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THERMAL BEHAVIOR OF HeLa AND KB CELLS IN SUSPENSION AND ATTACHED TO GLASS

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SUMMARY

HeLa S-3 and KB cells were grown in a LKB Batch Microcalorimeter under a variety of nutrient medium conditions and mixing intervals. These conditions produced rather large apparent endothermic and exothermic responses on mixing that could be correlated with the presence of suspended cells (unattached) as well as cells attached to the glass calorimeter vessel. Cells capable of being resuspended upon mixing of the calorimeter vessel produces first an endothermic followed by an exothermic signal while attached cells produced only an apparent endothermic response. The exothermic response is believed to be associated with increased metabolic heat on suspending the cells followed by partial suppression of the steady state metabolic heat on cell settling. Rates of cell settling correlated well with the rate of decay of the exothermic signal. The rapid appearance of endothermicity on mixing suggests it is associated with rapid events such as binding of nutrients to cell surfaces. The response in the endothermic direction on mixing is discussed in terms of the disruption of mechanisms which tend to exclude nutrients from the surface of the cell.

INTRODUCTION

Heat produced by living cells is composed of metabolic (chemical reaction) events as well as physical interactions both internal and external to the cell. It is therefore a very complex quantity, but nevertheless an observable characteristic of the cell itself. Much of the reported cellular microcalorimeter work has dealt with microorganisms. From such studies microcalorimetry has emerged as an analytical tool in microbiology [1].

Initially, we set out to define the heat profile of synchronized HeLa cells in suspension with the objective of assessing the heat output associated with G_1, S, G_2, and M phase metabolic events. Our initial experiments proved perplexing since the thermal response from mixing cells in culture was quite dependent on medium conditions and cell concentration. Under certain medium conditions large responses in the endothermic direction were observed on mixing while under other conditions exothermic behavior was exhibited.

The studies presented here were designed to investigate the behavior of HeLa and KB cells growing in the LKB Batch Microcalorimeter and to establish conditions suitable for batch calorimetric investigations of tissue cultured cells.

MATERIALS AND METHODS

Reagents

Joklik's modified minimal essential medium, non-essential amino acids and horse serum were obtained from Kansas City Biological Inc. (Lenexa, Kan.), tylocine and calf serum from Grand Island Biological Co. (Grand Island, N.Y.) and trypsin (1 : 250) from Difco Laboratories (Detroit, Mich.).

Cell cultivation

KB cells were a gift of Dr. Maurice Green, St. Louis University, and HeLa S-3 cells were obtained from American Type Culture Collection (Rockville, Md.). Cells were grown in suspension culture at 37 °C using Joklik's modified minimal essential medium and non-essential amino acids supplemented with tylocine (60 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and either 5 % horse serum or 10 % calf serum as indicated.

Log phase growth of stock suspension cultures was maintained by adding an equivalent volume of fresh medium (prewarmed to 37 °C) each day. Cell concentrations were routinely maintained between $2 \cdot 10^5$ and $5 \cdot 10^5$ cells/ml. Population doubling times for KB and HeLa cells were 24.5 h and 24.7 h respectively. Cultures were screened periodically for mycoplasma contamination according to Hayflick [2].

Cells were cultured in the calorimeter with rotation of the calorimeter chambers (mixing) every 30 or 60 min*. Rotation at these constant intervals permits the cells to grow at the same rate as cells in suspension. Aseptic conditions were monitored by incubating an aliquot of medium withdrawn from the calorimeter for seven days at 37 °C.

Instrumentation

Calorimetric measurements were performed at 37 °C in a Model 10700 LKB Batch Microcalorimeter equipped with glass vessels with an internal volume of 10 ml. The instrument was housed in a constant temperature environment room at 35.9 °C. Heat signals generated from the experiments were recorded and quantitated by a disc integrator. Turbidity measurements were made at 600 nm using a Beckman Acta M VI Spectrophotometer at 37 °C.

