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MICROBIAL CALORIMETRIC ANALYSIS (MCA): ANALYTIC METHOD FOR AQUEOUS ORGANIC COMPOUNDS *

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SUMMARY

When bacteria are adapted to metabolize organic compounds as carbon sources, in effect they become reactive reagents for analysis of specific compounds via calorimetry of their aerobic metabolic heat. From 2 to 5 mg. of adapted bacteria drive metabolism of a number of sugars, alcohols, fatty acids, phenols to completion in 4 to 10 minutes using 2 to 100 nanomoles of compound or analyte. Conditions are aerobic, ca. 25°C. The MCA general method simply uses microbial behavior normally occurring on a large scale in the environment, applying it to analysis of 0.5 to 2 ml. samples. Microbial aerobic turnover of carbon produces large molar heats, commonly half as much as oxygen bomb combustion heat of comparable samples, roughly 2X to 10X as much heat as most other classes of aqueous reactions.

INTRODUCTION

Microbial Calorimetric Analysis (MCA) can be outlined:

Compound in water (the analyte), limiting 'carbon' + Air $\xrightarrow[\text{Bacteria adapted to metabolize the analyte, excess cells.}]{}$ Metabolic products + Q_{heat}

Many analytic procedures involve use of a reagent, to react with the sought-for analyte to give a detectable 'signal'. In effect bacteria are the reagents in MCA, reacting with the analyte, using dissolved oxygen to drive the analytic reaction. Bacteria likely seem unorthodox analytic reagents. However if heat production and calorimetry have a future as a general method of analysis, a number of properties of bacteria make them not only attractive as reagents, but competitive.

Their range of applicability is very large. Bacteria can metabolize myriad compounds, except for the most perhalogenated compounds. Bacteria are often quite specific especially when adapted, able to metabolize particular sugars or alcohols or phenols, leaving closely related compounds and cogeners alone. Many bacteria are easy to grow, and adapt, so one can quickly create a large assortment of "reagents". Since their K_m values range down to 0.1 to 1.0 μM or even less (1), they readily detect analytes in micromolar concentrations. Bacteria

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work with optically dirty samples that completely block spectrophotometry, fluorimetry, etc. Bacteria are disposable, and mostly inexpensive. One 100 ml. culture of many bacteria provides enough cells for 10-20 MCA runs. Adaptation, which usually raises key receptor and enzyme levels X10 to X1000 fold over nonadapted cells (2), makes them vigorously reactive. Bacteria as 'reagents' are not necessarily slowly reacting, relative to many conventional chemical reagents used in analysis. Heat production from microbial uptake and aerobic metabolism usually is finished in 3 to 10 minutes after initial mixing, if analyte concentrations are kept in micromolar ranges. If analytes are millimolar, bacterial growth ensues after one or more doubling times. Heat production continues over much longer periods in that case, but conditions inciting growth are not used for MCA as we envision it.

One further question remains. How sensitive may such a method be, in relation to molar heats of metabolism of organic compounds like benzene, sugars, etc., and in relation to ranges of heat convenient to measure in current calorimeters? The intrinsic sensitivity of current instruments is in the microcalorie (heat), and microwatt (power) ranges, where signal/noise ratios become critical. We prefer to focus on the average, practical working ranges of batch mixing calorimeters. These are approximately 0-10 millicalories ± 8% as a lower range, 0-100 and 0-1000 millicalories of heat, ± 3% error as upper ranges for simple instruments (3) of moderate cost. Most aerobic microbial oxidations produce between (-)20 and (-)100 Kcal of exothermic heat/mole of carbon atoms (4), (-)20 to (-)1000 Kcal/mole lower M.W. compound (4,5). Dividing such working ranges by the molar heats, e.g. 20 millicalories range/200 Kcal of heat per mole, shows that 0.05 to 0.20 micromole of analyte should be comfortably in MCA range.

