Biocalorimetry: Past and Present. Some Views about the Future.

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INTRODUCTION

Biocalorimetric work is performed on different levels: model work, work on biochemical compounds and work on systems which have, more or less, retained their biological organization and life functions. Current studies include thermodynamic investigations as well as work in which the calorimeters are merely used as general analytical instruments. Thermodynamic studies on purified and well-defined biochemical compounds form a central part of the field. However, results are normally difficult to interpret on the molecular level and there is also a need for studies of model systems of differing complexity. Within a series of model compounds, the structure can be varied systematically and correlations can be made between thermodynamic data and structural features; this is usually not possible for the more complex biochemical compounds.

A range of non-thermodynamic calorimetric determinations are also carried out on biochemical compounds, in particular, relating to enzyme assays and determinations of substrate concentrations.

Biological systems are poorly defined from a thermodynamic point of view, and calorimetric measurements do not normally provide thermodynamic data which can be discussed on a molecular level. The most important use of calorimetric measurements on living systems is for purely analytical determinations. Often, the calorimeter is used as a general monitor of the biological process.

Space will not allow me to give a thorough review of the historical developments and the present status of biocalorimetry; therefore the present survey is rather sketchy. For more comprehensive summaries, the reader is referred to several published[1-3] as well as unpublished[4,5] review articles. Very useful bibliographies are found in the annual Bulletin of Chemical Thermodynamics[6].

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HISTORICAL DEVELOPMENTS

Biocalorimetry is a very old branch of science. Some of the first calorimetric experiments ever performed involved studies of biological systems[7,8]. As early as about 1780, Crawford, in England, and Lavoisier and Laplace, in France, calorimetrically measured the heat production in small animals. Lavoisier and Laplace deduced from their calorimetric measurements that at least the major part of animal heat production can be accounted for by formation of 'fixed air' (CO_2) from 'pure air' (O_2).

During the following 100 years, there was not much progress in the field, but toward the end of the 19th century, combustion calorimetry was developed and was employed on many biologically important compounds. For instance, in 1894, Rubner applied combustion calorimetry to animal materials in combination with respiration calorimetric studies on living animals. During the first decades of the present century, respiration calorimetry or 'whole body calorimetry' was rather extensively employed for measurements on animals and humans during rest and when performing mechanical work[7].

Another rather special branch of physiological calorimetry was initiated around 1910 by A. V. Hill, who used very sensitive thermopile instruments for measurements of heat production in nerves and in muscles.

During the first part of this century, numerous combustion calorimetric measurements were performed on food products, biological waste products and on purified, simple organic compounds known to take part in metabolic processes. Low temperature heat capacity calorimetry was also applied

on several biologically important compounds, but most Gibbs energy data now available for various steps of metabolic pathways have not been derived by calorimetric methods. Accurate combustion calorimetry and low temperature C_p work is of no particular importance for the biochemical field today.

A few reaction calorimetric investigations on biochemical systems were performed prior to 1950, in particular, by Meyerhof and his group, see ref. 9. Sturtevant and coworkers, and a few other investigators, reported results of some reaction calorimetric measurements during the fifties and the early sixties[9], but it was not until modern 'microcalorimeters' became available that the present area of biocalorimetry began. Calvet, starting from Tian's design of a thermopile heat conduction calorimeter, created the type of calorimeter which is now usually connected with his name. From a biological-calorimetric point of view, it was very important that H. Prat, using Calvet calorimeters, conducted a large number of 'demonstration experiments' which have been an inspiration for many recent studies on living organisms, see e.g. ref. 10.

Benzinger and Kitzinger were the first to devise an adequate mixing technique for a thermopile heat conduction calorimeter[11]. In addition, they demonstrated the use of their calorimeter for several well-defined biochemical processes. Sturtevant and his group, using an adiabatic shield calorimeter, were the first to start more systematic thermochemical studies on well-defined biochemical reactions.

As the middle and late sixties were approached, the field developed very rapidly, mainly due to instrumental developments and, in particular, due to the fact that commercially-produced instruments became available. The growth of biocalorimetry since that time is clearly seen from a comparative study between Sturtevant's review from 1962[9] and more recently given summaries of biocalorimetric studies[1-6].

INSTRUMENTATION

Several calorimetric principles and a large variety of different designs have been used in biocalorimetry. Design features and properties of most of the instruments have recently been discussed in some detail elsewhere[3] and the treatment here will be very brief.

