A MICROCALORIMETRIC STUDY OF ENERGY CHANGES DURING BACTERIAL GROWTH

A thesis presented for the degree of
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by

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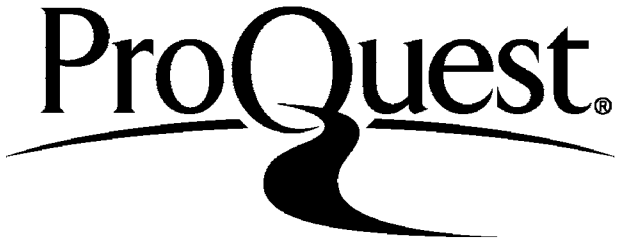

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CONTENTS

CHAPTER 4

VARIATION OF HEAT OUTPUT DURING GROWTH IN GLUCOSE-LIMITED MEDIA

CHAPTER 5

THE EFFECT OF TEMPERATURE ON HEAT OUTPUT OF K. AEROGENES

CHAPTER 6

THE EFFECT OF CHANGED CONDITIONS ON THERMAL AND GROWTH PARAMETERS DURING GROWTH OF K. AEROGENES IN STANDARD GLUCOSE MEDIA

ABSTRACT

Thermal events accompanying the growth of *K. aerogenes* in batch culture in C- and K^+-limited salts media were recorded using a flow-microcalorimeter; growth parameters (biomass, CO_2, pO_2 and pH) were also continuously monitored. Calorimetric data is discussed in terms of i) the power-time trace profile and ii) enthalpy changes associated with formation of cells and utilization of C-source.

Standard experimental and cultural conditions, which affect the measured thermal events, were established; at $P = 0.05$ the reproducibility of the heat output was 3%. The reproducibility of other growth parameters were similar.

The p-t trace accompanying aerobic growth in glucose-limited media consisted of a single peak, the maximum power output coincided with the cessation of exponential growth and exhaustion of glucose; at this point the CO_2 output was a maximum and pO_2 a minimum. The total heat output increased linearly with glucose concentration.

The enthalpy change for anabolic growth processes depends on the temperature and glucose-concentration. Generally the rate of heat output exceeded those for biomass and CO_2 production. Maintenance coefficients for batch growth in glucose- and α-methyl glucoside-limited media are calculated.

Addition of a second carbon source to cells growing in glucose had a characteristic effect on the p-t trace depending on its carbon content. The p-t trace of glucose-trained cells in media containing α-MG showed that it was metabolized in the presence of glucose. Results of thermal data for cells trained for growth in α-MG and mixed glucose/α-MG media are discussed in terms of known metabolic pathways.

The effects of metabolic inhibitors on thermal events are discussed in terms of their mode of action; azide inhibited growth in glucose but not in α-MG.

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Summary

Thermodynamic data, free energy, enthalpy and entropy values, have important practical applications in the fermentation industry as well as in chemical-biological research. In the fermentation industry important parameters which are calculated include the yield value, from mass-balance data, and hence the quantity of heat that will be generated by the culture from the enthalpy change for substrate oxidation. The heat generated by the culture is an important factor when considering the control of the growth temperature. Thermodynamic functions are important when investigating the feasibility of metabolic pathways. If free energy data is known for the different metabolic steps and the total energy available is known then energetics of the pathway can be calculated and used as supportive evidence for the pathways or aid explanation of known pathways.

Thermal and growth measurements were made during the growth of K. aerogenes in batch culture under carbon and K^+-limitation. Thermal measurements were made continuously throughout the growth cycle with an LKB 10700-1 flow microcalorimeter; growth parameters (CO_2, pO_2, biomass and pH) were also monitored continuously. The first reported systematic study of the effect of cultural and experimental conditions on the measured thermal events is presented. Six factors are considered to be the most important: (i) the level of aeration of the culture vessel and calorimeter flow line; (ii) the condition of the inoculum; (iii) the flow rate through the calorimeter cell; (iv) the recycling of the culture from the calorimeter back to the culture vessel; (v) the concentration of the growth limiting and/or carbon source and (vi) the growth and test temperature. These factors are inter-dependent and cannot be considered in isolation of each other.

The measured heat output was very dependent on the state and condition of the inoculum. Irreproducible results, ie. power-time profiles and total heat evolved, were obtained using inocula from routine daily sub-cultures or freeze-dried preparations. Cryogenically-stored standard inocula gave very reproducible growth and thermal parameters. At flow rates up to 70 $cm^3\ h^{-1}$ the exponential heat output, q(exp), increased becoming constant at higher flow rates (glucose concentration fixed). At a constant flow rate of 90 $cm^3\ h^{-1}$, the total heat output, q(tot), increased linearly with glucose concentration up to 5.4 mmol dm^{-3}; q(exp) was a linear function of glucose concentration up to 3.8 mmol dm^{-3}, both lines passed through the origin. The power-time trace became increasingly complex at glucose concentrations above 3.8 mmol dm^{-3} (flow rate 90 $cm^3\ h^{-1}$) and at lower flow rates with a fixed glucose concentration. The increased complexity of the p-t trace and the reduction in heat output were attributed to the loss of fully aerobic metabolism in the calorimeter cell; this is a function of the aeration conditions used. Such an investigation permitted the selection of standard experimental conditions. The transit time from culture vessel to calorimeter of 1.2 min is the shortest reported to date.

During aerobic growth in glucose-limited medium the maximum power output of the single p-t trace peak coincided with the end of exponential growth; also coincident were minima for the pO_2 and pH and maximum in CO_2 output, the values of these growth parameters subsequently quickly returned to their initial values. Such changes were typical for most growth-limited cultures irrespective of substrate. During growth in carbon-limited medium the specific power output was maximal during the early stages of growth attaining a constant lower

value during exponential growth which then increased a little near the point of substrate depletion, these changes were observed at all growth temperatures. The most energetically inefficient periods are the early and late stages of exponential growth, probably due to acceloratory processes and nutritional stress respectively.

The rate constant for heat output was generally greater than that for biomass increase, while those for CO_2 output was intermediate. The rate constants are substrate-concentration and temperature dependent; from 25 to 37°C all the rate constants increased but at higher temperatures they decreased and became equal. This is probably due to an inbalance in catabolic enzyme rates resulting in an uncoupling between catabolism and anabolism.

Heat output data was converted to various enthalpy change values, the most important was that for the consumption of 1 mol of carbon substrate, ΔH_{sub}, and for the production of 1 mol of cellular material, ΔH_p. Full appreciation and interpretation of measured power output cannot be achieved unless it is related to various growth parameters.

For most investigations the maintenance of fully aerobic conditions is essential, which enables thermal data to be interpreted in terms of relatively simple metabolic pathways. When the only products of metabolism are cell, CO_2 and water, mass-balance data can be calculated. For glucose-limited growth the measured CO_2 production agreed well with the calculated values and all substrate carbon could be accounted for as CO_2 and cell carbon.

From the mass-balance data an energy budget can be obtained. The enthalpy of oxidation, ΔH_{ox}, calculated from the fraction of glucose oxidised to CO_2 and H_2O can be compared with the determined value, ΔH_{gluc}. The difference between the values, ΔH_B, is attributed to the enthalpy changes associated with anabolic growth processes and accounts for

5-15% (depending on glucose concentration) of the total energy available from the glucose; this value also varies with temperature. With increasing glucose concentration the substrate energy stored as cellular carbon is constant, the wasted energy (heat) decreases with a concomitant increase in ΔH_B. This increase arises from the increased energy needed for the incorporation of small quantities of secondary metabolites into cellular components in place of the equivalent amount of glucose. From 25 to 39°C the stored and wasted energy remain constant, at higher temperatures the stored energy decreases and that wasted increases; ΔH_B approaches zero at extreme temperatures. The use of yield data and the heat of combustion of the cells permits the calorific efficiency to be calculated. This is similar for all glucose concentrations at 62% but decreases at temperatures above 39°C.

The first observed report of the use of α-methyl glucoside, α-MG, as a carbon and energy source by K. aerogenes as a result of training is of importance, as this compound is widely used in the study of transport mechanisms. The energy budget for the growth of α-MG-trained cells with α-MG as the sole carbon source provides an anomalous situation. At most α-MG concentrations the negative value of $\Delta H_{\alpha\text{-MG}}$ exceeds the theoretical maximum, while the calculated and experimental stoichiometric coefficients for CO_2 are in poor agreement. The results indicate that α-MG is not metabolised in the same manner as glucose. A feature of the p-t trace for growth in α-MG-limited medium is that the maximum power output occurs before the end of exponential growth, while changes in the other growth parameters (CO_2, pO_2) occur at the end of growth. The out-of-phase relationship is considered to be a function of metabolic activity and not due to oxygen limitation. Interpretation of the thermal data is hindered by a lack of knowledge

of the processes involved in the metabolism of α-MG.

The enthalpy associated with the production of cellular material, ΔH_p, does not vary significantly with glucose concentration, the mean value is -355 kJ (mol cell)$^{-1}$. This is equivalent to a mean specific heat output of -14.2 kJ (g cell)$^{-1}$. ΔH_p varies with the nature of the substrate, the condition of the inoculum and type of growth. In batch culture the heat output per generation, ΔH_g, varies with substrate, the condition of the inoculum and type of growth. In batch culture the heat output per generation, ΔH_g, varies with substrate concentration and temperature. For glucose concentrations 0.56 to 5.4 mmol dm^{-3} (at 37°C) the mean value is -2.6 kJ (g gen)$^{-1}$. With increasing specific growth rate ΔH_g tends to a lower negative value but never approaches that of cells growing in chemostat cultures at similar growth rates. Data accumulated during the growth of cells in media of different glucose concentrations and at different growth temperatures plotted as ΔH_g against Y_c, show that the value of ΔH_g increases linearly with increase in yield. Extrapolation of the data gives values for the theoretical maximum heat output and yield of 12.7 kJ (g cell)$^{-1}$ and 0.56 g g^{-1} respectively.

Extrapolation of the plot of ΔH_g against the number of generations for aerobic growth in glucose-limited medium gives a value of ΔH_g = -7.07 kJ (g gen)$^{-1}$ and a maximum number of generations as 8.62. If the theoretical maximum number of generations is that necessary to give the theoretical maximum yield then the ΔH_g value represents the efficiency of substrate conversion, ie. 55% of the available glucose energy. The slope of the line, 0.82 kJ (g gen)$^{-1}$ (or 0.05g glucose g^{-1} gen^{-1}) represents a maintenance energy term. Consideration of the doubling time gives a maintenance coefficient

of 0.066g glucose (g cell h)$^{-1}$. This is the first reported maintenance energy value for cells growing in batch culture. A similar value was also obtained for growth of α-MG-trained cells in α-MG media. The ΔH_g data and specific power output profiles provide an explanation as to why continuous (chemostat) growth is more efficient than batch growth.

The presence of excess oxygen during growth in glucose-limited medium caused a reduction in the yield and CO_2 output. The enthalpy changes were markedly less than those recorded for normal aerobic growth, the reason for this is unknown, but a theory is advanced. The addition of a second carbon source to the standard glucose medium did not affect the maximum power output, but the profile of the p-t trace was altered. The change in the profile was dependent on the carbon content of the second carbon source. Compounds containing 4 or less carbon atoms produced late secondary bursts of heat output while those with 4-6 carbon atoms produced a shoulder on the decay side of the single peak. Consideration of yield coefficients indicates that the efficiency of use of the available carbon is reduced when 2 carbon sources are present, the heat output being increased.

Glucose-trained cells did not metabolize α-MG when present as the only carbon source; no heat output was observed. Training of cells to α-MG as the sole carbon source was only possible after a series of sub-cultures in which the initial glucose concentration was reduced to zero. When α-MG was added to glucose-limited medium (glucose-trained cells) the maximum power output was lowered and the p-t profile altered; the shape depended on the α-MG concentration. Carbon dioxide output was increased, but the cell yield and heat output did not exceed those possible from glucose metabolism alone; there was a significant increase in the cell volume.

When α-MG trained cells were grown with glucose as the sole carbon source their growth rate was greater than that of glucose-trained cells and the thermal properties changed. The heat output increased above that calculated from mass-balance data. During mixed substrate experiments with glucose- and α-MG-trained cells diauxic growth occurred and glucose was metabolized first. The thermal results suggest that the uptake of α-MG and/or its subsequent metabolism is energy dependent for which a second energy source is needed. Since the affinity of α-MG-trained cells for glucose is increased it is possible that a second, inducible, metabolic pathway for α-MG metabolism has been developed which is not purely specific to α-MG, a modified metabolic pathway is suggested. Support for this comes from metabolic inhibitor studies, azide inhibits growth in glucose but not in α-MG.

Metabolic inhibitors (azide, DNP and FCCP) all had a profound effect on the p-t trace profile; that of sodium azide was very different to those of DNP or FCCP. Heat output in the presence of inhibitors was increased and biomass production decreased. The ATP content of the cell was markedly reduced upon addition of FCCP.

Cells trained to K^+-limitation, with excess glucose present, exhibited similar thermal and heat output parameters as for growth under glucose-limitation until K^+-depletion of the medium occurred. The remaining glucose was then slowly fermented with much heat dissipated. The addition of valinomycin reduced heat output and increased biomass production, indicating that valinomycin permits more efficient use of the available K^+.

SYMBOLS

Y_{sub}, Y_{gluc}	Yield of cells (g substrate)$^{-1}$ initially present.
Y_m	Yield of cells (mol substrate)$^{-1}$ initially present.
pO_2	Oxygen tension, % saturation
m	Biomass (dry weight),
m_r	Empirical formula wieght of cells
α-MG,	1-O-methyl-α-D-glucopyranoside
K	Rate constant
Δr, Δt,	Mean residence time of cell in calorimeter flow cell and transit time from culture vessel to flow cell (2.2.6).
CO_{2_p}	Carbon dioxide output as a function of biomass, equation 2.8.
$CO_{2_{gluc}}$, $CO_{2_{\alpha\text{-MG}}}$	Carbon dioxide output as a function of substrate concentration, equation 2.9.
p-t trace	Power-time trace
q, q(exp), q(tot)	Measured heat output, during exponential growth or until the p-t trace baseline is re-attained, for 1.16 cm^3 culture
ΔH_p, ΔH_{sp}	Enthalpy change accompanying the production of 1 mol or 1 g of cellular material, equation 3.1.
ΔH_{sub}, ΔH_{gluc}	Enthalpy change accompanying the consumption of 1 mol substrate, equation 3.2.
ΔH_{carbon}	Enthalpy change accompanying the consumption of 1 mol of substrate carbon.
ΔH_g	Enthalpy change accompanying the production of cellular material during one generation time, (4.2.1).

ΔH_B	Enthalpy change accompanying anabolic processes (biosynthesis), (9,10).
ΔH_{ox}	Theoretical enthalpy change accompanying the consumption of 1 mol substrate, (4.2.2).
ΔH_s, ΔH_c	Heat of combustion of substrate and cells respectively.

CHAPTER ONE

INTRODUCTION

1.1 Properties of Klebsiella aerogenes

K. aerogenes is a rod-shaped, Gram-negative, non-motile, non-sporing facultative anaerobe. The optimum growth temperature is 37°C, it readily grows in salts media supplemented with an organic carbon source. It is catalase-, KCN- and Voges-Proskauer-positive, methyl-red- oxidase- and phenylalanine-negative, it can reduce nitrate. Sugars are fermented with the production of gas, the fermentation pattern is to 2,3-butanediol. The latest taxonomic classification is given in Buchanan and Gibbons (1974).

The organism was chosen because of the immense bank of data accumulated on its nutritional requirements and metabolic processes. A limited amount of both qualitative and quantitative calorimetric work has been performed with this species.

1.2 Nutrition

K. aerogenes can obtain both energy and carbon from simple carbohydrates provided other essential nutrients are present (Tempest et al., 1965; Dicks et al., 1966); a wide range of carbon sources has been studied (Dean and Hinselwood, 1966; Hadjipetrou and Stouthamer, 1965). Generally, on transfer from glucose media the organism requires little or no adaption for growth on other carbohydrates; although for these carbon sources it does, glycerol and acetate; while some carbon sources will not support growth eg. formate or oxalate. The products of metabolism for aerobic growth depends on the carbon source.

Normally glucose is converted to pyruvic acid via the Embdem-Meyerhof pathway (glycolysis). Pyruvic acid then enters the Tricarboxylic acid (TCA) cycle. Oxygen is not required for glycolysis but it is for the conversion of pyruvic acid to useful products; under fully aerobic conditions the products are cells and CO_2. Dagley et al., (1951) showed that pyruvic acid accumulates during exponential aerobic growth,

in batch culture disappearing rapidly in the stationary phase. There is also evidence that added TCA cycle intermediates are probably converted to pyruvic acid before utilization.

Pyruvic acid undergoes oxidative decarboxylation to form acetyl- CoA; this then enters the TCA cycle. The net products of the TCA cycle are 2 moles CO_2, 1 mol ATP, 3 moles NADH and 1 mol $FADH_2$ per mol pyruvate. Reduced NAD and FAD formed during glycolysis and in the TCA cycle are oxidised by the electron chain; the oxidation of $FADH_2$ and NADH results in the production of 2 and 3 moles of ATP respectively. Overall 38 moles ATP are produced per glucose molecule; ATP is the energy coinage of the cell; 34 mol are produced by oxidation-phosphorylation (Stouthamer, 1978). The coupling of substrate oxidation and ATP formation has to be maximal for most efficient growth.

Oxygen is required for cell respiration as the final electron acceptor, it is also incorporated into cellular material. Cellular mechanisms influenced by oxygen are numerous; K. aerogenes adjusts its metabolism to the level of available oxygen. Harrison and Pirt, (1967) identified three phases of metabolism depending on the oxygen tension of the medium. When the oxygen tension was below 15% saturation anaerobic fermentation occurred. At very low oxygen tensions (<1%) low yields were obtained but increased oxygen uptake occurred although there was no increase in the extracellular organic carbon (Harrison and Loveless, 1971a,b).

Nitrogen is utilized by the cell to produce amino-acids, purines and pyrimidines. Harrison and Maitra (1969) suggested that adenine nucleotides may play a role in controlling respiration, cultures growing at low oxygen tensions showed an oscillatory respiration rate which was modified by adenine nucleotides. Proteins are specifically required for the coupling of phosphorylation of ADP to the electron flow in a number of heterotrophic bacteria (Baccarini-Melandri et al., 1970).

K. aerogenes, when grown in simple salts medium in which an ammonium salt provides the sole nitrogen source, employ a special mechanism for amino-acid synthesis (Tempest et al., 1970). The activities of enzymes in the TCA cycle vary according to the ammonia content of the medium (Brown et al., 1976).

Potassium is accumulated to high intracellular concentrations by many bacteria. It is important in certain enzyme-catalysed reactions (Dixon and Webb, 1964), especially those involved in phosphate transfer (Lardy, 1951); it also frequently participates in carbohydrate metabolism and respiration (Eddy and Hinshelwood, 1951). More recent theories link potassium ion gradients to energy coupling mechanisms (Harold, 1972).

In chemostat cultures of K. aerogenes with K^+- limitation a linear relationship exists between K^+ concentration and biomass production; the line did not pass through the origin; indicating that growth could be supported in the absence of K^+ (Tempest et al., 1966). Dicks and Tempest (1966) presented evidence that suggested K^+ and its balance with Mg^{++}, RNA and P is important in maintaining the functional state of ribosomal particles.

1.3 Transport Processes

It is self-evident that since the bacterial membrane is impermeable to the majority of polar molecules, eg. nutrients and metabolites, uptake is usually a matter of transport, rather than permeability. Transport depends upon interaction of the substrate with a component of the membrane, the component usually having some specificity towards the substrate, which translocates it across the membrane.

Microbial transport systems appear to involve protein molecules firmly embedded in the membrane, eg. the M protein of the β-galactoside transport system of E. coli (Kaback, 1972). Some results indicate that

the substrate combines reversibly with a carrier, the complex is then "mobile in" the osmotic barrier. Large apparent concentration gradients can be achieved, eg. sugars may attain a concentration of 0.1 - 0.2 mol dm^{-3} in the cytoplasm. Such high concentrative uptake implies the performance of work by the cells and coupling of the carrier centres with metabolic energy.

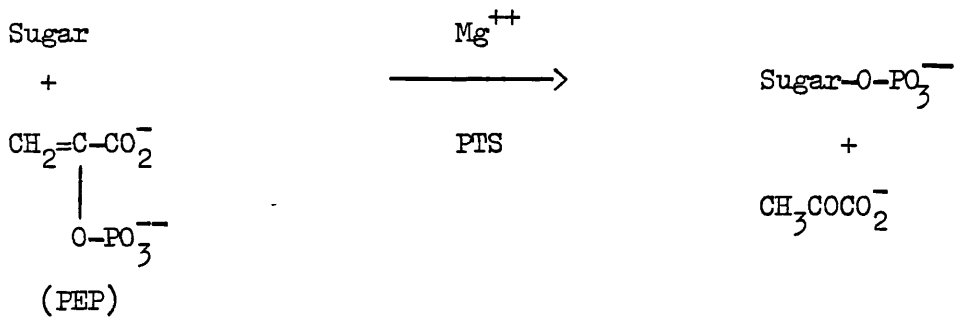
1.3.1 Sugar Transport

Sugars may be transported by "group translocation"; the substrate is chemically altered as it passes across the membrane. Thus it is a chemical group which transverses the membrane (Roseman, 1969). The most highly favoured and best understood transport system is vectorial phosphorylation of sugars; although, even here much remains unknown and conflicting reports are quite frequent.

The bacterial phosphotransferase system (PTS) was first identified in 1964, since then it has been found in widely different bacteria (Romano et al., 1970). The general sequence accounts for both transport and the initial step of sugar dissimulation. Evidence has been presented that the non-metabolizable analogue of glucose, α-D-methyl glucose (α-MG), is transported by the same mechanism as that for glucose. It is believed that glucose is transported across the membrane by the phosphoenolpyruvate: glucose phosphotransferase system; phosphoenolpyruvate (PEP) is the phosphate donor to the sugar. Most monosaccharide phosphate esters are the 6-phosphate esters; α-MG-6-phosphate has been identified in bacteria as well as the free sugar (Kessler and Rickenberg, 1963). A simple model to explain the PTS has been given by Roseman (1969). For Gram-negative organisms (K. aerogenes) the system consists of three protein fractions; Enzyme I, Enzyme II and a low molecular weight histidine- containing protein (HPr). Enzymes I and HPr are located in the cytoplasm and are not inducible; Enzyme II is located

in the membrane and is inducible; other protein components have been detected but not identified. It is thought that the sugar is phosphorylated as it transverses the membrane mediated by Enzyme II (Fig. 1.1).

FIGURE 1.1 Bacterial phosphotransferase reaction



Experimental evidence suggests that α-MG uptake only occurs in the presence of an oxidisable carbon source (Rapoport and Hagueuaver, 1971); it is generally reported that α-MG is excluded from steady-state cells upon addition of a second carbon source (Kessler and Rickenberg, 1963; Hoffee et al., 1964). These results indicate that an added energy source will increase translocation and exit processes of the sugar.

Hermandes-Asensio (1975) reported that inhibition of the transport of α-MG is mediated through energy derived from respiration, work with E.coli mutants have shown that cyclic-AMP is required for active transport. The uptake of K^+ is closely associated with sugar transport in Streptococci (Luoma and Tuompo, 1975). Evidence is accumulating that the PTS is more complex than thought with coupling mechanisms to metabolic energy and ion movements.

That the PTS is not the universal mechanism for sugar transport has been shown by two observations. In E. coli accumulation of β-galactoside is blocked by uncouplers whereas accumulation of α-MG is enhanced. Uncouplers stimulate PEP production (Kaback, 1970), this would seem to exclude coupling of the uptake of α-MG to the energy production of the cell. Barnes and Kaback (1970) have demonstrated that uptake of β-galactosides can be coupled directly to respiration under conditions that virtually exclude PEP.

1.3.2 Energy Coupling and Transport

Long et al., (1975, 1977) showed that active transport of α-MG or β-galactoside by E. coli (chloramphenicol-treated cells) released heat, further the kinetics of heat production varied with the type of transport system (the lactose or glucose system). Djavan (1980) showed that the addition of either sugar to growing chemostat cultures of K. aerogenes growing in glucose-limited media did not cause any significant change in the steady-state heat output. Many studies have been performed on chloramphenicol-treated resting cells; chloramphenicol inhibits protein synthesis, but it inhibits other enzymes too. Since sugar transport and dissimulation are connected to protein synthesis or inducible enzymes, the addition of inhibitor does not necessarily have a passive role with respect to those processes being investigated. Nevertheless the thermogenesis reported indicates that some energy was expended, the energy probably being derived from the maintenance store. When E. coli was subjected to carbon starvation conditions, in the presence of α-MG, uptake occurred and metabolic energy was wasted on fruitless transport processes, ie. ATP is not produced from α-MG, (Koch, 1971). When o-nitrophenyl galactoside was added it was

not hydrolysed, addition of trace amounts of glucose permitted hydrolysis. This implies that at least minimal metabolism or energy store is required to maintain a functional PTS.

Three conclusions have been made which bear directly upon the coupling of transport to energy generating metabolism (Kepes, 1971): (i) the exit of galactosides is a carrier-mediated process, probably the same site as for entry but a different mechanism; (ii) there is no evidence that phosphorylation or other modification of β-galactoside occurs during translocation, and (iii) coupling to metabolism is necessary only for accumulation against a concentration gradient.

Koch (1964) first postulated that β-galactoside transport occurs by facilitated diffusion with facultative energy coupling. This has been confirmed under a variety of conditions, primarily with metabolic inhibitors (azide, DNP, FCCP). The action of uncouplers is not solely confined to one specific action, a fact which must be borne in mind when interpretating results of inhibition studies. The effect of uncouplers on α-MG transport provides an anomalous situation and creates difficulties in presenting a general mechanism for translocation-energy coupling with the chemiosmotic theory of energy transformation and conservation (Harold, 1972).

Support for the proposal that energy coupling converts a facilitated diffusion system into an active transport system comes from the isolation of a mutant deficient in the energy-coupling step (Wilson et al., 1970). Kepes (1971) postulated that energy-coupling involves a covalent reaction with the energy donor and permease protein, thus forming an energized form of the donor. The identity of the energy donor is unknown, but ATP has been forwarded as a candidate. ATP can enhance β-galactoside translocation (West, 1969; Liberman and Topaly, 1968),

Other results indicate that the action of ATP may be an indirect one. Much more information is required about the intermediates present and how these are affected by inhibitors before the mechanism of energy coupling is fully understood.

1.4 Ion Gradients and Energy Coupling

The hypothesis has been forwarded that ion gradients are the link between transport and metabolism. This requires that the metabolic pathway catalyzes reactions which are oriented across the membrane thereby generating gradients of metabolites. Ions carrying an electrical charge are more favoured to serve energy coupling than are gradients of neutral molecules. Primary ion gradients support transport of metabolites against an electrochemical gradient by means of secondary carriers, these have an affinity for both the coupling ion and the particular metabolite. In the case of a symport, the ion and substrate are translocated in the same direction. The driving force on the ion could be electrical or due to a concentration gradient, this brings about active transport of the substrate molecule.

The ions most likely to be involved in coupling mechanisms and transport are H^+, Na^+ and K^+. All bacteria accumulate K^+ and it is possible that some of the potential energy stored in the gradient may be utilized to accumulate other metabolites. Many investigations have noted the apparent need for K^+ for various transport processes (Abrams, 1959; Packer and Perry, 1966; Wong et al., 1969), although the function of K^+ is not known. Much study is required into the role of K^+ and Na^+ in energy coupling processes in bacteria.

The gradient of protons was first considered as a linkage between transport and metabolism by Mitchell (1963). Mitchell observed that DNP inhibited the uptake of galactose and galactosides by E. coli and discharged any stored sugar. From this he postulated transport systems

as H^+-sugar symport and attributed the effect of uncouplers to their ability to facilitate diffusion of protons across lipid membranes. Mechanisms by which protons serve to couple metabolism to the uptake of nutrients have been developed by Mitchell (1970a,b).

1.5 Utilization of Energy for Growth and Maintenance in Batch Culture

The energy requirements of a growing culture are complex, many processes are occurring, each requires a different amount of energy to drive it. Apart from the energy needed by a growing culture, some is required by the cell, during growth or in the resting state, to maintain cell integrity and viability, this is referred to as the maintenance energy. This is shown by Bacillus species; if aeration of the culture is suddenly interrupted or the carbon source depleted the cells undergo autolysis; turnover of the cell wall material and the presence of ATP is essential for maintenance (Hadjipetrou and Stouthamer, 1963; Schulp and Stouthamer, 1970). For Bacillus species a large amount of the maintenance energy is required for cell wall biosynthesis; however, in other bacteria the energy requirement may be very low or non-existant (Higgins and Shockman, 1971).

Calorimetric measurements have been made to determine the enthalpy changes associated with anabolic processes; these were initially found to be negligible or smaller than the experimental error (Forrest, 1972), although recently Dermoun and Belaich (1979) reported a value of -137 kJ mol^{-1} for growth of E. coli on succinate.

Pirt (1965) derived an equation to calculate the maintenance energy from molar growth yields of bacteria growing at various specific growth rates in chemostat culture. At higher growth rates, the relative amount of energy needed for maintenance will be smaller than at lower growth yields. The amount of energy source used for maintenance is,

by the original definition, independent of growth, but it has since been concluded that this growth-independent consumption of energy is used for more processes than true maintenance processes.

Stouthamer (1977) modified Pirt's equation in the light of experimental evidence, by splitting the maintenance requirement into two terms, one growth-rate dependent (m_g) and the other growth-rate independent (m_e).

$$q_{ATP} = \frac{\mu}{Y_{ATP}^{max}} + m_e = \frac{\mu}{Y_{ATP}^{max}} + m_g\mu + m_e \qquad 1.1$$

where q_{ATP} is the rate of ATP production, μ the specific growth rate and Y_{ATP}^{max} the maximum yield per mol ATP.

Maintenance studies have been performed on continuous cultures; Hempfling and Mainzer (1975) suggested that the maintenance energy (for E. coli) varied with growth at the expense of different substrates and that lower yields would be obtained. Maintenance energy is also known to vary with temperature (Harrison and Loveless, 1971 a,b).

Growth in batch culture which is not the same as growth in the chemostat, more closely resembles growth in the chemostat where growth is limited by nutrient other than the energy source. It is only in the final stages of the utilization of the energy source that a similar situation exists as in the chemostat under conditions where the energy source limits growth. Stouthamer and Bettenhaussen (1973) concluded, after making various assumptions based on experimental chemostat data, that the influence of the maintenance energy on molar growth yields in batch growth had been seriously underestimated, and that coupling between energy production and growth rate is much tighter than assumed.

Attempts have been made to define the efficiency of growth of bacteria by using various yield coefficients, eg. Y_{ATP}, Y_{O2}, $Y_{av\ e^-}$, Y_{kcal}. Y_{ATP} was thought to be a constant (Bauchop and Elsden, 1960) for different microorganisms. Although the values show a considerable variation many lie close to the mean of 10.7 g per mol ATP for growth of heterotrophs, recent evidence has shown that this may not be correct (Payne and Wiebe, 1978). Y_{ATP} is usually calculated from values of ATP obtained by indirect methods, thus problems exist in the actual value due to difficulties in obtaining a precise value of the amount of ATP produced. Although Y_{ATP} is a useful concept, its value is very susceptable to growth conditions. Much work has been done in relating Y_{ATP} with maintenance energy in chemostat cultures but little in batch cultures.

In systems where the substrate provides both the energy and the carbon source most attention has been given to energy considerations (Payne, 1970). It may be more correct to consider the two together since the cell has to balance the utilization of the substrate so that each requirement is sufficiently satisfied to maintain growth. A review of how different yield coefficients vary with substrate and species is given by Bell (1972); he concluded that it was reasonable to consider the substrate firstly as a source of carbon and secondly as a source of energy.

When the only products are biomass, CO_2 and water the energy yield (Y_{kcal}) can be determined. If the conversion of carbon is the controlling factor the energy available for the formation of biomass is proportional to the heat of combustion of the substrate containing 1g of carbon. Y_{kcal} values for growth on alkenes are very much higher than those for sugars, but yield values are similar. Thus it seems that alkenes are oxidised to a level similar to that of sugars before the energy produced can be coupled to metabolism. The energy derived from the initial oxidation of the hydrocarbon is dissipated as heat.

Payne (1970) concluded that the energy obtained from electron transfer is significantly derived from the electrons available from the carbon atoms; electrons available from hydrogen thus playing a minor role in providing metabolic energy for growth. Mayberry et al., (1968) considered that the study of "budgeting" of electrons in the substrate could provide useful new data for evaluating aerobic growth in minimal media, although to date this has not received much attention.

1.6 Growth in Batch Culture

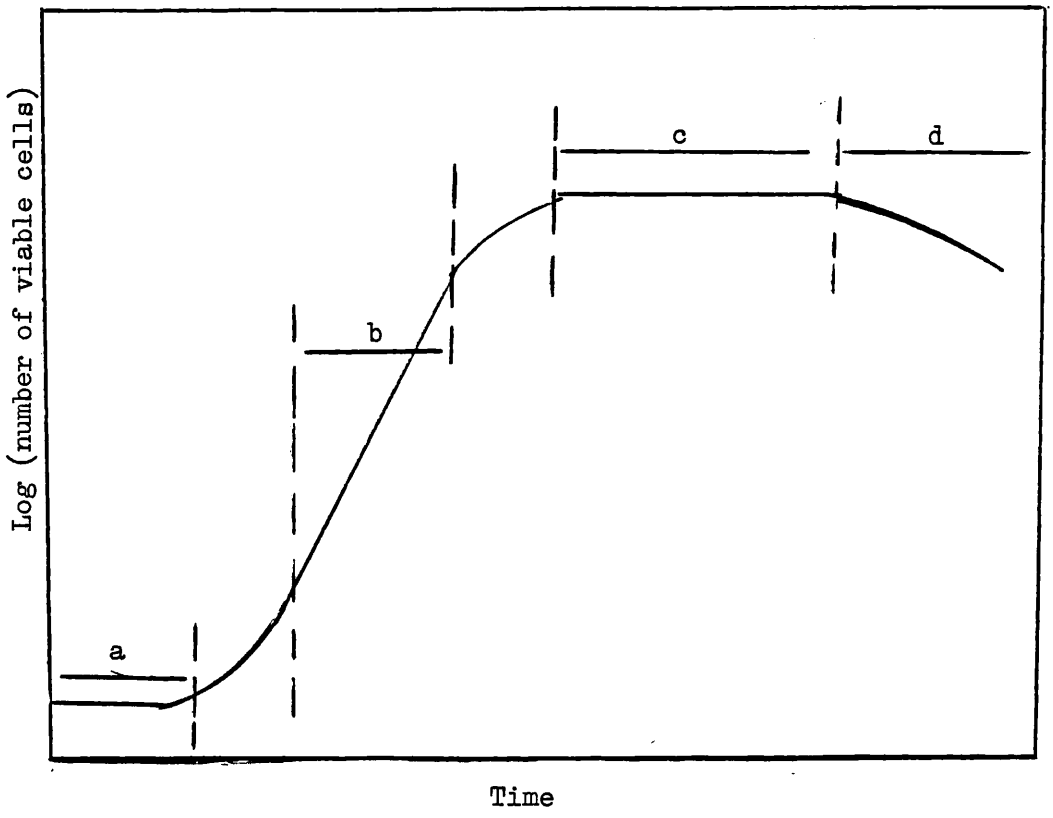
Growth in batch exhibits a characteristic growth cycle (Figure 1.2). As growth proceeds nutrients initially present in the medium, in excess, are reduced in concentration until one is depleted, or the accumulation of toxic compounds, cause growth to cease. The limiting compound can be a nutrient only or the combined energy-nutrient source eg. carbon.

At the beginning of growth there is often a lag phase, during this period no multiplication occurs; the cells adjust to the environment and synthesis of essential intermediates takes place. The length of the lag phase depends on the state and size of the inoculum. The cells then enter the exponential growth phase. Here division occurs at a maximum rate.

$$\frac{d(\ln m)}{dt} = \frac{\log_e 2}{t_d} = \mu$$

The biomass (m) increases with time (t), μ is the specific growth rate and t_d is the doubling time of the culture. The specific growth rate is affected by many environmental parameters, eg. temperature, pH, nutrients available and the presence of inhibitors. The specific growth rate is related to substrate concentration by a Michaelis-Menton type equation (Monod, 1942).

FIGURE 1.2 Typical bacterial growth cycle



a, lag phase
b, Exponential phase
c, Stationary phase
d, Death phase

When exponential growth ceases the cells enter the stationary phase, little or no further growth occurs and cell density is at a maximum; during this period metabolism of some nutrients may occur. When metabolism finishes the death phase is entered, here the viability of the culture slowly diminishes.

1.7 Microcalorimetry

On account of the widely diverse nature of calorimeter instrumentation and applications, consideration will be restricted to the present and potential significance of microcalorimetry in microbiology. During microbial metabolism heat is produced as a waste product, it is this heat that is measured in calorimetry. Such heat changes are small and require calorimeters of high thermal sensitivity ie. microcalorimeters.

1.7.1 Heat Production in Microbial Systems

Chemical reactions in biological systems may be considered to occur in solution at constant pressure and volume, under these conditions thermodynamic differences between enthalpy and energy can be ignored; the energy change which accompanies a reaction becomes the change in enthalpy or heat of reaction. The energy difference is dependent on the initial and final states of the system but independent of the complexity or the pathway of the reaction (the law of Hess).

The calorimetrically measured heat is related to the heat of reaction for a system in which no external work is done. Hence for a given reaction the quantity of heat evolved depends on the number of mols of reaction that have taken place. In a microbial cell many processes are occurring at the same time, the total heat of all the individual processes is recorded; thus microcalorimetry of microbial

systems is non-specific in the heat measured ie. the heat produced cannot, at present, be related to an individual process.

The heat of reaction is an unchangeable quantity, when the entropy change is also known the thermodynamic analysis of a reaction and of its driving force, Gibbs Free Energy (ΔG), is more complete. The increase in calorimetric data during the period 1960-1975 has been to a large extent concerned with determining enthalpy changes of reasonably well defined processes of biological interest. Microcalorimetric data has been presented with a view of contributing information on Prigogines hypothesis (Prigogine, 1956) that living organisms tend to a state of minimum entropy production (Stoward, 1962). Before the introduction of modern microcalorimeters, results were often contested, being based on data obtained from instruments of poor design and reproducibility (Forrest and Walker, 1962; Stoward, 1962).

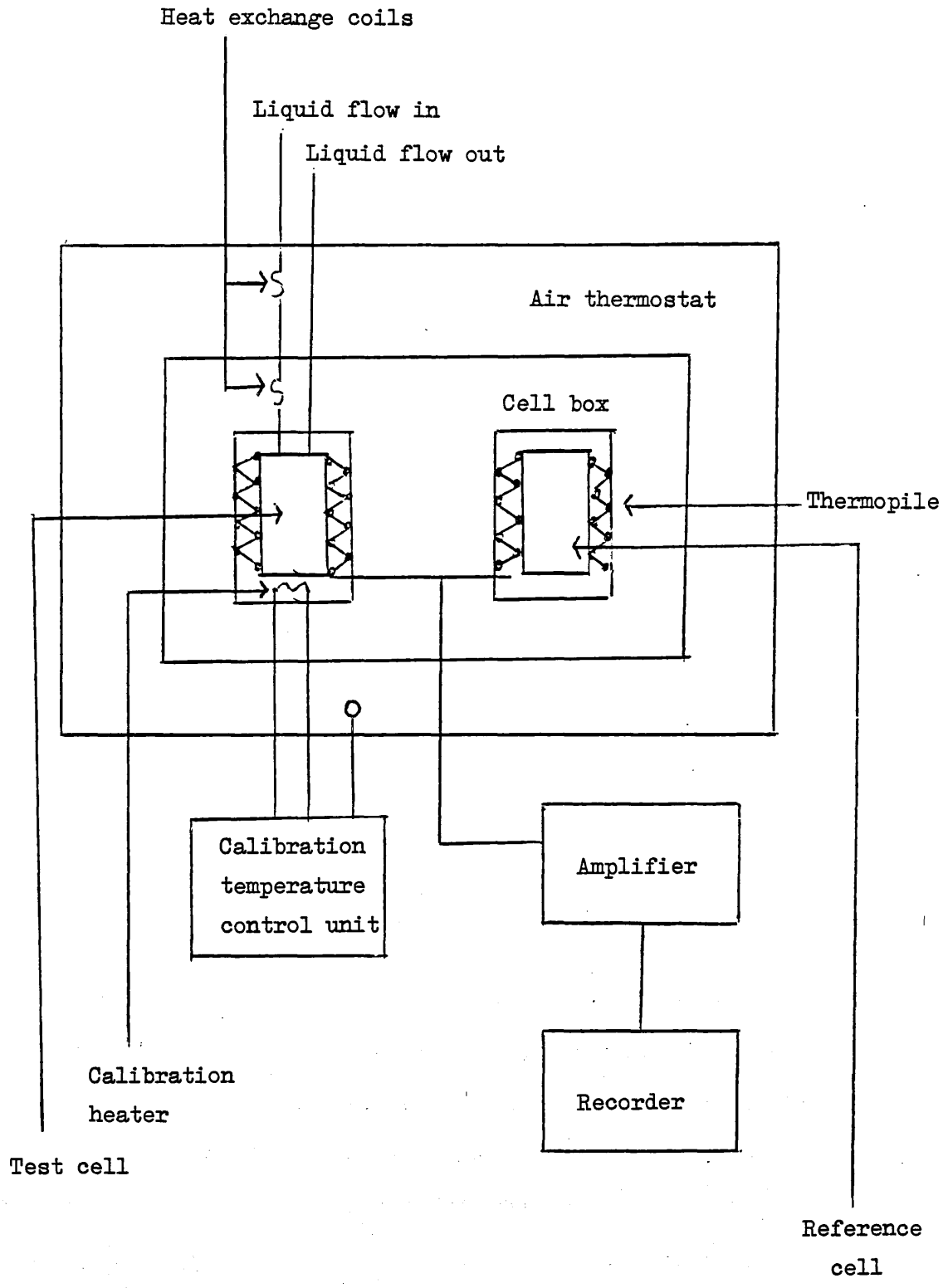
When applied to microbiological systems, due to the non-specificity, it is essential that supplementary biological and/or chemical data be recorded simultaneously with the calorimetric measurement, and preferably on the same system. This is essential if interpretation of the calorimetric data is to be meaningful.

1.7.2 Types of Calorimeter

Calorimeters may be classified according to the characteristics of the heat exchange balance between the calorimeter reaction vessel and the environment in which it is immersed. There are two main catagories of calorimeter in use (a) adiabatic, and (b) heat-conduction calorimeters.

In an ideal adiabatic calorimeter there is no heat exchange between the calorimeter proper and its surroundings. The temperature is kept constant at the calorimeter temperature by means of an electrically

FIGURE 1.3. The essential components of a heat-conduction microcalorimeter.



heated adiabatic jacket which completely surrounds the calorimeter proper. The heat absorbed or evolved is determined electrically by a feed-back system to a reference cell. The disadvantage of this type of calorimeter is that, unless complicated regulartory devices are included in the construction, there will always be leakage of heat to the surroundings. This type of calorimeter has limited application in microbiological studies.

Heat-conduction calorimeters are the principal type used in microbiological studies, and the type used in this work. The heat evolved or absorbed flows through a controlled path from the reaction cell to a heat sink (Fig. 1.3). A large thermostatted air bath is employed to give a constant background temperature. Located in this is a large metal block which acts as the heat sink. The reaction and reference cell are situated in the block, the cells are surrounded by thermopiles, connected in opposition to each other. This twin principle reduces background disturbances to the heat flow. The air bath and metal block also contain heat exchange coils which are required for establishing thermal equilibrium of the flowing bacterial culture suspension before entering the calorimeter cell.

The main thermal considerations that have entered into the design and construction of heat conduction microcalorimeters are: (Goldberg and Armstrong, 1974; Monk and Wadso, 1968) that (i) the thermal contact should be good between thermopile and reaction vessel; (ii) thermal contact should be poor between the heat sink and its surroundings and between the heat sink and reaction vessel; (iii) the temperature of the surroundings of the heat sink should be under control; (iv) the time constant for heat flow between reaction vessel and heat sink should be small; (v) the response of the thermopile should be independent of the reaction of the heat sink within the reaction vessel and (vi) any connections between the heat sink and reaction vessel should be

carefully positioned thermally at both locations.

The electrical output is a measure of the difference in voltage between the thermopiles surrounding the reaction and reference cells and so it is necessary to determine a calibration constant. A common method of calibration is to measure the electrical response for precise known amounts of heat. This is achieved by applying measured quantities of electricity to calibration heaters sited near the reaction cell; in a good calorimeter design the position of the calibration heaters within the mixing zone should not be critical (Monk and Wadso, 1968). An alternative method of calibration is to use a chemical reaction of accurately known enthalpy change, eg. heat of neutralization. This method is not usually employed, due to practical difficulties especially with flow-through cells.

The heat flowing from the cell to the heat sink or vice-versa passes through the thermopiles for each thermocouple the voltage, v_1, and the rate of heat flow, dq/dt are proportional to the temperature difference between the calorimeter cell and heat sink :

$$v_1 = C \frac{dq}{dt}$$

where C is a proportionality constant. Ideally all the heat passes through the thermocouples, therefore for all thermopiles the summed voltage and total heat flow are given by:

$$v_1 + v_2 + \ldots = V = C \frac{dQ}{dt} \qquad 1.3$$

The proportionality constant can be replaced by an effective value C_1 (Wadso, 1968) which is obtained by calibration; substitution of C_1 in equation 1.3 and integration gives

$$Q = \frac{1}{C_1} \int V dt \qquad 1.4$$

The total heat evolved is therefore proportional to the area under the voltage-time curve

$$Q = E \times \text{area}$$

where E is the calibration constant (2.2.1).

1.7.3 Presentation of Culture to Reaction Cell

A microcalorimeter may be used in one of several modes depending on the manner of mixing the culture suspension in the reaction cell. The most important types in microbiological work are batch and flow microcalorimetry.

Until the late 1960's most calorimeters were of the batch type. The closed calorimeter cell of limited volume (1-100 cm^3) may be one of two types. In the first type the reaction cell is divided into two compartments, the reactants are kept separate until brought together by mixing, this is achieved by rocking and rolling the entire heat sink assembly, i.e. the reaction and reference cells. This batch vessel is not convenient to use with microorganisms, since it is difficult to aerate or perform any manipulation on the culture. This type of batch calorimeter has been used to study interactions between resting bacteria and antibiotics (Ping, 1977).

The second type of batch cell consists of a single vessel in which the culture is grown. Early forms had similar restrictions as the first type; in that they were only suitable for anaerobic studies of short duration (Belaich and Belaich, 1976a,b). In 1961, Forrest designed a batch calorimeter in which the reaction vessel (200 cm^3) could be aerated and with provision for sample removal. This type suffered from cooling effects due to aeration of the culture, and also

when samples were removed the biomass (hence heat output) was reduced at the point of heat measurement. Never-the-less this was the first system which allowed the continuous study of microbial growth. More recently other types based on this design have been used, usually of smaller volumes allowing better aeration (Fujita et al., 1972; Dermoun and Belaich, 1979).

A microcalorimeter which has found many applications in batch studies is the Tian-Calvet instrument, an isothermal-twin calorimeter of the heat-flow type. A commercial model is available based on the design of Wadso (1968), it has a two compartment cell. Generally, the instrument is superior to others due to a number of unique features (Sturtevant, 1972). It has very good stability but requires more careful attention when cleaning the calorimeter cells.

Flow-microcalorimetry of microorganisms involves flowing the culture continuously through the reaction cell. The culture, growing outside the calorimeter, can be of unlimited volume since only a small volume flows through the calorimeter (<5 cm^3). The culture may be discarded, collected or recycled after passing through the reaction cell. Measurements, tests and other manipulations are possible on the culture without disturbing the thermal measurements.

The first successful flow-microcalorimeter was designed by Stoesser and Gill (1967); Monk and Wadso (1968) designed one specifically for microbiological studies based on a batch design, since 1968 several different designs have been reported (Wadso, 1976). In microbiological studies a number of factors can affect the results obtained, and much attention has been given to calorimeter sensitivity, stability and overall design. Unfortunately less attention has been paid to the experimental design and culturing conditions of the microorganisms. The lack of precise knowledge on how each set of conditions affects the

calorimetric measurement is often indicated in the literature; although, in general, when work has been done on the influence of experimental parameters on the results there is often a lack of control experiments or insufficient replicate experiments to determine the significance of the data.

The flow rate through the calorimeter cell has to be constant and known with high accuracy; thus the delivery device to the reaction cell has to be reliable. A number of delivery devices are available (Sturtevant, 1972; Picker et al., 1978). The temperature of the culture has to be equilibrated to that of the calorimeter before it reaches the reaction cell, temperature equilibrium is very important otherwise artefacts may appear in the recorded heat output. For aerobic cultures a compromise has to be made between a flow rate which allows thermal equilibrium and one which permits aerobic conditions to be maintained. Ideally the culture in the calorimeter cell should be in the same state as that in the external culture vessel. this goal is slowly being achieved by new types of calorimeter designs, but problems exist at the moment (Eriksson, 1980).

The flow rate affects the calibration constant (Monk and Wadso, 1968) because heat is carried away from the reaction cell by the suspension, this implies that heat can also be lost on the way to the reaction cell to the surroundings. Although the flow rate is important it is probably better to consider the transit and residence times, ie. the time taken for the culture to reach the reaction cell and the mean time a bacterium remains in the reaction cell respectively (3.3). With high flow rates and transit times <1 min temperature equilibrium may not be established, while at very low flow rates with transit and residence times >2 mins and >1 min respectively although thermal

equilibrium is established depletion of a substrate may occur in the flow line long before it does in the culture vessel.

In chemostat studies a situation of two limiting nutrients could seriously upset the steady-state growth producing an erroneous thermal response. It had been assumed that less heat will be measured than actually produced in the chemostat vessel because of the finite transit time (Ackland et al., 1976; Cardoso-Duarte et al., 1977); Djavan (1980) was able to extrapolate the heat output measured in the reaction cell to that of the culture at zero transit time, ie. in the chemostat.

For aerobically growing cultures reaction cells capable of handling mixed flow of air and culture have been developed (Eriksson and Wadso, 1971; Spink and Wadso, 1976). Such cells are useful if sedimentation is a problem and with yeast studies (Gustafsson and Lindman, 1977). They also reduce the risk of bacterial growth on the walls of the reaction cell which can occur in experiments of long duration or with high biomass levels. Aerobic flow cells were primarily designed to reduce thermal disturbances which occur from gas bubbles, transported with the culture, as they pass through the reaction cell. Also in the reaction cell vaporization may occur and CO_2, produced by metabolism, will go into solution; in extreme cases this can affect the measured thermal response.

1.7.4 Application of Microcalorimetry to the Study of Microbiological Systems

For complex biochemical and microbiological processes it may sometimes be advantageous to use a non-specific method by which an overall value of some property for the process(es) can be obtained. Unknown or unexpected phenomena might thereby be revealed which would not have been by a more specific technique. The flow-microcalorimeter

has proved to be a general analytical tool in the field of biochemistry. Applications involve enzyme kinetics (Tribout et al., 1976); enzyme assay (Yourtee et al., 1975; Beezer et al., 1974); determination of substrates (Goldberg and Armstrong, 1974) and investigations on living cells (Monk and Wadso, 1983). Some of the earlier work in this field has been reviewed by Levin (1977), Spink and Wadso, (1976) and Goldberg and Armstrong (1974).

Microbiological applications have increased in the last decade, particularly in the study of heat phenomena associated with the growth of bacteria. Early work has been reviewed by Calvet and Prat (1963), Brown (1969), Forrest (1972) and Belaich (1980). The following is an appraisal of the major applications reported during the last decade; the emphasis is on bacterial studies.

In a growing culture the overall heat flux arising from the catabolic and anabolic processes is recorded as a function of time and yields a power-time trace (p-t trace; thermogram). The deflection on the p-t trace is proportional to the intensity or the rate of the reactions, the integral gives the algebraic sum of the heats absorbed and evolved. The general shape and profile of the p-t trace can be interpreted in relation to changes in other biological and/or chemical properties, while the heat output for a given time interval can be determined from the area under the p-t trace for that time.

1.7.5 Characterization and Enumeration of Cultures by Microcalorimetry

Research in this field was not very active until Boling et al., (1973) published a short communication on bacterial identification, since then there has been more work in this field, less work has been performed on the characterization of yeasts.

Characterization and identification are based on the fact that microbiological metabolism is very complex, has a multiplicity of metabolic pathways and is dependent on the medium. This led to the view that p-t traces would be species-specific and this could be used for identification.

There are, however, a number of important points to consider. The metabolism of an organism, which strongly depends on the cultural conditions and nutrient supply, can shift from one metabolic pathway to another as a result of a small change in cultural or experimental conditions, this in turn can produce a distinct change in the profile of the p-t trace. Further it is often the case that different bacteria produce similar p-t traces, this is especially true when simple metabolic processes are occurring.

There are thus two basic requirements to be satisfied for identification: (1) the growth medium and cultural conditions must be accurately defined so that reproducibility is guaranteed; and (2) the growth medium and cultural conditions must be such that the metabolism is not simple but that individual metabolic pathways for each kind of organism are revealed. The amplitude of the maxima and minima on the p-t trace vary due to the amount of inoculum and also the length of the lag phase associated with the adjustment of the organisms to the growth medium. These are very important factors and, as will be shown later, have been neglected.

In the very few calorimetric investigations on characterisation of mixed populations in simple or complex media (cf. Schaarschmidt and Lamprecht, 1976) it was impossible to recognise individual profiles of any of the different growing organisms. A smoothing of the p-t trace is caused by superposition of the heat profiles of each growing species,

or by the unknown change in metabolism caused by the natural interference between the species.

