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Microbiological calorimetry

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INTRODUCTION

In several earlier studies, different key features of heat production and thermodynamic properties of microorganisms were identified, although at the time it was not always possible to study these items specifically and in detail. (i) The applicability of thermodynamic laws to microbial systems, in view of the complexity introduced by virtue of the nature of microbial systems and processes [1]; (ii) the dependence of the rate of heat production on the growth phase, (iii) on the rate of growth and (iv) on the type of metabolism were observed by Bayne-Jones and Rhees [2]; (v) the importance of taking into account side reactions to attain reliable heat data was already realized by Dubrunfaut in his work published in 1856 [3]; (vi) "basal metabolic rate" gives rise to extended low rates of heat production when growth has ceased. Giaja [4] showed in his work of 1920 that a very small quantity of energy was sufficient to maintain the viability of yeast cells. By referring to earlier reports, I wish to point out some items which are still of general interest and relevance for calorimetric studies of microorganisms, and which will be discussed in this review. Earlier calorimetric work, however, has been comprehensively and excellently reviewed by Battley [5].

For an extensive and specific overview of the calorimetric field, the reader is further referred to all the works in which the application of thermodynamics and calorimetry to biological systems has been discussed [5–20]. Since the elucidation of calorimetric data, and thereby the common usefulness of calorimetric measurements, has generally to be connected to other types of information for successful interpretation of acquired heat data, this review will not only be focused on calorimetric studies, but also, in part, on other connected fields.

THERMODYNAMIC PROPERTIES OF MICROBIAL SYSTEMS

The applicability of thermodynamic laws to biological systems has, for decades, been argued due to the high complexity of such systems and,

"several workers have felt that cells, in some way, may be able to bring about a net decrease in entropy" [1]. In contrast, Winzler and Baumberger [1] concluded that "Most workers, however, feel that the first and second laws of thermodynamics hold within cells just as in simpler systems".

The application of thermodynamics to biological systems requires both an understanding of classical thermodynamics, and the thermodynamics of irreversible processes, fields which, for many biologists, need advisably to be served in a digestible form [21,22]. In other words, as expressed by Warn [21]. "Thermodynamics, like classical music, is an acquired taste. The initiation must be sensitively carried out, otherwise the mathematical rigour, like the formal structure of the music, acts to discourage a deeper relationship. It is sad but true that some students, both of thermodynamics and of Bach, never recover from the initial shock. In this, we are all losers".

The complexibility of a thermodynamic treatment of cellular systems is characterized by the fact that such systems are open and exchange energy and matter with their surroundings, and that life processes are far from equilibrium. However, a thermodynamic treatment of the life processes requires a clear definition of the system's boundaries and of the initial and final states of the considered metabolic process(es): a prerequisite for required performance of energy transformation balances and external energy and mass transfer balances (for an extensive discussion see refs. 18, 22, 23). It also requires that thermodynamic variables are concisely used. Winzler and Baumberger [1] pointed out the importance of distinguishing between the Gibbs energy of reaction and the heat of reaction in dealing with the maximal energy available for work in metabolic processes. In line with this remark, Hill as long ago as 1912 [24] (cf. ref. 14) showed that Gibbs energy liberated in the hydrolysis of glycogen to lactic acid might be three times the heat of reaction, and suggested that this hydrolysis might be the source of energy of muscular contraction. Thus, metabolic reactions leading to cellular growth and maintenance, do not depend on heat but on work. However, as expressed by Roels, "any process needs a non-zero dissipation to proceed to a non-zero rate. Hence, an organism which transforms substrate energy into biomass energy needs to dissipate part of the substrate's energy to allow this process to proceed with a non-zero rate" [25]. Net microbial (growth) processes are characterized by their irreversibility, which means that the work term, dW, of the net process is zero. Therefore, for a net metabolic irreversible process, catalyzed by the cellular system which is considered to be at, or near, steady state, Gibbs energy (dG) is dissipated (dD) and the energy (enthalpy) change (dH) of the process may be measured as the heat change (dQ). The relation between these variables is expressed below and in Fig. 1

$$dQ = dD + dB \tag{1}$$

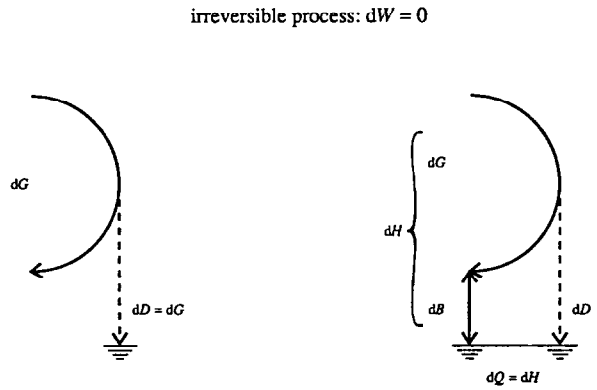

Fig. 1. Energy changes in an irreversible process occurring either in a closed or open system (dimension: energy per defined system). Left: "Ergodynamic" analysis of work (ergon) and Gibbs energy changes. Useful energy is either conserved as work or dissipated, $dG = dW + dD$ (irreversible process: $dW = 0$). Right: Thermodynamic analysis of heat (therme) and enthalpy changes (after ref. 26).

or in the more familiar form

$$dH = dG + dB \tag{2}$$

These relations and their applicability to biological systems have recently been extensively discussed [18,26]. Equation (1) explains the error in stating that useful energy which is not converted into work is lost as heat. Expressed correctly, the work is dissipated and the discrepancy between dissipated energy and heat, or between the two state functions, energy (enthalpy) and Gibbs energy, is due to the bound energy which is equal to the change in entropy times temperature ($dB = T dS$). However, the total energy expenditure of an isothermal cellular process, in other words the enthalpy change (dH), is at constant temperature and pressure equal to the net heat change (dQ or explicitly dQ_p with subscript p denoting constant pressure. In the following text dQ will be used without the subscript p, though a constant pressure is assumed). Microbial processes often take place in aqueous systems for which a constant volume is assumed to prevail in addition to constant temperature and pressure. This results in a negligable pressure–volume work. Consequently, for most practical purposes, the change in enthalpy in solution corresponds to the change in internal energy (dU), since $dH = dU + p dV$.

