CALORIMETRIC MEASUREMENT OF THE EFFECT OF pH ON THE ANAEROBIC AND AEROBIC CATABOLISM OF GLUCOSE BY STREPTOCOCCUS AGALACTIAE

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Flow microcalorimetry has been used to measure the anaerobic and aerobic rate of glucose catabolism by washed cell suspensions of Streptococcus agalactiae under flow and stopped flow conditions. The rate of glucose catabolism under aerobic and anaerobic conditions was similar, while heat production was greater under aerobic conditions due to divergence from a homolactic fermentation which was confirmed by enthalpy calculations. The pH of the buffer, in the range of 5.5 to 6.8, did not affect the rate of glucose catabolism or heat production. In the presence of 2,4-dinitrophenol at a concentration optimized for maximum stimulation of glucose catabolism, the increased amount of glucose catabolized appeared as lactic acid under anaerobic and aerobic conditions; buffer pH determined the rate of glucose catabolism, with the maximum rate occurring at pH 6.0. The significance of pH in restricting the growth of different strains of lactic acid bacteria is considered in terms of the provision of energy from carbohydrate catabolism.

Lactic acid bacteria of different genera have an optimum pH range for growth and a minimum pH below which they show little growth (1). This property is employed in the preservation of milk and meat products by encouraging the growth of lactic acid bacteria to lower the pH and hence inhibit the growth of organisms of public health significance. Fruit juice and wine have a low natural pH which allows spoilage or a desired fermentation by yeast or by a few species of lactic acid bacteria.

Wine is subject to bacterial fermentation (malolactic fermentation) by strains of Leuconostoc, Pediococcus and some lactobacilli. However, the preferred species L. oenos will grow at about pH 3 while strains of other genera can grow in the same wine only if the pH is increased to 3.6 or higher (2). Strains of Strepto-
coccus and other pathogens are unable to grow at wine pH. The ability of lactic acid bacteria to grow in a medium of adequate nutrition and pH depends upon the provision of metabolic energy from the anaerobic or aerobic catabolism of a sugar. The rate of sugar catabolism by washed cell suspensions of lactic acid bacteria is a well defined property (3), probably determined by the rate of sugar transport into the cell, the enzymes of the glycolytic pathway, and the pH of the supporting medium which influences the internal pH of the cell (4).

In this report, flow microcalorimetry (5, 6) has been used to determine the rate of anaerobic and aerobic catabolism of glucose by suspensions of Streptococcus agalactiae in phosphate buffer of varying pH. Anaerobic conditions were achieved by stopping the flow and allowing bacteria in suspension to exhaust the dissolved oxygen contained in the calorimetric measuring vessel. The measured rates have been compared with those determined in buffers of similar pH to which had been added the proton conductor 2,4-dinitrophenol (DNP) to equalize the pH of buffer and cell cytoplasm (4). The conditions required to maximize the effect of DNP have been determined.

MATERIALS AND METHODS

Bacteria. Streptococcus agalactiae a human faecal isolate, was utilized.

Cultivation of bacteria and preparation of washed suspensions. Streptococcus agalactiae was grown at 37° in a complex medium containing 10 g/l each of glucose, peptone, yeast extract and trisodium citrate. The incubation time was 16 hr. The cells from 1 l of medium were harvested by centrifugation and washed twice with 0.2 M (K2HPO4/KH2PO4) buffer at pH 6.2. The cell pellet was suspended in 50 ml of similar buffer and used to prepare cell suspensions ranging from 0.5 to 1.5 mg/ml dry weight of cells.

Calorimetric measurements. Cell suspensions containing 10 mM glucose were mixed, aerated at room temperature by bubbling with water-saturated compressed air and pumped through a flow calorimeter (5), which had a gold measuring vessel of 0.65 ml volume and was equilibrated at 37°. After obtaining a steady state thermal output, in about 15 min, the flow was stopped and another lower steady state was established after 30 min. The calorimeter was calibrated electrically during flow and stopped flow and the steady state values reported in watts.

Analytical methods. Cell suspensions were centrifuged immediately after the addition of 10 mM glucose and the pH of the supernatant measured with a Radiometer digital pH meter PHM 52 at 37°. Buffer pH was also measured after completion of the calorimetric measurements. Cell density was determined by measuring the optical density of suspensions at 650 nm in a Shimadzu spectrophotometer QV50; dry weight was obtained from a calibration curve prepared from stationary phase cells. One ml samples of ferments for glucose and lactic acid
RESULTS

Calorimetric record of aerobic and anaerobic heat production

A baseline was established by pumping 0.2 M, pH 6.2 phosphate buffer through the calorimeter followed by a suspension of *S. agalactiae* in similar buffer which caused a small deflection, *a* (Fig. 1), due to endogenous metabolism (7). On addition of 10 mM glucose to the cell suspension, the increased rate of heat production caused a new steady state, *b*, to be established. A further increase in heat production occurred, *c*, when 250 µM DNP was added to the cell suspension.

