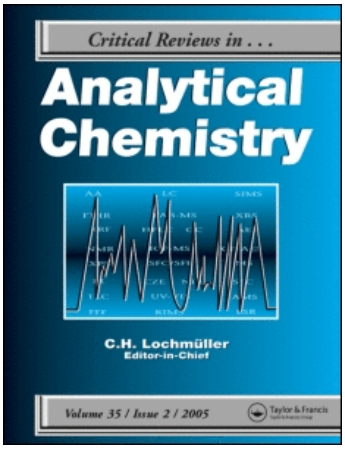

Critical Reviews in Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713400837

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To cite this Article Martin, Charles J. , Marini, Mario A. and Brown, Harry D.(1979) 'Microcalorimetry in Biochemical Analysis', Critical Reviews in Analytical Chemistry, 8: 3, 221 — 286
To link to this Article: DOI: 10.1080/10408347908542712
URL: http://dx.doi.org/10.1080/10408347908542712

MICROCALORIMETRY IN BIOCHEMICAL ANALYSIS

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I. INTRODUCTION

Recent years have seen the development of methodology transcending mere application of older techniques that could be applied to the rapid, precise determination of specific components in complex mixtures — usually of biologic origin. The development of ion-selective,[1] other than for protons, and enzyme-specific electrodes[2] are examples in point. Although the latter has its specific limitations of application, the generality of approach is limited only by miniaturization technology and the sophistication of the chemist/biochemist to attach enzyme molecules to insoluble matrices with retention and stability of biological activity. Solid surface fluorescence methods as developed by Guilbault and co-workers[3] are also of recent origin and offer great potential for routine and inexpensive assays in the clinical laboratory.

Calorimetry can be added to this list even though it is not new in either concept or theory, having been used by Lavoisier and Laplace in 1780.[4] Calorimetry, or more specifically microcalorimetry since we are interested in the measurement of small heat changes in small volumes, has as its prime attraction for analytical use, the fact that essentially all reactions occur with the absorption or evolution of heat. This also defines its greatest limitation for certain applications — the lack of specificity. This can either pose problems, e.g., the isolation or sorting-out of the heat change arising from a single reaction among others in a multireacting system, or be considered a blessing as in following the changes in metabolic activity of whole cells or tissues. In the field of biology, however, specificity is inherent in many of the very reactions that one wishes most to study, e.g., enzyme-substrate reactions, protein-protein reactions, protein-ligand binding, antigen-antibody interactions, etc., and the heat changes attendant with such reactions have been and can be related to the events that produced them.

With appropriate instrumentation then and assuming that a stoichiometric relation between a change in temperature and a particular event can be established, microcalorimetry can be utilized as an analytical tool. By measuring the change in only one intensive property, temperature, one can determine heat changes of microjoule* magnitude which relate in many cases to nanomole amounts or less of material. Furthermore, the magnitude of the temperature change that occurs is dependent on the concentration of reactants per unit volume and not on the total volumes involved. The lower limit of sample volume then is dependent solely on the practical degree of miniaturization of the calorimeter cell.

Aside from its analytical applications, heat change measurements of specific reactions can lead to values for integral enthalpy changes (ΔH). Provided that access to the equilibrium constant, K, of a reaction is also available and hence to a value for the Gibbs free energy change, ΔG, one can also calculate the change in entropy, ΔS, for the reaction. In many cases, proper design of the calorimetric procedure provides data from which both ΔG and ΔH, and hence, ΔS, can be obtained. Thus, both the analytical and thermodynamic uses of changes in temperature or its electrical equivalent will be considered in this review.

The analytical application of reaction microcalorimetry rests upon the relation

$$n = Q/\Delta H \qquad (1)$$

where Q, in calories or joules, is the heat released or absorbed per n moles of substance reacting or product formed, and ΔH is the integral molar enthalpy of the reaction. If an adiabatic calorimeter is used, then,

* One calorie equals 4.186 J; 1 W equals 1 J/sec.

$$Q = C\Delta T \quad (2)$$

i.e., the heat change, Q, is proportional to the temperature change, Δt, multiplied by the effective heat capacity, C, of the system.

If a heat-conduction microcalorimeter is used, Q is proportional to the heat flow, ϕ, integrated over the time of its duration, viz.,

$$Q = k \int \phi dt \quad (3)$$

Since any temperature change can be translated into a quantity Q for the reaction of concern and if ΔH is known, the concentration *n* can be determined. If ΔH is unknown, a calibration curve of Q ($\propto \Delta T$) vs. *n*, with *n* independently determined, can be used for the analysis of unknown samples. Alternately, kinetic rather than equilibrium measurements can lead to the quantitation of a given component. For example, if a component is present in trace amounts, such as an enzyme that catalyzes a given reaction, measurement of dQ/dt during the initial time course of the reaction can be equated to enzyme concentration (activity). The value of ΔH for the reaction need not be known. It need only be of sufficient magnitude to permit the detection of heat change with time. In certain cases, even this requirement can be obviated through coupling with other suitable reactions to provide the requisite amplification for detection (*vide infra*). Calorimetry thus shares with spectrophotometry (and other techniques) the equation of a physical parameter to the concentration of the component(s) generating the change in the observed parameter.

The measurement of a temperature (heat) change has several advantages over spectrophotometric procedures of assay. The necessity of optically clear solutions need not be met. The change in temperature of turbid solutions of microbial cultures, cell suspensions, etc., has been and can be determined. In fact, concentrations may be measured based upon the heat of precipitation. The requirement that the component to be determined has an accessible absorption band, a *sine qua non* in any spectral method, is of no relevance for microcalorimetric measurements.

Calorimetry as an analytic technique has another characteristic in its favor. It is essentially nondestructive and does not require additions of chromogenic agents to generate chemical species with workable absorption envelopes as needed for spectral assays. Considering that microcalorimetry also offers the potential for multiple sequential analysis on a single sample volume, the implication of this is obvious.

Given that the requisite specificity is experimentally attainable, the question of the sensitivity of microcalorimetry must be considered. Currently, batch and flow calorimeters are available with sensitivities of the order of 1 to 10 μcal ($\sim 10^{-5}$°C). Titration microcalorimeters presently in use in our laboratories are capable of detecting heat changes of less than 1 mcal with a high signal to noise ratio. Thus, if the ΔH for a reaction is 10 kcal/mol, the concentration of component required for detection (signal/noise $\geq$ 10) is approximately 30 nmol.

Thus, at the present state of development, calorimetry has proven to be useful for the resolution of a variety of biologic problems. With but few exceptions, this review will restrict itself to developments that have occurred since *circa* 1970 through 1977. It will also make no attempt to be inclusive in content. To do so would be redundant, and the reader is directed to a number of comprehensive reviews that have appeared by Wadsö,[5] Sturtevant,[6] Goldberg and Armstrong,[7] Rialdi and Biltonen,[8] Spink and Wadsö,[9] and Barisas and Gill.[10] Books by Tyrrell and Beezer,[11] Bark and Bark,[12]

Vaughn,[13] Barthel,[14] and the articles by Jordan et al.[15] and Marini and Martin[16] deal with the more specialized area of titration calorimetry.

Since this article involves the application of microcalorimetry to biochemical analysis, it dictates that we be concerned almost exclusively with solution calorimetry or the evaluation of heat changes occurring in solution and often called reaction solution calorimetry. This is appropriate since water is the universal environment for life processes. Furthermore, we shall be concerned primarily with those calorimetric constructions that are of sufficient sensitivity and undemanding of material that would permit them to be of practical application to the biologic sciences. Although what one would consider a practical instrument is necessarily contingent on the specific problem and the individual preference of the investigator, a desirable calorimeter can be specified.

Keeping in mind that design characteristics for biochemical applications should encompass the capability of both kinetic and endpoint (i.e., to a given extent of completion) measurements, the microcalorimeter would have the following specifications: (1) cell volume, 0.1 to 2.0 mℓ; (2) sensitivity, 1 to 10 μdegrees; (3) sensor response, 10 msec (99% actual), and (4) reproducibility, 0.1% maximum response. To our knowledge, this state has yet to be reached in a single instrument which would also permit rapid equilibration (approximately 5 min or less) and easy access to the actual reaction vessel and its contents.

II. TYPES OF MICROCALORIMETERS

A. Adiabatic

There are two main types of microcalorimeter constructions: adiabatic and heat conduction. A close approximation to the adiabatic state, i.e., no heat exchange of the reaction vessel with its surroundings, can be experimentally achieved by placing the reaction vessel within the envelope of an adiabatic shield. In the case of an exothermic reaction, the shield would be electrically heated in synchrony with the change in the reaction solution so that the condition of the temperature difference between vessel (T_1) and shield (T_0) remains essentially zero. In the case of an endothermic reaction, a heat-pump device can be used to keep the temperature of the reaction vessel constant and equal to that of the jacket or shield. Because of the complexity of design and the differential temperature control required, these types of calorimeters have not been utilized much for microcalorimetric constructions. Their overall design also dictates that they be used only for slow reactions.

B. Heat Conduction

A more common type of construction and one that in various designs, has been used for microcalorimetric work is the heat-conduction calorimeter. In this type, of which the Tian-Calvet design is a well-known example,[17,18] there is a transfer of heat through a thermopile to a heat sink. In practice, the heat capacity of the sink is of the order 10^3 greater than that of the reaction vessel. In such calorimeters the heat change, Q, is proportional to the voltage change, V, through the thermopile during the reaction

$$Q = k \int V dt \qquad (4)$$

where k is the effective proportionality constant. A representation of the form of a data output curves (Q in millivolts vs. time) for simulated pulse heat reactions is shown in Figure 1.* The curves were computed using the model reaction

All figures appear following the text.

$$A + B \rightarrow Products + Q$$

where A and B are reactants with Q, representing the transient observed voltage which is proportional to the heat change. The decay portion of the curve is exponential, and the total heat generated in the reaction in a heat-conduction calorimeter meeting certain conditions[17] is equal to the area under the curve.

When operated as a constant temperature calorimeter, the condition that $T_0 - T_1 = 0$ can be satisfied in different ways, and if the heat change is small, Joule heating and Peltier cooling can be used. High capacity heat pumps have been described by Walisch and Becker[19] using semiconductors.

1. Batch Microcalorimeters

A batch microcalorimeter of the heat-conduction type suitable for biochemical work has been described by Evans et al.[20] An exploded view of the aluminum calorimeter block is shown in Figure 2a. The twin-partitioned cells (Figure 2b) are machined from tantalum, and each part is charged with approximately 1.5 mℓ solution. Mixing occurs by rotation of the thermostatted assembly. For chemical reactions, the precision is about 1%.

A highly sensitive heat-conduction microcalorimeter has been designed by Prosen[21,22] primarily for analytical purposes (Figure 3). This calorimeter is sold commercially as the Prosen-Berger Batch Micro-biocalorimeter.* The partitioned cell (approximately 0.15 mℓ volume each side) is made of Kel-F plastic and is similar to that of Figure 2b. The response of this calorimeter is fast with a k constant of the order of 16 J/V/sec. The actual data output from this microcalorimeter in response to the input of 200 μJ electrical energy is shown in Figure 4.[23] Results obtained for the action of DPNase acting on DPN are shown in Figure 5. This figure also shows that after the reaction is over and equilibrium once again attained, that the action required for mixing leads to a barely detectable heat output.

2. Flow Microcalorimeters

A stopped-flow microcalorimeter has been designed for the study of enzyme kinetics by Berger et al.[24] It is of the Tian-Calvet type as modified by Evans et al.[20] and is shown in Figure 6. Sample sizes for each reagent can vary from 35 to 500 $\mu\ell$ and the sensitivity is 0.2 nmol for a reaction of 4×10^4 J/mol. Using a fluidic drive device as shown in Figure 7, cycle times between experiments are less than 5 min. Precision of response is within 2%, and Figure 8 shows repetitive runs for the mixing of HCl with NaOH. The k constant is 22.5 J/V/sec. In the data reduction process, finite element analysis[24,25] was used to reconstruct true kinetic curves.

A number of flow, as opposed to stopped-flow, microcalorimeters of the heat-conduction type have been described (cf. Spink and Wadsö[9]), and one of the most widely used is that due to Monk and Wadsö[26] and shown in Figure 9. A commercial version of this type is available.**

In a flow calorimeter, one or two liquids are pumped at a constant speed through a heat-exchange unit and then into either a flow-through cell or a mixing cell. The latter is used for measurement of various fast processes, heats of dilution, and other types of thermodynamic quantities. The flow-through cell, on the other hand, has been used primarily for analytical purposes, e.g., enzyme activity measurement, involving sub-

* Available from Thermonetics Corp., P. O. Box 9112, San Diego, Calif. 92109, in single or differential cell design.

** LKB Produkter AB, Bromma, Sweden. Sensitive batch and sorption microcalorimeters are also available.

strate-saturated enzyme solutions, cellular suspensions, etc. In this type of calorimeter, heat effect sensitivity is about 0.1 μW with precision of about 0.1% for reactions with heat effects of approximately 200 μW.

Heat-conduction flow calorimeters present problems in reconstructions of rate processes from the data output. Not only must one account for heat transfer through the thermopile, but also for the transit time the reacting solutions spend in the calorimeter cell. However, when the flow rate is adjusted so that the rate of heat flow from the cell is equivalent to the rate of heat production, then the measured response closely tracks the actual rate of heat generation.[27]

Beezer and Tyrrell[28] have treated the problems encountered in the extraction of kinetic data from flow microcalorimetric experiments. For enzyme-substrate reactions, data output from a flow-through cell will take the form shown in Figure 10. The heat output rate, dQ/dt, in calories or joules per second, will, for a zero order rate process (substrate saturation of the enzyme), be

$$dQ/dt = -k_o e_o V_c \Delta H_R \quad (5)$$

where k_o is a constant, e_o, the enzyme concentration; V_c, the volume of the flow cell; and ΔH_R the heat of the reaction. For first order reactions, wherein S_o (the substrate concentration) is much less than K'_m (the S_o giving one-half maximum velocity), one has

$$v_o = d(PROD)/dt = k_1 S_o \quad (6)$$

where v_o is the velocity of the reaction and k_1 equals $(e_o k)/K'_m$. If k_1 is sufficiently large, the quantity dQ/dt, now a more complicated function of enzyme concentration, substrate concentration, and residence time of the solution in the cell (inversely proportional to the flow rate), will be as shown in Figure 10. With S_o at values smaller than K'_m and as the only variable, dQ/dt at a constant time will be a linear function of S_o. At very high values of S_o, dQ/dt becomes a constant. For enzyme-substrate reactions showing adherence to Michaelis-Menten kinetics, the parameters of the reaction (V_{max} and K'_m) can be evaluated from flow calorimetric data,[28] but the process is cumbersome.

An adiabatic flow microcalorimeter of high precision and rapid response has been reported by Gill and Chen.[29] A diagrammatic cross-section is shown in Figure 11. Typical recorder tracings of the data output for mixing and electrical energy input experiments are shown in Figure 12. Precision of measurement is of the order of 0.1 μW.

C. Isoperibol Titration Microcalorimeters

A frequently used construction for microcalorimetry is of the isoperibol type, also called an isothermal jacket or constant-temperature environment calorimeter. The basic design can be elaborated in diverse ways and can be used in batch, flow, or titration mode. This variant of an adiabatic calorimeter has been widely used " . . . as the most precise construction(s) for fast processes . . . ".[5] When used as a titration microcalorimeter, it "can give faster operation than, and at least as good accuracy as, either batch or flow operation".[6]

In its most uncomplicated construction, there will be heat exchange between the reaction vessel and its surroundings during the course of any reaction, and such heat loss or gain must be corrected for. When used as a titration calorimeter, the reaction vessel will generally contain a stirrer, thermistor, titrant tips, and electrical heater. A

simple design of this type is shown in Figure 13.[30] In operation, such designs have the advantage of simplicity of operation, rapidity of response and fast cycle times between reactions. The construction shown in Figure 13 uses 2 to 4 mℓ reaction volumes, and with the use of the "thermo jet", pre-equilibrium conditions can be attained in less than 5 min. When such designs are operated in an ambient environment with the temperature of the jacket maintained by circulating tempered water, one approaches the desired combination of uncomplicated construction coupled with flexible and facile operation. Calibration is by neutralization of HCl (20 to 40 μmol) with standard KOH or electrically, and typical data output of such a reaction is shown in Figure 14. The graph also shows that with the addition of a combination pH electrode into the calorimeter cell, the simultaneous change in pH can also be recorded.[31]

Reproducibility of measurement as calculated from 100 (SD)/mean is 0.5% and can yield thermodynamic data in reasonable agreement with literature values.[30] For example, measurement of the protonation of Tris buffer (exothermic reaction) at 20°C gave −11.40 kcal/mol.[16] Literature values for the heat of ionization (reverse reaction) range from 11.35 to 11.39 kcal/mol.[32-34]

An example of the titration of a weak monoprotic acid (imidazole) is given in Figure 15. From the midheight of the thermal curve which occurs at the pK′ of the acid, one can readily calculate the heat of ionization (ΔH_i) from

$$\Delta H_i = \Delta H_F - (VC_sQ_{obs}/V_sN) \qquad (7)$$

where ΔH_F = heat of formation of water, approximately 13.5 kcal/mol at 20°C;[35] V_s = volume of standard used for determination of C_s, the calibration constant in cal/mV; V = volume of sample; Q_{obs} = height of thermal curve; and N = moles of acid titrated.

A "second generation" isoperibolic microcalorimeter now in use in our laboratories and designed and constructed in collaboration with R. L. Berger and L. Thibault of the National Heart, Blood and Lung Institute of the National Institutes of Health (NIH) and outfitted for the simultaneous measurement of both pH and temperature is shown in Figure 16. Maximum reaction volume is 2.0 mℓ, and the inner chamber is fitted with Teflon® stirrer, a 15-kΩ thermistor within hypodermic tubing, Leeds and Northrup pH electrodes (or Radiometer #GKS-73041 combination electrode), and titrant delivery tips. The increased sensitivity is indicated by the titration of 1.0 μmol HCl in a 2.0-mℓ volume by standard KOH using this calorimeter as shown in Figure 17.[36] The titration is complete in less than 10 sec, and 0.1 mV (1 in. pen deflection) equals 0.60 mdeg. The shape and treatment of the heat loss decay curve after completion of the reaction will be considered later. A block diagram of the total apparatus and the data acquisition and storage system is shown in Figure 18.

Titration calorimeters can also be operated isothermally, and one such construction is shown in Figure 19 and due to Christensen et al.[37] It represents a modification of an earlier design of an isoperibolic titration calorimeter[38] with reaction cup of approximately 100 mℓ volume. The entire device is immersed in a constant temperature bath, and a Peltier heat pump is connected to the reaction vessel. Through pulsed electrical heating and constant Peltier cooling, the reaction solution can be kept at a constant temperature. This isothermal state is maintained by equating the energy input from the variable heater to the sum of the heat from the reaction being studied and the energy removed by the constant Peltier cooler.

Assuming that other heat leakages can be experimentally compensated for, the energy required to maintain the reaction solution at a constant temperature can be

equated to Q. The authors[37] state that the inside calorimeter temperature can be controlled to $\pm 2 \times 10^{-4}$°C. This calorimeter, as constructed, can measure heats such as the ionization of water and the dilution of perchloric acid solutions to an accuracy of ±0.02 cal.

More recently,[39] a microversion of the Brigham Young's group isothermal titration calorimeter has appeared (Figure 20). The reaction cup has a 4-mℓ volume and is made of platinum. Temperature can be maintained constant to $\pm 2 \times 10^{-5}$°C, and heats as small as 3 mcal have been measured.* Titration calorimeters based on the designs of Christensen et al. can be obtained commercially.**

This same group has also designed and constructed an isothermal high-pressure flow calorimeter suitable for measuring heats of reaction and mixing at pressures up to 400 atm.[40] Energy changes of 0.036 to 7.17 cal/min are stated to be measured with a precision of 0.4%. For a calorimeter operated isothermally, heat capacity measurements and corrections for heat exchange with the surroundings are not necessary. For the microversion described in Reference 39, the data output is in the form of a derivative (dQ/dt) vs. time.[41] The response time of the system to changes in heat production is 2 to 3 min. This leads to "blind spots" in the output curve during the initial phase of a heat generating reaction. Of course, if kinetic information of fast processes is not desired or if reactions studied are very slow, this is not a disadvantage.

An instructive description of a total precision titration calorimeter system (manual or automatic operation) is given in Reference 14, page 185, and the titration assembly plus bath is shown in Figure 21. Temperature changes of the order of 10^{-5}°C can be measured, although for biochemical purposes, the reaction vessel volume (200 mℓ) is quite high.

Isoperibolic calorimeters based upon refined designs of Sunner and Wadsö[42] are commercially available.***

A novel and simple design of an analytical microcalorimeter has been described and tested by Pennington.[43,44] The construction is shown in Figure 22. For measurement of the heat change of a reaction, 5 to 50 μℓ of one solution is placed on the Peltier detector surface, and after thermal equilibration (15 to 30 sec), 5 to 50 μℓ of the preequilibrated second solution is added and the resultant heat change recorded. The data output is of the form of a heat-conduction calorimeter (cf. Figure 1), and typical response curves for the Tris-HCl reaction are shown in Figure 23. Heat changes of less than 5 mcal can be detected.

The device can be used for the measurement of enzyme-substrate reactions, chloride determinations, etc. with peak areas proportional to concentrations of substrate or other components of interest. Since reactions are diffusion controlled, enzyme activity cannot be quantitated except in the relative sense of time-for-completion of the reaction. Further refinement of this concept could prove a valuable analytical technique in the clinical laboratory from the standpoint of simplicity, rapidity, and ease of operation with both good precision and accuracy.

1. Heat Loss Corrections

Heat loss corrections from isoperibolic calorimeters have been approached in a variety of ways. All of them are approximations and vary from simple graphical procedures[11,16,45] to the more elegant heat balance equations proposed by the Brigham

* Design improvements now permit such calorimeters to maintain temperature constant to $\pm 2 \times 10^{-6}$°C.[195]

** Tronac, Inc., 1804 S. Columbia Lane, Orem, Utah 84057.

*** LKB Produkter AB, Bromma, Sweden. Sensitive batch and sorption microcalorimeters are also available.

Young group.[34,36] In macroconstructions, heat loss by conduction can be generally accounted for by the use of Newton's cooling law

$$dQ/dt = \frac{dT}{dt} = k\,(T_0 - T_1) + \theta \qquad (8)$$

where θ is the combined effect of heat of stirring and Joule heating by the thermistor. This is often used in the form of the Regnault-Pfaundler equations,[45] but is cumbersome to use. If the generation of heat is an exponential function, Dickinson's graphical method can be used and gives identical results to the Regnault-Pfaundler treatment for reactions of short duration.

