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Microcalorimetric techniques for characterization of living cellular systems. Will there be any important practical applications? [☆]

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Abstract

Microcalorimetric techniques for the investigation of living materials have been much improved during the past 25 years. A wide range of practical applications of such techniques has been envisioned. Following a brief discussion of principles and practice in microcalorimetry applied to living cellular systems some examples of such measurements are reviewed and examined with respect to their possible practical use.

Keywords: Cell characterization; Living organisms; Metabolism; Microcalorimetry

1. Introduction

Microcalorimetric instruments and working procedures for the measurement of living cellular systems have been much improved during the past 25 years. Examples of such work include investigations on microorganisms, cultured tissue cells, cells and tissue pieces prepared from human subjects and from animals, plant materials, small aquatic animals and insects. A wide range of practical applications has been envisioned in the fermentation and food industry, in clinical and pharmaceutical areas and in connection with development and control of medical techniques. Experiments on plant tissues have been presented as potentially important for agriculture and forestry, and many types of microcalorimetric investigation have been motivated by their import-

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ance to work in ecology. The main purpose with this paper is to examine where we stand today in respect of such goals. First a few important points concerning principles and practice of microcalorimetry applied to living systems will be briefly discussed. For a more detailed account of those topics see [1, 2].

2. Microcalorimetric measurements on living materials

The term microcalorimeter is not well defined. In the present paper the 'micro' prefix indicates that the thermal power sensitivity is about 1 μW or better. Vessels used in instruments designed for batch-wise measurements typically have a volume of 1–5 ml but much larger vessels are also used. The volume of a microcalorimetric flow vessel is usually smaller than 1 ml. Several measurement principles and many practical designs have been used in microcalorimetric measurements of living systems. Currently the most common type, by far, is the thermopile heat-conduction calorimeter. All examples of experiments discussed in this paper were performed with such instruments. Heat released (or absorbed) in the reaction vessel of a thermopile heat-conduction calorimeter is conducted to (or from) a surrounding heat sink via a thermopile. Normally the heat sink consists of a metal block and the thermopile is made up of one or more semiconducting thermocouple plates ('Peltier effect plates', manufactured primarily for use as thermoelectric coolers). The thermopile potential, U, is proportional to the heat flow through the thermopile. In practice, a significant fraction of the heat flow does not pass through the thermopile but that will not cause any systematic errors if an adequate calibration technique is used [3]. In most cases microcalorimeters are arranged as twin instruments.

'Peltier effect plates' have a high thermal conductance and the temperature difference between reaction vessel and the heat sink is normally very small, typically in the order of 10^{-3} K, when the 'thermal power' (the heat production rate), P, in the vessel is 100 μW, cf [4]. Microcalorimetric measurements on living systems are therefore usually conducted under essentially isothermal conditions and the calorimeters are often called 'isothermal microcalorimeters' in order to distinguish them from differential (temperature) scanning calorimeters, DSC.

During steady state conditions the thermal power, and thus the rate of the measured process, is directly proportional to the thermopile potential, Eq. (1)

$$P = \varepsilon U \tag{1}$$

where ε is a calibration constant. For microcalorimeters with 'normal' time constants ($\tau < 300$ s) Eq. (1) will often hold true, with a good approximation, also in measurements of non-steady state cellular processes. However, when P changes rapidly, for example when a stimulant is added to a cellular system, the thermal inertia of heat conduction calorimeters (the time constant, τ) must be taken into account. For a stirred vessel the simple Tian equation

$$P = \varepsilon(U + \tau \mathrm{d}U/\mathrm{d}t) \tag{2}$$

is usually adequate. It should be noted that most P–t curves reported for cellular systems in the literature are based on the simple Eq. (1) and kinetic information from the curves may then be unreliable.

In some investigations of living materials, plant tissues in particular, it has been proved useful to employ DSC in stepwise or in continuous scanning mode, see, e.g. [5].

