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High Precision Microcalorimetry: Apparatus, Procedures, and Biochemical Applications

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Apparatus and procedures used for high-precision microcalorimetric measurements are described. The calorimeter is of the heat-conduction type and utilizes semi-conductor thermoelectric modules. The bicompartmental reaction vessel is made of high-density polyethylene and holds about 0.5 mL of solution in each compartment. Imprecision of heat measurement is 0.2 percent when measuring 300 mJ of heat produced by a rapid chemical reaction. Three microcalorimeters are operated simultaneously using a microcomputer and a data acquisition system. Thermochemical and kinetic applications are described. The acquisition of data from an isoperibol solution calorimeter is also described.

Key words: biochemistry; chemical thermodynamics; enthalpy; enzyme-catalyzed reactions; heat-conduction microcalorimetry; isoperibol solution calorimetry; kinetics.

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1. Introduction

Microcalorimetry is an important technique for the measurement of heats of reaction in solution. Since small amounts of solution (typically less than one mL) are required, the technique finds many applications in the study of biochemical and biological systems [1,2][1]. It is the purpose of this paper to describe the microcalorimetric procedures and techniques currently used in the Chemical Thermodynamics Division at the National Bureau of Standards, with particular emphasis on the digital data acquisition system used for the collection and treatment of the experimental data. The simultaneous operation of three microcalorimeters as well as the treatment of experimental data is accomplished with this system. These microcalorimeters have been used in an extensive series of studies of the thermodynamics of enzyme-catalyzed reactions. The calorimeters and the associated data acquisition system can also be used to study the kinetics of enzyme-catalyzed reactions including the measurement of enzyme activity.

Recently, a multi-chambered microcalorimeter has been described by Prosen, Brown, Frohnsdorff, and Davis [3]. Since it contains four microcalorimeters in a single block, it has the advantage that one of the microcalorimeters can be used as a thermal reference or "tare" while the other three are used to monitor processes of interest. This arrangement allows one to carry out long-term studies, e.g., cement hydration. For reactions which are complete within a few hours, the use of a single microcalorimeter has proved successful.

The interfacing techniques used for the microcalorimeters are similar to those needed for the operation of an isoperibol solution calorimeter. This type of calorimeter is useful, in conjunction microcalorimeters described herein, for the study

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[1] Figures in brackets indicate literature references.

of processes in aqueous solution. The computer software used for the acquisition and treatment of experimental data from both the microcalorimeters and the solution calorimeter is described in a separate publication [4].

