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MICROCALORIMETRIC MEASUREMENT OF AEROBIC CELL METABOLISM IN UNSTIRRED CELL CULTURES*

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SUMMARY

Studies of cell metabolism in small-volume, unstirred calorimeters has been limited by the problem of oxygen diffusion to the cells. Many cell types settle to the bottom of the sample container and become essentially anaerobic. Cells subjected to these conditions generally die, or when capable, alter metabolism to anaerobic pathways. Metabolic heat measurements in such conditions do not reflect normal metabolism and provide little information about normal metabolic rates. To avert this problem, the densities of the media supporting growth of animal and plant cells were increased by addition of sterile solutions of Percoll, causing cells to float at the surface of the media where they could readily absorb oxygen. Under these conditions, a wide range of cell types were studied. Plant, microbial, and animal cells could be analyzed for responses to metabolic effectors or environmental factors which influence growth.

INTRODUCTION

Microcalorimetry is becoming an increasingly important tool for study of metabolic activities of cells and biological tissues. Isothermal heat rates have been measured for intact cells by both batch and flow calorimetry and related to such properties as growth rates, thermodynamic parameters, effects of activators and inhibitors, stress responses and changes within growth cycles (For general reviews see refs. 1, 2, 3).

Flow methods generally require larger quantities of material than do batch methods. In addition, flow studies of animal and plant cells are frequently complicated by tendencies of the cells to adhere to flow lines or to clump and settle. Batch studies of animal and plant cells are often limited by an inability to maintain cells and substrates fully dispersed during measurements. Most cells sediment in liquid culture unless stirred; local uneven distributions of pH, and depletion of substrates and oxygen may

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result. Even with ample air volume in the head space above the sample and with uniformly suspended cells, cultured cells may rapidly deplete local oxygen concentrations. Aerobic cells which sediment and become packed at the bottom of the calorimeter ampule rapidly become essentially anaerobic.

Stirring cells within batch calorimeter ampules to maintain even distributions of substrate creates technical difficulties. Heat flows associated with the stirring apparatus cause significant problems, particularly in small scale analyses (ref. 4).

In experiments where the liquid medium above the cells is shallow, oxygen diffusion rates may be adequate to allow oxidative metabolism of sedimented cells, even in unstirred vessels. However, restricted levels of available medium in these cases, when combined with the relatively large numbers of cells necessary to obtain adequate signal intensities for measurement, may place some very restrictive limitations on the lengths of experimental tests. All measurements must be completed before the cells deplete nutrients in the limited medium. Short-term metabolic heat rate measurements are possible, but longer-term biological studies, such as evaluation of the influences of effectors on growth and metabolism, are more difficult.

Alternatively, cell calorimetry studies may be carried out on solid media. Plant callus tissue on nutrient agar (ref. 5), and animal cells adhering to polystyrene disks (ref. 6), to plastic foil (ref. 7), or to microcarrier beads (ref. 8) have all been examined. Metabolic heat rates of plant callus cultures on agar are readily determined. Large numbers of cells may be used which are freely accessible to oxygen and other nutrients by simple diffusion. However, biological investigations of substances affecting growth of such cells suffer from the inability of experimenters to precisely alter and define concentrations of effectors in the solid medium. Well-defined dose-response curves are not readily obtainable.

Growth of animal cells on solid surfaces, such as glass or polystyrene disks or microbead carriers, is commonly contact inhibited. Consequently, growth is limited as a confluent monolayer of cells is approached. This limits the number of cells which can be fit into a calorimeter ampule and requires instrumentation capable of very sensitive measurements of metabolic heat rates. In addition, attached cell monolayers must be supplied with adequate nutrient solution to support metabolism and with oxygen which must diffuse through the medium covering the cells.

One class of aerobic cells which has been successfully examined in unstirred batch calorimeters is adipose cells (refs. 9, 10, 11). These cells form a floating layer at the surface of the medium. Availability of gas-phase oxygen and the large ratio of media to cell volume results in stable cell metabolism. This stable state allows relatively easy determination of metabolic rates.