In most microcalorimetric measurements reported for living cellular systems the reaction vessel has consisted of a closed, static ampoule. Such vessels can be suitable but, where possible, it is usually preferable to use stirred vessels (or vessels agitated by other means). For example, when a cell suspension is measured in a static vessel it is common that the cells will partly or completely sediment during the measurement. The cell concentration will not then be well defined and the concentration of the medium constituents, and the pH, will not be uniform. Tissue pieces in a liquid medium should preferably be kept in a rotating cage or some other holder bringing them in contact with a homogeneous medium [1].

Flow vessels (flow-through or flow-mixing) can be very useful for some systems but may pose problems if the cells tend to adhere to the walls of the flow vessel or the flow lines or if they are very heavy. Stirred flow-through ('perfusion') vessels are often ideal in work with tissue pieces [1,2]. For plant tissues or small animals kept in air it can be desirable to use a (wet) gas-perfusion technique [6]. Many cells are preferably studied in a state where they adhere to a solid surface. Techniques have been developed using stirred perfusion vessels with the cells adhering to a film or to micro carriers [1, 7].

The design of stirrers and of sample holders are very important instrument details in microcalorimetric work on cellular systems [1,2]. It is felt that stirred vessels with possibilites for perfusion of medium and the incorporation of injection devices ('perfusion–titration vessel') [1,2,8], electrodes [1,9], light-guides [1,10], etc, should be used more frequently than today. Simple static vessels are less expensive and can be easier to work with, but the information obtained from such vessels is usually inferior than that from the more complex vessel systems. Calorimetric work conducted on the microwatt level can easily be impaired by systematic errors. The choice of calibration technique should be given more attention and test processes should be used much more than today. In microcalorimetric work on living systems the possibilities of special types of systematic error must be considered [3]. However, it is felt that the development of microcalorimetry has now reached a level where such errors can largely be avoided.

2.1. Why calorimetry?

All kinds of living matter produce heat. The amount of heat is related to the sum of processes taking place in the material and calorimetry can therefore, in principle, always be used to monitor the overall process of a living system. However, a calorimetric 'process monitor' alone cannot identify the reaction system. The measured gross thermal power value is a complex property which depends on the net enthalpy changes for the different part-processes and of their rates. The thermal power–time curve for a biological sample can hardly be used as a general quantitative measure of its 'biological activity' unless this property is identified on the molecular level. It is, therefore, often desirable to employ one or more specific analytical techniques in

parallel with the calorimetric measurement. Why then use calorimetry at all? Why not monitor and describe the biological process solely by changes of specific properties like pH and the consumption or release of glucose, oxygen, carbon dioxide, etc.? In some practical cases it is certainly suitable to do that. However, one of the important features of calorimetry is that it can be used as sensor for all kinds of net changes of the reaction system, continuously and without any interference with the reaction system. Calorimetry is therefore an outstanding analytical technique for the discovery and quantitative assessment of unknown or unexpected part processes or phases of a complex reaction system. An expected thermal power can be calculated from analytical data describing net changes of the reaction system together with their molar or specific enthalpy values which normally are known ('indirect calorimetry') and be compared with results of a direct calorimetric determination. If there is a significant discrepancy between the direct and the indirect calorimetric value, it is concluded that the assumed model for the net reaction is not correct.

Chemical analyses should be made on a reaction system which is closely identical to that in the calorimetric vessel but it is not a trivial matter to arrange strictly parallel experiments with living material. The analyses should, therefore, preferably be made on the sample in the calorimetric vessel or, for flow systems, close to the calorimetric flow vessel. Different kinds of specific sensor, for example electrodes, positioned in the calorimetric vessel, Fig. 1, or in a flow line, are ideal. Fig. 2 shows results from the simultaneous determination of thermal power, pH and oxygen concentration (in the liquid phase) from an experiment with cultured T-lymphoma cells in a growth medium [9]. A 3 ml titration vessel equipped with electrodes (Fig. 1) was used. Fig. 2a summarizes the results of an experiment with a gas phase (air, 0.6 ml) above the stirred cell suspension. After the baseline was established with 2.7 ml of medium without cells, 0.1 ml of cell suspension was injected at the time indicated by the arrow. The corrected thermal power (Eq. (2)) increased almost linearly to twice its starting value during a 24 h period (the generation time was close to 24 h), whereas values for the oxygen concentration and the pH decreased. The non-exponential increase of P with time was caused by the effect of decreased pH [11]. The decrease of oxygen concentration in the liquid phase (expressed in terms of the equivalent gas phase equilibrium pressure) is a complex property when oxygen will diffuse over the liquid–gas interface. The main importance of oxygen measurement in this experiment is to ensure that the experiment is conducted under aerobic conditions.

