A thesis entitled:

MICROCALORIMETRIC studies of Chemostat Cultures of *Klebsiella aerogenes*

by

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for the degree of Doctor of Philosophy
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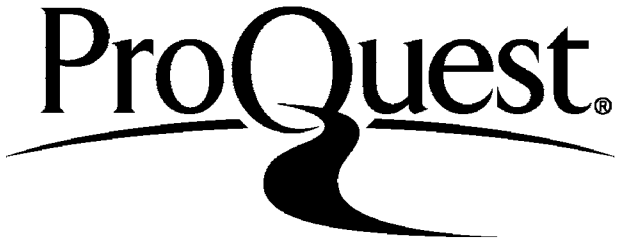

This thesis comprises a report of full-time research undertaken by the author in the Physical Chemistry Laboratories of Bedford College, University of London, from January 1978 to March 1980.

Ali Djavan

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Abstract

The heat output of steady-state cells of Klebsiella aerogenes growing in carbon-limited and C-sufficient media was measured with an LKB flow-microcalorimeter. Problems associated with oxygen shortage were eliminated by using low substrate concentrations, higher aeration rates, and high pump rates through the microcalorimeter.

The recorded heat output was extrapolated to the heat production by cells in the chemostat, and this was converted to specific heat output, ΔH_p/kJ $(g\ \text{cell})^{-1}$. i.e. the heat output of formation of 1 g cell during one generation time. This enabled direct comparison of the heat production for different dilution rates and growth under different conditions. In C-limited cultures both ΔH_p and molar growth yield increased with increasing dilution rate and attained constant values at $D > 0.5\ h^{-1}$. In C-limited media ΔH_p was the same irrespective of substrate concentration and carbon source. Although the specific heat output was much greater in C-sufficient than in C-limited chemostats, it was independent of the concentration of limiting nutrient and glucose, provided glucose was supplied in excess.

The increased heat output produced by adding small amounts of different substrates to glucose-limited chemostats depended on the added C-source; four types of substrate were recognised. The additional heat output increased linearly with the amount of added acetate, but not with glucose and pyruvate. Small amounts of uncouplers disturbed the steady-state heat output and the increased heat output was related to the stimulation of the ATPase system. The enhanced heat output when the pH was increased to 8, was associated with proton translocating ATPase activity.

The maintenance energy obtained from the variation of the growth yield and specific heat output with dilution rate for glucose- and glycerol-limited chemostats, was partly growth rate-dependent, m_g, and partly growth rate-independent, m'_s. These values were related to the maintenance coefficients reported in the literature.

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Thanks are also due to Mr. R.K. Mitchell for technical service and Miss M. Easton for elemental analyses and all the members of the Department of Chemistry, and my research colleagues for all their advice and co-operation.

I also wish to thank the technical staff of the Department of Botany, Bedford College, for all their assistance.

Dedicated

to my

Parents

List of Symbols

Symbol	Specification and Units
ΔH	Enthalpy change of a reaction (i.e. heat evolved).
Q	Total heat produced.
$\frac{dq_i}{dt}$, $\frac{dQ}{dt}$	Rate of heat output for thermocouple i; for all thermocouples.
V_i, V	Output voltage for thermocouple i, for all thermocouples.
ε	Proportionality constant between output voltage and heat output.
R_d	Chart deflection /μV (for heat output)
R	Chart deflection /mV (for CO_2 estimation)
V_{cell}	The volume of calorimeter cell /cm^3
V_f	Volume of the fermentor /cm^3
Δt	Transit time for bacterial suspension from fermentor to the calorimeter cell /min.
f	Flow rate of suspensions /cm^3 h^{-1}.
f_a	Flow rate of air /dm^3 min^{-1}.
$\Delta H'_p$	The recorded specific heat output /kJ $(g\ cell)^{-1}$
ΔH_p	The extrapolated specific heat output (i.e. heat evolved in the fermentor) /kJ $(g\ cell)^{-1}$.
m	Biomass /g cm^{-3}
s	Substrate concentration /mol dm^{-3}
S_r	Inflowing substrate concentration /mol dm^{-3}
μ	The specific growth rate /h^{-1}
μ_{max}	The maximum specific growth rate /h^{-1}
D	Dilution rate /h^{-1}
t_d	Doubling time of the culture /h.
Y_c	Yield coefficient /g cell per g substrate.
$Y_{sub,\ glu,\ gly,py,\ ac}$	Molar growth yield /g cell per mol of substrate, glucose, glycerol, pyruvate, acetate.
Y_{glu}^{ana}	Anaerobic molar growth yield / g cell per mol of glucose.
Y_c^{max}	Maximum yield coefficient /g cell per g substrate

Symbol	Specification and Units
Y_{glu}^{max}	Maximum molar growth yield /g cell per mol of glucose.
Y_{ATP}	ATP yield /g cell $(mol\ ATP)^{-1}$
q_{ATP}	Specific rate of ATP production /mol ATP $(g\ cell\ h)^{-1}$
$Y_{av.\bar{e}}$	g cell formed per available electron.
$P/2\bar{e}$	mol ATP per electron pair.
H^+/O	Number of proton ejected per atom O.
m_s	Maintenance coefficient /g glucose $(g\ cell\ h)^{-1}$.
m_e	Maintenance coefficient /mol ATP $(g\ cell\ h)^{-1}$.
M_e	Maintenance energy /kJ $(g\ cell\ h)^{-1}$.
m_s'	Growth rate-independent maintenance coefficient /g glucose $(g\ cell\ h)^{-1}$
m_g	Growth rate-dependent maintenance coefficient /g glucose $(g\ cell\ )^{-1}$

CHAPTER 1

INTRODUCTION

1. Introduction

Klebsiella aerogenes like any other chemoorganotrophic (non-exacting) organism can readily grow in simple salts media supplemented with an organic carbon source. Clearly in these media the carbon source is partly degraded to carbon dioxide and water for energy requirements and partly converted to cellular material. K. aerogenes due to its versatility to utilize a variety of carbon sources and its nonpathogenicity has been widely used for various metabolic and energetic studies. The particular strain of this organism was chosen for detailed study because of the well established literature on its growth in simple chemically defined media in batch and continuous cultures (Tempest et al., 1965; Dean and Hinshelwood, 1966).

1.1. Properties of Klebsiella aerogenes

K. aerogenes is a rod shaped, Gram negative, non-motile, non-sporing, facultative anaerobe which ferments sugars with production of gas. It is catalase, KCN, and Voges-Proskauer positive, but methyl red, oxidase, and phenyl-alanine negative.

1.2. Nutrition

K. aerogenes is one of the organisms capable of growth upon carbohydrates as sole source of carbon and energy, provided that the other essential nutrients such as; nitrogen, phophorous, magnesium, potassium, sulphur etc., are present, (Tempest et al., 1965, 1966, 1970; Dicks et al., 1966; Luria 1960).

1.2.1. Carbon source

A wide range of carbon sources in minimal growth media have been studied (Tempest et al.,1965; Dean and Hinshelwood, 1966; O'Brien et al., 1969; Hadjipetrou et al.,1965). These studies showed that while the

organism requires adaptation for some compounds such as acetic acid, glycerol, it is fully adapted for growth on other compounds such as glyceric acid, pyruvic acid, lactic acid, on transfer from a glucose medium. Compounds such as formic acid, oxalic acid, fail to support the growth.

In general glucose is normally converted to pyruvic acid by glycolysis (Embden-Meyerhof Pathway). Although oxygen is not required for this sequence of reactions the fate of pyruvic acid is dependent upon the availability of oxygen. Under aerobic conditions the major products are cells and carbon dioxide. Pyruvic acid undergoes oxidative decarboxylation to acetyl-CoA which then enters the Kreb's or tricarboxylic acid cycle (TCA). The net effect of the TCA is to produce 2 moles of CO_2, 1 mole of ATP, 3 moles of NADH, and one mole $FADH_2$ per mole of pyruvate. The reduced NAD, and FAD molecules formed during glycolysis and in TCA cycle are oxidised by the electron transport chain, in which the final electron acceptor is oxygen. The oxidation of one mole of $FADH_2$, and NADH results in the production of 2 and 3 moles of ATP respectively. Overall 38 moles of ATP are produced during the oxidation of one mole of glucose to carbon dioxide and water, of which 34 moles are produced through oxidative phosphorylation and the rest from substrate-level phosphorylation. The ATP produced during glucose degradation, is the energy store of the organism and it is utilised by hydrolysis ($\Delta G' = -31$ kJ mol^{-1}). Hence, a total of 1178 kJ of energy is produced by 1 mole of glucose to the organism. This is used for growth and the various anabolic processes occurring in the organism, any excess being liberated as heat. Glycolysis, fermentation and TCA cycle products are briefly summarised in Fig. 1.1.

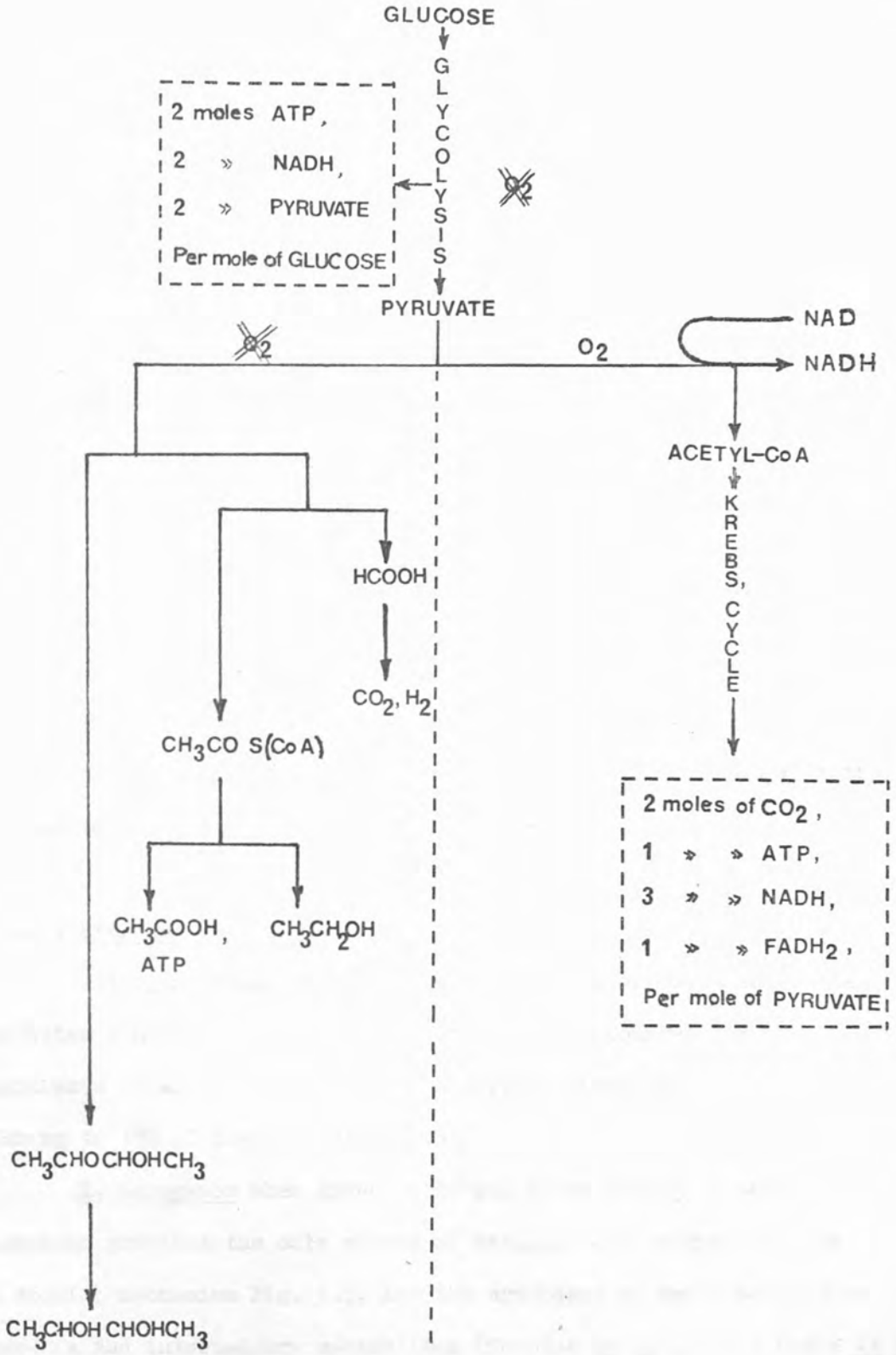

GLUCOSE
GLYCOLYSIS
2 moles ATP,
2 » NADH,
2 » PYRUVATE
Per mole of GLUCOSE
PYRUVATE
NAD
NADH
ACETYL-CoA
KREBS, CYCLE
HCOOH
CO2, H2
CH3CO S(CoA)
CH3COOH, ATP
CH3CH2OH
CH3CHOCHOHCH3
CH3CHOH CHOHCH3
2 moles of CO2,
1 » » ATP,
3 » » NADH,
1 » » FADH2,
Per mole of PYRUVATE

Fig. 1.1

1.2.2. Oxygen requirement

Oxygen is not only required for respiration (as final electron acceptor in respiratory chain), but also is incorporated in cell material (Hughes et al., 1969). The metabolic pathway of the organisms is adjusted by the presence or absence of oxygen (Gray et al., 1966a, 1966b). According to Harrison et al. (1967) there are three phases in relation to oxygen concentration. When the oxygen concentration is above 15% of saturation, the growth is fully aerobic, below this concentration there is a transition phase and further decrease to 5% of saturation gives rise to anaerobic fermentation. Furthermore, Harrison and Loveless (1971,a,b) working with K. aerogenes and E. coli, found that, reduced cell yields and increased oxygen uptake rates were obtained at low oxygen tension (2-3 mmHg) without any increase in the extra-cellular organic carbon. Oxidative phosphorylation in Enterobacter aerogenes occurs at two sites in the respiratory chain (Stouthamer, 1976 b) as shown in Fig. 1.2.

1.2.3. Nitrogen source

Nitrogen is usually provided as ammonium salts, amino acids or other simple N-containing compounds, it is required for the synthesis of amino acids, purines and pyrimidines and can account for up to 15% of the cell dry weight.

K. aerogenes when grown in simple salts medium in which ammonium provides the sole source of utilizable nitrogen, employs a special mechanism Fig. 1.3. for the synthesis of amino acids from ammonia and intermediary metabolites (Tempest et al., 1970; Meers et al., 1970). The enzymic activities vary according to the ammonia content in the culture (Brown et al., 1973, 1976). In carbon-limited cultures the glutamate dehydrogenase activity was increased many-fold, where

Fig. 1.2. Respiratory chain of Enterobacter aerogenes (Stouthamer 1976b), 1 and 2, indicate phosphorylation sites.

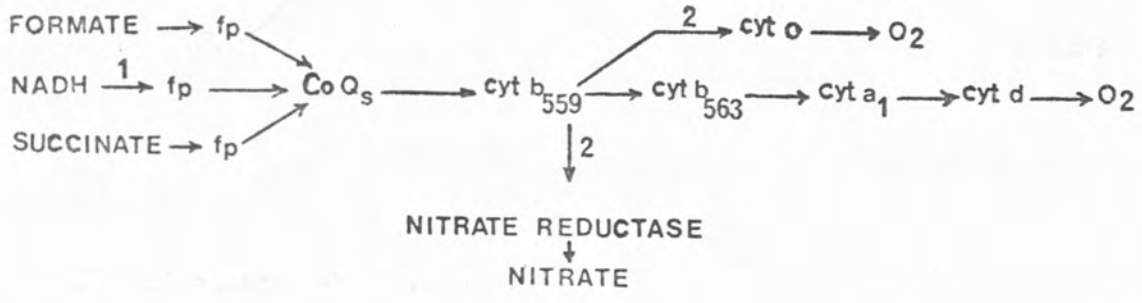
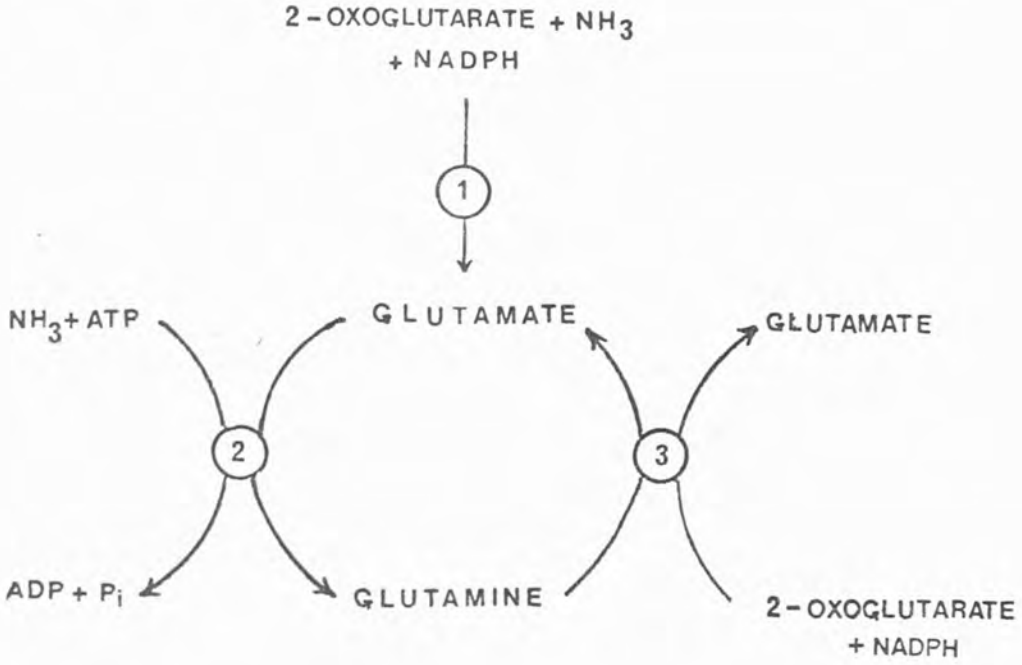


Fig. 1.3. Synthesis of glutamate in Aerobacter aerogenes (Tempest et al., 1970).



1. Glutamate dehydrogenase:

NH_3 + 2-oxoglutarate + NADPH $\longrightarrow$ glutamate + H_2O + $NADP^+$

2. Glutamate synthetase:

NH_3 + glutamate + ATP $\longrightarrow$ glutamine + ADP + P_i + H_2O

3. Glutamine amide-2-oxoglutarate aminotrasferase (oxidoreductase, NADP):

Glutamine + 2-oxoglutarate + NADPH $\longrightarrow$ 2 glutamate + $NADP^+$

Sum of reactions 2 and 3:

NH_3 + 2-oxoglutarate + ATP + NADPH $\longrightarrow$ glutamate + ADP + P_i + $NADP^+$ + H_2O

as in ammonia-limited cultures, glutamine synthetase and glutamate synthase activities were increased.

1.3. Kinetics of bacterial growth

1.3.1. Growth in batch cultures

This is the traditional method for studying the growth of bacteria, in that microorganisms generally exhibit a characteristic growth cycle (Fig. 1.4). Immediately after inoculation there may be a lag phase, a, during which the cells synthesize essential intermediates (Hinshelwood, 1946). The length of this phase is dependent upon the age and size of the inoculum, and the composition of the growth medium. No cell division occurs in this phase, although there is evidence of considerable enzymic activity and increase in size of the organisms.

Eventually cells will divide and enter into the exponential phase, b, during which there is a maximum rate of growth per organism. During this phase a certain minimum generation time is maintained.

The biomass (m) increases with time (t) according to the equation;

$$\frac{dm}{dt} = \mu\, m \quad \text{or} \quad \frac{1}{m}\frac{dm}{dt} = \mu \qquad 1.1$$

or

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

where μ is the specific growth rate and t_d is doubling time of growing culture.

Monod (1942) has shown that, the specific growth rate and mean generation time (t_d) are affected by several parameters, such as growth medium composition, temperature, pH, and substrate

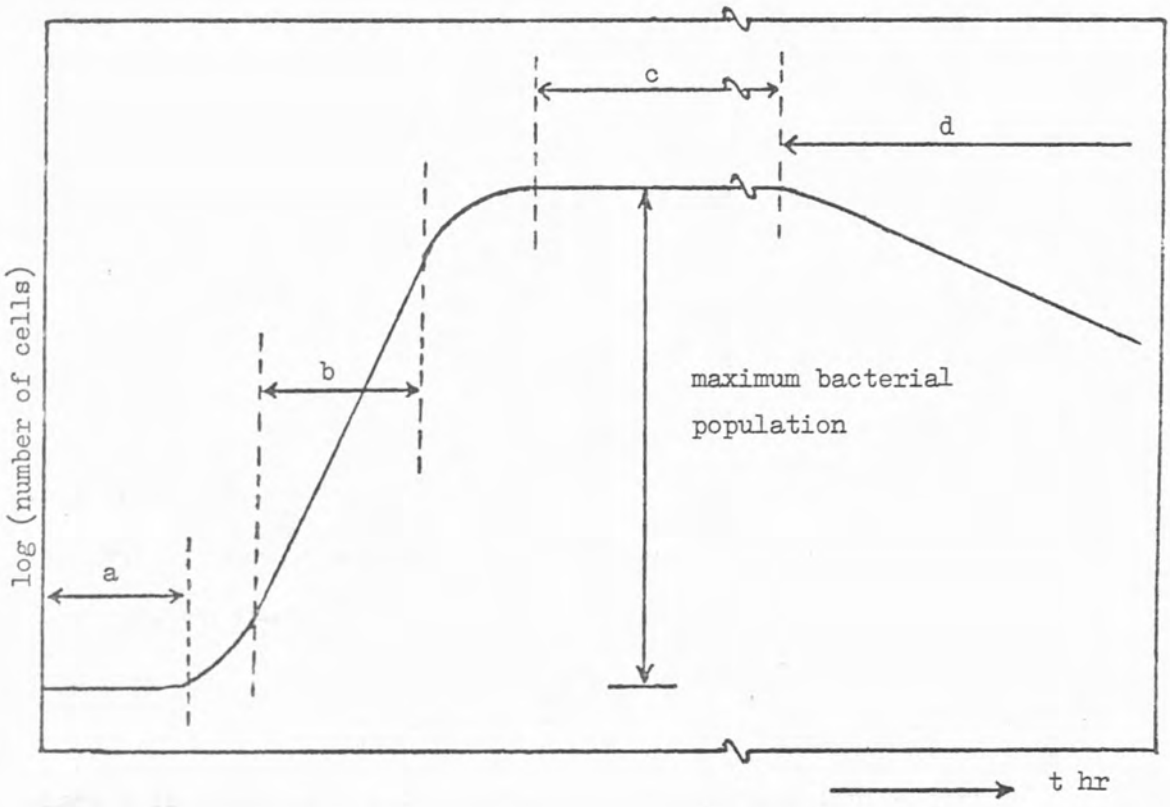


Fig. 1.4. A typical bacterial growth cycle.
a, b, c, d, indicate, Lag, Exponential,
Stationary, and Death phase respectively.

concentration. According to Monod, the specific growth rate is related to substrate concentration by a Michaelis-Menton type of equation;

$$\mu = \mu_{max} \left(\frac{s}{K_s + s} \right) \qquad 1.2$$

where μ_{max} is maximum specific growth rate, s the substrate concentration, and K_s the saturation constant (numerically equal to the substrate concentration at $\mu_{max}/2$). μ_{max} is attained during the exponential phase, where all nutrients are in excess. A simple relationship exists between growth and utilisation of substrate. This is shown in its simplest form in growth media containing a single organic substrate; under these conditions the growth rate is a constant fraction, Y, of the substrate utilisation rate:

$$\frac{dm}{dt} = - Y \frac{ds}{dt} \qquad 1.3$$

where Y is known as yield constant. Thus over any finite period of growth:

$$Y = \frac{\text{weight of bacteria formed}}{\text{weight of substrate used}}$$

If the values of the three growth constants, μ_{max}, K_s, and Y are known, equations 1.1, 1.2, 1.3, provide a complete quantitative description of the growth cycle in batch culture.

The exponential phase of growth comes to a halt as a result of exhaustion of an essential nutrient, development of toxic pH, and/or accumulation of toxic metabolites. This situation is called the stationary phase , c, where little or no growth occurs and the cell density has attained its maximum value. Shortly after the

onset of the stationary phase a phase of decline, d, occurs, where the number of viable cells decreases with time. The length of this period varies significantly with different organisms and media.

1.3.2. Growth in continuous culture (chemostat):

The growth of microorganisms in continuous culture takes place under steady-state conditions and the environmental factors, which can vary markedly during the growth cycle in batch culture, are maintained constant. Furthermore such culture parameters as pH, temperature, nutrient concentration and growth rate can be altered readily for experimental purposes.

Continuous cultivation of microorganisms has been used for many years (Felton et al; 1924; Rogers et al; 1930). Although these early reports demonstrated its fundamental importance, the theory of method and thus the possibility for its applications were not developed until 1950 when several fundamental papers were published which influenced further development and interests in this method (Monod, 1950; Novick et al., 1950 a, b; Adams et al., 1950).

Theoretical discussions of bacterial growth usually starts from the exponential equations 1.1, 1.2 and 1.3 (Monod 1942).

From the equation 1.1, it follows that exponential growth can occur at specific growth rates having any value between zero and μ_{max}, provided the substrate concentration can be held constant at an appropriate value; a fact of major importance in continuous culture (Monod, 1950; Novick et al., 1950 a, b).

The same equations and constants are equally applicable to the theoretical treatment of continuous culture. In continuous culture, fresh medium is added at a steady rate f $cm^3\ h^{-1}$ to a growing culture

whose volume V cm^3 is kept constant by means of an overflow. The ratio of the rate of inflowing medium to the working volume is known as the dilution rate, ie. $D = f/V$. The dilution rate is equal to the number of volumes of medium which pass through the culture vessel per hour. The reciprocal dilution rate, 1/D, gives a measure of the residence time of an organism in the culture vessel.

In a sufficiently aerated and stirred growth vessel changes in the concentration of organisms is given by:

$$\text{Increase} = \text{Growth} - \text{output}$$

$$\frac{dm}{dt} = \mu m - D m \qquad 1.4$$

Hence if $\mu > D$, dm/dt is positive and the concentration of organism will increase, while if $\mu < D$, dm/dt is negative and the concentration of organisms will decrease eventually to zero; ie. the culture will be washed out of the vessel. When $\mu = D$, $dm/dt = 0$ and m is constant, i.e. the culture is in its steady state, and the concentration of the organisms does not change with time.

This equation has been derived by several workers, but it has not always been realised that by itself the equation gives no information on, what dilution rates make a steady state possible.

On the basis of equation 1.2., Herbert *et al.*, (1956) have investigated the effects of dilution rate on the substrate concentration in the culture vessel. It was found that the net rate of change of substrate concentration is given by:

$$\text{Increase} = \text{Input} - \text{Output} - \text{consumption}$$

$$= \text{Input} - \text{Output} - \frac{\text{Growth}}{\text{Yield constant}}$$

$$\frac{ds}{dt} = D S_r - D s - \frac{\mu m}{Y} \qquad 1.5$$

Where S_r is the limiting substrate concentration entering the culture vessel and s is the substrate concentration in the overflow. Substitution of equation 1.2, into equations 1.4, and 1.5 gives:

$$\frac{dm}{dt} = m \left[\mu_{max} \left(\frac{s}{K_s + s} \right) - D \right] \qquad 1.6$$

and hence from the equation 1.3

$$\frac{ds}{dt} = D\ (S_r - s) - \frac{\mu_{max}\ m}{Y}\ \left(\frac{s}{K_s + s} \right) \qquad 1.7$$

The behaviour of continuous culture can be completely defined by the equations 1.5, 1.6, and 1.7.

Under steady state conditions $D = \mu$, $dm/dt = 0$ and $ds/dt = 0$. Solving equations 1.6, 1.7, for the condition $dm/dt = ds/dt = 0$, gives the steady state values of m and s, which are designated $\bar{m}$, $\bar{s}$;

$$\bar{s} = K_s \left(\frac{D}{\mu_{max} - D} \right) \qquad 1.8$$

$$\bar{m} = Y\ (S_r - \bar{s}\ ) = Y \left[S_r - K_s \left(\frac{D}{\mu_{max} - D} \right) \right] \qquad 1.9$$

From these the steady state concentration of bacteria and substrate in the culture vessel can be predicted for any value of the dilution rate and the concentration of inflowing substrate, provided the values of the growth constants K_s, μ_{max}, Y, are known.

In the steady state the specific growth rate (μ) is equal to the dilution rate (D);

$$\mu = \frac{\log_e 2}{t_d} = \mu_{max} \left(\frac{\bar{s}}{K_s - \bar{s}} \right) = D \qquad 1.10$$

The doubling time (t_d) is therefore equal to 0.693/D. If D = 1.0, one volume per hour is flowing through the culture vessel and the mass of organism will be doubling every 42 min. By varying S_r, and D an infinite number of steady states can be obtained, since steady state values of bacterial mass and substrate concentration depend solely on these values (μ_{max}, K_s , Y, are constant for a given organism and growth medium). The effect of varying the dilution rate on the steady state concentrations of bacteria, substrate, and mean generation time (t_d) are shown in Fig 1.5.

Theoretically the concentration of organisms has a maximum value when the dilution rate is zero, the substrate concentration then also being zero; this situation corresponds to the final stage of a batch culture just before the onset of the stationary phase. However, positive deviations occur in carbon-sufficient chemostats and negative deviations in carbon-limited chemostats as the D tends to zero. As the dilution rate increases the substrate concentration increases and the concentration of organisms falls until at a critical value of D, the concentration of organisms becomes zero and the substrate concentration ($\bar{s}$) becomes equal to S_r.

The critical value of the dilution rate, is obviously of great practical importance and its value is given by;

$$D_c = \mu_{max} \left(\frac{S_r}{K_s + S_r} \right) \qquad 1.11$$

When $S_r >> K_s$, which is usually the case, then $D_c \sim \mu_{max}$. The steady state concentration of organisms varies for different concentrations of inflowing substrate (equation 1.9). The concentration of substrate $\bar{s}$ is however independent of S_r, i.e. the curve relating dilution

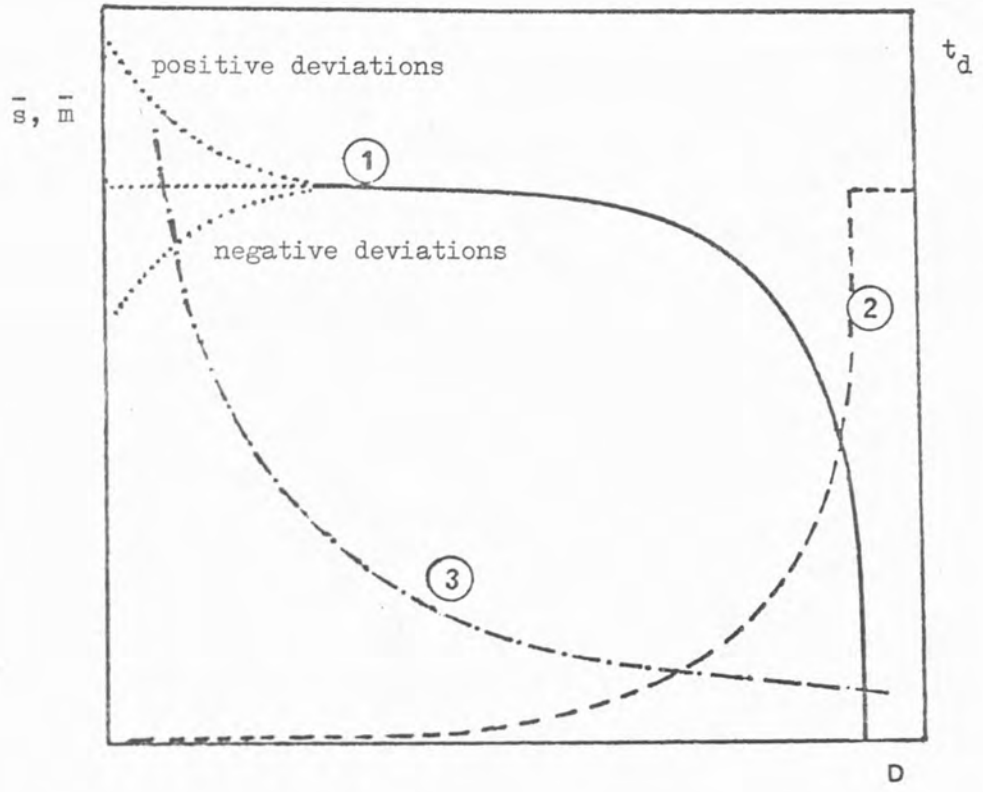


Fig. 1.5. Theoretical steady state relationships in continuous culture, where $\bar{m}$ is biomass, $\bar{s}$, substrate concentration, D, dilution rate, and t_d the doubling time. 1. $\bar{m}$; 2. $\bar{s}$; 3. t_d.

rate to the concentration of substrate in the culture vessel is the same whatever is the concentration of S_r (Novick et al. 1950 a,b).

The effective yield "Y_e" is defined as the ratio of bacteria formed to substrate supplied in the inflowing culture medium and is given by;

$$Y_e = \frac{\bar{m}}{S_r} = \frac{Y (S_r - \bar{s})}{S_r} \qquad 1.12$$

At all flow rates exceeding zero the effective yield is less than the yield constant owing to the substrate wasted in the outflow, $(\bar{s})$, which increases with the dilution rate.

The efficiency of utilization of the substrate supplied in the inflowing growth medium is given by;

$$\frac{Y_e}{Y} = \frac{S_r - \bar{s}}{S_r} = \frac{S_r - K_s \left(\frac{D}{\mu_{max} - D}\right)}{S_r} \qquad 1.13$$

It follows that for maximum utilization of substrate, the dilution rate should be as low as possible. However the shape of the curve relating $\bar{s}$ to D (Fig. 1.5), is such that, the loss of substrate in the outflow is in practice negligible up to quite high dilution rates.

1.4. Energetics of the growth of microorganisms

1.4.1. Biochemical assessments

It is generally agreed among biologists that the energy requirements of a living cell can be couched in terms of ATP (adenosine triphosphate). This compound; either directly or by some intermediate for coupling the energy - yielding reactions of

metabolism, provides the bond-forming power necessary for many synthetic reactions.

The hydrolysis of ATP with the release of stored energy and its formation from ADP and inorganic phosphate with the gain of energy may be written:

$$ATP + H_2O \rightleftharpoons ADP + P_i + H^+ \quad \Delta G' = -31 \text{ kJ mol}^{-1}$$

Several hypotheses of electron transport-linked phosphorylation have been proposed over the last three decades. These include; (a) chemical hypothesis (Lipmann, 1945; Slater, 1953), which involves a covalent high-energy intermediate between the electron-transport and the ATP-synthesizing system; (b) the conformational hypothesis (Boyer, 1965) which postulates a direct interaction between conformationally modified energy-transducing catalysts; (c) a hypothesis which proposes "intra-membrane proton fluxes" as a means of energy transduction (Williams, 1961); and (d) the chemiosmotic hypothesis (Mitchell, 1961), in which a transmembrane proton gradient is the basic energy transfer device.

At present, it is not known whether the form of potential energy produced in the coupling events is best described as a chemical intermediate, as a combination of membrane potential and pH gradient, or as a conformational state. However, the available evidence strongly supports the chemiosmotic hypothesis (Reed et al., 1975; Bakker et al., 1973; Cunarro et al., 1975).

