# VARIATIONS IN THE ENERGY OUTPUT AND ATP POOL DURING THE GROWTH OF *K. AEROGENES* IN DEFINED MEDIA

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by

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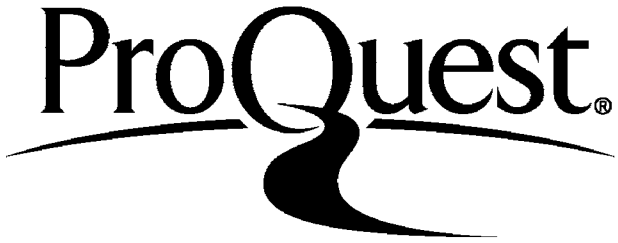

This thesis comprises a report of full-time research undertaken by the author in the Physical Chemical Laboratories of Bedford College, University of London, from October 1979 to September 1982

Dedicated to my parents

- for their support

during my seven years

of University study.

CONTENTS

## ABSTRACT

Thermal events accompanying the growth of K. aerogenes in batch culture in phosphate- and glucose-limited salts media were recorded using an LKB flow micro-calorimeter; changes in the cellular ATP content and growth parameters (biomass, $CO_2$) were also measured. Calorimetric data is discussed in terms of the specific power output (P) and ATP data in terms of the ATP pool.

Standard experimental and cultural conditions were established; at $p = 0.95$ the reproducibility of the heat output was 1%, and that of the ATP pool and other parameters was 3-4.5%.

The specific power and ATP pool are similar indicators of metabolic activity; the P-t trace generally followed the course of the ATP profile. The values of P and the ATP pool increased rapidly at the start of the exponential growth phase; and on cessation of growth they decreased rapidly. During exponential growth under glucose-limitation the ATP profile oscillated about a mean value; the specific power remained nearly constant.

The energy required for biosynthetic processes was calculated from mass and energy balances. Relative changes in this energy and the yield suggest that the biosynthetic energy is composed of the energy required for anabolism, for maintenance and for storage.

Three different and distinct types of consecutive growth occurred under phosphate-limitation (glucose 60% in excess); mass and energy balances were established for each. The specific power output and cellular ATP profile decreased with decreasing availability of phosphate. It is apparent that cellular phosphate exists in two forms; free and fixed phosphate.

The effect of increasing concentrations of sulphanilamide and nalidixic acid (NA) on thermal and growth parameters was similar. NA exhibited a dual dose-related antibacterial potency; this is attributed to the change in its mode of action on RNA, DNA and protein synthesis. Changes in the P-t profiles were observed during the development of cellular resistance to NA.

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## SUMMARY

Thermodynamic data has important practical applications in industrial biotechnology as well as in chemical and biological research. In fermentation processes important parameters which are calculated include the yield value from mass-balance data, and hence the quantity of heat that will be generated by the culture from the enthalpy change for substrate oxidation. The heat generated by the culture is an important factor when considering the control of the growth temperature. In particular the nature of flow-microcalorimetry lends itself to a useful process control instrument for industrial biotechnological reactions. Thermodynamic data when considered in conjunction with other biological data is an important research tool; flow-calorimetry can reveal information about the action of drugs upon the microbial cell.

Thermal, ATP and growth measurements were made during the growth of K. aerogenes in batch culture under conditions of glucose- and phosphate-limitation. Thermal measurements were made continuously throughout the growth cycle with an LKB 10700-1 flow microcalorimeter; growth parameters (biomass, $CO_2$) were also monitored continously. The bacterial ATP content was measured enzymically using the purified luciferase-luciferin system; this was not monitored continuously. The first reported systematic and quantitative study of changes in both thermal and ATP parameters under different limiting growth conditions is presented. Thermal and growth measurements were made during the batch growth of K. aerogenes in glucose-limited media containing a drug; sulphanilamide or nalidixic acid (NA). The first reported quantitative study of changes in thermal events due to drug action during bacterial growth is presented. The relatively slow time (1-2 s) taken to remove a sample of culture from the fermenter and mix with the bacterial

ATP extractant (trichloroacetic acid) did not result in erroneous ATP values. The stability of the bacterial ATP extract was excellent; enhanced by the presence of 18, crown, 6 as well as EDTA. Although the purified luciferin-luciferase used was a vast improvement on the crude luciferin-luciferase the ATP assay method was based on initial peak light readings, and a true internal standard could not be used. Even so the reproduciblity ($p = 0.95$) of the assay was good (±1-2%). The minimum detection limit of bacterial ATP as determined by the complete assay technique (sampling, extraction, assay) was $5 \times 10^{-11}$ mole, in the vial. The reproducibility at low concentrations was ±4.5% and at higher concentrations ±3% ($p = 0.95$).

The mean value of the ATP pool of K. aerogenes was $7.8 \times 10^{-6}$ mole $g^{-1}$ during exponential growth in glucose-limited media. This value is similar to that obtained by previous workers. During growth the ATP content of the cells oscillated about this mean value (±10% of the mean); these oscillations which were proved to be a real phenomenon, have not been reported by previous workers. This may be attributed to the failure of earlier workers to establish quantitatively the experimental errors of the complete assay technique, coupled with various misleading ways of representing the final data. The oscillations are believed to be due to the effects of two factors; changes in the cellular ATP content during the cell cycle caused by the operation of a slip-mechanism which controls metabolism and changes in the degree of synchrony caused by variances in the cell cycle time. Further the mean value of the ATP pool is the pool size which is required to drive metabolism.

Neither the ATP pool or the adenylate energy charge (EC) value is a complete explanation of cellular metabolic activity in every situation; indeed there are several disadvantages associated with the EC parameter. It was concluded that the ATP pool is a good metabolic indicator for

cells under the same environmental conditions so that the lag, logarithmic and stationary phases of growth can easily be detected. However comparisons of the ATP pool between cells trained to grow in different media or under markedly different conditions of growth (e.g. aerobic and anaerobic) ~~is~~ are more difficult to interpret, and is not as revealing of the comparative metabolic state of the cells. This is because such drastic changes are the cause of major metabolic changes both in catabolic and anabolic systems, which affects the degree of coupling of the two systems and hence the observed cellular ATP pool.

Previously published calorimetric data is unreliable and largely qualitative in nature; when quantitative data has been presented the failure to monitor and report other biological changes (biomass, carbon dioxide evolution, media analysis) has prevented a full and meaningful interpretation of the data. However the limited data cited for the growth of bacteria in glucose-limited media agrees well with that presented here.

Both the ATP pool and specific power (P) are reliable indicators of changes in metabolic activity under similar environmental conditions. However there are some major differences in the character of the two parameters and the way in which they respond to a given change in metabolic activity. The specific power is more responsive than ATP and therefore a better measure of rapid changes in catabolic activity. The cellular ATP pool is related to, and therefore a measure of, the cells' ability to maintain its integrity, (maintenance requirements), but the specific power is not. Therefore the ATP pool gives an indication as to the state (viability) of stationary state cells which the specific power does not.

The versatility of the bacterial cell is demonstrated by its adaptability to growth under conditions of deprivation. Training to phosphate-limited growth results is a change of cellular metabolism as

demonstrated by changes in the p-t, P-t and $CO_2$-t traces, and overall mass and energy balances. Relative changes in values of biosynthetic energy requirements and the yield suggest that the calculated value of this energy is composite: incorporating among others the energy of maintenance and of storage. The results also suggest that phosphate is present in two distinct forms in the bacterial cell; free and fixed phosphate.

Data on the specific power of a cell (i.e. a measure of its metabolic activity) can be interpreted in a meaningful manner, especially when accompanied by other physical data relating to growth. The anti-bacterial potency (per molecule) of NA on normal cells as determined calorimetrically was c. 180 times greater than that of sulphanilamide, (when evaluated at a NA concentration of 0.078 $mmol\,dm^{-3}$. The effects of increasing concentrations of sulphanilamide and NA on the growth of normal cells was similar. In general this resulted in decreasing yields and values of biosynthetic energy but increases in the amount of waste heat and carbon dioxide produced. Further the value of the specific power output immediately prior to the cessation of growth decreased with increasing drug concentration. The relationship between the yield and biosynthetic energy for increasing concentrations of NA was not linear. This was attributed to the unpredictable effect of NA upon the elements (energy required by anabolism, storage energy, maintenance energy) that contribute to the biosynthetic energy. NA exhibits a dual dose-related potency effect due to the relative effects of NA on DNA, RNA and protein synthesis at different concentrations. This change in the mode of action of NA is clearly shown by specific power measurements. The calori-metrically determined value of the concentration of NA at which this mode of action changes is 0.078 $mmol\,dm^{-3}$ a value similar to that obtained by previous investigators using different techniques. The calorimetrically

determined potency of NA at concentrations below 0.078 $mmol\,dm^{-3}$ was 18 times greater than at concentrations above 0.078 $mmol\,dm^{-3}$. Cells trained to grow in the presence of NA retain the capacity to maintain reasonably balanced growth in drug-free media, as observed by calorimetric studies. The development of resistance to NA by cells was also followed by monitoring changes in the p-t profiles. Cells trained and tested at a NA concentration of 0.086 $mmol\,dm^{-3}$ do not develop a complete resistance to NA, as do cells when trained and tested at 0.046 $mmol\,dm^{-3}$. Trained cells tested at a NA concentration of 0.690 $mmol\,dm^{-3}$ show the same degree of resistance to NA. These observations are explained in terms of the different modes of action of NA at different concentrations i.e. above and below that of 0.078 $mmol\,dm^{-3}$.

## SYMBOLS

| | |
|---|---|
| $CO_2$-t trace | Carbon dioxide-time trace |
| m-t trace | Biomass (dry weight) - time trace |
| Log M-t trace | Log biomass (dry weight) - time trace |
| p-t trace | Power/J $s^{-1}$ - time trace |
| P-t trace | Specific Power/J $s^{-1}$ $g^{-1}$ - time trace |
| $F_J$ | Factor to convert area ($cm^2$) under p-t trace to heat (J) |
| $F_{Js^{-1}}$ | Factor to convert chart displacement (cm) to power ($p/Js^{-1}$) |
| $\Delta H_T$ | Total heat evolved during test |
| $Y_{Gluc}$ | Yield of cells (g substrate)$^{-1}$ initially present |
| $\Delta H_{Gluc}$ | Heat evolved during the consumption of 1 mol of glucose |
| $\Delta H_B$ | Metabolised energy required for biosynthesis |
| $\Delta\%$ | Percentage of metabolised energy required for biosynthesis |

# CHAPTER ONE

# INTRODUCTION

## 1.1 Properties of Kelbsiella aerogenes

K. aerogenes is a rod-shaped, Gram-negative, non-motile, non-sporing facultative anaerobe. The optimum growth temperature is 37°C and the cells readily grow in salts media supplemented with an organic carbon source. Sugars are fermented with the production of gas, the fermentation pattern is to 2,3-butanediol.

The particular strain of this organism was chosen for detailed study because of the immense quantity of data accumulated on its nutritional requirements and metabolic processes. Some qualitative and quantitative calorimetric studies have been made with this species.

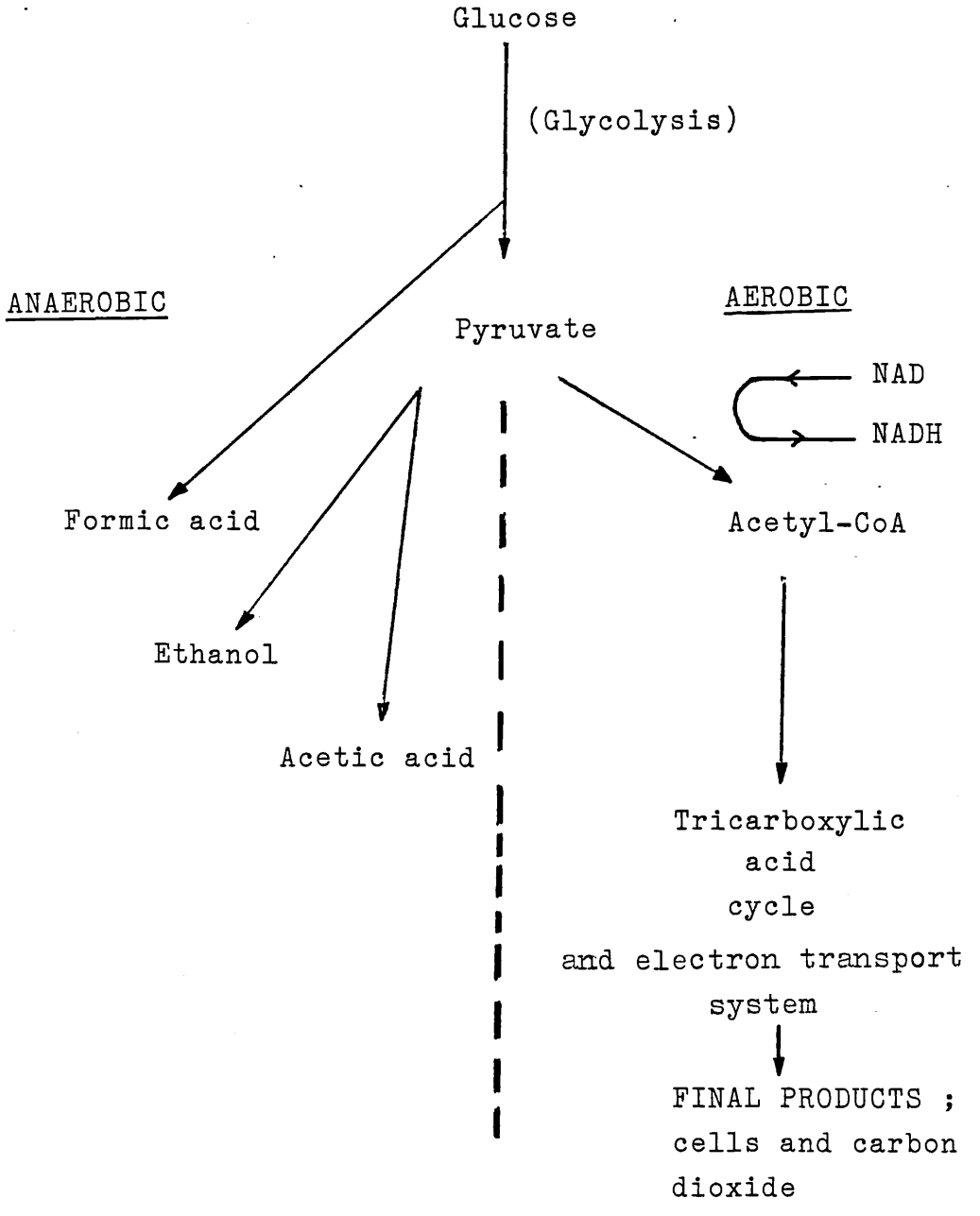
## 1.2 Nutrition

K. aerogenes can obtain both energy and carbon from simple carbohydrates provided other essential nutrients are present such as; nitrogen, phosphorous, magnesium, potassium, sulphur etc. (Tempest et al., 1965, 1966, 1970; Dicks et al., 1966; Luria, 1960).

A wide range of carbon sources in minimum 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 the use of such compounds as acetic acid and glycerol, it is fully adapted for growth on other compounds such as glyceric acid, pyruvic acid and lactic acid, on transfer from a glucose medium. Compounds such as formic acid do not support growth.

Normally glucose is converted to pyruvic acid via the Embden-Meyerhof pathway (glycolysis). Although oxygen is not required for this sequence of reactions the fate of pyruvic acid is dependent upon the availability of oxygen (Fig. 1.1). For the conversion of pyruvic acid to

FIGURE 1.1 Summary of Glycolysis, Fermentation and The TCA Cycle



useful products oxygen is required; under fully aerobic conditions the products are cells and carbon dioxide. Dagley et al., (1951) showed that pyruvic acid accumulates during exponential aerobic growth, disappearing rapidly in the stationary phase. There is also evidence that added tricarboxylic acid cycle intermediates are probably converted to pyruvic acid before utilisation.

Pyruvic acid undergoes oxidative decarboxylation to form acetyl-CoA; this then enters the Kreb's or Tricarboxylic acid (TCA) cycle (Fig. 1.2). The net products of the TCA cycle are 2 moles carbon dioxide, 1 mole ATP, 3 moles NADH and 1 mole $FADH_2$ per mole pyruvate. Reduced NAD (NADH) and FAD ($FADH_2$) formed during glycolysis and in the TCA cycle are oxidised by the respiratory electron chain (Fig. 1.3); the oxidation 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 (Stouthamer, 1978).

Under aerobic conditions oxygen is utilised for cell respiration as the final electron acceptor, it is also incorporated into cellular material. Cellular mechanisms influenced by oxygen are numerous; K. aerogenes adjusts its metabolism to the level of available oxygen. Harrison and Pirt, (1967) identified three phases of metabolism depending on the oxygen tension of the medium. When the oxygen tension was below 15% saturation anaerobic fermentation occurred, and above 15% aerobic fermentation. At very low oxygen tensions ($<1\%$) low yields were obtained.

K. aerogenes, when grown in simple salts medium in which an ammonium salt provides the sole nitrogen source, employs a special mechanism for amino-acid synthesis (Tempest et al., 1970). The activity

FIGURE 1.2 The Krebs or TCA Cycle and Glyoxylate Bypass

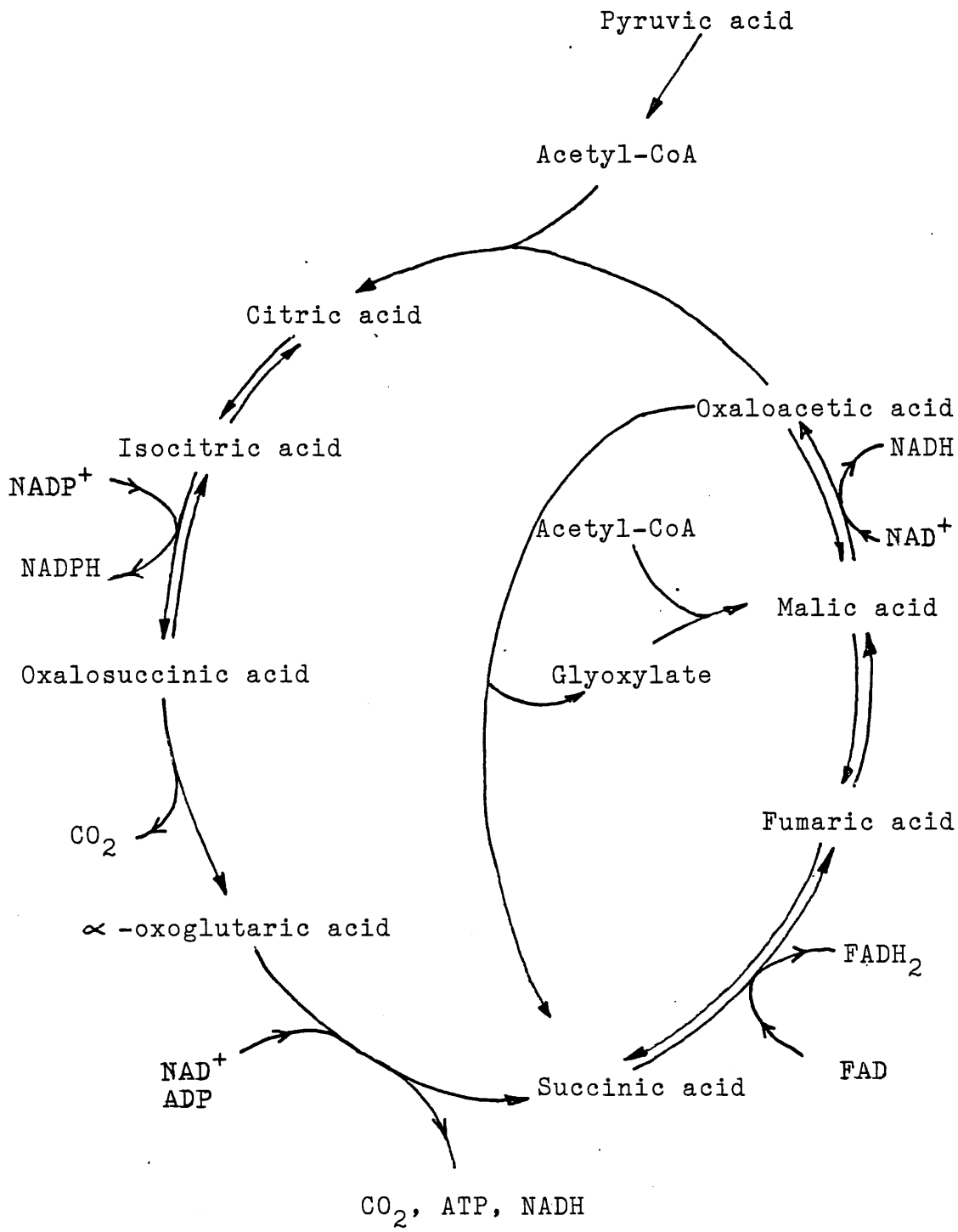
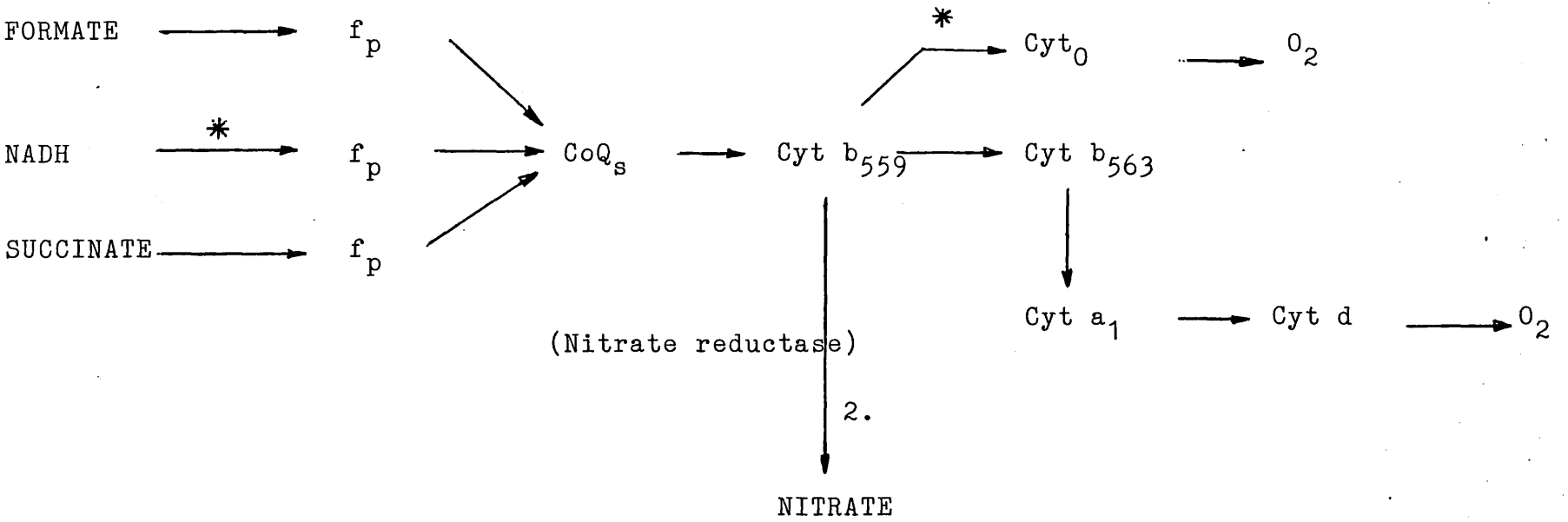


FIGURE 1.3 Respiratory Chain of E. aerogenes (Stouthamer 1976)

( * indicate sites of oxidative phosphorylation )



of enzymes in the TCA cycle vary according to the ammonia content of the medium (Brown et al., 1976).

## 1.3 ATP - Its Relevance

The reactions in a microbial cell can be divided into two basic types; catabolic - energy yielding break-down reactions, and anabolic - energy requiring building reactions. For the cell to maintain its internal processes (e.g. biosynthesis, active transport etc) the energy liberated by the catabolic reactions must be made available to the energy requiring anabolic reactions. ATP is the link between the energy-yielding and requiring reactions.

The $\Delta G$ value (+29 kJ) for the formation of ATP from ADP and pyrophosphate is large and positive, and therefore will not proceed spontaneously. One of the primary roles of catabolic processes is to supply the energy required for ATP synthesis.

For the reaction

$$\text{Glucose} + H_2PO_4^{2-} \rightarrow \text{Glucose-6-phosphate} + H_2O \qquad \Delta G = +12.6 \text{ kJ};$$

this reaction will therefore not occur to any great extent. If the following reaction

$$ATP + H_2O \rightarrow ADP + H_4PO_4 \qquad \Delta G = -29 \text{ kJ}$$

is coupled with the first then

$$ATP + \text{Glucose} \rightarrow \text{Glucose-6-phosphate} + ADP \qquad \Delta G = -16.7 \text{ kJ}$$

The overall $\Delta G$ value is now large and negative, hence the reaction is feasible.

In the bacterial cell the spectrum of processes served by ATP can be divided into two; biosynthetic reactions and active transport. Vital biosynthetic functions such as the synthesis of proteins, nucleic acids

and carbohydrates require energy and this is supplied directly or indirectly by ATP. Cells use ATP for cytoplasmic streaming movement and for the uptake and excretion of ionic materials against concentration gradients in active transport.

Several hypotheses of electron transport-linked phosphorylation have been proposed. These include; (a) the chemical hypothesis (Lipmann, 1945), which postulates a covalent high-energy intermediate between the electron-transport chain and the ATP-synthesising system; (b) the conformational hypothesis (Boyer, 1965) which postulates a direct interaction between conformationally modified energy-transducing catalysts. This hypothesis requires "intra-membrane proton fluxes" as a means of energy transduction (Williams, 1961); and (c) the chemiosmotic hypothesis (Mitchell, 1961), in which a transmembrane proton gradient is the basic energy transfer device. At present it is not known which theory best describes the process but 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-localised electron transport chain. ATP is not produced if reagents are present which inhibit the close interplay between substrate oxidation and ATP synthesis. As a consequence ATP-synthesis will cease because the necessary energy is not available, while the oxidation of substrate, uninhibited by respiratory control, proceeds at a maximal rate and produces heat instead of ATP (Baird et al., 1979).

## 1.4 Biochemical Targets for Drug Action

In general the action of a drug on a microbial cell can be

described by one or more of the following;

(i) enzyme exhibition;

(ii) inhibition of the transfer of information;

(iii) exlcusion and protection mechanisms.

(i) Enzymes are obvious targets for drug action due to their paramount role in microbial metabolism. This type of drug action may be due to; direct action at the substrate binding site; direct action at a cofactor binding site; the disorganisation of the active centre by non-specific action elsewhere in the enzyme.

A substrate binds to the active centre of an enzyme by virtue of the three dimensional and chemical correspondance between bond-forming groups in the substrate and complementary groups in the protein. A chemical analogue (isotere) of the substrate will also form a complex with the enzyme. If this complex is relatively stable it will prevent or reduce the access of the normal substrate to the enzyme and competitive inhibition will occur. An effective antibacterial competitive inhibitor is sulphanilamide - an analogue of p-aminobenzoic acid.

Competitive inhibition can occur at the cofactor binding site. Many cofactor analogues inhibit bacterial growth but their action is more often due to inhibition of cofactor synthesis (i.e. inhibition of enzymes with cofactor precursors as substrate) rather than by direct competition with the natural cofactor at the active centre.

Most drugs that inhibit enzymes by binding to groups outside the active centre are non-specific in action. Examples are - SH inhibitors and heavy metal ions.

(ii) The processes of DNA replication, and RNA transcription and translation are responsible for the transfer of information in the

microbial cell and involve enzymes and macromolecules. These macromolecules offer further targets for drug action since alteration of their conformation, masking of their active groups, occlusion of their surfaces or stabilisation of otherwise flexible sections may well stop the accomplishment of their function. Nalidixic acid inhibits the transfer of information by inhibiting DNA replication.

(iii) The cytoplasmic membrane and cell wall protect the substrates and metabolites in the cell from dilution or contamination. Surface active compounds have been studied which alter the interaction of lipid and protein within the membrane and render this protection mechanism inactive. The membrane itself has little strength and retains its structure and properties by virtue of the outer cell wall. The important structural component of the cell wall is peptidoglycan. Any impairment in peptidoglycan synthesis leads to membrane rupture and nullification of its protective role.

## 1.5 Kinetics of Bacterial Growth

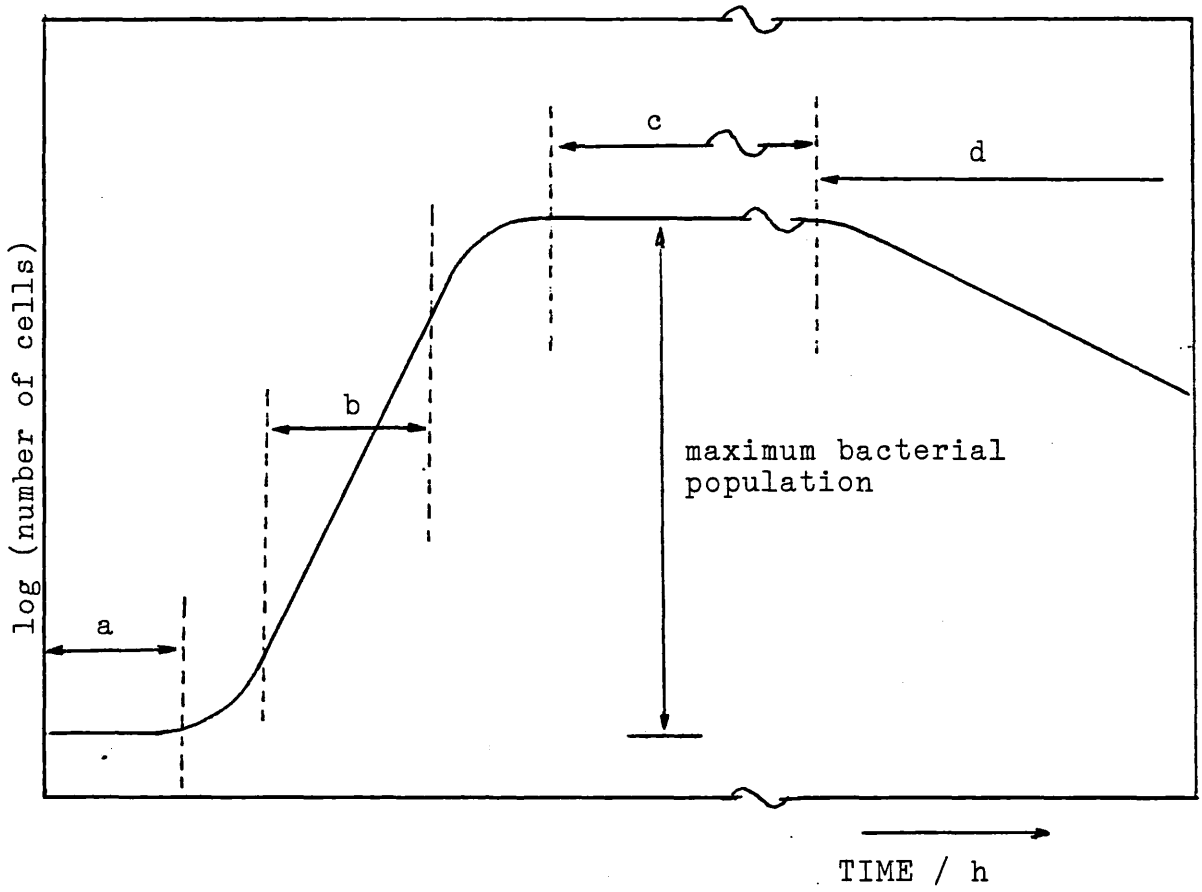
### 1.5.1 Growth in Batch Cultures

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 log phase (a) during which the cells synthesise essential intermediates (Hinshelwood, 1964). The length of this phase is dependent upon the age and size of the inoculum or the presence of inhibitors. 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

FIGURE 1.4 A Typical Bactrial Growth Cycle

(a,b,c,d, indicate Lag, Exponential, Stationary and Death phases respectively)



(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;

$$\frac{dm}{dt} = \mu m \quad \underline{or} \quad \frac{1}{m}\frac{dm}{dt} = \mu \qquad (1.1)$$

where $\mu$ is the specific growth rate. Monod (1950) showed that, the specific growth rate and mean generation time ($t_d$) are affected by such parameters as composition of the growth medium, temperature, pH, and substrate 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 $\mu_{max}$ is the maximum specific growth rate, s the substrate concentration, and $K_s$ the saturation constant (numerically equal to the substrate concentration at $\mu_{max}/2$). $\mu_{max}$ is attained during the exponential phase, where all nutrients are in excess. A simple relationship exists between growth and the utilisation of the 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:

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

when Y is known as the 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 $\mu_{max}$, $K_s$ and Y are known equations 1.1, 1.2 and 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 a 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 between different organisms and media.

### 1.5.2 Growth in Continuous Culture (Chemostat)

The growth of microorganisms in continuous cultures 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.

Theoretical discussions of bacterial growth usually starts from the exponential equations 1.1, 1.2 and 1.3 (Mond, 1950).

When a chemostat is operated under steady-state conditions the biomass of the chemostat culture is maintained at a constant value for a given dilution rate (D) and substrate concentration. ($D = f/V$ where $f/cm^3\ h^{-1}$ is the rate of addition of fresh medium to the culture, and $V/cm^3$ is the volume of the culture which is constant. Further, under

steady state conditions, the power (p) and specific power (P) outputs of the culture are maintained at a constant value for a given dilution rate and substrate concentration (Djavan, 1980).

## 1.6 Microcalormetry

In general all processes, physical, chemical or biological are accompanied by heat changes, 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), as regards sensitivity, temperature control, amount of material required etc. Recent developments have enabled these studies to become more quantitative and enabled the observed energy production to be correlated with thermodynamic data. Microcalorimetry is defined as the measurement of very small heat changes, but not necessarily with small quantities of material.

### 1.6.1 Heat Production in Microbial Systems

During microbial metabolism heat is produced as a waste product; it is this heat which is measured in calorimetry. Such heat changes are small and require calorimeters of high thermal sensitivity i.e. microcalorimeters.

Chemical reactions in biological systems may be considered to occur at constant pressure and volume, under these conditions thermodynamic differences between enthalpy and energy can be ignored; the energy change which accompanies a reaction becomes the change in enthalpy or heat of reaction. For a given reaction the energy difference is dependent upon the extent of the reaction and is independent of the reaction pathway (the law of Hess).

The measured heat is related to the heat of reaction for a system in which no external work is done. Hence for a given reaction the quantity of heat evolved depends on the number of moles of reaction which have taken place. In a microbial cell many processes occur simultaneously, the total heat of all the individual processes is recorded; thus calorimetry of microbial systems is non-specific i.e. the heat produced cannot, at present, be related to an individual process.

The heat of reaction is a defined quantity, when the entropy change is also known the thermodynamic analysis of a reaction, and of its driving force, Gibbs Free Energy ($\Delta G$), is more complete. Recent micro-calorimetric data has been presented with a view of contributing information on Prigogines hypothesis (Prigogines, 1956) that living organisms tend to a state of minimum entropy production (Stoward, 1962).

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

### 1.6.2 Types of Calorimeter

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

In an ideal calorimeter there is no heat exchange between the calorimeter proper and its surroundings. The temperature is kept constant at the calorimeter temperature by means of an electrically heated adiabatic jacket which completely surrounds the calorimeter proper. The heat absorbed or evolved is determined electrically by a

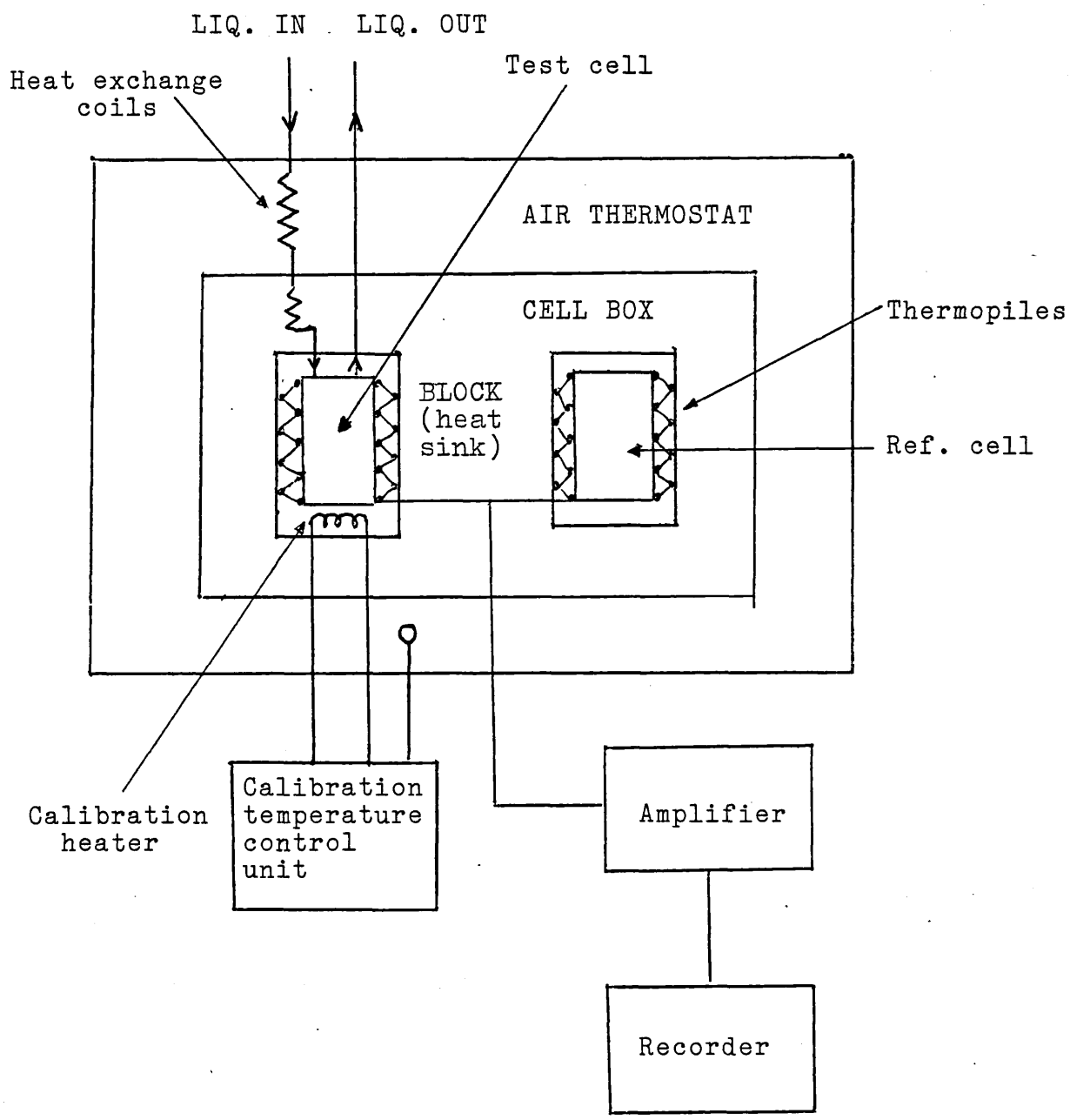
feed-back system to a reference cell. The disadvantage of this type of calorimeter is that, unless complicated regulatory devices are included in the construction, there will always be leakage of heat to the surroundings. This type of calorimeter has limited application in microbiological studies.

Heat conduction calorimeters are the principal type used for microbiological studies, and the type used in this work. The heat evolved or absorbed flows through a controlled path from the reaction cell to a heat sink (Fig. 1.5). A large thermostated air bath is employed to give a constant background temperature. Located in this is a large metal block which acts as the heat sink. The reaction and reference cells, which are situated in the block, are surrounded by thermopiles connected in opposition to each other. This twin principle reduces background disturbances to the heat flow. The air bath and metal block also contain heat exchange coils which are required for establishing thermal equilibrium of the flowing bacterial culture suspension before entering the calorimeter cell.

The main thermal considerations that have entered into the design and construction of heat conduction microcalorimeters are that; the thermal contact must be good between thermopile and reaction vessel; thermal contact must be poor between the heat sink and its surroundings and between the heat sink and reaction vessel; the temperature of the surroundings of the heat sink should be under control; the time constant for heat flow between reaction vessel and heat sink should be small; the response of the thermopile should be independent of the reaction of the heat sink within the reaction vessel; and any connections between the heat sink and reaction vessel should be carefully positioned thermally at both locations.

The electrical output is a measure of the difference in voltage

FIGURE 1.5 The Essential Components of A Heat-Conduction Microcalorimeter



between the thermopiles surrounding the reaction and reference cells, and so it is necessary to determine a calibration constant. A common method of calibration is to measure the electrical response for precise known amounts of heat. This is achieved by applying a measured quantity of electricity through calibration heaters sited near the reaction cell; in a good calorimeter design the position of the calibration heaters within the mixing zone should not be critical. An alternative method of calibration is to use a chemical reaction of accurately known enthalpy change, e.g. heat and neutralisation of a strong acid. This method is not usually employed, due to practical difficulties, especially with flow cells.

The heat flowing from the cell to the heat sink or vice-versa passes through the thermopiles, and for each thermocouple the voltage, $V_1$ and the rate of heat flow dq/dt are proportional to the temperature difference between the calorimeter cell and heat sink;

$$V_1 = C \frac{dq}{dt}$$

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

$$V_1 + V_2 + \ldots\ldots = V = C \frac{dQ}{dt} \qquad (1.11)$$

The proportionality constant can be replaced by an effective value $C_1$ which is obtained by calibration (2.3.2); substitution of $C_1$ in equation 1.11 and integration gives

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

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

$$Q = J_s \times A$$

where $J_s$ is the calibration constant (2.2.1).

## 1.7 Objectives of This Project

The objectives of the project were;

1. the establishment of standard conditions for the reproducible growth of K. aerogenes in salts media;
2. the establishment of sampling, extraction and analysis techniques to obtain quantitative, reproducible and physiologically representative values of the ATP content of metabolising bacteria;
3. the combination of a culture vessel, a flow calorimeter and a bacterial ATP sampling device, and establishment of optimum experimental conditions for the measurement of thermal events and bacterial ATP;
4. the determination of changes in energy and bacterial ATP during growth under conditions of;
   (a) glucose-limitation;
   (b) phosphate-limitation;
5. the correlation of changes in energy, $CO_2$ and biomass during the growths of cells in the presence of antibacterial agents.

# CHAPTER TWO

# EXPERIMENTAL TECHNIQUES

## 2.1 Bacteriological Methods

### 2.1.1 Organism

Klebsiella aerogenes (N.C.T.C. 418) was used in this investigation. It is a non-motile species of the Entero-bacteriacea. Colonies grown on agar are white, smooth, raised, convex and often mucoid. It is Gram-negative, rod shaped, non-spore forming, aerobic or facultatively anaerobic, catalase positive and Voges-Proskauer positive.

In recent years the taxonomic definition has changed and this is fully clarified in Bergey's Manual (Buchanan and Gibbons, 1974).

Recently a new species of Klebsiella has been isolated from soil samples, (Izard, et al., 1981).

### 2.1.2 Media

(a) Nutrient broth (Oxoid No. 1) was used for the maintenance and storage of stock cultures.

(b) Glucose-limited medium was prepared in glass distilled water from salts medium and glucose solution, (sterilised separately). The composition of the salts medium was; $KH_2PO_4$, 26 mmol $dm^{-3}$, (3.6 g $dm^{-3}$); $(NH_4)_2SO_4$, 8 mmol $dm^{-3}$, (1.0 g $dm^{-3}$); $MgSO_4.7H_2O$, 0.16 mmol $dm^{-3}$, (0.04 g $dm^{-3}$), this was adjusted to pH 7.00 with saturated NaOH solution. Aliquots (800 $cm^3$) of this salts medium were sterilised at 15 p.s.i. for 20 minutes. To the sterile salts medium (600 $cm^3$) was added (aseptically) sterile glucose solution (20 $cm^3$) of known concentration to provide glucose-limited medium of the required glucose concentration. The standard glucose concentration was 3.3 mmol $dm^{-3}$.

(c) Phosphate-limited medium was prepared from sterile salts medium (600 $cm^3$) to which was added aseptically sterile glucose solution

(20 $cm^3$) of known concentration and sterile $KH_2PO_4$ solution (20 $cm^3$) of known concentration. This provided phosphate-limited medium of the required glucose and phosphate concentrations. The composition of the salts medium was; NaCl, 5 mmol $dm^{-3}$, (0.3 g $dm^{-3}$); KCl, 4 mmol $dm^{-3}$, (0.3 g $dm^{-3}$); $(NH_4)_2SO_4$, 8 mmol $dm^{-3}$, (1.0 g $dm^{-3}$); $MgSO_4.7H_2O$, 0.16 mmol $dm^{-3}$, (0.04 g $dm^{-3}$); Tris(hydroxymethyl)aminomethane, 0.16 mmol $dm^{-3}$, (19 g $dm^{-3}$), this was adjusted to pH 7.37 with HCl (conc.). The resultant ionic strength of the phosphate-limited medium was the same as that of the glucose-limited medium. Tris(hydroxymethyl)aminomethane has a significant temperature coefficient (0.025 pH units per °C between 22°C and 37°C), hence the pH of the salts medium was adjusted to pH 7.37 at 22°C.

(d) Klebsiella aerogenes was grown in boiling tubes containing 25 $cm^3$ of the relevant limiting-medium for the purposes of culture maintenance and training. To the relevant sterile salts medium contained in the boiling tube was added (aseptically) sterile glucose solution (0.8 $cm^3$) or sterile glucose solution (0.8 $cm^3$) and sterile $KH_2PO_4$ solution (0.8 $cm^3$). The tubes were plugged with sterile cotton wool. The limiting media were aerated with sterile air passed down a sterile Pasteur pipette through the cotton wool plug.

### 2.1.3 Preparation of Cultures

The parent strain was maintained by a monthly subculture in 10 $cm^3$ nutrient broth, in screw capped universal bottles. These cultures were grown at 37°C for 15 h and then stored at 4°C.

Parent cells were adapted for growth in glucose- or phosphate-limited medium by repeatedly subculturing the cells in the relevant medium for a minimum of 15 subcultures. The cells were grown in the sterile plugged boiling tubes (2.1.2 d) at 37°C with vigorous aeration.

Cells of Klebsiella aerogenes did not metabolise tris(hydroxymethyl) aminomethane before or after training in phosphate-limited medium, since growth did not occur in phosphate-limited medium from which the glucose solution had been excluded.

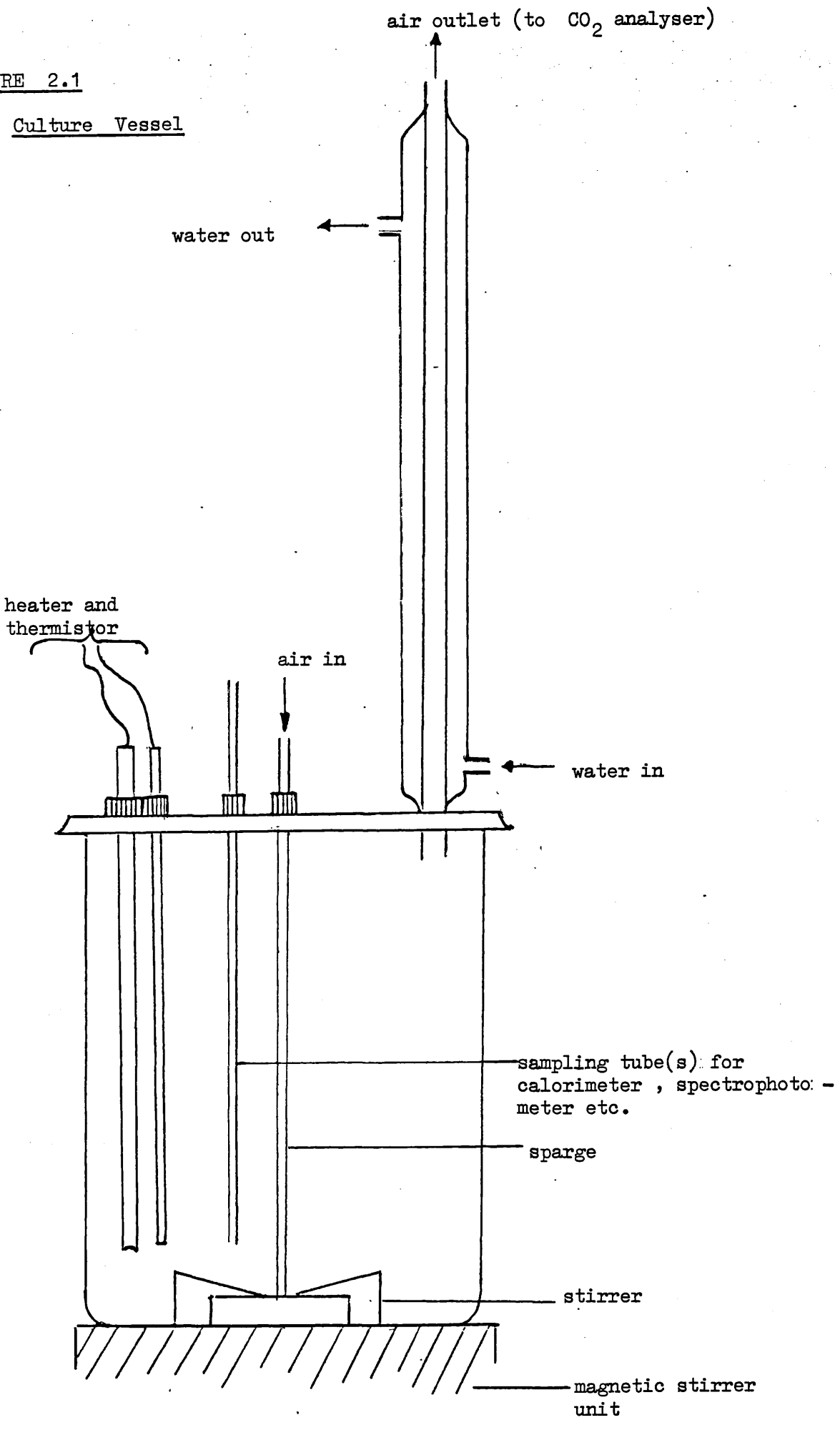
The adapted cells were used for the preparation of standard cryogenic inocula as follows: the adapted cells were inoculated into 620 $cm^3$ or 640 $cm^3$ of the appropriate limiting medium in a 1 $dm^3$ Gallenkamp Modular Fermenter System culture vessel, (Fig. 2.1). Sterile air was pumped in (1.5 $dm^3$ $min^{-1}$) via a sparger at the base of a magnetic stirrer operated at 1300 r.p.m. The temperature was maintained at 37°C ± 0.1°C. The cells were harvested aseptically 30-45 minutes prior to the termination of growth by centrifuging, (3500 r.p.m. for 15 mins). The sedimented cells were resuspended in salts medium (lacking carbon source or carbon and phosphate source) so that a 1.5 $cm^3$ aliquot gave a population of 3 x $10^7$ cells $cm^{-3}$ when inoculated into 620-640 $cm^3$ of medium. These 1.5 $cm^3$ aliquots were dispensed aseptically into 2 $cm^3$ sterile screw-capped polypropylene ampoules.

Previous workers had reported that cells stored in glass ampoules with plastic screw tops often exhibited a long lag phase (1.3 hr). This was found to be due to some phenolic monomer leaching from the phenolic resin screw tops of the ampoules during sterilisation, which inhibited growth.

A batch of ampoules (8-12) in a plastic cup was suspended above the surface (~2 mm) of liquid nitrogen, contained in a vivostat, for 30 minutes. They were then plunged into a liquid nitrogen and stored there until required. The whole process from the time of harvesting the cells from the fermenter and suspending the ampoules above the liquid nitrogen did not exceed 25 minutes. If the process took longer than 25 minutes the cells when inoculated into the relevant limiting medium exhibited an

FIGURE 2.1

Culture Vessel



unreproducible and unacceptably long lag phase (~1 h).

When an ampoule was required it was removed from the liquid nitrogen and dropped into a plastic beaker of freshly distilled water (500 $cm^3$) at a temperature of 37°C. Care was taken in this operation since there was the slight risk of an ampoule exploding. After exactly 3 minutes the ampoule was removed and its contents transferred by a sterile syringe, through a silicone-rubber membrane in the top of the fermenter, into the relevant medium which was maintained at 37°C with full aeration for at least an hour prior to inoculation.

### 2.1.4 Purity Checks

The purity of parent cultures and ampoule batches were checked by plating. Purity and identification was also determined using the A.P.I. 20E system.

### 2.1.5 Washing and Sterilisation

All contaminated glassware was either immersed in Lysol solution or autoclaved prior to washing. The decontaminated glassware was washed in tap water and then rinsed four times in distilled water. The use of detergents was avoided. Dry sterilisation was accompanied by drying glassware at 150°C for 2 hours; wet sterilisation with steam at 15 p.s.i. for 20 min. The electrical connections to the fermenter were wrapped to prevent water condensing on them. Also the condenser and other outlets of the fermenter were plugged with cotton wool and wrapped tightly in tin foil before autoclaving the complete fermenter assembly.

## 2.2 Measurement of Environmental Growth Parameters

### 2.2.1 Estimation of Biomass

The biomass was estimated from the measurement of the absorbance

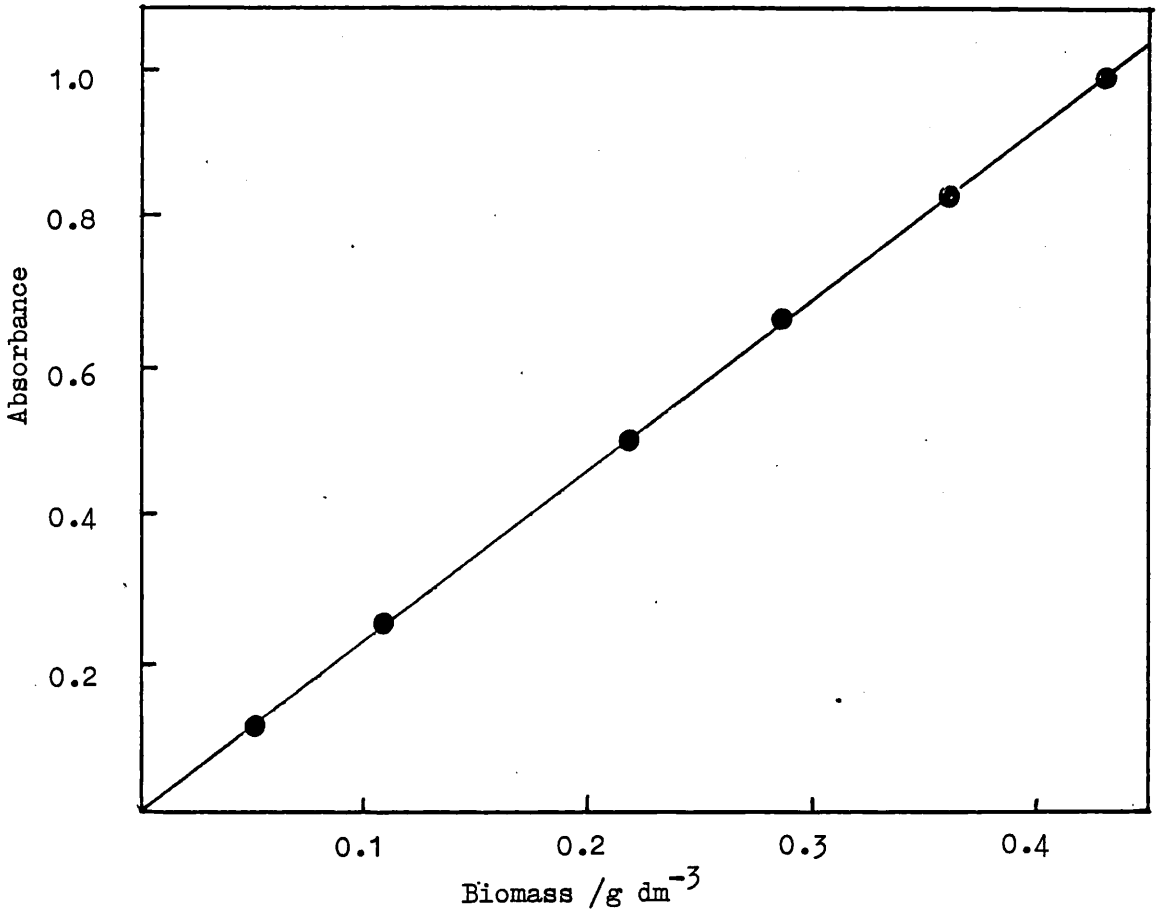
of the culture at 625 nm using a previously established biomass/ absorbance calibration curve. Bacterial size, density and refractive index change during the growth cycle, (Hadjipetrou and Stouthamer, 1963). This causes a change in the absorbance properties of the cells. For Klebsiella aerogenes $10^{10}$ cells, grown under a range of different culture conditions, have an equivalent dry weight of 11.3-12.9 mg, (Dean and Rogers, 1967).

It is not practical to produce a dry weight calibration curve for each stage of the growth cycle and so a standard was established with cells of a defined age. A new calibration curve was constructed for a change in culture conditions such as cells adapted to phosphate limitation growing in phosphate-limited medium or in standard glucose-limited medium, (i.e. phosphate sufficient).

The relevant medium (620 or 640 $cm^3$) was inoculated with the relevant cryogenically stored inoculum and grown under the chosen environmental parameters of growth, (37°C, full aeration, etc). These cells were harvested by centrifugation (3500 r.p.m. for 30 minutes) 40 minutes before the complete utilisation of the limiting nutrient. The cells were resuspended in distilled water and centrifuged. This washing was repeated twice more and the cells were resuspended in distilled water to give an absorbance of 0.85 at 625 nm against a water blank. Twelve aliquots (10 $cm^3$) were pipetted into beakers (25 $cm^3$) which had been previously dried at 96°C to constant weight. The twelve bacterial samples were dried at 96°C to constant weight.

A series of volumetric dilutions were prepared from the original bacterial suspension and the absorbance of each was measured and the bacterial dry weight versus absorbance curve was constructed, (Fig. 2.2). The plot in Fig. 2.2 refers to 1 cm path length cells in a Unicam SP600 spectrophotometer. Any change in the cells or the geometry of the

FIGURE 2.2 Bacterial Dry Weight Calibration Curve
(1 cm path length, 625 nm, SP 600 )




KEY ; —●— Bacteria trained for growth in glucose-limited medium and in phosphate-limited medium

spectrophotometer alter the slope of these curves. A difference of 17% was noted in the absorbance of a given bacterial suspension as measured on different spectrophotometers.

During a batch growth in the fermenter the growing bacterial suspension was pumped with a peristaltic pump through 'Teflon' tubing (I.D. 1 mm) lagged at 37°C to a 1 cm path length flow cell situated in a SP600 spectrophotometer. The suspension was then pumped back to the culture vessel again via lagged 'Teflon' tubing (Fig. 2.3). The flow rate of the suspension through the cuvette was 1.2 $dm^3\ h^{-1}$. The residence time for a bacterial cell in the spectrophotometer cell was approximately 8 seconds. Very small air bubbles are sucked up from the fermenter and passed through the spectrophotometer cell. This causes interference of the absorbance measurement which manifested itself as a broad line on the spectrophotometer chart recorder trace. This was overcome by selecting a standard position on the trace (usually the edge) of the broad line for initial zeroing and subsequent readings. The positioning in the fermenter of the intake tube for the bacterial suspension to the spectrophotometer to minimise this interference was found by trial and error.

The accuracy of any bacterial dry weight measurement using this set-up was ±2% ($p = 0.95$).

### 2.2.2 Estimation of Carbon Dioxide in Effluent Air

The effluent air emitted from the condenser of the fermenter was carbon dioxide enriched. This air was passed continuously through a drying tube containing calcium chloride to an I.R. gas analyser, (I.R.G.A. 20, G.P. Instrumentation).

The I.R. gas analyser was calibrated using B.O.C. special gases; in this case $CO_2$ in $N_2$ mixes. The concentrations of $CO_2$ used were 200,

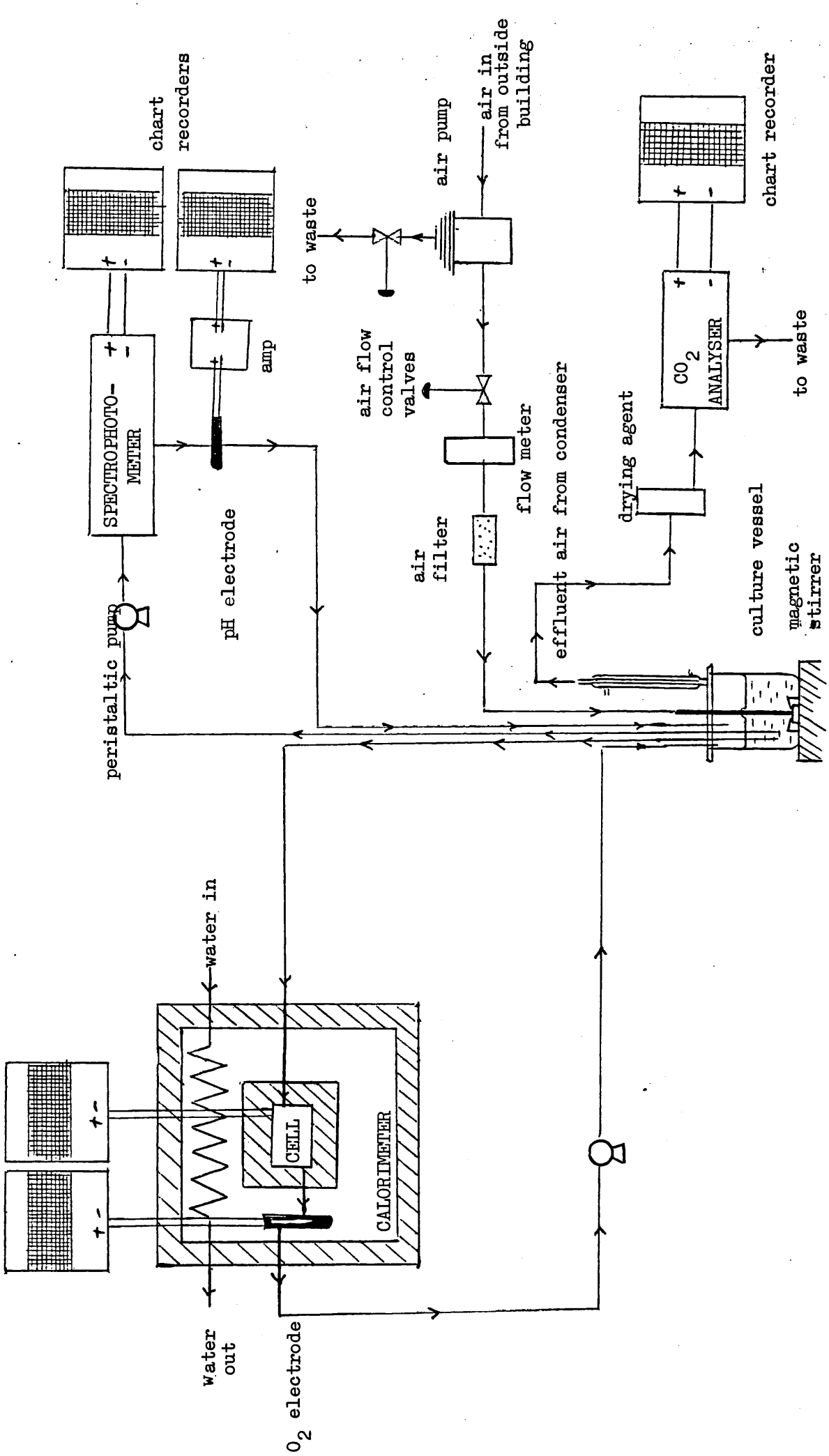



FIGURE 2.3 The Culture Vessel Assembly (Diagrammatic)

450 and 700 ppm. Filtered air drawn from outside the room was continuously pumped (1.5 $dm^3$ $min^{-1}$) through the reference cell of the analyser. In turn each of the special gases were pumped (1.5 $dm^3$ $min^{-1}$) through the analyser for a period of 1 hr. The electrical output of the analyser was fed to a flat bed chart recorder. The areas under the plateaux regions of Fig. 2.5 were linearly related to the number of moles of $CO_2$ passed and were used to calculate the factor $F_{CO_2}$ which converted area ($cm^2$) to moles of $CO_2$. The displacement on the recorder trace was linearly related to the concentration of $CO_2$.

The slope of the graph of area under the plateaux regions versus the total number of moles passed gave $F_{CO_2}$ as $2.146 \times 10^{-4}$ mole $cm^{-2}$. The slope of the graph of displacement from the zero position versus $CO_2$ concentration led to the conversion factor that 1 cm of displacement on the chart is equivalent to 51.5 ppm.

In practice the I.R. $CO_2$ analyser was used to monitor continuously the effluent gas (Fig. 2.3). The area under the time trace produced on the chart recorder was measured using a Hewlett Packard Digitiser. This area was multiplied by $F_{CO_2}$ to obtain the number of moles of $CO_2$ produced during growth or during any required time period.

### 2.2.3 Estimation of pH of Growth Medium

The pH of the culture medium was continuously monitored by pumping it through a small sheath (c. 2 $cm^3$) containing a glass calomel electrode assembly, (E.I.L. 1140200). The electrode was calibrated using buffer solutions of pH 4.50 and 7.00. The pH was measured with an E.I.L. pH meter (7020) and continuously recorded with an Oxford 3000 potentiometric recorder, (f.s.d. 10 mV).

### 2.2.4 Estimation of Oxygen Tension of Growth Medium

The oxygen tension of the culture medium during growth was

continuously monitored. The electrode was of the membrane type (Johnson et al., 1964) manufactured by Process Instruments Ltd of Sherbourne. The electrolyte consisted of an aqueous solution of acetic acid, sodium acetate and lead acetate. The electrode was mounted in a small sheath (c. 1 $cm^3$) through which the bacterial suspension flowed. The sheath/electrode was situated in the flow line immediately after the calorimeter cell within the calorimeter air bath, (Fig. 2.3). This avoided possible erroneous readings due to temperature fluctuations.

The electrode was calibrated by first pumping a 5% wt/wt sodium sulphite solution through the electrode for 40 minutes and the metering unit was set to zero. Secondly aerated growth medium was passed through the electrode for a period of 1.5 hr and the metering unit was set to the 100% level.

Since the accuracy of any given $O_2$ level was ±5% ($p = 0.95$) the $O_2$ measurement was only used qualitatively to indicate the possibility of oxygen depletion leading to partially anaerobic growth in the calorimeter cell. The 100% $O_2$ saturation level is only a relative value since differences in the composition of the media will result in changes in the maximum $O_2$ saturation levels.

### 2.2.5 The Complete Culture Vessel Assembly

The measurement of biomass, oxygen tension, pH and $CO_2$ evolution have been discussed. Figure 2.3 shows diagramatically how these form the complete monitoring assembly; also shown is the calorimeter for monitoring the power output.

The assembly consists of two flow lines for bacterial suspension and one air flow line. Each of the flow lines for medium and culture were sterilised by pumping a 5% v/v formaldehyde solution through for a period of 1 hr, followed by 300 $cm^3$ of sterile distilled water. These

flow lines were lagged in water jackets at 37°C to avoid erroneous results from the calorimeter and fermenter due to temperature fluctuations of the bacterial suspension.

The time taken for a bacterial cell to leave the culture vessel and pass through the calorimeter and oxygen electrode back to the culture vessel was 90 sec., and the time for a bacterial cell to reach the block of the calorimeter was 60 sec. These are the shortest times reported to date. The positioning of the oxygen electrode after the block of the calorimeter means that the oxygen tension in the calorimeter cell is always greater than the recorded value.

In the calorimeter flow line the bacterial suspension was sucked from the culture vessel with a variable speed peristaltic pump through the calorimeter and oxygen electrode back to the vessel. The output from the oxygen electrode after amplification was recorded on a flat bed chart recorder, as was the output of the microcalorimeter.

In the spectrophotometer flow line the bacterial suspension was pumped from the culture vessel to the flow cell contained in the spectrophotometer and then to the pH electrode back to the culture vessel. The time taken for a bacterial cell to be pumped around this line was 10 sec.

Air was sucked into the compressor/pump from outside the building and then by means of the controller valves and flow gauges a measured flow was fed through a sterilising filter into the culture vessel. The sterile air entered the culture vessel through the sparger, and into the medium through the base of the stirrer. The air ($CO_2$ enriched) left the culture vessel through the water condenser and passed through the drying agent into the I.R. $CO_2$ analyser. The output of the analyser was fed directly to a chart recorder.

Prior to inoculation the aerated medium in the culture vessel was pumped around the calorimeter and spectrophotometer lines for at least

1.5 hr, to obtain steady base lines for each of the measurements.

### 2.3.1 Power and Heat Measurement

An L.K.B. 10700-1 flow microcalorimeter fitted with an aerobic stainless steel cell (1.11 $cm^3$) was used for the measurement of power output. The position of the microcalorimeter in the complete culture vessel assembly is shown in Figure 2.3. Figure 2.4 shows a cross section through the calorimeter with the location of the oxygen electrode clearly shown.

### 2.3.2 Calibration

The aerobic cell is embedded in the block of the calorimeter between the thermopiles. The block also has a small resistor built into it, through which a small electrical current (0.2-4.0 mA) can be passed for a set period of time. The magnitude of the current and time for which it is passed is controlled by the control unit. The current when passed through this resistor produces a heating effect which simulates the rate of heat emission (power) from the bacterial suspension flowing through the calorimeter cell.

For calibration purposes a formaldehyde-killed bacterial suspension, maintained at 37°C with normal aeration in the culture vessel, was pumped at the required flow rate through the cell during the passage of the current through the resistor. The subsequent output from the calorimeter was recorded as a trace; the displacement from the zero position being a measure of the power output, ($Js^{-1}$). This is directly related to the resistance of the resistor and the (current)$^2$ flowing through;

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

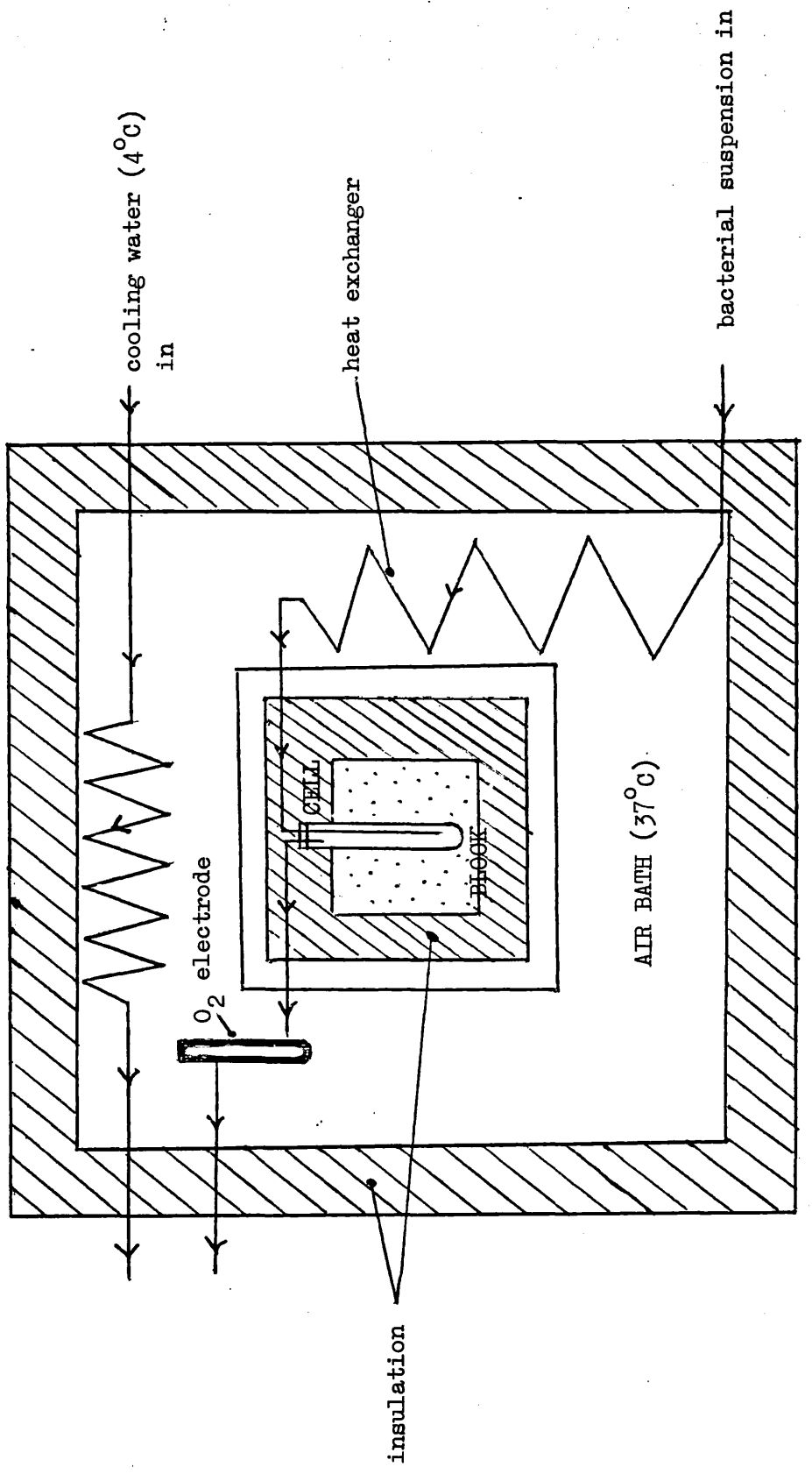



FIGURE 2.4 Diagrammatic Cross Section of The Calorimeter

where I is the applied current flowing and R is the heater resistance, (50 Ω). So any applicable current which was applied to the heating element produced a displacement which was directly proportional to the power output of the calorimeter which was proportional to the (current)$^2$.

Typical traces showing power as a function of time for cells growing in standard glucose-limited medium and the subsequent calibrations are shown in Figure 2.5.

