Thermochimica Acta, 88 (1985) 35—48
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

ISOTHERMAL BIOCALORIMETRY. A STATUS REPORT.

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ABSTRACT

A brief status report is given for the field of isothermal calorimetry applied to systems of biological interest. These include, for instance, water and simple model compounds, biopolymers, living cells, tissues and animals. Examples of recent thermodynamic work as well as investigations of a more general analytical nature are discussed. Special attention is given to recent developments in instrumentation and methodology.

INTRODUCTION

Calorimetric studies of biological interest include measurements on systems of different complexities: water, simple biochemical substances and model compounds, biopolymers, living cells and tissues, plant materials and animals. Experiments are performed under essentially isothermal conditions, as well as in temperature scanning experiments. Some studies can be characterized as thermodynamic investigations, not always performed on a molecular level, whereas others are of a more general analytical nature where the calorimeters are used mainly as "process monitors".

In this paper a brief status report will be given on isothermal calorimetry applied to different systems of biological interest. Special attention will be given to recent developments in instrumentation and methodology. Thermodynamic work as well as more general analytical investigations will be referred to but space will not allow comprehensive coverage of the field. References will be made to several reviews such as (1-6) whereas mention of specific investigations will be restricted mainly to very recent work.

WATER AND SIMPLE MODEL COMPOUNDS

The properties and functions of biological systems are intimately connected with the unique properties of water. The thermodynamic characterization of interactions between water and biochemical substances has attracted much interest during the past 20 years and has greatly influenced our views about the structure and function of biochemical systems. Calorimetric work in this field is currently performed on

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biopolymers, but, as earlier, the more systematic studies are conducted on the level of simple model compounds. Work of this kind has been of major importance for our present understanding of, for instance, biochemical ligand binding reactions and the structural properties of biopolymers. In addition, results of work on simple biochemical models are of course of direct importance for the general field of thermodynamics of aqueous solutions.

A main type of calorimetric experiment consists of dissolution at different temperatures of pure compounds into water and other solvents, leading to $\Delta_{sol}H$ and $\Delta_{sol}C_p$ values. By variation of the final concentration of solute or through separate dilution experiments, values for infinitely dilute solutions can be determined. Such experiments conducted over suitable concentration ranges will also lead to values for solute-solute interactions, see e.g. (7).

Dilution measurements are often best performed by flow-mixing microcalorimeters, whereas most solution calorimetric measurements are conducted by use of "macro" calorimeters. However, significant progress has recently been made in microcalorimetric solution calorimetry by which, in particular, slightly soluble compounds are studied. New instruments for dissolution of gases (8, 9), liquids (10) and solids (11) have been designed.

Thermodynamic values for the transfer of solutes from the gas phase to solution are important for discussions of solute-solvent interaction. It is thus surprising that very few laboratories presently perform vaporization calorimetry. A very valuable compilation of calorimetrically determined vaporization values for organic compounds has recently been prepared under the auspices of the Commission of Thermodynamics of the International Union of Pure and Applied Chemistry (12).

Heat capacity values for solutes in water are very important for the characterization of the "hydrophobic effect", which is believed to be essential for many biochemical binding reactions and for the structural properties of biopolymer systems. For instance, transfer of a hydrophobic group from water to a lipid phase or to a hydrophobic binding site is accompanied by a very large decrease in heat capacity, see e.g. (13). More recently, it has been shown that polyhydroxy alcohols and saccharides also have highly "abnormal" heat capacity values in water, see (14).

ΔC_p values for dissolution processes reflect the properties of the initial as well as the final system. If interest is focused upon the solvated state, the partial molar heat capacity for the solute at infinite dilution, $\Delta C_{p,2}^{\infty}$, is a more appropriate function. Such values can be derived from corresponding $\Delta_{sol}C_{p,2}^{\infty}$ and the heat capacity for the pure compound, $C_{p,2}^{*}$

$$C^{\infty}_{p,2} = \Delta_{sol}C^{\infty}_{p,2} + C^{*}_{p,2} \quad (1)$$

Unfortunately reliable $C^{*}_{p,2}$ values are often lacking, even for very simple compounds.

Some aspects of earlier model compound work were covered in (1). Recent systematic calorimetric studies on models and simple biochemical compounds includes work on amino acids and peptides, see e.g. (15, 16), amides, pyrimidines and purines (17, 18). The properties of alcohols and polyhydroxy compounds in aqueous solution have also been studied, see e.g. (20, 21). For the first time precise calorimetric measurements of solution of rare gases and hydrocarbon gases have been reported (22, 23).