On balance it appears that it is possible to identify a limited number of bacterial groups under exactly defined conditions, but that absolute identification is not possible. It is claimed that one advantage of microcalorimetric characterization is that results are ready in a shorter time than required by standard methods; but against this the organism has to be isolated before it can be identified. Unless defined conditions are laid down it will be impossible for different laboratories to obtain similar results.

The use of microcalorimetry for the enumeration of microorganisms with first suggested by Beezer et al.,(1974) who reported a method which could differentiate between 10^3, 10^4 and 10^5 bacteria cm^{-3}. Beezer et al.,(1978) published a modified method which could reliably detect 10^3 to 10^5 organisms cm^{-3} of fifteen bacterial strains important in clinical work. The enumeration of Saccharomyces cerevisiae in the range 5×10^4 to 10^7 organisms cm^{-3} (Beezer et al., 1976) and Mycoplasmatales (Ljungholm and Wadso, 1976) have been described. Bettelheim (1975) pointed out the potential of flow-microcalorimetry to identify, to enumerate, to determine antibiotic sensitivities and to distinguish between mixed and pure populations on a routine basis; however much refinement of techniques has to be made before these goals can be met.

1.7.6 Microbiological Metabolism and Related Topics

Most microcalorimetric studies have been qualitative in nature, mostly reporting p-t traces, although in recent years the tend has been towards quantitative measurements (4th Int. Sym. on Microcalorimetry in Biology 1980; abstracts). There have been few reports of the effects of metabolic modifiers on the heat output of microorganisms, although

this field has great potential. Before quantitative measurements can be made and the results interpreted the influence of the experimental conditions must be known so that proper conditions are established which will provide the required data. One of the major variables, for any calorimetric study, is the nature and character of the inoculum. Beezer et al.,(1976) described the preparation of a standard inoculum of two species of yeast by cryogenic storage. This was used in the development of assay procedures for various antibiotics. Until 1979 (Nichols et al., 1979) there had been no reports of the use of "standard inocula" for bacterial work; important advantages of using uniform inocula include good reproducibility of growth and thermal data and ease of handling.

Calorimetric antibiotic assay procedures have several advantages over routine agar plate diffusion methods (Beezer et al., 1977); most work has been with fungal or yeast antibiotics (Beezer et al.,1977; Jensen et al.,1976). Wadso (1976) described the effects of tetracyclines on E. coli. Semenitz and Tieffenbrunner (1976) carried out similar work using an homolytic E. coli. The different mode of action of 5-fluorocystosine from that of polyene has been demonstrated (Beezer et al.,1978). A semi-automated method for testing bacterial sensitivity to antibiotics has been developed for clinical applications (Binford et al., 1973).

Reports of the use of metabolic modifiers with a view of correlating calorimetric data with known physiological investigations have been scarce. Poole and Lloyd (1974) used an analogue of FCCP to uncouple oxidative-phosphorylation in the yeast, S. pombe; the calorimetric measurements confirmed that respiration is most sensitive to an uncoupler when the respiration rate is maximal. DNP has been used in investigations with microalgae (Tillberg et al., 1971) and lactic acid bacteria (Monk et al., 1977). The effects of DNP on heat output changes were correlated with known physiological events. Calorimetric

studies on energy metabolism during anaerobic fermentation of glucose by yeast using cyanide and azide as inhibitors have been made by Hoogerheide (1975).

Another way to modify respiration is to use genetically modified strains or mutants. Poole and Haddock (1975) used E. coli with genetically and phenotypically modified electron transport systems; the calorimetric measurements were correlated with oxygen uptake. p-trifluoromethyloxyphenyl hydrazone was also added to stimulate ATP production. Gustafsson (1979) attempted to relate heat output to ATP content and the osmotic condition of the cell.

In early calorimetric work the culture was grown in the calorimeter while a parallel culture was grown outside the calorimeter to provide metabolic data for comparison with the thermal changes of the other culture. Brettel et al., (1972) first demonstrated the combination of a flow microcalorimeter with an external culture vessel. In the early systems inefficient control of the two cultures would mean invalidated results. Even in modern flow systems some workers are not aware of the rigid control needed on experimental conditions and the necessity to correlate thermal data with basic growth parameters, eg. biomass, substrate concentration, if a meaningful and full interpretation of thermal data is to be achieved.

Eriksson and Wadso (1971) discussed the p-t trace during the aerobic growth of E. coli with reference to substrate and metabolites formed. The oxygen tension in the culture vessel and in the calorimeter flow cell had similar profiles which were inverse of the p-t trace. Few et al., (1976) carried out similar studies with K. aerogenes, here too a correlation between heat output and oxygen tension was apparent.

Attention has been given to studies of growth and metabolism in yeast, Platonov et al., (1970); Lamprecht et al., (1973); Schaarshmidt et al., (1973); Poole et al., (1974); Fujita et al., (1972). Lloyd et al., (1978) have reported some complex metabolic-calorimetric correlations in synchrous cultures of Tetrahymena pyrifermis.

Most calorimetric studies with bacteria have been with heterotrophs. The energy-consuming reactions for biosynthesis substract from the energy yielding reactions of catabolism. It has been stated that for heterotrophic organisms the energy of biosynthesis would not be expected to exceed 3% of the catabolic energy (Lloyd et al., 1978). Forrest (1972) pointed out that in these instances calorimetry mainly measures the enthalpy change associated with catabolism and that the enthalpy change associated with biosynthesis is too small to measure or smaller than the experimental error. However the improvement in experimental design and reproducibility has enabled Dermoun and Belaich (1979) to report an enthalpy of growth for E. coli. For autotrophes this measurement is not so easily attained (Dressers et al., 1970).

Microcalorimetry is ideal for studying a simple degradative pathway for carbohydrates; it has only recently been used for bacteria with complex fermentations, eg. the hexose fermentation by E. coli (Belaich and Belaich, 1976a). Although the p-t trace was complex, it was dependent on hydrogen lyase activity; when this enzyme was inhibited a simple p-t trace was obtained. Belaich and Belaich (1976b) used flow microcalorimetry to measure the affinity of E. coli for substrates. The rate of catabolic activity was generally a hyperbolic function of energy substrate concentration at low sugar concentrations.

The gathering of thermodynamic and/or analytical data has been the primary objective of most investigators while little attention has been paid to kinetics. Much kinetic information can be derived from calorimetric studies which may be performed for other reasons, eg. by suitable analysis of the heat output in the presence of an inhibitor it is possible to calculate the inhibitor constant. In the consideration of enzyme-substrate reactions the value of the Michaelis constant and maximum rate may be determined. A resume of the theoretical results is presented by Beezer (1976) with respect to kinetic data.

1.8 Objectives of this Project

From the literature review it is apparent that there has been no systematic study of the energy changes which accompany the growth of a simple organism under different cultural conditions. This means that there is no thermodynamic data relating to the production of biomass, the degradation of carbon source or the overall energy budget. The work reported in this thesis was undertaken, on a nutritionally simple organism growing in a chemically defined medium, in an attempt to rectify this deficiency and at the same time to show the importance of energy studies in understanding complex metabolic processes. The objectives can be sub-divided:

(1) the establishment of standard conditions for the reproducible growth of K. aerogenes in salts media;

(2) the combination of a culture vessel with a flow microcalorimeter and the establishment of optimum experimental conditions for the quantitative and reproducible measurements of thermal events;

(3) the determination of thermal and enthalpy changes occurring during glucose-limited growth and a study of how these parameters are affected by changed growth conditions, eg. growth and test temperature;

(4) a study of the influence of metabolic modifiers and uncouplers on glucose-limited growth and the relation of thermal events;

(5) an investigation of thermal and enthalpy changes which occur with other carbon sources;

(6) the measurement of thermal parameters when the energy source is not growth limiting.

CHAPTER TWO

EXPERIMENTAL TECHNIQUES

2.1. Bacteriological Methods

2.1.1. Organism

The strain of Klebsiella aerogenes N.C.T.C. 418 was used throughout this work. It is a rod shaped, Gram-negative, non-motile species of the Enterobacteriacea. In recent years the nomenclature and classification has undergone many changes, the current taxonomic position is discussed in Bergey's Manual (Buchanan and Gibbons, 1974).

2.1.2. Media

Oxoid nutrient broth (No.1) was used for maintenance and storage of parent cultures.

Chemically defined media was used for all experimental studies:

(a) Carbon-limited media prepared in glass distilled water of final composition: KH_2PO_4, 26 mmol dm^{-3}; $(NH_4)_2 SO_4$, 8 mmol dm^{-3}; $MgSO_4.7H_2O$, 0.16 mmol dm^{-3} and was adjusted to pH 7.0 with concentrated NaOH solution. Glucose was the main carbon source used, usually within the concentration range 0.55 - 5.6 mmol dm^{-3}. The salts solution and a concentrated glucose solution were sterilized separately at 15 p.s.i. for 20 minutes. The glucose solution was added aseptically to the salts solution to give the required concentration. Other carbon sources used were pyruvate (sodium salt and acid), sodium acetate, sodium oxyketoglutarate, sodium citrate, sodium succinate, ethanol and 1-O-methyl-α-D-glucopyranoside (α-MG).

(b) Potassium-limited salts medium was prepared using deionised water of final composition: $Na_2HPO_4 12H_2O$, 14.0 mmol dm^{-3}; $(NH_4)_2SO_4$, 8 mmol dm^{-3}; $MgSO_4 7H_2O$, 0.16 mmol dm^{-3} and the pH adjusted to 7.0. The necessary glucose (stock solution 0.17 mol dm^{-3}) was added to a final concentration of 3.3 - 6.6 mmol dm^{-3}. Aliquots of stock K_2SO_4 (0.25 mol dm^{-3}) were added to give a K^+ concentration of 0.3 - 5μg cm^{-3}.

Analytical grade reagents were used where available. Salts media, carbon source and K_2SO_4 stock solutions were sterilized separately and added aseptically.

2.1.3. Metabolic Inhibitors

Compounds used were selected on account of their known inhibition of specific processes: carboxylcyanid-p-trifluoromethyl-phenylhydrazon (FCCP), antimycin A, oligomycin, chloramphenicol and valinomycin from Boehringer and sodium azide and 2,4-dinitrophenol (DNP) from B.D.H.

2.1.4. Preparation of Cultures

The strain was maintained by monthly subculture in nutrient broth (10 cm^3 in screw capped universal bottle) and stored at 4°C.

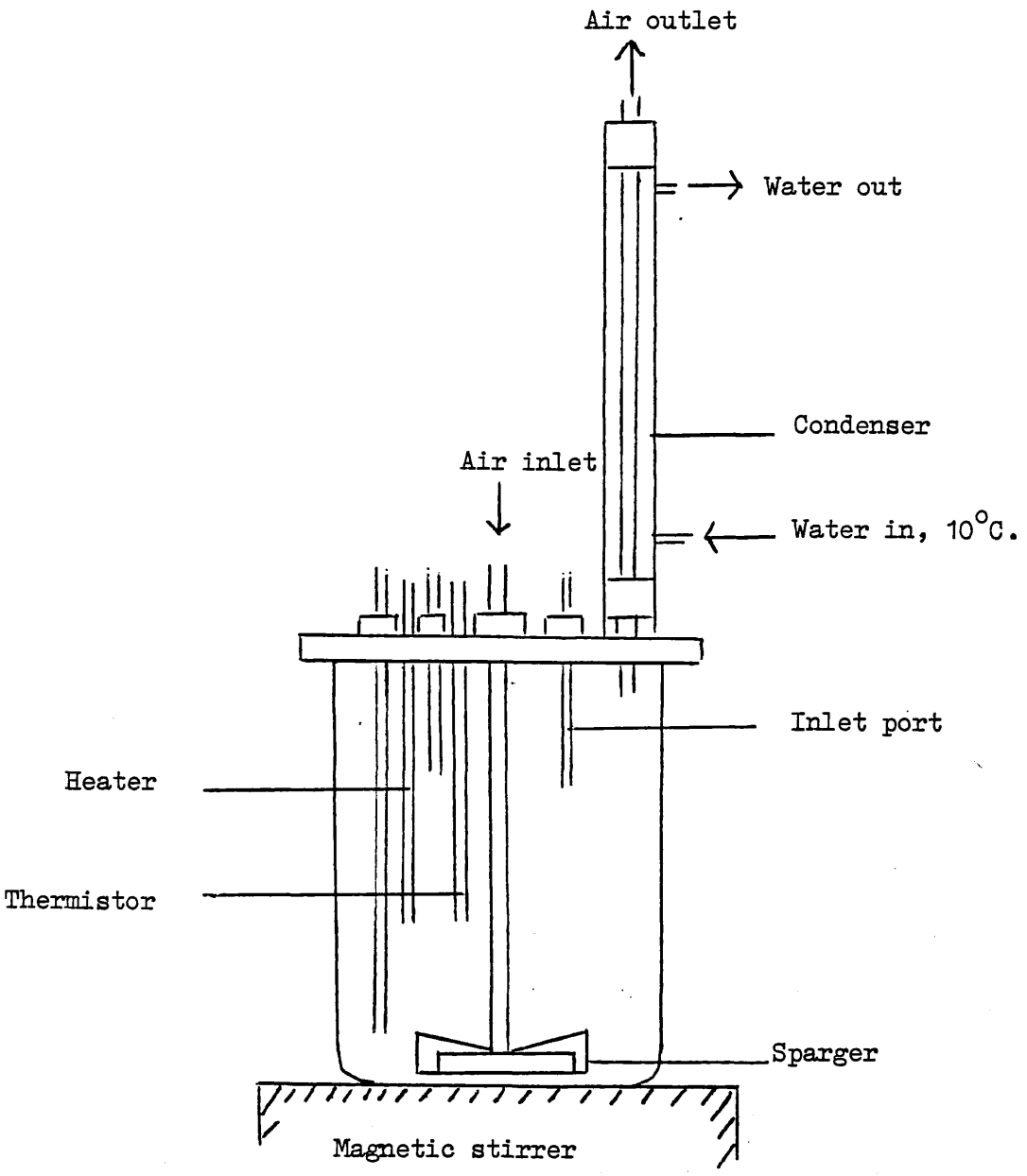
For routine purposes the cells were grown repeatedly in glucose-limited salts medium at 37°C with vigorous aeration. The sixth subculture, when fully grown, was stored in a 25 cm^3 medicine bottle at 4°C as a source of inocula.

Cells were adapted for growth in glucose- or potassium-limited medium by growing cells from the parent stock for a minimum of 15 subcultures in the appropriate medium. The limiting concentrations were: 3.3 mmol dm^{-3} glucose, and 1 μg cm^{-3} K^+ for potassium growth (with 5.6 mmol dm^{-3} glucose). Fully trained cells were characterized by their growth and thermal characteristics.

Cells were adapted for growth at different temperatures by at least three subcultures of parent stock cells in glucose limited salts medium at the required temperature (25 - 42°C).

For the preparation of stored inocula and for experimental work the cells were grown in 600 cm^3 of medium in a 1 dm^3 culture vessel of a Gallenkamp Modular fermentor system (Fig. 2.1). Sterile air was

FIGURE 2.1. Culture vessel



pumped in at the base of a sparger, usually at 1.6 dm^3 min^{-1}. The sparger, a magnetic stirrer operated at 1200 r.p.m. provided vigorous mixing and good dispersion of air bubbles for aeration of the culture. The temperature control was normally at 37.0 ± 0.2°C, unless otherwise specified.

2.1.5. Storage of Inocula for Routine Work

(a) Freeze-dried inocula. Glucose-trained cells were grown in the culture vessel (3.3 mmol dm^{-3} glucose) and harvested by centrifugation approximately 30 minutes before termination of exponential growth. The cells were resuspended and concentrated in 0.75% $^w/_v$ saline so that a $2cm^3$ aliquot would give a cell population of about 10^7 cells cm^{-3} on dilution in 600 cm^3 of medium. The concentrated cell suspension was dispensed into sterile glass ampoules and freeze-dried; the ampoules were septum-sealed aseptically and stored at 4°C. When required the cells were reconstituted by addition of 2 cm^3 medium and overnight incubation at 37°C to rehydrate cells, this suspension was then used as an inoculum.

(b) Cryogenic storage. This is the method which was finally selected for the preparation of the "standard" inocula. The required cells were grown in 600 cm^3 of the appropriate limiting medium. Cells were harvested by centrifugation about 30 minutes before the termination of exponential growth, except the K^+-limited cells which were harvested about 1 hour after exponential growth had finished. The sedimented cells were resuspended in a sterile volume of salts medium (lacking carbon source or K_2SO_4) so that a 1.5 cm^3 aliquot would give a population of about 3×10^7 cells cm^{-3} when diluted into 600 cm^3 of medium. Volumes of 1.5 cm^3 of the concentrated cell suspension were dispensed aseptically into 2 cm^3 sterile screw-capped

glass ampoules.

A batch of ampoules were suspended (in a small basket) just above the surface of liquid nitrogen, in a vivostat, for exactly 30 mins; they were then plunged directly into the liquid nitrogen and stored under the liquid nitrogen until required. When required an ampoule was removed and immediately immersed in a water bath at 37°C. After exactly 3 minutes the ampoule was removed and the cell suspension transferred immediately by a sterile syringe to the culture vessel (already aerated and at thermal equilibrium).

The cooling rate of the cell suspension over the liquid nitrogen was 24°C min^{-1} over the temperature range -10 to -60°C. The rate of cooling is critical for good viability (Beezer et al., 1976).

2.1.6. Purity and Viability of Cultures

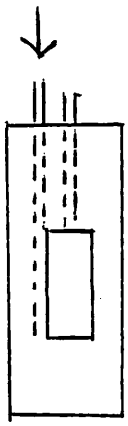
The purity of cultures was regulary checked by plating. Purity and identification were also determined using the A.P.1. 20E system (Appareils et pecedes d' identification, France).

Viability and cell numbers were determined by preparing a serial dilution of the cells in salts medium and plating onto agar. Total bacterial counts were also made microscopically using a haemocytometer.

2.1.7. Washing and Sterilization

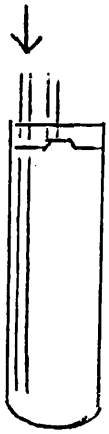
All glassware was washed with tap water and rinsed three times in glass distilled water. Glassware for potassium limitation studies was washed in deionised water. The use of detergents was avoided. Glassware was oven dried at 105°C and plugged with cotton wool where necessary.

FIGURE 2.2. Flow cells



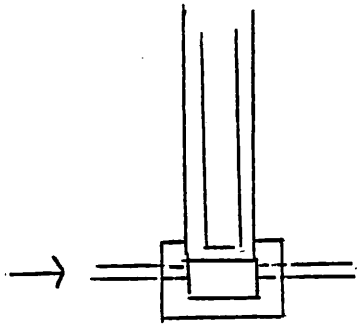
Flow-through cuvette

volume 1 cm^3



Aerobic flow cell

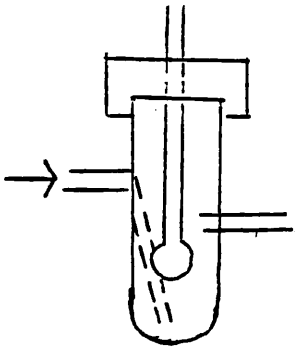
volume 1.16 cm^3



Flow-through oxygen

electrode

volume 0.1 cm^3



pH electrode assembley

volume 1 cm^3

⟶ = inlet

Sterilization of contaminated or clean equipment was performed by autoclaving at 121°C for 30 minutes. The empty culture vessel was sterilized by autoclaving, care was taken to cover the thermister and heater contacts, sterile medium was added aseptically.

2.2. Measurement of Physical Parameters during Bacterial Growth

2.2.1. Power and Heat output

Power-time (p-t) traces for growing cultures were obtained using an L.K.B. 10700-1 flow microcalorimeter fitted with an aerobic steel cell (Fig. 2.2) of 1.16 cm^3 volume. The culture was drawn through the calorimeter cell by a peristaltic pump.

The calorimeter response was calibrated by passing a culture, in which the bacteria had been killed, through the calorimeter cell under the same experimental conditions (i.e. those applying to the culture vessel and flow rate through the calorimeter cell) and applying an internal current of 2.5 mA for 30 minutes to produce a steady state power output (Fig. 2.3.).

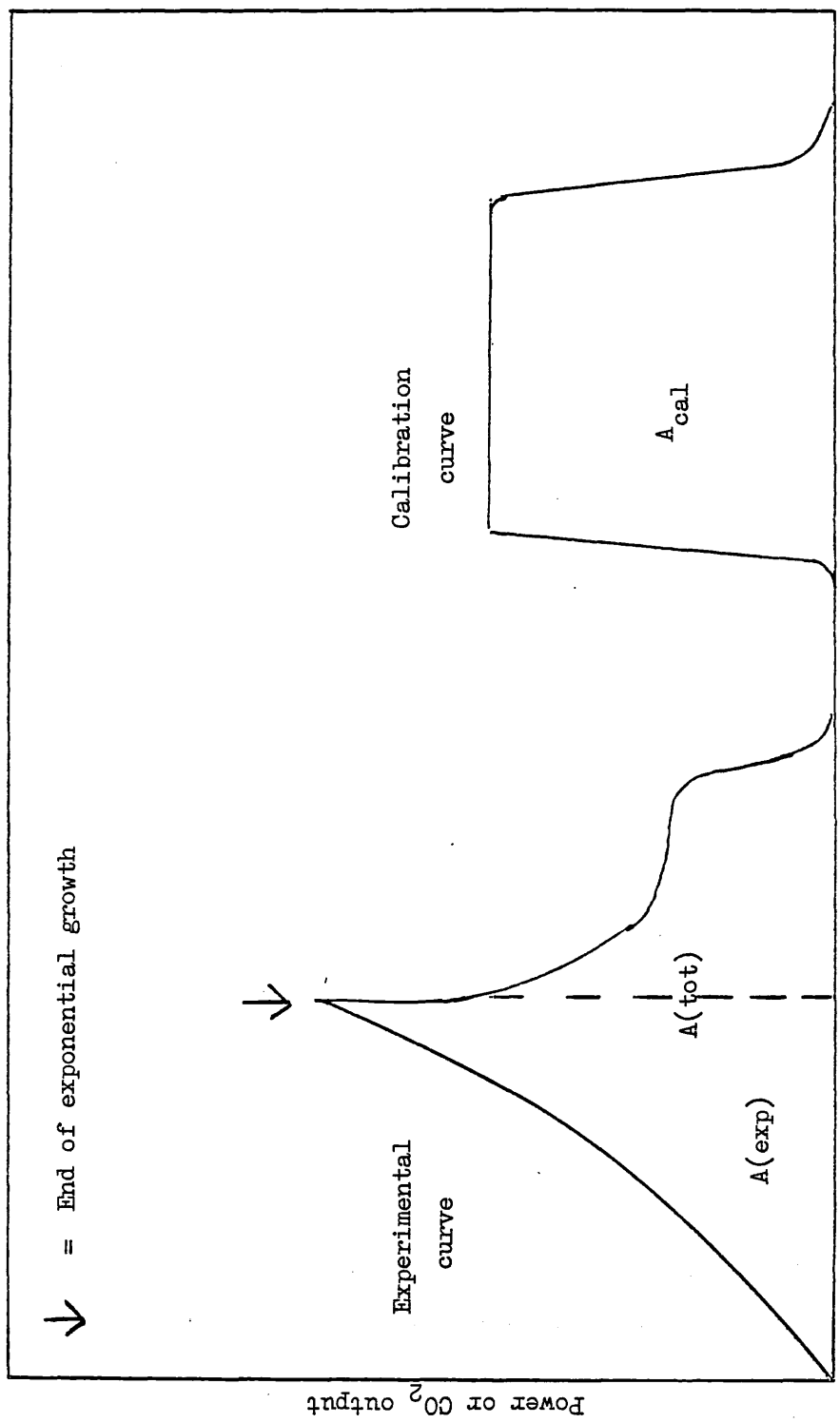
The calibration constant allows the power output and enthalpy (heat) change to be calculated from the power-time (p-t) trace. The output voltage from the calorimeter is proportional to the power output /J s^{-1}.

$$\frac{dQ}{dt} = I^2 R \qquad 2.1$$

where I is the applied current, R the heater resistance (50 chms).

The recorder deflection is proportional to the output voltage, hence the power output per unit deflection is obtained from the calibration p-t trace.

FIGURE 2.3. Schematic representation of experimental and calibration traces for power and CO_2 output



The electrical energy, Q_{cal} J, applied is given by

$$Q_{cal} = I^2 R t \qquad 2.2$$

where t is the time in seconds of the applied current.

The calibration constant, E, for heat output is obtained from equation 2.3.

$$E/J\ cm^{-2} = \frac{Q_{cal}}{A_{cal}} \qquad 2.3$$

where A_{cal} is the area/cm^2 under the calibration p - t trace (Fig. 2.3.).

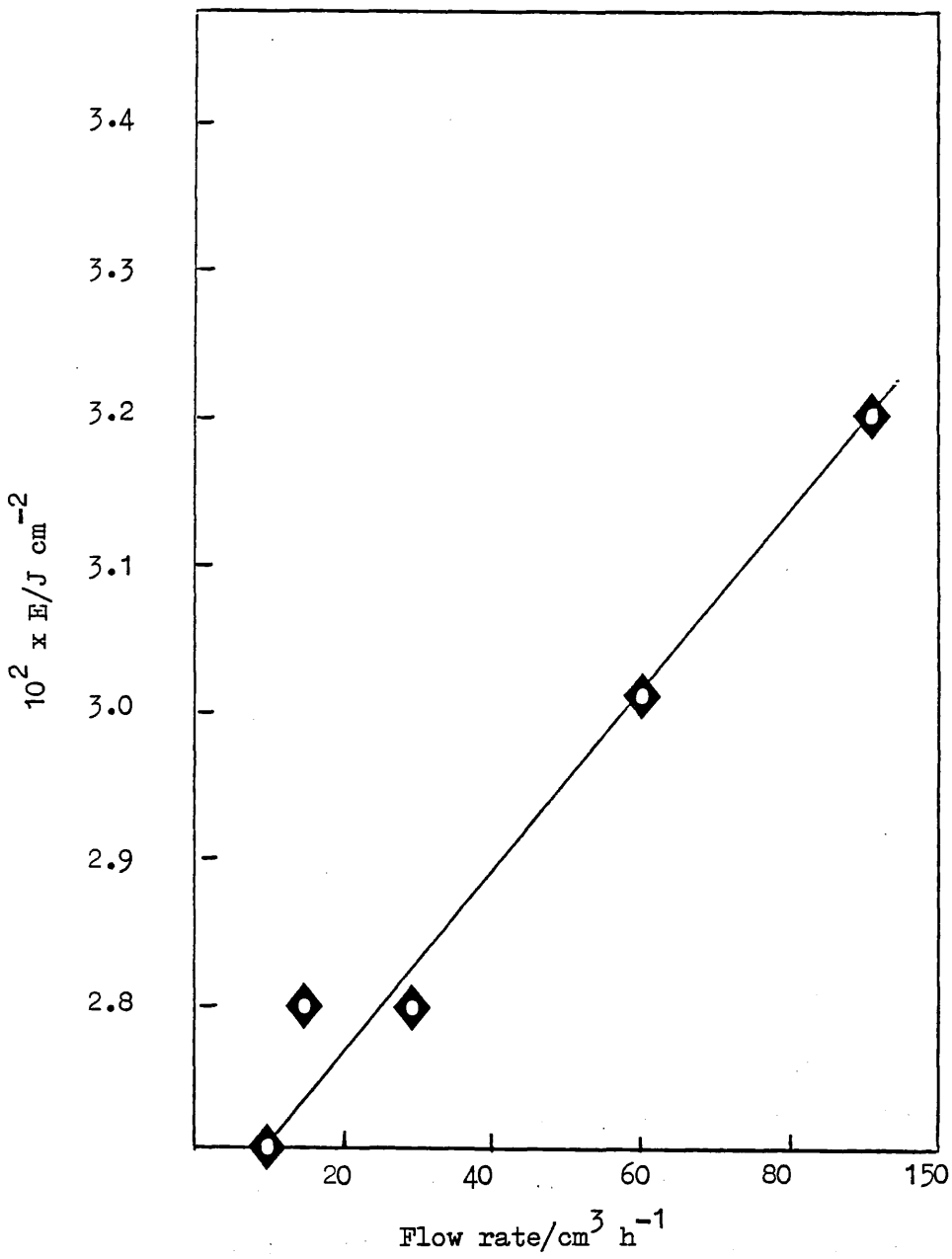
Multiplication of the integrated area, A(tot), under the experimental p - t trace (Fig. 2.3.) by the calibration constant gives the enthalpy (heat) change, ΔH.

$$\Delta H = E \times A(tot) \qquad 2.4$$

The experimentally measured heat output (q/J per 1.16 cm^{-3} culture) is considered in two ways, that during the period of exponential growth and the total heat output. Exponential heat output, q (exp), is calculated using the area, A(exp), under the p-t trace from zero time to a time coincident with the end of growth (Fig. 2.3.). Total heat output, q(tot) is calculated by using the total area under the p-t trace, A(tot), ie. from zero time until the baseline is re-established. (Fig. 2.3.).

The calibration constant, E, was constant at a fixed flow rate over the temperature range 25 - 42°C but varied linearly with flow rate at a fixed temperature (Fig. 2.4).

FIGURE 2.4. Variation of calibration constant with flow rate



The D.C. signal generated from the calorimeter was amplified and recorded on an Oxford 3000 potentiometric recorder (special 'P' input module) with normal deflection of 50μV. The calorimeter has a temperature stability of ± 0.01°C for a 0.05°C change in room temperature. The electron sensitivity was 0.1μV per μJ s^{-1} and a stability of 0.05 μV for a change of 0.01°C in the thermostatic air bath.

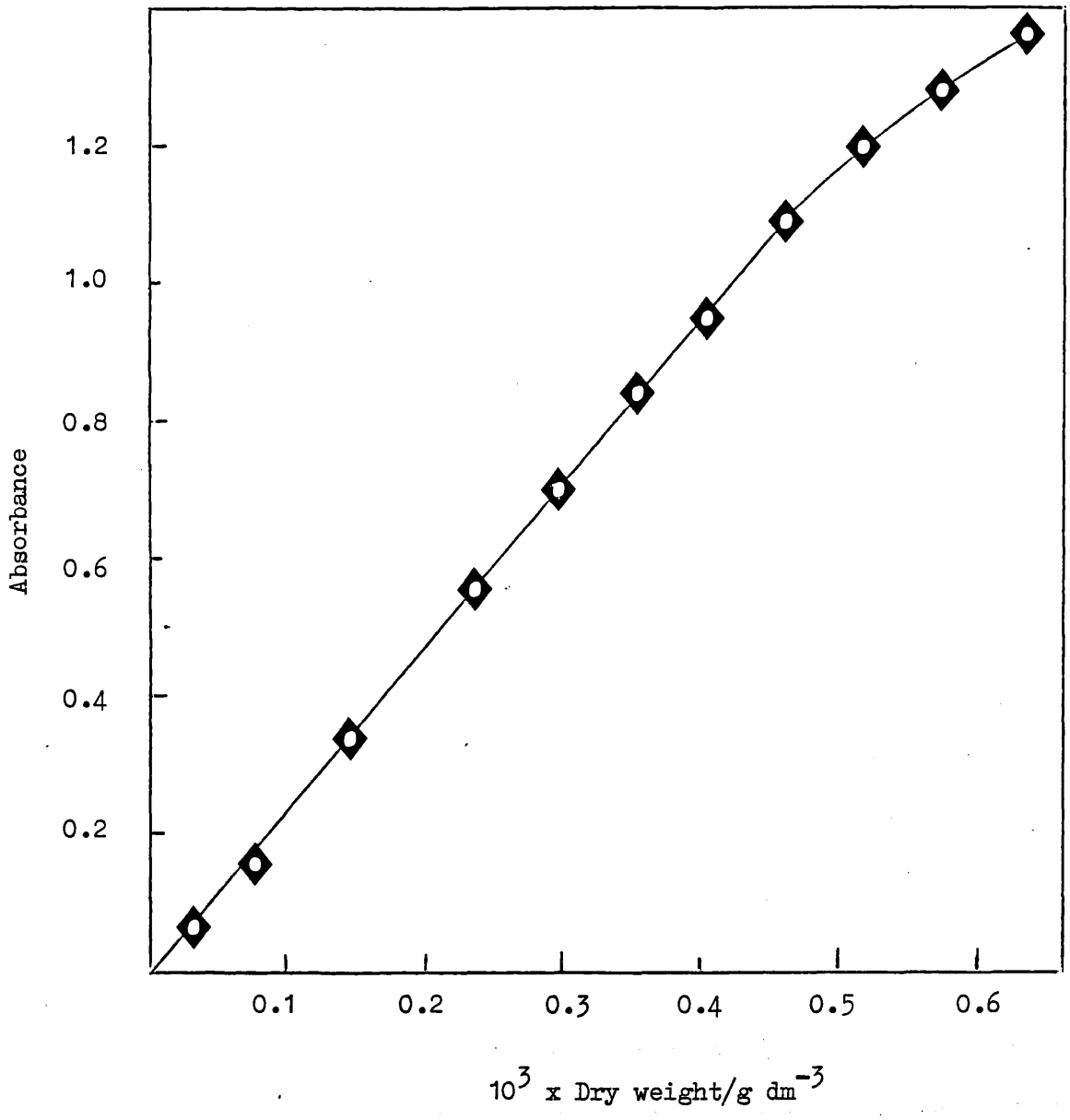
2.2.2. Estimation of Biomass

Biomass (dry weight) was determined by measuring the absorbance of the culture in a glass flow-through cuvette (Fig. 2.2.) at 625 nm (Unicam SP600 spectrophotometer). The dry weight was estimated from an absorbance/dry weight calibration curve (Fig. 2.5).

The calibration curve was prepared using "standard cells" as follows. Cells were grown in glucose-limited media (3.3 mmol dm^{-3}) at 37°C with full aeration and harvested 30 minutes before termination of exponential growth. The cells were washed twice with distilled water then resuspended in distilled water at an absorbance of about 1.4. Ten portions of this suspension each of 10 cm^3 volume were accurately pipetted into clean 25 cm^3 glass beakers which had been dried to constant weight. These aliquots were then oven dried at 105°C for two days and then over phosphorus pentoxide to constant weight. From the same stock suspension a series of accurately prepared dilutions were made into salts medium, the absorbance of each of these was then measured. Hence a biomass/absorbance calibration curve was prepared (Fig. 2.5).

Cells prepared in this manner are referred to as "standard cells". Bacterial size, density and the refractive index change during the growth cycle (Hadjipetrou and Stouthamer, 1963), causing a change in the absorbance properties of the cells. It was not practical to prepare dry

FIGURE 2.5 Bacterial dry weight calibration curve



weight calibration curves for each of the different stages of the cycle or for growth under other conditions and so all biomass data refers to cells prepared in the above manner.

2.2.3. Estimation of Oxygen Tension

The oxygen tension of the culture medium was monitored using Western Biological Oxygen electrodes, of the membrane type (Johnson et al.,1964). The electrolyte was an aqueous solution of composition: 5 mol dm^{-3} acetic acid, 0.5 mol dm^{-3} sodium acetate and 0.1 mol dm^{-3} lead acetate. An oxygen electrode was permanantly positioned in the return flow-line from the calorimeter (Fig. 2.6). Other oxygen electrodes, initially present in the culture vessel and in the spectrophotometer flow-line, were with-drawn later (3.1.). The electrodes were calibrated using aerated culture medium at the test temperature and a 5% $^{w}/_{v}$ sodium sulphite solution to give 100 and 0% saturation levels respectively. Oxygen tension was continuously displayed on an oxygen meter (Western Biological) and recorded (Oxford 3000 potentiometric recorder; 10 mV f.s.d.).

The oxygen tension (% saturation, pO_2) was used qualitatively to indicate the extent of aeration to ensure aerobic growth conditions were maintained. The maximum dissolved oxygen concentration varies with temperature and medium composition. Thus oxygen tensions with different conditions are not strictly comparable. The accuracy of a given value was about ± 5% of that value.

2.2.4. Measurement of pH

The pH of the culture medium was continuously monitored during growth by an in-line combined flow pH electrode, E.I.L. 1140200 (Fig. 2.2). The electrode was calibrated using buffer solutions of 4.5 and 7.0. The pH was measured with an E.I.L. pH meter (7020) and recorded with an Oxford 3000 potentiometric recorder (f.s.d. 10mV).

2.2.5. Estimation of Carbon Dioxide

Effluent air from the culture vessel was passed, via a drying agent, through a differential infra-red gas analyzer (I.R.G.A. G.P. Instrumentation). The scale of the analyzer was calibrated with known carbon dioxide-nitrogen mixes (B.O.C. special gases; 0.03, 0.05, 0.075 and 0.10% $^{v}/_{v}$ CO_2).

Calibration was made with 0.03% $^{v}/_{v}$ CO_2 in the reference beam and another calibration gas in the sample beam. A 0.03% $^{v}/_{v}$ CO_2 mixture was used in the reference beam since this concentration is similar to the concentration of CO_2 in the atmosphere; air was used as the reference for experimental purposes. The output voltage was recorded (Oxford 3000 potentiometric recorder, f.s.d. 10mV). The recorder deflection was proportioned to the CO_2 concentration (p.p.m.) in the effluent gas. From the area under the CO_2 concentration-time trace, Fig. 2.3, and the gas flow rate the quantity of carbon dioxide produced in a given time can be estimated. The calibration constant, ϕ, allows the carbon dioxide output to be expressed as a function of biomass or initial substrate concentration.

The volume of carbon dioxide passing through the analyser is given by:

$$V_{cal} = F\ t\ C \qquad 2.5$$

where F $dm^3\ h^{-1}$ is the gas flow rate, t the time interval and C dm^3 CO_2 per dm^3 of calibration gas.

The slope of the plot of the area under the calibration curve, A_{cal} (Fig. 2.3), against V_{cal} gives the volume of CO_2 per unit area under the trace (V_A).

At 25°C 0.0409 mol CO_2 occupies 1 dm^3 multiplication of molar volume by V_A 4.9×10^{-3} cm^2 $(dm^3 CO_2)^{-1}$ gives the calibration constant 0/mol CO_2 cm^{-2}, equation 2.6.

$$\begin{aligned} \phi &= V_A \times 0.0409 \\ &= 0.02 \times 10^{-3} \end{aligned} \qquad 2.6$$

Multiplication of the integrated area under the experimental curve, A(tot), for a given time interval (Fig. 2.3.) by the calibration constant gives the quantity of carbon dioxide produced in that time.

$$\text{Total amount of } CO_2/\text{mol} = \phi \times A(\text{tot}) \qquad 2.7$$

Carbon dioxide output as a function of biomass and glucose concentration are calculated from equations 2.8 and 2.9 respectively.

$$CO_{2_p}/\text{mol } CO_2 \text{ (g cell)}^{-1} = \frac{\phi \times A(\text{tot})}{V_f \times m \times 10^{-3}} \qquad 2.8$$

where V_f dm^3 is the culture volume and m mg cm^{-3} the final biomas.

$$CO_{2_{gluc}}/\text{mol } CO_2(\text{mol gluc})^{-1} = CO_{2_p} \times Y_m \qquad 2.9$$

where Y_m is the molar growth yield.

Errors in estimating the carbon dioxide output arose from difficulties in accurately determining the total volume passing through the analyzer and temperature changes during the passage of effluent air from the culture vessel to the analyzer. The total volume of effluent gas was obtained from the product of the flow-rate, assumed constant throughout a given experiment, and the time of the flow. This was not entirely satisfactory since there were variations in the flow rate due to losses from the culture vessel; further the short scale in the flow meter made accurate reading difficult. As far as possible the flow rate of air through the culture vessel was kept as constant as possible from one experiment to the next.

The overall accuracy of this measurement of CO_2 output was estimated to be $\pm 10\%$. To overcome some of the errors a second calibration method was used. The culture vessel containing 600 cm^3 distilled water and excess hydrochloric acid (final concentration 1 mol dm^{-3}) was equilibrated at 37°C using normal aeration conditions. Into the acid was pumped 8 cm^3 of a solution of sodium carbonate, prepared accurately by weight. This was added slowly and continuously so that the rate of carbon dioxide released was similar to that obtained during growth experiments.

From the ratio of the theoretical carbon dioxide content and that calculated using the above calibration procedure a correction to the carbon dioxide output value was calculated.

$$\frac{\text{Theoretical } CO_2}{\text{Calculated } CO_2} = \frac{1.40 \times 10^{-4} \text{ mol dm}^{-3}}{1.04 \times 10^{-4} \text{ mol dm}^{-3}} = 1.35 \quad 2.10$$

Multiplication of the measured CO_2 output value by this factor gave the corrected value for carbon dioxide output. All the experimental results quoted have been corrected.

2.2.6. The Complete Culture Vessel and Calorimeter Assembly

The culture vessel was positioned as close as possible to the inlet to the microcalorimeter. One flow-line passed from the culture vessel to the microcalorimeter (Fig. 2.6) and a second to the spectrophotometer and various electrodes.

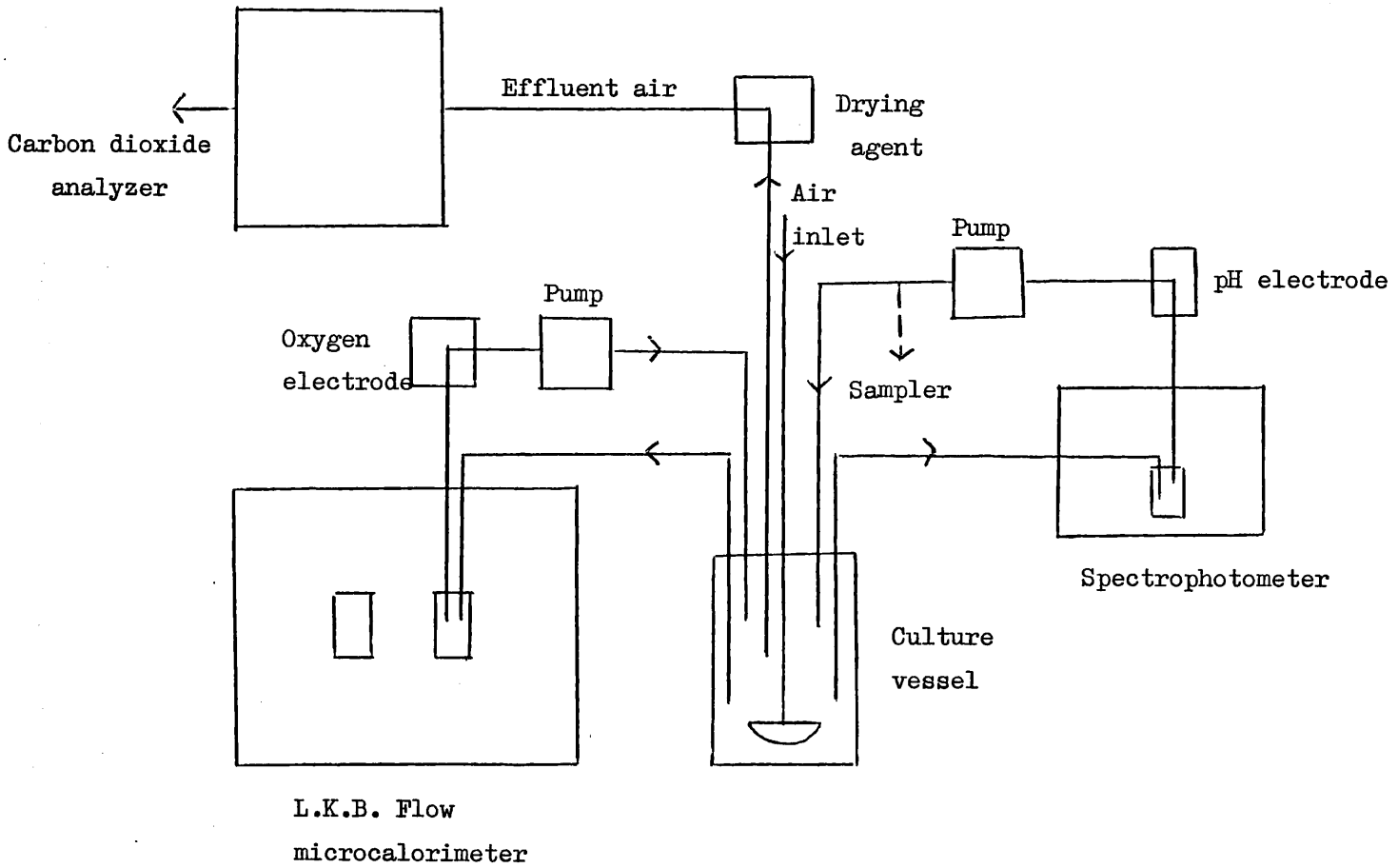
The spectrophotometer flow-line passed via the flow cell of a spectrophotometer and pH electrode through a pump and back to the culture vessel. Polypropylene tubing (i.d. 0.1 cm) connected the units in series, the distance in between each was kept as short as possible. The culture was drawn through the flow-line by a peristaltic pump at a flow rate of 400 $cm^3 h^{-1}$; the return length of tubing from the pump to culture vessel was lagged with a water jacket at the test temperature. The time for culture circulation was about 30 seconds.

An oxygen electrode was positioned in the return section of the flow-line to the microcalorimeter. The culture was drawn through the flow-line (teflon tubing i.d. 0.1 cm) by a peristaltic pump positioned after the oxygen electrode. The culture was then recycled to the culture vessel through a tube which was lagged with a heated water jacket at the test temperature.

The time taken for an organism to reach the microcalorimeter cell from the culture vessel is the transit time (Δt), this depends on the flow rate and length of connecting tubing. The mean time an organism remains in the flow cell is the residence time (Δr); this depends on the flow rate and flow cell volume.

The oxygen tension measured by the in-line oxygen-electrode after the calorimeter was considered to represent the lowest oxygen tension occurring in the calorimeter flow cell. The volumes of all the measuring units (Fig. 2.2) were kept as small as possible (3.1).

FIGURE 2.6 Experimental arrangement for measurement of thermal and growth changes



Provision was made for collecting samples of the culture from the calorimeter flow-line or from the culture vessel. After passing through the calorimeter cell, instead of being recycled, the culture was passed through a heater unit (80°C) to kill the cells, and collected in a fraction collector (30 minute periods).

Each flow-line was sterilized by pumping 5% $^{v}/_{v}$ formalin through for about 1 hour; this was followed by sterile water for about another hour. The ends of the tubing were transferred aseptically to the culture vessel and sterile medium pumped through the lines to waste. After sufficient time to allow displacement of the water with the medium the flow was returned to the culture vessel. Medium was pumped through the calorimeter to give a steady baseline for at least an hour before the culture vessel was inoculated.

2.3 Other Analytical Procedures

2.3.1 Glucose Analysis

Glucose analysis was carried out on the culture medium after centrifugation to remove bacterial cells. The method is based on that of Duboski (1962) as modified by Few *et al.*,(1976). A mixture of 3 cm^3 of 7% $^{v}/_{v}$ freshly distilled o-toluidine in glacial acetic acid and 1 cm^3 of standard glucose solution (0.1-0.3 g dm^{-3}) or sample in a boiling tube was placed in a boiling water bath for 15 minutes. After cooling, the absorbance was measured at 630 nm in a 1 cm path-length glass cuvette against a reagent blank in which distilled water replaced the sample. From the absorbance of an unknown sample the glucose concentration was obtained from the calibration curve. The curve was established for each series of analysis since oxidation of the reagent caused darkening with age. The concentration was estimated with a certainity of $\pm$ 0.003 mg cm^{-3}.

2.3.2 α-Methyl Glucoside Analysis

α-MG was estimated by an indirect method in which the glucoside was hydrolysed and the resulting glucose determined (2.3.1). α-MG is very sensitive to hydrolysis and vigorous control of the conditions was necessary for quantitative measurements.

When α-MG was the only carbon source present the following method was used. A mixture of 1 cm^3 α-MG (1-4.6 mmol dm^{-3}) or sample and 0.1 cm^3 concentrated HCl in a test tube was boiled for one minute. After cooling the free glucose was estimated.

When α-MG was present with glucose the glucose was determined on one sample and the total glucose after hydrolysis on another aliquot of the same sample. The glucose derived from the α-MG hydrolysis was determined by difference. The glucose standard curve was prepared with the addition of 0.1 cm^3 conc. HCl prior to boiling.

2.3.3 Acetate Analysis

The gas chromatographic method was based on the method of Laker and Mansell (1978). A glass column (6 ft x 4 mm i.d.) packed with 80/100 chromosorb WAW, with a stationary phase of 10% SP - 1200; 1% H_2PO_4 (Supleco Inc.) was used. A Pye-Unicam gas chromatograph (series 106) was used with a flame ionisation detecter. Attenuation was 200-2000; the output was recorded with a Philips potentiometric recorder (PM 8000).

Prior to use the column was conditioned for 24h at 190°C with nitrogen (B.O.C. oxygen-free) flowing through it. The experimental conditions were:- column temperature 117°C; nitrogen (carrier) 28 cm^3 min^{-1}; air 270 cm^3 min^{-1} and hydrogen 40 cm^3 min^{-1}. A standard acetate curve was prepared by injecting 1 μl (7 cm needle) of standard sodium acetate (1-5 mmol dm^{-3}). The standard acetate solutions were adjusted to a pH 1.5 with conc. HCl, they all contained 26 mmol dm^{-3} propionic acid.

Acetic acid came off the column first, the graph of the ratio of the peak height of acetic acid to the peak height of propionic acid against acetate concentration was linear. The filtered sample of the culture medium was treated in a similar manner as the standard acetate solution. The acetate content was determined from the standard curve; allowance was made for the dilution on acidification and addition of propionic acid.

2.3.4 Elemental Analysis of Bacterial Cells

Bacterial cells for analysis were harvested and washed three times in distilled water and oven dried ($105^{o}C$) to constant weight. The carbon, hydrogen, nitrogen and residue of the cells were determined with a Perkin-Elmer 240 elemental analyzer. Oxygen, phosphorus, sulphur and potassium were determined by Butterworth Laboratories. Values for oxygen, estimated by difference from the carbon, hydrogen and nitrogen analysis, agreed well with determined values.

From the elemental analysis it was possible to deduce an empirical formula for the bacterial cells. Cell formulae can be expressed in a number of ways but that advocated by Herbert (1976) is preferred. This has the general formula $CH_a O_x N_z$ ie. the formula contains one gram-atom of carbon, further the oxidation level of the cell carbon is of a similar level to that in the carbon substrates used in this work. From the empirical formula a formula weight can be calculated and used in the conventional way to describe a molar quantity of cellular material.

2.3.5 Bomb Calorimetry

The heat of combustion (ΔH_c) of washed and dried bacteria was determined in a Gallenkamp Autobomb Calorimeter at 25°C. Benzoic acid (B.S.C. 190K-thermochemical standard) was used for calibration. The heat of combustion was corrected for the formation of sulphur and nitrogen oxides.

CHAPTER THREE

ESTABLISHMENT OF STANDARD CONDITIONS FOR GROWTH AND THERMAL MEASUREMENTS

If quantitative growth and calorimetric measurements are to be made and interpreted in a meaningful manner then the influence of each experimental technique must be known. Although this has been recognised by several workers (eg. Belaich, 1980; Perry et al., 1979) there has been no fully systematic attempt to quantify the cultural and calorimetric conditions employed.

For any test medium the main factors which affect the calorimeter response are:

i) aeration of culture vessel and calorimeter line;

ii) condition of the inoculum;

iii) flow rate through the calorimeter cell;

iv) recycling of culture from calorimeter back to the culture vessel;

v) concentration of carbon and/or energy source;

vi) test and growth temperature.

These factors are inter-related and therefore cannot in practice be considered in isolation. However to establish standard conditions it is necessary to make some attempt to assess the importance of each in turn.

3.1 Aeration of Culture Vessel and Calorimeter Flow Line

Cells were grown in 2.8 mmol dm^{-3} gluoose (2.1.4) with various combinations of air inlet flow rates and mixing speeds. The effect of different levels of aeration of the calorimeter flow line were also investigated. Air was introduced in segments into the culture passing to the calorimeter by an additional peristaltic pump. The size of the air segments depended on the relative rates of flow of culture and air.

The results (Table 3.1) indicate several important points. The oxygen tension in the culture vessel is not affected by air flow rates in the range 1.6-3.0 $dm^3\ min^{-1}$, but high rates help in maintaining the oxygen tension (pO_2) in the calorimeter flow line. Although the pO_2 in the calorimeter is dependent on the flow rate through the calorimeter, it also depends on the rate of aeration of the culture vessel. The formation of a good culture-air bubble dispersion depended on stirring and aeration rates. Additional aeration of the calorimeter line (at 40 $cm^3\ h^{-1}$) does not cause any significant change in the oxygen tension in the calorimeter line. Additional aeration with a flow rate of 90 $cm^3\ h^{-1}$ is not feasible because a steady calorimeter baseline could not be attained due to lack of thermal equilibrium. The influence of the dead volume at the point of measurement is very important. With an electrode volume of 6 cm^3 the pO_2 minimum was about 2% saturation with a volume of 2 cm^3 the pO_2 only fell to 41%.

The profile of the p-t trace and the heat output varied markedly with the different aeration conditions. The p-t trace became more complex as the pO_2 decreases, the pH and cell yield values were reduced.

Since high flow rates were needed through the calorimeter (3.4) the following experiments were performed with an air inlet flow rate of 1.6 $dm^3\ min^{-1}$. No additional aeration in the calorimeter flow line was needed, many very small air bubbles were drawn out of the culture vessel with the culture into the flow line.

