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MICROCALORIMETRIC DETERMINATION OF GLUCOSE UTILIZATION BY LEISHMANIA *

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SUMMARY

Heat conduction calorimetry of Leishmania amazonensis parasites was carried out using 1 to 6 x 10^8 cells (promastigotes), dependent on glucose concentrations in the calorimeter and dependent on glucose used as carbon source in cell culture. Leishmania produce aerobic heat from endogenous sources even when given no exogenous carbon. However, depending on how cells were grown (adaptation) they respond to glucose addition with increased heat production up to a limit roughly around 25 μM glucose, plateauing thereafter. Heat production ranges from 8 to 135 x 10^{-4} millical/(sec)(10^8 cells) when dissolved oxygen is adequate.

INTRODUCTION

Microcalorimetry has been successfully used to study microbial metabolism, predator insect toxicology (1), fermentation control (2) as well as in basic chemistry, enzyme-ligand interaction (3), denaturation (4) and catalysis (5). The high sensitivity of conduction calorimeters enables one to measure heats of the order of 5 mcal. in reaction systems of 1 to 3 ml. volume containing ca. 10^7 to 10^8 cells, consuming nanomole amounts of metabolites in 5 to 15 minutes.

Such specifications make the technique attractive for metabolic and toxicologic research on microorganisms difficult to cultivate in vitro such as Leishmania and Trypanosoma (6). So far there are no reports of microcalorimetry with parasitic protozoa of this kind. We report now studies aimed at defining the profile of simple carbohydrate utilization by Leishmania, starting with glucose, known as an energy source for these organisms (7). Leishmania amazonensis, one of the pathogens causing New World leishmaniasis also known as muco-cutaneous leishmaniasis, was selected as the first organism to be worked with because of its relatively easy cultivation in vitro. An important extension of the work should be establishment of a fast screening

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test for drugs acting on both promastigote and amastigote forms of the numerous Leishmania species causing disease in man.

MATERIALS AND METHODS

Leishmania amazonensis strain PHB (IFLA/BR/67/PH8) was maintained in complex medium MD-29, plus 8% (v/v) bovine fetal serum, or in a chemically defined medium MD-29, prepared according to Melo et al. (6). Specific components including carbohydrates may be added, or deleted from this medium for metabolic study.

Promastigotes were collected by centrifugation at 2500 rpm for 15 minutes at 4°C., washed three times in phosphate/chloride buffer (PBS, 2.7 mM KCl, 0.137 M NaCl, 8.2 mM Na_2HPO_4, pH 7.2), and suspended in this PBS for calorimetry. Cells were counted in Neubauer chambers with a 4% standard error. Cells not used immediately after harvest were kept in an icewater bath, which they withstand well for several hours. After each calorimetric run cell motility was checked by microscopy as a criterion for viability.

Calorimetry was performed in a heat conduction batch mixing instrument similar to Wadsö's instrument (8) and calorimeters built in our respective laboratories in Brazil, and the U.S. (5). The Seebeck thermopiles have a figure of merit of 7 μW/μV. Calorimeter mixing vessels (gold) have a total volume of 5 ml. with the small compartment holding 1.0 ml. In typical runs Leishmania cells were added in 2.0 ml. total volume to the larger compartment. Other reagents were added to the smaller compartment in 0.1 ml. volume, all in PBS. The microcalorimeter was kept in a room controlled at 25°C. Manual mixing of the 'cylinder' or mixing vessel block for aeration was done at 5 minute or 10 minute intervals depending on the protocol. Equilibrium required from 10 to 25 minutes time before each run. Amplifier gains of X500 were used. Calibration was performed by neutralization of Tris by HCl based on a heat of (-)11,300 calories/mole of H^+ neutralized. Standard calibration plots were described by: $A = -92.52 + 299.46 \times q$ where A = integrated area under the output envelope and q = amount of heat released.

The number of Leishmania cells in each experiment was in a range 1 to 6 x 10^8 cells/2 ml. The average protein content is 4.3 mg/10^8 cells. All heat production values are exothermic quantities.