When the data output is in the form of a titration thermogram such as shown in Figure 15, the midheight of the curve measured at the extension of the lower and upper baselines is a relative measure of the total heat generated.[16] This assumes constancy of heat effects from stirring and Joule heating during the run and that the upper baseline has a constant slope. When coupled with similar treatment of the data obtained from the neutralization of HCl with KOH, the procedure can yield ΔH values with a reproducibility of 0.53% and in reasonable agreement with literature data.[30]

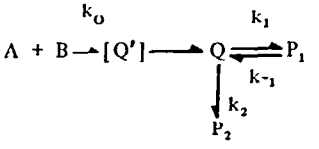
Eatough et al.[46] have carefully analyzed the results of thermal titrations. Equations have been presented applicable to calorimetric vessels larger than approximately 25 mℓ and with small heat leak moduli ($k<0.005$ min^{-1}). Their approach permits the calculation of (1) the total enthalpy change; (2) correction terms arising from heat exchange of the reaction vessel with surroundings, heat of stirring, Joule heating by the thermistor, and evaporation effects; (3) effects due to the temperature differential between titrant and reaction solution (if any); (4) heats of dilution; and (5) the enthalpy change from other chemical reactions. When heat flows due to these varied effects are calculated, the resultant heat balance permits evaluation of the enthalpy change of the chemical reaction of interest.

As Hansen et al.[34] have pointed out, however, heat loss to the surroundings from small vessels (approximately 5 mℓ or less) is proportionately much greater than from much larger vessels. This can be seen in Figure 24 where the heat leak modulus, k, is plotted against volume of Dewar reaction vessels. As the volume becomes smaller, k increases exponentially. The fact that k was found to be an inverse function of vessel volume required that the cooling correction be treated more exactly. Therefore, to correct for the heat exchange with the environment, it was necessary to determine how k varied with the volume of water in the Dewar vessel. This is shown in Figure 25. From such data and other considerations, expressions were derived which in concert with those used earlier,[46] permitted calculation of the heat loss to the surroundings from small cells. Their treatment was checked by measurement of ΔH for the ionization of water and the protonation of Tris buffer under conditions wherein approximately 25% of the total heat change was lost to the surroundings.[34] By the use of such cooling corrections, their ΔH values agreed closely with literature results.

A different approach to the same problem may be taken.[47] For thermal titrations done in 2.0 mℓ reaction volumes (cf. Figure 17) using the the microcalorimeter shown in Figure 16, the data output curves can be simulated by differential equation modeling. Curves closely resembling actual data obtained at differing titration rates (same reaction complete in 10 to 180 sec) are shown in Figure 26. Each curve is comprised of a "rise" in Q_{ob}, which is nonlinear even though dQ_a/dt (the "adiabatic" heat change with time) is a constant in each case, followed by an exponential decay curve. Each run done at a longer titration time results in its decay curve lying above the similar

portion of the shorter-timed reaction, although they all merge to a common point (the equilibrium point) at infinite time. This will differ slightly from the temperature of the surroundings due to stirring and Joule heating of thermistor effects.

The shape of the decay curves cannot be explained by simple Newtonian cooling. One must assume at least two exponential "holes" in the reaction cell equivalent to two major heat sinks if one is to reconstruct the "adiabatic" thermograms, i.e., the curves that would be obtained if there was no heat interchange with the surroundings. One such model which can correct all of the curves shown in Figure 26 to a common height Q_a (the "adiabatic" temperature rise) can be written:



where P_1 and P_2 represent the heat sinks; Q_a, the heat change without heat loss; Q, the observed heat change; k_0, a zero order rate constant; and k_1, k_{-1}, k_2, first order rate constants. The production of Q_a from the titration of A (HCl) with B (KOH), although the result of a second order reaction, is effectively zero order, since B is added to A at a constant rate and the reaction rate can be considered instantaneous.

Solution of the differential equations associated with this model[47] leads to the "adiabatic" heat curves shown as dashed lines in Figure 26. Since the identical reaction was assumed in the simulation of each curve, each corrected curve should have the same height as shown.

Heat loss corrections by this type of procedures do not require evaluation of the heat capacity of the system, but do require evaluation of the heat loss rate constants. This can be achieved by computerized iterative curve-fitting procedures (NIH-MLAB program)* using the decay curve of a fast reaction. This procedure is applicable to the "adiabatic" reconstruction of any kinetic curve, such as that shown for the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine ethyl ester in Figure 27.[16] It can also be applied to any titration thermogram wherein Q_{obs} as f(t) has been obtained as in the titration of the ionizable groups of proteins.

It should be remembered, however, that for larger reaction volumes, simple graphical procedures can serve the needs of an analytic procedure exceedingly well. If one calibrates the microcalorimeter by using the HCl-KOH reaction (or any other chemical reaction) so as to approximate all conditions in effect during the titration of a sample, then such multiple effects as Joule heating, stirring heat, the refrigerant effect, i.e., cooling of an exothermic reaction by titrant,[16] volume change, etc. are incorporated in the calibration constant. This rather simple approach can lead easily to analytical data good to within ±0.5% or better.[30]

III. ANALYTICAL APPLICATIONS

A. Clinical Assays

In the development of any approach to the determination of a given component in a multisolute solution by a routine procedure, there are several factors which ideally should be satisfied. The method should be

* An on-line modeling laboratory program obtainable from the Division of Computer Research and Technology, NIH, Bethesda, Md.

1. Specific for the desired component
2. Accurate and precise
3. Simple and rapid
4. Economical
5. Reliable

Present techniques of microcalorimetry have the potential to satisfy these varied requirements to a considerable degree, and microcalorimetric measurements have been used for the analytical determination of varied substituents, including individual components of mixtures. Its direct application to problems of the clinical laboratory, however, has been minimal.

Certainly, the heat change attendant to reactions capable of yielding clinically significant diagnostic information has been measured. By and large, however, such studies have been done with model systems, and only a scant few papers have appeared in the literature in which the usual patient-source biologic materials have been the object of study. This is due to a number of reasons which run the gamut from inertial resistance to change to real questions of concern, such as specificity of measurement, sensitivity, equilibration times, sample volumes, complexity of instrument design, and interpretation of results.

Some of these objections, in a conceptual sense, have already been addressed. There are a number of microcalorimeters on the market with the necessary sensitivity, small sample size requirements (0.15 mℓ), and precision to perform adequately. Rarely does the clinical chemist need data to better than ±2 to 3%, and for purely analytical assay work, these microcalorimeters are more than adequate.

In an early exploratory study, Monk and Wadsö[48] showed that one could readily detect both substrate and enzyme concentration levels using a flow microcalorimeter. Reactions studied were those involving glucose oxidase, cholinesterase, alkaline phosphatase, lactic acid dehydrogenase, and ATPase activity. The urea-urease system has been studied by Beezer et al.,[49] and a discussion of the data treatment of the kinetic results was given (cf. also Reference 28).

In the clinical laboratory, the assay of enzymes requiring the NAD-NADH or NADP-NADPH system as cosubstrates is routinely done. The change in absorbance at 340 nm is the detecting system. These methods also provide the basis for the determination of various metabolite levels.

Poe[50] has measured the enthalpy change of the reaction

$$NADH + H^+ + 1/2\ O_2 \rightleftharpoons NAD^+ + HOH$$

to be −61.6 ±1.1 kcal/mol. From the redox potential of this reaction ($E^{o\prime}$ = 1.136 V) and making a reasonable assumption for the quantity $(dE/dT)_p$, one can calculate a ΔH of −58 kcal/mol, in good agreement with that determined calorimetrically. Although we could not find comparable calorimetric data for the reaction

$$NAHD \rightleftharpoons NAD^+ + H^+ + 2e^-$$

from the value of $E^{o\prime}$ of 0.320 V, one can calculate a ΔH of ∼ −20 kcal/mol. With the assumption that this is reasonably correct, it justifies the premise that this magnitude of heat change will permit detection at the nanomole level of the oxidation or reduction of the NAD(H)-NADP(H) cofactors. In fact, for any reaction involving their oxidation, a proton is liberated. Therefore, if a Tris buffer is present, its protonation

would yield ΔH, −11.4 kcal/mol. Since heat changes are algebraically additive, the oxidation of NADH in the presence of a Tris buffer should yield an "effective ΔH" of approximately −30 kcal/mol. With the present design of the thermal titrator microcalorimeter in current use in our laboratories, this would permit the detection of approximately 10 nmol NADH (NADPH) oxidized. It is, therefore, apparent that enzyme and metabolite systems of biological materials which involve the pyridine nucleotides can be assayed using microcalorimetry. As a general principle, the heat change can be further augmented (amplification technique) when conditions permit a buffer to act as the proton acceptor.

In addition, any reaction that results in the formation of water ($\Delta H_F \cong -13.5$ kcal/mol) will contribute to the overall heat change observed, and in certain cases, serve as an amplifier of the reaction. This would apply to any reaction liberating hydrogen peroxide as a product of the reaction, viz., the glucose oxidase and the uricase enzyme systems. Coupling these reactions to catalase would result in the formation of water.

In other examples, the enzymatic conversion of cyclic-AMP to 5'-AMP proceeds with ΔH of 13 kcal/mol;[51] for the alkaline phosphatase-catalyzed hydrolysis of several orthophosphate esters, ΔH is of the order of −2 to −3 kcal/mol;[52] for the glucose to glucose-6-phosphate reaction catalyzed by hexokinase in the presence of Tris buffer, the "effective ΔH" has been determined to be −17.9 kcal/mol;[53] for the action of amylase on cyclooctaamylose, ΔH is approximately − 2 kcal/mol of 1:4 glycosidic bond hydrolyzed, from which the number of bonds hydrolyzed and the enzyme activity may be obtained.[54] Related to this potential usefulness in the assay for amylase activity is the report that complex formation between iodine and amylase yields an enthalpy change of −16.8 kcal/mol iodine bound.[55] Using thermal titration equipment, the selective precipitation of Ca^{++} with oxalate in the presence of Mg^{++} can be measured, and the concentrations of both ions may be obtained in a single run.[56] The effective ΔH for the reaction is −6.1 kcal.

Differential scanning calorimetry has also been studied in its application to clinical analysis. For example, Cassel and Ohnishi[57] have done scans on whole blood, serum, red blood cells, and isolated blood proteins. Differences in peak positions have been observed for hemoglobin-A and hemoglobin-S. The possibility of measuring the lecithin to sphingomyelin ratio in amniotic fluid was also explored.

Although the above comments do not encompass the total body of knowledge that could conceivably contribute to a methodology involving assay determinations by microcalorimetry, it indicates the feasibility of using this technique in its application to the development of clinical assay procedures. For those reactions using enzymes as specific reagents, insolubilized enzymes[58,59] would offer many advantages in microcalorimetric assays, and many enzymes are commercially available in this form.

The choice of microcalorimeter design for analytical purposes is primarily dictated by the sensitivity desired and the cycle times for repetitive assays. The latter incorporates such factors as equilibration times, simplicity of operation, and dexterity required of the operator. Microcalorimeters of the batch type require long equilibration times, but are extremely sensitive (of the order of microcalories) and in designs such as that due to Evans et al.[20] and Prosen and Goldberg,[21,22] the volumes can be 150 μl or less. As an alternative to greater complexity, size, and expense, the ingenious batch microcalorimeter of Pennington[43,44] represents a creative effort in the direction of simplified methodology.

Microcalorimeters of flow design are available that are as sensitive as good batch cell construction (e.g., the LKB-type 2107-020 based on Wadsö's design,[26] the Berger stopped-flow,[24] and the Gill and Chen model[29]), but require larger volumes of material.

A variation of the usual flow cell is the flow sorption microcalorimeter based on

the design by Wadsö[60] and available as the type 2107-30 LKB instrument. It has been extensively used in connection with immobilized enzyme columns.[61] The sensitivity is <1 μW for a continuing heat change and <1 μJ for a heat pulse reaction.

Although not as sensitive, titration microcalorimeters of either isoperibolic (cf. Figure 16) or of isothermal (cf. Figure 20) design are capable of detecting changes of <0.1 mcal, using sample volumes of 1 to 2 m*l*. They are suitable for the study of fast processes and can be used in either endpoint or kinetic mode. When used kinetically, i.e., for the assay of either metabolite or enzyme levels, the data output display from an isoperibolic cell is of the conventional format, similar to that obtained using a spectrophotometric assay method. This is a not unimportant point in ease of data interpretation.

Initial data output from a titration microcalorimeter operated isothermally is obscured as previously discussed, and thus such constructions are better suited to slow processes.

Very few enzyme-catalyzed reactions have been investigated in the detail necessary to permit evaluation of the thermodynamic parameters of the reaction. In some instances, the observed heat change has been related to the heats of formation of the reactants and the products which permit a greater confidence in the use of calorimetry for the thermodynamic evaluation of a biochemical reaction. Several practical difficulties are encountered in the use of this characterization. Although the heats of formation (derived from heats of combustion) of a number of simple organic materials are known with great accuracy, the heats of biochemical materials are not as well known, since they are rarely obtained in sufficiently pure form to yield meaningful data. Furthermore, the reactions in biochemical materials take place in a complex media for which a considerable amount of accurate data are required for a complete characterization. Added to this is the practical difficulty that the heats of reaction generally measured are considerably less than the heats of combustion involved.

As an example of these points, it is instructive to examine in some detail a recent paper by Rehak et al.,[62] who reported the determination of uric acid in serum using the Prosen-Berger Micro-biocalorimeter.

The uric acid sample (0.15 m*l*) was placed in one compartment of the partitioned calorimetric cell, and the uricase solution in Tris buffer (0.15 m*l*) was placed in the other compartment. The sealed cell was preheated to 30°C and inserted into the calorimeter for equilibration which occurred in about 15 min. The reaction was initiated by rotating the cell and allowing the contents to intermix. The heat change as a function of time was recorded on a digital tape cassette for data reduction and analysis via computerized programs. Cycle times were approximately 20 min, and an example of the data output obtained is shown in Figure 28. Note the similarity of the data to simulated heat flows from a reaction vessel into a heat sink as plotted in Figure 1.

The stoichiometry of the enzyme reaction involved is

$$\text{uric acid} + 1/2\ O_2 + 2H_2O \xrightarrow{\text{uricase}} \text{allantoin} + H_2CO_3 + H_2O_2$$

which releases 4.78 kcal/mol. The normal range of uric acid in serum is 20 to 60 mg/*l*, and for a sample which contains 40 mg/*l* (236 μ*M*), the heat produced will be 1.13×10^{-3} cal. The reaction may be amplified by coupling with catalase which regenerates oxygen by the catalytic conversion of the hydrogen peroxide, rapidly, quantitatively, and with a large heat change.[63] This "amplification reaction" raised the heat production to −35.76 kcal/mol. With catalase present in the reaction system,

a comparison of their microcalorimetric assay procedure was made with the continuous flow Auto Analyzer II® method for the determination of uric acid in serum (cf. Figure 29). The correlation coefficient between the two methods is 0.936.

The only interference noted in the assay of uric acid was due to the absorption of protein onto the calorimeter cell wall which was easily eliminated by a careful washing of the cell between runs. Other possible sources of error were the heats of dilution of sera and of the buffer. The contribution from the dilution of uric acid standards in the buffer was negligible, but was significant for the dilution of the sera with the buffer (-0.35 to $+0.21 \times 10^{-3}$ cal). This heat of dilution was dependent upon the protein concentration of the samples and was evaluated by mixing the sera and the buffer in the absence of uricase.

In the appendix to the article, Rehak et al.[62] showed that the ΔH they had determined for the uric acid-uricase system (conversion of uric acid to allantoin) could be accounted for by algebraic summation of known heats of formation. Their experimental ΔH for the reaction (-35.76 kcal/mol) was in very good agreement with the calculated value of -35.16 kcal/mol. Although this is a very satisfying result, it must be stressed that data for heats of formation of many biological compounds are not nearly as detailed nor extensive as to be of general utility in such calculations.

An extensive study of cholesterol oxidation has recently been completed by McGuinness et al.[64] The reaction studied was

$$\text{cholesterol} + O_2 \xrightarrow[\text{oxidase}]{\text{cholesterol}} \text{cholestenone} + H_2O_2$$

Heat measurements were made using the Evans type heat conduction microcalorimeter,[65] and the enthalpy of the reaction was found to be -27.0 ± 1.7 kcal/mol. From reported bond energies or heats of formation data, ΔH was calculated to be -23.9 to -32.0 kcal/mol and -29.4 kcal/mol, respectively. Better agreement between experimental and calculated heat of reaction was obtained using trigonal additivity procedures (-27.3 kcal/mol).

In any attempt to develop a new assay technique, there is the implicit requirement that it be comparable or preferably better than existing procedures. It, therefore, is not surprising that a good deal of effort has centered on the determination of glucose by microcalorimetry. Table 1 summarizes some of these efforts by various investigators.

In a thoughtful and rigorous account of the systematic and random errors encountered, Goldberg[70] has studied the calorimetric determination of glucose using the hexokinase reaction. The results obtained on five reference standards were compared with results of analysis by the spectrophotometric hexokinase - glucose-6-phosphate dehydrogenase method and isotope-dilution mass spectrophotometry. In most cases, the three methods were in agreement. The calorimetric assay would appear to have the advantage in ease of operation it provides for work on complex mixtures.

Monk and Wadsö[66] have shown that microcalorimetry can be used to measure the activity of ATPase, glucose oxidase, cholinesterase, and lactic acid dehydrogenase. Konickova and Wadsö[71] also demonstrated that inhibition of enzyme activity could be studied calorimetrically and examined the inhibition of cholinesterase by organophosphates (Figure 30). Changes in inhibition concentration at the microgram level could readily be detected. This same reaction has been studied more extensively by Beezer and Stubbs[72] as a direct means for the assay of organophosphates. For some inhibitors and using flow microcalorimetry, 0.02 μg organophosphate/mℓ could be determined.

Beezer et al.[49] performed a critical study of the urea-urease system by flow micro-

TABLE I

Glucose Determination by Microcalorimetry

Reagents	Accuracy (%)	Concentration range (μg/ml)	Method	Ref.
Glucose oxidase + peroxidase	—	1—40	Flow	66
ATP + hexokinase	2	300—10,000	DIE*	53
ATP + hexokinase	5	400—3,000	Batch	67
ATP + hexokinase (immobilized)	5	250—2,000	Flow	68
Glucose oxidase (immobilized) + catalase	5	90—180	Flow	69

* Direct injection enthalpimetry, total addition of reactants or reagents at zero time in a titration calorimeter.

calorimetry. They worked out the relationships of the heats observed for enzyme catalysis in the flow calorimeter and the enzyme concentration (zero- and first-order kinetics) and were able to estimate the urea concentration of urine. Analysis of the kinetics of the reaction also permitted an evaluation of K'_m, the substrate concentration (0.016 *M*) giving half the maximal velocity.

Rehak et al.[73] studied the relationship between activity and concentration of both soluble and immobilized forms (on glass beads) of uricase and lactic acid dehydrogenase using batch microcalorimetry. Data comparison of the soluble vs. insoluble enzyme forms are shown in Figures 31 and 32. Although the response of the thermal output to varying concentrations of reactant was excellent, they stated that " . . . microcalorimetric approach may be preferable for the determination of enzymes that do not produce changes in absorption (e.g., ATPase). Certainly the optical methods are preferable for enzymes like LDH."

Berger et al.[24] have used a stopped-flow microcalorimeter for the study of a number of enzymatic reactions, viz., creatine phosphokinase, DPNase, hexokinase, LDH, and uricase. Enthalpy changes for these reactions were determined and are given in Table 2. Since complete experimental details were not given, the explanation as to the difference of their value for the uric acid to allantoin conversion using uricase + catalase (ΔH, −27.55 kcal/mol), as compared to that of Rehak et al. (ΔH, −35.76 kcal/mol[62]), may be due to differences in the buffers used.

Using an LKB Batch Microcalorimeter, Hunt et al.[74] have studied the binding of Mg^{++} to glutamine synthetase. The heat change profile of this process (two protons released) was compared to that of an instantaneous reaction (buffer plus HCl) (Figure 33). Since more heat was released and in part at a slower rate with the enzyme system, it was assumed that there is one proton released instantaneously and one slowly. From graphical analysis, the $t_{1/2}$ of slow proton release at 37°C was calculated to be 55 sec.

A means of getting at rate constant data semiautomatically has been proposed by Evans et al.[75] The procedure was evaluated for the alkaline hydrolysis of ethyl acetate using a heat conduction type calorimeter.

Yourtee et al.[76] have been able to quantitate aldolase activity by measurement of the heat change that occurs in the conversion of fructose phosphate to products. A linear response of Q vs. substrate concentration was obtained. Interferences with

TABLE 2

Molar Enthalpies for Some Enzyme-catalyzed Reactions

Enzyme	Reaction	ΔH(kcal/mol)
Creatine phosphokinase	Creatine + ATP $\xrightarrow[pH = 9.0]{CPK}$ creatine phosphate + ADP	7.80
DPNase	$DPN^{(+)} + H_2O \xrightarrow[pH = 7.5]{DPNase}$ nicotinamide + ADP + $H^{(+)}$	6.80
Hexokinase	Glucose + ATP $\xrightarrow[pH = 7.6]{HK,\ Mg^{2+}}$ glucose-6-phosphate + ADP	11.75
Lactic acid dehydrogenase	Pyruvate + DPNH + $H^+ \xrightarrow[pH = 7.5]{LDH}$ L-lactate + $DPN^{(+)}$	3.33
	Urate + H_2O + $O_2 \xrightarrow[pH = 9.0]{uricase}$ allantoin + H_2O_2 + CO_2	
Uricase	$H_2O_2 \xrightarrow[pH = 9.0]{catalase}$ $H_2O + \frac{1}{2}O_2$	27.55

Note: The enthalpy values shown should be preceded by a negative sign since the reactions are exothermic. See, for example, Rehak et al.[62] who reported −35.76 kcal/mol for the uric acid-uricase system.