In this report we present procedures which extend capabilities for calorimetric examination of aerobic cells to a wide range of cell types. Cells in the calorimeter ampule are floated at the surface of medium supplemented with Percoll to increase solution density. With this procedure, metabolic properties of yeast, plant, insect, and mammalian cells have all been examined. The procedure allows: (a) use of large numbers of cells to ensure adequate signal intensities, (b) measurement of metabolism of unstirred cells without limitations due to rates of oxygen diffusion, (c) simple, quantitative addition of test substances for measurements of cell responses, and (d) use of media in quantities that allow long-term examination of cell changes without significant nutrient depletion.

MATERIALS AND METHODS

Calorimetric analyses were performed in a Hart differential scanning calorimeter Model 7707, run in the isothermal mode. Samples were contained in 1 cm^3 Hastelloy ampules with screw lids. The heat rate measured in the calorimeter is directly proportional to the metabolic rate. Isothermal measurements employed the methods of Criddle et al. (ref. 5). Measurement of metabolic rate by scanning calorimetry employed the methods of Hansen and Criddle (ref. 12).

Growth media for test cells were combined with sterile Percoll® (Pharmacia) solutions to obtain adequate densities to float cells. Percoll was chosen for this purpose since this PVP-coated silica sol has low viscosity, minimal effects on osmolality, little effect on pH and is generally non-toxic to growth of a wide variety of cell types. In general, Percoll (1.2 g/ml) was simply mixed with media in appropriate ratios to produce the correct density. It was necessary to prepare high-density fractions from the Percoll for studies with yeast because of the high density of these cells. High-density Percoll (approximately 1.3 g/ml) was prepared by centrifugation of stock Percoll solution at 20,000 rpm

for 40 minutes.

Fleischmann's Active Dry Yeast, Saccharomyces cerevisiae, was examined in early experiments because it is easily cultured under both aerobic and anaerobic conditions. Yeast were grown under aerobic conditions using 10% glycerol as the sole carbon source. Glucose-repressed, anaerobic growth of cells was obtained in 10% glucose. The remaining nutrient requirements were met using 1% Bacto-peptone (Difco) and 1% yeast extract (Difco). The yeast cultures were grown in Percoll-supplemented media of appropriate density.

The fibroblast cells used were baby hamster kidney fibroblasts cell line ATCC CCL10 grown in modified DB media. There were approximately 1 x 10^6 cells/ml. The tomato cells used were Tomato-45, grown on modified Murashige and Skoog medium (ref. 13). The insect cells were cultured from the fall army worm ATCC #1711 on Ex-Cell 400 media supplied by JRH Biosciences to a level of approximately 1 x 10^6 cells/ml. In experiments with these cell cultures, Percoll was added directly to the culture media in the appropriate concentrations just prior to calorimetric measurements.

RESULTS

Metabolic heat rates of brown adipose cells from hamsters are shown in Fig. 1. Because of their high lipid content, adipose cells are less dense than the medium and float to the surface in the calorimeter ampule. Accordingly, they absorb oxygen readily from the atmosphere, and with their high metabolic rates, produce large metabolic heat rates in the calorimeter. Fig. 1 also demonstrates that such floating cells can be used for measurements of the effects of added metabolic effectors. In this example, norepinepherine was added to the sample represented by the top curve in the figure, while the lower curve represents sample which was removed, opened, and reclosed without addition.

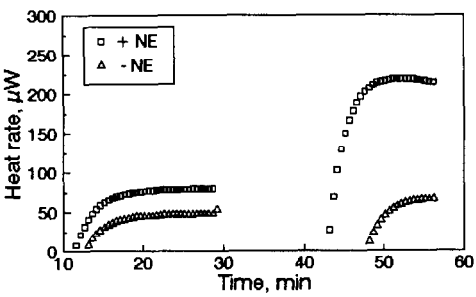

Fig. 1. Metabolic heat rates of hamster brown adipose tissue cells at 37°C.

