RESPIRATION AND ENERGY CONSERVATION IN BACTERIA

A thesis submitted for the degree of Ph.D.

by

ANDREW JOHN DOWNS
(Department of Biochemistry, University of Leicester)

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To Dawn

"We are a scientific civilisation: that means, a civilisation in which knowledge and its integrity are crucial. Science is only a latin word for knowledge..... knowledge is our destiny. Self knowledge, at last bringing together experience of the arts and the explanations of science, waits ahead of us."

(Bronowski, 1973)

The thesis entitled "Respiration and Energy Conservation in Bacteria" is the result of original work done mainly by me during the period of my registration at the University of Leicester.

A J Downs

Andrew J. Downs

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ABBREVIATIONS

Abbreviations in this thesis are those recommended by the Biochemical Journal. The following additional abbreviations are used:

TMPD	N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride
PIPES	piperazine N,N'-bis-2-ethane sulphonate
HQNO	2-n-alkyl-4-hydroxyquinoline-N-oxide
TCS	tetrachlorosalicylanilide

C H A P T E R I

GENERAL INTRODUCTION

CHAPTER I

GENERAL INTRODUCTION

1. Energy transfer in living organisms

With the exception of the chemosynthetic bacteria, the ultimate source of energy for all forms of life on the earth is the sun. Using the sun's energy, photosynthetic (autotrophic) cells produce organic compounds from CO_2 and H_2O and evolve O_2. Heterotrophic cells utilise the organic compounds from autotrophic cells and return the carbon as CO_2 to the atmosphere. Organic compounds such as glucose are potent energy sources for heterotrophic organisms because they are highly ordered molecules. When a glucose molecule is oxidised to form six CO_2 molecules and six H_2O molecules, a much higher degree of randomness is obtained since these small molecules can orient themselves in many different ways relative to each other. This increase in entropy is accompanied by a loss of free energy of the glucose molecule which is utilised in the cell to perform work. In addition to direct synthesis of ATP by substrate-level phosphorylation, oxidation of glucose in aerobic cells yields reduced pyridine and flavin nucleotides. These compounds are oxidised *via* the respiratory chain in a series of oxido-reduction steps which allows energy to be conserved in a biologically useful manner. This energy conservation predominantly involves the formation of ATP by the process of oxidative phosphorylation.

2. Oxidative phosphorylation

The way in which energy is conserved during the process of mitochondrial, bacterial and photosynthetic electron transport is still

unresolved in spite of many years' intensive research. However, there is considerable evidence that there is a common mechanism for all three systems. The following discussion on the possible mechanisms of oxidative phosphorylation relates to the mitochondrial system since this is most relevant to heterotrophic bacteria, on which studies are scant.

All theories of oxidative phosphorylation must be consistent with certain observations: (a) the stoichiometry of ATP synthesis relative to oxygen uptake necessitates 3 sites along the mitochondrial respiratory chain at which energy is conserved, (b) the energy from one site can be used at another, as indicated by the observation of reversed electron transport - this energy must therefore be interconvertible with a common intermediate other than ATP, (c) this high energy intermediate is relatively stable, since when its utilisation rate is low the speed at which it is broken down becomes the rate-limiting step of electron transport - this is known as respiratory control, (d) ion transport and transhydrogenation, apart from ATP synthesis and reversed electron transport, must be driven by this common intermediate, (e) uncoupling agents inhibit these energy-dependent processes and oligomycin inhibits ATP synthesis and also ion transport and transhydrogenation under conditions where ATP supplies the required energy. Any proposed mechanism of oxidative phosphorylation must therefore involve a 'high energy' intermediate, be it a chemical or physical phenomenon, and must define ways in which its energy can be used for the processes described above.

The three current hypotheses for the mechanism of oxidative phosphorylation are outlined briefly below:

(i) <u>The chemical coupling hypothesis</u>

Originally proposed in 1953 by Slater (see also Slater, 1971;

1972), the chemical hypothesis is based on an enzymatic mechanism of substrate-level phosphorylation. It is suggested that energy is transferred from respiratory chain components to postulated coupling factors. If A and B are adjacent respiratory carriers, the 'energy coupling' reaction which occurs is

$$AH_2 + B + I \rightleftharpoons A\sim I + BH_2 \qquad (1)$$

where I is a third component (not a respiratory carrier) and $A\sim I$ is a high energy intermediate. There is, however, no evidence that the intermediate is not $BH_2\sim I$ (Greville, 1969). $A\sim I$ gives rise to ATP via the following series of energy-transfer reactions:

$$A\sim I + X \rightleftharpoons A + X\sim I \qquad (2)$$

$$X\sim I + P_i \rightleftharpoons X\sim P + I \qquad (3)$$

$$X\sim P + ADP \rightleftharpoons X + ATP \qquad (4)$$

where $X\sim I$ is the common energy-transfer intermediate driving ion transport and transhydrogenation which is produced either by respiration or by breakdown of ATP. It is proposed that uncouplers exert their effect by either directly or indirectly causing the breakdown of $X\sim I$. Oligomycin (an inhibitor of both phosphorylation and electron transport) is proposed to block reaction (3).

Three lines of research have led to the identification of the three regions of energy conservation in the respiratory chain, viz the cross-over studies of Chance and Williams (1956), the determination of P/O or $P/2e^-$ for particular portions of the chain and redox potential measurements. The exact point of coupling at sites I and III is at

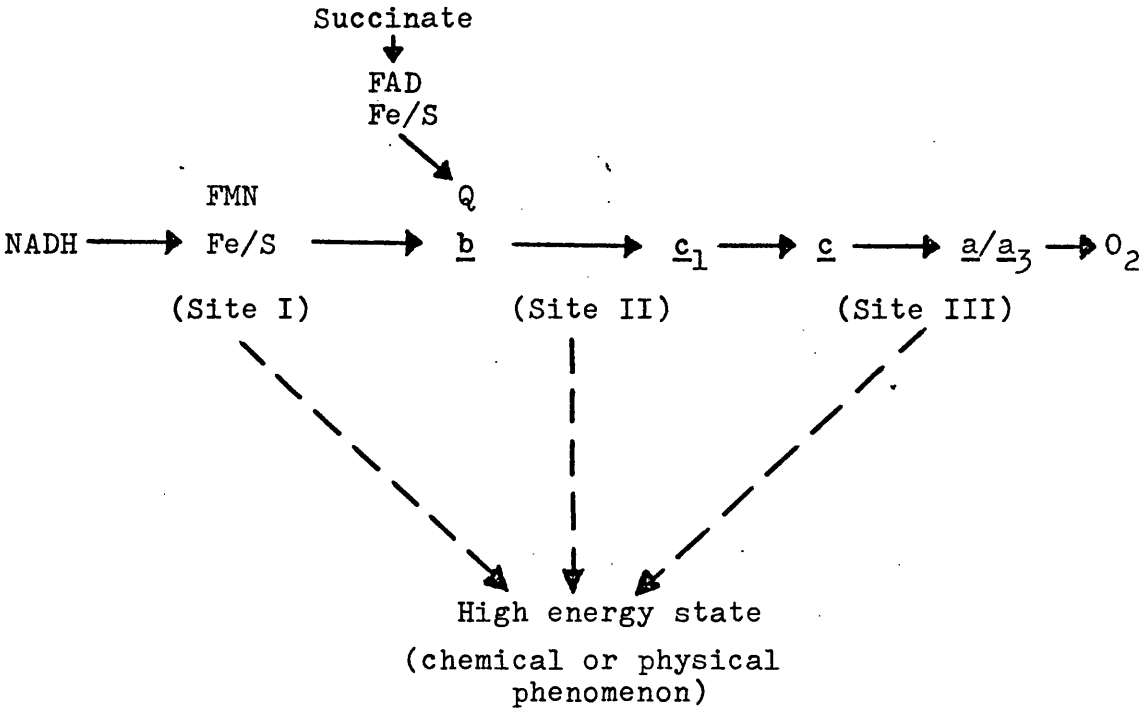
present unclear but the position of site II between the cytochrome b region and cytochrome c, is quite well established (Fig. I-1).

In terms of the chemical hypothesis, the formation of a high energy intermediate with a respiratory carrier would be expected to alter the redox potential of the carrier. This is because the redox potential of the couple $AH_2 + I \rightleftharpoons A\sim I + 2H^{\bullet}$ would be higher than that of the non-energised couple $AH_2 \rightleftharpoons A + 2H^{\bullet}$; i.e. $A\sim I$ would be more readily reduced than A (Wilson, 1974). To be involved in the coupling mechanism, the standard free energy change (ΔG^{o}) of the reaction $A\sim I \longrightarrow A + I$ would have to be approximately -8 kcal/mol, equivalent to a reduction in redox potential of 175 mV for a two-electron reaction.

In 1970, Wilson and Dutton demonstrated the presence of two types of cytochrome b in mitochondria, named cytochromes b_K and b_T by Chance et al. (1970), which exhibited different absorbance maxima and midpoint potentials. The midpoint potential of cytochrome b_T increased from -30 mV to +240 mV in the presence of ATP and this carrier was proposed by Chance et al. (1970) to be directly involved in energy transduction at site II due to the formation of $b_T\sim I$. However, Wilkström (1973) has presented evidence that the two forms of cytochrome b with different absorbance maxima both exhibit rises in their midpoint potentials in the presence of ATP and are hence both involved in energy transduction. Studies on energy conservation at site III (i.e. in the cytochrome oxidase region) have revealed that cytochrome a has a fixed midpoint potential whereas that of cytochrome a_3 decreases on addition of ATP (Wilkström and Saris, 1970; Wilson and Dutton, 1970; Lindsay et al., 1972). The results indicate that the decrease in midpoint potential may be great enough to allow formation of a high

Fig. I-1

Sites of energy conservation in the mitochondrial respiratory chain



energy intermediate with the reduced form of cytochrome $\underline{a}_3$, i.e. $\underline{a}_3^{2+} \sim I$. Investigations on energy transduction at the level of NADH dehydrogenase (site I) are a little confused due to the discovery of up to seven iron-sulphur centres in the enzyme complex (Orme-Johnson, 1973; Baltscheffsky and Baltscheffsky, 1974). Recently, midpoint potential measurements on these centres (Ohnishi and Pring, 1974) provided evidence that two of them may be involved in energy transduction since their midpoint potentials are altered in the presence of ATP.

Although the above experiments would seem to support the involvement of a chemical-type energy conserving mechanism at the three coupling sites, it is also possible to explain the midpoint potential shifts observed in terms of the chemiosmotic hypothesis (see below). This is because a membrane potential generated by respiration or other means would also be expected to alter the midpoint potentials of the respiratory carriers (Hinkle and Mitchell, 1970).

(ii) The chemiosmotic coupling hypothesis

Whereas in the chemical coupling hypothesis the initial energy conserving event is the formation of a covalently-bonded high energy intermediate, in the chemiosmotic hypothesis proposed by Mitchell (1961; 1968), energy from the oxidoreductions is conserved by translocation of protons from the inside to the outside of the inner mitochondrial membrane or bacterial plasma membrane. The pH and electrical gradient across the coupling membrane generated by respiration may then be used to drive ATP synthesis via a proton-translocating ATPase located in the membrane in a position physically unconnected with the respiratory chain.

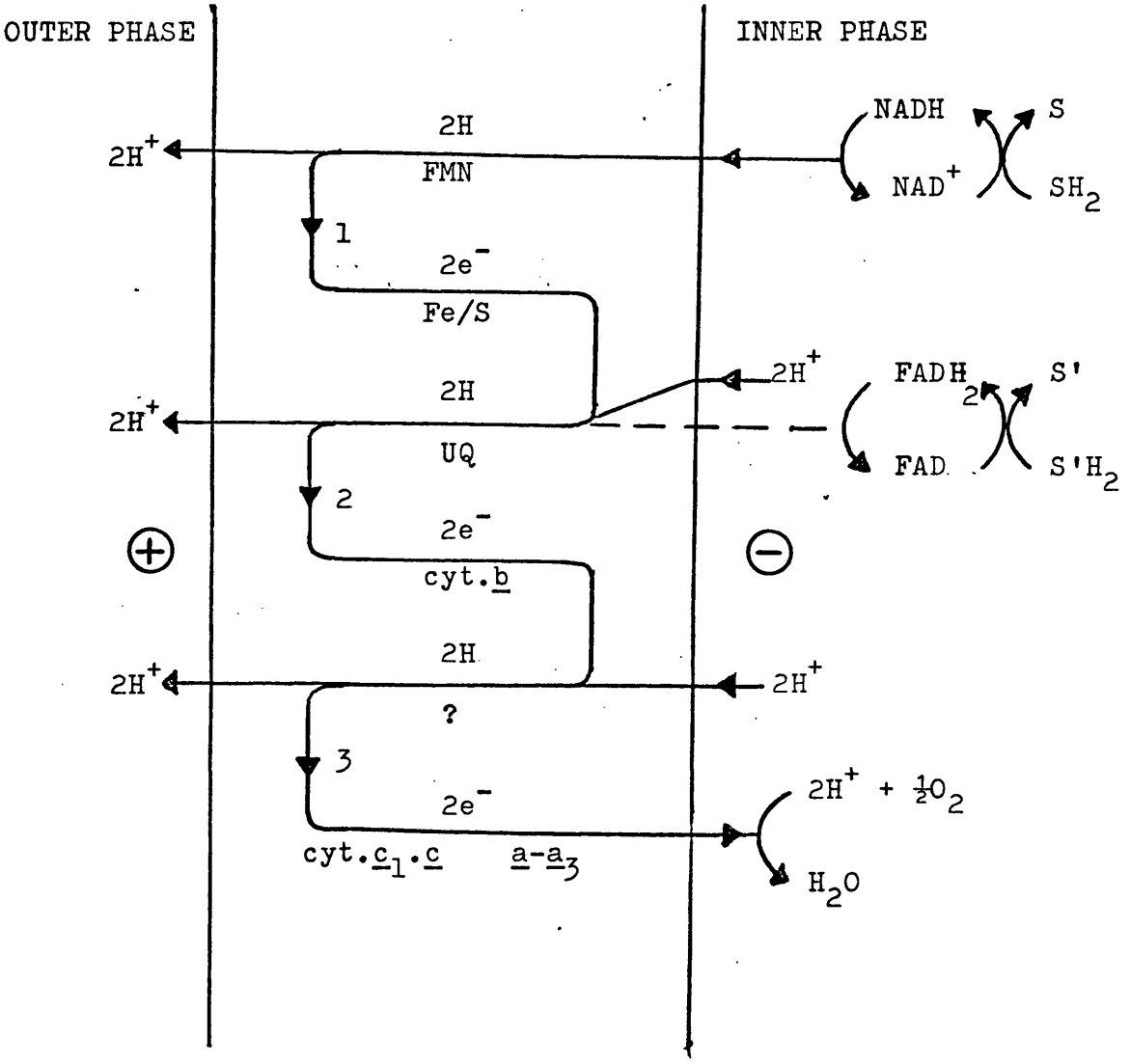
In order for this mechanism to work, the membrane must be topologically closed and of low permeability to H^+ and OH^-, otherwise the

proton 'current' would be short-circuited. In fact, it has been demonstrated by Mitchell and Moyle (1967a) that the mitochondrial membrane has a very low permeability to H^+ and OH^- and to ions in general unless the membrane possesses a specific porter system for the ion (e.g. the Ca^{++} porter) or the ion is sufficiently polarisable or large (e.g. SCN^- or NO_3^-) so that it is soluble in the lipid phase of the membrane.

The chemiosmotic hypothesis has very specific requirements for the orientation of respiratory carriers in the membrane since passage of reducing equivalents from substrate to oxygen must be accompanied by movement of protons from the inner to the outer phase. The proposed arrangement of carriers is shown in Fig. I-2 and consists of alternating hydrogen and electron carriers. There are three so-called proton translocating loops involved in the oxidation of NAD-linked substrates by mitochondria and two for flavin-linked substrates. Loop 1 is thought to be formed by the NADH dehydrogenase complex. A hydrogen carrier, presumably FMN (the prosthetic group of the enzyme) is reduced by two hydrogen atoms from $NADH_2$ near the inside of the membrane and subsequently becomes reoxidised by ejecting two protons outside the membrane, simultaneously transferring two electrons to the iron-sulphur constituent of the dehydrogenase. The iron-sulphur moiety is then reoxidised by donating two electrons to ubiquinone, which simultaneously takes up $2H^+$ from the inner phase and thus acts as a hydrogen carrier. Ubiquinone then completes another proton-translocating loop (loop 2) by reducing cytochrome b and ejecting two more protons into the outer phase. Cytochrome b donates electrons to an unidentified hydrogen carrier in loop 3 which completes the loop with the electron carriers cytochromes c_1, c and aa_3. In order for the respiratory chain in the region

Fig. I-2

The proposed arrangement of carriers in the mitochondrial respiratory chain according to the Chemiosmotic hypothesis



The possible identity of the hydrogen carrier in loop 3 is not established. S; NAD-linked substrates, S'; flavin-linked substrates, UQ; ubiquinone. The diagram illustrates the proposed proton-translocating properties of the respiratory chain. The signs represent the electrical gradient generated across the membrane during respiration.

cytochrome b to aa_3 to accomplish net proton translocation, the cytochrome oxidase (a_3) must donate electrons to oxygen on the inside of the membrane, since formation of a molecule of water involves consumption of two protons. Flavin-linked substrates donate reducing equivalents to the respiratory chain at the level of ubiquinone, thus bringing about proton translocation by loops 2 and 3 only.

Evidence for the stoichiometry of proton translocation is as follows. When a small pulse of oxygen is added to an anaerobic suspension of mitochondria, there is a temporary acidification of the external medium. If the quantity of H^+ expelled is expressed relative to the amount of oxygen reduced, an $\longrightarrow H^+/O$ ratio (g ion H^+ translocated per g atom oxygen reduced) of 6 is obtained for β-hydroxybutyrate oxidation (NAD-linked) and 4 for succinate oxidation (flavin-linked (Mitchell & Moyle, 1967b). According to the chemiosmotic hypothesis, these experiments indicate that the mitochondrial respiratory chain translocates protons such that two are ejected per pair of reducing equivalents transferred through each of the three regions of the respiratory chain associated with energy conservation. However, H^+ extrusion by mitochondria on addition of oxygen is accompanied by influx of Ca^{++} ions. Mitchell proposes that this ion movement passively compensates for the electrical potential built up across the membrane by proton translocation. Unfortunately for the chemiosmotic hypothesis, these observations can also be explained in terms of the chemical hypothesis since $X\sim I$ could drive a cation pump and the proton movement could then passively compensate for Ca^{++} uptake. Alternatively, and more plausibly, $X\sim I$ could drive a proton pump and the Ca^{++} movements could then be compensatory (see Chance et al., 1967; Greville, 1969).

Other investigations on the validity of the chemiosmotic hypothesis have attempted to determine the orientation of the respiratory carriers in the membrane. Mitchell's hypothesis has an absolute requirement for the carriers to be arranged in a sided fashion whereas in the chemical hypothesis this is not necessary. There is quite substantial evidence that cytochrome c is on the outside of the membrane, as demonstrated, for example, by the oxidation of exogenous cytochrome c by submitochondrial particles prepared by sonication ('outside-out' particles) but not by particles prepared by digitonin treatment ('inside-out' particles; Chance and Fugmann,1961). There is also some evidence that cytochrome oxidase reacts with oxygen on the inside of the membrane, as indicated by experiments involving the monitoring of proton movements during the reduction of exogenous ferrocyanide (Mitchell and Moyle, 1967c), TMPD (Mitchell, 1969) or cytochrome c (Mitchell, 1971) by mitochondria inhibited with Antimycin A. This is also supported by the observation that azide is a much more effective inhibitor of respiration when it is accumulated by mitochondria than under conditions where there is no accumulation (Palmieri and Klingenberg, 1967). However, the site of oxygen reduction on the oxidase may be different from the site of azide inhibition (Mitchell, 1969). Unfortunately, it is also possible to explain the above experiments involving artifical electron donors by means of the chemical hypothesis and an X~I-driven proton pump. Therefore the current evidence favouring the orientation of the respiratory carriers in the membrane in the manner predicted by Mitchell is somewhat flimsy.

The common high energy intermediate of the chemiosmotic hypothesis is the proton and electrical gradient across the membrane

generated by respiration. Respiratory control is assumed to be effected by the 'protonmotive force' (i.e. the electrochemical potential of protons) created by respiration causing a backward pressure in the respiratory chain which slows the rate of proton translocation and hence the rate of electron transport. The mechanism by which Mitchell proposes that the protonmotive force drives ATP synthesis is as follows. Since oxidative phosphorylation involves a dehydration reaction, i.e.

$$ADP + P_i \rightleftharpoons ATP + H_2O$$

a system which removes H_2O from the active centre of the ATPase would favour ATP production. This is postulated to be accomplished by the protonmotive force which causes H^+ to move to the inner phase and OH^- to the outer phase relative to the coupling membrane.

Proton translocation during ATP hydrolysis has been observed for mitochondria (Mitchell and Moyle, 1968; Moyle and Mitchell, 1973; Thayer and Hinkle, 1973), chloroplast grana (Carmeli , 1970), photosynthetic bacteria (Scholes et al., 1969) and for vesicle preparations of E. coli (West and Mitchell, 1974). ATP synthesis during application of a protonmotive force across the coupling membrane has been demonstrated in rat-liver mitochondria (Reid et al., 1966; Cockrell et al., 1967; Reid, 1970), chloroplasts (Jagendorf and Uribe, 1966; Uribe and Li, 1973) and photosynthetic bacteria (Jackson et al., 1968) but not, to date, in heterotrophic bacteria. However, these experiments are still open to interpretation in terms of the chemical hypothesis since the effects observed could be due to the action of a reversible X~I-driven proton pump. Measurements of the stoichiometry of ATP hydrolysis in mitochondria (Mitchell and Moyle, 1968; Mitchell, 1972) have indicated an $\longrightarrow H^+/P$

ratio (g ion H^+ translocated per mol ATP hydrolysed) of 2. Since $\longrightarrow H^+/O = \longrightarrow H^+/P \times P/O$ (Mitchell and Moyle, 1967b), this is consistent with a P/O ratio of 3 between NADH and oxygen.

The ATPase system is proposed to be located in the inner mitochondrial membrane subunits (Fernandez-Moran particles) projecting into the matrix spaces. In common with the chemical hypothesis, the ATPase of the chemiosmotic hypothesis involves a high energy compound, X~I. In one postulated mechanism for the ATPase reaction the pH gradient is used to convert X^- and IO^- into X~I in the membrane, H^+ being transported from the outside to the inside in the process. A reversible reaction then connects X~I with ADP, P_i and ATP (Fig. I-3). X and I may be thought of as ionisable groups in the proton translocating part of the ATPase system.

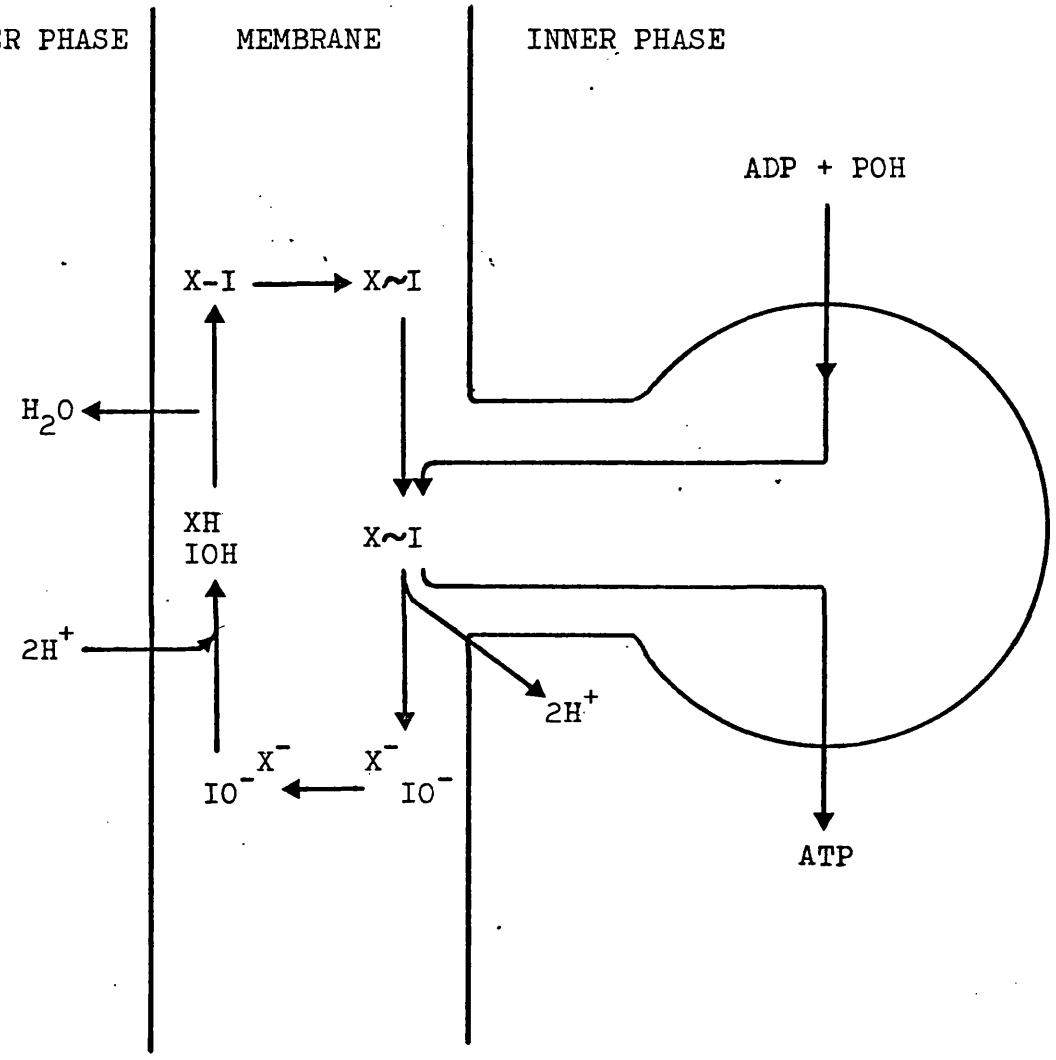
Uncouplers are postulated to have their effect by allowing proton permeation across the coupling membrane. This has been verified experimentally for mitochondria, chloroplasts and bacteria (see Greville, 1969; Scholes and Mitchell, 1970). Oligomycin would presumably act on the proton-translocating ATPase system.

(iii) The conformational coupling hypothesis

Instead of the energy derived from respiration being stored as a covalent compound (chemical hypothesis) or as a pH gradient and membrane potential (chemiosmotic hypothesis), in the conformational coupling hypothesis energy is stored in a conformational state of the macromolecules in the coupling membrane. As proposed by Boyer (1965), the energy is conserved as a conformational state of a respiratory carrier:

Fig. I-3

The proton-translocating ATPase system of the chemiosmotic hypothesis



The "ATPase II" system of Mitchell (1968) in which 2 g.ionH^+ are translocated inwards per mol ATP synthesised.

$$AH_2 + B \rightleftharpoons A^* + BH_2$$

$$A^* + ADP + P_i \rightleftharpoons A + ATP$$

In this case, the conformation hypothesis may be regarded as a special form of the chemical hypothesis, since both involve high-energy states of respiratory carriers.

On the basis of observations on the conformational states of mitochondria using electron microscopy, Green et al. (1968) suggested that the gross structural changes observed arose from conformational changes in the membrane subunits. These coincided well with the energy state of the mitochondria. More recently, Green and Ji (1972) suggested that the respiratory carriers developed an electric field in the membrane which resulted in a conformational change in the ATP synthesising system. This mechanism differed from the chemiosmotic hypothesis in that the field was not generated as a result of proton translocation. In both cases, however, the primary energy conserving event is charge separation. It is possible that the midpoint potential changes of respiratory carriers (see above) may be associated with conformational changes and that ATP synthesis may involve conformational changes at the coupling factor level (see Baltscheffsky and Baltscheffsky, 1974).

At present, it has not been ascertained which, if any, of these hypothesis is correct. Certain predictions of the chemiosmotic hypothesis, i.e. the impermeability of the coupling membrane to H^+, the

permeability-inducing properties of uncouplers and the movements of H^+ during ATP synthesis and hydrolysis have been confirmed. The ion-transporting properties of the mitochondria and a large body of work on the effects of the cation transport-mediating antibiotics (such as valinomycin, nigericin and gramicidin) can also be explained very satisfactorily in terms of the chemiosmotic hypothesis since only the presence of a pH and/or electrical gradient across the membrane is required (Greville, 1969; Henderson, 1971). Although these findings can also be explained in terms of the chemical or conformational hypothesis, a secondary assumption, i.e. the presence of a proton pump, is required.

It is quite possible that the true energy-coupling mechanism may involve more than one hypothesis, e.g. conformational changes might occur at the level of electron transport and ATP synthesis with the intermediary function fulfilled by either a chemical or chemi osmotic mechanism (Baltscheffsky and Baltscheffsky, 1974).

3. The efficiency of bacterial oxidative phosphorylation

It is now well established that energy conservation in isolated mitochondria is associated with three regions of the respiratory chain between NADH and oxygen (Slater, 1971) equivalent to a maximum P/O ratio of 3. There have been many attempts to establish whether similar efficiencies of energy conservation occur in bacteria. The techniques used can be divided into two groups, (a) assays on subcellular fractions and (b) assays on whole cells.

(a) Energy conservation in subcellular preparations

A number of studies on the phosphorylating efficiencies of bacteria have been carried out on phosphorylating subcellular membrane preparations. The methodology usually involves measurement of the rate of oxygen uptake and the concomitant rate of incorporation of $^{32}P_i$ into organic compounds (in the presence of an ATP trap such as glucose and hexokinase) on addition of an NAD- or flavin-linked substrate to an aerobic suspension of respiratory membrane in a suitable assay medium. Preparations of Gram-negative herotrophic bacteria, such as E. coli (Kashket and Brodie, 1963; Bragg and Polglase, 1963), Alcaligenes faecalis (Pinchot, 1953) and K. pneumoniae (Brice et al., 1974) yielded P/O ratios with NADH which rarely exceeded 0.5, although values of up to 1.1 were obtained with preparations of Azotobacter vinelandii (Ackrell and Jones, 1971a). However, with some Gram-negative chemoautotrophs such as Nitrobacter agilis (Aleem, 1968) and N. winogradskii (Kiesow, 1964), and with Gram-positive heterotrophs such as Mycobacterium phlei (Brodie, 1959) and Micrococcus lysodeicticus (Ishikawa and Lehninger, 1962) higher P/O ratios of up to 2.8 were obtained with NADH.

Low P/O ratios with some of the above preparations can probably be attributed to damage caused to the integrity of the energy conserving system during disruption of the cells. However, although P/O ratios can be measured with whole mitochondria, this is not possible with whole bacterial cells because of the impermeability of the plasma membrane to coenzymes and because of the many ATPase reactions occurring in the intact cells. In spite of the fact that measurements of P/O ratios using subcellular fractions from bacteria do not allow the true stoichiometry of the process to be elucidated, since such preparations

are bound to be partly uncoupled, they have nevertheless been useful in some cases in demonstrating the number of energy conservation 'sites' (equivalent to proton-translocating loops) present in the respiratory chains of certain bacteria. For example, John and Watley (1970) showed that Micrococcus denitrificans contained at least two energy conservation sites in its respiratory chain and Ackrell and Jones (1971a) demonstrated the presence of three sites in the redox chain of Azotobacter vinelandii (see Chapter II).

(b) Energy conservation in whole cells A number of whole-cell techniques have been employed in attempts to measure the efficiency of energy conservation in aerobic bacteria.

(i) Growth yields

P/O ratios for bacteria have been calculated by an indirect method involving measurements of the growth yield coefficients of the bacteria with respect to an energy-providing substrate (Y; e.g. g cells synthesised per mol glucose consumed). Measurements of Y for a number of heterotrophic anaerobes, in which the amount of ATP formed by catabolism was known from fermentation balances, allowed calculation of the efficiency of ATP utilisation for growth (Y_{ATP}: g cells synthesised per mol ATP generated by catabolism). Y_{ATP} was found to be a constant value for a number of bacteria at approximately 10.6 g cells.mol ATP^{-1} (Bauchop and Elsden, 1960;. Forrest and Walker, 1971). By measuring growth yields of aerobic bacteria and assuming that the above Y_{ATP} value applied also to aerobic organisms, various workers have calculated P/O ratios for growing cells. Applying this method, Gunsalus and Shuster (1961) and Wimpenny (1969) concluded that the P/O ratios of

aerobic bacteria were very low. However, their calculations may be invalid since no correction was apparently made for the fraction of the carbon source incorporated into cell material (Harrison, 1973).

Calculations of P/O ratios based on growth yields with respect to oxygen (Y_O, g cells synthesised per g atom oxygen consumed) instead of carbon substrate circumvents the necessity of knowing the amount of carbon incorporated into the bacterial cells (Whitacker and Elsden, 1963). The P/O ratio is then obtained by dividing Y_O by Y_{ATP}. Using this method, P/O ratios in the range 0.5 - 3 have been obtained; 0.5 for oxidative metabolism in Streptococcus faecalis (Smalley et al., 1968) and Acetobacter suboxidans (Whitacker and Elsden, 1963), 2 for Escherichia coli and Pseudomonas fluorescens (Whitacker and Elsden, 1963; Hernandez and Johnson, 1967) and approaching 3 for Klebsiella pneumoniae (formerly Aerobacter aerogenes, Hadjipetrou et al., 1964), Aerobacter cloaca (Stouthamer, 1969) and Proteus mirabilis (Stouthamer and Bettenhaussen, 1972).

In none of the above work was any allowance made for the proportion of energy derived from the substrate which was directed towards cell maintenance (see below). Hence the P/O ratios were presumablyunderestimated, the degree of underestimation depending on the maintenance coefficient. Harrison and Loveless (1971), who corrected for this factor, calculated a maximum P/O ratio of 2.4 for K. pneumoniae and subsequently maintenance-corrected P/O ratios of up to 3.4 were obtained for a number of other bacterial species by Meyer and Jones (1973). In spite of these corrections, the calculations were still based on the Elsden 'constant' of 10.6 g cells.mol ATP^{-1}. Recent work has questioned whether this value is correct (see section 4).

(ii) Change in nucleotide levels

A method for directly measuring the phosphorylating efficiency of whole bacterial cells was described by Hempfling (1970). By fluorimetrically monitoring the levels of NADH and adenine nucleotides in whole cells of E. coli before and after short bursts of respiration, mol ATP synthesised/mol NADH oxidised ratios of approximately 3 were obtained. Subsequently, similar values were obtained by Baak and Postma (1971) for A. vinelandii. However, van der Beek and Stouthamer (1973) presented evidence that this technique gives an overestimation of the P/O ratio and Harrison (1973) has suggested that the method cannot be regarded as giving an accurate measure of the P/O ratio due to a number of questionable assumptions involved in measurements of the NADH and adenine nucleotide pools under the experimental conditions employed.

(iii) $\longrightarrow H^+/O$ ratios

A possibly less equivocal method of directly measuring the phosphorylating efficiencies of whole bacteria is that of determining $\longrightarrow H^+/O$ quotients. According to Mitchell (1966), $P/O = \longrightarrow H^+/O \div \longrightarrow H^+/P$, hence P/O ratios can be calculated from $\longrightarrow H^+/O$ ratios if it is assumed that $\longrightarrow H^+/P$ for bacteria is 2, as for mitochondria (see section 2). Scholes and Mitchell (1970) pioneered the use of this technique by measuring $\longrightarrow H^+/O$ quotients of cell suspensions of Micrococcus denitrificans. Values of up to 8 were obtained with this organism, suggesting P/O ratios of up to 3 and the involvement, under these experimental conditions, of an H^+-translocating $NADPH \longrightarrow NAD^+$ transhydrogenase. $\longrightarrow H^+/O$ quotients have recently been measured on a number of other bacterial species; the results are discussed in Chapter V.

4. The efficiency of bacterial growth

(a) Growth yields Monod (1942) found a linear relationship between the extent of growth of E. coli, Bacillus subtilis and Salmonella typhimurium in batch culture and the initial concentration of carbon/energy substrate which ultimately limited growth. He expressed the relationship between growth and substrate utilisation as

$$\frac{\text{mass of bacteria formed}}{\text{mol of limiting substrate utilised}} = Y$$

where Y is the growth yield coefficient for the particular organism and substrate under study (g dry wt bacteria.mol substrate^{-1}). Bauchop and Elsden (1960) showed that it is possible to relate the growth yield coefficient to the energy, as ATP, made available by degradation of the substrate (allowing for the proportion of substrate carbon incorporated directly into cellular material), i.e.

$$Y = N \times Y_{ATP}$$

where N = mol ATP synthesised per mol substrate consumed and Y_{ATP} = g cells synthesised per mol ATP consumed. Substrate-level phosphorylation determines the value of N for anaerobic growth whereas both substrate-level and oxidative phosphorylation determine the value for aerobic growth. Strictly, N and Y_{ATP} should be expressed in terms of ATP equivalents in the case of aerobic growth, since a trans-membrane proton-motive force (or high energy intermediate) resulting from respiration may drive energy-requiring processes (e.g. uptake of nutrients) directly without the mediation of ATP (see above). Bauchop and Elsden (1960) calculated the value of Y_{ATP} for anaerobic growth of the bacteria

Streptococcus faecalis and Zymomonas mobilis and the yeast Saccharomyces cerevisiae. Y values were measured in batch cultures containing limiting quantities of energy source (e.g. glucose) and N values estimated by reference to the fermentation pathways known to be operating in the organisms. The Y_{ATP} values thus obtained were approximately constant at 10 g cells.mol ATP^{-1} (the Elsden 'constant'). A number of subsequent workers (see Stouthamer, 1969; Payne, 1970; Forrest and Walker, 1971) found similar values for a number of other anaerobic bacteria and assumed that Y_{ATP} was constant for different microorganisms at approximately 10.6 g cells.mol ATP^{-1}. However, there did seem to be small, reproducible differences in Y_{ATP} amongst the organisms studied; for example, Zymomonas mobilis gave an average Y_{ATP} of 8.5 ± 0.2 whereas Aerobacter cloacae gave a value of 11.9 ± 0.5 g cells.mol ATP^{-1} (both organisms grown on glucose; Forrest and Walker, 1971).

Forrest and Walker (1971) calculated the amount of ATP required for the polymerization of preformed monomers (glucose, amino acids, purine and pyrimidine bases and acetate) in the synthesis of 1 g of bacterial cells. They concluded that a Y_{ATP} value of approximately 27 g cells.mol ATP^{-1} might be expected (see also Stouthamer, 1973). Since the Y_{ATP} values observed experimentally were much lower than this, they suggested that most of the ATP generated by catabolism was used in ways which were not chemically defined. They considered that a Y_{ATP} of approximately 10 g cells.mol ATP^{-1} was the upper limit for cells of 'normal average' composition growing in complex media containing all of the required preformed monomers. However, Y_{ATP} values at variance with the Elsden 'constant' have recently been obtained for a number of other bacteria growing anaerobically. Thus, de Vries et al. (1970) obtained a Y_{ATP}

value of 20 for Lactobacillus casei whereas McGill and Dawes (1971) found a Y_{ATP} value of only 5.9 for Zymomonas anaerobia. In both of these studies, the fermentation pathways were known and values of N, and hence Y_{ATP}, could be calculated with certainty. Stouthamer and Bettenhaussen (1973) suggested that the different Y_{ATP} values obtained by the various workers might be due to the fact that the yield determinations were carried out in batch cultures in which no corrections were made for the proportion of energy used for cell maintenance (see below). They surmised that Y_{ATP} values of 10.6 and below were probably obtained with bacteria in which a high proportion of energy was directed to cell maintenance, by reason of slow growth rates and/or high maintenance coefficients, and that higher values might be expected for organisms with fast growth rates and low maintenance coefficients. They obtained a Y_{ATP} value, corrected for the maintenance requirement, of approximately 26 g cells.mol ATP^{-1} for A. aerogenes growing anaerobically in continuous culture. They consequently proposed that, during growth on complex medium, the Y_{ATP} value for different microorganisms of 'normal average' cell composition, corrected for maintenance, was a constant at approximately 25 g cells.mol ATP^{-1} (i.e. much higher than the Elsden 'constant').

At the start of the present work, no attempt had been made to measure Y_{ATP} values for aerobic bacteria because of the difficulty of obtaining reliable values for N. This is because N depends on the P/O ratios and there has been much uncertainty regarding the value of this parameter in different bacteria, as described in section 3. Indeed, measurement of Y has been used as an indirect means of calculating the P/O ratio by assuming that the Elsden 'constant' applies to aerobic as well as to anaerobic growth (see above).

(b) Energy of maintenance Although the existence of an 'energy of maintenance' for growing microorganisms was suggested as long ago as 1898 by Duclaux, the work of Monod (1942) indicated that the energy of maintenance of E. coli during growth was zero. However, Herbert (1958) found variations in the growth yield coefficient of bacteria when growing at different dilution rates in carbon-limited continuous culture and assumed this to be due to 'endogenous metabolism' of the organisms. Pirt (1965) considered this 'endogenous metabolism' to be the maintenance energy requirement of the bacteria, which was used for such purposes as the resynthesis of unstable macromolecules, the maintenance of concentration gradients and motility. He distinguished between the true growth yield coefficient of microorganisms, which would be obtained if all of the energy derived from the carbon substrate was directed towards growth, and the observed growth yield coefficient, which was a lower value taking account of the energy directed towards cell maintenance. Only at a hypothetical infinite growth rate, when the proportion of the total energy used for cell maintenance was zero, would the observed growth yield coefficient equal the true growth yield coefficient. He introduced and experimentally confirmed the following equation:

$$1/Y = 1/Y_G + m/\mu \qquad \text{I-1}$$

where Y is the observed growth yield coefficient (e.g. g cells.mol glucose^{-1}), Y_G is the true coefficient, m is the maintenance coefficient (e.g. mol glucose.g cells^{-1}.h^{-1}) and μ is the specific growth rate (h^{-1}). μ is related to the mean generation time, T, by the equation $\mu = \ln 2/T = 0.693/T$ (Hawker and Linton, 1971). Y and Y_G should be

corrected for the proportion of substrate carbon incorporated into cell material and not used for energy production. In view of the low Y_G values he obtained (e.g. for K. pneumoniae), Pirt (1965) probably did not make this correction (see also chapter IV Theory section).

Hadjipetrou et al. (1964) were the first workers to measure growth yield coefficients of aerobic bacteria with respect to oxygen (Y_O, g cells.g atom O^{-1}), but they failed to take account of the proportion of oxygen directed towards cell maintenance. Meyer and Jones (1973) pointed out that, for yields based on the consumption of oxygen rather than carbon source, equation I-1 becomes

$$1/Y_{O_2(obs)} = 1/Y_{O_2(true)} + m/\mu \qquad \text{I-2}$$

where $Y_{O_2(obs)}$ is the observed yield of cells on oxygen (g cells.mol O_2 consumed^{-1}), $Y_{O_2(true)}$ is the true yield and m is the maintenance respiration rate (mol O_2.g cells^{-1}.h^{-1}). Since m = M/N, where M is the maintenance coefficient with respect to ATP (mol ATP equivalents. g cells^{-1}.h^{-1}) and N is the efficiency of ATP synthesis from substrate (mol ATP equivalents.mol oxygen consumed^{-1}), from equation I-2 they derived

$$1/Y_{O_2(obs)} = 1/Y_{O_2(true)} + M/N\mu \qquad \text{I-3}$$

Herbert (1958) showed that, for aerobic microbial growth in continuous culture, the respiratory activity (QO_2, mol O_2 consumed.g cells^{-1}. h^{-1}) was made up of a term directly proportional to the growth rate and a constant term independent of the growth rate, i.e.

$$QO_2 = \alpha\mu + \beta \qquad \text{I-4}$$

where α and β are constants. The term β corresponds to the oxygen consumption for the supply of maintenance energy and is numerically equal to m in equation I-2. Expressing equation I-4 in terms of Y_{ATP} and M, Harrison and Loveless (1971) obtained the following equation:

$$QO_2 = {}^{\mu}/_{Y_{ATP.N}} + {}^{M}/_{N} \qquad \text{I-5}$$

Since $Y_{O_2(true)} = Y_{ATP.N}$ and $Y_{O_2(obs)} = \mu/QO_2$ (Harrison and Loveless, 1971), equation I-5 can also be easily derived from equation I-3.

Stouthamer and Bettenhaussen (1973) suggested that, for a single organism growing under defined culture conditions, Y_{ATP} was a variable which decreased as the growth rate decreased. They formulated a yield equation based on equation I-1 as follows:

$${}^{D}/_{Y_{ATP}} = {}^{D}/_{Y_{ATP}^{max}} + M \qquad \text{I-6}$$

where D was the dilution rate for continuous cultures (h^{-1}; numerically equal to μ, see Chapter IV), Y_{ATP} was the observed yield of organisms on ATP (g cells.mol ATP^{-1}, calculated from fermentation patterns of anaerobic bacteria) and Y_{ATP}^{max} was the yield corrected for energy of maintenance. The Y_{ATP} in this case should really be written $Y_{ATP(obs)}$ to distinguish it from the Y_{ATP} discussed above; the latter is numerically equal to Y_{ATP}^{max}. Equation I-6 can therefore be rewritten

$${}^{D}/_{Y_{ATP}(obs)} = {}^{D}/_{Y_{ATP}} + M \qquad \text{I-7}$$

or

$$^{1}/_{Y_{ATP(obs)}} \cdot N = {}^{1}Y_{ATP} \cdot N + {}^{M}/_{ND} \qquad I-8$$

now $Y_{ATP} \cdot N = Y_{O_2(true)}$, hence $Y_{ATP(obs)} \cdot N = Y_{O_2(obs)}$. Since $D = \mu$ for steady state continuous cultures, equation I-8 therefore reduces to equation I-3.

In the present work, the formulation of Harrison and Loveless (1971, equation I-5) was used to relate the consumption of oxygen by bacteria to their energy utilisation because the equipment employed measured QO_2 rather than $Y_{O_2(obs)}$.

5. The present work

The first part of the thesis (Chapter II) is concerned with the study of respiration and energy conservation in Azotobacter vinelandii, a bacterium which has the ability to fix atmospheric nitrogen. This obligate aerobe is known to have some peculiarities with regard to its respiratory system when grown under nitrogen-fixing conditions. An investigation of the properties of its respiratory system during growth in a medium supplemented with a source of combined nitrogen is described.

The second part of the thesis describes a study of the energy conservation efficiency of the respiratory chain of the aerobic bacterium Bacillus megaterium (Chapter III) and the development of a technique allowing the measurement of the growth yield with respect to ATP of the organism during chemostat culture (Chapter IV).

CHAPTER II

THE EFFECT OF A COMBINED NITROGEN SOURCE ON THE RESPIRATION AND ENERGY CONSERVATION OF AZOTOBACTER

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SUMMARY

1. Growth of the aerobic, nitrogen-fixing bacterium Azotobacter vinelandii in nitrogen-free medium (nitrogen-fixing conditions) was inhibited by high dissolved oxygen concentrations. There was no inhibition of growth in urea-containing medium (non-nitrogen-fixing conditions) presumably due to the absence of the oxygen-sensitive nitrogenase. Logarithmic growth of the organism in batch cultures was accompanied by a decrease in the respiratory activity (QO_2, mol O_2 consumed.g dry wt bacteria^{-1}.h^{-1}). This was probably in response to the decrease in dissolved oxygen concentration resulting from growth. The QO_2 of the urea cultures was lower than that of the nitrogen-free cultures at all regions of logarithmic growth.

2. The decrease in QO_2 during logarithmic growth was correlated with a simultaneous increase in the energy conserving efficiency of the respiratory chain occurring both at site I (NADH dehydrogenase) and on the oxygen side of the point of entry of reducing equivalents from malate.

3. The qualitative and quantitative cytochrome patterns of A. vinelandii grown in urea medium were very similar to those found for the organism grown in nitrogen-free medium (Ackrell and Jones, 1971b).

4. Respiratory membrane preparations of the organism grown in urea medium exhibited much lower NADPH oxidase activities than those reported for membranes derived from nitrogen-fixing cells (Ackrell et al. 1972). However, the allosteric kinetics of the NADPH dehydrogenase of the former membranes resembled those of the latter. There were some differences in the characteristics of inhibition by NAD^+ and adenine nucleotides.

5. The growth yield of A. vinelandii with respect to ATP (Y_{ATP}, g cells. mol ATP equivalents^{-1}) was much higher during growth on combined nitrogen medium than during growth on nitrogen-free medium, probably due to the energy requirements of nitrogen fixation.

6. In spite of its outstanding adaptations for growth in aerobic, nitrogen-deficient environments, A. vinelandii shows only a poor ability to modify its respiratory system for efficient growth on a source of combined nitrogen.

Introduction

1. The nitrogen-cycle

Nitrogen is an element which is recycled within ecosystems. In the perfect (closed) nitrogen cycle, green plants take up nitrogen from the soil chiefly in the form of nitrates, and convert it to organic nitrogen compounds. The plants may die or be eaten by animals, and dead organisms, together with waste and excretory products, are broken down by microbes which release the nitrogen as nitrates for further use. However, nitrates are continually lost from the soil due to leaching or convertion back to nitrogen gas by the action of denitrifying bacteria. Also, in agricultural ecosystems, where most of the above-ground parts of the plants are harvested and removed, the supply of dead organic matter to keep the cycle going is inadequate and the soils become depleted of nitrates (Varley, 1974). Although the weathering of rocks to release ammonia and the direct conversion of nitrogen gas in the atmosphere to nitrate (by the action of ultraviolet rays and lightning) contribute to a small extent to the replenishment of the fixed nitrogen content of soils, biological nitrogen fixation and the application of industrially-fixed nitrogen as fertilizers by man are most important in this respect.

It has been estimated (Dalton and Mortenson, 1972) that biological nitrogen fixation contributes nearly 5 times as much fixed nitrogen to the soil per annum as nitrogenous fertilizers. Most of the biological nitrogen fixation is attributed to the symbiotic nitrogen-fixing systems (involving bacteria) and the nitrogen-fixing systems of photosynthetic organisms (both blue-green algae and bacteria); free-living, non-photosynthetic nitrogen-fixing bacteria (e.g. Azotobacter

and Clostridium) seem to contribute little towards enriching the nitrogen content of soils (Jensen, 1965), although studies with such organisms have been rewarding in yielding information on the biochemistry of nitrogen fixation.

2. The distribution of nitrogen-fixing ability amongst free-living bacteria

Amongst the free-living bacteria the ability to fix nitrogen is more widespread in anaerobic than in aerobic organisms. Obligate anaerobes such as Clostridium pasteurianum, Chromatium, Desulphovibrio and Desulfotomaculum all include strains with the ability to fix nitrogen (Postgate, 1971). Members of the group able to grow both anaerobically and aerobically (the facultative anaerobes) can only fix nitrogen anaerobically; examples are Bacillus polymyxa, Bacillus macerans and Klebsiella pneumonia and, of the photosynthetic bacteria, Rhodospirillum. The Azotobacteriaceae comprise the most important family to fix nitrogen aerobically, i.e. Azotobacter, Azotomonas Beijerinckia and Derxia. There are very few organisms whose ability to grow aerobically by nitrogen fixation has been authenticated and which are not members of this family; such organisms include Mycobacterium flavum, Mycobacterium roseo-album and Mycobacterium azot-absorptum (Federov and Kalininskaya, 1961; L'vov and Lyubimov,. 1965; Biggins and Postgage, 1969). The introduction of the acetylene test (see below) has helped to eliminate several presumptive aerobic nitrogen fixers (Hill and Postgage, 1969).

3. The anaerobic nature of nitrogen fixation

The enzyme complex responsible for biological nitrogen fixation, nitrogenase, has the job of reducing the very stable $N \equiv N$ triple bond. The first reduction product which has been isolated is ammonia; although it has been postulated that N_2 may be reduced in two steps to diimide ($NH = NH$) and then to hydrazine ($NH_2 - NH_2$), no intermediates between N_2 and NH_3 have been detected. However, it is possible that the intermediates are enzyme-bound and that the final reduction product, ammonia, is released only after complete reduction of N_2 occurs (Dalton and Mortenson, 1972). Nitrogenase is also capable of reducing a number of non-physiological substrates (see Hardy and Burns, 1968). One of these, acetylene, is reduced to ethylene; the latter can be detected by gas chromatography thus forming the basis of a very sensitive assay for nitrogenase activity (Dilworth, 1966). Nitrogenases from both anaerobic and aerobic organisms have been purified to yield two, major, iron-sulphur-containing metalloprotein complexes, viz. azoferredoxin (M.W. approximately 50,000) and molybdoferredoxin (M.W. approximately 200,000; this component also contains molybdenum, see Dalton and Mortenson, 1972). The precise involvement of these subunits in the nitrogenase reaction is as yet unresolved, but the presence of both is required for the demonstration of nitrogen-fixation *in vitro* (Mortenson, 1965). Constituent proteins from different sources can in some cases cross-react and show restored enzymic activities; these cross-reactivities may be of evolutionary significance (see e.g. Murphy and Koch, 1971).

There is considerable evidence that the reductive process of nitrogen fixation requires anaerobic conditions. Both component proteins

from nitrogenase are oxygen sensitive, azoferredoxin particularly so (Bulen and Le Comte, 1966; Kelly, 1969). The requirements for enzymic activity *in vitro* are a source of ATP, a reductant and an anaerobic environment (Mortenson, 1964; Bulen *et al.*, 1964, 1965). Crude preparations which show nitrogenase activity in the presence of oxygen have been obtained from aerobic nitrogen fixers (Bergersen, 1966a,b; Stewart *et al.*, 1969; Yates and Daniel, 1970) but these results can be explained in terms of a particulate or compartmentalised nitrogenase protected from exposure to oxygen by respiration (Yates and Jones, 1974; see below). Growth of the aerobic nitrogen fixing bacteria, *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Azotomonas macrocytogenes* (Dalton and Postgate, 1969a; Drozd and Postgate, 1970a, b), *Mycobacterium flavum* 301 (Biggins and Postgate, 1969) and *Derxia gummosa* (Hill, 1972), is inhibited by excess oxygen when they are fixing nitrogen but not when they are supplied with a source of combined nitrogen. The acetylene test revealed that the nitrogenase was inactive under these conditions (Yates, 1970a; Drozd and Postgate, 1970a, b; Hill, 1972). From the above evidence it is reasonable to assume that nitrogen fixation is an anaerobic process catalysed by oxygen-sensitive proteins.

4. Protection of nitrogenase from oxygen in aerobic bacteria

The problem of how, in the obligately aerobic nitrogen fixers, the intracellular oxygen concentration is maintained at a level that is low enough to allow the nitrogenase to function and yet high enough to allow rapid cytochrome oxidase activity, and hence ATP synthesis and growth, has been the subject of much recent research. Postgate and his

coworkers have suggested two mechanisms by which Azotobacter, on being exposed to high oxygen concentrations, may (a) protect the nitrogenase from damage by oxygen (conformational protection) and (b) lower the intracellular oxygen concentration sufficiently to allow the nitrogenase to function and hence allow growth to resume (respiratory protection; see Dalton and Postgate, 1969a).

(a) Conformational protection As the nitrogenase in crude cell-free extracts from Azotobacter is oxygen-tolerant whereas the purified nitrogenase fractions are oxygen-sensitive (Kelly, 1969), it follows that the nitrogenase is protected from oxygen damage in crude extracts by being maintained in a conformational form in which it is oxygen tolerant. It is likely that nitrogenase is physically protected from oxygen damage in vivo since, on changing culture conditions from high to low oxygen solution rate, acetylene-reducing ability can "switch-on" immediately or after a short time lag, indicating that the functioning of the nitrogenase is very quickly restored (Yates, 1970a; Drozd and Postgate, 1970a, b). The failure of chloramphenicol to inhibit this "switch-on" process indicates that it is not associated with nitrogenase synthesis (Drozd and Postgate, 1970b).

Azotobacter nitrogenase is particulate in crude extracts (Bulen et al., 1964) whereas that of C. pasteurianum (an anaerobe) is soluble (Carnahan et al., 1960). Oppenheim and Marcus (1970) showed that nitrogen-fixing A. vinelandii had an extensive internal membrane network which was absent in ammonia-grown organisms. These observations, together with other evidence, suggested that the nitrogenase of Azotobacter is associated with membrane fractions which serve to protect it from oxygen damage by their close physical association and also by

their respiratory activity (see below). Conformational protection is envisaged, therefore, as being a reversible process, probably occurring *via* a rapid conformational change within the nitrogenase complex which causes the oxygen-sensitive nitrogen-fixing sites to become inaccessible to oxygen.

(b) *Respiratory protection* The growth and nitrogen-fixing efficiencies (expressed as yield.g carbon substrate^{-1}) of *A. chroococcum* in continuous culture are inversely proportional to the dissolved oxygen concentration (Dalton and Postgate, 1969a, b). These observations suggest that exposure to excess oxygen causes the organism to waste carbon source. Such wastage is also manifested by an increased respiratory activity (QO_2) of the cultures (Drozd and Postgate, 1970b). Nutrient conditions which prohibit the QO_2 increase (such as carbon-limitation) also result indamage to the nitrogenase system (Lees and Postgate, 1973). Enhanced QO_2 values have also been observed for *A. vinelandii* in nitrogen-free batch cultures on increasing the dissolved oxygen concentration by increasing the aeration rate (Jones *et al.*, 1973).

The phenomenon of very high respiratory activities for which the Azotobacteriaceae are famous (Williams and Wilson, 1954) is consequently thought to be the mechanism by which the organisms remove excess oxygen from the intracellular environment, hence allowing the reversal of the conformation protection process and nitrogen fixation and growth to proceed. Such enhancement of respiratory activity is known as respiratory protection and is discussed in greater detail in section 7 of this Introduction.

5. The respiratory system of Azotobacter

Extensive studies of the respiratory system of Azotobacter have allowed the mechanisms of the respiratory protection response to be largely elucidated. The respiratory system of Azotobacter growing on a source of atmospheric nitrogen is readily isolated in particulate form by disrupting whole cells (Alexander and Wilson, 1955). Such respiratory membranes exhibit a complex pattern of respiratory enzymes and carriers comprising several highly active dehydrogenases, a pyridine nucleotide transhydrogenase, ubiquinone and a complex cytochrome system terminated by three cytochrome oxidases.

(a) Dehydrogenases Unsupplemented respiratory membranes from A. vinelandii catalyse the oxidation of a number of substrates, viz. NADH, NADPH, succinate, malate and lactate (see Yates and Jones, 1974). Particulate oxidation of the latter three compounds is pyridine nucleotide independent. In terms of specific activity, the two most important respiratory chain dehydrogenases are those catalysing the oxidation of NADH and NADPH. Whereas respiratory membranes exhibit simple hyperbolic saturation kinetics with respect to NADH oxidation (K_m = 40 μM, Eilermann et al., 1970), sigmoidal saturation kinetics with a relatively poor affinity for the substrate have been found for NADPH oxidation ($S_{0.5}$ = 440 μM NADPH; Ackrell et al., 1972).

The NADPH dehydrogenase was also found to be inhibited by adenine nucleotides (pure competitive, AMP> ADP> ATP) and by NAD^+ (partially competitive, possibly allosteric inhibition; Ackrell et al., 1972). In spite of their extremely high specific activities, the NADH and NADPH dehydrogenases are the rate-limiting components of their respective oxidases (Jones et al., 1971a,b; Ackrell et al., 1972).

The NADH dehydrogenase has been partially purified and shown to contain an iron-sulphur protein and FMN (Der Vartanian, 1972) and the participation of iron in electron transport has been demonstrated by inhibitor studies during oxidation of NADPH and malate, as well as NADH (Jones et al., 1972).

(b) Pyridine nucleotide transhydrogenase The reversible pyridine nucleotide transhydrogenase which occurs in nitrogen-fixing Azotobacter (Kaplan et al., 1953) has been purified (Chung, 1970; van den Broek et al., 1971a) and shown to be a very high molecular weight enzyme containing FAD (van den Broek et al., 1971b). Two binding sites for NADPH, one catalytic and one regulatory, are present on the enzyme; ATP and possibly NAD^+ act as negative effectors on the regulatory site (van den Broek and Veeger, 1968).

(c) Cytochromes Reduced minus oxidised difference spectra of respiratory membrane preparations from nitrogen-fixing Azotobacter revealed the presence of b and c type cytochromes together with cytochrome a_2 (Smith, 1954) confirming earlier investigations by Negelein and Gerischer (1934). Reduced plus carbon monoxide minus reduced difference spectra further indicated that cytochromes a_1 and o were present (Caster and Chance, 1959). Photochemical action spectra of whole cells oxidising endogenous substrates have subsequently shown that cytochromes a_1, a_2 (also known as cytochrome d) and o can all act as terminal oxidases but that under these conditions, a_2 is of major importance (Caster and Chance, 1959; Jones and Redfearn, 1967a). The c-type cytochrome has been purified to yield two major components, cytochromes c_4 and c_5 (Tissières, 1956) which, in their reduced forms,

are readily oxidised by A. vinelandii respiratory membranes. Following a detailed investigation of the respiratory system of A. vinelandii using dual-wavelength spectrophotometry and cytochrome oxidase inhibitors, Jones and Redfearn (1967a) postulated the presence of a branched cytochrome system in the terminal regions of electron transfer. These workers suggested that the branch occurred at the level of ubiquinone to yield a major terminal pathway ($\underline{b} \longrightarrow \underline{a}_2$) and a minor terminal pathway ($\underline{c}_4 + \underline{c}_5 \longrightarrow \underline{a}_1/\underline{o}$). Subsequent investigators (Jurtshuk et al., 1969; Henderson, 1973) have presented evidence that the branch occurs at the level of cytochrome b (Fig. II-1).

(d) Quinones Azotobacter contains ubiquinone (Q-8; Temperli and Wilson, 1960; Jones and Redfearn, 1966) which has been found essential for NADH oxidase activity (Swank and Burris, 1969). It is probably, therefore, a main-chain carrier (Fig. II-1).

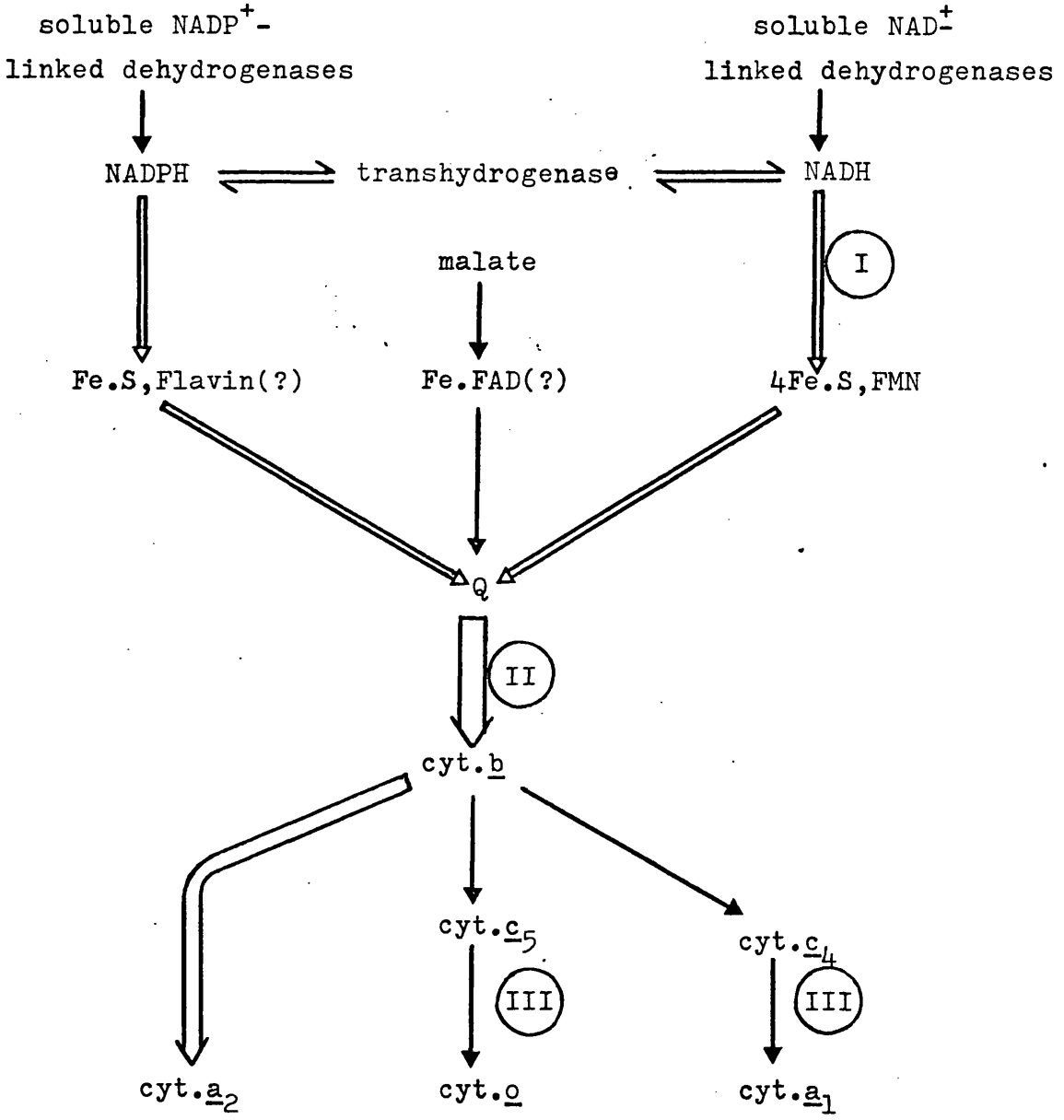
6. Energy conservation in Azotobacter

A number of workers have observed oxidative phosphorylation in particulate preparations from nitrogen-fixing Azotobacter (e.g. Hyndman et al., 1953; Tissières and Slater, 1955). The presence of phosphorylation at the level of NADH dehydrogenase (site I) and on the oxygen side of ubiquinone (site II) was first demonstrated by Eilermann et al., (1970) and Ackrell and Jones (1971a) detected the presence of a third phosphorylation site (III) of rather lower efficiency than sites I and II on the minor branch between the c-type cytochromes (probably $\underline{c}_5$; however, see below) and molecular oxygen (Fig. II-1).

Measurement of the rates of ATP synthesis and oxygen consumption

Fig. II-1

The respiratory system of A.vinelandii



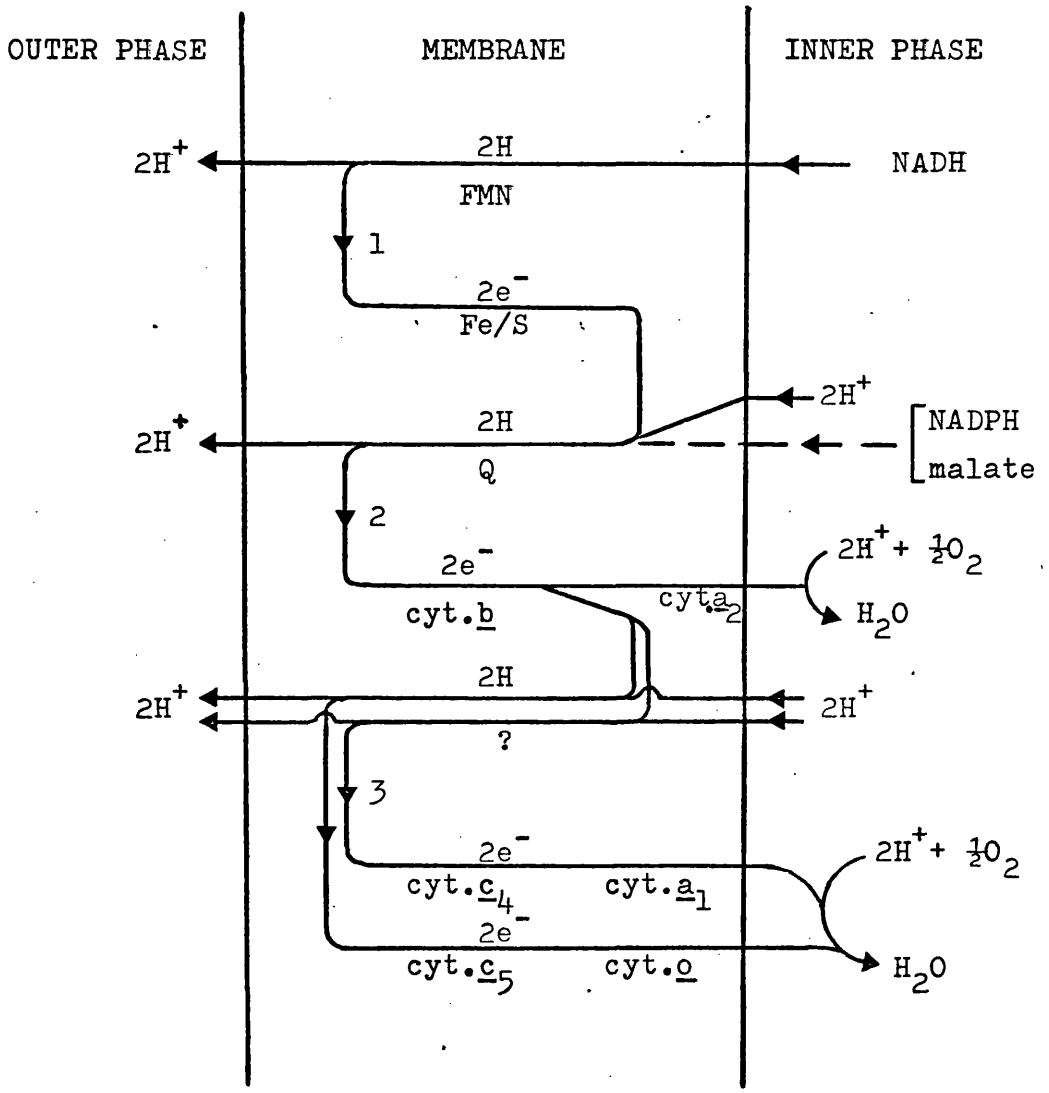
Phosphorylation sites are designated I,II,III and the approximate rates of electron transfer are denoted by the thickness of the arrows. The scheme has been demonstrated for the organism cultured under nitrogen-fixing conditions only (based on Jones & Redfearn,1967a)

in whole cells of A. vinelandii grown under nitrogen-fixing conditions yielded P/O ratios of approximately 2 for the NAD^+-linked oxidation of β-hydroxybutyrate (Knowles and Smith, 1970) and approximately 3 for oxidation of NADH (Baak and Postma, 1971). However, the latter value is not compatible with the observation of a maximum $\longrightarrow H^+/O$ quotient (Mitchell, 1966, 1967) of 3.5 obtained for whole cells of nitrogen-free-grown A. vinelandii oxidising endogenous substrates (Yates and Jones, 1974). If, as is implicit in the chemiosmotic theory for oxidative phosphorylation (Mitchell, 1966, 1967), the $\longrightarrow H^+/O$ quotient reflects the efficiency of oxidative phosphorylation (being the product of the P/O and $\longrightarrow H^+/P$ ratios), then the observed $\longrightarrow H^+/O$ quotient for Azotobacter was equivalent to a P/O of 1.75 (assuming $\longrightarrow H^+/P = 2.0$, as for mitochondria; Mitchell and Moyle, 1967b). Recent work by Dr. C.W. Jones (personal communication) indicates that proton translocation by whole cells of A. vinelandii, grown under nitrogen-fixing conditions, is associated with electron transport by both species of cytochrome c. Hence it is likely that both cytochromes c_4 and c_5 are involved in energy conservation at site III (i.e. loop 3). Fig. II-2 shows the respiratory chain components of Azotobacter arranged in accordance with the chemiosmotic hypothesis in a manner which is compatible with the terminal branches $b \longrightarrow a_2$ and $b \longrightarrow c \longrightarrow a_1/o$ having different efficiencies of energy conservation.