For a standard set of conditions, i.e. bacterial population, flow rate, stirring and aeration, different currents were passed and the displacement on the traces were plotted against power output, (eqn. 2.1). From the slope of this straight line graph (r = 0.999) a factor $F_{Js^{-1}}$ was calculated for the conversion of displacement (cm) into a power reading ($Js^{-1}$);

$$F_{Js^{-1}} = 0.4430 \times 10^{-4}\ Js^{-1}\ cm^{-1}$$

The area under the experimental or calibration trace for a measured time period represents the total amount of heat liberated during that time. The area under the calibration trace, $A_{calib}$, was measured using the digitiser. The total amount of heat liberated was calculated from

$$Q = I^2Rt \qquad 2.3$$

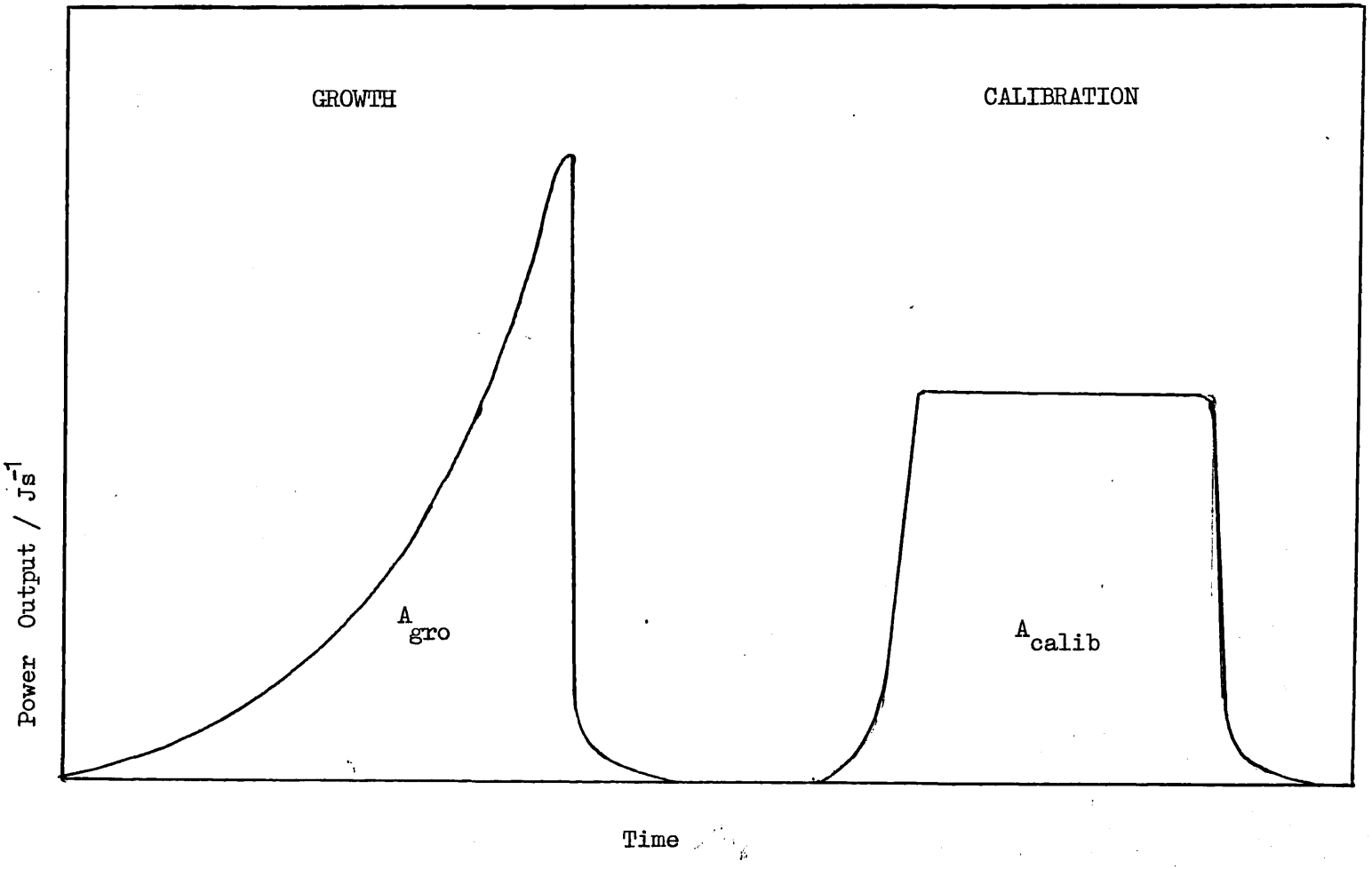
where t is the time in seconds for which the current was passed. A factor $F_J$ for the conversion of the area of chart paper ($cm^2$) to heat (J) was then calculated;

$$F_J = \frac{Q}{A_{calib}} = 1.550 \times 10^{-2}\ J\ cm^{-2} \qquad 2.4$$

### 2.3.3 Measurement of Heat Output during Bacterial Growth

The flow of the suspension through the calorimeter cell was 90 $cm^3$ $h^{-1}$. The flow rate and the effect of changing this rate on the p-t trace has already been established (Nichols and James, 1980).

FIGURE 2.5 Typical Power-Time Curve (P-t) for Growth in Glucose-Limited Medium and A Subsequent Calibration Curve



During a bacterial growth the heat was continuously monitored to produce the p-t trace. The position of zero heat output, i.e. the base line was established by pumping the stirred and aerated medium at 37°C for 1.5 hr through the calorimeter cell prior to inoculation.

The inoculum from cryogenic storage was thawed and injected into the culture vessel, (Section 2.1.3). As the cells started to divide and enter the growth phase there was an increase in the power output and hence the recorded p-t trace (Fig. 2.5). The power output continued to increase until the end of growth when there was usually a sudden fall. The experiment was continued until the p-t trace returned to the base line. Depending on the cells and medium used the attainment of the base line occurred 6-24 hr after the cessation of growth.

The displacement of the p-t trace from the base line was converted to power by using the factor $F_{Js-1}$ (Eqn. 2.3) and the area under the trace converted to heat output using the factor $F_J$ (Eqn. 2.4).

To obtain the total heat liberated from the total culture in the fermenter the following calculation was carried out;

$$\text{The total heat evolved } \Delta H_T = \frac{A_{gro}}{1.11} \times V \times F_J \qquad 2.5$$

where $A_{gro}$ is the heat measured by the calorimeter, i.e. area under the p-t trace, 1.11 is the effective volume of the calorimeter cell and V the initial total volume of the medium in the fermenter.

The effective volume of the aerobic steel cell was established as follows. The cell was dried and weighed. The cell was replaced in the calorimeter block and the flow lines connected. Water was pumped through the cell under the normal conditions of growth. The flow was then stopped, the flow lines disconnected and the cell and contents weighed. From the weight of the dry cell and the density of water the effective volume of the cell was calculated.

## 2.4 Analytical Enzymic Assay Methods

### 2.4.1 Preparation of Culture Suspension Prior to Analysis

A sample (30 $cm^3$) was drawn into a syringe via a sample port on the fermenter top. This was then forced through a Millipore filter (0.22 μm) and the cell free supernatant frozen in an acetone/solid $CO_2$ mulch and then stored at -20°C prior to analysis.

### 2.4.2 Validity of Enzymic Assays

To test the validity of all enzymic assays utilised in this thesis the following procedure was performed. The inhibitory effects of the media were tested by performing analyses for the relevant substances dissolved in medium and distilled water. A range of concentrations were analysed to test the linearity of the response (usually absorbance) of the assay, and its reproducibility. All assays were linear and the reproducibility was ±2% (p = 0.95) at the minimum detection limit of 0.2 mmol $dm^{-3}$.

### 2.4.3 Enzymic Assay of Acetate in the Culture Medium

#### Theory

In the presence of the enzyme acetyl-CoA synthetase (acetate thiokinase) acetate is converted with ATP and coenzyme A (CoA) to acetyl-CoA;

$$\text{Acetate} + \text{ATP} + \text{Co A} \xrightarrow[\text{thiokinase}]{\text{acetate}} \text{acetyle-CoA} + \text{AMP} + \text{pyrophosphate}$$

Acetyl-CoA reacts with oxaloacetate to form citrate in the presence of citrate synthase (CS);

$$\text{Acetyl-CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\text{CS}} \text{citrate} + \text{CoA}$$

The oxaloacetate required for the above reaction is formed from malate and NAD In the presence of malate dehydrogenase (MDH). In the reaction NAD is reduced to NADH;

$$\text{Malate} + \text{NAD} \xrightarrow{\text{MDH}} \text{oxaloacetate} + \text{NADH} + H^+$$

The determination of acetate is based on the formation of NADH measured by the increase in absorbance at 340 nm. Since a preceding indicator reaction is used the amount of NADH formed is not linearly proportional to the acetic acid concentration, (Bergmeyer, 1974).

Method

The following solutions were used:

Soln. 1; triethanolamine buffer solution (0.1 mol $dm^{-3}$ pH 8.4), containing L-malic acid (0.42 g $dm^{-3}$) and magnesium chloride (0.21 g $dm^{-3}$).

Soln. 2; ATP (25 g $dm^{-3}$), CoA (2.6 g $dm^{-3}$) and NAD (12.3 g $dm^{-3}$) in water.

Soln. 3; malate dehydrogenase (2,750 U $cm^{-3}$) and citrate synthase (675 U $cm^{-3}$).

Soln. 4; acetyl-CoA-synthetase (40 U $cm^{-3}$).

Solution 1 (0.80 $cm^3$), solution 2 (0.20 $cm^3$), water (1.60 $cm^3$) and sample (0.10 $cm^3$) were pipetted into a 1 cm path length U.V. spectrophotometer cell. The absorbance ($A_0$) of this was measured against an air reference at 340 nm. Solution 3 (0.01 $cm^3$) was added and after 3 minutes the new absorbance ($A_1$) was read. Solution 4 (0.01 $cm^3$) was added and after 10-15 minutes the absorbance ($A_2$) was read. A control was

performed by repeating the above procedure but replacing the sample (0.10 $cm^3$) with medium (0.10 $cm^3$) which had not been inoculated and therefore in which no growth had taken place.

Calculations

$$\Delta A = \left[ (A_2-A_0) - \frac{(A_1-A_0)^2}{(A_2-A_0)} \right]_{sample} - \left[ (A_2-A_0) - \frac{(A_1-A_0)^2}{(A_2-A_0)} \right]_{control}$$

Conc. of Acetate/mmol $dm^{-3}$ = 4.317 $\Delta A$

2.4.4 Enzymic Assay of Pyruvate in the Culture Medium

Theory

In the presence of the enzyme L-lactate dehydrogenase (LDH) pyruvate is reduced by nicotinamide-adenine dinucleotide (NADH) to L-lactate;

$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + NAD^+$$

The amount of NADH oxidised during the above reaction is stoichiometric with the amount of pyruvate. The decrease in NADH is determined by means of its absorbance at 340 nm, read against an air reference in a 1 cm cell, (Czok and Lamprecht, 1974).

Method

The following solutions were used;

Soln. 1; triethanolamine buffer solution (0.75 mol $dm^{-3}$, pH 7.6) containing EDTA (7.5 mol $dm^{-3}$).

Soln. 2; NADH (5 g $dm^{-3}$) and $NaHCO_3$ (10 g $dm^{-3}$) in distilled water.

Soln. 3; L-lactate dehydrogenase (5 g $dm^{-3}$).

Solution 1 (2.50 $cm^3$), solution 2 (0.20 $cm^3$) and sample (1.10 $cm^3$) or medium (0.1 $cm^3$) as the control, were pipetted into a 1 cm path length U.V. spectrophotometer cell and the absorbance ($A_0$) measured against an air reference at 340 nm. Solution 3 (0.02 $cm^3$) was added and after 6 minutes the absorbance ($A_1$) was measured.

Calculations

$$\Delta A = (A_0 - A_1)_{sample} - (A_0 - A_1)_{control}$$

Conc. of Pyruvate/mmol $dm^{-3}$ = 4.476 $\Delta A$

2.4.5 Enzymic Determination of Lactate in the Culture Medium

Theory

In the presence of L-lactate dehydrogenase (L-LDH), L-lactic acid (L-lactate) is oxidised by NAD to pyruvate;

$$\text{L-lactate} + NAD^+ \xrightarrow{\text{L-LDH}} \text{pyruvate} + NADH + H^+$$

The equilibrium of this reaction lies almost completely on the side of the lactate. However, by trapping the pyruvate in a subsequent reaction catalysed by the enzyme glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate the equilibrium can be displaced in favour of the pyruvate and NADH;

$$\text{Pyruvate} + \text{L-glutamate} \xrightarrow{\text{GPT}} \text{L-alanine} + \alpha\text{-ketoglutarate}$$

The amount of NADH formed in the above reaction is stoichiometric with the concentration of L-lactate. The increase in NADH is determined by means of its absorbance at 340 nm, with air as a reference in a 1 cm cell, (Noll, 1974).

Method

The following solutions were used;

Soln. 1; glycylglycine buffer solution (0.75 mol $dm^{-3}$, pH 10.0)

containing L-glutamic acid (14.5 g $dm^{-3}$).

Soln. 2; β-NAD (35 g $dm^{-3}$).

Soln. 3; glutamate-pyruvate transaminase (1600 U $cm^{-3}$).

Soln. 4; L-lactate dehydrogenase (5,500 U $dm^{-3}$).

Solution 1 (1.20 $cm^3$), solution 2 (0.20 $cm^3$), water (1.10 $cm^3$), solution 3 (0.02 $cm^3$) and sample (0.10 $cm^3$) or medium (0.10 $cm^3$) for the control, were pipetted into a 1 cm path length U.V. spectrophotometer cell. After 5 minutes the absorbance ($A_0$) was measured at 340 nm against an air reference. Solution 4 (0.02 $cm^3$) was added and after 10 minutes the new absorbance ($A_1$) was measured.

Calculations

$$\Delta A = (A_1 - A_0)_{sample} - (A_1 - A_0)_{control}$$

Conc. of Lactate mmol $dm^{-3}$ = 0.419 $\Delta A$

2.4.6 Enzymic Assay of Acetaldehyde in the Culture Medium

Theory

In the presence of aldehyde dehydrogenase (Al-DH) acetaldehyde is oxidised quantitatively by NAD to acetic acid;

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed in the above reaction is determined from its absorbance at 340 nm in a 1 cm path length cell against an air reference, (Lundquist, 1974).

Method

The following solutions were used;

Soln. 1; potassium diphosphate buffer solution (0.3 mol $dm^{-3}$, pH 9.0).

Soln. 2; NAD (34 g $dm^{-3}$).

Soln. 3; aldehyde dehydrogenase (80 U $cm^{-3}$).

Solution 1 (0.90 $cm^3$), solution 2 (0.10 $cm^3$), water (1.70 $cm^3$) and sample (0.10 $cm^3$) or medium (0.10 $cm^3$) as the control, were pipetted into a 1 cm path length U.V. spectrophotometer cell and the absorbance ($A_0$) measured at 340 nm against an air reference. Solution 3 (0.02 $cm^3$) was added and after 4 minutes the new absorbance ($A_1$) was read.

Calculations

$$\Delta A = (A_1 - A_0)_{sample} - (A_1 - A_0)_{control}$$

Conc. of Acetaldehyde/mmol $dm^{-3}$ = 4.476 $\Delta A$

2.4.7 Enzymic Determination of Ethanol in the Culture Medium

Theory

Ethanol is oxidised in the presence of the enzyme alcohol dehydrogenase (ADH) by NAD to acetaldehyde;

$$\text{Ethanol} + NAD^+ \xrightarrow{ADH} \text{acetaldehyde} + NADH + H^+$$

The equilibrium of this reaction lies on the side of ethanol and NAD. It can however be completely displaced to the right under alkaline conditions and by trapping of the acetaldehyde formed. Acetaldehyde is oxidised in the presence of aldehyde dehydrogenase (Al-DH) quantitatively to acetic acid;

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{Al\text{-}DH} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed in the above reaction is stoichiometric with half the amount of ethanol, (Beuter and Michal, 1977). NADH is determined from its absorbance in a 1 cm cell at 340 nm against an air reference.

Method

The following solutions were used:

Soln. 1; $Na_2HPO_4$ buffer solution (0.75 mol $dm^{-3}$, pH 9.0) containing NAD (4 g $dm^{-3}$), aldehyde-DH (3 U $cm^{-3}$).

Soln. 2; ADH (15,000 U $cm^{-3}$).

Solution 1 (1.00 $cm^3$), water (1.80 $cm^3$), and sample (0.10 $cm^3$) or medium (0.10 $cm^3$) for the control, were pipetted into a 1 cm path length U.V. spectrophotometer cell and after 2 minutes the absorbance ($A_0$) was measured at 340 nm against an air reference. Solution 2 (0.02 $cm^3$) was added and after 6 minutes the new absorbance ($A_1$) was read.

Calculations

$$\Delta A = (A_1 - A_0)_{sample} - (A_1 - A_0)_{control}$$

Conc. of Ethanol/mmol $dm^{-3}$ = 2.317 $\Delta A$

2.4.8 Enzymic Determination of Succinate in the Culture Medium

Theory

In the presence of the enzyme succinyl-CoA synthetase (SCS) succinate is converted with inosine-5'-triphosphate (ITP) and coenzyme A (CoA) to succinyl-CoA;

$$\text{Succinate} + \text{ITP} + \text{CoA} \xrightarrow{\text{SCS}} \text{IDP} + \text{succinyl} - \text{CoA} + P_i$$

The inosine-5'-diphosphate (IDP) formed reacts with phosphoenolpyruvate (PEP) to pyruvate in the presence of pyruvate kinase (PK);

$$IDP + PEP \xrightarrow{PK} ITP + pyruvate$$

Pyruvate is reduced by NADH in the presence of lactate dehydrogenase;

$$Pyruvate + NADH + H^+ \xrightarrow{LDH} L\text{-lactate} + NAD^+$$

The amount of NADH oxidised in the above reaction is stoichiometric with the amount of succinic acid originally reacted with ITP and CoA, (Michal et al., 1976). The NADH is determined from its absorbance measured at 340 nm in a 1 cm path length cell against an air reference.

Method

The following solutions were used;

Soln. 1; glycylglycine buffer solution (0.3 mol $dm^{-3}$, pH 8.4), containing $MgSO_4$ (4.0 mmol $dm^{-3}$).

Soln. 2; NADH-$Na_2$ (7.5 g $dm^{-3}$), $NaHCO_3$ (15 g $dm^{-3}$).

Soln. 3; CoA-$Li_3$ (10 g $dm^{-3}$), ITP-$Na_3$ (10 g $dm^{-3}$) and PEP-$(CHA)_3$ (10 g $dm^{-3}$).

Soln. 4'; PK (3 mg $cm^{-3}$), LDH (1 mg $cm^{-3}$).

Soln. 5 Succinyl-CoA synthetase (5 mg $cm^{-3}$) ≡ (0.36 U $cm^{-3}$).

Solution 1 (1.00 $cm^3$), solution 2 (0.10 $cm^3$), solution 3 (0.10 $cm^3$), water (1.4 $cm^3$), solution 4 (0.05 $cm^3$) and sample (0.10 $cm^3$) or medium (0.10 $cm^3$) for the control, were pipetted into a 1 cm path length U.V. spectrophotometer cell and after 5 minutes the absorbance ($A_0$) was

measured at 340 nm against an air reference. Solution 5 (0.02 $cm^3$) was added and after 20 minutes the new absorbance ($A_1$) was read.

Calculations

$$\Delta A = (A_0 - A_1)_{sample} - (A_0 - A_1)_{control}$$

Conc. of Succinate/mmol $dm^{-3}$ = 4.468 $\Delta A$

## 2.5 Analytical Chemical Assay of Culture Medium

### 2.5.1 Determination of Phosphate in the Culture Medium

Theory

Orthophosphate and molybdate ions condense in acidic solution to give molybdophosphoric acid which upon selective reduction with 2,4-diaminophenol hydrochloride in the presence of metabisulphate produces a blue colour due to molybdenum blue of uncertain composition. The complex exhibits a maximum absorption at 600 nm when in the presence of 5% perchloric acid. The intensity of the blue colour is proportional to the amount of phosphate initially incorporated in the heteropoly acid.

Method

The cell free sample (passed through a 0.22 μm Millipore filter) or standard phosphate solution (20 $cm^3$), perchloric acid (60%, 2 $cm^3$), amidol reagent (1% 2,4-diaminophenol hydrochloride in 20% sodium metabisulphite, 2 $cm^3$) and ammonium molybdate (8.3%, 1 $cm^3$) were mixed together and allowed to stand for 10 minutes. The intensity of the blue colour was measured at 600 nm against a water blank in 1 cm cells.

A calibration curve was constructed of concentration of phosphate versus absorbance which was found to be linear. From the slope of this line and an absorbance reading of the sample the concentration of

phosphate in the sample was calculated.

Results

The results expressed as μg P $cm^{-3}$ were found to have an accuracy of ±3% (p = 0.95) at the minimum detection limit of 0.1 μg P $cm^{-3}$.

### 2.5.2 Determination of Glucose in the Culture Medium

The method is based on that of Duboski (1962) as modified by Feu et al., (1976). Freshly distilled o-toluidine in glacial acetic acid (6%, 5 $cm^3$) and sample or standard solution (1 $cm^3$) were pipetted into boiling tubes. These were loosely sealed and placed in a boiling water bath for 15 minutes. The tubes were cooled in ice water and the intensity of the green colour measured at 630 nm against a blank reference using 1 cm cells. The concentration of an unknown can be obtained from a calibration curve constructed from standard glucose solutions.

It is important that freshly distilled o-toluidine is used. The o-toluidine in glacial acetic acid can be stored at 4°C, ready for use, for up to one week.

The accuracy of the method was found to be ±4% (p = 0.95) at the minimum detection limit of 0.04 mg $cm^{-3}$.

## 2.6 Other Analytical Procedures

### 2.6.1 Elemental Analysis of Bacterial Cells

Bacterial cells for analysis were harvested and washed three times in distilled water and oven dried (98°C) for 48 hr. The carbon, hydrogen, nitrogen and residue of the cells were determined with a Perkin-Elmer 240 elemental analyzer. Oxygen, phosphorus and sulphur were

determined by Butterworth Laboratories. Values for oxygen estimated by difference from the carbon, hydrogen and nitrogen analysis agreed well with determined values.

# CHAPTER THREE

## DEVELOPMENT OF A BACTERIAL - ATP EXTRACTION AND ANALYSIS TECHNIQUE

## 3.1 Introduction

### 3.1.1 Historical Background

Enzyme mediated light production, "bioluminescence" is a wide spread phenomenon in nature. Bioluminescent organisms are found in essentially all major phyla. Most systems consist of an enzyme catalysing the oxidation of a substrate by oxygen or hydrogen peroxide. The light emission is often of a high quantum yield and the colour varies from blue to red depending upon the system involved. The biological aspects of bioluminescence have been described in the very comprehensive monologue by Harvey (1952). Among insects, bioluminescence is found in a number of species, many of these belong to the Coleoptera family "Lambyridae"; the so called fireflies. These insects emit light from specialised abdominal organs as a mating signal (McElroy, et al., 1974).

Most of the present state of knowledge of the chemistry of firefly bioluminescence is due to the work of McElroy and his co-workers (McElroy, et al., 1976), with the North American firefly Photinus pyralis. This started in 1947 with the important discovery that firefly bioluminescence required ATP (McElroy, 1947).

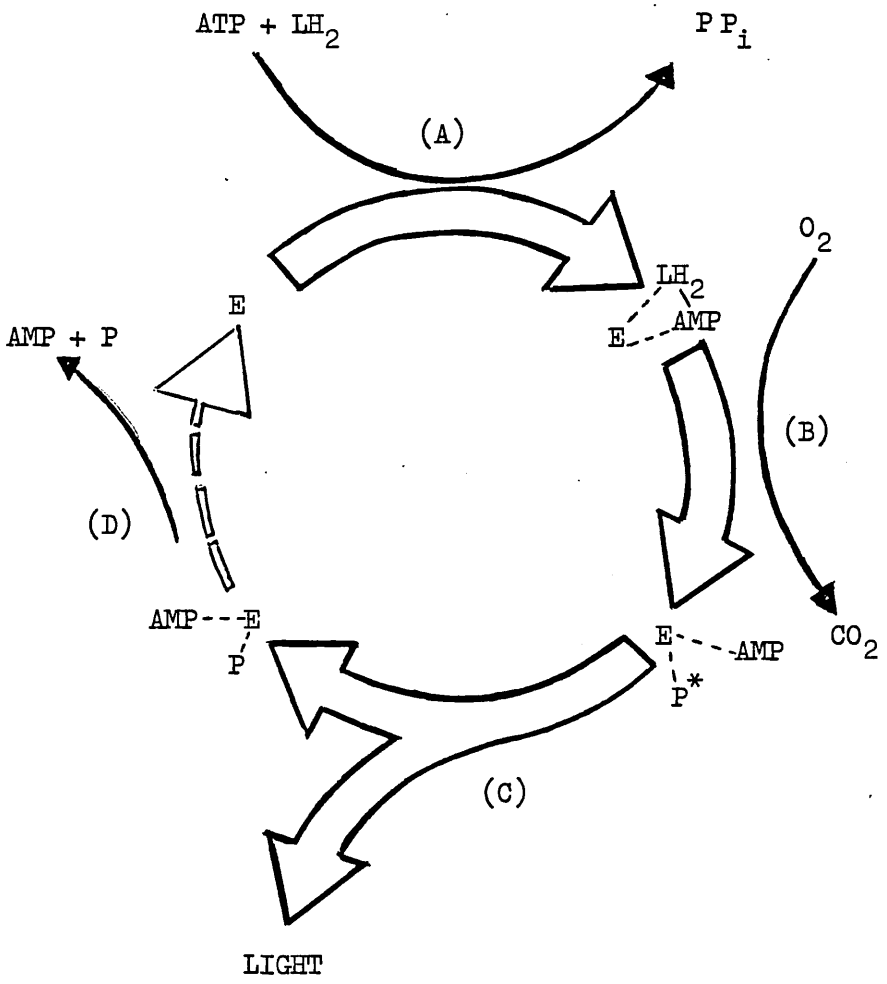
It soon became evident that the firefly system could be used for analytical purposes and in 1952 Stehler and Totler demonstrated some examples of assays based on the detection of adenosine-5'-triphosphate (ATP) by firefly bioluminescence (Stehler and Totler, 1952).

### 3.1.2 The Firefly Luciferase Reaction

The current knowledge regarding the firefly reaction is summarised in Figure 3.1. The basic steps involved are described below;

(a) In an initial activating step, similar to the activation of amino

FIGURE 3.1 A Schematic Summary of The Mechanism of The Firefly Luciferase Reaction




KEY ; $P^*$ : electronically excited oxyluciferin
P : oxyluciferin
E : luciferase
P $P_i$ : pyrophosphate
$LH_2$ : luciferin

acids in protein biosynthesis, luciferin, $LH_2$, is adenylated by ATP to form luciferyladenylate bound to the luciferase enzyme, E, with the release of pyrophosphate, $PP_i$, (Fig. 3.2). The structural formula of ATP is shown in Figure 3.3.

(b) In the next step the luciferyladenylate enzyme complex is oxidised by molecular oxygen resulting in the formation of carbon dioxide, adenosine-5'-monophosphate (AMP) and electronically excited oxyluciferin. AMP and oxyluciferin remain bound to the enzyme.

(c) On returning to the ground state the excited complex emits light. The last two steps can be expressed as shown in Figure 3.4. The light emission exhibits a maximum between 560 and 620 nm (McElroy, et al., 1974), depending upon the structure of the luciferase and the pH. The wavelength of maximum emission of the luciferase used for this work (L.K.B. Monitoring Reagent) was 562 nm at a pH of 7.75. Under favourable conditions quantum yields close to 100% have been observed (Seliger, et al., 1960).

(d) In the final step luciferin and AMP are released from the luciferase enzyme which is then free to enter another reaction cycle.

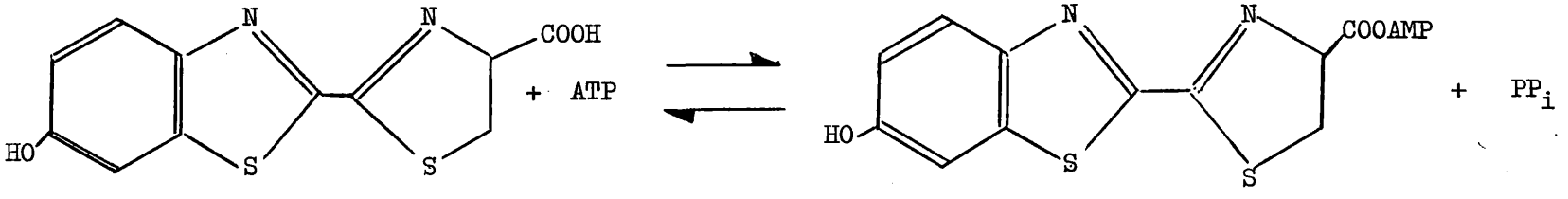
The overall reaction can be represented by the equation;

$$\text{ATP} + \text{LUCIFERIN} \xrightarrow[Mg^{2+}]{\text{LUCIFERASE}} \text{AMP} + PP_i + CO_2 + H_2O + \text{OXYLUCIFERIN} + h\nu$$

Magnesium ions are a co-factor required for the enzyme reaction.

The firefly luciferase enzyme is a complex protein. It is a dimer of two subunits of molecular weights of approximately 50,000. The

FIGURE 3.2 The Initial Activating Step of The Firefly Luciferase Reaction




(LUCIFERIN) (LUCIFERYL ADENYLATE)

FIGURE 3.3 The structural Formula of ATP

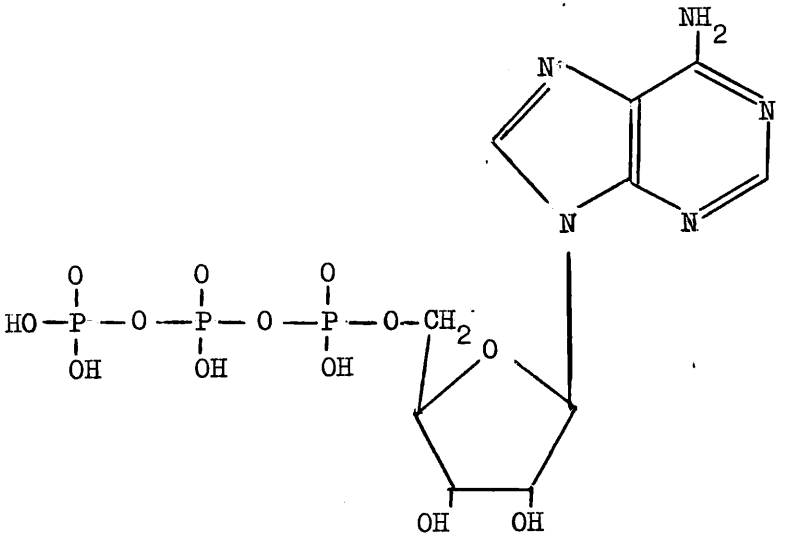
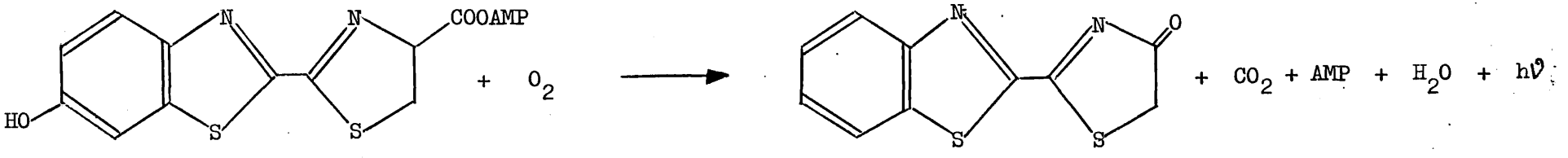


FIGURE 3.4 Steps B and C of The Firefly Luciferase Reaction




(OXYLUCIFERIN)

sulphydryl groups of the luciferase are essential for its catalytic action (DeLuca, 1969). The assay of ATP requires the presence in the luciferase/luciferin system of magnesium ions and oxygen. If the concentration of these components is held constant the system will emit light of intensity proportional to added low levels of ATP (Thore, 1976). The assay can detect as few as $10^{-17}$ moles of ATP (Chappelle, 1968).

### 3.1.3 Reaction Kinetics of the Firefly Luciferase Reaction

#### (i) Crude Luciferase

Crude luciferase is the name given to the unrefined luciferin/luciferase extracted from the insect commonly known as the 'Lantern Firefly'. Early workers using this extract to measure ATP levels found it difficult to obtain reliable results, because the crude extract had very complicated light emission kinetics due to the ATP-converting enzymes present, i.e. adenylate kinase, nucleoside diphosphate kinase and creatine kinase (Rasmussen, et al., 1968; Lundin, et al., 1975; Benny, et al., 1976). In the presence of substrates originating from the biological sample assayed and those present in the luciferin/luciferase reagent the contaminating enzymes continuously changed the ATP level in the system. This caused unpredictable light emission kinetics (Lundin, et al., 1975).

The other major factor which caused a change in the light output during measurement was the ratio of the concentrations of the luciferin and luciferase, which were incorrect. In the crude extract there was a relative excessively high concentration of luciferase with respect to luciferin. This enabled the reaction to progress at a relatively fast rate causing an appreciable drop in the ATP concentration during measurement. There was an initial peak light output at the start of the

reaction followed by a gradual decay as the reaction progressed (Fig. 3.5).

The rate limiting stage of the reaction (Fig. 3.1) is the removal of the AMP and oxyluciferin (P) from the luciferase (E), so that the enzyme can be recycled to combine with more luciferin and ATP (Lundin, et al., 1976).

In the crude extract because of the large amount of luciferase present the rate of the reaction was not dependent upon the recycling of luciferase and a flash of light was emitted (Fig. 3.5, A) followed by a rapid decay of the light output due to the consumption of the ATP. The consumption of the ATP was a result of the fast reaction rate.

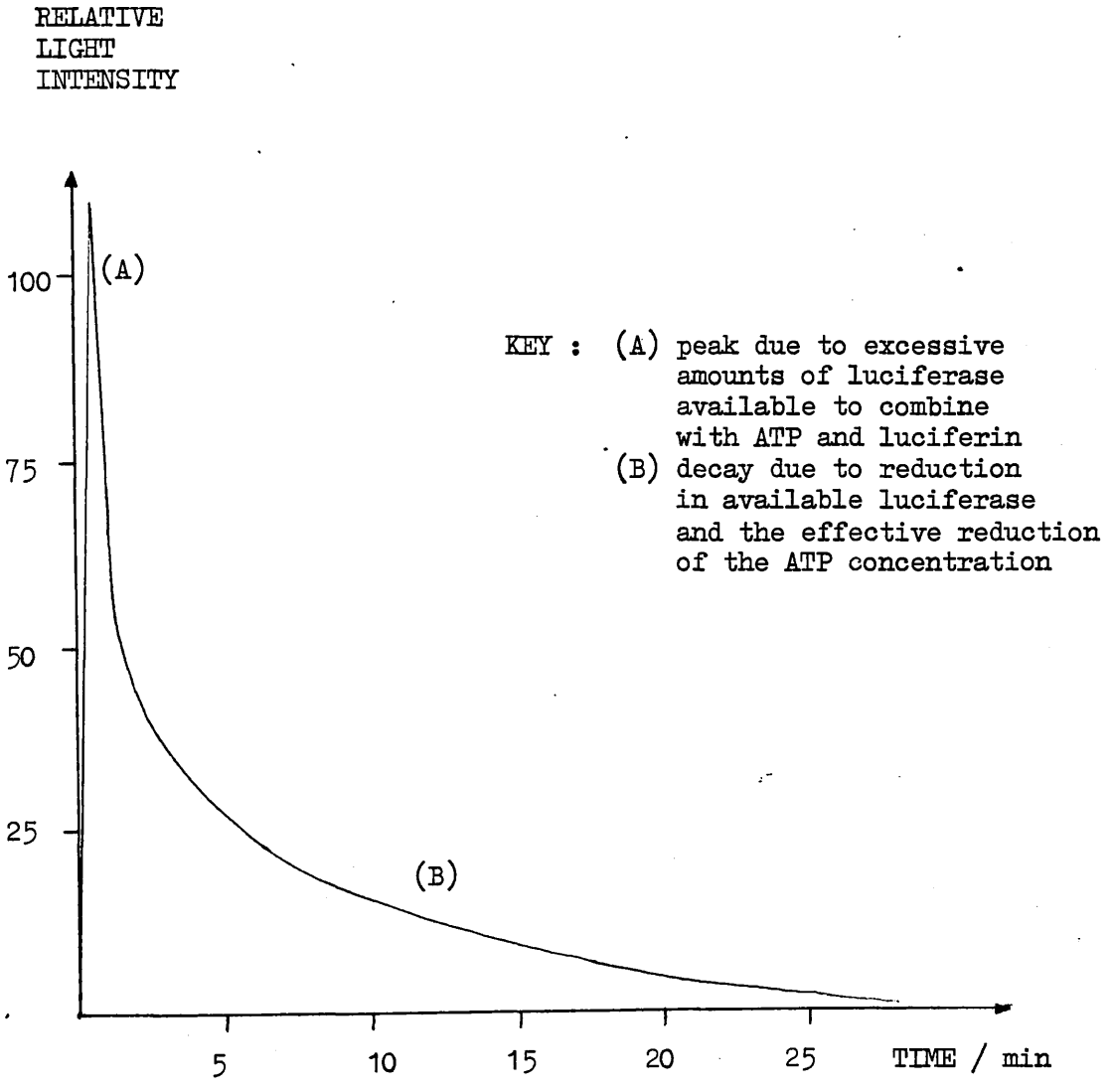
(ii) L.K.B.-Wallac Modified Luciferase

The crude extract and the flash produced meant that maximum analytical accuracy could only be obtained by controlled mixing at the measuring position, and further that only single point measurements of ATP could be obtained (Lundin, et al., 1975).

The L.K.B.-Wallac Modified Luciferase contains only a small amount of luciferase. Synthetic luciferin is added to the purified (ATP-ases removed) firefly extract, so that the concentration of luciferin is greater than that of the luciferase. The ratio of the luciferin/luciferase concentrations is such that the reaction is rate limiting with regards to the recycling of the luciferase, but that the ATP concentration, within prescribed limits, is directly proportional to the rate of the reaction and therefore to the intensity of the light emitted.

If the rate determining factor is the release of the luciferase and AMP, then at low concentrations of luciferase the concentration of ATP would not affect the reaction rate. Therefore another factor must be involved. The ATP concentration must affect the rate of removal of AMP

FIGURE 3.5 Typical Light Output when Crude Luciferin/ Luciferase Extract Is Used for The Measurement of ATP



and oxyluciferin from the luciferase. If this is the case then the light output will be proportional to the ATP concentration.

The use of the Modified Luciferase has resulted in increased specificity to ATP and time courses consistent with normal enzyme kinetics (Lundin, et al., 1976). This is substantiated by the fact that the light intensity (and therefore reaction rate) can be maintained essentially constant for a long period of time in response to a given low concentration of ATP. A prerequisite is that the rate of reaction and hence the rate of consumption of ATP during the light measurement is sufficiently low so as not to decrease the ATP concentration significantly. This is achieved by the use of controlled saturating concentrations of synthetic luciferin (Thore, 1980). A typical trace is shown in Figure 3.6 using a typical purified and luciferin saturated extract.

### 3.1.4 The L.K.B. Luminometer

In this instrument (Fig. 3.7), which has been specifically designed for bioluminescence measurements, the light emitted from the sample is reflected towards the photomultiplier tube where it is converted into a signal which is then amplified and presented as a digital display and print out. The digital display shows a new value and the printer prints out a new value every 10 seconds. The value is an integrated average value over the 10 second period.

The measuring head contains a radioactive sealed ampoule ($^{14}C$, activity 0.26 μCi) which can be used as a standard to check the electronics and stability of the instrument.

FIGURE 3.6 Typical Light Output when Purified Luciferin Saturated Extract Is Used for The Measurement of ATP

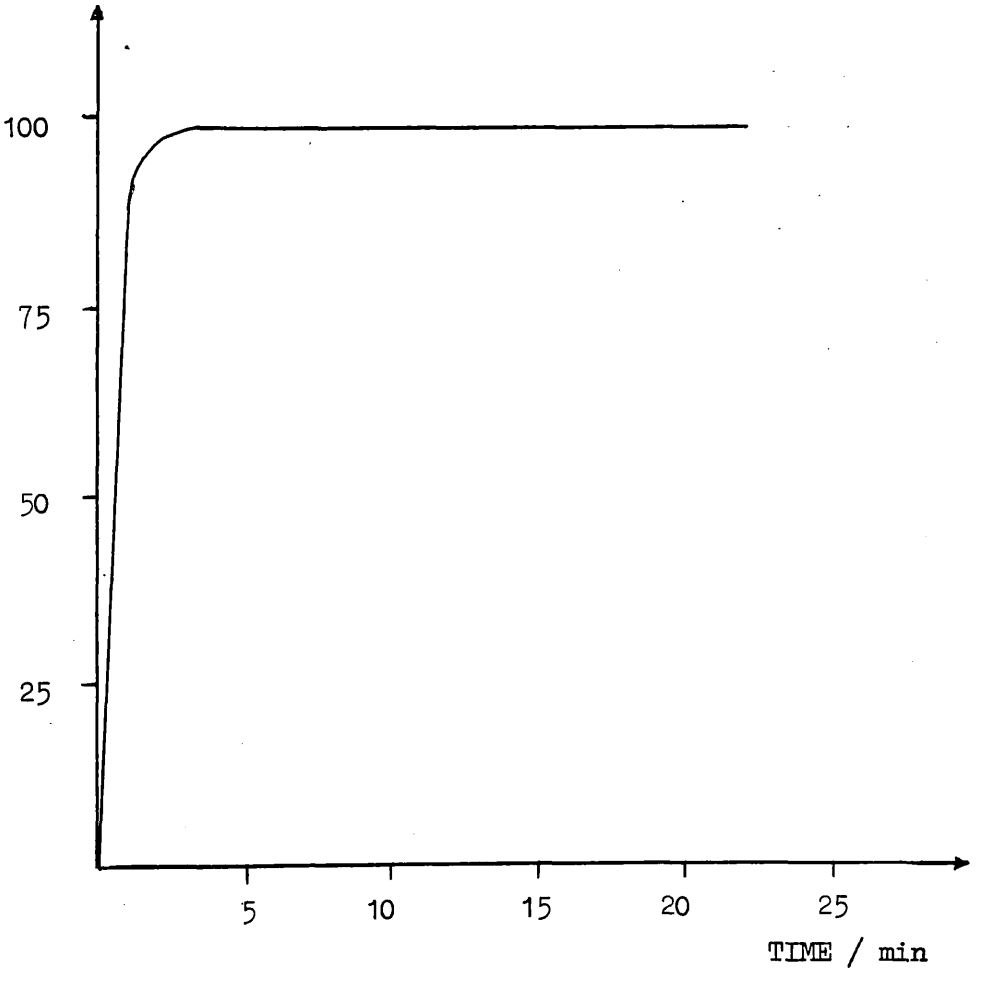
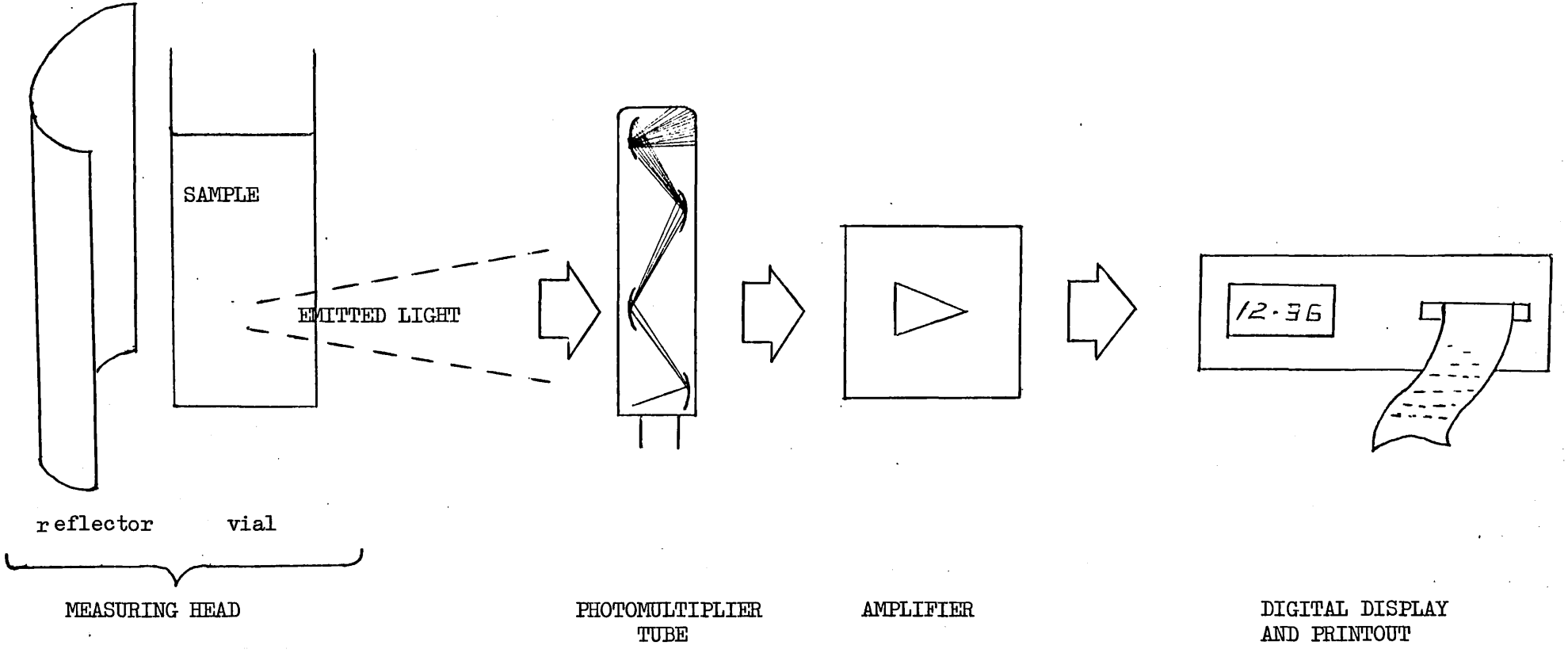


FIGURE 3.7 A Diagrammatic Representation of The L.K.B. Luminometer



## 3.2 Development of an Assay for ATP In the Bacterial Extract

### 3.2.1 Evaluation of the L.K.B. Assay

#### Introduction

The initial objective was to verify the manufacturer's claim that the change in intensity of the light emitted from an ATP sample containing monitoring reagent, (purified and luciferin enriched crude firefly extract containing magnesium and potassium salts), did not exceed 3% in 10 minutes.

The light output from the firefly bioluminescence reaction is affected by temperature (Fig. 3.8), pH (Fig. 3.9) and the inorganic and organic compounds present (Table 3.1), (Thore, et al., 1980).

The following procedure is recommended by L.K.B.

#### Reagents

(a) Reagent grade TRIS-acetic acid buffer solution (0.1 mmol $dm^{-3}$, pH 7.75) containing EDTA (2 mmol $dm^{-3}$). (TRIS is an abbreviation for Tris(hydroxymethyl)aminomethane).

(b) Stock solution of ATP ($10^{-5}$ mol $dm^{-3}$) in TRIS-acetate buffer. Serial dilutions made with TRIS-acetate buffer solution.

(c) Monitoring reagent.

#### Method

A vial containing TRIS buffer solution (0.8 $cm^3$) and an ATP solution (0.01 $cm^3$ - 0.005 $cm^3$) was placed in the measuring head of the luminometer, 0.2 $cm^3$ of monitoring reagent was added to the vial in the measuring head using a specially designed microsyringe.

FIGURE 3.8 Variation of Light Output with Temperature for The Firefly Bioluminescence Reaction

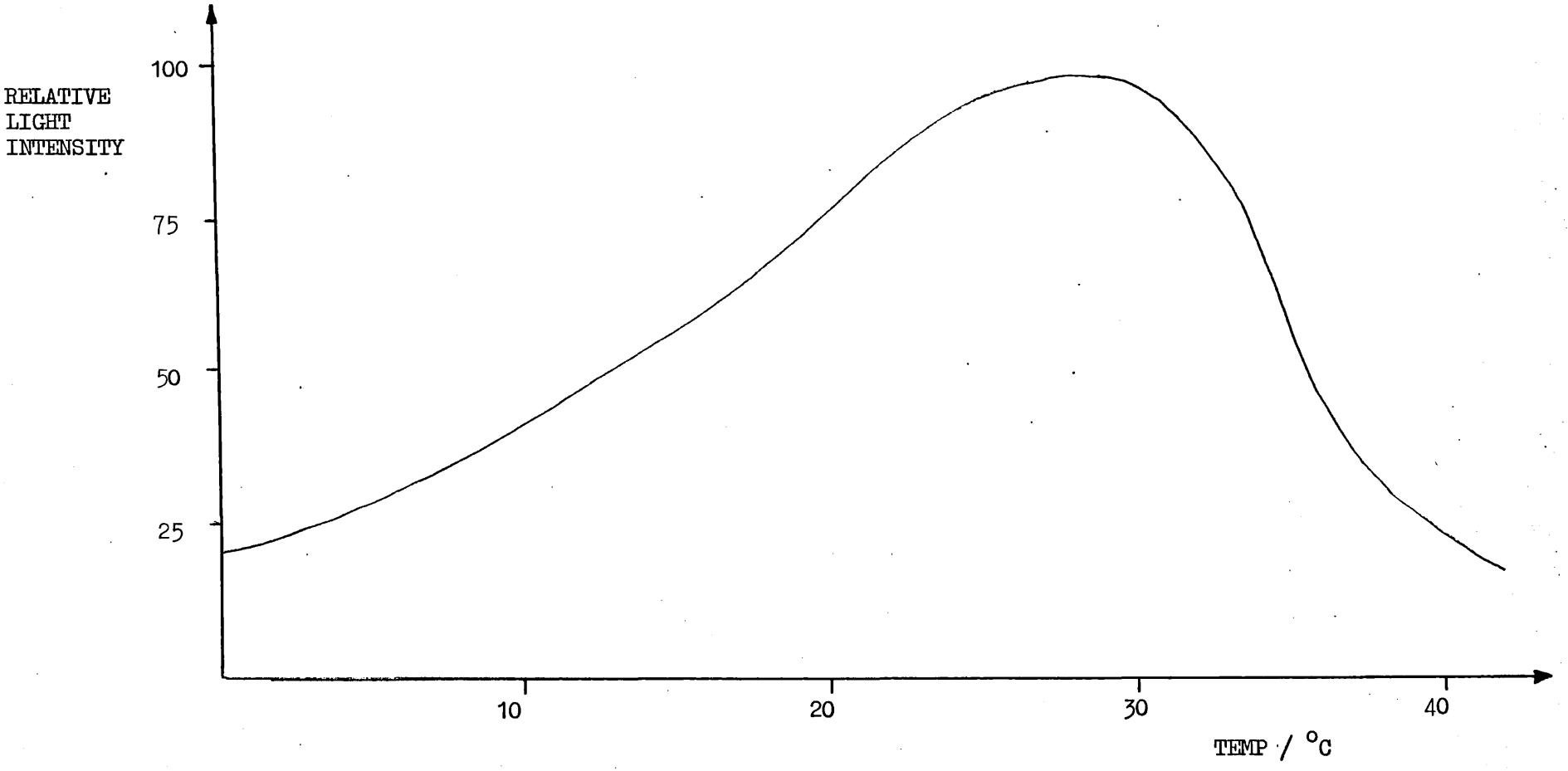


FIGURE 3.9 Variation of Light Output with pH for The Firefly Bioluminescence Reaction

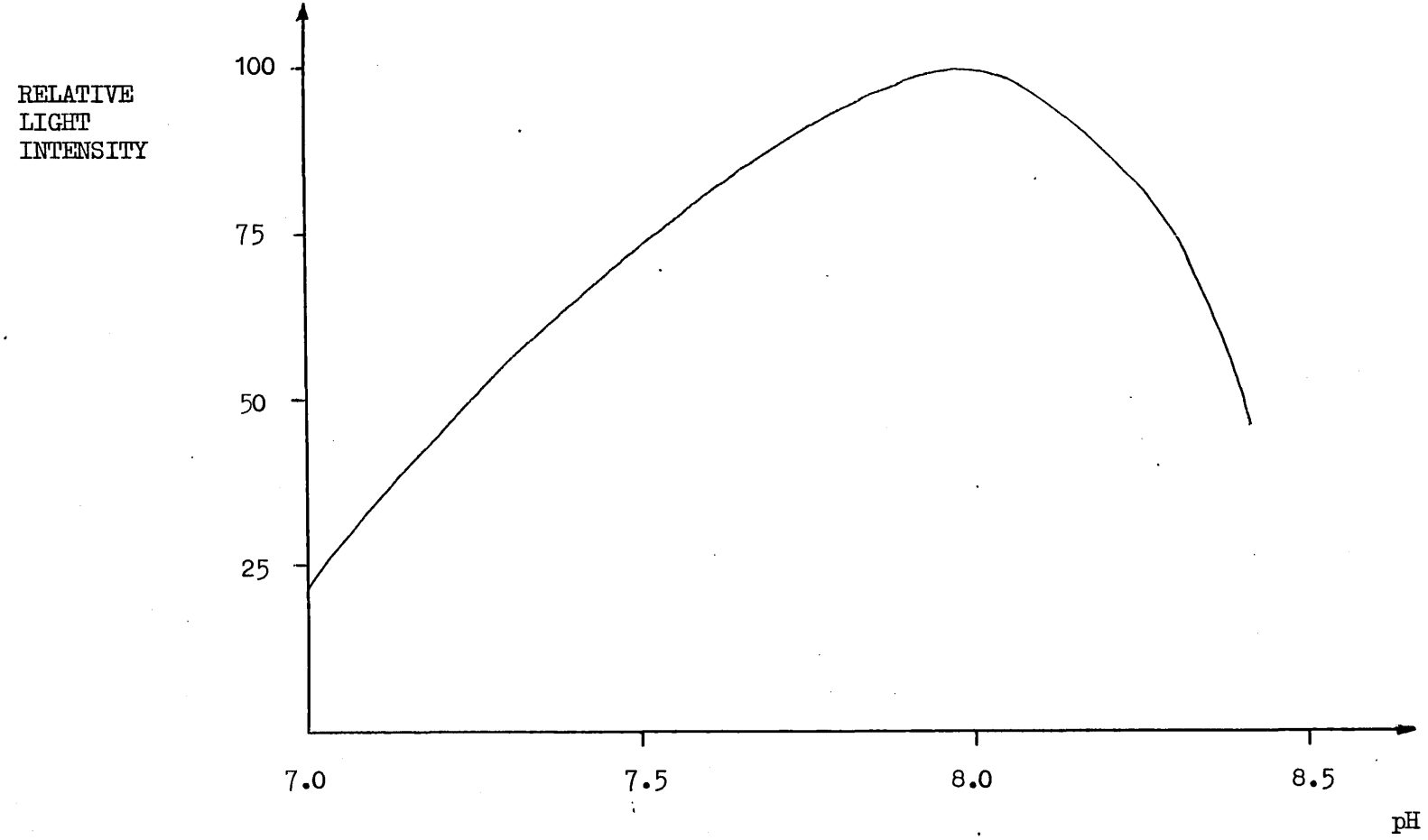


TABLE 3.1 Inhibitory Effects of Various Compounds on The Firefly Bioluminescence Reaction

| COMPOUND | APPROX. CONC. RESULTING IN A 50% REDUCTION OF THE RELATIVE LIGHT OUTPUT | |
|---|---|---|
| | / mmol $dm^{-3}$ | / vol. fraction |
| SALTS | | |
| $CaCl_2$ | 5 | |
| $Na_2SO_4$ | 11 | |
| $MgCl_2$ | 12 | |
| $NaN_3$ | 14 | |
| $CCl_3COONa$ | 15 | |
| NaCl | 20 | |
| KCl | 20 | |
| $(NH_4)_2SO_4$ | 21 | |
| BUFFERS | | |
| HEPES/Cl | 59 | |
| $CH_3COONa$ | 74 | |
| TRIS/Cl | 75 | |
| ORGANIC SOLVENTS | | |
| Diethyl Ether | 260 | 2.7 |
| Acetone | 610 | 4.5 |
| Ethanol | 1600 | 9.4 |
| Butanol | 153 | 1.4 |

Results and Discussion

When the addition of the monitoring reagent was rapid ( 0.5 sec) then the light output was a maximum 3-4 sec after mixing (Fig. 3.10). The average rate of decay of the light output (after 3 min) was 7.5% (Table 3.2).

This decay rate exceeded the specifications, i.e. 2% decay in 10 minutes. One of the reasons for using the L.K.B. monitoring reagent was that the decay rate over a 2-3 minute period was so small that it could be neglected. This would have permitted the use of a simple internal standard method. The vial containing TRIS buffer solution, ATP sample and monitoring reagent is removed from the measuring head and ATP standard (0.010 $cm^3$) added. After mixing the vial is returned to the head and the increase in light output measured.

The reproducibility of the initial peak value was unacceptable (±7%) at all ATP concentrations when rapid addition was used. In an attempt to reduce the decay rate several variations of the method were investigated. To test the theory that inorganic impurities such as $K^+$ and $Na^+$ were causing changes in the kinetics highest purity reagents were used as well as distilled-deionised water. This gave no decrease in the rate of decay. An increase in the EDTA concentration gave no reduction, but resulted in a proportional decrease in the light output (for a given ATP concentration) as the EDTA concentration increased. At a concentration of 4 mmol $dm^{-3}$ the light output was reduced to zero. This was the point at which the EDTA had chelated all the $Mg^{2+}$ (as well as $K^+$ and $Na^+$) which are required for the bioluminescence reaction. The use of TRIS-$H_2SO_4$ buffer solution instead of TRIS-acetate caused only a reduction in the overall light output due to the increased inhibitory effect of the ions (Table 3.1).

The one factor which affected the maximum value, its

FIGURE 3.10 Variation of Light Output with Time when Monitoring Reagent Is Rapidly Injected (ATP conc in vial $2 \times 10^{-7}$ mol $dm^{-3}$)

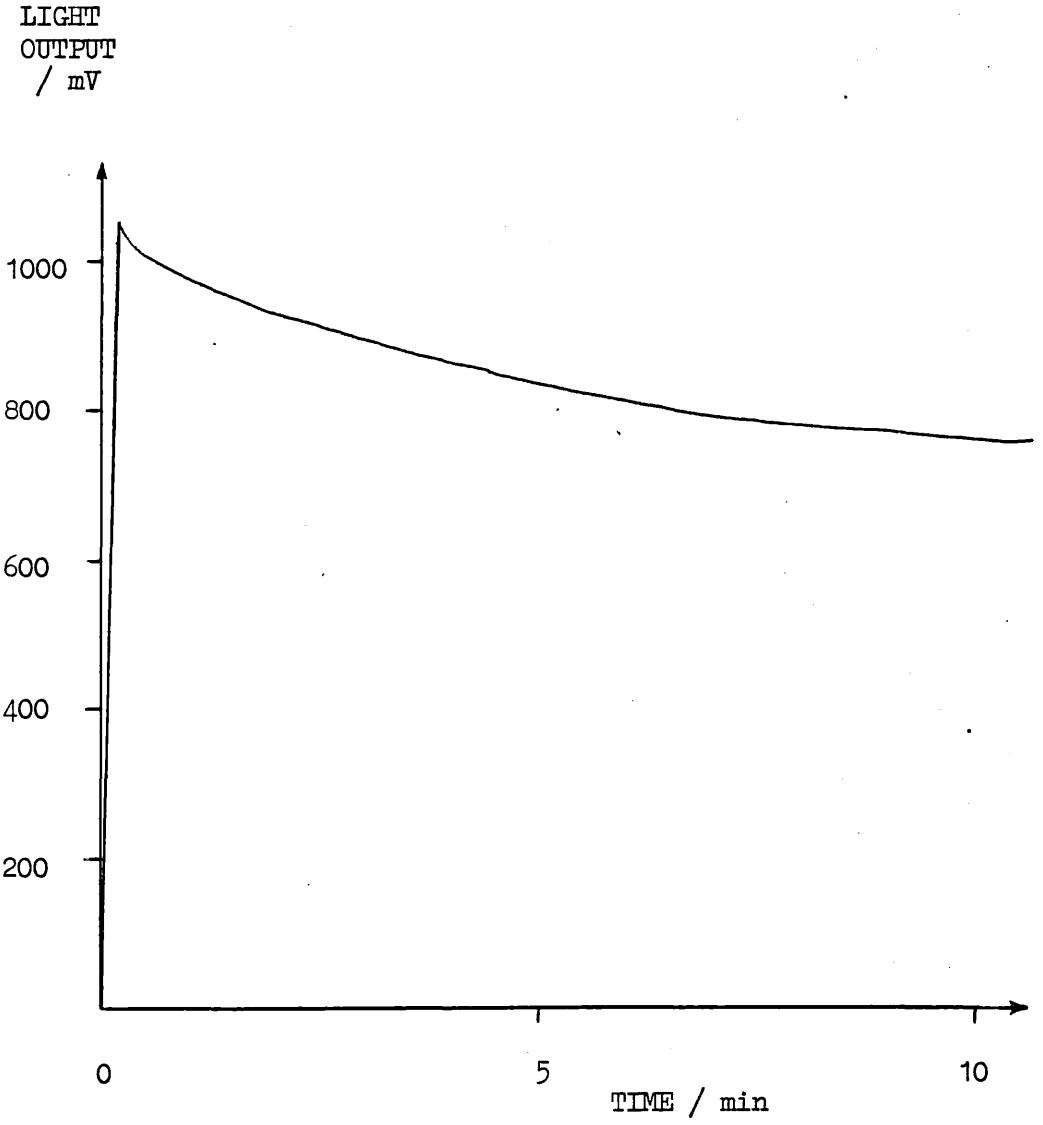



TABLE 3.2 Various Decay Rates for Different ATP Concs.

| CONC OF ATP IN VIAL / mol $dm^{-3}$ | $10^{-6}$ | $10^{-7}$ | $10^{-8}$ | $10^{-9}$ |
|---|---|---|---|---|
| AV. DECAY RATE / % reduction of peak value in 3 min | 7.7 | 7.6 | 7.9 | 8.0 |

reproducibility and the rate of decay was the rate of addition of the monitoring reagent, i.e. the effectiveness of mixing. As the mixing time increases (0.5-3 sec) the maximum light output decreases and the rate of decay decreases (Fig. 3.11). The reproducibility of the constant or plateau light output value for any mixing speed was unacceptable.

The optimum dispensing and mixing procedure was established to give the maximum reproducibility, this is described below.

### 3.2.2 The Assay Techniques for ATP in the Bacterial Extract

#### Method

An aliquot (0.8 $cm^3$) of the solution of ATP in TRIS-acetate buffer solution (pH 7.75) was pipetted into the vial. To this was added monitoring reagent (0.2 $cm^3$). The vial was placed in the measuring head of the luminometer. Exactly 10 seconds from the time of addition of the monitoring reagent the total light output over the next 10 seconds was integrated by the luminometer. This value was taken as the light output value for the given ATP concentration.

#### Results and Discussion

The integral of the light output over the second 10 second period represented the maximum light output, after which there was only a 1.5% decay over the next 10 minutes. This is better than the manufacturer's specifications. The type of mixing used gave a reproducible maximum value (±1%, p = 0.95). At low ATP concentrations ( $3 \times 10^{-9}$ mol $dm^{-3}$) the reproducibility was ±2% (p = 0.95). The maximum light output as determined by the above technique is a linear function of the ATP concentration (Fig. 3.12).

A background ATP level was established by determining the light

FIGURE 3.11 Variation of Light Output with Time for Different Mixing Speeds

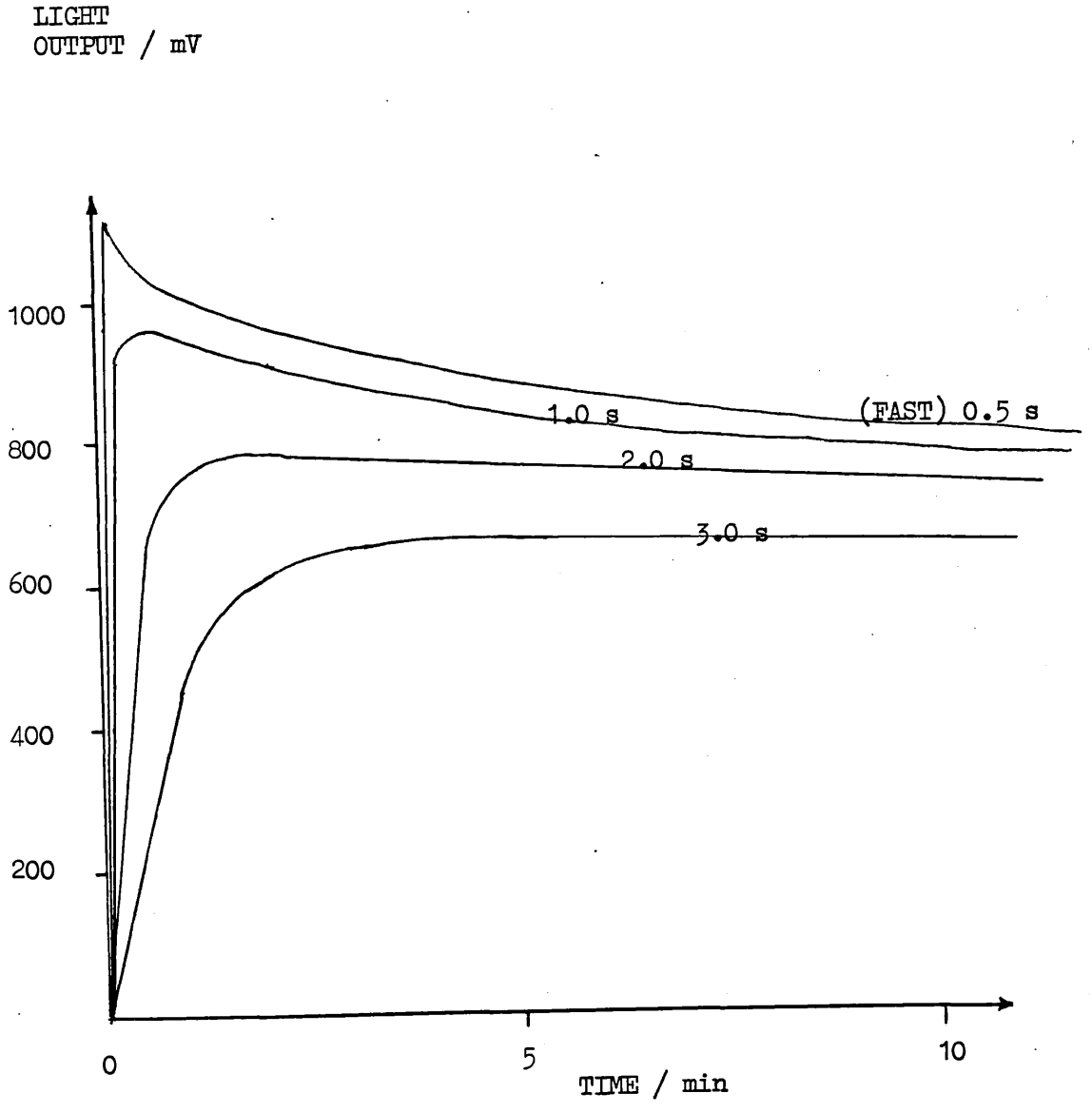
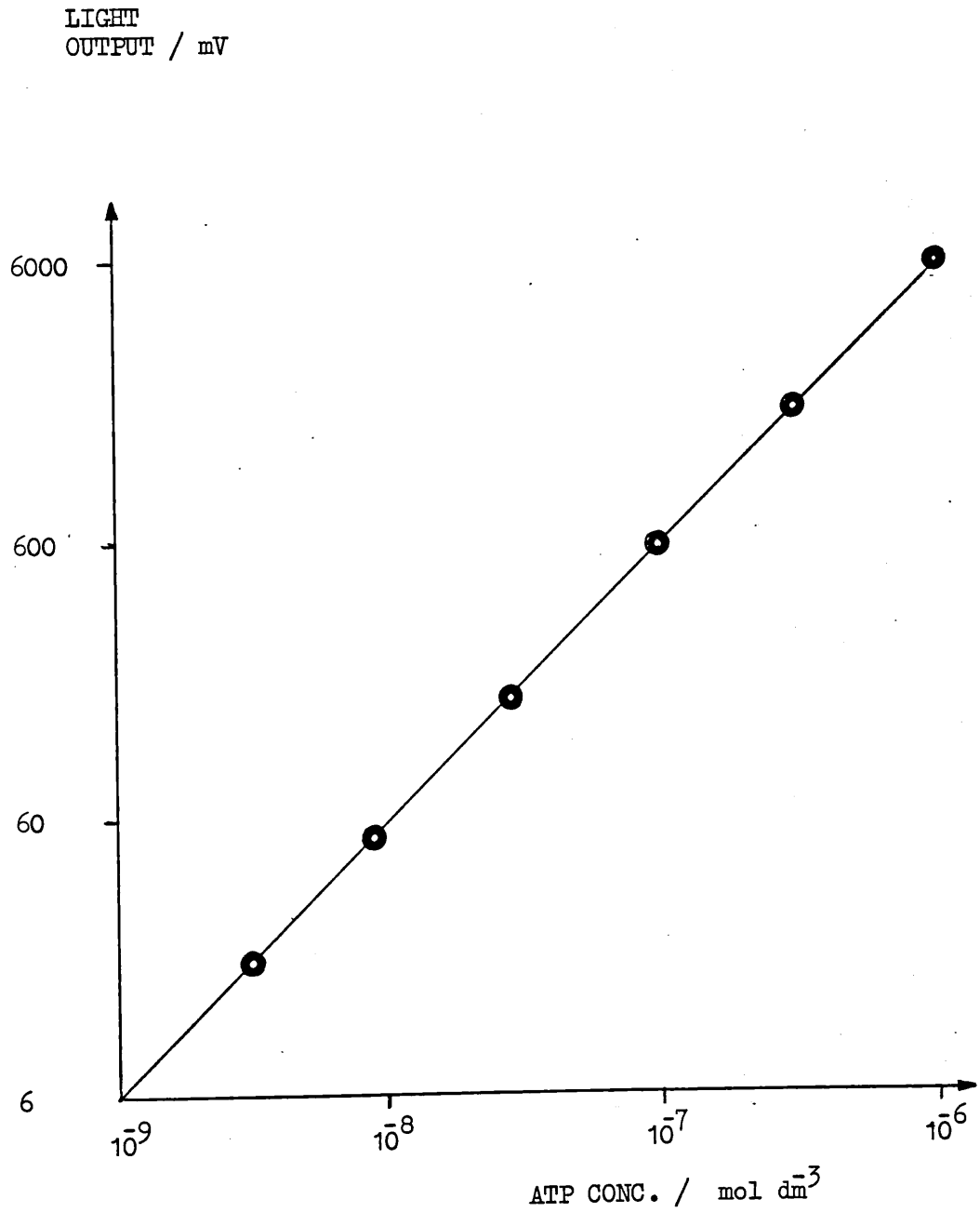


FIGURE 3.12 Variation of Light Output with ATP Concentration



output from TRIS-acetate buffer solution.

Although problems associated with the decay and reproducibility had been solved the use of a simple internal standard method was still not possible. It was found that once the vial had been removed from the measuring head (the requirement for the addition of the ATP standard) the light output was 20% lower when it was replaced, and further there was an increased decay.

To summarise the internal standard technique could not be used as the decay rate was too rapid or mixing after the addition of the internal standard caused erroneous light output readings. This behaviour may be due to changes in the oxygen available to the bioluminescence reaction caused by mixing. The more violent the mixing the higher the oxygen concentration the higher the maximum value and the greater the decay due to the increased rate of reaction. This assumes the reaction is occurring relatively rapidly so that the oxygen concentration is partially rate controlling.

Other research workers currently using the L.K.B.-Wallac Monitoring Reagent system (Wood, et al., 1981) have experienced high decay rates (6% in 3 minutes).

### 3.2.3 Development of an Internal Standard Assay Technique for ATP in the Bacterial Extract

#### Introduction

The factors which affect the light output for a given ATP concentration include

(i) temperature (Fig. 3.8);

(ii) pH (Fig. 3.9). TRIS-acetate buffer solution has a significant temperature coefficient of 0.5 pH units per 5°C;

(iii) monitoring reagent. The reconstituted freeze dried reagent deteriorates on storage and loses its light producing capacity for a fixed concentration of ATP (Fig. 3.13);

(iv) inhibitory effect of various compounds (Table 3.1).

It is possible that for a given bacterial extract all or some of these factors will be different, and the resultant deviation from the true light output will be significant. The deviation is accounted for by the use of some form of internal standard. In the technique which has been developed the sample is analysed in two parts. One part is analysed for ATP without pretreatment. To the other part is added a known amount of ATP before analysis. Both parts are analysed using the assay technique (3.2.2). The method is arranged such that the ATP in the sample is under exactly the same conditions as in the sample + standard. Measurement of the light output from both (and a blank) permits the amount of ATP in the sample to be calculated.

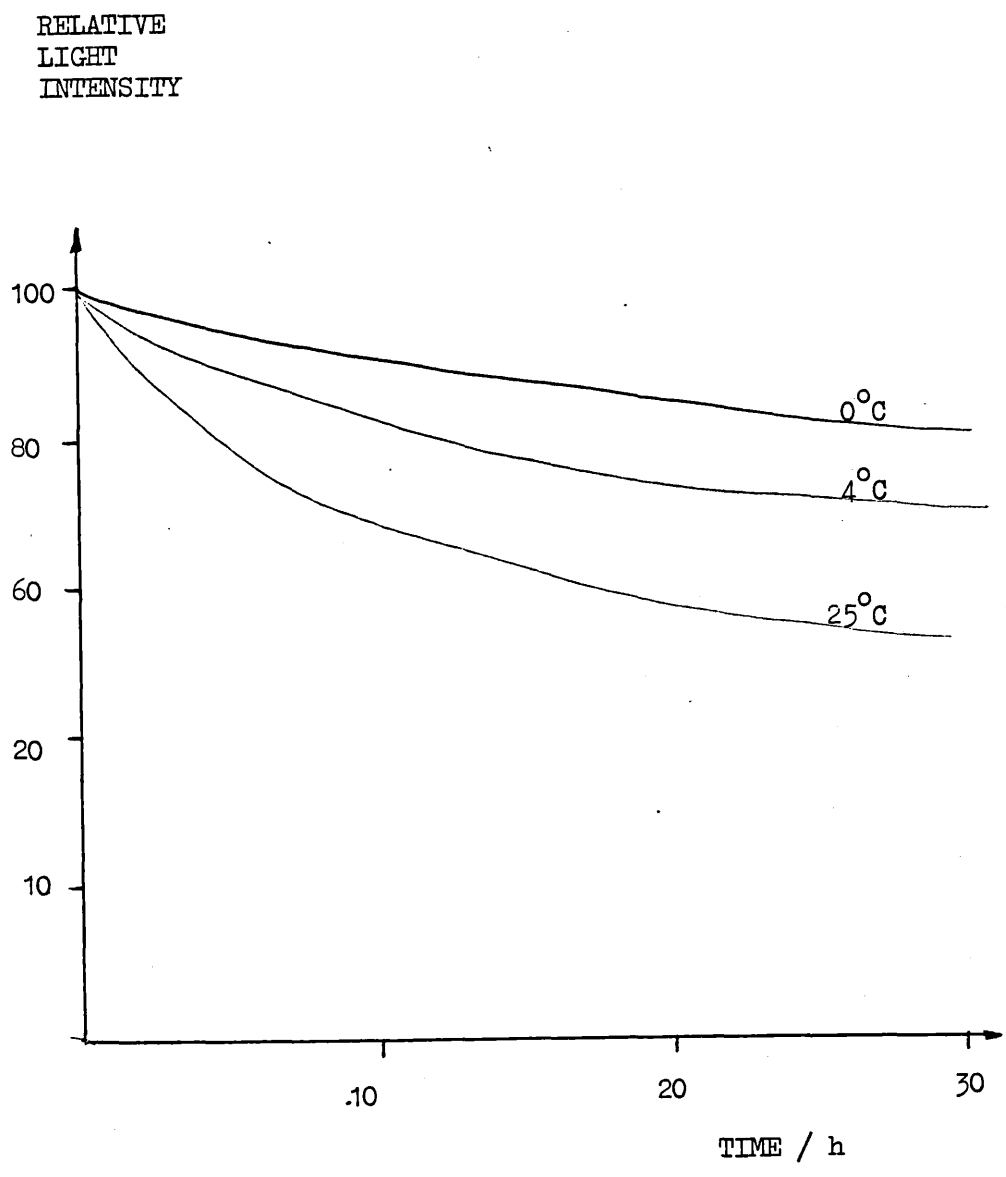
Method

To 10 $cm^3$ of ATP sample in TRIS-acetate buffer solution was added a known volume (0.5-1.0 $cm^3$) of ATP in TRIS-acetate buffer solution of known concentration; the STANDARD. To another aliquot (10 $cm^3$) of the ATP sample was added the same volume (0.5-1.0 $cm^3$) of TRIS-acetate buffer solution; the SAMPLE. The "BLANK" was TRIS-acetate buffer solution. The BLANK, SAMPLE and STANDARD were all analysed, in that sequence, using the assay technique (3.2.2). This sequence was repeated 5 times to calculate an averaged ATP concentration for the sample.