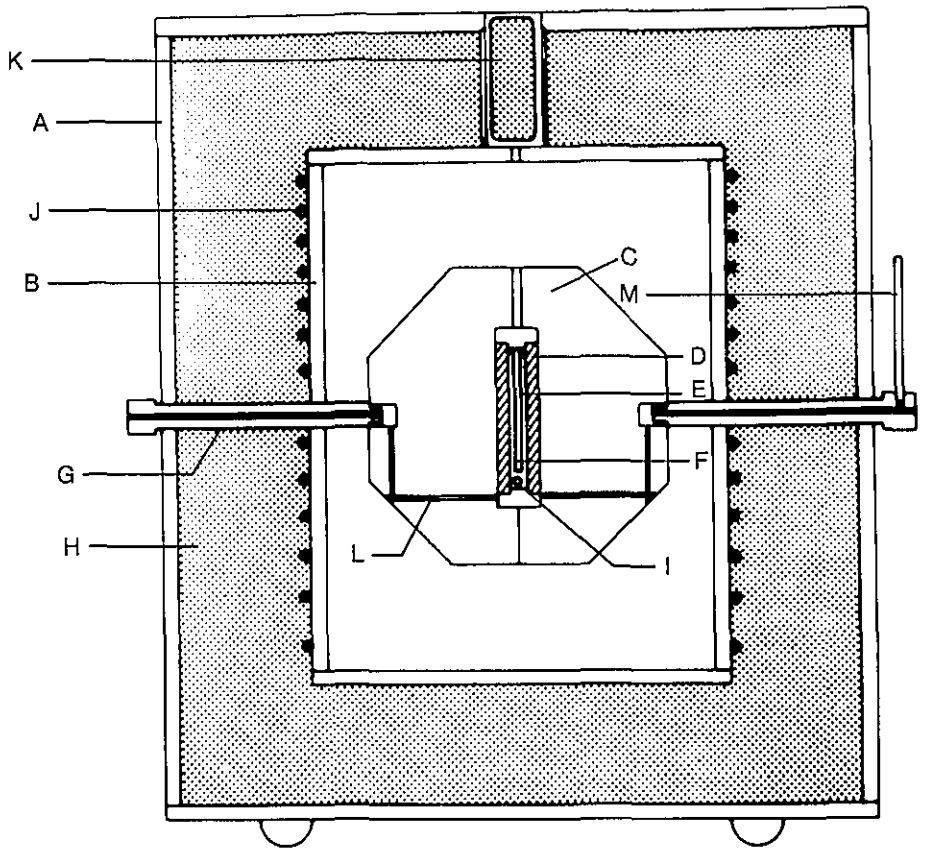
2. Apparatus

Our microcalorimeters are modelled after the one described by Prosen [5], a schematic of which is shown in figure 1. Essentially, it is a heat-conduction microcalorimeter[2] which accommodates a single bicompartmental reaction vessel. The thermopile assembly consists of a pair of bismuth selenide–bismuth telluride–bismuth antimonide thermoelectric modules (Cambion Corporation, model No. 3958-1)[3] electrically connected in series so that the resultant thermopile voltage is the sum of the voltages of the individual thermoelectric modules. Between the thermoelectric modules, D, is sandwiched a rectangular copper container, E, (3.10 cm wide by 3.10 cm high by 1.27 cm thick) which accommodates the reaction vessel. The thermoelectric modules and the copper container are held in place by means of adhesive. This assembly sits in the middle of a bevelled aluminum block, C, (17.8 cm long, 15.2 cm wide) which is supported by hollow nylon rods, G, (1.27 cm diameter, 0.32 cm wall thickness) within an inner aluminum cylinder, B, (25.1 cm diameter by 20.3 cm long with wall thickness of 1.27 cm and 1.27 cm thick end plates) that is thermostatted to within 0.2 mK per day. This inner aluminum cylinder resides within an outer aluminum cylinder, A, (32.1 cm diameter by 40.6 cm long with wall thickness of 0.95 cm and 1.27 cm thick endplates) and is separated from it by $\approx$2.5 cm of polyurethane insulation, H. The copper leads to the calibrating heater and to the thermopiles are carefully thermally tempered to the aluminum block and pass through holes drilled through that block before leaving the calorimeter via the hollow nylon rods, G.

The bicompartmental calorimetric reaction vessels (see fig. 2) are made of high-density polyethylene (0.762 cm thick by 2.54 cm high with 0.050 cm walls). The two compartments are not equal in size; one side holds 0.55 mL of solution

[2] In a heat-conduction calorimeter the reaction vessels is in good thermal contact with a thermopile which, in turn, is in good thermal contact with the heat-sink or block. Thus, most of the heat generated in the reaction vessel flows through the thermopiles to the block. The voltage generated by the thermopile is a measure of the power produced in the reaction vessel [6].

[3] Certain commercial materials and apparatus are identified in this paper to specify adequately the experimental procedures. Such identification does not imply recommendation or endorsement by the National Bureau of Standards.



A - Outer Aluminum Cylinder
B - Inner Aluminum Cylinder
C - Aluminum Block
D - Thermoelectric Module
E - Copper Container
F - Reaction Vessel
G - Nylon Rod
H - Polyurethane Insulation
I - Calibration Heater
J - Wires Wrapped on Inner Aluminum Cylinder
K - Entrance Port for Reaction Vessel
L - Hole in Aluminum Block for Wires
M - Handle for Rotation of Block

Figure 1–Schematic cross-section of microcalorimeter (c. f. [5]).

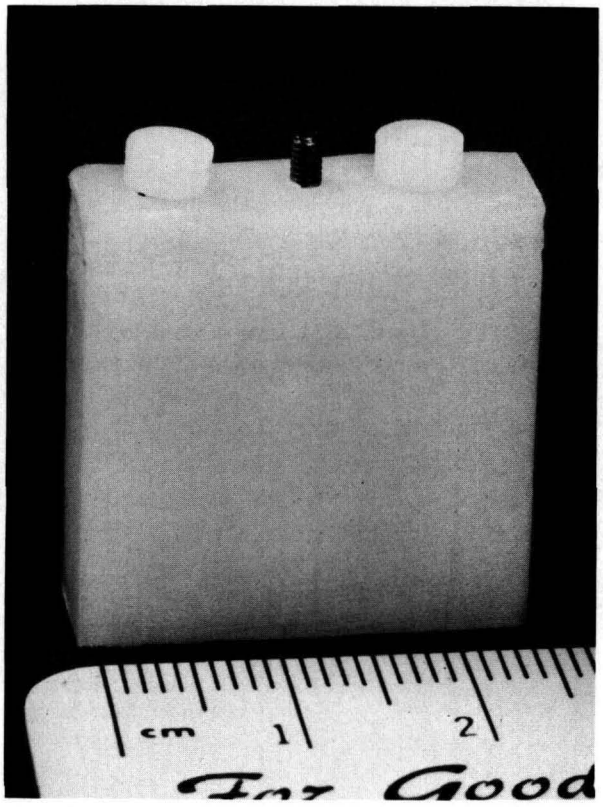


Figure 2–Photograph of high-density polyethylene reaction vessel with O-rings and Teflon plugs in place.

