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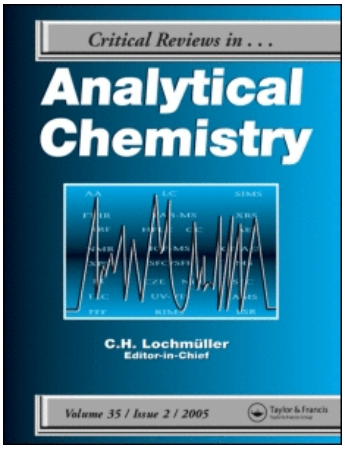

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ANALYTICAL CALORIMETRY IN BIOCHEMICAL AND CLINICAL APPLICATIONS

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I. INTRODUCTION

Calorimetry has played a fundamental historical role in the development of the basic thermodynamics of chemical systems. In this sense, calorimetry is one of the oldest methodologies in the field of chemistry. However, the application of calorimetric measurements to analytical problems has been much slower to develop, the most dramatic increases in use occurring within the last 15 to 20 years. In the fields of biochemistry and analytical biochemistry, only in the past 7 to 10 years has there been significant growth in the applications of calorimetery, largely as a result of new instrumentation capable of the high sensitivity required in dealing with the small quantities of reactants generally involved in biochemical systems. These recent developments have resulted in widespread interest in the capabilities of various calorimetric methods in the study of problems as diverse as enzyme substrate determination, the detailed structural information in lipid-bilayer thermal transitions, and the study of the behavior of living cells. Analytical calorimetry is a dynamic, growing field in biochemistry and is providing a basis for practical application to clinical, as well as to fundamental, research problems.

Calorimetry is a general term referring to the measurement of the quantities of heat evolved or absorbed in physical or chemical processes. Since most chemical processes do occur with measurable heat effects, calorimetry can be used to study a wide variety of chemical systems. The pervasiveness of thermal effects associated with chemical reactions and physical processes can be a disadvantage in an analytical sense because of the frequent lack of specificity of reactions that could be of analytical interest. However, by judicious control of reaction conditions and careful choice of reactants, specificity can be built into the analytical reaction. For example, enzymes, being catalysts for well-defined reactions, offer an inherent specificity to substrate under proper solution conditions. The binding of protons to specific basic sites in proteins can be studied by calorimetric methods, yielding information about the number of sites of each particular type. While specificity is usually analytically desirable, there are cases in which a general analytical probe can be very useful. For example, in the study of living cells it frequently is desirable to monitor the activity of cells under particular conditions in the environment of cells. Calorimetric methods can provide this direct probe of activity in media which would be difficult to examine by other procedures, e.g., in opaque media or in liquid dispersions.

For the purposes of this review, I have chosen to examine three major areas in which calorimetry is being used significantly in biochemical and biological investigations. These areas include (1) the analytical uses of enzymes and enzyme-substrate reactions, (2) the uses of scanning calorimetry to study the thermal behavior of biopolymers and lipids, and (3) the direct calorimetric investigation of in vivo cells, particularly the blood cells. While this choice of topics is somewhat arbitrary, there are some reasons for examining in some detail these particular general areas. First, this combination of topics illustrates the breadth of analytical applications of calorimetry in biological chemistry. Second, all of these areas have implications beyond fundamental biochemical interest. For example, all have basic consequences to the clinical and medical fields, either through direct application in clinical analysis or indirectly by providing tools of medical investigation. Another reason for considering these three particular areas is that they represent relatively new applications of calorimetry to biochemistry and biology. Reaction calorimetry and thermometric titration techniques have been more generally employed in biochemical problems than scanning calorimetry or methods using enzymes, but the more general methods have been reviewed relatively frequently.[1,4,7-11] It was felt that enzyme methods, scanning calorimetry, and studies of in vivo cells are areas of interest which provide some new and interesting applications in biochemical analysis.

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TABLE I

Analytical Calorimetry

Operational classification	Methodological classification	Ref.
Batch reaction	Batch calorimetry	1
	Direct-injection enthalpimetry (DIE)	2
	Thermokinetic analysis (TKA)	2
	Biological calorimetry	1, 5
Flow reaction	Flow calorimetry	1
	Flow enthalpimetry	3
	Peak enthalpimetry	4
	Biological calorimetry	6, 1
Titration reaction	Titration calorimetry	1
	Thermometric enthalpy titration (TET)	7—11
	Kinetic titrimetry	2
Temperature scanning	Differential scanning calorimetry (DSC)	1, 12
	Differential thermal analysis (DTA)	12

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This review will consider the three major areas with emphasis on the details of procedures, features of instrumentation, and performance characteristics of the calorimetric methods. Because a recent review[1] has provided a basic background to calorimetry as an analytical tool, attempts will be made to update and expand as much as possible on the material in that review. We will begin with a brief discussion of some new instrumentation which has recently been described, and follow that with the applications mentioned above. A summary section will include comments on other potentially interesting and useful areas of examination by calorimetric methods.

II. INSTRUMENTATION

A. Methodological Classifications

Calorimetric methods of analysis have frequently been classified according to the operational basis of the calorimetric experiment. For example, reaction calorimetry can be classified as batch (or direct injection) calorimetry or flow calorimetry, depending on the method used to introduce reactants into the calorimetric cell. In the batch experiment normally, the two reacting solutions are mixed together in one "shot," as for example, with a syringe injection of reactant into the cell containing the other component. In a flow experiment, the reaction mixture or reacting solutions are pumped through the calorimeter cell at a controlled rate, with the reaction occurring in a well-defined region of space within the cell. On the other hand in titration calorimetry, a titrant of known composition is pumped into the calorimetric reaction cell containing the titrand. In this case, the thermal response is used to determine the equivalence point in the titration reaction. These reaction calorimetry methods have been called by other names by different authors, and these are summarized in Table 1. Included in the table are methodological approaches for reaction conditions which are not under equilibrium control, but kinetic control. These methods can be used not only for analytical purposes, but also for extraction of kinetic information. Also indicated in Table 1 are the temperature-scanning methods in which the thermal behavior of solids or solutions is studied as a function of a carefully controlled temperature

change in the calorimeter cell. In this case, attention is focused on the heat effects generated as a consequence of specific transitions or reactions induced by temperature changes in the solid or solution. Finally, it should be mentioned that biological calorimetry, the study of living cells, can be performed with batch- or flow-type calorimeters, so these methodologies are included under the batch and flow classification. The living cells are placed in suitable ampules in the batch calorimeters or are flowed through a measuring cell of a flow-calorimeter to obtain the heat generated by the living cells.

In the material which follows, the operation classification of calorimetric methods will be used, since this approach seems more general. The majority of examples of reaction calorimetry use batch, flow or titration methods, and the temperature-scanning DSC method will be discussed in light of some of the new instruments capable of high sensitivity work on solutions.

In all of these methodological approaches, it will be useful to compare the types of instruments being used to obtain thermal and analytical information. So, let us first examine the basic design principles of calorimeters and what factors are involved in determining the sensitivity and other operational characteristics of the calorimeters being used.

B. Instrumentation Principles

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Instrumental design in calorimetry has centered around two basic principles of control of the heat flow in the calorimetric cell.[1] In an *adiabatic* cell, attempts are made to minimize any transfer of heat from the cell by appropriate shielding devices. With a *heat conduction* calorimeter, the cell is designed to transfer the heat to a heat sink (in a reproducibly controlled manner) usually through a thermopile heat sensor. Other calorimeter designs can be considered intermediate between these two. For example, in *isoperibolic* design, the calorimetric cell is placed in a constant temperature environment with the cell itself providing partial resistance to heat flow to the environment. The amount of actual heat loss from the cell is corrected for in the final calculations of the heat of the reaction process.[20] In many titration calorimetry experiments that have been reported, silvered Dewar flasks have been used as calorimeter cells. These flasks are close to adiabatic over a short time interval, but for longer reaction times, heat loss corrections must be used. Fortunately, for most titration experiments the reactions are fast, and the titrations complete within a few minutes.

For the study of biochemical or biological processes, an important factor in instrumental design is the sensitivity of the calorimeter. Because in many cases very small quantities (in the μM range) of substances, and thus, small quantities of heat, are involved in the reactions, it is necessary to scale the sensitivity to these microamounts. These experiments demand that microcalorimeters or equivalent devices are used. Let us examine, for the two basic calorimeter designs, what thermal sensitivities might be expected with available instrumentation.

For adiabatic calorimeters and, approximately, for Dewar-type vessels or isoperibolic designs, the heat effect is determined from the temperature change produced by reaction

$$Q = C \times \Delta T \qquad (1)$$

where Q is the heat liberated in the reaction, ΔT is the temperature change under adiabatic conditions, and C is an effective heat capacity of the system, which is largely determined by the quantities of reacting solutions. Thus, in adiabatic design, the sensitivity of the temperature measuring device and the effective heat capacity determine the minimum detectable heat effect. In a number of adiabatic and related calorimeter designs, thermistors are used as temperature sensors, and the temperature changes are

determined from the output of a Wheatstone-bridge circuit. A simple DC bridge of this sort can detect 10^{-3}°C relatively easily. With an effective heat capacity of 100 J/°gr, corresponding to about 25 mℓ solution volume, the minimum detectable heat would be about 100 mJ, which is typical of many titration calorimeters.[9,10] With smaller cell volumes, this detection limit can be decreased, e.g., with a 5 mℓ volume the limit is about 20 mJ.[19] Several authors[13,14] have reported AC Wheatstone bridge circuits with substantially increased temperature sensitivity of the order of 5 to 25 μ°C. With a cell volume of 15 mℓ and temperature sensitivity of 10μ°C, heat effects in the range of 1.0 mJ can be detected with batch calorimetric experiments. In an analytical sense, this level of thermal sensitivity must be translated into compositional information. The measured heat, Q, relates directly to the number of moles (n) of substance undergoing reaction and the enthalpy change (ΔHr) in the reaction

$$Q = n \times \Delta Hr \qquad (2)$$

With 1 mJ thermal sensitivity and an enthalpy change of 20 kJ/mol, it should be possible to detect 5×10^{-8} mol of reactant under proper conditions. This figure is for optimum conditions and is probably too low, but certainly this level of sensitivity is adequate for a number of applications in biochemical systems which will be discussed below.

The sensitivity characteristics of heat conduction calorimeters must be developed differently because of the nature of the thermal sensors normally used.[1,15] The thermopile sensor acts as a heat conducting link between the calorimeter cell and the heat sink. The thermopile produces a voltage, V, in proportion to the temperature gradient (ΔT) between the cell and the heat sink,

$$V = n \times e \times \Delta T \qquad (3)$$

where n is the number of junctions in the thermopile, and e is the thermoelectric constant for the average thermocouple in the pile. The temperature gradient in the cell relates to the rate of heat production from reaction in the cell, dQ/dt.

$$dQ/dt = n \times k \times \Delta T \qquad (4)$$

The constant, k, is the thermal conductivity of the average thermocouple in the pile. Using a heat conduction cell as in a batch calorimeter, the heat effect is obtained by integration of the voltage-time curve produced after reactants are mixed.

$$Q = k/e \int V dt \qquad (5)$$

From Equation 5, it is apparent that sensitivity is greatest with a large thermoelectric constant and small thermal conductivity for the thermocouples constituting the thermopile. (This combination will result in the largest area for a given heat input.) Note that the sensitivity for heat conduction through the thermopile is independent of the number of thermocouples, although if it were possible to increase n while keeping the total thermal conductivity constant, it would be possible to increase the sensitivity with increased n.*

* The sensitivity can be defined as the voltage produced per unit of heat flow, viz.,
Sensitivity = $V/(dQ/dt) = ne\Delta T/nk\Delta T = e/k$.
If the product, nk, could be made to remain constant, then as n increases, increased voltage would result as n × e increased.

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TABLE 2

Comparison of Characteristics of Several Reaction Calorimeter Designs

Calorimeter type	Operation	Heat level[a] (mJ)	Precision (% rsd)	Solution[b] volume (ml)	Ref.
Dewar-type	Titration	85	8	100	14
		830	0.4	100	14
Dewar-type	Titration	500	0.6	4	19
Isothermal	Titration	10	0.5	100	21
		50	0.2	100	21
Adiabatic	Flow	(10 μW)	0.9	2	22
Heat conduction	Flow	(40 μW)	1	2	17
		(400 μW)	0.1	2	17
Heat conduction	Batch	4	0.8	5	16
		300	0.5	5	16
Isoperibol	Batch	1000	0.05	100	1

[a] Typical experimental heat effect measured.
[b] Volume of calorimeter cell.

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The calorimeters developed by Wadsö,[16,17] Calvet,[18] and Prosen[15] are heat conduction calorimeters with thermopiles of the type discussed above. With electronic and digital integrators, these calorimeters can detect heat quantities of the order of 0.1 to 0.5 mJ, again in a range of thermal sensitivity that would make it possible to determine less than a μmol of reactant, provided the enthalpy change is in the 10 to 20 kJ/mol range. While these basic detection limits give an idea of the lower levels of detectability, it is perhaps best to examine calorimetric performance from the point of view of operational precision for specific levels of heat effects and for specific calorimeter designs. Table 2 gives such a comparison for several typical calorimeters. This comparison is not meant to be exhaustive, but rather to give some of the typical performance characteristics of the common types of calorimetric designs.

As the table indicates, Dewar-type titration calorimeters are used for larger volumes of solution and generally larger heat effects. The experiments can be completed in a few minutes, a definite advantage of this type of experiment. The isothermal titration calorimeter developed by Christensen, et al.[21] operates through a system of feedback loops and a Peltier cooling device to maintain the reaction solution at constant temperature. When heat is liberated in a reaction, the change in energy input to maintain constant temperature in the calorimeter is measured, which is proportional to the heat effect. This calorimeter is capable of higher precision and sensitivity than typical Dewar-titration calorimeters. Flow calorimeters measure heat per unit time, so are essentially wattmeters. Smaller volumes of solution are required, and experimental execution time is short. The batch heat-conduction calorimeters, developed by Wadsö[16] are very sensitive and require small volumes, but somewhat longer times are required to prepare and execute the calorimetric experiment. Good isoperibolic calorimeters usually require larger volumes and execution times, but are capable of the highest precision, values between 0.01 to 0.05% relative standard deviation being possible. This brief overview is meant to provide a simple comparison of some of the common types of reaction calorimeters. Let us now examine some new flow calorimeters that have been reported and also review some features of new temperature-scanning calorimeters.

C. Some New Calorimeters

Since the design and performance of a number of calorimeters that can be used in biochemical and biological work have been reviewed recently,[1] this section will present only several new instruments which have been developed for analytical purposes in the past several years.

1. Flow Calorimeters for Use with Immobilized Enzymes

As will be discussed below in the applications sections, the use of enzymes in calorimetric methods is very significant because specificity can be built into the analytical reactions through the generally rather selective ability of enzymes to react with specific substrates. In an analytical sense, the immobilized-enzyme reactors provide the additional advantage that the reactors can be reused for many analyses without changing the reactor columns.[25,26] Recently, there have been several groups applying immobilized-enzyme reactors combined with flow calorimetry to develop methods for the determination of enzyme substrates. This section will describe the instrumentation developed for those purposes.

Mosbach and co-workers have developed a series of devices they call "enzyme thermistors" for combining calorimetry and immobilized-enzyme reactors.[27,28] One of the first devices, shown in Figure 1, consists of a flow system containing a peristaltic pump which flows buffer solution (at 1 m*l*/min) continuously through an equilibration coil into a glass microcolumn containing about 1 m*l* of the immobilized-enzyme preparation. A thermistor probe is positioned approximately at the center of the microcolumn, and the column and probe are fixed in a perspex cylinder to provide mechanical support. A small air gap is left between the column and cylinder to provide a thermal barrier near the heat sensor where reaction occurs. The entire assembly is then submerged in a constant-temperature bath. For measurement of the temperature changes induced by enzyme-substrate reaction, the thermistor probe is connected to a high-precision bridge, the output of which is connected to a potentiometric recorder. On the most sensitive range of the bridge, 100 mV output corresponds to a temperature change of about 0.023°C. In an experiment, between 1.0 to 1.5 m*l* of a substrate solution is introduced into the flow line by a three-way-tap arrangement. A thermal response curve is obtained and completed within 6 to 12 min under these conditions. Figure 2 shows thermal response curves for differing concentrations of urea using an immobilized-urease column.[29] For most of the studies reported by Mosbach, the enzymes are immobilized on alkylamine glass beads after activation with glutaraldehyde in phosphate buffer.[25]

Because the microcolumn in the reactor is not truly adiabatic, heat losses, and thus thermal sensitivity losses, occur with this arrangement of the reactor column. To test the effect of these heat losses on linearity, some electrical calibration experiments were performed.[27] By simulating the temperature changes that occur in the reaction cell due to enzyme-substrate reactions by electrical heating with a heater wire wrapped around the column, it was shown that the temperature changes are linearly related to the power input from the heater. This result suggests that there is no change in the thermal efficiency as the higher temperatures are obtained. In addition, the absolute efficiency of the reaction cell was determined by comparing with experimental data, the expected temperature change for the reaction of immobilized trypsin with benzoyl-L-arginine ethylester, calculated from known enthalpy data for the reaction. This experiment indicated that about 50% of the theoretical temperature change was obtained under typical reaction conditions.

A modification of the basic enzyme thermistor is shown in Figure 3.[30] In this design, an additional reference thermistor is included in the flow stream prior to the immobilized-enzyme microcolumn, and the measuring thermistor is positioned at the end of

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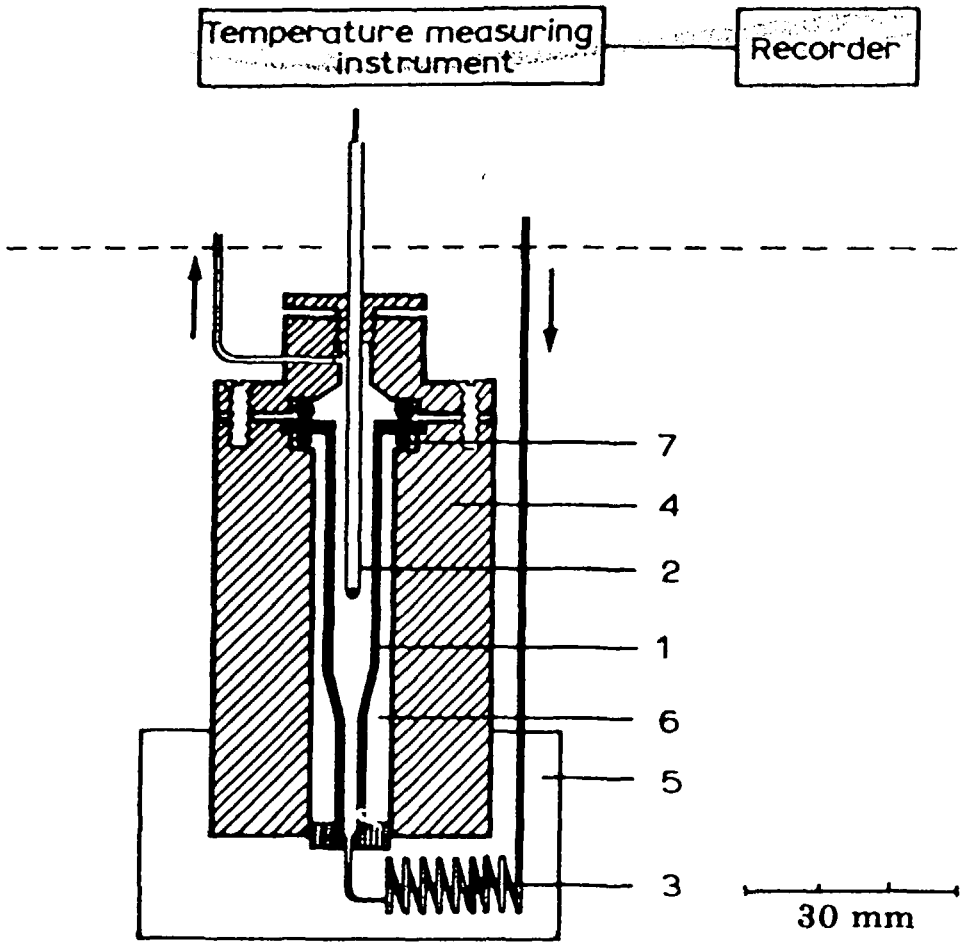

FIGURE 1. Enzyme thermistor flow calorimeter. The substrate solution is pumped through the heat exchanger (3) into the microcolumn (1) containing the immobilized-enzyme preparation. The thermistor (2) senses the temperature changes in the microcolumn. (Reprinted from Mosbach, K., Danielsson, B., Borgerud, A., and Scott, M., *Biochim. Biophys. Acta*, 403, 256 (1975). With permission.)

the column. With this differential measuring arrangement, better baseline stability is obtained, although the basic temperature sensitivity is not increased. A problem associated with the analysis of real samples using the enzyme thermistor is that when complex media, such as milk or urine, are injected on the columns, some nonspecific heat effects can be observed, particularly at low concentrations of substrate. Other sources of nonspecific heat effects arise when the pH, ionic strength, or viscosity of the sample medium is very different from the buffer of the flow system. While in some cases it is possible to minimize these effects by dilution of the sample substrate with flow buffer, this procedure is not always practical when substrate levels are low. The possibility of eliminating some of these nonspecific effects by using a reference column has been investigated by Mosbach and co-workers.[31] Figure 4 shows a diagram of the experimental arrangement of their split-flow enzyme thermistor. The enzyme column is essentially the same as the differential system discussed above. In the split-flow reactor, a second reference column, containing either inert glass beads or beads with catalytically inactive protein, is added to the flow system with thermistors located at the inlet and outlet of the reference column. A second pump is fixed at the effluent of the

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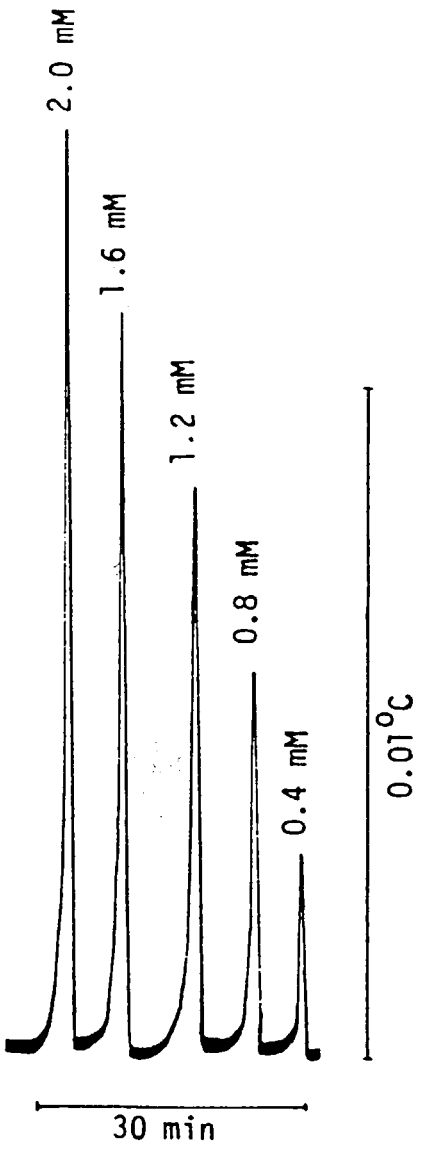