Calorimetric experiments

The effect of medium alone in the calorimeter was determined by injecting 6 ml of fresh medium into both the reference and sample vessels by using a sterile plastic disposable syringe fitted with a teflon needle. A volume of 6 ml was chosen so that the ratio of liquid volume to air space was consistent with the suspension culture conditions.

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* The LKB Microcalorimeter was modified to permit programmable mixing intervals. Circuit diagrams and a description of the modification will be supplied by the authors on request.

The 30 or 60 min mixing program was then initiated and the heat effect pattern recorded. This preconditioning of the calorimeter vessels with medium acclimates the glass surfaces of the vessels and provides a base line for comparison with the subsequent experiment with cells.

Approximately 15 h later the heat effect pattern produced by the calorimeter rotations was integrated and an electrical calibration performed. The medium was then extracted from both vessels and sterility checked as described above. The calorimeter was then prepared for a cell experiment by injecting 6 ml of cell free culture medium into the reference vessel. This medium was obtained from the stock suspension culture immediately prior to the daily dilution of the culture. The cells were removed by centrifugation ($400 \times g$, 6 min). In the sample vessel was placed 6 ml of a cell suspension (approximately $1.2 \cdot 10^6$ cells) obtained from the stock suspension culture at least 45 min after the daily dilution. The mixing interval (30 or 60 min) was then set and the heat effect pattern recorded.

Approximately 20 h later the heat effect pattern, after a mixing event, was integrated and the calorimeter contents assayed for cell growth. Cells in suspension were removed by extracting the medium fraction immediately after rotating the sample vessel. A 6 ml aliquot of trypsin (0.1 % in Hanks salts minus calcium, magnesium and glucose, pH 7.4) was added to the sample vessel and the calorimeter mixed every 2 min for 10 min, after which the trypsin fraction was immediately withdrawn. Cells were counted using a hemacytometer. Cells removed by trypsin treatment were assumed to have been attached to the glass vessel while the medium fraction was assumed to contain unattached cells or cells capable of being resuspended*.

At the conclusion of the experiment the calorimeter was cleaned by rinsing the vessels with 30 ml of water and 30 ml of a dilute sodium dodecyl sulfate solution. This was followed by three 30 ml water rinses, a methanol rinse for sterilization, and by air drying with a stream of filtered compressed air.

RESULTS

Fig. 1a gives a representative 24 h heat effect pattern of medium alone prior to introduction of KB cells. The perturbations represent the mechanical heat of mixing at 60 min intervals. The baseline itself is invariably very close to zero thermal output at the termination of the experiment. Figs. 1b and 1c show the heat effect pattern of KB cells grown in medium with 5 % horse serum at mixing intervals of 60 and 30 min respectively with deflections corresponding to the rotation (mixing) of the calorimeter vessel.

Normal operation of the LKB Batch Microcalorimeter requires the introduction of two different reactant solutions into separate compartments of the sample chamber. The two solutions must come to thermal equilibrium in order to establish a zero thermal output baseline prior to initiation of reaction by mixing. Healthy, growing tissue culture cells continually generate heat as a result of metabolic activity and no zero thermal baseline can be established after they are introduced into the calorimeter (Figs. 1b and 1c). Thus with the LKB batch instrument we cannot

* Cells that are attached to the calorimeter glass vessel are referred to as "attached cells" and cells capable of being resuspended upon mixing are referred to as "unattached cells".