In solution calorimetric work on simple model compounds, it is frequently advantageous to use 'macrocalorimeters', whereas in most work on biochemical and biological material, 'microcalorimeters' are usually employed. There is no sharp distinction between macro- and microcalorimeters, but in the present context, the 'micro' prefix usually indicates very sensitive instruments requiring small sample volumes. With modern micro-reaction calorimeters used for batchwise experiments, sample volumes typically are of the order of one or a few ml and the sensitivity is adequate for measurements of micromole quantities of substance. The heat effect sensitivity is usually of the order of 0.1–1 μW, and for instruments used in studies of slow processes, baseline stabilities often correspond to the order of 1 μW/day.

In calorimetric work on biochemical systems, as well as for work on cell suspensions, flow microcalorimeters started to appear about 10 years ago and have since become of great importance in biocalorimetry. A variety of flow vessels have been described whose volumes are normally of the order of 0.5–2 ml. Vessels can be of the flow-through type, used for steady-state processes and for suspension of cells, or they can be intended for measurements of processes initiated by the mixing of two liquid components.

Most microcalorimeters used in current biocalorimetric work are twin instruments. The twin principle is of particular importance in studies of low heat effects during long experimental periods. It is also a useful property that the heat effect produced by one sample can be directly compared with that from a reference sample.

Macrocalorimeters used for studies of simple model compounds are frequently of the isoperibolic type, employing thermistors as temperature sensors. Thermoelectric heat pump calorimeters and adiabatic shield calorimeters are also used in such work. The substance to be dissolved or reacted is usually enclosed in a glass ampoule prior to the start of the process. Alternatively, one reaction component, usually a solution, is pumped into the reaction vessel via a heat exchanger. Such instruments have found a wide use in titration

experiments such as those involving the determination of ΔH and K for ionization processes. Precise ampoule and titration calorimetric systems are marketed by LKB-Produkter, Bromma, Sweden, and by Tronac, Inc., Orem, Utah.

In studies of biochemical reactions and of cellular processes, it is thermopile conduction calorimeters which are in most frequent use, for example the Calvet microcalorimeter[10], manufactured by Setaram (Lyon, France), and the microcalorimetric system, made by LKB-Produkter. The latter system incorporates a batch mixing calorimeter[12], a flow instrument[13] and an ampoule calorimeter which is primarily used for measurements of heat effects from cellular systems under static conditions[14]. This latter type of instrument has also been used with perfusion vessels[14] and for flow cells designed for determination of slightly soluble model compounds in water, such as hydrocarbons[15].

Other thermopile heat conduction calorimeters used in biocalorimetry include the designs by Amaya *et al.*[16], Prosen[17], and Fujita *et al.*[18].

Gill and Chen[19] have described a differential adiabatic flow microcalorimeter characterized by a rapid response and a high sensitivity and precision. In another micro-reaction calorimeter designed by Gill and coworkers[20], it is possible to directly measure the heat of reaction between a gaseous compound and a substance in solution (*e.g.*, in reactions involving hemoglobin).

Nakamura[66] has very recently described a rapid response stopped-flow calorimeter using a 0.3 mmϕ thermistor as sensor, $t_{0.5}$ = 50 ms. In each experiment 2 x 1 ml is used. Nakamura and Matsuoka[67] used the same type of thermistor in a magnetically stirred dewar flask calorimeter, volume 5 ml, fitted with an oxygen electrode.

Picker[21] has described a sensitive mixing calorimeter using a thermistor as the temperature sensor. The instrument is available commercially (Setaram) but does not yet seem to have found use in biocalorimetry. The determination of heat capacities for model compounds in solution is often arrived at from precise measurements of solution enthalpies at different temperatures, but it can also be determined more directly. Picker[22] has designed an interesting flow instrument for measurements of the relative specific heat between two liquids, such as pure water and an aqueous solution. Partial molar heat capacities at zero concentration can be determined with a precision of *ca.* 1 J K^{-1} mol^{-1} for simple compounds. The instrument, which is marketed by Setaram, has recently been used for studies of many simple model compounds in aqueous solution and for protein solutions.

A novel drop heat capacity calorimeter[23] designed for measurements on 1 g samples of liquid or solid samples uses a heat conduction calorimeter as a receiver. With this instrument, heat capacities can be determined with a precision approaching 0.01%. The instrument has been used for model studies as well as for measurements of proteins in the solid state and in solution.