RESULTS AND DISCUSSION

Under the average conditions barometric pressure 917 mbar, 689 mm. Hg, 25°C, oxygen available in the calorimeter mixing vessels are calculated as 1.94 μmole dissolved in 2.1 ml. of liquid volume (PBS buffer) and 14140 μmole in the 3 ml. headspace above the suspension. We assumed O_2 solubility in PBS equals that for Ringer's solution having a Bunsen alpha value 0.0285 at 25°

using also equations developed by Umbreit et al. (9). Hence at any interval we could estimate available oxygen for metabolic combustion, using the conversion factor of 300 Kcal heat/mole glucose equivalent. This is supported by a widely ranging correlation for many organisms surveyed by Wang et al. (10), relating rate of heat production in Kcal/(l)(hr) and rate of oxygen consumption, Q_{O_2} in mole/(l)(hr), with a slope of 130 Kcal/mole O_2, together with an oxygen/hexose consumption ratio which is characteristically 2.5 to 3 mole/mole.

Since there is not available quantitative information concerning sources of energy in Leishmania deprived of any exogenous carbon source, as is the case in our endogenous experimental runs, we converted the heat values into glucose equivalents for comparison. There are indications of possible use of lipids or amino acids as carbon sources for metabolic combustion when carbohydrates are absent, but the literature is unclear about this.

The results to be reported now were obtained under two different sorts of conditions with respect to glucose. First Leishmania were cultivated in MD-29 medium without added carbohydrate but containing 8% v/v bovine fetal serum to assure growth. Second Leishmania were grown in the chemically defined MS-29 medium containing 10 mM glucose. Under both sets of conditions experiments were performed with cells collected on the fifth day, in the log period of growth. Microcalorimetric runs were obtained with three protocols: Without added glucose in the mixing vessels (to measure endogenous heat), and thereafter using two glucose concentrations, 25 μM and 2.5 mM glucose.

1. CARBOHYDRATE STARVED LEISHMANIA

1a. Endogenous heat generation

In absence of any external carbon source for immediate uptake, Leishmania releases heat slowly responding to oxygen depletion. In a typical experiment cells grown 5 days in MD-29 medium without carbohydrate but using 8% bovine fetal serum, release heat immediately after 100 seconds and stays there for some time, 400-500 seconds, then decays, shown in Figure 1-A. Remixing (new oxygen) restores heat production to its maximal level. These cyles may be repeated many times, up to several thousand seconds, 2 to 3 hrs. with no sign of energy depletion of cell endogenous energy source. When mixing is stopped a new, lower level of steady state heat generation is reached, about 1/3 lower (Figure 1B). However as before, mixing anew restores heat generation to its former higher level.

Using the steady state or plateau part of the heat output it was estimated heat flow is $35.0 \pm 4.9 \times 10^{-4}$ mcal/(sec)(10^8 cells) (Table 1). The amount of heat released after 1800 sec. was related to glucose turnover to estimate cellular weight loss. Approximately 122 nanomoles of glucose is combusted in

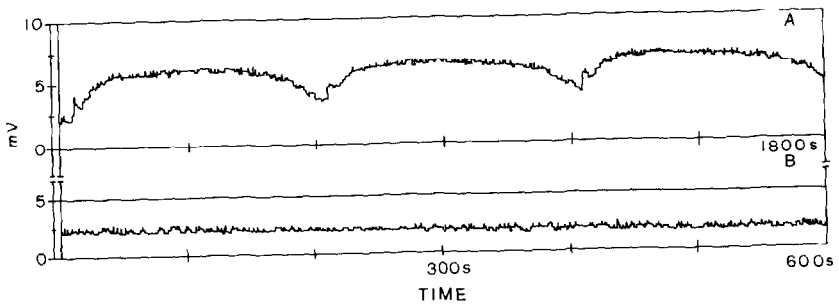

Figure 1. Endogenous heat production by starved Leishmania amazonensis; 5.8 x 10^8 cells/2.1 ml., PBS buffer, pH 7.2, 25°C. A: Mixing at 10 minute intervals. B: No mixing.