The net heat change (dQ) can be measured by calorimetry and is equal to the sum of the enthalpy change (dH) of all the reactions that occur during, for example, cellular growth. Thus, even though a growth process is composed of thousands of individual metabolic reactions, the net metabolic process comprising microbial growth can be described and thermodynamically treated as a rather simple chemical reaction [5,17,23,27–36].

Entropy changes accompanying microbial growth

A growth reaction representing balanced, exponential growth, was thermodynamically interpreted to be proceeding at a well-defined steady state [37]. At constant temperature the growing cells, which may be seen as an open isothermal system (at steady state: the growing cells are both identified as a constant catalytic unit — the system — and as biomass being part of the products of reaction), generating entropy internally at a constant rate, but as a result of the existing steady state, the entropy level of the system is constant. Consequently, there has to be an external flow of entropy equivalent to the internal production (see ref. 22). The negative internal entropy production multiplied by the temperature of the heat sink $-T\mathrm{d}_i S$ is equal to the dissipated energy $\mathrm{d}D$ of the growth reaction [18,26]. From eqn. (1) it follows that the entropy change of the total growth reaction times T ($T\mathrm{d}S = \mathrm{d}B$) is composed of two components, that is the dissipated energy ($\mathrm{d}D$) (at steady state, equal to the external change of entropy times T, $T\mathrm{d}_e S$) and the heat production ($\mathrm{d}Q$). Forrest [38] showed that during heterotrophic growth of three bacterial species, *Zymomonas mobilis, Streptococcus lactis* and *Streptococcus faecalis*, and one yeast specie, *Saccharomyces cerevisiae*, the flow of entropy, due to rejection of degraded products to the environment and the heat production of the catabolic process, is large enough to counteract the increase in information and order occurring during synthesis of new cellular material. Such studies show the applicability of the second law when also considering complex biological systems. This is in line with statements by Winzler and Baumberger [1].

Enthalpy changes accompanying catabolic versus anabolic processes

The enthalpy change of a growth reaction ($\mathrm{d}_g H$) can be divided into its catabolic ($\mathrm{d}_k H$) and anabolic parts ($\mathrm{d}_a H$) [1,5,15,34,39]

$$\mathrm{d}_g H = \mathrm{d}_k H + \mathrm{d}_a H \tag{3}$$

The enthalpy change of anabolism is low or may even be negligible compared to that of the catabolic process [5,38–43]. This statement, however, results from experiments performed in complex growth media or in defined media with sugars as the carbon and energy source. The difference in enthalpy change of a growth process between the substrates used for anabolism and the products of anabolism, which is mainly the biomass, is, in both these cases, small due to the relative similarity in degree of reduction [25,44,45] of the substrates (mainly the carbon source) and the biomass [5,46]. The small contribution of the anabolic part to the enthalpy change of the total growth reaction when a sugar is used as the carbon and energy source, can be illustrated by a growth process of the yeast *S.*

cerevisiae (calculated from data in a recent study by Larsson et al. [36]). In this experiment the yeast was aerobically grown with glucose as the only carbon and energy source and ammonium sulphate as the nitrogen source

$$\alpha,\beta\text{-D-}C_6H_{12}O_6(aq) + 0.298NH_4^+(aq) + 3.996O_2(g) \rightarrow$$
$$1.751CH_{1.71}O_{0.52}N_{0.17} + 4.075CO_2(g) + 4.766H_2O(l)$$
$$+ 0.298H^+(aq) + 0.010C_3H_8O_3(aq) + 0.071C_2H_4O_2(aq) \quad (4)$$

To calculate the enthalpy change of this reaction, tabulated thermodynamic data were used [23] together with the heat of combustion value of -522.8 kJ C-mol^{-1} of biomass [36]. The enthalpy change value attained is -1907.8 kJ mol^{-1} of glucose consumed.

The anabolic part of this growth reaction (4) can be written as

$$0.304\alpha,\beta\text{-D-}C_6H_{12}O_6(aq) + 0.298NH_4^+(aq) \rightarrow$$
$$1.751CH_{1.71}O_{0.52}N_{0.17} + 0.073CO_2(g) + 0.774H_2O(l) + 0.298H^+(aq) \quad (5)$$

The enthalpy change of this anabolic part of the growth reaction can be calculated to be -28.0 kJ (per amount of glucose given in eqn. (5)), which is equal to 1.5% of the enthalpy change of the total growth reaction. This value calculated for the anabolic part does include an uncertainty, since a small change in, for example, the heat of combustion of the biomass, will significantly affect such a low value and the calculated enthalpy change of the anabolic reaction may become positive or negative (see refs. 5, 39, 47). In fact, in current studies, variations of the combustion value and of the elemental composition of the biomass have been indicated in response to different physiological states or growth phases of the culture [47(a)] and as expected, combustion values vary largely depending on the type of organism and the environmental conditions. Measured heats of combustion of bacteria, yeasts, protozoa and algae range from -19 to -27 kJ per gram of ash-free biomass [48,49]. When comparing combustion values of the biomass, precautions have to be taken as to whether or not ash-free biomass is reported.

Regardless of whether it is positive or negative, the enthalpy change of the anabolic reaction is, as exemplified above, expectantly small and the catabolic part corresponds to as much as 98.5% of the total enthalpy change. However, for such metabolic processes for which a small total enthalpy change can be assumed, as in fermentative metabolism, the influence of the anabolic reaction on the total enthalpy change may be significant (see ref. 39). For anabolic reactions where there is a large difference in the degree of reduction between the anabolic substrates and the biomass, for example in autotrophic growth, the contribution of the anabolic reaction to the total enthalpy change of the process will be

substantial. The importance of the degree of reduction of the substrate was shown by Dermoun and Belaich [50] in their determination of the anabolic enthalpy change of aerobically grown *Escherichia coli* on succinic acid as the carbon and energy source. The enthalpy change was determined by two different methods to be +100.2 and +91.1 kJ mol^{-1} succinic acid, values which nicely closed the total enthalpy balance (see ref. 51).