When the flow was stopped, a small increase in heat production occurred, *d*, corrected for in calculations by using electrical calibration constants obtained under flow and stopped flow conditions. After several minutes of stopped flow the record of heat production decreased slowly, *e*, and then rapidly to a new steady state, *f*.

Measurement of heat production from glucose catabolism

Cell suspensions ranging from 0.5 to 1.5 mg of cells/ml in 0.2 M, pH 6.2 phosphate buffer containing 10 mM glucose were pumped through the calorimeter and the steady state flow and stopped flow heat production were measured. In Fig. 2 are shown the linear relationships between the flow and stopped flow specific power (heat production for unit volume or cell dry weight) for suspensions of different cell density. The slope of the linear regression for heat production under stopped flow and flow conditions gave 150 and 314 µW/mg dry weight of cells, respectively.

Together with the calorimetric measurements, two 100 ml cell suspensions of about 1 mg/ml in 0.2 M, pH 6.2 phosphate buffer were equilibrated at 37°. One suspension was made aerobic and the other anaerobic by bubbling water-saturated compressed air or oxygen-free nitrogen through the suspension. Catabolism was initiated by the addition of 10 µM glucose. Samples removed at 10 to 15 min intervals over a 2 hr period were analyzed for glucose and the rate of glucose catabolism was determined. The anaerobic and aerobic suspensions were found to degrade glucose at 0.082 and 0.085 µM/(min . mg of cells), respectively, indicating no effect of oxygen on the rate of glucose catabolism.

The stopped flow heat production of the 150 µW/mg of cells and the rate of glucose catabolism of the 0.082 µM/(min . mg of cells) indicates an enthalpy change of −110 kJ/mol of glucose; the calculated value for a homolactic fermentation in similar buffer is −117 kJ/mol of glucose (8). Similarly, the enthalpy change for
the catabolism of glucose by a cell suspension flowing through the calorimeter is \(-216\, \text{kJ/mol of glucose}\). The enthalpy change obtained under stopped flow conditions corresponds with the anaerobic homolactic fermentation of glucose by \(S.\ agalactiae\). Under flow conditions the enthalpy change corresponds with the aerobic catabolism of glucose, the increased heat production resulting from formation of degradation products in addition to lactic acid.

**Effect of DNP concentration on heat production from glucose catabolism by \(S.\ agalactiae\)**

Suspensions of stationary phase cells (1.2 mg/ml) in pH 6.2 phosphate buffer were treated with different concentrations of DNP ranging up to 300 \(\mu\text{M}\). Glucose was added at 10 mM and the aerobic and anaerobic heat production was measured at each concentration of DNP (Fig. 3). Heat production increased with DNP concentration, approaching a maximum rate between 200 and 300 \(\mu\text{M}\) DNP.

**Effect of DNP on the rate of glucose catabolism and formation of lactic acid**

Heat production by \(S.\ agalactiae\) catabolism of glucose was stimulated aero-
bically and, on addition of DNP, both anaerobically and aerobically (Figs. 1–3). This was investigated further by measuring the effect of DNP on the rate of glucose catabolism and the formation of lactic acid under both aerobic and anaerobic conditions.

Cells were suspended at about 1 mg/ml in 0.2 M phosphate buffer pH 6.2 containing 10 µM glucose and 5 replicates of 20 ml were incubated at 37° anaerobically under N₂ or aerobically by gassing with water saturated air, with and without the addition of 250 µM DNP. The yield of lactic acid was determined at the completion of fermentation. Anaerobic and aerobic rates of glucose catabolism were measured for similar cell suspensions.

The aerobic and anaerobic rates of glucose catabolism either with or without the addition of 250 µM DNP were similar, Table 1, indicating no effect of oxygen on the rate of glucose catabolism. The molar yield of lactic acid from glucose was essentially homolactic under anaerobic conditions being 1.93 and 1.89, without and with DNP present respectively. Aerobic fermentations showed 1 mol of glucose to be converted to 1.47 and 1.62 mol of lactic acid without and with the addition of DNP.

The rate of lactic acid formation can be calculated from the rate of glucose catabolism and molar yield of lactic acid, Table 1. The increase in rate of glucose catabolism due to the presence of 250 µM DNP shows essentially a homolactic conversion to lactic acid under both anaerobic and aerobic conditions. The fate of glucose not observed as lactic acid under aerobic conditions, and giving rise
to increased heat production (Fig. 2), was not determined.