Fig. 2b shows results of the corresponding experiment conducted without gas phase in the calorimetric vessel. In this case a fast linear decrease in the oxygen concentration will reach zero 5 h after the injection of the cells. The resulting changes of the P–t curve are complex as the cells will change their metabolism from aerobic to anaerobic. The pH will decrease more rapidly during the anaerobic phase. The oxygen measurements in this experiment can be used for the quntitative assessment of the oxygen consumed during the aerobic phase and thus for the comparison between results from direct and indirect calorimetry.

Criddle et al. [12] working on plant tissues in moist air (dark experiments) have developed a different technique for measurements of O_2 consumption and, simultaneously, CO_2 production. Two calorimetric vessels are connected by a gas-tight tube

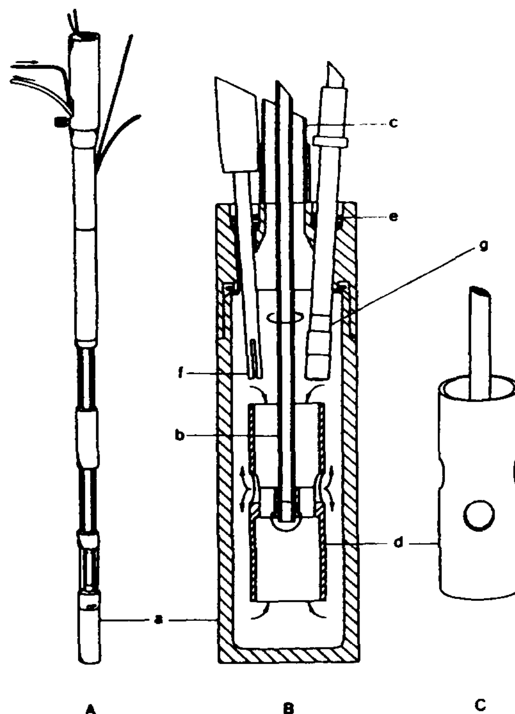

Fig. 1. (A) A titration/perfusion vessel equipped with a stirring device, a polarographic oxygen sensor and a combination pH electrode. (B) Section through the 3-ml sample compartment with electrodes mounted. The sample compartment is shown in the perfusion mode, with the turbine stirrer mounted on a hollow stirrer shaft. (C) Turbine stirrer made of Kel-F or stainless steel. (a) sample compartment; (b) hollow stirrer shaft; (c) steel tube; (d) turbine stirrer; (e) O-rings; (f) combination pH electrode with the glass membrane protected by a stainless steel tube; (g) polarographic oxygen sensor. Reproduced from [9].