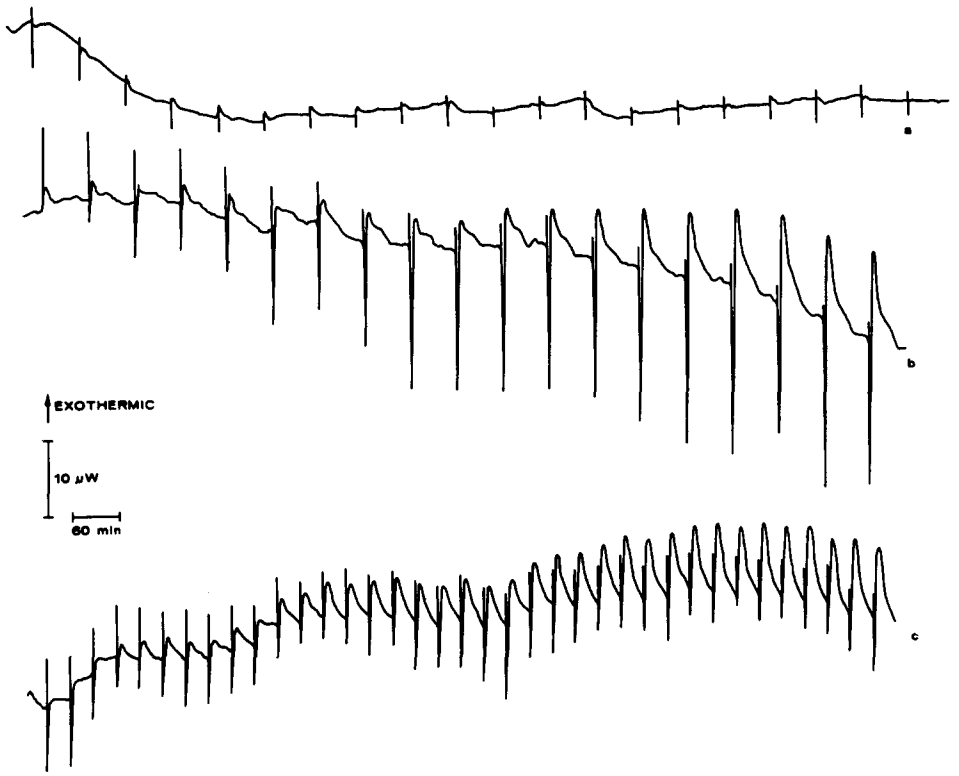


Fig. 1. Representative heat effect patterns due to mixing in a batch calorimeter: (a) medium alone, (b) KB cells in medium supplemented with 5 % horse serum. Over 95 % of the cells unattached with a mixing interval set at 60 min, and (c) KB cells present as described in Fig. 1b except that the mixing interval was set at 30 min.

accurately establish the thermal output of metabolizing cells. However, at the termination of the experiments we have used the μV output to estimate an average of 60 ± 4 μW (exothermic) for $2.4 \cdot 10^6$ cells or 25 pW/cell as the metabolic baseline heat.

Though there is variability in the steady state signals in the early hours of each experiment the final heat output ($\approx$ 20 h) appears remarkably constant. It should be fully understood that the signals we are concerned with in this work are the perturbations in heat output on top of this exothermic metabolic heat.

After several hours the perturbations due to mixing of cells are composed of two signals. The first signal appearing on mixing is an endothermic deflection of 3–4 min duration which is immediately followed by and sometimes overshadowed by an exothermic deflection with a duration of as much as 30 to 60 min. This heat effect pattern was obtained when the total number of cells, all in suspension, did not exceed $2.4 \cdot 10^6$. The magnitudes of the endothermic and exothermic signals are presented in Table I. Since the endothermic signal is closely followed by the exothermic, any overlap will result in underestimation of both types of heats. Hence, the heats listed in Table I represent minimum values.