FIGURE 2. Temperature response from 1 m*l* pulses of urea in phosphate buffer introduced into immobilized-urease column using enzyme thermistor flow calorimeter. (Reprinted from Danielsson, B., Gadd, K., Mattiasson, B., and Mosbach, K., *Anal. Lett.*, 9, 987 (1976). By courtesy of Marcel Dekker, Inc.)

reference column to provide control of flow through the reference side. This device was tested by using samples of glucose in media of differing pH, ionic strength, and viscosity from that of the flow buffer. The immobilized-enzyme column was glucose oxidase. Figure 5 shows thermal response curves for injection of glucose in a medium of pH 8.56 into a phosphate flow buffer at pH 7.00 using different methods of registering the thermal signal. With the split-flow arrangement, curve A is obtained, while curves B and C are registered for the differential and single thermistor methods, re-

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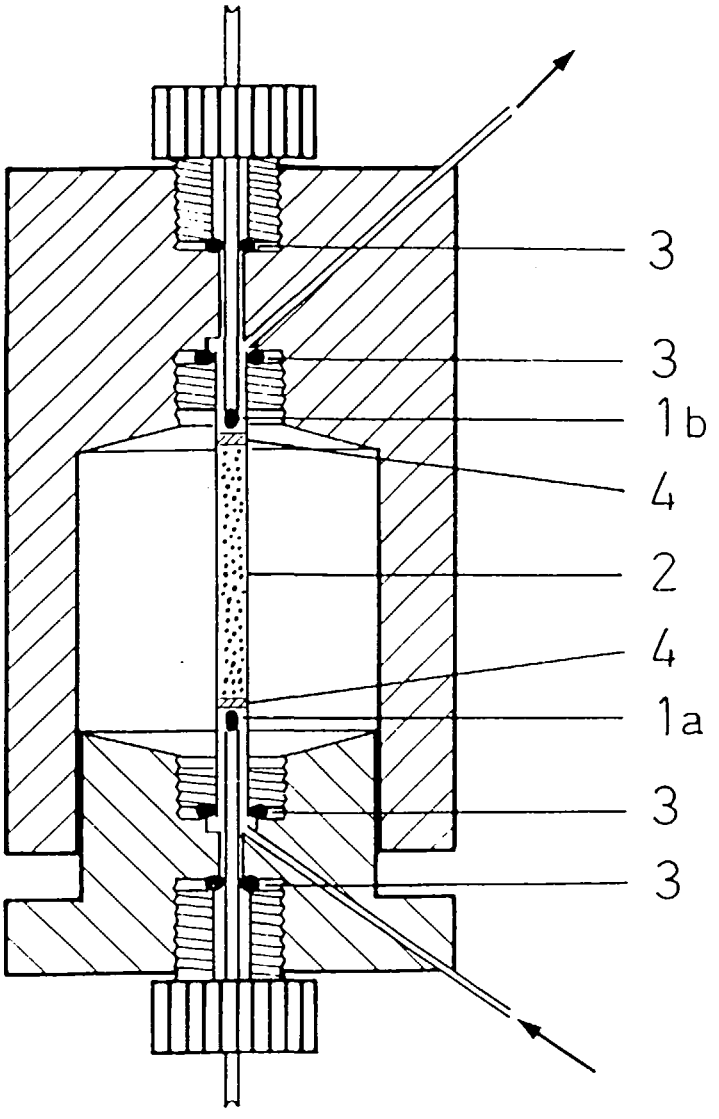

FIGURE 3. Differential enzyme thermistor unit. Reference thermistor, 1a. Sensing thermistor, 1b. Teflon column containing immobilized enzyme, 2. (Reprinted from Mattiasson, B., Danielsson, B., and Mosbach, K., *Anal. Lett.*, 9, 217 (1976). By courtesy of Marcel Dekker, Inc.)

spectively. Curves B and C show obvious enhanced heat effects as well as prepeaks that are associated with nonspecific heats resulting from the pH mismatch. It was also shown that the peak heights with the split-flow reactor changed very little from the height obtained when there was no pH mismatch. Thus, the split-flow design does seem to compensate for nonspecific heat effects due to differences between sample and the flow buffer. It was also demonstrated that nonspecific effects could be virtually eliminated from samples of urine and skim milk when testing for glucose in these biological samples.

A flow calorimeter for use with immobilized-enzyme columns has also been developed by Carr.[32,33] The design is similar in some respects to the differential enzyme thermistor discussed above. In this case, the enzyme column is surrounded by an evac-

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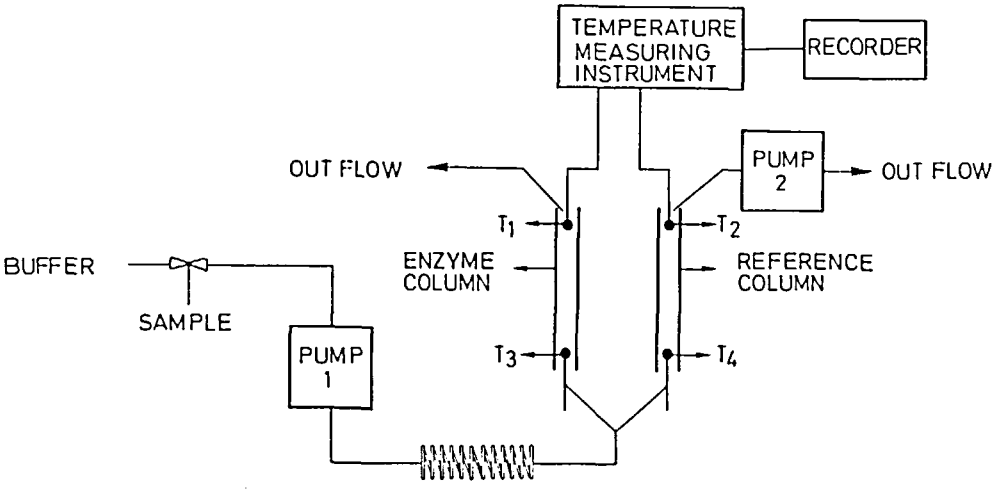

FIGURE 4. Experimental arrangement of split-flow enzyme thermistor. T_1 to T_4 are thermistors which can be used in different combinations. The pumps are peristaltic pumps. (Reprinted from Mattiasson, B., Danielsson, B., and Mosbach, K., *Anal. Lett.*, 9, 867 (1976). With permission.)

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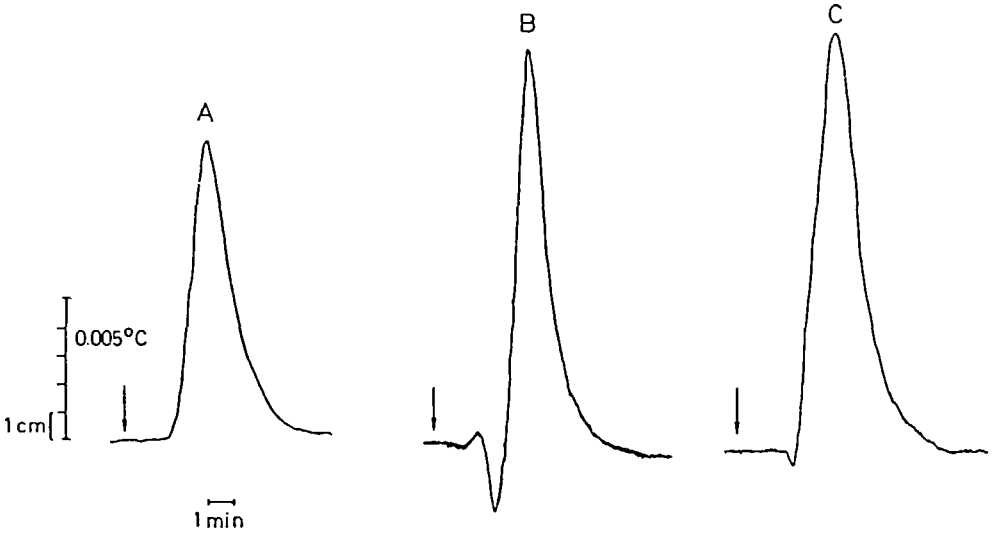

FIGURE 5. Experimental curves from the injection of 1 ml pulses of 0.5 mM glucose in phosphate buffer into glucose-oxidase immobilized-enzyme column. The thermal signals were obtained using A, the split-flow arrangement; B, the differential-thermistor arrangement; and C, the single-thermistor arrangement. (Reprinted from Mattiasson, B., Danielsson, B., and Mosbach, K., *Anal. Lett.*, 9, 867 (1976). With permission.)

uated space to cut down on thermal losses to the surroundings. The injection valve is a loop injector which delivers 120 μl of sanple solution when switched.

Temperature changes are measured by a sensitive, differential AC phase-lock bridge developed by the authors.[14] It appears, on the basis of comparison of data of Mosbach for temperature changes observed in the urea-urease system, that the sensitivity of the Carr system is somewhat higher, probably a result of the high-sensitivity temperature bridge and the better adiabaticity in the vicinity of the column. (See discussion below on the determination of urea.)

A study of the basic operational principles of immobilized-enzyme flow calorimeters has been published recently.[3] Various models for the dissipation of the thermal effect generated on the enzyme reactor are presented. Using basic ideas from chromatography theory, it was possible to find relationships for the dependence of the thermal-peak parameters (height, width, and area) on operational variables, such as sample concentration and volume, system flow rate, and enzyme-column size. The urea-urease enzyme-substrate reaction was studied to obtain experimental data on the effects of operational variables. Optimization of the operational variables indicated that, with 0.5 mℓ enzyme columns and a flow rate of 2 mℓ/min, approximately 60 samples/hr could be analyzed with sample volumes of 120 $\mu\ell$.

2. Temperature-Scanning Calorimeters

Temperature-scanning calorimeters are designed to allow a carefully controlled temperature scan to be imposed on a sample and a reference solid or solution. Through monitoring the temperature difference between the sample and reference and appropriate calibration procedures, a continuous record of heat capacity vs. temperature can be obtained. In the event that a thermally induced transition or reaction occurs in the sample, an increase or decrease in the heat-capacity function is noted, and the area of the transition peak is directly related to the enthalpy of the transition.

Instruments for these studies have traditionally been designed for the investigation of solids and solid-state reactions, which generally involve rather high temperature scans. Recently, however, there has been interest in using differential scanning calorimetry (DSC) for examining the properties of solutions, particularly aqueous solutions of biopolymers and lipidic materials. The study of aqueous solutions in the temperature range of 0 to 100°C and on rather dilute solutions of biopolymers requires high sensitivity and stability in the calorimeter design. In this section, we will briefly examine some new instruments specifically designed for studies on dilute aqueous solutions.

Two basic design principles have been applied to instruments for DSC. The difference in the two designs is a result of how the measured temperature difference between sample and reference containers is used to obtain the heat-capacity function vs. temperature. In the thermal null design,[1,24,49,113] electronic circuitry is used to supply power to the sample heater or to a separate reference heater in order to keep the temperatures of the two cells equal. Through this negative feedback control, the power input can be related to the thermal energy input to the sample at a particular temperature, and thus, is correlated with a heat capacity function. Calibration is obtained through scans of reference materials. The other basic design for DSC simply measures directly the temperature difference between sample and reference cell. Sample heat capacity is obtained from the temperature difference by using equations describing the thermal properties of the cells and associated measuring thermopiles. The basic equations describing the thermal properties of heat conduction cells are given in Calvet and Prat.[18] It should be pointed out that there are many instruments available, particularly for higher temperature scans, for which no attempt is made to obtain the heat-capacity function. These instruments simply record the temperature difference between sample and reference vs. the scanning temperature. They are usually called differential thermal analyzers (DTA). In order to obtain a true heat-capacity function, appropriate heat transfer calculations and calibrations are necessary for the definition of the function.

The design characteristics of several scanning calorimeters have been summarized in a recent review.[1] For our purposes here, let us examine the basic performance characteristics of three basic DSC instruments which use different principles for obtaining the heat-capacity data. The first, a commercially available unit from Perkin-Elmer Corporation, Norwalk, Conn., uses a thermal-nulling design with sample and refer-

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TABLE 3

Comparison of Some Temperature-Scanning Calorimeters

Specification	DSC-2[a]	Privalov[b]	Suurkuusk, Mountcastle, Biltonen[c]
Temperature range (°C)	−70—725°	0—100°	−20—125°
Temperature precision (°C)	±0.2	±0.1°	±0.05°
Temperature accuracy (°C)	±1°	±0.5°	±0.5°
Scanning rates (°C/hr)	19—4800	6—120	2—50
Heat capacity sensitivity (μcal/°C)	100	4	25
Calorimetric precision (%rsd)	1—3%	0.3%	0.5%
Sample cell volume (mℓ)	0.2	1.0	0.5

[a] Perkin-Elmer DSC-2, see References 1 and 113.
[b] See Reference 24.
[c] See References 53 and 54.

ence cups mounted in a constant-temperature environment.[1,113] Separate heaters and temperature sensors are associated with each cup, and the heaters are programmed to increase at a controlled rate. Heat capacity differences or a thermally induced transition cause temperature imbalances between sample and reference which are compensated for through feedback to the sample heater. This instrument was designed for a wider temperature range, and most of the reported applications are for solid samples. Its sensitivity suggests that it could be used for dilute solutions, if suitable sealed cells are employed. Table 3 summarizes some of the performance characteristics of the DSC-2.

A sensitive adiabatic scanning calorimeter has been reported by Privalov,[1,24] and its characteristics are also shown in Table 3. This instrument has a temperature sensor connected between an adiabatic shield and the reference cell which is used to control the environment of the cells and to keep the reference cell at the scanning temperature. The sample and reference cells have separate sensors and heaters, and a thermal-nulling technique is used to keep the two at equal temperature. Highly sensitive and precise measurements of heat capacities have been reported for 1 mℓ samples containing 1 to 2 mg/mℓ of protein. Other instruments similar to this basic design have been used by Sturtevant[49] and by Jackson and Brandts.[40]

The heat conduction DSC instrument of Suurkuusk et al., whose characteristics are shown in Table 3, has been described recently.[53] A more detailed account is forthcoming,[54] and the instrument will be commercially available through Tronac, Inc., Orem, Utah. The calorimeter consists of two rectangular cells, each sandwiched between a pair of thermoelectric modules. The thermoelectric modules are Peltier-effect devices which are equivalent to multijunction thermocouples. A large copper heat sink is in contact with the modules, and the heat sink is programmed to scan temperature during an experiment. The differential voltage from the sample and reference thermopiles is amplified and converted to digital form for computer processing of the ΔT data. The commercial unit will contain a microprocesser which will do corrections for the temperature dependence of the calorimetric constants and the temperature gradients in the measuring unit so that the true heat-capacity data as a function of temperature are presented. This calorimeter has been used to study gel-to-liquid crystal transitions in lipid bilayers, the solution concentrations being of the order of a few tenths of a percent lipid (see below).

Comparing the three types of scanning calorimeters shows that highly sensitive and precise heat-capacity data are possible, particularly with the Privalov and Suurkuusk

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designs. In making scanning-calorimeter measurements, baseline stability and reproducibility are fundamentally important to the operation. The ultimate sensitivity and calorimetric precision are largely determined by how carefully heat flow in the vicinity of the cells is controlled. Baseline reproducibility, then, is dependent on heat-flow parameters which must be known and enumerated. The DSC-2, which was designed for a larger temperature range, sacrifices some sensitivity and precision because of the difficulties in optimizing baseline stability under high-sensitivity conditions. Although not much work has been reported on solutions using the DSC-2, very reasonable data should be realizable for more concentrated solutions of biopolymers or related materials.

III. APPLICATIONS

A. Analytical Calorimetry Using Enzymes

1. Determination of Enzyme Activity

Enzyme activity is usually determined by spectrophotometric or potentiometric methods. In these approaches, the concentration of a particular substrate or product compound is monitored as a function of time, and the activity is evaluated as the number of μmol/min of substrate consumed or product produced by the reaction under zero-order-reaction conditions. (One unit of activity is defined as the amount of enzyme producing a conversion rate of 1 μmol/min.) For example, in the assay of lipase activity, tributyrin is hydrolyzed by the enzyme to butyric acid, whose concentration can be followed by the change in pH produced by the dissociation of butyric acid.[34] Many of these methods can be automated using pH-stats or Autoanalyzer® procedures.

There are some assays which are difficult to do using available techniques, requiring elaborate preparative steps to remove interfering turbidity or other optical interferences. Because calorimetric methods are not sensitive to the presence of suspended or dispersed phases in biological samples, the possibility that assay methods could be developed using the heat effects generated by enzymatic reactions with specific substrates seemed worth investigating. A number of such calorimetric assays have been studied.

One of the earliest systematic studies reported on enzyme assay by calorimetric methods is a flow method developed by Monk and Wadsö.[35] Using a flow-heat-conduction calorimeter,[1] the heat effects produced by appropriate substrates reacting with glucose oxidase, cholinesterase, alkaline phosphatase, and lactic acid dehydrogenase were examined. With the heat-conduction calorimeter, the thermal response recorded is related to reaction heat per second (dQ/dt), so the magnitude of the steady-state signal produced upon pumping the reaction mixture through the calorimeter relates directly to the number of moles reacting per second, and thus, to enzyme activity. In order to obtain absolute activity, calibration with standard samples is required in this technique. In this study, it was shown that sensitivity can be increased by using coupled reactions. For example, the determination of glucose oxidase activity can be done directly using a 1% glucose solution in phosphate buffer at pH 6.8. However, by adding *o*-dianisidine and peroxidase to the reaction mixture, resulting in oxidation of *o*-dianisidine by the peroxide produced from the glucose-oxidase reaction, the thermal response is more than doubled because of the combined heat effects of the two reactions. These results clearly show that careful choice of reaction conditions can lead to marked improvements in calorimetric response, and that knowledge of the basic thermodynamics of biochemical reactions can have useful consequences in an analytical sense.

Another approach to enzyme assay has recently been reported by McGlothin and Jordan.[36] Using a batch (direct injection) technique with a smaller Dewar cell, the

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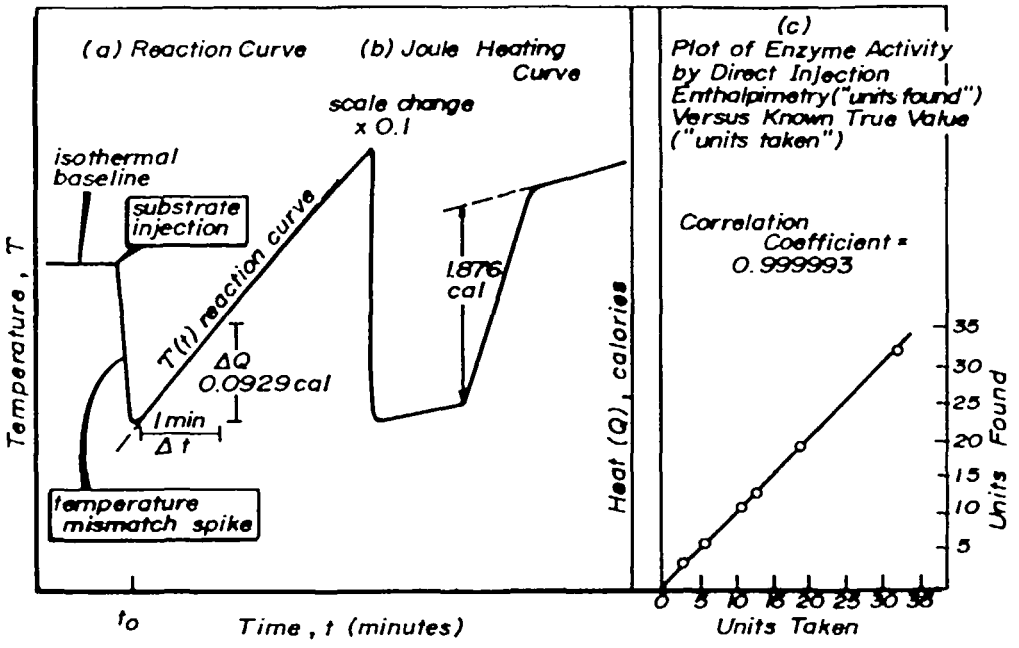

FIGURE 6. Enzyme activity determination of hexokinase using direct injection technique. (Reprinted from McGlothin, C. D. and Jordan, J., *Anal. Chem.*, 47, 1479 (1975). With permission. Copyright by the American Chemical Society.)

activity of hexokinase was determined using the phosphorylation of glucose reaction. In a typical experiment, 7 mℓ of an aqueous solution of ATP and $MgCl_2$ in Tris buffer at pH 8 are mixed with 100 $\mu\ell$ of the enzyme solution whose activity is to be measured. After temperature equilibration, a 1 mℓ aliquot of 20% aqueous glucose is added to the cell, and the time-temperature curve recorded for at least 100 sec. Figure 6 shows the results of a typical run. After an initial baseline displacement as a result of slight temperature mismatch between the cell and the glucose solution, a reaction curve results, which is essentially a linear temperature increase due to the zero-order enzymatic reaction. The slope, $\Delta Q/\Delta t$, of the linear portion of the reaction curve can be directly related to enzyme activity from knowledge of ΔH for the reaction:

$$\text{Enzyme activity } (\mu\text{mol/min}) = (\Delta Q/\Delta t) \times (1/\Delta H) \qquad (6)$$

The ΔH of reaction must be determined under conditions identical with the assay, and as shown in Figure 6, an electrical calibration must be executed in order to obtain the slope in caloric units. The curve in (c) of Figure 6 shows a correlation plot of the activity of hexokinase determined calorimetrically vs. the conventional spectrophotometric method, and as the figure shows, the correlation is excellent. The detection limit for hexokinase activity by this method is about 0.5 units, and the relative standard deviation for 3 to 5 replicate determinations is about 1%.

