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A stopped-flow calorimeter for biochemical applications

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A rapid-response stopped-flow calorimeter for small samples of reagents is described. The construction, performance characteristics and operational limitations are described, along with an example of its ability to resolve the kinetics of an enzyme-catalysed hydrolysis. It is thought likely that the method would find useful application in a variety of chemical and biochemical investigations.

INTRODUCTION

The calorimeter described in this paper has been developed for the study of rapid heat changes in solution, such as accompany many enzyme reactions. The use of heat, or temperature, changes to monitor rapid chemical reactions dates back to some of the earliest experiments with continuous-flow techniques (Roughton, 1930). The success of Roughton's investigations on the kinetics of haemoglobin–oxygen binding proved the value of such thermal kinetic studies, but also revealed the principal shortcoming of continuous-flow systems, namely that they are very extravagant in the use of reactants. Kodama & Woledge (1985) described two batch calorimeters that they designed and used for heat studies of different enzyme reactions. These required reactant volumes of only 1 ml and had a time resolution of 1 s or less. It seems likely that in these instruments Kodama & Woledge have pressed the development of the batch calorimeter close to its limit of speed, and thus, for still better time resolution, a different system is called for. A stopped-flow system would seem to be a promising option. Compared with continuous-flow systems, the stopped-flow technique is much more economical in the use of reagents and it has been successfully used for the study of transient kinetics by a variety of optical measurements. Consequently, there have been a number of proposals and attempts to adapt the stopped-flow method for the study of rapid enthalpy changes. Kodama & Kometani (1986) used this method in a trial study of ATP hydrolysis by myosin subfragment 1 but give only a preliminary outline description of the instrument. Other earlier essays in this field are reviewed by Berger *et al.* (1984), but, although the later instruments described there appear to have considerable promise, they do not seem to have been much exploited for the study of enzyme kinetics.

That such studies are likely to be rewarding is indicated by previous experience of investigating the same reaction by different transient kinetic techniques (Gutfreund, 1975). The non-specific nature of temperature measurements, although a drawback in some applications, can be turned to advantage in certain reaction systems by providing a means of monitoring those steps in the reaction sequence that are spectroscopically silent. Moreover, a powerful additional tool is presented by time-resolved heat measurements, namely reaction steps involving ionizations can be specifically characterized by performing the reaction in different buffers having high and low heats of ionization. This procedure has been successfully used by Millar *et al.* (1987) in a study of myosin ATPase.

For such measurements the requirement is for an instrument having a time resolution of a few milliseconds and a sensitivity in the range < 1 mK. This is similar to the specification required for measurement of heat changes in active nerve and muscle cells (Howarth *et al.*, 1979; Curtin *et al.*, 1983). In the present paper we describe such an instrument, together with tests of its performance characteristics and some features of its practical use. In the accompanying paper (Millar *et al.*, 1987) we report studies of the resolution of substrate binding, chemical catalysis and product dissociation during the reaction of myosin with ATP, results that further demonstrate the potential of the technique.

DESCRIPTION OF THE INSTRUMENT

Design considerations

The instrument has essentially the same configuration as an optical stopped-flow device but with a thermal detector positioned immediately downstream from the mixing chamber at the point where optical changes are normally observed. For the detection of thermal changes physical requirements are most easily seen if the system is though of as being in two parts. The first part comprises the two columns of fluid containing the reactants upstream from the mixing chamber. It is essential that these are at precisely the same temperature and that there are no persistent thermal gradients along their length. This is best achieved if both are in contact with a large thermal mass of high thermal conductivity. The second part comprises the reacting solution in the mixing chamber and the reaction chamber. Here the requirement is that the walls of the chamber and the detector itself should abstract the least possible heat from the reacting solution and that heat should be lost as slowly as possible to the environment. This requires that the reacting solution must be in contact with a very small thermal mass of low thermal conductivity. Thus it is seen that the two parts of the system have exactly the opposite requirements. These are satisfied in our apparatus by arranging for the initial fluid columns to be contained in narrow-bore syringes and 2 mm-bore delivery tubes that are in direct contact with a large-volume water thermo-