3.2 Condition of the Inoculum

Several workers have indicated that the history and condition, particularly the lag period, of the inoculum affects the thermal measurements (Perry *et al.*, 1979; Beezer *et al.*, 1976). For

Table 3.1 Minimum Oxygen Tensions Attained During Growth in 2.8 mmol dm^{-3} Glucose/Salts Media

Conditions	Aeration rate /dm^3 min^{-1}	Oxygen tension % Culture Vessel	Spectrophotometer Line	Calorimeter Line
Volume of sleeved electrode in Spectrophotometer line 6 cm^3; no aeration of calorimeter line; flow rate 40 cm^3 h^{-1}.	1.25	87.5	1.0	N.D.
	2.00	81.0	2.0	N.D.
	3.50	82.0	3.0	N.D.
Volume of sleeved electrode in Spectrophotometer line 2 cm^3; no aeration of calorimeter line; flow rate 40 om^3 h^{-1}	3.00	87.0	41.0	70.0
As above, except calorimeter line air segmented; flow rate 40 cm^3 h^{-1}	3.00	85.0	41.0	67.0
As above, except no aeration in calorimeter line, flow rate 90 cm^3 90 cm^3 h^{-1}	1.60	85.0	N.D.	40.0

N.D. Not Determined

Mixing Speed 1200 r.p.m.

Flow rate in Spectrophotometer line 400 cm^3 h^{-1}

quantitative measurements it is essential to have an inoculum which will give reproducible growth and heat outputs.

3.2.1 Types of Inocula Initially Used

Inocula from routine daily subcultures in salts/glucose media or freeze-dried cells grew with irreproducible lag periods and heat outputs which varied from one batch to another. The influence of the lag period on the p-t trace characteristics were more significant than the size of the inoculum (Fig. 3.1), a typical p-t trace is shown in Fig. 3.5.

The power output attained at the first maximum P_1, (see 3.5 for full details) varied little for lag periods up to 4.5 h, but thereafter it decreased. The power output at P'_2 varied erratically with the length of the lag period. The time interval between the appearance of the two peaks was nearly constant at about one hour for lag periods up to 3h. This time interval increased to 2-3 h when growth was further delayed. q(exp) increased with increasing lag from 2.2 for cells with no lag to 2.9 J per 1.16 cm^3 of culture with a 5.5 h lag; an increase of 24% which is larger than the experimental error. Similarly q(tot) increased with increasing length of lag period.

Since the thermal parameters varied so widely with the length of the lag period a "standard inoculum" was developed.

3.2.2 Standard Inoculum

Cryogenically-stored cells of *K. aerogenes* (2.1.5) fulfilled all requirements of a standard inoculum. The lag phase was absent, the detailed profile of the p-t trace, the heat output and growth parameters were reproducible over a period of many months, provided that the flow rate through the calorimeter and glucose concentration were fixed (Table 3.2). Considering all sources of error in measurements

FIGURE 3.1. The effect of the lag period on some thermal properties during aerobic growth of K. aerogenes

Glucose concentration 3.3 mmol dm^{-3}
Flow rate = 40 cm^3 h^{-1}

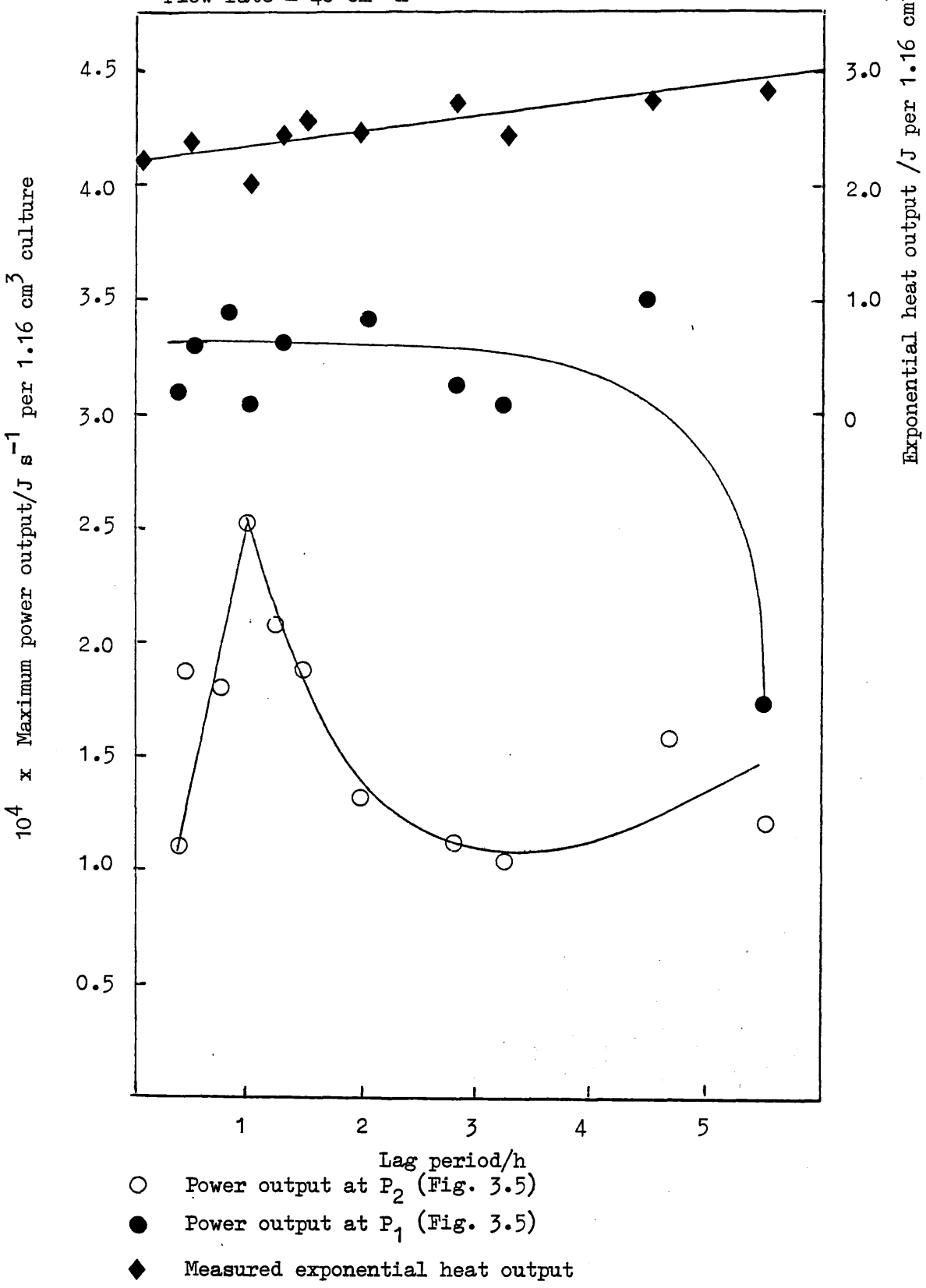


Table 3.2 Reproducibility of Calorimeter and Growth Parameters Using Cryogenically Stored Inocula

Glucose concentration 3.3 mmol dm^{-3};

Calorimeter Flow Rate: 40 cm^3 h^{-1} (no of experiments: 10)

Parameter	Mean($\bar{x}$)	St. deviation(s)	Mean**	$s/\bar{x}$ x 100
Power Output at P_1*	3.15 x 10^{-4} J s^{-1}	0.22	3.15 ± 0.14 x 10^{-4} J s^{-1}	7.0
Power Output at P_2*	1.77 x 10^{-4} J s^{-1}	0.42	1.77 ± 0.27 x 10^{-4} J s^{-1}	24.0
q(exp)	2.23 J	0.23	2.23 ± 0.14 J	10.0
Biomass at end of growth	0.227 mg cm^{-3}	0.036	0.227 ± 0.025 mg cm^{-3}	11.0
Time from inoculation to end of growth	4.6 h	0.20	4.6 ± 0.14 h	4.0

Calorimeter Flow Rate: 90 cm^3 h^{-1} (No of experiments: 10)

Power Output at P_1*	6.00 x 10^{-4} J s^{-1}	0.18	6.00 ± 0.18 x 10^{-4} J s^{-1}	3.0
q(exp)	3.22 J	0.05	3.22 ± 0.04 J	1.3
q(TOT)	3.97 J	0.14	3.97 ± 0.12 J	3.0
Time from inoculation to P_1	4.2 h	0.35	4.2 ± 0.30 h	8.0
Biomass at end of growth	0.257 mg cm^{-3}	0.013	0.257 ± 0.011 mg cm^{-3}	4.9

** Mean with 95% Confidence limits

* See Figs 3.4 and 3.5

in a biological system the reproducibility is very good. Cryogenically-stored inocula, which also possess the advantage of ready availability, were therefore adopted as the standard inoculum and used in all the following experiments.

3.3 The Variation of Heat Output with Flow Rate

Growing cultures were pumped through the calorimeter at flow rates in the range 10-105 $cm^3\ h^{-1}$ (glucose concentration: 3.3 mmol dm^{-3}). The profile of recorded p-t traces (corrected for changes in the calibration constant, Fig. 2.3) depends on the flow rate (Fig. 3.2). With increasing flow rate the profile becomes more simple tending to one peak, the first peak moving progressively towards the end of exponential growth. At the same time the second peak and the minimum value. disappearing and the baseline is re-attained sooner. At the higher flow rates the pO_2 in the flow line returns more quickly to near saturation level at the end of exponential growth.

q(exp) increased dramatically with increase in flow rate up to about 70 $cm^3\ h^{-1}$ (Fig. 3.3). At higher rates, with shorter transit and residence times, the heat output remained constant. At flow rates in excess of 110 $cm^3\ h^{-1}$ it was not possible to establish a steady baseline due to non-attainment of thermal equilibrium.

Rate constants for the increase in heat output, biomass and CO_2 output were calculated in the following manner. The value of each parameter, at 30 min intervals, was plotted logarithmically against time up to P_1, ie. during exponential growth. The plots are linear (Nichols *et al.*, 1979); the slope is the rate constant for the increase in that parameter.

Rate constants for heat output decrease with decreasing flow rate, except with the extreme flows, Table 3.3. At extreme flow rates the reproducibility of the measurements is poor (Table 3.2).

FIGURE 3.2. Variation of power-time trace with flow rate

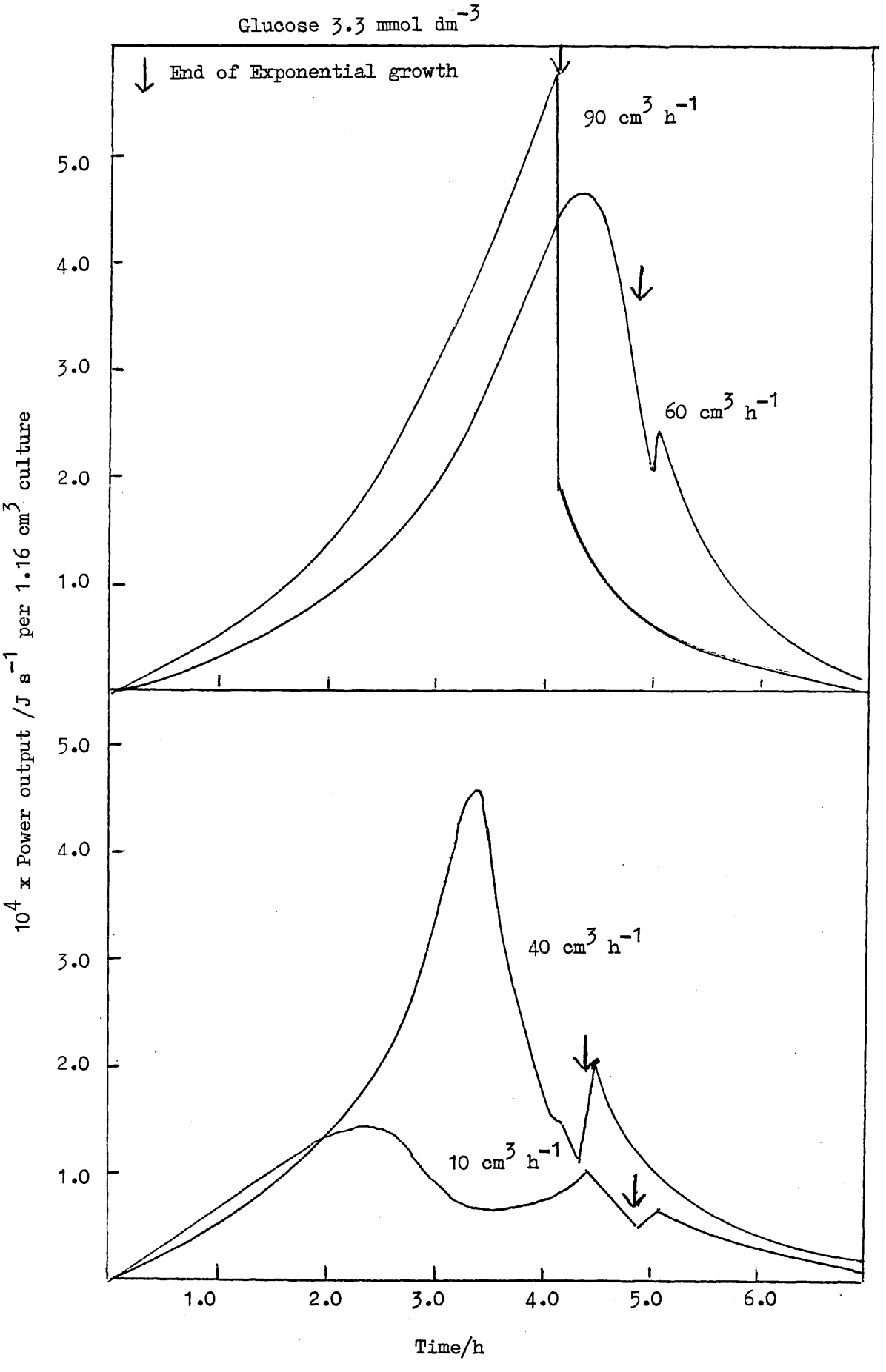


FIGURE 3.3 Effect of flow rate through the macrocalorimeter on heat output during exponential growth

q(exp)

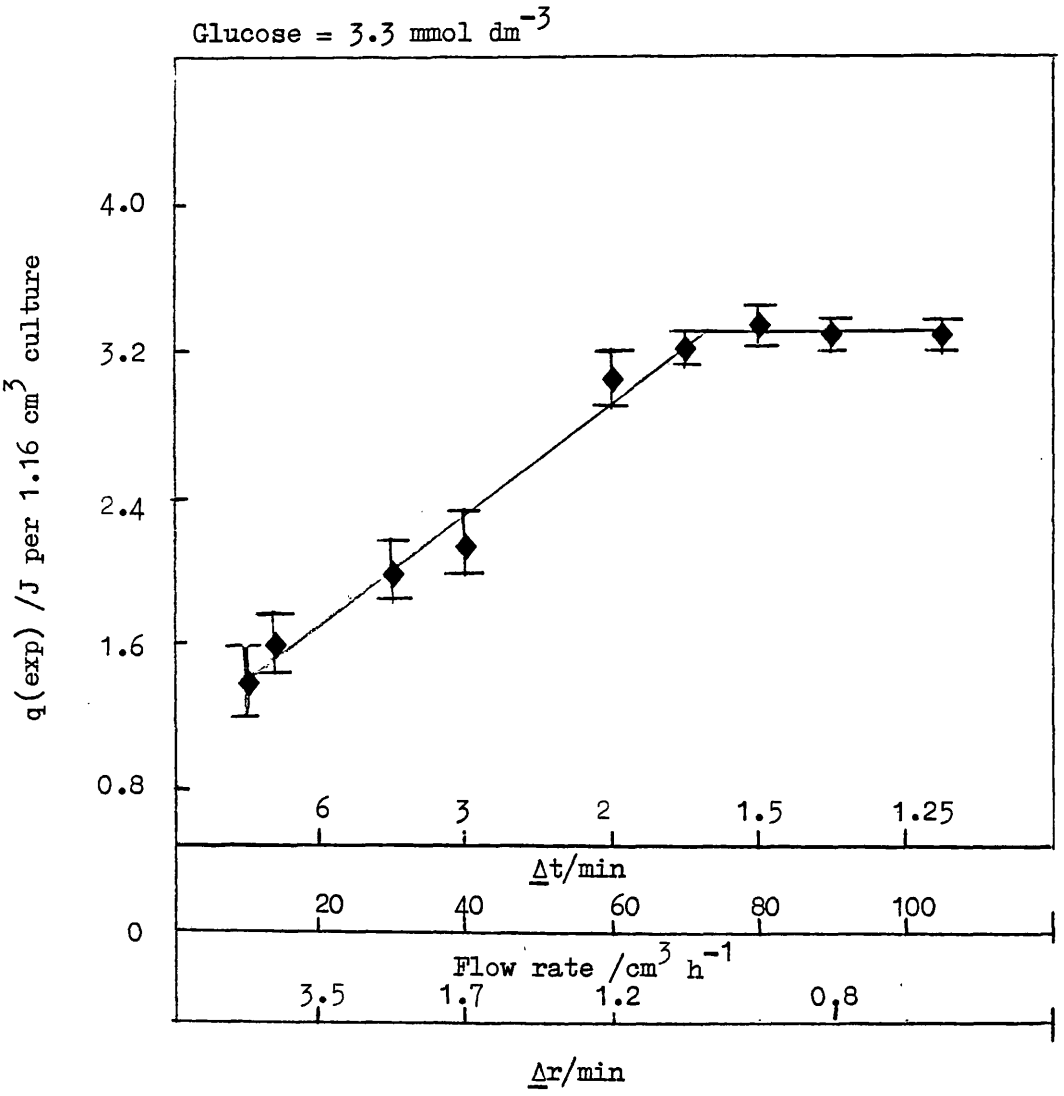


Table 3.3 Variation of Rate Constant with Flow Rate

Calorimeter flow rate /cm^3 h^{-1}	$10^3 k/min^{-1}$	
	Heat output	Biomass
10	9.00	N.D.
30	6.06	8.10
48	6.82	6.20
80	7.22	5.80
90	7.66	6.41
105	6.80	7.62

N.D. = Not determined

Since the error is large at the lower flow rates there is no significant difference between the rate constants at the different flows. At extreme flow rates the difference in the rate constants for biomass is probably due to the presence of secondary metabolites that form in the calorimeter line and are subsequently recycled to the culture vessel.

In Nichols et al., (1979) it is stated that the rate constants for heat output and biomass are in close agreement. This comment was made using the reproducibility possible at 40 cm^3 h^{-1}, this is now known not to be strictly correct. There is no significant difference in the two rate constants at low flow rates; with flow rates 90 and 80 cm^3 h^{-1} there is a difference, the rate of heat preceeds that for biomass.

The higher the pump rate the more nearly is the state of the organisms in the calorimeter cell the same as that of those in the culture vessel. Thus higher pump rates, compatable with the establishment of thermal equilibrium in the calorimeter, are most acceptable. The chosen pump rate for all further work, unless specified, was 90 cm^3 h^{-1}.

3.4 Effect of Recycling Culture from Calorimeter to Culture Vessel

When a flow microcalorimeter is coupled to a batch culture vessel it is usual to recycle the culture back to the culture vessel from the calorimeter. At a flow rate of 10 cm^3 h^{-1} when the culture was not recycled q(exp) was 15% less than when it was recycled. With a flow rate of 90 cm^3 h^{-1} q(exp) was the same whether the culture was recycled or not.

3.5 Standard Growth and Test Conditions

The following experiment "standard" conditions were these and were used in all the remaining experiments, unless otherwise stated:

i) cryogenically-stored inocula were used;
ii) Cultures were aerated at 1.6 dm^3 min^{-1} with a stirrer speed of 1200 r.p.m;
iii) the calorimeter flow rate was 90 cm^3 h^{-1}, Δt = 0.3 min, Δr = 0.8 min;
iv) there was no additional aeration of the calorimeter line;
v) the limiting glucose concentration was 3.3 mmol dm^{-3}
vi) the test temperature was 37.0 ± 0.2°C.

With these conditions the following reproducible growth and thermal changes were recorded during the growth cycle (Fig 3.4). As exponential growth proceeds power output increases to a maximum value (P_1) which coincides with the end of growth; after this point there is no detectable glucose in the culture medium. The carbon dioxide concentration passes through a maximum value at the end of growth paralleling the p-t trace while the pO_2, the inverse of the carbon dioxide output, passes through a minimum value. The pH decreases steadily throughout exponential growth reaching a steady value at the end of growth; during the early stationary phase the pH increases by a very small value then remains constant. After the abrupt change at the end of exponential growth, pO_2 and CO_2 quickly return to their initial values, while the power output attains a zero value within 10h.

The specific power output (Fig 3.4), ie. the power output per unit biomass (3.6), is maximal during early exponential growth, remaining approximately constant until near the end of growth when there is a small increase. With the onset of the stationary phase the specific power output becomes zero.

The carbon dioxide output, CO_2 , attains an initial high constant value, about 9.5 mmol CO_2 (g cell)$^{-1}$ during the first half of exponential growth. During the second half this falls to another constant value, 7.5 mmol CO_2 (g cell)$^{-1}$, which is maintained until the end of exponential growth when it quickly falls to zero.

The growth and thermal changes accompanying similar growth conditions but with a flow rate of 40 cm^3 h^{-1} through the calorimeter are illustrated for comparison in Fig 3.5. As with the higher flow rates abrupt changes occur at the end of exponential growth. The p-t profile is now more complex, consisting of two peaks, the second peak due to the oxidation of secondary intermediates which have accumulated in

FIGURE 3.4 Power-time curve and changes of growth parameters during glucose-limited growth

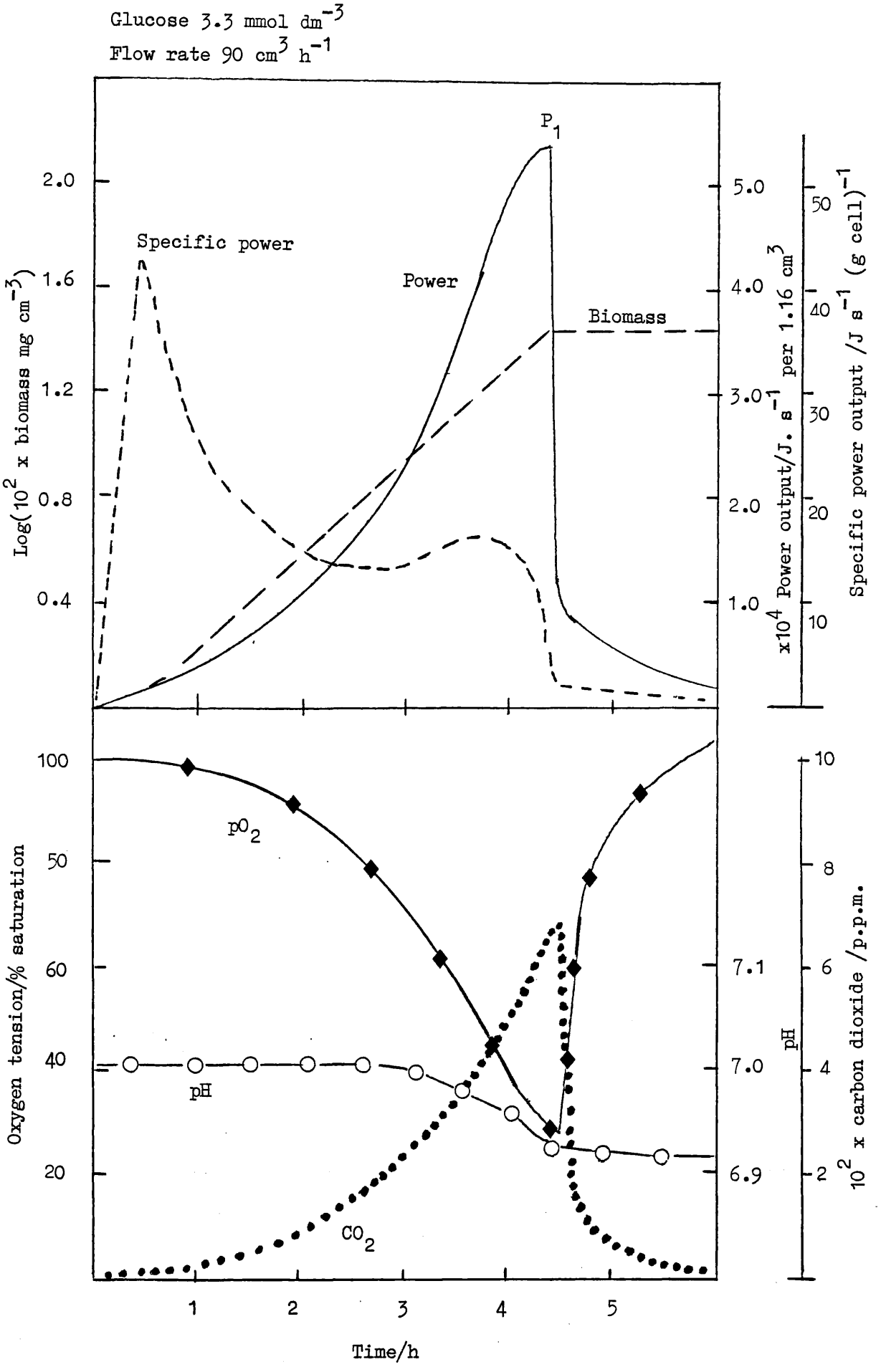
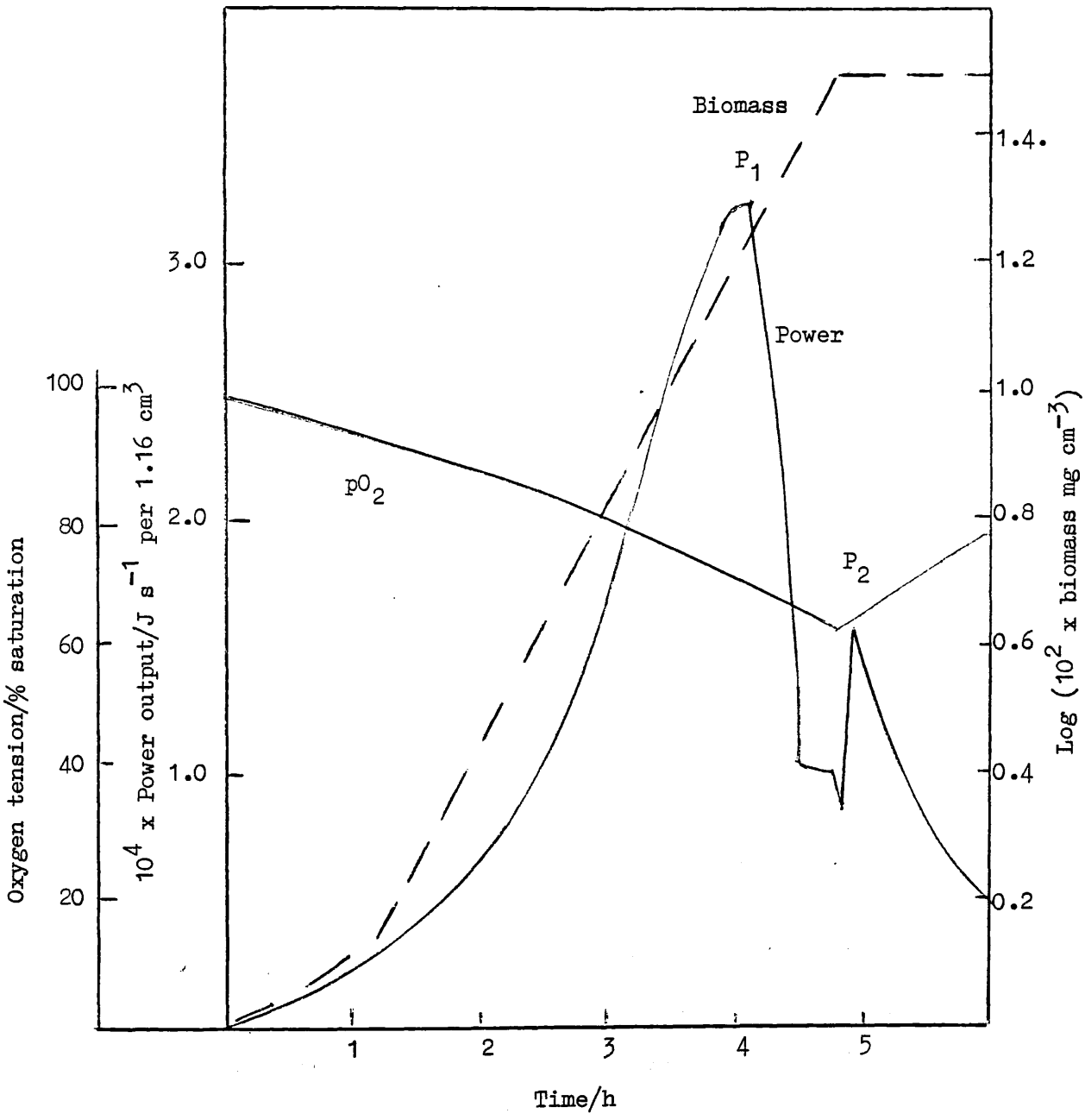


FIGURE 3.5. Power-time trace and changes of growth parameters during glucose-limited growth

Glucose = 3.3 mmol dm^{-3}

Flow rate = 40 cm^3 h^{-1}



the flow line due to oxygen deficiency. The reproducibility of the various parameters (Table 3.2.) is not so good as for higher pump rates. Generally, regardless of the experimental conditions, the changes in CO_2 output and pO_2 are parallel to or inverse of the p-t trace respectively.

Over the past 18 months standard test conditions have been used (flow rate 90 $cm^3\ h^{-1}$), the p-t profiles and total heat output have been reproduced at a level of $\pm$ 3% (cf Table 3.2).

3.6 Standard Calculation and Presentation of Enthalpy Values

For detailed discussion and comparison of results the heat output data must be related to measured changes in other parameters. The most convenient ways of achieving it is to convert the measured heat output into the enthalpy change occuring either during the production of 1 mol of cellular material or for the consumption of 1 mol of substrate (usually glucose).

The enthalpy change accompanying the production of 1 mol of cellular material is calculated from:

$$\Delta H_p/kJ\ (mol\ cell)^{-1} = \frac{q \times M_r}{\Delta m \times V} \qquad 3.1$$

where q is the measured heat output J per 1.16 cm^3 culture, M_r the empirical formula weight, Δm the increase in biomass (mg cm^{-3}), ie. final biomass-inoculum biomass and V the volume of the culture.

The 95% confidence limits for ΔH_p $\pm$ 6%.

The specific heat output, $\Delta H_{sp}/kJ\ (g\ cell)^{-1}$ is obtained by dividing ΔH_p by the empirical formula weight. Similarly the specific heat output per generation, $\Delta H_g/kJ\ (g\ cell)^{-1}\ gen^{-1}$, is obtained by dividing ΔH_{sp} by the total number of generations.

The enthalpy change accompanying the consumption of 1 mol of substrate is calculated from:

$$\Delta H_{sub}/kJ\ (mol\ sub)^{-1} = \frac{q}{V \times C} \qquad 3.2$$

where C/ mol dm^{-3} is the initial limiting substrate concentration, the 95% confidence limits for ΔH_{gluc} are ± 6%.

Values of ΔH_p and ΔH_{sub} are calculated using the total measured heat output values, q(tot). When the measured exponential heat output q(exp), is used this will be indicated ΔH_p (exp) or ΔH_{sub} (exp).

The enthalpy change accompanying the consumption of 1 mol of substrate carbon, used with mixed substrates is calculated from:

$$\Delta H_{carbon}/kJ\ (mol\ carbon) = \frac{q}{V(C_1 + C_2)} \qquad 3.3$$

where C_1 and C_2/mol dm^{-3} are mols of carbon available from each substrate.

Yield coefficients Y_{gluc}, Y_{carbon}, Y_{sub} are the dry weight of cells obtained from one gram of substrate or carbon initially present. Y_m is the molar growth yield and is the dry weight of cells obtained per mol of initial substrate utilized.

3.7 Summary

1. The condition of the inoculum effects the growth rate of the culture, this can be highly variable giving irreproducible thermal measurements.

2. Use of cryogenically-stored "standard" inocula allows very reproducible measurements to be made over a period of many months.

3. With fixed aeration conditions and decreasing the flow rate through the calorimeter the p-t trace becomes more complex as aerobic conditions are lost. Above a flow rate of 75 $cm^3\ h^{-1}$ the exponential heat output becomes constant.

4. With low flow rates, aerobic conditions are lost, recycled culture from calorimeter back to the culture vessel then influences future thermal events.

CHAPTER FOUR

VARIATION OF HEAT OUTPUT DURING GROWTH IN GLUCOSE-LIMITED MEDIA

4.1 Power-Time Traces and Growth Parameters for Glucose-Limited Aerobic Growth

The p-t traces of cells growing in media of glucose concentrations below 3.8 mmol dm^{-3} consisted of a single peak (Fig. 3.5 and 4.1). Above this concentration a shoulder occured on the decay side of the peak which developes into a second peak at 5.6 mmol dm^{-3} (Fig 4.1). The maximum power output (P_1) increased linearly with glucose concentrations up to 3.3 mmol dm^{-3} then levelled off. At low glucose concentrations the variability of the growth parameters were similar to those illustrated for 3.3 mmol dm^{-3} (Fig 3.5), the values depended on the glucose concentration. At high glucose concentrations (>3.8 mmol dm^{-3}) maximum carbon dioxide output and minimum oxygen tension of the medium coincided with the second peak of the p-t trace (P_2).

The plot of the logarithm of heat output (q), carbon dioxide output ($CO_{2_{gluc}}$) and biomass for 30 minute intervals against time during exponential growth are linear (Fig. 4.2). The rate constants, k, obtained from the slope, at 3.3 mmol dm^{-3} glucose are 7.66×10^{-3}, 6.88×10^{-3} and 6.66×10^{-3} min^{-1} for heat output, carbon dioxide and biomass respectively. As the initial concentration of glucose is increased k for the biomass production passes through a minimum value (2.2 mmol dm^{-3}). The rate constant for heat output over the middle concentration range (2.2 - 3.3 mmol dm^{-3}) is marginally less than that for biomass, while at the highest (lowest) concentrations studied the rate constants are significally greater (lower) than those for biomass (Table 4.1). The rate constant for carbon dioxide output was parallel to the rate constants for biomass and heat output (Table 4.1).

FIGURE 4.1. Variation of power-time trace with glucose concentration

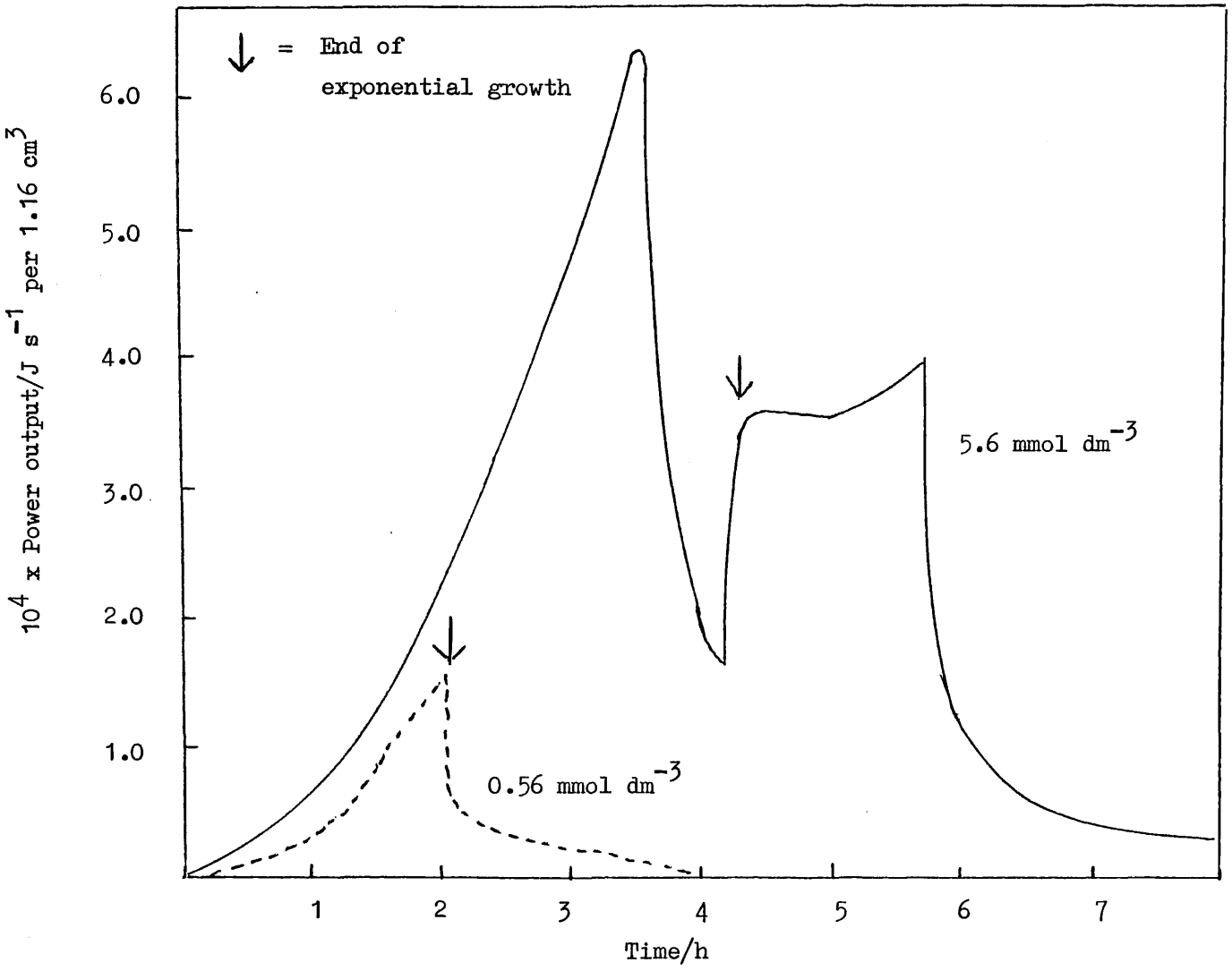


FIGURE 4.2. Heat output, carbon dioxide output and biomass as a function of time during exponential growth of K. aerogenes

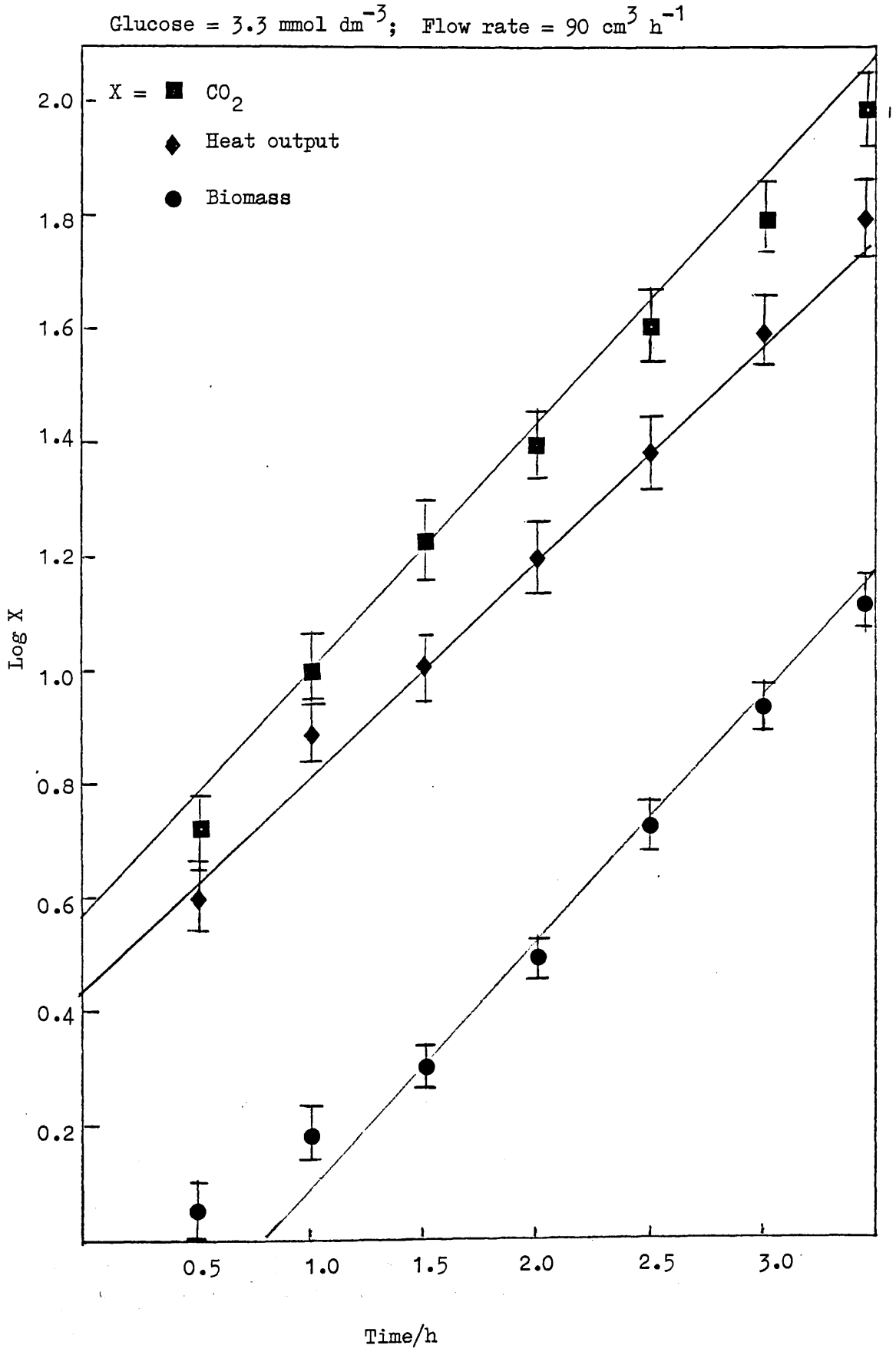


Table 4.1 Variation of Growth Parameters and Rate Constants with Initial Glucose Concentration

Glucose concentration /mmol dm^{-3}	Biomass $\Delta m/mg\ cm^{-3}$	Y_{gluc} /g g^{-1}	CO_{2_p} /mmol g^{-1}	$CO_{2_{gluc}}$ /mol (mol gluc)$^{-1}$	Minimum pO_2 %	10^3 x k/min^{-1} Heat output	CO_2	Biomass
0.56	0.044	0.44	N.D.	N.D.	90	9.57	N.D.	6.63
1.10	0.090	0.45	32.9	2.66	77	6.15	N.D.	7.29
1.70	0.130	0.43	37.4	2.89	63	N.D.	6.48	6.64
2.20	0.171	0.43	40.4	3.12	47	5.65	6.45	5.95
2.80	0.217	0.43	40.4	3.12	41	5.68	6.40	6.43
3.30	0.256	0.43	39.7	3.07	24	7.66	6.86	6.46
3.80	0.308	0.44	40.4	3.19	20	7.38	6.06	6.90
4.20	0.326	0.43	39.4	3.05	N.D.	7.11	6.11	6.96
5.40	0.410	0.42	N.D.	N.D.	25	7.16	N.D.	8.97

N.D. Not determined

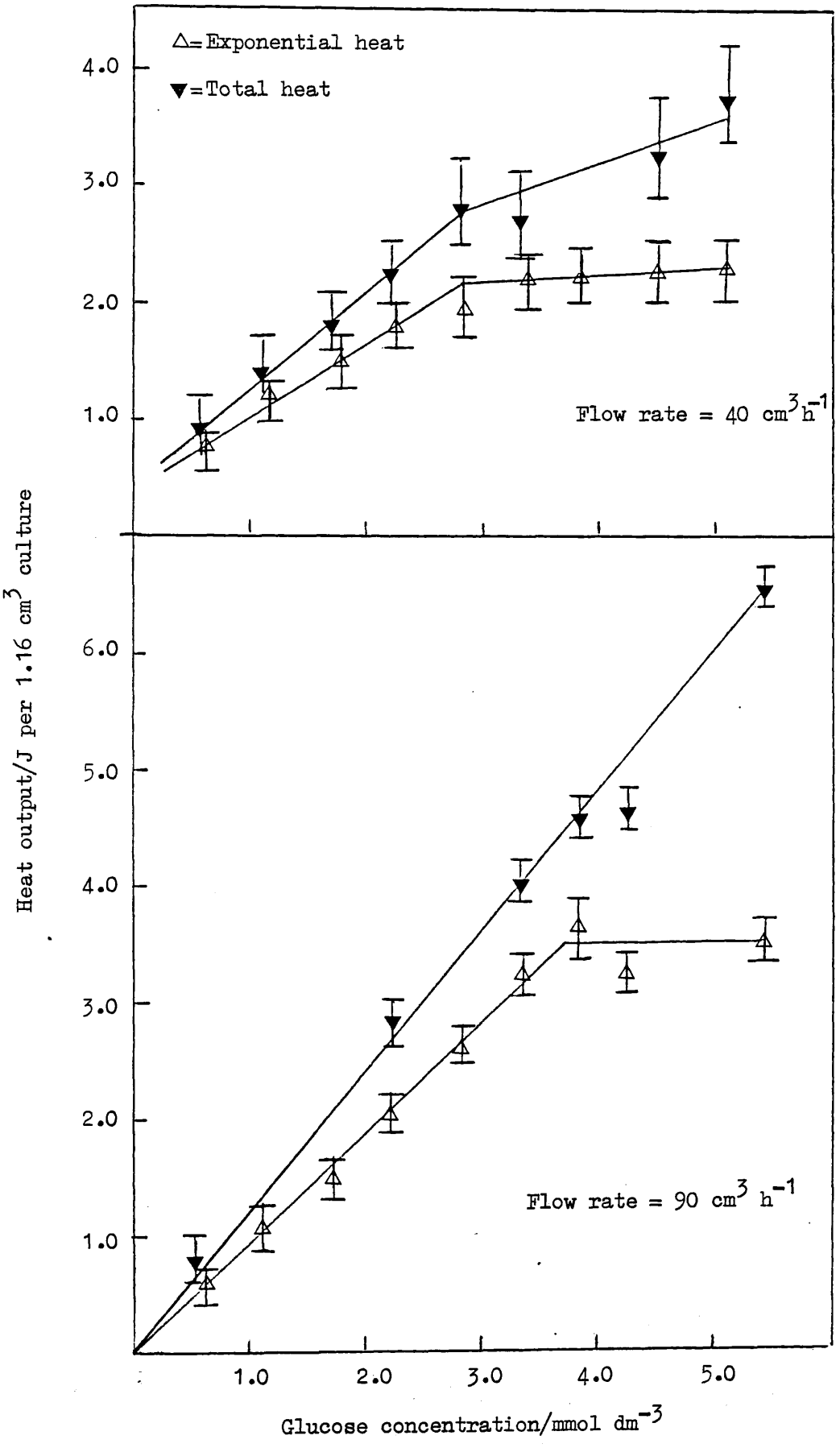
The yield coefficient (Y_{gluc}) is constant within the limits of experimental error for all glucose concentrations (Table 4.1). The minimum oxygen tension in the calorimeter line decreased with increasing glucose concentrations up to 3.8 mmol dm^{-3} and thereafter it was constant at a low level of about 24%. At glucose concentrations above 4.4 mmol dm^{-3} acetate (<1 mmol dm^{-3}) was detected.

4.2 Variation of Measured Heat Output with Initial Glucose Concentration

During growth with the glucose concentration range 0.56 - 5.4 mmol dm^{-3} the measured heat output, q (exp) and q (tot), increased with increasing concentration. When the pump rate through the calorimeter is 90 cm^3 h^{-1}, the exponential heat output was linear up to 3.8 mmol dm^{-3}, at higher glucose concentrations it levelled off (Fig. 4.3); the break coincided with the concentration at which there was an abrupt change in the profile of the p-t trace. The total heat output was linear over the whole concentration range, the linear ranges for q(exp) and q(tot) extrapolate through the origin. The difference between the values of q(tot) and q(exp) is the heat output after the end of exponential growth.

When the heat output was determined at a flow rate through the calorimeter of 40 cm^3 h^{-1} there was a break in plots of both q(exp) and q(tot) against glucose concentration. The breaks in the plots coincide with a marked change in the p-t trace profile, furthermore neither plot passed through the origin. Results and data made with a flow rate of 40 cm^3 h^{-1} presented in Nichols *et al.*, (1979) are discussed later (9.0).

FIGURE 4.3 Variation of measured heat output with flow rate and glucose concentration



4.2.1 Variation of ΔH_p and ΔH_{gluc} with Glucose Concentration

ΔH_{gluc} (equation 3.2) becomes more positive with increasing glucose concentration (Fig. 4.4); while ΔH_p (equation 3.1) remains constant within the limits of experimental error. ΔH_{gluc}, over the concentration range 0.56 - 5.4 mmol dm^{-3} glucose is in the range -1200 to -950 with a mean value of -1067 kJ $(mol\ gluc)^{-1}$. For ΔH_p the range is -318 to -397 with a mean of -355 kJ $(mol\ cell)^{-1}$; ie. the ΔH_{sp} is in the range -12.7 to -15.9 with a mean of -14.2 kJ $(g\ cell)^{-1}$.

Corresponding enthalpy changes during exponential growth show a similar trend, the values are lower (Table 4.2). The enthalpy for the production of cellular material per generation (ΔH_g) becomes more positive with an increase in the total number of generations that have occurred ie. with increasing glucose concentration.

4.2.2 Mass Balance for Aerobic Growth of K. aerogenes in Simple Salts/Glucose Media

The elemental composition of cells of K. aerogenes grown with glucose limitation (Table 4.3) did not vary with glucose concentration over the range 0.56 - 5.4 mmol dm^{-3}. Cells of this composition had a heat of combustion (ΔH_c) of -22.7 $\pm$ 0.9 kJ $(g\ cell)^{-1}$.

From the elemental composition the empirical formula for cells of K. aerogenes was derived, $CH_{1.77}O_{0.48}N_{0.25}P_{0.02}S_{0.01}$. Cells of this composition have a formula weight of 25.98. Generally the metals, S and P are neglected, cells then have a formula weight of 24.98. Unless otherwise indicated the latter formula weight is used in calculations of enthalpy value (eg. ΔH_p).

FIGURE 4.4. Variation of ΔH_p and ΔH_{gluc} with initial glucose concentration

Flow rate = 90 cm^3 h^{-1}

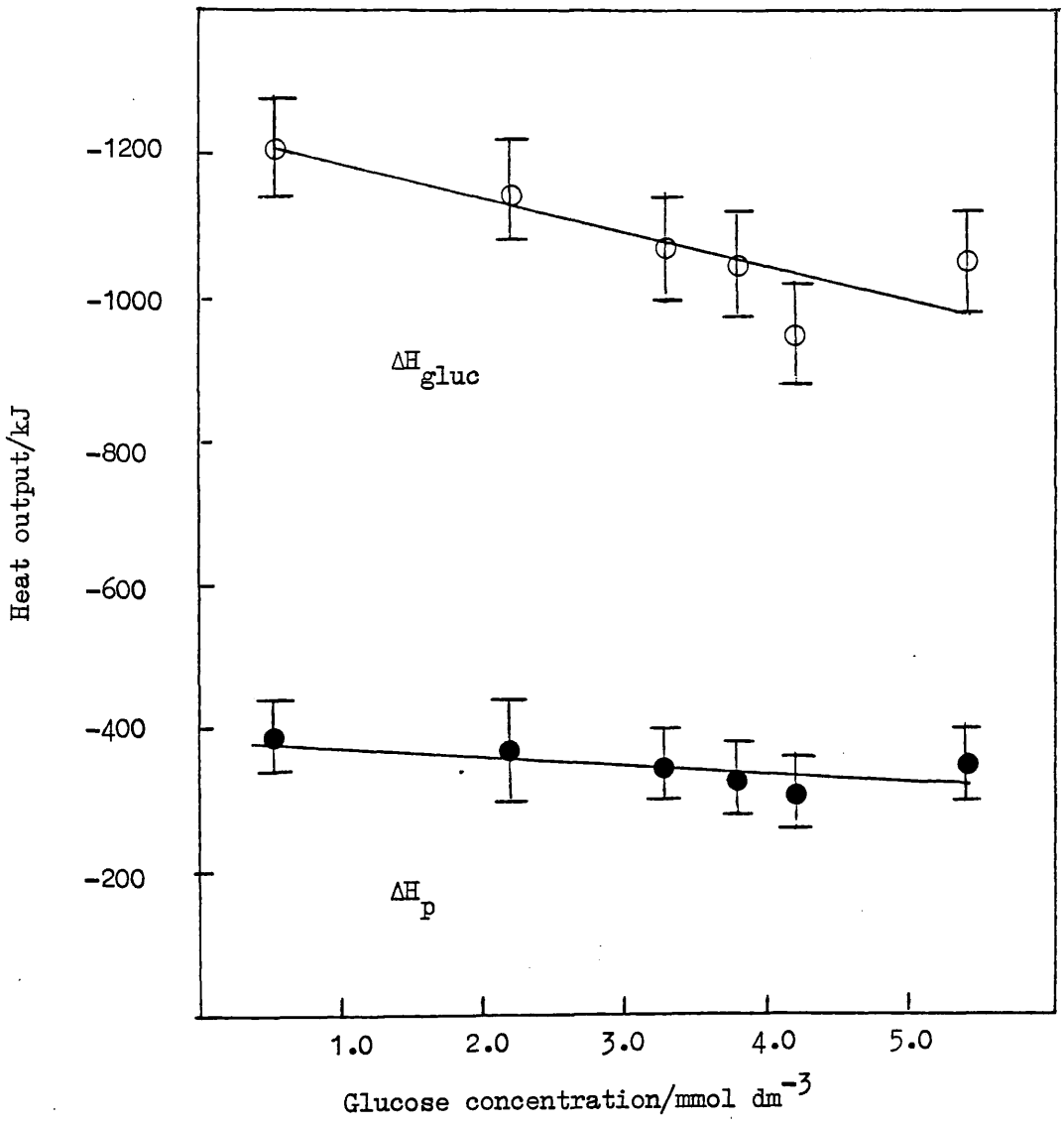


Table 4.2 Heat Output of K. aerogenes During Growth in Simple Salts/Glucose Media of Different Initial Glucose Concentrations

Glucose Concentration mmol dm^{-3}	Total No of Generations	Exponential Phase		Total Growth Cycle		
		ΔH_{gluc} /kJ (mol gluc)$^{-1}$	ΔH_p /kJ (mol cell)$^{-1}$	ΔH_{gluc} /kJ (mol gluc)$^{-1}$	ΔH_p /kJ (mol cell)$^{-1}$	ΔH_g /kJ (g cell)$^{-1}$ gen^{-1}
0.56	3.89	-815.8	-269.7	-1200.6	-397.0	-3.92
1.10	4.17	-854.2	-271.2	N.D.	N.D.	N.D.
1.70	4.75	-755.5	-256.7	N.D.	N.D.	N.D.
2.20	5.16	-795.4	-265.4	-1120.6	-374.6	-2.79
2.80	5.62	-803.5	-269.4	N.D.	N.D.	N.D.
3.30	5.67	-846.3	-283.5	-1037.9	-350.8	-2.38
3.80	5.70	-825.7	-264.7	-1045.5	-334.5	-2.26
4.20	6.01	-652.7	-214.3	- 950.3	-318.1	-2.04
5.4	6.10	-568.3	-194.5	-1048.8	-358.9	-2.26

Flow rate 90 cm^3 h^{-1}

ΔH_g = ΔH_p/No of generations

N.D. = Not determined

Table 4.3 Elemental Composition of K. aerogenes Grown in Simple Salts/Glucose Media

Element	Mean %	S* %	Mean* %
Carbon	45.80	0.46	45.80 ± 0.64
Hydrogen	6.78	0.09	6.78 ± 0.13
Nitrogen	13.10	0.15	13.10 ± 0.21
Oxygen	29.13	0.47	29.13 ± 1.32
Sulphur (1)	1.05		
Phosphorus (1)	2.42		
Residue	5.13	1.16	5.13 ± 3.24

n = 10

* Standard deviation

** Mean with 95% Confidence Limits

(1) One determination only

A theoretical mass balance equation can be set up for the oxidation of glucose to cellular material, carbon dioxide and water, assuming that these are the only products waste heat (ΔH_{ox}) is also evolved.

$$C_6H_{12}O_6 + X\,NH_3 + Y\,O_2 \longrightarrow Z\,CH_{1.77}O_{0.48}N_{0.25} + L\,CO_2 + N\,H_2O + \Delta H_{ox}$$

The stoichiometric coefficient for the cells (Z) is first determined from molar growth yields, Y_m, and empirical formula weight, M_r, (ie. Y_m/M_r). Once this has been fixed the other coefficients can be calculated.