The production of ATP in oxidative phosphorylation occurs by a complex process involving a membrane-localized electron transport chain. ATP will not be produced if "uncouplers" are present. These reagents have the ability to abolish the coupling of substrate oxidation to ATP-synthesis. As a consequence ATP-synthesis comes to a halt,

because the necessary energy is not available, while the oxidation of substrate, uninhibited by respiratory control, proceeds at maximal rate and produces heat instead of ATP (Poe et al., 1967; Hanstein, 1976; Baird et al., 1979).

Many attempts have been made to define the energy efficiency of the growth of microorganisms by various yield coefficients (Y_{sub}, Y_{ATP}, Y_O, Y_{O_2}, $Y_{av.}$, $\bar{e}$) in both batch and continuous cultures. For example values ranging from 4.7 to 20.9 have been obtained for Y_{ATP} (g dry weight of cells per mole of ATP) by several investigators working with different microorganisms in various media (Bauchop and Elsden, 1960). These data showed that Y_{ATP} is influenced by growth conditions, the values being lower in minimal media than in complex media. Thus the value of 10.5 suggested by some investigators for the estimation of ATP yield can give erroneous results.

The influenece of growth rate and maintenance energy on Y_{ATP} has been suggested by Pirt (1965). He derived an equation which relates the molar growth yield and the specific growth rate. It was assumed that during growth the consumption of the energy source is partly growth dependent and partly growth independent:

$$\frac{1}{Y_{glu}} = \frac{m_s}{\mu} + \frac{1}{Y_{glu}^{max}} \qquad 1.14$$

where Y_{glu} is the molar growth yield for glucose, m_s is maintenance coefficient which was assumed to be independent of the specific growth rate μ, Y_{glu}^{max} is the molar growth yield for glucose corrected for energy of maintenance. The amount of energy source which is used independently of growth is, by definition, called the maintenance energy.

It has been concluded, however, that this growth-independent consumption of energy source is used for more purposes than the true maintenance processes as originally defined (Stouthamer et al., 1975) which indicates that the use of the term maintenance coefficient for the mathematical parameter m_s in equation 1.14 may be misleading (Stouthamer, 1977).

Stouthamer et al., (1973) have modified equation 1.14 to give:

$$q_{ATP} = \frac{\mu}{Y_{ATP}} = \frac{\mu}{Y_{ATP}^{max}} + m_e \qquad 1.15$$

where Y_{ATP}^{max} is the growth yield per mol of ATP corrected for energy of maintenance; q_{ATP} is the specific rate of ATP production (mol ATP per g dry weight per hour), and m_e is the maintenance coefficient (mol ATP per g dry weight per hour).

It is clear from equation 1.15, that q_{ATP} is a linear function of μ and that Y_{ATP} is dependent on the growth rate. This has indeed been observed for Lactobacillus casei (de Vries et al., 1970), Saccharomyces cerevisiae (Watson, 1970), Enterobacter aerogenes (Stouthamer et al., 1973, 1975) Escherichia coli (Hempfling et al., 1975). It is concluded that both parameters (Y_{ATP}^{max}, m_e) are dependent on the growth conditions. The results obtained by these workers, showed that, estimates for Y_{ATP} are fairly precise.

Very different values of the maintenance coefficient for various microorganisms may be one of the factors contributing to the large variation of Y_{ATP} (Stouthamer et al., 1973).

For aerobic yield studies, Y_O, Y_{O_2}, values have been employed by several investigators (Hadjipetrou et al., 1964; Haddock et al., 1971; Poole et al., 1974 and 1975; Dorzd ., 1974; Downs et al., 1975; Stouthamer et al., 1975).

At the present time a chaotic situation exists in the interpretation of the results of aerobic growth experiments, because either Y_{ATP}^{max} must be known to calculate the $p/2\bar{e}$ ratio (mol ATP per electron pair), or vice versa.

Stouthamer (1977) has proposed two possible methods to determine growth parameters for aerobic cultures (a) a comparison of aerobic and anaerobic growth in facultative organisms (b) a comparison of substrate-and sulphate-limited growth in aerobic organisms.

1.4.2. Thermodynamic assessment

1.4.2.1. Free energy

The free energy efficiency (Bass-Becking et al., 1927); is defined by: $100\ \Delta G_b/\Delta G_c$, where ΔG_b is free energy usefully employed for biosynthesis of cell material and ΔG_c is the free energy produced by catabolism of energy source. The assumption made is that the free energy of oxidation of glucose to carbon dioxide and water is the reverse of the free energy required to reduce carbon dioxide and polymerize the reduced products to bacterial cell material at the level of oxidation of CH_2O. This was shown to be an oversimplification (Morowitz, 1968). It was assumed that the free energy change in the combustion of glucose and bacterial cells correspond closely, because the heats of combustion of glucose and bacterial cells are approximately the same. However, this neglects the very large entropy decrease in the production of cellular material. Values for free energy efficiency lie between 5% to 40%.

Theoretical calculations of ΔG_b by Morowitz (1968) give values below 418 J $(g\ cell)^{-1}$, and a free energy efficiency of about

5% for homolactic fermenters such as Strep. faecalis. Whilst these determinations show that the efficiency of growth on this criterion is low, thermodynamics indicates only what is energetically possible and gives no information about the details of the energetics of growth. The difference between thermodynamic and biochemical assessments is illustrated by considering the homolactic fermentation of glucose (Stouthamer, 1977) the ATP is produced with an efficiency of 50% while the ethanolic fermentation which, because of the difference in the balance of products, is only 40% efficient.

1.4.3. Microcalorimetry

In general all processes, physical, chemical or biological are accompanied by heat effects, heat being absorbed or liberated. Although the measurement of heat changes, by calorimetry, dates back a long time, early workers were handicapped by the limitation of their apparatus for biological purposes (Bayne-Jones, et al., 1929; Rahn 1932; Stoward, 1962; and Forrest and Walker 1964) as regards sensitivity, temperature control, amount of material required etc. An apparatus, developed by Calvet and Prat (1963) enabled them to show that thermogenesis in microbial cultures was systematically reproducible and characteristic of the particular organism and medium.

Recent developments have been mainly concerned with placing these studies on a more quantitative basis, and particularly in correlating the observed heat production with thermodynamic data (Battley 1960; Forrest et al., 1961; Belaich et al., 1963, 1968; Forrest, 1967a, Cooney et al., 1968).

The development of the flow microcalorimeter has been of special importance as it has facilitated the study of energy changes during bacterial growth (Delin et al., 1969; Eriksson and Wadsö, 1971). Microcalorimetry is defined as the measurement of very small heat changes, but not necessarily with small quantities of material.

1.4.3.1. Heat changes in biological processes

Biological reactions and processes normally take place in solution at a constant temperature and pressure and thus thermodynamic differences between energy and enthalpy can be discounted. The enthalpy change (ΔH), is therefore the change in energy of the process.

The experimentally measured heat evolution Q, during a reaction is related to ΔH by:

$$Q = -n \Delta H \qquad 1.16$$

where n is the number of moles of reaction taking place; the negative sign indicates the liberation of heat by the reaction (decrease in energy).

Where several simultaneous reactions occur the heat produced equals the sum of that produced in individual reactions;

$$Q = -\sum_{0}^{k} n_j \Delta H_j \qquad 1.17$$

The subscripts 0-k characterise K internal processes and n_j is the number of moles of reaction j and ΔH_j the enthalpy of that reaction per mole.

1.4.3.2. Types of calorimeter

The two principal types of calorimeters are adiabatic and heat conduction calorimeters. A detailed explanation and description of these is given elsewhere (Calvet and Prat, 1963). The specifications of different types are well described by the manufacturers (Calvet

and Prat, 1963, Benzinger et al., 1960; Monk and Wadsö, 1968; Wadsö, 1970), they have been reviewed recently by Levin (1977).

In adiabatic calorimeters there is no heat exchange between the calorimeter and its surroundings. The heat quantity, Q evolved depends on the temperature change and the heat capacity of the calorimeteric system C.

$$Q = C \Delta T \qquad 1.18$$

where ΔT is the observed experimental parameter.

In the heat conduction calorimeter the heat evolved is quantitatively transferred to the surroundings called the heat sink. With these calorimeters a property proportional to the heat flow is measured. The time-integral for the heat flow equals the total amount of heat released.

The microcalorimeter used in this work was of the heat-conduction type. Calorimeters of this type have certain common features and a plan of the essential components is shown in Fig. 1.6. A large air thermostat is employed, to give a constant background temperature control; the temperature of this, is maintained constant by a separate control unit. Located within this bath is a large metal block which acts as the heat sink. In the centre of this metal block are located two microcalorimeter cells one of which, the test cell, contains reactants or bacterial suspension; the other is the reference cell. These cells are surrounded by their own thermopiles and the thermopiles are connected in opposition to each other, thus one acts as blank. This twin principal thermal measurement is to prevent any disturbances from the surroundings. The air bath and the metal block also contain heat exchange coils which are necessary for establishing complete temperature equilibrium of the reactants or bacterial

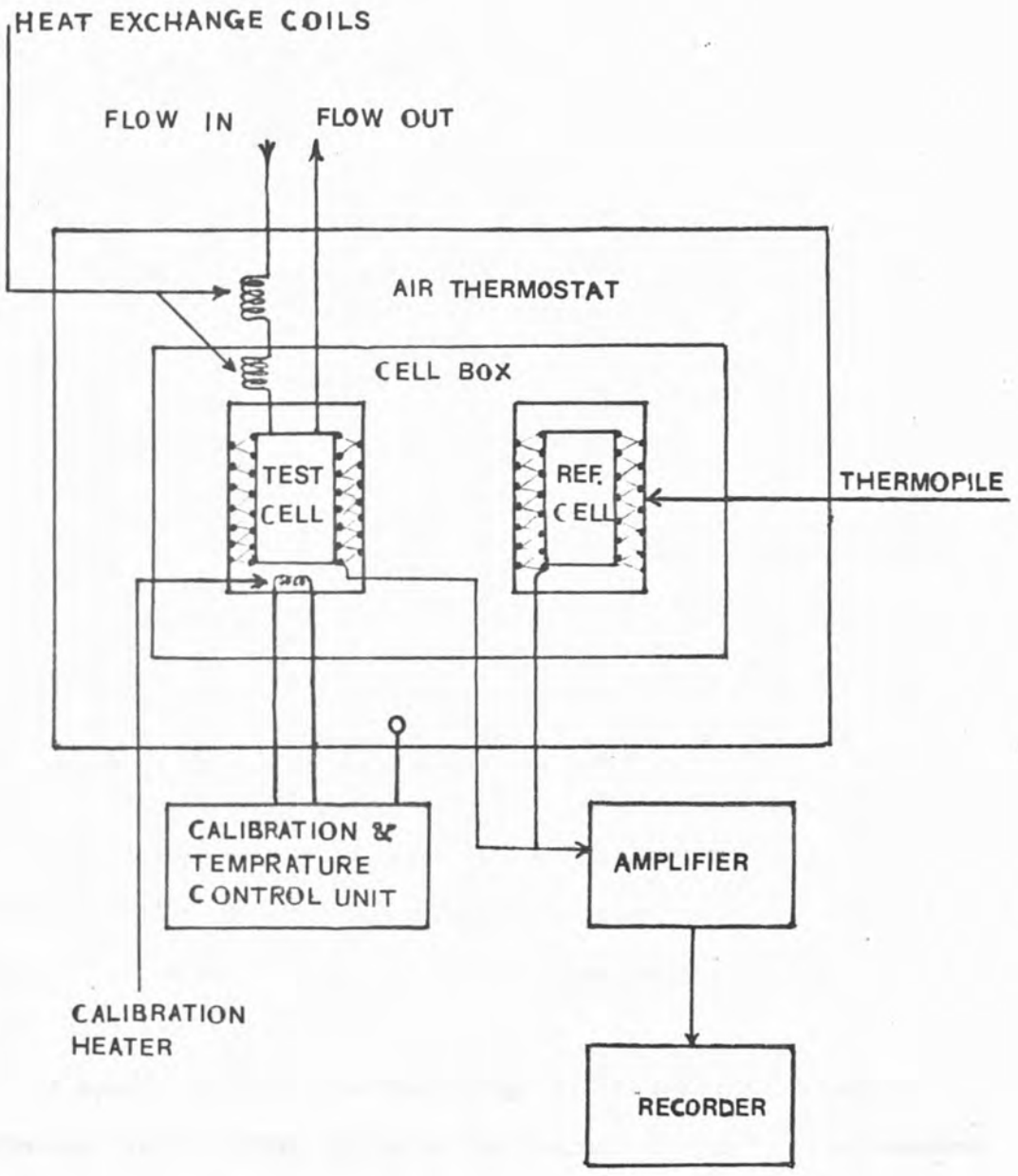


Fig. 1.6. THE ESSENTIAL COMPONENTS OF A HEAT-CONDUCTION CALORIMETER

suspension before entering the microcalorimeter cell. The exchange coil in the air bath was excluded in previous work (Ackland, et al., 1976), which failed largely because of the lack of attainment of temperature equilibrium. Although these heat exchange coils prolong the flow time, it is essential for temperature equilibrium.

In batch calorimeters the reaction vessel is split into two separate compartments (Fig 1.7.a). One reactant is placed in one of the two compartments and the second reactant placed in the other. When the temperature equilibrium is attained and a steady baseline established, the reactants are mixed by rotating the entire cell block. This type of cell is not suitable for bacterial studies because of difficulties associated with oxygen shortage, establishment of the baseline and monitoring of bacterial growth, etc. Even if these problems could be eliminated, this cell still cannot be adapted for use with continuous culture.

In the flow type of calorimeters, the cell is essentially a coil of gold tubing of narrow bore and thin walls (Fig. 1.7.b); this is mounted in a block to make thermal contact with the thermopiles. The passage of air bubbles through this cell causes thermal fluctuations; therefore, it is not suitable for the study of aerobic systems.

A special aerobic flow-cell (Fig. 1.7.c) has been developed by Monk and Wadsö (1968), in which the thermal fluctuations associated with the passage of air bubbles are eliminated. The aerobic cell is again mounted in a block to make thermal contact. In this cell it is possible to introduce alternate small volumes of air and bacterial suspension. This decreases the possibility of oxygen depletion at higher biomass (Eriksson et al., 1971).

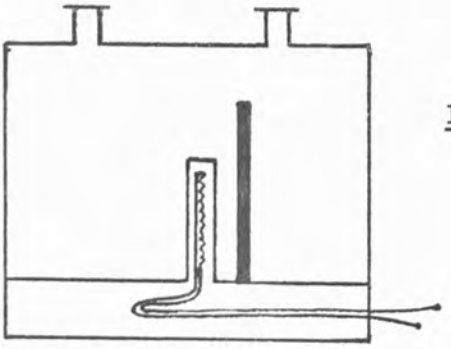


Fig. 1.7.a. Batch calorimeter cell.

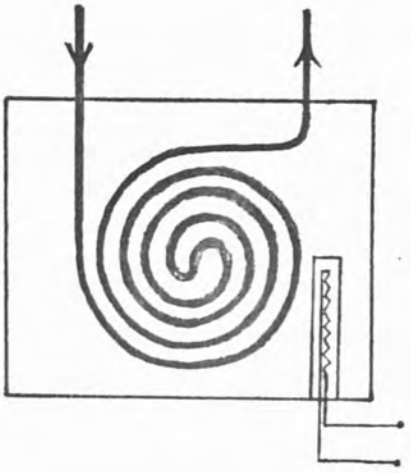


Fig.1.7.b.
The conventional flow cell.

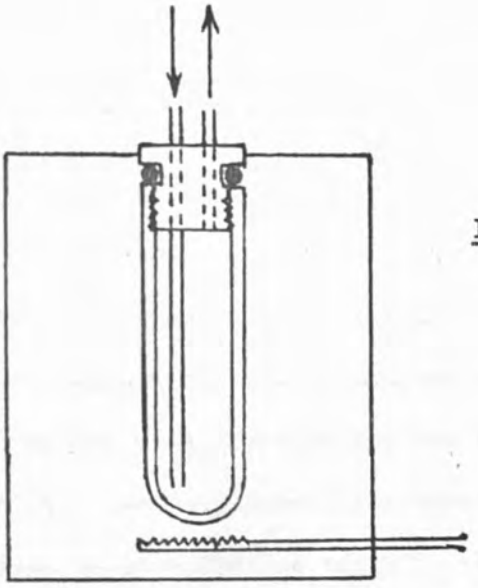


Fig. 1.7.c. The aerobic flow cell.

The total volume of such flow cells is between 0.7 and 1.2 cm^3. Solutions or suspensions under study are continually pumped through the flow-cell at a constant rate, throughout the course of an experiment, which can be carried out in an external vessel.

The heat flowing from the cell to the sink or vice-versa, passes through the thermopiles, which surround the microcalorimeter cell. For each thermocouple the voltage V, and the rate of heat flow dq_i/dt are proportional to the temperature difference between the calorimeter cell and the heat sink;

$$V = C \frac{dq_i}{dt} \qquad 1.19$$

where C is a proportionality constant. Ideally all heat is transported through the thermocouple leads, therefore for all thermocouples:

$$V_1 + V_2 + \ldots\ldots\ldots = C \frac{dq_1}{dt} + C \frac{dq_2}{dt} + \ldots\ldots \qquad 1.20$$

whence;

$$V = C \frac{dQ}{dt} \qquad 1.21$$

where V is the thermopile voltage and dQ/dt the total heat flow. In practice some of the heat does not pass through the thermocouples, (particularly at the narrow ends) but providing the thermocouples are well spaced around the cell each part of the recorded heat flow (dq_1/dt) can be associated with a certain thermocouple and the heat flow through it. The proportionality constant C in batch cells can be replaced by an effective value C_1 (Wadsö, 1968), which is obtained by calibration. Substitution of C_1 in equation 1.21 and integration gives:

$$Q = \frac{1}{C_1} \int V \, dt \qquad 1.22$$

The total heat evolved is therefore proportional to the area under the voltage-time curve; (Fig. 1.8.a), this may be written as:

$$Q = \varepsilon \times \text{Area} \qquad 1.23$$

where ε is the calibration constant.

The calibration constant is obtained by the application of a measured electric current for a given time through a heater of a known resistance built in the base of the calorimeter cell; the position of this heater is critical (Monk and Wadsö, 1968). The recorded deflection can then be assigned to a known heat output.

To establish the baseline for the heat output in a flow cell, blank solutions are initially passed through the flow cell. The test solution is then pumped through at the same rate, and the output voltage maintained and recorded. For all types of experiments involving continuous flow, the heat output is not equivalent to the area under the voltage-time curve, as it is with batch (calorimetric) method, but may be expressed as:

$$\frac{dQ}{dt} = \varepsilon R_d \qquad 1.24$$

where ε is calibration constant, and R_d μV (chart deflection) the difference between the baseline and the recorded trace. The calibration constant is obtained by flowing a blank solution or killed bacterial suspension through the flow cell, at the same rate as in the experiment and applying a known electrical current for a measured time through the heater (Fig. 1.8.b). The calibration constant is calculated from the known heat supplied and output voltage (deflection) for a given time ($J\ s^{-1}\ \mu V^{-1}$).

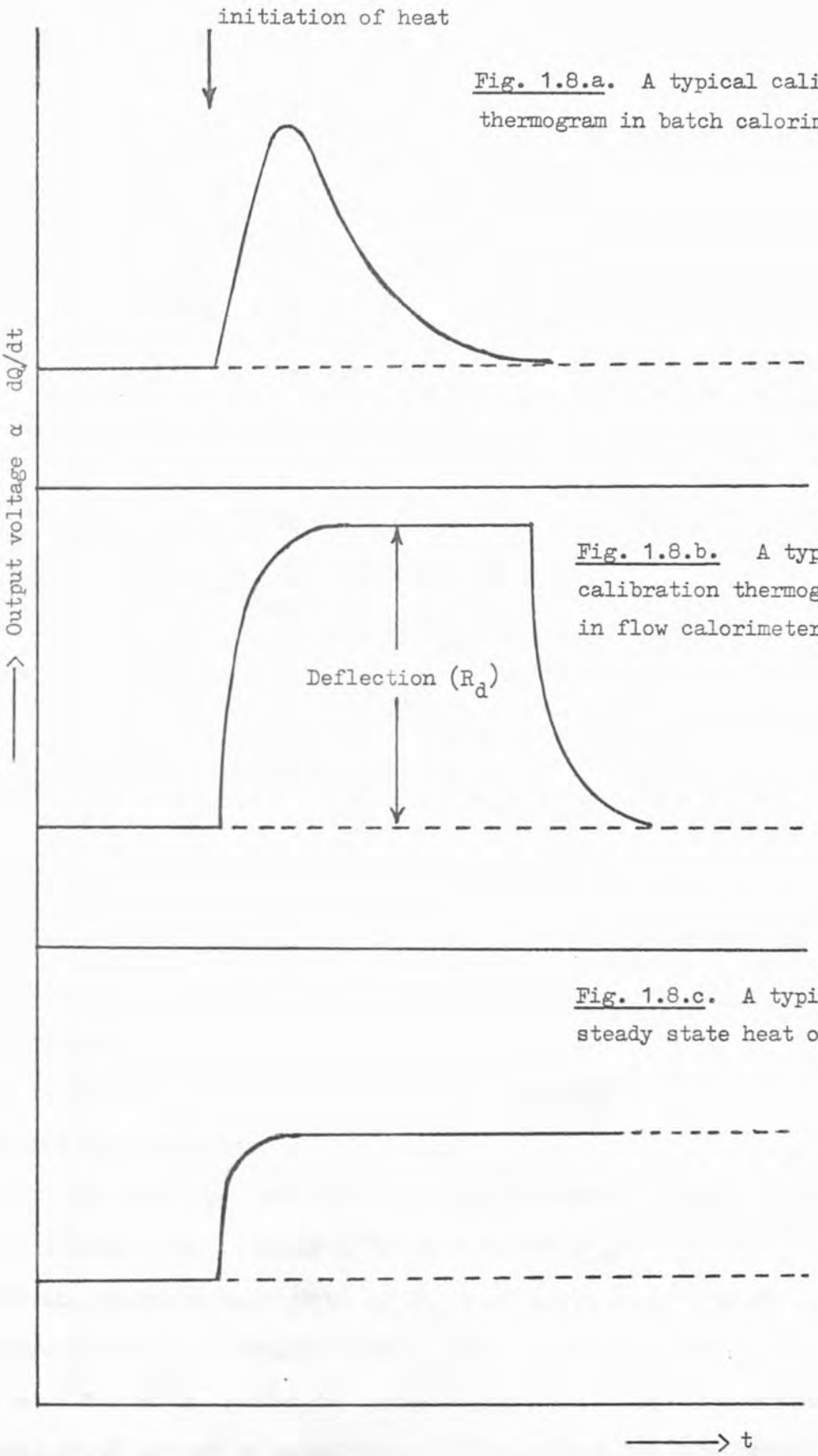


Fig. 1.8.a. A typical calibration thermogram in batch calorimeter.

Fig. 1.8.b. A typical calibration thermogram in flow calorimeter.

Fig. 1.8.c. A typical steady state heat output.

During an experiment involving a flow-cell it is not necessary to pump a reference solution through the reference cell; instead it is most convenient to keep this empty as heat effects associated with the flowing of solution or suspension through the flow cell are allowed for when the baseline is established.

1.4.3.3. Sensitivity of microcalorimeter

On account of the high sensitivity of microcalorimeters, there are some major factors which can affect the recorded heat output; to minimise errors arising from these, great care must be taken with the instrumentation. The most important of these are: thermal or electrical noise associated with instrumentation, interaction of the reaction cell with the solutions, vaporisation of water (in batch microcalorimeter due to leaks), unknown interactions between chemical species in the reaction cell. Viscous heating effects, as solutions are pumped through the microcalorimeter cell, are a particular problem with the flow-cell (Monk and Wadsö, 1968). The magnitude of this effect depends on the diameter of the flow lines, the flow rate and the viscosity of the solutions or suspensions. In bacterial studies, however, this is not a serious problem, since the baseline established with fresh medium at the start of the experiment, and the baseline, in the presence of formaldehyde-killed organisms, at the end of experiment are coincident.

The most important factor is the attainment of temperature equilibrium of the suspension in the heat exchanger coil before entering the microcalorimeter cell. This inevitably increases the flow time (i.e. transit time). At very high flow rates (i.e. $> 100\ cm^3\ h^{-1}$) when the transit time $\Delta t < 1.9$ min temperature equilibrium may not be established, while at very low flow rates

(i.e. < 40 cm^3 h^{-1}) the flow or transit time increases to more than 5 min. This is not suitable for bacterial suspensions, depletion of substrate and oxygen occurs in the lines and in the cell; this means that organisms in the microcalorimeter cell are in a totally different state to those in the fermentor.

In bacterial cultures only populations in excess of 10^7 cells cm^{-3} give out sufficient heat to be recorded by a microcalorimeter. Below this population the heat output of the culture is not detectable (Beezer et al., 1974) thus,with the present instrumentation, microcalorimetry is not applicable to the growth of bacterial cultures at very low populations.

The measure of reproducibility of heat measurements in microcalorimetry is between 0.2 and 2% for "clean" reactions. Electrical calibrations have a precision of better than 0.1% (Evans et al., 1968; Wadsö, 1968). Generally it may be concluded that the limits of error in the microcalorimetric heat measurement of chemical reactions is approximately $\pm$ 1%. Subsequent calculations using this thermal data, involve such quantities as biomass, which are not known so precisely, thus the final overall limits of error are $\pm$ 7%.

1.4.3.4. Microcalorimetric investigation of microorganisms in continuous culture.

The fundamental growth kinetics of the chemostat are well established. However, the thermodynamic equations are not so well catalogued, van Uden (1971) attempted to derive some basic equations for the thermodynamic of the chemstat, and concluded that the enthalpy of formation of one unit mass of cells (Hbi^f) growing in a chemostat under a specific steady state condition could be obtained from;

$$Hbi^f = \frac{\Sigma i\ Fi^s\ Hi^{fs} - \Sigma i\ Fi^p\ Hi^{fp} - K_q}{D} \qquad 1.25$$

where Fi^s, Fi^p are the transfer rates of a substrate and product; Hi^{fs}, Hi^{fp} the enthalpy of formation of one mole of substrate or product in the aqueous solution at its steady state concentration; K_q the specific heat output and D the dilution rate.

van Uden postulated that the flow microcalorimeter could be used to determine K_q and hence, since the other parameters could be calculated, it would be possible to calculate directly the enthalpy of formation of bacterial cells.

The use of a flow microcalorimeter to measure the specific heat output of chemostat cultures, however, presents several problems. When the culture from a chemostat is pumped into a microcalorimeter cell the measured heat output is invariant with time ($dQ/dt = 0$) providing the flow rate to the microcalorimeter is constant and the steady state of the fermentor is maintained.

Bacterial cells leaving the fermentor depart from their steady state, and the measured heat output is thus that of the bacteria respiring in the microcalorimetric cell and not that of cells in the fermentor. Eriksson and Holme (1973) established the problems associated with longer flow times (of the order of several minutes). The chemostat culture is limited by one nutrient, this means that in the flow line between the fermentor and microcalorimeter the concentration of this limiting nutrient will change and may even become zero. The growth rate will thus alter and the steady state of the fermentor no longer exists. In aerobic cultures, changes of oxygen tension in the flow line will intensify these problems. It was concluded that the use of a chemostat/flow microcalorimeter combination was not practical but that the use of a turbidostat

(in which the cell population is controlled) and a flow microcalorimeter could yield useful results.

The combination of turbidostat with a flow microcalorimeter was initially documented by Brettel et al.(1972), for investigating the growth of yeast S. cerevisiae. At dilution rates where glucose was only metabolised by oxidative degradation the specific rate of heat output (Q/x) could be described by:

$$\frac{Q}{x} = 0.26 \frac{J}{hx} + 6.52 \frac{J}{\mu x} \qquad 1.26$$

where J is the measured heat output in time h and μ the growth rate. Q/x increased linearly with the growth rate (Brettel, 1977). The first term in the expression for Q/x is believed to be due to energy of maintenance and the second due to cell growth.

Attempts have been made more recently to overcome the problems of combining a chemostat with a flow microcalorimeter. Cardoso-Duarte et al. (1977), working with a respiration-deficient mutant of S. cerevisiae, proposed a new method for converting heat production rates in the microcalorimetric cell into heat production rates in the fermentor. The rate of decay of glucose (the growth limiting nutrient) was measured at various times, up to a time corresponding to the flow time between fermentor and microcalorimeter, in a separate experiment. Glucose decay was exponential in the flow line and a glucose decay constant, K, was obtained. The rate of glucose consumption, K_c, in the calorimeter cell could be represented by:

$$K_c = V_c K S_{eq} \qquad 1.27$$

where V_c is the volume of the microcalorimeter cell, and S_{eq} the equivalent concentration of glucose (mean of the glucose concentration flowing in and out of the cell) which existed at an equivalent flow time, t_{eq}: this time can be calculated by the following equation;

$$t_{eq} = \frac{1}{K} \ln \left[\frac{V_c K}{f} \left(\frac{1}{e^{-K t_{in}} - e^{-K t_{out}}} \right) \right] \quad 1.28$$

where f is the flow rate between microcalorimeter and fermentor and t_{in} and t_{out} are the flow times from the fermentor to the inlet and exit of the calorimeter cell.

The rate of heat production q_c may be expressed by:

$$q_c = - \Delta H K_c \quad 1.29$$

in which ΔH is the enthalpy change when one mole of glucose is consumed by the population.

The rate of heat production per unit cell volume, q, can be obtained using the equation:

$$\ln q = \ln [- \Delta H K S_o] - K t_{eq} \quad 1.30$$

where S_o is the initial substrate concentration.

Hence by varying the flow rate, f, and plotting ln q against the calculated t_{eq} values a straight line was obtained. ΔH was calculated from the intercept from a knowledge of K and S_o.

The method presented assumes that the various thermodynamic quantities considered have the same value in the microcalorimetric cell as in the fermentor. This, as noted by the authors, is probably incorrect since the population in the fermentor is at a steady state

but that in the microcalorimeter cell is in a transient physiological state. Whether this problem causes a significant error is difficult to establish. The value of ΔH estimated in this way for the respiration deficient mutant of S. cerevisiae growing at $D = 0.079\ h^{-1}$, was -138.5 kJ per mol of glucose consumed. However using a batch calorimeter under anaerobic conditions Beliach et al., (1968) and Battley (1960) have reported values of $\Delta H = -119$ and $\Delta H = -96$ kJ per mol of glucose consumed for S. cerevisiae respectively.

Furthermore the method is strictly applicable only to an organism not requiring oxygen for growth. Aerobic systems cause further problems and the variation of measured heat outputs with oxygen tension would have to be allowed for.

Ackland et al., (1976), working with K. aerogenes in a glucose-limited chemostat, found that, the specific heat output of cultures growing under aerobic conditions, is the same irrespective of the glucose concentration in the inflowing medium, and it is decreased as the dilution rate increased. As noted by the authors, this was an artifact created by cooling effect of the inflowing medium (which caused baseline shift during the experiment).

To date, no one has succeeded in sucessfully combining a flow microcalorimeter with a chemostat of aerobically-respiring organisms.

1.5. Objectives of the project

The objectives of the project were:

1. to construct and operate a chemostat for use with aerobic organisms in combination with a flow microcalorimeter;

2. to investigate the effects of different growth conditions, and variables on the measured heat output of the growing cultures (*K. aerogenes* in glucose-limited chemostat) under steady-state conditions;

3. to study the heat output with other limiting nutrients;

4. to study changes in the steady state parameters caused by the addition of small amounts of different carbon sources, uncouplers, inhibitors, etc;

5. to interpret the results in terms of known metabolic pathways and if possible to attribute the measured heat output to a specific reaction sequence;

6. to determine the maintenance energy for such cultures and to relate it to the maintenance coefficients reported in the literature.

CHAPTER 2

EXPERIMENTAL TECHNIQUES

2.1. Bacteriological

2.1.1. Media and organisms.

The simple synthetic salts medium with an added carbon source, used in this investigation, consisted of KH_2PO_4, $(NH_4)_2\ SO_4$, $Mg\ SO_4$, $7\ H_2O$, with D-glucose, unless otherwise stated. The pH was adjusted to pH 7 by addition of sodium hydroxide solution. The exact quantities of these constituents are given in Table 2.1.

In practice the salts medium was prepared in 10 dm^3 bottles and sterilized at 115°C for 25 min. Glucose or other carbon sources were prepared separately and sterilized by heating at 100°C for 30 min, on three successive days; the required volume was added to the salts medium aseptically to give the desired concentration. Solutions of heat-sensitive substances, e.g. glucose-1-phosphate, glucose-6-phosphate, fructose-1-6, diphosphate ....etc., were prepared in sterile water under aseptic conditions and used in the last experimental run of the chemostat, after which, the whole system was washed and sterilized. Polypropylene glycol was used as an antifoaming agent at a concentration of 0.15 $cm^3\ dm^{-3}$ of medium. Uncouplers or any other reagents were added at required concentrations prior to sterilization, where necessary.

The uncoupling reagents and other substances used were, sodium amytal (Eli Lilly Co. Ltd), nalidixic acid (Winthrop), rotenone and 2-heptyl-4-hydroxyquinoline-N-oxide (Sigma), dicyclohexyl-carbodiimide (Aldrich Chemical), sodium barbitone, sodium azide, 2-4-dinitrophenol, malonic acid, guanidinium chloride, (BDH).

Klebsiella aerogenes (NCTC 418), used in this investigation; was maintained by bimonthly subculture in nutrient broth. The organisms were adapted to new nutrient prior to inoculation into chemostat cultures where necessary.

Table 2.1. Composition of growth media.

Limiting nutrient	glucose /g dm^{-3}	Carbon source other than glucose /g dm^{-3}	$MgSO_4$, $7H_2O$ /g dm^{-3}	KH_2PO_4 /g dm^{-3}	$(NH_4)_2SO_4$ /g dm^{-3}	Na_2SO_4 /g dm^{-3}
Carbon;						
glucose	0.35, 0.72	— — — —	0.04	5.04	1.00	— —
	0.89					
pyruvate	— — —	0.72 free acid	0.04	5.04	1.00	— —
acetate	— — —	0.72 " "	0.04	5.04	1.00	— —
glycerol	— — —	0.72 — — —	0.04	5.04	1.00	— —
Mg^{2+}	1.0	— — — —	0.002, 0.004	5.04	1.00	— —
Nitrogen	0.36, 0.72, 1.0	— — — —	0.04	5.04	0.10	0.93
	2.2, 2.8					
	2.2	— — — —	0.04	5.04	0.05	0.965

pH = 7.0

2.1.2. Sterilizing techniques

All the media and glass-ware were autoclaved for 25 min at 115°C, unless otherwise stated. The in-line tubing system to the microcalorimeter was sterilized by pumping 5% formalin followed by sterile distilled water for about 1 h. Grown cultures were killed by lysol in the waste container.

2.1.3. Continuous culture

The fermentor used was an inverted conical flask (250 cm^3), the base of which carried four port holes with ground glass joints. (14/23). A sintered glass disc was fused on the neck of the flask to disperse the air delivered by the side arm provided. The experimental lay-out and design of the fermentor are shown schematically in Fig. 2.1. and 2.2 respectively. The fresh medium was equilibrated to the growth temperature by passage through a water jacket at 37°C before entering the culture vessel. The medium was pumped by a variable peristaltic pump, d, to a T-piece, r, where it was met by a constant sterile air-flow delivered from a pump, and forced into the culture vessel. The overflow device provided, operated by the same pump, d, was adjusted at a level to maintain a constant working volume of 160 cm^3. As shown in Fig. 2.1., a 10 cm^3 pipette, c, was inserted in the flowline to measure the amount of inflowing medium (i.e. flow rate).