EXPERIMENTAL METHODS

Microorganisms

Escherichia coli K12 (ATCC 25240) for sugars analysis was grown, washed and adjusted in suspension concentration as described before (6). Adaptation of *E. coli* to various sugars required growth for a few hours at 37° on ca. 0.2% of the desired sugar, ca. halfway into log phase. Several strains of *E. coli* are not optimal for sucrose (7). Accordingly a scr^+ plasmid bearing *E. coli* was used, *Escherichia coli* DW2/PUR401 donated by Dr. R. Brooker (Minnesota) by mating strain DW2 with a DS405/PUR401 strain with a sucrose plasmid (Dr. T. Wilson, Harvard University). This *E. coli* was grown with (+ sucrose) or without (on glucose or fructose) depending on how it was to be adapted, in Davis-Mingioli minimal salts. *Pseudomonas putida* (ATCC 11172) adapts to several phenolics and other benzene aromatics. It was cultured at 30° using 0.04 to 0.05% carbon sources in minimal salts (4). As *Pseudomonas* cultures

grew, additional pulses of carbon sources, e.g. phenols were added to replace carbon consumed, but additions were held below 0.07% maximal levels. Microbial combustion of methanol and some aromatics used bacteria from unidentified, local soil isolates. They were enrichment cultures from continuous air circulation of aqueous soil suspensions grown for 1 day on 0.01% glucose, switched to 0.02-0.05% of the desired carbon source for 2-5 more days. Once adapted such cultures grow overnight on the chosen carbon source in shake flasks. Bacteria grown for use in MCA often can be stored in 50% glycerol in the deep freeze. They are retrieved by thawing, and centrifugally washing them twice (2).

Aerobicity and solvent conditions

Air is already dissolved in both the sample to be analyzed and the resting bacteria used to metabolize the carbon. Normally for analyte concentrations in the range 5-50 nanomoles/ml, using 2-6 mole O_2/mole analyte, there is enough dissolved oxygen in solution to provide some excess of oxygen (4). The mixing vessels contain from 3-6 ml. of additional air, roughly 40 micromoles of oxygen so that one or two added mixing cycles provide a large excess of oxygen for combustion of up to at least 200 nanomoles of most analytes.

If samples have buffer capacities and initial pH values very different from the cell suspension, that can be dealt with in several ways. First, by adjusting pHs to roughly match (± 0.2 pH) before mixing. Second, by diluting the sample into the same buffer used to handle and wash the cells. (Most real samples, e.g. food slurries, have such large analyte concentrations that they require large dilution). Third, H^+ transfer from pH mismatch occurs nearly

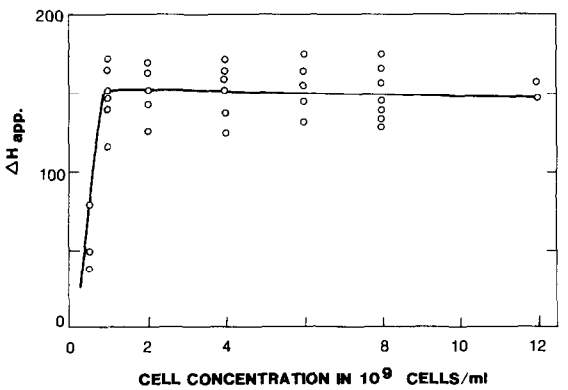

Figure 1. Cell concentration dependence of metabolic combustion of a fixed amount of acetate. Plateau occurs when cells are in excess, enabling consumption of all analyte in ∿6 to 8 minutes.

instantly on mixing. Heat from H^+ transfer decays out with a much different character than even the fastest microbial metabolism (1-2 minutes vs. 3-10 minutes). In practice therefore, H^+ transfer heats are easily subtracted or deconvoluted out of the overall thermogram, if not completely subtracted by the reference side of twin instruments (3).

Calorimetric instrumentation

Conventional twin batch mixing calorimeters (BRIC manufacture) for mixing 0.5 to 3 ml samples in both vessels were used. All of the calorimetric measurements were performed at 25°, essentially at ambient temperatures. When ambient temperatures deviate more than ±2° from set temperature, Peltier pumping (8) may be used for thermal protection.

Amount of bacteria required to drive metabolism to completion

Appropriately 2×10^9 cells, ca. one mg. dry weight usually suffices to drive aerobic metabolism of 50-100 nanomoles of substrates to completion in a few minutes. Sufficiency of cells is seen from plots represented by Figure 1, where it plateaus. Figure 1 plots $\Delta H_{apparent} = Q_{heat}$/moles acetate, using a fixed amount of acetate, varying the numbers of cells. Pseudomonas were used to consume acetate, 100 nanomoles, for 6-8 minutes after mixing. When cells are adapted to individual carbon sources, 3 to 5 mg. of cells usually provides an excess of cells for MCA.

