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PROGRESS AND PROBLEMS IN MICROCALORIMETRIC WORK ON MAMMALIAN CELL SYSTEMS

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ABSTRACT

Microcalorimetric techniques for measurements of processes under isothermal conditions have developed greatly during recent years, not least with respect to work on living cells. Some technical details will be reviewed briefly and some experimental problems in connection with work on mammalian cell systems will be discussed.

INTRODUCTION

Current calorimetric work on living systems includes studies of microorganisms, animal cells, pieces of tissues and intact animal organs, entire small and large animals, and materials from plants [1-3]. Within these areas the most work is currently being done on microorganisms and mammalian cells. Reports on measurements of tissue pieces and on intact organs are not very frequent. The same applies to small animals, mainly insects and small aquatic animals, and large animals, including humans. Surprisingly, very few calorimetric reports on material from plants have appeared.

The animal cells studied are usually of mammalian origin and mainly from humans. A significant part of these investigations has been performed on different fractions of blood cells. Other cell systems investigated include skin cells, fat cells, liver cells, tumor cells and cultured cells from established lines, sperm cells and muscle fibres [3-5].

Calorimetric studies on mammalian cells are undertaken for different reasons. Some investigations form parts of thermodynamic studies of energy

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balances for cell systems and in such work the calorimetric measurements are usually performed in parallel to careful biochemical analysis of metabolites formed. To a significant extent recent calorimetric work on mammalian cells has been methodological or has consisted of screening studies undertaken to explore the possible usefulness of calorimetry as an analytical technique in clinical and pharmacological investigations. Thus, many studies have been performed on cells (mainly blood cells) [3,4] obtained frm patients.

A MICROCALORIMETRIC SYSTEM

Calorimetric work on mammalian cellular systems is normally performed by using microcalorimeters operated under isothermal conditions, though there are cases where simple "macrocalorimeters" have been used successfully, see e.g. [6]. Such instruments will, however, require very large quantities of cells, typically two orders of magnitude more than the quantity required by a modern microcalorimeter.

Most of microcalorimeters used in work on living systems are of the heat conduction type [7]. The heat flow sensor normally consists of a thermopile, usually consisting of Peltier-effect plates made from semiconducting materials. For the past two decades our laboratory has been engaged in the systematic developments of such calorimeters, seeking more and more to design instrument systems which can be given several measurement functions with a minimum of mechanical and electronic components. The basic unit of our present system [8] consists of a 4-"channel" instrument where each channel is a thermopile twin heat-conduction calorimeter which can be operated with different insertion vessels or with permanently installed flow vessels. A commercial version was developed by LKB Produckter and is now marketed by Thermometric, Järfälla, Sweden.

Figs. 1 and 2 schematize some details which are of particular interest in the present context. In Fig. 1 A a twin calorimetric unit is shown. The two aluminium tubes (a) which are holders for cylindrical reaction vessels, are in contact with thermocouple plates (c). These conduct the heat *via* small aluminium blocks (b) to the surrounding aluminium cylinders (not shown) which act as main heat sinks. The reaction vessels can consist of simple closed steel or glass cylinders (1÷4 ml) or of the more complex vessels indicated in Fig. 2. Fig. 1 B shows a twin calorimetric unit fitted with ampoule holders and permanently installed flow vessels. The vessel in Fig. 2 A consists of a sample compartment (1÷3 ml), heat exchange units and devices for stirring