Indirect estimates of in vivo P/O ratios following measurement of growth efficiencies with respect to oxygen (Y_{O_2}; g cells.mol O_2 consumed^{-1}) gave values which were much lower than those obtained using the above direct methods (P/O $\leq$ 0.6, Nagai et al., 1969). However, the calculations were based on the possibly erroneous assumption that the

Fig. II-2

The respiratory system of A.vinelandii in relation to the chemiosmotic hypothesis



A scheme showing the possible arrangement in the plasma membrane of the respiratory carriers of nitrogen-fixing A.vinelandii which accounts for the observed respiration-induced proton translocation of whole cells of this organism(Yates & Jones,1974). The diagram shows the arrangement of all three cytochrome branches; only one branch would presumably be present in a single respiratory chain unit.(c.f. Fig.I-2).

yield of cells with respect to ATP (Y_{ATP}, g cells per mol ATP used for growth) was the same as for a number of other organisms, viz. 10.6 g cells.mol ATP^{-1} (Bauchop and Elsden, 1960; Forrest and Walker, 1971). Nagai et al. (1969) suggested that the Y_{ATP} for Azotobacter may be considerably less than this figure, due to the energy requirements of nitrogen fixation and respiratory protection. If this is true, the P/O ratio would be considerably higher than 0.6.

7. Respiratory Protection in Azotobacter

A number of changes occur in the respiratory system of Azotobacter coinciding with the increase in respiratory activity which accompanies an increase in the dissolved oxygen concentration of nitrogen fixing cultures. Thus there is an increase in the concentration of cytochrome a_2 (Drozd and Postgate, 1970b) which occurs by de novo synthesis (Jones et al., 1973). There is also a sharp decrease in energy conservation efficiency, particularly at site I, together with de novo synthesis of extra NADH and NADPH dehydrogenases (Jones et al., 1973). On the other hand, when the dissolved oxygen concentration of nitrogen fixing batch cultures of A. vinelandii fell from approximately saturating (222 μM) to nearly zero (< 10 μM) due to growth of the organism, there was a concomitant decrease in the concentration of cytochrome a_2 relative to the other oxidases and an increase in the P/O ratio, mainly as a result of an increase in phosphorylating efficiency at site I (Ackrell and Jones, 1971b). These observations appeared to signify a direct reversal of the respiratory protection procedure, under conditions of decreasing oxygen concentration.

It has consequently been proposed (Ackrell and Jones, 1971b; Ackrell et al., 1972; Jones et al., 1973) that, under highly aerobic conditions respiration of low energy conservation efficiency is promoted via an uncoupled NADH dehydrogenase and the non-phosphorylating branch of the cytochrome system terminated by cytochrome a_2. When the nitrogenase is "switched off" under conditions of very high oxygen concentrations, respiration may be augmented by the oxidation of NADPH (probably a primary donor in electron transport to nitrogenase in A. vinelandii; see Benemann et al., 1971) via the non-phosphorylating NADPH dehydrogenase (Erickson et al., 1972).

As the respiratory activity of bacterial cultures, to an approximation, is inversely proportional to the phosphorylating efficiency (Harrison and Loveless, 1971) it is likely that a large part of the increased respiratory activity exhibited by Azotobacter cultures subjected to oxygen stress is a direct consequence of the overall decrease in the P/O ratio brought about by the utilisation of pathways of electron transport of low energy conservation efficiency (Jones et al., 1973; Yates and Jones, 1974; see also Results and Discussion).

8. Properties of Azotobacter grown on a source of combined nitrogen

Wilson et al. (1943) showed that virtually all of the cell nitrogen of A. vinelandii was derived from the medium nitrogen when the organism was grown in urea-containing cultures. Nitrogen-fixing bacteria grown in the presence of a source of combined nitrogen, e.g.

ammonia, nitrate or urea, have long been known to be deficient in nitrogenase activity (Wilson, 1958). Circumstantial evidence that nitrogenase synthesis is a derepression and not an induction phenomenon has been presented for a number of nitrogen-fixing organisms including A. chroococcum (Dalton and Postgate, 1969 b). Brief studies on the respiratory system of Azotobacter by Lisenkova and Khmel (1967) and Dalton and Postgate (1969b) during growth on various sources of combined nitrogen indicated that it was not significantly different from that of nitrogen-fixing cells. However, Knowles and Redfearn (1968) reported that respiratory membranes prepared from A. vinelandii grown in urea-containing medium had depressed levels of cytochrome a_2 compared with membranes obtained from cells growing under nitrogen-fixing conditions or growing in medium containing glutamate or ammonium sulphate. The membranes prepared from nitrogen-free, glutamate- and ammonium sulphate-grown cells all contained similar levels of cytochromes c, b, a_1 and a_2. During growth on urea medium under an oxygen atmosphere no cytochromes c could be detected and the concentrations of the other cytochromes and ubiquinone were only 12-18% of those found for growth on nitrogen-free medium under high aeration conditions (Knowles and Redfearn, 1969). Drozd and Postgate (1970b), who utilised continuous culture maintained at a constant pO_2, did not find lower cytochrome a_2 concentrations in A. chroococcum growing in urea-containing medium compared with cells grown in nitrogen-free medium. This suggested that the differences in cytochrome a_2 concentration reported for A. vinelandii might have been due to different ambient oxygen concentrations in the nitrogen-free and urea-containing batch cultures.

9. The present work

Azotobacter seems well adapted to growing under conditions in which one obligatory growth nutrient (oxygen) controls the activity of an enzyme system (nitrogenase) that is itself obligatory for growth on atmospheric nitrogen. Thus, when the organism is subjected to oxygen stress (providing the ambient oxygen concentration is not too high; Dalton and Postgate, 1969b) conformational changes in the nitrogenase complex probably protect the enzyme from irreversible damage by oxygen while the respiratory protection response lowers the ambient oxygen concentration sufficiently to allow the nitrogenase to become reactivated and hence growth to resume.

The complex, branched cytochrome system of Azotobacter growing under nitrogen-fixing conditions is clearly capable of allowing wide variation in the energy conservation efficiency according to the prevailing oxygen concentration and the exact pathway of electron transfer employed. Thus, under conditions of high oxygen concentration the energy conserving efficiency is very low, eliciting respiratory protection, while under low oxygen concentrations, when there is a limited availability of electron acceptor, it is considerably higher.

When the organism is grown under conditions in which it contains no nitrogenase (i.e. during growth on a source of combined nitrogen), it might be anticipated that the respiratory system become adapted to give a high efficiency of energy conservation regardless of the oxygen status of the culture, since the respiratory protection response would no longer be required. This chapter describes experiments designed to answer the following questions. Does A. vinelandii exhibit an enhanced respiratory activity under excess

oxygen conditions during growth on combined nitrogen medium? Does the cytochrome system of cells grown on a fixed nitrogen source differ from that of cells grown under nitrogen-fixing conditions? Does the energy conserving efficiency differ? Lastly, do the properties of the NADPH dehydrogenase of fixed-nitrogen-grown cells differ from those of the NADPH dehydrogenase of nitrogen-free-grown cells (this enzyme probably contributes in part to respiratory protection in nitrogen-fixing cells)?

Materials and Methods

1. Culture of Azotobacter

Azotobacter vinelandii (N.C.I.B. 8660) was maintained on slopes containing nitrogen-free medium consisting of 822 mg Na_2HPO_4; 186 mg KH_2PO_4; 200 mg $MgSO_4.7H_2O$; 50 mg $CaCl_2$; 5 mg $NaMoO_4$; 140 mg $FeCl_3.6H_2O$; 15 g mannitol per litre, solidified with 2% w/v agar (Oxoid) and sterilised by autoclaving at 15 lb.in^2 for 15 min. The organism was subcultured onto fresh slopes at monthly intervals and, after 2 days' growth at 30°, was stored at 4°. For growth in liquid medium, loopsful of the bacterium from a slope were inoculated into 250 ml conical flasks, each containing 150 ml of the above medium. The cultures were then grown aerobically at 30° on a gyratory incubator. After approximately 18 h growth a standard inoculum (2.5 A_{680} units. 100 ml^{-1} growth medium; i.e. an initial concentration of 10 μg dry wt cells.ml^{-1}) was used for initiating growth in 2 l baffled conical flasks containing either 600 or 1500 ml medium. The gyratory incubator furnished the three aeration conditions used, viz. extra-high aeration (600 ml medium, oxygen transfer coefficient, K_LA = 300 x 10^{-6} mol O_2. $l^{-1}.h^{-1}.mm\ Hg^{-1}$), high aeration (600 ml medium, K_LA = 89.3 x 10^{-6}) and low aeration (1500 ml medium, K_LA = 1.41 x 10^{-6}). Oxygen-limited cells were usually obtained by allowing high aeration cultures to grow beyond the excess oxygen (logarithmic) growth phase. Occasionally low aeration conditions were used in order to obtain oxygen-limited cultures.

For growth on a source of combined nitrogen, 1.2 g.l^{-1} urea were added to the growth medium prior to inoculation. Inocula for fixed nitrogen experiments were always obtained from urea starter cultures. The latter were inoculated fresh from a slope to avoid

contamination, which the urea cultures were particularly susceptible to.

2. Techniques used for whole-cell experiments

(a) Measurement of growth characteristics Growth in batch cultures was following by monitoring the net absorbance at 680 nm and plotting as log A_{680} *versus* time. Culture densities (g dry wt.bacteria. l^{-1}) could be obtained by reference to a standard curve relating A_{680} to organism concentration. The dissolved oxygen concentration was measured using a polarographic Clark electrode, YSI 4004 (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA), inserted into the shaker flask *via* a Quickfit sidearm close to the base. The electrode was calibrated on uninoculated medium at 30° under the shaking conditions to be employed for the experiment. Although membrane electrodes (including the non-polarographic Mackereth type) strictly record the partial pressure of oxygen rather than the oxygen concentration (Siegell and Gaden, 1962; MacLennan and Pirt, 1966), the two were assumed to be equivalent for the purposes of the present work. Fresh medium at 30° was assumed to have an oxygen concentration of 222 μM (Chappell, 1964; equivalent to a partial pressure of 159 mm Hg). The dissolved oxygen concentration of growing cultures was continuously monitored using a recorder on the slowest chart-speed setting (30 $mm.h^{-1}$).

The respiratory activity of growing cultures was measured by rapidly transferring 2.0 ml samples from the culture flasks (or smaller samples diluted with uninoculated growth medium) into the reaction chamber of a pre-calibrated Clark electrode at 30°. Oxygen consumption was linear with respect to time for several minutes after sampling. QO_2 values (mol O_2.g $cells^{-1}.h^{-1}$) were then calculated after measurements

of culture density. These QO_2 values are defined as the 'potential' QO_2 (Harrison and Loveless, 1971), but when sampled from excess oxygen (> 10 μM), logarithmic phase cultures, the potential QO_2 was equal to the '*in situ*' QO_2. When sampled from oxygen-limited cultures, the potential QO_2 was greater than the *in situ* QO_2.

The pH of growth samples was measured using a precalibrated Pye-Unicam (model 290) pH meter.

(b) *Measurement of* $\longrightarrow H^+/O$ *quotients* Cells at the required stage of growth in batch culture were harvested by centrifugation at 11,000 x g for 10 min, washed in 140 mM KCl *plus* 1 mM Tris-HCl buffer pH 7.0, re-centrifuged and the pellet resuspended in the same very weak buffer. An apparatus similar to that described by Pressman (1967) was used for the measurement of small pH changes in the bacterial suspension to a high degree of sensitivity (see Mitchell and Moyle, 1967a). This consisted of a Rank (Cambridge) oxygen electrode of 6 ml capacity which, although not wired to measure oxygen concentrations, acted as an ideal temperature-controlled (30°) chamber for pH determinations performed by means of a Pye-Unicam (Cambridge) M5/E7 combination pH microelectrode in conjunction with a Pye-Unicam (model 290) pH meter, linked to a recorder. The cell suspension was added to the reaction chamber to give a density of 6 mg dry wt.ml^{-1} and a square of parafilm was carefully used to seal the top of the chamber except for a small slit through which a micro-syringe could be inserted. The cell suspension was allowed to become anaerobic by endogenous respiration and the pH was adjusted, when necessary, to 6.8 using 0.01 N NaOH. Addition of oxygen as air-saturation 140 mM KCl (30°) resulted in acidification of the medium due to respiration-

induced translocation of protons by the cells. Approximately 10 aliquots of KCl were added in the range 5-50 µl and the oxygen concentration in each was calculated from the data of Chappell (1964). The pH was maintained fairly constant by the addition of 5-20 µl 0.01 N NaOH; the latter also served to calibrate the oxygen-induced acidification. $\longrightarrow H^+/O$ quotients were expressed as g ions H^+ translocated per gatom O reduced.

In order to determine $\longrightarrow H^+/O$ quotients accurately and reproducibly on A.vinelandii, the conditions of assay were first carefully standardised. Particular attention was paid to the following assay conditions:

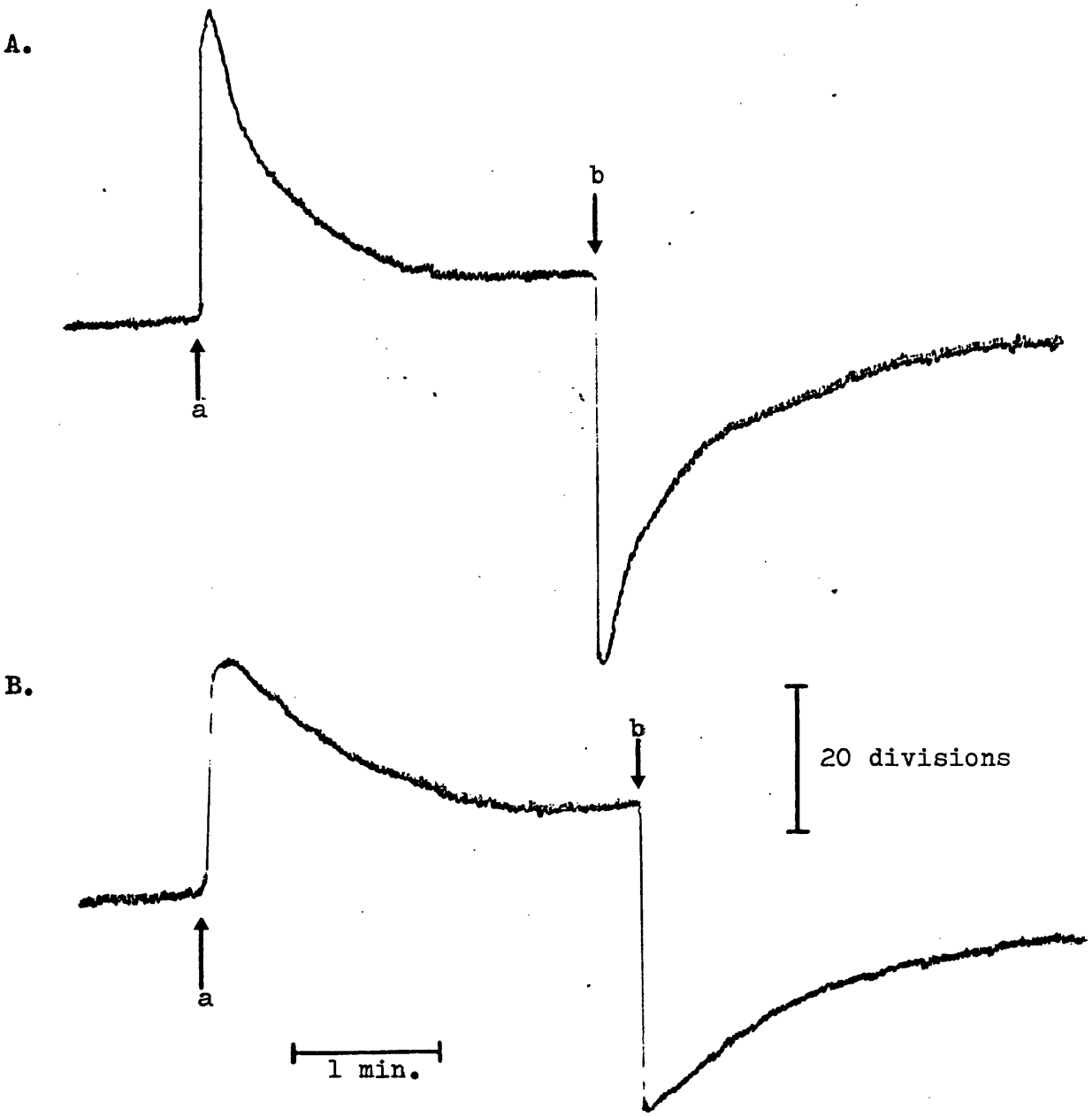
(i) Presence of carbonic anhydrase

The left hand part of Fig. II-3A shows the time-course of the observed outer pH change when an anaerobic suspension of nitrogen-free-grown A. vinelandii in an assay medium containing 100 mM KSCN (see below) was pulsed with a small quantity of oxygen. The medium was very quickly acidified due to respiration-induced proton translocation but there was also a subsequent rapid decay of the trace ($t_{\frac{1}{2}}$ of decay $\ll$ 30 sec). In the presence of 100 µg.ml^{-1} carbonic anhydrase (Scholes and Mitchell, 1970) there was a similar, rapid acidification of the medium on adding the oxygen but the decay rate of the trace was much slower ($t_{\frac{1}{2}} > 60$ sec; Fig. II-3B). The peak height was smaller than the corresponding pulse in the absence of carbonic anhydrase; this was presumably due to the almost instantaneous equilibration of the translocated protons with bicarbonate ions induced by the carbonic anhydrase:

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

Fig. II-3

Respiration-induced proton translocation in A.vinelandii; recorder traces



A.vinelandii was grown under high aeration conditions in nitrogen-free medium and harvested at A_{680}=1.5 (i.e. just oxygen-limited). Conditions in the electrode chamber ; 100mM KSCN, 6mg dry wt.cells. ml^{-1}. The figure shows the actual recorder traces on rapid addition to the anaerobic cell suspension of a) 22.2ng atom oxygen and b) 80.0 nmol. OH^-. A, no carbonic anhydrase present; B, 100 $\mu g.ml^{-1}$ carbonic anhydrase.

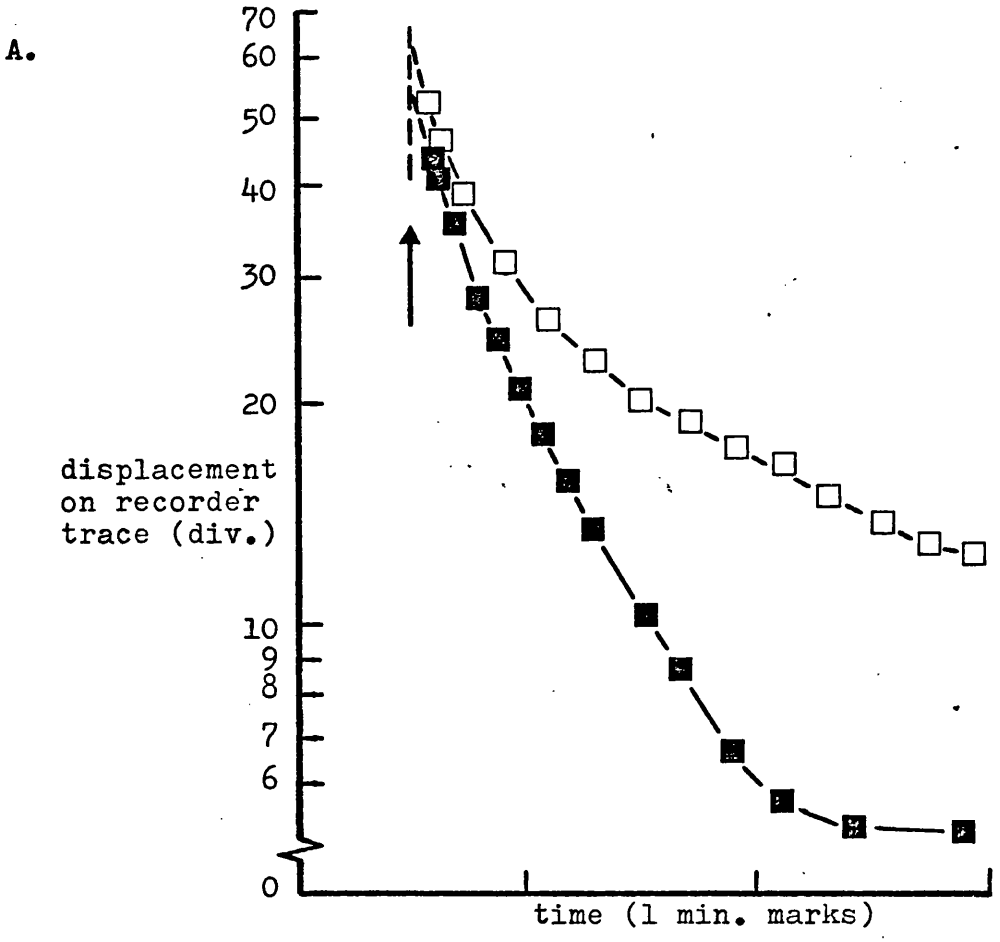
The subsequent decay of the proton pulse was probably due to the re-entry of the protons into the cell either by 'leakiness' or via the ATPase system. The rapid decay of the minus carbonic anhydrase pulse, however, was probably due to both a relatively slow reaction of the protons with bicarbonate and also to re-entry of protons into the cell.

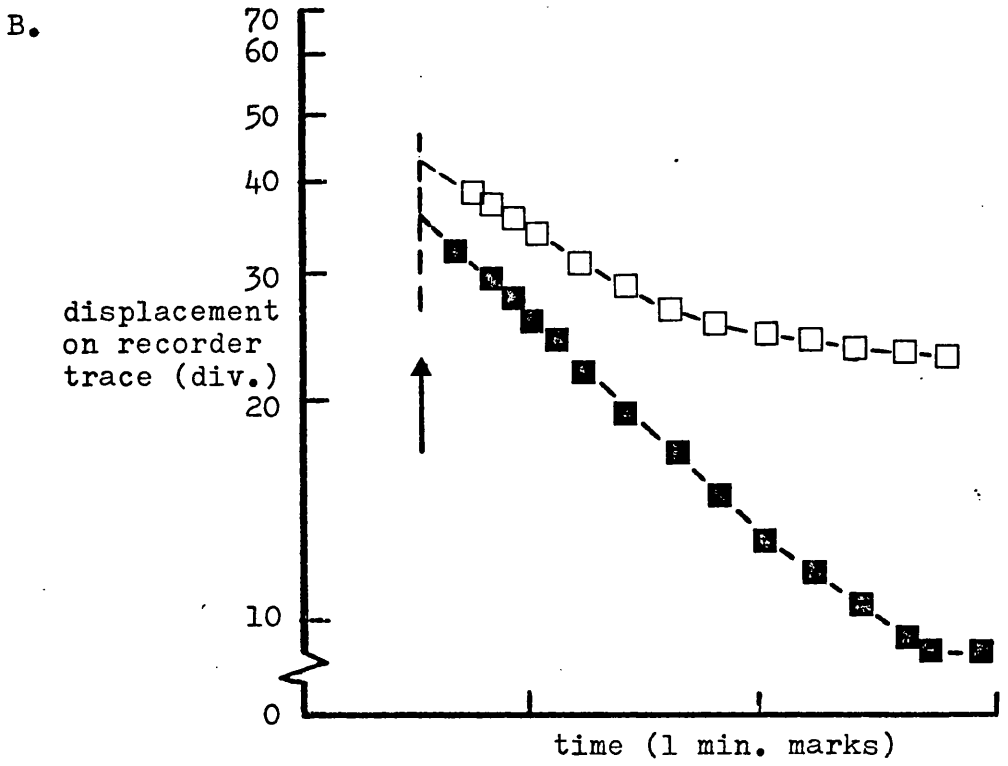
The characteristics of decay of the pH changes caused by additions of the anaerobic NaOH used for calibration purposes were similar to those of the oxygen pulses and likewise depended on the presence or absence of carbonic anhydrase (see right hand side of Fig. II-3).

Semi-logarithmic plots of the decay of the oxygen- and hydroxide-induced traces with respect to time are shown in Fig. II-4. Whereas the decay of the pulses in the absence of carbonic anhydrase was not exponential at any time (Fig. II-4A), the decay in the presence of this enzyme was exponential for > 1 min following its onset (Fig. II-4B). However, in no case did the traces return to the baseline, a situation which has also been reported for M. denitrificans (Scholes and Mitchell, 1970). The hydroxide calibration pulses decayed even less than the oxygen pulses, for reasons which are unclear. It was found to be very difficult to extrapolate traces minus carbonic anhydrase to zero time (assumed to be the same as $t_{\frac{1}{2}}$ of reduction of oxygen; see Scholes and Mitchell, 1970) because of the non-exponential decay. $\longrightarrow H^+/O$ quotients calculated for such experiments varied depending on whether extrapolations were performed directly on the recorder tracings or on the semi-log.plots (Table II-1). This uncertainty about the true $\longrightarrow H^+/O$ was not encountered with the plus carbonic anhydrase assays, however, as $\longrightarrow H^+/O$ quotients calculated directly from the traces and

Fig. II-4

Decay characteristics of the pulses shown in Fig. II-3





Decay of the pulses in Fig. II-3 relative to the baseline and with respect to time. 22.2 ngatom oxygen(■) or 80 nmole OH^-(□) added at the arrows. A, no carbonic anhydrase; B, 100 µg.ml c.anhyd.

Table II-1

Calculation of → H^+/O ratios from the data in Figs. II-3&4

	no carbonic anhydrase		100µg.ml^{-1} carbonic anhydrase	
	direct extrapolation from trace	extrapolation from semi-log. plot	direct extrapolation from trace	extrapolation from semi log. plot
acidification of medium due to addn. of 22.2ngatom oxygen (div.)	50.0	54.0	34.8	35.6
alkalination of medium due to addition of 80.0nmol.OH^- (div.)	61.3	61.0	41.5	42.5
→ H^+/O ratio (gion H^+.gatom oxygen)	2.96	3.18	3.02	3.02

from the semi-log. plots were the same (Table II-1). This was found to be the the case for A. vinelandii grown in both urea medium and nitrogen-free medium and also for B. megaterium (see Chapter III). 100 $\mu g.ml^{-1}$ carbonic anhydrase was therefore used routinely for all measurements of $\longrightarrow H^+/O$ ratios.

(ii) Presence of potassium thiocyanate

During respiration, the respiratory chain transports protons from the inside of the bacterial plasma membrane to the outside, thus generating a pH and electrical gradient across the membrane (see General Introduction). Thus the energy of the oxidoreductions is conserved, according to the chemiosmotic hypothesis, as a 'protonmotive force' (pmf, Mitchell, 1966) across the membrane such that

$$pmf = \Delta\psi - \frac{2.303\ R.T}{f}.\Delta pH$$

where $\Delta\psi$ = trans-membrane electrical differential and ΔpH = trans-membrane pH difference (outside pH minus inside pH, hence sign negative). In order to measure the pmf (as an $\longrightarrow H^+/O$ ratio) of the Azotobacter cells it was necessary to convert the $\Delta\psi$ portion of the pmf into a ΔpH, which could be measured. This was achieved by incubating the cells in the pH measuring chamber with potassium thiocyanate for 15 min prior to measurement of $\longrightarrow H^+/O$. ratios. The thiocyanate anions equilibrated across the bacterial plasma membrane, in which they are soluble (Mitchell, 1972), and on addition of an oxygen pulse returned to the outside medium along with translocated protons thus destroying the membrane electrical potential and allowing the maximum $\longrightarrow H^+/O$ ratio to be obtained. 100 mM KSCN was found to elicit the maximum stoichiometry for both

nitrogen-free-grown cells (Fig. II-5) and urea-grown cells (not shown). Potassium thiocyanate was chosen as a membrane potential-collapsing agent rather than valinomycin since Scholes and Mitchell (1970) obtained the highest $\longrightarrow H^+/O$ quotients for M. denitrificans at neutral pH's when using the former compound.

(iii) Cell density and pH

Variations in cell density between 6-24 mg dry wt.ml^{-1} produced a negligible change in the $\longrightarrow H^+/O$ ratio (Fig. II-6A) so a cell density of 6 mg dry wt.ml^{-1} was routinely used in the measuring chamber. Varying the initial pH in the assay chamber between 6.5 - 7.3 also had only a small effect on the $\longrightarrow H^+/O$ quotient (Fig. II-6B). However, to standardise the assay conditions, a pH of 6.7 - 6.8 was always achieved before measurements by the addition of small amounts of NaOH or HCl, if necessary. Cells grown in urea medium behaved identically to the nitrogen-free-grown cells on changing the cell density and pH of the assay medium.

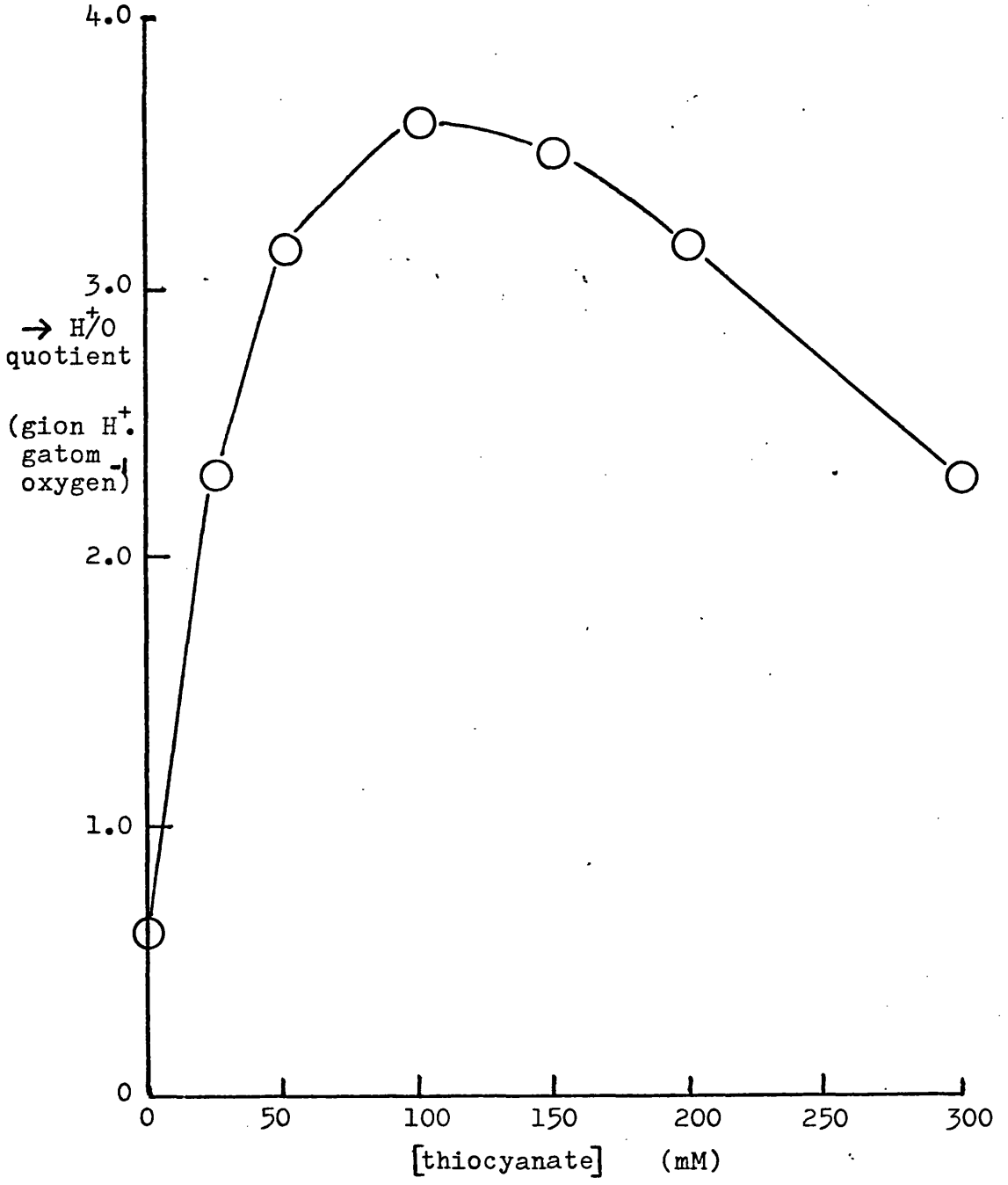
The change in the outer pH of the cell suspensions on pulsing with oxygen was virtually abolished in the presence of low concentrations (15 μM) of the uncoupler TCS.

3. Techniques used for sub-cellular experiments

(a) Preparation of respiratory membranes Cells were harvested at the desired phase of batch growth by centrifugation at 11,000 x g for 10 min. Preparation of phosphorylating respiratory membranes was by the method of Ackrell and Jones (1971a). Unwashed, packed cells were resuspended in freshly prepared 10 mM PIPES buffer

Fig. II-5

→ H^+/O quotients of A.vinelandii in the presence of potassium thiocyanate



A.vinelandii was grown under high aeration conditions in nitrogen-free medium and harvested in the oxygen-limited phase of growth (A_{680}=2.2). Conditions of assay of → H^+/O quotients; 6mg dry wt. cells ml^{-1}, 100µg.ml^{-1} carbonic anhydrase.

pH 6.4 (Sigma, St. Louis, Missouri, U.S.A.) plus 8 mM Mg acetate to an absorbance at 680 nm of 50. The cell suspension was disrupted by passage twice through a French pressure cell (American Instrument Co., Silver Spring, Maryland, USA) at 4000 $lb.in^{-2}$. Cellular debris and unbroken cells were removed by centrifugation at 28,000 x g for 15 min. Respiratory membranes were sedimented by a further centrifugation of the cell-free extract at 59,000 x g for 15 min and were resuspended at 15-20 mg $protein.ml^{-1}$ in the same buffer as above. The membranes were used immediately following preparation.

(b) Measurement of oxidase activities Oxidase activities were measured at 30^{o} using a Clark YSI 4004 oxygen electrode under respiratory state 3 conditions as defined by Chance and Williams (1956). The assay mixture containing 42 mM PIPES buffer pH 6.8, 8 mM Mg acetate, 0.55 mM ADP (Sigma) and 4.2 mM Na/K phosphate buffer pH 6.8 was placed in the electrode chamber of 2.4 ml capacity. Following temperature equilibration sufficient respiratory membranes to give a final oxidase activity of 25-75 µM oxygen $reduced.min^{-1}$ were added to the chamber followed, one minute later, by oxidisable substrate. The final concentrations of substrates were as follows: β-NADH (grade III, Sigma) 1 mM; D,L-malate 12 mM; NADPH (Boehringer Mannheim GmbH, Mannheim Germany) 2 mM. NADPH oxidase activity was measured under state 4 conditions using the above assay mixture minus ADP and inorganic phosphate, due to the inhibitory effect of ADP on the NADPH dehydrogenase (Ackrell et al., 1972). Oxidase activities, expressed as µgatom O consumed. $min^{-1}.mg\ protein^{-1}$, were calculated using the oxygen solubility data of Chappell (1964).

(c) Measurement of cytochrome concentrations In order to identify and quantitate the cytochromes present in the respiratory membranes, difference spectra were obtained using a Shimadzu MPS-50L split-beam recording spectrophotometer. The wavelength accuracy was occasionally checked using a didymium filter of known spectrum.

The concentrations of cytochromes *b*, *c*, a_1 and a_2(*d*) were determined from dithionite reduced minus oxidised difference spectra using the following data:

cytochrome *b* $mM\varepsilon$ = 17.5 for the α-peak (556-558 nm) to the trough on the red side (572-574 nm, from *E. coli* cytochrome *b*; Deeb and Hager, 1964). Since this gave the total concentration of cytochrome *b*, the concentration of non-autoxidisable cytochrome *b* was calculated by subtracting the concentration of cytochrome *o*.

cytochrome *c* $mM\varepsilon$ = 17.3 for the α-peak (551 nm) to the trough on the blue side (536-538 nm, from *A. vinelandii* cytochromes c_4 + c_5; Jones and Redfearn, 1966).

The concentration of cytochrome *o* was determined from dithionite reduced plus carbon monoxide minus dithionite reduced difference spectra. Because of interference from cytochrome a_1 the concentration of cytochrome *o* was measured by doubling the difference in absorbance between the Soret peak at 416-418 nm and the baseline on the blue side (395-400 nm) instead of using the peak to trough (428-432 nm) height. A $mM\varepsilon$ = 170.0 for the cytochrome *o* of *Acetobacter suboxydans* was used (Daniel, 1970).

The concentration of cytochrome a_1 was in all cases too low to allow its estimation from carbon monoxide spectra.

(d) Measurement of phosphorylating efficiencies The method used for measuring P/O ratios was that of Ackrell and Jones (1971a). Oxygen uptake was measured at 30° using a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, England) and an assay mixture containing 42 mM PIPES buffer pH 6.8; 8 mM Mg acetate; 0.55 mM ADP; 4.2 mM glucose; 4.2 mM Na/K phosphate buffer pH 6.8; 45 units yeast hexokinase (Sigma type III); 1.7 mg bovine serum albumin; $^{32}P_i$ to give a specific activity of approximately 1 x 10^4 counts.min^{-1}.μ mole inorganic $phosphate^{-1}$ and respiratory membranes sufficient to give an oxygen consumption rate of 0.5 - 1.5 μgatom.min^{-1}; total volume 4.0 ml. The mixture was incubated for 3 min and the reaction initiated by the addition of substrate; final concentrations, NADH 1.0 mM; malate 7.5 mM; NADPH 2.0 mM. Measurements of P/O (NADPH) ratios used assay mixture minus ADP and phosphate buffer (see (b) above). The reaction was stopped after 1 min by transferring 3 ml of the reaction mix into 1 ml of ice-cold 20% (w/v) trichloracetic acid. Denatured protein was removed by centrifugation and unesterified $^{32}P_i$ removed by the isobutanol-benzene extraction method of Avron (1960). Glucose 6-[^{32}P]phosphate was assayed in stainless steel planchets using a gas-flow counter (Tracerlab, Mechelen, Belgium). Corrections were made for the low incorporation of $^{32}P_i$ in minus-substrate controls. P/O ratios were then calculated from the phosphate (μmole) esterified during reduction of a known amount of oxygen (μgatom) in the electrode chamber.

(e) Miscellaneous Protein was assayed by the modified Biuret method (Gornall et al., 1948). Deionised, glass-distilled water was used throughout the preparation and examination of the respiratory membranes.

Results and Discussion

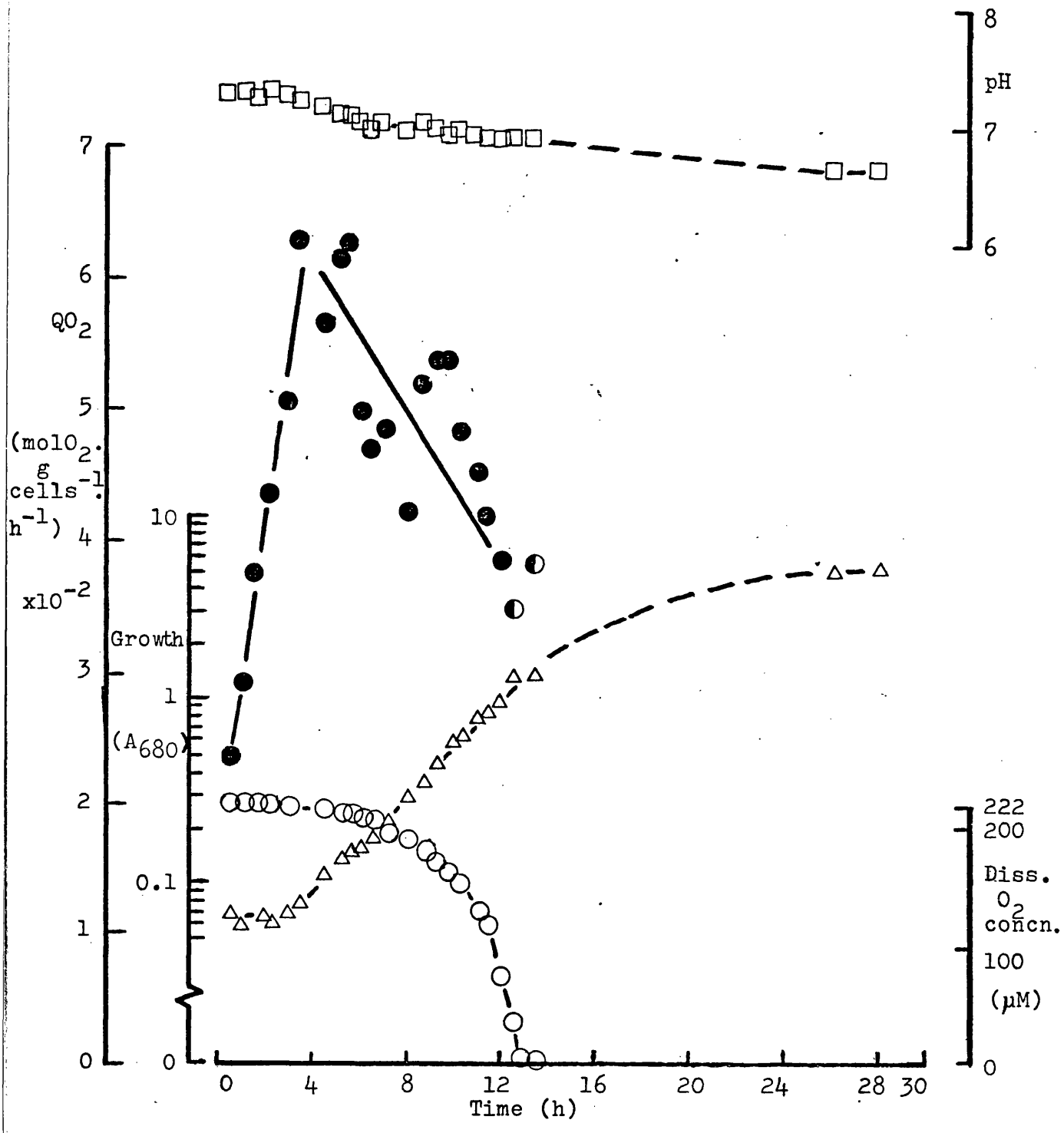
1. Growth and respiration of Azotobacter in batch culture

In order to compare the growth and respiration of A. vinelandii when growing in nitrogen-free medium and fixed nitrogen-containing medium, the technique of batch culture was used. This was because it was desired to investigate the response of the organism to different dissolved oxygen concentrations in the growth medium and a decrease in the dissolved oxygen concentration of the medium was a natural consequence of the increase in cell density during growth. Thus it was possible to follow the response of the cells to the oxygen environment from virtually oxygen-saturating (222 μM) to virtually zero oxygen (< 5 μM) conditions. Although continuous culture would have been more useful for this purpose, since steady state populations can be obtained under different environmental conditions, such facilities were not available at the time.

(a) Nitrogen-free cultures In the experiment shown in Fig. II-7, a flask containing nitrogen-free medium was inoculated from a nitrogen-fixing culture of A. vinelandii in the early oxygen-limited phase of growth and subjected to high aeration shaking conditions. There was a lag period of approximately 3 h before growth began. During this time, the dissolved oxygen concentration and pH of the culture remained virtually constant but the respiratory activity (QO_2) increased by approximately 2-fold from 0.0232 to 0.0504 mol O_2.g cells^{-1}.h^{-1}. The failure of the organism to grow immediately from the time of inoculation was attributed to the "switching-off" of the nitrogenase via conformational protection due to the high ambient oxygen concentration

Fig. II-7

Growth parameters of A.vinelandii on nitrogen-free medium



A flask containing 600ml nitrogen-free medium was inoculated at zero time with a nitrogen-free starter-culture of A.vinelandii in the late logarithmic phase of growth and subjected to high aeration shaking conditions. △, growth; ●, QO_2 (excess oxygen growth); ◐, potential QO_2 (oxygen-limited growth); ○, dissolved oxygen concn.; □, pH.

(220 μM). The increase in respiratory activity during the lag period was assumed to be the respiratory protection response of the organism since when the QO_2 reached approximately 0.050 mol O_2.g cells^{-1}.h^{-1} growth commenced. Presumably at this point the enhanced QO_2 had lowered the intracellular oxygen concentration sufficiently to allow "switch-on" of the nitrogenase and hence growth to begin. During the ensuing logarithmic phase of growth (specific growth rate, μ = 0.31 h^{-1}), there was a slight drop in pH and an increasingly rapid fall in the ambient oxygen concentration; the latter was immeasurably small at the end of logarithmic growth (< 5 μM) when the culture presumably became oxygen-limited at a culture density of approximately 0.48 g dry wt.l^{-1} ($A_{680} \simeq 1.0$). During the first two hours of logarithmic growth, the QO_2 was approximately 0.060 mol O_2.g cells^{-1}.h^{-1}. After this time the QO_2 decreased steadily to 0.038 mol O_2.g cells^{-1}.h^{-1} at the turnover point into oxygen-limited growth. It is known that cessation of logarithmic growth was not due to limitation by any other nutrient than oxygen because higher aeration rates allowed logarithmic growth to continue to correspondingly higher cell densities. It is, however, possible (though unlikely) that the growth rate became limited by the availability of N_2 rather than O_2 (Harrison, 1973). The culture continued to grow under oxygen-limited conditions reaching a maximum density of 2.5 g dry wt.l^{-1} ($A_{680} \simeq 5$) after 28 h. At this point the cells probably became carbon-limited. QO_2 values measured during the oxygen-limited phase of growth were "potential" rather than "in situ" (see Materials and Methods) and so were not comparable with the values obtained during excess oxygen growth.

The respiratory protection response of A. vinelandii can be

more clearly seen in the experiment shown in Fig. II-8. An oxygen-limited, nitrogen-fixing culture of Azotobacter growing under low aeration conditions (μ = 0.023 h^{-1}, "potential" QO_2 = 0.026 mol O_2.g $cells^{-1}.h^{-1}$) was suddenly subjected to extra-high aeration conditions resulting in an increase in the ambient oxygen concentration from <5 μM to 220 μM. Growth of the organism ceased, presumably due to "switch-off" of the nitrogenase, and during the ensuing 3 h lag period the QO_2 increased from 0.027 to approximately 0.062 mol O_2.g $cells^{-1}.h^{-1}$. This effect has been previously reported by Jones et al. (1973) under identical experimental conditions. When growth resumed, presumably as a result of respiratory protection allowing the reversal of the conformational protection of the nitrogenase, the QO_2 was found to decrease steadily, as found in the experiment shown in Fig. II-7. A decrease in the QO_2 of nitrogen-fixing Azotobacter when growing logarithmically in batch culture has not previously been reported.

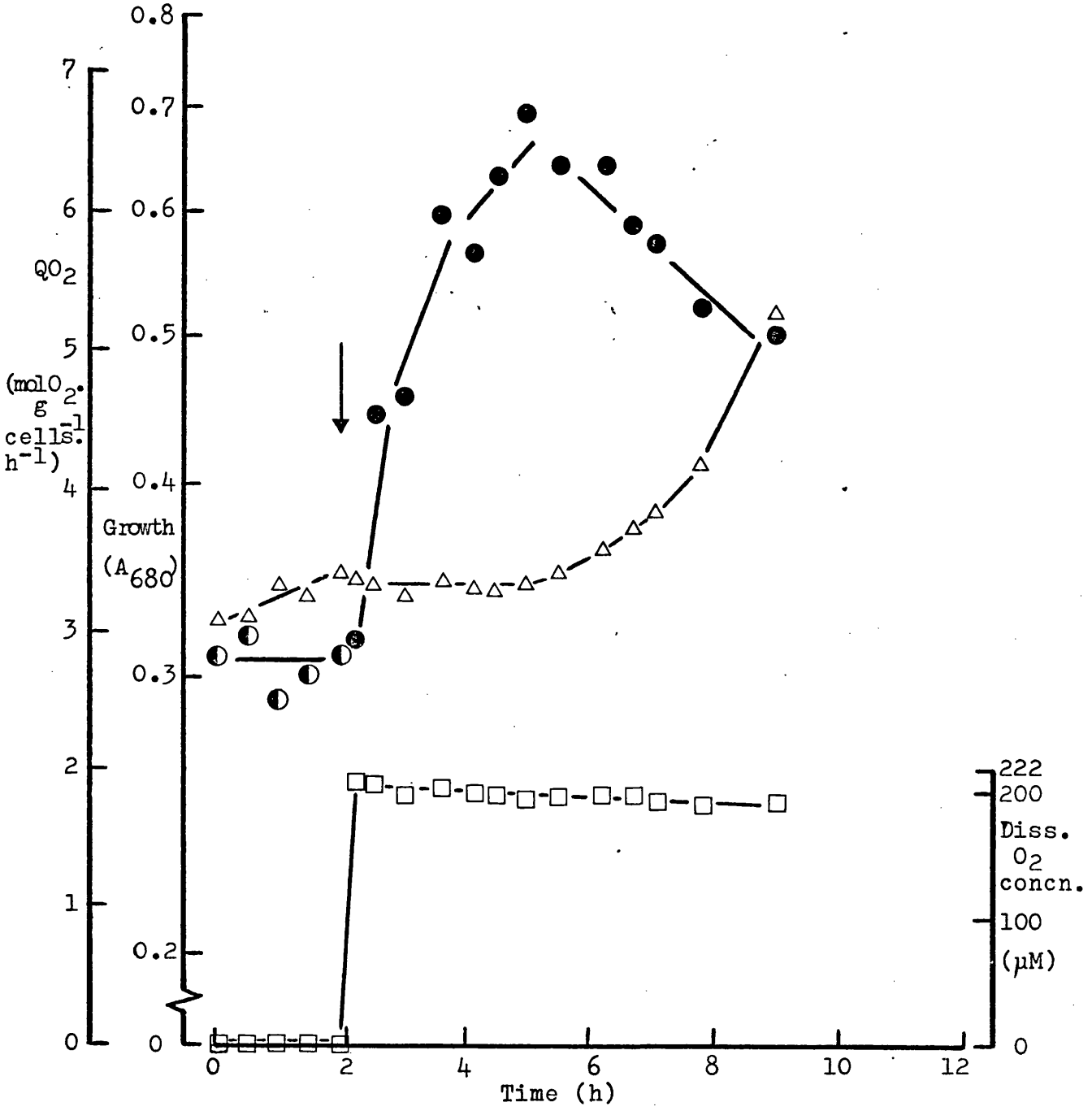
The QO_2 of bacterial cultures is related to the specific growth rate (μ,h^{-1}), the growth yield with respect to ATP (Y_{ATP}, g cells. mol ATP equivalents^{-1}), the maintenance coefficient (M, mol ATP equivalents. g $cells^{-1}.h^{-1}$) and the energy conservation efficiency (N, mol ATP equivalents.mol O_2^{-1}) by the equation

$$QO_2 = {}^{\mu}/_{Y_{ATP}}.N + {}^{M}/_{N} \quad \text{(see Chapter I)} \qquad \text{II-1}$$

During the lag phase following inoculation, in the experiment shown in Fig. II-7, or following an increase in the aeration rate, in the experiment shown in Fig. II-8, μ = 0 hence equation II-1 reduces to $QO_2 = {}^{M}/_{N}$. The increase in QO_2 observed during this period can therefore be attributed to an increase in M, a decrease in N, or both.

Fig. II-8

Growth parameters of A.vinelandii on nitrogen-free medium during an increase in the aeration rate of the culture



The aeration rate of a growing, oxygen-limited, nitrogen-fixing culture of A.vinelandii was suddenly increased at the arrow. △, growth; ◐, potential QO_2 (oxygen-limited growth); ●, QO_2 (excess oxygen growth); □, dissolved oxygen concn.

Jones et al. (1973) showed that, during the lag period in low to high aeration changeover experiments on nitrogen-fixing A. vinelandii similar to that described above, there was an increase in the activity of the NADH and NADPH dehydrogenases, an increase in the concentration of cytochrome a_2 and a loss of energy conservation at site I. These observations suggested a decrease in N during the lag period due to increased oxidation of the reduced pyridine nucleotides via their non-phosphorylating dehydrogenases followed by electron transfer predominantly via a non-phosphorylating branch of the respiratory chain (viz. cytochrome b $\longrightarrow$ cytochrome a_2). Using the techniques described in Chapter IV, Dr. C.W. Jones (personal communication) has recently found that the maintenance coefficient, M, is much higher during nitrogen (N_2)-limited chemostat culture of A. vinelandii (excess oxygen) than during oxygen-limited culture. Hence the increases in QO_2 observed during the lag periods in the batch culture experiments described above were probably due to a decrease in N and also to an increase in M, since in both types of experiment the cells were oxygen-limited prior to the lag phase.

During growth of A. vinelandii in nitrogen-free batch cultures under identical conditions to those used in the experiment shown in Fig. II-7, Ackrell and Jones (1971b) observed that the phosphorylating efficiency at site I of isolated respiratory membranes increased by about 5-fold between the early stages of logarithmic growth (when the P/O ratio was very low) and the turnover point into oxygen-limited growth. The energy conservation efficiency at site II also increased slightly during this period. The decrease in QO_2 of the culture shown in Fig. II-7 during the same period may therefore be explained as follows.

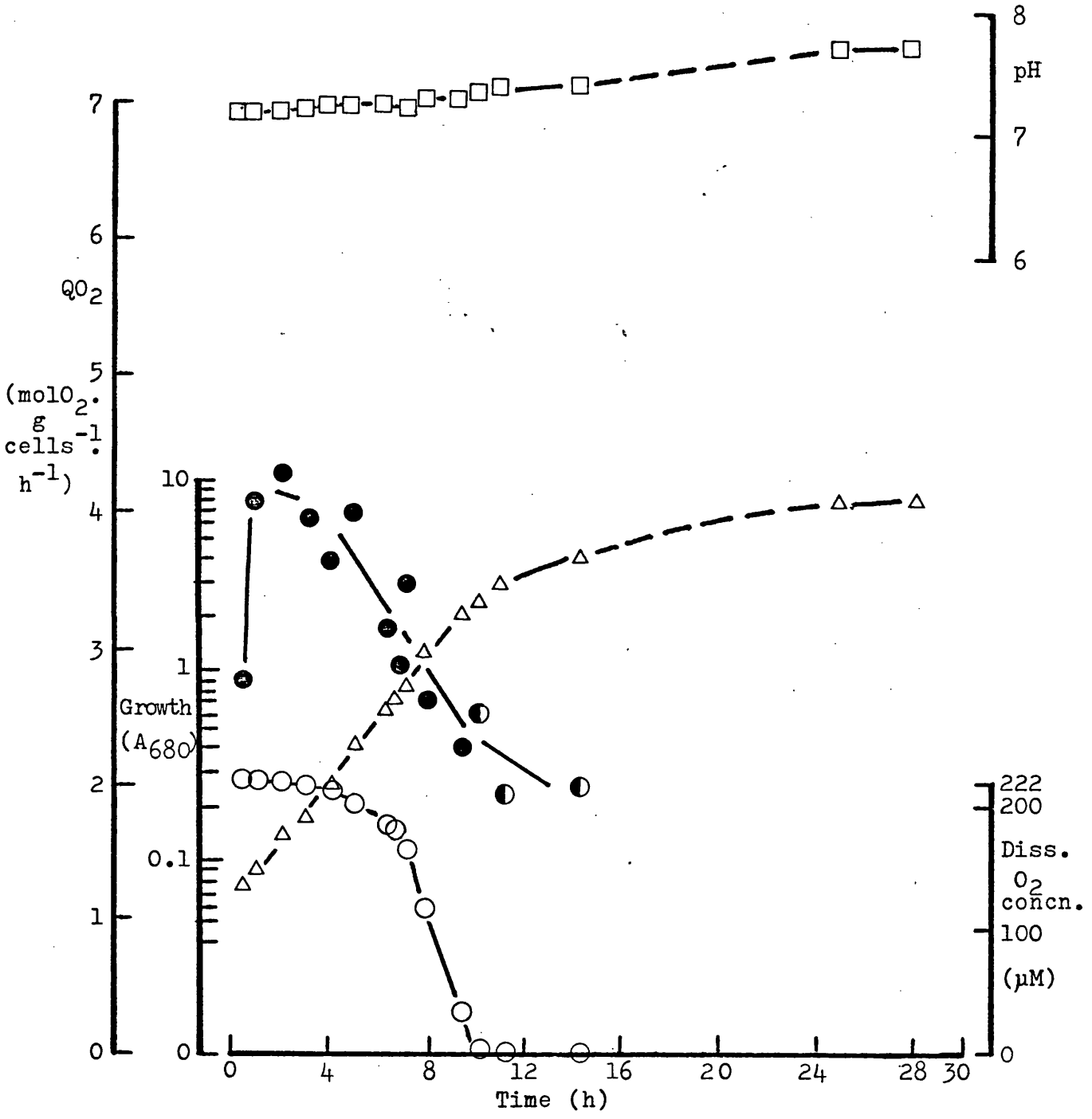
Since μ remained constant, and probably also M and Y_{ATP}, the increase in N resulting from an increase in the energy conservation efficiency predominantly at site I would have resulted (according to equation II-1) in a decrease in QO_2 during the logarithmic growth phase.

(b) Fixed-nitrogen cultures Urea was chosen as a suitable source of combined nitrogen. A number of ammonium salts (ammonium chloride, nitrate and acetate) were also tested but they brought about a drop in the pH of the medium which eventually caused growth to cease. This effect has been previously reported by Khmel and Andreeva (1967). In fact, urea induced a slight increase in the pH of the medium during growth.

There was no lag in growth when a flask containing urea medium was inoculated with urea-grown A. vinelandii in the early oxygen-limited phase of growth and subjected to high aeration conditions (Fig. II-9). This was presumably because growth of the organism under these conditions did not rely on the oxygen-sensitive nitrogenase. A lack of inhibition by high aeration rates of the growth of A. chroococcum in NH_4Cl-containing medium has been reported previously (Dalton and Postgate, 1969a). Logarithmic growth of A. vinelandii in the urea medium was accompanied by a steady decrease in the dissolved oxygen concentration. Unlike the nitrogen-fixing cultures, logarithmic growth occurred until a cell density of 1.0 g dry wt.l^{-1} ($A_{680} \simeq 2$) was reached, when the dissolved oxygen concentration was $< 5\mu M$. This was probably due to the lower respiratory activity of the urea cultures allowing a higher cell density to be reached before the onset of oxygen-limitation. However, as with the nitrogen-fixing cultures, there

Fig. II-9

Growth parameters of A.vinelandii on urea medium



A flask containing 600ml urea medium was inoculated at zero time with a urea starter-culture of A.vinelandii in the late logarithmic phase of growth and subjected to high aeration shaking conditions. △, growth; ●, QO_2 (excess oxygen growth); ◐, potential QO_2 (oxygen-limited growth); ○, dissolved oxygen concn.; □, pH.

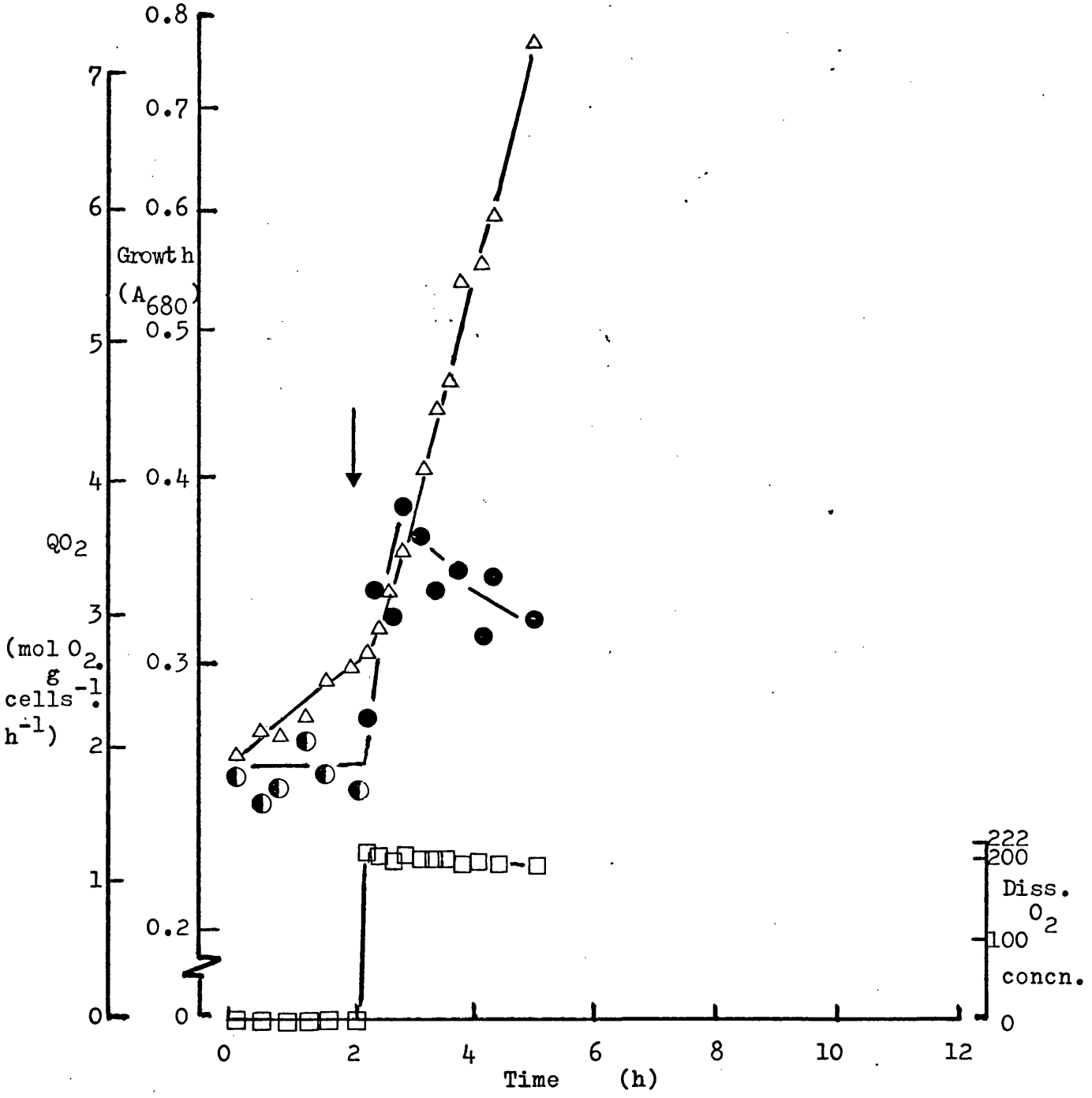
was a steady fall in the QO_2 during logarithmic growth, viz. 0.040 to 0.024 mol O_2.g cells^{-1}.h^{-1}. Oxygen-limited growth occurred up to a cell density of 4.0 g dry wt.l^{-1} ($A_{680} \simeq 8$) when the culture probably became carbon-limited. This cell density was somewhat higher than in the nitrogen-free experiments, probably reflecting the greater efficiency of energy utilisation in the urea culture (see later).

During a low aeration to extra-high aeration changeover experiment analogous to that performed on nitrogen-fixing cells, there was no noticeable lag period in growth of oxygen-limited, urea-grown cells (μ = 0.077 h^{-1}; "potential" QO_2 = 0.018 mol O_2.g cells^{-1}.h^{-1}). The growth rate immediately increased to that normally found during excess oxygen growth, viz. μ = 0.38 h^{-1} (Fig. II-10). This was presumably due to the absence of nitrogenase from these fixed-nitrogen-grown cells (Wilson, 1958). There was a much smaller increase in QO_2 than for the N-free cultures following the changeover, viz. 0.022 - 0.036 mol O_2.g cells^{-1}.h^{-1}, which occurred within the first hour. After this time, the QO_2 began to decrease as during logarithmic growth under high aeration conditions.

The respiratory activities of the nitrogen-free and fixed-nitrogen cultures of A. vinelandii at the different dissolved oxygen concentrations pertaining during logarithmic growth under high aeration conditions are compared in Fig. II-11. (data from Figs. II-7,9). In spite of the faster growth rate of the organism in urea medium than in nitrogen-free medium, the QO_2 of the urea culture was at all times lower than that of the nitrogen-fixing culture. This was considered to be due mainly to a lower Y_{ATP} in the nitrogen-free culture due to the energy requirements for nitrogen fixation (Nagai et al., 1969; Hill et al., 1972; see also

Fig. II-10

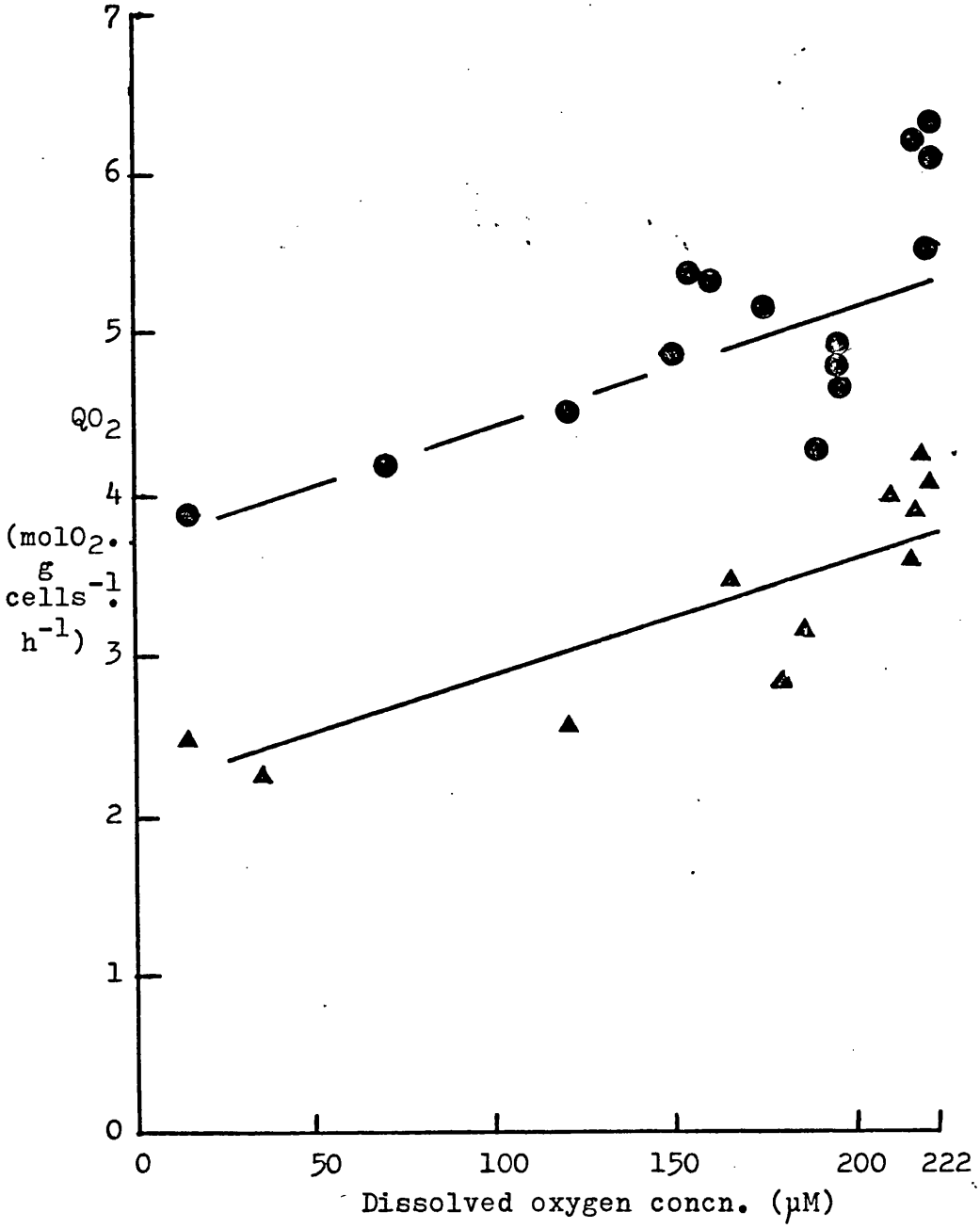
Growth parameters of A.vinelandii on urea medium during an increase in the aeration rate of the culture



The aeration rate of a growing, oxygen-limited, urea culture of A.vinelandii was suddenly increased at the arrow. △ , growth; ◐ , potential QO_2 (oxygen-limited growth); ● , QO_2 (excess oxygen growth); □ , dissolved oxygen concn.

Fig. II-11

A comparison of the respiratory activities of nitrogen-free and fixed nitrogen-grown cultures of A.vinelandii



Data replotted from Figs. II-7&9. ●, nitrogen-fixing culture; ▲, urea culture.

later). This difference in QO_2 between the two types of culture was reproducible.

The next step taken was to determine if the energy conservation efficiency of urea-grown cells differed from that of nitrogen-free-grown cells and also to find out if the decrease in QO_2 during logarithmic growth in batch culture occurred by the same mechanism in urea cultures as that postulated for nitrogen-free cultures.

2. Energy conservation

Two complementary techniques were used for measuring the energy conservation efficiency of A. vinelandii. The first, employing whole cells, allowed measurement of the overall efficiency and the second, employing respiratory membranes, allowed the relative phosphorylating efficiencies of the energy-conserving sites to be compared.

(a) Whole cells It was decided, both from a technical and theoretical standpoint, that the best way of measuring the energy-conserving efficiency of whole cells was to measure $\longrightarrow H^+/O$ quotients. These are related to the P/O ratio by the equation $P/O = \longrightarrow H^+/O \div \longrightarrow H^+/P$ (Mitchell, 1966, 1967). Even though such a procedure assumes that $\longrightarrow H^+/P = 2$ (a reasonable assumption by analogy with the oxidative phosphorylation system of mitochondria; see Chapter I), assumptions are also involved in other, elaborate, P/O ratio measurements on whole cells such as the procedure of Hempfling (1970).