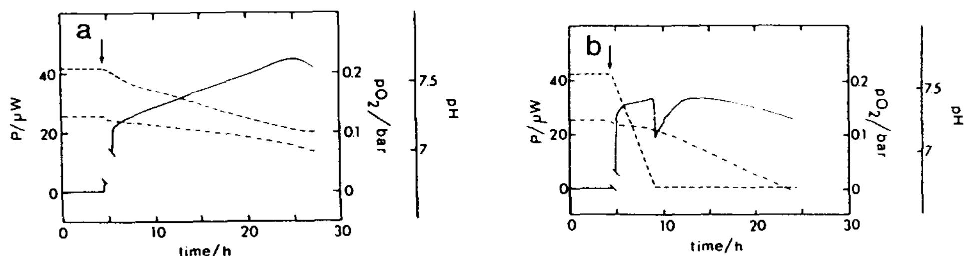

Fig. 2. Variation in heat production rate (——), oxygen activity (– – –) and pH (·–·–·) in a suspension of growing T-lymphoma cells. The experiments were conducted using the microcalorimetric vessel shown in Fig. 1. (a) After the baselines were established for the medium alone, cell suspension was added as indicated by the arrow. During the experiment, a gas phase (air) of 0.6 ml was present above the cell suspension. (b) The same experiment as in (a) except that no gas phase was present. Reproduced from [9].

and a pressure sensor is connected to the calorimetric system. The plant tissue is placed in one of the vessels and the other contains NaOH solution and serves as a CO_2 trap. The calorimetric signal from this vessel is related to the rate of release of CO_2 whereas the rate of O_2 consumption is obtained from measurements of the pressure drop in the closed system.

As yet, very few workers make use of combined microcalorimetric and chemical analytical techniques as described above. It is judged that much development effort will be conducted this area in the near future and that 'multifunctional' vessels soon will be in common use in microcalorimetric work on living material and on other complex reaction systems. However, as illustrated by the examples given below, microcalorimetry can provide the experimenter with useful analytical information on complex reaction systems even if the measurements not are conducted in combination with specific analytical measurements.

3. Some application areas

3.1. *Biocompatibility*

Isothermal microcalorimetry is presently used to a significant extent in industrial laboratories to evaluate the compatibility between different materials. Similar techniques ought to be useful in practical tests of the compatibility between living cells and artificial materials, e.g. polymers and metals. Monti, Ljunggren and coworkers have reported interesting results from work on the blood compatibility of a hemodialysis membrane, see, e.g., [13]. Fig. 3 shows results from measurements of human granulocytes. The vessels, static ampoules made from stainless steel, were lined on the inside surface with membranes made from fluorinated propylene (FEP, used as reference material) or with membranes obtained from commercially available hemodialyzers. The vessels were charged with a suspension of 10^6 cells. During the first part of the experiment (I) the cells were in their resting state and a low steady-state heat production rate is observed for the cells in contact with the reference polymer, FEP. Cells in contact with the hemodialysis membrane, a cellulose material (C), initially showed a heat burst which rapidly decreased, but for a long time the thermal power remained much higher than the corresponding value for the reference sample. The results were interpreted as an unwanted interaction between the granulocytes and the cellulose membrane material. The adverse nature of the effect was confirmed by the second part of the experiment (II) where the cells were stimulated by addition of zymosan. The result shows that the cells in the reference sample were strongly activated whereas cells in contact with the cellulose membrane gave a significantly smaller heat burst, a sign of decreased phagocytic response.

3.2. *Ecological system*

Ecologically interesting system like soil, sediments, waste water, small animals, etc, have been studied by microcalorimetric methods in several laboratories. Results of an

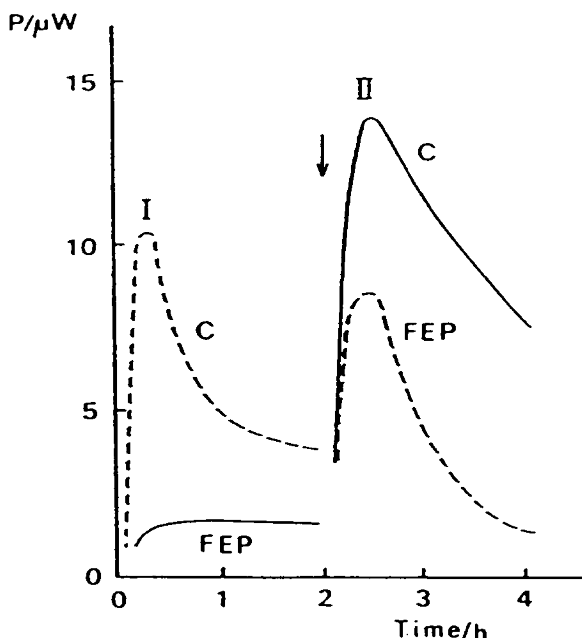

Fig. 3. Power–time curves obtained from experiments with human granulocytes in contact with the reference polymer FEP and with polymer C (regenerated cellulose). I, cells initially in resting state. II, cells stimulated with zymosan added at the time indicated with arrow. Adapted from [13].