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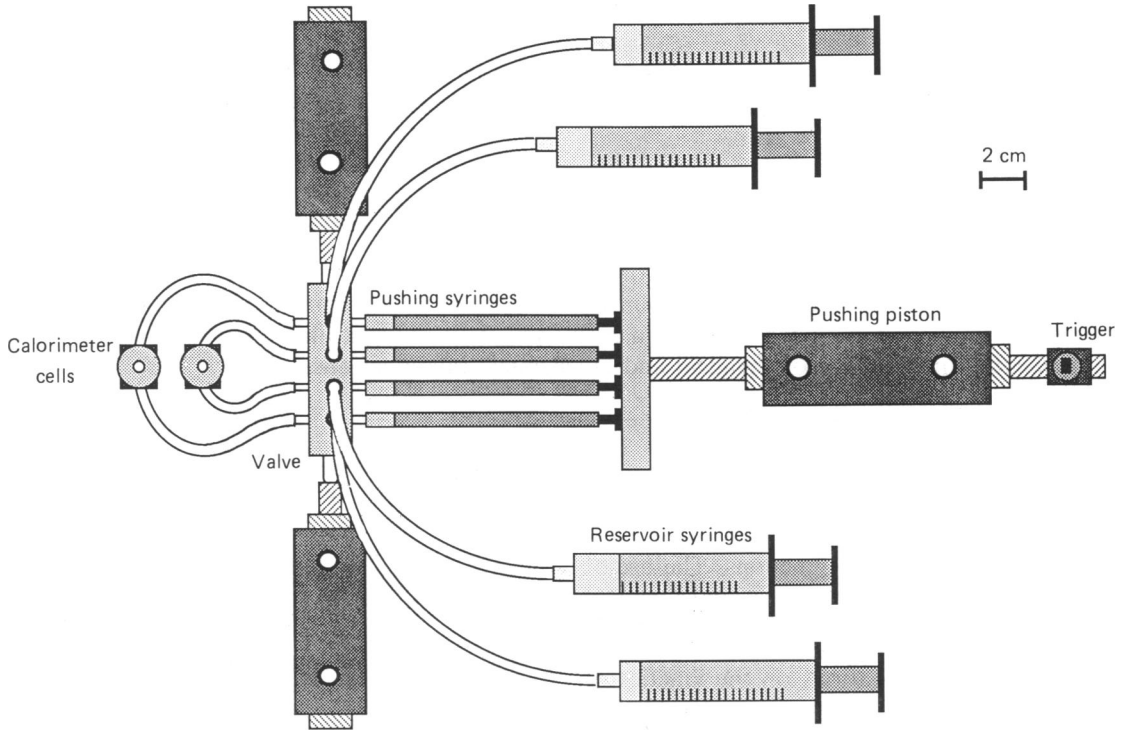

Fig. 1. Diagram showing lay-out of the stopped-flow calorimeter

The compressed air tubes to the pneumatic cylinders and leads to the thermopiles and trigger are omitted for clarity.

stat, and for the second part, by using for the reaction chamber, a very-thin-walled tube insulated from the thermostat by an air jacket. This led to the configuration as shown in Fig. 1.

The instrument described here is the one presently in use and all of the experiments described in the accompanying paper (Millar *et al.*, 1987) were made with this instrument.

Features of the instrument

In use the whole assembly on its base-plate is immersed in a well-stirred 80-litre water bath in a heavily insulated box. All operation of the instrument is from outside the box by remote control. Except at 0 °C, when there is ice present, no attempt is made to regulate the temperature of the bath. Rather, it is adjusted initially and then allowed to drift. In the course of a 4–5 h experiment the bath never drifts more than 0.1 K and then so slowly that the reference baseline for transient measurements is not affected.