Most heat capacity measurements on biochemical systems are performed as temperature scanning experiments, *i.e.*, the heat capacity is determined for a sample while its temperature is continuously increased. Thermal transition are seen as peaks on the C_p-temperature curve and the enthalpies of transition are proportional to the area under the transition peak. The most widely used instruments of this type are the differential scanning calorimeters (DSC) marketed by Perkin-Elmer (Norwalk, Conn.). Their main application area, which also applies to other commercial DSC-instruments, is in different kinds of analytical experiments, but they have also found use in thermodynamic measurements, such as thermal transitions of biopolymers in concentrated solutions. For DSC-measurements on dilute ($\leq$ 1%) biopolymer solutions, adiabatic shield instruments have been used in a large number of studies; in particular, the instruments designed by Privalov[24], Sturtevant[25], and Brandts[26]. The Privalov instrument, which is commercially available (Mashpriborintorg, Moscow), can be used for measurements on 1 ml samples containing 1–2 mg of biopolymer.

Recently, a DSC-instrument based on the heat conduction principle was designed by Suurkuusk *et al.*[27] and has been used in several studies involving lipid transitions. A commercial version is being prepared by Tronac, Inc. This instrument is judged to have approximately the same characteristics as the Privalov design. Another new and very sensitive DSC instrument, based on the heat conduction principle, is now marketed by Seikosha

Co., Tokyo.

Several simple thermistor-operated flow calorimeters for substrate determinations using immobilized enzymes have recently been developed by Mosbach and coworkers[28-30] (the somewhat unfortunate name 'enzyme thermistor' is used for these instruments), by Carr and coworkers[31,32], and by Schmidt *et al.*[33]. Typically, a substrate solution is pumped through an equilibration coil into a small column, volume *ca* 1 ml, containing immobilized enzyme. The column is surrounded by an airgap and the device is submerged in a constant temperature bath. In the most simple case, a thermistor is positioned in the column, where it will sense the difference in temperature between a solution containing a substrate and a reference solution that is pumped through the flow system. Alternatively, thermistors may be positioned before and after the reaction zone and the temperature change caused by the enzymatic process is measured. The samples can be injected in rapid succession (typically *ca* 15 samples/h) and the enzyme material can be used for many experiments. Attainable precision varies with the system investigated, but it appears to approach that obtained with more elaborated 'regular' microcalorimeters. However, the sensitivity is higher for these latter instruments.

WORK ON SIMPLE MODEL COMPOUNDS

Data from model studies are needed for discussion of results from biothermochemical work, and as a support for new theories and hypotheses in this field. In addition, thermochemical studies on biochemical models can usually be looked upon as part of the general field of thermochemistry. As such, the results will have their full value even if it turns out that a particular study is not of any immediate importance for the biochemical field. It is then required that the data be determined with adequate accuracy and it is thus desirable that studies on simple biochemical models be made with the highest possible accuracy, even if it is not judged to be necessary for a particular biochemical problem.

An area of special importance in current model studies is the one dealing with interactions between water and other solvents and solutes, including the thermodynamics of transfer of compounds and groups between different media. It is largely through the results of thermodynamic measurements on very simple compounds that it has been made clear that such transfer processes, taking place without rearrangements of any covalent bonds, are of greatest importance for the overall thermodynamic picture of most biochemical processes. One outstanding property in this connection is the very high (apparent) heat capacity for hydrophobic groups in aqueous solution. For instance, the partial molar heat capacity of a hydrocarbon molecule is about 3 times higher in water than in nonaqueous solvents. This property is sometimes taken as an index for the 'hydrophobic hydration' of a molecule. It is interesting to note that heat capacity group values for solutes in aqueous solution are highly additive[34-37].

During the last decade, a large number of calorimetric data (ΔH_{soln}, ΔC_p, $C_{p,2}$) has been produced, in particular, for alkylammonium compounds, amino acids, alcohols, phenols, carboxylic acids, amines, amides and ethers. Several heterocyclic compounds, including a few nucleotide bases, have also been studied, as well as some sugars and peptides. Properties of surfactants and other micelle-forming compounds have been studied rather extensively as models for lipid materials.

Few reliable data are available for hydrocarbons[35] and other slightly soluble compounds. With the emerging microcalorimetric techniques for determination of enthalpies of solution[15,38], there is high hope that this area will be better covered in the near future.

In addition to solvent-solute interactions, some association processes of simple compounds such as purines, pyrimidines and amides have also been studied[1,2].

THERMODYNAMIC INVESTIGATIONS ON BIOCHEMICAL SYSTEMS

A large variety of calorimetric-thermodynamic studies on biochemical compounds and reaction systems have been performed during the last 10 years, and the results have been summarized in several reviews[1-4], see also ref. 6.