Thermodynamic states and side reactions

In the reactions stated above describing a microbial growth process (Eqns. 4 and 5), the actual thermodynamic states (aqueous, gaseous, etc.) of the reactants and products were considered. The necessity to correct for side reactions has already been considered by Dubrunfaut [3] and Winzler and Baumberger [1] stated that it is necessary to, "evaluate the thermodynamic properties of substances in the physical form in which microorganisms actually metabolize them". Even more precisely, for a proper interpretation of experimental observations, the boundary of the system under study has to be clearly defined to be able to evaluate the thermodynamic state of the participating substances, and to correct for side reactions [5,8,14,28,36,41,42,52–60] which otherwise may considerably affect the final result (for an extensive discussion see ref. 23).

Energy balance analysis of microbial processes

Balance equations can be formulated for each of a system's extensive properties, which are characterized by the fact that they are additive [61]. Energy is an extensive property, which as stated in the first law of thermodynamics, is constant and has to be balanced at every stage of a process. When performing an energy balance analysis it is important to correct for side reactions and to use the actual thermodynamic states, mostly the aqueous state regarding the biological processes, except for gases, which due to actual conditions may persist in the gaseous state (for references see above). The calculated molar enthalpy change of a growth reaction ($d_g H$) is, as already discussed, at constant temperature and pressure equal to the heat change (dQ), provided that relevant corrections are made.

$$d_g H = dQ \tag{6}$$

Therefore, calorimetric measurement of heat production enables testing of the consistency of the data describing the reaction stoichiometry of the total growth process or of any phase of growth [5,23,27–29,30–36,39,41–43,51,57,61,62]. In several of these studies, a close agreement was reported between the measured heat change and the calculated enthalpy change based on biochemical analyses of participating substrates and products for

yeast and bacteria. Deviations from equality between $d_g H$ and dQ points to methodological errors or to the fact that the reaction stoichiometry is not correctly described; for example some product formation may have been neglected. An obtained imbalance may then be used for a proper interpretation of experimental data [52,59,66–69]. Traore et al. [70,71] verified by calorimetric measurements, a change of a suggested stoichiometry of the growth reaction of sulphate-reducing bacteria.

Balanced energy equations afford mathematical modelling of the growth process, which has been suggested for dynamic optimization of biotechnological processes [7,25,30,33–36,45,72].

Microbial growth efficiency

Owing to different definitions of the term "efficiency" (for a discussion see ref. 73), confusion has been caused and consequently a clarification is needed whenever the term is used. In their work Winzler and Baumberger [1] defined efficiency as the relation

$$\frac{\text{Free energy used in synthesis}}{\text{Free energy made available in breakdown}}$$

This definition corresponds to the "machine efficiency" [1] and was later explicitly defined and termed the thermodynamic efficiency [74,75] or ergodynamic efficiency [18]. The thermodynamic or ergodynamic efficiency of a chemical reaction is a power ratio or a flow times force ratio, since chemical power is the product of chemical flow and force. For example, the thermodynamic efficiency η_G of a microbial growth reaction, for which the input force is the molar Gibbs energy change of the catabolic reaction ($\Delta_k G$), the output force is the molar Gibbs energy change of the anabolic reaction ($\Delta_a G$) and the anabolic and catabolic flows are represented by $-J_a$ and J_k, respectively, then becomes

$$\eta_G = \frac{-J_a \, \Delta_a G}{J_k \, \Delta_k G} \tag{7}$$

The above defined efficiency is a "work efficiency", to be distinguished from a thermodynamic enthalpy efficiency. By analogy with the thermodynamic work efficiency, the thermodynamic enthalpy efficiency η_H is defined [18] as

$$\eta_H = \frac{-J_a \, \Delta_a H}{J_k \, \Delta_k H} \tag{8}$$

In eqn. (8), $\Delta_a H$ and $\Delta_k H$ are the molar enthalpy changes of the anabolic and catabolic reactions, respectively. The thermodynamic enthalpy efficiency can be calculated by measured data of a growth reaction, while the

calculation of the thermodynamic work efficiency of a growth reaction is complicated by the present lack of knowledge of the actual Gibbs energy change of the anabolic reaction [18]. To calculate η_G, the assumption is made that the Gibbs energy of the biomass is approximated by the enthalpy of the biomass. This is a weak point in efficiency calculations but may be a reasonable assumption [18,25]. Such an approach has been used in studies on microbial growth efficiencies [75,76].

Kedem and Caplan [74] showed that a thermodynamic work efficiency of less than 100% might be useful, since at 100% efficiency a system can only work at zero rate. This is why a system strives for optimal efficiency instead of maximal efficiency [14,73,77,78].

Microbial growth optimization was interpreted in terms of maximal growth rate when using a substrate more reduced than the biomass, resulting in a very low thermodynamic work efficiency, or in terms of maximal growth rate at an optimal thermodynamic work efficiency, when the substrate is more oxidized than the biomass [75,76]. The strategy tending towards a maximal growth rate was obtained for actively growing microorganisms. It would, however, be interesting to perform a microbial optimization analysis for organisms adapted to energy starving conditions, which is probably the "normal" state for most microorganisms in natural environments [79], a state for which an increased efficiency may be expected in response to the evolutionary pressure.

