STUDIES ON THE GROWTH PHYSIOLOGY OF *BENECKEA NATRIEGENS*

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ABSTRACT

Beneckea natriegens provides an interesting system for the study of respiratory control in bacteria as it apparently has four different recognisable cytochrome oxidases. From inhibitor studies on cell free extracts a branched electron transport chain has been proposed for this organism. The individual branches of the electron transport chain are distinguished by their differing affinity for oxygen and sensitivity to inhibition by cyanide. It has been suggested that the multiple oxidases present in this organism function at different medium dissolved oxygen tensions and may be associated with differing efficiences of phosphorylation.

The respiratory control and growth physiology of *Beneckea natriegens* was studied using continuous culture techniques. As a preliminary to this investigation the inorganic ion requirement of this organism was examined in batch culture and a basal salts medium formulated to facilitate the operation of a carbon limited chemostat culture at a medium glucose feed concentration of 4.0 gl^{-1}. The results obtained in batch culture were confirmed in continuous culture ($D = 0.34\ h^{-1}$) where a linear relationship between bacterial biomass and substrate concentration (0.5 to 4.0 gl^{-1} glucose) was observed, indicative of carbon limitation. The effect of growth rate on growth physiology was investigated and the molar growth yields from glucose and oxygen were found to be dependent on, and to increase with, increasing growth rate. The *in situ* respiration rate of *B. natriegens* was directly proportional to the dilution rate. The 'potential' respiration rate, however, was independent of the growth rate and remained at a value higher than the *in situ* respiration rate, the latter increased with increase in the dilution rate. The

cytochrome content of bacteria decreased with increasing growth rate, and no positive correlation between bacterial cytochrome content and respiration rate was observed.

To assess whether branching of the electron transport system occurred under physiological conditions, B. natriegens was grown at various medium dissolved oxygen tensions in glucose limited chemostat culture and the effect of medium dissolved oxygen tension on growth yield, respiration and cytochrome composition examined. The results indicated that the metabolic rate and growth efficiency of this organism, as indicated by the q_{O_2}, q_{CO_2} and Y_{glc}, was little affected by changes in the dissolved oxygen tension over the range <2 mm Hg to 134 mm Hg. The 'critical' dissolved oxygen tension in chemostat studies was <1.3 μM O_2 and is in good agreement with values of between 0.15 and 0.25 μM O_2 obtained for the Km for oxygen using the respirograph technique. These Km values are approximately an order of magnitude lower than those previously reported for this organism. These studies failed to show any physiological evidence of a switch in pathway of electrons from a coupled to an uncoupled branch. Moreover the different branches of the respiratory chain of B. natriegens are reported to be recognised by a difference of at least an order of magnitude in cyanide sensitivity of the respective terminal oxidases. Therefore, if there is a change in the relative importance of these branches in response to dissolved oxygen tension this would be reflected in the cyanide sensitivity of the bacteria. No such change in cyanide sensitivity over the dissolved oxygen range 8 to 140 mm Hg was observed.

If the branched respiratory chain proposed for B. natriegens operates under physiological conditions then growth of this organism in the presence of cyanide would be expected to cause a switch to the cyanide resistant respiratory pathway and change the growth efficiency. However, growth of B. natriegens in the presence of cyanide and carbon monoxide had no measurable effect on respiration rate or growth efficiency. But, at high growth rates, low concentrations of cyanide (24 μM) caused the culture to wash out although the cyanide sensitivity of harvested bacteria was independent of growth rate.

A detailed study was made of the effect of respiration rate on cyanide sensitivity of bacteria harvested from chemostat culture. The respiration rate was varied either by manipulating the initial substrate concentration or the incubation temperature. The sensitivity of respiration to KCN was found to be dependent on the initial respiration rate of the bacteria. At sub-maximal respiration rates the bacteria were insensitive to KCN concentrations below a threshold value which increased as the respiration rate decreased. The same effect was obtained when respiration rate was decreased by lowering the initial substrate concentration or incubation temperature. The results are consistent with the potential respiration rate being limited by a cyanide-sensitive component. At sub-maximal respiration rates, in the absence of KCN, another factor becomes rate limiting but addition of KCN at a concentration above a threshold value caused the cyanide sensitive component to become rate-limiting for respiration once more.

Growth in the presence of cyanide caused an increased production of the CO-binding c type cytochrome. As the increased synthesis of this cytochrome

did not cause an increased resistance to cyanide or an increase in respiration rate it was thought to be unrelated to cyanide sensitivity. Formate was oxidised constitutively by B. natriegens. In the methane utilising bacteria, a CO binding c type cytochrome has been implicated in the reaction that brings about the oxygenation of methane. It is postulated that formate is oxidised by B. natriegens via two separate systems which are distinguished by their differing 'apparent' Km for oxygen. One system is thought to involve a NAD-linked formate dehydrogenase, the other a CO binding c type cytochrome linked formate oxidase.

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LIST OF ABBREVIATIONS AND SYMBOLS

Symbol	Definition	Units
D	dilution rate	h^{-1}
M	maintenance coefficient, g glucose per g bacterial dry weight per hour	$gg^{-1}h^{-1}$
Mo	maintenance oxygen requirement, mmol oxygen per g bacterial dry weight per hour	mmol $g^{-1}h^{-1}$
N	number of mol ATP produced per mol oxygen taken up	mol ATP.mol O_2^{-1}
S_R	glucose concentration in input medium	mmol l^{-1}
S	glucose concentration in outgoing culture	mmol l^{-1}
td	generation time	h
μ	specific growth rate	h^{-1}
$q_{ac}{}^1$	specific rate of acetic acid production, mmol acetic acid per g bacterial dry weight per hour	mmol $g^{-1}h^{-1}$
$q_{ac}{}^2$	specific rate of acetic acid production, mmol acetic acid per g bacterial carbon per hour	mmol $g^{-1}h^{-1}$
$q_{CO_2}{}^1$	specific rate of CO_2 production, mmol CO_2 per g bacterial dry weight per hour	mmol $g^{-1}h^{-1}$
$q_{CO_2}{}^2$	specific rate of CO_2 production, mmol CO_2 per g bacterial carbon per hour	mmol $g^{-1}h^{-1}$
$q_{O_2}{}^1$	specific rate of oxygen consumption, mmol oxygen per g bacterial dry weight per hour	mmol $g^{-1}h^{-1}$

$q_{O_2}{}^2$	specific rate of oxygen consumption, mmol oxygen per g bacterial carbon per hour	mmol $g^{-1}h^{-1}$
$q_{glc}{}^1$	specific rate of glucose consumption, mmol glucose per g bacterial dry weight per hour	mmol $g^{-1}h^{-1}$
$q_{glc}{}^2$	specific rate of glucose consumption, mmol glucose per g bacterial carbon per hour	mmol $g^{-1}h^{-1}$
Y_{ATP}	molar growth yield for ATP, g bacterial dry weight per mol ATP	$gmol^{-1}$
$Y_{glc}{}^1$	molar growth yield from glucose, g bacterial dry weight per mol glucose	$gmol^{-1}$
$Y_{glc}{}^2$	growth yield from glucose, g bacterial carbon per mol glucose carbon	$gmol^{-1}$
Y_{gly}	growth yield from glycerol, g bacterial carbon per mol glycerol carbon	$gmol^{-1}$
$Y_{O_2}{}^1$	molar growth yield from oxygen, g bacterial dry weight per mol oxygen	$gmol^{-1}$
$Y_{O_2}{}^2$	growth yield from oxygen, g bacterial carbon per mol oxygen	$gmol^{-1}$
Y_{glc}^{max}	molar growth yield for glucose, corrected for energy of maintenance	$gmol^{-1}$
$Y_{O_2}^{max}$	molar growth yield for oxygen, corrected for energy of maintenance	$gmol^{-1}$

SECTION I

INTRODUCTION

1. The Genus Beneckea

   (i) General characteristics of marine bacteria

   (ii) Characteristics of Beneckea natriegens

   (iii) Metabolism of Beneckea natriegens

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3. The Effect of Cyanide and Carbon Monoxide on Growth Efficiency and Cytochrome Content

   (i) The mode of action of cyanide and carbon monoxide

   (ii) Effect of cyanide on metabolism in vivo

   (iii) Cyanide resistant respiration in eukaryotic organisms

   (iv) Cyanide resistant respiration in bacteria

INTRODUCTION

1. The Genus Beneckea

(i) General characteristics of marine bacteria

Bacteria of marine origin which grow on complex medium prepared with sea water but not on the same medium prepared with fresh water on initial isolation have been defined as marine bacteria (MacLeod, 1965). Bacteria isolated from such media mostly are Gram-negative, facultative anaerobic straight and curved rods and comprise the main chemoorganotrophic bacterial flora of the sea (MacLeod, 1965; 1968). These organisms have a specific requirement for sodium which is not replacable by equimolar amounts of other monovalent cations (MacLeod, 1965; 1968). Until recently the taxonomy of these organisms was poorly developed. Isolates of marine origin were assigned to the genera Vibrio, Aeromonas, Pseudomonas and Beneckea on the basis of few nutritional properties. Baumann, Baumann and Mandel (1971) subjected 146 Gram-negative, facultative anaerobic isolates of marine origin to extensive physiological and morphological characterisation. Subsequent numerical taxonomic analysis indicated that most of the marine strains previously assigned to Vibrio, Aeromonas, Pseudomonas and Beneckea should be placed into the redefined genus Beneckea.

(ii) Characteristics of Beneckea natriegens

On the basis of phenotypic similarity Baumann et al (1971) established nine groups. These groups could be distinguished from one another by multiple,

unrelated phenotypic traits. Six of these groups which had DNA containing 45-48 moles per cent guanine + cytocine were assigned to a redefined genus Beneckea. The genus Beneckea consists of non-pigmented, non-sporeforming Gram negative straight and curved rods of marine origin, which when grown on liquid medium have a single sheathed polar flagellum. When grown on solid medium, many strains have unsheathed, peritrichous flagella in addition to the sheathed, polar flagellum. All strains are chemoorganotrophic, facultative anaerobes which ferment glucose as well as some pentoses, hexoses, sugar alcohols and sugar acids with the production of acid but no gas. No strains denitrify, fix molecular nitrogen nor luminesce. Most strains reduce nitrates to nitrites. All are oxidase positive. All strains are capable of growing on a mineral medium containing artificial sea water with glycerol as the sole source of carbon and energy with ammonia as the sole source of nitrogen. Sodium ions are required for growth. No strains hydrolyse agar, utilise cellulose, C_6-C_{10} dicarboxylic acids, L-isoleucine, L-valine, L-lysine, aromatic amino acids, purines, pyrimidenes or n-hexadecane. The strains which comprise this genus are common inhabitants of open sea, coastal water and gills and guts of marine fish and shell fish.

Beneckea natriegens

Beneckea natriegens was first isolated from salt marsh mud, and its morphology and physiology were described by Payne (1958, 1960) and by McRorie, Williams and Payne (1959). Its specific epithet (Pseudomonas natriegens) was proposed by Payne, Eagon and Williams (1961). Six strains were isolated by enrichment methods by Baumann et al (1971). All were rods which had a single,

polar flagellum when grown on solid or liquid medium. All strains accumulated poly β hydroxybutyrate as an intracellular reserve product. This species was the nutritionally most versatile member of the genus Beneckea and utilised 65 different compounds as the sole source of carbon and energy. It was the only species reported to utilise L-rhamnose, benzoate, p-hydroxybenzoate, betaine and hippurate. Cells grown on p-hydroxybenzoate cleaved protocatechuate by means of a meta cleavage.

(iii) Metabolism of Beneckea natriegens

Using the 'fermentation train' technique described by Pelczar, Hansen and Konetzka (1955), Payne, Eagon and Williams (1961) examined the products of aerobic dissimilation of glucose by B. natriegens. In this study a mineral salts medium containing 10 gl^{-1} glucose, pH 7.5 was inoculated and incubated at 25-30°C for 72 h. Sterile CO_2 free air was bubbled continuously through the culture. Their results indicated that 94% of the glucose was recovered as metabolic products (Table I-1).

On the assumption that the remaining 6% of glucose carbon was incorporated into cell material a growth yield of 4.3 g bacterial carbon per mole glucose carbon was obtained.

In further studies using the 'fermentation train' technique, Eagon and Cho (1965) reported that CO_2, pyruvate, lactate and acetate were the main products of glucose metabolism by Beneckea natriegens when grown in mineral salts medium under aerobic and anaerobic conditions. When these experiments were carried out in shake flask culture, pyruvate and acetate were the only products of glucose metabolism. These results indicated that growth was probably oxygen limited in

Table I-1. Products of glucose dissimilation by Beneckea natriegens (Payne, Eagon and Williams, 1961).

mmoles glucose used per 100 ml	Products (mmoles produced per 100 ml)		% carbon atoms recovered
2.2	carbon dioxide	2.30	17
	acetic acid	1.95	30
	lactic acid	0.85	19
	pyruvic acid	1.25	28
			94

the 'fermentation train' experiments. Assuming that carbon not excreted as products was incorporated into cellular material an aerobic growth yield from glucose between 23 and 25 g bacterial carbon per mole glucose carbon was calculated (data from fermentation train).

Enzymes of the glycolytic and hexose monophosphate pathways have been detected in glucose grown cultures of B. natriegens. Under these conditions enzymes of the Entner Doudroff and phosphoketolase pathways were not detected (Eagon and Wang, 1962). Data from radiorespirometric experiments indicated that approximately 92% and 8% of glucose actually catabolised were routed via the glycolytic and hexose monophosphate pathways respectively.

Gluconate utilisation was inducable and radiorespirometric experiments revealed that gluconate was dissimilated predominantly (80%) via the Entner Doudroff pathway. A minor portion of substrate gluconate was catabolised by the organism via the hexose monophosphate pathway (Eagon and Wang, 1962). Eagon (1962b) suggested that the hexose monophosphate pathway was only of minor functional importance because of the existence of a sluggish system for the oxidation of reduced NADPH as no pyridine nucleotide transhydrogenase was detected in this organism.

All the enzymes of the Krebs cycle were shown to be present in cell free extracts of B. natriegens (MacLeod, Claridge, Hori and Murray, 1958). To explain the accumulation of pyruvate and acetate during growth on glucose under fully aerobic conditions, Eagon and Cho (1965) suggested that the Krebs cycle in B. natriegens is rate limited because isocitric dehydrogenase requires NADP as cofactor for activity. Thus metabolic products at the oxidation level of pyruvate

could accumulate due to a rapid initial metabolism of glucose and a sluggishly functioning Krebs cycle. This apparent wasteful aerobic utilisation of glucose is also found in E. coli and related facultative anaerobes. Amarisingham and Davis (1965) have suggested that this mode of metabolism has evolved to give these facultative anaerobes a selective advantage over their competitors. Incompletely oxidised intermediates to which competing organisms may not adapt quickly are produced from glucose and these remain in the environment for use as soon as the glucose supply is exhausted.

Role of Na^+ and K^+ in transport

The role of Na^+ and K^+ in the metabolism of B. natriegens has not been reported; however, the role of Na^+ and K^+ in the metabolism of marine bacterium B-16 has been extensively studied. Gram negative marine bacteria generally require Na^+ for growth and metabolism (MacLeod, 1965). Marine bacterium B-16 specifically requires Na^+ for the active transport of various metabolites into the cell (Drapeau and MacLeod, 1963; Drapeau, Matula and MacLeod, 1966; Wong, Thompson and MacLeod, 1969). Kinetic analysis of Na^+-dependent solute transport into marine bacterium B-16 demonstrated that Na^+ decreased the Km for the transport process, suggesting that the role of Na^+ is to increase the affinity of a specific carrier or binding protein for the compound to be transported (Wong, et al., 1969). Na^+ has been shown to control the porosity of the cytoplasmic membrane of marine bacterium B-16 and thus prevent the release of intracellular solutes from the cell (Drapeau and MacLeod, 1965).

As in most other types of cells, K^+ is accumulated against a concentration gradient in marine bacterium B-16 (Drapeau and MacLeod, 1963; Drapeau, Matula and MacLeod, 1966). K^+ is also required for the transport of amino acids into the cell. Thompson and MacLeod (1971) reported that the role of K^+ in amino acid transport was at the intracellular level. The accumulation of K^+ in the cell was dependent on the presence of Na^+ and was attributed to the ability of Na^+ to cause the membrane to retain the K^+ accumulated. The process of K^+ uptake is an active one requiring an energy source. Increase of the Na^+ concentration decreased the Km for the K^+ transport process. Hassan and MacLeod (1975) have suggested that K^+ transport is a carrier-mediated process and that Na^+ interacts with the carrier in some way to increase its maximal velocity and affinity towards the substrate, ie. K^+.

(iv) Respiratory system of Beneckea natriegens

Studies on the respiratory system of B. natriegens by Knowles and coworkers (Weston and Knowles, 1973; 1974; Weston, Collins and Knowles, 1974) were carried out on bacteria grown in batch culture. The organism was grown at 35°C in shake flask culture in minimal medium with lactate (25 mM) as the main carbon source and 2 mM citric acid which served as a chelating agent. This composition corresponds to a carbon value of 0.9 gl^{-1} for sodium lactate and 0.144 gl^{-1} for citric acid (Weston and Knowles, 1973). The bacteria were harvested from batch culture, disrupted by sonication and then centrifuged at low speed to remove the debris. The cell free extract was fractionated into particulate and supernatant fraction by centrifugation at 150,000 g for 90 min. The particles

were washed once in 10 mM $MgCl_2$-50 mM Tris-HCl (pH 7.5) before resuspension in the same buffer.

Cytochrome composition

The presence of at least six different b and c type cytochromes were demonstrated in the particulate fraction of B. natriegens harvested while in the stationary phase (15 h) of growth (Weston and Knowles, 1974). The wavelength optima of the b and c type cytochrome α-peaks in low temperature difference spectra (subscript) were:- cytochrome b_{557}, b_{562}, b or c_{554}, c_{547}, $c_{549.5}$ and a second c-type cytochrome absorbing at 549.5 nm, cytochrome $c_{549.5(CO)}$. The latter cytochrome was distinguished from the dominant cytochrome $c_{549.5}$ by its lower concentration, ability to bind CO and its presence in the supernatant fraction. Cytochrome b_{557} and $c_{549.5}$ represented 70% of the total b and c type cytochromes present in this organism (Weston and Knowles, 1974). The presence of a number of cytochromes that are capable of binding with CO and hence potentially capable of acting as terminal oxidases have been demonstrated in B. natriegens. These include cytochrome a_1, d, o and $c_{549.5(CO)}$ identified on the basis of CO-difference spectra and action spectra (Weston and Knowles, 1974). Cytochrome $c_{549.5(CO)}$ is the first non-variant, high potential (E'o = 0.32 V), CO binding c type cytochrome to be discovered (Weston and Knowles, 1973).

Action spectra of whole organisms indicated that cytochrome d functioned as a terminal oxidase although it was present only in very low concentrations (Weston and Knowles, 1974). The CO-binding c-type cytochrome was also found to have oxidase activity but the evidence for cytochrome o acting as an oxidase

Fig. I -1. The inhibition of succinate oxidase activity of Beneckea natriegens by cyanide. The reaction mixture was incubated at 35°C with cyanide for 5 min before addition of 0.8 mM succinate, ● - ●; or 30 mM succinate, o - o. The uninhibited oxidase activities were 0.08 and 0.20 μ atoms oxygen uptake per min per mg protein respectively (from Weston, Collins and Knowles, 1974).

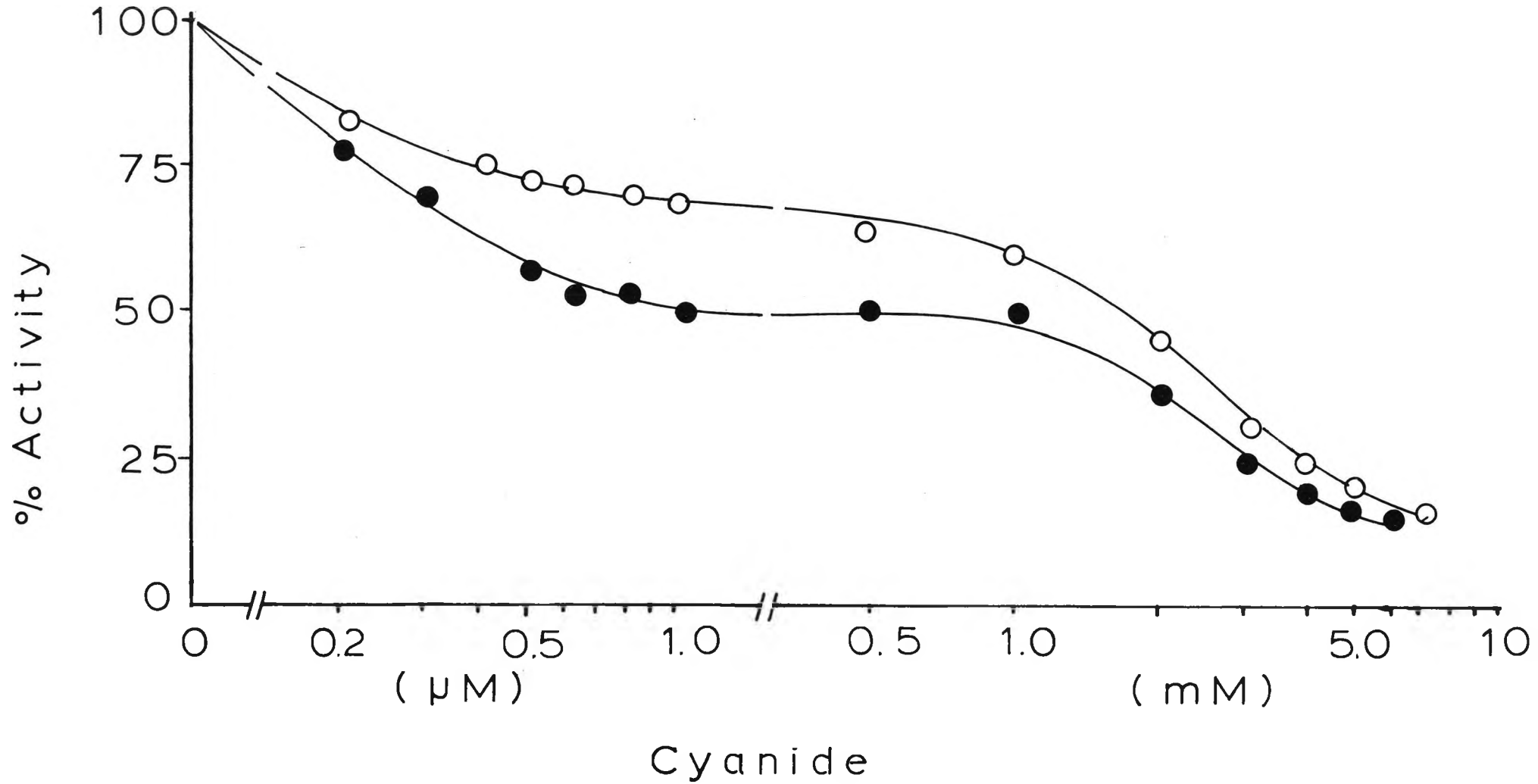

Fig. I-1

was inconclusive. In organisms cultured for periods longer than 16 h, cytochrome $\underline{a}_1$ appeared to be the major functional oxidase and cytochromes $\underline{d}$ and $\underline{o}$ and CO binding $\underline{c}$-type had minor oxidase functions (Weston and Knowles, 1974).

Effect of inhibitors on respiratory activity

Weston and coworkers reported that ascorbate N,N,N',N'-tetramethyl-phenylenediamine (TMPD) oxidase activity was completely inhibited by 10 μM KCN; succinate, NADH and lactate oxidase activities however, were only 50% inhibited by 10 μM KCN. Inhibition of NADH and succinate oxidase occurred in a biphasic manner (Fig. I-1), 10 mM KCN causing over 90% inhibition (Weston, Collins and Knowles, 1974). Ascorbate-TMPD oxidase activity was completely inhibited by 5 μM sulphide and 2 mM azide, which only partially inhibited the other oxidases. Based on these and further inhibitor studies on cell free extracts (Weston, Collins and Knowles, 1974) a branched electron transport chain has been proposed for B. natriegens (Weston, Collins and Knowles, 1974) in which ascorbate-TMPD would be oxidised by one pathway only, whereas NADH, succinate, D(-) lactate and L(+) lactate are oxidised via both pathways. However, Weston, Collins and Knowles (1974) proposed that respiration of succinate and lactate occurred preferentially via the pathway associated with ascorbate-TMPD oxidase activity. A schematic representation of the electron transport chain of B. natriegens proposed by Weston, Collins and Knowles (1974) is shown in Fig. I-2.

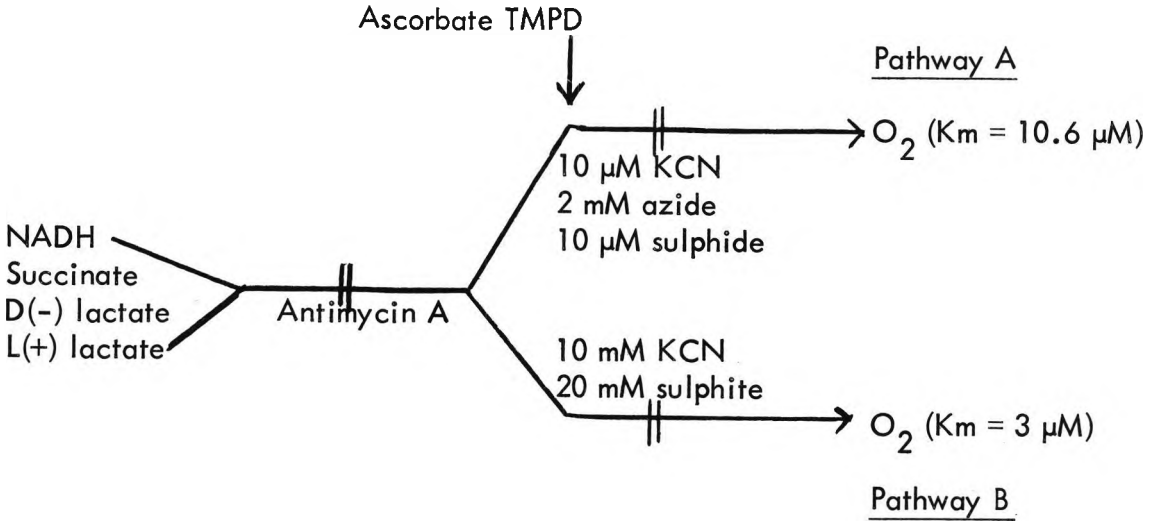

Fig. I-2. Proposed respiratory pathway of Beneckea natriegens (Weston, Collins and Knowles, 1974).

Affinity of the respiratory system for oxygen

Weston, Collins and Knowles (1974) attempted to measure the affinity for oxygen of each pathway shown in Fig. I-2. The cyanide sensitive, ascorbate TMPD pathway was found to have a Km value of between 8.0 and 17 μM oxygen. Measurement of the Km for O_2 in the presence of 10 μM KCN (to selectively inhibit the ascorbate TMPD pathway) yielded values ranging from 2.2 to 3 and 3.1 to 4.0 μM O_2 with NADH or succinate as substrate. However, the reliability of the method used by these workers to measure the apparent Km for O_2 is questionable as discussed fully in Chapter VIII.

2. Adaptation of the Respiratory System of Microorganisms to Environment and Physiological Changes

In the process of biological oxidation of an organic substrate energy is released by successive dehydrogenation of the carbon chain. The reduced equivalent so generated (normally two at a time) are transferred via a potentially graded series of reversible oxidation reduction sequences to a final acceptor which may be oxygen in the case of aerobic respiration, an inorganic compound other than oxygen as in anaerobic respiration, or an organic compound as in the case of fermentation. This transfer process is known as the electron transport system. The great diversity in the components of the electron transport system of bacteria is generally attributed to the vast range of metabolic types present in this group and is in contrast to that observed in the electron transport system of mammals.

A generalised scheme for the electron transport system in bacteria was proposed by Dolin (1961); Fig. I-3.

Thus hydrogen from the substrate is transferred to NAD^+, which in turn then donates electrons to the cytochrome system via a flavoprotein (FP). In bacteria the cytochrome components vary from species to species and even in the same species with growth condition. Thus, depending on the redox potential of the final acceptor, a wide range of carriers may have to intervene in the respiratory chain between the substrate and the terminal acceptor. The main difference between the respiratory chain of mammals and bacteria is that bacterial cytochromes are inducible (Harrison, 1975) and that bacteria are capable of possessing up to four different terminal oxidases simultaneously (Smith, 1968).

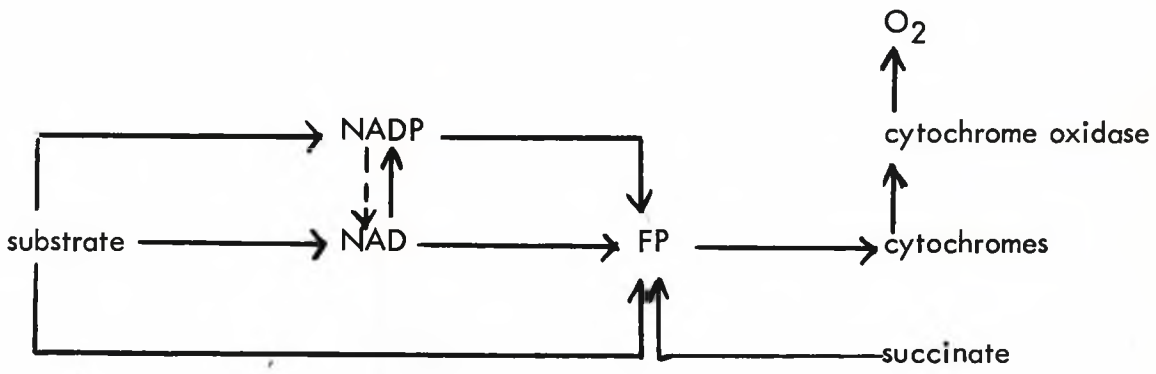

Fig. I-3. Generalised scheme for the electron transport system of bacteria (Dolin, 1961).

The significance of the distribution of cytochromes in bacteria in relation to the general physiological characteristics of these organisms has been reviewed by Meyer and Jones (1973c). These authors pointed out that the cytochrome profile $\underline{aa_3}.\underline{o}.\underline{b}.\underline{c}$ was found predominantly in Gram positive bacteria and the profile $\underline{a_1}.\underline{d}.\underline{o}.\underline{b}.\underline{c}.$ found predominantly in Gram negative bacteria. Cytochrome $\underline{a_1}$ and $\underline{d}(\underline{a_2})$ were always found together. These authors suggested that the various cytochrome profiles observed could be placed into one of four major groups.

(i) The aerobic and facultative anaerobic heterotrophic Gram positive group ($\underline{aa_3}.\underline{o}.\underline{b}.\underline{c}.$).

(ii) The aerobic and facultative anaerobic heterotrophic Gram negative group ($\underline{a_1}.\underline{d}.\underline{o}.\underline{b}.\underline{c}.$, $\underline{a_1}.\underline{o}.\underline{b}.\underline{c}.$ or $\underline{aa_3}.\underline{o}.\underline{b}.\underline{c}.$).

(iii) Anaerobic and microaerophilic heterotrophic group ($\underline{b}$ sometimes with $\underline{a}.\underline{d}.\underline{o}.$).

(iv) The chemo and photoautotrophic group ($\underline{c}$ plus $\underline{a_1}.\underline{aa_3}.\underline{o}.\underline{b}.$).

There were exceptions, eg. Arthrobacter globiformis, a Gram variable organism exhibited a cytochrome profile typical of a Gram positive heterotrophic aerobe ($\underline{aa_3}.\underline{o}.\underline{b}.\underline{c}.$) during logarithmic growth, but in the stationary phase cytochromes $\underline{aa_3}$ and $\underline{c}$ were replaced by $\underline{a_1}$ and $\underline{d}(\underline{a_2})$ yielding the profile typically exhibited by a Gram negative, heterotrophic facultative anaerobic bacterium ($\underline{a_1}.\underline{d}.\underline{o}.\underline{b}.\underline{c}.$). Thus the cytochrome profiles are not rigid and qualitative and quantitative changes may occur in response to environmental change.

Cytochrome $\underline{d}$ was mainly found in a small taxonomic group of organisms characterised by a high degree of adaptability to unstable habitats.

It is of interest that B. natriegens with a cytochrome profile a_1.d.o.b.c. fits into this group.

(i) The effect of growth rate on respiration, growth efficiency and cytochrome content of microorganisms

The chemostat has been used extensively to examine the regulatory response of bacteria to environmental change. In certain cases, for example the effect of growth rate on respiration, growth efficiency and cytochrome content, the chemostat technique is possibly the only meaningful experimental system. The growth rate can be varied independently of other environmental parameters by changing the dilution rate.

Assuming that the molar growth yield from oxygen was constant, Harrison (1975) derived an equation to predict the effect of dilution rate on the in situ respiration rate.

$$q_{O_2} = \frac{\mu}{Y_{O_2}} + M_o \qquad (1)$$

where q_{O_2} is the specific rate of oxygen consumption (mmol $g^{-1}h^{-1}$),

μ is the specific growth rate (h^{-1}) = D,

Y_{O_2} is the molar growth yield from oxygen (g mol^{-1})

M_o is the maintenance oxygen requirement (mmol $g^{-1}h^{-1}$)

A plot of the q_{O_2} values observed, against the experimental D value should give a straight line (eq. 1). Extrapolation of the straight line intersects the q_{O_2} axis at a value equivalent to the maintenance oxygen requirement rate M_o.

This relationship appears to be true for cultures where the energy source is growth limiting as found for Torula utilis by Tempest and Herbert (1965) and for Pseudomonas KB_1 with succinate as the sole carbon source (Rosenberger and Kogut, 1958). However the response observed for Klebsiella aerogenes cultured in a glucose limited chemostat deviated from the expected (Harrison and Loveless, 1971a). The potential q_{O_2} (ie. the respiration rate of bacteria harvested from the fermenter and supplied with excess substrate and oxygen in an oxygen electrode cell, Harrison and Loveless 1971b) of Pseudomonas KB_1 harvested from a succinate limited chemostat culture was observed to be higher than the in situ q_{O_2} over a dilution rate of 0.08 to 0.35 h^{-1}, but met the in situ curve at a dilution rate of 0.4 h^{-1}. Tempest and Herbert (1965) demonstrated a similar response for Torula utilis grown in carbon limited chemostat culture. When T. utilis was grown under nitrogen limitation, however, the potential q_{O_2} and in situ q_{O_2} decreased with decrease in the dilution rate. These authors suggested that this response may reflect differing metabolic priorities when protein synthesis is limited by energy supply as against the supply of a structural element.

The effect of growth rate on molar growth yield has been reviewed extensively (Stouthamer and Bettenhaussen, 1973; Abbott and Clamen, 1973) and will only be touched upon here. In chemostat culture under conditions of energy limitation a double reciprocal plot of Y against D yields a straight line the slope of which is the maintenance coefficient and the intercept at the ordinate the reciprocal of Y_{max}, the growth yield corrected for energy of maintenance (Stouthamer and Bettenhaussen, 1973). The effect of growth

rate of cytochrome composition has not received a great deal of attention. Rosenberger and Kogut (1958) examined the effect of growth rate on a succinate limited chemostat culture of Pseudomonas KB_1. The cytochrome level decreased with increase in the dilution rate. These authors suggested that the negative correlation between relative cytochrome content and growth rate in a carbon limited chemostat culture indicated that cytochrome synthesis was governed by external factor(s), eg. oxygen concentration, and not by the rate of general cell synthesis. The degree of oxidation of the electron transport chain was suggested as an alternative control mechanism. However, Harrison (1972a) reported that growth of Klebsiella aerogenes at low dilution rates (0.04 h^{-1}) was accompanied by the synthesis of high levels of cytochromes a_1 and a_2 normally observed during oxygen limited growth. Thus the mechanisms that operate to control the synthesis of cytochromes are complex and cannot be accounted for in terms of availability of oxygen or growth rate only.

(ii) The effect of dissolved oxygen tension on growth efficiency and cytochrome content

This subject has been reviewed extensively (Harrison, 1972b; 1973; 1975; Wimpenny, 1969; Hughes and Wimpenny, 1969) and the detailed response of the individual organisms is beyond the scope of this introduction, but some of the more important aspects will be discussed.

Examination of the effect of dissolved oxygen tension on the respiration rate of a wide range of procaryotic and eucaryotic organisms indicated a similar pattern of response (Gerard and Falk, 1931; Harvey, 1925; Shoup, 1929;

Kempner, 1939). Over a wide range of dissolved oxygen tensions respiration rate was independent of the dissolved oxygen tension but became dependent on the dissolved oxygen tension when the latter was lowered below a certain threshold value. The dissolved oxygen tension at which this occurred was termed the 'critical' dissolved oxygen tension (Gerard and Falk, 1931). The application of polarographic techniques facilitated a more accurate determination of the 'critical' dissolved oxygen tension. Workers employing this technique found that the relationship between dissolved oxygen concentration and respiration rate approximated to Michaelis-Menten type kinetics. Longmuir (1954) concluded that the critical dissolved oxygen tension reflected the affinity of a terminal oxidase for oxygen. Chance (1952) pointed out that the affinity of a cell for oxygen could not be treated as a simple Michaelis-Menten relationship. Kinetic studies of the cytochrome chain and simulated studies on an analogue computer enabled a model to be proposed which explained the sharp break in the dissolved oxygen-respiration rate relationship.

All the studies mentioned above were carried out on resting microbial suspensions. With the development of reliable probes for the measurement of dissolved oxygen a number of studies have been made of the effect of medium dissolved oxygen tension on the respiration rate of growing organisms. Chemostat studies on the effect of medium dissolved oxygen tension on bacterial respiration indicated that the response approximated to Michaelis-Menten kinetics (Johnson, 1967; Harrison and Pirt, 1967). The 'critical' oxygen tension observed for a number of organisms is shown in Table I-2.

Table I-2. Values reported for the 'critical' dissolved oxygen tension of growing microorganisms

Organism	Critical oxygen tension (mm Hg)	Reference
Klebsiella aerogenes	2-10	Harrison and Pirt (1967)
Aspergillus nidulans	1.75	Carter and Bull (1971)
Candida utilis	1.0	Moss et al (1969)
Pseudomonas AM1	28.0	MacLennan et al (1971)

The effect of dissolved oxygen tension on respiration rate in Azotobacter is a notable exception to the general response. In this organism the respiration rate increased with dissolved oxygen tension over the whole range to air saturation (Dalton and Postgate, 1968; Nagai et al. 1971). Increase in respiration rate was accompanied by a decrease in molar growth yield from the carbon substrate. Drozd and Postgate (1970) suggested that this response by the organism protected the nitrogenase system from inactivation by oxygen as the oxygen tension was increased. This hypothesis is supported by the findings of Ackrell and Jones (1971a,b) of a branched electron transport chain in this organism, one branch was reported to have lost a site of oxidative phosphorylation (site III; Downes and Jones, 1975) and to predominate at elevated dissolved oxygen tensions.

The response of microorganisms to dissolved oxygen tension has been studied in detail only in a small number of species. The results indicate a wide variation in response and at present no generalised pattern has emerged.

Effect of medium dissolved oxygen tension on bacterial cytochromes

Bacterial cytochromes are inducible, both qualitative and quantitative changes occur in response to changes in the growth environment. In many bacteria the terminal oxidases are produced maximally under oxygen limitation (Table I-3).

The reason for the increase in the terminal oxidases under oxygen limitation is not clear. One explanation is that the high content of terminal oxidase enables bacteria to maintain a high respiration rate at dissolved oxygen tensions below the Km for the cytochrome. The correlation between cytochrome

Table I-3. Examples of cytochromes that are produced maximally under conditions of oxygen limitation (from Harrison, 1976).

Organism	Cytochrome	Reference
Escherichia coli	a_1, d	Moss (1952)
Klebsiella aerogenes	a_1, d	Harrison et al. (1969)
Haemophilus parainfluenzae	b, c, a_1, d, o	Sinclair and White (1970)
Spirillum itersonii	b, c	Clark-Walker et al. (1962)
Azotobacter vinelandii	b, c	Lisenkova and Khmel (1967)
Candida utilis	b, c, aa_3	Moss et al. (1969)
Micrococcus dinitrificans	o	Sapshead and Wimpenny (1970)
Achromobacter strain D	o, a, d	Arima and Oka (1965)

oxidase content and Km for oxygen was found to be good in the case of H. parainfluenzae (White, 1963; Sinclair and White, 1970). Meyer and Jones (1973a) found a correlation between Km for oxygen and the nature of the cytochrome oxidase possessed by bacteria, however, as Harrison (1975) has pointed out, their definition of terminal oxidase and method of determining Km for oxygen is open to some criticism.

Meyer and Jones (1973a, b) suggested that multiple oxidases found in bacteria function at different medium dissolved oxygen tensions and are associated with different efficiences of phosphorylation. The work of Meyer and Jones (1973a, b) was carried out using cell free extracts and the operation of a similar system in whole bacteria under physiological conditions is yet to be established. Indeed, the field of bacterial physiology appears to be divided into two main schools, those employing whole bacteria and those using cell free systems. Few attempts have been made to demonstrate that data obtained from studies on cell free systems are a true reflection of what is taking place in the cell under physiological conditions.

3. The Effect of Cyanide and Carbon Monoxide on Growth Efficiency and Cytochrome Content

(i) The mode of action of cyanide and carbon monoxide

From the preceding account it is clear that in the differentiation of the electron transport system of bacteria, the use of cyanide and carbon monoxide as inhibitors of respiration played an important role. In this present work these inhibitors have been used. Therefore it is instructive to briefly review the literature on the mode of action of these compounds, and the mechanisms of cyanide resistant respiration reported for prokaryotic and eukaryotic organisms.

The effect of cyanide on living organisms is often associated with its importance as a respiratory poison and inhibitor of reactions involving metalloenzymes. However, cyanide has been shown to be very non-specific causing inhibition of a large number of enzymes which do not involve metalloenzymes (Dixon and Webb, 1964). Cyanide may inhibit enzymes by at least three mechanisms.

(a) Metalloenzymes:- cyanide is thought to inhibit metalloenzymes by complexing metals. Cytochrome oxidase has been reported to have the greatest known sensitivity to cyanide 10^{-8} M causing 50% inhibition (Dixon and Webb, 1964). Catalase and many peroxidases are also relatively sensitive and are usually strongly inhibited by concentrations of cyanide between 10^{-5} and 10^{-6} M (Dixon and Webb, 1964). The phenoloxidases and ascorbic acid oxidases are copper dependent metalloenzymes and are inhibited by cyanide but mainly at

higher concentrations (10^{-3} M). In certain cases cyanide can remove a particular protein-bound metal from enzymes, eg. copper from tyrosinase and ascorbic acid oxidase or molybdenum from nitrate reductase. The nature of the cyanide molecule concerned in the reaction may vary, Stannard and Hovecker (1948), and Chance (1952) concluded that the undissociated HCN molecule was involved in the inhibition of cytochrome oxidase. Keilin and Hartree (1939) reported that cyanide combined with both Fe^{3+} and Fe^{2+} states of cytochrome oxidase, however, the latter occurred under anaerobic conditions. The cyanide-ferric complex was reported to be the stable complex. Waino (1955) and Waino and Cooperstein (1957) however, concluded that cyanide combined with cytochrome oxidase in the ferrous form to form a stable complex. With regard to oxygen, cyanide is not a competitive inhibitor of cytochrome oxidase (Dixon and Webb, 1964).

(b) Carbonyl groups:- cyanide may combine with a carbonyl group in the enzyme, in a cofactor or prosthetic group or even in the substrate. Thus amino acid decarboxylases which require pyridoxyl phosphate as a coenzyme are inhibited by 10^{-3} M KCN (Dixon and Webb, 1964).

(c) Disulphide groups:- sodium cyanide may react with disulphide bonds irreversibly to give a thiol salt and a thiocyanate

$$RS = SR + NaCN \longrightarrow RSNa + RSCN$$

Keilin and King (1958) have suggested that cyanide inhibits succinic dehydrogenase by reacting with protein disulphide bonds.

Carbon monoxide has a much narrower range of action, inhibiting only enzymes containing iron and copper and not all of these. It inhibits enzymes that react directly with oxygen, with which it competes, combining with the reduced form of the enzyme. Owing to the competition with oxygen the inhibition by CO is determined by the ratio of the partial pressures of CO and O_2 to which the system is exposed (Dixon and Webb, 1964).

(ii) Effect of cyanide on metabolism in vivo

The effect of cyanide on isolated enzyme systems in vitro may not always resemble the effect observed in living organisms. This type of discrepancy has been noted on several occasions, and has led to the idea that cytochrome oxidase may mediate only part or even a small part of the total tissue respiration. A number of the earlier reports were contradictory. Warburg (1927) and Alt (1930) reported that respiration of various mammalian tissue were 95% inhibited by 10 mM KCN. Dixon and Elliott (1929), however, reported that inhibition of respiration by 3.3 mM KCN varied between 25 and 91%. Commoner (1940) reviewed much of the earlier work, providing an explanation for these apparent contradictions. He concluded that as cyanide sensitive respiration, which is normally mediated via cytochrome oxidase, is dependent on substrate availability the apparent ratio of cyanide sensitive to resistant respiration will vary with substrate supply. The absolute values of the cyanide resistant respiration were similar in all tissues examined, whereas the cyanide sensitive respiration varied and was dependent on substrate concentration (Table I-4). Observations of this type have led Hewitt and

Table I-4. Data taken from Commoner (1940) showing the relationship between cyanide resistant and sensitive respiration in a wide range of organisms

Tissue and organism	Respiration rate (ml O_2 per g fresh tissue per h)	Inhibition by KCN (%)	Respiration rate of cyanide resistant component (ml O_2 per g fresh tissue per h)
Kidney - rabbit	28	93.0	2.0
Kidney - rabbit	19.4	95.8	0.8
Kidney - pigeon	18.4	84.7	2.8
Kidney - guinea pig	16.7	88.0	2.0
Kidney - sheep	14.0	91.4	1.8
Diaphragm - rabbit	10.2	85.2	1.5
Liver - ox	8.0	87	1.0
Scenedesmus	6.0	78.3	3.3
Kidney - rabbit	5.5	78	1.2
Lathyrus embryo (10 days old)	3.0	66	1.0
Lathyrum embryo (starved)	0.8	0	0.8

Nicholas (1963) to conclude that "statements of relative cyanide sensitivity are therefore without meaning unless substrate saturation and potential activity are taken into account". These authors point out that there is little doubt that although some organs or during certain stages of development of particular organisms the proportion of respiration mediated through cytochrome oxidase may vary, in most circumstances cytochrome oxidase mediates the major proportion, or all of the respiratory oxygen uptake.

(iii) Cyanide resistant respiration in eukaryotic organisms

Although the respiratory systems of prokaryotic and eukaryotic organisms are strongly inhibited by cyanide (Slater, 1967; Ge'lman, Lukoyanova and Ostrovskii, 1967), cyanide resistant respiration has been reported in a wide range of eukaryotic organisms including plants (James and Beevers, 1950; Bahr and Bonner, 1973); fungi (Tissierer, Mitchell and Hawkins, 1953); protozoa (Hill and Cross, 1973) and algae (Sharpless and Butow, 1970). Cyanide insensitive respiration in eukaryotic organisms is the subject of an extensive review by Henry and Nyns (1975) and a summary of their findings will be given here.

Cyanide resistance in eukaryotic organisms is due to the presence of a cyanide resistant alternative oxidase in addition to cytochrome oxidase. The alternative oxidase can be specifically inhibited by hydroxamic acids (Schonbaum, Bonner, Storey and Bahr, 1971). Salicylhydroxamic acid, cyanide or antimycin A alone had little effect on respiration but salicylhydroxamic acid plus cyanide caused complete inhibition of respiration. The cyanide and antimycin A sensitive components of the electron transport system contained a normal complement of

cytochromes and is coupled to oxidative phosphorylation. The nature of the alternative oxidase is unknown but it may contain a non-heme iron protein (Schonbaum, Bonner, Storey and Bahr, 1971). This cyanide insensitive alternative oxidase, whenever present co-exists with the main respiratory chain and it is thought that branching of the respiratory system occurs at the flavoprotein level, before the site of action of antimycin A (Bahr and Bonner, 1973).

The physiological role of the alternative pathway is uncertain, but in a group of monocotyledonous plants, the Araceae (eg. genus Arum) the appearance of mitochondrial cyanide resistant respiration coincides with the generation of heat by the plant. The production of heat may facilitate blooming at the end of the winter when the environmental temperature is low, as observed for Symptocarpus foetidus. However, direct evidence that operation of the cyanide resistant alternative oxidase results in the generation of heat is lacking. Indeed the operation of the cyanide resistant pathway is not always accompanied by the generation of heat. Henry and Nyns (1975) have suggested a role of elimination of toxic substances or unnecessary metabolites from the cell.

(iv) Cyanide resistant respiration in bacteria

Cyanide resistant respiration has been reported for a number of bacteria (Oka and Arima, 1965; Arima and Oka, 1965; Skowronski and Strobel, 1969; MacFetters, Wilson and Strobel, 1970). In a number of cases complex media containing fermentable substrates were used and little attention was given to culture pH value or oxygen tension. Thus Bacillus cereus was reported to grow in the presence of 10^{-3} M KCN, (MacFetters, Wilson and Strobel, 1970),

however the growth rates observed in the presence of cyanide were much lower than observed for bacteria grown in the absence of KCN (fully aerobic) but very similar to those observed in the control grown at low aeration. Although good growth under anaerobic conditions is a characteristic of B. cereus (Wolf and Barker, 1968) these authors did not account for the possibility that growth occurred via fermentative metabolism. Ashcroft and Haddock (1975) demonstrated that growth of E. coli was completely inhibited by 250 μM KCN when a non-fermentable substrate such as succinate was the carbon source, whereas growth proceeded in the presence of 1 mM KCN when a fermentable substrate such as xylose was used.

MacFetters et al. (1970) reported that growth of B. cereus in the presence of 10 mM KCN caused a 50% increase in cytochrome b and a two-fold increase in a type cytochrome. These authors examined the effect of KCN on the respiration of bacteria harvested from batch culture in the presence and absence of KCN. Their results were expressed as percentage uninhibited respiration and bacteria grown in the presence of KCN appeared to be more resistant to this inhibitor. However, if absolute respiration rates are compared, the respiration rate was extremely low and not significantly different in bacteria grown in the presence and absence of cyanide (Table I-5).

Certain bacteria are capable of metabolising cyanide. Skowronski and Strobel (1969) reported that B. pumilus metabolised ^{14}C and ^{15}N labelled KCN to CO_2 and NH_3. Growth in the presence of 1 M KCN was accompanied by the formation of long filaments that did not revert to the normal form on the removal of KCN, suggesting the selection of a KCN resistant mutant.

Table I-5. Data recalculated from MacFetters, Wilson and Strobel (1970) showing the effect of growth in the presence of 10 mM KCN on respiratory rate and sensitivity to cyanide

KCN concentration	Growth in the absence of KCN		Growth with KCN	
	$^{q}O_2$ (mM O_2 per g dry wt per h)	Inhibition (%)	$^{q}O_2$ (mM O_2 per g dry wt per h)	Inhibition (%)
0	0.51	0	0.41	0
10 μM	0.40	21.6	0.34	17
100 μM	0.30	41.2	0.28	31.8
1 mM	0.15	70.6	0.23	44
10 mM	0.12	76.5	0.12	71

Growth of E. coli in the presence and absence of 150 μM KCN with succinate as the carbon source had no effect on NADH oxidase activity (Ashcroft and Haddock, 1975). However, the KCN concentration required to cause 50% inhibition of NADH oxidase increased from 75 μM in the control to 500 μM in cells grown in the presence of 150 μM KCN. Growth in the presence of KCN was accompanied by an increase in cytochrome b, a five fold increase in cytochrome d and a nine fold increase in the menaquinone level. These authors suggested that this was a direct consequence of the presence of KCN which caused a change in electron flow down the electrotransport system from a pathway involving ubiquinone-cytochrome b_{556} and cytochrome o in the absence of KCN to a cyanide resistant pathway involving the redox carrier menaquinone, cytochrome b_{558}, a and d. They concluded that cytochrome d was the cyanide resistant terminal oxidase. The induction of cytochrome d under fully aerobic conditions at low growth rates is well established (Harrison, 1972a) and this increase in the level of cytochrome d at low growth rates is a plausible explanation of the results reported by Ashcroft and Haddock as the mean generation time on succinate decreased from 2.4 h in the absence of KCN to 7 h in the presence of 150 μM KCN.

In a number of bacteria respiratory resistance to KCN has been reported to be inducible. Chromobacterium violaceum produces KCN during growth, indeed, this characteristic is used as a taxonomic marker in this organism (Sneath, 1966). Production of KCN during growth of this organism, is accompanied by the induction of a cyanide resistant respiratory system (Niven, Collins and Knowles, 1975). In Achromobacter strain D cytochrome o is the main oxidase when the

organism is cultured under high aeration and respiration is 50% inhibited by 10 μM KCN, but when grown in the presence of 1 mM cyanide cytochrome b, a and d increased and respiration was only 50% inhibited by 1 mM cyanide (Mizushima and Arima, 1960a,b). Cytochrome d was reported to be the cyanide insensitive oxidase in Achromobacter strain D (Oka and Arima, 1965; Arima and Oka, 1965).

Jones and Redfearn (1966; 1967) have reported the presence of a branched electron transport system in cell free extracts of Azotobacter vinelandii. The two pathways were distinguished by the substrate which acted as electron donors. Thus respiration of membrane particles with ascorbate N,N,N',N'-tetramethylphenylene-diamine (TMPD) as substrate was completely inhibited by 40 μM KCN, when NADH or succinate were the electron donors, however, 2 mM cyanide was required to inhibit respiration. The effect of hydroxamic acid on the KCN insensitive pathway has not been reported and the operation of a system similar to that found in higher plants cannot be ruled out, however, cytochrome d was suggested to be the cyanide insensitive oxidase in Azotobacter vinelandii. Weston, Collins and Knowles (1974) using the same techniques as Jones and Refearn (1966; 1967) have reported a similar branched electron transport system in cell free extracts of Beneckea natriegens. The pathway into which ascorbate TMPD feeds was completely inhibited by 10 μM KCN, however, when NADH or succinate was the respiratory substrate 3 mM KCN caused only 50% inhibition. The cyanide resistant pathway in this organism was not inhibited by hydroxamic acid (Weston, Collins and Knowles, 1974). In view of the findings of Commoner (1940) it is interesting to note that although the two respiratory systems in Azotobacter vinelandii exhibited

vastly differing cyanide sensitivities, the respiration rate via both systems was similar (Jones, 1973). In B. natriegens however (Weston, Collins and Knowles, 1974) the respiration rate via the NADH, succinate pathway was only 17% of that observed via the ascorbate TMPD pathway and so the difference in cyanide sensitivity may be due to the variation of respiration rate rather than a branching of the electron transport pathway.

Beneckea natriegens the metabolically most versatile member of the genus Beneckea (Baumann, Baumann and Mandel, 1971) has been reported as capable of growing faster than any other known organism (Eagon, 1962a). Rapid growth rate and metabolic versatility necessitate the operation of rapid and finely poised control mechanisms to enable this facultative anaerobic organism to co-ordinate its various functions. A branched electron transport system has been reported for this organism (Weston, Collins and Knowles, 1974) and it has been suggested that one of the ways this facultative anaerobic organism adapts to environmental change is by modulating the flow of electrons through either branch of the respiratory chain. Because this organism possesses these interesting characteristics it was decided to use it for investigating growth physiology and respiratory control.

The growth physiology of B. natriegens was studied using continuous culture techniques as these allowed a greater control over environmental parameters than afforded by conventional batch culture techniques. The growth conditions in chemostat culture were varied in an attempt to favour the operation of one or the other branch of the electron transport chain of this organism with an aim to

elucidate the factors controlling the flow of electrons via each pathway. Moreover, if the growth efficiency of the pathways differed this should be evident in the molar growth yield from glucose and oxygen.

A study of the factors that control growth yield and respiration in addition to having theoretical considerations have important industrial applications either for maximising yield when biomass production is important or minimising yield but maintaining a high rate of metabolism necessary, for example in effluent treatment.

SECTION II

MATERIALS AND METHODS

1. Organism, Media and Culture Methods

   (i) Organism

   (ii) Medium G

   (iii) Modified medium G

   (iv) Fermenter cultures

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2. Analytical Methods

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   (ii) Measurement of gaseous oxygen, carbon dioxide and hydrogen

   (iii) Measurement of gas flow rates

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(xi) Determination of ammonia nitrogen and orthophosphate

(xii) Determination of Mg^{2+} and K^{+}

(xiii) Determination of glucose, acetic acid, ethanol, pyruvic acid, lactic acid, citric acid and formic acid

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3. Measurement of the Respiration Rate of Harvested Bacteria Using a 'Closed' Oxygen Electrode Cell

(i) Calculation of respiration rate

(ii) Inhibitor studies

4. Measurement of the Respiration Rate of Harvested Bacteria Using an 'Open' Oxygen Electrode Cell

(i) Measurement of respiration rate

(ii) Determination of the 'apparent' Km for oxygen

5. Selection of a Formate Negative Mutant

MATERIALS AND METHODS

1. Organism, Media and Culture Methods

(i) Organism

Beneckea natriegens strain III, the gift of Dr. C. J. Knowles, University of Kent at Canterbury, was originally isolated and characterised by Payne, Eagon and Williams (1961).

(ii) Medium G

Medium for continuous culture experiments was made up in batches of ten, 30 litre amounts.

The basal medium contained (per litre):

(A) Na_2HPO_4, 1.5 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 3.0 g; NaCl, 23 g; KCl, 0.745 g; 1 ml trace element mixture from a stock solution containing per litre:- $CaCl_2 2H_2O$, 0.66 g; $ZnSO_4 7H_2O$, 0.18 g; $CuSO_4 5H_2O$, 0.16 g; $MnSO_4 4H_2O$, 0.15 g; $CoCl_2 6H_2O$, 0.18 g; H_3BO_3, 0.10 g; $Na_2MoO_4 2H_2O$, 0.30 g. The pH of A was adjusted to pH 7.3 with NaOH and the volume made up to 925 ml.

(B) Glucose or glycerol, 2 g; $MgSO_4 7H_2O$, 0.2 g in 50 ml distilled water.

(C) $FeCl_3 6H_2O$, 0.0167 g in 25 ml distilled water. Components A, B and C were autoclaved separately and mixed aseptically on cooling.

(iii) Modified medium G

A slight precipitate formed in medium G after combining components

A, B. and C. This precipitate was prevented from forming by the addition of 0.146 g trisodium citrate to component B. This measure allowed components B and C to be combined before autoclaving.

(iv) Fermenter cultures

An impeller-stirred Biotec fermentation vessel (LKB Co. Ltd., 232 Addington Road, Croydon) of working capacity approximately 2.3 l was used. The culture volume was kept constant by means of a stainless steel overflow tube (6 mm diam.), the bottom of which was connected to an effluent vessel by means of silicone rubber tubing. Effluent culture was pumped out of the vessel using a peristaltic pump (Northern Media Supply Ltd., Brough HU15 2PE) or by a diaphragm pump (F. A. Hughes & Co. Ltd., Epsom, Surrey). The temperature was maintained at $31.5^{\circ}C \pm 0.25^{\circ}C$ by controlling the circulation of hot and cold water through two separate fingers immersed in the culture. Culture pH was controlled at 7.23 ± 0.02 (unless otherwise stated) by the addition of 5N NaOH using a Titrator II (Radiometek A/S - Emdrupues 72-L 2400 Copenhagen, NV Denmark). Aeration was achieved by passing sterile air (1 l per min) through the vessel. The culture fluid was constantly stirred and the speed set between 500 and 1000 r.p.m. depending on the particular cultural conditions. Medium was pumped into the fermenter vessel with a diaphragm pump from a reservoir containing 30 litres of medium. When medium G was used the medium reservoir was stirred continuously with a magnetic stirrer to keep the slight precipitate, that formed, in suspension. A schematic diagram of the continuous culture assembly is shown in Fig. II-1.

Fig. II-1. Schematic representation of fermenter layout.

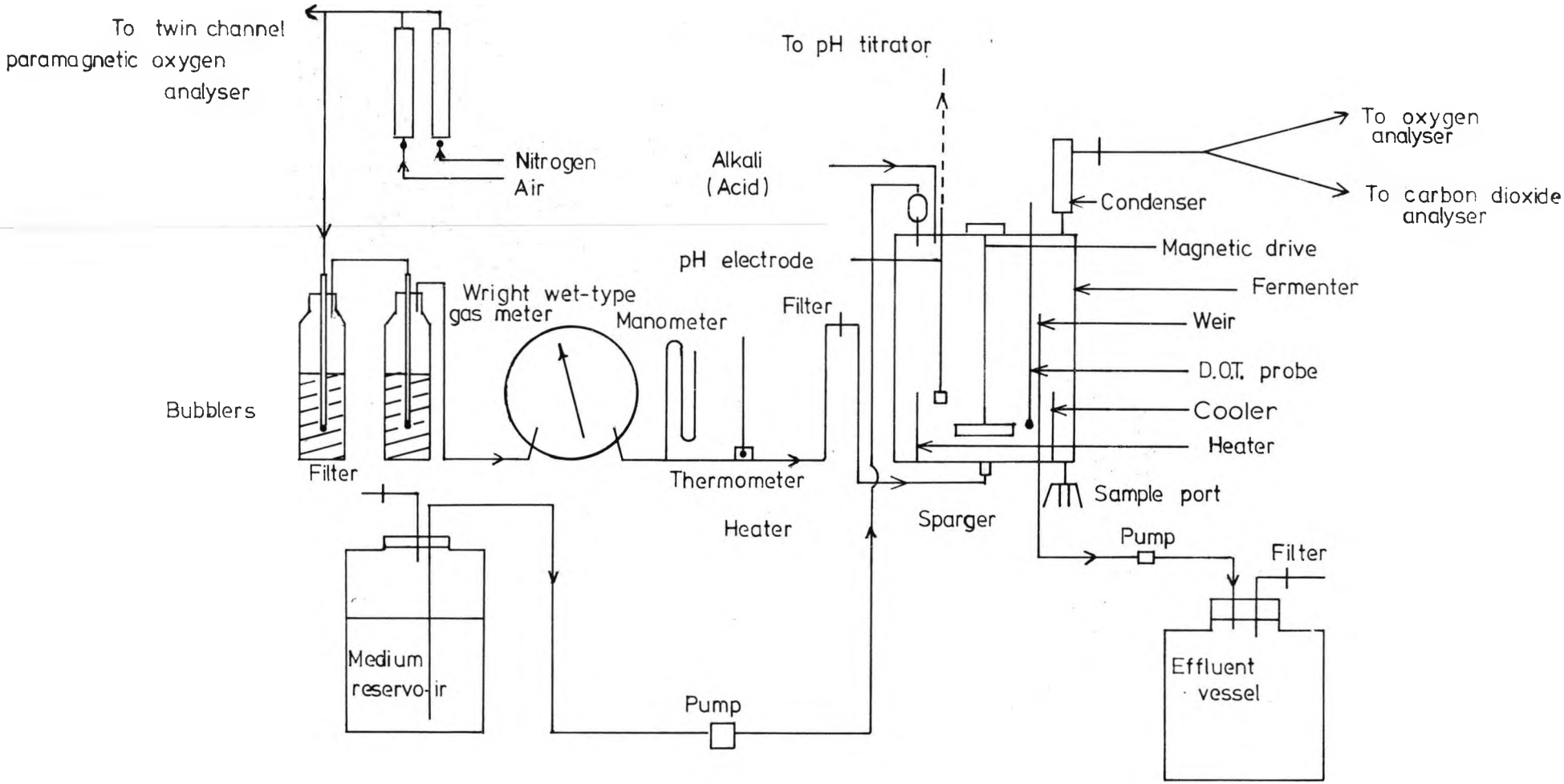


Fig. II-1. Schematic representation of fermenter layout

(v) Sampling procedure

In order to obtain accurate values of the concentration of intermediates that have a high turnover rate it is essential to ensure that the cells are killed and extracted as rapidly as possible (Harrison and Loveless, 1971a). In order to achieve quick mixing of culture sample and perchloric acid the sampling device shown in Fig. II-2(b) was constructed. A Pyrex test tube ($\frac{3}{4}$" diameter) was placed in the stainless steel air-tight chamber. Tap A was opened, no flow occurred until the syringe plunger was withdrawn to the required volume (10 ml). Culture was fired through the $\frac{1}{16}$" drain tube (B) into 0.8 ml of 0.85% perchloric acid in the test tube without touching the walls. Turbulence was thus maintained in the sample throughout the sampling time. Tap A was then closed, tap C opened and the plunger returned to its original position. The acid-extracted samples were left on ice for 30 min and then carefully neutralised with 5 N NaOH. The precipitate formed was spun down at 4° and the clear supernatant stored at -10°,

(vi) Inoculation of fermenter

The inoculum used throughout was 50 ml of a mid log phase culture. The inoculum was transferred directly from the shake flask into the fermenter using the arrangement shown in Fig. II-2(a). After inoculation, the rubber tubing connecting the inoculum vessel to the fermenter was clamped off and then cut.

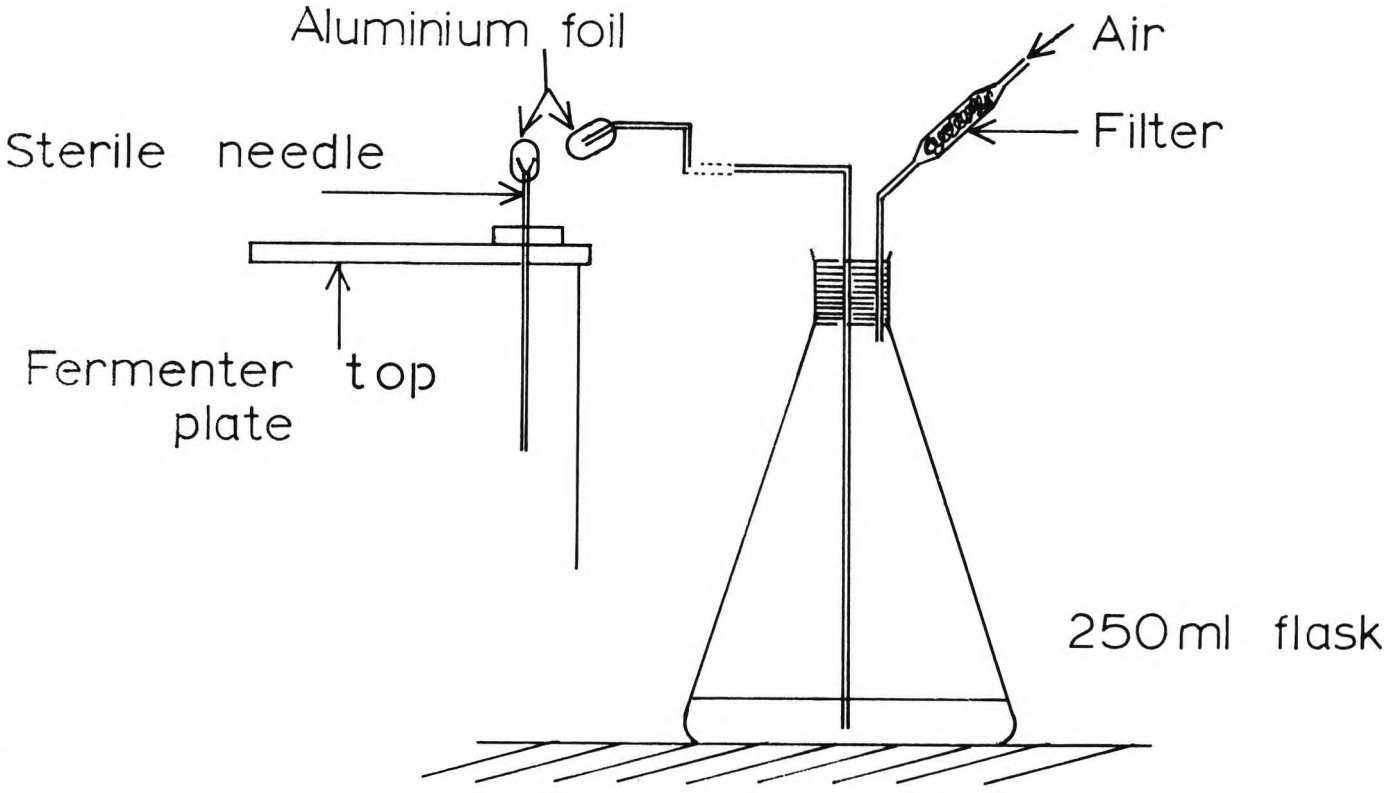
(vii) Contamination checks

A sample of culture was taken from the fermenter daily and streaked

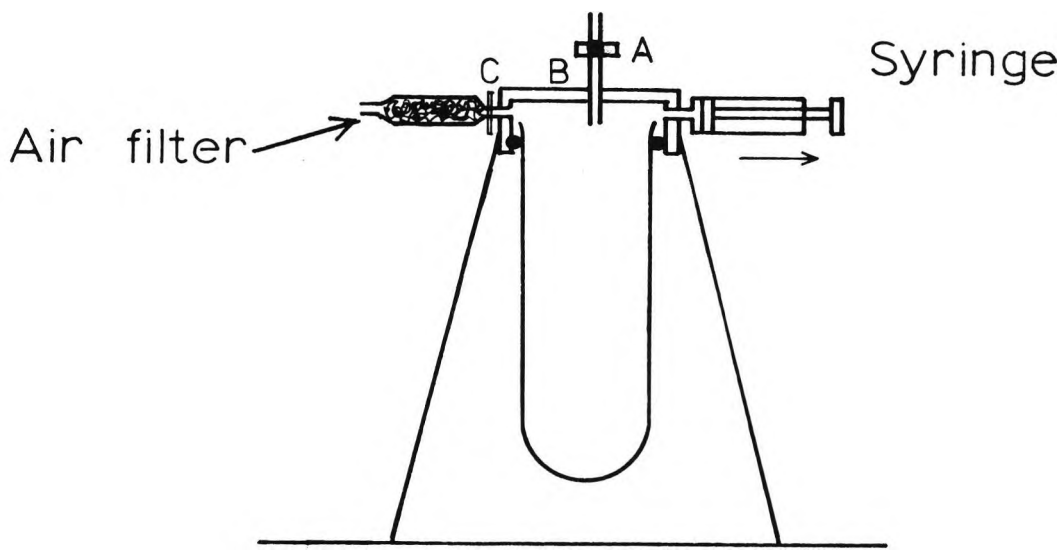
Fig. II-2(a) Method of inoculation of fermenter.

(b) Sampling port designed to eliminate regions of dead space and to ensure rapid sampling.

Fig. II-2.


Aluminium foil
Air
Filter
Sterile needle
Fermenter top plate
250ml flask

(a)


A
B
C
Syringe
Air filter

(b)

onto nutrient agar (Oxoid CM_3), Difco marine agar ('Difco' 2216) and Lab Lemco agar and incubated at 30°C for five days. Good growth on marine agar, growth on nutrient agar and no growth on Lab Lemco agar after 48 hours incubation was found to be characteristic of a pure culture of B. natriegens.

(viii) Maintenance of culture

The purity of the culture was examined by streaking samples onto the agars mentioned above. Culture was transferred from slope to slope at monthly intervals. To avoid the selection of a "fermenter strain" each fermenter inoculum was prepared from the stock culture which had not been grown in the chemostat. As a precautionary measure some experiments (effect of oxygen and experiments using KCN) were repeated with an inoculum grown directly from slope cultures obtained from Mrs. P. Colins, University of Kent at Canterbury.

2. Analytical Methods

(i) Measurement of dissolved oxygen tension

The dissolved oxygen tension was measured with a galvanic cell oxygen probe (L. H. Engineering Co. Ltd.) connected in series with a 5 k Ω variable potentiometer. The probe was calibrated in growth medium in equilibrium with known mixtures of air and nitrogen assayed by means of a Servomex oxygen analyser. The zero reading was obtained by bubbling nitrogen through the vessel for 24 h. Oxygen mixtures below 1% (v/v) oxygen were obtained by mixing nitrogen with a standard O_2/N_2 gas mixture containing 10 and 1% oxygen. A typical calibration curve obtained by plotting the μA generated at the oxygen electrode against the dissolved oxygen tension is shown in Fig. II-3. As the calibration indicated a linear response of the probe to oxygen, routine calibration was carried out by measuring the response at 100% nitrogen, 50% air + 50% nitrogen (v/v) and 100% air only.

(ii) Measurement of gaseous oxygen, carbon dioxide and hydrogen

Oxygen

Oxygen in the gas phase entering and leaving the fermenter vessel was continuously monitored by a twin channel paramagnetic oxygen analyser (Type OA 184 Servomex Controls Ltd., Crowborough, Sussex, England). The zero was set using "white spot" nitrogen, the maximum oxygen value was taken as 21% and the value set using air. The difference between the oxygen content of the inlet and outlet gas stream was a measure of the oxygen uptake of the fermenter culture.

Fig. II-3. A typical calibration curve obtained for the dissolved oxygen electrode.

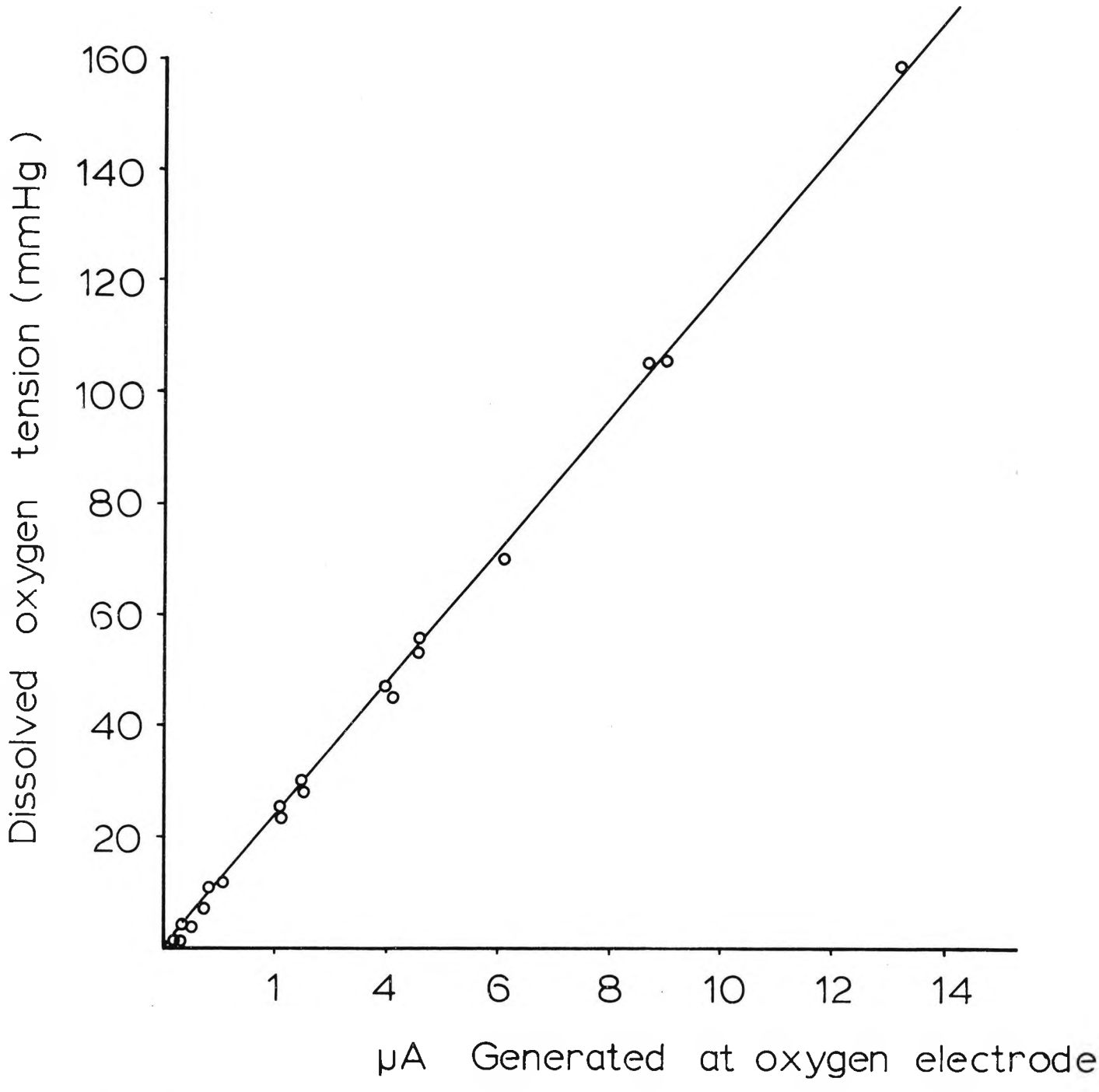

160
140
120
100
80
60
40
20
Dissolved oxygen tension (mmHg)
1
4
6
8
10
12
14
μA Generated at oxygen electrode

Fig. II-3.

Carbon dioxide

Carbon dioxide leaving the chemostat vessel was continuously monitored by an infrared analyser (M.S.A. Instrument Division, Glasgow E3). The instrument was calibrated with standard carbon dioxide obtained from British Oxygen.

In experiments in which carbon monoxide and cyanide were used the gas analysis was carried out by Mr. L. J. Barnes using the gas liquid chromatographic method of Deans, Huckle and Peterson (1971).

Hydrogen

Hydrogen was assayed using a katharometer control unit type GC 197 with a katharometer MK 158 (Servomex Controls Ltd.). The detection system relied on the thermal conductivity of hydrogen. The system was calibrated with helium which has a thermal conductivity close to hydrogen (336 compared with 406 cal lg sec^{o}C x 10^{6} for hydrogen).

(iii) Measurement of gas flow rates

Gas flow rates into the fermenter were measured on a wet type gas meter (Alexander Wright & Co., Westminster). The time taken for 1 litre of gas to pass through the meter was measured with a stop watch. The average of three readings was taken.

(iv) Measurement of atmospheric pressure (mmHg)

The atmospheric pressure was measured using a Fortin's barometer. A mercury manometer placed in the gas inlet stream was used to measure the pressure of the gas entering the fermenter.

(v) Measurement of temperature of inlet gas

The temperature of the inlet gas was measured directly from a mercury thermometer placed in the inlet gas stream.

(vi) Measurement of medium flow rates

The medium flow rate was measured by means of a calibrated arm on the medium reservoir. The mean value of three readings for the time taken to pump 20 ml from the side arm into the fermenter was recorded.

(vii) Measurement of fermenter volume

The fermenter volume was read off directly from a scale glued to the outside of the vessel when the impeller had been switched off after a given steady state. This was verified by draining the fermenter and measuring the volume when necessary.

(viii) Determination of bacterial dry weight

Duplicate samples of culture (10 ml) were centrifuged at 17,000 g for 35 min, washed twice in a solution of 0.4 M NaCl and finally taken up in distilled water and dried to constant weight at 98°C. For each steady state value two duplicate 10 ml samples were taken at intervals of at least five times the mean residence time.

(ix) Determination of bacterial carbon

Analysis of total culture carbon and supernatant carbon was made with a Beckman Model 915 Total Organic Carbon Analyser. Carbon analysis of whole

culture and of a Millipore (0.25 μm pore size) filtrate was made and the carbon content of the bacteria obtained from the difference. The carbon analyser was calibrated with standard glucose solutions. A typical calibration curve is shown in Fig. II-4.

(x) Determination of culture absorbance

Culture absorbance at 625 nm (CE 404 Colorimeter, Cecil Instruments Ltd.) was used as a guide to the constancy of the biomass content in steady state cultures. The culture absorbance was not used to compute carbon or dry weight of the culture.

(xi) Determination of ammonia nitrogen and orthophosphate

The amount of ammonium sulphate nitrogen and orthophosphate used by the culture was assayed by measuring (a) the total ammonia (ammonia plus ammonium ions in solution expressed as total ammonia gl^{-1}) and orthophosphate in the input medium and (b) the total ammonia and orthophosphate in the output medium. The ammonia and orthophosphate concentrations in samples were measured using a Technicon Autoanalyser II (Industrial Methods 160-73E and No. 155-71W respectively). (Determined by Mr. L. Barnes, Shell Research Limited, Sittingbourne, Kent).

(xii) Determination of Mg^{2+} and K^{+}

The magnesium and potassium content of culture medium and culture supernatant were measured using an SP-90 atomic absorption spectrophotometer (Pye Unicam Ltd.). (Determined by Mr. L. Barnes, Shell Research Limited, Sittingbourne, Kent).

Fig. II-4. Calibration of a Beckman Total Organic Carbon Analyser using known concentrations of glucose.

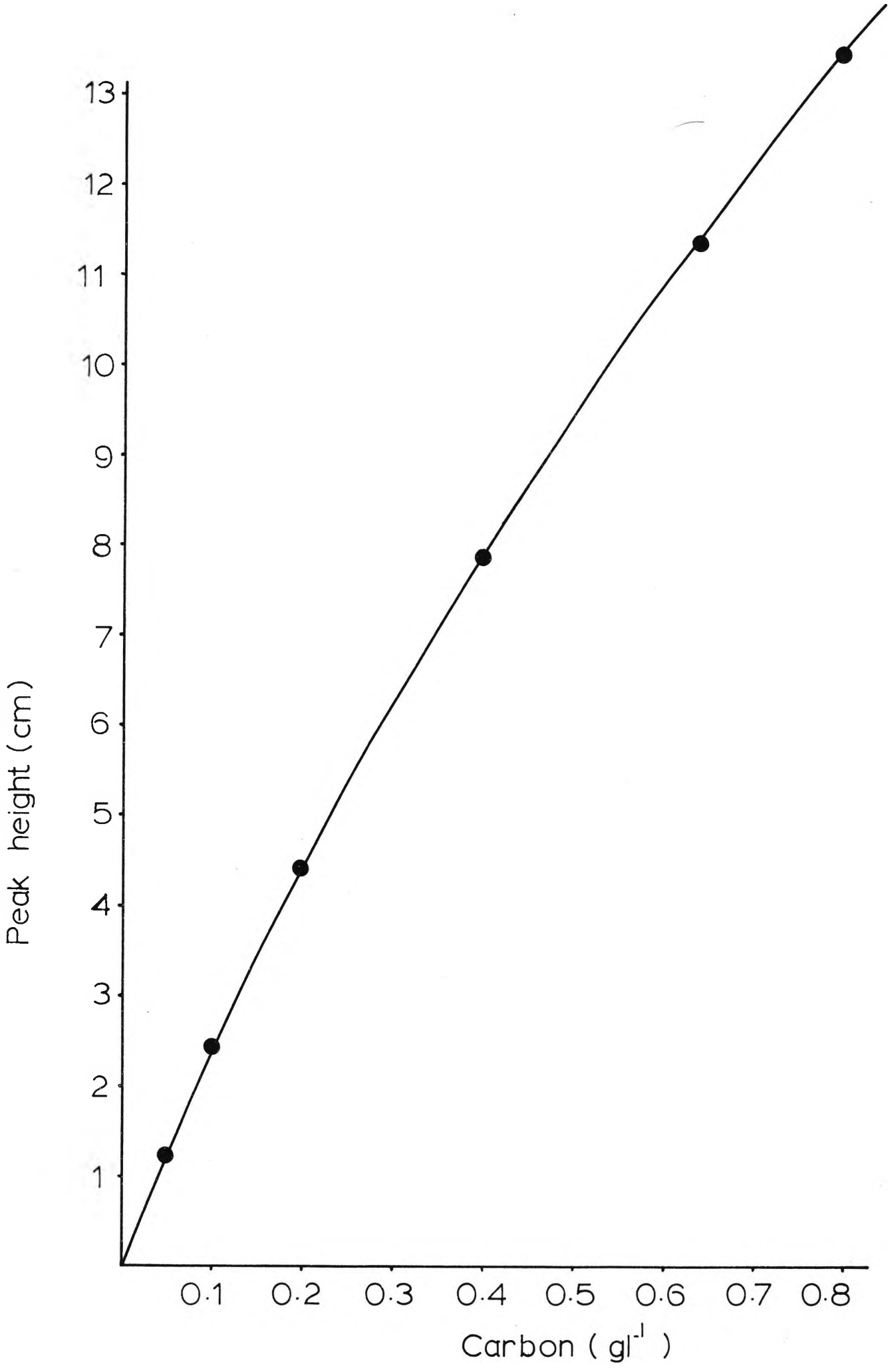

13
12
11
10
9
8
7
6
5
4
3
2
1
Peak height (cm)
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
Carbon (gl-1)

Fig. II-4.