**Effect of pH on the anaerobic and aerobic catabolism of glucose with and without DNP present**

Steady state heat production obtained by flow and stopped flow calorimetry (Fig. 1) with *S. agalactiae* catabolism of glucose was considered to be due to the establishment of aerobic and anaerobic conditions. Further, DNP at an optimum concentration of 250 µM stimulated the rate of glucose catabolism which was shunted to lactic acid under both anaerobic and aerobic conditions. This information allowed the investigation of buffer pH on the rate of glucose catabolism by washed cell suspensions using calorimetry.

A culture grown in complex medium until the late stationary phase was used to prepare washed cell suspensions in phosphate buffer ranging from 5.6 to 6.8 pH. Paired cell suspensions of about 1 mg/ml dry weight, with or without 250 µM DNP and containing 10 mM glucose, were measured for aerobic and anaerobic heat production. In Fig. 4 the results obtained with 3 different cell preparations are given. The anaerobic heat production remained constant at 142 µW/mg cells between pH 5.7 and 6.6; below pH 5.7 the heat production was negligible. The aerobic heat production remained constant at 230 µW/mg of cells over the same pH range as heat production under anaerobic conditions did and also began to decrease below pH 5.7.

In the presence of 250 µM DNP the maximum rate of heat production by cell suspensions degrading glucose occurred at pH 6.0 both aerobically and anaerobically giving 408 and 308 µW/mg cells, respectively. The rate decreased similarly both aerobically and anaerobically with increasing pH, and rapidly with decreasing pH, showing negligible heat production below pH 5.6. At pH 6.0 the addition of 250 µM DNP stimulated the anaerobic and aerobic heat production by 166 and 178 µW/mg cells respectively. Using the enthalpy data for anaerobic glucose catabolism shown in Fig. 2, the increased heat production corresponds to increased glucose catabolism of 0.09 and 0.097 µM/min·mg of cells anaerobically and aerobically. The similar stimulation in heat production and glucose catabolism due to

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* Rate of lactic acid formation calculated from the rate of glucose catabolism and molar yield of lactic acid from glucose.

Table 1. Rate of glucose catabolism, lactic acid formation and molar yield of lactic acid by *S. agalactiae* maintained aerobically and anaerobically with and without DNP.
DNP is in agreement with the previous observation that increased rate of glucose catabolism is diverted to lactic acid both anaerobically and aerobically.

**DISCUSSION**

Flow calorimetry can be applied to study metabolism by cell suspensions, both anaerobically and aerobically where the culture is able to utilize dissolved oxygen. Cell suspensions of *S. agalactiae* degrading glucose when flowing through the calorimetric cell resulted in a steady state heat production; when the flow was stopped, the microaerophilic culture used up the dissolved oxygen thus establishing anaerobic conditions, confirmed by the measured enthalpy change for homolactic glucose catabolism. The stimulated heat production in the presence of oxygen is the result of some glucose being oxidized to end products other than lactic acid. Under anaerobic and aerobic conditions the presence of DNP stimulates the rate of glucose catabolism which is diverted to lactic acid.

Over the pH range 5.7 to 6.7 the rate of glycolysis by *S. agalactiae* remained constant as indicated by heat production, demonstrating no effect of buffer pH on the ability of the cells to transport glucose or of the enzymes of the glycolytic pathway to degrade it. Under aerobic conditions an increased rate of heat production is observed due to diversion of some glucose carbon, about 27%, to prod-
ucts other than lactic acid. The aerobic and anaerobic heat production is constant over a similar pH range, indicating no influence of buffer pH on the aerobic component of glucose catabolism. At buffer pH of 5.7 the rate of glucose catabolism began to decrease rapidly, and at pH values less than 5.6 glucose catabolism was negligible. The lower pH level was critical in determining the integrity of the cell either with respect to glucose transport or glycolytic activity.

In the presence of DNP the heat production by cells degrading glucose was not constant over any pH range indicating the direct influence of buffer pH on glucose catabolism. The critical pH of 5.7 below which glucose catabolism was negligible was also found in the absence of DNP. Under conditions suitable for growth, the rate of ATP production available for biosynthesis depends upon the rate of glucose catabolism. Observations on washed cell suspensions of *S. agalactiae* indicate that growth in media of pH lower than 5.5 would be restricted due to the low rate of glucose catabolism.

Growth regulation by pH in strains of lactic acid bacteria is important in the preservation of some foodstuffs, and is related to the rate of carbohydrate catabolism determined by environmental pH. This property is subject to further study using strains of *Leuconostoc*, *Pediococcus* and *Lactobacillus* which show different minimum pH values below which they are unable to grow.

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REFERENCES