early study on soil conducted in our laboratory [14] seem to illustrate the potential practical usefulness of isothermal microcalorimetry in one of these areas. Experiments were made on four soil samples from the same source and of the same size which had been treated by the addition of (1) a small amount of water to bring the water content of the soil to 34%; (2) the same amount of water containing salts (0.1% $NaNO_3$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% K_2HPO_4); (3) the same amount of water containing 2% cellulose powder and (4) the same amount of water containing both with the salt mixture and the cellulose. The results, summarized in Fig. 4, were the same for series (1) and (2), for which a constant P value was observed for a period of more than 10 days. The heat production is believed to be caused mainly by the microbial activity of the soil. For series (3) a significant increase in P could be seen after one week. As judged by the rapid increase of P for sample (4) the soil investigated needed a supply of both salts and an energy source for a fast increase of its microbial activity. In a review article published almost 20 years ago [15], we optimistically commented on these results: "it is judged that model experiments of the type shown... can be developed to practically useful techniques in, for example, the field of agriculture, for pollution research and for routine pollution-testing procedures". However, despite significant method work and reports of many interesting applications, see, e.g., [2, 16–18] the technique does not as yet seem to have become established outside the circle of academic laboratories.

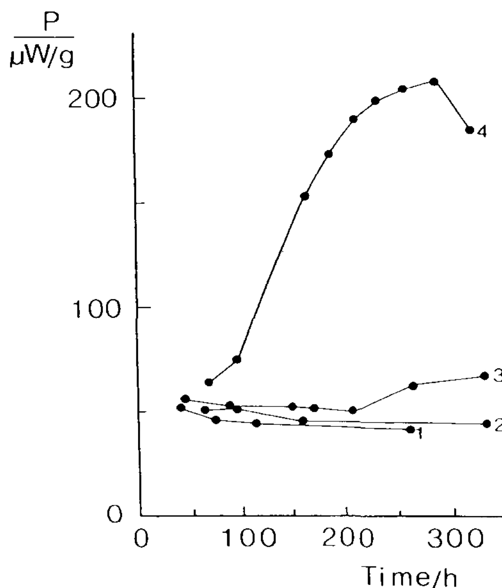

Fig. 4. Thermal power from soil samples from the same source to which was added water (1); water and salts (2); water and cellulose powder (3) water, salts and cellulose powder (4). Adapted from [14].

3.3. *Microbial calorimetric analysis of organic substances*

Several workers have shown results where isothermal microcalorimetry has been used for rapid assessment of the concentration of contaminating microorganisms in, for example, urine, blood and food but such methods have not generated any practical interest. In a somewhat related field, Lovrien and coworkers have more recently demonstrated an interesting analytical technique which might turn out to be competitive, see, e.g., [19]. These workers have shown that microcalorimetric measurements of the breakdown of organic substances by bacteria, specifically adapted for such energy sources, can be used as a sensitive technique for determination of such compounds under difficult conditions, e.g. in complex mixtures, including turbid suspensions and when the analytes are poor chromogens. Determinations on the level of 2–200 nmol can be completed in a few minutes.

3.4. *Living material from plants*

The use of isothermal and temperature-scanning microcalorimetric techniques for investigation of living plant tissue has been much developed during recent years, mainly by Hansen, Criddle, Breidenbach and coworkers, see, e.g., [5, 20]. It seems as if several important applications of microcalorimetry are close to being realized in areas like agriculture, forestry and environmental research and control. Hansen [21] has recently summarized the status of their calorimetric work on growing tissue from trees.