(xiii) Determination of glucose, acetic acid, ethanol, pyruvic acid, lactic acid, citric acid and formic acid

Glucose

The glucose concentration in the input medium and effluent culture was measured by the glucose oxidase system (Sigma Technical Bulletin No. 510, 1969).

Acetic acid and ethanol

Acetic acid and ethanol in culture Millipore filtrates were assayed by gas-liquid chromatography using a Varian 1800 series chromatograph and a Poropak Q column at 190°C.

Pyruvic acid and lactic acid

Pyruvic acid and lactic acid were assayed enzymically (Sigma Technical Bulletin No. 726-UV and No. 826-UV respectively 1968).

Citrate

Citrate was assayed by gas-liquid chromatography using a Varian 1800 series chromatograph and a Tenax-GC column.

Formic acid

Formic acid was determined by the thiobarbituric acid method (Dawes, McGill and Midgley, 1971).

(xiv) Amino acid analysis

The amino acid composition of samples of freeze dried bacteria were determined by Mr. N. Crabtree, Shell Research Limited, Sittingbourne, Kent.

Sample (100 mg) was refluxed for 24 h, with 180 ml 5.9 N hydrochloric acid in a round-bottomed flask. The condenser was washed with deionised water and the warm hydrolysate filtered through a Whatman No. 541 filter paper aided by gentle suction on a Buchner funnel. The hydrolysate was then transferred quantitatively to a 250 ml volumetric flask and made up to the mark with deionised water.

A sample of the hydrolysate (2 ml) was dried in vacuo over potassium hydroxide and sulphuric acid. The residue was redissolved in 2 ml 0.1 N HCl containing 0.1 μ moles norleucine per ml and 1 ml was put on the column. The analysis was carried out on a Technicon model NC-1 amino analyser, using the standard autograd composition for a 17 hour run. The column was 130 cm x 0.6 cm, filled with Technicon type A chromobeads. Elution was carried out at 60°C.

(xv) Cytochrome spectra

Cytochrome spectra were recorded at room and at low temperature (77°K), in a split beam spectrophotometer (Hitachi Perkin Elmer Model 356). The culture (800 ml) was rapidly drained from the fermenter at the end of a given steady state, centrifuged at 17,000 g for 35 min at 4°C and the pellet resuspended in 10 mM-$MgCl_2$ plus 50 mM-Tris-HCl buffer (pH 7.5), recentrifuged at 17,000 g and finally resuspended in 26 ml Tris-HCl buffer.

Oxidised minus reduced difference spectra were obtained by adding a few grains of $Na_2S_2O_4$ to one cuvette to obtain cytochromes in the reduced state and oxidising the cytochromes in the reference cuvette with a few grains of $K_3Fe(CN)_6$ or one drop of H_2O_2.

CO difference spectra were obtained by treating the contents of both cuvettes with $Na_2S_2O_4$ and bubbling a steady stream of CO through one cuvette for 1 min. The CO-treated cuvette was left in the dark for 10 min before the spectra were recorded at room temperature and at low temperature (77°K).

(xvi) Fluorimetry

The fluorimeter was a modification of the apparatus described by Harrison and Chance (1970). Modification of the electronic circuitry was carried out by Mr. C. Timms (Borden Laboratory, Shell Research Limited) and is shown in Appendix III. These modifications allowed the measurement of fluorescence at cell concentrations of 1 g per l dry weight. The glass wall of the Biotec fermenter was substituted by a stainless steel wall which eliminated the need for tedious black out procedures. Connections and ports on the fermenter were blacked out using black adhesive tape which was covered with aluminium foil and sealed with black adhesive tape. The culture in the fermenter was irradiated at 360 nm and the resultant fluorescence transmitted to the photomultiplier as shown in Fig. II-5.

The system was calibrated by injecting NADH into the fermenter and recording the resultant fluorescence. This is a very crude method as NADH dissolved in the medium has a much lower fluorescence yield than that inside the intact cell (Estabrook, 1962). Details of the fluorimeter apparatus are described in Appendix III.

Fig. II-5. Fluorimeter: Arrangement of optic fibres on fermenter base plate to facilitate direct irradiation of culture at 360 nm and detection of resultant fluorescence.

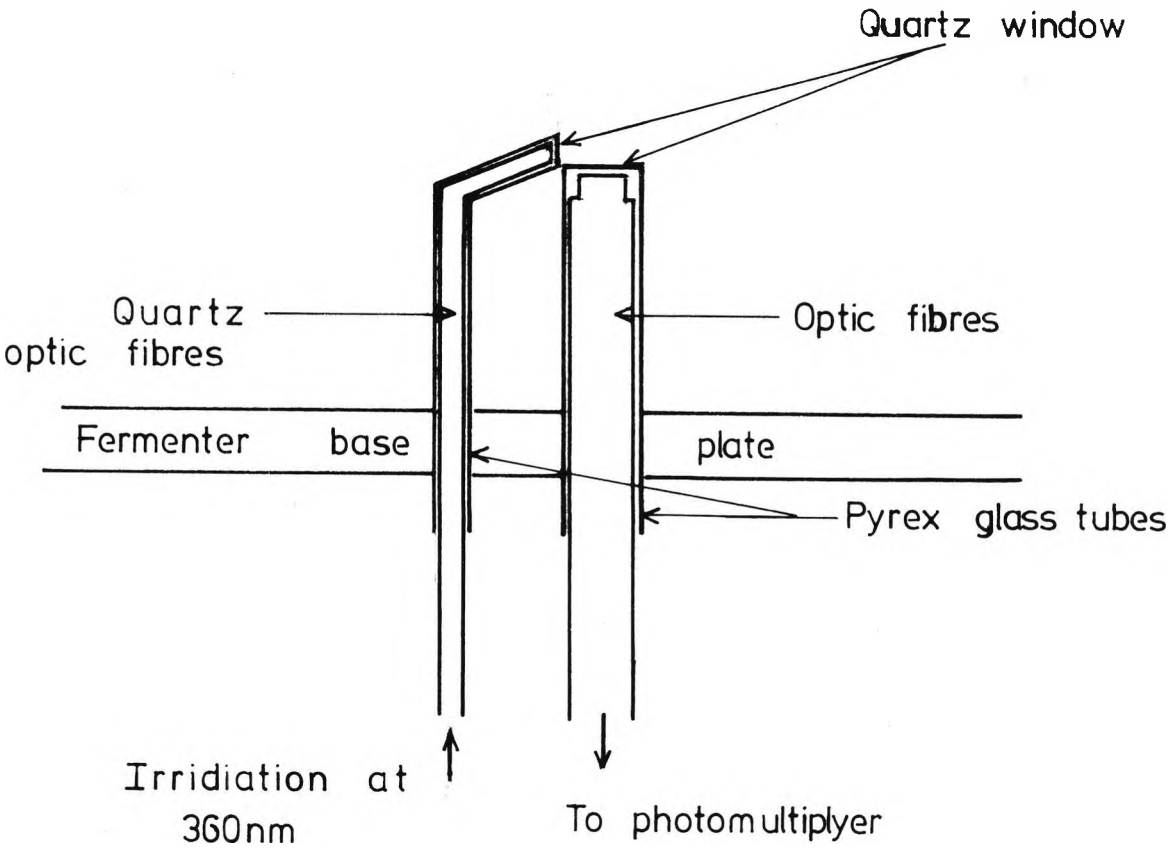


Fig.II-5. Fluorimeter

3. Measurement of the Respiration Rate of Harvested Bacteria Using a 'Closed' Oxygen Electrode Cell

(i) Calculation of respiration rate

The respiration rate of harvested bacteria was measured by diluting culture samples in complete basal medium minus the carbon and nitrogen sources, (pH 7.2), adding known quantities of glucose and following the oxygen uptake in an oxygen electrode respirometer (Rank Bros., Bottisham, Cambridgeshire). The potential respiration rate was calculated from the equation (Harrison and Loveless, 1971a):

$$q_{O_2} \text{ (mmol } g^{-1}h^{-1}) = \frac{r}{xd} \qquad (2)$$

where r = rate of depletion of oxygen concentration as measured in in the oxygen electrode cell (mmol $g^{-1}h^{-1}$)

d = dilution factor

x = concentration of organism (gl^{-1})

(ii) Inhibitor studies

Cyanide

Known concentrations of KCN were injected into the oxygen electrode cell containing cells buffered to pH 7.2. After a two minute incubation period substrate was injected into the cell and the oxygen uptake recorded. A pH value of 7.2 was used as the maximum inhibitory effect with cyanide was reported to occur at pH values two units less than the pK of cyanide. The pK value for

cyanide is 9.8, and the maximum inhibitory effect reported to occur between pH 7-8 (Hewitt and Nicholas, 1963).

Carbon monoxide

Carbon monoxide gas was bubbled through carbon and nitrogen deficient medium (pH 7.2) for 5 minutes. The saturated CO solution was diluted to give final concentrations of CO from 5 to 50% saturation. The bacteria were diluted with the CO solution in the oxygen electrode cell and left in contact with the CO for two minutes before the substrate was injected into the cell. A mixture of KCN plus CO was also used. CO saturated buffer was introduced into the oxygen electrode cell containing diluted culture, and the cell closed immediately KCN was then injected into the cell. A two minute incubation period was allowed before substrate was injected into the electrode cell.

4. Measurement of the Respiration Rate of Harvested Bacteria Using an 'Open' Oxygen Electrode Cell

This part of the work was carried out at the Institute of Biochemistry, Odense University, Denmark, under the guidance of Dr. H. Degn. The apparatus, the respirograph, on which these experiments were made was that built in the Institute (Degn and Wohlrab, 1971). The respirograph is an open system allowing oxygen to diffuse across the boundary between the gas and liquid phases. The optical cuvette used in the respirograph consists of a 12 mm x 12 mm x 35 mm hexagonal chamber fitted with a stirrer. The sensor end of a Clark electrode (Radiometek Ltd., Copenhagen, Denmark) is in contact with the liquid in the chamber. Another Clark electrode is placed in a chamber through which the effluent gas passes. The surface of the liquid in the cuvette is in contact with a gas mixture of N_2 and O_2 whose O_2 content is made to increase linearly with time by means of a linear oxygen gradient apparatus.

When the liquid contains a respiring sample the net transport of oxygen is in the direction from the gas to the liquid. Provided both the gas and liquid phases are homogenous the rate of transport of oxygen is linearly dependent on the difference in oxygen tension between the gas and the liquid. This relationship is expressed by the equation:

$$R = K (T_G - T_L) \qquad (3)$$

where R = rate of transport of oxygen from the gas to the liquid ($\mu m\ sec^{-1}$)

T_G = oxygen tension in the gas (μM)

T_L = oxygen tension in the liquid (μM)

K = oxygen transfer constant

The oxygen transfer constant depends on the surface area, the volume of the liquid and the temperature. If the gas mixture is allowed to flow rapidly through the volume above the liquid it is practically homogenous and its composition is not significantly affected by the exchange of oxygen with the liquid phase. As stirring is necessary to obtain homogeneity in the liquid phase, great care must be taken that this does not create bubbles because the latter will affect the surface area of the solution and change the oxygen transfer constant. The cuvette is adapted to fit a Perkin-Elmer 356 dual wavelength spectrophotometer. By selecting wavelength pairs that represent the isobestic point, ie. 558 nm minus 578 nm and 540 nm minus 553 nm for cytochrome b and c respectively of B. natriegens, the degree of oxidation of these respiratory pigments can be measured.

(i) Measurement of respiration rate

The measurement of the respiration rate by the respirograph depends on the relationship given by equation 3. At steady state, ie. when the oxygen tension in the liquid is constant, the rate of oxygen consumption by the respiring sample and the rate of transport of oxygen into the liquid are equal. If the oxygen tension in the gas and the oxygen transfer constant are known and the medium dissolved oxygen tension is measured polarographically, the rate of transport of oxygen (= respiration rate) can be calculated from equation 3.

Thus the respiration rate expressed as μ moles oxygen per g bacterial carbon per sec is given by equation (4):

$$\text{Respiration rate } (\mu \text{ mol } O_2\ g^{-1}\ sec^{-1}) = \frac{K(T_G - T_L)V}{\bar{x}} \qquad (4)$$

where V = liquid volume

$\bar{x}$ = g bacterial carbon in vol V.

The oxygen transfer constant (K) was determined experimentally at the start of each experiment and is numerically equal to:

$$K = \frac{\ln 2}{t_{\frac{1}{2}}} \qquad (5)$$

where $t_{\frac{1}{2}}$ is the half time of the oxygen saturation curve in the liquid obtained by passing pure oxygen over the cuvette that had previously been in equilibrium with N_2 in the gas phase.

(ii) Determination of the 'apparent' Km for oxygen

Ideally, a point by point determination of the steady state respiration rate as a function of the dissolved oxygen concentration should be made. However, with the use of the oxygen gradient mixer the oxygen tension in the gas is allowed to increase continuously at a very slow rate so that the system is at steady state. As the oxygen tension in the gas runs through a slow linear sweep, steady state values of oxygen tension in the liquid are recorded and the corresponding respiration rate calculated from equation (3). The 'apparent' Km for oxygen is obtained by a Lineweaver-Burke plot of respiration rate against the medium dissolved oxygen

tension. The respiration rate as calculated from equation (3) is given as μ molar per sec, and will not affect the value of the Km for oxygen. To obtain the rate in μ Moles per sec multiplication of equation (3) by the cuvette volume is necessary, thus:

$$R = K(T_G - T_L)V \qquad (6)$$

5. Selection of a Formate Negative Mutant

When B. natriegens is grown in minimal medium with glucose as the sole carbon source the culture pH value decreases, but when the organism is grown in medium containing a mixture of glucose plus sodium formate the culture pH value increases. Using this fact a simple selection procedure was devised. A pH indicator (BDH Universal pH indicator) was incorporated into marine agar containing glucose alone (2 gl^{-1}) or a mixture of glucose plus sodium formate (2 g of each). The wild type produced yellow colonies on medium containing glucose alone and purple colonies on medium containing a mixture of glucose plus sodium formate. A mutant that could not use formate would be expected to produce a yellow colony when grown on a mixture of glucose plus sodium formate.

Mutagenesis

A mid log phase shake flask culture of B. natriegens was exposed to N-methyl-N'-nitro-N-nitroso-guanidine (NTG, 500 $\mu g ml^{-1}$). Samples were withdrawn from the culture at various time intervals, diluted in carbon and nitrogen deficient medium, and then plated onto marine agar containing indicator and glucose plus formate. Five agar plates were used per serial ten-fold dilution. The percentage survival of the organism after exposure to NTG for various time intervals is shown in Fig. II-6. Plates containing yellow colonies were replicate-plated and the resultant yellow colonies isolated and streaked onto glucose plus formate medium. This particular experiment yielded three yellow colonies.

Fig. II-6. Percentage survival of Beneckea natriegens after exposure of a log phase culture to 500 μg ml^{-1} N-methyl-N'-nitro-nitrosoguanidine (NTG).

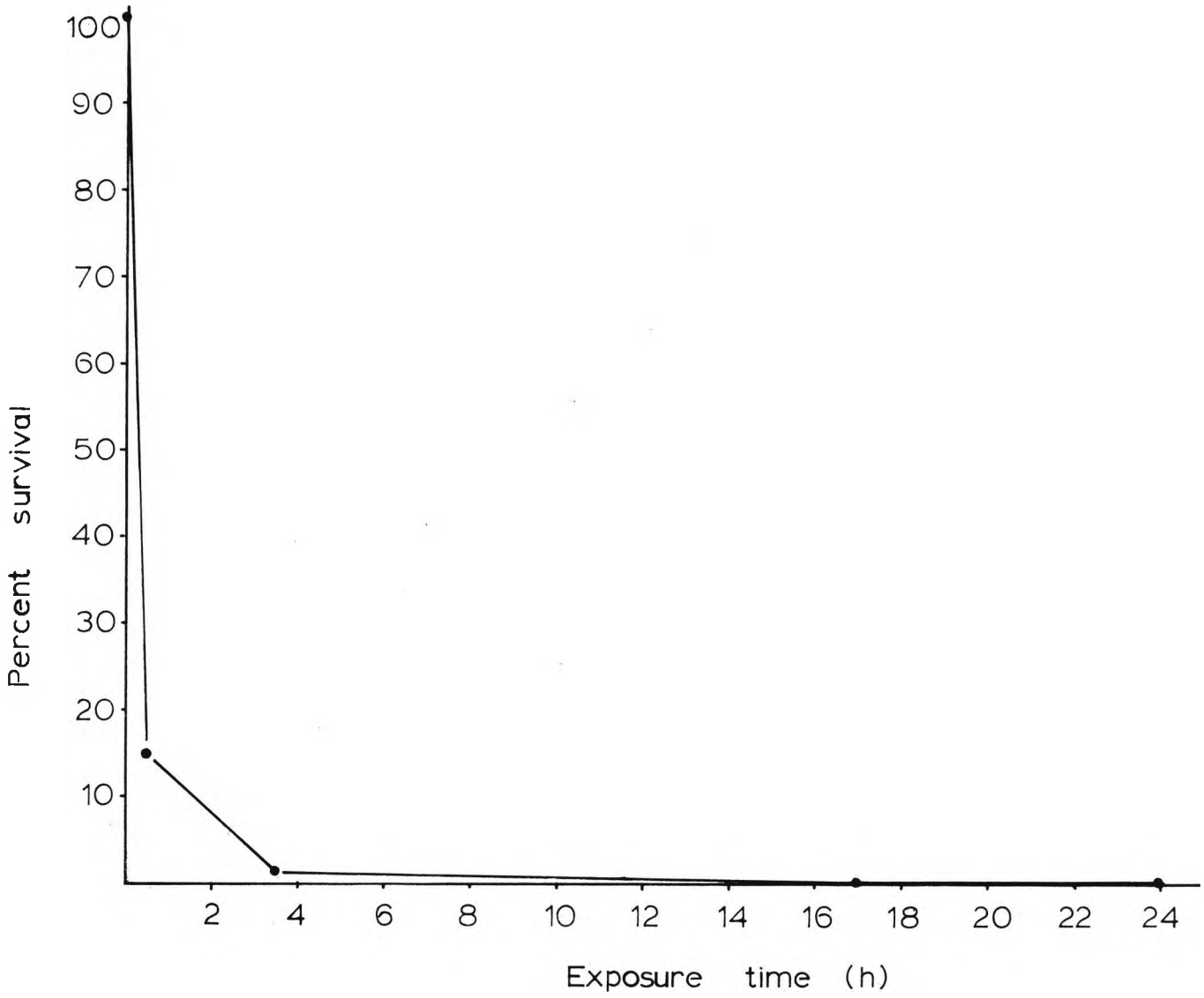

100
90
80
70
60
50
40
30
20
10
Percent survival
2
4
6
8
10
12
14
16
18
20
22
24
Exposure time (h)

Fig. II-6.

SECTION III

MOLAR GROWTH YIELDS, RESPIRATION AND CYTOCHROME PATTERNS OF BENECKEA NATRIEGENS WHEN GROWN UNDER CARBON LIMITATION IN A CHEMOSTAT

1. The Effect of Variation in the Substrate Feed Concentration ($D = 0.34\ h^{-1}$)
2. The Effect of Variation in the Dilution Rate
   (i) Unchelated medium G
   (ii) Medium G chelated with trisodium citrate
3. A Comparison of the Growth of Beneckea natriegens under Glucose and Glycerol Limitation in a Chemostat Culture
4. Discussion

MOLAR GROWTH YIELDS, RESPIRATION AND CYTOCHROME PATTERNS OF BENECKEA NATRIEGENS WHEN GROWN UNDER CARBON LIMITATION IN A CHEMOSTAT

The physiology of Beneckea natriegens was studied in carbon limited chemostat culture because high concentrations of glucose ($>2\ gl^{-1}$) inhibited the growth of this organism in batch culture (Appendix I). The range of glucose concentrations over which chemostat cultures were carbon limited was established by increasing the glucose concentration in the medium feed until the relationship between biomass and glucose concentration departed from linearity. Departure from linearity under these conditions is indicative of growth limitation by a nutrient other than the carbon and energy source. The growth yield from glucose and oxygen, respiration rate and cytochrome composition of B. natriegens was monitored at various specific growth rates in carbon limited chemostat culture. The data generated from these experiments would enable the calculation of the maintenance energy requirement for oxygen and glucose. The relationship between in situ and potential respiration rate and the cytochrome content of the organism could also be examined. Moreover these experiments would indicate a range of glucose concentrations and growth rates which could be employed in later experiments without the danger of the culture being limited by nutrients other than the carbon source. The growth of B. natriegens in glycerol limited chemostat culture was also examined.

1. The Effect of Variation in the Substrate Feed Concentration ($D = 0.34\ h^{-1}$)

Experiments carried out in batch culture (Appendix I) indicated that the constituents of medium G were sufficient to ensure that the culture was carbon limited at a glucose concentration of $4.0\ gl^{-1}$. To establish that the culture was indeed carbon limited, Beneckea natriegens was grown in continuous culture with medium containing various concentrations of glucose and the effect on yield and respiration rate examined.

Medium G without added trisodium citrate was used in this experiment. The glucose concentration in the medium feed was varied over the range 0.5 to $4.0\ gl^{-1}$. At each glucose concentration the culture was considered to be in a steady state after a minimum of seven culture volumes had passed through the vessel while the culture absorbance value remained constant. Two sets of results were recorded at each steady state, a minimum of four culture volumes being allowed to pass through the culture vessel between each set of readings. The steady state values obtained for bacterial carbon and bacterial dry weight as a function of the feed glucose concentration are shown in Fig. III-1. The total biomass of B. natriegens was found to bear a linear relationship to the glucose concentration.

The molar growth yield from glucose, expressed as g bacterial dry weight per mol glucose ($Y_{glc}1$) or g bacterial carbon per mol glucose carbon ($Y_{glc}2$), were independent of the medium feed glucose concentration, Table III-1.

Fig. III-1. Steady state values of bacterial dry weight and bacterial carbon as a function of the glucose concentration in the feed medium ($D = 0.34\ h^{-1}$).

Bacterial dry weight, ● - ●; bacterial carbon, o - o.

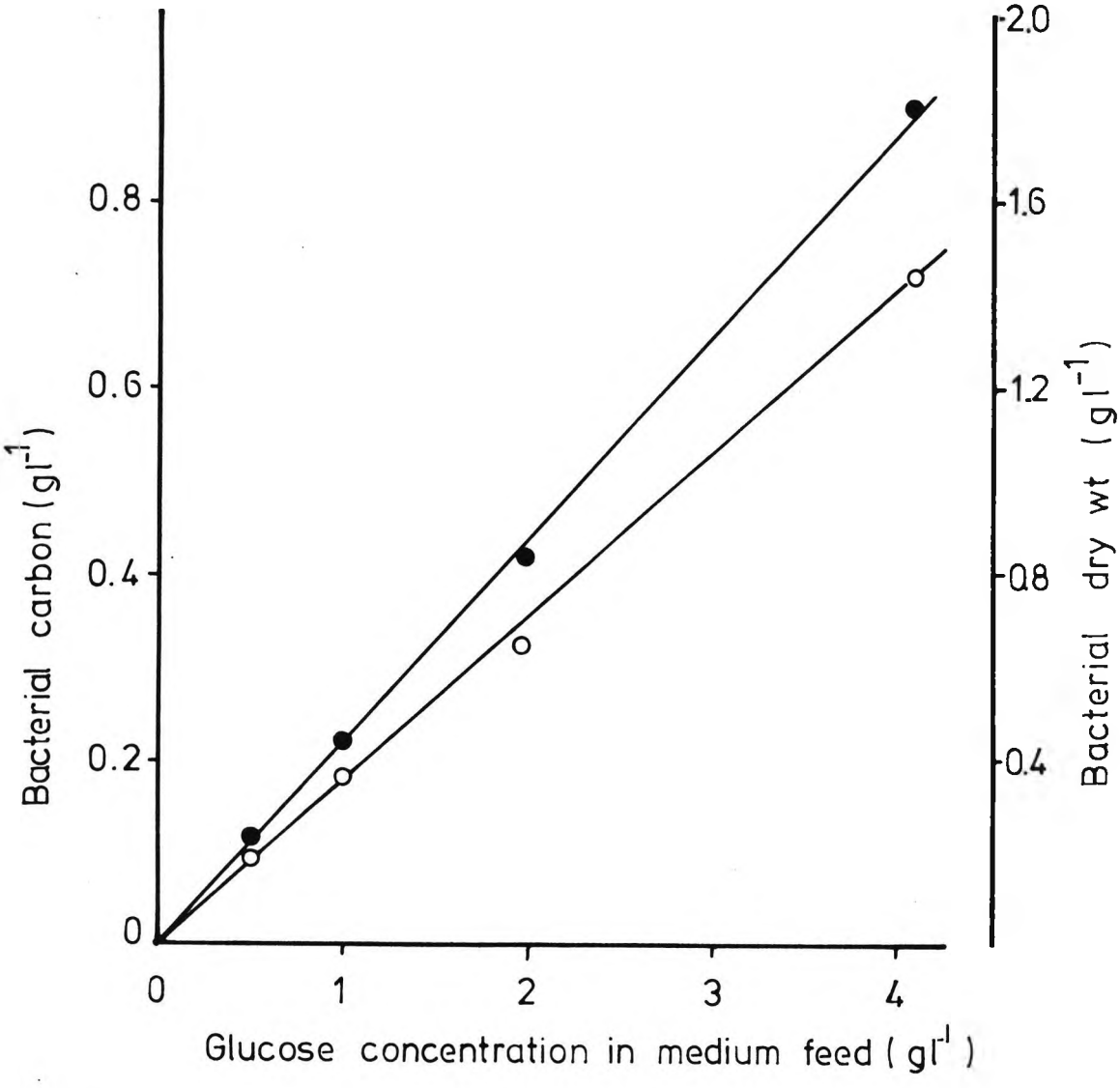

Bacterial carbon (gl^{-1})
0
0.2
0.4
0.6
0.8
Bacterial dry wt (gl^{-1})
0.4
0.8
1.2
1.6
2.0
0
1
2
3
4
Glucose concentration in medium feed (gl^{-1})

Fig. III -1.

Table III-1. A summary of data obtained for various physiological parameters as a function of the glucose concentration in the medium feed of a chemostat culture of Beneckea natriegens

Dilution rate (h^{-1})	Dissolved oxygen tension (mmHg)	Glucose conc. in feed medium (gl^{-1})	Molar growth yield		Specific rate of glucose consumption		Respiration rate, $q_{O_2}{}^{1*}$ (mmol $g^{-1}h^{-1}$)	Bacterial carbon (gl^{-1}) / Bacterial dry wt (gl^{-1})
			$Y_{glc}1^*$ (g mol^{-1})	$Y_{glc}2^*$ (g mol^{-1})	$q_{glc}1^*$ (mmol $g^{-1}h^{-1}$)	$q_{glc}2^*$ (mmol $g^{-1}h^{-1}$)		
0.34	159	0.51	81	33	4.3	10.4	10.0	40.7
0.34	153	1.01	78	32	4.3	10.2	9.8	41.0
0.34	140	1.95	78	30	4.4	10.2	8.5	38.5
0.33	123	4.08	80	34	4.1	10.2	8.0	42.5

*1 calculation based on g bacterial dry weight

*2 calculation based on g bacterial carbon

The specific rate of glucose consumption, expressed as mmol glucose per g bacterial dry weight per h, ($q_{glc}1$) or mmol glucose per g bacterial carbon per h, ($q_{glc}2$), was calculated from the equation:

$$q_{glc} = \frac{(S_R - \bar{S})D}{\bar{x}} \qquad (7)$$

S_R = glucose concentration in medium feed (mmol l^{-1})

$\bar{S}$ = steady state glucose concentration in medium effluent (mmol l^{-1})

D = dilution rate (h^{-1})

$\bar{x}$ = steady state bacterial dry weight (or carbon) gl^{-1}

The q_{glc} was fairly constant over the range of medium glucose concentration 0.5 to 4.0 gl^{-1} (Table III-1). The reasons for calculating the q_{glc} in terms of dry weight and carbon are: (1) as dry weight and carbon analysis are carried out independently it is a double check on the system; (2) so that results in this section may be compared with those given in the latter part of this thesis where results are expressed in terms of carbon only. The specific rate of oxygen consumption $q_{O_2}1$ (mmol oxygen per g bacterial dry weight per h) appeared to decrease slightly with increase in the feed glucose concentration (Table III-1).

As the amount of carbon dioxide produced per unit time was not measured a meaningful carbon balance could not be constructed.

2. The Effect of Variation in the Dilution Rate

Beneckea natriegens was cultured at various growth rates in a chemostat to enable the maintenance energy requirements and the growth yield from oxygen and glucose to be calculated.

The substrate concentration used was 2.0 gl^{-1} glucose, already demonstrated to limit growth in chemostat culture. The temperature and pH values were controlled at 31°C and 7.2 respectively. The experiment was carried out using (i) unchelated and (ii) chelated medium G in order to establish the effect of citrate on growth yield and respiration.

(i) Unchelated medium G

The values of bacterial dry weight and bacterial carbon as functions of the dilution rate are shown in Fig. III-2. The bacterial dry weight and carbon increased slightly as the dilution rate was increased, reaching a maximum value at a dilution rate between 0.52 h^{-1} and 0.64 h^{-1}. Further increase in the dilution rate caused a fall in bacterial dry weight and bacterial carbon concentration. The culture washed out at a dilution rate of 0.84 h^{-1}, which corresponds to a doubling time of 0.81 h. The glucose concentration in culture effluent remained low over the dilution rate range 0.12 h^{-1} to 0.64 h^{-1}, but increased as the dilution rate was further increased (Fig. III-2). Acetic acid was detected in culture effluent at dilution rates in excess of 0.52 h^{-1} reaching a maximum value of 0.16 gl^{-1} at a dilution rate of 0.79 h^{-1} (Fig. III-2).

The molar growth yield from glucose ($Y_{glc}1$ and $Y_{glc}2$) was found to increase with the dilution rate, reaching a maximum value at a dilution rate

Fig. III-2. Steady state values of bacterial carbon, dry weight, effluent glucose and acetic acid as a function of dilution rate.

Bacterial dry weight, o - o; bacterial carbon, ● - ●; glucose in effluent medium, △ - △ and acetic acid in effluent, ▲ - ▲.

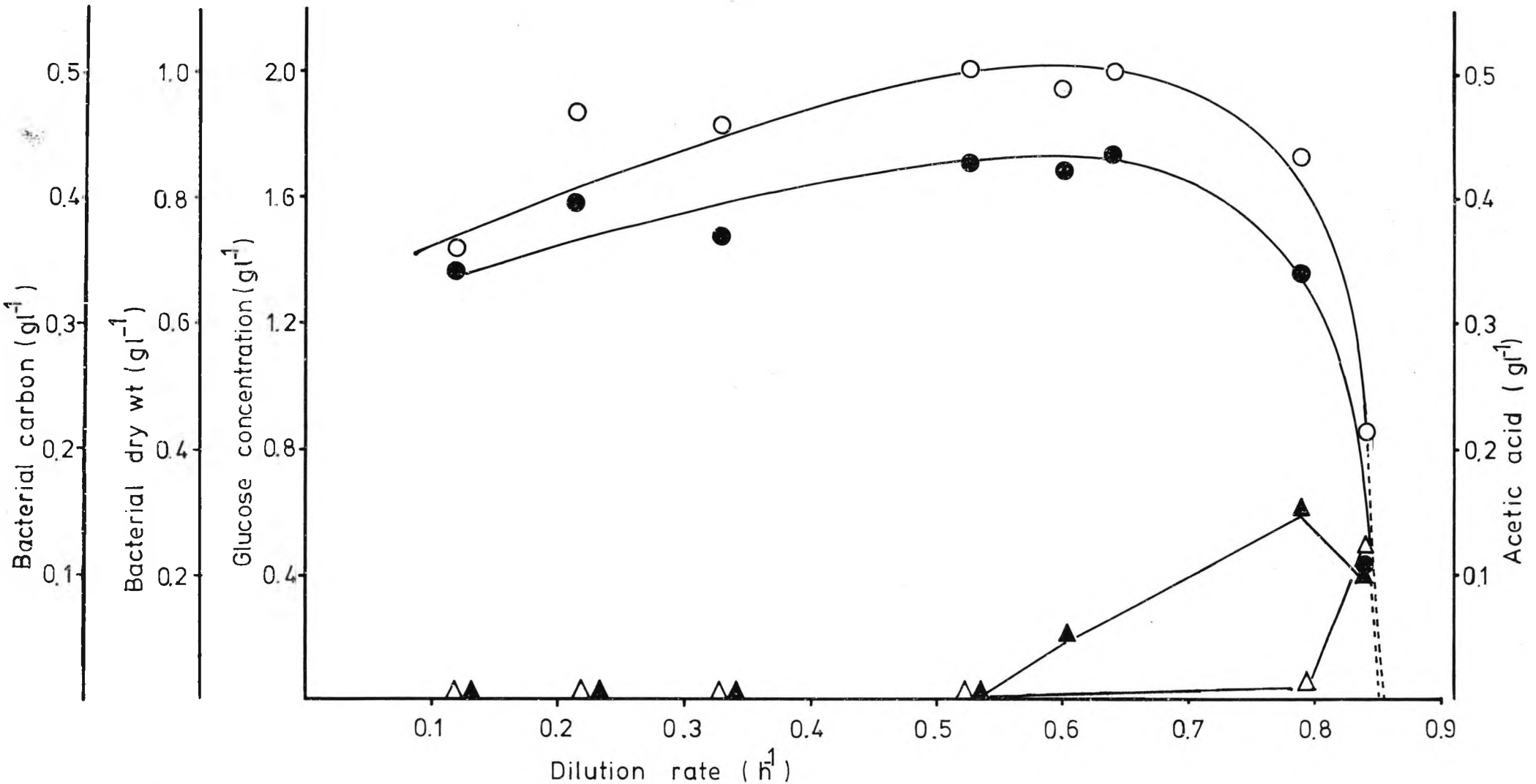

Bacterial carbon (gl^{-1})
Bacterial dry wt (gl^{-1})
Glucose concentration (gl^{-1})
Acetic acid (gl^{-1})
Dilution rate (h^{-1})
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Fig.Ⅲ-2.

near 0.64 h^{-1}. A plot of $Y_{glc}1$ and $Y_{glc}2$ against dilution rate is shown in Fig. III-3. The results are expressed in terms of dry weight and carbon because these were measured independently and there was no evidence to suggest a constant relationship between these two parameters.

The specific rate of glucose consumption, $q_{glc}1$ had a linear relationship to the dilution rate (Fig. III-4). The specific rate of acetic acid production $q_{ac}1$ (mmol acetic acid per g bacterial dry weight per h) also increased linearly at dilution rates above 0.5 h^{-1} (Fig. III-4).

The effect of dilution rate on the nitrogen, phosphorus and hydrogen (N, P and H) contents of freeze dried samples of B. natriegens is shown in Table III-2. The results indicate an increase in the P content of B. natriegens with growth rate. The N and H content appeared to be fairly constant.

The amino acid content (see Methods) of B. natriegens harvested from a fermenter at steady state under carbon limitation at various dilution rates was assayed. The results (Appendix II) indicated the presence of a full complement of amino acids that are nutritionally essential to man (F.A.O. 1967). As only one sample was assayed at each dilution rate the significance of the slight variation of the amino acid content could not be determined.

The Km of the bacterium for glucose at various growth rates was measured. Bacteria were harvested from the chemostat maintained under steady state conditions at various dilution rates and the effect of glucose concentration on the oxygen uptake rate was determined using a Rank oxygen electrode cell. From these data Lineweaver-Burk plots were constructed. The Km values obtained

Fig. III-3. Steady state values of molar growth yields from glucose, oxygen and ATP (estimated) as functions of dilution rate.

$Y_{glc}1$, Δ-Δ; $Y_{glc}2$, • - •; Y_{O_2}, o - o and Y_{ATP} (estimated) □ - □.

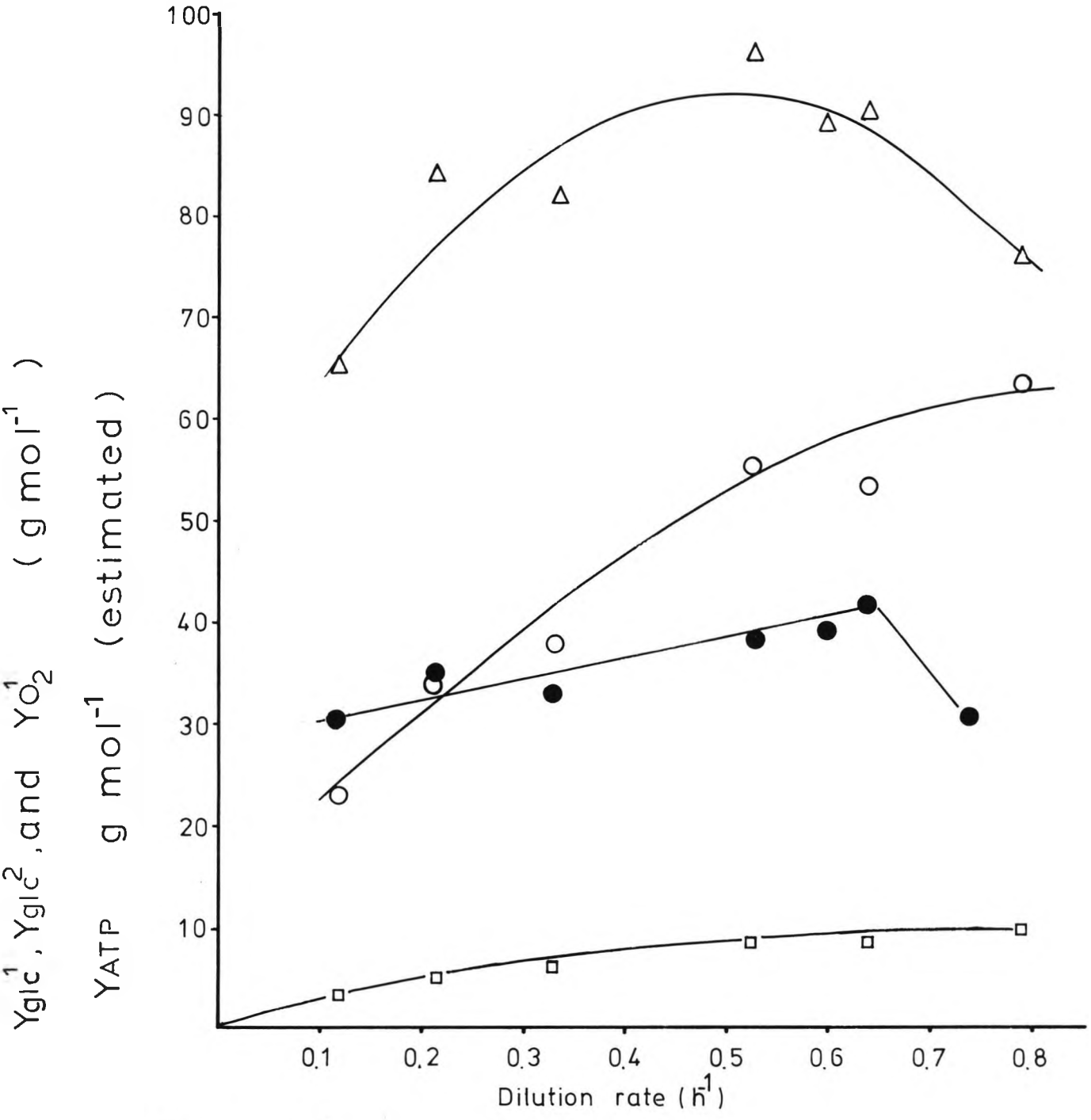

100
90
80
70
60
50
40
30
20
10
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Dilution rate (h^{-1})
Y_{GlC}^{1}, Y_{GlC}^{2}, and $Y_{O_2}^{1}$ (g mol^{-1})
Y_{ATP} g mol^{-1} (estimated)

Fig. Ⅲ-3.

Fig. III-4. Steady state values of glucose consumption and of acetic acid production as functions of the dilution value.

Glucose consumption, mmol glucose utilised per g bacterial dry weight per h, ● - ● and specific rate of acetic acid production mmol acetic acid per g bacterial dry weight per h, o - o.

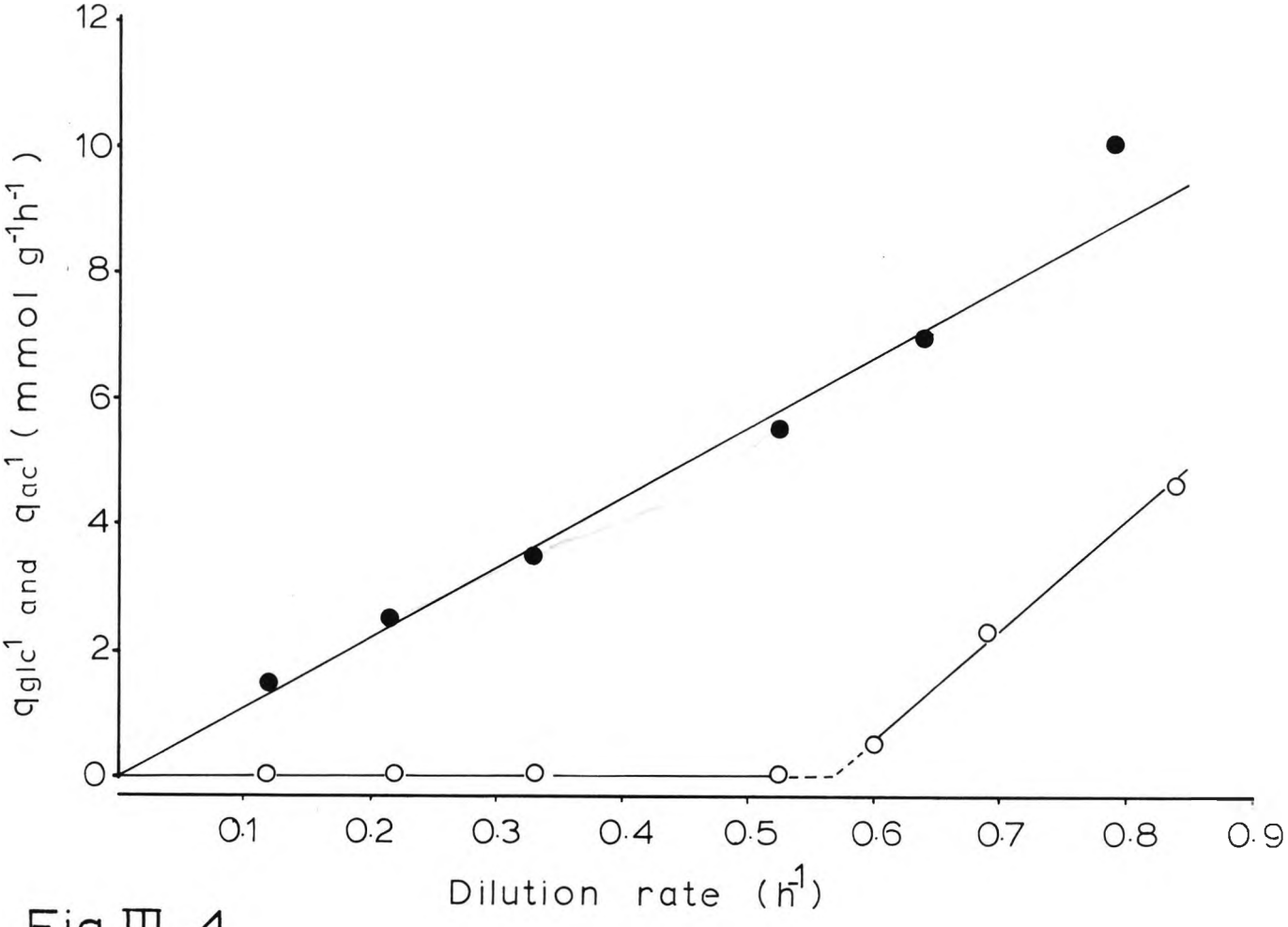

12
10
8
6
4
2
0
q_{glc}^{1} and q_{ac}^{1} (mmol $g^{-1}h^{-1}$)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Dilution rate (h^{-1})

Fig. III-4.

Table III-2. The effect of dilution rate on the N, H and P contents (g/100 g bacterial dry wt) of Beneckea natriegens

Dilution rate (h^{-1})	Nitrogen	Hydrogen	Phosphorus
0.37	10.4	5.4	1.6
0.37	10.3	5.4	1.8
0.40	10.0	5.2	2.0
0.40	9.9	5.4	-
0.64	10.9	5.0	3.0
0.64	10.7	-	-
0.72	10.0	4.5	-
0.72	10.3	-	-

Table III-3. Observed values for the Km for glucose of Beneckea natriegens as a function of the dilution rate

Dilution rate (h^{-1})	Km for glucose (μM)
0.12	32
0.21	28
0.33	33
0.64	26

at various dilution rates did not differ significantly. The mean value was found to be 29 $\pm$ 4 μ molar glucose (Table III-3).

The specific rates of oxygen consumption, q_{O_2} and of carbon dioxide production, q_{CO_2} (mmol per g bacterial dry weight per h) measured directly from the growing culture (in situ rate) increased linearly with increase in the dilution rate.

At zero growth rate the extrapolated line does not pass through the origin but cuts the q_{O_2} axes at 3.65 mmol O_2 $g^{-1}h^{-1}$ (Fig. III-5), this latter value represents the "maintenance requirement" for oxygen (Harrison and Loveless, 1971a). The respiratory quotient remained at unity throughout the range of dilution rates.

The "potential" respiration rate (the respiration rate of a sample taken from the fermenter and exposed to excess oxygen and substrate in an oxygen electrode cell) was independent of dilution rate and remained constant at a value higher than the in situ respiration rate which approached it with increase in the dilution rate (Fig. III-5). The endogenous respiration rate (oxygen uptake of freshly harvested bacteria in the absence of added carbon and nitrogen source) was found to increase with the dilution rate (Fig. III-5).

The molar growth yield from oxygen (Y_{O_2}) was calculated from the equation (Harrison and Loveless, 1971a):

$$Y_{O_2} = \frac{\mu}{q_{O_2}} \qquad (8)$$

Fig. III-5. Steady state respiratory activities as a function of dilution rate in a glucose limited chemostat culture of Beneckea natriegens.

qO_2 (x - x, m mol O_2 $g^{-1}h^{-1}$); qCO_2 (Δ - Δ, mmol CO_2 $g^{-1}h^{-1}$); potential respiration rate (o - o, m mol O_2 $g^{-1}h^{-1}$); endogenous respiration rate (● - ●, m mol O_2 $g^{-1}h^{-1}$) and medium dissolved oxygen tension (▲ - ▲, mmHg).

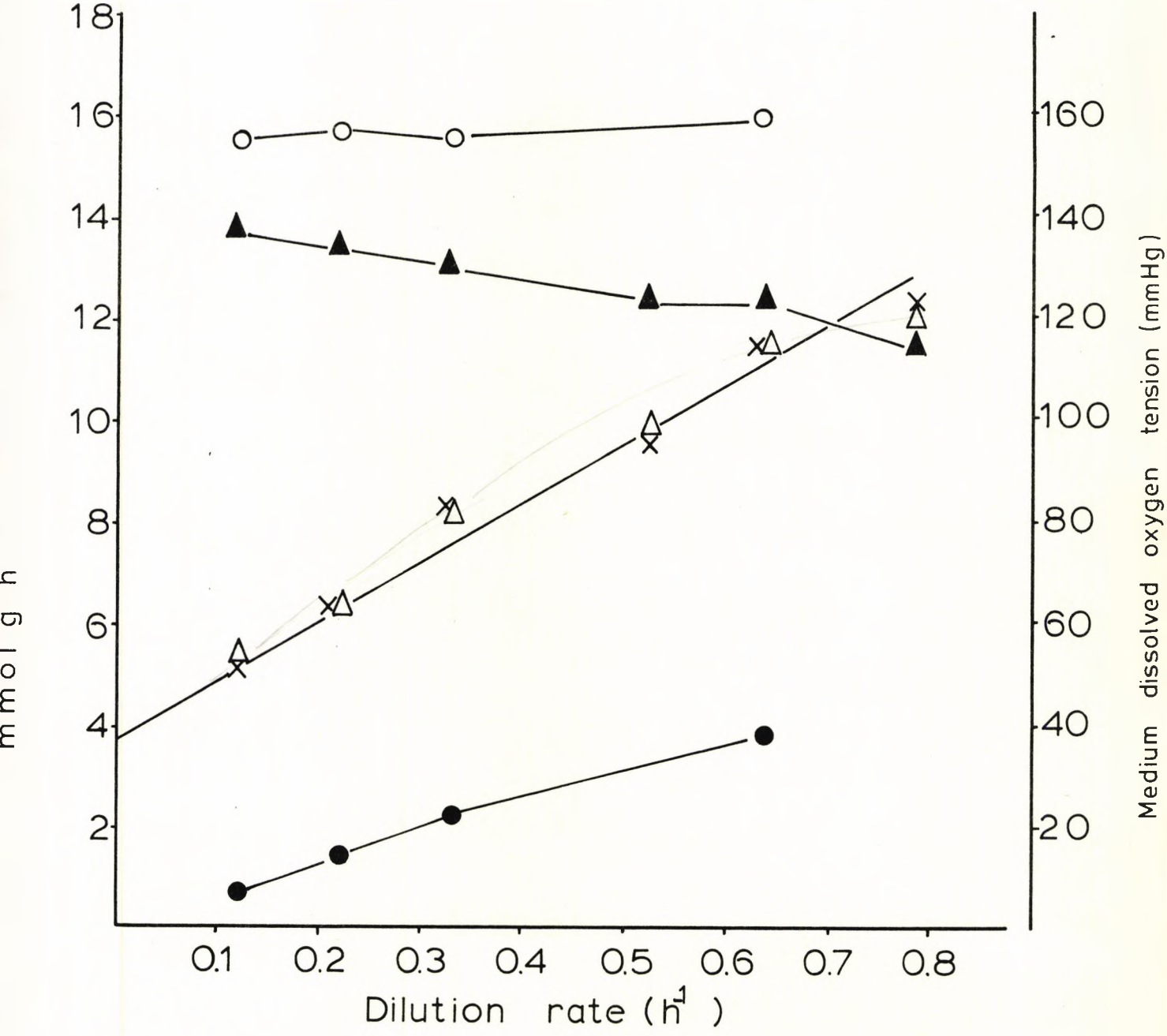

18
16
14
12
10
8
6
4
2
mmol g h
160
140
120
100
80
60
40
20
Medium dissolved oxygen tension (mmHg)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Dilution rate (h-1)

Fig.III-5.

The values of $Y_{O_2}1$ observed as a function of the dilution rate are shown in Fig. III-3. The $Y_{O_2}1$ increased markedly as the dilution rate was increased from a value of 23 g bacterial dry weight per mol oxygen at a dilution rate of 0.12 h^{-1} to 63 g per mol at a dilution rate of 0.79 h^{-1}.

The molar growth yield from ATP was calculated from the equation:

$$Y_{O_2}1 = Y_{ATP}N \qquad (9)$$

where $Y_{O_2}1$ = molar growth yield from oxygen

N = number of moles ATP produced per mole oxygen taken up

Assuming three sites of oxidative phosphorylation and two substrate level phosphorylations per glucose molecule, N will be equal to 6.3. The values of Y_{ATP} calculated in this way increased as a function of the dilution rate (Fig. III-3).

At various dilution rates the Mg^{2+} content of Millipore culture filtrates were assayed. The results obtained were expressed as g bacterial dry weight and g bacterial carbon per g Mg^{2+} utilised (Table III-4). The amount of Mg^{2+} utilised per g of bacterial mass increased markedly as the dilution rate was increased. At a dilution rate of 0.12 h^{-1} the Mg^{2+} utilised was 0.166 g/100 g bacterial dry wt, at a dilution rate of 0.79 h^{-1} the value increased to 0.47 g Mg^{2+}/100 g bacterial dry weight.

The medium in the reservoir had to be stirred continuously to keep a slight precipitate in suspension. [The constituents of this precipitate (probably a complex of Mg and Fe phosphates) were essential for growth]. When the medium

Table III-4. Biomass of Beneckea natriegens produced per g Mg^{2+} utilised as a function of the dilution rate

Dilution rate (h^{-1})	g bacterial dry weight per g Mg^{2+} utilised	g bacterial carbon per g Mg^{2+} utilised
0.12	601	283
0.21	461	195
0.33	546	220
0.51	341	136
0.64	344	145
0.79	212	84

reservoir was not stirred (at moderate growth rates) the culture absorbance decreased, the bacteria formed were typical of Mg^{2+} limited cultures being longer and fatter than normal carbon limited cells and the culture washed out at a dilution rate of 0.52 h^{-1}. Washout at this dilution rate was prevented by stirring the medium in the reservoir. To overcome this problem the pH value of the medium in the reservoir was lowered to pH 2 and this ensured that all the salts were held in solution, furthermore the magnesium content of the medium could be raised without precipitation of salts. However, this method proved to be ineffective as precipitation of Mg^{2+} salts appeared to occur in the fermenter where the pH value was controlled at 7.2, and the culture still washed out at a dilution rate similar to that observed when the slight precipitate was kept in suspension by stirring the medium in the reservoir..

A carbon balance for the growth of B. natriegens in chemostat culture at various dilution rates is shown in Table III-5. The carbon recovery appeared to be within acceptable limits.

Cytochrome analyses

Reduced minus oxidised difference spectra of whole cells (Fig. III-6) exhibited a absorption peaks at 558 nm and 553 nm indicating the presence of b and c type cytochromes respectively. As the growth rate of the organism was increased the relative height of the peaks due to cytochrome b and c changed. At a dilution rate of 0.12 h^{-1} the peak of the c-type was predominant but as the growth rate was increased the peak due to b type cytochrome(s) became evident. The relative increase in the b-type cytochromes was confirmed by the shift from

Table III-5. A carbon balance for the growth of Beneckea natriegens at various dilution rates in carbon limited chemostat culture using unchelated medium G.

Dilution rate (h^{-1})	Carbon input (gh^{-1})	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ Carbon dioxide carbon (gh^{-1})	Carbon recovery (%)	Unmetabolised glucose (g carbon h^{-1})
0.12	0.247	0.105	0.019	0.118	98.0	0.001
0.22	0.442	0.219	0.067	0.146	97.8	0.002
0.33	0.678	0.312	0.097	0.245	96.5	0.005
0.56	1.000	0.538	0.229	0.291	105	0.004
0.64	1.344	0.728	0.289	0.370	103	0.009
0.79	1.618	0.690	0.673	0.284	101	0.138

Fig. III-6. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a chemostat under steady state conditions at various dilution rates.

(a) Oxidised H_2O_2-reduced $Na_2S_2O_4$, (b) oxidised $K_3Fe(CN)_6$-reduced $Na_2S_2O_4$.

(1) Dilution rate, 0.12 h^{-1} (bacterial dry weight 10 mg ml^{-1})

(2) Dilution rate, 0.34 h^{-1} (bacterial dry weight 13.53 mg ml^{-1})

(3) Dilution rate, 0.52 h^{-1} (bacterial dry weight 14.7 mg ml^{-1})

(4) Dilution rate, 0.64 h^{-1} (bacterial dry weight 15.0 mg ml^{-1})

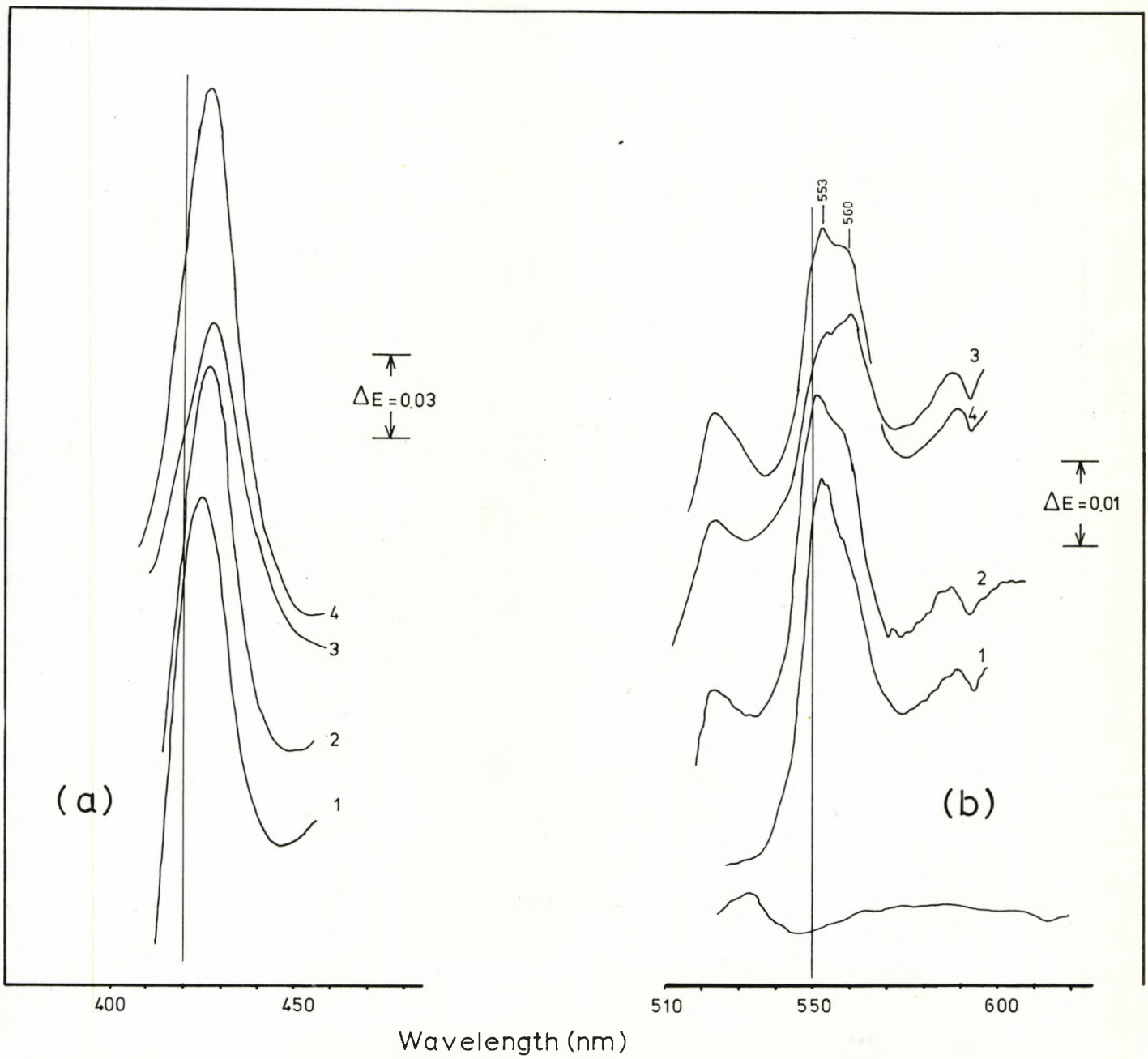

(a)
ΔE = 0.03
4
3
2
1
400
450
553
560
(b)
ΔE = 0.01
3
4
2
1
510
550
600
Wavelength (nm)

Fig. III-6.

Fig. III-7. Room temperature reduced plus CO minus reduced difference spectra of whole cells taken from a chemostat under steady state conditions at various dilution rates.

(1) Dilution rate, 0.12 h^{-1} (bacterial dry weight 7.22 mg ml^{-1})

(2) Dilution rate, 0.34 h^{-1} (bacterial dry weight 9.23 mg ml^{-1})

(3) Dilution rate, 0.52 h^{-1} (bacterial dry weight 9.02 mg ml^{-1})

(4) Dilution rate, 0.64 h^{-1} (bacterial dry weight 10.0 mg ml^{-1})

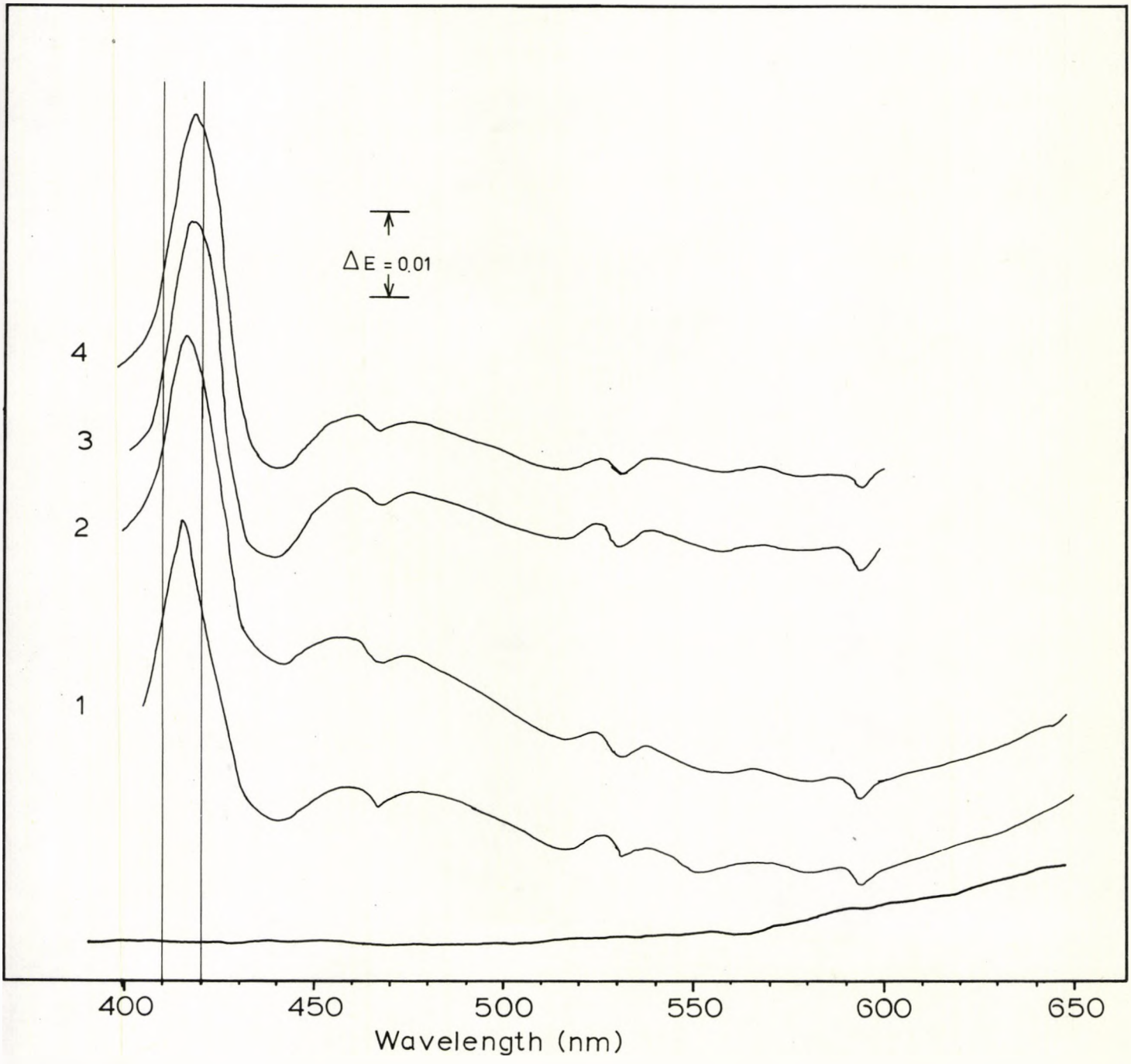


Fig.Ⅲ-7

425 nm to 428 nm in the absorption peak in the Soret region as the dilution rate was increased from 0.12 h^{-1} to 0.64 h^{-1} (Fig. III-6). Similarly CO difference spectra demonstrated an observable shift in the spectral peak from 415 nm to 419 nm (Fig. III-7) as the dilution rate was increased from 0.12 h^{-1} to 0.64 h^{-1}.

In order to assess the effect of growth rate on the relative amount of cytochrome present the absorbance per mg bacterial carbon was calculated (Fig. III-8). The results obtained from oxidised-minus-reduced difference spectra indicate that the level of both b and c type cytochromes appeared to fall as the growth rate was increased. The level of the CO-binding cytochrome(s) however appeared to remain fairly constant and independent of the dilution rate.

(ii) Medium G chelated with trisodium citrate

The inclusion of 0.146 gl^{-1} trisodium citrate in the medium prevented the formation of any visible precipitate. To ensure that the citrate did not affect the growth characteristics of Beneckea natriegens the molar growth yield, respiration rate and cytochrome patterns were examined at various dilution rates in citrate containing medium.

The steady state values of bacterial carbon increased with increase in the dilution rate to a maximum value at a dilution rate of 0.67 h^{-1}. Further increase in the dilution rate resulted in a fall in the bacterial carbon value, but steady states were maintained at dilution rates up to 1.04 h^{-1} (Fig. III-9). Increase in the dilution rate beyond 0.8 h^{-1} caused an increase in culture effluent glucose concentration (Fig. III-9).

Fig. III-8. Steady state values of cytochrome c, b and the ratio of cytochrome c to cytochrome b in organisms grown at various dilution rates. The level of CO binding cytochromes is also shown.

Cytochrome c (● - ●);ΔE/mg dry weight; cytochrome b (o - o,ΔE/mg dry weight); ratio cytochrome c/cytochrome b (Δ-Δ) and CO binding cytochromes (▲-▲,ΔE/mg dry weight).

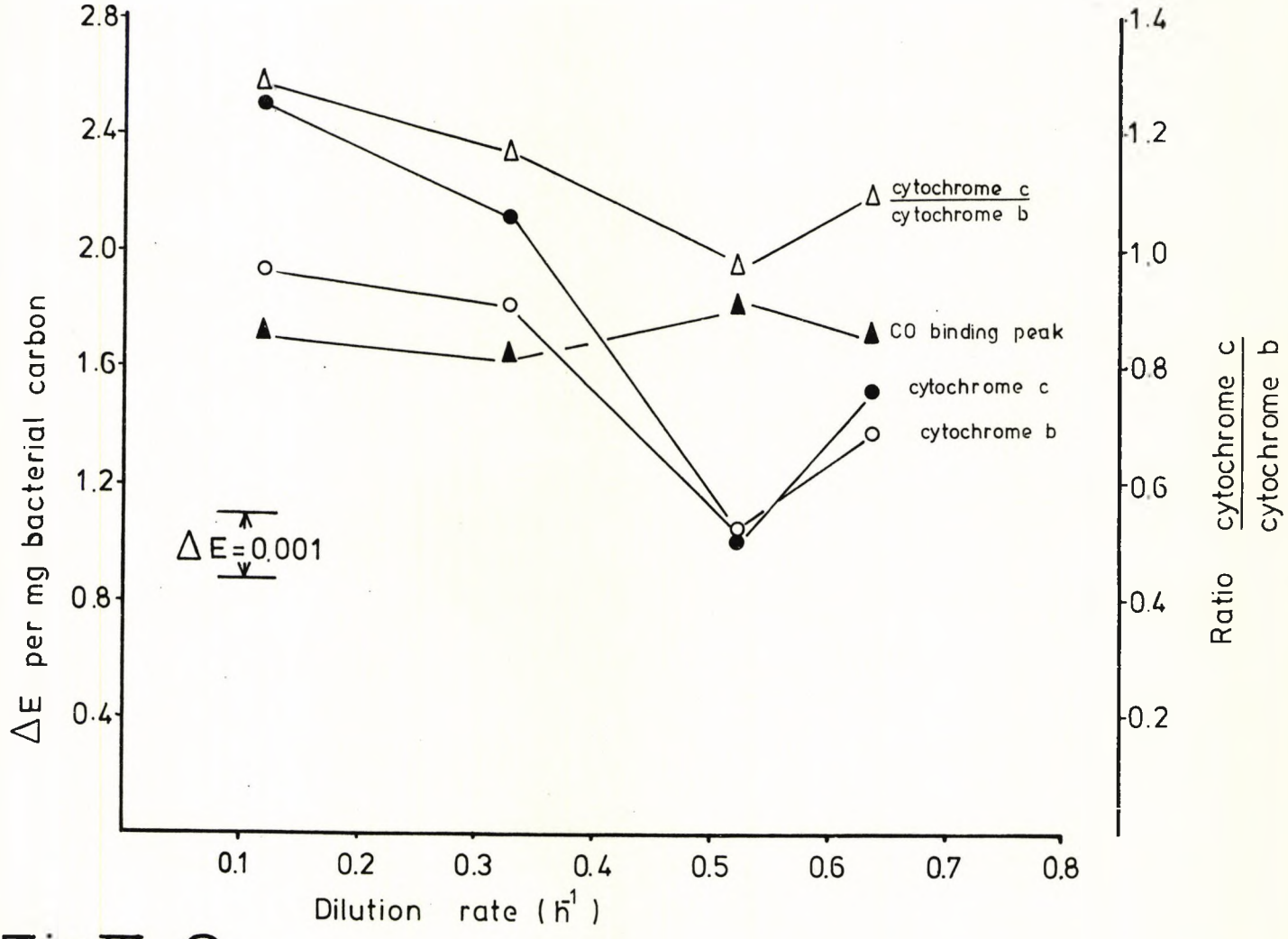

2.8
2.4
2.0
1.6
1.2
0.8
0.4
ΔE per mg bacterial carbon
ΔE=0.001
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Ratio cytochrome c / cytochrome b
Δ cytochrome c / cytochrome b
CO binding peak
cytochrome c
cytochrome b
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Dilution rate (h⁻¹)

Fig.III-8.

Fig. III-9(a) Steady state values of bacterial carbon and concentration of glucose and acetic acid in culture effluent as a function of dilution rate in citrate chelated medium G.

Bacterial carbon, ● - ●; effluent glucose concentration, Δ-Δ; effluent acetic acid concentration, ▲-▲.

(b) Steady state values of the specific rate of oxygen consumption and CO_2 production as a function of dilution rate in chelated medium G.

q_{CO_2}, □ - □; q_{O_2}, ■ - ■.

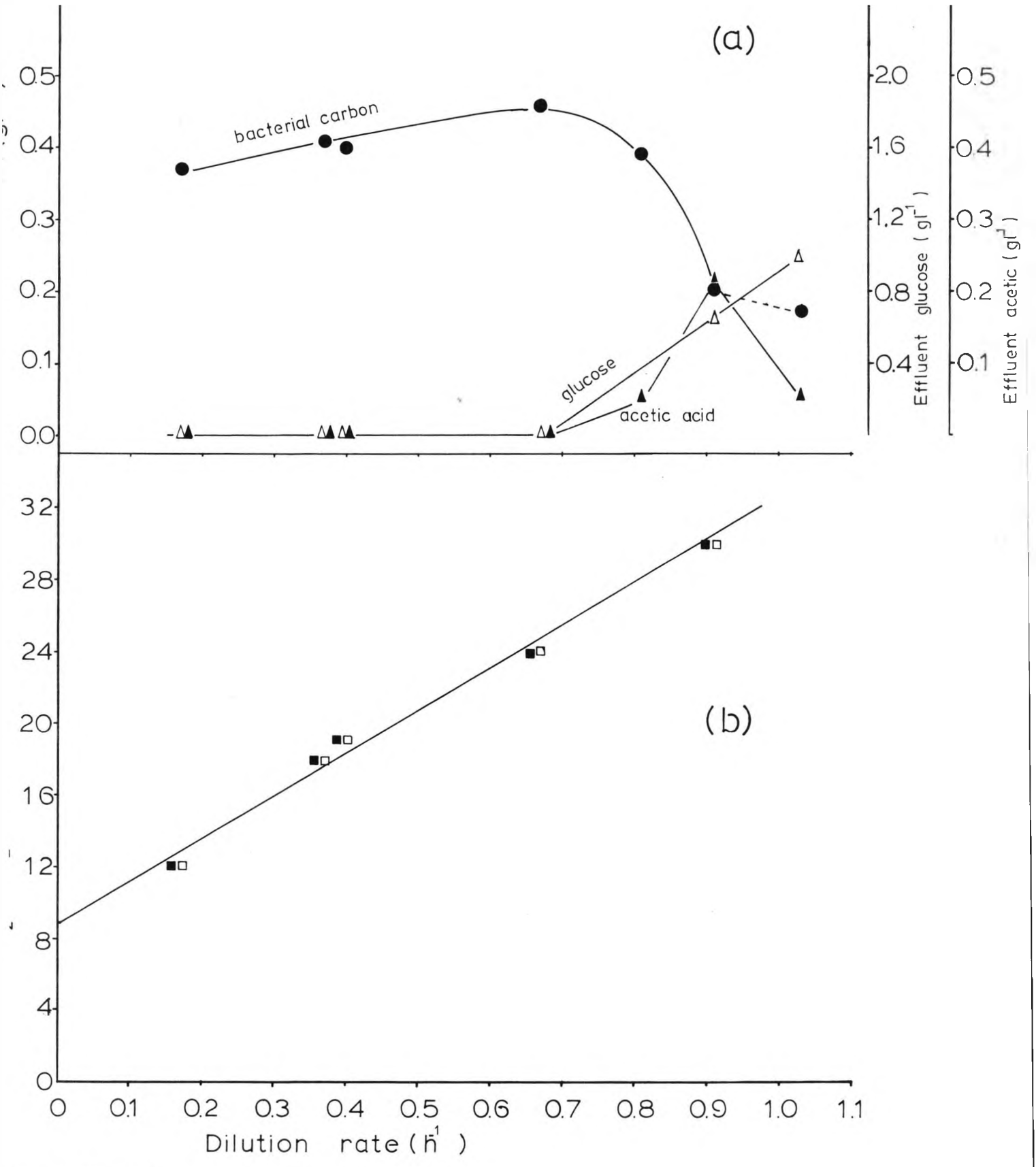

(a)
bacterial carbon
glucose
acetic acid
0.5
0.4
0.3
0.2
0.1
0.0
2.0
1.6
1.2
0.8
0.4
Effluent glucose (gl⁻¹)
0.5
0.4
0.3
0.2
0.1
Effluent acetic (gl⁻¹)
(b)
32
28
24
20
16
12
8
4
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Dilution rate (h⁻¹)

Fig.III-9.

Acetic acid was detected in culture effluent at dilution rates of 0.81 h^{-1}, 0.91 h^{-1} and 1.04 h^{-1}. The growth yield, $Y_{glc}{}^{2}$, increased as the dilution rate was increased from a value of 31.8 $gmol^{-1}$ at a dilution rate of 0.17 h^{-1} to 41 g mol^{-1} at a dilution rate of 0.67 h^{-1}. Further increase of the dilution rate caused a decrease in the growth yield from glucose (Table III-6).

The $q_{O_2}{}^{2}$ and $q_{CO_2}{}^{2}$ were found to increase linearly with increase in the dilution rate (Fig. III-9). The growth yield from oxygen $Y_{O_2}{}^{2}$ increased with an increase in the dilution rate (Table III-6). A carbon balance for the growth of B. natriegens at various dilution rates in the presence of citrate (Table III-7) indicated a satisfactory carbon recovery.

Cytochrome analysis

Oxidised minus reduced difference spectra were recorded at room temperature. The results indicated that the cytochrome content of B. natriegens decreased with increase in the dilution rate (Fig. III-10). The cytochrome content was qualitatively similar to that observed in cells grown in the absence of citrate (Fig. III-11). The shift in the cytochrome c to b ratio was not as marked but showed a slight decrease with increase in the dilution rate (Fig. III-10).

Table III-6. Growth yield and respiration of Beneckea natriegens as a function of dilution rate in carbon limited chemostat culture using chelated medium G (37°C, pH 7.2)

	Dilution rate (h^{-1})						
	0.17	0.37	0.40	0.67	0.81	0.91	1.04
$Y_{glc}{}^{2*}$ (g mole $^{-1}$)	31.8	39.0	37.4	41.0	33.0	28.5	29.0
$q_{O_2}{}^{2}$ (mmol $g^{-1}h^{-1}$)	12.0	18.1	19.1	24.0	-	30.0	-
$q_{CO_2}{}^{2*}$ (mmol $g^{-1}h^{-1}$)	12.0	18.1	19.1	24.0	-	30.0	-
Respiratory quotient	1	1	1	1	-	1	-
$Y_{O_2}{}^{2*}$ g $mole^{-1}$	14.1	20.4	20.9	27.0	-	29.0	-
$q_{glc}{}^{2*}$ (mmol $g^{-1}h^{-1}$)	5.2	-	10.7	16.1	-	31.6	-
$q_{ac}{}^{2*}$ mmol $g^{-1}h^{-1}$	*N.D.	N.D.	N.D.	N.D.	1.73	17.2	5.1
Effluent glucose (gl^{-1})	0.001	0.001	-	0.002	-	0.675	0.959

*2 = calculation based on g bacterial carbon. N.D. = not detected

Table III-7. A carbon balance for the growth of Beneckea natriegens at various dilution rates in carbon limited chemostat culture using citrate chelated medium.

D (h^{-1})	Carbon input (gh^{-1})	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ CO_2 carbon (gh^{-1})	Carbon recovery (%)
0.17	0.280	0.150	0.024	0.103	99.0
	0.280	0.147	0.024	0.108	99.6
0.37	0.694	0.360	0.076	0.210	93.0
0.40	0.662	0.327	0.083	0.213	94.0
0.67	1.068	0.622	0.110	0.256	92.5
	1.068	0.616	0.120	0.266	93.8

Fig. III-10. The effect of growth rate on the cytochrome patterns of Beneckea natriegens grown in citrate chelated medium G.

Cytochrome c, ● - ●; cytochrome b, o - o and ratio of cytochrome c:cytochrome b, Δ - Δ.

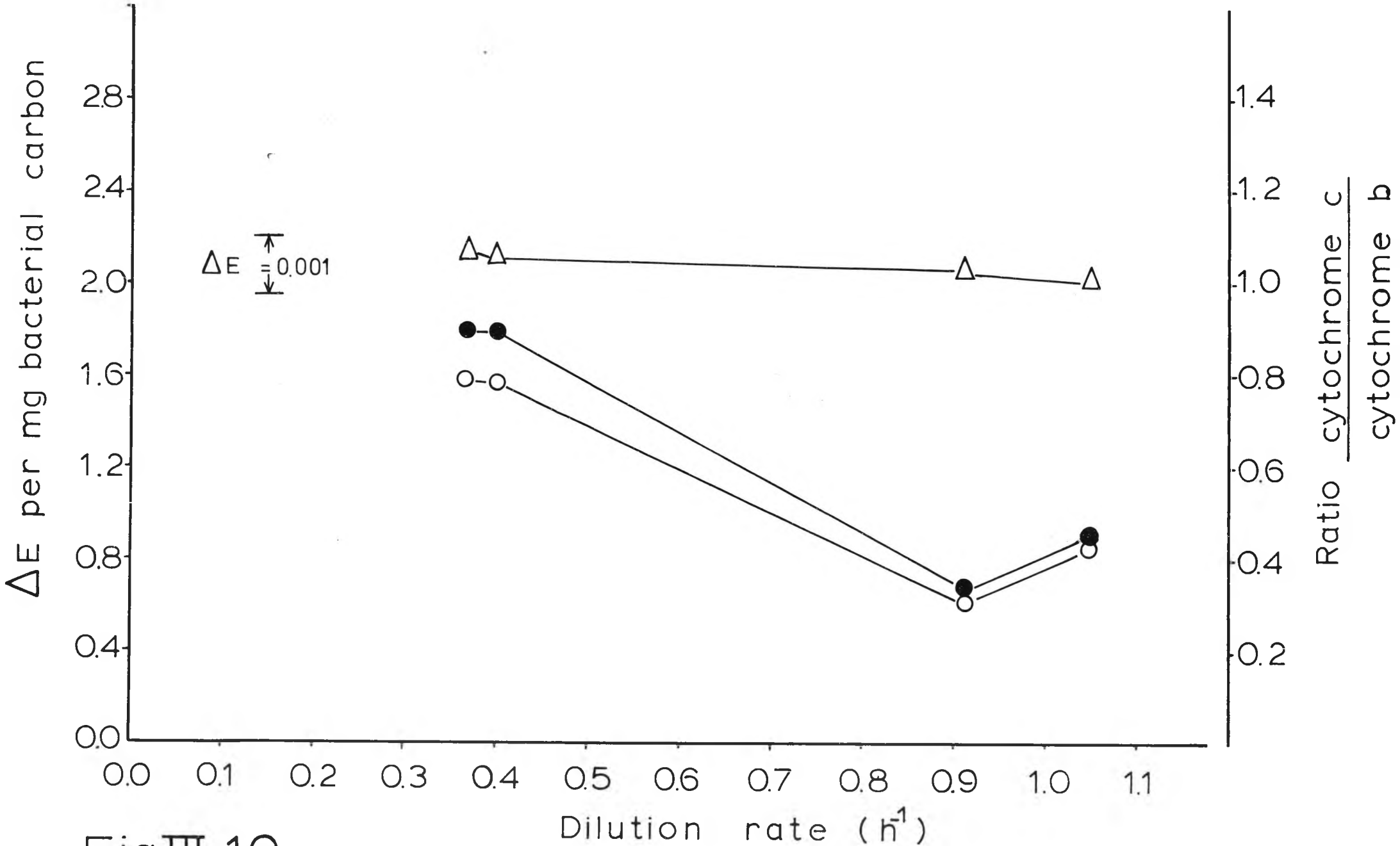

ΔE per mg bacterial carbon
ΔE = 0.001
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
Dilution rate (h⁻¹)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Ratio cytochrome c / cytochrome b

Fig.Ⅲ-10.

Fig. III-11. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a chemostat under steady state conditions at various dilution rates.

(a) Oxidised H_2O_2-reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$-reduced $Na_2S_2O_4$.

(1) Dilution rate, 0.37 h^{-1} (bacterial carbon 4.3 mg ml^{-1})

(2) Dilution rate, 0.40 h^{-1} (bacterial carbon 4.15 mg ml^{-1})

(3) Dilution rate, 0.91 h^{-1} (bacterial carbon 3.12 mg ml^{-1})

(4) Dilution rate, 1.04 h^{-1} (bacterial carbon 3.2 mg ml^{-1})

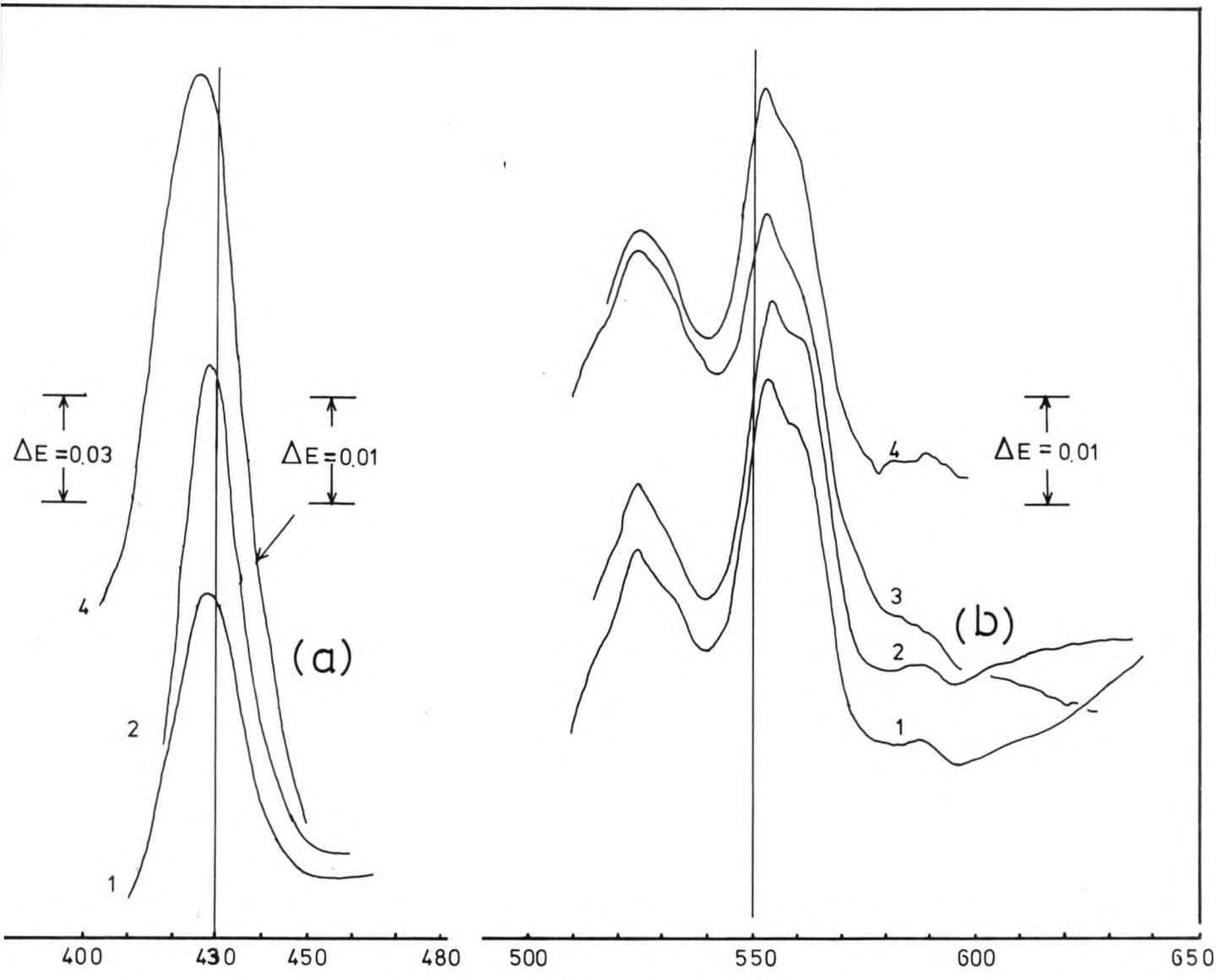

ΔE =0.03
ΔE =0.01
4
(a)
2
1
400
430
450
480
ΔE = 0.01
4
3
(b)
2
1
500
550
600
650
(Wavelength nm)

Fig III-11

3. A Comparison of the Growth of Beneckea natriegens under Glucose and Glycerol Limitation in Chemostat Culture

As glycerol grown bacteria were required for use in later studies, the physiology of B. natriegens grown in a chemostat under glycerol limitation was investigated, using chelated medium G.