HEAT PRODUCTION IN RELATION TO THE PHYSIOLOGICAL STATE

Phenotypic variations of microorganisms occur in response to a varying environment. In natural habitats the environment of microorganisms shifts continuously, rapidly or slowly depending on different internal and external biological, physical and chemical parameters, such as the prevailing biological flora, flow of potential substrates, temperature and osmotic pressure to mention just some of the affecting parameters. Also during "controlled" laboratory conditions, the environment of cultured microorganisms is rarely constant. At best, steady state conditions can be assumed to be established during continuous cultivation. However, long term adaptation of the microorganisms occurs in response to nutrient limitation and the actual adaptation time exceeds the theoretically expected and the practically accepted [80]. For example, Rutgers et al. [80] showed in their study with *Klebsiella aerogenes* NCTC 418 that at a dilution rate of 0.2 and 0.5 h^{-1}, 40 to 60 generations were needed to establish a steady state in terms of a constant limiting substrate concentration in the fermenter. Such a long term adaptation may have a phenotypic or genetic origin, or maybe both. In batch cultures, steady state conditions are impossible to reach due to a continuously changing environment. However, at a macroscopic level organisms can be approximated as constant catalytic units, exhibiting a

constant metabolism at a constant rate during the separate phases of growth. Such a constant catalytic system will, in this review, be used as a definition of a specific physiological state of a culture. In this context it is also understood that a culture at a specific physiological state produces a constant rate of heat per unit of biomass (e.g. specific rate of heat production, expressed in milliwatts per gram). Is such a constant heat production rate seen during logarithmic batch growth? Contradictory results have been reported in the literature. Bayne-Jones and Rhees reported in their early study [2] that young growing cultures of several bacteria species produced heat at a higher rate than older growing cultures. They were not, however, at that time able to conclude if these variations were due to cellular processes or to changing physical and chemical environmental factors. Similar results were obtained by Stoward [81] with growing cultures of *Aerobacter aerogenes*; these results were challenged [82] in terms of too long an instrumental response time. Stoward [83] responded to the criticism and argued that in his studies *A. aerogenes* probably changed its metabolic activity during growth. A non-constant rate of specific heat production during the exponential phase of growth was also obtained with *Klebsiella aerogenes* [67,84–86], *Escherichia coli* [34] and the yeasts *Saccharomyces cerevisiae* [46,63,88] and *Debaryomyces hansenii* [89–91]. On the contrary, Forrest et al. [52] showed in their studies with *Streptococcus faecalis* that during the exponential phase the heat evolved per unit mass of bacteria against time is constant (Fig. 2), while the heat per bacterial cell increased which was explained by an increased cell size during growth. A

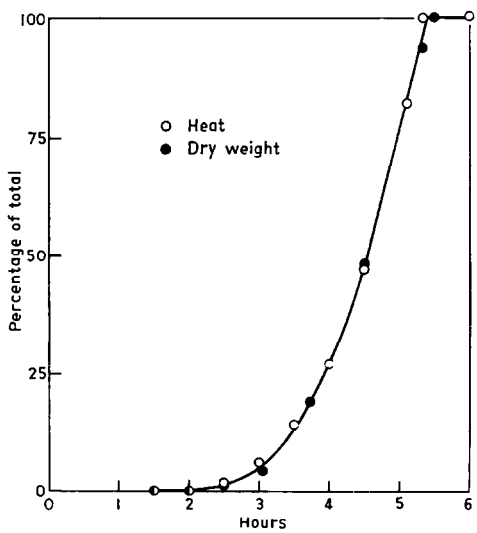

Fig. 2. Heat production by a growing culture of *Streptococcus faecalis*, with glucose as the energy source [52].

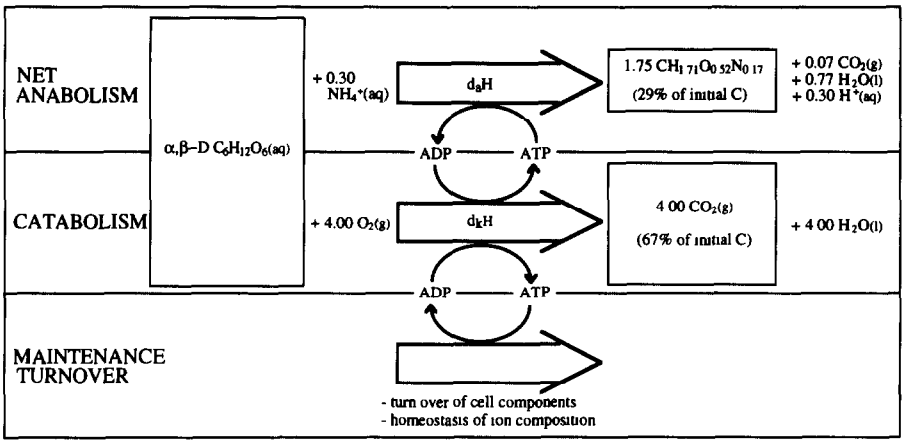

Fig. 3. Microbial metabolism exemplified by the aerobic growth process of *Saccharomyces cerevisiae* (eqns. (4) and (5)) (the small amounts of glycerol and acetate being produced in the catabolic reaction have been omitted in the figure). For simplicity, energy transformations are only exemplified by ATP turnover, i.e. the ATP/ADP cycle. The same initial and final state of the biomass is assumed, with the consequence that only the amount of biomass resulting from the growth reaction has to be considered. Maintenance reactions are defined to keep cell homeostasis (the initial state is equal to the final state), so the enthalpy change of the total growth reaction (d_gH) is the sum of the enthalpy changes of the anabolic reaction (d_aH) and of the catabolic reaction (d_kH): $d_gH = d_aH + d_kH$.

linear correlation between the rates of heat production and biomass production has been reported in a variety of studies with microorganisms [32–34,39,47,51,92,93].

Although there may be methodological explanations, not only instrumental but also medium compositional [34,36,94], for a varying specific rate of heat production of actively growing cultures, there are also metabolic explanations [see, for example, refs. 36,63,84,95]. As already discussed above, because of changing environmental conditions also during apparently balanced exponential growth, changes in the rates and types of metabolic reactions must be expected in a variety of systems. Confusing interpretations, however, are found in the literature regarding an explicit coupling between a reduced specific rate of heat production and an increased metabolic efficiency. Such conclusions may or may not be true, since a changed specific rate of heat production can also be the case even if the efficiency remains the same (read: thermodynamic work efficiency or ergodynamic efficiency). To understand the underlying mechanisms for a varying rate of heat production, a thorough analysis of the energy flows is necessary.

The total enthalpy change of a growth reaction (d_gH), measured as the heat change (dQ), can be divided into its anabolic (d_aH) and its catabolic (d_kH) parts (see above), which is illustrated in Fig. 3. In this context, the heat production will now be discussed systematically.