Results and Discussion

In this method the sample is ATP dissolved in TRIS-acetate buffer solution. In a real situation the sample is a bacterial extract

FIGURE 3.13 Stability of Reconstituted ATP Monitoring Reagent at Various Temperatures



containing various inhibiting compounds. In this case the blank is pure growth medium treated the same way as the bacterial suspension.

Contamination of glassware was a major problem, especially at low concentrations when contaminant ATP on glassware introduced considerable errors. All glassware was silated by rinsing with a 2% solution of dimethyldichlorosilane in carbon tetrachloride and then drying at room temperature. Prior to use all glassware was soaked in a 1% Decon (phosphate-free detergent) solution for 2 hours, rinsed with water, soaked in dilute HCl for 1 hour, washed with tap water and finally rinsed with distilled water. The use of plastic tops (polyethylene) was avoided as older oxidised tops and stoppers were found to adsorb bacterial ATP extracts, which even the most rigorous washing procedure could not remove. "Cling film" was therefore used for sealing the vessels and subsequent mixing.

The volume of ATP standard and its concentration was chosen so that the light output from the STANDARD was approximately twice that of the SAMPLE. A typical set of results and subsequent calculation is shown

| | BLANK | SAMPLE | STANDARD |
|---|---|---|---|
| Peak Value/mV | 1.20 | 82.35 | 169.8 |

Volume of TRIS-acetate buffer solution added to SAMPLE = 1.0 $cm^3$

Volume of ATP/TRIS-acetate buffer solution added to STANDARD = 1.0 $cm^3$

Conc. of ATP in TRIS-acetate buffer solution added to STANDARD = $1 \times 10^{-7}$ mol $dm^{-3}$

$\therefore$ Light output from SAMPLE (10 $cm^3$ of sample + 1.0 $cm^3$ of TRIS buffer solution)

$$= 82.35 - 1.20$$

$$= 81.15 \text{ mV}$$

$\therefore$ Light output from STANDARD (10 $cm^3$ of sample + 1.0 $cm^3$ of $1 \times 10^{-7}$ mol $dm^{-3}$ ATP in TRIS-acetate buffer solution)

$$= 169.8 - 1.20$$

$$= 168.6 \text{ mV}$$

$\therefore$ Light output from $\frac{1 \times 10^{-7}}{1000}$ mole of ATP in 11 $cm^3$

$$= 168.6 - 81.15$$

$$= 87.45 \text{ mV}$$

$\therefore$ No. of moles of ATP in SAMPLE

$$= \frac{81.15}{87.45} \times 1 \times 10^{-10}$$

$$= 9.279 \times 10^{-11}$$

The original 1 $cm^3$ sample of bacterial suspension is diluted by a factor of 6 during the extraction procedure.

$\therefore$ Total no. of moles of ATP in 1 $cm^3$ of the original bacterial suspension

$$= 9.279 \times 10^{11} \times 6$$

$$= 5.568 \times 10^{-10}$$

From the 5 calculated results an average was taken. The reproducibility varied with the concentration of the ATP in the sample;

| Conc. of ATP in Sample/mol $dm^{-3}$ | Reproducibility p = 0.95 |
|---|---|
| $1 \times 10^{-9}$ | ±4% |
| $3 \times 10^{-9}$ | ±2% |
| $5 \times 10^{-9}$ | ±2% |
| $1 \times 10^{-8}$ | ±2% |
| $1 \times 10^{-7}$ | ±2% |

When the SAMPLE consisted of known concentrations of ATP the experimentally measured value was within 2% of the expected, (at conc. 3 x $10^{-9}$ mol $dm^{-3}$).

For the determination of bacterial ATP the ATP-solution used in the above method is replaced by extract diluted with TRIS-acetate buffer solution. The BLANK is an extract of pure growth medium.

## 3.3 Method for Removal of a Sample of Growing Bacterial Culture from the Culture Vessel

### 3.3.1 Introduction

One of the most important criteria to be fulfilled was that the sample of bacterial suspension was representative of the growing culture in the culture vessel. The basic areas of consideration were as follows:-

(a) The correct sampling procedure governs the validity of the ATP value obtained following extraction and analysis, assuming that the extraction and analysis is correct.

(b) The time taken to remove the sample from the culture vessel and inject into the extractant medium may be crucial. The two factors which could change during this period to give an erroneous result are the temperature and oxygen content of the growth medium.

(c) The volume of sample must be reproducible and accurate.

### 3.3.2 The Sampling Method

The following method was used to remove a sample from the culture vessel during batch growth.

#### Method

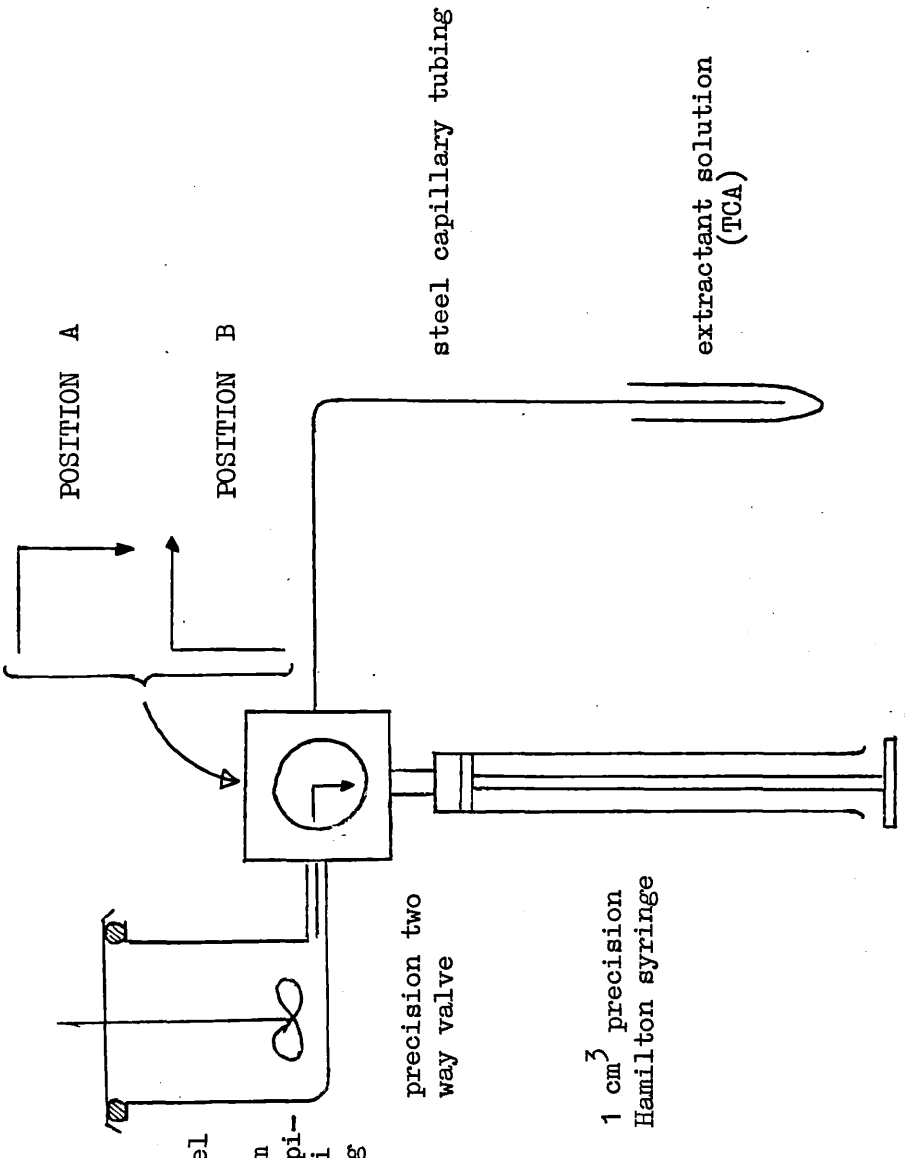
A 2 cm length of silicone rubber capillary tubing was sealed into the side of the culture vessel near the base. This was connected to a two way valve (Fig. 3.14). One outlet of this was connected to a precision Hamilton syringe and the other to a sample tube.

The valve was turned to position A and the plunger withdrawn to the 1 $cm^3$ stop mark. The sample was left in the syringe for 30 seconds to bring the temperature of the syringe barrel and tubing to that of the sample. The valve was turned to position B and the sample expelled from the syringe barrel through the steel capillary tubing. This sample was discarded. Four more samples were taken (without the 30 sec waiting period) in rapid succession. The first three of these were discarded and only the fourth sample injected into the extractant solution.

#### Results and Discussion

The reproducibility of sampling and delivering 1 $cm^3$ of bacterial suspension under the conditions of growth, established by weighing the 1 $cm^3$ aliquots delivered, was $\pm 0.1\%$ ($p = 0.95$).

FIGURE 3.14 A Schematic Representation of The Bacterial Suspension Sampling Apparatus



The time taken for the removal of a sample and its delivery into the extractant solution was 2-3 seconds. It has been suggested by Harrison and Maitra (1969) that the turnover of ATP in the bacterial cell is so rapid that if the time taken to transfer the sample to the extractant solution exceeds 1 second, then the ATP pool will change before it is extracted. The effect on the ATP content of a growing exponential bacterial culture caused by altering the sampling time was determined as follows.

Under the experimental conditions of growth the syringe barrel was filled with sample (1 $cm^3$) as described earlier, but only 0.5 $cm^3$ of this was injected into the extractant solution (for details of this see 3.4). The other 0.5 $cm^3$ was allowed to rest in the syringe for up to 10 seconds before this was injected into a further aliquot of extractant solution. There was no significant difference in the ATP pool value obtained from the 2 samples. The oxygen tension in the culture vessel during a batch growth never fell below 80% saturation. If aeration of the growing culture in the culture vessel was stopped the oxygen tension fell to 60% saturation in a period of 1 minute, even for the densest of cultures encountered. Work done initially by Pirt (1969) and subsequently verified by other workers using different techniques such as microcalorimetry (Nichols, 1981) has shown that the respiration rate (Kemper, 1979) and other metabolic processes are unaffected by oxygen tensions above 30% saturation. Below 30% saturation anaerobic growth processes may play a significant role in metabolism. It can then be concluded that with a sample time of 2-3 seconds any decrease in oxygen saturation as a factor altering the ATP pool may be ignored. The rapid successive sampling technique also ensured that ATP changes due to temperature changes did not occur.

## 3.4 Development of the ATP Extraction from Bacterial Cells

### 3.4.1 Introduction

The aims of an extraction technique which must be fulfilled are:-

(a) high degree of reproducibility;

(b) accurate detection of low levels of ATP (the lowest level encountered is 1 x $10^{-12}$ moles of ATP in the vial);

(c) accurate evaluation of errors;

(d) accurate evaluation of losses of ATP due to the technique, i.e. percentage recovery;

(e) the ATP value must be a true representation of the ATP in the bacterial sample;

To achieve these the following problems must be overcome:-

(a) the removal during the extraction procedure of luciferase inhibitors;

(b) the standardisation of the technique;

(c) the destruction of bacterial ATP-ases during extraction.

The most difficult problem was the destruction of the ATP-ases which cause changes in the concentration of the extracted bacterial ATP.

### 3.4.2 The Bacterial ATP Extraction Technique

The following reagents and techniques were used extensively to extract ATP from growing or stationary cultures of bacteria.

#### Reagents

(a) trichloroacetic acid (TCA) in distilled water (342 g $dm^{-3}$);

(b) EDTA (35 g $dm^{-3}$) and 18-Crown-6-ether (20 g $dm^{-3}$) in distilled water;

(c) ether (water saturated);

(d) TRIS-acetate buffer solution (0.1 mol $dm^{-3}$, pH 7.75) containing EDTA (2 mmol $dm^{-3}$).

Method

The bacterial sample was injected into the extractant solution (0.7 $cm^3$ of TCA solution and 0.7 $cm^3$ of EDTA/crown ether solution) contained in a 10 $cm^3$ centrifuge tube kept on ice. The mixed sample and extractant was maintained at 4°C for 12 minutes. The sample was centrifuged at 6°C for 5 minutes at 2050 g, and 2 $cm^3$ of the supernatant pipetted into a 10 $cm^3$ volumetric flask, taking care not to disturb the sedimented bacterial cell debris.

The extract was washed with ice-cold ether (3 x 5 $cm^3$). After each washing the ether was removed with a pasteur pipette so not to remove any of the lower extract layer. After the final washing the residual ether was removed by evacuating the flask (c. 2 mm Hg) for 5 minutes. The TRIS-acetate buffer solution (c. 5 $cm^3$) was then added and the flask immersed in an acetone/$CO_2$ mulch to quick freeze the contents. The extract could be stored at -20°C for up to 5 days prior to analysis (3.2.3).

Before analysis the extract was thawed and quantitatively transferred to a 50 $cm^3$ volumetric flask and the volume made up to the mark with TRIS-acetate buffer solution.

The blank was prepared by extracting 1 $cm^3$ of the uninoculated growth medium, and analysing the subsequent extract obtained.

Results

Five analyses were performed on each extract (sample); a typical set of results for one sample, showing the reproducibility of the measurement are

| | Light Output/mV | | | | |
|---|---|---|---|---|---|
| BLANK | 0.52 | 0.51 | 0.53 | 0.49 | 0.50 |
| SAMPLE | 82.54 | 82.01 | 81.99 | 82.36 | 82.41 |
| STANDARD | 165.2 | 165.3 | 165.0 | 164.8 | 165.8 |

Other data logged for this sample were

(a) volume of original sample taken (1 $cm^3$);

(b) volume of ATP of known concentration added to standard;

(c) absorbance of the original 1 $cm^3$ of bacterial suspension for biomass determination.

Utilising the above data 5 values of the ATP content can be calculated and hence an average value of the ATP content of the bacteria in the original 1 $cm^3$ sample can be obtained (units:mole of ATP per g dry wt of bacteria).

To determine the amount of ATP lost during the extraction, the culture vessel was filled with an ATP solution of known concentration. The solution was maintained under the conditions of growth and samples removed, extracted and analysed as described. Table 3.3 shows the percentage recovery and reproducibility of the ATP extraction technique for various concentrations of ATP in the culture vessel. Although the ATP extraction technique is a complex procedure the losses of ATP are very small (c. 1.4%) and the reproducibility is good (±4.5 - ±3.0%). The percentage loss is assumed to be the same for a bacterial suspension. This loss was corrected for every sample; the correction factor is dependent upon the concentration of ATP determined in the extract.

To test the linearity between bacterial mass (absorbance) and the ATP detected the following experiment was undertaken. The bacterial mass

TABLE 3.3 The Reproducibility and Percentage Recovery of The ATP Sampling,Extraction and Analysis Procedure for ATP Solutions

| CONC. OF ATP IN THE CULTURE VESSEL / mol $dm^{-3}$ | APPROX mV READING OBTAINED FROM LUMINOMETER | | OVERALL AV. % RECOVERY OF ATP | 95% CONFIDENCE LIMITS (t-test) |
|---|---|---|---|---|
| | SAMPLE | STANDARD | | |
| $5 \times 10^{-8}$ | 6 | 12 | 98.7 | ±4.5 |
| $1 \times 10^{-7}$ | 12 | 25 | 98.6 | ±4.0 |
| $2 \times 10^{-7}$ | 25 | 50 | 98.7 | ±3.0 |
| $5 \times 10^{-7}$ | 60 | 120 | 98.6 | ±3.0 |
| $1 \times 10^{-6}$ | 120 | 250 | 98.6 | ±3.0 |

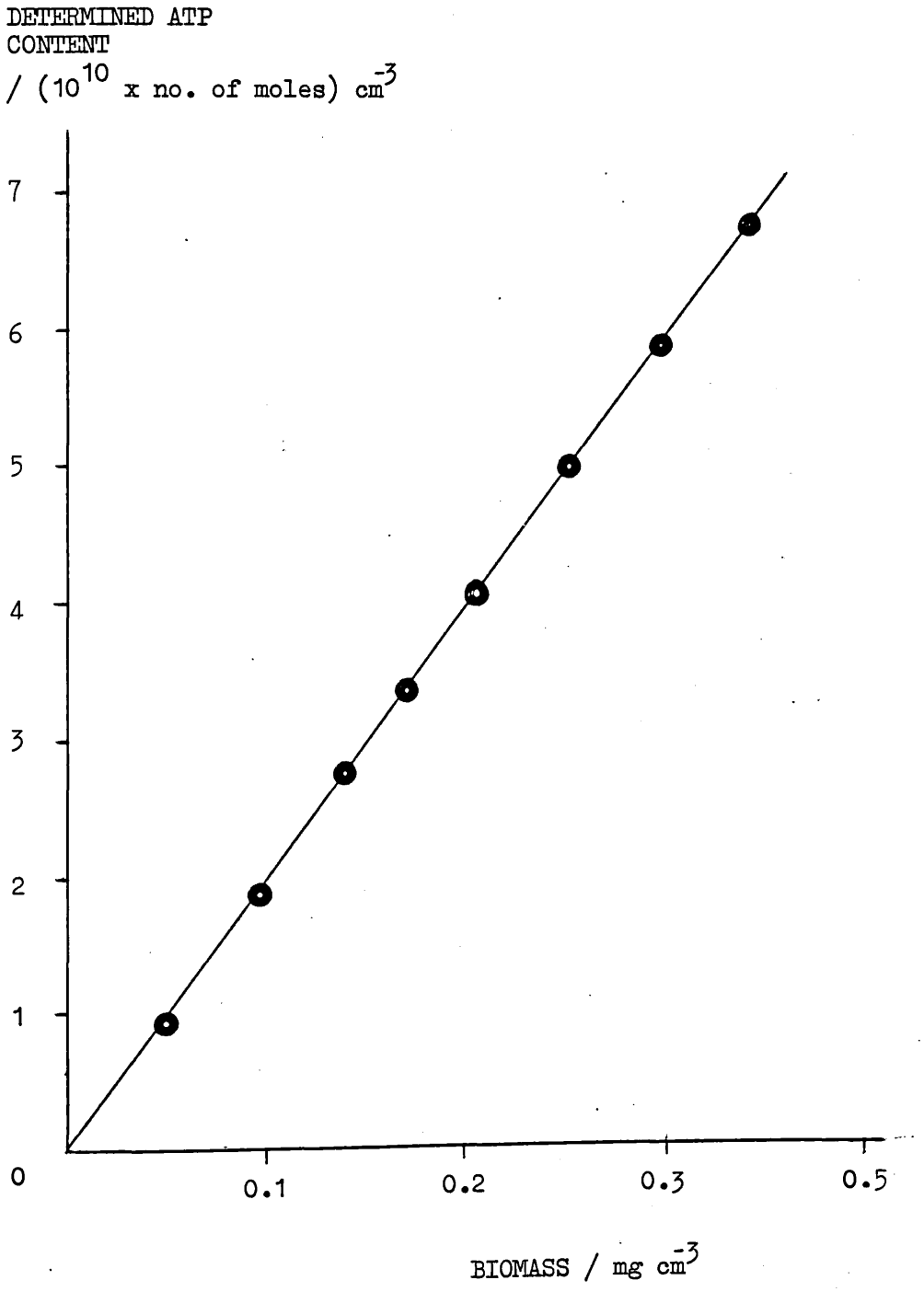
of a newly grown exponential culture was obtained by centrifuging and this was washed and resuspended in approximately the same volume of fresh salts medium (lacking carbon source). This was maintained under the conditions of growth (i.e. pH, aeration, temperature etc) in the culture vessel and 1 $cm^3$ samples removed, extracted and analysed as described. Samples were removed from bacterial suspensions of various concentrations for analysis. The linearity of the relationship between bacterial mass and ATP determined (Fig. 3.15) was good ($r = 0.998$). The reproducibility of the ATP already determined was similar to that shown in Table 3.3. The reproducibility is partly dependent on the bacterial mass, i.e. ATP concentration in the extract.

Discussion

When the cells were brought into contact with the extractant the cell walls were ruptured and precipitated and the cell contents (including ATP) passed into solution. The time of the extraction was established by extracting 1 $cm^3$ samples of a stationary bacterial suspension for various times. The ATP value reached a plateau value after 10 minutes and decreased only when the extraction time was increased to 25 minutes. The time of this established procedure is 17 minutes. If the temperature of the extracting mixture was allowed to exceed 7°C the rate of loss of ATP was greatly increased (there was only a 90% recovery at 10°C). This time period ensured 98.6% extraction of the ATP and optimum denaturing of the enzymes present especially ATP-ases.

During the extraction procedure, owing to the acidic pH, enzyme activity is very low. The pH of the ether washed extract is 4, i.e. still acidic enough to seriously inhibit enzyme activity. Enzyme activity is most apparent once the extract has been thawed and made up to

FIGURE 3.15 Linearity of The Determined ATP Content of Stationary Bacterial Suspensions of Varying Biomass



50 $cm^3$ with TRIS-acetate buffer solution as the pH is now 7.75. Enzyme activity is easily detected as consecutive analyses of the extract show a trend, i.e. decrease or increase. Enzymic interference became very noticeable when lower concentrations of TCA in the extractant were used. Exclusion of EDTA or 18-crown-6 or a failure to effectively centrifuge out cell debris resulted in unacceptably high enzyme activity, i.e. 30% drop in the determined ATP value when the diluted extract was left at room temperature for 10 minutes.

The diluted extract (50 $cm^3$) was so enzymically stable that if incubated at 37°C for 1 h there was no significant change in the ATP content. This is evidence that there is no enzymic interference in the extract which is vitally important.

The sampling time was 0.5 s and the use of the fine capillary tubing ensured immediate and effective mixing of the sample with the extractant.

The loss of ATP (1.4%) during extraction can be due to enzyme activity or chemical degradation. Nucleotide converting enzymes require $Mg^{2+}$ and $K^+$ co-factors. The presence of EDTA and 18-crown-6 are responsible in part for denaturing the enzymes by destabilising their tertiary structure. EDTA has been widely used for ATP extractions from plants (Guinn and Eiendenbook, 1972), blood platelets (Wiener, 1974) and bacteria (Chapelle, 1968; Klofat, 1969; Benny, 1976). The introduction of a crown ether in the extractant is a novel development. The use of alkali extractants such as KOH was avoided because this leads to increased ATP levels due to the degradation of $NAD^+$ (Kaplan, et al., 1951).

One of the main criteria for a successful extraction is a high yield of ATP which is accomplished by using TCA. At present most workers believe that TCA gives the most physiologically relevant result when

extracting from bacteria (Lundin and Thore, 1975). TCA has been used as the standard extraction by which all other extractions are compared. Other extraction methods such as TRIS-EDTA, KOH, ethanol, butanol, chloroform, $H_2SO_4$ and HCOOH were fully evaluated prior to the selection of TCA. The only drawback with the TCA method is the relatively long and tedious procedure. Recent work has also shown that 10% w/v TCA is the only extractant that precipitates 99-100% of bacterial protein (Dean, 1982).

## 3.5 Summary

1. The L.K.B.-Wallac modified luciferase is a vast improvement on the crude luciferase but its performance is not of a high enough standard to permit the use of a 'true' internal standard technique.

2. The use of initial maximum light output rather than plateau values resulted in a more reproducible assay.

3. The procedure gives a reproducible and physiologically representative ATP value. The problems of sampling time, temperature control and mixing with the extractant have been solved.

4. The use of TCA as the extractant gave the most physiologically representative ATP value. The introduction of 18-crown-6 resulted in an extremely stable extract.

5. A linear relationship between the total ATP recovered and bacterial mass has been established.

6. The minimum detection limit of ATP was $5 \times 10^{-11}$ mole of ATP in the vial, the reproducibility ±4.5% ($p = 0.95$). At higher ATP concentrations the reproducibility was ±3% ($p = 0.95$).

# CHAPTER FOUR

# EXPERIMENTAL RESULTS

## ATP LEVELS IN KLEBSIELLA AEROGENES GROWING IN GLUCOSE-LIMITED MEDIA

## 4.1 ATP Levels in Cells During the Exponential Phase of Growth in Glucose-Limited Medium

The bacteria were grown in 600 $cm^3$ of glucose-limited (3.3 mmol $dm^{-3}$) media under the standard conditions of growth, (37°C, aeration etc). A cryogenically stored bacterial suspension (2.1.3) was used to inoculate the media. Samples of the exponentially growing culture were removed, extracted and analysed to determine the ATP content, (Chapter 3). The samples were removed at 30 minute intervals. Twenty repeat experiments were conducted. Typical ATP levels (ATP profiles) during growth are shown in Figures 4.1, 4.2, 4.3 and 4.4.

Following inoculation there was an immediate and rapid increase in the bacterial ATP to a mean level of $7.8 \times 10^{-6}$ mole per g dry wt. During the large part of exponential growth there were oscillations ($\pm 0.75 \times 10^{-6}$ mole per g dry wt) about this constant mean level. Cessation of growth (glucose concentration zero) was accompanied by a rapid decrease of the ATP level to a value similar to that on inoculation ($2.5 \times 10^{-6}$ mole per g dry wt).

## 4.2 Comparison of the TCA Extraction Method with other Extraction Techniques

### 4.2.1 Low Temperature Extraction

In view of the results obtained which conflicted with earlier reports that the bacterial ATP level was constant during exponential growth, a low temperature extraction method was examined. This would test the theory that during the finite time taken for TCA to rupture the cells, changes in the ATP levels occur within the cell. It was considered that a reduction of the temperature of the extraction would

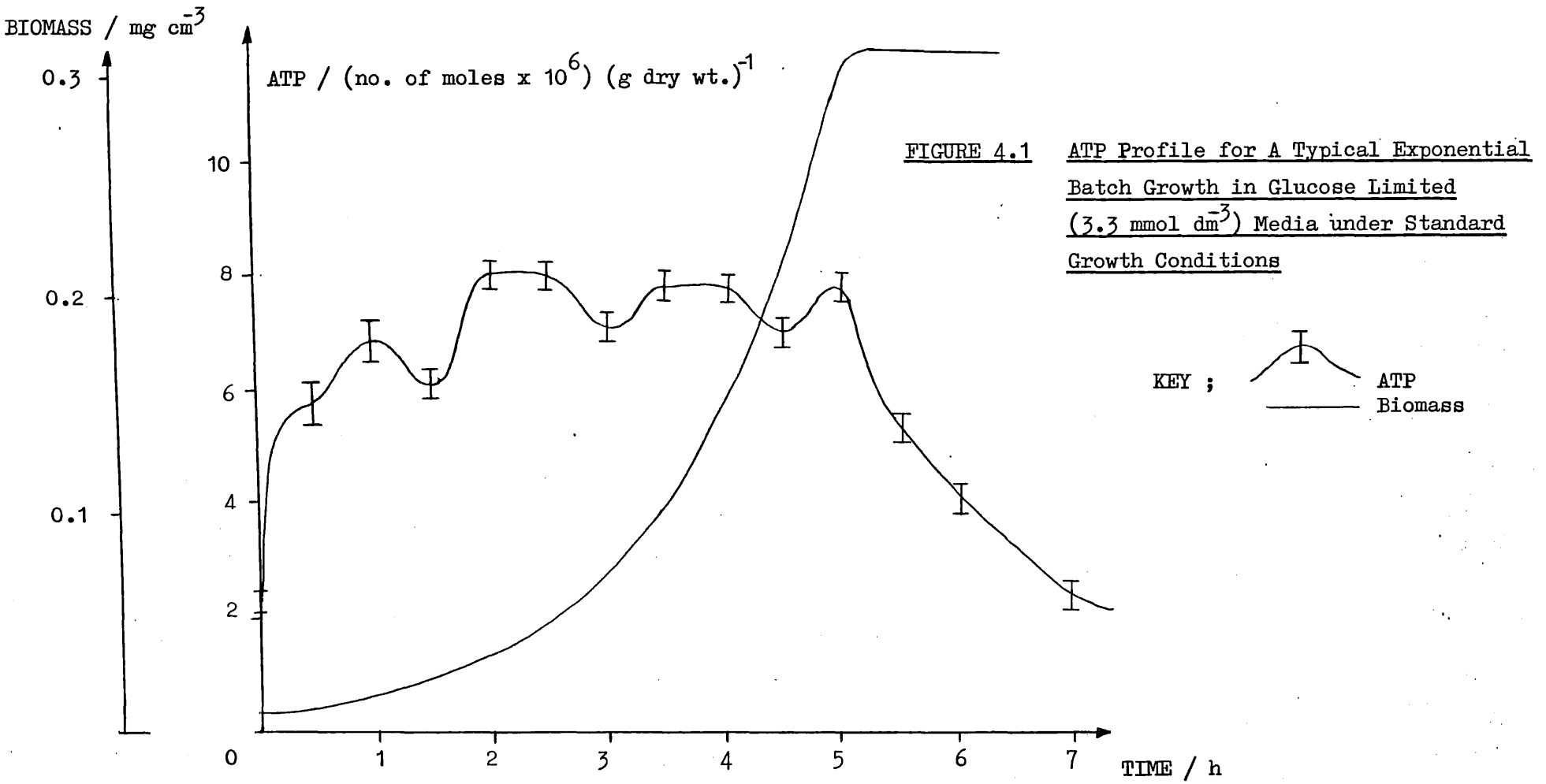



FIGURE 4.1 ATP Profile for A Typical Exponential Batch Growth in Glucose Limited (3.3 mmol dm$^{-3}$) Media under Standard Growth Conditions

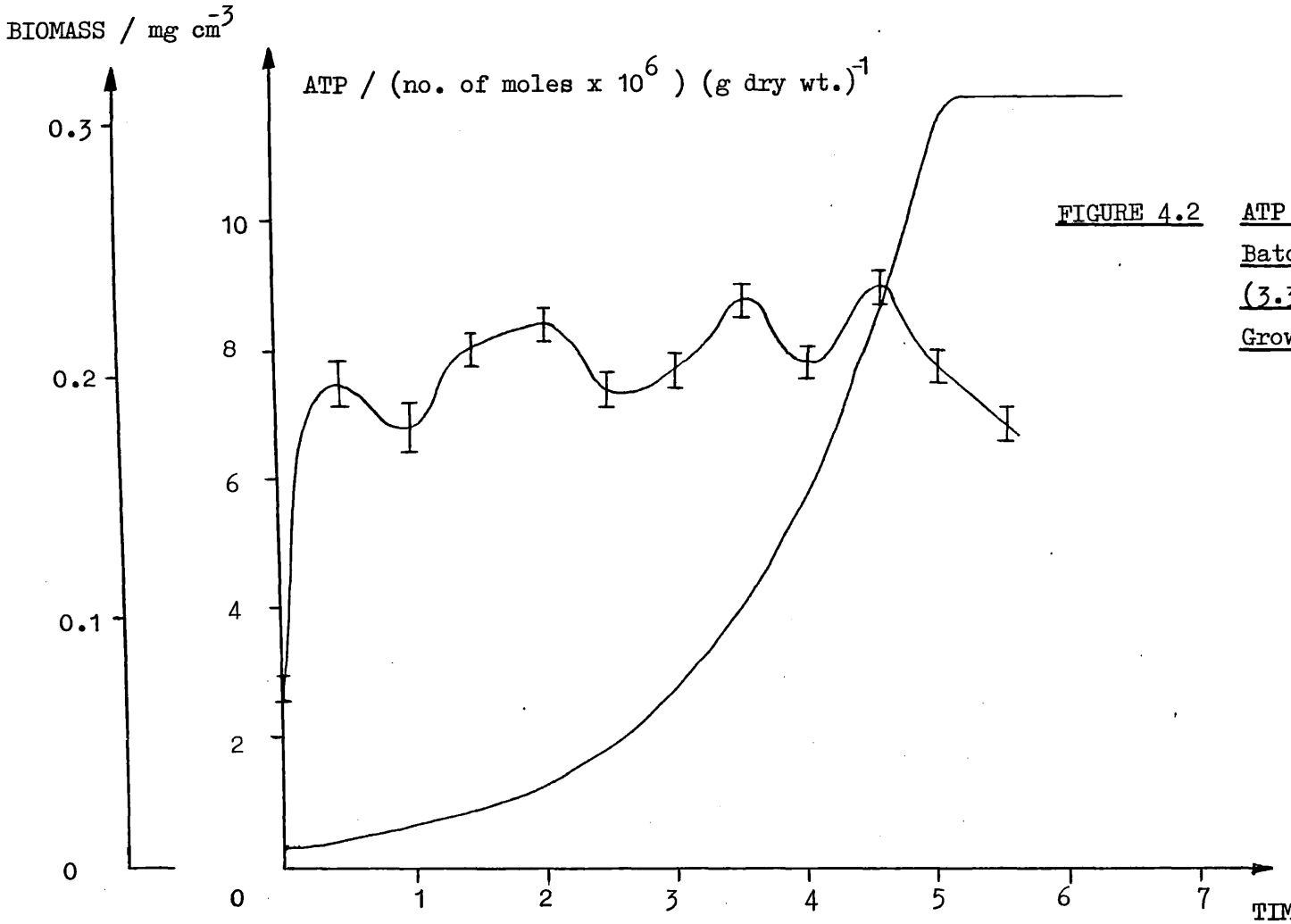



FIGURE 4.2 ATP Profile for A Typical Exponential Batch Growth in Glucose Limited (3.3 mmol $dm^{-3}$) Media under Standard Growth Conditions

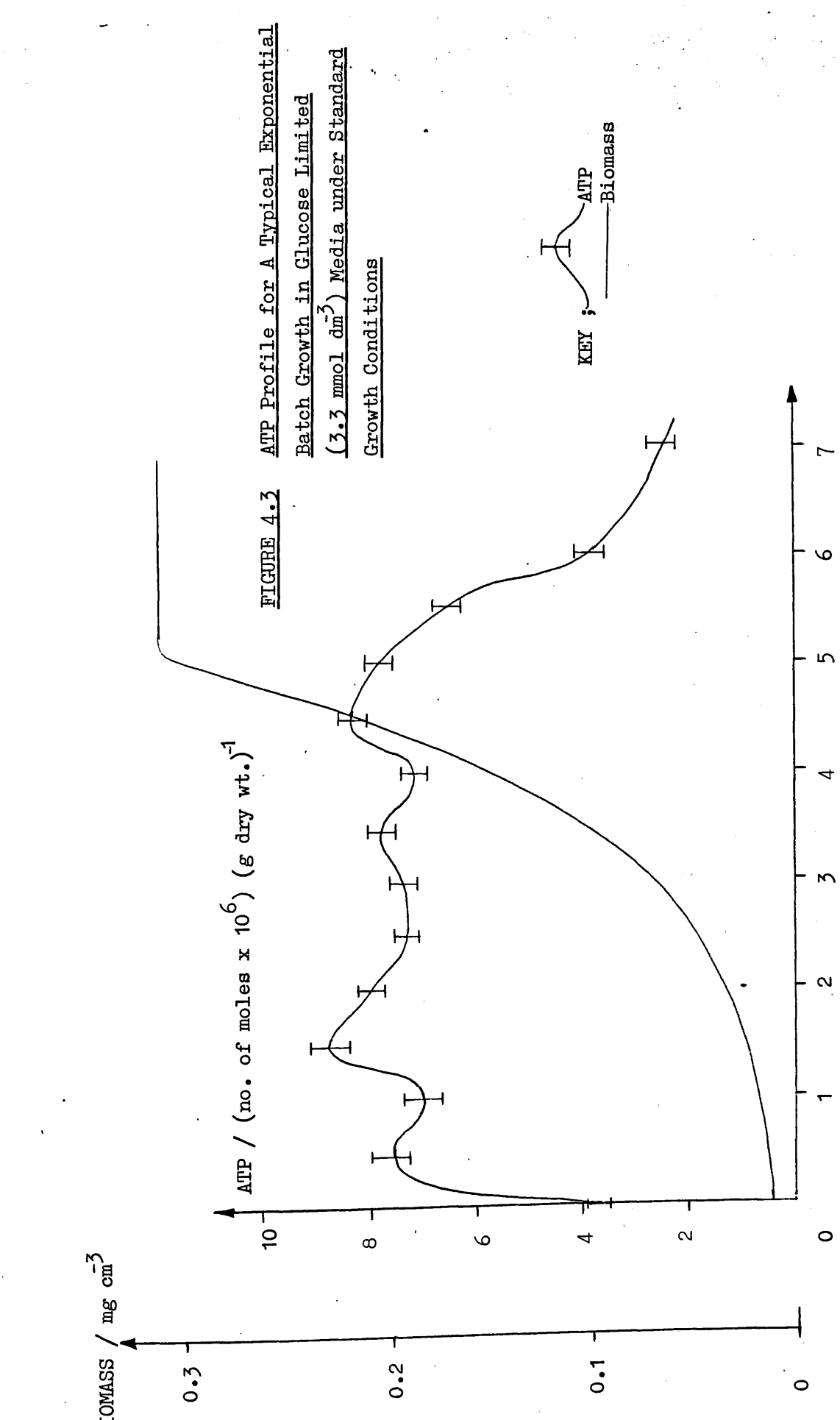



FIGURE 4.3 ATP Profile for A Typical Exponential Batch Growth in Glucose Limited ($3.3$ mmol dm$^{-3}$) Media under Standard Growth Conditions

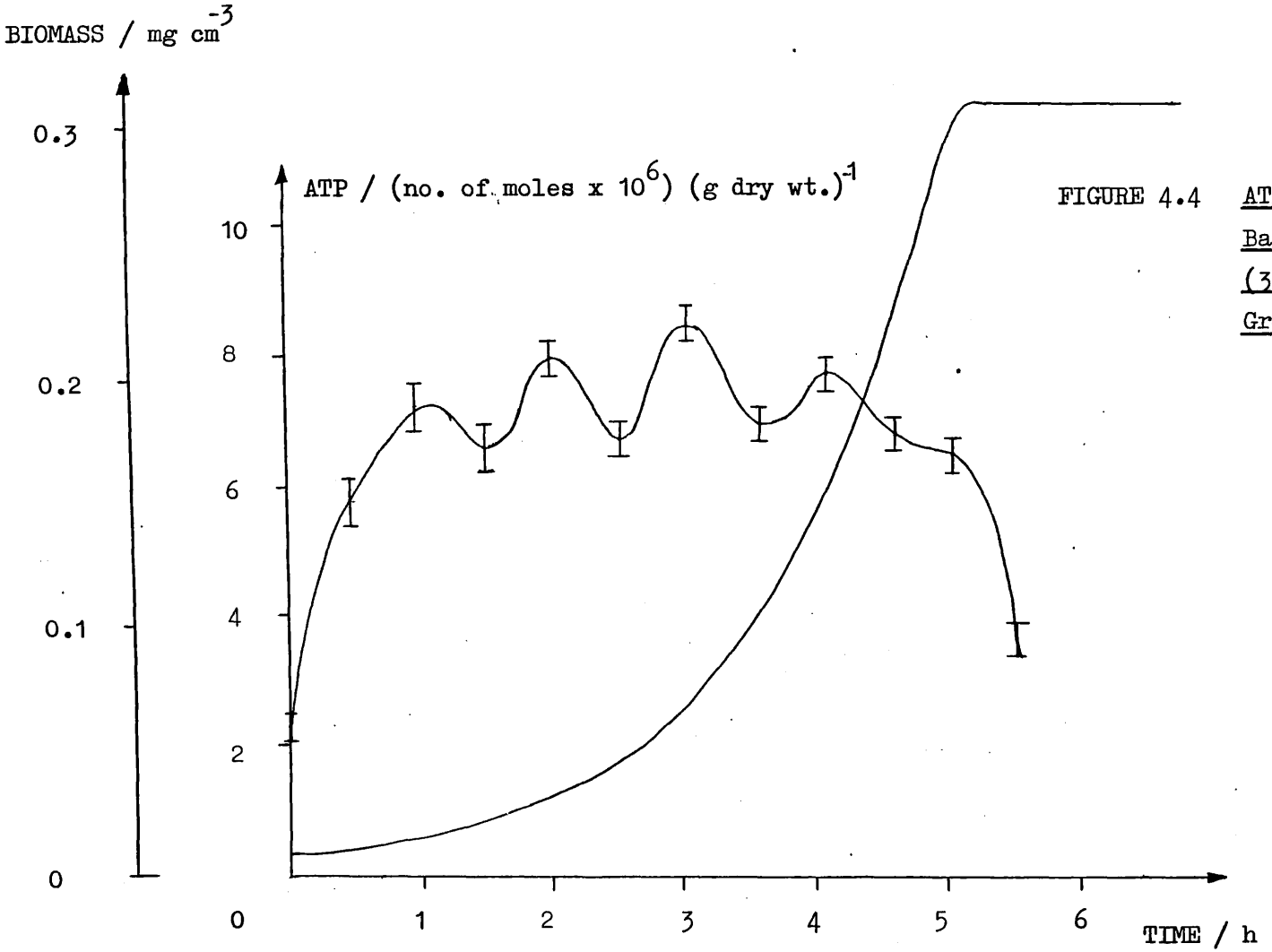



FIGURE 4.4 ATP Profile for A Typical Exponential Batch Growth in Glucose Limited (3.3 mmol $dm^{-3}$) Media under Standard Growth Conditions

KEY ; ATP
Biomass

minimise this effect. The sampling and analysis was exactly the same as that for the TCA technique, only the extraction was different.

Method

The bacterial sample (1 $cm^3$) was injected into 2 $cm^3$ of 10% TCA (maintained at -12°C) contained in a 3 $cm^3$ plastic cryogenic vial. The vial was sealed and immediately immersed in liquid nitrogen for storage. When required the sample was removed from the liquid nitrogen and transferred to an acetone/$CO_2$ bath maintained at -12°C for 20 minutes. The subsequent extraction and analytical procedures used were as described previously.

Results

During exponential growth the ATP levels obtained for duplicate samples, (removed in quick succession from the culture vessel) were not significantly different from those extracted with TCA at 4°C.

### 4.2.2 Sulphuric Acid Extraction

Bacterial ATP levels obtained using sulphuric acid for extraction were 9% lower than those obtained by the TCA extraction.

As one of the major criteria for a successful extraction is a high recovery of ATP, the results support the view that TCA gives the best physiologically representative bacterial ATP value.

## 4.3 Sample Duplication and Increased Frequency of Sampling for Glucose-Limited Batch Growth

It was necessary to establish the reproducibility of the changes in the ATP levels and demonstrate that the oscillation of ATP levels

during exponential growth were real and not due to an uncontrollable factor, i.e. rapidly changing ATP pool.

### 4.3.1 Duplication of Samples

At specified intervals during the exponential growth phase four 1 $cm^3$ samples were removed in quick succession ( 5 s). Each of these four samples were extracted and analysed separately.

There was close agreement between the 4 ATP values at a given time (Table 4.1).

### 4.3.2 Increased Frequency of Sampling

Samples were removed at 5 minute intervals; at the start of, at selected periods during, and at the end of the exponential growth phase.

The resultant ATP profile confirms the oscillatory nature of the ATP level, (Fig. 4.5). The plotted ATP values prove definite gradual changes in the ATP level at the start, during, and at the end of the exponential growth phase.

## 4.4 Degree of Synchrony During Glucose-Limited Growth

It is possible that the oscillations in the ATP level during exponential growth are due to the partially asynchronous nature of the growth. This would mean that the degree of synchrony is prominent enough to cause detectable changes in the exponential ATP value. Viable counts and total cell numbers were used to estimate the degree of synchrony.

### 4.4.1 Viability Counts

The number of viable cells in glucose-limited culture, (cryogenic inoculations) was monitored by plating. 1 $cm^3$ samples of the growing

TABLE 4.1 ATP Values Obtained for Duplicate Samples of An Exponentially Growing Glucose Limited ($3.3$ mmol $dm^{3}$) Batch Culture

| TIME FROM INNOCULATION / h | BIOMASS / mg $cm^{-3}$ | ATP VALUES /(no. of moles x $10^{6}$) $(g\ dry\ wt.)^{-1}$ DUPLICATE SAMPLES | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 2 | 0.038 | 7.90 | 8.05 | 7.95 | 8.17 |
| 3 | 0.080 | 6.21 | 6.06 | 6.12 | 6.38 |
| 4 | 0.175 | 6.62 | 6.67 | 6.66 | 6.68 |
| 5 | 0.340 | (END OF EXPONENTIAL GROWTH) | | | |
| 7 | 0.340 | 2.22 | 2.14 | 2.36 | 2.21 |
| 24 | 0.340 | 0.56 | 0.53 | 0.51 | 0.53 |

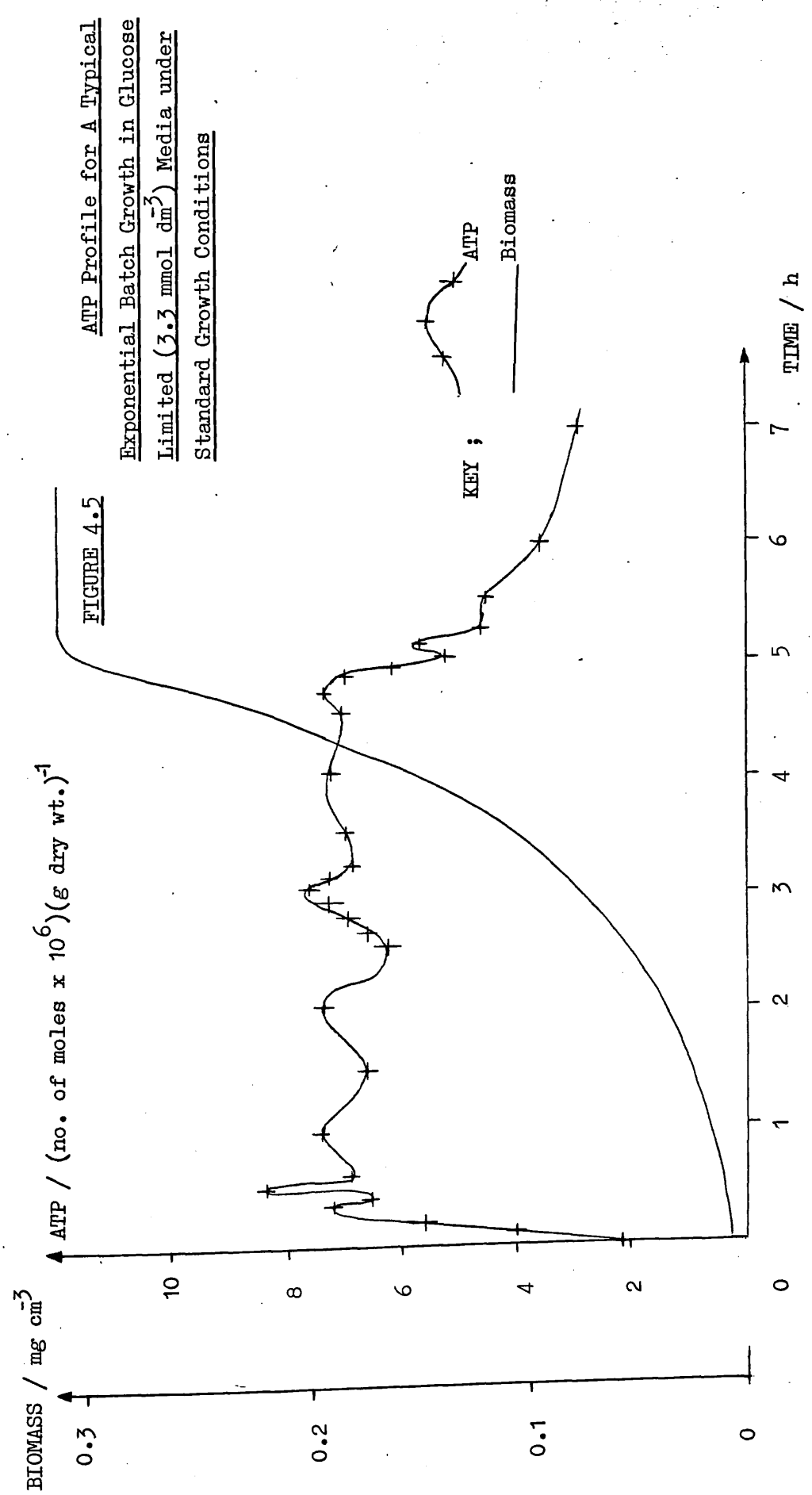



FIGURE 4.5

ATP Profile for A Typical Exponential Batch Growth in Glucose Limited (3.3 mmol dm$^{-3}$) Media under Standard Growth Conditions

culture were suitably diluted ($10^4$-$10^6$ times) and 0.1 cm aliquots spread onto agar plates. Each diluted 1 $cm^3$ sample was plated at each of three different dilutions on agar plates. These were incubated at 37°C for 24 h and the average count obtained.

At the same time duplicate 1 $cm^3$ samples of the growing culture were removed for ATP analysis. The biomass was also measured.

The linearity of the log(viable count)-time and log(biomass)-time plots proves the asynchronous nature of the growth (Fig. 4.6).

The two ATP profiles (Fig. 4.7) expressed as moles of ATP per unit mass and per viable cell, are very similar which confirms the oscillatory nature of the ATP level.

### 4.4.2 Total Cell Counts

Total cell counts were determined using a "Coulter Counter", which determines the number of cells suspended in an electrically conducting salts solution by detecting the change in resistance when a cell passes through a small aperture (30 μ). A voltage pulse of short duration is produced, of magnitude proportional to particle size. The series of pulses is then electrically scaled and counted.

At 30 minute intervals duplicate 1 $cm^3$ samples were taken; one for ATP analysis and the other for a total cell count. The sample for total cell count was injected into 50 $cm^3$ of filtered (Millipore filter) salts solution, 1000 $cm^3$ of which contains sodium chloride (8 g); potassium chloride (0.2 g); disodium hydrogen phosphate (1.15 g); potassium dihydrogen phosphate (0.2 g) and sodium azide (1 g); this fixes the growing suspension. This suspension was made up to 100 $cm^3$ with the salts solution. The background count of the salts solution was $5 \times 10^3$ $cm^{-3}$.

Again the linearity of the log(total cell count)-time and

FIGURE 4.6 Viability and Biomass Time Plots for Batch Growth in Glucose Limited (3.3 mmol $dm^{-3}$) Media under Standard Growth Conditions

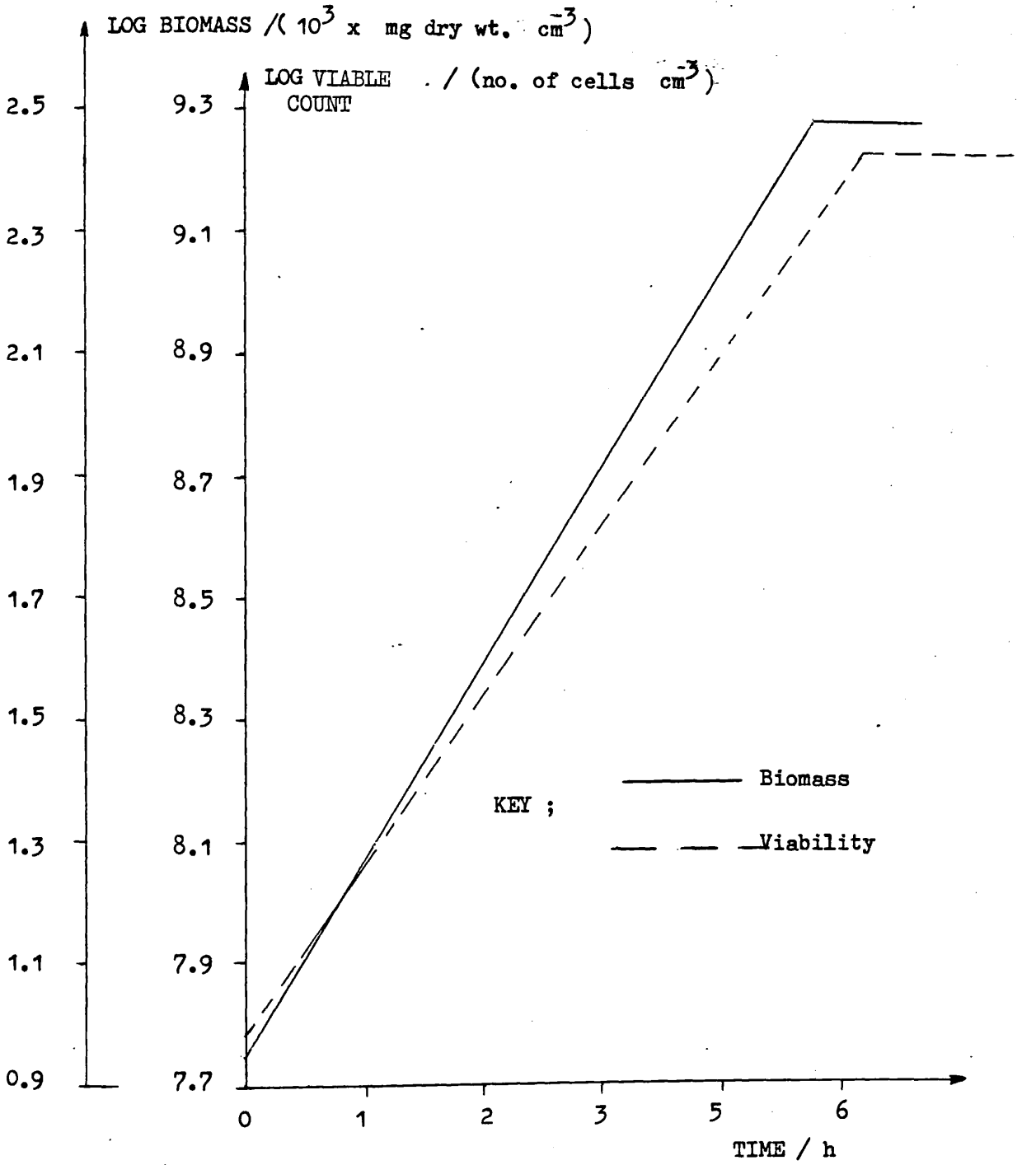
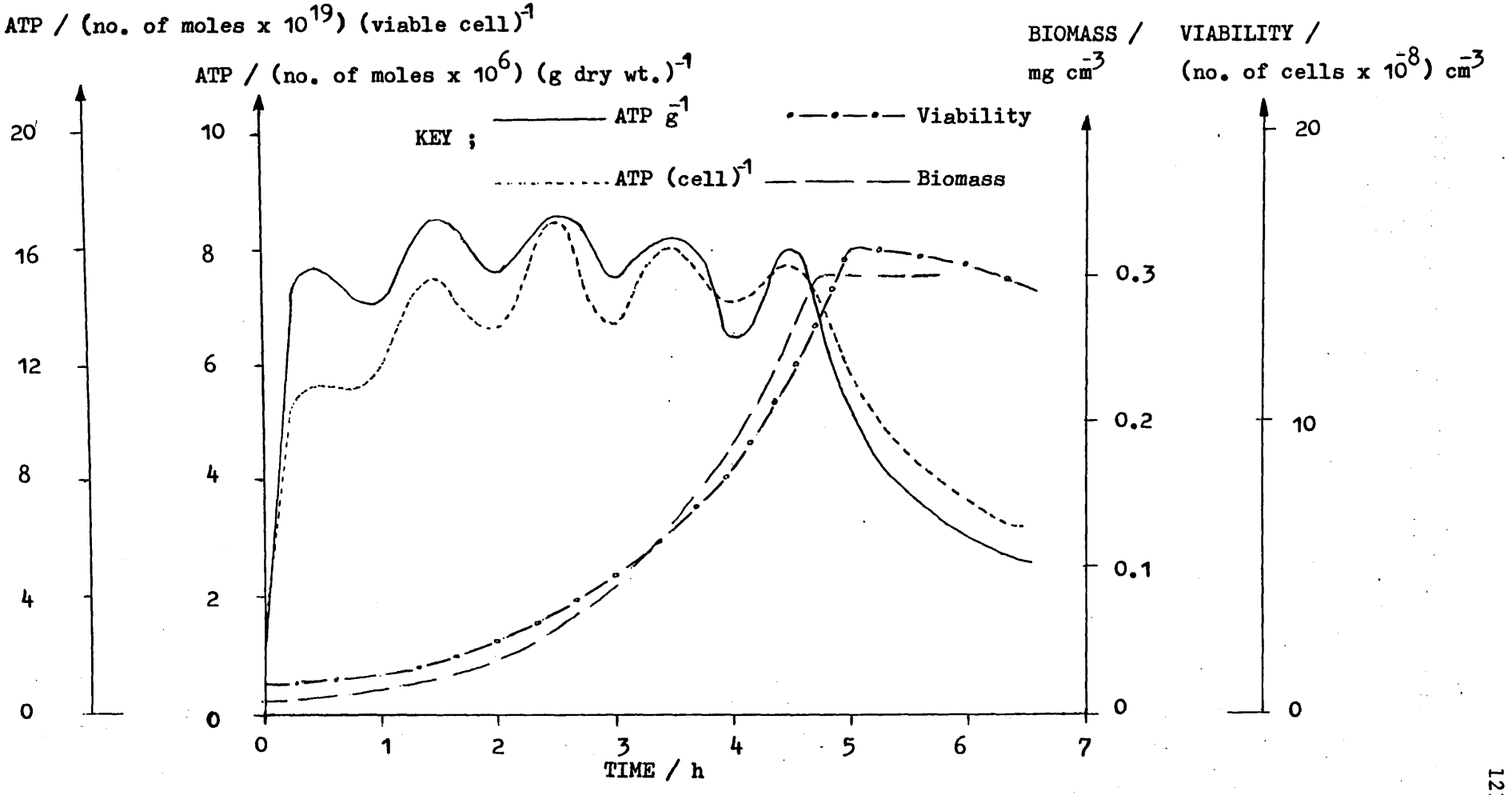


FIGURE 4.7 ATP Profiles with Viability and Biomass Curves for A Typical Exponential Batch Growth in Glucose Limited (3.3 mmol $dm^3$) Media under Standard Conditions



log(biomass)-time plots proves the asynchronous nature of the growth (Fig. 4.8).

Further the two ATP profiles (Fig. 4.9) expressed as moles of ATP per unit mass or per cell are similar, which further confirms the oscillatory nature of the ATP level.

## 4.5 ATP Levels for Batch Growth in Glucose-Limited Media Under Differing Conditions

### 4.5.1 Induced Lag Period

Previous studies of the ATP levels of growing cultures have not used cryogenically stored inocula. In an attempt to reproduce these earlier conditions an inoculum was taken from a culture which had been in the stationary phase for 36 h.

This induced a 2 h lag phase upon inoculation, however the resultant ATP profile was substantially the same as that for growth using a cryogenically stored inoculum (Fig. 4.10).

### 4.5.2 Growth under Anaerobic Conditions

The conditions of growth were exactly the same as the standard (aerobic) conditions except that the growth medium was purged with white spot nitrogen, (1 $dm^3$ $min^{-1}$) instead of air. The inoculum was from the standard cryogenic store of cells grown aerobically. Full anaerobic growth could not be sustained in the media unless it was purged with nitrogen for 24 h prior to inoculation.

The anaerobic conditions (initial 25 h) supported a poor growth and yield (Figs 4.11 (a) and (b)). During the initial lag phase there was a gradual increase in the ATP level until 15 h after inoculation when the ATP value reached 4.1 x $10^{-6}$ mole ATP per g dry wt. At this stage growth commenced, during this growth phase the ATP level rapidly rose to

FIGURE 4.8 Total Cell and Biomass Time Plots for Exponential Growth in Glucose Limited (3.3 mmol $dm^3$) Media under Standard Conditions

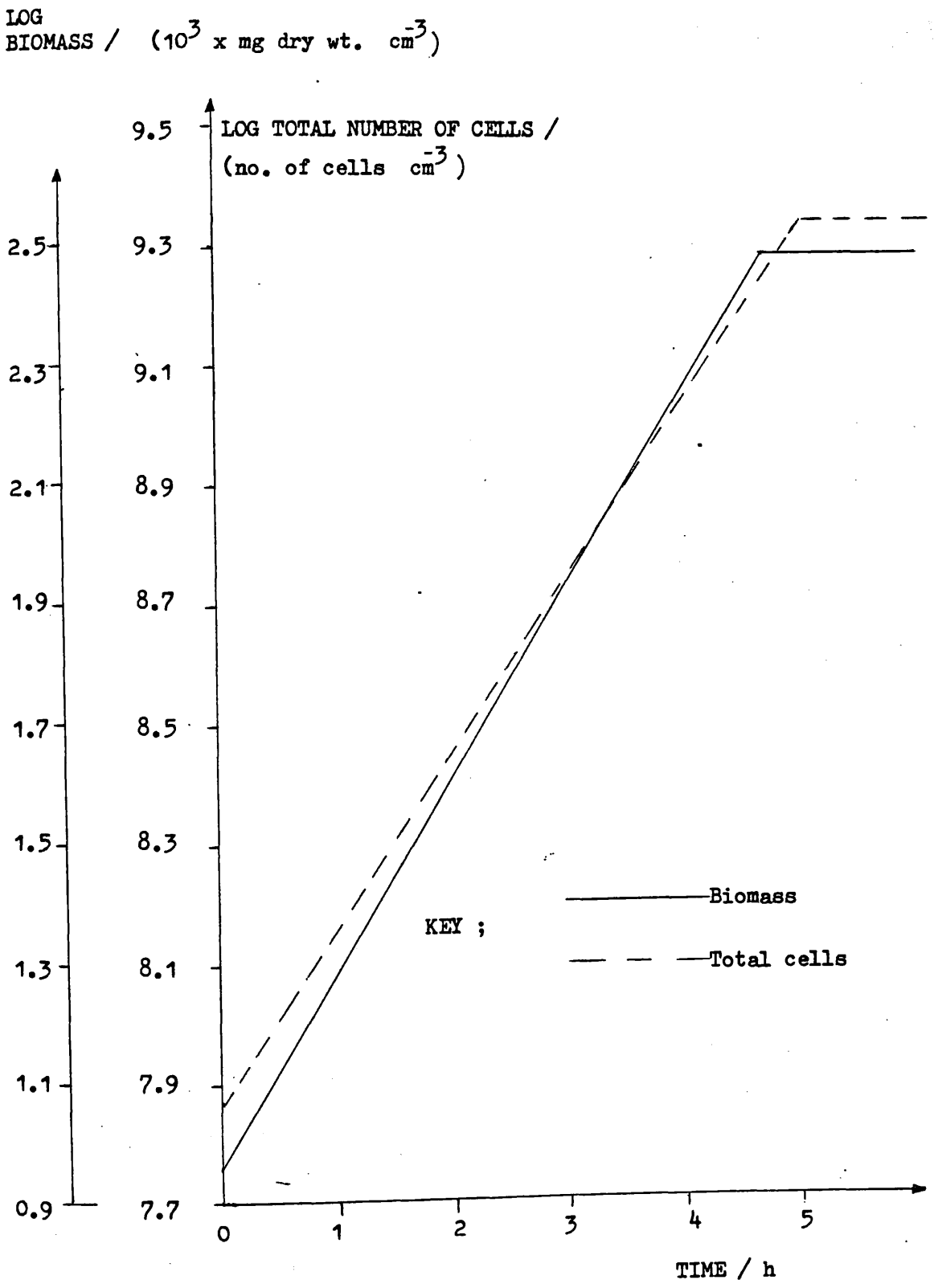


FIGURE 4.9 ATP Profiles with Viabilty and Biomass Curves for A Typical Exponential Batch Growth in Glucose Limited (3.3 mmol $dm^{-3}$) Media under Standard Conditions

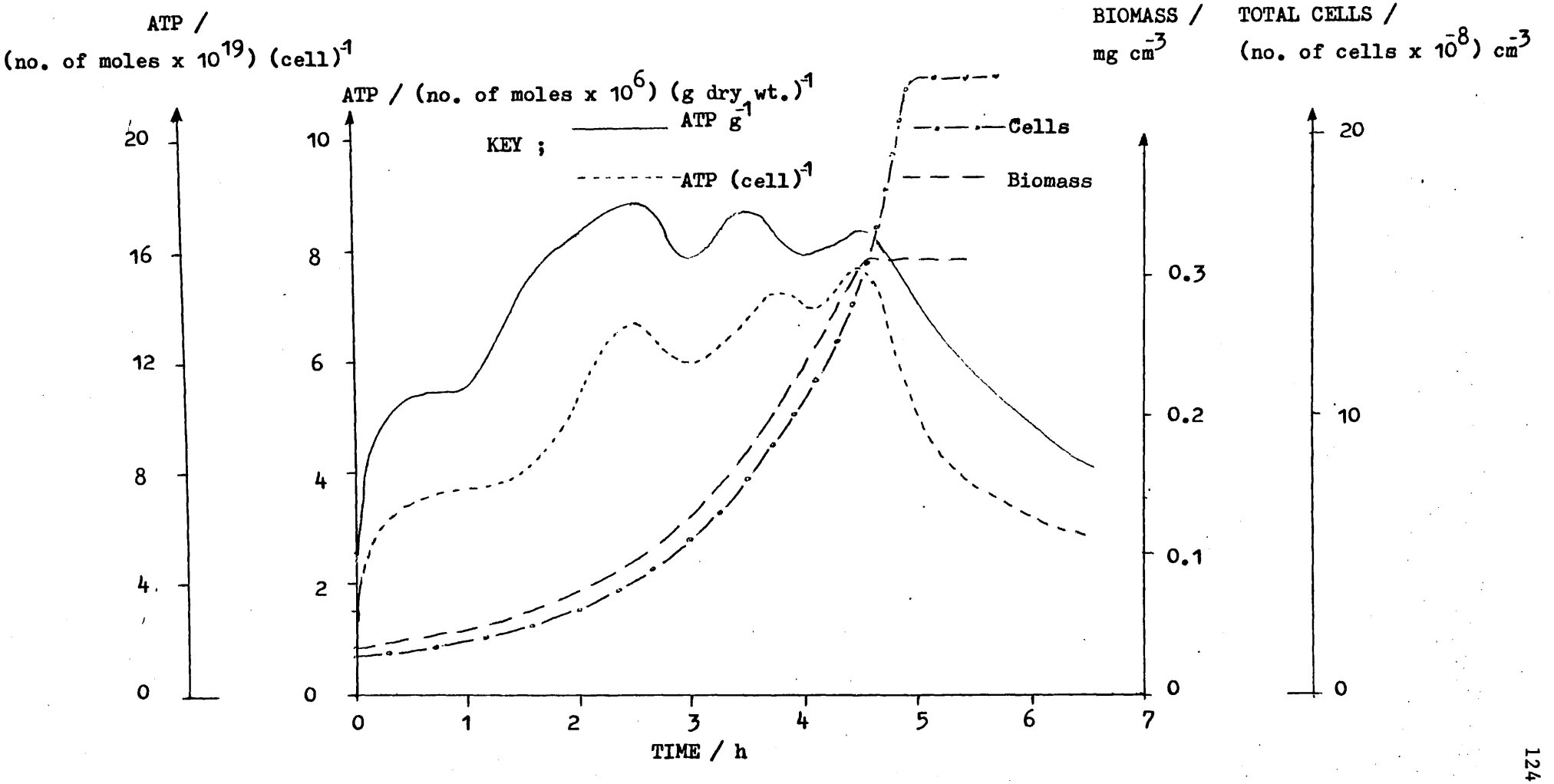


FIGURE 4.10 ATP Profile for An Exponential Batch Growth with An Induced Lag Phase in Glucose Limited ($3.3$ mmol $dm^{-3}$) Media under Standard Growth Conditions

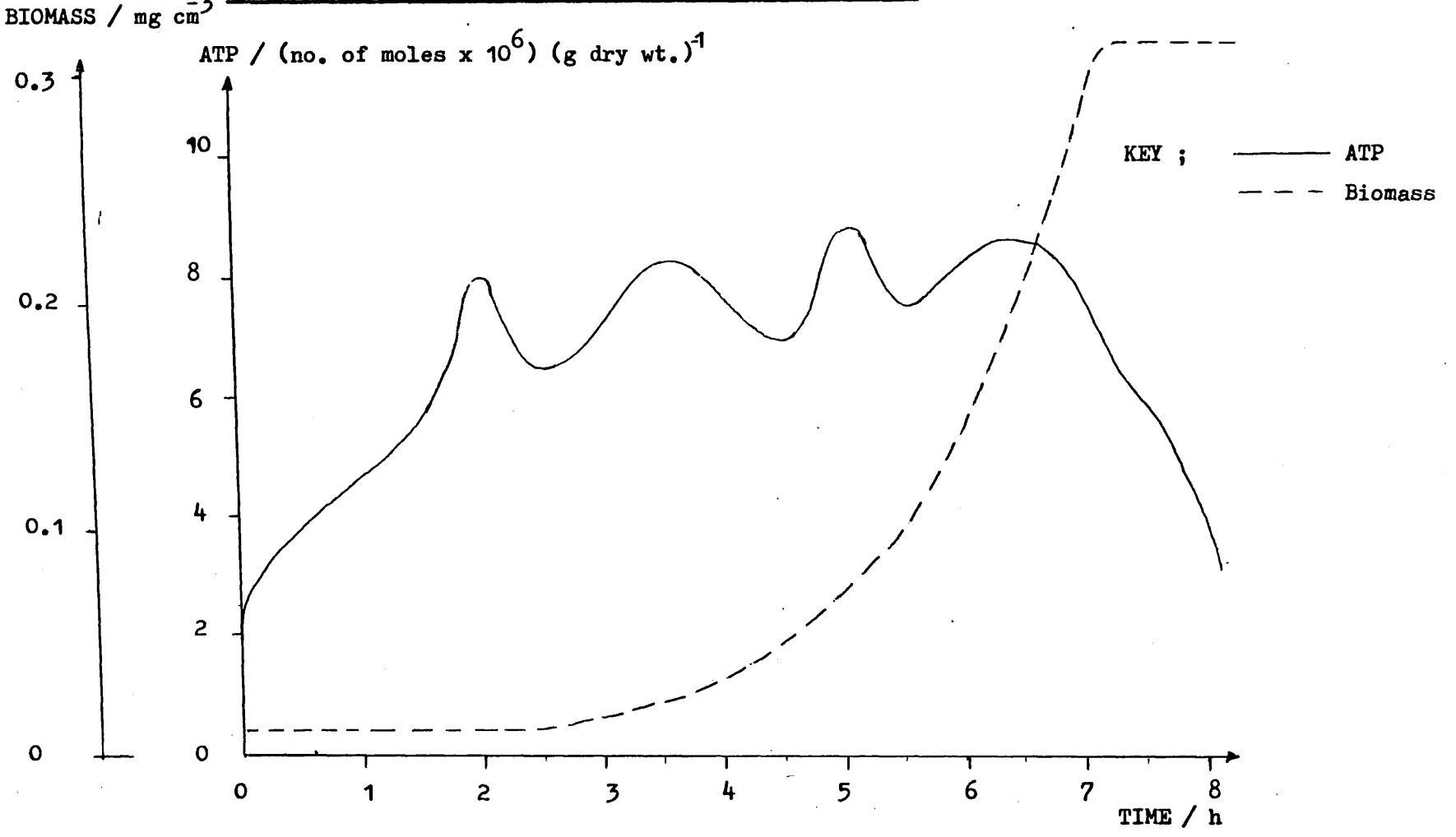


FIGURE 4.11(a) ATP Profile for Aerobically Trained Bacteria Growing under Anaerobic Conditions in Glucose Limited (3.3 mmol $dm^{-3}$) Media

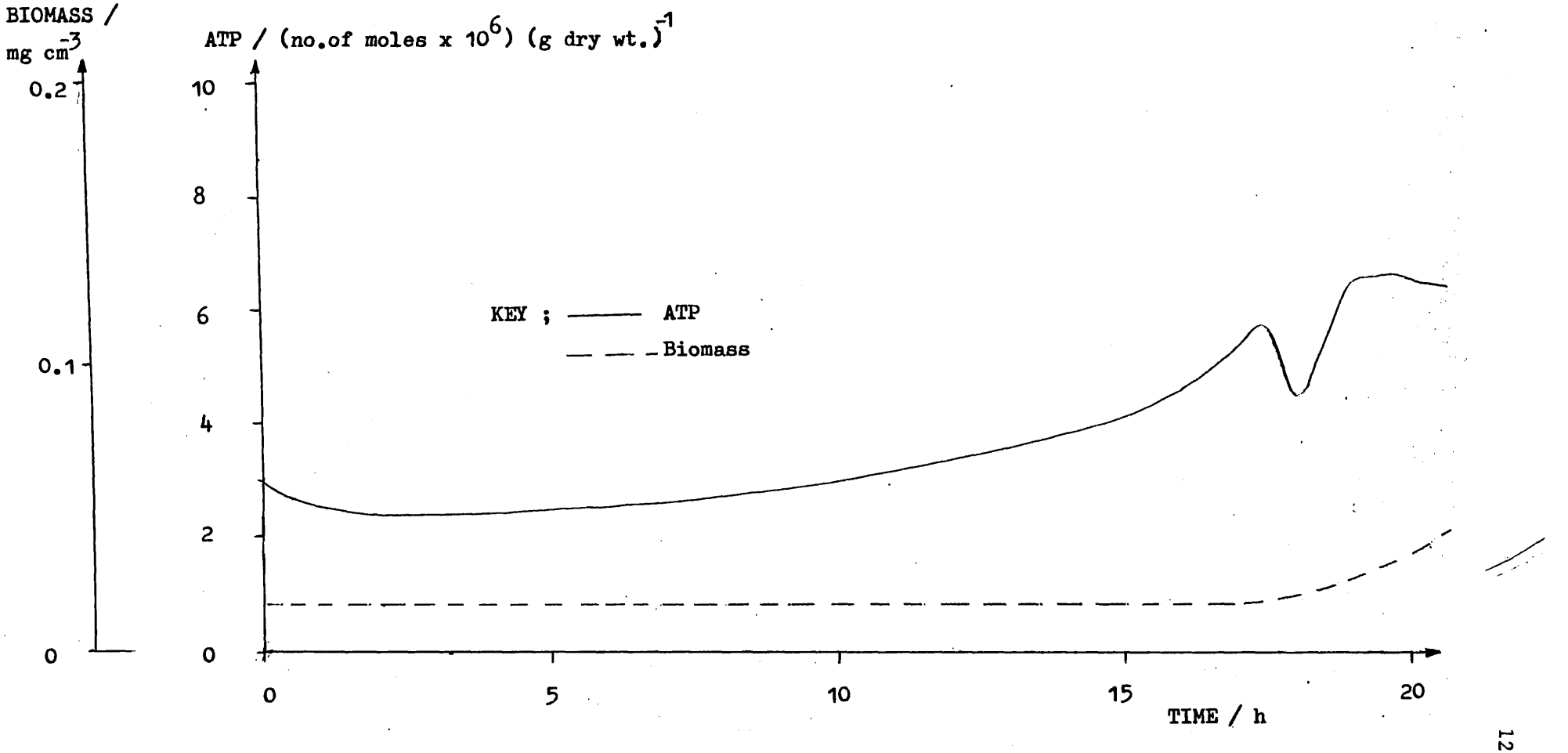
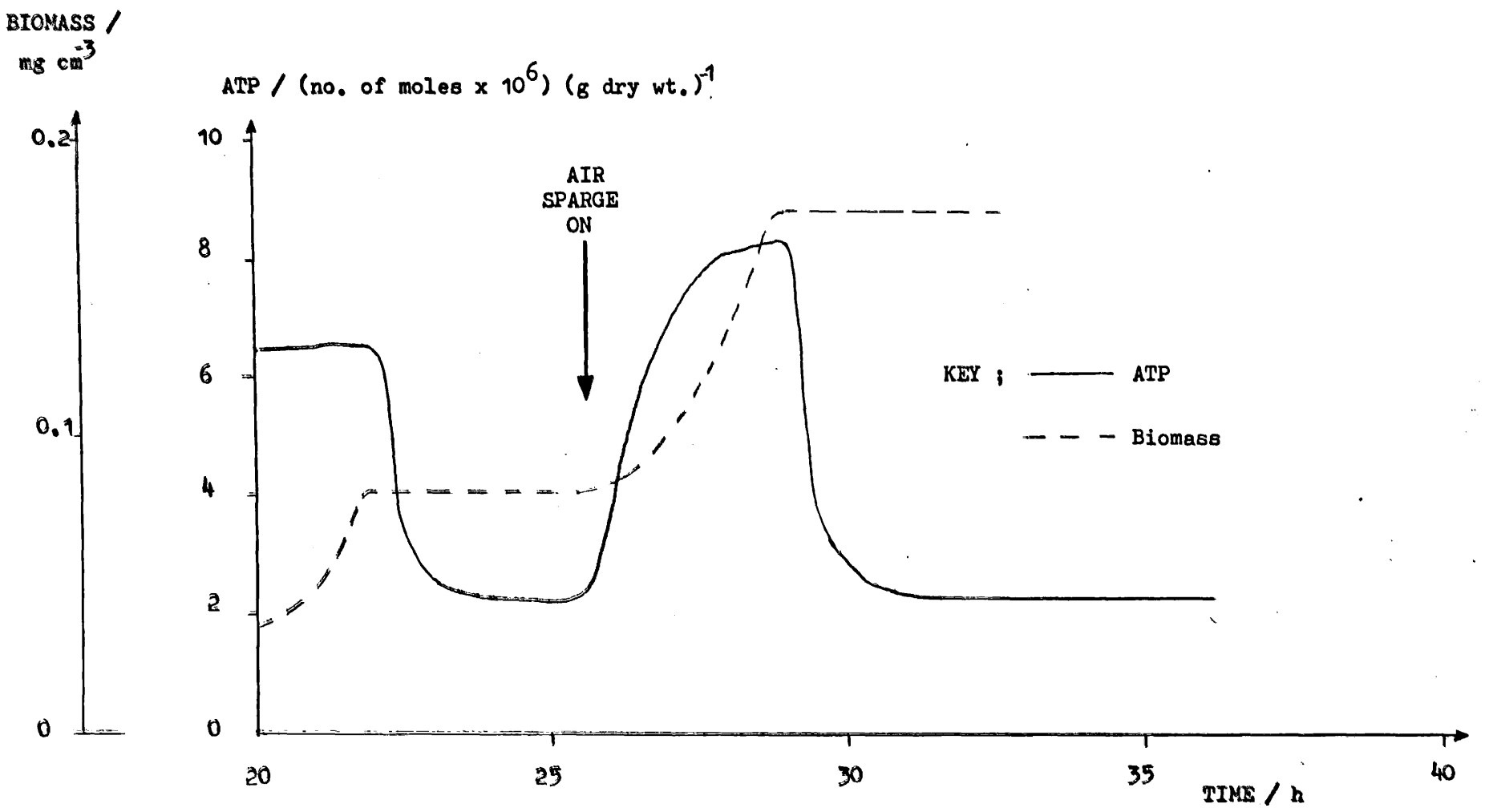


FIGURE 4.11(b) ATP Profile for Aerobically Trained Bacteria Growing under Anaerobic Conditions in Glucose Limited (3.3 mmol $dm^{-3}$) Media



a constant (anaerobic growth) value of 6.5 mole ATP per g dry wt. The complete consumption (anaerobic) of the glucose was accompanied by a rapid drop in the ATP level to the stationary phase value of 2.3 x $10^{-6}$ mole ATP per g dry wt and a cessation of growth. The introduction of air (25 h after inoculation) to establish aerobic conditions caused an immediate recommencement of growth and a rise in the ATP level to near the normal aerobic value for glucose-limited exponential growth (8.2 x $10^{-6}$ mole ATP per g dry wt). The complete consumption of the secondary metabolites (29.5 h after inoculation) was accompanied by a rapid drop in the ATP level to the stationary phase value and the cessation of growth.