and the other side holds 0.45 mL. Seals are made by compressing Teflon plugs thru the two No. 5-187 Buna-n O-rings which are seated in the filling holes of the reaction vessel. Each reaction vessel is provided with a male stud for connection to a threaded bakelite rod which is used for installation and removal of the cell within the calorimeter. Mixing of the solutions within the reaction vessel is accomplished by rotation of the aluminum block using a handle attached to the nylon support rods. During rotation, the reaction vessel is held in place in the rectangular copper container by means of a piece of phosphor-bronze sheet metal (0.025 cm thick) which is permanently attached to the inside of the rectangular copper container.

The temperature of the inner aluminum cylinder is controlled by means of a Wheatstone bridge circuit. Two arms of the bridge (No. 40 AWG formvar insulated manganin wire) are wrapped around the inner aluminum cylinder; the third arm (No. 40 AWG "99 alloy" formvar insulated wire) is also wrapped around this cylinder; and the fourth arm is a variable resistance box which is kept external to the calorimeter. This resistance box is used to set the temperature of an individual calorimeter. The voltage source for the Wheatstone bridge is a 1.5 V "Batt-sub." The bridge imbalance is amplified by an operational amplifier contained in a current-adjusting type controller (Leeds and Northrup Electromax V). The controller uses both proportional and reset model of operation which are adjusted to obtain a rapid control response with a minimum of overshoot or cycling. The current output from the controller is amplified using a dc power supply and a field-effect transistor amplifier network, and then passed through No. 30 AWG formvar insulated manganin wire which is wrapped around the inner aluminum cylinder. The manganin and the "99 alloy" wires are kept electrically insulated ($>10^8$ ohms) from each other and from the aluminum cylinder by wrapping the aluminum cylinder with a Kimwipe and then coating it with insulating varnish. A second Kimwipe is similarly attached and covers all of the wires that are wrapped around the inner cylinder. All wires are bifilarly wrapped.

A calibrating resistance heater (No. 42 AWG formvar insulated manganin, ≈ 160 ohms) is bifilarly wrapped around a piece of copper wire (No. 20 AWG heavy formvar insulated) which is then placed into a hole in the bottom of the copper container. The heater leads (No. 34 AWG enamelled copper wire) are soldered to the heater and thermally tempered to the copper container for a length of 8 cm. A similar amount of thermal tempering is also done to the copper heater leads at the aluminum block. Potential leads are attached to the copper wire at the point at which they first make contact with the aluminum block after coming from the copper container. Thus, a small heater lead correction (0.013%) is applied during electrical calibration of a calorimeter [7].

Electrical calibration of the calorimeter is performed by measuring (Hewlett Packard 3450A digital voltmeter, inaccuracy less than 0.002%) the voltage across the calibration heater located in the copper container and that across a 1000 ohm standard resistor connected in series with it. Current is supplied from a constant-current dc power supply (Sorensen QHS 40–.5, instability less than 0.001% over a one hour period). Time interval measurement is done using a digital counter (Hewlett Packard 5325B) having a resolution better than one millisecond. The calibration constant of a calorimeter, expressed in W V^{-1}, is measured [6] 1) as the ratio of the steady-state electrical power delivered via the calibrating heater to the steady-state voltage developed by the thermopile during calibration and 2) as the ratio of the total heat deliverd via the calibrating heater to the total area under the voltage-versus-time curve associated with the electrical heat input.

The thermopile voltage from each calorimeter is amplified using a nanovolt amplifier (Keithley model 140, gain linearity 5 ppm, gain accuracy 100 ppm, noise <30 nV peak-to-peak). The gain accuracy was verified using a voltage divider consisting of NBS calibrated 1Ω and 10 kΩ standard resistors in series. A potential difference of 10 V is maintained across these two resistors and the voltage across the 1 Ω standard resistor is amplified at a nominal gain of 10,000 using a nanovolt amplifier. Measurement of the voltage across the two standard resistors and of the amplifier output together with the values of the resistors yields the gain of the amplifier.

