Thermochimica Acta, 172 (1990) 123–130
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

EFFECTS OF EXPERIMENTAL FACTORS ON THE METABOLIC RATE OF T-LYMPHOMA CELLS AS MEASURED BY MICROCALORIMETRY *

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SUMMARY

The heat production rate of T-lymphoma cells (CCRF-CEM) cultured in suspension was measured as a function of several experimental parameters. Changes in temperature and pH induced, as expected, significant variations in the heat production rate per cell. No such effects were observed to result from variations in cell concentration and stirring rate. The preparation method used here, with standardized treatment of the sample 24 h prior to measurement, produced samples in a reproducible condition with minor day-to-day variations.

INTRODUCTION

Calorimetry is a well established tool in biological research and the rate of heat production($P = dQ/dt$) from a living system has often been used as a measure of the metabolic activity in, for example, clinical applications. For recent review on the subject, see (ref. 1).

Extensive instrument development has increased the possibility of obtaining reliable quantitative results in biocalorimetric applications. However, in order to obtain information of high precision in cellular systems, emphasis must also be focused on the development of experimental procedures. The evaluation of results should also benefit from increased understanding of the effects of various experimental factors.

The aim of the present study is to investigate the effects of several experimental factors believed to be influential during short-term batch-calorimetric experiments on cultured T-lymphoma cells. The reproducibility and condition of the samples after preparation is considered, as well as the influence of changes in temperature, pH, cell concentration and stirring rate on the heat production rate per cell (P_{cell}). Using these results, "reference" conditions have been suggested, together with methods of correcting the results for small variations in the measured experimental factors. Consideration has not been given to time-dependent changes in the general medium composition, such as reduction in substrate concentration, as it is believed that these variations are of minor importance during short-term experiments. An investigation of the effects of such time-dependent changes

* Presented at the 7th International Symposium on Microcalorimetric Applications in Biology, Egham, U.K., 9–11 April 1990, and Dedicated to Ingemar Wadsö on the Occasion of his 60th Birthday.

0040-6031/90/$03.50

during long-term experiments is in progress and will be published elsewhere (ref. 2).

EXPERIMENTAL

Cell culture

T-lymphoma cells, CCRF-CEM (ref. 3), were kindly provided by Dr C. Borrebaeck, Department of Immunotechnlogy, Wallenberg Laboratories, University of Lund. The cells were cultured at 37°C without stirring, in tissue culture flasks (Costar, Cambridge, MA) in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.3 g/l L-glutamine. The medium was buffered with 20 mM HEPES (pK_a = 7.3 at 37°C). Gentamicin was added at a concentration of 0.05 g/l. Medium and supplements were obtained from Flow Laboratories, Inc. (Rockville, MD), except for gentamicin, which was obtained from Gibco Ltd. (Uxbridge, England).

Sample preparation

The day before the measurement an exponentially growing suspension of lymphoma cells was counted and left to sediment for 1.5 h. The cell concentration was increased to $5 \cdot 10^6$ cells per cm^3 by removing cell-free supernatant. The concentrated cell suspension was then diluted 5 times with fresh medium.

On the day of the measurement, *i.e.* 24 h later, the above procedure was repeated. Changes in the final cell concentration were accomplished by adjusting the cell concentration in the concentrated suspension prior to dilution. The pH of the medium during the experiment was adjusted to the desired value by changing the pH of the fresh medium. Following the last dilution procedure the cell suspension was preincubated under experimental conditions for 3.5 h prior to the actual measurements.

Calorimetry

The commercial version of our 4-channel microcalorimeter ("Thermal Activity Monitor", LKB/Thermometric AB, Järfälla, Sweden) (ref. 4), equipped with four stirred vessels (ref. 5), was used in all calorimetric measurements.