Microbial stripping, spectrophotometric (Nelson-Somogyi reducing sugar) assay

These two auxiliary analytic methods were carried out as recently described (6). Microbial stripping is a technique also using adapted bacteria, for removing unwanted or interfering compounds before use of the principal analytic method. For example, to remove glucose from its mixtures with other sugars such as sucrose in yogurt, the sample slurry is mixed with glucose grown E. coli (0.5 to 1 minute). The cells are spun down and discarded, taking glucose with them. The supernate then is left with its sucrose, ready for analysis.

RESULTS AND DISCUSSION

Figure 2 shows heat production from 10 to 50 nanomoles of glucose, by E. coli grown on glucose. Glucose ('carbon) is limiting, cells are in excess. Remixing during midrun maintains oxygen concentrations so that all the cells which acquired glucose metabolize it at the maximum rate of which they are capable at 25°. Rates of such heat production, power, may be readily converted to overall velocities of carbon uptake plus metabolism. Observed power values are divided by the average molar heats of glucose metabolism determined under

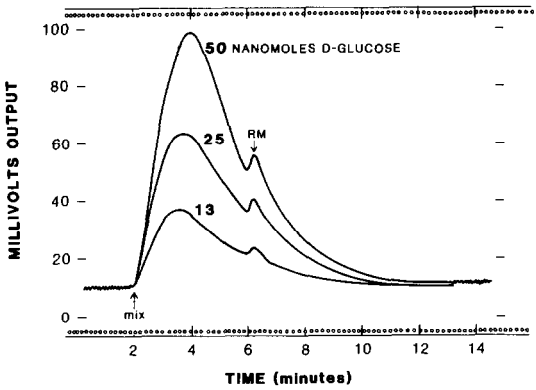

Figure 2. Microbial calorimetry (25°) of aerobic metabolism of glucose, Escherichia coli grown on glucose. RM = remixing: Renewal of dissolved oxygen from headspace air.

similar conditions (4). That is, conditions of limited carbon and excess cells. Such velocities compare well with initial velocities of glucose metabolism by E. coli estimated from oxygen respirometry, H^+ extrusion, etc. reported in the literature (9). Initially there occurs production of up to about 2 pW of heat/cell for glucose. This value falls under higher glucose concentrations when cells become saturated, to a few tenths of a picowatt per cell (9).

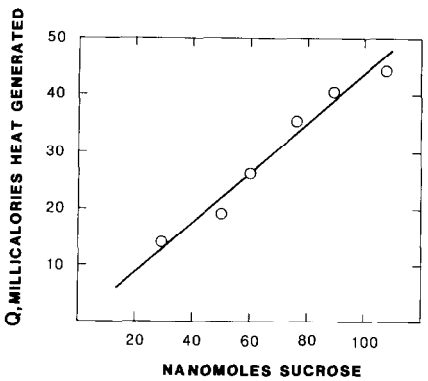

Figure 3. Standardization plot, heat production vs. amount of sucrose as an analyte, metabolized by a suc^+ E. coli.

Applied to analysis, Figure 3 plots Q_{heat} vs amounts of sucrose up to about 100 nanomoles of sucrose in MCA, using *suc*$^+$ *E. coli*, the DW2/PUR401 strain. The sucrose originally was part of a mixture of glucose and sucrose. The glucose was stripped out before calorimetry, by first treating the mixture with *E. coli* cells grown on glucose (a B/5 strain). Most strains of *E. coli* grow readily on glucose. Therefore most diluted mixtures of sugars are easily stripped of glucose (0.5 to 1 minute) by such cells. Stripping is quantitative down to approximately the K_m values for glucose uptake by *E. coli* which range from ca. 0.1 to 1.0 μM (7). The slope of Figure 3 is the apparent heat of aerobic molar metabolism over the range covered by the abscissa. The overall volume of cell suspension plus sample after mixing equals about 3 ml. Hence 0-100 nanomoles sucrose spans a concentration range 0-33 μM sucrose, in good relation to K_m values of *suc*$^+$ cells for rapid uptake. In an earlier paper we denoted heats gotten in this way - low carbon concentration, excess oxygen, excess cells - as $\Delta^\circ H$ values (4). Some authors may prefer other notation but in any case it is important to state cell and substrate 'carbon' concentrations, etc., together with the temperature. Slopes of calibration plots of kinds shown in Figure 3 are measures of basic parameters expressing the sensitivity of the analytic reaction. In Q_{heat} plots vs. concentration of this kind, the slope measures the exothermicity of the analytic reaction. In A_λ (absorbance) plots vs. concentration of analyte, i.e. in spectrophotometry, the slope measures the absorption coefficient or the intensity of the chromogenic analytic reaction.