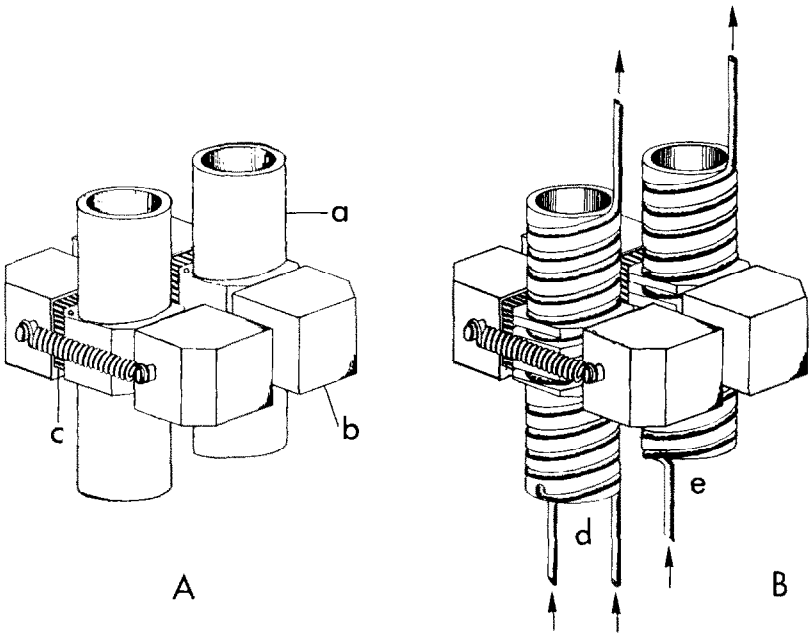

Fig. 1. A, Twin calorimetric unit: a, ampoule holder; b, aluminium block; c, Peltier effect plate. B, Combination unit: d, flow-mixing vessel; e, flow-through spiral.

and for sample injection. Sections through two sample compartments, are presented in Figs. 2B and 2C. Fig. 2B shows a 1 ml steel vessel fitted with a stirrer and an injection tube. Above the liquid there is a gas phase which may be decreased by insertion of a steel or teflon cone (not shown). Fig. 2B shows a 3 ml vessel operated as a flow-through or perfusion vessel (liquid flow rate $\leq$ 20 ml h^{-1}). A "turbine" stirrer, made from Kel F, is used. Measurements can be made with cells kept in uniform suspension. Alternatively, the cells can be anchored to the walls of the sample compartment, to a film inserted in the vessel, or to microcarriers kept in suspension, (see below).

For the determination of pH and pO_2, inserted into the sample compartment, are currently tested. They replace the plugs (h) indicated in the figure. The baseline stability is ±0.3 mW for a period of 10 h. The corresponding stability value for a simple closed ampoule is somewhat better ±(0.05÷0.1) μW.

The reproducibility of the baseline position is typically ±0.5 μW compared to 0.1 μW for a simple ampoule. Several other versions of these reaction vessels have been developed for other purposes.

