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Flow Microcalorimetry as an Analytical Tool in Biochemistry and Related Areas

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The use of flow microcalorimetry as a general analytical tool is discussed. Special reference is made to determinations of enzyme activity and substrate concentration and for analytical studies of slow biochemical and microbiological processes.

Enzyme activities have been determined by a flow calorimetric method for glucose oxidase, cholinesterase, alkaline phosphatase, lactic acid dehydrogenase, and for ATP-ase activity in a tissue homogenate. The method is also demonstrated on the determination of glucose concentration.

Practically every process — physical, chemical or biological — is accompanied by a heat evolution or a heat absorption. The heat quantities involved are quantitatively related to the extent of the processes and calorimetric methods are therefore of potential interest in all fields of chemical analysis.

Several analytical methods based upon calorimetric principles have been suggested and some of them have also found practical application. It may be concluded, however, that with the exception of various DTA-methods no calorimetric technique has found a wide application in chemical analysis. Several factors may be pointed out as reasons for this:

(1) Calorimetric analytical methods have in general a low specificity.

(2) Calorimetric methods have usually been complicated and time consuming in comparison with existing noncalorimetric methods.

(3) The sensitivity, in particular for systems in dilute solutions, has in general been lower than for methods in current use.

It is the purpose with this report to demonstrate that a recently developed flow microcalorimeter [1] has proved to be a general analytical tool characterized by a high sensitivity and a very simple analytical procedure.

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The present analytical technique is believed to be of particular interest in biochemistry and related areas. As for other calorimetric methods the specificity is low; however, in biochemical systems the desired specificity is often a property inherent to the compound or to the process to be investigated. Further, for complex biochemical or microbiological processes it will sometimes be advantageous to use a nonspecific analytical method by which a gross value for the process or sum of processes can be obtained. Unknown or unexpected effects might thereby be discovered which would not have been revealed by a more specific technique.

It is an important property of any calorimetric technique that one is not depending on clear solutions which often are required for current analytical procedures in biochemistry. Therefore, with a procedure like that demonstrated here one can usually avoid time consuming separation procedures.

ANALYTICAL USE OF FLOW MICROCALORIMETRY

If the analytical process involves a fast reaction as with an acid-base reaction or a complexometric coupling of metal ions a suitable analytical procedure may be identical with a "thermodynamic" heat of mixing experiment described earlier.[1] The solution to be analyzed and a reagent solution, with reagent in excess, are pumped with a constant flow rate through the calorimetric mixing cell. The resulting displacement of the voltage-time curve is at steady state proportional to the heat effect evolved and thus to the concentration of the substance reacted.

Care has to be taken to avoid, or to have under control, artefact heat effects occurring when the two reagents are mixed. In particular it is important not to record any uncontrolled heat of neutralization between two unequal buffer systems.

It is usually most convenient to have the instrument empirically calibrated by measurements on standard samples. For a wide range of concentrations there will normally be found a linear relationship between concentration and calorimetric response. For dilute solutions heats of dilution of reaction components will usually be insignificant. In such cases and in absence of artefact heat effects the calibration line will pass through the origin.

The procedure outlined above is for the present type of flow calorimeter suitable for processes which are shorter than the retention time of the flow cell. (*Cf.* discussion in Ref. 1.) The retention time for the present apparatus is 7 min at a flow rate of 20 ml/h. For slow reactions batch calorimetry (see, *e.g.*, Ref. 2) is usually the most accurate and sensitive calorimetric method, but as an analytical technique it is bound to be comparatively slow.[1] A flow calorimeter can be used more conveniently if a stopped flow technique is utilized; *cf.* Ref. 3. For the present type of flow calorimeter the area below the voltage-time curve will be proportional to the heat quantity evolved.

Alternatively a continuous flow (or a stopped flow) technique can be used on premixed reaction systems if the reactions are slow enough. This latter technique is illustrated by experiments reported here.

For slow biochemical or microbiological [4] processes a flow microcalorimeter forms a versatile and convenient analytical tool. Reaction solution, or suspension, is simply pumped through one of the calorimetric cells and the resulting voltage-time curve is recorded. Such a curve or a "thermogram" (*cf.* Ref. 5) will clearly give a gross value for the intensity of the reaction and this can be utilized both for exploratory studies and for control of, *e.g.*, technical processes.

In this paper the present analytical technique will be demonstrated by a general and simple method for the determination of enzymatic activities and by a procedure for determination of substrate concentrations.