The number of reports from this field, found in the literature each year, is presently of the order of 60–70. Of these, the majority are concerned

with proteins and protein reactions, mainly protein-ligand binding reactions. In most cases, the ligands are low molecular compounds such as ions, co-factors or inhibitors. From results of many of these calorimetric binding studies, both equilibrium constants and enthalpy changes (and thus entropy values) are derived. Several of the studies are extended over large enough temperature ranges to give precise ΔC_p values. In this connection, it is interesting to note the recent titration calorimetric instruments described by Woledge[39] and by Rüterjans and coworkers, *cf.* ref. 40. These workers have attached motor-driven syringes to LKB batch mixing calorimeters and have applied the instruments to studies of protein-ligand binding experiments. Such titration devices are judged to speed up the determination of a typical binding curve by an order of magnitude.

It was pointed out earlier that thermodynamic work on simple model compounds concentrates on studies of interactions between water and other solvents and solutes, and on the transfer processes between different media. Much of the discussion related to ligand binding and unfolding processes for biopolymers and to the properties of membranes are also closely connected with this area. As a characteristic example, we may look upon a simple enzyme-inhibitor reaction in aqueous solution:

At the binding process, the inhibitor molecule is transferred from bulk water to the active site of the enzyme, which often seems to have the nature of a cleft in the protein molecule. More or less specific non-covalent bonds are formed between the protein and the inhibitor and, in addition, the process may be accompanied by a conformational change of the protein. We may expect that (part of) the water shell initially surrounding the inhibitor molecule will be transformed to bulk water and that the water molecules in the active site cavity will be expelled and thus transformed to bulk water. A conformational change of the protein may, in addition, cause changes in the contacts between groups in the protein and the water. Many biochemical ligand binding reactions are characterized by large negative ΔC_p values which, therefore, are often taken as an indication of hydrophobic interactions between the molecules[1,2].

Another important group of studies with proteins involves transition or unfolding processes which are initiated thermally (DSC-experiments) or by mixing the protein solution with a denaturation agent. In the paper by Privalov (this issue) the capability of modern DSC in solving problems of intramolecular interactions and stabilization of structures of proteins and nucleic acids are discussed in some detail. Surprisingly few investigations are made on polynucleotides and nucleic acids (5–10 per year), about half of them being thermal transition studies.

Scattered studies involving properties and reactions of low molecular biochemical compounds are reported (about 10 reports per year), with just as many studies involving lipids and membrane materials. Very few calorimetric measurements are currently reported for carbohydrates.

A decade ago, calorimetric thermodynamic work on biochemical systems was confined to a few specialized laboratories. A large proportion of the work dealt with method development and general data collection. Today, instrumentation and measurement methods are much more developed and the studies have become increasingly problem-oriented. Calorimetric measurements are also more often conducted in parallel with other physicochemical measurements.

Derived biothermodynamic data may lead to conclusions concerning different properties such as stoichiometry, degree of association, molecular weight, reaction mechanism, identity between different binding sites or between compounds of different origin, method of preparation or treatment, interactions between biochemical compounds and buffer substances, *etc.* However, as was pointed out earlier, it is difficult to discuss biothermochemical results with the intention of 'explaining' the derived values in the same sense as may be possible for systems with simple compounds. Derived data are sometimes discussed in terms of honoured concepts like 'hydrogen bonds', 'hydrophobic interactions', 'conformational changes' or 'steric effects', *etc.*, but many interpretations of that type are, no doubt, highly speculative. It can be concluded that much systematic work remains to be done before a thorough analysis of such data can be performed. Different kinds of non-calorimetric techniques must be

involved in that work, as well as calorimetric studies on simple and on more complex models.

It is believed that calorimeters used for biothermodynamic work will not be much improved in the near future from the point of view of sensitivity or accuracy. But it can be expected that the technical development for some types of instruments will lead to much more convenient and rapid experimental procedures.

ANALYTICAL APPLICATIONS

Most processes are accompanied by heat evolution or heat adsorption. The heat quantities are related to the extent of the process, and the heat effect evolved for a given process is proportional to its rate. It is thus clear that calorimetry, in addition to its importance in thermodynamic work, also can serve as a general analytical tool.

The fact that calorimetric methods are so general, and thus nonspecific, is a serious limitation for many types of analytical problems. However, in biochemistry and biology the inherent specificity of the reaction systems often allows the use of a nonspecific analytical method. It should also be remembered that for some processes it is advantageous to use a nonspecific method rather than a very specific method for detection. It is then more likely that unknown phenomena will be discovered. If the sensitivity is sufficient, a life process can always be recorded by a calorimetric method, and it is often not necessary to disturb the biological system while its activity is measured. Another feature of importance to investigations on biochemical and biological systems is that calorimetric methods, in contrast to spectrophotometric methods, do not require optically clear objects but can be used on nontransparent systems, such as tissues, soil, and cell suspensions, or on crude solutions or suspensions of biochemical compounds.