The growth yields from glucose ($Y_{glc}2$) and glycerol ($Y_{gly}2$) were found to be 38.7 and 20.2 g bacterial carbon per mole glucose and glycerol carbon respectively.

The $q_{O_2}2$ and $q_{CO_2}2$ observed on glucose and glycerol are shown in Table III-8. The respiratory quotient on glucose was 1.0 while a value of 0.65 was observed with glycerol.

The growth yield from oxygen ($Y_{O_2}2$) was found to be 20.35 g bacterial carbon per mole oxygen on glucose and was considerably higher than the value of approximately 15 g bacterial carbon per mole oxygen when glycerol was the substrate. This would be expected as glycerol is a more reduced substrate than glucose.

A carbon balance for the growth of Beneckea natriegens in chemostat culture under glucose and glycerol limitation ($D = 0.37\ h^{-1}$) is shown in Table III-9.

Cytochrome measurements

Room temperature oxidised minus reduced difference spectra of whole cells indicated that the cytochrome content of glucose and glycerol grown bacteria were both qualitatively and quantitatively similar (Fig. III-12). Carbon monoxide difference spectra exhibited absorption maxima at 417.5 nm for glycerol grown cells, a value very similar to that observed with glucose grown cells (Fig. III-13).

Table III-8. A comparison of the growth of Beneckea natriegens under glucose and glycerol limitation in chemostats at a dilution rate of 0.37 h^{-1}

	Glucose limitation (2.0 gl^{-1} in feed)		Glycerol limitation (2.0 gl^{-1} in feed)	
$q_{O_2}{}^{2*}$ (mmol $g^{-1}h^{-1}$)	18.87	18.48	22.0	21.8
$q_{CO_2}{}^{2*}$ (mmol $g^{-1}h^{-1}$)	18.87	18.34	14.6	14.3
RQ	1.0	1.01	0.66	0.65
$Y_{glc}{}^{2*}$, $Y_{gly}{}^{2}$ (g mol^{-1})	38.13	39.4	20.25	20.27
$Y_{O_2}{}^{2*}$ (g mol^{-1})	20.2	20.5	14.4	15.9
Cytochrome contents (ΔE/mg cell carbon) b	1.56		1.52	
c	1.79		1.75	
Ratio c/b	1.14		1.15	
Unmetabolised substrate (gl^{-1})	0.002	0.002	not detected	
Acetic acid	not detected		not detected	

*2 calculation based on g bacterial carbon

Table III-9. A carbon balance for the growth of Beneckea natriegens in chemostat culture under glucose and glycerol limitation at a dilution rate of 0.37 h^{-1}

Growth condition	Carbon input (gh^{-1})	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ carbon dioxide carbon (gh^{-1})	Carbon recovery (%)
Glucose limitation ($2.0\ gl^{-1}$) pH 7.02	0.619	0.314	0.066	0.187	91.6
	0.617	0.319	0.062	0.185	91.7
Glycerol limitation ($2.0\ gl^{-1}$) pH 7.36	0.616	0.346	0.069	0.177	96.1
	0.645	0.363	0.074	0.177	95.2

Fig. III-12. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a chemostat under steady state conditions with glucose or glycerol as sole carbon and energy source.

(a) Oxidised H_2O_2-reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$-reduced $Na_2S_2O_4$.

(1) Glucose grown cells (bacterial carbon 3.9 mg ml^{-1})

(2) Glycerol grown cells (bacterial carbon 4.58 mg ml^{-1})

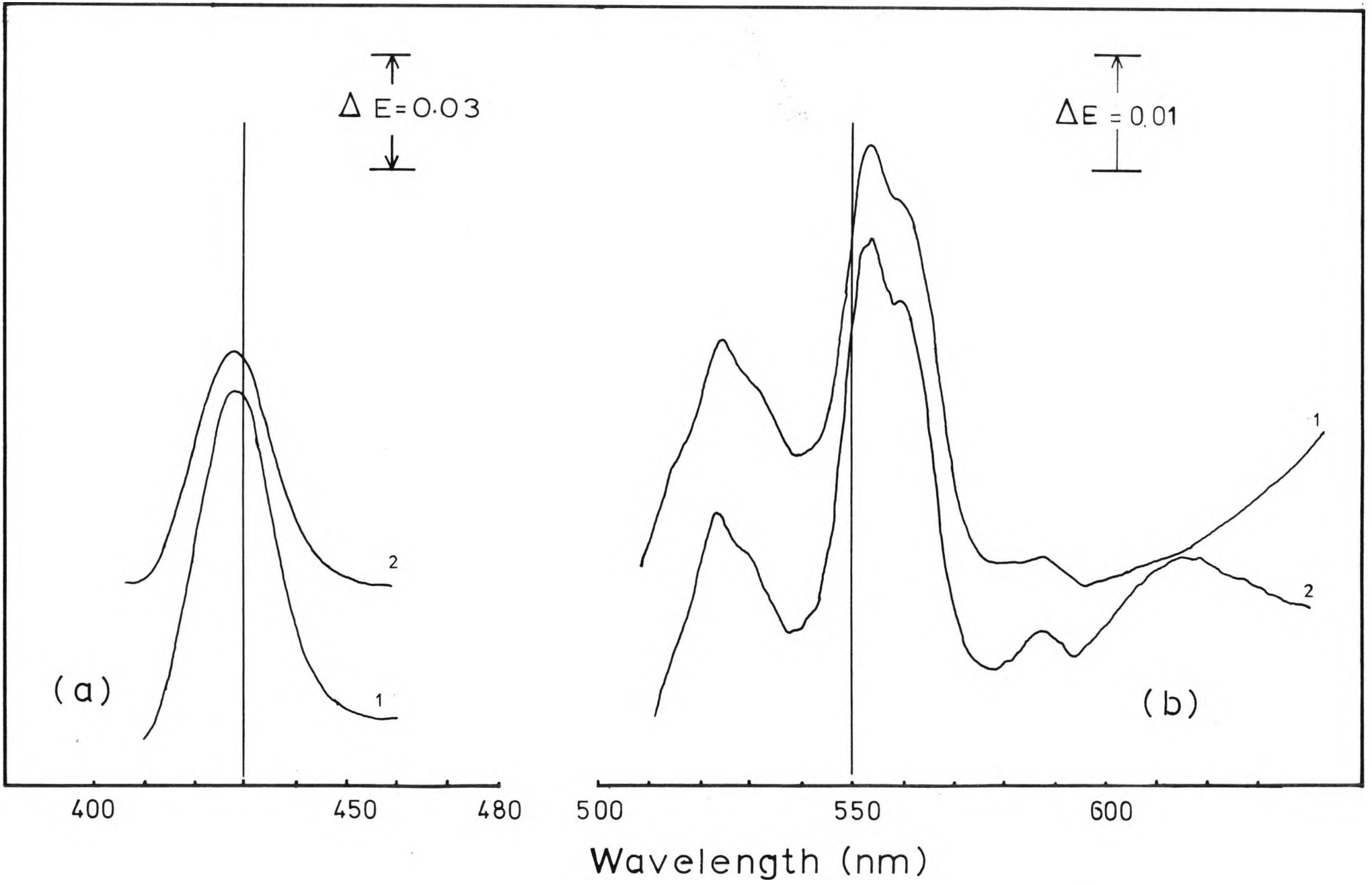


Fig.Ⅲ-12

Fig. III-13. Room temperature reduced plus CO minus reduced difference spectra of whole cells taken from glucose and glycerol limited chemostat cultures of Beneckea natriegens.

(1) Glucose limited culture; (2) glycerol limited culture. Bacterial concentrations as in Fig. 12.

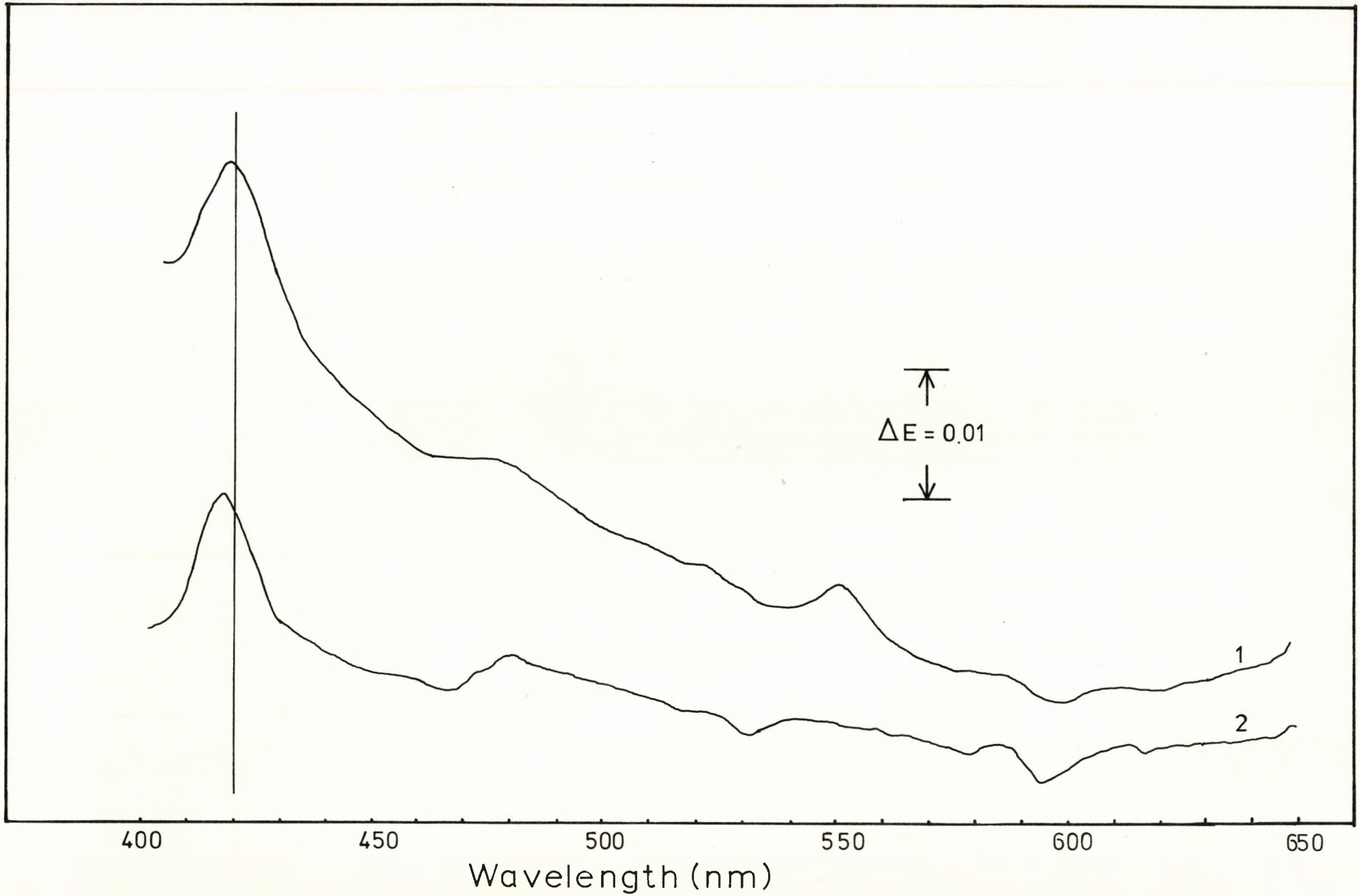


Fig.Ⅲ-13.

4. Discussion

A linear relationship between bacterial biomass and substrate concentration was first described by Monod (1942) who observed that in the presence of limiting amounts of energy source the amount of microbial growth was proportional to the amount of carbohydrate added. When the total biomass bears a linear relationship to the concentration of a particular nutrient, that nutrient may be assumed to be the sole growth limiting substrate. In the present experiments glucose was the sole limiting nutrient for Beneckea natriegens over a concentration range of 0.5 to 4.0 gl^{-1} at a dilution rate of 0.34 h^{-1}.

Growth yields from glucose (in the presence and absence of citrate) and glycerol

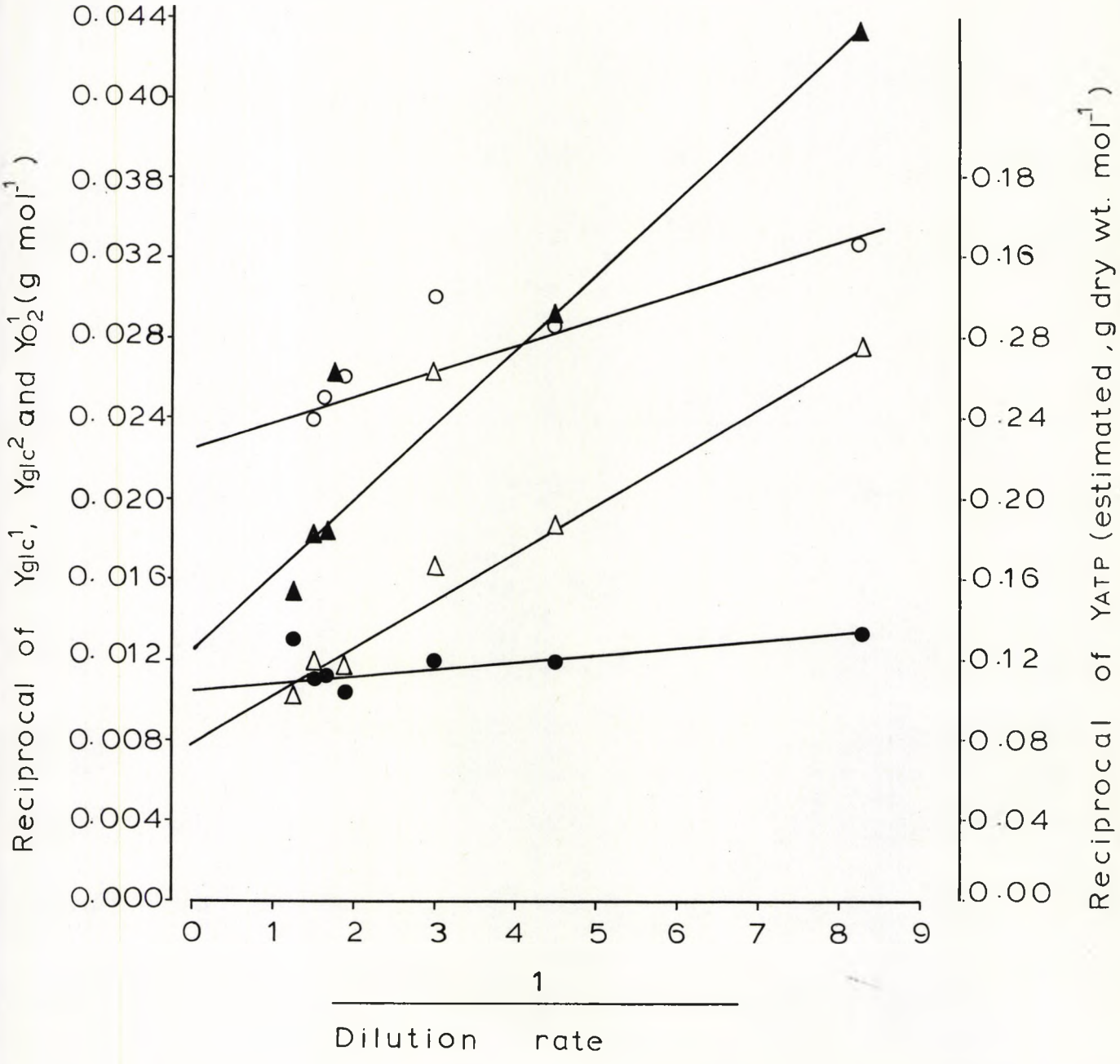
At a fixed dilution rate (0.37 h^{-1}) the $Y_{glc}1$ and $Y_{glc}2$ were independent of the glucose concentration (0.5 to 4.0 gl^{-1}). However, the yield of B. natriegens increased as the growth rate was raised. This type of response is commonly observed under carbon limitation, for example by Tempest and Hunter (1965) for Aerobacter aerogenes, by Palumbo and Witter (1969) and Mannett and Nakayama (1971) for Pseudomonas fluorescens. This response has been explained in terms of an energy requirement for maintenance purposes. Pirt (1965) derived an equation to calculate the maintenance energy from the molar growth yields of organisms growing at various specific growth rates and introduced the term maintenance coefficient (M) (the substrate, g, required for energy of maintenance per g bacterial dry weight per h). Assuming that the maintenance coefficient remains constant with dilution rate, the relative amount of energy needed for maintenance at higher growth rates

will be smaller than at lower growth rates. Therefore before the molar growth yield of B. natriegens (on a given substrate) can be compared with that obtained with other organisms a correction for the maintenance energy requirement should be made. This is achieved by making a double reciprocal plot of observed yield values against D (Pirt, 1965). This type of plot for growth in the absence of citrate gave a straight line for $Y_{glc}1$ and $Y_{glc}2$ (Fig. III-14). The intercept at the ordinate is the reciprocal of Y^{max}_{glc}, the growth yield corrected for energy of maintenance or "true growth yield" (Pirt, 1965). The $Y^{max}_{glc}1$ was found to be 96 g dry weight per mole glucose. To obtain the maintenance glucose requirement a double reciprocal plot of g dry weight per g glucose against D was constructed. The slope of the resultant straight line was the maintenance coefficient for growth (Pirt, 1965). A value of 0.094 g glucose per g dry weight per h was obtained, which is similar to that quoted by Pirt (1965) for Aerobacter cloacae.

The molar growth yield obtained in the presence of citrate, although slightly higher, gave a similar pattern of response. A plot of $1/Y_{glc}2$ against 1/D gave a straight line (Fig. III-15). The Y^{max}_{glc2} was found to be 46.5 g bacterial carbon per mol glucose carbon compared with a value of 41.0 g/mol $^{-1}$ for bacteria grown in the absence of citrate, Fig. III-14. No citric acid was detected by gas liquid chromatography (see Methods). Assuming a 50% conversion of citrate to biomass on a carbon to carbon basis (estimate from yield figures given by Hadjipetrov, Gerrits, Teulings and Stouthamer (1964), the bacterial molar growth yield would be expected to increase by approximately 1.6 g bacterial carbon per mole glucose carbon in the presence of 0.147 gl^{-1} trisodium citrate. As the average

Fig. III-14. A double reciprocal plot of experimental values obtained for $Y_{glc}1$, $Y_{glc}2$, Y_{O_2}, and Y_{ATP} (estimated) against D, in unchelated medium.

$Y_{glc}1$, ● - ●; $Y_{glc}2$, o - o; Y_{O_2}, ▲-▲; and Y_{ATP} Δ-Δ.


0.044
0.040
0.038
0.032
0.028
0.024
0.020
0.016
0.012
0.008
0.004
0.000
Reciprocal of Y_{glc}^{1}, Y_{glc}^{2} and $Y_{O_2}^{1}$ (g mol^{-1})
0.18
0.16
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
Reciprocal of Y_{ATP} (estimated, g dry wt. mol^{-1})
0 1 2 3 4 5 6 7 8 9
1
Dilution rate

ig. III - 1 4

increase of the molar growth yield in the presence of citrate was approximately 4 g bacterial carbon per mol glucose, citrate appeared to enhance the molar growth yield. This may have been due to the citrate concentration itself or to the increased availability of the ions chelated by citrate.

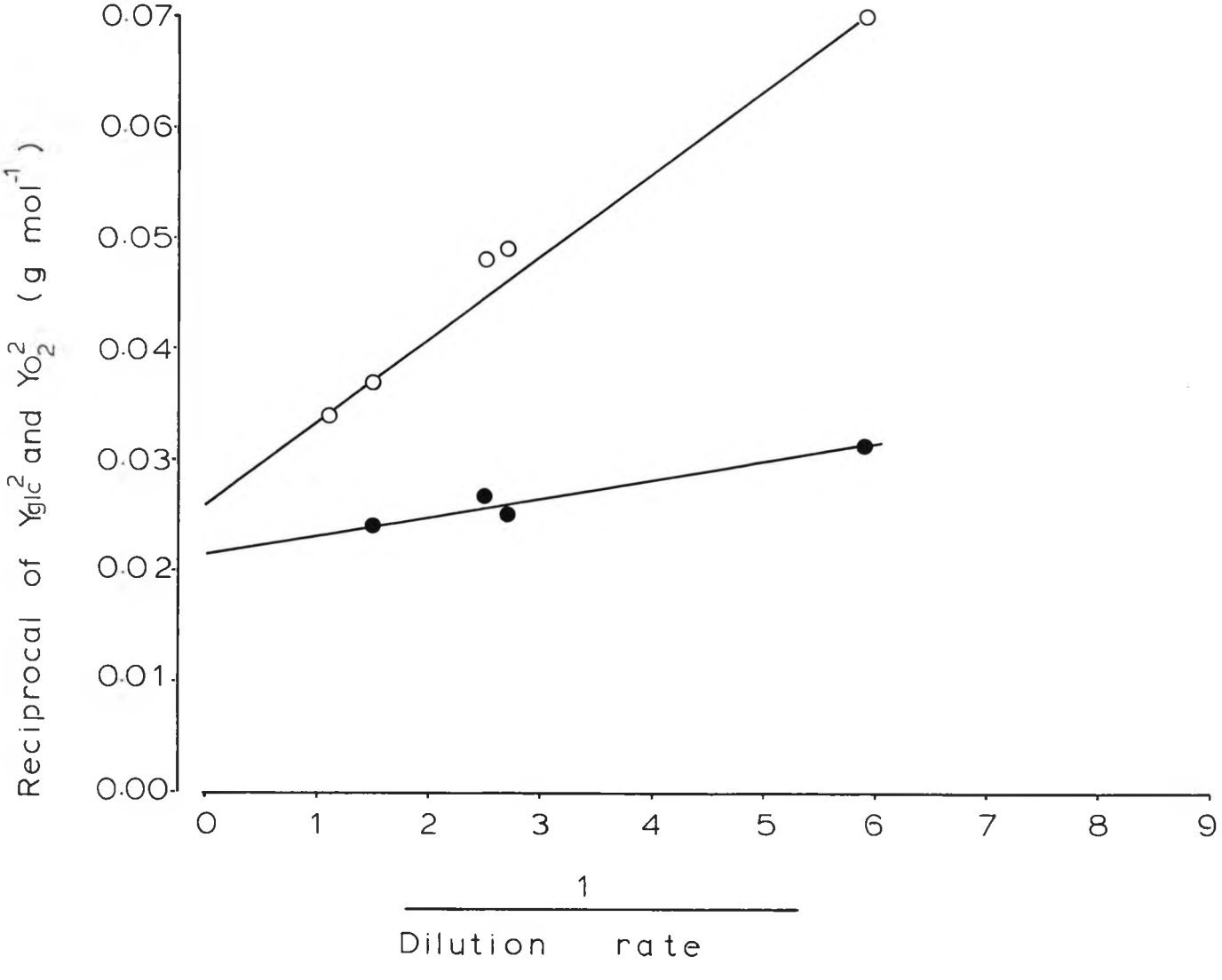
The molar growth yield from glycerol at a dilution rate of 0.37 h^{-1} was approximately 50% of that obtained for glucose at the same dilution rate. However, if a correction is made and the yield expressed on a carbon to carbon basis then the yield on glycerol is 0.56 g bacterial carbon per g glycerol carbon and that on glucose 0.53 g bacterial carbon per g glucose carbon at a dilution rate of 0.37 h^{-1}. Similarly, a marginally higher yield on glycerol was obtained for K. aerogenes grown in carbon limited chemostats (Neijssel and Tempest, 1975a), values of 0.56 and 0.54 g bacterial carbon per g glycerol and glucose carbon respectively were calculated from the data given by these authors.

The amount of Mg^{2+} utilised by B. natriegens (per g dry wt) increased with growth rate as reported for Klebsiella aerogenes by Tempest, Hunter and Sykes (1965). In this study no allowance was made for loosely bound Mg^{2+}. The increase in the Mg^{2+} 'content' of B. natriegens was accompanied by an increase in the P content, the nitrogen content, however, remained fairly constant. The results given in this section on the inorganic ion requirements of B. natriegens are not detailed enough to warrant a comparison with the extensive data published on this subject.

The affinity of B. natriegens for glucose was independent of growth rate, being 29 $\pm$ 4 μ molar a value similar to that reported for Klebsiella aerogenes grown in chemostat culture (Neijssel and Tempest, 1975a).

Fig. III-15. A double reciprocal plot of experimental Y_{O_2} and $Y_{glc}{}^2$ values against D, in citrate chelated medium.

$Y_{glc}{}^2$, ● - ● and Y_{O_2}, o - o.


0·07
0·06
0·05
0·04
0·03
0·02
0·01
0·00
Reciprocal of Y_{glc}^2 and $Y_{O_2}^2$ (g mol^{-1})
0
1
2
3
4
5
6
7
8
9
1
Dilution rate

ig. III - 15

The production of acetic acid and the increase in the $q_{ac}2$ above a dilution rate of 0.5 h^{-1} suggest a change in the metabolism of this organism. Although there was no effect on the yield at a dilution rate of 0.64 the yield dropped markedly at a dilution rate of 0.79 h^{-1} when the $q_{ac}2$ was at its maximum value. A similar response has been observed for Klebsiella aerogenes grown under glucose limitation (Harrison and Pirt, 1967; Stouthamer and Bettenhaussen, 1975). This response may be caused by some other nutrient or factor becoming growth limiting at growth rates near μ max (possible Mg or Fe).

As the culture was carbon limited over the range 0.5 to 4.0 gl^{-1} glucose the significance of the slight fall in the q_{O_2} with increase in the glucose concentration, is not understood. The linear relationship observed between the q_{O_2} and q_{CO_2} and dilution rate has been reported for a number of organisms. For example in K. aerogenes by Herbert (1958) and for Torula utilis by Tempest and Herbert (1965) and for K. aerogenes by Harrison and Loveless (1971a). At growth rates near μ max the q_{O_2} and q_{CO_2} did not markedly deviate from linearity as observed in K. aerogenes by Harrison and Loveless (1971a) where the q_{O_2} increased dramatically near μ max and by Stouthamer and Bettenhaussen (1975) who observed the opposite effect with the same organism (ie. a fall in the q_{O_2} at growth rates near μ max).

The fall in the total content of cytochromes with increase in the growth rate has been reported for other organisms (Rosenberger and Kogut, 1958). There appeared to be no correlation between the total cytochrome content and the potential respiration rate, in agreement with studies on K. aerogenes by Harrison,

MacLennon and Pirt (1969). However, there was some correlation between the total CO binding pigment and the potential respiration rate, both remaining fairly constant regardless of growth rate. This may suggest that the potential respiration rate could be limited by the quantity of terminal oxidase in this organism.

The very high growth rates observed in batch culture (doubling time 0.5 h) could not be attained in continuous culture. The maximum growth rate attained in medium without citric acid ($D = 0.84\ h^{-1}$) was probably limited by the availability of Mg^{2+} and/or Fe. The presence of trisodium citrate appeared to increase the μ max. This was probably a consequence of the increased availability of chelated ions. It has been proposed (Spencer, 1957) that cations in a chelated form are readily available as the adjustment of the equilibrium subsequent to the removal of iron from such a system is instantaneous in contrast to the time required for solution from a solid phase. Thus, although the medium contained 0.00975 g $Mg^{2+}\ l^{-1}$, and the Mg^{2+} requirement at a dilution rate of $0.79\ h^{-1}$ was 0.0039 g Mg^{2+}, the rate at which the Mg^{2+} re-dissolved from the precipitate probably was the limiting factor in the absence of citrate. The medium containing citrate had no visible precipitate but it was found that Millipore filters became clogged when large volumes (500 l) of this medium were sterilised by filtration. This suggests that the medium contained a fine precipitate that remains in suspension even in the presence of citrate. This fact may explain why the growth rate achieved in batch culture could not be attained in a chemostat, the culture becoming limited by some other nutrient.

The increase of the Y_{O_2} with increase in the growth rate has been reported by a number of workers, eg. by Harrison and Loveless (1971a) and by Abbott and Clamen (1973), and is explained in terms of the maintenance energy requirement. The influence on yield of the maintenance energy requirement is greatest at the lowest growth rates (Abbott and Clamen, 1973). A double reciprocal plot of $Y_{O_2}1$ in the absence of citrate and $Y_{O_2}2$ in the presence of citrate against D gave straight lines (Fig. III-14 and Fig. III-15 respectively). The $Y^{max}_{O_21}$ and $Y^{max}_{O_22}$ were found to be 80.5 g bacterial dry weight per mol O_2 and 38.4 g bacterial carbon per mol O_2 for B. natriegens grown in unchelated and chelated medium G respectively.

The calculated Y_{ATP} values (not corrected for maintenance) were dependent on the growth rate in a manner similar to that reported by Stouthamer and Bettenhaussen (1973). A double reciprocal plot of Y_{ATP} against D gave a straight line (Fig. III-14). The Y^{max}_{ATP} was found to be 13.15 g bacterial dry weight per mol ATP, which corresponds to an ATP requirement of 76.0 mmol per g bacterial dry weight. These values are similar to those reported for K. aerogenes by Stouthamer and Bettenhaussen (1975) where the Y^{max}_{ATP} and ATP requirement were found to be 14 g bacterial dry weight per mole ATP and 71.4 mmol per g bacterial dry weight respectively. However, the interpretation of this data is difficult as two assumptions were made:-

1) the organism had three sites of oxidative phosphorylation;

2) the maintenance energy requirement is constant.

The former cannot be measured directly and there is increasing evidence indicating that the latter (maintenance energy) varies with the condition of growth (Neijssel and Tempest, 1975b).

SECTION IV

RESPONSE OF *BENECKEA NATRIEGENS* TO CHANGES IN MEDIUM DISSOLVED OXYGEN TENSION

1. Molar Growth Yields, Respiration and Cytochrome Patterns at Different Medium Dissolved Oxygen Tensions
2. A Comparison of the Cyanide Sensitivity of Whole and Fractionated Bacteria Harvested from Oxygen Excess and Limited Chemostat Cultures
3. Discussion

RESPONSE OF *BENECKEA NATRIEGENS* TO CHANGES IN MEDIUM DISSOLVED OXYGEN TENSION

Beneckea natriegens has been reported to possess a branched electron transport system. The individual branches are identified by their differing Km for oxygen and sensitivity to cyanide. It has been suggested (Mayer and Jones, 1973a, b) that multiple oxidases found in bacteria function at different medium dissolved oxygen tensions and are associated with different efficiences of phosphorylation. In order to assess whether branching of the electron transport chain occurred under physiological conditions, *B. natriegens* was grown at various medium dissolved oxygen tensions in a glucose-limited chemostat (unchelated medium G) and the effect on growth, respiration and cytochrome patterns monitored. The dissolved oxygen tension was varied by changing the ratio of air to nitrogen in the inlet gas while keeping the total flow rate constant.

A dilution rate of 0.37 h^{-1} was chosen for this aspect of the work because it represented approximately 50% of the maximum specific growth rate. The qO_2 and qCO_2 have been shown to increase linearly with growth rate and metabolism to be unaffected between growth rates of 0.1 and 0.5 h^{-1}. Therefore, changes in the growth rate (between 0.1 and 0.5 h^{-1}) in this organism would be expected to have little or no effect on the value of the critical dissolved oxygen tension or on the pattern of response of the culture to dissolved oxygen tension.

1. Molar Growth Yields, Respiration and Cytochrome Patterns at Different Medium Dissolved Oxygen Tensions

The steady state values of molar growth yield from glucose (g bacterial dry weight per mol, Y_{glc}^{1} and g bacterial carbon per mol glucose carbon Y_{glc}^{2}) appeared to be independent of medium dissolved oxygen tension over the range 134 to <2 mmHg (partial pressure of oxygen in the gas phase 159-16 mmHg) (Fig. IV-1). However, reduction of the partial pressure of oxygen in the gas phase to 8 mmHg caused a marked fall in the molar growth yield from glucose from 92 ± 4 to 60 g bacterial dry weight per mol. At values of partial pressure of oxygen in the gas phase below 8 mmHg the output from the dissolved oxygen probe was virtually zero, the dissolved oxygen tension being below the lower limit of sensitivity of the probe (<2mmHg). Further reduction of the gaseous oxygen partial pressure to <0.5 mmHg (achieved by gassing with white spot nitrogen) caused a dramatic fall in the growth yield. Based on culture absorbance and bacterial carbon values the yield was approximately 5% of that observed under the excess oxygen phase (Fig. IV-1).

Dissolved oxygen tension

Reduction of the partial pressure of oxygen in the gas phase to 8 mmHg caused no detectable effect on medium dissolved oxygen as this was already below the lower limit of sensitivity of the probe, but the q_{O_2} of the culture decreased. The medium dissolved oxygen tension was found to be dependent on the partial pressure of oxygen in the effluent gas phase.

Fig. IV-1. Steady state values of bacterial molar growth yield from glucose and oxygen, culture absorbance and medium dissolved oxygen tension as a function of the partial pressure of oxygen in the gas phase of a carbon limited chemostat culture of Beneckea natriegens.

Medium dissolved oxygen tension mmHg; (o - o); molar growth yield from glucose, $Y_{glc}1$; (g bacterial dry weight per mol glucose, ■ - ■); and $Y_{glc}2$;(g bacterial carbon per mol glucose carbon, ● - ●); molar growth yield from oxygen $Y_{O_2}1$; (g bacterial dry weight per mol oxygen, □-□); culture absorbance at 625 nm (Δ-Δ).

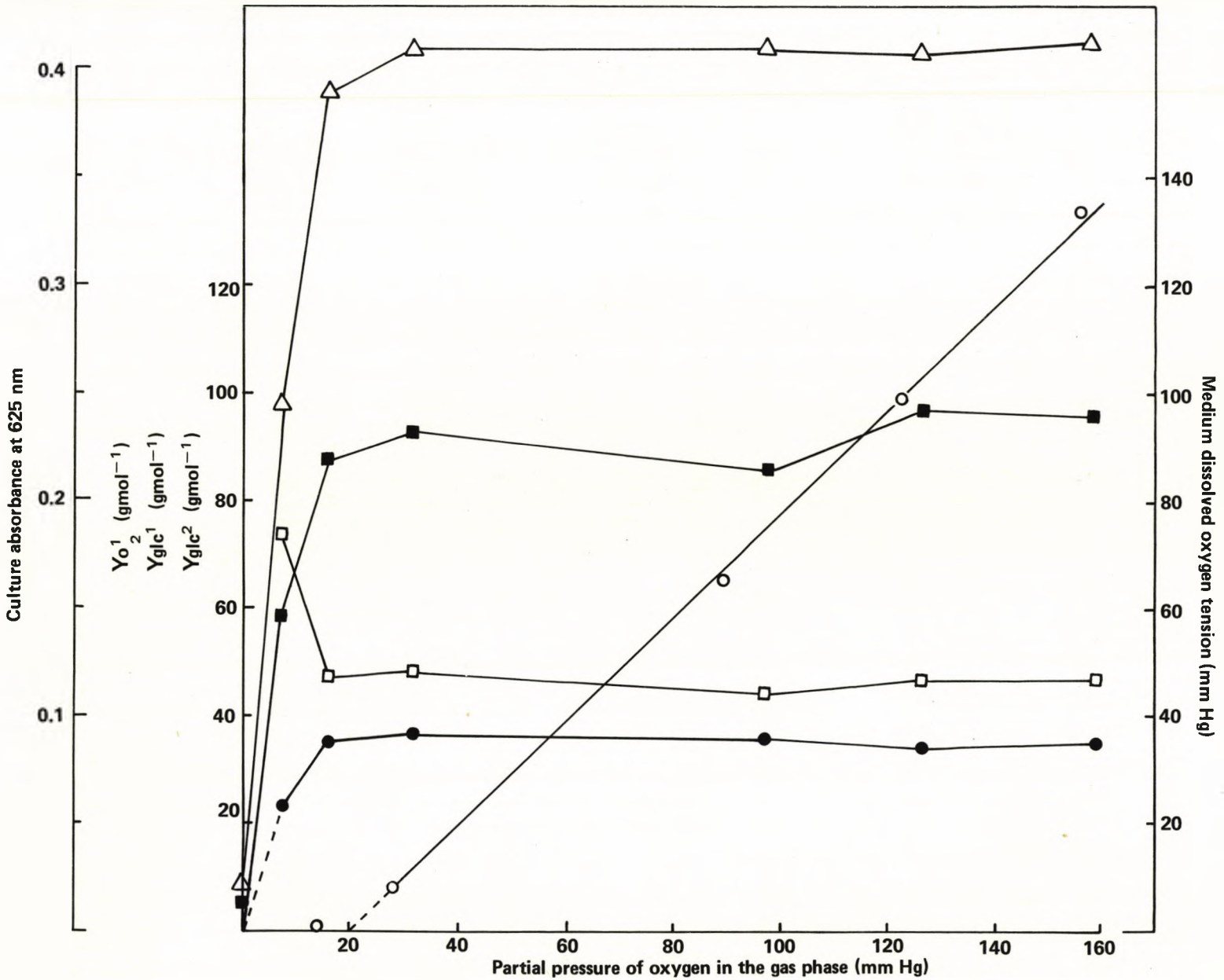


Fig IV – 1

The relationship between oxygen uptake, the partial pressure of oxygen in the gas phase and the medium dissolved oxygen tension is given by the equation:

$$N = K_L A\ (T_g - T_L) \qquad (10)$$

where N = oxygen uptake rate of culture (gh^{-1})

$K_L A$ = oxygen transfer coefficient ($gh^{-1}\ mmHg^{-1}$)

T_g = oxygen partial pressure in the gas phase (mmHg)

T_L = medium dissolved oxygen tension (mmHg)

As the oxygen uptake rate and the $K_L A$ value of the culture is constant the medium dissolved oxygen tension varies linearly with partial pressure of oxygen in the effluent gas phase (Harrison, 1973).

Glucose utilisation

The specific rate of glucose consumption $q_{glc}2$ was fairly constant over the range of medium dissolved oxygen tension 134 to <2 mmHg (gaseous partial pressure of oxygen 156-16 mmHg) but there appeared to be an approximate 50% increase in the $q_{glc}2$ when the gaseous partial pressure of oxygen was reduced to 8 mmHg (medium dissolved oxygen tension $\ll 2$ mmHg) (Fig. IV-2).

Nitrogen and phosphorus utilisation

Nitrogen and phosphorus utilisation by the culture was monitored and found to be independent of the dissolved oxygen tension between 134 and <2 mmHg (Fig. IV-2). Under anaerobic conditions the bacterial mass and the amount of nitrogen and phosphorus utilised could not be measured accurately.

Fig. IV-2. Steady state values of glucose, nitrogen and phosphorus consumption as a function of the partial pressure of oxygen in the gas phase of a carbon limited chemostat culture of Beneckea natriegens.

Specific rate of glucose consumption, q_{glc} (mmol $g^{-1}h^{-1}$, o - o); g nitrogen utilised per g bacterial dry weight, ● - ●; and g phosphate utilised per g bacterial dry weight, □ - □.

g Phosphorus utilised per g Bacterial dry weight
g Nitrogen utilised per g Bacterial dry weight
0.14
0.12
0.10
0.08
0.06
0.04
0.02
q_{glc} mmol$g^{-1}h^{-1}$
7
6
5
4
3
2
1
20
40
60
80
100
120
140
160
Partial pressure of oxygen in the gas phase (mm Hg)

Fig IV – 2

Specific rate of oxygen consumption (q_{O_2}) and CO_2 production (q_{CO_2})

The specific rate of oxygen consumption ($q_{O_2}1$) and of carbon dioxide production ($q_{CO_2}1$) (Fig. IV-3) were independent of medium dissolved oxygen tension over the range 134 to 8 mmHg. When the gaseous partial pressure of oxygen was reduced to 16 mmHg, the medium dissolved oxygen tension reading fell to zero (ie. <2 mmHg) but there was no effect on q_{O_2} and q_{CO_2}.

The values of $Y_{O_2}1$, obtained as a function of the gaseous partial pressure of oxygen, are shown in Fig. IV-1. The $Y_{O_2}1$ was independent of the partial pressure of oxygen in the gas phase over the range 159 to 16 mmHg. However, reduction of the gaseous partial pressure of oxygen to 8 mmHg caused a 37% increase in the $Y_{O_2}1$ value. The production of acetic acid under oxygen limitation was probably accompanied by ATP production via the phosphoroclastic cleavage reaction. This would account for the apparent increase in the Y_{O_2}.

The potential $q_{O_2}1$ was found to be higher than the *in situ* $q_{O_2}1$ at all oxygen tensions examined (Fig. IV-3) and increased as the partial pressure of oxygen was reduced. At dissolved oxygen tensions between 134 and 96 mmHg the potential $q_{O_2}1$ was approximately 10 mmol O_2 per g bacterial dry weight per h. When the medium dissolved oxygen tension was reduced to 65 mmHg, there was an increase in the potential respiration rate to 14 mmol $g^{-1}h^{-1}$ with no marked increase in the *in situ* $q_{O_2}1$. Lowering the medium dissolved oxygen tension to $\ll 2$ mmHg (gaseous partial pressure of oxygen 16 mmHg) caused a small increase in the potential respiration rate but further reduction of the partial pressure of oxygen in the gas phase to 8 mmHg caused a dramatic increase in

Fig. IV-3. Steady state values of the specific rates of oxygen consumption, CO_2 production, potential respiration rate and medium dissolved oxygen tension as functions of the partial pressure of oxygen in the gas phase of a carbon limited chemostat culture of Beneckea natriegens.

Medium dissolved oxygen tension mmHg, (o - o); specific rate of CO_2 production q_{CO_2} (mmol $g^{-1}h^{-1}$, • - •); specific rate of O_2 consumption, q_{O_2} (mmol $g^{-1}h^{-1}$, □ - □) and potential respiration rate (mmol $g^{-1}h^{-1}$, ■ - ■).

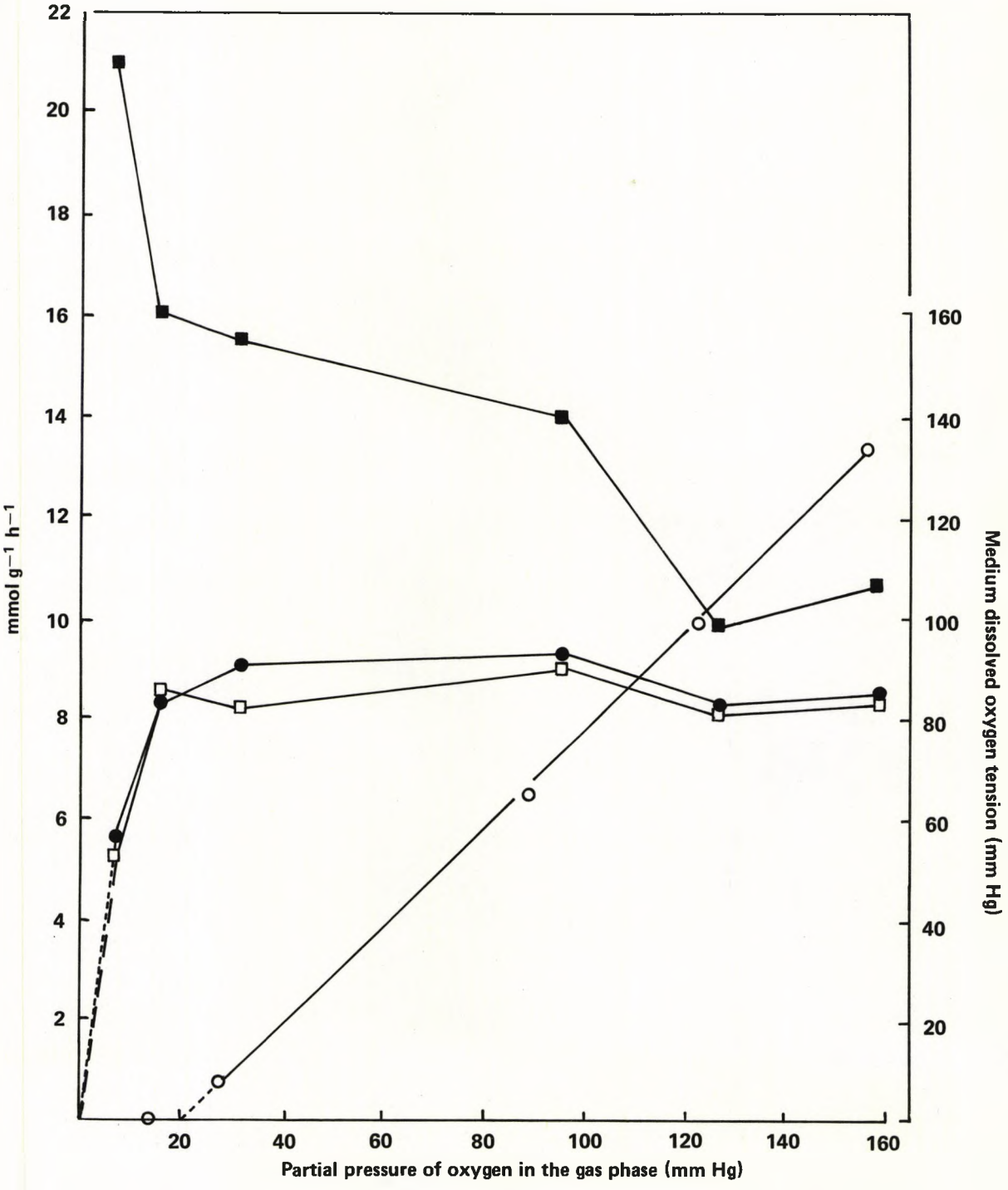

22
20
18
16
14
12
10
8
6
4
2
mmol g⁻¹ h⁻¹
160
140
120
100
80
60
40
20
Medium dissolved oxygen tension (mm Hg)
20
40
60
80
100
120
140
160
Partial pressure of oxygen in the gas phase (mm Hg)

Fig IV – 3

the potential respiration rate to 21 mmol $g^{-1}h^{-1}$, while the *in situ* q_{O_2} fell to 5.3 mmol $g^{-1}h^{-1}$.

Metabolic products

The formation of extracellular metabolites from glucose, as a function of the partial pressure of oxygen in the gas phase, is shown in Fig. IV-4. Acetic acid and ethanol were the two main products identified by gas liquid chromatography and their concentrations remained low and independent of changes in oxygen partial pressure until the medium dissolved oxygen tension was <2 mmHg, at which point a slight increase in the concentration of these compounds occurred. Both these metabolic products reached a maximum concentration when the partial pressure of oxygen in the gas phase was 8 mmHg. Pyruvic acid was found to be present at extremely low concentrations reaching a maximum of 3 mg l^{-1} when white spot nitrogen was passed through the fermenter. Only 10% of the carbon present in culture filtrates was identified. A carbon balance was constructed and the results obtained indicated a satisfactory carbon recovery (Table IV-1). A switch in metabolism was evident from the increase in the carbon in the culture filtrate when the gaseous partial pressure of oxygen was reduced to 8 mmHg (Fig. IV-4).

Cytochrome spectra

The results of oxidised minus reduced difference spectra indicated a qualitative change in the cytochrome contents of *Beneckea natriegens* over a medium dissolved oxygen tension range that caused relatively little change in the

Fig. IV-4. Steady state values of glucose, ethanol, acetic acid, culture supernatant carbon and medium dissolved oxygen tension as a function of the partial pressure of oxygen in the gas phase of a carbon limited chemostat culture of Beneckea natriegens.

Glucose (▲-▲, carbon gl^{-1}); ethanol (● - ●, carbon gl^{-1}); acetic acid (Δ- Δ, carbon gl^{-1}); culture supernatant carbon (o - o, gl^{-1}); and medium dissolved oxygen tension (□ - □, mmHg).

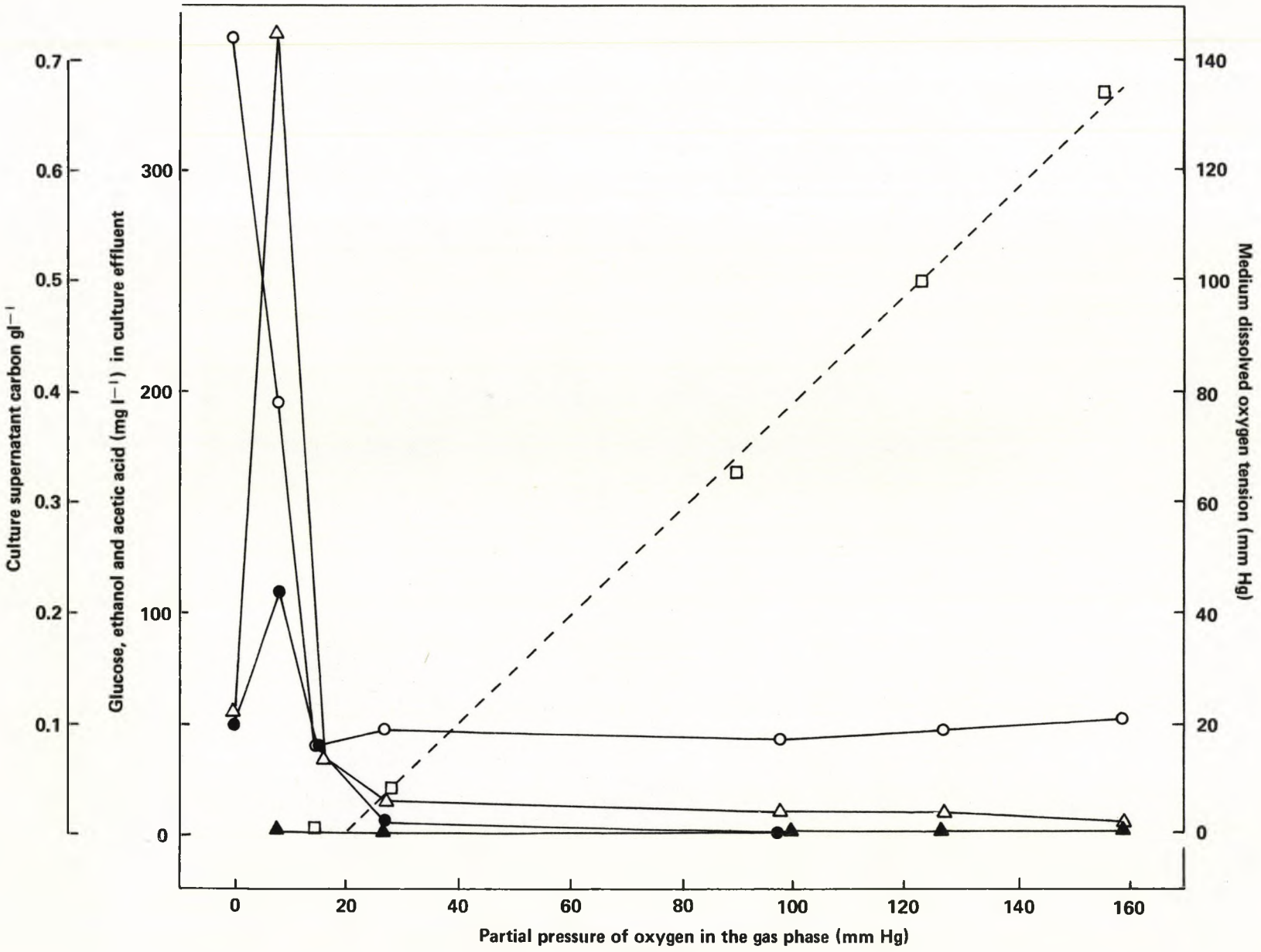

Culture supernatant carbon gl⁻¹
Glucose, ethanol and acetic acid (mg l⁻¹) in culture effluent
Medium dissolved oxygen tension (mm Hg)
Partial pressure of oxygen in the gas phase (mm Hg)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
300
200
100
0
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160

Fig Ⅳ − 4

Table IV-1. A carbon balance for the growth of Beneckea natriegens in chemostat culture. The values given represent averages obtained on analysis of duplicate samples taken from the chemostat at steady state at various medium dissolved oxygen tensions

Growth conditions			Carbon output (gh^{-1})			
Partial pressure of oxygen in gas supply to fermenter (mmHg)	Dissolved oxygen tension in fermenter (mmHg)	Carbon input (gh^{-1})	= Cell carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ Carbon dioxide carbon (gh^{-1})	Carbon recovery (%)
159.6	134	0.699	0.364	0.089	0.250	100.5
		0.699	0.323	0.100	0.243	95.2
127	96	0.690	0.341	0.082	0.237	95.6
		0.690	0.323	0.091	0.237	94.3
95	65	0.706	0.360	0.077	0.245	96.5
		0.692	0.346	0.077	0.245	96.5
32	8	0.706	0.353	0.084	0.259	98.5
		0.706	0.381	0.093	0.251	102.6
16	<2.0	0.706	0.344	0.074	0.224	91.0
		0.706	0.353	0.074	0.224	92.2
8	$\ll 1$	0.713	0.223	0.362	0.118	98.5
		0.718	0.251	0.353	0.118	100.5
0	0	0.798	0.060	0.738	-	100.0
		0.780	0.051	0.729	-	100.0

Fig. IV-5. Room temperature oxidised-minus-reduced difference spectra of whole organisms taken from a chemostat under steady state conditions at various medium dissolved oxygen tensions (a) oxidised H_2O_2-reduced $Na_2S_2O_4$ (b) oxidised $K_3Fe(CN)_6$-reduced $Na_2S_2O_4$.

(1) Medium dissolved oxygen tension 134 mm Hg, (bacterial dry weight 10.26 mg ml^{-1})

(2) Dissolved oxygen tension 96 mmHg, (bacterial dry weight 10.30 mg ml^{-1})

(3) Dissolved oxygen tension 65 mmHg, (bacterial dry weight 10.26 mg ml^{-1})

(4) Dissolved oxygen tension 8 mmHg, (bacterial dry weight 9.84 mg ml^{-1})

(5) Dissolved oxygen tension <1 mmHg, (bacterial dry weight 9.40 mg ml^{-1})

(6) Dissolved oxygen tension $\ll 1$ mmHg, (bacterial dry weight 6.33 mg ml^{-1})

(7) Dissolved oxygen tension $\ll 0.5$ mmHg

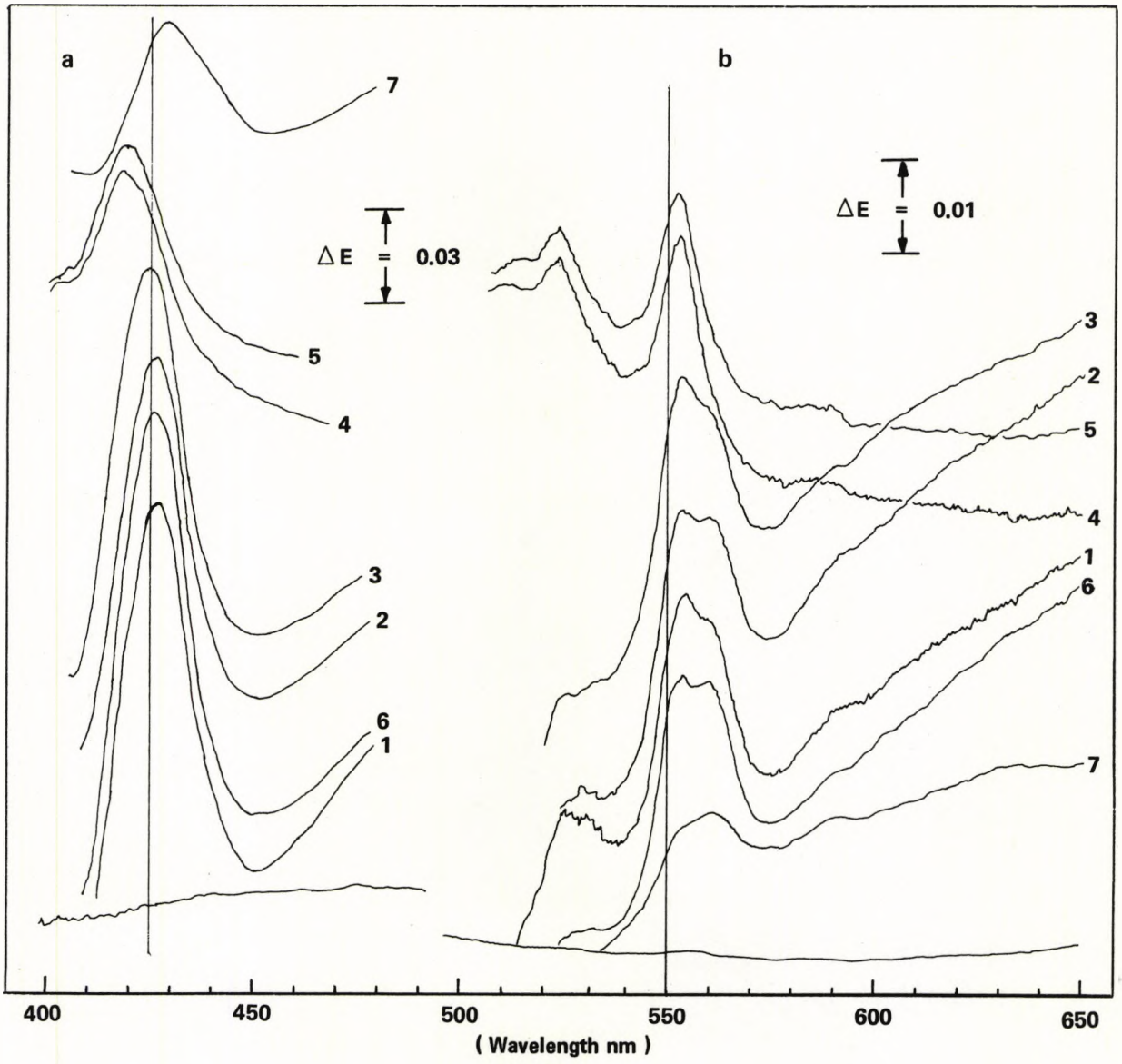


Fig IV – 5

in situ q_{O_2}, q_{CO_2}, Y_{glc} or Y_{O_2}. At dissolved oxygen tensions between 134 and 96 mmHg, peak absorbances at 560, 553 and 426.5 nm were observed indicating the presence of b and c-type cytochromes (Fig. IV-5). The position of the Soret peak at 426 nm indicated a mixture of cytochrome b and c. The ratio between cytochromes b and c changed as the dissolved oxygen tension was lowered to 65 mmHg. This is reflected in the position of the Soret peak which appeared at 424.0 nm indicating a relative increase in the c-type cytochrome content. Further reduction of the dissolved oxygen tension to 8 mmHg and 2 mmHg (partial pressures of oxygen in the gas phase 31 and 16 mmHg) caused a further shift towards the c-type cytochrome. This is clearly seen in the oxidised minus reduced spectra (Fig. IV-5) as a shift in the Soret peak to 418 nm. Further reduction of gaseous oxygen partial pressure to 8 mmHg, although causing no detectable change in the output of the dissolved oxygen probe, caused a change in the cytochrome content which appeared to revert to the pattern found in the excess oxygen phase, ie. peak absorbances at 560, 553 and 427 nm. Oxidised minus reduced difference spectra of bacteria grown in an atmosphere of nitrogen revealed small peak absorbances at 560, 553 and 428 nm.

The results of reduced-plus-CO minus reduced difference spectroscopy (Fig. IV-6) confirmed the qualitative changes in the cytochrome content observed in the oxidised minus reduced difference spectra. Peak absorbances in the Soret region were at 418 and 417 nm at dissolved oxygen tensions of 134 and 96 mmHg respectively, indicating the presence of mainly cytochrome o. Reduction of the dissolved oxygen tension to 65, 8 and $\leq$2 mmHg caused a shift in the Soret peak

Fig. IV-6. Room temperature reduced-plus-CO-minus-reduced difference spectra of whole cells taken from a chemostat under steady state conditions at various medium dissolved oxygen tensions.

(1) Dissolved oxygen tension 134 mmHg, (bacterial dry weight 10.26 mg ml^{-1})

(2) Dissolved oxygen tension 96 mmHg,(bacterial dry weight 10.30 mg ml^{-1})

(3) Dissolved oxygen tension 65 mmHg, (bacterial dry weight 10.26 mg ml^{-1})

(4) Dissolved oxygen tension 8 mmHg,(bacterial dry weight 9.84 mg ml^{-1})

(5) Dissolved oxygen tension 1 mmHg, (bacterial dry weight 9.40 mg ml^{-1})

(6) Dissolved oxygen tension 1 mmHg, (bacterial dry weight 6.33 mg ml^{-1})

(7) Dissolved oxygen tension 0.5 mmHg

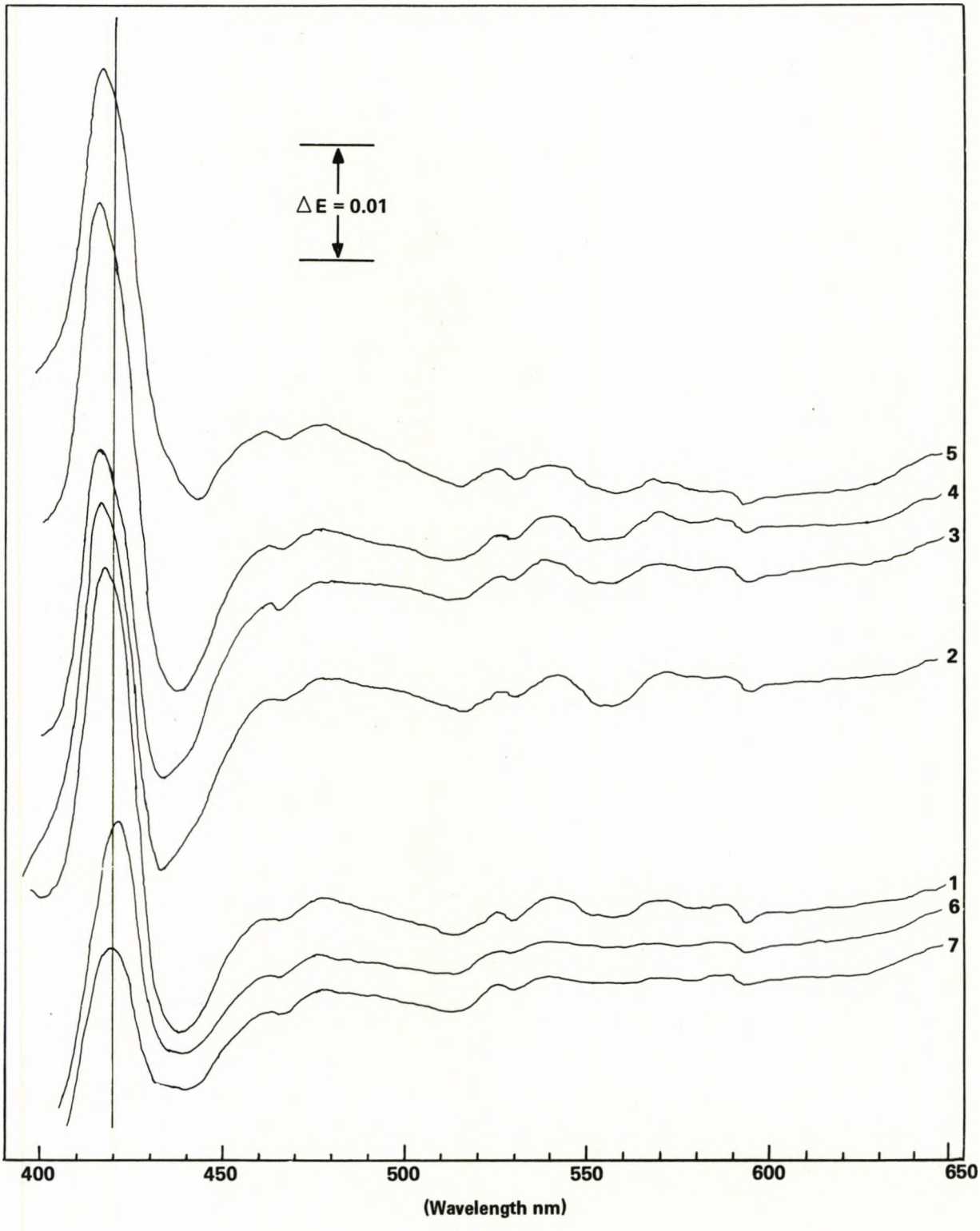


Fig Ⅳ – 6

to 416 nm, indicating a relative increase in the CO-binding cytochrome c. Further reduction in the gaseous partial pressure of oxygen to 8 mmHg, although causing no detectable change in the dissolved oxygen probe output which remained at zero, appeared to cause a change in the CO-liganded peak absorbance from 416 to 422 nm. Reduced-plus-CO minus reduced difference spectra of anaerobically grown bacteria revealed a peak at 420 nm.

Oxidised minus reduced difference spectra of whole bacteria were examined at low temperature (77°K) and the results supported those obtained at room temperature. Furthermore, cytochrome b was detected at dissolved oxygen tensions of 8 and <2 mmHg (as a shoulder at 555 nm on a peak at 551 nm) which was not evident in room temperature spectra.

In order to obtain an estimate of the relative change in the levels of particular cytochromes, the peak heights of room temperature oxidised minus reduced difference spectra were measured and expressed in terms of ΔE per mg bacterial dry weight (Fig. IV-7). The changes in the levels of cytochromes b and c showed some relationship to the three phases observed in the potential respiration rate (see Fig. IV-3). The level of cytochrome c appeared to remain constant over the medium dissolved oxygen tension range 134 to 65 mmHg, whereas a reduction in the dissolved oxygen tension from 65 to ≤ 2 mmHg appeared to cause a slight decline in the level of cytochrome c. At the partial pressure of oxygen in the gas phase that caused a switch in metabolism, the 'critical point', the level of cytochrome c increased indicating an apparent reversal to the original level. Similarly the level of cytochrome b appeared to remain fairly constant over the

Fig. IV-7. Steady state values of cytochromes c and b and the ratio of cytochrome c:cytochrome b in organisms grown at various medium dissolved oxygen tensions.

Cytochrome c (● - ●); ΔE per mg dry weight; cytochrome b (Δ - Δ, ΔE per mg dry weight) and ratio cytochrome c:cytochrome b (o - o).

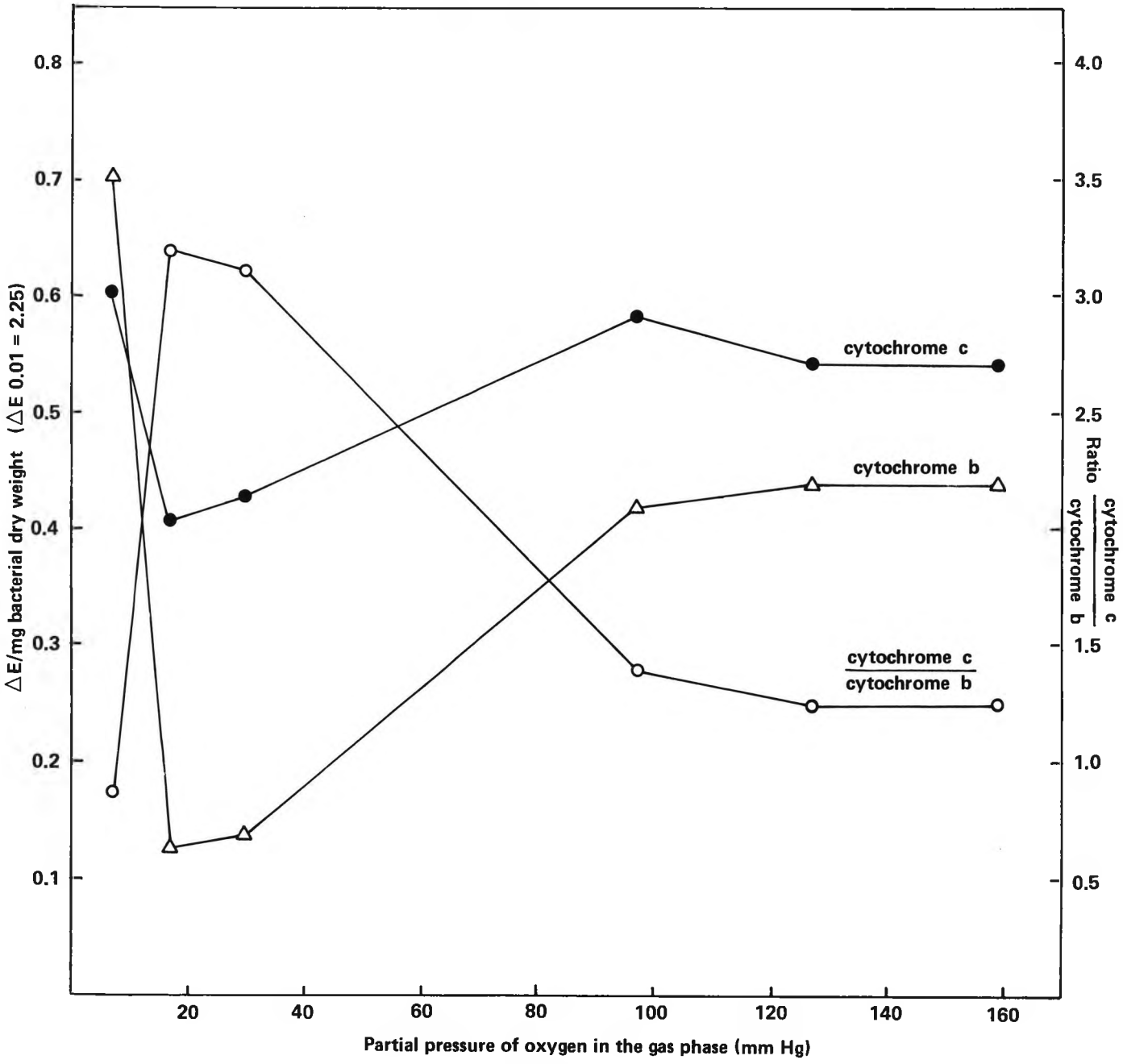

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ΔE/mg bacterial dry weight (ΔE 0.01 = 2.25)
4.0
3.5
3.0
2.5
1.5
1.0
0.5
Ratio cytochrome c / cytochrome b
cytochrome c
cytochrome b
cytochrome c / cytochrome b
20
40
60
80
100
120
140
160
Partial pressure of oxygen in the gas phase (mm Hg)

Fig Ⅳ – 7

Fig. IV-8. The effect of KCN on the potential respiration rate of organisms taken from a chemostat under steady state conditions at various medium dissolved oxygen tensions.

50 μmol KCN (● - ●); 75 μmol KCN (o - o).

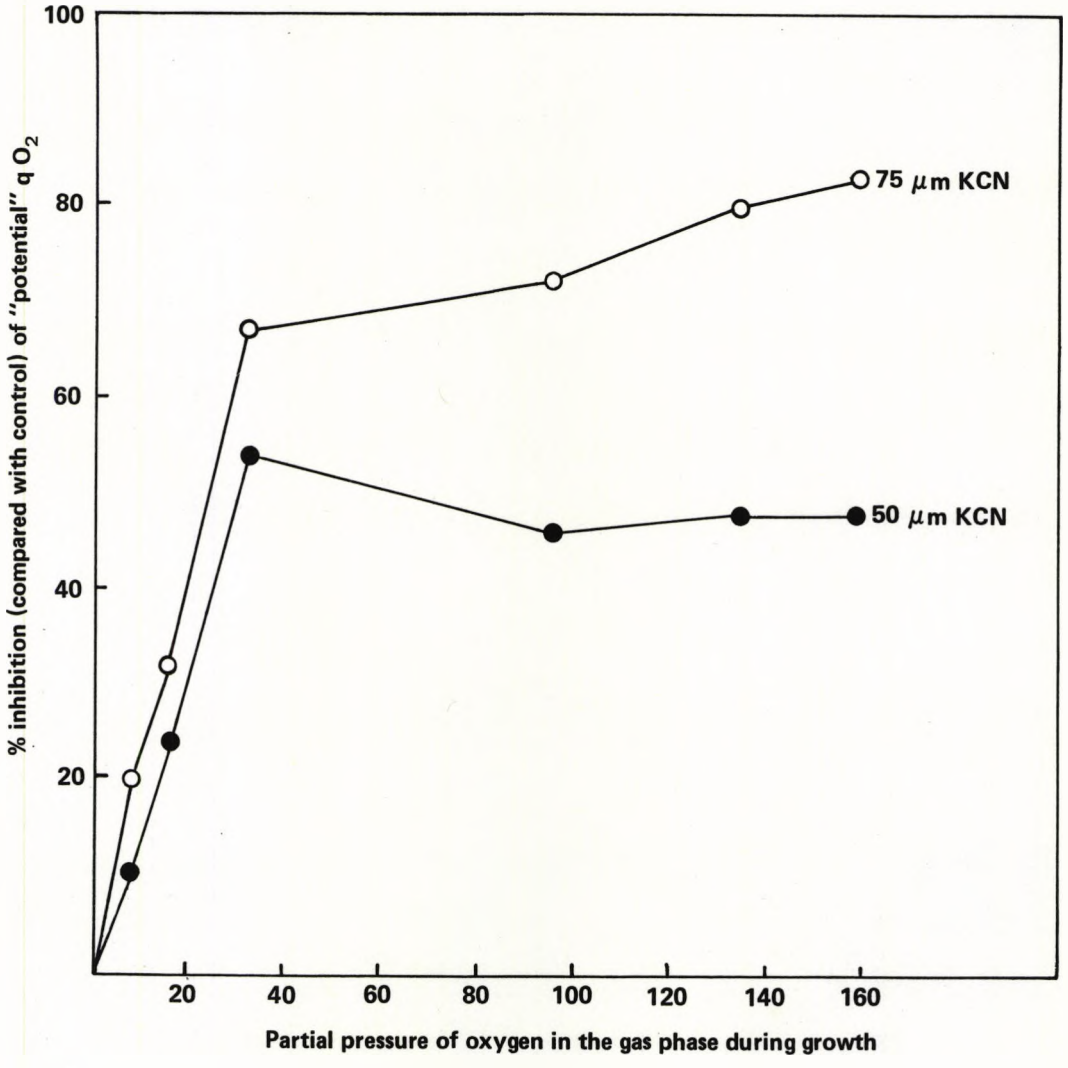

100
80
60
40
20
% inhibition (compared with control) of "potential" q O_2
75 μm KCN
50 μm KCN
20
40
60
80
100
120
140
160
Partial pressure of oxygen in the gas phase during growth

Fig IV – 8

range of dissolved oxygen tension 134 to 65 mmHg and declined sharply when the latter was reduced to 8 and <2 mmHg. Finally, below the critical point, the level of cytochrome b increased dramatically to a value higher than that observed under full aeration. These changes are seen more dramatically when the ratio of cytochrome c/cytochrome b is plotted (Fig. IV-7).

Cyanide sensitivity

The effect of 50 and 75 μM KCN on the respiration rates of bacteria taken from the fermenter at various steady state dissolved oxygen tensions is shown in Fig. IV-8. The cyanide sensitivity of the bacteria was almost independent of the dissolved oxygen tension over the range 134 to 8 mmHg but below a dissolved oxygen tension of 8 mmHg the bacteria exhibited a resistance towards cyanide.

NAD(P)H fluorescence

A carbon limited chemostat culture of B. natriegens was allowed to stabilise under fully aerobic conditions. A basal fluorescence reading was detected using the fluorimeter system (see Methods and Appendix III). The medium dissolved oxygen tension was then reduced from 134 mmHg to <1 mmHg in a stepwise manner by changing the ratio of air to nitrogen in the inlet gas phase while keeping the total gas flow constant. Reducing the dissolved oxygen tension from 134 to 4 mmHg appeared to have no effect on the fluorescence due to reduced pyridine nucleotides. Only when the culture was oxygen limited and the concentration fell below the level of detection of the oxygen electrode was there

any effect on fluorescence. At this point the oxygen demand of the culture exceeded the oxygen supply rate and an increase in fluorescence was detected (Fig. IV-9). This corresponded to the point at which fermentative metabolism was switched on. When white spot N_2 was passed through the fermenter a further increase in fluorescence was recorded. Restoration of the normal air flow rate caused an immediate reduction in fluorescence level to that observed under fully aerobic conditions. The response of the fluorimeter was immediate and had stabilised at a new value before the oxygen electrode had started to respond.

Fig. IV-9. The effect of a stepwise reduction of the medium dissolved oxygen tension on the culture fluorescence due to NAD(P)H + H^+ of a carbon limited chemostat culture (D = 0.36 h^{-1}) of Beneckea natriegens.

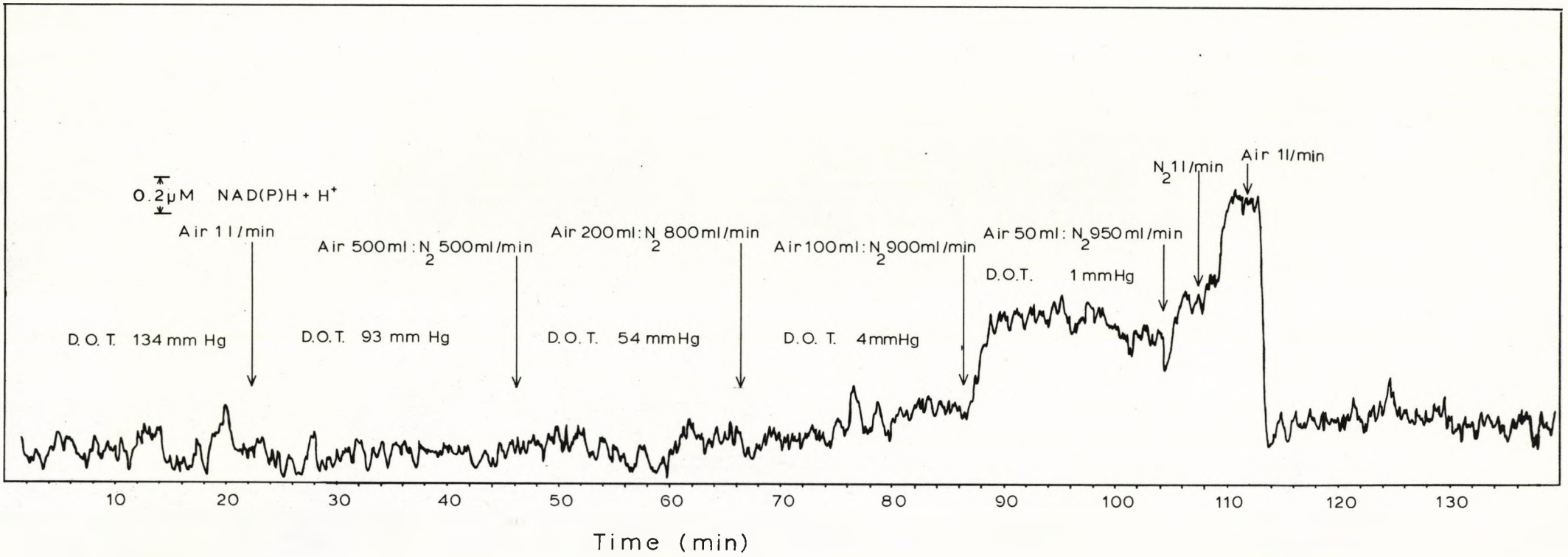

0.2μM NAD(P)H + H+
Air 1 l/min
Air 500ml : N2 500ml/min
Air 200ml : N2 800ml/min
Air 100ml : N2 900ml/min
Air 50ml : N2 950ml/min
N2 1l/min
Air 1l/min
D.O.T. 134 mm Hg
D.O.T. 93 mm Hg
D.O.T. 54 mmHg
D.O.T. 4mmHg
D.O.T. 1 mmHg
10
20
30
40
50
60
70
80
90
100
110
120
130
Time (min)

Fig. IV-9

2. A Comparison of the Cyanide Sensitivity of Whole and Fractionated Bacteria Harvested from an Oxygen Excess and Limited Chemostat Cultures

The potential respiration rate of Beneckea natriegens grown in continuous culture under carbon limitation appeared to be at least an order of magnitude more sensitive to KCN than that reported by Weston, Collins and Knowles (1974) for organisms grown in batch culture on a similar medium. To clarify this situation B. natriegens was grown under conditions of oxygen excess and oxygen limitation at a dilution rate of 0.26 h^{-1}. Bacteria were harvested from the chemostat at steady state and extracts of the respiratory system prepared and examined by Dr. J. A. Weston (University of Kent at Canterbury).

A summary of the physiological parameters monitored is shown in Table IV-2. Under oxygen limitation the molar growth yield, q_{O_2} and q_{CO_2} decreased, while the q_{glc}, Y_{O_2} and potential respiration rate increased. These findings are in agreement with those reported earlier.

A carbon recovery for the growth of B. natriegens under oxygen excess and limitation is shown in Table IV-3.

Bacteria freshly harvested from the chemostat were sensitive to inhibition by KCN; 100 μM KCN caused 80% and 40% inhibition of the potential respiration rate of bacteria taken from a chemostat under excess and limited oxygen conditions respectively (Table IV-2). After sonication and fractionation into particulate and supernatant fractions the potential respiration of the same bacteria was markedly more resistant to KCN. Thus 3 mM KCN caused only 57% and 80% inhibition of the NADH oxidation rates of bacteria respectively grown under

Table IV-2. The effect of oxygen excess and oxygen limitation on the respiration and growth of Beneckea natriegens in chemostat cultures operating at $D = 0.26\ h^{-1}$.

Growth Condition	Excess Oxygen	Limited Oxygen
Partial pressure of oxygen (mmHg)	159 mmHg	8 mmHg
Dissolved oxygen tension (mmHg)	133 mmHg	<1 mmHg
q_{O_2}2* (mmol $g^{-1}h^{-1}$)	15.42	12.25
$qCO_2$2* (mmol $g^{-1}h^{-1}$)	15.21	13.43
Potential q_{O_2}2* (mmol $g^{-1}h^{-1}$)	34	52
RQ	0.99	1.09
Y_{glc}2* (g mol^{-1})	39.1	29.6
Y_{O_2}2* (g mol^{-1})	16	21
Inhibition of potential q_{O_2} by 100 μM KCN (%)	80	40
q_{glc}2* (mmol $g^{-1}h^{-1}$)	6.7	9.0

2* Calculation based on g bacterial carbon.

Table IV-3. A carbon balance for the growth of Beneckea natriegens grown under oxygen excess and oxygen limitation ($D = 0.26\ h^{-1}$) in chemostat culture

Growth condition	Carbon input (gh^{-1})	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ Carbon dioxide carbon (gh^{-1})	Recovery (%)
Excess oxygen (Dissolved oxygen tension 134 mmHg)	0.477 0.477	0.258 0.265	0.057 0.050	0.178 0.178	103.3 103.3
Limited oxygen (Dissolved oxygen tension <1 mmHg)	0.477 0.477	0.196 0.196	0.138 0.152	0.119 0.119	95.0 98.0

oxygen excess and limitation (J. A. Weston, 1974). Oxygen uptake rates of oxygen excess and oxygen limited grown Beneckea natriegens, recalculated from the data of Weston (1974), are shown in Table IV-4. These results indicate a large loss of respiration rate on cell fractionation. Assuming a 65% protein content, the respiration rate is approximately 14% of that obtained with whole cells respiring glucose.