During the last decade, solution calorimeter work on simple compounds has greatly increased our knowledge about solute-solvent interactions relevant for biochemical systems. However, it is noteworthy that there still appears to be no work done on several essential groups of compounds such as simple phosphates, sulphides and disulphides, imidazoles and indoles. Further, few investigations on biochemical models are conducted over a wide range of temperatures. Reliable data for partial molar heat capacities are still quite rare and there is virtually no information about the variation of heat capacities with temperatures. The latter kind of data is judged to be of critical importance for a better understanding of, e.g., structural effects in water caused by e.g. hydrophobic groups.

BIOCHEMICAL SYSTEMS

Current isothermal calorimetric work on purified biochemical compounds and assemblies of compounds mainly deal with different kinds of non-covalent binding and association processes involving proteins. In a wide sense such processes are often referred to as protein-ligand binding. This field has recently been thoroughly reviewed by Hinz (5, 6), who also presented compilations of calorimetrically derived thermodynamic data. See also the reviews by Jones and Skinner (4) and by Rialdi and Raffanti (24). Ligands can be small molecules or ions (protons, metal ions, co-enzymes, enzyme inhibitors) or polymers or aggregates (proteins, nucleic acids, lipids). Microcalorimetric work is typically carried out on the level of $\leq$ 1 µmol of protein. Different batch and flow microcalorimeters are used. Frequently the protein-ligand complexes formed are rather weak and the measurements then have the character of titration experiments. This will make the experiments more demanding, but on the other hand they can then lead to simultaneous determination of both the enthalpy change and the equilibrium constant and thus also of the entropy change.

Protein-ligand binding reactions studied are usually highly specific,

which makes them well-suited for thermodynamic characterization. In some cases it is possible to discuss the thermodynamic data using information from model compound work together with structural information obtained by, in particular, X-ray crystallography, see e.g. (25). One approach used in the analysis of thermodynamic data for protein-ligand binding has been to divide the measured process into two hypothetical steps (26). In the first step the individually hydrated species are brought together and non-polar surfaces interact, giving a partially immobilized complex and "de-structured" water. In a second step, further interactions which do not involve the solvent are considered: electrostatic interactions, hydrogen bond formation and van der Waals forces. In summary it can be concluded that progress is being made in the analysis of thermodynamic data for protein binding reactions and the important role of water has been very clearly demonstrated. However, we are still far from a stage when discussion concerning various contributions to the measured values can be held on a truly quantitative level.

The list of calorimetric studies of protein-ligand binding reactions (5, 6) is quite impressive compared to the situation a decade ago. Still, it is felt that the activity in this very central field of bio-thermodynamics is presently quite low. Ligand-binding work involving nucleic acids and lipids is indeed very scarce. However, as will be reported below, there have recently been significant developments in instrumentation and working procedures which are of immediate interest for microcalorimetric studies of biochemical ligand binding processes. These developments may lead to an increased activity in this field.

Methodological developments in ligand binding studies.

A few years ago Woledge and Kodama reported the outline of a new twin microcalorimeter (27), see also (28), using 2 ml reaction vessels. The twin vessels were connected with a 30 junction silver-constantane thermopile which had a low thermal conductivity leading to a slow decay of the differential power signal. Stirring was achieved by means of vertical oscillations of a teflon disc. During an experiment a few μl of reagent was injected into the reaction vessel by use of a motor-driven syringe. A complete calorimetric titration curve can be produced within 3 min once the thermal equilibration has been established (which requires 1 - 2 h). However, the instrument appears to be less sensitive than traditional thermopile heat-conduction calorimeters using semi-conducting Peltier-effect plates as heat-flow sensors. The design of the instrument does not seem to have been reported in any detail.

The LKB batch microcalorimeter which has been used extensively in

ligand-binding work has been fitted with a titration unit (29) suitable for automatic titration procedures (30). Titration experiments can be performed much more rapidly and with significantly less material than in the original batch calorimetric procedure.

Recently a new titration and perfusion vessel was developed in our laboratory (31). It is used as an insertion vessel with the LKB "Bio-Activity Monitor" (32). These instruments will be discussed later in this report. It is typical that significantly less than 1 µmol of protein is needed in experiments where a complete ligand binding curve is obtained by an automatized procedure.