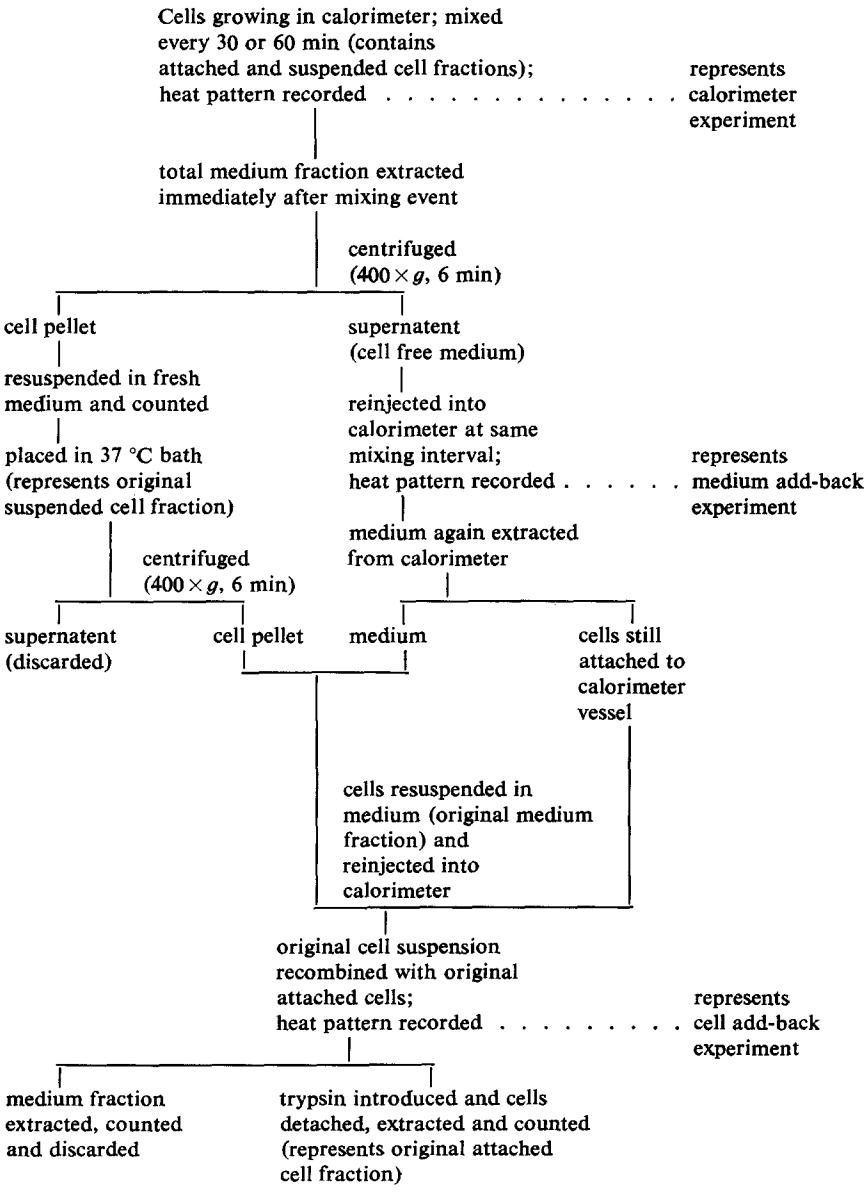
Because it is possible that the medium, and not the cells, could be producing

TABLE I

Average heat values of the final mixing deflection at approximately 24 h using KB cells grown in medium supplemented with 5 % horse serum. At least 95 % of the cells were unattached. In the worst case the errors are no greater then ±70 %.

Mixing interval (min)	Endothermic (mJ)	Exothermic (mJ)	Number of measurements
30	1.0	6.2	5
60	2.5	12.3	3

Scheme 1



the heat effect pattern, "medium add-back" and "cell add-back" experiments were performed as outlined in Scheme 1. In a typical medium add-back experiment the initial procedure is the same as the one used to generate the data in Fig. 1. At the conclusion of the calorimeter experiment, the medium fraction is removed from the sample vessel and centrifuged. The resulting cell pellet is resuspended in fresh medium, counted and then placed in a 37 °C bath. The cell free supernatant is reinjected into the sample vessel and the heat effect pattern recorded using the same programmed mixing cycle. Centrifugation and reinjection takes about 10 min. This procedure constitutes a medium add-back experiment in which only unattached (suspended) cells are removed from the calorimeter vessel.