Table IV-4. Oxygen uptake rates of oxygen excess and oxygen limited grown Beneckea natriegens recalculated from the data of Weston (1974)

Growth condition	NADH oxidase activity of particulate fraction (mmol O_2 per g bacterial protein per h)	Inhibition by 3 mM KCN of NADH oxidase activity (%)	Ascorbate-TMPD oxidase activity of particulate fraction (mmol O_2 per g bacterial protein per h)
oxygen excess	1.02	57	0.72
oxygen limited	1.74	80	26.7

3. Discussion

The metabolic rate and growth efficiency of Beneckea natriegens, as indicated by the q_{O_2}, q_{CO_2} and $q_{glucose}$, when growing in continuous culture, was little affected by changes in dissolved oxygen tension over a range from below the sensitivity of the oxygen probe used (< 1 mmHg) to 134 mmHg. In contrast, the potential q_{O_2} of the culture increased by 50% as the dissolved oxygen tension was decreased from 100 to 65 mmHg. There was a small, but hardly significant rise in the in situ respiration rate over this range of dissolved oxygen although an increase in the cytochrome c content, relative to cytochrome b, was evident. The insensitivity of metabolism to changes in the dissolved oxygen content above a certain 'critical' level is the usual response of aerobic and facultative bacteria (Harrison, 1973). However, the changes in cytochrome content and potential respiration rate at dissolved oxygen tensions within this range, when the metabolism of the cell growing in situ in the fermenter was unaffected, was an entirely unexpected result. It would seem that the mechanisms for induction of the cytochrome and control of the potential respiration rate are unrelated to the actual respiration rate of the organism. Also, the actual respiration rate under carbon-limited growth is not a function of the respiratory potential of the culture or of the cytochrome content.

After the dissolved oxygen tension had fallen to a level below that which could be detected by the probe, the oxygen supply could be reduced still further. Reducing the oxygen supply under these conditions had a profound effect on the metabolism of the culture. A similar finding has been reported for Klebsiella aerogenes (Harrison and Pirt, 1967). When the partial pressure of oxygen was

reduced and the dissolved oxygen tension decreased to less than 1 mmHg, there was a switch to fermentative metabolism. This was reflected in an apparent rise in the Y_{O_2}, a marked increase in the $q_{glucose}$, an increase in fluorescence due to NAD(P)H, and a decreased yield coefficient from glucose. The potential q_{O_2} under these conditions increased dramatically to its highest value, while the cytochrome pattern reverted to that obtained at dissolved oxygen tensions above 96 mmHg. Thus, not only was there no quantitative correlation between cytochrome content, potential q_{O_2} and q_{O_2} *in situ*, but there appeared to be no qualitative correlation, these parameters changing independently of each other.

Beneckea natriegens did not demonstrate the phenomenon of an increased *in situ* respiration rate, in response to low dissolved oxygen tension that has been found to occur in *Klebsiella aerogenes* and *Escherchia coli* (Harrison and Pirt, 1967; Harrison and Loveless, 1971a), and which is accompanied by a switch to a less efficient pathway of glucose oxidation.

The different branches of the electron-transport chain reported for cell-free preparations of *Beneckea natriegens* (Weston, Collins and Knowles, 1974) and also for Azotobacter (Ackrell and Jones, 1971a) are distinguished by a difference in cyanide sensitivity of the respective terminal oxidase of at least an order of magnitude. Therefore, if there is a change in the relative importance of these branches in response to changing dissolved oxygen tension, as has been proposed for *Azotobacter* (Ackrell and Jones, 1971b), this should be reflected in the cyanide sensitivity of the bacteria. In the present work no such change in cyanide sensitivity over the dissolved oxygen range (8 to 140 mmHg) was observed.

As the dissolved oxygen tension fell below the sensitivity of the probe there was a steep fall in cyanide sensitivity although the in situ and potential q_{O_2} showed little change. As the oxygen supply was reduced still further, cyanide sensitivity decreased in parallel with q_{O_2}. Thus, again there is no clear correlation between cyanide sensitivity and respiratory activity or yield coefficient. Moreover, the observed changes in cytochrome content could not be correlated with the cyanide sensitivity.

These studies failed to show any physiological evidence of a switch in pathways of electrons from a "coupled" to an "uncoupled" branch of the electron transport system such as has been suggested for Azotobacter (Ackrell and Jones, 1971a) and also for E. coli and K. aerogenes (Harrison and Maitra, 1969). Also, no correlation was found between induction of CO-binding cytochrome c and cyanide sensitivity. Whatever is the significance of the multiplicity of terminal oxidase cytochromes in Beneckea natriegens it does not appear to relate to adaptation to dissolved oxygen changes. The induction of increased cytochrome content and potential respiration rate at dissolved oxygen tensions well above the 'critical' value raises the question how do these organisms detect changes at dissolved oxygen tensions which have little effect on cell metabolism or on the in situ respiration rate? The reason for these changes in cytochrome content and respiratory potential remains obscure.

This study demonstrated that fractionation of Beneckea natriegens has a marked effect on respiration rate and cyanide sensitivity. Although different substrates were employed, for whole and fractionated bacteria, the respiration

rate of fractionated bacteria was reduced greatly compared with whole bacteria. Moreover the cyanide insensitivity increased by a factor of approximately 30 times. The reasons for this difference in cyanide sensitivity of fractionated and whole cells are discussed fully in Section VI.

SECTION V

THE EFFECT OF CYANIDE AND CARBON MONOXIDE ON THE GROWTH OF *BENECKEA NATRIEGENS* IN CHEMOSTAT CULTURE

1. Excess Oxygen Conditions (Carbon Limitation, $D = 0.36\ h^{-1}$)

   (i) Effect of 24 μM KCN, 20% CO and a combination of KCN plus CO (unchelated medium G)

   (ii) Effect of 50 μM KCN (chelated medium G)

2. Oxygen and Carbon Limitation ($D = 0.36\ h^{-1}$)

3. High Growth Rate ($D = 1.04\ h^{-1}$) with Oxygen and Carbon in Excess

4. Discussion

THE EFFECT OF CYANIDE AND CARBON MONOXIDE ON THE GROWTH OF *BENECKEA NATRIEGENS* IN CHEMOSTAT CULTURE

Experiments reported in the previous section (Section IV) failed to show any physiological evidence of a switch in pathway of electrons when the dissolved oxygen tension was gradually reduced from air saturation (159 mmHg) to $\ll 1$ mmHg.

The potential respiration rate of bacteria freshly harvested from the chemostat at steady state under oxygen excess and limitation was sensitive to KCN, 100 μM KCN causing 80% and 40% inhibition respectively. The same batch of cells upon fractionation into particulate and supernatant fraction exhibited a 30 fold increase in resistance to KCN. It was decided therefore to examine the effects of KCN and CO on growing bacteria. If branching of the electron transport chain occurred under physiological conditions in the chemostat then growth in the presence of excess oxygen would facilitate the operation of the KCN sensitive pathway with a higher Km for O_2. The presence of 10 μM KCN in the growth medium might therefore cause a switch to the KCN resistant pathway. If these pathways differ in their relative growth efficiency then a change in yield and respiration rate will be evident. Moreover, the potential respiration rate of bacteria grown in the presence of excess oxygen and 10 μM KCN would become markedly more resistant to KCN compared with those grown in the absence of KCN. The presence of 10 μM KCN in the growth medium under oxygen limitation might be expected to have no effect on yield or respiration.

1. Excess Oxygen Conditions (Carbon Limitation, $D = 0.36\ h^{-1}$)

(i) Effect of 24 μM KCN, 20% CO and a combination of KCN plus CO (unchelated medium G)

As a precaution against the toxic effects of KCN and CO all experiments that involved the use of these substances were carried out in a flame proof unit specially set up for the chemostat study of methane utilising organisms, this ensured that all effluent gases were vented to the exterior of the laboratory. The oxygen consumption and CO_2 production of fermenter cultures were measured by Mr. L. J. Barnes using a modification of the gas liquid chromatography system described by Deans, Huckle and Peterson (1971). Carbon monoxide was obtained from standard CO cylinders (British Oxygen Company) and mixed with air before the gas entered the fermenter. The flow rate and thus the concentration of CO in the gas phase was controlled at 20% v/v by monitoring the flow through a rotameter. Potassium cyanide was weighed and immediately dissolved in culture medium and the pH adjusted to 7.5. The solution was then Millipore filtered directly into the medium reservoir.

Growth of B. natriegens under fully aerobic conditions ($D = 0.36\ h^{-1}$) in the presence of 24 μM KCN in the growth medium (pH 7.3), 20% CO in the inlet gas phase or a combination of 24 μM KCN plus 20% CO had no effect on the growth yield from glucose (Table V-1). The specific rate of oxygen consumption, q_{O_2}, was found to be unaffected by the presence of 24 μM KCN in the growth medium, but bacteria grown in the presence of 24 μM KCN plus 20% CO appeared to have a slightly higher q_{O_2} value (Table V-1). The specific rate of carbon dioxide production was unaffected by growth in the presence of these inhibitors (Table V-1).

Table V-1. The effect of growth in the presence of KCN and CO on the yield, respiration and cytochrome content of a carbon limited chemostat culture of Beneckea natriegens (oxygen excess, unchelated medium G, D = 0.36 h^{-1})

Gas flow rate (ml min^{-1})	Inhibitor concentration	Y_{glc}^{2*} ($gmol^{-i}$)	In situ qO_2^{2*} (mmol $g^{-1}h^{-1}$)	Potential qO_2^{2*} (mmol $g^{-1}h^{-1}$)	In situ qCO_2^{2*} (mmol $g^{-1}h^{-1}$)	R.Q.	Dilution rate (h^{-1})	Cytochrome (ΔE/mg bacterial carbon) b	c	c/b ratio	Effluent glucose (gl^{-1})	Carbon recovery (%)
Air 800 N_2 200	-	35.3	16.79	32.78	17.22	1.025	0.35	1.41	1.65	1.17	<0.003	91.2
Air 800 N_2 200	24 μM KCN	35.2	16.22	33.3	17.38	1.075	0.35	1.54	1.68	1.09	<0.003	94
Air 800 N_2 0	CO 200 ml per min + 24 μM KCN	34.98	18.57	35.7	17.75	0.96	0.38	1.21	1.24	1.02	<0.003	95
Air 800 N_2 200	-	34	-	32.2	-	-	0.39	-	-	-	<0.003	89

*2 = Calculation based on g bacterial carbon.

Fig. V-1. The effect of KCN and CO on the potential respiration rate of Beneckea natriegens harvested from a carbon limited chemostat culture at steady state under (1) oxygen excess, o - o; (2) oxygen excess plus 24 μM KCN in the medium feed, • - •; (3) oxygen excess with 24 μM KCN in the medium feed plus 20% CO in the gas phase, Δ - Δ.

Effect of KCN (a); effect of CO (b) and effect of KCN plus CO (c).

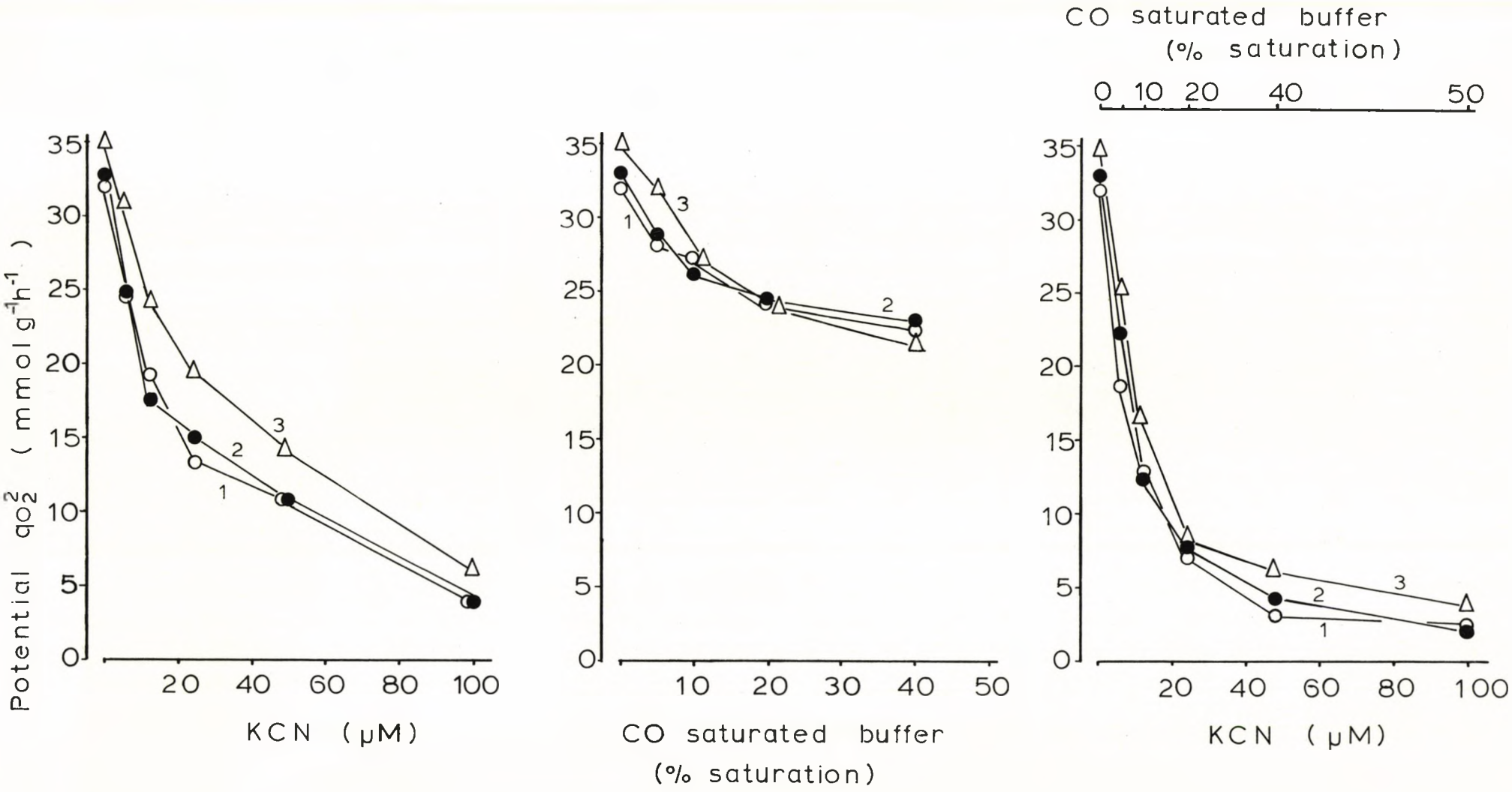

Potential qo_2^2 (mmol $g^{-1}h^{-1}$)
KCN (μM)
CO saturated buffer
(% saturation)
CO saturated buffer
(% saturation)
0 10 20 40 50
KCN (μM)
1
2
3

Fig. V-1

Growth in the presence of 24 μM KCN or a combination of 24 μM KCN plus 20% CO had no marked effect on the respiration rate (potential q_{O_2}) of harvested bacteria (Table V-1). Moreover the sensitivity of the respiration rate of harvested cells to KCN and CO was not markedly altered after growth in the presence of these inhibitors separately. However, after growth in the presence of both KCN and CO together the respiration rate of harvested bacteria was slightly more resistant to inhibition by KCN than the control (Fig. V-1(a)). The sensitivity of the respiration rate to carbon monoxide also appeared to be unaffected by growth in the presence of KCN and CO. A 40% CO saturated buffer caused approximately 32% inhibition of respiration in all cases (Fig. V-1(b)).

The combined effect of KCN plus CO on the respiration rate of harvested bacteria was greater than the effect observed with either inhibitor alone (Fig. V-1(c)). A combination of 20% CO saturated buffer plus 24 μM KCN caused approximately 75% inhibition of the respiration rate of harvested bacteria whether grown in the absence or presence of these inhibitors.

Cytochrome spectra

Oxidised minus reduced difference spectra of whole cells were recorded at room temperature. Absorbance maxima occurred at 553 and 559 nm and a Soret peak was observed at 426 nm indicating the presence of c and b type cytochromes respectively (Fig. V-2). The presence of 24 μM KCN or a combination of 24 μM KCN in the medium feed plus 20% CO in the gas phase did not appear to change the relative amounts of cytochrome present. The amount of cytochrome b and c present in cells grown in the presence of 24 μM KCN was not significantly different from

Fig. V-2. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a chemostat under steady state conditions with excess oxygen in the presence and absence of KCN and CO.

(a) Oxidised H_2O_2-reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$-reduced $Na_2S_2O_4$.

(1) Oxygen in excess, (bacterial carbon 3.92 mg ml^{-1})

(2) oxygen excess plus 24 μM KCN in the medium feed, (bacterial carbon 3.90 mg ml^{-1})

(3) oxygen excess plus 24 μM KCN in the medium feed and 20% CO in the gas phase, (bacterial carbon 3.80 mg ml^{-1}).

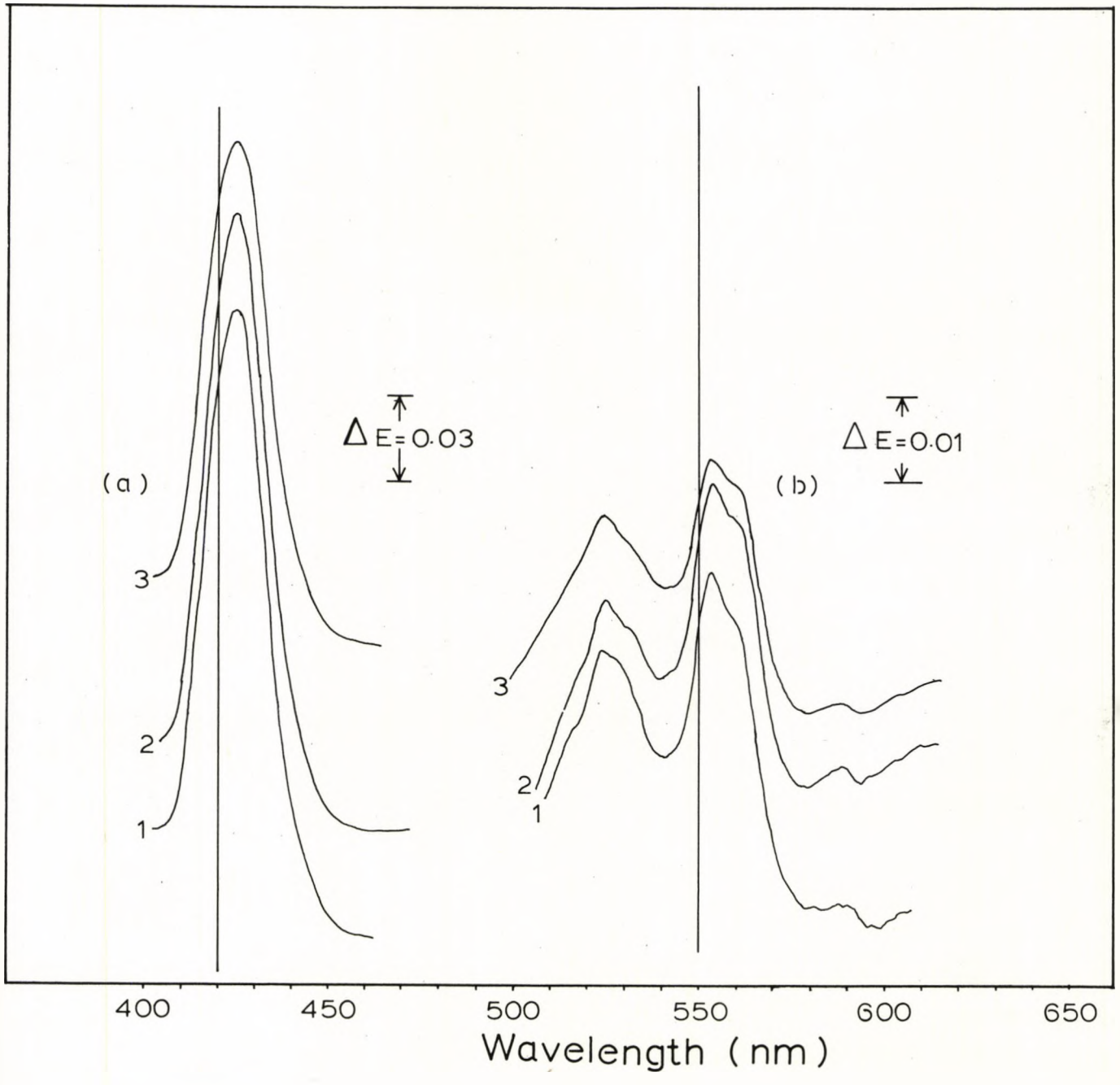


Fig. V-2

Table V-2. A carbon recovery for the growth of Beneckea natriegens under carbon limitation in chemostat culture in the presence and absence of KCN and CO (oxygen excess, $D = 0.36\ h^{-1}$)

Growth condition	Carbon input (gh^{-1})	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ Carbon dioxide carbon (gh^{-1})	Recovery (%)
Air 800 ml + 200 ml N_2	0.720	0.324	0.110	0.207	89.0
Air 800 ml + 200 ml N_2 + 24 μM KCN	0.729	0.357	0.125	0.209	94.7
Air 800 ml + 200 ml CO + 24 μM KCN	0.727	0.354	0.127	0.213	95.4
Air 800 ml + 200 ml N_2	0.808	0.407	0.142	0.188	91.2

that of the control. Growth of B. natriegens in the presence of 24 μM KCN plus 20% CO in the gas phase appeared to cause a slight decrease in the total amount of both b and c type cytochromes (Table V-1). The ratio of cytochrome c:cytochrome b, however, remained fairly constant at approximately 1.10 ± 0.07 in all three cases.

Carbon balance

A carbon balance for the growth of B. natriegens in chemostat culture under oxygen excess in the presence and absence of KCN and CO is shown in Table V-2.

(ii) Effect of 50 μM KCN (chelated medium G)

The exposure of the culture to 50 μM KCN was achieved by injecting a solution of KCN (pH 7.5) into the fermenter and simultaneously changing the medium feed to one containing 50 μM KCN. This caused an immediate inhibition of respiration (Fig. V-3). The oxygen uptake, as indicated by the difference in the oxygen content of the inlet and effluent gas measured on a twin channel paramagnetic oxygen analyser fell from approximately 1% to a minimum value of 0.62% O_2 after 30 min. The respiration then recovered and overshot the original value reaching a maximum of about 1.2% O_2. This increase in the oxygen consumption was probably brought about by the metabolism of glucose or fermentation products accumulated during the initial inhibition of respiration. The respiration then returned to a steady state uptake value of 1.10% O_2. Inhibition of CO_2 output was not as severe as that observed for oxygen uptake (Fig. V-3) but

Fig. V-3. The effect of injecting 50 μM KCN into a carbon limited chemostat culture ($D = 0.36\ h^{-1}$) of Beneckea natriegens, on oxygen uptake and CO_2 output. The medium reservoir was simultaneously changed to one containing 50 μM KCN in addition to the normal constituents.

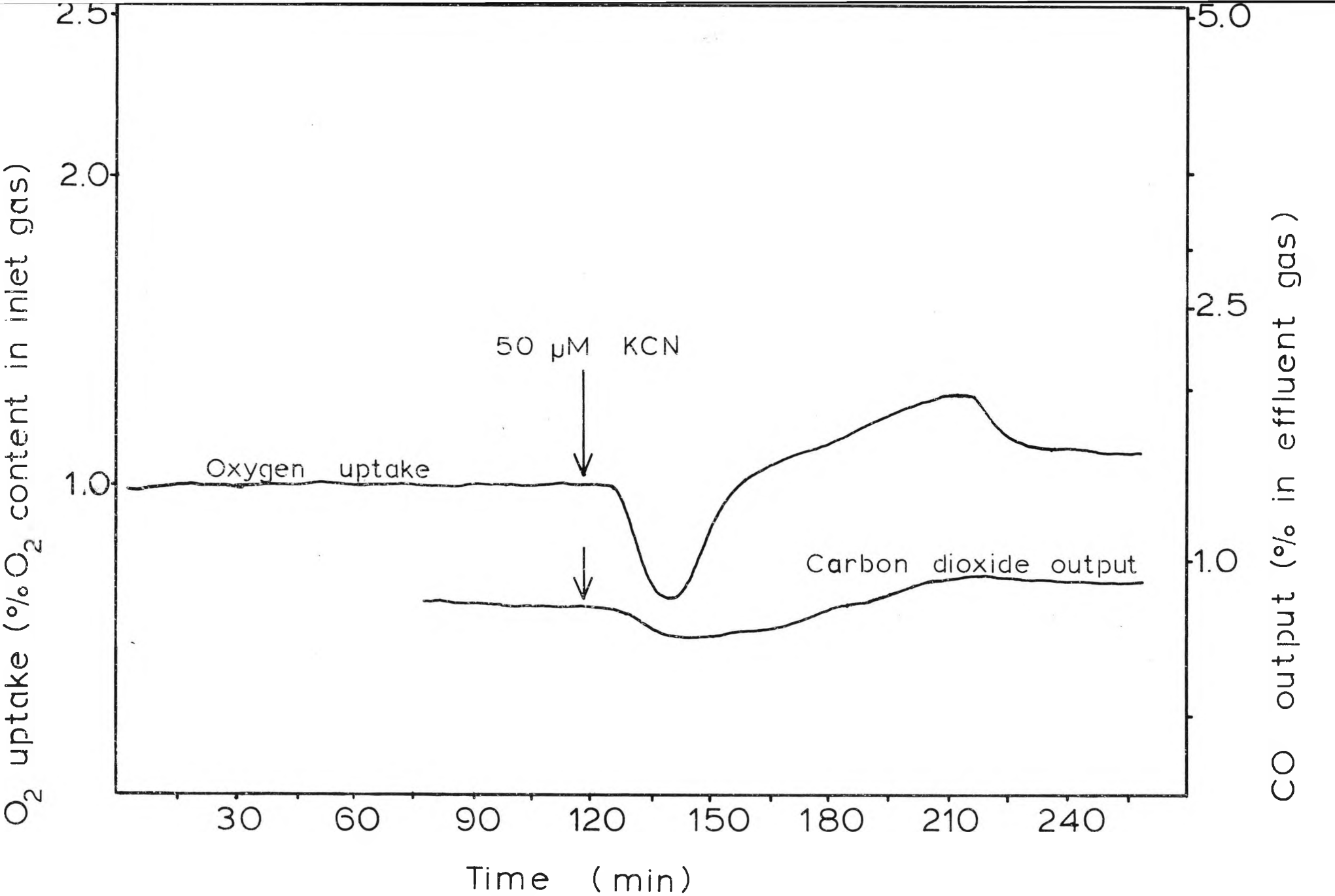

2.5
2.0
1.0
5.0
2.5
1.0
50 μM KCN
Oxygen uptake
Carbon dioxide output
30
60
90
120
150
180
210
240
Time (min)
O2 uptake (% O2 content in inlet gas)
CO output (% in effluent gas)

Fig.V-3

decreased to a minimum value after approximately 25 mins. The CO_2 output then increased to a maximum value after 60 mins and overshot the original value before returning to a steady state value. The culture stabilised in the presence of 50 μM KCN after approximately 2 h. The presence of 50 μM KCN in the growth medium had no marked effect on the growth yield from glucose after a steady state was achieved, but appeared to cause a slight increase in the q_{O_2} (Table V-3). This was reflected in the molar growth yield from oxygen which decreased from 18.4 g bacterial carbon per mole oxygen in the control to 16.5 g/mole in the presence of KCN.

The specific rate of CO_2 production was not greatly affected by the presence of 50 μM KCN (Table V-3).

The value for the q_{glc} obtained in the presence of 50 μM KCN was not significantly different from that of the control (Table V-3).

A satisfactory carbon recovery for the growth of B. natriegens in the presence of 50 μM KCN was obtained (Table V-4).

Cytochrome spectra

Oxidised minus reduced difference spectra were recorded at room temperature (Fig. V-4). The results indicated that growth in the presence of 50 μM KCN caused an increase in the level of both b and c type cytochromes (Table V-3). Furthermore, the ratio of cytochrome c:cytochrome b appeared to have increased from 1.14 in the control to 1.54 in the cells grown in the presence of 50 μM KCN. This change in the cytochrome c:cytochrome b ratio is reflected in the wavelength of the peak of absorbance in the Soret region of the spectrum which was 427 nm in cells grown in the presence of 50 μM KCN compared to a value of 428.5 nm in the control.

Table V-3. The effect of the presence of 50 µM KCN in the medium feed on the growth, respiration and cytochrome pattern of a fully aerobic, carbon limited, chemostat culture of Beneckea natriegens ($D = 0.37\ h^{-1}$)

	Control without KCN	In the presence of 50 µM KCN
Y_{glc}^{2*} (g mol^{-1})	39.0	40.2
$q_{O_2}^{2*}$ (mmol g^{-1}h^{-1})	19.75	22.40
$q_{CO_2}^{2*}$ (mmol g^{-1}h^{-1})	17.95	18.47
q_{glc}^{2*} (mmol g^{-1}h^{-1})	9.20	9.32
$Y_{O_2}^{2*}$ (g mol^{-1})	18.40	16.50
Cytochromes (ΔE/mg bacterial carbon)		
cytochrome c	1.79	2.64
cytochrome b	1.56	1.71
Ratio $\frac{\text{cytochrome c}}{\text{cytochrome b}}$	1.14	1.54
Unmetabolised glucose (gl^{-1})	<0.001	<0.001

*2 = Calculation based on g bacterial carbon.

Table V-4. A carbon balance obtained for the growth of Beneckea natriegens in carbon limited chemostat culture in the presence and absence of 50 μM KCN ($D = 0.37\ h^{-1}$)

	Carbon input (gh^{-1})	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1})	+ Carbon dioxide carbon (gh^{-1})	Recovery (%)
Control	0.694	0.361	0.097	0.214	96.8
Growth in the presence of 50 μM KCN	0.706	0.379	0.099	0.227	99.8

Fig. V-4. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a carbon limited chemostat under steady state conditions in the presence and absence of 50 μM KCN.

(a) Oxidised H_2O_2-reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$-reduced $Na_2S_2O_4$.

(1) Growth in the presence of 50 μM KCN, (bacterial carbon 4.4 mg ml^{-1})

(2) Growth in the absence of KCN, (bacterial carbon 4.3 mg ml^{-1})

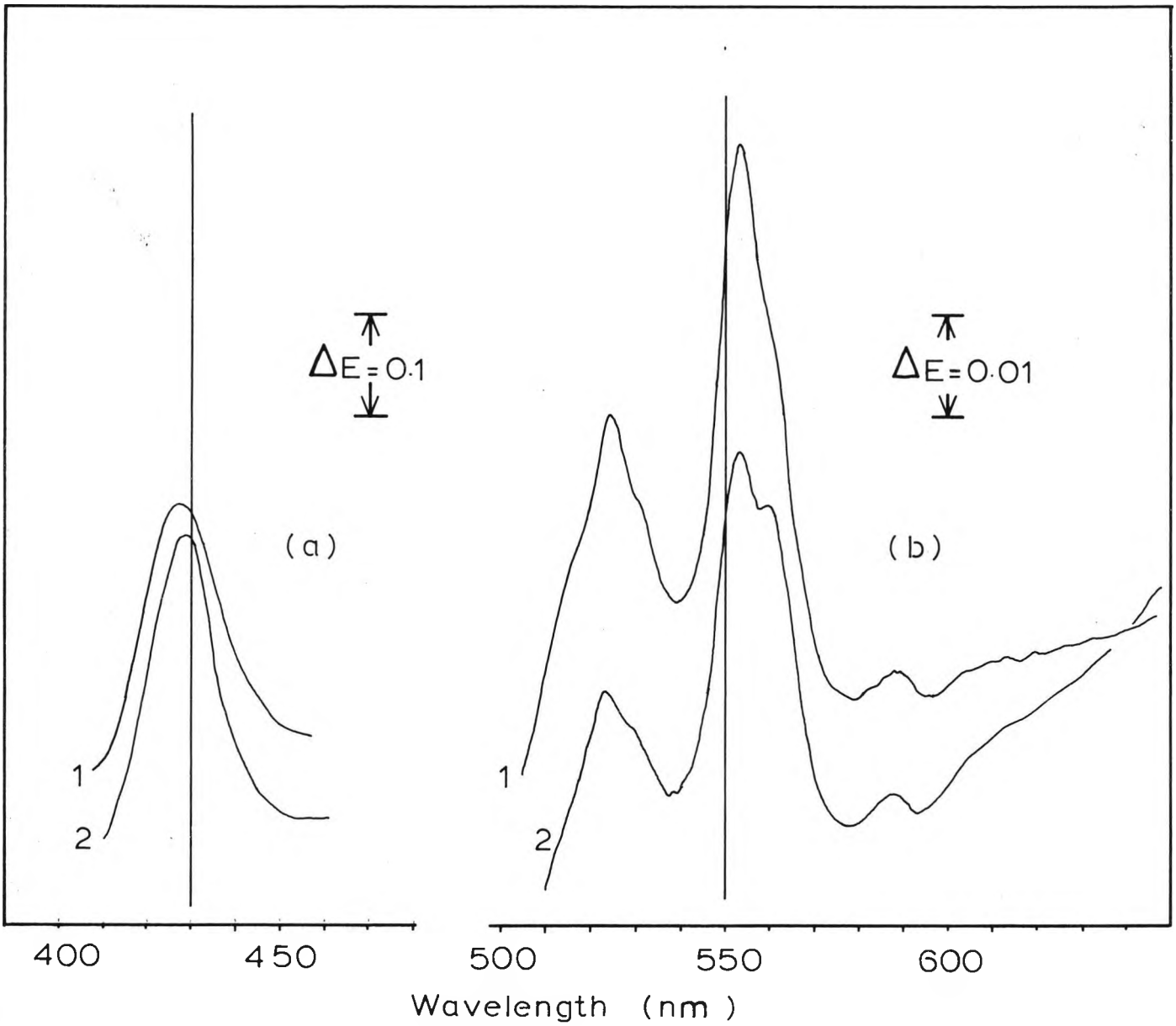


Fig. V-4

Reduced plus CO minus reduced difference spectra revealed a shift in the predominant peak of absorbance from 419 nm in the control to 414.5 nm with a shoulder at 419.5 nm in the cells grown in the presence of 50 μM KCN (Fig. V-5). This is indicative of a relatively higher content of CO-binding cytochrome c in the cyanide-grown cells and a relatively lower content of cytochrome o.

Fig. V-5. Room temperature reduced plus CO minus reduced difference spectra of whole cells taken from a chemostat under steady state conditions under carbon limitation ($D = 0.36\ h^{-1}$) in the presence and absence of 50 μM KCN.

(1) Control; (2) growth in the absence of 50 μM KCN, (bacterial carbon 4.3 mg ml^{-1}); (3) growth in the presence of 50 μM KCN, (bacterial carbon 4.4 mg ml^{-1}).

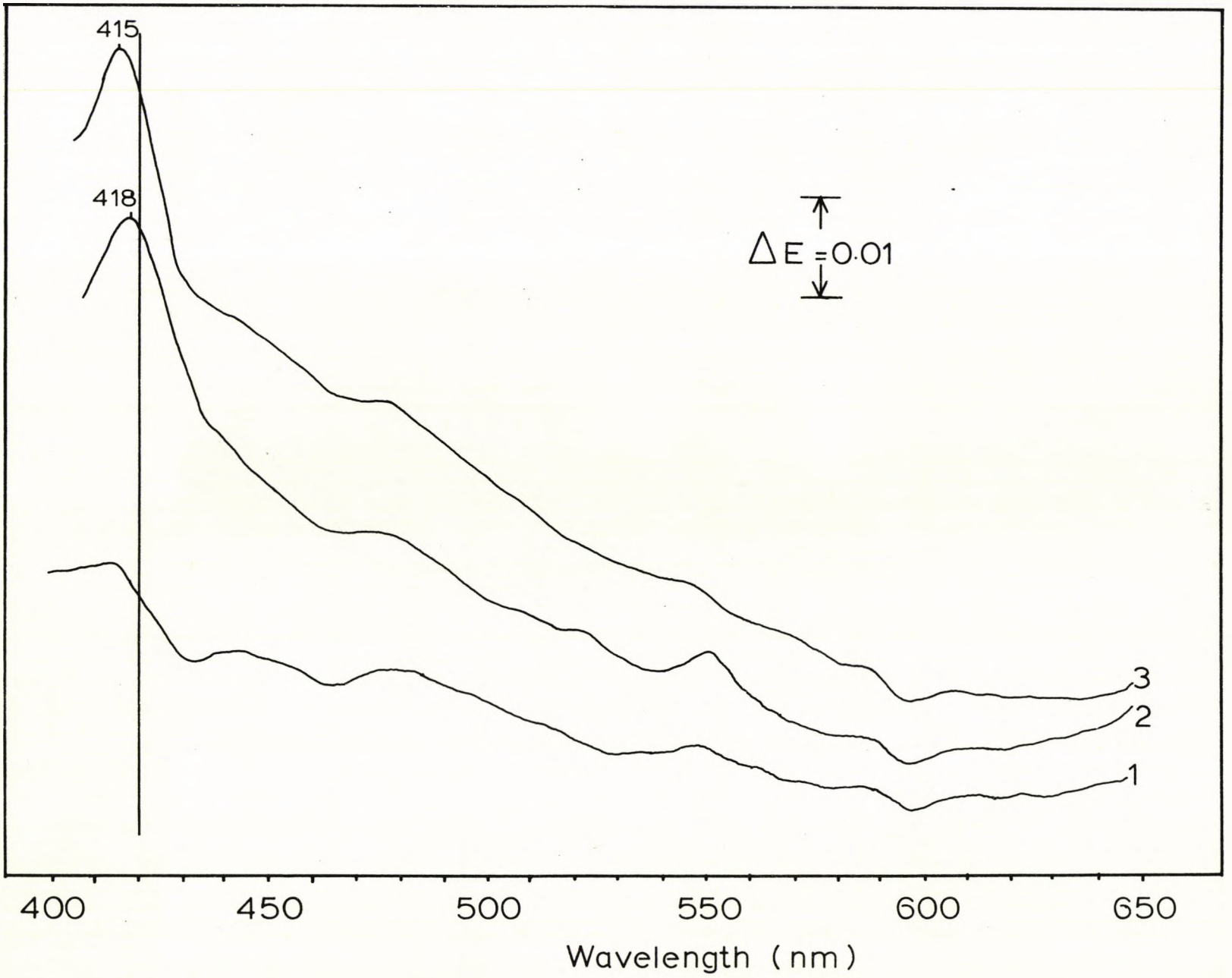


Fig. V- 5

2. Oxygen and Carbon Limitation ($D = 0.36\ h^{-1}$)

Effect of 24 μM KCN, 20% CO and a combination of KCN plus CO (chelated medium G)

The partial pressure of oxygen in the gas phase was reduced to approximately 8 mmHg by mixing air (50 ml min^{-1}) with nitrogen (950 ml min^{-1}). These gas flow rates have been shown to cause oxygen and carbon limitation at medium feed glucose concentrations of 2.0 gl^{-1} (Section IV). KCN was added to the growth medium as described previously and CO concentrations of 20% (v/v) were obtained by adding CO to the inlet gas phase while simultaneously reducing the N_2 flow rate so that the total gas flow rate remained constant.

The presence of 24 μM KCN in the medium feed appeared to have no significant effect on the growth yield which was found to be 21.6 g bacterial carbon per mole glucose carbon (Table V-5). The presence of either 20% CO in the gas phase or 20% CO plus 24 μM KCN in the medium feed caused the culture to immediately wash out (Table V-5). The culture recovered if the CO was removed from the inlet gas phase. The respiration rate could not be measured on this culture as the method available was not sufficiently sensitive (oxygen uptake and carbon dioxide output were measured by Mr. L. J. Barnes using a modified gas liquid chromatography system described by Deans, Huckle and Peterson, 1971, see Methods).

Although the sensitivity of the respiration rate of harvested bacteria (potential q_{O_2}) to KCN and CO was unaffected by growth under oxygen limitation

Table V-5. The effects of 24 μM KCN in the medium feed, of 20% CO in the gas phase and of a combination of KCN plus CO on the growth and respiration of Beneckea natriegens under oxygen limitation in a chemostat (D = 0.36 h^{-1})

Gas flow rate (ml min^{-1})	Inhibitor concentration	Y_{glc2}^{*} (g mol^{-1})	Culture absorbance (625 nm)	Dilution rate (h^{-1})	Unmetabolised glucose (% of glucose supplied)	Cytochrome level (ΔE/mg bacterial carbon) cyt b	cyt c	Ratio cytochrome c / cytochrome b
Air 50 N_2 950	-	19.38	0.24	0.35	0	2.4	2.97	1.24
Air 50 N_2 950	24 μM KCN	21.60	0.25	0.35	0	2.4	3.02	1.26
Air 50 N_2 750	CO (200 ml min^{-1})	Wash out	0.07	0.35	100	-		-
Air 50 N_2 750	CO (200 ml min^{-1}) + 24 μM KCN	Wash out	0.04	0.38	100	-		-
Air 50 N_2 950	-	20.92	0.25	0.35	0	-		-

in the presence of 24 μM KCN (Fig. V-6), the bacteria were markedly more resistant than cells grown under fully aerobic conditions (Fig. V-1).

Cytochrome spectra

Under oxygen limitation the total b and c type cytochrome content increased by approximately 70% (Table V-5) which is in good agreement with data given in Section IV. However the ratio of cytochrome c:cytochrome b remained fairly similar to that observed under fully aerobic conditions (Table V-1). The presence of 24 μM KCN in the medium feed during growth under conditions of oxygen limitation did not affect the cytochrome content quantitatively or qualitatively (Fig. V-7).

Fig. V-6 The sensitivity of the potential respiration rate, of bacteria grown under oxygen limitation, to KCN and CO.

Bacteria grown under oxygen limitation, o - o; bacteria grown under oxygen limitation in the presence of 24 μM KCN, ● - ●; and carbon limited fully aerobic culture included for comparison, Δ -Δ .

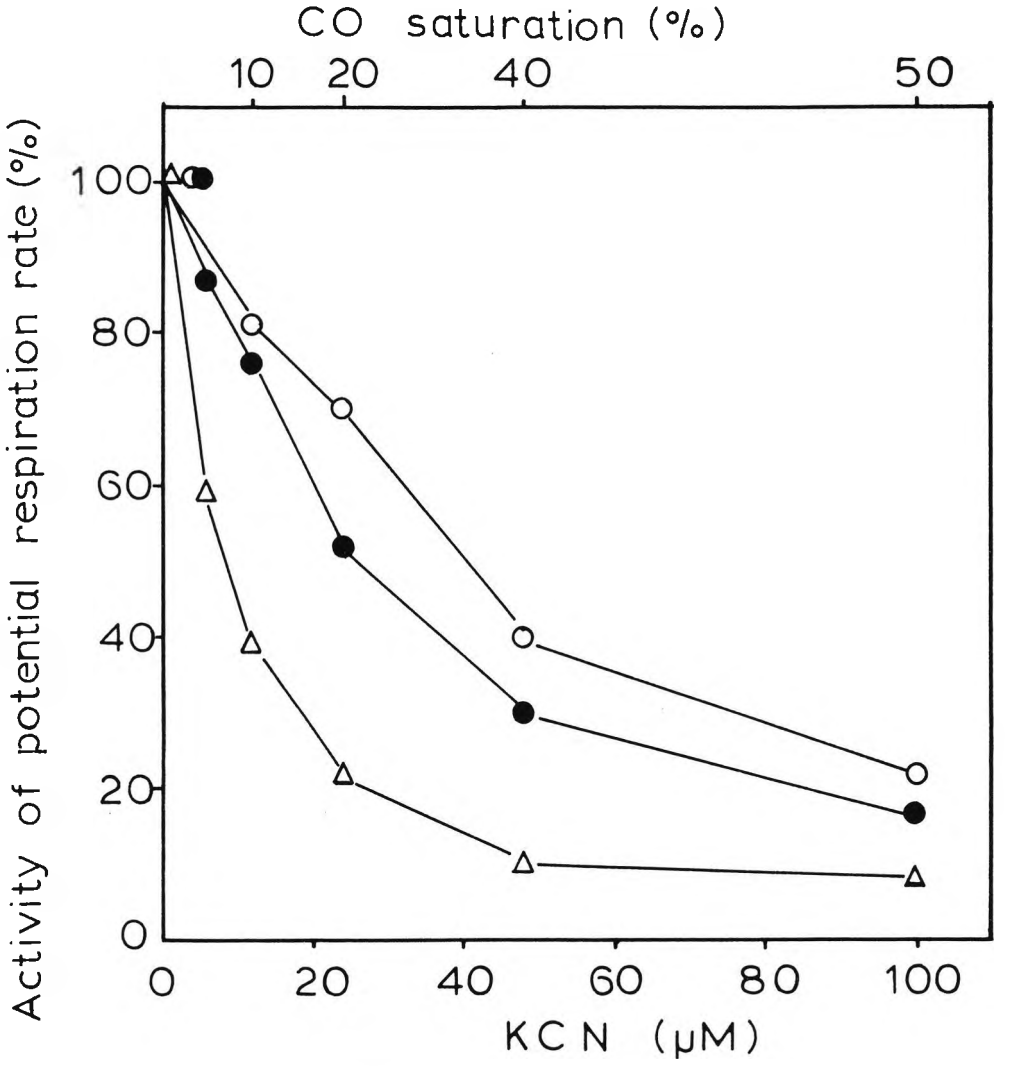

CO saturation (%)
10
20
40
50
Activity of potential respiration rate (%)
100
80
60
40
20
0
0
20
40
60
80
100
KCN (μM)

Fig. V-6

Fig. V-7. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a chemostat at steady state under (1) oxygen and carbon limitation, (bacterial carbon 4.6 mg ml^{-1}); (2) oxygen and carbon limitation in the presence of 24 μM KCN, (bacterial carbon 4.8 mg ml^{-1}).

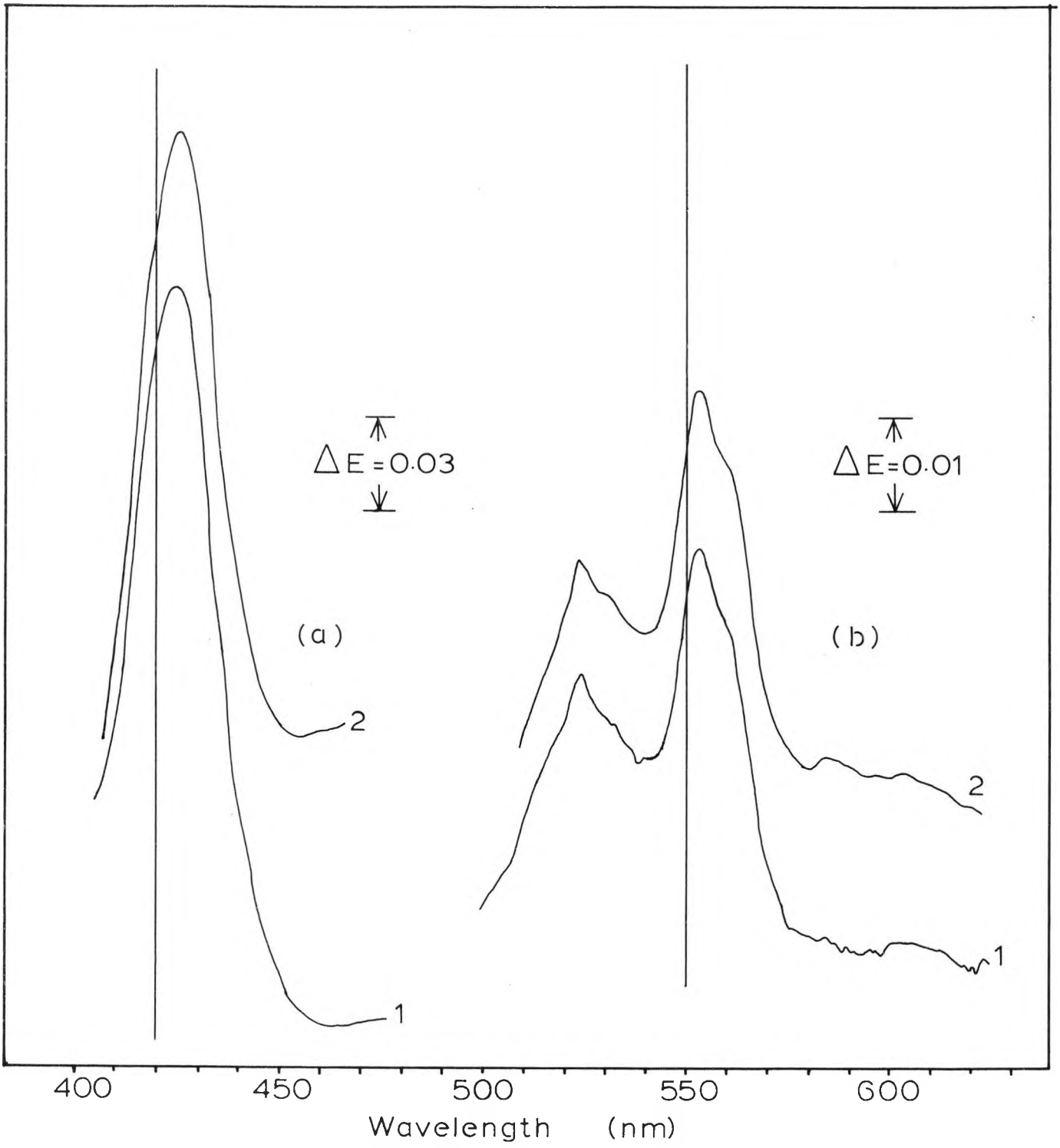


Fig. V-7

3. High Growth Rate (D = 1.04 h^{-1}) with Oxygen and Carbon in Excess

Effect of 24 μM KCN, 20% CO and a combination KCN plus CO (chelated medium G)

At a dilution rate of 1.04 h^{-1} approximately 50% of the input glucose was utilised. The molar growth yield from glucose decreased to 29.5 g bacterial carbon per mol glucose carbon utilised. In the presence of 20% CO in the inlet gas phase the molar growth yield decreased further to 26.2 g bacterial carbon per mol glucose carbon utilised. The presence of 24 μM KCN in the medium feed caused the culture to wash out. Similarly a combination of KCN plus CO caused the culture to wash out.

The sensitivity of the potential respiration rate to inhibition by KCN plus CO was measured and found to be similar in bacteria grown in the presence and absence of CO (Fig. V-8). Furthermore the sensitivity of the potential respiration rate to KCN plus CO was very similar to that observed with cells grown under fully aerobic conditions at a dilution rate of 0.36 h^{-1}.

Cytochrome analysis

As expected (see Section III) the cytochrome content of bacteria grown at a dilution rate of 1.04 h^{-1} was lower than that observed at lower dilution rates. Peak absorbances at 553 nm and 560 nm were observed and indicated the presence of c and b type cytochromes (Fig. V-9). The cytochromes present in bacteria grown in the presence of 20% CO in the inlet gas phase although qualitatively similar to that of the control appeared to be quantitatively less (Table V-6). Moreover the ratio of cytochrome c:cytochrome b absorption peaks increased indicating a relative increase in the amount of cytochrome c in cells grown in the presence of 20% CO in the inlet gas phase.

Fig. V-8. The effect of a combination of KCN plus CO on the potential respiration rate of bacteria harvested from a chemostat at steady state ($D = 1.04\ h^{-1}$) with excess oxygen in the presence and absence of 20% CO in the gas phase.

Growth in the absence of CO, o - o; growth in the presence of CO, • - •.

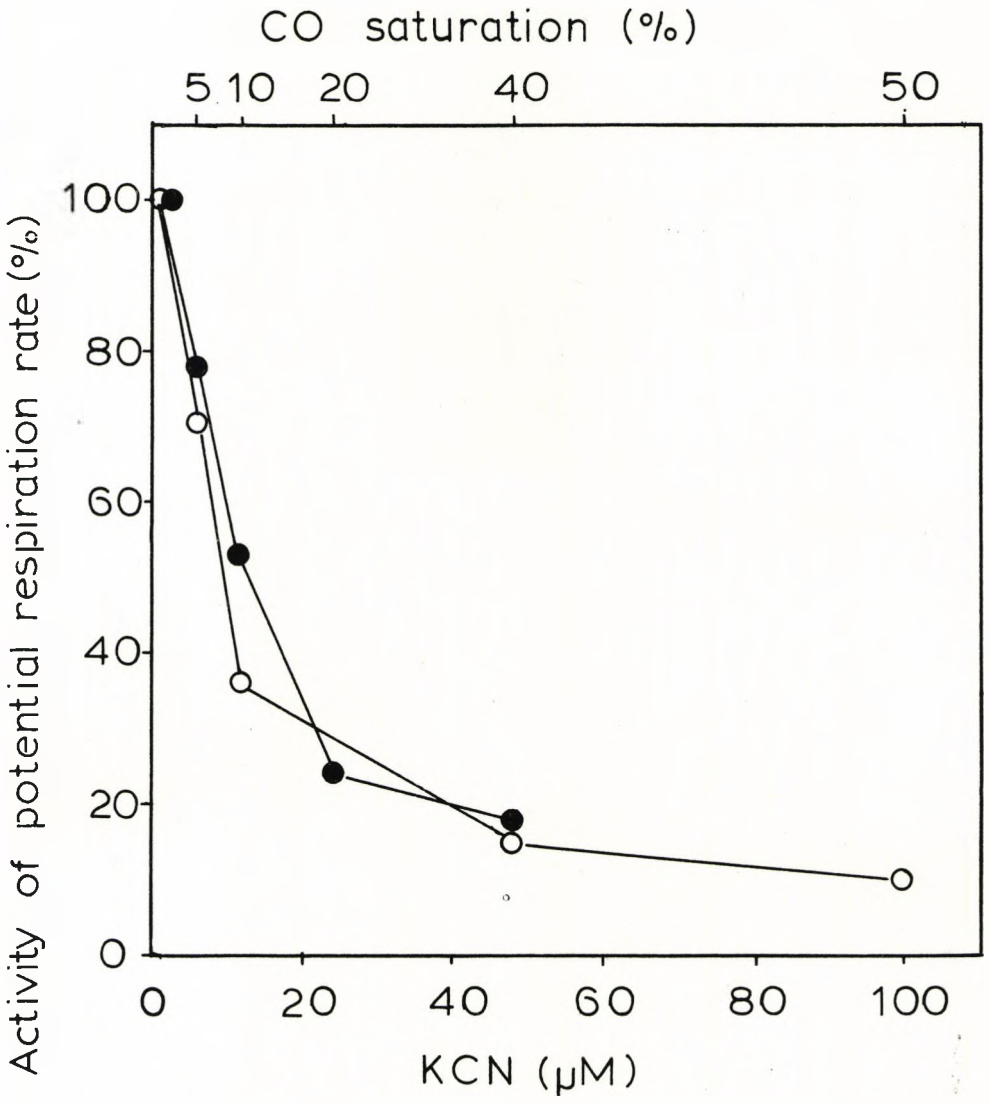

CO saturation (%)
5 10 20 40 50
100
80
60
40
20
0
Activity of potential respiration rate (%)
0 20 40 60 80 100
KCN (μM)

Fig.V-8

Fig. V-9. Room temperature oxidised minus reduced difference spectra of whole organisms taken from a chemostat at steady state with oxygen in excess ($D = 1.04\ h^{-1}$) in the presence and absence of 20% CO in the gas phase.

(1) Growth in the absence of CO, (bacterial dry weight 6.4 mg ml^{-1}); (2) growth in the presence of 20% CO in the gas phase, (bacterial dry weight, 5.68 mg ml^{-1}).

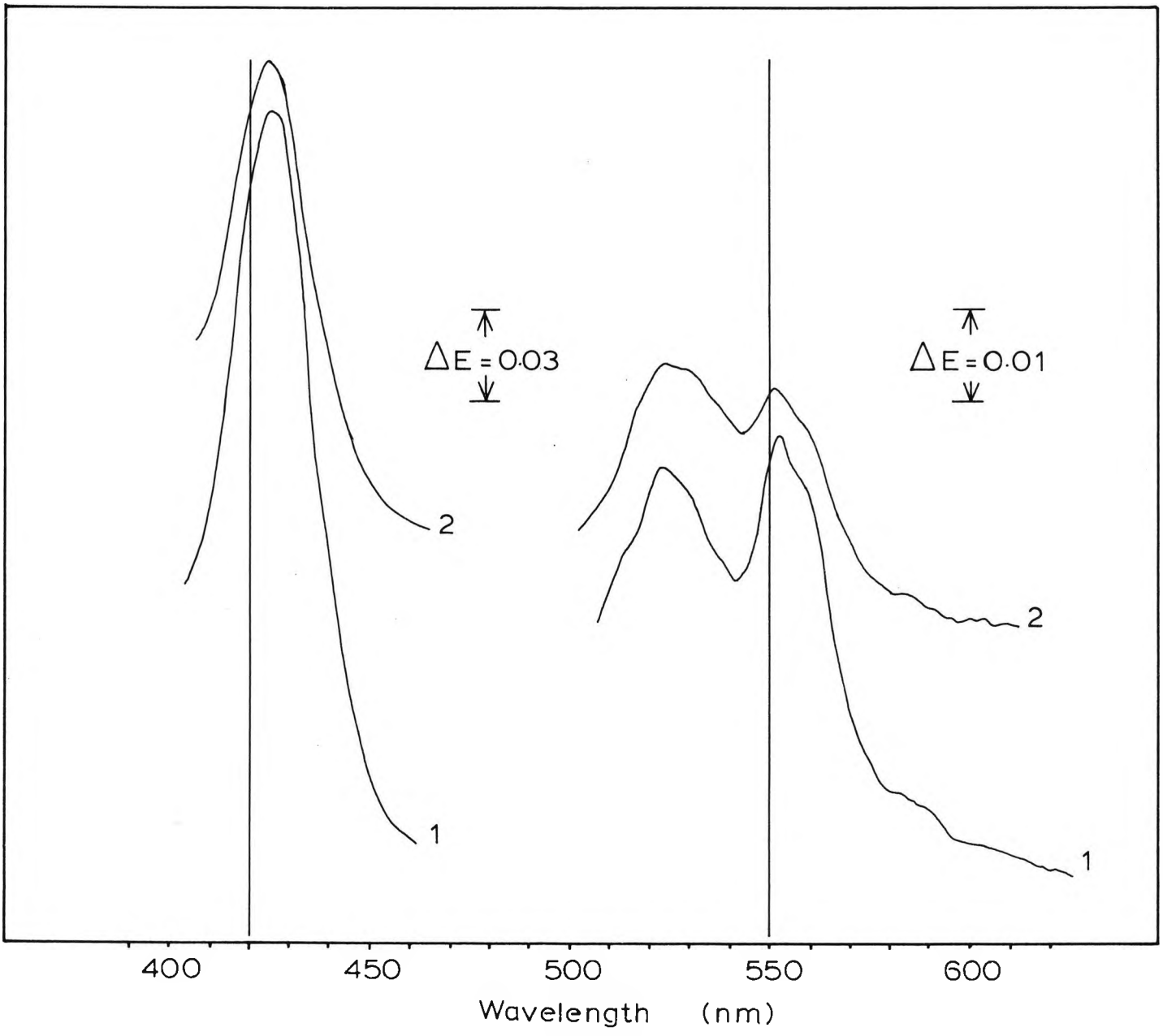


Fig.V-9

Table V-6. The effect of 20% CO in the inlet gas phase on the cytochrome content of Beneckea natriegens cultured at a dilution rate of 1.04 h^{-1}.

	Cytochromes (ΔE/mg bacterial cell carbon)		
	cytochrome b	cytochrome c	Ratio $\frac{\text{cyt c}}{\text{cyt b}}$
Control	0.76	0.84	1.105
Growth in the presence of 20% CO	0.30	0.40	1.333

4. Discussion

Under fully aerobic conditions at a dilution rate of 0.36 h^{-1} the presence of 24 μM KCN in the medium feed, 20% CO in the inlet gas phase or a combination of these two inhibitors during growth, had no effect on $Y_{g|c}$, *in situ* respiration rate, or cytochrome pattern. Furthermore, growth in the presence of KCN and CO did not induce a cyanide insensitive respiratory pathway. The sensitivity of the respiration rate of harvested bacteria (potential respiration rate) grown in the presence and absence of these inhibitors were indistinguishable.

Under oxygen limitation the presence of 24 μM KCN in the growth medium had no effect on respiration, molar growth yield or cytochrome pattern. However the presence of 20% CO in the gas phase caused culture wash out. This might be expected as CO, but not KCN binds preferentially with the cytochrome in the reduced state and, under oxygen limitation, the cytochromes would be in a more reduced state (Dixon and Webb, 1964).

When the growth rate was increased (D = 1.04 h^{-1}) 24 μM KCN in the medium feed caused the culture to wash out, although the respiration rate of cells harvested from the fermenter exhibited no greater sensitivity to cyanide than that observed at lower growth rates (D = 0.36 h^{-1}). This observation suggests that the degree of cyanide sensitivity is dependent on the physiological state of the culture and that the effect of KCN on respiration is greatest when the organism is growing rapidly and thus the respiratory system is in a high state of flux.

There was no indication in these experiments that the presence of cyanide or carbon monoxide during growth had caused the operation of an alternative branch of the electron transport chain as might have been expected if a cyanide resistant pathway existed.

SECTION VI

THE EFFECT OF RESPIRATION RATE ON SENSITIVITY TO CYANIDE AND CARBON MONOXIDE IN BENECKEA NATRIEGENS

1. Results

   (i) Respiration on glucose

   (ii) Respiration on glycerol

   (iii) The effect of temperature

   (iv) Effect of cyanide on bacteria from batch culture

   (v) Cyanide sensitivity of bacteria grown in the presence of cyanide

   (vi) Respiration of other CO binding cytochrome c containing bacteria

2. Discussion

THE EFFECT OF RESPIRATION RATE ON SENSITIVITY TO CYANIDE AND CARBON MONOXIDE IN BENECKEA NATRIEGENS

At growth rates approximately 35% of μ_{max} ($D = 0.36\ h^{-1}$) the growth and respiration of a carbon limited chemostat culture of B. natriegens was unaffected by the presence of 24 μM KCN in the medium feed. However, at growth rates near μ_{max} the same concentrations of cyanide caused culture wash out although the sensitivity of harvested bacteria to KCN was similar and independent of growth rate. A possible explanation of these results may lie in the relationship between growth rate and the in situ and potential respiration rate. The potential respiration rate (of harvested bacteria in the presence of excess oxygen and glucose) was found to be independent of growth rate (see Section III) and higher than the in situ respiration rate which approached it with increasing growth rate. At a dilution rate of $0.36\ h^{-1}$ the in situ q_{O_2} is approximately 50% of the potential q_{O_2}, however, at growth rates near μ_{max} the in situ and potential q_{O_2} are very similar. Thus when the organism is respiring maximally, either during growth at values of D near μ_{max} or when presented with excess substrate and oxygen during measurement of the potential q_{O_2}, respiration is sensitive to cyanide. A second factor that may contribute to respiratory sensitivity to cyanide is substrate concentration; glucose is present in excess at growth rates near μ_{max} and during measurement of the potential respiration rate. It was decided therefore to examine the effect of respiration rate and substrate concentration on respiratory sensitivity to cyanide. The respiration rate was varied either by changing the substrate concentration or by lowering the temperature of incubation in the presence of excess substrate.

Growth of B. natriegens in chemostat culture in the presence of 50 μM KCN caused an increase in the level of CO binding c type cytochrome. This cytochrome has been found in a number of organisms that are resistant to cyanide or produce cyanide during growth (Weston, Collins and Knowles, 1974; Niven, Collins and Knowles, 1975). A CO binding c type cytochrome has been shown to be involved in methane metabolism of Methylosinus trichosporium (Tonge, Harrison, Knowles and Higgins, 1975). Respiration of this organism has been reported to be resistant to cyanide (Weston, 1974). To establish the relationship between this CO binding c type cytochrome and respiratory resistance to cyanide the cyanide sensitivity of Methylococcus capsulatus and Pseudomonas extorquens (CO binding c containing organisms) and B. natriegens after growth in the presence of 50 μM KCN was examined.

1. Results

(i) Respiration on glucose

The effect of cyanide concentration on the respiration rate of Beneckea natriegens freshly harvested from a glucose-limited chemostat culture and supplied with excess (133 μM) glucose is shown in Fig. VI-1(a). Respiration was sensitive to cyanide, over the whole range of cyanide concentrations tested down to 5 μM. The experiment was repeated using a limiting glucose concentration of 44 μM which gave an initial respiration rate of only 85% of the maximum. In this case 6 μM cyanide had no effect on respiration rate but higher concentrations caused inhibition. With 22 μM glucose, giving a respiration rate of 70% of the maximum, cyanide concentrations of up to 12 μM had no effect and with 11 μM glucose, giving a respiration rate of 45% of the maximum, cyanide had little effect at concentrations below 24 μM. Above these 'critical' cyanide concentrations the curves were almost completely superimposable.

The effect of carbon monoxide at various glucose concentrations is shown in Fig. VI-1(b) and it can be seen that it had little effect on respiration rate. However, carbon monoxide in combination with cyanide caused much greater inhibition than cyanide alone (Fig. VI-1(c)). Once again, at sub-maximal respiration rates there was a range of cyanide concentrations below which cyanide had no effect and above which the response curve was superimposable on that of the maximum respiration rate.

Fig. VI-1. The effect of respiration rate on the sensitivity of respiration of cells harvested from a glucose grown culture of Beneckea natriegens to cyanide and carbon monoxide.

(a) The effect of KCN; (b) the effect of CO; (c) the effect of a combination of KCN and CO. The respiration rate was varied by injecting glucose at the following concentrations:- x - x, 133 μM, o - o, 44 μM; ● - ●, 22 μM and ▲-▲, 11 μM into the oxygen electrode cell.

Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
20
40
60
80
100
μM KCN
(a)
Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
10
20
30
40
50
% CO saturation
(b)
Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
20
40
60
80
100
μM KCN
5 10 20 40 50
% CO saturation
(c)

Fig VI – 1

Fig. VI-2. The effect of respiration rate on the sensitivity of the respiration of a glycerol grown culture of Beneckea natriegens to cyanide and carbon monoxide.

(a) The effect of KCN; (b) the effect of CO; (c) the effect of a combination of KCN and CO. The respiration rate was varied by injecting glycerol:- x - x, 352 μM; ● - ●, 85 μM; o - o, 44 μM and ▲-▲, 22 μM into the oxygen electrode cell.

Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
20
40
60
80
100
μM KCN
(a)
Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
10
20
30
40
50
per cent CO saturation
(b)
Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
20
40
60
80
100
μM KCN
(c)
5
10
20
40
50
% CO saturation

Fig VI – 2

(ii) Respiration on glycerol

In order to investigate whether the phenomena observed above were unique to glucose, and possibly associated with its active transport, the experiment was repeated with bacteria grown on glycerol (reported to be taken up by facilitated diffusion (Sanno, Wilson and Lin, 1968)). The pattern of results was very similar to that obtained with glucose (Fig. VI-2).

(iii) Effect of temperature

A series of experiments were made in order to test whether the change in sensitivity was a function of respiration rate itself or of substrate concentration. The respiration rate was altered, in the presence of excess (133 μM) glucose, by changing the temperature of the oxygen electrode cell. Fig. VI-3 shows that lowering the temperature, and thus the respiration rate, rendered the bacteria more insensitive to cyanide. Again at sub-maximal respiration values there was a range of low cyanide concentrations to which the bacteria were completely insensitive.

(iv) Effect of cyanide on bacteria from batch culture

Earlier reports of insensitivity to cyanide by B. natriegens were based on bacteria grown in batch culture (Weston, Collins and Knowles, 1974). Therefore, B. natriegens was grown on glucose in batch culture and the effect of cyanide tested on bacteria harvested from the logarithmic phase and the early and late stationary phases. Fig. VI-4 shows that the bacteria harvested in the stationary phase had a much lower maximum respiration rate and were also less sensitive to cyanide.

Fig. VI-3. The effect of incubation temperature on the sensitivity of the respiration of glucose grown cells to KCN.

x - x, 31°C; ● - ●, 25°C; o - o, 20.5°C.

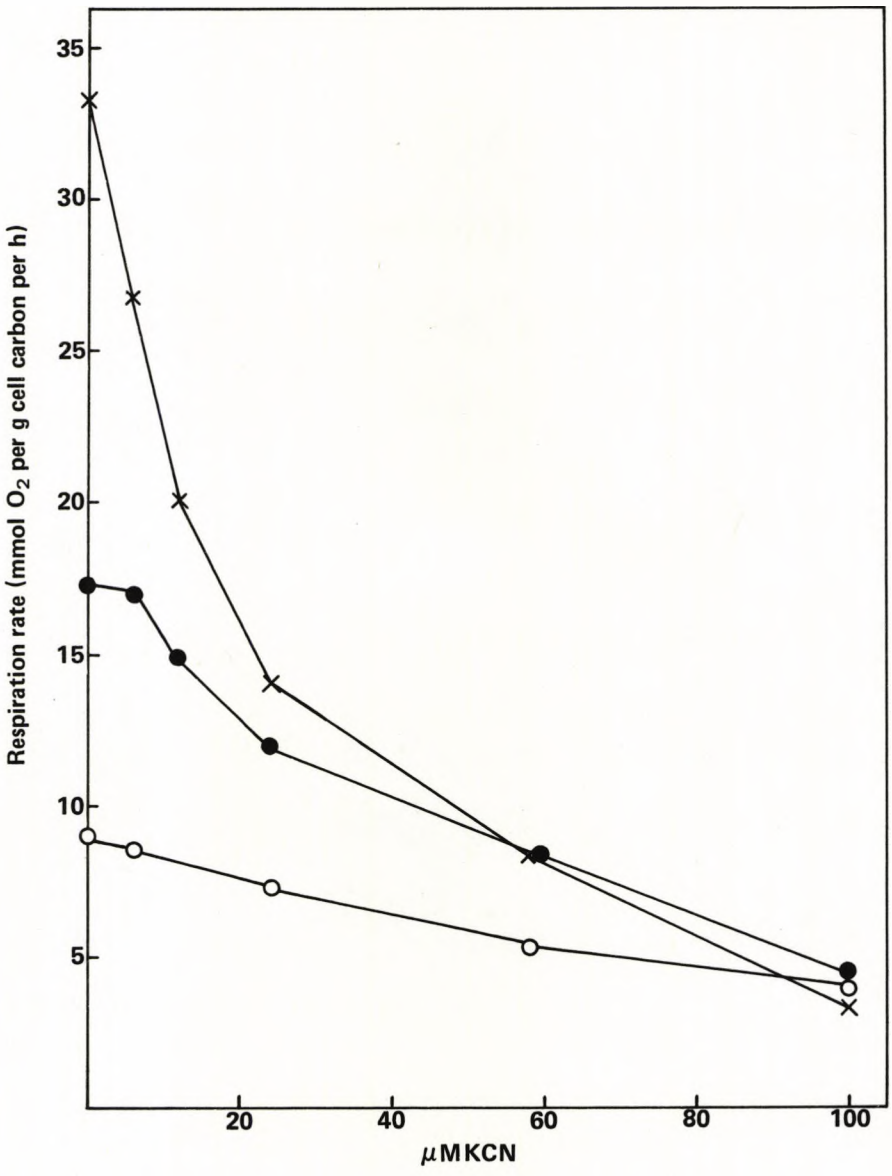

Respiration rate (mmol O_2 per g cell carbon per h)
35
30
25
20
15
10
5
20
40
60
80
100
μMKCN

Fig VI – 3

Fig. VI-4. Cyanide sensitivity of the respiration of Beneckea natriegens taken from log phase, early and late stationary phase batch culture.

Log phase, x - x; early stationary phase (16 h), ● - ●; late stationary phase (24 h), o - o.

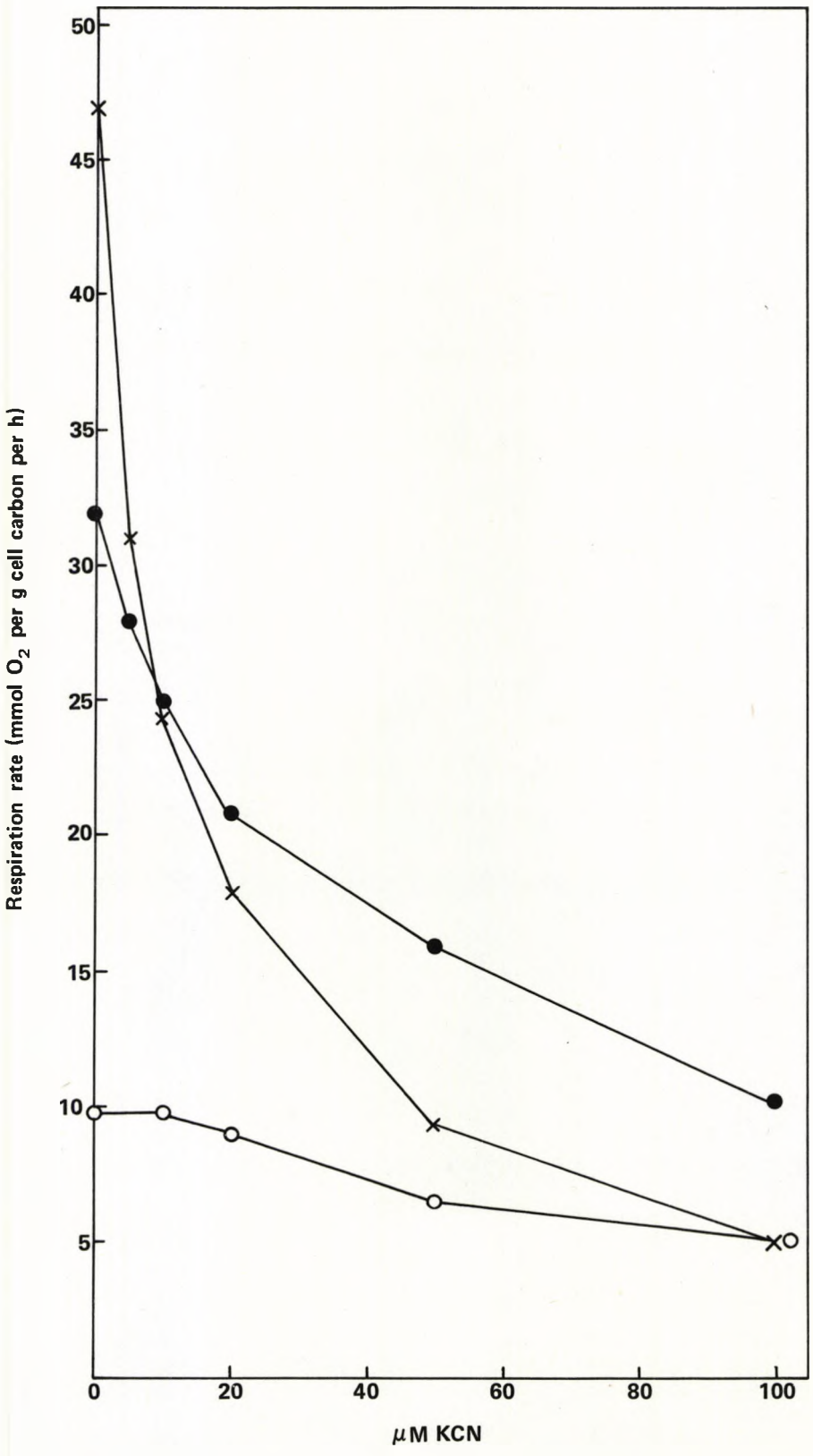

Respiration rate (mmol O_2 per g cell carbon per h)
50
45
40
35
30
25
20
15
10
5
0
20
40
60
80
100
μM KCN

Fig VI – 4

(v) Cyanide sensitivity of bacteria grown in the presence of cyanide

The effect of exposure to cyanide during growth was investigated by adding 50 μM KCN to the medium supplied to a glucose-limited chemostat culture. It has been shown previously that growth and in situ respiration in chemostat culture in the presence of excess oxygen is unaffected by 50 μM KCN but the level of CO-binding cytochrome c in the bacteria is increased significantly (see Section V). The maximum potential respiration rate of harvested bacteria grown in the presence of KCN was very similar to that obtained in the absence of KCN (compare 133 μM glucose rate in Fig. VI-1(a) and VI-5). A similar pattern of sensitivity to cyanide and to combined cyanide and carbon monoxide was also obtained, this being dependent on the respiration rate of the bacteria. However, the extent of sensitivity to the combined effect of carbon monoxide and cyanide was somewhat lower in the cyanide-grown bacteria (Fig. VI-1(c) and VI-5(c)).

The effects of KCN and CO singly and in combination at maximal respiration rates indicated that the combined effect was additive (Table VI-1).

(vi) Respiration of other CO binding cytochrome c containing bacteria

The presence of a CO binding c type cytochrome has been demonstrated in a number of microorganisms that either produce cyanide during growth or are resistant to cyanide (Weston, Collins and Knowles, 1974; Niven, Collins and Knowles, 1975). Cyanide resistance has been observed in the methane utilisers, Pseudomonas methanica and Methylosinus trichosporum and in methanol utilisers, Hyphomicrobium and Pseudomonas extorquens (Weston, 1974). These organisms have been shown to contain a CO binding c type cytochrome. As the presence

Fig. VI-5. The effect of respiration rate on the sensitivity of the respiration of Beneckea natriegens culture in the presence of 50 μM KCN to KCN and CO. The respiration rate was varied by injecting glucose x - x, 133 μM and • - •, 11 μM into the oxygen electrode cell.

Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
20
40
60
80
100
μM KCN
(a)
Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
10
20
30
40
50
per cent CO saturation
(b)
Respiration rate (mmol O_2 per g cell carbon per h.)
5
10
15
20
25
30
35
20
40
60
80
100
μM KCN
5 10 20 40 50
% CO saturation
(c)

Fig VI – 5

Table VI-1. The effect of KCN and CO on the maximum respiration rate of Beneckea natriegens harvested from continuous cultures grown on glucose in the presence and absence of cyanide, and on glycerol

KCN concentration (μM)	Inhibition (%) (A)	CO concentration (% saturation)	Inhibition (%) (B)	KCN + CO (μM,%)	Inhibition observed (%)	Inhibition A + B (%)
			Glucose grown bacteria (in presence of 50 μM KCN)			
6.0	21.0	5.0	3.0	6 + 5	24.0	24.0
12.0	35.0	10.0	15.0	12 + 10	54.0	50.0
24.0	56.0	20.0	18.0	24 + 20	76.0	74.0
48.0	75.0	40.0	20.0	48 + 40	89.0	95.0
100.0	91.0	50.0	24.0	100 + 50	93.0	115.0
			Glucose grown bacteria			
6.0	24.0	5.0	4.5	8 + 5	39.0	28.5
12.0	34.0	10.0	11.2	12 + 10	69.0	45.2
24.0	52.5	20.0	18.0	24 + 20	79.0	70.5
48.0	66.0	40.0	30.25	48 + 40	91.0	96.2
100.0	82.0	50.0	34.0	100 + 50	92.0	116.0
			Glycerol grown bacteria			
6.0	21.4	5.0	2.0	6 + 5	24.0	23.4
12.0	36.0	10.0	15.4	12 + 10	53.4	51.4
24.0	57.0	20.0	18.4	24 + 10	76.7	75.4
48.0	75.4	40.0	19.4	48 + 40	88.0	94.8
100.0	90.5	50.0	24.7	100 + 50	90.0	115.2

of increased levels of CO binding c type cytochrome did not alter the sensitivity of respiration to KCN in B. natriegens it was decided to re-examine the respiratory sensitivity of Pseudomonas extorquens and Methylococcus capsulatus to KCN.

Pseudomonas extorquens was grown in batch culture in mineral salts medium with succinate or methanol as the sole carbon and energy source. Bacteria were harvested during the logarithmic phase of growth and the sensitivity of the potential respiration rate to KCN was examined. Pseudomonas extorquens oxidised methanol rapidly when grown with methanol or succinate as the carbon source. In both cases respiration on methanol was strongly inhibited by KCN, 20 μM causing approximately 60% inhibition of respiration (Fig. VI-6(a)). Succinate grown Ps. extorquens oxidised succinate at approximately 50% the rate observed during methanol oxidation. Respiration on succinate appeared to be more resistant to KCN (Fig. VI-6(a)).

The respiration of methanol by Methylococcus capsulatus, freshly harvested from a chemostat under methane limitation, was strongly inhibited by KCN. A concentration of 20 μM KCN caused 50% inhibition of respiration (Fig. VI-6(b)).

Fig. VI-6. The effect of KCN on Pseudomonas extorquens respiring methanol and succinate and Methylococcus capsulatus respiring methanol in an oxygen electrode cell.

(a) Pseudomonas extorquens grown in batch culture on methanol as the sole carbon and energy source, respiring methanol (98 μM), o - o; and Ps. extorquens grown on succinate as the sole carbon and energy source, respiring methanol (98 μM), x - x; and succinate (178 μM), ● - ●.

(b) Methylococcus capsulatus harvested from a methane limited chemostat culture, respiring methanol (98 μM) x - x.

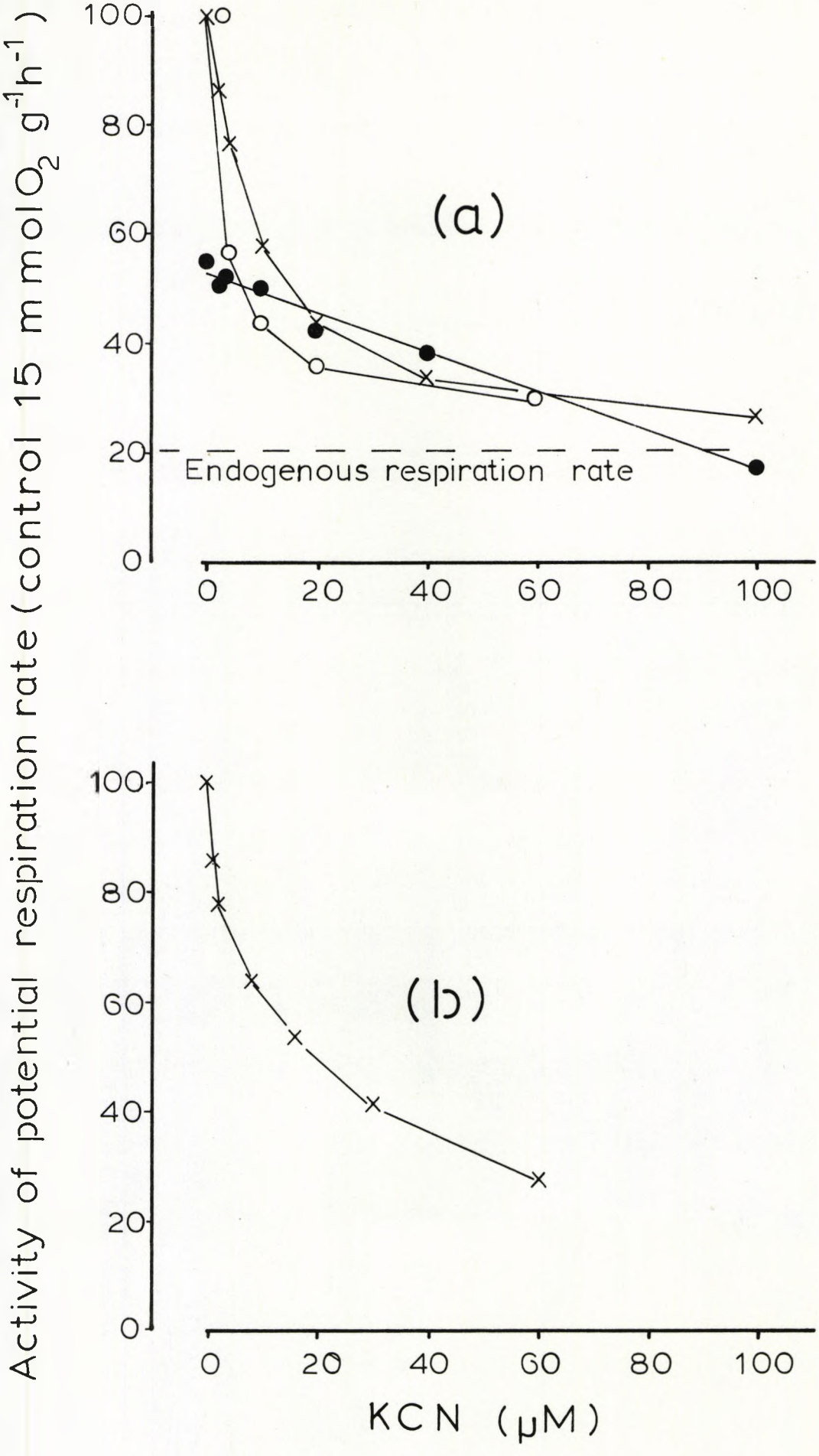

Activity of potential respiration rate (control 15 m mol O_2 $g^{-1}h^{-1}$)
100
80
60
40
20
0
(a)
Endogenous respiration rate
0
20
40
60
80
100
100
80
60
40
20
0
(b)
0
20
40
60
80
100
KCN (μM)

Fig. VI-6

2. Discussion

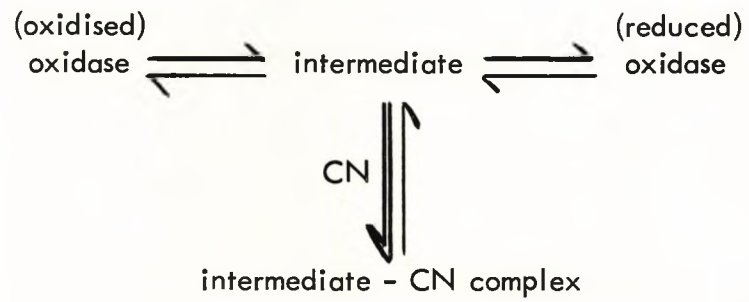
The results reported here indicate a far greater dependence of respiratory sensitivity to cyanide on the actual respiration rate than has been reported previously for whole cells of any species. At maximum respiration rates the effect of KCN and carbon monoxide appeared to be at least additive,(Table VI-1). That is, the combined effect of KCN and CO was equal to a summation of their separate effects. This type of response is usually found when the two inhibitors act on the same enzyme (Nijs, 1967).