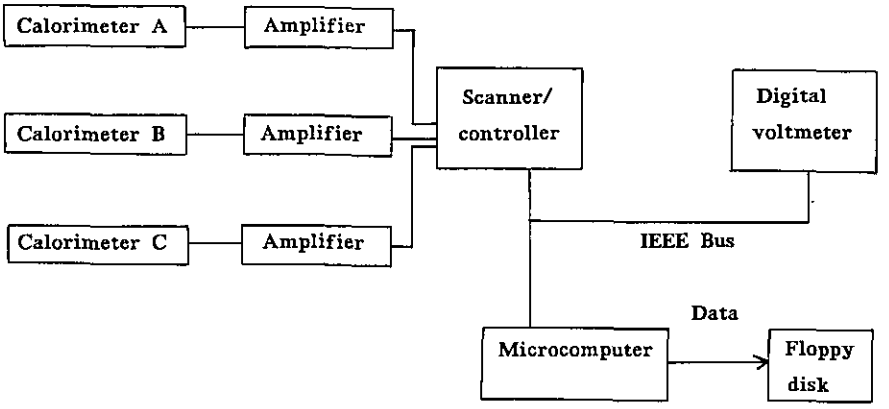


Figure 3–Schematic of data acquisition system for microcalorimeters.

The data collection system is shown schematically in figure 3. The microcomputer is an Apple IIe with two floppy disk drives, a dot matrix printer, a color video monitor, and several cards. The cards in the microcomputer are: 1) clock, parallel and serial interfaces, 2) IEEE-488 communications bus controller, 3) 128 kbyte RAM, 4) 80 column display, and (5) Z–80 microprocessor. All data recording electronics are IEEE-488 bus instruments. They are a digital voltmeter (Hewlett Packard 3456A, inaccuracy <0.002%) and a scanner/data acquisition/control unit (Hewlett Packard 3457A). In-house software was written to control the experiments and to analyze the data.

The temperatures of the microcalorimeters are measured using either a platinum resistance thermometer and a resistance bridge (0.00001 Ω sensitivity) or with mercury-in-glass thermometers having a readability of about 0.02 K. In either case, the thermometer is placed directly into the rectangular copper container in which a reaction vessel is normally placed.

In principle, the calorimeters can operate up to the temperature at which the thermopile units will fail (≈120 °C). A problem which arises at higher temperatures (≈60 to 70 °C) is that the high-density polyethylene reaction vessels expand and thus, if a vessel is introduced into a calorimeter at a lower temperature, that vessel will be extremely difficult to remove after it has reached the temperature of the calorimeter. This problem can be remedied by reducing the thickness of the reaction vessels.

The isoperibol solution calorimeter has been previously described [8]. The principal modification to it is that a digital counter (Hewlett Packard 5334A) is now used to measure the frequency of the quartz-crystal oscillator used for temperature measurement in the calorimeter vessel. The counter is interfaced via the data acquisition/control unit and the IEEE bus to the microcomputer. A schematic of the data acquisition system used for the solution calorimeter is shown in figure 4.

All measuring apparatus were calibrated using standards traceable to NBS. Specifically, the standard resistors and standard cells used in our instrument rack were calibrated by the Electricity Division at NBS, the counter used for time interval

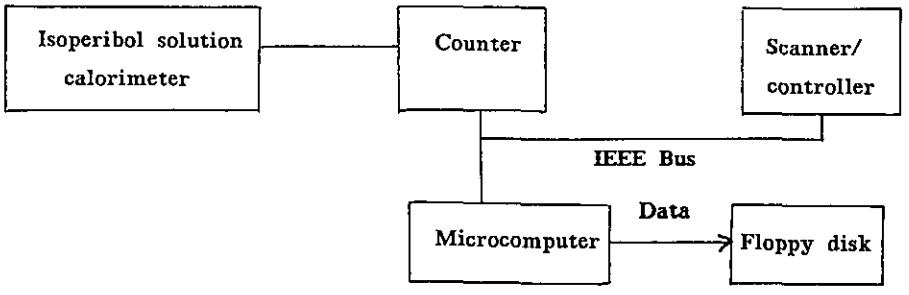


Figure 4–Schematic of data acquisition system for isoperibol solution calorimeters.

measurement was calibrated using the NBS 10 kHz standard frequency, and a capsule-type platinum resistance thermometer was calibrated by the Temperature and Pressure Division at NBS. The resistance bridge used for temperature measurement and the digital voltmeters were calibrated using, respectively, the calibrated resistors and standard cells. The balance used for mass determinations was calibrated using an NBS calibrated set of class M weights.