A cell add-back experiment could be performed by extending the above described operations. At the conclusion of a medium add-back experiment, the unattached cells are again pelleted and resuspended in medium extracted from the sample vessel. This cell suspension is reintroduced into the sample vessel that still contains the attached cell fraction. The mixing interval (30 or 60 min) is set and the heat effect pattern recorded.

Figs. 2a, 2b and 2c show the mixing deflections from a normal calorimeter experiment, a medium add-back experiment and a cell add-back experiment respectively. The corresponding cell counts for these experiments are listed in Table II. It can be seen from the data that the mixing deflection from the calorimeter experiment almost disappears in the medium add-back experiment where 93 % of the cells were removed (as suspended cells) and that the original endo-exo signal was substantially recovered in the cell add-back experiment.

It was noted that as the number of unattached cells increased, a larger exothermic signal was produced upon mixing. To further explore the possible connection between the exothermic signal and unattached cells, the rate at which cells settle out of solution was compared with the rate of decay of the exothermic signal.

The rate of decay of the exothermic signal was measured by introducing an excessive amount of HeLa cells ($3.0 \cdot 10^6$ cells) into the sample vessel as in a normal

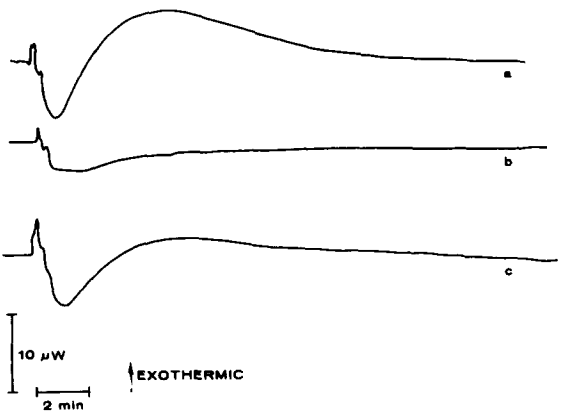


Fig. 2. Representative heat effect patterns due to mixing: (a) calorimeter experiment, (b) medium add-back experiment and (c) cell add-back experiment. Cells were grown in medium supplemented with 5 % horse serum. See Table II for corresponding cell counts.

TABLE II

Cell counts for a representative calorimeter experiment, medium add-back experiment and cell add-back experiment.

Experiment type	Counts ($\times 10^{-5}$ cells)		
	Medium fraction (unattached)	Trypsin fraction (attached)	Total cells*
Calorimeter	22.0	—	23.0
Medium add-back	0.8	—	1.8
Cell add-back	22.6	1.0	23.6

* Assuming $1.0 \cdot 10^5$ cells were attached to the vessel during the course of all experiments.

calorimeter experiment. The mixing interval was set at 30 min and the heat effect pattern recorded. The logarithmic decay portions of the exothermic signals produced after 17 h were used to construct a first order rate plot. A representative signal is shown in Fig. 3. An apparent rate constant of 0.13 ± 0.02 min^{-1} was calculated from four successive rotations. It should be noted that the rate of decay of the exothermic signal far exceeds the time constant of the LKB Batch Microcalorimeter.

The first order rate constant of cell settling was determined from a plot of the decrease in absorbance (light scattering) versus time. In this experiment 3 ml of a HeLa cell suspension ($3.4 \cdot 10^5$ cells/ml) were placed in a cuvette and rotated every 30 min for 17 h. The cuvette was then rotated and placed in the spectrophotometer as described (see Methods). The average rate constant for three successive rotations was 0.12 ± 0.02 min^{-1}. It can be seen that the apparent rate constants indicate a direct relationship between cells settling out of solution and the decay of the exothermic signal.