The relationship between cyanide concentration and respiration rate at different initial respiration rates was unexpected. There have been few systematic studies reported on the effect of respiration rate on cyanide sensitivity but Jones (1973) reported pure uncompetitive inhibition for physiological substrates, but non-competitive inhibition for ascorbate dichlorophenolindophenol oxidation via cytochrome o in Azotobacter vinelandii. The relationship found in B. natriegens in this work was neither that of simple competitive nor non-competitive inhibition. At sub-maximal respiration rates there is a range of cyanide concentrations to which respiration is completely insensitive but at the point where these curves meet that obtained in the presence of excess glucose, respiration becomes sensitive and followed the same curve as that obtained with excess glucose.

A similar family of curves was obtained with glycerol as the substrate and it is suggested, therefore, that the effect was not due to inhibition of glucose transport by cyanide. Moreover, decreasing the respiration by lowering the incubation temperature also gave a similar series of curves from which it may be

concluded that respiration rate rather than substrate concentration is the important factor. Although not strictly comparable to experiments carried out with B. natriegens, variation of the respiration rate of Pseudomonas extorquens observed when methanol or succinate was the substrate gave a similar pattern of response.

These results indicate an apparent titration of cyanide against a limiting component of the respiratory chain. At maximal respiration rates this component is limiting for respiration rate, therefore low cyanide concentrations cause inhibition. At sub-maximal rates there is an excess of this component which can be titrated with and probably binds to cyanide before any restriction of respiration occurs. This would suggest that the potential respiration rate is limited by a cyanide binding terminal oxidase in this organism. Kauffman and Van Gelder (1973a, b) have suggested that an intermediate species of cytochrome d reacted with cyanide in Azotobacter vinelandii. A similar scheme was proposed by Pudek and Bragg (1974, 1975) for Escherichia coli, thus:-



At high respiration rates a greater proportion of the oxidase would be in the intermediate state and thus cyanide would exert its maximum effect under these conditions. This scheme provides an explanation for the action of cyanide and is consistent with respiration being limited by a cyanide binding terminal oxidase.

Beneckea natriegens grown in the presence of 50 μM KCN contained more CO-binding c-type cytochrome than when grown in its absence, but the maximum respiration rate was not increased. If CO-binding cytochrome c was the rate-limiting component, then either a higher respiration rate or an increased resistance to cyanide would be expected when it is induced. Therefore, this CO-binding c-type cytochrome would not appear to be related to the cyanide-sensitivity or the rate-limiting component in the respiratory system of this organism.

The results reported here do not substantiate earlier reports (Weston, 1974) that the CO binding cytochrome c containing organisms Pseudomonas extorquens and Methylococcus capsulatus have respiratory systems resistant to cyanide. Thus this CO binding cytochrome c does not appear to be related to cyanide resistance in these organisms.

Cytochrome d has been shown by Weston and Knowles (1974) to be a major terminal oxidase in B. natriegens even though it is barely detectable in cytochrome difference spectra. In fact, the turnover number of terminal oxidases is generally so high that respiration may be routed entirely through a cytochrome which is barely detectable in most instruments presently available (Harrison, 1975). Thus, it is possible that cytochrome d was the main terminal oxidase in the cells studied here but that it was present at such low concentrations that the maximum respiration rate was limited by this component. This cytochrome then, would bind with cyanide and respiration rate would depend on the number of unbound sites.

The results reported here are not consistent with an alternative cyanide-insensitive pathway functioning as a major respiratory pathway, as all the cells tested were cyanide-sensitive at high respiration rates. However, there was in each case, a residual cyanide-insensitive respiration amounting to some 10% of the maximum. This may represent the cyanide-insensitive pathway that Weston and Knowles (1974) detected in cell free extracts of B. natriegens.

The results reported in this section indicate that a great deal of caution must be exercised when cyanide is used as an inhibitor in studies of the electron transport system. As Commoner (1940) and Hewitt and Nicholas (1963) have pointed out statements of relative cyanide sensitivity are without meaning unless substrate saturation and potential respiratory activity are taken into account. These considerations have been entirely neglected by workers in the field of bacterial electron transport.

SECTION VII

GROWTH AND RESPIRATION IN THE PRESENCE OF SODIUM FORMATE

1. Metabolism of Formate by *Beneckea natriegens*

   (i) Oxidation of sodium formate by glucose grown bacteria

   (ii) Effect of pH on respiration rate in the presence of formate

   (iii) Utilisation of formate as a carbon and/or energy source

   (iv) Effect of inhibitors on formate oxidation

      (a) Effect of KCN and CO on respiration of formate in an oxygen electrode cell

      (b) Effect of amytal, rotenone and 2-N-heptyl-4-hydroxy quinoline N-oxide on formate oxidation

   (v) Growth on a mixture of glucose and formate in the presence of 10 μM KCN

   (vi) Fluorescence changes observed during metabolism of pulses of formate injected into a glucose limited chemostat culture

   (vii) Measurement of hydrogen evolution during formate oxidation

2. A Formate Non-Oxidising Mutant of *Beneckea natriegens*

   (i) Comparison of respiration, growth yield and cytochrome content of mutant and wild type *Beneckea natriegens* grown in carbon limited chemostat culture

   (ii) Effect of 10 μM KCN on growth and respiration of the formate non-oxidising mutant on a mixture of glucose and formate in chemostat culture

3. Discussion

GROWTH AND RESPIRATION IN THE PRESENCE OF SODIUM FORMATE

Results of experiments reported in Section V indicated that exposure of a glucose limited chemostat culture ($D = 0.37\ h^{-1}$) to 50 μM KCN caused immediate inhibition of respiration, while gradual recovery to the level of the control occurred in approximately 2 hours. Recovery of the respiration rate coincided with an increase in the level of CO-binding c type cytochrome. Although the bacteria were capable of growth in the presence of 50 μM KCN, growth under these conditions caused no significant increase in the potential respiration rate or in resistance to cyanide. A number of workers have demonstrated the presence of CO-binding c type cytochromes in various microorganisms that are either resistant to cyanide or produce KCN during growth (Weston, Collins and Knowles, 1974; Niven, Collins and Knowles, 1975) implying a link between this cytochrome and cyanide resistance (Weston, 1974). The respiration of the methane and methanol utilisers, Pseudomonas methanica, Methylosinus trichosporum, Pseudomonas extorquens and Hyphomicrobium has been reported to be resistant to KCN (Weston, 1974). These organisms have been shown to contain CO-binding c type cytochrome (Tonge, Harrison, Knowles and Higgins, 1975). However, when the sensitivity of respiration on methanol to KCN was examined (Section VI) the results indicated that the respiration of these organisms is strongly inhibited by low KCN concentrations (10 μM KCN). Thus the presence of the CO-binding c type cytochrome does not appear to be related to KCN resistance in these organisms. Tonge, Harrison, Knowles and Higgins (1975) have suggested the involvement of a CO-binding c type cytochrome in one-carbon metabolism. As the increased level of this CO-binding

c type cytochrome did not confer increased resistance to KCN in Beneckea natriegens it is possible that KCN acts as an inducer of one carbon compound metabolism in this organism.

The pathway of glucose dissimilation in Beneckea natriegens is similar to that reported for Escherichia coli (Wang, Stern, Gilmor, et al. 1958). Under oxygen limitation the products of glucose metabolism include ethanol, acetate, pyruvate, lactate and CO_2. The most probable one carbon compound to be encountered by B. natriegens during growth on glucose would be formate. Formate is produced by E. coli and may be metabolised to CO_2 and H_2 under oxygen limited or anaerobic conditions. Therefore, it was decided to investigate the possibility that B. natriegens could utilise formate as a source of energy and carbon.

1. Metabolism of Formate by Beneckea natriegens

(i) Oxidation of sodium formate by glucose grown bacteria

Bacteria harvested from a glucose limited chemostat culture were found to be capable of oxidising sodium formate without prior induction. Injection of 500mg sodium formate into a chemostat at steady state under glucose limitation (pH 7.2) resulted in immediate increases in oxygen uptake and carbon dioxide output by the culture (Fig. VII-1(a)). When this experiment was repeated with a glucose limited culture maintained at steady state at pH 6.1 a much more rapid oxygen uptake and CO_2 output rate was observed (Fig. VII-1(a)). Similar results were observed with a glycerol limited chemostat culture (Fig. VII-1(b)). These effects are unlikely to represent stimulation of respiration on glucose or glycerol as these cultures were strictly carbon limited. Moreover, the evolution of substantial amounts of CO_2 suggested that formate was being oxidised.

(ii) Effect of pH on respiration rate in the presence of formate

Beneckea natriegens was grown in shake flask culture containing 2 gl^{-1} glucose plus 2 gl^{-1} sodium formate (pH 7.2). Bacteria were harvested, washed and resuspended in carbon- and nitrogen-free medium G. The effect of pH on sodium formate oxidation was examined in an oxygen electrode cell. A plot of oxygen uptake rate against pH value is shown in Fig. VII-2. A peak in oxygen uptake rate at pH 5.5 and a shoulder at pH 6.75 were observed; this result may indicate the presence of more than one formate oxidising system in this organism.

Fig. VII-1. The effect of injecting 500 mg sodium formate into a chemostat culture of Beneckea natriegens maintained at steady state under carbon limitation.

(a) Under glucose limitation at pH 6.25 and 7.2

(b) Under glycerol limitation at pH 6.15.

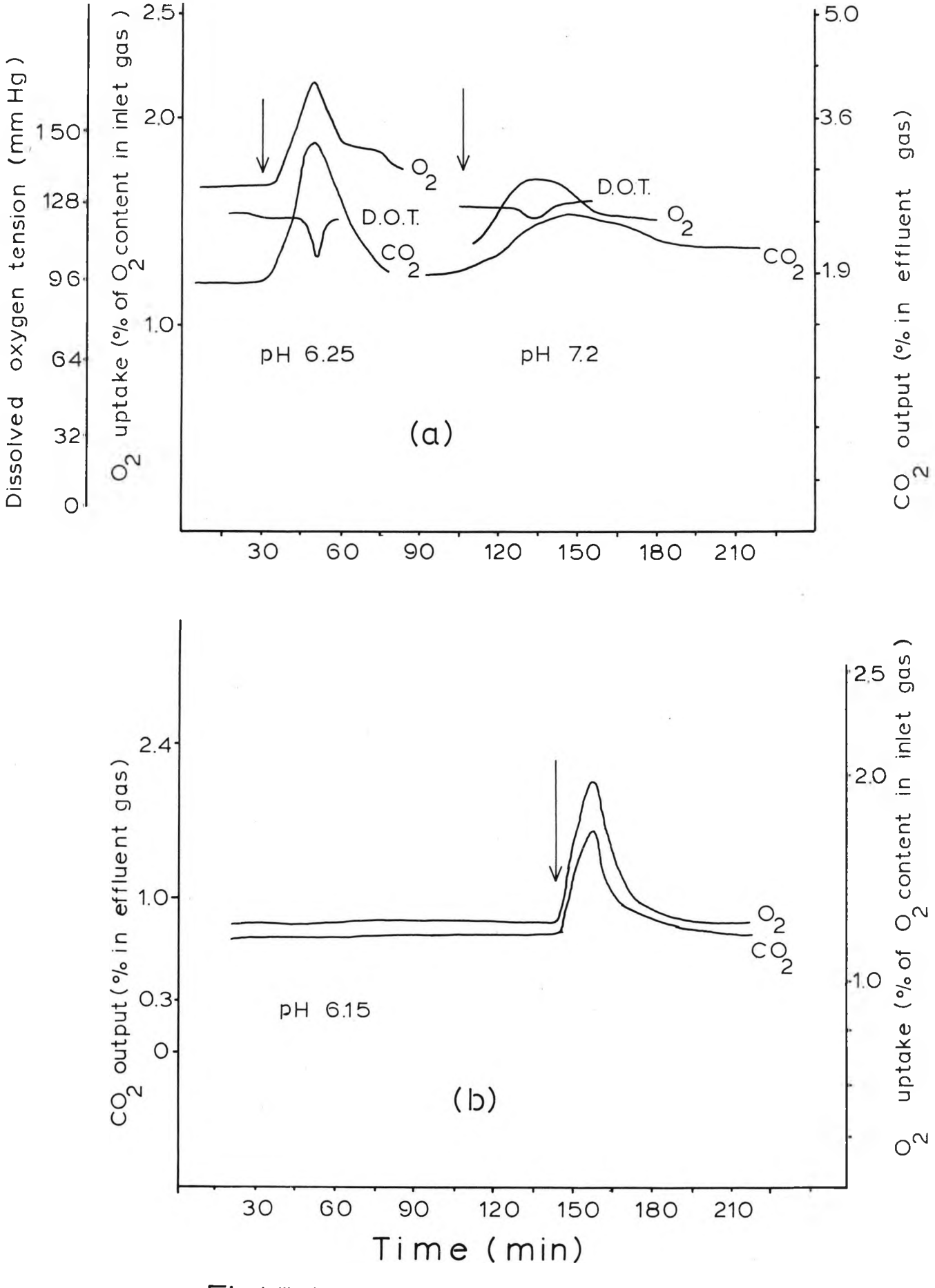

Dissolved oxygen tension (mm Hg)
150
128
96
64
32
0
O2 uptake (% of O2 content in inlet gas)
2.5
2.0
1.0
5.0
3.6
1.9
CO2 output (% in effluent gas)
O2
D.O.T.
CO2
pH 6.25
pH 7.2
(a)
30
60
90
120
150
180
210
CO2 output (% in effluent gas)
2.4
1.0
0.3
0
O2 uptake (% of O2 content in inlet gas)
2.5
2.0
1.0
O2
CO2
pH 6.15
(b)
30
60
90
120
150
180
210
Time (min)

Fig. VII-1

Fig. VII-2. The effect of pH on the rate of oxygen uptake of Beneckea natriegens respiring sodium formate in an oxygen electrode cell. Bacteria were grown in batch culture at 30°C, pH 7.2 in the presence of glucose ($2\ gl^{-1}$) and sodium formate ($2\ gl^{-1}$).

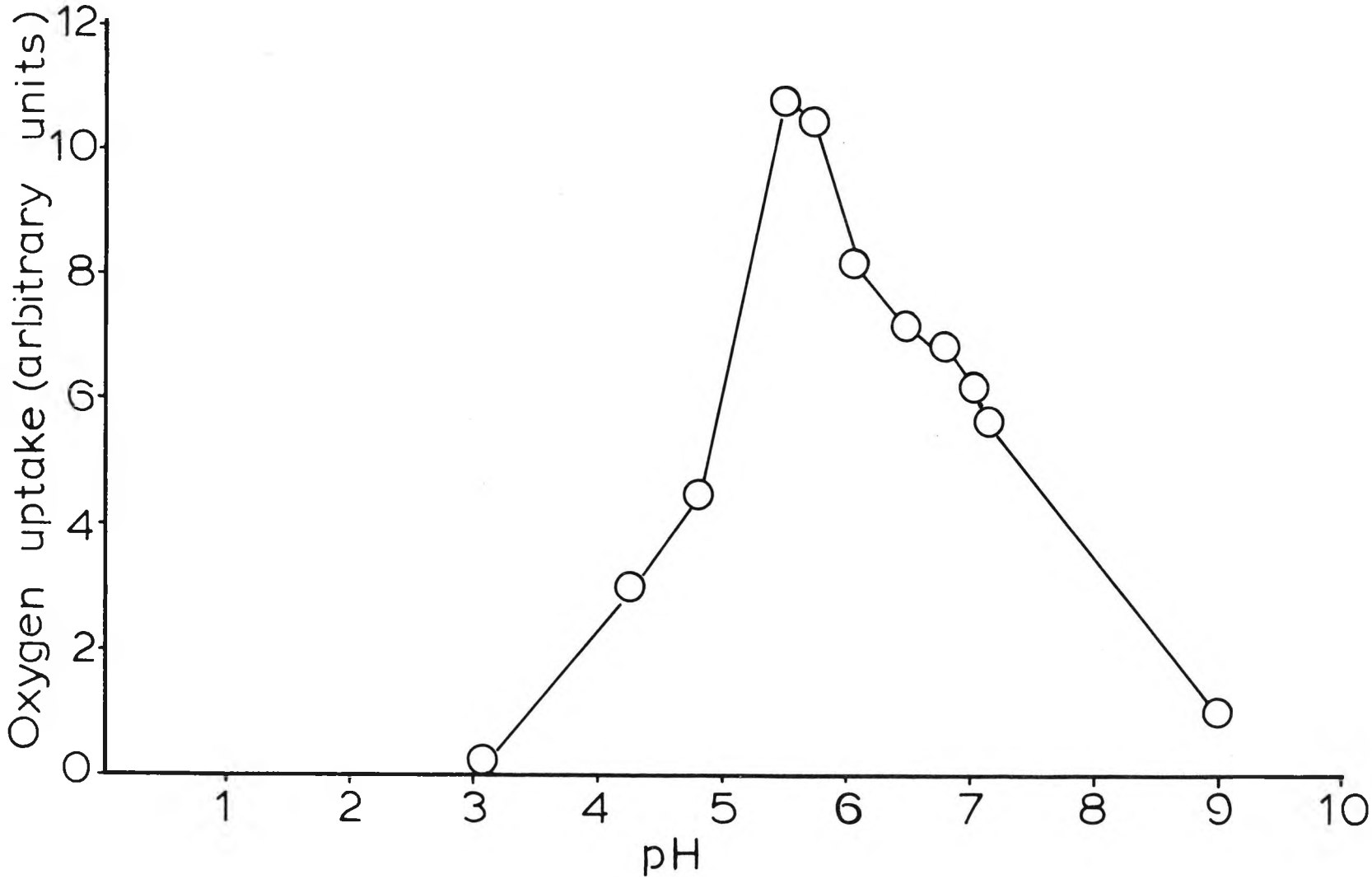


Fig.VII-2

(iii) Utilisation of formate as a carbon and/or energy source

As sodium formate was readily oxidised by B. natriegens the possibility that formate could be utilised as a carbon and energy source was investigated. No growth was obtained in shake flasks containing medium G and sodium formate as the sole carbon and energy source, but Beneckea natriegens grew readily on a mixture of glucose plus sodium formate. A chemostat culture was set up with a mixture of glucose and sodium formate (2 gl^{-1} of each) in the medium feed. Metabolism of the mixed substrates resulted in a rise in the pH which subsequently was controlled by the addition of 5N HCl (metabolism on glucose alone resulted in the production of acid). The molar growth yield, calculated on a basis of glucose only and ignoring the formate used, increased from 39.0 g bacterial carbon per mol glucose to between 42.5 and 43.2 g bacterial carbon per mol on a mixture of glucose plus sodium formate (2 gl^{-1} of each) under carbon limitation in a chemostat at a dilution rate of 0.37 h^{-1} (Table VII-1). The increase in the Y_{glc} was accompanied by a decrease in the q_{glc} 2 from 9.3 in the control to 8.45 mmol glucose per g bacterial carbon per h. There was a significant decrease in the molar growth yield from oxygen (Y_{O_2} 2) from 18.4 g bacterial carbon per mole oxygen in the control to between 14.9 and 15.5 g per mole (Table VII-1). Bacteria grown on the mixture of carbon substrates exhibited 20% increase in the q_{O_2} and a 100% increase in the q_{CO_2}.

To investigate whether the organism could incorporate carbon derived from formate, the formate concentration was increased to 6 gl^{-1} while the glucose concentration was maintained at 2 gl^{-1}. A sudden increase in the sodium

Table VII-1. The effect of sodium formate and glucose on the yield, respiration, cytochrome pattern and cyanide sensitivity of a chemostat culture of Beneckea natriegens (D = 0.37 h^{-1})

	Glucose (2.0 gl^{-1})	Glucose (2.0 gl^{-1}) HCOONa (2.0 gl^{-1})		Glucose (2.0 gl^{-1}) HCOONa (6.0 gl^{-1})	Glucose (2.0 gl^{-1}) HCOONa (4.95 gl^{-1}) + 10 µM KCN	Glucose (2.0 gl^{-1}) HCOONa (6.0 gl^{-1}) + 10 µM KCN
		pH 7.65	pH 6.1			
q_{O_2}* (mmol $g^{-1}h^{-1}$)	19.75	23.7, 23.5,	23.8, 23.8	43.2, 42.6, 43.13, 43.61	56.27 51.12	
q_{CO_2}* (mmol $g^{-1}h^{-1}$)	20.95	38.3, 38.04,	42.0, 42.0	79.2, 78.0, 85.5, 86.5	91.1 82.0	
q_{glc_2}* (mmol $g^{-1}h^{-1}$)	9.3	8.33, 8.26,	8.59, 8.61	8.9, 8.8, 9.09, 9.19	14.7 13.2	
Y_{glc_2}* ($gmol^{-1}$)	39.0	42.37, 42.7,	43.0, 43.2	40.2, 40.8, 44.0, 43.5	24.1 26.9	W A S H O U T
Y_{O_2}* ($gmol^{-1}$)	18.4	14.89, 15.02,	15.5, 15.5	8.31, 8.43 - -	6.31 6.95	
Cytochrome b	1.568	1.830		1.78	0.252	
Cytochrome c	1.792	1.812		1.72	0.280	
Ratio c/b	1.142	0.990		0.96	1.11	
Unmetabolised glucose (gl^{-1})	0.002	0.001, 0.001,	0.004, 0.001	0.004, 0.004, 0.002, 0.002	0.0025 0.0025	1.40 1.84 1.84
Acetic acid (mgl^{-1})	-	- -	- -	- - - -	- -	126 20 10
Ethanol (mgl^{-1})	-	-	-	-	-	10

2* - Calculation based on g bacterial carbon

formate concentration to 6 gl^{-1}, (produced by injecting a solution of sodium formate into the fermenter and simultaneously changing the medium reservoir to one containing 6 gl^{-1} formate), caused an immediate inhibition of growth, the culture absorbance decreased but regained a steady state within 24 h. At this high sodium formate concentration no significant increase in the molar growth yield, expressed in terms of glucose utilised, was found, values between 40.2 and 44 g bacterial carbon per mole glucose carbon being obtained (Table VII-1). The q_{O_2} and q_{CO_2}, however, increased dramatically by 118% and 192% compared to that observed in the control (Table VII-1).

Cytochrome analysis

The relative contents of the different cytochromes of B. natriegens grown on a mixture of glucose and formate was similar to that observed in glucose grown bacteria (Fig. VII-3) with the exception of cytochrome b which appeared to have increased in glucose plus formate grown cells (Table VII-1). Carbon monoxide difference spectra of cells grown on glucose alone or on a mixture of glucose plus formate exhibited peak absorbances at 418 nm indicative of a predominance of cytochrome o.

(iv) Effect of inhibitors on formate oxidation

(a) Effect of KCN and CO on respiration of formate in an oxygen electrode cell

Oxidation of sodium formate by B. natriegens was found to be very sensitive to inhibition by KCN (Fig. VII-4), 10 μM causing approximately 60% inhibition of

Fig. VII-3. Room temperature oxidised minus reduced difference spectra of whole bacteria taken from a chemostat under steady state conditions under carbon limitation.

(a) Oxidised H_2O_2 minus reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$ minus reduced $Na_2S_2O_4$.

(1) Glucose grown bacteria (4.3 mg bacterial carbon ml^{-1})

(2) Glucose plus formate grown bacteria (4.6 mg bacterial carbon ml^{-1}).

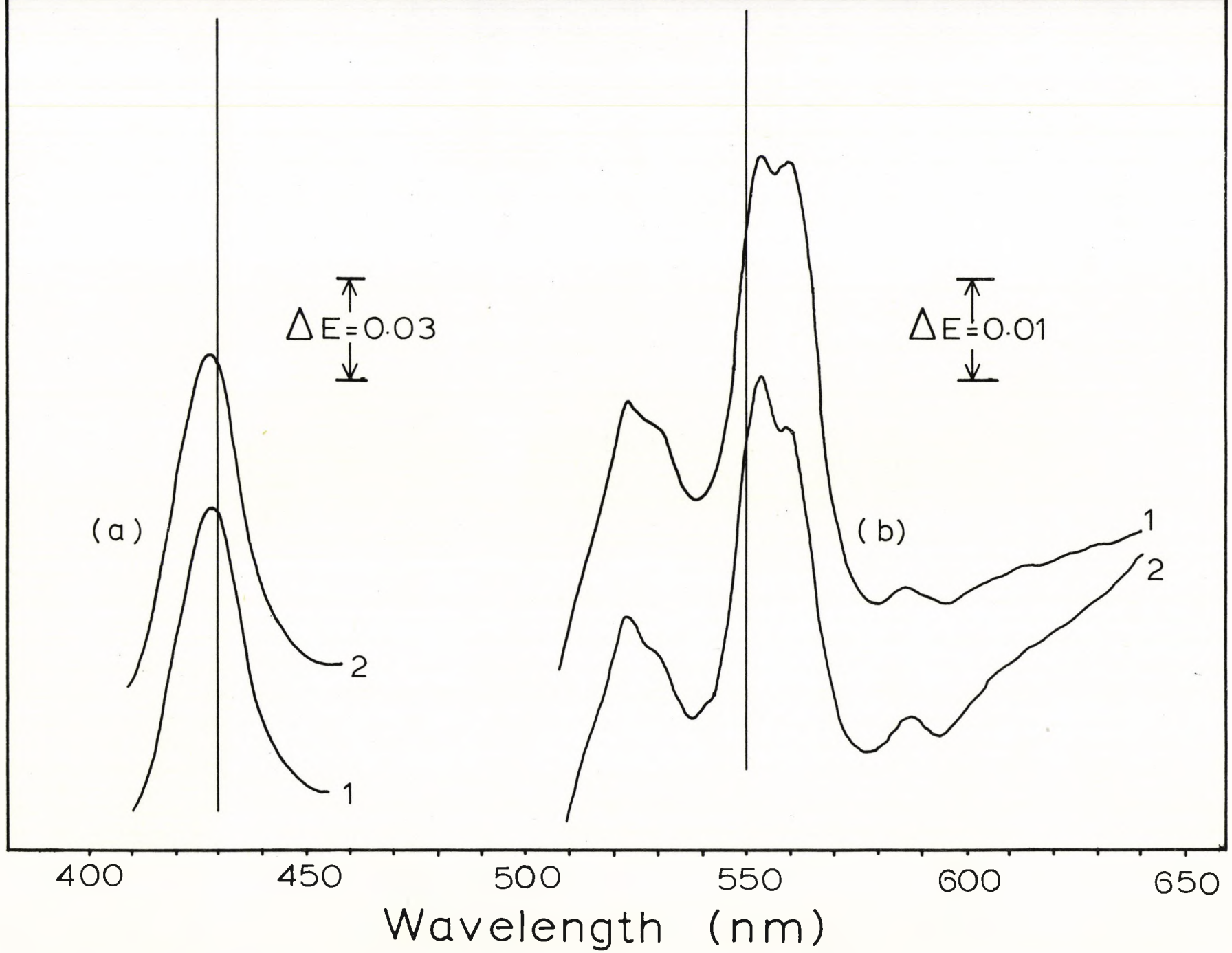


Fig. VII-3

Fig. VII-4. The effect of KCN and CO on the oxidation of sodium formate (1.5 mM) by Beneckea natriegens in an oxygen electrode cell.

Bacteria were harvested from a batch culture grown on a mixture of glucose plus sodium formate (2 gl^{-1} of each) pH 7.2, 30°C. The effect of KCN, x - x; CO, • - • and KCN plus CO, o - o.

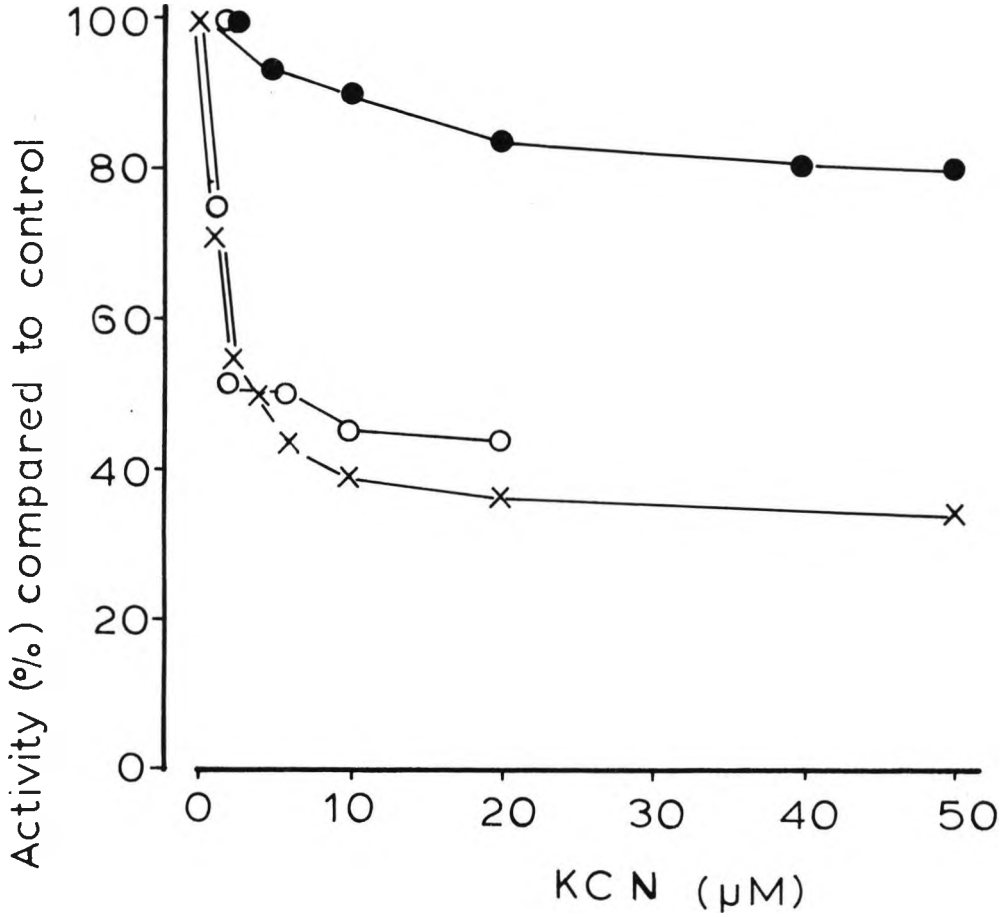

100
80
60
40
20
0
Activity (%) compared to control
0
10
20
30
40
50
KCN (μM)

Fig. VII-4

respiration. Carbon monoxide had a less marked effect, a 50% CO saturated buffer caused 20% inhibition of respiration. A combination of KCN and CO did not have an additive effect as seen when glucose was the substrate (see Section VI). Indeed the combined effect of KCN and CO appeared to be less than that observed with each inhibitor alone (Fig. VII-4).

(b) Effect of amytal, rotenone and 2-N-heptyl-4-hydroxyquinoline N-oxide on formate oxidation

The oxidation of sodium formate and NADH + H^+ by a sonicated bacterial suspension of B. natriegens (glucose plus formate, batch grown) was unaffected by high concentrations of amytal and rotenone but was completely inhibited by low concentrations of 2-N-heptyl-4-hydroxyquinoline N-oxide (Table VII-2).

(v) Growth on a mixture of glucose and formate in the presence of 10 μM KCN

A glucose limited chemostat culture of B. natriegens has been shown to be unaffected by 10 μM KCN in the medium feed (Section V). However, as 10 μM KCN caused approximately 60% inhibition of respiration on formate in the oxygen electrode system, its effect on respiration and yield of a culture growing on a mixture of glucose and sodium formate was examined. KCN was added (to give a final concentration of 10 μM) to a culture maintained at steady state on a mixture of glucose (2 gl^{-1}) and sodium formate (4.95 gl^{-1}) and 10 μM KCN was added to the medium supplied to the culture. An immediate effect on respiration was evident but the culture attained a steady state within 24 h in the presence of 10 μM KCN (Fig. VII-5).

Table VII-2. The effect of amytal, rotenone and 2-N-heptyl-4-hydroxyquinoline N-oxide on respiration of NADH and formate by a sonicated suspension of *Beneckea natriegens*

Substrate	Respiration rate (μmoles O_2 min^{-1})	Inhibitor	Inhibition (%)
NADH (2 μmoles)	191	Rotenone (0.6 mM)	36
Sodium formate (45 mM)	90	Rotenone (0.6 mM)	0
NADH (7 μmoles)	336	Amytal (20 mM)	2
Sodium formate (45 mM)	92	Amytal (20 mM)	0
NADH (2 μmoles)	210	2N-Heptyl-4-hydroxyquinoline N-oxide (2.8 μM)	100
Sodium formate (45 mmoles)	90.65	2N-Heptyl-4-hydroxyquinoline N-oxide (2.8 μM)	100

Fig. VII-5. The effect of 10 μM KCN on the respiration of a chemostat culture of Beneckea natriegens growing on a mixture of glucose plus formate. KCN was injected into the chemostat culture of Beneckea natriegens at steady state, on a mixture of glucose plus formate (2 gl^{-1} of each) to give a final concentration of 10 μM. The medium feed was simultaneously changed to one containing 10 μM KCN.

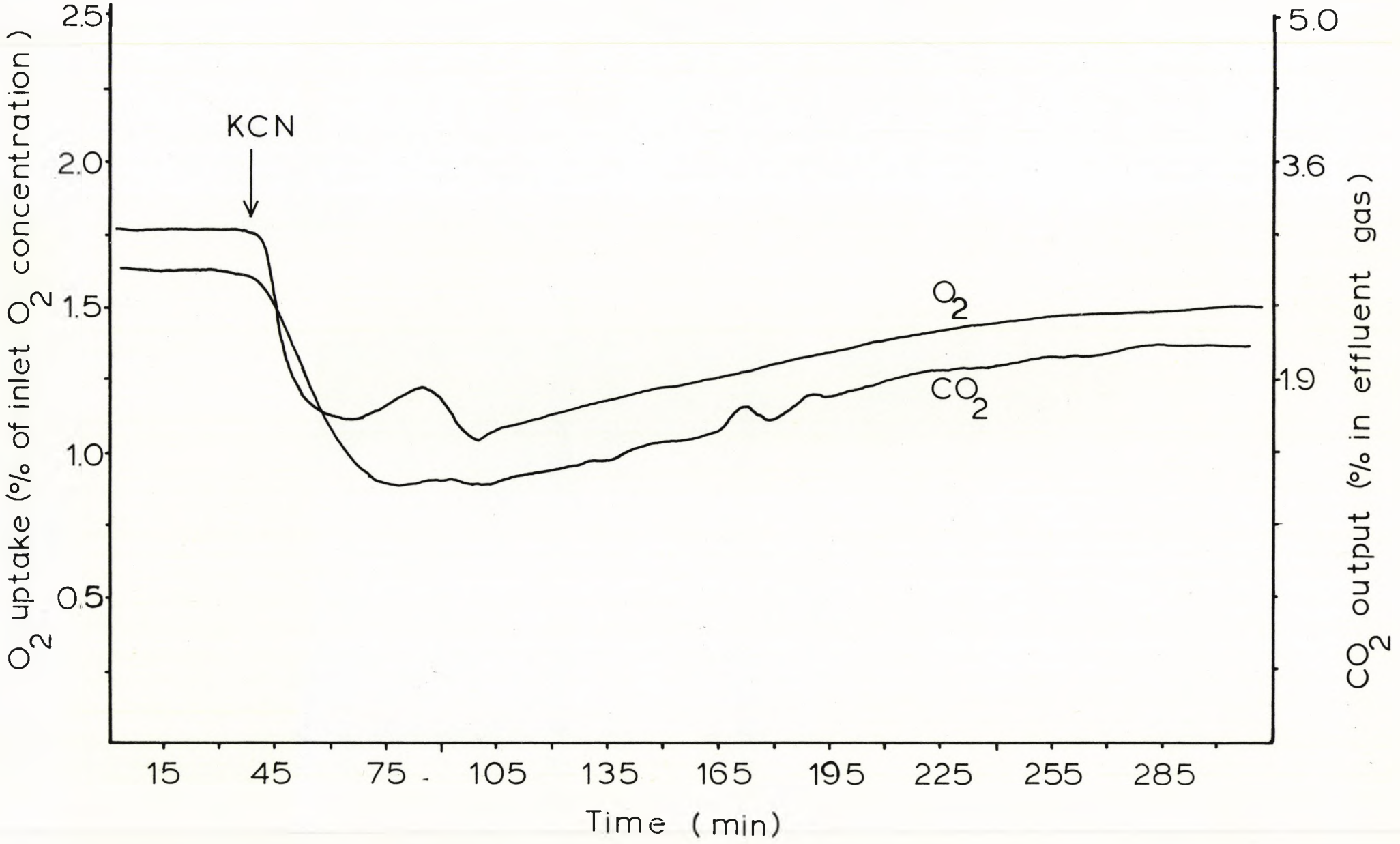

2.5
2.0
1.5
1.0
0.5
O_2 uptake (% of inlet O_2 concentration)
KCN
O_2
CO_2
5.0
3.6
1.9
CO_2 output (% in effluent gas)
15
45
75
105
135
165
195
225
255
285
Time (min)

Fig.VII-5

In the presence of 10 μM KCN the molar growth yield, expressed on the basis of the glucose utilised and ignoring the formate, decreased markedly to 25.5 g bacterial carbon per mol glucose carbon compared with 43 g per mol in the control. The culture utilised virtually all the glucose and this was accompanied by a marked increase in the respiration rate. No acetate or ethanol was detected in the culture effluent. The $q_{O_2}2$ and $q_{CO_2}2$ increased to values between 51 and 56 and 82 and 91 mmol $g^{-1}h^{-1}$ respectively. This was reflected as a decrease in $Y_{O_2}2$ to approximately 6.5 g mol^{-1} (Table VII-1). Similarly the q_{glc} increased reflecting a diminished yield on glucose.

When the formate concentration (of the glucose plus formate mixture) was increased to 6 gl^{-1} in the presence of 10 μM KCN, growth was inhibited and the culture washed out. Acetic acid and ethanol were detected initially but these decreased as the glucose concentration in the effluent increased to that present in the medium feed. A carbon balance for the growth of B. natriegens in a mixture of glucose and formate in the presence and absence of KCN is shown in Table VII-3.

Cytochrome analysis

The cytochrome content of bacteria grown in the presence of 10 μM KCN (on a mixture of glucose plus sodium formate) decreased by 85% compared with the control (Fig. VII-6). However, the ratio of cytochrome c:cytochrome b was similar to that observed in the absence of KCN (Table VII-1).

Table VII-3. A carbon recovery for the growth of Beneckea natriegens on a mixture of glucose plus sodium formate in the presence and absence of 10 μM KCN in chemostat culture

Growth substrate(s)	Carbon input (gh^{-1}) glucose	formate	citrate	= Bacterial carbon (gh^{-1})	+ Millipore filtrate carbon (gh^{-1}) product + dissolved CO_2		Carbon dioxide carbon (gh^{-1})	Recovery (%)
Glucose 2.0 gl^{-1} pH 7.5	0.664	-	0.030	0.361	0.003	0.037	0.214	88.6
	0.677	-	0.030	0.379	0.060	0.037	0.227	94.2
Glucose 2.0 gl^{-1} HCOONa 2.0 gl^{-1} pH 7.65	0.649	0.289	0.029	0.382	0.072	0.103	0.386	97.5
	0.649	0.289	0.029	0.385	0.072	0.119	0.386	99.4
Glucose 2.0 gl^{-1} HCOONa 2.0 gl^{-1} pH 6.1	0.684	0.305	0.030	0.411	0.063	0.001	0.504	96.0
	0.684	0.305	0.030	0.411	0.063	0.001	0.504	96.0
Glucose 2.0 gl^{-1} HCOONa 6.0 gl^{-1}	0.453	0.789	0.027	0.252	0.297	0.026	0.668	97.9
	0.453	0.789	0.027	0.256	0.293	0.026	0.668	97.9
	0.453	0.789	0.027	0.263	0.293	0.026	0.668	98.5
	1.547			0.377	0.207		0.965	100.0
Glucose 2.0 gl^{-1} HCOONa 4.95 gl^{-1} + 10 μM KCN	0.551	0.642	0.026	0.184	0.470	0.026	0.565	102.0
	0.551	0.642	0.026	0.206	0.473	0.026	0.575	105.0
Glucose 2.0 gl^{-1} HCOONa 6.0 gl^{-1} + 10 μM KCN	1.531			1.59				103.85

Fig. VII-6. Room temperature oxidised minus reduced difference spectra of whole bacteria taken from a chemostat under steady state conditions in the presence of glucose alone, glucose + formate and glucose + formate + 10 μM KCN.

(a) Oxidised H_2O_2 minus reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$ minus reduced $Na_2S_2O_4$.

(1) Bacteria taken from a glucose limited chemostat (4.3 mg bacterial carbon ml^{-1})

(2) Bacteria taken from a chemostat at steady state with glucose 2 gl^{-1} and formate 4.95 gl^{-1} in medium feed (4.6 mg bacterial carbon ml^{-1})

(3) Bacteria taken from a chemostat with glucose 2 gl^{-1} and formate 4.95 gl^{-1} + 10 μM KCN in medium feed (10.7 mg bacterial carbon ml^{-1}).

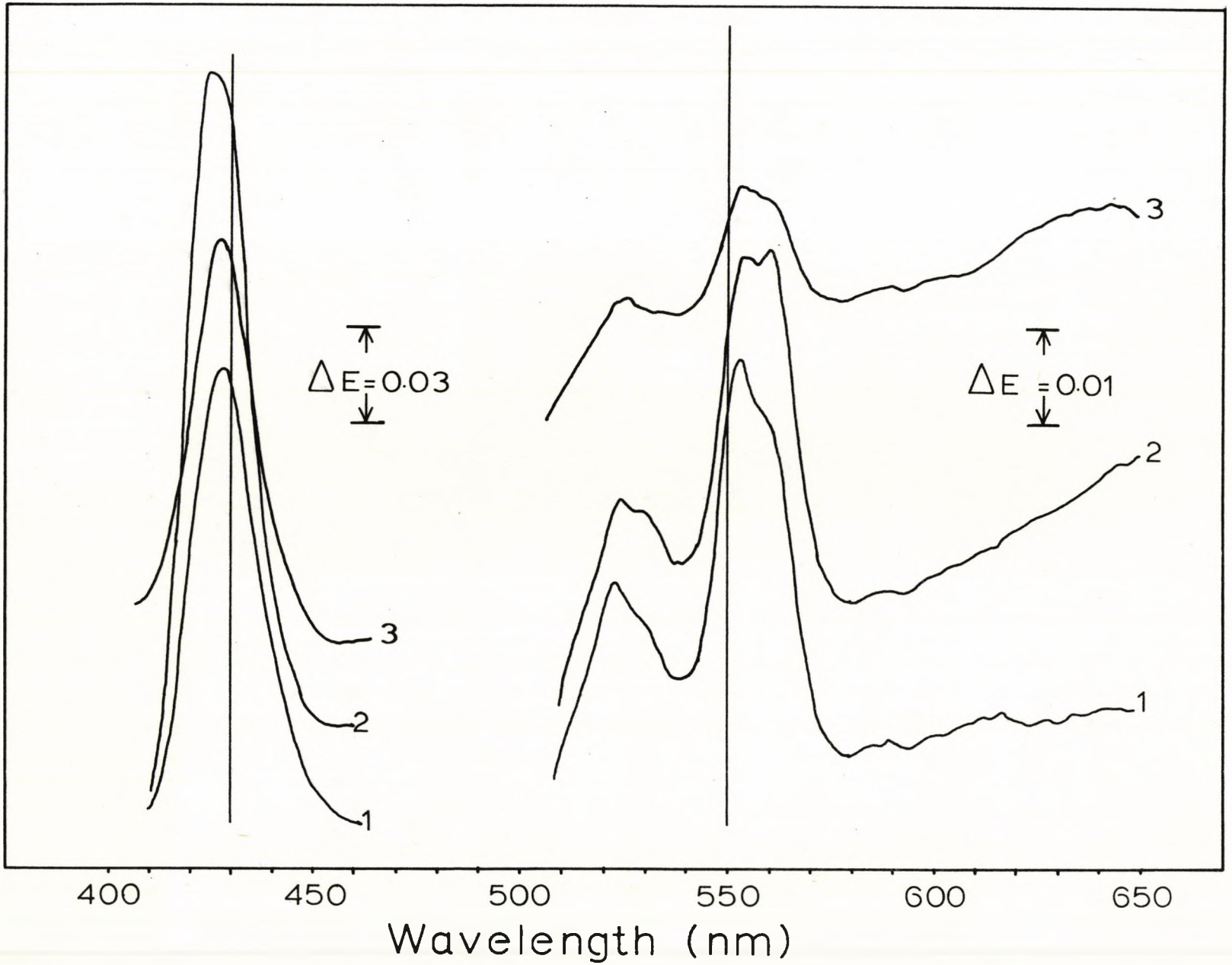


Fig.VII-6

(vi) Fluorescence changes observed during metabolism of pulses of formate injected into a glucose limited chemostat culture

In aerobic bacteria growing on oxalate or C_1 compounds the only formate dehydrogenases reported are NAD linked (Quayle, 1972). Therefore, it was decided to examine the effect of formate on the NAD(P)H fluorescence of a glucose limited chemostat culture of B. natriegens.

The injection of 1 g sodium formate directly into a fermenter maintained under glucose limitation caused immediate changes in oxygen uptake and CO_2 output; return to the original levels occurred after approximately 50 mins (Fig. VII-7). These changes were accompanied by an increase in NAD(P)H + H^+ fluorescence in the culture (Fig. VII-8). The level of fluorescence returned to the base line in about 50 min. When KCN (to give a final concentration of 10 μM) was injected simultaneously with 1 g sodium formate no significant change in fluorescence was observed, but oxygen uptake and CO_2 output were inhibited by approximately 40% (Fig. VII-7).

(vii) Measurement of hydrogen evolution during formate oxidation

In Escherichia coli formate may be metabolised under anaerobic conditions via formic hydrogenlyase complex to H_2 and CO_2. The possibility that this reaction occurred under aerobic conditions in B. natriegens was investigated.

A catharometer system (see Methods) was set up to measure the presence of hydrogen in the effluent gas phase. Hydrogen was not detected during formate metabolism, either when formate was injected into the fermenter or during steady state conditions. The limit of detection was approximately 0.01% hydrogen in the gas phase.

Fig. VII-7. The effect of injecting 1 g sodium formate directly into a chemostat maintained under glucose limitation on oxygen uptake and CO_2 output and the effect of 10 μM KCN on this oxidation.

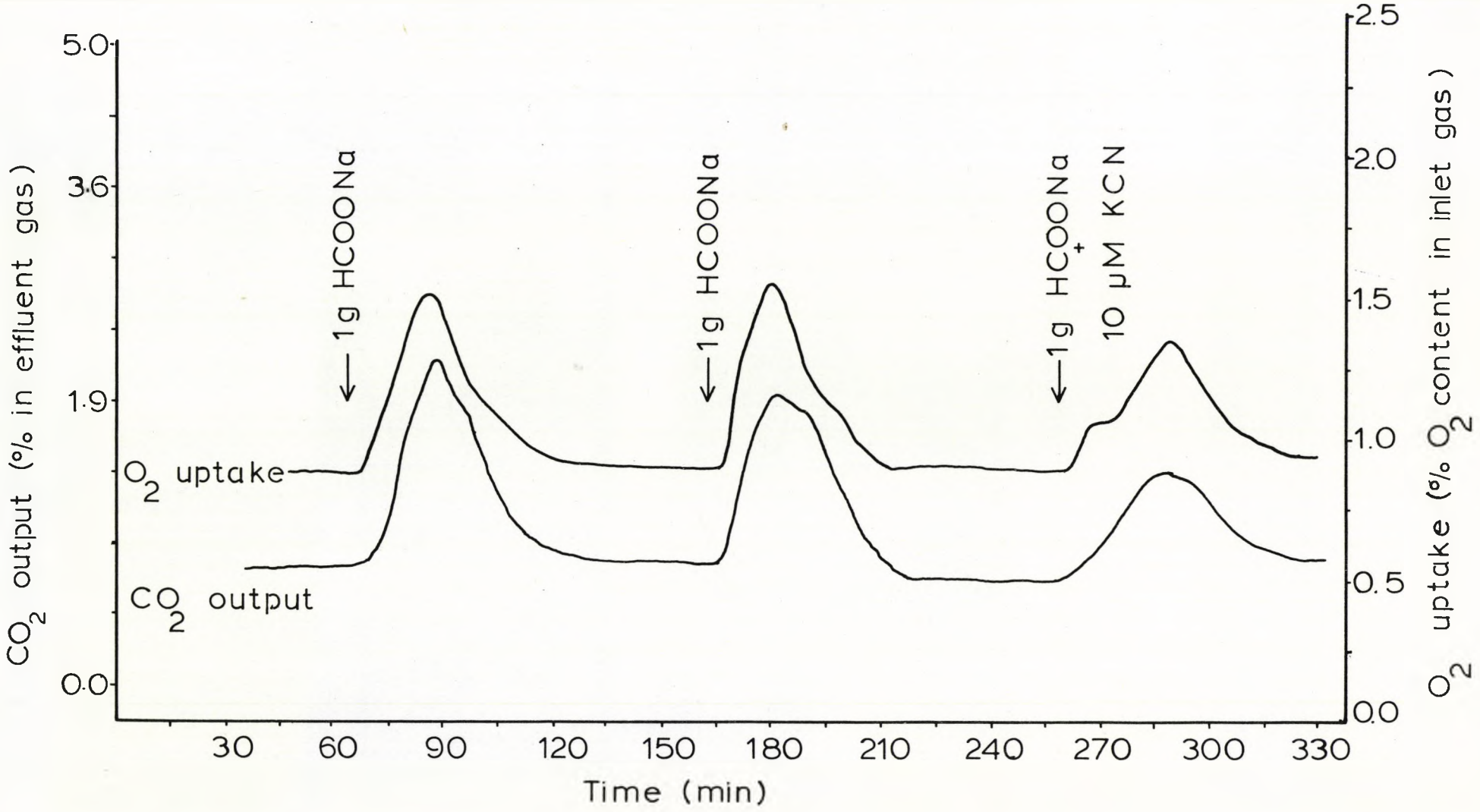

5.0
3.6
1.9
0.0
CO2 output (% in effluent gas)
2.5
2.0
1.5
1.0
0.5
0.0
O2 uptake (% O2 content in inlet gas)
1g HCOONa
1g HCOONa
1g HCOONa
10 μM KCN
O2 uptake
CO2 output
30
60
90
120
150
180
210
240
270
300
330
Time (min)

Fig.VII-7

Fig. VII-8. The effect of injecting 1 g sodium formate directly into a chemostat, maintained under glucose limitation, on fluorescence measured as NAD(P) H generated.

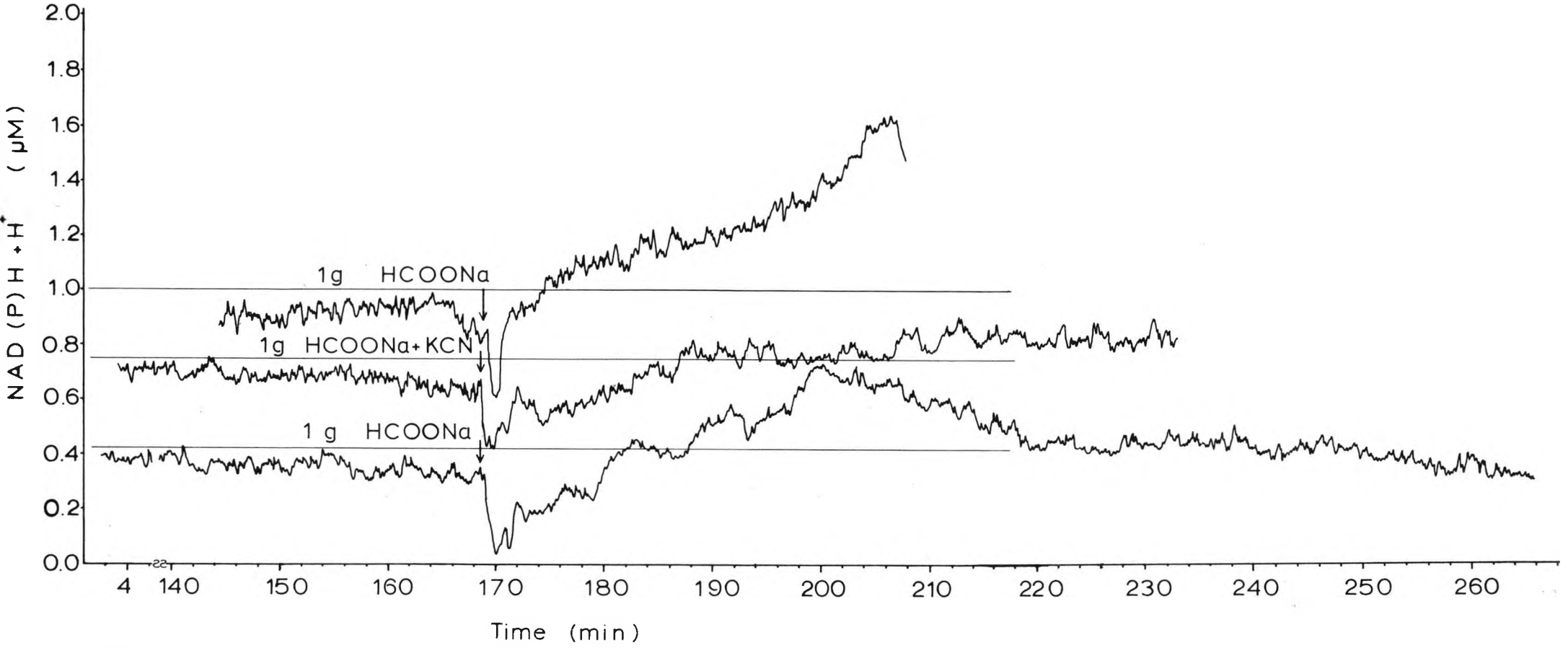

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
NAD (P) H + H+ (μM)
1g HCOONa
1g HCOONa+KCN
1 g HCOONa
4
140
150
160
170
180
190
200
210
220
230
240
250
260
Time (min)

Fig.VII-8

2. A Formate Non-oxidising Mutant of Beneckea natriegens

In the presence of sodium formate the growth of Beneckea natriegens was inhibited by 10 μM KCN. A mutant that could not metabolise formate was isolated (see Methods), in order to find out whether it was formate per se, the metabolism of formate, or, a complex between formate and KCN, which caused increased sensitivity to KCN. The formate negative mutant was grown in the presence of formate and KCN.

(i) Comparison of respiration, growth yield and cytochrome content of mutant and wild type Beneckea natriegens grown in carbon limited chemostat culture

The mutant was grown in a chemostat under glucose limitation, at a dilution rate of 0.37 h^{-1}. The molar growth yield, the q_{O_2}, qCO_2, q_{glc} and Y_{O_2} of the mutant were similar to that observed for the wild type bacterium (Table VII-4). The potential respiration rate of the mutant appeared to be similar to that of the control, however it was slightly more resistant to the effect of KCN (Fig. VII-12).

Cytochrome analysis

Cytochrome analysis indicated that the mutant grown on glucose had a cytochrome complement indistinguishable from that of the wild type (Fig. VII-9). The ratio of cytochrome c:cytochrome b were also similar (Table VII-4).

Injection of sodium formate into the fermenter at steady state under glucose limitation did not result in increased oxygen uptake or CO_2 output by the mutant.

Fig. VII-9. Room temperature oxidised minus reduced difference spectra of whole bacteria taken from a chemostat under steady state conditions in the presence of glucose alone and glucose + formate.

(a) Oxidised H_2O_2 minus reduced $Na_2S_2O_4$; (b) oxidised $K_3Fe(CN)_6$ minus reduced $Na_2S_2O_4$.

(1) Wild type, glucose grown (4.30 mg ml^{-1} bacterial carbon)

(2) Formate-negative mutant, glucose grown (4.37 mg ml^{-1} bacterial carbon)

(3) Formate-negative mutant, glucose and formate-plus cyanide grown (4.25 mg ml^{-1} bacterial carbon).

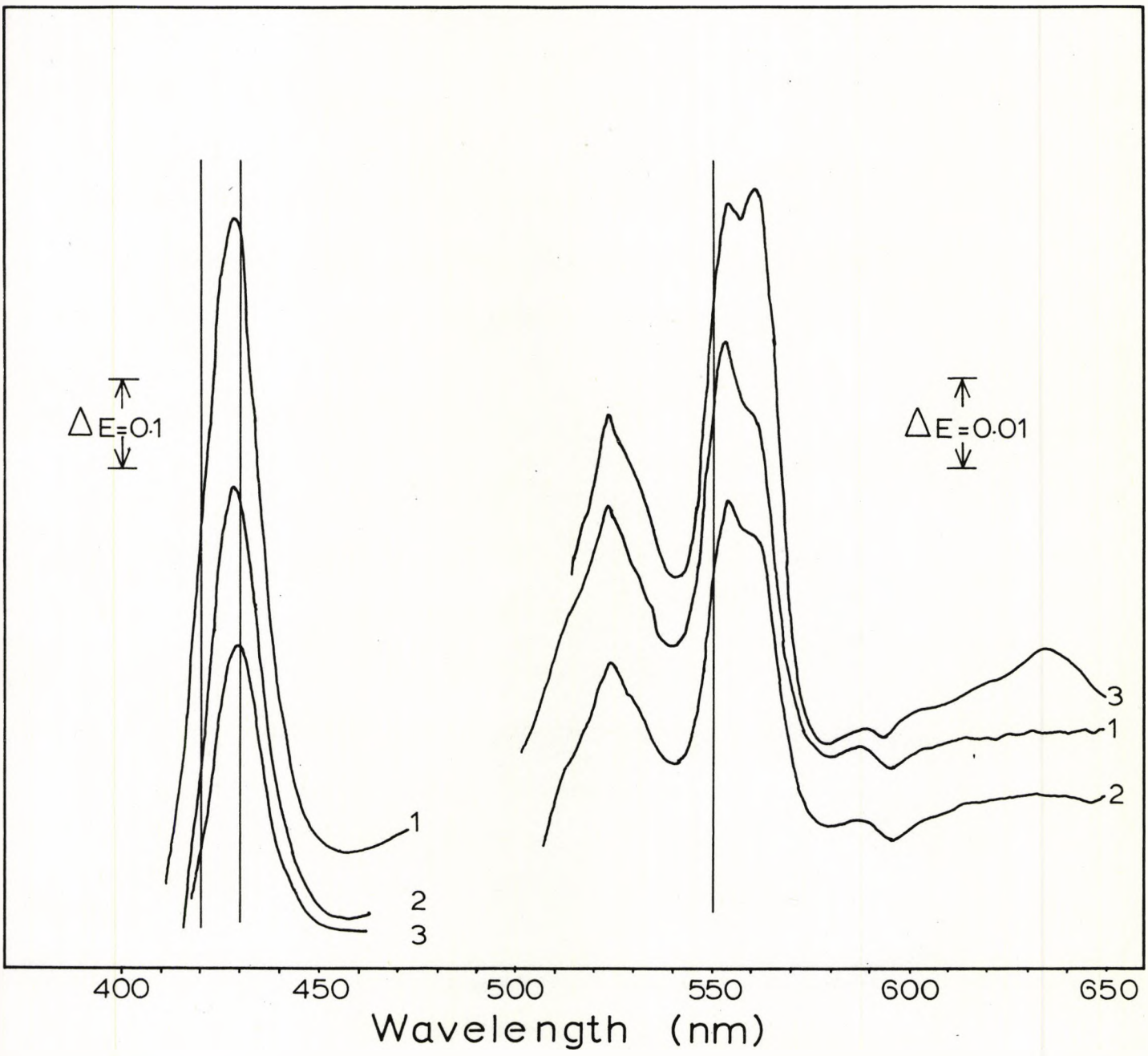


Fig. VII-9

Table VII-4. The growth of a formate negative mutant of Beneckea natriegens in a chemostat on glucose alone and on a mixture of glucose, formate and 10 μM KCN ($D = 0.37\ h^{-1}$)

Physiological parameters	Substrate concentration and growth conditions	
	Glucose 2.0 gl^{-1}	Glucose, 2.0 gl^{-1}; sodium formate, 6 gl^{-1}; 10 μM KCN (After 54 h exposure)
q_{O_2}2* (mmol $g^{-1}h^{-1}$)	18.87 18.48	22.2
q_{CO_2}2* (mmol $g^{-1}h^{-1}$)	18.87 18.34	23.4
q_{glc}2* (mmol $g^{-1}h^{-1}$)	9.82 9.62	10.5
Y_{glc}2* (g mol^{-1})	38.13 39.4	38.3
Y_{O_2}2* (g mol^{-1})	20.16 20.54	18.13
Cytochrome b (ΔE/mg bacterial carbon)	1.7	2.9
Cytochrome c (ΔE/mg bacterial carbon)	1.85	2.64
Ratio c/b	1.08	0.91
Glucose in effluent (gl^{-1})	0.001	0.001

2* - Calculation based on g bacterial carbon.

(ii) Effect of 10 μM KCN on growth and respiration of the formate non-oxidising mutant on a mixture of glucose and formate in chemostat culture

A mixture of cyanide and sodium formate was injected into a glucose limited culture of the mutant to give a final concentration of 6 gl^{-1} sodium formate and 10 μM KCN respectively. The medium reservoir was simultaneously switched to one containing 6 gl^{-1} sodium formate, 10 μM KCN and 2 gl^{-1} glucose. This procedure was adopted, although it is not strictly comparable with the experiment made with the wild type organism, because growth of the formate non-utilising mutant in the presence of sodium formate would facilitate selection of the wild type. The sudden exposure of the culture to formate (6 gl^{-1}) and 10 μM KCN caused depression of the molar growth yield. After 16 h exposure the Y_{glc} was 27.5 g bacterial carbon per mole glucose, and this increased beyond that of the control before reaching a stable value similar to that of the control (mutant on glucose alone) after approximately 54 h (Fig. VII-10). The q_{O_2} and q_{CO_2} did not exhibit the large increases observed for the wild type. There was a small decrease in the respiration rate but this recovered after 54 h to a value close to that found on glucose alone (Fig. VII-10).

The molar growth yield from oxygen, $Y_{O_2}2$, was 15 g bacterial carbon per mole oxygen after 16 h exposure to 10 μM KCN plus 6 gl^{-1} sodium formate but recovered nearly to the control (mutant on glucose alone) value after 54 h (Fig. VII-10).

Fig. VII-10. The effect of injecting cyanide and formate into a glucose limited chemostat culture of the formate-negative mutant of Beneckea natriegens. Final concentrations were 10 μM KCN and 6 gl^{-1} formate. The medium was simultaneously changed to one containing 10 μM KCN, 2 gl^{-1} glucose and 6 gl^{-1} formate.

Y_{glc}, • - •; q_{O_2}, x - x; q_{CO_2}, Δ-Δ; Y_{O_2}, o - o; and q_{glc}, □-□.

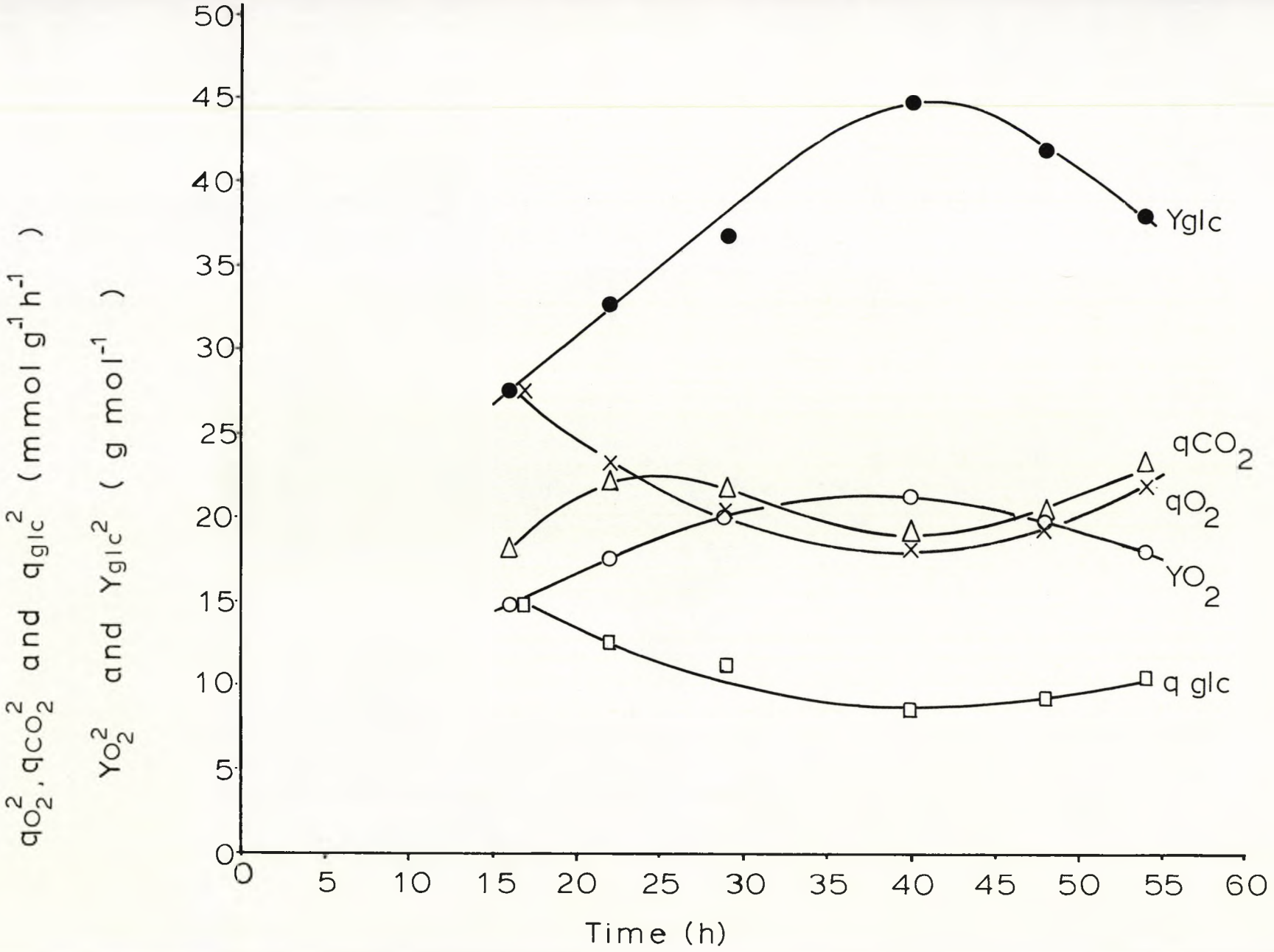

qo_2^2, qco_2^2 and q_{glc}^2 (mmol g^{-1} h^{-1})
Yo_2^2 and Y_{glc}^2 (g mol^{-1})
Time (h)
Yglc
qCO_2
qO_2
YO_2
q glc
0
5
10
15
20
25
30
35
40
45
50
55
60

Fig. VII-10

The initial fall in the Y_{glc} was accompanied by an increase in the q_{glc}, however the q_{glc} returned to a value similar to that of the control after 40 h exposure to formate plus cyanide.

Cytochrome analysis

Exposure of the formate negative mutant to 10 μM KCN plus 6 gl^{-1} sodium formate caused a large increase in both b and c type cytochrome levels (Fig. VII-9). The ratio of cytochrome c to cytochrome b decreased to 0.91 (Table VII-4), a value similar to that observed when the wild type was grown on a mixture of glucose plus formate (Table VII-1).

Carbon monoxide difference spectra demonstrated absorbance peaks at 417.5 nm, 416 nm and 417.5 nm for the wild type grown on glucose, the mutant grown on glucose and the mutant grown on a mixture of glucose, formate and 10 μM KCN respectively (Fig. VII-11).

A carbon balance for the growth of the mutant on glucose alone and in a mixture of glucose, formate and 10 μM KCN is shown in Table VII-5. The carbon analysis indicated that the formate was not utilised significantly.

Fig. VII-11. Room temperature reduced plus CO minus reduced difference spectra of whole cells taken from a chemostat under steady state conditions in the presence of glucose alone and glucose + formate.

(1) Wild type bacteria, glucose grown (4.30 mg ml^{-1} bacterial carbon)

(2) Mutant bacteria, glucose grown (4.37 mg ml^{-1} bacterial carbon)

(3) Mutant bacteria, glucose and formate plus cyanide grown (4.25 mg ml^{-1} bacterial carbon)

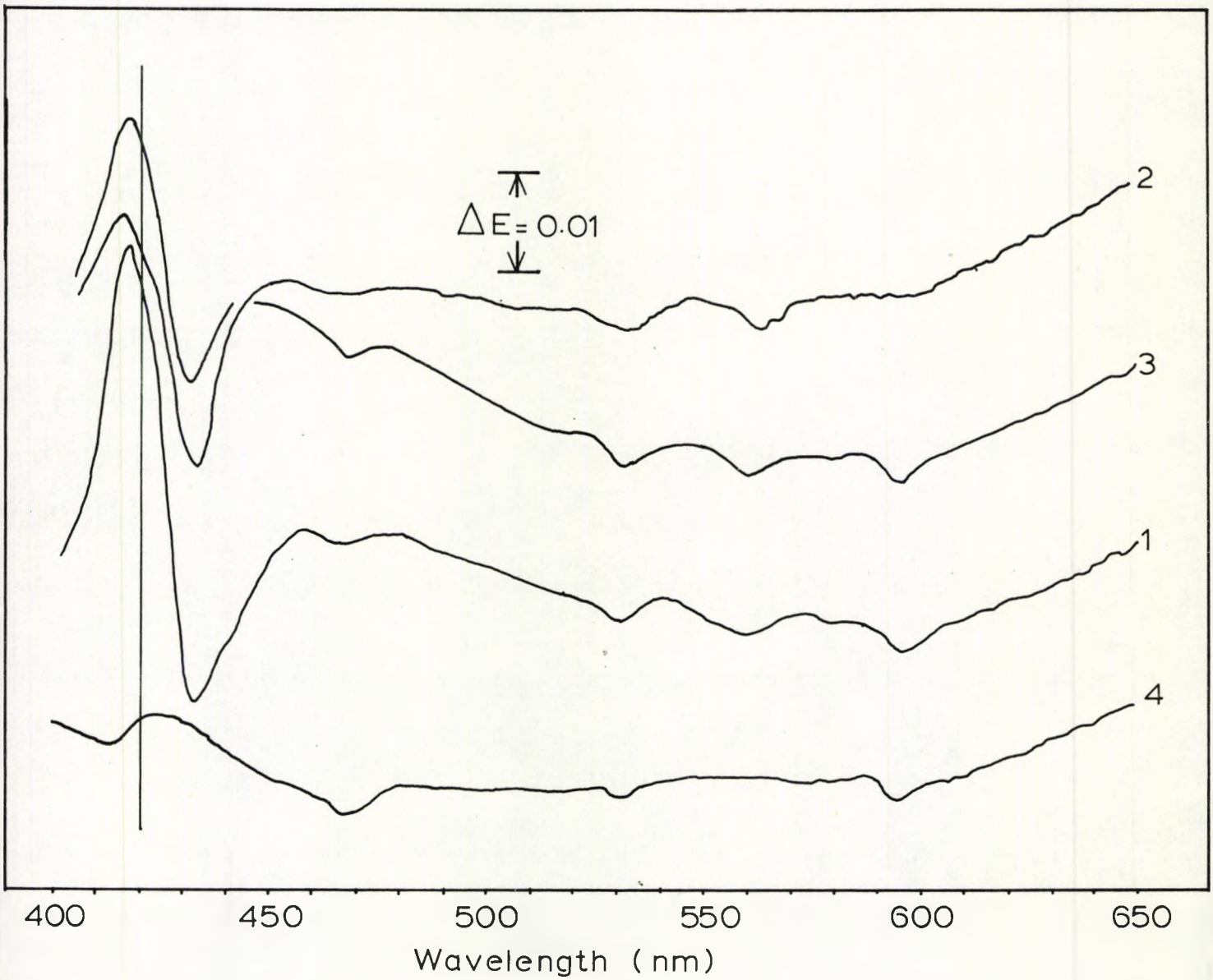


Fig. VII-11

Fig. VII-12. The effect of KCN on the respiration rate of harvested bacteria.

(1) Glucose grown wild type bacteria

(2) Glucose grown formate-negative mutant.

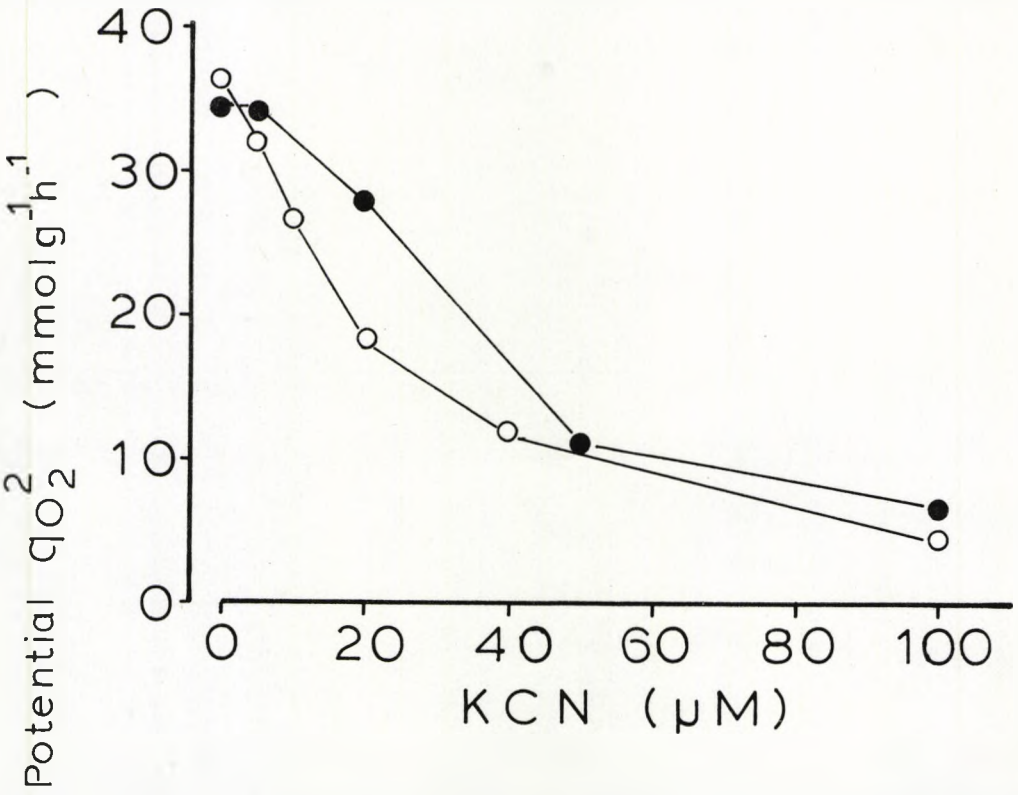


Fig. VII-12

Table VII-5. Carbon recovery for the growth of the formate non-oxidising mutant of Beneckea natriegens on glucose alone and on a mixture of glucose plus formate

Substrate	Carbon input (gh^{-1})				= Cell carbon (gh^{-1})	Product carbon + unmetabolised carbon (gh^{-1})	Carbon dioxide carbon (gh^{-1})	Recovery (%)
Glucose (2 gl^{-1})	0.619				0.314	0.066	0.187	91.5
	0.617				0.319	0.062	0.185	91.7
	Time (a) (h)	Glucose	Formate	Citrate				
Glucose (2 gl^{-1})	16	0.639	0.909	0.030	0.210	1.199	0.110	96.3
HCOONa (6 gl^{-1})	22	0.639	0.909	0.030	0.253	1.139	0.163	98.5
+	29	0.646	0.904	0.030	0.326	0.989	0.200	95.8
10 μM KCN	40	0.665	0.846	0.028	0.378	0.845	0.224	94.0
	48	0.605	0.846	0.028	0.354	0.861	0.225	97.4
	54	0.626	0.876	0.029	0.333	0.882	0.233	94.5

(a) Time after addition of formate plus cyanide to a steady state, glucose limited chemostat culture of the mutant.

3. Discussion

Beneckea natriegens was found to be capable of oxidising sodium formate without prior exposure to the compound. The formate oxidising system appears to be constitutive when this organism is cultured either on glucose or glycerol. Although the pH otpimum for this reaction is fairly low (pH 5.5) resembling the anaerobic formate hydrogenlyase system of E. coli, no H_2 was detected and the reaction proceeded under fully aerobic conditions.

Amytal, rotenone and 2-N-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) cause inhibition at specific points in the electron transport chain of eukaryotic organisms but their action on microbial systems is variable (Heinen, 1971). Therefore the results reported here using these inhibitors on the respiration of formate on a batch culture grown B. natriegens does not indicate the precise site of entry of electrons from formate. However, as amytal and rotenone are reported to inhibit flavoproteins (Heinen, 1971) and as these inhibitors did not affect respiration on NADH + H^+ or formate, it is suggested that a flavoprotein is unlikely to be involved. Moreover, as 2-N-heptyl-4-hydroxyquinoline-N-oxide, an inhibitor reported to act either between cytochrome b and c or between flavoprotein and cytochrome b, caused complete inhibition of NADH + H^+ and formate respiration, it is suggested that electrons from formate may enter the electron transport system before cytochrome b and probably at the level of NADH + H^+. Further evidence of the involvement of NAD in formate oxidation was obtained using fluorimetry, oxidation of pulses of formate caused an increase in fluorescence when the culture was irradiated at 360 nm indicative of a reduction of $NAD(P)^+$

to NAD(P)H + H^+. This increase in fluorescence was not observed when 10 μM KCN plus sodium formate were injected simultaneously into the fermenter, although oxidation (as measured by oxygen uptake and CO_2 output) proceeded at 60% of the control rate. It has been reported that all C_1-utilising microorganisms that have been studied to date oxidise formate via an NAD linked formate dehydrogenase that is strongly inhibited by cyanide. This is consistent with the data presented here, and it is suggested that formate is oxidised via an NAD linked formate dehydrogenase system in B. natriegens. The presence of 10 μM KCN inhibited this reaction, however, oxidation of formate still occurred and it is suggested that a second formate oxidation mechanism may function in this organism. The presence of more than one mechanism for the oxidation of formate has been reported for Alcaligenes LOx grown on oxalate. This organism contains an NAD linked formate dehydrogenase and an NAD independent formate oxidase activity (Chandra and Shethna, 1975).

The molar growth yield based on glucose alone increased when B. natriegens was grown on a mixture of glucose (2 gl^{-1}) plus formate (2 gl^{-1}). However as further increase in the formate concentration to 6 gl^{-1} caused no further increase in yield it is suggested that formate may be used as an energy but not as a carbon source. The presence of 10 μM KCN in the growth medium of a glucose limited chemostat culture was previously shown to have no effect on growth yield or respiration (see Section V), however, 10 μM KCN caused a very marked decrease in the yield from glucose of B. natriegens grown on a mixture of glucose plus formate (2 gl^{-1} and 4.95 gl^{-1} respectively). Further increase in the formate

concentration to 6 gl^{-1} caused culture wash out. Thus the degree of sensitivity to KCN of a culture of B. natriegens grown on a mixture of glucose plus formate was related to the formate concentration. As the presence of a mixture of glucose, formate and cyanide (2 gl^{-1}, 6 gl^{-1} and 10 μM respectively) had no effect on a formate non-oxidising mutant, it is not the formate concentration per se but the metabolism of formate that is related to cyanide sensitivity.

To explain these observations it was postulated that formate oxidation in B. natriegens proceeded via two separate systems:-

(1) Formate dehydrogenase

$$2\,HCOO^- + O_2 + NAD \underset{10\ \mu M\ KCN}{\rightleftharpoons} NADH + H^+ + 2CO_2$$

(2) Formate monooxygenase

$$2\,HCOO^- + O_2 + XH + H^+ \rightleftharpoons 2CO_2 + 2H_20 + X^+$$

Although there is no conclusive evidence to support the operation of a formate oxygenase reaction the circumstantial evidence points to the operation of a mechanism of this type.

The oxygenase reaction was postulated to explain the reduction in the yield when the organism was grown on a mixture of glucose (2 gl^{-1}) and sodium formate (4.95 gl^{-1}) in the presence of 10 μM KCN. The KCN would be expected to inhibit the NAD linked dehydrogenase (preventing an increase in $NADH + H^+$ fluorescence). The oxidase reaction would then utilise an unknown electron donor (eg. $NADH + H^+$) derived from glucose metabolism to reduce X^+ to $XH + H^+$ which would be consumed in the formate oxidase reaction. A CO

binding c type cytochrome is involved in the oxygenase reaction of C_1 utilising organisms (Tong et al., 1975). As the CO binding c type cytochrome of B. natriegens is similar it is suggested that this cytochrome is involved in the oxygenase reaction of formate oxidation. This depletion of reducing power would bring about a reduction in yield.

On the assumption that:

(1) 1 mole glucose produces 36 moles ATP (via Embden Meyerhoff and Krebs cycle);

(2) 1 mole NADH + H^+ is utilised per 2 moles HCOONa oxidised (indirectly via XH + H^+); and

(3) 3 ATP per NADH + H^+ are produced via oxidative phosphorylation;

then 2 gl^{-1} glucose (11 mM) will yield 396 mM ATP, and 4.95 g HCOONa (72.7 mM) will utilise 36.4 mM NADH + H^+. Therefore, an equivalent of 109 mM ATP will be lost, and the reduction in Y_{glc} of bacteria grown on HCOONa plus glucose plus 10 μM KCN would be 27.5% or a Y_{glc} of 29.0 g bacterial carbon per mole glucose carbon.

The experimental data gave yields that were extremely close to this calculated value (24.1 to 26.9 g bacterial carbon per mole glucose). These values would be expected to be lower than the calculated values as the latter was based on the assumption that all the glucose was utilised to product ATP. Further support for the operation of two formate oxidising systems in B. natriegens was obtained from studies of the 'apparent' Km for oxygen (see Section VIII). However, confirmation of the two formate oxidising systems must await the isolation and characterisation of the two enzyme systems involved.

SECTION VIII

DETERMINATION OF THE APPARENT Km FOR OXYGEN AND KINETICS OF CYTOCHROME b AND c CHANGES OF BENECKEA NATRIEGENS USING A RESPIROGRAPH

1. Respiration of NADH + H^+ by Sonicated Bacterial Suspensions

   (i) Bacteria harvested from a glucose limited chemostat culture, $D = 0.2\ h^{-1}$

   (a) Respiration of NADH + H^+

   (b) Respiration of NADH + H^+ in the presence of 2 mM azide

   (ii) Bacteria harvested from a chemostat culture grown on a mixture of glucose ($2.0\ gl^{-1}$) and sodium formate ($2.0\ gl^{-1}$, $D = 0.4\ h^{-1}$)

2. Respiration of Ascorbate TMPD by Sonicated Bacteria (Harvested from a Glucose Limited or Glucose plus Formate Limited Chemostat Culture)

   (i) Respiration of ascorbate TMPD

   (ii) Effect of 2 mM azide on respiration of ascorbate TMPD

3. Respiration of Sodium Formate

   (i) Bacteria harvested from a glucose limited chemostat culture, $D = 0.2\ h^{-1}$ (sonicated and whole cells respiration)

   (ii) Bacteria harvested from a chemostat culture grown on a mixture of glucose plus formate, $D = 0.4\ h^{-1}$ (respiration of sonicated bacteria)

4. Respiration of Endogenous Substrate

5. Discussion

DETERMINATION OF THE APPARENT Km FOR OXYGEN AND KINETICS OF CYTOCHROME b AND c CHANGES OF BENECKEA NATRIEGENS USING A RESPIROGRAPH

Using cell-free extracts of Beneckea natriegens, Weston, Collins and Knowles (1974) reported different values for the 'apparent' Km for oxygen in the presence and absence of cyanide and attributed them to the different branches of the electron transport pathway which they postulated. The cyanide (and azide) resistant branch in the electron transport system had the greatest affinity for oxygen with an 'apparent' Km value between 2.2 and 4 μM oxygen. The cyanide (and azide) sensitive branch had an 'apparent' Km for oxygen between 10 and 17 μM O_2. These values were obtained on cell free suspensions by bubbling nitrogen into the reaction chamber until the oxygen concentration had been reduced to the desired level, then adding particles and substrate with a microsyringe and measuring the respiration rate over a short period of time. Errors may be included in measuring the Km by this method because the response time of the oxygen electrode cell is longer when the oxygen tension is reduced towards zero oxygen tension (Fig. VIII-1). The error may be reduced if the buffer is equilibrated with nitrogen and then exposed to oxygen, the response of the probe is more rapid and remains linear in response to increase in oxygen tension over the range 0-100 μM oxygen (Fig. VIII-1). Ideally the respiration rate should be recorded when the oxygen in the gas phase is kept constant until a steady state respiration rate is reached. By varying the oxygen concentration in the gas phase, and hence the steady state dissolved oxygen tension and respiration rate, a series of respiration rates at different dissolved

Fig. VIII-1. The response of a Rank oxygen electrode to gassing and degassing with N_2 and air.