3. Procedures

The cleaning and loading of the bicompartmental reaction vessels has been found to be an important aspect in achieving good reproducibility. Thus, an ultrasonic cleaner is used for cleaning the reaction vessels following the initial removal and/or inactivation of the solutions in the vessel; for example, a saturated aqueous solution of urea is added to a vessel which contained an active enzyme in solution. Following ultrasonic cleaning, the vessels are rinsed 10 times with distilled water, air dried for several minutes, and then stored in a vacuum desiccator over a drying agent.

The loading of the reaction vessels with solutions is done using a Teflon tipped syringe to avoid problems with droplets which can adhere to metal needles. Avoiding the use of metal objects also helps to mini-mize the chance of damage to the O-ring seats on the reaction vessels. Since the amounts of solution are determined gravimetrically, the vessels are allowed to stand in the balance case in which the weighings are done to minimize both thermal and electrostatic effects. A bottle containing a uranium salt is also kept in the balance case to aid in dissipating any charges. Following loading of a vessel, it is placed into an aluminum block thermostatted at a temperature close to that of the aluminum block in the calorimeter. This pre-equilibration helps to minimize the thermal shock to the calorimeter block. Finally, the reaction vessels are loaded into the calorimeters via the entrance ports and allowed to equilibrate for about one hour. Following equilibration, the calorimeter blocks are rotated several times to mix the contents of the reaction vessels.

The acquisition of data can be broken down into three periods: a fore-period during which the block and reaction vessel are in thermal equilibrium and during which no process heat is produced; a reaction or main-period during which process heat is liberated and flows thru the thermopiles to the block; and an after-period during which no process heat is produced and by which time the reaction vessel and block are again in thermal equilibrium. Recording of the data is typically commenced a few minutes prior to the initiation of the reaction being studied and is continued at 5-second intervals until the conclusion of the after-period. A typical microcalorimetric thermogram is shown in figure 5. During an experiment the amplified voltage data from each microcalorimeter is displayed in a different color on the video monitor. At the conclusion of an experiment, the voltages are recorded on a floppy disk and then analyzed using appropriate computer codes [4].

4. Performance Characteristics

The performance characteristics of the instrumentation are summarized in table 1. Note that the principal limitation on the measurement of small quantities of heat is the magnitude and reproducibility of the "blank" heat of mixing, i. e., the heat effect associated with the mixing of a solution with itself. This effect is dependent upon the solutions being mixed and thus should be determined for each separate investigation in appropriate control experiments.

In table 1 the quantity F is the calibration constant of a calorimeter. Thus, the measured power (dq/dt) is given by

$$dq/dt = F \cdot E \tag{1}$$

where E is the measured voltage relative to a baseline voltage. The total heat measured is given by integration of the area under the curve in a microcalorimetric thermogram [7].

The variation of the calibration constant with temperature can be described by the equation:

$$F = A + B\,T + C\,T^2 \tag{2}$$

where T is the temperature in Celsius. Typical values of the temperature coefficient parameters are: A, 19 to 23 $W\,V^{-1}$; B, -0.07 to -0.08 $W\,V^{-1}\,K^{-1}$; and C, 0.0004 to 0.0005 $W\,V^{-1}\,K^{-2}$.

5. Thermochemical Applications

These microcalorimeters have been used in a series of investigations of the thermodynamics of enzyme-catalyzed reactions (see table 2 and the references contained therein). In addition to their inherent biochemical importance, several of these studies involved reactions of either current or potential industrial importance such as the manufacture of fructose, which is the principal constituent

of corn syrup, and aspartate which is used in the sweetener "Aspartame."

In most of these studies, equilibrium constants have been determined as a function of temperature using chromatographic techniques. Thus, chromatography has been used to measure the extents of the reactions and to detect the presence of any side reactions. Direct heat measurements provide an accurate and direct determination of the enthalpy change for a process. Thus, the combination of equilibrium constants, determined via chromatography or other methods, and enthalpy changes determined from calorimetric measurements are needed to establish and improve the accuracy of the final set of thermodynamic parameters (ΔG, ΔH, ΔS and ΔC_p) for a given process. A knowledge of these thermodynamic parameters allows one to accurately model the behavior of the chemical process of interest over a wide range of temperatures. The combined measurement techniques, microcalorimetry, and chromatography, thus complement each other and are a valuable combination in studying biochemical processes.