Data taken from paper by Berger, R. L., Davids, N., and Panek, E.; *Journ. Calorim. Anal. Therm.*, 6, 1(1975) and uncorrected for proton liberation heats.

serum present were not noted and they suggest the method's application for clinical isoenzyme determination.

O'Farrel et al.[77] have made comparisons between cholinesterase activity in human serum as measured calorimetrically and spectrophotometrically. Provided that heats of mixing and of dilution are properly corrected for, both methods gave concordant results.

Using an LKB sorption microcalorimeter, Johansson et al.[61] have measured the heat changes occurring in enzyme-substrate reactions using immobilized enzyme columns. Two different calorimeter cells were used: one was a heat-conduction design operated isothermally (Figure 34a), and the other was used with the column suspended in a constant temperature jacket (Figure 34b). In the latter, the difference in temperature of solution entering and leaving the column was sensed by two thermistors. The former design was considered the more advantageous, since heat flux from the reaction was directly proportional to moles substrate converted/time multiplied by ΔH per mole.

Volume of the columns used was approximately 0.5 mℓ, and about 4 samples per hour could be processed. Using both heat pulse and steady state modes of operation, they examined the trypsin (immobilized) hydrolysis of the substrate benzoyl-L-arginine ethyl ester (BAEE). Their results by both methods are shown in Figures 35 and 36. The steady state method, with heat flows measured in the microwatt range, was the more sensitive.

Danielsson and Mosbach[78] discuss the use of what they call "enzyme thermistor" devices. Single and differential flow units are shown in Figure 37. Using flow rates of 60 mℓ/hr with sample volumes of 1 mℓ, precision of measurement was of the order of ±2%. As they state, this is good enough for clinical assay purposes, and one can do assays at the rate of about one per minute.

A variant on the same theme is discussed by Weaver et al.[79] They relate their experiences thus far with the "thermal enzyme probe" (TEP) as one type of immobilized enzyme calorimeter. The detection of temperature changes of the order of 10^{-4}°C are currently possible with their device. Cogent arguments are presented for the possible application of these devices, not only for clinical assay work, but also as devices for use in the *in situ* monitoring of biological reactions.

For additional and general surveys of the developments and uses of the technology of immobilized enzymes, one can consult the review by Ambrus[80] and Volume 44 of *Methods in Enzymology*.[81] The use of enzymes (various methods) in analytical chemistry has been recently summarized by Fishman and Schiff.[82]

Although not intended to be comprehensive, Table 3 presents a listing of enzymatic reactions that have been examined, some only casually, others rather intensively, by calorimetric means. It is the feeling of the authors that this list will continue to grow and that advances in instrument design will make calorimetry a more attractive alternative to certain existing methods and will be used for the assay of components for which there is currently no satisfactory procedure.

Determination of chloride by reaction with mercuric nitrate ($\Delta H = -13.4$ kcal/mol) using a titration calorimeter with direct injection of the reagent permits the determination of 5 to 17 μmol with an accuracy of 5%.[90] Although this variation does not compare favorably with the conventional techniques (2% error), the authors feel that the procedure may be easily automated and that the precision can be improved. Pennington[44] also determined chloride with mercuric nitrate in 10-$\mu\ell$ samples which contained 0.5 to 2 microequivalents.

Hansen et al.[91] have reported a procedure for the determination of nitrite in air samples by direct injection of sulfamic acid with a precision of 5% and with a limit of detection of 3 nmol. Under their conditions, none of the normally occurring interfer-

TABLE 3

Enzyme Reactions Examined by Microcalorimetry

Enzyme	Type of Microcalorimeter	Ref.
Acetylchloinesterase	Batch	88
Acetylcholinesterase + organophosphates	Batch	89
Aldolase	Batch	76
Alkaline phosphatase	Flow	66
	—	52
Amylase	—	54
ATPase	Flow	66
	—	83
Catalase	Titration	16
	—	63
	Batch	59
Cholesterol oxidase	Batch	64
Cholinesterase	Flow	66
Cholinesterase + inhibitors	Flow	71
Cholinesterase + inhibitors	Batch	77
	Flow	72
Chymotrypsin + acetyl-L-tyrosine ethyl ester	Titration	16
Creatinephosphokinase	Stopped-flow	24
DNAse	Batch	88
DPNase	Batch	23
	Stopped-flow	24
Glucose oxidase	Batch	84
Glucose oxidase + peroxidase	Flow	67
Glucose oxidase + catalase	—	69
Glucose oxidase (inhibited by Ag^{+} and Pb^{+2})	Batch	87
Hexokinase + ATP	Stopped-flow	24
	Titration	53
	Batch	67
	Flow	68
	Batch	70
	Thermal enzyme Probe (flow)	79
Hyaluronidase	Batch	88
Lactic acid dehydrogenase	Stopped-flow	24
	Flow	69
	Flow	66
	Batch	79
Peroxidase	Batch	88
	Stopped-flow	85
Trypsin	Flow (sorption)	61
Urease	Flow	49
	Batch	88
	Batch	69
	Batch	86
Uricase	Stopped-flow	24
	Batch	73

ences found for the spectrophotometric determination of nitrite were found to cause any analytical problems.

Smith and.Carr[92] and Carr et al.[93] have used titration calorimetry to determine protein concentration in sera by titrating with diphosphotungstic acid. Although the heat evolved is a function of the anions bound and the number of anionic sites, they showed a good correlation of the calorimetric data with Kjeldahl nitrogen determination (Figure 38). Protein concentrations from 0.1 to 1.5% could be determined with a precision of 0.5%.

B. Ligand Binding and Proteins

1. Proton Binding

The nature of the ionization processes of proteins is of critical importance in any explanation of their intra- and intermolecular reactions. Although extensive studies of the ionization behavior of proteins by titration potentiometry have yielded considerable empirical information, proton binding or exchange, as in ligand binding, is as yet incompletely understood, primarily because of a lack of knowledge of the thermodynamics of the reactions involved.

Earlier calorimetric studies essentially compared the heat of binding of protons to the various group-sets (i.e., carboxyl, imidazoyl, α-amino, sulfydryl, phenolic and ϵ-amino). The average heat of ionization (ΔH_i) of each set was obtained as the total liberated heat per mole divided by the number of groups titrated. This is, in essence, a direct calorimetric measure of the process initially proposed by J. Wyman.[94] His original analysis of hemoglobin using the van't Hoff relationship was confirmed by titration calorimetry.[95] However, this type of analysis of thermal data cannot yield values for individual ΔH_i values, since in a protein, the pH ranges in which the various groups and group-sets ionize are overlapping. The heat change observed in each region reflects (1) the number of groups titrating in that region; (2) their ionization constants; and (3) their heats of ionization. Despite these difficulties, attempts to evaluate the heats of ionization of the ionizable groups of proteins by calorimetry have been made.

Comparative studies of a protein and its diazotized derivative by calorimetry have been used by Jesperson and Jordan[96] to evaluate the number of groups on ovalbumin which had reacted with *p*-aminobenzoic acid. They reported an increase of 13 carboxyl groups and a reduction of 6 amino groups. Since seven imidazoles also were diazotized, the reaction was easily characterized.

Bjurulf[97] described the continuous enthalpy titration of lysozyme using a flow system coupled with continuous pH measurements (Figures 39 and 40). Solutions of lysozyme (1%) at pH 4.59 and varying concentrations of HCl and KOH were pumped through the calorimeter at 250 μl/min, and the heat generated was obtained at 10 to 15 min after the start of pumping. The pH of the effluent solutions was monitored, and the potentiometric curves were calculated. These were found to be in reasonable agreement with published values except for two additional titratable carboxyl groups. The heats of ionization of the groups were calculated by averaging the heat liberated per group. His results are given in Table 4.

The assignment of ΔH_i values to the individual residues cannot be regarded as definitive, and Bjurulf states that only in regions where a few groups are titrated can the values be regarded with certainty. Actually, even this may be misleading, since the value for the single imidazole which titrates in the pH 6.5 to 7.5 region will have contributions from four carboxyls with pK 4.5 and with $\Delta H_i \cong 0.5$ kcal/mol and from the α-amino group (pK'$\sim$8; ΔH_i, 10.5 kcal/mol).

Bjurulf also points out the difficulties of relating thermodynamic values obtained with model compounds to the side chain residues of proteins. There is also the possi-

TABLE 4

Apparent Heats of Ionization for the Amino Acid Residues of Lysozyme at 25°C in 0.15 *M* KCl

pH Range	Groups ionized	ΔH_i (kcal/mol)
2.0—4.59	1 α-carboxyl	0.35
	4 Asp	1.00
	1 Glu	0.11
4.59—6.5	1 Asp	1.07
	1 Glu	0.11
	2 unknown	0.83
6.5—7.5	1 His	6.0
7.5—8.25	1 α-amino	10.5
8.25—10.25	1 Tyr	6.0
	4 Lys	12.42
10.25—12.0	1 Tyr	6.0
	2 Lys	12.66

Data from paper by Bjurulf, C., *Eur. J. Biochem.*, 30, 33, (1972).

bility that subtle conformational changes are reflected in the measured heat changes. These difficulties notwithstanding, his work clearly demonstrated that the combined potentiometric and calorimetric titration of proteins could yield thermodynamic data.

Biltonen et al.[98] performed a calorimetric study of the carboxyl ionizations of α-chymotrypsin and chymotrypsinogen in which they were able to estimate the heat associated with the attendant conformational changes observed at low pH. In the titration of α-chymotrypsin from pH 4 to less than 2, 10 carboxyl groups are titrated with the liberation of 9 kcal/mol protein. The ΔH_i is therefore −0.9 kcal/mol carboxyl group. They find, however, that chymotrypsinogen has an average ΔH_i of 4.4 kcal/mol carboxyl group. Since this value is much too high for any carboxylic acid ionization, they attribute the abnormal heat observed to a single carboxyl group (possibly ASP-194) with pK′ ∼2.5 and ΔH_i of 30 kcal/mol.

In the evaluation of any titration curve (potentiometric and/or calorimetric), one contends with both systematic and random errors. Random errors are generally within acceptable limits, and in our laboratories, reproducibility of any given experiment is about 0.5%. Cabani and Gianni[99] have concluded, however, on the basis of error (random and systematic) propagation analysis, that attempts to simultaneously determine both ionization constants and heats of ionization from calorimetric data will lead to large errors in these values. They assumed systematic errors ranging from 3 to 28% of total heat liberated and random errors of 1%. Their assumptions have been contested by Christensen et al.,[100] who proceeded to show that prudent, experienced investigators would in reality be dealing with systematic errors of less than 1%. This would cause errors of only 0.01 units or less in log K and 0.09 to 0.04 kcal/mol in ΔH values of approximately 3 to 4 kcal/mol.

These errors are almost trivial compared to the error that can be introduced in the routine estimation of protein concentration. This is quite often done using a molar extinction coefficient that is variable in its acceptance. If impurities are present, the errors are multiplied. In fact, Bolen et al.[101] describe an "enthalpy ratio test" for determining the purity of RNAse. Although other techniques are also plagued by these errors, calorimetry is especially affected because of its sensitivity and because the generation of heat is a universal and distinct property of a reaction. The presence of 10%

inert protein would yield a 10% error in, say, the specific biological activity. However, with calorimetry, the 10% impurity could contribute to much more than 10%, and perhaps even the majority, of the total observed heat change.

During a titration, there may also be reversible association or dissociation of the protein. This can, in some cases, be independently verified and conditions selected which will assure the state of the protein species. However, subtle changes may still occur which will contribute to the heat observed. Experiments should be run at a variety of protein concentrations, if possible, and concentration dependence of the heat effects should be observed. Irreversible association (aggregation) is also possible, but it is generally a slow process which is particularly annoying for long-term equilibrations and determinations, such as those used in batch calorimeters. It is not a problem for flow or titration calorimetry, since these are run for shorter periods of time. In fact, precipitation reactions can be measured after the heat of a reaction has occurred,[92,93] and since protonic processes are essentially instantaneous, this does not constitute a source of error. The contributions to the observed heat from stirring, mixing, dilution, and Joule heating of the thermistor can be readily corrected for by running the appropriate control experiments. Cooling corrections, when required, can be estimated as previously discussed.

The values derived for ΔH_i values of the ionizable groups of proteins are, with some exceptions, in agreement with the values found for the individual amino acids (compare data of Bjurulf [Table 4] with that of Marini et al.[30]). The calorimetric data of the individual amino acids are also in general agreement with heats of ionization data obtained by the van't Hoff method.

Flogel and Biltonen[102] used flow calorimetry to determine the heat change produced during the ionization of RNAse from pH 8 to 4 (Figure 41). In this region, 5 groups are titrated, and a total of 50 kcal/mol protein were liberated. By using the literature pK's for the four histidine residues as determined by NMR, they analyzed for the "best fit" pK' values to their potentiometric curve. Three of the imidazoles were then assigned a normal heat of ionization of 6.5 kcal/mol, and the ΔH_i for a group with pK' of 6.7 was allowed to vary until a good fit to the data was achieved. This best-fit value was found to be 24 kcal/mol. In the RNAse-3'CMP complex, ΔH_i for this imidazole was reported to be 20 kcal/mol (Table 5). These values are extraordinary and appear to be nearly of the same magnitude as that reported for the second ionization of imidazole (pK', 14.2; ΔH_i, 17.6 kcal/mol; Hanania et al.[103]). This high value is probably the result of the arbitrary assignment of both pK' and ΔH_i to the other groups, and they report that an assignment of 14 kcal/mol to the ΔH_i of both His-48 and His-105 would also be in accord with the data. Equally possible would be the assignment of approximately 10 kcal/mol to each of the 4 histidine residues. It is apparent that their data does not permit assignment of unique values and that interpretations based upon the values used[104,105] must necessarily be restrained and accepted with caution.

In a paper by Martin et al.,[36] studies were reported on the potentiometric and calorimetric titration of RNAse from pH 3.0 to 11.5 at 25°C in 0.05 *M* KCl. Their data can be fitted to ΔH_i values for the 4 histidine residues ranging from 4.4 to 6.5 kcal/mol (Figure 42). When one histidine residue (pK', 7.2) is assigned a value of 24 kcal/mol, the deviation of the calculated Q vs. pH curve from the experimental data is seen to be severe.

Shiao and Sturtevant[106] noted that the earlier reports dealing with calorimetric studies of proton binding (Hermans and Rialdi,[107] Kresheck and Scheraga[108]) to myoglobin and ribonuclease appeared to indicate that heats of ionization of the normal side chain groups were similar to those of model compounds. Utilizing flow calorimetry, they investigated the ionic behavior of chymotrypsinogen, lysozyme, and oxidized cyto-

TABLE 5

Apparent Heat of Protonation and pK′ Values for the Histidine Residues of Ribonuclease A and of Its Complex with 3′-CMP

	RNAse		RNAse - 3′-CMP	
Residue	pK′	$-\Delta H_p$* (kcal/mol)	pK′	$-\Delta H_p$ (kcal/mol)
His_{12}	5.8	6.5	7.2	6.5
His_{48}	6.7	24	7.1	20
His_{105}	6.6	6.5	6.6	6.5
His_{119}	5.0	6.5	7.2	6.5
Lys_1	8.0	11.0	8.0	11.0

* Heat of protonation is equal to heat of ionization, but with opposite sign.

Data from paper by Flögel, M. and Biltonen, R., *Biochemistry*, 14, 2603 (1975).

chrome *c* at 25°C in 0.15 *M* KCl. The values for pK′, ΔH_i, and N used to calculate the thermal potentiometric curves are shown in Table 6. The thermal curve of chymotrypsinogen (Figure 43) in the acid region pH 4 to 2 is different from that reported by Biltonen et al.[98] in that the titration is endothermic (ΔH, +24 kcal/mol), whereas Biltonen finds an exothermic ΔH (−37.4 kcal/mol). There is no apparent explanation for this discrepancy, although both authors suggest a conformational change in this region. The other parameter values for chymotrypsinogen (pK′ and N) are in agreement with the potentiometric results of Marini and Wunsch,[109] but they do not agree with the heats of ionization reported by Martin et al.[31,110-112] Shiao and Sturtevant state, however, that their derived parameter values are not the only possible combinations which will match the experimental data.

Their curve for cytochrome *c* (Reference 106) appears to contain two unknown ionizable groups. In addition, only one imidazole group of the three known to be present appears to be titratable. Their group, "X", (cf. Table 6) appears to have the characteristics of an α-amino group which is not known to be present in horse heart cytochrome *c*, and they tentatively attribute this ionization to a stabilized carboxyl group. The group designated "Y" appears to be the group responsible for the conformational transition of ferro- to ferricytochrome *c*.[113] A group of this type has also been found to be present in ferricytochrome *c*, but not in ferrocytochrome *c* by Marini et al.[114] In their data treatment, Shiao and Sturtevant[106] assumed four titratable phenolic groups when only one titrates normally by the usual spectrophotometric assay.[115] Further, their potentiometric titration curve shows approximately 25% more groups titrating than are actually present.[116]

Their values for lysozyme (cf. Table 6) are in agreement with those reported by Bjurulf,[97] and the thermal titration curve appears to be qualitatively and quantitatively similar.

Izatt et al.[41] used a titration calorimeter for the titration of insulin, and the slopes of the thermal curve were used to obtain ΔH_i values for each region of the curve. These were then compared to known values of model compounds, since it was assumed that ΔH_i is dependent on the type of functional group and not on the structure of the rest of the molecule. They also report the thermal titrations of native and heat denatured human serum albumin (Figure 44).

TABLE 6

Parameter Values Used to Compute the Thermal Titration Curves of Proteins at 25°C in 0.15 *M* KCl

Type of group	Oxidized cytochrome *c*			Chymotrypsinogen			Lysozyme		
	N	pK′	ΔH_i	N	pK′	ΔH_i	N	pK′	ΔH_i
ε-Amino	19	10.5	10.5	13	9.9	10.0	6	10.3	11.0
Phenolic	4	10.2	6.0	2	10.0	6.0	3	10.2	7.0
α-Amino	0	—	—	1	7.8	10.0	1	7.6	10.0
Imidazole	1	6.5	6.0	2	6.5	6.0	1	6.5	7.0
X	1	7.8	10.0	0	—	—	0	—	—
Y	1	9.4	18.0	0	—	—	0	—	—

Data from paper by Shiao, D. D. F. and Sturtevant, J. M., *Biopolymers*, 15, 1201, (1976).

An approach which avoids the need to average the heats of ionization was suggested by Christensen et al.,[117] who obtained the heat of ionization and the ionization constant, K, for HSO^-_4 in a single thermometric titration. Their values for K and hence ΔG, ΔH_i, and ΔS were in good agreement with reported literature values.

Titration calorimetry is ideally suited to the evaluation of the ionization constant and the heat of ionization (or protonation) of acids and bases. During the addition of acid or base, each point on the continuously generated titration curve corresponds to a single run in a batch calorimeter or a flow calorimeter using a mixing cell. In the reaction

$$A^- + H^+ \rightleftharpoons HA + Q$$

The amount of heat, Q, liberated is the product of the molar enthalpy and the concentration of acid HA as given by Equation 1.

In the general case of the up-scale titration (base addition) of a series of monoprotic acids such as are found in proteins, the groups titrated, $\bar{r}$, is given by

$$\bar{r} = \sum_i N_i K_i / (K_i + H) \qquad (9)$$

where N_i is the concentration of the ith group with ionization constant K_i, and H is the hydrogen ion concentration. The activity coefficients are set equal to unity. The heat change at any pH is given by

$$Q_c = 1/C_s \sum_i [N_i K_i/(K_i + H)] \; [\Delta H_F - (\Delta H_i)_i] \qquad (10)$$

where Q_c is the heat change corrected for heat loss, volume changes, and the heat due to the titration of free protons.

For mixtures of acids in which the pK's are separated by 2 pK′ units or more, straightforward slope analysis[16] will yield each group's pK′ and ΔH_i. For mixtures in which overlapping of the ionization makes such analysis either impossible or fraught

with considerable error, solution of the appropriate simultaneous equations can lead to an evaluation of the parameters.[118] This latter method of analysis has been applied to both model mixtures and the thermal titration of chymotrypsinogen and δ-chymotrypsin.

A more elaborate approach has been taken by Marini et al.[119,120] wherein data of heat change as a function of pH for three mixtures was analyzed using a computer-assisted iterative curve-fitting procedure (the NIH-MLAB program). Thermograms for three different mixtures containing formic acid, pyridine, glycylglycine, imidazole, β-mercaptoethanol, phenol, and methylamine are shown in Figure 45. These mixtures were formulated to mimic the range of pK′ values of the ionizable group-sets found in proteins. The results of detailed analysis of one mixture are given in Table 7. Curve-fitting was done for each N, pK′, and ΔH_i; a total of 24 parameter values. The values obtained were in excellent agreement with data obtained from the individual model compounds. Only in the case of β-mercaptoethanol (pK′ 9.73) and phenol (pK′ 10.02) was it not possible to resolve the individual heats of ionization. The total heat, however, was in good agreement with the sum of both heats of ionization (12.0 kcal/mol). A number of other mixtures were tested as representative of a variety of protein ionizations and in each case, the parameter values were in excellent agreement with the known values.

This technique was applied to proteins, but suffered from the requirement that separate thermal and potentiometric titrations were needed. In addition to the inconvenience, there was the real danger of amplifying the errors in each determination. Sreenathan et al.[31] provided for the simultaneous monitoring of pH and heat by introducing a pH electrode into the calorimetric cell. This greatly simplified the technique, reduced the time and the amount of protein required, and obviated the errors that arose from separate runs. In addition, many more data points were available which greatly simplified the curve-fitting procedure and gave greater confidence in the values obtained from the data analysis.

Sreenathan et al.[31,110] have reported their studies on chymotrypsin. A partial sorting-out of the contributions of the various group-sets and group sub-sets to the pK′ and ΔH_i values that described the experimental data was achieved through the use of group-modified derivatives. For example, nitration of the two normally ionizing tyrosine residues enabled their differentiation from the ε-amino groups.[110] Similar studies have been done with the protein RNAse in the pH range 3.5 to 11.5[36] (cf. Figure 42). The ionization behavior was completely reversible between these pH extremes, and the entire curve was generated in less than 2 min. Reproducibility was better than 1%.

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The potentiometric and thermal titration curves for oxidized horse heart cytochrome c are not reversible due to an hysteresis effect.[114] The acid titration curve does not match that obtained by downscale titration, although each directional titration is reproducible. This effect complicates analysis of the thermal data.