Fig. 2 shows metabolic heat rates of S. cerevisiae aerobically grown under three experimental conditions: no Percoll, 90% stock Percoll, and 90% high density Percoll. Glycerol-grown cells

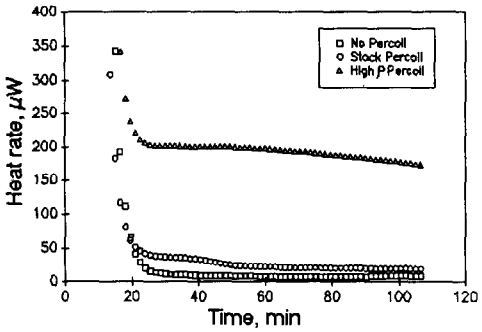

Fig. 2. Effects of medium density on metabolic rates of S. cerevisiae growing aerobically on glycerol at 25°C.

produce little heat when not floated on high density medium. When floated on high density Percoll, a dramatic increase in the metabolic heat rate of the yeast cells was observed. Table 1 lists the percent increase of metabolic heat rate from the addition of Percoll. Increases of 2.6- and 26-fold in metabolism were observed for growth of cells in stock and high density media, respectively. Increasing the density of the medium, to float the cells to the top of the ampule effectively eliminates the metabolic limiting factor of oxygen diffusion, while meeting the remaining metabolic requirements.

Anaerobic metabolism of glucose grown cells was much less dependent on the presence of Percoll (Fig. 3). Yeast grown without Percoll had a large heat output rate, thus showing its independence from oxygen diffusion. Anaerobic yeast showed a 1.5-and 1.7-fold increase with the addition of stock and high-density Percoll, respectively, over yeast grown without Percoll (Table 1). The increase results from elimination of the effects of cells settling to the bottom of the ampule. By suspending the cells throughout the medium, local depletion of nutrients, changes in pH and buildup of waste products do not limit metabolism in the unstirred cultures.

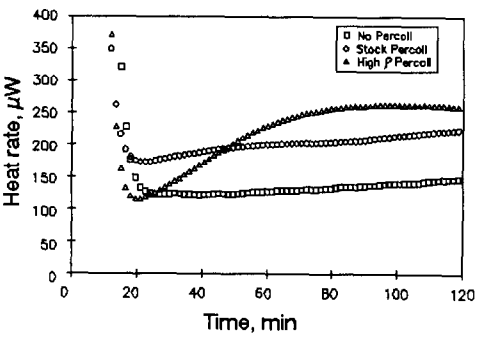

Fig. 3. Effects on medium density on metabolic heat rates of S. cerevisiae growing anaerobically on 10% glucose.

Mammalian fibroblast cells were tested with the addition of 20% and 50% Percoll directly to the culture (Fig. 4). Fibroblast heat rates cannot be readily measured in the absence of Percoll. Heat rates were essentially zero for cultures measured with either no or 50% Percoll. With no Percoll, oxygen became limiting to

cells on the bottom of the calorimeter ampule. Dilution with 50% Percoll caused excessive changes in medium osmolality and cells were killed. The addition of 20% Percoll provided enough density to bring a large number of cells in contact with air without any detrimental osmotic effects on the cells. Floating cells on the high density media allowed the long-term examination of cell changes without significant nutrient depletion.

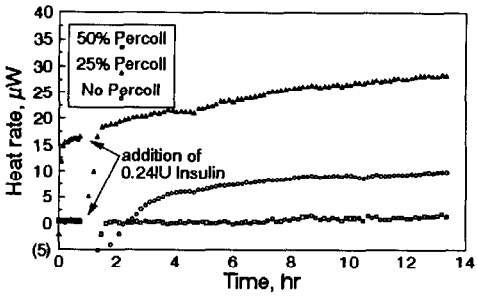

Fig. 4. Effects of medium density on metabolic heat rates of mammalian fibroblasts at 37°C.