Fig. 1 shows the instrument as it is normally used, with two calorimeters operated differentially. The valve block is made from aluminium alloy with a slot to take a close-fitting polytetrafluoroethylene slider, which is drilled to form a four-ganged three-way tap so that in one position the delivery syringes are in straight-through communication with the delivery tubes and thence to the calorimeter cells, and in the other position the delivery syringes connect to the storage syringes for re-charging. Actuation of the slider is by the two air cylinders. The third air cylinder actuates the delivery syringes. All three air cylinders are 25 mm-bore double-acting cylinders and are supplied with air at 0.8 MPa (8 bar) pressure from outside the box via nylon tubes (which are not shown in the illustration). Speed of the cylinders is controlled in both directions of movement by needle valves restricting the exhaust side. The excursion of the main pushing cylinder is restricted by two pieces of aluminium channel clipped over the through-rod at the front and rear ends. The rear one carries a piezo-electric wafer, sealed into the cut-off end of a plastic disposable syringe, which provides a trigger signal at the precise moment when the rod hits the stop. Movement of the piston can be monitored by a conducting plastic linear transducer that is also coupled to the rear extension of the through-rod but not shown in the diagram.

In this arrangement the flow is arrested upstream from the calorimeter, and after each push the delivery syringes are re-charged with a single portion of reactant solution (usually 150 μl) ready for the next push. More commonly in stopped-flow systems the flow is arrested by stopping a collecting syringe on the downstream side of the observation cell. This method was not open to us because it leads to a large pressure jump at the moment of stop, which would cause a large temperature change due to thermo-elastic heat released in the fluid (and more than likely rupture the thin walls of the observation tube). Bowen *et al.* (1980) describe another solution to this problem using a push-pull flow arrangement. We have not tried this but it could be added to this instrument and may be better than the simple upstream stop.

Calorimeter cells

A calorimeter cell of the current type is illustrated in Fig. 2. It has four parts: a mixing chamber, a reaction chamber, a thermopile and an air jacket.

Mixing chamber. The requirement of the mixing chamber is that it provides for rapid and complete mixing but with the least amount of mechanical work,

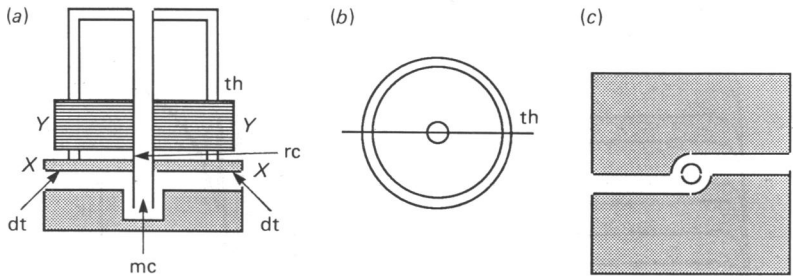

Fig. 2. Diagram of a calorimeter cell showing the mixing chamber and the reaction chamber in section

(*a*) A single calorimeter cell. Key: th, thermopile; mc, mixing chamber; rc, reaction chamber; dt, delivery tubes. The two reactant solutions enter the mixing chamber at the sides and exit via the thin-walled tube that forms the reaction chamber. The thickness of the thermopile is exaggerated in relation to the diameter of the reaction chamber. (*b*) Cross-section through the thermopile at the level *Y–Y*. (*c*) Cross-section through the mixing chamber at the level *X–X*.

and hence heat, put into the fluid. We do not know of any established theoretical treatment that would provide a basis for such a design, so the form and dimensions of the present chambers have evolved by intuition rather than by design. Mixing chambers may be made by drilling in Perspex [poly(methyl methacrylate)]. This is satisfactory for single cells, but for paired cells to be used in a differential mode it is important to have the two chambers exactly alike and it is difficult to machine them with the necessary precision. The two mixing chambers in the present cells were made by casting liquid epoxy resin, both in the same mould. This method has given the best results to date. It is important to ensure that the reaction chamber tube is truly central where it projects into the mixing chamber and that the gap between the end of the tube and the back of the mixing chamber is exactly the same in the two cells. It is our practice to view the mixing chambers under 20× magnification and to remove surface irregularities at this level.