6. Application to Measurement of Enzyme Activity and Kinetics

The discussion that follows assumes that the enzyme-catalyzed reaction being studied follows Michaelis-Menten kinetics. The assumed mechanism is:

$$E+S=E\cdot S=E+P \tag{3}$$

where the enzyme, substrate, and products in aqueous solution are designated as E, S, and P, respectively. E·S is the enzyme-substrate complex and the k_i are rate constants. There are three processes to be considered:

$$E+S=E\cdot S \tag{4}$$

$$E\cdot S=E+P \tag{5}$$

and

$$S=P\,. \tag{6}$$

Process (6) is the sum of processes (4) and (5). If at the start of a reaction the enzyme concentration is

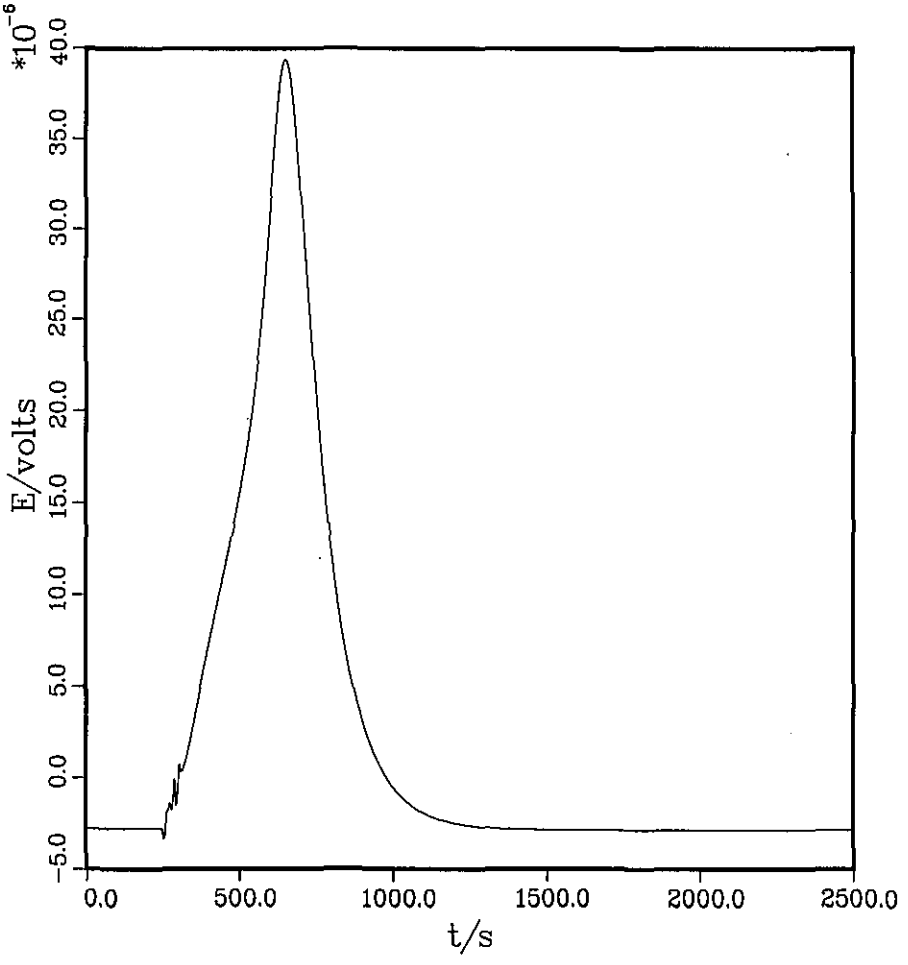


Figure 5–Microcalorimetric thermogram of an enzyme-catalyzed reaction: the hydrolysis of adenosine 5'-triphosphate (ATP) to adenosine 5'-diphosphate (ADP) and inorganic phosphate using *meromyosin*. The irregularities at the beginning of the main or reaction period are attributable to mixing effects. The measured heat effect is 238.0 mJ.

Table 1. Performance characteristics of microcalorimetric instrumentation at 25 °C.