Isothermal measurements of metabolic heat rates of tomato cells in the presence of 25% and 50% Percoll are shown in Fig. 5. Addition of greater than 25% Percoll had no added benefit in increasing the heat rate. With the addition of 25% Percoll, the density of the medium was sufficient to float cells and the fully aerobic metabolic heat rate for this volume of cells was measured. Percoll has no thermally induced reactions in the temperature range of interest for most biological studies, and therefore can also be used in experiments in which the temperature is scanned continuously. Results from scanning calorimetric measurement of the metabolic heat rate of a sample of tomato cells in culture supplemented with 25% Percoll are shown in Fig. 6 as an example. Measured isothermal data points are also indicated. These data points were collected before starting the downward temperature scan of the same sample. The agreement between the scanning and isothermal results and the lack of any

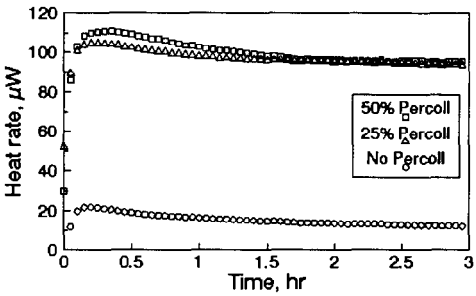

Fig. 5. Effects of medium density on metabolic heat rate of tomato cells growing at 25°C.

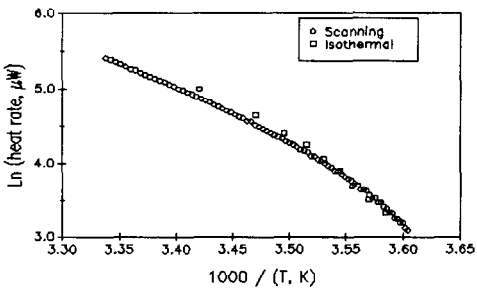

Fig. 6. Scanning and isothermal calorimetric measurement of the metabolic heat rate of tomato cells.

thermotic events shows the absence of thermally induced reactions in Percoll. The nonlinearity of the Arrhenius plot is the characteristic response to cold temperature stress on these cells. Finally, insect cell metabolism was measured isothermally using 20% and 50% Percoll added directly to the cell culture. Results are given in Fig. 7. At 20% Percoll, the density of the medium was not great enough to bring the cells to the top of the ampule and no increase in heat rate was observed. At 50% Percoll the density was great enough to float cells.

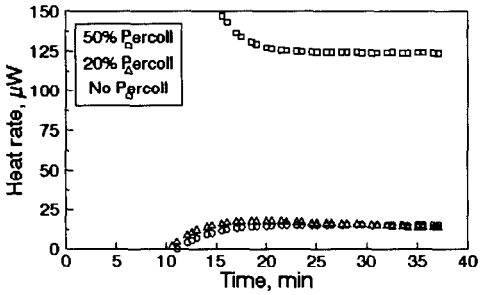

Fig. 7. Effects on medium density on metabolic rate of fall army worm cells grown on Ex-Cell 400 media.

Table 1 summarizes Percoll concentrations used in the tests of cell flotation for measurement of cellular metabolic rates.

TABLE 1

Cell flotation results.

Cell type	Heat rate μW	Fold increase in metabolic heat rate
Anaerobic yeast		
Control	132	
Stock Percoll	204	1.5
High density Percoll	225	1.7
Aerobic yeast		
Control	5	
Stock Percoll	18	2.6
High density Percoll	139	26
Tomato Cells		
Control	12	
25% Stock Percoll	94	6.6
50% Stock Percoll	95	6.7
Insect cells		
Control	15	
25% Stock Percoll	15	1.0
50% Stock Percoll	124	8.0
Fibroblast Cells + Insulin		
Control	10	
20% Stock Percoll	28	2.8
50% Stock Percoll	1	Inhibition

CONCLUSIONS

A technique has been developed for microcalorimetric measurement of metabolic heat rates of aerobic cells by floating cells on media with densities increased by the addition of Percoll. Cells float at the medium-air interface where oxygen diffusion is not limiting to cell respiration. The technique has been shown to be effective for studies of yeast, plant, and animal cell cultures. Flotation of cells on aqueous media allows long-time studies of cell metabolism in response to growth factors, inhibitors, stress factors, and other chemical and physical effectors.

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