These experiences of various laboratories point out that a number of problems involved in the interpretation of the thermal titration data of proteins must still be considered:

1. Even though all titrations are run at essentially constant temperature (rarely will

TABLE 7

Evaluation of the Parameter Values of a Mixture by Calorimetry and Their Comparison with Those Determined Independently

Compound	Found*			Literature values	
	N	pK′	ΔH_i (kcal/mol)	pK′	ΔH_i (kcal/mol)
Glycylglycine pK′$_1$	0.99	3.09	1.41	3.09—3.29	0.32—1.60
pK′$_2$	1.02	8.38	10.47	8.23—8.46	10.60—11.60
Formic	0.99	3.79	−0.35	3.75	−0.40—1.36
Pyridine	0.99	5.35	4.75	5.17—5.30	4.28—5.23
Imidazole	1.01	7.19	8.65	6.99—7.20	7.10—8.79
β-Mercaptoethanol	1.02	9.73	12.99	9.72	6.21
Phenol	1.01	10.02		9.88—10.00	4.80—5.66
Methylamine	1.03	11.04	12.45	10.62—10.79	12.72—13.50

* *From the titration of an equimolar mixture (0.004 M) at 20°C in 0.15 M KCl.*

Data from paper by Marini, M. A., Martin, C. J., Berger, R. L., and Forlani, L., *Analytical Chemistry*, Vol. 3, Porter, R. S. and Johnson, J. F., Eds., Plenum Press, New York, (1974), 407.

there be more than a 0.05°C rise), the effect of a heat capacity change with pH may be significant.

2. Subtle conformational changes may alter thermodynamic values. To determine the nature and extent of these changes appears to be a formidable problem.
3. The effect of a change in pK′ (ΔG) on the heat of ionization. Or conversely, will the pK′ change if ΔH_i changes? There appears to be a feeling that the heat of ionization will remain constant for any perturbation in pK′. This would appear to be the case for a number of symmetrical dibasic acids which have the same ΔH_i, but with pK′ values that vary as much as six units. However, in proteins there are a number of examples which would indicate that both parameters may vary.
4. Resolution of the parameter values (pK′ and ΔH_i) by curve-fitting is not an entirely unequivocal solution. It would be desirable to constrain the possible solutions for multiequilibria by the application of independently obtained information of a good degree of precision.
5. The validity of the use of the electrostatic interaction parameter for the analysis of protein ionization should be reexamined. This may require a strict convention, since a constantly changing pK′ requires another set of parameters to be fitted. At this time, it has been largely ignored in the analysis of calorimetric data.
6. Data obtained with buffers present should be viewed with considerable skepticism. Buffer ion binding is dependent on pH and any such process that exchanges protons will complicate analysis of the data. Compensation for such exchanges requires knowledge of the number of protons involved, the heat of ionization of the buffer, and the heat of ionization of the group which releases it. This latter point has invariably been ignored.

In spite of all the real and possibly imaginary difficulties involved with the thermal titration of proteins and with the calorimetric studies of ligand binding, the knowledge gained will be invaluable in quantitatively describing the relationship of conformation to biological activity. Even when the reaction heats cannot be separately identified and measured, the measurement itself has considerable empirical and phenomenological value.

The extension of these types of studies dealing with the ionization reactions of proteins will be also aided by advances in instrumentation. In this regard, it is anticipated that a recently constructed differential isoperibolic pH microcalorimeter will be most helpful.[121]

2. Other Ligands

Binding of ligands to biological polymers has principally been studied by a variety of techniques which provide an evaluation of the binding constants. Thermodynamic parameters for binding may then be derived by an observation of the effect of temperature on the binding constants as forumulated by van't Hoff:

$$d \ln K/dt = \Delta H_B/RT^2 \quad (11)$$

where K is the association constant from whence ΔG_B may be obtained, and ΔH_B is the binding enthalpy. This is usually evaluated from the slope (equal to $-\Delta H_B/R$) of a ln K vs. 1/T plot. This procedure leaves much to be desired since the range of temperature that can be applied to biological systems is rather narrow. Furthermore, the data collected is often "noisy", leading to slope evaluations with poor precision and an almost unfailing faith in their independence of temperature. This is frequently not true, and appropriate corrections must be made for changes in the heat capacity of the system which requires most careful calorimetric measurement.

The van't Hoff equation has proven to be useful for studies on the reversible conformational changes of biopolymers, and its use is valid when macromolecules exist in only two thermodynamic states.[122-125] Calorimetry has been used to test the two-state nature of the native-to-unfolded protein state transition, and the agreement between such studies and van't Hoff type data for ribonuclease A,[126] chymotrypsinogen,[98,127] and cyanomethylmyoglobin[128] has provided support for the theory.

In simple systems, calorimetric measurement of the heat change that occurs with ligand binding can lead directly to ΔH_B, K_B, and number of ligands bound.[117,129,130] In biological systems, one has multiple contributions, such as (1) heat generated by the binding of the ligand; (2) the changes in solvation of the polymer and the ligand; (3) heat produced by changes in protonation; (4) heat produced by conformational changes of the polymer; and (5) changes in degree of association and bonding of multimeric species to contend with. The following examples illustrate how some of the various heat processes involved may be identified.

Evans and Frampton[131] investigated the interactions of galactose, lactose, and myo-inositol with calcium in aqueous solution by batch calorimetry. They found that the increase in the heat of binding was linear as the sugar concentration was increased at a constant salt ($CaBr_2 \cdot 2H_2O$) concentration. This they interpret as a 1:1 interaction. A similar stoichiometry is observed with $Ca(NO_3)_2 \cdot 4H_2O$, but there is a greater evolution of heat which may be the result of the competition of the nitrate anion for the sugar. For this reaction, the stability constants are less than one which in view of the exothermic heat of binding, means that the changes in the free energy are small and positive and that the entropy change is negative. If the binding is attributed to a simple replacement of the hydration shell of the calcium salt by the hydroxyl groups of the sugar, then the negative entropy would indicate that the release of water molecules from the shell must be compensated for by a decrease in the degrees of freedom of the sugars upon complex formation.

Holt et al.[132] studied the binding of calcium to the milk protein α_{s1}-casein by batch calorimetry in an attempt to determine those factors that influence casein micelle formation. A previous calorimetric study by Silberzahn[133] had indicated that the binding was endothermic and that milk had a capacity to bind 90 meq Ca^{++} per liter. Holt et

al. found that the heat of solution of calcium chloride at the concentrations used was negligible. A small dilution heat of the protein was corrected for by adding the protein solution to a buffer solution without calcium. Typical thermograms are shown in Figure 46. The initial reaction of calcium with the protein (curve A) is endothermic and is the result of specific binding as well as any rapid conformational changes resulting from the binding. At about 4 m*M* $CaCl_2$, there is a large increase in the turbidity of the solutions which corresponds to the endotherm observed as a result of desolvation and multimer formation. They provisionally conclude from an analysis of heats of binding as a function of $CaCl_2$ concentration, that the entire process is a resultant of calcium binding and cross-linking, intramolecular hydrogen bonding, and finally protein association through hydrophobic bonding (endothermic).

Binding of sodium *n*-dodecyl sulfate to β-lactoglobulin as a function of pH is also a very complex problem.[134] At low concentrations, the interaction is ionic and is accompanied by a conformational change. At higher concentrations, the binding appears to be of hydrophobic character which (at pH 3.5) is not influenced by 8 *M* urea. The surfactant appears to cause aggregation under these conditions. At pH 5.5, the interaction is primarily ionic, and 8 *M* urea weakens the hydrophobic bonds of the protein.

Rosseneu et al.[135,136] examined the binding enthalpy of phospholipids with the apoprotein from human high-density lipoproteins. Because of the incompleteness of the physical and chemical data of the components of the system, they were unable to perform a rigorous thermodynamic analysis of the calorimetric data. They were, however, able to determine that binding at low concentrations is exothermic, and at higher concentrations of phospholipid, a desegregation occurs which is endothermic. The binding affinities deduced from the enthalpy of binding appear to be apo AII ≃ apo CIII > apo AI. They also estimated the enthalpy due to the change in the apoprotein helicity during binding. Although the work is largely descriptive, they feel that microcalorimetry can be used to obtain the thermodynamic parameters when only one equilibrium is involved as in the binding of phosphatidylcholine to apo HDL or apo AII. Where more than one binding process is involved, the binding constants, the saturation number per binding class, and the mean enthalpy per bound ligand may be obtained by methods discussed by Fletcher et al.[137]

In an excellent series of papers,[138-142] calorimetry was used to isolate and determine each component of a series of binding reactions. The binding of nicotinamide-adenine dinucleotide (NAD) to both rabbit skeletal muscle and yeast glyceraldehyde 3-phosphate dehydrogenase was found to be consistent by both equilibrium and calorimetric techniques.[139] The thermal curves are shown in Figure 47. The yeast enzyme was found to have four identical independent sites with $\Delta G = -7.4$ kcal/mol, $\Delta H = -12.4$ kcal/mol, and $\Delta S = -16.8$ cal/K·mol at 25°C. The rabbit enzyme showed strong site interaction of an anticooperative nature.

Binding of S-peptide to S-protein to form ribonuclease S′ over a temperature range of 5 to 40°C showed that all of the thermodynamic parameters for the reaction were temperature dependent which was largely due to conformational transitions[140] (Figure 48). The binding enthalpy, ΔH_B, −24 kcal/mol and entropy, ΔS_B, −42 cal/K·mol at 5°C cannot be quantitatively related to the free energy change because the three-dimensional solution structures of S-peptide and S-protein are not known. Quantitative information on the contributions of the forces establishing protein structure are also not universally agreed upon. Obviously, a good deal of quantitative data on the structural components are needed before such processes are understood.

The importance of estimating the change in heat capacity (ΔC_p) on the interpretation of binding was shown for the interaction of the inhibitor D-hexitol-1,6-diphosphate with rabbit muscle aldolase[141] (cf. Figure 49). Although the calorimetric data are similar to that reported by Lehrer and Barker[143] for the binding of D-arabinitol-1,5-diphos-

phate, their interpretation for the nonlinear van't Hoff or Arrhenius plots based on an assumed temperature-dependent equilibrium between two forms of an enzyme can "in general be replaced by the simpler assumption of a nonvanishing ΔC_p".

Binding of oxygen to hemerythrin is represented by $\Delta H = -9.2$ kcal/mol at pH 7 and 25°C[142] (Figure 50). Only a slight positive enthalpy was found for the association of subunits in agreement with the van't Hoff estimate.[144] Values for the heat of binding of oxygen or carbon monoxide to a number of respiratory proteins have also been reported.[145,146]

A considerable number of studies on the binding of cations (Mg^{++} and Mn^{++}) to nucleotides and nucleic acids have been reviewed and summarized by Rialdi and Biltonen.[8] The general results for the nucleotides indicate that the enthalpy and entropy change is positive, and the binding appears to be determined by the release of bound water from the free ion. For the nucleic acids, the results are understandably more complex, but again the driving force appears to be a large positive entropy change, although the enthalpy is negative or zero.[147]

Nucleotide binding to tRNA is apparently enthalpy driven ($\Delta H = -16$ to -20 kcal/mol) for the complementary nucleotide to phenylalanine specific tRNA.[148] Binding of amino acids to L-isoleucine tRNA ligase of *Escherichia coli* showed (Figure 51) that the enthalpy of binding is the same (−3.7 kcal/mol) and has the same temperature dependence.[149] Because all the amino acids studied contributed equally to the free energy which is responsible for the specificity, then the only possible variation in this term must stem from the entropy. The largest ΔS found was 11.7 cal/K·mol for isoleucine binding.

Interaction of hormone with receptor sites has been observed calorimetrically to demonstrate the changes in the binding capacity of rat prostate cytosol to testosterone, dihydrotestosterone, and progesterone at the time of hormone-receptor complex formation.[150] Chiu et al.[151] studied the adsorption of human native fibrinogen on glass and charcoal. The adsorptivities did not follow a simple Langmuir isotherm, but could best be described by the sorption of a monolayer followed by multilayer adsorption.

Interaction of ADP with myosin has been reported to be exothermic on the basis of the temperature dependence of the binding constant.[152] Yamada et al[153] confirmed this by a calorimetric study of the binding of ADP to heavy meromyosin (HMM), but the results were not quantitative. Goodno and Swenson[154] conducted a careful calorimetric study in which they attempted to isolate the various heat processes, but at low molar ratios of ADP (2 to 4 to 1), the reaction is only slightly exothermic ($\Delta H = -1$ to -3 kcal/mol ADP bound) which they explain as being due to proton release and neutralization of the buffer. At higher mole ratios, the exothermic reaction is as great or greater than that reported by Yamada et al.,[153] but this heat is slowly released and is not characteristic of a fast binding process. They attribute the heat to the contaminants myokinase and AMP-deaminase and the HMM-catalyzed hydrolysis of ATP produced from myokinase-induced ADP dismutation. The results and the interpretation are contested, however, by Kodama and Woledge,[155] who used batch calorimetry to determine the binding constant, the number of sites, and the change in enthalpy. Their data were corrected for heat due to adenylate kinase and deaminase and for the ATP present. At 12°C they found that the heat corrections were negligible for all but the ATP hydrolysis by myosin and that the heat of binding of ADP to myosin is exothermic (ΔH, −17.5 kcal/mol; log K, 6.08; N, 1.74 at 12°C, pH 7.8). Protons released by the reaction contribute about 2 kcal/mol to the observed heat. At 25°C, the reaction is also strongly exothermic, but they were unable to obtain quantitative results because of the instability of myosin at this temperature. They reason that the results of Goodno and Swenson[154] at 20°C may reflect the gradual reduction of the ADP binding activity of

myosin. Resolution of these observed discrepancies is of interest, since the thermodynamics of the reaction should aid in the understanding of both myosin ATPase activity and the energetics of muscular contraction.

Binding of nucleotides to ribonuclease A and T1 has been the subject of calorimetric study by several investigators.[101,105,156,157] Monovalent salts reduce the binding affinity from an apparent enthalpy change of −15 kcal/mol at ionic strength, 0.05 to −6 kcal/mol at ionic strength, 3. This is not due to changes in the binding constant and apparently is the result of a salt effect on intrinsic binding ability of the nucleotide and the ionic behavior of the protein. The Biltonen group[101,105,157] has attempted to dissect the binding of cyclic nucleotide derivatives and mononucleotide inhibitors for the contributions due to van der Waals and electrostatic forces. They conclude that the electrostatic interaction is the significant portion. On the basis of a mechanism which involves the formation of a dianionic pentacoordinated phosphate transition state intermediate, it is calculated that the electrostatic interaction could effect a rate enhancement of 2×10^2 to 10^6. Considerably more fundamental calorimetric and kinetic studies on the influence of ionic strength on enzymic catalysis are needed before a proper explanation is possible. Nonetheless, the work is an excellent example of the attempts to delineate the nature and the sources of heat arising from complex biological interactions.

C. Cells and Tissues

In no other studies has the universality of heat production by physical and chemical processes proved to be as difficult to interpret as those dealing with cellular and subcellular systems. Although calorimetry can be used for the study of energy metabolism and was used extensively by Meyerhof[158] for an elucidation of the metabolism and function of muscle, it suffers from a number of disadvantages. Interpretation of the overall heat production in cells requires a fundamental knowledge of all cellular processes. This has yet to be attained. Preparation of cell suspensions by differing procedures influences in a remarkable and unpredictable manner cell metabolism and hence the heat production observed. Because of these difficulties, application of calorimetric techniques for the study of living cells is descriptive rather than quantitative. Despite this, such efforts have provided information not previously available by other means.

Forrest[159,160] has used batch calorimetry to provide a thermal profile of bacterial metabolism. These thermal profiles have been shown to be reproducible for a particular medium and bacterial culture.[161-164] Since a variety of differing bacteria exhibit characteristic thermogenesis curves, this has been suggested as the basis for their classification and identification.[165-168] A listing of 200 thermograms of clinically significant microorganisms has been compiled by Russell et al.[169] Calorimetry has also been used to diagnose urinary infections,[167] bacterial contamination in milk,[170] and stored food.[171] The viability of yeast stored at liquid nitrogen temperatures has also been examined calorimetrically.[172]

One of the exploratory studies using microcalorimetry to monitor the growth and respiration of cultures was conducted by Kemp on *Enterobacter aerogenes, Clostridium pasteurianum,* and *Schizosaccharomyces pombe.*[173] Heat production for *Enterobacter* generally followed an exponential growth curve for about 8 hr after which there was a lag phase even though growth continued. Initial heat production for *Clostridium* grown from spores under anaerobic conditions was linear for the first 3 hr, followed by an exponential increase that appears to be triggered by a short exothermic burst. Heat production was unchanged at 6 to 7 hr of growth after which another exponential evolution of heat occurred (Figures 52 and 53).

Since heat production follows growth only for a limited time, Kemp postulated in

agreement with Delin et al.[174] that depletion of certain nutrients required an induction period for the bacteria to adapt to the utilization of other components of the media. In synchronously dividing cultures of the yeast *S. pombe* grown under anaerobic conditions, heat production was linear with no evidence of a plateau between doublings (Figure 54). They suggest that this may reflect heat production due to cellular elongation which occurs prior to division. In the presence of the uncoupling agent, 2-oxomalononitrile *m*-chlorophenylhydrazone, heat production oscillated in synchrony with the oxygen uptake.[175]

Kemp[173,175] has also demonstrated that calorimetry may be used to estimate respiratory metabolism in embryonic chick cells. He was less successful with tissue culture cells in monolayer on glass beads because the preparations were not viable after 30 hr, although sharp exothermic peaks at 4-hr intervals could be observed up to 16 hr. Hydrolysis of 8.5 μM ATP in 1 hr by 10^6 chick muscle cells could be inhibited by 4,4′-dithiodi(nicotinic acid) (112 μM) to 22% of the original activity. Since this inhibitor does not enter the cell, it was postulated that the hydrolytic activity resides on the cell surface.

This type of study illustrates well that calorimetric techniques have an advantage over spectrophotometry for the study of surface-localized enzymes. It is not destructive and does not require the harsh conditions needed for cellular rupture to eliminate turbidity. Calorimetry can thus provide previously unknown information on much studied systems. However, interpretation and assimilation of the results will require a reevaluation of the way we tend to look at cellular processes. As Kemp states,[173] " . . . the slow development of analytical microcalorimetry in biology can be traced to a distrust of thermodynamics, even though no more advanced understanding [of it] is required than is for optics in spectrophotometry".

Use of bacteria as the assay reagent for inhibitors such as antibiotics was suggested by Prat,[176] who showed that streptomycin reduced the heat output of a growing culture of *E. coli*. Calorimetric assays for sulfonamide,[15,177] chlorpromazine and aminophylline,[178] penicillin G,[179] and tetracycline[180] have been reported. Beezer et al.[181,182] have presented a carefully considered microcalorimetric procedure for the assay of a wide range of antimycotic agents which can be perfomed in a much shorter time (1 hr) than the classical plate diffusion method (16 hr). The reproducibility of the calorimetric technique (±3%) compared to ± 5 to 10% for the plate method is also superior. Its ease of operation will also permit the assay of antibiotic combinations to determine the "potentiation" effect. In addition to the analytical results achieved, the shape of the thermograms also indicated differences in the mode of action of the antibiotics. Perhaps this ancillary finding may prove beneficial in delineating the mode of action and the design of additional antibiotic agents.

Binding of D-glucose to red cell ghosts has been studied by flow calorimetry.[183] Specific binding was estimated as the differences in the heat production of D- and L-glucose binding for which they estimated 8.33 ±2.3 mcal/g nonhemoglobin protein (NHP) which would be about 22 kcal/mol (Figure 55).

Cerny et al.[184] have used microcalorimetry to differentiate blood with sickle cell trait (SA) from normal blood by measuring the heat of dilution in hypotonic (0.0659 *M*) urea solution. Oxygenated SA blood gave a heat of +13.1 mcal/10^9 erythrocytes, and deoxygenated SA blood gave −87.5 mcal/10^9 cells. Changes in the heat signals were caused by the more extensive shape changes observed in the deoxygenated sample. Normal deoxygenated blood produced a heat of −63.6 mcal/10^9 cells which had little or no distortion. They propose that microcalorimetry be used to evaluate sickle cell therapy and for the prediction of therapeutic drug dosages.

Platelet metabolism has been followed by microcalorimetry, but despite the many

attempts at standardizing the preparations, the data do not appear to be sufficiently precise to permit more than a qualitative interpretation.[185,186]

Human red blood cells have been extensively studied, and their metabolic processes are well known.[187] Because of this and the ease with which samples may be obtained, prepared, and analyzed simultaneously for heat production and metabolism, calorimetric studies with red cells are much more quantitatively defined. Monti and Wadsö[188] have compared preparation techniques, suspension media, storage conditions, pH, temperature, and glucose concentration by a variety of calorimetric techniques and have concluded that the calorimetric results obtained by other investigators[189,190] were in fair agreement with their values if the proper corrections for the various conditions were applied.

Minakami and deVerdier[191] reported a calorimetric study of glycolysis in red blood cells under a variety of metabolic conditions. Red cell suspensions in buffered saline at 37°C were maintained at a constant pH (±0.02) by the addition of NaOH (0.3 *M*) until no further pH changes were noted. The suspension was then pumped through a flow calorimeter at a rate of 16.7 mℓ/hr. When the suspension entered the calorimeter cell (approximately 5 min), heat from the suspension caused an upward shift to a new heat production rate. When this new rate had stabilized, a compound to be studied could be added to the calorimeter cell, and its effect on heat production recorded. A typical thermogram is shown in Figure 56.

Of all the heat produced by 1 mℓ blood (70 to 100 mcal/hr), only about half is associated with glycolysis, since heat is still evolved after lactate production has ceased or if glycolysis is inhibited. The source of this heat is unknown, although it may partially involve the production of methemoglobin.[188] From the lactate production and the heat liberated (Figure 57), the estimate for the heat produced is about 17 kcal/mol lactate. The expected heat production from glucose to lactate is 13 kcal/mol as given in an appendix to the paper. The additional 4 kcal/mol is attributed to the production of a proton which neutralizes the buffers of the cell (hemoglobin, phosphate, and bicarbonate). An exact calculation for this effect was not attempted but a value of 4 kcal/mol is not unreasonable. Interestingly enough, the hemolysate of a red cell suspension mimicked the behavior of whole cells although NAD^+ was required to allow lactate formation (Figure 58). The authors acknowledge that their calculations are based on a steady state assumption, and they have measured the concentration of a number of metabolites during the course of the calorimetric observations. However, since the major source of heat is the conversion of pyruvate to lactate, their assumption seems justified. It would, nonetheless, be of interest to observe the heat production kinetically.