The technique may lead to a valuable method for the prediction of the rate of biomass production of a tree from measurements conducted on tissue samples from very young plants.

Another promising area in this field is the correlation between the rate of heat production from plant tissues and different stress factors such as temperature and salt concentration [5]. As an example, Fig. 5 shows the effect of different concentrations of NaCl on the root tissue from three different barley cultivars [22]. Root tissues were placed in a static microcalorimetric vessel on a disc of filter paper wetted with NaCl solution. Results of the calorimetric measurements on the different samples were normalized by setting the heat production rate at low salt concentration to 100%. A sudden decrease in the thermal power values to about half the maximum value was observed when the salt concentration was increased. The concentrations at which this inhibition takes place reflect the different salt tolerances of the different root tissues. When the NaCl concentration was increased above 150 mM, a second inhibition step was observed but in that case no significant difference between the cultivars was obtained.

3.5. Clinical applications

Much microcalorimetric work has been conducted in the clinical field, largely by Monti and coworkers at Lund University Hospital [23, 24]. Many investigations have been concerned with comparison of cellular material, in particular fractions of blood

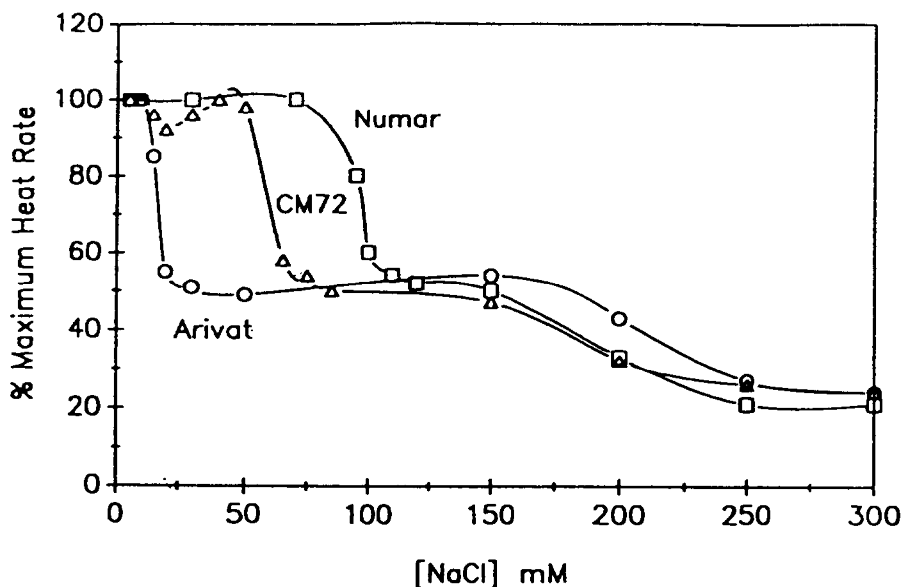

Fig. 5. Effect of NaCl concentration on root tissues of three barley cultivars: Arivat (○); CM 72 (Δ) and Numar (□). Reprinted from [22].

cells, obtained from healthy subjects and from patients. Some of these have been primarily aimed at information on a fundamental level but to a large extent the goal has been to explore the possible use of isothermal microcalorimetry as a diagnostic or a prognostic analytical tool.

A certain type of cell obtained from a healthy individual and measured calorimetrically under standardized conditions has a characteristic average value for its thermal power (expressed in terms of watt per cell, or watt per mass unit of protein, etc.). Unfortunately, the influence of biological variations is quite large and the spread in the values, determined under standardized conditions, is much larger than expected from the precision of the instrument and the experimental procedure employed. Still, it has been shown that such standard P values for many types of cell are significantly different from corresponding values determined for cells obtained from certain groups of patients. Such ΔP values are of fundamental medical interest but the metabolic state of cells prepared from a patient, and thus their P value, typically have even larger variation than the standard P value determined with materials from healthy subjects. The ΔP value determined for a certain type of cell obtained from a patient can therefore only be of limited value for the identification of a desease. However, isothermal microcalorimetry may well be used in practice to grade the state of a patient relative to some reference state or to follow a change in the P value for a certain cell type resulting from a medical treatment or caused by the progress of the disease.