Work on the biochemical level

Enzyme assays. When flow calorimetric procedures are used for enzyme assays, enzyme preparations are normally mixed with an excess of substrate to give a zero-order reaction system, which is then pumped through a flow-through cell of the calorimeter[41]. Steady-state heat effects observed are directly proportional to the enzyme activities. The possibilities for 'chemical amplification' should be considered, in particular the use of a suitable buffer system. A typical example is provided by hydrolysis of an ester, *e.g.* of acetyl choline, ROAc.

$$(ROAc + H_2O \rightarrow ROH + Ac^- + H^+)_{aq}$$

The enthalpy change of the reaction is close to zero, $\Delta H = 1.2$ kJ mol^{-1}. However, if the proton released is taken up by an amine buffer with a highly exothermic enthalpy of protonation (*e.g.* tris with $\Delta H_{prot} = -47.5$ kJ mol^{-1}), the reaction will become strongly exothermic. If the hydrolysis process is carried out in a phosphate buffer ($\Delta H_{prot} = -4.7$ kJ mol^{-1}), the measured reaction will be only weakly exothermic.

Several flow calorimetric studies of enzyme activity measurements have been reported[3]; see also the recent batch calorimetric studies by Rehak *et al.*[42], involving enzymes in solution as well as immobilized enzymes, and studies by McGlothlin and Jordan[43].

Determination of substrate concentration. It has been shown that nearly the same flow calorimetric technique as was used for enzyme assays can be adopted for substrate determination, the difference being that the substrate concentration should be rate-limiting. In principle, however, it is easier to determine substrate quantities in a batch or a stopped-flow experiment. The heat quantity evolved in the experiment, after small corrections have been applied, is directly proportional to the quantity of substrate in the sample and the result is thus independent of the activity of the enzyme preparation used. Goldberg *et al.*[44] have applied a batch calorimetric procedure to the determination of glucose in serum, using the hexokinase reaction. These workers have also looked into the thermochemistry of this process in some detail. McGlothlin and Jordan[45] employed the same reaction for glucose determination in samples of serum, plasma or whole blood, using an injection procedure with a simple Dewar vessel calorimeter.

The thermistor-operated flow instruments for substrate determinations by use of immobilized enzymes[28–33] have recently been used for many clinically important compounds such as glucose, urea, cholesterol and penicilline. It is believed that

this technique soon will be established as a routine bio-analytical technique. Problems due to non-specific heat effects caused by such factors as the presence of various components in complex biological samples, have been observed. Such effects can probably be overcome by use of 'split-flow' techniques[30] or by more sensitive instruments which will allow the use of more dilute reaction solutions.

Fig. 1 shows a schematic representation of the split-flow technique and results of a comparison between parallel determinations of serum glucose using a split-flow thermistor instrument and a spectrophotometric technique. In an interesting variation of the immobilization technique, antigen-antibody complexes are used[45]. Whole cells have also been immobilized and used with this analytical technique[48,49].

Reaction kinetics. Calorimetry has repeatedly been suggested as a tool for obtaining reaction kinetic data in biochemistry. Several model reactions have been studied using different instruments and methods[3], but the technique appears not to have obtained any practical importance. Calorimetry most probably has a great potential in this field, although we may have to await further instrumental developments before a breakthrough will come. In the recent methodological work by Johnson and Biltonen[50], it was demonstrated that moderately fast flow calorimeters can be used to obtain accurate kinetic data for reactions whose half-lives exceed a few seconds, *cf.* Fisher *et al.*[51].

Cellular systems

Work on calorimetric methods for studies of microorganisms is currently being conducted in several laboratories. Among the most promising practical applications are the identification procedures for bacteria by 'fingerprint-thermogram', antibiotic sensitivity tests, characterization of inocula, determination of values for microbial activity in soil and the use of calorimeters as general monitors in basic as well as applied studies of fermentation processes. Rather extensive calorimetric studies have also been made with preparations of the major types of blood cells. A few studies on tissue cells and with pieces of tissue have been reported. Heat production from small and large animals, including humans, are also being reported, but the activity in this area is surprisingly low.