Another enzyme-assay method has been reported by Rehak et al., using the NBS-NIH batch microcalorimeter.[37] The enzymes studied were uricase and lactate dehydrogenase (LDH-H_4). In the batch experiment, 150 $\mu\ell$ of enzyme solution is mixed with 150 $\mu\ell$ of substrate solution, both in appropriate buffers, yielding thermograms similar to that shown in Figure 7. The data are acquired in digital form, so that the true thermogenesis curve can be calculated by an appropriate computer program from the experimental curve to obtain a concentration-time curve, also shown in Figure 7. The

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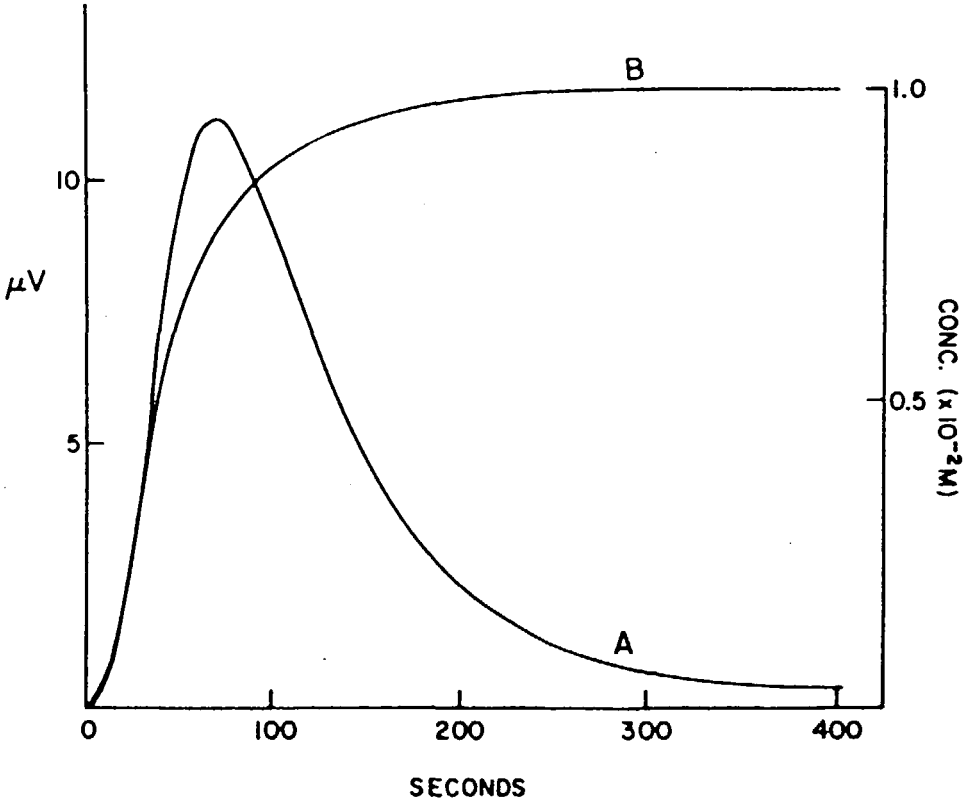

FIGURE 7. Experimental thermogram (A) and calculated concentration curve (B) for the determination of lactate dehydrogenase activity using a batch heat-conduction microcalorimeter. (Reprinted from Rehak, N. N., Everse, J., Kaplan, N. D., and Berger, R. L., *Anal. Biochem.*, 70, 381 (1976). With permission.)

initial slope of the concentration-time curve is directly related to the amount of substrate reacted per unit time, and thus, to the enzyme activity. Linear relations were observed between the enzyme concentration and turnover rate for ranges of enzyme concentration of 1 to 10 μM. Specific activity calculated from the data agreed within a few percent of the activities determined spectrophotometrically. As a part of this work, some experiments were carried out on the determination of the activity of enzymes bound directly to glass beads. The LDH and uricase were covalently linked to alkylamine glass beads using the glutaraldehyde procedure mentioned above,[25] the amounts of enzymes immobilized being 18 μg/mg of beads (uricase) and 72 μg/mg of beads (LDH). The beads were introduced into one compartment of the calorimeter cell just as is the case with the soluble enzymes. Again, linear curves were obtained relating the amount of enzyme to turnover rate, and the results for activity determinations showed good agreement with the spectrophotometric method. These experiments clearly demonstrate the applicability of calorimetry to activity measurements on enzymes which are particulate in nature, e.g., membrane-bound enzymes. The activity could thus be measured without solubilizing the enzyme, which can lead to undesirable changes in structure and activity. Because calorimetric measurements do not require optically clear solutions, direct examination of intact membranes or tissue homogenates becomes possible. (See the paper of Monk and Wadsö[35] for a flow-calorimetric experiment using a tissue homogenate with ATPase activity.)

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The determination of the activity of proteolytic enzymes by flow calorimetry was recently demonstrated by Konickova and Wadsö.[38] In studies of trypsin and pronase (a mixture of proteolytic enzymes), the hydrolysis reactions of *p*-tosyl-L-arginyl methyl ester·HCl (TAME) and *N*-benzoyl-L-arginine ethyl ester·HCl (BAEE) in Tris buffers were used to generate the calorimetric signal. In the experiments, solutions of enzyme and substrate were flowed into a mixing cell in the calorimeter, and the deflection of the thermal signal from baseline was related to enzyme concentration. Equilibrium was attained in about 8 min. It required 3 m*l* each of enzyme and substrate solutions. Linear calibration curves were obtained in the range of 0.5 to 5 μg/m*l* for trypsin and 1 to 10 μg/m*l* for pronase, with TAME as substrate. BAEE gave somewhat lower sensitivity than TAME. Some experiments with trypsin and pronase were also carried out using casein as substrate, with somewhat decreased sensitivity. The activity of pepsin with hemoglobin substrate was also investigated by the flow method, yielding linear calibration curves in the range of about 10 to 150 μg/m*l*. All of the above calorimetric methods were compared with conventional spectrophotometric assays. It was judged that the trypsin-TAME method gave comparable sensitivity to the conventional method, while in the other cases the calorimetric methods showed somewhat reduced sensitivity.

2. Determination of Substrates with Soluble Enzymes

In the classical Michaelis-Menten formulation of enzyme catalysis, the dependence of the rate of reaction on substrate concentration is zero order at high concentrations, but becomes first order when the substrate concentration is small relative to the Michaelis constant for the reaction. For enzyme-assay procedures as described above, it is convenient to operate in the zero-order region of excess substrate. The rate is then dependent only on total enzyme concentration. On the other hand, enzymatic reactions can be used for determination of substrate concentrations if the reaction is carried out under first-order conditions. This approach has been used in developing some of the classical enzyme-substrate methods, as well as in calorimetric techniques for substrate analysis.[58,59]

Perhaps the most widely studied substrate determined by combined calorimetric-enzymatic methods is glucose. One of the earliest reports uses a flow calorimetric method.[35] Substrate solutions of glucose are mixed with an enzyme solution containing glucose oxidase, peroxidase, *o*-dianisidine, and buffer outside the calorimeter. The reaction scheme involves oxidation of glucose to gluconic acid followed by the peroxidase catalyzed decomposition of H_2O_2 produced in the first step. The reaction mixture is pumped into a flow-through calorimeter cell, and the heat signal generated during the contact time in the cell is related to glucose concentration. This method is suitable for glucose concentrations in the 3 to 30 μg/m*l* range.

Batch calorimetric approaches for glucose determination have also been reported recently. Using the NBS batch, heat-conduction microcalorimeter[15] the hexokinase-catalyzed phosphorylation reaction of ATP with glucose in Tris buffer was studied both in the aqueous buffer and in human serum samples.[39] The reaction involves the conversion of glucose to glucose-6-phosphate by ATP in the presence of Mg^{++} ion, which yields ADP and a hydrogen ion. The hydrogen ion then protonates the basic component of the buffer, producing a rather strongly exothermic reaction. The thermodynamics of this reaction has been worked out under slightly differing conditions by McGlothlin and Jordan.[55] The reaction

$$\text{glucose} + \text{Mg(ATP)}^{-2} \xrightarrow{\text{hexokinase}} \text{glucose-6-phosphate}^{-2} + \text{Mg(ADP)}^{-} + \text{H}^{+} \qquad (7)$$

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has an enthalpy change of -27.6 kJ·mol^{-1} when the Mg^{+2} ion concentration is stoichiometrically equivalent to the ATP present. The protonation reaction in Tris,

$$\text{Buffer-NH}_2 + \text{H}^+ \longrightarrow \text{Buffer-NH}_3^+ \qquad (8)$$

was found to have an enthalpy change of -47.3 kJ·mol^{-1}. Thus, the total heat of reaction is made up of a substantial contribution from the protonation of buffer. Obviously, the choice of buffer system will have an important effect on the sensitivity of the calorimetric method in this case.

In the NBS study of this reaction for the determination of glucose,[39] a 1 mℓ aliquot of glucose-substrate sample was mixed with 0.1 mℓ of buffer containing ATP and magnesium chloride. Weighed amounts of the substrate-buffer mixture and a hexokinase solution in buffer were mixed in the colorimetric cell, and the area of the heat-conduction curve was evaluated relative to standard curves obtained from known glucose concentrations in the range of 0 to 1350 mg/ℓ. Linear calibration was obtained over this range of concentration. Glucose was also determined in 45 samples of serum from human patients in the range of 420 to 4000 mg/ℓ. These same samples were analyzed by the standard glucose-oxidase colorimetric procedure, and the results correlated very closely. The repeatability of measurements in serum was about 24 mg/ℓ in the range studied.

A calorimetric method for glucose also has been reported by McGlothlin and Jordan.[55,56] The method uses a direct-injection technique in which a 500 μℓ sample of glucose solution is injected into 7 mℓ of a phosphorylation mixture consisting of ATP, $MgCl_2 \cdot 6H_2O$, and Tris buffer at pH = 8. The reaction cell is a small Dewar similar to the one described above used for enzyme-activity determinations. A reaction curve like the one shown in Figure 6 is obtained, except that because the reaction is in the first-order region, the exothermic heat effect stops when glucose is completely consummed. The height of the exothermic deflection is proportional to the quantity of glucose in the sample. An electrical calibration step is performed after the reaction is complete, and the concentration of glucose determined from the known heat of reaction under the conditions of the experiment. Aqueous glucose standards gave a linear calibration curve in the range of about 50 to 1000 mg/100 mℓ with a precision of about 2% in that range. Glucose determinations in plasma, serum, and whole blood were also studied, and comparisons were made with other glucose methods. In serum and plasma samples, the calorimetric values for glucose were within ±2% of the values obtained by the glucose-oxidase colorimetric procedure. It is important to point out that in the colorimetric procedures, blood protein must be precipitated before the determination, but in the calorimetric method this step is not necessary. Glucose assay and recovery experiments were carried out on whole-blood samples. Again, the assays were within 2% of the glucose-oxidase procedure, and recoveries were 100 ± 1.5%. The colorimetric method seems to have several advantages over other procedures, including the observations that protein, sample color, particulate matter or viscosity effects do not seem to hamper the results. This method also would be fairly simple to automate.

Another microcalorimetric method using a combination of enzyme-catalyzed reactions is the determination of uric acid, reported by Rehak et al.[57] Uricase is an enzyme specific to the conversion of uric acid to allantoin, but the heat of reaction is quite small (about 20 kJ·mol^{-1}). However, the addition of catalase to the reaction mixture catalyzes the conversion of hydrogen peroxide, produced in the uric acid reaction, to oxygen and water. The latter reaction is strongly exothermic, thus providing the added thermal response needed for the calorimetric determination. The enthalpy change for the combined reactions is -149 kJ·mol^{-1}. Using an NBS-type heat-conduction micro-

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calorimeter,[15] uric acid solutions in either aqueous buffer or in human serum samples were mixed with a uricase reagent containing catalase and Tris buffer. The integrated heat effect for standards was linearly related to Autoanalyzer® results in the range of about 10 to 70 mg/l uric acid. Replicate analysis of uric acid in buffer gave a precision of about 3%. The results of recovery experiments from serum gave mean values of 102.2 ± 2.3%, and the comparison of serum results with the Autoanalyzer® procedure gave good linear correlation. A mixing artifact was observed in the serum samples causing a positive displacement of baseline after the uric acid reaction was complete. The displacement was attributed to a slow reaction between serum protein adsorbed on the calorimeter cell walls and other protein in solution. The effect could be eliminated by washing the cell with a solution of NaOH or a proteolytic enzyme.

In closing this section, it should be mentioned that enzyme inhibitors can be determined by calorimetric methods. Several of these studies have been summarized in a previous review.[1] Cholinesterase activity with acetyl choline substrate was measured using the LKB flow calorimeters, and the effect on activity was monitored as organophosphate pesticides were added. In the study of Dimefox® pesticide inhibition of cholinesterase, concentrations as low as 0.5 $\mu g \cdot ml^{-1}$ Dimefox® produced measurable diminution of the thermal signal for the reaction.[60] Beezer and Stubbs have reported a similar study of cholinesterase inhibition by tetraethylpyrophosphate (TEPP) and by parathion.[61] These two inhibitors are of different types, TEPP being a direct inhibitor, and parathion a latent variety. The latent inhibitors are activated in vivo to produce a product which is a direct inhibitor. In this case, parathion is converted to paraoxan which is a potent inhibitor to cholinesterase activity. Again, calorimetric response is diminished in proportion to the concentration of inhibitor in the reaction mixture. For parathion, the method could be used in the range of 5 to 100 $\mu g \cdot ml^{-1}$, and for TEPP, reduction in activity resulted from concentrations of 0.02 to 1 $\mu g \cdot ml^{-1}$ of inhibitor.

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3. Determination of Substrates Using Immobilized Enzymes

As was mentioned in the Instrumentation section (see Section II.C.1), there has been considerable activity in the application of immobilized-enzyme flow reactors coupled with calorimetric measurements to determine substrates specific to individual enzymes. There are several distinct advantages of this approach over the solubilized-enzyme methods. First, using a flow reactor speeds up the analytical process. The flowing buffer contains necessary reactants for initiation of reaction in the enzyme column, so that sample substrate can be injected into the flow stream directly. The thermal measurement occurs as the substrate enters the reactor column. Since no extensive thermal equilibration steps are required, samples can be injected in rapid succession. The enzyme reactor column can be used repeatedly after many injections, thus, the expense of fresh enzyme preparations for each analysis is cut down. Also, because the enzyme column is a component of the measurement system, the response can be calibrated for incomplete conversion or other chemical processes which might affect the extent of conversion. These factors make the immobilized-enzyme reactor an attractive alternative to solubilized enzyme methods in calorimetry.

Mosbach and Danielsson reported one of the first applications of immobilized-enzyme reactors in analytical calorimetry.[28] In this study, immobilized trypsin and apyrase were reacted with benzoyl-L-arginine ethyl ester (BAEE) and adenosine triphosphate (ATP), respectively. The calorimeter instrument was the first "enzyme thermistor" developed by Mosbach and coworkers (see above in the Instrumentation section). In the study of the BAEE-trypsin reaction, solutions of about 1 to 8 mM BAEE in buffer at pH = 8 were injected into the immobilized-trypsin flow reactor and the time-temperature curves recorded. Figure 8 shows the thermal-response curves obtained. Curve "a" shows the experimental curve for the injection of 1 ml of 6 mM

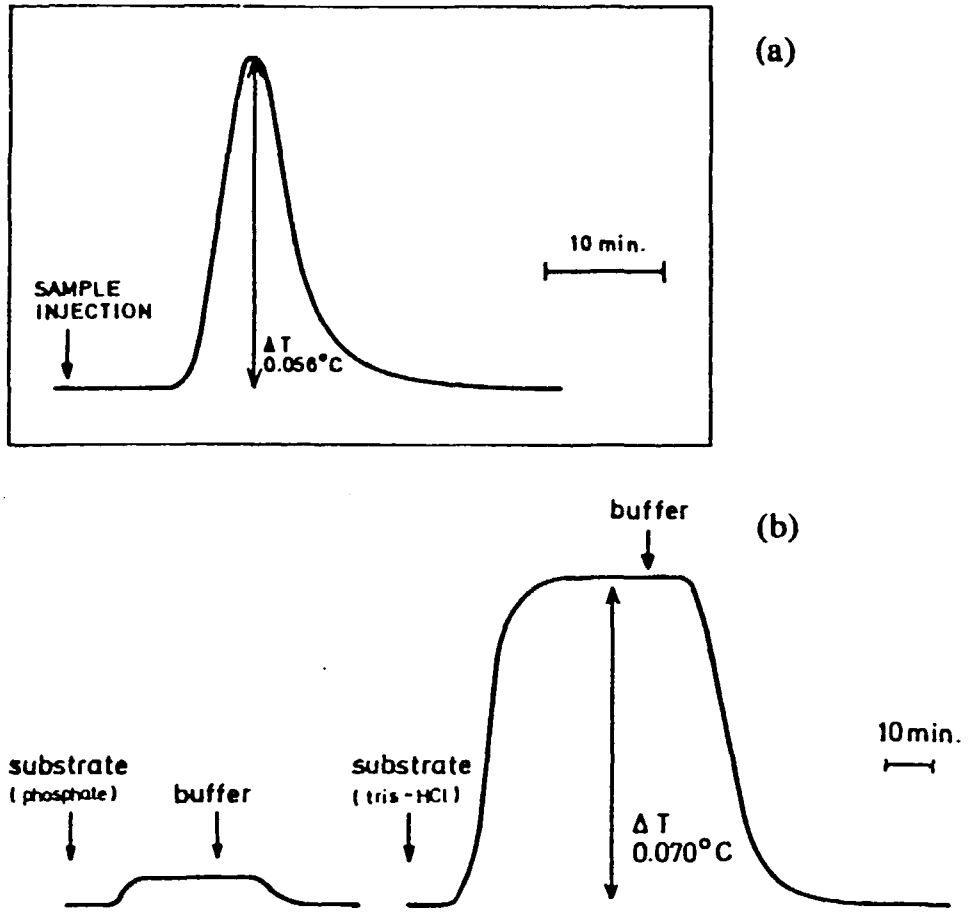

FIGURE 8. (a) Experimental heat effect obtained after injection of 1 m*l* pulse of 6 m*M* BAEE into immobilized-trypsin column. (b) Experimental curve obtained for continuously flowing substrate, 6 m*M* BAEE, in phosphate and Tris-HCl buffers. (Reprinted from Mosbach, K. and Danielsson, B., *Biochim. Biophys. Acta*, 364, 140 (1974). With permission.)

BAEE in Tris-HCl buffer into the flow reactor. Plots of either the area or peak height gave linear behavior over the range of 1 to 8 m*M* BAEE under these conditions. The curves in "b" of Figure 8 show the effect of changing buffer conditions. Since the hydrolysis of BAEE produces an acid product, a substantial part of the observed heat effect results from protonation of the buffer. The heat of protonation of phosphate is about 10 times less than that of Tris, so there is a large difference in the two heats of reaction produced in the enzyme reactor. For the study of ATP hydrolysis, the glass-bound apyrase enzyme contained 5′-ATPase, 5′-ADPase, and 5′-AMPase, so the hydrolysis reactions converted ATP to AMP. The flow buffer in this case was veronal-HCl at pH = 6.7. Again, linear calibration curves were obtained for 1 m*l* injections in the range of 1 to 8 m*M* ATP. No diminution of thermal response was found over a 10-day period of use of the enzyme columns.

Using a redesigned reactor column which placed the thermistor directly in the buffer flow stream, Mosbach et al.[27] studied the behavior of several other immobilized enzyme-substrate reactions. The glucose oxidase-glucose system, penicillinase-penicillin G, urease-urea, and again, the trypsin-BAEE enzyme-substrate pairs were investigated. With repositioning of the thermistor, several advantages are realized. The system is

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easier to handle, higher flow rates can be used, and because the thermistor is closer to the site of reaction, higher sensitivity results. In the penicillin G experiments, linear calibration curves (ΔT in °C vs. concentration) were obtained in the range of about 1 to 100 m*M* when 1.5 mℓ aliquots were injected into the flow buffer moving at 60 mℓ/hr. Calibration curves obtained on successive days were found to be identical, and the enzyme column could be stored for several weeks at room temperature with no observed diminution of thermal response. Similar results were obtained for urea with linear response in the range of 2.5 to 7 m*M*, the range normally found in human blood serum. For the glucose-glucose oxidase system, linear response was noted from about 0.06 to 1 m*M*, but there was a pronounced leveling off of the thermal response above 1 m*M*. The limiting effect was ascribed to either limited amounts of O_2 in the medium for complete reaction or a limiting glucose oxidase activity on the column.

Danielsson et al. have applied the immobilized-enzyme flow calorimeter to determination of urea in serum.[29] Using the same system as described above, calibration curves for known amounts of urea in aqueous buffer were obtained. Again, the linear range extended from about 0.1 m*M* to 200 m*M*, with the coefficient of variation about 1% at 0.1 m*M*. Because of the sensitivity of the system to urea, samples of serum could be diluted at least 10-fold. This dilution eliminates some possible heat effects due to nonspecific factors such as solvation or viscosity differences between sample matrix and the circulating buffer. A comparative study of the calorimetric method with an Autoanalyzer® method for urea using diacetyl monoxime showed excellent correlation of results. Within-day precision on the serum sample determinations was about 1.5%, and recovery experiments accounted for 98 to 100% of the urea. With serum samples, the enzyme columns were suitable for over 100 analyses, there being a tendency for clogging after prolonged use. About 12 to 15 samples per hour could be processed through the calorimetric method.

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Carr and coworkers have reported a similar study of the urea-urease system, both in aqueous buffer[32] and in serum.[33] Using their version of the immobilized enzyme flow reactor,[3] 100 $\mu\ell$ samples of urea in the 1 to 100 m*M* range of concentration were studied in aqueous buffer. The precision obtained was about 1 to 3% over the entire range, and at least 30 samples per hour could be analyzed. Figure 9 shows typical thermal-response curves obtained in the urea determinations. These particular samples contained bovine serum albumin and NaCl as well as buffer to simulate conditions in serum. The effect of sample volume on the thermal response was investigated. Larger sample volumes produced both an increased peak width as well as peak height. It was proposed that the changes in width and height with increased sample volume are due to heat-transport effects, the larger volumes heating a larger portion of the column. This increased dispersion of heat in the column would result in broader peaks, and ultimately, in lowered peak height when the entire column approaches an isothermal condition. In Figure 9, the blank injection produces a double peak with positive and negative deflections. This injection effect can be exaggerated by mismatch of electrolyte concentration or by pH mismatch between sample and flow buffer. It was found that the injection artifacts do not produce a significant error in measurement of peak heights because the double peak crosses the zero temperature axis quite near the maximum in a true sample peak. Stability of the enzyme column was tested by daily injections of a urea standard for a period of 18 days. It was found that an average decrease of about 0.4%/day occurred over this time period. Since the enzyme concentration was in large excess over substrate under the conditions of the study, it was assumed that the decrease in thermal response is a result of changes in column packing, and thus, the efficiency of mixing of sample with the enzyme packing.