Reaction chamber. The reaction chamber, where the measurements are made, is a tube that carries the reaction solution from the mixing chamber. The requirements are that the heat capacity of the wall must be small compared with that of the contained fluid and that the wall must come rapidly into temperature equilibrium with the fluid. Both of these are met by having a thin-walled tube. Our tubes are made by spiral wrapping a tape, cut from 10 μm polyester film, around a 2 mm polythene former, the width of the tape and the pitch of the spiral being calculated to give a double thickness of film at all points along the wall. The tape is coated with epoxy resin as it is wound and excess resin is removed. When the resin has hardened the polythene former is withdrawn. These tubes weigh 2.37 mg for 5 mm length (i.e. the length of the thermopile) and have a heat capacity of about 2.76 mJ·K^{-1}, or about 4% that of the contained solution.

Thermopile. The thermopiles are made from 25 μm-diameter constantan and chromel wire. The wires are embedded in epoxy resin and insulated by sandwiching between two layers of 10 μm polyester film. The thermopile consists of 100 couples in series, has an electrical resistance of 2300 Ω and extends 5 mm along the line of flow. The heat capacity of the thermopile in contact with the solution is 0.8 mJ·K^{-1}, which is close to 1% of that of the solution. The thermo-electromotive force of constantan/chromel couples is 59.1 μV·K^{-1} at 273 K. Thermopiles made in this way give the full theoretical output provided that none of the couples is short-circuited. The thermopile is inserted through slits cut in the side of the tube, arranged so that the 'hot' junctions lie along the midline, and is then sealed in with a small amount of epoxy resin.

Air jacket. The air jacket is simply the end cut from a 5 ml plastic disposable syringe. The thermopile passes through, and is sealed into, slits cut in the wall so that the electrically insulated 'cold' junctions protrude into the bath water.

Characteristics of the instrument

Three characteristics determine the usefulness of the instrument in a particular application. They are sensitivity, speed of response and rate of heat loss.

Sensitivity. Fig. 3(*a*) shows a series of records obtained by reaction of different concentrations of HCl with Tris buffer at pH 8. In Fig. 3(*b*) the output voltage is plotted against temperature change calculated from the known enthalpy change of the reaction. The sensitivity is given by the slope of the regression line, giving a value of 5.95 mV·K^{-1}, exactly the same as the expected theoretical value for 100 thermojunctions. This procedure gives the true temperature sensitivity of the instrument, but (unlike a batch calorimeter) it slightly overestimates the heat calibration because it does not take account of the heat capacity of the thermopile and the wall of the reaction chamber. This is because the neutralization reaction is so quick that all the heat is released before the flowing solution reaches the thermopile. Typically the flow continues for 30 ms or more, giving plenty of time for the thermopile and wall to come into temperature equilibrium. Thus, when the flow finally stops, the fluid in contact with the thermopile does not lose any heat to these 'inert' elements. On the other hand, the enzyme reactions that the instrument is designed to investigate are not so fast and have scarcely got underway before the solution reaches the thermopile after mixing. Thus, when the flow is arrested, all subsequent reaction heat is shared with the inert elements, leading, in the present instrument, to a 5% reduction in temperature change.