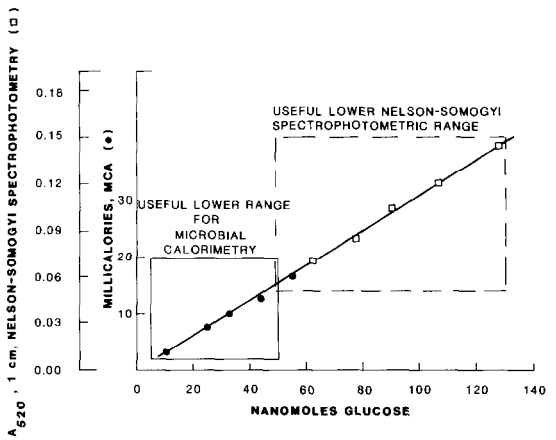

Figure 4. Comparison of lower practical analytic ranges for glucose, in the MCA method and in reducing sugar spectrophotometry.

Figure 4 compares glucose analyzed by microbial calorimetry (MCA) and glucose analyzed by the Nelson-Somogyi spectrophotometric method for reducing sugars. Using glucose, the lower practical ranges for both methods are displayed by two ordinate axes; A_{520} absorbances, and observed calorimetric heats. Definitions of 'practical lower operating range' are somewhat arbitrary. Nevertheless MCA at least brackets and may surpass the lower analyte ranges for which spectrophotometry is valid, in this case where glucose is the analyte. The slope for Nelson-Somogyi glucose's data equals a molar absorption coefficient $\varepsilon_{520} \cong 8000\ M^{-1}cm^{-1}$, reasonably large for spectrophotometric analytic practice.

Table 1

Table 1. Molar aerobic heats at 25° of metabolism of analytes having poor or no chromogenic character. Combustion via bacteria adapted to each analyte by growth on it as a carbon source.

Compound	Organism	Apparent molar heat, $(-)\Delta°H$
Methanol	Soil isolate	102 Kcal
Ethanol	*P. putida*	145
Acetate	*E. coli* B/5, *P. putida*	128
Propanol	*P. putida*	232
Propionate	*P. putida*	265
Glycerol	*E. coli* B/5	201
Butanol	*P. putida*	216
Succinate	*P. putida*	120
Benzene	*Acinetobacter*	191
Toluene	*Acinetobacter*	188
Benzoate	*P. putida*	485
Glucose	*E. coli*	305
Fructose	*E. coli*	320
Sucrose	*E. coli*	470
Maltose	*Klebsiella*	530

Table 1 summarizes apparent molar heats of metabolism of important analytes having 'poor chromogenic character'. These are compounds which by themselves are not derivatized by a prochromogen, having ε values $\leqq 500$. Many of the compounds in Table 1 are u.v. transparent. They are transparent for reasons connected with the fact that they are maximally reduced and so produce large heats of oxidation. Benzene and toluene (Table 1) are often casually called water insoluble. However they are generously soluble - in water - on the scale needed for microbial combustion of them in MCA (4). Benzene has a water solubility at 25° of 2.3×10^{-2} M; toluene, 0.8×10^{-3} M (10). These aromatic hydrocarbons as well as naphthalene and biphenyl (water solubilities 25° = 2.5×10^{-4} M and 4.9×10^{-5} M respectively (11)) have been microbially combusted by

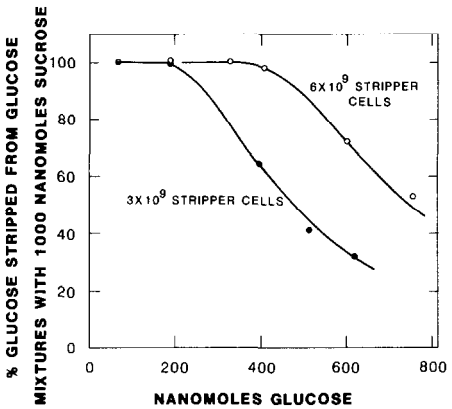

Figure 5. Stripping glucose from glucose-sucrose mixtures using glucose grown E. coli cells, 25°.

