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ENERGY RESERVES AND STORAGE POLYMERS IN INTACT BACTERIA ANALYZED BY METABOLIC CALORIMETRY *

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SUMMARY

Bacteria grown under nitrogen limitation and excess carbon accumulate energy storage polymers such as PHB, poly β-hydroxybutyrate. On mixing 1 to 2 ml. suspensions of such cells (2-20 mg. of cells) with air but no additional carbon in a calorimeter large heats evolve until cellular internal energy reserves are consumed. Division of the overall heat by the specific heat content of monomer aerobic metabolism - estimated as (-)200 Kcal/mole PHB monomer - gives the apparent weight of energy reserve content of the cells. The calorimetric method is easier to carry out than conventional respirometric or oxygen uptake methods that require tight control of oxygen concentration baselines. In calorimetry any roughly set oxygen concentration suffices if there is an excess of oxygen sufficient to exhaust the storage materials residing in the cells.

INTRODUCTION

Many cells build stores of 'energy supplies', apparently to sustain them when external supplies are scarce. Both prokaryotes (bacteria) and eukaryotes (mammals) do this. Several questions pertain: What kind of compounds? How much? How can they be measured: What triggers the building up, and drawing down of such reserves? Dawes (1), and Dawes and Senior (2) wrote two notable reviews discussing these questions in relation to bacterial growth. Lately the question, 'How much?' has taken on new urgency because some of the bacterial energy stores are, or may soon become, industrial commodities. Bacterial inclusion body polymers likely will be useful as a basis for biodegradable plastics. Internal polymers - storage polymers - are decisive in the longer term basic problem of life and death of cells, and how they respond to stress (1).

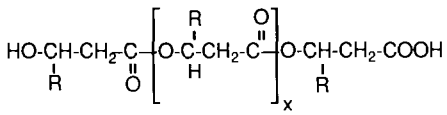

Figure 1. PHB, poly β-hydroxybutyrate. The R group is $-CH_3$ in linear PHB.

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Our work pertains to the narrower question of how to measure microbial energy reserves, without having to break open the cells, extract the polymers, isolate them, and perform conventional "wet" analysis (3). We propose an independent means, aerobic microbial calorimetry. If microorganisms can be prodded to catabolize their energy reserves, they should simultaneously produce heat, measurable by calorimetry. This appears to work, so far at least with four bacteria including Pseudomonas. Before reviewing experimental details, we briefly describe how we were prompted to develop the method. We were trying to use Pseudomonas putida as 'combustion reagents' to analyze micromolar concentrations of compounds in the MCA method, Microbial Calorimetric Analysis. The MCA method has been described for analysis of phenols and lignin related fragments (4), and analysis of sugars obtained from cellulose by cellulase enzyme action (5). Although the general MCA method works well for several exogenous sugars when Escherichia coli are the combusting or metabolizing organism, certain Pseudomonas gave what at first seemed severe difficulty in analysis for acetate, propionate, etc. Control experiments, growing Pseudomonas, harvesting and centrifugally washing cells, then mixing them under aerobic conditions with simply minimal salts and no exogenous carbon at all, produced huge, sustained heat production on each calorimetric mixing cycle. Eventually it dawned on us that the heat must be coming from carbon sources inside the bacteria, endogenous carbon. We also observed that the overall heat production was directly proportional to the number of cells. The overall time and the number of mixing cycles needed to exhaust all the 'endogenous heat', ca. 2 hours and 20-30 cycles, was independent of the number of cells. Battley's treatise (6) describes the basis for such behavior of heat production dependent on storage materials, polymers in particular. Several species of Pseudomonas are especially able to accumulate PHB polymers represented in Figure 1. Grown under nitrogen limitation, also under oxygen limitation and with excess carbon, many organisms accumulate PHB (or related polymers), sometimes as much as 60-80% of their own dry weight (2). If PHB or similar polymers accumulated for example 30% of the dry weight of bacteria ordinarily used in MCA, how much heat can be expected from aerobic metabolism of PHB?