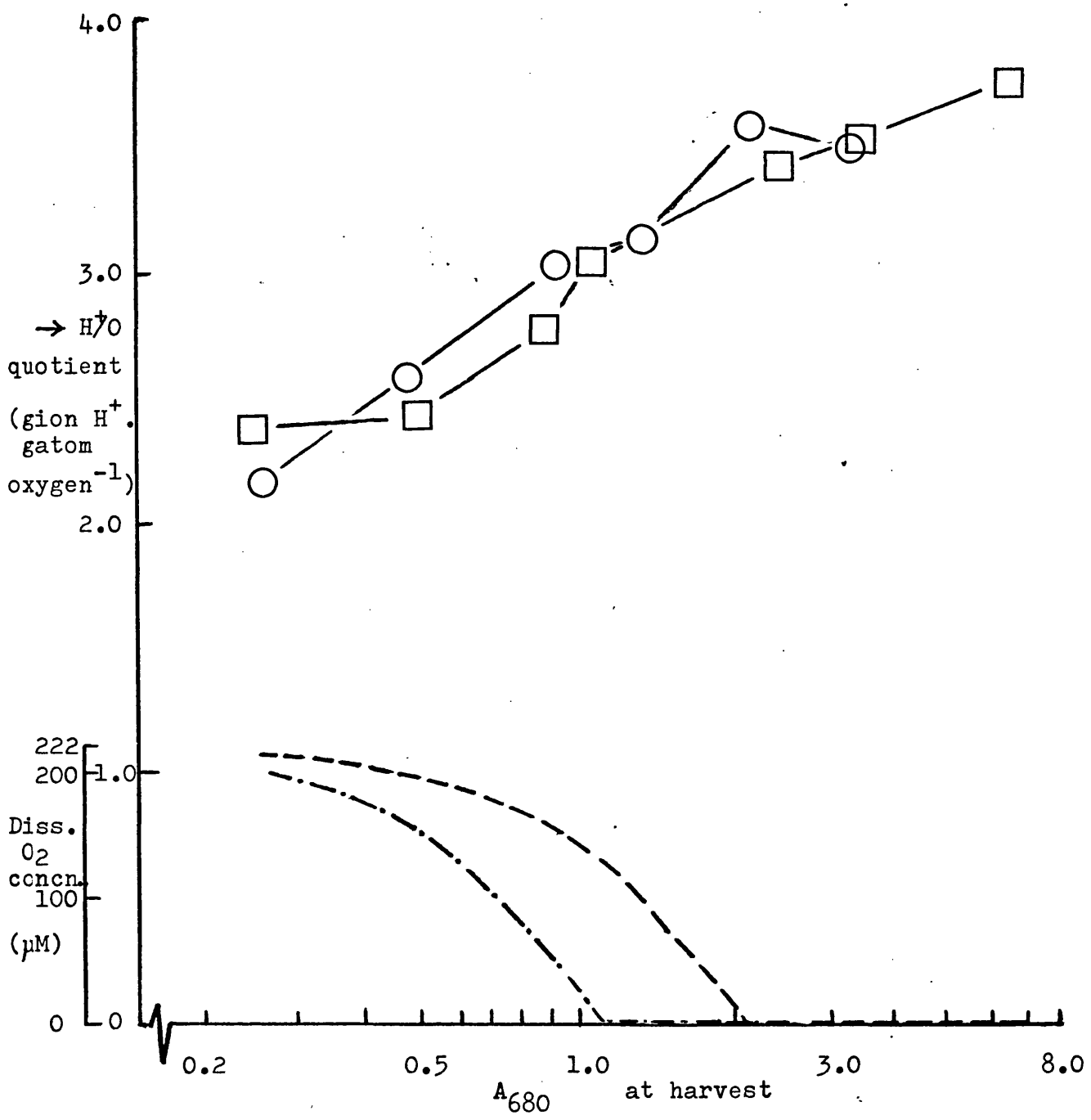
$\longrightarrow H^+/O$ quotients of A. vinelandii harvested at various stages of high aeration growth, assayed under the optimum conditions described in Materials and Methods and oxidising endogenous substrates

are shown in Fig. II-12. It can be clearly seen that the $\longrightarrow H^+/O$ quotient increased with the culture density at harvest for A. vinelandii growing under both nitrogen-fixing and fixed nitrogen conditions. This increase in the P/O ratio ($\equiv \longrightarrow H^+/O \div 2$) reflected the decrease in QO_2 observed under identical growth conditions (Figs. II-7,9) and was assumed to be related to the fall in dissolved oxygen concentration of the growth medium. The increases in the $\longrightarrow H^+/O$ ratios between early logarithmic growth (excess oxygen) and oxygen-limited growth were equivalent to an increase in the P/O ratio from approximately 1.0 to 1.7, in the case of the nitrogen free-grown cells, and from approximately 1.2 to 1.9, in the case of the urea-grown cells. The increase in the $\longrightarrow H^+/O$ quotient exhibited by the nitrogen-free-grown cells was compatible with what is known about the respiratory system under these conditions. At high dissolved oxygen concentrations (low A_{680}) site I would be largely uncoupled and a large proportion of the electron flux would pass to oxygen via the b $\longrightarrow a_2$ cytochrome branch. Most energy conservation would then occur at site II (loop 2 in Fig. II-2) and hence give a P/O ratio of approximately 1. At low dissolved oxygen concentrations and during oxygen limited growth, not only would coupling at site I be higher but also preferential synthesis of cytochrome o would result in a larger proportion of the total electron flux being routed via the minor, phosphorylating, b $\longrightarrow$ c $\longrightarrow$ o cytochrome branch (Ackrell and Jones, 1971b). These two factors could lead to the observed increase in the $\longrightarrow H^+/O$ ratio. It was therefore necessary to find out if such changes were also responsible for the increase in the whole cell $\longrightarrow H^+/O$ ratios observed during growth on urea medium.

(b) Respiratory membranes Unfortunately, the relatively

Fig. II-12

→ H^+/O quotients of A.vinelandii harvested at various stages of high aeration growth



A.vinelandii was grown under high aeration conditions on either nitrogen-free (○) or urea (□) medium. Following harvest at various stages of growth the cells were prepared for the assay of → H^+/O quotients. Conditions of assay; 6 mg dry wt. cells.ml^{-1}; 100 μg.ml^{-1} carbonic anhydrase; 100 mM KSCN. Dissolved oxygen concn.; —·—·—·—, nitrogen-free medium; -----, urea medium.

simple technique of measuring the $\longrightarrow H^+/O$ quotient of starved bacterial cells loaded with specific substrates, which gives information concerning the efficiency of energy conservation at different sites ($\equiv$ loops), had not been developed at the time of this work (see Chapter III). It was therefore necessary to study the phosphorylating efficiency of the individual sites using respiratory membranes. Fortunately, a procedure for preparing respiratory membranes of A. vinelandii with high phosphorylating efficiencies had already been described (Ackrell and Jones, 1971a).

The malate and NADH oxidase activities of respiratory membranes prepared from urea-grown cells were found to be very high (Table II-2) and comparable to those of membranes prepared from nitrogen-fixing cells (Ackrell and Jones, 1971b). The activities were approximately twice as high in membranes prepared from oxygen-limited urea-grown cells as in membranes from excess oxygen (logarithmic) cells. The NADPH oxidase activity, however, was less than 1/6th of that found for membranes prepared from nitrogen-fixing cells by Ackrell et al. (1972). There was little change in the activity of this oxidase during passage of the organism from the excess-oxygen to the oxygen-limited phase of growth. The rate of oxidation of NADH plus malate was faster than the rate of oxidation of NADH alone (not shown), indicating that both the NADH and the NADPH dehydrogenases were the rate-limiting steps in the oxidation of their respective substrates. This was also the case for the dehydrogenases of membranes prepared from nitrogen-fixing cells (Jones et al., 1971a,b; Ackrell et al., 1972).

Having established that respiratory membranes prepared from

Table II-2

Oxidase activities of respiratory membranes prepared from *A. vinelandii* harvested at various stages of growth on urea medium.

Growth conditions	Specific oxidase activity (μgatom $O.min^{-1}.mg\ protein^{-1}$)		
	Malate	NADH	NADPH
excess oxygen (A_{680} at harvest = 0.4-1.0)	0.94	1.09	0.36
oxygen-limited (A_{680} at harvest = 3.0-3.5)	2.04	2.17	0.29

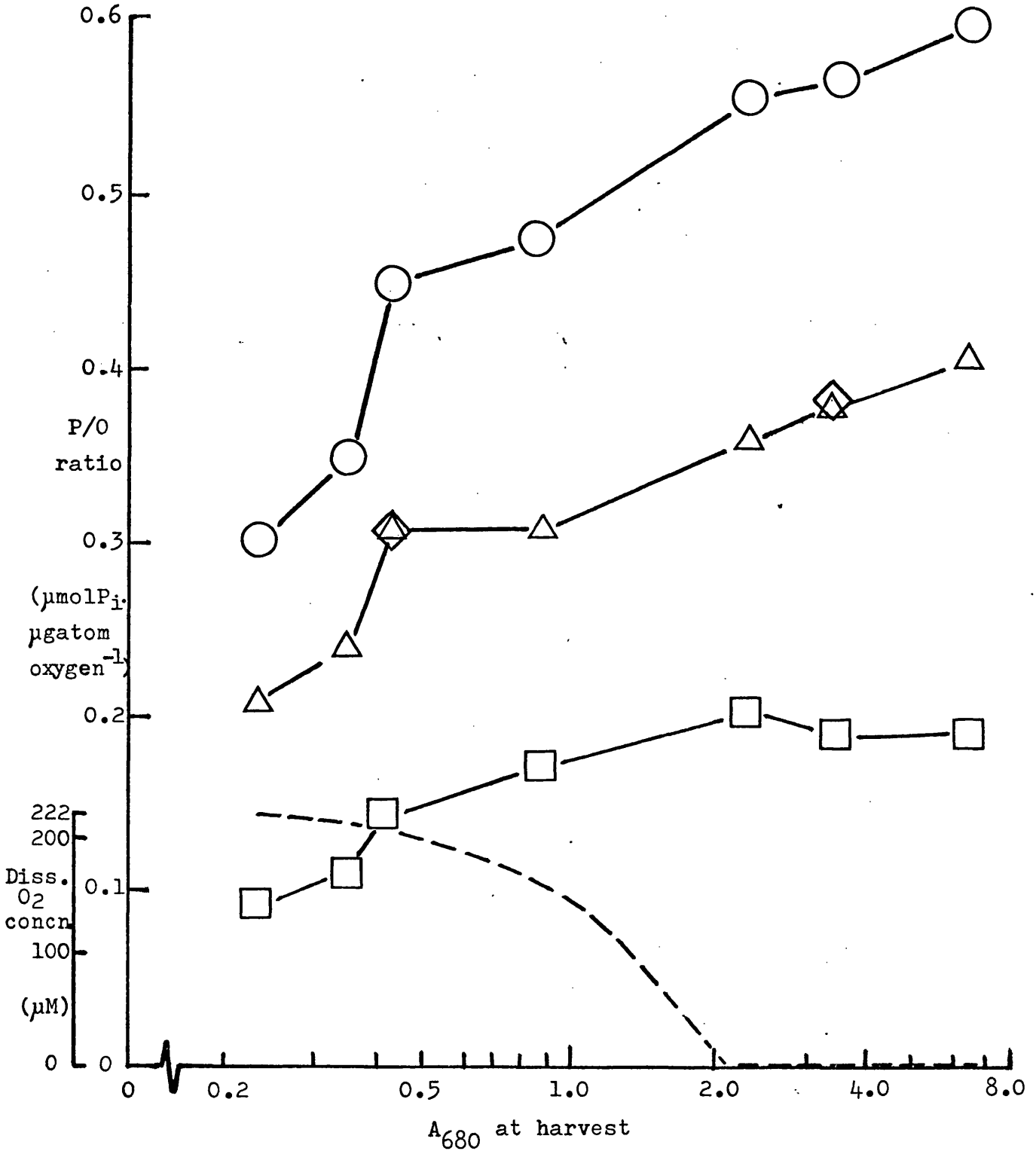
A.vinelandii was grown under high aeration conditions on urea medium and harvested at various stages of growth. Respiratory membranes were prepared and oxidase activities measured as described in Materials and methods.

urea-grown cells contained these three particulate, and presumably respiratory chain-linked, oxidase activities, P/O ratios with these substrates were measured on cells harvested at various stages of high aeration growth (Fig. II-13). The fact that the P/O ratio with NADH as substrate was considerably higher than with malate, although yielding virtually the same rates of oxygen uptake, indicated that the NADH dehydrogenase was, like that of nitrogen-free-grown cells (Eilermann et al., 1970), associated with site I energy conservation. The P/O (NADH-malate) ratio is plotted as site I P/2e^-. There was no phosphorylation associated with the NADPH dehydrogenase since the P/O (NADPH) ratio was the same as the P/O (malate) ratio. During excess oxygen (logarithmic) growth the P/2e^- ratio at site I increased considerably but no further increase occurred during the oxygen-limited growth phase. The P/O ratio with malate also increased during excess oxygen growth and continued to increase during oxygen limitation. These results are very similar to those found for A. vinelandii grown in nitrogen-free medium; under these conditions there was likewise no phosphorylation associated with the NADPH dehydrogenase (Erickson et al., 1972). There was also an increase in the site I P/2e^- ratio and an increase in the P/O (malate) ratio for nitrogen-fixing A. vinelandii during growth under high aeration conditions (Ackrell and Jones, 1971b). The increase in phosphorylating efficiency appeared to be associated with the decrease in dissolved oxygen concentration of the growth medium for membranes prepared from both nitrogen-free-grown and urea-grown cells.

The increase in the whole cell $\longrightarrow H^+/O$ ratios observed for A. vinelandii during high aeration growth in urea medium can therefore be correlated with the increases in phosphorylating efficiency at site I

Fig. II-13

P/O ratios of respiratory membranes prepared from A.vinelandii harvested at various stages of growth on urea medium



A.vinelandii was grown under high aeration conditions on urea medium and harvested at various stages of growth. Phosphorylating respiratory membranes were prepared and P/O ratios measured as described in Materials and methods. ○, P/O(NADH) ratio; △, P/O(malate) ratio; □, P/O(NADH—mal) ratio = site I; ◇, P/O(NADPH) ratio; – – – , dissolved oxygen concn..

and between the malate dehydrogenase and oxygen. In order to determine which region of the respiratory chain the latter increase in phosphorylating efficiency was associated with, it was necessary to determine the cytochrome composition of the urea-grown cells.

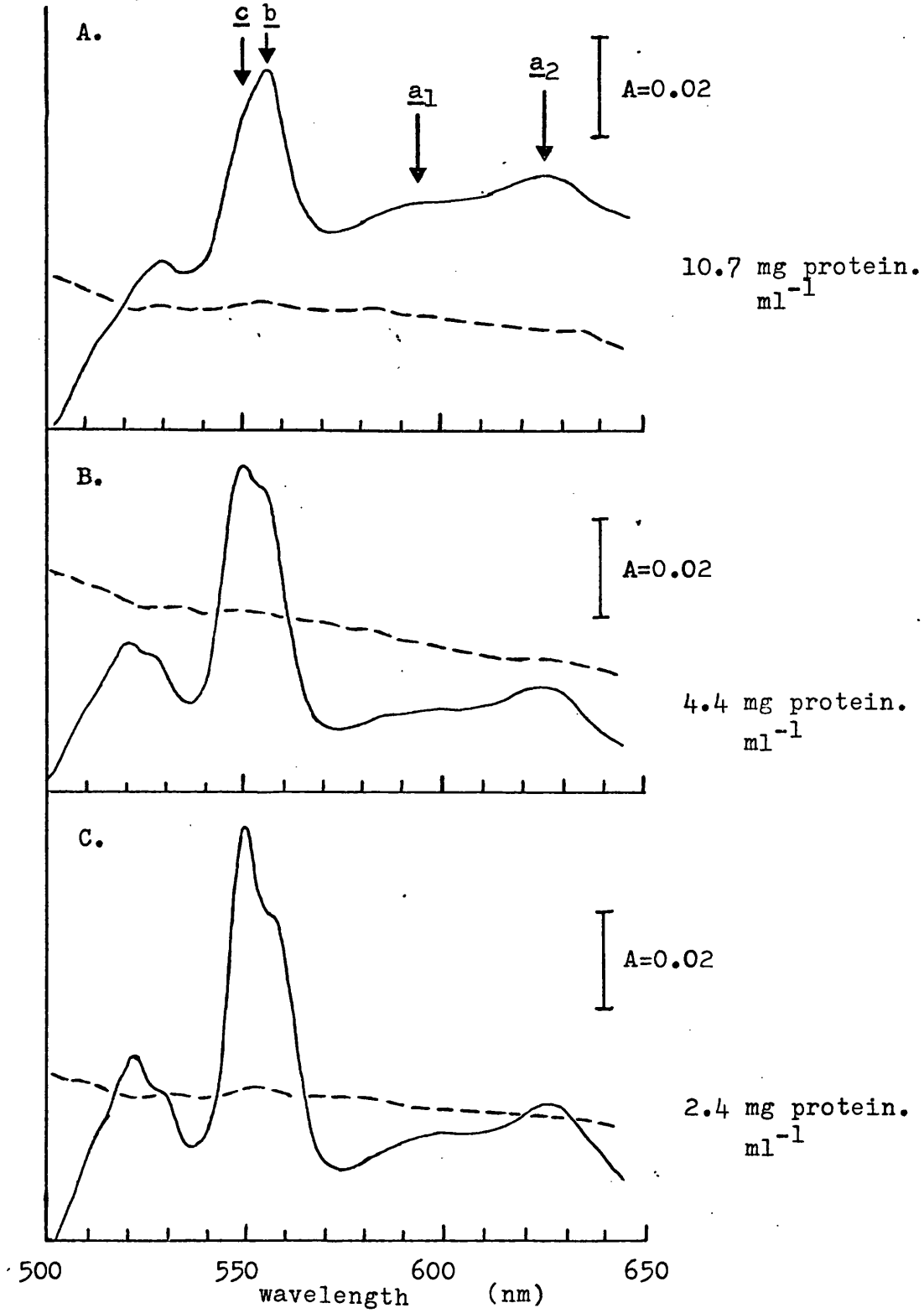
3. Cytochromes

In order to identify and quantitate the cytochromes present in A. vinelandii grown in urea medium, difference spectrophotometry was performed on respiratory membrane preparations. Dithionite-reduced minus oxidised spectra in the visible region revealed the presence of cytochromes b, c, a_2 and also a_1. The latter was not detected in whole cell reflectance spectra. The c-type cytochrome was probably a mixture of types c_4 and c_5, as for nitrogen-fixing cells (Tissières, 1956) but there is no evidence in the literature that this is the case for the cytochrome c of combined nitrogen-grown cells. The cytochrome c became much more apparent during the oxygen-limited region of growth compared with the excess oxygen region (Fig. II-14). Dithionite-reduced plus carbon monoxide minus dithionite-reduced spectra in the Soret region revealed a characteristic w-shaped spectrum (Fig. II-15). This has been previously reported for membrane preparations from nitrogen-free-grown cells and was due to the interference of the cytochrome o spectrum (peak 418 nm, trough 430 nm) by the a_1 spectrum (peak 428 nm, trough 441 nm; Castor and Chance, 1959). However, it was still possible to measure the concentration of cytochrome o (see Materials and Methods). The cytochrome o of Azotobacter had a low affinity for CO (or a low rate of binding of CO) as was found for the cytochrome o of Bacillus megaterium KM (Broberg and Smith, 1967) and it was found necessary to bubble the

Fig. II-14

Difference spectra of respiratory membranes prepared from A.vinelandii

Dithionite-reduced minus oxidised spectra

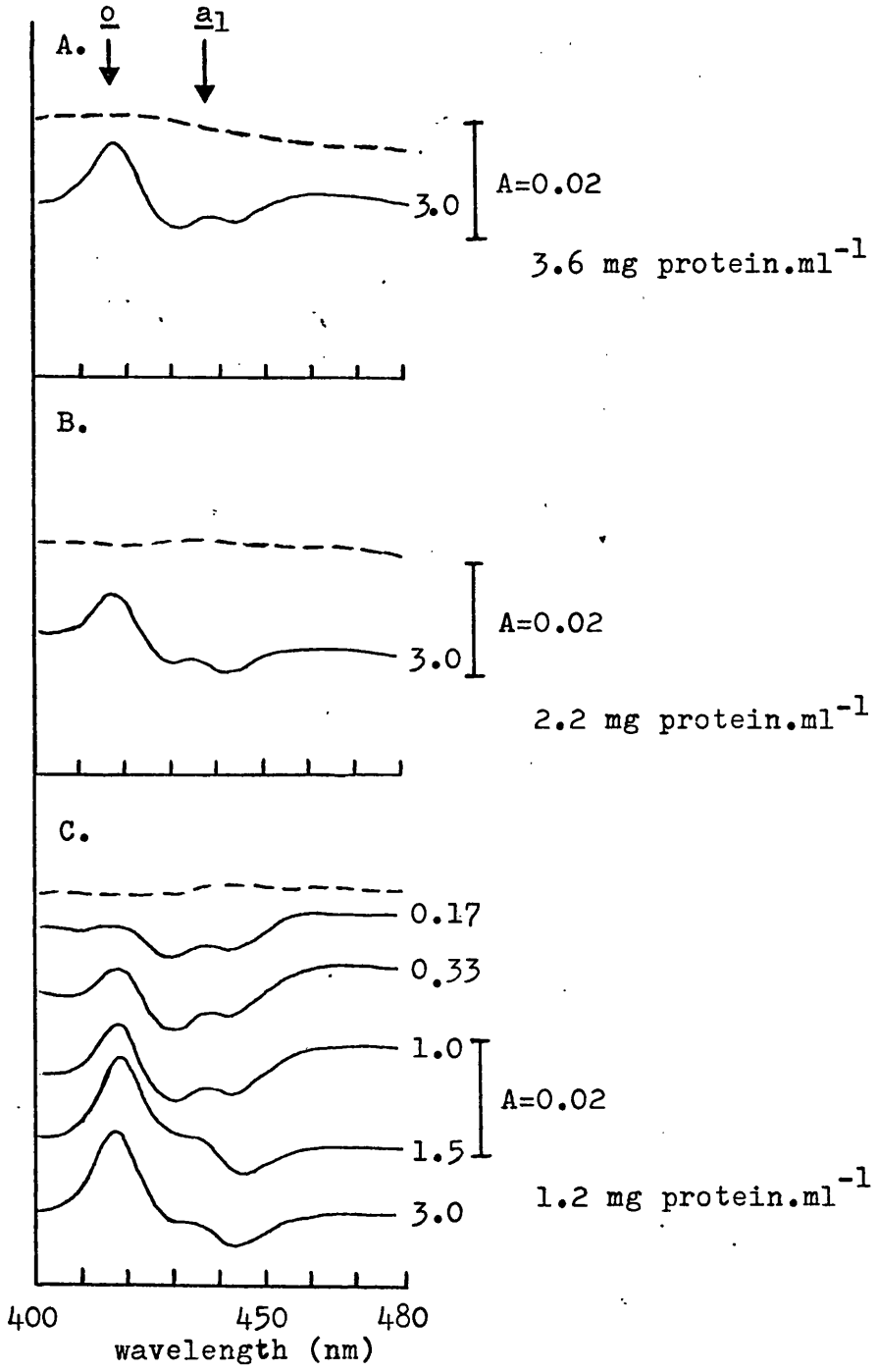


Respiratory membranes were prepared from A.vinelandii harvested at various stages of growth on urea medium. ——— , dithionite-reduced minus oxidised; ------ , oxidised minus oxidised. A_{680} at harvest; A, 0.25 (excess oxygen); B, 3.3 (just oxygen-limited); C, 6.5 (oxygen-limited).

Fig. II-15

Difference spectra of respiratory membranes prepared from A.vinelandii

Dithionite-reduced plus carbon monoxide minus dithionite-reduced spectra



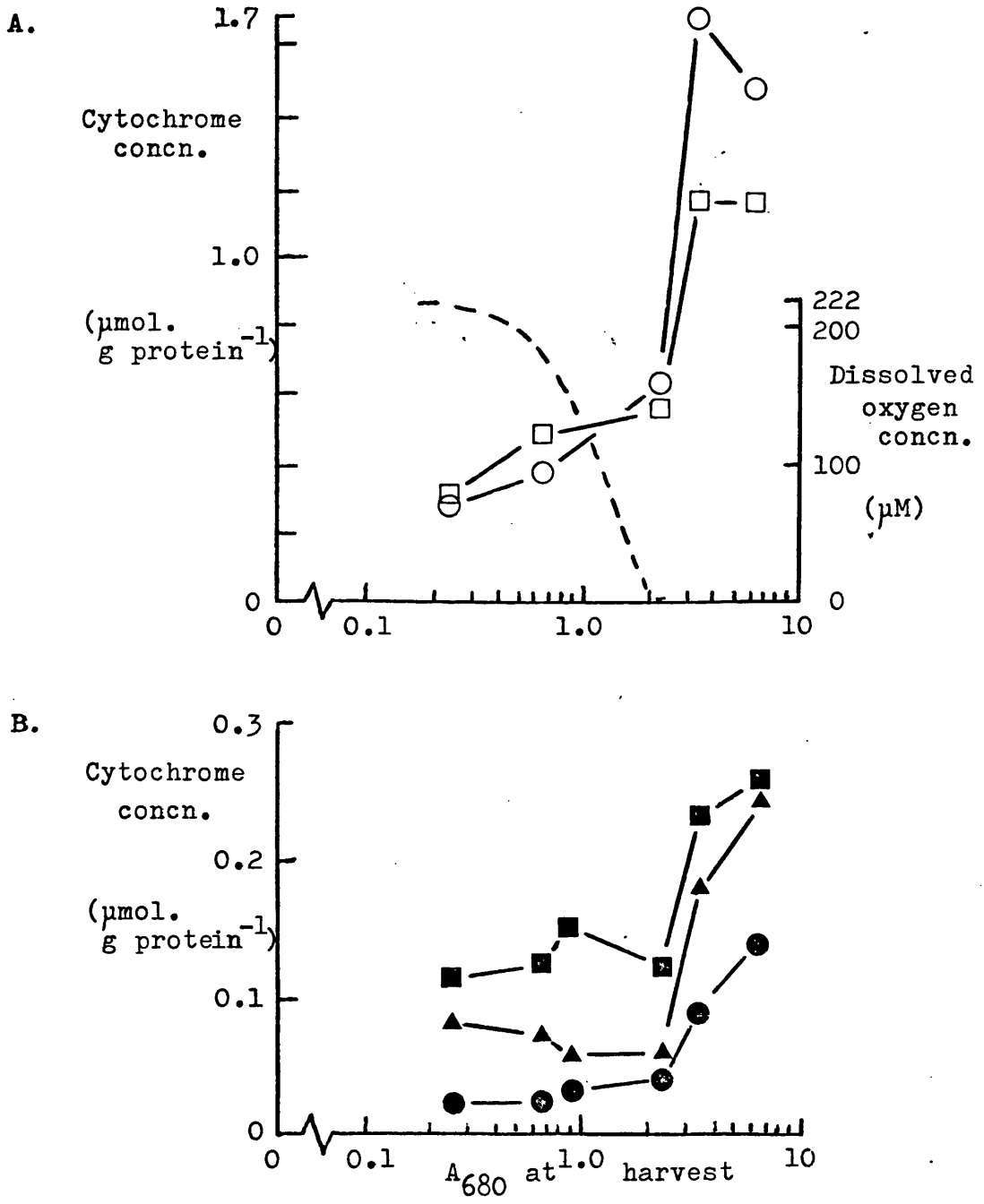
The respiratory membrane preparations used were identical to those in Fig. II-14. ——, dithionite-reduced plus CO minus reduced; ---, reduced minus reduced. The figures alongside the spectra denote the time (min) for which the membrane suspension was bubbled with CO.

membrane suspensions with CO for at least 2 min in order to achieve maximum binding (Fig. II-15C).

There was no evidence for depressed levels of cytochrome $\underline{a}_2$ in urea-grown cells compared with nitrogen-free-grown cells as reported by Knowles and Redfearn (1969). As pointed out by Yates and Jones (1974), these workers used preparations which were derived from cells growing under differing dissolved oxygen concentrations. The concentrations of cytochromes in the membranes prepared from the urea-grown cells used in the present work were similar to the concentrations in membranes prepared from nitrogen-fixing cells, when derived from the same region of the growth curve (Ackrell and Jones, 1971b). The concentrations of the intermediate $\underline{b}$ and $\underline{c}$ type cytochromes and cytochromes $\underline{a}_1$, $\underline{a}_2$ and $\underline{o}$ (which presumably acted as terminal oxidases, as in nitrogen-fixing cells; Castor and Chance, 1959; Jones and Redfearn, 1967a) were all found to increase with the A_{680} of the culture at harvest, especially following the onset of oxygen-limited growth (Fig. II-16). This was also observed for membrane preparations of nitrogen-fixing cells (Ackrell and Jones, 1971b). The concentration of cytochrome $\underline{c}$ increased relative to cytochrome $\underline{b}$ during the oxygen-limited phase of growth (Fig. II-16A). This, together with the apparent increase in the cytochrome $\underline{o}$ concentration relative to the concentrations of cytochromes $\underline{a}_1$ and $\underline{a}_2$ (Fig. II-16B), may have caused a greater proportion of the total electron flux to pass $\underline{via}$ the minor, possibly phosphorylating, branch of the respiratory system ($\underline{b} \longrightarrow \underline{c} \longrightarrow \underline{o}$), assuming the respiratory chain of urea-grown cells to be branched, as in nitrogen-fixing cells (Jones and Redfearn, 1967a). If this occurred, it may possibly have resulted in the higher P/O (malate)

Fig. II-16

Cytochrome concentrations of respiratory membranes prepared from A.vinelandii



Respiratory membranes were prepared from A.vinelandii harvested at various stages of growth on urea medium. Cytochrome concentrations were calculated from difference spectra. □ , cytochrome b; ○ , cytochrome c; ▲ , cytochrome a_1; ■ , cytochrome a_2; ● , cytochrome o; -----, dissolved oxygen concn..

ratios observed for membranes prepared from oxygen-limited cells than for membranes prepared from excess oxygen cells (Fig. II-13).

These experiments therefore showed that there was very little difference quantitatively in the cytochrome composition of A. vinelandii grown in nitrogen-free medium and urea medium.

4. The NADPH dehydrogenase

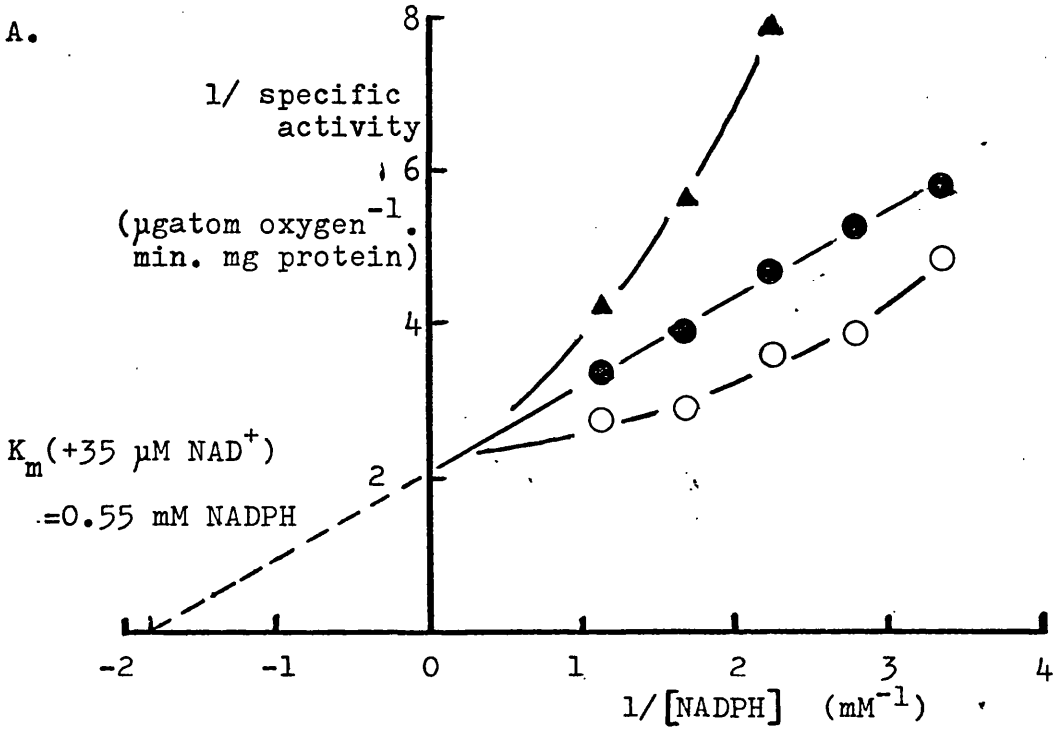
Oxidation of NADPH by respiratory membranes prepared from nitrogen-fixing A. vinelandii is subject to several types of non-classical respiratory control. The sigmoidal saturation kinetics exhibited by the NADPH dehydrogenase ensure that small changes in the intracellular NADPH/$NADP^+$ ratio could significantly affect the rate of NADPH oxidation (Ackrell et al., 1972). This is probably important for the respiratory protection response since at particularly high oxygen concentrations in cultures of Azotobacter, nitrogenase is "switched off" and respiration is presumably augmented by oxidation of NADPH via the non-phosphorylating NADPH dehydrogenase (Erickson et al., 1972). Furthermore, the NADPH dehydrogenase is competitively inhibited by adenine nucleotides and thus responds to "energy charge" as defined by Atkinson and Walton (1967); minimum activity of this non-phosphorylating dehydrogenase was observed under conditions of low energy charge (Ackrell et al., 1972). It is likely that, under such conditions or in the presence of the potent inhibitor NAD^+, NADPH is preferentially converted to NADH via the NADPH $\longrightarrow$ NAD^+ transhydrogenase and thence oxidised via the more efficiently phosphorylating NADH dehydrogenase. It was consequently of interest to determine if the same types of respiratory control operated on the NADPH

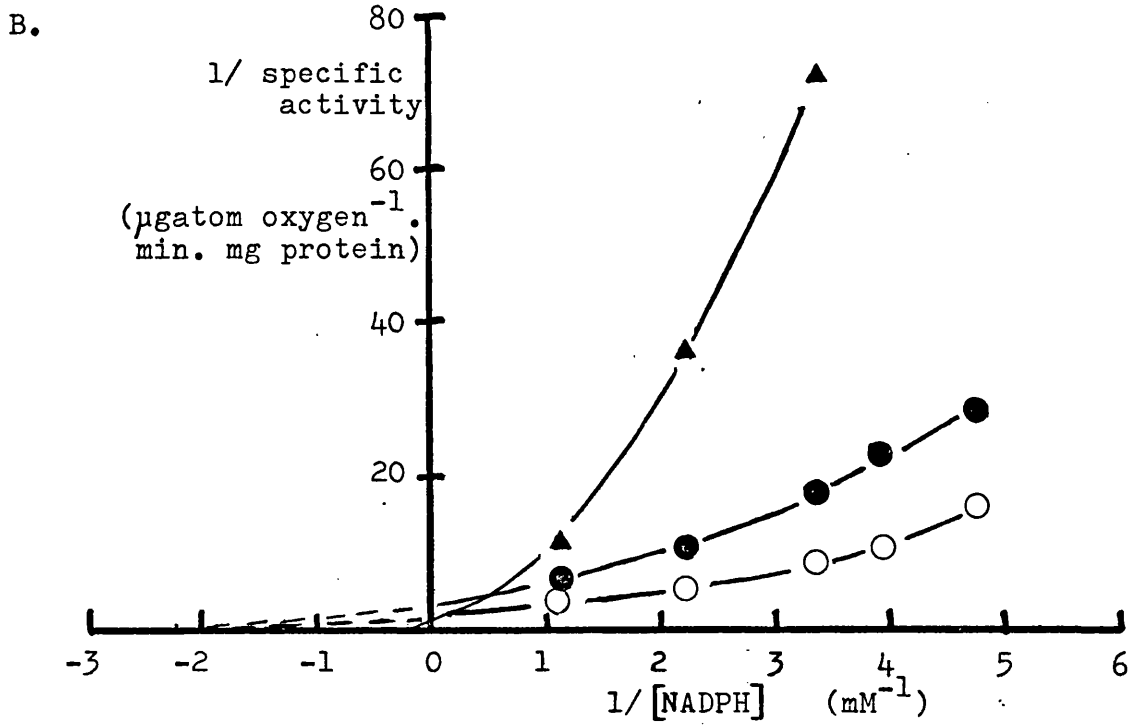
dehydrogenase of urea-grown A. vinelandii.

Ackrell et al. (1972) reported that respiratory membrane preparations of A. vinelandii grown under nitrogen-fixing conditions exhibited very high NADPH oxidase activities (V_{max} up to 3.3 μgatom O consumed.min^{-1}.mg $protein^{-1}$). Respiratory membrane preparations of the organism grown on urea medium exhibited much lower activities, as already mentioned (Table II-2); Lineweaver-Burk plots revealed much lower V_{max} values than with the membranes of the above workers, irrespective of whether the cells were derived from the excess oxygen phase of growth (Fig. II-17A; V_{max} = 0.40 μgatom O.min^{-1}.mg $protein^{-1}$) or the oxygen-limited phase (Fig. II-17B; V_{max} = 0.36). However, the saturation kinetics of NADPH oxidation were sigmoidal, a feature shared by membranes obtained from nitrogen-fixing cells (Ackrell et al., 1972). This was revealed by the upwardly curving Lineweaver-Burk plots (Fig. II-17) and the n_{NADPH} values of approximately 2 obtained from Hill plots (Fig. II-18). NADPH oxidation by these membranes was inhibited by AMP and NAD^+ (AMP less potent than NAD^+; Fig. II-17). The sigmoidal saturation kinetics observed with the unmodulated NADPH dehydrogenase were maintained in the presence of 4 mM AMP, as revealed by the n_{NADPH} values of approximately 2 obtained from Hill plots (Fig. II-18), regardless of whether the membranes were derived from cells harvested during the excess oxygen or early oxygen-limited phase of growth on urea medium. This was also the case for membranes prepared from nitrogen-fixing cells (Ackrell et al., 1972). With membranes prepared from excess oxygen urea-grown cells, 35 μM NAD^+ completely abolished the sigmoidal saturation kinetics to yield a rectangular hyperbola, seen as a straight line on the Lineweaver-Burk plot (Fig. II-17A), with a Hill coefficient of approximately

Fig. II-17

Saturation kinetics of the NADPH dehydrogenase of respiratory membranes prepared from urea-grown A.vinelandii

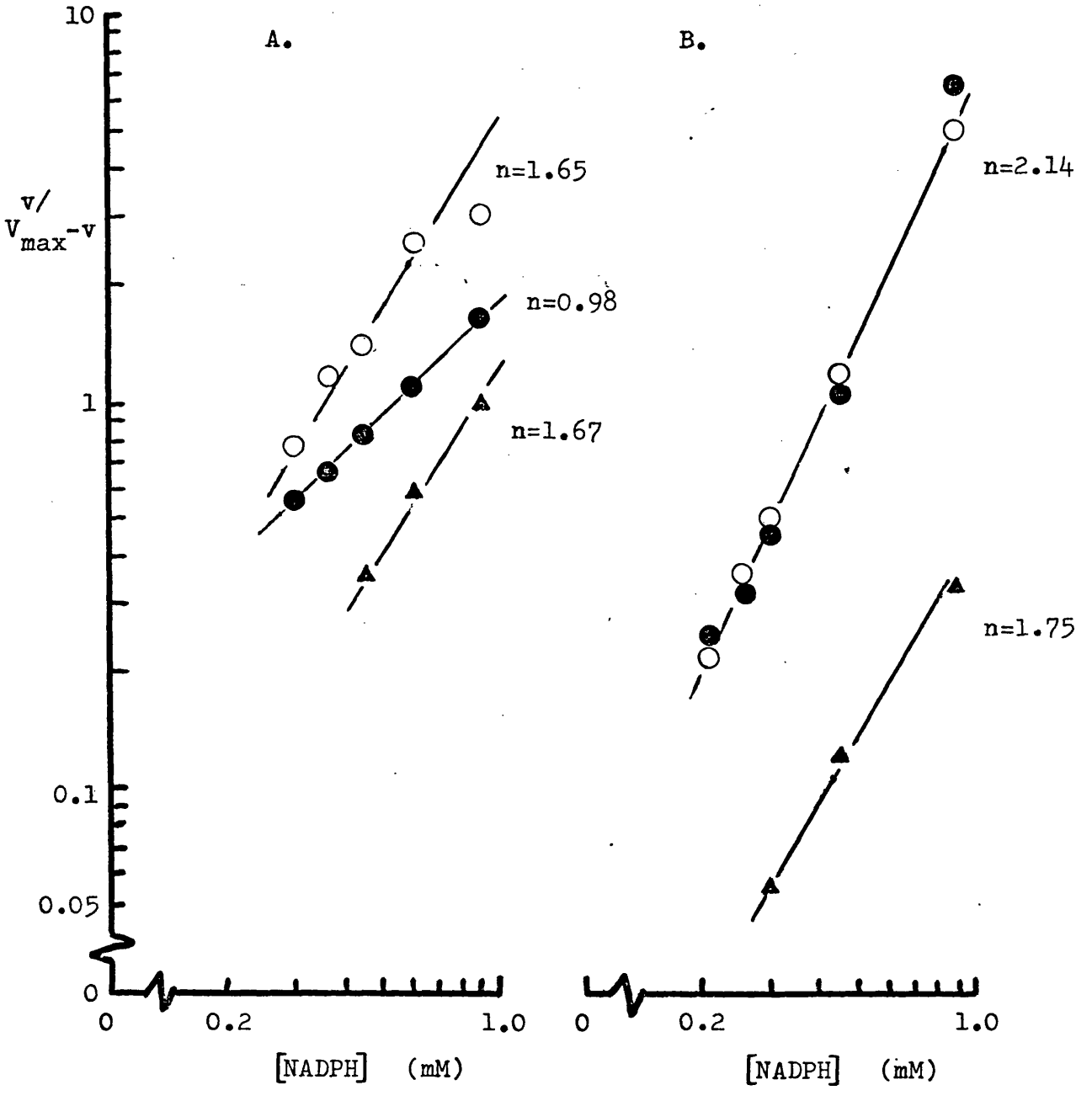




A.vinelandii was harvested at different stages of high aeration growth on urea medium. The A_{680} of the cells at harvest was A,0.45 and B,3.24. Respiratory membranes were prepared and specific oxidase activities were measured with varying concentrations of NADPH. A, membranes prepared from excess oxygen cells; B, membranes prepared from oxygen-limited cells. ○ , no inhibitor; ● , plus 35μM NAD^+; ▲ , plus 4.0mM AMP.

Fig. II-18

Hill plots of the data shown in Fig. II-17



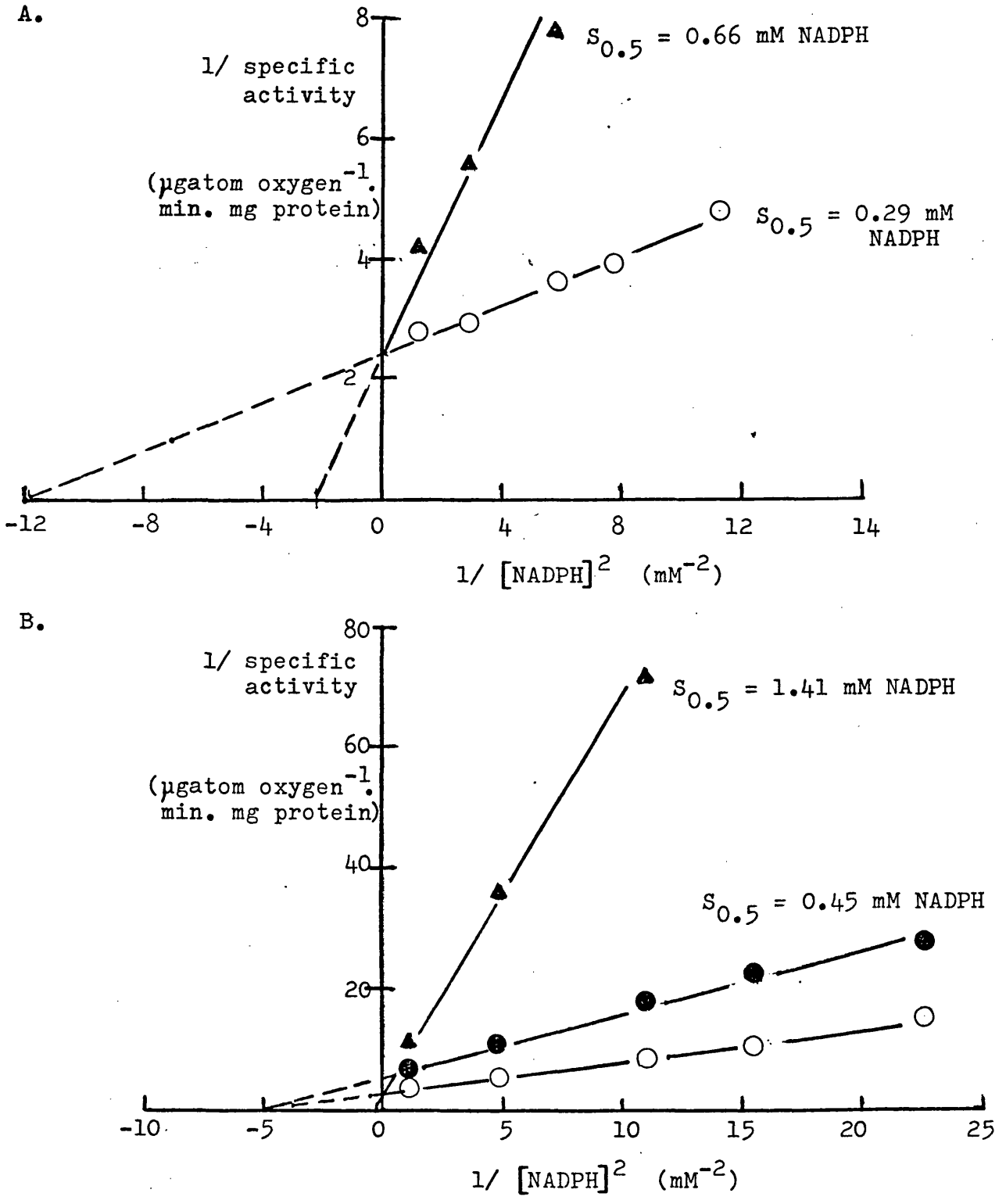
A, membranes prepared from excess oxygen cells; B, membranes prepared from oxygen-limited cells. ○ , no inhibitor; ● , plus 35μM NAD^+; ▲ , plus 4.0mM AMP.

1 (Fig. II-18A). The Lineweaver-Burk plot revealed that there was no decrease in V_{max} in the presence of NAD^+, indicating that the inhibition was competitive. Similar characteristics for NAD^+ inhibition of the NADPH dehydrogenase of membranes prepared from nitrogen-fixing cells were found by Ackrell *et al.* (1972). Rather surprisingly, with membranes obtained from urea-grown cells in the early oxygen-limited phase of growth, the sigmoidal saturation kinetics with respect of NADPH oxidation were maintained in the presence of 35 μM NAD^+ (Fig. II-17B; $n_{NADPH} \simeq 2$, Fig. II-18B)

Straight lines were obtained when the data from Fig. II-17 were replotted as $^1/v$ against $^1/[NADPH]^2$ (Fig. II-19; NAD^+-modulated NADPH oxidation by membranes from excess oxygen cells excluded). The unmodulated NADPH dehydrogenase yielded $S_{0.5}$ values of 0.29 and 0.45 μM NADPH for membranes prepared from excess oxygen cells and oxygen-limited cells respectively. These values were similar to that obtained by Ackrell *et al.* (1972) for the unmodulated enzyme of membranes derived from nitrogen-fixing cells ($S_{0.5}$ = 0.44 mM NADPH). In the presence of AMP, there was no change in V_{max} but an increase in $S_{0.5}$ to 0.66 and 1.41 mM NADPH for membranes prepared from excess oxygen and oxygen-limited urea-grown cells respectively. This indicated that AMP acted as a competitive inhibitor with respect to NADPH. A plot of $^1/v$ against $^1/[NADPH]^2$ for NADPH oxidation by membranes derived from oxygen-limited cells in the presence of 35 μM NAD^+ also yielded a straight line, but in this case there was a decrease in V_{max} from 0.36 to 0.18 μgatom $O.min^{-1}.$ mg $protein^{-1}$ while the $S_{0.5}$ remained the same as for uninhibited NADPH oxidation (i.e. 0.45 mM NADPH). This was indicative of non-competitive inhibition by NAD^+ of NADPH oxidation. The kinetic parameters of the

Fig. II-19

1/v versus $1/S^2$ plots of the data shown in Fig. II-17



A, membranes prepared from excess oxygen cells; B, membranes prepared from oxygen-limited cells. ○ , no inhibitor; ● , plus 35µM NAD^+; ▲ , plus 4.0mM AMP.

NADPH dehydrogenase of respiratory membranes obtained from nitrogen-free-grown and urea-grown A. vinelandii are summarised in Table II-3.

Plots of $v/V_{max} - v$ against inhibitor concentration showed that both AMP and NAD^+ exhibited Hill coefficients ($n_{inhibitor}$) of approximately 1 (Fig. II-20). The I_{50} value for NAD^+ inhibition of the NADPH dehydrogenase of membranes prepared from excess oxygen cells was somewhat higher than that of membranes prepared from oxygen-limited cells (I_{50} = 125 μM, cf. 36 μM NAD^+). The same was true for inhibition by AMP (I_{50} = 7.8 mM, cf. 1.7 mM AMP).

Dixon plots confirmed that inhibition of the NADPH dehydrogenase by AMP was competitive and, further, showed that the K_i for AMP was higher for membranes prepared from excess oxygen cells than for membranes prepared from oxygen-limited cells (1.15, cf. 0.37 mM AMP; Fig. II-21). This reflected the differences in I_{50} discussed above. Dixon plots for NAD^+ inhibition confirmed that, for membranes prepared from excess oxygen cells, the inhibition with respect to NADPH oxidation was competitive (K_i = 69 μM NAD^+; Fig. II-22A) whereas for membranes prepared from oxygen-limited cells the inhibition was non-competitive (K_i = 33 μM NAD^+; Fig. II-22B).

The effect of NAD^+ and AMP on NADPH oxidation by membranes prepared from oxygen-limited urea-grown cells was further analysed by measuring the NADPH oxidase activity at various inhibitor concentrations. Plots of ^{1}/fractional inhibition (i) versus ^{1}/[inhibitor] (Fig. II-23) were linear, showing that inhibition of the NADPH dehydrogenase was a hyperbolic function of the AMP and NAD^+ concentration (Webb, 1963). This suggested that only one molecule of each inhibitor reacted with the dehydrogenase, a conclusion that was compatible with the $n_{inhibitor}$ values

Table II-3

The effect of NAD^+ and AMP on the kinetics of NADPH oxidation

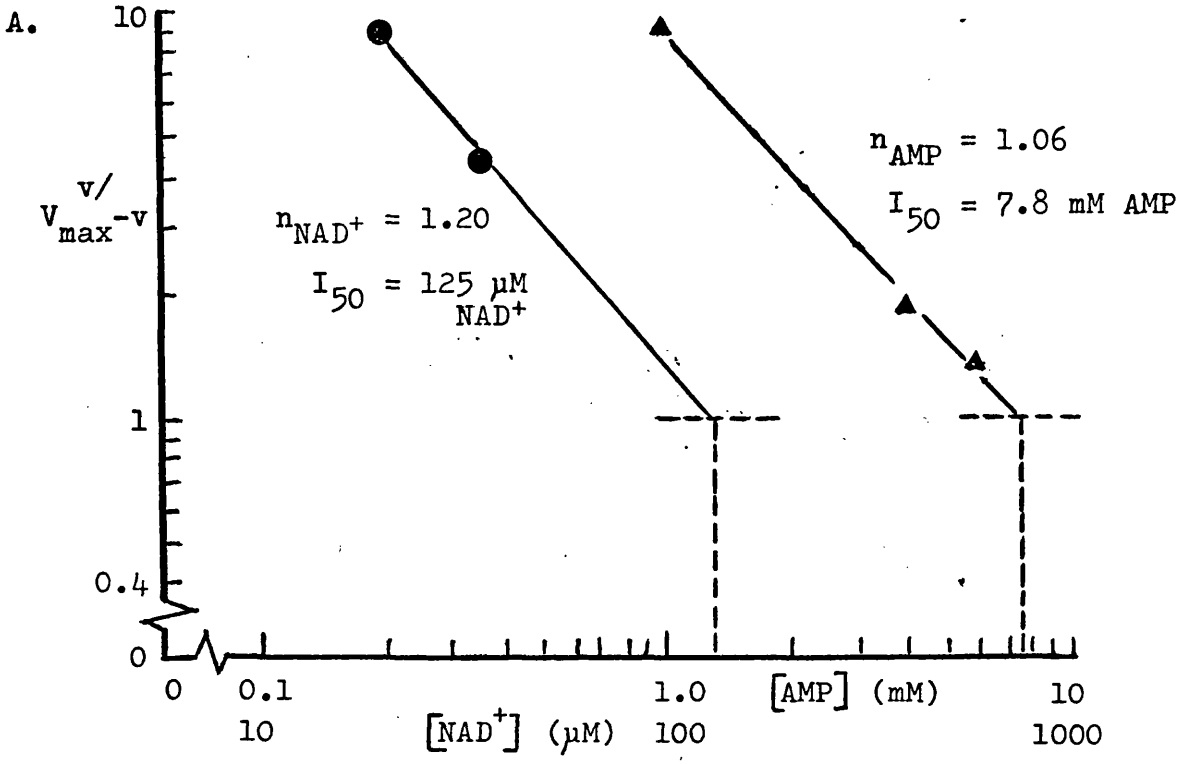
Nitrogen source	Growth conditions	Inhibitor	V_{max} (μg atom O.min^{-1}. mg protein^{-1})	$S_{0.5}$ (mM NADPH)	K_m (mM NADPH)	n_{NADPH}
Atmospheric nitrogen*	early oxygen-limited	none	3.3	0.44		1.85
		+NAD^+	3.3		1.28	0.98
		+AMP	3.3	1.15		1.78
Combined nitrogen (urea)	excess oxygen	none	0.40	0.29		2.25
		+NAD^+	0.40		0.55	1.26
		+AMP	0.40	0.66		1.97
	early oxygen-limited	none	0.36	0.45		2.12
		+NAD^+	0.18	0.45		2.12
		+AMP	0.36	1.41		1.72

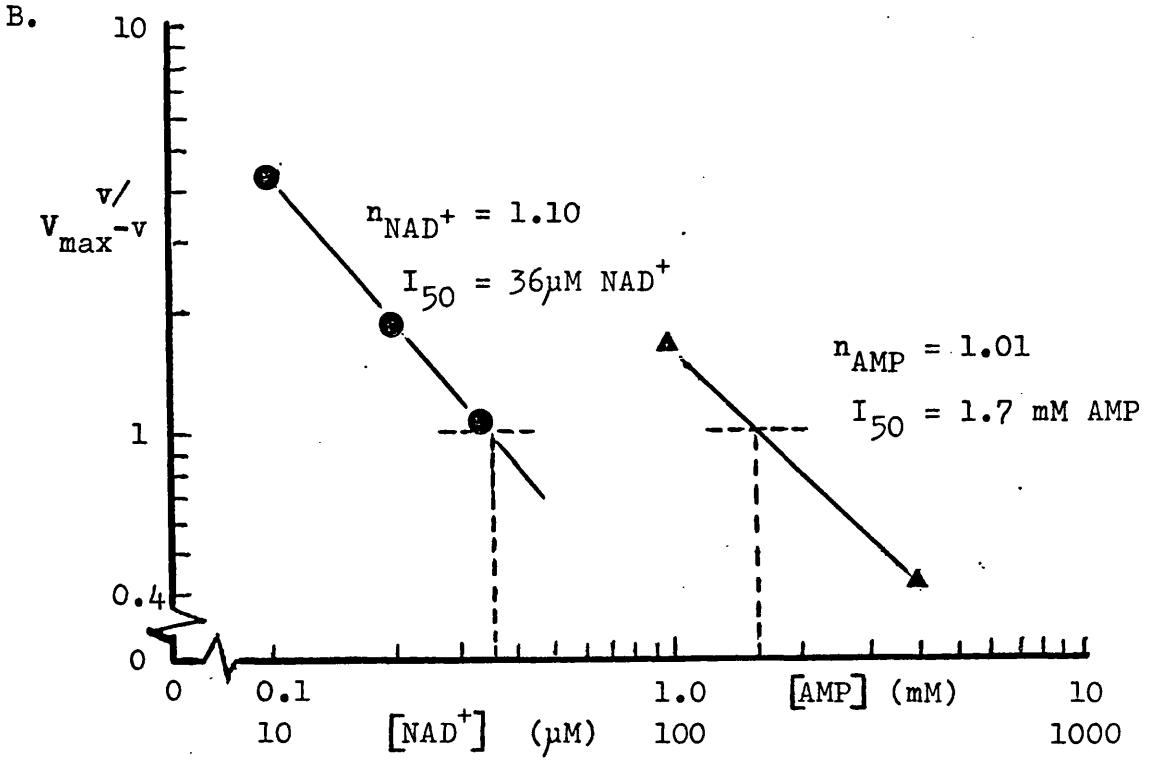
*Data from Ackrell et al.(1972)

V_{max} values were obtained from plots of 1/v versus $1/[NADPH]^2$. n_{NADPH} values were obtained using the V_{max} values in the table rather than those from 1/v versus 1/[NADPH] plots, for reasons of accuracy. Inhibitor concns.; NAD^+ 35 μM, AMP 4.0 mM.

Fig. II-20

Hill plots of inhibition by NAD^+ and AMP of the NADPH dehydrogenase of respiratory membranes prepared from urea-grown A.vinelandii

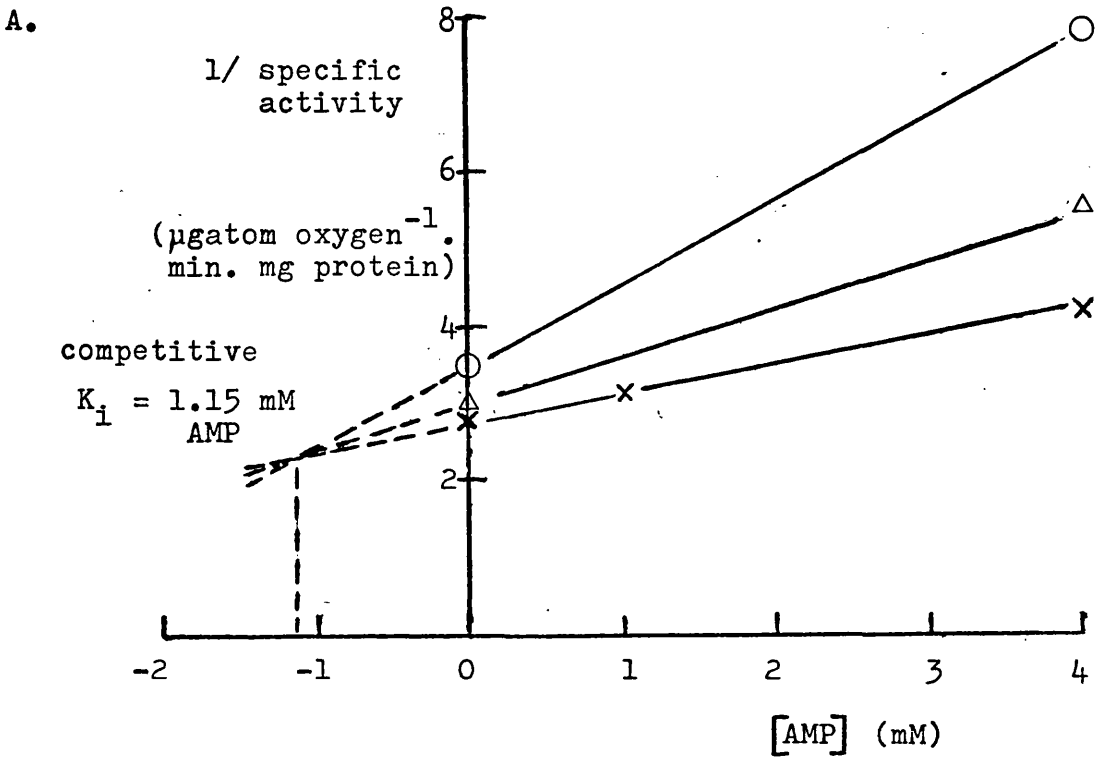


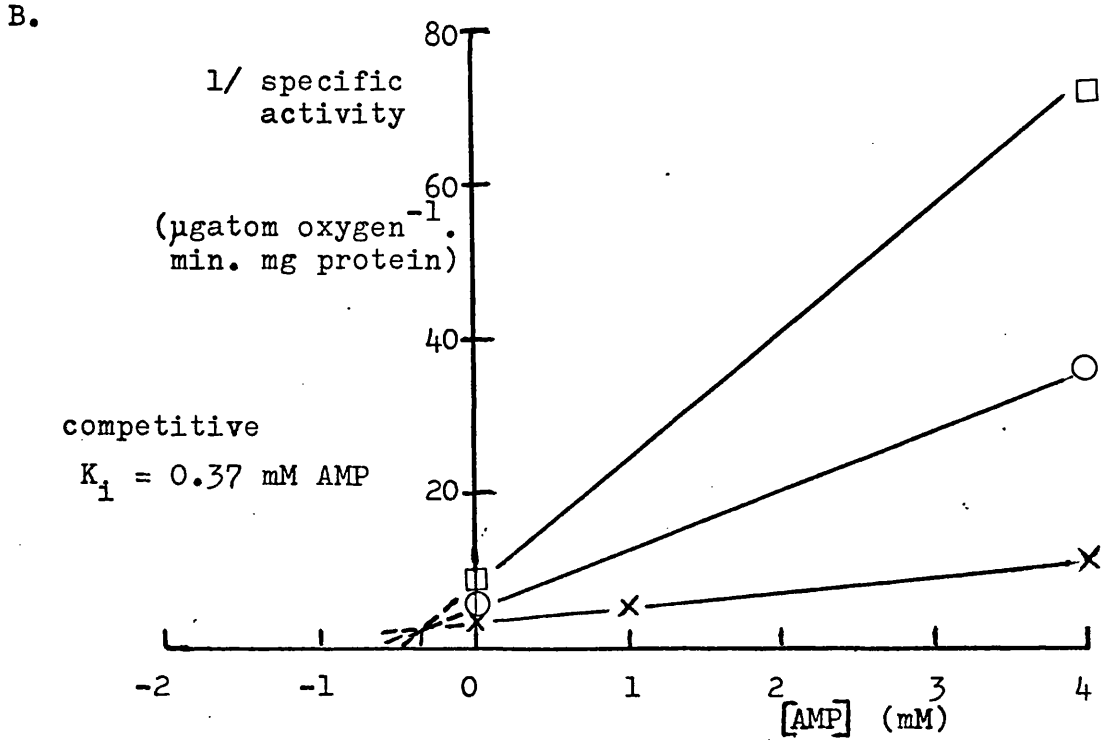


A, membranes prepared from excess oxygen cells; B, membranes prepared from oxygen-limited cells. NADPH concentration = 0.90 mM.

Fig. II-21

Dixon plots of the inhibition by AMP of the NADPH dehydrogenase

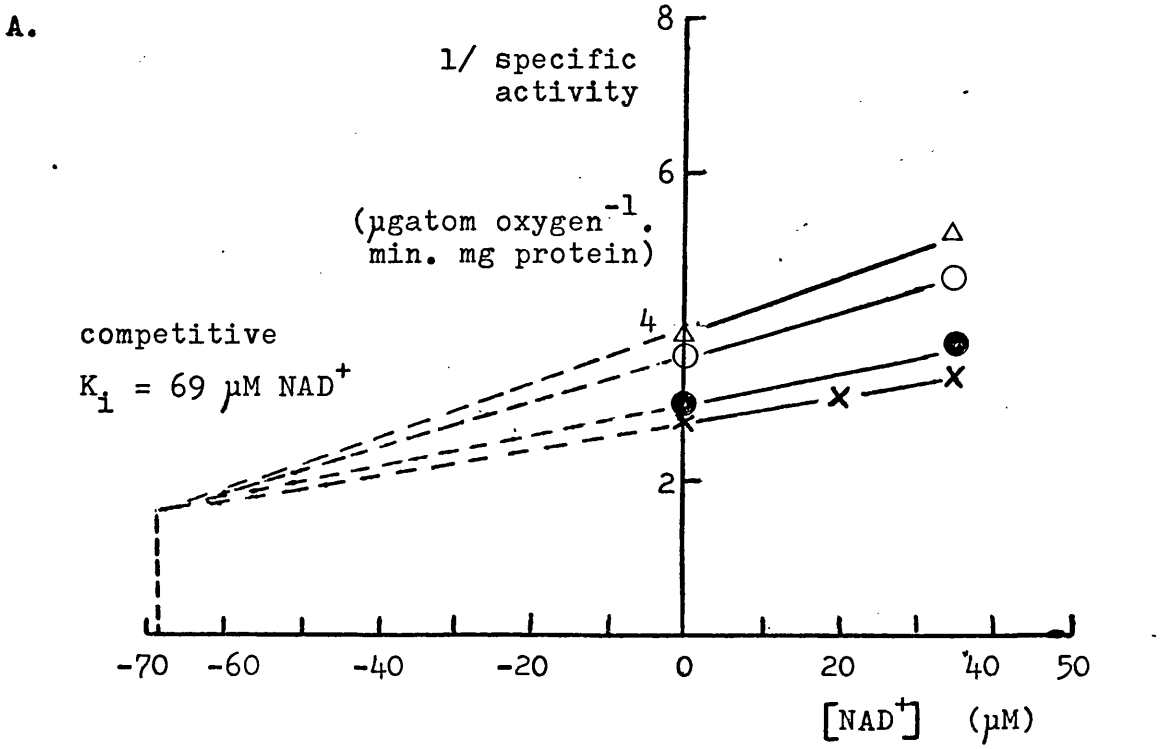


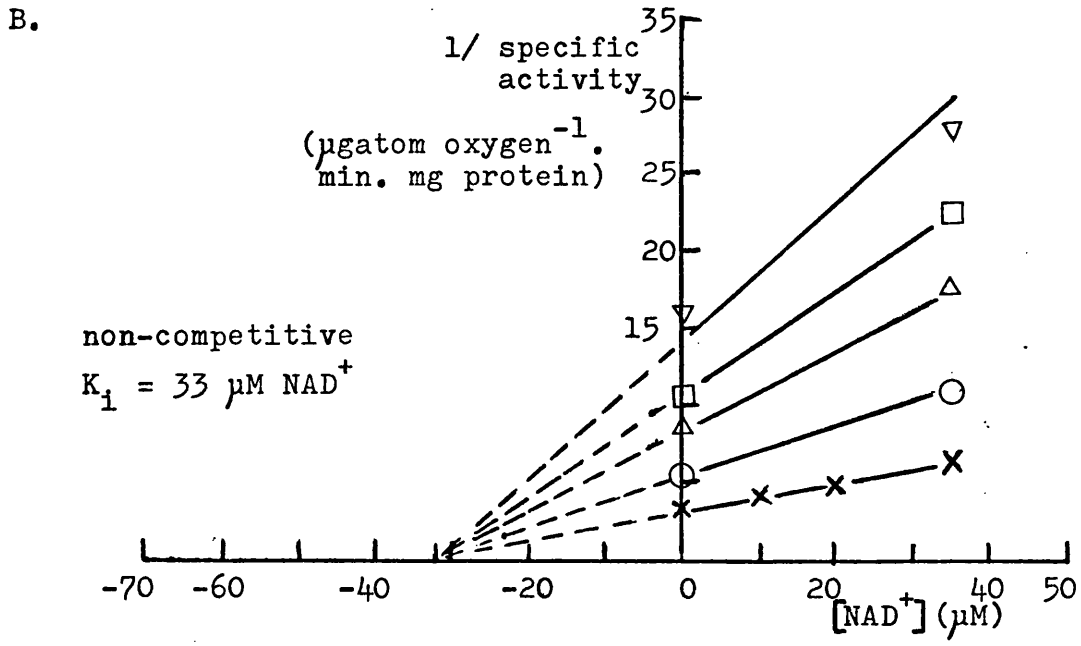


A, membranes prepared from excess oxygen cells; B, membranes prepared from oxygen-limited cells. NADPH concentration; □ , 0.30 mM; ○ , 0.45 mM; △ , 0.60 mM; × , 0.90 mM

Fig. II-22

Dixon plots of the inhibition by NAD^+ of the NADPH dehydrogenase

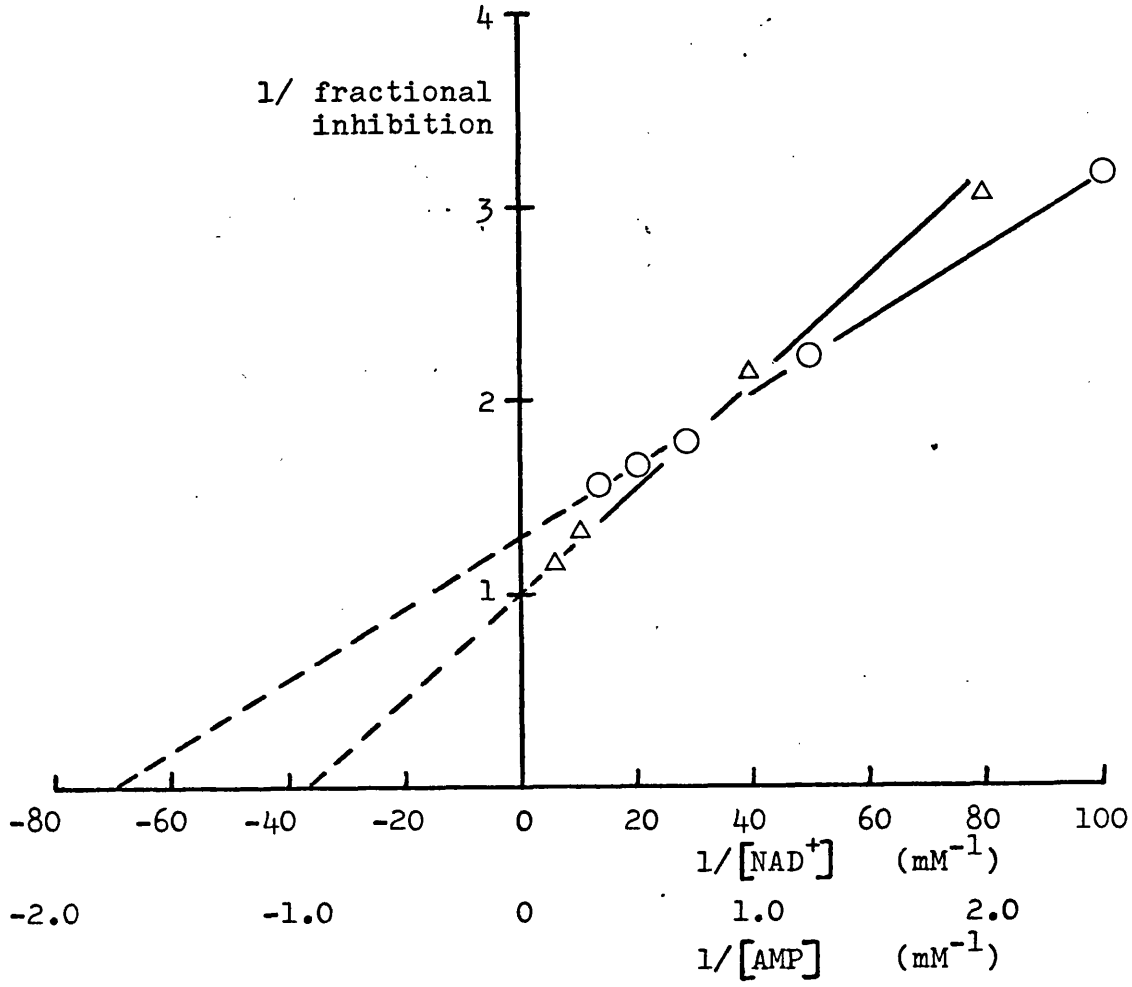




A, membranes prepared from excess oxygen cells; B, membranes prepared from oxygen-limited cells. NADPH concentration; ▽ , 0.21 mM; □ , 0.26 mM; △ , 0.30 mM; ○ ,0.45 mM; ● , 0.60 mM; × , 0.90 mM.