Results of the urea determination in commercial quality-control sera showed excellent correlations with the urease-indophenol and diacetyl monoxime methods.[33] The

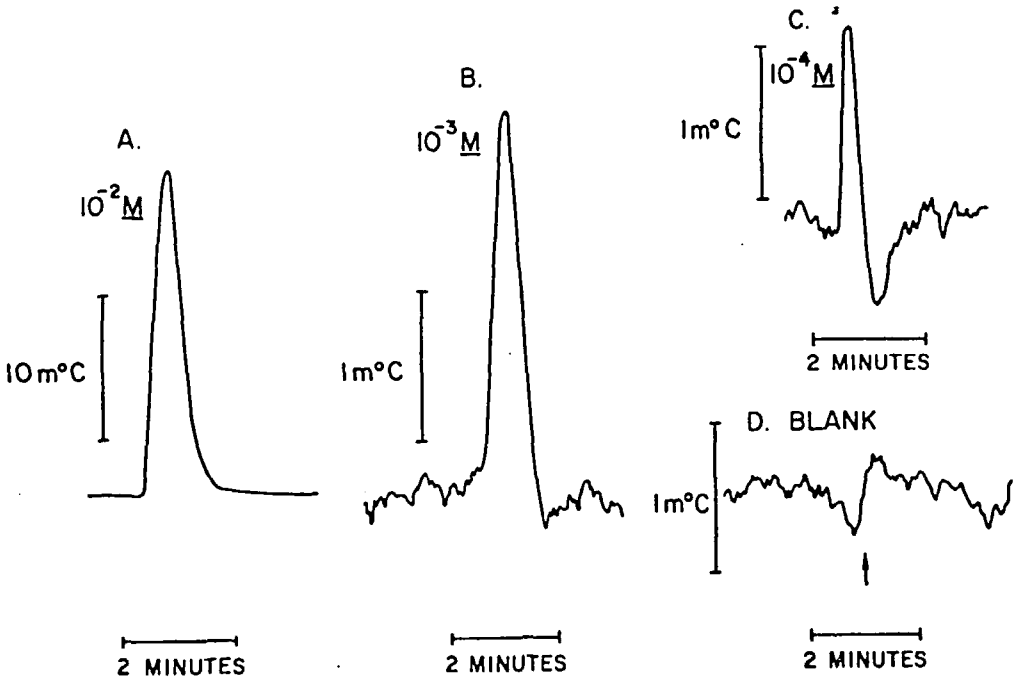

FIGURE 9. Thermochemical signal traces for the injection of 100 μl samples of urea onto an immobilized-urease column. Samples contained bovine serum albumin, NaCl,and EDTA in phosphate buffer. (Reprinted from Canning, L. M. and Carr, P. W., *Anal. Lett.*, 8, 359 (1975). By courtesy of Marcel Dekker, Inc.)

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within-day precision based on five sample determinations was about 0.5% relative standard deviation. Day-to-day repeatability was about 9% over an 18-day period. Recovery and interference studies were performed on serum samples which had been dialized against an NaCl-Tris buffer system at pH = 7.4. Recoveries of 99 to 104% were realized, and no significant interference resulted from addition of NH_4Cl, hemoglobin, or bilirubin to the serum samples.

A variation of the usual immobilization of enzymes on glass beads has been reported recently.[72] The procedure involves first immobilization of antihuman serum albumin on Sepharose® CL-4B. Next, samples of enzyme-albumin conjugates from human albumin (HSA) and enzyme are prepared by glutaraldehyde coupling. The HSA-enzyme conjugate is then added to the immobilized anti-HSA column, yielding an immobilized enzyme complex specific to the enzyme substrate. Using this antigen-antibody interaction, the enzyme is bound reversibly, and can be removed by washing with a glycine buffer at pH = 2.2. The antibody column can then be reloaded with a fresh enzyme preparation or a completely different enzyme-HSA conjugate. Figure 10 shows the sequence of an assay cycle which determines one substrate, removes its enzyme conjugate, replaces it with a new enzyme-HSA complex, and determines the new substrate. The interesting and potentially very useful aspect of this procedure is that the exchange of enzyme on the column can be accomplished in as little as 10 min, provided the same buffer can be used. Thus, a single flow-calorimeter system could be used for a number of different assay procedures. The immobilized antibodies are stable for up to a month at 27°C with continuous buffer or sample flow.

In this study using the antibody-antigen immobilization, hydrogen peroxide, penicillin G, phenol, tyrosine, sucrose, and glucose were determined using their appropriate enzyme conjugates on the column. The sensitivities and linear working ranges were similar to glass-bound preparations, except in the case of phenol and sucrose where

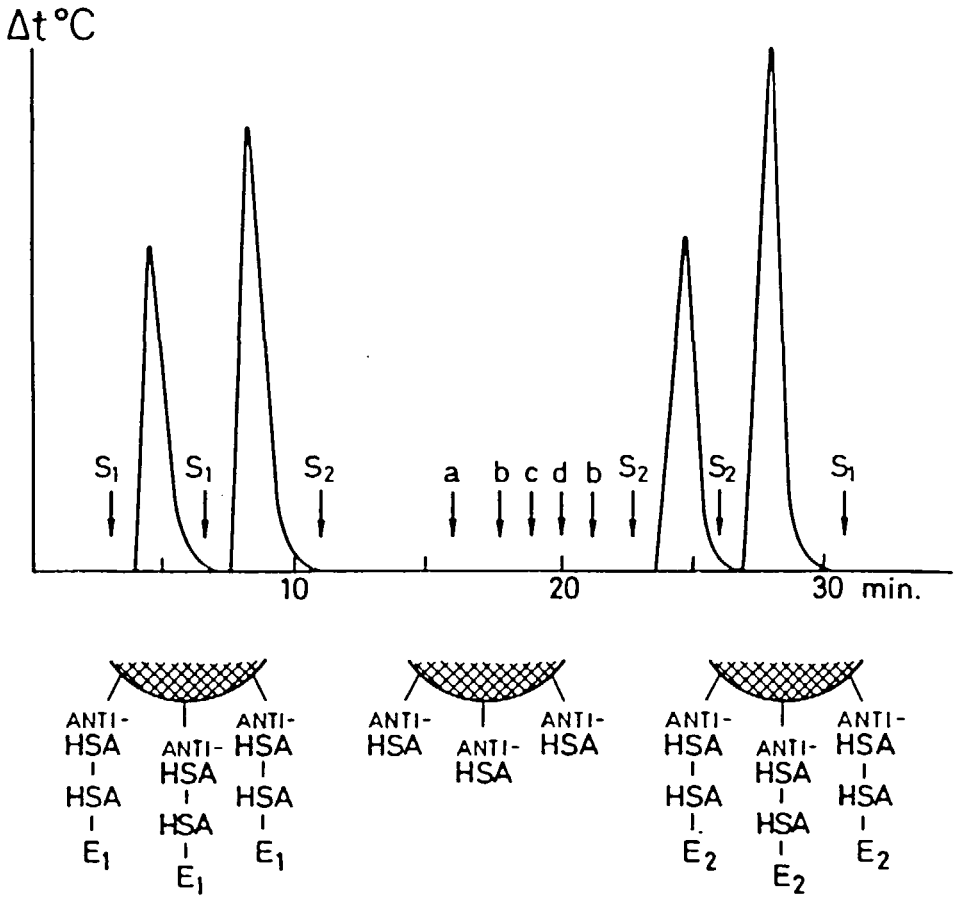

FIGURE 10. Schematic diagram of antigen-antibody assay cycle. Arrows indicate changes in perfusion medium. Cycle starts with an enzyme-antigen (E_1-HSA) bound to antibody (anti HSA)-containing support material. At the arrows marked, S_1,substrate for enzyme E_1 is introduced in pulses, and at arrows marked, S_2, substrate for enzyme E_2 is introduced. At the arrow (a), a 2 min pulse of glycine-HCl buffer is introduced to split the complex and wash the system. After a pulse of buffer at (b), new enzyme-antigen complex (E_2-HSA) is introduced (c), followed by a pulse of phosphate buffer containing KCl (d). Finally, the flow buffer (b) is introduced. (Reprinted from Mattiasson, B., *FEBS Lett.*, 77, 107 (1972). With permission.)

there was about an 100-fold decrease in sensitivity. It was suggested that the lower sensitivity results from a lower specific activity for the albumin-conjugated proteins.

The use of the immunosorbent technique has also been applied to the determination of human serum albumin itself.[73] Anti-HSA is again bound to Sepharose® to form the antibody column. A known amount of a preparation of a catalase-HSA conjugate was mixed with the sample containing an unknown amount of HSA. This mixture was added to the column, resulting in competitive binding of HSA and the HSA-catalase complex. Next, an aliquot of 1 m*M* H_2O_2 was added to the column, resulting in a large thermal signal as the peroxide reacted with the immobilized catalase. Calibration curves were constructed showing the percentage decrease in the temperature signal with increased concentration of HSA in the samples. Although the curves are not linear, samples in the range of 0.1 to 200 mg/m*l* could be assayed with a precision of around 1%. After the reaction is complete, the column can be washed with glycine-HCl buffer to regenerate the antibody column for another sample. A new determination could commence in about 10 min. The results reported show that the possibility clearly exists

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for assaying metabolites down to $10^{-10}M$ using this thermometric enzyme-linked immunosorbent assay.

Table 4 shows a summary of recently reported substrates which have been studied using immobilized-enzyme flow calorimeters. Also shown in the table are some examples of soluble enzyme methods using flow or batch calorimetric procedures. In comparing the soluble- vs. immobilized-enzyme methods, the most significant difference is in the number of samples which can be analyzed per hour. Generally, because batch procedures require time for equilibration of the reactants to calorimeter temperature, fewer samples can be run per hour. While the equilibration time is lessened using flow calorimeters, the time required for establishment of a steady-state signal can limit the expediency of the determination. With immobilized-enzyme reactors, the thermal signals are obtained in a short time after injection of the sample with as many as 30 to 40 samples per hour possible. This feature can be a definite advantage in clinical analysis.

While it is not immediately apparent in Table 4, it should be mentioned that the general level of sensitivity of most batch microcalorimeters is higher than an immobilized-enzyme flow calorimeter. Thus, lower detection limits are, in principle, possible with solubilized enzyme methods. The total advantage of increased sensitivity depends on the enzymatic system being studied, so it is difficult to generalize this feature. However, increased sensitivity can mean that generally lower linear working ranges can be used, thus requiring less soluble enzyme for the reaction mixture. This can offset somewhat the favorable reusability characteristic of the immobilized reactors.

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In working with biological samples, such as serum, urine, or other complex media, the samples can produce nonspecific heat effects due to the presence of protein or other biochemical components in any calorimetric methodology.[1] In the flow calorimeters and in immobilized-enzyme reactors, viscosity or media-matching factors also produce unwanted thermal effects in high sensitivity operation, as has been mentioned in the discussions above. While these effects can be corrected for by running blank experiments, reproducibility of blanking procedures is a problem in high-sensitivity work. With batch heat-conduction calorimeters, the actual transient response in the blank is not so critical, since areas are used to evaluate the total heat effect. Blanking is thus quite repeatable if the reacting system itself is constantly repetitive.

The use of a split-flow design, as reported by Mattiasson, Danielsson, and Mosbach seems to provide a solution to the artifacts produced by nonspecific heat effects in immobilized-enzyme reactors.[31] In the split-flow reactor the second column introduced to compensate for nonspecific effects must be close to identical with the active column, except that it must lack the enzymatic activity. Packing the reference column with glass beads appears to eliminate most of the thermal artifacts, as is shown in Figure 11. In this example, skim milk (which has been diluted 1:20 in distilled water) was injected into the split-flow reactor, and the response was compared with the simple single thermistor or differential thermistor arrangements. (See Instrumentation Section for details.) As can be seen, the split-flow design eliminates virtually all of the artifacts observed with the other arrangements. The glucose content of urine samples was also examined with the split-flow configuration, and again, the peak heights and shapes indicated elimination of distortions seen in the differential or single thermistor designs.

In summary, the immobilized-enzyme flow calorimeters seem to offer very useful alternative methods for determination of biochemically significant molecules. The relatively low-cost, fast, and precise measurements possible make the methods quite attractive for clinical and other medical investigations.

B. Differential Scanning Calorimetry Studies

The application of temperature-scanning calorimetry to biochemical problems has

TABLE 4

Comparison of Immobilized Enzyme and Soluble Enzyme Calorimetric Methods

Substrate	Enzyme	Medium	Reported linear range (mM)	Estimated precision (%)[a]	Samples/hr	Ref.
			Immobilized Enzyme Methods			
BAEE[b]	Trypsin	Buffer	1—8	2	3	28
ATP[c]	Apyrase	Buffer	1—8	2	3	28
Tyrosine[d]	Tryosinase	Buffer	0.1—1	1	12	72
Penicillin G	Penicillinase	Buffer	1—100	2	6	27
Penicillin G[d]	Penicillinase	Buffer	0.1—10	1	12	72
Urea	Urease	Buffer	2.5—7	2	6	27
Urea	Urease	Serum	5—60	1	15	29
Urea	Urease	Buffer	0.1—100	0.5	30	32
Urea	Urease	Serum	5—70	0.5	40	33
Glucose	Glucose oxidase	Buffer	0.06—1	2	6	27
Glucose	Hexokinase	Serum	0.1—25	5	40	70
Glucose	Glucose oxidase-catalase	Serum	0.01—0.45	0.6	12	71
Glucose[d]	Glucose oxidase	Buffer	0.05—0.5	1	10	72
Lactose	Lactase-glucose oxidase-catalase	Skim milk	1—3	4	4	30
Sucrose	Invertase	Buffer	1—50	1	12	72
Cholesterol	Cholesterol oxidase	Buffer	0.03—0.15	4	4	30
H_2O_2[d]	Catalase	Buffer	0.05—10	1	12	72
Phenol[d]	Tryosinase	Buffer	0.1—1	1	12	72
			Soluble Enzyme Methods			
Glucose	Glucose oxidase-peroxidase	Buffer	0.02—0.2	5	4	35
Glucose	Hexokinase	Serum	0.7—7	3	8	39
Glucose	Hexokinase	Serum, plasma, whole blood	2—55	2	6	56
Urea	Urease	Buffer	5—25	2	2	59
Uric acid	Uricase-catalase	Serum	0.06—0.6	2	5	57
Parathion	Cholinesterase[e]	Buffer	0.04—3	2	2	61
TEPP[f]	Cholinesterase[e]	Buffer	0.0002—0.006	2	2	61
Dimefox[g]	Cholinesterase[e]	Buffer	0.0003—0.06	2	1	60

[a] Precision estimate is % relative standard deviation. When not specifically stated, the prevision is estimated from data given.
[b] Benzoyl-L-arginine ethyl ester.
[c] Adenosine triphosphate.
[d] These methods used immunosorbent immobilization rather than glass beads.
[e] These experiments were enzyme inhibition studies.
[f] Tetraethyl pyrophosphate.
[g] Tetramethylphosphorodiamidic fluoride.

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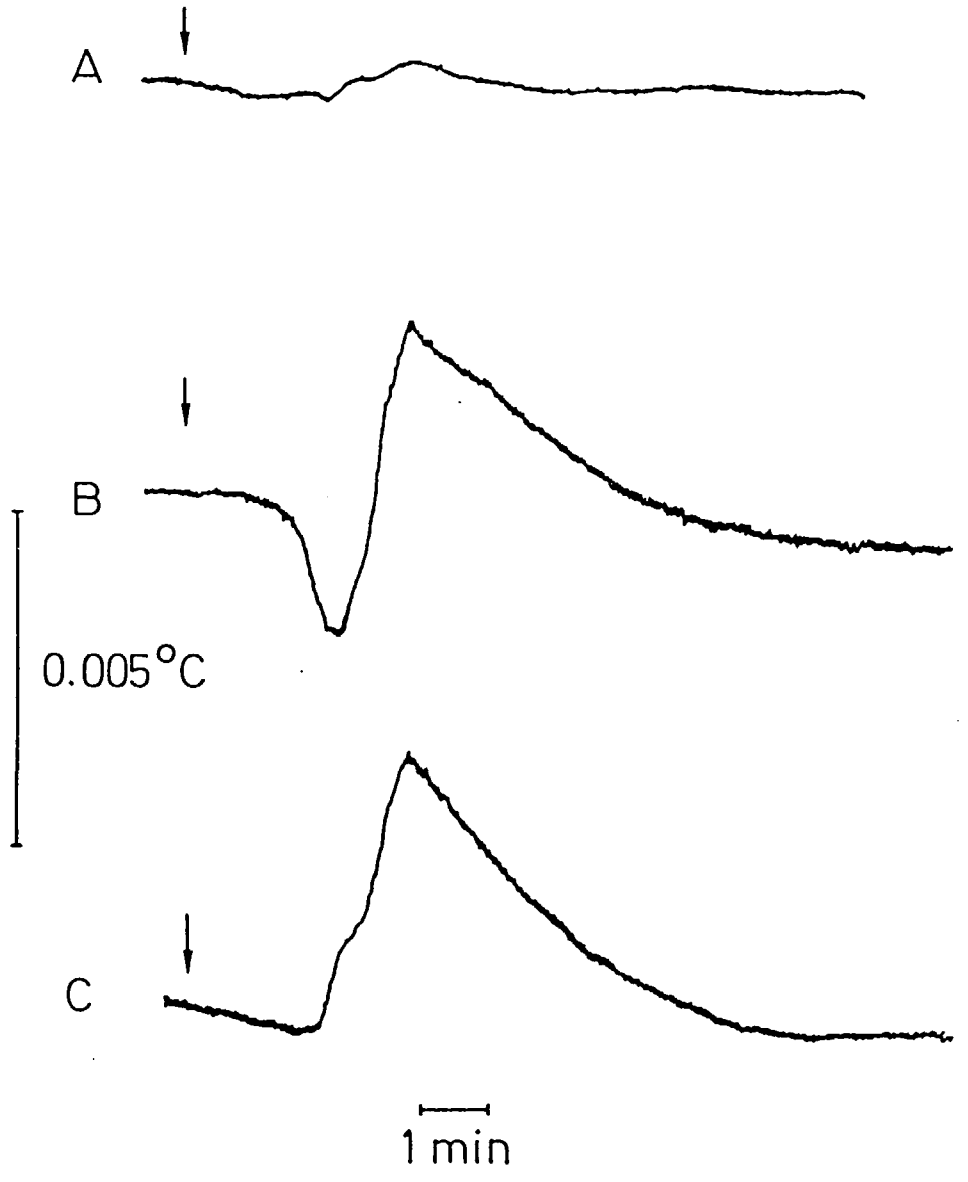

FIGURE 11. Thermal effects obtained from injection of 1 m*l* pulses of skim milk (1:20 dilution with distilled water) using split-flow enzyme thermistor. Curve A, split-flow arrangement. Curve B, differential arrangement. Curve C, single thermistor arrangement. (Reprinted from Mattiasson, B., Danielsson, B., and Mosbach, K., *Anal. Lett.*, 9, 867 (1976). With permission.)

been largely concentrated on studies of biopolymers. The characterization of the thermal denaturation process for globular proteins in dilute aqueous solutions has been of major interest, as have investigations of conformational transitions in nucleic acids and polynucleotides. The thermal behavior of lipid bilayers has recently been studied by application of the DSC technique. Let us first examine the type of information which can be obtained from scanning calorimetry as applied to problems involving biopolymers or lipids in solution.

In the normal DSC experiment, a solution of the biopolymer is placed in one cell of the DSC instrument and a reference solution in the other. The temperature is then

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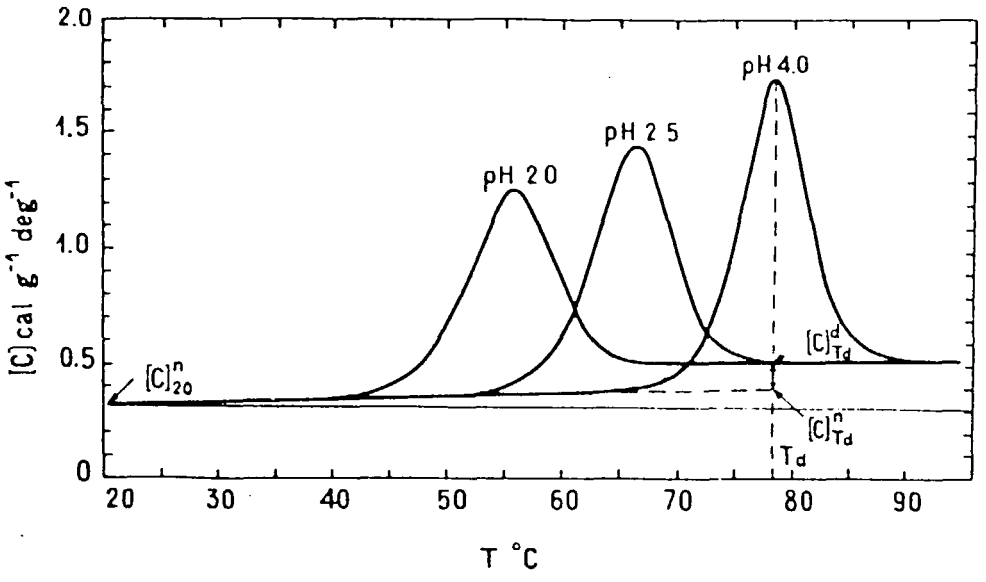

FIGURE 12. DSC thermogram showing partial heat capacity of lysozyme at different pH values. "Td" is denaturation temperatures. $[C]^{n}_{Td}$ and $[C]^{d}_{Td}$ are partial heat capacities of native and denatured states at the denaturation temperature. (Reprinted from Privalov, P. L. and Khechinashvili, N. N., *J. Mol. Biol.*, 86, 665 (1974). With permission. Copyright by Academic Press, Inc. (London) Ltd.)