1800 sec., equivalent to 22 µg glucose loss by 5.8 x 10^8 cells. From the average dimensions of Leishmania promastigote body not including flagellum, 14 microns length, 2.4 micron width, the cell volume (ellipsoid) is calculated as 46 x 10^{-12} cm^3. Assuming density of 1.00, each cell body weighs 46 picogram. Metabolized glucose equivalent is 0.037 picogram/cell, a cellular weight loss ca. 0.08% in 1800 seconds, from cellular endogenous energy stores. During the same interval 732 nmoles O_2 is consumed, ca. 38% of the initial dissolved oxygen. This fits the decreased heat generation seen in each peak after ca. 500 seconds in Figure 1A with return to the former, higher steady state plateau after mixing.

If mixing is carried out at 5 minute intervals one can maintain the steady state heat production for very long times (data not shown), showing again that oxygen is required for endogenous heat production by Leishmania. The lower steady state level seen in Figure 1B when no mixing was applied reflects a restricted oxygen supply to the cell suspension, limited by diffusion from the air headspaces.

1b. Heat production from 25 µM glucose; exogenous metabolite

Under exactly the same experimental conditions except now using 25 µM glucose in the mixing vessels, overall heat in 1800 seconds approximately doubles. There are produced sharper slopes in the thermograms after mixing, and accelerated heat production shown in Figure 2. Heat output at the upper, steady state level was ca. 65 x 10^{-4} mcal/(sec)(10^8 cells), roughly twice the endogenous (no added carbon) value. As expected from oxygen availability, duration of steady state phase of power generation was shortened, followed by a decrease in heat flow on oxygen depletion, again returning to the highest plateau on remixing and

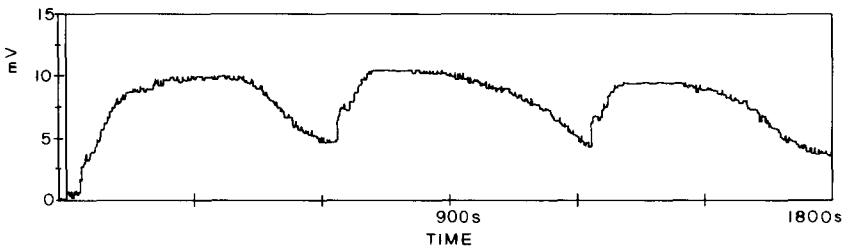

Figure 2. Heat generation by initially starved Leishmania mixed with 25 μM glucose.

replenishing oxygen. On stopping mixing, a lower steady level was reached, very stable in magnitude, producing 29 x 10^{-4} mcal/(sec)(10^{8} cells), values rather smaller than the 'endogenous' case.

Total heat released in the experiment illustrated in Figure 2, 47 mcal, is somewhat larger than the sum of endogenous heat, 28 mcal., plus heat generated from total combustion of the added glucose, ca. 16 mcal. As the process goes beyond 1800 seconds, possibly low glucose concentrations even stimulate endogenous heat liberation. It appears likely that in 25 μM glucose Leishmania not only use the added glucose but also maintain their endogenous heat production.

1c. Heat release from 2.5 mM glucose, exogenous metabolite

Again under the same experimental conditions but now using 2.5 mM glucose, far larger than 25 μM glucose, heat released by Leishmania decreases. There appears an inhibitory effect by the large glucose concentrations on cell metabolism, the "glucose effect" seen with other microorganisms (11).

Figure 3A shows a much shorter duration of the steady state phase of heat production relative to the foregoing examples, lasting only about 100 seconds. This is followed by decay to the baseline; again mixing and reoxygenation returns heat production to higher steady state or plateau level. As in other cases this process continues for a long time provided oxygen is supplied. Interrupting mixing reduces heat generation to very low levels, far lower than the normal endogenous level. In separate experiments we saw that upon decreasing mixing intervals to 5 minutes or even to 2 minutes generates a practically flat steady state line, or heat, confirming the essential role of oxygen. On average, heat generation at steady state was 45.4 (±4.1) x 10^{-4} mcal/(sec)(10^{8} cells).