Fig. II-23

1/ i versus 1/ [I] plots of the inhibition by AMP and NAD^+ of the NADPH dehydrogenase



Respiratory membranes were prepared from A.vinelandii harvested in the oxygen-limited phase of growth on urea medium (A_{680} = 3.24). △ , AMP; ○ , NAD^+. NADPH concentration 0.90 mM.

of approximately 1 obtained in Fig. II-20. With AMP as inhibitor, the intercept on the ordinate was 1.0 which indicated complete inhibition of the NADPH dehydrogenase at infinite AMP concentration. Thus AMP acted as a pure (dead-end) competitive inhibitor. With NAD^+ as inhibitor, the $^1/i$ intercept value was > 1.0 indicating that NAD^+ was a partially non-competitive inhibitor. Ackrell et al. (1972) also found that AMP and NAD^+ were pure and partial inhibitors respectively of the NADPH dehydrogenase of membranes prepared from nitrogen-fixing cells. It was therefore assumed that these inhibitors acted similarly on the NADPH dehydrogenase of excess oxygen urea-grown cells. The characteristics of the inhibition by NAD^+ and AMP of the NADPH dehydrogenase of membranes prepared from A. vinelandii grown under the different culture conditions are summarised in Table II-4.

The NADPH dehydrogenase of membranes prepared from combined-nitrogen-grown cells was inhibited by ADP and ATP as well as by AMP. The inhibition was assumed to be pure, competitive. There was only slight inhibition by inorganic phosphate. The percentage inhibition of NADPH oxidation in the presence of 4 mM AMP/ADP/ATP and also inorganic phosphate is shown in Table II-5. Minimum activity of the NADPH dehydrogenase was observed under conditions of high energy charge with membranes prepared from excess oxygen urea-grown cells but under conditions of low energy charge for membranes prepared from early oxygen-limited cells. Membranes from nitrogen-fixing cells resembled the latter preparation in this respect (Ackrell et al., 1972).

In the presence of high concentrations (400 μM) of NAD^+, there was a stimulation in the rate of NADPH oxidation, especially at low substrate concentrations (< 0.06 mM NADPH), by membranes prepared

Table II-4

A summary of the characteristics of inhibition by NAD^+ and AMP of the NADPH dehydrogenase

Nitrogen source	Growth conditions	Inhibitor	I_{50} (μM)	K_i (μM)	$n_{inhibitor}$	Type of inhibition
Atmospheric nitrogen*	early oxygen-limited	NAD^+	19	7.1	approx. 1	partially competitive
		AMP	2200	730	approx. 1	pure competitive
Combined nitrogen (urea)	excess oxygen	NAD^+	125	69	1.20	competitive (probably partial)
		AMP	7800	1150	1.06	competitive (probably pure)
	early oxygen-limited	NAD^+	36	33	1.10	partially non-competitive
		AMP	1700	370	1.01	pure competitive

*Data from Ackrell et al.(1972)

Table II-5

Inhibition of the NADPH dehydrogenase by adenine nucleotides

Nitrogen source	growth conditions	% inhibition			
		AMP	ADP	ATP	Pi
Atmospheric nitrogen*	early oxygen-limited	74	47	28	-12
Combined nitrogen (urea)	excess oxygen	34.4	52.6	89.5	10.0
	early oxygen-limited	75.2	74.7	58.0	0.5

*Data from Ackrell et al.(1972).

Concentrations of effectors; preparations from nitrogen-fixing cells, 5 mM; preparations from urea cells, 4 mM.

from both excess oxygen and oxygen-limited urea-grown cells. This probably reflected the formation of NADH *via* an NADPH $\longrightarrow$ NAD^+ transhydrogenase followed by its subsequent rapid oxidation by NADH oxidase, which was much more active in these preparations than the NADPH oxidase (Table II-2). Thus the transhydrogenase appears to be active in urea-grown *A. vinelandii* as well as in the nitrogen-fixing organism (Ackrell *et al.*, 1972).

The properties of the NADPH dehydrogenase of urea-grown *A. vinelandii* are therefore summarised as follows. NADPH acted as an allosteric activator of its own respiratory chain dehydrogenase. The adenine nucleotides AMP, ADP and ATP were inhibitors with respect to NADPH and the inhibition by AMP, at least, was pure, competitive. NAD^+ was a more potent inhibitor of NADPH oxidation by membranes prepared from oxygen-limited cells than by membranes prepared from excess oxygen cells. NAD^+ inhibition with the former membranes was partially non-competitive whereas with the latter membranes it was (partially) competitive. Only one molecule of AMP/NAD^+ was bound on the dehydrogenase. These results can be interpreted in the following manner. The NADPH dehydrogenase probably contained (a) a catalytic site which bound one molecule of NADPH or adenine nucleotide, (b) a further site (or sites) for the binding of one molecule of NADPH (homotropic allostery) or, in the case of excess oxygen cells, one molecule of NAD^+ (heterotrophic allostery). NAD^+ may or may not have been bound at the same site as NADPH. These characteristics are exactly those proposed for the NADPH dehydrogenase of nitrogen-fixing cells (Ackrell *et al.*, 1972). The enzyme of oxygen-limited, urea-grown cells presumably differed from the above since the presence of NAD^+ did not decrease the Hill coefficient with respect

to NADPH (n_{NADPH}) below 2. Thus NAD^+ must have acted at a site functionally unconnected with the allosteric NADPH binding site.

A possible rationale for the characteristics of the inhibition by NAD^+ and adenine nucleotides of the NADPH dehydrogenase of urea-grown A. vinelandii is as follows. Under excess oxygen conditions NAD^+ was a relatively poor inhibitor. Since the inhibition under these conditions was competitive with respect to NADPH, high concentrations of the latter could substantially overcome the inhibition and NADPH oxidation could proceed via the dehyrogenase. Under oxygen-limited conditions, NAD^+ was a more potent inhibitor (lower K_i) and since the inhibition was non-competitive, high concentrations of NADPH would be less effective at overcoming the inhibition of the dehyrogenase. NADPH would therefore tend to be oxidised via the NADPH $\longrightarrow$ NAD^+ transhydrogenase yielding NADH which would be oxidised via the energy-conserving NADH dehydrogenase. There would be no energetic advantage in this procedure under excess oxygen conditions since the NADH dehydrogenase was then only poorly phosphorylating. Indeed, trans-hydrogenation of NADPH instead of direct oxidation would probably have limited the rate of electron transport to sites II and III under these circumstances and would hence have limited the rate of ATP synthesis and growth (the NADH and NADPH dehydrogenases were the rate-limiting components of their respective oxidases; see section 2 above).

The observed maximum inhibition of the NADPH dehydrogenase of membranes prepared from oxygen-limited cells under conditions of low energy charge would likewise have causedpreferential, and energetically advantageous, oxidation of NADPH via the transhydrogenase and phosphorylating NADH dehydrogenase. There would be no advantage in

this under excess oxygen conditions due to the poorly coupled NADH dehydrogenase and it is therefore not too surprising that minimum activity of the NADPH dehydrogenase of membranes prepared from cells grown under these conditions occurred in the presence of high energy charge. This situation is analogous to classical respiratory control.

Essentially the same pathways of NADPH oxidation were postulated to occur in nitrogen-fixing A. vinelandii (Ackrell et al., 1972). Under conditions of oxygen stress, when growth was inhibited due to inactivation of the nitrogenase, the major NADPH utilising systems (i.e. nitrogen fixation and biosynthesis) would have been inoperable and the resultant, high intracellular NADPH concentration would have overcome the potent (competitive) inhibition by NAD^+ and allowed rapid oxidation of NADPH by its dehydrogenase. During nitrogen fixation and growth, when the concentration of NADPH was presumably much lower, inhibition of the NADPH dehydrogenase by NAD^+ and low energy charge would have ensured NADPH oxidation predominantly via the transhydrogenase and the phosphorylating NADH dehydrogenase.

Because of the much lower activity of the NADPH dehydrogenase in membranes prepared from urea-grown cells than in membranes prepared from nitrogen-fixing cells, this enzyme probably plays a more important role during nitrogen-free growth. Indeed, it is probably responsible for allowing nitrogen-fixing cells to respire much faster than urea-grown cells under excess oxygen conditions (see section 1 above), thus in part eliciting the respiratory protection response.

5. Growth yields

Chemostat studies on a number of nitrogen-fixing bacteria have shown that the efficiency of conversion of carbon/energy source into cell material ($Y_{substrate}$) is decreased when molecular nitrogen replaces ammonia as the nitrogen source. This is true of the aerobic organism A. chroococcum (Dalton and Postgate, 1969a, b), the facultative anaerobe Klebsiella pneumoniae (Daesch and Mortenson, 1968) and the anaerobes Clostridium pasteurianum and Desulphovibrio desulphuricans (Hill et al., 1972). These studies suggest that the efficiency of synthesis of cell material from ATP equivalents (Y_{ATP}) is lower during growth on nitrogen-free medium than during growth on combined nitrogen medium, presumably because of the energy requirements of nitrogen fixation. From the whole cell chemostat work, Hill et al. (1972) calculated that at least 18 mol ATP were required for reduction of 1 mol N_2, except in the case of Azotobacter which appeared to carry out N_2 reduction more efficiently. Cell-free preparations yielded an average value of 15 mol ATP consumed.mol N_2 reduced^{-1} (Burris, 1971).

Unfortunately, it was not possible to obtain Y_{ATP} values for A. vinelandii grown under nitrogen-free and fixed nitrogen conditions from the data presented in section 1 above. This would have required measurement of the respiratory activity at different growth rates using a chemostat which was not available at the time (see Chapter IV Introduction) It is only feasible to measure Y_{ATP} for A. vinelandii under oxygen-limited conditions in chemostat culture, since under any other nutrient-limiting conditions the dissolved oxygen concentration would be variable (unless the chemostat is equipped with an oxystat) and hence give variable N values (mol ATP equivalents synthesised per mol O_2 consumed) due to the

oxygen-sensitive respiratory system. This in turn would lead to spurious plots of QO_2 *versus* D (from which Y_{ATP} values are obtained; see Chapter IV) as found by Nagai and Aiba (1972) for glucose-limited cultures.

For *A. vinelandii* ATCC 9046 growing under oxygen-limited, nitrogen-fixing conditions in chemostat culture, Nagai and Aiba (1972) obtained a $Y_{O_2(true)}$ value (see Chapter I) of 13 g cells.mol O_2^{-1} and an m_{O_2} value of 0.0055 mol O_2.g $cells^{-1}.h^{-1}$. Using the chemostat equipment described in Chapter IV, Dr. C.W. Jones (personal communication) has recently measured $Y_{O_2(true)}$ values (= $Y_{ATP}.N$) and m_{O_2} values (= M/N) for *A. vinelandii* NCIB 8660 (the strain used in the present work) growing under oxygen-limited conditions on both nitrogen-free and urea medium. The results, together with those of Nagai and Aiba (1972) are shown in Table II-6. The $Y_{O_2(true)}$ values of the two strains growing under nitrogen-fixing conditions were quite similar; the $Y_{O_2(true)}$ value of urea-grown NCIB 8660 was much higher. Rather surprisingly, the maintenance coefficient (m_{O_2}) of NCIB 8660 was much lower under both growth conditions than that of ATCC 9046.

Determinations of $\longrightarrow H^+/O$ ratios were also performed on the NCIB 8660 cultures and were found to be virtually invariant over the range of dilution rates employed. Both nitrogen-free and urea cultures gave $\longrightarrow H^+/O$ quotients in the range 3.6-3.8, which were close to the values obtained for oxygen-limited batch cultures (Fig. II-12). In order to calculate Y_{ATP} values for this strain, it was necessary to obtain values for N. These were not numerically equal to the $\longrightarrow H^+/O$ quotients obtained under the two growth conditions because account had to be taken of ATP generated by substrate-level phosphorylation (see Chapter IV).

Table II-6

Growth yields and maintenance coefficients of A.vinelandii

Strain	Nitrogen source	Y_{O_2} (true) (= Y_{ATP}.N) (g cells.mol O_2^{-1})	m_{O_2} (= M/N)	$\rightarrow H^+/O$ quotient (g ion H^+. g atom O^{-1})	N (mol ATP equivs. mol O_2^{-1})	Y_{ATP} (g cells. mol ATP equivs.$^{-1}$)	M (mol ATP equivs. g cells^{-1}.h^{-1})
ATCC 9046*	N_2	13	0.0055	—	—	—	—
NCIB 8660	N_2	11.7	0.00080	3.79 ± 0.11(11)	4.15	2.82	0.0033
	urea	27.4	0.00194	3.63 ± 0.18(10)	3.99	6.86	0.0078

*Data from Nagai and Aiba(1972).

Conditions of assay of $\rightarrow H^+/O$ quotients as in Fig. II-12. Results expressed as the average ± S.E.M. (no. of determinations).

A. vinelandii contains enzymes of both the Entner-Doudoroff and phosphogluconate pathways, both of which are probably major metabolic routes (Mortenson and Wilson, 1954, 1955; Johnson and Johnson, 1961). Complete oxidation of 1 mol mannitol via the Entner-Doudoroff pathway and the tricarboxylic acid cycle would yield 3 mol ATP (net) by substrate-level phosphorylation whereas oxidation via the phosphogluconate pathway (yielding fructose 6-phosphate and glyceraldehyde 3-phosphate) and the tricarboxylic acid cycle would yield $3\frac{1}{3}$ mol ATP (net). Assuming that the latter pathway is used predominantly and that 1 mol ATP equivalent is required for uptake by the bacterium of 1 mol mannitol, complete oxidation of the latter would yield $2\frac{1}{3}$ mol ATP (net) by substrate-level phosphorylation, i.e. $2\frac{1}{3} \div 6.5 = 0.36$ mol ATP per mol O_2 consumed. This figure must therefore be added onto the average $\longrightarrow H^+/O$ ratios in order to obtain values for N (i.e. total mol ATP equivalents synthesised per mol O_2 consumed). The resultant N values for the nitrogen-free and urea cultures are given in Table II-6, together with the calculated Y_{ATP} and M values.

As anticipated by other workers (Nagai et al., 1969; Hill et al., 1972) the Y_{ATP} for Azotobacter growing on nitrogen-free medium (2.82 g cells.mol ATP equivalents^{-1}) was much lower than for growth on fixed nitrogen medium (6.86 g cells.mol ATP equivalents^{-1}), presumably due to the energy requirements of nitrogen fixation. Rather unexpectedly, the maintenance coefficient (M) for growth on nitrogen-free medium was lower than for growth on urea medium (0.0033, cf. 0.0078 mol ATP equivalents.g cells^{-1}.h^{-1}).

It is apparent that this large difference in Y_{ATP} could readily account for the lower respiratory activity exhibited by

A. vinelandii growing logarithmically in batch culture on urea medium compared with that observed during growth on nitrogen-free medium (see secion 1, Fig. II-11).

Conclusions

It might be expected that A. vinelandii, when growing on a source of combined nitrogen, would adapt its respiratory system to give the highest possible efficiency of energy conservation whatever the dissolved oxygen concentration of the culture (with the exception of severely oxygen-limited conditions; see Ackrell and Jones, 1971b), as there is no need for respiratory protection in the absence of nitrogenase. Thus one might anticipate that, under highly aerobic conditions, energy conservation at site I would be preserved, that high concentrations of the phosphorylating cytochrome branch $\underline{c} \longrightarrow \underline{a_1}/\underline{o}$ would be present and that, perhaps, a phosphorylating NADPH dehydrogenase with normal (hyperbolic) saturation kinetics would be present. In order to consider why these changes from the respiratory system of nitrogen-fixing cells were not observed for urea-grown cells, it is necessary to discuss the evolution of nitrogen fixation.

Nitrogen fixation is regarded as being a phylogenetically old property (De Ley and Park, 1966). However, the primitive environment probably contained considerable amounts of free ammonia (Bada and Miller, 1968) and it would seem uneconomic for primaeval organisms to have fixed nitrogen under such conditions, since it is a highly endergonic process (Burris, 1971). This led Silver and Postgate (1973) to propose that the original function of nitrogenase may have been to reduce substrates other than nitrogen gas which were toxic to the primitive organisms, possibly cyanide and cyanogen; these compounds are both reduced by contemporary nitrogenases (see Dalton and Mortenson, 1972; Silver and Postgate, 1973). If this was the case, nitrogenase was presumably used for reduction of nitrogen gas only when the ammonia content of the primitive biosphere

had dropped to such a low level that nitrogen fixation conveyed a selective advantage to the organisms concerned, in spite of its high energy requirements. In Azotobacter, this must have been accompanied by the development of the conformational and respiratory protection responses eliciting nitrogen fixation under aerobic conditions. Azotobacter was consequently able to occupy aerobic, nitrogen-deficient environmental niches in which it was dominant over other organisms unable to fix nitrogen or else able to do so only under anaerobic conditions (this assumes that the ability to fix nitrogen was a crucial factor in the survival of Azotobacter, as is likely). The poor energy-conserving efficiency of the respiratory system of Azotobacter due to respiratory protection was presumably of little consequence since the organism was not competing with other organisms for carbon/energy source under these conditions.

Hence, the species of Azotobacter found on earth today are presumably organisms which have survived by competing effectively with other organisms under aerobic, nitrogen-deficient conditions. It is perhaps not too surprising, therefore, that A. vinelandii does not adapt its respiratory system to conserve energy efficiently during growth on a source of combined nitrogen since, in the past, its ability to fix atmospheric nitrogen has presumably favoured its adaptation to nitrogen-deficient environments.

CHAPTER III

THE RESPIRATORY SYSTEM OF *Bacillus megaterium*

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THE RESPIRATORY SYSTEM OF *Bacillus megaterium*

SUMMARY

1. The respiratory systems of two strains of *B. megaterium* (D440 and M) were studied following growth in batch culture on a glycerol/minimal-salts medium under excess oxygen and oxygen-limited conditions.

2. Both strains of the organism contained cytochromes *b*, *a* and *o*. Strain D440 also contained cytochrome a_3 but appeared to lack cytochrome *c* whereas strain M contained cytochrome *c* but lacked cytochrome a_3. The concentrations of all of the cytochrome species were higher in the respiratory membranes prepared from cells grown under oxygen-limited conditions.

3. Respiratory membrane preparations from both strains exhibited NADH, malate, glutamate, succinate, glycerol 3-phosphate and ascorbate/TMPD oxidase activities. In addition, membranes of strain M oxidised NADPH, probably *via* a respiratory chain dehydrogenase rather than *via* a NADPH $\longrightarrow$ NAD^+ transhydrogenase. Malate oxidation was NAD-linked in membranes of strain D440 but predominantly flavin-linked in membranes of strain M. Glycerol 3-phosphate oxidation was flavin-linked and glutamate oxidation NAD-linked in both strains. The V_{max} was much higher and the K_m much lower for ascorbate/TMPD oxidation by membranes of strain M than by membranes of strain D440, reflecting the presence of cytochrome *c* in the former organism.

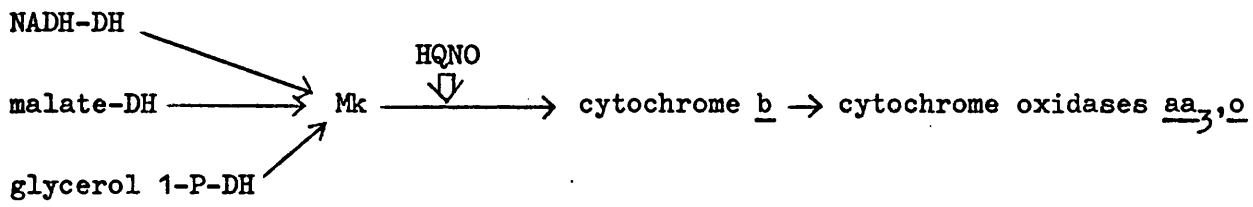
4. Maximum $\longrightarrow H^+/O$ quotients of approximately 4 were obtained for whole-cell preparations of strain D440 grown under excess oxygen conditions and for preparations of strain M grown under both excess oxygen and oxygen-limited conditions. Substrate-loading experiments similar to those described by Lawford and Haddock (1973) demonstrated the presence of proton translocating loops 1 and 2 in strain D440 and loops 2 and 3 in strain M. Strain M appeared to lack a functional loop 1 and this was probably partially inoperative in strain D440 grown under oxygen-limited conditions.

Introduction

At the commencement of this research work there had been no reports in the literature of measurements of the yield coefficient, Y_{ATP} (g dry weight cells produced per mol ATP generated from catabolism) for growth of aerobic bacteria. This was because of the uncertainty regarding the value of the P/O ratio for aerobic bacteria, which is required for the calculation of Y_{ATP} from growth yield measurements (see Chapter I). Since a technique was available for the measurement of the energy conservation efficiency of aerobes, in the form of $\longrightarrow H^+/O$ ratio determinations, an opportunity presented itself to calculate Y_{ATP} values for an aerobic bacterium.

Azotobacter vinelandii was not considered suitable for such studies since it has a very complex branched cytochrome system whose energy conservation efficiency varies according to the growth conditions, as described in the preceding chapter. Instead, Bacillus megaterium was chosen for this purpose. This organism is a Gram-positive, obligately aerobic, spore-forming bacterium. Two early investigations into its cytochrome system, using reduced minus oxidised difference spectrophotometry of membrane preparations of the organism revealed the presence of cytochromes b, c and a in strain M (Weibull and Bergström, 1958) and also in an unspecified strain (Vernon and Mangum, 1960). Later investigations of strain KM (Broberg and Smith, 1967) and ATCC 14581 (Kröger and Dadák, 1969) confirmed the presence of cytochromes b and a and, by the use of carbon monoxide difference spectra, further demonstrated the presence of cytochromes a_3 and o, which presumably acted as terminal oxidases in these bacteria. However, cytochrome c was either missing (strain KM) or present in very low concentrations (strain ATCC 14581) in these particular organisms.

Kröger and Dadák (1969) found NADH, malate and glycerol 3-phosphate dehydrogenase activities in B. megaterium strain ATCC 14581 and also showed that menaquinone is an essential redox component in the oxidation of endogenous substrates by respiratory membranes of this organism. They further showed by studies with the inhibitor HQNO that menaquinone is located in the respiratory chain on the substrate side of the point sensitive to HQNO whereas the cytochromes are located on the oxygen side;



The cytochrome system of B. megaterium was therefore considered to be simpler than that of A. vinelandii since it was apparently unbranched and contained only cytochrome oxidases aa_3 and o (cf. a_1, a_2 and o in A. vinelandii). Moreover, the evidence available at the time of the initiation of this work suggested that these two enzymes terminated respiratory chains of identical energy conservation efficiency (see Meyer and Jones, 1973a), hence any variation in the relative amounts of these oxidases during growth (as reported by Broberg and Smith, 1967) would not be expected to alter the P/O ratio. It therefore appeared that the investigation of the energy conservation efficiency and growth efficiency and also the development of the required techniques would be easier and simpler with B. megaterium than with A. vinelandii.

This chapter describes an investigation into the respiratory system of B. megaterium, which was a necessary prelude to the studies on growth efficiency described in the next chapter. Two strains of

B. megaterium were acquired for the present work, one containing and one lacking cytochrome *c*, since it was considered desirable to investigate the possibility that the presence or absence of this component might affect the energy conserving ability of the respiratory chain.

Materials and Methods

1. Culture of B. megaterium

Bacillus megaterium strain D440 was a gift of the Medical Research Council Microbial Systematics Unit, University of Leicester, and B. megaterium strain M was a gift of Dr. C. Weibull. The bacteria were maintained on nutrient agar (Oxoid) slopes in 110g McCartney bottles at 4° following growth for 2 days at 30°. Each month the slopes were subcultured and the purity checked by streaking on nutrient agar plates. The two strains formed colonies of an identical morphological type which became brown on aging at 30°.

The bacteria were grown in liquid culture initially on nutrient broth medium (150 ml broth in 250 ml baffled conical flasks) by shaking overnight on a gyratory incubator at 30°. The broth starter cultures were then used to inoculate minimal-salts starter cultures which were also allowed to grow overnight on the incubator. This procedure avoided the long lag period which preceded growth when minimal-salts cultures were inoculated directly from slopes. The minimal-salts medium contained 4.65 g K_2HPO_4, 0.90 g KH_2PO_4, 2.0 g NH_4Cl, 200 mg $MgSO_4.7H_2O$, 10 mg NaCl, 10 mg $FeSO_4.7H_2O$, 10 mg $MnSO_4.4H_2O$, 2 mg NH_4MoO_4 per litre (Gary and Bard, 1952). The carbon/energy source was glycerol (66 mM). Minimal-salts starter cultures were used to initiate growth in 2 l baffled flasks containing either 600 ml or 1500 ml minimal-salts medium. The two aeration rates routinely used were: high aeration (600 ml medium, oxygen transfer coefficient, $K_LA = 300 \times 10^{-6}$ mol $O_2.l^{-1}.h^{-1}.mm\ Hg^{-1}$) and low aeration (1500 ml medium, $K_LA = 1.41 \times 10^{-6}$).

2. Preparation of respiratory membranes

Cytochrome concentrations and oxidase activities were measured using respiratory membranes prepared from the two strains of B. megaterium by the method of Meyer and Jones (1973a). Following growth to the desired cell density, cells were harvested by centrifugation at 4^{o}, homogenised in glass-distilled water, recentrifuged and resuspended in 25 mM sodium phosphate buffer, pH 7.4, to an absorbance at 680 nm of approximately 50. 10 ml portions were disrupted by sonication for 5 min at 4^{o} using a M.S.E. 100 watt ultrasonic disintegrator at an amplitude of 8 μm peak to peak. The suspensions were re-cooled in ice every 60 sec. Cell debris and unbroken cells were removed by centrifugation at 7000 g for 10 min and the resultant cell-free extract was either used directly for enzyme assays or the respiratory membranes were sedimented by further centrifugation at 160,000 g for 1 h. The membranes were then resuspended in the above phosphate buffer to a protein concentration of 10-30 $mg.ml^{-1}$. The membranes were always used immediately following preparation for measurement of oxidase activities and cytochrome concentrations.

3. Measurement of oxidase activities

Oxidase activities were measured as described in Chapter II. The final concentrations of oxidisable substrates in the reaction chamber were: β-NADH (Grade III, Sigma), 1 mM; NADPH (Boehringer), 2 mM; D,L-malate, 12 mM; sodium D-iso-ascorbate, 2 mM plus TMPD (Fisons), 7.6 mM, L-glycerol 3-phosphate, 12 mM; L-glutamate, 12 mM; succinate, 12 mM.

4. Measurement of cytochrome concentrations

The concentrations of cytochromes b and c were determined from dithionite-reduced minus oxidised difference spectra of respiratory membrane suspensions as described in Chapter II. The concentration of cytochrome a was determined from the same spectra assuming a $m\mathcal{M}\varepsilon$ = 19.4 for the α-peak (602 nm) to the trough on the red side (620-625 nm). The contribution to this α-peak of cytochrome a_3 was first calculated using a $m\mathcal{M}\varepsilon$ = 4.6 at these wavelengths, the a_3 concentration having been calculated from the reduced plus carbon monoxide minus reduced spectrum (see below). The net absorption was then used to calculate the concentration of cytochrome a. The extinction coefficients used were those obtained by Van Gelder (1966) who investigated pure mitochondrial cytochrome oxidase.

The concentrations of cytochromes o and a_3 were determined from dithionite-reduced plus carbon monoxide minus dithionite-reduced difference spectra using the following data:

Cytochrome o, $m\mathcal{M}\varepsilon$ = 170.0 for the Soret region peak (415-418 nm) to trough (430 nm, from Ac. suboxydans; Daniel, 1970). The peak-to-trough measurement could only be used when cytochrome o occurred alone (B. megaterium strain M). When cytochromes o and a_3 occurred together (strain D440), cytochrome o appeared as a shoulder on the cytochrome a_3 peak. Its concentration was therefore calculated by doubling the difference in absorbance between the shoulder at approximately 418 nm and the baseline on the blue side (395-400 nm).

Cytochrome a_3, $m\mathcal{M}\varepsilon$ = 148.0 for the Soret region peak (430 nm) to trough (443 nm, from mitochondrial cytochrome a_3; Vanneste and Vanneste, 1965). Since cytochrome a_3 occurred together with cytochrome o

in B. megaterium strain D440, and the latter interfered with the a_3 peak, the cytochrome a_3 concentration was measured by doubling the difference in absorbance between the trough at 443 nm and the baseline on the red side (460-470 nm).

5. Preparation of cells for substrate-loading experiments

In order to starve cell samples of B. megaterium of endogenous substrates so as to measure $\longrightarrow H^+/O$ quotients when the cells were oxidising added substrates, the method which Lawford and Haddock (1973) utilised for E. coli was tried. The cells were grown in the normal minimal salts medium plus glycerol, harvested during the exponential growth phase and washed twice with medium minus ammonium chloride and glycerol. The cells were then resuspended in this medium to a cell density of approximately 0.15 mg dry wt.ml^{-1} and shaken vigorously in a 2 l baffled flask at 30° in order to deplete the cells of endogenous substrates. However, even up to 6 h shaking was found to be insufficient to reduce the respiratory activity (QO_2) of the cells to < 200 $\mu l O_2 . min^{-1} . mg$ dry wt. $cells^{-1}$, as measured using a Clark electrode (see Chapter II). Added substrates failed to produce a significant stimulation of QO_2 of such cells and measurement of $\longrightarrow H^+/O$ quotients would presumably have reflected oxidation of endogenous rather than of added substrates.

In order to achieve adequate starvation, the following procedure was adopted. Cells were grown overnight under high aeration conditions in minimal-salts medium containing 13 mM glycerol (0.2 x normal concentration) After several hours of exponential growth, the cells became carbon/energy limited on reaching an A_{680} of 1.5-2.0 and consequently became starved of glycerol. The following morning, the cells exhibited endogenous QO_2

values of < 30 $\mu l O_2 . min^{-1} . mg$ dry wt. $cells^{-1}$ which could be significantly stimulated by adding oxidisable substrates. The cells were harvested and prepared as usual for the assay of $\longrightarrow H^+/O$ quotients, as described in Chapter II. These were measured either with no additions (endogenous substrates) or in the presence of one of the following oxidisable substrates: 20 mM glycerol, 20 mM glutamate, 10 mM succinate, 10 mM malate or 12 mM ascorbate plus 7.6 mM TMPD. These were incubated with the cells for 15 min prior to assay; the cells thus became "loaded" with substrate. Respiratory activities of the cells were measured at the time of the $\longrightarrow H^+/O$ ratio determinations either with no additions (endogenous respiration) or in the presence of one of the above substrates at the same final concentration.

Results and Discussion

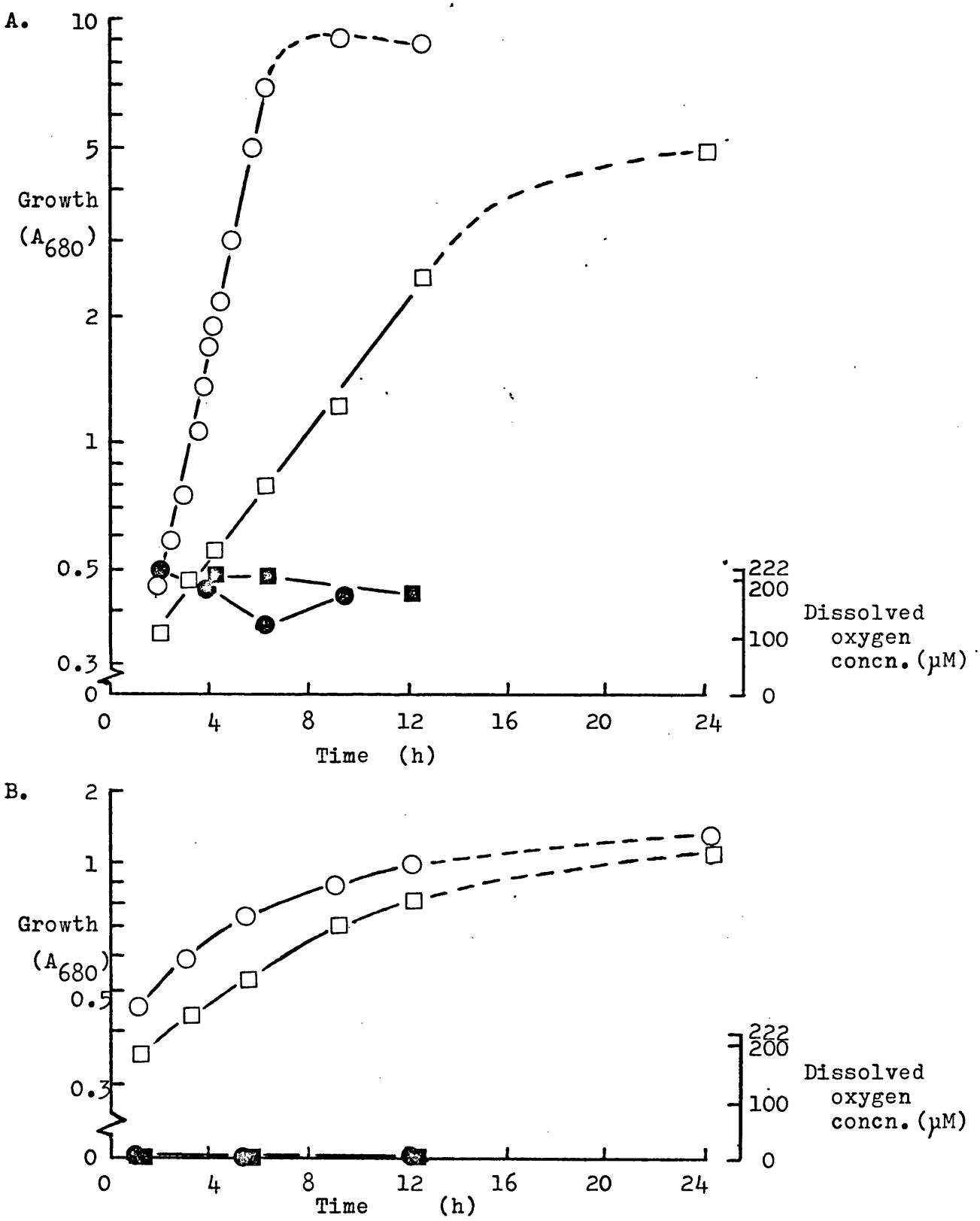
1. Growth characteristics of B. megaterium in batch cultures

B. megaterium strains D440 and M were grown in batch culture under both high aeration and low aeration conditions. Throughout high aeration growth, oxygen was in excess, as shown by measurements of the dissolved oxygen concentration, which did not drop below 130 μM. It was found in other experiments that dissolved oxygen concentrations of $\leq$ 10 μM were required to induce a decrease in growth rate when all other nutrients were in excess (this was therefore the critical oxygen concentration; Harrison, 1973). Growth under high aeration conditions was logarithmic for both strains until high cell densities were reached (Fig. III-1A). The specific growth rate of strain D440 was much higher than that of strain M (μ = 0.63 h^{-1} and 0.17 h^{-1} respectively). At high cell densities an unknown factor (probably glycerol) became growth-limiting. This was accompanied in cultures of strain D440 by an increase in the dissolved oxygen concentration from 130 μM to 190 μM. The final cell density of the cultures of strain D440 was higher than that of the cultures of strain M (3.2 cf. 2.2 mg dry wt ml^{-1}), probably reflecting the higher proportion of available energy (glycerol) diverted towards cell maintenance rather than growth in the slower-growing strain M. For all experiments the cells were harvested in the exponential growth phase between A_{680} = 0.8 (0.30 mg dry wt ml^{-1}) and A_{680} = 2.0 (0.80 mg dry wt ml^{-1}).

Under low aeration conditions, the dissolved oxygen concentration was immeasurably small (<5 μM) soon after inocculation for both strains

Fig. III-1

Growth curves of B.megaterium in batch culture



Growth of B.megaterium strains D440 (circles) and M (squares) on minimal-salts medium plus glycerol. A, high aeration conditions (excess oxygen); B, low aeration conditions (oxygen-limited). Open symbols, growth; closed symbols, dissolved oxygen concn..

of the organism (Fig. III-1B). The growth rates were not exponential but steadily declined, indicating that the cells were becoming progressively more oxygen-limited. The cells were harvested for experimental purposes between A_{680} = 0.7 (0.25 mg dry wt.ml^{-1}) and A_{680} = 1.1 (0.45 mg dry wt. ml^{-1}).

2. Characterisation of the respiratory systems of the *B. megaterium* strains

(a) Cytochromes In order to ascertain the cytochrome compositions of the two strains of *B. megaterium*, difference spectrophotometry was performed on respiratory membrane preparations of these organisms.

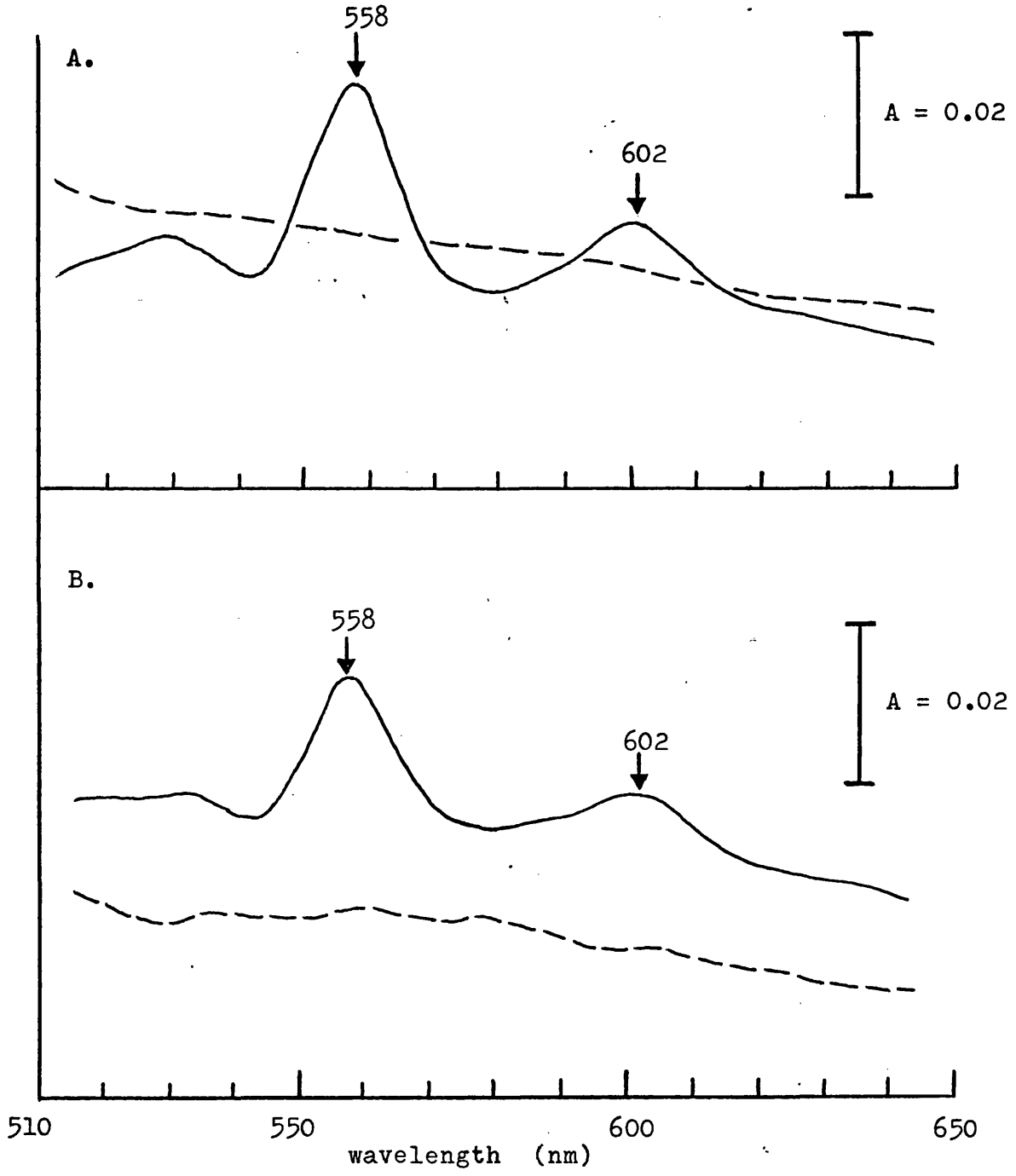
Respiratory membranes prepared from batch cultures of *B. megaterium* strain D440 grown under both excess oxygen and oxygen-limited conditions yielded dithionite-reduced minus oxidised difference spectra which indicated the presence of a *b*-type cytochrome and cytochrome *a* (peaks at approximately 558 nm and 602 nm respectively; Fig. III-2). The spectra were very similar to those reported by Broberg and Smith (1967) and Kröger and Dadák (1969) for *B. megaterium* strains KM and ATCC 14581 respectively. All three strains appear to lack cytochrome *c* (although low temperature spectra of Kröger and Dadák, 1969, suggested a trace of a *c*-type cytochrome in their strain).

In common with strain D440, dithionite-reduced minus oxidised difference spectra of respiratory membranes prepared from strain M indicated the presence of cytochrome *a* (peak at approximately 602 nm; Fig. III-3). However, the α-peak of cytochrome *b* at approximately 560 nm appeared as a shoulder on a cytochrome peaking at approximately 550 nm. This suggested the presence of a *c*-type cytochrome, which has

Fig. III-2

Difference spectra of respiratory membranes prepared from B.megaterium strain D440

Dithionite-reduced minus oxidised spectra

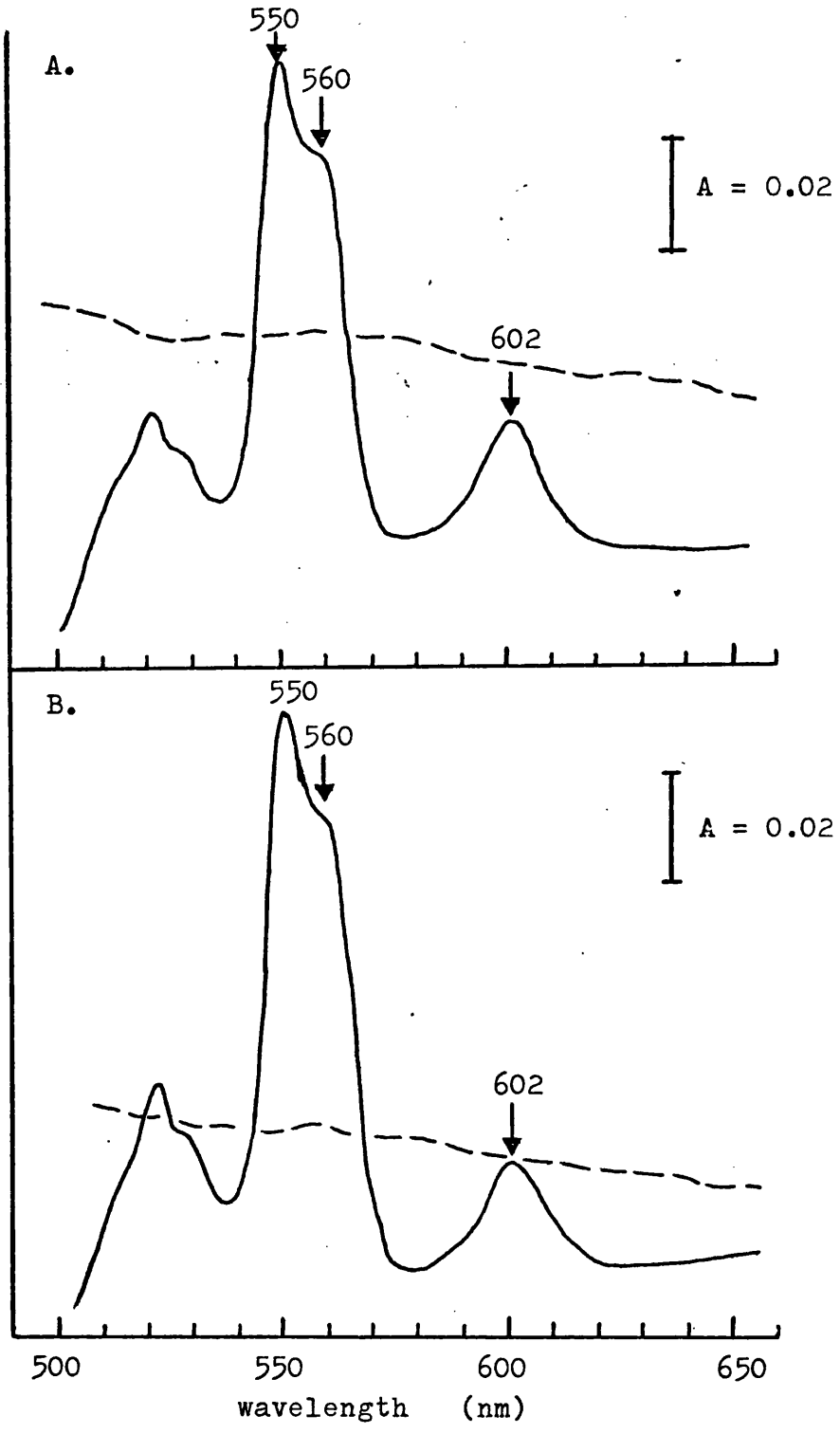


Respiratory membranes were prepared from B.megaterium strain D440 grown under excess oxygen conditions(A, harvest A_{680} = 0.71) or oxygen-limited conditions (B, harvest A_{680} = 0.66). ——— ,dithionite-reduced minus oxidised; ------, oxidised minus oxidised. Protein concentration; A, 8.3 mg.ml^{-1}; B, 3.3 mg.ml^{-1}.

Fig. III-3

Difference spectraof respiratory membranes prepared from B.megaterium strain M

Dithionite-reduced minus oxidised spectra



Respiratory membranes were prepared from B.megaterium strain M grown under excess oxygen conditions (A, harvest A_{680} = 1.11) or oxygen-limited conditions (B, harvest A_{680} = 0.63).——, dithionite-reduced minus oxidised; ------, oxidised minus oxidised. Protein concentration; A, 6.4 $mg.ml^{-1}$; B, 4.9 $mg.ml^{-1}$.

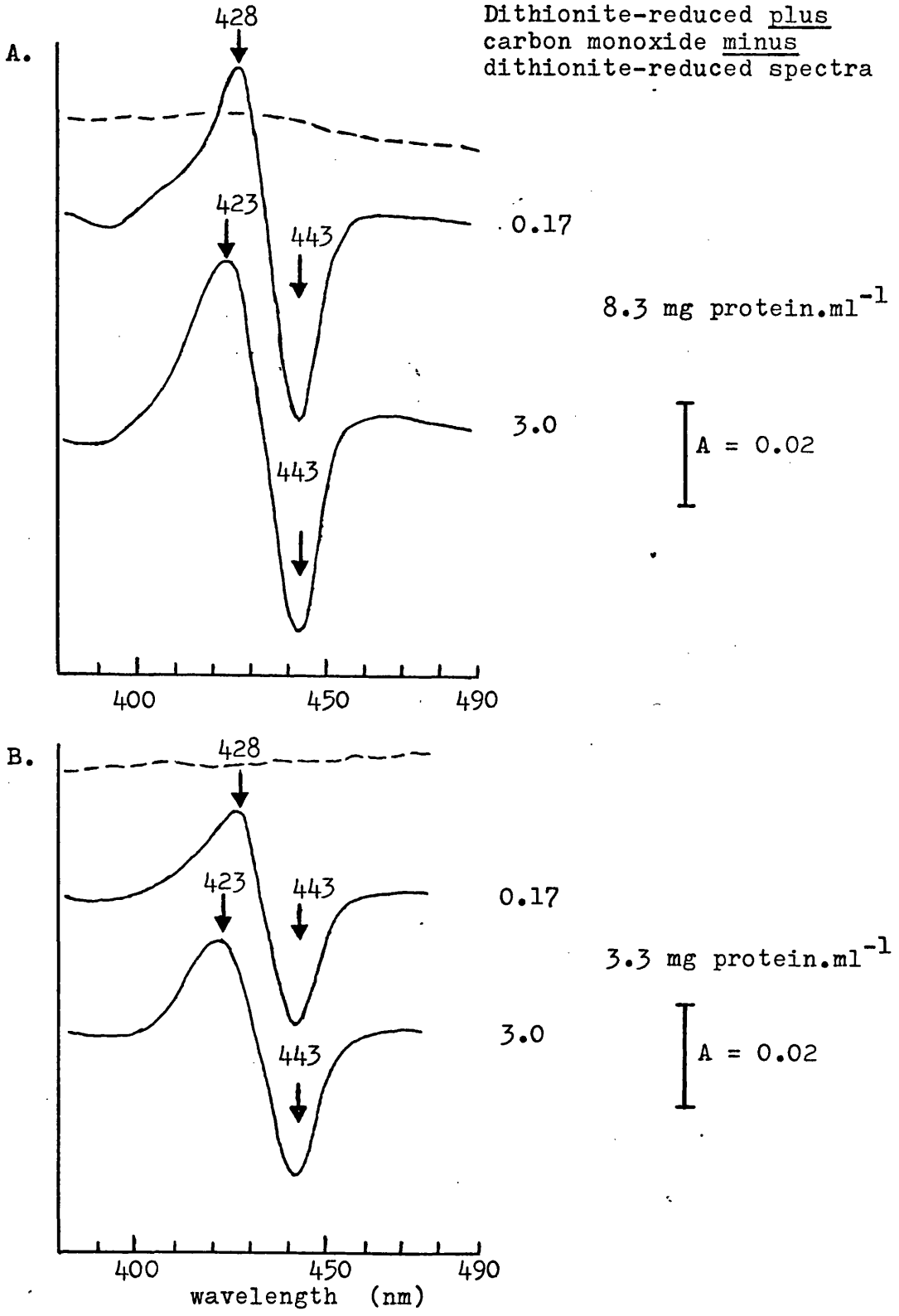
been previously reported for strain M by Weibull and Bergström (1958).

Dithionite-reduced + carbon monoxide minus dithionite-reduced difference spectra were performed in order to determine which CO-binding pigments (i.e. potential cytochrome oxidases) were present. The spectra for strain D440 showed a peak at approximately 428 nm which shifted to approximately 423 nm on increasing the exposure time of the membrane suspension to CO from 10 sec to 3 min. The peak height was simultaneously increased (Fig. III-4). The trough position (443 nm) and size was, however, unaffected by the length of exposure to CO. These effects were assumed to be due to the presence of cytochrome $\underline{a}_3$ (peak 430 nm, trough 443 nm) which had a high affinity for CO, together with cytochrome $\underline{o}$ (peak 418 nm, trough 430 nm) which had a low affinity for CO, as reported by Broberg and Smith (1967) for these oxidases in strain KM. Exposure of the membrane suspensions to CO for greater than 3 min had no further effect on the spectra. The probable mode of interaction of the spectra of these two cytochromes yielding a single peak between their own absorbance maxima is shown in Fig. III-5. The spectrum of cytochrome $\underline{a}_3$ was first drawn in by assuming its peak (at 430 nm) to be symmetrical with its trough (at 443 nm). The difference between the spectrum of cytochrome $\underline{a}_3$ alone and the observed spectrum gave a curve of similar shape to the reduced plus carbon monoxide minus reduced difference spectrum of cytochrome $\underline{o}$ (peak 418 nm, trough 430 nm). This analysis helped to confirm that there were, indeed, two distinct CO-binding pigments, i.e. cytochromes $\underline{a}_3$ and $\underline{o}$. In spite of the interference between the spectra of these two cytochromes, it was still possible to measure their concentrations, as described in the Methods section.

Carbon monoxide difference spectra of membranes of strain M

Fig. III-4

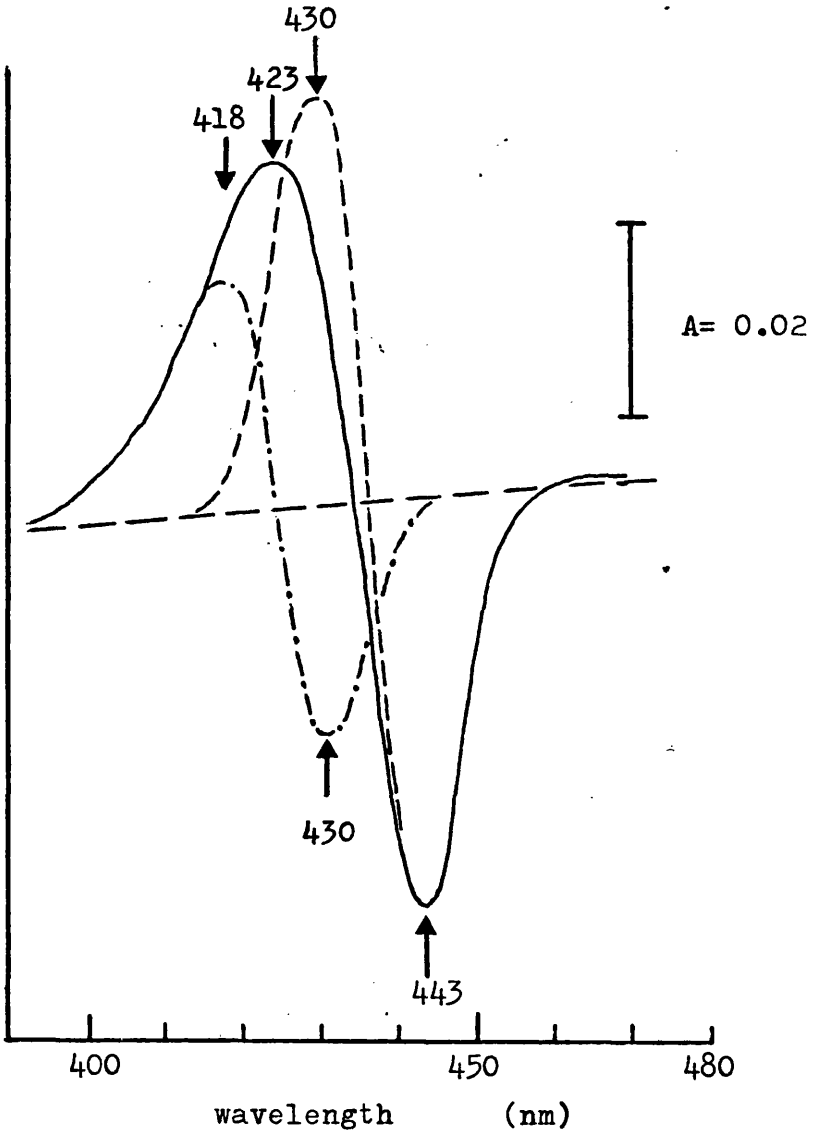
Difference spectra of respiratory membranes prepared from B.megaterium strain D440



The respiratory membrane preparations used were the same as those in Fig. III-2. —— , dithionite-reduced plus CO minus reduced; – – – –, reduced minus reduced. The figures alongside the spectra denote the time (min) for which the membrane suspension was bubbled with CO.

Fig. III-5

Analysis of the CO difference spectrum obtained with respiratory membranes of B.megaterium strain D440



The observed reduced plus CO minus reduced difference spectrum (solid line) of membranes prepared from cells grown under excess oxygen conditions is redrawn from Fig. III-4A (3.0 min CO spectrum).-----, estimated contribution of cytochrome a_3 (peak 430 nm, trough 443 nm) to the observed spectrum; -·-·-·-, resultant spectrum obtained by subtracting ----- from —— (corresponds to the spectrum of cytochrome o ; peak 418 nm, trough 430 nm).

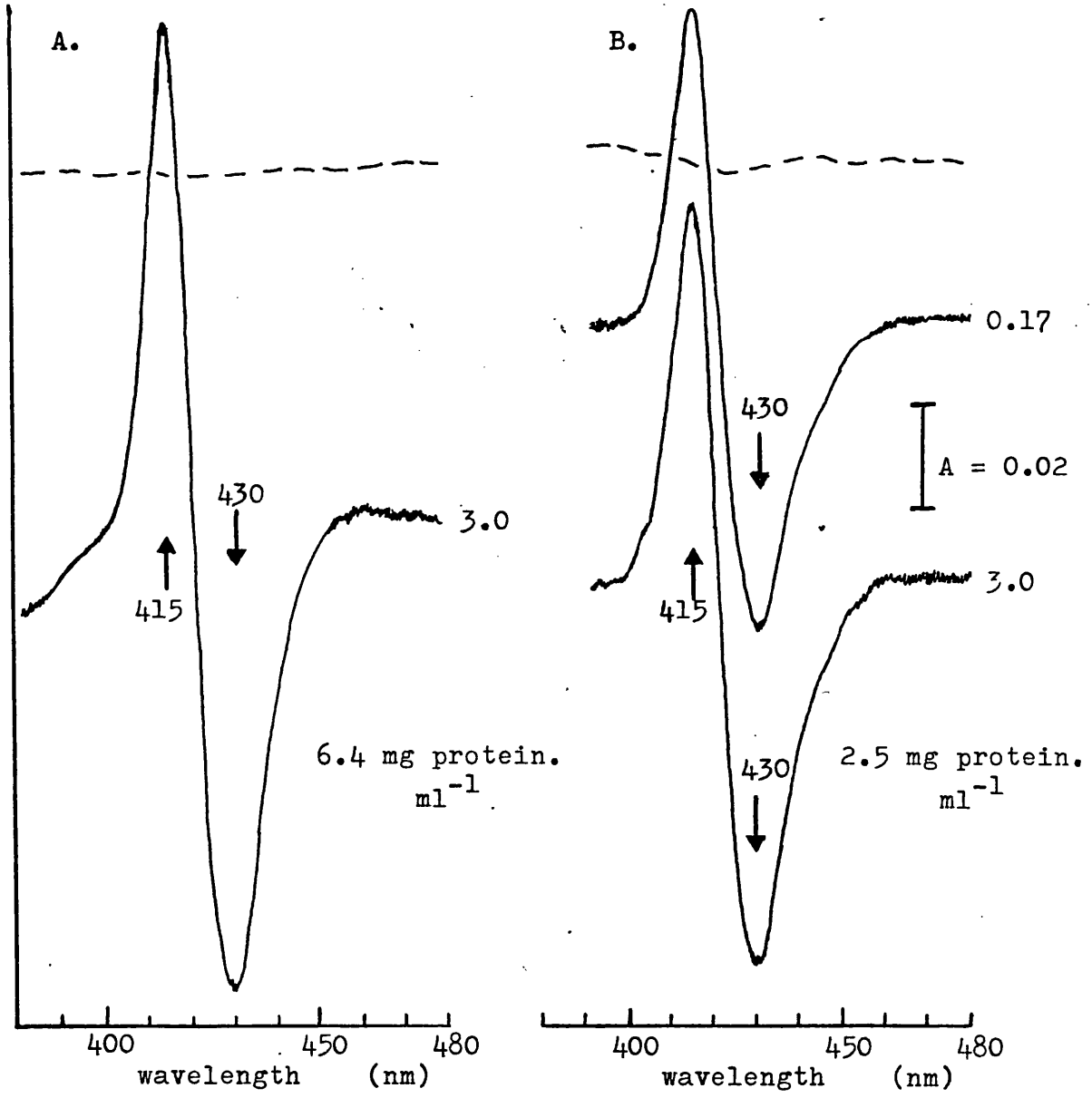
were distinct from those of strain D440 since neither the position of the peak at approximately 415 nm nor the trough at 430 nm changed on increasing the exposure of the membrane suspensions to CO (fig. III-6). The wavelengths of the peak and trough indicated the presence of cytochrome o and the symmetry of the spectra irrespective of the time for which the samples were bubbled with CO suggested that no cytochrome a_3 was present. This was rather surprising considering the presence of an a-type cytochrome in the reduced minus oxidised spectra. As far as is known, the presence of cytochrome a without a_3 has not previously been reported. The affinity for CO of the cytochrome o of strain M (or possibly the rate of binding of CO by the cytochrome) was considerably greater than that of the cytochrome o of strain D440. This was borne out by the observation that increasing the time of bubbling of the membrane suspensions with CO from 10 sec to 3 min induced an increase in absorbance of only 10-12% for the cytochrome o of strain M compared with an increase of 75-80% for that of strain D440. The cytochrome o of strain D440 seems to resemble the cytochrome o of A. vinelandii in its low affinity for CO (or low rate of binding of CO; see Chapter II).

The cytochrome concentrations of respiratory membranes prepared from B. megaterium grown under different culture conditions are shown in Table III-1. Membranes prepared from oxygen-limited cells contained higher absolute concentrations of all cytochromes compared with membranes from excess oxygen cells, a phenomenon which is common to many bacteria (Hughes and Wimpenny, 1969). However, the cytochrome concentrations expressed relative to cytochrome b show much less variability, suggesting an increase in the number of respiratory chain units per cell during oxygen-limited growth. This was presumably to help counteract the very low availability of electron acceptor (O_2) under these conditions.

Fig. III-6

Difference spectra of respiratory membranes prepared from B.megaterium strain M

Dithionite-reduced plus carbon monoxide minus dithionite-reduced spectra



The respiratory membrane preparations used were the same as those in Fig. III-3. ——, dithionite-reduced plus CO minus reduced;-----, reduced minus reduced. The figures alongside the spectra denote the time (min) for which the membrane suspension was bubbled with CO.

Table III-1

The cytochrome concentrations of respiratory membranes prepared from B.megaterium grown in batch culture

Strain	growth conditions	absolute cytochrome concentrations (nmol.g protein^{-1})					relative cytochrome concentrations (nmol.g protein^{-1})				% of total oxidase		ratio a_3/a
		b	c	a	a_3	o	c	a	a_3	o	a_3	o	
D440	excess oxygen	128	-	42	69	33	-	0.33	0.54	0.26	68	32	1.64
	oxygen-limited	211	-	68	120	57	-	0.32	0.57	0.27	68	32	1.77
M	excess oxygen	422	700	159	-	167	1.66	0.38	-	0.40	0	100	-
	oxygen-limited	581	1032	175	-	341	1.78	0.30	-	0.59	0	100	-

Cytochrome concentrations were measured from the spectra shown in Figs. III-2,3,4&6.

In strain D440, the percentage of each oxidase relative to the total oxidase was virtually constant irrespective of the growth conditions (in contrast to the work of Broberg and Smith, 1967, on strain KM) with cytochrome $\underline{a}_3$ present in an approximately two-fold excess over cytochrome $\underline{o}$. That oxygen-limitation did not induce preferential synthesis of one oxidase relative to the other possibly suggests that these oxidases have similar affinities for oxygen. Indeed, Meyer and Jones (1973b) found that the K_m's with respect to oxygen of the cytochromes $\underline{a}_3$ and $\underline{o}$ of other bacterial species were quite similar. Strain D440 exhibited an $\underline{a}$: $\underline{a}_3$ ratio of 1.6 : 1.7 which is in contrast to the mammalian $\underline{a}$: $\underline{a}_3$ ratio of unity (Van Gelder and Muijsers, 1966). Although the $\underline{a}$: $\underline{a}_3$ ratio of 1.6 : 1.7 may reflect a true difference from the mammalian system (i.e. $2\underline{a}$: $\underline{a}_3$), it is probable that it is due to inaccurate mMε values for cytochromes $\underline{a}$ and $\underline{a}_3$ of bacteria. Since no cytochrome $\underline{a}_3$ could be detected in strain M, the $\underline{a}$: $\underline{a}_3$ ratio in this organism was infinity.

(b) Oxidase activities Respiratory membrane preparations from both strains of B. megaterium exhibited NADH, malate, succinate, glycerol 3-phosphate, glutamate and ascorbate/TMPD oxidase activities. Oxidase activities were expressed in terms of both the protein content and the cytochrome $\underline{b}$ content of the membranes (Table III-2). Values expressed in the latter way were useful since they were a measure of the oxidase activity per respiratory chain unit; as shown above, the cytochrome concentrations of the membranes relative to protein varied with the growth conditions.

The NADH oxidase activity of strain D440 membranes was much

Table III-2

The specific oxidase activities of respiratory membranes prepared from B.megaterium grown in batch culture

Strain	growth conditions	Specific oxidase activity (ngatom oxygen. min^{-1}.mg $protein^{-1}$)									Specific oxidase activity (ngatom O. min^{-1}. µmol $\underline{b}^{-1}$)	
		NADH	malate ± NAD^+	NADH + mal.	glutamate	succinate	glycerol -3-P	NADH + G-3-P	NADPH ± NAD^+	ascorbate /TMPD	NADH	malate ± NAD^+
D440	excess oxygen	200	43	197	8	4	33	225	0	104	781	168
	oxygen-limited	152	30	148	N.D	N.D.	N.D.	N.D.	0	257	720	142
M	excess oxygen	47	20	58	5	7	15	57	29	1085	444	184
	oxygen-limited	62	116	130	N.D.	N.D.	N.D.	N.D.	34	1483	423	800

N.D. = not determined

higher than that of strain M membranes when compared on a protein basis, but the difference was much less when compared on a cytochrome b basis, suggesting that the activity per respiratory chain unit was fairly similar.

The malate oxidase activities of membranes of strain D440 derived from both excess oxygen and oxygen-limited cells and also of membranes of strain M derived from excess oxygen cells were considerably lower than the corresponding NADH activities. The fact that the malate oxidation rate was not enhanced in the presence of NAD^+ with these preparations suggested either that malate oxidation was flavin-linked via a membrane-bound malate oxidase or that sufficient NAD^+ was initially present to stimulate malate oxidation via a soluble malate dehydrogenase present in the unwashed membrane preparations. Alternatively, both of these pathways might have occurred. It was found that the malate oxidase activity of strain M membranes derived from oxygen-limited cells was approximately twice the NADH activity, indicating that some or all of the malate oxidation occurred via its respiratory chain dehydrogenase rather than via a soluble NAD^+-linked enzyme present in these membrane preparations.

Oxidation of NADH plus malate by strain D440 membranes was at a rate approximately equal to the rate of NADH oxidation alone. This suggested that malate was oxidised by an NAD-linked dehydrogenase rather than by its own respiratory chain dehydrogenase, in which case the oxidase activity in the presence of the two substrates would have been greater than the rate of NADH oxidation alone. With membranes of strain M, the rate of oxygen uptake in the presence of NADH plus malate was higher than the malate or NADH rates alone, indicating that a large proportion of the malate oxidation was NAD-independent, via its respiratory chain dehydrogenase (this was also the case for malate oxidation by strain

ATCC 14581; Kröger and Dadák, 1969).

Both strains exhibited low glutamate and succinate oxidase activities. Glycerol was not oxidised at a significant rate by membrane preparations of either strain in spite of the fact that it was the carbon/energy source of the growing cells. Since it was considered likely that glycerol would be assimilated by means of glycerol kinase in the growing cells, the membrane preparations were tested for glycerol 3-phosphate oxidase activity. They oxidised this compound at a substantial rate, as shown in Table III-2. The dehydrogenase responsible in the two strains was presumably membrane-bound and flavin-linked rather than soluble and NAD-linked since a mixture of NADH and glycerol 3-phosphate was oxidised at a rate nearly equal to the sum of the individual oxidation rates for these substrates (Table III-2). Strain ATCC 14581 of Kröger and Dadák (1969) also exhibited flavin-linked glycerol 3-phosphate oxidase activity.

There was no detectable NADPH oxidase activity exhibited by respiratory membrane preparations of strain D440. Strain M membranes, on the other hand, contained substantial NADPH oxidase activity. Since the activity was not stimulated by addition of NAD^+, the NADPH was either oxidised *via* its respiratory chain dehydrogenase or it underwent transhydrogenation to NADH followed by oxidation by the NADH dehydrogenase, in which case there was sufficient NAD^+ present in the membrane suspension to allow maximum transhydrogenase activity. Dialysis of strain M membranes to free them of any traces of NAD^+ did not elicit any NAD^+-dependent NADPH oxidase activity (Table III-3), suggesting that NADPH oxidation by these membranes was *via* its respiratory chain dehydrogenase. There was considerable overall loss of oxidase activities with the strain M membranes following dialysis; this loss was complete with strain D440 membranes.

Table III-3

Specific oxidase activities of dialysed respiratory membranes of B.megaterium strain M

specific oxidase activity (ngatom oxygen. min^{-1}. mg $protein^{-1}$)				
NADH	NADPH	NADPH $+NAD^+$	malate	malate $+NAD^+$
9.1	6.2	5.9	8.3	8.2

Identical respiratory membranes were used to those in Table III-2 (strain M, excess oxygen). The membranes were dialysed against 25 mM Na phosphate buffer (pH 7.2) for 18h at 4^o prior to measurement of oxidase activities.

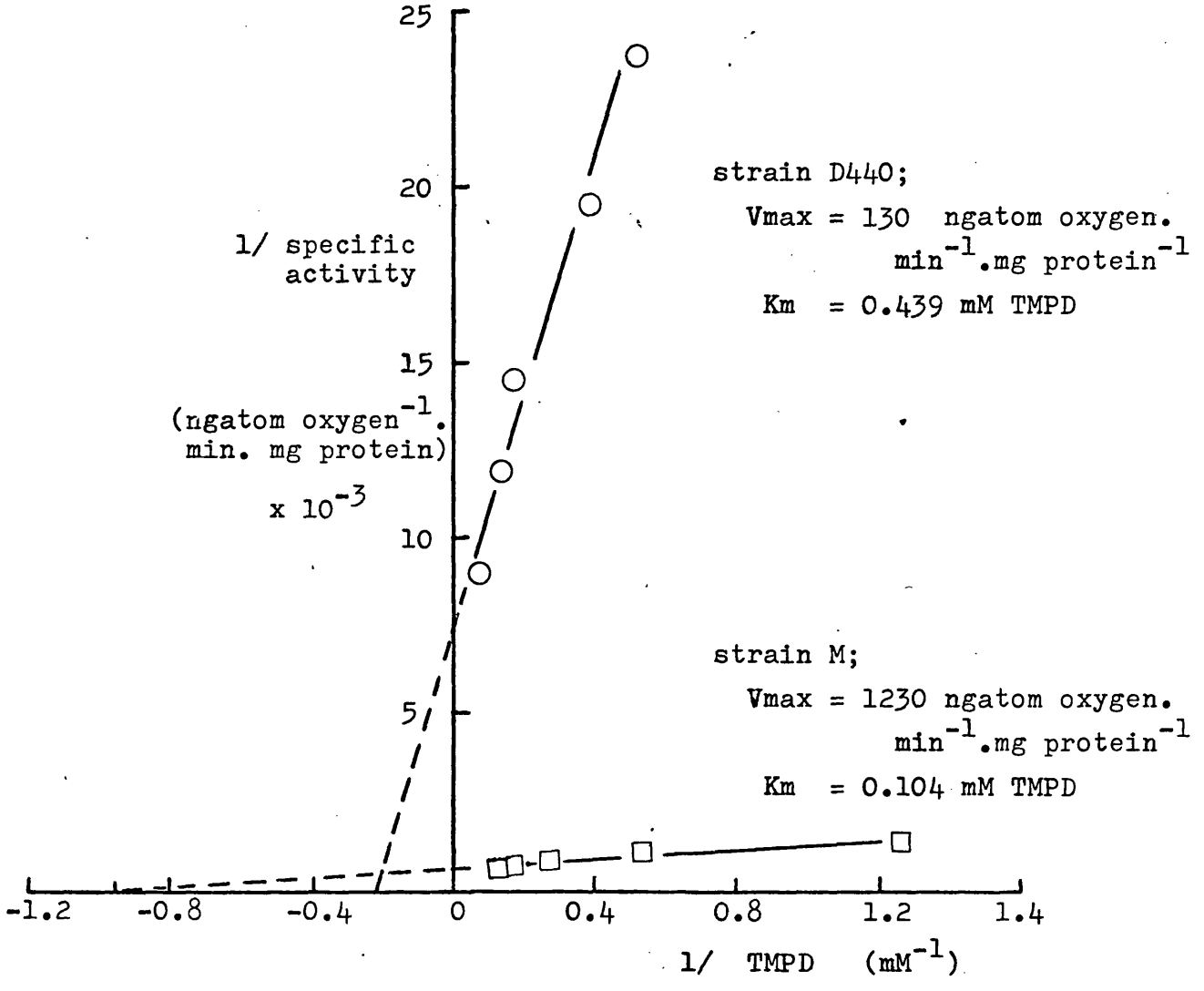
The reasons for the lability of the membrane preparations during dialysis is unclear.