Gill and coworkers have recently reported the design of a new twin titration microcalorimeter using Peltier-effect thermocouple plates as sensors (33). The design of this instrument is based on the experience gained with another titration calorimeter recently reported from the same laboratory (34). The new instrument uses the heat conduction principle but in addition computer-controlled electrical compensation is employed, which significantly reduces the measurement time in comparison with a "passive" heat conduction instrument of comparable design. The volume of the reaction vessel is 0.2 ml and the sensitivity is reported to be 1 µJ. The instrument is used with completely filled vessels.

Analytical and kinetic applications

Some years ago there was significant optimism concerning the potential for isothermal calorimetric methods applied to analytical problems in biochemistry (1). There is ongoing methodological work in the field of calorimetric determination of enzyme activity and substrate concentration. However, few groups are involved in this work and it appears as if there are as yet very few practical applications. The most promising methods are believed to be those which use simple "thermal probes" which probably should not be called calorimeters, cf. e.g. Danielsson et al (35) and Fulton et al (36).

Another area where microcalorimetry may not have been applied to the extent which was expected some years ago is in enzyme kinetics. A recent example from this field is given by Lindberg et al (37), who studied the enzymatic degradation of starch microspheres, using a batch instrument.

Berger and co-workers have reported a microcalorimeter design for measurements of the kinetics of fast reactions. In a stopped-flow apparatus, where a thermistor was used as the sensor, kinetics of processes with reaction times of a few ms could be studied (38). This group has also described a new sensitive batch instrument optimized for thermal kinetic measurements (39). This instrument is a twin thermopile heat conduction

calorimeter in which mixing is achieved by rotation of the calorimeter.

LIVING SYSTEMS

Calorimetric work is performed on a large variety of living systems: microorganisms, mammalian tissue cells and organs, small and large animals. Notably, work on living plant materials is very scarce.

Calorimetric measurements on living material are often undertaken as a result of a (potential) practical importance in areas like clinical analysis, biotechnology or ecology. It is therefore not surprising that to a large extent the calorimeters are used more as general analytical tools, "process monitors", than as thermodynamic instruments. Thermal powers recorded vary from a few μW in microcalorimetric work on mammalian tissue cells to 100 W or more in "whole body calorimetry" applied on humans and other large animals.

Cellular systems

Calorimetric work on microorganisms and mammalian cells reported before 1978 or 1979 is well covered in the monographs edited by Jones (2) and Beezer (3). Sources of systematic errors and other problems in connection with calorimetric work on living cells have been discussed in some detail (40). Flow instruments used as power meters in work on, for instance, cellular systems are inherently difficult to calibrate electrically, which has called for suitable test and calibration reactions. Recently one such process was described where triacetin is hydrolyzed in imidazole/acetic acid buffer (41). The nearly constant power level is determined by the buffer composition.

Setaram (Lyon, France) and LKB Produkter (Bromma, Sweden) have recently introduced new commercial microcalorimeters which are of interest for studies of cells but also in other fields of bio-calorimetry. Both instruments are thermopile heat conduction calorimeters.

The Setaram instrument ("Bio-DSC") (42) is designed for temperature scanning measurements as well as for isothermal batch and flow calorimetry. The calorimeter is used with different removable vessels. The power sensitivity when used in the isothermal mode is stated to be 0.2 μW. No detailed report on the design and the performance of the instrument seems to have been published.

The LKB instrument, "BioActivity Monitor", is based on the design reported in (32). The instrument consists of a precise water thermostat ($\pm$ $1 \cdot 10^{-4}$ K) which can house up to 4 independently operated calorimeters ("channels"). They include twin and single calorimeters using sealed ampoules as insertion reaction vessels (1-20 ml) or permanent flow vessels

(mixing and flow-through). The flow vessels consist of gold tubes which are spiralized around aluminium tubes which also can serve as receivers for insertion vessels. The long term (day) stability of the baseline signal is better than 0.1 μW when static ampoules are used as reaction vessels or when a low flow rate (c:a 10 ml/h) is used. At high flow rate (60 ml/h) the baseline stability is still high but short time fluctuations become more pronounced ($\pm$ 0.5 μW).

A new insertion vessel which fits into the LKB instrument was recently developed in our laboratory (31). It is primarily intended for use with living cells and in titration experiments. Reagents can be added by use of a programmable micro-syringe. Cells can be in suspension or they can be adhered to supports such as a polystyrene film or microcarriers. Liquid medium (or gas) can be perfused through the vessel during the measurement. The baseline stability is typically $\pm$ 0.3 μW over 10 h (stirring rate 50 rpm, perfusion rate 20 ml/h).