Determination of enzymatic activity. A quantitative assay for an enzymatic activity can be obtained by determination of the heat effect generated by the substrate saturated enzymatic system. The calorimetric procedure adopted consists simply of pumping an enzyme solution, or suspension, mixed with an excess of a suitable substrate, through the calorimetric cell. If the kinetics of the process follows zeroth order for some time there will be established a voltage-time curve parallel to the instrument baseline (curve a, Fig. 1). The baseline displacement, Δ, is directly proportional to the heat effect and will thus form a direct measure for the enzymatic activity under the conditions of the experiment.

Sometimes product inhibition or instability of the enzyme will cause the voltage signal to descend towards the baseline while substrate is still in excess, curve b, Fig. 1. In such cases one can for instance choose the maximum Δ-value or any Δ-value at a given time to be compared with corresponding values determined by use of standard samples.

If the investigated system is a solution, pumping might be stopped after the reaction cell has been filled as the steady state value is independent of flow rate (instrument baseline will slightly vary with flow rate [1]). If suspensions are investigated it is preferable to continue the pumping to prevent settling of the material. In the experiments described here the sample was continuously pumped.

Enzymatic determination of substrate concentration. The heat quantity evolved when a substrate is reacted under given experimental conditions is directly proportional to the substrate quantity. Such a heat quantity may be determined as in a regular "thermodynamic" experiment. A convenient

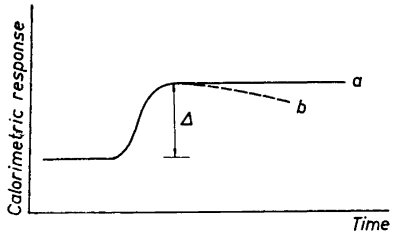

Fig. 1. Calorimetric curves from determination of enzyme activities.

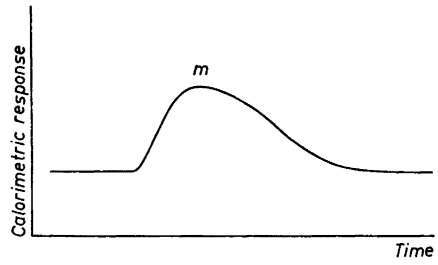

Fig. 2. Calorimetric curve from the determination of substrate concentrations.

method for slow processes, using a premixed sample will be described here for the enzymatic determination of glucose. The method is similar to the enzyme activity determinations, but substrate concentration should be rate limiting and enzyme activities should be the same at calibration and analysis experiments.

Substrate and enzyme systems are mixed and immediately pumped through the calorimeter. The decreasing reaction rate combined with the thermal inertia of the calorimetric system including the filling time of the cell will cause the calorimetric curve to show a maximum, m, before descending towards the baseline value, Fig. 2.

As a premixed solution will be pumped through the cell neutralization effects etc. from the mixing process will not be observed, which will be of value in cases where it is difficult to have such effects under control.

At this type of experiment a considerable part of the heat connected with the substrate reaction will not be measured, as the reaction is started outside the calorimetric cell. During the filling process of the calorimetric cell an increasing fraction of the total heat effect is being evolved in the calorimeter. Finally, from the time when the cell is filled with reaction solution a constant fraction of the total heat effect evolved will be measured.

In order to make a quantitative evaluation of the calorimetric results the calorimetric curves should be compared with those obtained at calibration experiments run under identical conditions. Several characteristics of the voltage-time curve may be used for this purpose. Here the maximum value chosen for comparison (identical kinetics has to be assumed for calibration and analytical reactions, respectively). In cases where kinetic identity cannot be taken for granted a safer method would be to run the reactions to completion (or near completion) and compare surface areas below the voltage-time curves which are proportional to the heat quantities evolved in the cell.

Steady state values can be obtained for substrate analysis if substrate and enzyme are mixed at constant flow rates in or outside the calorimeter.

EXPERIMENTAL

Calorimeter and experimental procedure. The flow microcalorimeter used has recently been described in detail elsewhere.[1] The apparatus is a twin calorimeter utilizing the "heat conduction principle". Heat evolved in one of the reaction cells is conducted through the surrounding thermopile before reaching a metal block acting as a heat sink. Analytical results are obtained from the amplified differential voltage signal of the two thermopiles.

The premixed reaction solution was pumped through the heat exchange unit and from there through one of the calorimeter cells. The flow cell used in the present experiments was slightly modified compared to that described in Ref. 1. It consisted of a metal plate made from 24 K gold and with a channel system similar to that described earlier. The present flow cell, however, was without constrictions and had a single inlet tube instead of a T-piece. The channels had a circular section and formed a cell volume of 2.3 ml.

A Keithley 150 B Microvolt Ammeter was used as an amplifier. The amplified signal was recorded with a Sargent SR recorder, 125 mV.