Speed of response. Fig. 4 shows a record obtained by passing a 2 ms pulse of current between electrodes placed

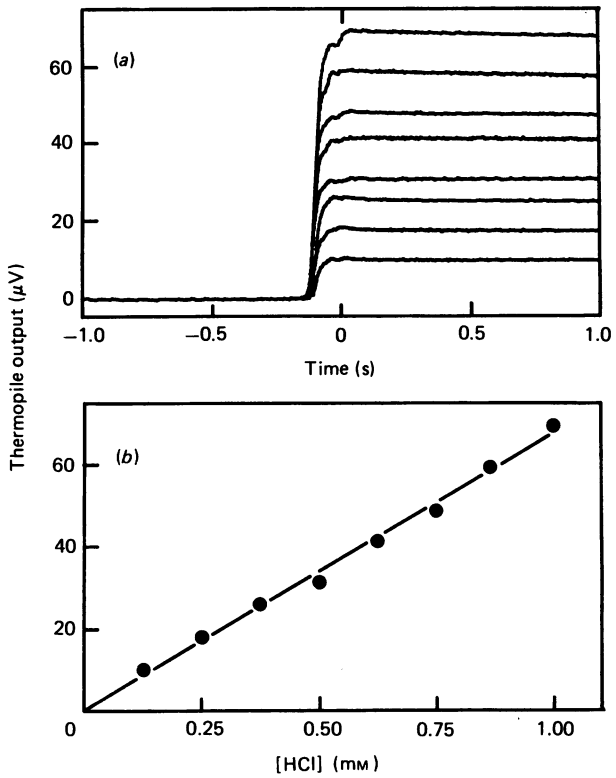

Fig. 3. Calibration with Tris versus HCl

(*a*) Calorimeter records corresponding to the eight HCl concentrations shown in (*b*). Time zero marks the time at which flow stops. (*b*) Peak thermopile output versus HCl concentration. Slope of fitted line = 67.2 $\mu V \cdot mM^{-1}$. Since ΔH for ionization of Tris = −47.5 $kJ \cdot mol^{-1}$, this corresponds to 5.95 $mV \cdot K^{-1}$. Conditions: 25 mM-Tris, [HCl] as indicated (concentrations refer to solution in reaction chamber after mixing), pH 8.0, 5 °C.

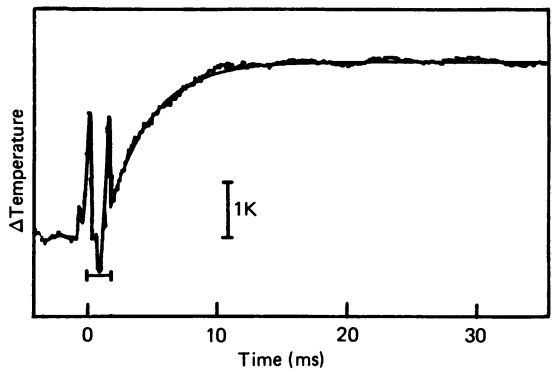

Fig. 4. Response time of the calorimeter

A 2 ms pulse of heat (marked by the horizontal bar) was produced by passing an electric current pulse through an electrolyte solution in the reaction chamber. This is the average of six records with the pulse polarity reversed between successive records. Time zero marks the time at which the pulse starts. The fitted exponential is fitted from 2 ms and has a rate constant of 302 s^{-1}.

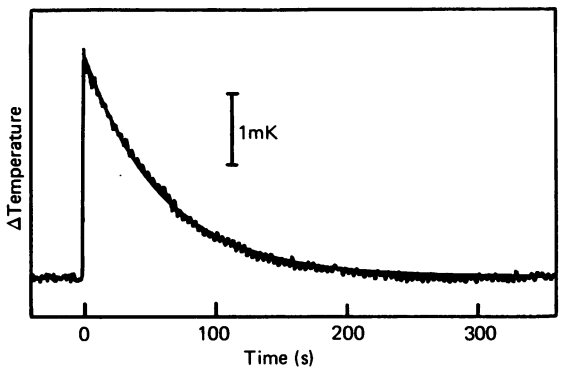

Fig. 5. Cooling curve showing rate of heat loss from the calorimeter

This is one of the records from the experiment illustrated in Fig. 3 but on a much slower time scale. Conditions: 250 μM-HCl versus 25 mM-Tris, pH 8.0, 5 °C. Time zero marks the time at which flow stops. The fitted exponential has a rate constant of 0.0168 s^{-1}.