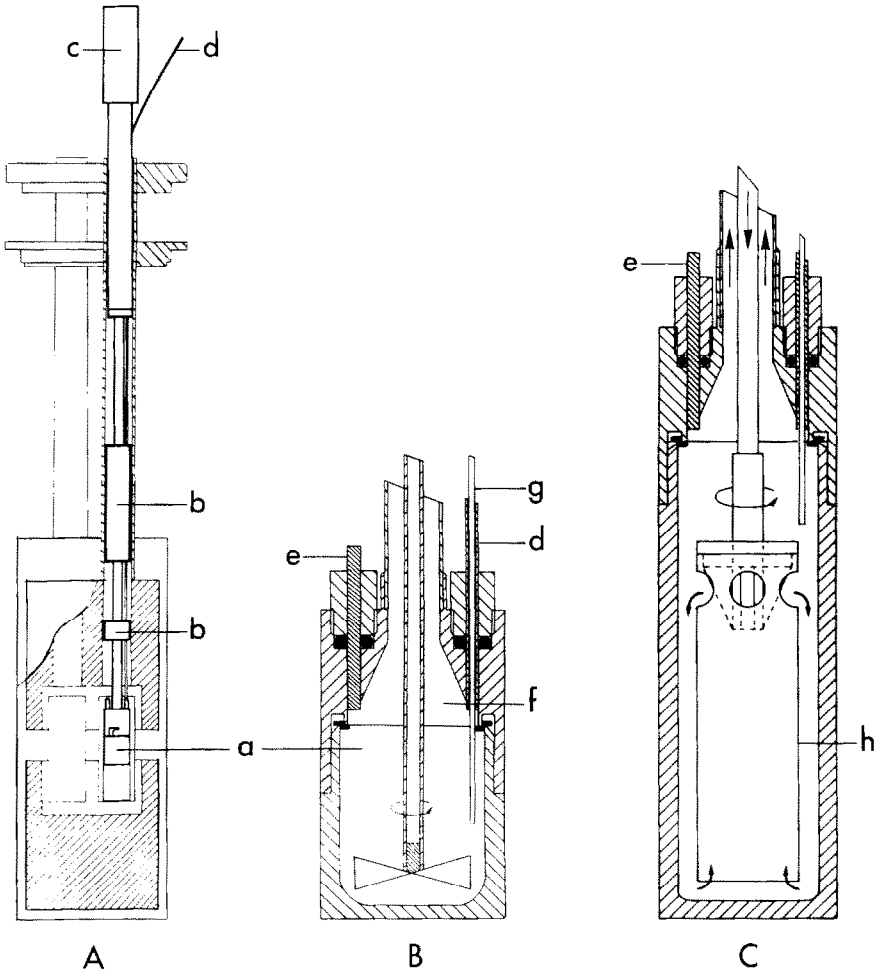

Fig. 2. Schematic view of microcalorimetric vessels used for measurements on mammalian cells. A, Vessel positioned in twin calorimetric channel: a, sample compartment; b, heat exchange bolts; c, stirring motor; d, titration tube. B, 1 ml steel vessel fitted with simple stirrer: e, plug; f, gas phase; g, injection needle. C, 3 ml perfusion vessel: h, "turbine" stirrer.

SOME EXPERIMENTAL PROBLEMS IN CALORIMETRIC MEASUREMENTS OF MAMMALIAN CELLS

It is felt that we have now reached a level of instrument development where it is possible to perform accurate measurements on the heat production rates for a wide variety of mammalian cells under acceptable and adequately controlled physiological conditions. However, there are many experimental parameters which can lower the precision or the accuracy of the

measurements. Some of these problems will be discussed below.

Preparation methods, sample storage

Samples of non-cultured cells are prepared from tissues; for practical reasons they must be stored some time before the calorimetric measurements can start. As one may expect, the method of preparation and the skill with which this is done can affect the results to a very significant extent. For instance, the cells can be damaged or the fraction of contaminating cells can vary. A striking example was given by Ikomi-Kumm *et al.*'s work on human lymphocytes [11]. Pure cell suspensions ($0.5 \div 5 \times 10^6$ cells ml^{-1} suspended in autologous plasma, static vessel) gave good steady-state power-time curves. If the cell sample was contaminated with phagocytic cells, a higher but rapidly declining power value was obtained.

As may be expected, the thermal power for a non-growing cell sample tends to decrease with storage time. The temperature during preparation and even a short storage period (1÷2 h) can also affect the power value significantly. For instance, erythrocytes [12] and muscle fibre bundles [13] stored at 4 °C for a few hours will initially give significantly higher power values when measured at 37 °C than those found in samples stored at this temperature. The effect can be explained by a build-up of the ATP pool which had been decreased during the low-temperature storage.

Cell count

It is not an easy task to assess accurately the number of cells present in a sample of mammalian cells - often the calorimetric power determination is far more precise than the cell count value. For calorimetric work in this field, it is therefore very desiderable to improve cell count methods. Special problems are met in flow experiments with cell suspensions where the cells tend to sediment or adhere to the flow lines and to the wall of the calorimetric vessel [7]. The number of cells actually measured may thus be no more than approximate.

Sedimentation, adhesion

Most mammalian cells in suspension will sediment quite rapidly if measured by use of a static calorimetric vessel. This will lead to poor definition of both cell concentrations and extracellular components. In particular, pH gradients can significantly interfere with the results of the calorimetric measurements. Poorly defined "crowding effects" and partial oxygen starvation of the cells can take place even if the average cell

concentration would not give rise to such effects. For quantitative thermochemical investigations on cell suspensions, it is thus important to ensure - by stirring or other means - that the cell concentration is uniform in the calorimetric vessel. However, care should be taken not to use too high a stirring rate, or a stirrer which otherwise might damage the cells. We have found that our turbine stirrer, Fig. 2, works very satisfactorily in this respect [9,10].