In a few experiments at 2.5 and 5 mM glucose there was observed a tendency toward heat output oscillation. However this was not very consistent. Possibly

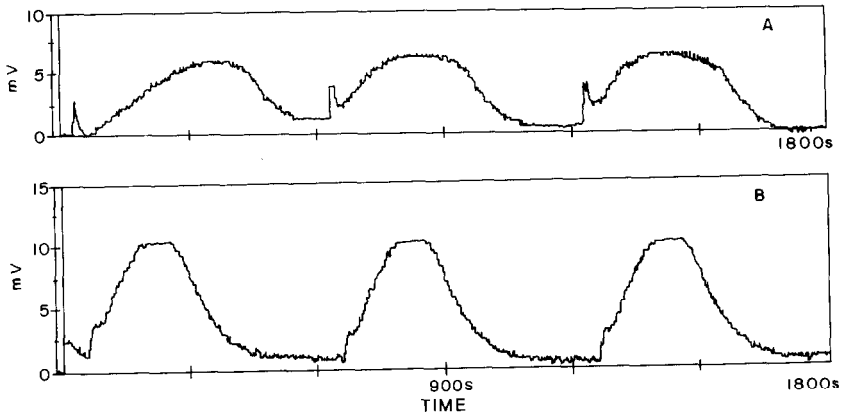

Figure 3. Heat generation by Leishmania amazonensis mixed with 2.5 mM glucose in PBS, pH 7.2, 25°C, 5.8 x 10^8 cells/2.1 ml. in both cases. A: Carbohydrate starved (nonadapted) cells. B: Cells grown in 10 mM glucose.

the metabolic state or condition of the protozoan cells is not exactly reproduced in different batches of cells, although care was taken to perform corresponding steps alike, in each experiment.

2. LEISHMANIA GROWN IN 10 mM GLUCOSE

2a. Endogenous heat production

In these experiments Leishmania were grown in MS-29 medium with 10 mM glucose, with the other conditions similar to the foregoing examples. Cells were collected on the fifth day of culture and processed as described above.

A pattern similar to that observed for starved cells was seen. However the steady state heat flow production was almost three times higher, 105 ± 11 x 10^{-4} mcal/(sec)(10^8 cells) (Table 1). Using calculations similar to those outlined above under 1a, 45 nmoles of glucose were 'burnt' by 2.8 x 10^8 cells in 1800 sec., ca. 0.2% of cell mass and about 14% of dissolved oxygen was consumed. Again mixing at shorter intervals, 5 minutes, keeps upper level or plateau heats maximized. However longer intervals engender decreases in heat production rates after 500 seconds.

2b. Heat release with 25 μM glucose

Heat flow curves were similar to those of Figure 2 but steady state heats were higher than the endogenous case, 111 ± 5 vs 105 ± 11 x 10^{-4} mcal/(sec)(10^8 cells). But such differences of ca. 5% are relatively small, and in some

contrast to the case for starved cells where 25 μM glucose nearly doubles plateau heats. This strongly implies a ready-to-go cell machinery to use glucose, adaptation of cell metabolism to intake of sugar in the culture medium.

2c. Heat release with 2.5 mM glucose

At considerably higher glucose concentrations on mixing a larger steady state heat flow, 135 (±4.2) x 10^{-4} mcal/(sec)(10^8 cells) was observed. No "glucose effect" could now be detected by quantitating the plateau heats, alone. Analysis of the time envelopes showed that although more heat was generated giving a higher plateau, it was quite short lived, lasting only about 50 seconds. Some of such data are shown in Figure 3B following the standard protocol. Thus the 2.5 mM glucose generated plateaus gave increased power values. However there was in effect an inhibition of glucose utilization seen from calculation of total glucose equivalents actually consumed by the cells. These data are shown in last column of Table 1. It compares the maximal consumption data calculated from the steady state flows in the adjoining column of Table 1.

CONCLUSIONS

Heat conduction microcalorimetry can, indeed, be used for investigation of Leishmania metabolism, expressed by their heat generation as a function of time. Numbers of cells required for measurement, ca. 10^8 cells, promastigotes, are easily within the capacity of most research laboratories specializing in culture of these cells. Their physical resistance, as well as the non-aggressiveness of calorimetry renders the general method quite applicable to screening research, a major aim of our work.