in the column of fluid a few millimetres away from the thermopile at each end. At the beginning and end of the pulse the record is disturbed by capacitive coupling of the pulse into the thermopile circuit, but it can be seen that the record takes off during the 2 ms and at the end of the pulse the record continues to its maximum value in an exponential fashion with a time constant of 4.5 ms. The calorimeter itself is thus very fast. In normal use the effective speed of response of the system is determined by the bandwidth set in the amplifier to achieve an acceptable signal-to-noise ratio.

Rate of heat loss. Heat released into the reaction chamber escapes to the bath by three, approximately equal, parallel routes: upstream and downstream along the fluid column and laterally via the conductors of the thermopile. Heat loss across the air space is insignificant. Fig. 5 shows a cooling curve and demonstrates that it is fitted well by a single exponential having a rate constant of 0.017 s^{-1} (time constant 58.8 s). This remains the same in different experiments, and enables the heat loss to be corrected with sufficient precision to allow observations to be made over many minutes.

The instrument in use

The above specification characteristics meet most of the requirements of the original project. In use, however, there are a number of practical factors that need always to be borne in mind. Some of these are outlined below.

Work heat. Considerable mechanical work is performed on the solutions as they are driven through the delivery tubes and the mixing chambers, resulting in the release of heat. Fig. 6 shows a record of this work heat resulting from driving water alone through the calorimeter. Much of the development work has been aimed at minimizing this work heat, but some is unavoidable and therefore has to be treated as the baseline of measurement. When a reaction is to be measured the heat due to the reaction can be observed by subtracting a control trace obtained with solutions that mimic the physical properties of the reacting solutions, in particular the viscosity and concentration, but with no reaction present.

There are two ways to subtract the control traces. In the first, the control records are made in separate pushes

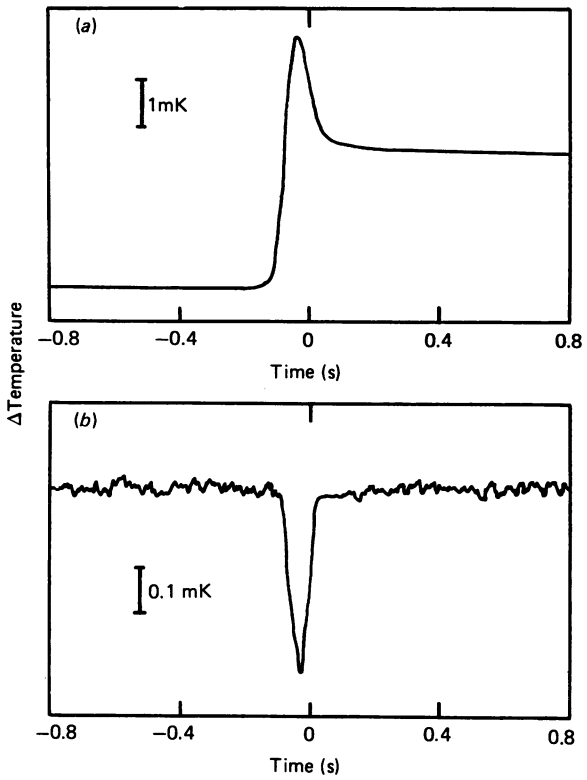

Fig. 6. Records of work heat

(*a*) Output from a single calorimeter cell due to water versus water. Time zero is the time at which flow stops. The change in level between the baseline and the horizontal part of the record following the push is the heat produced by the mechanical work done on the fluid to overcome viscous resistance. (*b*) The difference signal seen at higher gain with the use of the paired calorimeter cells, i.e. (water versus water) − (water versus water). The negative spike is the result of imperfect symmetry between the two halves of the system, but the work heat is cancelled 25 ms after the stop. This is the average of three traces. Conditions: flow velocity 2 m/s, distilled water in all four syringes, 5 °C.

before or after the reaction records. This needs only one calorimeter but it requires that the work heat must be the same in all pushes. In our system this is not found to be the case. The variation is probably largely due to inconsistency in the speed of operation of the air cylinders. These cylinders are not designed for precise control of speed, and it is likely that they would be improved by hydraulic damping or, perhaps better, they could be replaced by hydraulic actuators.