The dilution rate, D, was then calculated by the expression;

$$D = \frac{\text{Rate of flow of fresh medium } (cm^3\ h^{-1})}{\text{Working volume } (160\ cm^3)}$$

The dilution rate is equal to the specific growth rate (μ/h^{-1}).

The culture vessel was inoculated with 10-15 cm^3 of 18 h old cells of K. aerogenes grown in the same medium and aerated at a constant rate of 1.0-1.2 $dm^3\ min^{-1}$ by sterile air dispersed through

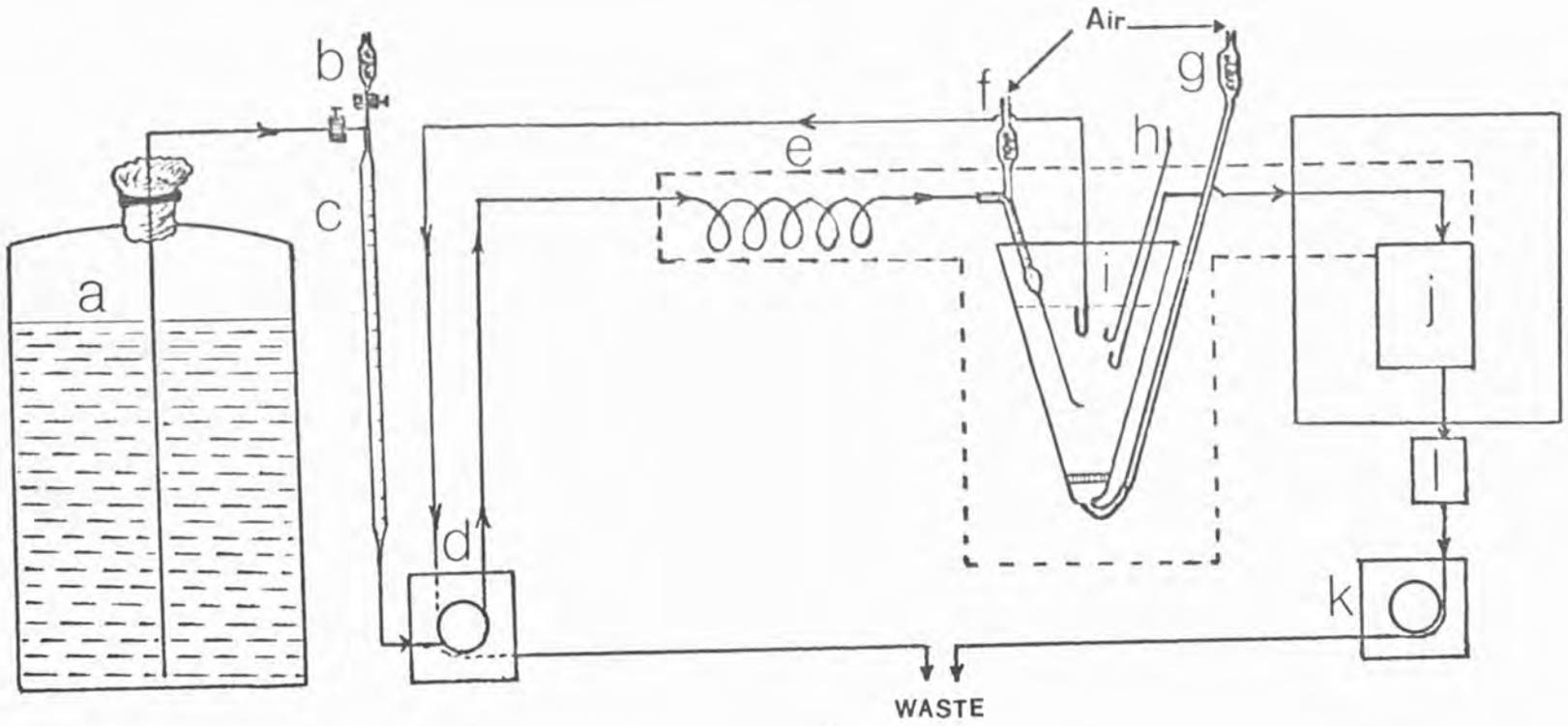


Fig. 2.1: Schematic layout of chemostat and microcalorimeter; a, medium reservoir; b, f and g air filters; c, a 10 cm^3 pipette; d and k peristaltic pumps; e, the heating coil for inflowing medium; h, sampling port; i, culture vessel (fermentor); j, microcalorimeter; l, oxygen electrode. Area within broken line indicates the thermostatted parts.

Fig. 2.2: The fermentor

m, fresh medium inlet; n, overflow system; h and p, sampling port and microcalorimeter connection; g, fermentor air inlet; o, condenser and air outlet; r, T-piece where inflowing medium and air meet; f, air filter; e, the thermostatted jacket for inflowing medium; q, sintered glass disc.

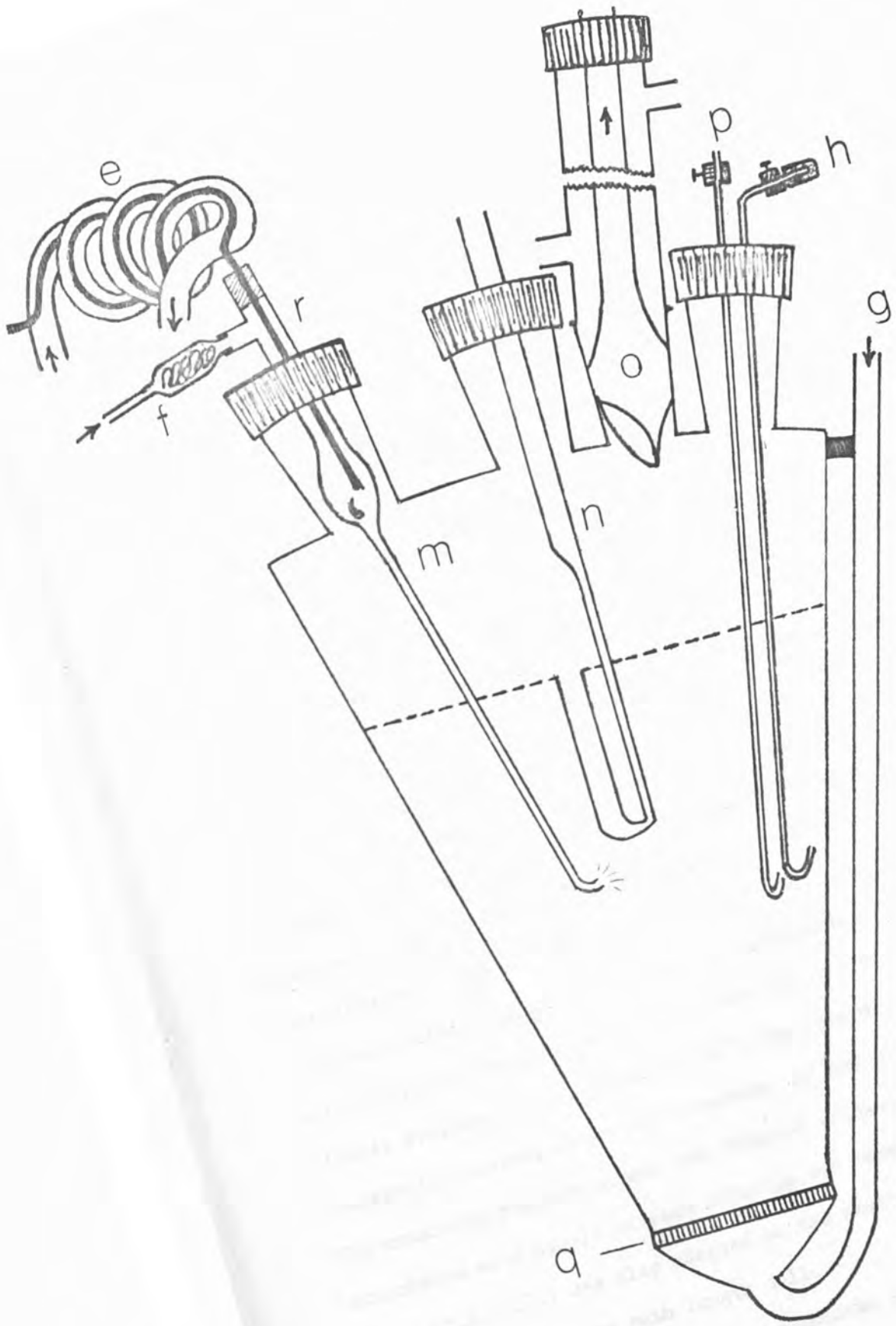


Fig. 2.2

the sintered glass disc. This provided sufficient stirring and aeration of the culture to maintain a high oxygen tension. The air was sterilized by passing through cotton wool filters and saturated before entering the culture vessel. The effluent gas was passed through a condenser, o , at the top of the culture vessel and bubbled into 5% formalin solution. Two to three hours after inoculation, continuous pumping was commenced at the desired dilution rate. Normally, a period of 8-10 h was sufficient for the establishment of the steady state with respect to biomass. However, all sampling and heat measurements were made after about 18 h.

2.1.4. Estimation of biomass.

The bacterial mass was measured spectrophotometrically using a P.E. 124 Spectrophotometer. The absorbance of the bacterial suspension was measured at 625 nm in a 1 cm path length glass cell against a blank of distilled water.

A calibration curve of dry weight of organisms (mg cm^{-3}) against absorbance was constructed. A large volume of culture grown in glucose-limited (4 mmol dm^{-3}) chemostat, at a dilution rate of 0.51 h^{-1}, was collected. The suspension was centrifuged, the cells washed twice with distilled water and finally resuspended in sufficient water to give a suspension of absorbance about 1.2. 10 cm^3 aliquots of this suspension were dried at 100°C for several days in weighed beakers to constant weight to determine the dry weight of the organism. The remaining suspension was then diluted volumetrically and the absorbance of a sample of each dilution was measured; the calibration curve (Fig. 2.3.) was also adapted to the absorbance readings obtained with 0.5 cm path length cell.

These curves apply only to organisms grown, sampled and treated under the stated conditions. Since the shape, size and

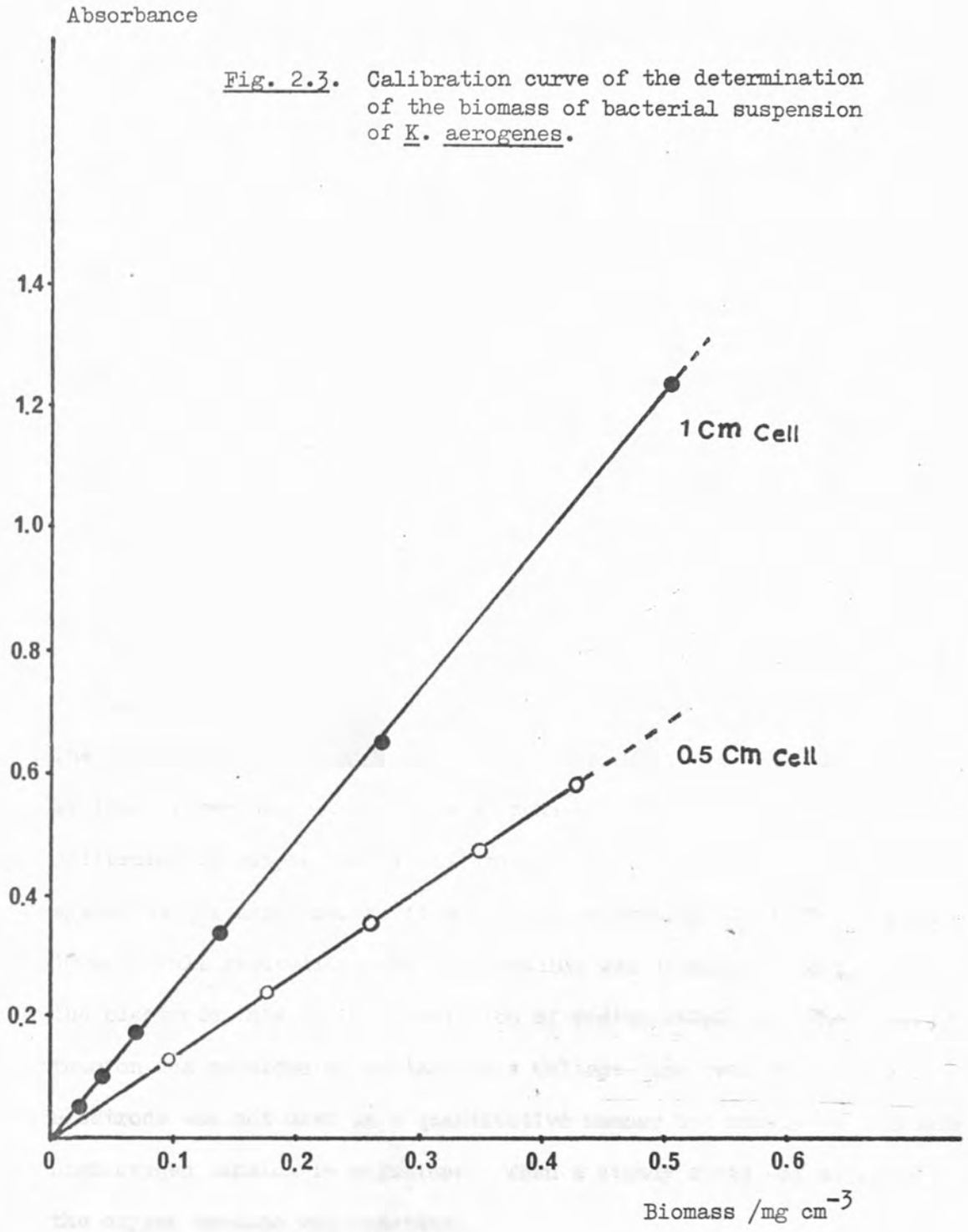


Fig. 2.3. Calibration curve of the determination of the biomass of bacterial suspension of K. aerogenes.

absorbance properties of the cells depend on the culture conditions and the age, the dry weight refers to the number of cells having the same absorbance as the "standard" sample and not necessarily the real dry weight of organisms present. Dean and Rogers (1967) working with K. aerogenes, have shown that, 10^{10} cells, grown under a variety of conditions have an equivalent dry weight of 11.3 - 12.9 mg.

Samples were taken by a syringe from the fermentor and the cells killed by formalin for the measurement of absorbance and hence biomass. It was necessary to cool the samples to the room temperature, because polypropylene glycol present in the growth medium was turbid above 25° C and gave erroneous results.

2.2. Environmental parameters.

2.2.1. Measurement of oxygen tension

The oxygen tension of a growing culture was measured using a flow-type oxygen electrode (process Instrumentation Ltd., Sherbourne) included in the flow-line immediately after the microcalorimeter. The electrode was mounted in as small sheath as possible; this avoided oxygen depletion in the suspension. The electrode was calibrated by pumping water, saturated with air, through the flow system at the experimental flow rate and adjusting the meter to give 100% of full saturation; the zero reading was obtained by dipping the electrode in a 5% (w/v) solution of sodium sulphite. The oxygen tension was recorded as voltage on a voltage-time recorder. This electrode was not used in a quantitative manner but merely to indicate high oxygen tension in solution. When a steady state was attained the oxygen tension was constant.

2.2.2. Measurement of pH

The bacterial suspension was pumped through a small sheath containing a glass electrode/calomel electrode assembly, the pH was measured with an EIL 7020 pH meter (Electronic Instruments Ltd).

2.2.3. Estimation of carbon dioxide

Carbon dioxide evolved during the growth of the organisms, was estimated by an infra-red gas analyser (G.P. Instrumentation, IRGA 120,).

The instrument was calibrated using different concentrations (500, 750, 1000 ppm) of CO_2 in N_2 gas (B.O.C. standards) using a sample of 300 ppm CO_2 in N_2 as reference. This reference was chosen for its close value to the atmospheric CO_2 concentration; air was always used as reference gas in all subsequent experiments. The zero was adjusted when the reference gas was passing through both test and reference cell; the other gas mixtures were then passed through the test cell. The differential reading (in mV) is a linear function of CO_2 concentration (Fig. 2.4). The calibration constant i.e. the slope of the line, was 9.75 ppm per mV.

The flow rate was measured with the flow meter incorporated in the apparatus. The effluent gas from the fermentor was dried by passing through a column of solid $CaCl_2$ and then pumped through the test cell; air from outside the building was pumped through the reference cell. The total volume of effluent air could not be pumped through the analyser since it disturbed the steady-state conditions in the chemostat due to pressure build up in the tubing and analyser. Consequently only a part of the effluent gas was passed through analyser but at the end of the experiment all the gas was passed through the flowmeter to permit the calculation of the total volume of gas passing in the doubling time. The recorded concentration of CO_2 was converted to mmol of CO_2 produced per g

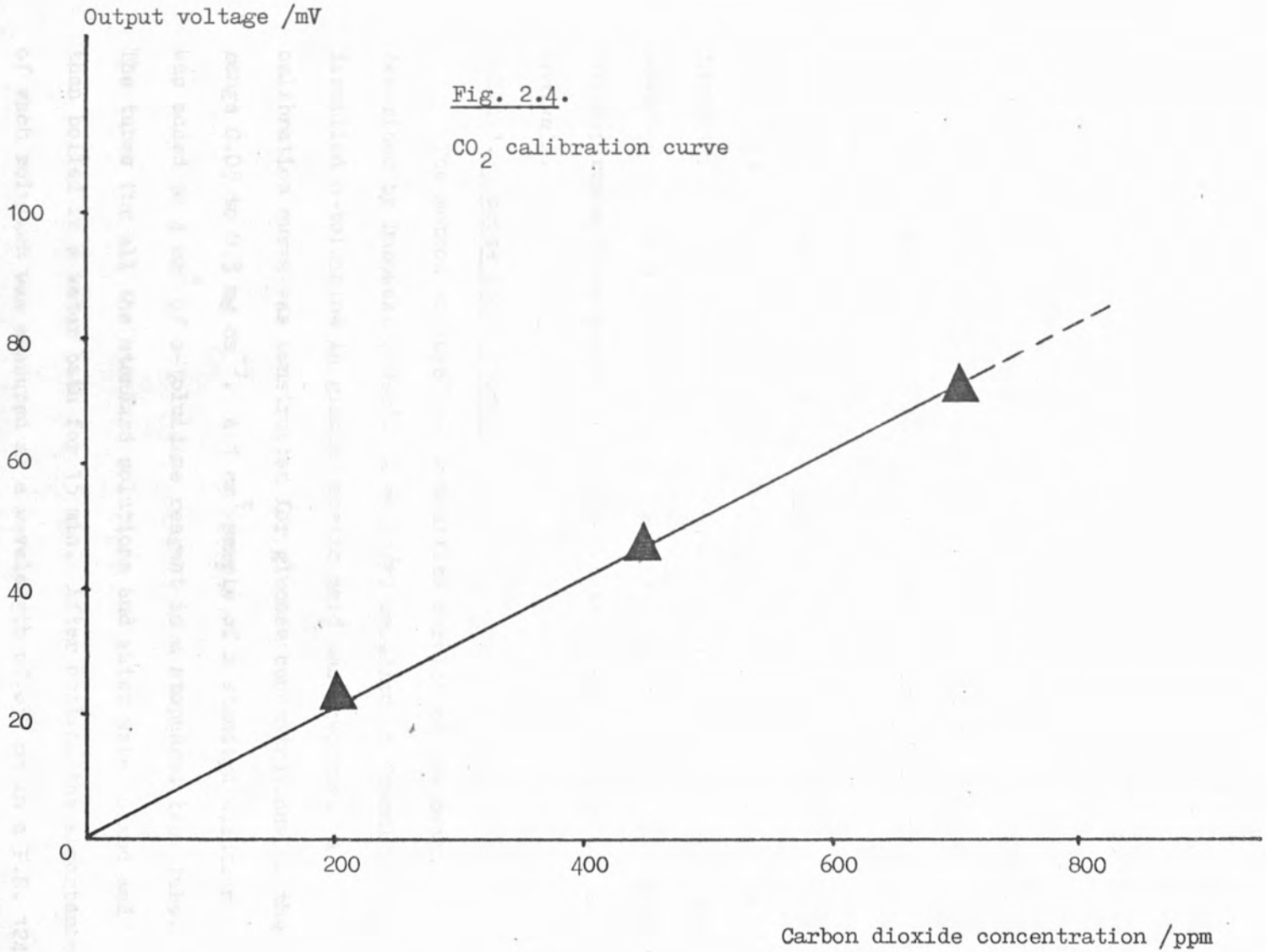


Fig. 2.4.

CO_2 calibration curve

of cells using the following expression:

$$\text{Concentration of } CO_2 \text{ /mmol } CO_2 \text{ (g cell)}^{-1} = \frac{R\ C\ f_a\ t_d}{10^6 \times V_f \times m \times 24.2} \qquad 2.1.$$

where, $f/dm^3\ h^{-1}$ the rate of air flow; t_d/h the doubling time; C the calibration constant, R/mV the chart reading; V_f/cm^3, fermentor volume (normally 160 cm^3); $m/g\ cm^{-3}$ the biomass, the gram molecular volume at 22° C = 24.2 dm^3. The largest source of error in this estimation was in the knowledge of the total volume of air passing through the flow-meter during the period of doubling time (± 10%) and from the temperature change during the passage of air through the fermentor and subsequently to and through the analyser.

2.2.4. Chemical analysis of supernatant

Samples were collected aseptically with a syringe from the fermentor, and immediately (5–10 s) filtered through a cellulose acetate membrane (grade 0.22 nm, Oxoid) on a water pump to remove the cells. The supernatant was stored in a deep-freeze at -20° C for chemical analysis.

2.2.4.1. Glucose concentration

The method employed was a modified version of the method described by Dubowski (1962). A 6% (v/v) solution of freshly distilled o-toluidine in glacial acetic acid was prepared. A calibration curve was constructed for glucose concentrations in the range 0.05 to 0.3 mg cm^{-3}. A 1 cm^3 sample of a standard solution was added to 4 cm^3 of o-toluidine reagent in a stoppered test tube. The tubes for all the standard solutions and water were mixed and then boiled in a water bath for 15 min. After cooling the absorbance of each solution was measured at a wavelength of 630 nm in a P.E. 124 spectrophotometer, against the blank of distilled water which had

been similarly treated. A typical calibration curve is shown in Fig. 2.5. It was necessary to re-establish the calibration curve for every set of analyses, because the o-toluidine reagent darkened with age.

Test samples (1 cm^3) were treated as standard samples. The glucose concentration of the samples could be estimated with an accuracy of $\pm$ 0.03 mg cm^{-3}.

2.2.4.2. Acetate concentration

The determination of volatile fatty acids (VFA) has been investigated by different methods, and gas liquid chromatography (GLC) has been found to be a reliable and sensitive technique. Some investigators have employed extraction prior to GLC examination (Kevim et al., 1975; Carlsson, 1973; Hauser, 1975). Direct analysis of aqueous culture supernatant fluids has been successfully made by Rogosa et al.(1968), for the quantitative separation of lower fatty acids (C_2 - C_6).

In the determination of acetate in plasma by GLC, using a porous polymer stationary phase, reported by Laker et al. (1978), samples were mixed with an internal standard (propionic acid). It was found that the area ratio (acetate/propionate) was a linear function of the acetate concentration.

A modified version of Rogosa's method was adopted for the estimation of acetate in the culture media. A Pye-Unicam (Series 104) gas chromotograph was used. The electrical response of the hydrogen flame detector was recorded on a potential-time recorder (Philips, PM 8000). A 6 ft. x 4 mm (ID) glass column was packed with 10% SP - 1200, 1% H_3PO4 on 80/100 chromosorb WAW (supplied by Supelco Inc). It was conditioned for a period of 24h, at 190° C in a stream of oxygen-free nitrogen.

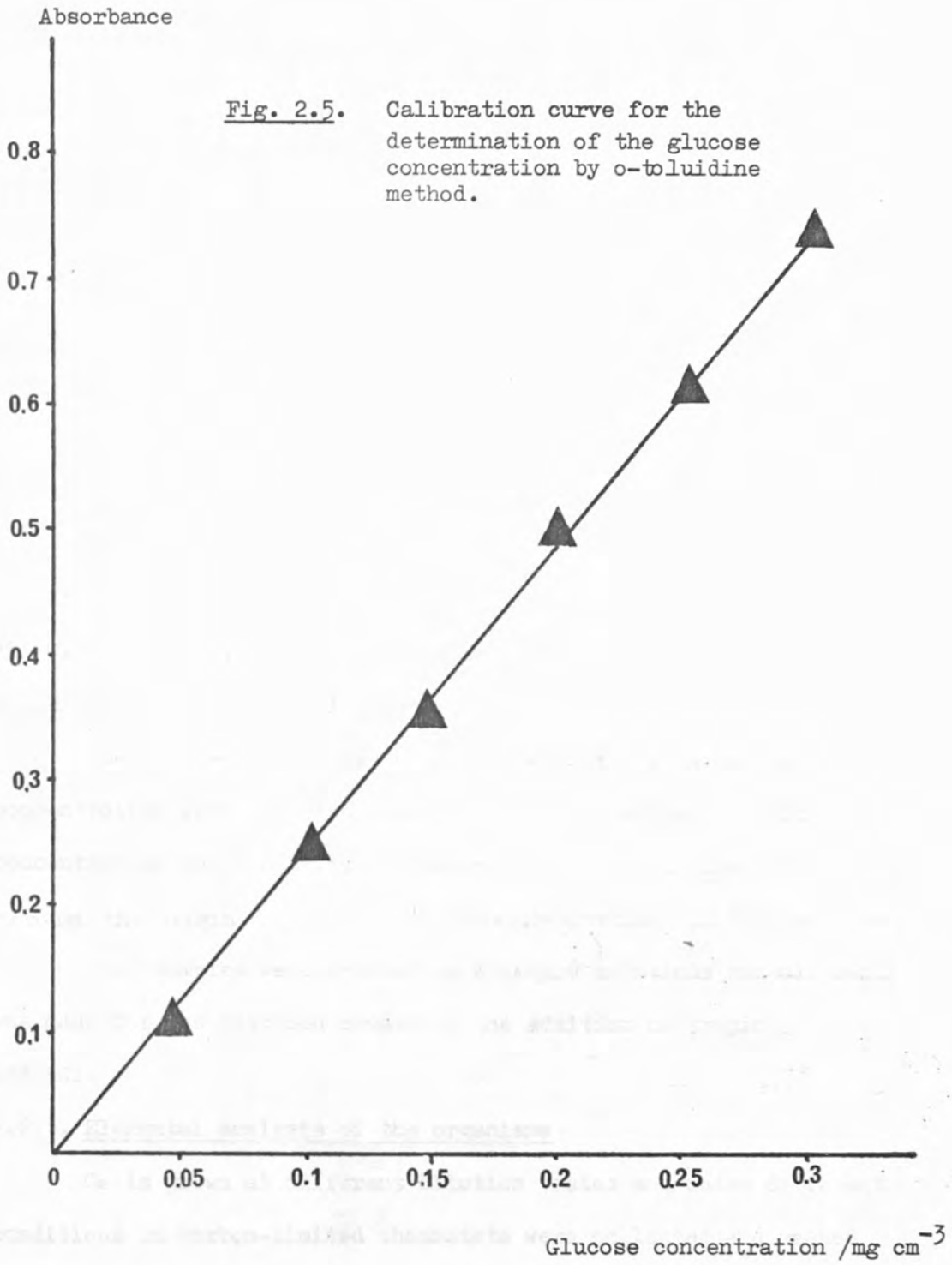


Fig. 2.5. Calibration curve for the determination of the glucose concentration by o-toluidine method.

The standard conditions established were; the flow rate of the carrier gas (N_2) 28 cm^3 min^{-1}, for air and hydrogen 370 and 40 cm^3 min^{-1} respectively; oven temperature of 117° C, and attenuation of 200 - 2000 times.

Standard solutions of sodium acetate (3.12 - 25 mmol dm^{-3} as free acetic acid) were prepared containing propionic acid as an internal standard at a fixed concentration of 26 mmol dm^{-3}; the pH was adjusted to 1.2 - 1.5, by adding concentrated HCl; allowance was made for the dilution caused by the addition of HCl. A 1 nm^3 sample from each solution was injected by a 5 nm^3 syringe (with a 7 cm needle) into the injection port of the chromatograph. The acetic acid comes off the column before the propionic acid (Fig. 2.6).

A calibration curve was constructed by plotting the peak height ratio of acetic acid/propionic acid against acetate concentration, this was linear from 0 to 14 mmol dm^{-3} acetate (Fig. 2.7) with a coefficient variation of less than 2%.

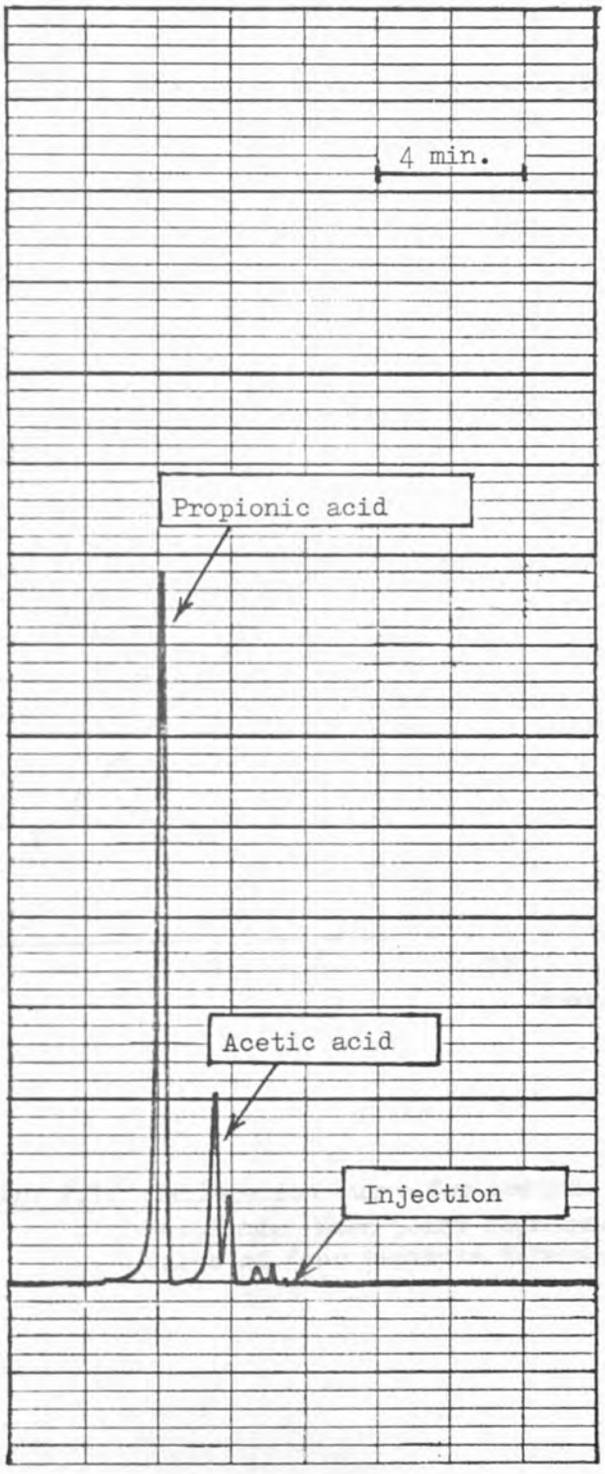
When different volumes (0.5 - 5 nm^3) of a given acetate concentration were injected instead of a fixed volume of different concentration the linearity was maintained, but the line did not pass through the origin. This supports the observations of Rogosa (1968).

Test samples were treated as standard solutions and allowance was made for the dilution caused by the addition of propionic acid and HCl.

2.2.5. Elemental analysis of the organisms

Cells grown at different dilution rates and under different conditions in carbon-limited chemostats were collected and washed twice with distilled water and dried at 90-100° C for 24 h. The dried cells were analysed for C, H and N; the oxygen was obtained by difference.

Fig. 2.6. Typical chromatogram



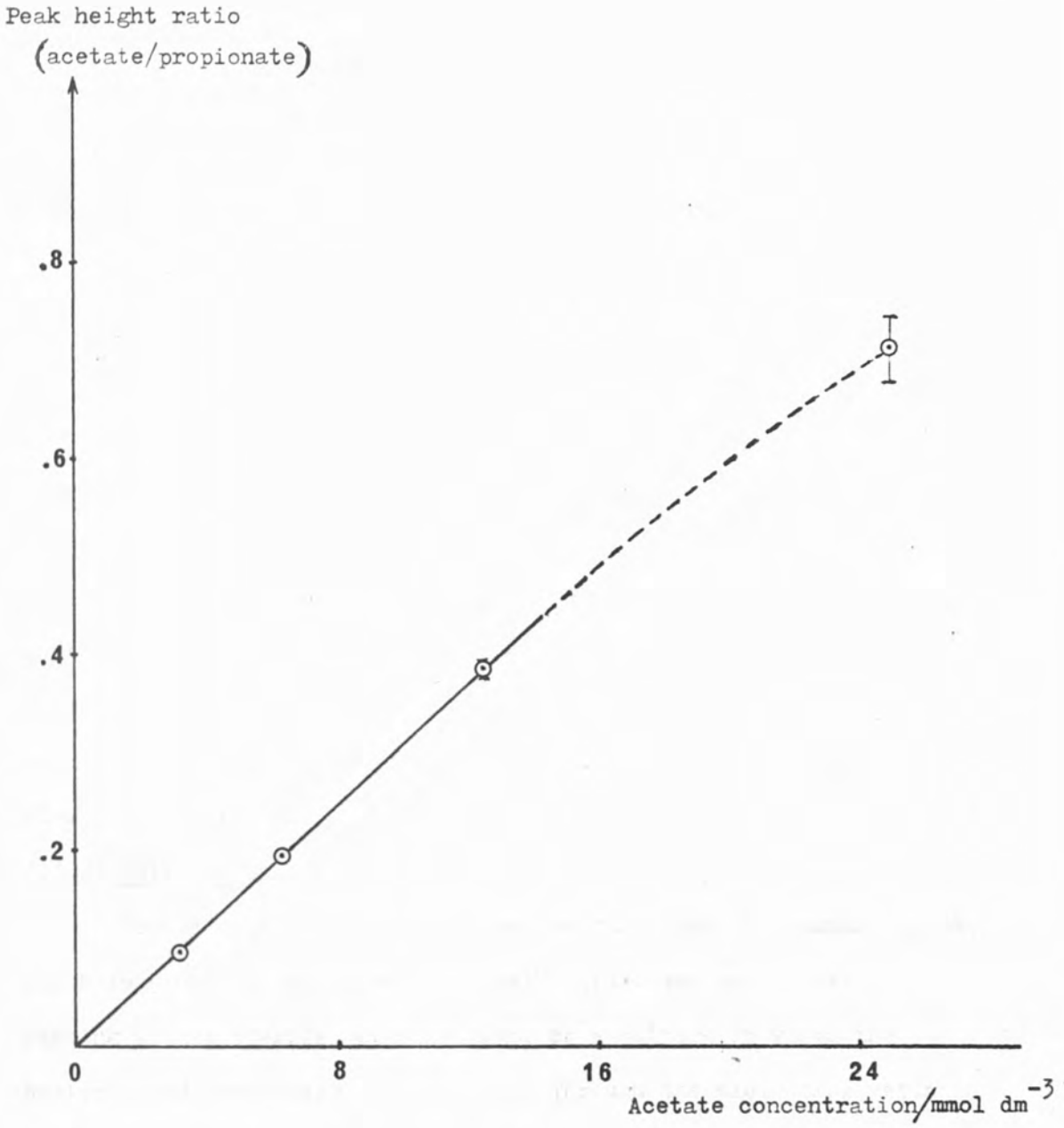


Fig. 2.7. Calibration curve for acetate in aqueous solution. Each point represents the average value of four separate determinations.