Calorimeters which are suitable for the study of tissue preparations have long been known[192] and have been used for muscles, seeds, plants, vertebrates, and invertebrates.[18] The thermograms obtained are complex and make similar studies with cells seem simple by comparison. Indicative of studies in this direction, Lörinczi and Futó[193] have designed a heat conduction microcalorimeter for the study of muscle (Figure 59) and Lovrien and Wensman[194] have studied the heat emanating from insects with and without stimulants in a specially constructed cell.

In no case have such thermograms been interpreted in terms of their underlying causes, but it would seem to be only a question of time along with the accumulation of additional data before such studies become more meaningful.

IV. CONCLUDING REMARKS

The use of calorimetry for the direct measurement of heat changes accompanying

biological reactions has increased markedly within the past decade. In no small part, this has been the result of changing technology in microcalorimeter design and in their commercial availability. The best of current instrumentation can detect heat changes of less than 1 μJ, equivalent to a temperature change of the order of 10^{-5}°C, using microliter volumes of material. Measurements can be made in 15 min or less, depending on the design of the microcalorimeter, and with a reproducibility of within 1%.

These state-of-the-art developments translate directly into the means by which calorimetry can be used as an analytical tool. They make possible the determination of various substances at the nanomole concentration level and in the area of biological application, the assay of enzymes and metabolites of clinical significance. Quantitation of the heat changes of isolated reactions can also lead to an evaluation of the thermodynamic parameters of the reaction. On still another application level, microcalorimetry, as a noninvasive and nondestructive probe, is admirably suited to follow the heat changes of complex life processes exhibited by cells, tissues, and whole organisms. As a sensing instrument for the monitoring of the growth of bacterial cultures, the "thermal print" obtained is sufficiently unique to qualify as a means for their classification and identification.

In a strictly analytical sense, probably the greatest potential use of microcalorimetric techniques lies in the area of clinical chemistry. Admittedly, there has not been a stampede by clinical chemists to embrace such procedures. It is felt, however, that this will accelerate, particularly so once automated and simple to operate microcalorimeters of fast recycle timee make their appearance. In fact, a parallel can be drawn between the development of biochemical microcalorimetry today and that of spectrophotometry about 40 years ago. The introduction of the Cary-Beckman spectrophotometer changed forever the procedures for measurement of light absorption and led to its preeminence as the instrument for the identification and quantitation of biologically important compounds. This stage was not reached overnight and was the laborious result of many laboratories making many analyses and developing procedures for quantitation. By analogy then, one can expect that the future will see microcalorimetry become of almost routine use in many and varied analytical applications.

It can also be anticipated that microcalorimetry will be used with increasing frequency to further our understanding of biological reactions and processes. It will not be as some would have it, the panacea for all problems. Rather, its use will undoubtedly contribute its fair share to the varied interpretations that arise from using multiple approaches to complex problems. For calorimetry offers the investigator still another window through which the results observed for a particular process must be either in accord with prior thought or stand as a challenge to it being wrong.

We can thus expect that the use of calorimetry will bring new insights into many phenomena, whether it be to further probe the various energy steps in enzyme catalysis or to delineate and define the purity of a system. These activities in turn will bring realization that although the change in free energy of a system is a better criterion than the change in enthalpy for the occurrence of a spontaneous process, the evaluation of both and hence of the entropy, will lead to a more fundamental understanding of the system.

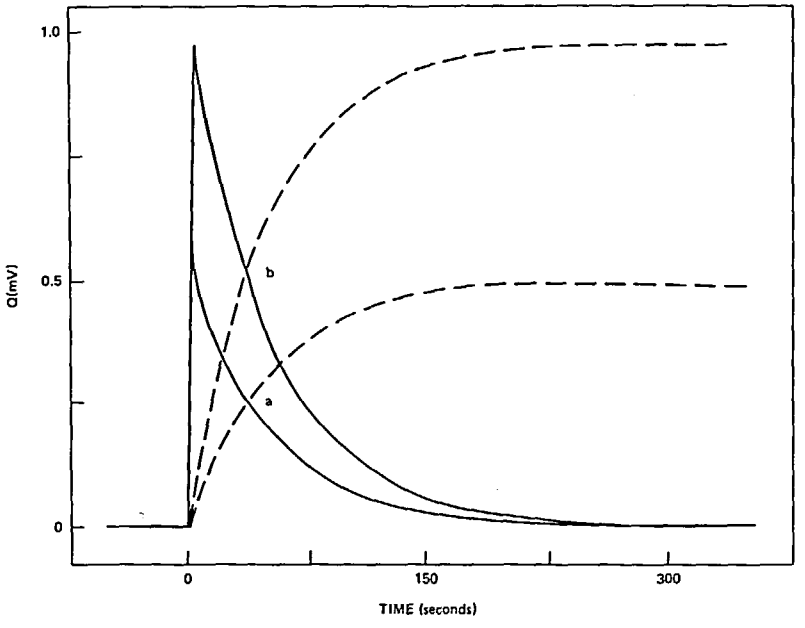


FIGURE 1. Simulated heat flow using heat conduction microcalorimeter with pulse heat (voltage) charges of (a) 0.1 mV/sec/5 sec and (b) 0.2 mV/sec/5 sec (solid lines). The integral forms representing the adiabatically reconstructed kinetic curves are given by the dashed lines. The Newtonian cooling (transfer) constant was taken as 0.02 sec^{-1}.

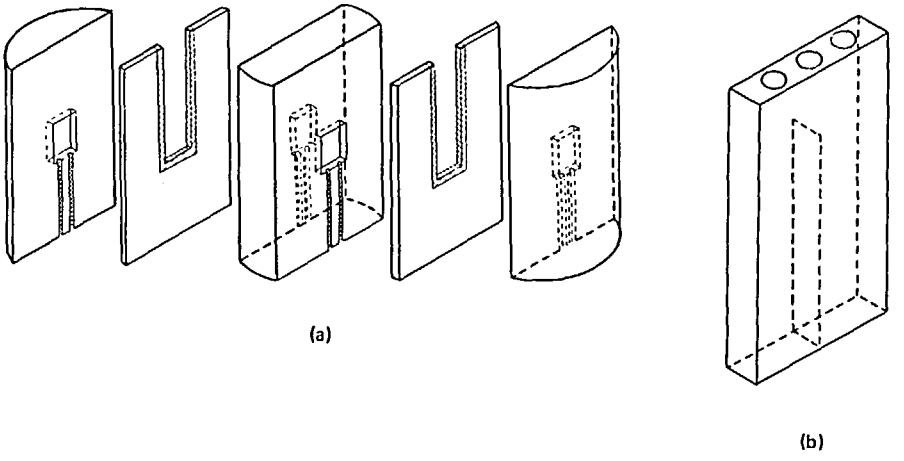


FIGURE 2. The Evans et al.[20] batch heat conduction microcalorimeter. (a) Exploded view of aluminum calorimeter block sections with recesses to accept semiconductor sensors and twin partitioned cells of the type shown in (b). Mixing of the two solutions is by rotation of the entire assembly contained within a large insulated can. (From Evans, W. J., McCourtney, E. J., and Carney, W. B., *Chem. Instrum.*, 2, 249 (1969). With permission.)

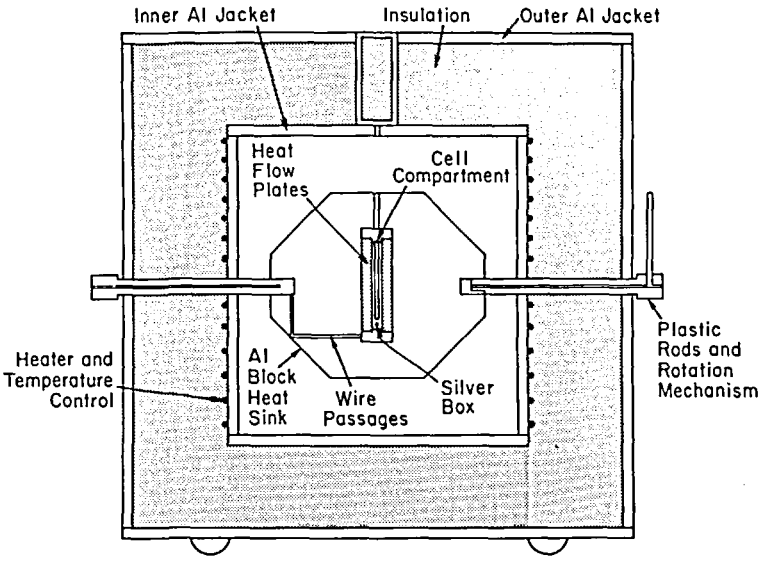


FIGURE 3. The Prosen-Berger batch microbiocalorimeter. A small partitioned cell of the type shown in Figure 2(b) resides in a large aluminum heat sink as shown with mixing achieved by rotation. The original design is due to Prosen[21] and is often referred to as the National Bureau of Standards (NBS) batch microcalorimeter. (Drawing obtained through the courtesy of R. L. Berger, National Heart, Lung and Blood Institute. Reproduced by permission of Thermonetics Corp., Box 9112, San Diego, Calif., 92109.)

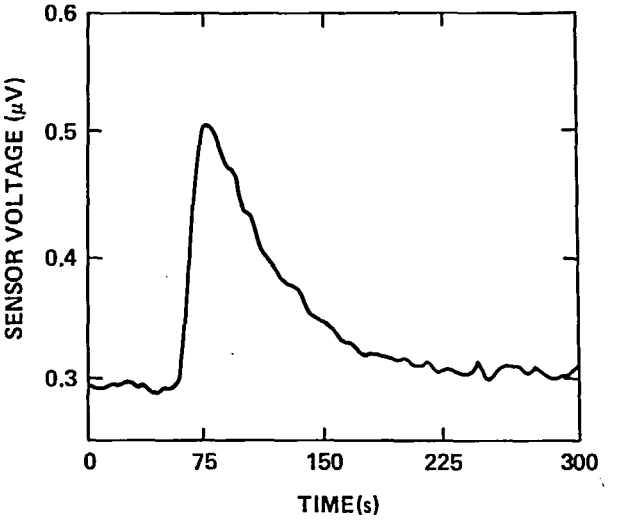


FIGURE 4. Response of the NBS batch microcalorimeter to a heat pulse of 200 μ J. (From Armstrong, G. T., *Calorimetry, Thermometry and Thermal Analysis,* The Society of Calorimetry and Thermal Analysis, Kagaku Gijitsu-Sha, Tokyo (1973). With permission.)

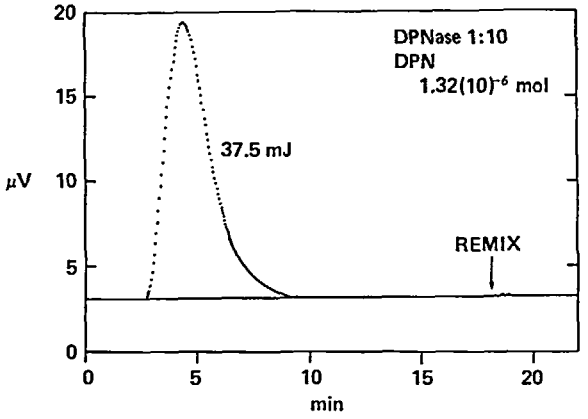


FIGURE 5. The heat change observed during the enzyme-catalyzed hydrolysis of nicotinamide adenine dinucleotide (DPN) by DPNase. Reaction carried out in the NBS batch microcalorimeter. Note that rotation of the calorimeter and thus remixing after completion of the reaction contributes negligible energy. (From Armstrong, G. T., *Calorimetry, Thermometry and Thermal Analysis*, The Society of Calorimetry and Thermal Analysis, Kagaku Gijitsu-Sha, Tokyo, (1973). With permission.)

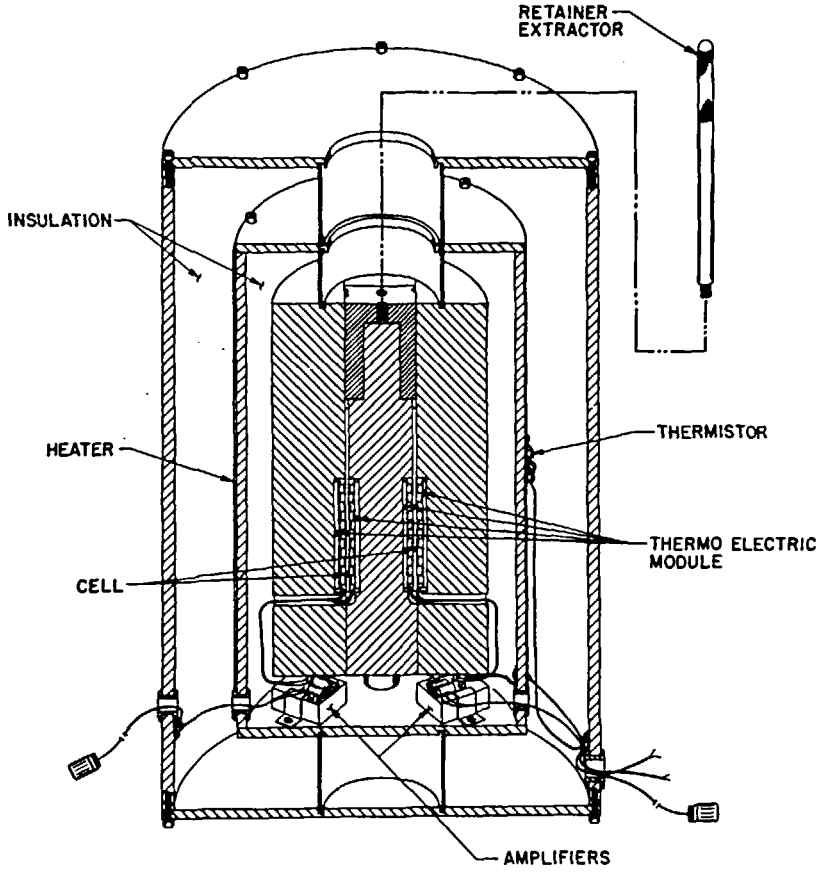


FIGURE 6. The Berger stopped-flow microcalorimeter. The flow cell is made of tantalum with a six-jet, large-bore mixing unit to provide complete mixing 2 to 3 cm downstream. The drawing and permission for its reproduction is through the courtesy of R. L. Berger, National Heart, Lung, and Blood Institute, NIH. Details of its operation and application are to be found in Reference 24.

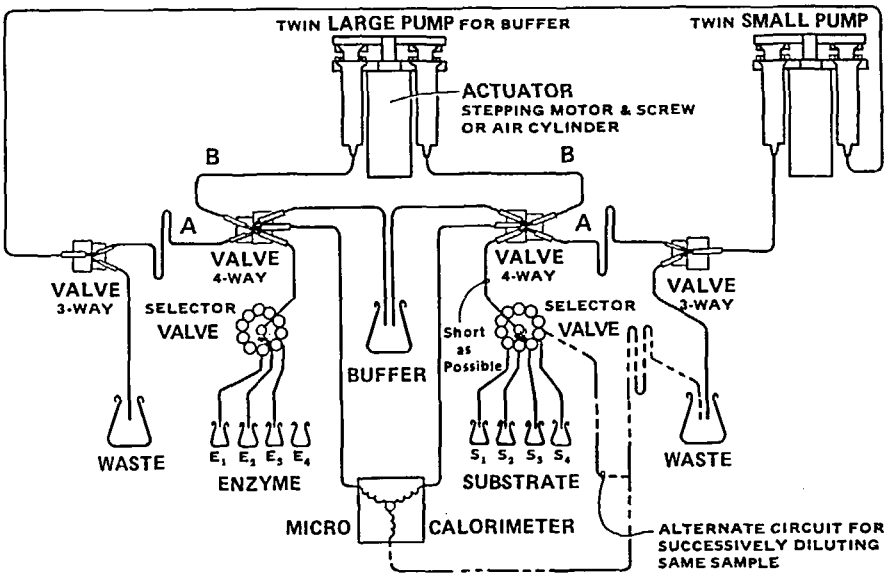


FIGURE 7. Schematic of the fluidic drive mechanism for enzyme-substrate assays in the Berger stopped-flow microcalorimeter. The system can be operated in automatic, semiautomatic, and flush modes. In automatic, buffer is sent through the system to thoroughly flush out the previous reactants followed by introduction of a selected enzyme-substrate set. Time periods for preheating of the reactants and duration of heat measurement can be preselected. At completion of any one period of observation, the cycle can be repeated. (from Berger, R. L., Davids, N., and Panek, E., *J. Cal. Anal. Therm.*, 6, 1 (1975). With permission.)

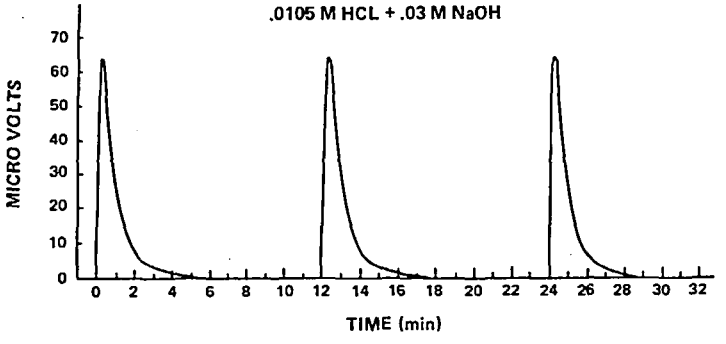


FIGURE 8. Successive runs in the Berger stopped-flow microcalorimeter using the neutralization reaction of HCl with NaOH. Reproducibility (chemically and electrically) is within 2%. (From Berger, R. L., Davids, N., and Panek, E., *J. Cal. Anal. Therm.*, 6, 1 (1975). With permission.)

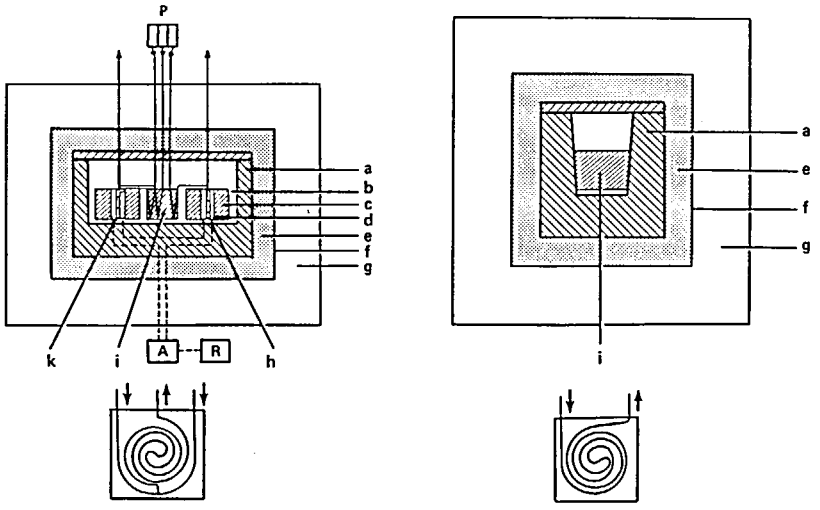


FIGURE 9. LKB Flow Calorimeter based on a design by a Monk and Wadsö.[26] Upper left, cross-section of microcalorimeter: (a) main heat sink, (b) air space, (c) aluminum block, (d) semiconductor thermopiles, (e) styrofoam insulation, (f) stainless steel container, (g) thermostated water bath, (h) flow-through cell, (i) heat-exchange unit, (k) mixing reaction cell. A is an amplifier and R is a recorder. Upper right, transverse section through the flow microcalorimeter; lower left, the mixing cell; lower right, the flow-through cell. (From Spink, C. and Wadsö, I., *Methods of Biochemical Analysis*, Vol. 23, Glick, D., Ed., John Wiley & Sons, New York, 1976, 1. With permission.)

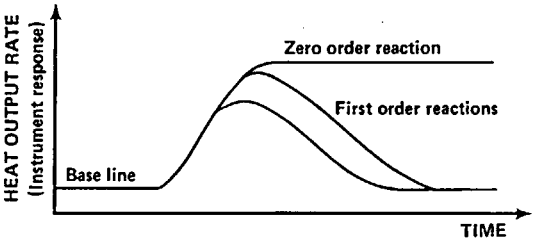


FIGURE 10. Heat output rate from a flow microcalorimeter (flow-through cell) vs. time for an enzyme-catalyzed reaction kinetically either zero or first order. (From Beezer, A. E. and Tyrrell, H. J. V., *Sci. Tools*, 19, 13 (1972). With permission.)

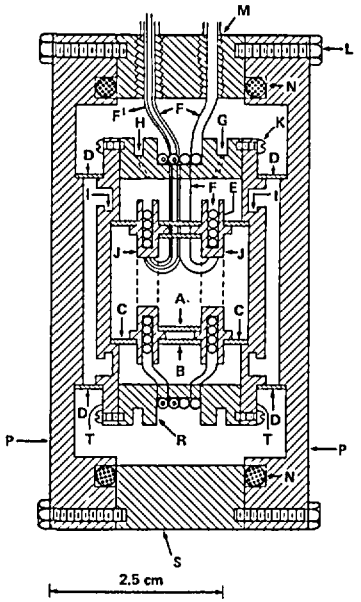


FIGURE 11. The Gill and Chen adiabatic flow microcalorimeter: (A,B) main thermopile, (C) shield thermopile, (D) bath thermopile, (E) pulse heaters, (F) cell tubing, (F') small cell tubing, (G) heater leads' groove, (H) thermopile leads groove, (I) shield control heaters, (J) cell spool, (K,L) screws, (M) cell tubing, heater wires, thermopile wires, (N) O-ring, (P) end plates, (R) adiabatic shield ring, (S) thick ring, (T) end plates. (From Gill, S. J. and Chen, Y. J., *Rev. Sci. Instrum.*, 45, 774 (1972). With permission.)