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scanned from ambient or slightly below to the appropriate maximum temperature, which is usually not above 90°C for aqueous solutions. As mentioned in the section on instrumentation, the thermal signal output from the DSC is directly related to the heat capacity of the sample solution and, thus, to the partial heat capacity of the biopolymer in solution. In the absence of any thermally induced transitions, one obtains a record of the heat capacity as a function of temperature. In the event that a transition occurs, such as denaturation or unfolding of the biopolymer, there will be an enthalpic contribution to the output, giving rise to a peak whose area is proportional to the enthalpy of the transition process. Normally, there is a heat-capacity change upon transition of the biopolymer from its initial to final state. Therefore, there is a baseline shift from which the ΔCp for the transition can be obtained directly. Finally, as the temperature is continuously scanned beyond the transition, the temperature dependence of the heat capacity of the post-transitional state of the biopolymer is obtained. Thus, from a single scan of the biopolymer or lipid solution, considerable thermodynamic information on the various material states of the biopolymers can be directly measured. It should be emphasized that the calorimetrically determined enthalpic effects may or may not be the same values as obtained from the temperature dependence of equilibrium properties — the van't Hoff enthalpies. This is an important point in the discussion of conformational changes of state. In the case of a simple two-state transition, the van't Hoff and calorimetric enthalpies are identical, provided the states have been properly defined. For some classes of biopolymers, however, the identity of initial and final states is not always clear, and caution is necessary in the interpretation of the thermodynamic values in terms of a simple two-state model (see below). Let us now look at some specific examples of the use of DSC in several problem areas in biochemistry.

1. Thermal Transitions in Globular Proteins

The important property that many globular proteins have of undergoing reversible thermal denaturation has been of interest to protein chemists for many years. Until

sensitive scanning calorimeters became available, the majority of studies of thermal denaturation were done using spectroscopic or potentiometric techniques. However, since about 1970, there has been a significant number of DSC studies on globular proteins. Most of these have used sensitive adiabatic scanning calorimeters with capabilities for measuring solutions containing as low as a few tenths of a percent of protein.[40,23,24] Figure 12 shows DSC data for lysozyme at several different pH values using 0.04 *M* glycine buffers. The data were obtained with the Privalov scanning calorimeter.[41,24] The actual experimental curves, which resemble those in Figure 12, are recorded directly in temperature coordinates on an X-Y recorder. After electrical calibration and a baseline scan, the Y coordinate can be converted to partial heat-capacity units, as shown in the figure.

DSC data have been reported for chymotrypsin,[41] chymotrypsinogen,[40,42] lysozyme,[43,41] myoglobin,[41,42] ribonuclease,[23,41,42] and cytochrome c.[41] the results can be summarized as follows, based on the paper by Privalov.[41]

1. The partial heat capacities of all proteins in their native states are the same within experimental error, the value at 20°C being, $C_{p,2} = 1.30 \pm 0.08$ J $g^{-1}K^{-1}$.
2. As temperature increases, the partial heat capacity increases linearly up to the denaturation transition, the increase being about the same for all proteins, and independent of pH.
3. Denaturation leads to a considerable increase in partial heat capacity, and is different for each protein. The ΔC_p^d for a given protein seems to be insensitive to temperature.
4. The thermal stability of the proteins increases with increased pH, as do the specific enthalpies of denaturation. In addition, the enthalpies of denaturation of all the proteins increase with increased temperature, and the slopes of the (d Δ h/dT) lines are equal to the ΔC_p^d values for the various proteins.
5. The van't Hoff enthalpies and the calorimetric enthalpies of denaturation (assuming a two-state model) are within about 5% of each other. The 5%-greater calorimetric enthalpy is outside the experimental error, so it is thought to be a real difference.

The interpretation of these results has led to several conclusions about globular-protein denaturation.[41] First, because the calorimetric and van't Hoff enthalpies are quite close, the denaturation transition can be considered a cooperative, two-state transition between the native and denatured states to a first approximation. The 5% deviation can be explained assuming the presence of intermediate states, but because the deviations are small, the states must be quite unstable. The change in heat capacity of the native protein with temperature does seem to indicate some changes of state of the protein, but these changes cannot be interpreted as cooperative transitions between actual definable states, since no enthalpic contributions were ever found. A loosening of structure, allowing increased thermal motion, could explain the gradual increase in $C_{p,2}$ with temperature. Interpretation of the enthalpies of denaturation has been attempted using models of hydrogen and hydrophobic bonding in the native structures. Of particular interest is the dependence of the enthalpy on temperature (identical with the ΔC_p^d). Assuming coordinate positions of the atoms based on X-ray data, it is possible to estimate the number of potential hydrogen and hydrophobic bonding sites in the native structure of the protein.[41] Then, if in the denatured state the side chains and hydrogen-bonded groups are all allowed contact with water, the conclusion seems apparent that the ΔC_p^d is mainly due to the changes occurring as the nonpolar side chains interact with water. This kind of estimate of the structural features of protein molecules important to the thermodynamic behavior is a significant step in understanding both function and evolution of protein behavior. Privalov has recently published

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three extensive papers on lysozyme, consolidating data from DSC, isothermal calorimetry, and potentiometric titrations, which provides a complete thermodynamic characterization of the protein under a wide scale of experimental conditions.[44] The complete thermodynamic standard functions for native and denatured lysozyme are presented for the temperature range 0 to 100°C and pH = 1.5 to 7.0.

2. Thermal Transitions in Lipidic Materials

Biomembranes are thought to be constituted of bilayers of phospholipids in which the hydrocarbon tails of the fatty acids are directed toward the center of the bilayer, and the polar phosphatidylcholine ends are in contact with water on the two outer surfaces of the bilayer. The membrane bilayers are known to undergo gel-to-liquid crystal transitions, which have proved very useful in the study of the membranes.[45] Scanning calorimetry seems to offer an extremely valuable tool for examining these transitions, particularly with the availability of new, sensitive instrumentation.

Some attempts have been made to study by DSC techniques the intact membranes of simple organisms. Reinert and Steim, using a Perkin-Elmer DSC-1B instrument, have reported a study of living cells of *Mycoplasma laidlawii*.[46] First, samples of whole cells suspended in buffer were scanned from about 0 to 80°C. The cells were in the logarithmic growth phase. DSC scans of the cells showed two endothermic peaks, one beginning at 20°C and the other at about 50°C. Cell membranes were then isolated from the cells, and DSC scans obtained over the same temperature range. The same two peaks were observed with the membranes, and the areas per gram of lipid were the same as with the intact cells. A rescan of the membrane preparation showed only the first transition, which led the authors to conclude that the higher temperature transition is due to irreversible denaturation of membrane-bound protein. This conclusion was strengthened by preparing a sample of protein-free lipid material and observing that the DSC scan of this material showed only the low temperature transition. Another DSC study of membrane- bound proteins has been reported recently, in this case the investigation of the transitions in aqueous suspensions of human erythrocyte ghosts.[47] It was shown that the activity of ATPase could be correlated with transitions observed in the DSC scan of the membrane-bound enzyme.

The study of the behavior of phospholipids in bilayers has been facilitated by the examination of the properties of synthetic liposome dispersions. The dispersions of synthetic phospholipids are considered as models whose basic physical properties are similar to the properties of biological membranes. Hinz and Sturtevant have reported DSC studies on dilute aqueous suspensions of synthetic L-α-lecithins.[48] The measurements were made on solutions of less than 1% lipid in water using the sensitive calorimeter developed by the Sturtevant group.[49,23] Dimyristoyl, dipalmitoyl, and distearoyl L-α-lecithins were studied in the temperature range of about 5 to 60°C. Each lipid showed two endothermic transitions, one a very intense, sharp peak, and the other a small, broad peak about 5 to 10°C below the larger peak. The larger peak was associated with the gel-to-liquid crystal transition, which demonstrated a very high degree of cooperativity. Cooperativity estimates are made by comparing the van't Hoff enthalpy with the calorimetric enthalpy of the transition. Units of from 70 to 200 were obtained from the comparison, depending upon which lecithin was involved. The exact nature of the lower temperature transition was not clear, but a suggestion that it could be due to rotation of the polar heads of the lipid was made. In this study of the gel melting transition, the ΔC_p for the transition was found to be very small. This result suggests that only a very small percentage of the hydrocarbon must come in contact with water in the transition.

The influence of cholesterol on the thermal transitions of lecithin bilayers was also reported by Hinz and Sturtevant.[50] It was found that the transition enthalpy decreases

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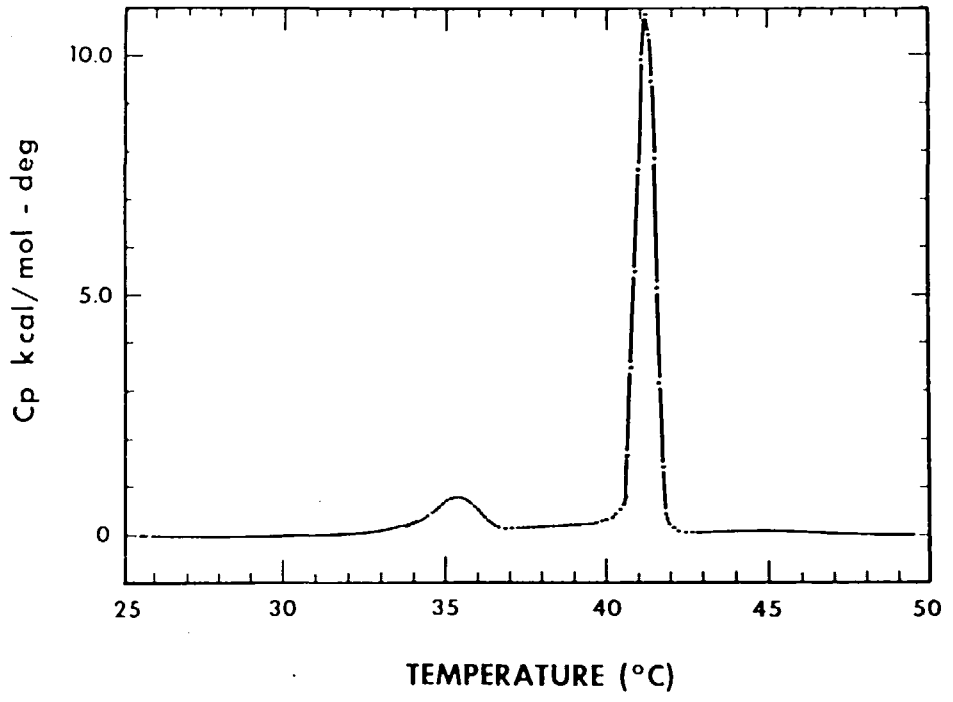

FIGURE 13. DSC scan of fresh dispersion of multilamellar, Bangham-type liposomes prepared from dipalmitoylphosphatidylcholine in 50 m*M* KCl. (Reprinted from Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., and Thompson, T. E., *Biochemistry*, 15, 1393 (1976). With permission. Copyright by the American Chemical Society.)

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linearly with increasing concentrations of cholesterol, with the transition gone by 33 mol% cholesterol. These results indicate that each cholesterol molecule removes 2 molecules of lipid from the hexagonal gel phase which undergoes transition to the liquid cyrstalline state.

It should be mentioned that studies very similar to those reported above have been carried out using the Perkin-Elmer DSC-1B.[51,52] In these studies, concentrations of lipidic material were considerably higher, about 30 to 50% by weight. Although the general conclusions about the transitions were similar, there are discrepancies in the breadth of the transitions and, thus, cooperativity, most likely attributable to the higher concentrations and different scanning conditions employed in the two studies.

A rather thorough study of dipalmitoylphosphatidyl-choline bilayer vesicles has been reported recently.[53] Using a newly designed DSC instrument[54] and fluorescence-probe methods, these authors made a distinction between the behavior of larger multilamellar vesciles as compared with single-lamellar, spherical vesicles. Dispersions of single-lamellar vesicles were prepared by sonification of the lipid material contained in 50 m*M* KCl and subsequent centrifugation of the larger liposomes. Figures 13 and 14 show the DSC scans of the two types of vesicle preparations. The multilamellar vesicles show the sharp peak at 41.2°C and a smaller peak at 35.4°C, both of which are in good agreement with the results of Hinz and Sturtevant.[48] The scan of the single-lamellar vesicles showed quite distinctively different behavior. Two maxima are observed at 36.9°C and 41.2°C. It was found that these maxima changed with time, upon repeat scanning of the preparation the peak at 41°C grew at the expense of the 37°C peak. The repeat scan after several cycles appears to resemble very much the multilamellar preparations, although not in exact detail. An experiment in which the single-

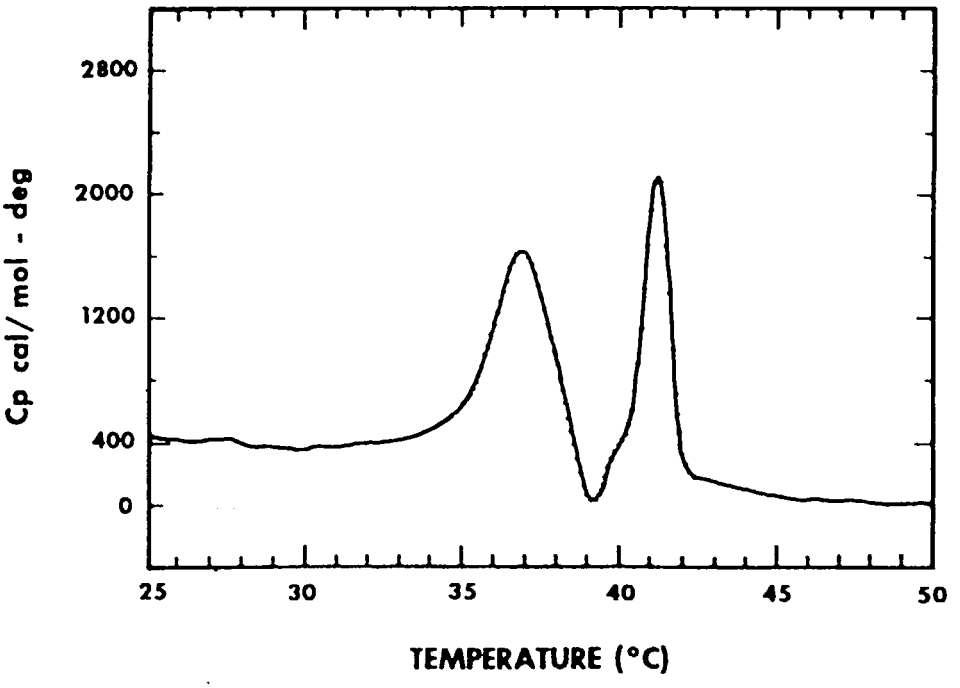

FIGURE 14. DSC scan of small single-lamellar vesicles prepared from dipalmitoylphosphatidylcholine. Preparation was in calorimeter at 0°C for 3.0 hr before commencing scan. (Reprinted from Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., and Thompson, T. E., *Biochemistry*, 15, 1393 (1976). With permission. Copyright by the American Chemical Society.)

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lamellar vesicles were cooled to −10°C for about 80 hr before the DSC scan showed peaks again resembling the multilamellar liposomes. Comparison of fluorescence polarization studies at several temperatures with the calorimetric scans allowed several conclusions to be drawn about the behavior of the vesicles. First, the single-lamellar vesicles show a single transition at 37°C, with a ΔH of transition of 6.3 kcal/mol, and as long as the temperature is above the transition temperature (48 to 51°C), the small vesicles are stable, although prolonged storage at the higher temperature seems to produce aggregation as evidenced by increased turbidity in the solutions.

Secondly, dispersions of multilamellar structures show a transition at 41° with ΔH = 35.1 kJ/mol. At temperatures below the phase transition, the large forms are more stable than the small vesicles, so that at the lower temperatures aggregation to the multilamellar form is favored. The DSC scans of the single-lamellar vesicles from lower to higher temperature therefore show composite contributions of both forms. With these analyses of the data, it is possible to formulate a model to describe the thermodynamics and kinetics of the transitions between forms of the two types of vesicles, and to describe the gel-to-liquid crystal transitions for each. The results indicate that the properties of the two forms are quite different, and that the single-lamellar vesicles are probably better models for most biological membranes.

Another study of the thermal behavior of lipidic materials has been reported recently.[62] In this case, liposomes of various natural and synthetic sphingomyelin lipids were examined by DSC and fluorescence techniques. The sphingomyelins are lipids found extensively in brain tissue, and are particularly interesting from a calorimetric point of view because they undergo thermotropic phase transitions in the physiological temperature range. The liposomes prepared for these studies were multilamellar vesicles suspended in 50 m*M* KCl at concentrations of lipid ranging from about 10 to 30

mg ml^{-1}. For the natural sphingomyelins derived from sheep or bovine brain preparations, the DSC scans showed complex, but reversible, gel-liquid crystal transitions. For sheep-brain liposomes, two maxima are observed at 31.2 and 37.1°C. Bovine-brain liposomes showed three maxima at about 30, 32, and 38°C, the actual values depending somewhat on the preparation. The total enthalpy change, estimated from the transition curves for all of the preparations regardless of source, was 29.3 kJ/mol. In an earlier study of bovine-brain sphingomyelins, the suggestion was made that the several maxima appearing in the heat-capacity function might result from phase separation of the individual components of the natural mixture.[63] Thus, it was of interest to examine synthetic mixtures of the individual sphingomyelin liposomes.

DSC thermograms of four synthetic samples showed transitions very similar to the multilamellar liposomes of the synthetic lecithin discussed above. Rather sharp gel-liquid crystal transitions with temperature maxima in the range of 40 to 50°C, depending on the sphingomyelin structure, were observed. Some interesting results were found for mixtures of the synthetic compounds, as shown in Figure 15. Curve A shows the heat capacity function for a 1:1:1 molar mixture of *N*-palmitoyl-, *N*-stearoyl-, and *N*-lignocerylsphingomyelins. The dashed lines show curves calculated assuming the components behave independently of each other. The results show that the mixed dispersions are, in fact, unfused and thermally independent. However, if the dispersions are prepared from a mixture which has been colyophilized prior to suspension in the KCl medium, curve "B" is obtained. Here a prominent maximum occurs at about 40°C, and the enthalpy change for the total transition is 28.5 kJ/mol, very close to the value obtained for the two natural sphingomyelin preparations. These results are quite significant because they show that the thermotropic behavior of colyophilized sphingomyelins is a complex function of the composition, in which a homogeneous phase persists with all components contributing equally to the thermal behavior. It thus seems unlikely that the complexities in the transition curves for the natural sphingomyelins result from actual phase separaion of the individual components of the mixtures. These DSC studies show very clearly the power of the calorimetric technique in unraveling rather subtle properties of biologically significant molecules. With the added sensitivity of the new scanning calorimeters, which can be used for dilute aqueous solutions, many new problems in protein and lipid biochemistry can be examined. The more difficult problems associated with the thermal behavior of polynucleotides and nucleic acids will be discussed in the next section.

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3. Thermal Transitions in Polynucleotides and Nucleic Acids

Experimentally, the nucleic acids (DNA and RNA) and synthetic polynucleotides exhibit rather simple thermal behavior. Normally, there are melting transitions which correspond to the unravelling of double or triple helical structures, or for single-stranded nucleotides, the breaking of stacked, base-pairs in the helix. The melting transitions are pH- and ionic-strength dependent. However, they usually show well-defined, single maxima for each type of transition. The complexity of these systems arises in the interpretation of the thermodynamics of the transition curves in terms of basic molecular processes. For example, DNA consists of high molecular weight, double-stranded chains of nucleotide residues, hydrogen bonded in specific unit sequences. The melting transition of such structures includes thermal contributions from the basic separation of the strands plus any base-pair stacking which occurs in the single strands after separation. Thus, in addition to the pH- and ionic-strength dependence, the specific number and sequence of bases in the DNA structure influence the melting temperature and the enthalpy changes involved.

There have been a number of scanning calorimetric studies of DNA and related nucleotides which confront the above problems.[64,65] These and other more recent stud-

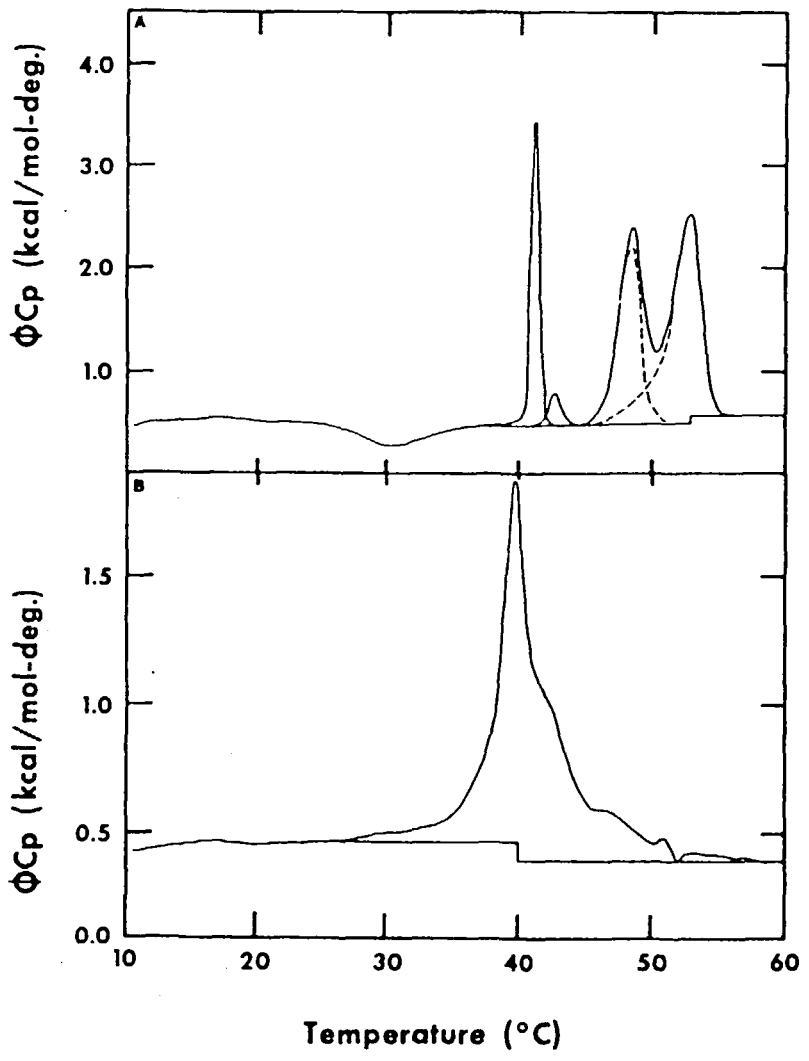

FIGURE 15. Apparent heat capacity vs. temperature for 1:1:1 *N*-palmitoylsphingosinephosphorylcholine and *N*-stearoyl- and *N*-lignocerylsphingosinephosphorylcholine liposomes. (A), mixed; (B), colyophilized. Broken curves in A are estimated contributions for the pure sphingomyelins, assuming each behaved independently. (Reprinted from Barenholz, Y., Suurkuusk, D., Mountcastle, T. E., and Biltonen, R. L., *Biochemistry*, 15, 2441 (1976). With permission. Copyright by the American Chemical Society.)

ies on polynucleotides have been reviewed recently.[66] Somewhat simpler to interpret are the melting transitions in synthetic oligonucleotides for which the specific composition and sequence of bases are known. So for this review, the behavior of a specific oligonucleotide system will be presented as an example of the methods and approaches to interpretation recently developed for such molecules.