From several analyses the average percentages of the elements were; C = 46.0 H = 6.76, N = 12.2, O = 29.12. There was a residue of 6.11%. From these values an empirical formula of $CH_{1.72}\ O_{0.47}\ N_{0.23}$ was obtained which is in good agreement with $CH_{1.73}\ O_{0.43}\ N_{0.24}$ reported by Herbert (1977) for cells of K. aerogenes grown in a glycerol-limited chemostat at a dilution rate of 0.85 h^{-1}. The only significant difference is for oxygen which also includes the P, S content of the cells (not measured in the present work).

2.3. Thermal measurements

2.3.1. The apparatus

An LKB 10700/1 flow microcalorimeter was used. The microcalorimeter was fitted with an aerobic steel cell (Fig. 1.7c) of volume of 1.18 cm^3, which eliminates thermal fluctuations associated with the passage of air bubbles through a conventional flow cell (Monk et al.,1968).

2.3.2. Calibration of response

The calorimeter response, i.e. voltage from the thermocouples which is proportional to rate of heat output, was calibrated by passing either sterile water, medium, or a culture in which the bacteria had been killed by formalin, through the microcalorimeter cell under the same experimental conditions as used for test, and applying a current of 2 mA for 30 min to the internal heater to produce a steady heat output which was recorded (Fig. 1.8b). The electrical heat was calculated from the expression RI^2t, where R = 50 ohms, I = 2 mA, and t = 30 min. (i.e. heat = 0.36 J). Using the equation:

$$\text{Rate of heat output} = \frac{dQ}{dt} = \varepsilon R_d$$

where ε is the calibration constant and R_d is chart deflection, therefore;

$$\varepsilon R_d = 0.36 \text{ (J)}$$

$$\therefore \varepsilon = \frac{0.36}{R_d}$$

Since this value is obtained for 1800 s, dividing this value by 1800, gives, ε, in the units of rate of heat out-put per unit chart deflection, i.e. J s^{-1} per 1μV.

For a given flow rate ε was the same for sterile water, medium and killed culture, but varied linearly with the flow rate (Fig. 2.8) according to the equation:

$$\varepsilon = \varepsilon_o + k_\varepsilon f$$

where ε_o is the intercept (1.45×10^{-5} J s^{-1} μV^{-1}) f/cm^3 h^{-1}, the flow rate and k_ε a constant. This variation of ε is supported by the work of Monk et al. (1968).

2.3.3. Measurement of heat output

The environmental temperature to measure the heat output accompanying the growth of K. aerogenes was chosen as 37° C, unless otherwise stated.

Before any measurements were made, the calorimeter cell and the inline polypropylene tubing (1mm id) were sterilized by passing through 5% (v/v) formalin solution and then sterile distilled water for about an hour. On the establishment of the baseline and its maintenance for at least 1h, the connecting tubing was transferred aseptically to the chemostat vessel. Since the equipment was not in a constant temperature room, the connecting tubing, which was kept as short as possible was passed through a water jacket maintained at the growth temperature (Fig. 2.1.). For a fixed flow rate through the microcalorimeter, the position of the base-line was independent

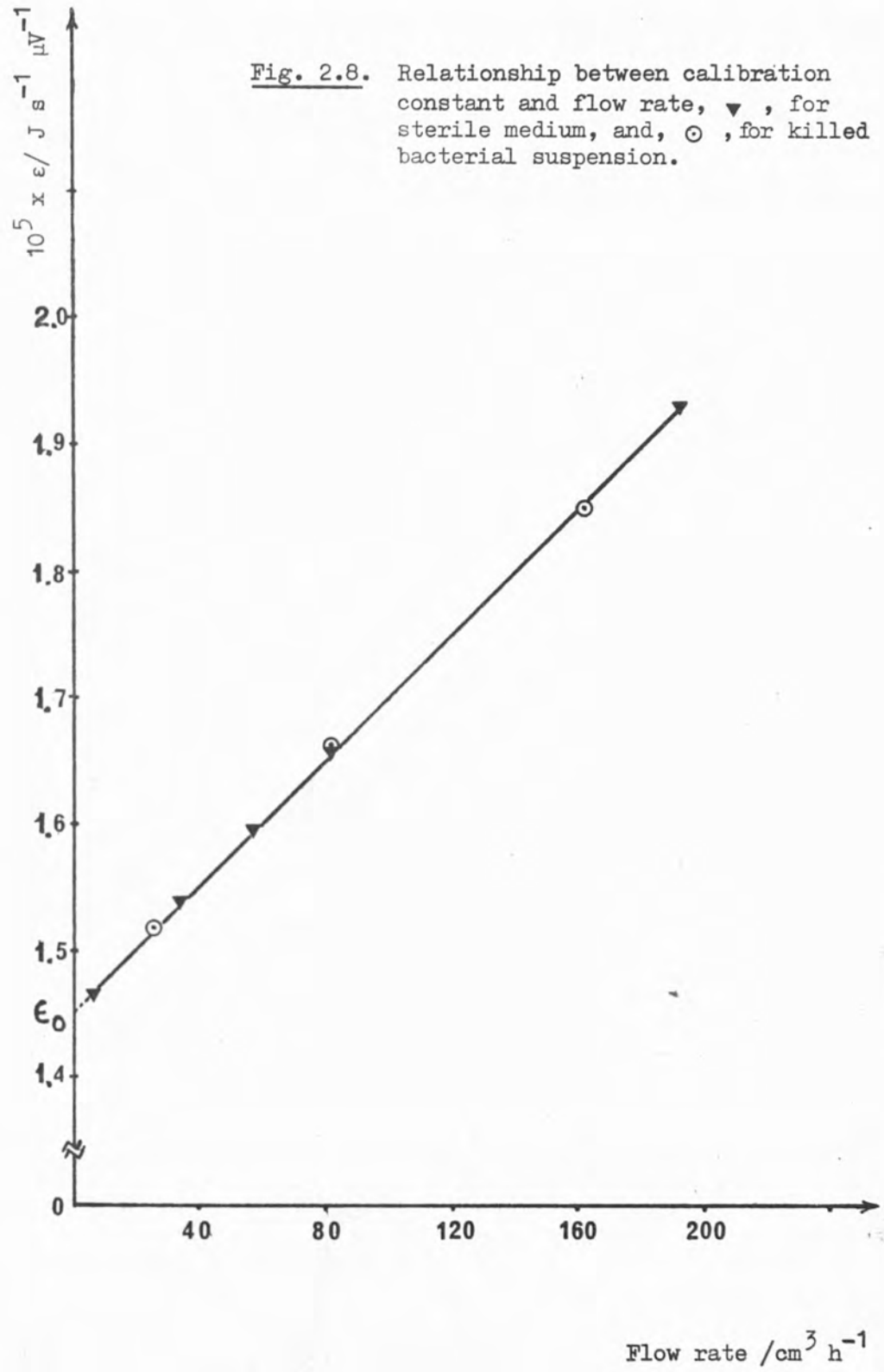


Fig. 2.8. Relationship between calibration constant and flow rate, ▼ , for sterile medium, and, ⊙ , for killed bacterial suspension.

of the dilution rate (i.e. the rate of inflowing medium), and a steady baseline could be maintained for periods in excess of 24h for a given set of conditions (i.e. D = 0.25 - 1.0 h^{-1}, and flow rate f = 10 - 100 cm^3 h^{-1}). Previous work in this laboratory (Ackland et al., 1976) failed due to base-line drift caused by the cooling effect of the inflowing growth medium, into the fermentor and hence into the microcalorimeter cell. Such effects were eliminated in the present work by an efficient pre-heating system of the medium immediately before entering the culture vessel.

The growing culture was pumped from the chemostat (Fig 2.1. pump K,) through the tubing to the microcalorimeter cell. The heat evolved by the cells was recorded as a volt/time trace (50 μV full scale deflection), for some hours to ensure a constant steady-state heat-flow established in the microcalorimeter. This electrical response was converted to the rate of heat output by multiplying by the appropriate calibration constant.

CHAPTER 3

EXPERIMENTAL RESULTS

Establishment of standard procedure for measurement of heat output and environmental parameters.

3.1. Development of chemostat and its combination with microcalorimeter.

3.1.1. The design of the fermentor.

The fermentor used in the preliminary experiments was an inverted conical flask (250 cm^3) with a sintered glass disc fused in the neck. The neck of the flask was drawn out and attached to an 8 mm bore glass tube, through which was pumped sterile air (1.0 - 1.2 dm^3 min^{-1}). A thick-walled capillary tube was blown into the side of the flask to provide the overflow from the fermentor. The base of the flask carried four portholes. Fresh medium was pumped into the fermentor via the central porthole which was sealed with a rubber teat. The other portholes were sealed with cotton wool and used for taking samples for measurement of biomass, substrates and pH and for an oxygen electrode.

The gradual condensation of water vapour on the cotton wool (in the portholes) caused a pressure build up and this decreased the working volume of the fermentor, by pushing the culture out of the fermentor through the overflow. This considerable change in the working volume of the fermentor, did not allow the maintenance of steady-state growing culture. Even when the steady-state conditions were maintained by frequent changing of these cotton wool plugs, the biomass was much higher than expected, due to evaporation. Another problem associated with wet cotton wool plugs was the possibility of contamination of the culture.

These difficulties were overcome in a new design of fermentor which carried four portholes now with ground glass joints (14/23). A water cooled (10 - 15° C) condenser mounted on to one of these portholes allowed the escape of effluent air with the condensation of water vapour. This eliminated the problems encountered with evaporation.

The fresh medium was delivered to a specially blown Pasteur pipette (Fig 2.2.) fixed on a glass joint. When the medium arrived in the break bulb, it was forced into the culture vessel by a constant flow of sterile air. This constant air flow had an important role in preventing blockage of the Pasteur pipette with the growing organisms and hence infection of inflowing medium. The side arm (overflow device) was discarded and replaced by a J-shaped glass tube inserted through the centre porthole; the position and height of this were adjusted to give a working volume of 160 cm^3. Surplus culture was pumped from this J-tube with the same pump as that used for the inflowing growth medium. The internal diameter of the over-flow tubing in the pump was larger than that of the inflowing tubing to ensure a steady working volume. (Figs. 2.1. and 2.2). The other portholes carried two lengths of stainless steel tubing, one was used for sampling the culture and the other for connection to the micro-calorimeter.

3.1.2. Coupling of the chemostat to the microcalorimeter

The connecting tubing (polypropylene, 1 mm diameter) between the microcalorimeter and the fermentor, and between the medium reservoir and the chemostat were kept as short as possible.

The fresh medium, stored in a 10 dm^3 bottle, was equilibrated to the growth temperature by passage through a water jacket at 37° C, before entering the culture vessel. In this way difficulties encountered previously due to cooling effects of the medium were eliminated (Ackland, Prichard and James, 1976), and a steady baseline could be maintained for periods in excess of 24 h at any dilution rate (0.25 to 1.0 h^{-1}) and at any flow rate (40 - 100 cm^3 h^{-1}).

The medium was pumped by means of a variable speed peristaltic pump to a T-piece where it met a constant flow of sterile air (in the medium break bulb, Fig. 2.2.) and was forced into the culture vessel through the specially blown Pasteur pipette.

The microcalorimeter cell and the in-line tubing were sterilized by passing 5% formalin and sterile water through for an hour; the base-line was established by pumping sterile distilled water for at least an hour at the required flow rate. The connecting tubing to the microcalorimeter was then transferred aseptically to the fermentor and cells of K. aerogenes growing in the chemostat in their steady state, were pumped into the microcalorimeter cell at the same flow rate as used for establishing the baseline.

Recycling of the culture (after passing through the microcalorimeter cell and pH and oxygen electrode assemblies) disturbed the steady state condition in the chemostat and this, in turn, produced erratic changes in the heat output. It was therefore essential that the culture should pass to waste after the microcalorimeter.

In the preliminary experiments the connecting tubing between fermentor and microcalorimeter was not thermostatted. Since the equipment was not in a constant temperature room. The bacterial suspension cooled down to room temperature in the connecting tubing and entered the microcalorimeter at this lower temperature (18 - 25^{o} C). At very low flow rates this cooling had no effect on thermal measurements because the temperature equilibrium was established in the heat exchange coils in the microcalorimeter. Such flow rates (below 40 $cm^3\ h^{-1}$) were not suitable for studies of growing bacterial suspensions because of substrate and oxygen

depletion in the flowline and in the cell. High flow rates (80 - 100 cm^3 h^{-1}) which were more suitable for the bacterial suspensions, created problems which were reflected in irreproducible heat responses. The problems were due to the non-attainment of temperature equilibrium of the suspension before entering the calorimeter cell. To overcome these problems associated with temperature equilibrium, the bacterial suspension was kept at 37.0$\pm$ 0.5° C, through the connecting tubing between microcalorimeter and the fermentor by passing it through a thermostatted water jacket, maintained at 37° C.

In consequence the technique finally adopted was to pump the fresh medium, equilibrated to the growth temperature, into the culture vessel at the required rate. The bacterial suspension was then pumped to the microcalorimeter through the thermostatted (37.0$\pm$ 0.5°C) connecting tubing which was also kept as short as possible. Finally the organisms leaving the microcalorimeter cell passed through the oxygen and pH electrode assemblies to waste.

The final design of the fermentor/chemostat assembly (Fig. 2.1. and 2.2) and standard technique improved the reproducibility for the rate of heat output. Over a period of more than a year with different chemostat cultures in limiting glucose medium the day to day reproducibility was better than $\pm$ 7%.

3.2. Heat output of chemostat culture

When the growing culture from the chemostat in the steady state, was pumped at a constant flow rate through the microcalorimeter cell in place of the sterile medium, there was an immediate increase in the rate of heat output from the baseline to a constant value.

The steady-state heat output was maintained until the dilution rate was changed, an increase (decrease) in dilution rate (i.e. specific growth rate) produced an increase (decrease) in heat output, which lasted for a few hours until the organisms regulated their metabolism to the new conditions when the original rate of heat output was reestablished. At low values of D, when the biomass decreased there was a reduction in the rate of heat output. Thus it was apparent that the rate of heat evolved depended on the biomass. When formalin was added to the culture the rate of heat output decreased and the baseline was reestablished. When sterile medium was pumped instead of the culture the baseline was reattained (Fig. 3.1.).

The heat output for a given set of conditions was recorded only after steady-state conditions had been maintained for several hours; over a period of more than a year this output was very reproducible for different chemostat cultures. From replicate experiments it was found that for cells growing in 4 mmol dm^{-3} glucose, D = 0.65 to 0.85 h^{-1}, and at a pump rate of 80 cm^3 h^{-1}, the mean value of the rate of heat output (with 95% confidence levels) = $1.84 \pm 0.13 \times 10^{-4}$ J s^{-1} (W) per 1.18 cm^3 of culture and the biomass = 0.372 ± 0.015 mg cm^{-3}.

The measured heat output depended on such factors as glucose concentration and hence biomass, dilution rate, and pump rate. To assess the effect of such factors, the measured rate of heat output must be converted to a quantity which is comparable for different conditions of growth and test. The quantity chosen was the specific heat output, $\Delta H'_p$, the heat evolved during the formation of 1g of cells during the time of 1 generation.

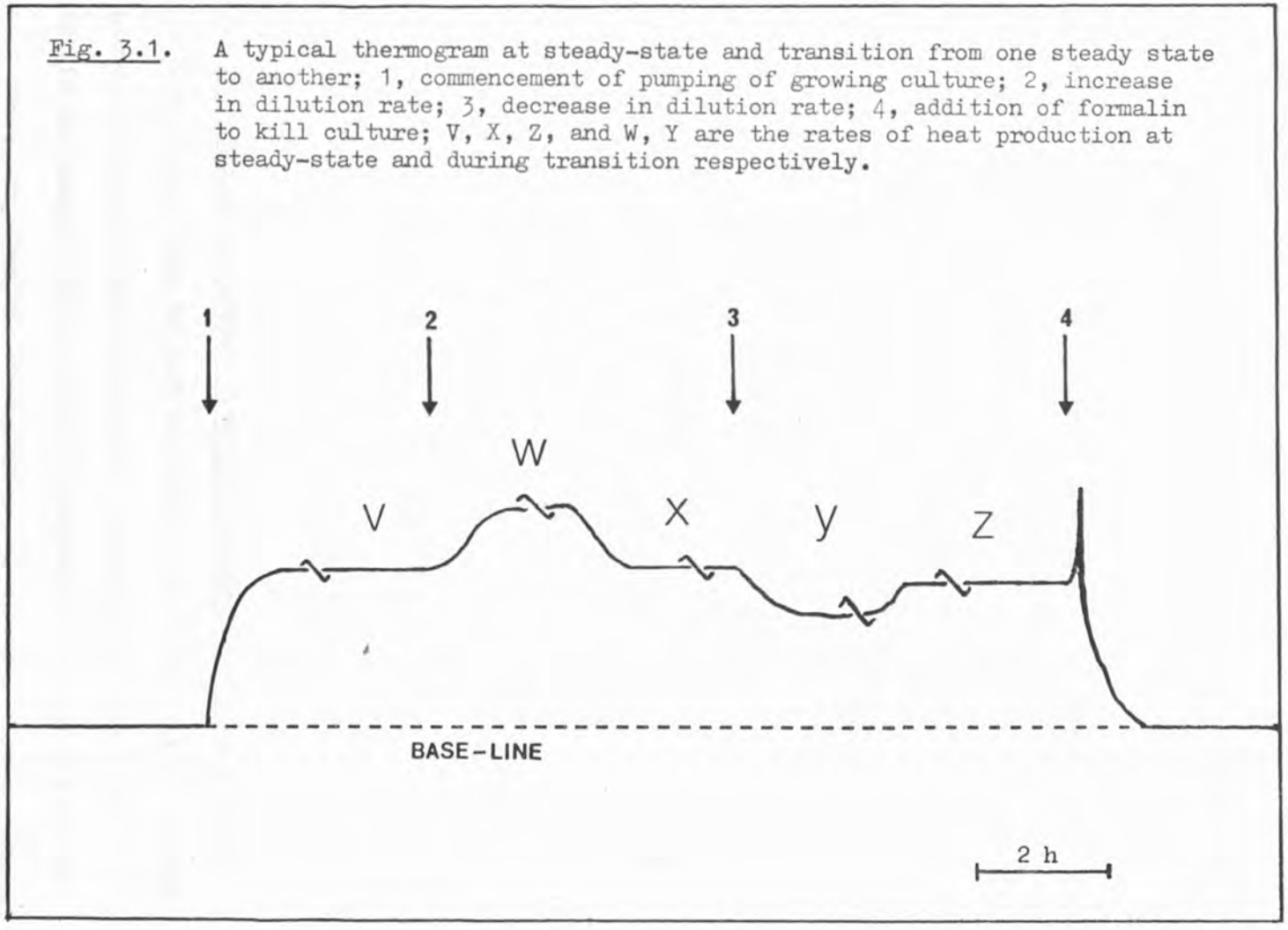


Fig. 3.1. A typical thermogram at steady-state and transition from one steady state to another; 1, commencement of pumping of growing culture; 2, increase in dilution rate; 3, decrease in dilution rate; 4, addition of formalin to kill culture; V, X, Z, and W, Y are the rates of heat production at steady-state and during transition respectively.

The recorded voltage was first converted to the rate of heat output per unit volume of suspension by multiplying by the appropriate calibration constant (section 2.3.2), and dividing by the volume of microcalorimeter cell, this gives the rate of heat output per cm^3 of bacterial suspension (i.e. J cm^{-3} s^{-1} or W cm^{-3}).

$$\frac{dQ}{dt} = \varepsilon R_d$$

$$\therefore \quad \text{Rate of heat output per unit volume} = \frac{\varepsilon R_d}{V_{cell}} \qquad 3.1$$

where ε is the calibration constant, $R_d/\mu V$, the chart deflection, V_{cell}, the volume of the microcalorimeter cell (1.18 cm^3).

If m g cm^{-3} is the biomass in the calorimeter cell then:

$$\text{the rate of heat output per } g \text{ cells} = \frac{\varepsilon R_d}{V_{cell}\ m}$$

If the doubling time, t_d $\left(= \frac{\ln 2}{D} \right)$ is the standard time for measurement, then the heat output per g of cells is given by

$$\Delta H'_p = \frac{\varepsilon R_d t_d}{V_{cell}\ m}$$

$$= \frac{\varepsilon R_d \ln 2}{V_{cell}\ m D} \qquad 3.2$$

3.3. The effects of transit time (Δt) on the rate of heat output:

The rate of heat production during the growth of K. aerogenes in glucose-limited medium (4 mmol dm^{-3}) at different dilution rates (in the range 0.65 to 0.85 h^{-1}) was measured after different transit times, Δt, from the chemostat to the microcalorimeter, where Δt is defined by the expression:

$$\Delta t = \frac{t_{in} + t_{out}}{2} \qquad 3.3$$

t_{in} and t_{out} are the flow times from the chemostat to the entrance and to the exit of the calorimeter cell respectively. This was achieved by varying either the flow rate or the length of tubing between the microcalorimeter cell and the chemostat.

The flow rate through the microcalorimeter cell could not exceed the dilution rate, otherwise the culture was completely pumped out of the chemostat to waste. The flowing culture was not recycled since this disturbed the steady state and affected the biomass and heat output. This inevitably meant that thermal measurements could not be achieved in the apparatus available at low dilution rates ($<0.25\ h^{-1}$). The rate of heat output increased linearly with decreasing values of Δt (Fig. 3.2), extrapolation to $\Delta t = 0$ gives a value for the rate of heat production in the chemostat as 4.8×10^{-4} kJ s^{-1} (g cell)$^{-1}$. This graph was used in all subsequent calculations to convert the recorded rate of heat output in the calorimeter to that in the fermentor. Where possible a flow rate of 80 $cm^3\ h^{-1}$ was used; this minimized the extrapolation.

In summary, the specific heat output in the fermentor per g cell produced, ΔH_p, was calculated using the expression;

$$\Delta H_p = \Delta H'_p + C\ \Delta t\ t_d \qquad 3.4$$

where, $\Delta H'_p$, is the recorded specific heat output, $C = 3.60 \times 10^{-5}$ kJ s^{-1} (g cell)$^{-1}$ per min (Fig 3.2).

3.4. Standard conditions of test

As a result of the preliminary experiments a standard set of conditions was chosen for all subsequent experiments.

Cells of K. aerogenes were grown in glucose-limited chemostat, (4 mmol dm^{-3}) at pH = 7, an environmental temperature of 37.0°,

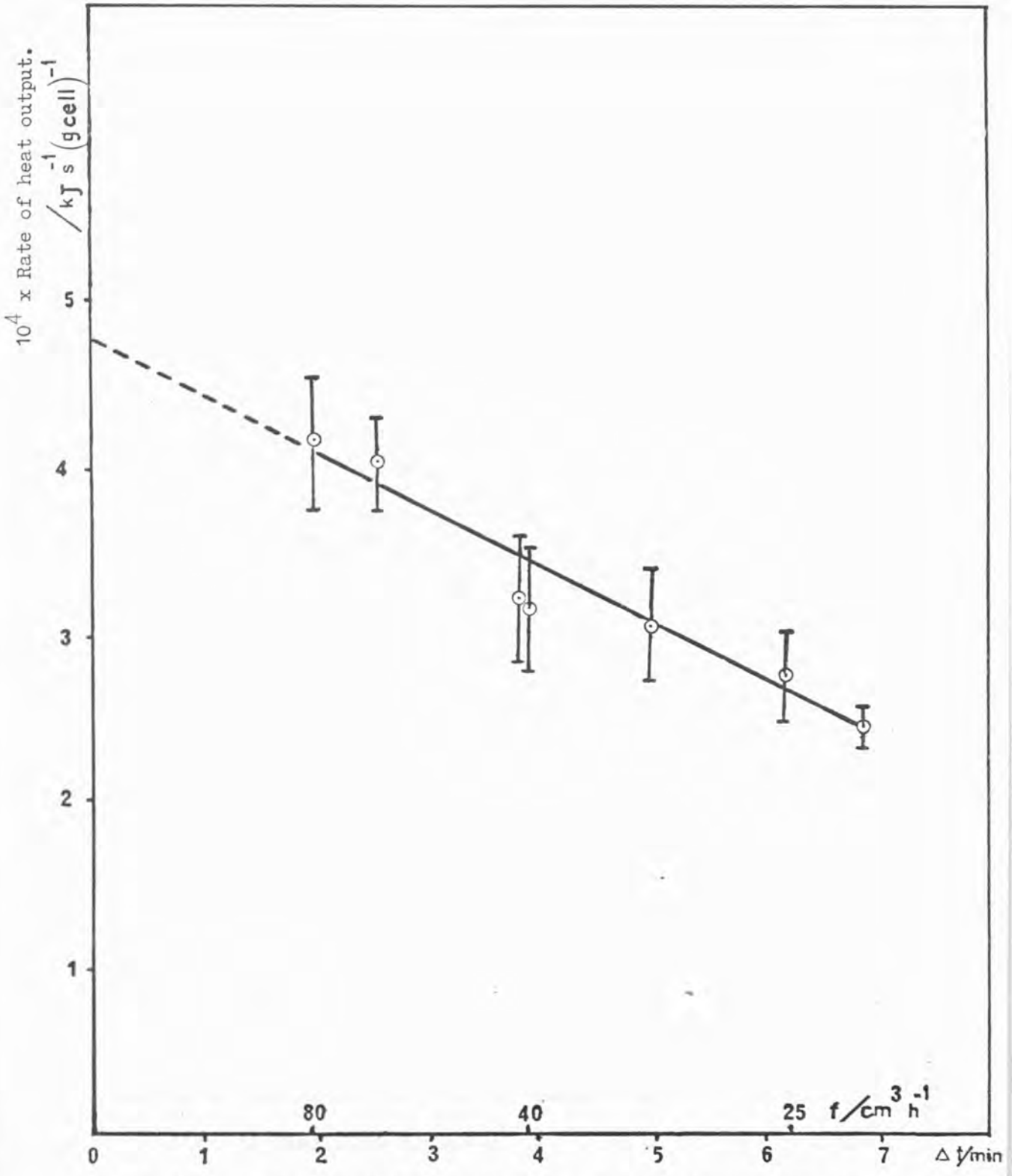


Fig. 3.2. The rate of heat production as a function of Δt, of K. aerogenes in standard glucose-limited chemostat at different dilution rates (0.65 - 0.85 h^{-1}). The biomass was 0.374± 0.015 mg cm^{-3}. The best line was calculated through 66 separate experimental points and the bars (I) indicate the scatter of these points. C = 3.60 x 10^{-5}kJ s^{-1} (g cell)$^{-1}$ per min, the slope of the line.

with an aeration rate of the culture vessel of 1.0 - 1.2 dm^{-3} min^{-1}. This provided sufficient stirring and aeration of the culture to maintain a high oxygen tension. The pumping of alternate pulses of air through the microcalorimeter cell did not cause any change in the steady-state heat output; this suggested that oxygen is not limiting in the microcalorimeter. Steady-state growth in the chemostat was maintained for 8 - 10 generation times before any thermal or biomass measurements were made.

The fresh medium was equilibrated to the growth temperature by means of a water jacket before entering the culture vessel and the bacterial suspension was kept at 37.0 $\pm$ 0.5° C through the connecting tubing between microcalorimeter and the fermentor by means of a thermostatted water jacket. The connecting tubing was kept as short as possible.

The flow rate through the microcalorimeter was adjusted according to the dilution rate. For values of D above 0.5 h^{-1} a fixed flow rate of 80 cm^3 h^{-1} was employed. The base-line was established by passing sterile distilled water through the microcalorimeter cell at the required flow rate. The connecting tubing was then transferred aseptically to the chemostat culture in its steady state, and the bacterial suspension pumped into the microcalorimeter cell at the required flow rate. The bacterial suspension leaving the calorimeter, passed through the in-line oxygen and pH electrodes to waste. For a given dilution rate the heat output was maintained for 2 - 3 h to ensure a constant reproducible response.

Bacterial sedimentation in the microcalorimeter cell, occurred after prolonged pumping through the cell, this disturbed

the steady-state heat output. When the measurement period exceeded 24 h, the oxygen tension dropped to zero, and the heat output was no longer steady due to oxygen deficiency. The steady heat output was resumed on removal of the sediment from the cell.

Samples for measurement of biomass, glucose, acetate, etc. were collected at regular time interval (1 h) and the other environmental parameters such as oxygen tension, pH and carbon dioxide were recorded during the thermal measurement.

CHAPTER 4

EXPERIMENTAL RESULTS

Glucose - limited medium

4.1. Growth in glucose - limited chemostat

Cells of K. aerogenes were grown in standard glucose - limited chemostats, under standard conditions (section 3.4).

The environmental parameters were estimated (Section 2.2), when the cells were growing at various dilution rates in the range 0.25 to 1.0 h^{-1}. The pH, which did not vary with dilution rate, was in the range of 6.8 - 6.9. The oxygen tension measured at the outlet of the calorimeter varied with varying dilution rate and flow rate, but was never less than 35% of saturation. Although not an accurate value of pO_2, it was an indicator of aerobic growth.
No glucose or acetate was detected in samples taken from the steady-state growing chemostats at various dilution rates.

The molar growth yield and specific heat output depended on the dilution as described in the following sections.

4.1.1. Variation of molar growth yield with dilution

The maximum observed yield coefficient of K. aerogenes growing aerobically in a glucose - limited chemostat at different glucose concentrations (2 - 5 mmol dm^{-3}) was 0.52 ± 0.02 (i.e. molar growth yield Y_{glu} = 94 ± 3.6 g cell per mol of glucose). The yield coefficient was constant at D> 0.5 h^{-1}, but decreased at lower dilution rates, i.e. growth rates (Fig. 4.1).

4.1.2. Variation of specific heat output (ΔH_p) with dilution

The heat output of K. aerogenes grown under standard conditions (glucose conc. = 4 mmol dm^{-3}) was measured at various dilution rates at steady-state conditions. Over a wide range of values of D the rate of heat output was independent of the dilution rate, this is because the biomass was constant. At low dilution rates, when the biomass decreased the rate of heat output decreased (cf. Fig. 3.1)

At high dilution rates, the extrapolated specific heat output in the chemostat, ΔH_p, approached a constant value of -1.09 kJ $(g\ cell)^{-1}$, but below 0.6 h^{-1} this value decreased rapidly with decreasing D. The lowest value recorded at D = 0.25 h^{-1} (the lowest dilution rate attainable) was-5.85 kJ $(g\ cell)^{-1}$. This variation of ΔH_p with D is quite unlike the variation of biomass with dilution rate (Fig. 4.1). This indicates that at low dilution rates, when the biomass is lower and the doubling time is high, the growth process is very inefficient. The conversion of glucose to cellular material is low and so the unused glucose is converted to carbon dioxide and water with the liberation of heat, which is recorded in the calorimeter.

4.1.3. Variation of ΔH_p with glucose concentration

The specific heat output of K. aerogenes grown in chemostat cultures of different glucose concentration (1.9 to 4.9 mmol dm^{-3}) was measured under standard conditions at various dilution rates. Within the limits of experimental error, ΔH_p was independent of glucose concentration (Fig. 4.1).

4.2. The steady-state heat output of cells in limiting - glucose media under other conditions

4.2.1. Variation of ΔH_p with the pH and temperature of the medium

Cells of K. aerogenes were grown in several glucose - limited standard chemostat cultures at various pH values ranging from 6 to 8 at a fixed D = 0.52 h^{-1}. ΔH_p decreased with increase in pH over the range stated, while the value of Y_{glu} passed through a maximum and the carbon dioxide output through a minimum at a pH of 7.15 (Table 4.1). These variations suggest that at pH values removed from 7.15 more glucose is fermented to give carbon dioxide.

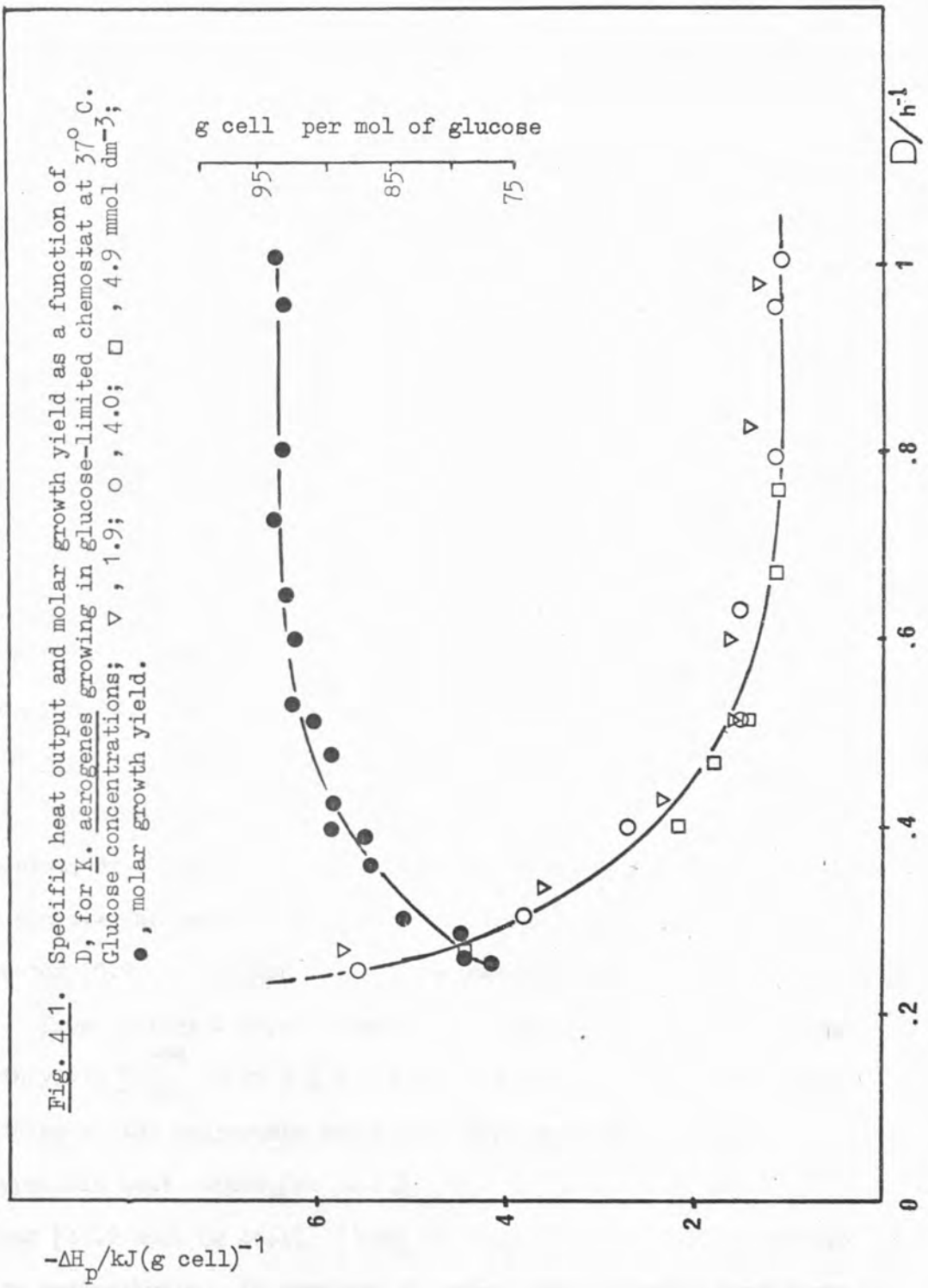


Fig. 4.1. Specific heat output and molar growth yield as a function of D, for K. aerogenes growing in glucose-limited chemostat at 37° C. Glucose concentrations; ▽ , 1.9; ○ , 4.0; □ , 4.9 mmol dm^{-3}; ● , molar growth yield.