The waste heat or enthalpy of oxidation, ΔH_{ox}, is estimated as follows. The total energy available is taken to be the heat of combustion of glucose, $\Delta H_s = -2862.1$ kJ mol^{-1}. The quantity of carbon converted to cell material is the cell stoichiometric coefficient (Z); the difference of this to that initially available (ie. 6) is the proportion of carbon oxidised. It is thus possible to calculate the proportion of the initial total energy available that could be lost as heat (ΔH_{ox}).

Experimental stoichiometric coefficients for cell and carbon dioxide are given in Table 4.4. The theoretical stoichiometric coefficient for carbon dioxide (L) is in good agreement with that determined experimentally. The recovery of substrate carbon is good, within the limits of experimental error all can be accounted for as cell carbon and carbon dioxide. ΔH_{gluc} is significantly less than the theoretically predicted value, ΔH_{ox}, at all glucose concentrations.

4.3 Summary

1. Using fixed experimental conditions thermal events accompanying glucose-limited growth were recorded. At concentrations below 3.8 mmol dm^{-3} glucose the p-t trace was a single peak, this

was coincident with the end of exponential growth.

2. The rate constants for biomass, heat and CO_2 output were determined; generally the rate of heat output exceeded that for biomass.

3. q(exp) increases linearly with glucose concentrations up to 3.8 mmol dm^{-3}; q(tot) is linear over the whole concentration range; both lines extrapolate through the origin. When standard conditions are not used the lines do not pass through the origin.

4. Over the glucose concentration range 0.56 to 5.4 mmol dm^{-3} the yield and ΔH_p are constant, ΔH_{gluc} decreases.

5. Mass balance data for aerobic growth is given. Experimental values show that substrate carbon can be accounted for as cell and CO_2. The measured enthalpy change ΔH_{gluc} is significantly below the theoretical value.

Table 4.4 Mass Balance Data for the Aerobic Growth of K. aerogenes with Glucose Limitation

Glucose Concentration /mmol dm^{-3}	Y_m /g(mol gluc)$^{-1}$	Stoichiometric coefficients for mass balance						CO_2	Experimental Values	
		NH_3 X	O_2 Y	Cell Z	CO_2 L	H_2O M	ΔH_{ox} /kJ (mol gluc)$^{-1}$	L	ΔH_{gluc} /kJ (mol gluc)$^{-1}$	% Substrate carbon recorded
0.56	79.2	0.79	2.78	3.17	2.83	4.38	-1350.0	N.D.	-1200.6	N.D.
2.20	77.4	0.77	2.86	3.09	2.91	4.42	-1388.1	3.12	-1120.6	101.6
3.30	77.4	0.77	2.86	3.09	2.91	4.42	-1388.1	3.07	-1037.7	100.8
3.80	79.2	0.79	2.78	3.17	2.83	4.38	-1350.0	3.19	-1045.5	106.4
4.20	77.4	0.77	2.86	3.09	2.91	4.42	-1388.1	3.05	- 950.3	101.8
5.40	73.8	0.74	3.01	2.95	3.05	4.50	-1453.9	N.D.	-1048.8	N.D.

N.D. = Not determined

ΔH_s = -2862.1 kJ (mol gluc)$^{-1}$

ΔH_{ox} = % carbon oxidised x ΔH_s

Mass balance; $C_6H_{12}O_6 + X\,NH_3 + YO_2 \longrightarrow Z\,CH_{1.77}O_{0.48}N_{0.25} + L\,CO_2 + M\,H_2O + \Delta H_{ox}$

Standard deviation of determined values Z and L CO_2 ± 5%.

CHAPTER FIVE

THE EFFECT OF TEMPERATURE ON HEAT OUTPUT OF K. AEROGENES

The growth and test temperature was varied in the range 25 to 42°C, the cells were all grown in a standard medium of glucose concentration 3.3 mmol dm^{-3}, the pump rate through the calorimeter was 90 cm^3 h^{-1}. Care was taken to ensure that the growth and test temperature (ie. the calorimeter block) were the same; any difference would give rise to heating or cooling of the flowing culture and this would produce artefacts in the p-t trace.

5.1 Variation of Growth Parameters with Temperature

With increasing temperature the doubling time decreased to a minimum value at 37°C, thereafter increasing greatly (Table 5.1). The yield coefficient decreased marginally from 25 to 37°C and significantly at higher temperatures. The maximum oxygen tension decreased with increasing temperature, a minimum value occurring at 37°C. At higher temperatures the minimum oxygen tension was initially greater, but at 42°C a value of zero was attained during late exponential growth. It is important to note that the initial saturation level (ie. 100%) is not equivalent for each temperature with respect to the dissolved oxygen concentration. The solubility decreases with increasing temperature, a value of, say, 37% pO_2 is thus a function of the initial saturation level.

Carbon dioxide production increases over the whole temperature range, at 42°C it is twice that at 25°C (Table 5.1). The theoretical stoichiometric coefficient for CO_2 is significantly lower than the measured value, with the exception of 37°C. There is no significant difference in the minimum pH values recorded during growth at different temperatures. Acetate was detected in the culture medium at temperatures above 37°C; 1.1 and 0.4 mol acetate $(\text{mol gluc})^{-1}$ were recorded at 40.5 and 42°C.

Table 5.1 Variation of Growth Parameters with Temperature

Temperature /°C	Doubling time /h	Yield coefficient /g g^{-1}	Minimum pO_2 /%	Minimum pH	CO_{2_p} /mmol g^{-1}	$CO_{2_{gluc}}$ /mol (mol gluc)$^{-1}$	Theoretical $CO_{2_{gluc}}$ /mol (mol gluc)$^{-1}$
25.0	1.20 ± 0.15	0.48 ± 0.03	40.7 ± 9.0	6.82 ± 0.04	33.6 ± 1.27	2.90	2.61 ± 0.13
29.3	0.97 ± 0.04	0.45 ± 0	38.3 ± 8.2	6.86 ± 0.01	35.5 ± 0.91	3.09	2.80 ± 0.14
33.0	0.78 ± 0.10	0.46 ± 0.02	18.3 ± 5.0	6.85 ± 0.07	40.5 ± 3.8	3.35	2.71 ± 0.14
37.0	0.76 ± 0.04	0.43 ± 0.02	18.8 ± 4.9	6.83 ± 0.06	39.7 ± 5.0	3.07	2.90 ± 0.15
39.4	0.96 ± 0.06	0.46 ± 0.02	40.5 ± 10.6	6.80 ± N.D.	40.8 ± 2.30	3.38	2.69 ± 0.13
40.5	1.04 ± 0.07	0.41 ± 0	41.0 ± 2.6	6.88 ± 0.03	49.7 ± 2.90	3.67	3.05 ± 0.15
41.4	1.30 ± N.D.	0.41 ± 0.10	58.0 ± 7.6	N.D.	59.4 ± 6.6	4.38	3.05 ± 0.15
42.0	1.79 ± 0.32	0.27 ± 0.05	0	6.72 ± 0.05	75.6 ± 7.5	3.67	4.06 ± 0.20

* Expressed as % of full saturation at specified temperature

N.D. = Not determined

To overcome the oxygen starvation at 42°C the culture was oxygenated with pure oxygen. This increased the yield (Y_{gluc} = 0.29) but the oxygen tension was below 10% for a considerable period.

5.2 Elemental Composition of Cells Grown at Different Temperatures

Elemental analysis of cells grown at 27, 32 and 42°C were not significantly different to that of cells grown under standard conditions at 37°C, (Table 5.2). Using these elemental compositions a plot of formula weight against temperature was prepared from which formula weights were obtained for all temperatures used; these were used for calculating ΔH_p and mass balance data.

5.3 Variation of Power-Time Trace Profiles with Temperature

The p-t trace for cells growing under standard conditions at 37°C consisted of a single peak with rapid re-attainment of the baseline. Growth at temperatures below 37°C produced a shoulder on the decay side of the peak (Fig. 5.1). At 25°C the shoulder extended for about 0.5 h and for about 1 h at 33°C. At 39°C the shoulder was present but with increasing temperature the shoulder became a period of constant but low power output. At 40.5°C this near constant region lasted for about 2 hours (Fig. 5.1), while at 42°C it extended up to 9 hours. During this period there was considerable oxygen demand and carbon dioxide output. With oxygenated growth at 42°C a similar p-t trace was obtained.

The specific power output for the different temperatures showed the same characteristics, during exponential growth, as that illustrated for standard growth at 37°C (Fig. 3.4). The end of exponential growth was marked by a very rapid fall in power output. The maximum power output attained (peak height) increased linearly with temperature up to 37°C (Fig. 5.2), hereafter decreasing rapidly in a biphasic manner.

Table 5.2 Elemental Composition of K. aerogenes Grown in Salt Media/Glucose Limiting at Different Temperatures

Temperature °C	Carbon* %	Hydrogen* %	Nitrogen* %	Residue* %	Oxygen* %
27	45.05 ± 0.17	6.75 ± 0.17	12.85 ± 0.06	2.50 ± 2.01	32.85 ± 2.6
32	45.70 ± 0.31	6.77 ± 0.06	12.93 ± 0.66	4.07 ± 2.50	30.3 ± 2.8
42	45.88 ± 0.50	6.93 ± 0.09	11.6 ± 0.17	4.30 ± 0.37	31.23 ± 0.5

* n = 4; Mean with standard deviation

FIGURE 5.1. Effect of temperature on power-time trace profile

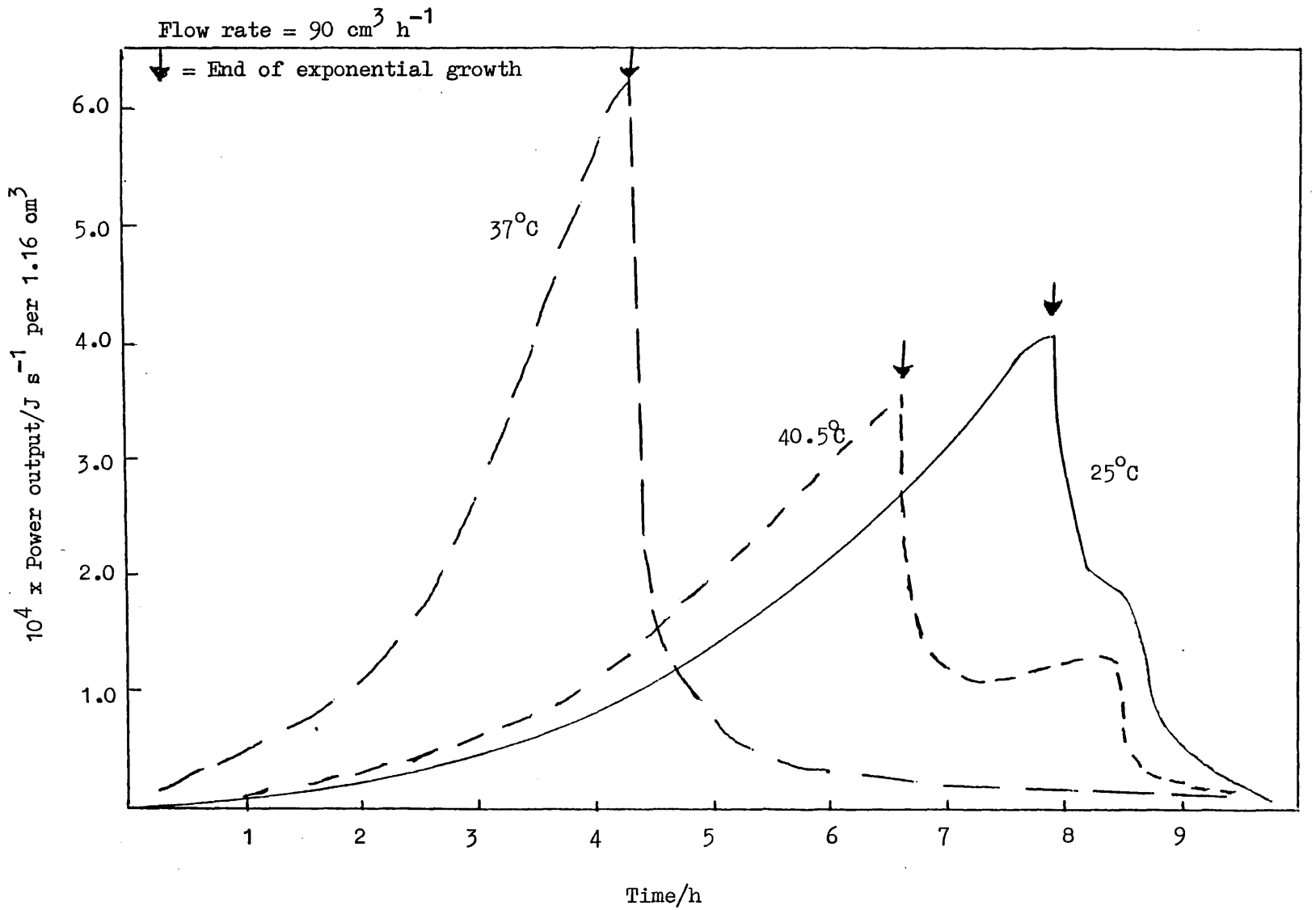
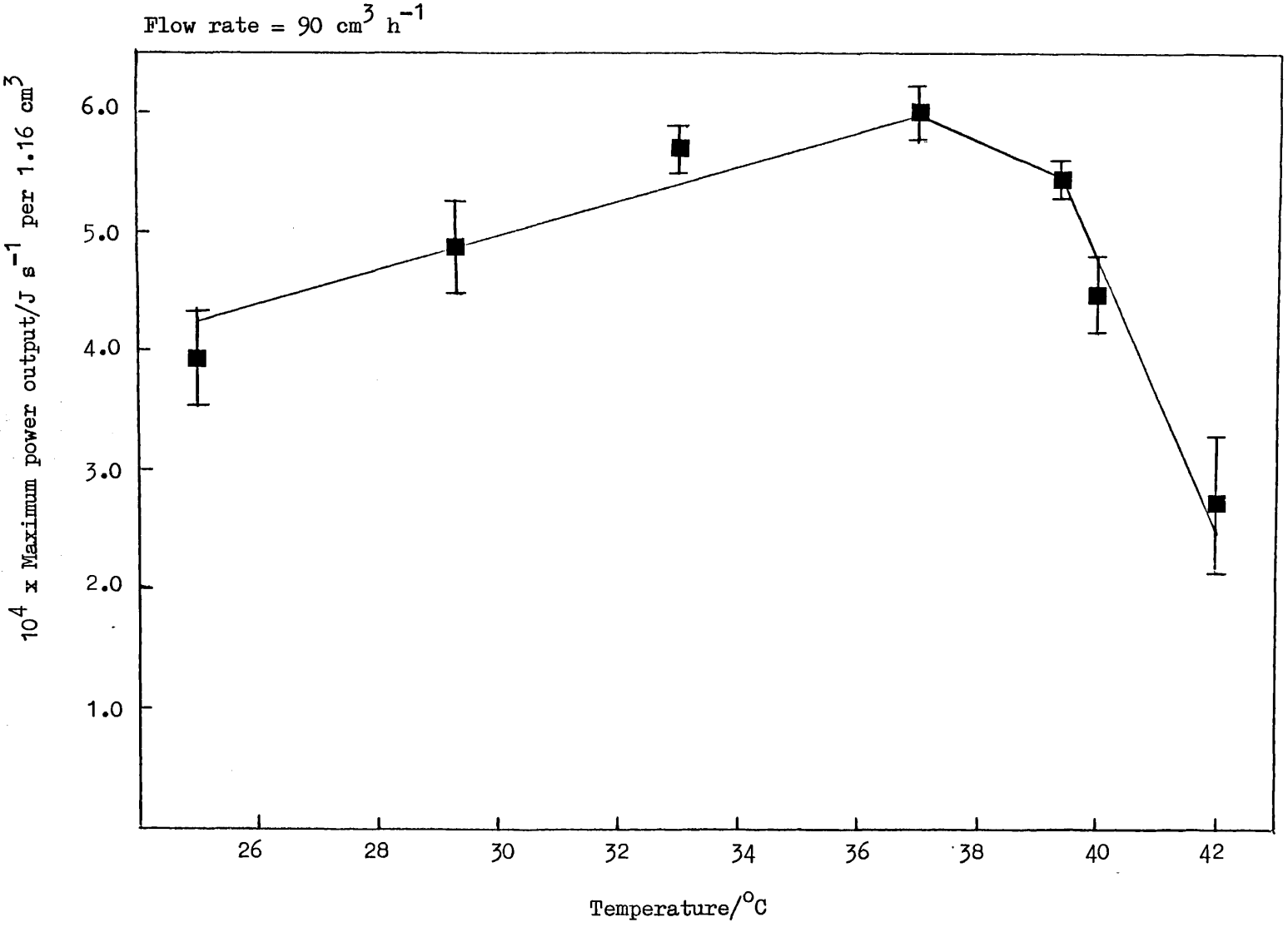


FIGURE 5.2. Effect of temperature on the value of maximum power output



5.4 Variation of Rate Constants with Temperature

Rate constants, from exponential growth log plots, for the increase in heat output, biomass and CO_2 output were determined (Fig. 5.3). All rate constants reached a maximum value at 37°C, thereafter decreasing very rapidly.

Over the whole temperature range the rate constants for power and CO_2 output are similar, except at 37°C where carbon dioxide is marginally lower. Below 37°C the rate constants for biomass is lower than those for power and CO_2 output. At temperatures above 37°C there is no significant difference in the value of the three rate constants.

5.5 Variation of Heat Output with Temperature

The enthalpy changes, ΔH_{gluc} and ΔH_p, show similar trends with temperature (Fig. 5.4), becoming marginally more positive from 25 to 37°C. Between 37 and 40.5°C there is a small decrease, followed by a rapid decrease at 42°C. The values of ΔH_{gluc} and ΔH_p at 42°C were 1.7 and 2.8 times larger than the corresponding values at 37°C.

5.6 Summary

1. Y_{gluc} decreases, CO_2 output increases and the oxygen tension decreases with increasing growth rate and test temperature.

2. The elemental composition of the cells is not significantly affected by growth temperature.

3. The profile of the p-t traces varies with temperature; a shoulder occurs on the decay side except at 37°C. At high growth temperatures the shoulder becomes a region of constant low power output.

4. The rate constants for biomass, CO_2 production and heat oxygen increase in parallel from 25 to 37°C thereafter decreasing abruptly.

5. Enthalpy values, ΔH_{gluc} and ΔH_p, change marginally from 25° to 40.5°C, at higher temperatures they rapidly become more negative.

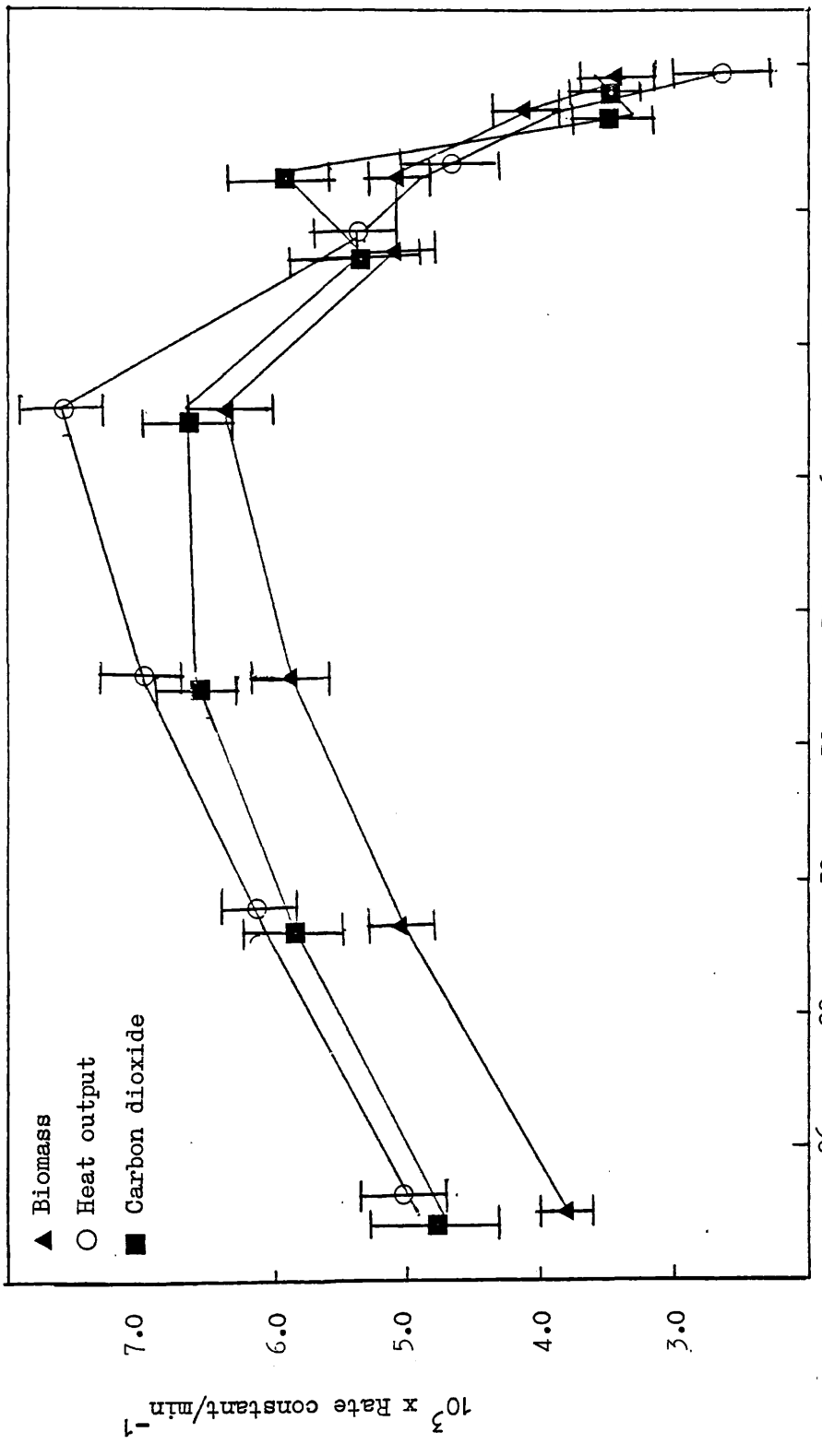
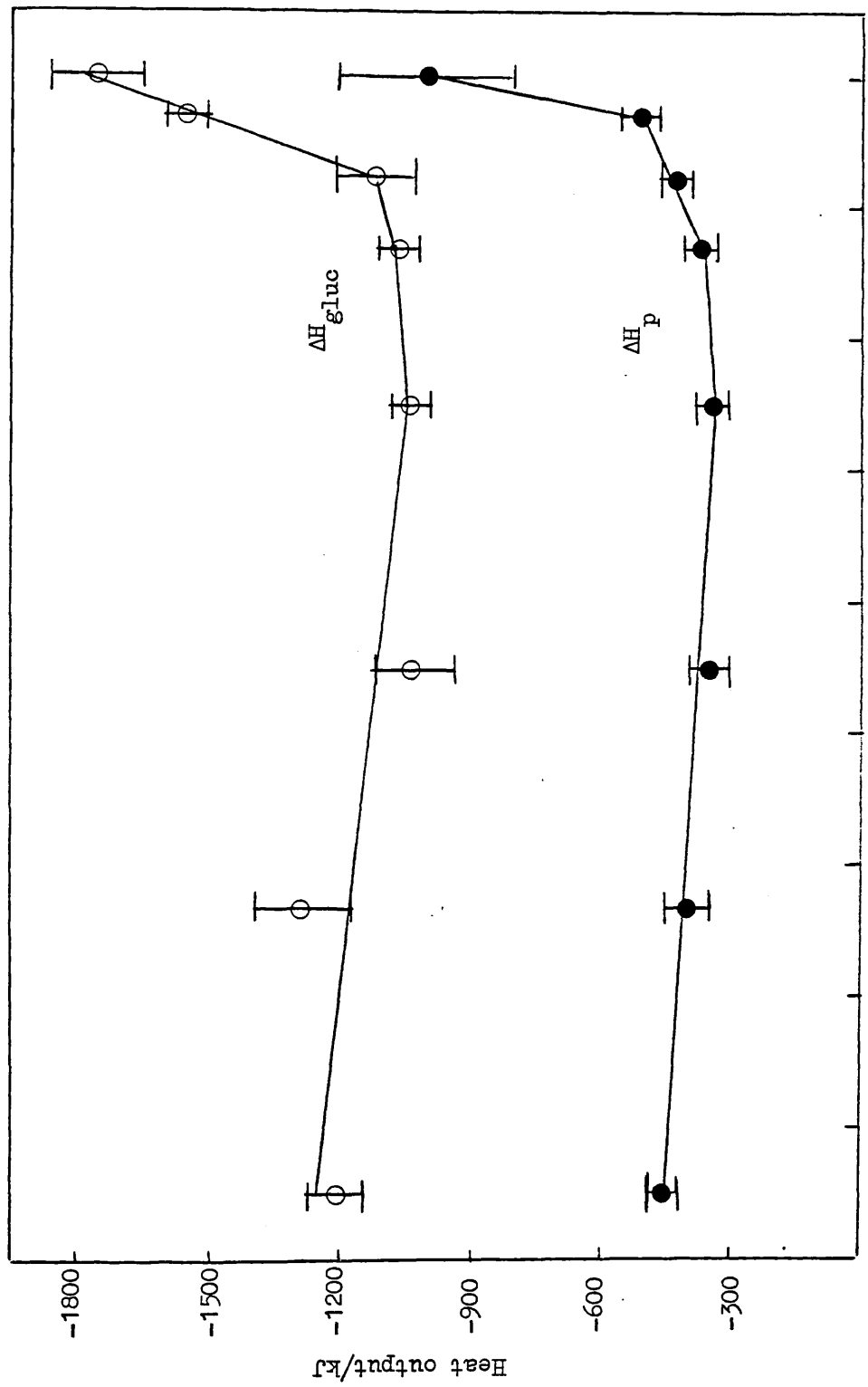


FIGURE 5.2. Rate constants for heat output, biomass and carbon dioxide during exponential growth as a function of temperature

FIGURE 5.4. Variation of enthalpy changes with temperature for glucose-limited growth



CHAPTER SIX

THE EFFECT OF CHANGED CONDITIONS ON THERMAL AND GROWTH PARAMETERS DURING GROWTH OF K. AEROGENES IN STANDARD GLUCOSE MEDIA

6.1 Growth in Presence of Oxygen

There are several reports that the presence of excess oxygen is growth inhibitory and changes metabolic processes. Little work has been done on this topic; it was felt that enthalpy changes could provide an insight into metabolic changes occurring in the presence of excess oxygen.

The culture vessel was aerated with pure oxygen (B.O.C. nitrogen free), other growth and test conditions were as for standard growth (3.5).

6.1.1 The Effect of Pure Oxygen on p-t Trace and Growth Parameters

The p-t trace during growth in pure oxygen at a flow rate of 0.8 dm^3 min^{-1} was similar to those reported previously (Fig. 3.4), consisting of a simple peak; the maximum power output, 5.7×10^{-4} J s^{-1}, coincided with the end of exponential growth. With increased oxygenation (1.2 dm^3 min^{-1}) the p-t trace changed and consisted of two broad peaks, the first peak occurred during exponential growth and large amounts of heat were evolved after the cessation of growth.

With an oxygen flow rate of 0.8 dm^3 h^{-1} and glucose 3.3 mmol dm^{-3} Y_{gluc} was greater than for standard aerobic growth. (Table 6.1), but at higher oxygen levels the Y_{gluc} was less. The doubling time increased dramatically with increasing oxygen. Carbon dioxide produced was less than that for aerobic growth and carbon recovery was poor. Although pure oxygen was supplied the oxygen tension in the calorimeter line fell to a low level; the decrease was greatest at higher oxygen levels remaining below 10% saturation for a considerable time with an oxygen inlet flow rate of 1.2 dm^3 min^{-1}. Acetate was detected during oxygen-sufficient growth, 0.9 mol acetate (mol gluc)$^{-1}$ was recorded with initial glucose 3.3 mmol dm^{-3}.

Table 6.1 Thermal and Growth Parameters of K. aerogenes During Growth Under Aerobic, Oxygenated and Anaerobic Conditions

Growth conditions	Gluoose concentration /mmol dm^{-3}	†* Increase in biomass /mg cm^{-3}	*Y_{gluc} /(g cell)g^{-1}	Doubling time /h	CO_2 gluc /mol (mol gluc)$^{-1}$	Carbon recovery /%	ΔH_p /kJ (mol cell)$^{-1}$	ΔH_{gluc} /kJ (mol gluc)$^{-1}$
Standard aerobic growth	3.3	0.256	0.43	0.7	3.07	100.8	-350.8	-1037.9
	1.7	0.124	0.43	0.7	N.D.	N.D.	N.D.	N.D.
Oxygen Flow rate								
0.8 dm^3 min^{-1}	3.3	0.300	0.50	1.3	N.D.	N.D.	-527.4	-1906.8
0.8 dm^3 min^{-1}	1.7	0.126	0.42	0.8	1.53	85.3	-1237.1	-3539.3
1.2 dm^3 min^{-1}	3.3	0.228	0.38	3.0	N.D.	N.D.	-3176.3	-8742.8
Standard aerobic growth (1)	3.3	0.256	0.43	0.7	3.07	100.8	- 283.5	- 846.3
	1.7	0.124	0.43	0.7	N.D.	N.D.	- 256.7	- 755.5
Anaerobic (1)	3.3	0.102	0.17	1.6	N.D.	N.D.	- 248.0	- 391.0
	1.7	0.048	0.16	1.9	1.15	38.2	- 723.0	- 785.0
	1.1	0.036	0.18	2.7	1.89	63.6	-1163.0	-1465.0

* At end of exponential growth phase

† Initial biomass 0.006 mg cm^{-3}

(1) Enthalpy changes for exponential growth only

6.1.2 Effect of Pure Oxygen on Enthalpy Changes

ΔH_p and ΔH_{gluc} are considerably lower than those for standard aerobic growth (Table 6.1) ie. increase in heat evolved during the formation of one mol of cells or consumption of one mol of glucose. At high oxygen levels very large amounts of heat were dissipated.

6.2 Anaerobic Growth

K. aerogenes is a facultative anaerobe, little is known about anaerobic growth energy changes. Anaerobic growth was carried out so that calorimetric data could be determined and compared with aerobic growth.

6.2.1 Power-Time Trace and Growth Parameters Under Anaerobic Conditions

The culture vessel was flushed with pure nitrogen (B.O.C. oxygen free) at a flow rate of 1.6 dm^3 min^{-1}. The complete apparatus, culture medium and all flow lines were equilibrated with nitrogen flushed medium for at least 3 hours.

The p-t trace consisted of a small peak during exponential growth, followed by a period of lower power output and then a smaller second peak which coincided with the end of exponential growth. After the second peak there was a considerable period of lower (near constant) power output. During growth on 3.3 mmol dm^{-3} glucose this period lasted for 20 hours; the maximum power output was 1.34 J s^{-1}.

Y_{gluc} was about a third of that during standard aerobic growth (Table 6.1). The doubling time was long, and increased with decreasing glucose concentration. Carbon dioxide production was low and carbon recovery poor.

During anaerobic growth acetate was produced in large quantities; a maximum of 0.7 mol acetate per mol glucose was attained in the early stationary phase. After termination of exponential growth on 1.1 mmol dm^{-3} glucose the biomass increased by 0.030 mg cm^{-3} over a period of about 15 hours.

6.2.2 Enthalpy Changes During Anaerobic Growth

ΔH_p and ΔH_{gluc} were very low (Table 6.1), during growth in 1.1 mol dm^{-3} glucose they are 16 and 7 times lower than the respective values for aerobic growth (Table 6.1). At higher glucose concentrations since the total heat output was so large, exceeding the full scale deflection of the recorder, only the exponential heat output was measured and tabulated (Table 6.1). For glucose concentrations 3.3, 1.7 and 1.1 mmol dm^{-3} $\Delta H_p(exp)$ is always much lower (and increases with glucose concentration) than the corresponding average value, -395 kJ $(mol\ cell)^{-1}$, for standard aerobic growth. However the $\Delta H_{gluc}(exp)$ (Table 6.1) are mostly greater than the mean for aerobic growth (-1183 kJ $(mol\ gluc)^{-1}$). The constancy of ΔH_p and ΔH_{gluc} under anaerobsis with respect to glucose concentration is lost. Comparison of q(exp) and q(tot) clearly shows that most heat output occurred during the stationary phase.

6.3 Growth in the Presence of an Added Second Carbon Source

Several investigators have showed that intermediates of the tricarboxylic acid (TCA) cycle can affect the production of certain TCA cycle enzymes. It was considered that the enthalpy changes could be altered by an enzyme in-balance.

Quantitative thermal and growth data were gathered with a flow rate through the calorimeter of 90 $cm^3\ h^{-1}$, and standard conditions of aeration (3.5). Some qualitative data was gathered with a flow rate of 40 $cm^3\ h^{-1}$. The cells were always grown in medium of glucose

concentration 3.3 mmol dm^{-3} to which a second carbon source was added to give the required concentration. Where possible the second carbon source was added as a sterile solution.

6.3.1 Ethanol, Acetate and Succinate

The addition of these substrates to the media at zero time produced a change in the standard p-t trace (Fig. 6.1 A). On the decay side of the peak, which occurred at the end of exponential growth, a shoulder or period of near constant power output occurred; the extent of this period was dependent on the concentration of the added substrate. ΔH_p was significantly lower than that for standard growth. Yield coefficients with respect to glucose, Y_{gluc}, indicate that some of the additional substrate was used (Table 6.2). In terms of carbon the yield coefficients are lower than for growth on glucose alone. Y_{gluc} decreased and the carbon dioxide output increased with an increase in the amount of ethanol added.

6.3.2 α-Ketoglutorate and Citrate

The presence of either of these two carbon sources produced a shoulder on the decay side of the p-t trace peak (Fig. 6.1 A). Citrate ΔH_p values were significantly less than in glucose while Y_{gluc} values indicated possible conversion of added substrate to cellular material; Y_{carbon} was well below the value for growth in glucose alone (Table 6.2).

FIGURE 6.1. Diagramatic power-time traces of standard growth in the presence of a second carbon source

A and C second carbon added at zero time

B only second carbon added at end of growth

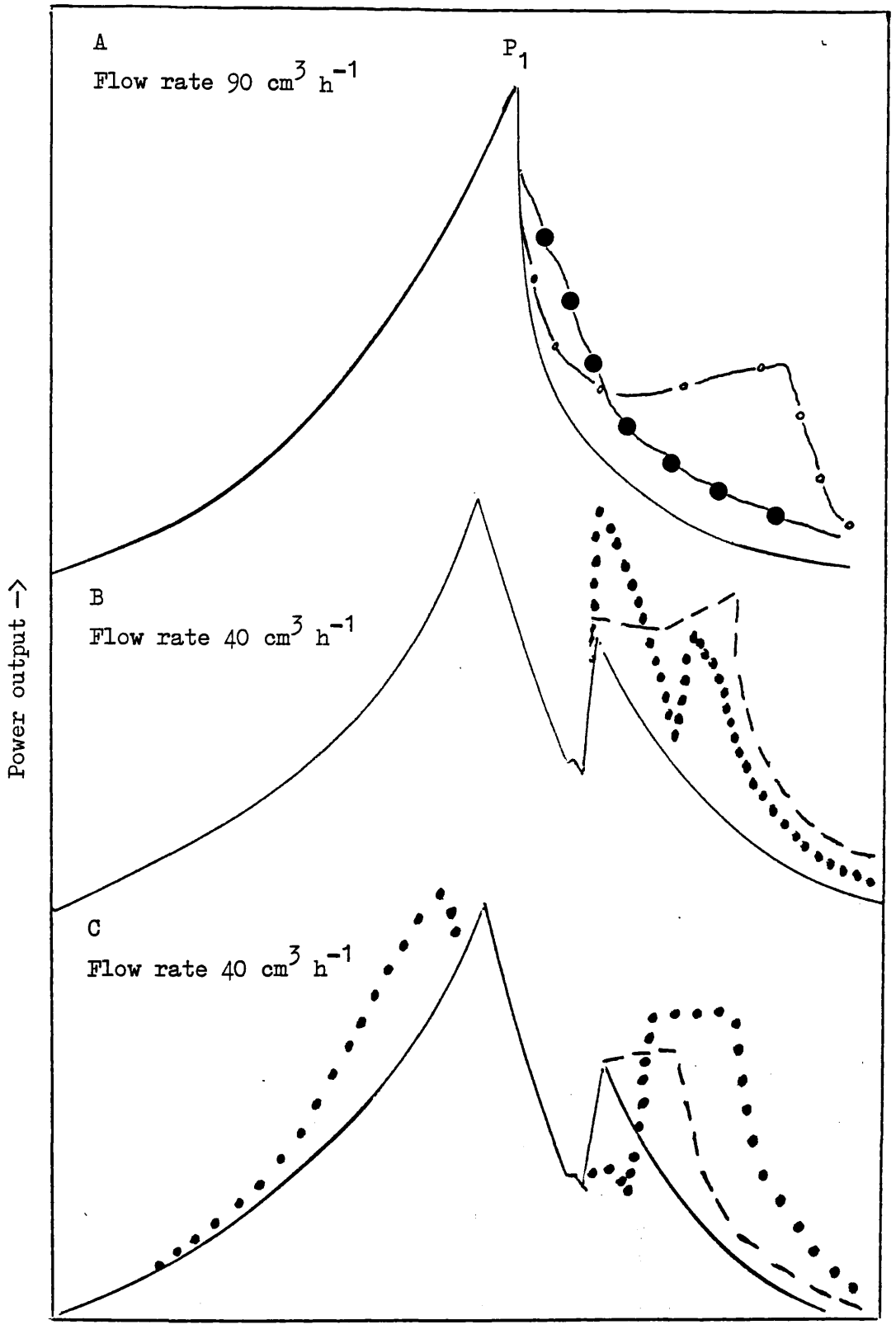


Table 6.2 Growth and Thermal Parameters During Growth in Standard Glucose Media in the Presence of a Second Carbon Source (added at zero time)

Pump rate 90 cm^3 h^{-1}

Second substrate	Concentration /mmol dm^{-3}	Y_{gluc} /g cell (g gluc)$^{-1}$	Y_{carbon} /g cell (g carbon)$^{-1}$	CO_2(gluc) /mol (mol gluc)$^{-1}$	10^4 x Maximum power output /J s^{-1}	ΔH_p /kJ (mol cell)$^{-1}$
None	0	0.43	1.07	3.05	6.00	-350 ± 30
α-Ketoglutarate	1.1	0.52	0.78	5.63	6.01	-405.7
Citric acid	2.0	0.55	0.52	6.01	5.95	-407.2
	1.0	0.52	0.69	N.D.	5.45	-209.7
	0.5	0.43	0.74	3.52	5.98	-363.9
Succinic acid	2.0	0.50	0.63	3.19	6.16	-347.6
Ethanol	7.6	0.53	0.76	8.49	5.63	-405.7
	1.7	0.43	0.96	3.65	6.05	-398.9

N.D. = Not determined

In all the above examples the p-t trace profile was paralleled by concomitant changes in the effluent carbon dioxide and oxygen tension profiles. The maximum power output did not vary significantly (Table 6.2). Only in the presence of ethanol was any significant biomass increase observed during the second period of heat output (ie. the normal stationary phase).

6.3.3 Pyruvate and Acetate

The thermal data for these substrates was collected with a flow rate of 40 cm^3 h^{-1} through the calorimeter. The presence of pyruvate (0.7 mmol dm^{-3}) from zero time caused an increase in growth rate, the doubling time was 36 mins; normally 45 mins. The rate of increase in heat output was 8.25×10^{-3} min^{-1} compared with 7.66×10^{-3} min^{-1} for growth in glucose only. When pyruvate was depleted the p-t trace returned to that for standard glucose growth (Fig. 6.1 c). The second peak (P_2) was considerably broadened presumably due to increased power output accompanying the oxidation of secondary intermediates such as acetate.

When acetate was present in the media from zero time the only observed change in the p-t trace was a broadening of the second peak (Fig. 6.1 C).

When pyruvate or acetate was added at a time coincident with the end of exponential growth there was additional power output manifested either as two peaks or a broadening of the second peak respectively (Fig. 6.1 B). The addition of pyruvate always resulted in further biomass production.

6.4 Thermal Changes During the Growth of K. aerogenes in the Presence of Uncoupling Agents and Inhibitors

ATP production by oxidative phosphorylation occurs by a complex process involving a membrane - localized electron transport chain. The presence of certain compounds, uncouplers, will halt the production of ATP, oxidation of the substrate will proceed and produce heat instead of ATP. The aims of these experiments were to determine the thermal and enthalpy changes that occur when uncouplers are present, also to determine these values when growth inhibitory compounds are added eg. chloramphericol which inhibits protein synthesis. Uncouplers of oxidative phosphorylation are FCCP and DNP. Sodium azide inhibits cytochrome oxidase and possibly ATPase. Antimycin A (uncouples the first coupling site) and oligomycin (inhibits ATPase) do not generally affect bacterial growth, it was considered that thermal events may show some unknown effect.

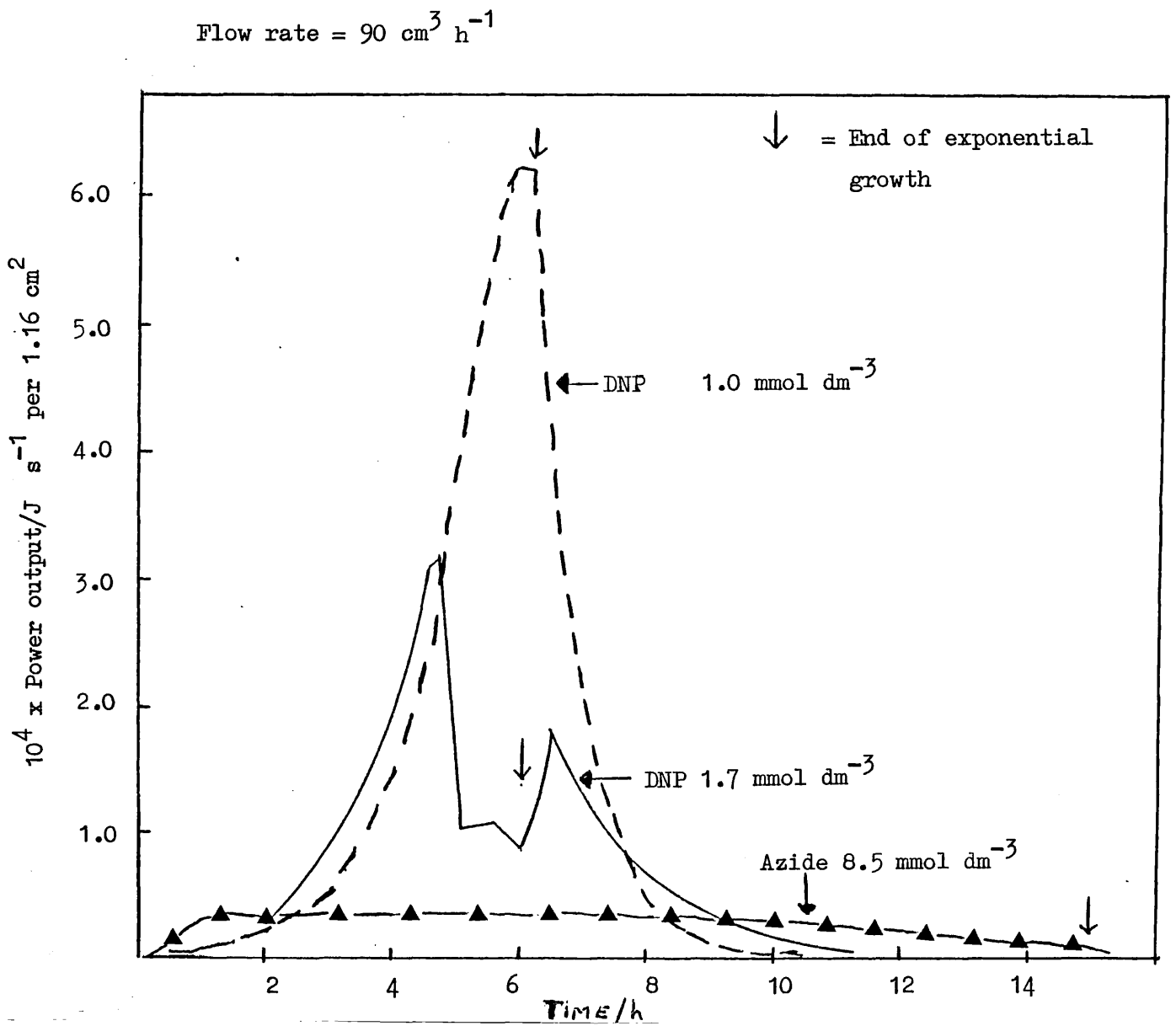
All the experiments were made using "standard" test conditions, ie. 3.3 mmol dm^{-3} glucose; pump rate through calorimeter = 90 cm^3 h^{-1}. In one set of experiments inhibitor was added at zero time, while in the second set the inhibitor was added at a biomass of 0.113 mg cm^{-3} dry weight, ie. about halfway through exponential growth.

Only important differences from standard growth are described. The carbon dioxide output in the effluent gas had a similar profile to the p-t trace, while the oxygen tension in the medium was inverse.

6.4.1 Power-Time and Growth Parameters

A. Addition of inhibitors at zero time, DNP (1.1 mmol dm^{-3}) caused no change in the p-t trace, but at higher concentrations a marked change occurred (Fig. 6.2). The exponential heat output continued for about 4.5 h before there was an abrupt decrease, followed by a period

FIGURE 6.2. The effect of inhibitor on the power-time trace; addition at zero time



of near constant power output and then a second maximum power output. At a concentration of 1.7 mmol dm^{-3} DNP these features were more accentuated than at 1.3 mmol dm^{-3}.

Yields were lowered with increasing DNP concentration (Table 6.3). The oxygen tension attained a lower minimum earlier during exponential growth than for standard growth; further it remained low (about 10% saturation) until growth ended. The pH decrease was also greater, in 1.7 mmol dm^{-3} DNP the minimum was 6.5 with a small rise (about 0.05) during the stationary phase.

Sodium azide at a concentration of 8.5 mmol dm^{-3} was very inhibitory to growth, $Y_{gluc} = 0.03$. The power output was of a low constant value until near depletion of glucose where it decreased slowly (Fig. 6.2). Carbon dioxide output was at a low level (40 p.p.m.); the oxygen tension never fell below 95% saturation, but the pH fell considerably, 6.75.

Chloramphenicol inhibited biomass production (Table 6.3); the p-t trace was similar to that for azide. Carbon dioxide output was low (20 p.p.m.) and the final CO_2 produced, $CO_{2_{gluc}}$, was very low; in contrast for azide it was high. Oligomycin (0.016 g dm^{-3}) produced no changes in the growth or p-t trace parameters.

B. Addition of inhibitors during exponential growth. The addition of sodium azide (8.5 mmol dm^{-3}) caused cessation of exponential growth and an immediate and dramatic decrease in the power output (Fig. 6.3). The power output then remained constant at a lower level until the depletion of glucose, during which time there was a small increase in biomass (0.019 mg cm^{-3}). The effluent carbon dioxide also decreased to a constant value of 40 p.p.m. until glucose depletion. With 1 mmol dm^{-3} DNP or 0.01 mmol dm^{-3} FCCP the p-t traces were similar to that of the control. Higher concentrations of either inhibitor

Table 6.3 *The Enthalpy Changes of Cells of K. aerogenes Grown in Standard Glucose Medium in the Presence of Inhibitors*

Inhibitor	Concentration /mmol dm^{-3}	*Increase of biomass /mg cm^{-3}	Y_{gluc} g cell (g gluc)$^{-1}$	ΔH_p /kJ (mol cell)$^{-1}$	ΔH_{gluc} /kJ (mol gluc)$^{-1}$	$CO_{2_{gluc}}$ /mol (mol gluc)$^{-1}$
A. Addition of inhibitor at zero time						
Control	0	0.256	0.43	- 350.8	-1037.7	3.07
DNP	1.0	0.226	0.38	- 647.2	-1705.7	2.62
	1.3	0.150	0.25	- 645.1	-1128.4	N.D.
	1.7	0.143	0.24	- 567.0	- 945.6	N.D.
Sodium azide	8.5	0.020	0.03	-1369.5	- 339.6	1.01
Chloramphenicol	1.0	0.036	0.18	-2180.3	-2750.1	0.13
B. Addition of inhibitor during exponetial growth						
DNP	1.0	0.195	0.33	- 699.6	-1590.8	3.98
	2.0	0.147	0.25	- 598.9	-1026.6	3.80
Sodium azide	7.9	0.130	0.22	- 884.9	-1426.2	4.34
FCCP	0.01	0.258	0.43	- 393.3	-1243.4	2.32
	0.03	0.258	0.43	- 386.7	-1222.5	3.25
	0.06	0.195	0.33	- 462.5	-1128.4	3.81
	0.13	0.143	0.24	- 644.0	-1136.3	4.10
Antimycin A	0.07	0.270	0.45	- 329.4	-1167.6	2.03

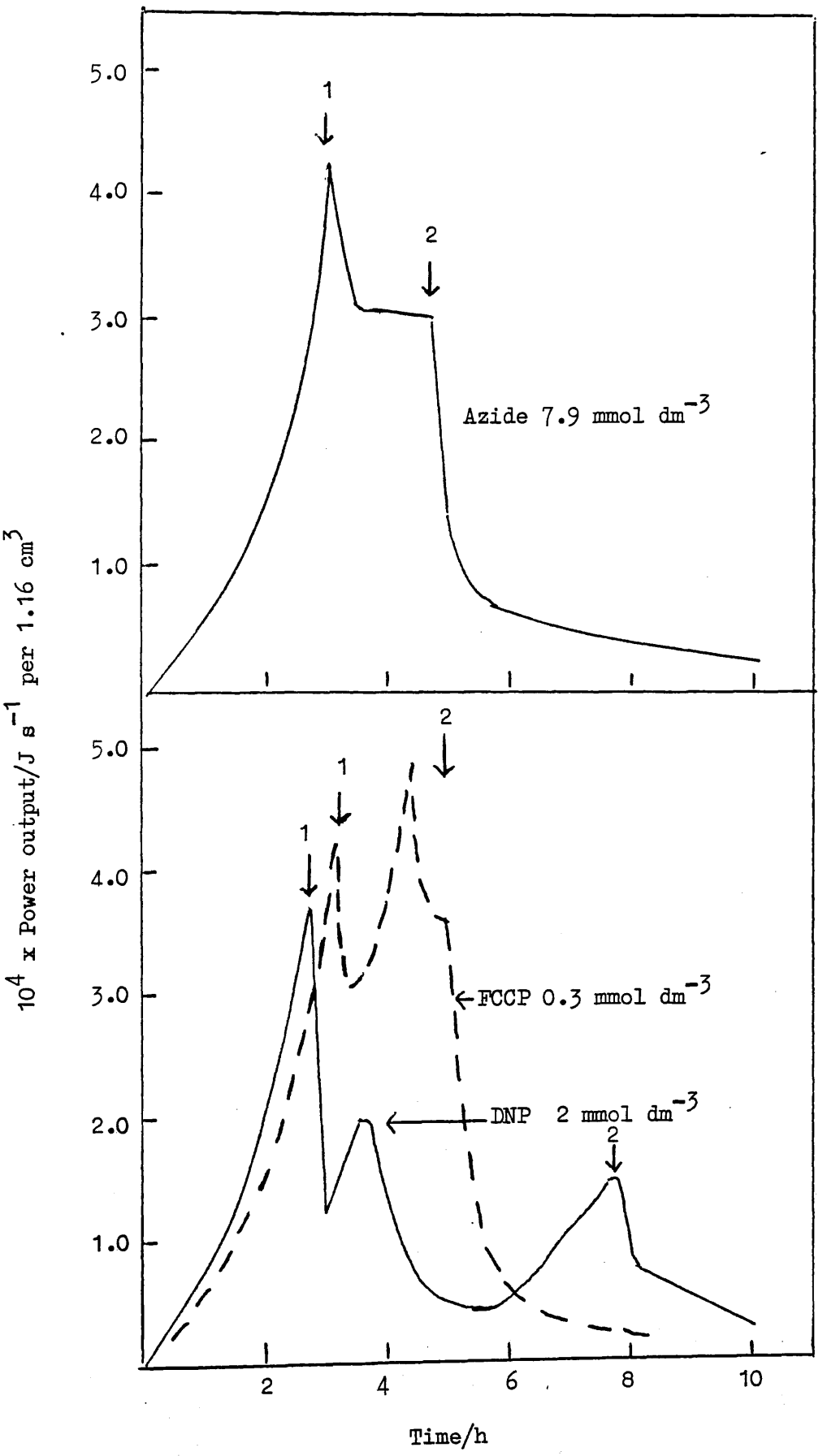
* Initial biomass 0.006 mg cm^{-3}

N.D. = Not determined

FIGURE 6.3 The effect of inhibitor on the Power-time trace; addition during exponential growth

1. = Addition of inhibitor

2. = Depletion of glucose



resulted in a decrease of the power output for about 0.5 h after which it increased again to give the second peak (Fig. 6.3). The extent of the decrease and increase depended on inhibitor concentration. At a concentration of 2 mmol dm^{-3} DNP there was a third maximum in power output after 7.5 h; a similar peak occurred with 0.13 mmol dm^{-3} FCCP after about 14h.