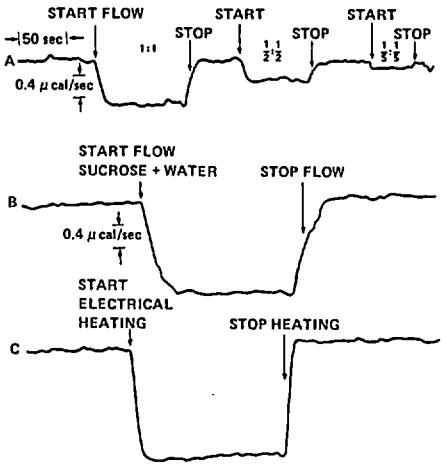


FIGURE 12. Test experiments (heat/sec vs. time) with the Gill and Chen flow microcalorimeter. (A) heat of water-water mixing at differing flow rates (1:1 at 0.0025 m*l*/sec); (B) heat of dilution, water and sucrose (0.3 *M*); (C) response curve to extended pulse electrical heating. (From Gill, S. J. and Chen, Y. J., *Rev. Sci. Instrum.*, 43, 774 (1972). With permission.)

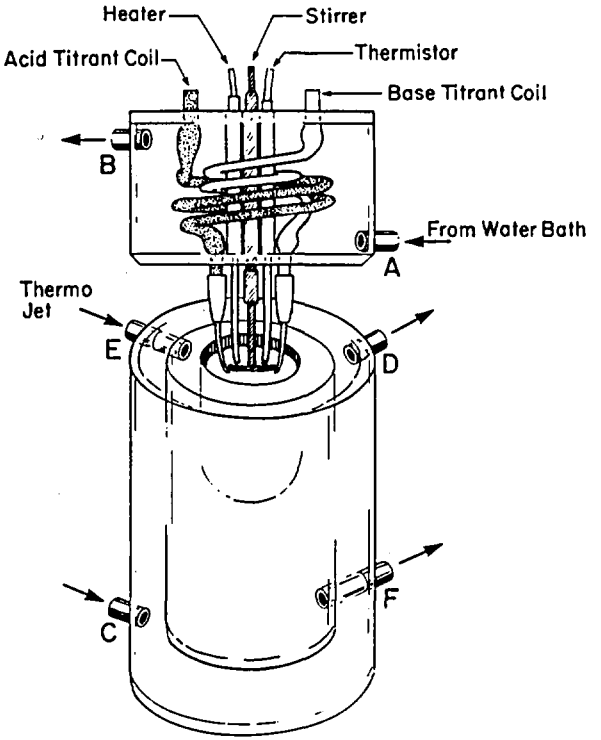


FIGURE 13. Isoperibolic titration microcalorimeter reaction cell assembly. (From Marini, M. A., Berger, R. L., Lam, D. P., and Martin, C. J., *Anal. Biochem.*, 43, 188 (1971). With permission.)

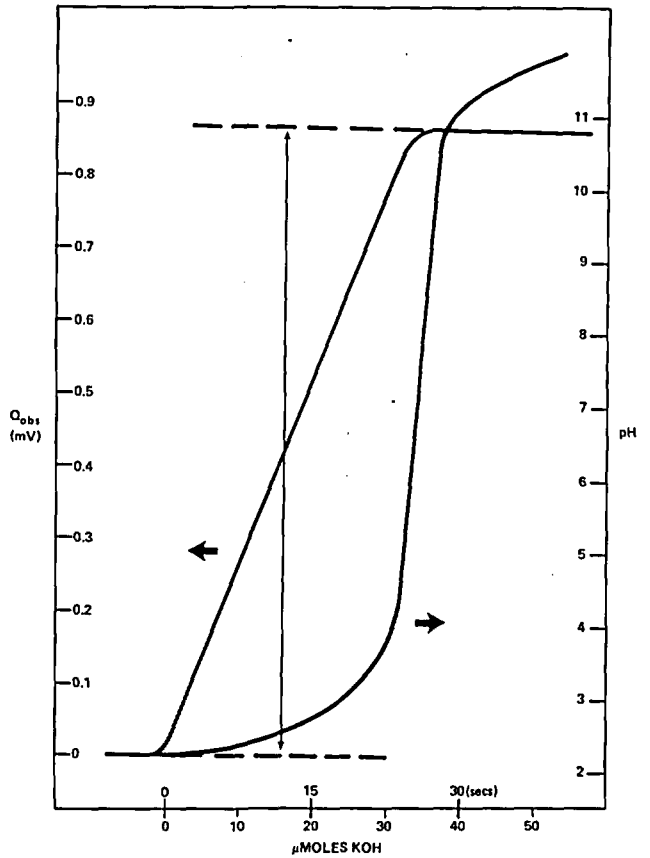


FIGURE 14. Simultaneous recording of heat change (Q_{obs}) and pH during the neutralization of 32 μmol HCl in 0.15 *M* KCl (4.0 m*l* volume) with N/1 KOH at 20°C using the microcalorimeter shown in Figure 13.[31]

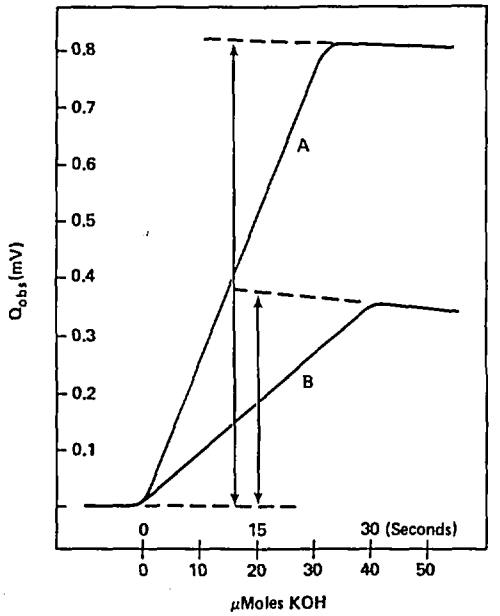


FIGURE 15. Recorder tracings of heat change (Q_{obs}) obtained using a titration microcalorimeter of type shown in Figure 13. (A) neutralization of HCl (32 μmol with N/1 KOH; (B) titration of 40 μmol imidazole. (From Martin, C. J., Sreenathan, B. R., and Marini, M. A., in *Analytical Calorimetry*, Vol. 3, Porter, R. S. and Johnson, J. F., Eds., Plenum Press, New York, 1974, 425. With permission.)

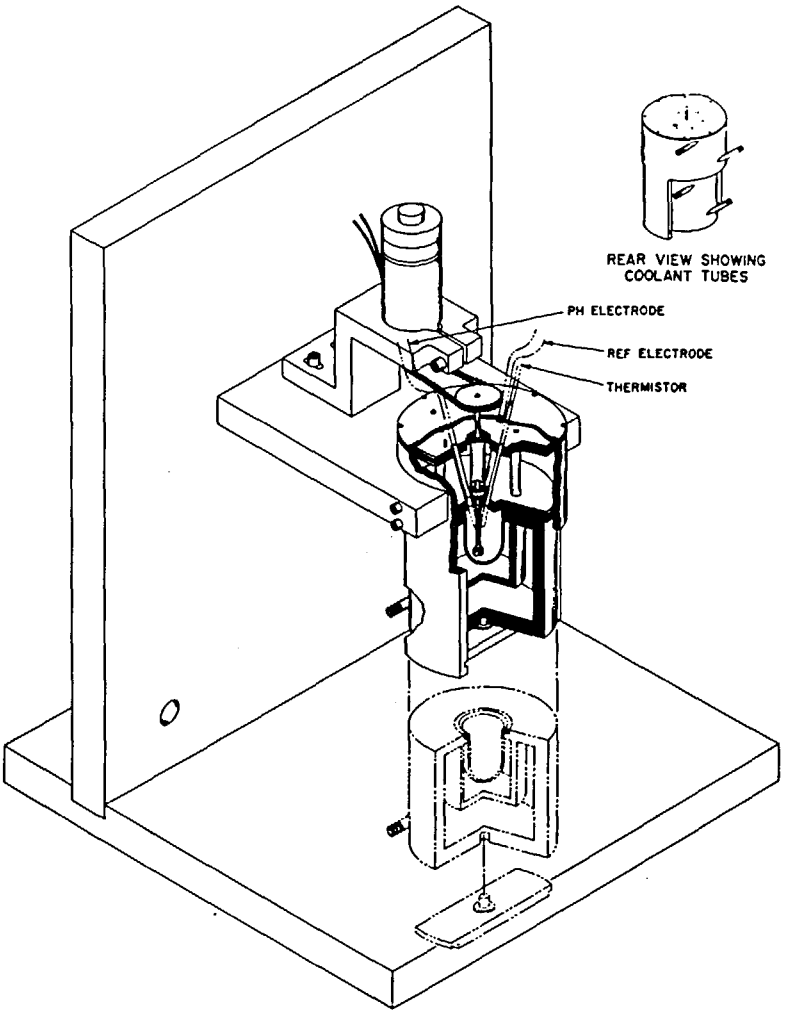


FIGURE 16. The Berger-Thibault-Marini isoperibolic titration microcalorimeter. The drawing is through the courtesy of R. L. Berger, National Heart, Lung, and Blood Institute, NIH.

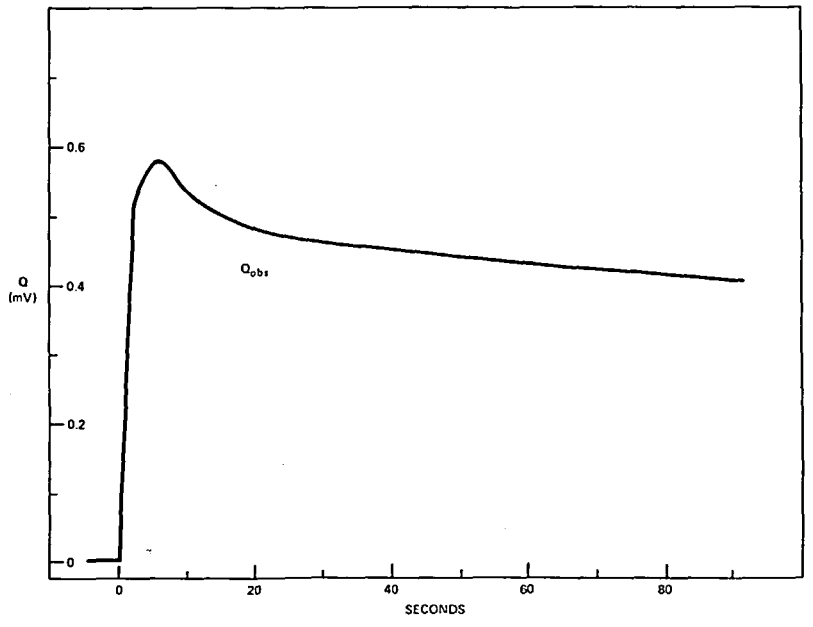


FIGURE 17. The thermal titration of 1.0 μmol HCl in 2.0 m*l* 0.15 *M* KCl with N/1 KOH using the microcalorimeter shown in Figure 16. Data of Martin, C. J., Sreenathan, Berger, R. L., and Marini, M. A., 32nd Annu. Calorimetry Conf., Sherbrooke, Quebec, Canada, July 6 to 8, 1977.

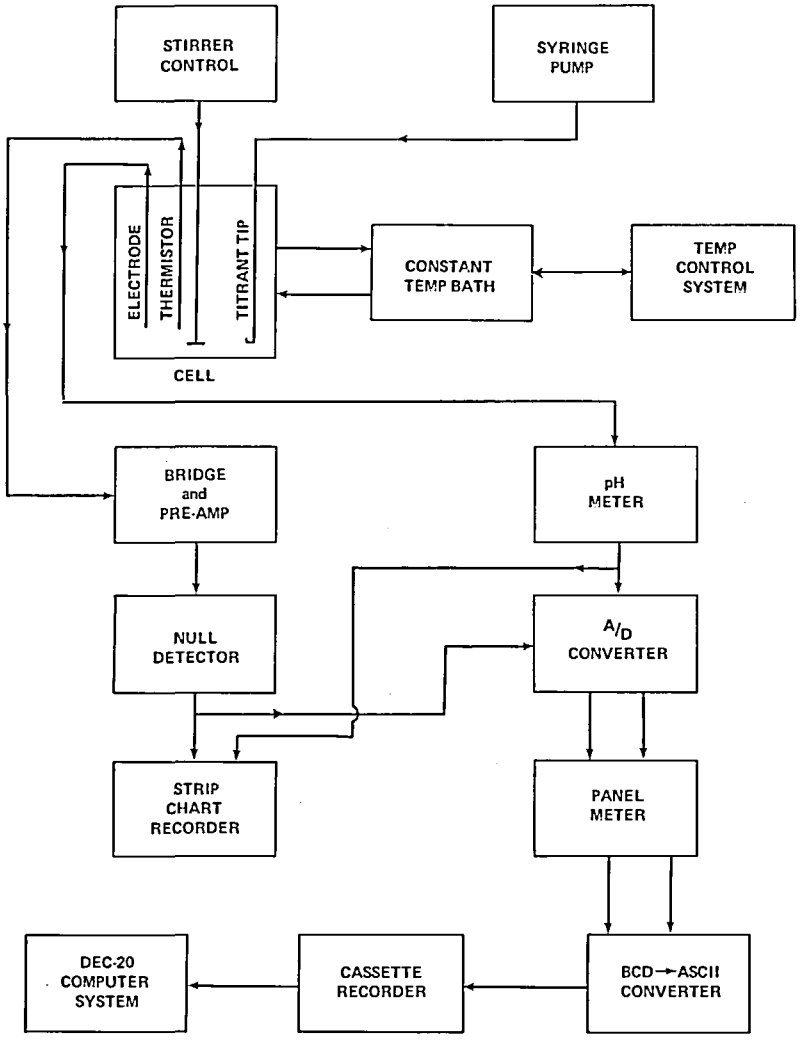


FIGURE 18. Block diagram of the components of the author's thermal and potentiometric titration assembly and the data acquisition, display and storage system.

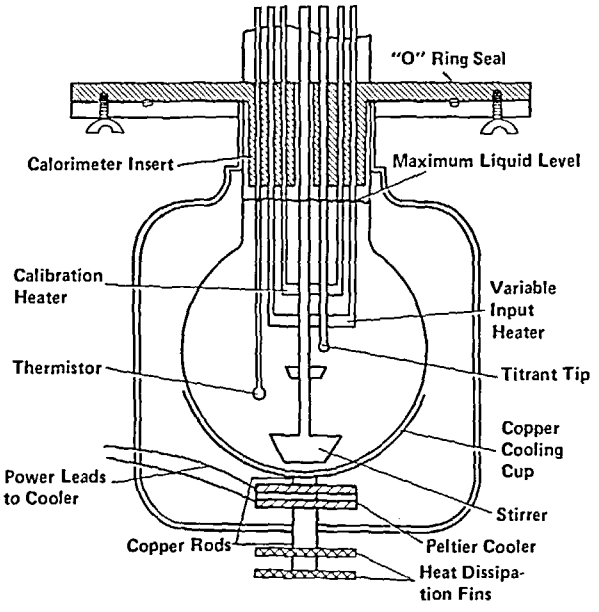


FIGURE 19. Isothermal titration calorimeter of Christensen and co-workers.[37] (From Christensen, J. J., Johnston, H. D., and Izatt, R. M., *Rev. Sci. Instrum.*, 39, 1356 (1968). With permission.)

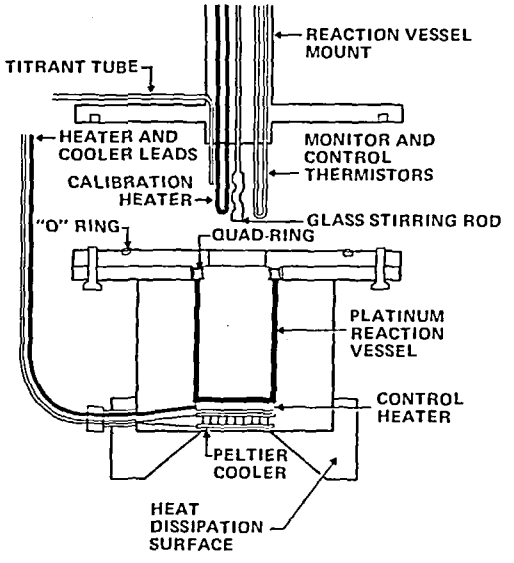


FIGURE 20. Micro version of an isothermal titration calorimeter. Reaction cell, 4.0 m*l* volume. (From Christensen, J. J., Gardner, J. W., Eatough, D. J., Izatt, R. M., Watts, P. J., and Hart, R. M., *Rev. Sci. Instrum.*, 44, 481 (1973). With permission.)

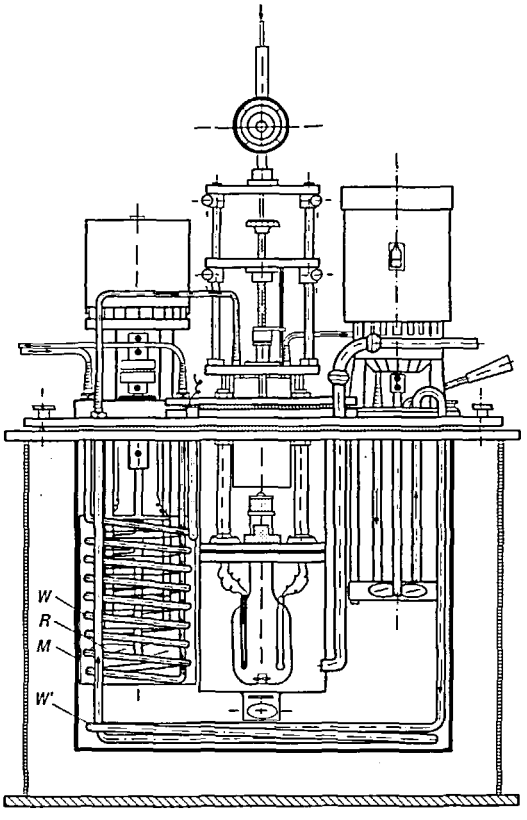


FIGURE 21. Precision thermometric titration assembly and bath designed by R. Wachter, J. Barthel, and K. Wachter-Lenz. The reaction vessel volume is 200 m*l* and temperature changes of 10^{-5} °C can be detected. (From Barthel, J., *Thermometric Titrations*, John Wiley & Sons, New York, 1975. With permission.)

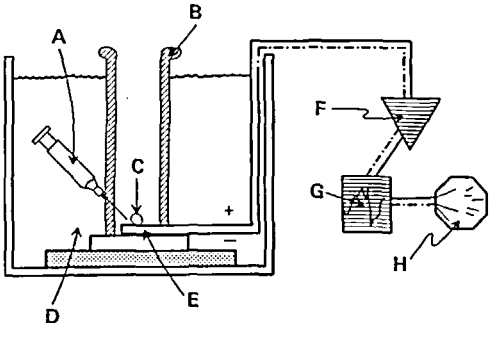


FIGURE 22. The analytical microcalorimeter of Pennington: (A) 250-μl syringe (reservoir for reagent "B"); (B) 24/40 standard taper glass fitting cut to 3 cm; (C) drop containing reagent "A"; (D) water bath; (E) Peltier unit detector (actual size 0.9 × 0.9 cm); (F) microvolt amplifier; (G) recorder; (H) data storage and integration. (From Pennington, S. N., *Anal. Biochem.*, 72, 230 (1976). With permission.)

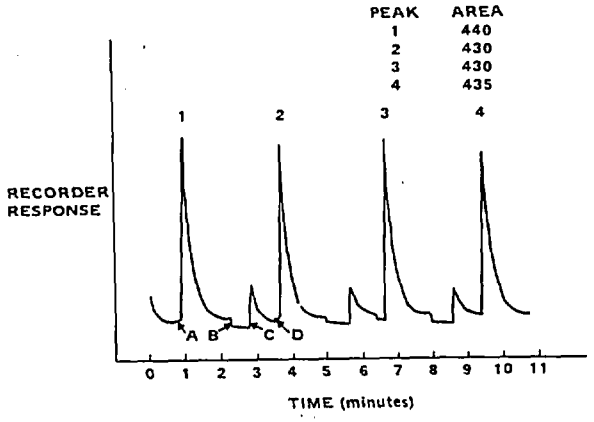


FIGURE 23. Typical response curves of the Pennington microcalorimeter to the protonation of 10$\mu\ell$ 0.515 *N*Tris buffer. Point "A" indicates initiation of reaction; "B" indicates completion of reaction; "C", new sample added; and "D", equilibrium established and second reaction initiated. Peak heights do not match because of diffusion-controlled mixing, but peak areas are comparable. Detector output sent directly to a strip-chart recorder operated at 10 mV full-scale sensitivity. (From Penington, S. N., *Anal. Biochem.*, 72, 230 (1976). With permission.)

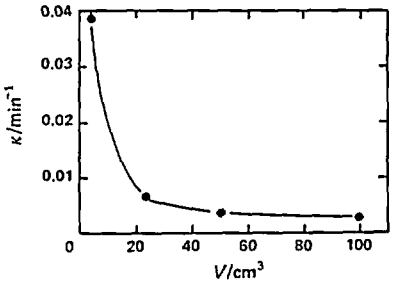


FIGURE 24. Effect of Dewar reaction vessel volume on heat leak constant, κ. (From Hansen, L. D., Jensen, T. E., Mayne, S., Eatough, D. J., Izatt, and Christensen, J. J., *J. Chem. Thermodyn.*, 7, 919 (1975). With permission.)

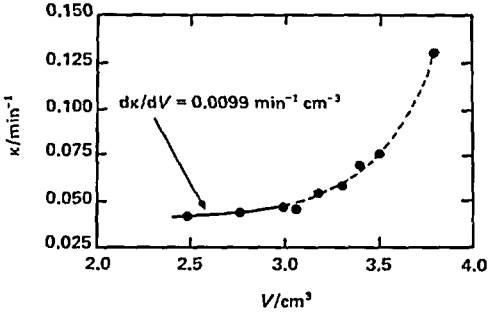


FIGURE 25. Effect of water volume in Dewar vessel of 3-mℓ volume on heat leak constant, κ. (From Hansen, L. D., Jensen, T. E., Mayne, S., Eatough, D. J., Izatt, R. M., and Christensen, J. J., *J. Chem. Thermodyn.*, 7, 919 (1975). With permission.)