Table 4.2: ΔH_p, Y_{glu} and CO_2 evolution during the growth of K. aerogenes in media of different pH (D = 0.52 h^{-1}, glucose concentration 4 mmol dm^{-3}).

pH		Y_{glu}	$-\Delta H_p$	CO_2 produced
initial	during growth	/g cell mol^{-1}	/kJ(g cell)$^{-1}$	/mmol (g cell)$^{-1}$
6.0	5.70	83.7	1.30	22.1
6.5	6.15	85.0	1.30	21.4
7.0	6.80	93.7	1.55	18.7
7.4	7.15	95.0	1.58	18.0
7.5	7.30	92.5	1.57	20.4
8.0	7.80	90.7	1.66	23.2

The growth and test temperature of growing cultures of K. aerogenes under standard conditions, was increased to 42° C. The specific heat output and other parameters measured at various dilution rates (Fig. 4.2.) were similar to those obtained under standard conditions (cf.Fig. 4.1.).

4.2.2. Heat output under anaerobic conditions

The organisms were grown in standard glucose - limited chemostat, at a fixed dilution rate of0.52 h^{-1}. The anaerobic conditions were achieved by purging "white spot" nitrogen through both the fermentor (0.9 - 1 dm^3 min^{-1}), and the medium reservoir.

From replicate experiments it was observed that although the growth yield [Y^{ana}_{glu} = 32.9 ± 0.5 g cell (mol glucose)$^{-1}$] was about one third of the comparable value for cells growing aerobically, the specific heat output [ΔH_p =-2.9 ± 0.2 kJ (g cell)$^{-1}$] and CO_2 evolved [47.2 mmol (g cell)$^{-1}$] were 2- and 2.5-fold that for aerobic growth respectively. In contrast to growth under aerobic conditions, acetate was produced in large amounts and achieved a steady-state concentration 0.6 mol per mol of glucose.

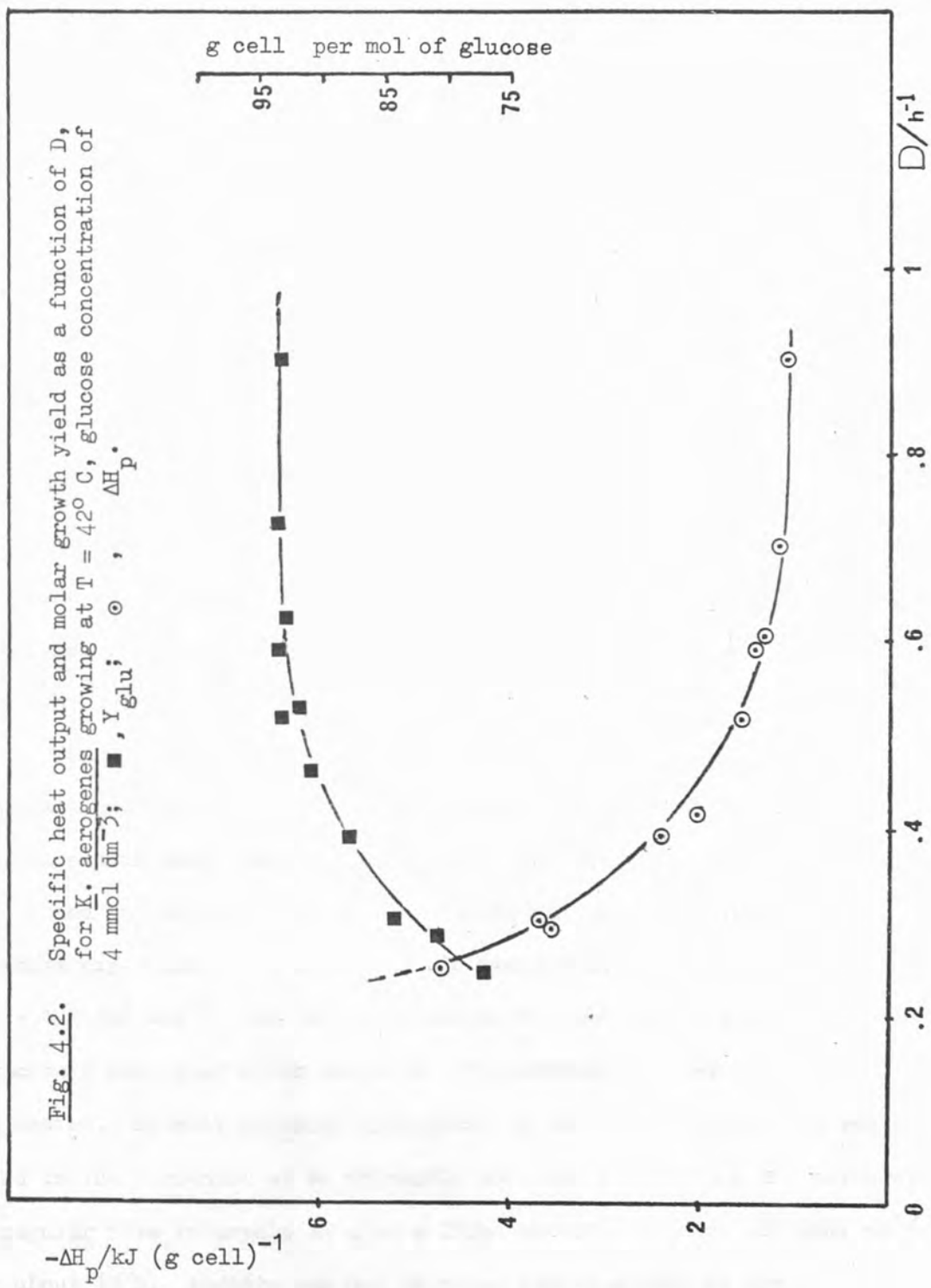


Fig. 4.2. Specific heat output and molar growth yield as a function of D, for K. aerogenes growing at T = 42° C, glucose concentration of 4 mmol dm^{-3}; ■, Y_{glu}; ⊙, ΔH_p.

The rate of heat output increased several-fold on mixing alternate volumes of air and growing culture through the calorimeter cell; this was due to the oxidation of accumulated secondary metabolites. A similar response was obtained during the transition period from anaerobic to aerobic condition in the fermentor when the nitrogen was replaced with air.

4.2.3. Growth of K. aerogenes in the presence of uncoupling agents and inhibitors of oxidative phosphorylation

The production of ATP in oxidative phosphorylation occurs by a complex process involving a membrane - localized electron transport chain. ATP will not be produced if uncouplers are present. These reagents have the ability to abolish the coupling of substrate oxidation to ATP synthesis. As a consequence ATP synthesis comes to a halt, because the necessary energy is not available, while the oxidation of substrate, uninhibited by respiratory control, proceeds at maximal rate and produces heat instead of ATP (Poe et al., 1967; Hanstein, 1976; Baird et al., 1979).

The organisms were grown in glucose-limited (4 mmol dm^{-3}) chemostat cultures in the presence of added sodium malonate, sodium amytal, sodium barbitone, rotenone, nalidixic acid, 2-4-dinitrophenol (DNP), and sodium azide, at various concentrations and growth rates. In these experiments the fermentor was aerated at a rate of 1.6 - 1.8 dm^3 min^{-1}, and the measurements of heat output and other parameters were made after about 20 - 25 generation times in the new medium. Because rotenone aggregated in the growth medium, it was added to the fermentor as an ethanolic solution (20 cm^3 per dm^3 culture) at regular time intervals to give a final concentration of 200 μmol dm^{-3} for about 12 h. Acetate was not detected during growth in the presence of any of the added reagent.

Sodium malonate (20 mmol dm^{-3}) was utilized by the organisms at this concentration and the biomass increased several-fold, the oxygen tension dropped to zero and the rate of heat output was very low due to anaerobic conditions in microcalorimeter cell. Pulses of air bubbles through the calorimeter caused a sharp increase in the rate of heat output. Further addition of malonate up to 150 mmol dm^{-3} caused partial cessation of the growth, which was characterized by a sharp decrease in CO_2 production, heat output and oxygen uptake rate.

In the presence of sodium amytal (70 and 150 μmol dm^{-3}) the biomass was increased slightly (5 - 10%) due presumably to the utilization of the amytal, but ΔH_p was the same as that for control, however the CO_2 output was marginally lower than that evolved in control (Table 4.2).

Sodium barbitone produced a marked decrease in the value of ΔH_p. The yield value depended both on the concentration of the inhibitor and the dilution rate, decreasing with increased concentration and lower dilution rate. Doubling the concentration of inhibitor at a fixed dilution rate caused only a small decrease in ΔH_p (Table 4.2).

Nalidixic acid (430 μmol dm^{-3}) decreased the Y_{glu} value drastically and at concentration of 860 μmol dm^{-3} and dilution rate of 0.52 h^{-1} the culture washed out from the fermentor (Table 4.2). Despite the low value of Y_{glu},-ΔH_p was only marginally lower than that for the control. The reduction in Y_{glu} and-ΔH_p was more marked at the lower dilution rate.

The presence of DNP at a concentration of 0.04 mmol dm^{-3} had very little effect on Y_{glu}, ΔH_p and the CO_2 produced. At concentrations in the range 0.24 to 1.0 mmol dm^{-3} ($D = 0.52\ h^{-1}$), Y_{glu} was reduced to a constant value (10% below the control) and ΔH_p decreased

Table 4.2. Thermal and environmental properties during the growth of *K. aerogenes* in the presence of uncouplers and inhibitors (glucose conc. = 4 mmol dm^{-3}).

Uncoupler or inhibitor	Conc. /mmol dm^{-3}	D /h^{-1}	Y_{glu} /g cells (mol glucose)$^{-1}$	$-\Delta H_p$ /kJ(g cell)$^{-1}$	CO_2 evolved /mmol (g cell)$^{-1}$	glucose /mmol dm^{-3}	acetate /mmol dm^{-3}
None	———	0.25	76.9	5.85	38.2	nd	nd
"	———	0.30	84.5	4.18	ND	nd	nd
"	———	0.40	89.5	2.65	28.7	nd	nd
"	———	0.52	93.7	1.55	18.7	nd	nd
"	———	0.63	93.8	1.50	19.4	nd	nd
"	———	0.80	94.7	1.25	ND	nd	nd
"	———	0.95	93.3	1.20	21.3	nd	nd
"	———	1.04	94.2	1.09	ND	nd	nd
DNP	0.04	0.52	90.4	1.70	22.2	nd	nd
	0.24	0.52	84.7	2.60	27.8	nd	nd
	0.51	0.52	83.9	2.46	26.8	nd	nd
	1.00	0.52	85.5	2.51	29.6	nd	nd
	1.00	0.29	76.2	4.69	37.5	nd	nd
Sodium azide	0.62	0.35	59.5	3.98	33.8	nd	nd
	"	0.42	58.5	3.00	27.3	nd	nd
	"	0.52	75.0	2.17	30.7	nd	nd
	"	0.69	75.9	1.91	28.5	nd	nd
	"	0.85	77.8	1.39	29.6	nd	nd

(Table 4.2. continued)

Nalidixic acid	0.43	0.33	50.9	2.97	26.5	nd	nd
	"	0.52	65.5	1.41	18.4	nd	nd
	0.86	0.52	The culture washed out		—	—	—
Sodium amytal	0.07	0.52	98.5	1.56	16.5	ND	ND
	0.15	0.52	103.8	1.60	15.3	ND	ND
Sodium barbitone	2.40	0.52	83.7	2.58	23.6	ND	ND
	4.80	0.29	68.8	4.68	29.3	ND	ND
	"	0.52	74.9	2.81	34.7	ND	ND
Rotenone *	0.20	0.52	102.5	3.35	28.5	ND	ND
Control **	None	0.52	103.5	3.43	26.9	ND	ND

* Ethanolic solution of rotenone was added at intervals to the fermentor (200 μ mol in 20 cm^3 absolute alcohol per dm^3 of culture).

** The culture contained 20 cm^3 ethanol per dm^3., ND, not determined, nd, not detectable.

correspondingly. At lower dilution rates the yield value was slightly (5%) lower than that of the control and ΔH_p correspondingly lower (Table 4.2).

The effect of sodium azide on growing cells of K. aerogenes in glucose - limited (4 mmol dm^{-3}) chemostat, was investigated at a fixed concentration of 620 μmol dm^{-3} at various dilution rates (0.35 - 0.85 h^{-1}). There was a significant decrease in Y_{glu}, and consequently a decrease in ΔH_p value in comparison to the controls at all dilution rates. The carbon dioxide output was much higher than that of the controls for all dilution rates (Table 4.2); it is thus apparent that in the presence of sodium azide less of the carbon source is converted to biomass, the remainder being degraded to carbon dioxide and water by exothermic processes. When the azide concentration was doubled by the addition of 0.5 cm^3 of sodium azide solution directly into the fermentor at the steady-state, the rate of heat output was increased by a further 10%.

Rotenone was added to the fermentor as an ethanolic solution (20 cm^3 per dm^3 of culture) at regular time intervals to maintain a final concentration of 200 μmol dm^{-3}, until a steady heat output was recorded. In the control experiment carried out under the same conditions with the addition of 20 cm^3 ethanol per dm^3 of culture to the fermentor there was a marked increase in the rate of heat output due to the oxidation of ethanol. The oxygen tension oscillated between 40% and 60% of saturation in both cases. No additional change was observed in the biomass or specific heat output due to the presence of rotenone (Table 4.2).

4.3. Changes in steady-state heat output associated with changes of environmental parameters

It is common knowledge that, steady-state conditions are disturbed by changes in environmental parameters. A change in dilution rate (Section 3.2), or the addition of extra substrates, nutrients, inhibitors, uncouplers, etc... to the growing culture in the steady-state, will produce marked changes in the steady-state conditions. Some investigations of these transient-states are described in the following sections.

4.3.1. Addition of extra substances to the growing culture of K. aerogenes in the steady-state

4.3.1.1. Addition of extra carbon source

Cells of K. aerogenes were grown in standard glucose-limited (4 mmol dm^{-3}) chemostat at a fixed dilution rate 0.52 h^{-1}. The aeration of the culture was increased to 1.6 - 1.8 dm^3 min^{-1}, and a fixed flow rate of 80 cm^3 h^{-1} (i.e. Δt = 1.9 min) through the microcalorimeter was used. All the environmental parameters were measured under the steady-state conditions. A small volume (0.5 cm^3) of a solution containing different carbon sources (0.9% w/v) were added to the fermentor (i.e. 160 cm^3 of growing culture) and changes in all parameters were monitored for a sufficient period of time to ensure that steady-state growth in glucose-limited medium was reestablished.

A wide range of carbon sources were added under the same conditions. In general the superimposed peaks on the steady-state heat output following the addition of these carbon sources may be divided into four groups (Table 4.3), thermograms of these four groups are shown in Fig. 4.3. The rate of oxygen uptake and the carbon dioxide

Table 4.3. Effect of adding various carbon sources to cells of *K. aerogenes* growing in standard glucose-limited chemostat under steady-state conditions, Δt = 1.9 min, D = 0.52 h^{-1}, substrates added; 4.5 mg per 160 cm^3 of growing culture (C-sources other than glucose derivatives, sucrose, glycerol, this weight is calculated as free-acid), see also Fig. 4.3.

Group	Substrate	Additional heat output over the steady-state output.
1	glucose pyruvate	Sharp increase in the rate of heat output, passing through a maximum reattaining steady-state value after 30 min.
2	glucose-1-phosphate glucose-6-phosphate sucrose malate succinate fumarate	Gradual increase and decrease in the rate of heat output passing through a maximum. Subsequent addition causes a sharp increase in the rate of heat output which is similar to Group 1 response.
3	formate acetate oxaloacetate glycerol malonate	Sharp increase in the rate of heat output which was maintained for long periods before returning to normal value. Similar pattern on subsequent addition.
4	α-ketoglutarate citrate glycine	Slight increase (10-20%) in the rate of heat output, which lasted for several hours before reattaining normal value. No change on subsequent addition.

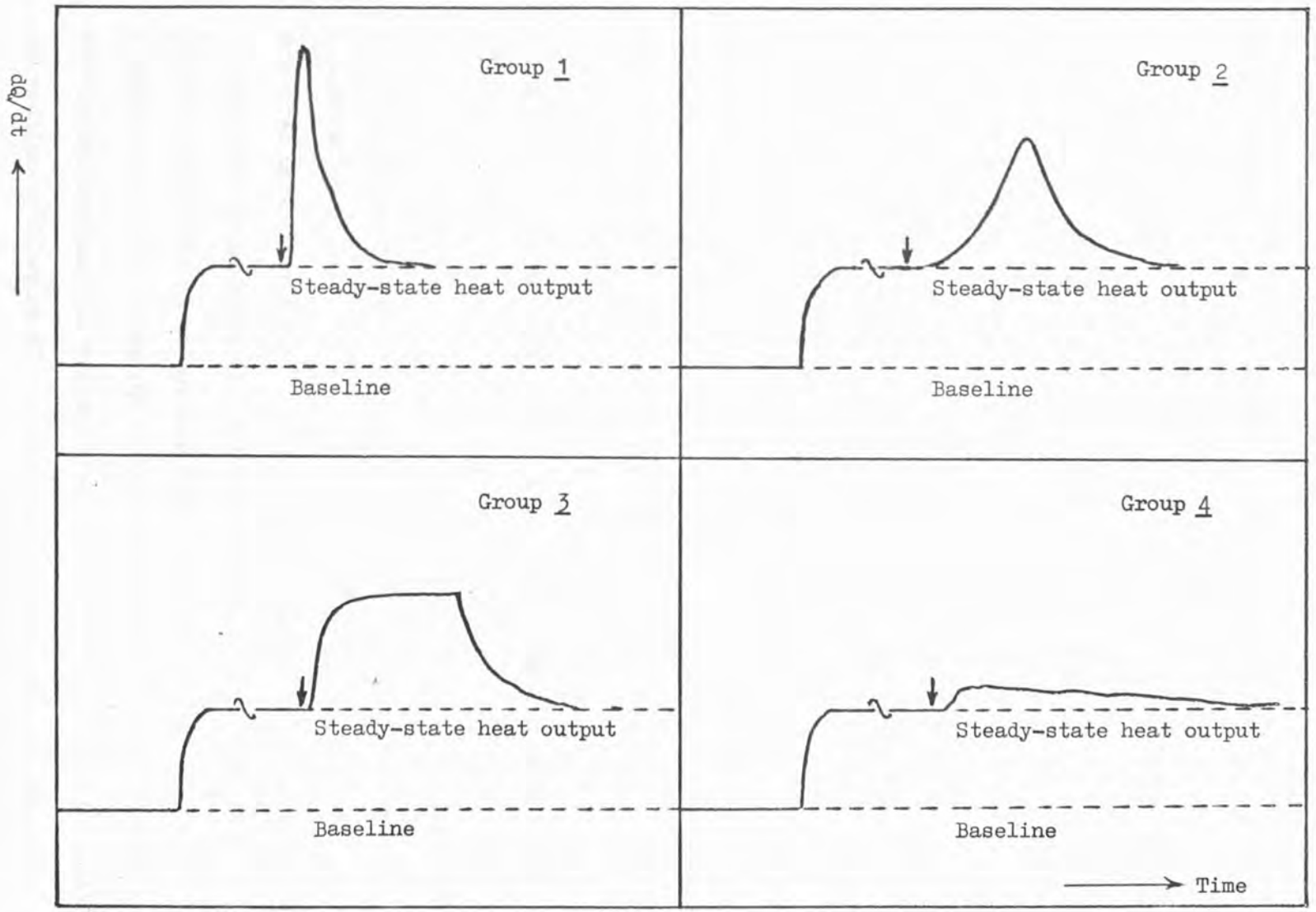


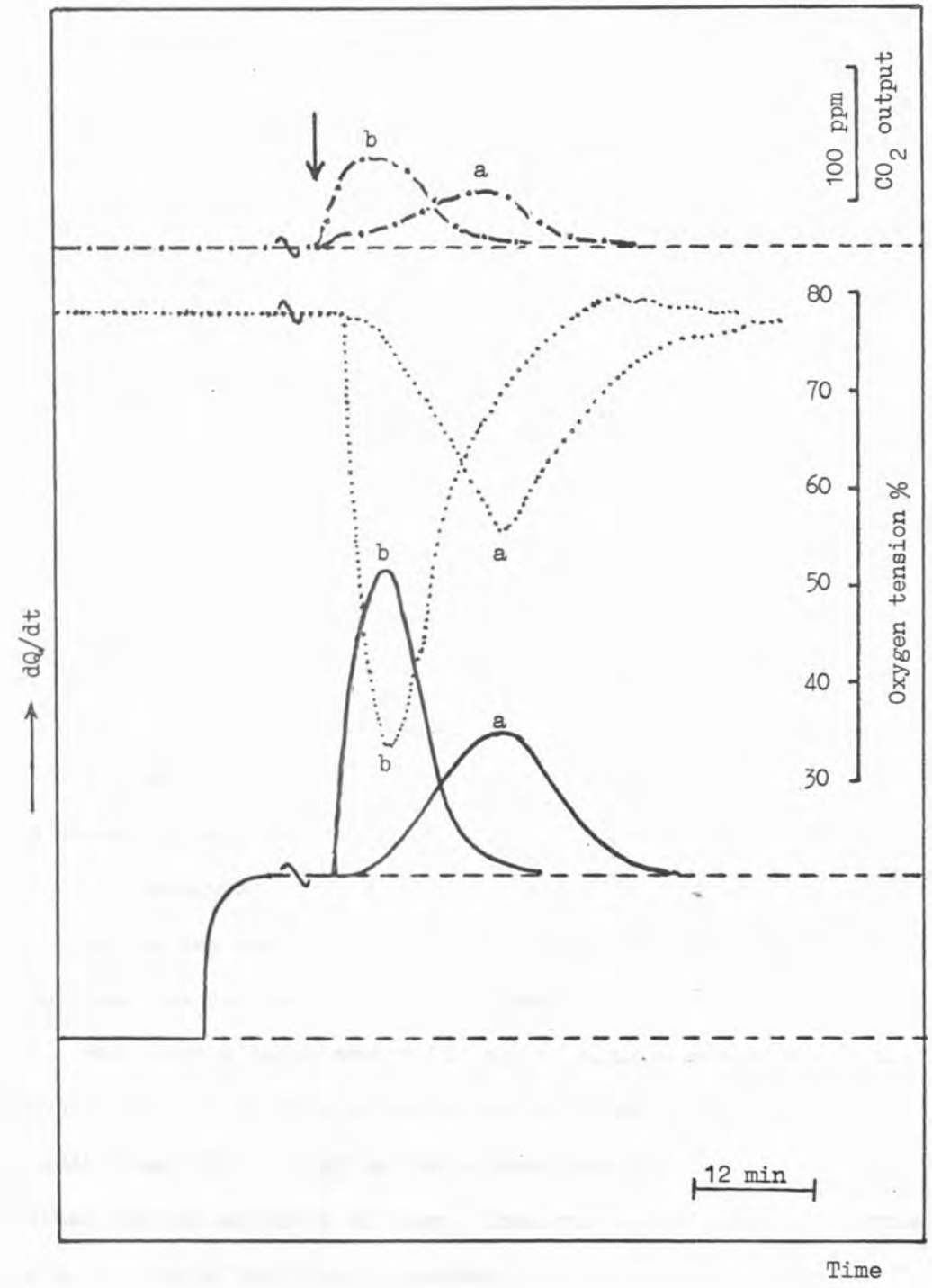
Fig. 4.3. The heat response caused by the addition of extra carbon source (↓) to the steady-state growing culture of K. aerogenes in glucose-limited (4 mmol dm^{-7}) chemostat, at D = 0.52 h^{-1}; Δt = 1.9 min.

production both increased in parallel with the rate of heat output. Since the amount of the added substrate was small, its maximum concentration was small in comparison to the concentration of glucose in the medium, there was no detectable change in the biomass of the culture.

On repeated addition of the substrates of Group 2 at 30 min intervals into the culture vessel, the bell-shaped heat response (in the first addition) was replaced with a sharp response similar to that shown by Group 1 substrates (Fig. 4.4.), parallel changes in the production of CO_2 and oxygen tension were also recorded. This suggested that the organisms were becoming adapted to the additional carbon source. This phenomenon was no longer evident if the time interval between successive additions of substrate exceeded several generation times; during this additional time interval any adaptation was lost. Subsequent addition of substrates of Groups 3 and 4, however, produced changes in heat output, carbon dioxide output and oxygen tension which were similar to those recorded on the first addition.

In a separate series of experiments the same volume (0.5 cm^3) of solutions of different concentrations (0.22, 0.45, 0.9 and 1.8% w/v) of various substrates were added at a time, the amount added produced an immediate concentration which was always very much less than the concentration of glucose in the growth medium. The heat output was monitored until it returned to the minimal value for steady-state growth and the area of superimposed peak on the steady-state heat output (which is proportional to the additional heat output) was measured. Because the calculation of the quantitative heat output requires a complicated mathematical treatment associated with flow time (i.e. Δt) and continuous dilution of the added substrate, only the areas (expressed as cm^2) under the curves were measured. Typical thermograms

Fig. 4.4. A typical response of, ——, heat output; —·— , CO_2 production; ....., oxygen tension for the addition (↓) of sucrose, a and b indicate two successive addition of 0.5 cm^3 of 0.45% w/v, solution of sucrose. D = 0.52 h^{-1}, Δt = 1.9 min.



obtained with different amounts of added substrate are shown in Fig. 4.5, the plot of the areas of the superimposed peaks on the steady-state heat output, against the added substrate is shown in Fig. 4.6.

Of the substrates investigated (glucose, pyruvate and acetate) it was only for acetate that the additional heat output was a linear function of the amount added. This may be due to a limited acetate uptake of the organisms in the presence of glucose. The non-linearity of the graphs for glucose and pyruvate, is probably due to a more rapid utilization of these substrates (than acetate) at lower amounts and disappearance of the added substrate by the time the suspension reaches the calorimeter.

For higher amounts of glucose and pyruvate there was evidence of oxygen depletion (less than 15% saturation) in the microcalorimeter cell. In the case of glucose two distinct peaks (Fig. 4.5.f) were superimposed on the steady-state heat output at higher amounts (9 mg in 160 cm^3 culture). The one immediately after the addition was due to the added substrate, while the second was due to the oxidation of a secondary metabolite eg. acetate. Although the amount of the added substrate was large enough to cause oxygen deficiency, its final concentration was small and consequently any secondary metabolite was at too low a concentration to be detected.

When a very large amount (72 mg) of glucose was added to the fermentor (weight of glucose in 160 cm^3 of fresh medium = 115 mg), the additional heat output for both peaks was much higher than that obtained for the addition of 9 mg. Chemical analysis of the culture medium now showed that acetate accumulated and had attained a concentration of 1.5 - 1.8 mmol dm^{-3}. There was a decrease of pH

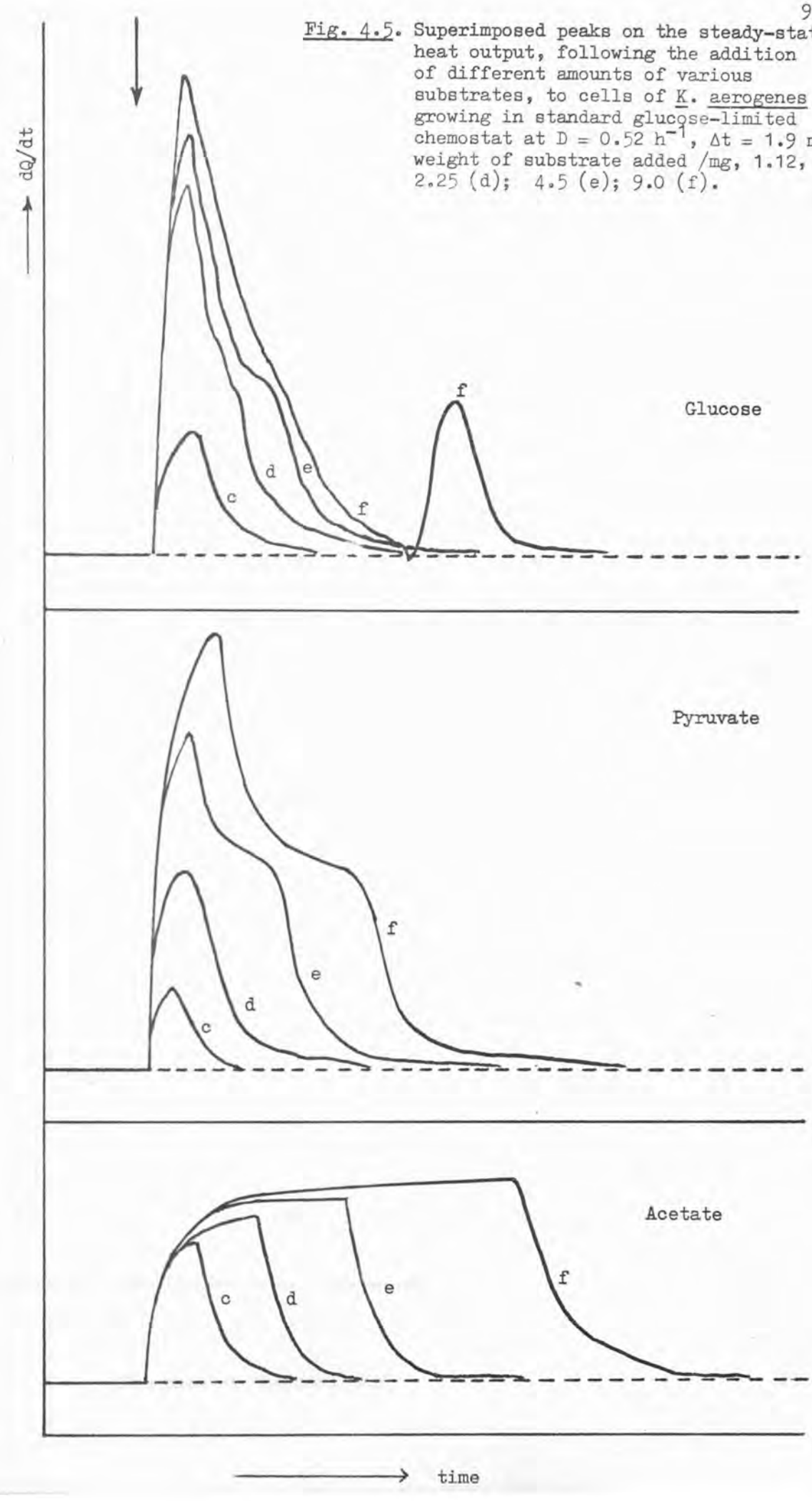


Fig. 4.5. Superimposed peaks on the steady-state heat output, following the addition of different amounts of various substrates, to cells of K. aerogenes growing in standard glucose-limited chemostat at D = 0.52 h^{-1}, Δt = 1.9 min, weight of substrate added /mg, 1.12, (c); 2.25 (d); 4.5 (e); 9.0 (f).

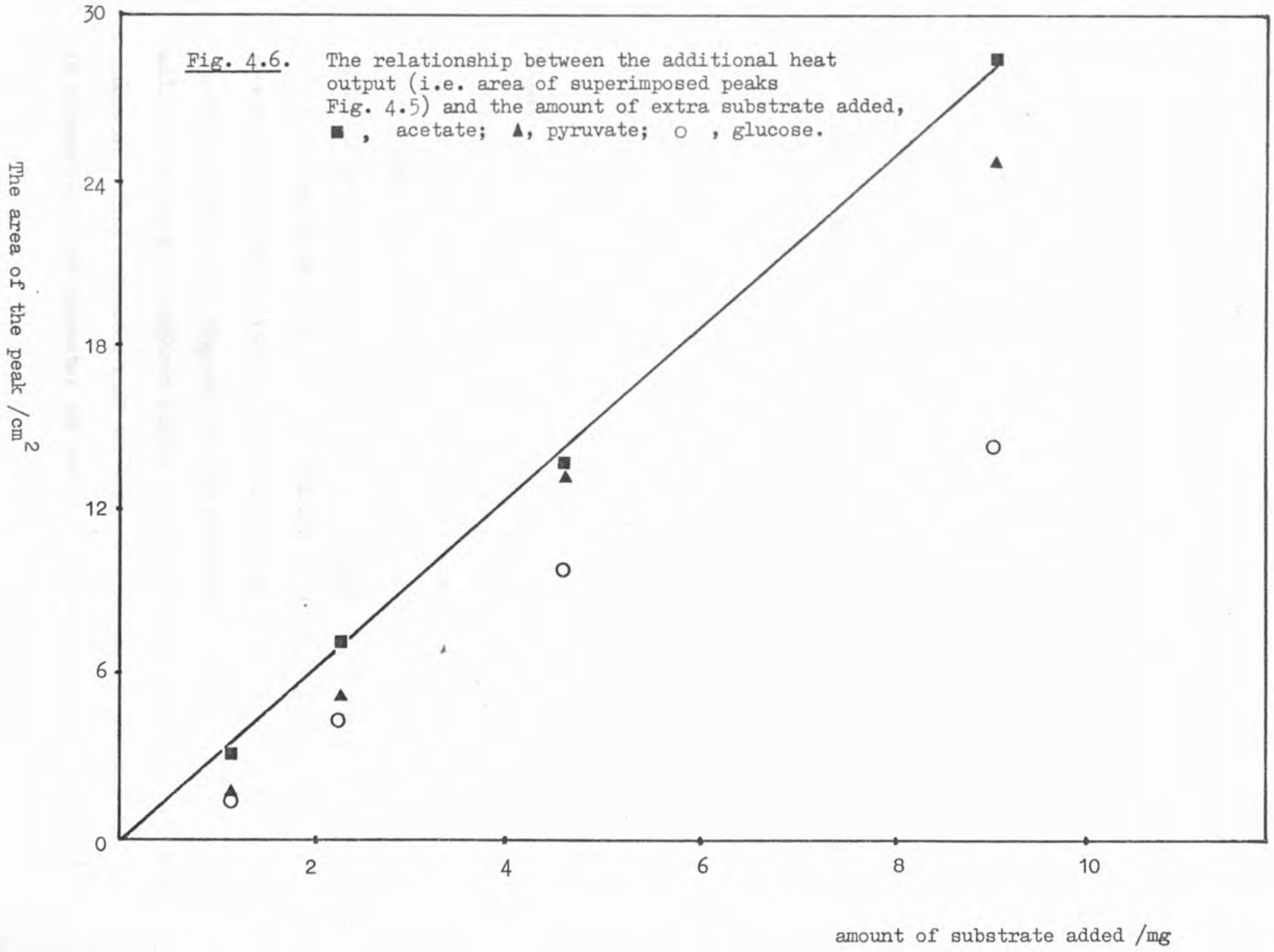


Fig. 4.6. The relationship between the additional heat output (i.e. area of superimposed peaks Fig. 4.5) and the amount of extra substrate added, ■ , acetate; ▲, pyruvate; ○ , glucose.

to 6.4 and a marked increase in the biomass (25 - 30%). The oxygen tension was below 10% of saturation and only resumed its steady-state value after a few hours, when all the acetate had been oxidized.

The measured area under the peak (i.e. the additional heat output), for a given amount of added substrate, under given set of conditions was very reproducible (± 2% at 95% confidence level). As expected the shape and the area of the peaks on the steady-state heat output for a given amount of substrate varied with flow time, and growth conditions.