## 4.6 Cellular ATP Levels During Growth in Glucose-Limited Chemostats

For comparison ATP levels were measured during the growth of cells of Kelbsiella aerogenes in a continuous culture fermenter under standard conditions (Djavan, 1980).

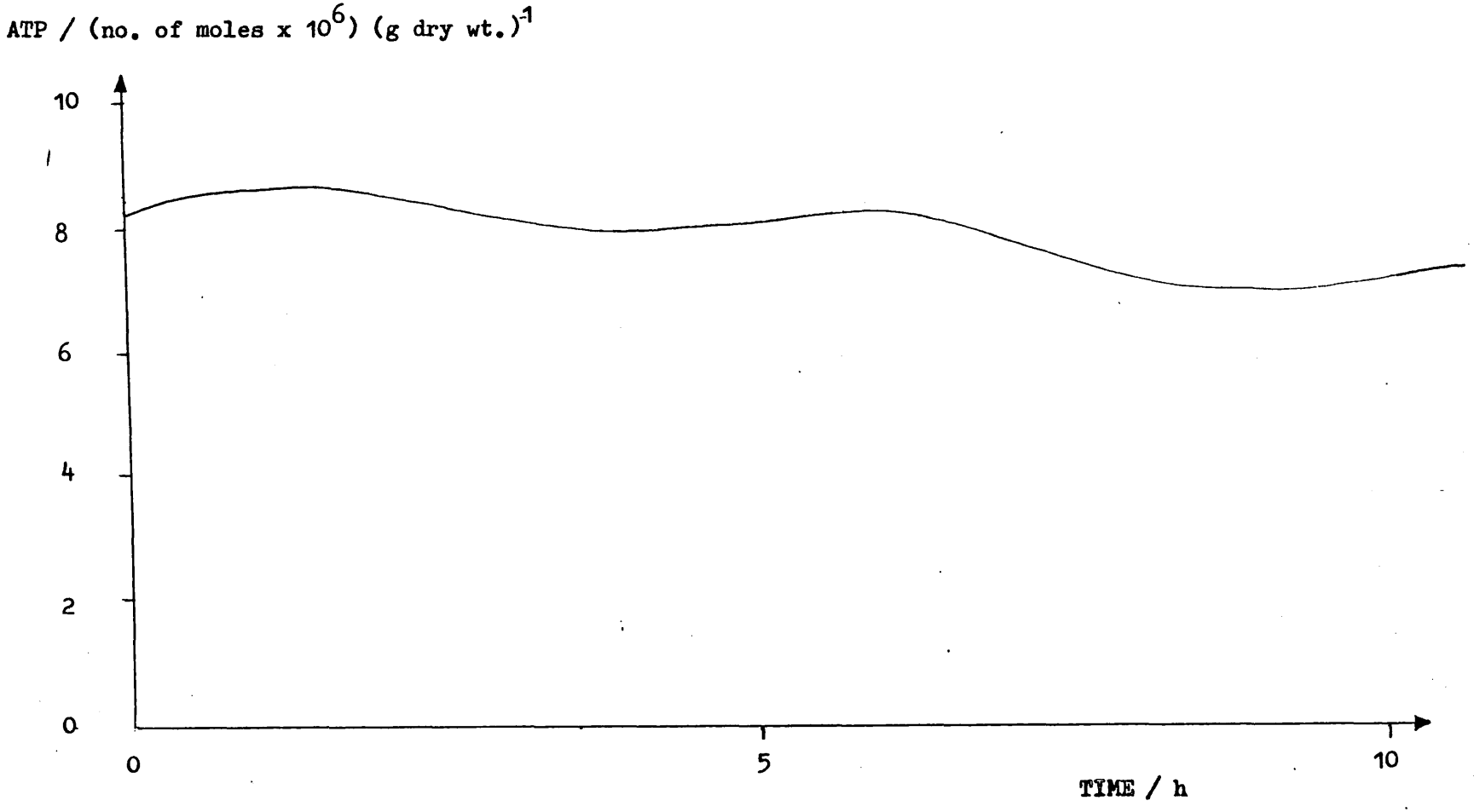
### 4.6.1 Experimental

A glucose-limited chemostat (glucose concentration; 4.00 mmol $dm^{-3}$) was operated aerobically at 37°C at a dilution rate of 0.52 $h^{-1}$ (Djavan, 1980). After 24 h of operation steady conditions were established and 1 $cm^3$ samples were taken at 30 minute intervals from the overflow.

The samples were extracted and the ATP content determined as described previously. The biomass was a constant during this period which averaged 0.370 mg $cm^{-3}$ ±3%.

Over a 10 h period the ATP content of the cells was in the range 7-8 x $10^{-6}$ mole per g dry wt. (Fig. 4.12); the value is comparable to that observed during the exponential phase of cells growing aerobically

FIGURE 4.12 A Typical ATP Profile for A Glucose Limited ($4.0$ mmol $dm^{-3}$) Chemostat ($D = 0.52\ h^{-1}$) under Standard Growth Conditions



under glucose-limitation.

## 4.7 Summary

1. During the exponential growth phase there were oscillations of the bacterial ATP content ($\pm 0.75 \times 10^{-6}$ mol $g^{-1}$) about a mean level ($7.8 \times 10^{-6}$ mol $g^{-1}$).

2. Bacterial ATP levels obtained using other extraction techniques were similar or significantly lower than those obtained by TCA.

3. The oscillations of the ATP content are real and not a result of the sampling or a semi-synchronous growth.

4. The ATP profiles per viable cell and per cell are similar to ATP profiles per unit mass.

5. Under anaerobic growth the ATP content is a mean value of $6.5 \times 10^{-6}$ mol $g^{-1}$.

6. During growth in a glucose-limited chemostat the ATP content was in the range of $7\text{-}8 \times 10^{-6}$ mol $g^{-1}$.

# CHAPTER FIVE

## ESTABLISHMENT OF STANDARD CONDITIONS FOR POWER MEASUREMENTS OF GROWING BACTERIAL CULTURES

## 5.1 The Theoretical (Ideal) Power Output

Theoretically for an exponentially growing culture consuming glucose to produce bacterial mass and carbon dioxide only, the power should increase exponentially with time (Fig. 5.1). Exhaustion of the glucose is accompanied by cessation of growth, and the power output rapidly falls to zero (without any secondary power output).

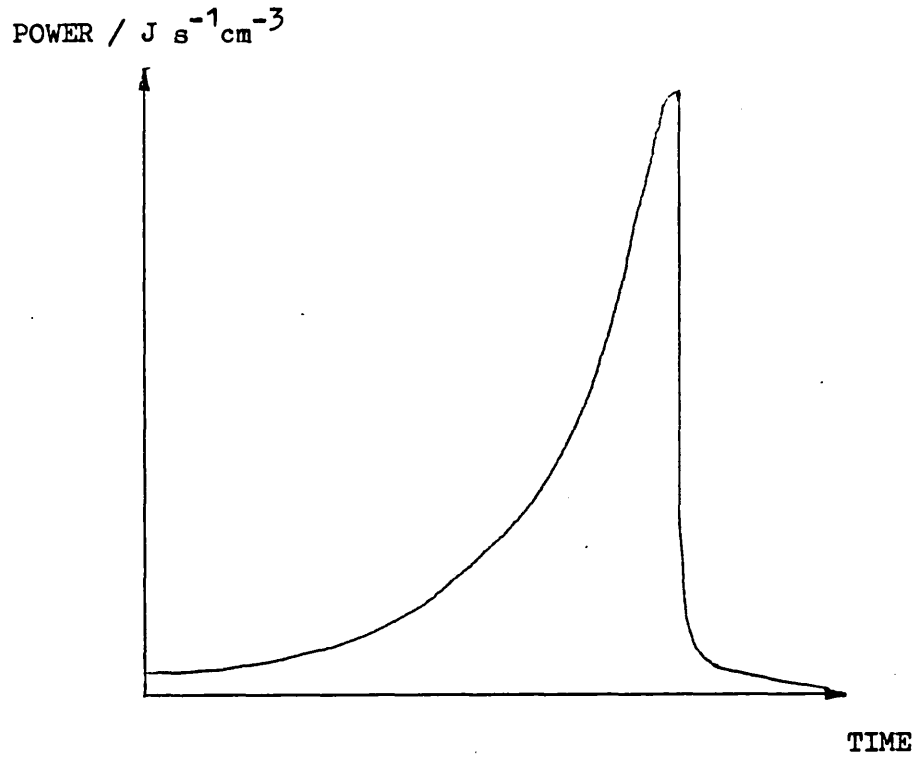
The first objective was to establish standard and reproducible conditions for growth, and for the measurement of power output during bacterial growth under aerobic conditions in glucose-limited (3.3 mmol $dm^{-3}$) media. These standard conditions can then be used subsequently for cells growing in other media of different compositions, (which produce power-time curves different to that in Figure 5.1).

### 5.1.1 The Experimental Power Output

The early power-time (p-t) traces obtained (Fig. 5.2) during experimental growth exhibited a secondary power (and carbon dioxide) output after cessation of growth and complete consumption of the glucose (A). Acetate was present in the culture during the exponential phase of growth (2.4). Following the cessation of growth the acetate was metabolised with the formation of carbon dioxide and the power output remained constant until this secondary metabolite had been removed. The heat evolved during acetate metabolism ranged from 0 to 11% of the total heat and the acetate concentration (as measured at point A) ranged from 0 to 1.09 mmol $dm^{-3}$).

The p-t and carbon dioxide-time ($CO_2$-t) traces are identical in shape throughout the complete growth cycle.

FIGURE 5.1 Theoretical Power Output from An Exponential Glucose Limited Culture Grown under Ideal Aerobic Conditions



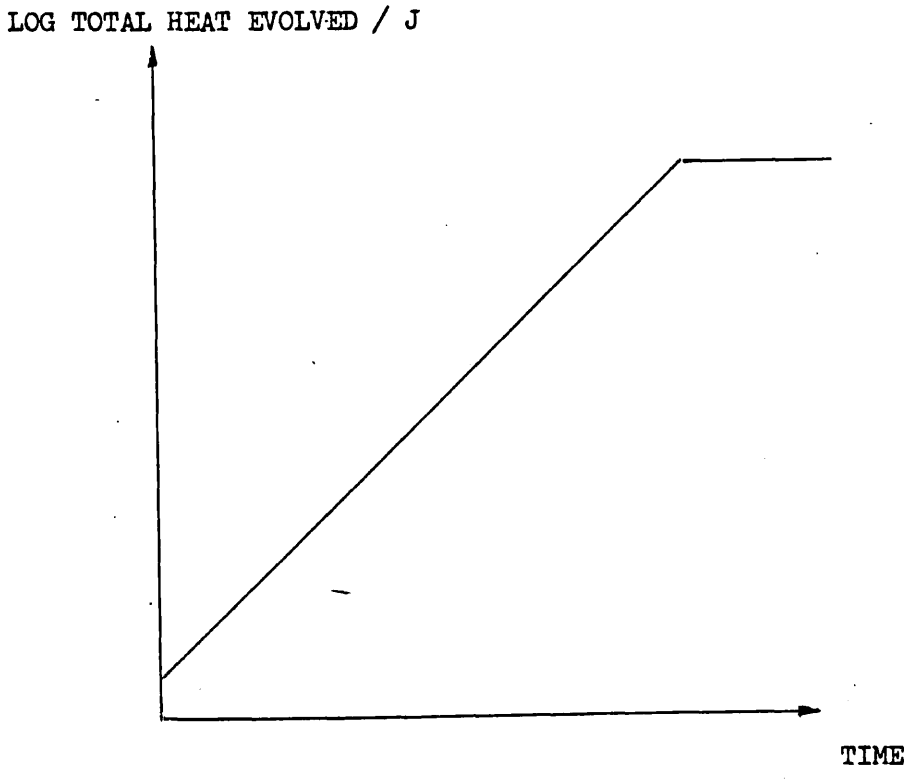
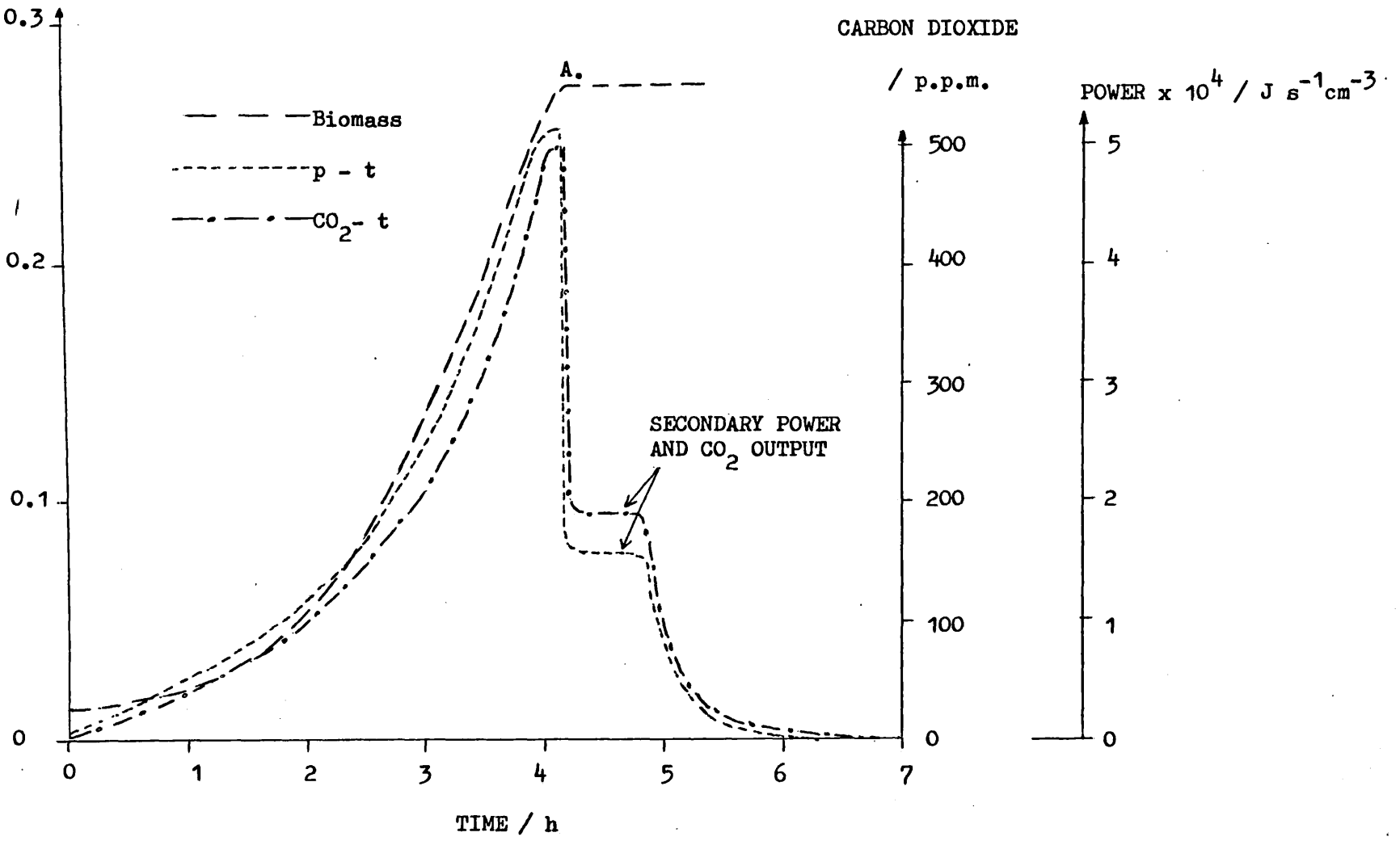



FIGURE 5.2 p - t Trace and Changes of Growth Parameters during Glucose Limited (3.3 mmol $dm^{-3}$) Growth under Non-standard Conditions. (Cal. flow : 90 $cm^3 h^{-1}$, stirrer setting : 210).



## 5.2 Investigation of the Cause of Secondary Power Output

Previous workers (Nichols, 1980) attributed the secondary power output to oxygen starvation in the calorimeter line and cell. It was thought that in the line the oxygen tension was below 30% saturation conditions under which anaerobic metabolism becomes prominent producing acetate and ethanol (Pirt, 1969). It has been reported that up to 20% of the total heat measured during aerobic growth is evolved during the metabolism of the anaerobically produced acetate and ethanol (Nichols, 1980). It has been suggested that these products are returned to the culture vessel where they accumulate. It is the metabolism of these anaerobically produced products which gives rise to the secondary power output at the end of growth. There are two arguments which conflict with this explanation.

(a) The total heat evolved during the metabolism of the secondary intermediates (which is a measure of the amount accumulated in the culture vessel) is relatively too large to be solely attributed to the effect of passing the culture through the calorimeter. The volume of bacterial suspension contained in the calorimeter cell and line is 2.5 $cm^3$ i.e. c. 0.4% of the total medium in the culture vessel. Further it is unlikely that oxygen starvation in the line is totally responsible for the relatively large amounts of accumulated secondary metabolites.

(b) The shape of the p-t trace (Fig. 5.2) is dissimilar from those made under almost anaerobic conditions in the cell (Fig. 5.4 a). The initial power output increases exponentially with time supporting the view that this is representative of the aerobic growth in the culture vessel.

FIGURE 5.3 p - t Trace and Changes of Growth Parameters in Glucose Limited (3.3 mmol $dm^{-3}$) Media

(Cal. flow : 90 $cm^3 h^{-1}$, stirrer setting : 190)

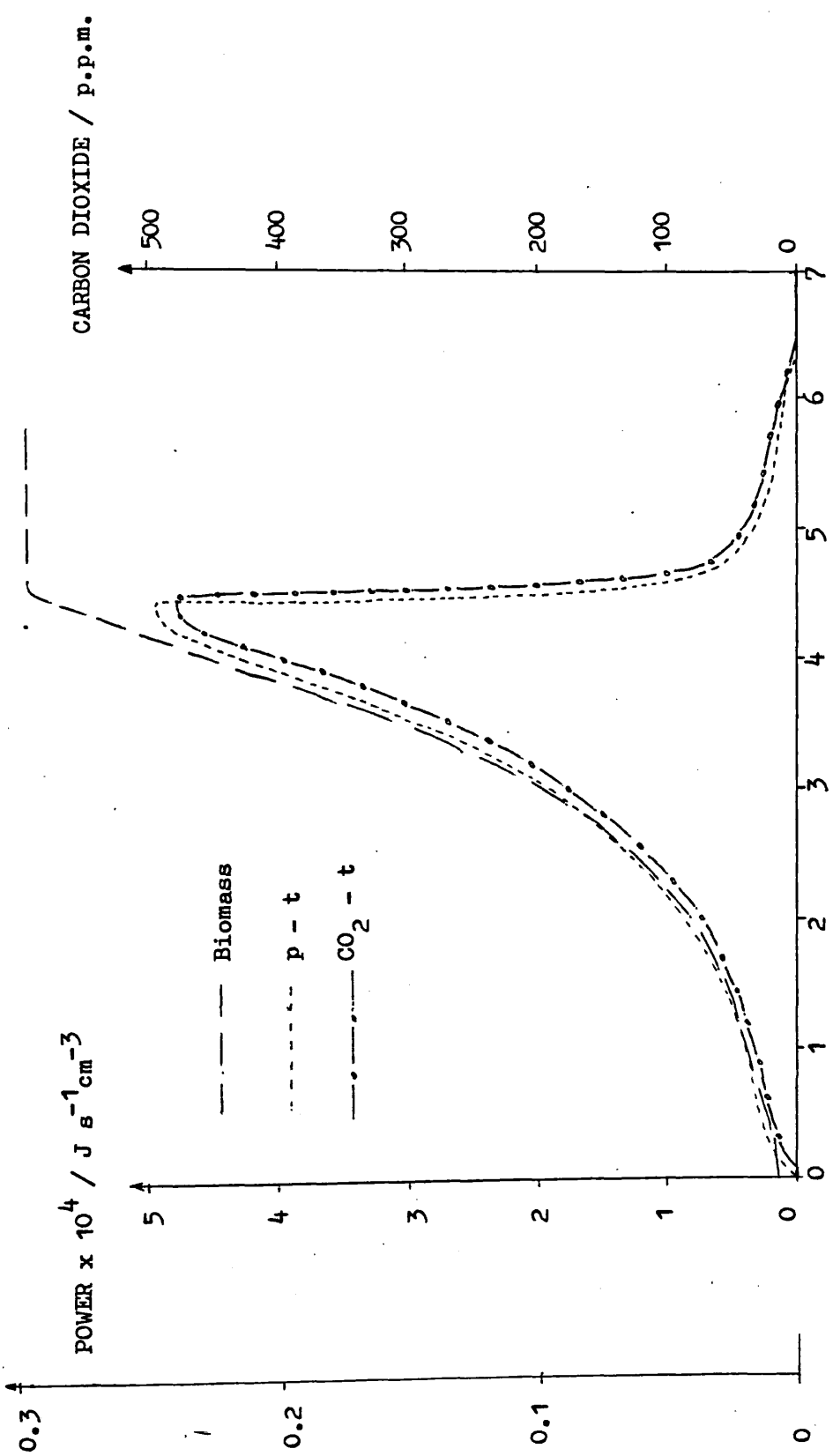
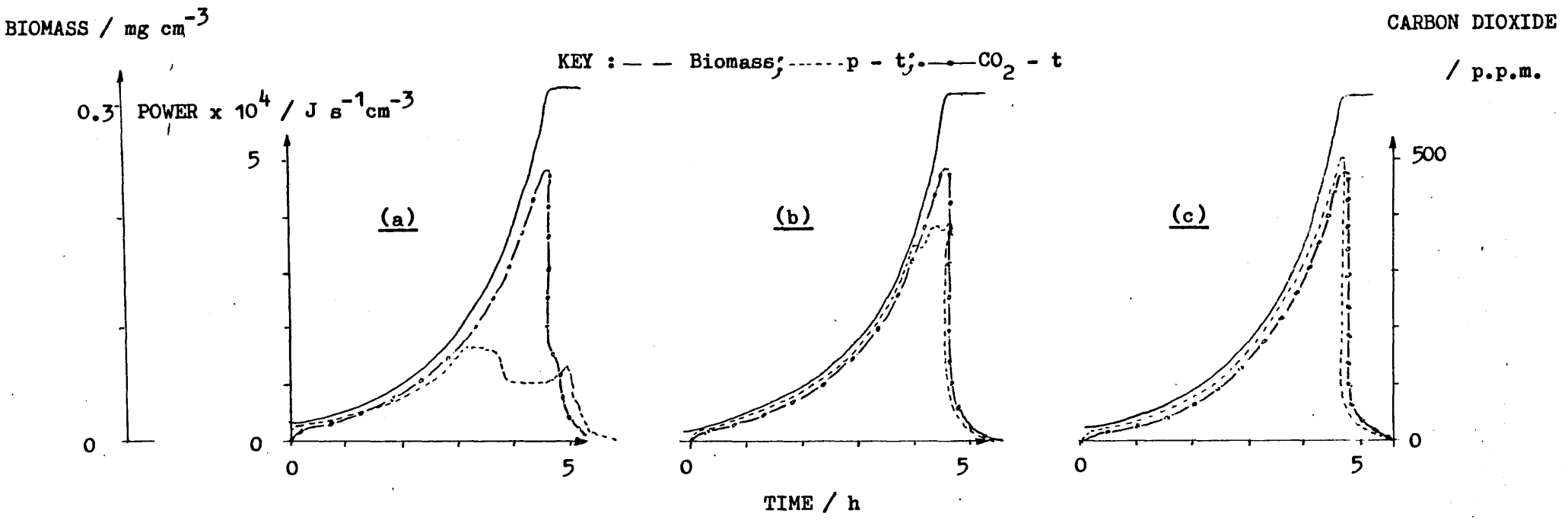


FIGURE 5.4 p - t and $CO_2$ - t Traces at Various Calorimeter Flow Rates in Glucose Limited Media

(Stirrer setting : 190)




| | | | | |
|---|---|---|---|---|
| Calorimeter flow | : | 20 cm$^3$ h$^{-1}$ | 40 cm$^3$ h$^{-1}$ | 90 cm$^3$ h$^{-1}$ |
| Min. $O_2$ tension in cal. line | : | 0 % | 5 % | 30 % |
| Total heat evolved | : | 1.118 KJ | 1.894 KJ | 1.903 KJ |

There are four factors which determine the oxygen tension encountered by the bacteria in the microcalorimeter cell and line;

(a) the rate of growth - the greater the growth rate, the greater will be the demand for oxygen per unit time;

(b) the density of the culture - the greater the biomass the greater will be the demand for oxygen per unit time;

(c) the rate of flow of the culture through cell and line - the faster the flow the greater will be the value of the minimum oxygen tension;

(d) stirring (aeration) of the bacterial culture in the culture vessel - the greater the aeration the greater will be the oxygen tension in the culture.

The $CO_2$-t trace is a measure of the change of $CO_2$ content in the effluent gas and is therefore an independent representation of the growth in the culture vessel. The shape of the $CO_2$-t trace (stirrer setting: 210) when the culture is not being pumped through the calorimeter is similar to that when it is being pumped through (Fig. 5.2). This proves that the secondary $CO_2$ output (and therefore power output) is not the result of pumping the culture through the calorimeter, but occurs in the culture vessel due to another factor. When the stirrer setting was reduced to 190 (flow rate = 90 $cm^3\ h^{-1}$) there was no secondary power or $CO_2$ output, and the p-t and $CO_2$-t traces were similar to those expected theoretically (Fig. 5.1). The minimum oxygen tension (end of exponential growth) was 30% saturation in the line and 90% in the culture vessel.

The secondary output (Fig. 5.2) occurred when the oxygen tension in the culture vessel at the end of the growth was in excess of 95%. This high value (at a stirrer speed of 210) appeared to be the cause of acetate formation and the subsequent secondary power and $CO_2$ outputs; this metabolic change at abnormally high oxygen tensions has not been reported before. Previous workers (Nichols, 1980) did not have the advantage of a $CO_2$-analyser which made it difficult to identify the cause of the secondary outputs.

Further, reducing the flow rate through the calorimeter did not result in an excessively large secondary $CO_2$ output (Fig. 5.4). This proves that there was not an accumulation of appreciable amounts of secondary metabolites in the culture vessel due to the anaerobic conditions encountered in the calorimeter. Even at extremely low flow rates (20 $cm^3\ h^{-1}$) the secondary $CO_2$ output was only just recognisable (Fig. 5.4 a); and barely significant (4% of the total $CO_2$ evolved). At this low flow rate the p-t trace bears no resemblance to the $CO_2$-t trace as anaerobic metabolism in the cell and line is prominent and is <u>not</u> representative of metabolism in the culture vessel. The total heat evolved was less as the <u>rate</u> of growth was slower in the flow line and cell due to the anaerobic conditions, although the total heat evolved per g of substance was greater for anaerobic than aerobic growth.

At a flow rate of 40 $cm^3\ h^{-1}$ (Fig. 5.4 b) the shape of the p-t trace is similar to that of the $CO_2$-t trace except during the later stages of exponential growth. During the early stage of growth when the number of bacterial cells and therefore oxygen requirement is relatively low, the time (3.5 min) during which the cells are subjected to a given low oxygen tension ( 30%) is not severe enough to cause a significant change to anaerobic growth in the cell. Hence the p-t trace is representative of the aerobic growth in the culture vessel. Towards the

end of growth the denser culture has a greater oxygen demand which results in a change to anaerobic growth in the cell and line; under these conditions the shape of the p-t trace is not similar to that of the $CO_2$-t trace. Hence the p-t trace towards the end of growth is not representative of the growth in the vessel.

At 90 $cm^3$ $h^{-1}$ (Fig. 5.4 c) the effect of oxygen starvation on the bacteria in the cell is insignificant (minimum oxygen tension :30%) and the shape of the p-t trace is similar to that of the $CO_2$-t trace. Hence the p-t trace is representative of the aerobic growth in the culture vessel.

## 5.3 The Standard Conditions

Considering factors discussed (5.2) the standard conditions of growth and measurement chosen were;

| | |
|---|---|
| Calorimeter flow rate | :90 $cm^3$ $h^{-1}$ |
| Stirrer setting | :190 (950 rpm) |
| Temperature | :37°C |
| Air flow | :1.5 $dm^3$ $min^{-1}$ |

in glucose-limited (3.3 mmol $dm^{-3}$) media. The p-t, $CO_2$-t traces and biomass curves for such growth have already been described (Figs 5.3 and 5.4 c). These chosen conditions were maintained for all subsequent work.

The standard conditions are only applicable to experiments in which the bacteria in the calorimeter cell are subjected to no less than 30% oxygen saturation for a duration of 1.5 minutes. This is controlled by the growth rate and total biomass.

A reliable indicator to the validity of the p-t trace is that it

must be similar in shape to that of the $CO_2$-t trace unless alternative and identified metabolic activity dictates otherwise, i.e. conversion of ethanol to acetate which releases heat but not $CO_2$.

## 5.4 Typical Energy and Mass Balance Calculations for Glucose-Limited Growth under Standard Conditions

This is a typical calculation of mass and energy balances for aerobic growth in glucose-limited medium. The following experimental data is logged for the growth;

(a) total heat evolved (area under p-t trace),

$\Delta H_T$ = 1.890 kJ (2.3.3);

(b) initial and final biomass value (2.2.1)

initial $m_i$ = 0.0118 mg $cm^{-3}$

final $m_f$ = 0.3090 mg $cm^{-3}$;

(c) total $CO_2$ evolved (area under $CO_2$-t trace)

= 5.351 x $10^{-3}$ mol (2.2.2);

(d) initial volume of growth media = 620 $cm^3$;

(e) initial concentration of glucose = 3.3 mmol $dm^{-3}$;

### 5.4.1 Mass Balance

The yield, $Y_{Gluc}$, is defined as the weight of cells formed per gram of glucose initially present.

Total weight of bacteria formed

$$= \frac{(0.3090 \times 620) - (0.0118 \times 620)}{1000}$$

$$= 0.1843 \text{ g}$$

Weight of glucose present initially

$$= \frac{180.16 \times 3.3 \times 620}{1000 \times 1000}$$

$$= 0.3686 \text{ g}$$

$$\therefore \quad Y_{Gluc} = \frac{0.1843}{0.3686} = 0.50 \text{ (g cell) } g^{-1}$$

The carbon content of bacteria

$$= 45.11\% \text{ (wt/wt) (Table 5.1)}$$

$\therefore$ Total amount of C stored in bacteria

$$= \frac{0.1843 \times 45.11}{100 \times 12}$$

$$= 6.928 \times 10^{-3} \text{ mol}$$

The C in $CO_2 = 5.351 \times 10^{-3}$ mol

Total carbon available initially = $12.277 \times 10^{-3}$ mol

Hence percentage carbon recovery, $C_{rec}$

$$= \frac{6.928 \times 10^{-3} + 5.351 \times 10^{-3}}{12.277 \times 10^{-3}} 100$$

$$= 100\%$$

TABLE 5.1 Elemental Composition of K. aerogenes Grown in Glucose Limited Media

| ELEMENT | CONTENT / % Wt./Wt. | 95% CONFIDENCE LIMITS |
|---|---|---|
| C | 45.11 | ± 1.5% |
| H | 6.77 | ± 2.0% |
| N | 12.51 | ± 1.6% |
| O | 25.73 | ± 5.1% |
| S | 1.06* | |
| P | 2.45* | |
| Residue | 6.40* | |

( *One determination only)

5.4.2 Energy Balance

Heat of combustion of glucose

$H_{Gluc} = -15.88 \text{ kJ } g^{-1}$

∴ Total energy available initially

$= 15.88 \times 0.3686$

$= 5.855 \text{ kJ}$

∴ Percentage waste heat

$$= \frac{H_T}{5.855} \times 100$$

$$= \frac{1.890}{5.855} \times 100$$

$$= 32.3\%$$

It is assumed that the oxidation state of carbon in cellular material is the same as that in glucose. This enables the energy stored in the bacteria formed to be calculated on the basis of the percentage of the initial carbon incorporated. This assumption is supported by the fact that there is no significant difference in the stored energy value when calculated from the heat of combustion of glucose trained cells (Nichols, 1980).

∴ Percentage energy stored in the bacteria formed

$$= \frac{\text{mol C in bacteria}}{\text{mol C present initially}} \times 100$$

$$= \frac{6.928 \times 10^{-3}}{12.277 \times 10^{-3}} \times 100$$

$$= 56.4\%$$

The energy required for biosynthesis (anabolic reactions), $H_B$, is defined as

$$H_B = \left|\begin{array}{l}\text{total energy}\\ \text{available}\\ \text{initially}\end{array}\right| - \left|\begin{array}{l}\text{total}\\ \text{energy}\\ \text{stored}\end{array}\right| + \left|\begin{array}{l}\text{total}\\ \text{waste}\\ \text{energy}\end{array}\right|$$

∴ Percentage energy required for byosynthesis, Δ%

$= 100 - (56.4 + 32.3)$

$= 11.3\%$.

5.4.3 Experimental Results

The stored energy and waste heat for 10 replicate experiments (Table 5.2) shows the mean values to be significantly different from those reported previously; stored : 49%, waste : 38%, biosynthesis : 13% (Nichols, 1980). These earlier results may have been due to less efficient conditions of growth (probably aeration) producing more waste heat and a lower yield (0.44 g $g^{-1}$) than those used in this work. The proportion of energy required for biosynthesis is not significantly different to that of Nichols and is similar to values reported by other workers; 9% for growth of E. coli on succinate (Dermoun and Belaich, 1979). The evaluation of an energy value for biosynthesis discounts earlier reports that it was too small to measure or smaller than the

experimental error (Forest, 1972). The reproducibility of the results (Table 5.2) is excellent considering they refer to a biological system involving data from several different techniques.

## 5.5 Effect of Aeration in the Line on the p-t Trace

One situation not considered so far is the measurement of the waste heat from a growing culture when the oxygen tension in the calorimeter cell and line is similar to that in the culture vessel. The only practical way to achieve this is to aerate the line and cell by feeding alternate segments of sterile air into the line with the culture. There are several factors to be considered:-

(a) the introduction of air into the line reduces the actual volume of culture contained in the cell. Recalibration of the effective cell volume for a given set of aeration conditions is required;

(b) the introduction of air into the line (i.d. : 2 mm) causes the formation of culture segments separated by air bubbles. The maintenance of a relatively high oxygen tension (80%) in the segments is dependent on the diffusion of oxygen from the air bubbles; this is a slow process. For this to succeed the segments must not be longer than 2 mm;

(c) the ratio of the relative volumes of air and liquid not only alters the effective cell volume but also the base line of the p-t trace.

The criteria which must be fulfilled for an accurate measurement of the waste heat from a growing culture, while aerating the line, are that the air to liquid ratio in the line is constant and that the liquid

TABLE 5.2 Energy and Mass Balances for Growth in Glucose Limited Media (3.3 mmol $dm^{-3}$)

| | TOTAL HEAT EVOLVED $\Delta H_T$ /kJ | YIELD /g $g^{-1}$ | TOTAL $CO_2$ EVOLVED /mol x $10^3$ | % CARBON RECOVERY $C_{rec}$ | % ENERGY STORED | % ENERGY WASTED | % ENERGY FOR BIOSYNTHESIS $\Delta$% |
|---|---|---|---|---|---|---|---|
| | 1.877 | 0.49 | 5.597 | 101 | 55.33 | 32.0 | 12.7 |
| | 1.930 | 0.49 | 5.692 | 102 | 55.3 | 33.0 | 11.7 |
| | 1.868 | 0.48 | 5.626 | 100 | 54.2 | 31.9 | 13.9 |
| | 1.879 | 0.49 | 5.288 | 98 | 55.3 | 32.1 | 12.6 |
| | 1.918 | 0.48 | 5.419 | 98 | 54.2 | 32.7 | 13.1 |
| | 1.894 | 0.49 | 5.156 | 97 | 55.3 | 32.3 | 12.4 |
| | 1.901 | 0.50 | 5.549 | 102 | 56.4 | 32.5 | 11.1 |
| | 1.857 | 0.50 | 5.314 | 100 | 56.4 | 31.7 | 11.9 |
| | 1.911 | 0.50 | 5.556 | 102 | 56.4 | 32.6 | 11.0 |
| | 1.869 | 0.40 | 5.357 | 99 | 55.3 | 31.9 | 12.8 |
| Mean $\bar{x}$ ± 95% confidence limits | 1.890 ±1% | 0.49 ±1% | 5.546 ±2.2% | 100 ±1.3% | 55.41 ±1% | 32.27 ±1% | 12.32 ±1% |

segments are ≤2 mm.

In practice the attainment of segments of 2 mm in length was possible, but maintenance of a constant air to liquid ratio was not. To obtain segments of 2 mm the liquid:air ratio was 1:4. The air was either pumped into the culture (which was already in the line) using a peristaltic pump or drawn in by the vacuum in the line via a glass capillary. The geometry of the junction where the liquid and air mix is critical to the continued formation of segments of the correct length. An oxygen tension of 80% saturation (as measured by the oxygen electrode in the line, Fig. 2.4) could be maintained during growth although the liquid/air ratio was not stable.

The reproducibility of the measured total heat was poor (±12%, $p = 0.95$) due to the instability of the liquid/air ratio which caused cell volume and base line fluctuations. The mean value of several measurements was not significantly different to that for a non-aerated line (Table 5.2).

For this work an aerated line is not required, but if in subsequent work a higher substrate concentration is used resulting in a higher biomass and oxygen demand aeration in the line will be required. It will then be necessary to investigate the problems encountered in more detail.

## 5.6 Summary

1. The secondary heat and carbon dioxide outputs are due to abnormally high oxygen tensions in the culture vessel and not to the effect of pumping the culture through the calorimeter.

2. Standard conditions of growth and measurement produce a p-t trace

representative of the growth in the culture vessel.

3. The optimised standard conditions result in more efficient growth so the measured waste heat and yield in glucose-limited medium are therefore significantly different from previous workers. The energy required for biosynthesis is similar to that reported earlier.

4. Aeration of the flow line did not seriously effect the value of the measured waste heat.

# CHAPTER SIX

# EXPERIMENTAL RESULTS

## SPECIFIC HEAT OUTPUT AND ATP PROFILES FOR EXPONENTIALLY GROWING BACTERIA IN GLUCOSE-LIMITED MEDIA

## 6.1 The Significance of the Specific Heat Output

The conversion of the calorimeter trace to the total heat evolved ($\Delta H_T$/J) and the rate of heat evolution (power, p/J $s^{-1}$ $cm^{-3}$) during bacterial growth is familiar (2.3.3). The bacterial ATP content as calculated (3.1 and 3.2) is an instantaneous one, as it is based on instantaneously measured values of the total ATP content of a suspension and the bacterial mass. If the heat evolution from a bacterial suspension is to be compared with the calculated ATP content then it must also be instantaneous in nature, i.e. the specific power, P/J $s^{-1}$ $g^{-1}$ defined as;

$$\frac{\text{instantaneous power output at time t}}{\text{biomass at time t}} = \frac{p}{m} \qquad 6.1$$

The biomass at time t is calculated from the absorbance measurement (2.2.1); this value is used in the calculation of the bacterial ATP content.

Since the power and biomass of the growing bacterial suspension are measured continuously (2.2.1 and 2.3) a value of the specific power can be calculated at regular intervals during growth. The interval chosen was 10 minutes.

## 6.2 p-t and P-t Traces and ATP Profiles During Aerobic Growth in Glucose-Limited Medium

The power output, biomass and $CO_2$ evolution were monitored continuously (2.2.1 and 2.3) during growth in glucose-limited (3.3 mmol $dm^{-3}$) medium under standard conditions.

The specific power-time (P-t) traces and ATP profiles are very similar (Figs 6.1, 6.2, 6.3). The shape of the p-t trace and the

## Legends for Figures

FIGURES 6.1, 6.2, 6.3,

p - t and P - t Traces, and ATP Profiles for Growth in Glucose - Limited Medium

KEY ;

| | | |
|---|---|---|
| — — —Biomass | / mg $cm^{-3}$ | (m) |
| -------- Power x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| •——•——• Specific Power | / J $s^{-1}g^{-1}$ | (P) |
| ———— ATP Profile | / ($10^6$ x mole ATP) $g^{-1}$ | ATP |

(By defn. P = p / m )

FIGURE 6.4

( As above at Faster Stirrer Speed )

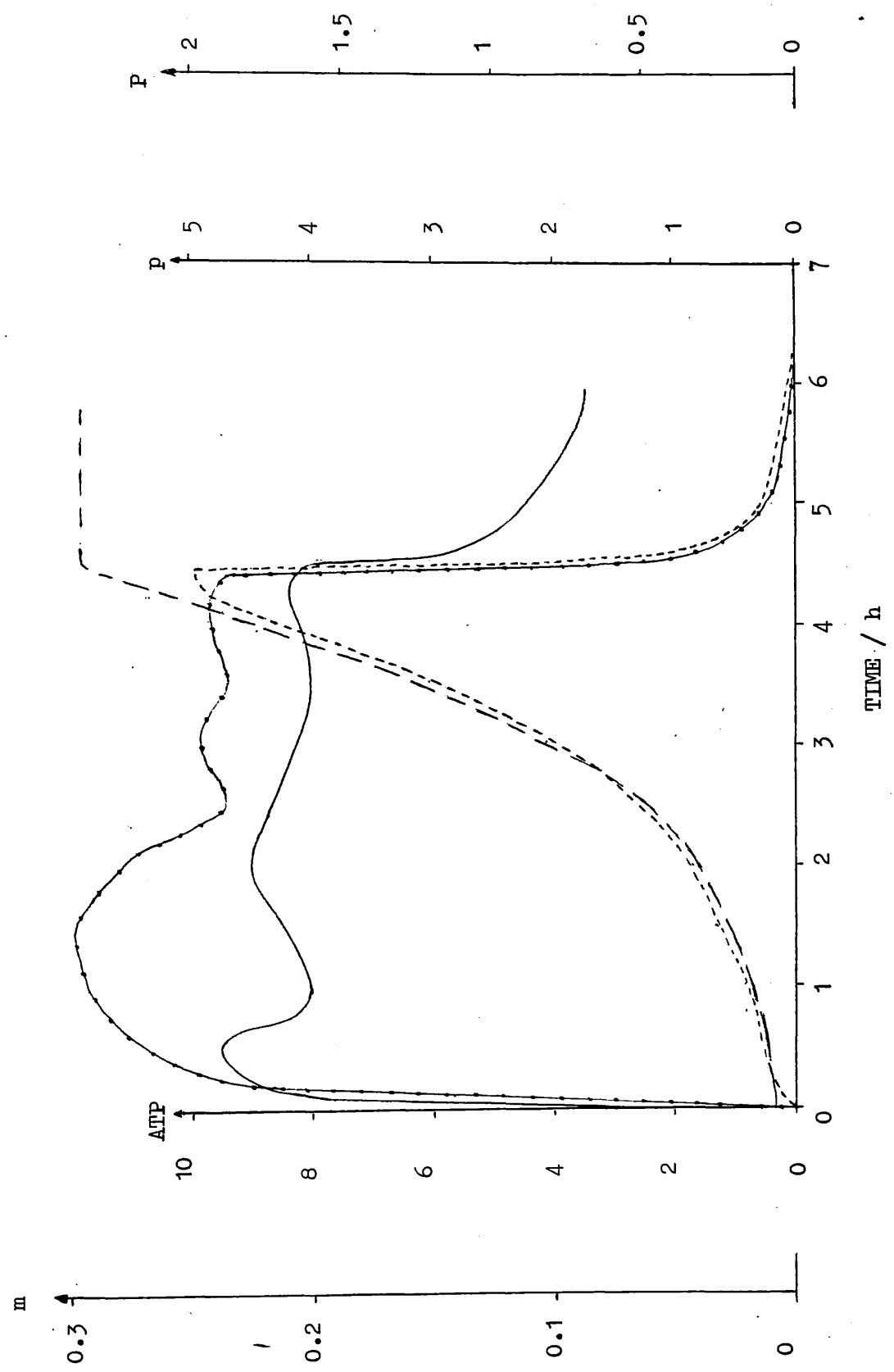



FIGURE 6.1

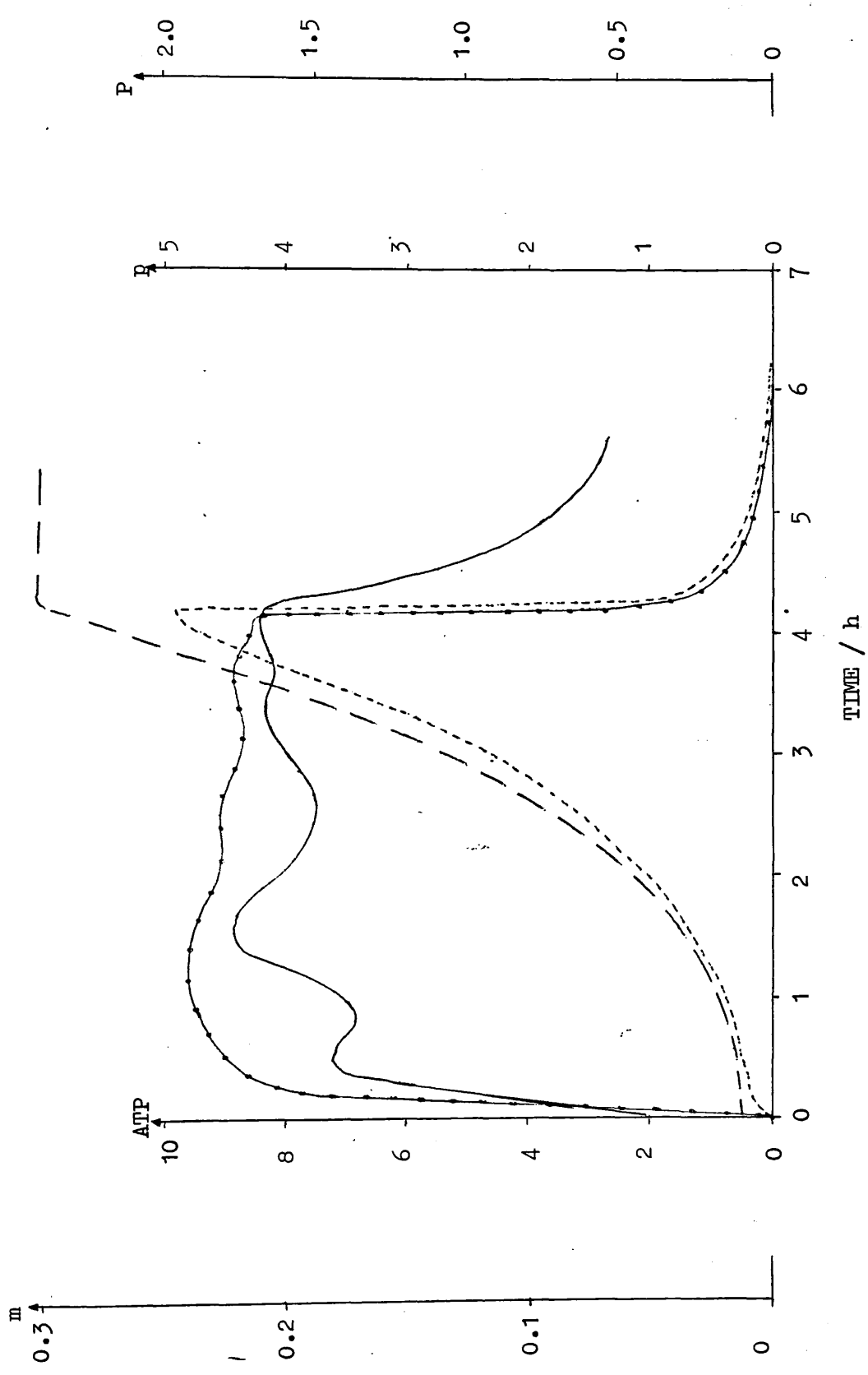



FIGURE 6.2

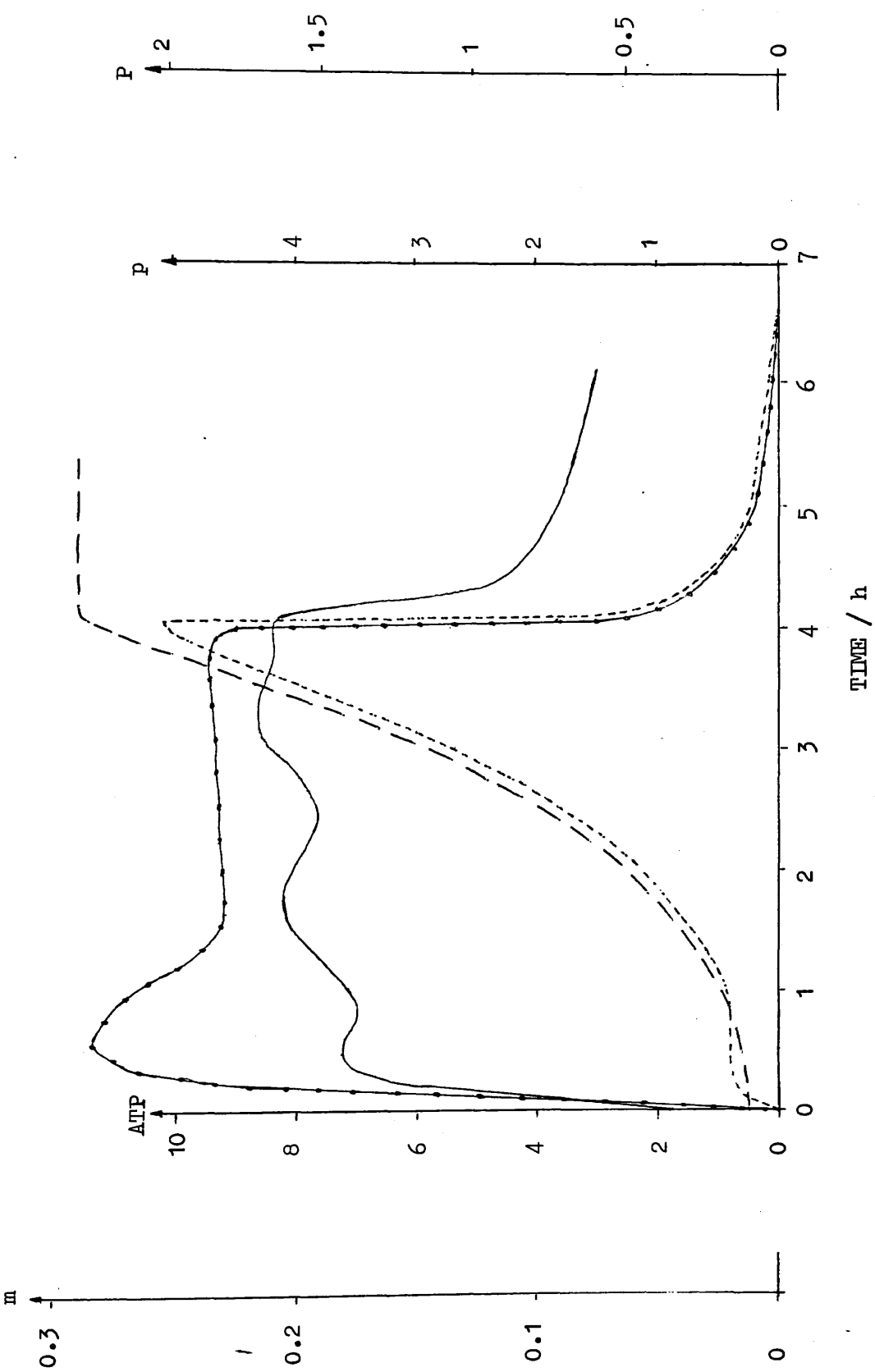



FIGURE 6.3

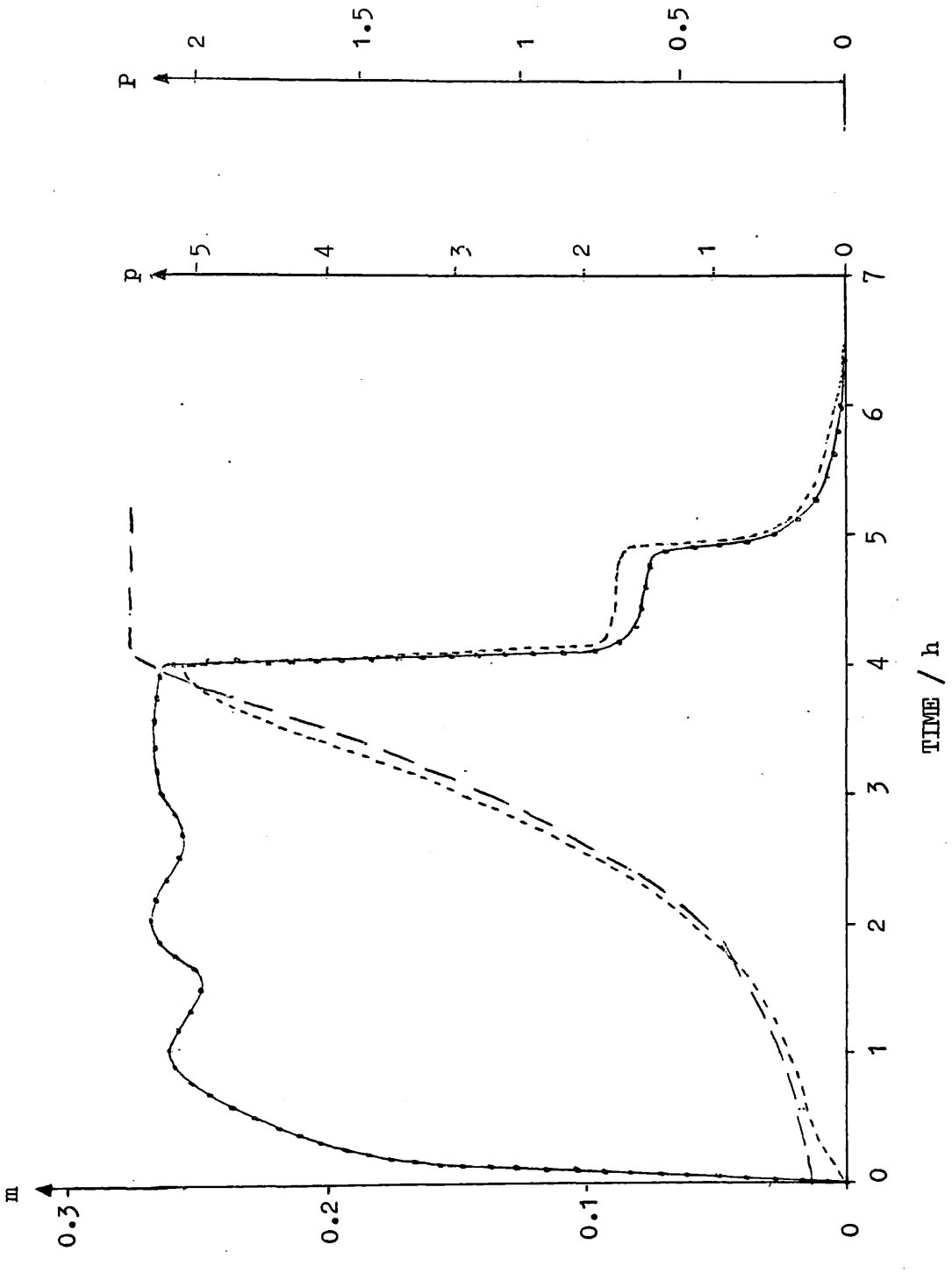



FIGURE 6.4

calculated values of $\Delta H_T$, $Y_{Gluc}$ and $t_d$ for each growth are similar to those obtained for cells growing under standard conditions (Figs 5.3, 5.4 c). Further the shape of the $CO_2$-t traces (not shown) mimics that (i.e. follows closely) that of the p-t traces. Following inoculation there was a rapid rise in the bacteria ATP to the usual level of c. $8 \times 10^{-6}$ mol $g^{-1}$ (4.1). This was accompanied by a rapid rise in the specific power to a variable level during the earlier stages of exponential growth (1.9-2.4 J $s^{-1}$ $g^{-1}$). In the later stages of exponential growth the specific power was more constant (1.8-1.95 J $s^{-1}$ $g^{-1}$) and there was evidence of oscillations similar to that of ATP. These oscillations however tended to be in opposition to (out of phase with) the oscillations exhibited by ATP. On cessation of growth with the complete consumption of the glucose there was a rapid drop in both the ATP and P to values similar to those of the inoculum.

At faster stirrer speeds (1200 rpm) the p-t and P-t traces were different (Fig. 6.4) to those obtained under standard conditions of growth. This was due to the presence of acetate, produced during glucose metabolism. During exponential growth the specific power was constant at a higher value of 2.1 J $s^{-1}$ $g^{-1}$, lowering during acetate metabolism to a value of c. 0.6 J $s^{-1}$ $g^{-1}$. On complete metabolism of the acetate P decreases to a value similar to that of the cells of the inoculum. The $CO_2$-t trace again mimics that of the p-t trace, also showing a short region of low but constant output after growth had ceased.

## 6.3 Summary

1. The ATP profiles and P-t traces are closely similar; there is a marked increase in value of ATP and P during the early stages of growth, steady values during the later stages, and rapid decrease on cessation of growth.

2. The ATP profiles and P-t traces show oscillations about mean values; the ATP oscillations tending to be out of phase with the P-t oscillations.

# CHAPTER SEVEN

# EXPERIMENTAL RESULTS

## ATP, ENERGY AND MASS BALANCE FOR GROWTH IN PHOSPHATE-LIMITED MEDIA

## 7.1 Selection of Suitable Phosphate Concentrations

Cells maintained repeatedly in glucose-limited media were grown in phosphate-limited medium contained in boiling tubes (2.1.3). The initial concentration of phosphate in each was in the range 0-12 x $10^{-3}$ g $dm^{-3}$ but the initial glucose concentration was constant (3.3 mmol $dm^{-3}$). The absorbance of each culture after cessation of growth was recorded (Fig. 7.1) to determine the maximum phosphate concentration which maintained phosphate-limited growth, this concentration was 6.15 x $10^{-3}$ g $dm^{-3}$.

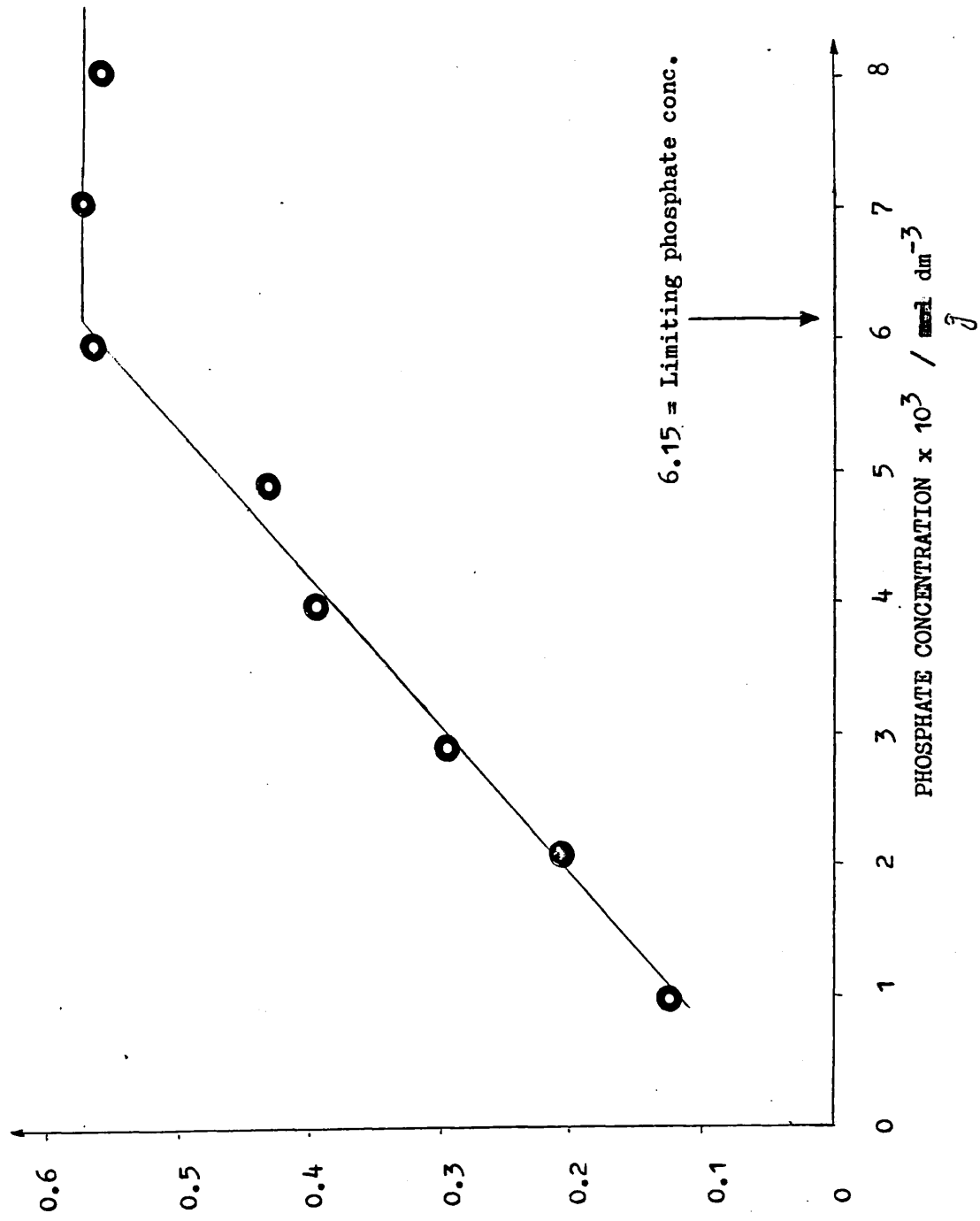
Two phosphate concentrations (2.5 and 5.5 x $10^{-3}$ g $dm^{-3}$) were chosen for training and subsequent study. When cells were grown in phosphate-limited media (initial glucose conc. :3.30 mmol $dm^{-3}$) containing phosphate at an initial concentration of 2.5 or 5.5 x $10^{-3}$ g $dm^{-3}$ then phosphate-limited growth resulted. Further at the phosphate concentration of 2.5 x $10^{-3}$ g $dm^{-3}$ the phosphate in the medium was exhausted when approximately 40% of the glucose had been consumed, and at the concentration of 5.5 x $10^{-3}$ g $dm^{-3}$ the phosphate was exhausted when approximately 90% of the glucose had been consumed.

Cells were adapted to phosphate-limitation by subculturing (20 times) in the appropriate phosphate-limited medium. Cryogenically stored cells, stored in phosphate-limited salts medium (2.1.2 c), were prepared from 640 $cm^3$ cultures grown in media of the relevant phosphate concentration.

### 7.1.1 Elemental Composition of Phosphate Trained Cells

Elemental analysis of the cells (2.6.1) grown in phosphate-limited media, (glucose conc. : 3.3 mmol $dm^{-3}$, phosphate conc. : 2.5 or 5.5 x $10^{-3}$ g $dm^{-3}$), revealed the following composition:

FIGURE 7.1 Absorbance after Cessation of Growth versus Initial Phosphate Concentration



| Element | Content /%wt/wt | 95% Confidence Limits |
|---|---|---|
| C | 45.20 | ±1.5% |
| H | 6.81 | ±2.0% |
| N | 12.64 | ±1.6% |
| O | 26.82 | ±5.1% |
| S | 1.07* | |
| P | 0.85* | |
| Residue | 5.21* | |

(* : One determination only)

The results are not significantly different to those of cells grown in glucose-limited media (5.5).

## 7.2 Growth of Phosphate-Limited Cells ($2.5 \times 10^{-3}$ g $dm^{-3}$) in Phosphate-Limited Medium (Glucose conc. : 3.3 mmol $dm^{-3}$)

Cultures were grown aerobically under the standard conditions of growth and measurement (5.3). The p-t and P-t traces for one such growth (Fig. 7.2) were more complex than those for growth in glucose-limited medium (Fig. 5.3). Enzymic (2.4) and chemical (2.5) assays of the medium during growth revealed three distinctly different growth sections; A, B and C (Fig. 7.3).

### Section A

During this 2.5-3.0 h period extracellular phosphate was present in the medium. The initial phosphate present in the medium at (a) was

## Legend for Figure 7.2

FIGURE 7.2

p - t and P - t Traces and Changes of Growth Parameters in Phosphate Limited ($2.5 \times 10^{-3}$ g $dm^{-3}$) Medium

KEY ;

| | | | |
|---|---|---|---|
| — — — | Log Biomass | / mg $dm^{-3}$ | (LOG M) |
| — • — • — | Carbon Dioxide | / p.p.m. | ($CO_2$) |
| - - - - - - - - - - | Power x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| ———— | Specific Power | / J $s^{-1}g^{-1}$ | (P) |

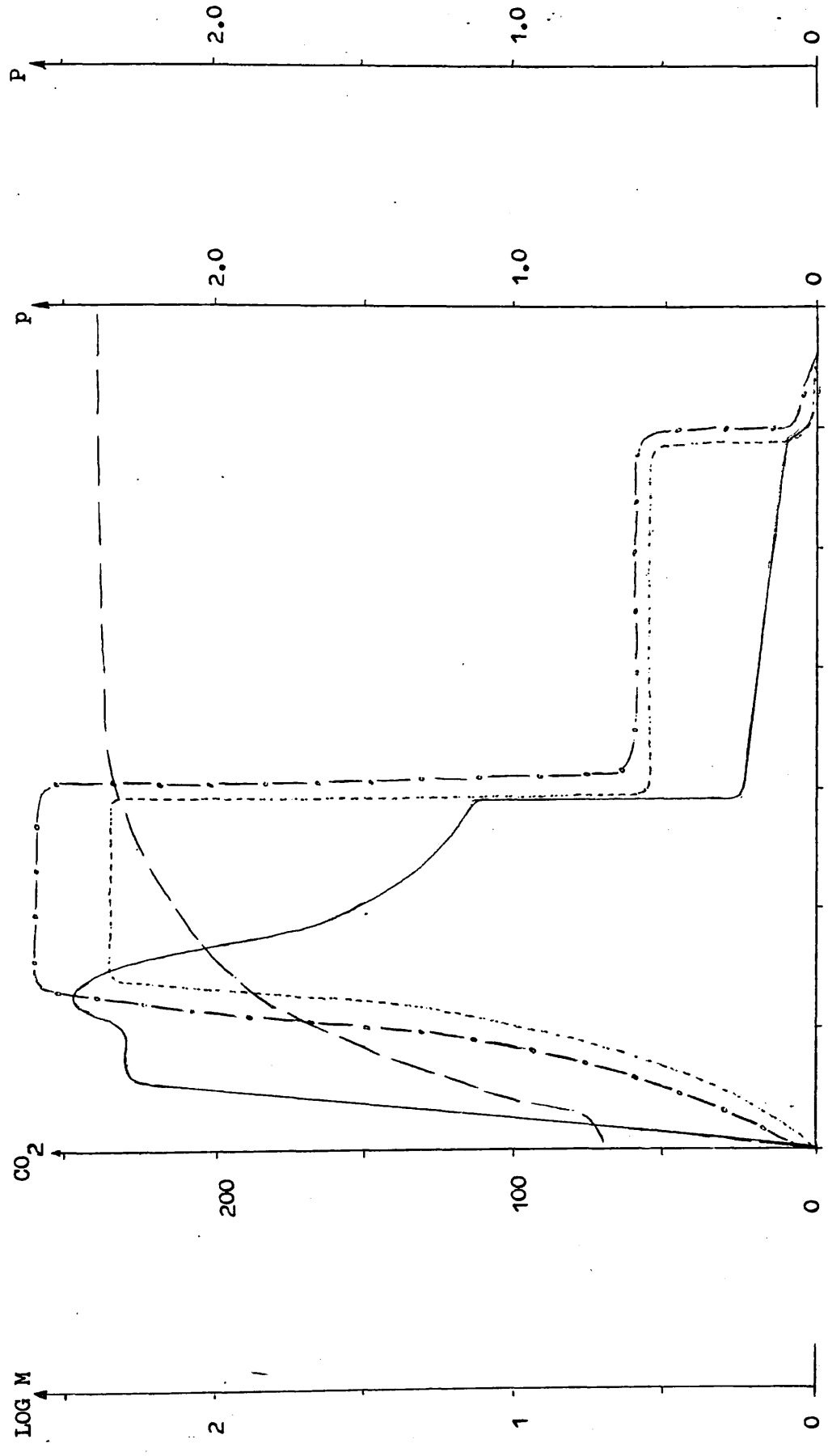



FIGURE 7.2

## Legend for Figure 7.3

FIGURE 7.3

Phosphate Limited ($2.5 \times 10^{-3}$) Growth

KEY ;

— — — Log Biomass / mg $dm^{-3}$ (LOG M)

— • — • — Carbon Dioxide / p.p.m. ($CO_2$)

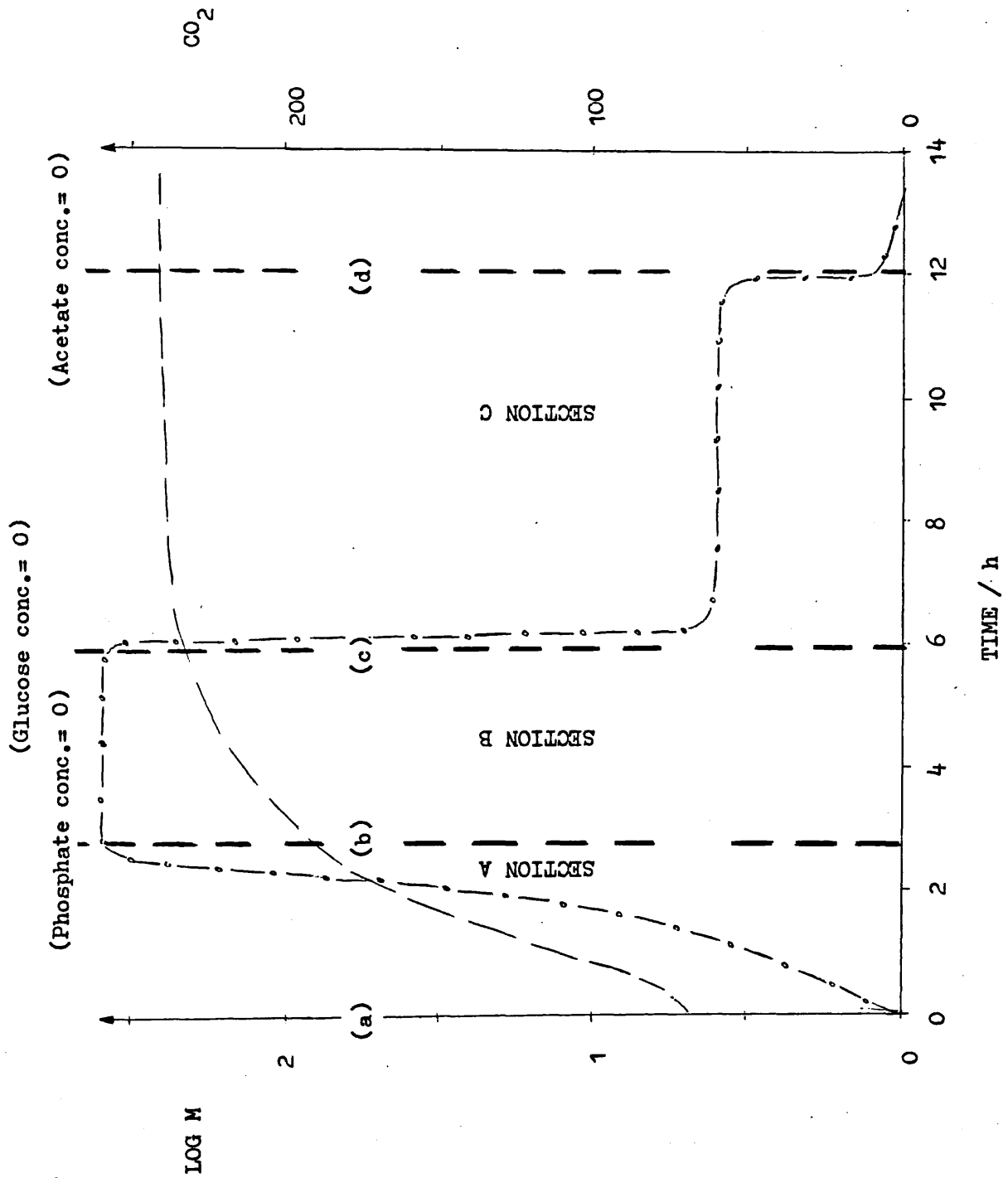

$CO_2$
200
100
0
14
12
10
8
6
4
2
0
TIME / h
(Acetate conc.= 0)
(Glucose conc.= 0)
(Phosphate conc.= 0)
(a)
(b)
(c)
(d)
SECTION A
SECTION B
SECTION C
2
1
0
LOG M

FIGURE 7.3

consumed during growth until it was exhausted at (b). During this period some of the initial glucose was converted to acetate (conc. at (b) c. 0.7 mmol $dm^{-3}$), as well as to biomass and carbon dioxide.

