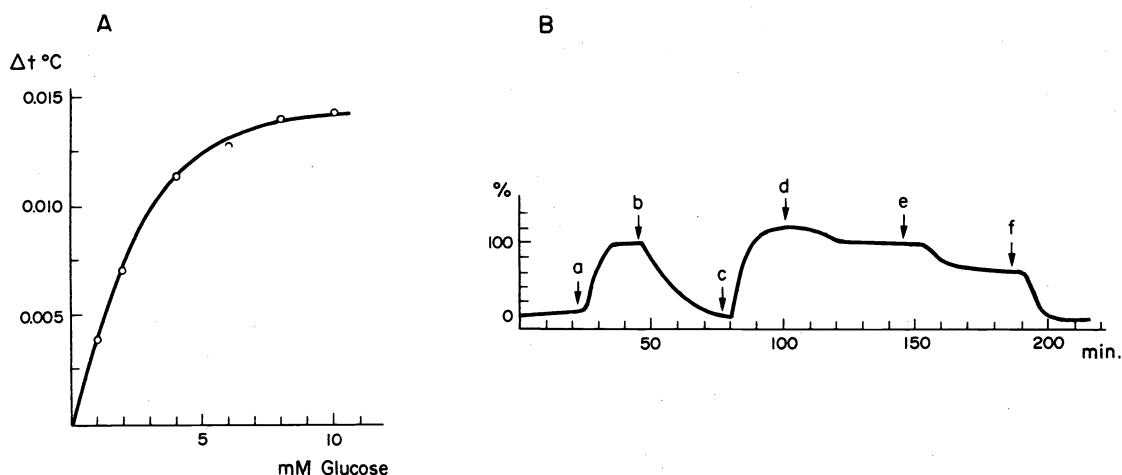


Fig. 9 A. Measured temperature peak height as a function of glucose concentration. The glucose substrate was dissolved in 0.1 M potassium phosphate buffer, pH 7.0, and introduced as 1 min. pulses into the microbe thermistor.

B. Thermogram obtained with the microbe thermistor following introduction of various metabolites and inhibitors dissolved in 0.1 M potassium phosphate buffer, pH 7.0, into the flow. The arrows indicate addition of a) 1 mM glucose; b) buffer; c) 1 mM glucose + 1 mM 2,4-dinitrophenol; d) 1 mM glucose; e) 1 mM glucose + 2 mM arsenate; f) buffer. The steady-state response to 1 mM glucose is set as 100 %. (Reproduced from Ref. 50 with permission from the publisher).

The cell preparation was also studied when exposed to different metabolic inhibitors. Thus, when 2,4-dinitrophenol was introduced during continuous introduction of glucose, an increase in heat production was registered, whereas the addition of arsenate had the opposite effect; see thermogram in Fig. 9 B. These effects are consistent with what is known from cell metabolism.

#### ENZYME ACTIVITY DETERMINATION

Calorimetry as a tool for enzyme activity determination has long been recognized (Ref. 51 & 52). The instrumentation used has generally been conventional microcalorimeters. Numerous applications exist, however, where the general features of calorimetry should be advantageous, but where less complicated and accurate instrumentation would be sufficient.

Calorimetric determinations of enzyme activities in solution can readily be made with a slightly modified enzyme thermistor equipment. The enzyme column is replaced with a piece of teflon tubing forming a "reaction coil" with a volume of about 1 ml in which the soluble enzyme to be analyzed reacts with its substrate present in excess. The sample solution and an appropriate substrate solution (with the substrate in excess) are each passed through a heat exchanger. The solutions are mixed and rapidly passed through a new short heat exchanger to eliminate heat generated by mixing, before entering the reaction coil. The temperature at the outlet is continuously measured with a thermistor. Linear correlation has been found between temperature response and enzyme activity for a variety of enzymes as long as the substrate is in excess. Fig. 10 exemplifies the technique showing the temperature response at different urease activities. This enzyme has given a linear response in the activity range 0.01-100 I.U./ml. Amylase (E.C. 3.2.1.1) activities have also been measured although with lower sensitivity (Ref. 53).

Alternatively, the enzyme present in the sample solution can first be enriched by using an affinity binding step (e.g. binding the enzyme to Con A-Sepharose or an adsorbent containing hydrophobic groups). Such an arrangement resembles the conventional enzyme thermistor and the enzymic activity is determined by introducing substrate in excess. The adsorbent is then regenerated and a new sample can be introduced (Ref. 54).