In order to establish whether the physical action of particles settling out of solution contributes to the exothermic signal, an experiment using HeLa cells fixed in ethanol/acetic acid (3 : 1, v/v) was performed. HeLa cells ($2.2 \cdot 10^6$ cells) were washed twice in phosphate buffered saline before being suspended in ethanol/acetic acid for 5 min. The cells were again washed twice in phosphate buffered saline, resuspended in medium, and injected into the calorimeter. The heat effect pattern, which was recorded over 17 h, was essentially identical to one produced by medium

Fig. 3. Heat effect pattern of a single mixing event after 17 h with an excess number of HeLa S-3 cells grown in medium supplemented with 10 % calf serum.

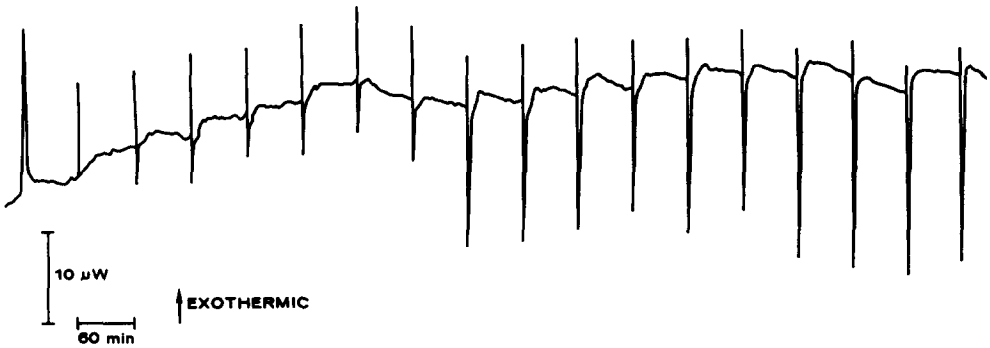


Fig. 4. Heat effect patterns due to mixing of Hela S-3 cells grown in medium supplemented with 10 % calf serum. Mixing interval set at 60 min.

alone (Fig. 1a). Thus the exothermic signal appears to depend on the presence of viable cells.

To further explore the origin of these signals an experiment was designed in which a majority of the cells was attached to the glass surface of the calorimeter vessel. This was accomplished by using HeLa cells in medium supplemented with 10 % calf serum. Fig. 4 shows a representative 24 h heat effect pattern; the corresponding cell counts are given in Table III. It can be seen that with 71 % of the cells attached to the glass surface a response in the endothermic direction (2.8 mJ) is produced with little evidence of any exothermic behavior. Apparently, there is an insufficient number of unattached cells to produce a detectable exothermic signal.

This experiment was carried a step further by performing a medium add-back experiment (see Scheme 1) resulting in 100 % of the cells being attached to the glass surface. Upon mixing, the heat pattern produced an apparent endothermic response of 1.8 mJ (Table III). The reduction in the signal is likely a result of the removal of the unattached cells which also contribute to the apparent endothermic signal as was seen in the initial mixing events in Figs. 1b and 1c.

TABLE III

Cell counts and heat values of a representative calorimeter and medium add-back experiment with HeLa S-3 cells grown in medium supplemented with 10 % calf serum.

Experiment type	Cell count ($\times 10^{-5}$)		
	Medium fraction	Trypsin fraction	Endothermic signal (mJ)
Calorimeter	6.1	14.8	2.8
Medium add-back	0.0	14.8	1.8

DISCUSSION

Metabolizing HeLa and KB cells under our conditions evolve heat at what we estimate to be 25 pW/cell as mentioned in the results. Wadsö (private communication) has obtained similar values with HeLa cells by a more direct method using a drop

ampoule microcalorimeter. Our interest in these experiments has been to characterize the thermal behavior of HeLa and KB cells on top of this exothermic metabolic heat.

As previously stated most experiments have initially $1.2 \cdot 10^6$ cells in 6 ml medium. Under these conditions with KB and HeLa cells we get population doubling in 24 h regardless of whether the cells are mixed at 30 or 60 min intervals.