After the addition of DNP or FCCP at a biomass of 0.113 mg cm^{-3} exponential growth ceased and biomass production continued at a very slow rate; final yields were very low (Table 6.3). In general the carbon dioxide profile was similar to the p-t trace except at 2 mmol dm^{-3} DNP, where an additional small trough, superimposed on the broad trough of the CO_2 trace, coincided with the broad minimum of the p-t trace profile (Fig. 6.3).

FCCP at concentrations less than 0.03 mmol dm^{-3} produced a slight broadening of the standard peak (P_1) in the p-t trace. No change in growth rate was observed but the rate of carbon dioxide output increased. However the final CO_2 (gluc) was similar to that found for the control. At higher concentrations of FCCP carbon dioxide production increased (Table 6.3). Preliminary measurements of ATP showed that within 10 minutes of adding FCCP (0.13 mmol dm^{-3}) the ATP concentration was reduced to a third of the value at the time of addition.

The addition of antimycin A (0.07 mmol dm^{-3}) did not affect the p-t trace. The only observed change was a decrease in the rate of carbon dioxide output; the total carbon dioxide produced was lower than that for standard growth, but the yield was a little higher.

6.4.2 The Influence of Inhibitors on the Elemental Composition of the Cells

Cells from inhibitor calorimetric studies were harvested after exponential growth had ceased; washed three times with distilled water and then oven dried.

Significant differences ($P = 0.05$) were found between the composition of standard cells and cells grown in the presence of FCCP and antimycin A with respect to their oxygen and carbon contents (Table 6.4). From the elemental composition empirical formulae and formula weights were calculated, the latter values used in calculations of ΔH_p.

6.4.3 Enthalpy Changes in the Presence of Inhibitors

DNP (1 mmol dm^{-3}) stimulated heat output but at higher concentrations ΔH_{gluc} was slightly greater than the standard glucose value. Sodium azide when present from zero time produced dramatic increases in ΔH_{gluc} but produced a decrease if added during exponential growth (Table 6.3). Chloramphenicol produced a marked decrease in ΔH_{gluc}.

ΔH_p values during growth in the presence of DNP, sodium azide FCCP and chloramphenicol were always less than that of the control standard glucose (Table 6.2). Chloramphenicol had the most dramatic effect decreasing ΔH_p by a factor of 5.

6.4.4 The Effect of Inhibitors on the Fate of Substrate Carbon and Energy

The amount of substrate carbon converted to cellular material decreased with increasing DNP and FCCP concentration (Table 6.5). Sodium azide and chloramphenicol caused a large decrease in conversion of substrate to cellular material, while antimycin A produced a small increase. The recovery of substrate carbon as cell material and carbon dioxide

Table 6.4 Mean Elemental Composition of Cells of K. aerogenes Grown in the Presence of Inhibitors

Cells	Carbon %	Hydrogen %	Oxygen %	Nitrogen %	Residue %	Emprical formula	Formula weight
Control	45.8	6.8	29.1	13.1	5.1	$CH_{1.77}O_{0,48}N_{0.25}$	24.98
Sodium azide $7.9\ mmol\ dm^{-3}$	47.3	6.8	27.1	13.0	5.6	$CH_{1.72}O_{0.48}N_{0.24}$	24.46
FCCP $0.13\ mmol\ dm^{-3}$	47.1	7.2	30.2	12.8	2.7	$CH_{1.82}O_{0.48}N_{0.24}$	24.93
Antimycin A	49.0	6.7	24.7	13.6	6.0	$CH_{1.64}O_{0.38}N_{0.24}$	23.03

Table 6.5 Fate and Recovery of Substrate Carbon During Growth of K. aerogenes in Standard Glucose Media in the Presence of Inhibitors

Inhibitor	Concentration /mmol dm^{-3}	Fate of substrate carbon: Converted to cell * %	Oxidised (Theoretical)* %	Recovered as CO_2 %	Total recovered %	Recovery of total substrate energy as heat %
A. Addition of inhibitor at zero time						
Control	0	51.7	48.3	50.6	102.3	43.0
DNP	1.0	43.9	56.1	43.2	87.1	60.0
1.3	1.3	28.9	71.1	N.D.	N.D.	39.4
	1.7	27.7	72.3	N.D.	N.D.	33.0
Sodium azide	8.5	3.7	96.3	16.7	20.4	11.9
Chloramphenicol	1.0	21.6	78.4	2.1	23.7	96.7
B. Addition of inhibitor during exponential growth						
DNP	1.0	38.8	61.2	65.7	104.5	55.6
	2.0	29.4	70.6	62.7	92.1	35.9
Sodium azide	7.9	27.0	73.0	71.7	98.6	49.8
FCCP	0.01	55.9	44.1	38.3	94.1	43.4
	0.03	55.9	44.1	53.6	109.5	42.7
	0.06	40.0	60.0	62.9	102.9	39.4
	0.13	29.1	70.9	67.7	96.8	39.7
Antimycin A	0.07	58.5	41.5	33.5	92.0	40.8

* Section 4.2.2.

N.D. Not determined

was low in the presence of all inhibitors, particularly for sodium azide and chloramphenicol. Glucose was never detected at the end of growth, acetate was detected during growth in the presence of DNP.

The amount of carbon for oxidation processes can be equated with the proportion of the total energy available from combustion of the substrate (glucose). In most cases the quantity of heat recovered (ΔH_{gluc}) is less than the theoretical maximum, ΔH_{ox} (Table 6.5).

6.5 Summary

1. Aeration with pure oxygen causes marked changes in the p-t trace and growth parameters of glucose-limited growth. Enthalpy changes are very reduced, ie. greater heat output.

2. Anaerobic growth results in very low yields and CO_2 production. Enthalpy changes are very much below the expected values.

3. The presence of a second carbon source affected the growth and thermal parameters of standard glucose growth. Only pyruvate affected the exponential growth period. Succinate, ethanol and acetate increased the stationary phase power and heat output more than α-ketoglutarate or citrate.

4. The presence of uncouplers of oxidative-phosphorylation reduced yields of standard glucose growth, CO_2 output was increased. Sodium azide and chloramphenicol inhibited growth, but not glucose metabolism. Antimycin increased the yield, oligomycin had no effect.

5. Enthalpy changes in the presence of uncouplers or inhibitors were low, ie. heat output increased. Recovery of available substrate energy as heat was poor.

CHAPTER SEVEN

MICROCALORIMETRIC STUDIES DURING THE GROWTH OF K. AEROGENES IN α-METHYL GLUCOSIDE

α-Methyl glucoside (α-MG) is used as a non-metabolizable substrate for studying sugar transport mechanisms across bacterial membranes, it is an analogue of glucose. Long et al., (1975, 1977) showed that thermal events were associated with the transport of α-MG. It was considered that enthalpy changes associated with the uptake of α-MG could be useful in defining more clearly the transport mechanism involved.

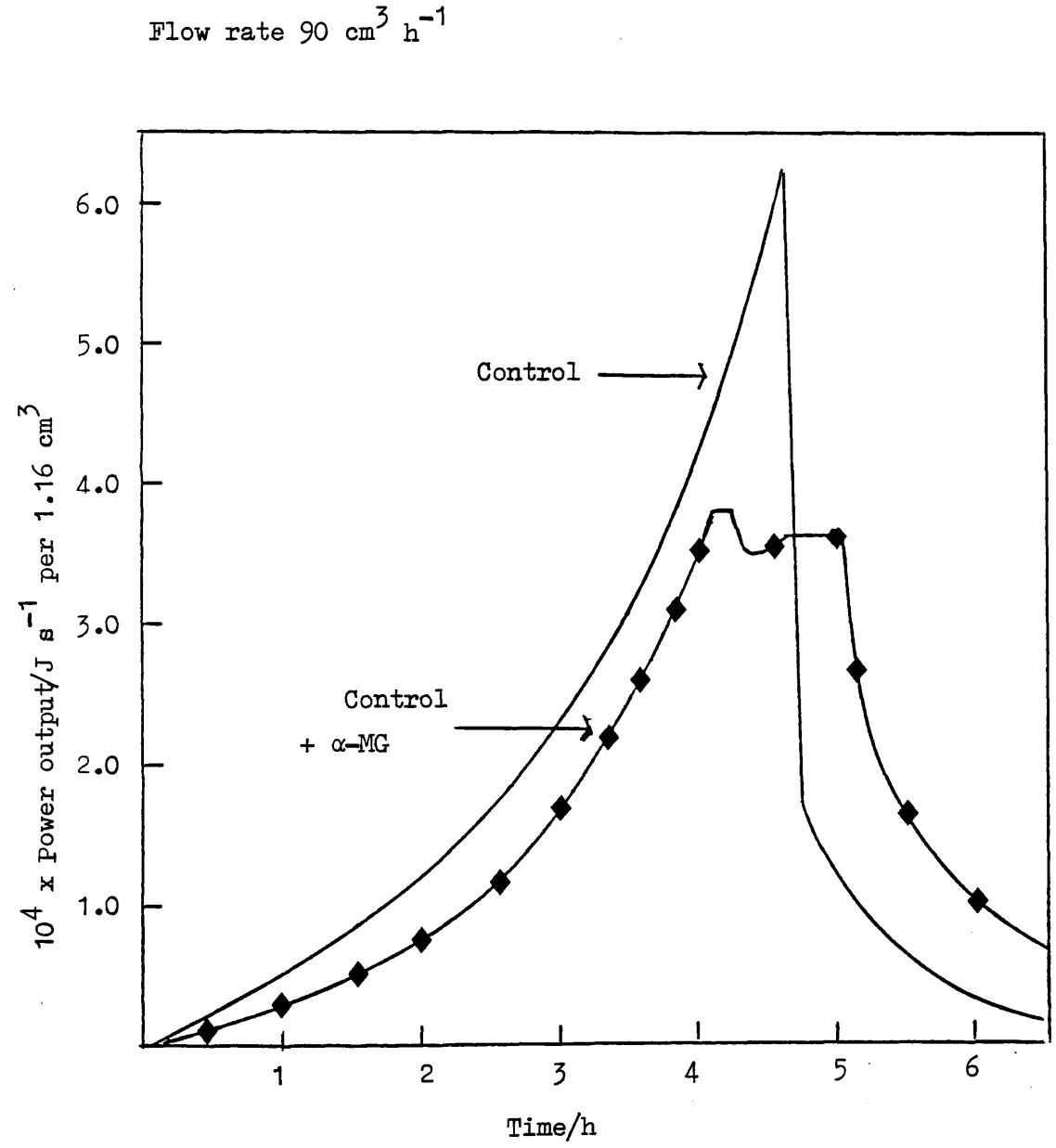
7.1 The Influence of α-MG on Growth in Glucose-Limited Media

At zero time (ie. just before inoculation of the culture vessel) a volume of concentrated α-MG stock solution was added to give the desired concentration. Standard growth conditions (3.3 mmol dm^{-3} glucose) and cryogenically stored glucose-trained cells were used as inocula.

7.1.1 Power-Time Trace and Growth Parameters

The presence of α-MG caused a change in the standard p-t trace profile expected during growth in glucose. With increasing α-MG concentration the exponential power and heat output ceased increasingly earlier, the power output then became constant until the remaining glucose was depleted, whereafter it decreased rapidly (Fig. 7.1). The value of the maximum power output was reduced by the presence of increasing amounts of α-MG. When exponential growth ceased glucose was no longer detected, but α-MG was present (at a reduced concentration from that added initially to the medium). At the end of exponential growth the p-t trace and growth profiles (CO_2, pO_2) returned more slowly to their initial values than during growth in standard glucose media.

FIGURE 7.1 Power-time trace during growth in glucose in the presence of (4.6 mmol dm^{-3}) α-methyl glucoside



The exponential rate constants for the increase in heat output and biomass decreased with increasing concentrations of α-MG (Fig. 7.2). The yield coefficient, calculated on the basis of glucose as the sole carbon source, Y_{gluc}, was not signifcantly altered by the presence of α-MG in the medium (Table 7.1). Carbon dioxide production was increased at all α-MG concentrations, in general it increased with increasing concentration.

When α-MG (9 mmol dm^{-3}) was present at zero time, but glucose withheld and added 1h later (during this period no growth occurred) the yield was very reduced and carbon dioxide production very high (Table 7.1). When the situation was reversed and α-MG added about halfway through the normal period of exponential growth the yield was significantly higher, but carbon dioxide production was also high. At the point of adding α-MG the effluent CO_2 concentration decreased (400 to 100 p.p.m.) and then increased again over the next 30 minutes. The pO_2 decreased normally until after a 30 minute lapse when there was a sudden increase in oxygen uptake which was maintained until the end of growth. The p-t traces for these two variations also differ (Fig. 7.3). When glucose was added 1 h after inoculation the growth rate was decreased and a single peak on the p-t trace recorded. Rate constants for biomass and heat output were 6.22×10^{-3} and 4.19×10^{-3} min^{-1} respectively. When α-MG was added after growth had started in glucose the power decreased slowly from the point of addition until glucose was depleted, and then more abruptly. The rate constant for biomass increase was 6.22×10^{-3} min^{-1}, similar to that for standard growth.

The minimum pO_2 attained was raised by the presence of α-MG, while the minimum pH attained was lower than for standard growth in glucose (Table 7.1).

FIGURE 7.2 The effect of α-Methyl glucoside on rate constants for increase in heat output and biomass during standard growth

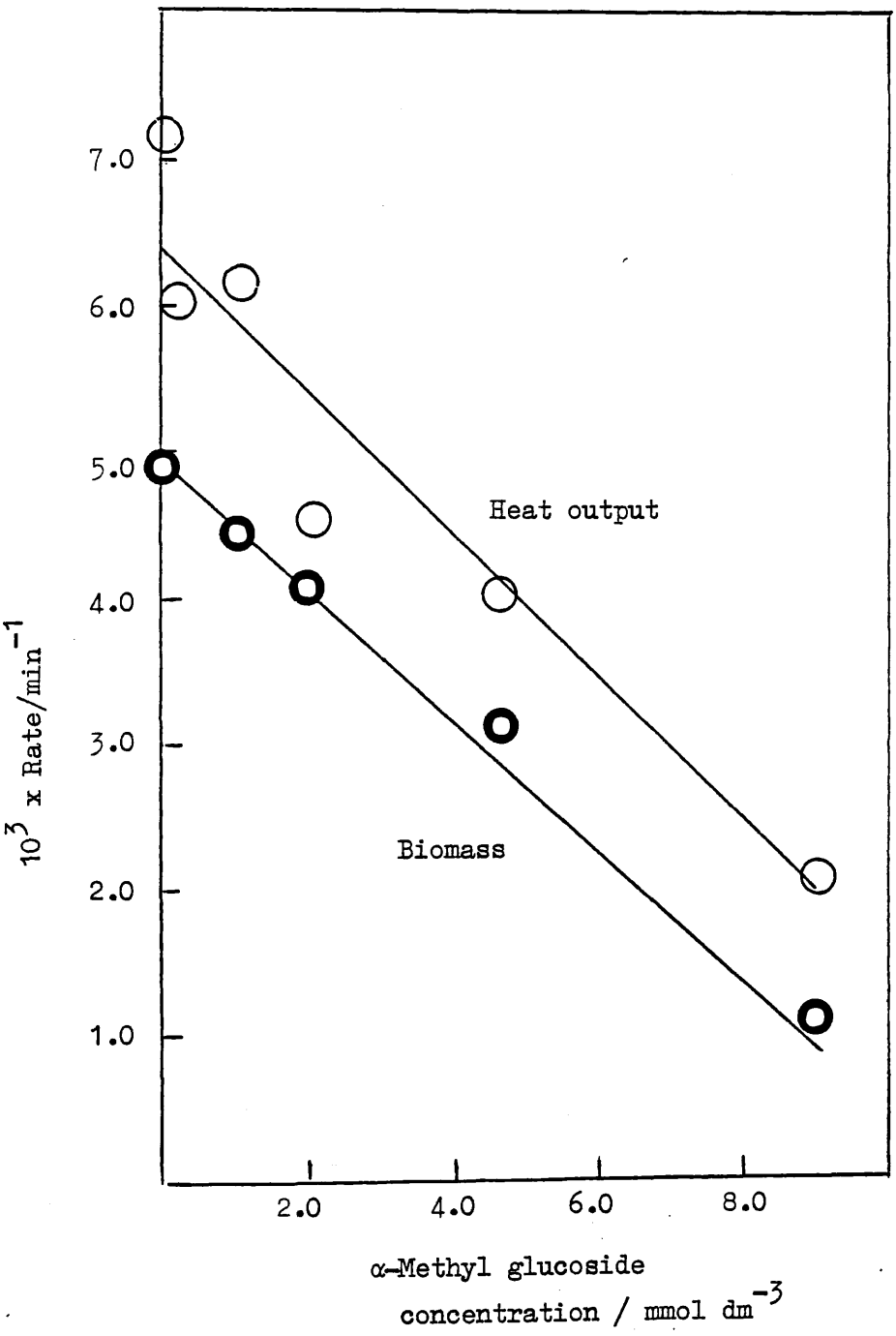


Table 7.1 The Influence of α-MG on the Growth of K. aerogenes in Glucose Limited Media

α-MG concentration /mmol dm^{-3}	Doubling time /h	Y_{gluc} /(g cell)g^{-1}	Minimum pO_2 %	Minimum pH	$CO_2{}_{gluc}$ /mol (mol gluc)$^{-1}$	10^4 x Maximum power output J s^{-1}	Measured heat output q(exp) /J per	q(tot) 1.16 cm^{-3}	Enthalpy changes ΔH_p /kJ(mol cell)$^{-1}$	ΔH_{gluc} /kJ(mol gluc)$^{-1}$
0	0.7	0.43	28	6.83	3.07	7.66	3.22	3.97	-350.8	-1037.7
0.3	0.6	0.36	16	N.D.	3.93	5.90	3.34	4.56	-452.5	-1191.1
1.0	0.7	0.40	14	N.D.	3.64	4.97	3.23	4.07	-368.2	-1063.0
2.0	0.7	0.43	27	N.D.	4.31	4.58	3.17	4.23	-353.1	-1104.0
4.6	0.8	0.43	38	6.70	4.31	4.09	3.45	4.86	-405.6	-1209.0
8.8	0.9	0.43	39	6.80	4.36	4.05	3.71	5.30	-442.4	-1384.0
9.0	1.13	0.43	44	6.74	3.85	3.48	3.67	4.63	-385.0	-1209.0
*9.0	1.0	0.35	48	N.D.	4.78	6.20	N.D.	4.28	-434.8	-1118.6
**4.6	N.D.	0.52	11	6.70	3.88	4.66	4.31	8.02	-555.3	-2092.9

* Glucose added at 1 hour after inoculation

** α-MG added during exponential growth

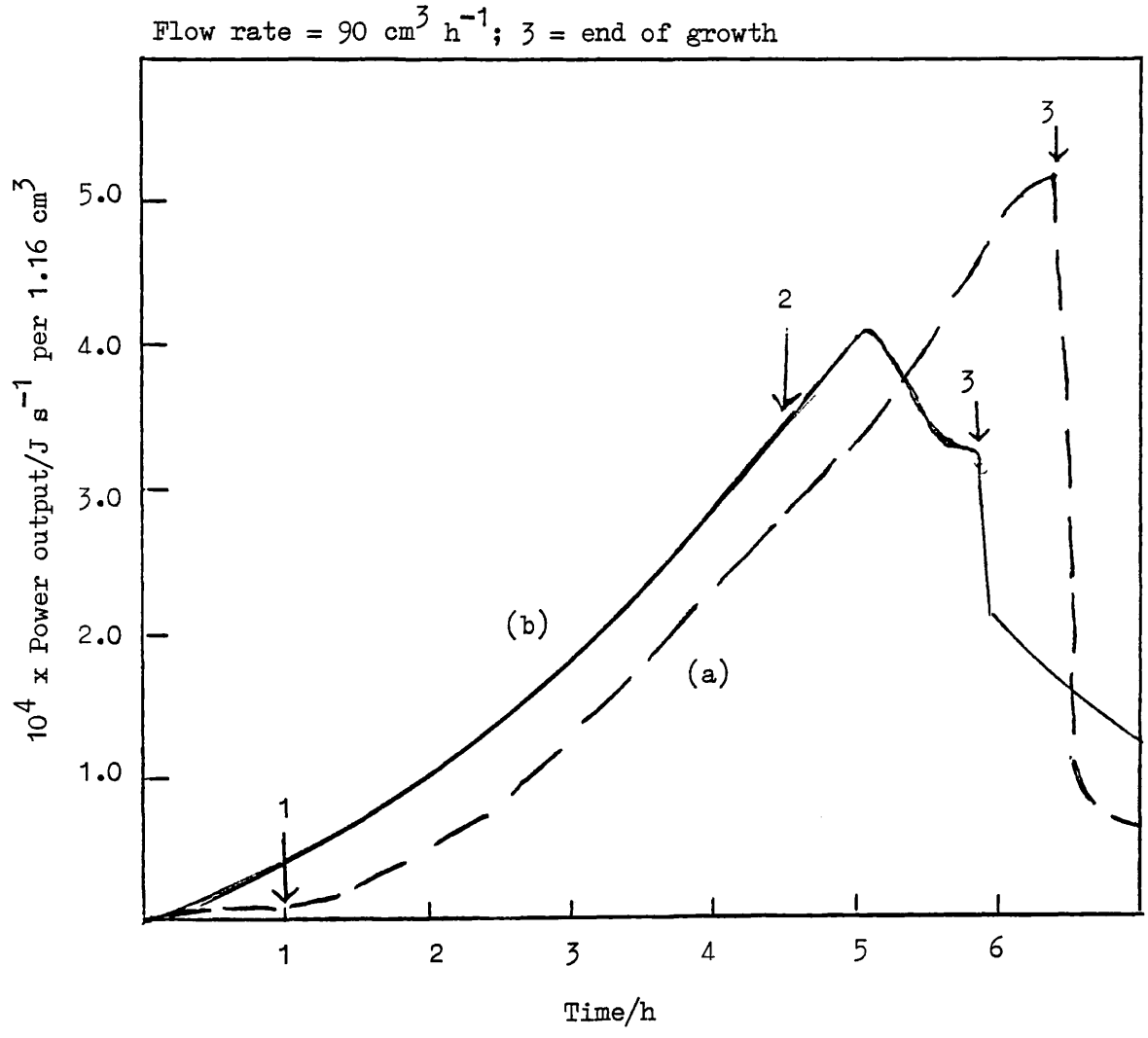
Yields and enthalpy changes calculated on the assumption that glucose is the sole carbon and energy source.

N.D. Not determined

FIGURE 7.3 Power-time traces of glucose-trained cells in salt medium

(a) containing α-MG (4.6 mmol dm^{-3}) with glucose (3.3 mmol dm^{-3}) added at 1.

(b) containing glucose (3.3 mmol dm^{-3}) with α-MG (4.6 mmol dm^{-3}) added at 2.



If α-MG was used as the only substrate with glucose-trained cells (ie. lacking the presence of glucose) no growth or heat output was detected.

7.1.2 Measured Heat Outputs and Enthalpy Changes

The values of q(exp) and q(tot) increased with increasing α-MG concentration, values of q(exp) were higher than for growth in glucose alone (Table 7.1). At concentrations above 2 mmol dm^{-3} α-MG q(tot) increased linearly with concentration.

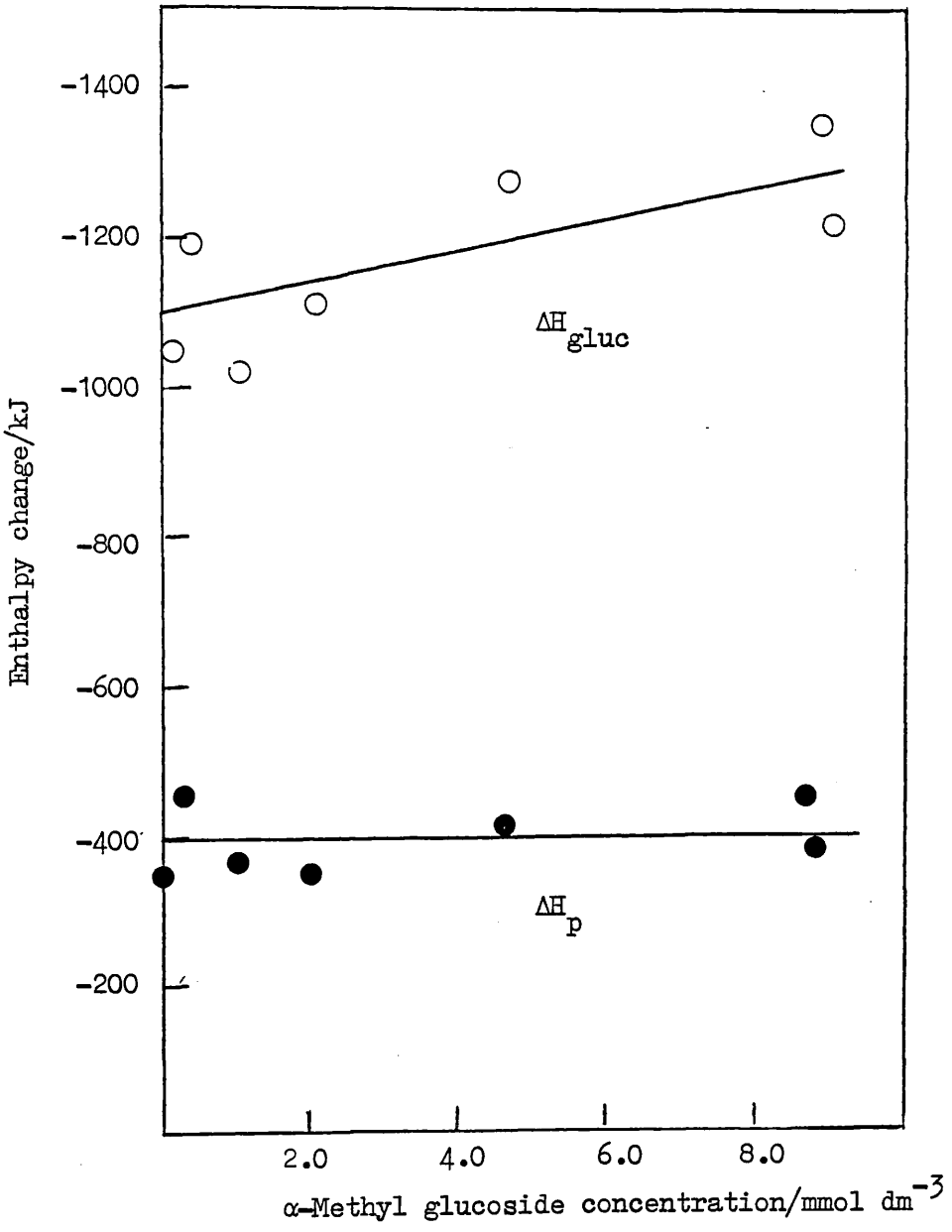
ΔH_p values in the presence of α-MG varied erractically (Fig. 7.4); the mean and range for ΔH_p were -402 and -352 to -452 kJ $(mol\ cell)^{-1}$ respectively. The mean value is significantly above the mean ΔH_p for glucose growth (-356 kJ $(mol\ cell)^{-1}$). Values for ΔH_g range from -2.60 to -2.22 kJ g^{-1} gen^{-1}.

ΔH_{gluc} calculated on the assumption that glucose was the sole carbon and energy source varied over the whole α-MG concentration range (Fig. 7.4). At high α-MG concentrations it was outside the values for growth in glucose alone.

7.2 Variation of Growth and Thermal Parameters for Aerobic Growth of K. aerogenes with α-MG as Limiting Carbon Source

Cells of K. aerogenes were trained to grow in media with α-MG (4.6 mmol dm^{-3}) as the sole carbon source. Training was achieved by growing cells in a mixture of glucose and α-MG; then gradually reducing the glucose concentration to zero, further subcultures in medium containing α-MG only were made before inocula were prepared for cryogenic storage. All conditions of growth, harvesting etc were as for growth in glucose (2.1.4., 2.1.5.).

FIGURE 7.4 Variation of enthalpy change for standard glucose-limited growth in the presence of α-Methyl glucoside



7.2.1 Power-Time Trace and Growth Parameters

Growth of α-MG-trained cells in α-MG medium was much slower than glucose-trained cells in glucose; a doubling time of 1.0 h compared with 0.7 h. Growth proceeded exponentially until the depletion of α-MG (2.3.2). At a point coincident with the end of exponential growth effluent carbon dioxide passed through a maximum value and the oxygen tension a minimum value (Fig. 7.5). The return of these parameters to their initial values took considerably longer than in standard glucose media.

The power increased exponentially during exponential growth to a maximum value (P_1) which occurred just prior to the end of growth. The power then decayed but not as abruptly as for growth in glucose (Fig. 7.5) while the specific power output, ΔH_{sp}. showed similar trends to that obtained during glucose limited growth. The rate constants for the increase in heat output and biomass during exponential growth in 4.6 mmol dm^{-3} α-MG were 3.59×10^{-3} and 4.10×10^{-3} min^{-1} respectively. The rate constant for heat output is lower than that for biomass; this is reverse of the position during standard glucose growth (cf. 4.1).

The yields varied with α-MG concentration and the carbon dioxide produced decreased with increasing yield (Table 7.2). Acetate was detected in the medium, the maximum was 0.6 mol acetate per mol α-MG at an initial α-MG concentration of 4.6 mmol dm^{-3}. The elemental composition of α-MG-trained cells, which differed little from that of standard glucose cells was: carbon 46.8%, hydrogen 6.8%; nitrogen 12.7% and oxygen 26.7%. The empirical formula is $CH_{1.73}O_{0.43}N_{0.23}$ and formula weight 28.83.

FIGURE 7.5 Typical p-t trace and changes in growth parameters during growth in α-Methyl glucoside (4.6 mmol dm^{-3})

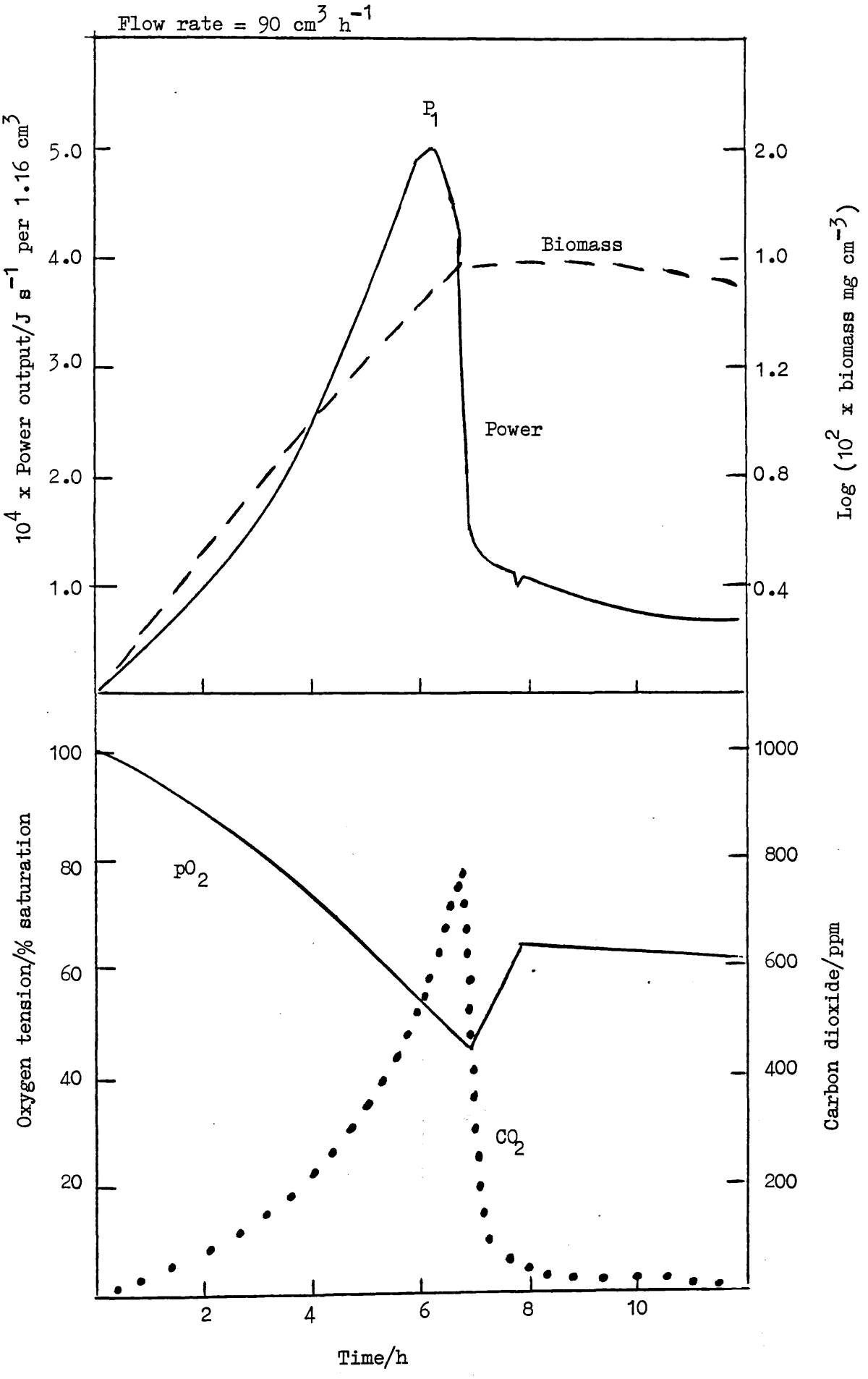


Table 7.2 Growth Parameters for Growth of K. aerogenes in α-methyl Glucoside Limited Media

α-MG concentration /mmol dm^{-3}	No of generations	$Y_{\alpha\text{-MG}}$ /(g cell)g^{-1}	Y_{carbon} /(g cell)g^{-1}	Minimum pH	Minimum pO_2 /%	$CO_{2\,\alpha\text{-MG}}$ /mol (mol α-MG)$^{-1}$
0.7	4.07	0.42	0.87	6.95	85	2.62
1.4	4.54	0.57	1.33	N.D.	46	2.38
2.0	5.61	0.51	1.20	N.D.	16	1.86
2.7	6.23	0.49	1.15	6.81	28	3.83
3.4	6.69	0.49	1.10	6.75	24	3.53
4.0	7.46	0.44	1.03	6.70	18	3.68
4.6	N.D.	0.49	1.14	6.80	N.D.	3.87

N.D. Not determined

7.2.2 Measured Heat Outputs and Enthalpy Changes

q(exp) and q(tot) increased linearly with α-MG concentration up to about 3.3 mmol dm^{-3} (Table 7.3). Both lines on the q against α-MG concentration plot extrapolate back through the origin (cf. glucose, Fig. 4.3).

ΔH_p and $\Delta H_{\alpha\text{-MG}}$ varied over the whole concentration range (Fig. 7.6); with increasing initial α-MG concentration, $\Delta H_{\alpha\text{-MG}}$ first decreased and subsequently increased in an apparantly linear manner. ΔH_p increased and passed through a maximum value at 2.8 mmol dm^{-3} α-MG; at higher concentrations ΔH_p was constant at about -590 kJ $(mol\ cell)^{-1}$.

The specific heat output (ΔH_{sp}) which varied with concentration was just over twice that found for glucose-limited growth. The heat output per generation, ΔH_g, decreased with increasing α-MG concentration, this value is considerably higher than that for growth of glucose-trained cells in glucose (Table 7.3).

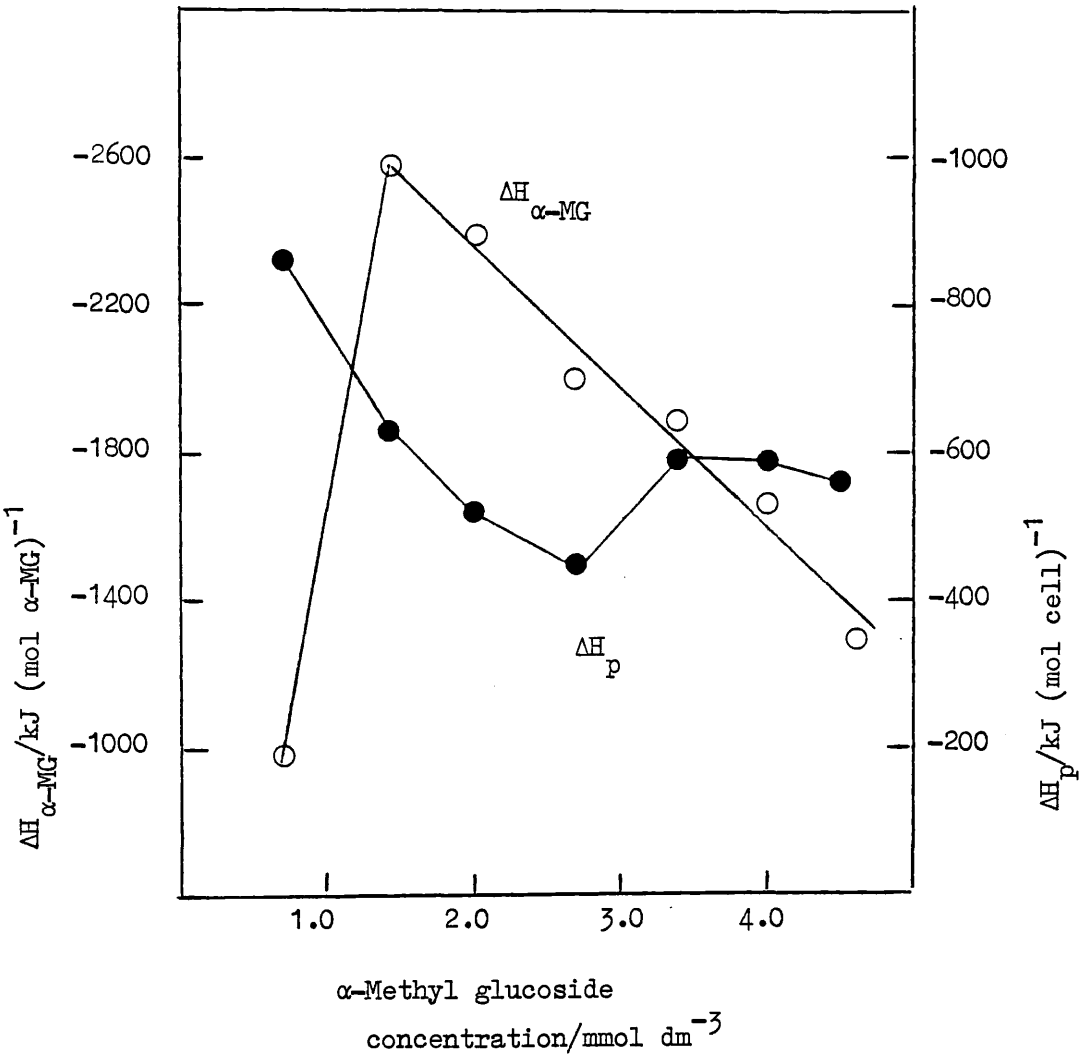
7.2.3 Growth of α-MG-trained Cells in Glucose Media

When α-MG cells were grown in medium containing glucose (3.3 mmol dm^{-3}) as the carbon and energy source the p-t trace consisted of a single peak, (cf. standard p-t trace in glucose Fig. 3.4), the maximum power output (5.8×10^{-4} J s^{-1}) occurred about an hour before the end of exponential growth. The power fell sharply at the end of growth (Fig. 7.8). The rate constant for biomass increase, 7.65×10^{-3} min^{-1}, was greater than that for glucose-trained cells in standard glucose medium.

Table 7.3 Thermal Parameters for Growth of K. aerogenes in α-methyl Glucoside Media

α-MG concentration /mmol dm^{-3}	10^4 x maximum power output /J s^{-1}	Measured heat q/exp) /J per	q(tot) 1.16 cm^3	$\Delta H_{\alpha\text{-MG}}$ /kJ (mol α-MG)$^{-1}$	ΔH_p /kJ (mol cell)$^{-1}$	ΔH_{sp} /kJ (g cell)$^{-1}$	ΔH_g /kJ (g gen)$^{-1}$
0.7	1.32	1.32	2.06	-1391.5	-829.7	-34.8	-8.54
1.4	3.22	3.20	4.81	-2961.6	-637.4	-26.7	-5.88
2.0	2.20	4.92	6.49	-2797.2	-524.8	-22.0	-3.92
2.7	4.09	5.65	7.50	-2394.4	-449.8	-18.9	-3.03
3.4	4.88	7.25	9.20	-2332.4	-607.6	-25.5	-3.81
4.0	4.40	6.27	9.91	-2135.7	-596.9	-25.0	-3.35
4.6	5.72	6.95	9.32	-1746.5	-533.7	-18.5	N.D.

FIGURE 7.6. Heat output during growth of α-MG-trained K. aerogenes in α-MG-limited media



Measured heat outputs were q(exp) = 3.40 J and q(tot) = 5.66 J and yield, Y_{gluc} = 0.48, giving values for ΔH_{gluc} and ΔH_p of -1478.6 kJ (mol gluc)$^{-1}$ and-414.4 kJ (mol cell)$^{-1}$ respectively.

7.2.4 Growth of α-MG-trained Cells in Media Containing Glucose and α-MG

When grown in media containing glucose (3.3 mmol dm^{-3}) and α-MG (4.6 mmol dm^{-3}) diauxic growth was obtained (Fig. 7.7). The p-t trace has two peaks; both maxima occurring during the exponential growth periods. The first peak occurred about an hour before glucose depletion and the second before α-MG depletion. The rate constants for the increase in biomass for the first and second periods of diauxic growth were 6.96 x 10^{-3} and 4.33 x 10^{-3} min^{-1} respectively. The heat output was not diauxic, the rate constant for heat output up to the first peak was 8.29 x 10^{-2} min^{-1}. The measured heat outputs under the p-t curve a-b and b-c (Fig. 7.6) were 3.93 J and 4.00 J respectively. Enthalpy changes (Table 7.4) are compared with those values obtained for growth in the separate substrates.

7.3 Thermal Changes During Growth of α-MG-trained Cells in the Presence of Uncouplers

α-MG-trained cells were grown in medium containing α-MG as the sole energy and carbon source, varying amounts of inhibitors were added either at zero time or during exponential growth. The power and other growth parameters were continuously monitored.

DNP. Although the addition of DNP (final concentration 6.5 mmol dm^{-3}) during exponential growth caused no change in the growth rate, there was a reduction in the yield ($Y_{\alpha\text{-MG}}$ = 0.34) and an increase in the carbon dioxide output from 3.87 to 4.66 mol CO_2 (mol α-MG)$^{-1}$.

FIGURE 7.7 Power-time trace and changes in growth parameters during growth of α-MG trained cells in a mixture of 4.6 mmol dm^{-3} α-MG and 3.3 mmol dm^{-3} glucose

Flow rate 90 cm^3 h^{-1}

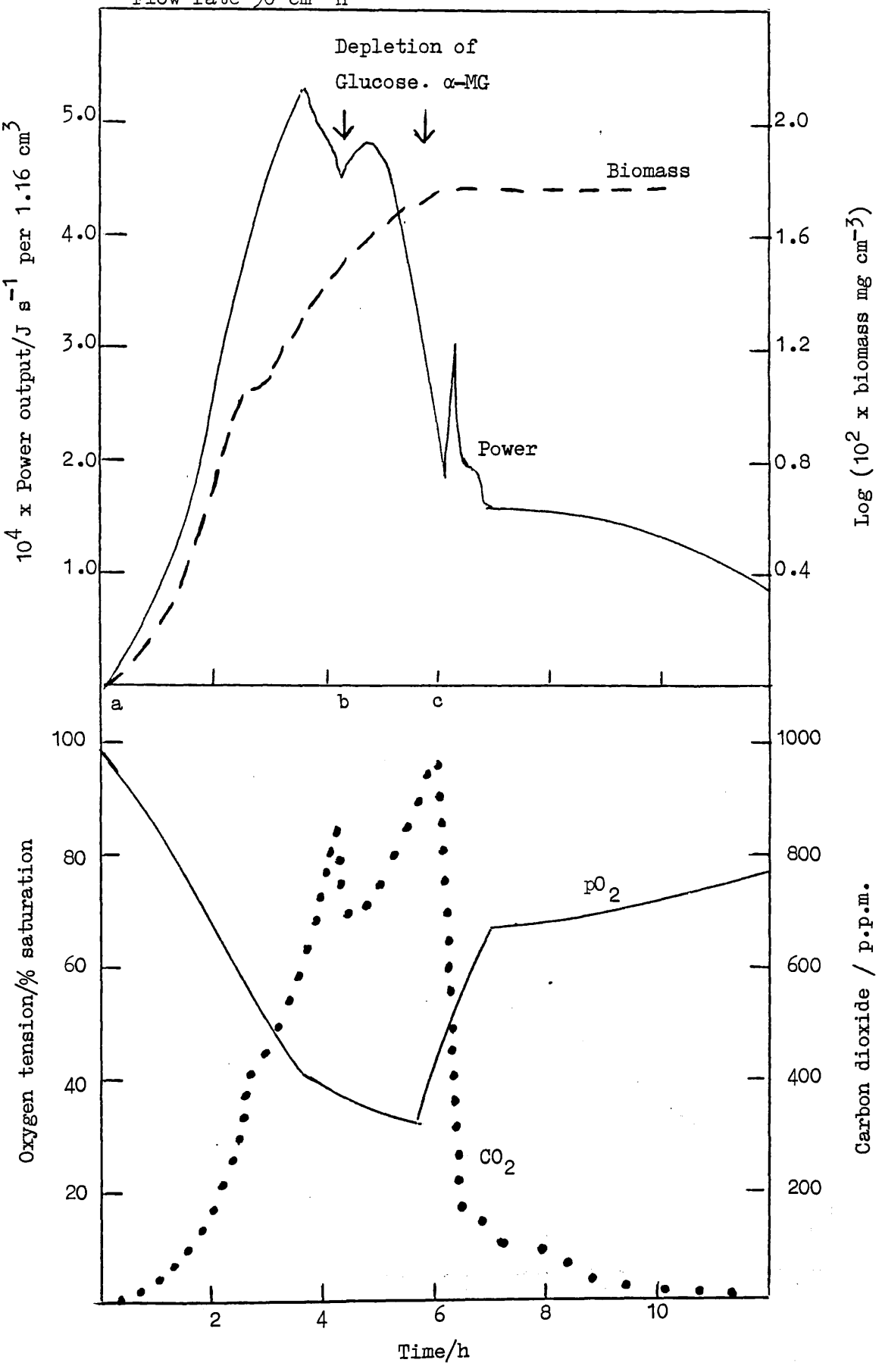


FIGURE 7.8 Schematic p-t traces of K. aerogenes trained and grown in salts media of different carbon sources

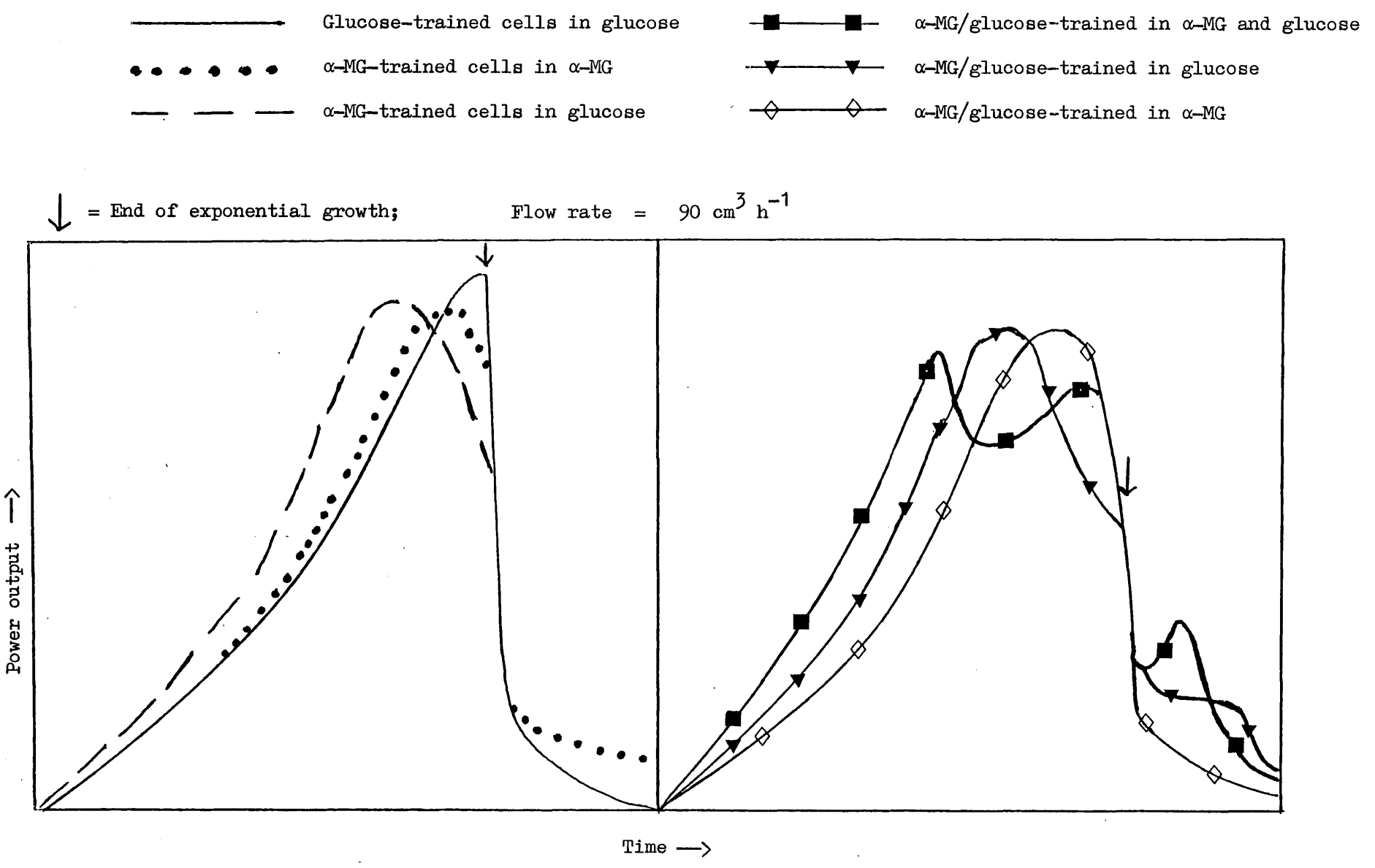


Table 7.4 Enthalpy Changes during Growth of K. aerogenes in the Presence of α-methyl Glucoside and/or Glucose

Cells trained to	Growth	Concentration /mmol dm^{-3}	Y_{carbon} /(g cell) g^{-1}	ΔH_p /kJ(mol cell)$^{-1}$	ΔH_{carbon} /kJ(mol carbon)$^{-1}$	CO_{2_p} /mmol g $cell^{-1}$
Glucose	Glucose	3.3	1.07	350.8	173.0	39.7
α-MG	α-MG	4.6	1.14	533.7	249.5	40.7
α-MG	Glucose	3.3	0.76	239.9	501.1	47.5
	α-MG	4.6				
α-MG	Glucose	1.2	1.40	386.6	549.6	22.6
	α-MG	0.7				
α-MG/ Glucose	Glucose	3.3	1.28	349.3	215.7	37.2

The power increased to a maximum value (4.2×10^{-4} J s^{-1}) about 30 minutes before the end of exponential growth before attaining a low near constant power output, 1.5×10^{-4} J s^{-1}, which was maintained for 3h. The CO_2 and pO_2 profiles were parallel to and the inverse of the p-t trace respectively.