The reaction components were mixed in a room thermostated at 25°C and then pumped to the calorimeter by a Perpex pump at a flow rate of 0.33 ml/min.

Experiments reported in this paper depend upon the kinetics of the process being investigated and therefore it is important to keep the temperature of the reaction mixture under control and equal at both calibration and reaction experiments. The calorimeter is operated under very nearly isothermal conditions, and the temperature of the cell contents is almost the same as that of the thermostated water bath in which the calorimeter is contained, *i.e.* 37°C, unless otherwise indicated.

A temperature gradient exists along the flow path from the tube containing the reaction mixture to the pump and calorimeter. At constant temperature and flow rate this gradient is reproducible and in enzyme analysis is useful in extending the steady state. For substrate analysis both temperature and flow rate must be carefully controlled to ensure reproducible results.

Materials. Glucose oxidase (Activity: 15 μmoles glucose/min, pH 5.1, 35°C) was kindly supplied by Kabi, Stockholm, and "Glox", which is a preparation containing glucoseoxidase, peroxidase, *o*-dianisidine, and buffer, was obtained from the same company. All other purified enzymes were obtained from Sigma, St. Louis, Mo: peroxidase from horseradish, type I; cholinesterase from horse serum, type IV; alkaline phosphatase from calf mucosa, type I, and lactic acid dehydrogenase from beef heart, type III.

The buffer used in the ATP-ase experiments contained imidazol, 25 mM; ATP, 4 mM; Mg^{2+}, 5 mM; Na^{+}, 78 mM; K^{+}, 5 mM; EDTA, 0.13 M. pH of the buffer was 7.4.

RESULTS

Determination of glucose oxidase activity. A 1 % glucose substrate was prepared in 1/15 M phosphate buffer at pH 6.8 and to 10 ml aliquots were added different amounts of glucose oxidase. The reaction mixture was pumped through the calorimetric cell and the heat effect measured. The reaction followed zeroth order kinetics and a calorimetric steady state curve was obtained. Curve a, Fig. 3 shows the calorimetric response, Δ, plotted *versus* enzyme concentration. It is seen that a linear relationship is obtained giving arbitrary activity numbers for the enzyme solutions.

At another series of experiments peroxidase and *o*-dianisidine was added to the glucose substrate in the concentrations used by Raabo and Terkildsen.[6]

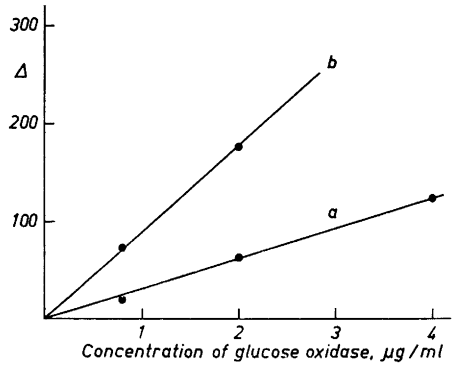

Fig. 3. Determination of glucose oxidase activity. Calorimetric response, Δ, is plotted *versus* enzyme concentration. Amplification = 10^4.

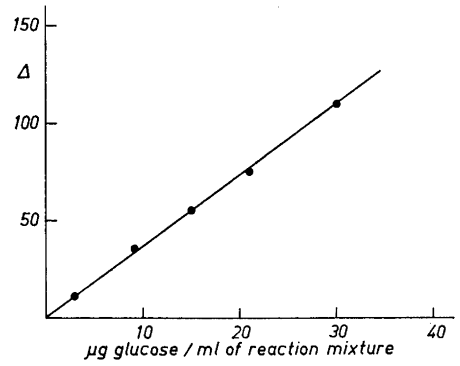

Fig. 4. Determination of glucose concentration. The calorimetric response, Δ, taken as the peak value (*m* in Fig. 2). Amplification = 10^4.

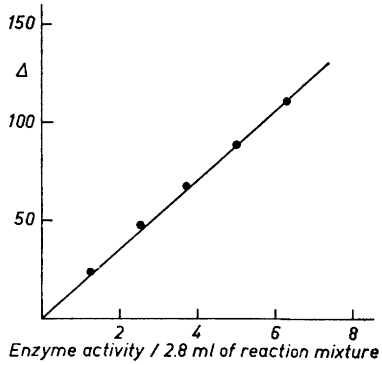

Fig. 5. Determination of cholinesterase activity. Calorimetric response, Δ, is plotted *versus* the enzyme activity given in units of Rappaport *et al.*[7] Amplification = 10^4.