The steady-state heat output was not affected on addition of acetate to the cultures of K. aerogenes growing either aerobically in the presence of sodium azide or under anaerobic conditions. For a given amount of added glucose, the additional heat output was higher (100%) when the cells were growing in the presence of azide and lower (50%) when growing under anaerobic conditions than the additional heat output for the growth under aerobic conditions.

The heat response following the addition of glucose-6-phosphate to glucose-limited chemostats of K. aerogenes growing in the presence or absence of sodium barbitone was the same; in both cases the organisms required adaptation. This suggests that the high glucose-6-phosphate dehydrogenase activity (Dean and Moss, 1971) recorded in the presence of sodium barbitone had no effect on utilization of glucose-6-phosphate.

The addition of glycerol or glucose to cells of K. aerogenes growing in a glycerol-limited chemostat (See sect. 5.1.1.) culture showed a similar heat response to that obtained on the addition of Group 1 compounds (Table 4.3) to cells in a glucose-limited chemostat. This indicates that the organisms adapted to and growing in glycerol-limited chemostat can readily utilize glucose.

4.3.1.2. Addition of uncoupling agents and inhibitors

The object of this study was to observe (1) the immediate changes in heat output, biomass, pO_2, CO_2, etc... when cells in a steady-state were challenged with numerous uncouplers and inhibitors, and (2) if the steady-state conditions could be re-established after the inhibitor had been washed out. This work differs from that reported previously (Sect. 4.2.3) where the cells were grown in medium containing the inhibitors at sub-lethal concentrations.

Cells of K. aerogenes were grown in standard glucose-limited (4 mmol dm^{-3}) chemostat at a fixed dilution rate of 0.52 h^{-1} and Δt = 1.9 min. All environmental parameters were measured at steady-state and a 0.5 cm^3 aliquot of solutions of uncoupler or inhibitor (containing 100 μmol) was added to the fermentor (160 cm^3), changes in the thermal and other environmental parameters were monitored for a sufficient time to ensure the reattainment of steady-state conditions.

The added compounds were: rotenone, dicyclohexyl-carbodiimide (DCCD), guanidinium chloride, sodium amytal, sodium azide and 2-heptyl-4-hydroxy-quinoline-N-oxide (HQNO).

Rotenone and DCCD were dissolved in ethanol and ethanolic solutions of these compounds were added to the chemostat in its steady-state. The additional heat response superimposed on the steady-state heat output was compared to the control with the added ethanol (0.5 cm^3). All the other compounds were added as aqueous solutions.

There was a significant increase (150%) in the rate of heat output when DCCD or sodium azide was added; the steady-state heat output was reattained after several hours when the added reagents were diluted out from the culture. The biomass was decreased subsequently (20 - 30%)

following the addition of DCCD or sodium azide. After making allowance for the alcohol there was no further change in the rate of steady-state heat output on the addition of rotenone (Fig. 4.7), the CO_2 production (10 - 15%) and oxygen uptake rate increased.

Sodium amytal produced an immediate decrease in the rate of CO_2 production and oxygen uptake (20 - 30%), the steady-state values of both were reattained within several hours when the added reagent was diluted out of the culture vessel. The rate of heat output increased gradually by 50% and the biomass was decreased by 15 - 20%.

Guanidinium chloride and HQNO had no effect on any of the steady-state values of the environmental or thermal parameters.

The addition of sodium azide (100 μmol) to an anaerobic chemostat culture, caused an increase (35%) in the rate of heat output, and a marked decrease (80%) in CO_2 production.

In acetate-limited chemostats (Sect. 5.1.3) the rate of heat output, and oxygen uptake increased several-fold as a result of the addition of sodium azide. The oxygen tension dropped to zero and the heat output decreased due to anaerobic conditions and as a consequence carbon dioxide production decreased by 30 - 25%. Alternate air pulses through the microcalorimeter cell caused a sharp peak on the rate of heat output.

In glucose-sufficient chemostat cultures, Mg^{2+} or N-limited, (Sect. 5.2) there was an increase of 20% in the rate of steady-state heat output as a result of the addition of sodium azide.

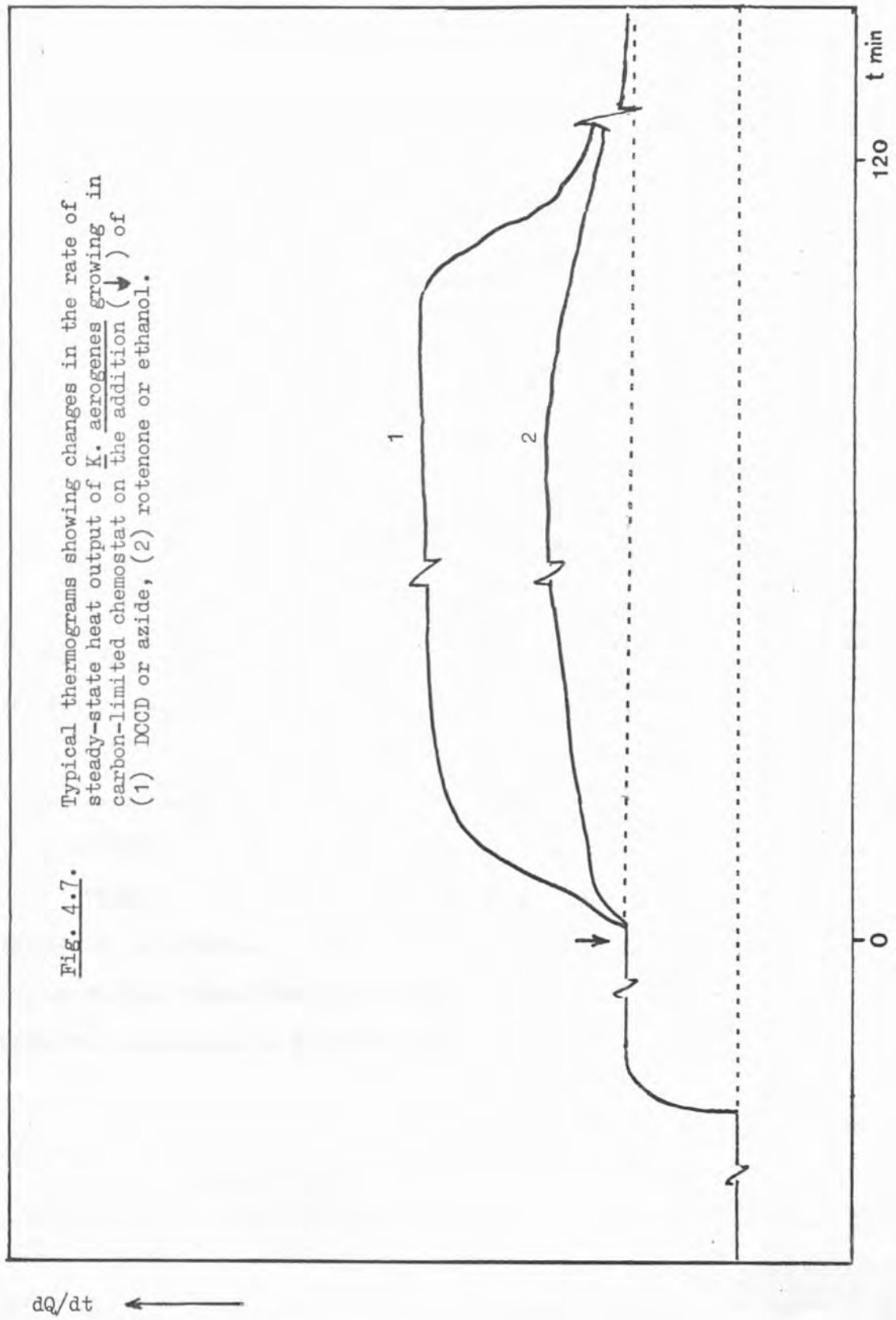


Fig. 4.7. Typical thermograms showing changes in the rate of steady-state heat output of K. aerogenes growing in carbon-limited chemostat on the addition (↓) of (1) DCCD or azide, (2) rotenone or ethanol.

4.3.2.3. Addition of other compounds

Cells of K. aerogenes were grown in standard glucose-limited (4 mmol dm^{-3}) chemostat at D = 0.52 h^{-1}. All environmental parameters were measured at steady-state and 0.5 cm^3 (4.5 mg) of solutions of methyl-β-D-thio-galactopyranoside (MTG), α-methyl-D-glucoside (MG), and adenosine triphosphate (ATP) were added. Changes in heat output and other environmental parameters were monitored for a sufficient time to allow re-establishment of the steady-state conditions. ATP and MTG had no effect on the steady-state conditions. There was a small but insignificant decrease (5%) in the rate of heat output, CO_2 production and oxygen uptake, as a result of the addition of MG, the steady-state values were reattained after about 30 min. This appears to be due to some disruption of glucose uptake by the added MG.

4.4. Transient pH change in the growing culture

The organisms were grown in standard glucose-limited (4 mmol dm^{-3}) chemostat at D = 0.52 h^{-1}. All the environmental parameters were measured at steady-state and changes in these were monitored on the addition of 0.5 cm^3 of solutions of either sodium hydroxide or sulphuric acid (of sufficient concentration to bring about a change of 1 pH unit).

There was no change in the rate of heat output or oxygen uptake following the addition of sulphuric acid, but a sharp increase in carbon dioxide production was observed. This could be associated with the low solubility of carbon dioxide at low pH values.

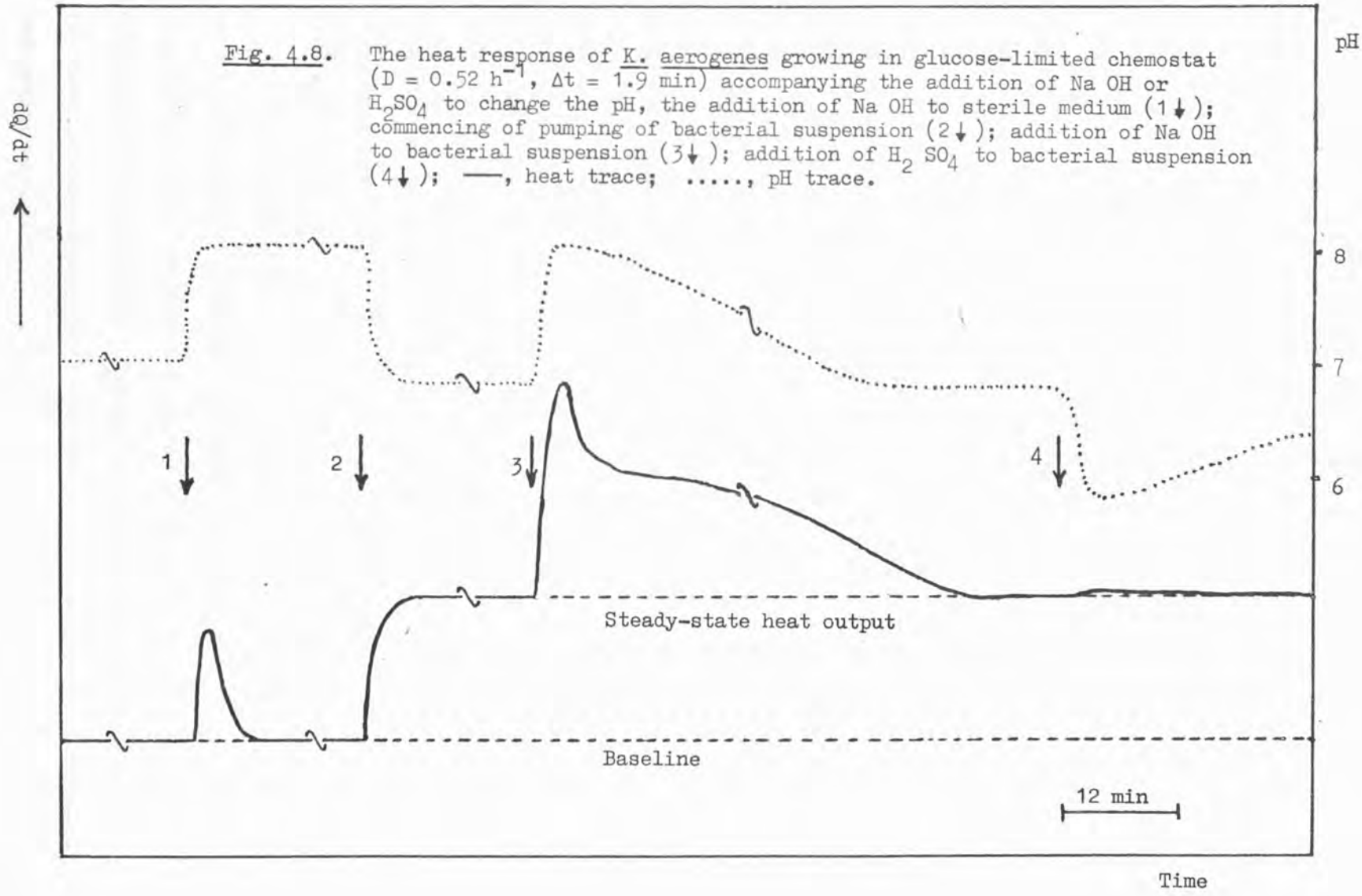


Fig. 4.8. The heat response of K. aerogenes growing in glucose-limited chemostat ($D = 0.52\ h^{-1}$, $\Delta t = 1.9$ min) accompanying the addition of Na OH or H_2SO_4 to change the pH, the addition of Na OH to sterile medium (1↓); commencing of pumping of bacterial suspension (2↓); addition of Na OH to bacterial suspension (3↓); addition of H_2SO_4 to bacterial suspension (4↓); ——, heat trace; ....., pH trace.

A sharp peak with a shoulder was superimposed on the steady-state heat output as a result of adding sodium hydroxide to the fermentor (Fig. 4.8). The carbon dioxide output was abruptly decreased to zero and only gradually resumed its steady-state value. This is due to the increased solubility of carbon dioxide at higher pH. The addition of sodium hydroxide to the killed culture or sterile medium, caused a small peak in the heat output.

4.5. Summary

1. The molar growth yield and specific heat output were constant at dilution rates greater than 0.5 h^{-1}. At lower dilution rates the molar growth yield decreased and the specific heat output increased

2. ΔH_p was independent of the glucose concentration and the growth and environmental temperature (37 - 42° C).

3. ΔH_p increased with increasing pH of the growth medium, while Y_{glu} passed through a maximum and the CO_2 through a minimum value at pH 7.15.

4. Under anaerobic conditions of growth there was a marked reduction in the molar growth yield, increase in-ΔH_p and CO_2. On transition from anaerobic to aerobic growth the heat output increased several-fold.

5. In the presence of sodium barbitone, sodium azide and DNP, in the medium there was a marked reduction in the value of Y_{glu} and consequently an increase in the values of-ΔH_p and carbon dioxide production. No significant change was observed in the values of these parameters as a result of growth in rotenone, nalidixic acid, and sodium amytal.

6. The additional heat output following the addition of extra substrate to the steady-state cells in carbon-limited chemostat for various carbon sources was divided into four groups. There was some evidence of adaptation to the additional carbon source.

7. The additional heat output was a linear function of the amount of added acetate but not for glucose and pyruvate.

8. The rate of heat output increased and biomass decreased on adding sodium azide or DCCD to the steady-state growing K. aerogenes in glucose-limited chemostat. The steady-state conditions were reattained after several hours, when the added reagent washed out of the fermentor.

9. Transient increase of pH to 8 by addition of NaOH, caused a significant increase in the rate of heat output. There was no change in the heat output when the pH was decreased to 6.

CHAPTER 5

EXPERIMENTAL RESULTS

Steady-state heat output in other nutrient-limited media

5.1. Carbon-limiting chemostats

5.1.1. Glycerol-limited chemostat

Glucose-grown cells of K. aerogenes were adapted to glycerol by 9 subcultures in simple salts medium containing glycerol prior to growth in a glycerol-limited (7.8 mmol dm^{-3}) chemostat. The heat output and other parameters were measured at various dilution rates (0.29 - 0.88 h^{-1}) under the same conditions as for standard glucose-limited chemostats. The measured yield coefficient was 0.54 $\pm$ 0.04 (Y_{gly} = 49.7g cell per mol of glycerol); this is marginally higher than that for glucose.

As reported for glucose-limited chemostats, the molar growth yield was constant at D> 0.5 h^{-1} but decreased at lower dilution rates. At high dilution rates the value of ΔH_p approached a constant value of -1 kJ $(g\ cell)^{-1}$, but below 0.5 h^{-1} this value decreased rapidly with decreasing D. The lowest value recorded at D = 0.29 h^{-1} (the lowest dilution rate investigated) was -4.4 kJ $(g\ cell)^{-1}$, (Fig 5.1). The variations of ΔH_p and Y_{gly} with D are similar to the variation of these parameters in glucose-limited chemostats (cf. Fig. 4.1). The graphs of ΔH_p against D were coincident for both carbon sources that; the values of ΔH_p was independent of the carbon source. Thus for these two carbon sources an equal amount of heat is dissipated during the formation of a unit mass of cells. The pH was 6.85 - 6.90 and the carbon dioxide production was the same as that in glucose-limited chemostats (Table 5.1).

When the inflowing medium to the chemostat was stopped the rate of heat output immediately fell to a very low value. This indicated the absence of residual glycerol and other secondary metabolites (section 5.3).

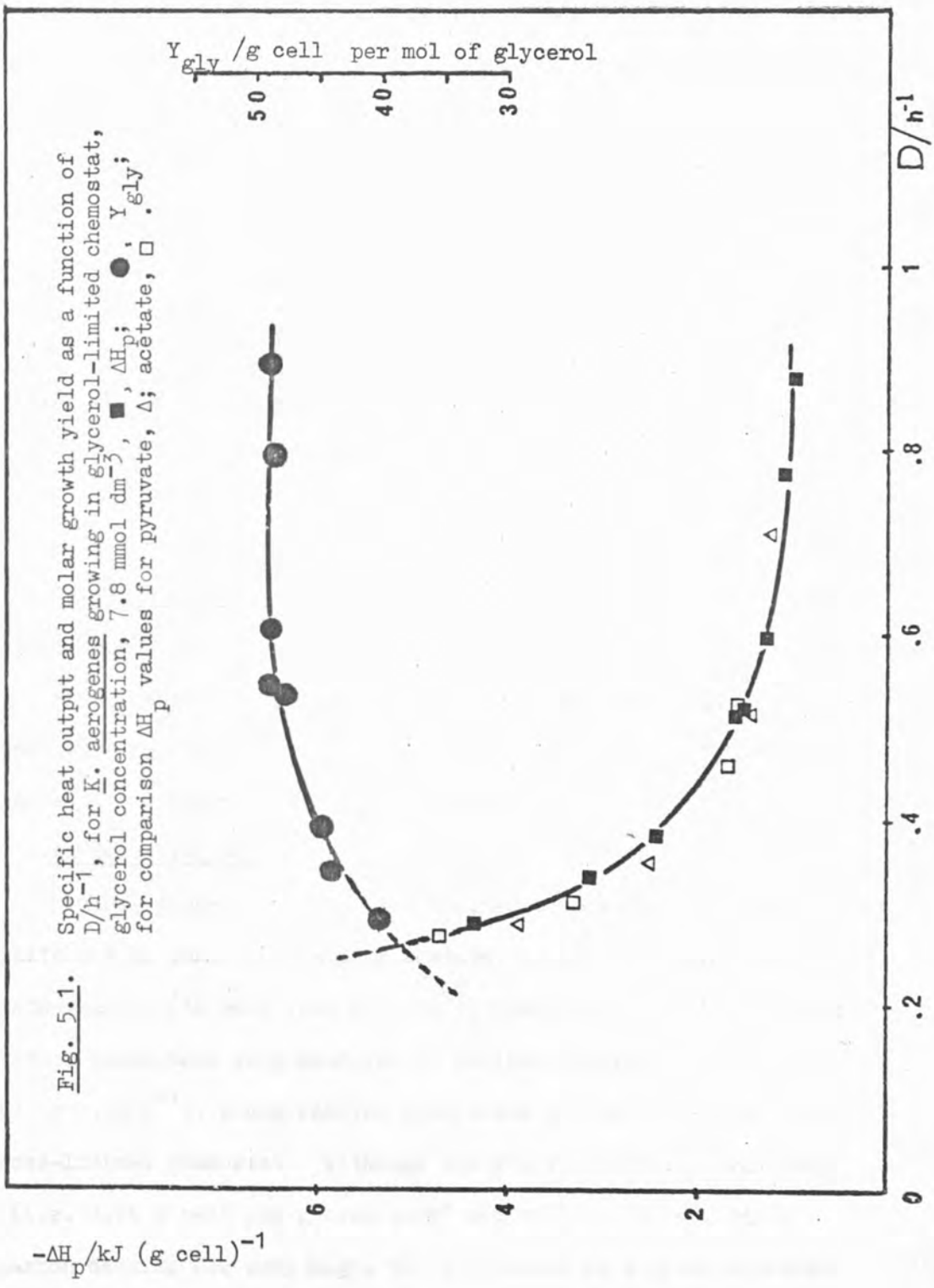


Fig. 5.1. Specific heat output and molar growth yield as a function of D/h^{-1}, for K. aerogenes growing in glycerol-limited chemostat, glycerol concentration, 7.8 mmol dm^{-3}, ■, ΔH_p; ●, Y_{gly}; for comparison ΔH_p values for pyruvate, Δ; acetate, □ .

5.1.2. Pyruvate-limited chemostat

Glucose-grown cells of K. aerogenes were adapted to pyruvate by 7 subcultures in salts medium containing sodium pyruvate, prior to growth in a pyruvate-limited (8.2 mmol free acid dm^{-3}) chemostat. The heat output and other parameters were measured at various dilution rates (0.29 to 0.71 h^{-1}) under the same conditions as for standard glucose chemostats. Despite a low yield coefficient (0.38 g cell per g of pyruvic acid) which varied only slightly with D (Table 5.1) and a higher carbon dioxide production, the ΔH_p values were, within the limits of experimental error, the same as those of glucose or glycerol, and varied in a similar manner with D (Fig. 5.1). Acetate was produced at D> 0.5 h^{-1}, and unlike the other chemostats the pH of the culture was 7.1 to 7.2. The increase in pH appears to be due to uptake of pyruvate as free acid by the organisms. A considerable increase in pH for Pseudomonas aeruginosa growing in citrate-limited chemostat has been reported by Hamlin et al. (1967).

When the inflowing medium to the chemostat was stopped the rate of heat output decreased in two steps to a low value due to the presence of secondary intermediates (section 5.3).

5.1.3. Acetate-limited chemostat

Glucose-grown organisms were adapted to acetate by 15 subcultures in salts medium containing sodium acetate, prior to growth in an acetate-limited (12 mmol free acid dm^{-3}) chemostat. The heat output and other parameters were measured at various dilution rates (0.27 to 0.52 h^{-1}), under similar conditions to that of a standard glucose-limited chemostat. Although the yield coefficient was very low (i.e. 0.26 g cell per g free acid) and the rate of evolution of carbon dioxide was very high, the ΔH_p values at a given dilution

rate were identical to those of other carbon-limited chemostats, and varied with D in a similar manner. (Table 5.1). Residual acetate was detectable at D> 0.3 h^{-1}, the pH value of the culture was 7.15 to 7.2.

In acetate medium there are considerable fluctuations in the growth rate during repeated subculture of organisms (Dean and Hinshelwood, 1954). Although the organisms are adapted, the growth rate is not necessarily as rapid as in glucose medium. This is compatible with the residual acetate in chemostat culture medium.

5.1.4. Mixed glucose-acetate-limited chemostat

The organisms adapted to growth in acetate, were grown in acetate-limited chemostat (12 mmol free acid per dm^{-3}), at a fixed dilution rate D = 0.52 h^{-1}. After several generation times, when the steady state (with respect to biomass and the heat output) was attained, the chemostat was fed with the new medium containing mixed glucose and acetate, at concentrations of 2 and 6 mmol dm^{-3} respectively. The heat output and other parameters were measured regularly for several days. The initial biomass (0.12 mg cm^{-3}) in acetate-limited chemostat increased gradually and reached a steady high value (0.27 mg cm^{-3}) after 148 h. The specific heat output, ΔH_p which decreased markedly during the first 24 h, increased steadily to -1.69 kJ $(g\ cell)^{-1}$ after 148 h growth in mixed substrate medium; the evolution of carbon dioxide decreased from 77 to 26 mmol $(g\ cell)^{-1}$ correspondingly (Table 5.2).

Table 5.1. Thermal and environmental properties during the growth of K. aerogenes in glycerol-, pyruvate-, and acetate-limited chemostats.

Substrate	conc. /mmol dm^{-3}	D /h^{-1}	Y_{sub} /g cell (mol of sub)$^{-1}$	$-\Delta H_p$ /kJ (g cell)$^{-1}$	CO_2 evolved /mmol (g cell)$^{-1}$	Acetate mmol dm^{-3}
glycerol	7.8	0.29	41.7	4.4	ND	nd
		0.34	43.4	3.2	26.0	nd
		0.38	44.7	2.5	ND	nd
		0.51	47.9	1.56	ND	nd
		0.52	48.5	1.49	19.3	nd
		0.60	49.2	1.32	18.1	nd
		0.78	48.9	1.08	ND	nd
		0.88	49.7	1.04	ND	nd
pyruvate	8.2	0.29	29.8	3.9	48.8	nd
		0.35	31.1	2.6	43.9	nd
		0.52	32.7	1.5	ND	0.6
		0.52 *	22.5	1.78	ND	2.2
		0.71	33.5	1.31	36.0	0.9
Acetate	12	0.27	10.4	4.97	ND	nd
		0.30	11.0	3.13	106.2	0.4
		0.46	14.2	1.77	81.4	2.2
		0.52	15.8	1.57	76.9	3.7

* Complete medium including pyruvate was autoclaved. ND: not determined; nd: not detected.

Table 5.2. Growth of acetate-adapted cells of K. aerogenes in a mixed acetate (6 mmol dm^{-3}) and glucose (2 mmol dm^{-3}) medium, at D = 0.52 h^{-1}.

time /h	biomass /mg cm^{-3}	$-\Delta H_p$ /kJ (g cell)$^{-1}$	Conc./mmol dm^{-3} of acetate	glucose	CO_2 evolution /mmol (g cell)
0	0.12	1.57	3.7	0	76.9
24	0.19	2.2	1.3	nd	49.9
48	0.21	2.08	0.9	nd	51.5
120	0.26	1.73	0.5	nd	27.0
148	0.27	1.69	0.3	nd	26.0

nd: not detected

The data obtained suggested that, glucose was consumed before the acetate and the utilization of mixed substrate was as efficient as each carbon source alone, once the organisms have regulated their metabolic processes to the new environment.

5.2. Carbon-sufficient chemostats

5.2.1. Nitrogen-limited chemostat

The organisms were grown in N-limited medium (glucose-sufficient) prior to growth in N-limited chemostat (Table 2.1). The nitrogen source was ammonium sulphate and glucose was the carbon and energy source; this was supplied at different concentrations (2-15 mmol dm^{-3}). Glucose was detected in the samples taken from the fermentor, at all concentrations above 3 mmol dm^{-3}. The heat output and other parameters were measured under similar conditions to those used for standard glucose-limited chemostats. Since the rate of heat output did not vary with Δt (in the range 1.9 - 4 min), the heat output in the calorimeter cell $\Delta H'_p$, was recorded; this was not extrapolated back ($\Delta t = 0$) to give a value in the chemostat.

For dilution rates in the range 0.48 to 0.52 h^{-1}, the specific heat output at steady-state was independent of the ammonium sulphate concentration and also the glucose concentration, provided that glucose was present in excess. At a lower dilution rates $\Delta H'_p$ was markedly lower and this was accompanied by a higher carbon dioxide output. The biomass, unlike carbon-limited chemostats, was greater at lower dilution rates, it depended on ammonium sulphate concentration, and independent of glucose concentration (Table 5.3).

Table 5.3. Growth of cells of K. aerogenes in nitrogen-limited chemostat.

$(NH_4)_2 SO_4$ /mmol dm^{-3}	glucose Conc. /mmol dm^{-3}	D /h^{-1}	biomass /mg cm^{-3}	$-\Delta H'_p$ /kJ(g cell)$^{-1}$	CO_2 evolved /mmol (g cell)$^{-1}$
0.76	2.0	0.52	0.187	3.69	ND
0.76	4.0	0.52	0.185*	4.24	ND
0.76	5.5	0.52	0.180	4.35	39.6
0.76	12.2	0.52	0.189	4.49	40.7
0.76	15.5	0.52	0.185	4.29	ND
0.38	12.2	0.52	0.105	4.40	37.0
0.38	12.2	0.48	0.109	4.85	ND
0.38	12.2	0.32	0.125	7.28	58.3

* The corresponding biomass in glucose-limited chemostat was 0.374 mg cm^{-3}

ND: not determined.

In general, the specific heat output was high at all glucose concentrations, however it decreased markedly when the glucose concentration approached a value of 2 mmol dm^{-3}. This clearly indicates that the environment is also becoming carbon-limited. Acetate (1 to 2 mmol dm^{-3}) was produced in all chemostats and the pH of the cultures was 6.5 to 6.6.

5.2.2. Magnesium-limited chemostat

The organisms were grown in magnesium-limited medium (glucose-sufficient) prior to growth in a magnesium-limited chemostat (Table 2.1). Glucose was supplied in excess (6 mmol dm^{-3}) as carbon and energy source. The heat output and other parameters were measured at steady-state conditions; glucose was detectable at different dilution rates and at different concentration of magnesium sulphate.

The recorded heat output was not extrapolated to give a value in the chemostat since in glucose-sufficient chemostats the rate of heat output did not vary with Δt. The specific heat output as for N-limited chemostats, was higher than that for glucose-limited chemostats by a factor of 3 (Table 5.4).

Table 5.4. The growth of K. aerogenes in magnesium-limited chemostat (glucose conc. = 6 mmol dm^{-3})

Conc. of $MgSO_4$ /mmol dm^{-3}	D /h^{-1}	biomass /mg cm^{-3}	$-\Delta H'_p$ /kJ (g cell)$^{-1}$	CO_2 evolved /mmol (g cell)$^{-1}$
0.008	0.28	0.23	9.14	ND
0.008	0.53	0.19	4.90	28.5
0.016	0.52	0.28	4.74	30.6

ND: not determined.

An increase in D resulted in a marginal decrease in the rate of heat output; this is due to positive deviations in biomass at low dilution rates in these cultures. This behaviour is quite unlike that in glucose-limited chemostats.

5.3. Step down to zero growth rate

If the supply of fresh medium to a chemostat in its steady-state is stopped, then the dilution rate falls to zero (i.e. $\mu = D = 0$). It was of interest to monitor and record changes in the heat output of cells growing in carbon-limited and carbon-sufficient chemostats when the flow of medium was stopped. This should provide information about residual substrate and accumulated secondary intermediates.

To maintain a constant volume in the fermentor when the supply of fresh medium was stopped, the bacterial suspension was recycled through the microcalorimeter and back to the fermentor. The suspension leaving the microcalorimeter was equilibrated to the growth temperature by passing through a water jacket at 37°C before re-entering the fermentor. Changes in the rate of heat output, carbon dioxide production etc...., due to stopping the flow of fresh medium were continually monitored for 2-3 h from the cut off time of the fresh medium (Fig. 5.2). It was not possible to monitor the heat change for long periods due to bacterial sedimentation in the calorimeter cell (section 3.4); this was especially serious for glucose-sufficient cultures, where heavy sedimentation and much higher heat outputs were observed.

The heat response observed for carbon-limited chemostats was significantly different to that for carbon-sufficient chemostats.

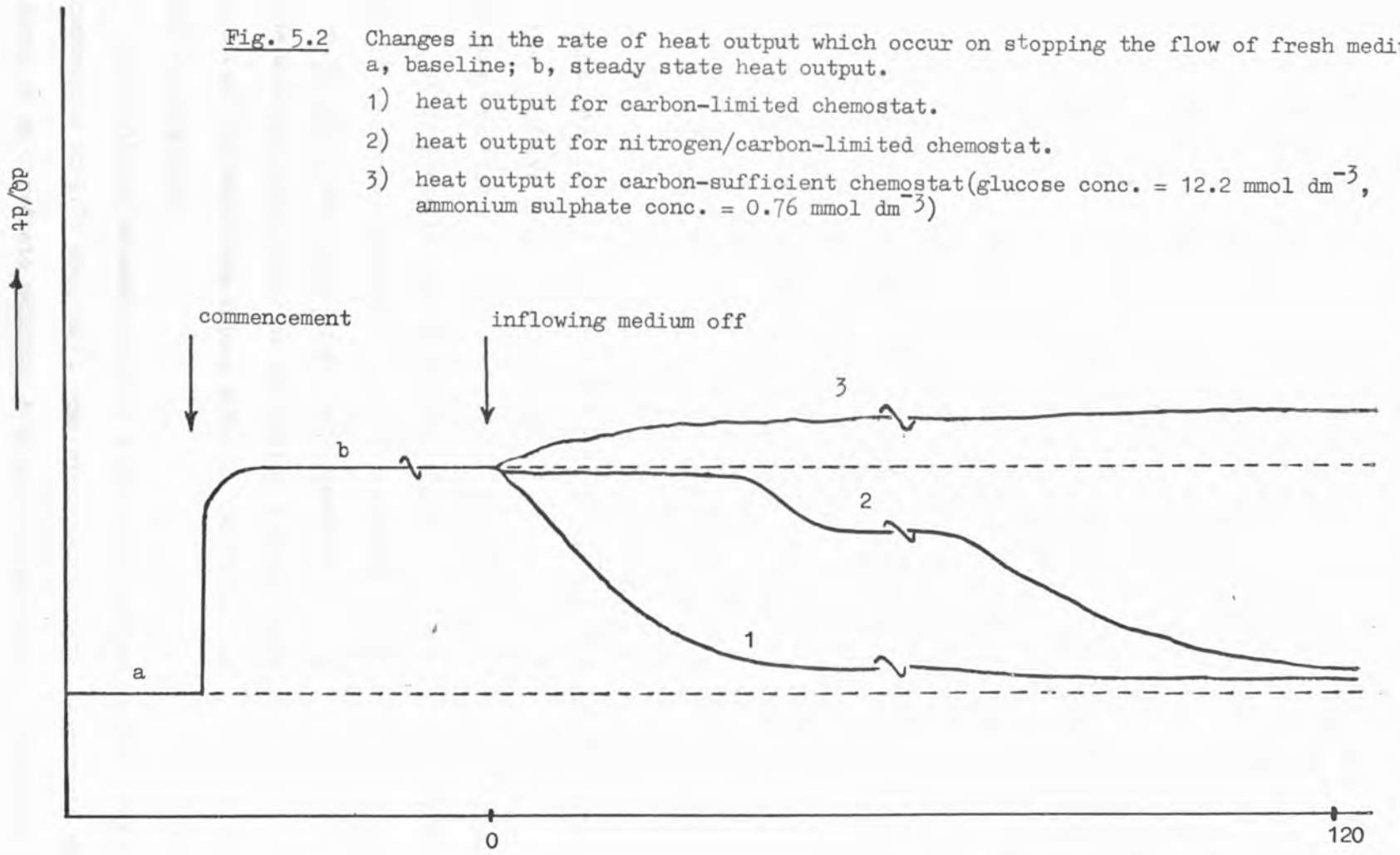
For glucose- and glycerol-limited chemostats, the rate of carbon dioxide production and heat output immediately began to decrease and within 15 min were 5 - 10% and 15 - 25% respectively of their initial steady-state values (Curve 1 in Fig. 5.2); indicating the absence of residual substrate and any secondary metabolite. Eventually the rate of heat output approached the base-line value. In pyruvate-limited chemostats the heat output decreased immediately but levelled off at

Fig. 5.2 Changes in the rate of heat output which occur on stopping the flow of fresh medium, a, baseline; b, steady state heat output.