The inhibited values of q(exp) and q(tot) were higher, 7.59 and 11.08 J per 1.16 cm^3 culture respectively, than for uninhibited growth. $\Delta H_{\alpha\text{-MG}}$ at -2076.5 kJ (mol α-MG)$^{-1}$ is lower than the uninhibited value and ΔH_p at-917.9 kJ (mol cell)$^{-1}$ is about twice that for uninhibited growth (cf. Table 7.3).

Sodium azide. The addition of sodium azide (final concentration 2.5 mmol dm^{-3}) to the growing culture (1.4 mmol dm^{-3} α-MG) during exponential growth caused a reduction in the yield ($Y_{\alpha\text{-MG}} = 0.39$) and increased CO_2 production to 2.38 mol CO_2 (mol α-MG)$^{-1}$, cf. Table 7.2. The rate constant for the increase in biomass was not altered significantly, 6.04×10^{-3} and 6.52×10^{-3} min^{-1} for inhibited and uninhibited growth respectively. This is in marked contrast to growth of glucose trained cells in glucose-limited media where the addition of sodium azide caused exponential growth to cease.

After the addition of sodium azide power output continued at a slightly lower value, the maximum (2.85×10^{-4} J s^{-1}) occurred near the end of exponential growth. No secondary period of heat output was recorded, (cf. DNP), and the baseline was quickly re-established.

The values of q(exp) and q(tot) for inhibited growth are greater (6.51 and 8.45 J per 1.16 cm^3 culture respectively) than for uninhibited growth. The enthalpy changes $\Delta H_{\alpha\text{-MG}}$ and ΔH_p are -5203.2 kJ (mol α-MG)$^{-1}$ and -1962.7 kJ (mol cell)$^{-1}$ respectively. These values are 1.8 and 3.1 times greater than the values obtained for uninhibited growth.

7.4 Thermal Properties of Cells Trained to Grow in Mixed Substrates (3.3 mmol dm^{-3} glucose and 4.6 mmol dm^{-3} α-MG)

The cells were trained to grow in a medium containing α-MG and glucose (4.6 and 3.3 mmol dm^{-3} respectively) by repeated subculture (15 times) in this medium.

7.4.1 Growth on α-MG as Sole Carbon Source

The p-t trace for cells trained to the mixed substrate, grown in media containing α-MG (4.6 mmol dm^{-3}) only consisted of a broad maximum; the maximum value occurring about 1 h before the end of exponential growth (Fig. 7.8). The rate constants for heat output and biomass increase were 3.2×10^{-3} and 2.8×10^{-3} min^{-1} respectively. The rate constants are lower than those for growth of glucose-trained cells in standard glucose media or α-MG trained cells in α-MG media The exponential heat output, q(exp) was very high, 6.95 J per 1.16 cm^3 culture. α-MG was not detected at the end of exponential growth.

7.4.2 Growth on Glucose as Sole Carbon Source

The p-t trace, for growth in 3.3 mmol dm^{-3} glucose, was a broad single peak, the maximum occurred during exponential growth, this was followed by a period of lower power output in the early stationary phase (Fig. 7.8). Rate constants were similar to those recorded for growth of glucose-trained cells in standard glucose. Glucose was not detected at the end of exponential growth.

7.4.3 Growth on Mixed Substrates

The p-t trace profile accompanying growth on glucose and α-MG (3.3 and 4.6 mmol dm^{-3} respectively) consisted of two peaks, the first

of higher power output; followed by the second coincident with the end of growth (Fig. 7.8). Acetate was detected at the end of growth. Heat outputs under each p-t peak were 3.69 and 6.13 J respectively. (cf. Fig. 7.7). Diauxic growth was not observed.

7.5 Elemental Composition of Cells Grown in the Presence of α-MG

Whenever cells were grown in the presence of α-MG, irrespective of their training, their carbon contents were above that for cells grown in glucose only. No other significant differences were found. The empirical formulae and weights are given in Table 7.5.

7.6 Size Distribution of Cells Grown in the Presence of α-MG and/or glucose

The various trained cells were grown in media containing different carbon sources (glucose and/or α-MG). At the end of exponential growth the cells were harvested, washed and resuspended in isopon. The size distribution was measured in a Coulter Counter (model ZB1, with a C-1000 channelyzer).

There was no significant difference in the mean volume of glucose trained cells and α-MG trained cells grown in their respective media and glucose or α-MG as the sole carbon source respectively (Table 7.6). Glucose trained cells grown in a mixture of glucose and α-MG have a larger mean volume than standard cells (7.1). Cells trained to the mixed substrates, α-MG and glucose, when grown back in glucose only, have a much larger mean volume but the standard deviation of the mean is large.

Table 7.5 Elemental Composition of K. aerogenes Trained to Grow to Different Carbon Sources

Cells trained to	Growth substrate	Carbon %	Hydrogen %	Nitrogen %	Oxygen %	Residue %	Empirical formulae	Formula weight
α-MG	Glucose ($3.3\ mol\ dm^{-3}$)	46.3	6.7	13.2	28.4	5.4	$CH_{1.74}O_{0.46}N_{0.24}$	24.46
α-MG glucose	α-MG ($4.6\ mmol\ dm^{-3}$)	47.7	6.7	12.8	26.2	6.8	$CH_{1.68}O_{0.41}N_{0.22}$	23.32
α-MG/ glucose	Glucose ($3.3\ mmol\ dm^{-3}$)	46.9	6.6	13.2	26.5	6.8	$CH_{1.69}O_{0.42}N_{0.24}$	23.77
α-MG	Glucose ($3.3\ mmol\ dm^{-3}$) and α-MG ($4.6\ mmol\ dm^{-3}$)	47.9	6.6	13.2	25.4	6.7	$CH_{1.65}O_{0.39}N_{0.24}$	23.25

Table 7.6 Size Distribution of Cells of K. aerogenes Trained and Grown to Different Carbon Sources

Trained to	Growth substrate	Mean volume μm^3	Model volume μm^3	Median volume μm^3	Standard deviation μm^3	Relative skewness μm^3
Glucose	Glucose (3.3 mmol dm^{-3})	0.402	0.264	0.335	0.232	1.09
α-MG	α-MG (4.6 mmol dm^{-3})	0.406	0.311	0.358	0.200	1.21
α-MG	Glucose (3.3 mmol dm^{-3})	0.360	0.312	0.346	0.120	0.58
Glucose	*Glucose (3.3 mmol dm^{-3}) and α-MG (4.6 mmol dm^{-3})	0.534	0.296	0.450	0.039	1.42
Glucose/ α-MG	Glucose (3.3 mmol dm^{-3})	0.578	0.370	0.496	0.309	0.95

* cf. 7.1

7.7 Summary

1. The presence of increasing amounts of α-MG at zero time to standard glucose-limited growth causes a change in the p-t trace. Enthalpy changes were increased and rate constants for heat output and biomass decreased. Y_{gluc} was unchanged but CO_2 output increased.

2. α-MG-trained cells grown in α-MG as the sole carbon source had a p-t trace and growth parameter profiles similar to those obtained for glucose growth. The growth rate was reduced.

3. q(exp) and q(tot) increased linearly with α-MG concentration, the extrapolated values passed through the origin of a q-α-MG concentration plot. ΔH_p and $\Delta H_{\alpha\text{-MG}}$ varied over the whole concentration range.

4. α-MG-trained cells grown back onto glucose-limited media had a higher growth rate than standard glucose-trained cells. α-MG-trained cells grown in mixed substrates exhibited diauxic growth. Rate constants for the exponential growth periods correlate with those for glucose and α-MG growth only.

5. Cells trained to grow in mixed substrates exhibited altered power output characteristics. These cells did not exhibit diauxic growth when grown in mixed substrates.

CHAPTER EIGHT

MICROCALORIMETRIC STUDIES DURING THE GROWTH OF K.AEROGENES IN POTASSIUM-LIMITED MEDIA

Calorimetric studies were made with K^+-limited growth with two objectives; (1) to determine whether the enthalpy changes associated with growth are similar when the energy source is not growth limiting as those when it is and (2) to determine enthalpy changes associated with enhanced K^+ transport and to possibly relate these to the chemiosmotic theory.

These were preliminary experiments to determine the problems and feasibility of the investigations.

8.1 Growth Parameters During K^+-limited Growth

These results refer in particular to changes which occurred during growth of K^+-trained cells (2.1.4) in potassium-limited media, 0.3 $\mu g\ cm^{-3}$, (glucose concentration 3.3 $mmol\ dm^{-3}$); the overall changes are similar for any K^+/glucose combination.

More complex changes were recorded than those for glucose-limited growth (Fig. 8.1). Exponential growth continued until the depletion of K^+ from the medium, after which slower, non-exponential growth was recorded. Although glucose was depleted after about 7 hours growth still continued at a very slow rate for about 20 h. Acetate was increasingly detected during the first 8 h, reaching a maximum just after the depletion of glucose of 0.3 mol acetate per mol glucose. The final yield was lower than that for glucose-limited growth (Table 8.1).

The carbon dioxide concentration of the effluent gas varied continually during the whole growth cycle, during exponential growth the carbon dioxide output was also exponential. At the onset of K^+-limitation it passed through a maxmimum value (Fig. 8.1), then remained at a high level passing through a series of peaks and troughs until there was an abrupt decrease when glucose became depleted. During the remaining period of slow growth carbon dioxide output increased steadily to a maximum value coincident with the end of growth.

FIGURE 8.1 Power-time and growth parameter changes during growth in K^+-limited media

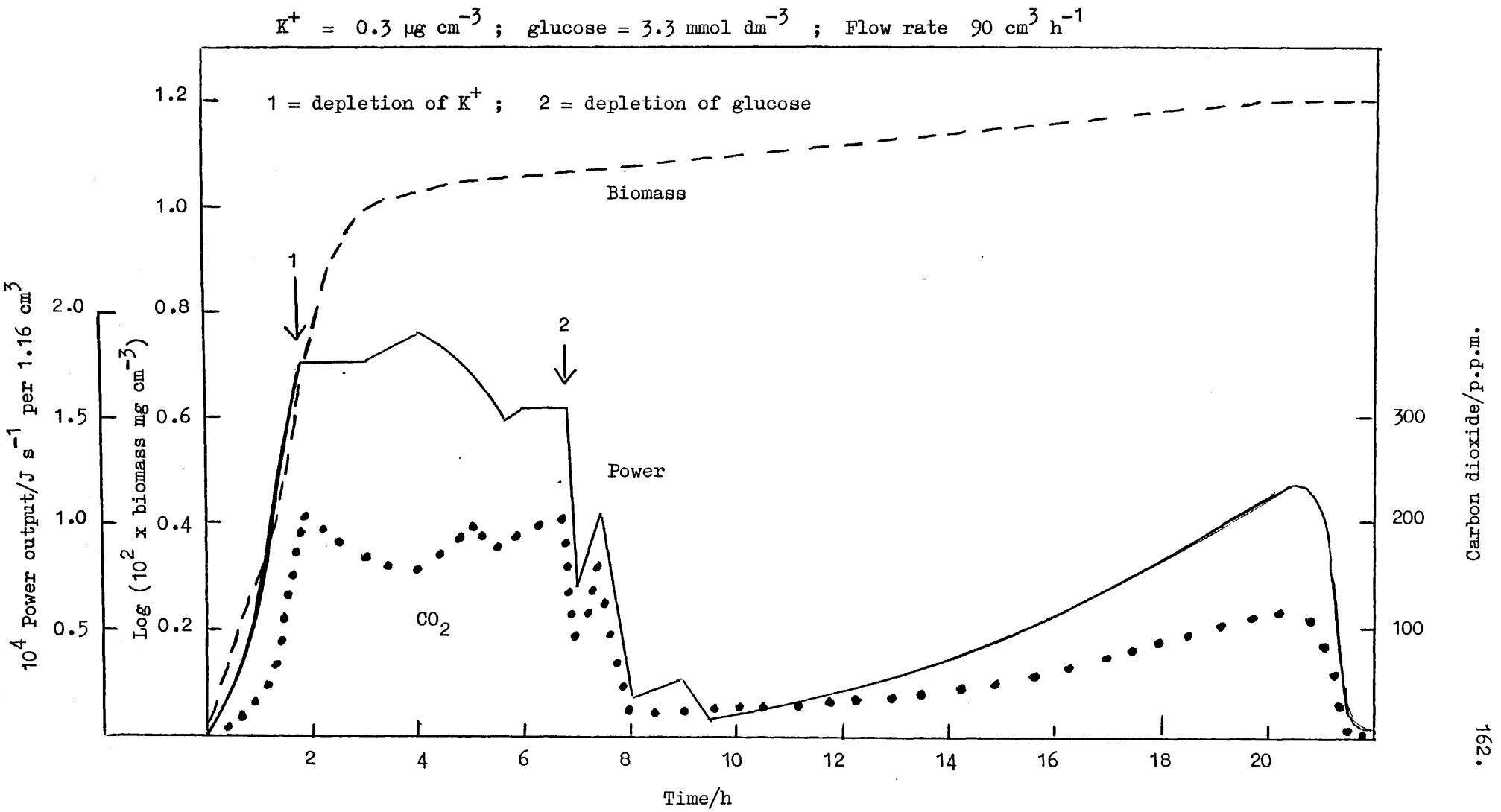


Table 8.1 Enthalpy Changes and Growth Parameters During Growth in Potassium-Limited Media

Glucose concentration /mmol dm^{-3}	Added K^+ /$\mu g\ cm^{-3}$	Y_{gluc} /(g cell)g^{-1}	ΔH_p /kJ(mol cell)$^{-1}$	ΔH_p^k /kJ(mol cell)$^{-1}$	ΔH_{gluc} /kJ(mol gluc)$^{-1}$	$CO_{2_{gluc}}$ /mol(mol gluc)$^{-1}$	* pO_2 minima % saturation
6.7	1.5	0.49	-586.0	-496.2	-2045.6	4.43	0/0
6.7	0.9	0.18	N.D.	-392.2	N.D.	3.79	25/N.D.
6.7	0.6	0.38	-763.1	-245.2	-2088.1	5.17	10/0
6.7	0.3	0.49	-566.1	-219.3	-1973.7	4.16	N.D.
6.7	0.01	[0.23]	[-633.6]	-221.7	[-1044.7]	[2.49]	N.D.
3.3	0.3	0.28	-736.0	-258.4	-1517.6	5.91	65/34
3.3	0.3	[0.23]	[-633.6]	-243.1	[-2121.0]	[2.06]	40/N.D.
1.7	0.3	0.37	-781.1	-282.4	-2023.2	4.36	65/34

ΔH_p values determined with M_r = 24.98

ΔH_p^k values for first 7 h period

values enclosed [ ] are incomplete measurements made after 14 h.

* minima at K^+ depletion and end of growth respectively.

Oxygen tension reached a minimum value coincident with K^+ depletion, then remained at a low level during the first period of high carbon dioxide output before it increased sharply. The pO_2 then fell to a second minimum (Table 8.1) coincided with the end of slow growth, thereafter it increased rapidly to near saturation level.

During the period that potassium was present the pH decreased very slightly; but during the remaining period when glucose was still present the pH dropped markedly (about 6.4). During the following lengthy period of carbon dioxide output the pH increased substantially, 6.7, but remained below the initial pH value.

8.1.1 Power-time Traces During Potassium-Limited Growth

These results refer specifically to the same experimental conditions as described above. The power output was at a relatively low level compared to that recorded for growth in glucose limited media. The p-t trace consisted of a number of peaks and troughs that were coincident with the carbon dioxide output (Fig. 8.1). At the point of potassium limitation the power output ceased to be exponential; it then remained at a similar level until glucose was depleted when it decreased abruptly. During the long period of slow growth and slow increase in carbon dioxide output the power output increased and reached a maximum value which was coincident with the maximum carbon dioxide output and end of growth. Subsequently the baseline was very quickly reattained.

8.1.2 Variation of p-t Trace Profiles and Growth Parameters with Different Levels of Potassium

With fixed glucose concentration and increasing K^+ concentration the following changes were noted: (a) the period of exponential growth lengthened and the following period of slow growth decreased; (b) the p-t trace profile and CO_2 output profile became more simple and (c) the early period of relatively high power and carbon dioxide output was decreased. In general, the final yield (which tended to be variable) increased, but the yield was below that for growth under glucose limitation except at high K^+ levels. The yield at the point of K^+ limitation was dependent on the K^+ concentration of the medium (Table 8.1). The addition of K^+ near the end of exponential growth extended this phase.

8.1.3 Variation of p-t Traces and Growth Parameters With Different Glucose Concentrations During K^+-Limitation

At a fixed K^+ concentration and increasing glucose concentration the p-t trace and growth changes were similar to those occurring at a fixed glucose concentration but with decreasing K^+ concentration ie. the profiles became more complex.

8.1.4 Enthalpy Changes During Potassium-Limited Growth

ΔH_p may be considered in two ways: ΔH_p, calculated from the total measured heat output varied randomly with K^+ concentration within the range -586 to -786 kJ $(\text{mol cell})^{-1}$. These values are very significantly greater than those for glucose-limited growth (-355 kJ $(\text{mol cell})^{-1}$). ΔH_p^k, determined using the appropriate measured heat output up to the point of K^+-limitation, are very similar to those for glucose limited growth (Table 8.1). Moreover the values of ΔH_p^k increase linearly with decreasing K^+ concentration.

The very large amount of heat evolved during the consumption of glucose, (ΔH_{gluc} = -1518 to -2086 kJ (mol gluc)$^{-1}$), is greater than that during glucose-limited growth (ΔH_{gluc} = -1067 kJ (mol gluc)$^{-1}$). This enthalpy change varies randomly with both the glucose and the K^+ concentration.

8.1.5 Growth and Thermal Changes in Potassium Salts Medium Lacking Added K^+

Since growth continued, albeit at a very slow rate, after potassium depletion an experiment was performed as normal but in which no K_2SO_4 was added. Growth occurred very slowly (doubling time about 10 h), the yield (Y_{gluc}) after 15 hours was 0.17 (glucose was still present). The p-t trace consisted of a low broad peak and up to this time ΔH_{gluc} = -1068 kJ (mol gluc)$^{-1}$.

8.2 Growth of Cells at Low K^+ in the Presence of Valinomycin

Cells of *K. aerogenes* trained to grow at a low K^+ concentration were grown in 600 cm^3 of medium (glucose 3.3 mmol dm^{-3}) with K^+ limitation (0.3 μg cm^3) and valinomycin added (in a small volume of ethanol) at a time coincident with the expected depletion of K^+ from the medium. A control was performed in which only ethanol was added at a similar time.

8.2.1 The Effect of Valinomycin on Power Output and Growth Parameters

The presence of increasing amounts of valinomycin caused marked changes in growth parameters and p-t trace changes. The yield and carbon dioxide produced increased (Table 8.2). Immediately after the addition of valinomycin growth continued at a faster, non-exponential, rate. Similarly the rate of carbon dioxide output increased and oxygen tension decreased.

Table 8.2 Growth and Enthalpy Changes for Growth in Potassium-Limited Media in the Presence of Valinomycin

Valinomycin/$\mu g\ cm^{-3}$	0	6.0	16.0
K^+ / $\mu g\ cm^{-3}$	0.3	0.3	0.3
Glucose / $mmol\ dm^{-3}$	2.20	1.70	1.70
Y_{carbon} /g cell g^{-1}	1.04	0.91	1.59
*Y_{carbon} /g cell g^{-1}	0.52	0.60	0.51
Final biomass /$mg\ cm^{-3}$	0.267	0.260	0.591
CO_2 /mmol $(g\ cell)^{-1}$	26.5	65.8	52.5
CO_2 /mol $(mol\ carbon)^{-1}$	0.33	0.72	1.00
ΔH_p /kJ $(mol\ cell)^{-1}$	-963.8	-1529.9	N.D.
ΔH_p /kJ $(mol\ cell)^{-1}$	-626.0	-588.3	-470.4
ΔH_{carbon} /kJ $(mol\ carbon)^{-1}$	-483.6	-439.9	N.D.
*ΔH_{carbon} /kJ $(mol\ carbon)^{-1}$	-155.4	-169.2	-115.4

* Measurements after a period of 7 h

N.D. not determined

The addition of valinomycin to a final concentration of 6 $\mu g\ cm^{-3}$ caused a small decrease in the power output but quickly increased to continue as the control experiment (Fig 8.2). The power output decreased sharply when glucose was depleted and then followed by a slow increase due to the oxidation of secondary metabolites and ethanol. The addition of valinomycin to give a final concentration of 16 $\mu g\ cm^{-3}$ produced a dramatic change in the p-t trace profile (Fig. 8.2). The carbon dioxide output profile reflected the p-t trace profile.

8.2.2 The Effect of Valinomycin on Enthalpy Changes

Due to the presence of two substrates (glucose and ethanol) enthalpy has to be expressed per mol of carbon. With increasing valinomycin concentration the enthalpy changes associated with consumption of carbon, $\Delta H\ (\text{mol carbon})^{-1}$ and the production of cellular material (ΔH_p) increase and decrease respectively.

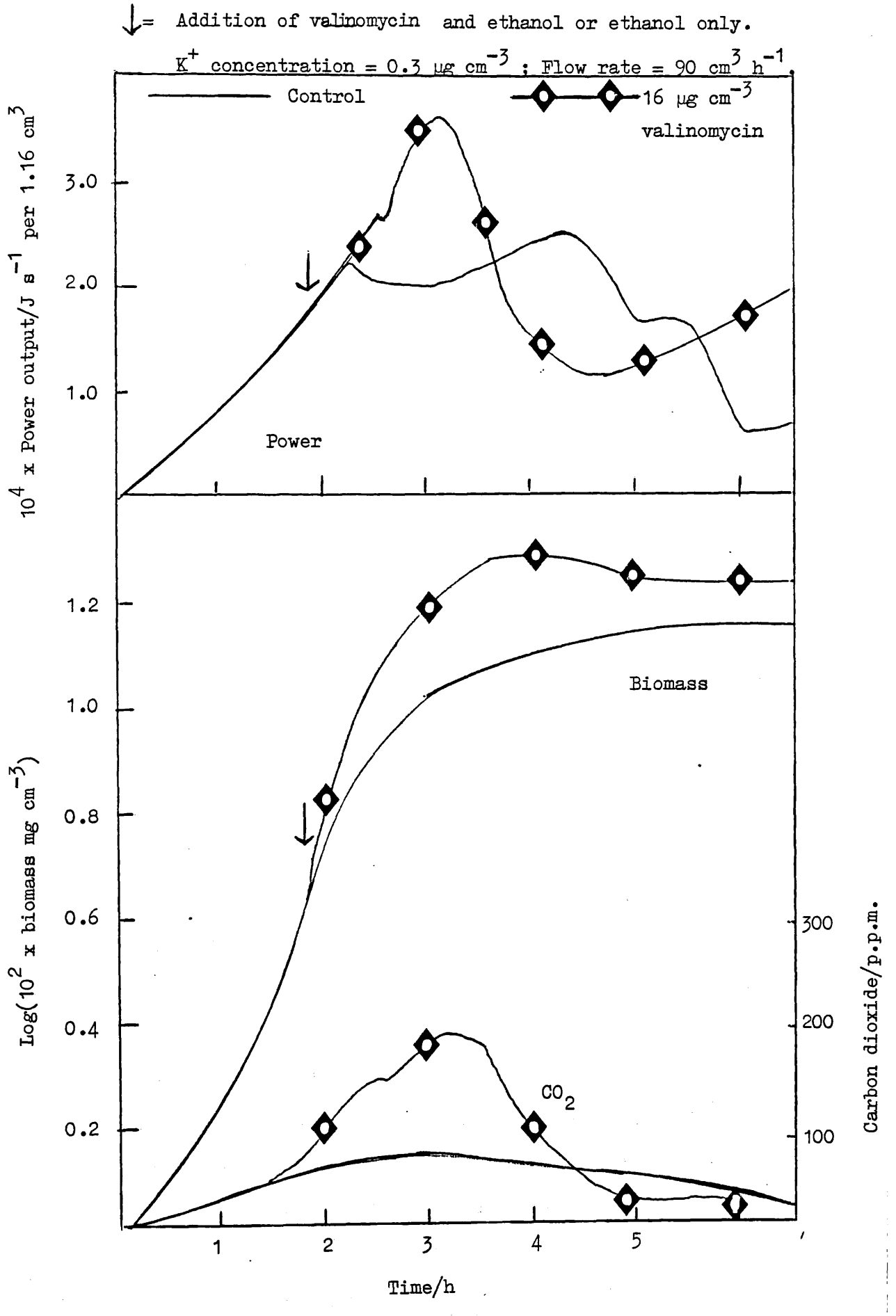
The enthalpy changes were also calculated using the appropriate data for the first 7 h from inoculation; the metabolism of secondary metabolites and ethanol was therefore reduced but the action of valinomycin was most marked. The enthalpy changes now increase with increasing valinomycin concentration (Table 8.2).

When valinomycin (final concentration 8 $\mu g\ cm^{-3}$) was added to glucose-limited salts medium with excess K^+; no changes in growth or thermal parameters were observed.

8.2.3 Elemental Composition of Cells Grown in the Presence of Valinomycin

With the increasing concentration of valinomycin the carbon content of the cells increased, while the K^+ and hydrogen contents decreased. There was no detectable change in the oxygen content,

FIGURE 8.2 Power-time and changes in growth parameters for growth in potassium limited media in the presence of valinomycin



(Table 8.3). The analysis was performed on washed stationary phase cells harvested at the end of a calorimetric experiment.

Table 8.3 Elemental Composition of Cells Grown in the Presence of Valinomycin Under K^+-limitation

Valinomycin concentration $\mu g\ cm^{-3}$	K^+-limitation $\mu g\ cm^{-3}$	Carbon %	Hydrogen %	Nitrogen %	Oxygen %	Potassium %	Residue %
0	0.3	40.8	8.0	14.0	29.5*	1.15	8.6
6	0.3	47.5	6.9	13.0	28.9*	1.16	3.7
16	0.3	48.0	6.7	13.4	29.8**	1.09	6.5

* Determined

** By difference

8.3 Summary

1. Exponential growth ceased when K^+ was depleted from the media; growth continued at a very slow rate until glucose and its oxidation products were depleted.

2. The p-t trace was rather complex, during exponential growth power output increased exponentially, from the point of K^+-limitation it remained high at a near constant level until glucose was depleted; power then fell rapidly; during the period of secondary metabolite oxidation power increased reaching a maximum value coincident with the end of growth. The p-t trace became more simple or complex if the K^+ concentration was increased or decreased (relative to the glucose concentration) respectively.

3. ΔH_p varied randomly with K^+ concentration and was greater than that in glucose-limited growth. ΔH_p^k increased lineraly with K^+ concentration and was of similar values to glucose-limited growth.

4. The addition of valinomycin to K^+-limited growth caused marked changes in growth parameters and the p-t trace profile. The yield and CO_2 produced increased. Enthalpy changes for the first 7 h period of growth increased with increasing valinomycin concentration.

CHAPTER NINE

DISCUSSION

Throughout the course of these experimental studies emphasis was placed on the establishment and use of standard conditions of growth and test to obtain reproducible quantitative data. Only when all experimental variables and their influence on the experimental measurements are known can reliable data be collected and a meaningful interpretation made. In this respect the use of chemically defined media with a growth limiting nutrient aids interpretation of data Using the experimental arrangement described (3.5) and a flow microcalorimeter, for any bacterial species and medium, there are six main factors which can affect the measured thermal and growth data (3.0). Although these factors are inter-dependent each must be investigated to obtain the optimum experimental conditions.

It has been shown that, with continuous monitoring systems, very reproducible calorimetric and growth measurements can be made. The use of standard conditions, particularly the state of the inoculum, gave heat output values, q(exp) and q(tot), which, at the 95% confidence level, have limits of ± 1.3% and ± 3% respectively; and for the biomass ± 3.0%. The reproducibility of other parameters and observed enthalpy changes are similar (Table 3.2). Quantitative data in the literature rarely have reproducibility levels quoted; thus the significance of the results may not be easily assessed.

For discussion and comparison of the heat output data the results must be related to changes in growth parameters. This was achieved by considering the enthalpy change accompanying the utilization of 1 mol of glucose, ΔH_{gluc}; the formation of 1 mol of cells, ΔH_p, and the formation of 1 g of cells during one generation, ΔH_g(3.6). Values of these combine a series of errors from thermal and growth data, but with standard conditions their reproducibility is good;

for ΔH_{gluc} ± 6% and ΔH_p ± 10%. A very serious criticism of published calorimetric work is the lack of data on growth and other physical parameters; this means that there can be no correlation with thermal data. Earlier papers tend to consider only qualitative changes in the shape of the p-t trace.

The dry weight measurements were particularly prone to a number of sources of error and the method of monitoring growth is open to criticism (2.2.2); the bacterial volume changes appreciably during the growth cycle. In preliminary experiments during growth in the batch culture the mean bacterial volume of freshly inoculated cryogenically stored cells was 0.577 μm^3; after 90 minutes it had increased to 0.725 μm^3 subsequently decreasing to 0.690 μm^3 for the remainder of the exponential phase. After 2 hours in the stationary phase the mean volume was 0.461 μm^3. Not only do such changes affect the optical properties of the culture but changes of cell volume may have a thermal component, albeit small. The biomass of "standard cells" was a linear function of the absorbance up to an absorbance of 1.1 and since this property of the culture could be continually monitored this was the method chosen to record growth of the various cultures.

Cells grown in the presence of α-methyl glucoside were considerably larger than glucose-limited cells (Table 7.6), their refractive index and absorbance properties may be substantially different and this may lead to an increased error in biomass and ΔH_p data; ideally a special dry weight calibration is needed for growth in α-MG.

For most investigations the maintenance of fully aerobic conditions was essential. This enabled thermal data to be correlated to known, relatively simple, metabolic pathways. Whatever the dissolved oxygen concentration in the culture vessel it is very important to ensure that the oxygen concentration in all parts of the experimental assembly is similar. Thus it can be assumed that similar metabolic processes are occurring in bacteria in all parts of the flow system and the thermal events can therefore be related to metabolic events occurring in the culture vessel. For aerobic glucose metabolism the oxygen tension should exceed 15% saturation (Harrison and Pirt, 1967), in the calorimeter flow line, for standard growth the minimum pO_2 was 25%. The products of aerobic glucose metabolism are cells, CO_2 and water, secondary metabolites must be kept to negligible concentrations so that reliable mass-balance data can be obtained, this then permits the budgeting of substrate carbon and energy.

Mass-balance data for aerobic growth is presented (4.2.2), using the molar growth yield and the empirical formula weight the stoichiometric coefficient (Z) is determined first, the remaining coefficients can then be fixed (Table 4.4). Within the limits of experimental error the measured values for CO_2 production agreed well with the calculated values, all substrate carbon was accounted for as cell carbon and CO_2. The elemental composition of glucose cells, which was similar to that of published values (Wang et al., 1976; Herbert, 1976), did not vary significantly with growth temperature (5.2) but was altered as a result of growth in the presence of matabolic inhibitors (5.4.2 and 7.2.1). The carbon content of the cells was significantly greater when α-MG was used as a substrate (Table 7.5).

The heat output (q)-glucose concentration plots, (Fig. 4.3) which are linear, extrapolate through the origin. q(tot) is linear over the whole concentration range and q(exp) up to 3.8 mmol dm^{-3}. It is important that this type of plot is linear passing through the origin; intersection of the y-axis is indicative of the presence of a second energy source or secondary metabolites (Nichols et al., 1979).

With glucose concentrations below 3.8 mmol dm^{-3} when aerobic conditions are maintained throughout the assembly, ie. pO_2 > 40% saturation, the p-t trace consists of a single peak, the point of maximum power output coincides with the termination of exponential growth (Fig. 3.4) and the exhaustion of glucose. At higher glucose concentrations or with low flow rates (Figs. 3.2 and 4.1) the p-t trace shows secondary periods of heat output; during these periods changes in CO_2 output and O_2 uptake indicate the presence of oxidisable secondary metabolites; this period of oxidation does not result in further biomass production. Thus accumulated secondary metabolites are oxidized to CO_2 and water with the concomitant evolution of heat.

q(exp) is low at low flow rates (Fig. 3.3) due to loss of aerobic conditions in the calorimeter flow circuit, glucose is converted to secondary metabolites (some of which are volatile and lost from the system). The secondary metabolites are utilised after the depletion of glucose and are responsible for the second peak in the p-t trace (Fig. 3.2). At high flow rates aerobic conditions are maintained in the calorimeter circuit, glucose is fully oxidised to cell material, CO_2 and water, the heat output, q(exp), becomes constant and the p-t trace consists of a single peak.

Another feature of the change of the p-t profiles with increasing glucose or decreasing flow rate is that the first peak (P_1) occurs increasingly earlier during the exponential growth phase. This indicates that oxygen becomes growth limiting (in the calorimeter cell only) before the depletion of glucose, this is the result of the fixed aeration conditions used. Clearly in this type of system the thermal measurements cannot be strictly related to growth, because secondary products formed in the calorimeter will be recycled to the culture vessel which will affect metabolic processes occurring in the culture vessel and hence the heat output (3.4). By changing only one of two parameters (flow rate or glucose concentration) a considerable variety of p-t trace profiles can be obtained. Thus claims that microcalorimetry is suitable for routine identification purposes are probably over-stated (Boling et al., 1973; Monk, 1978) unless standard and controlled conditions are established and followed. Identification of species in a mixed culture would only be possible after a number of growth experiments (cf. Schaaschmidt and Lamprecht, 1976) thereby losing any advantages over established techniques. This application of flow microcalorimetry has recently been rejected by workers who once advocated the method (Beezer et al., 1978).

Several workers have attempted to overcome the problems associated with oxygen-starvation by additional aeration of culture in the calorimeter line. The use of alternate air-culture segments may not be as effective as once believed (Eriksson and Wadso, 1971; Few et al., 1976). The efficiency of this type of aeration depends on the relative size and dispersion of the air segment in the culture, ie. the interface area. In good oxygen transfer to the culture the air should be homogeneously dispersed as very small bubbles.

The aeration and stirring of the culture in the culture vessel used in the present work created a large number of very small air bubbles which were sucked into the calorimeter line, this coupled with a high flow rate gave good aeration over a tenfold glucose concentration range.

It is only recently that high flow rates, compatible with the attainment of thermal equilibrium, have been used. The choice of flow rate was based not only on the p-t profile but, more importantly, on the total heat output (Fig. 3.3). A flow rate of 90 cm^3 h^{-1} gave a transit time from the culture vessel to the calorimeter of 1.3 minutes, this is the shortest time reported to date. Because of the importance of this factor it is recommended that it is always reported, similarly the residence time of the organisms in the calorimeter cell. This time is rarely considered, the short time (0.8 min) used in this work is favourable when compared with so called improved flow cell design which give values of Δt and Δr of 1.5 and 0.8 mins respectively (Gustafsson, 1980).

The use of cryogenically-stored bacteria as a standard inoculum for calorimetry has not been reported previously, although Froud (1972) advocated the general advantages of standard cryogenic inocula. Beezer et al., (1976) used cryogenically-stored yeast for calorimetric investigations; initially the method was used to reduce variable inoculum response to antibiotics, the improvement in thermal data subsequently becoming apparent. A variable inoculum gives irreproducible thermal measurements (Fig. 3.1), this can be due to variable inocula size, viability levels and/or physiological states of individual cells. When cells from a growing culture were used a lag period developed, the length of which varied greatly.

During a lag period bacterial metabolism can be different from that during exponential growth, cellular damage must be repaired and essential metabolites synthesised before cell growth and division occurs. This means possible changes in the composition of the medium and this affects future metabolic events; such changes have been known for a long time (Sherman and Albus, 1924; Dagely et al., 1951). It is interesting to note the preference of some workers (Lamprecht, personal communication) to use small inocula for thermal studies; their reasoning is that during the long lag and adjustment period variability of the cell population is reduced. Considering the problems encountered in this work the question to be asked is: whether measured thermal changes are really due to the attributed metabolism when a lag occurs? While this procedure is not recommended for quantitative work it has serious limitations in qualitative studies too.

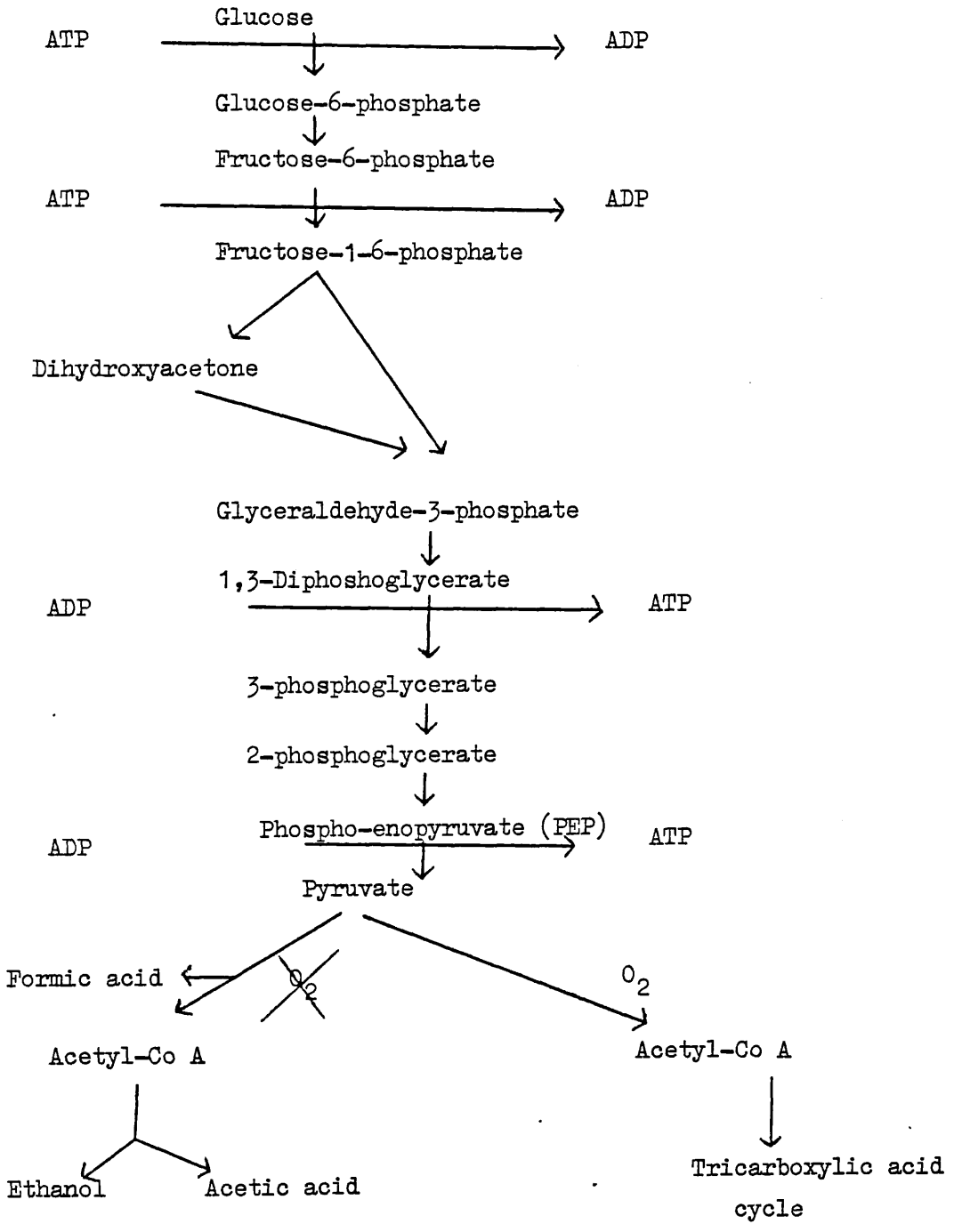
Cryogenically-stored inocula for calorimetric work have a number of other advantages, the main one being the production of reproducible growth and thermal data. They also make possible reliable comparative studies between laboratories since they are easy and convenient to handle. The only restraint is the undesirability of adding a cryoprotectant which would be transferred to the culture vessel and could affect the metabolism of the culture. For the simple organism used in this work the absence of a cryoprotectant had no adverse effect on the viability and other properties of the cells; however, this may not be the situation with more fragile and more nutritionally demanding organisms. The cooling velocity ($24^{\circ}K\ min^{-1}$) is above that regarded as optimum for microorganisms (Beezer et al., 1976), nevertheless there was no detectable loss of viability after several months of storage.

The specific power output has a characteristic profile during the exponential growth phase, (Fig. 3.4), irrespective of the glucose or α-MG concentration or the growth temperature. The maximum value which occurs during early exponential growth confirms the observation of Schaarchmidt et al., (1975) that acceloratory processes have a high rate of energy dissipation. The small increase near the end of exponential growth could be due to nutritional stress or deceloratory processes which are energetically inefficient. Gustafsson, (1979), presented evidence that nutritional stress may increase heat dissipation.

The rate constants for heat output, biomass production and CO_2 output during growth in a fixed concentration of glucose vary with temperature (Fig. 5.3) and with glucose concentration at constant temperature (Table 4.1). Generally, at low glucose concentrations (<3.3 mmol dm^{-3}), the rate constants for biomass production are greater than those for heat output; at higher glucose concentrations the trend is reversed; (exceptions occur at the extreme glucose concentrations used). Previous reports (Forrest and Walker, 1962; Belaich et al., 1968) indicate that the rate of degradation of energy source, biomass production and heat output are similar. This suggests that in the present work, the rate of glucose degradation is not equal to the rate of biosynthesis. Glucose is catabolised by the Embdem-Meyerhof pathway (Fig. 9.1a) and the Tricarboxylic acid (TCA) cycle (Fig 9.1b).

Catabolic processes are exogonic; although Forrest (1972) showed that calorimetry measures the heat output associated with catabolic processes, it must be realized that it is waste heat which is measured. This is the difference between the energy supplied and

FIGURE 9.1a Embden-Meyerhof glycolytic pathway



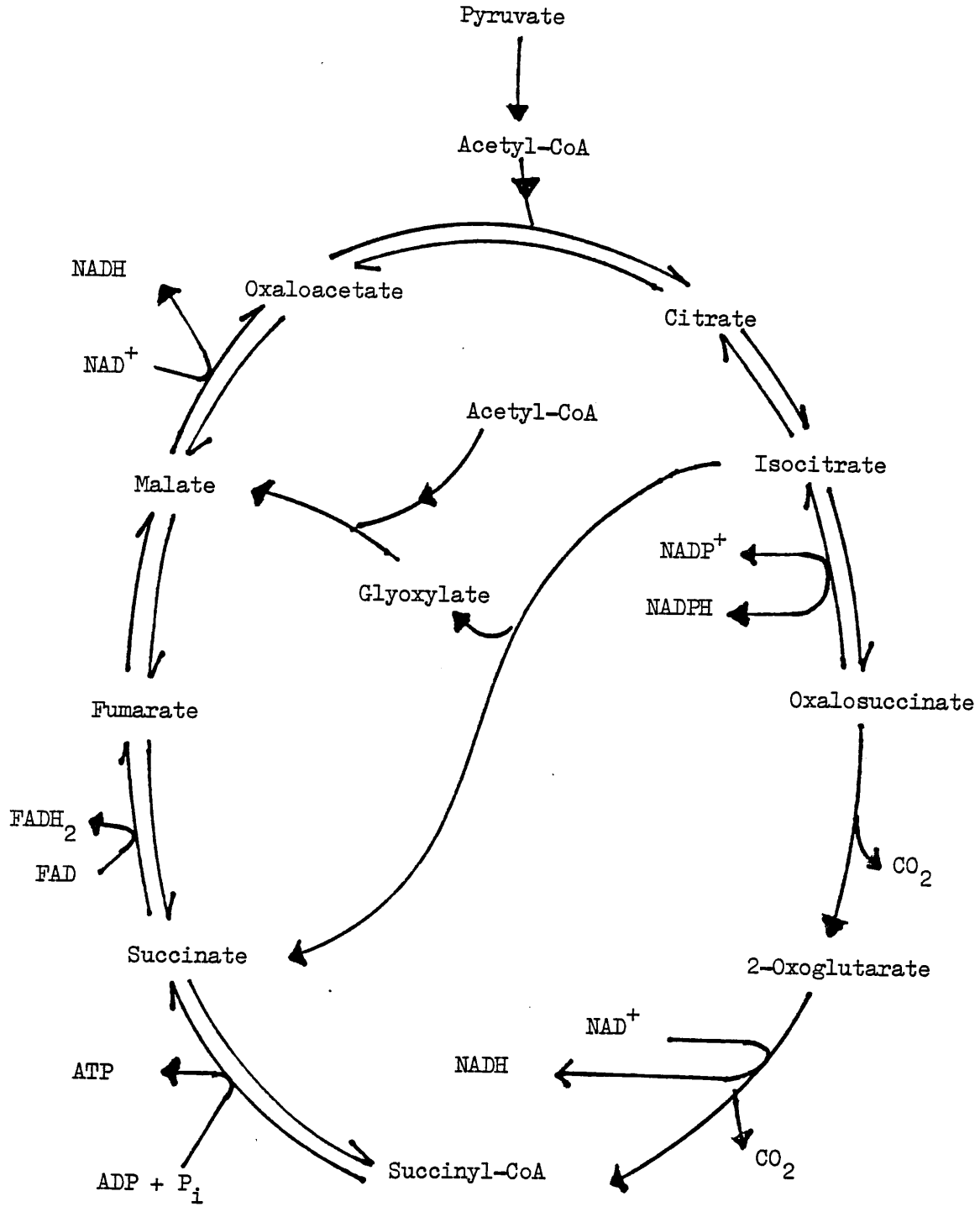


FIGURE 9.1b The Tricarboxylic Acid Cycle and Glyoxylate Bypass

that evolved in catabolic and absorbed in anabolic processes. Since energy yielding reactions must occur before biosynthsis it is more likely that there should be a difference in the rates, similar to those obtained at high glucose concentrations. If the coupling between energy production and biosynthesis is very 'tight' then similar rates would be expected, irrespective of glucose concentration (Forrest, 1972). At a fixed initial glucose concentration the rate constants increased in parallel with temperature over the range 25-37°C (Fig. 5.3), at higher temperatures (37-39°C) the rate constants became similar. At still higher temperatures changes in the metabolism occur and more secondary products are formed, this reduces energy production hence heat output during exponential growth. Thus the conclusion from Forrest's (1972) proposal is that "tighter" coupling has occurred, in effect the reverse is more likely to have occurred.

Neijssel et al., (1976a,b) have proposed slip reactions during growth of K. aerogenes in glucose sufficient chemostats. In the slip mechanism the energy source, in excess, is degraded at a much higher rate than necessary for biosynthesis, ie. energy is lost as extra heat resulting in low yields. It is important to recall that during growth in batch culture glucose is in excess until near the point of limitation. A slip mechanism would give a rate constant for heat production in advance of that for biomass production. This is observed above 3.8 mmol dm^{-3} glucose, but there is no dramatic decrease in the yield (Table 4.1). Many factors could affect the rate constants, eg. transport processes, extracellular glucose concentration (which is continually decreasing during batch growth); so a full explanation of the observed changes is not possible. The experimental conditions are particularly important if the biomass and heat output data are to be comparable. At low flow rates through the calorimeter (40 cm^3 h^{-1}) the rate constants (Nichols et al., 1979), and yields (Table 9.1) meet the requirements of the proposed

Table 9.1 Heat Output of Cells of K. aerogenes in Simple Salts/Glucose Media of Different Initial Glucose Concentrations; Flow rate = 40 $cm^3\ h^{-1}$

Glucose concentration /mmol dm^{-3}	Yield coefficient /g g^{-1}	q(tot) /J 1.16 cm^{-3}	ΔH_p /kJ (mol cell)$^{-1}$	ΔH_{gluc} /kJ (mol gluc)$^{-1}$	ΔH_{ox} /kJ (mol gluc)$^{-1}$	ΔH_B /kJ (mol gluc)$^{-1}$	$\Delta H_B/\Delta H_s$ x 100 %
0.55	0.42	0.73	-374.3	-1159	-1417	-258	9.0
1.10	0.41	1.40	-372.2	-1102	-1468	-366	12.8
1.60	0.39	1.66	-308.2	- 897	-1531	-634	22.2
2.20	0.37	2.14	-309.3	- 839	-1579	-741	25.8
2.80	0.40	2.77	-301.3	- 855	-1488	-633	22.1
3.30	0.34	2.60	-271.8	- 681	-1683	-1002	35.0
5.00	0.31	3.25	-328.0	- 562	-1812	-1249	43.7
5.50	0.29	3.76	-281.1	- 589	-1866	-1277	44.6

$\Delta H_B = \Delta H_{ox} - \Delta H_{gluc}$

$\Delta H_s = 2862$ kJ (mol gluc)$^{-1}$

slip-mechanism, ie. heat output in advance of biomass production and low yields. But it is known that low oxygen tension is responsible for changed metabolism in the calorimeter circuit.

Cells grown in a substrate which provides both energy and carbon for growth have to control the balance of catabolism and anabolism, ie. the conversion of substrate to cellular material while having sufficient energy to do so. Battely (1960) defined growth reactions as convervative or non-conservative with respect to the available energy. In non-conservative reactions all the available energy from the substrate is converted into enthalpy and entropy, as a result the measurable heat will be maximal. Because of the need for this control, several suggestions have been made as to the existence of an 'energy budget' (Senez, 1962; Mennett and Nakayama, 1971).

Two methods have been proposed to express the efficiency of bacterial growth, caloric efficiency (McCarty, 1965) and yield-mass balance data. The expression: $Y\ \Delta H_{c/\Delta H_s} \times 100$ has been accepted as valid for expressing caloric efficiency of heterotrophs (Mennett and Nakayama, 1971). Where ΔH_c is the heat of combustion of cells, ΔH_s the heat of combustion of glucose and Y a yield factor. The percentage waste heat can be obtained from a similar expression in which ΔH_c is replaced by ΔH_p (Table 9.2).

The heat of combustion of cells of K. aerogenes was 22.7 kJ $(g\ cell)^{-1}$ irrespective of glucose concentration. This compares favourably with the literature values of 22.3 and 23.4 kJ $(g\ cell)^{-1}$ (Wang et al., 1976; Prochazka et al., 1973). The oxidation of the cellular material (equation 9.1) in the bomb calorimeter actually measures changes in the internal energy of the process.

Table 9.2 Caloric Efficiency and Waste Heat for Cells of K. aerogenes Growing on Glucose Limited Media

Glucose concentration /mmol dm^{-3}	No. of generations	Yield coefficient /g g^{-1}	ΔH_p /kJ g^{-1}	ΔH_g /kJ g^{-1} gen^{-1}	Caloric[1] efficiency %	Waste[2] heat %
0.56	3.99	0.44	-15.28	-3.83	63.4	42.8
2.20	5.16	0.43	-14.42	-2.79	62.0	39.5
3.30	5.67	0.43	-13.50	-2.38	62.0	32.0
3.80	5.70	0.44	-12.88	-2.26	63.4	36.1
4.20	6.01	0.43	-12.24	-2.04	62.0	33.5
5.40	6.10	0.42	-13.81	-2.26	60.6	36.9
$\bar{x}$						

(1) Caloric efficiency $100\ (\Delta H_c/\Delta H_s)Y_c$

(2) Waste heat $100\ (\Delta H_p/\Delta H_s)Y_c$

ΔH_g = ΔH_p/No of generations

ΔH_c = -22.7 kJ $(g\ cell)^{-1}$

ΔH_s = -15.7 kJ $(g\ glucose)^{-1}$

$$CH_{1.77}O_{0.48}N_{0.25} + 1.02O_2 \longrightarrow CO_2 + 0.51H_2O + 0.25\,NH_3 \quad 9.1$$

The enthalpy change is calculated from equation 9.2.

$$\Delta H = \Delta U + \Delta nRT$$

Where Δn, the difference between the number of molecules of gaseous products and gaseous reactants, is 0.25. Thus for the combustion of the cells $\Delta n\,RT = 0.62$ kJ and is within the limits of experimental error thus $\Delta H = \Delta U$.

The caloric efficiency is similar for all glucose concentrations, the waste heat accounts for all the remaining available energy from the glucose (Table 9.2). From the total available energy from glucose (2862 kJ mol^{-1}), 1780 kJ mol^{-1} are stored and 1082 kJ dissipated as heat. In his review Payne (1970) noted that 55-65% of available energy was stored as cell biomass irrespective of substrate or organism, the mean value obtained in this work of 62% is in agreement with these earlier values. Although it has been reported that the chemical composition of bacterial cells depends on the growth temperature (Tempest and Hunter, 1965), no significant difference was observed over the temperature range 25-42°C. The finding that the heat of combustion of cells of P. fluorescens was constant when grown at temperatures in the range 20-35°C (Mennett and Nakayama, 1971) is not too surprising. Nevertheless for a wide range of bacterial species with significantly different chemical compositions the heats of combustion are quite similar with a mean of 22.6 kJ $(g\ cell)^{-1}$ (Prochazka et al., 1973). Thus although the chemical composition of the cell can vary with species and temperature, ΔH_c is invariant, the possibility of considering an energy budget is favourable. Since the yield varies with growth temperature (Table 5.1) it follows that the caloric efficiency (which is yield-dependent) also varies with temperature. At temperatures below 40°C the yield and

hence caloric efficiency are not markedly affected by growth temperature; however at higher temperatures there is a significant decrease in caloric efficiency from 59% at 41.5°C to 49% at 42°C.