Heat production related to catabolism

Different types of catabolism relate to different rates of heat production. For example, the energy (enthalpy) change or the heat change of ethanol fermentation (in line with what has already been discussed, energy change, enthalpy change and heat change will be used synonymously in the following) is small compared to the heat of respiration with glucose as the energy source. Theoretically, the former gives -100 kJ mol^{-1} of glucose catabolically consumed and the latter -2814 kJ mol^{-1}, when calculated for real biological processes taking place in an aqueous environment [23,26]. Keeping a respiratory catabolism, but changing to another energy source may also be reflected by a different enthalpy change of the reaction. For example respiration of ethanol gives -1357 kJ mol^{-1} of ethanol or -679 kJ C-mol^{-1} compared with -469 kJ C-mol^{-1} of respired glucose. These values imply only catabolism, i.e. when the energy source catabolically consumed is totally fermented or oxidized.

Heat production of coupled versus uncoupled metabolism

The catabolic reaction, exemplified in Fig. 3 by the respiratory metabolism and the given thermodynamic states of the substances (gaseous, aqueous, etc.), results in an enthalpy change of -2814 kJ mol^{-1} of glucose consumed irrespective of whether energy transforming reactions, such as ATP turnover or production/consumption of membrane potentials occur or not. In other words, assuming that all energy transforming intermediates are kept in a steady state (for example, a complete ATP turnover occurs, which results in constant concentrations of ATP and ADP, and constant membrane potentials prevail), then the heat change resulting from the catabolic reaction per unit of glucose catabolically consumed is the same if efficient coupling to anabolic reactions occurs or not. For instance, zero coupling between catabolism and anabolism because of futile cycling would give the same heat change per unit of glucose catabolically consumed as full coupling (for discussion see refs. 14, 60). This is a result of enthalpy being a state variable, independent of the path, and only dependent on the initial and final states of the reaction (e.g. ref. 9).

In contrast the metabolic enthalpy change per mol of glucose totally consumed is of course growth yield dependent. The growth yield ($Y_{X/S}$) of eqn. (4) is 0.26 g dry mass per gram of glucose consumed. If the growth yield is doubled to approximately 0.5 g g^{-1}, a value common for many respiratory bacteria and yeasts, the enthalpy change of the total growth reaction will decrease to about -1100 kJ mol^{-1} glucose compared to about -1900 kJ mol^{-1} glucose related to the lower growth yield of the respiro-fermentative *S. cerevisiae*. The value of -1100 kJ mol^{-1} is in agreement with the enthalpy change of -1200 kJ mol^{-1} glucose ($Y_{X/S} = 0.50$) at-

TABLE 1

Characterization of different physiological states for glucose-grown cultures of *Saccharomyces cerevisiae* (freely from ref. 88).

Phase	Carbon/ energy source	μ (h^{-1})	Specific dQ/dt (mW g^{-1} dry mass)	dQ/dX (kJ g^{-1} dry mass formed)
Respiro-fermentative	Glucose	0.29	-763 ± 89(SD)	-9.4 ± 0.8(SD)
Respiratory	Ethanol	0.03	-423 ± 21(SD)	-51 ± 3(SD)

tained for the respiratory yeast *Debaryomyces hansenii* [90] and -1000 kJ mol^{-1} glucose ($Y_{X/S} = 0.45$) during respiratory growth of the yeast *Kluyveromyces fragilis* (calculated from data given in ref. 33). These data are also, as expected, in line with data obtained from other respiring yeasts and bacteria ($Y_{X/S} \approx 0.5$), growing on glucose or substrates of the same degree of reduction as glucose when expressed in kilojoules per C-mole of the substrate (calculated from data given in ref. 34). The value of the growth yield is strain and substrate dependent, relying on the actual metabolism, futile cycling, uncoupling, by-passing sites or modifications of the respiratory chain, overflow metabolism and maintenance requirements [60,63,94,96–106].

The implication of a changed metabolism from mainly fermentative (mixed respiratory–fermentative) to respiratory growth of *S. cerevisiae* (growth implicates catabolism coupled to anabolism) can be seen in Table 1. The respiro-fermentative metabolism of *S. cerevisiae* results from the well documented (for a review see refs. 107, 108) mixed respiratory and fermentative metabolism during aerobic batch cultivation with glucose as the energy source (Fig. 4). During the respiro-fermentative catabolism, glucose is mainly fermented to carbon dioxide and ethanol, while ethanol is

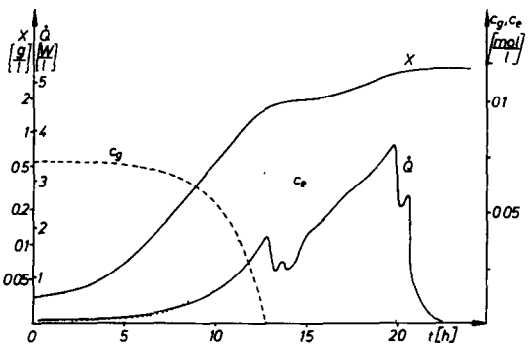

Fig. 4. Batch culture of the yeast *Saccharomyces cerevisiae* growing in a synthetic glucose medium. $\dot{Q}$, rate of heat production; X, dry mass; c_g, glucose concentration; c_e, ethanol concentration of the culture [62]. ($\dot{Q}$ in the text dQ/dt).

subsequently fully oxidized to carbon dioxide and water during the respiratory phase. The data in Table 1 point to several distinct features of metabolic heat production.

Firstly, as a result of the small energy change of the fermentative process compared with the energy change of the respiratory process per C-mole of energy source consumed, the heat yield dQ/dX (defined as the quantity of heat produced per unit of biomass formed and symbolized as $\Delta Q/\Delta X$ [68], $Y_{Q/X}$ (see review, ref. 17), dQ/dX [88] or ΔQ_X [36]), is low during the respiro-fermentative metabolism as compared to the respiratory metabolism, i.e. in this example about -9 kJ g^{-1} compared to -51 kJ g^{-1} dry mass formed. In the former case only 2–3% of the glucose was respired, while 80% of the glucose was fermented to ethanol [23]. However, with an increasing proportion of respiratory catabolism, the large enthalpy change value of the respiratory metabolism (large negative value) will outweigh the contribution of the fermentative metabolism (small negative value) (see ref. 35). By performing an energy balance analysis, the energy flow distribution can be deduced [36,51,68,94]. During the respiro-fermentative phase for the case given in Table 1, only 6% (percent of the total product formation expressed in kJ l^{-1}, which is equal to the total substrate consumption when the energy balance analysis is completed) of the energy change of the total growth reaction was dissipated as heat, only 14% was conserved as biomass, but as much as 75% was lost to, or conserved in, the environment as ethanol plus small amounts of glycerol (4%) and acetate (1%) [94] (Fig. 5).