In order to determine whether malate oxidation in strain D440 was pyridine nucleotide-linked, and also to ascertain if this was the case for glutamate oxidation in both strains of B. megaterium, the rate of reduction of coenzyme ($NAD^+/NADP^+$) was measured at 340 nm following its addition to an assay mixture containing the appropriate cell-free extract (prepared as described in Materials and Methods) plus substrate together with 1 mM cyanide to block reoxidation of the reduced coenzyme by the respiratory chain. NAD^+-linked (but not $NADP^+$-linked) malate and glutamate oxidation was detected with cell-free extracts of strain D440 and also NAD^+-linked (but not $NADP^+$-linked) glutamate oxidation with cell-free extracts of strain M. Malate oxidation was not tested with the latter preparations but was assumed to be principally particulate flavin-linked from the data of Table III-2.

Strain M membranes exhibited much higher ascorbate/TMPD oxidase activities than strain D440 membranes (Table III-2). A Lineweaver-Burk plot of the oxidase activity measured at different concentrations of TMPD (fig. III-7) revealed that the strain M membranes not only had a much higher V_{max} for ascorbate oxidation than strain D440 membranes (strain M membranes, V_{max} = 1230 ngatom $O.min^{-1}.mg\ protein^{-1}$; strain D440 membranes, V_{max} = 130) but also that the affinity of the former membranes for TMPD was higher (strain M membranes, K_m = 0.104 mM TMPD; strain D440 membranes, K_m = 0.439 mM TMPD). These results confirm that the cytochrome c observed spectrophotometrically in the strain M membranes was a functional part of the respiratory chain, since reduced TMPD is generally considered to act as an artificial electron donor to endogenous cytochrome c (Green and Baum, 1970). It is possible that the small ascorbate/TMPD oxidase

Fig. III-7

Specific oxidase activities of respiratory membrane preparations of B.megaterium in the presence of varying TMPD concentrations



Respiratory membranes were prepared from cells in the logarithmic phase of growth under excess oxygen conditions. ○ , strain D440 (1.53 mg protein per assay); □ , strain M (0.33 mg protein per assay).

activity observed with the membranes of strain D440 was due to electron donation by reduced TMPD either to cytochrome b or directly to the cytochrome oxidases (aa_3 and o).

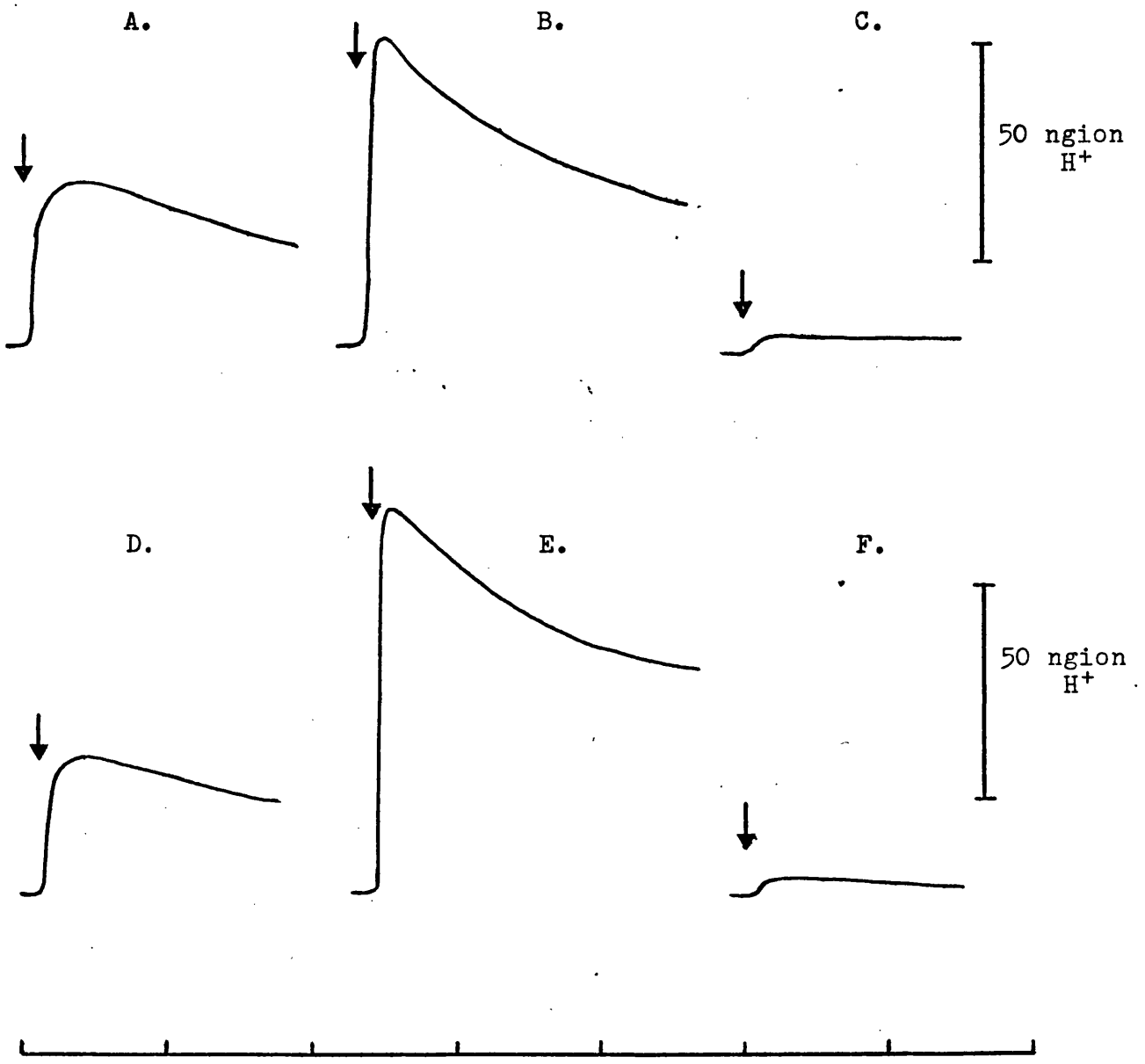
3. Energy Conservation

(a) Measurement of $\longrightarrow H^+/O$ quotients Before $\longrightarrow H^+/O$ quotients, determined as described in Chapter II, could be measured accurately and reproducibly for B. megaterium, it was first necessary to optimise and standardise the assay conditions. For the reasons described in Chapter II, all measurements of $\longrightarrow H^+/O$ quotients were performed in the presence of 0.5 $mg.ml^{-1}$ carbonic anhydrase. Typical recorder tracings of respiration-induced acidification of whole cell suspensions of B. megaterium strains D440 and M, when oxidising endogenous substrates, are shown in fig. III-8 A and D respectively. The respiration-driven proton translocation was enhanced in the presence of KSCN, for reasons described in Chapter II, when the $t_{\frac{1}{2}}$ of decay of the acid pulse was approximately 2 min for both strains (fig. III-8 B, E; KSCN concentration, 150 mM). The oxygen-induced increase in acidity of the suspension medium was virtually abolished by low concentrations (15 μM) of the uncoupler TCS, indicating that the proton movements were associated with respiration (fig. III-8 C, F).

Variations in cell density or pH of the assay medium produced negligible changes in the $\longrightarrow H^+/O$ quotient exhibited by either strain irrespective of whether the cell samples were derived from excess oxygen, logarithmically growing cultures (fig. III-9 A, B) or oxygen-limited cultures (fig. III-10 A, B). However, to standardise the assay conditions, a cell density of 6 $mg.ml^{-1}$ and a pH of 6.7 - 6.8 were routinely used for all measurements of $\longrightarrow H^+/O$ quotients. 30 mM KSCN elicited maximum $\longrightarrow H^+/O$ ratios for strain M (figs. III-9C, III-10C) and also for strain D440 when the latter was grown under excess oxygen conditions (fig. III-9C). Rather unexpectedly, a much higher KSCN

Fig. III-8

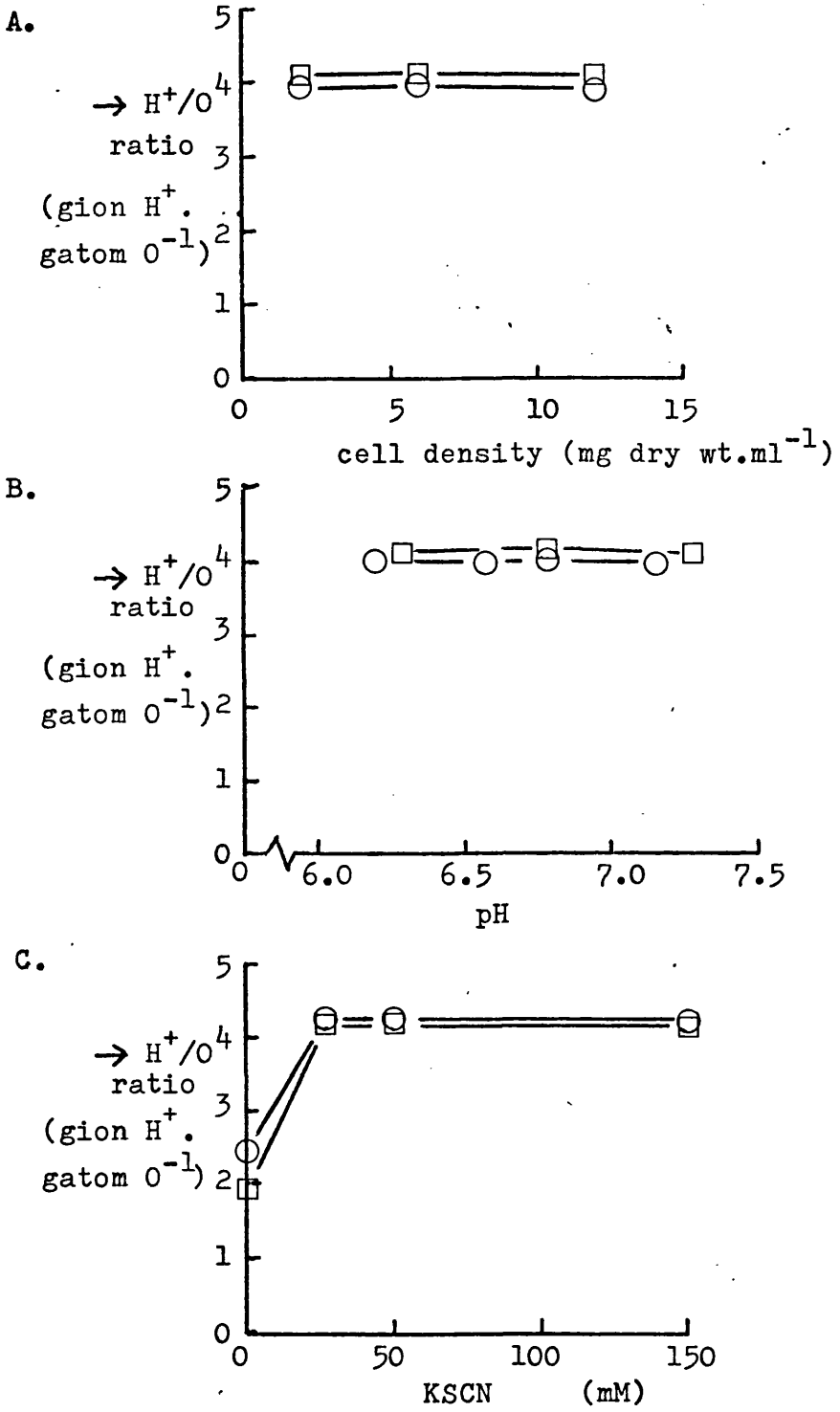
Respiration-driven proton translocation in B.megaterium



Time-course of respiration-driven acidification of cell suspensions of B.megaterium strain D440 (A-C) and strain M (D-F) derived from excess oxygen cultures. The figure shows the actual recorder traces on addition to the anaerobic cell suspensions of 22.2 µgatom oxygen (arrows). Increase in acidity of the outer phase is shown upwards. Assay conditions; 0.5 mg.ml^{-1} carbonic anhydrase; pH 6.6-6.8; cell density 6 mg dry wt.ml^{-1}; A and D, no further additions; B and E, 150 mM KSCN; C and F, 150 mM KSCN, 15 µM TCS.

Fig. III-9

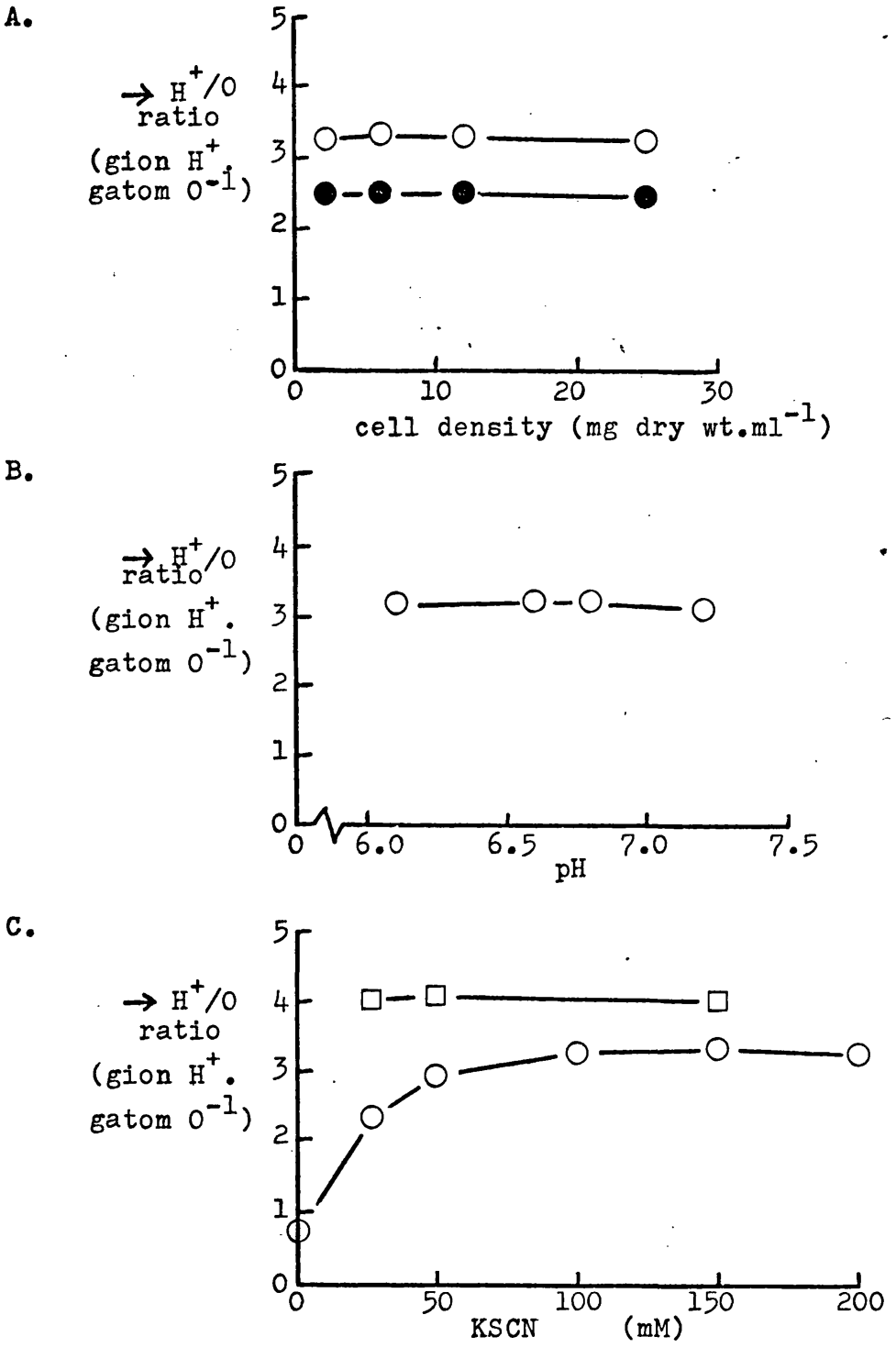
The effect of assay conditions on the → H^+/O quotients of B.megaterium grown under excess oxygen conditions



B.megaterium strain D440 (○) or M (□) was harvested in the logarithmic phase of growth under excess oxygen conditions (harvest A_{680} = 0.8-2.0). Conditions of assay; A, pH = 6.8 , 150 mM KSCN (cell density varied); B, cell density = 6 $mg.ml^{-1}$, 150 mM KSCN (pH varied); C, pH = 6.7, cell density = 6 $mg.ml^{-1}$(KSCN concn. varied). A single cell preparation was used for each set of measurements.0.5 $mg.ml^{-1}$ carbonic anhydrase used throughout.

Fig. III-10

The effect of assay conditions on the $\rightarrow H^+/O$ quotients of B.megaterium grown under oxygen-limited conditions



B.megaterium strain D440(○,●) or M(□) was harvested following growth under oxygen-limited conditions (harvest A_{680} = 0.7-1.1). Conditions of assay; A, pH = 6.8, ● 25 mM KSCN, ○ 150 mM KSCN; B, cell density = 6 $mg.ml^{-1}$, 150 mM KSCN; C, pH = 6.8, cell density = 6 $mg.ml^{-1}$. A single cell preparation was used for each set of measurements. 0.5 $mg.ml^{-1}$ carbonic anhydrase used throughout.

concentration (150 mM) was required for maximum $\longrightarrow H^+/O$ quotients to be realised for strain D440 grown in oxygen-limited culture (fig. III-10C). The reason for this is not clear. However, for the sake of standardisation, 150 mM KSCN was routinely used with all preparations.

(b) $\longrightarrow H^+/O$ quotients of cells oxidising endogenous substrates

Whole cell $\longrightarrow H^+/O$ quotients of B. megaterium grown in batch culture were assayed using the standardised conditions described above. The results are shown in Table III-4. When oxidising endogenous substrates, $\longrightarrow H^+/O$ quotients of approximately 4 were obtained for strain D440 grown under excess oxygen conditions and also for strain M grown under both excess oxygen and oxygen-limited conditions. Oxygen-limited cells of strain D440 exhibited rather lower $\longrightarrow H^+/O$ quotients of approximately 3.1. Assays performed in the presence of valinomycin (8.3 $\mu g.ml^{-1}$) instead of KSCN, in the normal Tris/KCl buffer did not result in higher $\longrightarrow H^+/O$ quotients being observed with these oxygen-limited cells. Neither was any increase achieved by use of 1 mM glycyl-glycine buffer (pH 6.8) instead of the normal Tris buffer. Hence it was assumed that the $\longrightarrow H^+/O$ quotients of the oxygen-limited strain D440 cells were genuinely low compared with those exhibited by the other cells and that the assay conditions were not at fault. The dissolved oxygen concentration of the cultures from which the oxygen-limited cells were derived was always less than 5 μM. When the dissolved oxygen concentration of logarithmically growing, excess oxygen cells was varied between 220 μM and 15 μM by manipulation of the aeration rate or the culture density, the $\longrightarrow H^+/O$ ratios remained constant at approximately 4 (fig. III-11). This suggested that the $\longrightarrow H^+/O$ quotients of the cells fell only when the cells became growth-limited with respect to oxygen. A possible

Table III-4

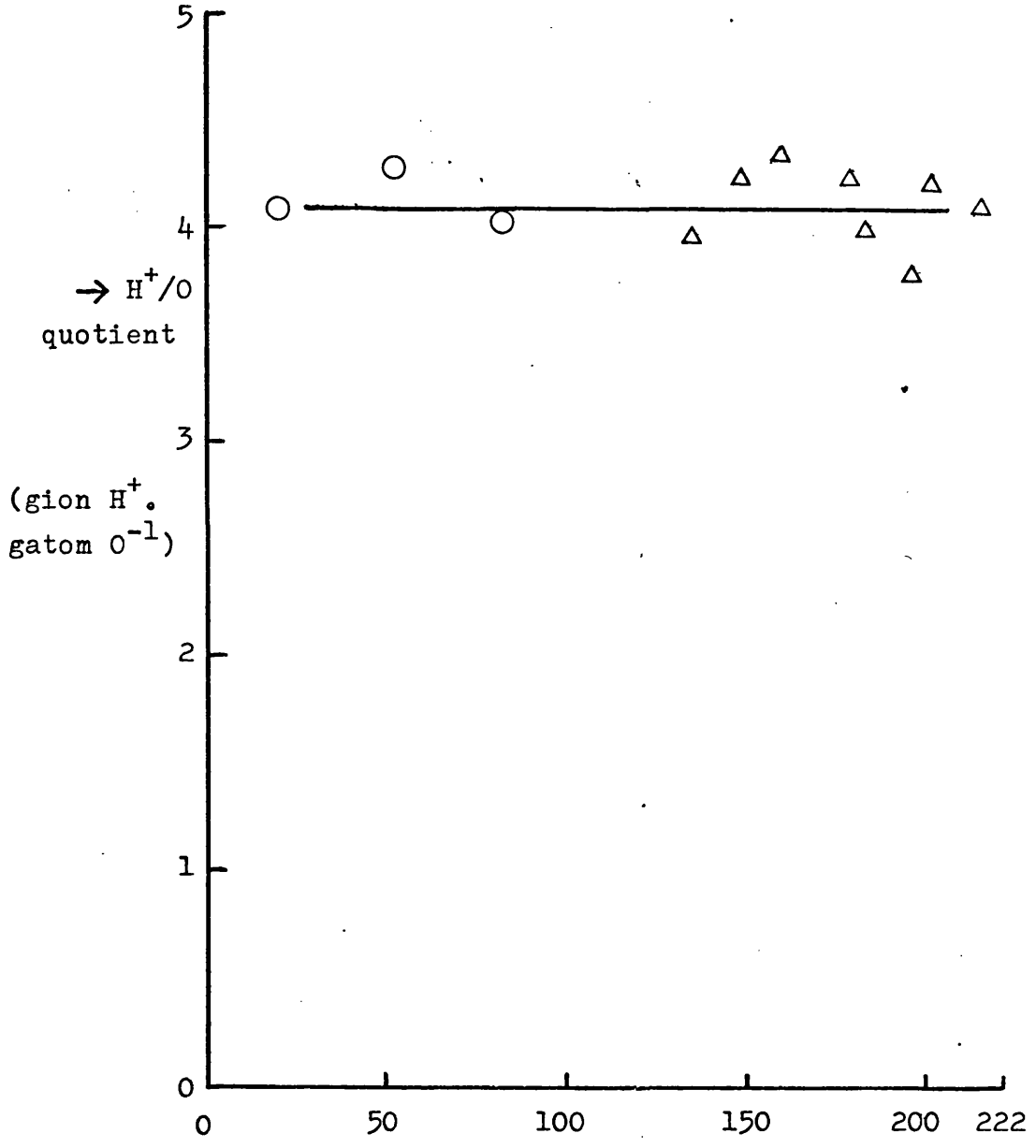
→ H^+/O quotients of whole cells of B.megaterium oxidising endogenous substrates

Organism	Growth conditions	→ H^+/O quotient (gion H^+. gatom oxygen^{-1}; ±S.E.M., no.of determinns)
strain D440	excess oxygen	3.95 ± 0.15 (5)
	oxygen-limited	3.14 ± 0.21 (6)
strain M	excess oxygen	4.19 ± 0.18 (3)
	oxygen-limited	4.25 ± 0.20 (2)

A_{680} at harvest; excess oxygen cells, 0.5-2.0; oxygen-limited cells, 0.7-1.1. Conditions of assay of → H^+/O quotients; 0.5 mg.ml^{-1} carbonic anhydrase; cell density 6 mg dry wt.ml^{-1}; 150 mM KSCN; pH 6.6-6.8.

Fig. III-11

→ H^+/O quotients of B.megaterium strain D440 in relation to the dissolved oxygen concentration of the batch cultures at harvest



B.megaterium strain D440 was grown in batch culture under excess oxygen conditions. △ , normal high aeration conditions (harvest A_{680} = 0.5-6.0); ○ , medium aeration conditions (greater culture volume, same shake-rate; harvest A_{680} = 1.5-3.0). The dissolved oxygen concn. of the cultures at harvest decreased with increasing A_{680}. Conditions of assay of → H^+/O quotients; 0.5 $mg.ml^{-1}$ carbonic anhydrase; cell density 6 $mg.ml^{-1}$; 150 mM KSCN; pH 6.6-6.8.

explanation for this phenomenon is presented later.

Since, according to Mitchell's chemiosmotic hypothesis of oxidative phosphorylation (Mitchell, 1966, 1968), the P/O ratio (mol P_i esterified.gatom O^{-1}) = $\longrightarrow H^+/O \div 2$, the $\longrightarrow H^+/O$ quotients of approximately 4 obtained for the two strains of B. megaterium (ignoring for the moment oxygen-limited strain D440) indicated P/O ratios of approximately 2 and suggested the presence of 2 proton translocating oxido-reduction loops in the respiratory chains of these organisms. In order to establish which two loops of loops 1, 2 and 3 (see Chapter I) were present in the two strains of B. megaterium under examination (N.B. the absence of detectable transhydrogenase activity in these organisms eliminated the possible involvement of loop 0), substrate loading experiments similar to those described by Lawford and Haddock (1973) were performed.

(c) $\longrightarrow H^+/O$ quotients of cells oxidising added substrates

Lawford and Haddock (1973) found that whole cells of E. coli oxidising predominantly flavin-linked substrates (such as succinate, D-lactate, and glycerol 3-phosphate) yielded $\longrightarrow H^+/O$ quotients of approximately 2 whereas cells oxidising predominantly NAD(P)-linked substrates (such as malate) exhibited values of approximately 4. These experiments established the presence of proton-translocating loops 1 and 2 in the respiratory chain of this organism.

A series of analogous experiments was performed on the two strains of B. megaterium. In order to measure $\longrightarrow H^+/O$ quotients corresponding to oxidation of added substrates, it was first necessary to deplete the store of endogenous substrates in the cells. A procedure was devised, slightly different from that of Lawford and Haddock (1973),

which adequately starved the cells of endogenous substrates so that their endogenous respiration rate was low (see Materials and Methods).

(i) B. megaterium strain D440

Unstarved cells of B. megaterium strain D440 grown in batch culture under excess oxygen conditions and subsequently prepared for measurement of proton translocation exhibited an endogenous respiratory activity (QO_2) of 171 µl $O_2.min^{-1}.mg$ dry wt $cells^{-1}$ and an $\longrightarrow H^+/O$ quotient of 3.95 gion $H^+.gatom.O^{-1}$ (average values; Table III-5). Starved cells exhibited an endogenous QO_2 of 23.3 and an endogenous $\longrightarrow H^+/O$ quotient of 2.83 (average values). The substrates glycerol, succinate, L-malate and glutamate were all oxidised by the starved cells, but at different rates; thus glycerol induced a 140% increase in QO_2 over the endogenous respiration rate but the increases with succinate, glutamate and malate were only 19%, 57% and 36% respectively. However, in spite of this, glycerol and succinate induced a slight decrease in the $\longrightarrow H^+/O$ quotient relative to the endogenous value whereas glutamate and malate brought about an increase in $\longrightarrow H^+/O$ quotient (Table III-5).

An attempt was made to normalise the results by calculating the $\longrightarrow H^+/O$ quotients which would have been observed if the contribution of the endogenous substrates to respiration-induced proton translocation was zero. This involved the assumption that the relative rates of oxidation of endogenous substrates and the added substrates were the same in the pH-measuring chamber as in the oxygen electrode chamber. The following equation was then used to calculate 'corrected' $\longrightarrow H^+/O$ quotients, i.e. $\longrightarrow H^+/O_{substrate}$ quotients:

Table III-5

→ H^+/O quotients of B.megaterium during substrate-loading experiments (strain D440)

Preparation	added substrate	observed → H^+/O quotient (gion H^+.gatom O^{-1})	QO_2 ($\mu l O_2 . mg\ cells^{-1} . h^{-1}$)	→ $H^+/O_{substrate}$ quotient (gion H^+. gatom O^{-1})
Unstarved	endogenous	3.95 ± 0.15(5)	171 ± 6(5)	
Starved	endogenous	2.83 ± 0.19(5)	23.3 ± 1.8(5)	
	glycerol	2.66 ± 0.23(5)	56.4 ± 8.5(5)	2.54
	succinate	2.56 ± 0.32(5)	27.8 ± 1.1(5)	—
	glutamate	3.24 ± 0.16(5)	36.6 ± 2.0(5)	3.96
	malate	3.19 ± 0.27(5)	31.6 ± 3.0(5)	4.20

B.megaterium strain D440 was grown under excess oxygen conditions. Unstarved cells, harvest A_{680} = 0.5-2.0. Starved cells, harvest A_{680} = 1.7-2.2. Conditions of assay of → H^+/O quotients as in Table III-4. Results expressed as average ± S.E.M. (no. of determinations).

$$(\longrightarrow H^+/O_{obs.} \times QO_{2\ obs.}) = (\longrightarrow H^+/O_{endog.} \times QO_{2\ endog.}) + (\longrightarrow H^+/O_{substrate} \times QO_{2\ substrate})$$

$$\text{whence} \longrightarrow H^+/O_{substrate} = \frac{(\longrightarrow H^+/O_{obs.} \times QO_2) - (\longrightarrow H^+/O_{endog.} \times QO_{2\ endog.})}{QO_{2\ substrate}}$$

where $\longrightarrow H^+/O_{endog.}$ and $QO_{2\ endog.}$ are the values with no added substrate, $\longrightarrow H^+/O_{obs.}$ and $QO_{2\ obs.}$ are the observed values in the presence of substrate and $QO_{2\ substrate}$ is the respiratory activity due to oxidation of added substrate only, i.e.

$$QO_{2\ substrate} = QO_{2\ obs.} - QO_{2\ endog.}$$

$\longrightarrow H^+/O_{substrate}$ quotients are shown in Table III-5. No figure was calculated for succinate oxidation as it was considered that the QO_2 stimulation with this substrate was not high enough to allow an accurate calculation to be made.

The $\longrightarrow H^+/O_{substrate}$ ratios for glutamate and malate oxidation were found to be close to 4.0 whereas the value for glycerol oxidation was only 2.54. Since glutamate and malate oxidation in strain D440 are NAD-linked rather than flavin-linked (see above), it seemed likely that the $\longrightarrow H^+/O_{substrate}$ ratio of approximately 4 observed during oxidation of these substrates was due to the involvement of the whole respiratory chain from NADH to oxygen which must therefore have encompassed two proton translocating loops. The $\longrightarrow H^+/O_{substrate}$ quotient of approaching 2 observed during glycerol oxidation, which was probably flavin-linked (see above), suggested that this substrate donated reducing

equivalents to the respiratory chain at a more positive redox potential than NADH and hence elicited proton translocation *via* a single loop only. Thus the NADH dehydrogenase of this strain is implicated as a proton translocating enzyme complex, i.e. loop 1. The other functional proton translocating loop was presumably loop 2 since the involvement of loop 3 was precluded by the absence of cytochrome *c* from this strain.

The results also indicate that the unstarved preparations which gave $\longrightarrow H^+/O$ quotients of approximately 4 oxidised NAD-linked endogenous substrates rather than flavin-linked substrates. During the starvation procedure, the substrates depleted most effectively were presumably NAD-linked rather than flavin-linked, thus resulting in the $\longrightarrow H^+/O$ quotients of only 2.83 (average) obtained for starved cells oxidising endogenous substrates. Under these conditions, more flavin-linked substrates were presumably available for oxidation than NAD-linked substrates. A decrease in the $\longrightarrow H^+/O$ quotient following starvation has also been observed for a number of other organisms with a functional loop 1 when oxidising endogenous substrates, presumably for the same reasons (e.g. *Acinetobacter lwoffi* and *Pseudomonas ovalis*; Brice *et al.*, 1975).

As mentioned above, unstarved cells of strain D440 obtained from oxygen-limited batch cultures also gave $\longrightarrow H^+/O$ quotients of less than 4 (average 3.14; Table III-4). This could have been due either to oxidation of a mixture of NAD- and flavin-linked substrates or to a partial loss of coupling of oxidation of NAD-linked substrates to proton translocation. In order to distinguish which of these mechanisms was responsible, attempts were made to starve the cells and load with NAD-linked substrates. If $\longrightarrow H^+/O$ quotients of approaching 4 were then observed, this would indicate that both proton translocating loops were

entirely present in these cells. On the other hand, if, in spite of a stimulation of QO_2 caused by the presence of NAD-linked substrates, there was no stimulation of the $\longrightarrow H^+/O$ quotient, this would be indicative of a partial loss of looping. Unfortunately, it was not possible to starve the oxygen-limited cells by the same method as that used for excess oxygen cells. This was because excess oxygen cells were starved by allowing the growing cultures to run out of carbon/energy source (glycerol). Had the same procedure been used for oxygen-limited cells, as the glycerol concentration fell in the growth medium, the cells would eventually have become glycerol-limited and the dissolved oxygen concentration in the culture would have risen, thus nullifying the experiment because the cells would no longer have been oxygen-limited. Instead of this method, the original technique of Lawford and Haddock (1973) was used (see Materials and Methods), even though this involved the assumption that subjecting the washed cells to high aeration shaking conditions in growth medium minus ammonium chloride and glycerol would starve the cells but not alter the (possibly) different respiratory chain properties of the oxygen-limited cells to the properties of the excess oxygen cells, as described above. However, as was the case with excess oxygen cells, this starvation procedure was inadequate and little or no stimulation of QO_2 above the endogenous rate was observed on addition of NAD-linked substrates to the cell suspensions.

It was therefore impossible to determine whether the low $\longrightarrow H^+/O$ ratios found with oxygen-limited cells were due to a change in the ratio of the intracellular flavin-linked and NAD-linked substrate concentrations or dehydrogenase activities or to a mixture of respiratory chain types containing one or two functional proton translocating loops. In view of the fact that strain M appeared to completely lack a

functional proton translocating loop 1 in its respiratory chain (see below), it seems likely that under oxygen-limited conditions loop 1 also becomes partially inoperative in strain D440.

(ii) B. megaterium strain M

Unstarved cells of B. megaterium strain M grown in batch culture under excess oxygen conditions and subsequently prepared for measurement of proton translocation exhibited an endogenous QO_2 of 105 $\mu l\ O_2.min^{-1}.mg\ dry\ wt\ cells^{-1}$ and an $\longrightarrow H^+/O$ quotient of 4.19 gion $H^+.gatom\ O^{-1}$ (average values; Table III-6). Starved cells exhibited a QO_2 of 16 and an $\longrightarrow H^+/O$ quotient of 4.23 (average values) when oxidising endogenous substrate. Significant stimulation of QO_2 was observed in the presence of succinate, glutamate and glycerol (36%, 46% and 340% stimulation of the endogenous QO_2 respectively). However, the corresponding $\longrightarrow H^+/O$ ratios were little altered from the endogenous ratio and, even after applying the correction procedure (see above), $\longrightarrow H^+/O_{substrate}$ quotients were $\geqslant$ 3.50 (Table III-6). Malate failed to significantly increase the QO_2 over the endogenous value, in spite of the fact that it was oxidised by respiratory membranes prepared from excess oxygen cells (see Table III-2). This was probably because the glycerol-grown cells were not induced for malate uptake.

Glutamate oxidation by the starved cells yielded a calculated $\longrightarrow H^+/O_{substrate}$ quotient of 3.50 (Table III-6). Since glutamate oxidation in strain M was shown to be NAD-linked (see above), this indicated the presence of 2 proton translocating loops in the respiratory chain between NADH and molecular oxygen. However, oxidation by starved cells of glycerol and succinate, both of which were flavin-linked, also gave $\longrightarrow H^+/O_{substrate}$ quotients of approximately 4. This suggested that

Table III-6

→ H^+/O quotients of glycerol-grown B.megaterium strain M during substrate-loading experiments

Preparation	added substrate	observed → H^+/O quotient (gion H^+.gatom O^{-1})	QO_2 ($\mu l O_2$.mg cells^{-1}.h^{-1})	→ H^+/O substrate quotient (gion H^+.gatom O^{-1})
Unstarved	endogenous	4.19 ± 0.18(3)	105 ± 5(3)	
Starved	endogenous	4.23 ± 0.08(4)	16.2 ± 2.0(4)	
	glycerol	4.23 ± 0.16(4)	71.2 ± 4.1(4)	4.23
	succinate	4.05 ± 0.11(4)	22.0 ± 0.8(4)	3.55
	glutamate	3.98 ± 0.13(4)	24.6 ± 1.5(4)	3.50
	malate	4.02 ± 0.10(4)	17.5 ± 1.8(4)	—
	asc./TMPD	3.19 ± 0.01(4)	340 ± 21(4)	3.13

B.megaterium strain M was grown under excess oxygen conditions on glycerol medium. Unstarved cells, harvest A_{680} = 0.8-2.0. Starved cells, harvest A_{680} = 1.3-1.7. Conditions of assay of → H^+/O quotients as in Table III-4. Results expressed as average ± S.E.M. (no. of determinations).

the NADH dehydrogenase in this organism, in constrast to that in strain D440, was not associated with proton translocation; i.e. loop 1 was inoperative.

In an attempt to verify these results, cells were grown in batch culture under excess oxygen conditions in the presence of limiting amounts of succinate or glutamate as carbon/energy source, instead of glycerol. The rational behind this procedure was that the subsequent addition of the growth substrate to starved cells should induce a large increase in respiration rate relative to the endogenous rate, since the cells would be induced for the uptake of that substrate. This was found to be the case with starved, succinate-grown cells. When succinate was added to these cells, there was an increase in QO_2 of 330% relative to the endogenous rate, compared with an increase of only 36% when succinate was added to starved, glycerol-grown cells (Table III-7). In spite of the much larger stimulation in QO_2, the $\longrightarrow H^+/O$ quotient exhibited by starved, succinate-grown cells in the presence of added succinate was not significantly different from the endogenous value. The calculated $\longrightarrow H^+/O_{substrate}$ ratio was 3.68, i.e. nearly 4.0. This supported the concept of two proton translocating loops between the point of entry of reducing equivalents from flavin-linked substrates and molecular oxygen, in contrast to the presence of only 1 such loop in strain D440. Unfortunately, glutamate did not induce a greater stimulation in QO_2 relative to the endogenous QO_2 when added to starved, glutamate-grown cells rather than starved, glycerol-grown cells, even though the former cells were presumably induced for glutamate uptake. It was therefore not possible to obtain a more accurate value for the $\longrightarrow H^+/O_{substrate}$ quotient for glutamate oxidation in strain M than that

Table III-7

→ H^+/O quotients of succinate-grown B.megaterium strain M during substrate-loading experiments

Preparation	added substrate	observed → H^+/O quotient (gion H^+.gatom O^{-1})	QO_2 ($\mu l O_2$.mg cells^{-1}.h^{-1})	→ $H^+/O_{substrate}$ quotient (gion H^+.gatom O^{-1})
Starved	endogenous	3.71 ± 0.19(4)	19.2 ± 1.2(4)	
	succinate	3.69 ± 0.17(4)	82.1 ± 4.1(4)	3.68

B.megaterium strain M was grown under excess oxygen conditions on minimal-salts medium containing 13 mM succinate. Succinate-limited (starved) cells were harvested at A_{680} = 1.6-1.8. Conditions of assay of → H^+/O quotients as in Table III-4. Results expressed as average ± S.E.M. (no. of determinations).

shown in Table III-6. However, the latter value shows fairly conclusively that loop 1 is inoperative in this organism.

There was a very large stimulation of QO_2 when ascorbate/TMPD was added to starved cells of strain M (approximately a 2000% increase; Table III-6). Mitchell (1969) proposed that reduced TMPD donates electrons to cytochrome c on the outer side of the coupling membrane, at the same time releasing protons, and that the electrons are donated to the cytochrome oxidase on the inner side of the membrane where reduction of oxygen occurs. Under such circumstances, an $\longrightarrow H^+/O$ quotient of 2 would be expected. Since the $\longrightarrow H^+/O$ quotient exhibited by starved cells of strain M in the presence of ascorbate/TMPD was considerably lower than the endogenous $\longrightarrow H^+/O$ quotient (3.19 cf. 4.23; Table III-6), some oxygen was presumably reduced by electrons from TMPD as well as from the endogenous substrates. This suggested that the cytochrome c of strain M is associated with electron transfer at loop 3.

The fact that the $\longrightarrow H^+/O_{substrate}$ ratio for oxidation of ascorbate/TMPD was considerably higher than 2 (viz 3.19) may have been due to the very high QO_2 for ascorbate/TMPD oxidation observed in the oxygen electrode chamber. At the low oxygen concentration prevailing in the pH-measuring chamber the QO_2 may have been much lower, hence the $\longrightarrow H^+/O_{substrate}$ ratio for ascorbate/TMPD oxidation may have been overestimated.

4. The proton-translocating loops of B. megaterium

The substrate-loading experiments on the two strains of B. megaterium presented above appear to suggest the presence of two functional proton-translocating loops in both organisms. The NADH dehydrogenase of strain D440 (ignoring the oxygen-limited cells) is

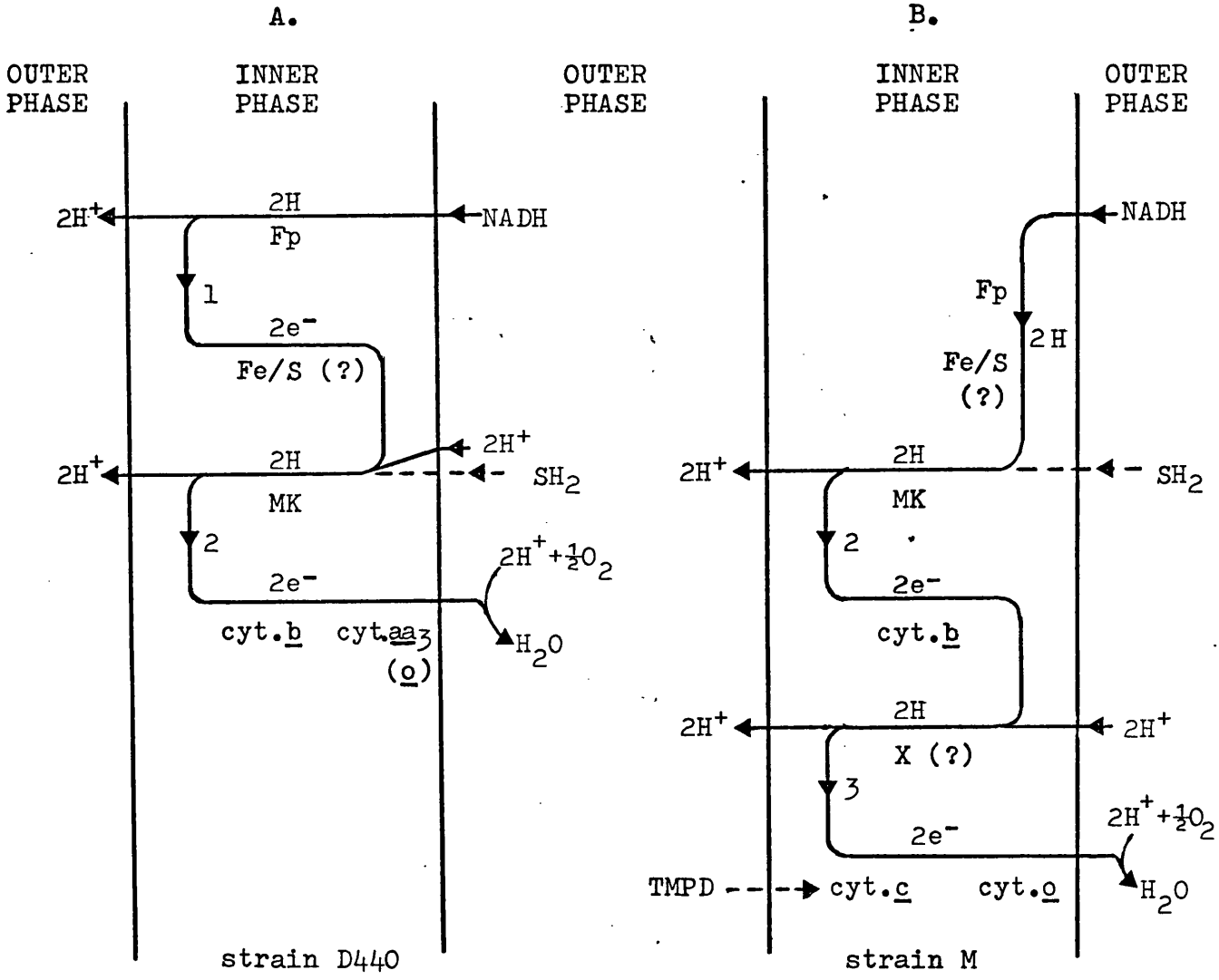
apparently associated with proton translocation (loop 1 operative) whereas that of strain M is not (loop 1 inoperative). Both strains presumably contain loop 2, but loop 3 is probably present only in strain M, since strain D440 lacks cytochrome c.

Attempts were made to confirm the presence of a functional proton translocating loop 1 in strain D440 and its absence in strain M by measurement of phosphate esterification in respiratory particles. Procedures which have been successfully employed to prepare phosphorylating respiratory membranes of A. vinelandii, by the method of Ackrell and Jones (1971a; see Chapter II) and M. denitrificans, by the method of John and Whatley (1970) were utilised for B. megaterium. However, values of $^{32}P/O$ ratio for NADH oxidation, measured by the method of Ackrell and Jones (1971a; Chapter II), were very low (< 0.1) for respiratory membranes of B. megaterium prepared by either of these techniques. Because of the low phosphorylating efficiencies, it was impossible to distinguish the presence or absence of site I phosphorylation by the use of different substrates. In view of the lack of time available, it was not considered feasible to systematically modify the membrane preparation procedures or assay conditions in the hope of obtaining higher phosphorylating efficiencies.

The probable sequences of the carriers in the respiratory chains of the two strains of B. megaterium, which are in accordance with the studies on the proton translocating properties of the organisms, are shown in fig. III-12. Loop 1 of the respiratory chain of strain D440 (fig. III-12A) is assumed to be formed by the NADH dehydrogenase complex, as is the loop 1 of mitochondria (see Chapter I), and to contain flavoprotein (H-carrier) and iron-sulphur protein(s) (electron carrier). The sequence menaquinone $\longrightarrow$ cytochrome b $\longrightarrow$ cytochrome oxidases is in

Fig. III-12

Possible arrangements in the plasma membrane of the respiratory carriers of the two strains of B.megaterium



1,2 and 3; proton translocating loops. Fp, flavoprotein. Fe/S , iron-sulphur protein. MK, menaquinone. X, hydrogen carrier of unknown identity. SH_2, flavin-linked substrates (glycerol and succinate).

accordance with the work of Kröger and Dadák (1969). The scheme requires reduction of oxygen to occur on the inside of the plasma membrane in order for proton translocating loop 2 to be formed by menaquinone (H-carrier), cytochrome b (electron carrier) and the oxidases. Loop 2 is hence very similar to the loop 2 of mitochondria (fig. I-2) which contains cytochrome b but has ubiquinone in place of menaquinone as the hydrogen carrier.

Fig. III-12B shows the probable sequence of carriers in strain M. Here the two operative loops are 2 and 3. The non-proton translocating NADH dehydrogenase donates hydrogen atoms to menaquinone which, together with cytochrome b, forms loop 2 which is identical to the postulated arrangement of loop 2 in strain D440. However, instead of donating electrons to cytochrome oxidase, the cytochrome b of strain M probably donates them to a second hydrogen carrier, X, of unknown identity, which forms proton translocating loop 3 with cytochromes c and o (electron carriers). Again, the cytochrome oxidase (o) is required to reduce oxygen on the inside of the plasma membrane in order to complete the loop, and there is some evidence from the above experiments with ascorbate/TMPD that this is the case. The two schemes in fig. III-12 also show the point of entry of reducing equivalents from flavin-linked substrates (glycerol and succinate). In each organism, oxidation of one pair of reducing equivalents from NADH by an oxygen atom is accompanied by uptake of 4 protons from the inner phase of the membrane and secretion of 4 protons into the outer phase, thus resulting in the maximum stoichiometry of approximately 4 for the $\longrightarrow H^+/O$ quotients observed with these bacteria.

C H A P T E R IV

GROWTH YIELDS AND MAINTENANCE COEFFICIENTS OF *Bacillus megaterium*

CHAPTER IV

GROWTH YIELDS AND MAINTENANCE COEFFICIENTS OF B. megaterium

SUMMARY

1. The development of a technique for the measurement of the *in situ* respiratory activity of bacterial chemostat cultures is described. Such measurements on B. megaterium allowed calculation of the growth yields with respect to ATP (Y_{ATP}) and maintenance coefficients (M) during growth under various culture conditions.

2. Values for Y_{ATP} of 13.1 and 10.4 g cells.mol ATP equivalents^{-1} were obtained for strains D440 and M respectively during glycerol-limited chemostat growth. Similar values were obtained during NH_4Cl-limited and oxygen-limited growth. Glutamate-limited growth of strain M gave a lower value of Y_{ATP} than glycerol-limited growth, suggesting that the nature of the carbon/energy source can affect the magnitude of Y_{ATP}. The maintenance coefficients obtained during NH_4Cl-limited culture of both strains were considerably higher than the values obtained under the other culture conditions. This may have been due to energy-dependent uptake of NH_4^+ under NH_4Cl-limiting conditions.

3. Growth of both strains of B. megaterium in batch culture under excess oxygen conditions was probably of similar efficiency to glycerol-limited growth in continuous culture.

Introduction

As there have been no reports in the literature of Y_{ATP} measurements on aerobic bacteria, it was considered desirable to measure the Y_{ATP} values of the two strains of B. megaterium. Since the energy conservation efficiencies of the respiratory chains of the two strains were known (see Chapter III), it was considered possible to measure growth yields of the bacteria and thereby arrive at values for Y_{ATP}, as outlined in Chapter I. Before Y_{ATP} values could be obtained, it was first necessary to select the best technique for determining growth yields of aerobic bacteria.

Since growth yields with respect to carbon/energy source require correction for the proportion of carbon incorporated into cell material, which must be measured accurately, it was considered that the measurement of growth yields with respect to oxygen (either as Y_O; g cells.gatom O consumed^{-1} or Y_{O_2}; g cells.mol O_2 consumed^{-1}) would be the most straightforward method for obtaining Y_{ATP} values for B. megaterium. As far as is known, B. megaterium does not exhibit any enzymic reactions, apart from cytochrome oxidase, which incorporate oxygen at a significant rate (as is seen, for example, with Methanomonas which oxidatively assimilates methane forming methanol; see also Hughes and Wimpenny, 1969), hence no correction need be made when measuring growth yields with respect to oxygen of this organism. Y_O measurements were first undertaken by Hadjipetrou et al. (1964) for the estimation of P/O ratios in K. pneumoniae. The technique has been described by Stouthamer (1969). In the present work, the slightly modified approach of Harrison and Loveless (1971), viz the measurement of the in situ respiratory activity (QO_2) of growing cultures, was used

for the calculation of Y_{ATP}. Although these workers used their method to calculate P/O ratios (by assuming a value for Y_{ATP}), the same technique can be applied for the calculation of Y_{ATP} values if N (i.e. mol ATP equivalents synthesised.mol O_2 consumed^{-1}) can be measured by an independent technique. The equation derived by Harrison and Loveless (see Chapter I) relates the QO_2 (mol O_2 consumed.g dry wt cells^{-1}.h^{-1}) of growing, energy-limited bacterial cells to Y_{ATP} as follows:

$$QO_2 = {}^{\mu}/_{Y_{ATP}.N} + {}^{M}/_{N} \qquad \text{IV-1}$$

where μ = specific growth rate (h^{-1}), M = maintenance coefficient (mol ATP equivalents.g cells^{-1}.h^{-1}). Stouthamer and Bettenhaussen (1973) pointed out that the equation derived by Pirt (1965), relating the observed yield with respect to carbon/energy source to the true yield corrected for maintenance energy (see Chapter I), which was supposed to be valid only under conditions where the energy source was also the growth-limiting nutrient (i.e. under carbon- or oxygen-limited conditions) could, in fact, also be applied to conditions where growth is limited by nutrients other than the energy source. This presumably also applies to equation IV-1, since it is derived directly from Pirt's equation (see Chapter I).

Brice et al. (1974) employed a batch culture technique to correct for the maintenance energy when calculating Y_{ATP} for K. pneumoniae and E. coli. Their method consisted of allowing bacteria to grow in Warburg manometer flasks and measuring the QO_2 of the cells at different specific growth rates. Use of equation IV-1 then allowed calculation of M and Y_{ATP} (see also Meyer and Jones, 1973a). However, this technique,

appealing in its simplicity, had the drawback that the growth rate of the cells was controlled by the oxygen solution rate of the cultures; i.e. the cells were always oxygen-limited. It is not possible using this method to achieve growth limitation by any other nutrient, e.g. carbon source. In addition to this, the cells were not in a steady state as the growth rate of the cells in the manometer flasks was continually slowing due to progressive oxygen-limitation and also the cells were subjected to unavoidable manipulations prior to the growth yield measurements. It subsequently became apparent (Jones et al., 1975) that the Y_{ATP} value obtained by Brice et al. (1974) for E. coli was considerably over-estimated, thus emphasising the inaccuracies of this technique.

In deciding on the culture technique to use for B. megaterium, it was necessary to bear in mind that there have been suggestions in the literature that the Y_{ATP} value of a particular organism may be affected by the use of different carbon/energy sources (Hadjipetrou et al., 1964; Stouthamer and Bettenhaussen, 1973) and that the maintenance coefficient (M) may be affected by excess carbon/energy substrate and by variation in the ammonium chloride concentration of the growth medium (Stouthamer and Bettenhaussen, 1973). In order to ascertain if similar variations occurred with B. megaterium, it was necessary to grow the organism under different substrate-limiting conditions. It was considered that continuous culture was the only practicable way of achieving this. Continuous culture also has the advantage that, at each chosen dilution rate (i.e. specific growth rate; see the Theory section of this Chapter), steady-state growth of the microorganism occurs, thus making QO_2 measurements and hence Y_{ATP} calculations more accurate than with batch culture techniques.

Theory of continuous culture

A brief outline of the theoretical aspects of continuous culture is present below (see Herbert et al., 1956; Dawes,1969; Bull,1973). Monod (1942) first observed that the specific growth rate, μ, of bacteria is governed by the concentration of the growth limiting substrate, s, in the growth medium such that

$$\mu = \mu_m \left(\frac{s}{K_s + s}\right) \qquad \text{IV-2}$$

where μ_m and K_s are constants for the particular system under study; μ_m is the growth rate when excess substrate is present and K_s is the substrate concentration at which $\mu = 0.5\ \mu_m$, called the "saturation constant". It follows from equation IV-2 that exponential growth can occur at specific growth rates having any value between zero and μ_m provided the substrate concentration can be held constant at a given value. Apparatus for the continuous cultivation of microorganisms is designed to enable this condition to be met.

There is a simple relationship between growth and utilisation of substrate such that

$$\frac{\text{mass of bacteria formed}}{\text{mass of substrate used}} = \frac{dx}{ds} = Y \qquad \text{IV-3}$$

where x is the concentration of organisms (dry weight per unit volume) and Y is the yield coefficient. The yield coefficient for microorganisms growing on many substrates is constant with respect to growth rate (e.g. amino acids, vitamins, etc.); the special case of growth on nutrients which act as both a carbon and energy source, when a variation in Y with

growth rate is observed, will be discussed later.

The continuous culture apparatus consists of a culture vessel into which sterile growth medium is fed at a flow rate f ; growth occurs in the vessel and the microbial culture also emerges at flow rate f. The volume of the culture (v) is kept constant by a levelling device. Efficient stirring is necessary so that the entering medium approaches the ideal condition of instantaneous mixing and uniform distribution throughout the vessel - the so-called 'completely-mixed' vessel. The wash-out rate, i.e. the rate at which organisms initially present in the vessel would be washed out if growth ceased but the flow of medium continued, is given by

$$-\frac{dx}{dt} = \frac{f}{v} \cdot x = D\,x \qquad \text{IV-4}$$

The ratio f/v is known as the dilution rate, D.

In the growth vessel the organisms grow at a rate μx and are simultaneously washed out at a rate described by equation IV-4. Hence, the net rate of change of concentration of organisms in the culture vessel is given by

$$\frac{dx}{dt} = \begin{array}{c}\text{rate of increase}\\ \text{due to growth}\end{array} - \begin{array}{c}\text{rate of disappearance}\\ \text{due to efflux}\end{array}$$

$$= \mu x - Dx \qquad \text{IV-5}$$

When $\mu = D$, $dx/dt = 0$ and x is constant and independent of time, i.e. there is a steady state concentration of organisms in the vessel. If $\mu > D$, then dx/dt is positive and the concentration of organisms will increase, while if $\mu < D$, dx/dt is negative and the concentration of organisms will decrease, eventually to zero, corresponding to the washing out of the culture vessel. Under steady state conditions, therefore, the specific growth rate, μ, of the organisms in the culture

vessel is exactly equal to the dilution rate, D.

In the growth vessel, substrate enters at a concentration s_R, is consumed by the organisms, and flows out at concentration s. Now, the rate of change of substrate concentration in the effluent medium

= rate of input - rate of output - rate of consumption.

Therefore, since rate of consumption = growth/yield coefficient,

$$\frac{ds}{dt} = Ds_R - Ds - \frac{\mu x}{Y} \qquad \text{IV-6}$$

As μ is a function of s (equation IV-2), we have from equation IV-5,

$$\frac{dx}{dt} = x[\mu_m \left(\frac{s}{K_s + s}\right) - D] \qquad \text{IV-7}$$

and from equation IV-6

$$\frac{ds}{dt} = D(s_R - s) - \frac{\mu_m x}{Y} \left(\frac{s}{K_s + s}\right) \qquad \text{IV-8}$$

These two equations define completely the behaviour of a continuous culture in which the fundamental growth relationships are those described by equations IV-2 and IV-3.

From a consideration of equations IV-7 and IV-8 it will be apparent that if s_R and D are held constant and D does not exceed a certain critical value (see equation IV-11), then unique values of x and s exist for which both dx/dt and ds/dt are zero, i.e. the system is in a steady state. If equations IV-7 and IV-8 are solved for dx/dt = 0 and ds/dt = 0, the steady state values of x and s, designated $\bar{x}$ and $\bar{s}$ are given by the expressions

$$\bar{s} = K_s \left(\frac{D}{\mu_m - D}\right) \qquad \text{IV-9}$$

$$\bar{x} = Y (s_R - \bar{s}) = Y\left[s_R - K_s \left(\frac{D}{\mu_m - D}\right)\right] \qquad \text{IV-10}$$

With these equations, and knowing the values of the constants μ_m, K_s and Y for a given organism and growth medium, the steady state concentrations of organisms and substrate in the culture vessel can be predicted for any value of the dilution rate and concentration of inflowing substrate. If these predicted values for $\bar{x}$ and $\bar{s}$ are then plotted as a function of the dilution rate, D, a graphical representation of the operation of the system is obtained, as shown in Fig. IV-1.

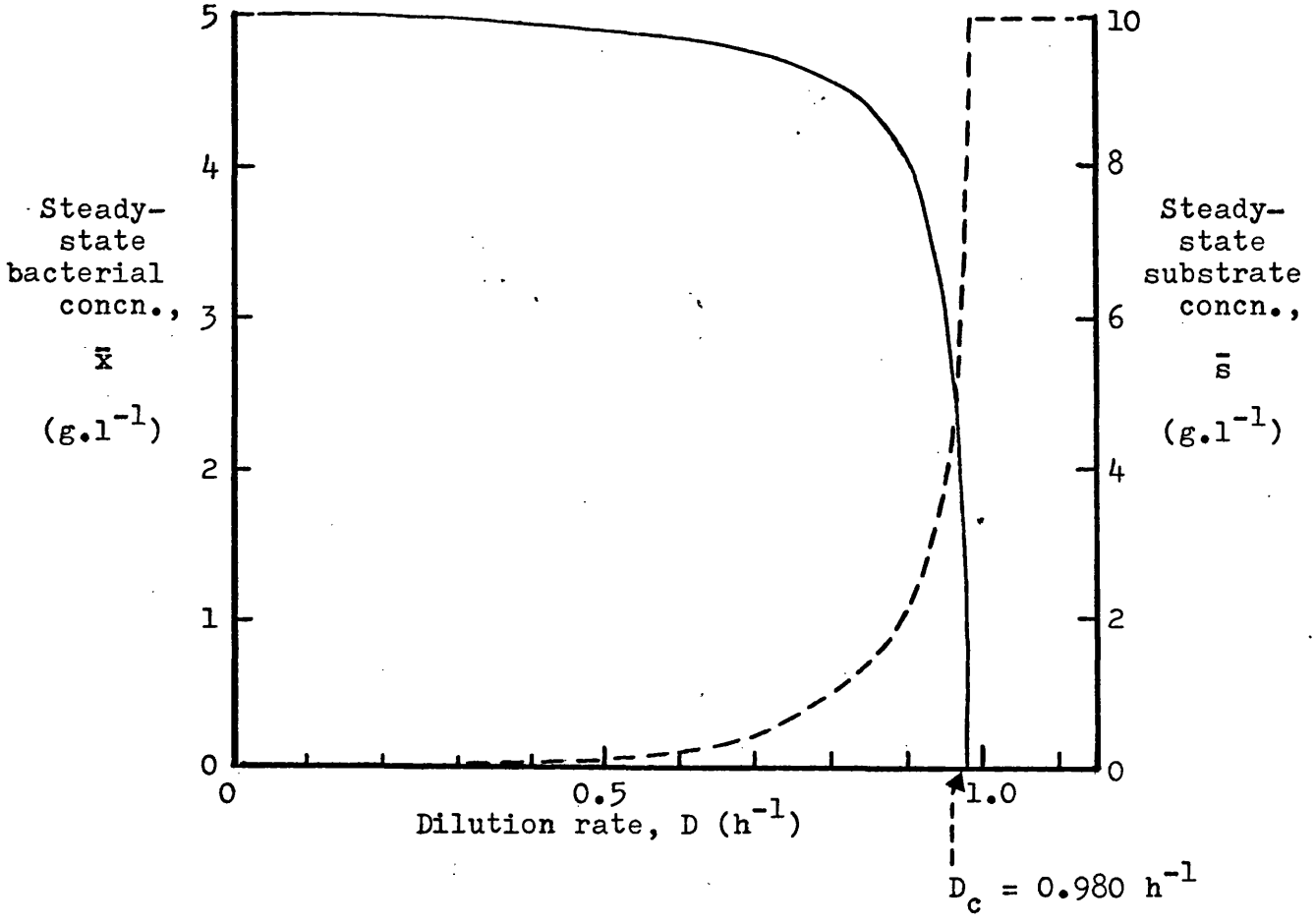
A consideration of Fig. IV-1 reveals that over most of the range of possible dilution rates the steady state concentration of substrate in the culture vessel is very low, i.e. the substrate is almost completely consumed by the organisms. Only when the dilution rate approaches a critical value, designated D_c, corresponding to that at which washout occurs, does unused substrate appear in the culture; at D_c the substrate concentration becomes equal to s_R. The concentration of organisms has a maximum value when the dilution rate is zero and as the dilution rate increases, the cell concentration falls (reflecting the increase in the ambient substrate concentration), eventually becoming zero at dilution rate D_c. . The critical dilution rate, D_c, can be calculated from equation IV-9 since it is equal to the highest possible value of μ which is attained when $\bar{s}$ is at its maximum value s_R. Thus

$$D_c = \mu_m \left(\frac{s_R}{K_s + s_R}\right) \qquad \text{IV-11}$$

Fig. IV-1

Theoretical steady-state relationships in continuous culture

(yield coefficient constant)



The steady-state values of bacterial concentration and substrate concentration for chemostat growth when the yield coefficient (Y) is invariant with respect to dilution rate. The concentrations are calculated using equns. IV-9 &10 for an organism with the following growth constants; μ_m = 1.0 h^{-1}, Y = 0.5 g cells. g substrate^{-1}, K_s = 0.2 $g.l^{-1}$. The substrate concentration in the inflowing medium is taken to be 10 $g.l^{-1}$.(After Herbert et al., 1956)

and when $s_R \gg K_s$, which is usually the case, $D_c \triangleq \mu_m$.

Variation in the yield coefficient (Y) with dilution rate for bacterial growth in continuous culture limited by the supply of carbon/energy source was first reported by Herbert (1958). He attributed the variation to the 'endogenous metabolism' of the bacteria. Pirt (1965) considered this factor to be equivalent to the maintenance coefficient of the cells (m, mol substrate consumed.g dry wt cells^{-1}.h^{-1}) which he related to the observed growth yield (Y, g dry wt cells.mol substrate consumed^{-1}) and the true growth yield (Y_G) by the following equation:

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_G} \quad \text{(see Chapter I)} \qquad \text{IV-12}$$

The true growth yield is therefore the yield corrected for cell maintenance. Since Y is dependent on growth rate, Pirt (1965) modified equation IV-10 of Herbert *et al.* (1956) to give the steady state value of the organism concentration ($\bar{x}$) for continuous cultures limited by carbon/energy source.

$$\text{since} \quad Y = \frac{D\,Y_G}{m\,Y_G + D} \quad \text{(from equation IV-12)} \qquad \text{IV-13}$$

equation IV-10 becomes

$$\bar{x} = \frac{D\,Y_G}{m\,Y_G + D}\left[s_R - K_s\left(\frac{D}{\mu_m - D}\right)\right] \qquad \text{IV-14}$$

However, this formulation is not strictly accurate since Y in equation IV-10 is an *observed* yield which includes the proportion of substrate carbon incorporated into cellular material. Equation IV-10 should therefore be written

$$\bar{x} = Y_{obs} \left[s_R - K_s \left(\frac{D}{\mu_m - D}\right)\right] \qquad \text{IV-15}$$

Y_{obs} is related to Y by the equation

$$Y_{obs} = \frac{r.Y}{p.Y + r} \qquad \text{IV-16}$$

where r = g carbon contained per mol carbon source ÷ molecular wt in g of carbon source, p = fraction by weight of carbon in the bacterial cells. Equation IV-15 then becomes

$$\bar{x} = \frac{r.Y}{p.Y + r} \left[s_R - K_s \left(\frac{D}{\mu_m - D}\right)\right] \qquad \text{IV-17}$$

The steady state bacterial concentration of a continuous culture limited by a substrate which acts as both an energy source and a carbon source is thereby given by (combining equations IV-13 and IV-17):

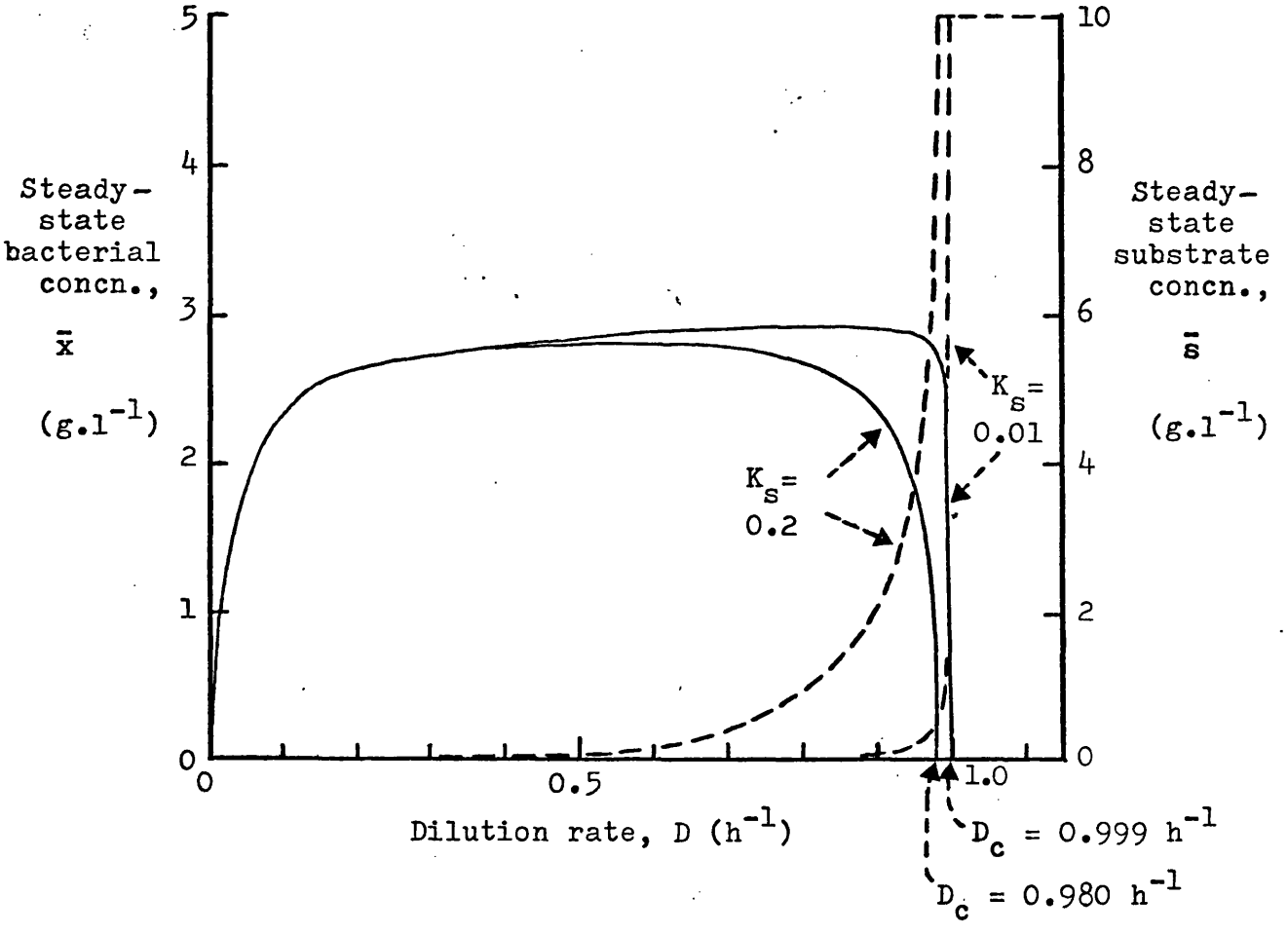
$$\bar{x} = \frac{D.Y_G.r}{D.Y_G.p + r(m\ Y_G + D)} \left[s_R - K_s \left(\frac{D}{\mu_m - D}\right)\right] \qquad \text{IV-18}$$

Predicted values of $\bar{x}$ and $\bar{s}$ as a function of dilution rate can also be plotted for carbon/energy limited cultures in the same manner as Fig. IV-1 by choosing suitable values for Y_G, p, r and m; for the sake of simplicity, values for s_R, K_s and μ_m are kept the same as before. The results are shown in Fig. IV-2. As $\bar{s}$ is independent of the yield coefficient, the plot of $\bar{s}$ against D is identical to that of Fig. IV-1. However, the plot of $\bar{x}$ against D is considerably different. The maximum value of $\bar{x}$ in Fig. IV-2 is much lower than in Fig. IV-1, emphasising the proportion of substrate carbon incorporated into cell material, and $\bar{x}$ decreases to 0 at D = 0 in Fig. IV-2 as a result of the substrate

Fig. IV-2

Theoretical steady-state relationships in continuous culture

(yield coefficient variable)



The steady-state values of bacterial concentration and substrate concentration for chemostat growth when the limiting substrate is both a carbon and energy source and the yield coefficient (Y) decreases with decrease in dilution rate. The concentrations are calculated from equns. IV-9 &18 for an organism with the following growth constants; μ_m = 1.0 h^{-1}, Y_G = 0.5 g cells.g substrate^{-1}, m = 0.1 g substrate.g cells^{-1}.h^{-1}. The substrate is assumed to be glycerol, hence r = 36/92 = 0.391. S_R = 10 $g.l^{-1}$. p is taken as 0.50 (Luria, 1960)

requirement for cell maintenance. As in Fig. IV-1, $\bar{x}$ decreases to 0 as D approaches D_c. This decrease in $\bar{x}$ occurs at lower dilution rates when K_s is high then when K_s is low - compare the plots with two different values for K_s. The plot with $K_s = 0.01\ g.l^{-1}$ is similar to that expected for carbon/energy limited cultures since the affinity of bacterial cells for the carbon substrate under these conditions is usually very high (e.g. $K_s = 0.012\ g.l^{-1}$ for Aerobacter cloacae growing in continuous culture under glycerol-limited conditions; Herbert et al., 1956).