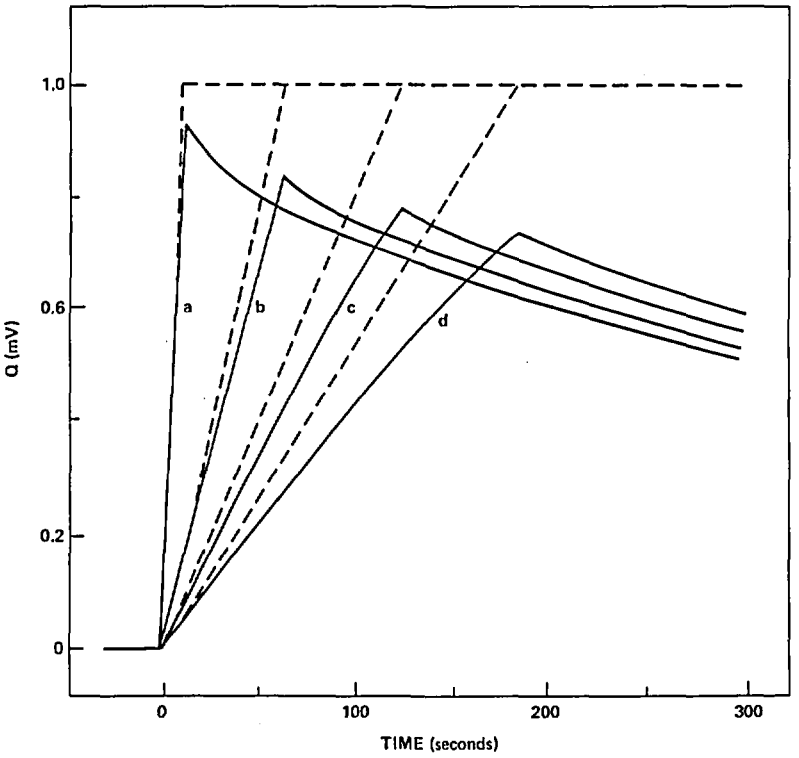


FIGURE 26. Solid lines: simulation by differential equation modeling of the observed heat assuming heat generation rates of (a) 0.1 mV/sec/10 sec; (b) 1.667×10^{-3} mV/sec/60 sec; (c) 8.33×10^{-3} mV/sec/120 sec; and (d) 5.55×10^{-3} mV/sec/180 sec. Decay constants for the model used (see text) were k_1, 0.01 sec^{-1}; k_{-1}, 0.06 sec^{-1}; k_2, 0.002 sec^{-1}. The dashed lines represent the "adiabatic" reconstructions of the heat generated. (Data of Martin, C. J., Sreenathan, B. R., and Marini, M. A.)

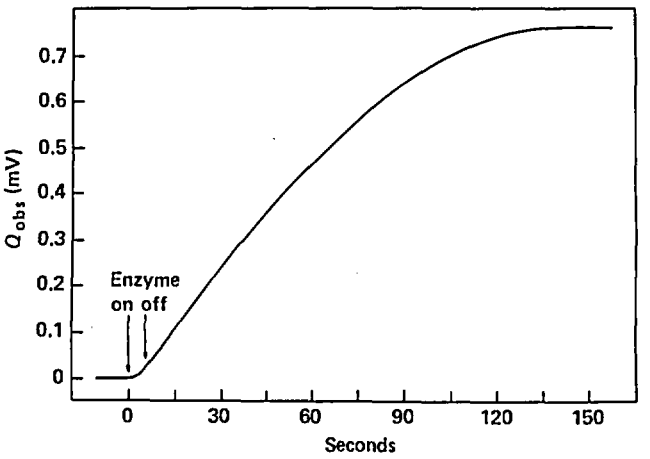


FIGURE 27. Reaction rate curve for the chymotrypsin-catalyzed hydrolysis of acetyl-L-tyrosine ethyl ester. The reaction volume was 4.0 m*l* containing 40 μmol of the ester and was 0.04 *M* in Tris buffer at pH 8.0 and at 25°C. The chymotrypsin solution (approximately 5 g/*l* was in the glass coils of the upper chamber of the reaction assembly shown in Figure 13 and was added for 6 sec (approximately 30 μg chymotrypsin) to initiate hydrolysis at the point indicated. The heat evolved with time essentially represents the change associated with the addition of a proton to the unprotonated Tris buffer component. This curve has been corrected for slope deviations of the upper and lower temperature base lines. (From Marini, M. A. and Martin, C. J., in *Methods in Enzymology*, Vol. 27, Part D, Hirs, C. H. W. and Timasheff, S. N., Eds., Academic Press, New York.)

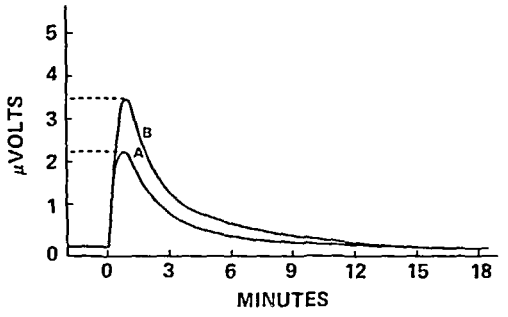


FIGURE 28. Recorder trace of voltage for the calorimetric determination of uric acid in serum. Concentration of uric acid in curve A = 47 mg/*l* and in curve B = 69 mg/*l*. (From Rehak, N. N., Janes, G., and Young, D. S., *Clin. Chem.*, 23, 195 (1977). With permission.)

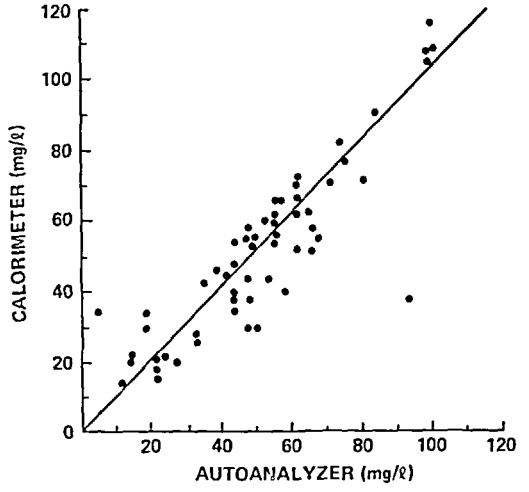


FIGURE 29. Correlation between serum concentrations of uric acid as determined calorimetrically and by continuous-flow (Auto Analyzer II®). (From Rehak, N. N., Janes, G., and Young, D. S., *Clin. Chem.*, 23, 195 (1977). With permission.)

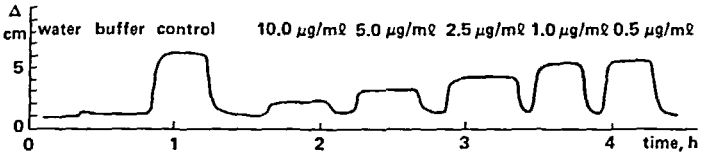


FIGURE 30. Recorder trace of the calorimetric determination of cholinesterase activity in the presence of tetramethylphosphorodiamidic fluoride at 25°C. The inhibitor in the concentrations indicated was preincubated with the enzyme for 30 min prior to reaction. (From Konickova, J. and Wadso, I., *Acta Chem. Scand.*, 25, 2360 (1971). With permission from Acta Chemica Scandinavica.)

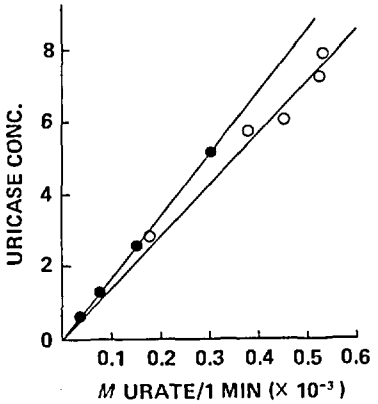


FIGURE 31. Relationship between uricase concentration and the observed turnover rate. Soluble uricase (—●—); uricase immobilized on glass beads (—○—). The concentrations are expressed as micrograms of enzyme in total volume in the calorimeter cell. (Rehak, N. N., Everse, J., Kaplan, N. O., and Berger, R. L., *Anal. Biochem.*, 70, 381 (1976). With permission.)

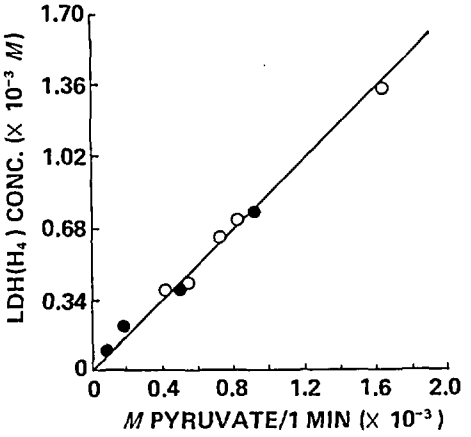


FIGURE 32. *Relationship of lactic dehydrogenase* concentration and the observed turnover rate in the calorimeter for soluble (—●—) and the immobilized enzyme (—○—). (From Rehak, N. N., Everse, J., Kaplan, N. O., and Berger, R. L., *Anal. Biochem.*, 70, 381 (1976). With permission.)

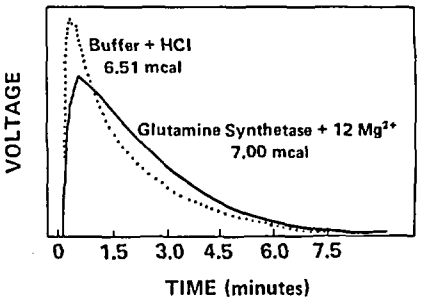


FIGURE 33. Heat released during ~2% neutralization of Hepes buffer at 37°C (dashed curve) and the heat release produced upon the binding of 12 molar equivalents of Mg^{++} by glutamine synthetase in 0.05 *M* Tris/0.1 *M* KCl at pH 7.19 (solid curve). The heat released is proportional to the area under each curve. (Reprinted with permission from Hunt, J. B., Ross, P. D., and Ginsburg, A., *Biochemistry*, 11, 3716 (1972). Copyright by the American Chemical Society.)

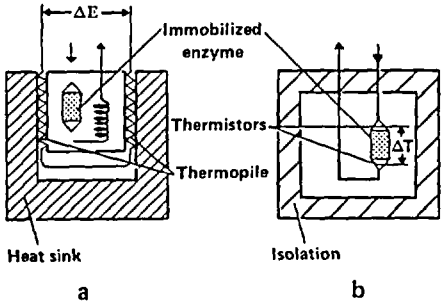


FIGURE 34. Calorimeter constructions for measurement of heat of reaction of immobilized enzymes: (a) Heat flow measurement. The heat flows from the reaction cell via thermopiles to the heat sink, generating a voltage, ΔE, over the thermopiles, which is directly proportional to the heat flow. (b) Temperature measurement. The reaction cell is thermally insulated from its surroundings, and the temperature increase, ΔT, of the fluid passing the cell is measured. (From Johansson, A., Mattiasson, B., and Mosbach, K., in *Methods in Enzymology*, Vol. 44, Mosbach, K., Ed., Academic Press, New York, 1976, 659. With permission.)

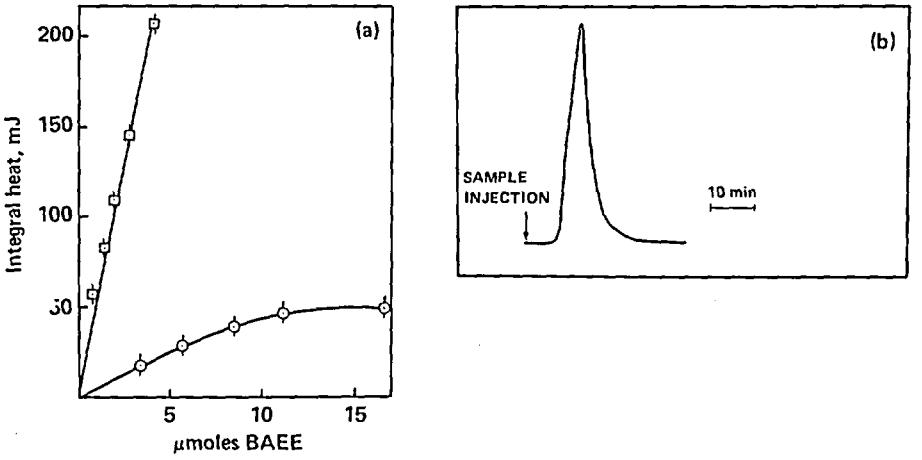


FIGURE 35. Heat pulse experiments on immobilized trypsin. (a) Integral heat as a function of the amount of substrate injected. ⊙, Phosphate buffer; ⊡, Tris buffer. (b) Experimental curve obtained after injection of 135 μl of a 0.041 *M* benzoyl-L-arginine ethyl ester (BAEE) solution. Integral heat corresponds to 28.2 mJ. (From Johansson, A., Mattiasson, B., and Mosbach, K., in *Methods in Enzymology*, Vol. 44, Mosbach, K., Eds., Academic Press, New York, 1976, 659. With permission.)

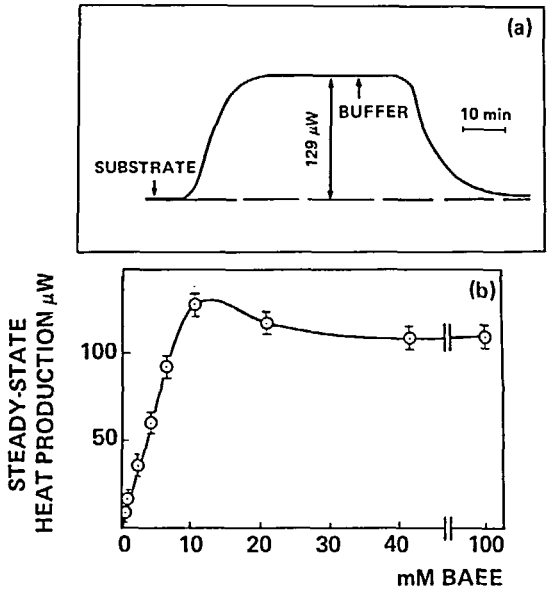


FIGURE 36. Steady-state experiments on immobilized trypsin. (a) Recorded calorimetric response at a continuous flow of 10 m*M* benzoyl-L-arginine ethyl ester (BAEE) in phsophate buffer. (b) Steady-state heat production as a function of BAEE concentration in phosphate buffer. (From Johansson, A., Mattiasson, B., and Mosbach, K., in *Methods in Enzymology*, Vol. 44, Mosbach, K., Ed., Academic Press, New York, 1976, 659. With permission.)

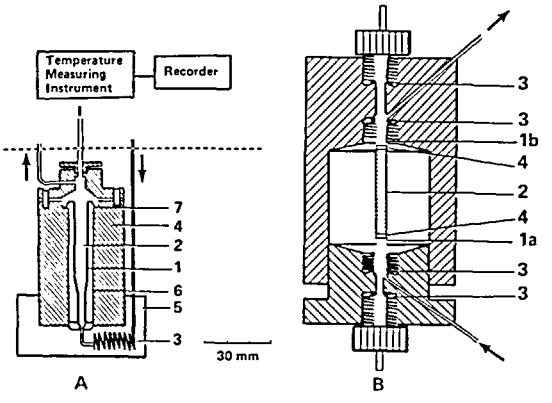


FIGURE 37. A. Single thermistor unit. 1, Microcolumn containing the immobilized enzyme preparation; 2, steel tube with thermistor; 3, heat exchanger; 4, Perspex® cylinder; 5, water jacket; 6, air space, 7, O-rings. B. A differential enzyme thermistor unit. 1a, Reference thermistor; 1b, sensing thermistor; 2, Teflon® column containing the immobilized enzyme; 3. O-rings; 4, porous polyethylene disks. (From Danielsson, B. and Mosbach, K., in *Methods in Enzymology*, Vol. 44, Mosbach, K., Ed., Academic Press, New York, 1976, 667. With permission.)

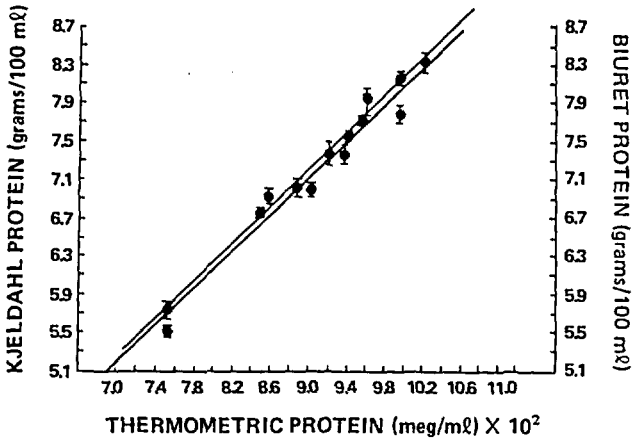


FIGURE 38. Correlation between total serum protein determined calorimetrically and by Kjeldahl analysis (—Δ—) and by biuret (—O—). The bars represent the range of values found in duplicate experiments. (Reprinted with permission from Smith, E. B. and Carr, P.W., *Anal. Chem.*, 45, 1688 (1973). Copyright by the American Chemical Society.)

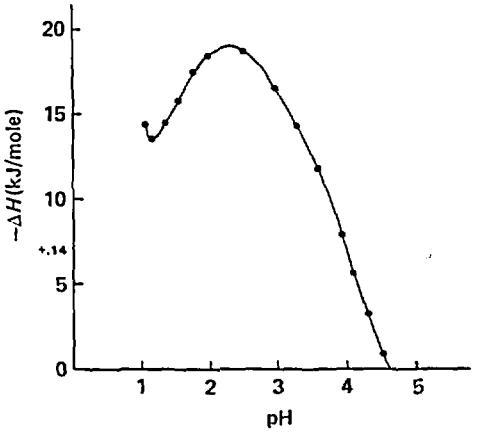


FIGURE 39. Enthalpy titration curve of lysozyme in the acidic pH range at 25°C in 0.15 *M* KCl: (From Bjurulf, C., *Eur. J. Biochem.*, 30, 33 (1972). With permission.)

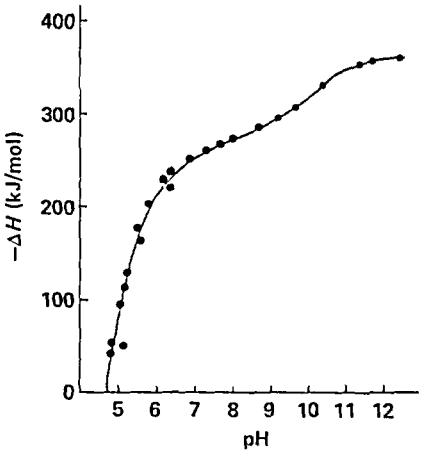


FIGURE 40. Enthalpy titration curve of lysozyme in the alkaline pH range at 25°C in 0.15 *M* KCl. The experimental heat shown is the sum of the individual heats of ionization of the amino acid residues and the heat of formation of water. (From Bjurulf, C., *Eur. J. Biochem.*, 30, 33 (1972). With permission.)

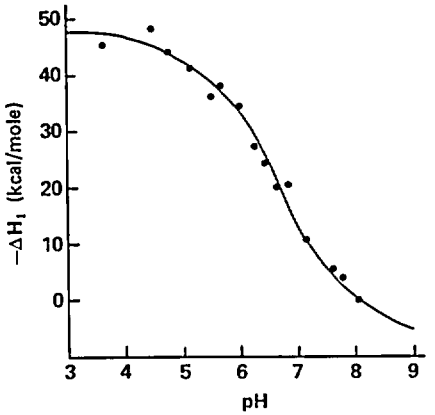


FIGURE 41. Enthalpy titration of ribonuclease A at 25°C and ionic strenght, 0.05. The enthalpy has arbitrarily been set to zero at pH 8. The solid line is a curve calculated from the data given in Table 5. (Reprinted with permission from Flogel, M. and Biltonen, R., *Biochemistry*, 14, 2603, (1975). Copyright by the American Chemical Society.)

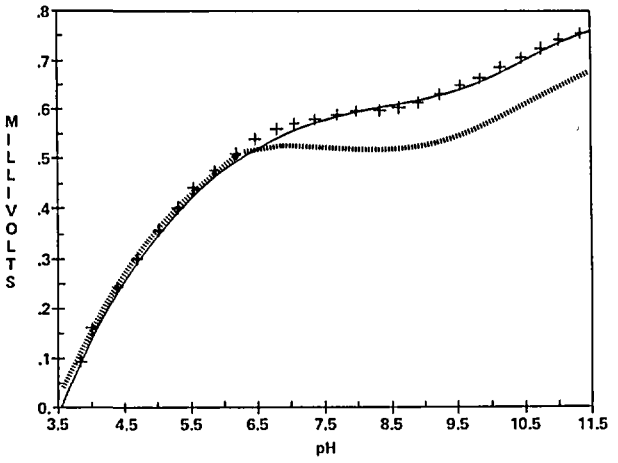


FIGURE 42. Combined thermal and potentiometric titration of ribonuclease A in 0.05 *M* KCl at 25°C. The experimental points (+) have been fitted (solid line) with the parameter values derived by iterative curve fitting. The heats of ionization of the four histidine residues present range from 4.4 to 6.5 kcal/mol.[36] The lower curve is the thermal titration calculated for one of the histidine residues assigned pK′, 7.2 and ΔH_i, 24 kcal/mol.