In contrast, during the early respiratory phase, as much as 69% of the energy was dissipated as heat, but still the energy conserved as biomass was raised (22%) in comparison with the respiro-fermentative metabolism [36]. From this example it is seen that, despite a high energy dissipation as heat during the respiratory metabolism, more energy was conserved as biomass

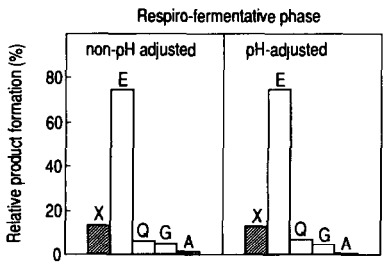

Fig. 5. Relative product formation, calculated as the energy content of each product (kJ l^{-1}) in percentage of the total energy content of the products (kJ l^{-1}) of a non-pH adjusted and a pH adjusted culture of *Saccharomyces cerevisiae* in the respiro-fermentative phase during aerobic batch growth in defined medium with glucose as the carbon and energy source. X, biomass; E, ethanol; Q, heat; G, glycerol; A, acetate [94].

per amount of substrate consumed in comparison to the respiro-fermentative metabolism. This was also the result when glucose was used as the respiratory substrate during fed-batch and continuous cultivation [94,109], cf. [62]. Thus, the respiratory metabolism in this example is more efficient in terms of biomass conservation than the fermentative metabolism. However, in terms of thermodynamic work efficiency (η_G), fermentative and respiratory catabolic coupling to ADP phosphorylation may show about the same efficiency during active metabolism [14,18,78], an analysis which has not yet been performed on microorganisms.

Secondly, by using the heat yield (dQ/dX), normalization of the heat production rate (dQ/dt) has been made towards both the present amount of biomass (X) and towards the growth rate (μ), since reformulating by including the growth equation ($dX/dt = \mu X$ or $dX = \mu X dt$) results in $dQ/dX = (dQ/dt)(1/\mu X)$. The specific rate of heat production (specific dQ/dt), in contrast, is not normalized for the growth rate [86,88] and the relation between dQ/dX and specific $dQ/dt (= dQ/(dtX))$ is therefore $(dQ/dX) \cdot \mu = dQ/(dtX)$. The highest growth rate of aerobically glucose-grown *S. cerevisiae* is attained with the respiro-fermentative metabolism (Table 1), which results in a high specific dQ/dt (Table 1). In other words, a low heat-yield, dQ/dX, imposed by the type of catabolism may, as in this case, be counterbalanced by a high metabolic activity, resulting in a high growth rate, and thereby yielding a high specific dQ/dt. The opposite prevails in the respiratory phase of *S. cerevisiae*.

Heat production in relation to maintenance

Maintenance is generally defined to include costs required to maintain cell status quo, without any contribution to a net increase of biomass (Fig. 3). However, not all processes are easily attributed to either maintenance processes or anabolic processes [110].

A small maintenance requirement has been suggested for growing microorganisms in comparison to energy flows related to the growth reaction (e.g. refs. 38, 106, 111). Maintenance requirements may, however, increase considerably due to environmental factors [94,99,100,106], or, conversely, approach zero in "dormant" cells [98]. Maintenance energy demands have been considered growth rate independent according to Pirt's classical concept [97], but more recently generalized concepts of maintenance include both a growth rate dependency and growth rate independency [98,110,112,113] (reviewed in ref. 73).

If the status quo of the cell is kept because of maintenance metabolism, maintenance turnover reactions per se do not result in an enthalpy change or experimentally measured heat change. Again, this is due to the fact that enthalpy is a state variable, only dependent on the initial and final states of the reaction. Maintenance metabolism, however, includes heat production

in the sense that a part of the energy source, via catabolism and energy transforming reactions, has to be used in driving the maintenance turnover reactions (Fig. 3). Thus, if maintenance requirements are increased, more energy will be transformed in the ATP/ADP cycle or via membrane potentials to meet this increased energy requirement to, keep, for example, a constant intercellular ion composition. Thus a smaller part of the energy source will be left to furnish the anabolic reactions via catabolic energy transforming reactions, and a smaller part of the carbon and energy source will consequently be conserved as biomass. Calorimetrically, increased maintenance requirements, without any change in the type of catabolic or anabolic metabolisms, will be seen as an increased heat yield, dQ/dX.

Maintenance processes may comprise a large part of the available energy [94,100,106], at extremes resulting in no available energy for synthesis of biomass and probably in cell death. For example, for an anaerobically grown culture of *S. cerevisiae* at a low environmental pH less than 2.8, Verduyn et al. [106] calculated that the fermentation rate limits the supply of ATP for maintenance purposes (cf. ref. 114). A major role was ascribed to the pH effect on the plasma membrane ATPase and to an increased proton gradient across the plasma membrane, resulting in an increased passive proton uptake rate. These measurements were optimized by the use of continuous culture experiments and by using a respiratory-deficient strain of *S. cerevisiae*, making possible a calculation of the ATP production. In batch experiments, Gustafsson and Larsson [94] studied increased maintenance energy requirements of yeasts in response to reduced environmental pH and osmotic potential. In response to a decreasing pH, the metabolic rates of *S. cerevisiae* were strongly influenced (Fig. 6).