Samples of 2.7 ml of cell suspensions prepared as above were transferred to four vessels, which were inserted into the calorimeter. A gas phase of 0.8 ml was present above the suspension, which was stirred by means of a turbine stirrer. Unless otherwise stated, the experiments were performed under "standard" conditions: at 37.0°C, with a stirring rate of 90 rpm, the initial cell concentration close to $1 \cdot 10^6$ cells per cm^3, and pH 7.2. The measurements were terminated two hours after insertion of the vessel into the calorimeter and the cell concentration and pH were determined immediately for each vessel.

The viable cell count was determined in a Bürker chamber by measuring the capacity of the cells to exclude trypan blue. Measurements of pH were made with a G297/62 capillary pH electrode (Radiometer A/S, Copenhagen, Denmark). Instrument baselines were established before and after the experiment with 2.7 ml water in the vessel. The calorimeter was calibrated with an electrical heater which could be inserted into the vessels. For a more detailed description of the calorimetric technique, see Schön and Wadsö (ref. 6).

Most values of P_{cell} are given as the mean of four parallel measurements with twice the standard deviation of the mean.

RESULTS AND DISCUSSION

The viability of the cells after the final dilution was typically above 90-95%. In the cases where the viable cell count decreased below this value, the precision of the final result decreased.

A lag-phase of about 0.5 h immediately following the dilution procedure was observed; the lag phase could last up to 2.5 h. A pre-incubation period before taking measurements is therefore often necessary. The lag-phase increased if the cells were centrifuged and washed in the dilution process and resuspended in fresh medium. This procedure was therefore avoided and the medium was renewed by the more gentle procedure described above. The existence of a lag-phase following dilution is consistent with the "leakiness" of the CCRF-CEM cells discussed below.

No significant differences between the results of experiments performed during different days were found. It is therefore concluded that only minor day-to-day variation in the sample condition existed.

No significant heat production rate was found for new or used growth medium without cells present, $P = 0.1 \pm 0.3\ \mu W/cm^3$. Thus, the observed heat production rates reported below can be entirely attributed to cell-related processes.

Variations in the gentamicin concentration in the range of 0-0.5 g/l caused no significant changes in P_{cell}. It is therefore likely that the gentamicin concentration used here, 0.05 g/l (as recommended by the manufacturer), has no significant effect on the cellular metabolism. A more thorough investigation of the effects of various antibiotics on T-lymphoma cells is in progress in this laboratory.

The pH has a well-known effect on cellular metabolism, as well as on *in vitro* enzyme activity. The relatively large pH effects found here (*cf.* figure 1) were therefore not unexpected. The maximum P_{cell} was found to be 13.0 pW

per cell at pH 7.15.

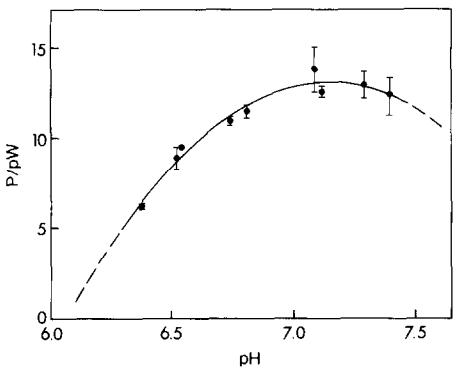

Fig. 1. The heat production rate per cell, P_{cell}, as a function of the medium pH.

From the appearance of the polynomial regression curve calculated for these values, it is tempting to draw parallels to the pH-dependence of single enzymes. However, attempts to refer the pH-dependence found here to a single enzymatic step meet with the same difficulties mentioned in the discussion of the E'_a-values below. The relative pH-dependence of P_{cell} has been measured for erythrocytes (ref. 7), lymphocytes (ref. 8) and CCRF-CEM (under non-growing conditions) (ref.6). The values found in these investigations were 120%, 78 % and 21% per pH-unit, respectively. With a similar treatment based on linear regression, a relative pH-dependence of 61% was calculated for the present system.