1) heat output for carbon-limited chemostat.

2) heat output for nitrogen/carbon-limited chemostat.

3) heat output for carbon-sufficient chemostat(glucose conc. = 12.2 mmol dm^{-3}, ammonium sulphate conc. = 0.76 mmol dm^{-3})



about 55% of its initial value after 15 min. Since acetate was present in such cultures, the residual heat output could be due to the consumption of the accumulated acetate. When acetate consumption was complete the heat output then decreased towards the base-line. There was no marked change in biomass in these cultures. When the flow of fresh medium to acetate-limited chemostat cultures was stopped, the rate of heat output gradually decreased, the extent depended on the residual acetate. The biomass increased slightly (7-10%) in these cultures presumably due to the utilization of residual acetate to form more cellular material.

In carbon-sufficient chemostats (glucose conc. = 12.2 mmol dm^{-3}; ammonium sulphate conc. = 0.76 mmol dm^{-3}), when the flow of fresh medium was stopped the rate of heat output increased (15 - 20%) and continued at the increased rate for some time (Curve 3, in Fig. 5.2). Carbon dioxide production was at 50 - 60% of its steady-state value within 15 - 20 min, and continued at this rate until the C-source was depleted. There was 25 - 30% increase in the biomass due to conversion of excess glucose into cellular material; this is in agreement with the positive deviations in these cultures. The changes of heat output were only monitored for a limited period (2 - 3 h); after this time heavy sedimentation of bacterial cells in the microcalorimeter cell gave erratic and irreproducible heat output. On the complete utilization of the carbon source, the heat output will attain the base-line value, after an initial arrest while residual acetate is degraded.

At a glucose concentration of 2 mmol dm^{-3} and ammonium sulphate concentration of 0.76 mmol dm^{-3}, the step down to zero growth rate was followed by an immediate marginal decrease in heat output. Within a period of about 2 h this decreased drastically in two distinct phases

(Curve 2 in Fig. 4.2). Glucose was present only as a trace apparently the carbon-sufficient condition was approaching the carbon-nitrogen-limited condition. The first fall in heat output, which occurred after about 30 min was clearly due to the depletion of extracellular carbon source (glucose), while the second was the result of the breakdown of secondary matabolites, (acetate) as confirmed by GLC analysis.

5.4. Summary

1. The specific heat output is constant and independent of the carbon source (glucose, pyruvate, glycerol, acetate) provided that the organisms are fully adapted to the carbon source. The yield coefficient and its variation with D, depended on the substrate.

2. The specific heat output in carbon-sufficient chemostats (Mg or N-limited) was higher than that obtained in carbon-limited chemostats and was independent of limiting nutrient and carbon source concentrations provided that, the carbon source was supplied in excess.

3. In all carbon-limited chemostats the heat output increased when D increased, while in carbon-sufficient chemostats the heat output decreased with increase in D.

4. On step-down to zero growth rate, the heat output was decreased immediately in carbon-limited culture whereas in carbon-sufficient chemostats, the heat output was increased slightly and continued at this rate until the residual substrate was used.

CHAPTER 6

DISCUSSION

A reproducible technique has been developed and established for the measurement of energy changes associated with the growth of cells of K. aerogenes in chemostat cultures. For a given set of growth and pumping conditions the reproducibility of the heat output is ± 7%; this is acceptable for a biological system involving two separate experimental techniques. Thus a change of more than 10% in the heat output is significant.

Among the other environmental parameters; pH was measured with an uncertainity of ± 0.05 pH unit; the reproducibility of molar growth yield (Y_{sub}) was ± 4% (therefore a change of more than 6% is significant). The concentration of acetate (GLC) and glucose (o-toluidine), were measured with an accuracy of less than ± 2% and ± 1% respectively. The dilution rate and flow rate were measured by collecting samples for known time, with an accuracy of better than ± 1%. The poor reproducibility of carbon dioxide production (± 10%) is due partly to the measurement of the total volume of effluent air from the fermentor and partly to temperature changes of the air which occurred on passing from the fermentor to the gas analyser. To avoid errors due to carbon dioxide changes within the laboratory all the air for the fermentor and for the reference beam of the analyser was taken from outside the building.

The problems associated with oxygen depletion in the microcalorimeter cell (Eriksson and Holme, 1973; Cardoso-Duarte et al., 1977) were eliminated by using lower and limiting substrate concentrations (i.e. lower biomass), higher flow rates and adequate aeration of the culture vessel. Under these circumstances a fully aerobic culture was maintained in the calorimeter cell as well as in the fermentor.

This was confirmed by the fact that when alternate pulses of air were passed through the microcalorimeter cell no change in the steady-state heat output was detected, in marked constrast, a sharp peak was superimposed on the steady-state heat output in oxygen deficient cultures. Under standard conditions the oxygen tension, which was constant once steady-state conditions had been established, depended on biomass and concentration of carbon source, however it never dropped below 35% of full saturation.

The development of limited oxygen state in carbon-limited chemostats of K. aerogenes, was accompanied by complex oscillations in the dissolved oxygen tension (Harrison et al., 1967). Similar changes occurred in the work described here when the oxygen limitation was initiated by the sudden addition of large amounts of extra substrate into the steady-state growing culture. These oscillations lasted for several generation times.

The difficulties encountered with the lack of temperature control of the fresh medium (Ackland, Prichard and James, 1976) and the growing culture in the connecting tubing to the microcalorimeter have been successfully overcome using an efficient heating coil and thermostatting the connecting tubing ($37.0 \pm 0.5^{\circ}$ C). It is of great importance to ensure that temperature equilibrium in the microcalorimeter is attained, otherwise the measured heat output is in error due to heat absorbed or evolved by the culture to bring it to temperature equilibrium.

Recycling of the culture (after passing through the microcalorimeter cell, the pH and oxygen electrode assemblies) disturbed the steady state conditions in the chemostat and this, in turn, produced erratic changes in the heat output. In consequence the technique adopted was to pump the culture through the

microcalorimeter to waste, thus the flow rate could not exceed the dilution rate. For the chemostat vessel available, (160 cm^3 of culture) measurements were not possible at dilution rates below 0.25 h^{-1}. Lower dilution rates could be achieved with a larger volume of culture in the chemostat and hence a larger fermentor. This would introduce further problems associated with adequate aeration and stirring of larger volumes of culture.

The rate of heat output of a chemostat culture in its steady-state was constant (i.e. $dQ/dt = 0$). However after pumping the culture through the microcalorimeter for several hours there was a gradual increase in the rate of heat output. This was associated with wall growth and/or sedimentation of bacteria in the microcalorimeter cell (Eriksson and Holme, 1973; Gustafsson and Lindman, 1977). In experiments which involved taking measurements over an extended period it was necessary to disconnect the fermentor from the microcalorimeter and wash out the calorimeter cell and re-establish the baseline. On reconnecting the fermentor the original steady state heat output was reattained.

The heat output of steady-state cells is a linear function of the transit time, Δt, from the chemostat to the microcalorimeter cell (Fig 3.2.). The higher the pump rate (i.e. lower Δt values) the nearer is the state of the cells in the calorimeter cell to that of cells in the chemostat. At the very high pump rates great care must be taken to ensure that thermal equilibrium is established in the microcalorimeter, for this reason all connecting tubes were thermostatted and kept as short as possible; flow rates in excess of 90 $cm^3\ h^{-1}$ were not used. Although thermal equilibrium is well

established at very low flow rates (below 40 cm^3 h^{-1}) these low flow rates are not suitable for studying bacterial suspensions because depletion of substrates, oxygen etc. occurs in the flow lines and in the measuring cell. By extrapolation to $\Delta t = 0$, the rate of heat output by the cells in the chemostat can be estimated; over a range of dilution rates this was 4.8×10^{-4} kJ s^{-1} (g cell)$^{-1}$.

The specific heat output of cells in the fermentor (ΔH_p) was calculated by the equation;

$$\Delta H_p = \Delta H'_p + C \ \Delta t \ td \qquad 3.4$$

where, $\Delta H'_p$, is the recorded specific heat output, calculated using equation 3.2 and C a constant equal to 3.60×10^{-5} kJ s^{-1} (g cell)$^{-1}$ per min (Fig. 3.2). The time interval chosen for the calculation of ΔH_p was the doubling time (i.e. ln 2/D). This enables direct comparison of the heat changes of cells growing at different dilution rates and under different conditions (Table 6.1).

The growth rate, molar yield values and the specific heat output for cells growing in glucose medium were the same over the small temperature range (37 - 42° C) investigated. This supports the results of Hunter and Rose (1972), and indicates that over this limited range the change in entropy (ΔS) for all the processes is zero (i.e. $\Delta S = 0$). Thus since:

$$\Delta G = \Delta H - T\Delta S \qquad 6.1$$

it follows that:

$$\Delta G = \Delta H$$

Table 6.1. Thermal and environmental properties of the growth of *K. aerogenes* grown under different conditions in chemostat cultures, $D = 0.52\ h^{-1}$, pH = 7, $T = 37^{\circ}$ C.

Limiting nutrient and conc. /mmol dm^{-3}	Carbon source and conc. /mmol dm^{-3}	inhibitor or uncoupler and conc. /mmol dm^{-3}	Y_{sub} /g cell per mol	$-\Delta H_p$ /kJ(g cell)$^{-1}$	CO_2 evolved /mmol (g cell)$^{-1}$	Y_{carbon} /g cell per g C
Acetate 12.00	-	none	15.8	1.57	76.9	0.66
Pyruvate 8.20	-	"	32.7	1.50	ND	0.91
Glycerol 7.80	-	"	48.5	1.49	19.3	1.35
Glucose/acetate (2 , 6)	-	"	-	1.69	26	-
$(NH_4)_2 SO_4$ 0.38	glucose 12.2	"	-	4.40	37	-
" 0.76	" 12.2	"	-	4.49	40.7	-
Mg SO 0.016	" 6	"	-	4.74	30.6	-
Glucose 1.90	-	"	92.6	1.62	20.8	1.29
" 4.90	-	"	94.5	1.57	20.0	1.31
" 4.00	-	"	93.5	1.60	18.7	1.30
"	-	DNP 0.04	90.4	1.70	22.2	1.25
" "	-	" 0.24	84.7	2.60	27.8	1.18
" "	-	" 0.50	83.9	2.46	26.8	1.16
" "	-	" 1.00	85.5	2.51	29.6	1.19
" "	-	Azide 0.62	75.0	2.17	30.7	1.04
" "	-	Nalidixic acid 0.43	65.5	1.41	18.4	0.91

Table 6.1 (continued)

Limiting nutrient and conc. /mmol dm^{-3}	Carbon source and conc. /mmol dm^{-3}	inhibitor or uncoupler and conc. /mmol dm^{-3}		Y_{sub} /g cell per mol	$-\Delta H$ /kJ(g cell)$^{-1}$	CO_2 evolved /mmol (g cell)$^{-1}$	Y_{carbon} /g cell per g C
Glucose 4	-	Na amytal	0.07	98.5	1.56	16.5	1.37
" "	-	"	0.15	103.8	1.60	15.3	1.44
" "	-	Na barbitone	2.40	83.7	2.58	23.6	1.16
" "	-	"	2.80	74.9	2.81	34.7	1.04
" "	-	Rotenone	0.20	102.5	3.35	28.5	-
" "	-	Control	none	103.5	3.43	26.9	-
" "	growth pH = 6.0	-		83.7	1.30	22.1	1.16
" "	growth pH = 8.0	-		90.7	1.66	23.2	1.26
" "	growth temp. 42°C	-		91.5	1.60	ND	1.27
" "	anaerobic growth	-		32.9	2.90	47.2	0.46

ND, not determined

This implies that, within the limits of experimental error, the measured enthalpy change (ΔH_p) can be identified with the free energy change. The free energy changes for different steps of glycolysis are shown in Fig. 6.1.

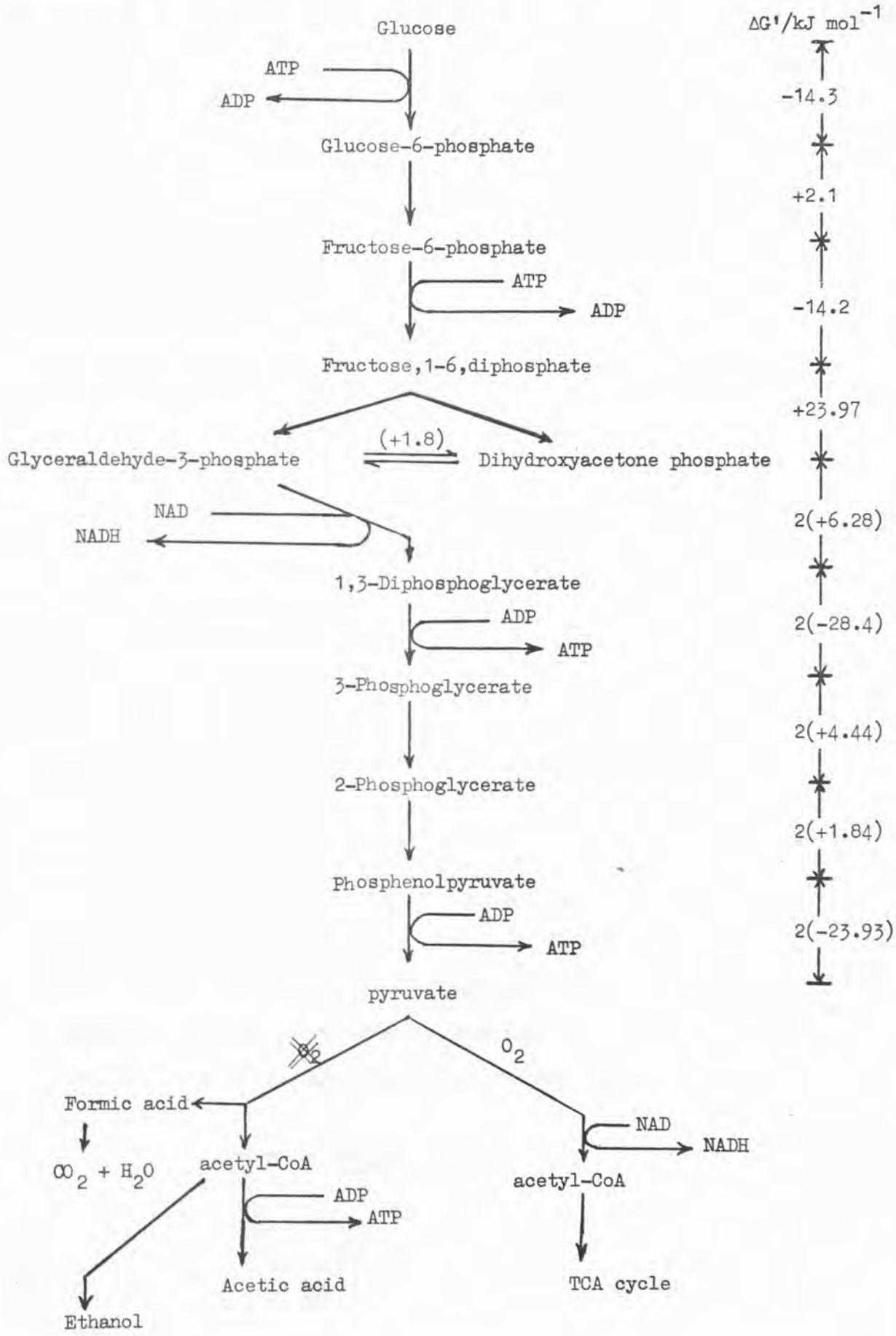
Under fully aerobic conditions in carbon-limited chemostats, growing at various glucose concentrations (2-5 mmol dm^{-3}), all the carbon source is converted to cellular material and carbon dioxide (Table 6.1). The yield coefficient of K. aerogenes growing under these conditions was 0.52 ± 0.02 (i.e. Y_{glu} = 94 ± 3.6 g cells per mol of glucose). The yield coefficient decreased at lower growth rates (D) in agreement with the results of other investigators (Herbert, 1961; Harrison et al., 1971; Tempest et al., 1967). The equation:

$$\frac{1}{Y_c} = \frac{m_s}{\mu} + \frac{1}{Y_c^{max}} \qquad 1.14$$

derived by Pirt (1965), assumes a constant maintenance coefficient (m_s) for all growth rates; the validity of this has been questioned by some workers (Stouthamer et al., 1973; Neijssel et al., 1976) In a number of cases no linear relationship was obtained (Pirt, 1965; de Veries et al., 1970; Watson, 1970); this was shown to be due to an influence of μ (D) on the metabolic pattern and Y_{ATP} of the organism. Equation 1.14, was modified by replacing Y_c and Y_c^{max} by Y_{ATP} (yield per mol of ATP) and Y_{ATP}^{max} and multiplying by μ (Stouthamer and Bettenhaussen, 1973) to give:

$$\text{Rate of ATP production} = q_{ATP} = \frac{\mu}{Y_{ATP}} = \frac{\mu}{Y_{ATP}^{max}} + m_e \qquad 1.15$$

Fig. 6.1. Glycolysis or Embden Meyerhof pathway



In relating the experimental and theoretical value of Y_{ATP}^{max} Stouthamer *et al.* (1977) obtained;

$$q_{ATP} = \frac{\mu}{Y_{ATP}^{max}} + m_e = \frac{\mu}{Y_{ATP(theor)}^{max}} + m_g\mu + m_e \qquad 6.2$$

In this equation the maintenance requirement is split into two terms: m_g the growth-rate-dependent energy requirement; and m_e the growth-rate-independent energy requirement, for purposes other than formation of new cell material.

The double reciprocal plot of yield coefficient and growth rate (equation 1.14) for all concentrations of glucose consists of two linear portions (Fig. 6.2), with calculated maintenance coefficients of 0.19 and 0.048 g glucose (g cell h)$^{-1}$ for dilution rates greater than or less than 0.5 h^{-1} respectively; Y_c^{max} = 0.54 g cell (g glucose)$^{-1}$. Glycerol-limited chemostat cultures also behaved in a similar manner, the calculated maintenance coefficients were 0.14 and 0.055 g glycerol (g cell h)$^{-1}$ for dilution rates greater than and less than 0.5 h^{-1} respectively (Fig. 6.3); Y_c^{max} = 0.56 g cells (g glycerol)$^{-1}$. Erickson *et al*, (1979) in an analysis of data presented by Herbert (1959) for glycerol-limited chemostat culture of *K. aerogenes* using equation 1.14, obtained a maintenance coefficient of 0.076 g glycerol (g cell h)$^{-1}$ and Y_c^{max} = 0.56. These authors developed consistency tests on the basis of material and energy balances to investigate growth and maintenance requirements of a culture. The equations derived were analogous to;

$$\frac{\mu}{Y_c} = \frac{\mu}{Y_c^{max}} + m_s \qquad 6.3$$

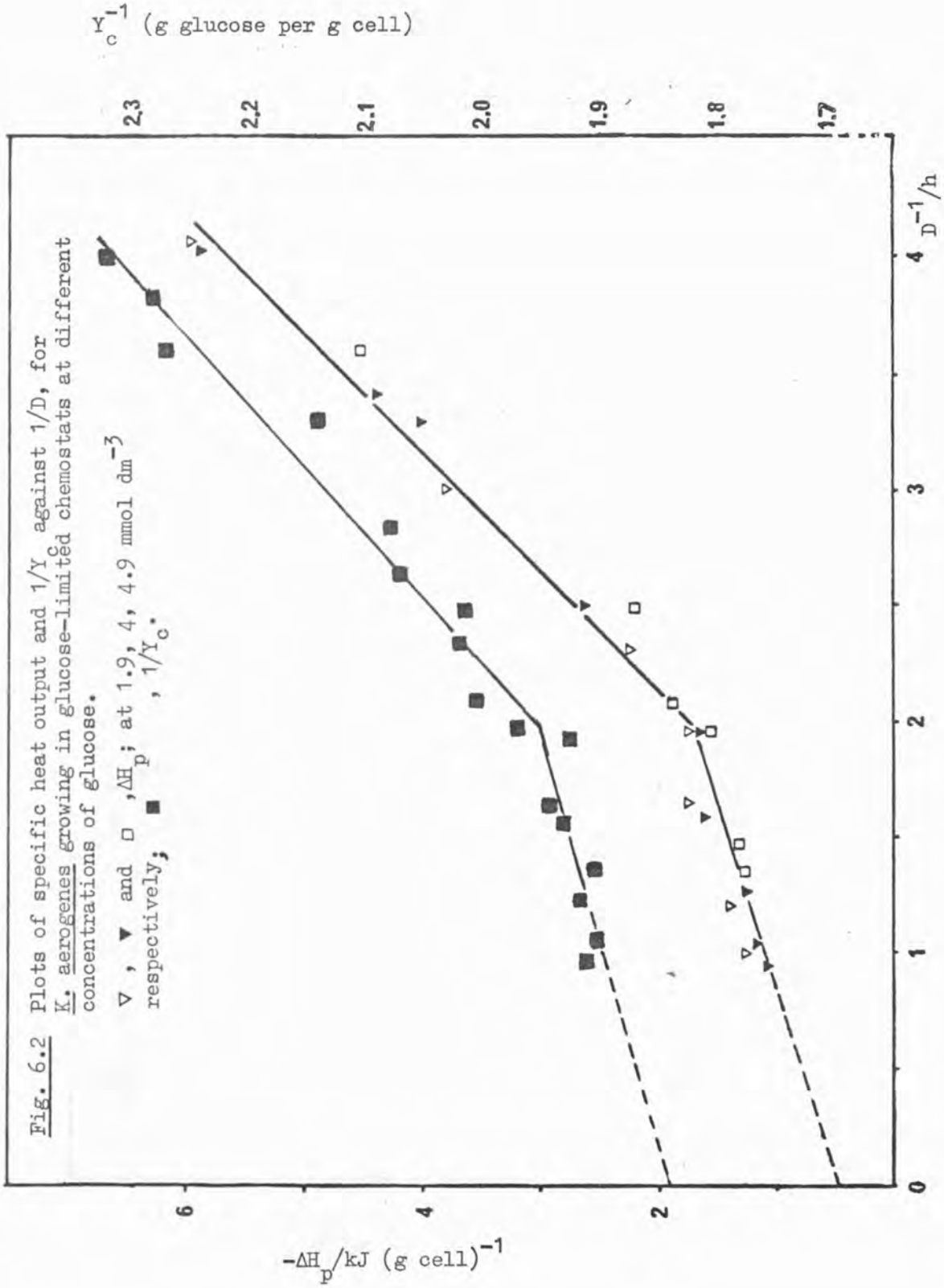


Fig. 6.2 Plots of specific heat output and $1/Y_c$ against $1/D$, for *K. aerogenes* growing in glucose-limited chemostats at different concentrations of glucose.

▽, ▼ and □, ■; at 1.9, 4, 4.9 mmol dm^{-3} glucose, respectively; ▽, □, $-\Delta H_p$; ▼, ■, $1/Y_c$.

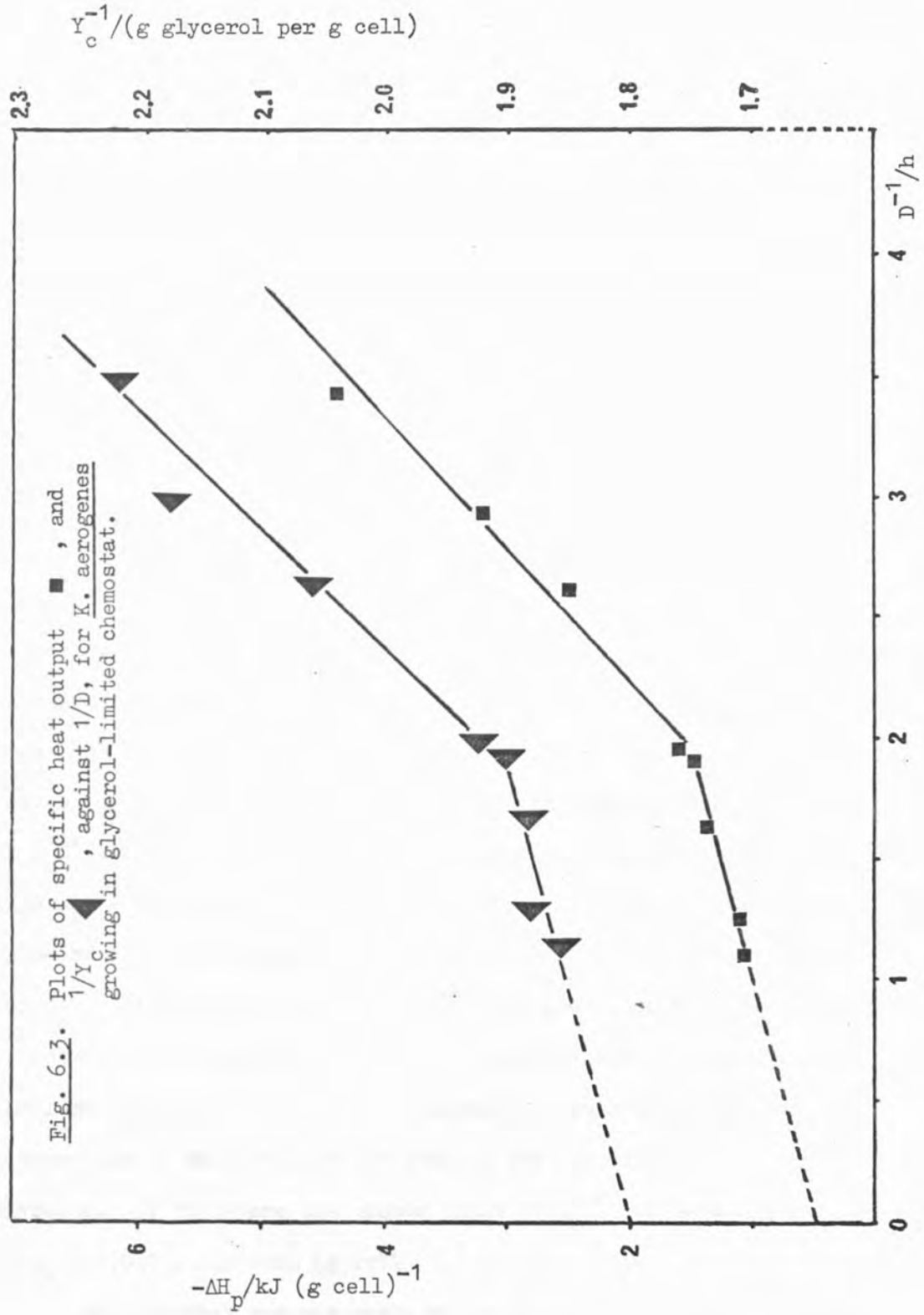


Fig. 6.3. Plots of specific heat output ■, and $1/Y_c$ ▲, against 1/D, for K. aerogenes growing in glycerol-limited chemostat.

This is the Pirt equation in which every term is multiplied by μ (i.e. D). Replacing m_s in equation 6.3, by a growth rate-dependent and a growth rate independent term we obtain:

$$\frac{D}{Y_c} = D\left[\frac{1}{Y_c^{max}} + m_g\right] + m'_s \qquad 6.4$$

where m_g, is growth rate-dependent and m'_s is growth rate independent maintenance coefficient.

The plots of D/Y_c against D for glucose and for glycerol are linear (Fig. 6.4); this clearly indicates that part of the maintenance coefficient is growth rate-dependent. The intercept, m'_s of the lines, were 0.11 and 0.14 g sub (g cell h)$^{-1}$ and the slopes of the lines $[1/Y_c^{max} + m_g]$, 1.79, and 1.68 for glucose and glycerol respectively. Substituting the values of Y_{sub}^{max} obtained from double reciprocal plots (Figs. 6.2 and 6.3.) gave negative values for m_g for both glucose and glycerol. However substituting of $Y_c^{max} = 0.58$ obtained from the plot of ΔH_p against Y_c (Fig. 6.7.), gave $m_g = 0.061$ g glucose (g cell)$^{-1}$ for glucose. Using the value of Y_c^{max} for glycerol of 0.565 (Fig. 6.7) a negative value for m_g in glycerol culture was still obtained. This value was rejected, since maintenance cannot be negative. Moreover, the error involved in the estimation of Y_c values is higher than that for glucose due to difficulties involved in the quantification of glycerol. If therefore the higher limit of Y_c^{max} is used a value of $m_g = 0.041$ g glycerol (g cell)$^{-1}$ is obtained.

The observed maximum yield value in acetate-limited chemostat was $Y_{ac} = 16$ g cell per mol of acetate, which is close to the value of 18.9 reported, elsewhere (Bolton, et al., 1972), and much higher

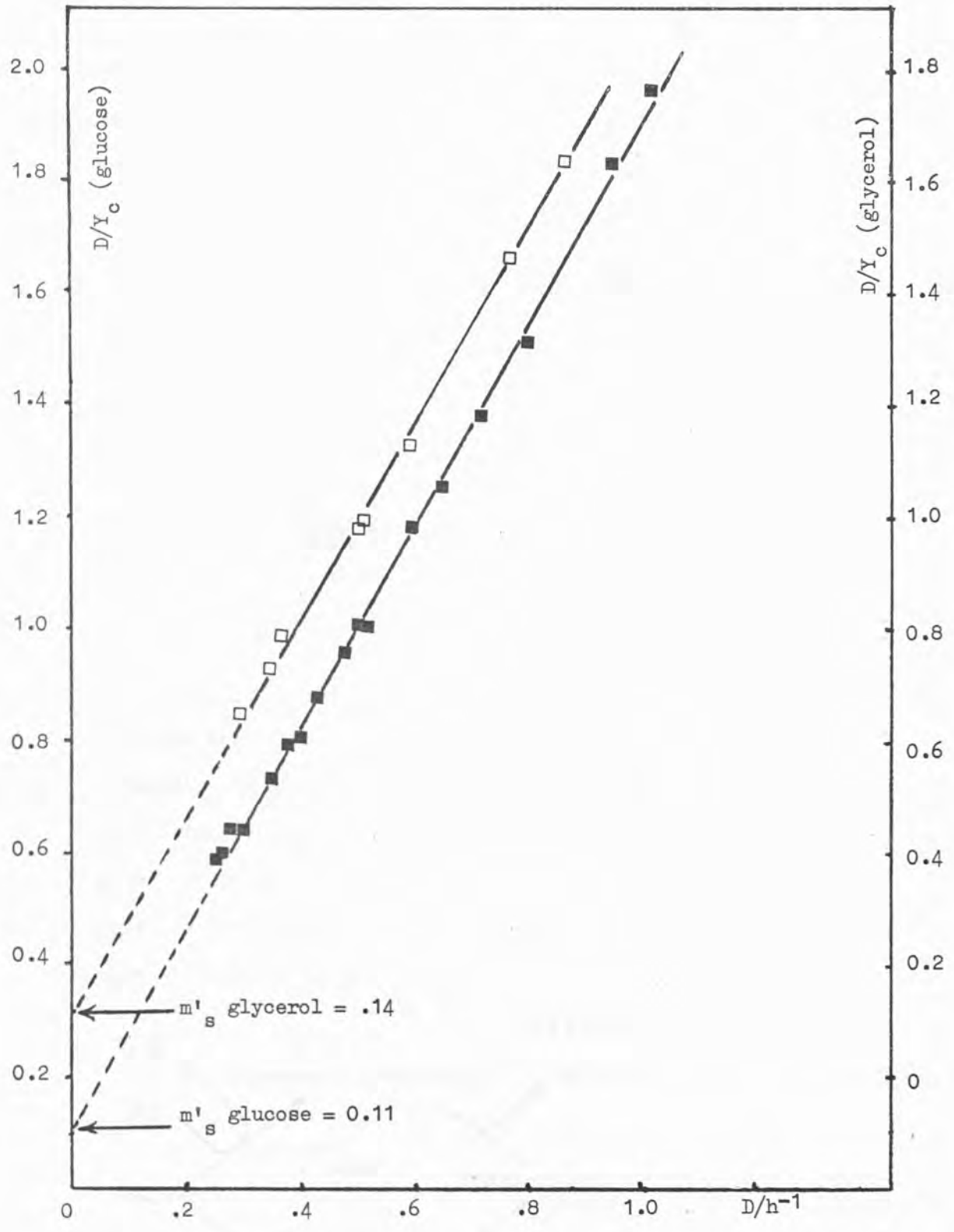


Fig. 6.4. The plot of D/Y_c against D for K. aerogenes grown in glucose-limited, ■ , and glycerol-limited, □ , chemostats.

than 10.5 reported by Hadjipetrou et al., (1964). The molar growth yield in pyruvate-limited chemostat (Y_{py} = 34 g cell per mol of pyruvate) was, however, much higher than the values 12 - 17.9 reported by Hadjipetrou et al., (1964) and Bolton et al., (1972) respectively. This large discrepency may be attributed to the growth conditions in particular in the preparation of the medium, since a lower yield value, Y_{py} = 22 was obtained when the complete pyruvate medium was autoclaved.

Among the substrates investigated (Table 6.1), the order of efficiency in terms of observed yield values was; glycerol $\gg$ glucose $>$ pyruvate $>$ acetate.

The high efficiency of glycerol is due presumably to the existence of dual pathway for its assimilation in these organisms (Neijssel et al., 1975), which is represented in Fig. 6.5. These two pathways are glycerol dehydrogenase and glycerol kinase with extremely low and high affinities respectively. Glycerol dehydrogenase is seemingly induced in the excess glycerol environment, whereas glycerol kinase is induced under glycerol-limited conditions. This dual pathway enables the organisms to utilize the energy source more efficiently.

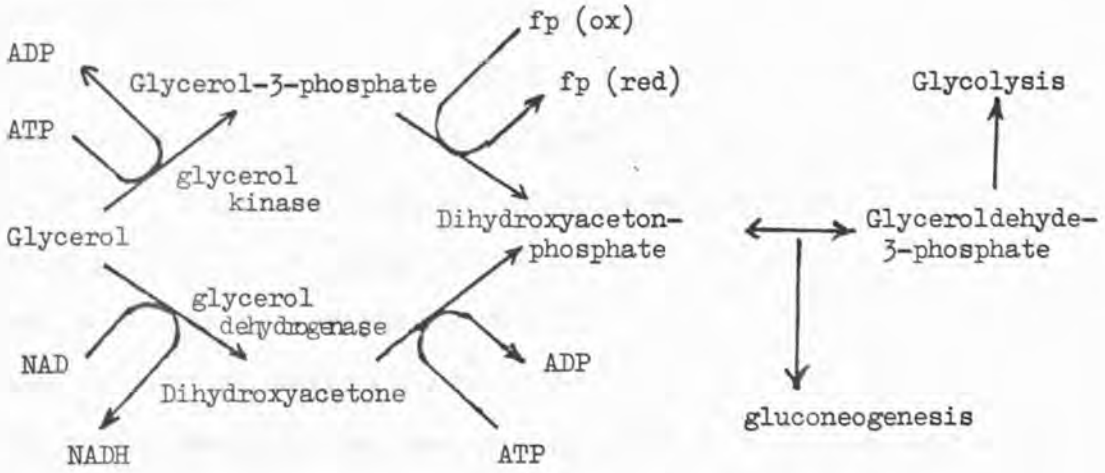


Fig. 6.5. Dual pathway for glycerol assimilation in K. aerogenes (Neijssel et al., 1975)

Although acetate is in the same oxidation state as glucose nevertheless it has the lowest efficiency. This is most probably due to the fact that, the first step in acetate metabolism is the conversion to acetyl-Co A by acetyl-Co A synthetase, an AMP forming process (Webster, 1965) and also gluconeogenesis processes which are all energy consuming. When the organisms are growing on short chain fatty acids as a sole carbon and energy source, there is a draining off of intermediates of TCA cycle; for biosynthetic purposes. This continual tapping off of these intermediates is replenished by a cyclic mechanism called the "glyoxylate cycle" Fig. 6.6., (Kornberg et al., 1957; and 1958; Wegner et al; 1968). Therefore the potential energy source (TCA cycle) is partially lost; the lower recorded yield values are consistent with these mechanisms.