The synthetic polynucleotides polyriboadenylic acid (poly A) and polyribouridylic acid (poly U) are used as models for some of the structural components of natural nucleic acids. Poly A and poly U can associate to a double helical structure and also to a triple helix, poly (A + 2U), depending on the conditions. Poly A alone seems to form a single-stranded helix with stacked bases, while poly U does not show this behavior. All of these conclusions have been reached from studies of equilibrium shifts

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in the spectra of oligonucleotides as the composition of solution and temperature are changed.

One of the first scanning calorimetric studies of poly A and poly U solutions was reported by Neumann and Ackerman.[67] Using an adiabatic scanning calorimeter, buffered salt solutions at pH = 6.8 containing weighed amounts of poly A and poly U were scanned through the transitions corresponding to the dissociation of the double helix. Figure 16 shows the experimental calorimetric scan, as well as the degree of conversion obtained by integration of the calorimetric scan. Shown in the figure are optical-density data, which also relate to the extent of conversion from double helix to single-stranded forms.

$$\text{poly (A + U)} \longrightarrow \text{poly A + poly U} \qquad (9)$$

Because poly A is known to undergo base-stacking interactions, the actual state of poly A at the temperature of melting of poly (A+U) is unknown. The measured enthalpy of double to single strand conversion contains an added re-coiling effect as poly A forms the base-stacking structures. It has been estimated that at about 95°C poly A exists as a random coil, so it was felt that increasing the salt concentration, which raises the double to single strand conversion temperature, could be used to evaluate the re-coiling effect of poly A. Unfortunately, it was found that at the higher ionic strengths (greater than about 0.2 *m*) two melting transitions occur corresponding to the following reactions:

$$\text{poly (A + U)} \longrightarrow \tfrac{1}{2}\text{poly (A + 2U)} + \tfrac{1}{2}\text{poly A} \qquad (10)$$

$$\text{poly (A + 2U)} \longrightarrow \text{poly A + 2 poly U} \qquad (11)$$

The poly (A+U) undergoes strand separation and then formation of a triple helix involving two poly U strands. The higher temperature transition corresponds to the complete unraveling of the triple helix. However, by making some simple assumptions about the additivity of the reaction sequences, it was possible to estimate the base-pair interaction enthalpies for dissociation of the double and triple helix structures. The enthalpy change for dissociation of the base-pair (A+U) was found to be 38.9 ± 2 kJ/mol of base pair, while the triple-helical dissociation enthalpy was 56.5 kJ/mol of (A+2U). At the transition temperatures for the dissociation of the double or triple helices, the base-stacking enthalpy for poly A must be accounted for in the actual observed enthalpies of the transitions. A value of −18 ± 8 kJ/mol (A) was derived for the stacking enthalpy in poly A.

A study of heat-capacity changes in the conformational transitions of poly A, poly U, and poly (A+U) has been reported recently.[68] Using a new scanning calorimeter (see Instrumentation section), as well as absolute heat-capacity measurements, the temperature dependence of the heat capacities was determined over temperature ranges above and below the transition involved in strand separation or base-unstacking. The results indicate that double-stranded configurations have relatively small apparent heat capacities, while single-stranded polynucleotides have heat capacity values approaching those of free nucleotides in an aqueous medium. Thus, the enthalpy changes for nucleic acid transitions should be highly temperature dependent, an observation which is consistent with the relatively large ΔC_p of melting found for the poly (A+U) transition. (The ΔC_p was about 418 J/mol K per base pair.) The heat capacity data for single-stranded poly U was found to be consistent with other observations that above 15°C it exists in a totally random coil configuration. On the other hand, the temperature dependence of the heat capacity of poly A showed significant base stacking between

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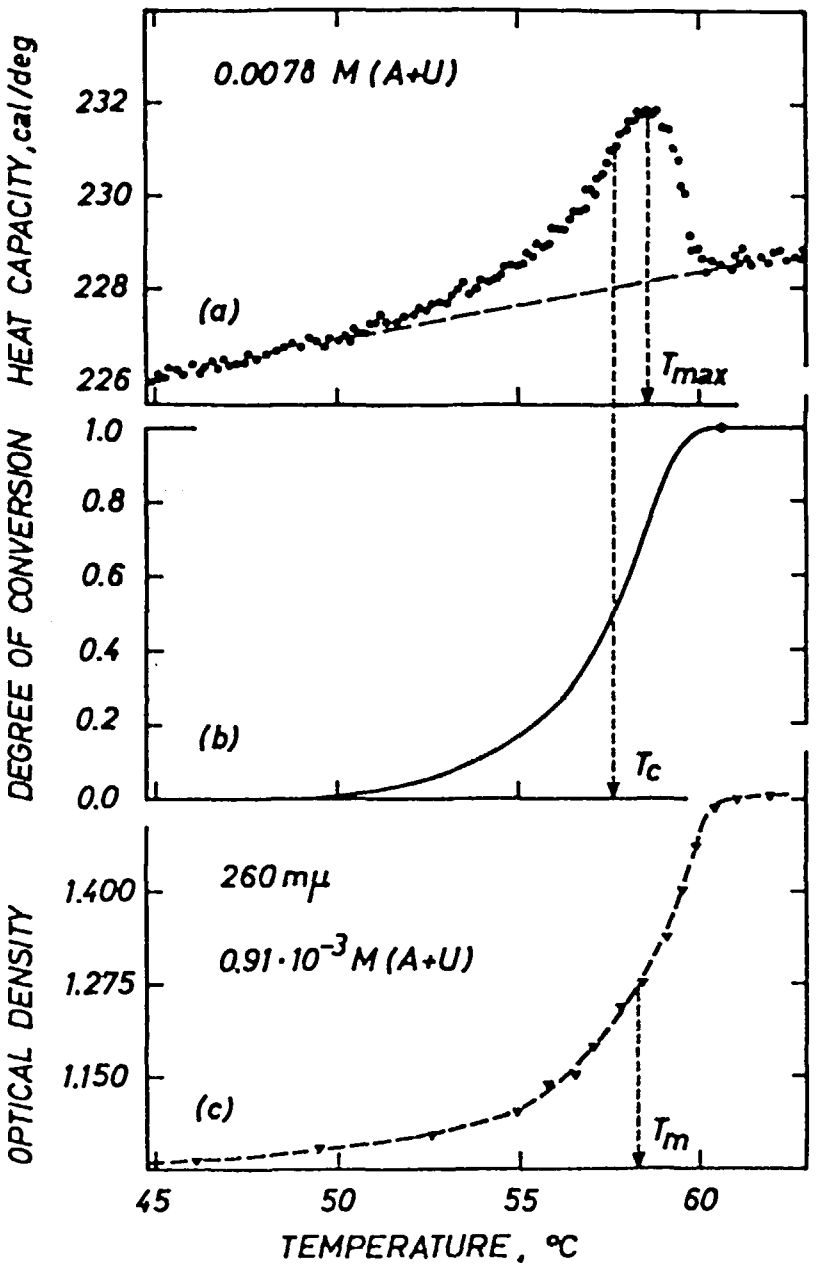

FIGURE 16. Course of helix-coil transition of poly(A + U). Heat capacity (a), degree of conversion (b), and optical density at 250 nm (c), as a function of temperature. (Reprinted from Neumann, E. and Ackermann, T., *J. Phys. Chem.*, 73, 2170 (1969). With permission. Copyright by the American Chemical Society.)

10 and 70°C. An enthalpy change for stacking estimated from the scanning calorimeter data was −39.7 kJ/mol, and the cooperativity parameter was found to be 0.57 ± 0.03 for the stacking of adenine bases.

Interpretation of the calorimetric melting transitions for poly (A + U) in statistical mechanical terms has been attempted by both Neumann and Ackermann[67] and, more recently, by Freire and Biltonen.[69] In these approaches, attempts are made to fit statis-

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tical models to the unraveling process in terms of the cooperativity parameter and the degree of conversion in the thermal transition. In the deconvolution method of Freire and Biltonen, it is shown that the partition function can be directly calculated from scanning calorimeter data, and that parameters such as size of cooperative unit, helical-segment lengths, and coil-segment lengths can be obtained from the theoretical treatment. When applied to the poly (A + U) melting transition, it was found that the mean helical-segment length decreases with increasing temperature well before strand separation occurs. However, the mean coil-segment length remains almost constant until the temperature is very close to the melting transition temperature. Thus, the results indicate that the unfolding process proceeds through the formation of many short helical sequences. The cooperative unit for strand separation was calculated to be 120 base pairs, independent of salt concontration. It was also found that the minimum helical-segment length is about 10 base pairs in the double-stranded form. Application of these methods to more complex DNA structrues should provide interesting insight into the unravelling processes for these fundamentally important biopolymers.

C. Analytical Calorimetry of Living Cells

In any living system, the various metabolic events occurring within the cells are reactions which produce heat. Thus, with calorimeters that are sensitive enough, monitoring the heat effects provides a way of studying organisms in vivo. Living cells in a particular medium produce thermal energy which is a reflection of the basic activity and viability of the cells. In addition, the time dependence of the observed thermal effect (the thermogenesis curve) reflects changes in growth patterns with time, and provides basic correlations to the type of organism and to the conditions under which they are growing. Physical or chemical changes in the environment of the cells can have an effect on a particular metabolic path and, thus, cause changes in total heat production. Thus, the calorimeter is a general tool for observation of living cells that could provide information on the activity, the identity, and the response to chemicals of various cellular systems. Because the measurements can be obtained with virtually no disturbance of the system, such as addition of chemicals, use of light, or use of separation steps, calorimeters are particularly adaptible for in vivo investigations. The lack of specificity of the heat effect to a particular chemical or process can be a definite advantage in studying living systems, since a number of different variables can be altered in the medium or in the physical environment (e.g., temperature) which affect particular processes and not others. Changes in the total heat effect will reflect alteration in that specific aspect of the total cell activity.

Calorimeters have been designed for the study of many types of living systems, ranging from microorganisms to man. From the point of view of calorimetry, it is perhaps experimentally simplest to study microorganisms, since the microcalorimeter instruments used are very similar to those used in fundamental biochemical studies. Flow calorimeters or batch-mixing devices can be used for bacterial and other simple unicellular systems (see Reference 1, and also the more detailed description of a microcalorimeter designed specifically for biological systems in Reference 74).

For multicellular plants or animals, special calorimeters are often required. However, a number of studies have been accomplished on insects and small animals in the Calvet-type microcalorimeters.[75] For larger animals, unique measuring systems have been devised for calorimeters which can contain whole animals.[76]

Considerable work has been reported in recent years on calorimetric investigations of microorganisms and blood cells. Since this literature is of direct interest in clinical and medical fields, some of the recent applications of calorimetry in these areas will be reported here.

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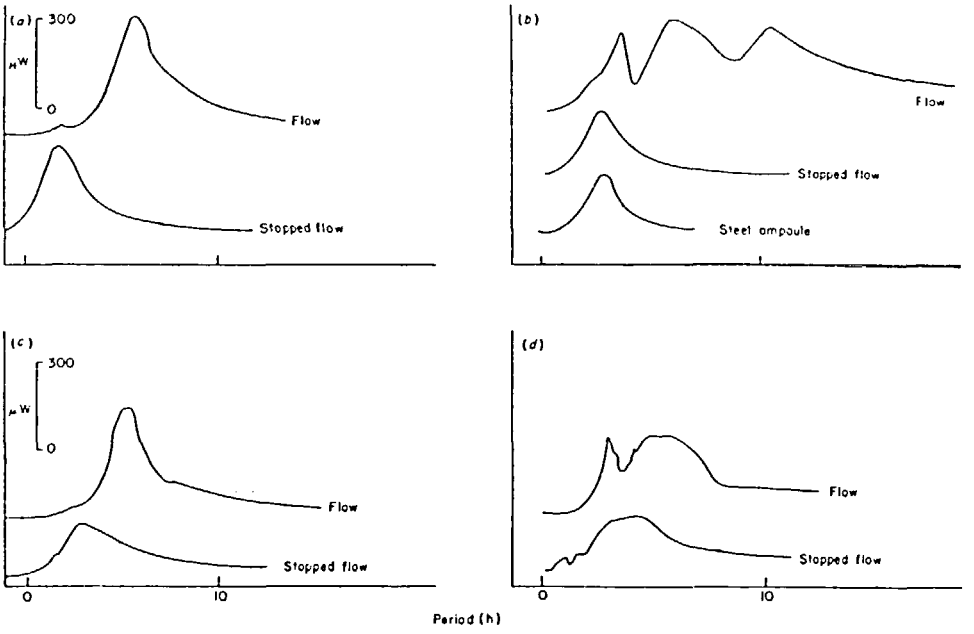

FIGURE 17. Thermograms of bacteria obtained under different oxygen concentrations. In flow experiments, growth was aerobic. In stopped-flow and in ampule experiments, growth was anaerobic. (a) *Streptococcus faecium* (b) *S. faecalis* var. *liquefaciens*, (c) *S. agalactiae*, and (d) *Escherichia coli* 281. (Reprinted from Monk, P. and Wadsö, I., *J. Appl. Bacteriol.*, 38, 71 (1975). With permission.)

1. Microorganisms

a. Fundamental Growth Studies

Bacterial calorimetry has been extensively reviewed by Forrest,[77,78] from which several general comments can be drawn relative to microcalorimetry as a tool for the study of bacterial growth. First, there is generally very little heat production during the lag phase of growth. This is due to the relatively small number of cells and the relatively low level of catabolic activity in the lag phase. During exponential growth, the heat production, rate of synthesis of new cellular material, and the appearance of products of catabolism all follow the same exponential function. Then upon entering the stationary phase, heat production drops drastically per unit weight of bacteria, as does the catabolic activity and rate of cell division. This decreased activity is a consequence of the limitation of some nutrient or energy source, or a result of the accumulation of toxic products in the growth medium. For aerobic organisms, oxygen depletion may be a limiting factor.

Thus, the general shape of a bacterial thermogenesis curve is bell-shaped, as is shown in Figure 17. There are some differences in the thermal growth curves among the various organisms, particularly as they enter the stationary phase. Some bacteria show marked oscillations and complex structural features in their thermogenesis curves which are quite reproducible.[78,79] Figure 17 shows some of these complexities and also shows that the same bacteria grown anaerobically produce rather different patterns, usually much simpler bell-shaped patterns. It is clear that the oxygen concentration in the medium plays a significant role in determining the observed behavior. The curves shown in Figure 17 were obtained with a flow microcalorimeter equipped with a flow-through cell. The bacteria are growing in an aerated vessel outside the calorimeter at 37°C, and samples are continuously pumped through the calorimeter cell so that heat generated by the cells during the residence time in the calorimeter is recorded. By stop-

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ping the flow, anaerobic conditions can be generated, since the walls of the calorimeter cell are impermeable to oxygen. Thus, the flow calorimeter, combined with stopped-flow mode of operation, can generate the two extremes of oxygen requirements for bacterial growth.

The exact nature of the complexities of the thermogenesis curves and the role of oxygen is not clearly understood. However, a recent study of lactic acid bacteria shows some interesting aspects of the problem.[80] Using a flow calorimeter, a suspension of *Streptococci* in a phosphate buffer was first pumped through the calorimeter, producing a small steady-state heat effect due to the endogenous metabolism of the cells. Next, glucose was added to the medium, and the heat effect increased to a new value due to the catabolism of glucose and an oxidative heat effect. If the flow is then stopped, producing an anaerobic condition, the heat effect decreases to a new steady state corresponding to anaerobic glycolysis. From earlier studies on fermentation, it is known that under anaerobic conditions the heat effect must correspond to rate of degradation of glucose to lactic acid in homolactic fermentation. In the presence of oxygen, the heat effect is the sum of the oxidative component plus a decreased anaerobic heat effect. In the presence of oxygen, the conversion of glucose to products can be represented by:

$$\text{Glucose} + XO_2 \longrightarrow (2 - X)\ \text{lactate} + X(\text{acetate} + \text{carbonate}) \qquad (12)$$

Under anaerobic conditions, X = 0, corresponding to the formation of 2 mol of lactate per mole of glucose. It is possible to calculate the enthalpy change of the above reaction from data in the literature. This comes out to be $\Delta H = -(117 + 509X)$ kJ/mol of glucose in phosphate buffer at pH = 6.2. Thus, the calorimetric determination of the ratio of the anaerobic ($\Delta H = -117$ kJ/mol) to aerobic heat effect allows a measure of X, the number of moles of oxygen reacting under the specified aerobic condition. It was found that for a particular experiment the calorimetric ratio was 2.35, leading to a value of X of 0.31 mol. The medium was analyzed for lactate, and yielded 1.67 mol. The value of X obtained from this stoichiometry, (2−X) = 1.67, is 0.33 mol, in excellent agreement with the calorimetrically determined value. Thus, by simple calorimetric measurements, the oxygen status and glucose-conversion stoichiometry, can be determined. Since the various strains of *Streptococci* exhibit differences in their ratio of oxidative to anaerobic conversion of glucose, the ratio of the aerobic to anaerobic heat effect could be a useful taxonomic feature with these particular organisms.

Growth curves for microorganisms other than bacteria have been reported. The extensive work by Lamprecht and co-workers on yeast cells has been reviewed recently.[1] Again, bell-shaped growth curves are common for the thermal effects of growing yeast cultures. In these cases, the oxygen status of the medium also is very important, as is dramatically shown in Figure 18. Under conditions of high oxygen pressure, the thermograms of a *Saccharomyces* strain show oscillations, which are also observed in turbidity measurements on the same system.[81] The oscillations were interpreted as due to density fluctuations of the cells caused by changes in CO_2 and water uptake. It is assumed that cells containing CO_2 resulting from glucose metabolism increase their volumes and, thus, become less dense. The cells begin to float to the surface of the liquid, where they eventually lose the CO_2 and sink again. When the oxygen pressure is high enough, the cells produce a significant increase in heat effect due to respiration, also causing rapid build-up of CO_2. Thus, the oscillations are very pronounced under high oxygen pressures. Again, the shapes of the thermogenesis curves depend very significantly on the conditions under which the microorganisms are growing.

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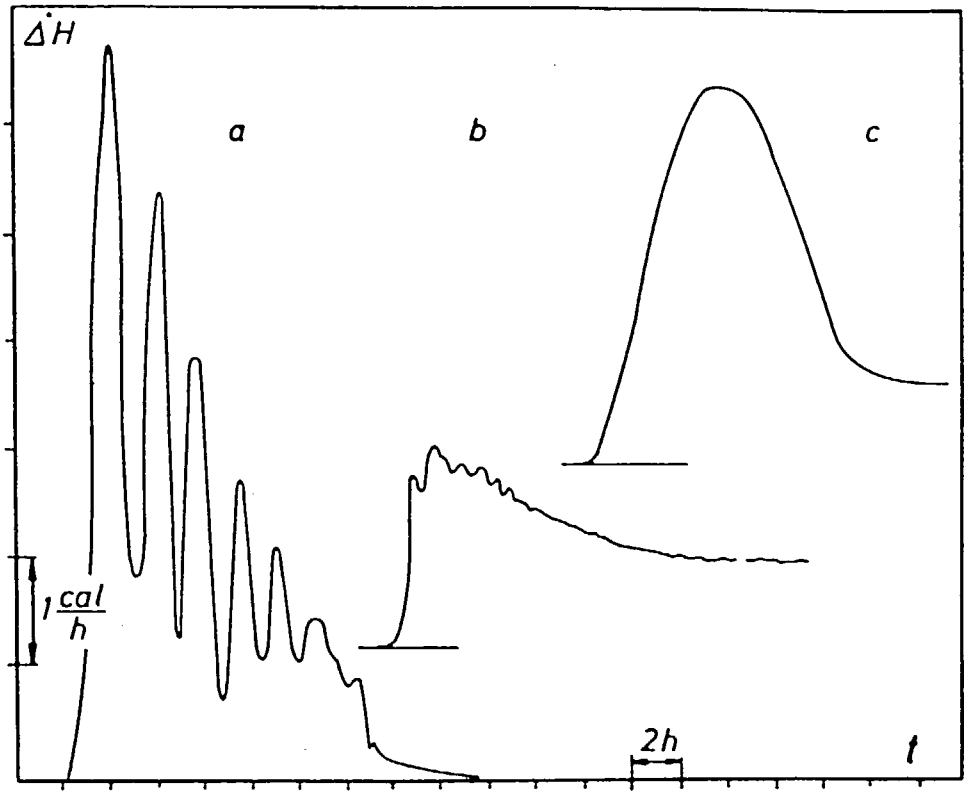

FIGURE 18. Thermograms of a strain of *Saccharomyces* in unstirred glucose-phosphate buffer under different oxygen pressures. (a) 90 m*M* liquid depth, 5 kP/cm² oxygen pressure; (b) 10 m*M* liquid depth, 2kP/cm²; (c) 50m*M* liquid depth and atmospheric pressure. (Reprinted from Schaarschmidt, B., Lamprecht, I., and Stein, W., *Biophysik*, 9, 349 (1973). With permission. Copyright by Pergamon Press, Ltd.)