Figure 2a shows the heat production rate per cell (P_{cell}) at temperatures between 25°C and 42°C. The temperature dependence at 37°C was found to be approximately 1 pW/°C, which corresponds to 8 % per °C. Values are corrected to pH 7.2 using the pH-dependence of P_{cell} shown in figure 1. Although temperature is easily controlled during most calorimetric measurements, it may cause problems in measurements were calorimetric results are compared with results obtained under conditions were temperature control is less strict, e.g. biochemical analysis. The present results indicate that temperature control should be on the order of 0.1°C.

A plot of ln P_{cell} vs. $1/T$, using the results from figure 2a, is shown in figure 2b. Based on the similarities between this plot and an Arrhenius plot, an apparent activation energy (E'_a) was calculated to be 87 kJ/mol from the

slope between 25°C and 37°C. At temperatures above 37°C, E'_a began to decline rapidly. Referring E'_a to the activation energy of some specific rate-limiting step might not be possible (ref. 9). Conclusions regarding the nature of the metabolic processes based on E'_a-values should therefore be avoided unless they can be confirmed by more specific analytical methods. The linearity observed here is nevertheless remarkable and has also been observed for other cellular systems, such as lymphocytes (ref. 8) and erythrocytes (ref. 7). See also Lönnbro and Schön (ref. 10) for a thorough discussion of temperature effects on cellular metabolism in fibroblasts.

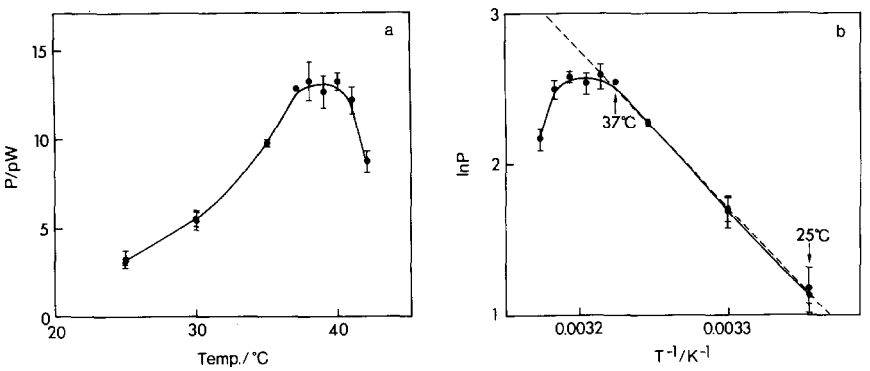

Fig. 2. (a) Changes in P_{cell}, as a function of temperature. (b) A plot of ln P_{cell} *vs.* $1/T$ based on the values in fig 2(a). All values are corrected to pH 7.2 using the results from figure 1.

Figure 3 shows P_{cell} as a function of the cell concentration in the range of 0.5 to 2.2 • 10^6 cells per cm^3. All values are corrected to pH 7.2 using the results from figure 1. P_{cell} shows no significant variation with the cell concentration. A "crowding effect" has been reported by Hedeskov & Esmann for normal human lymphocytes (ref 11). These workers found inhibition in the oxygen uptake at high cell concentrations, which they attributed to limitations in the oxygen supply. Their observation does not conflict with the present results, as the oxygen supply during the present experiments is found to be sufficient (see discussion below). A similar effect on respiration was observed by Sand *et al.* (ref. 12) in cultured cell lines derived from lymphocytes and lymphoma cells. However, they claimed that oxygen limitations did not cause this effect, suggesting "a possible regulatory mechanism by which cells (in suspension) sense the presence of nearest neighbors". If such a regulation exists, it has no significant impact on the present system. It

should be noted that the term "crowding effect" discussed here has no direct relation to the contact inhibition observed in cultures of anchorage-dependent primary cell lines.

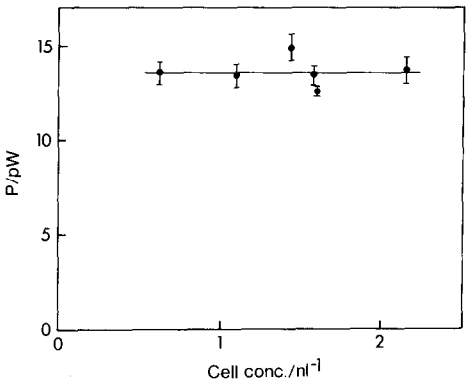

Fig. 3. P_{cell} as a function of the cell concentration. All values are corrected to pH 7.2 using the results from fig. 1.