N_2 bubbled through buffer in cell previously saturated with air, x - x; air bubbled through buffer previously saturated with N_2, o - o.

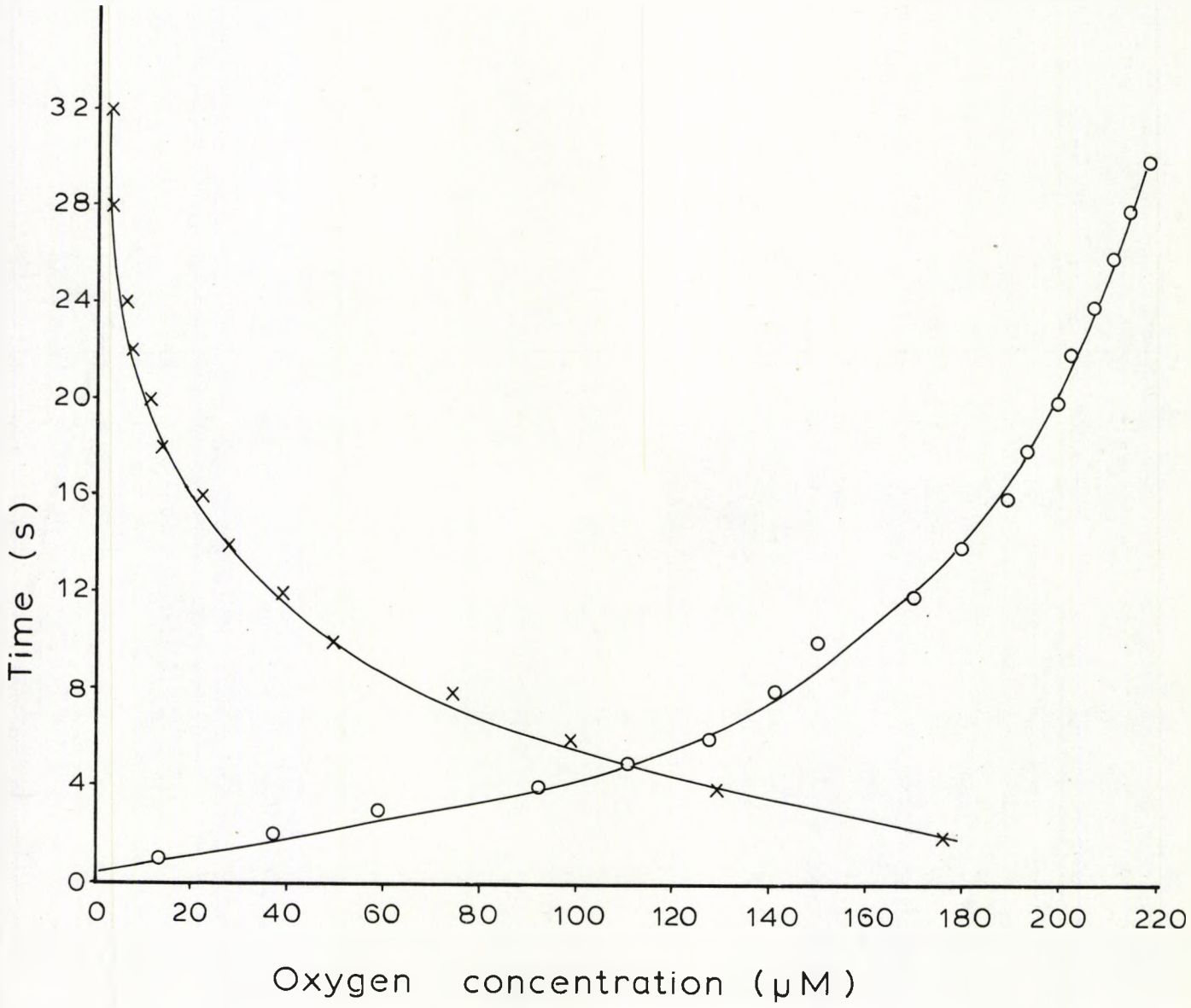

Time (s)
0
4
8
12
16
20
24
28
32
0
20
40
60
80
100
120
140
160
180
200
220
Oxygen concentration (μM)

Fig. VIII-1

oxygen tension values may be obtained. Degn and Wohlrab (1971) have developed a simplified system by using a linear oxygen gradient mixer. The apparatus consists of an optical cuvette fitted with a magnetic stirrer, and thermostatically maintained at 29°C by water circulation (see Methods for details). The surface of the liquid sample in the cuvette is in contact with a gas mixture containing N_2 and O_2. The gases are mixed by a linear oxygen gradient apparatus producing mixtures whose oxygen content increases linearly with time. The rate of change of oxygen tension can be controlled and even maintained constant at a predetermined value. The respiration rate is calculated at various dissolved oxygen tensions and the 'apparent' Km for oxygen obtained by a Lineweaver Burk plot of respiration rate against oxygen tension in the liquid (see Methods). The cuvette is situated in a dual wavelength spectrophotometer. The kinetic changes of cytochromes may be monitored by using carefully selected wavelength pairs (see Methods).

The measurement of the 'apparent' Km for oxygen of glucose and glucose plus formate grown bacteria respiring NADH + H^+, ascorbate plus N, N, N^1, N^1-tetramethylphenylenediamine (TMPD), formate and glucose in the presence and absence of azide might facilitate the identification of a branched electron transport system with differing affinities for oxygen. Moreover the operation of two formate oxidation systems in glucose plus formate grown bacteria should be evident by an alteration in the Km for oxygen during respiration on formate in the presence of azide. (This is the condition under which this system was thought to operate in the glucose plus formate + 10 μM KCN chemostat culture. See Section VII).

Bacteria were harvested from a carbon limited chemostat culture of B. natriegens growing on glucose (2.0 gl^{-1}, D = 0.2 h^{-1}) or on a mixture of glucose (2.0 gl^{-1}) and sodium formate (2.0 gl^{-1}, D = 0.4 h^{-1}). The 'apparent' Km for oxygen was measured for whole bacteria and for sonicated bacterial suspensions respiring NADH + H^+, formate, ascorbate-TMPD and glucose. Weston, Collins and Knowles (1974) measured the Km for the cyanide sensitive pathway with ascorbate-TMPD and the cyanide resistant pathway with NADH + H^+ plus 10 μM KCN. These workers also reported that 2 mM azide had the same effect as 10 μM KCN (ie. complete inhibition of ascorbate-TMPD oxidase but only partial inhibition of NADH oxidase activity.) Although KCN has been used in all the previous work reported in this thesis azide was used because the constant stream of gas over the cuvette in the respirometer may strip off KCN, azide would not be lost from the system at an appreciable rate.

1. Respiration of NADH + H^+ by Sonicated Bacterial Suspensions

(i) Bacteria harvested from a glucose limited chemostat culture, $D = 0.2\ h^{-1}$

(a) Respiration of NADH + H^+

A sonicated bacterial suspension (see Methods) of B. natriegens readily oxidised NADH + H^+. Kinetic changes in cytochrome b were measured using the wavelength pairs 558 nm minus 578 nm. The wavelengths represented the isobestic point for cytochrome(s) b. At submaximal respiration rate (1.5 mM O_2 per g bacterial carbon per h) the absorbance of cytochrome b exhibited two marked oxidation steps before reaching a steady oxidation level at a dissolved oxygen tension in excess of 4 μM (Fig. VIII-2). At maximum respiration rate (5.78 mM O_2 per g bacterial carbon per h) only one step was observed and absorbance due to cytochrome b remained reduced in the presence of excess oxygen (Fig. VIII-3). The ratio of ΔE obtained from fully aerated (equilibrated with oxygen in the gas phase) minus anaerobic (equilibrated with nitrogen in the gas phase) to the total ΔE obtained by reducing the contents of the cuvette with dithionate minus oxidation with potassium ferricyanide was only 45%.

A Lineweaver-Burk plot of respiration rate against the oxygen tension in the liquid for B. natriegens respiring NADH + H^+ is shown in Fig. VIII-4. The apparent Km for oxygen was found to be between 0.15 and 0.20 μM oxygen.

(b) Respiration of NADH + H^+ in the presence of 2 mM azide.

A concentration of 2 mM sodium azide caused approximately 16% inhibition of respiration rate of B. natriegens respiring NADH + H^+, however

Fig. VIII-2. Kinetic changes in cytochrome b (558 nm minus 578 nm) of a sonicated suspension of Beneckea natriegens (glucose grown) respiring at submaximal rate on NADH + H^+ in a respirograph.

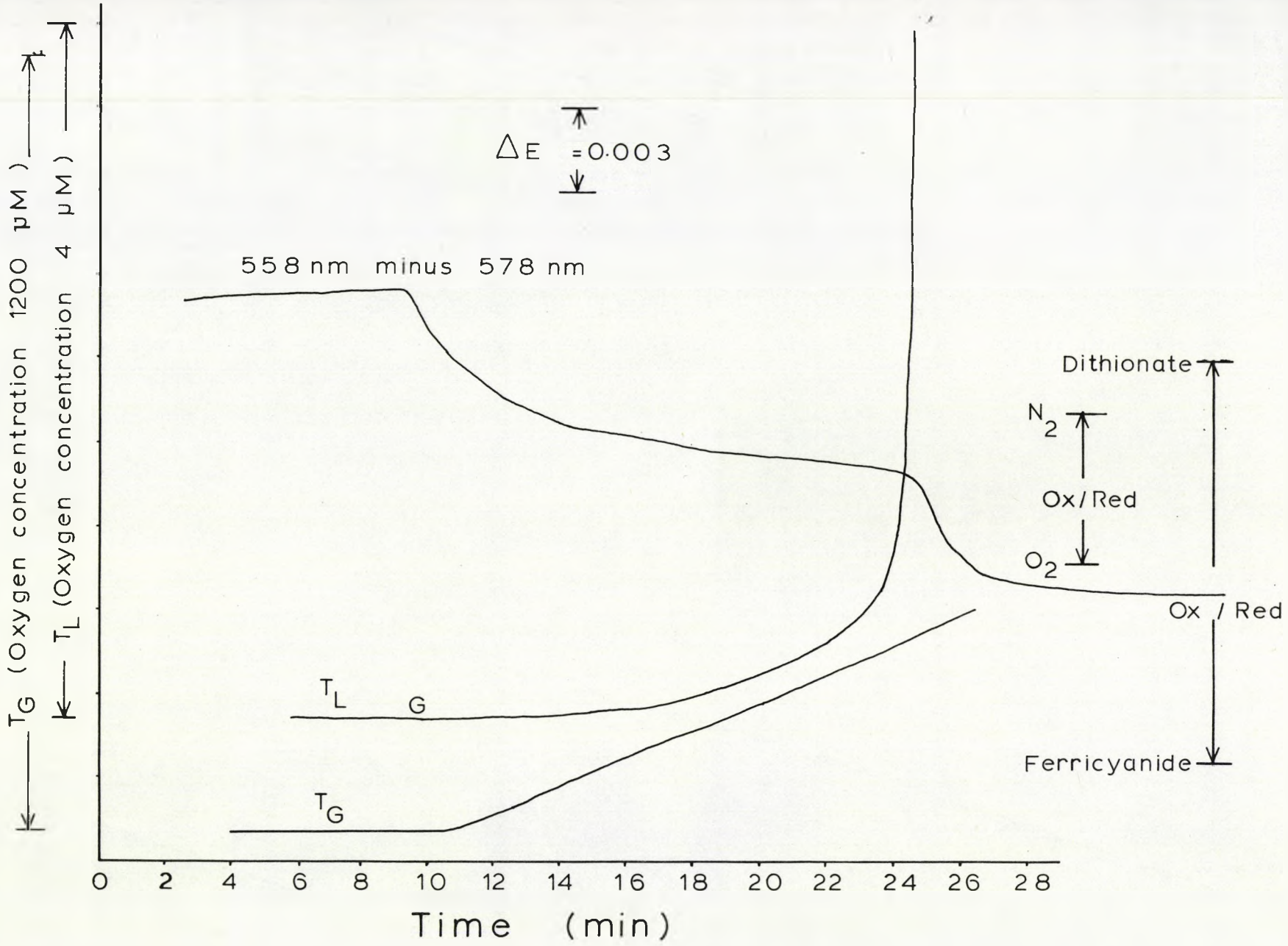

ΔE =0.003
558 nm minus 578 nm
Dithionate
N2
Ox/Red
O2
Ox / Red
Ferricyanide
TL G
TG
TG (Oxygen concentration 1200 μM)
TL (Oxygen concentration 4 μM)
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (min)

Fig. VIII-2

Fig. VIII-3. Kinetic changes in cytochrome b of a sonicated suspension of Beneckea natriegens (glucose grown) during respiration at maximal rate on NADH + H^+ in a respirograph.

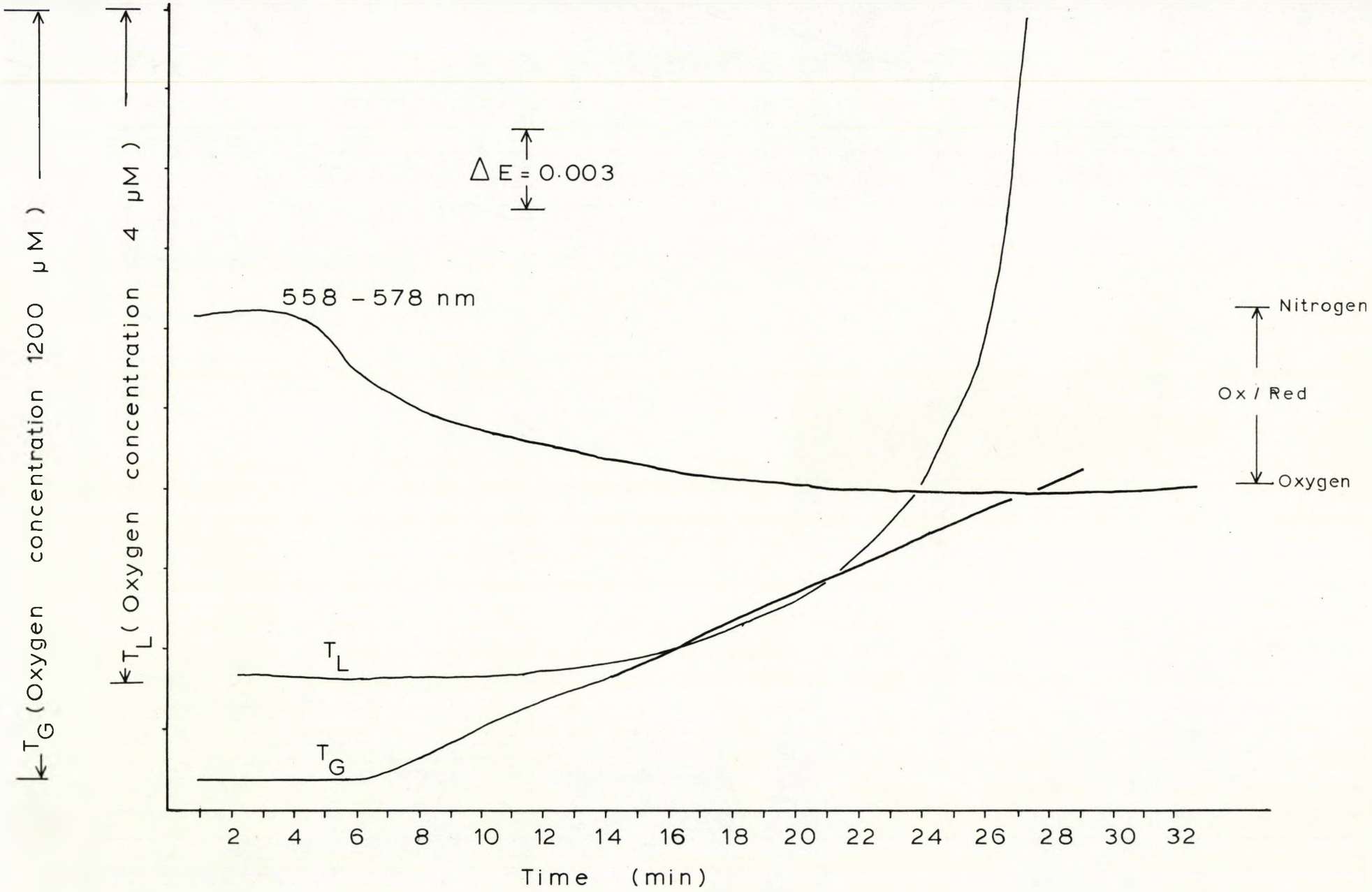

T_G (Oxygen concentration 1200 µM)
T_L (Oxygen concentration 4 µM)
ΔE = 0.003
558 – 578 nm
Nitrogen
Ox / Red
Oxygen
T_L
T_G
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (min)

Fig. VIII-3

Fig. VIII-4. A Lineweaver-Burk plot of respiration rate against the oxygen tension in the liquid for Beneckea natriegens respiring NADH + H^+ (glucose grown).

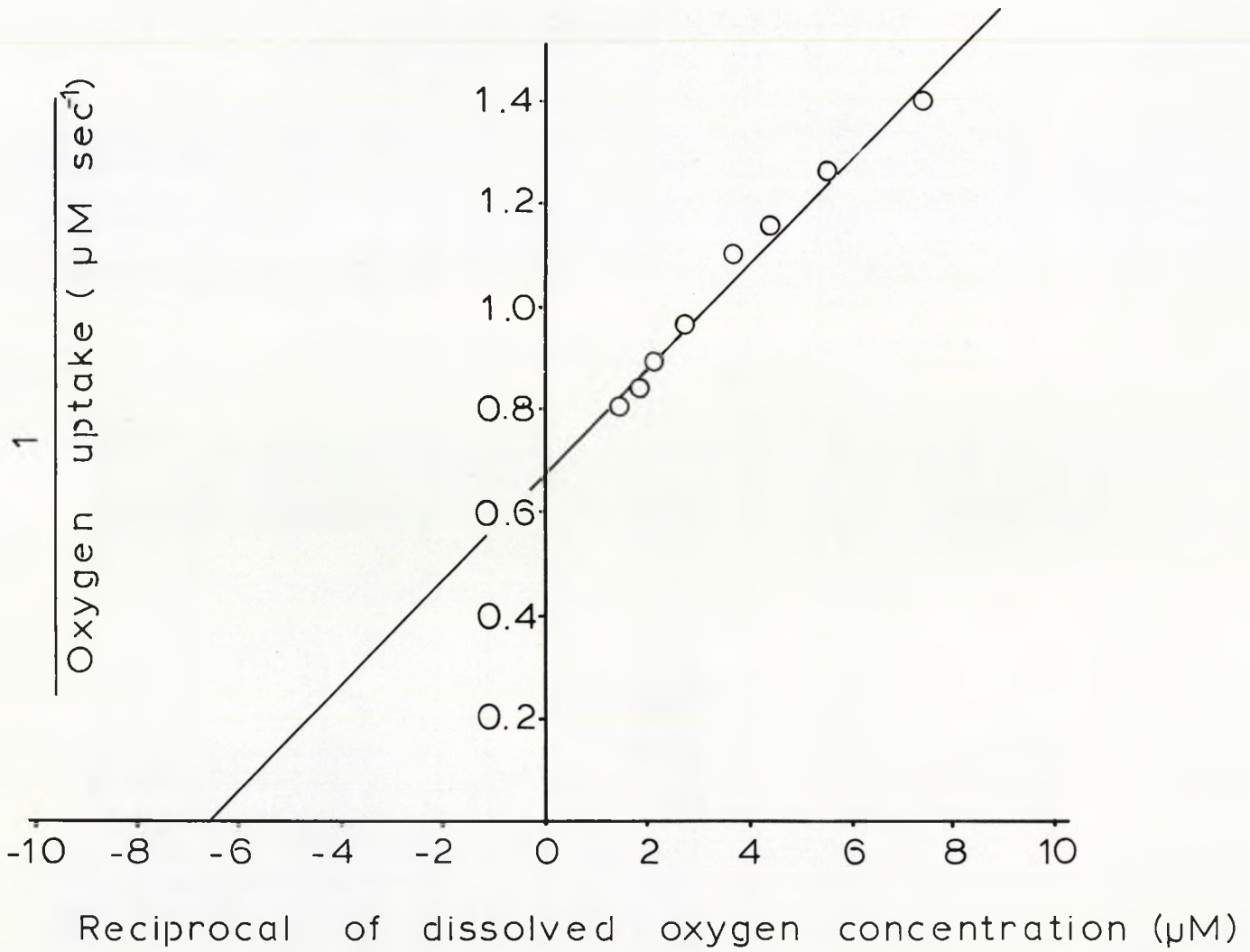

1
Oxygen uptake (μM sec⁻¹)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-10
-8
-6
-4
-2
0
2
4
6
8
10
Reciprocal of dissolved oxygen concentration (μM)

Fig. VIII - 4

there was no effect on the absorbance of cytochrome b. The 'apparent' Km for oxygen in the presence of azide could not be measured accurately as the dissolved oxygen tension increased too rapidly over the range 0-4 μM oxygen.

(ii) Bacteria harvested from a chemostat culture grown on a mixture of glucose (2.0 gl^{-1}) and sodium formate (2.0 gl^{-1}) D = 0.4 h^{-1}

Respiration of NADH + H^+ in the presence and absence of azide.

The Km for oxygen of B. natriegens respiring NADH + H^+ in the presence and absence of 2 mM azide is shown in Fig. VIII-5. The presence of 2 mM azide caused approximately 20% inhibition of respiration rate on NADH + H^+ (Table VIII-4), and an increase in the Km for oxygen from 0.25 to 0.48 μM oxygen.

Fig. VIII-5. The effect of 2 mM azide on the 'apparent' Km for oxygen of a sonicated suspension of Beneckea natriegens (glucose plus formate grown) respiring $NADH + H^+$.

$NADH + H^+$, ● - ●; $NADH + H^+$ plus 2 mM azide, o - o.

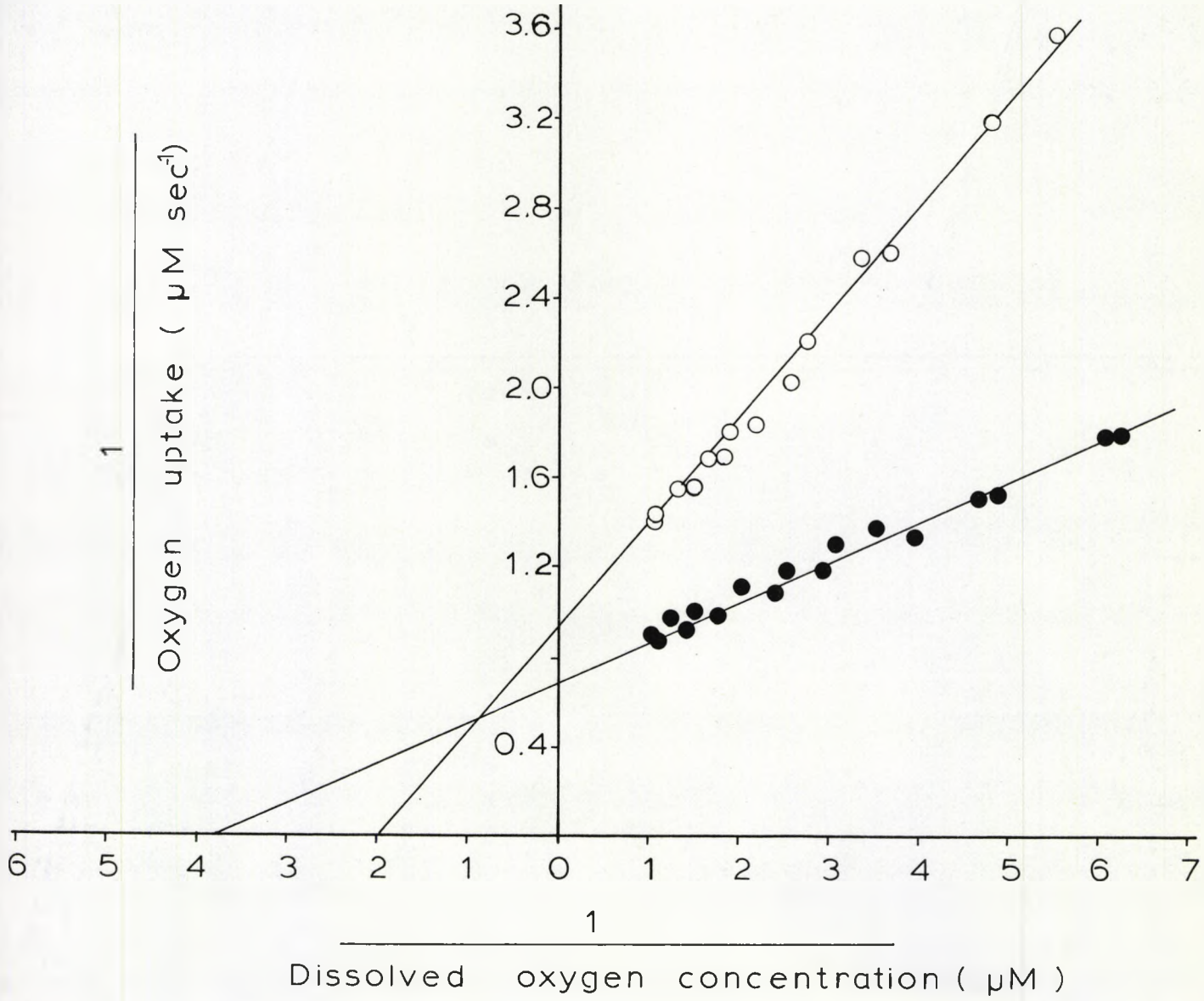

3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.4
1
Oxygen uptake (μM sec-1)
6 5 4 3 2 1 0 1 2 3 4 5 6 7
1
Dissolved oxygen concentration (μM)

Fig. VIII- 5

2. Respiration of Ascorbate TMPD by Sonicated Bacteria (Harvested from a Glucose Limited or Glucose plus Formate Limited Chemostat Culture)

(i) Respiration of ascorbate TMPD

Bacteria were harvested from a glucose limited (2.0 gl^{-1}) and glucose plus formate limited chemostat culture (2.0 gl^{-1} of each), concentrated and then sonicated. Sonicated bacterial suspensions readily oxidised ascorbate-TMPD. The respiration rate on ascorbate-TMPD of glucose and glucose plus formate grown bacteria was the same (2.8 mM O_2 per g bacterial carbon per h). The Km for oxygen of glucose and glucose plus formate grown bacteria were not significantly different and were found to be 0.21 and 0.16 μM oxygen respectively (Table VIII-1, Fig. VIII-7).

The degree of oxidation of a particular cytochrome during respiration of a substrate compared to the oxidation of the particular cytochrome observed with ferricyanide may be indicative of the involvement of that cytochrome during oxidation of the substrate. The ratio of ΔE at 540 nm minus 553 nm (cytochrome c) obtained from fully aerobic (equilibrated with oxygen in the gas phase) minus anaerobic (equilibrated with N_2 in the gas phase) to the total ΔE obtained by reducing the contents of the cuvette with dithionate minus oxidation with ferricyanide (x 100) was only 15%. One 'step' was observed in the cytochrome absorbance (at 540 nm minus 553 nm) and this occurred immediately the oxygen gradient was started at a dissolved oxygen $\ll$0.04 μM (Fig. VIII-6(a)).

Fig. VIII-6. Kinetic changes in cytochrome c (540 nm minus 553 nm) during oxidation of ascorbate TMPD by a sonicated suspension of Beneckea natriegens.

(a) In the absence of azide

(b) In the presence of 2 mM azide.

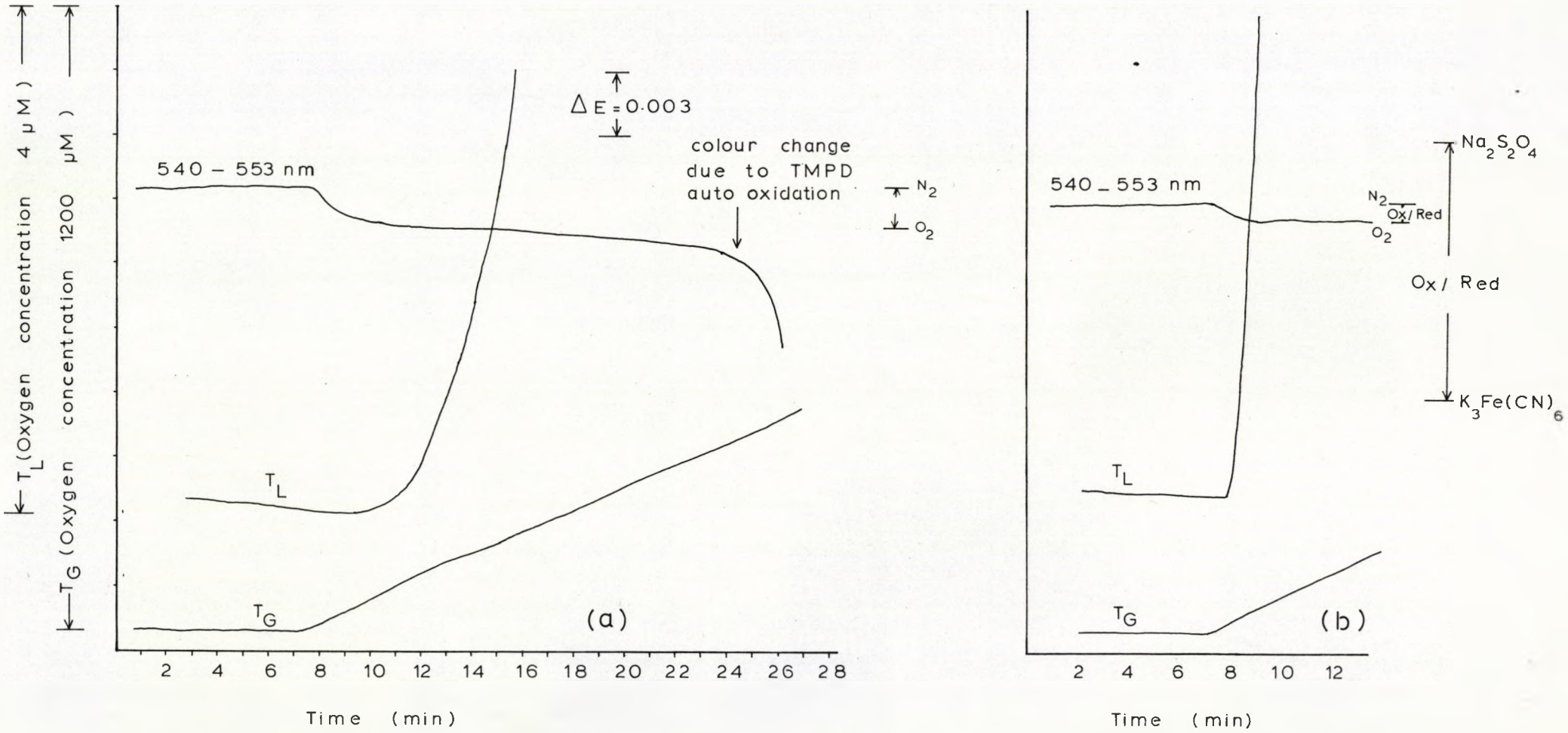

T_L (Oxygen concentration 4 µM)
T_G (Oxygen concentration 1200 µM)
ΔE = 0·003
540 – 553 nm
colour change due to TMPD auto oxidation
N_2
O_2
T_L
T_G
(a)
2 4 6 8 10 12 14 16 18 20 22 24 26 28
Time (min)
$Na_2S_2O_4$
540 _ 553 nm
N_2
Ox/Red
O_2
Ox / Red
$K_3Fe(CN)_6$
T_L
T_G
(b)
2 4 6 8 10 12
Time (min)

Fig. VIII - 6

Fig. VIII-7. The 'apparent' Km for oxygen of glucose and glucose plus formate grown bacteria respiring ascorbate-TMPD.

Bacteria harvested from glucose limited chemostat culture, Δ-Δ; bacteria harvested from glucose plus formate chemostat culture, o - o.

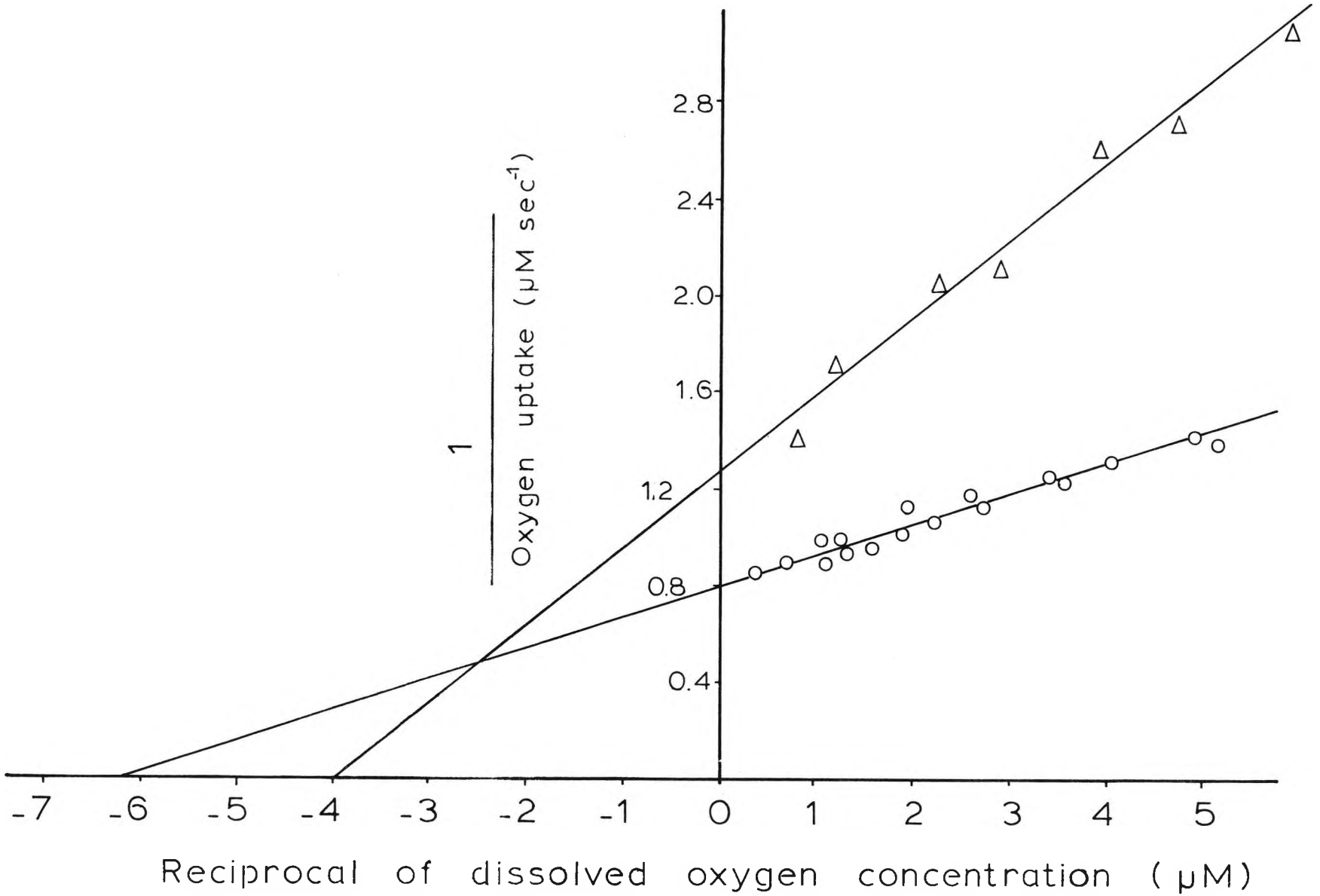

2.8
2.4
2.0
1.6
1.2
0.8
0.4
1 / Oxygen uptake (μM sec⁻¹)
-7
-6
-5
-4
-3
-2
-1
0
1
2
3
4
5
Reciprocal of dissolved oxygen concentration (μM)

Fig. VIII-7

Table VIII-1. The effect of azide on the 'apparent' Km for oxygen of Beneckea natriegens respiring ascorbate-TMPD

Condition of growth	Azide concentration (mM)	Ascorbate-TMPD (1 mM + 5 mM)	Respiration rate (mM O_2 per g bacterial carbon per h)	'Apparent' Km for oxygen (μM)
Glucose limited chemostat ($D = 0.2\ h^{-1}$)	0.0	+	2.80	0.25, 0.22, 0.17
Glucose limited chemostat ($D = 0.2\ h^{-1}$)	2.0	+	≏0.28	not measurable
Glucose + formate limited chemostat ($D = 0.4\ h^{-1}$)	0.0	+	2.82	0.14, 0.14, 0.20
Glucose + formate limited chemostat ($D = 0.4\ h^{-1}$)	2.0	+	≏0.28	not measurable

(ii) Effect of 2 mM azide on respiration of ascorbate-TMPD

The inclusion of 2 mM sodium azide in the reaction mixture caused 90% inhibition of ascorbate-TMPD. The remaining respiration rate was too low for Km determination. The ratio of ΔE at 540 minus 553 nm obtained from fully aerobic minus anaerobic to the total ΔE obtained by reducing the contents of the cuvette with dithionate minus oxidation with ferricyanide (x 100) was 8%. Moreover the single 'step' in the cytochrome absorbance of cytochrome c (540 nm minus 553 nm) was not observed (Fig. VIII-6(b)).

3. Respiration of Sodium Formate

(i) Bacteria harvested from a glucose limited chemostat culture, $D = 0.2\ h^{-1}$ (sonicated and whole cells respiration)

Sodium formate was readily oxidised by a suspension of whole bacteria. A value of 9.41 mM O_2 per g bacterial carbon per h was observed, sonicated bacteria demonstrated a 32% loss of respiratory activity. The 'apparent' Km for oxygen of whole bacteria respiring sodium formate was 0.18 µM (Fig. VIII-9).

The ratio of ΔE of cytochrome c obtained from fully aerobic minus anaerobic to the total ΔE obtained by reducing the contents of the cuvette with dithionate minus oxidation with ferricyanide (x 100) was 15% (Fig. VIII-8). The absorbance of cytochrome c (540 - 553 nm) exhibited only one 'step' which occurred immediately the oxygen gradient was started (Fig. VIII-8) when the dissolved oxygen tension was below 0.04 µM.

The inclusion of 2 mM azide in the reaction mixture caused 27% inhibition of the respiration rate, however the Km for oxygen was not affected and an average value of 0.15 µM oxygen was observed (Fig. VIII-9, Table VIII-2). The presence of azide eliminated the single 'step' observed in the cytochrome c absorbance (Fig. VIII-10).

(ii) Bacteria harvested from a chemostat culture grown on a mixture of glucose plus formate $D = 0.4\ h^{-1}$ (respiration of sonicated bacteria)

The oxidation of sodium formate by a sonicated cell suspension of B. natriegens in the respirograph was accompanied by a single oxidation step in

Fig. VIII-8. Kinetic changes in cytochrome c (540 nm minus 553 nm) of Beneckea natriegens (whole bacteria) respiring formate.

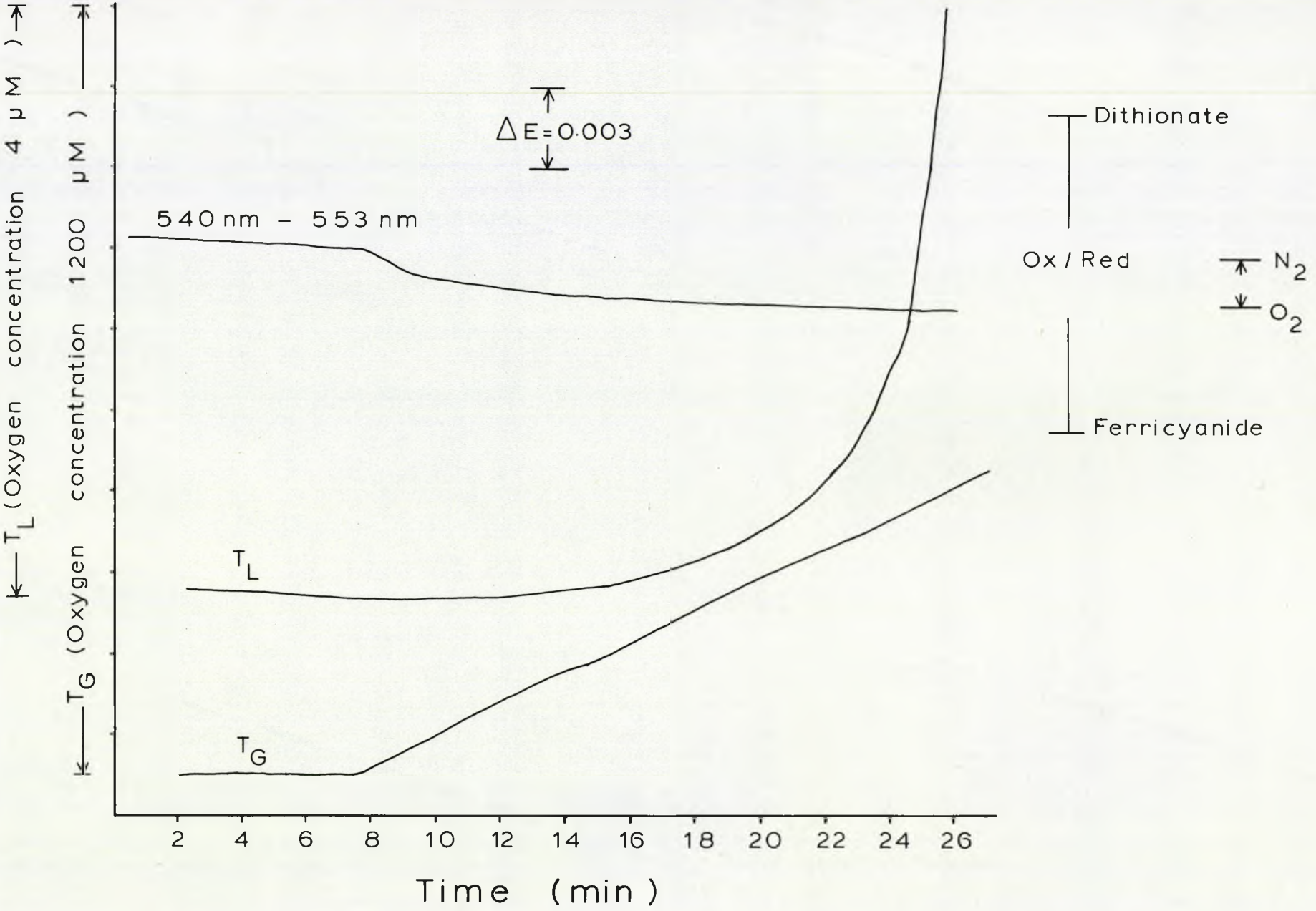

T_L (Oxygen concentration 4 μM)
T_G (Oxygen concentration 1200 μM)
ΔE = 0.003
540 nm – 553 nm
T_L
T_G
Dithionate
Ox / Red
N_2
O_2
Ferricyanide
2 4 6 8 10 12 14 16 18 20 22 24 26
Time (min)

Fig. VIII - 8

Fig. VIII-9. The 'apparent' Km for oxygen of whole bacteria respiring formate in a respirograph in the presence and absence of azide (glucose grown).

Formate alone, ● - ●; formate plus azide, o - o.

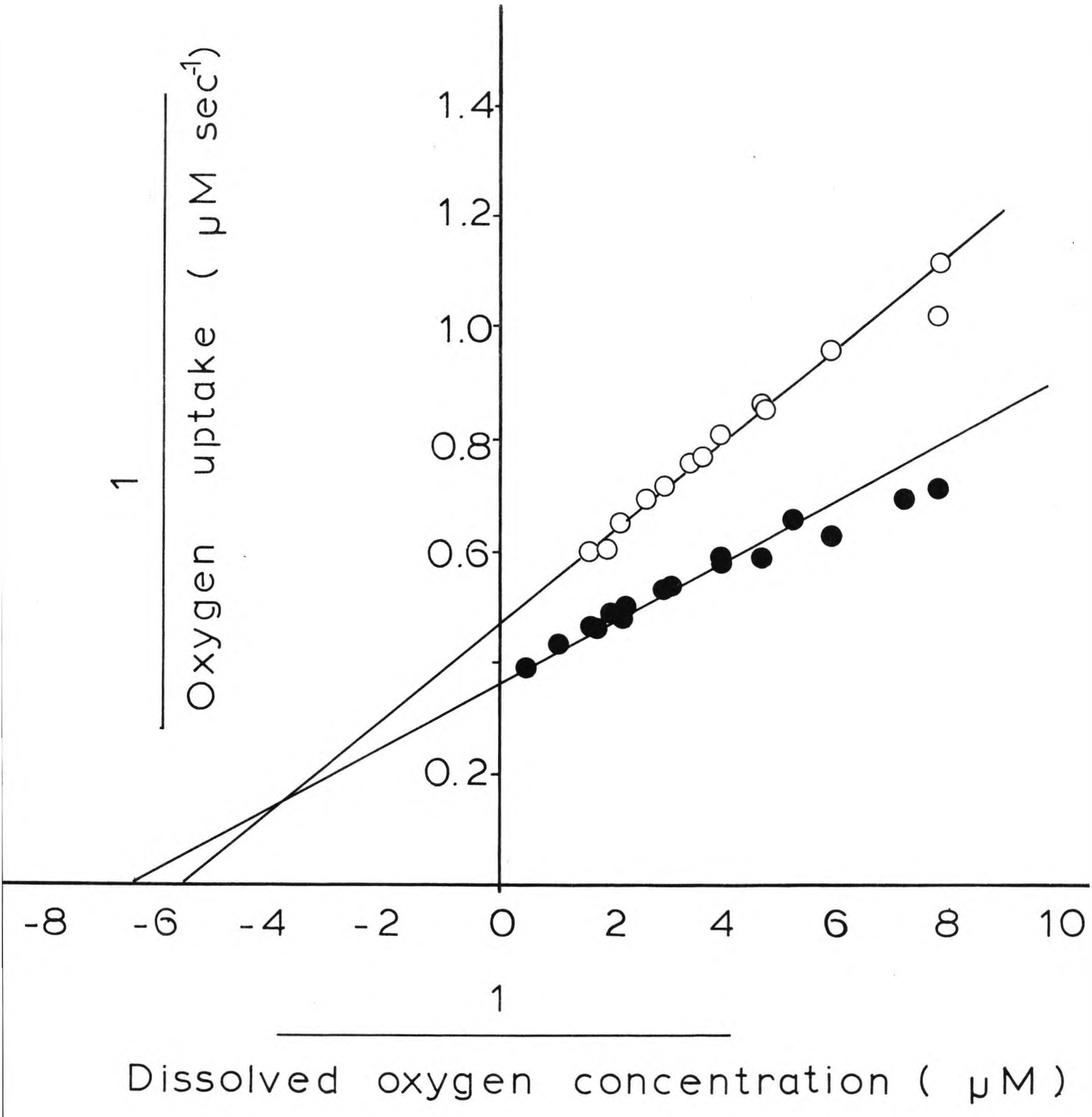

1
Oxygen uptake (μM sec-1)
1.4
1.2
1.0
0.8
0.6
0.2
-8
-6
-4
-2
0
2
4
6
8
10
1
Dissolved oxygen concentration (μM)

Fig.VIII-9

Fig. VIII-10. The effect of azide on the kinetic changes in cytochrome c of Beneckea natriegens (whole bacteria, glucose grown) respiring formate.

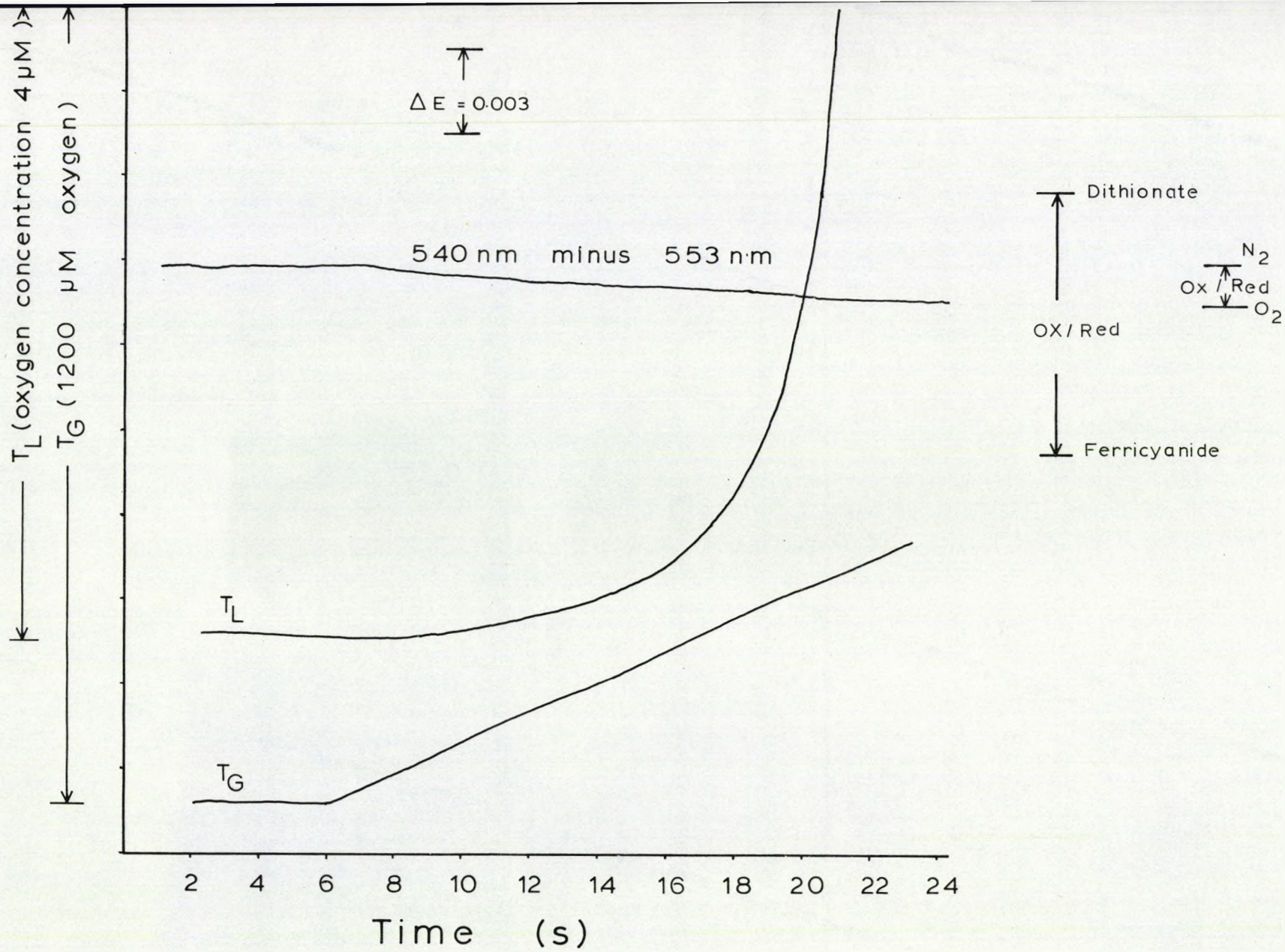

T_L (oxygen concentration 4 µM)
T_G (1200 µM oxygen)
ΔE = 0.003
540 nm minus 553 nm
T_L
T_G
2 4 6 8 10 12 14 16 18 20 22 24
Time (s)
Dithionate
N_2
Ox / Red
O_2
OX / Red
Ferricyanide

Fig. VIII-10

Table VIII-2. The effect of azide on respiration rate and 'apparent' Km for oxygen of Beneckea natriegens respiring sodium formate (whole bacteria harvested from a glucose limited chemostat culture, $D = 0.2\ h^{-1}$)

Sodium formate conc. (mM)	Azide conc. (mM)	Respiration rate mM O_2 per g bacterial carbon per h	'Apparent'Km for oxygen (μM)	ΔE of aerobic and anaerobic states as % of total cytochromes*
64	0.0	9.41	0.148, 0.143, 0.176, 0.173, 0.190	15
64	2.2	6.70	0.178, 0.178, 0.148, 0.155	15.5

* Calculated on the ratio of ΔE at 558 nm minus 578 nm obtained from fully aerated (equilibrated with oxygen in the gas phase) minus anaerobic (equilibrated with nitrogen in the gas phase) to the total ΔE obtained at 558 nm minus 578 nm by reduction of the contents of the cuvette with dithionate and oxidation with ferricyanide.

the cytochrome c absorbance, as observed with glucose grown cells. Similarly the inclusion of 2 mM azide in the reaction mixture eliminated this step.

Effect of azide on the 'apparent' Km for oxygen of Beneckea natriegens respiring formate

The respiration rate of glucose plus formate grown bacteria on sodium formate was 7.9 mM O_2 per g bacterial carbon per h (Table VIII-3). The inclusion of 2 mM azide in the reaction mixture caused 33% inhibition of respiration rate (Table VIII-3). These values are similar to those observed with glucose grown bacteria. However, unlike the response observed with glucose grown bacteria, the presence of 2 mM azide caused a marked change in the 'apparent' Km for oxygen from 0.16 μM to 0.63 μM (Fig. VIII-11).

Fig. VIII-11. The effect of 2 mM azide on the 'apparent' Km for oxygen of a sonicated suspension of Beneckea natriegens (glucose plus formate grown) respiring sodium formate.

Formate, o - o; formate plus azide, ● - ●.

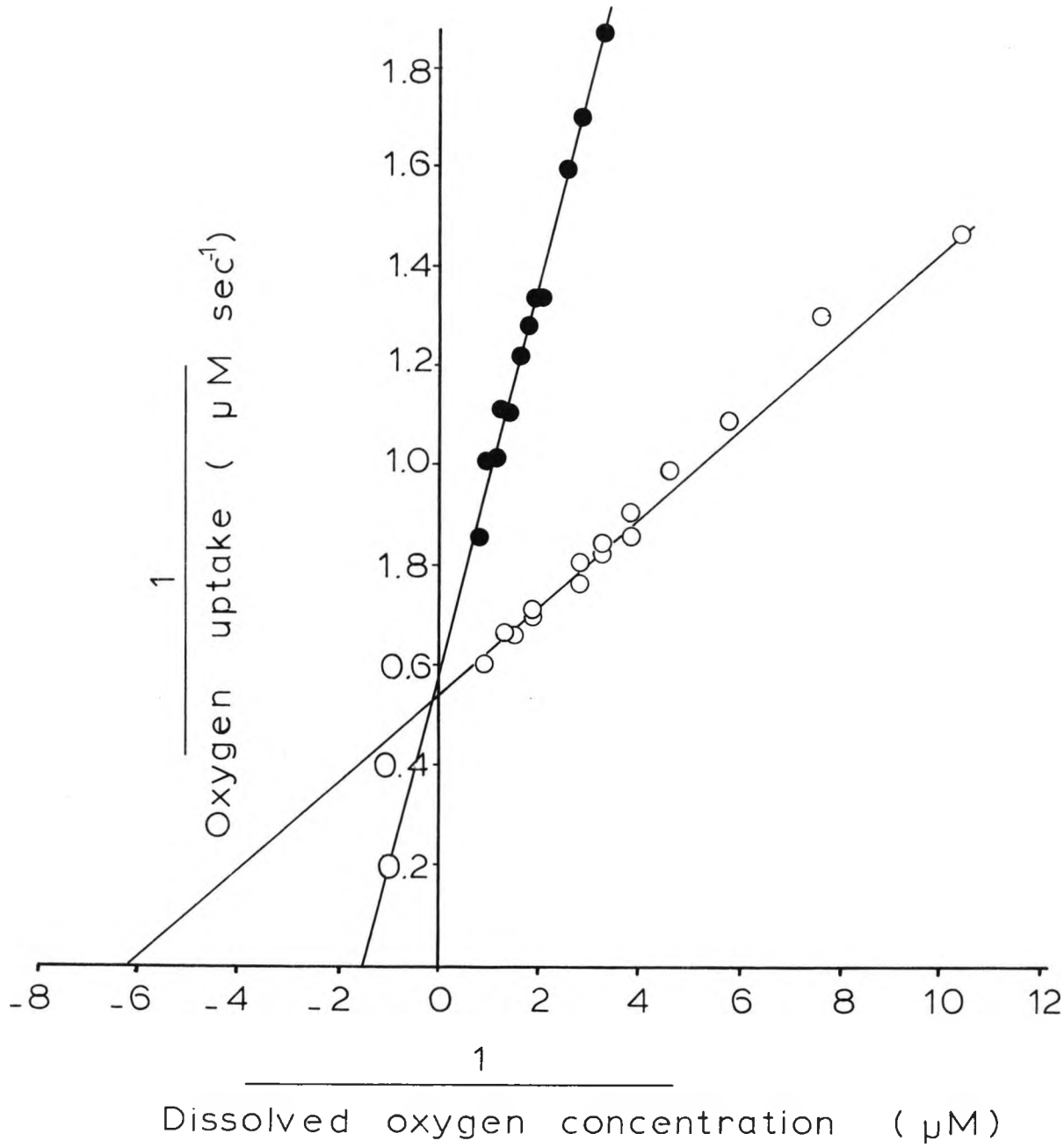

1.8
1.6
1.4
1.2
1.0
1.8
0.6
0.4
0.2
-8
-6
-4
-2
0
2
4
6
8
10
12
1
Oxygen uptake (μM sec-1)
1
Dissolved oxygen concentration (μM)

Fig.VIII-11

Table VIII-3. The effect of 2 mM azide on respiration and 'apparent' Km for oxygen of Beneckea natriegens respiring sodium formate. (Sonicated bacteria harvested from a carbon limited chemostat culture on glucose plus sodium formate, $D = 0.4\ h^{-1}$)

Sodium formate conc. (mM)	Azide conc. (mM)	Respiration rate mM per g bacterial carbon per h	'Apparent' Km for oxygen (μM)	ΔE of aerobic and anaerobic states as % of total cytochrome
64	0.0	7.9	0.15, 0.17,	29
64	2.2	5.3	0.64, 0.62	34
Endogenous substrate	0.0	0.28	not measured	51

4. Respiration of Endogenous Substrate

Bacteria harvested from chemostat culture grown on a mixture of glucose plus formate (2 gl^{-1} of each, carbon limited, D = 0.4 h^{-1})

Whole bacteria harvested from a glucose limited chemostat culture did not exhibit a measurable endogenous respiration rate. Glucose plus formate grown bacteria (whole cells) exhibited an endogenous respiration rate of 3.0 mM O_2 per g bacterial carbon per h. The 'apparent' Km for oxygen of glucose plus formate grown whole bacteria respiring endogenous substrate was 0.50 μM. After sonication the Km for oxygen could not be measured because the respiration rate was approximately 40% lower than that observed with whole bacteria.

The ratio of Δ E of cytochrome b (558 nm minus 578 nm) obtained from fully aerobic minus anaerobic to the total ΔE obtained by reducing the contents of the cuvette with dithionate minus oxidation with ferricyanide (x 100) was 59%.

5. Discussion

A summary of the various values obtained for the 'apparent' Km for oxygen of Beneckea natriegens is given in Table VIII-4. The values for the 'apparent' Km for oxygen of glucose grown bacteria were similar regardless of respiratory substrate. Although ascorbate TMPD oxidation was strongly inhibited by 2 mM azide the 'apparent' Km for oxygen was indistinguishable from that observed with NADH + H^+, formate or glucose. These results do not support the report of Weston, Collins and Knowles (1974) that Beneckea natriegens has a branched electron transport system, the individual branches of which can be distinguished by their apparent Km for oxygen. These authors obtained values of 10-17 μM O_2 for the Km for oxygen of B. natriegens respiring ascorbate TMPD, and values between 2 and 4 μM O_2 for the Km for oxygen during respiration of NADH + H^+ in the presence of 10 μM cyanide.

Azide (2 mM) caused 90% inhibition of respiration on ascorbate TMPD but only between 16 and 20% and 23 and 33% of respiration on NADH + H^+ and formate respectively. The possibility that ascorbate TMPD is oxidised via a separate pathway cannot be ruled out, however this pathway does not have a different 'apparent' Km for oxygen.

The values for the 'apparent' Km for oxygen of bacteria harvested from a glucose limited chemostat culture varied between 0.15 and 0.25 μM. These values support the data given in Section IV, where respiration rate and metabolism of a carbon limited chemostat culture of B. natriegens was unaffected until the dissolved oxygen tension was lowered to <1 mmHg (1.3 μM oxygen). These

values of 'apparent' Km for oxygen are at least an order of magnitude lower than those reported by Weston, Collins and Knowles (1974) for B. natriegens.

The 'apparent' Km for oxygen of glucose plus formate grown bacteria was similar to that of glucose grown bacteria. However, unlike that observed with glucose grown bacteria the 'apparent' Km for oxygen of glucose plus formate grown bacteria was affected by 2 mM azide. Although the respiration rate on NADH + H^+ and formate was inhibited to the same extent (as glucose grown bacteria) the 'apparent' Km for oxygen increased markedly in the presence of azide from 0.16 to 0.63 μM and 0.25 and 0.48 μM in the case of formate and NADH + H^+ respectively. Thus growth on a mixture of glucose plus formate appeared to have induced a separate electron transport system with a lower affinity for oxygen. In the presence of 2 mM azide electrons derived from respiration on NADH and formate (glucose plus formate grown bacteria) may be directed through this pathway.

Glucose grown bacteria did not exhibit a measurable endogenous respiration rate. Glucose plus formate grown bacteria exhibited a marked endogenous respiration rate when whole bacteria were examined. When sonicated bacterial suspensions were examined the respiration rate was not measurable. This was probably caused by dilution of the endogenous substrate upon cell sonication. The Km for oxygen of glucose plus formate grown whole bacteria respiring endogenous substrate was 0.5 μM O_2. The endogenous substrate is probably poly-β-hydroxybutyrate as this is the main storage polymer of B. natriegens (Baumann, Baumann and Mandel, 1972). Bacteria capable of

accumulating poly-β-hydroxybutyrate have been shown to possess a constitutive NAD-specific D-(-)-3-hydroxybutyrate dehydrogenase. In all cases the products are NADH + H^+ and acetoacetate.

To explain the results reported in Section VII the operation of two routes of formate oxidation were proposed for B. natriegens when grown on a mixture of glucose plus formate. Formate either entered the respiratory pathway via formate dehydrogenase at the level of NADH + H^+ or was oxidised via an oxygenase reaction requiring NADH + H^+. The results reported in this section are consistent with this hypothesis. The 'apparent' Km for oxygen of glucose grown bacteria respiring formate or NADH + H^+ were indistinguishable from one another and remained unaltered in the presence of azide. However, in glucose plus formate grown bacteria the Km for oxygen during respiration on formate increased markedly from 0.16 μM to 0.63 μM O_2 in the presence of azide. These results are indicative of the presence of two formate oxidation pathways which can be distinguished by their differing affinities for oxygen.

The changes in the absorbance of the cytochromes is difficult to interpret, however a number of points were observed:

1. At maximal respiration rate cytochrome b (or c) was never completely oxidised and the ΔE of aerobic minus anaerobic, expressed as a percentage of the total ΔE of dithionate reduced minus ferricyanide oxidised, was never more than 50%. Values obtained during glucose oxidation and oxidation of endogenous substrate suggested that electrons from glucose and endogenous substrate enter the

Table VIII-4. A summary of the respiration rates and 'apparent' Km values for oxygen of Beneckea natriegens respiring various substrates

Growth condition	Nature of bacterial suspension: Whole bacteria	Nature of bacterial suspension: sonicated	Substrate	Respiration rate (mmol $g^{-1}h^{-1}$)	Inhibition by 2 mM azide (%)	'Apparent Km' for oxygen (μM)	ΔE at aerobic and anaerobic states as % of total cytochrome*
Bacteria harvested from a glucose limited chemostat culture ($D = 0.2\ h^{-1}$)		+	ascorbate TMPD	2.80		0.21	15 cytochrome c
		+	ascorbate TMPD + 2 mM azide	-	90	-	8 cytochrome c
		+	NADH + H^+	5.78		0.17	45 cytochrome b
		+	NADH + H^+ + 2 mM azide	4.85	16	not measured	45 cytochrome b
		+	sodium formate	6.35		0.15	15 cytochrome c
		+	formate + 2 mM azide	4.85	23.7	0.17	15 cytochrome c
	+		formate	9.41		0.18	not measured
	+		formate + 2 mM azide	6.70	28.8	0.15	not measured
	+		glucose	4.30	-	0.18	42 cytochrome b see App. III
	+		endogenous	not measurable			
Harvested from a glucose + formate limited chemostat culture ($D = 0.4\ h^{-1}$)		+	ascorbate TMPD	2.82		0.16	15
		+	ascorbate TMPD + 2 mM azide		90	-	8
		+	NADH + H^+	10.16		0.25	not measured
		+	NADH + H^+ + 2 mM azide	8.13	20.2	0.48	not measured
		+	formate	7.9		0.16	29
		+	formate + 2 mM azide	5.3	33	0.63	39
	+		endogenous	3.0		0.50	59
		+	endogenous	0.28		-	

* Calculated on the ratio of △E obtained of fully aerobic minus anaerobic to the total △E obtained by reducing the contents of the cuvette with dithionate and oxidation with ferricyanide.

electron transport system at the level of NADH + H^+ (Chance, 1957) (Table VIII-4). The steady state cytochrome c reduction with ascorbate TMPD or formate was only 15% (glucose grown bacteria). Beneckea natriegens has been shown to contain at least two b type and four c type cytochromes (b_{557}, b_{562}, c_{554}, $c_{549.5}$, $c_{549.5(CO)}$. Absorbance of cytochrome b and c at 558 nm minus 578 nm and 540 nm minus 553 nm is a measure of the total b and c type cytochromes. Thus, ascorbate TMPD and formate may donate electrons to particular components within the total c type cytochrome whereas NADH + H^+ and glucose may donate electrons to more than one component causing a greater degree of reduction.

2. When glucose (see Appendix IV) or NADH + H^+ were the respiratory substrates, complex step changes were observed in the cytochrome b absorbance. With ascorbate TMPD and formate only one step was observed and this was eliminated when azide was added. These steps may indicate the operation of more than one component within the total b cytochrome fraction during respiration on NADH + H^+ and glucose.

SECTION IX

CONCLUSIONS

CONCLUSIONS

These studies have failed to show any physiological evidence of a switch in the pathway of electrons from a coupled to an uncoupled branch of the respiratory system either during growth at different medium dissolved oxygen tensions or during growth in the presence of cyanide. Whatever the significance is of the multiplicity of terminal oxidase cytochromes in Beneckea natriegens, it does not appear to be related to dissolved oxygen changes or cyanide concentration. The reason for, and the mechanism(s) involved in causing the increased cytochrome content and potential respiration rate at dissolved oxygen tensions well above the 'critical' value remain obscure.

The cyanide sensitivity of growing bacteria was dependent on the respiration rate of the culture. Bacteria harvested from chemostat culture exhibited cyanide sensitivity that was independent of growth conditions. These results are not consistent with an alternative cyanide insensitive pathway functioning as a major respiratory pathway, as all cells tested were cyanide sensitive at high respiration rate. However, there was, in each case, a residual cyanide insensitive respiration amounting to some 10% of the maximum. The latter may represent the cyanide insensitive pathway that Weston, Collins and Knowles (1974) detected in cell free extracts of this organism, particularly as the respiration rate of fractionated bacteria represented approximately 10% of the value observed with whole bacteria (Chapter IV).

The cyanide sensitivity of B. natriegens harvested from chemostat culture was a function of the respiration rate. Consequently statements commonly

found in the literature of relative cyanide sensitivity, expressed as percentage inhibition, or activity, are without meaning unless substrate saturation and potential activity are taken into account. For example, although the actual respiration rate supported by succinate was only 4.2% of that supported by ascorbate TMPD Weston, Collins and Knowles (1974) suggested that the biphasic curve obtained by plotting percentage activity against cyanide concentration for B. natriegens respiring succinate indicated a branched electron transport system. The cyanide insensitive pathway consitituted, at maximum, 10% of the total bacterial respiration rate (Table IX-1).

Cyanide has been used extensively as a probe in the study and elucidation of the electron transport system of bacteria. The results reported here indicate that a great deal of caution must be exercised when interpreting data derived from experiments of this type. The respiration rate of chemostat cultures of B. natriegens was not limited by the terminal oxidase until the growth rate approached μ_{max} where the in situ and potential respiration rates were similar. Experiments using cyanide indicated that the potential respiration rate was limited by the terminal oxidase.

Beneckea natriegens was found to metabolise formate constitutively. It is suggested that the CO-binding c type cytochrome is not related to cyanide sensitivity as implied by Weston (1974) but rather is involved in formate metabolism. Studies on the 'apparent' Km for oxygen with formate as the respiratory substrate indicated the presence of two separate systems of formate oxidation. From yield coefficient data and NADH + H^+ fluorescence studies it is postulated that one system involves an NAD linked formate dehydrogenase and the other is a formate

Table IX-1. The effect of KCN on the respiration of ascorbate TMPD and succinate by cell free extracts of Beneckea natriegens. Data recalculated from Weston, Collins and Knowles (1974)

KCN concentration	Respiration rate on ascorbate TMPD* (mM O_2 per g protein per h)	Inhibition (%)
0	57.00	0.00
0.1 µM	31.90	44.10
0.5 µM	17.30	69.60
1.0 µM	14.25	75.00
10.0 µM	7.12	87.60
	Respiration rate on succinate (mM O_2 per g protein per h)	
0	2.40	0.00
0.2 µM	1.94	19.20
0.5 µM	1.34	44.20
1.0 µM	1.20	50.00
500 µM	1.20	50.00
1.0 mM	1.20	50.00
2.0 mM	0.84	65.00
3.0 mM	0.74	69.20
5.0 mM	0.33	86.25

oxygenase possibly linked with the CO binding c type cytochrome, a similar CO binding c type cytochrome has been implicated in the methane oxygenase reaction (Tong et al., 1975).

APPENDIX I

THE INORGANIC ION REQUIREMENTS OF MARINE BACTERIA

1. Introduction

   (i) Requirement of Na^+

   (ii) Requirement of K^+

   (iii) Requirement of Mg^{2+} and Ca^{2+}

   (iv) Requirement of chloride

   (v) Requirement of trace elements

2. Materials and Methods

   (i) Media

   (ii) Shake flask experiments

   (iii) Inoculum

   (iv) Measurement of growth

3. Results

   (i) Medium development

   (ii) The effect of variation of the concentration of glucose in the medium on the growth of Beneckea natriegens

   (iii) Determination of the optimal NaCl concentration required for growth of Beneckea natriegens

   (iv) The effect of variation of the ammonium sulphate concentration on the growth of Beneckea natriegens

(v) The effect of variation of the magnesium sulphate concentration on the growth of Beneckea natriegens

(vi) The effect of variation of the phosphate concentration on the growth of Beneckea natriegens

(vii) The effect of variation of the concentration of trace elements on the growth of Beneckea natriegens

4. Discussion

APPENDIX I

1. Introduction

An investigation into the requirements of Beneckea natriegens for inorganic ions was considered a necessary preliminary to chemostat studies. The aim of this study was to provide a basic salts medium optimal for the growth of the organism under carbon limitation in a chemostat.

A large number of investigations have been made of the inorganic ion requirements of bacteria; however, comparatively few of these studies have yielded quantitative data. This is particularly so in the case of studies reported of the inorganic ion requirements of marine bacteria. Much of the earlier work on the mineral requirements of marine bacteria was made with complex laboratory media such as nutrient broth and fish broth (MacLeod, 1968) which were probably heavily contaminated with inorganic ions. This has resulted in a great deal of confusion regarding the absolute requirements of these organisms for various individual ions. By using special precautions to avoid the introduction of inorganic contaminants into the medium Richter (1928) and Murdak (1933) demonstrated that luminous marine bacteria required Na^+ for growth and for luminescence.

(i) Requirement of Na^+

Initially it was thought that the requirement for sodium chloride, or sea water, of marine bacteria merely reflected a requirement of these organisms for an environment of suitable osmotic pressure. This conclusion was supported by the observations of Harvey (1915) and Hill (1929) who demonstrated lysis

of marine bacteria when suspended in distilled water. The first report indicating a specific requirement for Na^+ by marine bacteria is generally attributed to Richter (1928). Using chemically defined medium MacLeod and Onofrey (1956a) demonstrated that of six marine organisms examined all required Na^+ for growth and that a concentration between 0.2 M and 0.3 M was optimal for growth. Although growth occurred after prolonged incubation at concentrations of Na^+ between 0.02 M and 0.03 M, growth did not occur in the absence of added Na^+. All marine bacteria that have been examined in detail have a specific requirement for Na^+ (MacLeod, 1968) which cannot be replaced by Li^+, Rb^+ or Cs^+, or by osmotic agents (MacLeod and Onofrey, 1956a; Payne, 1960; Tomlinson and MacLeod, 1957)

Bacteria that require Na^+ for growth vary in their quantitative requirement for Na^+. Extreme halophilic species require an optimum of approximately 2 M to 5 M Na^+ (Brown and Gibbons, 1955) and growth ceases if the Na^+ concentration is below 1.5 M. Christian (1956) reported that Vibrio costicoles, a moderate halophilic bacterium, had a non-specific requirement of approximately 0.4 M NaCl but the specific requirement was only 0.017 M Na^+. The marine bacteria examined to date have optimal requirements for Na^+ ranging from 0.05 to 0.4 M depending on the species. Reports that marine bacteria can be trained to grow in fresh water media (Zobell and Michener, 1938; Zobell, 1946) must be viewed with caution as these studies were carried out on complex media, the organic components of which have been shown to reduce the Na^+ requirement (MacLeod and Onofrey, 1963; Merkel, Traganza, Mukherjee, Griffin and Prescott, 1964).

(ii) Requirement of K^+

Bukatch (1956) was one of the first workers to demonstrate that a marine bacterium required K^+. Subsequently a large number of marine bacteria have since been shown to have this requirement (Payne, 1960; Tyler, Bielling and Pratt, 1960). The requirement for K^+ could not be spared or replaced by Rb^+ (MacLeod and Onofrey, 1957). Tempest, Dicks and Hunter (1966) demonstrated that a plot of bacterial concentration against the medium K^+ concentration gave a straight line but, as this did not pass through the origin, they suggested that other substances present in the medium replaced the requirement for K^+ to some extent. The minimal requirement for K^+ has been shown to vary with growth rate and an interdependence between RNA and K^+, similar to that between RNA and Mg^{2+}, has been demonstrated in Aerobacter aerogenes (Tempest et al., 1966).

(iii) Requirement of Mg^{2+} and Ca^{2+}

Using a chemically defined medium, MacLeod and Onofrey (1956a, b) studied the effect of various concentrations of Mg^{2+} and Ca^{2+} on the growth of marine bacteria after 70 h and demonstrated a requirement of between 4 and 8 mM Mg^{2+} for maximal rate and extent of growth. In view of the poor experimental design, these values must be treated with caution. Tempest, Hunter and Sykes (1965) have shown that when A. aerogenes is grown under Mg^{2+} limitation the yield of bacteria is directly proportioned to the Mg^{2+} content of the medium. Moreover the extrapolation of the line obtained by plotting the Mg^{2+} concentration against the bacterial yield passed through the origin indicating that Mg^{2+} could not be functionally replaced and that almost all the Mg^{2+}

present in the culture was contained in the organism. The intracellular Mg^{2+} requirement varied greatly with growth rate, being greatest at the maximum rate. Present evidence indicates that the large requirement for Mg^{2+} is mainly due to its requirement as a stabiliser of ribosomal structure.

A marked interaction between Mg^{2+} and Ca^{2+} was reported by MacLeod and Onofrey (1956b); thus the presence of 2.5 mM Ca^{2+} in the medium reduced the Mg^{2+} requirement of a particular marine bacterium from 8 to 0.04 mM but did not eliminate the need for this ion. High levels of Ca^{2+} (50 mM) on the other hand increased the requirement for Mg^{2+}. Norris and Jensen (1957) reported that several species of Azotobacter had a requirement for Ca^{2+} for growth. Humphrey and Vincent (1962) examined the walls of Rhizobium trifolii that had been grown under Ca^{2+} depleted conditions. Since nearly all the Ca^{2+} present in the Ca^{2+}-depleted culture was present in the wall they suggested that Ca^{2+} was an essential component not replacable by Mg^{2+} or any other divalent cation. The Ca^{2+} content of Aerobacter aerogenes grown under glycerol, and magnesium limitation and of Bacillus subtilis var. niger grown under Mg^{2+}-limitation at various growth rates showed no correlations with the RNA, Mg^{2+}, K^{+} or phosphorus content (Tempest, 1969). However, as the composition of the bacterial walls has been reported to change with growth rate (Ellwood and Tempest, 1967a, b; 1968) these results are difficult to interpret.

(iv) Requirement of chloride

The chloride concentration of sea water is approximately 0.53 M. It is not surprising, therefore, to find that the growth of marine bacteria is

affected by the chloride concentration. MacLeod and Onofrey (1956b) examined six marine isolates, three of which were found to have a specific requirement for chloride and the growth of the remainder was enhanced in the presence of chloride ions. Bromide was equally as active as chloride but iodide was toxic. The requirement of marine bacteria for chloride and its effect on growth resembled the response of these organisms to Na^+ (MacLeod and Onofrey, 1956b). The requirement for halides by moderate and extreme halophilic bacteria varies from a specific requirement for Cl^- to no demonstratable requirement (Larsen, 1962). All bacteria that have been shown to require Cl^- have also required Na^+ though the converse is not true. An exception to this is Desulphovibrio desulfuricans as it requires NaCl for growth; Na^+ but not Cl^- may be replaced by other ions.

(v) Requirement of trace elements

When sea water was used to dilute chemically defined media the growth of a number of marine bacteria was limited by iron (MacLeod and Onofrey, 1956b). Menzel and Ryther (1961) reported that the growth of coccolithophorid flagellates and diatoms in the Sargasso Sea was limited by the availability of iron which was found almost entirely as particulate ferric complexes. The addition of EDTA led to rapid multiplication of these organisms. The gradual growth of marine organisms under these conditions (accelerated by EDTA) raises the possibility that adaptation might depend on the production of chelating agents as is commonly found in fungi (Davies, 1970).

Although the total amount of trace metals may appear adequate they are poorly available in tropical seas and the North Sea because these seas are

lacking in critical organic chelating agents. This was clearly demonstrated by the addition of EDTA to sea water which caused an increase in the growth of a diatom, Skeletonema and various phytoplankton (Barber and Ryther, 1969).

The macro and trace element composition of a number of bacteria expressed as mg element per g cell dry weight is given in Appendix I, Tables 1 and 2. The amount of a given trace element per g cell dry weight varies considerably according to the bacterial species examined. The specific requirement of bacteria for various trace elements probably varies. However, the values quoted by different workers are not strictly comparable as different methods of assay have been used. The trace metals commonly found in sea water also are shown in Appendix I, Tables 1 and 2.

Appendix I - Table 1. The macro-elemental composition of bacteria expressed as mg element per g bacterial dry weight

Element	C	N	P	S	Na	K	Mg	Ca	Reference
Escherichia coli	500	103-150	32.0	11	-	14.7	3.6	7.6	Luria (1960)
Batch cultured.									
Escherichia coli			42.8	8.6	0.074	11.7	6.2	0.14	
Micrococcus roseus			32.5	7.9	0.63	9.2	12	0.71	
Sphaerotilus natans			21.3	5.7	0.74	1.66	5.7	1.6	Rouf (1964)
Bacillus cereus			32.3	6.9	0.55	45.8	10.3	0.2	
Bacillus cereus (spores)			12.2	7.0	0.37	18.8	5.7	0.07	
Aerobacter aerogenes									
Mg^{++} limited D = 0.1 h^{-1}							1.01		
0.8 h^{-1}							2.74		
Glycerol limited dilution rate = 0.1 h^{-1}							1.51		
0.8 h^{-1}							2.36		Tempest and
K^+ limited D = 0.1 h^{-1}							1.49		Strange (1966)
0.8 h^{-1}							3.02		
NH_4 limited D = 0.1 h^{-1}							2.06		
0.8 h^{-1}							3.10		
Aerobacter aerogenes									
NH_4 limited D = 0.1 h^{-1}		98		9.2			1.8		
0.8 h^{-1}		110.8		10.4			2.9		
SO4 limited D = 0.1 h^{-1}		110.2		10.3					
0.8 h^{-1}		112.6		10.5					
K^+ limited D = 0.1 h^{-1}		116.6	13.1	10.9			1.2		
0.8 h^{-1}		108.1	25.6	10.14			2.4		
Carbon limited D = 0.1 h^{-1}		116.8		10.95			1.3		
0.8 h^{-1}		108.8		10.20			3.0		
Mg^+ limited D = 0.1 h^{-1}		110.2					1.1		
0.8 h^{-1}		99.36		9.3			2.6		
PO_4 limited D = 0.1 h^{-1}		120		11.25			1.0		
0.8 h^{-1}		111		10.87			3.1		
Bacillus subtilis									
Mg^{++} limited dilution rate = 0.1 h^{-1}		99.3		9.3			1.4		
0.21 h^{-1}		84.9		7.96			1.69		
0.41 h^{-1}		90.7		8.50			2.12		Tempest, Dicks
0.56 h^{-1}		88.4		8.29			-		and Meers
0.57 h^{-1}							2.52		(1967)
Bacillus subtilis						49	3	0.10	
B. megaterium						40	2	-	
B. megaterium						21	11	11	
B. subtilis spores						9	5	16	
B. cereus spores						2	3	19	Tempest (1969)
B. megaterium spores						6	5	10	
B. macerans						5	4	2	
Aerobacter aerogenes						16	3	2.2	
Rhizobium trifolii						-	2	2.0	
Bacillus megaterium		63							
B. megaterium spores		92-138							
B. cereus var. mycoides		106							
var. anthracis		68							
var. anthracis (spores)		86-133							Murrel (1969)
B. subtilis strain I		106.4							
II		105.8							
Bacillus species (27 strains) (spores)			0.45-2.7						
Clostridium bifermentans						16	2.5	64	
Bacillus species (27 strains)			11		0.8	6.5	4.5	22.7	
Pseudomonas C D = 0.32 h		110	36			6.2	7.8	1.2	Mateles and Battat 1974
Aerobacter aerogenes 0.05 h^{-1}			15.0			9.2	1.24		Tempest and
Chemostat D = 0.7 h^{-1}			19.0			16.2	2.6		Meers 1968

Appendix I - Table 2. The trace element composition of bacteria expressed as mg element per g dry weight

Organism / Element	B	Al	Tl	Cr	Mn	Ni	Cu	Sr	Ag	Sn	Ba	Pb	V	Mo	Co	Fe	Zn	Reference
Escherichia coli	0.012	0.023	0.0012	0.002	0.020	0.0047	0.0335	0.0033	0.00055	0.0039	0.0044	0.011	-	0.0026	-	0.2	0.08	
Micrococcus roseus	0.0077	0.033	0.0037	0.0013	0.0069	0.0055	0.0143	0.0042	0.00065	0.011	0.0044	0.0037	-	0.0046	-	0.13	0.24	
Sphaerotilus natans	-	0.019	0.0042	0.00068	0.0041	0.0070	0.0247	0.0067	0.00093	0.023	0.0044	0.0134	0.0061	-	-	18.1	0.24	
Bacillus cereus	0.0024	0.016	0.0017	0.00034	0.479	0.0023	0.0343	0.0033	0.0018	0.0039	0.00179	0.0074	-	-	-	0.2	0.24	Rouf (1964)
Bacillus cereus (spores)	-	0.015	0.0017	0.0014	1.40	0.0031	0.748	0.0084	0.00055	0.0047	0.0044	0.0037	0.0028	-	-	0.27	2.5	
Minimal detectable amount	0.00093	-	0.00029	0.00006	0.00041	0.00031	-	0.00084	0.000093	0.00007	0.00053	0.000092	0.00028	0.00013	0.0005	-	-	
Bacillus subtilis		-			-		-									-	-	
Bacillus megaterium		-			-		-									-	-	
B. megaterium		0.1			0.1		0.1									0.1		
B. subtilis spores		0.1			0.1		0.1									0.1		Tempest (1969)
B. cereus spores		0.4			0.1		0.2									0.2		
B. megaterium spores		0.1			0.1		0.2									0.1		
B. macerans		0.2			0.1		0.1									0.2		
Bacillus species Av. of 27 strains			0.21		2.3												0.7	
Clostridium bifermentans					0.1													
Pseudomonas D = 0.32 h^{-1}	0.0006					0.0013	0.0041							0.0018		0.59	0.0036	Mateles and Battat (1974)
Medium G. Conc (mg l^{-1})	0.0175				0.0369		0.04				0.23			0.1189	0.0446	3.79	0.04	
Sea water (mg g^{-1} sea water)	0.005	0.00001			0.000002	0.000002	0.000003							0.00001	0.0000005	0.00001	0.00001	

2. Materials and Methods

(i) Media

Medium A. (Modification of that used by Baumann, Baumann and Mandel, 1971).