Estimation of heat of metabolic consumption of storage polymer PHB

Bacterial heats of aerobic metabolism vary with the bacteria, the detailed pathway they use, and of course the carbon source. Nevertheless one can make rough estimations starting from work pertaining to lower M.W. carbon sources (6,7). For example aerobic metabolism of glucose by many bacteria usually generates (-)305 ± 20 Kcal of heat per mole for conversion to acetate and three CO_2 molecules (8). This heat is in reasonable agreement with calculations

based on conventional thermochemical calculations with 45% of glucose's carbon forming CO_2, about 16% forming acetate or ethanol and the remainder of the carbon remaining in the cell as biomass or intermediates. If one estimates via the 'reductance' methods (γ values) reviewed by Battley (6) taking into account the carbon source and the metabolic oxidation products weighted by their stoichiometry of formation, 305 exothermic Kcal of heat/mole glucose fits fairly well with estimates from the reductance degree or 'available electrons' calculations. By two somewhat differing means of such calculations, 281, and 308, Kcal heat were predicted per mole glucose. (All such heats are exothermic, (-) sign, 4.18 Joule/calorie).

Applying these estimation methods to the PHB monomer $C_4H_6O_2$ we arrived at 150 Kcal, and 230 Kcal of heat expected per mole of monomer, assuming half the carbon forms CO_2. On average therefore PHB is expected to produce ca. 200 ± 50 Kcal heat/mole of monomer, aerobically metabolized. In our earlier instrument (8) cell suspension volumes are 2 ml. They contain approximately 1 or more mg. of cells (dry weight) and 5 ml. of headspace filled with air. If 1 mg. cells had 30% PHB, monomer M.W. = 86.1 Daltons:

$$10^{-3}\text{gm. cells} \times \frac{0.30\ \text{gm.PHB}}{\text{gm. cells}} \times \frac{1\ \text{mole PHB monomer}}{86.1\ \text{gm. PHB}} \times (-)2\times10^{8}\ \frac{\text{mcal heat}}{\text{mole}} =$$

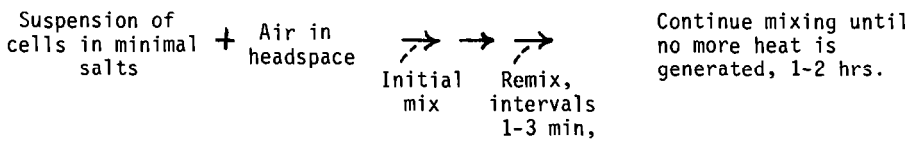
$$Q_{heat} = (-)700\ \text{mcal}$$

Such heat production is more than adequate for measurement. Normally ranges of 10-100 mcal. heat are conventionally used for analysis in the MCA method (4) for low M.W. compounds.

Is there enough oxygen to actually drive PHB to complete metabolic combustion? Approximately 4 to 6 oxygen molecules/PHB monomer are likely required as a minimum. In the foregoing example we have 3.5 µmoles PHB monomer. Hence a minimum of ca. 20 µmoles of O_2 are needed. If the 2 ml. of suspension is initially saturated with air the suspension contains only about 0.5 µmole of oxygen, not nearly enough. However the headspace of about 5 ml. of air has 44 µmole of oxygen. This is not a large excess but if stirring is sufficient it should bring PHB metabolism to completion if there are not also severe rate limitations.

METHODS

Calorimetry of bacterial storage polymers can be outlined:



The reference vessel in the calorimeter (conventional twin batch instrument (4,9)) was loaded with a volume of minimal salts-buffer, equal to the volume

of cell suspension in the sample vessel.

An external control experiment was carried out at the bench, preaeration of the cells tumbled in a sealed tube with excess (10-20 volumes) air for ca. 2 hours. After preaeration they were loaded in the calorimeter and measured in their ability to produce heat in the same way sample cells (not preaerated) were examined. Evolution of very little or no heat from vigorously preaerated cells, in contrast to heat production from cells not preaerated, supports the central tenet. Namely that newly harvested cells kept oxygen poor retain their energy reserve polymers which subsequently evolve heat upon exposure to oxygen in the calorimeter. All calorimetric measurements were made at 25°C.