Despite this influence, this strain of *S. cerevisiae* did not significantly change the size distribution of energy flows until a pH value as low as 2.8 was reached (Figs. 5 and 6), which resulted in an increased heat yield from -47 kJ g^{-1} biomass formed to about -72 kJ g^{-1}. For *Debaryomyces hansenii*, the heat yield was already significantly increased at an external pH below 3.7, and at a pH of around 3 the energy expenditure for growth had more than doubled. This means that the ATP requirement per unit of biomass formed more than doubled if unchanged coupling coefficiency and P/O ratio is assumed.

However, both the marine and highly halotolerant yeast *D. hansenii* [115] and baker's yeast *S. cerevisiae* changed the size-distribution of energy flow in response to a decreased osmotic potential caused by high sodium chloride concentrations (0.68–1.35 M), by shifting an increased part to catabolism compared to anabolism and an increased part of the glycolytic flux to polyol (glycerol) production [89,90,91,116]. The intracellular part of the glycerol produced is used for osmoregulation [89,117,118]. The shift in energy flow resulted in increased heat yields, which with regard to *S. cerevisiae* corresponded to -44, -50 and -57 kJ g^{-1} biomass formed in

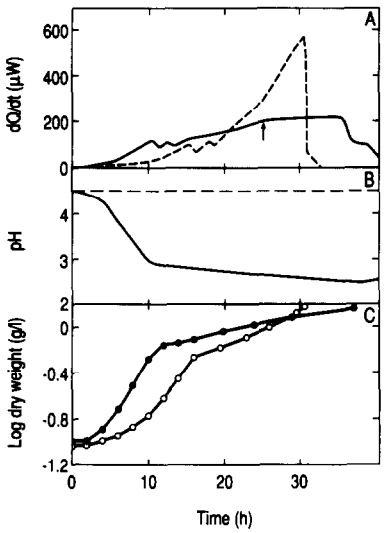

Fig. 6. (A) Rate of heat production, dQ/dt; (B) pH and (C) dry mass during aerobic batch growth of *Saccharomyces cerevisiae* in pH adjusted (broken lines and open circles) and non-pH adjusted cultures (full lines and closed circles) in defined media with glucose as the carbon and energy source. The arrow denotes a shift in heat yield during the respiratory phase of the non-pH adjusted culture; see text for explanation [94].

0%, 2% and 4% NaCl, respectively, calculated from the total growth period with glucose as carbon and energy source [87]. Consequently, a dual change of the catabolic flow in response to the decreased environmental osmotic potential was shown, both the increase in glycerol production and an increased energy transformation to meet the increased energetic cost of maintenance reactions, probably to keep an acceptable intracellular ion composition towards a steep salt gradient [99,119].

By adopting the concept of maintenance by Pirt [97], it was calculated that the increased maintenance energy requirement for *D. hansenii* during growth at pH 3 compared to growth at pH of approximately 4 was -330 mW g^{-1} biomass, which was calculated to be more than 50% of the total specific dQ/dt at pH 3. This high maintenance energy dissipation was one of the main factors which caused reduction in growth rate from 0.2 to 0.07 h^{-1}. The increased maintenance in response to the lowered pH from 4 to 3 was indicated to be of the same order as the increased maintenance for growth at 1 M NaCl, which is in accordance with reported data for chemostat grown *S. cerevisiae* under the same pH and salinity conditions [99,106]. These values of increased maintenance can be compared with values of maintenance energy dissipation for growth during optimal cultivation conditions, being reported to be -25 mW g^{-1} for *K. fragilis* [120], -100 and -60 mW g^{-1} for *S. cerevisiae* depending on the medium (defined and complex, respectively) [62] and -80 mW g^{-1} for *Escherichia coli* [31].

Maintenance discussed so far refers to requirements during growth. However, heat production during endogenous metabolism, occurring as a result of energy source starvation or total nutrient starvation, may result both from energy transformations coupled to maintenance processes (cell component recycling and homeostasis of the intracellular ion composition) as well as from de novo synthesis of cell components. The transition from active growth to starvation–survival, the latter distinguished by a physiological state at low metabolic activity, has been shown to be an active process resulting in changed relative rates between different metabolic reactions and de novo synthesis of proteins specific for the state of starvation–survival [121,122]. The energy source during this process has to be of endogenous origin. The degradation of energy reserves may be monitored and quantified by calorimetry [123].

Calorimetric studies of starving cultures are scarce, but as expected, heat production rate of endogenous metabolism is low (reviewed in refs. 124, 125). Forrest and Walker [111] and Schaarschmidt (see ref. 125) reported values of the specific rate of heat production of about -10 mW per gram dry mass for starving *Streptococcus faecalis* and *S. cerevisiae*, which is an even lower value than the maintenance energy dissipation values of optimally growing cultures as given above.

It is obvious that in response to severe environmental stress conditions, all the available energy may be diverted to meet the highly increased maintenance energy requirements. By contrast, at starving conditions without additional stress factors even small differences in maintenance energy requirement may be of profound importance for the competition-pressure during starvation survival (see ref. 118). This is an area of great interest from an ecological and biotechnological point of view, and may stimulate future energetic studies.

DIRECT VERSUS INDIRECT CALORIMETRY

A direct correlation between the measured rates of heat production and oxygen uptake during growth of a variety of microorganisms has been reported (e.g. refs. 33, 34, 62, 126–130).

This correlation is relevant only for aerobic respiratory metabolism and the basis for this correlation is the oxycaloric equivalent, $\Delta_k H_{O2}$, which is the calculated enthalpy change per unit amount of oxygen consumed. For the catabolic reaction exemplified in Fig. 3, for which the enthalpy change was calculated as -2814 kJ per mole of glucose consumed, the oxycaloric equivalent is one sixth of $-2814 = -469$ kJ per mole O_2 consumed. For a variety of different conditions and substrates, such as carbohydrates, lipids and proteins, the oxycaloric equivalents range from -430 to -480 kJ (mol O_2)$^{-1}$ [57,60]. From this it follows that simultaneous calorimetry and respirometry provides a tool in physiological, ecological and biotechnologi-