As an example, the extensive studies by Monti and coworkers on non-Hodkin (NHL) cells and on peripheral blood lymphocytes from NHL patients will be referred to [25–27]. It was shown that P values were significantly correlated, both for lymphoma cells and for lymphocytes, with the degree of malignancy of the disease, although there was a significant overlap between P values for patients with progressive disease as compared with patients who improved during the treatment. Median survival time for a group of 76 NHL patients with normal P values for their peripheral blood lymphocytes was 39 months whereas the corresponding time for the group of patients with increased P values was only 8.5 months. Fig. 6 shows results of a statistical analysis of survival time for NHL patients based on P values for lymphoid cells obtained from their lymph nodes [26]. The results suggest that microcalorimetry can be used to assess the prognosis of NHL (in particular taking into account that this disease includes a heterogenous group of tumours) which can lead to optimum therapy for each patient.

3.6. *Cell–drug interactions*

There are many reports in the literature on microcalorimetric investigations of the effect of drugs and drug-like substances on cellular systems. Microorganisms as well as animal cells and tissues (mainly of human origin) have been studied; for reviews and discussions see, e.g., [28–29] (microorganisms) and [23, 24, 30] (animal cellular systems).

The effect of antibiotics on microorganisms has been studied by several groups. As an example, Fig. 7 shows results from an early investigation [31] in our laboratory on the effect of a group of related antibiotics on the same strain of *E. coli* isolated from

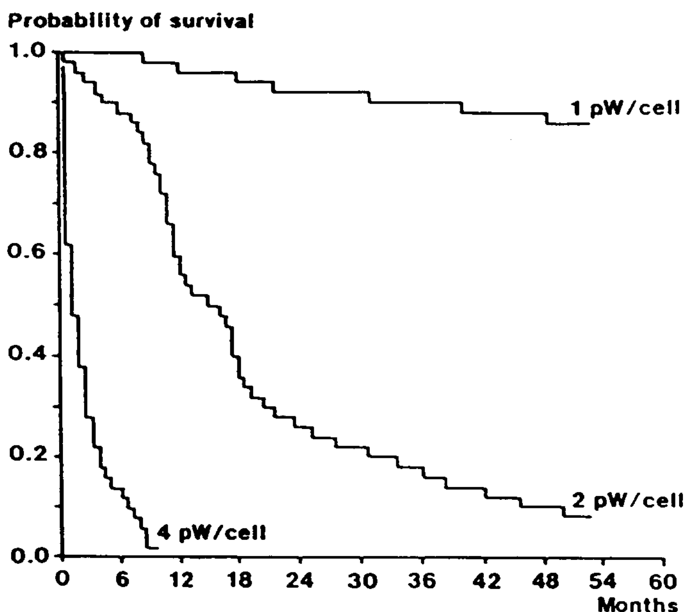

Fig. 6. Results from a statistical treatment of results from microcalorimetric measurements on lymphoma cells from NHL patients. Estimated probability of survival in relation to heat production rate of lymphoma cells for hypothetical patients aged 65 and with high grade NHL-stage III–IV. Constructed by multivariate modelling of the hazard rates. Reprinted from [26].