Bacteroides (10), Pseudomonas and unidentified soil isolates from methanol enrichment raised under the same conditions as Pseudomonas (4), were grown with conventional minimal salts and trace metals. Alcaligenes eutrophus (ATCC no. 17699) was cultured in ATCC media no. 425 (11) except that fructose instead of succinate was used as the carbon source. Concentrations of sugars, alcohols, etc. as carbon sources during culture were kept between 0.5 and 2%. Nitrogen, i.e. NH_4^+ was made limiting by diluting minimal salts into additional carbon substrate with ammonium. After growth, cells were spun down twice, centrifugally washed in isotonic saline. Cell concentrations and adjustment were measured by an average turbidity or spectrophotometry parameter of $2 \times 10^9 \times A_{660} \times cm^{-1}$ to give the number of cells/ml. in a 1 cm. cuvette (12).

It is necessary to refrain from using a large excess of cells in calorimetry if headspaces are small. An excess of cells exhausts all oxygen available in the headspace before cellular energy reserves can be consumed. A batch mixing calorimeter with larger mixing vessels was constructed, providing about 10 ml. air to drive combustion, for Bacteroides, Alcaligenes and Pseudomonas.

RESULTS

Figure 2 shows two kinds of power production outputs from A. eutrophus cells mixing with air (intact cells) and a third, control experiment using cells that were well preaerated before insertion in the calorimeter. Each small vertical arrow ↑ signifies remixing, renewing air dissolved in the cell suspension from headspace air. Remixing intervals in trace 1 were 5 minutes apart. Upon remixing, cells renew combustion of their internal polymers. But they soon starve for oxygen and appreciably slacken heat production until the next remixing cycle and jolt of oxygen. After 40 minutes, about 8 cycles of remixing-resting, considerable energy stores have been consumed. However some energy stores remain; appreciable heat is still generated after 40 minutes.

Trace 2, remixing performed each 1-2 minutes, clearly shows metabolic "burning" occurs at a higher rate than in the first example and is sustained

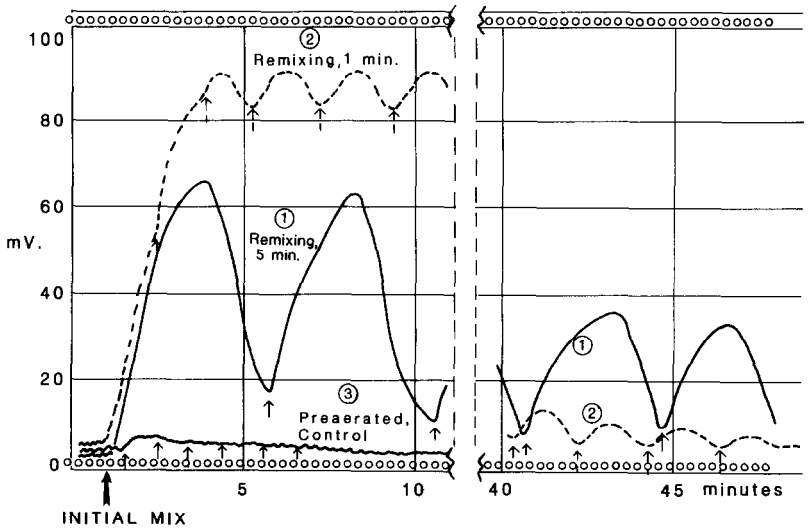


Figure 2. Heat production of "soil isolate" bacteria metabolizing their interal energy stores (4), using oxygen but no exogenous carbon, on remixing cells several times with headspace air in heat conduction calorimetry, 25°. Trace 1: Five minute mixing intervals. Trace 2: 1 minute intervals. Trace 3: external control experiment; cells were preaerated at the bench 2 hours before insertion in the calorimeter.

more steadily. After 40 minutes, sample 2 was more thoroughly exhausted than sample 1 after 40 minutes. Thereafter sample 2 has little energy reserve left. Sample 3 shows calorimetric behavior of well preaerated cells which have little carbon reserves to metabolize from the start of calorimetry and generate negligible heat throughout.

Figure 2 also indicates that renewed oxygen quickly triggers metabolism of stored carbon, as well as sustains it. Not all bacteria consume their energy stores so rapidly as Figure 2 indicates. For example, Pseudomonas putida carry out metabolic combustion for up to 2 hours and about 20 remixing cycles, at 25°.