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b. Bacterial Taxonomy Using Calorimetry

As has been discussed in the previous section, the anaerobic growth of bacteria generally produces rather simple thermal patterns. On the other hand, very highly characteristic and reproducible growth patterns are observed under aerobic conditions. The complex thermal patterns for aerobic growth would, therefore, seem to provide a valuable taxonomic tool for classification of bacterial strains. Boling et al., have published a study of a large number of different members of the family Enterobacteriace using a multichannel batch calorimeter.[82,84] In this case, a small volume of brain-heart infusion medium was innoculated with about 500 cells, and growth was observed for 8 to 14 hr. Characteristic patterns were found to be reproducible even though the oxygen status is not clearly defined in such a system. Russel and coworkers have published a summary of growth patterns for a large number of organisms by this technique.[84]

Monk and Wadsö, using the flow calorimeter mentioned above, obtained the curves shown in Figure 17 for several *Streptococcus* spp., and *Escherichia coli.*[79] Again, it is the aerobic patterns which show the complex behavior that would be useful for taxonomic studies. In one experiment with *Streptococcus faecalis* a sealed, steel ampule was used in a batch-type calorimeter,[74] yeielding a thermogram very similar to the stopped-flow pattern (see Figure 17b). It would therefore seem that any closed-batch calorimeter would be less suitable for general classification studies for aerobic orga-

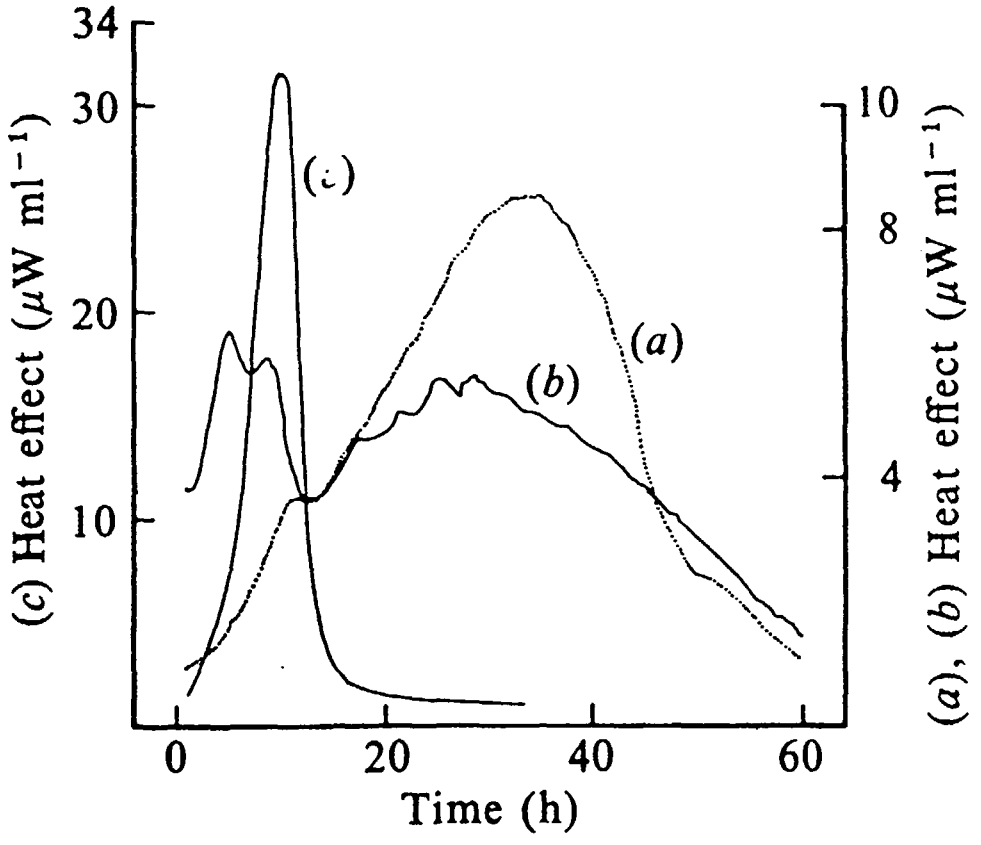

FIGURE 19. Thermograms of (a) *Acholeplasma laidlawii*, (b) *A. granularum*, and (c) *Ureaplasura urealyticum*. (Reprinted from Ljungholm, K., Wadsö, I., and Mardh, P.-A., *J. Gen. Microbiol.*, 96, 283 (1976). With permission. Copyright by the Cambridge University.)

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nisms since many of the structural subtleties in the thermograms disappear under the anaerobic conditions.

For organisms whose major energy sources involve fermentation, oxygen control is not so critical. In a study of several microorganisms of the order Mycoplasmatales it was shown that a static, batch-type calorimeter could be used to obtain distinctive growth curves.[83] Figure 19 shows thermograms of three of the organisms. The patterns were obtained from cultures grown in sealed ampules which contain an air space above the surface of the liquid. It was found that identical patterns were obtained whether the space was filled with air or nitrogen. The *Acholeplasma* spp. ferment glucose, while the *Ureaplasma* is unique in its ability to hydrolyse urea. In these cases, the growth medium is particularly critical because of the need for specific nutrients. For example, the mycoplasmas have an obligate need for sterols, and the ureaplasmas show very small heat effects unless urea is present in the medium.

From these studies on bacterial growth, it is apparent that in order to use calorimetry for classification and identification purposes, careful study of growth media and oxygen requirements are necessary. In addition, it is useful to have information obtained by conventional microbiological methods, e.g., plate counts, pH changes during growth, and glucose consumption, so that quantitative correlations of calorimetric thermograms to growth conditions can be made. Once appropriate growth media and oxygen requirements have been established, it is then possible to set up the necessary conditions for obtaining reproducible and characteristic thermograms of the microor-

ganisms. It would seem that the greatest value of calorimetry in this field would be in "fingerprinting" the microorganisms. The use of a thermogram to confirm the identity of a suspected organism would seem of great value for clinical methods. The system described by Russel[82,84] with many calorimetric channels would allow testing of an organism in several media, and the resulting thermograms could be directly compared with files of patterns from known species. The possibility of using a computerized identification system combined with calorimetry has recently been suggested by Johansson et al.[85]

c. Cytotoxic Agents and Calorimetric Growth Patterns

Several years ago Binford et al., published a method for qualitatively determining the sensitivity of several strains of bacteria to various antibiotic agents.[86] Using a flow microcalorimeter, an initial thermogram was run on the bacteria in an appropriate growth medium for about 10 min, in which time there was an increased heat effect indicative of growth. Subsequent experiments were conducted using the same culture and medium, except that an antibiotic was added to the medium. If the heat effect increased as in the pure culture, the bacteria were considered resistant, while an unchanging or decreasing heat effect was taken to mean that the organism was sensitive to the chemical agent. Correlations were made with the standard disk-agar-diffusion method, and the calorimetric sensitivity tests agreed in 87% of the samples with the disk-agar method if the cultures were saturated with oxygen prior to the calorimetric experiment.

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Since the thermograms do reflect growth patterns, it seems reasonable that any agent that can alter growth of the microorganisms will also alter the heat effects associated with growth. A detailed study of the effects of tetracyclines on the thermal growth patterns of *E. coli* has been reported by Mardh et al.[87,88] The drugs were added in both the lag phase and during exponential growth. A batch calorimeter was used for the lag-phase studies, and a flow calorimeter was used for the log-phase work. Figures 20 and 21 show thermograms obtained in typical experiments in the two phases of growth. The effect of the antibiotic added during the lag phase was to postpone the onset of exponential growth, as well as to cut down the total heat production relative to control, as shown in Figure 20. The time required to reach a heat effect of 2 μW/mℓ was 12.5 hr for minocycline, 7.3 hr for doxycycline, 6.6 hr for oxytetracycline, and 4.5 hr for tetracycline when the concentration of antibiotic was 0.4 μg/mℓ. When the antibiotics were added during exponential growth, there was an immediate alteration of the heat effect curve, doxycycline causing a heat decrease which remained without marked change for about 16 hr. For tetracycline, the change in heat production was less pronounced. First there was a small reduction which remained constant for about an hour, followed by a general increase in heat production (See Figure 21). Increasing the concentrations of the antibiotics produced a corresponding decrease in heat production. The larger doses caused an almost total cessation of thermal activity. These results show that there is a definite correlation between heat production and the rate of cell metabolism, and that there are clear differences in kinetic and total effectiveness on growth among the different antibiotics. Because the time-response behavior can be directly monitored by calorimetry, these studies can provide information on the kinetics of action of antibacterial agents that cannot be obtained by conventional techniques. The application to the establishment of optimum dose levels and intervals seems most interesting for the fields of pharmacy and medicine.

Because the metabolic processes in cells can be altered by addition of chemicals, the possibility of developing bioassays for such agents using calorimetric monitoring has been investigated. Jensen et al. have studied the thermogenesis curves for *Streptococcus faecalis* cells in the presence of several cytotoxic agents.[89] Using an isothermal

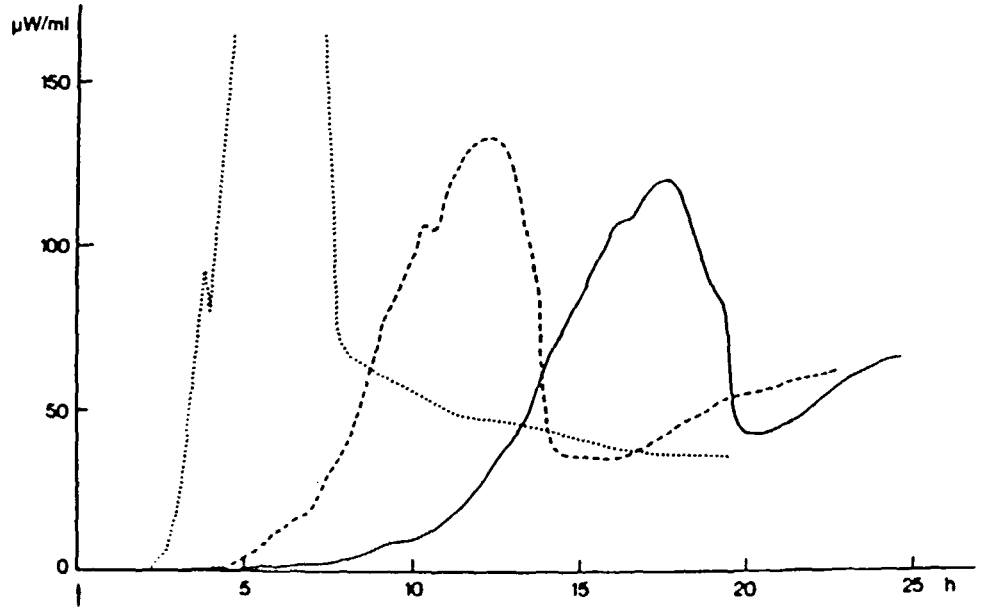

FIGURE 20. Thermograms of *Escherichia coli* grown in presence of doxycycline (—) and tetracycline (----) at a concentration of 0.4 μg/ml (½MIC). A nonantibiotic containing culture is also shown (....). (Reprinted from Mårdh, P.-A., Andersson, K.-E., Ripa, T., and Wadsö, I., *Scand. J. Infect. Dis. Suppl.*, 9, 12 (1976). With permission.)

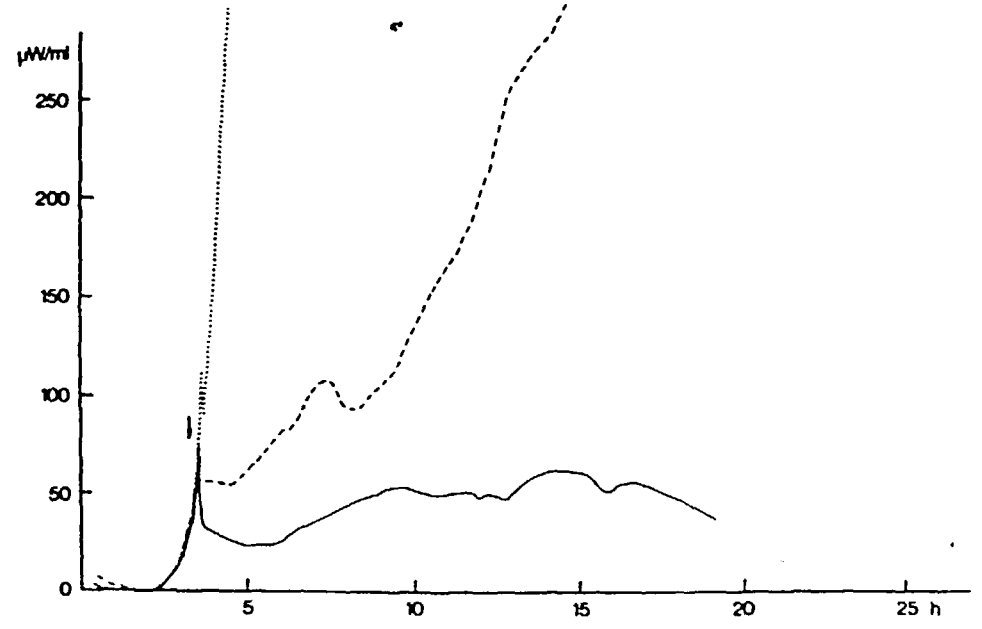

FIGURE 21. Thermograms of *Escherichia coli* to which doxycycline (—) and tetracycline (----) were added at the same level of heat production during logarithmic-growth phase. Concentration of antibiotic was 1.6 μg/ml. Reference thermogram is indicated by (.....). (Reprinted from Mårdh, P.-A., Andersson, K.-E., Ripa, T., and Wadsö, I., *Scand. J. Infect. Dis. Suppl.*, 9, 12 (1976). With permission.)

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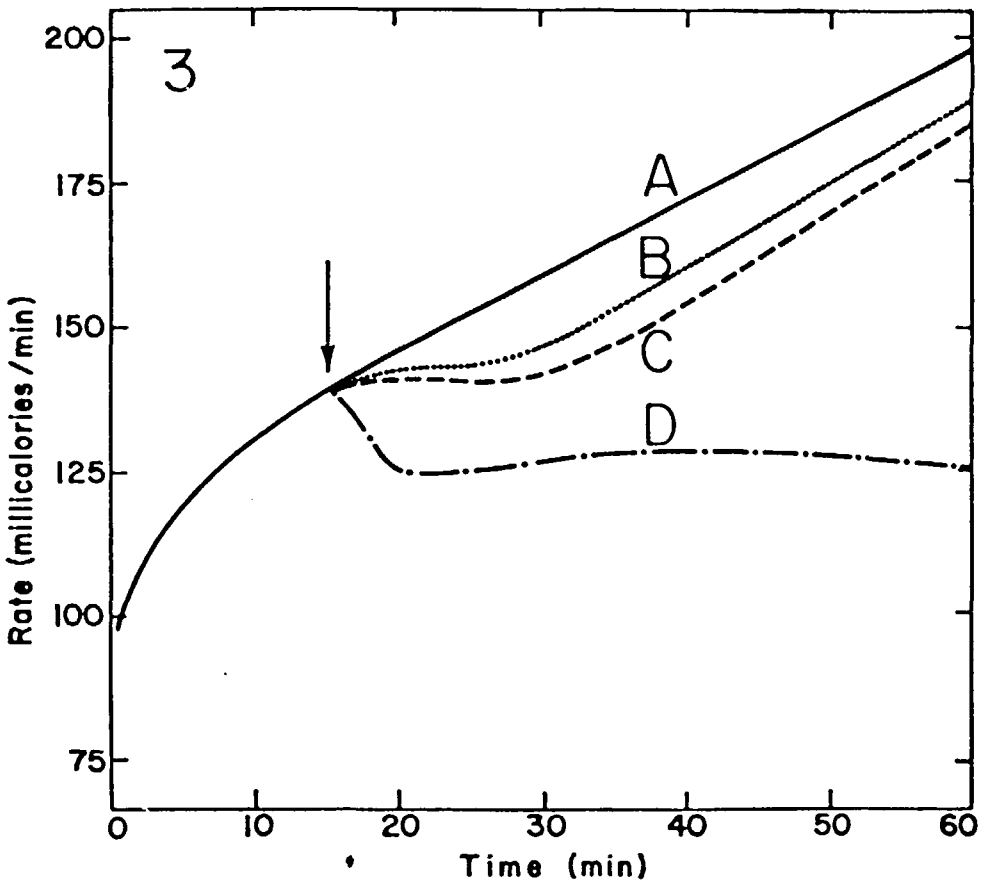

FIGURE 22. Plot of the rate of heat production vs. time for the addition to *Streptococcus faecalis* culture. (A), standard curve. (B), 200 μl of heat-inactivated human serum. (C), 200 μl of normal human serum. (D), 200 μl of 0.28 μg of tetracyline-HCL/μl normal human serum. Samples were injected into 25 ml of 4% DMSO broth at the arrow. (Reprinted from Jensen, T. E., Hansen, L. D., Eatough, D. J., Sagers, R. D., Izatt, R. M., and Christensen, J. J., *Thermochem. Acta*, 17, 65 (1976). With permission.)

calorimeter[90] thermal growth curves were obtained on the organism in 25 ml of growth medium consisting of tryptone, yeast extract, K_2HPO_4, NaCl, and glucose. After mixing the organism with growth medium, the heat production was monitored for about 1 hr. A steady, linear increase in heat production occurred after about 15 min. When the various chemical agents were added, departures from the curve corresponding to the untreated samples were observed. For example, addition of 250 μl of a 0.63M KCN solution caused a sharp decrease in heat production, followed by a rapid return to an almost constant value. Sodium fluoride produced only a minor perturbation of the growth curve. Antibiotics, such as penicillin G or tetracycline caused heat production to decrease or become constant, the particular level dependent upon the drug. Figure 22 shows the behavior of the thermogenesis curves in the presence of human serum and human serum with tetracycline. The amount of tetracycline would correspond to about 280 ppm of tetracycline in the serum. Also shown is the curve for heat-treated serum, which would be presumed to have fewer cytotoxic agents. Human serum causes a temporary reduction in heat production, but the curve soon returns to a line parallel to the untreated samples. The heat-treated serum sample caused less displacement from the standard curve. Serum with tetracycline produced a curve similar to

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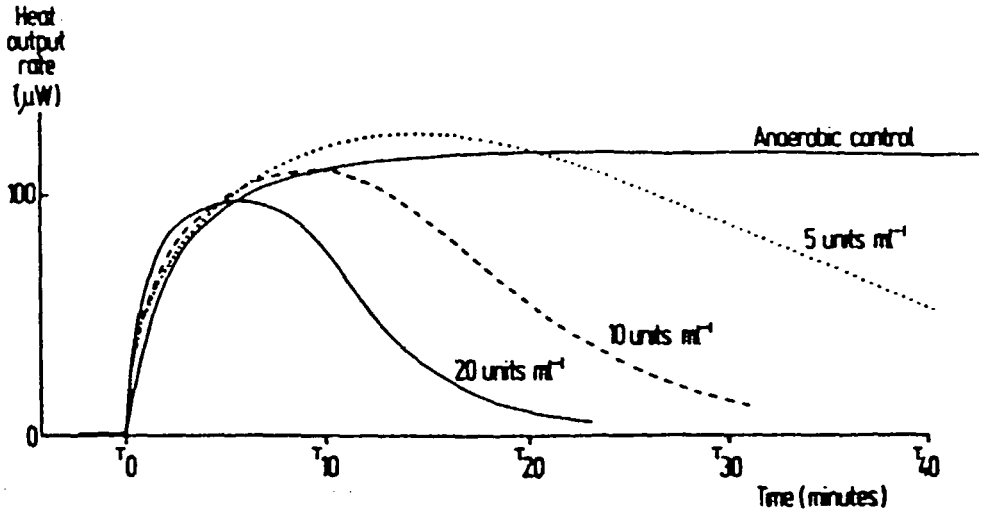

FIGURE 23. Thermograms of *Saccharomyces cerevisiae* containing increasing amounts of nystatin bulk material. (Reprinted from Beezer, A. E., Newell, R. D., and Valentine Tyrell, H. J., *Anal.Chem.*, 49, 34 (1977). With permission. Copyright by the American Chemical Society.)

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the tetracycline alone, a decrease in heat production followed by a leveling off. The rate of decrease seemed to relate to the concentration of the drug, so the measurement of the half-life of the thermal decrease was proposed as the basis of a bioassay for tetracycline in serum.

A bioassay for nystatin bulk material, a polyene antifungal agent, has been developed by Beezer et al.[91] In this procedure, which uses a flow calorimeter with flow-through cell, heat production in a yeast culture of *Saccharomyces cerevisiae* is examined in the presence and absence of the antibiotic. Figure 23 shows typical thermograms of yeast suspensions containing varying amounts of nystatin. Anaerobic conditions were maintained by flushing the incubation medium with nitrogen. The shapes of the curves show a definite relationship to the concentration of antibiotic, but the functional dependence is rather complex. The decay of thermal activity does not follow any obvious kinetic law, so there is no simple parameter to relate to dose. It was decided that the response parameter which gave the best dose-response correlation was the time required for the signal to rise and then fall to some arbitrary percentage over baseline. The zero time was chosen as the time at which the first calorimetric response is detected. Plots of this response time vs. log dose gave straight lines when the 30 or 40% value over baseline was chosen as the parameter. Response time values ranged from about 6 to 36 min in the range of dosages studied. It was found that the yeast cells are more susceptible to nystatin as the preparation age decreases. This affects the sensitivity range accessible for calorimetric measurements. For example, with cells which are 4 hr old, the sensitivity range is 0.5 to 5 units/m*l*, while for 12-hr-old cells, the range is 10 to 75 units. Comparisons were made with the agar-plate-diffusion method for nystatin assay, and compatible results were obtained if the values derived from each method were within the standard deviations of the individual measurements. Reproducibility of the calorimetric measurements is ±3.5%, compared with ±5 to 10% for the agar-plate method. The sensitivity is about 0.5 un/m*l*, compared with 20 un/m*l*. The results can be obtained in about 1 hr, while the agar-plate method requires about 16 hr per sample. Thus, this bioassay procedure seems to have definite advantages over conventional methods for nystatin assays.

Beezer et al. have applied this bioassay procedure for other antifungal agents.[92] Can-

dicidin, amphotericin B, filipin, pimaricin, lycensomycin, and clotrimazole were studied in the μmol range. Some basic differences in thermal behavior were noted for several of these antibiotics. Pimaricin, clotrimazole, and lucensomycin gave curves similar to the control suspension (Figure 23), except at lower levels of thermal activity. This behavior contrasts with that shown by nystatin and others in the series, which cause a decreased heat effect with time, presummably due to cessation of cellular activity. It thus appears that the inhibitory effects of these different classes of antibiotics are a result of different mechanistic details in the interaction with the cells. That these subtleties can be directly observed calorimetrically illustrates the power of the technique in studies of pharmacokinetics. As in the nystatin bioassay discussed above, the reproducibility of results is about ±3%, and the assays are in general agreement with the agar-plate diffusion method. The calorimetry method allows a classification of potency to be established on the basis of the time required to inhibit respiration, the most potent being the drug which inhibits respiration in the shortest time.

It appears from these investigations on cytotoxic agents that calorimetry has a valuable role to play in antibiotic-sensitivity testing, in studies of the kinetics and mechanism of drug-host interactions, and in the development of bioassay procedures not only for drugs and antibiotics, but also for any chemical agents which might effect the growth of microorganisms.