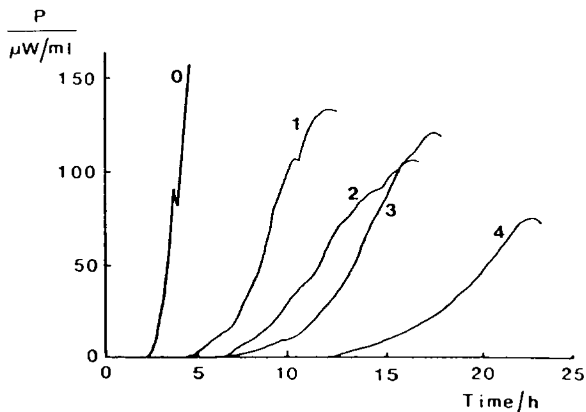

Fig. 7. Results of microcalorimetric growth experiments with *E. coli* cultured in the presence of tetracycline (1); oxytetracycline (2); doxycyline (3); and minocycline (4). The result from a control experiment (0) is also shown (cf the text). Adapted from [31].

a patient with acute infection of the urinary tract. Experiments were carried out by use of a static calorimetric vessel. The growth medium (trypticase soy broth) containing antibiotics was incubated at time zero with 10^4 bacteria ml^{-1}. Experiments were conducted with tetracycline, doxycycline, oxytetracycline and minocycline, in each

case the concentration was 0.4 μg ml^{-1}, equal to 0.5 MIC (minimum inhibitory concentration). A control experiment was conducted with the same growth medium without the presence of antibiotics. It is seen that minocycline prolonged the onset of the growth for a longer time than did the other tetracyclines tested. A large number of experiments of this kind has been reported, see, e.g., [32] (bacteria) and [33] (yeasts). Usually results have been reported on the phenomenological level as in the example above, but in some cases results also have been analyzed quantitatively, see e.g., the work of Takahashi et al. [34] and Beezer et al. [35].

Experiments on the effect of drugs on animal (human) cellular systems include several investigations on T-lymphoma cells, used as models for tumour cells in exploratory studies of the possible use of microcalorimetry in tests to predict the action of anticancer drugs [36–39]. Other recent examples of studies of cell–drug interactions include the systematic work by Monti and coworkers on the effect of β-blockers on skeletal muscle and on other cell systems, see, e.g., [40–43]. Although there are many reports on animal (human) cell–drug interactions, it is striking that only a few laboratories have been involved in such studies. As for work with microorganisms, the investigations have focused on the changes of the metabolic heat-production rate caused by the drugs. In a different kind of study, Aki and Yamamoto [44] used a flow microcalorimetric technique to obtain values for the binding of some drugs to human erythrocytes.

Isothermal microcalorimeters well suited for different types of investigation of cell–drug interactions are now commercially available. It is surprising that the pharmaceutical industry so far has shown only marginal interest for the use of microcalorimeters for such work, noting that many instruments of a suitable kind are presently employed for other tasks in their laboratories, see, e.g., [45, 46].

4. Conclusions

For a long time—about 40 years—it has been stated that microcalorimetry is a promising analytical tool for the characterization of living cellular systems in several areas of practical importance, cf Prat in [47]. Recent examples include results from pharmaceutical and the clinical areas, different medical techniques, agriculture, forestry and ecology. Microcalorimetric work in these areas has resulted in important fundamental knowledge but we must conclude that there has not yet been any significant breakthrough from a practical application point of view in any of these areas. Potential applications have been illustrated by a large number of model experiments and instruments, and working procedures have been much improved. Most of the experimental problems which we were facing 10 years ago have now been solved or at least identified and under control. Part of the applications and method work reported in this field has been conducted with laboratory-made equipment, but commerical microcalorimeters are now available which cover most experimental situations described for cellular materials. However, they are expensive and have been designed mainly as (thermodynamic) research instruments and not as routine analytical tools. It is felt that these two factors significantly restrain the use of micro-

calorimetry in the applied areas mentioned above. A more important hindrance to a breakthrough is probably connected with the basic properties of all calorimetric measurements—their non-specific nature. Keeping in mind the usefulness of the non-specific signal, I believe that 'multifunctional' instruments, i.e. microcalorimeters equipped with specific sensors (cf Fig. 1), or combined with separate instruments which provide specific analytical information, will make microcalorimetry much more competitive as an anlytical instrument for metabolic systems. It is predicted that such combined techniques will develop rapidly during the next few years.

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