Figs. IV-1 and 2 were therefore useful for predicting the effect of dilution rate on the culture density during chemostat growth of B. megaterium under different nutrient-limiting conditions.

Materials and Methods

1. Construction of the Chemostat

In view of the high cost of commercial continuous culture systems, the apparatus chosen utilised standard Quickfit components which could be assembled for less than 10% of the normal retail cost of chemostats (Baker, 1968; Fig. IV-3). Connections between the chemostat and its associated equipment were by means of autoclavable silicone-rubber tubing. Sterile medium from a 10 l aspirator was supplied to the chemostat vessel (working volume 200-300 ml depending on the aeration rate) at a rate which was controlled by a H.R. flow-inducer (Watson-Marlow Ltd., Marlow, Bucks.) and which could be measured by means of a 10 ml pipette in a branch of the medium line (see Fig. IV-4). The culture in the chemostat vessel was maintained at 30° by a fixed-contact thermometer in conjunction with a relay and a mains heating lamp and was kept at constant volume by means of a weir-type overflow, the effluent being collected in a 5 l flask. The culture volume was measured for each experiment from markings on the side of the vessel by stopping the stirrer (a captive, PTFE-covered magnetic flea) and allowing the vortex to subside. The volume of the culture was required for calculation of respiratory activity (see below) and dilution rate (D h^{-1}). The latter was computed as follows:

$$D\ (h^{-1}) = \frac{\text{rate of addition of fresh medium } (l.h^{-1})}{\text{culture volume } (l)}$$

Oxygen was supplied to the culture in air which was saturated with water using a sintered-glass sparger prior to passage through the chemostat vessel; this was to prevent evaporation from the culture which

Fig. IV-3

The chemostat and associated equipment

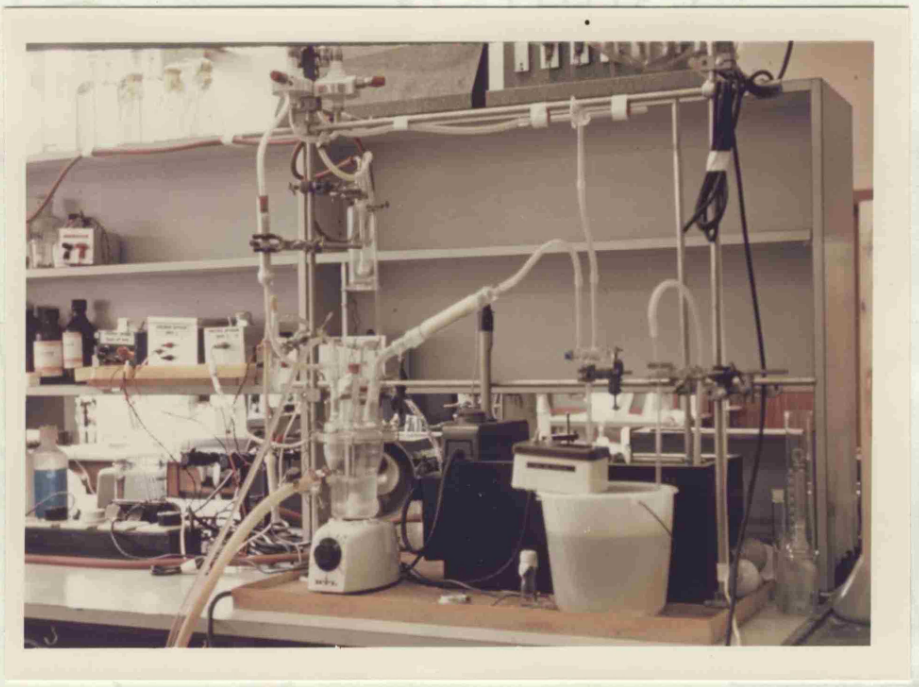
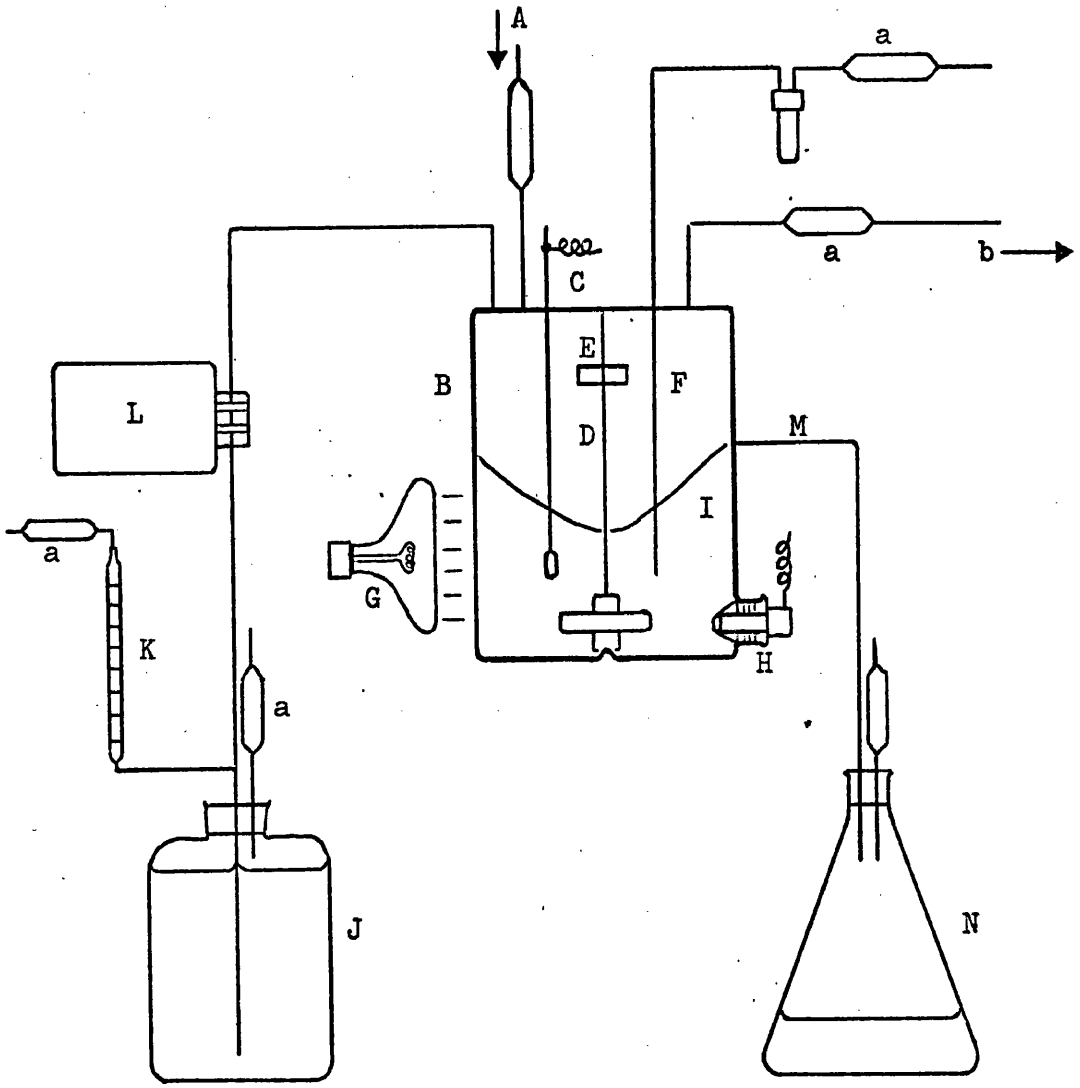


Fig. IV-4

A schematic diagram of the chemostat



A, air supply; B, chemostat vessel; C, thermometer; D, captive stirrer; E, vane on stirrer shaft to ensure adequate air turbulence; F, sampling tube; G, culture heating lamp; H, oxygen electrode; I, culture; J, medium reservoir; K, medium flow-rate meter; L, medium pump; M, culture overflow; N, collecting flask; a, sterile cotton-wool air-filters; b, effluent air to oxygen analyser.

would have altered the culture conditions, especially at low dilution rates. The sparging process also helped to remove traces of dirt and oil vapour from the air supply. A variable-speed magnetic drive unit under the chemostat vessel controlled the speed of rotation of the magnetic stirrer in the vessel and hence the aeration rate of the culture. No baffling was required apart from that achieved by protrusion of the thermometer and sampling tube into the culture on either side of the stirrer shaft (Fig. IV-4). The drive unit could be set to give the three aeration rates routinely employed, viz: low aeration (oxygen transfer coefficient, $K_L A$ = 4.9 μmol $O_2 . l^{-1} . h^{-1} . mm\ Hg^{-1}$), high aeration ($K_L A$ = 55) and extra-high aeration ($K_L A$ = 132). The $K_L A$ for each aeration condition was calculated as follows. Arnold and Steel (1958) derived the following relationship describing the rate of solution of oxygen from a gas into culture medium:

$$S = K_L A (P_G - P_L) \qquad \text{IV-19}$$

where S is the rate of oxygen transfer to the culture (mol $O_2 . l^{-1} . h^{-1}$), P_G is the partial pressure of oxygen in the gaseous phase and P_L is the partial pressure of oxygen in the culture medium (mm Hg). Now, at any particular time in the growth of an aerobic organism, the rate of oxygen transfer to the culture medium from the gaseous phase is equal to the rate of oxygen uptake by the bacteria, i.e. $S = QO_2 . x$, where QO_2 is the respiratory activity (mol $O_2 . g\ cells^{-1} . h^{-1}$) and x is the organism concentration (g dry wt. l^{-1}). Therefore, equation IV-19 becomes

$$QO_2 . x = K_L A (P_G - P_L)$$

whence $$K_L A = \frac{QO_2 . x}{(P_G - P_L)} \qquad \text{IV-20}$$

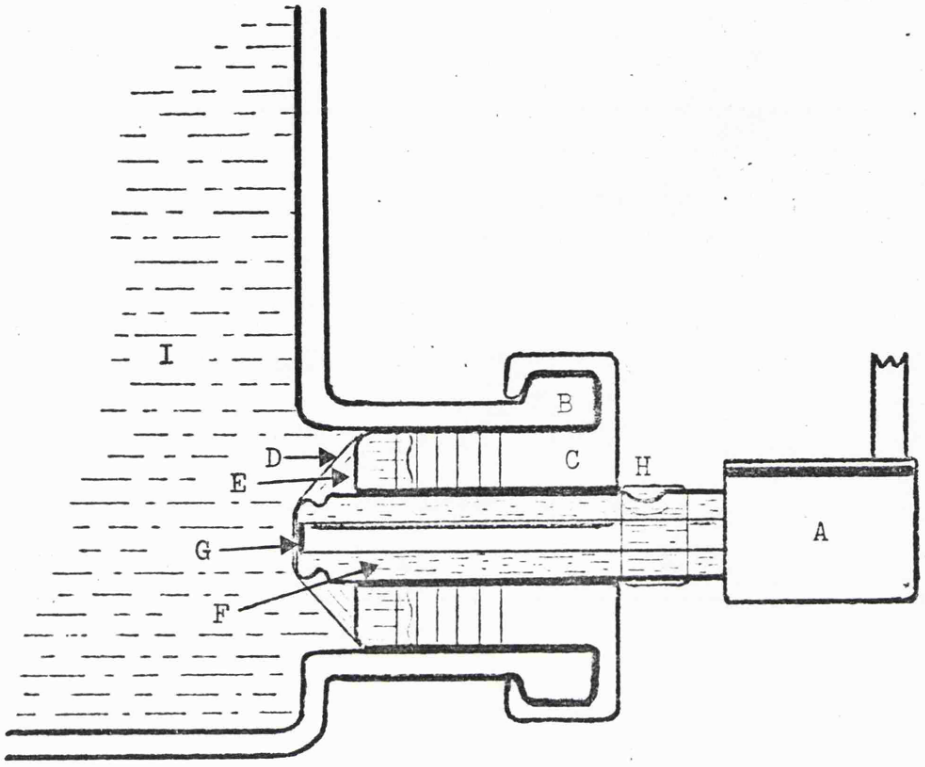
Since the values of QO_2, x and P_L of the culture in the chemostat varied according to the dilution rate, organism used and culture conditions, an average value of $K_L A$ for each aeration condition could be calculated. The partial pressure of oxygen in the gaseous phase (P_G) was assumed to be 159 mm Hg.

The dissolved oxygen concentration (assumed to be equivalent to the partial pressure of oxygen) of the culture was measured by means of a Clark electrode (YSI 4004). As the electrode was not autoclavable, it was necessary to use an alternative method of sterilisation. Immersion for 1 h in 100% ethanol was unreliable in preventing contamination and eventually a technique for autoclaving the electrode membrane in situ was developed (Fig. IV-5). The procedure for achieving this was as follows: a circle of membrane of the appropriate size was wrapped round the end of a subaseal which was then inserted into a port near the base of the chemostat vessel. After autoclaving the apparatus, the electrode was pushed through a hole in the centre of the subaseal so that the membrane was stretched around the tip of the electrode and the electrode protruded slightly into the cavity of the chemostat vessel. Electrolyte was then added as normal. Using this method, the side of the membrane facing the culture was sterile prior to inoculation.

The electrode was calibrated in sterile, air saturated medium at 30°. As the electrode design is such that it consumes oxygen at a slow rate, calibration was performed with the stirrer on in order to avoid local oxygen depletion in the vicinity of the electrode tip (cathode) which would have resulted in low oxygen readings. Measurements

Fig. IV-5

The electrode used for the measurement of the dissolved oxygen concentration of chemostat cultures



A, Clark YSI 4004 oxygen electrode; B, glass wall of chemostat vessel; C, Suba seal; D, electrode membrane (0.001 in. Teflon); E, air space; F, electrolyte; G, cathode; H, port for addition of electrolyte (sealed with parafilm); I, chemostat culture.

of oxygen concentrations were made on a recorder using the 2 mV setting. With the output of the electrode corresponding to air-saturated medium amplified to 1.8 mV, the current corresponding to zero oxygen (with the chemostat containing a growing culture and the stirrer operating at very low speed, approximately 2 $rev.sec^{-1}$) was too small to be measurable on the recorder. The electrode was re-calibrated after each experiment with a particular culture by removing all but approximately 50 ml of culture from the vessel and turning the stirrer up to a high speed setting. It was then assumed that the electrode output approximated to the air-saturation value, because of the very high oxygen solution rate compared with oxygen uptake rate of the bacteria. The drift in output of the electrode was less than $\pm$ 5% per week.

2. Growth of B. megaterium in the chemostat

(a) Concentrations of growth-limiting substrates Steady state bacterial growth in continuous culture is always limited with respect to the concentration of a particular nutrient. It was desired to grow B. megaterium under a variety of nutrient-limitation conditions in the chemostat and, in order to do this, it was necessary to select the appropriate limiting-substrate concentration in order that this substrate be growth-limiting rather than any other.

For all of the chemostat work, the same minimal-salts medium was employed as that used for batch culture (see Chapter III). In order to determine the ideal concentration of a particular substrate, a number of shake flasks, each containing a different concentration of the substrate, were inoculated with the desired strain of B. megaterium and aerated on a gyratory incubator at 30° until no further growth occurred.

The final bacterial concentration in each flask was then measured and the results plotted against the initial substrate concentrations. There was an approximately linear relationship between total growth and glycerol/NH_4Cl concentration for strain D440 (Fig. IV-6) and also between total growth and glycerol/glutamate/NH_4Cl concentration for strain M (not shown). From such graphs, the appropriate substrate concentration for the supply medium was chosen which would give a bacterial density in the chemostat of between 0.3 - 0.5 mg dry wt.ml^{-1}. 0.5 mg dry wt.ml^{-1} was the upper limit for culture density in the chemostat because of the absence of pH control on the chemostat apparatus. The buffering capacity of the medium was adequate only for culture densities below this value.

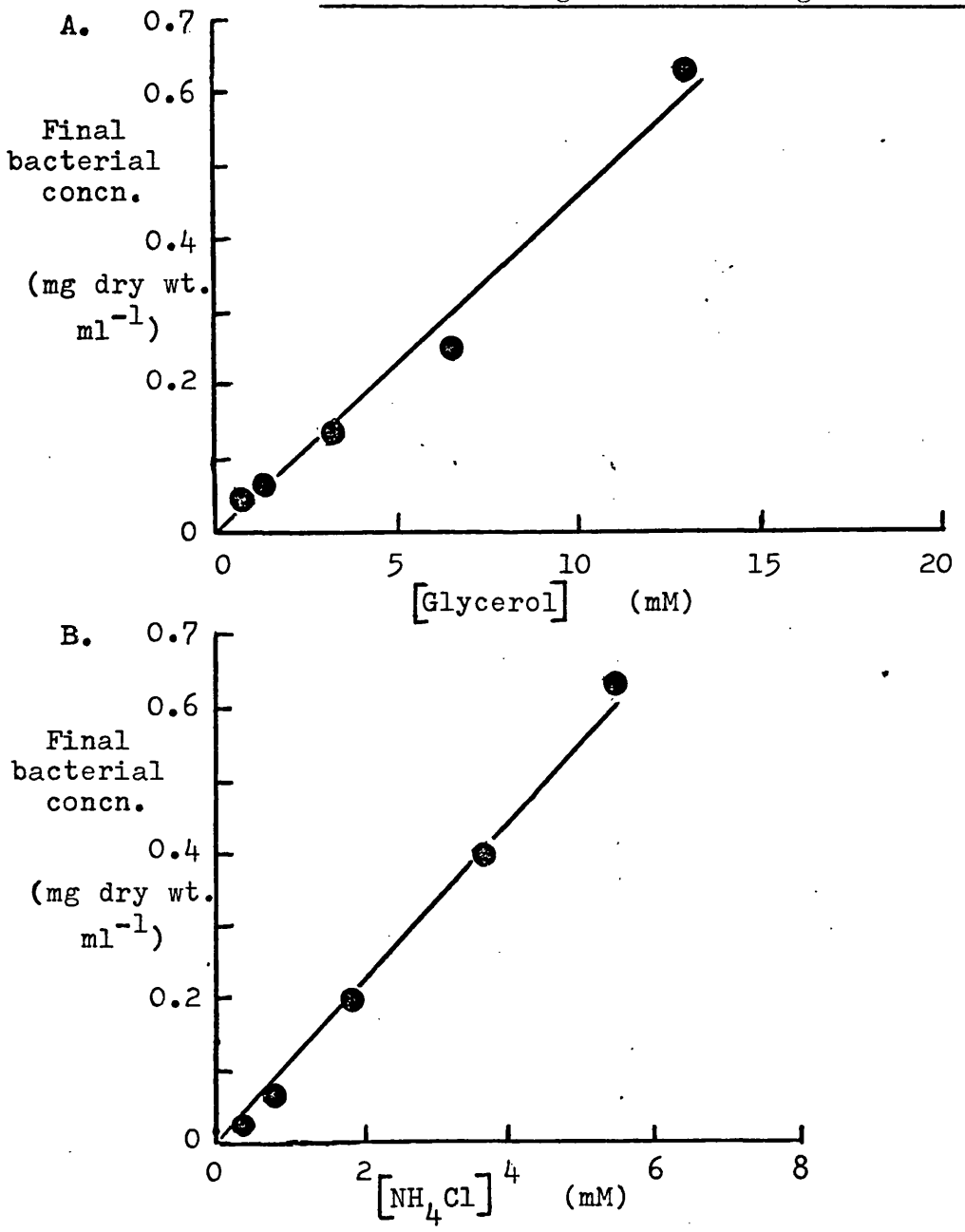
Oxygen-limited cultures were obtained by using a low aeration rate together with normal, batch culture concentrations of nutrients in the supply medium.

The nutrient concentrations in the input medium under the various nutrient-limitation conditions used were as follows:
strain D440: glycerol-limited (high aeration), [glycerol] = 7.60 mM; glycerol-limited (extra high aeration), [glycerol] = 1.52 mM; nitrogen-limited, [NH_4Cl] = 3.70 mM.
strain M: glycerol-limited, [glycerol] = 5.44 mM; glutamate-limited, [glutamate] = 5.44 mM; nitrogen-limited, [NH_4Cl] = 3.70 mM.
Under glycerol-, glutamate- and oxygen-limited conditions [NH_4Cl] = 37 mM. Under nitrogen- and oxygen-limited conditions [glycerol] = 33 mM.

(b) Setting up and operation of the chemostat The chemostat apparatus was split into three sections for autoclaving; the aspirator

Fig. IV-6

Determination of the appropriate limiting-substrate concentration for chemostat growth of B.megaterium strain D440



B.megaterium was grown under excess oxygen conditions in batch cultures containing varying concentrations of a selected nutrient. Final bacterial concentrations were calculated from measurements of A_{680} and reference to a dry weight curve. A, limiting substrate glycerol ($[NH_4Cl]$ = 37 mM). B, limiting substrate ammonium chloride ([glycerol] = 66mM). Concentrations of nutrients selected for chemostat growth of strain D440; glycerol limitation, 7.6 mM glycerol; nitrogen limitation, 3.7 mM NH_4Cl.

containing the medium, the chemostat with its ancilliary equipment and the effluent flask. Following autoclaving at 15 lb.in^2 for 1 h, the desired quantities of trace elements (see Chapter III) and carbon/energy source were added to the medium aspirator in the sterile room.

After reassembling the equipment, the chemostat vessel was filled with sterile medium from the aspirator and inoculated with 5 ml of the desired strain of B. megaterium from a nutrient broth starter culture. Such starter cultures were always inoculated fresh from a slope to avoid contamination of the chemostat. The bacteria in the culture vessel were allowed to grow for several hours until they had reached a sufficiently high cell density for the medium pump to be turned on. At least 4 volume changes of the culture at the required dilution rate were always allowed before any experiments were performed. The culture was assumed to be in a steady state when measurements of A_{680} of effluent samples were constant with respect to time.

The pH of the culture was measured on effluent samples using a Pye-Unicam (model 290) pH meter.

For measurement of cytochrome concentrations, the effluent was collected overnight in the effluent flask maintained at 0-4° by ice. After harvesting the cells, respiratory membranes were prepared and cytochrome concentrations determined as described in Chapter III.

$\longrightarrow H^+/O$ quotients were measured on samples removed directly from the chemostat (i.e. on growing cells) via the sampling port. The cells were harvested, washed and assayed as described in Chapter II.

3. Measurement of the in situ respiratory activity of chemostat cultures

It was necessary to measure the respiratory activity (QO_2) of

chemostat cultures of B. megaterium in order to calculate the growth yield of the organism with respect to ATP (i.e. Y_{ATP}) under different culture conditions. Unfortunately, this was not a simple procedure. Harrison and Loveless (1971) showed that QO_2 measurements on samples removed from the chemostat ('potential' QO_2 values) were invariably higher than direct measurements on the chemostat culture ('in situ' QO_2 values), at least with the carbon/energy limited cultures they were studying. Similar errors are likely to occur for cultures limited by other substrates because steady-state conditions no longer apply to cell samples removed from the chemostat (N.B. the 'potential' QO_2 is probably identical to 'in situ' QO_2 in the case of excess oxygen batch cultures, in which all nutrients are present in excess; see Chapter II). Therefore, the simple technique of measuring the oxygen uptake rate of cell samples in an oxygen electrode chamber (see Chapter II) could not be used for QO_2 measurements on chemostat cultures of B. megaterium. Instead, 'in situ' QO_2 values were obtained by calculation after measuring the following parameters: (a) the difference in oxygen concentration between fresh air and effluent air from the chemostat ($\Delta\%\ O_2$), (b) the flow rate of air through the chemostat, and (c) the bacterial density in the chemostat.

(a) Measurement of $\Delta\%\ O_2$ This was the most difficult parameter to measure, because of the small value of $\Delta\%\ O_2$. Although for a given respiring culture in the chemostat the $\%\ O_2$ in the effluent air could be decreased, and hence $\Delta\%\ O_2$ increased by (1) increasing the culture density (by increasing the concentration of growth-limiting substrate in the supply medium) and/or (2) decreasing the air flow rate through the chemostat , there were limits in the present work to which

these parameters could be changed. This was because cell densities of greater than 0.5 mg dry wt bacteria.ml^{-1} were undesirable (see above) and because air flow rates below approximately 20 ml.min^{-1} were difficult to measure accurately (see below). These limitations, together with the small culture volume, necessitated measurements of $\Delta\%\ O_2$ of less than 2%.

The most commonly employed technique for determining oxygen concentration in gases makes use of the paramagnetic properties of oxygen (see e.g. Tempest et al., 1967). Such paramagnetic analysers are, however, expensive, especially when modified to measure accurately small differences in oxygen concentration. An attempt was therefore made to employ the much cheaper polargraphic Clark electrode (YSI 4004) for this purpose. Because of its design, the Clark electrode can be used to measure oxygen concentration in gases as well as the more usual application for liquids. Oxygen, which diffuses through the Teflon membrane at the tip of the electrode, undergoes electroreduction to water at the platinum cathode. The cathode, which is maintained at 0.80 V, is depolarised by this oxygen allowing a current to flow which is proportional to the oxygen in contact with the cathode surface. The reference anode, together with the electrolyte, is also behind the membrane so that no current flows through the material being measured. For this reason, the electrode can be used to measure oxygen in non-conducting liquids or gases. The reactions which occur at the electrodes are as follows:

At the silver anode:

$$4\ Ag + 4\ Cl^- \longrightarrow 4AgCl + 4\ e^-$$

At the platinum cathode:

$$2\,H^+ + 2\,e^- + O_2 \longrightarrow H_2O_2$$

$$H_2O_2 + 2\,H^+ + 2\,e^- \longrightarrow 2H_2O$$

overall:

$$4\,Ag + 4\,Cl^- + 4\,H^+ + O_2 \longrightarrow 4\,AgCl + 2H_2O$$

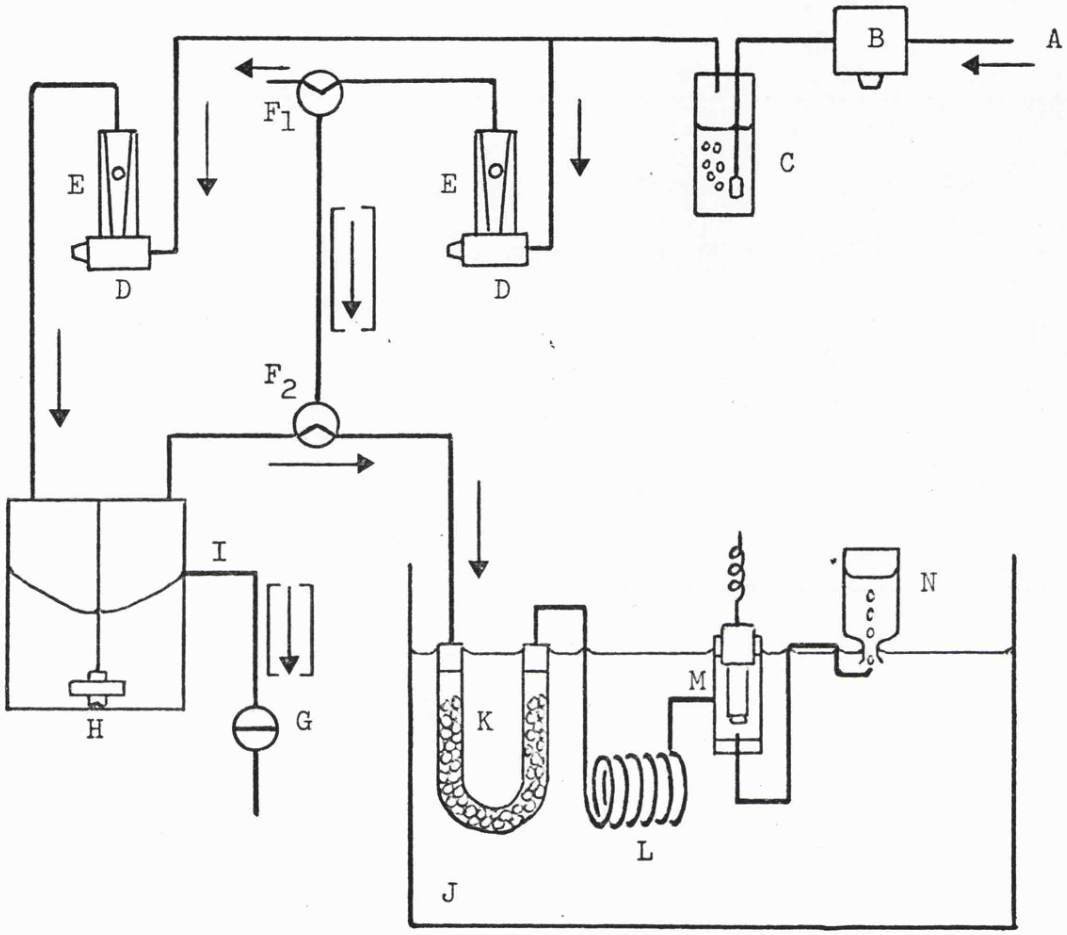
The equipment which supplied the polarizing voltage to the electrode in the present work also converted the variable current produced by the electrode in response to varying oxygen concentrations into a variable voltage which could be amplified and recorded on the 100 mV setting of a recorder. Because of the sensitivity of membrane electrodes to temperature (the output increases as the temperature increases) it was necessary to undertake measurements at constant temperature. The electrode, in its air-tight holder (adapted from Quickfit apparatus), was therefore kept in a water-bath maintained at 30°. In order to ensure that the gas to be measured was temperature-equilibrated, a thin-walled glass coil was also included in the water-bath on the input side of the oxygen analyser (Fig. IV-7).

It was found that oxygen measurements on the damp effluent air from the chemostat resulted in condensation forming on the electrode membrane which seriously impaired its performance due to the slow rate of oxygen diffusion through the water layer. This problem was overcome by drying the air by passage through a U-tube containing self-indicating silica-gel.

For accurate measurement of $\Delta\%\ O_2$ it was essential to maintain

Fig. IV-7

A schematic diagram of the chemostat oxygen analyser and associated equipment



A, air supply; B, Manostat pressure regulator; C, sparger; D, Flostat control units; E, GAP flowmeters; F_1 & F_2, three-way taps; G, two-way tap; H, chemostat vessel; I, culture (effluent air) overflow; J, analyser water bath; K, drying-tube (containing silica gel); L, temperature-equilibrator (glass coil); M, oxygen electrode in holder; N, collecting bottle for air flow-rate determinations; arrows, direction of air flow. In the diagram taps F_2 and G are set to direct effluent air into the analyser.

a steady air flow rate through the chemostat and also to have a steady fresh air supply for reference purposes. Both air supplies came from a common source: a compressed-air tap. A Manostat pressure regulator (John Watson & Smith Ltd., Leeds) was used to even out gross irregularities in air pressure from the tap and the air line was then split into two, one line for the chemostat and the other for the fresh air supply. Both lines were fitted with Flostat control units (G.A. Platon Ltd., Croydon, Surrey) which eliminated any residual irregularities in air pressure and which allowed adjustment of air flow rates. Either fresh air or effluent air was directed through the analyser by means of three-way taps in the air streams (F_1 and F_2 in Fig. IV-7). When fresh air was passed through the analyser, tap F_2 was closed to the effluent air and the latter passed out of the chemostat via the culture overflow, I, and the two-way tap G. When effluent air was directed through the analyser, tap G was closed and tap F_2 opened to the chemostat. At the same time, tap F_1 was opened to the outside air, so that back-pressure in the fresh air line did not alter the effluent air flow rate, since both fresh air and effluent air came from a common source. The chemostat overflow was of wide bore (2 cm) so that effluent air could be passed to the analyser for at least 20 min before the culture effluent in the blocked-off overflow rose to the weir level and destroyed the steady state conditions in the chemostat vessel.

It was essential to ascertain if the electrode output was linear with respect to oxygen concentration for the range of oxygen concentrations to be measured. Various mixtures of oxygen and "white-spot"nitrogen were passed, at known flow rates, through the analyser at a constant total flow rate. The output of the electrode was then

measured in the range 0-100% O_2. The electrode output was linear with respect to oxygen concentration (partial pressure) over the range 0-60% O_2 but there was some tailing-off in output above this value (Fig. IV-8). As the oxygen concentrations measured in this work were $\leqslant$ 20.9% and as the zero current (with "white-spot" nitrogen) was only 1.5% of the value with air, it was assumed that the oxygen concentration of air passing through the analyser was directly proportional to the electrode output.

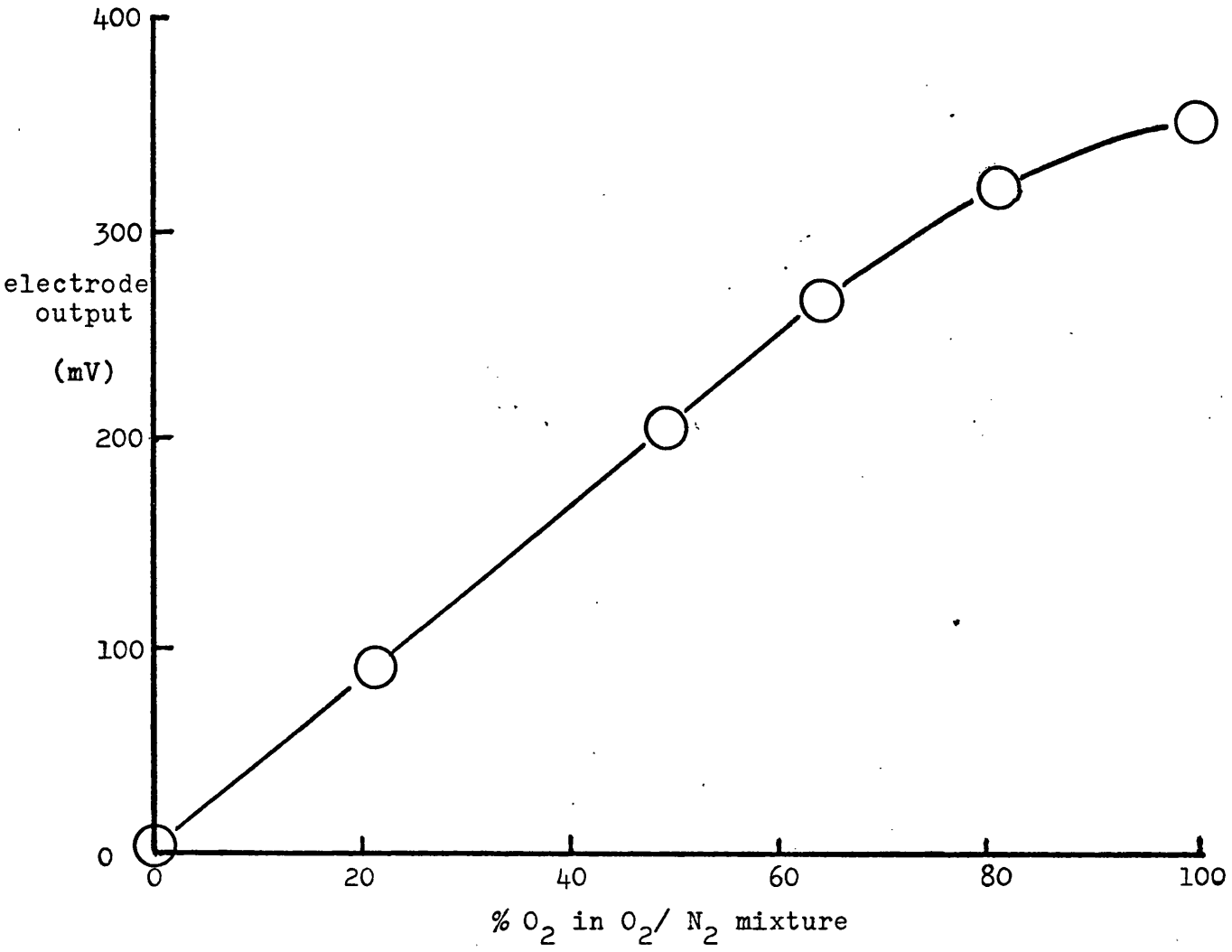
Unfortunately, Δ% O_2 could not be measured accurately with the electrode connected to the normal polarographic circuit plus amplifier. If the amplifier was set to give an output of 90 mV for fresh air, then the maximum drop in output on changing to effluent air (assuming Δ% O_2 $\simeq$2%, corresponding to chemostat cultures of high respiratory activity) was approximately 8 mV. This difference could not be accurately measured with the recorder on the 0-100 mV range.

Hayes et al. (1968) used a Clark electrode to measure low respiration rates of insects by amplifying and then 'backing off' a large proportion of the output signal so that only the variable region was recorded. As far as is known, this idea has not been exploited for measurements of the respiratory activity of chemostat cultures.

A backing-off circuit was therefore constructed according to the design of Hayes et al.(1968) and set against a standard potentiometer to give an output of 90.0 mV. This was then connected in series with the electrode amplifier and recorder (Fig. IV-9). As the current from the backing-off circuit travelled through the equipment in a direction opposite to that of the amplified electrode current, the difference in potential between the electrode amplifier output and the back-off output

Fig. IV-8

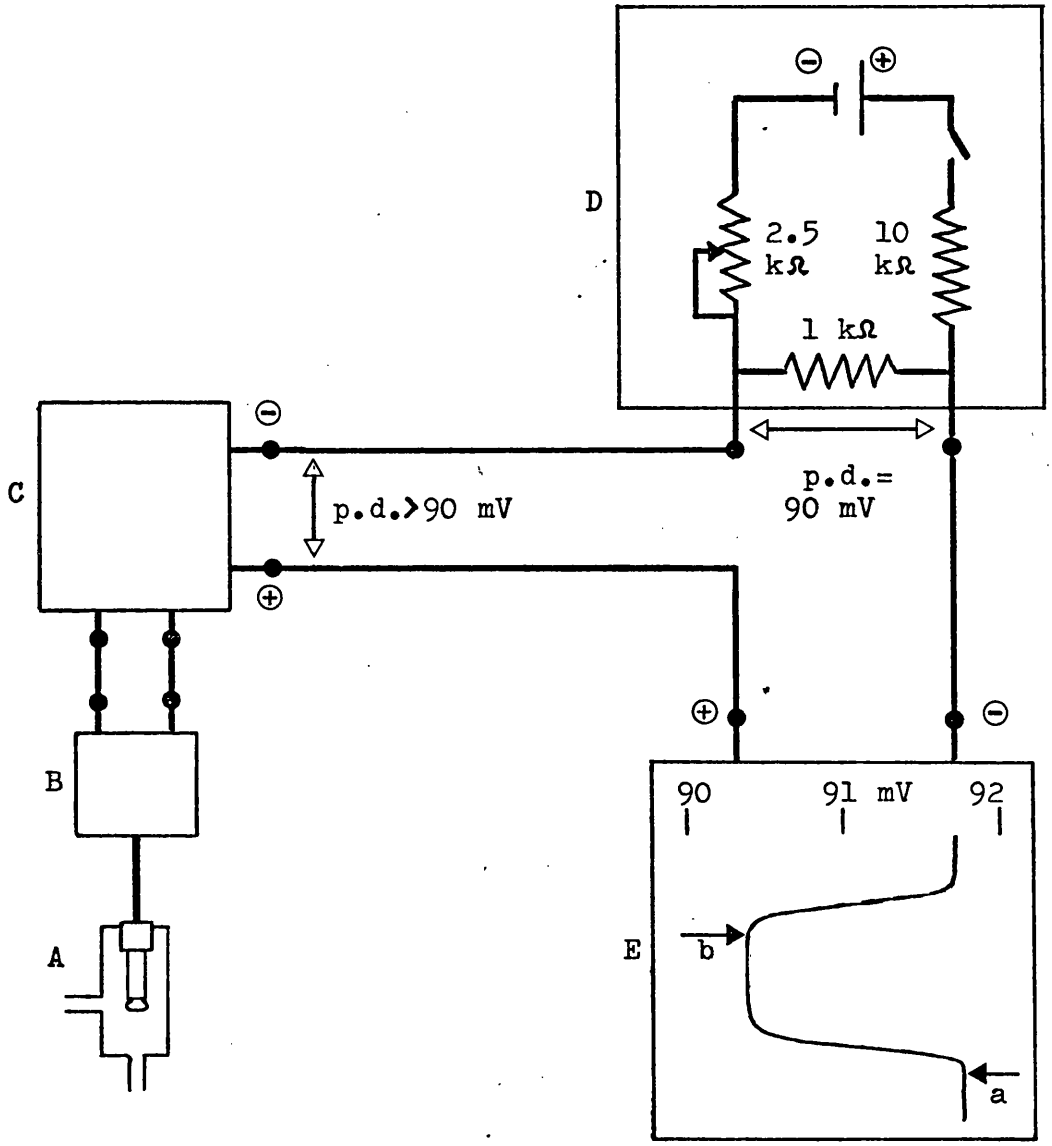
The output of the analyser electrode on varying the P_{O_2} of the gas.



Known amounts of oxygen and 'white-spot' nitrogen were mixed and passed through the analyser at a combined flow rate of 60ml.min^{-1}. Circuit employed; variable amplifier set to give an output of 90 mV with air. When used for measuring the respiratory activity of bacterial cultures, the oxygen concentration was about 20%.

Fig. IV-9

The electrical equipment used in conjunction with the oxygen analyser



A, oxygen electrode in holder; B, polarographic circuit; C, variable amplifier; D, backing-off circuit; E, recorder with trace. a, analyser air supply changed from fresh air to effluent air; b, vice-versa. Recorder range 0-2 mV (f.s.d. = 0.454 % O_2).

was registered on the recorder (the variable electrode amplifier was always adjusted so that the output corresponding to effluent air was >90 mV). When the recorder was on the 2 mV range, full scale deflection corresponded to a $\Delta\%\ O_2$ of 0.454. Values of $\Delta\%\ O_2$ larger than this could be measured on the 5 mV range (f.s.d. ≡ 1.10% O_2) or the 10 mV range (f.s.d. ≡ 2.09% O_2). By means of the variable amplifier, the electrode output could be amplified by a suitable amount such that the outputs corresponding to both fresh and effluent air appeared on the appropriate recorder range, as shown in Fig. IV-9. In general, the 0-2 mV range was used for cultures with low respiratory activities ($\Delta\%\ O_2$ small) and the higher ranges for cultures having high respiratory activities ($\Delta\%\ O_2$ large).

The sensitivity of the oxygen analyser used in conjunction with the backing-off circuit is compared, in Fig. IV-10, with the sensitivity without this equipment being attached. With the backing-off circuit connected and the recorder on the 0-2 mV range (Fig. IV-10B), the $\Delta\%\ O_2$ in this case was calculated as follows:

electrode output due to fresh air = 91.19 mV

electrode output due to effluent air = 90.08 mV

$$\%\ O_2 \text{ in effluent air} = \frac{90.08}{91.19} \times 20.90 = 20.62\%$$

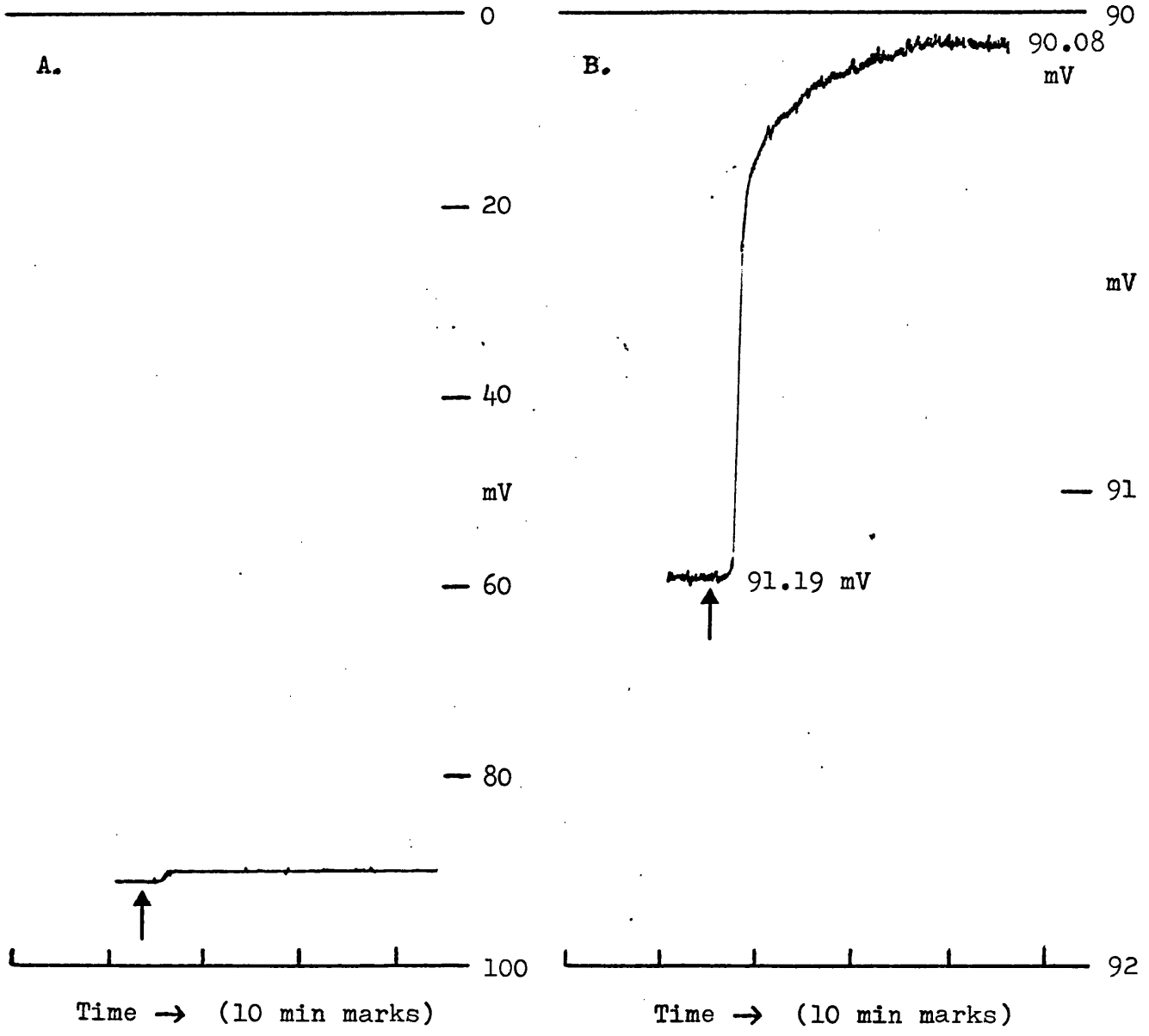
$$\therefore\ \Delta\%\ O_2 = 20.90 - 20.62 = 0.28\%$$

The noise level was equivalent to an oxygen concentration of less than 0.005% O_2 and was less obtrusive on the high recorder ranges than on the low range.

Although the analyser was simple to use in theory, there were,

Fig. IV-10

The output of the analyser electrode on changing the air supply from fresh air to effluent air



The chemostat contained a steady-state, glycerol-limited culture of B.megaterium strain D440 ($D = 0.2\ h^{-1}$; $QO_2 = 3.9$ mmol O_2. g cells^{-1}.h^{-1}) The air supply to the analyser was changed (arrows) from fresh air to effluent air. Two recorder tracings are shown; A, electrode amplifier adjusted to give an output of 91-92 mV for fresh air; B, as A but used in conjunction with a 90 mV backing-off circuit. Recorder ranges; A, 0-100 mV; B, 0-2 mV. Fresh air and effluent air flow rates, 30 ml.min^{-1}.

in practice, a number of technical difficulties resulting from the high amplifications used, which had to be overcome before reproducible measurements of QO_2 could be routinely observed.

(i) Temperature variations in the water bath

Fluctuations in electrode output not corresponding to a change in % O_2 of the air passing through the analyser were traced to the waterbath heater (Fig. IV-11). Although this unit maintained the water temperature between 30.60° and 30.83°, as measured with a thermometer, such a variation was too great for the electrode operating at high amplifications. A more sensitive heating unit was subsequently employed (Grant Instruments Ltd., Cambridge, England) which resulted in an invariant output signal from the electrode. Changes in the temperature of the water bath were then undetectable, using a thermometer. Variation in the electrode output was found to be approximately linear with respect to temperature (Fig. IV-12).

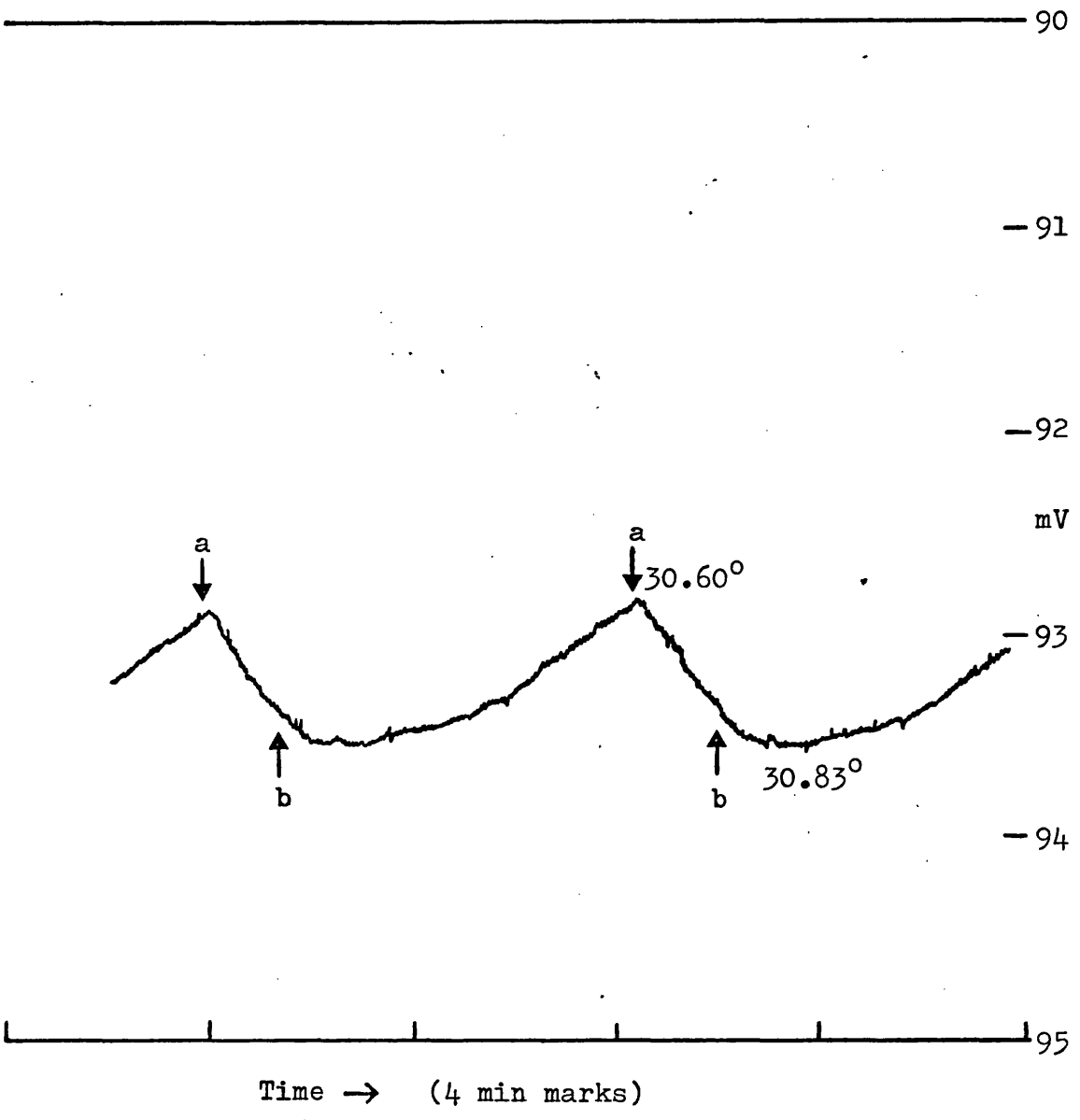
(ii) The effect of different air flow-rates

In order to achieve maximum accuracy in measurements of $\Delta\%\ O_2$ it was necessary, following each measurement of the electrode output corresponding to effluent air, to measure the output corresponding to fresh air. This was because there was always a small drift in electrode output with respect to time.

When fresh air was passed through the analyser at different flow rates, it was found that the electrode output increased slightly with increasing flow rate, in spite of the constant % O_2 of the air (Fig. IV-13). Thus the electrode output was about 0.6% greater at a flow rate of 100 $ml.min^{-1}$ than at a flow rate of 5 $ml.min^{-1}$. This can be explained by the fact that, in operating, the electrode consumes a small amount of oxygen. At low air flow rates, the turbulence in the

Fig. IV-11

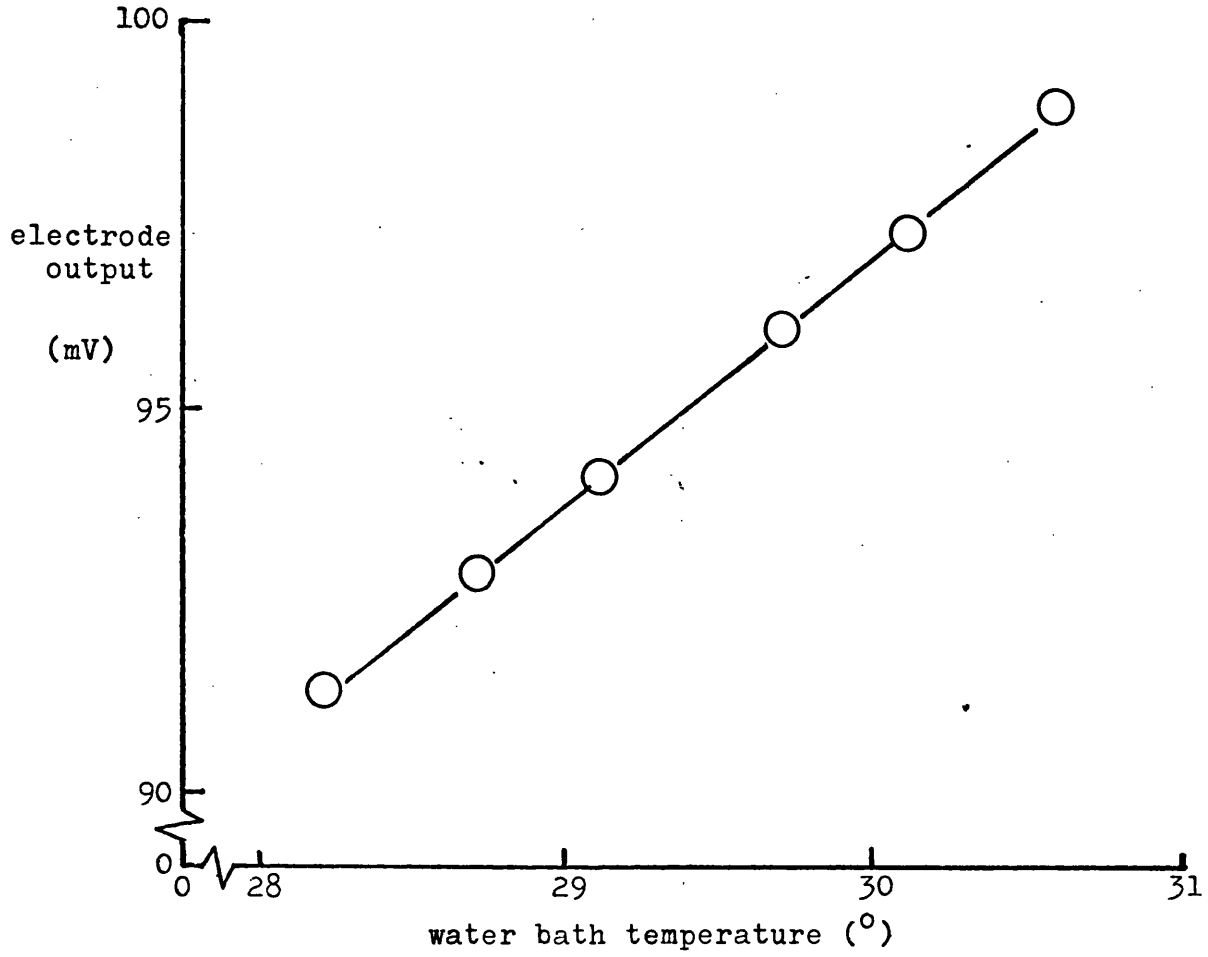
The output of the analyser electrode during inadequate temperature-control of the water bath



Fresh air with a constant oxygen content (20.90 %) and flow rate (30 ml.min^{-1}) was passed through the analyser. The analyser water bath was maintained at approximately 30° by a Techne TE1 water heater. A recorder tracing is shown of the output of the electrode corresponding to fluctuations in the temperature of the water bath. Recorder range 5 mV. Arrows; a, heater on; b, heater off.

Fig. IV-12

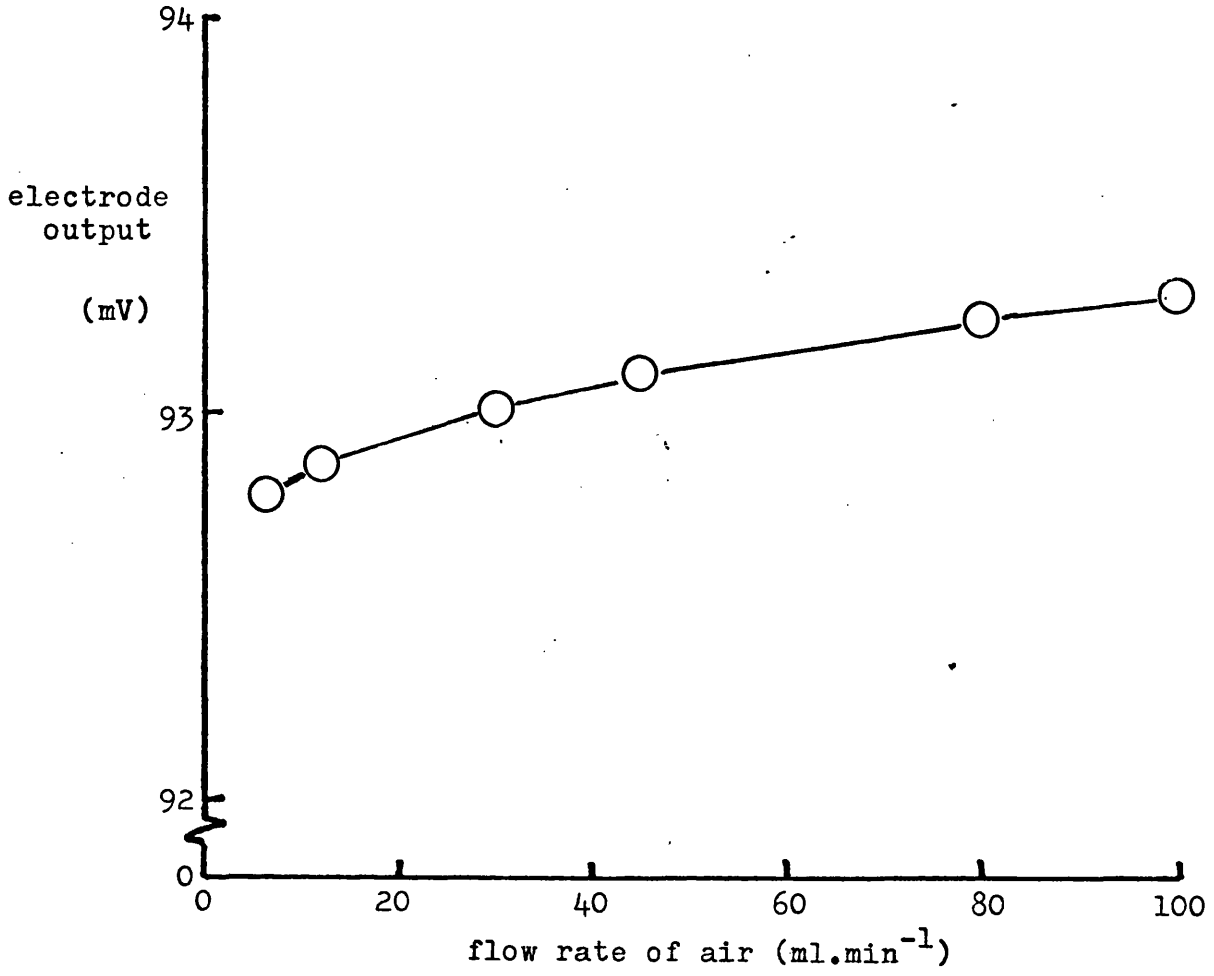
The output of the analyser electrode on varying the temperature of the analyser water bath.



Fresh air with a constant oxygen content (20.90%) and flow-rate (30ml.min^{-1}) was passed through the analyser whose temperature was varied by means of thermostat controls on the water bath heater. Circuit employed; variable amplifier in conjunction with a 90mV backoff circuit. When used for measuring the respiratory activity of bacterial cultures, the temperature of the analyser water bath was approximately 30.0°.

Fig. IV-13

The output of the analyser electrode on varying the air flow-rate through the analyser.



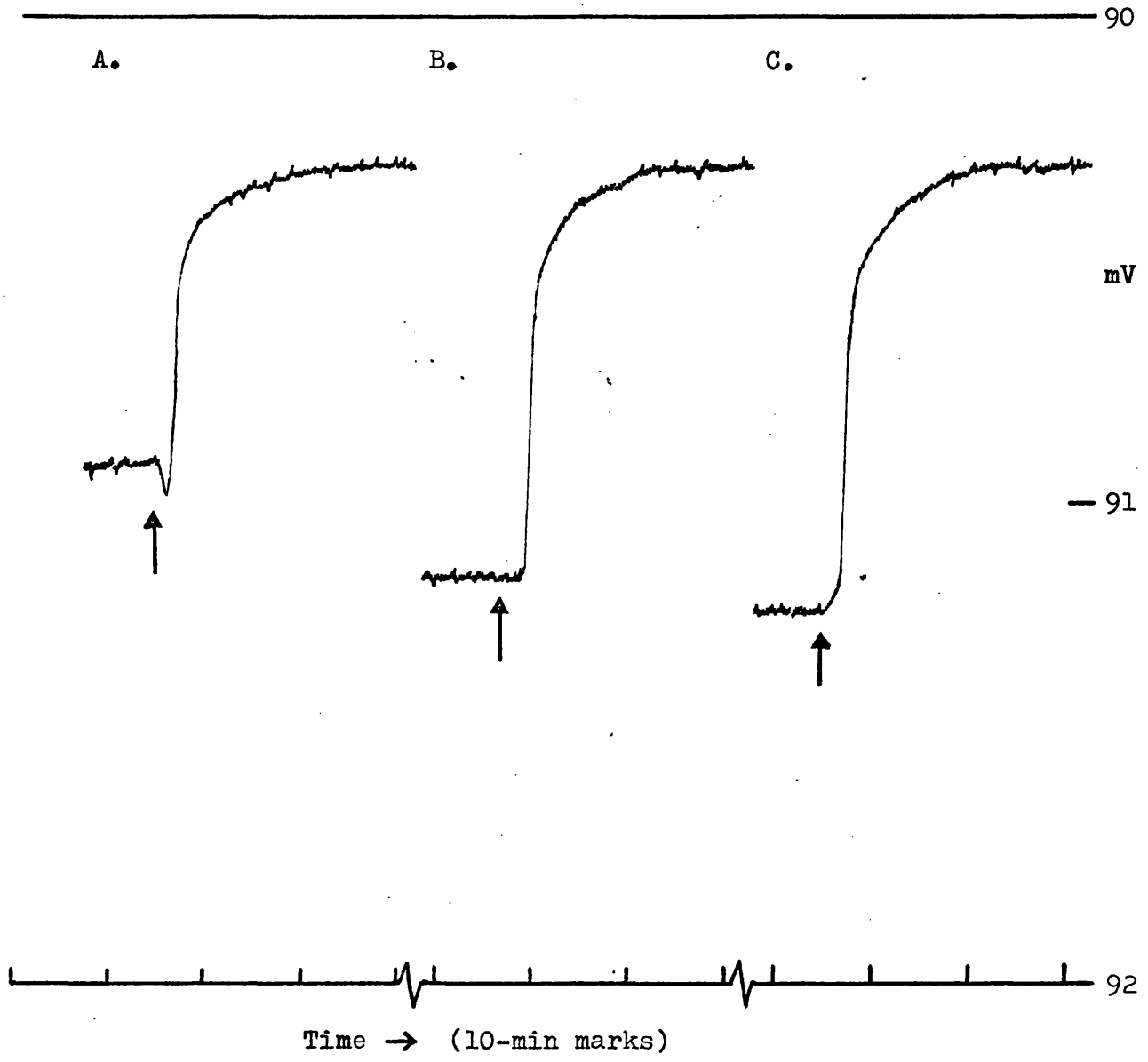
Fresh air with a constant oxygen content (20.90%) was passed through the analyser at various flow-rates. Circuit employed; variable amplifier in conjunction with a 90mV backoff circuit. When used for measuring the respiratory activity of bacterial cultures, the air flow-rate was approximately 30ml.min^{-1}.

region of the electrode membrane was probably insufficient to prevent slight oxygen depletion at the cathode, leading to a lowered output compared with higher flow rates (this effect is also observed when using these electrodes to measure oxygen concentration in inadequately stirred liquids, although the effect is then greater due to the much slower diffusion of oxygen in liquids than gases). Apparently, higher flow rates than 100 $ml.min^{-1}$ were required to overcome the depletion effect, but this was considered impracticable since flow rates of between 20-30 $ml.min^{-1}$ were considered to give an optimum balance between the accuracy of measurement of $\Delta\%\ O_2$ (most accurate at low flow rates because $\Delta\%\ O_2$ large) and the accuracy of measurement of the flow rate itself (most accurate at high flow rates).

A consequence of this property of the electrode was that if the flow rates of the effluent and fresh air through the analyser differed, the resultant electrode readings did not give the true value of $\Delta\%\ O_2$. In the experiment shown in Fig. IV-14 the effluent air flow rate was constant and the culture in the chemostat was in a steady state, so the $\Delta\%\ O_2$ was invariant with respect to time. However, if the fresh air flow rate was less than the effluent air flow rate (trace A), the apparent $\Delta\%\ O_2$ was less than the true $\Delta\%\ O_2$, observed when the flow rates were equal (trace B). On the other hand, when the fresh air flow rate was higher than the effluent rate, the apparent $\Delta\%\ O_2$ was higher than the true $\Delta\%\ O_2$ (trace C). For this reason, great care was taken in setting the fresh air flow rate to match the effluent flow rate. The fresh air supply to the analyser, as well as the air supply to the chemostat, was fitted with an air flow regulator and a GAP flowmeter (G.A. Platon Ltd., Croydon, Surrey). This allowed the fresh air flow

Fig. IV-14

The effect of unbalanced air flow rates on the electrode output



The chemostat contained a steady-state glutamate-limited culture of B.megaterium strain M ($D = 0.055\ h^{-1}$, $QO_2 = 3.0$ mmol $O_2 \cdot g\ cells^{-1} \cdot h^{-1}$). Three recorder tracings of the analyser electrode output are shown; the fresh air flow rates were A 5 $ml.min^{-1}$, B 30 $ml.min^{-1}$ and C 40 $ml.min^{-1}$. The effluent air flow rate was 30 $ml.min^{-1}$ in each case. The air supply to the analyser was changed at the arrows from fresh to effluent air. Recorder range 0-2 mV.

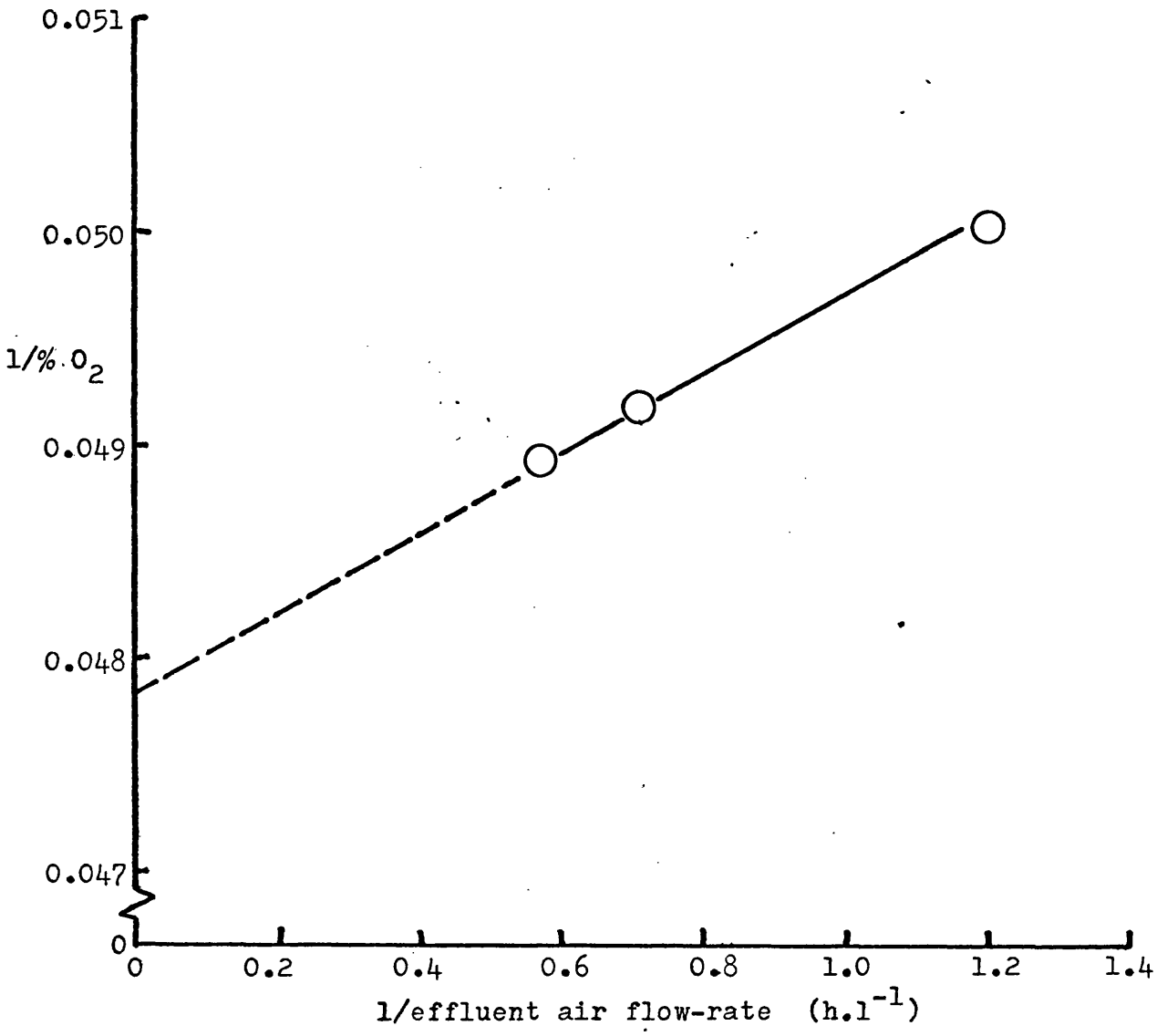
rate to be set to the effluent flow rate. However, the best indication that the flow rates were equal was the lack of immediate response of the electrode output on changing the air supply from fresh to effluent air (or vice versa) as shown in Fig. IV-13, trace B (compare with traces A and C). The lag in electrode response in this case was due to the slow flushing out of the old air from the analyser, which took 1-2 min.