It was frequently observed that the cells did not grow at cell concentrations below $0.3 \cdot 10^6$ cells per cm^3. This phenomenon might be due to dilution of intermediary metabolites, as cultured cells are frequently "leaky" (ref. 13).

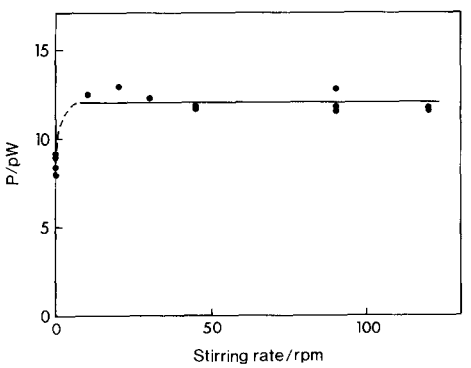

Fig. 4. P_{cell} as a function of the stirring rate. All values are corrected to pH 7.2 using the results in fig. 1.

No significant variation in P_{cell} with stirring rate was observed in the

range of 10-120 rpm (*cf.* figure 4). This indicates that the cells are not damaged by the turbine stirrer, a conclusion which is supported by the fact that no significant changes in viability could be observed in this range.

Without oxygen supply from the gas phase, the oxygen dissolved in the cell suspension (approx. 0.2 mmol/l) can be expected to last for approximately 3 to 4 h under "standard" conditions ("standard" refers here to the conditions described under Calorimetry above). In a static ampoule, the mechanism of oxygen transport to the cells is limited to diffusion. The decrease in P_{cell} observed in the unstirred vessels (figure 4), where these cells sediment during the first hour, might therefore be caused by insufficient oxygen supply. The medium oxygen concentration was measured at the end of a "standard" short-term experiment using a polarographic oxygen electrode inserted into the calorimetric vessel (ref. 14). Since the oxygen concentration was above 0.15 mmol/l the cell suspension can be assumed to have an adequate oxygen supply. A difference in oxygen activity exists between the medium and the gas phase, as oxygen is continuously consumed in the medium. The magnitude of this difference is related to the rate of oxygen consumption and the stirring efficiency. As rate limiting effects have been found at medium oxygen concentrations as high as 0.02 mmol/l (ref. 15), it is sometimes advisable to measure the actual medium oxygen activity during the experiment, especially when high cell concentrations and/or low stirring efficiencies are used.

In order to compare quantities measured, they should be referred to a common and defined state. This poses a problem in complex systems such as cell suspensions where the number of potentially influential experimental parameters is likely to be large. One way to solve, or circumvent, this problem is to assure that the initial sample condition is constant at a reproducible physiological state and use this state as a "reference" state (ref. 7). Based on the results found in this report it is suggested that the reference state should be defined with respect to pH, temperature, and the procedure used for preparing the sample, provided that cell concentration and stirring rate are kept within the investigated range. A suitable reference state might therefore be the state of the cell suspension at the time of preparation, with a temperature of 37°C and a pH of 7.2. This is a relatively constant and physiologically suitable state where minor variations in pH have little effect and it is approximately equal to the final state after a "standard" short-term measurement. The mean value of P_{cell} = 12.8 ± 0.8 pW per cell, found at this state, is therefore nearly equal to the reference value. Small deviations in pH and temperature can be corrected for using the

relationships found in figures 1 and 2a. However, it is desirable to keep the pH-value above 6.8, as the largest pH-effects on P_{cell} were found at lower pH-values with the present system.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Ingemar Wadsö for his guidance and support throughout this work. I would also like to thank Ms. G.Gräntz for preparing the drawings. This work has been supported by a grant from The Swedish Natural Science Research Council.

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