A third possibility based on the enzyme thermistor is an indirect method in which the sample solution is incubated with a known substrate amount for a fixed time and the remaining substrate is determined with an enzyme thermistor using the same enzyme in immobilized form. The useful range of measurement is about one order of magnitude and the sensitivity is affected by incubation time and substrate amount. Urease activities down to 0.01 I.U./ml are easily determined by this method.

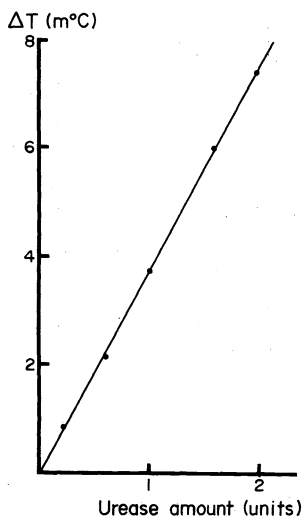


Fig. 10. Temperature peak heights for different amounts of urease dissolved in buffer and introduced as 0.5 min. pulses at a flow rate of 1.5 ml/min. into an enzyme thermistor unit modified for direct enzyme activity determination. Substrate (0.2 M urea) was introduced at a flow rate of 0.2 ml/min. and the volume between mixing and temperature measurement (the residence volume) was about 1 ml.

This modified enzyme thermistor may also be used to follow other events accompanied by an enthalpy change, including inorganic reactions of interest in environmental control. It is also useful as a tool in microbiological studies in obtaining thermograms analogous to what has been demonstrated with conventional microcalorimeters (Ref. 1).

#### DISCUSSION

In recent years considerable interest has been paid to the combination of enzymes and calorimetry. Several groups have designed low cost instruments having a complexity much lower than that of conventional microcalorimeters. One simple approach to enzymic analysis of substrates in this field is the direct injection enthalpymetry (DIE) in which an enzyme solution is placed in a small Dewar vessel (Ref. 55). Then the sample is added in a small volume and the temperature change is registered with a thermistor. After re-equilibration a new sample can be introduced and measured; this procedure can be repeated several times. The technique is simple but continuous analysis cannot be performed and the enzyme has to be renewed often in contrast to other devices in which immobilized enzymes are used.

One instrument utilizing immobilized enzymes employs a small solid state Peltier unit placed in a waterbath as detector. The reaction takes place on the surface of the Peltier unit which is covered by a thin, anodized aluminium foil to which the enzyme has been coupled (Ref. 56). Alternatively, the enzyme and its substrate are placed in a small droplet on the detector unit (Ref. 6). This instrument has the advantages of simple sample handling and comparatively rapid operation but its sensitivity is lower than that of some other devices discussed here. Furthermore, continuous analysis operation does not seem feasible at the present stage.

Another instrument that in principle offers very simple operation is the thermal enzyme probe (TEP) (Ref. 8), which consists of two thermistors with an immobilized enzyme preparation attached to one of them. The other one is used as reference. The probe is dipped into the sample solution and the small temperature change produced by the enzymic reaction is measured with a Wheatstone bridge. However, diffusional limitations and heat escape lead to a low sensitivity. The originators of the device recommend a flow-through cell with laminar sample flow as one way of increasing the sensitivity (Ref. 57), although this means a detraction from the simple concept of the original TEP.

A flow system in which the sensor resembles the TEP has also been described (Ref. 58). Here the enzyme(s) is entrapped in fibres (Ref. 59) wrapped around one of the thermistors and again the other thermistor is used as a reference. The thermistors are mounted within a glass-cell which is thermostatted in a waterbath. The sample solution is continuously

pumped through the unit and the sensitivity seems to be somewhat higher than that of the simple TEP.

At present the most promising type of device is the enzyme thermistor described here or the similar immobilized enzyme flow - enthalpimetric analyzer (Ref. 4). The reaction occurs in a small column or cell containing the immobilized enzyme. Much higher catalytic capacity can be achieved in these devices than in the previous ones. The temperature of the solution that is continuously pumped through the unit is registered with the aid of a thermistor placed at the outlet of the column. The limit of detection is normally in the concentration range  $10^{-6}$  -  $10^{-5}$  M and it can be used for the analysis of discrete samples as well as in continuous analysis. Furthermore, the operational stability of the enzyme thermistor is superior due to the high concentration of enzyme used.

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