Acinetobacter and Pseudomonas putida (4). Taken up by excess adapted bacteria, they combust and produce heat nearly as rapidly as sugars do, on average.

Several of the analytes or compounds in Table 1 are not easy to derivatize when they are dilute and in water. That is, to prepare them for GC analysis, or attach a chromogen for spectrophotometry. Even if they were easy to derivatize, in practical problems as in fermentation and food industries these analytes usually appear in raw, optically dirty samples, in slurries akin to soil.

Figure 5 illustrates how many bacterial stripper cells are needed to scavenge out (to strip) glucose from mixtures of sugars as in yogurt. We find that microbial stripping as outlined above is a rapid, simple technique that considerably expands MCA's scope. Many E. coli and Klebsiella can be grown that both strip out, and microbially combust, selected sugars such as maltose and cellobiose (6). Several Pseudomonas can be quickly raised to scavenge selected phenols plus numbers of the compounds listed in Table 1. Figure 5 indicates how a few mg. of stripper E. coli cells (ca. 2 x 10^9 cells/dry mg (9)) remove glucose in 1 minute, completely removing 200-400 nanomoles of glucose from glucose-sucrose mixtures.

In nature, in the environment, microbial stripping constantly occurs. Neither stripping nor heat production by bacteria are new in principle. Both are ancient. They turn over huge tonnages of carbon, nutrients, pollutants, detritus, feedstocks in fermentation and in soil. The techniques in MCA simply are transfer of those processes to the laboratory, aided by heat conduction

calorimetry pioneered by Wadsö and collaborators (12). Our paper is intended to illustrate not only what calorimetry can do in analysis, but also the capabilities of bacteria as reagents. They deserve more employment.

ACKNOWLEDGEMENT

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REFERENCES

1 J.L. Ingraham, O. Maaloe and F.C. Neidhardt, Growth of the Bacterial Cell, Sinauer Associates, Publisher, Sunderland, Massachusetts (1983) pp. 155-173.
2 R.E. Lovrien, M.L. Ferrey, T.S. Magnuson and R.A. Blanchette, Microbial calorimetric analysis: Lignin related compounds in micromolar concentrations, in N.G. Lewis and M.G. Paice (Eds.), Plant Cell Wall Polymers, A.C.S. Symposium Series 399, American Chemical Society, Washington, D.C. (1989) pp. 544-558.
3 R.H. Hammerstedt and R.E. Lovrien, Calorimetric techniques for metabolic studies of cells and organisms under normal conditions and stress. J. Exp. Zool. 228 (1983) 459-469.
4 R. Lovrien, G. Jorgenson, M. Ma and W. Sund, Microcalorimetry of micro-organism metabolism of monosaccharides and simple aromatic compounds. Biotechnol. Bioeng. 22 (1980) 1249-1269.
5 A.M. James, Growth and metabolism of bacteria, in A.M. James (ed.), Thermal and Energetic Studies of Cellular Biological Systems. IOP Publishing Ltd., Bristol (1987) pp. 1-9.
6 R.E. Lovrien, K.K. Williams, M.L. Ferrey and D.A. Ammend, Calorimetric versus growth microbial analysis of cellulase enzymes acting on cellulose. Appl. and Environ. Microbiol. 53 (1987) 2935-2941.
7 E.C.C. Lin, Dissimilatory pathways for sugars, polyols and carboxylates, in F. Neidhardt, J. Ingraham, B. Magasanik, K.B. Low, M. Schaechter and H.E. Umbarger (eds.), Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, Vol. I, Am. Soc. Microbiology Publications, Washington, D.C. (1987) 244-284.
8 P.C. Anderson and R.E. Lovrien, A flow calorimeter for assay of hormone and metabolite induced changes in steady state heat production by tissue. Analyt. Biochem. 100 (1979) 77-86.
9 I.N. Boe and R.E. Lovrien, Cell counting and carbon utilization velocities via microbial calorimetry. Biotechnol. Bioeng. 35 (1990) 1-7.
10 R.L. Bohon and W.F. Claussen, The solubility of aromatic hydrocarbons in water. J. Am. Chem. Soc. 73 (1951) 1571-1578.
11 W.E. May and S.P. Wasik, Determination of the solubility behavior of some polycyclic aromatic hydrocarbons in water. Analyt. Chem. 50 (1978) 997-1000.
12 I. Wadsö, Microcalorimeters. Quart. Rev. Biophys. 3 (1970) 373-427.