The specific power output increased to a value of 2.35 J $s^{-1}$ $g^{-1}$ when it was fairly constant for a short period before starting to decrease after exhaustion of the phosphate (Fig. 7.2), and the p-t and $CO_2$-t traces increased in parallel. During this period growth was exponential with a doubling time of about 35 minutes, towards the end however the doubling time increased with the near exhaustion of the phosphate.

Section B

During this 3-3.5 h period extracellular phosphate was not present in the medium. Some of the residual glucose, c. 2.10 mmol $dm^{-3}$ at (b), was converted to acetate, conc. at (c) : c. 2.10 mmol $dm^{-3}$, the remainder appearing as biomass and carbon dioxide. The concentration of glucose at (c) was zero.

The power and $CO_2$ outputs were constant while the specific power decreased gradually due to the increase in biomass. The rate of production of biomass decreased throughout this period.

Section C

During this 6 h period the acetate formed earlier was converted to carbon dioxide and a small amount of biomass (Fig. 7.3).

The power and $CO_2$ outputs fell dramatically at (c) to constant levels. These were accompanied by an abrupt decrease in the specific power output followed by a gradual decrease during the latter part of this period (Fig. 7.2). At (d) when the acetate concentration was zero the p-t, P-t and $CO_2$-t traces reattained their respective base lines.

Other secondary metabolites (acetaldehyde, succinate, lactate, ethanol) were not detected in the medium during growth. More frequent sampling during section B, for acetate analysis, showed the rate of formation of acetate to be similar to that of the biomass (Fig. 7.4).

In a subsequent experiment it was established that growth occurred in phosphate-limited medium containing acetate as the sole carbon source, (30% increase in the biomass when the initial concentration of acetate was 3.3 mmol $dm^{-3}$). In the absence of any carbon source (glucose or acetate) there was no growth.

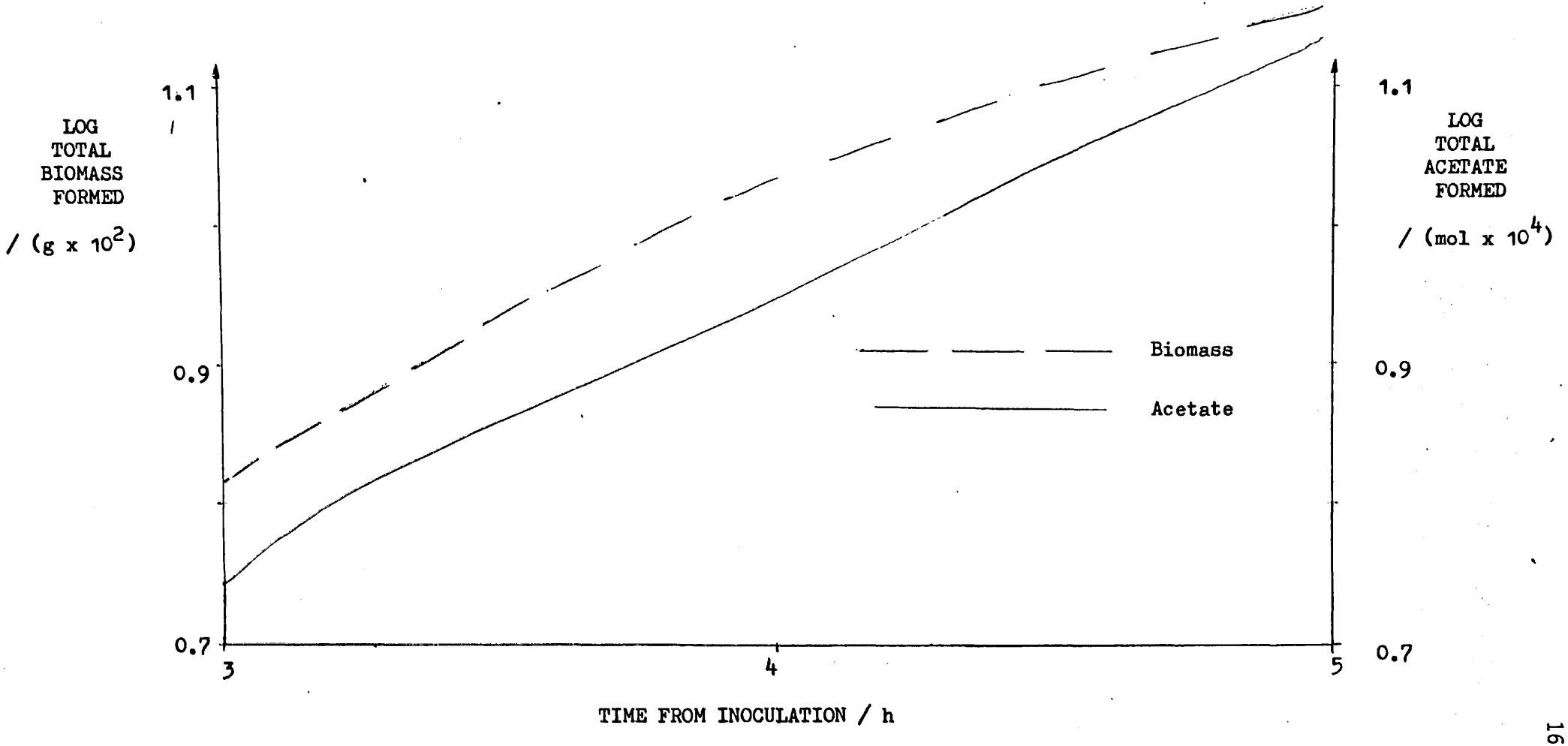
### 7.2.1 Cellular ATP Content

During growth in phosphate-limited media the ATP profile was parallel to the P-t trace (Fig. 7.5). The ATP content after inoculation increased to a near constant value (c. 10 x $10^{-6}$ mol $g^{-1}$) which was maintained while the extracellular phosphate was present in the medium (Section A). Once the phosphate was exhausted the ATP level decreased gradually (Section B) until a value of c. 2 x $10^{-6}$ mol $g^{-1}$ was reached upon commencement of acetate metabolism (Section C). During acetate metabolism a constant ATP level was maintained (c. 2 x $10^{-6}$ mol $g^{-1}$) which fell rapidly after the exhaustion of the acetate.

### 7.2.2 Energy and Mass Balances - A Worked Example

The balance calculations are based on the division of the growth into 3 sections (Fig. 7.3). The calculations utilise the experimental data for "GROWTH ONE" and the results are shown in Tables 7.1 and 7.2. The notation used refers to that in Fig. 7.3.

FIGURE 7.4 Acetate Formation during Section B of Phosphate Limited ($2.5 \times 10^{-3}$ g $dm^{-3}$) Growth



## Legend for Figure 7.5

FIGURE 7.5

ATP Profile and P - t , p - t Traces for Growth in Phosphate Limited ($2.5 \times 10^{-3}$ g $dm^{-3}$) Medium

KEY ;

| | | |
|---|---|---|
| — — — Biomass | / mg $dm^{-3}$ | (LOG M) |
| ———— ATP | / (mol $\times 10^{6}$) $g^{-1}$ | (ATP) |
| - - - - - - - - Power $\times 10^{4}$ | / J $s^{-1}$ $cm^{-3}$ | (p) |
| —•—•—•— Specific Power | / J $s^{-1}$ $g^{-1}$ | (P) |

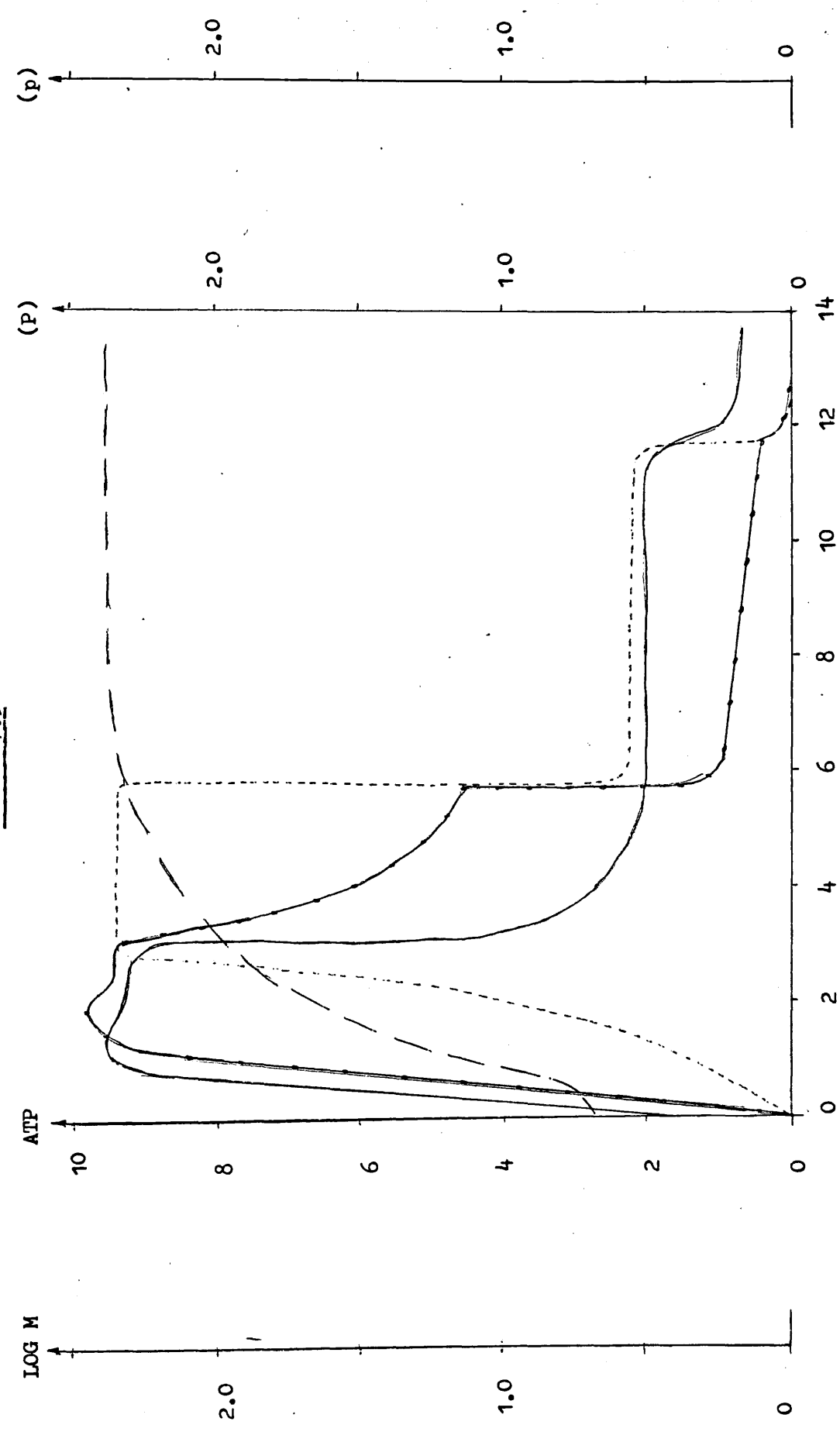



FIGURE 7.5

TABLE 7.1 Mass Balance Data for Three Growths in Phosphate Limited ($2.5 \times 10^{-3}$ g $dm^{-3}$) Media

| DESCRIPTION OF DATA | GROWTH ONE | GROWTH TWO | GROWTH THREE |
|---|---|---|---|
| **SECTION A** | | | |
| % of metabolised C stored as acetate | 19 | 26 | 23 |
| % " " C " " biomass | 45 | 46 | 44 |
| % " " C incorporated in $CO_2$ | 36 | 32 | 36 |
| % " " C recovered, $C_{rec}$ | 100 | 104 | 103 |
| **SECTION B** | | | |
| % of metabolised C stored as acetate | 22 | 20 | 20 |
| % " " C " " biomass | 40 | 42 | 43 |
| % " " C incorporated in $CO_2$ | 40 | 40 | 39 |
| % " " C recovered, $C_{rec}$ | 102 | 102 | 102 |
| **SECTION C** | | | |
| % of metabolised C stored as acetate | 0 | 0 | 0 |
| % " " C " " biomass | 32 | 30 | 29 |
| % " " C incorporated in $CO_2$ | 71 | 72 | 75 |
| % " " C recovered, Crec | 103 | 102 | 104 |
| **OVERALL** | | | |
| Yield / g $g^{-1}$ | 0.431 | 0.444 | 0.432 |
| % of metabolised C stored as biomass | 48.8 | 50.2 | 48.9 |
| % " " C incorporated in $CO_2$ | 50.5 | 52.8 | 52.5 |
| % " " C recovered, $C_{rec}$ | 99 | 103 | 101 |

TABLE 7.2 Energy Balance Data for Three Growths in Phosphate Limited ($2.5 \times 10^{-3}$ g $dm^{-3}$) Media

| DESCRIPTION OF DATA | GROWTH ONE | GROWTH TWO | GROWTH THREE |
|---|---|---|---|
| **SECTION A** | | | |
| Total waste heat / kJ | 0.560 | 0.555 | 0.556 |
| % of metabolised energy wasted | 25 | 23 | 24 |
| % " " " stored as acetate | 17 | 23 | 20 |
| % " " " " " biomass | 45 | 46 | 44 |
| % " " " required for biosyn. | 13 | 8 | 12 |
| **SECTION B** | | | |
| Total waste heat / kJ | 1.484 | 1.340 | 1.421 |
| % of metabolised energy wasted | 39 | 39 | 38 |
| % " " " stored as acetate | 20 | 18 | 18 |
| % " " " " " biomass | 40 | 42 | 43 |
| % " " " required for biosyn. | 1 | 1 | 1 |
| **SECTION C** | | | |
| Total waste heat / kJ | 0.815 | 0.845 | 0.902 |
| % of metabolised energy wasted | 72 | 71 | 77 |
| % " " " stored as acetate | 0 | 0 | 0 |
| % " " " " " biomass | 32 | 31 | 28 |
| % " " " required for biosyn. | -4 | -2 | -5 |
| **OVERALL** | | | |
| Total waste heat / kJ | 2.859 | 2.740 | 2.879 |
| % of metabolised energy wasted (WASTE) | 47.3 | 46.3 | 47.7 |
| % " " " stored as biomass (STORED) | 48.8 | 50.2 | 48.9 |
| % " " " required for biosyn. (BIOSYNTHESIS) | 3.9 | 3.5 | 3.4 |

## Section A

### Mass Balance

During this section some of the glucose present initially at (a) was metabolised to acetate, bacterial mass and carbon dioxide.

(i) Glucose

Total original carbon available in 640 $cm^3$ of culture given that the initial glucose concentration at (a) was 3.30 mmol $dm^{-3}$

$= 12.672 \times 10^{-3}$ mole

The concentration of glucose at (b) was 2.075 mmol $dm^{-3}$ which is equivalent to $7.969 \times 10^{-3}$ mole carbon.

Hence amount of carbon metabolised

$= (12.672 - 7.969) \times 10^{-3}$

$= 4.703 \times 10^{-3}$ mole

Thus amount of original carbon metabolised

$$= \frac{4.703 \times 10^{-3}}{12.672 \times 10^{-3}} \times 100$$

$= 37.11$

$= \underline{37\%}$

(ii) Acetate

Concentration of acetate at (b) was 0.711 mmol $dm^{-3}$. This is equivalent to $0.455 \times 10^{-3}$ mole of acetate or $0.909 \times 10^{-3}$ mole of carbon present as acetate in the medium (640 $cm^3$) at (b).

Hence amount of metabolised carbon ($4.703 \times 10^{-3}$ mole) incorporated in acetate

$$= \frac{0.909 \times 10^{-3}}{4.703 \times 10^{-3}} \times 100$$

$$= \underline{19\%}$$

(iii) Biomass

Incremental increase in the biomass was 0.0878 mg $cm^{-3}$. This is equivalent to

$$\frac{0.0878 \times 640 \times 45.20}{1000 \times 100 \times 12}$$

$= 2.117 \times 10^{-3}$ mole of carbon

(where carbon content of cells = 45.20% wt/wt)

Hence amount of metabolised carbon ($4.703 \times 10^{-3}$ mole) incorporated in biomass

$$= \frac{2.117 \times 10^{-3}}{4.703 \times 10^{-3}} \times 100$$

$$= \underline{45\%}$$

(iv) Carbon Dioxide

Carbon dioxide evolved was $1.670 \times 10^{-3}$ mole.

Hence amount of metabolised carbon ($4.703 \times 10^{-3}$ mole) incorporated in carbon dioxide

$$= \frac{1.670 \times 10^{-3}}{4.703 \times 10^{-3}} \times 100$$

$$= \underline{36\%}$$

(v) Carbon Recovery

Hence amount of metabolised carbon ($4.703 \times 10^{-3}$ mole) recovered/accounted for ($C_{rec}$) as acetate, biomass and carbon dioxide formed

$$= (19 + 45 + 36)$$

$$= \underline{100\%}$$

Energy Balance

Total energy available from the glucose (3.30 mmol $dm^{-3}$) initially present in the medium (640 $cm^3$)

$$= 6.042 \text{ kJ } (5.4)$$

Since 37.11% of the initial substrate was consumed during this period then available energy (provided by metabolism) from this source

$$= 6.042 \times 0.3711$$

$$= 2.242 \text{ kJ}$$

(i) Waste

Total measured heat evolved from the medium (640 $cm^3$)

$$= 0.560 \text{ kJ}$$

Hence amount of energy provided by metabolism (2.242 kJ) wasted as heat

$$= \frac{0.560}{2.242} \times 100$$

$$= \underline{25\%}$$

(ii) Acetate

Amount of acetate formed

$= 0.455 \times 10^{-3}$ mole

Hence the energy stored as acetate

$= 0.455 \times 10^{-3} \times 840$

$= 0.382$ kJ

(Assuming $\Delta H_{comb}$ at 37°C of acetate, soln. = 840 kJ $mol^{-1}$)

Hence amount of energy provided by metabolism (2.242 kJ) stored as acetate

$$= \frac{0.383}{2.242} \times 100$$

$= \underline{17\%}$

(iii) Biomass

Since 45% of the metabolised carbon was incorporated in biomass then the percentage of the energy provided by metabolism stored as biomass is 45 (5.4).

(iv) Biosynthesis

The amount of energy provided by metabolism required for biosynthesis (5.4) during this section

$= 100 - (25 + 17 + 45)$

$\Delta\%_A = \underline{13\%}$

Section B

During this section the remainder of the glucose at (b) was

converted to acetate, biomass and carbon dioxide. The calculations are similar to those of section A.

Mass Balance

(i) Glucose

The concentration of glucose at (b) = 2.075 mmol $dm^{-3}$ and at (c) = zero

Hence the total carbon metabolised during this section

$$= \frac{2.075 \times 10^{-3} \times 640 \times 6}{1000}$$

$$= 7.968 \times 10^{-3} \text{ mole}$$

(ii) Acetate

The concentration of acetate at (c) = 2.103 mmol $dm^{-3}$.

Thus the incremental increase in acetate concentration

= 2.103 - 0.711 (i.e. conc. at (b))

= 1.392 mmol $dm^{-3}$

Thus amount of acetate formed

$$= \frac{1.392 \times 10^{-3} \times 640}{1000}$$

$$= 0.891 \times 10^{-3} \text{ mole}$$

Hence amount of carbon incorporated in acetate

$= 0.891 \times 10^{-3} \times 2$

$= 1.782 \times 10^{-3}$

Hence amount of metabolised carbon ($7.968 \times 10^{-3}$ mole) incorporated in acetate

$$= \frac{1.782 \times 10^{-3}}{7.968 \times 10^{-3}} \times 100$$

$= \underline{22\%}$

(iii) Biomass

The incremental increase in biomass was 0.1320 mg $cm^{-3}$.

Hence amount of carbon incorporated in biomass

$$= \frac{0.1320 \times 640 \times 45.20}{1000 \times 100 \times 12}$$

$= 3.182 \times 10^{-3}$ mole

Thus amount of metabolised carbon incorporated in biomass

$$= \frac{3.182 \times 10^{-3}}{7.968 \times 10^{-3}} \times 100$$

$= \underline{40\%}$

(iv) Carbon Dioxide

Amount of carbon dioxide evolved during this section was $3.164 \times 10^{-3}$ mole.

Hence amount of metabolised carbon ($7.968 \times 10^{-3}$ mole) in carbon dioxide

$$= \frac{3.164 \times 10^{-3}}{7.968 \times 10^{-3}} \times 100$$

$$= \underline{40\%}$$

(v) Carbon Recovery

The amount of metabolised carbon ($7.968 \times 10^{-3}$) recovered/accounted for ($C_{rec}$) as acetate, biomass and carbon dioxide formed

$$= (22 + 40 + 40)$$

$$= \underline{102\%}$$

Energy Balance

(i) Glucose

Energy available from the glucose during section B

$$= 6.042 - 2.242$$

$$= 3.800 \text{ kJ}$$

(When 6.042 kJ is the initial energy available at (a) and 2.242 kJ is the energy used during section A).

This is the energy available (provided by metabolism) during this section.

(ii) Waste

Total energy wasted as heat from 640 $cm^3$ of medium during this section was 1.484 kJ.

Hence amount of energy provided by metabolism (3.800 kJ) wasted as heat

$$= \frac{1.484}{3.800} \times 100$$

$$= \underline{39\%}$$

(iii) Acetate

The amount of acetate formed = $0.891 \times 10^{-3}$ mole.

Hence amount of energy stored as acetate

$= 0.891 \times 10^{-3} \times 840$

$= 0.748$ kJ

Hence amount of energy provided by metabolism (3.800 kJ) stored as acetate

$$= \frac{0.748}{3.800} \times 100$$

$$= \underline{20\%}$$

(iv) Biomass

Since 40% of the metabolished carbon was incorporated in biomass then the percentage of energy provided by metabolism stored as biomass is 40 (5.4).

(v) Biosynthesis

The amount of energy provided by metabolism required for biosynthesis (5.4)

$$= 100 - (39 + 20 + 40)$$

$$\Delta\%_B = \underline{1\%}$$

## Section C

During this section the acetate at (c), conc. = 2.103 mmol $dm^{-3}$, is metabolised to form biomass and carbon dioxide. The calculations are similar in principle to those of section A.

### Mass Balance

(i) Acetate

Amount of acetate metabolised

$$= \frac{2.103 \times 10^{-3} \times 640}{1000}$$

$$= 1.346 \times 10^{-3} \text{ mole}$$

Hence amount of carbon metabolised

$$= 1.346 \times 10^{-3} \times 2$$

$$= 2.692 \times 10^{-3} \text{ mole}$$

(ii) Biomass

The incremental increase in the biomass = 0.0360 mg $cm^{-3}$.

Hence the amount of carbon incorporated in biomass

$$= \frac{0.0360 \times 640 \times 45.20}{100 \times 100 \times 12}$$

$= 0.868 \times 10^{-3}$ mole

Thus amount of metabolised carbon ($2.692 \times 10^{-3}$ mole) incorporated in biomass

$$= \frac{0.868 \times 10^{-3}}{2.692 \times 10^{-3}} \times 100$$

$= \underline{32\%}$

(iii) Carbon Dioxide

Amount of carbon dioxide which was evolved during this section = $1.918 \times 10^{-3}$ mole

Hence the amount of metabolised carbon ($2.692 \times 10^{-3}$ mole) incorporated in carbon dioxide

$$= \frac{1.918 \times 10^{-3}}{2.692 \times 10^{-3}} \times 100$$

$= \underline{71\%}$

(iv) Carbon Recovery

The amount of <u>metabolised carbon</u> ($2.692 \times 10^{-3}$ mole) recovered/accounted for ($C_{rec}$) as biomass and carbon dioxide formed

$= (32 + 71)$

$= \underline{103\%}$

<u>Energy Balance</u>

(i) Acetate

Energy available (provided by metabolism) from acetate

$= 1.346 \times 10^{-3} \times 840$

$= 1.131$ kJ

(ii) Waste

The energy wasted as heat from 640 $cm^3$ of culture = 0.815 kJ

Hence amount of energy provided by metabolism (1.131 kJ) wasted as heat

$$= \frac{0.815}{1.131} \times 100$$

$= 72\%$

(iii) Biomass

Since 32% of the metabolised carbon was incorporated in biomass then the amount of energy provided by metabolism (1.131 kJ) stored as biomass is 32% (5.4).

(iv) Biosynthesis

The amount of energy provided by metabolism required for biosynthesis (5.4) during this section

$= 100 - (72 + 32)$

$\Delta\%_C = \underline{-4\%}$

Overall

The calculations are the same as those described earlier (5.4) for cells grown in glucose-limited medium, where the growth is not sectionalised. The calculated mass balance (Table 7.1) and energy

balance values (Table 7.2) are shown under the heading "GROWTH ONE".

## 7.3 The Growth of Phosphate-Limited Cells ($2.5 \times 10^{-3}$ g $dm^{-3}$) in Glucose-Limited Medium

The p-t and $CO_2$-t traces for phosphate trained cells grown in glucose-limited (3.3 mmol $dm^{-3}$) media were irreproducible and the variation of the total waste heat (1.534 - 2.188 kJ) and yield (0.46 - 0.55 g $g^{-1}$) was outside the normal limits of experimental error (Fig. 7.6). Cessation of growth always accompanied the exhaustion of the glucose, and no further growth occurred during the metabolism of the secondary metabolites (acetate or acetate and ethanol) which were formed during glucose metabolism. The concentration of these metabolites as measured at the end of growth varied; acetate : 0.90 - 1.85 mmol $dm^{-3}$; ethanol : 0 - 0.15 mmol $dm^{-3}$. The calculated overall percentage energy for biosynthesis ($\Delta$%) varied widely (5-15%). The shapes of the p-t traces when only acetate was formed (Fig. 7.6 a, b, c, d) were markedly different to those when both acetate and ethanol were produced (Fig. 7.6 e, f, g, h). Apart from the experiments in which ethanol was formed the $CO_2$-t and p-t traces were very similar in shape.

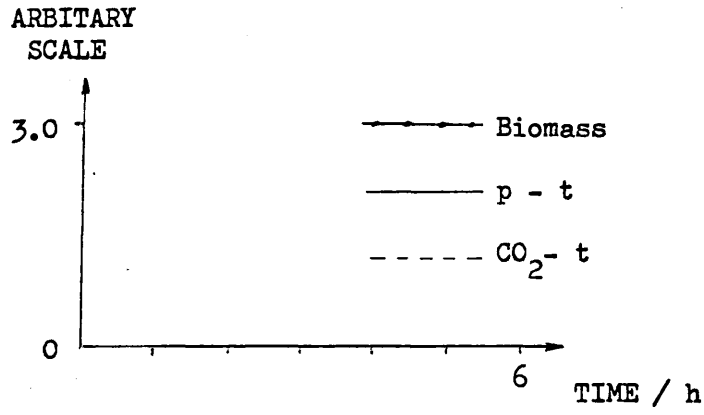
It is difficult to understand why cells from the same cryogenic stores, when inoculated into glucose-limited media and grown and tested under the same standard conditions should show such a wide range of metabolic pathways. These differences were reflected in the p-t and $CO_2$-t traces and in the differing secondary metabolites.

Phosphate-trained cells which had been subcultured five times in glucose-limited media, and then grown in glucose-limited medium in the culture vessel, produced p-t, P-t and $CO_2$-t traces similar to that of glucose-trained cells (Fig. 5.3). Further, the mass and energy balances

## Legend for Figure 7.6

FIGURE 7.6

p - t and $CO_2$- t Traces with Energy and Mass Balances for Growths of Phosphate Trained Cells in Glucose Limited Media



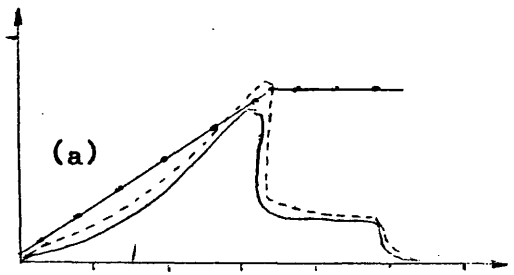

A value of 3.0 on the ARBITARY SCALE corresponds to ;

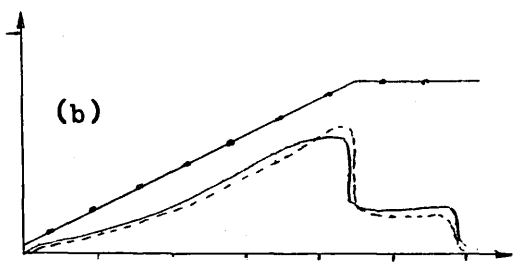
3.0 for LOG M / mg $dm^{-3}$ ;

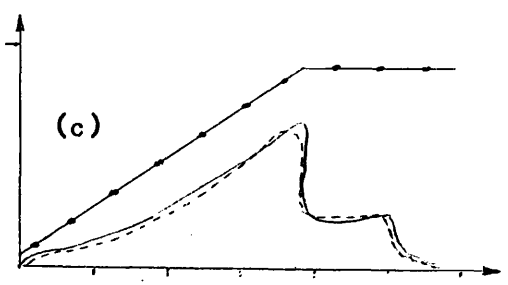
9.0 for POWER x $10^4$ / J $s^{-1}cm^{-3}$ ;

9.0 for CARBON DIOXIDE / p.p.m. .

FIGURE 7.6




| TOTAL HEAT EVOLVED / kJ | YIELD / g $g^{-1}$ | CARBON AS BIOMASS / mmol | CARBON AS $CO_2$ / mmol | % CARBON RECOV. ( $C_{rec}$ ) | % WASTE ENERGY | % STORED ENERGY | % ENERGY FOR BIOSYNTHESIS $\Delta$ % |
|---|---|---|---|---|---|---|---|
| 1.996 | 0.46 | 6.934 | 5.561 | 99 | 33. 0 | 54.7 | 12.3 |
| 2.126 | 0.46 | 6.663 | 6.408 | 103 | 35.8 | 52.6 | 11.6 |
| 2.188 | 0.54 | 7.751 | 4.670 | 98 | 36.2 | 61.2 | 2.6 |

FIGURE 7.6 (Cont.)

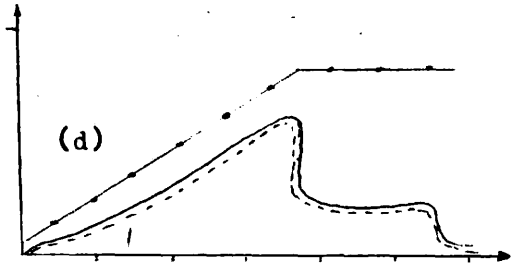
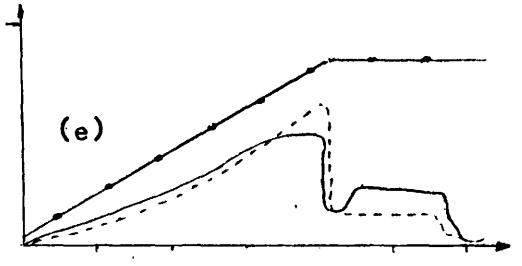
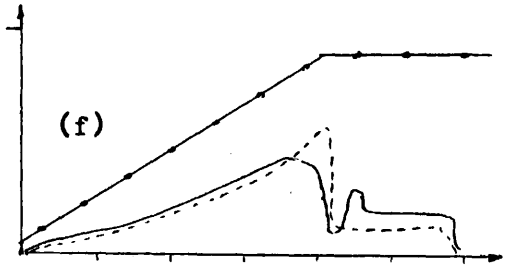
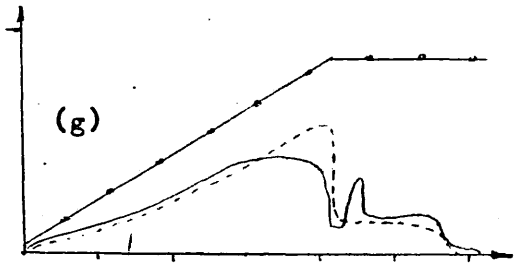
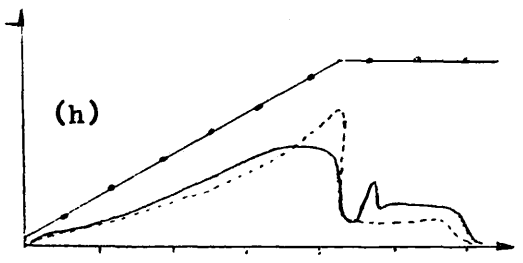
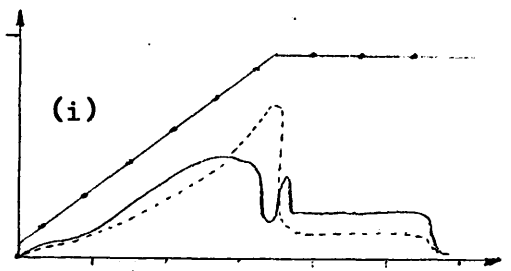
| | TOTAL HEAT EVOLVED / kJ | YIELD / g $g^{-1}$ | CARBON AS BIOMASS / mmol | CARBON AS $CO_2$ / mmol | % CARBON RECOV. ( $C_{rec}$ ) | % WASTE ENERGY | % STORED ENERGY | % ENERGY FOR BIOSYNTHESIS $\Delta$ % |
|---|---|---|---|---|---|---|---|---|
|  (d) | 1.869 | 0.48 | 6.865 | 4.991 | 94 | 30.9 | 54.2 | 14.9 |
|  (e) | 1.862 | 0.48 | 6.903 | 6.389 | 105 | 30.8 | 54.4 | 14.8 |
|  (f) | 2.029 | 0.55 | 7.837 | 5.194 | 103 | 33.6 | 61.8 | 5.1 |

FIGURE 7.6 (Cont.)

| | TOTAL HEAT EVOLVED / kJ | YIELD / g $g^{-1}$ | CARBON AS BIOMASS / mmol | CARBON AS $CO_2$ / mmol | % CARBON RECOV. ( $C_{rec}$ ) | % WASTE ENERGY | % WASTE ENERGY | % ENERGY FOR BIOSYNTHESIS $\triangle$ % |
|---|---|---|---|---|---|---|---|---|
|  (g) | 1.783 | 0.53 | 7.519 | 5.125 | 100 | 29.5 | 59.0 | 11.5 |
|  (h) | 1.720 | 0.50 | 7.155 | 5.101 | 96 | 28.5 | 56.5 | 15.0 |
|  (i) | 1.534 | 0.55 | 7.860 | 4.557 | 98 | 25.4 | 62.0 | 12.6 |

were similar (5.5) to those of glucose-trained cells. It therefore appears that cells subjected to long periods of phosphate-limitation can recover their normal metabolic processes after a few subcultures in normal glucose-limited medium.

## 7.4 The Growth of Phosphate-Limited Cells ($5.5 \times 10^{-3}$ g $dm^{-3}$) in Phosphate-Limited Medium (Glucose conc. : 3.3 mmol $dm^{-3}$)

Cells repeatedly grown in phosphate-limited media ($5.5 \times 10^{-3}$ g $dm^{-3}$) and stored cryogenically were grown in media (640 $cm^3$) of this phosphate concentration for measurement of power, biomass etc, under standard conditions.

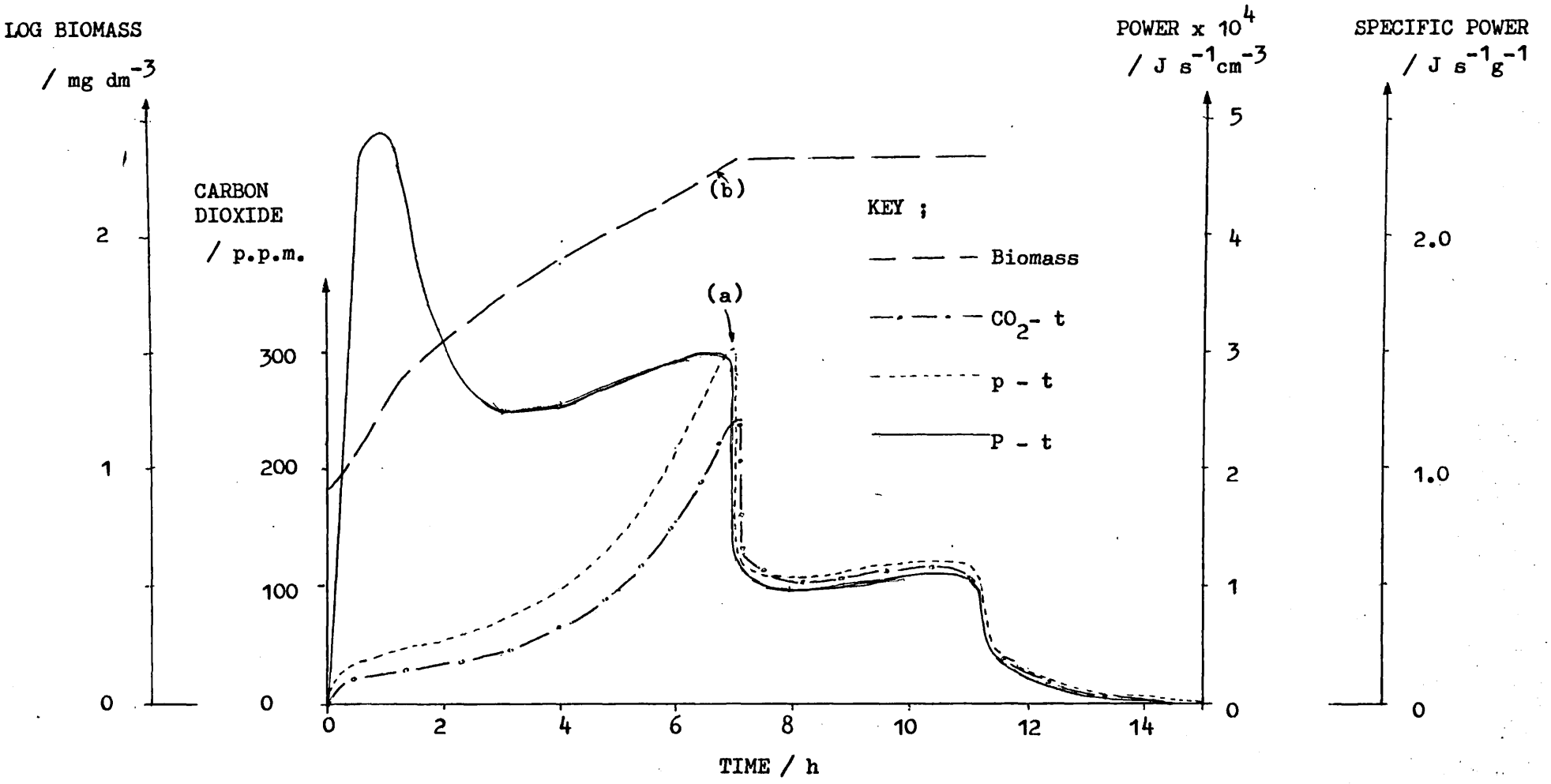
The phosphate in the medium was exhausted (b) when 93-95% of the glucose had been consumed (Fig. 7.7), however growth did not stop until the glucose was exhausted (a).

The p-t and $CO_2$-t traces varied in a similar manner, the power and $CO_2$ outputs increased gradually to (a) and then decreased rapidly to constant values which were maintained during secondary metabolite consumption. Acetate was the only secondary metabolite detected (2.4).

After inoculation the specific power increased extremely rapidly to a peak value (2.5 J $s^{-1}$ $g^{-1}$) and then dropped to an approximately constant value (1.5 J $s^{-1}$ $g^{-1}$) for the rest of growth. On cessation of growth the specific power fell to a steady value (0.5 J $s^{-1}$ $g^{-1}$) during consumption of the secondary metabolite.

The elemental analysis of the cells revealed the following composition;

FIGURE 7.7 P - t, p - t Traces and Changes of Growth Parameters during Phosphate Limited ($5.5 \times 10^{-3}$ g $dm^{-3}$) Growth



| Element | Composition /%wt/wt |
|---|---|
| C | 45.12 |
| H | 6.71 |
| N | 12.59 |
| O | 26.04 |
| S | 1.10 |
| P | 1.87 |
| Residue | 5.96 |

The calculated mass and energy balance data for the overall growth are as follows;

Mass Balance

| | | |
|---|---|---|
| YIELD | : | 0.35 |
| C in biomass | : | $4.906 \times 10^{-3}$ mole |
| C in $CO_2$ | : | $7.588 \times 10^{-3}$ mole |
| $C_{rec}$ | : | 99% |

Energy Balance

| | | |
|---|---|---|
| % waste | : | 47.7 |
| % stored | : | 38.7 |
| % biosynthesis (Δ%) | : | 13.6 |

## 7.5 Summary

1. During the growth of trained phosphate-limited cells ($2.5 \times 10^{-3}$ g $dm^{-3}$) in phosphate-limited medium ($2.5 \times 10^{-3}$ g $dm^{-3}$) three distinct sections were recorded, corresponding to the consumption of phosphate and glucose, glucose, and the metabolism of the secondary products. Mass and energy balances were established for each section of growth and for the overall growth.

2. Growth of phosphate-trained cells ($2.5 \times 10^{-3}$ g $dm^{-3}$) in glucose-limiting media was irreproducible, producing acetate or ethanol and acetate; marked differences in the p-t and $CO_2$-t traces were observed.

3. Growth of phosphate-trained cells ($5.5 \times 10^{-3}$ g $dm^{-3}$) in phosphate-limited ($5.5 \times 10^{-3}$ g $dm^{-3}$) medium was reproducible. Acetate was produced during growth and glucose metabolism.

# CHAPTER EIGHT

# EXPERIMENTAL RESULTS

## THE EFFECT OF SULPHANILAMIDE ON ENERGY AND MASS BALANCES DURING THE GROWTH OF K. AEROGENES

## 8.1 Experimental

To establish the minimum inhibitory concentration (MIC) of sulphanilamide on cells growing in glucose-limited (3.3 mmol $dm^{-3}$) medium growth was initiated in each of 15 boiling tubes containing the medium (2.1.3) with different known amounts of sulphanilamide in the range 0 to 8.14 mmol $dm^{-3}$. After 24 h with aeration the absorbance of each was measured.

A sterile sulphanilamide solution (10 $cm^3$) was prepared which when added to the culture (620 $cm^3$) gave the required concentration of this drug. The sulphanilamide solution was injected into the growing culture (620 $cm^3$) through a rubber septum in the top of the culture vessel. The medium was inoculated with a cryogenic ampoule and the culture aerated (5.3), the biomass (2.2.1), evolution of carbon dioxide (2.2.2) and power (2.3) were monitored continuously. The sulphanilamide solution was added when the biomass of the culture reached 0.082 mg $cm^{-2}$. At this point 21% of the glucose initially present had been consumed and the specific power was c. 1.9 J $s^{-1}$ $g^{-1}$ i.e. the relatively constant exponential growth value (6.2, Figs 6.1, 6.2, 6.3).

## 8.2 Results

The MIC for cells growing in glucose-limited medium was 4.65 mmol $dm^{-3}$; a value greater than that (2.32 mmol $dm^{-3}$) obtained when grown in nutrient broth (Yau, 1977).

The p-t, P-t and $CO_2$-t traces for cells growing in glucose-limited (3.3 mmol $dm^{-3}$) media containing sulphanilamide at different concentrations are shown in Figs 8.1, 8.2 and 8.3 Prior to the addition of drug the power, biomass and carbon dioxide concentration increased

FIGURE 8.1 The Effect of Sulphanilamide (9.3 mmol $dm^{-3}$) on Growth in Glucose Limited Medium

FIGURE 8.2 As above except Sulphanilamide (11.5 mmol $dm^{-3}$)

FIGURE 8.3 As above except Sulphanilamide (14.1 mmol $dm^{-3}$)

KEY ;

| | | | |
|---|---|---|---|
| ——— ——— | Log Biomass | / mg $dm^{-3}$ | (LOG M) |
| . — . — . | Carbon Dioxide | / p.p.m. | ($CO_2$) |
| - - - - - - - - | Power x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| ——————— | Specific Power | / J $s^{-1}g^{-1}$ | (P) |

DRUG ↓ Introduction of Sulphanilamide into Growing Culture

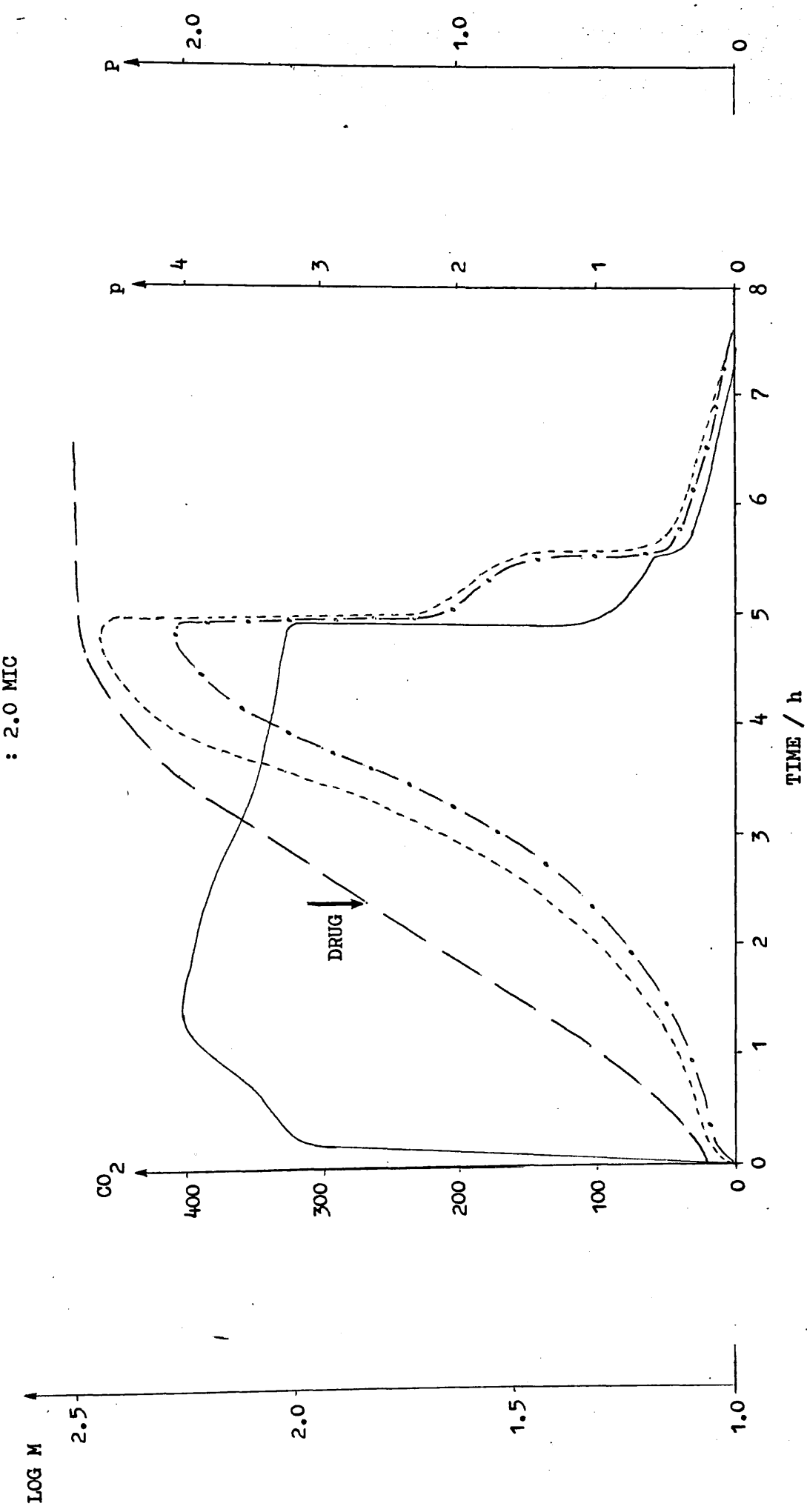



FIGURE 8.1 Sulphanilamide conc. : 9.3 mmol $dm^{-3}$
: 2.0 MIC

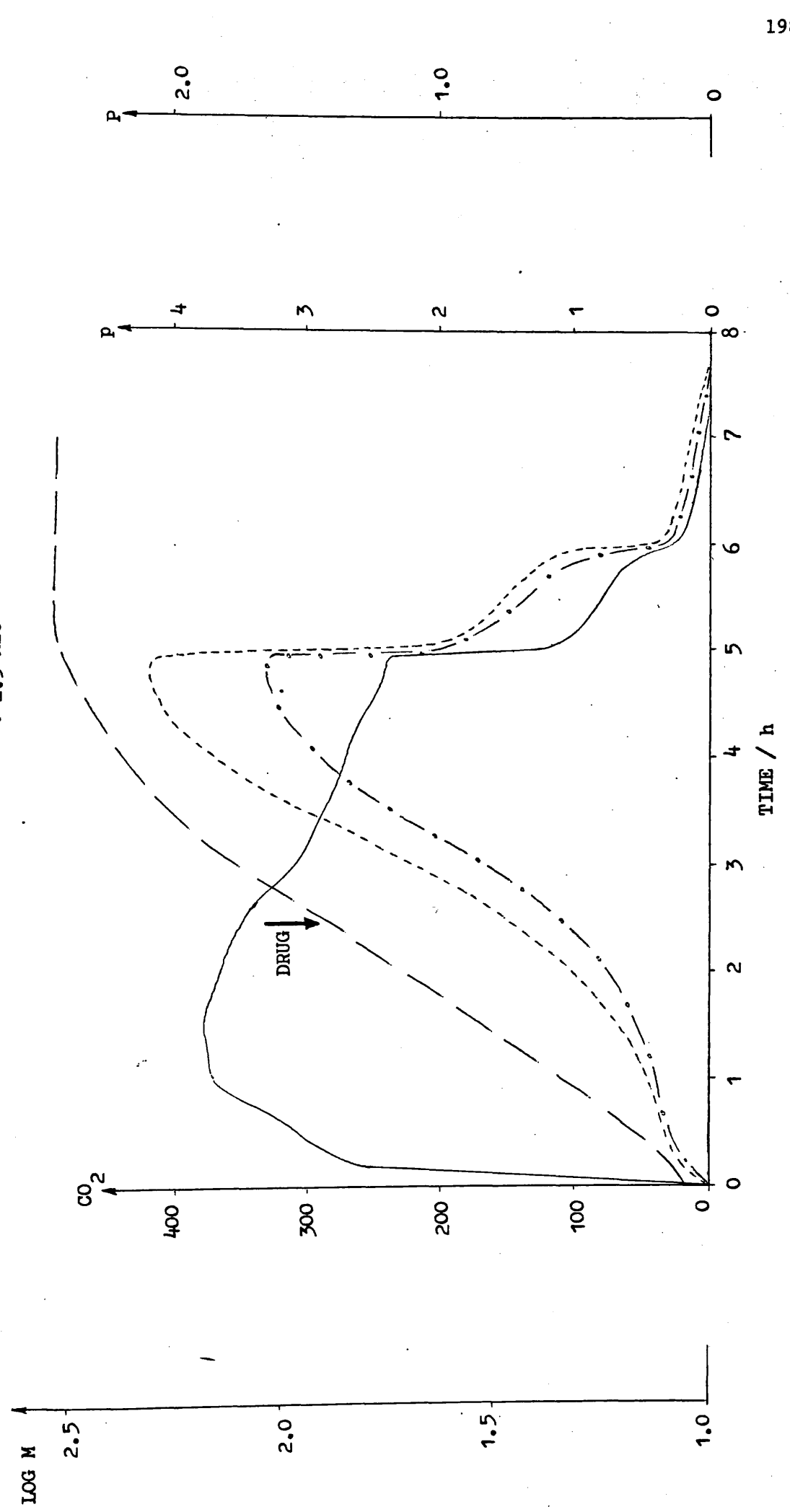



FIGURE 8.2 Sulphanilamide conc. : 11.5 mmol $dm^{-3}$
: 2.5 MIC

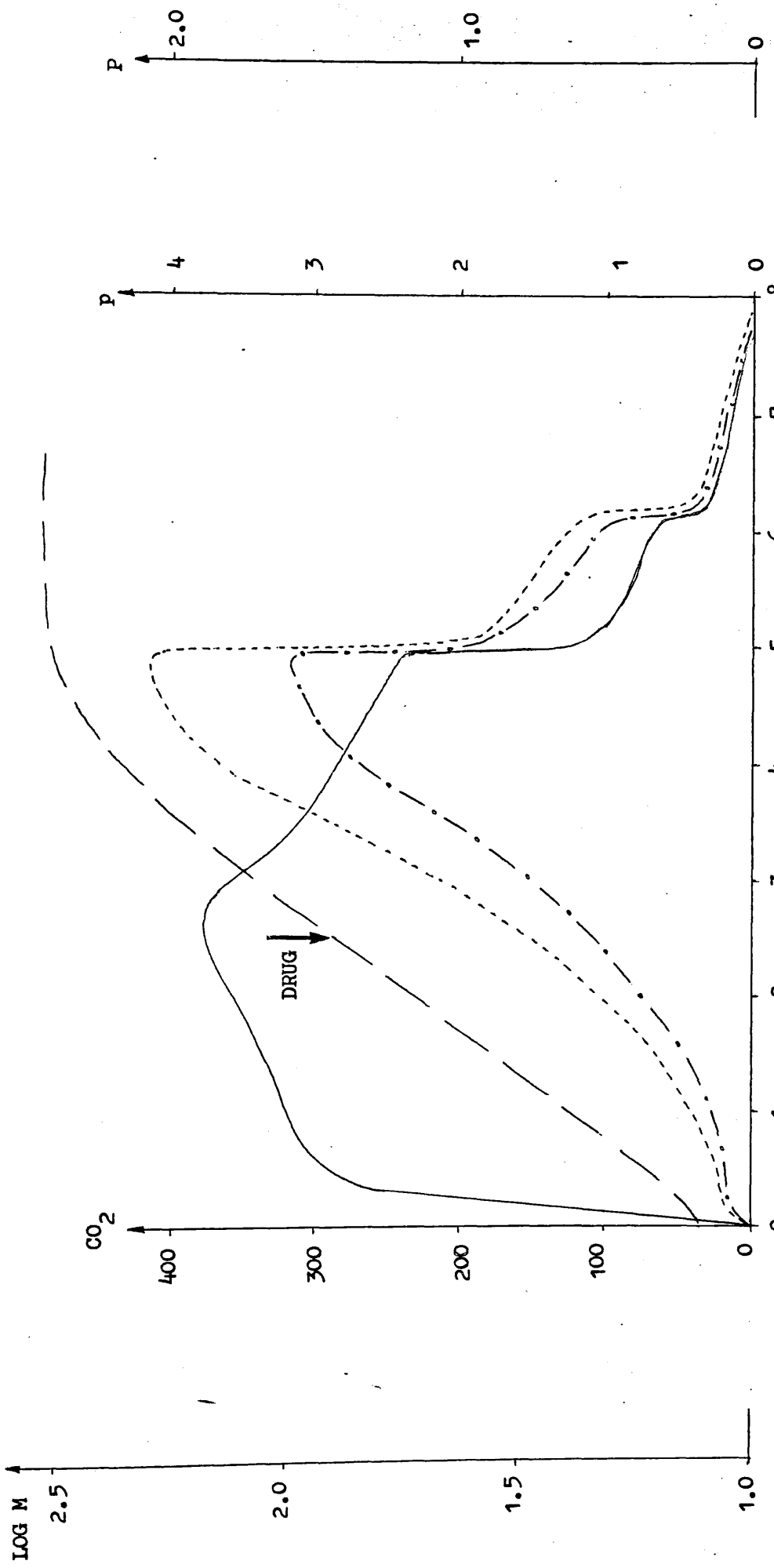



FIGURE 8.3 Sulphanilamide conc. : 14.1 mmol $dm^{-3}$
: 3.0 MIC

exponentially during glucose metabolism. Further the specific power attained a value of c. 1.90 J $s^{-1}$ $g^{-1}$.

After the addition of the drug and during glucose metabolism the rate of increase of the power, biomass and carbon dioxide concentration decreased gradually. The greater the drug concentration the greater was the extent of this decrease. The specific power also decreased gradually. The specific power value immediately prior to the exhaustion of the glucose was markedly dependent on the drug concentration (Table 8.1), as was the power. The power and specific power values were less than those obtained for cells grown in ordinary glucose limited medium; the greater the sulphanilamide concentration the lower the values. Since the drug has little effect on the final biomass or yield (Fig. 8.2) this decrease in specific power is attributable to the decrease in power.

During this growth period in the presence of drug, acetate was produced; the greater the drug concentration the greater the amount of acetate produced in the range 0.3-0.7 mmol $dm^{-3}$, (as measured near the end of growth).

After the glucose had been exhausted the p-t, P-t and $CO_2$-t traces decreased rapidly to lower levels and during this period acetate was metabolised, (producing carbon dioxide only). During acetate metabolism the secondary power and carbon dioxide outputs recorded were similar to those observed when cells were grown in glucose-limited medium under non-standard conditions (Fig. 5.2).

The corresponding energy and mass balances (Table 8.2) for growth in the presence of the three different drug concentrations are based on calculations described earlier (5.4). With increasing drug concentration the total heat evolved ($\Delta H_T$) increased significantly; at 3 MIC 18% more waste heat was produced as compared with the control (drug conc. : zero). With increasing drug concentration the proportion of energy stored (and

TABLE 8.1 The Effect of Sulphanilamide on The Power and Specific Power at The End of The Growth Phase

| RELATIVE SULPHANILAMIDE CONC. /MIC | POWER x $10^4$ /J $s^{-1}cm^{-3}$ | SPECIFIC POWER / J $s^{-1}g^{-1}$ |
|---|---|---|
| 0 | 5.00 | 1.95 |
| 2.0 | 4.62 | 1.60 |
| 2.5 | 4.20 | 1.22 |
| 3.0 | 4.14 | 1.18 |

TABLE 8.2 The Effect of Sulphanilamide on The Energy and Mass Balances during Growth in Glucose Limited Media

| CONC. OF SULPHAN. / MIC | CONC. OF SULPHAN. / mmol $dm^{-3}$ | TOTAL HEAT EVOLVED ($\Delta H_T$) / kJ | TOTAL $CO_2$ EVOLVED / mol x $10^3$ | YIELD / g $g^{-1}$ | % CARBON RECOVERY | % ENERGY WASTED | % ENERGY STORED | % ENERGY FOR BIOSYNTHESIS $\Delta$% |
|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 1.90 | 5.55 | 0.50 | 102 | 32.5 | 56.4 | 11.1 |
| 2.0 | 9.3 | 2.09 | 5.88 | 0.46 | 100 | 35.7 | 55.0 | 9.3 |
| 2.5 | 11.5 | 2.19 | 5.61 | 0.48 | 100 | 37.4 | 54.3 | 8.3 |
| 3.0 | 14.1 | 2.24 | 6.64 | 0.48 | 100 | 38.2 | 54.1 | 7.7 |

therefore yield) decreased slightly. Further there was a decrease in the energy required for biosynthesis; at 3 MIC 37% less energy was required for biosynthesis. It is interesting to note that the relatively large drop in the biosynthesis value was accompanied by a small drop (4%) in the yield.

## 8.3 Summary

1. Sulphanilamide (>3 MIC ≡ 14.1 m mol $dm^{-3}$) substantially increased (18%) the proportion of energy wasted and decreased (37%) that required for biosynthesis.

2. The yield and proportion of energy stored decreased only slightly (max : 4%) at these sulphanilamide concentrations.

# CHAPTER NINE

# EXPERIMENTAL RESULTS

## THE EFFECT OF NALIDIXIC ACID ON ENERGY AND MASS BALANCES DURING THE GROWTH OF K. AEROGENES

## 9.1 Inhibition of Growth and MIC of Nalidixic Acid

To establish the minimum inhibitory concentration (MIC) of nalidixic acid (NA) on cells growing in glucose-limited (3.3 mmol $dm^{-3}$) medium growth was initiated in each of 15 boiling tubes containing the medium (25 $cm^3$) with different known concentrations of NA in the range of 0 to 0.216 mmol $dm^{-3}$. After 24 h with aeration the absorbance of each of the suspensions was measured. Growth did not occur above 0.052 mmol $dm^{-3}$ and hence the MIC for NA was established as 0.052 mmol $dm^{-3}$ (0.012 g $dm^{-3}$, 12 ppm).

Sterile NA solution (10 $cm^3$) was injected through a rubber septum in the top of the culture vessel into 620 $cm^3$ of growing culture (glucose-limited media : 3.3 mmol $dm^{-3}$) to give the required NA concentration. The NA was added to the growing culture approximately 1.7 h after inoculation (2.1.3) at a specified absorbance reading (biomass : 0.082 mg $cm^{-3}$); corresponding to c. 21% consumption of the glucose present initially. The choice of this point in the growth cycle fulfilled the following criteria:

(a) under the standard conditions of growth and measurement (5.3) there was an appreciable amount of growth. The addition of NA prior to inoculation prevented appreciable growth for an unacceptably long time;

(b) the specified power output had attained its exponential value of c. 2.0 J $s^{-1}$ $g^{-1}$. The P-t traces for growth at different NA concentrations were then easier to compare as the reference value of c. 2.0 J $s^{-1}$ $g^{-1}$ was initially exhibited by each;

(c) the effect of the different concentrations of NA on the

measured parameters of growing cells was comparable i.e. the power, specific power, biomass and carbon dioxide output for different growths were comparable. The standardisation of the inoculum was of paramount importance as variations in the lag phase and inoculum size caused variations in the overall energy balance.

The selection of suitable NA concentrations to which the growth of cells were subjected fulfilled the following criteria:

(a) the incremental increases in the NA concentration resulted in a significant change in the growth parameters i.e. power etc;

(b) the NA concentrations were bacteriostatic and not bactericidal.

In this text the NA concentration is expressed in three different units; g $dm^{-3}$, mmol $dm^{-3}$ and the number of MIC's, (assuming the MIC for NA is 0.052 mmol $dm^{-3}$). This allows for a more meaningful comparison to be made between the results, especially between those obtained from cells grown in the presence of different drugs. Further the biomass, as in the biomass-time traces, is expressed in two different forms; biomass, m/mg $cm^{-3}$ or log biomass, LOG M/log(mg $dm^{-3}$). The logarithmic notation was used when the raw data showed a change in the growth rate, as this form best illustrates this effect.

## 9.2 The Effect of Adding Nalidixic Acid to Growing Cultures

### 9.2.1 Changes in Growth Parameters

In general the log biomass-time (LOG M-t), p-t, P-t and $CO_2$-t

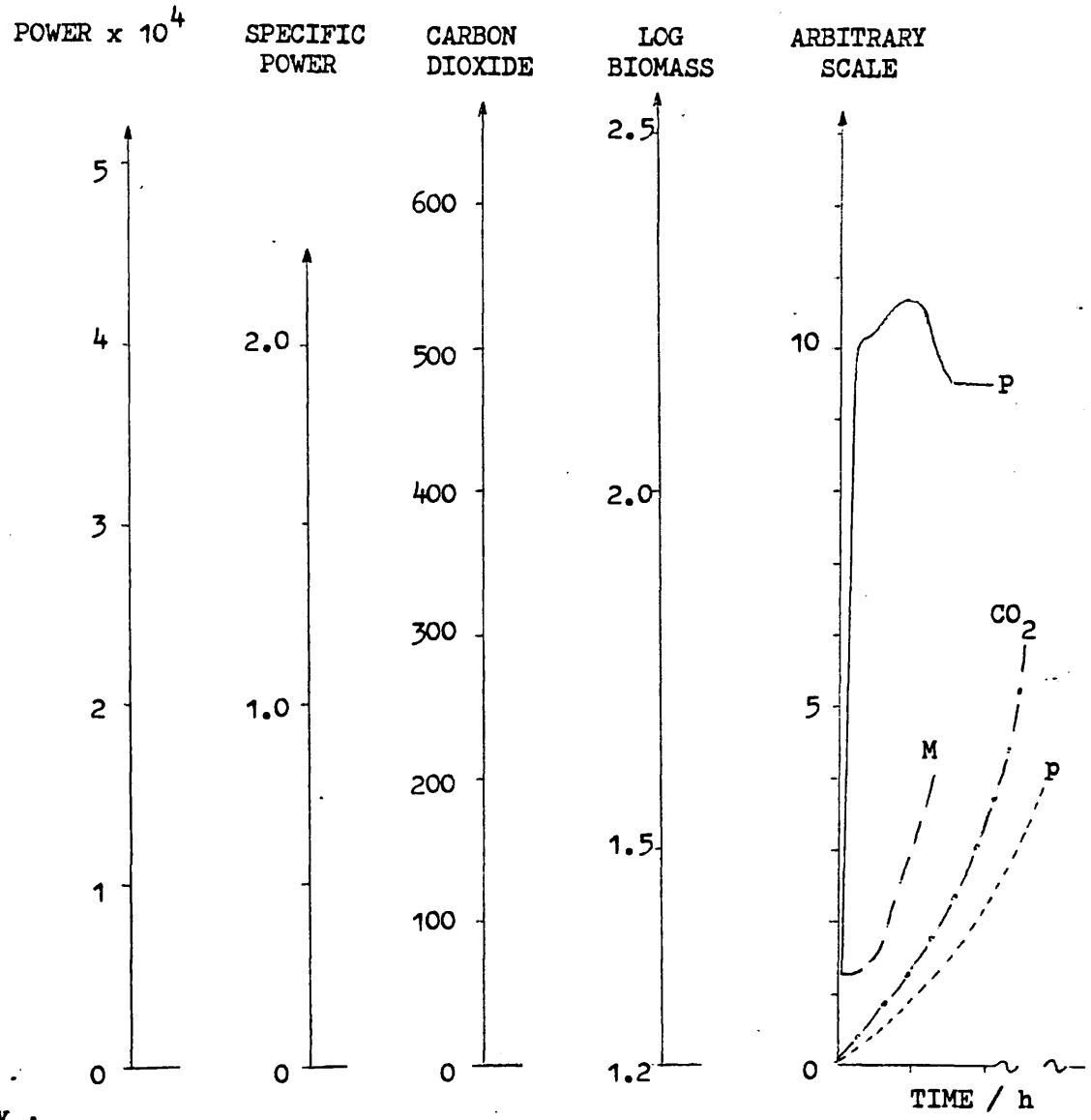
traces for cells growing in glucose-limited 3.3 mmol $dm^{-3}$) media containing NA (Fig. 9.1 b-h) were very different from those of cells growing in glucose-limited media (Figs 5.3, 5.4 c, 9.1 a). Further these differences became progressively greater as the concentration of NA was increased.

During growth acetate and ethanol were detected in the medium. The highest concentrations (1.40 and 0.11 mmol $dm^{-3}$ respectively) were found during the growth in the presence of the highest NA concentration (Fig. 9.1 h), immediately prior to the cessation of growth. The concentration of acetate in the medium during the growth of cells in the presence of NA (0.690 mmol $dm^{-3}$; Fig. 9.1 f) is shown in Fig. 9.2 On cessation of growth and exhaustion of the glucose the cells began to consume the secondary metabolites. This was reflected in the gradual decrease in the acetate concentration after exhaustion of the glucose.

When NA was present in the medium the growth rate decreased gradually during glucose metabolism, until immediately prior to the cessation of growth, exhaustion of the glucose and commencement of secondary metabolite consumption (G), the growth rate was at its minimum, and subsequently, the doubling time ($t_d$) at its maximum. As the concentration of NA increased (Fig. 9.1 b-h) its effect on the (LOG M)-t traces became more apparent. As a general rule the decrease in the growth rate during glucose metabolism, and the time required for the exhaustion of the glucose and cessation of growth became greater as the concentration of NA was increased. At the higher NA concentrations (Fig. 9.1 f, g, h) as the last of the glucose was metabolised the growth rates were very low.