For cells of K. aerogenes growing anaerobically in glucose-limited (4 mmol dm^{-3}) chemostat cultures at D = 0.52 h^{-1}, the yield value Y^{ana}_{glu} = 32.7 g cell per mol of glucose and ΔH_p = - 2.92 kJ $(g\ cell)^{-1}$. The main fermentation products are, acetate, ethanol, formate and lactate (Hadjipetrou et al., 1964). In this type of fermentation, pyruvate is decomposed by a thioclastic reaction to acetyl-Co A and formate. Acetyl-Co A is converted to acetate by means of phosphate-acetyltransferase and acetate-kinase. Thus above the net gain of 2 mol of ATP per mol of glucose produced during glycolysis, an additional mol of ATP is generated for each mole of acetate formed. Ethanol which is also formed in this fermentation is produced by reduction of acetyl-Co A (Dawes et al., 1956) and energy from acetyl-Co A is lost during its conversion to ethanol. Presumably this reaction is responsible for the high value of ΔH_p recorded under anaerobic conditions. The net gain of ATP can only be calculated when the amount of acetate produced

during the fermentation is known (Hadjipetrou et al., 1964).

The amount of acetate produced per mol of glucose under anaerobic conditions was 0.6 mol (i.e. 0.6 mol of ATP). Thus under these conditions the net gain of ATP is 2.6 mol per mol of glucose and $Y_{ATP} = 32.9/2.6 = 12.6$ g cell $(\text{mol ATP})^{-1}$ in good agreement with results of Hadjipetrou et al., (1964).

The concept of Y_{ATP} was first introduced by Bauchop and Elsden (1960), who reported a mean value of $Y_{ATP} = 10.5$ for a wide range of organisms. This concept has proven very useful in interpreting the energetics of anaerobic growth, where the glycolytic system of glucose utilization prevails (Hadjipetrou, et al, 1964; Stouthamer, 1969; Stouthamer et al., 1976), but turned out to be very unsatisfactory for aerobic cultures (Neijssel et al., 1976; Forrest and Walker 1964). This is because the complicated nature of oxidative phosphorylation in microorganisms has not yet been established.

Several investigators attempting to relate ATP-production and cell yield in aerobes have resorted to estimates of ATP-gain based on oxygen consumed; values for $p/2\bar{e}$ (van der Beck, et al., 1973; Hempfling, 1970; Knowles et al., 1970; Ackrell and Jones, 1971); H^+/O ratios (Brand et al., 1976; Garland, 1977; Jones, 1977) and at times on fragmentary knowledge of the components of the electron chain in the organism under study.

Although a chaotic situation exists on potential oxidative phosphorylation in microorganisms, there is evidence in support of two phosphorylation sites in K. aerogenes (Harrison and Loveless 1971a; Stouthamer et al., 1976).

Assuming a similar value of Y_{ATP} = 12.6 under aerobic conditions, 7.46 mol of ATP should be realized for the production of 94 g cell per mol of glucose utilized. (i.e. 94/12.6 = 7.46). From the elemental analysis and carbon dioxide evolution about 40% of glucose is degraded for energy requirements and the rest is converted to cellular material. If there is only one site of phosphorylation in the respiratory chain only 6.4 mol of ATP is obtained per mol of glucose consumed. (i.e. 16 mol per mol of glucose degraded). Therefore there must be more than a single phosphorylation site to meet the amount of cellular material produced and also to meet the maintenance requirements. The actual number of phosphorylation sites is still obscure in microorganisms. However there is evidence (Bordie et al., 1965; Rose, 1965) that, in bacteria the chain is shorter with fewer catalysts, for P/O ratios of 1.0 and below are usually obtained with bacterial cell free extracts. This supports the presence of one phosphorylation site in the electron transport chain, but it is possible that the chain operates fully in intact cells and that ATP production is restricted by the shortage of reduced respiratory components. In support of this is the important role of TCA cycle in the synthesis of cell material (Roberts et al., 1955; Gilverg et al., 1956; Trivett et al., 1971), during which components of the TCA cycle are continually drained off. These components are replenished by the glyoxylate bypass (Kornberg et al., 1957 and 1958; Kornberg, 1958), in which only 1 mole of NADH is produced in comparison to 3 mole in the complete TCA cycle (Fig. 6.6).

Under aerobic conditions of growth one mole of glucose is converted to 2 mol of pyruvate and thus enters the TCA cycle and provides the energy and biosynthetic precursors for cell growth and

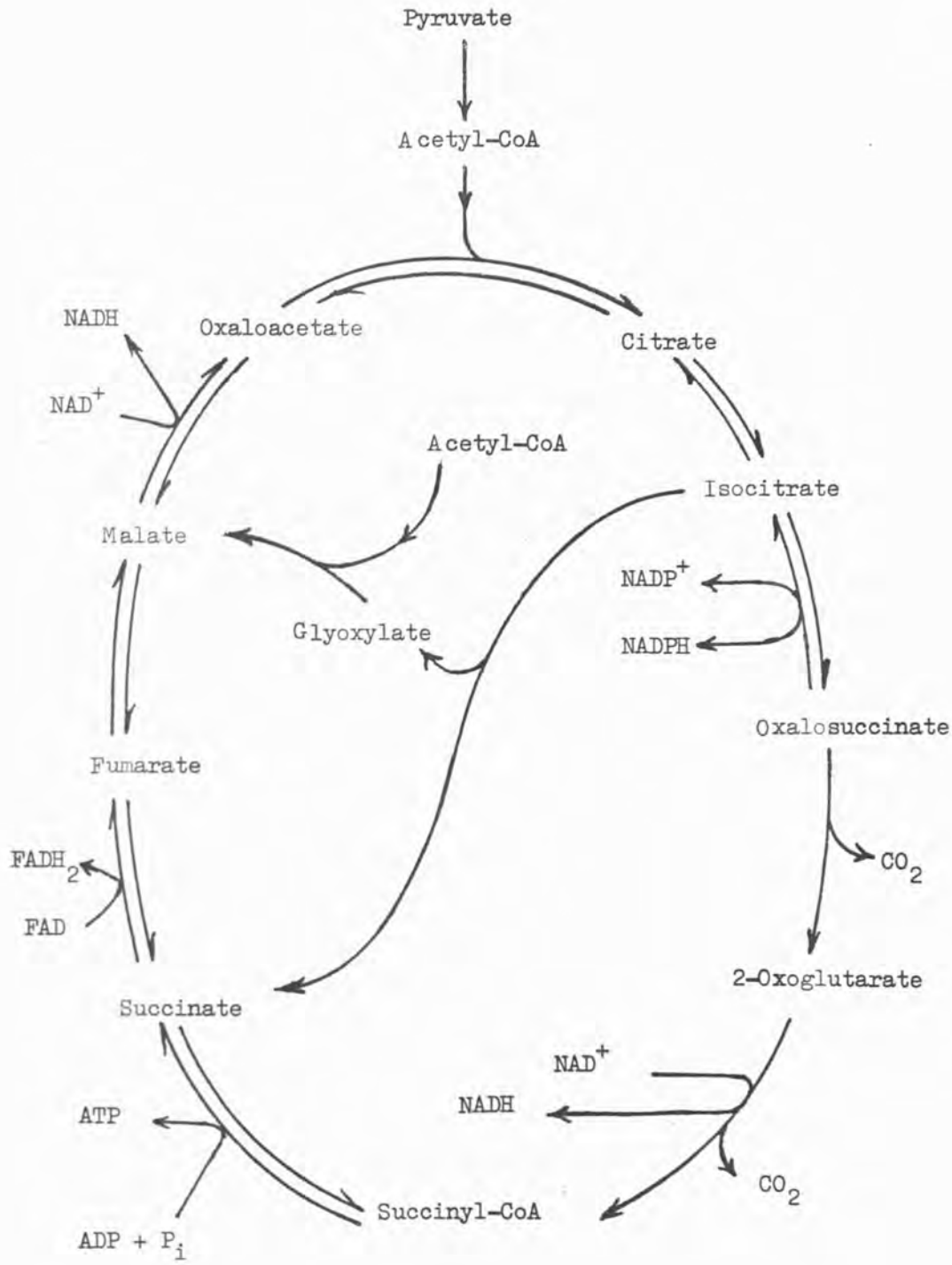


Fig. 6.6. The Kreb's (TCA) cycle; and Glyoxylate by-pass.

division. From the measured data during growth at D = 0.52 h^{-1}: Y_{glu} = 93.5; Y_{glu}^{ana} = 32.9; Y_{ATP} = 12.6; the amount of acetate formed during the anaerobic growth = 0.6 mol per mol of glucose; the pyruvate yield may be calculated as follows:

$$Y_{glu} = Y_{glu}^{ana} - 0.6\ Y_{ATP} + 2\ Y_{py} \qquad 6.5$$

The term, $Y_{glu}^{ana} - 0.6\ Y_{ATP}$, is the yield value for only glycolysis and $2\ Y_{py}$ is the yield of 2 mol of pyruvate.

From equation 6.5 it follows:

$$Y_{py} = \frac{Y_{glu} - Y_{glu}^{ana} + 0.6\ Y_{ATP}}{2}$$

$$= \frac{93.5 - 32.9 + (0.6 \times 12.6)}{2}$$

$$= 34.28\ g\ \text{cell (mol pyruvate)}^{-1}$$

This value is in good agreement with the experimental value 32.7 There is thus support for the above argument.

Having established the molar growth yield balance it should be possible to establish a molar heat output balance in a similar manner:

$$\Delta H_{glu} = \Delta H_{glu}^{ana} + 2\Delta H_{py} - \Delta H_{ac} \qquad 6.6$$

where ΔH_{glu} and ΔH_{glu}^{ana} are the heat evolved per mol of glucose under aerobic and anaerobic conditions respectively; ΔH_{py}, is the heat evolved per mol of pyruvate, ΔH_{ac}, the additional heat evolved due to extra phosphorylation during anaerobic growth [i.e. $0.6\ Y_{ATP} \times \Delta H_{p}^{ana}$ where ΔH_{p}^{ana} is the anaerobic specific heat output /kJ $(g\ \text{cell})^{-1}$], substituting the appropriate values (Table 6.1)

in equation 6.6. gives;

$$\Delta H_{glu} = (\Delta H_p^{ana} \times Y_{glu}^{ana}) + 2\ (\Delta H_p \times Y_{py}) - 0.6\ Y_{ATP}\ \Delta H_p^{ana}$$

$$= -95.4 - 98.1 + 21.9$$

$$= -171.6\ kJ\ mol^{-1}$$

This value is greater than the heat evolved per mol of glucose under aerobic conditions ($\Delta H_{glu} = -148.5$). This difference may be attributed to the energy lost during the formation of ethanol (Dawes et al., 1956) under anaerobic conditions. Since the amount of ethanol formed is equal to the amount of acetate (0.6 mol per mol of glucose), assuming free energy of hydrolysis of ATP = -31 kJ mol^{-1} (where $\Delta S = 0$), then about 18.7 kJ is lost during this process. If this value (- 18.7 kJ) is now subtracted from the left hand side $\Delta H_{glu} = -153$ kJ $(mol\ glucose)^{-1}$, which is, within the limits of experimental error, in good agreement with the experimental value obtained during aerobic growth.

This suggests that the amount of energy lost (as heat) per mol of glucose during glycolysis is the same under aerobic and anaerobic conditions. The higher specific heat output under anaerobic conditions accounts for the energy loss during the fermentation of pyruvate in particular in the production of ethanol.

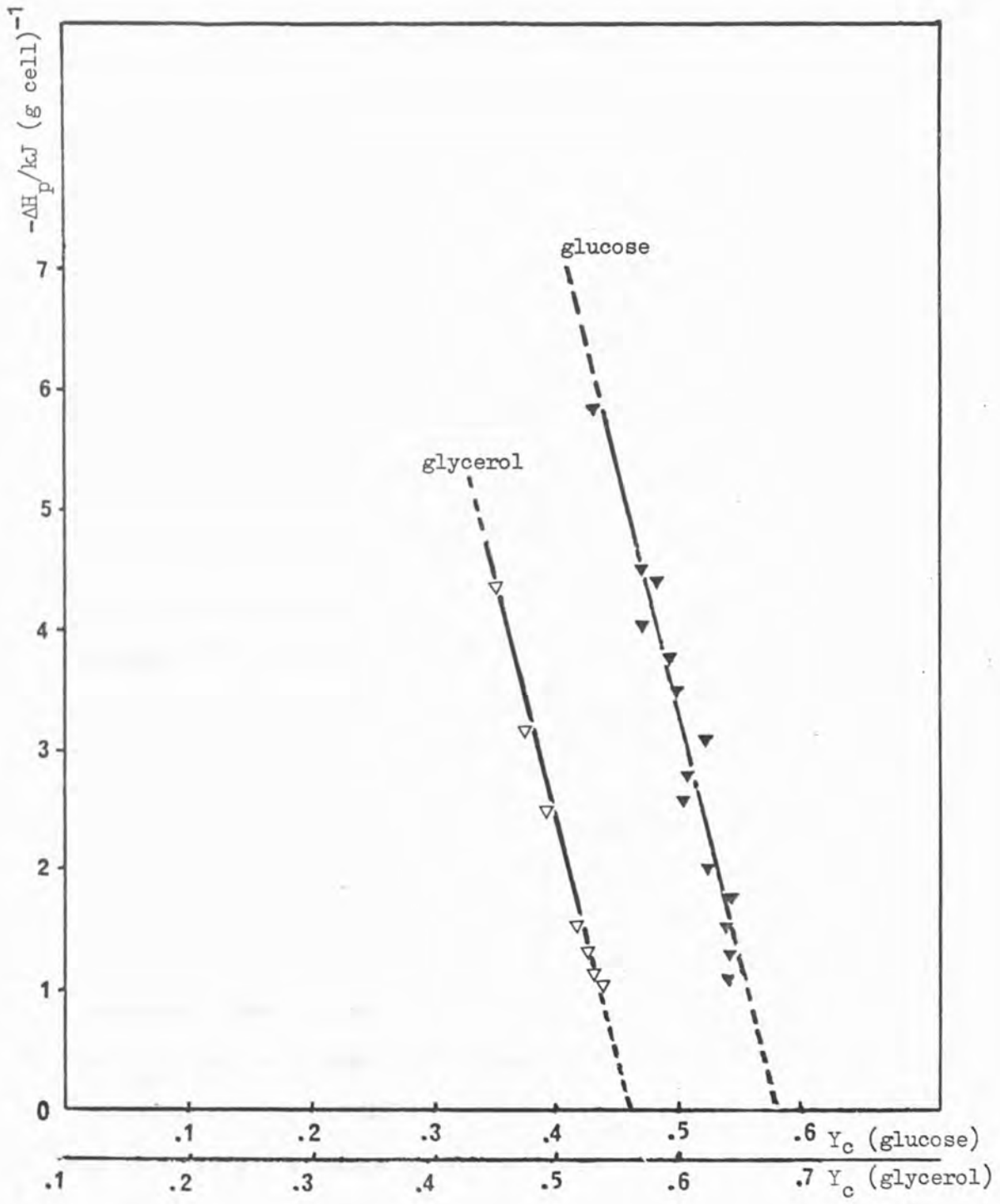
The decrease in yield coefficient at low dilution rates implies that the organisms are energetically less efficient and unused energy is wasted as heat (Bauchop et al., 1960; Harrison et al., 1967). This was confirmed by measurements of the specific heat output at different dilution rates for growth in a range of glucose concentrations. The values of ΔH_p were strictly dependent on D (Figs. 4.1. and 4.2) and were independent of substrate concentration and carbon source (Table 6.1).

The specific heat output of cells in carbon-limited chemostats under standard conditions increased linearly with decrease in the yield coefficient (Y_c); Fig. 6.7., shows this for glucose- and glycerol-limited chemostats. This plot can be extrapolated in both directions; as ΔH_p approaches zero, the maximum yield coefficient approaches a value of 0.58 and 0.565 for glucose and glycerol respectively. However the error involved in the case of glycerol is much higher than that for glucose. The maximum yield coefficient is the situation when there are no heat losses and the efficiency of utilization of the substrate is 100%. At the other extreme when there is no growth (i.e. $Y_c = 0$) and glucose is degraded to carbon dioxide and water ΔH_p should approach the heat of combustion of 1.84 g glucose (i.e. $1/Y_c^{max}$, when $1/D = 0$; Fig 6.2) i.e. -28.78 kJ. When $Y_c = 0$ the heat evolved is about 29 kJ $(g\ cell)^{-1}$, although this is a large extrapolation agreement with the predicted value is good. The same argument is also applicable for growth in glycerol-limited chemostat.

At high dilution rates ΔH_p approached a low constant value of -1.09 kJ $(g\ cell)^{-1}$, but below $D = 0.6\ h^{-1}$ this value increased rapidly with decreasing dilution rate. The greater value of this specific heat output recorded at $D = 0.25\ h^{-1}$, (the lowest dilution rate attainable) was -5.85 kJ $(g\ cell)^{-1}$. This variation of ΔH_p with dilution rate is quite unlike the variation of biomass with dilution rate (Figs. 4.1., 5.1.)

The plot of ΔH_p against 1/D for glucose and glycerol limitations consists of two linear portions and are strictly comparable with the double reciprocal plots (Figs. 6.2 and 6.3).

Fig. 6.7. Specific heat output plotted against the yield coefficient of K. aerogenes growing in glucose-limited, ▼ ; and glycerol-limited, ▽ ; chemostats.



The slopes of these two lines give a measure of the maintenance energy (M_e) for growth in two distinct dilution ranges. The values of maintenance energy obtained from the slopes of these lines are 0.56 and 1.98 kJ (g cell h)$^{-1}$ for glucose and 0.51 and 1.77 kJ (g cell)$^{-1}$ for glycerol for values of D greater than or less than 0.52 h^{-1} respectively. At the maximum theoretical growth rate (1/D = 0) ΔH_p = 0.5 kJ (g cell)$^{-1}$ for both glucose and glycerol. Using the heat of combustion of glucose (15.64 kJ g^{-1}) and of glycerol (18.05 kJ g^{-1}), these values of the maintenance energy (M_e) can be converted to give maintenance coefficients (m_s) of 0.036 and 0.13 g glucose (g cell h)$^{-1}$ and 0.028 and 0.098 g glycerol (g cell h)$^{-1}$ for D greater than or less than 0.52 h^{-1} respectively. These figures compare favourably with those obtained from the variation of the yield coefficient with D, viz. 0.048 and 0.19 g glucose (g cell h)$^{-1}$. For the plots of ΔH_p or $1/Y_c$ against 1/D the intersection of the two linear portions (Figs. 6.2 and 6.3). occur at the same dilution rate (0.52 h^{-1}).

For a dilution rate of 0.52 h^{-1}, under standard conditions, Y_{glu} = 94 g cell (mol glucose)$^{-1}$; any wall growth in the fermentor will affect the value of Y_{glu}, the value of ΔH_p, however is not affected and is independent of wall growth in the fermentor. Consider an error of 1 g of cells in the value of Y_{glu}; this will mean a deficiency in the glucose degraded amounting to 1.85 g. Since the heat of combustion of glucose is 15.64 kJ g^{-1} a change in ΔH_p amounting to 0.31 kJ (g cell)$^{-1}$ would be expected. The average measured value of ΔH_p (at the 95%

confidence level) = -1.56 ± 0.07 kJ $(g \text{ cell})^{-1}$. Since the limits of error of ΔH_p measurements are considerably less than those predicted from errors in Y_{glu}; it is apparent that the heat measurements are more reliable and more accurate than are measurements of the molar growth yield.

The shape and position of the ΔH_p-D curves are the same irrespective of the environmental temperature (37 and 42° C), substrate concentration, and the carbon source. This means that, for the formation of a unit mass of cells from a variety of carbon sources (glucose, glycerol, pyruvate, acetate) at the same growth rate the amount of heat dissipated is constant. However this is contrary to that expected from the variations of the values of yield coefficients with different substrates. The low values of Y_c are usually attributed to energy-requiring processes such as uptake and biosynthetic processes. With low yield substrates, the amount of carbon dioxide evolved per unit mass of cells is much higher than that for glycerol or glucose. This indicates that a large proportion of the substrate is used as energy source. For example, the uptake of acetate as an AMP-forming process (Webster, 1965) and gluconeogenesis (which exists in acetate metabolism) is an energy-requiring process in contrast to glycolysis.

Cells grown in glucose-limited chemostat cultures at high dilution rates are energetically more efficient than cells growing in batch cultures at a similar growth rate [$\Delta H_p = -1.09$ kJ $(g \text{ cell})^{-1}$ in chemostat culture; $\Delta H_p = -9.5$ kJ $(g \text{ cell})^{-1}$ in batch culture (Nichols, Prichard, and James, 1980)]. The yield was the same

under both conditions of cultivation. The higher value of ΔH_p in batch culture is expected, since in this type of culture all components of growth medium including the energy source (in this case glucose) are in excess during the exponential growth phase until the limiting nutrient (glucose) truly becomes limiting and depleted during the final division before the onset of the stationary phase. Although not strictly comparable, the conditions during exponential growth in batch culture and during growth in glucose-sufficient chemostats are similar in that the energy source is present in excess. In both cases the excess energy source is wasted with the evolution of heat and production of carbon dioxide.

In glucose-sufficient chemostats at the same growth rate, the specific heat output is 3 times greater than that in glucose-limited chemostats (Table 6.1). This is in good agreement with slip-reaction mechanisms proposed by Neijssel et al., (1975, 1976). That is, when the energy source is in excess the organisms degrade the energy source at a much higher rate than required for biosynthetic processes, i.e. the growth is uncoupled. Thus under these circumstances in glucose-sufficient chemostats and in batch cultures, a low value of Y_{glu} would be expected; this is not supported by experimental evidence (Nichols et al., 1980).

The addition of extra substrate to a standard chemostat culture of K. aerogenes growing at steady-state, disturbed the steady heat output depending on how readily these substrates were utilized. On the basis of heat response these substrates could be divided into four groups (Table. 4.3). Since the heat response

to the addition of pyruvate was similar to that for extra glucose, it may be deduced that the cells growing in glucose-limited chemostat are also adapted to growth on pyruvate. The heat response for citrate and α-ketoglutatrate was very poor and did not improve with successive additions; this suggests that organisms cannot readily metabolize these compounds and do not easily become adapted to utilize them in the presence of glucose metabolism. All C_4 intermediates of the TCA cycle malate, succinate, fumarate fell into the second group to which the organisms showed the potential for adaptation. The initial heat response was a broad peak superimposed on the steady-state heat output for the first addition, this became sharper on successive addition (at 30 min intervals) and was now comparable to the peak exhibited on the addition of extra glucose. If the time interval between successive additions exceeded few generation times the organisms were no longer adapted to the substrate under investigation and the heat response reverted to a broad peak. This is compatible with the theory of continuous culture; that is the only organisms which survive in a chemostat are those which are fully adapted to the environment.

On the addition of acetate; the heat output continued steadily at an increased rate until the added acetate was consumed; oxygen defficiency was not observed. The prolonged heat output was due to the restricted uptake and utilization of acetate by the organisms, in the presence of glucose metabolism. A linear relationship existed between the additional heat output and the added acetate. The additional heat output on addition of more

glucose or pyruvate was, however, not a linear function of the amount of substrate added (Fig. 4.6). It is possible that, for the very small quantities added, the added substrate is largely used by the time the bacterial suspension reaches the microcalorimeter cell; additional heat output is therefore smaller and liable to be variable. On the other hand for larger amounts of added glucose the culture in the microcalorimeter cell was deficient in oxygen.

Under standard conditions when more than 6 mg of glucose was added to the culture the additional heat output was now as two distinct peaks superimposed on the steady-state heat output. The first peak was due to the oxidation of glucose and the second to the oxidation of acetate which was detected in the culture after the addition of larger amounts of glucose.

When a mixture of glucose and acetate was added, the heat response superimposed on the steady-state heat output was a sharp peak with a shoulder; the extent of the shoulder depended on the amount of acetate added. A possible explanation for the absence of the second peak, is that simultaneous utilization of both carbon sources occurs although the rate of acetate utilization is much slower than that of glucose. A similar heat response was observed when a larger amount of pyruvate was added. A similar reasoning applies in this case since pyruvate breaks down to acetate and carbonate.

Although the quantitative studies of the heat output following the addition of extra substrate might prove very useful in terms of energetics, it involves very complicated mathematical equations associated with continuous dilution of the added substrate in the fermentor, and the heat losses during transit time (Δt).

These mathematical treatments were avoided in this work and should be sought in future investigations. The general conclusions arrived at are in no way invalidated by lack of this treatment.

The ATP production in oxidative-phosphorylation occurs by a complex process involving a membrane-localized electron-transport chain. Although the molecular mechanism of phosphorylation remains to be established (Boyer et al., 1977), the chemiosmotic hypothesis provides a unifying picture of the overall mechanism (Mitchell, 1961, 1966). In this hypothesis proton excitation or oxidation of organic molecules, induces a flow of electrons within the membrane; this is accompanied by a unidirectional movement of protons across the membrane. This creates an electric potential and a proton gradient across the membrane. In electrical units the proton motive 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. Accordingly $\Delta\mu_H$ 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 reverse flow of protons provides the energy for a membrane associated enzyme, the coupling factor, to synthesize ATP.

A transient change in environmental pH to pH = 8, by addition of sufficient sodium hydroxide, caused an increase of about 90 - 100% in the steady state heat output (section 4.4.). This change in heat output may be explained by ATP hydrolysis according to the reaction (Garland, 1977):

$$ATP^{4-} + H_2O + x\,H^+_{in} \rightleftharpoons ADP^{3-} + \text{phosphate} + xH^+_{out}$$

in which an alkaline pH favours the forward reaction (i.e. ATP hydrolysis) a process which is accompanied by a decrease in free energy. However there is some evidence that, reduction of cytochrome b_{565} can be obtained in the absence of energy conservation, when the mitochondial membranes are brought to a pH of about 8 (Chance et al., 1970; Sato et al., 1971; Wikstrom et al., 1971; Williams, 1970; Azzi et al., 1971). In the absence of energy conservation a higher heat output would be expected.

Furthermore, proton ejection induced by oxygenation of anaerobic mitochondria has been reported by Papa et al., (1975), and Penniston (1973) has shown that this rapid proton release is accompanied by a burst of oxygen consumption, The rapid phase of oxygen consumption and proton release can be ascribed to oxidation of reduced respiratory carriers.

A high respiration rate and a low cell yield during the transition period from anaerobic to aerobic growth for K. aerogenes have been reported by Harrison et al., (1971 b), who suggested that uncoupling between growth and energy conserving mechanisms occurs in this period. Several-fold enhancement in the heat output during this transition period (section 4.2.2) is in support of the oxidation of reduced respiratory carriers (NADH), similar large enhancements in the heat output were never observed following the addition of fermentation products (e.g. ethanol, acetate).

According to Mitchell's hypothesis (West and Mitchell, 1973); if the proton-translocating ATPase and proton-translocating oxido-reduction reactions are valid, then if the two reactions occur in one membrane they can be coupled by virtue of their common substrate, the translocated proton (Garland, 1977).

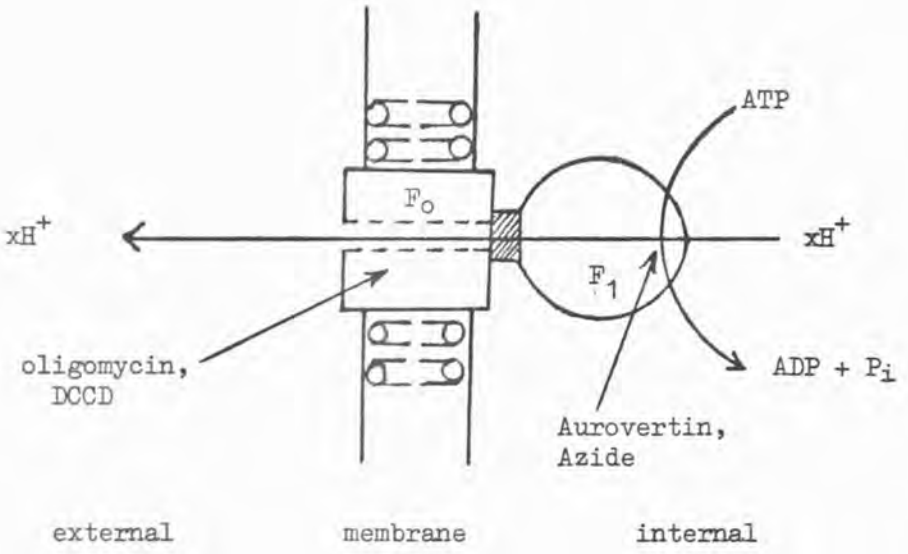
The overall reaction for ATP formation is thus:

$$AH_2 + B + (n/x)\ ADP^{3-} + (n/x)\ \text{phosphate} \rightleftharpoons A + BH_2 + (n/x)\ ATP^{4-} + (n/x)H_2O$$

Harrison et al., (1969) found that, immediately after the addition of glucose or succinate to a glucose-limited chemostat culture of K. aerogenes, the ATP content of the cells increased slightly, then fell to a value below the steady-state until the extra substrate was exhausted. During this period the increased heat output suggests an enhanced ATPase activity due to proton-translocating reactions.

Moreover the addition of non-metabolizable substrates such as methyl-β-D-thiogalactoside had no effect on the steady-state heat output and a negligible decrease was observed with α-methyl-D-glucoside. These observations support the fact that, substrate transportation processes are not involved with ATPase activity. This is supported by the work of Schairer et al., (1972), who found that, β-galactoside, could be taken up by an ATPase-deficient mutant of E. coli. There is also evidence (Nieuwenhuis et al., 1973) that several mutants of E. coli, deficient in oxidative phosphorylation, lack different components of the ATPase complex.

The structure and composition of the ATPase for coupling membranes appear to be similar in chloroplasts, mitochondria and bacteria (Postma et al., 1976). However, Baird et al. (1979) concluded that, despite many striking similarities, a universal model for the structure of coupling factor complex operative in oxidative- and photophosphorylation can not yet be presented. A schematic representation of an ATPase complex is given by Garland (1977):



The complex consists of F_1- and F_0- ATPase fractions, the F_1 fraction is soluble in water. ATP synthesis and hydrolysis is catalysed by the coupling factor.

The possibility of extra-cellular ATPase activity was ruled out by the fact that there was no change in the rate of heat output as a result of the addition of ATP to the growing culture of K. aerogenes, since the ATP, due to the large size of the molecule, cannot be transported into the cells.

ATP will not be produced in the presence of (a) uncouplers, which act independently to dissipate the proton gradient or (b) energy transfer inhibitors, which prevent proton movement through the coupling factor. The potential energy in the presence of uncouplers would be lost as heat (Poe et al., 1967).

The F_1-ATPase is the site of action of azide and aurovertin and F_0-ATPase is the site of action of DCCD and oligomycin (Garland, 1977).

In the presence of sodium azide (0.62 mmol dm^{-3}), the specific heat output for K. aerogenes growing in carbon-limited chemostat (glucose concentration = 4 mmol), was significantly higher and Y_{glu} was markedly lower than the corresponding values in the absence of azide at all growth rates. This result is consistent with the uncoupling activity of azide (Wilson, 1969; Garland, 1977).

The addition of azide or DCCD (100 μmol) to the glucose-limited chemostat culture of K. aerogenes in its steady-state, caused an increase in the respiration rate and carbon dioxide production, which was accompanied by 2.5- fold increase in the rate of heat output. (Fig. 4.7). The biomass decreased 30% within 30 min, indicating the wasteful utilization of the carbon source. The steady-state values resumed after several hours only when the added reagent was diluted out of the fermentor.

In glucose-sufficient (nitrogen-limited) chemostats where the specific heat output was higher than in a carbon-limited chemostat by a factor of 3, the addition of azide (100 μmol) caused an increase of only 20%. This difference in heat output between the two types of chemostats suggests, there is a high ATPase activity in carbon-sufficient chemostats under normal conditions of growth, and supports the "slip-mechanism" in carbon-sufficient chemostats proposed by Neijssel et al., (1975; 1976 a; 1976 b).

The addition of azide (100 μmol) to a carbon-limited chemostat of K. aerogenes growing under anaerobic conditions, increased the rate of heat output only 20%. The rate of carbon dioxide production decreased drastically to 15% of its steady-state value. Under anaerobic growth ATP production is restricted to

substrate level phosphorylation. In the presence of azide the available ATP is hydrolyzed by ATPase and glycolysis comes to partial halt.

When the azide concentration was doubled by the addition of 100 μmol of azide to a carbon-limited chemostat of K. aerogenes growing in the presence of azide (0.62 mmol dm^{-3}), the rate of heat output increased only by a further 10%.

In addition, the changes in the rate of heat output (2.5-fold increase) were the same on addition of 100 μmol DCCD to the carbon-limited chemostat cultures growing in the presence or absence of azide (620 μmol dm^{-3}). This suggests that, the stimulation of F_1- or F_0-ATPase results in the uncoupling of growth.

Although the exact mechanisms of heat dissipation under normal conditions of growth are largely unknown, there is, however, evidence for naturally occuring ATPase- inhibitors in mitochondria (Pullman et al., 1963), chloroplasts (Nelson et al., 1972) and in E. coli (Nieuwenhuis et al., 1974) which regulate the ATPase activity under normal conditions of growth.

In conclusion it has been demonstrated that the combination of a flow-microcalorimeter with a chemostat is a very useful and powerful tool in the study of the energetics of processes associated with cellular growth and division. To interpret the experimental results as fully as possible and obtain meaningful data it is essential to monitor other properties of the growing culture, e.g. biomass, oxygen tension, carbon dioxide formation, and the concentration of the substrate and secondary metabolites. The study of cells in chemostat culture by this method is of great

interest since the cells are growing in the steady state under optimum growth conditions and the heat output is very reproducible and invariant with time. Although previous workers realized that two maintenance coefficients were neccessary to describe bacterial growth in chemostat culture, they were unable to evaluate these. From energy studies, values of the two coefficients have been obtained for growth in glucose-and in glycerol- limited media. Preliminary studies of changes in the steady-state heat output resulting from the addition ofmore or other substrate or specific inhibitors or uncouplers have been interpreted quantitively in terms of changes in biochemical processes ocurring in the cells. This is perhaps the more useful aspect to study in greater detail, for by using specific inhibitors it will be possible to determine the relative importance (energetic) of the different processes which occur during growth and division. (See Appendix).

Appendix

Suggestions for further work:

1. Confirmation of identical values of ΔH_p for a wider range of C-source.

2. Investigation of the heat output in the presence of electron acceptors other than O_2 and under anaerobic conditions with pyruvate and other C-sources.

3. Study of other nutrient-limiting components of the medium e.g. phosphate, sulphate.

4. Investigation of carbon-sufficient chemostats other than glucose, e.g. glycerol, mannitol, pyruvate.

5. Investigation of the changes of the heat output for a wider range of growth and environmental temperature. In the small range (37 - 42° C) investigated in the present work the growth rate is unchanged, however at lower temperature when the growth rate is lower (Hunter and Rose, 1972), additional thermodynamic data may be obtained.

6. Quantitative study of heat changes during transient-state (i.e. addition of a substrate, uncoupler).

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