Component (a) NaCl, 23 g; KCl, 0.745 g; $MgSO_4$ $7H_2O$, 2.46 g were dissolved in 190 ml distilled water.

Component (b) Tris (hydroxymethyl) aminomethane (Tris) hydrochloride, 7.88 g; NH_4Cl, 1.33 g; K_2HPO_4, 0.0448 g; $FeSO_4$ $7H_2O$, 0.027 g; citric acid, 0.45 g; glucose, 4.5 g; was dissolved in 800 ml distilled water and the pH adjusted to 7.5.

Component (c) 0.146 g $CaCl_2$ $2H_2O$ was dissolved in 10 ml distilled water.

Components (a), (b) and (c) were autoclaved separately at 110° for 20 mins, allowed to cool and then mixed.

Medium B

Medium B differed from medium A with respect to the phosphate concentration only, which was increased from 0.0448 g to 1.36 g K_2HPO_4 l^{-1}.

Medium C

Component (a) NaCl, 23 g; KCl, 0.745 g; $MgSO_4$ $7H_2O$, 2.46 g was dissolved in distilled water and made up to 250 ml, pH 7.5.

Component (b) NH_4Cl, 1.33 g; KH_2PO_4, 1.16 g; Na_2HPO_4, 1.4 g; citric acid, 0.45 g; glucose 4.5 g; was dissolved in 700 ml distilled water, pH 7.5.

Component (c) (i) $FeCl_3$ $6H_2O$, 0.0167 g was dissolved in 20 ml distilled water. (ii) 1 ml of a trace element mixture (from a stock solution) was made up to 20 ml with distilled water. (iii) $CaCl_2$ $2H_2O$, 0.146 g was dissolved in 10 ml distilled water.

Trace element stock solution(gl^{-1})

$CaCl_2$ $2H_2O$, 0.66; $ZnSO_4$ $7H_2O$, 0.18; $CuSO_4$ $5H_2O$, 0.16; $MnSO_4$ $4H_2O$, 0.15; $CoCl_2$ $6H_2O$, 0.18; H_3BO_4, 0.10; Na_2MoO_4 $2H_2O$, 0.30.

Components (a), (b) and (c) were autoclaved separately at 110°C for 20 mins and mixed on cooling.

Medium D

Component (a) NaCl, 23 g; KCl, 0.745 g; $MgSO_4$ $7H_2O$, 2.46 g was dissolved in distilled water and made up to 200 ml.

Component (b) Na_2HPO_4, 1.5 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 3.0 g; glucose, 4.5 g; citric acid 0.420 g was dissolved in distilled water and made up to 760 ml (pH 7.5).

Component (c) (i) $FeCl_3$ $6H_2O$, 0.0167 g was dissolved in 20 ml distilled water. (ii) 1 ml of a trace element mixture was made up to 20 ml with distilled water. Autoclaved as in medium C.

Medium E

Component (a) NaCl, 23 g; KCl, 0.745 g; $MgSO_4$ $7H_2O$, 0.2 g were dissolved in distilled water and made up to 200 ml.

Component (b) Na_2HPO_4, 3.0 g; KH_2PO_4, 3.0 g; $(NH_4)_2SO_4$, 3.0 g; citric acid, 0.45 g; glucose, 4.5 g were dissolved in distilled water and made up to 760 ml (pH 7.5).

Component (c) 1 ml $FeCl_3$ and 1 ml trace element mixture as in medium D.

Components (a), (b) and (c) were autoclaved separately at 110°C for 20 mins and mixed on cooling.

Medium G

Component (a) NaCl, 23 g; KCl, 0.745 g; $MgSO_4$ $7H_2O$, 0.1 g were dissolved in distilled water and made up to 200 ml.

Component (b) Na_2HPO_4, 1.5 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 3.0 g; glucose 0.5 g (unless otherwise stated) were dissolved in distilled water and made up to 760 ml (pH 7.5).

Component (c) $FeCl_3$ and trace elements as in D.

Components (a), (b) and (c) were autoclaved separately and mixed on cooling.

(ii) Shake flask experiments

Experiments on the growth of B. natriegens were made in 250 ml Erlenmeyer conical flasks fitted with side arms to facilitate direct absorbance measurements. The flasks contained 50 ml of culture medium. The absorbance of the side arms varied but, by rotating the flasks to a set position in the colorimeter, they could be calibrated and set to a value near to the zero reading of a control flask. The variation that occurred between flasks had little effect on the results

because (i) the difference between flasks was small and (ii) the difference remained constant so that relative changes in absorbance could be measured with reasonable accuracy. The flasks were incubated at 32°C on an orbital shaker (Gallenkamp), set at 280 r.p.m. with a 4" throw.

(iii) Inoculum

A 0.5% (v/v) inoculum of mid-log phase cells (absorbance 1.0 at 625 nm) was used in all shake flask experiments. All experiments were carried out in duplicate.

(iv) Measurement of growth

Growth was measured as culture absorbance at 625 nm on a Cecil EEL colorimeter or at 640 nm on a Klett Summerson nephelometer.

3. Results

(i) Medium development

On the basis of work described by Baumann et al. (1971) a range of different media were empirically formulated. A comparison of the media developed and their ability to support the growth of Beneckea natriegens is summarised in Appendix I, Table 3.

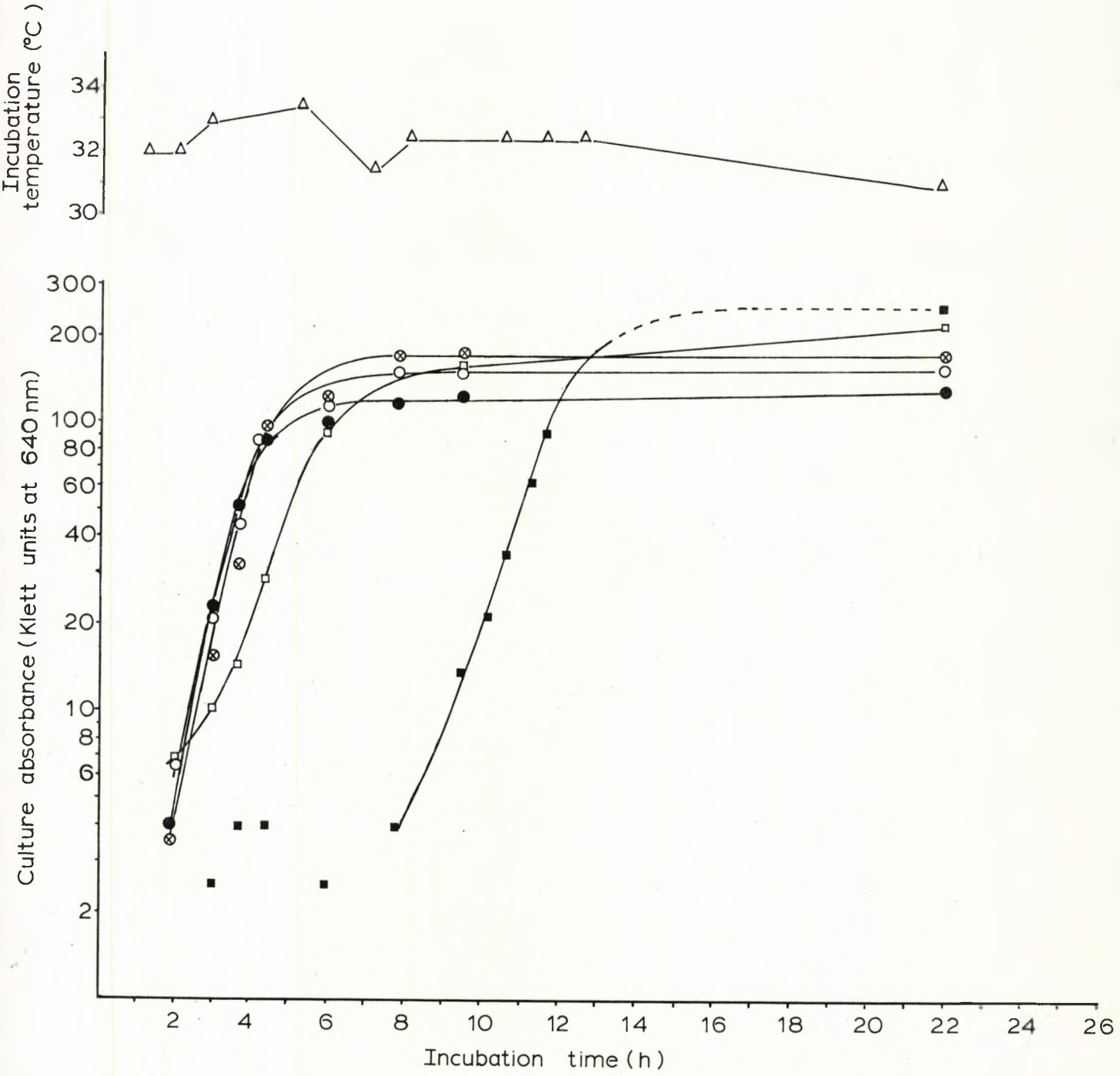
The growth rate of B. natriegens in medium A, B, C and E appeared to be very similar (Appendix I, Figure 1) and that in medium D was somewhat lower. Although the glucose concentration in all the media was 4.5 gl^{-1} the final culture absorbance varied considerably. The modified medium of Baumann et al. (medium A) was not used as a basis for further development as it supported a much lower culture density than medium E, indicating that growth was probably limited by a nutrient other than the carbon source. Medium E was chosen as the basis for further development.

The presence of a metabolisable chelating agent in continuous culture medium could interfere with the metabolism of the organism and lead to difficulty in interpretation of results. It was therefore decided to reformulate medium E omitting the citric acid. This was achieved by lowering the phosphate and magnesium concentrations by 50%. This unchelated medium, was designated medium G.

In order to assess the effect of citric acid and EDTA on the growth of B. natriegens in media G, shake flasks were set up containing various combinations of glucose, citric acid and EDTA. The growth of B. natriegens under these conditions is shown in

Appendix I, Fig. 1. Growth of Beneckea natriegens in various media.

Medium A, ● - ●; B, ⊛ - ⊛; C, o - o; D, □ - □; E, ■ - ■. The variation of the temperature of the incubation room is shown Δ - Δ.


Incubation temperature (°C)
34
32
30
Culture absorbance (Klett units at 640 nm)
300
200
100
80
60
40
20
10
8
6
2
2
4
6
8
10
12
14
16
18
20
22
24
26
Incubation time (h)

App. I. Fig.-1

Appendix I, Table 3. A comparison of the media developed and their ability to support the growth of Beneckea natriegens

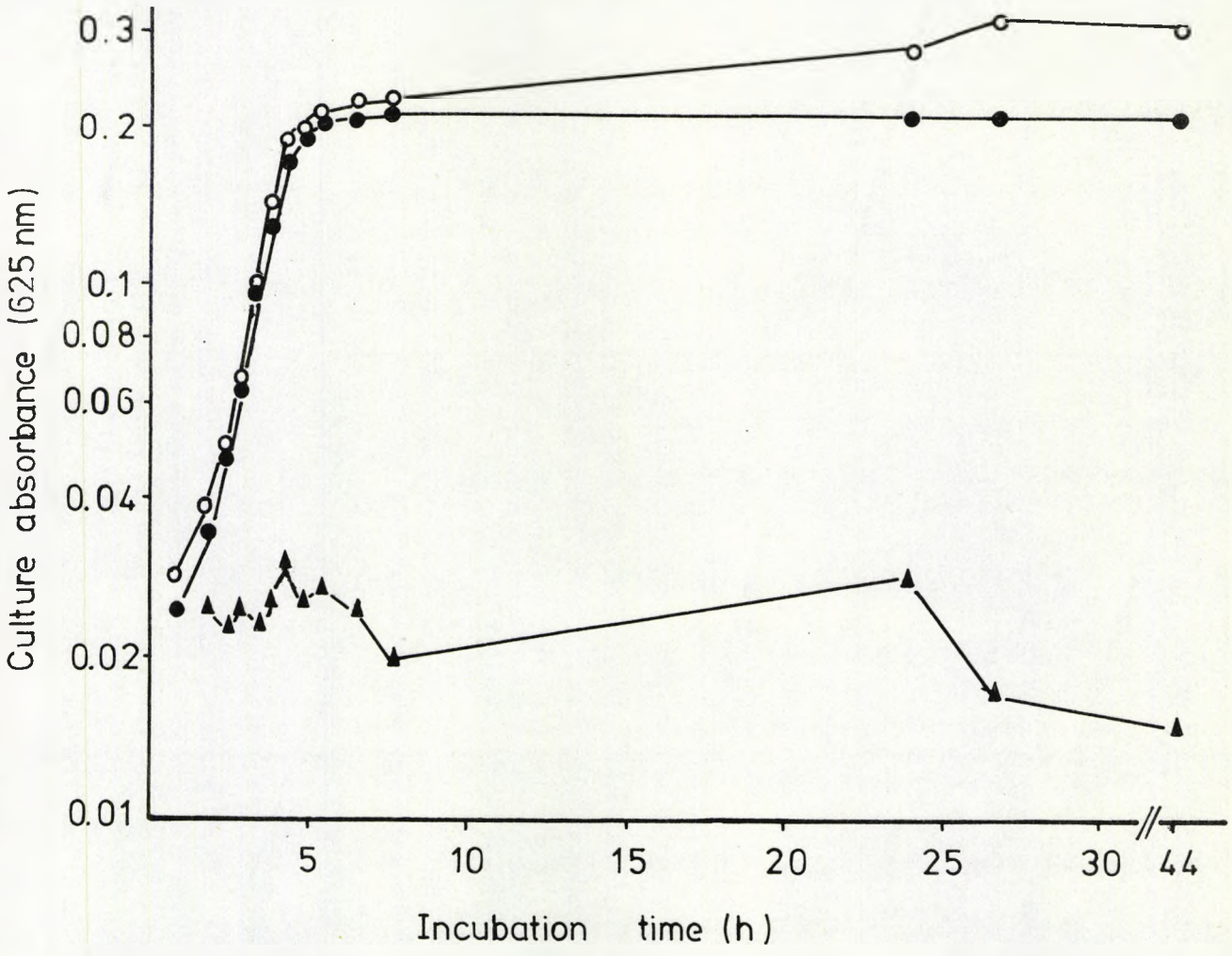
Medium	A	B	C	D	E
Phosphate source (gl^{-1})					
Na_2HPO_4	-	-	1.409	1.40	2.81
KH_2PO_4	0.0448	1.36	1.17	1.17	2.31
Nitrogen source (gl^{-1})					
NH_4Cl	1.33	1.33	1.33	-	-
$(NH_4)_2SO_4$	-	-	-	2.90	2.90
Glucose (gl^{-1})	4.5	4.5	4.5	4.5	4.5
Magnesium source (gl^{-1})					
$MgSO_4$ $7H_2O$	2.46	2.46	2.46	2.46	0.20
Iron source (gl^{-1})					
$FeSO_4$ $7H_2O$	0.027	0.027	-	-	-
$FeCl_3 6H_2O$	-	-	0.0167	0.0167	0.0167
Calcium source (gl^{-1})					
$CaCl_2$ $2H_2O$	0.146	0.146	0.146	0.00066	0.00066
Trace element mixture	-	-	+	+	+
Citric acid (gl^{-1})	0.45	0.45	0.45	0.45	0.45
Doubling time (h)	0.6	0.53	0.61	0.78	0.66
Final culture absorbance (Klett units)	130	179	158	220	253
Lag phase (h)	<0.5	<0.5	<0.5	$\simeq 1.0$	$\simeq 6$

Appendix I, Fig. 2. The growth rates of B. natriegens in medium G containing either glucose alone or a mixture of glucose and citric acid were not significantly different. The citric acid appeared to be used in diauxic manner. Glucose-grown cells exhibited a long lag period before growth occurred in medium containing citric acid as the sole carbon source. EDTA did not support growth of the organism and it was too toxic to be considered as an alternative chelating agent. The final culture absorbance produced in medium G containing various combinations of citric acid, glucose and EDTA indicated that citric acid was utilised by this organism, Appendix I, Fig. 3.

(ii) The effect of variation of the concentration of glucose in the medium on the growth of Beneckea natriegens

The initial lag period before growth commenced increased as the concentration of glucose in the medium was raised. The lag period was virtually non-existent at a medium glucose concentration of 0.5 gl^{-1} but increased to approximately 17 h at a medium glucose concentration of 15 gl^{-1} (Appendix I, Fig. 4). The increase in the glucose concentration of the medium also appeared to cause a decrease in growth-rate. This effect was small at concentrations of glucose below 4 gl^{-1} but increased markedly as the medium glucose concentration was further increased. The final culture pH value decreased with increasing glucose concentration and reached a minimum value of pH 4.9 at a medium glucose concentration of 6 gl^{-1}. Further increase in the glucose concentration did not cause a further decrease in the final culture pH value (Appendix I, Fig. 4). The final culture absorbance of B. natriegens as a

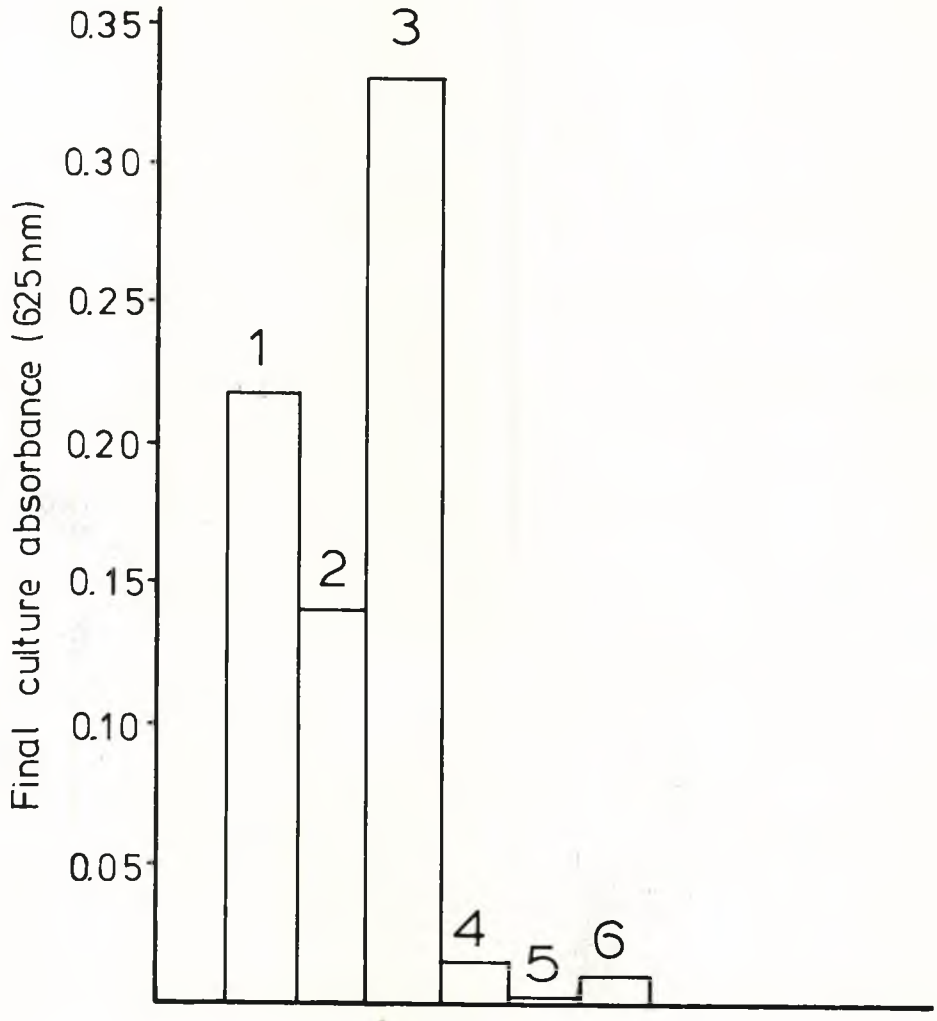
Appendix 1, Fig. 2. The effect of citric acid and ethylene diamine tetracetic acid (EDTA) on the batch growth of Beneckea natriegens in medium G. Glucose 0.5 gl^{-1}, ● - ●; glucose 0.5 gl^{-1} plus citric acid 0.5 gl^{-1}, o - o; and glucose 0.5 gl^{-1} plus EDTA 0.5 gl^{-1}, Δ-Δ.


0.3
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Culture absorbance (625 nm)
5
10
15
20
25
30
44
Incubation time (h)

App. I. Fig.-2

Appendix 1, Fig. 3. The final culture absorbance of Beneckea natriegens produced in medium G containing various combinations of glucose, citric acid and EDTA.

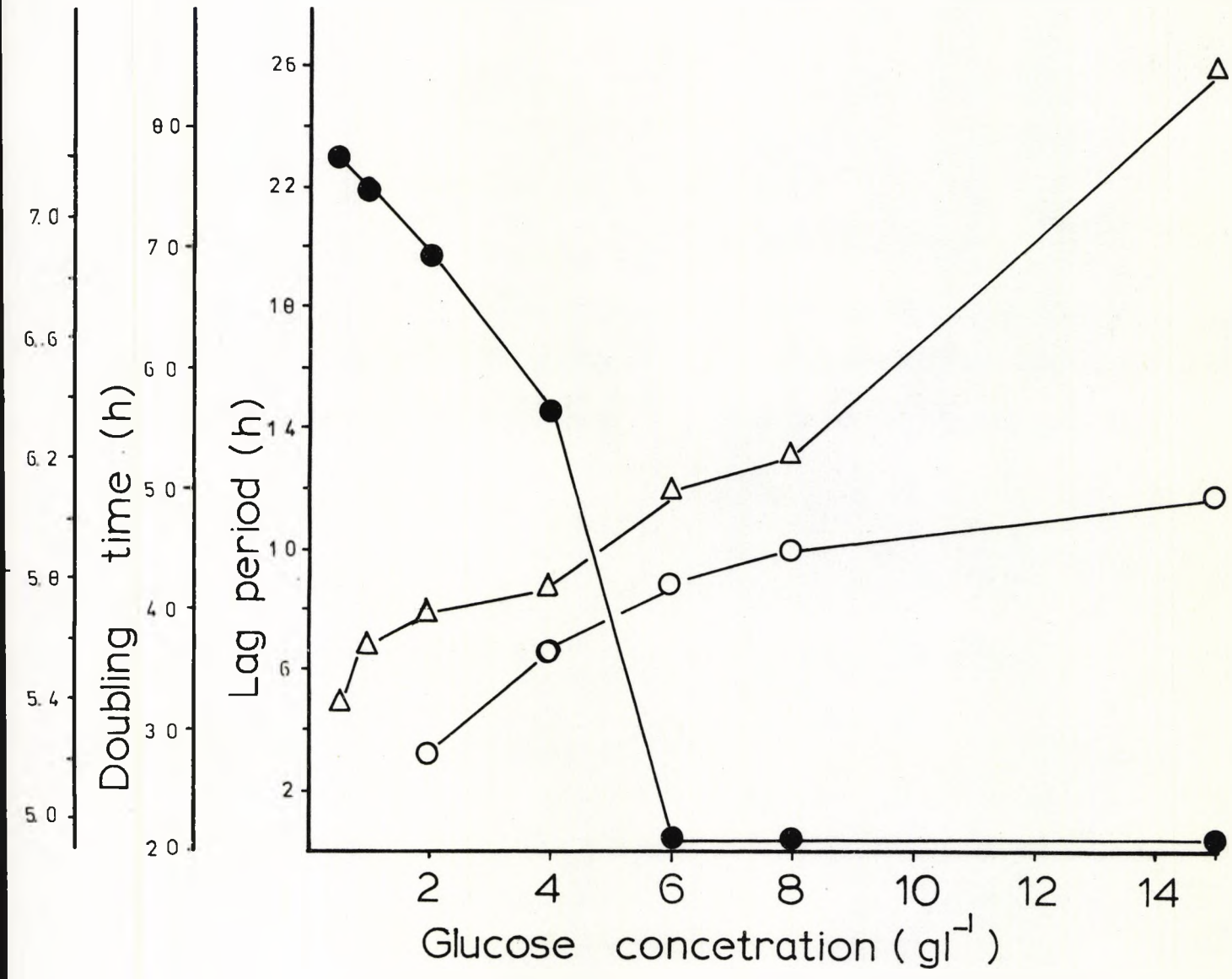
(1) Glucose, 2.77 mM; (2) citric acid, 2.37 mM; (3) citric acid, 2.37 mM + glucose, 2.77 mM; (4) glucose, 2.77 mM + EDTA, 0.25 gl^{-1}; (5) citric acid, 2.37 mM + EDTA, 0.25 gl^{-1}; (6) EDTA, 0.25 gl^{-1}.


0.35
0.30
0.25
0.20
0.15
0.10
0.05
Final culture absorbance (625nm)
1
2
3
4
5
6

App.I. Fig.-3

Appendix I, Fig. 4. The effect of increase in the medium glucose concentration on the lag period, growth rate and final culture pH of Beneckea natriegens grown in medium G.

Lag period, o - o; doubling time, Δ - Δ; and final culture pH value, ● - ●.


Doubling time (h)
7.0
6.6
6.2
5.8
5.4
5.0
80
70
60
50
40
30
20
Lag period (h)
26
22
18
14
10
6
2
2
4
6
8
10
12
14
Glucose concetration (gl^{-1})

App. I. Fig.-4

function of the glucose concentration is shown in Appendix I, Fig. 5. The culture absorbance of B. natriegens appeared to be independent of the glucose concentration when the latter was increased beyond 4 gl^{-1}.

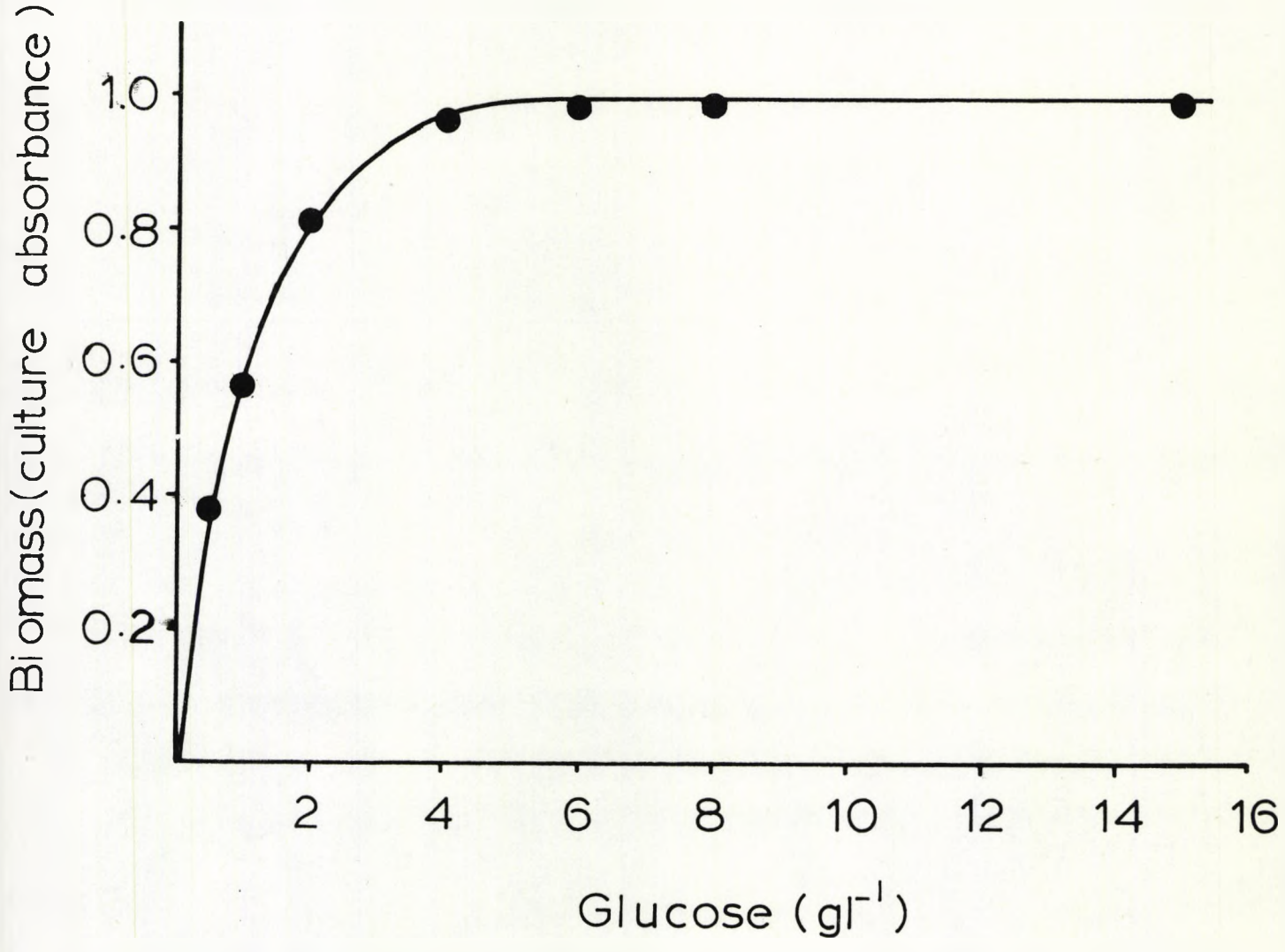
(iii) Determination of the optimal NaCl concentration required for growth of Beneckea natriegens

Shake flasks were set up using medium A containing 0.5 gl^{-1} glucose and either lacking NaCl, or, containing 0.1, 0.2, 0.3 or 0.4 M NaCl. The growth curves of B. natriegens observed in the presence of various concentrations of NaCl is shown in Appendix I, Fig. 6. The growth rate and final culture absorbance of Beneckea natriegens appeared to be independent of the NaCl concentration over the range 0.2 to 0.4 M NaCl, but was marginally lower in medium containing 0.1 M NaCl. The organism failed to grow in the absence of added NaCl. Although 0.2 M NaCl would have been optimal for growth, a concentration of 0.4 M NaCl was used in both batch and chemostat work as it was thought that the higher NaCl concentration would help to reduce the risk of contamination.

(iv) The effect of variation of the ammonium sulphate concentration on the growth of Beneckea natriegens

The growth of B. natriegens in medium G containing 0.5 gl^{-1} glucose and various levels of $(NH_4)_2SO_4$ is shown in Appendix I, Fig. 7. The growth rate of the organism was independent of the $(NH_4)_2SO_4$ concentration over the range 0.50 to 3.0 gl^{-1}. At an ammonium sulphate concentration of 6.0 gl^{-1} slight inhibition of the growth rate was observed.

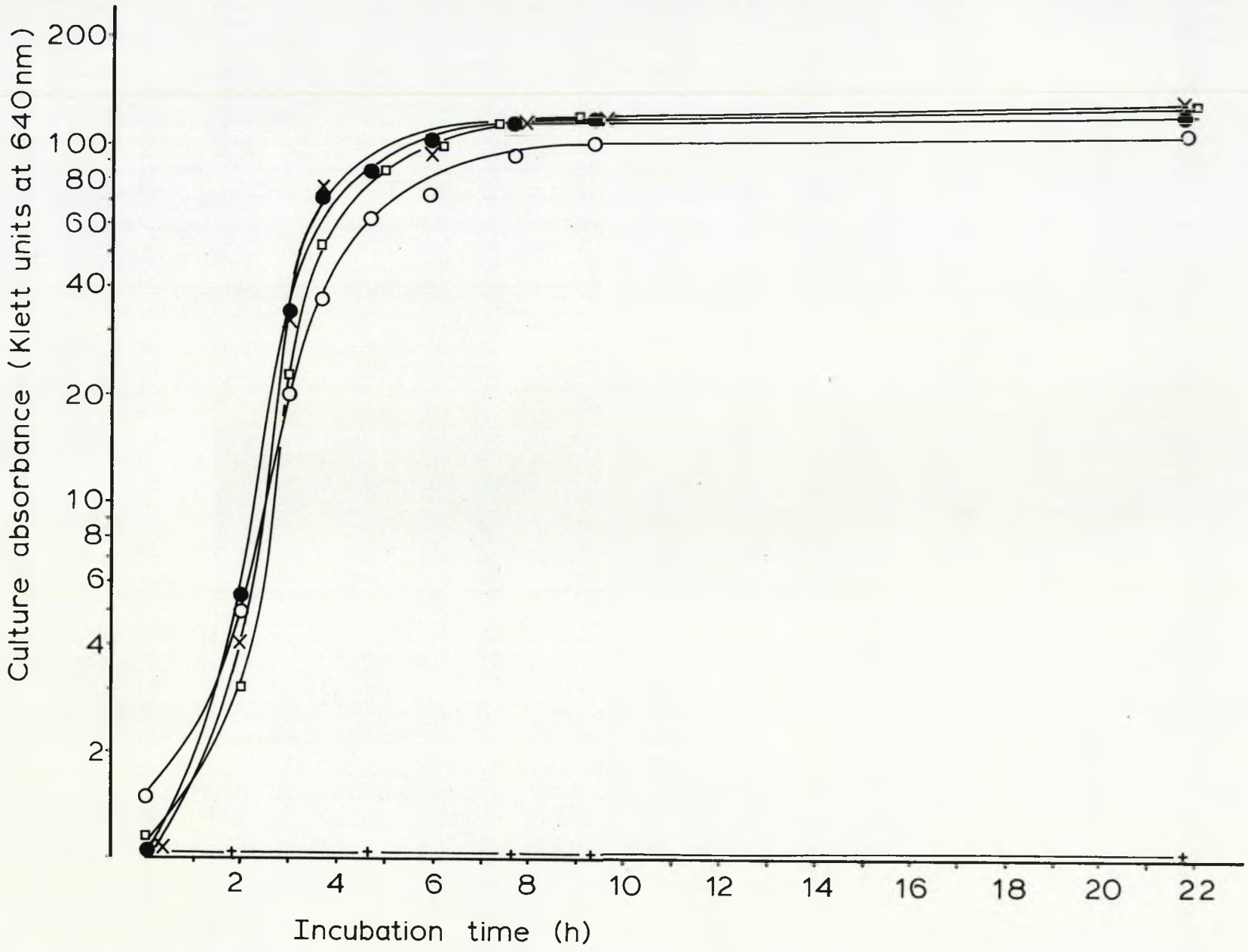
Appendix 1, Fig. 5. The final culture absorbance of Beneckea natriegens supported in medium G as a function of the medium glucose concentration.


Biomass (culture absorbance)
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
14
16
Glucose (gl⁻¹)

App. I. Fig.- 5

Appendix 1, Fig. 6. The effect of sodium chloride concentration on the growth of Beneckea natriegens in batch culture.

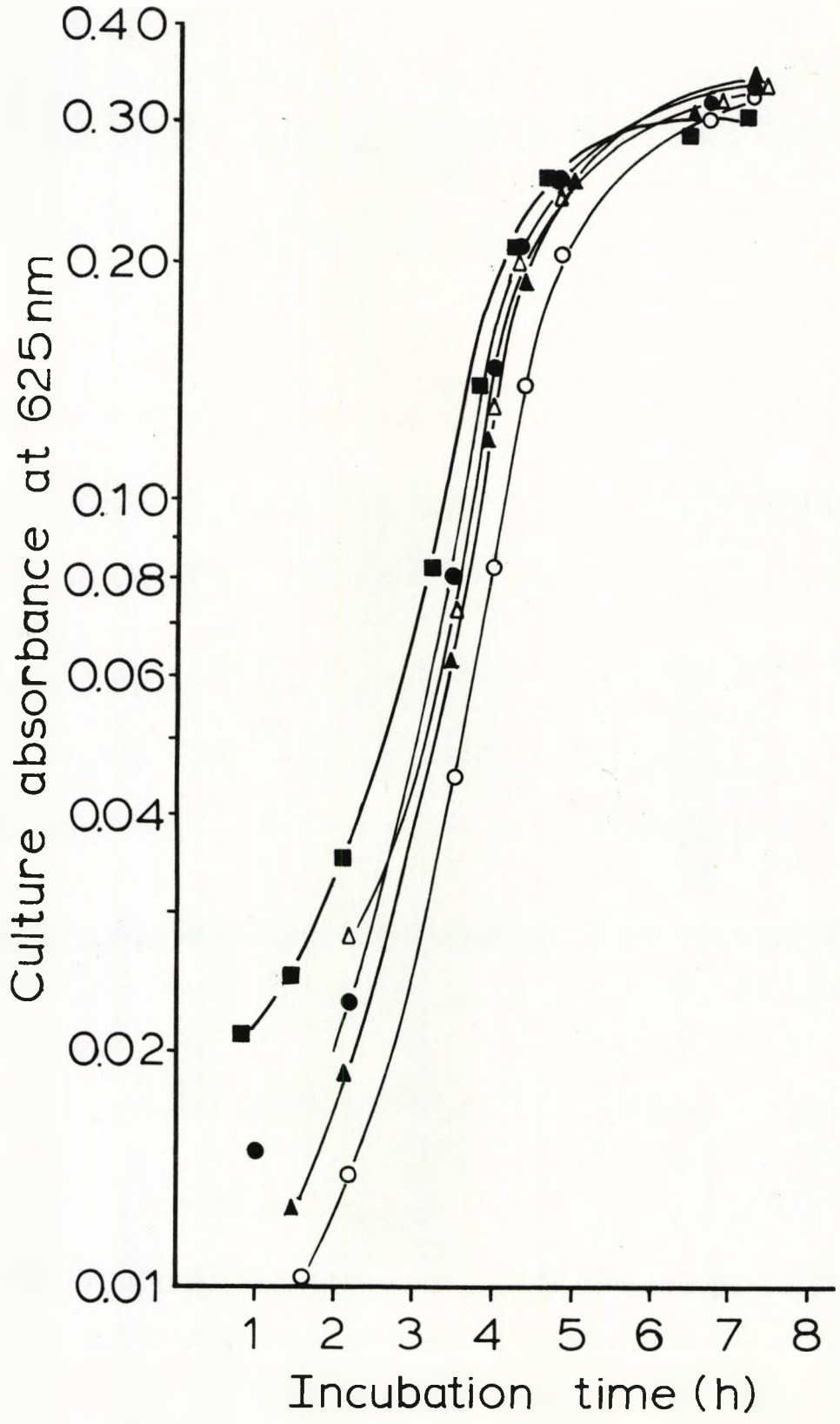
Medium NaCl concentrations:- x - x, 0.4 M; ● - ●, 0.3 M; □-□, 0.2 M; o - o, 0.1 M and + - + no NaCl.


Culture absorbance (Klett units at 640nm)
200
100
80
60
40
20
10
8
6
4
2
2
4
6
8
10
12
14
16
18
20
22
Incubation time (h)

App. I. Fig.-6

Appendix 1, Fig. 7. Growth of Beneckea natriegens in medium G containing various concentrations of ammonium sulphate.

Medium $(NH_4)_2SO_4$ concentrations:- o - o, 0.25 gl^{-1}, Δ - Δ, 0.5 gl^{-1}; ● - ●, 1.0 gl^{-1}; ▲ - ▲, 2.0 gl^{-1} and ■ - ■, 6.0 gl^{-1}.


0.40
0.30
0.20
0.10
0.08
0.06
0.04
0.02
0.01
Culture absorbance at 625nm
1
2
3
4
5
6
7
8
Incubation time (h)

App. I. Fig.-7

(v) The effect of variation of the magnesium sulphate concentration on the growth of Beneckea natriegens

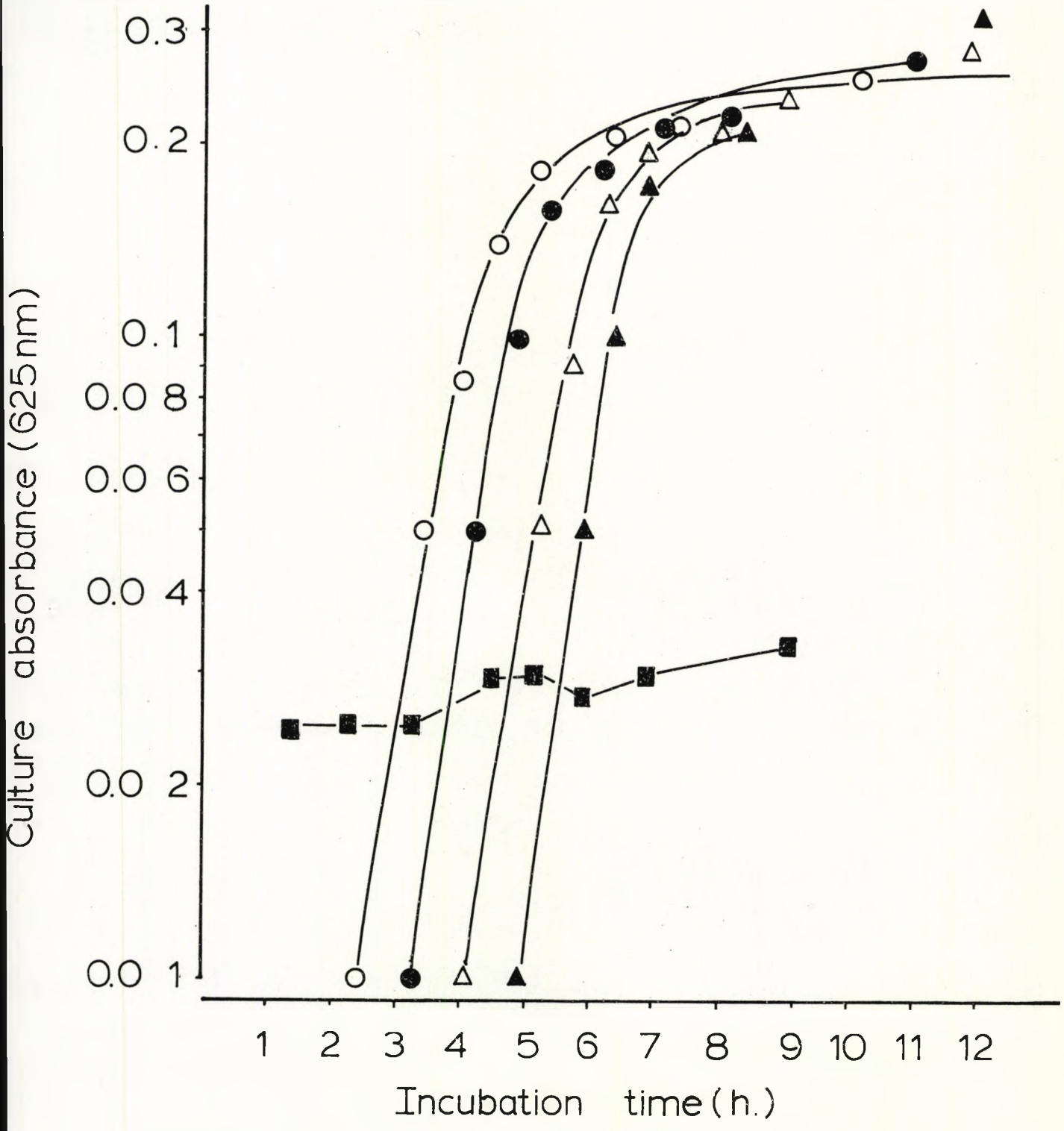
Poor growth of B. natriegens was observed in medium G lacking magnesium (Appendix I, Fig. 8). The final culture absorbance at a glucose concentration of 0.5 gl^{-1} was found to be independent of the $MgSO_4\ 7H_2O$ concentration above 0.1 gl^{-1} (Appendix I, Fig. 9). The growth rate was also found to be independent of the $MgSO_4\ 7H_2O$ concentration above 0.1 gl^{-1} provided the medium contained 0.4 M NaCl (Appendix I, Fig. 8). High $MgSO_4\ 7H_2O$ concentrations (1.0 gl^{-1}) appeared to compensate for the effect of low and high NaCl concentrations, restoring the growth rate of cells grown at 0.2 and 0.8 M NaCl to a value close to that observed in the presence of 0.4 M NaCl. The doubling time of the organism did not show any clear relationship to the $MgSO_4\ 7H_2O$:NaCl ratio (Appendix I, Fig. 10)

(vi) The effect of variation of the phosphate concentration on the growth of Beneckea natriegens

The growth of B. natriegens in medium G containing 2.0 gl^{-1} glucose and various concentrations of phosphate is shown in Appendix I, Fig. 11. Above a concentration of 0.5 gl^{-1} phosphate ($Na_2HPO_4 + KH_2PO_4$ 1:1) the growth rate appeared to be independent of the phosphate concentration. The period of logarithmic growth was extended as the concentration of phosphate was increased from 0.060 g to 3.0 g phosphate (Na_2HPO_4:KH_2PO_4 1:1), beyond this value no effect was evident. The pH value of the culture after growth had ceased was found to be approximately 4.5 over a phosphate concentration 0.06 g to 2.0 gl^{-1}

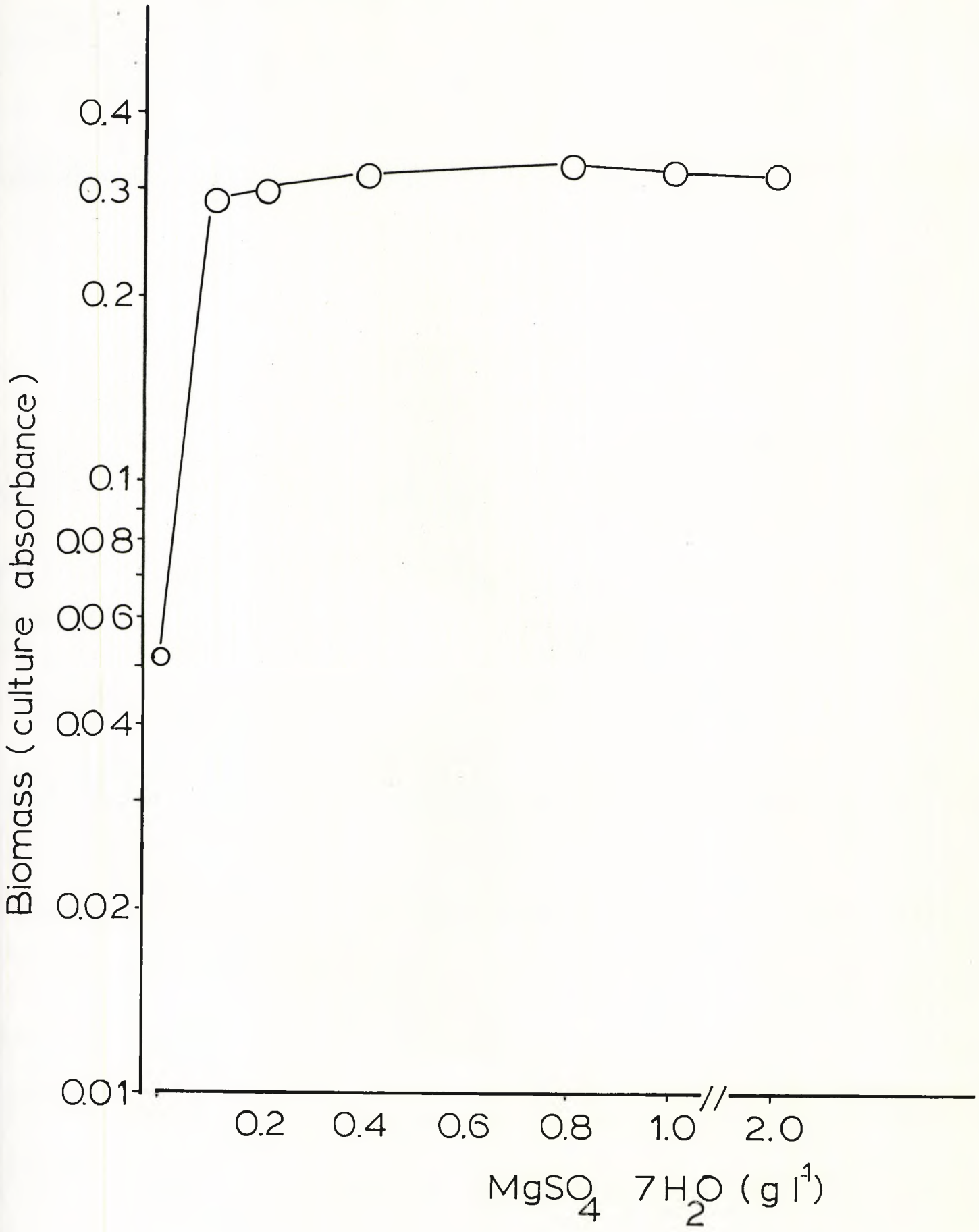
Appendix 1, Fig. 8. The effect of magnesium sulphate concentration on the growth rate and final culture absorbance of Beneckea natriegens in medium G containing 0.4 M NaCl.

$MgSO_4 \cdot 7H_2O$ concentrations:- 0.1 gl^{-1}, o - o; 0.2 gl^{-1}, ● - ●; 0.4 gl^{-1}, Δ - Δ; 1.0 gl^{-1}, ▲ - ▲; and no $MgSO_4 \cdot 7H_2O$, ■ - ■.


0.3
0.2
0.1
0.0 8
0.0 6
0.0 4
0.0 2
0.0 1
Culture absorbance (625nm)
1 2 3 4 5 6 7 8 9 10 11 12
Incubation time (h.)

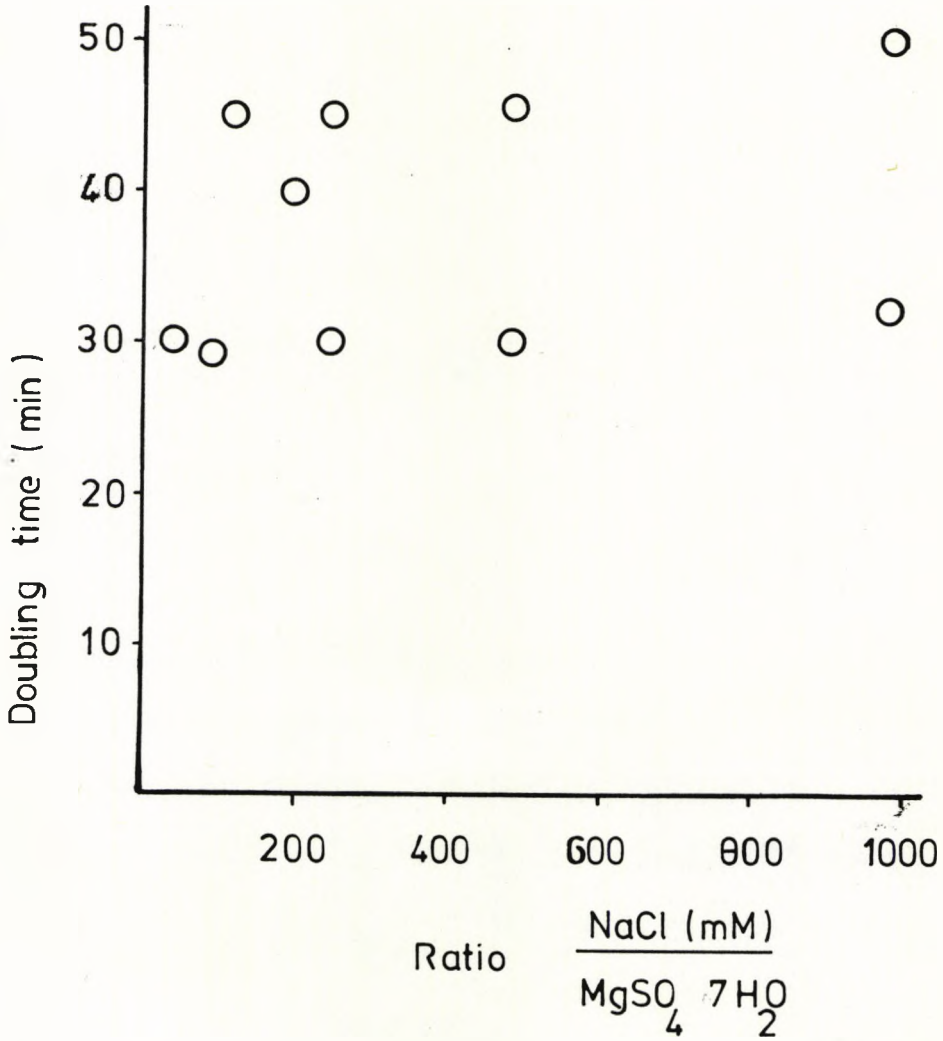
App. I. Fig.-8

Appendix 1, Fig. 9. The final culture absorbance of Beneckea natriegens supported in medium G containing varying concentrations of magnesium sulphate.


0.4
0.3
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Biomass (culture absorbance)
0.2
0.4
0.6
0.8
1.0
2.0
MgSO4 7H2O (g l-1)

App. I. Fig.-9

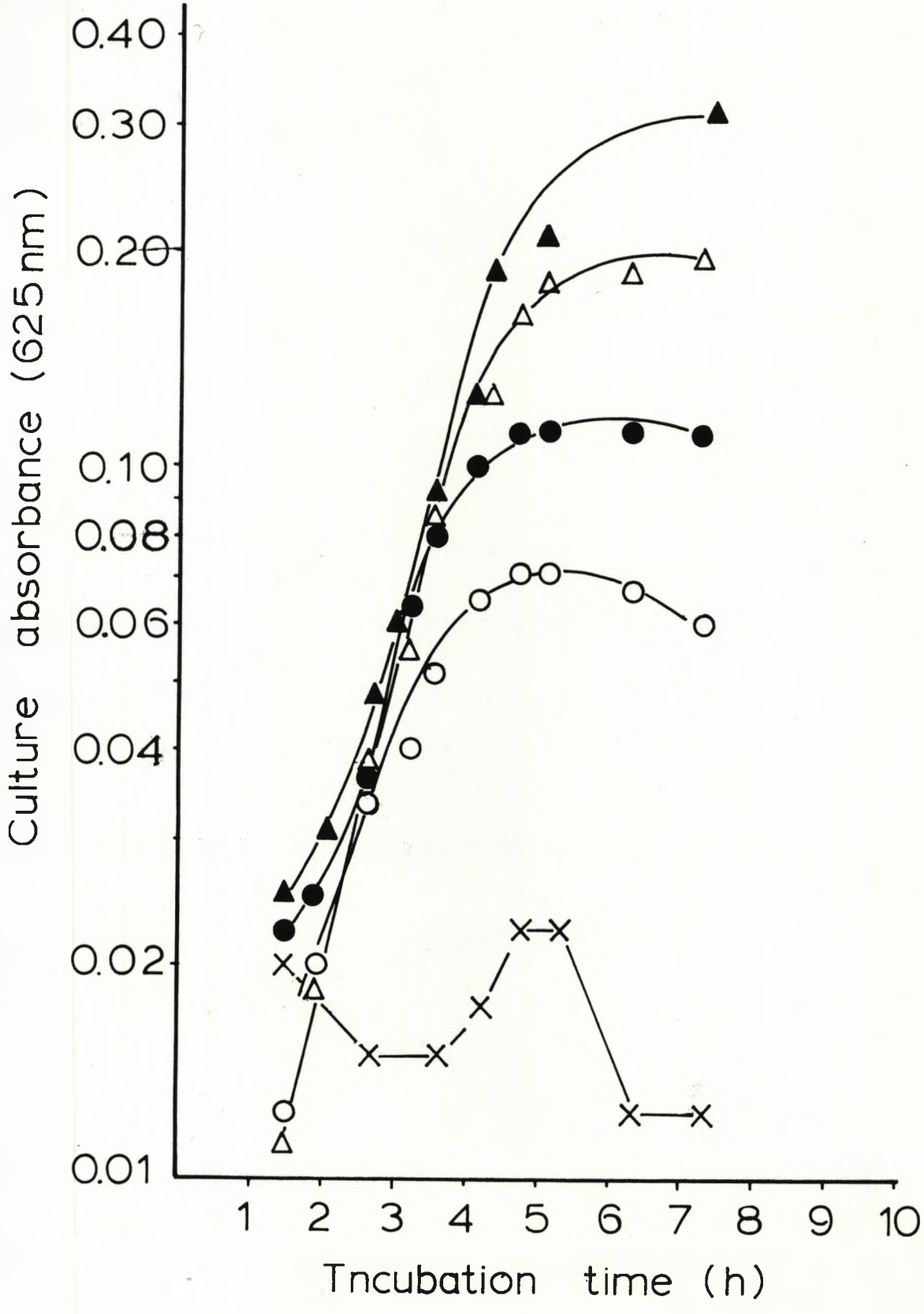
Appendix 1, Fig. 10. The effect of the sodium chloride:magnesium sulphate ratio on the growth rate of Beneckea natriegens in medium G.


50
40
30
20
10
Doubling time (min)
200
400
600
800
1000
Ratio
NaCl (mM)
MgSO4 7H2O

App.I. Fig.-10

Appendix 1, Fig. 11. The effect of variation of the phosphate concentration on the growth of Beneckea natriegens in medium G.

Phosphate concentration, Na_2HPO_4:KH_2PO_4 1:1:- x - x, 0.06 gl^{-1}; o - o, 0.25 gl^{-1}; ● - ●, 0.5 gl^{-1}; Δ - Δ, 1.0 gl^{-1} and ▲ - ▲, 3 gl^{-1}.


0.40
0.30
0.20
0.10
0.08
0.06
0.04
0.02
0.01
Culture absorbance (625nm)
1
2
3
4
5
6
7
8
9
10
Tncubation time (h)

App. I. Fig.-11

phosphate. At phosphate concentrations of 3.0 gl^{-1} and 4.0 gl^{-1} the pH appeared to remain at pH 6.5 (Appendix I, Fig. 12). The final bacterial concentration (carbon gl^{-1}) supported in medium G containing various concentrations of phosphate and 0.5 and 2.0 gl^{-1} glucose is shown in Appendix I, Fig. 12. At a glucose concentration of 0.5 gl^{-1} the final bacterial concentration (carbon gl^{-1}) appeared to be independent of the phosphate concentration above 1.0 gl^{-1} phosphate. At a glucose concentration of 2.0 gl^{-1} however the final bacterial concentration (carbon gl^{-1}) was dependent on the phosphate concentration over the range 0-3 gl^{-1}.

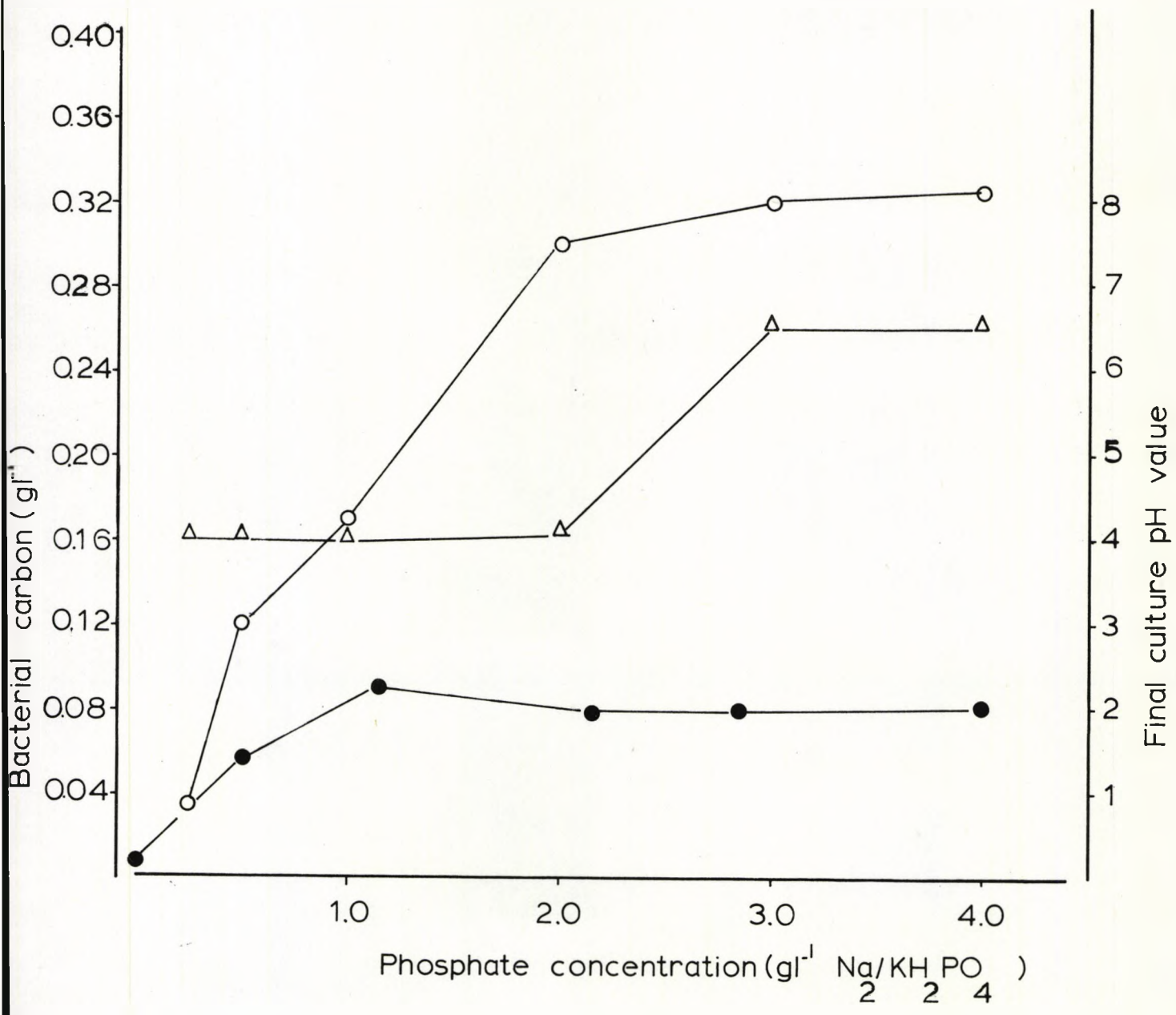
(vii) The effect of variation of the concentration of trace elements on the growth of Beneckea natriegens

The final bacterial concentration (carbon gl^{-1}) supported in medium G containing 0.5 gl^{-1} glucose was independent of the trace element concentration (Appendix I, Fig. 13), obtained in the absence of added trace elements values were not significantly different from that observed in the presence of added trace elements. An increase in the trace element concentration by a factor of four did not appear to have any toxic effect on the culture.

Appendix 1, Fig. 12. The effect of the phosphate concentration on the final culture absorbance of Beneckea natriegens and the final culture pH value of medium G, at a glucose concentration of 0.5 gl^{-1}.

Concentration of 0.5 gl^{-1}, ● - ● and 2.0 gl^{-1}, o - o.

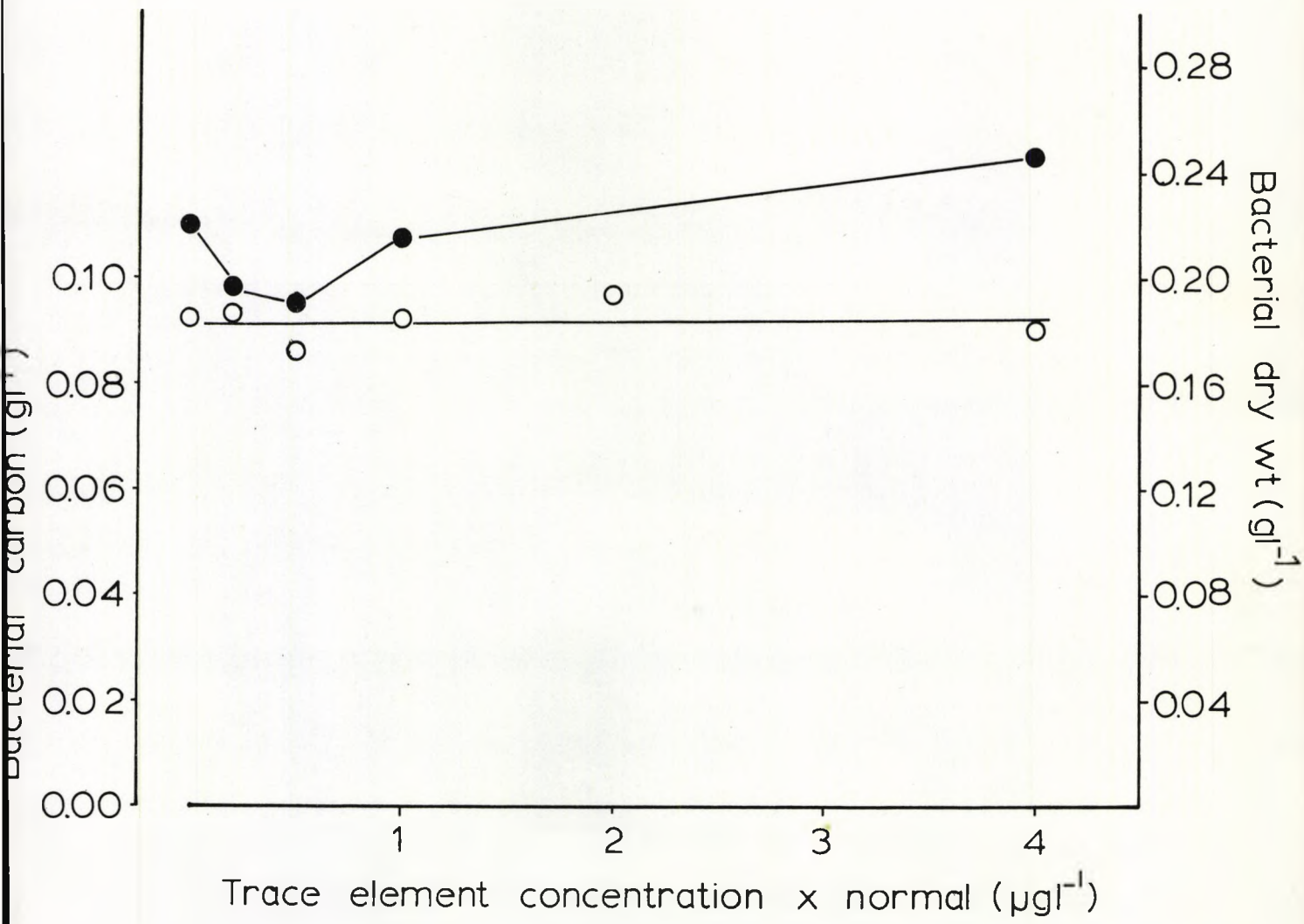
Final culture pH value at a glucose concentration of 2.0 gl^{-1}, Δ-Δ.


0.40
0.36
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
Bacterial carbon (gl⁻¹)
8
7
6
5
4
3
2
1
Final culture pH value
1.0
2.0
3.0
4.0
Phosphate concentration (gl⁻¹ Na/KH PO)
2 2 4

App. I. Fig. - 12

Appendix 1, Fig. 13. The effect of variation in the trace element concentration on the final culture absorbance of Beneckea natriegens supported in medium G.

● - ●, g cell carbon l^{-1}; o - o, g bacterial dry weight l^{-1}.


0.10
0.08
0.06
0.04
0.02
0.00
0.28
0.24
0.20
0.16
0.12
0.08
0.04
Bacterial dry wt (gl^{-1})
1
2
3
4
Trace element concentration x normal (µgl^{-1})

App. I. Fig. -13

4. Discussion

The total biomass of B. natriegens support by medium G was dependent on the glucose concentration in the range 0.5 to 4 gl^{-1}. Further increase in the glucose concentration did not result in an increase in the culture biomass, indicating that growth was probably limited by some factor(s) other than the carbon source. Increase in the glucose concentration of the medium had a marked effect on the growth characteristics of Beneckea natriegens. The growth rate decreased and the lag phase increased with increasing glucose concentration. The inhibition of the growth rate and increase in the lag phase caused by increase in the medium glucose concentration may be explained to some extent by the rapid fall of the culture pH value as the glucose concentration was increased. Beneckea natriegens has been shown to produce acidic media when grown on glucose (Eagon and Cho, 1965). However, inhibition of the growth rate and increase in the lag phase cannot be explained solely in terms of a fall in the pH value because the growth rate decreased and lag phase increased over a glucose concentration (6-15 gl^{-1}) over which there was no further fall in the pH value. It is possible that glucose itself has an inhibitory effect, high levels of glucose causing suppression of the respiratory pathway of B. natriegens in a manner analogous to the Crabtree effect demonstrated in yeast (Crabtree, 1929, Swanson and Clifton, 1948; De Deken, 1966).

Beneckea natriegens did not grow in the absence of added NaCl. The growth rate of B. natriegens in medium A was found to be independent of the NaCl concentration over the range 0.2 - 0.4 M. These results are contradictory

to those obtained in medium G where the growth rate was found to decrease when the NaCl concentration was either lower or higher than 0.4 M NaCl. This apparent contradiction can be explained by the high $MgSO_4\ 7H_2O$ (2.5 gl^{-1}) of medium A which appeared to compensate to some extent for Na^+ deficiency. The magnesium requirement of B. natriegens grown in shake flask experiments was found to considerably lower (0.4 mM Mg^{2+}) than the values quoted for marine bacteria by MacLeod (1965) (4 - 8 mM). On closer examination of the original paper (MacLeod and Onofrey, 1956b) it was found that the Mg^{2+} requirements were measured by incubating various organisms for 70 h with different concentrations of Mg^{2+} and then measuring the percent light transmission. No attempt had been made to measure growth rate at different Mg^{2+} concentrations or to relate the Mg^{2+} requirement to the quantity of bacteria present.

As Na^+ has been shown to compete with Mg^{2+} for electrostatic interaction with components of the cell envelope of a marine isolate (Devoe and Oginsky, 1969) the effect of variation in the Mg^{2+}:Na^+ ratio on growth rate was examined. Although no clear correlation between Mg^{2+}:Na^+ ratio and growth rate was observed, high $MgSO_4\ 7H_2O$ concentrations (1 gl^{-1}) appeared to compensate for the effect of both high and low NaCl concentrations in the growth medium. At a NaCl concentration of 0.4 M the growth rate was independent of the $MgSO_4\ 7H_2O$ concentration above 0.1 gl^{-1}. Consequently a concentration of 0.4 M NaCl was used in the medium developed for chemostat studies. The nitrogen source used throughout was ammonium sulphate. At high concentrations (6 gl^{-1}) of $(NH_4)_2SO_4$ there appeared to be a reduction of

the growth rate of B. natriegens. This is uncommon in heterotrophic organisms but is well documented for methane utilising bacteria (Whittenbury, Phillips and Wilkinson, 1970; Eccleston and Kelly, 1972). The main effect of the phosphate concentration on growth rate and biomass of B. natriegens was probably caused through its effect on final pH value. At low phosphate concentrations (0.25 to 2.0 gl^{-1} $Na_2HPO_4 + KH_2PO_4$ 1:1 mixture) the buffering capacity of the medium was low. An increase in the glucose concentration and the consequent fall in the pH value caused the apparent dependency of the biomass on the phosphate concentration. Although the medium of Baumann et al. (1971) was buffered with Tris.hydrochloric acid, growth was probably limited by the phosphate concentration which was only 0.0448 g KH_2PO_4 l^{-1}. The use of such a low concentration of phosphate is rather surprising since MacLeod and Onofrey (1956a) demonstrated that of the six marine bacteria examined the minimum requirement was 1.13 mM phosphate.

The biomass of B. natriegens supported in medium G appeared to be independent of added trace elements. This is not surprising as much of the trace element requirement is probably met by the contaminants present in the other salts added.

On the basis of the data shown in Appendix I, Tables 1 and 2, the quantity of bacterial dry weight potentially supported per g of element was calculated and is shown in Appendix I, Table 4. On the basis of these values the biomass potentially supported by each constituent of medium G was compiled. The results (Appendix I, Table 5) indicate that at a concentration of 4.0 g

glucose l^{-1} all the major constituents would be in excess and the culture would be carbon limited. From the experimental data (Appendix I, Table 5) the absolute requirement for each inorganic ion cannot be calculated. However, the data indicate that the culture would be carbon limited at a glucose concentration of 3.4 gl^{-1}. The medium developed provides a basis for further development in the chemostat.

Appendix I, Table 4. The quantity of bacterial dry weight that may be produced per g of element (based on the data given in Tables 1 and 3)

	g dry weight per g element		Values quoted by Harrison (1965)
	Range	Average	
Carbon			1.1
Nitrogen	6.60 - 14.7	9.6	8.0
Phosphorus	23 - 47	30.48	28.5
Sulphur	88 - 175	109	100
Magnesium:			
Chemostat culture	322 - 900	432	510
Batch culture	83 - 500	192	
Potassium	20 - 602	73	
Calcium	833 - 14285	4098	
Iron	1694 - 10000	4784	

Appendix I, Table 5. The quantity of bacterial dry weight that could be supported in a culture of Beneckea natriegens growing on medium G. (Based on the data given in Appendix I, Table 4).

Inorganic nutrients in medium	Potential g bacterial dry weight supported			
	Range	Average	Harrison (1965)	Experimental values
Carbon source Glucose 2.0 gl^{-1}(0.8 g C) 4.0 gl^{-1}(1.60)	- -	- -	0.88 0.176	0.85 -
Nitrogen source $(NH_4)_2SO_4$ 3.0 gl^{-1}(0.6363 g N)	4.19-9.35	6.10	5.08	not less than 2.56
Phosphorus source Na_2HPO_4 1.5 gl^{-1}(0.3273 g P) KH_2PO_4 1.5 gl^{-1}(0.3414 g P)	15.38-31.4	20.38	19.05	not less than 1.708
Sulphur source $(NH_4)_2SO_4$ 3.0 gl^{-1}(0.7283 g S) $MgSO_4$ $7H_2O$ 0.1 gl^{-1}(0.0131 g S)	65.2-129.7	80.8	74.1	not less than 1.708
Magnesium source $MgSO_4$ $7H_2O$ 0.1 g (0.00986 g Mg)	3.17-8.87	4.25	5.02	not less than 1.708
Potassium source KH_2PO_4 1.5 gl^{-1}(0.3310 g K) KCl 0.745 g (0.392 g K)	14.4-314.6	52.77	-	not less than 1.708
Iron source $FeCl_3$ $6H_2O$ 0.0167 g (0.00361 g Fe) contaminant Fe 0.00018	6.11-36.1	17.27 0.894	-	not less than 19.817
Calcium source $CaCl_2$ $2H_2O$ 0.00066 gl^{-1} Ca contaminant 0.000675 gl^{-1}	- -	0.742 0.760	- -	not less than 3.45

APPENDIX II

THE AMINO ACID PROFILE OF BENECKEA NATRIEGENS AS A FUNCTION OF GROWTH RATE

In view of its nutritional versatility, B. natriegens could be considered for use in the treatment of industrial effluent and the cells produced used as a source of protein. The organism could be grown on sea water supplemented with a cheap nitrogen and phosphorus source. For this reason the amino acid profile of B. natriegens was examined (Appendix II, Table 1). No significant change in the amino acid profile was observed as the growth rate was increased (Appendix II, Fig. 1). The amino acid content of B. natriegens compared favourably with the values quoted for soya bean meal, fish meal, Toprina and the F.A.O. reference protein. The sulphur amino acid (methionine and cystine) content of B. natriegens was lower than the F.A.O. standard, being 2.52 compared with 4.2 g per 16 g N respectively. However, this value compares favourably with the sulphur amino acid content of soya bean meal, Toprina and the protein content of the methane utiliser Methylococcus capsulatus (see Section III).

Appendix II - Table 1. A comparison of the amino acid content of freeze dried samples of Beneckea natriegens harvested from a carbon limited chemostat at various dilution rates with standard F.A.O. protein, soya bean meal, B.P. yeast protein ('Toprina') and Methylococcus capsulatus protein. The data is expressed as g amino acid/16 g N.

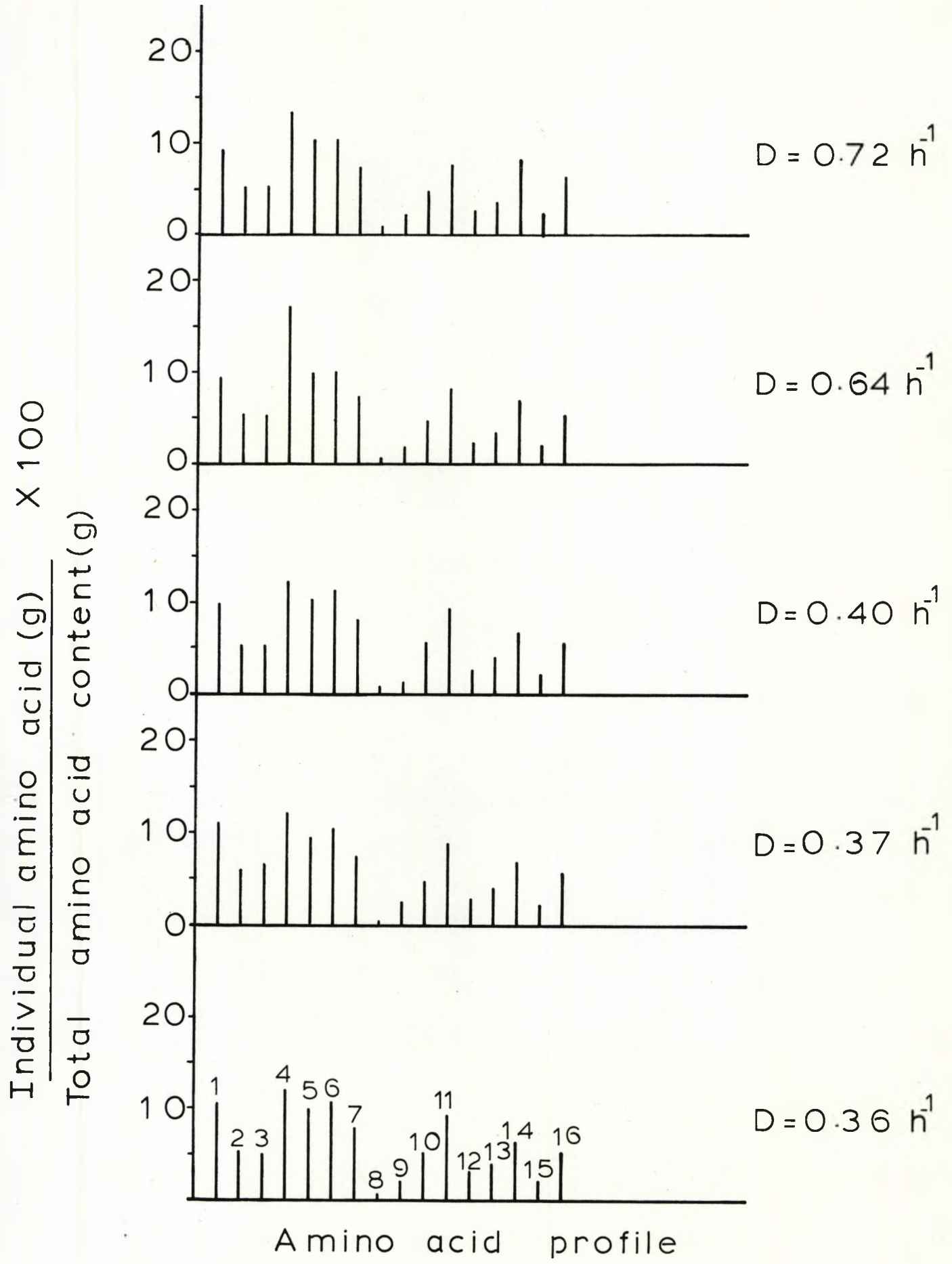
Dilution rate (h^{-1})	Beneckea natriegens (carbon limited)				Beneckea natriegens (growth in the presence of glucose + sodium formate)		Soya bean meal (Shacklady, 1969)	F.A.O. reference protein F.A.O. 1967	Fish meal (Wilkinson, 1972)	'Toprina' (B.P. Protein Limited) (yeast protein)		Methylococcus capsulatus (D'Mello 1972)
	0.36	0.37	0.64	0.72	0.4	0.4				G	L	
Amino acids												
Aspartic acid	8.66	9.27	6.85	7.48	7.58	7.23	N.D.					8.0
Threonine	4.01	4.45	3.31	3.72	3.66	3.44	4.0	2.8	4.2	4.9	5.4	4.2
Serine	3.34	4.35	2.84	3.35	3.23	3.26	N.D.					3.2
Glutamic acid	10.8	11.37	13.5	11.98	10.5	9.39	N.D.					8.8
Glycine	4.62	4.63	3.88	4.72	4.5	3.85	7.5		5.8	4.7	4.5	4.5
Alanine	5.87	5.92	4.69	5.61	5.86	4.90	N.D.					6.2
Cystine*	0.88	0.48	0.64	1.32	1.14	0.72	1.4	2.0	1.0	1.1	0.9	0.7
Valine*	5.71	5.48	4.48	3.36	5.46	4.67	5.0	4.2	5.2	5.8	5.8	5.5
Methionine*	1.96	1.54	1.55	2.01	0.95	1.94	1.4	2.2	2.6	1.8	1.6	1.9
Isoleucine*	4.16	4.06	3.14	3.81	4.3	3.37	5.4	4.2	4.6	5.0	5.3	4.0
Leucine*	7.12	7.4	5.64	6.13	7.28	5.99	7.7	4.8	7.3	7.4	7.8	6.7
Tyrosine*	3.44	3.37	2.32	2.88	2.92	2.36	2.4		2.9	3.6	4.0	3.5
Phenylalanine*	4.08	4.29	3.11	3.54	3.94	3.34	5.1	2.8	4.6	4.3	4.8	4.0
Lysine*	5.93	6.32	5.54	7.37	5.86	6.11	6.5	7.0	7.0	7.4	7.8	4.8
Histidine*	1.85	1.92	1.49	2.06	1.75	1.47	2.4		2.3	2.1	2.1	1.7
Arginine*	5.77	6.24	4.85	6.84	5.68	5.09	7.7		5.0	5.1	5.0	4.8
Glutamine*	0.20	0.15	0.22	0.20	0.41	0.16	N.D.					
Tryptophan*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.5	1.4				
Crude protein (%)	62.5	64.68	72.9	63.43	57.5	62.18	45		65.0			65.0

N.D. not determined

* amino acids essential for human nutrition

Appendix II, Fig. 1. The amino acid profile of Beneckea natriegens (expressed as percent of total amino acid fraction) as a function of dilution rate.