To demonstrate conclusively that the small changes in electrode output, observed on changing the air supply to the analyser from effluent air to fresh air (or vice versa) under the standardised conditions described above, really were due to a difference in oxygen concentration of the gases, the following experiment was performed. The % O_2 in the effluent air from the chemostat, which contained a steady-state bacterial culture (i.e. the oxygen uptake rate was constant), was measured at three different effluent air flow rates. Sufficient time was allowed following each change in flow rate for the system to equilibrate and, during each measurement of % O_2, the flow rate of the fresh air through the analyser was carefully matched to the flow rate of the effluent air. As was expected, the % O_2 in the effluent air increased as the flow rate decreased. A double reciprocal graph of % O_2 in the effluent air against effluent air flow rate (Fig. IV-15) gave a straight line plot which cut the ordinate at approximately $^1/_{20.90\%}$ O_2, i.e. the % O_2 of fresh air; this would be the % O_2 in the effluent air at infinite flow rate. This experiment provided evidence that the electrode responded reliably to small changes in % O_2 of the air.

A typical recorder trace of a sequence of analyser air-supply changes, from fresh air to effluent air and vice versa, as was routinely obtained for measurements of the in situ respiratory activity of

Fig. IV-15

The % O_2 of the effluent chemostat air on varying the effluent air flow-rate.



The effluent air from the chemostat was passed through the analyser at various flow-rates. The chemostat contained a glutamate-limited culture (μ=0.35h^{-1}) of B. megaterium strain M with a constant respiratory activity (Q_{O_2} = 11.6mmol O_2.gcells^{-1}.h^{-1}). After each change in flow-rate, the air flowing through the chemostat was allowed to equilibrate with the oxygen uptake rate of the culture. The $\%O_2$ in the effluent air for each flow-rate was calculated from the corresponding electrode output (mV). The results are shown plotted as reciprocals. The intercept on the y-axis corresponds to the $\%O_2$ of the influent chemostat air (20.90%).

chemostat cultures, is shown in Fig. IV-16. Also shown is the method of correction for the small drift in electrode output in the calculation of $\Delta\%\ O_2$.

(b) Measurement of air flow rates Measurement of the effluent air flow rate was also necessary for calculation of the in situ respiratory activity of a particular chemostat culture. Since it was considered that the G.A.P. flowmeter was insufficiently accurate for this purpose, a gravimetric procedure was employed. A dropping bottle with a ground-glass stopper (capacity 100 ml) was filled with distilled water and temperature equilibrated in a 30° water bath for 20 min. After drying, the bottle was weighed. Effluent air was then collected in the bottle, by displacement of water, for a measured period of time and the bottle re-equilibrated in the water bath for a further 20 min. With the stopper in the open position, the air in the bottle was adjusted to atmospheric pressure by lining up the water level in the inverted bottle with the water level in the bath. After closing the stopper, the bottle was removed, dried and reweighed.

Hence:

$$\text{vol. of gas in collecting bottle} = (g_1 - g_2)/d \text{ ml}$$

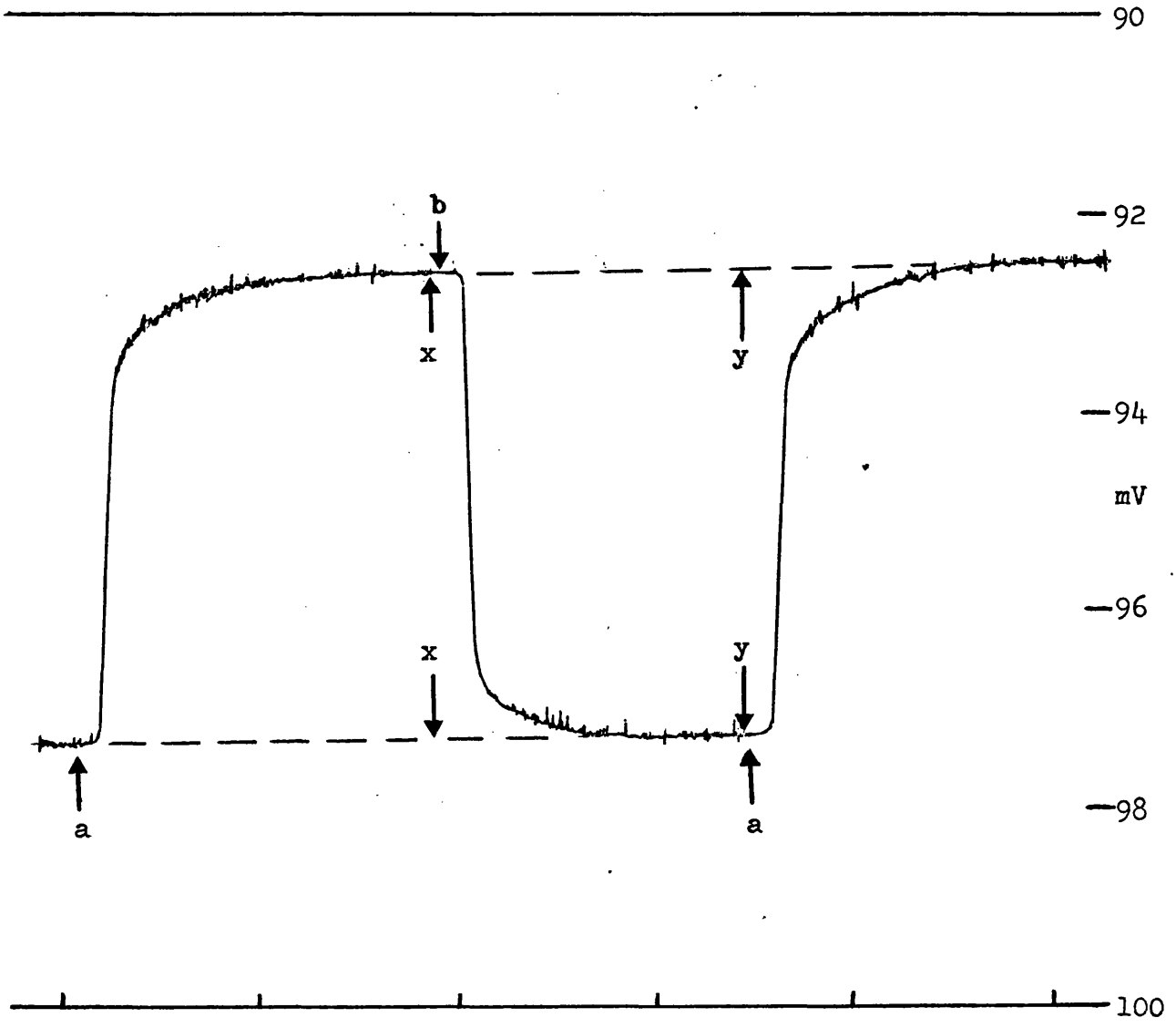
where g_1 and g_2 are the mass of the bottle before and after the air collection respectively (g) and d = density of water at 30°, 760 mm Hg = 0.9957 $g.ml^{-1}$ (it was assumed that the atmospheric pressure was constant at 760 mm Hg). Now, the vapour pressure of water at 30° = 32 mm Hg. Therefore:

$$\text{vol. of air in collecting bottle} = \frac{760 - 32}{760} \cdot \frac{(g_1 - g_2)}{d}$$

$$= 0.958 \cdot \frac{(g_1 - g_2)}{d} \text{ ml}$$

Fig. IV-16

A typical recorder trace used for the calculation of QO_2.



The chemostat contained a steady-state glycerol-limited culture of B.megaterium strain D440 ($D = 0.42\ h^{-1}$). The figure shows a typical recorder tracing used for the calculation of $\Delta\%\ O_2$ and hence QO_2. The fresh air and effluent air flow rates were 30 $ml.min^{-1}$. The air supply to the analyser was changed at a, from fresh to effluent air and at b, from effluent to fresh air. Drift in the electrode output was corrected for by extrapolation as shown. The electrode outputs corresponding to fresh and effluent air were obtained either at x or at y.

If t is the time for which the air sample was collected, then

$$\text{flow rate of air} = \frac{0.958}{t} \cdot \frac{(g_1 - g_2)}{d} \quad \text{ml.min}^{-1}$$

$$= \frac{0.0575}{d.t} \cdot (g_1 - g_2) \quad \text{l.h}^{-1}$$

A correction must be made for the medium dripping into the chemostat vessel during the flow rate measurement, since this increased the apparent effluent air flow rate by a small amount (by a maximum of +6% depending on the dilution rate). If D = dilution rate (h^{-1}) and v = culture volume (l^{-1}):

$$\text{effluent air flow rate} = \frac{0.0575}{dt} \cdot (g_1 - g_2) - Dv \quad \text{l.h}^{-1}$$

It was established that collection of air by displacement of water did not decrease the apparent effluent air flow rate due to back pressure in the air supply, since gas collection at different outlet levels in the water bath resulted in almost identical calculated flow rates. No decrease in the apparent flow rate was observed until the air was collected at depths below 5 cm water. However, flow rates were routinely measured by collection as near to the water surface as possible (i.e. depth of collection $\leqslant$ 0.5 cm).

(c) Measurement of culture density Measurement of culture density, also required for calculation of *in situ* QO_2, was not based on turbidometric readings and reference to a dry weight curve, since this procedure can give erroneous results due to the variation in bacterial size and shape under different growth conditions (Pirt, 1965; Hill *et al.*

1972). Instead, following measurements of $\Delta\%\ O_2$ and effluent air flow rate, a 100 ml culture sample was removed from the chemostat vessel and centrifuged at 11,000 x g for 10 min. The cell pellet was washed with glass-distilled water, recentrifuged and dried to constant weight.

Having measured the $\Delta\%\ O_2$, the effluent air flow rate and the culture density for a particular bacterial population in the chemostat, the respiratory activity of the culture was calculated as follows:

$$QO_2 = \Delta\%\ O_2 \times {}^{C}/_{Z} \times {}^{273}/_{T} \times {}^{1}/_{22.4} \text{ (mol } O_2 \text{ consumed. g dry wt bacteria}^{-1}.h^{-1})$$

where C = effluent air flow rate ($l.h^{-1}$), Z = dry weight of bacteria in the chemostat vessel, T = the absolute temperature at which the effluent air flow rate was measured (i.e. $303^{\circ}A$).

4. Calculations

(a) Growth yields and maintenance coefficients with respect to ATP

Growth yields with respect to ATP (Y_{ATP}; g cells.mol ATP equivalents^{-1}) were measured on both batch and continuous cultures of *B. megaterium*.

It was not possible to correct the calculations made on batch cultures for energy used for cell maintenance, hence $Y_{ATP(obs)}$ values (uncorrected for maintenance) were obtained rather than Y_{ATP} values (corrected for maintenance, see below). From measurements of respiratory activity (QO_2, mol O_2 consumed.g cells$^{-1}.h^{-1}$) and specific growth rate

($\mu.h^{-1}$) of logarithmically growing batch cultures, the apparent growth yield of the organism with respect to oxygen, $Y_{O_{2(obs)}}$ (g.cells.mol O_2 consumed^{-1}) was calculated as follows:

$$Y_{O_{2(obs)}} = {}^{\mu}/_{QO_2} \quad \text{(Harrison and Loveless, 1971)} \qquad \text{IV-21}$$

whence $$Y_{ATP\ (obs)} = Y_{O_{2(obs)}}/N \qquad \text{IV-22}$$

where N = mol ATP equivalents synthesised per mol.O_2 consumed.

Y_{ATP} values, corrected for maintenance, were obtained for chemostat cultures under various substrate-limiting conditions by measuring the QO_2 of cultures growing at different dilution rates. QO_2 is related to Y_{ATP} by the Harrison and Loveless (1971) equation (IV-1):

$$QO_2 = {}^{\mu}/_{Y_{ATP}.N} + {}^{M}/_{N}$$

where M is the maintenance coefficient (mol ATP equivalents.g cells^{-1}.h^{-1}) and μ is the specific growth rate (h^{-1}). Since, for steady-state chemostat cultures μ = D, where D is the dilution rate (h^{-1}), then

$$QO_2 = {}^{D}/_{Y_{ATP}} + {}^{M}/_{N} \qquad \text{IV-23}$$

Hence a plot of QO_2 <u>versus</u> D will give a straight line of gradient $^{1}/_{Y_{ATP}}.N$ and intercept $^{M}/N$ provided that Y_{ATP}, M and N remain constant during changes in D.

$Y_{ATP(obs)}$ values for batch growth and Y_{ATP} and M values for chemostat growth were calculated using N values derived from the following equation:

$$N \text{ (mol ATP equivalents synthesised.mol } O_2 \text{ consumed}^{-1}) = \frac{n_{fp} \cdot l_{fp} + n_{NAD} \cdot l_{NAD} + SLP}{R} \quad \text{IV-24}$$

where l_{fp}/l_{NAD} = number of proton translocating loops in the respiratory chain between the point of entry of reducing equivalents from flavin-linked substrates/NAD-linked substrates and molecular oxygen; n_{fp}/n_{NAD} = number of flavin-linked substrates/NAD-linked substrates oxidised per mol carbon substrate consumed; SLP = mol ATP synthesised by substrate-level phosphorylation per mol carbon substrate consumed; R = mol O_2 completely oxidising 1 mol carbon substrate.

For glycerol oxidation, n_{fp} = 2 (enzymes responsible: glycerol 3-phosphate dehydrogenase, succinate dehydrogenase), n_{NAD} = 5 (glyceraldehyde 3-phosphate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, malate dehydrogenase), SLP = 2 (net : glycerol kinase -1, phosphoglycerate kinase +1, pyruvate kinase +1, succinyl thiokinase +1), R = 3.5.

For glutamate oxidation, n_{fp} = 2 (2 x succinate dehydrogenase), n_{NAD} = 7 (glutamate dehydrogenase, 2 x α-ketoglutarate dehydrogenase, malic enzyme, pyruvate dehydrogenase, isocitrate dehydrogenase, malate dehydrogenase), SLP = 2 (2 x succinate dehydrogenase), R = 4.5.

It was shown in Chapter III that l_{NAD} = 2 and l_{fp} = 1 for *B. megaterium* strain D440 and l_{NAD} = 2 and l_{fp} = 2 for *B. megaterium* strain M. Substituting these values into Equation IV-24 gave the following results:

For B. megaterium strain D440:

glycerol oxidation: $N = \frac{2.1 + 5.2 + 2}{3.5}$

$= 4.00$

For B. megaterium strain M:

glycerol oxidation: $N = \frac{2.2 + 5.2 + 2}{3.5}$

$= 4.57$

glutamate oxidation: $N = \frac{2.2 + 7.2 + 2}{4.5}$

$= 4.45$

(b) Percentage of carbon in chemostat cells Although not measured directly, approximate values for the percentage carbon in carbon/energy-limited chemostat cells could be obtained by calculation as follows:

oxygen uptake rate of culture $= QO_2 . x$ (mol $O_2 . l^{-1} . h^{-1}$)

where x = organism concentration (g dry wt bacteria.l^{-1}).

$\therefore$ rate of oxidation of carbon substrate = (glycerol or glutamate)

$\frac{QO_2 . x}{R}$ (mol C-substrate.$l^{-1} . h^{-1}$)

where R = mol O_2 oxidising 1 mol carbon substrate. Now,

rate of C-substrate addition to culture in fresh medium =

$[\text{C-substrate}]_{input} . D$ (mol C-substrate.h^{-1})

where $[\text{C-substrate}]_{input}$ = concentration of C-substrate in fresh medium. The ambient C-substrate concentration in the culture medium was not measured. For the purposes of this work it was assumed to be zero (i.e. the cells were assumed to completely utilise all added C-substrate). Although only an approximation, the concentration was almost certainly negligible compared with $[\text{C-substrate}]_{input}$ since the affinity of chemostat-grown bacterial cells for the limiting substrate is very high (see e.g. Herbert et al., 1956; Neijssel et al., 1974). Therefore,

$$\begin{pmatrix}\text{rate of incorporation}\\ \text{of C-substrate into new}\\ \text{cell material}\end{pmatrix} = [\text{C-substrate}]_{input}.D - \frac{QO_2.x}{R}$$

$$(\text{mole C-substrate}.l^{-1}.h^{-1})$$

From this, the rate of incorporation of carbon into new cell material (L, g carbon.l^{-1}.h^{-1}) was calculated. Now, since

$$\begin{pmatrix}\text{rate of synthesis of new}\\ \text{cell material}\end{pmatrix} = D.x \ (\text{g dry wt}.l^{-1}.h^{-1})$$

$$\therefore \quad \underline{\%\ \text{carbon in cells}} = \underline{L/D.x}$$

Results and Discussion

1. Cytochrome concentrations of chemostat-grown cells

Difference spectrophotometry was performed on respiratory membranes prepared from the two strains of B. megaterium grown in continuous culture under various nutrient-limitation conditions. Membrane preparations of glycerol-limited and nitrogen-limited cultures of strain D440 and glycerol-limited, glutamate-limited and nitrogen-limited cultures of strain M contained very similar qualitative and quantitative cytochrome patterns to membrane preparations of the respective organisms grown under excess oxygen (logarithmic) growth conditions in batch culture (Table IV-1; compare with Table III-1). Thus membrane preparations of strain D440 contained cytochromes b, a, a_3 and o and appeared to lack cytochrome c. The ratio of the concentration of cytochrome oxidase a_3 to that of cytochrome o was approximately the same as in membranes prepared from batch-grown cells (i.e. approximately 2 : 1) and the ratio a_3/a was again considerably greater than unity. Membrane preparations of strain M contained cytochromes b, c, a and o and appeared to lack cytochrome a_3, as was observed in membranes prepared from batch-grown cells. Membranes prepared from oxygen-limited chemostat cells of both organisms contained higher absolute cytochrome concentrations then those of the other preparations examined. However, the concentrations were very similar when compared on a cytochrome b basis, suggesting the presence of a larger number of respiratory chain units per cell under oxygen-limited conditions in the chemostat compared with the number per cell under the other nutrient-limitation conditions examined. A parallel situation was observed with membranes prepared from oxygen-

Table IV-1

The cytochrome concentrations of respiratory membranes prepared from B.megaterium grown in chemostat culture

Strain	Nutrient limitation	Absolute cytochrome concentrations (nmol.g protein^{-1})					Relative cytochrome concentrations (nmol.nmol $\underline{b}^{-1}$)				% of total oxidase		ratio $\underline{a_3}/\underline{o}$
		$\underline{b}$	$\underline{c}$	$\underline{a}$	$\underline{a_3}$	$\underline{o}$	$\underline{c}$	$\underline{a}$	$\underline{a_3}$	$\underline{o}$	$\underline{a_3}$	$\underline{o}$	
D440	glycerol-limited	134	-	38	61	28	-	0.28	0.46	0.21	69	31	1.61
	nitrogen-limited	151	-	45	63	30	-	0.30	0.42	0.20	68	32	1.40
	oxygen-limited	252	-	60	95	43	-	0.24	0.38	0.17	69	31	1.58
M	glycerol-limited	650	750	149	-	342	1.15	0.23	-	0.53	0	100	-
	nitrogen-limited	690	775	157	-	359	1.12	0.23	-	0.52	0	100	-
	oxygen-limited	930	1045	166	-	375	1.12	0.18	-	0.40	0	100	-
	glutamate-limited	638	735	135	-	333	1.15	0.21	-	0.52	0	100	-

The dilution rates of the cultures from which cell samples were derived for the preparation of respiratory membranes were; strain D440, D = 0.3-0.4 h^{-1}; strain M, D = 0.07-0.12 h^{-1}(except glutamate limited, D = 0.30 h^{-1}).

limited batch cells when compared with membranes prepared from excess-oxygen batch cells (see Table III-1).

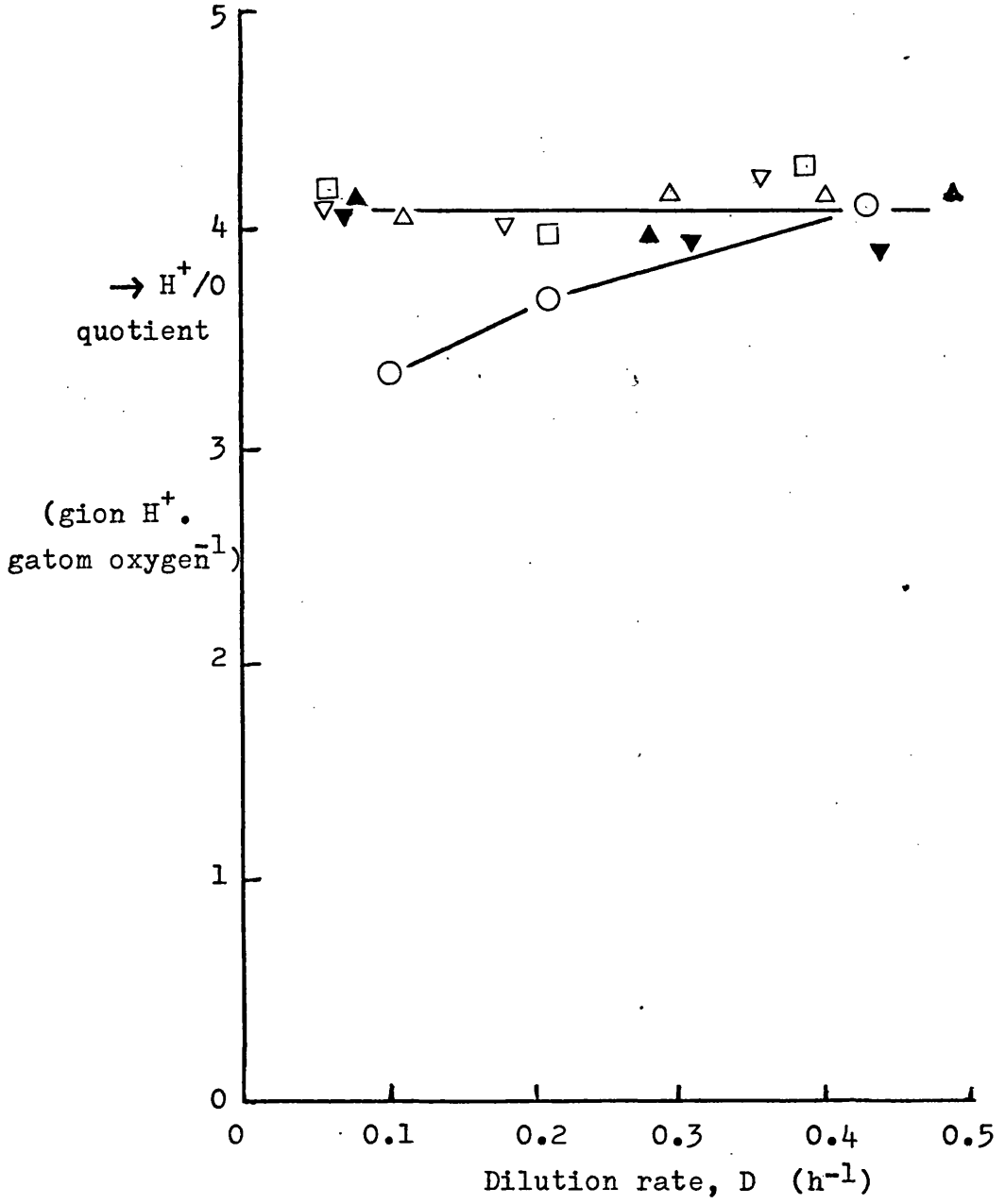
2. $\longrightarrow H^+/O$ quotients of chemostat-grown cells

$\longrightarrow H^+/O$ quotients were measured on cell suspensions of the two strains of B. megaterium obtained from continuous cultures under various nutrient-limiting conditions. With the exception of oxygen-limited cells of strain D440, all of the other preparations examined exhibited $\longrightarrow H^+/O$ quotients of approximately 4 when oxidising endogenous substrates (Figs. IV-17; IV-18). This indicated that two proton-translocating loops were functioning in the respiratory chains of each organism when grown in chemostat culture, as well as when grown in batch culture. Since the cytochrome patterns of the batch-grown cells and chemostat-grown cells were very similar (see above), it was assumed that the operative proton translocating loops were the same in the chemostat-grown cells as in the batch-grown cells, i.e. loops 1 and 2 in strain D440 and loops 2 and 3 in strain M (see Chapter III).

In order to calculate Y_{ATP} values for chemostat cells, it was necessary to obtain values for N, i.e. the number of mols ATP equivalents synthesised per mol oxygen consumed. The value of N pertaining in growing cells in the chemostat was not necessarily numerically equal to the $\longrightarrow H^+/O$ quotients measured on non-growing cell samples, for the following reasons. Firstly, the $\longrightarrow H^+/O$ quotients did not account for ATP generated by substrate-level phosphorylation. Secondly, in the case of B. megaterium strain D440, the in vitro $\longrightarrow H^+/O$ quotient (i.e. the value measured experimentally) was almost certainly not the same as the in vivo $\longrightarrow H^+/O$ quotient (i.e. that operating in growing cells).

Fig. IV-17

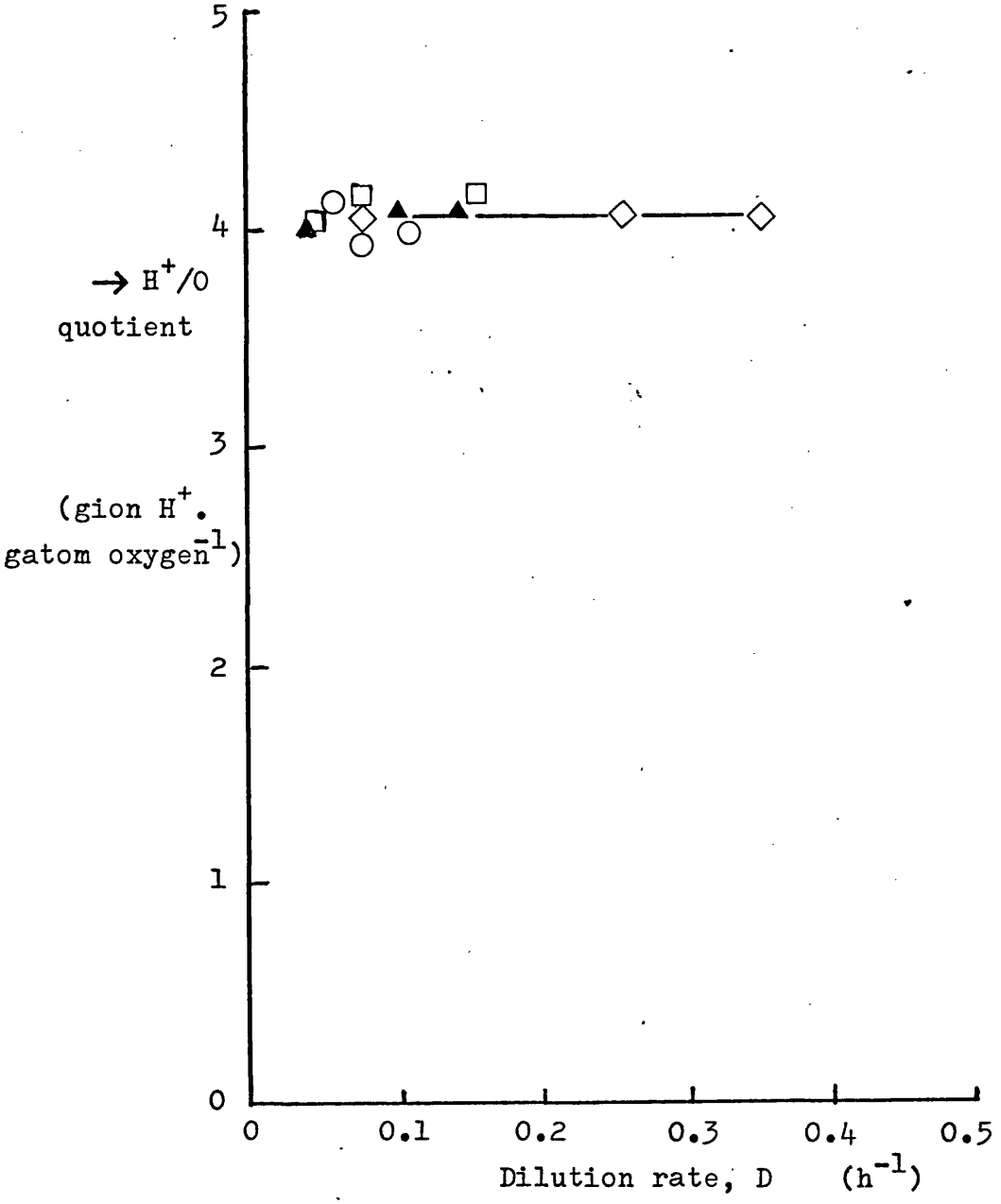
→ H^+/O quotients of whole cells of chemostat-grown B.megaterium strain D440 oxidising endogenous substrates



→ H^+/O quotients were measured on cell suspensions of B.megaterium strain D440 obtained from continuous cultures growing at various dilution rates. The growth-limiting nutrients were; ▲ glycerol (high aeration), ▼ glycerol (extra-high aeration), △ glycerol (pH = 7.5-7.8), ▽ glycerol (pH = 5.8-6.1), □ nitrogen (NH_4Cl), ○ oxygen. Conditions of assay; 0.5 $mg.ml^{-1}$ carbonic anhydrase, cell density 6 mg dry $wt.ml^{-1}$, 150 mM KSCN, pH 6.6-6.8.

Fig. IV-18

→ H^+/O quotients of whole cells of chemostat-grown B.megaterium strain M oxidising endogenous substrates



→ H^+/O quotients were measured on cell suspensions of B.megaterium strain M obtained from continuous cultures growing at various dilution rates. The growth-limiting nutrients were; ▲ glycerol, □ nitrogen (NH_4Cl), ◇ glutamate, ○ oxygen. Conditions of assay as in Fig. IV- 17.

This was because the $\longrightarrow H^+/O$ quotients of approximately 4 obtained with chemostat-grown cells of this organism (ignoring for the moment the oxygen-limited cultures) represented oxidation of only NAD-linked substrates. Flavin-linked substrates were not oxidised *in vitro* either because of preferential oxidation of NAD-linked substrates in the measuring chamber or because the cells became depleted of flavin-linked substrates during the preparation procedure for measurement of $\longrightarrow H^+/O$ quotients. If flavin-linked substrates had been oxidised *in vitro* by cell suspensions of strain D440, the $\longrightarrow H^+/O$ quotients obtained would have been < 4 since their oxidation by this organism elicits proton translocation only at loop 2 whereas oxidation of NAD-linked substrates elicits proton translocation at loops 1 and 2 (see Chapter III). In growing cells, both flavin-linked and NAD-linked substrates were presumably produced during glycerol catabolism. The resultant, *in vivo*, $\longrightarrow H^+/O$ quotient would therefore have depended on the relative amounts of flavin-linked and NAD-linked substrates available for oxidation. The probable amounts are given in the Materials and Methods section where values for N are calculated.

Such corrections of the measured $\longrightarrow H^+/O$ quotients were not necessary for strain M since proton translocation occurred at loops 2 and 3 during oxidation of both flavin-linked and NAD-linked substrates (see Chapter III). The relative rates of oxidation of these substrates would therefore not affect the $\longrightarrow H^+/O$ quotients; hence the *in vivo* quotients were presumably the same as the *in vitro* values.

Oxygen-limited chemostat cultures of strain D440 were different from the other cultures examined since the $\longrightarrow H^+/O$ quotients exhibited by the cell samples decreased with the dilution rate of the

culture from which they were derived (Fig. IV-17). Thus, although an $\longrightarrow H^+/O$ quotient of approximately 4 was obtained for cells harvested from a high dilution rate culture ($D = 0.43\ h^{-1}$), the $\longrightarrow H^+/O$ quotient was only 3.37 for cells derived from a low dilution rate culture ($D = 0.10\ h^{-1}$). It was suggested in Chapter III that the low $\longrightarrow H^+/O$ quotient (average value = 3.14) exhibited by preparations of strain D440 grown under oxygen-limited conditions in batch culture was due to a partial loss of coupling of the NADH dehydrogenase complex to proton translocation at loop 1. If this explanation is correct, a gradual loss of looping can be observed with the oxygen-limited chemostat-grown cells as the dilution rate decreased, corresponding to an increase in the oxygen-limitation of the cells (Fig. IV-17). The batch-grown cells, with a lower $\longrightarrow H^+/O$ quotient than that exhibited by the chemostat cells growing at the lowest dilution rate, were presumably more oxygen-limited than the latter since they exhibited a lower average specific growth rate ($\mu = 0.03\ h^{-1}$).

3. Growth yields and maintenance coefficients of *B. megaterium* in continuous culture

(a) Glycerol-limited cultures *B. megaterium* strain D440 was grown on a minimal-salts medium in continuous culture under high aeration, glycerol-limited conditions. The dilution rate was varied between 0.076 - 0.490 h^{-1} and the culture density, pH, dissolved oxygen concentration and respiratory activity (QO_2) were measured for each growth condition. Although the culture washed out at $D = 0.60\ h^{-1}$, no attempt was made to determine accurately the critical dilution rate (D_c),

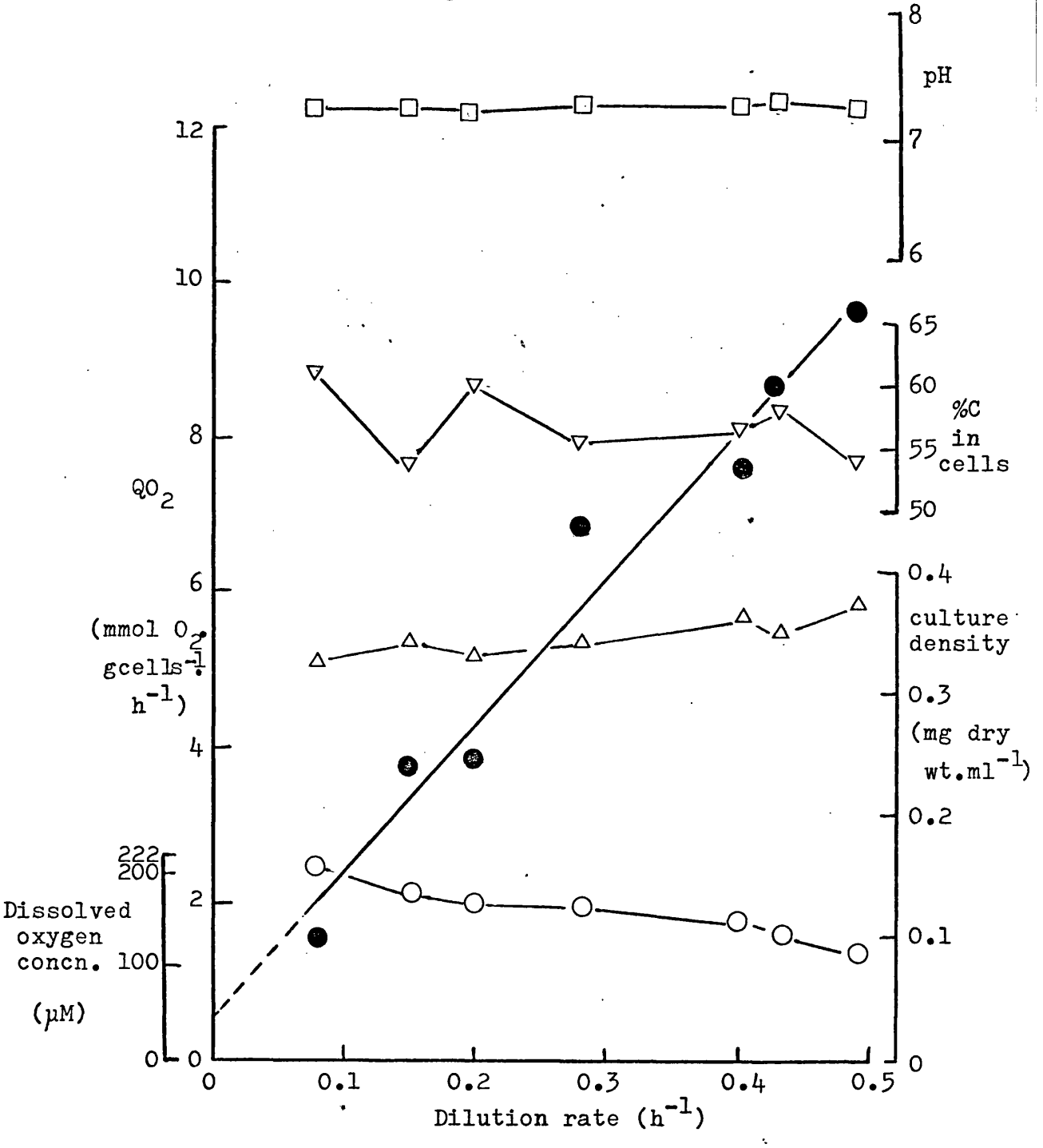
which was between D = 0.49 h^{-1} and D = 0.60 h^{-1} (the logarithmic specific growth rate in batch culture on minimal salts medium plus glycerol under excess oxygen conditions, i.e. μ_m = 0.63 h^{-1}). The pH of the culture at different dilution rates was almost invariant at 7.2 - 7.3. This indicated that the buffering capacity of the minimal salts medium was adequate for the culture densities employed (0.3 - 0.4 mg dry wt.ml^{-1}).

The QO_2 of the cultures varied greatly with the dilution rate, having a maximum value at high dilution rates. A plot of QO_2 versus D approximated to a straight line (Fig. IV-19); the best straight line through the points was calculated by linear regression analysis using a Hewlett-Packard 1600B bench-top computer and the fit shown to be fairly good (correlation coefficient 0.98). The line gave a positive intercept on the ordinate, indicating that this organism had a maintenance energy requirement. Values for $^{M}/_{N}$ (0.00060 mol O_2.g $cells^{-1}$.h^{-1}) and $Y_{ATP}.N$ (53.8 g cells.mol O_2^{-1}; $\equiv Y_{O_2}$) were hence obtained from the intercept and slope of the line respectively. Using a value of N (4.00 mol.ATP equivalents synthesised.mol O_2 $consumed^{-1}$) calculated as described in Materials and Methods, values were calculated for Y_{ATP} (13.5 g cells.mol ATPequivalents^{-1}) and M (0.00240 mol ATP equivalents. g $cells^{-1}.h^{-1}$).

Along with the variation in QO_2 with dilution rate, there were also variations in the culture density and dissolved oxygen concentration. The culture density increased slightly with increasing dilution rate; thus, at D = 0.076 h^{-1} the culture density was 0.325 g dry wt.l^{-1} whereas at D = 0.490 h^{-1} it was 0.370 g dry wt.l^{-1}. The effect of a falling growth yield (equivalent to a falling culture density) with

Fig. IV-19

Some growth parameters of glycerol-limited continuous cultures of B.megaterium strain D440 (high aeration)



B.megaterium strain D440 was grown in continuous culture under high aeration, glycerol-limited conditions in the pH range 7.2-7.3.

● QO_2, ○ dissolved oxygen concn., △ culture density, □ pH, ▽ %C in cells. Correlation coefficient for QO_2 versus D plot = 0.98.

decreasing dilution rate has been reported previously for carbon/energy limited bacterial chemostat cultures (see Herbert, 1958; Harrison and Loveless, 1971) and was anticipated from theoretical considerations (see the Theory section of this chapter). The fact that there was no decrease in the culture density at high dilution rates (D = 0.4 - 0.5 h^{-1}) indicated that the affinity of the cells for the limiting substrate (glycerol) was high (i.e. K_s low: see Fig. IV-2)

In contrast to the culture density, the dissolved oxygen concentration was found to decrease as the dilution rate increased. Thus at D = 0.076 h^{-1} the dissolved oxygen concentration was 210 μM whereas at D = 0.490 h^{-1} it was 120 μM (saturating concentration = 222 μM; Chappell, 1964). This effect can be explained as follows. In the Materials and Methods section it was shown that (Equation IV-20):

$$K_L A = \frac{QO_2 . x}{P_G - P_L}$$

where $K_L A$ = oxygen transfer coefficient (mol $O_2 . l^{-1} . h^{-1} . mm\ Hg^{-1}$), x = organism concentration (g dry wt.l^{-1}), P_G = partial pressure of oxygen in the gaseous phase (mm Hg), and P_L = partial pressure of oxygen in the culture medium (mm Hg). Therefore,

$$P_L = P_G - \frac{QO_2 . x}{K_L A} \qquad \text{IV-25}$$

Now, as the stirring rate of the culture was not altered and the culture volume was constant, $K_L A$ was presumably constant with respect to change in dilution rate. Likewise, P_G was presumably virtually constant (it may have altered slightly since the % O_2 in the air in the chemostat vessel was presumably the mean of the % O_2 of the fresh air and the

% O_2 of the effluent air). Therefore, since both QO_2 and x increased as D increased, the dissolved oxygen concentration was required simultaneously to decrease in order that equation IV-25 balance. This is the effect that was observed experimentally. It was a general phenomenon with all of the culture conditions investigated, with the exception of the oxygen-limited cultures.

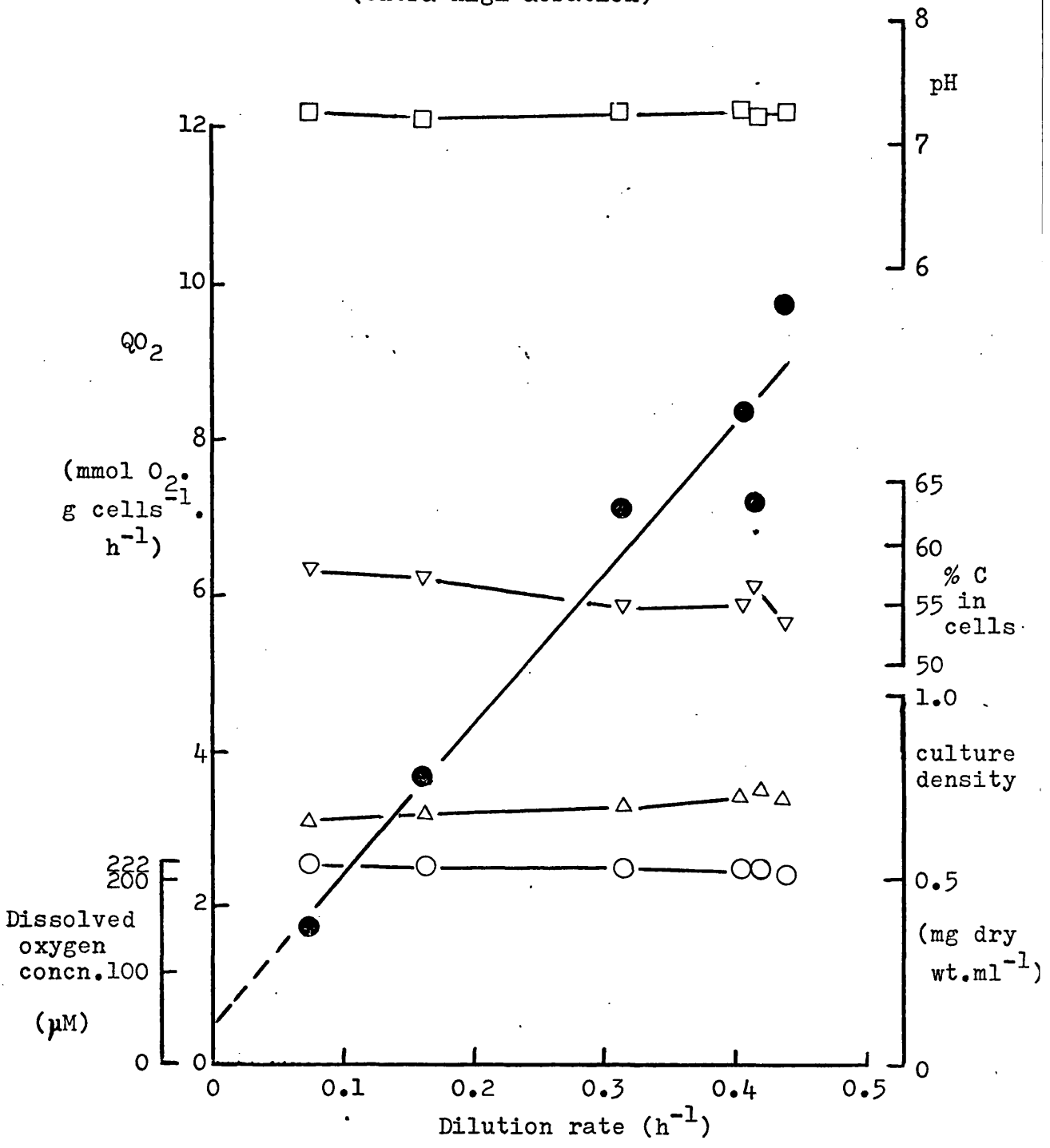
The significance of the values for % carbon of the cells, calculated for each dilution rate and found to be in the range 54-61%, will be discussed later along with the results obtained for the other carbon-limited culture conditions.

It was considered desirable to ascertain whether the variation in dissolved oxygen concentration with dilution rate, observed under high aeration, glycerol-limited culture conditions, had a simultaneous effect on the QO_2 of the cultures hence leading to spurious measurements of Y_{ATP} and M. To do this it was necessary to hold the dissolved oxygen concentration at a constant value. This was achieved by the use of extra-high aeration conditions (stirrer speed faster), together with a lower glycerol concentration in the input medium (20% of the concentration employed under high aeration conditions). The resultant high K_LA and low culture density resulted in a dissolved oxygen concentration which remained between 200-218 μM regardless of the dilution rate of the culture (Fig. IV-20). The pH was again approximately constant at 7.2-7.3. A plot of QO_2 versus D gave a straight line with a correlation coefficient = 0.97. From the gradient and intercept of the line, values for Y_{ATP} (12.9 g cells.mol ATP equivalents^{-1}) and M (0.00188 mol ATP equivalents.g cells^{-1}.g^{-1}) were obtained which were close to those obtained under high aeration conditions.

Fig. IV-20

Some growth parameters of glycerol-limited continuous cultures of *B.megaterium* strain D440

(extra-high aeration)



B.megaterium strain D440 was grown in continuous culture under extra-high aeration, glycerol-limited conditions in the pH range 7.2-7.3. Symbols as in Fig. IV-19. Correlation coefficient for QO_2 *versus* D plot = 0.97.

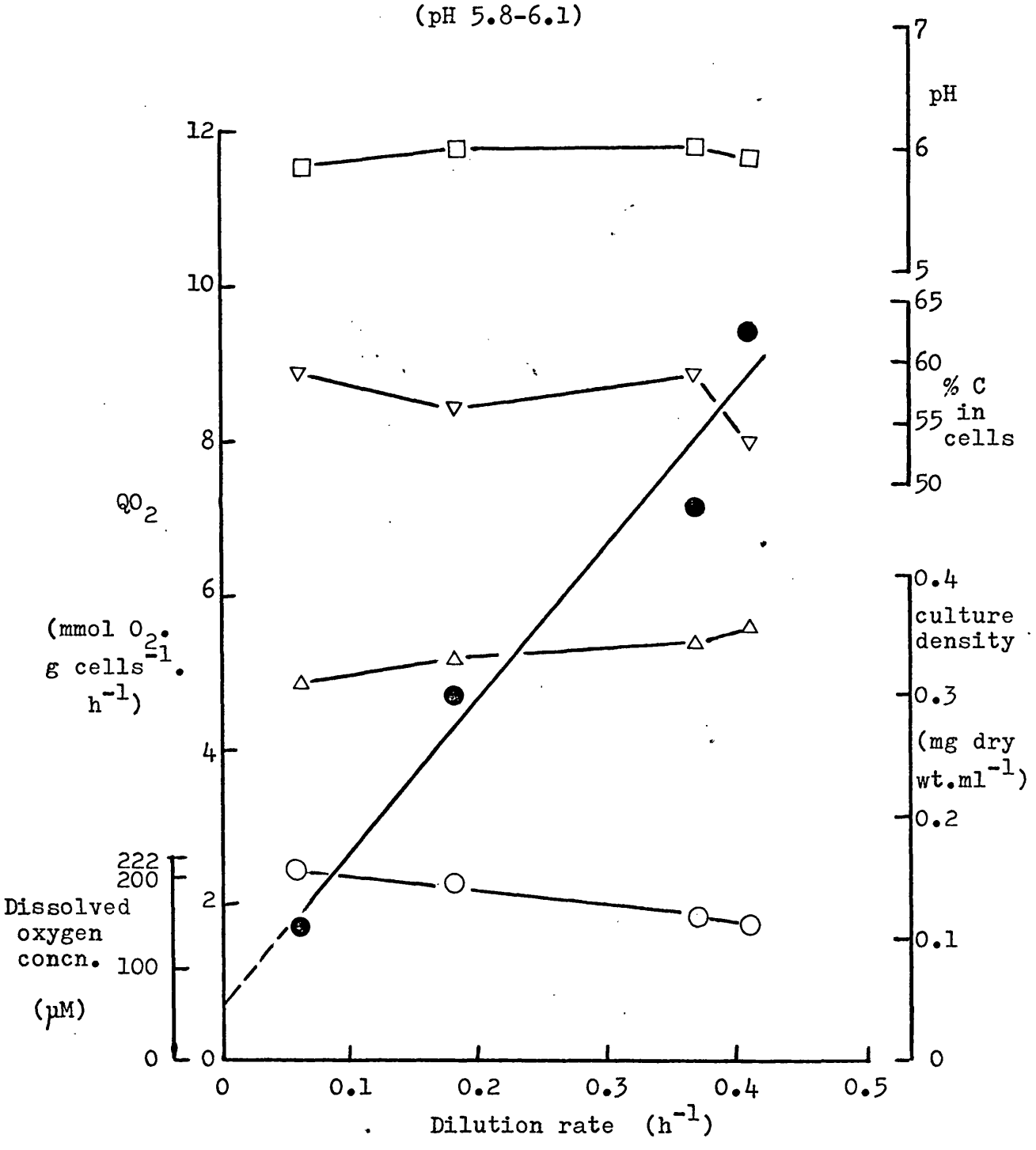
Thus a variation in the dissolved oxygen concentration of chemostat cultures of strain D440 between 120-218 μM O_2 had no effect on the growth efficiency of the organism. Although it would have been preferable to run the other culture conditions employed under conditions of constant dissolved oxygen concentration, i.e. under extra-high aeration conditions (oxygen-limited cultures excepted), it was considered that QO_2 measurements were more accurate under normal high aeration conditions because of the greater culture volume (slower stirrer speed) and higher culture density (higher glycerol concentration in input medium). Thus high aeration conditions were used for all nutrient-limiting conditions (except oxygen) for both strain D440 and strain M. Although not checked, a variation in dissolved oxygen concentration was also assumed to cause no change in growth efficiency of chemostat cultures of strain M.

It was considered possible that growth of B. megaterium in continuous culture at different pH values might cause a change in growth efficiency. To test if this was the case, strain D440 was grown in glycerol-limited continuous culture under high aeration conditions with the pH of the input medium adjusted so that growth occurred in the pH range 5.8 - 6.1 (Fig. IV-21) and 7.5 - 7.8 (Fig. IV-22). The corresponding Y_{ATP} values, calculated from QO_2 measurements, were 12.5 and 13.5 g cells.mol ATP equivalents^{-1} for the high acidity and low acidity cultures respectively, and the corresponding M values were 0.00276 and 0.00272 mol ATP equivalents.g cells^{-1}.h^{-1} respectively. Hence there was no significant difference in Y_{ATP} or M from the high aeration, pH 7.2 - 7.3 cultures. It is conceivable that more extreme pH conditions could have caused a change in growth efficiency (probably a decrease) but this

Fig. IV-21

Some growth parameters of glycerol-limited continuous cultures of B.megaterium strain D440

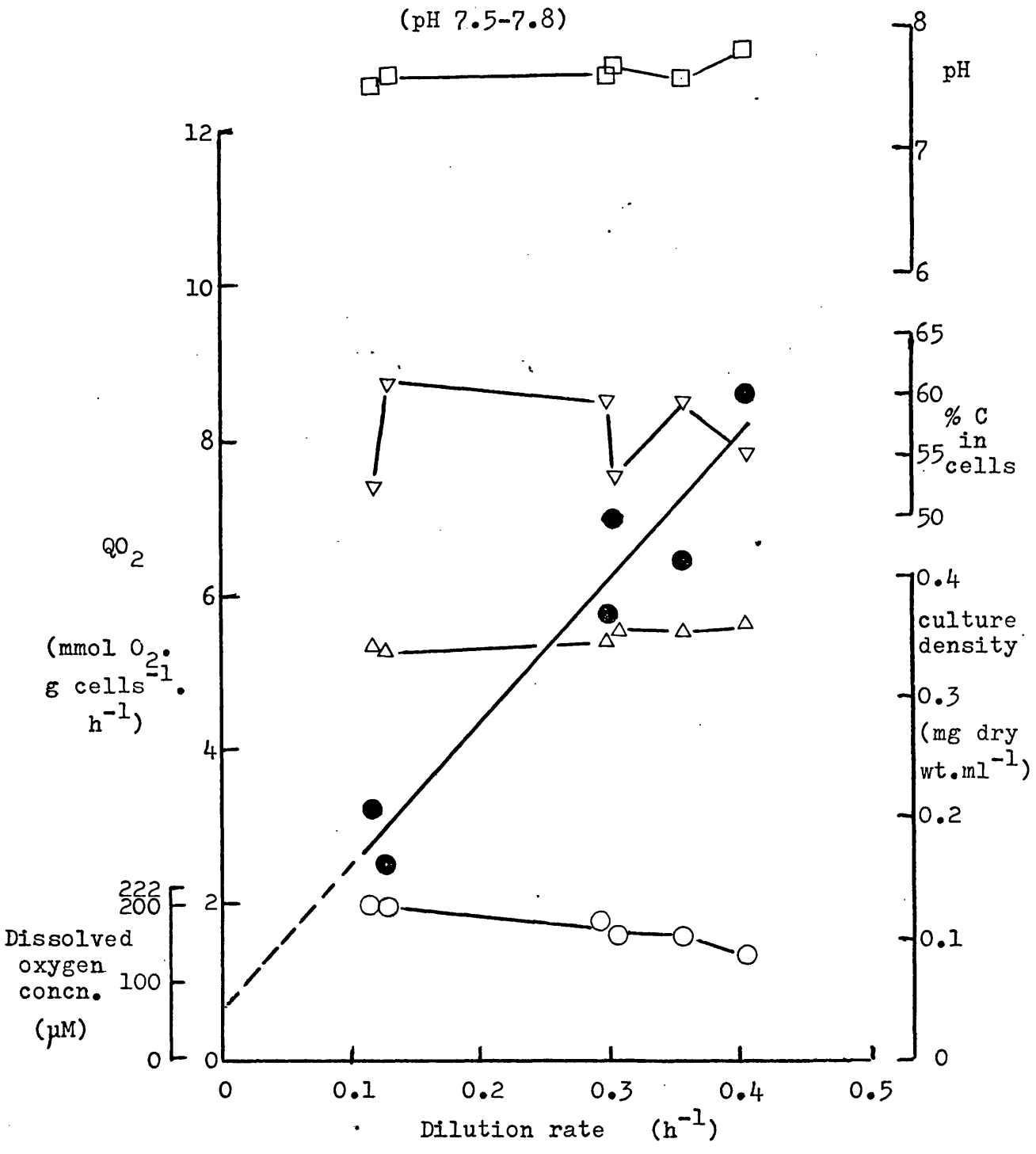
(pH 5.8-6.1)



B.megaterium strain D440 was grown in continuous culture under high aeration, glycerol-limited conditions in the pH range 5.8-6.1. Symbols as in Fig. IV-19. Correlation coefficient for QO_2 versus D plot = 0.98.

Fig. IV-22

Some growth parameters of glycerol-limited continuous cultures of *B.megaterium* strain D440



B.megaterium strain D440 was grown in continuous culture under high aeration, glycerol-limited conditions in the pH range 7.5-7.8. Symbols as for Fig. IV-19. Correlation coefficient for QO_2 *versus* D plot = 0.96.

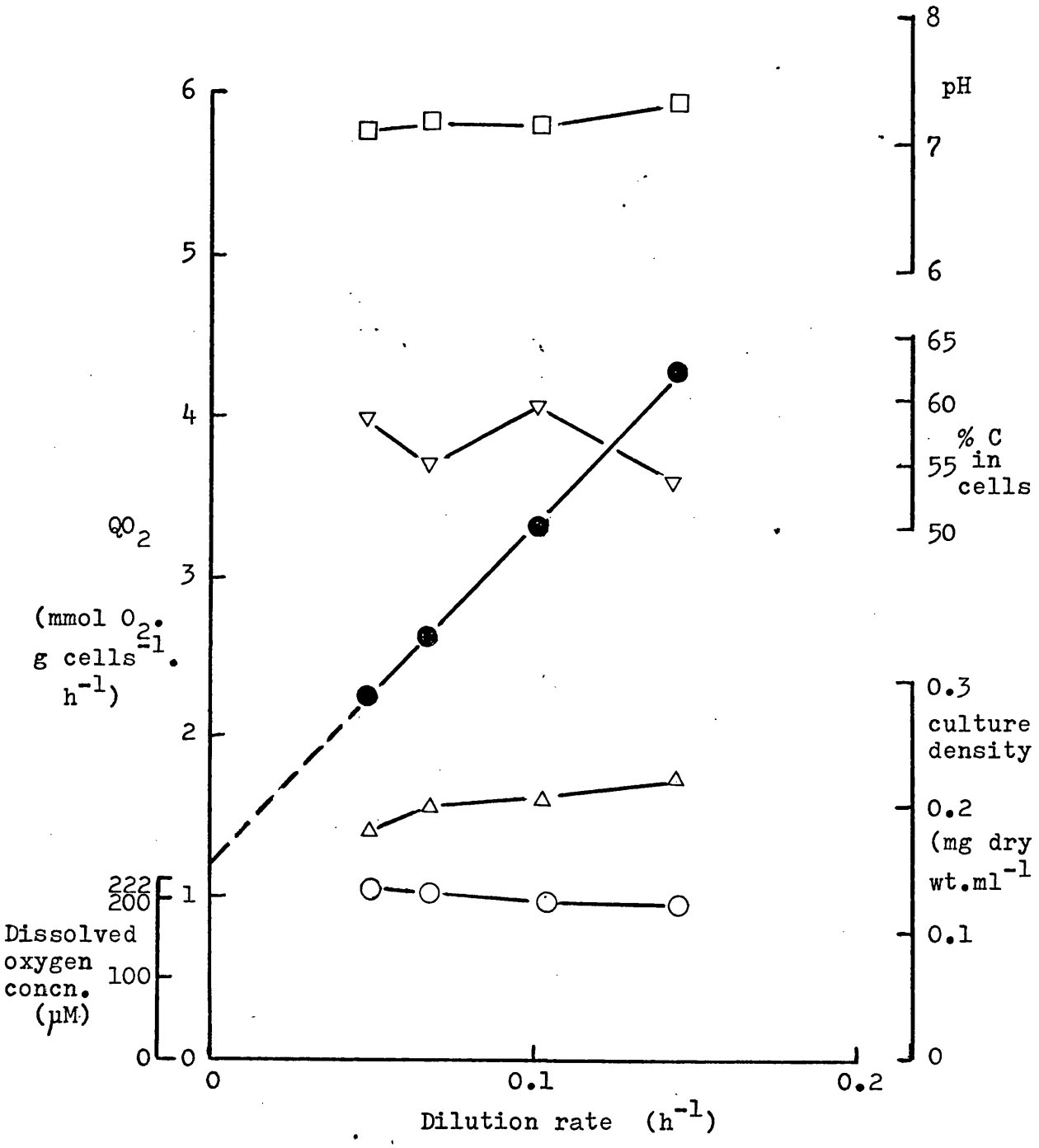
possibility was not tested.

The maximum attainable dilution rate during high aeration, glycerol-limited continuous culture of B. megaterium strain M was low; the culture washed out at D = 0.16 h^{-1} (hence $D_c \leqslant 0.16\ h^{-1}$). This was anticipated in view of the low logarithmic growth rate of the organism in minimal-salts plus glycerol medium under excess oxygen, batch culture conditions ($\mu_m = 0.173\ h^{-1}$). The pH of the continuous cultures of strain M was approximately constant at 7.1 - 7.3 when the dilution rate was varied between D = 0.045 h^{-1} and D = 0.140 h^{-1} (Fig. IV-23). $Y_{ATP}.N$ and M/N were measured from a plot of QO_2 versus D, as for strain D440 and, using a value of N calculated for glycerol oxidation by strain M as described in Materials and Methods, a Y_{ATP} value of 10.4 g cells. mol ATP equivalents^{-1} and an M value of 0.00562 mol ATP equivalents.g cells^{-1}. h^{-1} were calculated.

The Y_{ATP} values obtained for the two strains of B. megaterium growing in continuous culture under glycerol-limited conditions are therefore fairly similar at 13.5 and 10.4 g cells.mol ATP equivalents^{-1} for strains D440 and M respectively. Stouthamer and Bettenhaussen (1973) stated that a Y_{ATP} of 25 g cells.mol ATP equivalents^{-1}, which is close to the theoretical maximum yield of approximately 27 calculated by Forrest and Walker (1971), might be expected for bacteria growing in complex medium in which all of the preformed monomers required for polymerization into cell components are present (i.e. glucose, amino acids, purine and pyrimidine bases and acetate). It is therefore quite likely that the two strains of B. megaterium growing in complex medium would have yielded higher Y_{ATP} values than in the minimal-salts medium employed in this work. However, this possibility was not tested due to the lack of time available.

Fig. IV-23

Some growth parameters of glycerol-limited continuous cultures of B.megaterium strain M



B.megaterium strain M was grown in continuous culture under high aeration, glycerol-limited conditions. Symbols as for Fig. IV-19. Correlation coefficient for QO_2 versus D plot = 0.99.

Interestingly, the Y_{ATP} values obtained for B. megaterium are close to the old Elsden 'constant' of 10.6 g cells.mol ATP^{-1} obtained for anaerobic bacteria (see Bauchop and Elsden, 1960; Forrest and Walker, 1971). However, this comparison is not strictly valid since the Elsden 'constant', unlike the values in the present work, was obtained from batch culture experiments in which no correction was made for energy directed to cell maintenance. Thus it is really a $Y_{ATP(obs)}$ value. Had corrections for energy of maintenance been made, a Y_{ATP} value of considerably greater than 10.6 g cells.mol ATP^{-1} would probably have been obtained, since the micro-organisms involved were grown in rich media.

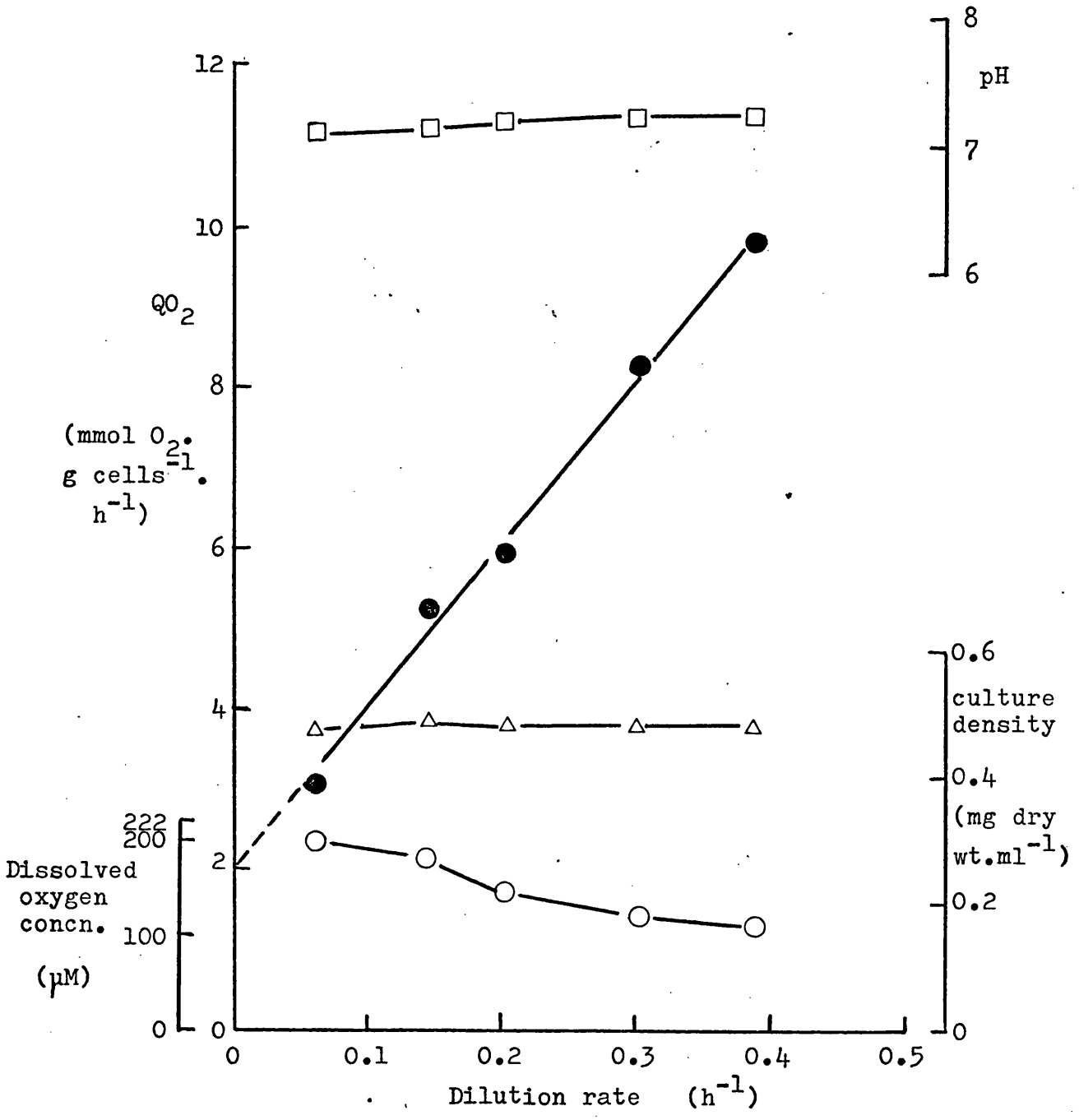
The maintenance coefficients obtained for the two strains of B. megaterium grown under high aeration conditions in glycerol-limited continuous culture at pH 7.1 - 7.3 were 0.00240 and 0.00562 mol ATP equivalents.g $cells^{-1}.h^{-1}$ for strains D440 and strain M respectively. There are relatively few values of M for bacteria quoted in the literature for comparison. During anaerobic growth, values of approximately 0.003 mol ATP.g $cells^{-1}.h^{-1}$ were obtained for Propionibacterium pentosaceum and P. freudenreichii (Stouthamer and Bettenhaussen, 1973), 0.0015 for Lactobacillus casei (de Vries et al., 1970), 0.028 for P. mirabilis (Stouthamer and Bettenhaussen, 1972; 1973) and 0.0387 for K. pneumoniae (Stouthamer and Bettenhaussen, 1973). For aerobic growth, no reliable values of M have been published (those of Brice et al., 1974 are suspect due to the batch culture technique employed; see Introduction to this chapter). However, Herbert (1958), Pirt (1965) and Harrison and Loveless (1971) have all calculated maintenance coefficients for K. pneumoniae, growing aerobically in carbon-limited continuous culture,

in terms of the rate of substrate utilisation (oxygen, glycerol and oxygen respectively). Since this organism probably contains proton translocating loops 1 and 2 (Brice et al., 1974), values for N can be calculated for each of the culture conditions employed and hence values for M derived. The resultant values for M are 0.0134 (Herbert, 1958), 0.0116 (Pirt, 1965) and 0.0056 mol ATP equivalents.g cells^{-1}.h^{-1} (Harrison and Loveless, 1971). The average value of M for K. pneumoniae growing under aerobic conditions (0.0102 mol ATP equivalents.g cells^{-1}.h^{-1}) is therefore somewhat higher than for the two strains of B. megaterium, but there is no order of magnitude difference.

(b) Nitrogen-limited cultures The two strains of B. megaterium were also grown in continuous culture under conditions in which the input medium contained only 10% of the normal ammonium chloride concentration (3.7 mM instead of 37 mM) but excess of glycerol (33 mM instead of 6.6 mM, which was employed for glycerol-limited cultures). Such cultures were nitrogen-limited, as revealed by increasing the concentration of NH_4Cl in the supply medium, when there was a concomitant increase in culture density. The pH of the cultures was maintained between 7.0 - 7.3 by the buffering capacity of the medium and high aeration conditions were employed so that the dissolved oxygen concentration was always > 100 µM O_2. Unlike the glycerol-limited cultures, the bacterial densities of the nitrogen-limited cultures were almost invariant with respect to dilution rate; no decrease occurred at the lower dilution rates employed (Figs. IV-24, -25). This suggested that the yield coefficients of the organisms with respect to NH_4Cl remained constant as the dilution rate was altered; i.e. the steady-state bacterial concentration versus

Fig. IV-24

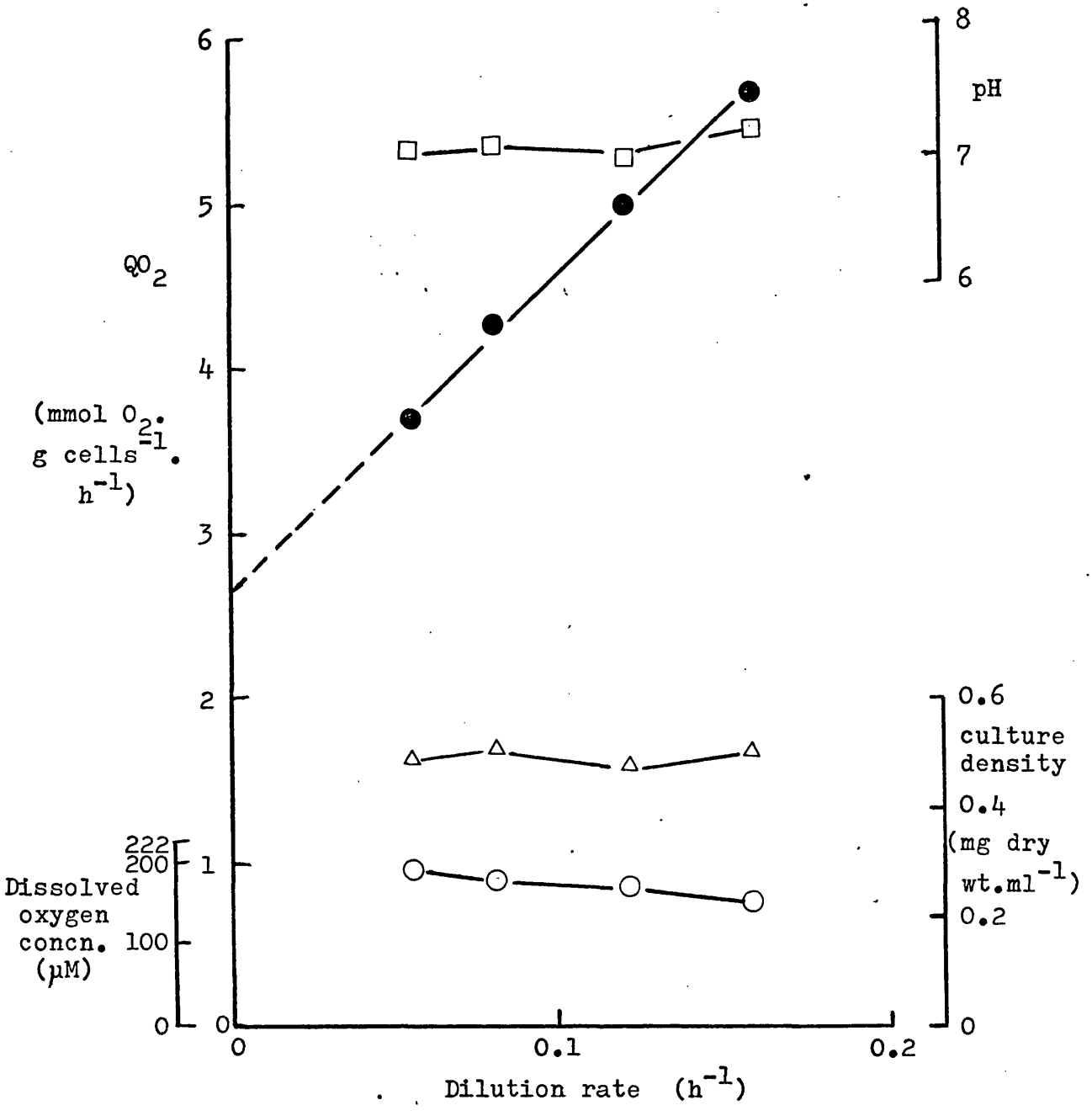
Some growth parameters of nitrogen(NH_4Cl)-limited continuous cultures of B.megaterium strain D440



B.megaterium strain D440 was grown in continuous culture under NH_4Cl-limited conditions. Symbols as for Fig. IV-19. Correlation coefficient for QO_2 versus D plot = 0.99.