Below, a few typical examples of recent calorimetric measurements on cellular systems will be summarized. A few problem areas will also be touched upon. For more comprehensive reviews, see ref. 3 and various chapters in the monographs

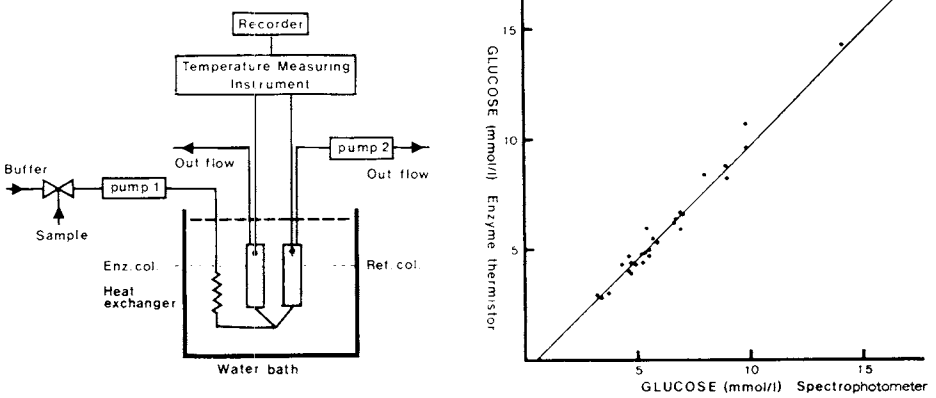

Fig. 1 (a) Schematic representation of the split-flow technique. After temperature equilibration in the heat exchanger the buffer/sample flow is split equally on the enzyme and the reference column. The temperature difference between the two columns is measured with thermistors placed at the ends of the columns.
(b) Serum glucose as measured by the split-flow method versus conventional spectrophotometric technique (hexokinase/glucose-6-phosphate dehydrogenase).
[From Danielsson *et al.*[46]]

edited by Lamprecht and Schaarschmidt[52], Jones[4] and Beezer[5].

Aerobic growth processes. In many types of calorimetric experiments with aerobic cells, it is a difficult problem to arrange for a sufficient supply of oxygen. A direct aeration of the medium in the calorimetric vessel is usually not possible, as it is difficult to avoid disturbing evaporation effects. Flow calorimetry seems to be the most reliable and convenient calorimetric method in this connection. However, the transport time for the cells between the aerated reservoir and the calorimeter vessel, together with the residence time in the flow vessel, can be significant, typically of the order of 5 min. For concentrated and fast growing aerobic cell suspensions, the dissolved oxygen may thus be already consumed in the flow line or during the passage through the calorimetric vessel. A higher oxygen consumption can be tolerated if a mixed flow of liquid suspension and gas is used[53]. An experimental assembly used for this purpose is shown schematically in Fig. 2. The cell suspension is pumped from a fermentor to a T-piece, where it is met by a constant flow of air or another gas mixture. A mixed flow of suspension and gas will first pass through the heat exchanger, where gas-liquid phase equilibrium is established at the proper temperature, and it will then pass to the calorimetric vessel. At the exit of the flow calorimeter, the oxygen pressure of the suspension can be tested, after which the suspension, for instance, may be pumped to a fraction collector kept at a low enough temperature to essentially stop the microbial activity.

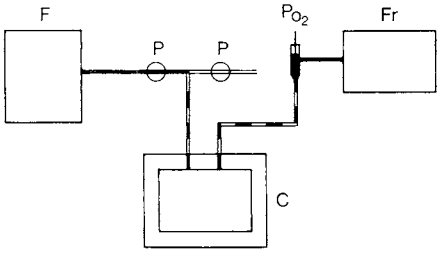

Fig. 2 Schematic diagram showing an arrangement for segmented gas-suspension flow in aerobic microbial growth experiments.
F, fermentor; P, pump; C, calorimeter; P_{O_2}, oxygen electrode; Fr, fraction collector (preferably kept at low temperature).