In the second method a second calorimeter is used to record the control trace at the same time as the reaction trace. This method avoids the problem of variation in pushing speed, but it requires that the two calorimeters are exactly alike in their characteristics. This is the method that has yielded the best results in our experiments (see Fig. 6*b*).

The most obvious and elegant method would be to use a truly differential system by placing the opposite ends of a thermopile in a pair of parallel reaction tubes in the same enclosure, one carrying the reaction mixture and the other the blank. We have made some good recordings using this system, but the arrangement is not at all easy to use and if something goes wrong in the course of an experiment it is impossible to know which of the two sides is at fault.

It is, of course, important to minimize the work heat as far as possible by the design of the system. It is of obvious advantage to preserve laminar flow in the delivery tubes. For this they must have a smooth interior surface and the flow should not be subjected to rapid changes of diameter or direction. Their dimensions are arranged to keep the Reynolds number at the expected flow rates in the region below 2300, the critical value above which laminar flow becomes unstable. The delivery tubes between the valve and the mixing chambers hold somewhat more fluid than is ejected in a single push. This means that fluid that may have been heated by being forced out of the syringes and through the valve does not reach the mixing chambers until the following push. The half-time of temperature equilibration between the tubes and the bath is calculated to be 1.7 s, so that there is plenty of time in the interval between pushes (usually 8 min) for any temperature gradients in the tubes to be dissipated.

Flow rate. Work heat is very sensitive to flow rate, and to keep the former to convenient levels it is necessary to operate the instrument at flow rates considerably less than is usual in optical stopped-flow systems. However, if the flow is too slow problems may arise with mixing and scouring.

Mixing was tested at different speeds by using acid–base reactions to obtain records such as those of Fig. 3(*a*). There it can be seen that no heat is produced after the stop, as it would be if incompletely mixed solutions continued to react by diffusion in the stationary fluid in the reaction chamber.

Scouring is the term used in the laboratory to refer to the displacement of the old fluid in the reaction chamber by the incoming fluid during the push. This was also tested by using the acid–base reaction, by making a series of pushes in quick succession. If scouring is complete the records all peak at the same level, but if there is incomplete scouring the first record is low because of the extra heat capacity of the remnants of the old solution. The second record, if made before there has been appreciable heat loss, goes higher, and so on in a diminishing staircase to the final value.

By these tests we found that a flow rate of 2 ms^{-1} in the reaction chamber and a push volume of 300 μl (150 μl from each syringe) give good results.

Push artifacts. In addition to the work heat, Fig. 6 also displays a spike-like artifact during the push. This artifact has manifested itself in all versions of the instrument, and although other artifacts have been identified and eradicated this one has so far proved resistant to treatment. Although it is mostly cancelled in the subtraction, there is invariably some small residual, due to imperfect symmetry, which persists for a short time after the stop and may interfere with measurements in the first 20 ms. For reactions lasting longer than this it does not get in the way. The relative magnitude of the artifact, the difference signal and a reaction trace under typical experimental conditions can be seen by comparing Figs. 6 and 7.