cal studies and applications. For purely aerobic metabolism, calorimetric measurements may substitute oxygen measurements (indirect calorimetry) or vice versa. It must, however, be kept in mind that the heat measurements of a growth process include both the heat production related to anabolism and catabolism, although the former may be negligibly small when substrates of about the same degree of reduction as the biomass are respired (see above). Oxygen uptake, however, relates to the catabolic respiratory reaction, but may also include some involvement from biosynthetic reactions. Maybe more important is the possible combination of the two types of measurements to identify possible anaerobic metabolism solely or in addition to aerobic metabolism [60,63,68,131]. By using the combination of respirometry and calorimetry on a synchronized chemostat culture of *S. cerevisiae*, it was interestingly indicated that during the period of the cell cycle just before the maximal budding period, the respiring capacity was too small to fulfil the required energy flow, which was then supplemented by fermentative metabolism [65,95]. In a toxicology study, using complex natural river sediments, qualitatively the same result was obtained with calorimetry and respirometry. The combination, however, of the two types of measurements had the advantage of indicating the dominating nature of aerobic respiratory metabolism in these samples and its sensitivity to the toxicant [132]. The calorimetric method used in this study (ampoule calorimetry; for a technical description see ref. 133) was later improved by using perfusion calorimetry [134] (for a technical description see ref. 135). A correlation between the calorimetric and respirometric measurements was also shown in the triggering of heat production and oxygen consumption by a surface in starving *Vibrio* DW1 [136]. This triggering effect shown on catabolism, is in line with statements that surfaces may "trigger" a burst of activity leading to optimization of survival configuration.

Poole and Haddock [129] point to the possibility of using combined oxygen uptake and calorimetric measurements for a rapid assessment of the mode of glucose metabolism and the recognition of respiratory-deficient strains. The mode of glucose metabolism was tested at our laboratory by a rapid screening method, but without making use of oxygen measurements. Instead, calorimetric measurements were combined with the use of respiratory chain (azide) and glycolytic (iodoacetate) inhibitors in the type of experiments shown in Fig. 7.

A stationary phase culture of *S. cerevisiae* responded immediately upon glucose addition. The addition of azide caused rapid decrease in activity, although there was still some activity until glucose was depleted. This activity was obviously due to fermentative metabolism, since additional glucose pulsing increased the heat production rate to 10% of the value obtained before azide addition, activity being lost upon iodoacetate addition. Further glucose pulsing did not result in any measurable activity [46]. Assumed respiratory-deficient strains were tested in the same way, a

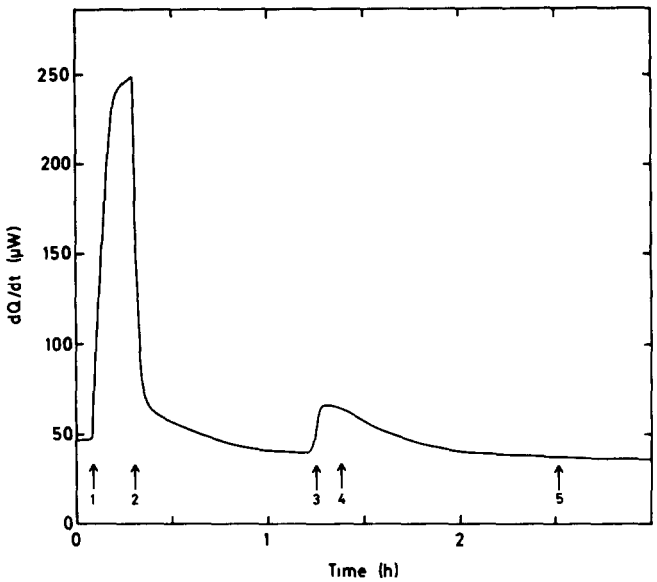

Fig. 7. The effect of azide and iodoacetate on glucose metabolizing *Saccharomyces cerevisiae*. Figures indicate the addition of glucose (0.2 g l^{-1}) (1, 3 and 5), azide (1 mM) (2) and iodoacetate (1 mM) (4) [46].

method which seems promising in rapid screening for respiratory-deficient mutants, but which equally well could be performed by combining calorimetry and respirometry.

These types of measurements point also to the potential of calorimetry, alone or in combination with other techniques, in the field of pharmacology and toxicology. For further information in this field, I refer to a review on pharmaca in this special issue.

CONCLUDING REMARKS

In biology, calorimetry can be used both for quantification of energy transformations and for kinetic studies of biological processes. The combination of the two areas may result in an understanding of a dynamically changing biological process. For instance in the example being used throughout this review, the aerobic growth of the yeast *S. cerevisiae* with glucose as the only carbon and energy source, the kinetics of the heat production rate in relation to the kinetics of the growth rate indicated a continuously changing metabolism. During the mixed respiratory-fermentative metabolism these changes in the physiological state of the culture are explained by a continuously changing proportion of the respiratory catabolism in relation to fermentative catabolism [63,88]. Calorimetry in combination with biochemical analyses verified the suggested stoichiometry of the different physiological states by closing the energy balance [36,65,68].

In this way an improved stoichiometry of the growth reaction was verified for the anaerobic respiratory metabolism of sulphate-reducing bacteria [70,71], and an understanding was reached for the successive use of available extracellular and of intermediary produced electron acceptors of anaerobic dissimilatory nitrate-reducing bacteria [64,137]. Also in the process of an understanding of the energetics and stoichiometry of the complex interspecies, hydrogen transfer in growing mixed cultures of the sulphate-reducing bacterium *Desulfovibrio vulgaris* and the methane-producing bacterium *Methanosarcina barkeri*, kinetic energy flow studies and energy balance calculations were used [138] and of the degradation of complex carbohydrates by *Cellulomonas* sp. 21399 [139].

Finally, by shifting to the even more complex energy flow studies of cultures growing in complex ill-defined media or mixed microbial communities of natural samples, calorimetry is still a useful tool, due to its unspecificity. This is because all types of metabolism will result in heat production and therefore calorimetry is generally applicable to every kind of biological system. However, the success in interpreting the experimental data to yield valuable and unique information will generally be dependent on combining the calorimetric data with other data obtained using more specific techniques.

For a review in the ecological field the reader is referred to papers on this field in this special issue and to earlier works referred to at the beginning of this paper.

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