Microorganisms. Reference is made to the following general review articles: studies of growth and metabolism in bacteria by Krescheck (43) and Belaich (44) and in yeast by Lamprecht (45). Many reports have since been published in this field, some of which will be mentioned below.

In a series of papers, James and coworkers have reported results from systematic studies of the thermochemistry of growth of Klebsiella aerogenis in a simple media (40). Batch cultures as well as chemostate cultures were investigated by use of a flow calorimetric technique. Belaich and co-workers have investigated the energetics of sulphate-reducing bacteria (42) and the aerobic growth of a Cellulomonas strain (48). Ishikawa et al (49) studied the thermochemistry of E. coli grown on synthetic medium under different limiting conditions. They used a twin heat conduction calorimeter (50) in which the culture could be agitated and aerated. Nunomura and Fujita have made several studies of metabolism of yeast (51) in a rotating twin calorimeter.

Lovrien et al (52) used a twin batch calorimeter for studies of microbial metabolism of different monosaccharides and simple aromatic compounds. The instrument was of the thermopile heat conduction type using vessels with two open compartments. The sample vessel was loaded with 1 ml bacterial suspension and with 0.5 ml of a solution containing the carbon source. Mixing was achieved by rotating the calorimeter. The gas phase atmosphere in the vessels was controlled by use of a gas flow line.

The use of calorimetrically observed power-time curves for the identification (or characterization) of microorganisms has been discussed by Newell (53). Recent reports on this subject include work by Herman et al (54) on strains of the Enterobacteriaceae family and by Perry et al (55) on

commercial yeast strains. In another study on yeast by Perry et al (56), the crucial relationship between innoculum density and glucose concentration on the metabolic activity, and thus on the power-time curves, was investigated. Itoh and Takahashi (57) recently investigated the growth of bakery yeast on a liquid synthetic medium. A kinetic and thermochemical analysis was made.

Microcalorimetric studies of the action of antibiotics on microorganisms have been reviewed by Beezer and Chowdhry (53). For more recent work in this field, see (58-62).

Gordon and Millero (63) used an LKB sorption microcalorimeter in a study of bacteria attached to microcarriers . The column of the sorption calorimeter (volume 0.5 ml) was filled with microcarrier beads to which bacteria were attached. During the measurement, medium was perfused through the column. Lock and Ford (64) have designed a simple flow calorimeter for measurements of heat production of attached and sedimentary aquatic microorganisms. The instrument design is based on the split flow instrument used with, e.g., immobilized enzymes ("enzyme thermistor"), see e.g. (35).

The use of flow microcalorimetry for the assessment of toxicity effects on microbial systems by environmental chemicals has recently attracted a marked interest, see (65-69). Lasserre and Tournié (70, 71) have used a flow calorimetric method to characterize the properties of the metabolic activities in marine microcosms at the water-sediment surface. A methodology was developed by which rapid and global changes in metabolism and energy flow could be assessed in experimental microcosms.

A methodological study on the use of microcalorimetry for the characterization of microbial activity in soil was made by Ljungholm et al (72). The microbial activity in different soils has been assessed in recent studies by Sparling (73, 74). Kawabata et al (75) have studied the effect from different pollutant chemicals on glucose metabolism in soil and Yamano and Takahashi (76) used a multichannel calorimeter to measure the temperature effect of the activity of microbial degradation of several carbon sources added to soil.

Another example of the use of microcalorimetry for studies of complex microbial systems is given by the work by Fardeau et al on the biodegradation of straw (77) and by Redl and Tiefenbrunner (78) on the degradation of substrates in waste-water systems.

Blood cells and other tissue cells. Blood cells are convenient to collect and to prepare and their metabolic properties are of great importance in clinical analysis - a field in which microcalorimetry appears to be developing into a practically useful tool. Substantial methodolo-

gical work on the calorimetric characterization of blood cells has been done during the last 10 years and several investigations have been concerned with differences between cells prepared from patients and healthy subjects (79, 80); for more recent examples see e.g. (81-84).

A methodological study on human blood lymphocytes has recently been made by Ikomi-Kumm et al (85). Nanri and Minakami (86) investigated the heat production by pig platelets in relation to glycolysis and respiration by use of a flow microcalorimeter and simultaneous determination of oxygen consumption.