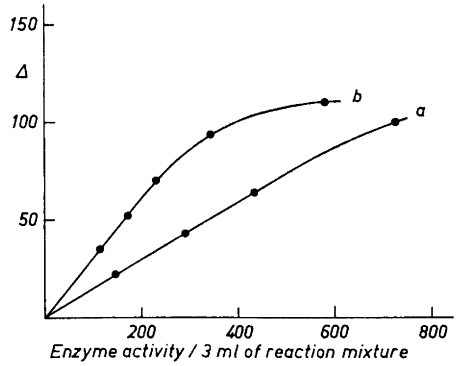

Fig. 6. Determination of alkaline phosphatase activity at 25°C (curve a) and 37°C (curve b). Calorimetric response, Δ, is plotted *versus* enzyme activity as given in units of Amador *et al.*[8] Amplification = 10^4.

This coupled enzyme system resulted in a highly exothermic reaction causing a three fold amplification in the heat effect; curve b, Fig. 3.

Determination of glucose concentration. To 10 ml aliquots of the coupled enzyme reagent "Glox", were added different amounts of glucose and the reaction mixture was pumped directly through the calorimeter. The maximum value m (see Fig. 2), achieved 10 min after initiating the reaction, plotted *versus* glucose concentration gave a linear relationship. Results are summarized in Fig. 4.

Determination of activities for cholinesterase, alkaline phosphatase, and lactic acid dehydrogenase. Calorimetric experiments were controlled in accordance with procedures used in clinical investigations.[7–9] The spectrophotometric methods described in the cited references were used to determine the activity of the pure enzymes measured. Data obtained were used to adjust enzyme concentrations to give zeroth order rates for a suitable time to record steady state values in the calorimeter. A steady state value of 13—15 min was sufficient at a flow rate of 20 ml/h. Results are summarized in Figs. 5—7 where calorimetric responses (Δ-values) are plotted *versus* measured enzyme activities.

For cholinesterase a Tris-buffer was used instead of the phosphate buffer recommended. At a process like this where acid is produced during the reaction there will be a heat effect from its neutralization by the buffer. In the present case where the reaction is exothermic there will be a substantial "amplification effect" with this choice of buffer as the heat of protonation of Tris is highly exothermic compared to that of a phosphate buffer.

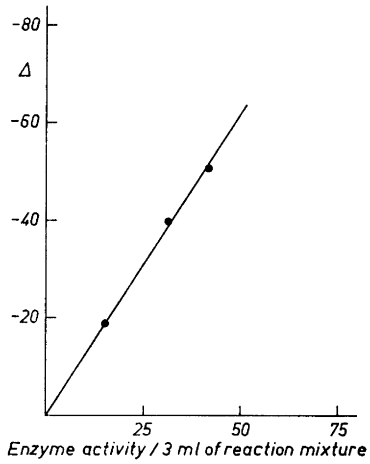

Fig. 7. Determination of lactic acid dehydrogenase activity. Calorimetric response, Δ, is plotted *versus* the LDH activity expressed in units of Amador *et al.*[9] Amplification $1/3 \times 10^5$.

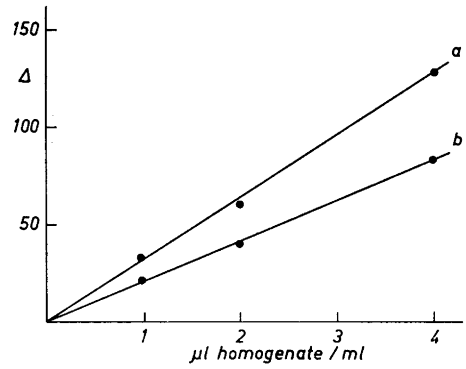

Fig. 8. Determination of ATP-ase activity in a tissue homogenate. Calorimetric response, Δ, is plotted *versus* concentration of homogenate. 1 μl of the homogenate will release 2.8 μmoles P_i/min if no inhibitor is added, curve *a*. Curve *b* was obtained when the suspension contained 1.0 μmole Ouabain per ml. Amplification $= 1/3 \times 10^5$.

*Determination of ATP-ase activity in a tissue homogenate.** A homogenate prepared from rat kidney cortex [10] was added to an imidazole buffer containing ATP in excess and the suspension was pumped through the calorimeter cell. Steady state values were obtained and a plot of calorimetric response values *versus* amount of homogenate formed a linear relationship, Fig. 8. Experiments were repeated with Ouabain (an ATP-ase inhibitor) added to the buffer and considerably lower activities were found. The decrease in activity is in agreement with results obtained through conventional analytical methods.[11]

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