The data in Figs. 1b and 1c reveal both an endothermic and exothermic response upon mixing. Furthermore, under certain conditions we get only an endothermic response (Fig. 4) and under other conditions we observe signals that are entirely exothermic (Fig. 3).

The endothermic and exothermic perturbations produced upon mixing are qualitatively the same whether KB or HeLa cells are used. The quantitative differences in signals arise from a difference in the number of cells attached versus unattached in the sample vessel.

The differences in the degree of cell attachment is dictated by cell type and serum content. It is known that 3T3 mouse fibroblast cells avidly attach to glass surfaces and very few free cells are found suspended. When these cells are grown in the calorimeter they exhibit only endothermic responses on rotation of the chamber (mixing). Furthermore, the HeLa medium add-back experiment in which only attached HeLa cells are present also gives only an endothermic response (Table III). These endothermic responses appear to be a manifestation of the physical state of the cell (attached) independent of cell line. Thus, their origin may be thought of in terms of a cell's response to its environment.

In experiments with a large proportion of attached cells the endothermic signal is rapid, having a duration of 3–4 min. From the data in Table III and Fig. 4 it is seen that the endothermic signal is produced by attached as well as unattached cells. Being the earliest measurable thermal response after mixing, it is likely that the endothermic behavior reflects such rapid processes at the cell surface as binding of nutrients to the cell and subsequent transport. Though endothermic binding is much less frequent than exothermic binding such behavior can and does occur [3]. In fact, the binding of metabolic substrates to bacterial cells has been observed to be an endothermic process (Lovrien, R., private communication).

We suggest two possible mechanisms which would result in greater cell surface binding of nutrients on mixing. Cell crowding diminishes access of medium to cell surfaces and mixing disrupts cell interaction and exposes greater surface area to the medium. This explanation is supported by experiments in Figs. 1b and 1c which show the rapid appearance of an endothermic response. The second mechanism involves the establishment of a diffusion boundary layer about cells which is perturbed on mixing [4–6]. This mechanism would prevail in experiments with a large majority of the cells attached, e.g. a medium add-back experiment.

Prior to mixing, the cells in the calorimeter are in a relatively static environment. Under these conditions the flow of liquid at the cell surface is at a minimum and the transfer of molecules to and from the cell surface is largely diffusion dependent. This produces a concentration gradient (diffusion boundary layer) in which a layer of medium at the cell surface has nutrient concentrations less than that of the bulk medium. Disruption of these concentration gradients can occur on mixing, resulting in a heat response. Stoker [4] has indicated that establishment of diffusion boundary layers in 3T3 mouse fibroblast cultures is responsible for the inhibition of growth of

the monolayer. Upon perturbing this layer by gentle flow of medium, cell division of a confluent monolayer took place. If this interpretation is correct then the boundary diffusion layer exerts significant control over cellular behavior and is likely to manifest itself initially as a rapid thermal response on mixing.

From our observations, we have found that an exothermic signal is produced only when there are reasonably large numbers of cells present which can be resuspended upon mixing (Figs. 1 and 4, Tables II and III). Thus, there seems to be a correlation between cells not attached to the glass surface and the exothermic signal. Prior to mixing, we find that the majority of the cells have settled to the bottom of the calorimeter vessel. Because of crowding, they have poorer contact with medium but nonetheless are metabolizing to some degree and producing heat. This crowding (or layering) can be observed in an external glass sample vessel with dimensions of the calorimeter vessel. Also, we have calculated that a cell grown in suspension occupies an area of roughly $2.4 \cdot 10^{-6}$ cm^2. With $2.4 \cdot 10^6$ cells in the calorimeter vessel, which has a bottom surface area of less than 2.6 cm^2, one can easily see that cells are layered and in poor contact with the medium. Upon mixing however, many of the cells are resuspended, placing them in intimate contact with needed medium components. The cells appear to react to this medium contact by a burst of metabolism in the form of an exothermic signal. This burst signal is transient and ultimately the magnitude is suppressed as the cells settle to the bottom of the vessel and crowding begins to suppress their metabolic activity. This medium stimulation and subsequent suppression is substantiated by the fact that one obtains the same apparent rate constant for decay of the exothermic signal and the rate of cells settling out of solution. Since cells are already producing a steady state exothermic baseline heat the effect of mixing crowded cells would be to release them from a suppressed state by bathing all cell surfaces in the medium.