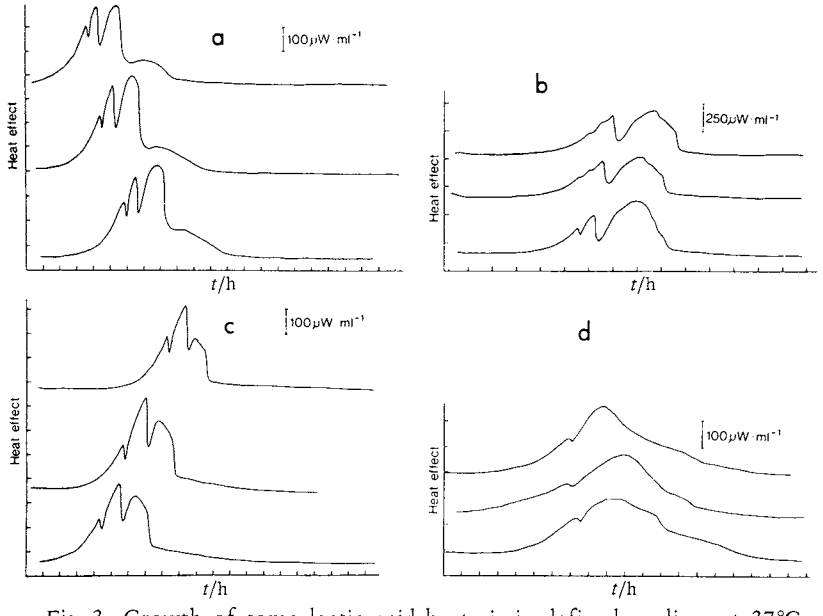

Fig. 3 Growth of some lactic acid bacteria in defined medium at 37°C.
(a) *Streptococcus cremoris* 703, (b) *Str. diacetylactis* 65, (c) *Str. lactis* 38, (d) *Leuconostoc cremoris* 80.
Three replicate thermograms are shown for each strain and are displaced on the response axis for clarity. Time of inoculation is shown as zero hours and the units of increase in heat effect are shown on each figure. [From Fujita *et al.*[57]]

Identification and numeration of microorganisms. The identification of bacteria by calorimetric recording of their growth processes was first demonstrated by Boling *et al.*[54], *cf.* Russel *et al.*[55] and review articles by Schaarschmidt and Lamprecht[56], Kresheck in ref. 4 and Newell in ref. 5. The highly profiled growth thermograms obtained when a complex medium was innoculated with clinically important bacterial strains was found to be sufficiently unique, allowing them to serve as fingerprint patterns for their identification. Recently, Fujita *et al.*[57] developed a similar method for the identification of a group of lactic acid bacteria of importance in the dairy industry. In this case, a well-defined synthetic growth medium was used, Fig. 3.

In the recent work by Beezer *et al.*[58], a flow calorimeter was used for the numeration of bacteria in culture media and in clinical specimens of urine. Bacteria at concentration levels from 10^3–10^5 cells ml^{-1} could be reliably determined for strains causing urinary infections.

Testing of antibiotic activity. In several recent studies, static and flow microcalorimetric methods were used to assess antibiotic activity, see *e.g.* Mardh *et al.*[59], Semenitz and Tiefenbrunner[60], and Beezer *et al.*[61]. Result suggest that such methods soon will find practical value in such areas as pharmacological studies and in clinical work.

Fig. 4 shows some results from one of these studies, in which it is evident that the calorimetric method can uncover essential properties that are not revealed by traditional assay methods. Results shown in Table 1 suggest that calorimetry in several critical respects is superior to plate-diffusion methods in the testing of antibiotic activity.

Blood cells. During the past few years, several groups have performed calorimetric investigations on blood cells; for reviews, see chapters by Monti and Wadsö in ref. 4 and by Levin in ref. 5. The object has usually been to investigate the potential use of calorimetric techniques as a diagnostic tool in clinical hematology. In several studies, measurements have been made with cells prepared from patients.

Most work has been made on erythrocytes and thrombocytes, for which the effects of variation of several experimental parameters has been investigated in some detail: cell preparation techniques, suspension media, pH, temperature, glucose concentration, storage conditions and the calorimetric technique. By use of data for variation in heat effect with experimental conditions, it is possible to recalculate well-documented experimental values that correspond to a chosen set of standard conditions. The use of such 'standard' heat effect values is believed to be essential when results of different investigations are compared (*e.g.*, when comparing data for cells from patients with normal values).

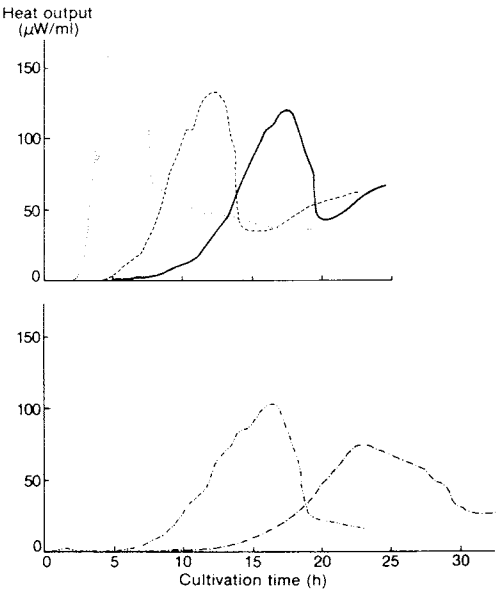

Fig. 4 Upper panel. Heat output of *E. coli* cultured in the presence of tetracycline (– – –), doxycycline (——), or in the absence of antibiotic (·····). Lower panel. Heat output of *E. coli* in the presence of oxytetracycline (—·—) or minocycline (—··—). Each antibiotic was present at a concentration of 0.4 μg/ml (0.5×MIC). All experiments were carried out in an ampoule microcalorimeter. [From Mårdh *et al.*[59]]