We are not altogether sure as to the cause of this artifact. Various different tests have shown that it is not

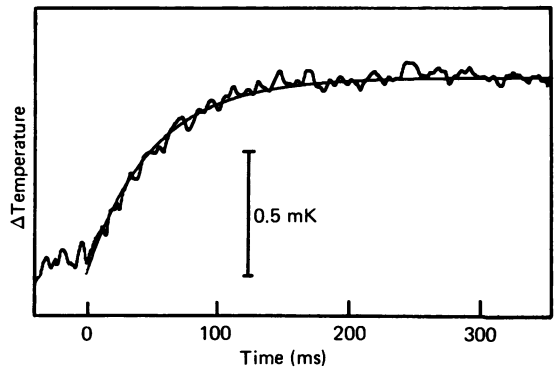

Fig. 7. Hydrolysis of acetyltyrosine ethyl ester by chymotrypsin

Conditions: 250 mM-chymotrypsin, 90 μM-acetyltyrosine ethyl ester, 0.1 M-imidazole buffer (reaction chamber concentrations), pH 7.0, 22 °C. Time zero marks the time at which flow stops. The observed temperature rise is due to the release of 1 mol of H^+/mol of acetyltyrosine ethyl ester as the acyl-enzyme complex breaks down to form free acetyltyrosine and enzyme. The fitted exponential has a rate constant of 19.6 s^{-1} and an amplitude 0.79 mK, which corresponds to an enthalpy change of -37 kJ·mol^{-1}.

due to rapid heat loss to some inert heat capacity such as the walls of the chamber, nor is it caused by distortion of the thin-walled tubes. Also, it is not an electrical artifact such as might be induced in the thermopile circuit electro-magnetically or by some form of charge separation during the flow. A special 'calorimeter' having an element made exactly as a themopile save that all its conductors were of constantan (instead of constantan–chromel couples), and therefore insensitive to temperature, did not show any sign of this artifact. Therefore the artifact must be a temperature, and hence a heat, change. The falling phase after the stop is many times faster than the rate of heat loss from the system and therefore must be due to heat absorption. This, and some other properties of the artifact, suggest that it is caused, at least in part, by pressure changes. If it is due to thermoelastic heat in the fluid it would require pressure changes of the order of 1 atm to account for the observed magnitude of the artifact.

Data acquisition. The output from each calorimeter is in the region of 1 μV and is amplified by a low-noise d.c. differential instrumentation amplifier, based on the AD-524-AD, followed by an OP-07C, giving an overall gain of 10^5. The amplifier suffers no discernible baseline drift over a period of several minutes, long enough for the slowest observations ever likely to be made with this type of calorimeter. The amplified signals from the two channels, or difference signal as required, are taken to a Nicolet 3091 digital oscilloscope, and the output, digitized as 4000 12-bit points, is displayed and stored on an Apple IIe computer. In records of short duration heat loss is insignificant, but for recordings exceeding about 10 s it is corrected in the computer according to the relation:

$$\Delta T_{t(\text{corrected})} = \Delta T_{t(\text{observed})} + \sum_{t}^{0} [\Delta T_{t(\text{observed})} \cdot (1 - e^{-k\Delta t})]$$

where ΔT_t = temperature change at time t, Δt = time increment and k = rate constant of heat loss. Analysis of the recorded traces was done in the computer using standard non-linear fitting programs (Edsall & Gutfreund, 1983).

CONCLUSION

Fig. 7 shows an experimental record of the time course of heat production during a single turnover of the enzymic hydrolysis of acetyltyrosine ethyl ester by chymotrypsin. The fitted exponential gives a rate constant close to 20 s^{-1} for the reaction under the stated conditions. It is clear from this that the calorimeter in its present form is a practical working research instrument, and further examples of its application are given in the accompanying paper (Millar *et al.*, 1987). At the slow end of its range it reaches and overlaps well into the range where batch calorimeters become a better choice (Kodama & Woledge, 1985), and at the quick end it extends the useful range of thermal kinetic studies *in vitro* into a hitherto inaccessible region. Because the development of the device has been associated with a research project we have not been able to take time to optimize systematically all of the components of the system but have instead progressed by experience and intuitive short cuts using components and materials that came readily to hand. Further development of the system should certainly be possible.

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