An obvious feature of the heat effect patterns in Fig. 1 is the greater magnitude of the exothermic and endothermic responses as the experiment progresses. These results support our interpretation of the origin of the heat responses. During the intervals between mixing, a static (unperturbed) environment is produced and the local nutrient medium surrounding the cells becomes increasingly depleted resulting in a larger exothermic signal upon mixing (desuppression of metabolism). Increases in cell number also bring about greater crowding and depletion of nutrients from the medium thus increasing the exothermic response. By the same token, the greater need for medium components will result in greater binding and transport of nutrients producing a larger endothermic response. Since the number of cells in contact with the bulk medium is constant under static conditions, the endothermic response due to the diffusion boundary effect should remain constant.

To maximize the access of medium components at the cell surface we performed a calorimetric experiment using a 5 min mixing interval. By mixing this frequently the static environment necessary for establishing diffusion boundary layers is greatly reduced and cell crowding is also minimized. As seen in Fig. 5, the endothermic and exothermic responses do not have an opportunity to develop and the thermal responses of mixing events are quite similar to the first two mixing events in Figs. 1 and 4, i.e. without occurrence of significant endothermic or exothermic responses.

The gentle and frequent (5 min) mixing of KB cells approximates normal suspension culture conditions. The cells double in 24 h and when removed from the

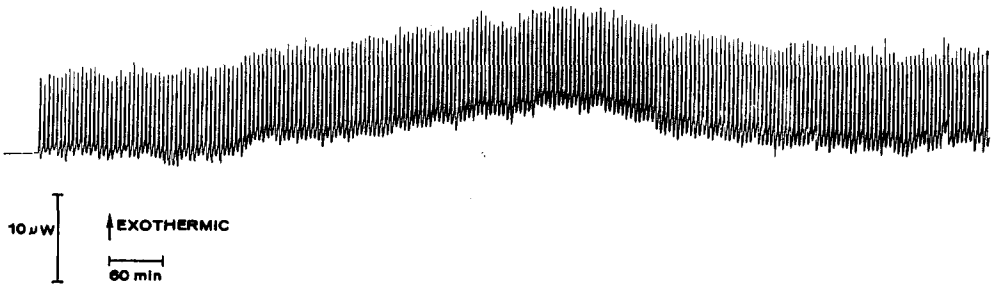


Fig. 5. Heat effect patterns due to mixing of KB cells as described in Fig. 1. Mixing interval was set at 5 min.

calorimeter and cultured they continue to thrive. We expected to see an increase if not a doubling in the exothermic baseline heat of about 60 μW over the 24 h period but this was not apparent in any of our experiments. Wadsö (private communication), using a drop ampoule microcalorimeter has also experienced nonproportional behavior of HeLa cell number with heat output. Kemp on the other hand, has found a linear increase in heat output for 16 h using embryonic chick muscle cells attached to glass beads and flowing medium around the cells [7]. Since the use of continually fresh medium produced the expected behavior, it is possible that accumulation or depletion of some component in the medium may be affecting the thermal response in our batch experiments. Regardless of the mechanism, the effect does not retard the growth of the culture.

The experiments performed here illustrate that the complex thermal behavior of tissue culture cells on mixing reflects their physical state (attached or suspended) as well as their metabolic behavior. The dissection of these thermal responses and the possible origins of the endothermic and exothermic effects, provide a basis for interpreting the thermal profiles of tissue culture cells in the batch microcalorimeter.

ACKNOWLEDGEMENTS

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