Table 1. Comparison of determinations of nystatin by flow calorimetry and agar plate diffusion method [from Beezer *et al.*[61]]

	Calorimetry	Plate method
Reproducibility	±3%	±5–10%
Sensitivity	0.5 units ml^{-1}	20 units ml^{-1}
Time required	1 h	16 h
Automation	possible	not possible

For erythrocytes and thrombocytes, good steady-state heat effects are usually observed and the measurements can be performed with good precision, but there still remain many methodological problems to be investigated. For the leucocyte fractions (mainly lymphocytes and granulocytes), the heat effects are small and problems occur due to sedimentation and crowding effects. For granulocytes, adhesion to the calorimetric vessel or to the tubes in flow calorimeters provide very significant experimental difficulties, *cf.* chapter by Monti and Wadsö in ref. 4.

In order to increase the specificity of the calorimetric method or to amplify a certain property of the cellular system, it can be useful to add substances which activate or inhibit the metabolic processes. Several examples of this experimental approach have been used with blood cells: stimulation by phagocytosis, addition of methylene blue (stimulation of the pentose shunt) and stimulation by mitogenic agents.

Tissue cells. Calorimetric studies with tissue cells are made in suspension or with the cells attached as monolayers to a solid support, such as the walls of the reaction vessel or to glass beads contained in the vessel. For a recent review, see chapter by Kemp in ref. 4.

It is believed that calorimetric methods applied to preparations of tissue cells can be of very significant importance for cell biology, including applications in the medical field. However, calorimetric techniques and working procedures used are, as yet, in an early stage of development.

Biotechnical processes. For systems with a very high oxygen consumption, such as many aerobic microbial growth processes of biotechnical interest, there appears, as yet, to be no suitable calorimeter available. In addition to problems with oxygenation, such cultures are often very viscous, which makes the conventional flow or agitation procedures, used with existing calorimeters, unsuitable. In such cases where the heat effects produced normally are large, typically of the order of a few $W\,l^{-1}$, it may be preferable to use a simple thermometric technique such as that described by Mou and Cooney[62].

Soil. Microbial heat production in soil can vary within wide limits, but typically it is in the range of $5-50\,\mu W\,g^{-1}$ (25°C) and can thus easily be measured in a modern microcalorimeter. However, there are many methodological problems involved[63-65]. One of them relates to problems with oxygen deficiency and high levels of carbon dioxide in the calorimeter. In order to avoid significant changes in the concentration of these gases during long term experiments, the following insert-ampoule technique was recently developed[65]. Soil samples are enclosed in plastic ampoules with top and bottom consisting of membranes made from 1 mm silicone rubber. This latter material has a high permeability both for oxygen and for carbon dioxide. During an extended experimental period (weeks or months), the plastic ampoule can be exposed to a controlled atmosphere outside the calorimeter, except for the brief calorimetric observation periods during which the plastic insert ampoule is enclosed by the calorimetric ampoule made from steel.

Some conclusions. Calorimetric investigations on living cells represent a vast experimental area, where many specialized instrumental properties and working procedures are needed. Requirements for adequate sensitivity, stability and precision of the calorimetric instruments are usually satisfied but the important characterization of the physiological conditions of the cells, during the calorimetric measurements, is frequently neglected. It is felt that further attention must be given to the design of specialized calorimetric vessels, where the conditions of the cells (*e.g.* supply of oxygen, sedimentation, adhesion, cell concentration in a flow vessel, pH, *etc.*) can be conveniently confirmed. More attention should also be given to the development of processes suitable for tests and calibrations of microcalorimeters used for these studies. Multichannel instruments will be needed in order to permit suitably large series of measurements to be investigated.

The rate of publication in this field is as yet quite low. However, it is envisioned that within a few years, most studies in biocalorimetry will be performed on cellular material, as this is an area where calorimetry has a unique potential as an analytical tool. Many new and specialized calorimetric-analytical instruments will probably appear and play important roles in basic biological research, as well as in applied areas, such as clinical investigations, food technology and fermentation,

and in ecology and agriculture.

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