Characteristic	
calibration constant (F)	16 to 22 W V^{-1}
sensitivity (1/F)	0.045 to 0.059 V W^{-1}
noise level of baseline	20 nV peak-to-peak
detectability (1/3 × noise level/sensitivity)	0.15 μW
stability of baseline	100 nV over 24 hours
linearity of calibration constant	0.1 percent from 0.01 mW to 2 mW
time constant[a]	63 s
stability of calibration constant	0.2 percent over 2 years
imprecision[b]:	
q ≈ 10 mJ	0.3%
q ≈ 100 mJ	0.1%
q ≈ 500 mJ	0.05%
q ≈ 300 mJ (chemical heat)	0.2%
"blank" heat effects:	
no reaction vessel in place	0.023±0.03 mJ
empty vessel in calorimeter	−0.065±0.06 mJ
H_2O	0.67±0.22 mJ
human serum	−0.75±1.0 mJ
glucose isomerase (T=25 °C)	−101±4 mJ
inaccuracy[c]	<0.2%

[a] A reaction vessel containing 1.0 mL of water was in the copper container.
[b] Based upon experiments in which electrical heat was introduced for periods of 10 to 100 seconds. All uncertainties are standard deviations.
[c] The inaccuracy was assessed by the performance of both heater placement tests [1] and by measurements of heats of neutralization of HCl with excess NaOH.

e_0 mol L^{-1} and the substrate concentration is s_0 mol L^{-1}, at time t the concentrations are given in terms of the extent of reaction variables (ξ) for the above processes:

$$[E] = e_0 - \xi_4 + \xi_5 \quad (7)$$
$$[S] = s_0 - \xi_4 \quad (8)$$
$$[E{\cdot}S] = \xi_4 - \xi_5 \quad (9)$$
$$[P] = \xi_5 . \quad (10)$$

The rate equations are:

$$d[E{\cdot}S]/dt = k_1[E][S] - k_{-1}[E{\cdot}S] - k_2[E{\cdot}S] + k_{-2}[E][P] \quad (11)$$
$$d[S]/dt = -k_1[E][S] + k_{-1}[E{\cdot}S] \quad (12)$$
$$d[P]/dt = k_2[E{\cdot}S] - k_{-2}[E][P] . \quad (13)$$

At any given time, the instantaneous rate of heat production (dq/dt) is given by:

Table 2. Reactions in aqueous solution which have been studied in our microcalorimeters.

Process	Enzyme	Reference
glucose + ATP^{4-} = glucose 6-phosphate^{2-} + ADP^{3-} + H^+	*hexokinase*	[9,10]
mannose + ATP^{4-} = mannose 6-phosphate^{2-} + ADP^{3-} + H^+	*hexokinase*	[9]
fructose + ATP^{4-} = fructose 6-phosphate^{2-} + ADP^{3-} + H^+	*hexokinase*	[9]
glucose = fructose	*glucose isomerase*	[11]
xylose = xylulose	*glucose isomerase*	[12]
ribose = ribulose = arabinose	*glucose isomerase*	[13]
fumarate^{2-} = malate^{2-}	*fumarase*	[14]
aspartate$^-$ = fumarate^{2-} + NH_4^+	*aspartase*	[15]
$ATP^{4-} + H_2O$ = $ADP^{3-} + HPO_4^{2-} + H^+$	*meromyosin*	[16]

$$dq/dt = k_1[E][S](\Delta H_A) + k_{-1}[E{\cdot}S](-\Delta H_A) + k_2[E{\cdot}S](\Delta H_B) + k_{-2}[E][P](-\Delta H_B) . \quad (14)$$

In general the rate of heat production is a function of four individual rate constants and of two enthalpies in addition to the initial concentrations of enzyme and substrate. Since the time constants of the microcalorimeters are 63 s, the power (dq/dt) must be obtained by deconvolution of the experimental thermogram. If however, slow processes are being studied the deconvolution corrections become negligible. A simplification can be obtained by consideration of the two limiting cases that are now discussed. If the concentration of substrate is much larger than that of the enzyme, the rate of formation of product is proportional to the concentration of the (active) enzyme:

$$d[P]/dt = \lambda_1[E] \quad (15)$$

where λ_1 is an empirical rate constant. If a microcalorimetric experiment is performed where a small amount of enzyme is mixed with a large excess of substrate, the power evolved is:

$$dq/dt = (d[P]/dt)(dq/d[P]) = -[E]\, V \Delta H_6 \quad (16)$$

where V is the volume of solution present and ΔH_6 is the molar enthalpy difference between P and S. The enzyme activity is a measure of the number of moles of substrate converted to product in a given period of time. It is given by:

$$\begin{aligned} dn/dt &= (dq/dt)/(dq/dn) \\ &= -(dq/dt)/\Delta H_6 . \end{aligned} \tag{17}$$

Thus the enzyme activity can be conveniently determined in a one-step procedure if the enthalpy change is known for the reaction of interest.