When NA was present in the medium not only did the growth rate decrease but so did the rate of increase in the power output as compared with that in the absence of NA (Fig. 9.1 a). At low NA concentrations

FIGURE 9.1 P - t, p - t and $CO_2$-t Traces for Cells Growing in Glucose Limited (3.3 mmol $dm^{-3}$) Media Containing Different Concentrations of Nalidixic Acid




KEY ;

- --------- POWER x $10^4$ / J $s^{-1}cm^{-3}$ (p)
- ———— SPECIFIC POWER / J $s^{-1}g^{-1}$ (P)
- — • —•— CARBON DIOXIDE / p.p.m. ($CO_2$)
- — — — LOG BIOMASS / mg $dm^{-3}$ (LOG M)

DRUG ↓ : Introduction of nalidixic acid into growing culture

G : End of growth and exhaustion of glucose

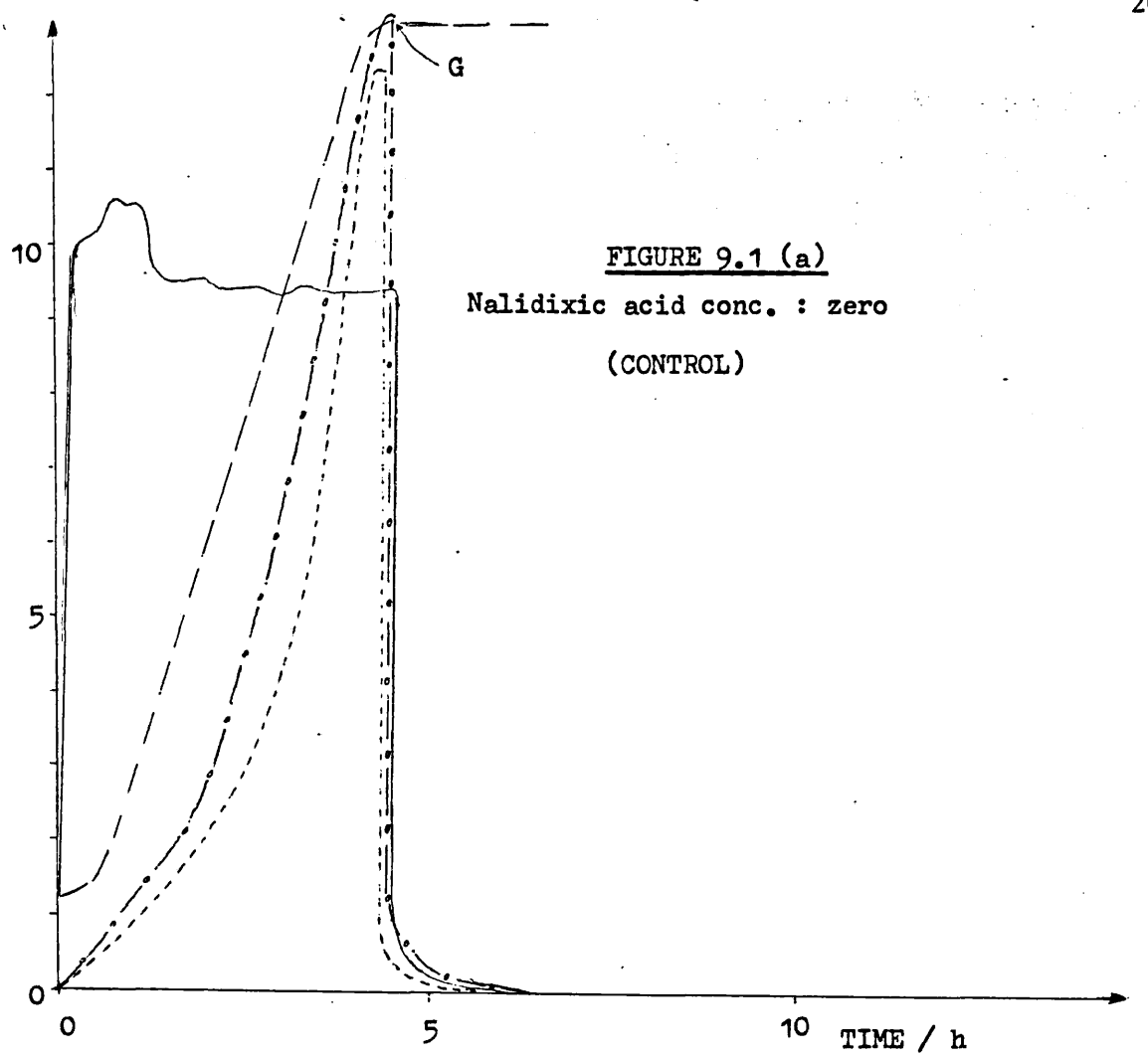



FIGURE 9.1 (a)

Nalidixic acid conc. : zero

(CONTROL)

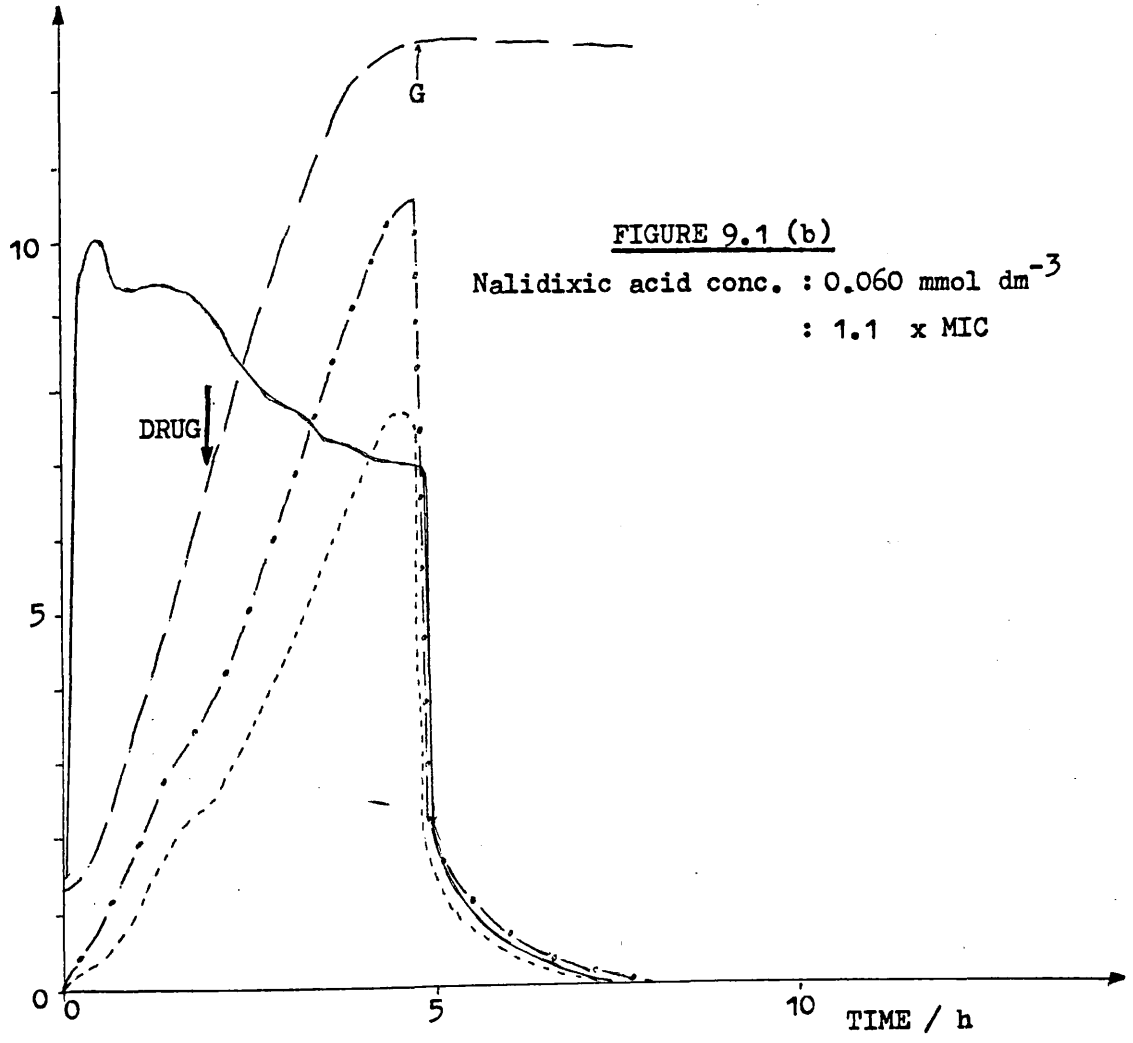



FIGURE 9.1 (b)

Nalidixic acid conc. : 0.060 mmol $dm^{-3}$

: 1.1 x MIC

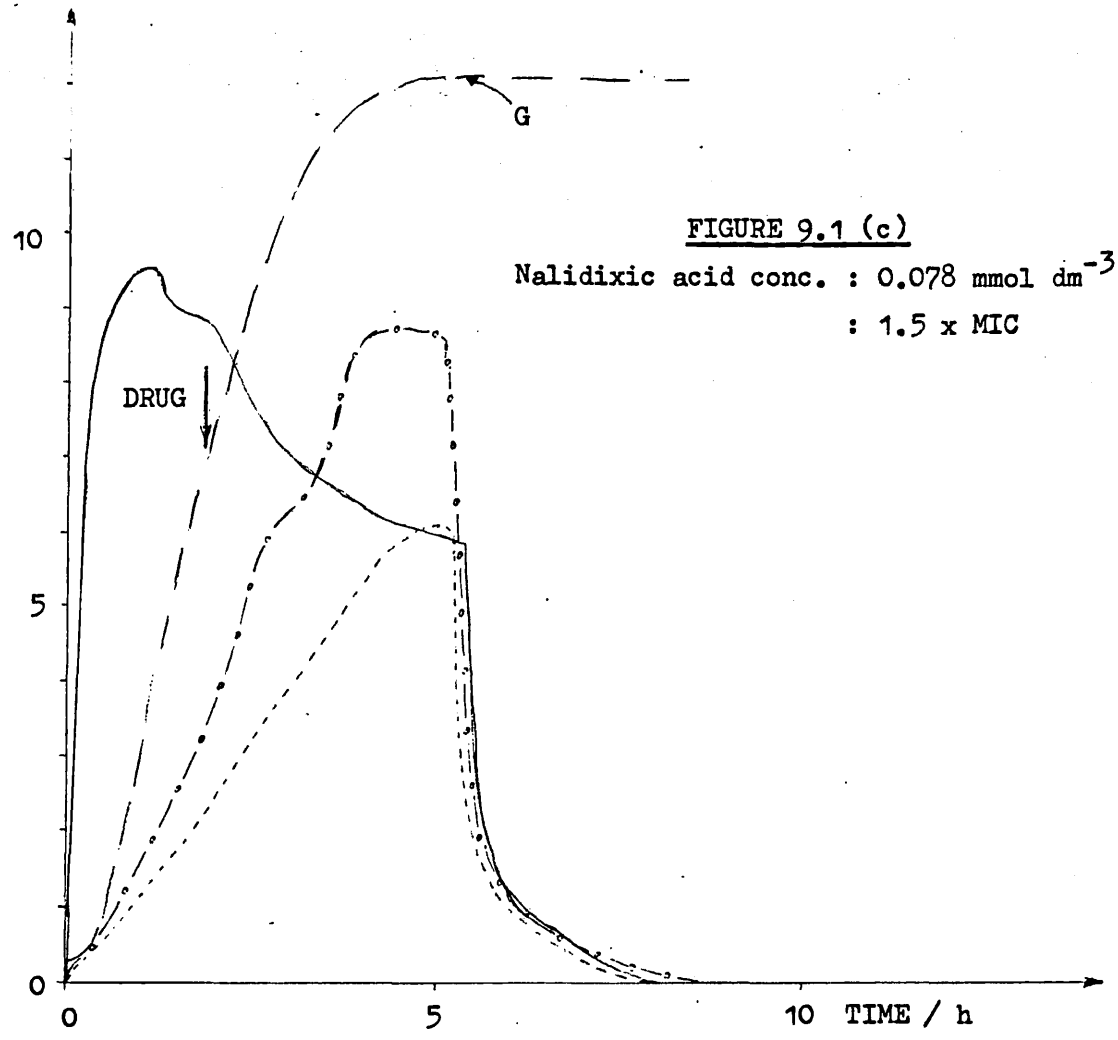



FIGURE 9.1 (c)

Nalidixic acid conc. : 0.078 mmol $dm^{-3}$

: 1.5 x MIC

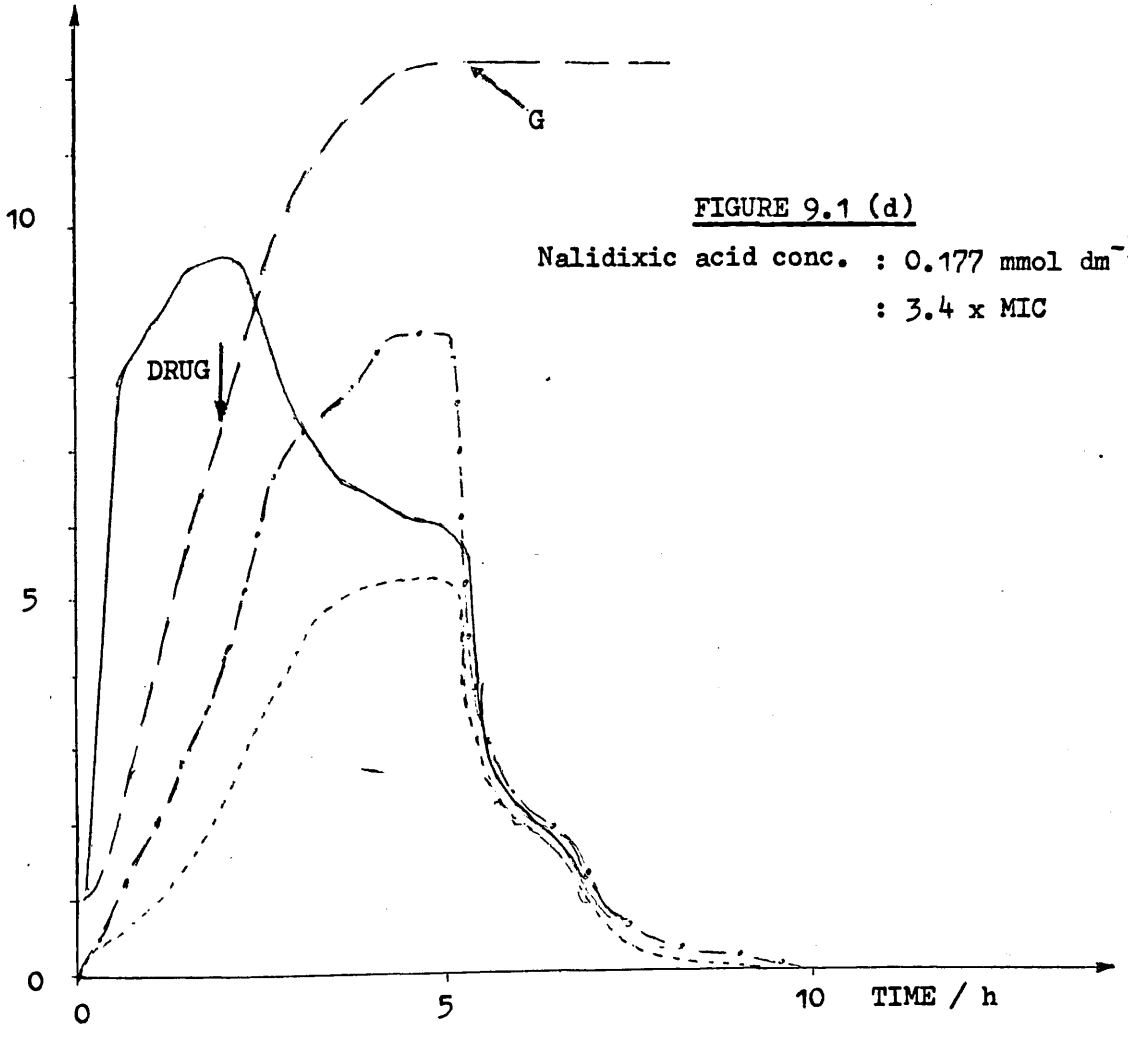



FIGURE 9.1 (d)

Nalidixic acid conc. : 0.177 mmol $dm^{-3}$

: 3.4 x MIC

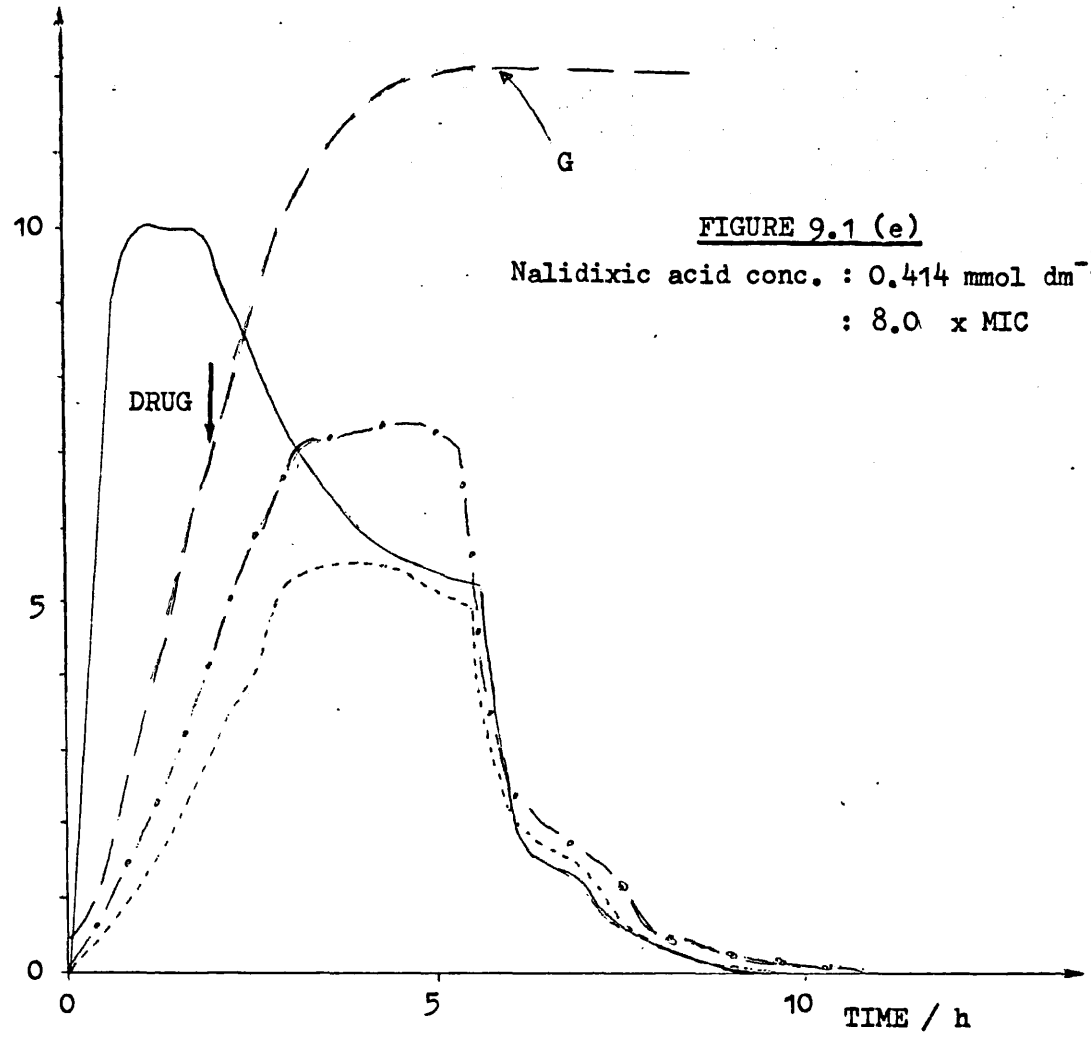



FIGURE 9.1 (e)

Nalidixic acid conc. : 0.414 mmol $dm^{-3}$

: 8.0 x MIC

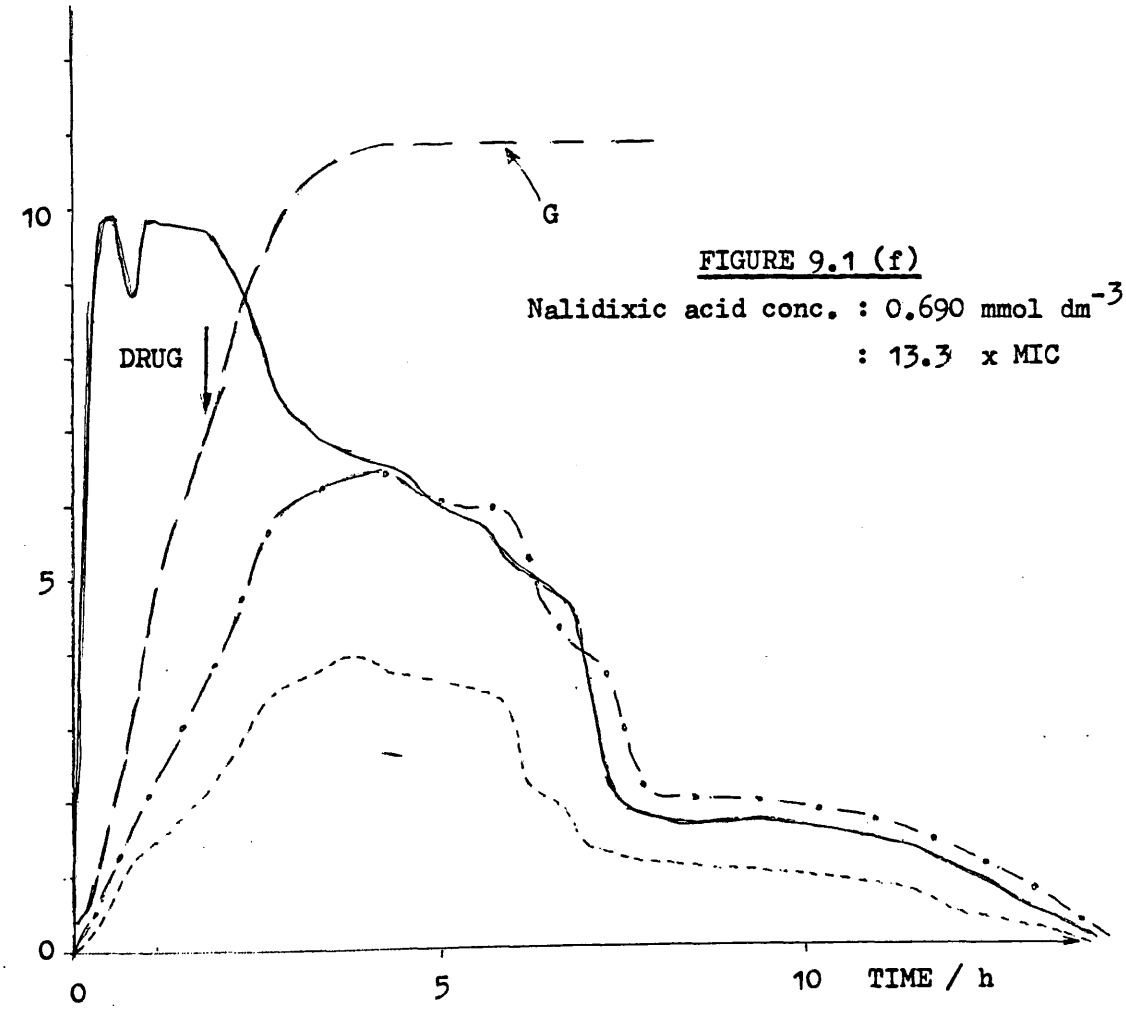



FIGURE 9.1 (f)

Nalidixic acid conc. : 0.690 mmol $dm^{-3}$

: 13.3 x MIC

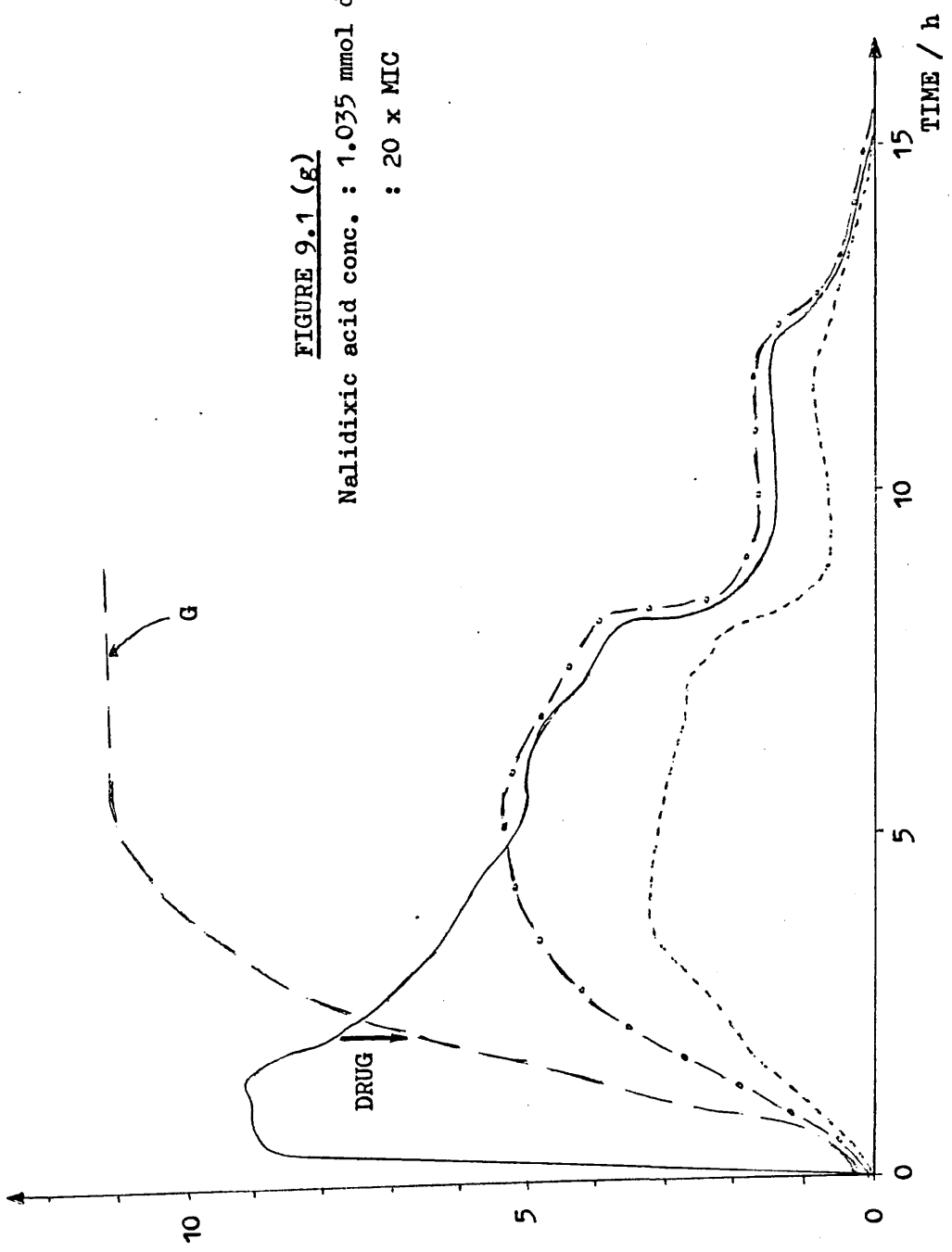



FIGURE 9.1 (g)

Nalidixic acid conc. : 1.035 mmol $dm^{-3}$

: 20 x MIC

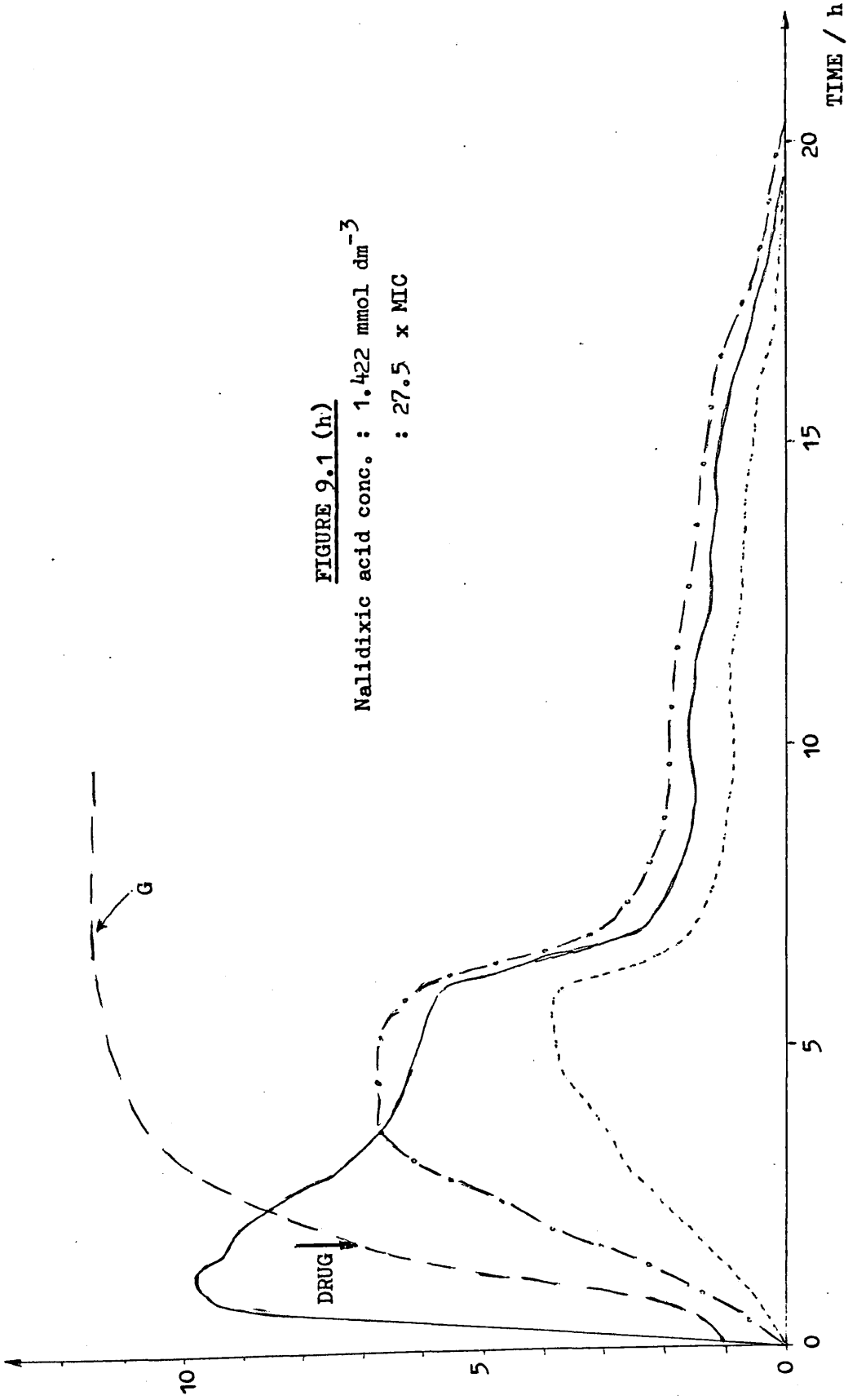



FIGURE 9.1 (h)

Nalidixic acid conc. : 1.422 mmol $dm^{-3}$

: 27.5 x MIC

(Fig. 9.1 b, c) there was a gradual rise in the power outputs during growth. On cessation of growth the power output decreased rapidly to zero; there was no significant secondary power output as relatively small amounts of acetate and ethanol were produced during growth. The p-t traces (even though they are more rounded in shape) resemble those produced by cells grown in drug free medium (Fig. 9.1 a). At higher NA concentrations (Fig. 9.1 d-h) the power output increased during the early stages of growth but during the latter stages, where substantial reductions in growth rate occurred, the power output remained approximately constant. On cessation of growth (G) and commencement of secondary metabolite consumption the power output decreased to a new value ($0.5 \times 10^{-4}$ J $s^{-1}$ $cm^{-3}$), which remained constant for a period depending on the NA concentration. As a general rule with increasing NA concentration the amount of secondary metabolite produced increased, and in consequence the total waste energy released (area under the p-t trace) during secondary metabolite consumption also increased. On exhaustion of the secondary metabolites the power output decreased to zero (Fig. 9.1 b-h). The time required for the p-t trace to reattain its base line after inoculation increased dramatically as the amount of NA present in the medium increased; 6.5 h for the control, 7.5 h at 0.060 mmol $dm^{-3}$ (Fig. 9.1 b), 20 h at 1.442 mmol $dm^{-3}$ (Fig. 9.1 h).

The $CO_2$-t traces for cells grown in the presence of NA (Fig. 9.1 b-h) were similar to the p-t traces described earlier. Parallel changes in the $CO_2$-t and p-t traces for growth caused by different concentrations of NA were observed.

The presence of NA in the medium reduced the specific power output (Fig. 9.1 b-h) from that value (c. 2.0 J $s^{-1}$ $g^{-1}$) obtained during the exponential growth of cells in glucose limited medium <u>not</u> containing NA i.e. the control (Fig. 9.1 a). On inoculation the specific power output

increased extremely rapidly, and during growth prior to the addition of the drug (NA) a value of c. 2.0 J $s^{-1}$ $g^{-1}$ was reached and maintained for approximately 1 h. After addition of the drug, during growth (and glucose metabolism), the specific power decreased gradually. The rates at which these decreases occurred (see the P-t traces) and the value of the specific power reached at the end of growth was dependent on the NA concentration (Table 9.1). As the NA concentration increased the rate of reduction in the specific power increased and the value of the specific power immediately prior to the cessation of growth decreased. An exception occurred at the highest NA concentration (Fig. 9.1 h) where the action of NA may be more bactericidal than bacteriostatic. Further the lower concentrations of NA had a relatively greater effect on specific power at the end of growth than did the higher concentrations (Table 9.1). On cessation of growth and glucose metabolism (Fig. 9.1 a, b, c) the specific power output decreased rapidly to zero. When significant amounts of secondary metabolites were formed (Fig. 9.1 d-e) the specific power output decreased to a lower value (c. 0.4 J $s^{-1}$ $g^{-1}$) which remained approximately constant during secondary metabolite consumption. The time for which secondary specific power output was maintained increased with increasing NA concentration : c. 1 h at 0.177 mmol $dm^{-3}$ (Fig. 9.1 d), c. 13 h at 1.422 mmol $cm^{-3}$ (Fig. 9.1 h). On exhaustion of the secondary metabolites the specific power output decreased to zero; the P-t trace reattained the base line. The P-t trace for the growth of cells at the highest NA concentration used (Fig. 9.1 h) reattained the base line c. 20 h after inoculation, compared with 6.5 h for the control (Fig. 9.1 a).

Cells grown in the presence of NA were elongated compared to the control cells. The greater the NA concentration the greater the elongation; at the higher concentrations (Fig. 9.1 g, h) the cells were approximately twice the length of control cells. The elongated cells

TABLE 9.1 Specific Power at The End of Growth as A Function of NA Concentration

| CONC. OF NA / MIC | SPECIFIC POWER / J $s^{-1}g^{-1}$ |
|---|---|
| 0 | 1.90 |
| 1.1 | 1.40 |
| 1.5 | 1.20 |
| 3.4 | 1.16 |
| 8.0 | 1.08 |
| 13.3 | 0.90 |
| 20.0 | 0.74 |
| 27.5 | 1.08 |

were slightly "dumbell" in shape suggesting that NA inhibits cell division. Further cells grown in the presence of NA produced large quantities of mucus compared to the control cells, causing the formation of foam in the culture vessel during growth.

### 9.2.2 Mass and Energy Balances During Growth

The mass (Table 9.2) and energy (Table 9.3) balances for cells grown in the presence of differing amounts of NA have been calculated from the p-t, P-t, (LOG M)-t and $CO_2$-t data of Figs 9.1 a-h.

As the concentration of NA increased (except at the two highest concentrations) the yield and the carbon incorporated in biomass decreased while the carbon incorporated in carbon dioxide increased (Table 9.2). At the two highest concentrations these trends were reversed. The lower concentrations of NA had a relatively greater effect on the incremental reduction of yield and the incremental increase in the carbon incorporated in carbon dioxide than did the higher concentrations. The carbon recovered ($C_{rec}$) was close to the 100% value except at the highest NA concentration when a value of 113 was obtained.

As the NA concentration increased so did the energy wasted as heat (Table 9.3); at the highest concentration encountered the waste heat was approximately double that for the control. The relationship between waste heat and NA concentration was not linear, and this is illustrated graphically (Fig. 9.3). As the concentration increased the energy stored decreased, except at the two highest concentrations, in a non-linear manner. Further as the NA concentration increased the energy required for biosynthesis ($\Delta$%) decreased, and at the highest NA concentration an unrealistic negative biosynthesis value was obtained (Table 9.2 and Fig. 9.3). As the concentration increased the change in the energy stored was mirrored by the change in the energy wasted (Fig. 9.3) except at the

TABLE 9.2 Mass Balance for Cells Growing in Glucose-Limited Media Containing Different Amounts of Nalidixic Acid

(Data from Figures 9.1 a→h)

| CONC. OF NALIDIXIC ACID / MIC | CONC. OF NALIDIXIC ACID / g $dm^{-3}$ | YIELD / g $g^{-1}$ | % CARBON AS $CO_2$ | % CARBON AS BIOMASS | % CARBON RECOVERY ( $C_{rec}$ ) |
|---|---|---|---|---|---|
| 0 | 0 | 0.496 | 42.4 | 56.0 | 98 |
| 1.1 | 0.060 | 0.449 | 51.7 | 50.7 | 102 |
| 1.5 | 0.078 | 0.418 | 51.2 | 47.2 | 98 |
| 3.4 | 0.177 | 0.412 | 55.9 | 46.5 | 102 |
| 8.0 | 0.414 | 0.390 | 66.1 | 44.1 | 110 |
| 13.3 | 0.690 | 0.294 | 70.0 | 33.1 | 103 |
| 20.0 | 1.035 | 0.319 | 67.5 | 36.1 | 104 |
| 27.5 | 1.422 | 0.352 | 73.8 | 39.6 | 113 |

TABLE 9.3 Energy Balance for Cells Grown in Glucose Limited Media Containing Different Amounts of Nalidixic Acid (Data from Figures 9.1 a→h)

| CONC. OF NALIDIXIC ACID / MIC | CONC. OF NALIDIXIC ACID / ~~g dm$^{-3}$~~ mmol dm$^{-3}$ | TOTAL HEAT EVOLVED / kJ | % WASTE ENERGY | % STORED ENERGY | % ENERGY REQUIRED FOR BIOSYNTHESIS Δ% |
|---|---|---|---|---|---|
| 0 | 0 | 2.291 | 31.9 | 56.0 | 12.1 |
| 1.1 | 0.060 | 2.337 | 39.9 | 50.7 | 9.4 |
| 1.5 | 0.078 | 2.501 | 42.7 | 47.2 | 10.1 |
| 3.4 | 0.177 | 2.842 | 48.6 | 46.5 | 4.9 |
| 8.0 | 0.414 | 2.769 | 47.3 | 44.1 | 8.6 |
| 13.3 | 0.690 | 3.431 | 58.6 | 33.1 | 8.3 |
| 20.0 | 1.035 | 3.607 | 61.6 | 36.1 | 2.3 |
| 27.5 | 1.422 | 4.467 | 76.3 | 39.6 | -15.9 |

FIGURE 9.2 Production of Acetate by Cells Growing in Glucose Limited Medium Containing Nalidixic Acid (0.690 mmol $dm^{-3}$)

(For the key and scale of the "ARBITRARY SCALE" see Figure 9.1)

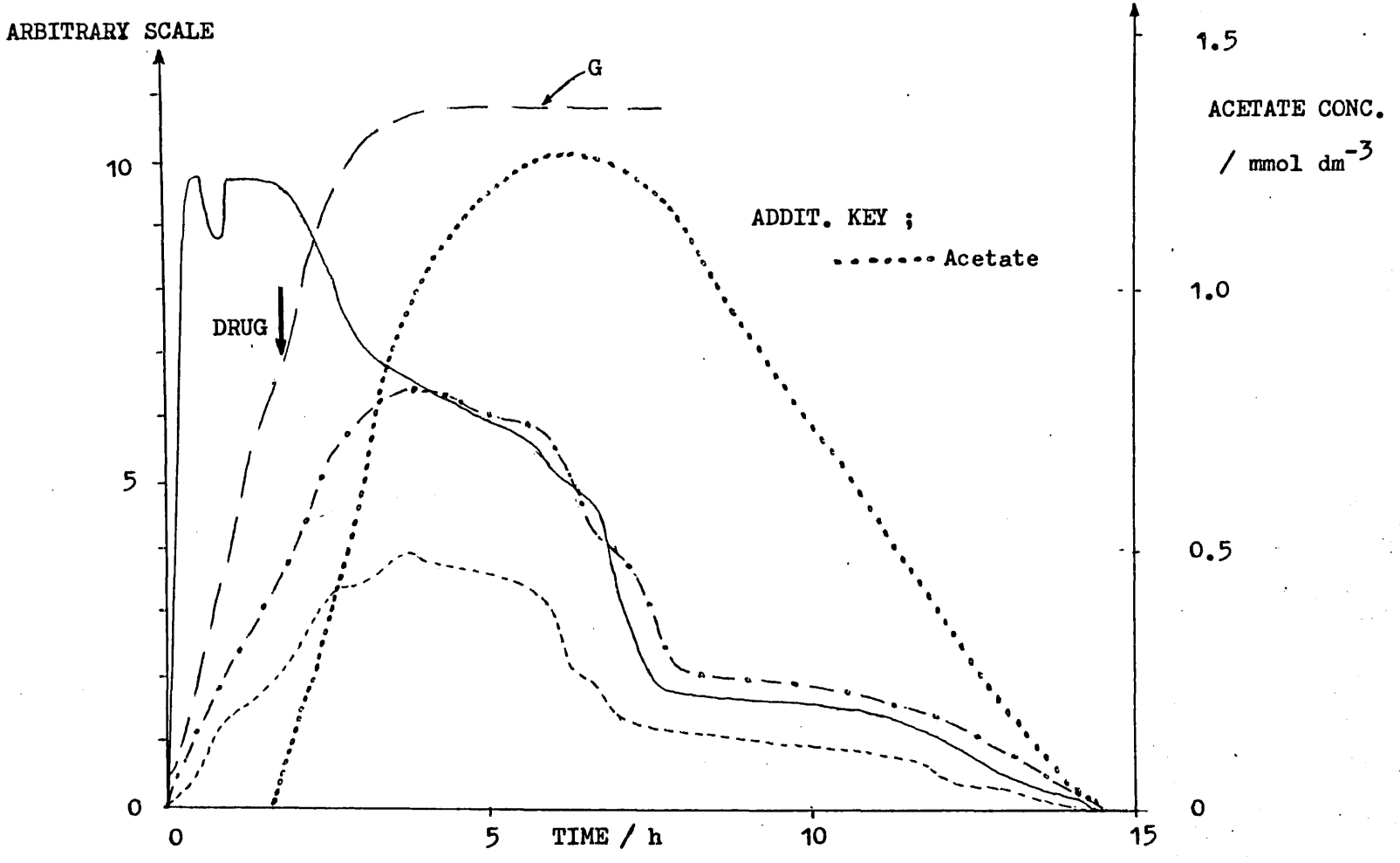
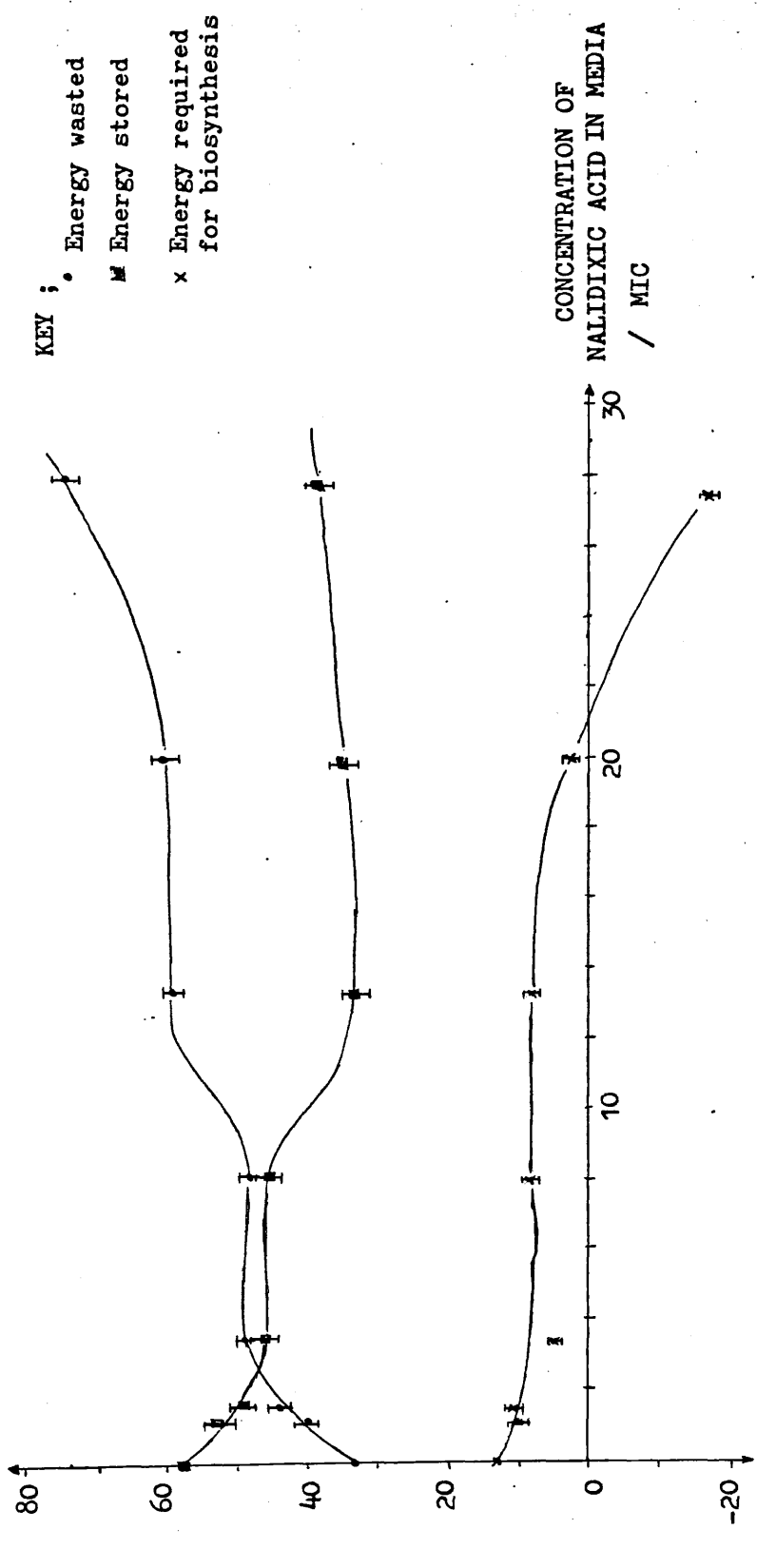


FIGURE 9.3 Energy Balance as A Function of Nalidixic Acid Concentration for Cells Grown in Glucose Limited Media



highest NA concentration.

It is indeed strange that at the two highest NA concentrations the yield increased as on theoretical grounds it should have continued to decrease. This increase in yield may be due to the use of an erroneous absorbance/biomass calibration due to the different light scattering and absorption properties of elongated cells when compared to those of control cells; the elongation being at its maximum at these two concentrations. If this is the case then the calculated percentage of carbon stored in biomass (36.1 and 39.6%) would have been too high and would account for the high $C_{rec}$ values (104 and 113% respectively). Further this theory would also account for the increase in stored energy (based on the stored carbon values) and the subsequent smaller negative biosynthesis values. This argument was disproved in a subsequent experiment in which a new calibration curve (2.1.2) was constructed for bacterial cells grown in the presence of NA (1.422) mmol $dm^{-3}$). This calibration curve was coincident with that for the standard glucose-limited cells.

The changes in light-scattering and absorption properties of elongated cells may have been attributable to the associated mucus formed; most of which would have been removed during washing of the cells in the subsequent absorbance/biomass calibration procedure (2.1.2). Therefore the new calibration curve for elongated cells may have been coincident with that for control cells due to the loss of mucus.

## 9.3 The Growth of Cells Adapted to Nalidixic acid

Cells which had been maintained in glucose-limited media were adapted for growth in the presence of NA by subculturing (5 times) in glucose-limited (3.3 mmol $dm^{-3}$) media containing NA (0.022 mmol $dm^{-3}$).

These cells were then subcultured 10 times in medium of NA concentration 0.043 mmol $dm^{-3}$. At this point the culture was divided into two; the cells in one set were subcultured a further 10 times at 0.043 mmol $dm^{-3}$ and cells in the other set were subcultured 10 times at 0.086 mmol $dm^{-3}$. The relatively low training concentrations were chosen so that training did not take an unacceptably long period of time, and that growth during training could be maintained easily. Cryogenically stored inocula in salts medium (2.1.2) were prepared from each of the two trained cultures. The trained cells were finally grown in medium (620 $cm^3$) containing the appropriate concentration of NA for the preparation of the cryogenic inocula.

Cells adapted to NA were then grown in glucose-limited (3.3 mmol $dm^{-3}$) media (630 $cm^3$), sometimes containing NA, under the standard conditions of growth for the measurement of thermal and environmental parameters.

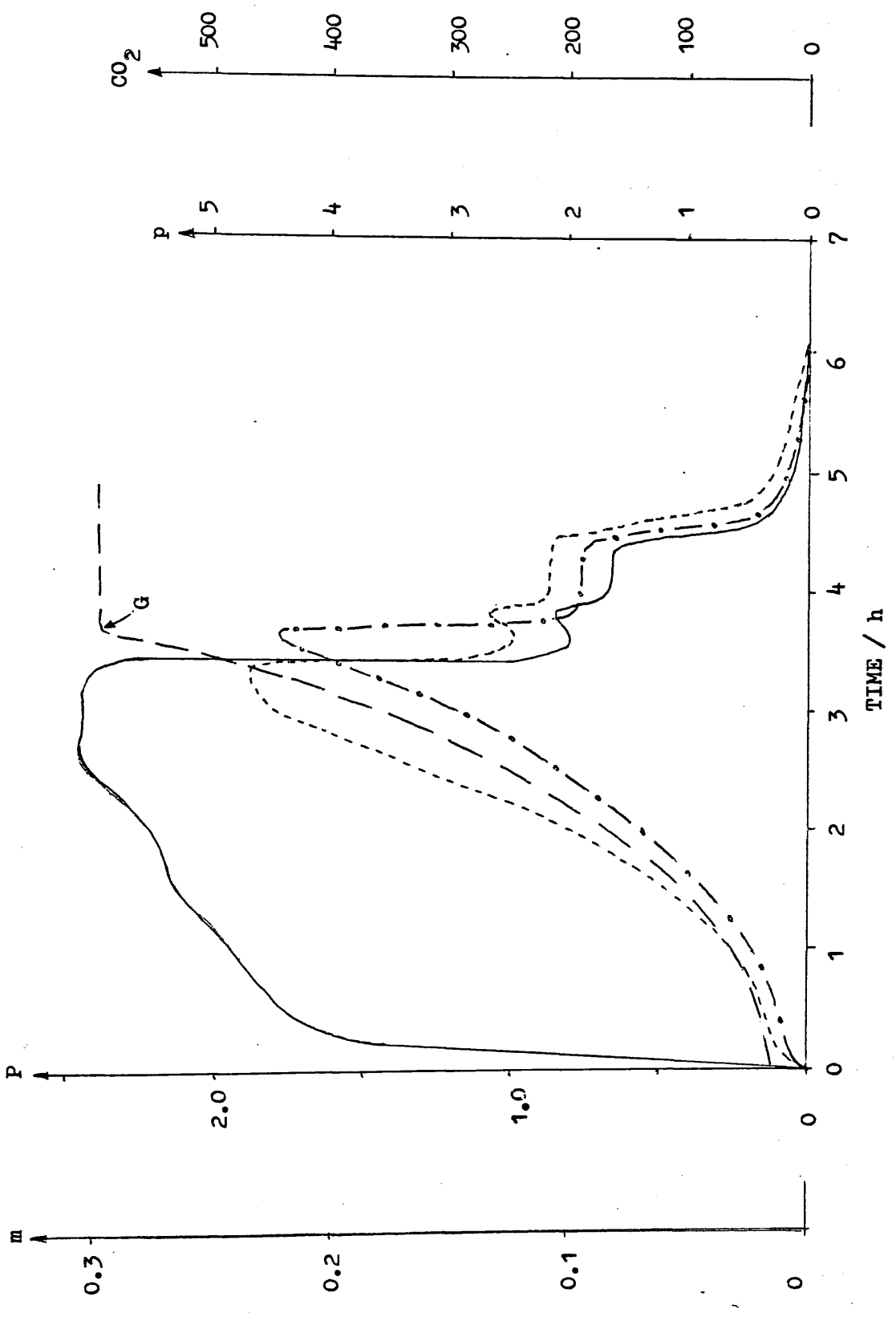
### 9.3.1 Changes in Growth Parameters when NA-Adapted Cells Are Grown in The Absence of the Drug

Typical p-t, P-t, m-t and $CO_2$-t traces for cells adapted to NA (0.043 mmol $dm^{-3}$) grown in glucose-limited medium under standard conditions are shown in Fig. 9.4. During exponential growth ($t_d$ = 48 min) secondary metabolites were produced and acetate was detected in the medium. On cessation of growth and exhaustion of the glucose these were metabolised. During growth the power increased at a greater rate than did the carbon dioxide output; the p-t and $CO_2$-t traces were not exactly similar and were displaced along the time axis. The specific power output increased rapidly on inoculation and during growth it increased gradually until immediately prior to cessation of growth it had reached a value of c. 2.4 J $s^{-1}$ $g^{-1}$. This is 20% more than that (2.0 J $s^{-1}$ $g^{-1}$) obtained during the growth of normal cells in glucose-limited medium.

FIGURE 9.4 The p - t, P - t and $CO_2$ - t Traces for Cells Adapted to Nalidixic Acid (0.043 mmol $dm^{-3}$) Grown in Drug Free Medium

KEY ;

| | | | |
|---|---|---|---|
| - - - - - - | POWER x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| ———— | SPECIFIC POWER | / J $s^{-1}g^{-1}$ | (P) |
| — · — · — | CARBON DIOXIDE | / p.p.m. | ($CO_2$) |
| — — | BIOMASS | / mg $cm^{-3}$ | (m) |


m
0.3
0.2
0.1
0
P
2.0
1.0
0
G
TIME / h
0 1 2 3 4 5 6 7
p
0 1 2 3 4 5
$CO_2$
0 100 200 300 400 500

On cessation of growth the power, specific power, and carbon dioxide outputs decreased rapidly to new values which were maintained during the consumption of the acetate and other secondary metabolites. (The specific power decreased to a new value of 0.7 J $s^{-1}$ $g^{-1}$). On exhaustion of these the p-t, P-t and $CO_2$-t traces reattained their respective base lines.

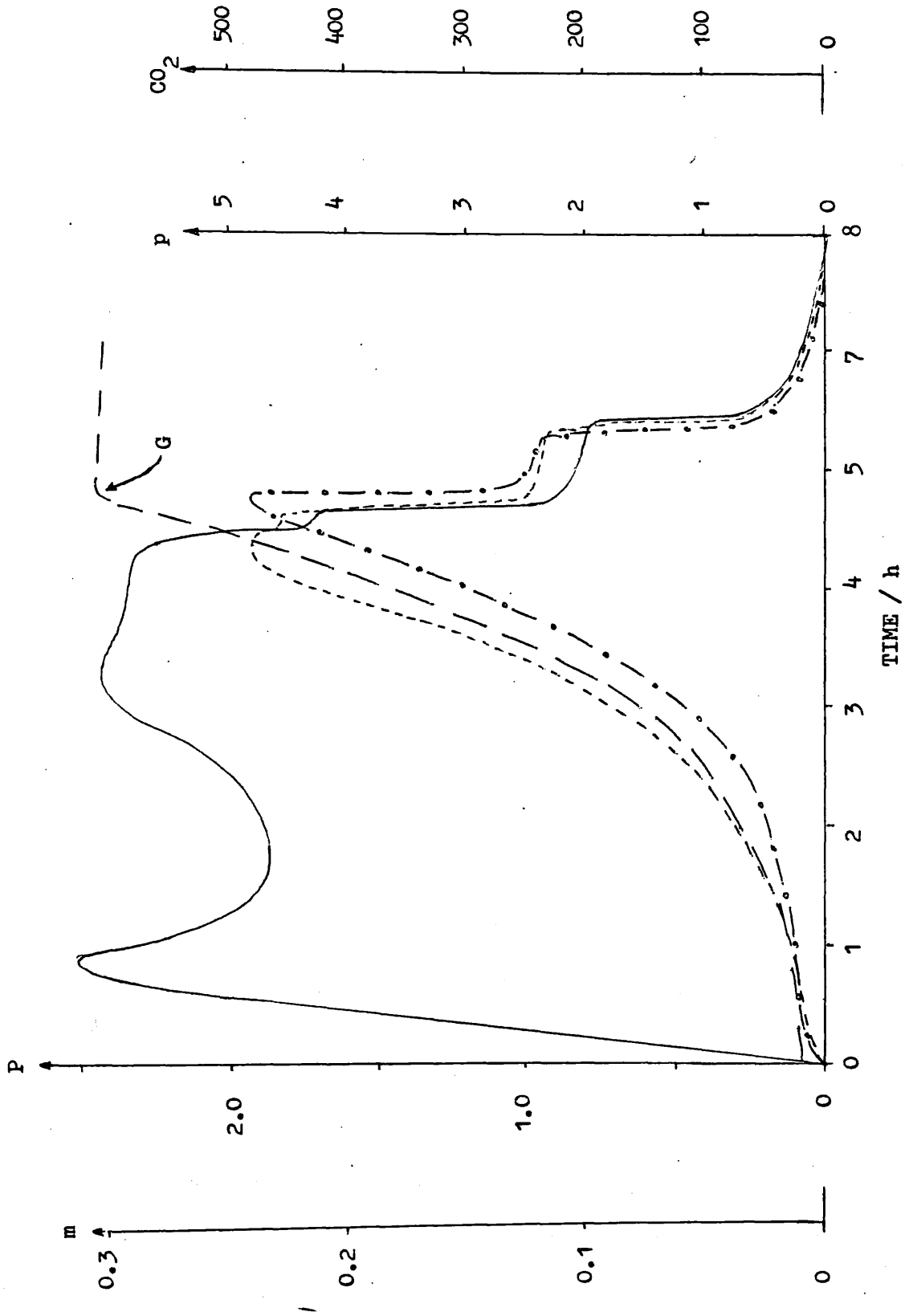
Typical p-t, P-t, m-t and $CO_2$-t traces for cells adapted to NA (0.086 mmol $dm^{-3}$) and grown in glucose-limited medium are shown in Fig. 9.5. During exponential growth ($t_d$ = 46 min) acetate was detected in the medium which was metabolised when the glucose was exhausted and growth had ceased. During growth the p-t and $CO_2$-t traces were similar to those for cells adapted at 0.043 mmol $dm^{-3}$ (Fig. 9.4), the power again increased at a greater rate than the carbon dioxide output. After inoculation the specific power output increased rapidly to c. 2.5 J $s^{-1}$ $g^{-1}$ and during the early stages of exponential growth gradually decreased to 2.0 J $s^{-1}$ $g^{-1}$. The specific power output then increased to 2.5 J $s^{-1}$ $g^{-1}$; a value which was maintained during the latter stages of growth. On cessation of growth the power, specific power and carbon dioxide outputs decreased rapidly to new values which were maintained during the consumption of the secondary metabolite(s). (The specific power maintained a value of c. 0.8 J $s^{-1}$ $g^{-1}$.) On exhaustion of the secondary metabolite(s) the p-t, P-t and $CO_2$-t traces reattained their respective base lines.

For both sets of adapted cells the p-t and $CO_2$-t traces did not resemble those for normal cells grown in glucose-limited medium (Figs 5.3, 5.4 c, 9.1 a); the traces for adapted cells were more rounded in shape and during growth the rate of increase in the power output was greater than that of the carbon dioxide. Further the P-t traces were unlike those for normal cells; the specific power output reached and

FIGURE 9.5 The p - t, P - t and $CO_2$ - t Traces for Cells Adapted to Nalidixic Acid (0.086 mmol $dm^{-3}$) Grown in Drug Free Medium

KEY ;

| | | |
|---|---|---|
| POWER x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| SPECIFIC POWER | / J $s^{-1}g^{-1}$ | (P) |
| CARBON DIOXIDE | / p.p.m. | ($CO_2$) |
| BIOMASS | / mg $cm^{-3}$ | (m) |


m
0.3
0.2
0.1
0
P
2.0
1.0
0
G
$CO_2$
500
400
300
200
100
0
p
5
4
3
2
1
0
0
1
2
3
4
5
6
7
8
TIME / h

sometimes maintained values of c. 2.5 J $s^{-1}$ $g^{-1}$. The specific power value for the growth of normal cells was 1.9-2.0 J $s^{-1}$ $g^{-1}$ (Figs 5.3, 5.4 c, 9.1 a).

The mass and energy balances for both sets of adapted cells (Table 9.4) are not significantly different from each other. The yields (0.490 g $g^{-1}$), carbon recoveries (98 and 96%), waste heats (33.3 and 32.5%) and biosynthesis values, Δ%, (11.4 and 12.2%) are not significantly different. Further the values of these different parameters do not differ significantly from those obtained for the growth of standard glucose-cells grown in glucose-limited media (control).

### 9.3.2 Changes in Growth Parameters when NA-Adapted Cells Are Grown in The presence of The Drug

The adapted cells (0.043 or 0.086 mmol $dm^{-3}$) were grown in glucose-limited media containing NA (0.043 or 0.086 mmol $dm^{-3}$ respectively); the NA was incorporated in the medium prior to inoculation.

The p-t, P-t, m-t and $CO_2$-t traces for adapted cells (0.043 mmol $dm^{-3}$) were closely similar to those for ordinary cells growing in glucose-limited media i.e. control (Figs 5.3, 5.4 c, 9.1 a) but were quite unlike those for the adapted cells grown in glucose-limited medium not containing NA (Fig. 9.4). There were no secondary metabolites formed during exponential growth ($t_d$ = 48 min) and therefore no secondary power or carbon dioxide output was detected.

For cells trained and grown at the higher concentration (0.086 mmol $dm^{-3}$) the power and carbon dioxide outputs rose exponentially during growth; the release of power and carbon dioxide being in phase with each other (Fig. 9.6) in contrast to those out of phase for adapted cells grown in glucose-limited media (Figs 9.4, 9.5). On cessation of growth

TABLE 9.4 Mass and Energy Balance for Cells Adapted to Nalidixic Acid

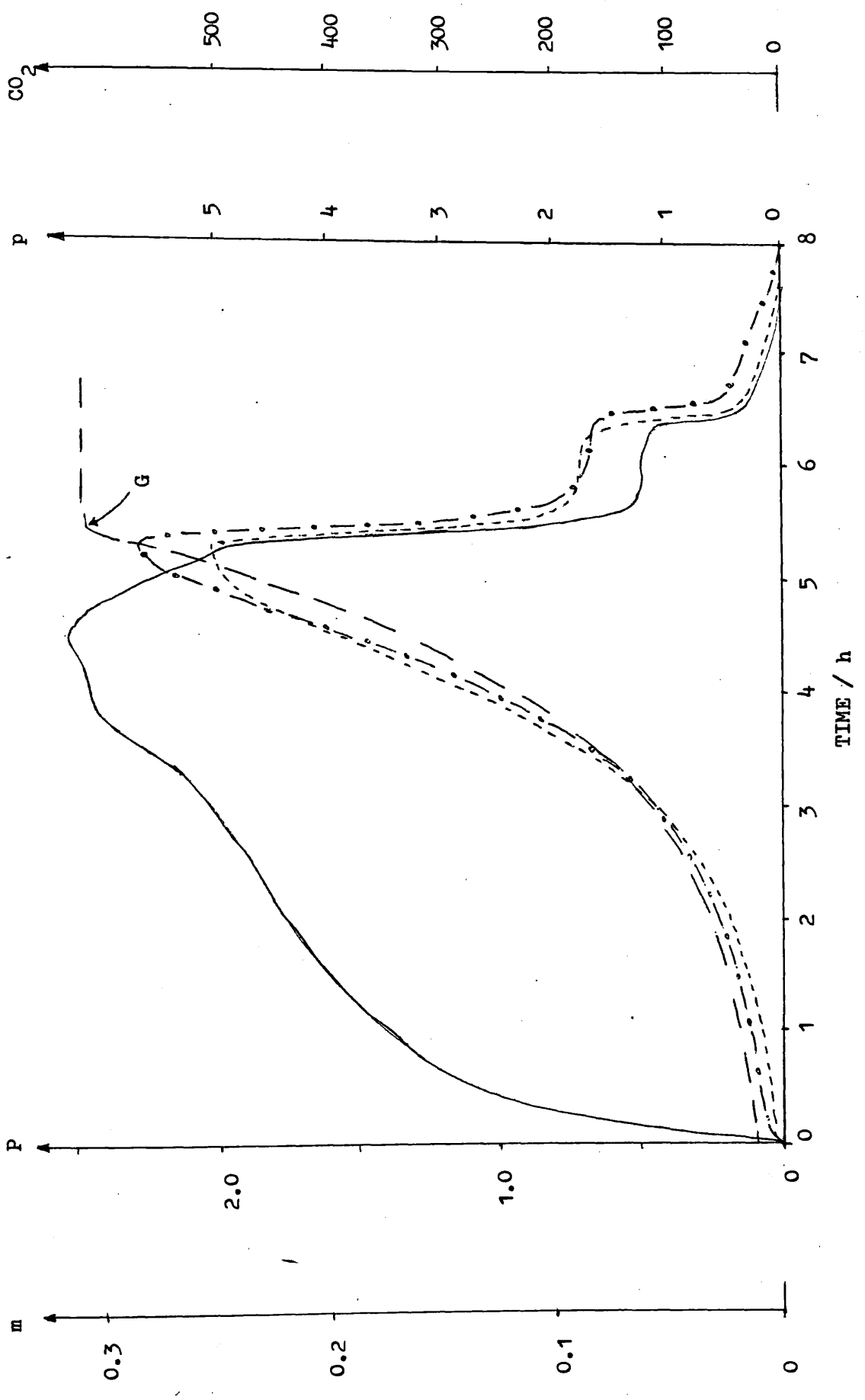
| CONC. OF NA AT WHICH CELLS WERE ADAPTED /mmol $dm^{-3}$ | CONC. OF NA IN TEST MEDIA /mmol $dm^{-3}$ | YIELD /g $g^{-1}$ | % CARBON | | | % ENERGY | | |
|---|---|---|---|---|---|---|---|---|
| | | | AS $CO_2$ | AS BIOMASS | $C_{rec}$ | WASTED | STORED | BIOSYN. Δ% |
| 0+ | 0+ | 0.496 | 42.4 | 56.0 | 98 | 31.9 | 56.0 | 12.1 |
| 0.043 | 0 | 0.490 | 42.3 | 55.3 | 98 | 33.3 | 55.3 | 11.4 |
| 0.086 | 0 | 0.490 | 40.2 | 55.3 | 96 | 32.5 | 55.3 | 12.2 |
| 0.043 | 0.043 | 0.490 | 42.3 | 55.3 | 101 | 32.0 | 55.3 | 12.7 |
| 0.086 | 0.086 | 0.503 | 48.4 | 56.3 | 105 | 36.1 | 56.3 | 7.6 |
| 0.043 | 0.069* | 0.471 | 42.7 | 53.6 | 96 | 37.8 | 53.6 | 8.6 |
| 0.086 | 0.069* | 0.492 | 49.1 | 55.1 | 104 | 36.4 | 55.1 | 8.5 |

* NA added after inoculation at a biomass of 0.0823 mg $cm^{-3}$ +Control

FIGURE 9.6 p - t, P -t and $CO_2$- t Traces for Cells Adapted to Nalidixic Acid (0.086 mmol $dm^{-3}$) Grown in Glucose Limited Medium Containing Nalidixic Acid (0.086 mmol $dm^{-3}$)

KEY ;

| | | | |
|---|---|---|---|
| - - - - - - | POWER x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| ——— | SPECIFIC POWER | / J $s^{-1}g^{-1}$ | (P) |
| —. —. — | CARBON DIOXIDE | / p.p.m. | ($CO_2$) |
| — — | BIOMASS | / mg $cm^{-3}$ | (m) |


m
0.3
0.2
0.1
0
P
2.0
1.0
0
p
5
4
3
2
1
0
$CO_2$
500
400
300
200
100
0
G
0
1
2
3
4
5
6
7
8
TIME / h

and the exhaustion of the glucose the power and carbon dioxide outputs decreased rapidly to new values which were maintained during consumption of the secondary metabolite(s); including acetate which was produced during growth. On inoculation the specific power increased rapidly to a value of c. 1.0 J $s^{-1}$ $g^{-1}$ and then gradually during the rest of the growth phase to reach a value of c. 2.5 J $s^{-1}$ $g^{-1}$. On cessation of growth the specific power fell rapidly to attain a lower constant value (c. 0.5 J $s^{-1}$ $g^{-1}$) during acetate metabolism. On exhaustion of the acetate the P-t trace reattained its base line.

The mass and energy balances (Table 9.4) for cells adapted to 0.043 mmol $dm^{-3}$ NA grown in medium containing NA (0.043 mmol $dm^{-3}$) are similar to those obtained for ordinary cells grown in glucose-limited drug free medium.

The mass and energy balances for adapted cells (0.086 mmol $dm^{-3}$) grown in medium containing NA (0.086 mmol $dm^{-3}$) are dissimilar to those obtained for ordinary cells grown in glucose-limited medium in the absence of the drug (Table 9.4). The cells trained to 0.086 mmol $dm^{-3}$ produced the same yield as for the control but the waste energy increased (31.9 to 36.1%) and consequently the biosynthesis value (Δ%) decreased (12.1 to 7.6%).

### 9.3.3 Changes in Growth Parameters when NA is Added to NA-Trained Cells Growing in Drug Free Medium

The adapted cells (0.043 or 0.086 mmol $dm^{-3}$) were grown in glucose-limited media; the NA solution was injected into the medium at a biomass of 0.082 mg $cm^{-3}$ (9.1). These experiments were a repeat of those when ordinary cells were grown in glucose-limited media containing NA (0.690 mmol $dm^{-3}$) (Fig. 9.1 f) except that the cells used were trained to NA.

For cells adapted to 0.043 mmol $dm^{-3}$ NA the power and carbon dioxide outputs (Fig. 9.7) increased steadily during growth; during the latter stages of growth the doubling time ($t_d$) increased and consequently the rate of increase was reduced. The rate of increase of the power output was greater than that of the increase in carbon dioxide production. On cessation of growth and exhaustion of the glucose the power and carbon dioxide outputs decreased rapidly to maintain constant new values during consumption of the secondary metabolite(s); acetate, which had been produced during growth. On inoculation the specific power increased rapidly to a value of c. 2.4 J $s^{-1}$ $g^{-1}$ and then during growth to c. 2.8 J $s^{-1}$ $g^{-1}$. Towards the end of growth the specific power output decreased to a value of c. 1.0 J $s^{-1}$ $g^{-1}$ which was maintained during consumption of the secondary metabolite(s). On exhaustion of this the p-t, P-t and $CO_2$-t traces reattained their respective base lines.

For cells adapted to 0.086 mmol $dm^{-3}$ NA the power and carbon dioxide outputs (Fig. 9.8) rose steadily during growth; except during the latter stages when the doubling time ($t_d$) increased. The cells exhibited a greater rate of increase in the heat output than in the carbon dioxide output. On cessation of growth and exhaustion of the glucose the power and carbon dioxide outputs decreased rapidly to maintain constant values during consumption of the secondary metabolite(s); acetate which had been produced during growth. The rate of growth slowed continually after the introduction of the drug. On exhaustion of the secondary metabolite the power output decreased rapidly to zero. The overall shape of the p-t trace is not exactly similar to that for cells trained at the lower concentration (Fig. 9.7) but they are similar in character.

The specific power (Fig. 9.8) rose rapidly after inoculation to a maximum value of c. 3.7 J $s^{-1}$ $g^{-1}$, thereafter decreasing rapidly to c. 2.8 J $s^{-1}$ $g^{-1}$ which was maintained for the rest of growth. On cessation

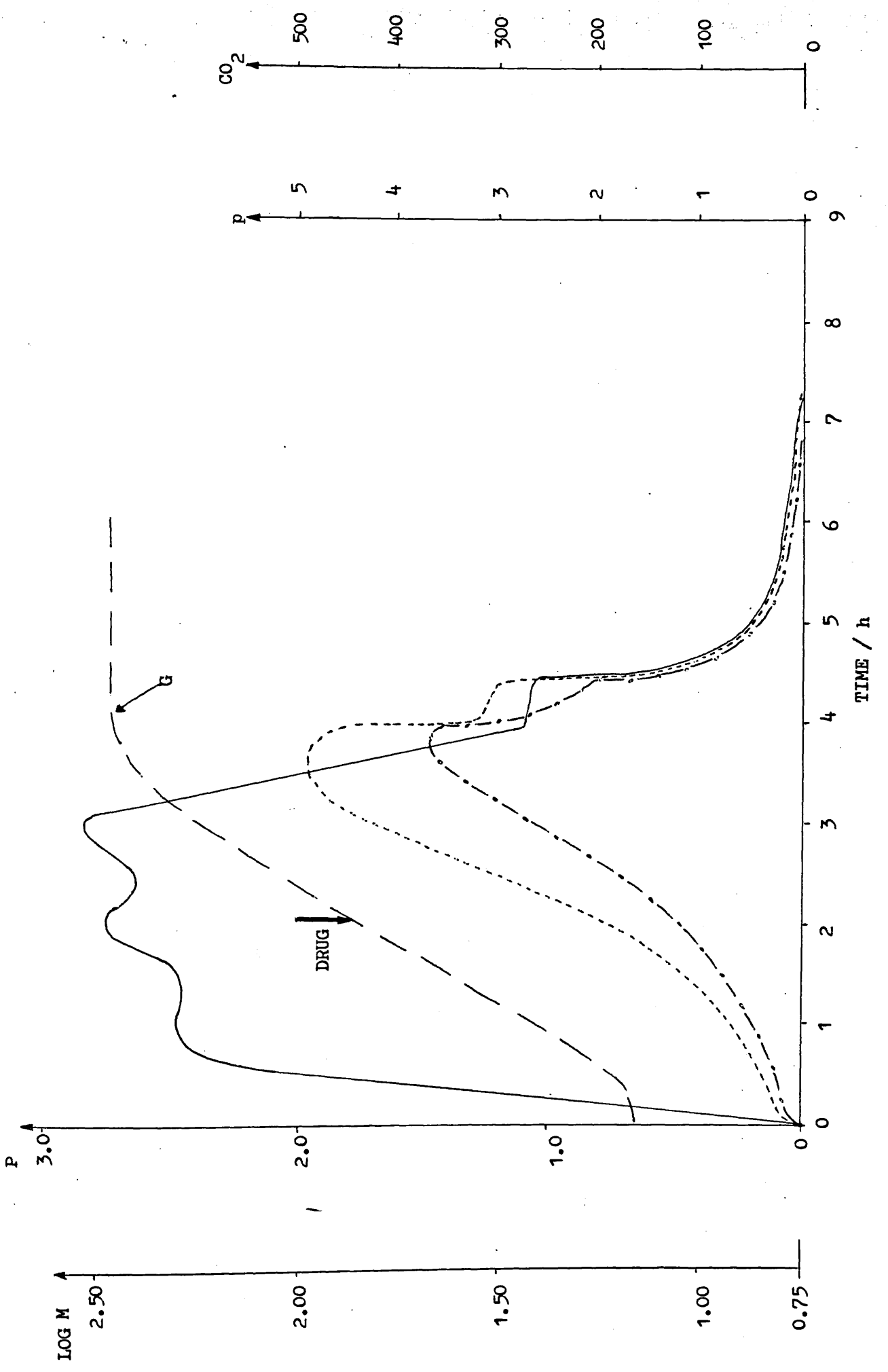
FIGURE 9.7 p - t, P - t and $CO_2$ - t Traces for Cells Adapted to Nalidixic Acid (0.043 mmol $dm^{-3}$) Grown in Glucose Limited Medium Containing Nalidixic Acid (0.690 mmol $dm^{-3}$)

KEY ;

| | | | |
|---|---|---|---|
| - - - - - | POWER x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| ——— | SPECIFIC POWER | / J $s^{-1}g^{-1}$ | (P) |
| ,— . —. | CARBON DIOXIDE | / p.p.m. | ($CO_2$) |
| — — | LOG BIOMASS | /mg $dm^{-3}$ | (LOG M) |

DRUG ↓ : Introduction of nalidixic acid into growing culture

G : End of growth and exhaustion of the glucose


LOG M
2.50
2.00
1.50
1.00
0.75
P
3.0
2.0
1.0
0
G
DRUG
0
1
2
3
4
5
6
7
8
9
TIME / h
p
0
1
2
3
4
5
$CO_2$
0
100
200
300
400
500

of growth the specific power decreased rapidly to a value of c. 0.8 J $s^{-1}$ $g^{-1}$ which was maintained during consumption of the secondary metabolite(s). On exhaustion of these the specific power rapidly decreased to zero. The initial peak in the specific power output after inoculation was probably due to the perceptable lag phase incurred. The numerical values of the specific power output (2.5-3.0 J $s^{-1}$ $g^{-1}$) for cells adapted to 0.043 mmol $dm^{-3}$ NA during growth was similar to that (2.4-2.9 J $s^{-1}$ $g^{-1}$) for the cells trained to the higher drug concentration (Figs 9.7, 9.8).