Leishmania have a clearly measurable endogenous metabolism which releases heat when the cells are suspended in simple solution, PBS buffer, without carbon sources. This activity lasts a long time when oxygen is kept provided. Given glucose, their heat production patterns vary according to how the cells were cultivated beforehand. There is a clear difference between carbohydrate-starved and carbohydrate-fed Leishmania cells concerning their glucose utilization. In starved cells, low added glucose concentrations, of the order of 25 μM, stimulate glucose consumption measured both by their steady state heat flow and by glucose equivalents actually consumed. Much larger glucose concentrations, 2.5 mM, about 100X as large, displays a clear glucose effect, decreasing the steady state heat flow to lower levels instead of to higher levels, also decreasing actual glucose utilization during a fixed interval.

TABLE 1.

Parameters of heat generation by Leishmania amazonensis dependent on growth conditions and on added glucose concentrations in mixing calorimetry.

Calorimetric mixing components	Heat Production (mcal/(sec)(10^8 cells) x 10^4	Maximal glucose utilization	Experimental average glucose utilization
		(nmole/10^8 cells/1800 sec.)	
CELLS GROWN IN DEFINED MEDIUM, NO CARBOHYDRATE			
Endogenous	35 (4.9)[a]	21.0	16.1
25 μM glucose	60.5 (3.0)[b]	36.2	27.0
Ditto, no mixing	29.0 -	-	-
2.5 mM glucose	45.4 (4.1)[c]	27.2	8.0
CELLS GROWN IN DEFINED MEDIUM WITH 10 mM GLUCOSE			
Endogenous	105 (11)	63.0	50.0
25 μM glucose	111 (5.0)[b]	66.2	48.6
2.5 mM glucose	135 (4.2)[c]	81.0	35.9

[a]Standard error in parentheses

[b]Glucose added, 52.5 nmole

[c]Glucose added, 5.3 μmole

On the other hand, in case of cells grown in 10 mM glucose, quite large concentrations, a different pattern is seen. Steady state heat flows are much larger in both the endogenous case and in the presence of exogenous glucose, increasing with the glucose concentration (Table 1). A glucose effect is observed however, on calculation of how much glucose is actually used as demonstrated in Table 1.

A more detailed discussion of these data shall rest on work with other simple carbohydrates that Leishmania can use, as well as some of the biochemical indicators being carried out in parallel to further calorimetry.

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REFERENCES

1 R.H. Hammerstedt and R. Lovrien, Calorimetric techniques for metabolic studies of cells and organisms under normal conditions and stress. J. Exper. Zool. 228 (1983) 459-469.

2 R. Bar, Fermentation calorimetry versus microcalorimetry. Trends Biotech. 6 (1988) 55-56.

3 R. Turner, I.E. Liener and R. Lovrien, Equilibria of Bowman-Birk inhibitor association with trypsin and α-chymotrypsin. Biochemistry 14 (1975) 275-278.
4 P. Bro and J.M. Sturtevant, The heat content of bovine serum albumin in acid solution. J. Am. Chem. Soc. 80 (1958) 1789-1793.
5 R. Lovrien, K.K. Williams, M.K. Ferrey and D.A. Ammend, Calorimetric versus growth microbial analysis of cellulase enzymes acting on cellulose. App. Environ. Microbiol. 53 (1989) 2935-2941.
6 M.N. Melo, H.P. Azevedo, I. Roitman and W. Mayrink, A new defined medium for culturing Leishmania promastigotes. Acta Tropica 42 (1985) 137-141.
7 M.N. Melo, Cultivo de Leishmania em meio definido estudo de seus requerimentos nutricionais. Ph.D. thesis, Federal University of Minas Gerais, Belo Horizonte M.G., Brazil (1982) 1-133.
8 I. Wadsö, Microcalorimeters. Quart. Rev. Biophys. 3 (1970) 383-427.
9 W.W. Umbreit, R.H. Burris and J.F. Stauffer, Manometric and Biochemical Techniques. Burgess Publishing Co., Minneapolis, MN, Fifth ed. (1972) 5-11.
10 D.I.C. Wang, C.I. Cooney, A.L. Demain, P. Dunhill, A.E. Humphrey and M.D. Lilly, Fermentation and Enzyme Technology. John Wiley and Sons, New York (1979) p. 84.
11 A. Fiechter, G.F. Fuhrman and O. Kappeli, Regulation of glucose metabolism in growing yeast cells, in: A.H. Rose and J.G. Morris, eds. Adv. in Microbial Physiol. 22 (1981) 123-186.