If, however, the enzyme is present in large excess over the amount of substrate, the rate of formation of the product can frequently be described by:

$$d[P]/dt = \lambda_2[S] . \tag{18}$$

The power evolved is:

$$dq/dt = -\lambda_2[S]\, V\Delta H_6 . \tag{19}$$

The quantity λ_2 is a measure of the optimal rate of conversion of substrate(s) to product(s). Thus, a knowledge of the enthalpy change also allows one to conveniently determine the value of λ_2 from a microcalorimetric experiment. Application of this methodology to the assay of a sample of *glucose isomerase* is shown in figures 6 and 7.

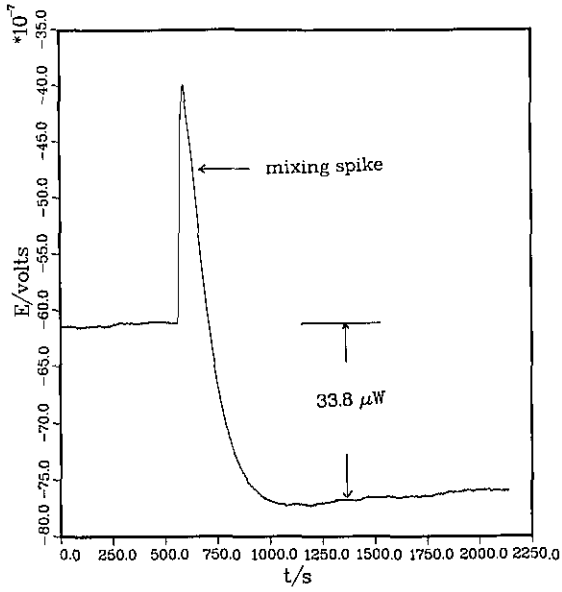


Figure 6–Microcalorimetric determination of the activity of a sample of *glucose isomerase*. The conditions of measurement were: T=50.0 °C, pH=7.4, and phosphate buffer (300.3 mM Na_2HPO_4 and 8.7 mM KH_2PO_4). The measured power was -33.8 μW and the amount of enzyme used was 25.8 mg of solution. Using this data and an enthalpy change of 4.68 kJ mol^{-1} [11] in equation (17) yields an activity of 2.8×10^{-7} mol s^{-1} (g solution)$^{-1}$ or 16.8 μmol min^{-1} (g solution)$^{-1}$. The deconvoluted curve is shown in the figure. Note that although there is a large mixing spike, it does not enter into the final measurement result.

We thank Edward Prosen for his helpful discussions on calorimetry and Irving Price and Nisan Altstein for their help with the electronic circuits.

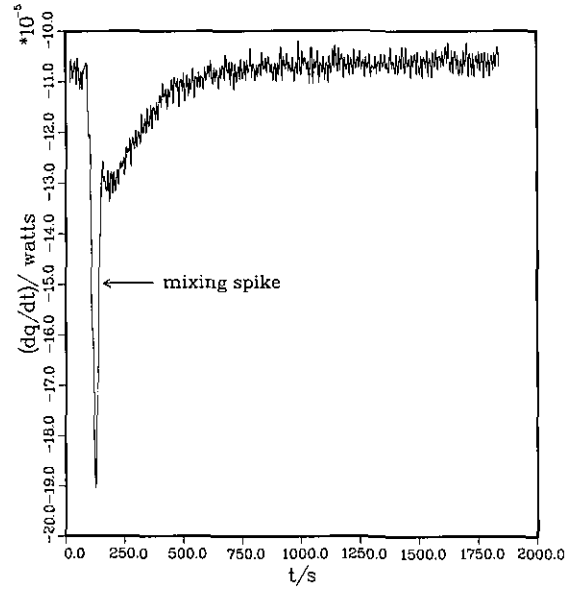


Figure 7–Microcalorimetric determination of the optimal rate of conversion of glucose to fructose using *glucose isomerase*. The conditions of measurement were: T=50.0 °C, pH=7.4, and phosphate buffer (30.3 mM Na_2HPO_4 and 8.7 mM KH_2PO_4). The initial concentration of the glucose was 4.02 mmol L^{-1}, and the total volume of solution present was 0.087 mL. The *deconvoluted curve is shown in this figure. Extrapolation of* the measured power to the start of the experiment yields a value of -42.0 μW. Use of this value, the initial concentration of the glucose, $\Delta H = 4.68$ kJ mol^{-1}, and eq. (19) leads to $\lambda_2 = 0.0026$ s^{-1}. As in the previous example (fig. 6), there is also a large mixing spike.

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