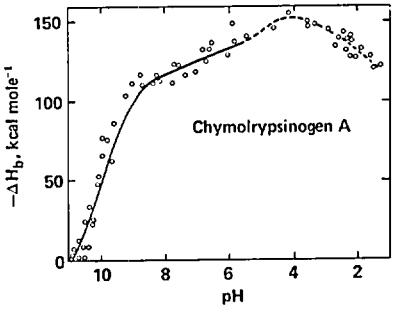
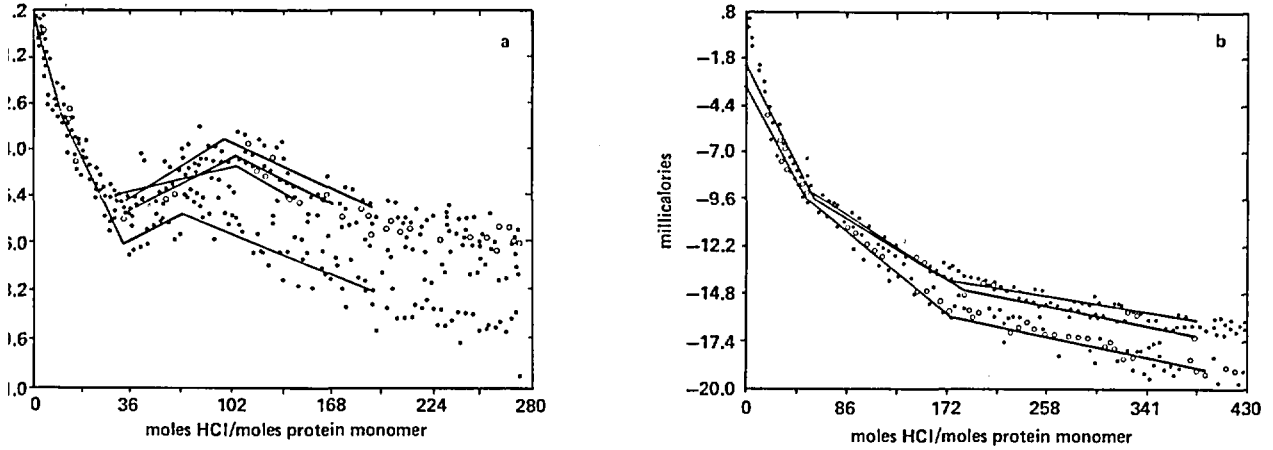


FIGURE 43. Enthalpy titration of chymotrypsinogen A at 25°C in 0.15 *M* KCl. The solid line represents the curve calculated on the basis of the constants given in Reference 106, Table 1. (From Shiao, D. D. F. and Sturtevant, J. M., *Biopolymers*, 15, 1201 (1976). With permission.)



URE 44. Calorimetric titration of HCl (0.16 *M*) into: (a) native human serum albumin (4 mg/m*l*) (4 runs) and (b) heat-denatured an serum albumin (4 mg/m*l* solution which was previously placed in boiling water for 10 min) (3 runs). (From Izatt, R. M., Hansen,)., Eatough, D. J., Jenson, T. E., and Christensen, J. J., in *Analytical Calorimetry*, Vol. 3, Porter, R. S. and Johnson, J. F., Plenum s, New York, 1974, 237. With permission.)

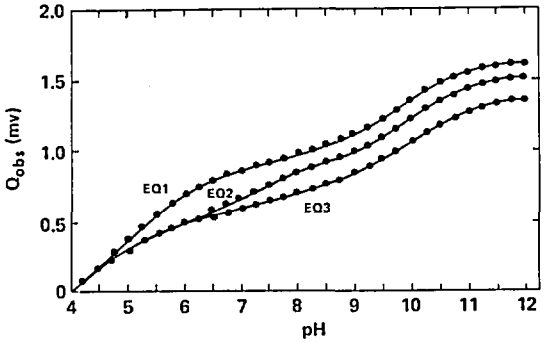


FIGURE 45. Calorimetric titration of a mixture. EQ_3 contains glycylglycine, formic acid, pyridine, imidazole, β-mercaptoethanol, phenol, and methylamine each in 0.004 *M* concentration. EQ_2 contains twice the concentration of imidazole, and EQ_1 contains twice the concentration of pyridine. The solid lines are calculated from the constants given in Table 7. (From Marini, M. A., Martin, C. J., Berger, R. L., and Forlani, L., in *Analytical Calorimetry*, Vol. 3, Porter, R. S. and Johnson, J. F., Eds., Plenum Press, New York, 1974, 407. With permission.)

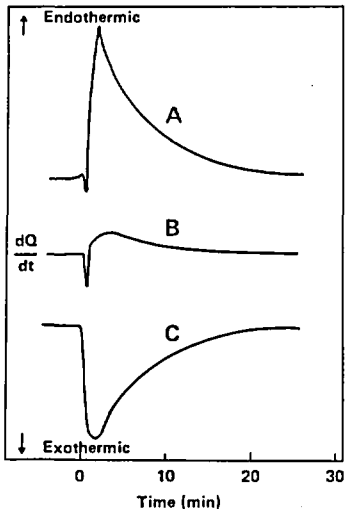


FIGURE 46. Experimental curves showing the rate of exchange of heat when α_{s1}-casein reacts with calcium chloride. A, 3.68 m*M* $CaCl_2$; B, 7.30 m*M* $CaCl_2$; C, 4.68 m*M* $CaCl_2$. (From Holt, C., Parker, T. G., and Dalgleish, D. G., *Biochem. Biophys. Acta*, 379, 638 (1975). With permission.)

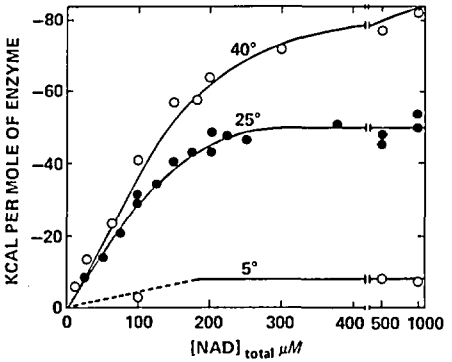


FIGURE 47. Thermal titration of yeast glyceraldehyde 3-phosphate dehydrogenase with NAD in 0.05 *M* potassium phosphate buffer (pH 7.32) containing 0.05 *M* KCl and 0.002 *M* EDTA. Enzyme concentrations: 46×10^{-6} *M* at 5° and 25°, 56×10^{-6} *M* at 40°. The curves at 25° and 40° are drawn using the values of K and ΔH given in Table I of reference 139. (Reprinted with permission from Velick, S. F., Baggott, J. P., and Sturtevant, J. M., *Biochemistry*, 10, 779 (1971). Copyright by the American Chemical Society.)

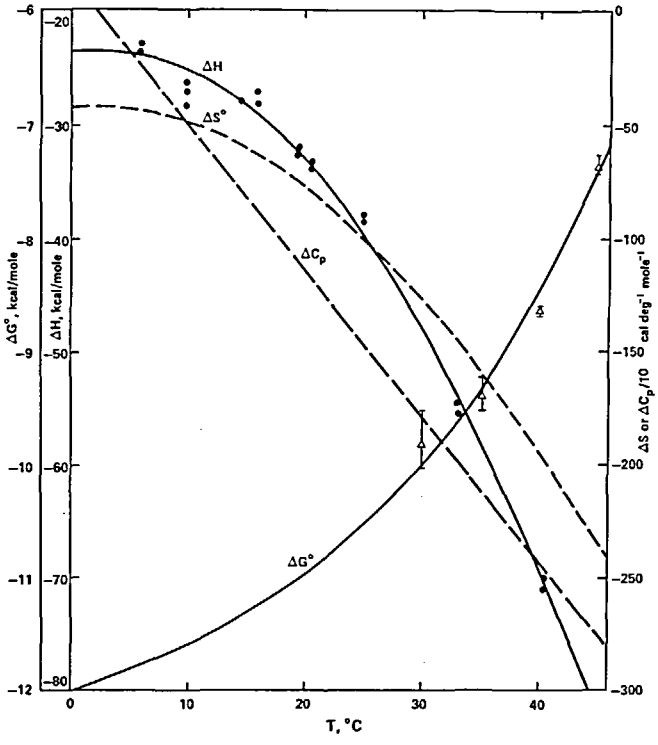


FIGURE 48. Summary of the thermodynamic parameters for the reaction of S-peptide with S-protein. The curve for ΔH is a least-squares fit of the experimental data (circles) to a quadratic in the temperature. The curve for ΔC_p is the temperature derivative of the ΔH curve. The curve for ΔG° was obtained by integration of the Gibbs-Helmholtz equation, the integration constant being determined by comparison to the experimental values (triangles). (Reprinted with permission from Hearn, R. P., Richards, F. M., Sturtevant, J. M., and Watt, G. D., *Biochemistry*, 10, 806 (1971). Copyright by the American Chemical Society.)

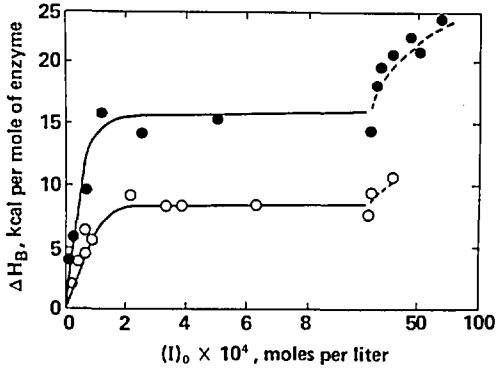


FIGURE 49. Calorimetric titration curves of muscle aldolase with hexitol-1,6-diphosphate at pH 7.5, 25°C. The heat absorbed, corrected for dilution heats, when the inhibitor is bound is plotted as a function of the total inhibitor concentration. (○) Titration in 0.01 *M* Herpes buffer; enzyme concentrations, 26-52 μM. (●) Titration in 0.1 *M* glycylglycine buffer; enzyme concentrations, 24-60 μM. All solutions contained 0.001 *M* EDTA. The solid curves are calculated on the basis of $K = 1.8 \times 10^5\ M^{-1}$ and the observed value for ΔH_B at inhibitor saturation ($(I)_o \sim 0.6$ m*M*) with the assumption of 2.7 combining sites per molecule of enzyme. (Reprinted with permission from Hinz, H. J., Shiao, D. D. F., and Sturtevant, J. M., *Biochemistry*, 10, 1347 (1971). Copyright by the American Chemical Society.)

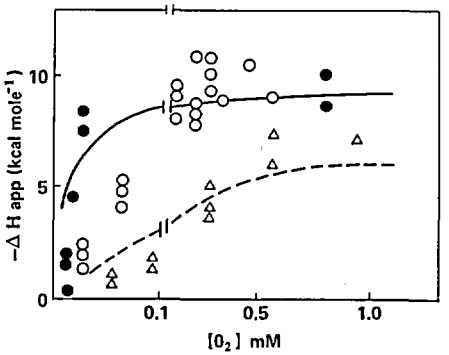


FIGURE 50. Enthalpy of binding of oxygen to hemerythrin at 25°C in Tris-cacodylate buffer, pH 7.0 (●) and pH 9.2 (○). Similar data (△) at pH 7.0 in the presence of 0.03 *M* $NaClO_4$. The solid line was calculated using $\Delta H = -9.2$ kcal/mol and $K = 10^5\ M^{-1}$ and the dashed line using $\Delta H = -6.9$ kcal/mol and $K = 10^4\ M^{-1}$. The scatter in the data in the presence of perchlorate precludes any interpretation of the significance of these values (other than the overall binding enthalpy) as well as any suggestion as to the need for more than one equilibrium constant to fit the data in the presence of perchlorate. (Reprinted with permission from Langerman, N. and Sturtevant, J. M., *Biochemistry*, 10, 2809 (1971). Copyright by the American Chemical Society.)

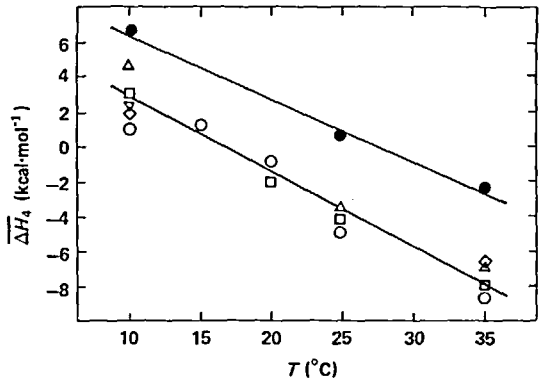


FIGURE 51. Temperature dependence of the enthalpies associated with binding of various amino acids and L-isoleucinol to L-isoleucine: tRNA ligase (*Escherichia coli* MRE 600). Buffer conditions: 50 m*M* potassium phosphate containing 1 m*M* dithioerythritol at pH 7.5. L-isoleucine, (○);L-leucine, (△);L-valine, (□);L-norvaline, (◇);L-2-amino-3,4-dimethyl pentanoic acid, (▽);L-isoleucinol, (●). (From Hinz, H. J., Weber, K., Flossdorf, J., and Kula, M.-R., *Eur. J. Biochem.*, 71, 437 (1976). With permission.)

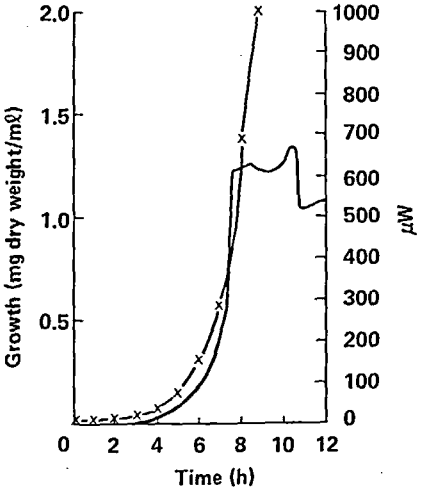


FIGURE 52. Heat produced by a culture of *Enterobacter aerogenes* at 30°C, pumped (20 m*l*/h) through the flow microcalorimeter (100 or 300 μV amplification), when compared with the exponential growth curve (x———x). (From Kemp, R. B., *Pestic. Sci.*, 6, 311 (1975). With permission.)

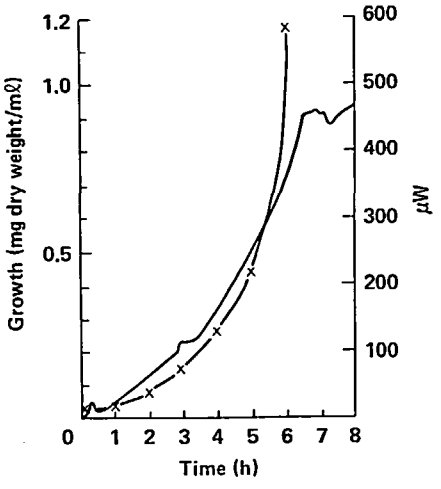


FIGURE 53. Heat production of a growing culture of *Clostridium pasteurianum* flowing (20 m*l*/hr) through the microcalorimeter (100 or 300 μV amplification) from the culture at 37°C. Growth (x————x) is estimated turbidimetrically and converted to milligrams per milliliter using a calibration curve. (From Kemp, R. B., *Pestic. Sci.*, 6, 311 (1975). With permission.)

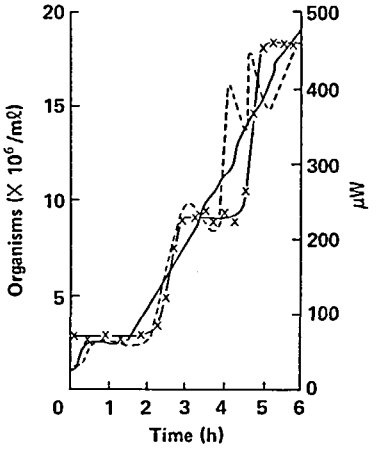


FIGURE 54. Heat production of aerated suspension of *Schizosaccharomyces pombe* at 30°C through two successive doublings in cell numbers (x————x). Rate of heat production (μW) measured by flow microcalorimetry (total pumping rate 80 m*l*/hr) of a control suspension (————) and one treated with 16.2 μ*M* 2-oxomalononitrile *m*-chlorophenyl-hydrazone (----------). Instrument amplification 100 μV. (From Kemp, R. B., *Pestic. Sci.*, 6, 311 (1975). With permission.)

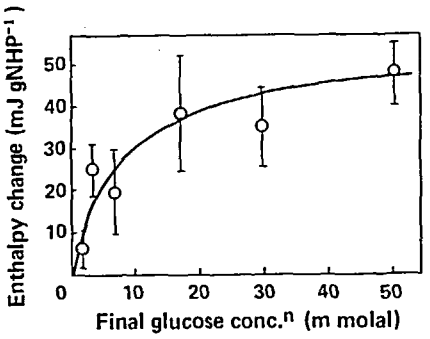


FIGURE 55. The enthalpy of specific binding of D-glucose to human erythrocyte ghosts at 15°C as a function of the final glucose concentration. Enthalpy is given in mJ/g nonhemoglobin protein. The error bars denote the standard deviations. (From Zala, C. A., Jones, M. N., and Levine, M., *FEBS Lett.*, 48, 196 (1974). With permission.)

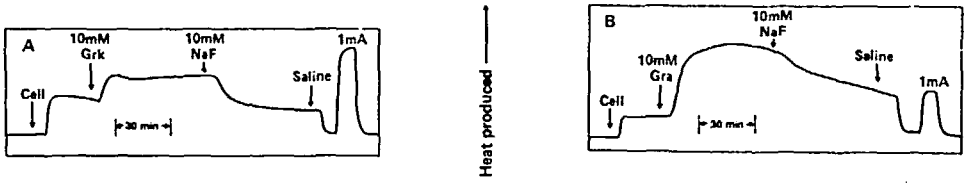


FIGURE 56. Thermograms showing the effect of (A) dihydroxyacetone and (B) D-glyceraldehyde on red cells. (A) Red cells (citrated blood stored for 2 days) were suspended in an isotonic saline containing 5 m*M* KCl, 5 m*M* inorganic phosphate, 1 m*M* $MgCl_2$, and 5 m*M* glucose and were then incubated at pH 7.4, 37°C. The hematocrit value was 30%. The suspension was pumped into the calorimeter at the point indicated by an arrow labeled "Cell". Dihydroxyacetone (Grk) and sodium fluoride (NaF) solutions were added successively to a final concentration of 10 m*M* at points indicated. The rates of lactate formation before and after the addition of dihydroxyacetone were 3.3 and 5.5 μmol/hr per 1 m*l* cells, respectively. (B) The conditions were the same as in (A) except that red cells stored for 3 days were used. D-glyceraldehyde (Gra) was added to a final concentration of 10 m*M*. The sensitivity of the instrument was reduced to half as shown by the calibration, and the rates of the lactate formation before and after the addition of Gra were 3.6 and 3.0 μmol/hr per 1 m*l* cells, respectively. Glycerol was formed after the addition, at the rate of 0.74 μmol/hr per 1 m*l* cells. (From Minakami, S. and de Verdier, C.-H., *Eur. J. Biochem.*, 65, 451 (1976). With permission.)

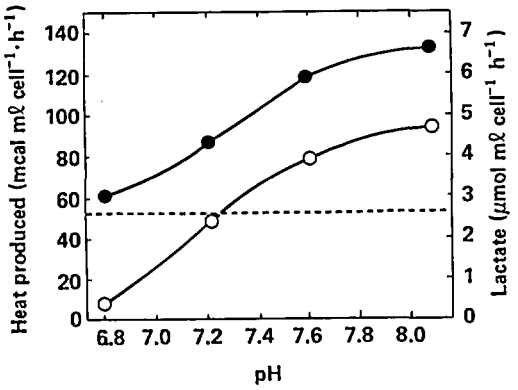


FIGURE 57. Relationship between the heat production (—●—) and lactate concentration (—○—) as a function of pH. Red cells stored for 2 days were suspended in isotonic saline containing 5 m*M* KCl, 2 m*M* inorganic phosphate, 1 m*M* $MgCl_2$, and 10 m*M* glucose. The hematocrit value was 30%. The broken line represents the heat production rate not associated with glycolysis which was obtained from the steady state rate in the presence of fluoride. (From Minakami, S. and de Verdier, C.-H., *Eur. J. Biochem.*, *65*, 451 (1976). With permission.)

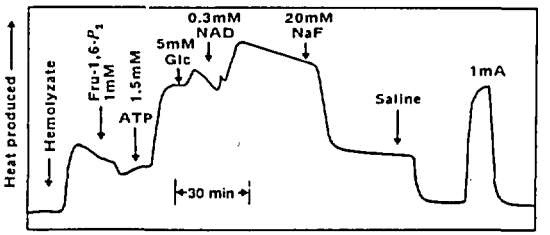


FIGURE 58. Thermogram showing the effect of substrates and coenzymes on a hemolyzate. Red cells from 4-day-old citrated blood were washed with isotonic KCl solution and hemolyzed by addition of about 2 volumes of water and subjected to freezing and thawing by liquid nitrogen. Concentrated salt solutions were added to make final concentrations in the hemolyzate, 50 m*M* KCl, 2 m*M* NaCl, 2 m*M* inorganic phosphate, and 1 m*M* $MgCl_2$ (without consideration of the salt concentrations coming from the cell suspensions). The concentration of hemoglobin was 116 g/*l*. The pH was kept at 7.6 (37°C) by the addition of 0.3 *M* KOH. Substrates and coenzymes were added to the incubation vessel at the points indicated. No lactate formation was observed before the addition of NAD^+. After this addition, the rate of lactate formation increased to 3.3 μmol/hr/m*l* hemolyzate. (From Minakami, S. and de Verdier, C.-H., *Eur. J. Biochem.*, *65*, 451 (1976). With permission.)

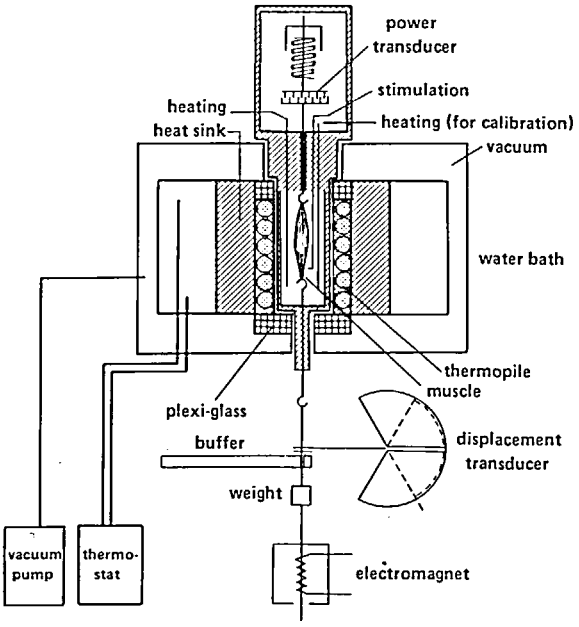


FIGURE 59. Schematic view of the microcalorimeter designed for the study of heat production in muscle. (From Lörinczi, D. and Futó, Z., *Acta Biochim. Biophys. Acad. Sci. Hung.*, 9, 371 (1974). With permission.)

ACKNOWLEDGMENTS

The authors wish to thank Dr. Robert L. Berger of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Md., Dr. William J. Evans of the Southern Regional Research Laboratory, New Orleans, La., and Dr. Lee Hansen, Brigham Young University, Provo, Utah for their helpful suggestions.

We also wish to thank Mrs. Bertha Freeman for her patience and invaluable assistance during the preparation and typing of this manuscript.

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