Eftimiadi and Rialdi have used flow microcalorimetry to investigate the heat production of human granulocytes (neutrophiles) activated with phorbol-12-myristate-13-acetate (87) and during phagocytosis of different strains of staphylococcus (88). Oxygen consumption and formation of metabolites were determined, enabling the authors to analyze the results on a molecular thermochemical level.

Batch mixing microcalorimetry has been used to study the activation of human blood cells by antigen-antibody complexes (89). It was shown that most of the activation heat is produced by the granulocyte fraction. It was also shown that granulocytes from patients with acute leukemia in remission have a decreased response to immune complexes (90).

Thorén et al (91) have reported preliminary results from an investigation on rabbit alveolar macrophages.

Calorimetric work on mammalian tissue cells has been reviewed by Kresheck (43) and Kemp (92).

Heat production by adipocytes obtained from collagenase-treated fat tissue has been investigated in several studies. Monti et al (93) used a static calorimetric ampoule technique in a methodological study on human adipocytes. Sörbris et al (82) found a significantly lower power value per cell for preparations from fat tissue in obese patients than for cells from a control group. After weight reduction, cells from obese patients showed increased power values, but these remained significantly lower than for the controls. Kuroshima et al (94) have studied activation of heat production caused by glucagone and noradrenaline in rat adipocytes.

Several calorimetric studies on human skin cells have been reported (95, 96). The cells were attached to a polystyrene film which was inserted into the static reaction vessel of a twin heat conduction calorimeter (Bioflux, Thermanalyse, Grenoble, France). Methodological aspects were investigated as well as the influence of drugs on the metabolic activity.

Loesberg et al (97, 98) used a simple Dewar vessel calorimeter (thermistor used as sensor, volume 100 ml) in their studies of suspensions of cultured tissue cells (rat hepatoma cells and neuroblastoma cells).

Measurements were made during different stages of the cell cycle (98).

Inskeep and Hammersted (99), cf. (100), used a thermopile heat conduction calorimeter (Microcalorimeters, Inc., St. Paul, Minn., USA) in a study of bovine sperm in the presence of C^{14}-labelled glucose. Metabolite analyses were made and over 95% of glucose carbon equivalents were identified as metabolic products. Elia et al (101) used an LKB batch calorimeter to study the sperm-egg interaction in the Ascadion Ciona intestinalis. Spermatozoa were bound to receptor sites on the vitelline coat of glycerol-treated eggs ("ghost" eggs). It was concluded that only monomeric spermatozoa undergo metabolic activation.

Intact organs and tissues

Calorimetric work on organs and tissues was reviewed by Woledge a few years ago (102). Only a few studies have been reported during the last couple of years.

Niesler and Axon (103) and Coulson (104) measured the thermal powers produced by perfused small animal hearts. Ponce-Hornos et al (105) measured myocardial heat production simultaneously with mechanical and metabolic parameters by use of a newly designed perfusion calorimeter. Lorinczi (106) has studied the heat effects connected with contraction of frog striated muscle.

Hansen and Knudsen (107) used a perfusion vessel adapted to an LKB flow calorimeter to study the metabolism of white adipose tissue from obese and lean mice. On a weight basis the thermal power was significantly lower for tissue from obese mice than from lean mice, but not when expressed on a DNA basis, cf. experiments with isolated human adipocytes (82).

Very few calorimetric studies have been reported for plant materials. Anderson and Lovrien (108) have described a perfusion calorimeter which was used to monitor the thermal power produced by corn coleoptile tissue and its response to indoleacetic acid. In each experiment 250 mg of tissue was used. Heytler and Hardy (109) used a simple Dewar vessel calorimeter for measurements of heat changes connected with N_2 fixation processes in isolated soybean nodules. During the experiments, 1 g of nodules was flushed by a humidified gas stream.

Animals

Several groups employ microcalorimetry in their metabolic studies of small aquatic animals. For references see, e.g., Gnaiger (110). Instrumental and methodological aspects of this field have recently been discussed by Gnaiger (111) and by Pamatmat (112).

Lovrien et al (113) have described a twin calorimeter for use with

individual insects. The two calorimetric vessels are connected by a stopcock to give the insect the option of crawling from the sample chamber to the reference chamber (attracted by e.g. a pheromone). Air is flown through the instrument and vaporized compounds may be injected in the flow.

Currently there appear to be few calorimetric studies conducted on medium-sized animals, whereas human "whole-body calorimeters" are used in several laboratories, see e.g. (114, 115).

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