The special problems of sedimentation and adhesion in flow calorimetric experiments have been pointed out in the preceeding paragraph.

Most cultured mammalian cells require a solid support, e.g. the vessel wall, a film positioned in the vessel or microcarriers kept in suspension by a suitable stirring method. Schaarschmidt and Reichert [14] introduced the use of a specially prepared polystyrene film (Sterilin, Teddington, UK) in their work with human skin fibroblasts. A static calorimetric vessel was employed (Bioflex microcalorimeter, Thermanalyse, Grenoble, France), cf our work with melanoma cells, where the film technique was used in a stirred medium-perfused vessel [9]. In our work on green-monkey kidney fibroblasts [15], the cells were allowed to adhere to microcarriers (Cytodex, Pharmacia, Uppsala, Sweden), kept in uniform suspension with a "turbine" stirrer (Fig. 2h). Medium could be perfused through the vessel without any loss microcarriers - a sharp boundary between the suspension and the clear medium was formed immediately above the stirrer.

Oxygen concentration

Water in equilibrium with air dissolves about 0.2 μmol oxygen per ml at 37 °C. 1 ml of aqueous medium will thus contain enough oxygen for the complete oxidation of about 33 nmol glucose. This corresponds to about 10 μW during 3 h for a steady-state process. With the dilute cell suspensions normally used in work with mammalian cells, and with very sensitive calorimeters, it is thus possible to make rather long experiments without any transfer of oxygen to the calorimetric liquid during the experiment.

In experiments where the liquid medium is shallow and in cases where the cellular material is floating (adipose tissue, fat cells) significant amounts of oxygen can be transferred from a gas phase even if the medium is not stirred [7]. Oxygen can be supplied more actively to the calorimetric liquid by perfusing gas equilibrated with the medium or with an equivalent liquid (temperature, vapour pressure). The perfusion vessel shown in Fig. 2 can be used for this, but as the enthalpy of vaporization of water is very large, it is not easy to perform the equilibration precisely enough and the noise level will

TABLE 1

pH sensitivity for some mammalian cells at 37 °C. A, expressed as the increase in thermal power (%) at an increase of one pH unit at pH 7.40.

Cell type	A	Reference
Erythrocytes	120[a]	12
	150[b]	18
Thrombocytes	20[a]	20
Lymphocytes	78[a]	11
T-lymphoma cells	21[c]	10
Adipocytes	61[a]	21

[a] Static vessel; [b] Stirred vessel; [c] Stirred vessel, non-growing cells

tend to increase. A suitable gas perfusion arrangement has been described in [16], cf [17].

For cells anchored to a solid support, a satisfactory oxygen supply can usually be arranged by continuous perfusion of medium through a vessel, such as that shown in Fig. 2 C.

Often the enthalpy of solution of oxygen cannot be neglected and it is then important to know if the net consumption of oxygen relates to dissolved or gaseous oxygen [7].

We have very recently designed an oxygen electrode (a Clark cell) which can be positioned inside the reaction vessels shown in Fig. 2 [18]. The electrode signal serves as a warning of oxygen depletion and in principle one can measure the amount of oxygen consumed (our tests of the electrode have not yet been completed). Obviously, one cannot accurately measure oxygen consumption if there is a significant gas phase present in the vessel. On the other hand, if the cells are anchored to a support and medium is perfused through the vessel, it is possible to place the oxygen electrode outside the calorimeter. The twin electrode system designed by Gnaiger [19] and commercially available from Cytobios, Innsbruck, Austria, could prove interesting if tested for this purpose in experiments with mammalian cells. It has been successfully used for quantitative measurements of oxygen consumption in calorimetric perfusion experiments with small aquatic animals [19].