The p-t, P-t and $CO_2$-t traces for adapted cells (0.043 and 0.086 mmol $dm^{-3}$) grown in media containing NA (0.690 mmol $dm^{-3}$) were unlike those obtained when ordinary cells were grown in glucose-limited media i.e. the control (Figs 5.3, 5.4 c, 9.1 a). The p-t and $CO_2$-t traces for adapted cells showed secondary power and carbon dioxide outputs; traces for ordinary cells did not. The specific power outputs during growth maintained a value of c. 2.7 J $s^{-1}$ $g^{-1}$ whereas the value for normal cells was 1.9 J $s^{-1}$ $g^{-1}$.

NA (0.690 mmol $dm^{-3}$) had a much greater effect on the growth of untrained cells (Fig. 9.1 f) than on trained cells (Figs 9.7, 9.8). For adapted cells the rate of growth in the presence of NA was greater than that for ordinary cells, and the amount of secondary metabolites formed was less, hence the secondary power and carbon dioxide outputs were smaller. Further the specific power (c. 2.7 J $s^{-1}$ $g^{-1}$) of adapted cells during growth was fairly constant, which was in contrast to that of standard cells when the specific power decreased from c. 1.9 J $s^{-1}$ $g^{-1}$ at the start of growth to c. 0.9 J $s^{-1}$ $g^{-1}$ at the end.

The mass and energy balances for the growth of the two adapted cells in media containing NA (0.690 mmol $dm^{-3}$) (Table 9.4) were similar; the yields (c. 0.48 g $g^{-1}$), waste energies (c. 37%) and biosynthesis

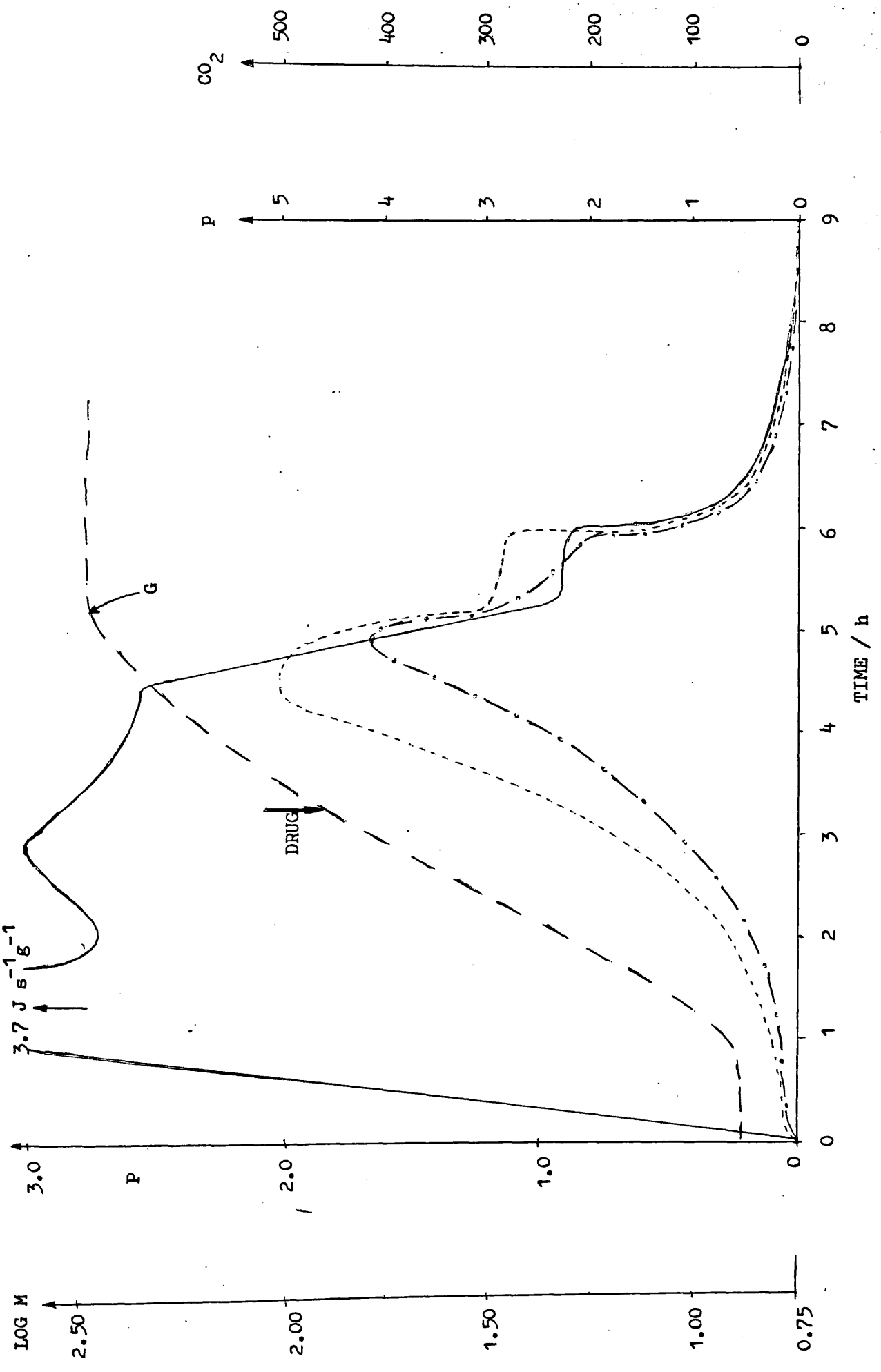
FIGURE 9.8 p - t, P - t and $CO_2$ - t Traces for Cells Adapted to Nalidixic Acid (0.086 mmol $dm^{-3}$) Grown in Glucose Limited Medium Containing Nalidixic Acid (0.690 mmol $dm^{-3}$)

KEY ;

| | | | |
|---|---|---|---|
| - - - - - | POWER x $10^4$ | / J $s^{-1}cm^{-3}$ | (p) |
| ——— | SPECIFIC POWER | / J $s^{-1}g^{-1}$ | (P) |
| .—.—.—. | CARBON DIOXIDE | / p.p.m. | ($CO_2$) |
| — —— | LOG BIOMASS | / mg $dm^{-3}$ | (LOG M) |

DRUG ↓ : Introduction of nalidixic acid into the growing culture

G : End of growth and exhaustion of the glucose


LOG M
2.50
2.00
1.50
1.00
0.75
P
3.0
2.0
1.0
0
3.7 J s⁻¹g⁻¹
G
DRUG
0
1
2
3
4
5
6
7
8
9
TIME / h
p
5
4
3
2
1
0
CO2
500
400
300
200
100
0

values, Δ%, (c. 8.5%) were not significantly different. It is immediately apparent that although the yield was not substantially different from that of the control the waste heat had increased from 31.9 to C. 37% and the biosynthesis values (Δ%) decreased from 12.1 to c. 8.5%. Further a comparison of mass and energy balances for normal and trained cells grown in the presence of NA (0.069 mmol $dm^{-3}$) (Tables 9.2, 9.3, 9.4) revealed an increase in the yield (0.319 to 0.48 g $g^{-1}$), a decrease in the waste heat (61.6 to 37%) and an increase in the biosynthesis value (2.3 to 8.5%).

## 9.4 Summary

1. The addition of NA to a growing culture of untrained cells reduced the growth rate and produced secondary metabolites. The specific power output and biosynthesis value were reduced, and the waste heat increased compared to the values for cells grown in drug free medium (control). The greater the NA concentration the greater the effect on each parameter.

2. Trained cells grown in drug free media exhibited different metabolic pathways to that of the control; secondary metabolites were produced and the p-t, P-t and $CO_2$-t traces were dissimilar to those of the control although the mass and energy balances were similar.

3. The growth of cells trained to 0.043 mmol $dm^{-3}$ NA in media of the same drug concentration was similar to that of control cells but the growth of cells adapted to the higher concentration in media containing NA (0.086 mmol $dm^{-3}$) was not; the waste energy increased and the biosynthesis value decreased as compared to the control.

4. The effect of NA (0.069 mmol $dm^{-3}$) on the growth of adapted cells was less drastic than that on the growth of ordinary cells, although the growth of adapted cells in the presence of NA did not fully revert to that of ordinary cells in glucose-limited media (control).

# CHAPTER TEN

# DISCUSSION

The crucial role of ATP in living organisms has been clear since 1941 (Lipmann). However it was not until 1961 that an attempt was made to measure the ATP pool of a growing micro-organism (Franzen and Binkley). This had been made possible by the recent development of a luciferin-luciferase system which facilitated the estimation of low concentrations of ATP (Strehler and McElroy, 1957). During the 1960's many such studies were undertaken which culminated in the then provocative concept of metabolic regulation by adenine nucleotides, proposed by Atkinson (1968). This now generally accepted theory is described numerically by the adenylate energy charge (EC), which

$$EC = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$$

is a linear function from 0 (all AMP) to 1 (all ATP) of the proportion of the total adenylate pool that contains anhydride-bound phosphate of high free energy of hydrolysis. Substrates are catabolised by microbial cells to a limited number of small molecules. These are interconverted, as necessary, and serve as precursors for synthesis of the macromolecular constituents of the cell via the anabolic or biosynthetic pathways. However, catabolism and anabolism are not linked only by these building block molecules. Net synthesis of ATP from ADP occurs during catabolism. The ATP is used to drive endergonic biosynthetic pathways, and is converted to ADP and AMP. The adenine nucleotides are also intercoverted by the action of adenylate kinase (Noda, 1973). ATP, ADP and AMP therefore act as metabolic "energy" mediators and are ideally placed to regulate the whole metabolic economy of the cell. In general, catabolic sequences contain regulatory enzymes that are activated by ADP or AMP, or inhibited by ATP: catabolism of substrates therefore proceeds maximally

only when ATP needs to be regenerated. Conversely, many regulatory enzymes of ATP-consuming biosynthetic pathways are activated by ATP or inhibited by ADP or AMP. During the exponential growth of K. aerogenes in glucose limited media (3.3 mmol $dm^{-3}$) the ATP pool value averages 7.8 x $10^{-6}$ mol $g^{-1}$ (4.1), which corresponds to c. 0.4% by weight of all the compounds in the cell. It has been estimated (Roberts et al., 1957) that small molecular weight metabolites represent about 8% of the dry weight of E. coli. Assuming a similar figure for K. aerogenes then ATP is present as 5% of all the small molecules. Values for the volume of water contained in various bacteria average at 3 $cm^3$ $g^{-1}$ (Winkler and Wilson, 1966). Consequently the intracellular ATP concentration for aerobically growing cells of K. aerogenes is 2.6 mmol $dm^{-3}$. The relatively large amounts of ATP in the microbial cell clearly indicates its vitally important role in metabolism. Much work in the past has been concerned with correlating the EC value with the growth of bacteria, rather than the ATP pool with metabolic activity. However it has been shown in this work and by others that under different conditions the ATP pool varies greatly (x 10) and is therefore indicative of overall metabolic activity. Another indicator of the overall metabolic activity of the microbial cell is the waste heat produced as revealed by microcalorimetric measurements. This work utilises (in tandem) both these indicators (ATP analysis, microcalorimetry) to investigate some aspects of bacterial metabolism.

A prominent character of microbial metabolism is the generation of waste heat. The heat as measured by the microcalorimeter represents the sum of heat from the overall complex metabolism, rather than the heat of some isolated metabolic reaction. Thus calorimetry is non-specific by nature (as is the ATP pool) and to be meaningful has to be supplemented by other biological data, i.e. carbon dioxide evolution, biomass formation, chemical/enzymic analysis of growth medium etc. The p-t trace

of growing microbial cells has been widely acclaimed to be characteristic of the cells and therefore useful as an identification technique. The use of p-t traces for the identification of microorganisms was implied by Rubner (1906) and later by Prat (1953). The recent availability of sensitive commercial calorimeters has confirmed this claim (Weber 1966; Boiling et al., 1973; Staples et al., 1973; Russel et al., 1975; Monk and Wadso 1975; Johansson et al., 1975; Schaarschmidt and Lamprecht, 1976; Kallings and Hoffner, 1977; Fujita et al., 1978). There are only a few instances in the cited literature where reservations have been expressed about the use of calorimetry for identification purposes. These are based on observations of the effects on the p-t trace of inoculum density (Weber, 1966; McClaire and Pilsworth, 1976) and history and maintenance of the organism (Russel et al., 1975). But even with such reservations these authors are of the opinion that under strictly controlled conditions, identification is possible. The only author who is of the opinion that identification is not possible and that "characterisation" of micro-organisms under restricted conditions might be possible is Beezer et al., (1976), Beezer (1977) and Beezer et al., (1978). It was established that inoculum size and the state (past history) of the inoculum does effect the shape of the p-t trace and the overall mass and energy balances calculated for the growth. Therefore in order to establish a standard identification procedure the inoculum must be in a standard state. I believe the problems associated with producing a standard inoculum from clinical/biological samples far outweigh the advantages of microcalorimetry over other more classical identification techniques. Further even if these problems are overcome. I also believe that only "characterisation", rather than identification will be possible.

Many microcalorimetric investigations on the growth of various

yeasts and bacteria have been reported over the last decade. These have dealt with metabolic activities and energy balances during growth on various substrates under widely different conditions, e.g. aerobiosis, drug influence etc. Most experiments were carried out in batch calorimeters, under ill-defined "standard conditions", with the cell cultures enclosed within the calorimetric vessel (Yasufumi, 1981); in which the culture vessel is contained in the calorimeter and serves the dual function of both fermenter and calorimeter cell. The disadvantage of these systems is that the culture is not easily accessible, without thermal disturbance for other measurements (i.e. carbon dioxide output, biomass, growth medium analysis etc), which are required to interpret heat measurements in a meaningful manner. Most early work involved the study of anaerobic growth where the problems associated with aeration and oxygen starvation can be ignored. The use of a flow calorimeter combined with a fermenter was first introduced by Eriksson and Wadsö (1971) to monitor the heat output of bacterial cells growing exponentially. Problems associated with oxygen starvation in the calorimeter flow line during aerobic growth studies have perpetuated the use of batch calorimeters, even though this presents some major disadvantages when compared to flow calorimetry. The problems of oxygen deficiency in the flow lines to the cell in the flow calorimeter resulted in studies of the power output during the anaerobic growth of batch cultures, or aerobic/ anaerobic chemostat cultures where oxygen starvation is not a problem. Brettel (1980) is the only worker to have successfully measured the heat output by flow microcalorimetry of cells growing exponentially under truly aerobic conditions using high initial substrate concentrations (100 mmol $dm^{-3}$ glucose). This was achieved by aerating the calorimeter flow line and cell. In this work and in previous work in this laboratory (Nichols, 1980) low initial substrate concentrations ( 5 mmol $dm^{-3}$) have

been used for aerobic growth in batch culture to avoid oxygen starvation and semi-aerobic growth in the calorimeter flow line and cell (5.3, 5.5).

In general flow microcalorimetry as a tool to record the growth and metabolism of microbial cultures has major advantages over other classical techniques i.e. turbity (lower sensitivity and slower response); oxygen consumption (limited to aerobic cultures); infrared determination of carbon dioxide (variable response under different growth conditions); cell mass (lower sensitivity and slower response). Further flow calorimetry has the advantages that measurements are independent of the culture volume, are continuously monitored, and they are not disturbed through the stirring of, and the addition of materials to the culture. As well as the use of microcalorimetry to identify micro-organisms and to study their metabolism there are several other major applications. As the heat measured (under standard conditions of metabolism and test) is proportional to the number of microorganisms present, microcalorimetry has been used to give bacterial populations in clinical culture media and urine samples (Beezer et al., 1979a), and milk (Cliffe et al., 1973). Microcalorimetry has long been used to study the effects of a wide variety of stimulants, inhibitors, drugs and anti-biotics on microorganisms; the effect of penicillins on E. coli (Mardh, 1979), the effect of salt on yeasts (Gustafsson and Norkrans, 1976), antibacterial action of tetracyclines (Moberg, 1977). Some recent developments in the application of microcalorimetry include the measure-ment of the heat produced from intact organs and tissues such as rat livers (Baisch, 1978) and human epidermis (Anders et al., 1979).

Only one author (Gastafsson, 1979) has utilised microcalorimetry (flow) and ATP analysis in tandem to study the metabolism of growing microbes. As in this work the specific power output (6.1) and ATP pool of metabolising cells has been correlated with each other. It is

certainly curious that besides this work there is only one other example of metabolic studies, utilising both heat and ATP measurements; as both are indicative of overall metabolism and therefore ideal tools to use together to elucidate cell metabolism. The many practical and technical problems which have to be overcome to successfully establish and operate both techniques in tandem may be the reason for this.

Emphasis has been placed on the establishment and use of standard and defined conditions of growth and test, in order to obtain quantitative and reproducible data. Further, so that this data can be interpreted meaningfully, several different types of measurement have been used together. In this respect the use of chemically defined and simple media with a growth limiting nutrient is essential.

The linearity of the ATP measurement over the tested range both for standard ATP solutions (Fig. 3.12) and bacterial suspensions (Fig. 3.15), as well as the reproducibility (±3-4.5%, $p = 0.95$) and recovery of ATP (98.7%) (Table 3.3) is excellent for a biological system, considering the complexity of the overall procedure, (sampling, extraction and analysis). In the literature recovery of ATP from standard solutions (using TCA extraction) is nearly always near to 100%, but rarely does bacterial ATP data have reproducibility levels quoted (Chapelle and Levin, 1968; Bomsel and Pradet, 1968; Sharp et al., 1970; Chapman et al., 1971; Holms et al., 1972; Nilsson et al., 1977; Gustafsson, 1979). When they are stated it is usually impossible to determine the reproducibility levels for an ATP measurement under the experimental conditions of growth and test (Gadkari and Stolp, 1975). When the reproducibility levels ($p = 0.95$) under the experimental conditions of growth and test can be calculated from the stated statistical data, the levels are very much

worse when compared to those obtained in this work i.e. ±20% (Cole and Wimpenny, 1967), ±13% (Lloyd et al., 1978). Some reproducibility levels are better such as ±7% (Lundin and Thore, 1975). This value refers to the extraction/analysis of standard ATP solutions and therefore excludes the errors likely to be introduced by a sampling technique performed under the conditions of growth, and the instability of the resultant bacterial ATP extract. Accurate knowledge of the reproducibility levels for the overall technique under the conditions of growth and test are necessary if changes of the ATP pool during growth are to be evaluated correctly. Quantitative microcalorimetric data for microbial growth in the literature rarely gives reproducibility levels, thus the significance of the results may not be easily assessed. The shape of the p-t curves are usually adequately illustrated so that a reasonably accurate interpretation can be made of the real conditions of growth and test. A very serious criticism of published calorimetric work is the lack of data on growth and other physical parameters; therefore there can be no correlation with thermal data and interpretation is difficult. Earlier papers tend to consider only qualitative changes in the shape of the p-t trace.

The reproducibility level (p = 0.95) for the detemrination of the total heat evolved, $\Delta H_T$/kJ (2.3.3) during aerobic growth in glucose-limited media was ±1% (Table 5.2), which is excellent for a biological system. This result is better than that of Nichols (1980) who quotes a level of ±3%. This increased reproducibility is probably due to use of more accurately defined standard conditions of growth and test. The assumption that this high degree of reproducibility (±1%) is applicable to heat measurements made under more complex conditions of growth, e.g. phosphate-limitation (Table 7.2), and in the presence of sulphanilamide (Table 8.1) or NA (Table 9.3), may not be fully valid. The amount of repetitive work carried out under the more complex growth conditions does

not allow a full statistical analysis, but indications are that the reproducibility is at a level of ±3%.

The reproducibility of a determined dry weight measurement is ±2% (2.2.1). The use of a spectrophotometer to monitor growth has already been discussed and is open to criticism (2.2.1). The biomass of "standard" cells was a linear function of the absorbance within the limits encountered, and as this property of a culture could easily be monitored continuously this method was chosen to record growth under various conditions. Although the shape and size (Hadjipetrou and Stouthamer, 1963) and internal volume (Nichols, 1980) of the cells change during the growth cycle and under different conditions of growth, the dry weight absorbance calibration curve (Fig. 2.2) constructed for glucose-limited standard cells was used throughout this work. This curve was found to be coincident with those constructed for phosphate-limited growth and for cells grown in the presence of a drug; which has already been discussed (9.2.2).

The reproducibility of the estimation of the carbon dioxide evolved (2.2.2) was ±2% (Table 5.2) for glucose-limited growth. This improved reproducibility (e.g. 10% Djaven, 1980; Nichols, 1980) is due to the improved accuracy of the measurement and control of the air entering the culture vessel under the experimental conditions of growth and test. Previous workers had erratic control of the air flow entering the culture vessel and erroneous flow measurements were made of the carbon dioxide enriched effluent gases. This improvement is reflected in the near complete recovery of initial carbon ($C_{rec}$) and the excellent reproducibility (±1.3%) (Table 5.2).

The elemental composition of the cells when grown in glucose-limited media (Table 5.1) is similar to that of published values (Wang et al., 1976; Herbert, 1976; Djavan, 1980; Nichols, 1980). Further the

composition is similar (except for phosphorous) to that of cells grown in phosphate-limited media (7.1.1 or 7.4). The phosphorous content of cells grown in phosphate-limited media ($2.5 \times 10^{-3}$ g $dm^{-3}$: 0.85% wt/wt; $5.5 \times 10^{-3}$ g $dm^{-3}$: 1.87% wt/wt) is lower when compared to that of cells grown in glucose-limited media (2.45% w/w). Thus under phosphate-limited conditions less phosphorous is available to be incorporated into cellular material.

The relevance, validity and physiological representability of the intracellular ATP pool value, as determined by the extraction technique has already been discussed in Section 3. The problems associated with the three integral steps of the technique; sampling (3.3), extraction (3.4) and assay (3.2) have been solved. The reproducibility levels for the technique were statistically calculated from results obtained using washed bacterial suspensions, in which cells were not metabolising. The reproducibility levels estimated from the results obtained using duplicate sets (x 4) of bacterial suspensions taken during the exponential growth phase (4.3.1, Table 4.1) is the same as that for washed suspensions. This is of importance as it proves that the bacterial ATP extraction technique is reproducible even during growth when the cells are in a dynamic state of metabolic activity.

Slow (4-10 s) sampling techniques were used in many of the estimates of intracellular ATP content during the 1960's. It was not appreciated until the end of that decade that the ATP pool of metabolising/growing microorganisms turns over many times per second (Harrison and Maitra, 1969; Holms et al., 1972; Miovic and Gibson, 1973). In view of this emphasis was placed upon extremely fast sampling techniques so that the metabolising bacterial sample was effectively

mixed with the extractant in a short time (0.1 s), to instantaneously quench the metabolic activity of the cells (Harrison and Maitra, 1969).

In this work the sampling technique is relatively slow; 1-2 s (3.3.2), when compared to earlier workers. Even so the intracellular ATP pool values obtained during the exponential phase of growth in aerated glucose media is similar to that of previous workers. Knowles (1977) suggested that in practice the conditions in the growth medium are usually maintained during the few seconds required to remove a sample from the growth medium and mix it with the extractant, but it is dangerous to rely on very slow ( 4 s) sampling techniques.

The data collected suggests that a change in the ATP Pool of metabolising/growing cells (which can be detected by the assay technique) caused by a change in the external environment takes 30-60 s. The environmental changes can be changes in the aeration or temperature of the medium, a change of the metabolisable substrate, introduction of a substrate or exhaustion of a substrate. It may well be true that the ATP turnover rate changes immediately in response to an environmental change but this obviously does not seem to affect the ATP pool initially. The evidence for the slow reaction to change (stability) of the ATP pool to changes in the external environment can be summarised thus:

(a) bacterial samples removed from the growth medium during exponential growth can remain in the sampling device for up to 10 s prior to injection into the extractant, without any significant change in the measured ATP pool. This not only illustrates the stability of the intracellular ATP pool when under stress due to changes in the temperature and aeration of the medium, but also that fast sampling times are unnecessary;

(b) changes in the ATP pool caused by changes in the external environment can be faithfully monitored using 5 minute sampling intervals (4.3.1, Fig. 4.5). On introducing the inoculum into the aerated glucose-medium (37°C) the rapid rise in the intracellular ATP pool upon commencement of growth can be monitored; as well as its fall on exhaustion of the glucose and cessation of growth. This shows that even with such drastic changes in the external environment endured by the cells the change in the ATP pool is relatively slow (30-60 s).

The reason for this relative stability of the ATP pool in respect to environmental changes may be entirely due to purely physical phenomena resulting from cell structure. A finite time is obviously required for molecular diffusion and transport across the cell wall and membrane. This is a prerequisite so that the effects of changes in the external environment can manifest themselves in the metabolism of the cell and hence eventually in the ATP pool. Another less probable reason for this stability may be the existence within the cell of a regulatory mechanism which "opposes" rapid changes in the cellular ATP content. This could be achieved by enzyme mediated processes which slow down the rate of utilisation of ATP (except for maintenance) and replenish the ATP pool by the breakdown of other phosphonucleotides such as $NAD^+$.

The EDTA incorporated in the TCA extractant chelates with the divalent metal ions present; most importantly magnesium. The removal of magnesium associated with enzyme systems partially destroys their tertiary structure and renders them irreversibly inactive. The inactivation of these enzymes, particularly ATP-ases by EDTA is well known (Chappelle and Levin, 1968; Pirt, 1969; Wettermark *et al.*, 1970; St. John, 1970; Sharpe *et al.*, 1970; Bagnare and Finch, 1972; Dhople and Hanks, 1973; Lundin and Thore 1975; Lloyd *et al.*, 1978). It is important

that the inactivation be irreversible as reversibly inactivated enzymes may become reactivated when magnesium is added; a requirement for the firefly luciferase assay. The magnesium is contained in the LKB monitoring reagent (3.2.1). The stability of the extract was increased by the addition of a crown ether (18, crown, 6) which chelates with univalent cations and may well inactivate enzymes in the same way as EDTA. Even though the stability constant for 18, crown, 6 and potassium is only 2-3 in aqueous media, (EDTA-magnesium has a stability constant of 8.7), the crown ether is still successful in removing monovalent cations from enzyme systems. This is because the stability constant between enzymes and monovalent cations is also small. The extreme stability of the bacterial ATP extract was illustrated in an experiment when after incubating the extract at 37°C for 20 min the ATP concentration was unchanged. The use of TCA to extract ATP from Gram negative bacteria like K. aerogenes with cell walls which are resistant to chemical and physical disintegration is regarded as the most acceptable method (Thore, 1979). The use of sulphuric acid as an extractant (4.2.2) resulted in a lower ATP recovery; this is regarded as the main criterion for assessing the success of an extraction procedure (Lundin and Thore, 1975). Other less successful and tested methods are boiling buffer solution (Chappelle and Levin, 1968; Holm-Hansen and Booth, 1966; Patterson et al., 1970), boiling ethanol (St. John, 1970), butanol (Sharpe et al., 1970) chloroform (Dhople and Hanks, 1973), sulphuric acid (Forrest and Walker, 1965; Lee et al., 1971) and formic acid (Klofat et al., 1969).

The solution of the problems associated with the assay have already been discussed (3.2.1 and 3.2.2). In conclusion although the LKB monitoring reagent was a vast improvement on the crude luciferase (3.1.3 (i), Fig. 3.5) it did not fully realise the specifications of the purified luciferase (3.1.3 (ii), Fig. 3.6). Consequently the assay

method used was based on initial peak light readings, and a true internal standard could not be used. Even so the reproducibility ($p = 0.95$) of the method was good (±1-2%). Recently with the advent of sophisticated nuclear magnetic spectrophotometers and the use of new techniques (TMR), NMR has been used as an "assay method" to study the *in vivo* levels of ATP, ADP and AMP in the intact cells of limb muscles (Wilkie, 1982) and kidneys (Radcliffe, 1982). Wilkie found that the differences in the ATP, ADP and AMP pools in resting muscle cells starved of oxygen and in metabolising cells under aerobic conditions, as compared to resting aerated cells were similar. And yet the rate of glycolysis (conversion of glucose to lactate) was 300 times greater in the metabolising cell compared to that of the resting cell. Wilkie suggested that glycolysis is not controlled by the cellular levels of ADP; in conflict with present thinking. It seems likely that TMR/NMR studies of this kind on *intact* microbial cells will throw doubt on the many theories of metabolic control over the coming years. This will apply especially to the regulatory role of ATP, ADP and AMP.

The mean value of the ATP pool of *K. aerogenes* was $7.8 \times 10^{-6}$ mol $g^{-1}$ (Figs 4.1-4.4 and 6.1-6.3). The literature reveals that only early papers specify ATP pool values for microorganisms under different growth conditions as later work was orientated to determining the EC value and it is this parameter that is usually quoted. Further it is usually impossible to calculate an ATP pool value from the given EC data.

For exponentially growing cells of *E. coli* in aerated glucose-limited media the following average values have been quoted for the ATP pool; $6.6 \times 10^{-6}$ (Franzen and Binkley, 1961); $9.5 \times 10^{-6}$ (Cole *et al.*, 1967): $6.7 \times 10^{-6}$ (Lowry *et al.*, 1971); $7.5 \times 10^{-6}$ (Holms *et al.*, 1972);

$7.0 \times 10^{-6}$ mol $g^{-1}$ (Chapman and Atkinson, 1973). For K. aerogenes a value of $9.0 \times 10^{-6}$ mol $g^{-1}$ has been reported (Harrisson and Maitra, 1969). These values are similar to that obtained in this work. The variance in the values can be attributed to the use of different extractants and inaccurate evaluation of the ATP lost during the extraction procedure; a view supported by others (Lundin and Thore, 1975).

The size of the ATP pool for the different phases of growth (lag, logarithmic, and stationary) in aerated glucose media (Figs 4.1-4.4 and 6.1-6.3) changes dramatically. The average value of the ATP pool for the inoculum (i.e. time = 0) is $2.5 \times 10^{-6}$ mol $g^{-1}$, during the minimal lag phase this rises to $7.8 \times 10^{-6}$ mol $g^{-1}$, which is maintained during growth. On exhaustion of the glucose the ATP level decreases to approximately the value of the inoculum. The ATP pool of the inoculum is representative of resting, fully viable cells, and that of growing cells is representative of metabolising cells with an ATP turnover rate of 300-450 per minute. Here then are two contrasting states, and this is reflected in the relative sizes of the ATP pools. This information suggests that high growth/metabolic rate, i.e. a highly energetic state, is indicative of a large ATP pool. The ATP pool has been long regarded as a suitable parameter for characterising the energy state of micro-organisms (Strange et al., 1963; Forrest, 1965; Kelly and Syrett, 1966; Cousin and Belaich, 1966; Cole et al., 1967; Lazdurski and Belaich, 1972, Gadkari and Stolp, 1975).

The ATP pool expressed as mole per gram of protein or nitrogen (rather than mol $g^{-1}$) of a wide variety of bacteria growing in batch culture is constant throughout the logarithmic growth phase (Chapman et al., 1971; Holms et al., 1972; Bachi and Ettlinger, 1973; Miovic and Gibson, 1973; Dietzler et al., 1974; Gadkari and Stolp, 1975). In

contrast however Forrest (1975) claimed that the ATP pool of Streptococcus faecalis decreased throughout the logarithmic growth phase. Cole et al., (1967) suggtested that the ATP pool was either in balance, decreased or increased during growth depending on culture conditions. Einger (1975) reported that the ATP content of the chemoautotroph Nitrobacter winogradskyi increased during logarithmic growth. Knowles and Smith (1970) suggested that the ATP pool of Azobacter vinelandii also increased during the growth phase.

Most workers do not quote reproducibility levels for the ATP pool values, and further few express the results in a tabulated or graphical form in terms of the ATP pool. Further much work has been carried out in complex media under ill-defined conditions of growth and test. Therefore it is difficult to make comparisons between the published data and the quantitative results obtained in this work, which have been obtained under standard and defined conditions of growth and test in simple media.

The published data of ATP contents of two microorganisms during growth (Figs 10.1 a, b, c) as determined by three different workers is a typical illustration of the use of various units and graphical representations. The ATP pool (mole $g^{-1}$) of E. coli as determined by Cole et al., in 1967 (Fig. 10.1 a) shows initially a gradual increase in the ATP pool during growth and then a decrease. Certainly even taking into account the errors of the technique (bar lines shown) this over and under production of ATP during growth is a real phenomenon and is recognised as being so by the author. In contrast, by way of comparison, the typical ATP profile for K. aerogenes grown in glucose-limited media under aerobic conditions (Fig. 10.2 a) illustrates the osicllatory nature of the ATP pool and the smaller limits of error. The ATP content (expressed as moles per unit volume of culture) for E. coli during exponential growth is shown in Fig. 10.1 b (Holms et al., 1972). The scale used to

FIGURE 10.1(a) The ATP Pool of E. coli during Aerobic Growth in Synthetic Medium Containing Glucose (22 mM) (Cole et al., 1967)

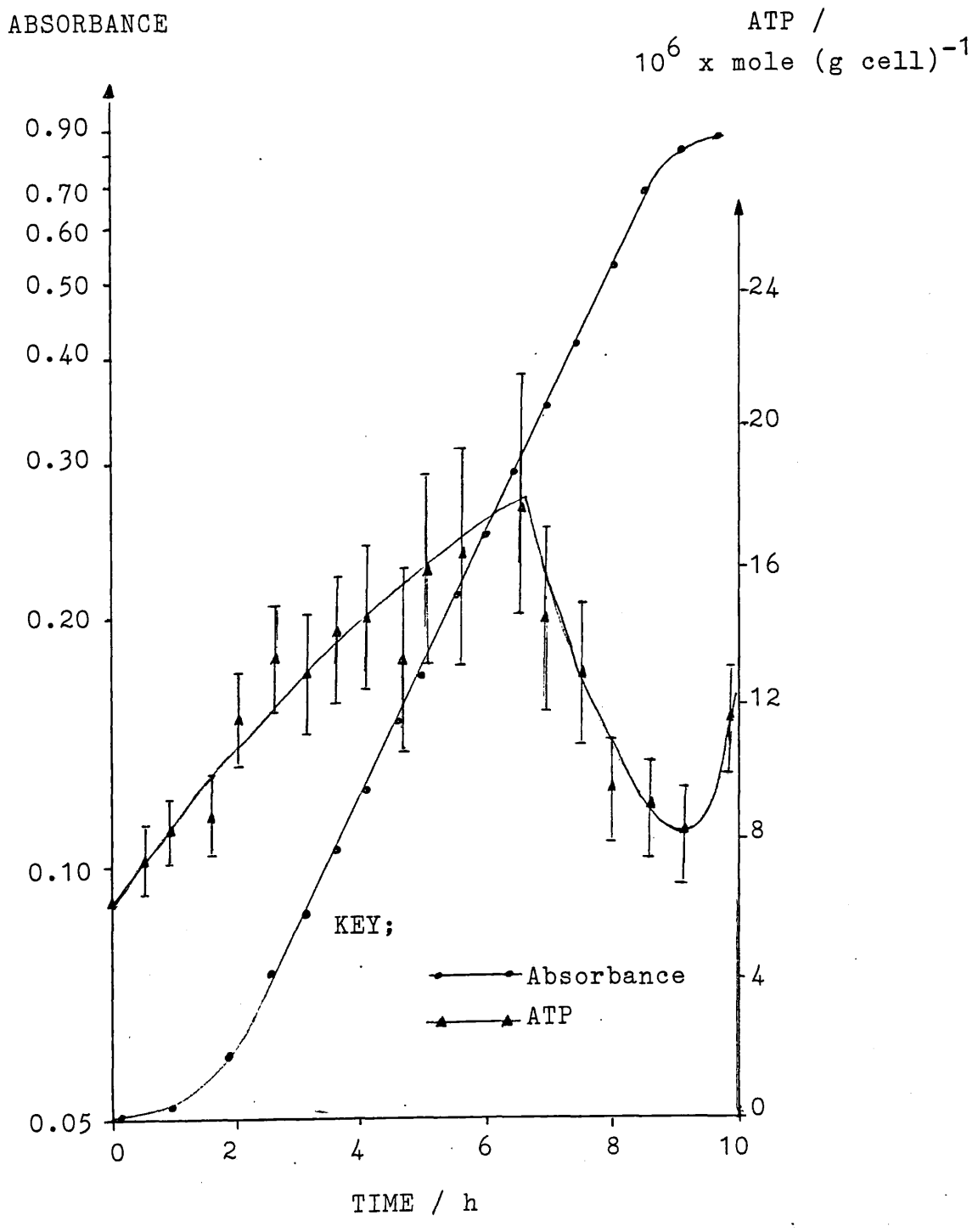


FIGURE 10.1(b) ATP Content of E. coli during Aerobic Growth in Simple Salts Medium Containing Glucose (2 mM ) (Holms et al., 1972)

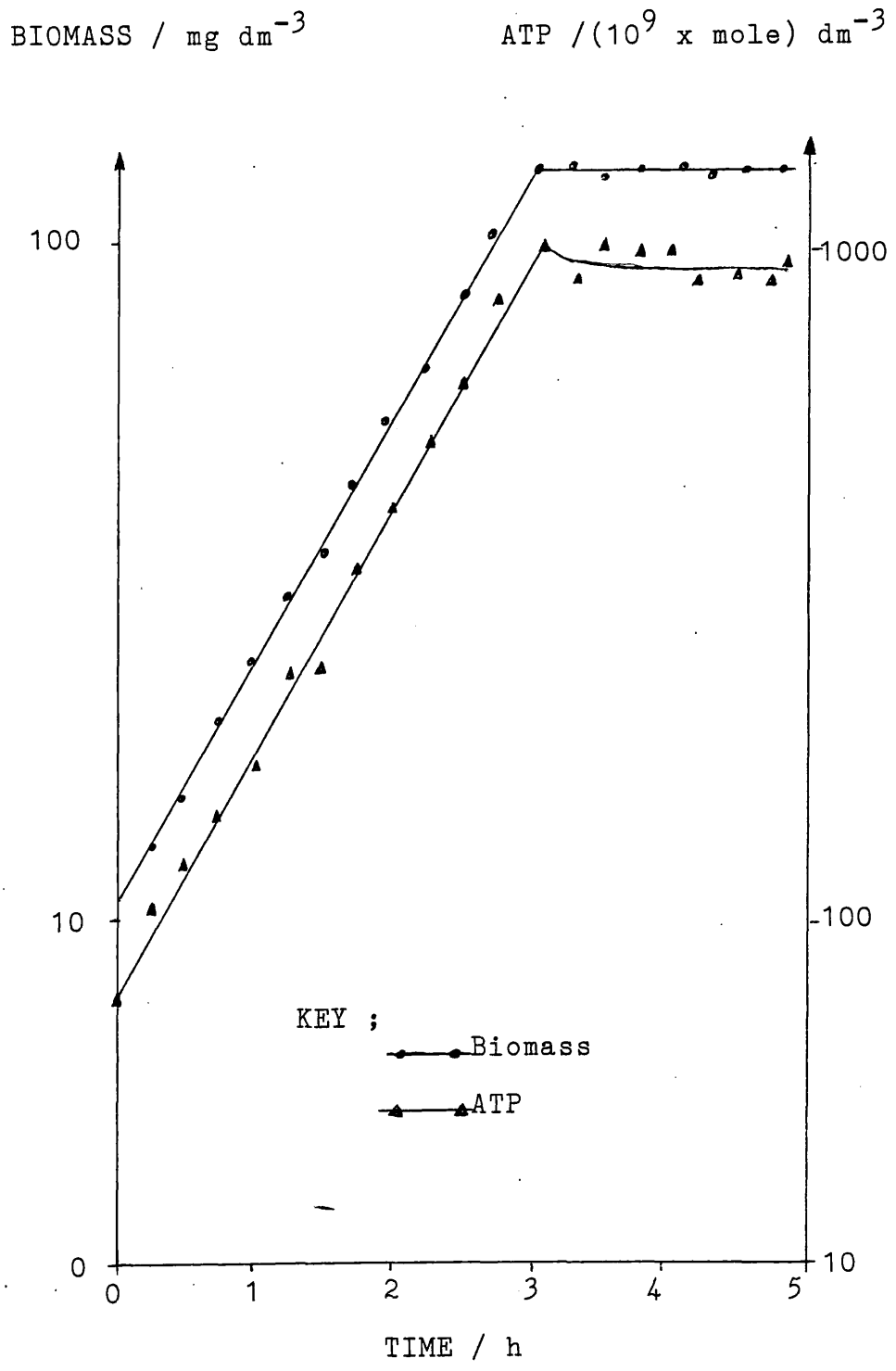
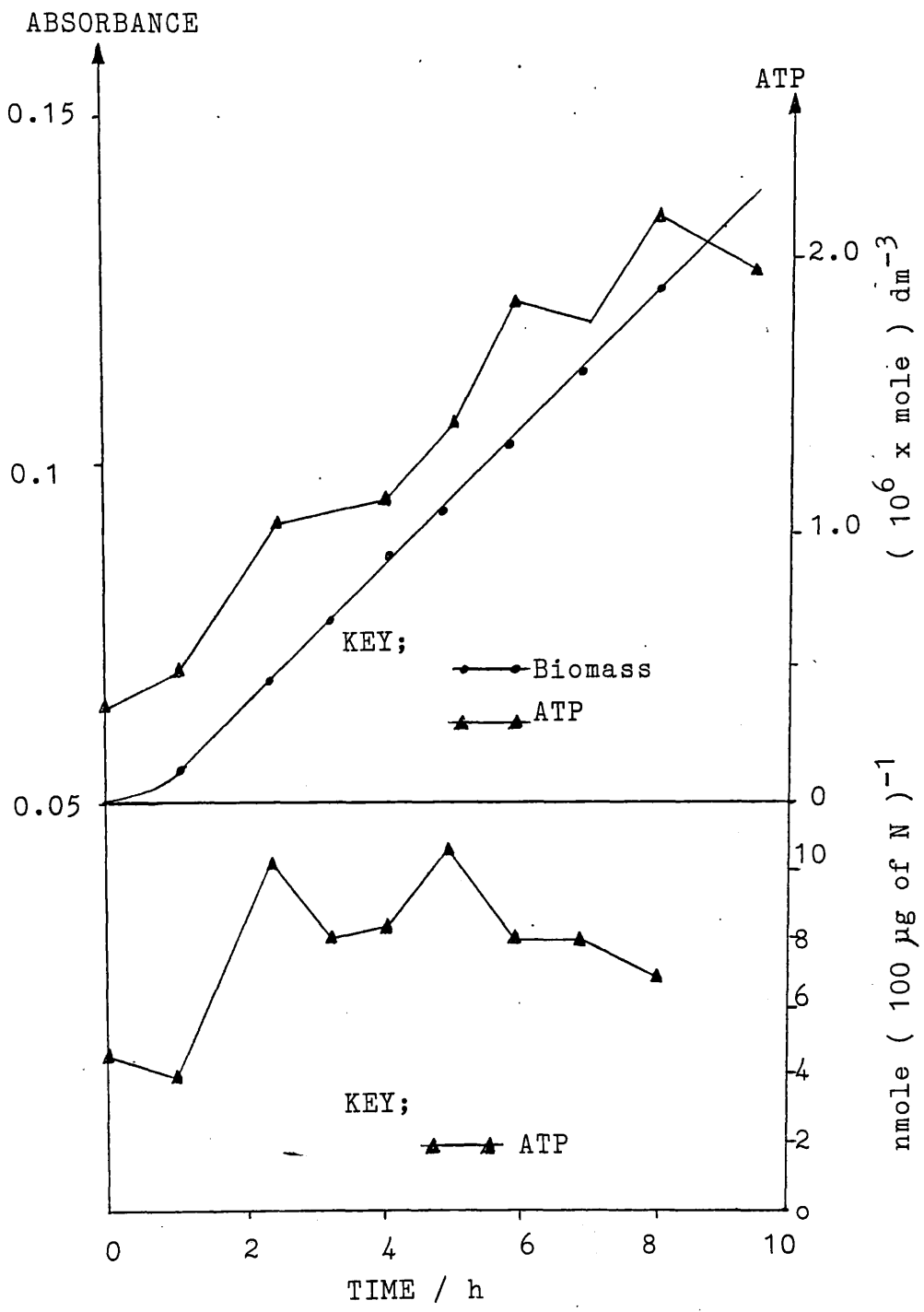


FIGURE 10.1(c) The ATP Pool of Bdellovibrio bacteriovorus during Aerobic Growth in Peptone Medium (Gadkari and Stolp, 1974 )



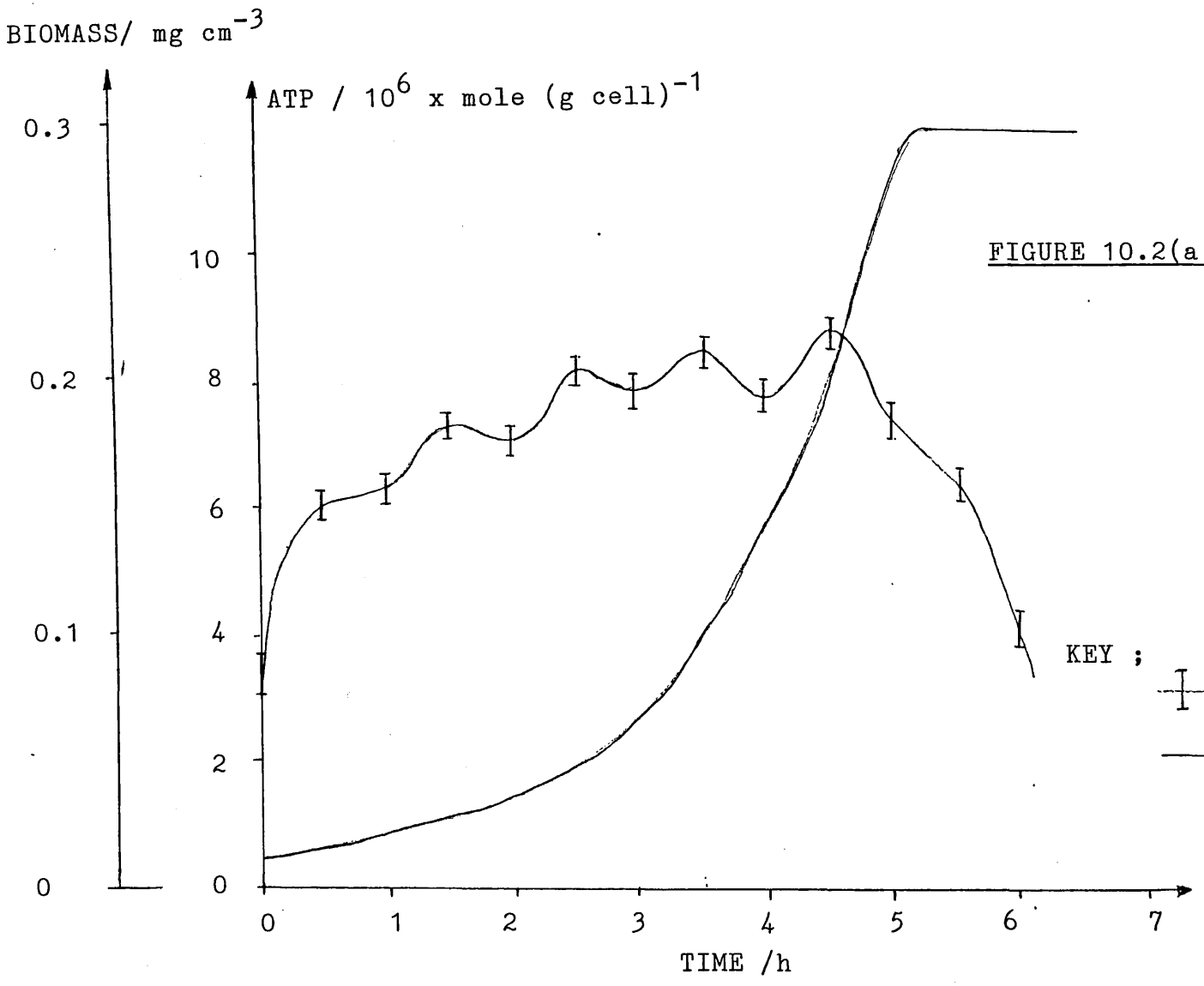
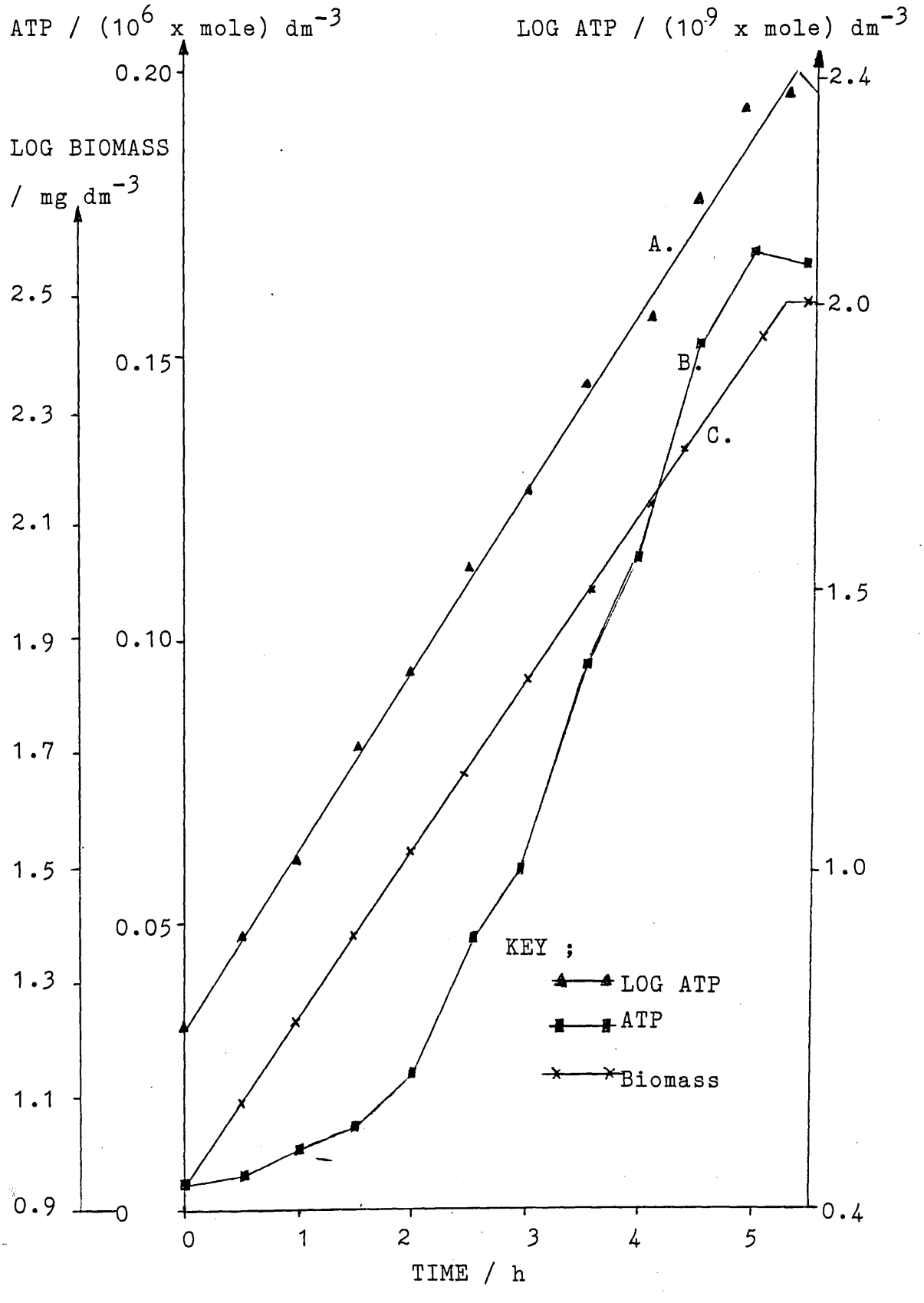



FIGURE 10.2(a) ATP Profile for A Typical Exponential Batch Growth in Glucose-Limited (3.3 mmol dm$^{-3}$) Media under Standard Growth Conditions

FIGURE 10.2(b) The ATP Content of K. aerogenes during A Typical Exponential Growth in Glucose-Limited Media



represent the change of the ATP pool during growth is logarithmic; variations in the ATP content of the cells will be disguised and not as clear or dramatic as using a linear scale. The ATP level of K. aerogenes (Fig. 10.2 a) is plotted logarithmically (line A Fig. 10.2 b). This is similar to the log ATP plot for cells of E. coli. When the data is presented in this way (Fig. 10.2 b, curve A) the ATP content of the cells of K. aerogenes appears to be constant throughout growth; indeed Holms interpreted his data (Fig. 10.1 b) in this way. A likely reason for this is that any variations of the ATP content which occurred during growth can be accounted for by experimental errors, which in this paper have not been quoted. An even worse possible situation is that the reproducibility of the complete ATP assay technique was never determined quantitatively. The linear presentation of the ATP pool (mole $(gN)^{-1}$) and the ATP content (mole per unit volume of culture) for growing cells of Bdellovibrio bacteriovorous (Fig. 10.1 c), (Gadkari and Stolp, 1975), in peptone-limited medium shows large variations. The authors suggest that the ATP content of the cells during growth is effectively constant. This seems strange; the only explanation for this interpretation is that the variation in the ATP level did not exceed the experimental error attributable to the complete assay technique. The errors were not quoted.

To summarise, the failure to establish quantitatively and analytically the experimental errors of the complete assay technique, coupled with the various misleading ways of representing the final data, has resulted in the majority of authors not recognising changes in the ATP pool of microorganisms during growth. The change in the cellular ATP pool during exponential growth of K. aerogenes (Figs 4.1-4.4 and 6.1-6.3, 10.2 a) is relatively small (c. ±10% of the mean value) and under less strictly defined conditions than those used may well have not been

significant. In consequence the ATP pool would have been considered to be constant during growth. Some workers have identified continual and large changes in the ATP pool during growth, and most have interpreted the results in terms of a constant pool. No one has identified a periodic oscillation about a mean value; made possible by the well defined and small limits of error of the complete bacterial ATP assay technique.

In view of the novel oscillatory nature of the bacterial ATP pool during aerobic growth in glucose-limited media (Figs 4.1-4.4 and 6.1-6.3) an investigation was completed to establish the validity of this finding. An increase in the frequency of sampling (4.3.2, Fig. 4.5) and sample duplication unequivocally shows that changes in the ATP pool during growth were real and reproducible (4.3.1, Table 4.1). This is imortant evidence in support of the validity of the oscillatory nature of the ATP pool during growth.

It was considered that the use of cryogenic inocula (2.1.3), not widely used by other workers, and the resultant minimal lag phase (0.5 h) induced the oscillations in the ATP pool. Changes in the ATP pool during the growth of cells with a lag phase of 2 h (4.5.1, Fig. 4.10) were similar to that of an aerobic culture in glucose-limited medium where growth was initiated with cryogenically stored cells (Figs 4.1-4.4 etc).

Consideration was given to the possibility that the oscillatory nature of the ATP pool could be due to the synchronous nature of the culture. Synchronous cultures can be obtained by various techniques and the degree of synchrony is assessed by the synchrony index (F) of Blumenthal and Zahler (1962);

$$F = (N/N_0) - 2^{t/g}$$

where F has a maximum value of 1.0 for a culture exhibiting perfect

synchrony. N is the number of organisms at time t, $N_0$ is the number of organisms at time zero, and g is the mean generation time. Generally synchronous cultures are difficult to obtain and maintain. The maximum value of F obtainable at present is 0.8. As each cycle is monitored the synchronous nature of the culture is lost at an alarmingly fast rate (Table 10.1).

TABLE 10.1. - Variation of F During Synchronous Growth

| Cell Cycle | F Value |
|---|---|
| 1st | 0.80 |
| 2nd | 0.42 |
| 3rd | 0.20 |
| 4th | 0.10 |
| 5th | 0.08 |

Large changes in the ATP levels of synchronous cultures of Tetrahymena pyriformis (Lloyd et al., 1977) occurred during a cell cycle (Fig. 10.3), which are certainly outside the limits of experimental error (±3%, p = 0.95).

To estimate the degree of synchrony of cells of K. aerogenes growing in aerated glucose-limited media, growth was monitored by viable counts (4.4.1), and by total cell counts (4.4.2). During growth both the viable counts (Fig. 4.6) and the total cell counts (Fig. 4.8) increased logarithmically, and therefore deviations from exponential (asynchronous) growth were not detected. In both cases the ATP profiles expressed as mole ATP per viable cell (Fig. 4.7), or per cell (Fig. 4.9) were similar to the normal ATP profiles expressed in units of mole $g^{-1}$. Thus the

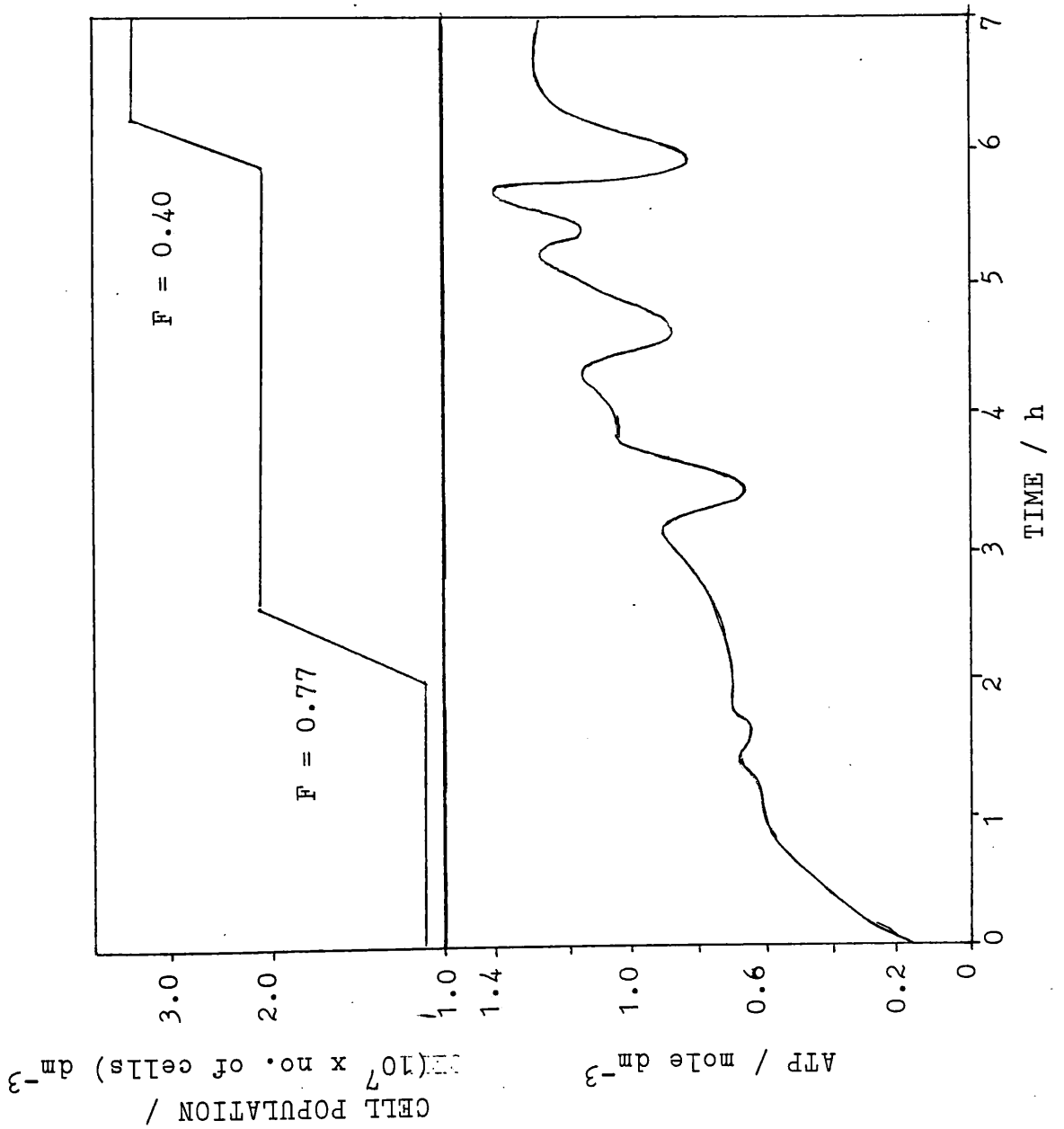



FIGURE 10.3 Changes in The ATP Content of T. pyriformis during Growth in Aerated Media (Lloyd et al., 1977)

observed oscillations in the ATP pool during growth are most probably not due to synchronous growth.

Even though synchronous growth was not detected by viable cell and total cell counts the "synchrony variation" theory may still be a possible explanation as to the cause of the oscillatory nature of the ATP pool. A degree of synchrony may be present during exponential growth that is too small to be detectable by cell counts, but is still substantial enough to be detected by variations in the ATP pool. The only explanation for this is that the time taken by each individual cell to complete a cycle is variable. If it is impractical to obtain and maintain a purely synchronous culture, then it can be argued statistically that it is also impractical to obtain and maintain a purely asynchronous culture. If a large population of growing bacterial cells is considered ($10^{12}$ cells $dm^{-3}$) as encountered then it is highly improbable that at any moment in time every cell will be at a different stage in the cell cycle, (i.e. purely asynchronous, F = 0). It is more probable that a degree of synchrony is exhibited, and further due to differences in the growth cycle time for each individual cell the degree of synchrony will change. This change in synchrony will perhaps result in a change in the measured ATP pool value during exponential growth, as the cellular ATP content of cells varies during the growth cycle (Fig. 10.3). Further the almost continual change in the size of the ATP pool during exponential growth is supported by the fact that the F value of a synchronous culture changes rapidly (Table 10.1).

The absence of oscillations in the ATP pool during the lag and stationary phases for the various growths considered (aerobic, anaerobic, glucose-limited, phosphate-limited, etc) does not conflict with this possible explanation as to the cause. During these phases of cell metabolism cell division (if any) is very slow and therefore variations

in the synchrony (if any) will be relatively small. Further during the lag phase the metabolism is directed to repair and reorganisation of the cell, and during the stationary phase to maintenance of cell integrity. The oscillations of the ATP pool are most prevalent during aerobic exponential growth in glucose-limited media (Figs 4.1-4.4 and 6.1-6.3). Under aerobic conditions in glucose-limited media in a chemostat (Fig. 4.12), anaerobic exponential growth in glucose-limited media (Fig. 4.11), and under aerobic growth in phosphate-limited media (Fig. 7.5) the oscillatons in the ATP pool are smaller. In these cases growth is slower and it may be that under these conditions where the cell cycle time is greater the change in the degree of synchrony and hence the ATP pool is relatively less compared to short cell cycle times.

It is possible that the oscillatory nature of the ATP pool is the result of a mechanism to control cellular metabolism. The ATP pool size and hence the cellular ATP concentration changes in order to maintain a relatively constant metabolic rate. In a cell where catabolism (ATP producing) is tightly coupled to anabolism (ATP requiring) changes in the ATP pool are indicative of a "slip mechanism" whereby the relative rates of the two (catabolism and anabolism) are varied. Whether the change in ATP is a result of the change in the rates of the two metabolic systems or vice versa is debatable. Even so it is generally accepted that a high cellular ATP concentration slows the rate of catabolism and increases the rate of anabolism. A low concentration of ATP would have the opposite effect. Indeed this over- and under-production of the ATP pool has been identified before (Cole et al., 1967, Fig. 10.1 a) but the nature of the change in the ATP content was interpreted as a gradual one rather than oscillatory. It seems strange that there should be a gradual change in the ATP pool of exponentially growing cells. Therefore it is suggested that any gradual changes observed are erroneous and are most likely due

to changes in the ATP standard during measurement of the ATP contents of the bacterial extracts, rather than cellular control mechanisms. The absence of oscillations in the ATP pool during growth under other conditions (i.e. phosphate-limited, anaerobic etc) is in conflict with the slip mechanism theory as it should still operate under these conditions and cause fluctuations in the ATP pool.

The absence of oscillations during the lag and stationary phases can be explained by this slip mechanism. During these phases the slip mechanism is not required as one or both of the metabolic systems (catabolism or anabolism) is not operational and therefore oscillations in the ATP pool will not be present. During the lag phase the ATP increases gradually (Fig. 4.10) due to catabolic activity, (carbon dioxide is produced which is the end product of catabolism) and there is little growth; anabolic activity is minimal. Once the ATP pool size has reached a certain value (c. $7.8 \times 10^{-6}$ mol $g^{-1}$) growth (anabolism) commences. This value represents the minimum cellular concentration of ATP that is required to fully activate (drive) anabolism, so that new cellular material is produced in glucose-limited media under aerobic conditions. During the stationary phase neither catabolism or anabolism operate and therefore the slip mechanism is not necessary and hence no oscillations. The gradual fall in the ATP level is due to the utilisation of ATP for cellular maintenance.

A more feasible explanation for the oscillations observed during exponential growth is the combination of both theories; the slip mechanism and the synchrony variation. The oscillations in the ATP content observed during synchronous growth is the result/cause of the operation of the slip mechanism to control metabolism during the cell cycle. In consequence, because there are oscillations of the cellular ATP content during the cycle, and because the cell cycle time is variant,

then during exponential growth (asynchronous) the average observed ATP pool will vary.

It has been generally assumed that the ATP pool is a suitable parameter for characterising the energetic/metabolic state of micro-organisms. The ATP pool of K. aerogenes under different growth conditions varied greatly; 0.5 to 9.7 x $10^{-6}$ mol $g^{-1}$ (Table 10.2). There is no simple relationship between the size of the pool and experimental indicators of metabolic activity such as growth rate, carbon dioxide output etc. Indeed this fact has been noted by previous workers and is one of the reasons for the general interest shown in the EC value. This was because it was thought that the metabolism of the cells responds to variations in the mole fraction of ATP rather than to the concentration of the nucleotide itself. However a degree of correlation between the cellular ATP content and the value of the EC has been reported (Fynn and Davison, 1976; Nivel et al., 1977; Skjoldhal and Bamstedt, 1977), and therefore it may be that both parameters are unable to fully account for all possible metabolic activity in a direct and relatively simple way. A survey of the value obtained for both the ATP pool and the EC (Knowles, 1977) for a vast array of different conditions of metabolism and growth reveals that the ATP pool of fully viable cells was in the range 3 to 11 x $10^{-6}$ mol $g^{-1}$, while the EC was between 0.7 to 0.9 in all cases, and usually 0.8 to 0.9.; this highlights the disadvantages of the use of the EC value as a metabolic indicator as small changes in its value (usually within the limits of experimental error as three nucleotides have to be assayed), disguise large changes in the ratios of the ATP, ADP and AMP

TABLE 10.2 The ATP Pool of Cells of K. aerogenes under Different Growth Conditions

| STATE OF CELLS | CORRESPONDING FIGURE IN TEXT | AVERAGE ATP POOL ($10^6$ x mole)$g^{-1}$ |
|---|---|---|
| 1. Cells in the stationary phase for c. 36 h | —— | 0.5 |
| 2. Cells of inoculum; immediately after adding to any growth media | MANY | 2.0 |
| 3. P-trained cells in P-limited medium (extracellular P conc. = 0) metabolising (with some growth) acetate | 7.5 | 2.0 |
| 4. Fully viable cells in the stationary phase for c.2 h (glucose-limited) | MANY | 2.5 |
| 5. Cells growing anaerobically in glucose-limited media | 4.11 | 6.5 |
| 6. Cells growing aerobically in glucose-limited media | 4.1-4.4 | 7.8 |
| 7. Cells growing aerobically in glucose-limited chemostat | 4.12 | 7.8 |
| 8. Cells metabolising ethanol and acetate aerobically | 4.11 | 8.2 |
| 9. Cells in P-limited media (extracellular P available) growing on glucose | 7.5 | 9.7 |

pools, as well as metabolic activity/state and growth.

From the results presented here it is more likely that the changes in the relative size of the cellular ATP pool during the different phases of growth, in a particular medium, (glucose limited (Figs 4.1-4.4 and 6.1-6.3; phosphate limited (Fig. 7.5)), are a reflection of the relative changes in the rates of both catabolism and anabolism. Further the changes in the ATP pool during the metabolism (growth) of (on) the same substrate in different media, (i.e. on glucose in glucose-limited medium (Figs 4.1-4.4) and in phosphate-limited medium (Fig. 7.5, Section A)), and under different growth conditions, (i.e. aerobic or anaerobic (Fig. 4.11)), are a result of changes in metabolism and therefore the degree of coupling between anabolism and catabolism. This is not in conflict with the operation of the slip mechanism, as this mechanism, which causes small changes in the rates of catabolism and anabolism and in the degree of coupling of the two to control the overall metabolic rate, possibly operates to some extent (more so in aerated glucose-limited media) under all conditions in every type of media. It may be argued that changes in the cellular ATP pool during different phases of growth may be due not only to changes in the relative rates of catabolism and anabolism but to changes in the degree of coupling as well.

In some cases it may be difficult to distinguish between the two processes as they may operate in tandem or one may be the result of the other. The definition of the degree of coupling for a bacterial cell is the degree to which the two systems; catabolism and anabolism operate in conjunction with each other and the degree to which both systems are sympathetic to each other via linking biochemical reactions, which include the dephosphorylation of ATP and the phosphorylation of ADP. In this way an efficient, balanced and controlled overall metabolism is obtained. A tightly coupled (efficient) metabolism will be the result of

a good balance between the two systems so that there is neither a massive accumulation or near depletion of required metabolic intermediates, such as ATP. In a loosely coupled system the opposite may well be true. The degree of coupling may well be affected by both changes in the relative rates of specific types of catabolism and anabolism as well as changes in the type of the two systems.

A high ATP pool could be indicative of efficient catabolism and inefficient anabolism; loose coupling. A low ATP pool could be indicative of inefficient catabolism and efficient anabolism; still loose coupling. However the size of the ATP pool and hence the degree of coupling need not be indicative of the overall rate of metabolism as measured by the rate of formation of heat, biomass, carbon dioxide or by the rate of oxygen uptake. This is because the ATP pool is possibly more a measure of the degree of coupling between catabolism and anabolism rather than the actual rates of the two systems and therefore the overall metabolic rate, which may well be different under different growth conditions and in different media. This argument leads to the statement that the ATP pool is a reliable indicator of the relative metabolic activity for cells growing under the same conditions in the same media. As a comparative metabolic indicator for cells growing under different conditions or in different media the pool size is difficult to interpret and therefore unreliable. This is not to say that there is no correlation of the ATP pool for cells growing under different conditions or in different media; in this work a low ATP pool ( $2.0 \times 10^{-6}$ mol $g^{-1}$) is always indicative of resting cells.

For K. aerogenes no simple relationship has been shown between the ATP pool and other possible indicators of metabolic activity; rate of biomass or carbon dioxide formation. However there are some general observations to be made and some more specific ones concerning ATP pool

values under specific conditions. Generally the ATP pool of stationary cells (0.5-2.5 x $10^{-6}$ mol $g^{-1}$) is smaller than that of metabolising cells (2.5-9.7 x $10^{-6}$ mol $g^{-1}$), with the exception of cells growing on acetate in phosphate-limited media (Figs 7.3, Section C, 7.5) in the absence of extracellular phosphate (2.0 x $10^{-6}$ mol $g^{-1}$) (Table 10.2). This unusually low value may be due to the fact that phosphate has to be scavenged from other cells merely limiting the regeneration of ATP from ADP. It is surprising that growth takes place at all under these conditions (albeit slow), and it is an example of the adaptability of the microbial cell achieved through training. The facility for the production of biomass from acetate at low ATP levels must be due to considerable changes in the metabolism of the cell from that of cells trained to grow in glucose-limited media. In this case the small ATP pool is indicative of an overall slow metabolic rate as the rate of growth and carbon dioxide evolution is much slower than that of normal cells (glucose trained) growing in glucose-limited media. The reduced efficiency/rate of catabolism and hence ATP production is due to two factors; the lack of phosphate and the utilisation of acetate as the substrate. The relative rate of consumption of ATP may have increased, since acetate fails to provide many intermediate molecules required for anabolism. Thus new ATP requiring anabolic pathways may be brought into use to account for this failure. The overall effect of both will be to reduce the ATP pool. It is difficult to interpret the ATP pool in terms of the degree of coupling, however a small pool would be expected as well as loose coupling due to the adverse conditions of growth.

The stability of the ATP pool and its reluctance to fall below a certain value in resting cells (2.0 x $10^{-6}$ mol $g^{-1}$) even after 36 h (0.5 x $10^{-6}$ mol $g^{-1}$) is remarkable. Presumably when all of the carbon source is used up the supply of ATP and demands for its use in biosynthesis

cease simultaneously. However the results suggest the existence of mechanisms whereby the maintenance of the ATP pool is given priority with the obvious selective advantage to the cell of being able to make a sudden response to more favourable growth conditions after a period of deprivation.

The cellular ATP pool for cells growing aerobically in glucose-limited chemostat ($7.8 \times 10^{-6}$ mol $g^{-1}$) was similar to that of cells growing exponentially in batch culture. This agrees with that value observed by Harrison and Maitra (1969) for the continuous growth of K. aerogenes in glucose-limited media ($7.6 \times 10^{-6}$ mol $g^{-1}$). The similarity of the two values is not surprising since under both conditions the degree of coupling will be similar as will be the reaction pathways of catabolism and anabolism.

The ATP pool value for anaerobic growth (Fig. 4.11; $6.5 \times 10^{-6}$ mol $g^{-1}$) is lower than that for aerobic growth (Figs 4.1-4.4; $7.8 \times 10^{-6}$ mol $g^{-1}$) in glucose-limited media. This lower anaerobic value compared for the aerobic value for growth on the same substrate has been noted by previous workers (Harrison and Maitra, 1969; Chapman et al., 1971). Even though the rate of aerobic growth is twice that for anaerobic the cellular ATP pool of aerobic cells is only c. 25% greater than that for cells growing anaerobically. This is due to growth taking place under different conditions. The lower ATP pool for anaerobic growth is possibly due to the restriction of catabolism and therefore ATP production as a result of less efficient oxidative phosphorylation since an electron acceptor other than oxygen has to be found.

The cellular ATP pool for cells during the aerobic metabolism of ethanol and acetate (Fig. 4.11; $8.2 \times 10^{-6}$ mol $g^{-1}$) is higher than for cell growth aerobically in glucose-limited media (Figs 4.1-4.4; $7.8 \times 10^{-6}$ mol $g^{-1}$). This may be due to the over production of ATP caused by

the cells still using the ATP-generating pathways developed for the previous anaerobic growth as well as oxygen dependent ATP generating pathways. Further as catabolism is operational so also is ATP production, but anabolism and hence ATP consumption is dormant; this would lead to an increase in the cellular ATP pool. The highest value of the ATP pool ($9.7 \times 10^{-6}$ mol $g^{-1}$) was found for cells growing on glucose in phosphate-limited media while extracellular phosphate is still available (Fig. 7.3, section A) (Table 10.2). During training of the cells in phosphate-limited media due to the shortage of available phosphate extra ATP generating capacity is probably developed, especially to aid growth on glucose when extracellular phosphate is not available (Fig. 7.3, section B). This extra ATP generating capacity developed for growth when extracellular phosphate is <u>not</u> present results in an over-production of ATP (hence a large ATP pool) when extracellular phosphate is present. This accounts for the extremely high ATP pool value when compared to that of ordinary cells growing in glucose-limited media. Thee cells have a similar rate of growth and carbon dioxide production to that of phosphate trained cells during growth on glucose in the presence of extracellular phosphate.