The energy budget can be examined in more detail using mass-balance data. The enthalpy of oxidation, ΔH_{ox}, calculated from the fraction of glucose oxidised to CO_2 and H_2O can be compared with the determined value, ΔH_{gluc} (Table 9.3). The difference between these values, ΔH_B, which is greater than the experimental error can be attributed to the enthalpy changes associated with anabolic growth processes. This accounts for 5-15% (depending on the glucose concentration) of the total energy available from the glucose. Early reports indicated that the enthalpy of growth, ΔH_B, was too small to measure, or smaller than the experimental error (Forrest, 1972) are therefore not substantiated. A value of 9% for the enthalpy of growth of *E. coli* on succinate has been reported (Dermoun and Belaich, 1979).

The total energy available from glucose can be accounted for in terms of energy stored, energy for anabolic processes and waste energy (heat). While the proportion of energy stored remains constant with increasing glucose concentration, there is a decrease in the waste energy and an increase in the anabolic energy (Fig. 9.2). The increased energy required for anabolic processes at higher glucose concentrations is possibly needed for the incorporation of small quantities of secondary metabolites into cellular components in place of the equivalent amount of glucose (Whitaker and Elsden, 1963); also other processes may be more energy demanding at higher glucose concentrations, eg. transport processes. Kinetics of glucose permeation determined from calorimetric data (Belaich *et al.*, 1968) indicates that a single transport system, as envisaged by Hoffee *et al.*, (1964) is present; in this the affinity

FIGURE 9.2 Energy budget for cells of K. aerogenes growing in glucose-limited media at 37°C

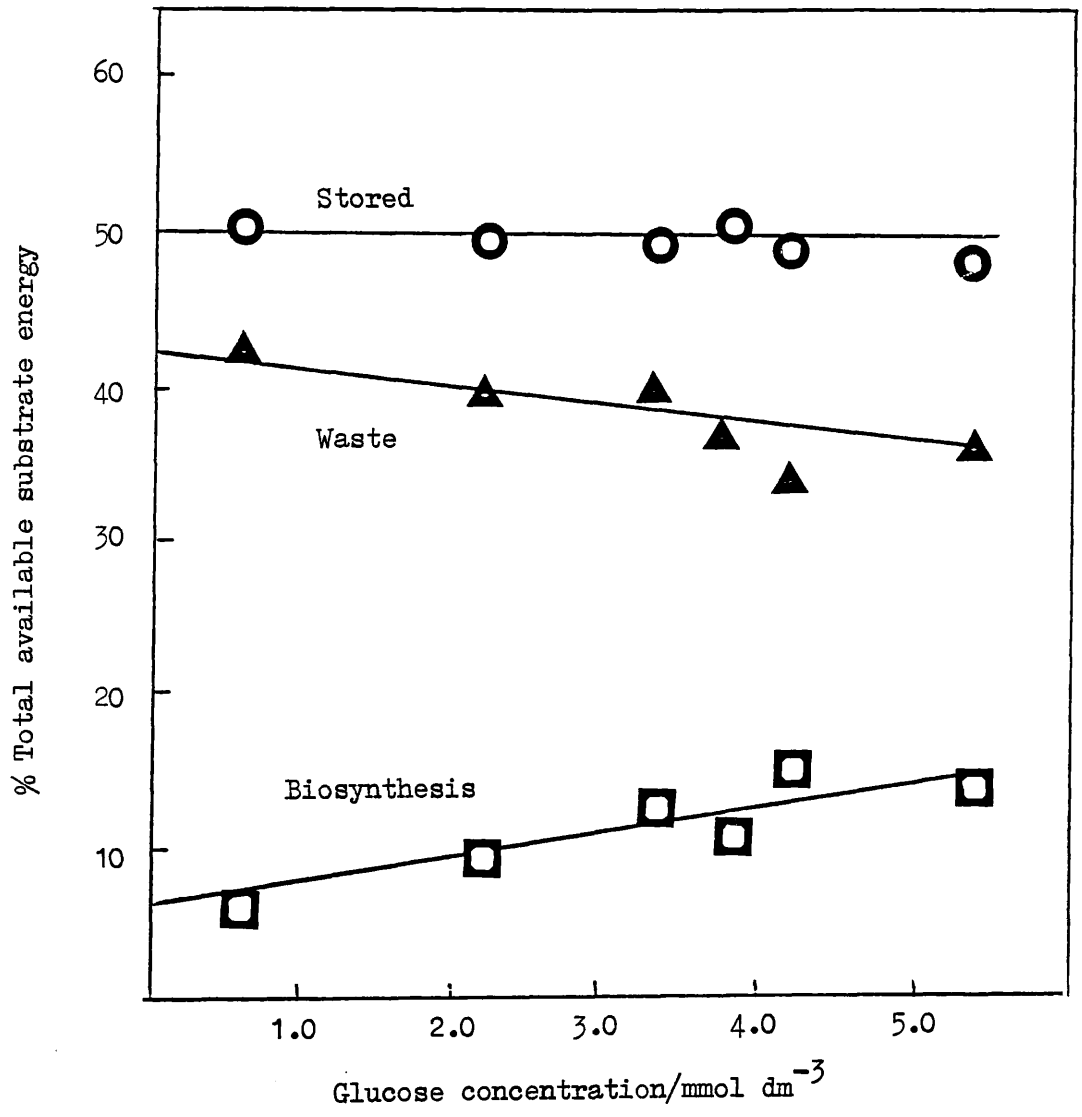


Table 9.3 Proportion of Enthalpy of Oxidation of Glucose Recovered as Heat (Flow rate 90 cm^3 h^{-1})

Glucose concentration /mmol dm^{-3}	ΔH_{ox}(1) /kJ(mol gluc)$^{-1}$	ΔH_{gluc} /kJ(mol gluc)$^{-1}$	ΔH_B(2) /kJ(mol gluc)$^{-1}$	$\Delta H_B / \Delta H_s$ x 100 %
0.56	-1350.0	-1200.6	-149.4	5.2
2.20	-1388.1	-1120.6	-267.5	9.3
3.30	-1288.1	-1037.7	-350.4	12.2
3.80	-1350.0	-1045.5	-304.5	10.6
4.20	-1388.1	- 950.3	-437.8	15.2
5.40	-1453.9	-1048.8	-405.1	14.2

(1) ΔH_{ox} = % carbon oxidised x ΔH_s

(2) ΔH_B = ΔH_{ox} - ΔH_{gluc}

ΔH_s = 2862.1 kJ (mol gluc)$^{-1}$

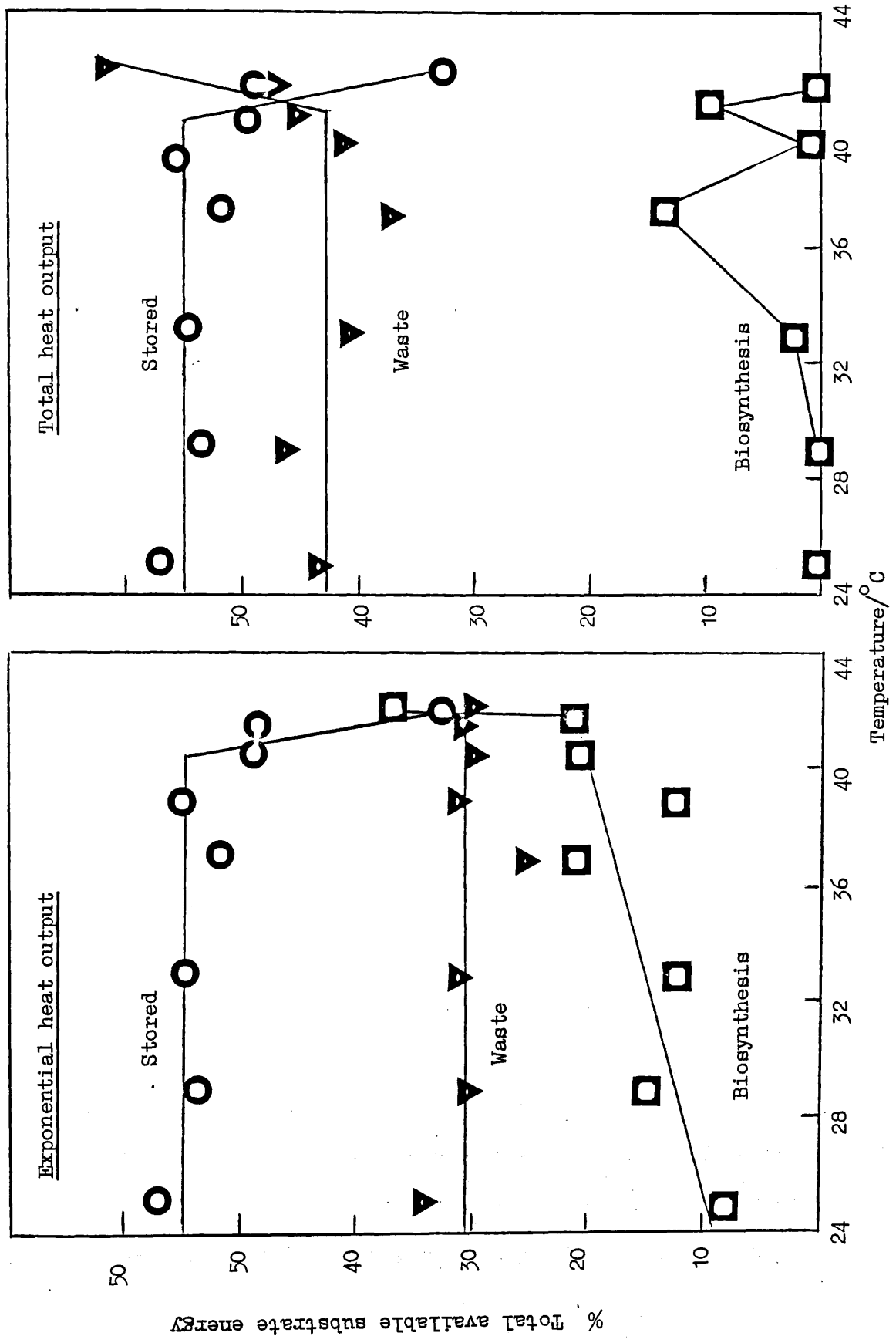
for glucose depends on the extracellular glucose concentration with the transport mechanism being coupled to an energy source, ie. the energy for the transport of glucose would be part of the enthalpy of growth term.

A similar trend in ΔH_B occurs when large quantities of secondary products are produced, some of which will be volatile and/or not further metabolised, thus their energy is lost to the cell (Table 9.1). It is only during growth at the two lowest glucose concentrations at the lower pump rate that the term, ΔH_B, approaches the values for aerobic growth (recorded at a flow rate of 90 $cm^3\ h^{-1}$).

When the energy budget is considered for growth at constant initial glucose concentration but at different growth temperatures the stored energy is constant for temperatures in the range 25-39°C, thereafter falling dramatically (Fig. 9.3). At growth temperatures 25-33°C, the wasted energy accounts for the remaining energy from the substrate, thus ΔH_B is zero, while at temperatures of 37°C and above significant values for ΔH_B were recorded.

The waste energy during the exponential growth period only, is almost constant for all temperatures (Fig. 9.3); the difference between this and the total waste energy is that produced during the stationary phase. From 25-39°C the difference is constant at about 8%; at about 39°C it increases to about 30%. This indicates that large amounts of glucose have been converted to secondary products which are subsequently oxidised by exothermic processes during the stationary phase as is indicated by O_2 uptake and CO_2 production. These products are responsible for the difference in the ΔH_{ox} and ΔH_{gluc} values, ie. the enthalpy of growth. The reason for the variation in the value of ΔH_B at temperatures below 37°C is unknown.

FIGURE 9.3 Energy budget for cells of K. aerogenes at different growth temperatures: glucose concentration 3.3 mmol dm^{-3}



The energy budget for the growth of α-MG-trained cells with α-MG as sole carbon substrate provides an anomalous situation. With the exceptions of growth in media of 0.7 and 4.6 mmol dm^{-3} α-MG the negative value of $\Delta H_{\alpha\text{-MG}}$ exceeds the theoretical maximum, ΔH_{ox} obtained from mass-balance data. The heat of combustion of α-MG was determined by bomb calorimetry and found to be 3582.3 kJ $(mol\ \alpha\text{-MG})^{-1}$. The calculated and experimental stoichiometric coefficients for CO_2 are in poor agreement, (Table 9.4), particularly below 2.7 mmol dm^{-3} α-MG. Koser and Saunders (1934) reported that for K. aerogenes α-MG is fermented with the production of a variety of secondary products, although the level of aeration is unclear in their work; the results presented here indicate that α-MG is not metabolized in the same manner as glucose. In making the calculations it has been assumed that the cells were fully trained to growth in α-MG; this may not be true. It would be desirable to continue training for a much larger number of generations and then to repeat the thermal and growth measurements. An interesting feature of the p-t trace profile (Fig 7.4) is that the first maximum of power output occurs before the end of exponential growth, while changes in the other parameters (CO_2, pO_2) occur at the end of exponential growth, as for growth of glucose-trained cells in glucose (Fig 3.4).

Similar p-t traces have been recorded during the growth of glucose-trained cells in glucose medium, eg. at low flow rates through the microcalorimeter cell. When the peak power output occurs before the end of exponential growth, however, this is always accompanied by abrupt changes in other parameters. These data were interpreted in terms of oxygen limitation, but oxygen limitation does not occur during growth in α-MG. The out-of-phase relationship between power and the growth

Table 9.4 Mass Balance Data for Aerobic Growth of K. aerogenes in α-MG Medium

α-MG concentration /mmol dm^{-3}	$\underline{Y}_m$ g (mol α-MG)$^{-1}$	Fate of Substrate Carbon: Converted to cell %	Oxidised %	Stoichiometric coefficients for mass balance (1) cell $\underline{Z}$	CO_2 $\underline{L}$	Expt value of $\underline{L}$	ΔH_{ox}(2) /kJ(mol α-MG)$^{-1}$	$\Delta H_{\alpha\text{-MG}}$ /kJ(mol α-MG)$^{-1}$
0.7	81.5	40.4	59.6	2.83	4.17	2.62	-2135.1	-1391.5
1.4	110.6	54.9	45.1	3.84	3.16	2.38	-1615.6	-2961.6
2.0	98.9	49.0	51.0	3.43	3.57	1.86	-1815.6	-2797.2
2.7	95.1	47.1	52.9	3.30	3.70	3.83	-1895.0	-2394.4
3.4	95.1	47.7	52.9	3.30	3.70	3.53	-1895.0	-2332.4
4.0	85.4	42.3	57.7	2.96	4.04	3.68	-2067.0	-2135.7
4.6	95.1	47.1	52.9	3.30	3.70	3.87	-1895.0	-1746.5

(1) $C_7H_{14}O_6 + NH_3 + O_2 \longrightarrow \underline{Z}\ CH_{1.73}O_{0.43}N_{0.23} + L\ CO_2 + H_2O + \Delta H_{ox}$

(2) ΔH_{ox} = % carbon oxidised x ΔH_s

ΔH_s = 3582.3 kJ (mol α-MG)$^{-1}$

Mr = 28.83

parameters is probably a function of metabolic activity. It could be conjected that, because of the low growth rate and the near depletion of α-MG, a proportion of the population is not able to obtain sufficient α-MG to maintain their metabolic functions, thus a slowing down occurs with a decrease in power output. This may be because of poor transport across the cell membrane, when sufficient α-MG is present the rate of heat output is in advance of that for biomass, similar to that observed for growth in most glucose concentrations.

Interpretation of the thermal data is hindered by the lack of knowledge on the processes involved in the metabolism of α-MG. α-MG has been used in sugar-transport studies as a glucose analogue, for those strains which can metabolise α-MG chloramphenicol is added, this inhibits protein synthesis. Free α-MG and α-MG-6-phosphate have been found inside the cell, but how the α-MG is metabolised, once in the cell is unknown, two possibilities are advanced (Fig. 9.8); these will be described later.

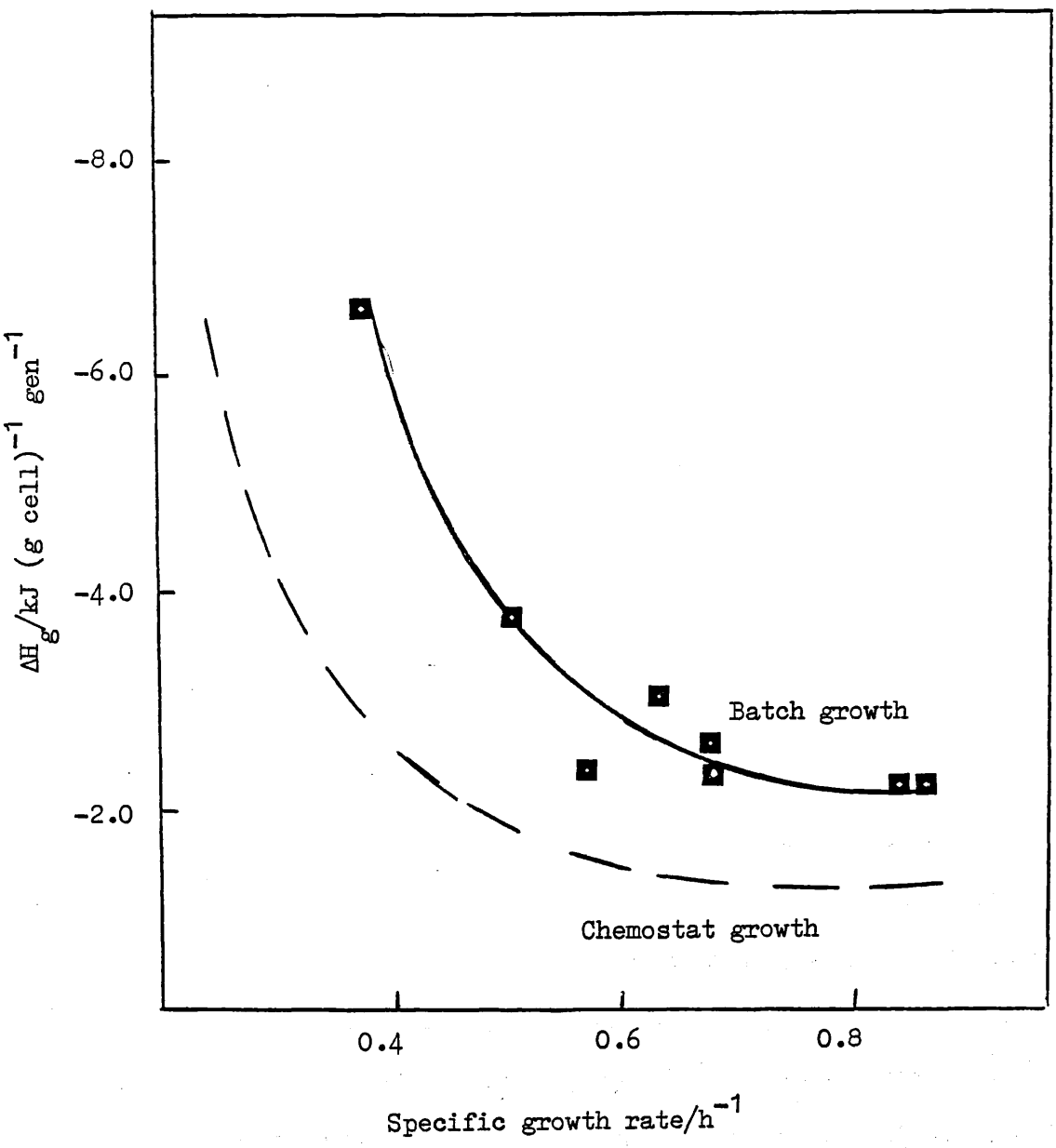
The enthalpy of production of 1 mol of cells, ΔH_p, does not vary significantly with the glucose concentration (Fig. 4.4), the mean value for ΔH_p is -355 kJ (mol cell)$^{-1}$. This is equivalent to a mean specific heat output, ΔH_{sp}, = -14.2 kJ. The lower values are similar to the value of -11.0 kJ (g cell)$^{-1}$ reported by Grangetto (1963) for K. aerogenes growing aerobically in glucose in batch culture. ΔH_{sp} varies with the nature of the substrate, the condition of the inoculum and type of growth. For K. aerogenes grown on succinate ΔH_{sp} = -16 kJ (g cell)$^{-1}$, (Grangetto, 1963) and E. coli on succinate ΔH_{sp} = -19.7 kJ (g cell)$^{-1}$ (Derman and Belaich, 1979). Unfortunately the condition and history of the inoculum and the growth conditions are not always reported, so results between laboratories are not strictly comparable.

The similarity of the results does indicate that the substrate energy wasted as heat is similar; this would be expected if an overall general energy budget exists.

In batch culture the heat output per generation, ΔH_g, varies with glucose (substrate) concentration and growth temperature. For glucose concentrations 0.56 to 5.4 mmol dm^{-3} the mean value is -2.6 kJ g^{-1} gen^{-1}; for the same doubling time (or specific growth rate) for K. aerogenes growing in a chemostat in medium of the same composition $\Delta H_g = -1.09$ kJ g^{-1} gen^{-1}, a value which is independent of glucose concentration (Djavan, 1980). Additionally the yield in a chemostat is greater than in batch growth, thus the caloric efficiency is higher in chemostat culture.

Djavan (1980) found that ΔH_g (his ΔH_p) depended on the dilution rate (ie. specific growth rate), at high dilution rates ΔH_g tends to a constant low negative value. Comparison of values obtained for chemostat culture with ΔH_g values obtained during growth in batch culture at different growth rates, (produced by growth at different temperatures), indicates that growth in batch culture always results in more heat production (Fig. 9.4). Djavan found that for a fixed growth rate there was no difference in ΔH_g or yields when the chemostat was at 37°C or 42°C; this is not the case for batch growth. This implies that different metabolic control mechanisms may occur in batch and chemostat growth. Nutritional needs can be influenced by growth temperature (Ingram, 1965) but this is more important with nutritionally demanding organisms growing in complex media. The thermal and growth properties of K. aerogenes grown from cryogenically stored cells, at a given temperature were independent of the temperature at which the cells were grown for inoculum preparation; ie. cryogenic inocula prepared from cells at 37°C or 25°C when grown at 29°C gave identical p-t traces and growth curves.

FIGURE 9.4 Variation of ΔH_g with growth rate for cells of K. aerogenes growing in glucose-limited media



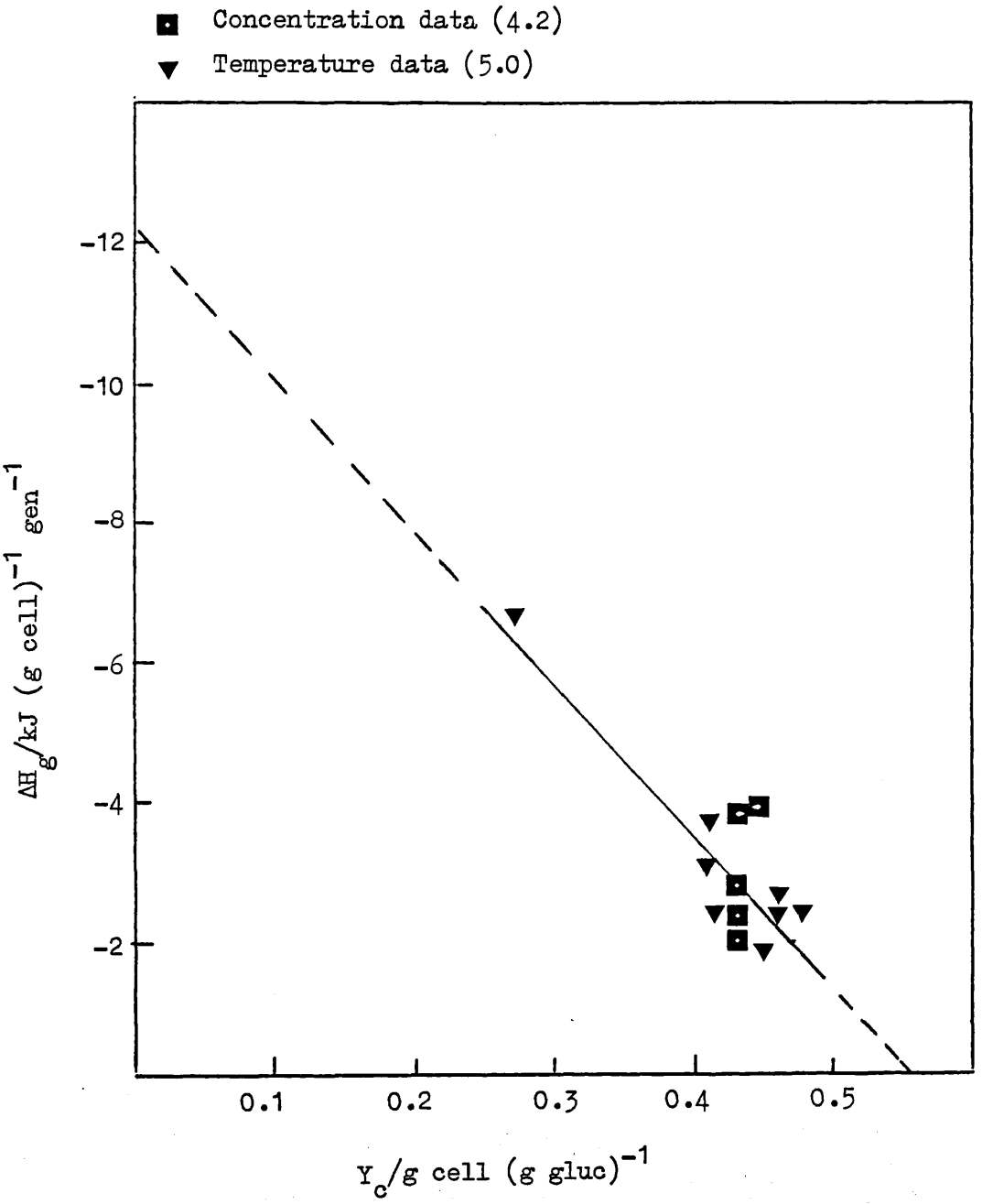
There is evidence that at some temperatures growth and respiration become uncoupled (Senez, 1962), at temperatures above 37°C for K. aerogenes both the yield and growth rate decreased to zero at 42°C, whereas the respiratory activity increased and was progressively inhibited above 42°C. This suggests that in this temperature region growth and respiration have become uncoupled. The changed p-t trace and changes in the other growth parameters (Table 5.1) during aerobic growth at different temperatures indicates that the products of metabolism vary with temperature. Marked changes in the relative rate constants for heat and biomass production, and CO_2 evolution occur at temperatures in excess of 37°C, and in the range 40.5 to 42°C these become equal at a very low value (Fig. 5.3). Yields were low although glucose was metabolised; this may represent a form of uncoupling. Since energy-growth coupling will be mediated by enzymes, the activities of which are temperature dependent, it is not possible to envisage an overall simple uncoupling mechanism. It is possible that reduced enzyme activity accounts for changes over the temperature range 25-39°C, while at higher temperatures complete loss of activity of specific enzymes through denaturation may occur. Oxidation (respiration) of glucose could still occur without biomass production, this would require large amounts of oxygen, as is found experimentally (5.1).

The concept of maintenance energy has been used in studies of the energetics of bacterial growth. This is the energy required to maintain the structural integrity and composition of a bacterial cell (Stouthamer, 1977). To account for the variation of yield with dilution rate in continuous culture, Pirt (1965) assumed that the maintenance energy was the same for resting and growing cells. Later studies have revealed the existence of two components (Stouthamer, 1977), one a growth-rate-dependent term and the other a growth-rate-independent

the latter applies to processes other than formation of new cell material. Values for these two terms have been calculated from calorimetric data (Djavan, 1980) for aerobic growth of K. aerogenes in chemostat culture. There is little data on maintenance energy for cells growing in batch culture, possibly because accurate calorimeters have not been available. Data accumulated during the growth of cells in media of different glucose concentrations or at different temperatures plotted as ΔH_g against Y_c, (Fig 9.5) show that the heat evolved for the formation of 1 g of cells during one generation time decreases linearly with increase in yield. When no biomass is formed and glucose is oxidised to CO_2 and water only the heat output will be maximal, ie. for glucose 15.7 kJ g^{-1}, this theoretical value should be attained when $Y_c = 0$. At the other extreme if all the substrate is converted to cellular material the extrapolated value, as ΔH_g approaches zero, should be the theoretical maximum yield possible, Y_c^{max}. Although these quantities are of limited value because of the large extrapolations needed; nevertheless some interesting conclusions and comparisons with other results can be obtained.

The calculated extrapolated values are $Y_c^{max} = 0.56$ g cell (g glucose)$^{-1}$ and $\Delta H_g = 12.7$ kJ g^{-1}. The theoretical maximum yield is very close to that found by Djavan (1980) of 0.58 g cell (g glucose)$^{-1}$ for cells growing in chemostat culture. The extrapolated value of ΔH_g is 3.5 kJ less than the heat of combustion of glucose, this is equivalent to the combustion of 0.22g glucose. A similar plot can be made of ΔH_{gluc} against Y_c; the extrapolated values are $\Delta H_{gluc}^{max} = -2511.4$ kJ (mol gluc)$^{-1}$ and $Y_c^{max} = 0.89$ g cell (g glucose)$^{-1}$, the expected values are -2862 kJ (mol gluc)$^{-1}$ and 0.9 g cell (g glucose)$^{-1}$ if assuming glucose

FIGURE 9.5 Plot of ΔH_g against Y_c for cells of K. aerogenes grown in glucose-salts media

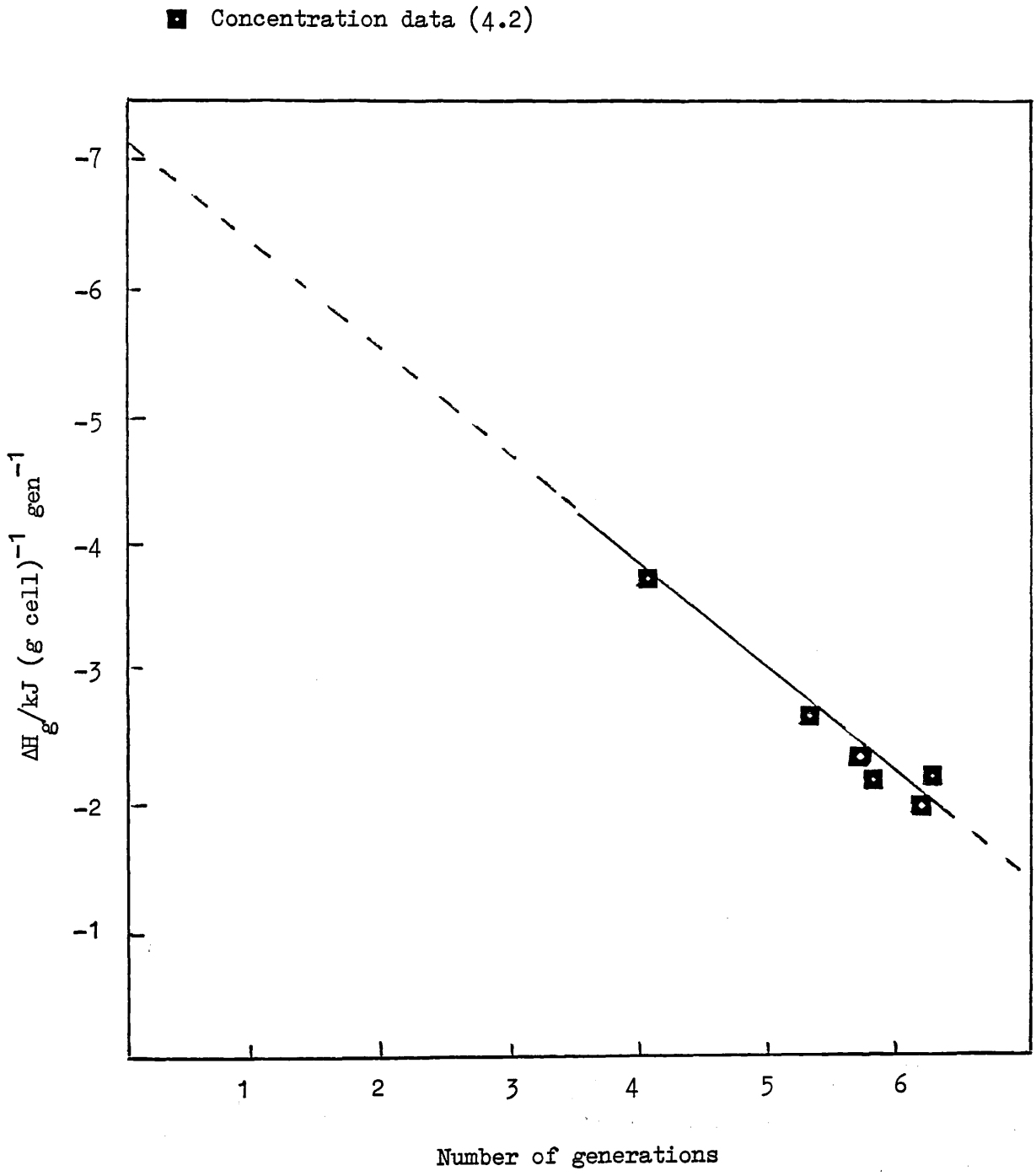


is completely oxidised to CO_2 and water or is completely converted to cell material respectively.

The plot of ΔH_g against the number of generations, for aerobic growth in glucose media, is linear (Fig. 9.6). Extrapolation of this line to zero generations gives $\Delta H_g = -7.07$ kJ g^{-1} gen^{-1}, and to zero heat output, gives the maximum number of generations as 8.62. If the theoretical maximum number of generations is that necessary to give the theoretical maximum yield (for aerobic growth with a doubling time of 45 mins), then the intercept -7.07 kJ g^{-1} gen^{-1} represents the efficiency of substrate conversion, ie. -7.07 kJ $(g\ cell)^{-1}$ is wasted heat, thus 55% of the available energy is available to be converted into cellular material. The slope of the line, 0.82 kJ g^{-1} gen^{-1} (or 0.05 g glucose g^{-1} gen^{-1}), represents a maintenance energy term. Considering the doubling time this can be converted to give a maintenance coefficient of 0.066 g glucose $(g\ cell\ h)^{-1}$. This is similar to the value given by Djavan (1980) of 0.061 g glucose $(g\ cell\ h)^{-1}$ for the growth-rate-dependent term, m_g. Taking into account the errors involved in measurement and the extrapolation to give these small energy values, the agreement between the two results is good.

For growth in α-MG (7.2) the corresponding values are ΔH_g^{max} = 12.7 kJ g^{-1}, maximum number of generations = 9.01 and slope = 1.41 kJ g^{-1} gen^{-1}. The latter value is equivalent to 0.08 g α-MG $(g\ cell)^{-1}$ gen^{-1} or a calculated maintenance coefficient (m_g) of 0.062 g α-MG $(g\ cell\ h)^{-1}$, a value very similar to that obtained for glucose growth. Erikson et al., (1979) obtained a combined maintenance coefficient of 0.076 g glycerol $(g\ cell\ h^{-1})$ for K. aerogenes. At the present time it is not possible

FIGURE 9.6 Variation of ΔH_g with the number of generations during growth of cells of K. aerogenes in glucose-salts media

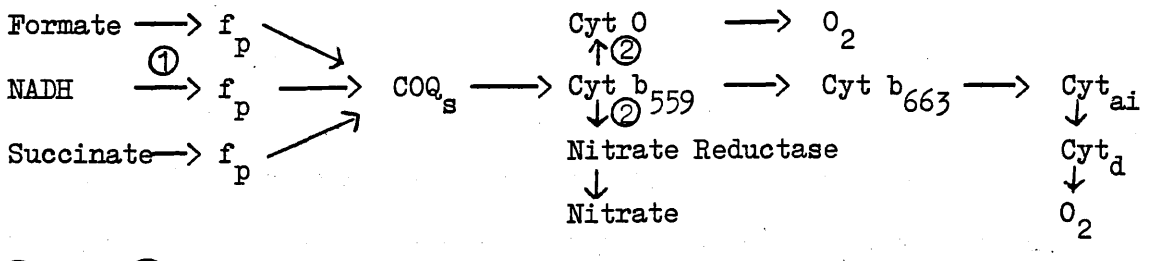


to say whether the slope represents the growth-rate-dependent term or an overall maintenance coefficient. The latter seems unlikely since ΔH_g is dependent on growth rate (as indicated from temperature studies, Fig. 5.3).

One question that may be posed is why should ΔH_g vary with the number of generations? Consideration of the specific power-time profile (Fig 3.4) shows that the most energetically inefficient period is the early part of the exponential growth period, ie. the first generation time. As further generations occur the growth becomes more energetically efficient, with more generations the large initial energy requirement is spread over more generations thus reducing the value, until at the maximum number of generations possible the value should approach zero. This value will never be attained in practice because the chemical reactions will always result in heat some of which will be lost from the system. The natural progression is from batch to continuous growth where an infinite number of generations is possible, thus continuous growth will always be more energetically efficient than batch growth.

Cellular mechanisms influenced by oxygen are numerous, oxygen is a component of cellular material and it is the final acceptor in the respiratory chain (Fig. 9.7).

FIGURE 9.7 Respiratory Chain of Enterobacter aerogenes



① and ② sites of phosphorylation

(from Stouthamer, 1977)

Minkevich and Eroshin (1973) concluded that oxygen uptake was proportional to heat production, while Poole and Haddock (1975). showed a strong correlation between heat output and oxygen uptake. The heat (and power) output should be expected to mirror the oxygen uptake, ie. pO_2 profile. This was always found to be the case, indicating that heat output was due to oxidative processes; further since the CO_2 profile paralleled heat output the oxygen must have been for the oxidation of some of the carbon substrate.

During growth in the presence of excess oxygen, although the yield and CO_2 output were reduced, the enthalpy changes were markedly less than those recorded for normal aerobic growth (Table 6.1). Over-aeration inhibits multiplication and also reduces the levels of enzymes in the TCA cycle (Gray et al., 1966 a,b). Reasons for oxygen toxicity are unknown although several hypotheses have been forwarded (Cole, 1974). Two which are most favoured relate to CO_2 deficiency or an oxidation-reduction potential of the medium which is too high for growth. Carbon dioxide is an essential growth factor and aeration with oxygen would reduce the CO_2 concentration in the medium due to a lower partial pressure, further the lowered CO_2 production would reduce that available even more.

There is evidence that excess oxygen may result in cytochrome destruction or a change in the order of their oxidation. If these changes are occurring coupled with reduced activity of the TCA cycle enzymes, then the effect would be very similar to that expected with anaerobic growth, in which glucose is converted to a wide variety of secondary metabolites, eg. acetate, ethanol. Oxidation of these would then account for the increased oxygen uptake, but not necessarily the large enthalpy changes, since there would not be the sufficient quantity

of secondary metabolites, which upon oxidation would produce the necessary heat output.

The large negative enthalpy values can only be attained if another energy source is available. Two possible energy sources are the ammonium salt and the cell. It would be necessary to know whether (a) the cells can form nitrate from ammonium salts and then reduce it and/or (b) if nitrate is formed chemically when excess oxygen is present. Clearly the TCA cycle enzymes are not working as for normal aerobic growth and this will reduce the quantity of ATP produced. Pirt (1968) pointed out that failure in the energy needed for maintenance results in lysis of the cell, thus alternative complex substrates are provided.

The breakdown of pyruvate via the TCA cycle (Fig. 9.1b) requires oxygen, however, oxygen is not required for glycolysis which results in a wide variety of products (Hadjipetrou et al., 1964). This is the situation during anaerobic growth, the low molar growth yield obtained (30.6 g cell (mol gluc)$^{-1}$) is in agreement with other values reported for K. aerogenes during glucose-limited growth (Hadjipetrou et al., 1964). Large quantities of acetate and ethanol are formed during anaerobic growth, ethanol is formed by reduction of acetyl-Co-A; this process having a high dissipation of energy as heat (Dawes et al., 1956). The production of CO_2 during anaerobic growth suggests that the oxygen comes from that provided in the substrate, eg. from glucose. If oxygen had entered the system higher yields or increased CO_2 production could have been expected.

Although anaerobic growth results in the recovery of large amounts of the available energy as heat, the enthalpy changes reported here cannot be readily explained in terms of the available substrate energy. An indication that other, unknown, changes were occurring was apparent from the varying doubling times during anaerobic growth in glucose; as the glucose concentration decreased the doubling time increased

(Table 6.1). One possibility is that cell lysis occurred at some stage during the growth cycle, possibly towards the end of the exponential growth period.

The addition of a second carbon source to cultures growing in glucose (3.3 mmol dm^{-3}) did not affect the maximum power output (Table 6.2) but the p-t trace profiles were altered. When pyruvate was added at zero time an increased growth rate was observed; the pyruvate was used at the same time as the glucose, but a second power peak developed. When pyruvate was added at the end of growth a p-t trace similar to that for growth in glucose, but with a low flow rate of 40 cm^3 h^{-1} was recorded (Fig 3.5), there was a further increase in the biomass. Thus under these conditions, pyruvate metabolism results in the production of biomass and secondary products even though the recorded oxygen tension is high. The second power peak caused by the pyruvate was similar in profile to that obtained upon addition of acetate at the end of growth. Acetate, however, did not result in the production of extra biomass unless present in excessive quantities.

The addition of the other carbon sources all increased the yield coefficient, Y_{gluc}. From a consideration of the yield coefficient, Y_{carbon}, it is apparent that the efficiency of use of the available carbon is lower than that of the control, ie. glucose only (Table 6.2) and that the CO_2 output is high. ΔH_p is less than that of the control, ie. there is more heat output, further indicating that the efficiency of growth is reduced by the presence of the second carbon source.

Compounds containing 4 or less carbon atoms produce late peaks on the p-t trace, whereas those with 4-6 carbon atoms only produce a shoulder on the decay side of the peak of the p-t trace (Fig. 6.3).

It is probable that the intermediates nearest to pyruvate enter the TCA cycle more easily, requiring less energy to do so. TCA cycle intermediates affect the early phase of growth in batch cultures (Dagely et al., 1951); succinate, α-ketoglutarate, oxalacetate and glutamine all increase the early growth rate; whereas pyruvate, fumarate or malate had no effect or were inhibitory. Tempest et al., (1970) showed that K. aerogenes employs a special mechanism to synthesise amino-acids from α-ketoglutarate when ammonium is the only nitrogen source; this process may be affected by increased α-ketoglutarate levels.

The effects of a second carbon source on growth and metabolism can be many and varied and will depend on the stage of growth at which it is added. It was expected that TCA cycle intermediates would have been able to enter the cycle easily thereby increasing growth efficiency, in fact they tend to depress it. The argument of Belaich (personal communication) that it is better to use succinate rather than glucose as a substrate because secondary products are not formed does not necessarily mean that the TCA cycle has not been modified.

Long et al., (1975, 1977) showed that thermal events occurred during the uptake of α-MG by E. coli in the presence of chloramphenicol. The possibility was considered that the enthalpy change accompanying the uptake of α-MG could be determined for K. aerogenes in the absence of chloramphenicol. Glucose-trained cells did not metabolise α-MG when it was the only carbon source, no heat or CO_2 output was observed. It has been observed that α-MG will only be taken up in the presence of another oxidisable carbon source (Rapoport and Hagueuauer, 1971). The addition of a second carbon source to resting cells, in suspension in α-MG, also causes a reduction in the intracellular α-MG concentration (Hoffee et al., 1964; Kesseler and Rickenberg, 1963). Thus in the presence of glucose it was considered that uptake of α-MG would occur,

any change in the thermal parameters being due to the uptake of α-MG.

When α-MG and glucose were present in the growth medium from zero time a change in the p-t trace profile occurred which was dependent on α-MG concentration; the change was not due to oxygen limitation. Although the enthalpy change increased, ΔH_{gluc}, the yield value remained constant and similar to that for glucose growth (Table 7.1). At first sight it seemed that α-MG had not been metabolized, but this was not the case. During growth in the mixed substrate the CO_2 output was increased; the extent of the increase was such that the theoretical amount from the metabolism of glucose only was exceeded (Table 7.1). This leads to the possibility either that some α-MG has been partially metabolised to CO_2 but not biomass or that some α-MG has been converted to biomass and CO_2 while the conversion of glucose has been suppressed. The significant increase in cell volume during growth in α-MG (Table 7.6) could be indicative that α-MG has been taken up and stored but not metabolised. The most probable explanation is that the additional enthalpy change is mainly due to oxidation of α-MG and production of CO_2 and not solely due to the uptake of α-MG.

This is the first reported observation that glucose-trained cells can be adapted to grow on α-MG as the sole carbon source. This leads to some interesting questions on the uptake and metabolism of α-MG, about which little is known. Although the questions cannot be fully answered, further insight is obtained from the results of growth and thermal studies of α-MG-trained cells (7.0).

When α-MG-trained cells were grown with glucose as the sole carbon and energy source their growth rate and thermal parameters were changed; their affinity for glucose was increased. The p-t trace had a single peak, the maximum power output occurs before the end of

exponential growth; although this trace was similar to the trace obtained with oxygen limitation, oxygen was not limiting. From mass-balance data the expected heat evolved is 1144.8 kJ $(\text{mol gluc})^{-1}$, however the measured heat evolved is greater than this by 333.8 kJ $(\text{mol gluc})^{-1}$.

When glucose-trained cells were inoculated into salts/α-MG medium, there was no detectable heat or CO_2 output or increase in biomass. On the addition of glucose growth proceeded, again the CO_2 evolved exceeded the value expected for oxidation of glucose alone whereas the enthalpy change, ΔH_{gluc}, was the calculated value; the conversion of substrate to cell material was considerably reduced. In the reverse experiment, when α-MG was added to cells growing in glucose there was an increase in the final yield, the CO_2 production and the heat output over the values for the control (Table 7.1). There was an immediate increase in the oxygen uptake on the addition of α-MG indicating increased respiratory activity. For all cultures, growth ceased when glucose was depleted even though α-MG was still present in the media.

These results suggest that the uptake of α-MG and/or its subsequent metabolism is energy dependent for which a second energy source has to be present. Since the affinity for glucose of α-MG-trained cells increased it is possible that a second, inducible, metabolic pathway for α-MG metabolism has been developed which is not purely specific to α-MG. Further support for this lies in the ability to train cells to grow on α-MG only, but only after a series of subcultures with glucose present. When α-MG-trained cells are grown in a mixture of α-MG and glucose the growth parameters indicate diauxic growth (Fig. 7.6). This type of growth occurs when catabolic repression

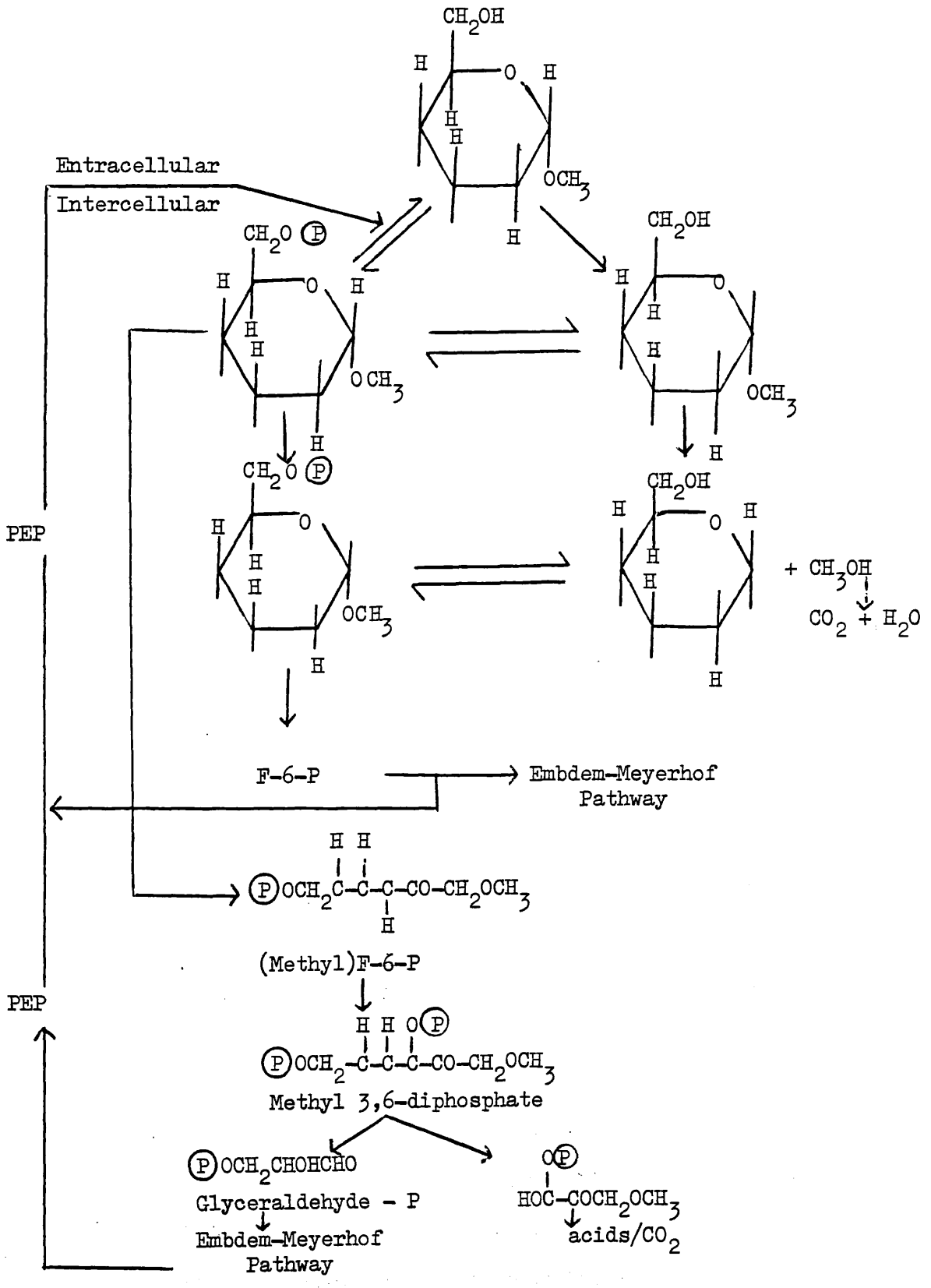
occurs, metabolism of glucose takes place first followed by the metabolism of α-MG. The sugar uptake system and/or following metabolism shows a preference for glucose. α-MG-trained cells always show a greater affinity for glucose than do glucose-trained cells, however, glucose-trained cells growing on glucose in the presence of α-MG have reduced rate constants (Fig. 7.2); this suggests that α-MG is a repressor.

Although there is conflicting evidence, sugar transport occurs via the phosphoenolpyruvate (PEP): glucose phosphotransferase system (PTS); in which the sugar is phosphorylated and transported across the cell membrane, this is probably an endogonic process. The phosphorylated sugar then enters the Embdem-Meyerhof pathway, PEP is generated here; both free and phosphorylated α-MG have been found in bacterial cells. Cohen and Monod (1957) have shown that extra CO_2 production results from the oxidation of stored α-MG. It is possible that α-MG enters the cell by two mechanisms; active transport and diffusion. Brocklehurst and Gardner (1977) found that α-MG stimulated proton uptake in yeasts and concluded that α-MG was a substrate of a proton symport (ie. influx) and not phosphorylation. The addition of inhibitors of oxidative-phosphorylation (DNP or sodium azide) did not halt the growth of α-MG-trained cells growing on α-MG although yields were reduced. The addition of sodium azide to cells growing in glucose was inhibitory, thus it seems that at least two mechanisms operate for the transport of sugars. Further these uncouplers are known to stimulate proton efflux, this is contrary to the suggestion that α-MG uptake is due solely to a proton-driven mechanism. Further, uncouplers of oxidative phosphorylation are known to stimulate the rate of formation of PEP (Davies and Gibson, 1967), the molecule which provides energy for transport (Carter and Dean, 1977).

Transported sugars are usually metabolised via the Embdem-Meyerhof pathway (glycolysis), since this is the main source of PEP it can be inferred that glycolysis and sugar transport are coupled. When a sugar is the sole carbon source available, the constituents of the cell have to be formed from cleavage products of the sugar, however when the carbon source also provides the energy the cells needs to conserve as much energy as possible and not waste it on transport processes. The use of one molecule of PEP to transport a sugar molecule against a gradient is not wasteful when considered against the large yield of ATP per sugar molecule. Any reduction in ATP or PEP produced will affect yields and/or transport processes.

There are therefore two possible routes, or a combination, by which α-MG is metabolised. α-MG is first transported into the cell, mainly as the phosphate sugar, where it can then be dephosphorylated or left as the phosphorylated sugar. The methyl group is then removed by a demethylating enzyme in an energy requiring process to give methanol, which is then degraded to CO_2 and water (Fig. 9.8). The hydrolysis of methanol would yield 714.4 kJ mol^{-1} as heat. For the demethylation, a type of β-glucosidase (probably present) is required since removal of the methyl group is similar to hydrolysis of maltose which K. aerogenes can break down to glucose and utilise readily. After the removal of the methyl group the glucose residue is then metabolised by normal processes (Fig. 9.1a). The rate limiting step is probably the hydrolysis of the methyl group. If the methanol was not further metabolised it would become toxic. If these pathways are correct then the yield values for the growth in a mixture of glucose and α-MG should be higher (Table 7.1), since this is not the case it is supportive evidence of catabolic repression.