Variation in pH

As one may expect, the thermal power produced by a cell varies with the

pH of the medium. As shown in Table 1, however, there is a large variation in the pH-sensitivity between different mammalian cells.

It can be seen that erythrocytes in particular are extremely sensitive to variations in pH. A medium often used for erythrocytes and for other blood cells is plasma, for which bicarbonate is an important buffering substance. It should be observed that when plasma is in contact with a gas phase, it may easily lose CO_2, which may lead to a significant increase in pH and thus in thermal power. With other buffers, the pH for mammalian cell samples will decrease with time, primarily due to the formation of lactic acid. As pointed out earlier, it is important that the cells form a uniform suspension in experiments where one wants to determine an accurate thermal power value. In order to control the pH continuously during a calorimetric measurement, we have recently incorporated a pH electrode into the reaction vessel [18].

Infection

In calorimetric work on blood cell preparations, for instance, the power value sometimes increases exponentially after a few hours. One then has reason to suspect a bacterial infection. For short experiments (1÷2 h) bacterial infection is usually not a problem even if no special precautions have been taken to sterilize the calorimetric vessel.

In our laboratory we usually sterilize the calorimetric vessels with 70% ethanol (the Kell F stirrer by hydrogen peroxide solution),.followed by extensive washing with sterile water. For experiments lasting several hours, we normally add gentamicin, a broad-spectrum antibiotic, to the medium. Nassberger and Monti [22] have recently shown that this drug does not induce any thermal effects on erythrocytes, granulocytes or lymphocytes when used in concentrations normally employed in calorimetric experiments, about 0.05 g l^{-1}. In our laboratory we obtained the same result for growing T-lymphoma cells [23].

Biological variation

In work on non-cultured cells (blood cells in particular), one will usually observe a much larger variation in the thermal power values than expected from the combined imprecision of the calorimetric measurement and the cell count. In the case of calorimetric measurements linked to clinical analytical problems, it is a disadvantage that this "biological variation" is so significant. If the state of the cells could be more "normalized", it seems likely that the diagnostic value of the calorimetric measurement would increase.

Calorimetric techniques. Calibrations

It is felt that for accurate measurements on most mammalian cell systems, stirred vessels with means for injection of reagents and perfusion of the medium are the most suitable. For pieces of tissue, a rotating cage has proved to be useful as a sample holder [13]. However, it should be observed that results of calorimetric measurements on a certain cellular system can be very different depending on the calorimetric technique used. Sometimes this can be due to systematic errors in the calorimetric measurements, but the difference is probably more frequently due to differences in physiological conditions for the cells, see for instance [13].

In the calorimetric work on physical or chemical systems, it is sometimes meaningful to aim at an accuracy of 0.1÷0.01%, whereas in work on living cells, errors of a few percent are usually not significant. However, much larger systematic calorimetric errors can easily be introduced - it is difficult to combine the design requirements of an ideal calorimeter with the requirements for adequate physiological conditions and other desiderable properties for experiments with living cells carried out at the microwatt level. When accurate results are aimed at, it is therefore important to check that the (electrical) calibration value is not impaired by unacceptable systematic errors. One useful method is to place a calibration heater inside the reaction vessel, in good thermal contact with the liquid medium. Such heaters may not be convenient for routine use, but a series of test experiments with different heater positions will give the experimenter a realistic feeling for possible systematic errors and a correction factor for use with the regular heater may be derived.

It is very difficult to calibrate a flow-through vessel used with cell suspensions not retained in the calorimeter vessel. For such vessels, as well as for other vessels used for power measurements of cellular materials, a chemical test reaction seems to be the best choice. A few years ago we purpose-designed a process based on the hydrolysis of triacetin in imidazol-acetate buffer [24]. Different mixtures giving power values in the range of 7÷90 $\mu W\ ml^{-1}$ (25, 37 °C) were described. The power values for the mixtures can be calculated accurately (±0.5%) as a function of time during extended reaction periods, 20 h or more.

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