Figure 3 plots power production as a function of cell mass for A. eutrophus, using larger calorimeter mixing vessels of ca. 12 ml. of volume. Again when cells were well preaerated, even 20 mg. of cells generate little heat in the calorimeter on aeration (lower plot of Figure 3). Four kinds of bacteria have been examined in this way, Pseudomonas putida, Bacteroides fragilis, Alcaligenes eutrophus, and an unidentified soil isolate grown on 0.1-0.5% methanol.

Dividing the overall heats, the integrated power envelopes from calorimetry, by 200 Kcal heat/mole, the estimated average heat for metabolizing PHB monomer, between 5% and 50% of the dry weight of these cells may be PHB, depending on bacteria, growth conditions, carbon source. Such analyses are in good general

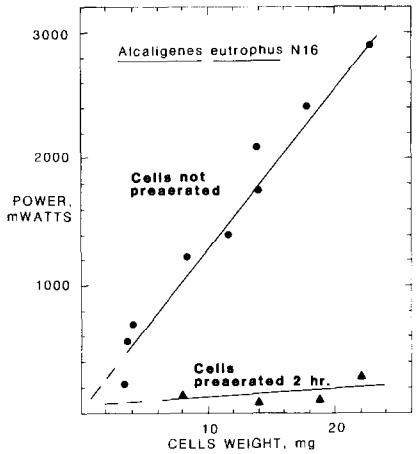


Figure 3. Power production measured by calorimetry of Alcaligenes eutrophus, vs. cell biomass measured by spectrophotometric turbidity (12). Upper plot: cells were not preaerated and therefore contain their energy reserve materials. Lower plot: preaerated cells, lacking their reserves when loaded in the calorimeter.

agreement with what a number of PHB-storing bacteria are capable of, including Pseudomonas (1). Obviously more data, and comparison with conventional extractive-chemical degradative methods, need be made.

DISCUSSION

Microbial calorimetry is an underused tool for estimating bacterial cell 'energy stores' without having to break them open. Conventional extractive analysis of PHB from bacteria is a rather lengthy procedure (3). The advantage of microbial calorimetry is that it is rapid and easy to do. One simply inserts the cell suspension washed X1 or X2 of any metabolizable carbon, in a batch mixing calorimeter having enough headspace volume to provide adequate air.

Whether such calorimetry is completely precise and accurate for assay of cellular energy stores is not yet known. To be sure all energy storage polymers are not necessarily linear PHB. Some organisms make PHB copolymers. Glycogen and other polymers are energy reserve materials in some cells. Yet other energy reserves are lipids, lower M.W. compounds. A calorimetric technique of this kind does not answer all the questions posed in the introduction, particularly not the biochemical identity of energy reserves. However microbial calorimetry can provide a rather easily gotten estimate of whatever 'heat content' is contained in bacterial energy reserves that are easily drawn on by adding oxygen. The general technique is analogous to

calorimetric techniques developed for biocombustion of cellulose and soil polymers by Gustafsson (13). Because in a technique of this kind, wherein a rather roughly varying supply of oxygen suffices to drive the analytical reaction so long as there is a surplus of oxygen, there is no need to precisely hold oxygen baseline concentrations to a narrow tolerance during 1-2 hours. Hence calorimetry may have advantages over electromeric oxygen respirometry which requires careful control of oxygen concentration baselines. In short, respirometry relies on measurement of oxygen concentration differences with respect to a baseline concentration, whereas calorimetry with excess oxygen is only 'carbon limited', not dependent on rigorous control of oxygen tension.

One other aspect of the technique should attract interest. A question frequently arising in cell metabolism is: What triggers metabolic drawdown of energy reserves? Here, air (oxygen) is the "trigger", but actually oxygen is simply the ultimate electron acceptor and part of the fuel. More significantly uncoupling agents such as dinitrophenol are potential triggers or signals that can call on glycolysis and make it accelerate. Any signal such as glutathione deficiency that demands restoration of ATP, NADPH, the Atkinson energy charge, should act similarly. Such agents, uncouplers and prooxidant chemical stressor compounds (14) may be mixed with cells in addition to mixing with air. Hence the general method likely has use for assaying the impact of uncouplers and toxic compounds on cells. Their effects are amplified when they force the cells to use extra oxygen and endogenous carbon reserves (15), to produce large metabolic heats.

Acknowledgement

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