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d. Measurement of Microbial Activity in Mixed Cultures

In many natural systems, such as soils, foods, water, or biological fluids, mixed cultures of microorganisms are active. In such cases, heat production is a consequence of the total activity of the entire mixed population of organisms. One might expect that such systems would show rather complex thermal behavior because of the potentially very diverse metabolic events occurring as the organisms grow. Under carefully controlled conditions, however, it has been found that thermal activity in some natural systems is relatively simple, even though the interpretation of the activity might not be so.

A number of years ago, Walker and Forrest studied fermentation in the rumen of sheep.[93] In spite of the number of different organisms and possible number of concurrent reactions proceeding, the kinetics of heat production was simple first order over long time periods. Differing samples did show different total heat production, but thermal activity seemed to correlate very well with total amounts of solid substrates present. Because of the simple behavior, effects of perturbations, such as addition of reagents or metabolic intermediates, could be directly examined. For example, additions of sodium ion or lactate, neither of which are normal intermediates in the breakdown of carbohydrate in the rumen, strongly inhibit heat production. On the other hand, cellobiose causes a jump in heat production. Cellobiose is an intermediate in the breakdown of cellulose.

Studies of soils have been reported by Mortensen et al.[94] Using a batch calorimeter designed for biological samples,[74] an ampule containing a soil sample was introduced into the calorimeter. The measured heat production remained constant for many hours in the calorimeter under these conditions. Experiments were conducted on the effects of nutrients on the rate of heat production. Addition of powdered cellulose alone was found to have no effect on heat production over a period of 10 days. Upon addition of a mixture of salts plus cellulose, after a 3-day lag period, heat production steadily increased for 7 to 8 days. The salt mixture, containing $NaNO_3$, $MgSO_4 \cdot 7H_2O$ and K_2HPO_4, had no effect on thermal activity by itself. The results indicated that microbial activity, as well as specific decomposition reactions, are involved in total heat production in such a system. The use of calorimetric data of this kind for monitoring the effects of possible pollutants in the soil seems very feasible. Any deleterious effect

on microbial activity caused by pesticides or other undesireable chemicals could be directly observed through comparison of the thermal production of control and suspect samples.

The use of microcalorimetry for actual bacterial count determinations in urine has been reported by Beezer et al.[95,96] First, studying the heat production in a flow calorimeter as a function of cell count for 15 strains of bacteria which commonly cause urinary infection, it was found that within 2 hr of incubation in a nutrient-broth, samples containing originally greater than 10^5 cells/mℓ gave easily detectable signals. The common infecting strains showed linear correlation of plate count to heat production. Examination of simulated urine samples showed that the components of urine did not interfere with heat production by the cells. Applying the method to 299 actual urine samples showed that significant bacteriuria was detected by the microcalorimetric method with a false negative rate of 1% and a false positive rate of 15%. While longer incubation times would make the method more sensitive, it seems quite useful to find that 36% of the urine samples studied could be eliminated from further investigations within 2½ hours of receipt in the laboratory. Most common bacteriological techniques require about 18 hr for quantitative culture counts.

In closing this section on microorganisms, it is apparent that many very useful qualitative and quantitative applications of microcalorimetry are possible in the fields bacteriology, pharmacology, clinical medicine, and in pollution control. Both as a general analytical probe and as a specific tool for determining cellular vitality, calorimetry has many advantages over some of the available techniques for studying microorganisms. In addition, the nature of the origins of thermal signals makes it possible to examine phenomena not normally accessible by other available methods.

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2. *Blood Cells*

The other major category of living cells which have been investigated by calorimetric methods is the blood-cell system. Again, as with microorganisms, the cells can be studied with available microcalorimeters, thus requiring no major design changes to accomodate the cells. Some of the early work in this field by Levin and Wadsö and coworkers has been reviewed recently.[1] One of the major concerns of these investigations is to find typical values of the heat production in normal blood by the major blood cell components, erythrocytes, leukocytes, thrombocytes, and lymphocytes. The thermal activity of these blood components can then be used in studies of metabolism and other fundamental biochemical problems. In addition, there has been interest in the use of the heat production by the individual blood cells, as well as by whole blood, to investigate blood disorders and disease. So, from the point of view of clinical chemistry, calorimetric studies of blood cells are of basic interest. Let us first examine some of the work on the individual blood components, and then look at the more complex problems of whole blood and blood disorders.

a. *Calorimetric Studies on Erythrocytes*

The most extensive calorimetric work on blood cells has been with erythrocytes, the red blood cells. Levin has reported several studies on erythrocytes using flow calorimeters.[97,98,99] Measurement of heat production was accomplished by flowing a washed suspension of human erythrocytes in heparin plasma through the calorimeter after establishing a baseline with plasma alone. The deflection of the baseline was calibrated in terms of heat production/mℓ of packed cells. Duplicate determinations were reproducible to about 5%, and day-to-day precision on samples from one individual was about 10% relative standard deviation. The mean heat production from 59 healthy persons was 116 μW/mℓ of packed erythrocytes with a standard deviation of about 18 μW/mℓ. Heat production was also examined in two synthetic media, one containing

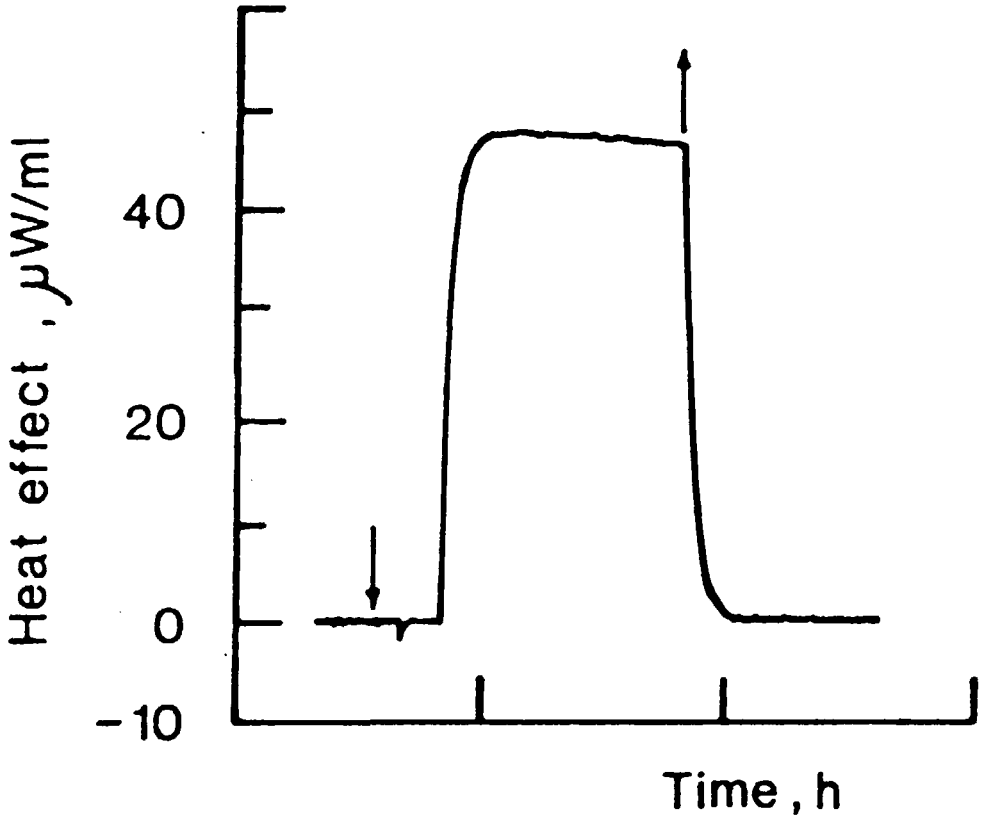

FIGURE 24. Thermogram for erythrocytes suspended in plasma. Arrows indicate introduction and removal of sample ampoule from calorimeter. (Reprinted from Monti, M. and Wadso, I., *Scand. J. Clin. Lab. Invest.*, 36, 573 (1976). With permission.)

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amino acids in addition to the basic constituents of albumin, salt, and glucose.[99] Incubations of the cells for up to 96 hr at 37°C were periodically examined by the flow-calorimeter method. Heat production was found to be higher during early stages of incubation, but after about 12 hr a steady state was reached. The excess heat production in the early stages of incubation was closely correlated with glucose consumption and lactate production in the samples. Changes in the concentrations of other expected metabolites caused only minor effects on total heat production.

An extensive investigation of heat production by erythrocytes has been reported by Monti and Wadsö.[100-103] Using their batch calorimeter,[74] heat production was measured on the erythrocytes suspended in plasma and under a variety of other conditions in order to determine the effects of various experimental conditions on the observed heat effects. A typical experimental curve is given in Figure 24. The measured heat effect is obtained approximately 40 min after introduction of the cell suspension into the calorimeter. It was found that heat production by this method was about 79 ± 11 μW/mℓ of packed cells in plasma, compared with the 116 μW/mℓ value reported by Levin above. However, in subsequent studies of variations in experimental conditions, the heat production per cell was found to be very sensitive to several important parameters.[102,103] For example, the heat effect was found to change about 1.2%/0.01 pH unit, and when the erythrocytes were originally stored at 4°C, the heat effect increased initially by about 6%/hr. After about 24 hr of storage, the heat effect was 50% higher than the initial value. Heat production was also very dependent on temperature. The

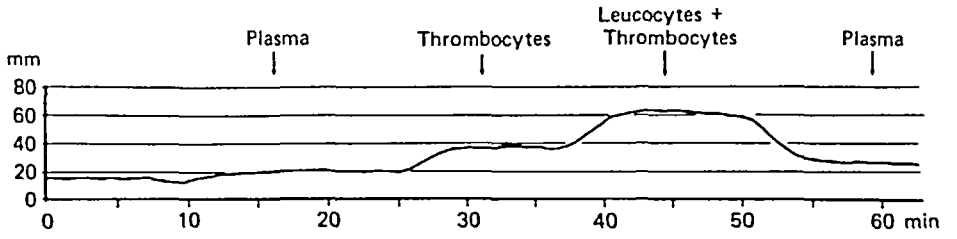

FIGURE 25. Thermograms from flow calorimetric experiments of heat production by plasma, thrombocytes, and leukocytes and thrombocytes mixed in plasma. (Reprinted from Levin, K., *Clin. Chem. Acta*, 32, 87 (1971). With permission.)

value at 25°C was 47% of the effect at 37°C and 170% at 41°C. A comparison of results using flow vs. batch calorimeter measurements showed no significant difference in heat effect, but there was an effect caused by the medium in which the cells were suspended. Higher heat effects were found in glucose-phosphate buffer than in plasma. These results show clearly that in order to obtain a standard heat effect for erythrocytes, conditions of preparation and storage of the cells, pH and media composition, and temperature must be carefully specified and standardized. With samples prepared by an adsorption method, not stored at low temperature, at pH = 7.40, and at 37°C, the heat effect in plasma is 99.6 ± 9.6 μW/mℓ of packed cells for cells from healthy males.[103] Interestingly, the corresponding value for females is 91.8 ± 6.5 μW/mℓ. Slightly higher values are obtained in glucose-phosphate buffer under the same conditions, 101.7 ± 11.4 μW/mℓ for male subjects.

Some metabolic stimulation effects on erythrocytes have been studied by microcalorimetry.[104] Erythrocytes are known to metabolize glucose mainly by the Emden-Meyerhof anaerobic pathway. In the presence of methylene blue, however, the aerobic, pentose phosphate pathway is stimulated, and the conversion of glucose to CO_2 and H_2O results. Because the enthalpy associated with conversion of glucose to CO_2 and H_2O is about 25 times that for production of lactate, an increased heat production would be expected. It was found that the ratio of the heat effect with methylene blue added was 7.8 times greater than without in plasma samples. In serum and in buffer, the ratios were 7.4 and 10.2, respectively. The pH dependence of the heat effect was found to be smaller with methylene blue than in pure plasma samples.

b. Leukocytes and Thrombocytes

Several systematic studies of leukocytes and related blood components have been reported. Levin has measured heat production by thrombocytes and mixtures of leukocytes and thrombocytes.[105] Figure 25 shows an experimental curve with various levels of heat production in the leukocyte-thrombocyte system. The final plasma baseline was found to be above the origianl due to leukocytes adhering to the calorimeter cell walls. As high as 50% of the leukocytes were lost in a typical experiment because of this adhesive effect. A redesigned flow system which allows air bubbles to be introduced into the flow line ensures a uniform movement of the cells through the calorimeter cell.[106]

Batch-type calorimetric measurements have been made on thrombocytes under a variety of conditions of pH, temperature, and preparative methods.[107] The effect of pH on the heat effect was considerably less than with erythrocytes. Heat production was not changed by cell concentration, or by storage for several hours at room temperature. The value of the heat effect at pH = 7.40 was found to be 61 ± 10 fW/cell. Thus, as with erythrocytes, there seems to be a characteristic heat effect associated with normal thrombocytes from healthy individuals.

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In connection with studies of heat production in whole blood, Bandmann et al. have determined standard heat effects for lymphocytes and polymorphonuclear leukocytes as well as for erythrocytes and thrombocytes.[108] In the measurements on leukocytes and thrombocytes in plasma using the batch calorimeter, curves like those in Figure 24 were obtained, except that there is an initially high heat production which decreases rapidly to steady-state values after 30 to 40 min. These peaks were not observed in buffer, and their origin is not understood. The heat effect values reported for leukocytes and thrombocytes were obtained after reaching the steady-state condition. Leukocytes in plasma showed higher heat effects than when suspended in buffer, the values being 3.5 pW/cell in plasma and 1.2 pW/cell in buffer. Lymphocytes also gave different values in buffer (4.6 pW/cell) and in plasma (2.2 pW/cell) suspensions.

c. Whole Blood

Having evaluated the heat production contributions from the individual cell fractions, Bandmann et al. used these values to calculate the expected heat effect for whole blood.[108] If there is simple additivity of the heat effects, the measured values for whole blood should agree with calculated heat production. Using the mean values for the fractions measured for 11 samples, the following summation resulted:

Cell fraction	Heat effect (mW/*l* whole blood)
Erythrocytes	34 ± 2
Leukocytes	12 ± 3
Lymphocytes	5 ± 1
Thrombocytes	12 ± 2
Plasma	0.5 ± 0
Total	63.5 ± 5

The average measured value on whole blood for the 11 subjects was 62 ± 7 mW/*l* of blood, in excellent agreement with the expected value. Thus, measurements on whole blood do reveal the total activity of the various cell fractions. This factor suggests that some studies on blood-cell metabolism or the behavior of the various fractions can be studied directly on whole blood, especially for erythrocytes, leukocytes, and thrombocytes, which are the major heat producers in whole blood. For example, it has been shown that leukocytes show enhanced heat production during phagocytosis of latex particles (see Reference 1). This same stimulation of heat production was observed in whole-blood samples diluted with buffer containing latex particles. Because the effects can be observed in whole blood, the preparative steps to isolate individual fractions are eliminated. This could be a definite advantage in many studies.

d. Blood Cell Heat Production and Disease

A number of observations have been made on the erythrocytes of patients with anemic disorders. In their original work on heat production by erythrocytes, both Levin[98] and Monti and Wadsö[100] noted significant increases in heat effect from cells of anemic patients. For example, the average value of heat production with various types of anemia was found to be 126 ± 29 mW/*l* of packed cells, compared with 79 ± 11 mW/*l* for normal subjects.[100] While the origin of the excessive heat production is not clear, increased glycolytic activity has been demonstrated in several pathological conditions with anemia.[98] Whether this is due to a change in basic metabolism by the cells or to the presence of more young cells, often observed with hemolytic disease, remains to be determined.

A study of heat production by the erythrocytes of patients suffering from chronic

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uremia has been reported recently by Monti.[109] Anemia often is found in cases of renal failure, thought to be a result of abnormalities in erythrocyte metabolism. One theory is that the anemia results from a decreased activity of the pentose-phosphate-pathway in the erythrocyte metabolism of glucose. Since the pentose shunt can be stimulated by methylene blue (as discussed above), microcalorimetric measurements on the erythrocytes of uremic patients might provide insight into the origins of the anemia. It was found that methylene blue stimulation of the pentose shunt produced a higher heat production in uremic patients, the ratio to normal subjects being 1.18 in plasma. However, the unstimulated cells also indicated a higher heat production in the uremic patients, the ratio being 1.21 in this case. These results seem to indicate no major decrease in erythrocyte aerobic metabolism in the pentose shunt. A similar study of erythrocytes in anemic patients with liver disease came to the same conclusion, again no decreased activity in the pentose shunt in those patients with anemia.[110] In both of these studies, calorimetry has provided a method for examination of rather subtle metabolic processes in the blood cells.

The red blood cells of hyperthyroid patients are known to show increased oxygen and glucose consumption. Thus, calorimetry has been applied to a study of the erythrocytes of patients with hyperthyroidism, before, during, and after treatment for the condition.[111] With patients that exhibited definite clinical symptoms of hyperthyroidism, the mean heat production of their erythrocytes was 111 ± 20 mW/*l* of cells. After treatment, patients in euthyroid states showed mean heat production values of 95 ± 12 mW/*l*. These values compare with 86 ± 8 mW/*l* obtained for normal subjects. During these calorimetric tests, there was a definite correlation of heat production with CDI, the clinical diagnostic index, a measure of the clinical state of the patient. There was a pronounced decrease in heat effect as treatment commenced. This study illustrates the value of using heat production as a test for clinical condition and the peripheral effects of the thyroid hormones.

Another calorimetric testing procedure has been reported by Levin for diagnosis of systemic lupus erythematosus, a syndrome of unknown cause or causes characterized by multisystem involvement.[112] Flow calorimetry is used to measure heat production in leukocytes stimulated by addition of homogenate prepared from the subjects own leukocytes. In normal patients, little stimulation occurs, but in 12 out of 13 cases of systemic lupus erythematosus, increased heat production was noted, the highest value being a 400% increase in thermal effect. The stimulation of leukocyte heat production in these cases is regarded as a manifestation of phagocytosis upon addition of the autohomogenate. A relation was found between the extent of stimulation and the activity of the disease in the patients.

While these applications of calorimetry for the study of blood disorders and disease activity are rather new, the relative ease with which measurements can be made and the general utility of the information seem of great value to clinical and diagnostic medicine. Also, as a tool for the investigation of basic metabolic events in the cells, calorimetry seems highly practical in many cases.

IV. SUMMARY

In this review, I have attempted to present a perspective of three major application areas in which calorimetry can be used to obtain valuable analytical and basic biochemical information. The feasibility of using specific enzyme-substrate reactions coupled with calorimetric measurements has been demonstrated clearly in a number of biochemically and clinically significant applications. Particularly with the immobilized-enzyme flow calorimeters, rapid, reproducible, accurate, and relatively low-cost analytical measurements can be obtained for several important substrates. Specific meth-

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ods for determination in serum of glucose, urea, uric acid, and other clinically important substances have been demonstrated.

Another fundamental calorimetric technique, temperature-scanning calorimetry, was shown to be a powerful tool for the study of proteins, polynucleotides, and lipidic materials in aqueous solution. In addition to giving basic thermodynamic information on conformational transitions, DSC provides a technique for characterizing structural variations in biopolymers and complex liposomes under varying conditions in solution. The investigation of protein denaturation, polynucleotide unraveling or base-pair stacking, and the behavior of biomembranes and lipid bilayers by DSC methods has provided a basis for fundamental interpretations of the behavior of these complex systems.

Basic flow and batch microcalorimetry have been demonstrated as valuable techniques for the examination of living cells. Calorimetric growth studies on bacteria have provided a basis for the use of thermograms in bacterial classification and in the determination of total microbial activity in mixed cultures. Heat production by bacteria has been clearly related to the effectiveness and time-dose response of antibiotic agents, thus making it feasible to use calorimetry for antibiotic sensitivity testing and for developing bioassay methods for cytotoxic agents. Investigations of heat production by blood cells have proven that basic biochemical information about blood-cell metabolism can be generated from calorimetric experiments. Also, because heat production by the cells can be altered by disease, calorimetric measurements can be used to examine disease states involving the blood cells. In addition, as a monitor of patient condition in clinical treatment, blood-cell calorimetry has opened new possibilities in this important medical field.

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In looking to the future, several points should be made. First, in order for enzyme-calorimetry methods to compete with other methods in biochemical and clinical analysis, execution time and automation are important factors. Certainly, flow calorimeters, and particularly the immobilized-enzyme flow calorimeters, are capable of rapid enough execution to be competitive with present procedures in clinical analysis. The flow calorimeters are also quite easy to automate with existing technology. Although calorimetric methods are not widely employed in present clinical, analytical procedures, the best possibilities for exploitation are in those problems where sample preparations to remove turbidity or dispersed phases are cumbersome, or where spectral interferences prevail in the medium of interest. Batch calorimetric measurements can be made on dispersions or turbid solutions, and the presence of spectral absorbers is of no consequence in the calorimetric enzymatic methods, It would be of interest to see if calorimetry could be combined with enzymatic reactions for the determination of fats and other related lipids, since these are troublesome analytical problems.

Differential scanning calorimetry is a unique tool in itself for studies of biopolymers and other complex, aggregated systems. DSC measurements on proteins and polynucleotides have provided a basis for understanding the energetics and conformational status of these biopolymers, so it would seem that fruitful future applications might be in studying the effects that alteration of media, or chemical alteration of the biopolymer itself, might have on their basic properties. For example, how thermal transitions in DNA or related nucleotides would be effected by mutagenic agents should be studied. Any alternations in the basic helical structures of DNA ought to be reflected in both the position and size of the melting transitions of the biopolymer.

The study of living cells by calorimetry promises to offer a number of future possibilities both in basic biochemical studies and in clinical chemistry. Investigations of heat production by other cellular systems and further studies on the effects of environmental conditions of the cells on the thermograms would seem very fruitful. The development of new bioassay procedures using living cell cultures ought to be examined

for clinically important substances. The possibility of using cellular heat production by other than blood cells to indicate disease states would be potentially very valuable.

Analytical calorimetry is a relatively new technique in biochemistry, and in addition to the development of new analytical methods for clinical chemistry, future uses in pharmacokinetics, medical biology, and pollution control seem considerable. This review has examined only a few major biochemical problem areas which have used the calorimetric technique. Numerous other experimental problems have been attacked using various calorimetric methods, including a considerable number of protein reactions other than enzyme-substrate reactions. The use of the enthalpimetric titration technique in biochemical analyses has been extensive. Then, of course, the wide use of DSC for the characterization and study of solid substrates is well known. Thus, we see that calorimetry is a broad-based, established analytical technique which has contributed and will contribute much valuable scientific information to a variety of basic fields.

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