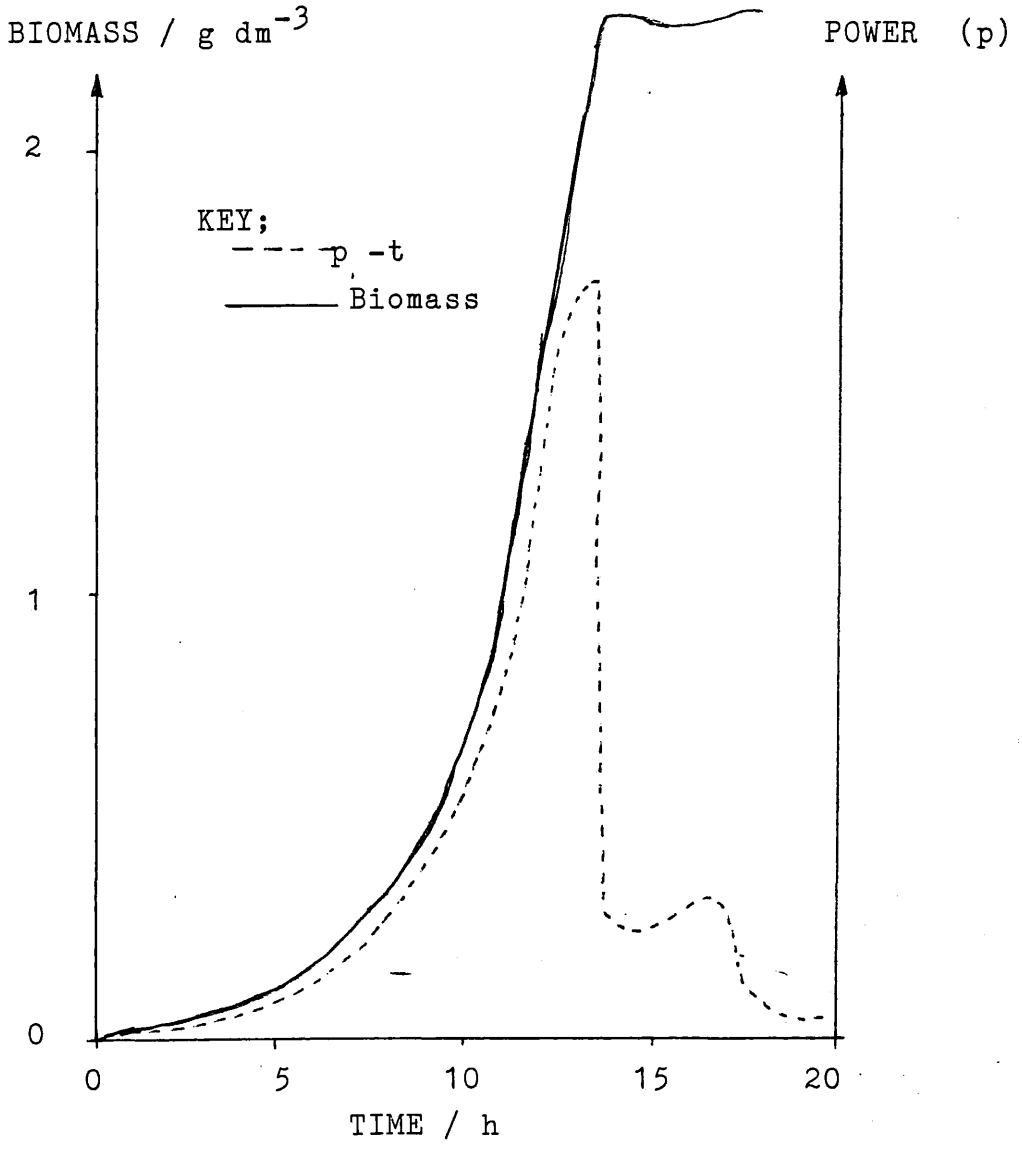
Neither the ATP pool nor the EC give an indication as to the turnover rate of ATP in the cell. The turnover rate is the number of times per minute that the ATP pool is emptied and subsequently refilled by the production and utilisation of ATP. Further information is required concerning oxygen consumption, carbon dioxide production and/or substrate utilisation during growth to enable this turnover rate to be calculated. The conditions of growth and test used were not suitable for the determination of oxygen-consumption and unsubstantiated assumptions have to be made to calculate the turnover rate from oxygen, carbon dioxide or substrate utilisation data. The values of ATP turnover rates

obtained by previous workers vary greatly; for K. aerogenes values of 50, 150 and 200 $min^{-1}$ have been reported (Stouthamer and Battenhaussen, 1973), for E. coli values of 273-461 $min^{-1}$ (Holms et al., 1972.) For batch growth of various microbes in defined media under glucose-limited conditions it has been estimated that maintenance of cell integrity requires a turnover rate of c. 4 mmol $g^{-1}$ $h^{-1}$ (Stouthamer and Battenhausse, 1973). If an ATP turnover rate for K. aerogenes of 300 $min^{-1}$ is assumed and an ATP pool of $7.8 \times 10^{-6}$ mol $g^{-1}$ then the total throughput of ATP per hour = $300 \times 60 \times 7.8 \times 10^{-6} = 140$ mmol $g^{-1}$ $h^{-1}$. Therefore about 3% of the total ATP generated during growth in aerated glucose-limited media is required to maintain the integrity of the cell.

The first use of a flow calorimeter in conjunction with a fermenter to monitor the growth (anaerobic) of microorganisms was by Monk and Wadsö (1968). The first example of the use of a flow calorimeter, equipped with an aerated cell and flow line, in conjunction with a fermenter to monitor the aerobic batch growth was that of Eriksson and Holme (1973). E. coli was grown in simple salts media containing glucose (22 mmol $dm^{-3}$) as the limiting nutrient. The power-time (p-t) trace obtained (Fig. 10.4) is similar to that for growth in glucose-limited (3.3 mmol $dm^{-3}$) media obtained in this work (Fig. 5.2). The p-t curve for growth at this higher glucose concentration shows a secondary power output although the causes are not clear.

The problems of over aeration of the growing culture in the vessel and the subsequent secondary power output and change in shape of the p-t trace have already been discussed (5.2). Changes in the shape of the p-t trace under highly aerated conditions of growth; conditions of high

FIGURE 10.4 p - t Trace for E.coli Grown Aerobically in Glucose-Limited (22 mmol $dm^{-3}$) Media
(Eriksson and Holme, 1973)



dissolved oxygen tension (DOT), has been observed before (Hughes and Wimpenny, 1969; Harrison, 1972; Schaarschmidt et al., 1974). The toxic effect on growth of extremely high DOT's due to the repression of enzymes and the formation of free radicals is well known (Haugaard, 1968). The evidence suggests that cytochrome destruction takes place. The effect of that and the reduced activity of the tricarboxylic acid cycle enzymes would result in growth similar to anaerobic growth (4.5.2) in which glucose is converted to secondary metabolites. The secondary metabolites produced would include acetate; which was detected in highly aerated glucose-limited media (5.1.1), this intermediate was the cause of the secondary heat output.

Quantitative work prior to that of Eriksson and Holme (1973) mainly involved the use of batch calorimeters to study the growth of microbes under anaerobic conditions (Belaich, 1963; Belaich and Senez, 1965; Belaich and Murgier, 1971; Lamprecht et al., 1971; Schaarschmidt, 1973). Since 1973 there have been many studies of microbial growth using flow microcalorimetry, but only a few have been concerned with aerobic growth (Ackland et al., 1976; Few et al., 1976; Wadsö, 1976; Monk, 1978; Wang et al., 1978). Further only a few investigations were made to determine the cause of changes in the p-t traces in relation to the underlying metabolic reactions (Few et al., 1976; Wang et al., 1978). In these studies there is evidence of oxygen starvation in the calorimeter cell and line, as these were not aerated, and this gave rise to more complex p-t traces when compared to those reported here for aerobic growth in glucose-limited media (Fig. 5.3). Recent quantitative studies utilising flow microcalorimetry to monitor aerobic batch growth (Nichols, 1980) were achieved without oxygen starvation due to the use of relatively low concentrations of the limiting substrate and high pump rates through the calorimeter. In another recent study (Brettel et al.,

1980) the calorimeter cell and line were aerated so that high substrate concentrations (10-100 mmol $dm^{-3}$) could be used. Although some of the practical problems associated with the aeration of the cell and line have been solved by Brettel the reproducibility of the power output was seriously affected; this was also observed in this study (5.5). There have also been many successful quantitative studies of the power output from aerobic batch growth using modified batch calorimeters (Denmon and Ishikawa et al., 1981(a), Ishikawa et al., 1981(b)).

Early reports (Forest and Walker, 1962; Balaich et al., 1968) have indicated that the rate of degradation of energy source, the rate of production of biomass and waste heat are similar. Nichols (1980) found that the rate of glucose degradation (catabolism) is not equal to the rate of biosynthesis (anabolism), and that the actual and relative rate of each varies, as does the waste heat evolved per mole of substrate consumed under different conditions. This change in the relative rates of catabolism and anabolism under different conditions is a result of a change in the metabolic pathways used and hence the degree of coupling. This is reflected in the change in the amount of waste heat evolved.

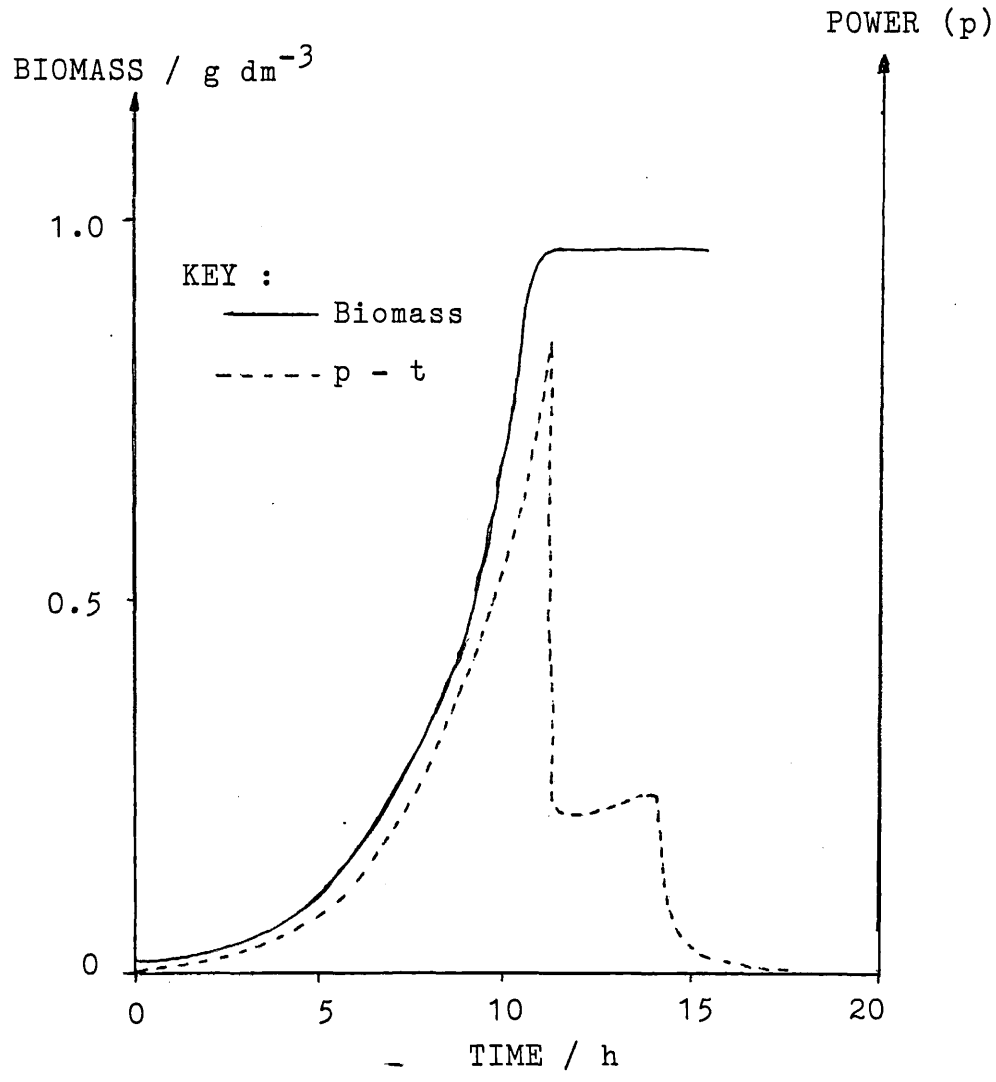
Many previous workers have obtained quantitative calorimetric data for the batch growth of bacteria in aerated glucose-limited media, and therefore this is a suitable standard (reference) for the comparison of results. In this work a value for $\Delta H_{glu}$ of -923.2 kJ $mol^{-1}$ was obtained for the growth of cells in aerated glucose-limited (3.3 mmol $dm^{-3}$) media. This measured enthalpy change is in good agreement with other previously reported values for similar conditions of growth; -817.6 kJ $mole^{-1}$ (Eriksson and Holmes, 1973); -1016.4 kJ $mol^{-1}$ (Cooney et al., 1976); -1063.2 kJ $mol^{-1}$ (Nichols, 1980); -1074 kJ $mol^{-1}$ (Ishikawa et al., 1981). Variations in the values can be attributed to variations in the conditions of growth and test. Generally the value obtained tended to be

numerically smaller (less waste heat evolved) compared to that of previous workers. This is probably due to the better defined conditions of growth, particularly aeration of the culture resulting in more efficient cellular metabolism (and therefore greater yields of biomass). During the growth of E. coli (Ishikawa et al., 1981) acetate was formed, this resulted in the low yield (0.41 g of cells per g glucose) when compared to that of K. aerogenes (Table 5.2; 0.49 g $g^{-1}$). Other workers (Nichols, 1980) have also reported lower yields (0.44 g $g^{-1}$).

Under defined conditions of growth and test changes in the p-t trace are governed by changes in the metabolism of the cells. The simplest cases encountered are the p-t traces for the growth of cells in glucose-limited media under non-standard (Fig. 5.2) and standard (Fig. 5.3) conditions. The heat output from metabolising cells in these cases and more complex ones will be discussed later in terms of the specific power output.

A comparison of the p-t traces between those obtained previously is of limited value. Only a small proportion of calorimetric data is concerned with bacterial growth in simple chemically defined media. The comparison of the p-t traces obtained for the growth of other types of microbes such as yeasts, or in complex media can be misleading due to the unidentified differences in metabolism. Further most data for bacterial growth in glucose-limited media has been obtained using high initial glucose concentrations (10-100 mmol $dm^{-3}$). Therefore work where aerobic conditions have been maintained by aeration of the calorimeter cell (Eriksson and Holme, 1973) or by the use of batch calorimetry where a vessel acts both as calorimeter cell and fermenter (Ishikawa, 1981), is sparse. The p-t traces recorded under such conditions utilising high glucose concentrations (11 mmol $dm^{-3}$, Fig. 10.4; 22 mmol $dm^{-3}$, Fig. 10.5) show a secondary heat output. Further these p-t traces (Figs 10.4, 10.5)

FIGURE 10.5 p - t Trace for E. coli Grown Aerobically in Glucose - Limited (11 mmol $dm^{-3}$) Media (Ishikawa et al., 1981)



are similar to the p-t trace obtained during growth in glucose-limited (3.3 mmol $dm^{-3}$) media under non-standard conditions (Fig. 5.2). In both the examples (Figs 10.4, 10.5) the power output rises exponentially during growth and on the depletion of the glucose decreases rapidly to a lower secondary power output. The secondary power output is due to the metabolism of acetate formed during the consumption of the glucose.

The formation of acetate could be due to a high dissolved oxygen tension in the culture vessel, as was obtained with K. aerogenes , or as a direct result of the use of high initial glucose concentrations. It is impossible to attribute the cause to either factor, owing to the limited amount of data presented.

Previous workers (except Nichols, 1980) believed that the calorimetric studies of metabolising microbial cells perceives only catabolic reactions and that the effect of anabolism (if any) may well be too small to measure (Belaich et al., 1968; Forest, 1969; Beezer et al., 1978; Dermoun and Belaich, 1980; Redl and Tiefbrunner, 1981). However Nichols (1980) was able to calculate the energy associated with anabolism i.e. the energy required for biosynthesis ($\Delta H_B$), (5.4.2). Therefore the assumption that the measured waste heat of metabolising cells is due to catabolism only, is misleading. The measured waste heat is the difference between the energy supplied and that evolved in catabolic and absorbed in anabolic processes. In other words the waste heat (like the ATP pool) is an overall measure of activity dependent upon the extent of both catabolism and anabolism and therefore most probably the degree of coupling. Brettel et al., (1980) quoted a reproducibility of ±8% for the determination of the difference between the energy input (available from the substrate) and output (recovered as heat and biomass) i.e. for the determination of $\Delta H_B$, or $\Delta\%$ i.e. the percentage of metabolised/

available energy required for biosynthesis. Therefore the identification of an anabolic element in the energy balance was overlooked as the missing energy could almost always be accounted for by experimental errors. The large experimental errors incurred by Brettel were due to the use of an aerated calorimeter cell and line. Indeed the reproducibility of the heat measurement (±12%, $p = 0.95$) when the cell and line was aerated (5.5) was much worse than when it was not (±1%). Further the reproducibility of the overall balance (i.e. input minus output) and therefore the percentage energy required for biosynthesis ($\Delta\%$) was ±1% (Table 5.2). For E. coli, Ishikawa et al., (1981) recovered 90% of the input energy as biomass and waste heat. The remaining 10% (12.3% for K. aerogenes)(5.5) was attributed to the energy stored in non-utilisable metabolites which were present in the medium after metabolic activity and hence heat output had ceased. These metabolites were never identified by analysis and the assumption is unsubstantiated. If the evolution of carbon dioxide during growth had been measured a full carbon balance could have been calculated, and possibly a 100% carbon recovery ($C_{rec}$) obtained. This would have disproved the assumption that the missing energy was stored as secondary metabolites which consequently would have led the investigator to the incorporation of a biosynthetic element in the energy balance.

It has already been suggested that the waste heat from, and the cellular ATP content of metabolising cells are similar measures, as they are both indicative of metabolic activity. Changes both in power (heat) output and ATP content of metabolising cells reflect the changes in overall metabolism caused by relative changes both in catabolism and

anabolism. If changes in the output and ATP content of cells are to be correlated then the most useful interpretation will be obtained when both are in comparative units. For this purpose the (instantaneous) ATP pool (mole of ATP per gram dry weight of bacteria) and the (instantaneous) specific power (6.1)(watts per gram dry weight of bacteria) have been chosen. Further the specific power-time (P-t) trace is a superior parameter in interpreting underlying metabolic changes than the power-time (p-t) trace as the specific power is a measure of the metabolic activity per cell while power is a measure of metabolic activity per unit volume of culture.

Only one other investigator (Gustafsson, 1979) has expressed calorimetric data of metabolising cells in terms of an instantaneous measure of specific power. Some investigators (Nichols, 1980; Brettel et al., 1981) have expressed the results in terms of a non-instantaneous specific power; this is a measure of the power per gram dry weight of bacteria formed during a given time interval. The interpretation of such non-instantaneous specific power measurements is more difficult and less revealing of the real nature of the underlying metabolic change.

During the later stages of the exponential growth of cells in glucose-limited media (Figs 6.1-6.3) the specific power was constant (1.80-1.95 J $s^{-1}$ $g^{-1}$) under the standard conditions; a value characteristic of aerobic, glucose metabolism forming only biomass, carbon dioxide and water. The over production of power (1.90-2.4 J $s^{-1}$ $g^{-1}$) during the early stages of exponential growth is most probably the result of ATP requiring and exothermic reactions activated during this period to repair and/or replace cellular components in the cells of the inocula. This overproduction could also be the result of inefficient coupling due to enzymes damaged during cryogenic storage.

The specific power during exponential growth of D-lansenii in

glucose-limited media (Gustafsson, 1979; Fig. 10.6) is relatively constant (c. 0.35 J $s^{-1}$ $g^{-1}$). This value is low compared to the value obtained for K. aerogenes (1.8-1.95 J $s^{-1}$ $g^{-1}$) under similar conditions (Figs 6.1-6.3). The growth rate of the yeast (D. lansenii) is much less than that of K. aerogenes. The ATP pool is constant at c. 8 x $10^{-6}$ mol $g^{-1}$ (Fig. 10.6) which is very similar to that for the growth of K. aerogenes under similar conditions (Figs 4.1-4.3 and 6.1-6.3). The drastic difference between the growth rates of D. lansenii and K. aerogenes is reflected in the difference between the values of the specific power output but not in the values of the ATP pool as these have remained very similar. This highlights one of the major differences between specific power and the cellular ATP pool as indicators of metabolic activity. The value of the specific power is more dependent on the rate of catabolism rather than the degree of coupling; the opposite is true for the value of the cellular ATP pool. Therefore when comparing the specific power values for different microbes growing under the same conditions or for the same microbe growing under vastly different conditions consideration has to be given to the relative difference in the growth rates (i.e. rate of overall metabolism and hence catabolism). However the value of the specific power is still a reliable indicator of comparative metabolic activity for cells under the same environmental conditions.

The specific power responds dramatically to a change in metabolism (Figs 6.1-6.3). Immediately after inoculation of the cells into pure medium there was a rapid rise in the specific power due to the start of catabolism and its increase in rate. The relatively constant value during growth represents a state of dynamic equilibrium between catabolism and anabolism. On exhaustion of the glucose and cessation of catabolism the specific power outputs drops rapidly to zero. After

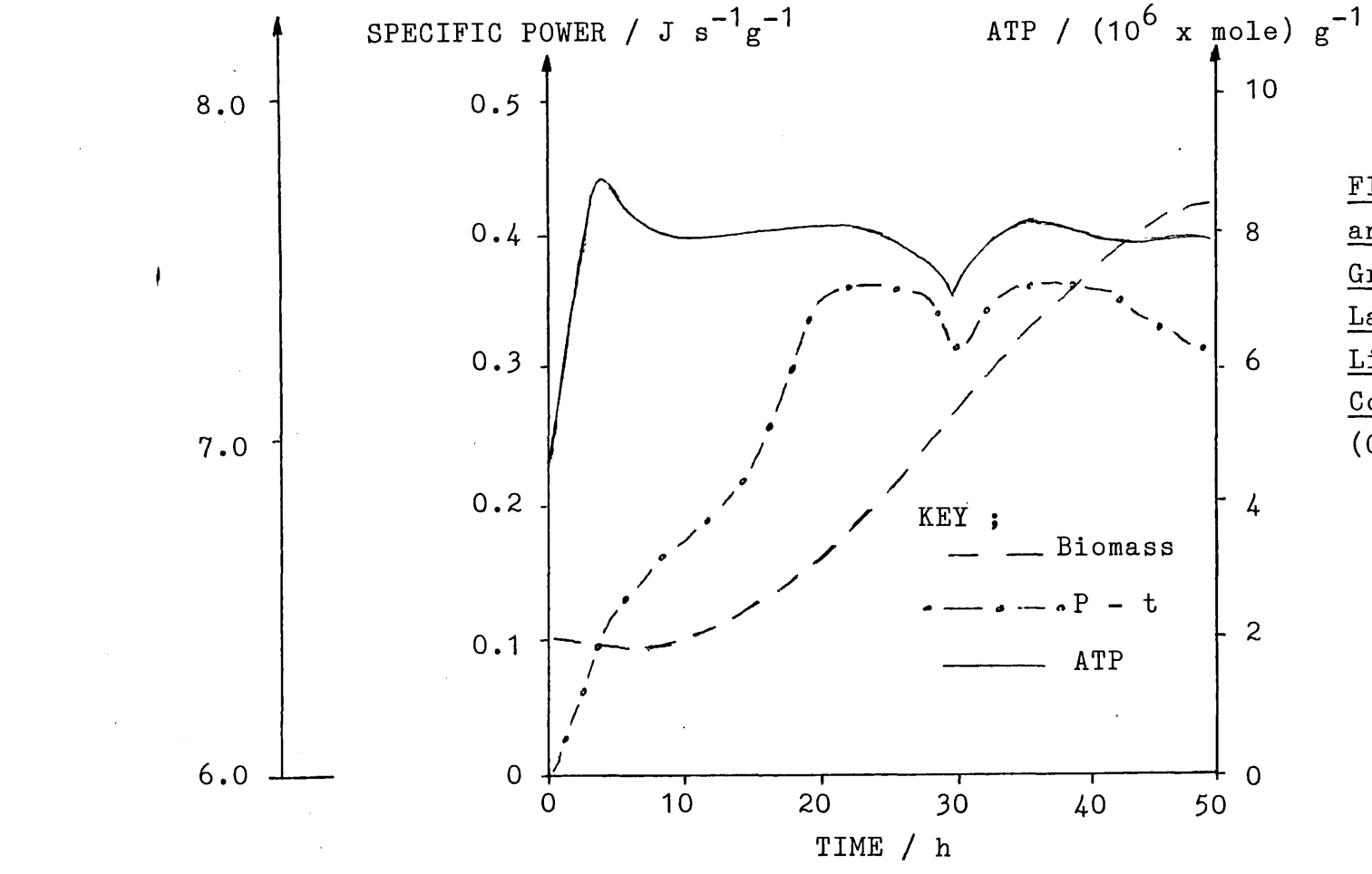



FIGURE 10.6 P - t Trace and ATP Profile for Aerobic Growth of Debaryomyces Lansenii in A Glucose - Limited (5.5 mmol dm$^{-3}$) Complex Medium (Gustafsson, 1979)

c. 2 h in the statinary phase the cells are still fully viable and maintenance processes are taking place, but there is no production of waste heat, (or it is too small to measure), as catabolic activity has ceased.

The specific power output during the later stages of exponential growth in glucose-limited media under non-standard conditions (Fig. 6.4) is greater (c. 2.5 J $s^{-1}$ $g^{-1}$) than that (c. 1.9 J $s^{-1}$ $g^{-1}$) obtained under standard conditions (Figs 6.1-6.3). This is due to the increased rate (throughput) of catabolism possibly caused by the intermediate metabolites usually destined for anabolic processes under standard conditions being catabolised to acetate. Under these non-standard conditions after exhaustion of the glucose the specific power decreases to a low value (0.6 J $s^{-1}$ $g^{-1}$) which is maintained during the complete oxidation of acetate when anabolism has ceased.

During aerobic growth in glucose-limited (Figs 6.1-6.3) and phosphate-limited media (Fig. 7.5) the ATP profile always followed the course of the P-t trace. This supports the suggestion that the cellular ATP pool and the specific power are indicative of similar metabolic processes. During the stationary phase the specific power output decreases to zero, but the ATP pool does not. This highlights a further difference between specific power and the cellular ATP pool as metabolic indicators. During the stationary phase maintenance processes are occurring and the finite size of the ATP pool demonstrates that it is probably partly determined by maintenance requirements; maintenance energy is too small to be determined and so the specific power is zero.

Gustafsson (Fig. 10.6) also observed that the ATP and p-t traces are similar in shape during exponential growth. This author is the only previous investigator to monitor power and ATP measurements in tandem on the same growing culture. Only one other author has used both (Lloyd

et al., 1978) and then not in tandem during a study of the synchronous growth of Tetrahymena pyriformis.

Oscillations of the specific power during exponential growth in glucose-limited media observed are in general not as frequent or as large as those of the ATP pool (Figs 6.1-6.3). The oscillations in the ATP pool have been attributed to changes in the ATP content of the cells during the growth cycle accompanied by a variable growth cycle time which caused changes in the degree of synchrony of an exponentially growing culture. During the synchronous growth of T. pyriformis (Lloyd et al., 1981) oscillations in the ATP pool (Fig. 10.3) were very much greater than those of the waste heat (Fig. 10.7). In view of this, then oscillations of the heat output (specific power) during exponential growth would be smaller than those of the ATP pool. During exponential growth variations in the P-t trace about the mean value (Figs 6.1-6.3) and oscillations in the ATP pool are difficult to correlate. However there does appear to be a tendency for changes in the specific power to be in opposition to those of the ATP pool. It is possible that the change in the cellular ATP concentration is a mechanism to maintain a constant rate of catabolism.

The cellular ATP pool size and the specific power output for the different growth conditions are shown in Table 10.3 The data illustrates further the differences between the two parameters when they are used as indicators of metabolic activity. When the catabolic activity is zero (i.e. stationary cells) the specific power is zero but the ATP pool never falls below $0.5 \times 10^{-6}$ mol $g^{-1}$. The relationship between changes in the specific power and the size of the ATP pool observed under different conditions is not linear, but an increase in the specific power output is accompanied by an increase in the ATP pool. During the consumption of acetate in phosphate-limited media when extracellular phosphate is not

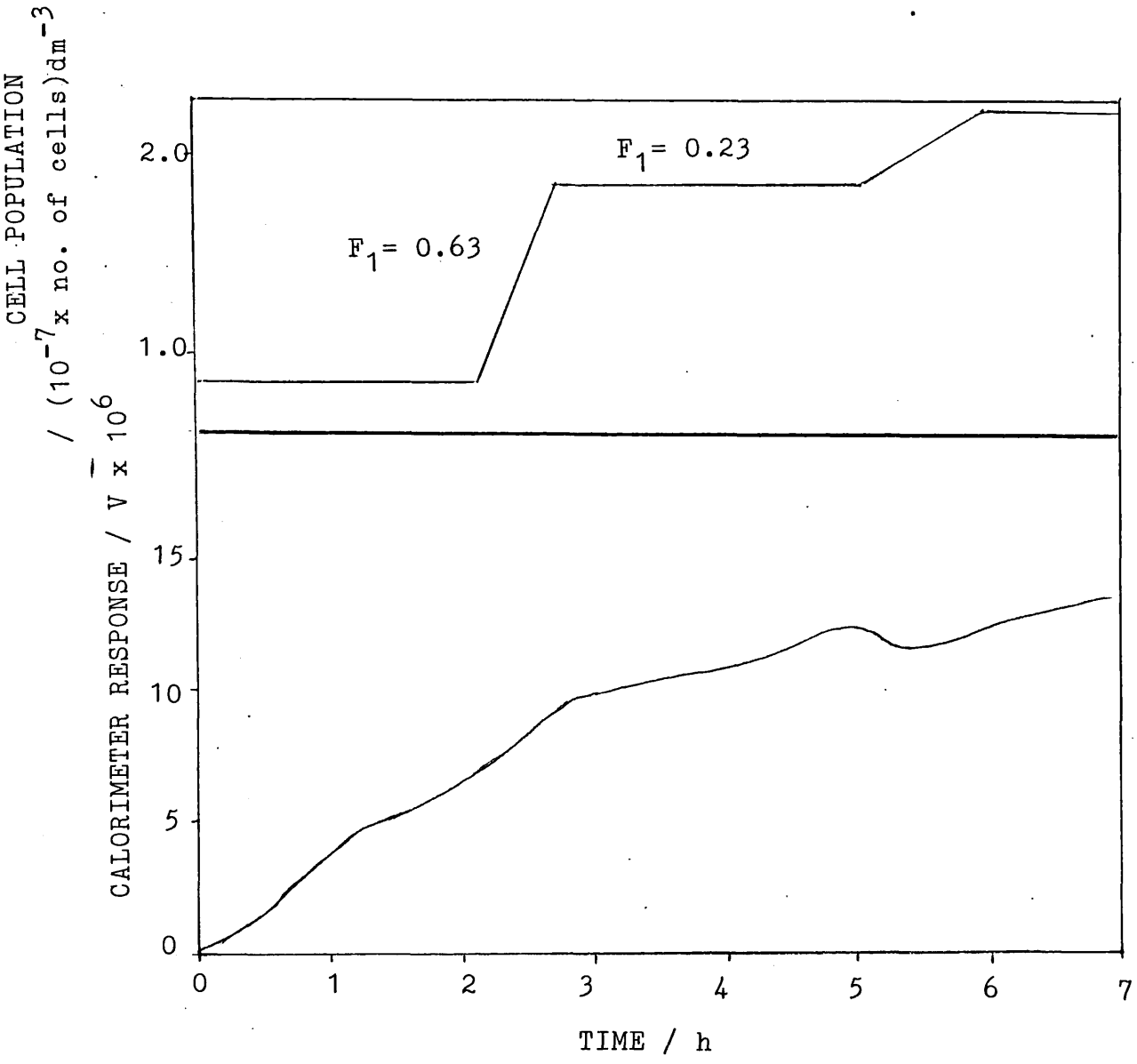



FIGURE 10.7 Changes in The Waste Heat Output of T. pyriformis During Synchronous Growth in Aerated Medium (Lloyd et al., 1977)

TABLE 10.3 The ATP Pool and Specific Power Output of Cells of K. aerogenes under Different Growth Conditions

| STATE OF CELLS | CORRESPONDING FIGURE IN TEXT | POWER / $J\ s^{-1}\ g^{-1}$ | ATP POOL/ $(10^{6}$ xmole$)\ g^{-1}$ |
|---|---|---|---|
| 1. Cells in the stationary phase for c. 36 h | —— | 0 | 0.5 |
| 2. Cells of inoculum; immediately after adding to any growth media | 6.1-6.4, 7.5 | 0 | 2.0 |
| 3. Fully viable cells in the stationary phase for c. 2 h (glucose - limited) | 6.1-6.4 | 0 | 2.5 |
| 4. P-trained cells in P-limited medium (extracellular P conc. = 0) metabolising (with some growth) acetate | 7.5 | 0.2 | 2.0 |
| 5. Cells growing aerobically in glucose-limited media | 6.1-6.3 | 1.95 | 7.8 |
| 6. Cells in P-limited media (extracellular P available) growing on glucose | 7.5 | 2.25 | 9.7 |

available (Fig. 7.3 section c, and Fig. 7.5) the specific power output is low (0.2 J $s^{-1}$ $g^{-1}$) and the cellular ATP pool is at a value (2.0 x $10^{-6}$ mol $g^{-1}$) similar to that of fully viable stationary cells. This is indicative of a low rate of catabolism and ATP generation due to the lack of readily available phosphate. The specific power output (2.25 J $s^{-1}$ $g^{-1}$) for cells growing on glucose in phosphate-limited media when extracellular phosphate is available (Fig. 7.3 section A and Fig. 7.5) is greater than the value (1.95 J $s^{-1}$ $g^{-1}$) for exponential growth in glucose-limited media (Figs 6.1-6.3). This is indicative of the formation of acetate during growth; which in turn results in less of this intermediate metabolite from catabolism being available for anabolism; the rate (throughput) of catabolism is therefore greater. Some of the linking metabolic pathways between catabolism and anabolism are not functioning as efficiently as in cells growing in glucose-limited media i.e. the degree of coupling in phosphate-limited media is looser.

There have been very few studies of the cellular ATP pool of microbes growing in phosphate-limited media (Damoglau and Dawes, 1967). Under conditions of phosphate deprivation the size of the ATP pool is considerably reduced. In contrast there is no documentation of a calorimetric study of bacterial growth in phosphate-limited media. Tempest and his colleagues have observed alterations in the chemical composition of bacteria as a result of training to growth in phosphate-limited media. Under phosphate-limited conditions the wall polymer teichoic acid ceases to be synthesised. Instead there appears in the wall a phosphate-free polymer, a teichuronic acid (a polymer of glucoronic acid residues). Thus even when phosphate is in short supply the cell still continues to

synthesise an anionic polymer in the wall which like the teichoic acid polymer has the capacity to bind with divalent ions. During phosphate-limited conditions cells synthesise very small amounts of phospholipid and produce instead phosphate-free glycolipids. Almost nothing is known however, of the way in which glycolipids take over the physiological functions of phospholipids in the membrane.

The exact mode of transport of phosphate (pyrophosphate) across the cell wall and membrane has not been fully established as has the mode of transport of other nutrients. There is still some debate as to its nature. Present thinking suggests the existence of two major systems of phosphate transport first introduced by Willsky et al., (1973); phosphate inorganic transport (PIT) and phosphate specific transport (PST). It has been suggested that PIT is coupled to a proton motive force and PST is possibly energised by phosphate bond energy (Rosenberg et al., 1977). In support of this active transport it has been observed (Rosenberg et al., 1979) that high concentration gradients (x 500) were established by the two systems. In this the phosphate is esterfied upon entry to the cell and de-esterfied by phosphøtases with phosphotransferase activity on the internal side of the membrane. Thus phosphate transport into the cell is believed to be closely linked to that of the transport of carbon substrate, particularly sugars.

The overall energy balance for growth reveals a small percentage of the metabolised energy unaccounted for; this has been attributed to that required by biosynthesis/anabolism ($\Delta\%$). If this is true then it could be suposed that there is a direct linear relationship between this and the yield, so that a reduction in the yield would result in a similar reduction in $\Delta\%$. A reduction in yield is equivalent to a reduction in the extent to which anabolic processes have taken place; and therefore a reduction in $\Delta\%$ would be expected to accompany this change. From the

results obtained a decrease in yield is accompanied by a decrease in the value of Δ%, but the relationship between the two is not linear. For the growth of cells in glucose-limited media the yield was 0.49 g $g^{-1}$ and 12.32% of the available energy was required for biosynthesis, (Δ%)(Table 5.2). The overall energy balance for the growth of cells in phosphate-limited media reveals an average biosynthesis value (Δ%) of 3.6% (Table 7.2) and an average yield of 0.44 g $g^{-1}$ (Table 7.1). Growth in phosphate-limited media resulted in a 60% reduction in the value of Δ% but only a 10% reduction in the yield compared to growth in glucose-limited media. Further the reduction in yield observed during growth in the presence of nalidixic acid (NA), (Table 9.2) is accompanied by a reduction in the value of Δ% (Table 9.3) but again there is no linear relationship between the reduction in yield and that of Δ%. The conditions of growth in these latter examples are very much different to that for glucose-limited media, and hence the metabolism is very different. However the non-linear relationship between the changes in yield and Δ% cannot be justified using the present definition of Δ%. Δ% as measured and calculated is most probably a composite term and its value is due to the additive effect of several elemental quantities that can vary in value. One element in the composite Δ% value may well be associated with the maintenance requirements of the cell, another may be associated with a cellular energy storage system which is operative under favourable conditions of growth but inoperative under less favourable conditions. The existence of an energy storage system would be advantageous to the cell as during periods of deprivation it could be used to supplement cellular metabolism and cell repair. Energy is probably only stored under favourable growth conditions when surplus substrate/secondary metabolites is/are available for processing into metabolites for storage. The intermediates used for storage could be high energy molecules (e.g. ATP) which are then utilised during periods

of stress caused by the starvation of nutrients or some other deprivation or adverse environmental conditions. The storage of such intermediates under such unfavourable growth conditions as in phosphate-limited media is less likely than in glucose-limited media where there is a plentiful supply of phosphate. This is substantiated by the observation that the ATP pool ($0.75 \times 10^{-6}$ mol $g^{-1}$) of stationary cells grown in phosphate-limited media (Fig. 7.5) is less than that ($2.5 \times 10^{-6}$ mol $g^{-1}$) of stationary cells grown in glucose-limited media (Figs 6.1-6.4). In consequence that part of the value of $\Delta\%$ which can be attributed to the energy storage element will be less for cells grown in phosphate-limited media than in glucose-limited media. There are no theoretical or practical reasons to suggest that there has been an increase in the maintenance element of cells grown in phosphate-limited media compared to that of cells grown in glucose-limited media. Therefore the value of $\Delta\%$ for growth in phosphate-limited media will be substantially less than that for growth in glucose-limited media, as was observed. Due to the composite nature of the value of $\Delta\%$ there will be no linear relationship between the relative reductions (changes) in the yield and $\Delta\%$ when a comparison is made between cells grown in glucose- and phosphate-limited media, (or for growth in some other media or under different growth conditions).

Rationalisation of the values of $\Delta\%$ obtained for the different types of metabolism outlined in Fig. 7.3 (sections A, B and C) during growth in phosphate-limited ($2.5 \times 10^{-3}$ g $dm^{-3}$) media can also be achieved by explanations based on the composite nature of the biosynthesis value ($\Delta\%$). It may be presumed that under the conditions of growth experienced by the cells in section C (extracellular acetate present but glucose and phosphate not present) the cells will be in a more deprived hence stressful situation when compared to that of cells in section B (extracellular glucose and acetate present but phosphate not

present) and even more so compared to that of cells in section A (extracellular glucose and phosphate present). Therefore under the least favourable growth conditions (section C) energy storage will be at a minimum; greater quantities of energy may well be stored during section B and even more during section A. This is substantiated by the relative size of the cellular ATP pools for cells in each section (Fig. 7.3 section A 9.5 x $10^{-6}$; section B, 9.5-2.5 x $10^{-6}$ (decreasing); section C, 2.5 x $10^{-6}$ mole $g^{-1}$). Part of the ATP contained in the pool probably represents some of the stored high energy phosphate bond intermediates that are ultimately utilised by the cell during periods of deprivation. Due to the relative contribution of the stored energy element to the value of $\Delta$% for each section, $\Delta$% will be smallest for section C (av. -3.7%), for section B (av. 1.0%) and largest for section A (av. 11%) as shown in Table 7.2. The observation of negative biosynthesis values (Table 7.2, section C) is impossible to explain theoretically utilising the composite nature of $\Delta$%. If $\Delta$% is partly composed of a maintenance element this should always be present in metabolising or viable cells, and therefore the value of $\Delta$% will always be positive. The cause of negative $\Delta$% values could be atributed to experimental errors in the absorbance measurement of biomass and hence stored energy, due to changes in the light scattering properties of the cells.

When cells trained to grow in phosphate-limited media are grown in glucose-limited media (7.3) the p-t, P-t and $CO_2$-t traces are widely different for each growth as are the mass and energy balances (Fig. 7.6). This is indicative of different metabolic processes occurring in the cell. The particular pathways chosen by the cells can be accounted for in terms of the availability of several different overall metabolic routes. During repeated growths in phosphate-limited media the cells develop the capacity to produce greater quantities of certain enzymes and intermediates to maintain growth; these are not required in such large

quantities for growth in glucose-limited media. The capacity of the phosphate trained cells to produce enzymes and intermediates required for growth in glucose-limited media, and hence maintain the operative status of glucose-limited metabolic pathways is still present. This is supported by the observation that phosphate trained cells grow normally in glucose-limited media after five sub-cultures in glucose-limited media (7.3). Consequently the presence and operation of several major pathways results in a poor control of the degree of coupling between catabolism and anabolism, and hence overall metabolic activity. This is reflected in the difference between the mass and energy balances. The degree to which each pathway is utilised is poorly regulated and the final balance (a measure of the extent each is utilised) may almost be a matter of statistical chance.

During the growth of cells in phosphate-limited media ($2.5 \times 10^{-3}$ g $dm^{-3}$) during section B (Fig. 7.5) the power output is constant while the P-t trace falls gradually. The successful operation of catabolism requires the presence of available (free) phosphate (pyrophosphate) so that ADP can be converted to ATP. The constant power output during this section can be accounted for as follows. The increase in biomass (the rate of increase decreases gradually during this period) which causes a gradual increase in the power output is exactly balanced by a gradual change in metabolism of the cells; this would tend to cause a gradual decrease in power output, so that the measured power output remains constant. This gradual change in metabolism could be due to the free phosphate becoming progressively scarcer during this period. Once free phosphate has been exhausted it can be "scavenged" from other sources such as that "fixed" in organic phosphoesters or from a cellular storage pool of inorganic phosphate. Ent. aerogenes stores a pool of inorganic phosphate in the form of polyphosphates which is utilised during phosphate deprivation (Harold, 1966). A gradual change in the metabolism

of the cells can usually be detected by a gradual change in the relative rates of formation of products. The only products detected during section B, Fig.7.3 (also Fig. 7.5) were acetate, biomass, carbon dioxide and water. The rate of formation of biomass was similar to the rate of formation of acetate (Fig. 7.4) suggesting that metabolism is unchanged during this period. The probability that the effect on the power output of a change in metabolism accompanied by a change in the rate of formation of biomass results in a constant measured power output is highly unlikely. It would be more probable that the effects of each do not balance and to observe a gradual change in the power output during this section. A closer evaluation of the results suggests only one plausible explanation for the constant value of the power during this section. During section A, Fig. 7.3 (also Fig. 7.5) phosphate available in the medium has been incorporated into biomass in two probable forms; as organic phosphesters (structural polymers, nucleic acids etc) i.e. fixed phosphate, and as unbound ionic phosphate i.e. free phosphate. The postulation of the presence of fixed and free phosphate in the microbial cell is not a novel one (Medveczky, 1971). It is possible that this pool of free phosphate is in fact a membrane-associated one. During growth in phosphate-deprived medium (section B) when extracellular phosphate is not present phosphate is scavenged. The use of the word scavenged in some contexts can be misleading, and a more appropriate word to use is "shared". The rate of growth during section B decreases gradually due to the decreasing availability of phosphate. It can be assumed that catabolism and hence power and specific power output is also rate limited by the availability of phosphate. Further it is safe to assume that only free phosphate can take part in catabolism. Therefore as the power output during section B is constant then so must be the rate of catabolism and hence the size of the free-phosphate pool per unit volume

of culture. This hypothesis is supported by the observation that rate of evolution of carbon dioxide (an end product of catabolism) also remains constant throughout this period (Fig. 7.3). This does not conflict with the observation that the specific power output decreases gradually during section B. During growth the amount of free-phosphate per cell decreases as during each cell cycle the phosphate present in the parent cell has been shared between the two newly formed cells. Further as the power output remains constant and therefore also the free-phosphate pool per unit volume of culture, then there must be no exchange of phosphate between the free and fixed forms. Certainly as the power is constant the total amount of free-phosphate present per unit volume of culture is constant. However there may be an exchange of fixed phosphate from one molecule to another such as the movement of phosphate from cell wall and membrane polymers (for which substitute phosphate free polymers can be synthesised), to nucleic acids (for which there is no evidence that the cell can synthesise a substitute). Thus the free phosphate in a parent cell (when extracellular phosphate is not available) is shared during cell division, between the two new cells only as free phosphate and the fixed phosphate is shared only as fixed phosphate. This general theory is supported by the observation that the ATP pool also decreases gradually during section B, as the size of the free-phosphate pool per cell (which regulates the regeneration of ATP from ADP) also decreases gradually.

The application of flow microcalorimetry to microbial systems to monitor growth or as a method for identifying and characterising micro-organisms has already been discussed. A recent application not so far

discussed is the use of flow microcalorimetry for the bioassay of metabolic modifiers. Binford et al., (1973) were the first investigators to show that microcalorimetry could be used to demonstrate the sensitivity of microorganisms to various antibiotics. Subsequent investigators have almost exclusively concentrated upon the microcalorimetric bioassay of antifungal antibiotics. There have been no publications describing a full quantitative study of the action of an antibacterial drug using microcalorimetry. Moreover, the few reports that do exist on the semi-quantitative bioassay of antibiotics mostly describe microcalorimetric observations on the affects of drugs upon non-growing (respiring) yeast cells (Beezer, 1977; Beezer et al., 1977(a); Beezer et al., 1977(b); Beezer and Chowdhry, 1980(a)), growing yeasts (Beezer et al., 1979; Beezer and Chowdhry 1980(b); Beezer and Chowdhry, 1981), and some upon growing bacteria (Mardh et al., 1976; Mardh et al., 1978; Beezer et al., 1978; Beezer et al., 1980). The phrase "semi-quantitative" in the context of the above cited literature means that the investigators have analysed quanitatively relative changes in the value of the power output (p-t trace) caused by the drug. Few investigators have monitored the power output of a microbial culture containing the drug until metabolism had ceased. Further, none have studied or quantified the secondary metabolites formed during growth in the presence of the drug, or utilised the calorimetric measurement in conjunction with other biological measurements (biomass formation, carbon dioxide evolution). The presentation of fully quantitative data which includes mass and energy balances has not been possible. No investigators have used the specific power parameter (P-t trace) to determine the potency/action of drugs on microbial cells. In the studies described when growing cells were used the drug was introduced during the logarithmic growth phase. The subsequent change in the power output (p-t

trace) was evaluated usually by measuring the time taken for the power output to reduce to a specified value or by measuring an area encompassed by the p-t trace. The evaluated changes in the power output obtained for growth in the presence of different concentrations of the same drug have in some cases been linearly related to the concentration of the drug or the logarithm of the concentration. The general character of the changes observed in the shape of the p-t trace with increasing drug concentrations are similar to those observed (Figs 8.1-8.3, 9.1 a-h). The evaluated changes in the power output obtained for the growth of a specified organism in the presence of the same concentrations of different drugs has been used to obtain drug potency ratings. In most cases the rating did not agree with that obtained by classical techniques such as MIC or diffusion assay.

The complete lack of real quantitative data can be attributed to the fact that most of the research has been directed towards the establishment of microcalorimetry as a rapid and easy technique for the bioassay of drugs, and not as a reliable and quantitative tool to elucidate the full effects of drug action upon metabolising mocribes. The reasons that have prompted previous workers to direct their research in this direction are indeed valid; many of the classical techniques to establish drug potency are tedious, unreliable and inaccurate.

The use of specific power as the calorimetric measurement takes into account changes in biomass, and any changes are a measure of the effect of the drug per microbial cell. Hence specific power is more of a standard measure than power and therefore superior for comparative work. Thus specific power should be used to evaluate the drug-microbe interaction even when developing calorimetric bioassays, so that a meaningful interpretation of the results can be made. There have been no previous calorimetric studies of the interaction of sulphanilamide or NA with bacteria.

Sulphalilamide inhibits the synthesis of folic acid. Folic acid is a co-enzyme concerned with the metabolism of groups containing one carbon atom; the transfer of methyl groups and the utilisation of formate in the synthesis of amino acids and nucleotides. Sulphanilamide is very similar in structure to p-aminobenzoic acid which is required for folic acid synthesis. The antibacterial action of sulphanilamide is a result of it competitively inhibiting the incorporation of p-aminobenzoic acid into folic acid.

The bactericidal action of NA can be correlated with the onset of DNA degradation. Nevertheless the inhibitory action of NA is reversible; on transfer to drug free medium the cells immediately resume DNA synthesis and further killing is arrested (Deitz et al., 1966). The exact mechanism by which NA inhibits DNA synthesis is not fully understood. Direct binding of the negatively charged NA molecule to the strongly negatively charged DNA molecule is most unlikely due to the electrostatic repulsive forces. Further DNA extracted from NA treated cells is not cross-linked (Boyle et al., 1969). Evidence that DNA degradation in NA treated cells appears to commence at the replicating fork and to proceed sequentially along the chromosome to involve progressively older DNA, and that both the new strands and the parental template strands are degraded together, suggests that NA has some direct action of the DNA replicating apparatus of the bacterial cell. The situation is evidently complex and its solution must await a more detailed understanding of the nature of the DNA-replicating enzyme system.

Changes in the cellular ATP pool were not monitored during the microcalorimetric study of the antibacterial effects of sulphanilamide and NA. This was due to the lack of time and not to the fact that additional information about the action of the drugs would not have been forthcoming if ATP had been monitored. A literature survey reveals only

one study (Thore et al., 1977) of the effect of drugs (ampicillin) on the intracellular levels of ATP in bacterial cells. The ATP content of the culture was directly proportional to its viability (as measured by colony-forming-units); a decrease in viability due to drug action was accompanied by a reduction in the ATP content. It seems likely that the effect of sulphanilamide and NA of the ATP content of cells of K. aerogenes would be similar to that of ampicillin and the higher the drug concentration the greater the reduction in ATP content. Although the ATP profiles for cells growing in drug-free media followed the course of the P-t trace (Figs 6.1-6.3, 7.3), it is debatable whether the same will be true for cells grown in the presence of drug.

The value (4.65 mmol $dm^{-3}$) of the MIC for sulphanilamide (8.2) and that (0.052 mmol $dm^{-3}$) of NA (9.1) were established by the media dilution technique. It has been noted that the conditions encountered by the cells growing in media in boiling tubes may not be exactly similar to those encountered in the 600 $cm^3$ culture vessel. The conditions in the culture vessel due to the more efficient aeration are probably more favourable to growth than those encountered in boiling tubes. Therefore MIC values determined by media dilution in the boiling tubes may be lower in value than if they had been determined in the culture vessel under the standard conditions of growth. This is supported by the observation that relatively high concentrations of sulphanilamide (3 x MIC) and NA (27.5 x MIC) were bacteriostatic and not bactericidal (Figs 8.3 and 9.1 h). Further, MIC values were determined by inoculating stationary phase cells (exhibiting a lag phase) into media already containing the drug, while during test the drugs were added to rapidly metabolising exponentially growing cells. The reasons for adding the drugs to exponentially growing cells and not to the media prior to inoculation was to avoid long lag phases, to obtain a measurable amount of growth and therefore to avoid

long periods of experimental measurement.

Previous workers using calorimetric methods have observed that concentrations of drugs in excess of the MIC (determined by media or broth dilution) were still bacteriostatic (Mardh et al., 1976; Mardh et al., 1978; Semenitz, 1978; Beezer and Chowdhry, 1981).

The antimicrobial potency (per molecule of drug) of sulphailamide (MIC: 4.65 mmol $dm^{-3}$), based on MIC values is about 90 times less than that of NA (MIC: 0.052 mmol $dm^{-3}$). This difference is reflected in the different changes of the P-t, p-t and $CO_2$-t traces, and mass and energy balances for growth in the presence of sulphanilamide and NA. Probably the best calorimetric measure of the antibacterial potency of drugs on growing cells is the specific power output immediately upon exhaustion of the glucose and cessation of growth. The output decreases from 1.95 J $s^{-1}$ $g^{-1}$ (observed with the beginning of growth in the absence of drug) with increasing drug concentration (Figs 8.1-8.3, 9.1 b-h). This is because the value of the specific power immediately on cessation of growth represents the metabolic state of the cell as a result of the action of a given concentration of drug for a specified period of time. This calorimetric measure of antibacterial potency can be used to compare the action of different drugs or the action of different concentrations of the same drug.

The concentration of sulphanilamide (14.1 mmol $dm^{-3}$) required to reduce the specific power to the same value (1.20 J $s^{-1}$ $g^{-1}$) on cessation of growth (Fig. 8.3) is c. 180 times greater than that concentration of NA (0.078 mmol $dm^{-3}$)(Fig. 9.1 c). This microcalorimetric evaluation of the difference in potency between the two drugs (x 180) is twice that obtained from MIC data. The relatively greater potency of NA compared to that of sulphanilamide may be due to the nature of their action in the cell. The action of sulphanilamide by incorporation into folic acid may

be more easily overcome by the adaptation of cellular metabolism than the possible enzymic interaction of NA on DNA replication.

The effects of increasing concentrations of sulphanilamide (Table 8.1) and of NA (Tables 9.2, 9.3) on the mass and energy balances of growing cells are similar. In general increasing the drug concentration causes a decrease in the yield (and hence the energy stored) and the value of Δ%, and an increase in the amount of waste heat and carbon dioxide produced. As the concentration of the drug increases the coupling between catabolism and anabolism (i.e. the efficiency with which the cell produces biomass) is increasingly affected; the degree of coupling decreases. The two drugs restrict anabolic processes and probably leave catabolism relatively unaffected. Becaue anabolism is not functioning to its full capacity in the presence of drug, then intermediates from catabolism usually destined for anabolism are further catabolised to produce relatively greater quantities of waste heat and carbon dioxide. Further due to the reduced extent of anabolism in the presence of drug, the yield is smaller. As the concentration of the drug increases so will the differences between the mass and energy balances as compared to those for growth in drug free media.

A detailed study of the effect of increasing concentrations of sulphanilamide on the mass and energy balances for growth is not possible due to the limited amount of data (Table 8.1). The use of higher concentrations of sulphanilamide was not possible on account of the solubility of the drug in the concentrated solution injected into the growing culture. However the data available for increasing concentrations of NA is more plentiful; changes in the percentage of available energy stored, used for biosynthesis (Δ%) and wasted, with increasing concentrations of NA are shown in Tables 9.2, 9.3 and Fig. 9.3. Over the concentration range (0-20 MIC) although there is a non-linear

relationship between the yield and Δ% (Fig. 10.8), nevertheless a lower yield is accompanied by a lower value of Δ%. Even allowing for errors in estimating the biomass the deviation from linearity is in excess of experimental error. A graphical representation of the percentage carbon stored or the percentage energy stored versus Δ% observed for increasing concentrations of NA gives an identical scatter of the points as in Fig. 10.8. This non-linear relationship between the yield and Δ% can be attributed to the composite nature of the calculated value of Δ%; the effect of different concentrations of NA will not be manifested in the same way in the value of Δ% as it will in the value of the yield. This is because changes caused by different drug concentrations on the stored and maintenance energy terms which in part contribute to the calculated value of Δ% are probably not related to drug concentration in a simple manner. However as in phosphate-limited media where the cells are under stress due to the deprivation of phosphate the cells growing in the presence of drug (NA) are also under stress. This results in a reduction in the contribution of the stored and maintenance energy terms to Δ%, and hence a reduction in the value of Δ%. A lower yield (which is a measure of the extent of anabolism) is generally accompanied by a decrease in Δ% (Δ% is in part due to the energy required by anabolism). It is logical that a decrease in the yield will be accompanied by a decrease in the contribution of the energy required for anabolism to the value of Δ%. This decrease accompanied by a decrease in the contribution from the stored and maintenance energy terms will result in a general decrease in the value of Δ% as the yield decreases due to the action of increasing concentrations of NA. It is important to realise that when referring to the stored energy term in the context of contributing to the value of Δ%, the energy stored is in the form of high energy phosphate-bond - containing-molecules and not as in biomass, which is concerned with the

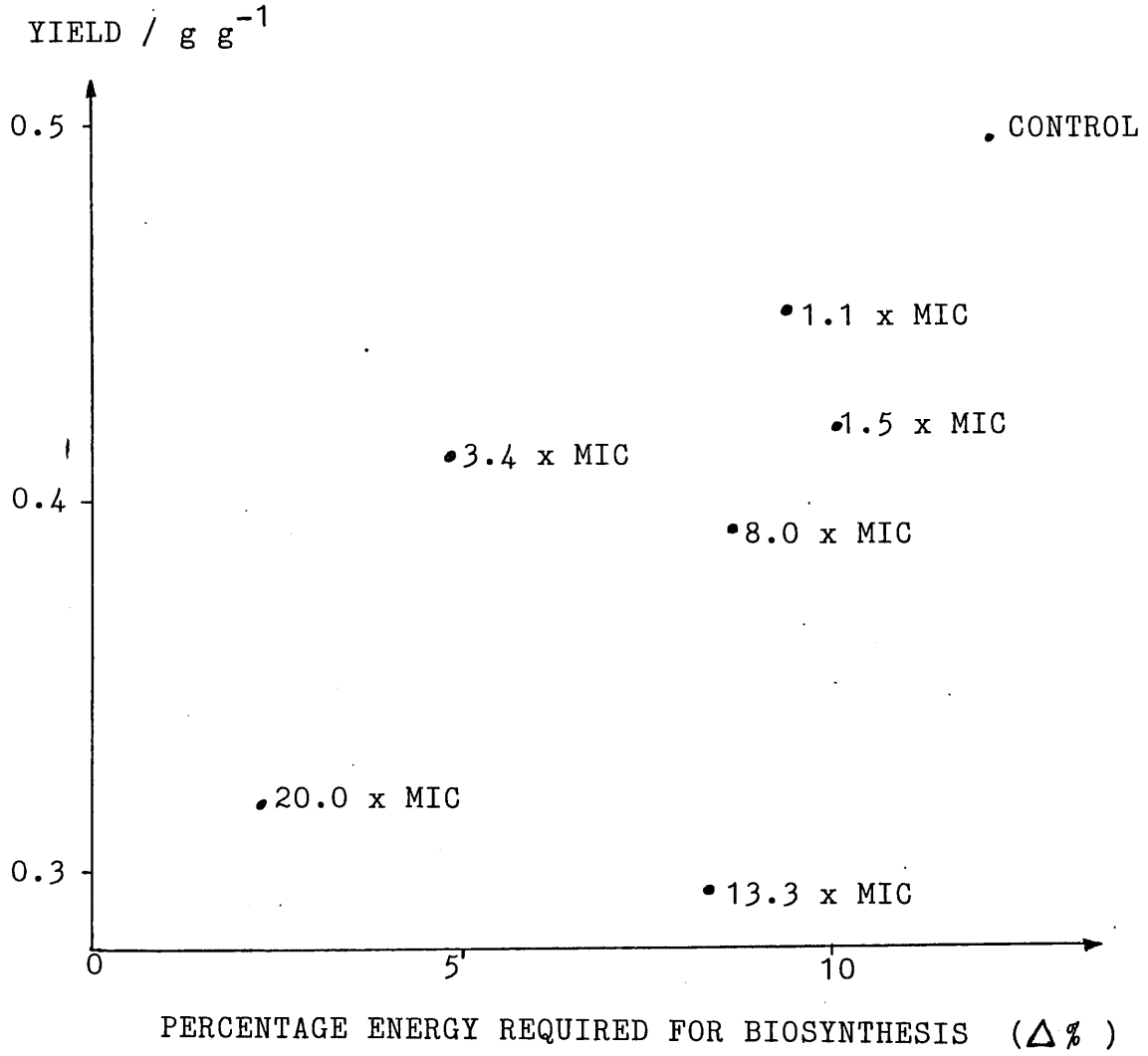


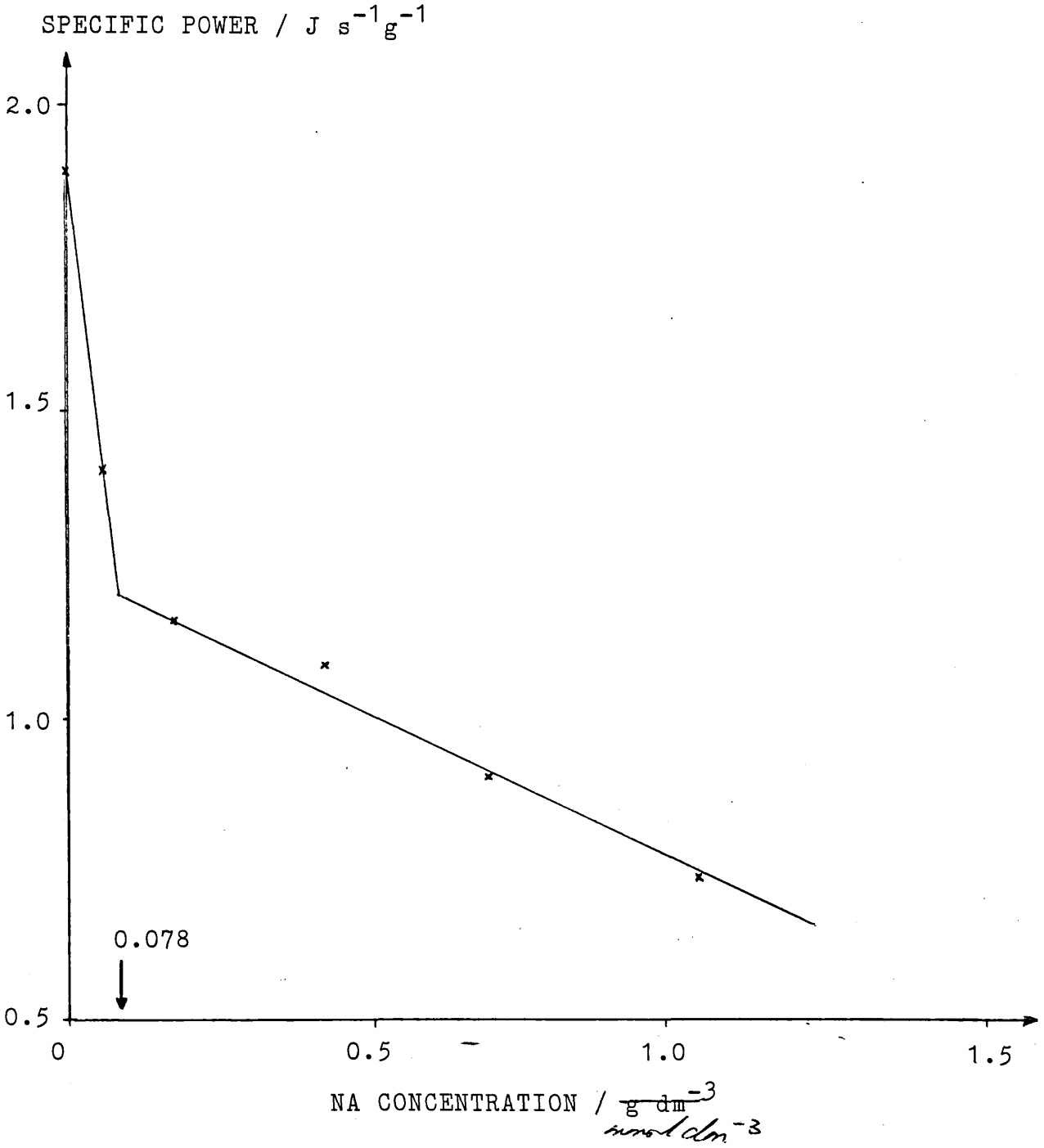

FIGURE 10.8 The Variation in The Values and The Percentage Energy Required for Biosynthesis (Δ% ) during Growth in The Presence of Different Concentrations (Shown) of NA

overall mass and energy balances.

The total exlusion from the data presented in this discussion of the results obtained for growth in the presence of the highest concentration (1.422 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) of NA (Tables 9.2, 9.3 and Fig. 9.1 h) is due to the erroneous carbon balance ($C_{rec}$ = 113%) and energy balance ($\Delta$% = 15.9%). These erroneous values may be attributed to changes in the light scattering properties of the cells causing changes in the absorbance-biomass calibration curve. Any change in the light scattering may be due to the formation of elongated cells or excess mucus produced when the cells grow in the presence of NA. Cell elongation has been observed before (Dean and Moss, 1970; Stevens, 1980) but the formation of mucus has not, when cells are grown in the presence of NA. Dean and Moss reported that NA did not effect the relationship between turbidity and cell mass. However these two investigators used a relatively low concentration of NA (~~0.065 g dm$^{-3}$~~ 0.28 mmol dm$^{-3}$) compared to the highest NA concentration (1.422 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) used.

The variation of specific power (at the end of growth) with NA concentration (Fig. 10.9, using data of Table 9.1) shows two distinct regions of antibacterial action/potency. As the NA concentration is increased up to a value of 0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ the potency per molecule of the drug for this lower region is constant. At higher concentrations (0.078-1.035 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) there is a constant but reduced antibacterial potency. As the two regions show a linear relationship between specific power and NA concentration the antibacterial potency exhibited by NA is constant for each region. As the gradient of the line for each region is different the antibacterial potency of NA (per molecule of NA) is different. Utilising the gradients of these two lines as a measure of the antibacterial potency, the potency of NA in the concentration range of 0-0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ is 18 times greater than at concentrations above

FIGURE 10.9 Specific Power at The End of Growth as A Function of NA Concentration



0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$. The curves of the waste energy and stored energy expressed as a function of the NA concentration (Fig. 9.3) show a break at this same concentration in support of the dual potency of NA, (especially if the data from the two highest NA concentrations is ignored). Further the curves of the yield or the percentage carbon stored expressed as a function of NA concentration are identical in shape to the curve of stored energy versus NA concentration. This emphasises the point that when specific power is used as the parameter for the estimation of the potency of NA (Fig. 10.9) the resultant data is far more explicit than for other parameters (Fig. 9.3). Further any interpretation of the p-t trace as a parameter for the measurement of potency did not reveal this dual dose-related phenomena. This reinforces the importance of the use of specific power as a parameter of measurement when the drug-microbe interaction is monitored calorimetrically.

This dual dose related potency of NA as an antibacterial agent against various species of Gram-negative bacteria has been noted previously (Goss et al., 1964; Winshell and Rosenkranz, 1970; Crumplin and Smith, 1975). A more recent and detailed investigation with growing cells of E. coli (Stevens, 1980) showed that not only did NA exhibit the dual potency but so did some closely related analogues (oxolinic acid). Stevens showed (by viability counts) that with increasing concentrations of NA up to c. ~~0.08 g dm$^{-3}$~~ 0.34 mmol dm$^{-3}$ NA was increasingly bactericidal but further increases in the NA concentration (~~0.08-1.00 g dm$^{-3}$~~ 0.34-3.4 mmol dm$^{-3}$) dramatically reversed this trend. The concentration of NA at which this potency change occurred is similar to that observed in this work (Fig. 10.9). Stevens also showed that at increasing concentrations of NA up to ~~0.08 g~~ 0.34 mmol dm$^{-3}$ DNA synthesis was increasingly inhibited while at higher concentration (~~0.08-1.00 g dm$^{-3}$~~ 0.34-3.4 mmol dm$^{-3}$) the inhibition of DNA synthesis remained constant

but now inhibition of the synthesis of RNA and protein commenced, (and increased with increasing concentration). He concluded that NA is bactericidal at concentrations below c. ~~0.08 g dm$^{-3}$~~ 0.34 mmol dm$^{-3}$ when inhibition of DNA replication occurs in the absence of any marked effect on RNA and protein synthesis, but is bacteriostatic at concentrations above c. ~~0.08 g dm$^{-3}$~~ 0.34 mmol dm$^{-3}$ when DNA, RNA and protein synthesis are all inhibited.

The more lethal effect of NA at concentrations below 0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ when only DNA synthesis is inhibited arises as the result of an imbalance between DNA and protein synthesis. The imbalance presumably leads to inhibition of cell division but continued cell growth causing elongation and then filamentation (Stevens, 1980) and hence cell death. Extensive cell death was not detected during the growth in the presence of NA concentrations below 0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$. This may be due to the fact that Stevens measured the viability of the cells after long periods of contact (4 h) with NA; the period of contact during growth in the presence of NA below 0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ were shorter, not exceeding 3 h (Figs 9.1 c, d). Further the cells were probably under more favourable growth conditions than in the previous investigation and hence the antibacterial potency of NA may well have been less. However this difference in the growth conditions does not interfere with the identification of the dual close-related potency effect of NA or the determination of the concentration of NA at which the potency changes.

The clinical importance of the dual close-related potency of NA is that the readily attainable urinary concentrations of NA used to treat bacterial infections of the urinary tract far exceeds 0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ (McChesney et al., 1964). Hence when treating urinary tract infections with NA the dose should be controlled in order to maintain a NA concentration in the urine of just less than 0.078 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ to obtain the most lethal antibacterial effect. Further the fact that the dual dose-related potency of NA has been identified by other investigators using different

techniques illustrates the potential of microcalorimetry in investigations of microbe-drug interaction.

When NA-trained cells (0.043 and 0.086 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) were grown in drug free media the mass and energy balances resembled those of ordinary cells grown in drug free media i.e. the control (Table 9.2). This shows that trained cells still retain the capacity to maintain balanced growth in the absence of the drug. It may be assumed that the enzymes associated with DNA, RNA and protein synthesis have not been radically changed by training and are able to function in the absence of the drug. However the fact that the p-t and P-t traces for growth of trained cells in drug free media (Figs 9.4, 9.5) are different to those of the control (Fig. 9.1 a) and that acetate is also produced during growth (9.3.1) shows that some disturbance of the metabolism of the cells has occurred due to the training.

When NA-trained cells (0.043 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) are grown in media containing NA (0.043 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) the p-t and P-t traces are similar to those of control cells as are the energy and mass balances (Table 9.4). This shows that cells trained to grow in lower NA concentrations are resistant to the effect of NA at this concentration (0.043 ~~g dm$^{-3}$~~ mmol dm$^{-3}$). However the p-t and P-t traces (Fig. 9.6) and mass and energy balances (Table 9.4) for the growth of trained cells (0.086 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) in media containing NA (0.086 ~~g~~ mmol dm$^{-3}$) are not similar to those of the control. This shows that the cells trained at this higher concentration are not fully resistant; as are those trained at the lower concentration (0.043 ~~g dm$^{-3}$~~ mmol dm$^{-3}$). It is important to appreciate that at the training concentration of 0.043 ~~g dm$^{-3}$~~ mmol dm$^{-3}$ NA exhibits a higher potency than at the training concentration of 0.086 ~~g~~ mmol ~~dm$^{-3}$~~ dm$^{-3}$ (Fig. 10.9). At the lower concentration (0.043 ~~g dm$^{-3}$~~ mmol dm$^{-3}$) the cells only have to modify DNA synthesis to maintain balanced growth which is easier than having to modify DNA, RNA and protein synthesis in order

to maintain balanced growth at the higher NA concentration (0.086 ~~g~~ mmol $dm^{-3}$). Whether NA-trained cells (0.043 ~~g $dm^{-3}$~~ mmol $dm^{-3}$) are fully resistant to NA concentration of 0.086 g $dm^{-3}$ is a matter of debate as this was not tested. The answer is probably not as cells trained at a NA concentration of 0.043 ~~g $dm^{-3}$~~ mmol $dm^{-3}$ will not have developed any mechanism to overcome the effects of NA on RNA and protein synthesis which would be required for balanced growth at 0.086 ~~g $dm^{-3}$~~ mmol $dm^{-3}$.

Cells repeatedly grown at either concentration of NA and then grown in media containing NA (0.690 ~~g $dm^{-3}$~~ mmol $dm^{-3}$) show a more balanced metabolism as indicated by the p-t and P-t traces (Figs. 9.7, 9.8) and mass and energy balances (Table 9.4) than do ordinary cells grown in the presence of NA (0.690 ~~g $dm^{-3}$~~ mmol $dm^{-3}$)(Fig. 9.1 f, Tables 9.2, 9.3). Trained cells grown at a NA concentration of 0.690 ~~g $dm^{-3}$~~ mmol $dm^{-3}$ exhibit a higher yield and $\Delta$% value, and a lower waste heat output compared to the corresponding values of normal cells grown in the same concentration of NA: this is indicative of more balanced growth. This repeated growth of cells in NA at either concentration induces some resistance. The mass and energy balances for cells trained at either concentration (0.043 or 0.086 ~~g~~ mmol $dm^{-3}$) and then grown in media containing NA (0.690 ~~g $dm^{-3}$~~ mmol $dm^{-3}$) are similar. From the data it is impossible to determine the relative changes in the synthesis of DNA, RNA and protein due to training in the presence of different concentrations of NA. Further investigations utilising the techniques of Stevens to establish the amount of synthesis of DNA, RNA and protein combined with flow microcalorimetry could provide an even deeper understanding as to the mode of action of NA on the bacterial cell.

This investigation has shown that reproducible quantitative thermal and growth data can be obtained from a system employing continuous monitoring techniques. Strict control of the conditions of growth and test is paramount if thermal data are to be correlated with growth measurements and those of cellular ATP pool. The quantitative data presented show that such controls are necessary if significant measurements are to be obtained, particularly if small values are involved e.g. $\Delta\%$, oscillations in the ATP pool.

Calorimetry is a reliable and powerful technique in the study of the energetics associated with cellular metabolism, and particularly in the investigation of cell-drug interaction. However the full potential of this technique cannot be realised unless and until standard and hence comparative parameters are employed. The combination of microcalorimetry with other continual monitoring techniques will in the years to come yield a deeper understanding of all aspects of cellular metabolism.

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