I	Aspartic	9	Methionine
2	Threonine	I0	Isoleucine
3	Serine	II	Leucine
4	Glutamic	I2	Tyrosine
5	Glycine	I3	Phenylalanine
6	Alanine	I4	Lysine
7	Valine	I5	Histidine
8	Cystine $\frac{1}{2}$	I6	Arginine


Individual amino acid (g) / Total amino acid content(g) X 100
20
10
0
D = 0.72 h-1
D = 0.64 h-1
D = 0.40 h-1
D = 0.37 h-1
D = 0.36 h-1
1
2 3
4
5 6
7
8
9
10
11
12
13
14
15
16
Amino acid profile

App.II - Fig. 1

APPENDIX III

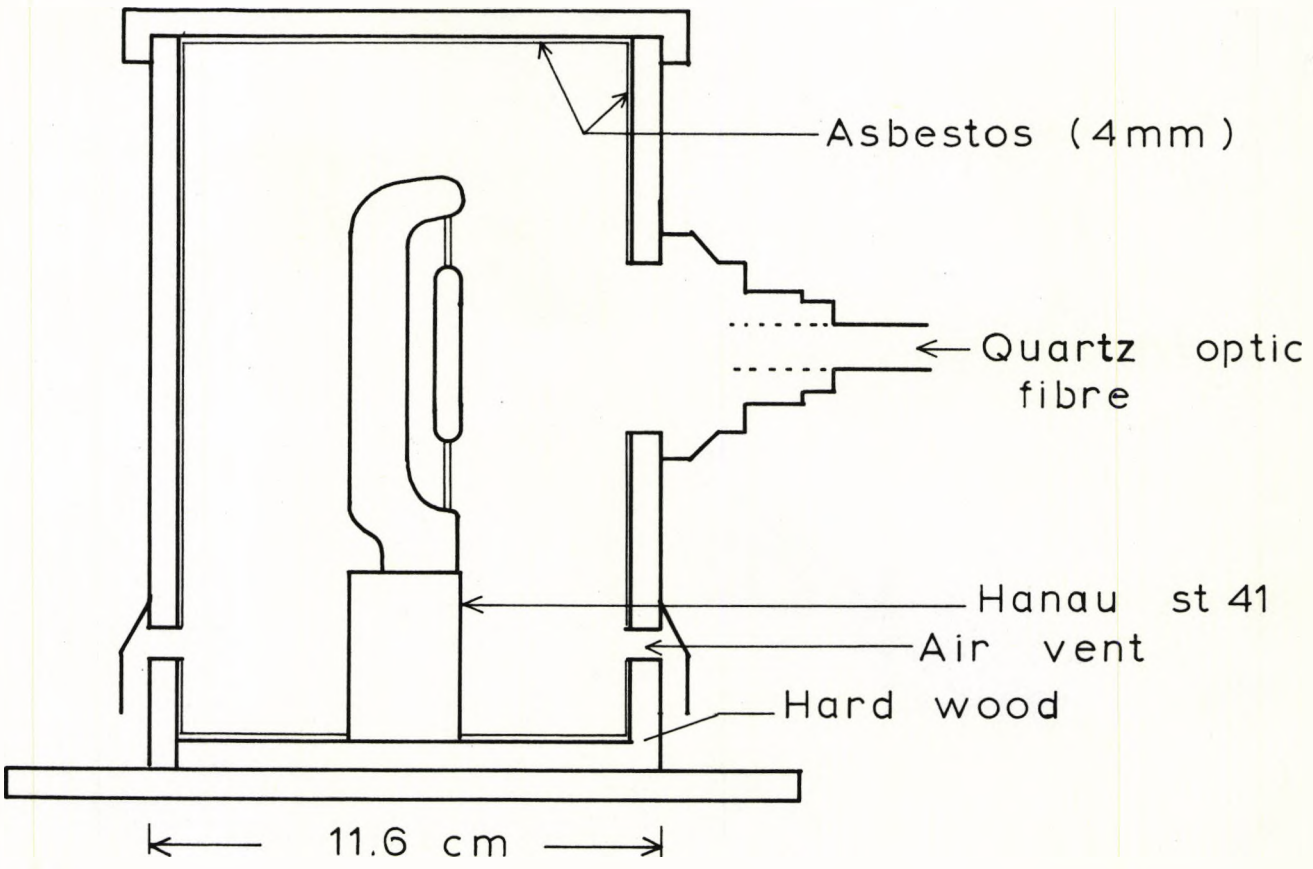
FLUORIMETER

The fermenter was irradiated at 360 nm by a suitable light source using quartz optic fibres (see Materials and Methods). The resultant fluorescence due to NAD(P)H was detected by a photomultiplier coupled to the fermenter by an optic light pipe. The photomultiplier signal was amplified and recorded. The light source (Hanau ST47 quartz lamp) was housed in a ventilated, asbestos-lined, hard wood box with a clamp to attach the quartz light pipe, Appendix III-Fig. 1. To ensure complete stability the lamp was supplied by a Hanau spectral lamp power supply.

The photomultiplier (EMI, EM 9524B) was housed as shown in Appendix III-Fig. 3 and protected against radiation by a μ metal shield. The base of the photomultiplier tube consisted of an array of three separate plates, Appendix III-Fig. 4. The housing back plate with HT and output connection via 2 PET 100 sockets is shown in Appendix III-Fig. 4(1). The HT was connected to KEPCO regulated dc power supply and adjustable from 0-1500 volts. The complete dynode potential dividing resistor chain was constructed on resin bonded insulating board, Appendix III-Fig. 4(2). The photomultiplier 14 pin socket and μ metal shield is shown in Appendix III-Fig. 4(3).

The current output of the photomultiplier was fed into the amplifier where a series of switched load resistors R_1 to R_5 were provided to enable a variable conversion of photomultiplier current to voltage (Appendix III-Fig. 2). This voltage was fed into a high impedance (10^{11} ohms) non-inverting FET amplifier (A)

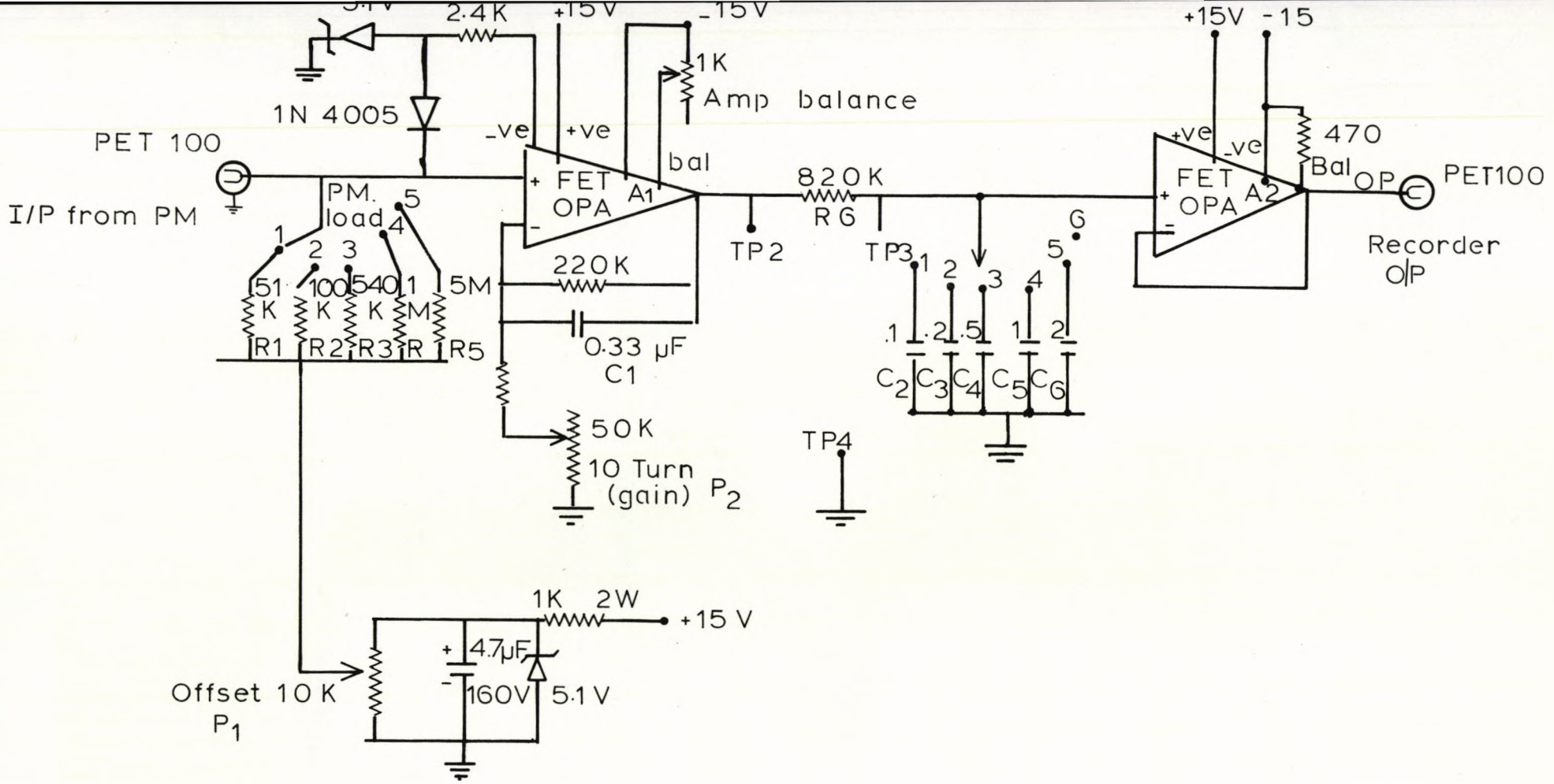
Appendix III, Fig. 1. Construction of the housing for the quartz lamp used to irradiate the fermenter culture at 360 nm.



Housing for light source

App. III. Fig. 1

Appendix III, Fig. 2. Fluorimeter amplifier.



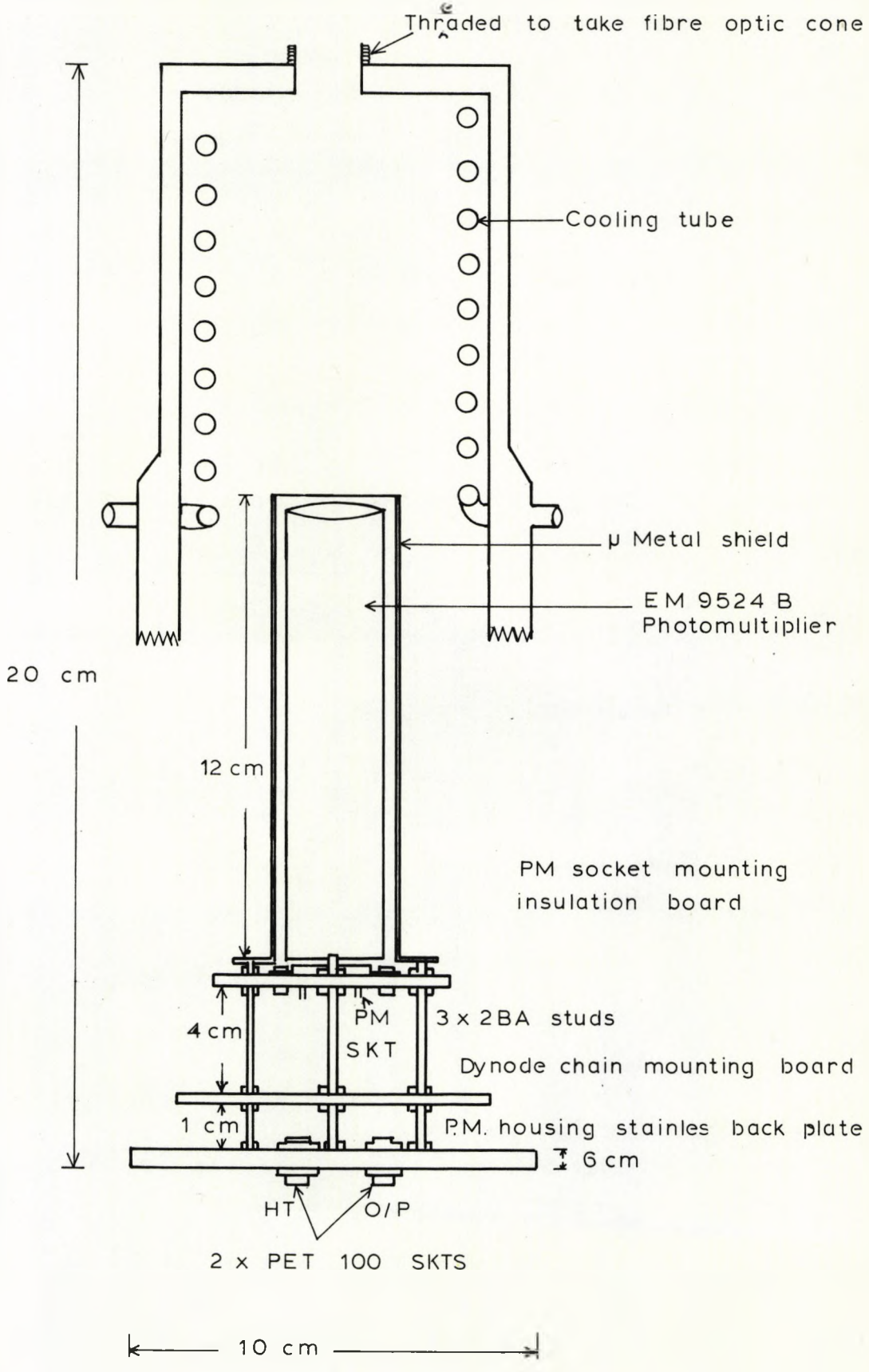
Flourimeter Amplifier

App. III - Fig. 2

with a variable gain potentiometer (P_2) and integral action via capacitor C_1. The output of the amplifier was passed through a simple first order fitter network consisting of R_6 and C_2 to C_6 giving time constants from 0.08 to 1.65 sec in five settings. This reduced the problem of noise due to bubbles in the fermenter.

The final stage was a 1:1 buffer amplifier (A_2) to enable connection of various recording facilities without loading the filter. The input impedance of the Servoscribe chart recorder used was sufficient to make A_2 redundant. To enable the examination of the peak of the photomultiplier signal an offset was provided by potentiometer P_1.

Appendix III, Fig. 3. Photomultiplier housing.



Photomultiplier

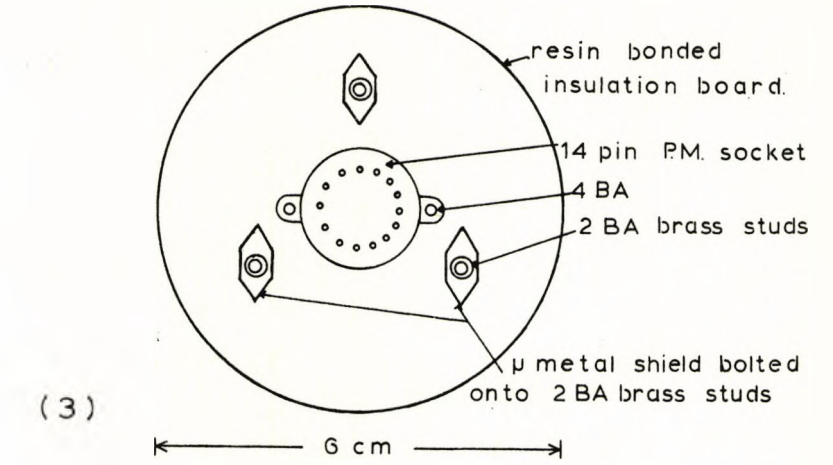
App III - Fig. 3

Appendix III, Fig. 4. Construction of photomultiplier.

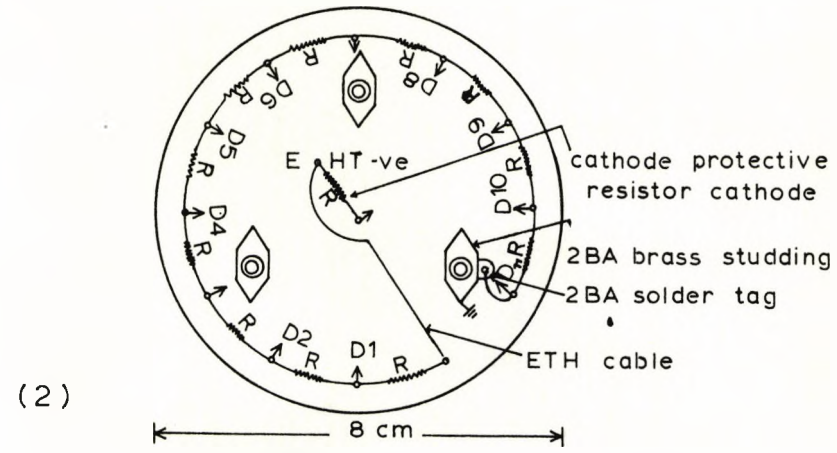
(1) Housing back plate with HT and output connections

(2) Dynode potential dividing resistor chain on insulating board

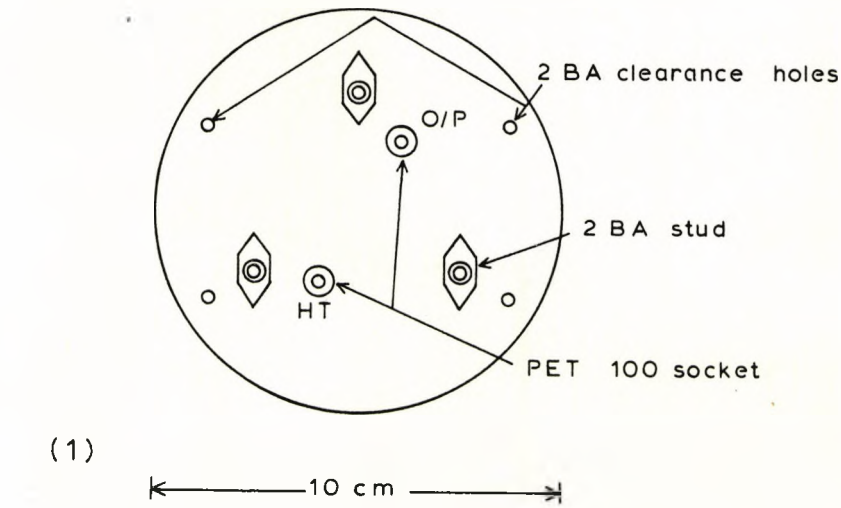
(3) Photomultiplier 14 pin socket and μ metal shield.



(3)

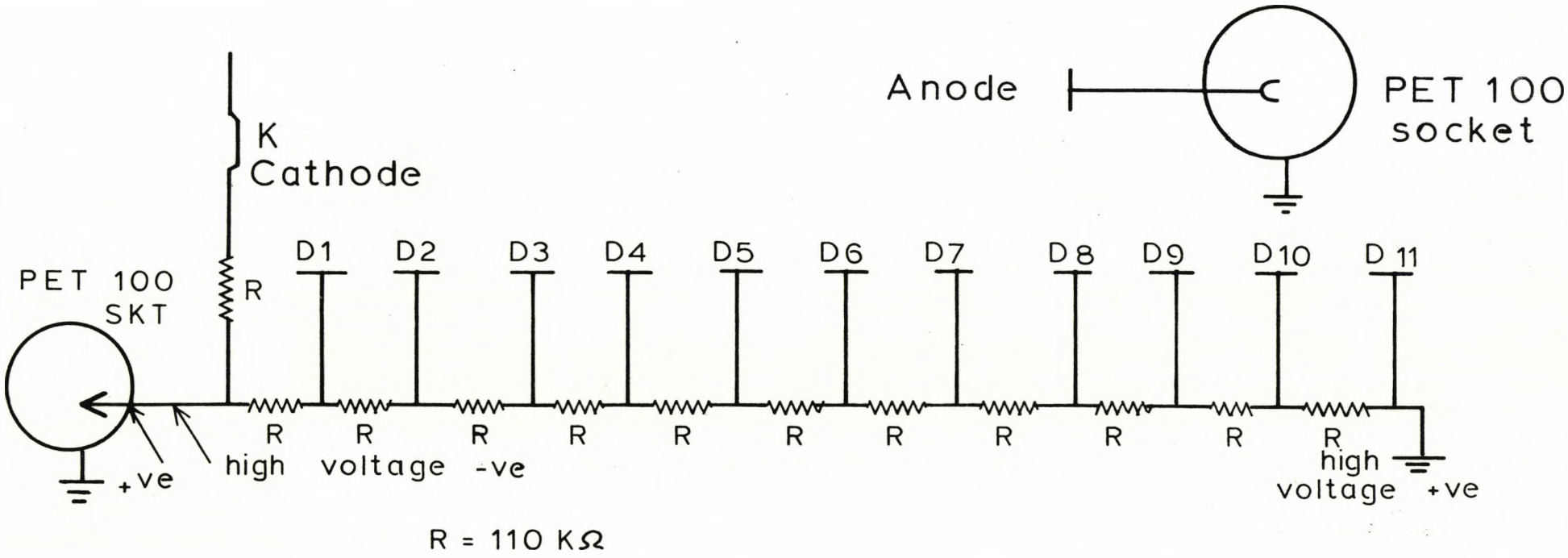


(2)



(1)

App III - Fig. 4



Dynode chain for photomultiplier socket

App III - Fig. 5

APPENDIX IV

THE EFFECT OF GLUCOSE CONCENTRATION ON RESPIRATION RATE, 'APPARENT' Km FOR OXYGEN AND CYTOCHROME ABSORBANCE OF BENECKEA NATRIEGENS AS MEASURED USING THE OPEN OXYGEN ELECTRODE SYSTEM

Increase in the glucose concentration in the open oxygen electrode cell had a marked effect on the respiration rate and absorbance at 558 nm minus 578 nm. At a glucose concentration of 0.4 gl^{-1} the respiration rate was 4.3 mMoles O_2 per g cell carbon per h. Increase in the glucose concentration to 0.8 gl^{-1} caused a 59.4% decrease in respiration rate. Further increase in the glucose concentration to 4.0 and 8.0 gl^{-1} caused a 62.8% and 69.8% decrease in respiration rate respectively (Appendix IV, Table 1). The immediate inhibition of respiration rate was evident by the rapid rise of the dissolved oxygen tension as the glucose concentration was increased (Appendix IV, Fig. 1).

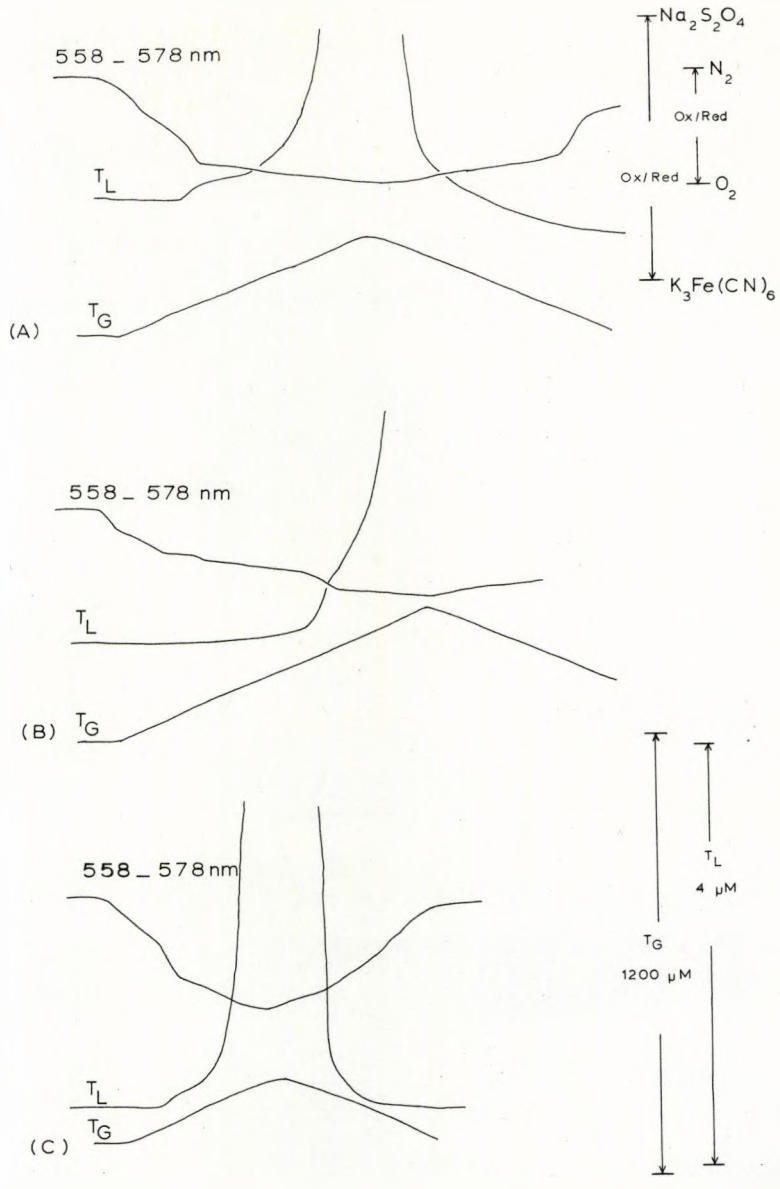
The cytochrome b absorbance (at 558 nm minus 578 nm) exhibited oxidation immediately the oxygen gradient was started although the dissolved oxygen tension was considerably lower than the Km for oxygen ($\ll$0.004 μM). As the concentration of oxygen in the liquid approached 4 μM the cytochromes reached a stable oxidation state. The number of 'steps' that occurred before this state was reached appeared to depend on the respiration rate. At maximum respiration rate four 'steps' were evident (Appendix IV, Fig. 1) as the respiration rate decreased (because of the increase in glucose concentration) the number of 'steps' before the cytochromes reached a stable oxidised state decreased (Appendix IV, Table 1). At maximum

Appendix IV, Table 1.

Glucose concentration (gl^{-1}) (mM)	ΔE of aerobic and anaerobic states as % of total cytochrome*	Absorbance 'steps' at 558-578 nm	Respiration rate (mM per g bacterial carbon per h)	Inhibition of respiration rate by glucose (%)	Apparent Km for oxygen (μM)
0.1 0.55	42	3	2.8	-	not measurable
0.4 2.2	40	4	4.3	0	0.18, 0.18, 0.15, 0.24
0.8 4.4	37	3	1.75	59.4	not measurable
4.0 22	36	2	1.60	62.8	not measurable
8.0 44	36	2	1.30	69.8	not measurable

* Calculated on the ratio. of ΔE at 558 nm minus 578 nm obtained from fully aerated (equilibrated with oxygen in the gas phase) minus anaerobic (equilibrated with nitrogen in the gas phase) to the total ΔE obtained by reduction with dithionate minus oxidation with ferricyanide.

Appendix IV, Fig. 1. The effect of glucose concentration on the respiration and kinetic changes in cytochrome b (558 nm minus 578 nm) of Beneckea natriegens in a respirograph.

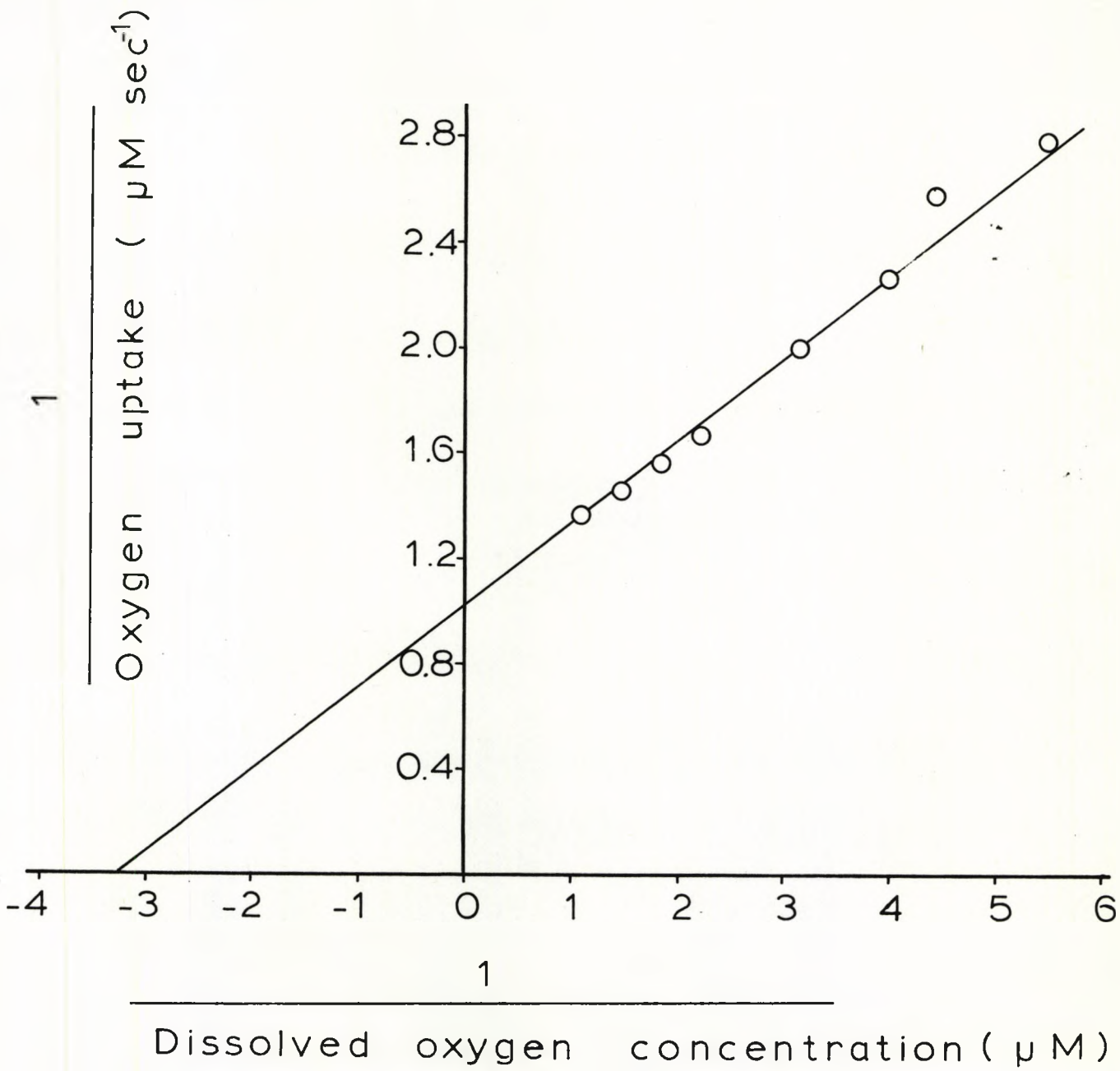

558 _ 578 nm
T_L
T_G
(A)
$Na_2S_2O_4$
N_2
Ox/Red
Ox/Red
O_2
$K_3Fe(CN)_6$
558 _ 578 nm
T_L
T_G
(B)
558 _ 578 nm
T_L
T_G
(C)
T_L
4 µM
T_G
1200 µM
558_578 nm
(D)
558_578 nm
(E)

App. IV Fig 1

respiration rate the cytochrome(s) remained reduced even in the presence of excess oxygen ($\ll$ 4 μM). However, when the respiration rate was inhibited (by increase in the glucose concentration) the cytochromes attained a stable oxidised state when the dissolved oxygen tension was approximately 4 μM. The significance of these 'steps' is not known but may reflect the operation of more than one cytochrome component within the 558-578 nm absorbance band.

The ratio of ΔE obtained from fully aerated (equilibrated with oxygen in the gas phase) minus anaerobic (equilibrated with N_2 in the gas phase) to the total ΔE, obtained by reducing the contents of the cuvette with dithionate minus oxidation with potassium ferricyanide, is shown in Appendix IV, Table 1. The results indicate that the cytochromes were only 36-42% reduced, a value similar to that observed with NADH as substrate (see Section VIII) suggesting that electrons from glucose feed into the electron transport system at the level of NADH. The 'apparent' Km for oxygen of B. natriegens respiring glucose (0.4 gl^{-1}) varied between 0.18 and 0.24 μM. Increase in the glucose concentration caused a change in the 'apparent' Km for oxygen. The altered Km could not be measured because the dissolved oxygen tension increased too rapidly (Appendix IV, Figs. 1 and 2). These results cannot be explained in terms of a change in the K value with increase in the glucose concentration as the experimental value for K was 0.005 sec^{-1} at 0 and 8.0 gl^{-1} glucose. These results suggest that in addition to causing an increase in the lag period and a decrease in the growth rate of a batch culture of B. natriegens (Appendix I), high glucose concentration ($>$0.8 gl^{-1}) has an immediate inhibitory effect on respiration rate. This is a rapid control mechanism of respiration rate when compared with the Crabtree effect (De Deken, 1966).

Appendix IV, Fig. 2. The apparent Km for oxygen of Beneckea natriegens (glucose grown) respiring glucose in a respirograph.


1 / Oxygen uptake (µM sec-1)
2.8
2.4
2.0
1.6
1.2
0.8
0.4
-4
-3
-2
-1
0
1
2
3
4
5
6
1 / Dissolved oxygen concentration (µM)

App. IV. Fig. 2

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Molar Growth Yields, Respiration and Cytochrome Patterns of *Beneckea natriegens* when Grown at Different Medium Dissolved-oxygen Tensions

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SUMMARY

The effect of medium dissolved-oxygen tension on the molar growth yield, respiration and cytochrome content of *Beneckea natriegens* in chemostat culture (D 0·37 h^{-1}) was examined. The molar growth yield (Y), the specific rate of oxygen (q_{O_2}) and glucose consumption, and the specific rate of carbon dioxide evolution were independent of the dissolved-oxygen tension above a critical value (< 2 mmHg). However, the potential respiration rate increased with reduction in the dissolved-oxygen tension at values of the dissolved-oxygen tension well above the critical value. Changes in the cytochrome content occurred at dissolved-oxygen tensions well above the critical value. An increase in cytochrome *c* relative to cytochrome *b* was observed as the dissolved-oxygen tension was decreased. Reduction of the dissolved-oxygen tension to less than 1 mmHg caused a switch to fermentative metabolism shown by the apparent rise in Y_{O_2} and decrease in the molar growth yield from glucose. At this point the potential respiration rate (q_{O_2}) increased to its highest value, while the cytochrome pattern reverted to that observed at dissolved-oxygen tensions above 96 mmHg. There appeared to be no correlation between cytochrome content, potential q_{O_2}, *in situ* q_{O_2}, and cyanide sensitivity of the organism at various dissolved-oxygen tensions.

INTRODUCTION

Beneckea natriegens, a facultative anaerobic, Gram-negative marine bacterium (Baumann, Baumann & Mandel, 1971) provides an interesting system for the study of respiratory control in bacteria, as it apparently has four different, recognizable, cytochrome oxidases. Cytochromes *o*, a_1 and *d*, and a CO-binding *c*-type cytochrome, have been identified on the basis of CO difference spectra and action spectra (Weston & Knowles, 1974). From inhibitor studies on cell-free extracts, a branched electron transport chain has been proposed for *B. natriegens* (Weston, Collins & Knowles, 1974). Action spectra of whole organisms indicated that cytochrome *d* functioned as a terminal oxidase although present only at very low concentration (Weston & Knowles, 1974). The CO-binding *c*-type cytochrome was also found to have oxidase, activity but the evidence for cytochrome *o* acting as an oxidase was inconclusive. In organisms cultured for periods longer than 16 h cytochrome a_1 was the major functional oxidase and cytochromes *d* and *o* and CO-binding *c*-type had minor oxidase functions (Weston & Knowles, 1974). Meyer & Jones (1973*a*, *b*) suggested that multiple oxidases found in bacteria function at different medium dissolved-oxygen tensions

and are associated with different efficiencies of phosphorylation. To assess whether branching of the electron transport system occurred under physiological conditons, *B. natriegens* was grown at various medium dissolved-oxygen tensions in a glucose-limited chemostat culture and the effects on growth, respiration and cytochrome content were monitored. A preliminary report has already been published (Linton, Harrison & Bull, 1974).

METHODS

Organism and medium. Beneckea natriegens strain Baumann 111, the gift of C. J. Knowles, University of Kent, was originally isolated and characterized by Payne, Eagon & Williams (1961).

The basal medium contained, in a final volume of 925 ml: Na_2HPO_4, 1·5 g; KH_2PO_4, 1·5 g; $(NH_4)_2SO_4$, 3·0 g; NaCl, 23 g; KCl, 0·745 g; trace element mixture, 1 ml. The trace element mixture was from a stock solution containing (g/l): $CaCl_2.2H_2O$, 0·66; $ZnSO_4.7H_2O$, 0·18; $CuSO_4.5H_2O$, 0·16; $MnSO_4.4H_2O$, 0·15; $CoCl_2.6H_2O$, 0·18; H_3BO_3, 0·10; $Na_2MoO_4.2H_2O$, 0·30; the pH was adjusted to 7·3 with NaOH. Solutions of glucose (2·0 g) with $MgSO_4.7H_2O$ (0·2 g) in a final volume of 50 ml and of 1 ml $FeCl_3$ (from a stock solution containing 16·7 g $FeCl_3.6H_2O$/l) in a final volume of 25 ml, were also prepared. The three components of the complete medium were autoclaved separately and mixed aseptically after cooling.

The glucose concentration of 2·0 g/l in the feed medium has been previously shown to be growth limiting.

Fermenter cultures. An impeller-stirred, Biotec fermentation vessel (LKB) of working capacity 2·3 l, was used. The culture volume was kept constant by means of a stainless-steel overflow tube (6 mm diam), the bottom of which was connected to an effluent vessel by silicone rubber tubing. Effluent culture was pumped out of the vessel using a peristaltic pump (Northern Media Supply Ltd, Brough HU15 2PE). The temperature was maintained at $31{\cdot}50 \pm 0{\cdot}25$ °C by controlling the circulation of hot and cold water through two separate fingers immersed in the culture. Culture pH was controlled at $7{\cdot}23 \pm 0{\cdot}02$ by adding 5 M-NaOH using a Titrator II (Radiometek A/S-Emdrupues, Copenhagen, Denmark). The medium, stirred at 600 rev./min, was aerated by passing sterile air through it at 1·0 l/min. Medium was pumped into the fermenter vessel with a diaphragm pump (F. A. Huges & Co. Ltd, Epsom, Surrey), from a reservoir containing 30 l which was stirred continuously with a magnetic stirrer to keep in suspension a slight precipitate which formed in the complete medium. The dilution rate was maintained at 0·37 h^{-1}, which represented approximately 50 % of the maximum growth rate. The specific rate of oxygen consumption (q_{O_2}) and of carbon dioxide production (q_{CO_2}) were shown to increase linearly with growth rate (Linton, unpublished) and metabolism to be unaffected between growth rates of 0·1 and 0·8 h^{-1}. Therefore, change in the growth rate of this organism would be expected to have no effect on the value of the critical dissolved-oxygen tension or the pattern of response of the culture to dissolved-oxygen tension.

The dissolved-oxygen tension was varied by changing the ratio of air to nitrogen in the inlet gas while keeping the total flow rate constant. The dissolved-oxygen tension was measured with a galvanic-cell oxygen probe (L.H. Engineering Co. Ltd, Stoke Poges, Buckinghamshire), connected in series with a 5 kΩ variable potentiometer. The probe was calibrated in growth medium in equilibrium with mixtures of air and nitrogen assayed by means of a Servomex oxygen analyser. The zero reading was obtained by bubbling nitrogen through the medium for 24 h. Oxygen mixtures below 1 % oxygen were obtained by mixing

nitrogen with standard O_2–N_2 gas mixtures containing 10 and 1 % oxygen. Oxygen in the gas, entering and leaving the vessel was continuously monitored by a twin-channel, paramagnetic oxygen analyser (Type OA 184, Servomex Controls Ltd, Crowborough, Sussex). Carbon dioxide leaving the vessel was continuously monitored by an infrared analyser (M.S.A. Instrument Division, Glasgow). Gas flow rates through the fermenter were measured on a wet-type gas meter (Alexander Wright and Co., London).

Analytical methods. Duplicate samples of culture (10 ml) were centrifuged at 17000 ***g*** for 35 min. The sediments were washed twice in a solution of 0·4 M-NaCl and finally taken up in distilled water and dried to constant weight at 98 °C. For each steady-state value duplicate 10 ml samples were taken at intervals of at least five times the mean residence time.

Total culture carbon and supernatant carbon were determined with a Beckman Model 915 total organic carbon analyser. Carbon analyses of the whole culture and of a Millipore (0·25 μm pore size) filtrate were made and the carbon content of the bacteria obtained from the difference.

Glucose concentrations in the input medium and effluent culture were measured with the glucose oxidase system (*Sigma Chemical Bulletin No.* 510, 1969). Acetic acid and ethanol in Millipore filtrates of the culture were assayed by gas–liquid chromatography, using a Varian 1800 series chromatograph and a Poropak Q column at 190 °C. Pyruvic acid and lactic acid were assayed enzymically (*Sigma Chemical Bulletin No.* 726-*UV* and *No.* 826 *UV*, respectively, 1968).

Culture extinction, measured at 625 nm, was used as a guide to the constancy of the biomass content in steady-state cultures. The extinction was not used to compute carbon content or dry weight of the culture.

The amount of ammonium sulphate nitrogen used was measured from the total ammonia in the input and output media. The ammonia and orthophosphate concentrations in samples were measured using a Technicon Autoanalyser II (*Industrial Methods Nos.* 160–73*E* and 155–71*W*, respectively).

Cytochrome spectra were recorded at room temperature and at low temperatures (77 °K), in a split beam spectrophotometer (Hitachi Perkin Elmer Model 356). The culture (800 ml) was rapidly drained from the fermenter at the end of a given steady state and centrifuged at 17000 ***g*** for 35 min at 4 °C. The pellet was resuspended in 10 mM-$MgCl_2$ plus 50 mM-tris-HCl buffer pH 7·5, then centrifuged at 17000 ***g*** and finally resuspended in 26 ml tris-HCl buffer. Oxidized minus reduced difference spectra were obtained by adding a few crystals of $Na_2S_2O_4$ to one cuvette to obtain bacterial cytochromes in the reduced state, and oxidizing the bacterial cytochromes in the reference cuvette with a few crystals of $K_3Fe(CN)_6$ or one drop of H_2O_2. Carbon monoxide difference spectra were obtained by treating the contents of both cuvettes with $Na_2S_2O_4$ and bubbling a steady stream of CO through one cuvette for 1 min. The CO-treated cuvette was left in the dark for 10 min before the spectra were recorded at room temperature and at low temperature (77 °K).

The potential respiration rate of the bacteria was measured by diluting culture samples in complete basal medium minus the carbon and nitrogen sources (pH 7·2), adding known quantities of glucose and following the oxygen uptake in an oxygen-electrode respirometer (Rank Bros, Bottisham, Cambridgeshire). The potential respiration rate (q_{O_2}), in mmol/g/h, was calculated from the equation (Harrison & Loveless, 1971):

$$q_{O_2} = r/xd,$$

where r is the rate of depletion of oxygen concentration as measured in the oxygen electrode cell (mmol/g/h), d the dilution factor, and x the concentration of the organism (g/l).

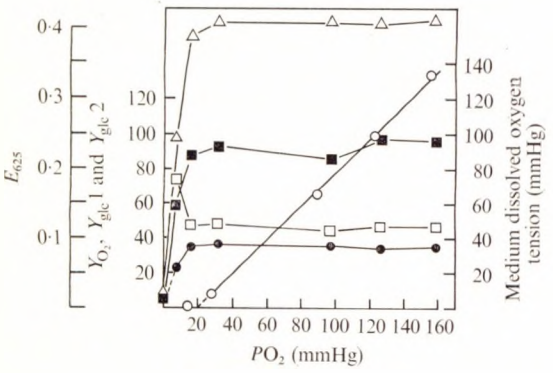

Fig. 1

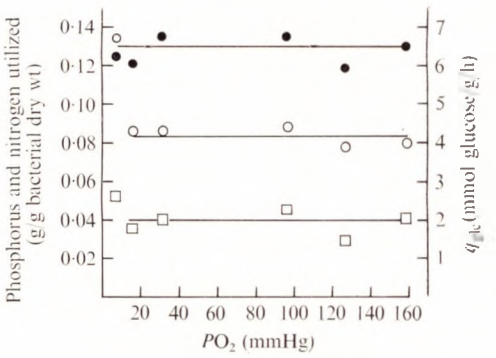

Fig. 2

Fig. 1. Steady-state values of molar growth yields from glucose and oxygen, culture extinction and medium dissolved-oxygen tension as a function of the PO_2 of a carbon-limited chemostat culture of *B. natriegens*. ○, Medium dissolved-oxygen tension (mmHg); ■, Y_{glc} 1 (g bacterial dry wt/mol glucose); ●, Y_{glc} 2 (g bacterial carbon/mol glucose carbon); □, Y_{O_2} (g cell dry wt/mol oxygen); △, culture extinction at 625 nm.

Fig. 2. Steady-state values of glucose, nitrogen and phosphorus consumption as a function of the PO_2 of a carbon-limited chemostat culture of *B. natriegens*. ○, q_{glc}; ●, nitrogen utilized; □, phosphorus utilized.

RESULTS

Growth physiology

The steady-state values of molar growth yield from glucose [g bacterial dry wt/mol (Y_{glc} 1), and g bacterial carbon/mol glucose carbon (Y_{glc} 2)] appeared to be independent of medium dissolved-oxygen tension over the range 134 to < 2 mmHg (partial pressure of oxygen in the gas phase PO_2, 159 to 16 mmHg) (Fig. 1). However, reduction of the PO_2 to 8 mmHg caused a marked fall in the molar growth yield from glucose, from 92 ± 4 to 60 g bacterial dry wt/mol. At PO_2 values below 8 mmHg the output from the dissolved-oxygen probe was virtually zero, the dissolved-oxygen tension being below the lower limit of sensitivity of the probe (< 1 mmHg). Further reduction of the PO_2 to < 0·5 mmHg (achieved by gassing with 'white spot' nitrogen) caused a dramatic fall in the molar growth yield. Based on culture extinction and bacterial carbon values, the yield was approximately 5 % of that observed under the excess oxygen phase (Fig. 1).

Reduction of the PO_2 to 8 mmHg caused no detectable effect on medium dissolved oxygen, as this was already below the lower limit of sensitivity of the probe, but the q_{O_2} of the culture decreased. The medium dissolved-oxygen tension was found to be dependent on PO_2. The relationship is shown in Fig. 1 and is given by the equation:

$$N = K_L A\,(T_g - T_L),$$

where N is the oxygen uptake rate of the culture (g/h), $K_L A$ the oxygen transfer coefficient (g/h/mmHg), T_g the oxygen partial pressure in the gas phase (mmHg), and T_L is the medium dissolved-oxygen tension (mmHg).

The medium dissolved-oxygen tension varied linearly with partial pressure of oxygen in the effluent gas phase (Harrison, 1973).

The specific rate of glucose consumption (q_{glc}; mmol glucose/g bacterial dry wt/h) was calculated from the equation:

$$q_{glc} = (S_R - S)\,D/\bar{x},$$

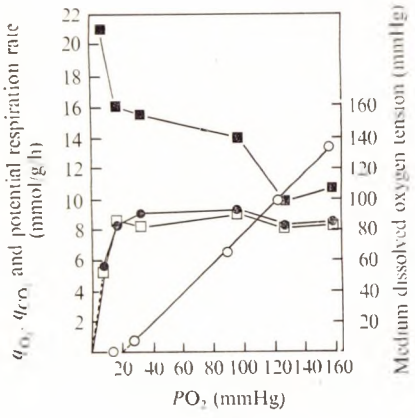

Fig. 3

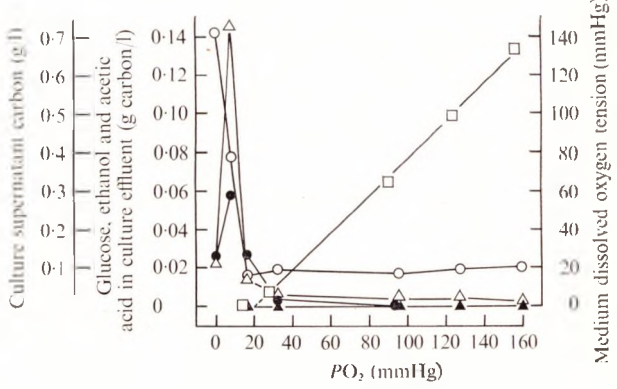

Fig. 4

Fig. 3. Steady-state values of the specific rates of oxygen consumption and CO_2 production, potential respiration rate and medium dissolved-oxygen tension, as functions of the PO_2 of a carbon-limited chemostat culture of *B. natriegens*. ○, Medium dissolved-oxygen tension; ●, q_{CO_2}; □, q_{O_2}; ■, potential respiration rate.

Fig. 4. Steady-state values of glucose, ethanol, acetic acid, and culture supernatant carbon, and medium dissolved-oxygen tension, as functions of the PO_2 of a carbon-limited chemostat culture of *B. natriegens*. ▲, Glucose carbon; ●, ethanol carbon; △, acetic acid carbon; ○, culture supernatant carbon; □, medium dissolved-oxygen tension.

where S_R is the glucose concentration in the medium feed (mmol/l), S the steady-state glucose concentration in the medium effluent (mmol/l), D the dilution rate (h^{-1}), and $\bar{x}$ is the steady-state bacterial dry wt (g/l).

The q_{glc} was fairly constant over the range of medium dissolved-oxygen tension of 134 to < 2 mmHg (PO_2 156 to 16 mmHg) but there appeared to be about a 50% increase in the q_{glc} when the PO_2 was reduced to 8 mmHg (medium dissolved-oxygen tension ≪ 2 mmHg) (Fig. 2).

Nitrogen and phosphorus utilization by the culture was monitored and found to be independent of the dissolved-oxygen tension between 134 and < 2 mmHg (Fig. 2). Under anaerobic conditions the bacterial mass and the amount of nitrogen and phosphorus utilized could not be measured accurately.

Both q_{O_2} and q_{CO_2} were independent of medium dissolved-oxygen tension over the range 8 to 134 mmHg (Fig. 3). When the PO_2 was reduced to 16 mmHg, the medium dissolved-oxygen tension reading fell to zero (i.e. < 2 mmHg), but there was no effect on q_{O_2} or q_{CO_2}.

The molar growth yield from oxygen (Y_{O_2}), in g bacterial dry wt/mol O_2, was calculated from the equation (Harrison & Loveless, 1971):

$$Y_{O_2} = \mu/q_{O_2},$$

where μ is the specific growth rate (h^{-1}). The values of Y_{O_2} obtained, as a function of the PO_2, are shown in Fig. 1. The Y_{O_2} was independent of the PO_2 over the range 159 to 16 mmHg. However, reduction of the PO_2 to 8 mmHg caused a 37% increase in the Y_{O_2} value. The potential q_{O_2} was found to be higher than the *in situ* q_{O_2} at all oxygen tensions examined (Fig. 3) and increased as the partial pressure of oxygen was reduced. At dissolved-oxygen tensions between 134 and 96 mmHg, the potential q_{O_2} was approximately 10 mmol O_2/g bacterial dry wt/h. When the dissolved-oxygen tension was reduced to 65 mmHg,

Table 1. *A carbon balance for the growth of B. natriegens in chemostat culture*

The values given represent averages obtained on analysis of duplicate samples taken from the chemostat at steady state at various medium dissolved-oxygen tensions.

			Carbon output (g/h)			
PO_2 (mmHg)	D.o.t. (mmHg)	Carbon input (g/h)	Cell carbon	Product carbon	Carbon dioxide carbon	Carbon recovery (%)
159·6	134	0·699	0·364	0·089	0·250	100·5
		0·699	0·323	0·10	0·243	94·0
127	96	0·690	0·341	0·082	0·237	95·6
		0·690	0·323	0·091	0·237	94·3
95	65	0·706	0·36	0·077	0·245	96·5
		0·692	0·346	0·077	0·245	96·5
32	8	0·706	0·353	0·084	0·259	98·5
		0·706	0·381	0·093	0·251	102·0
16	< 2·0	0·706	0·344	0·074	0·224	91·0
		0·706	0·353	0·074	0·224	92·2
8	≪ 1	0·713	0·223	0·362	0·118	98·5
		0·718	0·251	0·353	0·118	100·5
0	0	0·798	0·06	0·738	—	100
		0·780	0·051	0·729	—	100

D.o.t., dissolved-oxygen tension.

the potential respiration rate increased to 14 mmol/g/h with no marked increase in the *in situ* q_{O_2}. Reduction of the dissolved-oxygen tension to $<<$ 1 mmHg (PO_2 16 mmHg) caused a small increase in the potential q_{O_2}, but further reduction of the PO_2 to 8 mmHg caused a dramatic increase in the potential respiration rate to 21 mmol/g/h, while the *in situ* q_{O_2} fell to 5·3 mmol/g/h.

The formation of extracellular metabolic products from glucose, as a function of the PO_2, is shown in Fig. 4. Acetic acid and ethanol were the two main products identified by g.l.c. and their concentrations remained low and fairly constant until PO_2 was reduced to 16 mmHg (medium dissolved-oxygen tension < 2 mmHg), when there was a slight increase in the concentration of these compounds. Both these products reached a maximum concentration when the PO_2 was 8 mmHg. Pyruvic acid was found at extremely low concentrations (a maximum of 3 mg/l) when 'white-spot' nitrogen was passed through the fermenter. The carbon balance indicated a satisfactory carbon recovery (Table 1). A switch in metabolism was shown by an increase in the culture-filtrate carbon when the PO_2 was reduced to 8 mmHg (Fig. 4). Only 10% of the carbon present in culture filtrates was identified.

Cytochrome analysis

The results of oxidized minus reduced difference spectra indicated a qualitative change in the cytochrome contents of *B. natriegens* over a range of medium dissolved-oxygen tensions that caused relatively little change in the *in situ* q_{O_2}, q_{CO_2}, Y_{glc} or Y_{O_2}. At dissolved-oxygen tensions between 134 and 96 mmHg, peak extinctions at 560, 553 and 426·5 nm were observed, indicating the presence of *b*- and *c*-type cytochromes (Fig. 5). The position of the Soret peak at 426 nm indicated a mixture of cytochromes *b* and *c*. The ratio between cytochromes *b* and *c* changed as the dissolved-oxygen tension was lowered to 65 mmHg. This is

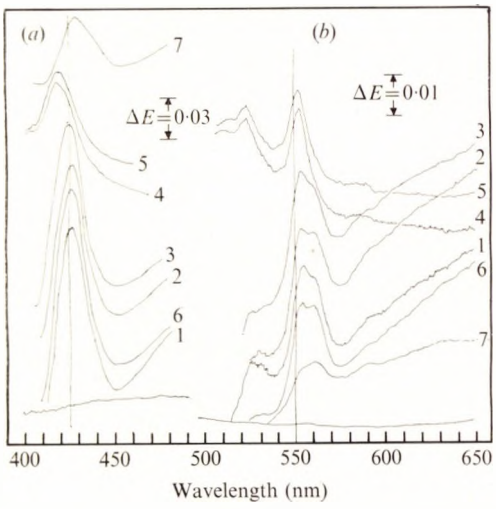

Fig. 5

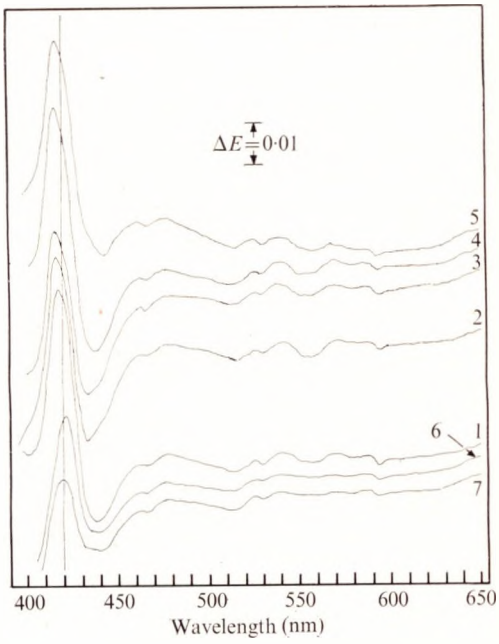

Fig. 6

Fig. 5. Room-temperature oxidized minus reduced difference spectra of whole bacteria taken from a chemostat under steady-state conditions at various medium dissolved-oxygen tensions. (*a*) Oxidized H_2O_2 minus reduced $Na_2S_2O_4$; (*b*) oxidized $K_4Fe(CN)_6$ minus reduced $Na_2S_2O_4$. (1) Medium dissolved-oxygen tension (d.o.t.) 134 mmHg, bacterial dry weight 10·26 mg/ml; (2) d.o.t. 96 mmHg, bacterial dry weight 10·30 mg/ml; (3) d.o.t. 65 mmHg, bacterial dry weight 10·26 mg/ml; (4) d.o.t. 8 mmHg, bacterial dry weight 9·84 mg/ml; (5) d.o.t. < 1 mmHg, bacterial dry weight 9·40 mg/ml; (6) d.o.t. ≪ 1 mmHg, bacterial dry weight 6·33 mg/ml; (7) d.o.t. ≪ 0·5 mmHg, bacterial dry weight 2·40 mg/ml.

Fig. 6. Room-temperature reduced-plus-CO minus reduced difference spectra of whole cells taken from a chemostat under steady-state conditions at various medium dissolved-oxygen tensions. (1) d.o.t. 134 mmHg; (2) d.o.t. 96 mmHg; (3) d.o.t. 65 mmHg; (4) d.o.t. 8 mmHg; (5) d.o.t. < 1 mmHg; (6) d.o.t. ≪ 1 mmHg; (7) d.o.t. ≪ 0·5 mmHg. Bacterial dry weight as in Fig. 5.

shown by the position of the Soret peak which appeared at 424 nm, indicating a relative increase in the *c*-type cytochrome content. Further reduction of the dissolved-oxygen tension to 8 and < 2 mmHg (PO_2 values 31 and 16 mmHg) caused a further shift towards the *c*-type cytochrome. This is clearly seen in the oxidized minus reduced spectra (Fig. 5) as a shift in the Soret peak to 418 nm. Further reduction of PO_2 to 8 mmHg, although causing no detectable change in the output of the dissolved oxygen probe, caused a change in which the cytochrome content appeared to revert to the pattern found in the excess-oxygen phase (peak extinctions at 560, 553 and 427). Oxidized minus reduced difference spectra of bacteria grown in an atmosphere of nitrogen revealed small peak extinctions at 560, 553 and 428 nm.

Reduced-plus-CO minus reduced difference spectra (Fig. 6) supported the qualitative changes in the cytochrome content observed in the oxidized minus reduced difference spectra. Peak extinctions in the Soret region were at 418 and 417 nm, at dissolved-oxygen tensions of 134 and 96 mmHg respectively, indicating the presence of mainly cytochrome *o*. Reduction of the dissolved-oxygen tension to 65, 8 and < 2 mmHg caused a shift in the

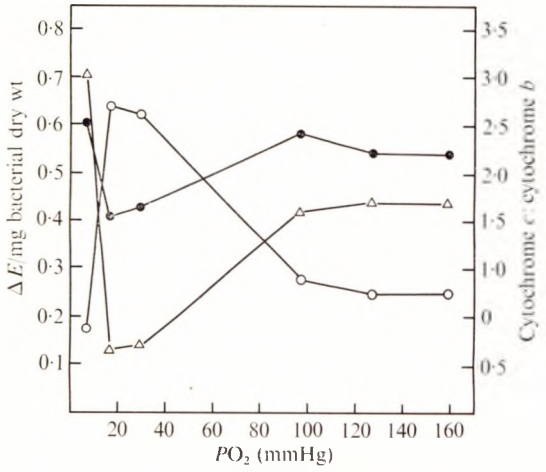

Fig. 7

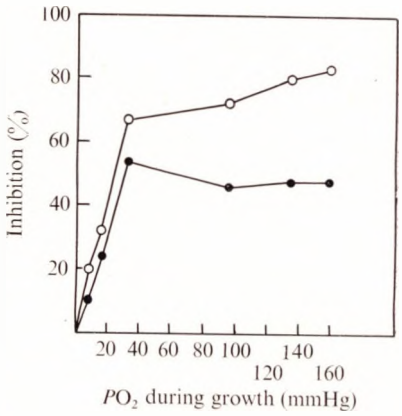

Fig. 8

Fig. 7. Steady-state values of cytochromes *c* and *b* and the ratio of cytochrome *c* to cytochrome *b* in bacteria grown at various gaseous oxygen partial pressures. ●, Cytochrome *c* (as ΔE/mg dry wt); △, cytochrome *b* (as ΔE/mg dry wt); ○, ratio cytochrome *c*:cytochrome *b*.

Fig. 8. The effect of KCN (as inhibition compared with a control without KCN) on the potential q_{O_2} of bacteria taken from a chemostat under steady-state conditions at various gaseous-oxygen partial pressures. KCN was at (●) 5 μmol or (○) 75 μmol.

Soret peak to 416 nm, indicating a relative increase in the CO-binding cytochrome *c*. Further reduction in the PO_2 to 8 mmHg, although causing no detectable change from zero on the dissolved-oxygen probe output, appeared to cause a change in the CO-liganded peak extinction from 416 to 422 nm. Reduced-plus-CO minus reduced difference spectra of anaerobically-grown bacteria revealed a peak at 420 nm.

Oxidized minus reduced difference spectra of whole bacteria were examined at low temperature (77 °K) and the results supported those obtained at room temperature. Cytochrome *b* was detected at dissolved-oxygen tensions of 8 and < 2 mmHg as a shoulder at 555 nm on a peak at 551 nm; this cytochrome *b* was not evident in room-temperature spectra.

To obtain an estimate of the relative change in the levels of particular cytochromes, the peak heights of room-temperature oxidized minus reduced difference spectra were measured and expressed in terms of ΔE/mg bacterial dry wt (Fig. 7). The changes in the levels of cytochromes *b* and *c* showed some relationship to the three phases observed in the potential respiration rate (see Fig. 3). The level of cytochrome *c* appeared to remain constant over the medium dissolved-oxygen tension range 134 to 65 mmHg, whereas a reduction in the dissolved-oxygen tension from 65 to < 2 mmHg appeared to cause a slight decline in the level of cytochrome *c*. At the PO_2 that caused a switch in metabolism, the 'critical point', the level of cytochrome *c* increased, indicating an apparent reversal to the original level. Similarly the level of cytochrome *b* appeared to remain fairly constant over the range of dissolved-oxygen tension 134 to 65 mmHg, and declined sharply when the range was reduced to 8 and < 2 mHg. Finally, below the 'critical point' the level of cytochrome *b* increased dramatically to a value higher than that observed under full aeration. The ratio cytochrome *c*:cytochrome *b* (Fig. 7) remained constant over the medium dissolved-oxygen range 134 to 65 mmHg. Reduction in the dissolved-oxygen tension to 8 and < 2 mmHg appeared to cause an increase in the relative amount of cytochrome *c* until the 'critical

point' was reached, when it reverted to a ratio near that found in bacteria grown under fully aerobic conditions.

Cyanide sensitivity

The effect of 50 and 75 μmol KCN on the potential respiration rates of bacteria taken from the fermenter at various dissolved-oxygen tensions is shown in Fig. 8. The cyanide sensitivity of the bacteria was almost independent of the dissolved-oxygen tension over the range 134 to 8 mmHg, but below a dissolved-oxygen tension of 8 mmHg the bacteria exhibited a marked resistance towards cyanide.

DISCUSSION

The metabolic rate and efficiency of *B. natriegens*, as indicated by the q_{O_2}, q_{CO_2} and q_{glc} when growing in continuous culture, was little affected by changes in dissolved-oxygen tension over a range from below the sensitivity of the oxygen probe used (< 2 mmHg) to 134 mmHg. In contrast, the potential q_{O_2} of the culture increased by 50% as the dissolved-oxygen tension was decreased from 100 to 65 mmHg. There was a barely significant rise in the *in situ* q_{O_2} over this range of dissolved oxygen, although an increase in the cytochrome *c* content relative to cytochrome *b* was evident. The insensitivity of metabolism to changes in the dissolved-oxygen content above a certain 'critical' level is the usual response of aerobic and facultative bacteria (Harrison, 1973). However, the changes in cytochrome content and potential respiration rate at dissolved-oxygen tensions within this range, when the metabolism of the bacteria growing *in situ* in the fermenter was unaffected, was an entirely unexpected result. The mechanisms for induction of the cytochrome and potential respiration rate appear to be unrelated to the actual respiration rate of the organism. Also, the actual respiration rate under carbon-limited growth is not a function of the respiratory potential of the culture or of the cytochrome content.

Even after the dissolved-oxygen tension had fallen to a level below that which could be detected by the probe, the oxygen supply could be reduced further. Reducing the oxygen supply under these conditions had a profound effect on the metabolism of the culture, as has been reported for *Klebsiella aerogenes* (Harrison & Pirt, 1967). As the PO_2 was reduced, with dissolved-oxygen tension less than 1 mmHg, there was a switch to fermentative metabolism. This was shown in an apparent rise in the Y_{O_2}, a marked increase in q_{glc} and a decreased yield coefficient from glucose. The potential q_{O_2} under these conditions increased dramatically to its highest value, while the cytochrome pattern reverted to that obtained at dissolved-oxygen tensions above 96 mmHg. Thus, not only was there no quantitative correlation between cytochrome content, potential q_{O_2} and *in situ* q_{O_2}, but also, apparently, no qualitative correlation: the values changed independently of each other.

The phenomenon of increased respiration rate in the fermenter (*in situ* rate) in response to low dissolved-oxygen tension, as found with *Escherichia coli* and *K. aerogenes* (Harrison & Pirt, 1967; Harrison & Loveless, 1971), and which is accompanied by a switch to a less efficient pathway of glucose oxidation, was not found with *B. natriegens*.

The different branches of the electron-transport chain reported for cell-free preparations of *B. natriegens* (Weston & Knowles, 1974) and Azotobacter (Ackrell & Jones, 1971*a*) are distinguished by a difference in cyanide sensitivity of the respective terminal oxidase of at least an order of magnitude. Therefore, if there is a change in the relative importance of these branches in response to changing dissolved-oxygen tension, as has been proposed for Azotobacter (Ackrell & Jones, 1971*b*), this should be reflected in the cyanide sensitivity of

the bacteria. No such change in cyanide sensitivity over the dissolved-oxygen range 8 to 140 mmHg was observed here. As the dissolved-oxygen tension fell below the sensitivity of the probe there was a steep fall in cyanide sensitivity, although the *in situ* and potential q_{O_2} changed little. As the oxygen supply was reduced further, cyanide sensitivity decreased along with q_{O_2}. Thus, again there is no clear correlation between cyanide sensitivity and respiratory activity or yield coefficient. Moreover, the observed changes in cytochrome content could not be correlated with the cyanide sensitivity.

These studies failed to show any physiological evidence of a switch in pathways of electrons from a 'coupled' to an 'uncoupled' branch of the electron-transport system such as has been suggested for Azotobacter (Ackrell & Jones, 1971*b*) and for *E. coli* and *K. aerogenes* (Harrison & Maitra, 1969), Also we found no correlation between the induction of CO-binding cytochrome *c* and cyanide sensitivity. Whatever the significance is of the multiplicity of terminal-oxidase cytochromes in *B. natriegenes*, it does not appear to relate to adaptation to dissolved-oxygen changes. The induction of increased cytochrome content and potential respiration rate at dissolved-oxygen tensions well above the 'critical' value raises some questions on how these organisms detect changes in dissolved oxygen which have little effect on cell metabolism or the *in situ* respiration rate. The reason for changes in cytochrome and respiratory potential is obscure.

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1. Introduction

Respiration of cell-free extracts of the marine bacterium *Beneckea natriegens* was reported to be inhibited by 10 μM cyanide when ascorbate-TMPD* was the substrate but, with succinate as the electron donor, 1 mM KCN caused only 50% inhibition [1]. These results were explained in terms of a terminal branching of the electron transport system. *Beneckea natriegens* had been shown to possess 4 different potential terminal oxidases: cytochrome a_1, cytochrome *d*, cytochrome *o* and a CO-binding cytochrome *c* [2], and it was suggested by Weston and Knowles [2] that this may be further indication of a branching of the respiratory chain at the terminal oxidases, although they did not ascribe any particular cytochrome to the cyanide-insensitive pathway. Studies of cyanide sensitivity of whole cells of *B. natriegens* grown at different oxygen concentrations in chemostat culture failed to reveal changes in the sensitivity of the maximum potential respiration rate (i.e. respiration in the presence of excess substrate) to cyanide [3], although the relative content of cytochromes *o* and CO-binding *c* varied [4]. Further experiments showed that growth of glucose-limited cultures was resistant to inhibition by cyanide while harvested cells, supplied with excess substrate, were sensitive (Linton, Harrison and Bull, in preparation). This could be explained if it were the actual electron flux through the respiratory system on which the sensitivity to cyanide depended. This latter possibility has been investigated in the present work using harvested cell suspensions.

* TMPD = *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride.

2. Materials and methods

2.1. *Organism and media*

Beneckea natriegens strain III was provided by C. J. Knowles, University of Kent at Canterbury. The organism was grown in continuous culture under glucose or glycerol limitation in a minimal medium containing (per litre) (A) Na_2HPO_4, 1.5 g; KH_2PO_4, 1.5 g; $(NH_4)_2SO_4$, 3.0 g; NaCl, 23 g; KCl, 0.745 g; 1 ml trace element mixture from a stock solution containing $CaCl_2 2H_2O$, 0.66 g; $ZnSO_4 7H_2O$, 0.18 g; $CoSO_4 5H_2O$, 0.16 g; $MnSO_4 4H_2O$, 0.15 g; $CaCl_2 6H_2O$, 0.18 g; H_3BO_3; 0.10 g; $Na_2MoO_4 2H_2O$, 0.30 g pH 7.3 (925 ml). (B) Glucose or glycerol 2.0 g; $MgSO_4 7H_2O$, 0.2 g; trisodium citrate 0.146 g; 0.020 g $FeSO_4 7H_2O$ pH 7.3 (75 ml). Components A and B were autoclaved separately and mixed on cooling. Details of the growth conditions in continuous culture have been described [4]. Erlenmeyer flasks fitted with side arms to facilitate direct absorbance measurements were used to grow the organism in batch culture and the minimal medium described above was used with glucose as the sole source of carbon and energy.

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2.2. *Measurement of respiration rate of harvested bacteria*

Respiration rates were measured by diluting fresh samples from the chemostat in complete basal medium minus the carbon and nitrogen source (pH 7.2), adding known quantities of glucose and following the oxygen uptake in an oxygen electrode respirometer cell (Rank Bros., Bottisham, Cambridgeshire [5]). Measurements were made at 31°C unless stated otherwise.

2.3. *Inhibitor studies*

Solutions of known concentrations of KCN were injected into the oxygen electrode cell containing bacteria suspended in a solution buffered to pH 7.2. After a 2 min incubation period substrate was injected into the cell and the oxygen uptake recorded. Preliminary experiments had shown that between 0.5 and 1.0 mins were required after exposure to cyanide, to attain a constant rate of respiration.

Carbon monoxide gas was bubbled briskly through the buffer (pH 7.2) for 5 min which was sufficient to saturate the solution. The saturated CO solution was diluted to give final concentrations of CO from 5 to 50% saturation. The bacteria suspended in aerated saturated solution at pH 7.2 were diluted with the CO solution in the oxygen electrode cell, darkened by covering with aluminium foil and left in contact with the CO for 2 min before the substrate was injected into the cell.

For studying the combined effect of KCN and CO, CO saturated buffer was introduced into the oxygen electrode cell containing diluted culture and the container closed immediately. KCN was then injected into the suspension. A 2 min incubation period was allowed before substrate was injected into the electrode cell.

3. Results

3.1. *Respiration of glucose*

The effect of cyanide concentration on the respiration rate of *Beneckea natriegens* freshly harvested from a glucose-limited chemostat culture and supplied with excess (133 μM) glucose is shown in fig.1a. Respiration was sensitive to cyanide, over the whole range of cyanide concentrations tested down to 5 μM. The experiment was repeated using a limiting glucose concentration of 44 μM which gave an initial respiration rate of only 85% of the maxi-

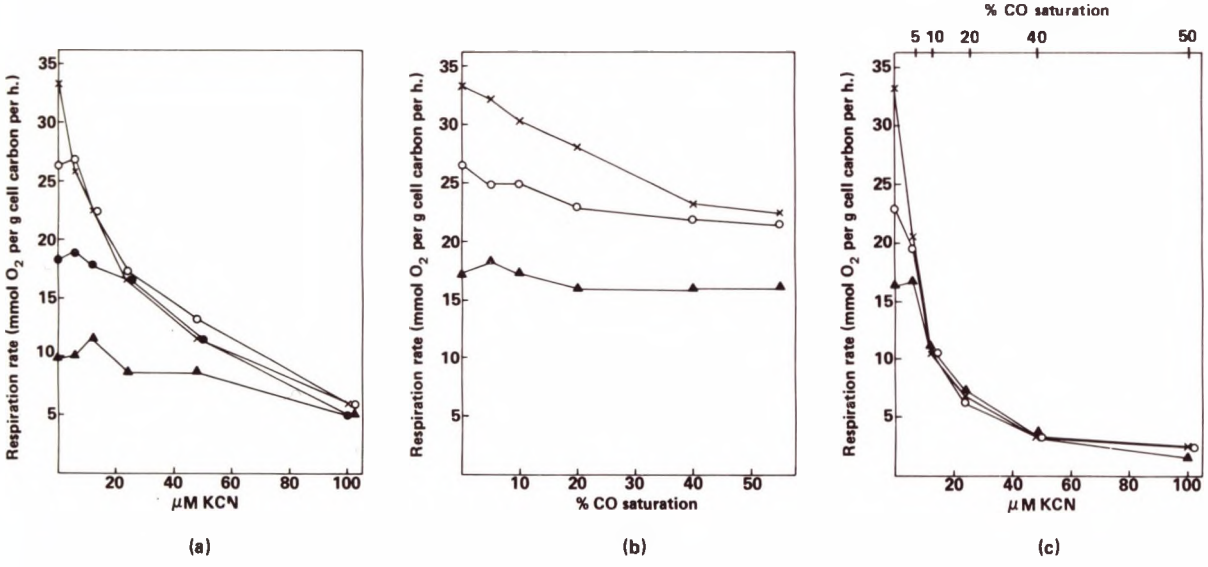

Fig.1. The effect of respiration rate on the sensitivity of respiration of cells harvested from a glucose grown culture of *B. natriegens* to cyanide and carbon monoxide. (a) The effect of KCN. (b) The effect of CO. (c) The effect of a combination of KCN and CO. The respiration rate was varied by injecting glucose at the following concentrations (X——X) 133 μM; (○——○) 44 μM; (●——●) 22 μM; and (▲——▲) 11 μM into the oxygen electrode cell.

mum. In this case 6 μM cyanide had no effect on respiration rate but higher concentrations caused inhibition. With 22 μM glucose, giving a respiration rate of 70% of the maximum, cyanide concentrations of up to 12 μM had no effect and with 11 μM glucose, giving a respiration rate of 45% of the maximum, cyanide had little effect at concentrations below 24 μM. Above these 'critical' cyanide concentrations the curves were almost completely superimposable (except the 11 μM glucose case).

The effect of carbon monoxide at various glucose concentrations is shown in fig.1b and it can be seen that it had little effect on respiration rate. However, carbon monoxide in combination with cyanide caused much greater level of inhibition than cyanide alone (fig.1c). Once again, at sub-maximal respiration rates there was a range of cyanide concentrations below which cyanide had no effect and above which the response curve was superimposable on that of the maximum respiration rate.

3.2. *Respiration on glycerol*

In order to investigate whether the phenomena observed above were unique to glucose, and possibly associated with its active transport, the experiment was repeated on bacteria grown on glycerol (reported to be taken up by facilitated diffusion) [6]. The pattern of results was very similar to that obtained with glucose: The maximum respiration rate (obtained with 352 μM glycerol) was the same and concentrations of glycerol of 85, 44 and 22 μM gave a similar family of curves as that shown in fig.1a for glucose. The combination of cyanide and carbon monoxide also gave a remarkably similar pattern of curves to that obtained with glucose.

3.3. *Effect of temperature*

A series of experiments made in order to test whether the change in sensitivity was a function of respiration rate itself or of substrate concentration. The respiration rate was altered, in the presence of excess (133 μM) glucose by changing the temperature of the oxygen electrode cell. Fig.2 shows that lowering the temperature, and thus the respiration rate, rendered the bacteria more insensitive to cyanide. Again at sub-maximal respiration values there was a range of low cyanide concentrations to which the bacteria were completely insensitive.

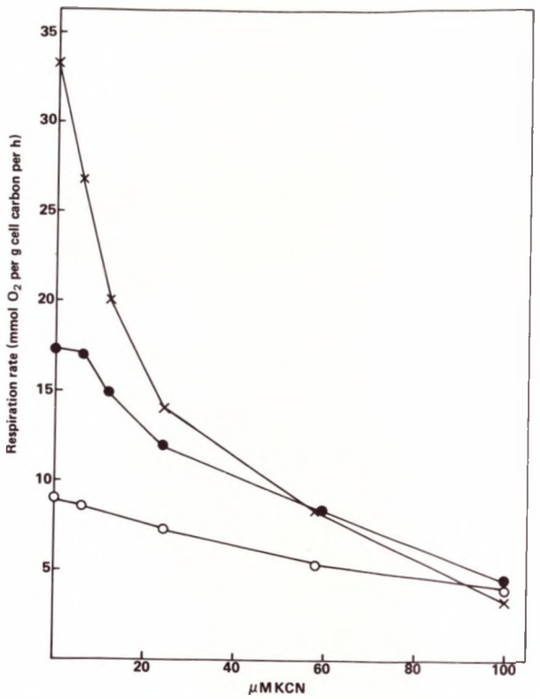

Fig.2. The effect of reduced incubation temperature on the sensitivity of the respiration of glucose grown cells to KCN. (X——X) 31°C; (●——●) 25°C; (○——○) 5°C.

3.4. *Effect of cyanide on bacteria from batch cultures*

Earlier reports of insensitivity to cyanide by *B. natriegens* were based on bacteria grown in batch culture [1]. Therefore, *B. natriegens* was grown on glucose in batch culture and the effect of cyanide tested on bacteria harvested from the logarithmic phase and the early and late stationary phases. Fig.3 shows that the bacteria harvested in the stationary phase had a much lower maximum respiration rate and were also less sensitive to cyanide.

3.5. *Cyanide sensitivity of bacteria grown in the presence of cyanide*

The effect of exposure to cyanide during growth was investigated by adding 50 μM KCN to the medium supplied to a glucose-limited chemostat culture. We have shown previously that growth and in situ

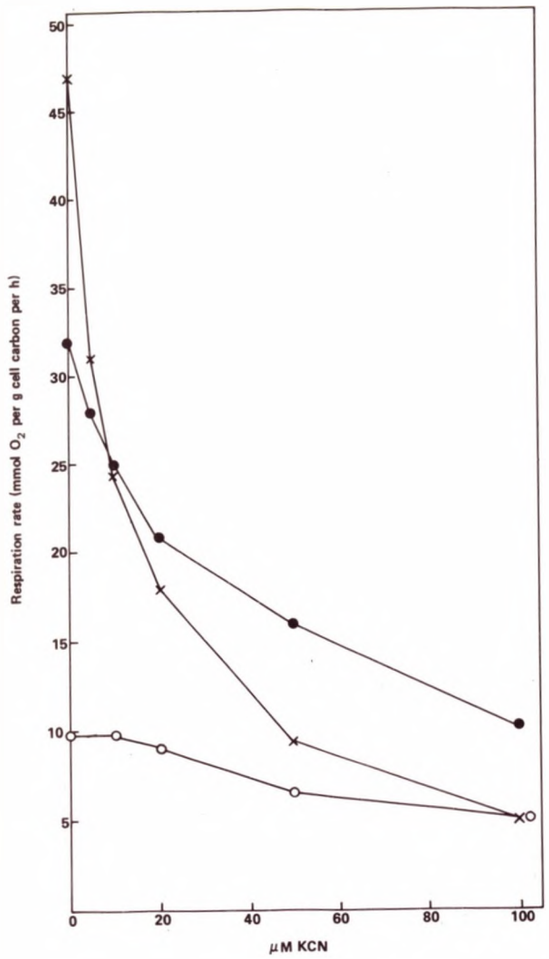

Fig.3. Cyanide sensitivity of the respiration of *B. natriegens* taken from log phase early and late stationary phase batch culture. Log phase (X——X); early stationary phase (16 h) (●——●); late stationary phase (24 h) (○——○).

respiration in chemostat culture in the presence of excess oxygen is unaffected by 50 μM KCN but the level of CO-binding cytochrome *c* in the bacteria is increased significantly [4]. The maximum potential respiration rate of harvested bacteria grown in the presence of KCN was very similar to that obtained in the absence of KCN and a similar pattern of sensitivity to cyanide and to combined cyanide and carbon monoxide was also obtained, this being dependent on the respiration rate of the bacteria.

The effects of KCN and CO singly and in combination at maximal respiration rates indicated that the combined effect was additive (table 1).

4. Discussion

The results reported here indicate a far greater dependence of respiratory sensitivity to cyanide on the actual respiration rate than has been reported previously for whole cells of any species. At maximum respiration rates the effect of KCN and carbon monoxide appeared to be at least additive, that is the combined effect of KCN and CO was equal to a summation of their separate effects. This type of response is usually found when the two inhibitors act on the same enzyme [7].

The relationship between cyanide concentration and respiration rate at different initial respiration rates was unexpected. There have been few systematic studies reported on the effect of respiration rate on cyanide sensitivity but Jones [4] reported pure uncompetitive inhibition for physiological substrates, but non-competitive inhibition for ascorbate dichlorophenolindophenol oxidation via cytochrome *o* in *Azotobacter vinelandii.* The relationship found in *B. natriegens* in this work was neither that of simple competitive nor of non-competitive inhibition. At sub-maximal respiration rates there is a range of cyanide concentrations to which respiration is completely insensitive but at the point where these curves meet that obtained in the presence of excess glucose, respiration becomes sensitive and followed the same curve as that obtained with excess glucose.

A similar family of curves was obtained with glycerol as the substrate and it is suggested, therefore, that the effect was not due to inhibition of glucose transport by cyanide. Moreover, decreasing the respiration by lowering the incubation temperature also gave a similar series of curves from which it may be concluded that respiration rate rather than substrate concentration is the important factor.

These results indicate an apparent titration of cyanide against a limiting component of the respiratory chain. At maximal respiration rates this component is limiting for respiration rate therefore low cyanide

Table 1
The effect of KCN and CO on the maximum respiration rate of *B. natriegens* harvested from continuous cultures grown on glucose in the presence and absence of cyanide and on glycerol

KCN concentration (μM)	Inhibition (%) (A)	CO concentration (% saturation)	Inhibition (%) (B)	KCN + CO (μM %)	Inhibition observed (%)	Inhibition A + B (%)
			Glucose-grown bacteria (in presence of 50 μM KCN)			
6	21	5	3	6 + 5	24	24
12	35	10	15	12 + 10	54	50
24	56	20	18	24 + 20	76	74
48	75	40	20	48 + 40	89	95
100	91	50	24	100 + 50	93	115
			Glucose-grown bacteria			
6	24	5	4.5	8 + 5	39	28
12	34	10	11.2	12 + 10	69	45
24	52.5	20	18	24 + 20	79	70.5
48	66	40	30.25	48 + 40	91	96
100	82	50	34	100 + 50	92	116
			Glycerol-grown bacteria			
6	21.4	5	2.0	6 + 5	24	23.4
12	36	10	15.4	12 + 10	53.4	51.4
24	57	20	18.4	24 + 20	76.7	75.4
48	75.4	40	19.4	48 + 40	88	94.8
100	90.5	50	24.7	100 + 50	90	115.2

concentrations cause inhibition. At sub-maximal rates there is an excess of this component which can be titrated with and probably binds to cyanide before any restriction of respiration occurs. This would suggest that the potential respiration is limited by a cyanide binding terminal oxidase in this organism. *Beneckea natriegens* grown in the presence of 50 μM KCN contained more CO-binding *c*-type cytochrome than when grown in its absence, but the maximum respiration rate was not increased. If CO-binding cytochrome *c* were the rate-limiting component then either a higher respiration rate or an increased resistance to cyanide would be expected when it is induced. Therefore, this CO-binding *c*-type cytochrome would not appear to be related to the cyanide-sensitivity or the rate-limiting component in the respiratory system of this organism.

Cytochrome *d*, has been shown by Weston and Knowles to be a major terminal oxidase in the *B. natriegens* even though it is barely detectable in cytochrome difference spectra [2]. In fact, the turnover number of terminal oxidases is generally so high that respiration may be routed entirely through a cytochrome which is barely detectable in most instruments presently available [9]. Thus, it is possible that cytochrome *d* was the main terminal oxidase in the cells studied here but that it was present at such low concentrations that the maximum respiration rate was limited by this component. This cytochrome then, would bind with cyanide and respiration rate would depend on the number of unbound sites.

The results reported here are not consistent with an alternative cyanide-insensitive pathway functioning as a *major* respiratory pathway, as all the cells tested were cyanide-sensitive at high respiration rates. However, there was in each case, a residual cyanide-insensitive respiration amounting to some 10% of the

maximum. This may represent the cyanide-insensitive pathway that Weston and Knowles [2] detected in cell free extracts of *B. natriegens*.

So far this phenomenon has not been investigated fully for any other organisms but we have found that a similar relationship between cyanide sensitivity and respiration rate may exist for *Pseudomonas extorquens* grown on methanol (J. Linton, unpublished data). There is no reason to assume that *Beneckea natriegens* would be unique in this feature and thus cyanide sensitivity of micro-organisms should always be investigated in relation to their respiration rate.

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