FIGURE 9.8 Proposed pathways of α-MG metabolism



An alternative route is for α-MG to enter the Embdem-Meyerhof pathway as the phosphorylated sugar, the second phosphorylation would now occur at a different position and fructose-1,6 diphosphate would not be formed, unless demethylation or transmethylation occurred at an intermediate point (Fig. 9.8). The subsequent breakdown of the diphosphate would yield glyceraldehyde-3-phosphate in smaller quantities, thereby reducing the PEP formed. This would reduce the rate of uptake by transport, hence yields. The other 3 carbon intermediates, normally converted to glyceraldehyde-3-phosphate, would be wasted and oxidised to CO_2 and other products. A modified pathway of this type could explain changes in the observed growth parameters; to explain changes in the enthalpy values it is necessary to know the path way and amounts of all products of metabolism. The first scheme is most probable for α-MG-trained cells growing in α-MG only, and the latter scheme when mixed substrates are used.

The effects of uncouplers of oxidative-phosphorylation on bacterial metabolism has not received the attention that has been given to their effects on mitrochondria, the two systems are different and extrapolation of data is done at risk. Questions that have been posed include: do uncouplers inhibit metabolic processes in bacteria by draining of the available energy as heat, by collapsing the electrochemical gradient of H^+ as envisaged by the chemiosmotic hypothesis or by acting, eg. blocking, at separate sites for each metabolic process involved?

Several hypotheses have been proposed to account for energy conserving mechanisms (Harold, 1972). The theory which has gained most favour is the chemiosmotic theory (Mitchell, 1966, 1968); uncouplers have been used extensively in an attempt to test its validity. ATP production in oxidative phosphorylation occurs by an, as yet, unknown mechanism involving a membrane-bound electron transport chain. The hypothesis predicts that the membrane has a low electrical conductivity,

that the respiratory chain is an alternating sequence of hydrogen carriers and electron carriers arranged across the membrane in loops. Oxidation of the substrate results in translocation of protons from one side of the membrane to the other, in any loop two protons are passed across. The membrane has to be impervious to stop immediate back-flow of protons. The unidirectional flow of protons creates an electrical potential and proton gradient across the membrane. The protomotive force, $\Delta\mu_H$, is expressed:

$$\Delta\mu_H = \Delta\psi - Z\Delta pH$$

where $Z = 2.3\ RT/F$, $\Delta\psi$ is the membrane potential. The protomotive force is made up of an electrical component, $\Delta\psi$, and an osmotic component, ΔpH, where ΔpH is the pH difference between the two sides of the membrane. The gradient of pH and of electrical potential generated by the respiratory chain reverses the direction of an ATPase so that it brings about a net synthesis of ATP. Thus destruction of the proton gradient will uncouple oxidative-phosphorylation.

A protomotive force may also be generated by the hydrolysis of ATP by ATPase; a mechanism used to explain the protomotive force in anaerobes or facultative bacteria metabolizing anaerobically in which ATP is synthesised is by substrate level phosphorylation (Decker et al., 1977).

Many uncouplers act by conducting protons thereby dissipating the proton gradient (Decker and Long, 1978); these and other uncouplers have secondary effects (Nicholas and Ordal, 1978). Weinbach and Garbus (1969) presented evidence that uncouplers inhibit metabolic processes through proton-ligand interactions which cause a protein-conformational change whereby the proteins are stablilized in "unproductive" conformations. This reduces the energy available for amino-acid transport.

The inhibitors (sodium azide, DNP and FCCP) all had a profound effect on the p-t trace profiles, that of sodium azide was very different to that of DNP or FCCP (Fig. 6.3). At the concentrations used sodium azide almost completely inhibited biomass production, while DNP and FCCP reduced it markedly. The heat output in the presence of any of the inhibitors was increased, ie. a decreased enthalpy change, irrespective of the phase of growth when added. Azide inhibition causes a reduced value of ΔH_p ie. an increase in waste heat, this inhibitor not only conducts protons but inhibits cyctochrome oxidase and possibly ATPase. These effects mean that glucose cannot be converted to cellular material, but is broken down to secondary products. Experimental support for this is the low pH attained and small CO_2 output despite glucose depletion. Once glucose is depleted the power output returns to the base-line and CO_2 output ceases, indicating that the secondary products are not further metabolised. More energy would be required for the metabolism of these products, which probably includes acetate (Webster, 1965), than for the equivalent amount of glucose; this energy is no longer available when azide is present. Azide did not drain off the available energy as heat, since the heat evolved was very much below that for uninhibited growth, (Table 6.3).

With DNP and FCCP all the substrate-carbon can be accounted for as cellular material and CO_2 (Table 6.5). The p-t trace, in the presence of high concentrations of inhibitor, consisted of several peaks indicating the presence of secondary products; the pattern of O_2 uptake and CO_2 evolution supported this conclusion. Acetate was detected during growth in the presence of DNP. Since the carbon recovery is almost complete, (decreasing marginally at higher concentrations) and the heat evolved is very much below that expected from oxidative processes (Table 6.5), the implication is that some energy-demanding processes

have come into operation. These may include the transport processes, biosynthetic processes, which may occur by alternate routes, and alternative phosphorylation pathways resulting in low ATP yields. Neijssel (1977) using K. aerogenes growing in glucose-limited chemostat concluded that DNP does not prevent ATP formation by oxidative-phosphorylation, but this was only true if the rate of oxygen consumption was not impeded by a very low rate of supply of energy source.

The possible difference of ATP production indicated by the use of inhibitors between batch culture and chemostat cultures and also that different metabolic control mechanisms may exist (p 198) may be causes for the different efficiences and maintenance requirements of the two culture types. In batch culture the supply of nutrient (glucose) becomes reduced because transport processes are inhibited and the fixed aeration conditions limits the supply of oxygen at high biomass levels. In contrast, α-MG metabolism is not affected by DNP or sodium azide, in the same manner as glucose. This supports the suggestion of different metabolic pathways that for α-MG is catalyzed by PEP while that for glucose involves phosphorylation of the sugar by PEP but also requiring an energy input for translocation.

ATP production was reduced greatly upon addition of FCCP, thus the energy content of the cell was reduced. Poe et al., (1967) showed that ATP is not produced in the presence of (a) uncouplers which dissipate the proton gradient or (b) those which prevent proton movement through the coupling factor. The potential energy would then be lost as heat.

Djavan (1980) found that the addition of α-MG or methyl-β-D-thiogactoside to growing chemostat cultures of K. aerogenes in glucose-limited media caused no change in the heat output, and concluded that tranport processes did not involve ATPase; this supports previous observations (Schaner and Haddock, 1972; Nieuwenhuis et al., 1973). The significance of ATPase is that it is considered to be a coupling factor (Harold, 1972), mediating energy-transfer which culminate in ATP formation. The nature and functions of coupling factors are unclear at the moment. Oligomycin which inhibits ATPase, is unfortunately not active in bacterial systems. Antimycin A is a specific inhibition of the second coupling site but it does not conduct protons and is generally considered inactive with bacteria. The small differences between uninhibited and inhibited growth by antimycin A are probably not significant, but further investigation is needed.

Valinomycin can uncouple oxidative-phosphorylation processes under some conditions; its main mode of action is the transport of K^+ across lipid membranes. Valinomycin forms a clathrate with K^+, the complex carries a positive charge, net k^+ movement is electrogenic. The movement generates and responds to an electrical potential, this induces k^+ uptake, translocation of k^+ against a large concentration gradient is energy-linked and can be supported by respiration or ATP. Uptake of k^+ is electrically compensated by ejection of protons or concurrent uptake of anions. The simplest interpretation for the mechanism of energy input is based on the chemiosmotic theory.

There is an hypothesis that ion gradients are links between transport and metabolism. Since all bacteria accumulate k^+ it is possible that some of the potential energy stored in the gradient is used to transport other metabolites. Further investigation is necessary before firm conclusions can be made.

In media containing k^+ as the main cation (in excess) the addition of valinomycin did not cause any observable changes in thermal and growth parameters. When the k^+ concentration was reduced to be growth-limiting (sodium in excess), however, the addition of valinomycin had a marked affect on growth and thermal parameters (8.2). The presence of valinomycin increased both growth rate and yield; in contrast, Harold and Baarda (1967) observed inhibition of the growth of Streptococcus faecalis. At a concentration of 6μg cm^{-3} valinomycin did not change the parameters from the control, but at 16 μg cm^3 there were marked changes. The enthalpy change was higher, ie. lower heat output, in the first seven hours from inoculation. Less heat and increased yields indicates that growth was more efficient during this period.

The initial period of the p-t trace resembles that for glucose-limited growth. When the p-t trace recorded during growth in 16 μg cm^{-3} valinomycin is compared with that of the control, or with that in 6 μg cm^{-3} valinomycin, (Fig. 8.1), it is apparent that the heat output after K^+-limitation (ie. during glucose fermentation) has been, in effect moved forward by the presence of valinomycin and more glucose is oxidised to cell material. This implies that although the same calculated amount of K^+ is originally present, the valinomycin present has enabled the cell to use the cation in a manner which permits more efficient growth. This must be due, inpart, to a higher intracellular K^+ concentration before K^+-limitation occurs, thus more energy is produced and stored to be drawn on later when K^+ should have become growth limiting, thus further transport and aerobic oxidation of glucose is possible. Alternatively, since valinomycin can conduct K^+ in either direction, as oscillating flux between intracellular and extracellular K^+ may be set up dependent on the internal K^+ concentration, in this respect it should be recalled that cells (harvested at the end of growth have a

decreased potassium concentration when growth occurs in the presence of valinomycin (Table 8.3). In effect an ion pump is formed which could be coupled to energy transformation processes (Harold, 1972).

In K^+-limited growth, aerobic oxidation of glucose occurred until the depletion of K^+, the remaining glucose was fermented to some biomass with much secondary metabolite formation. The secondary metabolites were inturn metabolised with the formation of some biomass (Fig. 8.1). Some growth occurred in K^+-limiting media when no K^+ was added (8.1.6), Tempest et al., (1966) have presented evidence that theoretically this could occur. One reason why K^+ depletion did not become fully growth limiting may be due to the high Na^+ concentration in the medium which the cell may be able to use. Values of ΔH_p^k (ie. before K^+-limitation) are similar to values of ΔH_p for aerobic glucose growth (Fig. 4.4). Total ΔH_p values are large and negative due to the large quantity of heat evolved during the oxidation of secondary metabolites. Full interpretation of the thermal data requires quantitative analysis of the secondary products formed.

In conclusion, this course of experimental study has shown that reproducible quantitative thermal and growth data can be obtained from system employing continuous monitoring techniques. Strict control of inocula and experimental conditions are essential if thermal data is to be correlated with growth measurements and metabolic events occurring in the culture vessel. Quantitative thermal data is presented which show that such controls are necessary if significant measurements are to be obtained, particularly if small values are involved, eg. ΔH_B.

The consideration of enthalpy change data with growth cycle events, eg. ΔH_g, leads to the estimation of a maintenance coefficient for batch growth. Comparison of thermal data for aerobic, anaerobic and excess oxygen growth states indicates that thermal events differ significantly between batch and continuous growth although yield data are similar; batch culture always results in greater heat dissipation. Addition of other, or sole use of, various carbon sources to provide both energy and carbon for growth shows the importance of the derived thermal data in any when consideration of growth efficiency and energy budgets. Many workers have indicated that flow microcalorimetry has great potential in metabolic studies; although much has been done in the last decade the full potential of the technique will only be realised if reliable quantitative data is collected.

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Microcalorimetry studies of energy changes during the growth of *Klebsiella aerogenes* in simple salts/glucose media
1 Establishment of standard conditions

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Abstract

The shape of the thermogram and the total heat output of aerobically growing cultures of *Klebsiella aerogenes* depend on the nature and state of the inoculum, the composition of the growth medium, aeration in the fermentor and in the calorimeter, and the pump rate of the culture through the microcalorimeter cell. Standard conditions of growth and heat measurement have been established which give reproducible thermograms and total heat output. Experimental results using different glucose concentrations are reported and the enthalpy changes for the consumption of glucose and the production of cells have been calculated.

Introduction

The cultural conditions and supply of nutrients determine not only the metabolic processes in a growing bacterial culture (Neijssel and Tempest, 1975), but also the rate of heat production and total heat output. In any study of energy changes occurring during bacterial growth it is essential to consider the influence of all the experimental conditions of growth, the method of measurement of the heat changes on the profile of the recorded thermogram, and, where possible, control these so that reproducible and reliable data can be accumulated. Although it has been reported that the heat flux and heat output depend (a) on the cultural conditions, e.g. size and age of inoculum, nature of the growth medium, aeration of the culture; (b) on the type of calorimeter, e.g. ampoule or flow calorimeter; and (c) for flow-calorimeters on the pump rate, aeration and substrate concentration in the calorimeter circuit, there has been no systematic quantitative study of these factors.

Any interpretation of the results of thermal measurements in a flow-calorimeter system can only be meaningful if the sample of organisms actually in the microcalorimeter cell are, as far as possible, in the same state as those in the culture vessel outside the calorimeter. For aerobic organisms it is essential that the oxygen tension in the calorimeter cell, and in the flow line to this cell, is high enough to avoid changes in metabolic processes due to oxygen starvation (Harrison and Pirt, 1967). Care must also be taken to ensure that none of the substrates becomes limiting in the flow line and before that in the culture vessel. Both these factors are controlled by the transit time taken for an organism to pass from the fermentor to the microcalorimeter cell. Changes in the oxygen tension have been correlated with features of the thermogram profile (Eriksson and Wadsö, 1971, Few *et al.*, 1976), and a strong correlation between oxygen uptake and heat evolution has been established (Poole and Haddock, 1975).

The work to be described was undertaken to establish standard growth conditions (inoculum, medium, aeration, stirring, etc) and test conditions in a flow microcalorimeter (aeration, flow rate, transit and residence times), and to test the reproducibility

of the heat profile and total heat output under these conditions. In the first instance it was decided to concentrate mainly on changes occurring during the exponential growth of *Klebsiella aerogenes* in a simple salts/glucose medium in which glucose was limiting. This reduced energy changes due to fermentation and breakdown of secondary metabolites from any excess carbon source. Using these defined conditions, data can then be accumulated on specific metabolic processes by specific modification or inhibition of these processes.

Materials and methods

Strain and growth medium

The strain of *Klebsiella aerogenes* (NCTC 418) used throughout this work was maintained by monthly subculture in nutrient broth. For experimental purposes the organisms were grown in glucose-limited medium of pH 7.0, prepared in glass distilled water, of final composition: KH_2PO_4 26 mmol dm^{-3}; $(NH_4)_2SO_4$ 8 mmol dm^{-3}; $MgSO_4.7\,H_2O$ 0.16 mmol dm^{-3}. To this salts solution was added glucose to give concentrations in the range of 0.5 to 5.5 mmol dm^{-3}. The salts solution in the fermentor vessel and the appropriate glucose solution in a medical flat bottle were autoclaved separately and mixed aseptically.

Cultural conditions

The cells were grown at 37°C as a batch culture in 640 cm^3 of growth medium in the 1 dm^3 culture vessel of a Gallenkamp Modular Fermentor system. The culture was stirred by a magnetic stirrer (c. 1,200 rpm) and aerated vigorously (3.5 dm^3 sterile air per min). An oxygen electrode (Western Biological FL) was incorporated in the fermentor; the pH of the growing culture was not kept constant.

Measurement of pH, oxygen tension and biomass

The pH and bacterial growth were each recorded from a flow system, in which the culture was recirculated at 400 $cm^3\ h^{-1}$ through a microcombination pH electrode (EIL 1140 200), and a 1 cm-path length flow-cuvette (in a Unicam SP 600) connected in series. Growth was monitored by recording the absorbance of the suspension at 625 nm against a blank of salts medium. A calibration curve of dry weight of organisms (mg cm^{-3}) against absorbance was constructed in separate experiments using a suspension of washed cells grown under standard conditions.

After leaving the flow-cuvette the culture passed through the peristaltic pump and then back to the fermentor vessel by polypropylene tubing enclosed in a water jacket maintained at 37°C. This ensured that the returning culture was at the same temperature as that of the growing culture in the fermentor. The total time for the complete cycle from and back to the fermentor was 30 s.

A flow-type oxygen electrode, included in the flow line immediately after the microcalorimeter cell gave a measure of the oxygen tension in the microcalorimeter cell. This electrode was calibrated using aerated medium at 37°C and 5% w/v sodium sulphite solution to give the 100 and 0% levels respectively. The pH and oxygen electrodes were sleeved in such a way that only a small volume of liquid surrounded each electrode.

Thermal measurements

Thermograms accompanying the growth of *K. aerogenes* at 37°C were obtained using a LKB 10700-1 flow microcalorimeter fitted with an aerobic steel cell to eliminate thermal fluctuations associated with the passage of air bubbles. The measured volume of this aerobic cell at different flow rates (10 to 105 $cm^3\ h^{-1}$) was 1.16 ± 0.02 cm^3. The bacterial suspension was drawn through the microcalorimeter cell from the fermentor and additional aeration in the line was not necessary (Schaarschmidt and Lamprecht, 1976).

The length of polypropylene tubing (id 0.1 cm), lagged with a water jacket at 37°C (to avoid cooling of the growing culture), used to connect the fermentor to the microcalorimeter was kept as short as possible. The flow rate was measured by collecting a sample over a timed period at the start and at the end of an experiment. At 40 $cm^3\ h^{-1}$ the time taken, Δt, for an organism to reach the microcalorimeter cell from the fermentor was approximately 3 min, and the total time for return to the fermentor was approximately 6 min.

The calorimeter response was calibrated by passing a culture, in which the bacteria had been killed by formalin, through the cell under the same experimental conditions (i.e. those applying to the culture vessel, aeration, stirring and the flow rate used) and applying an internal current of 2.5 mA for 30 min to produce a steady heat output. The calibration constant varied linearly with the flow rate (Monk and Wadsö, 1968); the full scale deflection on the recorder was 100 μV. Areas enclosed under thermograms were measured with a Hewlett-Packard Digitizer Pac (Mode 10).

All the connecting tubing, microcalorimeter cells and electrodes were sterilized by pumping 10% v/v formalin and then sterile water through the systems for about 1 h. The ends of the tubing were transferred aseptically to the culture vessel and, sterile medium, aerated and at 37°C, was pumped through the microcalorimeter to give a steady base line. After the baseline had been maintained for at least 1 h the fermentor was inoculated with the standard inoculum.

Preparation of standard inoculum

Cells of *K. aerogenes* growing in 640 cm^3 of glucose-limiting medium (3.3 mmol dm^{-3}) in the fermentor were harvested by centrifugation approximately 30 min before the end of exponential growth. The sedimented cells were resuspended in a sterile volume of salts medium (lacking glucose) so that a 1.5 cm^3 aliquot, on dilution to 640 cm^3 in culture medium would give a cell population of about 3×10^7 cells cm^{-3}. Volumes of 1.5 cm^3 of this suspension were transferred aseptically to 2 cm^3 screw-capped glass ampoules. A batch of these ampoules was suspended just above the surface of liquid nitrogen in a vivostat for exactly 20 min and then plunged directly into the liquid nitrogen. The ampoules were stored under liquid nitrogen until required. When required, an ampoule was removed from storage and immediately immersed in a water bath at 37°C. After exactly 3 min the ampoule was removed and the suspension transferred immediately to the culture medium (already at 37°C) by a sterile syringe.

Analytical methods

Glucose analysis was carried out on extracellular fluid obtained by centrifugation of 4 cm^3 samples of bacterial suspension, which had been heated to 80°C to kill the organisms. The glucose analysis was based on the formation of a coloured compound with *o*-toluidine (Few *et al.*, 1976; Dobowski, 1962).

Results

In all the growth experiments the oxygen tension in the fermentor never fell below 80% full saturation (even at the highest glucose concentration used); the oxygen tension of the culture after passing through the microcalorimeter cell (the value plotted with the thermograms) was never less than 40% full saturation. Thus at all times the cells were under good aerobic conditions.

(1) Standardization of inoculum and growth conditions

Cells were grown in glucose-limiting medium (3.3 mmol dm^{-3}) under the standard conditions of stirring and aeration and the growing culture pumped through the microcalorimeter cell at 40 cm^3 h^{-1} (Δt = 3 min). Initially cells from an 18 h-old culture, grown in the same medium, were used as inoculum. The length of the lag phase, the shape and position (with respect to time) of the thermogram, and the total heat output, varied from experiment to experiment. The first prerequisite for any detailed quantitative study of energy changes is the reproducibility of the results; this can only be achieved by standardizing the inoculum.

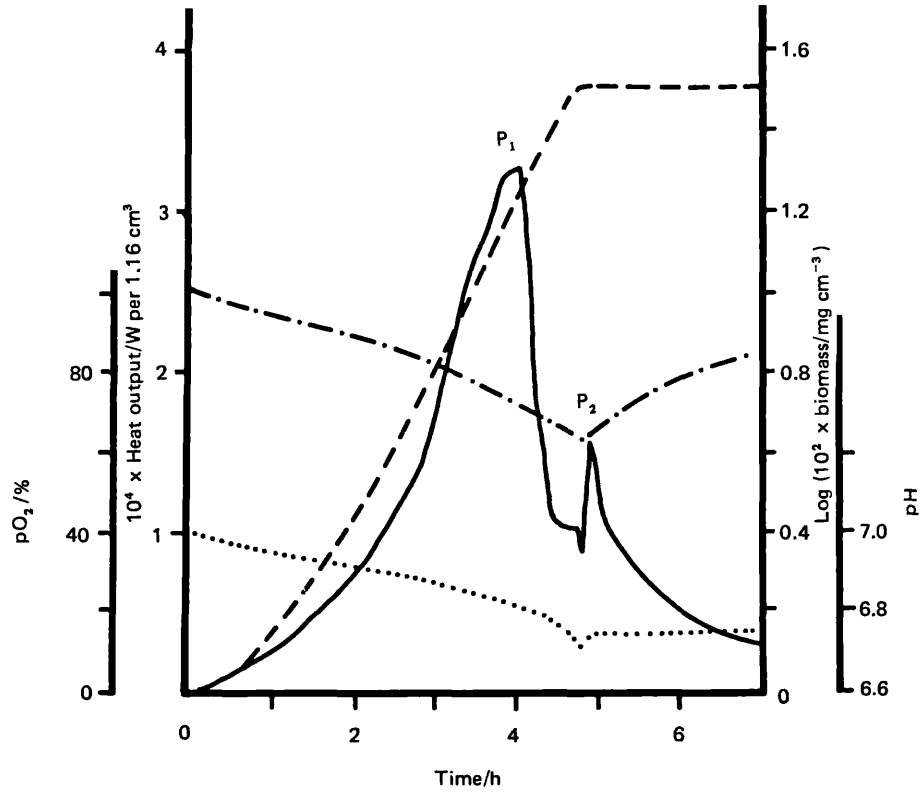

Figure 1 Typical thermogram and changes of other environmental parameters accompanying growth of *Klebsiella aerogenes* in simple salts/glucose-limited medium at 37°C. Glucose concentration = 3.3 mmol dm^{-3}; flow rate = 40 cm^3 h^{-1}; Δt = 3 min. ——— measured thermogram; ..... pH; —·—·—· pO_2; - - - - - - - log (10^2 x biomass/mg cm^{-3}).

Table 1 Reproducibility of calorimetric and growth parameters using cryogenically stored standard inocula

Parameter	Mean ($\bar{x}$)	Standard deviation (S)	Mean**	$S/\bar{x}$ (%)
Heat output at P_1 *	3.15×10^{-4} W	0.22	$3.15 \pm 0.14 \times 10^{-4}$ W	7
Heat output at P_2 *	1.77×10^{-4} W	0.43	$1.77 \pm 0.27 \times 10^{-4}$ W	24
Heat evolved during exponential growth (q_{exp})	2.23 J	0.23	2.23 ± 0.14 J	10
Time from inoculation to P_1	3.6 h	0.22	3.6 ± 0.14 h	6
Time from inoculation to end of growth	4.6 h	0.20	4.6 ± 0.14 h	4
Biomass at P_1	0.149 mg cm^{-3}	0.02	0.149 ± 0.012 mg cm^{-3}	13.5
Biomass at end of exponential growth	0.324 mg cm^{-3}	0.036	0.324 ± 0.022 mg cm^{-3}	11
Minimum pH	6.77	0.03	6.77 ± 0.02	0.4

* Heat flow from cells in 1.16 cm^3 volume calorimeter cell.
** Mean with 95% confidence limits.
Glucose concentration = 3.3 mmol dm^{-3}. Number of samples = 10. Flow rate = 40 cm^3 h^{-1} (Δt = 3 min).

Cells were freeze-dried in media of different composition, in the absence of cryoprotective substances to avoid changes in the final composition of the growth medium. The use of such preparations as inocula proved unsatisfactory due to increased and variable lag phase and loss of viability of the cells.

Cryogenically-stored cells fulfilled all the requirements, the lag phase was absent, the detailed shape and position of the recorded thermogram and the total heat output was reproducible over a period of many months. During growth in 3.3 mmol dm^{-3} glucose media the rate of heat output increased rapidly during the early part of exponential growth (Figure 1), and attained a maximum value (P_1) when growth was approximately 50% complete. During the final divisions of the cells there was a marked decrease in the heat output. This was followed by a short period during which the heat output was constant before another decrease and increase to a second smaller maximum value at P_2 (about 56% of the value at P_1). This second maximum value coincided with the onset of the stationary phase, and thereafter the rate of heat output decreased and the baseline of the thermogram was attained some 12 h after inoculation. The onset of the stationary phase was also characterized by a minimum value in the pH and oxygen tension of the culture medium and the complete utilization of glucose. The pH of the medium increased slightly (c. 0.02) during the stationary phase but never attained the original value. In contrast, the oxygen tension which had decreased steadily during the exponential growth phase increased rapidly to 100% after a few hours in the stationary phase.

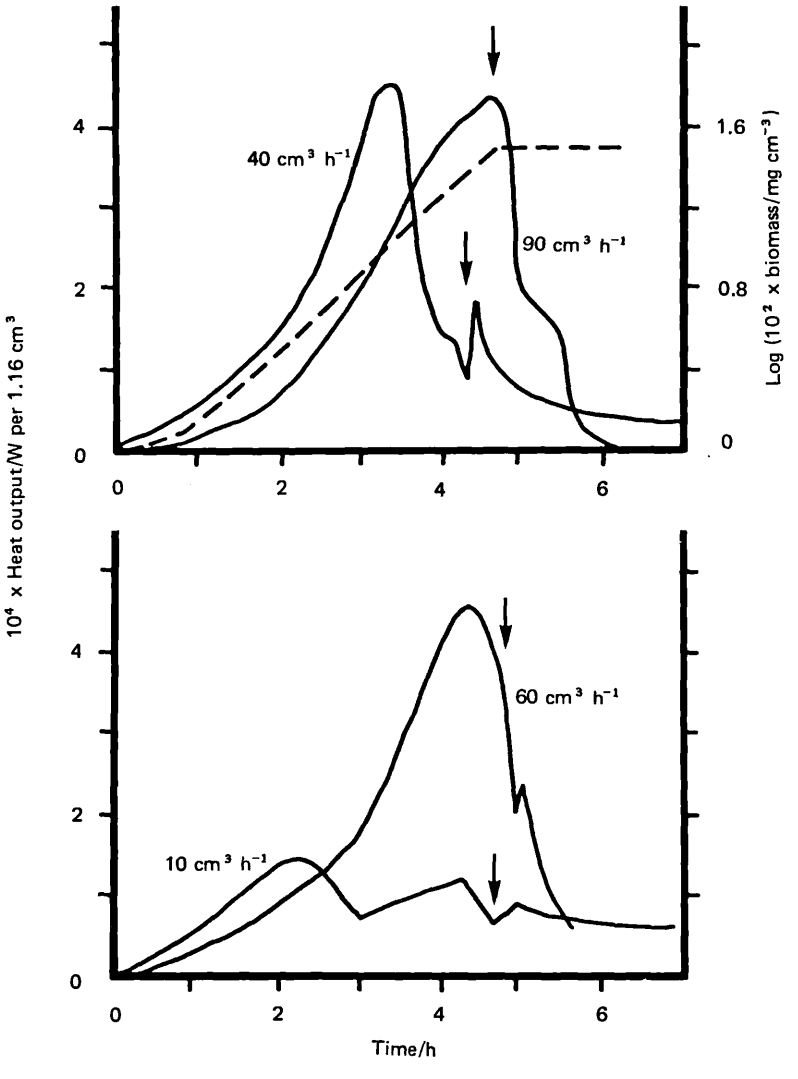

Figure 2 Variation of thermogram profile with flow rate during growth of *K. aerogenes* in simple salts/glucose-limited medium at 37°C. Glucose concentration = 3.3 mmol dm^{-3}. Flow rates 10, 40, 60 and 90 cm^3 h^{-1}; ------ log (10^2 x biomass/mg cm^{-3}). The vertical arrow indicates the end of exponential growth.

The reproducibility of the heat output, thermogram profile and other growth parameters which was achieved using cryogenically-stored inocula growing under the standard conditions (Table 1) is good for a biological system. Using these standard conditions more detailed studies of the energetics of bacterial growth can be undertaken with confidence.

(2) Standardization of flow rate through the microcalorimeter

Cultures growing in glucose-limiting medium (3.3 mmol dm^{-3}) from cryogenically-stored inocula were pumped through the microcalorimeter cell at different flow rates in the range 10 to 105 $cm^3\ h^{-1}$. The profiles of the recorded thermograms, corrected for variation of calibration constant (Figure 2), show a trend with change of flow rate. With increasing flow rate the profile becomes more simple tending to one large peak with a shoulder on the decay side; the first value of the maximum heat output occurs nearer the end of exponential growth (the plateau value and second maximum gradually disappearing). The baseline was re-attained much sooner at the higher flow rates.

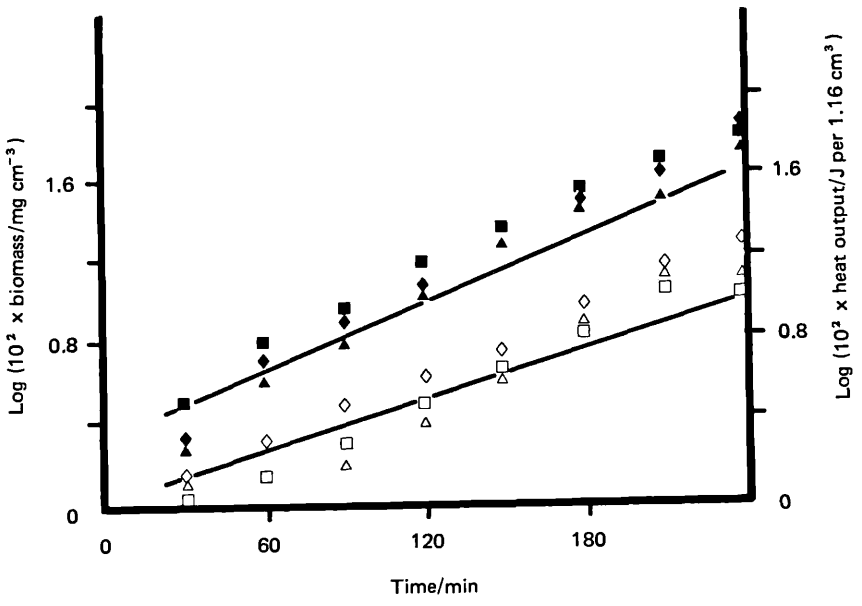

Figure 3 Heat output and biomass during exponential growth of *K. aerogenes* at different flow rates. Solid symbols represent heat output for 1.16 cm^3 suspension; open symbols represent the biomass. ■, □, 40 $cm^3\ h^{-1}$; ▲, △, 60 $cm^3\ h^{-1}$; ◆, ◇, 80 $cm^3\ h^{-1}$. (Lines plotted are the calculated lines for heat output and biomass for all flow rates).

Table 2 Heat output of cells of *K. aerogenes* during growth in simple salts/glucose media of different initial glucose concentrations (flow rate = 40 cm^3 h^{-1}, Δt = 3 min)

Glucose concn c/mmol dm^{-3}	Total biomass m/mg cm^{-3}	Total no. of generations	Yield value Y/g cell per g glucose	Exponential phase: q_{exp}/J (1)	ΔH_{exp}/kJ (mol gluc)$^{-1}$ (3)	ΔH_p/kJ (mol cell)$^{-1}$ (4)	Total growth cycle: q_{total}/J (2)	ΔH_{total}/kJ (mol gluc)$^{-1}$ (3)	ΔH_p/kJ (mol cell)$^{-1}$ (4)
0.55	0.046	3.89	0.46	0.65	−1032	−329	0.73	−1159	−369
1.10	0.102	4.17	0.51	1.16	− 913	−265	1.40	−1102	−319
1.60	0.155	4.75	0.52	1.52	− 822	−228	1.66	− 897	−249
2.20	0.208	5.16	0.52	1.72	− 675	−192	2.14	− 839	−239
2.80	0.288	5.62	0.58	1.99	− 614	−161	2.77	− 855	−224
3.30	0.300	5.67	0.50	2.15	− 563	−167	2.60	− 681	−202
3.90	0.307	5.70	0.44	2.20	− 487	−167			
4.40	0.380	6.01	0.48	2.20	− 431	−135			
5.00	0.385	6.03	0.43	2.33	− 402	−141	3.26	− 562	−197
5.50	0.404	6.10	0.40	2.30	− 361	−132	3.76	− 589	−217

(1) q_{exp} is heat evolved by 1.16 cm^3 of growing culture during exponential growth phase.
(2) q_{total} is heat evolved by 1.16 cm^3 of growing culture from inoculation until thermogram re-establishes the base line.
(3) ΔH/kJ (mol gluc)$^{-1}$ = q/(cell vol × c × 10^{-6}) = 8.62 × 10^2 q/c.
(4) ΔHp/kJ (mol cell)$^{-1}$ = q × M_r (cell)/(Δm × cell vol) = q × 26.98/(Δm × 1.16) = 23.26 × $q/\Delta m$.
Δm = biomass at end of exponential phase − initial biomass.

The actual heat output over any given time interval during growth was obtained by measuring the area under the recorded thermogram between the appropriate times. The heat output was measured at 30 min intervals from the thermograms recorded at different flow-rates and log (heat output per 1.16 cm^3 of growing culture) was plotted against time (Figure 3). Within the limits of experimental error the rate of heat output was exponential and independent of the flow rate. Furthermore, the rate of heat output is in close agreement with the rate of increase of biomass, confirming that at any given time the heat output is proportional to the biomass (Forrest and Walker, 1962).

The heat output during exponential growth, q_{exp}, was obtained from the area under the thermogram from the onset of growth to the start of the stationary phase. This increased dramatically with increase of flow rate to 60 $cm^3\ h^{-1}$; at higher flow rates, i.e. lower values of Δt, q_{exp} remained approximately constant (Figure 4). The heat output after the onset of the stationary phase decreased with increasing flow rate.

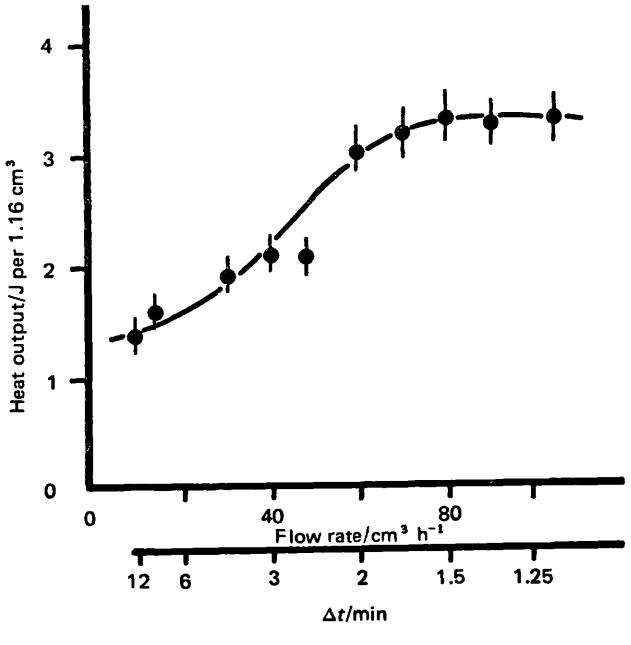

Figure 4 Effect of flow rate through the calorimeter on the heat output during exponential growth.

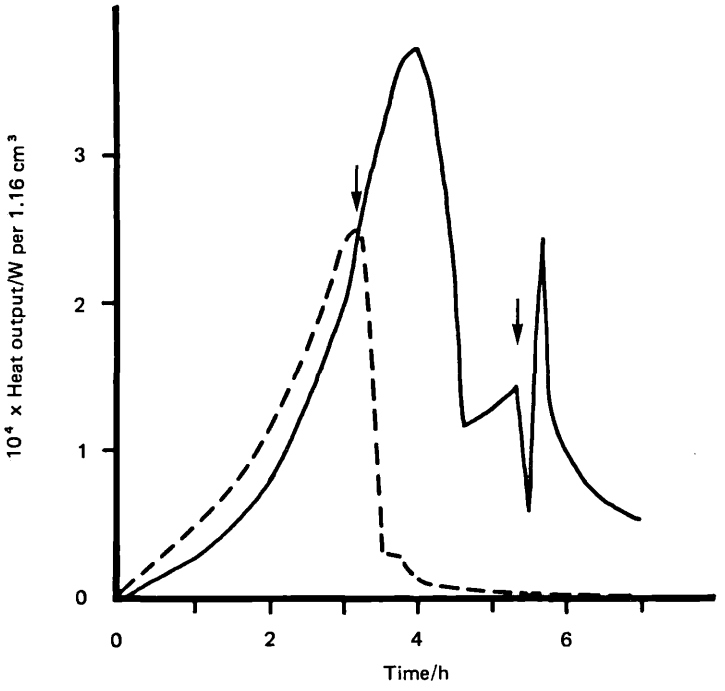

Figure 5 Variation of thermogram profile with glucose concentration during the growth of *K. aerogenes* in simple salts/glucose-limited medium at 37° C. Flow rate = 40 cm^3 h^{-1}; Δt = 3 min. ——— 5.0 mmol dm^{-3} glucose; - - - - - 1.1 mmol dm^{-3} glucose. The vertical arrow indicates the end of exponential growth.

As the flow rate is decreased the conditions in the microcalorimeter cell become less like those in the fermentor. The residence time of an organism in the microcalorimeter cell is increased and deficiencies in oxygen and glucose cause the onset of different metabolic processes. The most changed conditions occur at the lowest flow rate; and on recycling, secondary metabolites not present in the fermentor will be introduced into the growing culture. Preliminary experiments at a flow rate of 10 cm^3 h^{-1}, in which the culture was not recycled (after the microcalorimeter it was pumped to waste) showed a further reduction of about 15% in the heat evolved during exponential growth.

(3) Variation of the heat output and profile of the thermogram with glucose concentration of the culture medium

Cultures growing in glucose-limiting media (0.55 to 5.5 mol dm^{-3}) from cryogenically-stored inocula were pumped at a fixed rate of 40 cm^3 h^{-1} through the microcalorimeter cell. The profile of the recorded thermograms (Figures 1 and 5) varied with the initial glucose concentration. At low concentrations (< 2.2 mmol dm^{-3}) the thermogram was a single peak with a shoulder, which became more pronounced with increasing

concentration, on the decay side. At concentrations above 2.8 mmol dm^{-3}, the thermograms became more complex; they all had similar profile characteristics which were increasingly accentuated with increasing glucose concentration. The rate of increase of heat output which is independent of the profile of the thermogram and the rate of increase of biomass were approximately the same for all glucose concentrations. The rate constants for heat output and biomass were $1.67 \pm 0.17 \times 10^{-2}$ min^{-1} and $1.34 \pm 0.24 \times 10^{-2}$ min^{-1} respectively.

During growth at all concentrations the pH and oxygen tension changed in a similar manner to that shown in Figure 1, the concentration of glucose decreased during exponential growth, and glucose was completely depleted at the onset of the stationary phase.

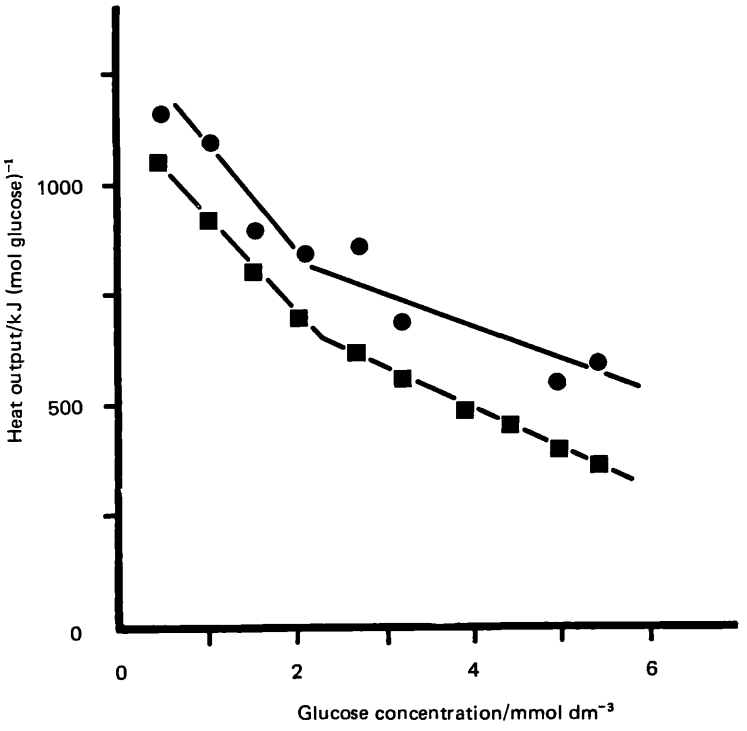

Figure 6 Variation of heat evolved per mol of glucose consumed with the initial glucose concentration. ■, ΔH_{exp}; ●, ΔH_{total}.

Table 3 Variation of heat output and ΔH_p with pump rate

Pump rate/cm^3 h^{-1}	Δt/min	q_{exp}/J per 1.16 cm^3 culture	ΔH_p/kJ (mol cell)$^{-1}$
105	1.1	3.31*	−257*
90	1.3	3.32	−257
80	1.5	3.38	−262
70	1.7	3.18	−247
60	2.0	3.08	−239
48	2.5	2.10	−163
40	3.0	2.15	−167
30	4.0	1.95	−151
14	8.6	1.61	−125
10	12.0	1.40	−109

* 95% confidence limits ± 6%.
Glucose concentration = 3.3 mmol dm^{-3}.

With increasing glucose concentration, the heat output during exponential growth, q_{exp}, and the total heat output, q_{total}, (measured from inoculation to the time the thermogram attained the baseline) increased rapidly, but at the higher concentrations both tended to constant values (Table 2). The heat evolved per mol of glucose consumed during the exponential phase, ΔH_{exp}, and the total heat evolved per mol of glucose, ΔH_{total}, increased with decreasing glucose concentration (Figure 6). There is a marked break in both the ΔH-glucose concentration plots between 2.2 and 2.8 mmol dm^{-3}, and this is the concentration range in which there is a marked change in the profile of the recorded thermogram.

Discussion

To avoid difficulties experienced by other workers in maintaining high oxygen tensions in the fermentor and in the microcalorimeter cell, the cells were grown in limiting glucose medium (at concentrations not exceeding 5.5 mmol dm^{-3}) with vigorous stirring and aeration. This concentration of glucose was selected as the maximum value because there was little increase in biomass at higher concentrations; in fact the biomass/glucose concentration plot is only linear up to 4.4 mmol dm^{-3}. At concentrations in excess of this the residual glucose, not used for growth, is broken down to intermediate metabolites and eventually to carbon dioxide and water by processes which require large amounts of oxygen and which dissipate much heat.

The oxygen tension in the fermentor at the highest concentration of glucose was never less than 80% full saturation. The factors which govern the oxygen tension in the microcalorimeter cell are the amount of dissolved oxygen and bubbles of air in the suspension, the pump rate and the length of tubing connecting the fermentor to the microcalorimeter. The length of tubing was reduced to a minimum by mounting the fermentor very close to the microcalorimeter, and so the transit time, Δt, depended on the pump rate. There was a considerable heat loss during the passage through the polypropylene tubing from the fermentor to the microcalorimeter (there was a temperature drop of 8 to 10°C) due to the fact that the equipment was not in a temperature controlled room. This heat loss was eliminated by enclosing the connecting tubing in a water-jacket maintained at 37°C by circulating water.

A similar heated jacket, surrounding the tubing through which the culture was recycled from the pH electrode and flow cuvette to the fermentor, prevented cooling the culture in the fermentor. Since so many small air bubbles were drawn from the fermentor with the culture into the microcalorimeter the introduction of air segments into the line was not necessary to maintain good aerobic conditions in the microcalorimeter cell. The introduction of segments of air, a process which does not necessarily maintain a high oxygen tension in the centre of the alternate bacterial segments at room temperature gives difficulties with the establishment of thermal equilibrium; this problem was avoided in our work (Few *et al.*, 1976; Eriksson and Holme, 1973).

Although the conditions of growth, medium, aeration, stirring, etc, were kept constant, the effect of the state of the cells in the inoculum on the thermogram profile and the total heat evolved from the cultures was most dramatic. Cells, grown for 18 h in the standard synthetic medium, and used as inocula proved most unsatisfactory. On account of the divergence of age of individual cells within such an inoculum and the variation of the viability and total bacterial population between such inocula, the length of the lag phase of the daughter cultures was very variable and the rate of and pattern of heat output was not reproducible. Freeze-dried cells proved unsatisfactory as inocula for similar reasons.

Cryogenically-stored cells used as inocula grew with no lag phase and gave reproducible thermogram profiles. An analysis of the essential features (Table 1) of cultures grown from such inocula over a period of many months revealed a high level of reproducibility, the confidence limit for a value of the total heat output during exponential growth at the 95% level is ± 7%. The cells for cryogenic storage must be harvested before the end of exponential growth to ensure maximum viability, must be washed free of growth medium and waste products, and must be suspended in simple salt solution to avoid altering the composition of the growth medium. Once frozen it is essential that the frozen pellets be kept under liquid nitrogen otherwise changes in the length of the lag phase and thermogram profiles occur. Apart from the excellent reproducibility of the results, the use of this stored material avoids continual maintenance of growing stock cultures and provides standard inocula for other studies under different growth conditions.

Another important consideration in the experimental arrangement is the residence time of an organism in the microcalorimeter cell, this is determined solely by the pump rate (varying from 7 min at 10 $cm^3\ h^{-1}$ to 0.77 min at 90 $cm^3\ h^{-1}$). At very low pump rates (Few *et al.*, 1976) when Δt = 12 min, there is considerable depletion of oxygen and the possibility exists of substrate exhaustion in the flow line in addition

to increased residence time of an organism in the microcalorimeter cell. As a result there will be changes in the metabolism of the cells with the production and fermentation of secondary metabolites, processes which will be accompanied by energy changes.

Although pumping at very high rates ($\Delta t < 1$ min) has the advantage that the state of the organisms in the microcalorimeter cell is more nearly the same as that in the fermentor, it suffers from the disadvantage that thermal equilibrium may not be established within the heat exchanger of the microcalorimeter. It is, therefore, necessary to establish an optimum flow rate intermediate between these two extremes. For cells grown under standard conditions there were marked differences in the profiles of the thermograms (Figure 2) and the total heat evolved for the different pump rates (Figure 4). The heat was evolved exponentially over a large part of the exponential growth phase; both the mass and amount of heat evolved doubling every 45–50 min (Figure 3).

An increase in the flow rate from low values, initially resulted in an increase in the heat output during the exponential growth phase, q_{exp}; at flow rates between 70 and 105 $cm^3\ h^{-1}$ ($\Delta t = 1.7$ and 1.1 min) q_{exp} remained constant within the limits of experimental error. The optimum flow rate for pumping in the microcalorimeter circuits is in the range 70 to 90 $cm^3\ h^{-1}$. Over this range thermal equilibrium is established in the microcalorimeter and the low transit and residence times mean that the organisms in the microcalorimeter cell are more nearly in the same state, as regards oxygen tension and substrate concentration, as that of cells in the fermentor. The use of high pump rates has the additional advantage that sedimentation of organisms in the calorimeter cell and in the line is reduced to negligible proportions; this consideration is of greater importance for heavier cells such as yeasts (Gustafsson and Lindman, 1977). In reporting thermograms and thermal data, measured with a flow system, it is essential that the flow rate and transit time be stated.

Using the standard cultural conditions and a fixed flow rate of 40 $cm^3\ h^{-1}$ ($\Delta t =$ 3 min) the profile of the recorded thermogram was very dependent on the glucose concentration (Figures 1 and 5). At low concentrations, there was only a single peak with a shoulder on the decay side, such a profile is expected if all the carbon source is converted to cellular material and carbon dioxide, with no accumulation of secondary intermediates. At higher glucose concentrations, there is a secondary heat pulse at the onset of the stationary phase when all the glucose has been consumed. As there is no further increase in biomass, the heat evolved arises from the breakdown of intermediate metabolites, e.g. acetate, which have accumulated during exponential growth. This breakdown is oxidative but since there is an adequate supply of oxygen, the oxygen tension of the medium increases to full saturation in contrast to previous observations when a second minimum value of oxygen tension was reported. With decreasing glucose concentration the heat evolved per mol of glucose (ΔH_{exp} and ΔH_{total}) increased; there is a distinct break in the ΔH-concentration plots at a concentration between 2.2 and 2.8 mmol dm^{-3}. It is in this concentration range that there is a marked change in the profile of the recorded thermogram (Few *et al.*, 1976).

These changes in ΔH and in the profile of the thermograms are due to changes in metabolic processes whereby all the glucose, at higher concentrations, is not converted directly into cellular material but instead is broken down to secondary metabolites. These metabolites, which are not used as a carbon source for growth, are subsequently

oxidised to carbon dioxide and water by exothermic processes. The decrease in pH during growth which becomes larger the higher the glucose concentration, is evidence to support the fact that these intermediates are acidic in nature. In some preliminary experiments acetate has been detected in the culture medium towards the end of the exponential growth phase; this is in agreement with the observation of Eriksson and Wadsö (1971). The number of bacterial generations during exponential growth, which increases with increasing glucose concentration, reaches a limit of about six for the three highest glucose concentrations. This limit and the very low molar growth yield at these high glucose concentrations suggest incomplete conversion of the carbon source into cellular material.

In arriving at a reliable value for the heat evolved per mol of glucose consumed or the heat evolved for the production of 1 mol of cells (Herbert, 1976), figures obtained for the three lowest glucose concentrations (Table 2) were rejected because of the small extent of growth, and those for the two highest concentrations were rejected on account of the low yield values and the formation of secondary metabolites. From the results for the intermediate glucose concentrations (2.2–4.4 mmol dm^{-3}) and at a pump rate of 40 cm^3 h^{-1} the average value for the consumption of 1 mol of glucose in the exponential phase is given by $\Delta H_{exp} = -555$ kJ mol^{-1} and for the production of 1 mol of cells of elementary formula $CH_{1\cdot72}\ O_{0\cdot56}\ N_{0\cdot24}\ P_{0\cdot02}\ S_{0\cdot01}$ and relative formula mass 26.98, $\Delta H_p = -164$ kJ mol^{-1}.

Since at a constant glucose concentration the heat output, q_{exp}, increases with increasing pump rate to a constant value (Figure 4), the heat evolved during the exponential phase of growth both for the consumption of 1 mol of glucose and the production of 1 mol of cells will follow a similar pattern of variation. The results for the variation of the heat output for cells grown in 3.3 mmol dm^{-3} glucose at different pump rates are collected in Table 3. Since at the higher pump rates, i.e. lower transit and residence times, the state of the organisms in the microcalorimeter cell will be close to that of the cells in the fermentor as regards growth, metabolism and environmental conditions, the more correct values of q_{exp} to use for subsequent calculations are those obtained at the higher pump rates. Further the concentration of 3.3 mmol dm^{-3} glucose is an optimal concentration to consider as there is adequate growth with minimal formation of secondary metabolites. Thus taking the values of q_{exp} at the four highest pump rates the value of $\Delta H_p = -257$ kJ $(\text{mol cells})^{-1}$. This value which may be expressed as −9.5 kJ per gram of cells is near the value of −11.0 kJ per gram of cells of *K. aerogenes* (Grangetto, 1963), reported previously.

The total heat evolved during the consumption of 1 mol of glucose and the production of 1 mol of cells calculated from the measured value of q_{total} (Table 2), are higher than the corresponding values for the exponential phase of growth, especially during growth at the higher concentrations of glucose. This increase is due to the heat evolved in the stationary phase during the oxidation of secondary metabolites which are present in much higher concentrations in the media of high glucose concentration.

It is of interest that the thermogram profiles at high flow rates are, in general, similar to those of cultures grown in media of low glucose concentration. At lower flow rates or higher glucose concentrations the thermograms become more complex. In both cases there is an increase in the formation of secondary intermediates and the demand on the available oxygen will be maximal. It is possible that under both these

conditions there is local depletion of oxygen in the flow line leading to a loss of fully aerobic conditions.

In conclusion we have demonstrated that before any systematic study of the energetics of bacterial growth is undertaken it is essential to establish standard conditions for growth and thermal measurements which give reproducible results. The most important factors in heat measurements with a flow calorimeter are (a) the nature and state of the inoculum, bearing in mind that cryogenically-stored cells are very suitable and convenient; (b) the composition of the growth medium which for preference should be growth-limiting with respect to one substrate (in this way exothermic processes associated with fermentation and breakdown of excess nutrients are reduced); (c) adequate aeration in the fermentor, flow lines and calorimeter cell; and (d) optimum pump rate, since the higher the pump rate (compatible with the attainment of thermal equilibrium) the closer will be the state of the organisms in the calorimeter cell to that of those in the fermentor with respect to substrate and oxygen concentration. The use of nutrient rich media (Schaarschmidt and Lamprecht, 1976), although possibly allowing good growth, results in complex thermograms in which it is difficult to distinguish between energy changes due to growth and division and those due to secondary degradative processes.

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