Fig. IV-25

Some growth parameters of nitrogen(NH_4Cl)-limited continuous cultures of B.megaterium strain M



B.megaterium strain M was grown in continuous culture under NH_4Cl-limited conditions. Symbols as for Fig. IV-19. Correlation coefficient for QO_2 versus D plot = 0.99.

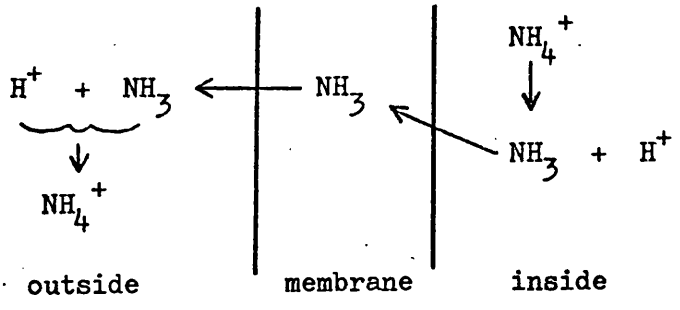
dilution rate followed the theoretical curve shown in Fig. IV-1. This was in contrast to the glycerol-limited cultures which exhibited bacterial concentration *versus* dilution rate plots similar to the theoretical curve shown in Fig. IV-2, indicating that the yield coefficient of the cells with respect to glycerol changed as the dilution rate was altered (due to their maintenance energy requirements).

Values of Y_{ATP} for the NH_4Cl-limited cultures, obtained from plots of QO_2 *versus* D, were very close to the values obtained for glycerol-limited cultures (for strain D440, Y_{ATP} = 12.3 g cells.mol ATP equivalents^{-1}; strain M, Y_{ATP} = 11.4), hence the efficiency of ATP utilisation for growth was very similar under the two culture conditions. Harrison and Hamer (1971) found that growth of the methanol-utilising organism *Pseudomonas extorquens* in the presence of excess methanol resulted in decreased cell yields compared with methanol-limited cultures. Harrison (1973) consequently suggested that depressed growth yields might occur in the presence of excess energy source because of wasteful energy utilisation, but expressed doubt as to whether this uncoupling of growth and energy processes occurred at the level of energy generation (i.e. oxidative phosphorylation) or energy utilisation for growth (i.e. Y_{ATP}) in the case of *Ps. extorquens*. The present work indicates that with *B. megaterium*, growth in the presence of excess energy source (glycerol) in nitrogen-limited cultures causes neither a reduction in the efficiency of energy generation (as measured by $\longrightarrow H^+/O$ ratios) nor in the efficiency of energy utilisation for growth (as shown by the Y_{ATP} values).

Rather surprisingly, the maintenance coefficients of the two strains of *B. megaterium* growing in nitrogen-limited culture were much

higher than during growth in glycerol-limited culture. Thus, for strain D440, M = 0.00240 and 0.00792 mol ATP equivalents.g cells^{-1}.h^{-1} for glycerol-limited and nitrogen-limited cultures respectively and for strain M, M = 0.00562 and 0.01220 mol ATP equivalents.g cells^{-1}.h^{-1} for glycerol-limited and nitrogen-limited cultures. A scrutiny of the work of Harrison and Pirt (1967) reveals that the maintenance coefficient for nitrogen-limited growth of K. aerogenes was also higher than that for glucose-limited growth of this organism, since the respiratory activity of excess oxygen, nitrogen-limited cells was much higher than the respiratory activity of excess oxygen, glucose-limited cells growing in continuous culture at a similar dilution rate (see Harrison and Pirt, 1967; Figs 1a and 5).

A possible explanation for the higher maintenance coefficient of nitrogen-limited cells compared with carbon/energy-limited cells is as follows. During growth of the bacteria, respiration induces a proton-motive force across the plasma membrane which tends to drive protons inwards (see Chapter 1). The pmf has a pH component such that the exterior of the cells is more acid than the interior. Assume that NH_4^+ ions are present inside the cells, and that the concentration of these ions is low outside (as would occur during nitrogen-limited growth); the plasma membrane is probably impermeable to NH_4^+ ions but permeable to NH_3 (see Henderson, 1971) and hence NH_4^+ ions will disappear from the inside of the cells and appear on the outside by the following mechanism (see also Mitchell,1970; Harold,1972):



This process will be accelerated by the pH gradient and the concentration gradient of NH_4^+ ions. In order for nitrogen-limited cells to maintain a sufficiently high intracellular NH_4^+ concentration for metabolic processes, an ATP (or high energy intermediate)-utilising system for uptake of NH_4^+ ions may be required. If NH_4^+ entry is ATP linked under NH_4Cl-limited conditions, then the rate of ATP utilisation will increase with an increase in dilution rate (i.e. the faster the growth rate, the faster the uptake rate of NH_4^+ ions required). This would both increase the maintenance coefficient and decrease the apparent Y_{ATP}. However, it is possible that at fast growth rates the outward flow of NH_3 would be slower than at low growth rates due to rapid biosynthetic reactions and hence the energy requirement for NH_4^+ uptake may tend to a constant irrespective of the dilution rate. This would have the effect of increasing the maintenance coefficient of the NH_4Cl-limited cells rather than decreasing the Y_{ATP}, as was observed with the two strains of B. megaterium.

Stouthamer and Bettenhaussen (1973) found that high concentrations of NH_4Cl in the input medium caused an increased maintenance coefficient for tryptophan-limited growth of K. pneumoniae. At first sight this seems contradictory to the results quoted above for B. megaterium; however, it is quite possibly that an ATP (or intermediate)-driven outwardly-directed 'pump' for NH_4^+ ions may be required to prevent the build up of inhibitory intracellular concentrations of NH_4^+ when NH_4Cl is present in high concentrations externally.

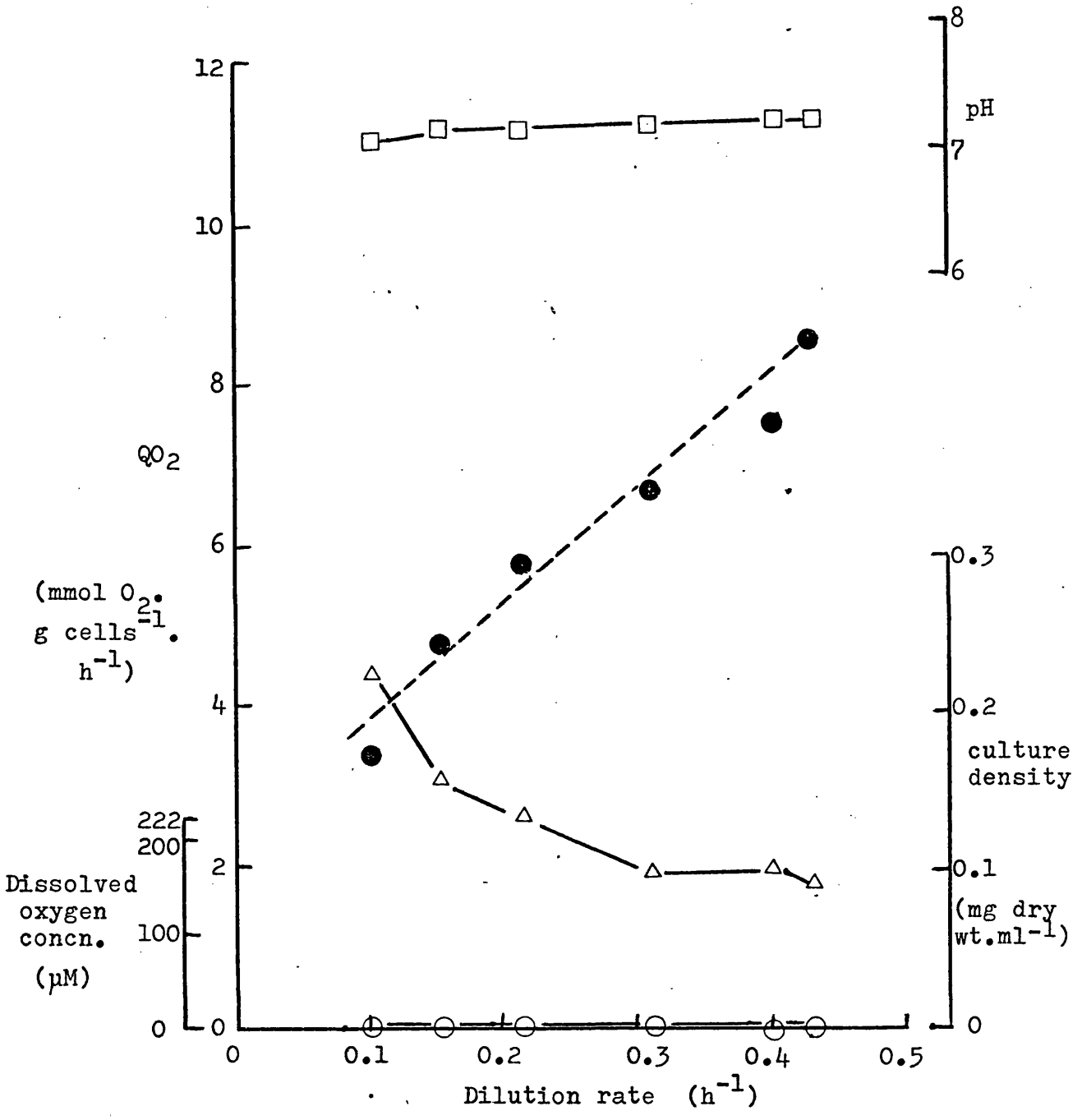
(c) Oxygen-limited cultures The two strains of B. megaterium were grown in continuous culture under low aeration conditions. Glycerol and ammonium chloride were present in excess in the input medium (33 mM

and 37 mM respectively) and the cultures were known to be oxygen-limited since increasing the stirring rate (and adjusting the flow rate of the input medium to maintain the same dilution rate) resulted in an increase in culture density. Another indication of the oxygen-limited status of the cultures was the immeasurably small dissolved oxygen concentration at all dilution rates employed (see Figs. IV-26; -27). The pH of the cultures was maintained in the range 7.1 - 7.3 for strain D440 and 6.9 - 7.1 for strain M by the buffering capacity of the medium.

The gradient of the plot of QO_2 *versus* D for strain D440 (Fig. IV-26) was less than the gradient for the glycerol- and nitrogen-limited cultures of this organism which suggested, at first sight, that the Y_{ATP} value was higher under oxygen-limited conditions. However, as described above, the $\longrightarrow H^+/O$ quotients exhibited by samples from these cultures decreased with the dilution rate of the culture. This was attributed to the gradual loss of coupling at loop 1 as the degree of oxygen-limitation of the cells increased. It was also supported by the observation that, at a dilution rate of 0.43 h^{-1}, the oxygen-limited cells exhibited a similar QO_2 to glycerol-limited cells at the same dilution rate (see Fig. IV-19). Both types of cells gave $\longrightarrow H^+/O$ ratios of approximately 4. On the other hand, at a dilution rate of 0.1 h^{-1}, the oxygen-limited cells exhibited a considerably higher QO_2 than glycerol-limited cells at the same dilution rate. This fitted in with the lower $\longrightarrow H^+/O$ quotient obtained for the oxygen-limited cells than the glycerol-limited cells under these conditions, since the oxygen-limited cells would be required to respire faster in order to synthesise ATP equivalents at the necessary rate.

Fig. IV-26

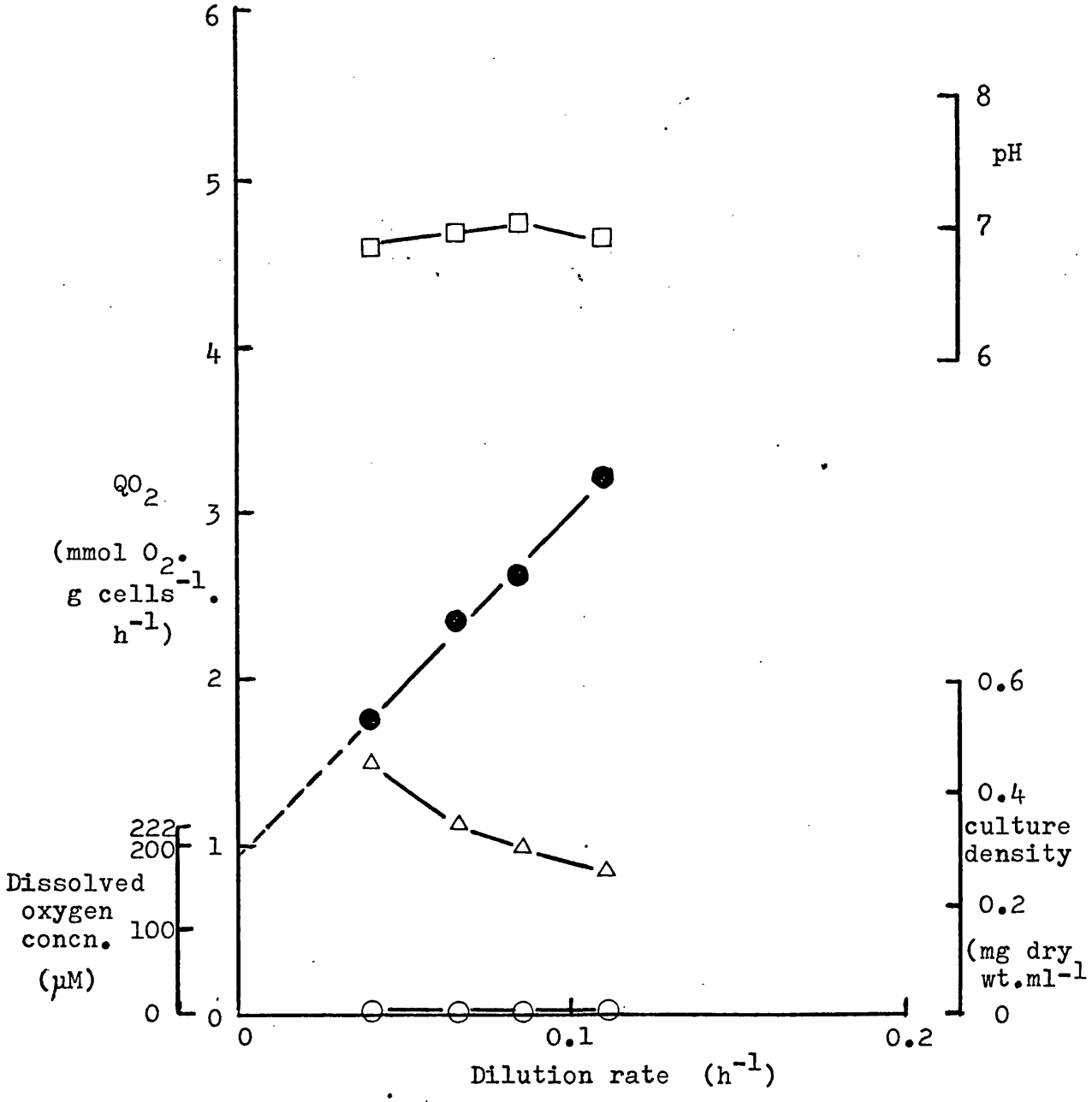
Some growth parameters of oxygen-limited continuous cultures of B.megaterium strain D440



B.megaterium strain D440 was grown in continuous culture under oxygen-limited conditions. Symbols as for Fig. IV-19.

Fig. IV-27

Some growth parameters of oxygen-limited continuous cultures of B.megaterium strain M



B.megaterium strain M was grown in continuous culture under oxygen-limited conditions. Symbols as for Fig. IV-19. Correlation coefficient for QO_2 versus D plot = 0.99.

Although it was not possible to obtain a value of Y_{ATP} for oxygen-limited growth of strain D440 directly from Fig. IV-26, because the $\longrightarrow H^+/O$ quotient and hence the value of N changed with dilution rate, it was possible to do so indirectly by the following procedure. At the lowest dilution rate examined (D = 0.104 h^{-1}), the $\longrightarrow H^+/O$ quotient = 3.37 g ion H^+.gatom O^{-1}. Assuming that in these cells there was a partial loss of loop 1, n_{fp} = 1 and n_{NAD} = 3.37/2 = 1.69 (see Materials and Methods). Substituting these values into equation IV-24 gave N = 3.56 mol ATP equivalents.mol O_2 consumed^{-1}. From equations IV-21 and -22 a value for $Y_{ATP(obs)}$ of 8.70 g cells.mol ATP equivalents^{-1} could be calculated for these cells. This procedure was repeated for the other two cultures from which samples were taken for $\longrightarrow H^+/O$ ratio measurements (i.e. D = 0.215, 0.430 h^{-1}), thus giving $Y_{ATP(obs)}$ values at each dilution rate (Table IV-2). A plot of $^1/Y_{ATP(obs)}$ against $^1/D$ (see equation I-7) approximated to a straight line (Fig. IV-28). From the intercept on the ordinate the Y_{ATP} value (corrected for maintenance energy) for oxygen-limited growth of strain D440 was obtained, viz 13.1 g cells.mol ATP equivalents^{-1}. This was very close to the value obtained under high-aeration glycerol-limited conditions (13.5 g cells.mol ATP equivalents^{-1}). The maintenance coefficient, M, obtained from the slope of the graph in Fig. IV-28, was 0.0040 mol ATP equivalents.g cells^{-1}.h^{-1}. This was rather higher than the value of M obtained for glycerol-limited growth (0.00240) but less than the value obtained for NH_4Cl-limited growth (0.00792). Why the maintenance coefficient for oxygen-limited growth of strain D440 should be higher than that for glycerol-limited growth is unclear.

In contrast to strain D440, strain M gave a plot of QO_2 versus

Table IV-2

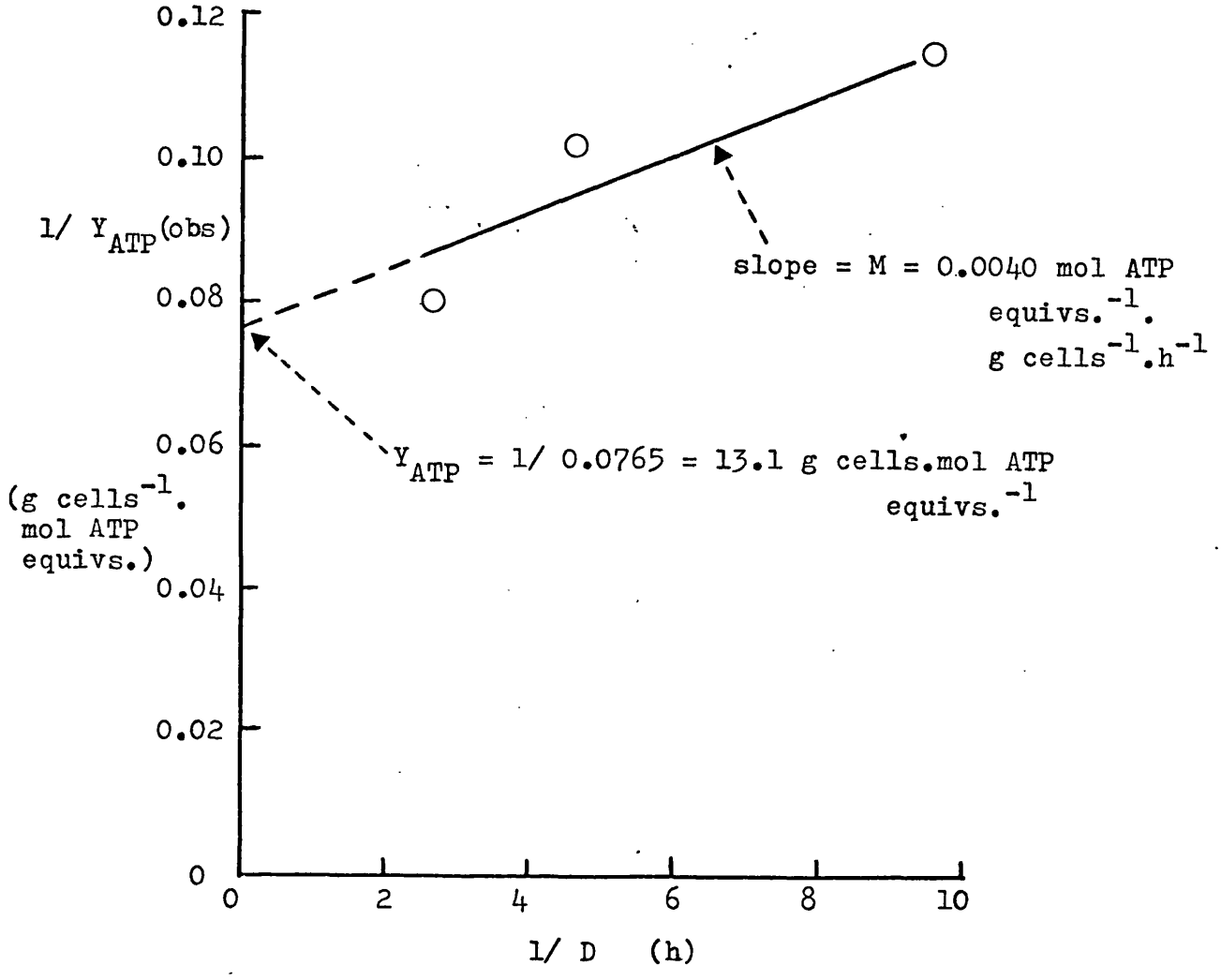
Calculation of Y_{ATP}(obs.) values for oxygen-limited chemostat growth of B.megaterium strain D440

Dilution rate (D, h^{-1})	1/ D (h)	QO_2 (mol O_2. $g.cells^{-1}$. h^{-1})	$\rightarrow H^+/O$ quotient (gion H^+. gatom O^{-1})	N (mol ATP equivs. mol O_2 $consumed^{-1}$)	Y_{ATP}(obs.) (g cells.mol ATP $equivs^{-1}$)	1/ Y_{ATP}(obs.) ($g\ cells^{-1}$.mol ATP equivs.)
0.104	9.61	0.00336	3.37	3.56	8.70	0.115
0.215	4.65	0.00578	3.70	3.78	9.84	0.102
0.430	2.32	0.00860	4.13	4.00	12.5	0.080

$\rightarrow H^+/O$ data obtained from Fig. IV-17. Corresponding QO_2 data obtained from Fig. IV-26.

Fig. IV-28

A plot of $1/ Y_{ATP}(obs.)$ versus $1/ D$ for oxygen-limited continuous cultures of B.megaterium strain D440



Data obtained from Table IV-2.

D for oxygen-limited growth which yielded a gradient very similar to that obtained for glycerol- and nitrogen-limited cultures (Fig. IV-27). This was anticipated in view of the virtual invariance of the $\longrightarrow H^+/O$ quotients exhibited by cell samples derived from oxygen-limited cultures growing at various dilution rates (see Fig. IV-18). It was therefore possible to obtain values for Y_{ATP} and M directly from Fig. IV-27. The Y_{ATP} value for oxygen-limited growth was 10.7 g cells.mol ATP equivalents^{-1} which was close to the value obtained under glycerol-limited conditions (10.4). In contrast to oxygen-limited strain D440, the maintenance coefficient for oxygen-limited growth of strain M, viz 0.00439 mol ATP equivalents.g cells$^{-1}h^{-1}$ was slightly less than the value obtained under glycerol-limited conditions (0.00562). There is no obvious explanation for these differences. However, it is concluded that a change in the culture conditions from growth-limitation by the supply of electron donor (glycerol) to growth-limitation by the supply of electron acceptor (oxygen) did not affect the true growth efficiency of either strain of B. megaterium, as shown by the Y_{ATP} measurements.

Both strains exhibited a rather unexpected decrease in culture density with increase in dilution rate (see Figs. IV-26, -27), a phenomenon which was not explained by the theoretical aspects of continuous culture considered earlier in this chapter. Tempest and Dicks (1967) reported that potassium-, magnesium- and phosphorus-limited growth of K. pneumoniae in continuous culture was characterised by an increase in culture density at low dilution rates, and this was attributed to an increased yield of the organism on these substrates at the lower growth rates employed. Such a situation is opposite to that observed with carbon/energy limited cultures, where the yield decreases as the

growth rate decreases. However, there is an alternative explanation to a variation in growth yield which explains the variation of culture density with change in dilution rate of oxygen-limited B. megaterium. Since (equation IV-20)

$$K_L A = \frac{QO_2 . x}{(P_G - P_L)}$$

hence $$x = \frac{K_L A (P_G - P_L)}{QO_2} \qquad \text{IV-26}$$

As the stirring rate of the cultures was not altered and the culture volume was constant, $K_L A$ was presumably constant with respect to change in dilution rate. Likewise, P_L was approximately constant at almost zero mm Hg and P_G was presumably constant at 159 mm Hg . Since QO_2 decreased with decrease in dilution rate, the culture density increased in order to balance equation IV-26. Further support for this explanation was obtained by calculating the oxygen uptake rate of the culture at each dilution rate in terms of mol $O_2 . l^{-1} . h^{-1}$. This was found to be virtually constant, as was expected since P_G and P_L were constant (i.e. $QO_2 . x$ = a constant)

(d) Glutamate-limited cultures There is evidence in the literature that low growth yields are obtained for bacteria during growth on substrates of low molecular weight such as acetate, formate, oxalate and methane (see Stouthamer, 1969; Harrison, 1973). In addition, Hadjipetrou et al. (1964) found that pyruvate and the tricarboxylic acid cycle intermediates succinate, fumarate, malate, and also glutamate (enzymically converted to α-ketoglutarate in the cells) resulted in low Y_O values for growth of K. pneumoniae compared

with those obtained for a number of sugars (e.g. D-glucose, D-galactose) and glycerol.

It is likely that in the case of the low molecular weight carbon substrates considerable energy is required for their conversion to biosynthetic monomers (especially in the case of the C_1 compounds methane and formate). Synthesis of many bacterial cellular components (e.g. lipids, structural polysaccharides and nucleic acids) requires quite highly reduced intermediates (e.g. fatty acids and glycerol for lipid biosynthesis, simple sugars for polysaccharide biosynthesis and ribose for purine synthesis). Hence it is likely that more energy must be expended during synthesis of these compounds if the carbon substrate is relatively oxidised compared with, say, glucose. Acetate, formate and oxalate clearly fall into this category as do pyruvate and the tricarboxylic acid cycle intermediates. One would therefore expect to see low Y_{ATP} values for bacterial growth on both low molecular weight carbon substrates and relatively oxidised carbon substrates.

It is now well established that uptake of the uncharged sugars lactose, D-galactose and L-arabinose by E. coli is associated with inward proton movements, probably with a mol sugar : gion H^+ stoichiometry of 1 : 1 (West and Mitchell, 1972; Henderson, 1974). Uptake of these sugars is energy requiring since it is postulated that the proton gradient generated during respiration is used as a driving force (West and Mitchell, 1972). It is also likely that uptake of negatively-charged carbon substrate molecules by bacteria is energy requiring. It is known that transport of the acetate anion into the cells of higher plants and animals is accompanied by proton uptake; the transported species is probably the neutral acid (see Henderson, 1971). Uptake

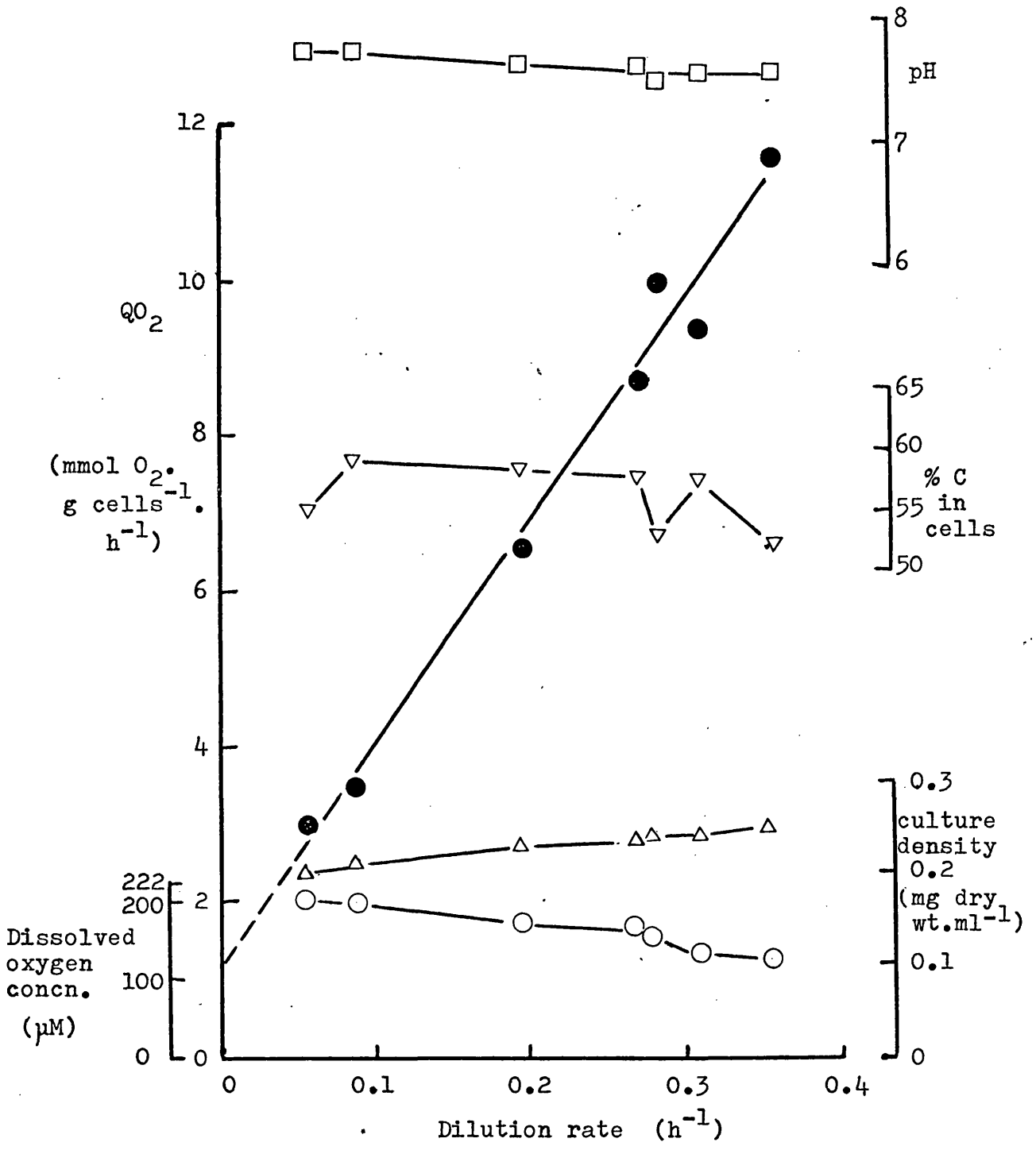
of neutral, basic and acidic amino acids by Staphylococcus aureus can also be driven by transmembrane pH and electrical gradients (Niven and Hamilton, 1974). Hence it is probable that growth yields of bacteria on carbon substrates such as oxalate are very low because not only is the Y_{ATP} low but also the overall efficiency of synthesis of ATP equivalents from the substrate is low due to the energy required for transport of the substrate into the cell.

Since no allowance was made for maintenance energy requirements in the growth yield experiments cited above, it is possible that the growth yields of, for example, K. pneumoniae on tricarboxylic acid cycle intermediates were low solely because of a slower growth rate of the organism on these compounds than on glucose. In that case, low Y_O values may not have reflected low Y_{ATP} values. In order to ascertain if the Y_{ATP} value of B. megaterium was altered by the choice of carbon/energy substrate, strain M was grown in continuous culture limited by glutamate rather than glycerol. Glutamate was chosen since it was known from personal observation that the specific growth rate of the organism in batch cultures containing glutamate was high (μ_m = 0.42 h^{-1}) and since glutamate is presumably converted directly to α-ketoglutarate (a tricarboxylic acid cycle intermediate) in the cells by glutamate dehydrogenase. The cells were grown in continuous culture under high aeration conditions. Although the pH of the input medium to the chemostat was the same as that for glycerol-, nitrogen- and oxygen-limited growth (i.e. pH 7.2), the actual pH of the cultures was in the range 7.6 - 7.8 rather than approximately 7.2 as for the other culture conditions. This was probably due to the release of NH_4^+ ions into the culture medium following catabolism of glutamate.

A plot of QO_2 *versus* D (Fig. IV-29) gave a Y_{O_2} (= $Y_{ATP}.N$) value of 34.8 g cells.mol O_2^{-1} from which a Y_{ATP} value of 7.82 was obtained using the value of N (4.45 mol ATP equivalents.mol O_2 consumed^{-1}) calculated as described in Materials and Methods. This Y_{ATP} value was somewhat lower than the value obtained for glycerol-limited growth (10.4 g cells.mol ATP equivalents^{-1}). An attempt was made to correct the value of N (and hence Y_{ATP}) of the glutamate-limited cells for the transport of glutamate, assuming this to be linked to proton uptake. At the pH of the culture medium (7.7) the glutamate was presumably present almost entirely as $R\text{-}NH_3^+[COO^-]_2$ (assuming pka_2 = 4.25, pka_3 = 9.67; Segel, 1968). As it would presumably be transported across the plasma membrane as $R\text{-}NH_2[COOH]_2$, this would require the uptake of 1 gion H^+ per mol glutamate transported. Since the *in vivo* $\longrightarrow H^+/O$ quotient = (2 x 2) + (7 x 2)/4.5 = 4.00 (see Materials and Methods), during oxidation of 1 mol glutamate, 9 x 4 = 36 gion H^+ were translocated. However, 1 gion H^+ would be needed for uptake of 1 mol glutamate, hence the 'effective' *in vivo* $\longrightarrow H^+/O$ ratio = 35/9 = 3.89. With substrate-level phosphorylation (2/4.5 = 0.45) a 'corrected' N value of 4.34 was obtained. This resulted in a Y_{ATP} value, corrected for glutamate uptake of 8.02 g cells.mol ATP equivalents^{-1}, which was still rather lower than the value obtained during glycerol-limited growth. Even this comparison may not be strictly valid, however, since glycerol uptake under glycerol-limited conditions may have been energy requiring, thus having a small effect on the Y_{ATP} value for glycerol-limited cultures. However, it seems quite possible that the lower Y_{ATP} value obtained for glutamate-limited growth was due largely to the greater amount of energy required for monomer biosynthesis from the relatively oxidised glutamate (or α-ketoglutarate) molecule than from the glycerol molecule.

Fig. IV-29

Some growth parameters of glutamate-limited continuous cultures of B.megaterium strain M



B.megaterium strain M was grown in continuous culture under glutamate-limited conditions. Symbols as for Fig. IV-19. Correlation coefficient for QO_2 versus D plot = 0.99.

The maintenance coefficient for glutamate-limited growth (0.00530 mol ATP equivalents.g cells^{-1}.h^{-1}) was only slightly lower than for glycerol-limited growth (0.00562). From these results it can be calculated that, while the Y_{ATP} for glutamate-limited growth was 25% lower than the Y_{ATP} for glycerol-limited growth, the maintenance coefficient was only 6% lower. Abbott and Clamen (1973) stated that maintenance energy is required for such functions as resynthesis of unstable macromolecules, motility and maintenance of concentration gradients. The relatively small decrease in maintenance coefficient compared with the decrease in Y_{ATP}, obtained by altering the culture conditions from glycerol- to glutamate-limited, suggests that most of the energy used for maintenance purposes in B. megaterium strain M was not used in a biosynthetic capacity. As this organism (like strain D440) was found to be only slightly motile, it is possible that most of the maintenance energy requirement was for preservation of the right ionic composition of the cells. Stouthamer and Bettenhaussen (1973) presented evidence that this is also the case for K. pneumoniae.

A summary of the Y_{ATP} and M values obtained for the two strains of B. megaterium under the various culture conditions employed is given in Tables IV-3 and 4.

(e) The effect of energy storage compounds on the measured Y_{ATP} values Under certain growth conditions, the carbon/energy source supplied to micro-organisms may to some extent be assimilated instead of degraded, resulting in the formation of reserve polymers. Such polymerization reactions are characterised by the highly efficient use of ATP (Walker, 1968) with the result that the laying down of reserve materials can cause large increases in the dry weight of the organisms

Table IV-3

A summary of the Y_{ATP} and M values calculated for growth of B.megaterium strain D440 in continuous culture

Growth-limiting substrate	Carbon/energy source	Other growth conditions	Y_{ATP}.N (g cells. mol O_2^{-1})	M/N (mol O_2.g cells^{-1}.h^{-1})	$\rightarrow H^+/O$ quotient (gion H^+. gatom O^{-1})	N (mol ATP equivs. mol O_2^{-1})	Y_{ATP} (g cells. mol ATP equivs.$^{-1}$)	M (mol ATP equivs. g cells^{-1}.h^{-1})
glycerol	glycerol	high aeration (pH 7.2-7.3)	53.8	0.00060	4.09 ± 0.05(3)	4.00	13.5	0.00240
glycerol	glycerol	extra-high aeration (pH 7.2-7.3)	51.6	0.00047	3.97 ± 0.04(3)	4.00	12.9	0.00188
glycerol	glycerol	high aeration (pH 5.8-6.1)	50.0	0.00069	4.10 ± 0.05(3)	4.00	12.5	0.00276
glycerol	glycerol	high aeration (pH 7.5-7.8)	54.0	0.00068	4.12 ± 0.03(3)	4.00	13.5	0.00272
NH_4Cl	glycerol	high aeration (pH 7.1-7.3)	49.2	0.00198	4.15 ± 0.08(3)	4.00	12.3	0.00792
oxygen	glycerol	low aeration (pH 7.1-7.3)	(see Table IV-2, Fig. IV-28)			4.00	13.1	0.0040

Y_{ATP} and M/N values are obtained from the gradient and intercept of the plot of QO_2 *versus* D for each culture condition. $\rightarrow H^+/O$ ratios are obtained from Fig. IV-17 and are expressed as the average ± S.E.M.(no. of determinations).

Table IV-4

A summary of the Y_{ATP} and M values calculated for growth of *B.megaterium* strain M in continuous culture

Growth-limiting substrate	Carbon/energy source	Other growth conditions	$Y_{ATP}.N$ (g cells. mol O_2^{-1})	M/N (mol O_2.g $cells^{-1}.h^{-1}$)	$\rightarrow H^+/O$ quotient (gion H^+. gatom O^{-1})	N (mol ATP equivs. mol O_2^{-1})	Y_{ATP} (g cells. mol ATP $equivs^{-1}$)	M (mol ATP equivs. g $cells^{-1}.h^{-1}$)
glycerol	glycerol	high aeration (pH 6.9-7.1)	47.6	0.00123	4.06 ± 0.03(3)	4.57	10.4	0.00562
NH_4Cl	glycerol	high aeration (pH 7.0-7.2)	52.0	0.00267	4.11 ± 0.03(3)	4.57	11.4	0.0122
oxygen	glycerol	low aeration (pH 6.9-7.1)	48.9	0.00096	4.03 ± 0.04(3)	4.57	10.7	0.00439
glutamate	glutamate	high aeration (pH 7.4-7.6)	34.8	0.00119	4.08 ± 0.02(3)	4.45	7.82	0.00530

Y_{ATP} and M/N values are obtained from the gradient and intercept of the plot of QO_2 *versus* D for each culture condition. $\rightarrow H^+/O$ ratios are obtained from Fig. IV-18 and are expressed as the average ± S.E.M. (no. of determinations).

(Doudoroff, 1966). In the present work, the Y_{ATP} values calculated for the two strains of B. megaterium were based on measurements of QO_2, i.e. mol O_2 consumed.g dry wt bacteria^{-1}.h^{-1}. If these bacteria contained significant quantities of storage polymers it is possible that the Y_{ATP} values obtained were inaccurate.

A number of micro-organisms can accumulate glycogen as an energy-storage compound, e.g. E. coli (Holme, 1957) and yeast (Herbert, 1958). In addition to glycogen, Wilkinson and Monroe (1967) reported that B. megaterium strain KM accumulated poly-β-hydroxybutyrate (PHB). They found that glycogen was accumulated in significant amounts only at low growth rates under nitrogen-limited conditions in continuous culture whereas PHB was accumulated under nitrogen-, potassium-, sulphur- and even carbon/energy-limited conditions. Under nitrogen- and carbon/energy-limitation, the extent of accumulation of PHB increased with increase in dilution rate.

No attempt was made in the present work to measure the concentrations of possible storage polymers in the two strains of B. megaterium employed. However, calculation of the percentage carbon of the cells under glycerol- or glutamate-limited conditions (see Materials and Methods) showed that this parameter was approximately constant at 54-61% in both strains, regardless of dilution rate (see Figs. IV-19-23, 24). This was in contrast to the work of Wilkinson and Monroe (1967), which indicated an increase in the percentage carbon of glucose-limited chemostat cells of B. megaterium strain KM with increase in the dilution rate at which the cells were grown. This discrepancy could be a result of strain differences.

It is therefore unlikely that the strains used in the present

work accumulated significant quantities of storage polysaccharides, at least under carbon/energy limited conditions, so the Y_{ATP} values obtained are probably fairly accurate. However, it must be admitted that the %C values obtained in the present work, being calculated values, are open to some error since it was assumed that no carbon (either as glycerol or other metabolites) appeared in the effluent medium.

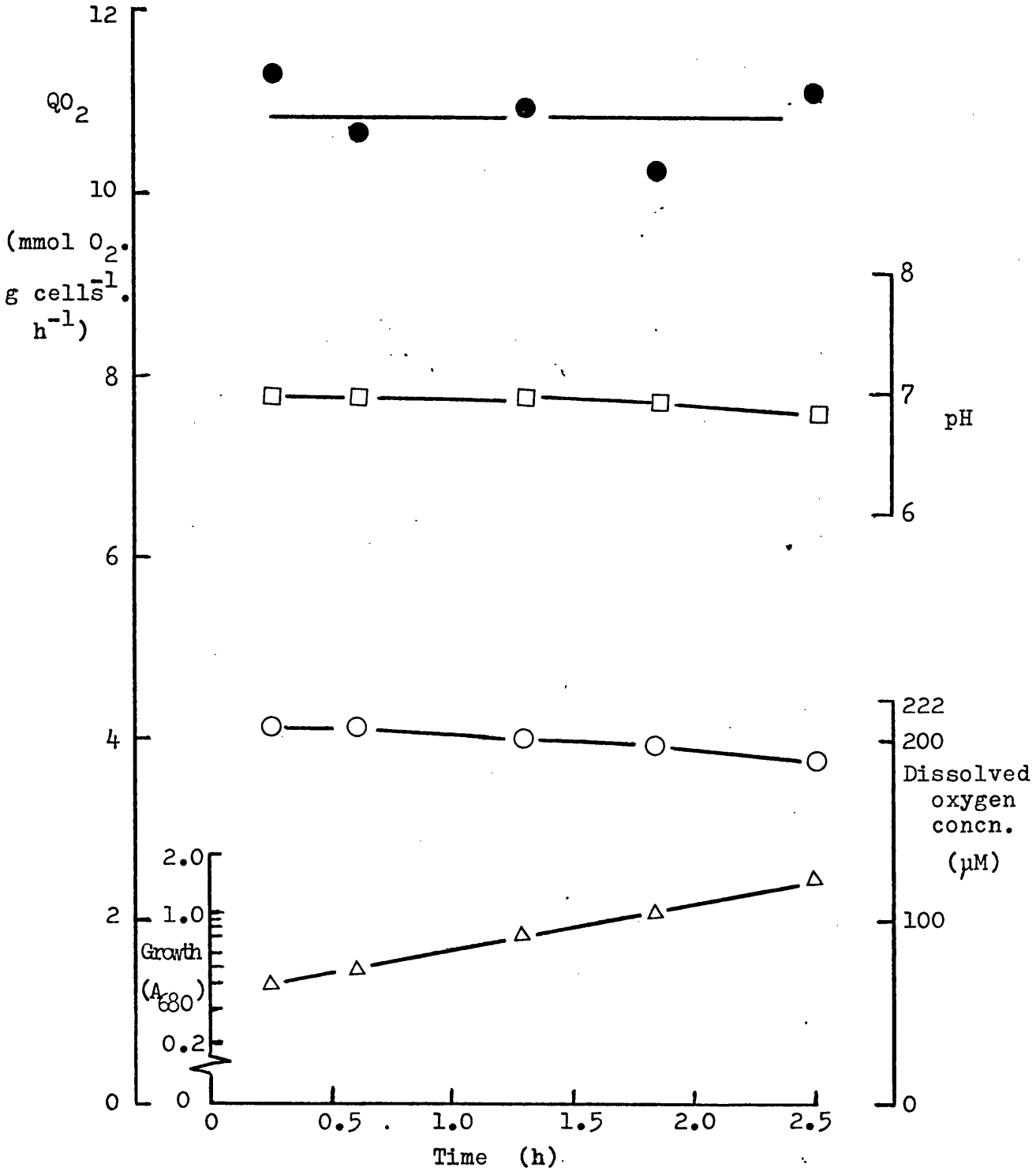
Unfortunately, it was not possible to calculate the %C of the bacteria growing under nitrogen- and oxygen-limited conditions.

4. Growth yields of B. megaterium in batch culture

Various growth parameters of the two strains of B. megaterium were measured in batch culture under excess oxygen conditions with glycerol as the carbon/energy source (strain D440, Fig. IV-30; strain M, Fig. IV-31) in order to obtain values of $Y_{ATP(obs)}$. As noted previously (Fig. III-1), the logarithmic growth rate of strain D440 under these conditions in batch culture was higher than that of strain M (strain D440, specific growth rate, $\mu = 0.630\ h^{-1}$; strain M, $\mu = 0.173\ h^{-1}$). This was reflected in the higher respiratory activity of strain D440 cultures than strain M cultures (average respiratory activities in the experiments illustrated; strain D440, $QO_2 = 0.0109$ mol O_2.g cells^{-1}.h^{-1}; strain M, $QO_2 = 0.00455$ mol O_2.g cells^{-1}.h^{-1}). The dissolved oxygen concentration and pH of the cultures were fairly constant for both organisms during the early stages of batch growth ($A_{680} < 2.0$) but the values of both these parameters decreased during the later stages. For this reason, growth up to $A_{680} = 2.0$ only is shown. Although these batch cultures were not, by definition, in steady state, it was possible to calculate the apparent growth yields with respect to ATP ($Y_{ATP(obs)}$)

Fig. IV-30

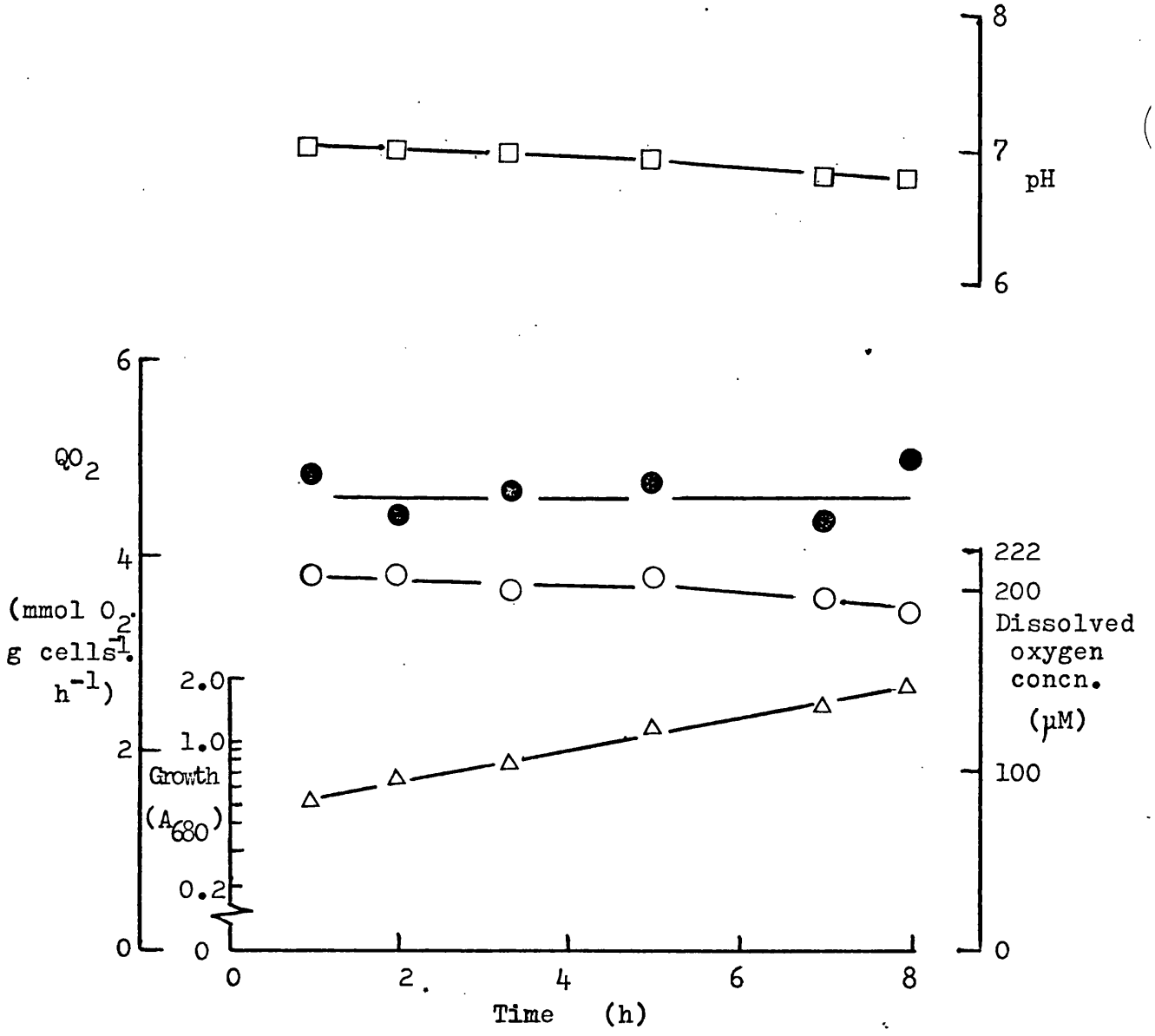
Growth parameters of B.megaterium strain D440 in batch culture



B.megaterium strain D440 was grown under batch culture conditions in a minimal-salts medium containing glycerol (66 mM). ● , QO_2; □ , pH; ○ , dissolved oxygen concn.; △ growth.

Fig. IV-31

Growth parameters of B.megaterium strain M in batch culture



B.megaterium strain M was grown under batch culture conditions in a minimal-salts medium containing glycerol (66 mM). Symbols as for Fig. IV-30.

as described in Materials and Methods. $Y_{ATP(obs)}$ values of 14.4 and 8.3 g cells.mol ATP equivalents^{-1} were obtained for B. megaterium strains D440 and M respectively (Table IV-5). However, although this appeared to suggest that strain M was less efficient at synthesising new cell material than strain D440, the former organism grew much more slowly on the minimal salts/glycerol medium employed than did the latter organism. Hence a larger proportion of the available ATP equivalents were presumably directed towards cell maintenance rather than synthesis of new cell material in strain M than in strain D440. It was not possible to obtain values for the maintenance coefficient (M) during batch growth of these bacteria and hence obtain Y_{ATP} values corrected for the energy of maintenance. Since growth of the organisms was not limited by the availability of any nutrient, none of the M values obtained during continuous culture could strictly be applied. The cultures were certainly not oxygen- or NH_4Cl-limited, nor were they glycerol-limited (at least, not by the availability of glycerol; their growth rates may possibly have been limited by the uptake rate of glycerol). Bearing this in mind, it was assumed that, for these experiments, the M values were the same as those obtained for glycerol-limited chemostat growth. Y_{ATP} values for batch growth were hence obtained by substituting the appropriate values of μ, QO_2, M and N into equation IV-1. This procedure gave a Y_{ATP} for batch growth of strain D440 of 15.3 g cells.mol ATP equivalents^{-1} which was fairly close to the value obtained for glycerol-limited chemostat growth (viz. 13.1), and a Y_{ATP} for batch growth of strain M of 11.4 g cells.mol ATP equivalents^{-1} which was very similar to the chemostat value (viz. 10.4).

These calculations therefore provide evidence that growth in

Table IV-5

Calculation of Y_{ATP}(obs.) and Y_{ATP} values for logarithmic, batch growth of B.megaterium

Strain	Logarithmic specific growth rate (μ, h^{-1})	QO_2 (mol O_2. g cells^{-1}.h^{-1})	Y_{O_2}(obs.) (g cells. mol O_2^{-1})	N (mol ATP equivs. mol O_2^{-1})	Y_{ATP}(obs.) (g cells. mol ATP equivs.$^{-1}$)	M (mol ATP equivs. g cells^{-1}. h^{-1})	Y_{ATP} (g cells. mol ATP equivs.$^{-1}$)	Y_{ATP} (glycerol-limited cont. culture; g cells mol ATP equivs.$^{-1}$)
D440	0.630	0.0109	57.6	4.00	14.4	0.00240	15.3	13.1
M	0.173	0.00455	38.0	4.57	8.3	0.00562	11.4	10.4

The specific growth rate and average QO_2 for batch growth of the organisms are obtained from Figs. IV-30&31. The values for maintenance coefficient(M) are those obtained for glycerol-limited continuous cultures (Tables IV-3&4).

batch cultures at high specific growth rates in the presence of excess carbon/energy source is of an efficiency at least as great as growth in carbon/energy-limited chemostat culture, at least for B. megaterium. In addition, they underline the importance of correcting for the maintenance energy requirements of bacteria in order to obtain accurate values of Y_{ATP}.

CHAPTER V

GENERAL DISCUSSION

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It is evident from the studies on A. vinelandii and B. megaterium described in this thesis that the measurement of $\longrightarrow H^+/O$ ratios, as first described by Mitchell (1966), offers a rapid and useful method for determining the efficiency of energy conservation in whole bacteria.

$\longrightarrow H^+/O$ quotients have recently been measured for a number of other bacterial species harvested during the exponential phase of growth and oxidising endogenous substrates. Jones et al. (1975) grouped these organisms according to their respiratory chain composition and correlated the latter with the $\longrightarrow H^+/O$ quotients they exhibited. Organisms which were classified in Group I on the basis of their lack of both membrane-bound NADPH $\longrightarrow NAD^+$ transhydrogenase activity and cytochrome c yielded $\longrightarrow H^+/O$ ratios of approximately 4. Such organisms were Escherichia coli (Lawford and Haddock, 1973; Brice et al., 1974), Klebsiella pneumoniae (Brice et al., 1974), Bacillus licheniformis and B. subtilis. These organisms therefore appeared to contain only two proton translocating loops in their respiratory systems. The presence of either cytochrome c in Micrococcus lysodeicticus (designated Group II) or an active, membrane-bound NADPH $\longrightarrow NAD^+$ transhydrogenase in Acinetobacter lwoffi (Group III), in addition to the basic respiratory chain of Group I organisms, resulted in the endogenous $\longrightarrow H^+/O$ ratios significantly increasing towards 6 (equivalent to an average of nearly three proton translocating loops per respiratory chain unit). The

addition of both cytochrome c and transhydrogenase to the basic respiratory chain in the Group IV organisms Pseudomonas ovalis and Hydrogenomonas eutropha (also Micrococcus denitrificans; Scholes and Mitchell, 1970) further increased the endogenous $\longrightarrow H^+/O$ ratios into the range 6-8 (equivalent to 3-4 proton translocating loops). Jones et al. (1975) concluded from the observed $\longrightarrow H^+/O$ ratios of cells loaded with specific substrates that proton translocating loops 1 and 2 were present in all of the organisms investigated, but that loops 0 and 3 were dependent on the presence of transhydrogenase and cytochrome c respectively. The substitution of menaquinone for ubiquinone (which are both hydrogen carriers) or cytochrome oxidase aa_3 for o (which are both electron carriers) appeared to have no significant effect on the efficiency of respiration-linked proton translocation.

In the scheme of Jones et al. (1975), B. megaterium strain D440 can be classified together with several other bacilli as a conventional Group I organism containing a basic respiratory system unsupplemented with either transhydrogenase or cytochrome c and exhibiting proton translocating loops 1 and 2. Strain M, which contained cytochrome c but no transhydrogenase is classified as a Group II organism and therefore might be expected to exhibit proton translocating loops 1, 2 and 3. Since proton translocation at loop 1 is not observed, this implies in terms of the chemiosmotic hypothesis of oxidative phosphorylation (see Chapter I) that the hydrogen and electron carriers of the NADH dehydrogenase were not looped across the membrane and hence did not translocate two protons outwards per pair of reducing equivalents transferred. Partial loss of ability of the NADH dehydrogenase of strain D440 to translocate protons was also observed following oxygen-

limited growth of the organism.

A. vinelandii cannot easily be classified in the scheme of Jones et al. (1975) since the pathways of electron transfer in the respiratory chain of this organism depend on the ambient oxygen concentration of the growth medium, as described in Chapter II. Under highly aerobic growth conditions, the organism contained only low concentrations of cytochrome c. Thus it may be classified in this case as a Group I organism since it contains a basic respiratory chain and the transhydrogenase is normally soluble rather than membrane bound (Chung, 1970). However, instead of an $\longrightarrow H^+/O$ quotient of 4, a value of approximately 2 was obtained. This was probably because the components of the NADH dehydrogenase were not looped across the membrane (i.e. Site I uncoupled). During oxygen-limited growth, the concentration of cytochrome c was much higher. However, since the respiratory system contains the cytochrome branches $\underline{b} \longrightarrow \underline{a}_2$ and $\underline{c}_4 + \underline{c}_5 \longrightarrow \underline{a}_1/\underline{o}$ (Jones and Redfearn, 1967a), the organism under these growth conditions cannot satisfactorily be classified in Group II, for which $\longrightarrow H^+/O$ quotients of 6 may be anticipated. In spite of this, the $\longrightarrow H^+/O$ quotients of approaching 4 which were obtained probably reflected an increased use of the terminal electron transport pathway containing cytochrome c, as well as an increase in the efficiency of proton translocation by the NADH dehydrogenase complex.

The advent of a technique for measuring the $\longrightarrow H^+/O$ ratios of cells loaded with specific substrates (Lawford and Haddock, 1973) allows the number of energy coupling sites in the respiratory chain of

a particular bacterium to be easily determined. From measurements of the oxygen consumption rate of the growing organism, Y_{ATP} can then be readily calculated. Using this approach (Chapter IV), values of 13.5 and 10.4 g cells.mol ATP equivalents^{-1} were obtained for B. megaterium strains D440 and M growing in glycerol-limited continuous culture. Recently, I.S. Farmer and C.W. Jones (personal communication) have determined Y_{ATP} for E. coli W growing aerobically in continuous culture using the techniques described in Chapter IV. A value of 10.0 g cells.mol ATP equivalents^{-1} was obtained for glycerol-limited growth, which is close to the values obtained for B. megaterium.

These values are considerably lower than the theoretical maximum value for Y_{ATP} of 28.8 - 31.9 g cells.mol ATP equivalents^{-1} predicted by Gunsalus and Shuster (1961), Forrest and Walker (1971) and Stouthamer (1973). As the amount of energy used for monomer biosynthesis is considered to be quite small , at least when glucose is the carbon source, growth rate-dependent utilisation of energy for processes other than biosynthesis presumably occurs, e.g. transport of nutrients, motility, maintenance of osmotic balances or turnover of macromolecules (Stouthamer and Bettenhaussen, 1975).

The Y_{ATP} of only 2.82 g cells.mol ATP equivalents^{-1} obtained for nitrogen-fixing A. vinelandii (Chapter II) clearly reflects a situation in which considerable energy must be expended in the synthesis of monomers from simple precursors, in this case due to the fixation of atmospheric nitrogen. Under conditions in which there was a supply of combined nitrogen to the cells, the Y_{ATP} was much higher at 6.86 g cells.mol ATP equivalents^{-1}. However, the fact that this figure is still considerably lower than the values quoted above for B. megaterium

and E. coli may indicate that A. vinelandii has to expend more energy than the latter organisms for growth rate-dependent energy-utilising purposes such as those cited above.

Current evidence therefore indicates that Y_{ATP} for aerobic bacteria lies in the approximate range 7-13 g cells.mol ATP equivalents^{-1}. Measurements of Y_{ATP} of other aerobic organisms would be useful since bacteria exhibiting high Y_{ATP} values (and also high efficiencies of respiratory chain energy conservation) would presumably be most economical for the production of single cell protein (see e.g. Kihlberg, 1972) for animal or human consumption. The identification of the energy-dependent processes which cause the experimentally observed Y_{ATP} values to be lower than the theoretical values may also be rewarding in this respect.

REFERENCES

Abbott B.J. & Clamen A. (1973) Biotech. Bioeng., XV, 117.

Ackrell B.A.C., Erickson S.K. & Jones C.W. (1972) Eur.J.Biochem., 26, 387.

Ackrell B.A.C. & Jones C.W. (1971a) Eur.J.Biochem., 20, 22

Ackrell B.A.C. & Jones C.W. (1971b) Eur.J.Biochem., 20, 29

Aleem M.I.H. (1968) Biochim.Biophys.Acta, 162, 338.

Alexander M. & Wilson P.W. (1955) Proc.Nat.Acad.Sci. USA., 41, 843

Arnold B.H. & Steel R. (1958) in "Biochemical Engineering" (Ed. R. Steel) Heywood & Co. Ltd., London p.149

Atkinson D.E. & Walton G.M. (1967) J.Biol.Chem., 242, 3239

Avron M. (1960) Biochim.Biophys.Acta, 40, 257

Baak J.M. & Postma P.W. (1971) FEBS Letters, 19, 189

Bada J.L. & Miller S.L. (1968) Science, N.Y., 159, 423

Baker K. (1968) Lab.Practice, 17, 817

Baltscheffsky H. & Baltscheffsky M. (1974) Ann.Rev.Biochem., 43, 871

Bauchop T. & Elsden S.R. (1960) J.Gen.Microbiol., 23, 457

Benemann J.R., Yoch D.C., Valentine R.C. & Arnon D.I. (1971) Biochim. Biophys.Acta, 226, 205

Bergerson F.J. (1966a) Biochim.Biophys.Acta, 115, 247

Bergerson F.J. (1966b) Biochim.Biophys.Acta, 130, 304

Biggins D.R. & Postgate J.R. (1969) J.Gen.Microbiol., 56, 181

Boyer P.D. (1965) in "Oxidases & Related Redox Systems" (Eds. T.E. King, H.S. Mason & M. Morrison) John Wiley, New York, p. 994

Bragg P.D. & Polglase W.J. (1963) J.Bact., 86, 1236

Brice J.M., Law J.F., Meyer D.J. & Jones C.W. (1974) Biochem.Soc.Trans., 2, 523.

Broberg P.L. & Smith L. (1967) Biochim.Biophys.Acta, 131, 479

Brodie A.F. (1959) J.Biol.Chem., 234, 398

Bronowski J. (1973) "The Ascent of Man", B.B.C. London, p.437

Bulen W.A., Burns R.C. & Le Comte J.R. (1964) Biochem.Biophys.Res. Comm., 17, 265

Bulen W.A., Burns R.C. & Le Comte J.R. (1965) Proc.Nat.Acad.Sci.,USA. 53, 532

Bulen W.A. & Le Comte J.R. (1966) Proc.Nat.Acad.Sci., USA, 56, 979

Bull A.T. (1973) in "Companion to Biochemistry" (Eds. A.T. Bull, J.R. Lagnado, J.O. Thomas, K.F. Tipton)

Burris R.H. (1971) in "The Chemistry and Biochemistry of Nitrogen Fixation" (Ed. J.R. Postgate) Plenum Press, London, p. 106

Carmeli C. (1970) FEBS Letters, 7, 297

Carnahan J.E., Mortenson L.E., Mower H.F. & Castle J.E. (1960) Biochim.Biophys.Acta, 44, 520

Castor L.N. & Chance B. (1959) J.Biol.Chem., 234, 1587

Chance B. & Fugmann U. (1961) Biochem.Biophys.Res.Comm., 4, 317

Chance B., Lee C-P. & Mela L. (1967) Federation Proc., 26, 1341

Chance B. & Williams G.R. (1956) Advan.Enzymol., 17, 65

Chance B., Wilson D.F., Dutton P.L. & Erecinska M. (1970) Proc.Nat.Acad. Sci., USA, 66, 1175

Chappell J.B. (1964) Biochem.J., 90, 225

Chung A.E. (1970) J.Bact., 102, 438

Cockrell R.S., Harris E.J. & Pressman B.C. (1967) Nature, 215, 1487

Daesch G. & Mortenson L.E. (1968) J.Bact., 96, 346

Dalton H. & Mortenson L.E. (1972) Bact.Reviews, 36, 231

Dalton H. & Postgate J.R. (1967) J.Gen.Microbiol., 48, v

Dalton H. & Postgate J.R. (1969a) J.Gen.Microbiol., 54, 463

Dalton H. & Postgate J.R. (1969b) J.Gen.Microbiol., 56, 307

Daniel R.M. (1970) Biochim.Biophys.Acta, 216, 328

Dawes E.A. (1969) "Quantitative Problems in Biochemistry", Livingstone, London, p. 228

Deeb S.S. & Hager L.P. (1964) J.Biol.Chem., 239, 1024

De Ley J. & Park T.W. (1966) Antonie van Leeuwenhoek, 32, 6

Der Vartanian D.V. (1972) Zeitschrift für Naturforshung, 27b, 1080

Dilworth M.J. (1966) Biochim.Biophys.Acta, 127, 285

Doudoroff M. (1966) in "Biochemical Energetics" (Eds. N.O. Kaplan & E.P. Kennedy) Academic Press, New York, p. 385

Drozd J.W. & Postgate J.R. (1970a) J.Gen.Microbiol., 60, 427

Drozd J.W. & Postgate J.R. (1970b) J.Gen.Microbiol., 63, 63

Drozd J.W., Tubb R.S. & Postgate J.R. (1972) J.Gen.Microbiol., 73, 221

Duclaux E. (1898) Traite de microbiologie, 1, 208, Masson, Paris

Eilermann L.J.M., Pandit-Hovenkamp H.G. & Kolk A.H.J. (1970) Biochim. Biophys.Acta, 197, 25

Erickson S.K., Ackrell B.A.C. & Jones C.W. (1972) Biochem.J., 127, 73P

Federov M.F. & Kalininskaya T.A. (1961) Mikrobiol., 30, 9

Forrest W.W. & Walker D.J. (1971) Advan.Microb.Physiol., 5, 213

Gary N.D. & Bard R.C. (1952) J.Bact., 64, 501

Gornall A.G., Bardawill C.S. & David M.M. (1948) J.Biol.Chem., 177, 751

Green D.E., Asai J., Harris R.A. & Penniston J.T. (1968) Arch.Biochem. Biophys., 125, 684

Green D.E. & Baum H. (1970) "Energy & the Mitochondrion", Academic Press, New York, p. 90

Green D.E. & Ji S. (1972) Proc.Nat.Acad.Sci.,USA, 69, 726

Greville G.D. (1969) Curr.Top.Bioenerget., 3, 1

Gunsalus I.C. & Shuster C.W. (1961) in "The Bacteria" Vol. 2 (Eds. I.C. Gunsalus & R.Y. Stanier) Academic Press, New York. p.1

Hadjipetrou L.P., Gerrits J.P., Teulings F.A.G. & Stouthamer A.H. (1964) J.Gen.Microbiol., 36, 139

Hardy R.W.F. & Burns R.C. (1968) Ann.Rev.Biochem., 37, 331

Hardy R.W.F., Burns R.C., Hebert R.R., Holsten R.D. & Jackson E.K. (1972) Plant Physiol., 43, 1185

Harold F.M. (1972) Bacteriol.Rev., 36, 172

Harrison D.E.F. (1973) Crit.Rev.Microbiol., 2, 185

Harrison D.E.F. & Hamer G. (1971) Biochem.J., 124, 78

Harrison D.E.F. & Loveless J.E. (1971) J.Gen.Microbiol., 68, 45

Harrison D.E.F. & Pirt S.J. (1967) J.Gen.Microbiol., 46, 193

Hawker L.E. & Linton A.H. (1971) in "Microorganisms: Function,Form & Environment" Edward Arnold, London, p. 175

Hayes D.K., Schechter M.S., Mensing E. & Horton J. (1968) Anal.Biochem., 26, 51.

Hempfling W.P. (1970) Biochim.Biophys.Acta, 205, 169

Henderson P.J.F. (1971) Ann.Rev.Microbiol., 25, 393

Henderson P.J.F. (1973) Biochem.J., 135, 101

Henderson P.J.F. (1974) in "Comparative Biochemistry & Physiology of Transport" (Eds. L. Bolis, K. Bloch, S.E. Luria & F. Lynen) North Holland, Amsterdam, p. 409

Herbert D. (1958) Rec.Prog.Microbiol., VII Intern.Congr., p. 381

Herbert D., Elsworth R. & Telling R.C. (1956) J.Gen.Microbiol., 14, 601

Hernandez E. & Johnson M.J. (1967) J.Bact., 94, 991

Hill S. (1972) J.Gen.Microbiol., 67, 77

Hill S., Drozd J.W. & Postgate J.R. (1972) J.Appl.Chem.Biotechnol., 22, 541

Hill S. & Postgate J.R. (1969) J.Gen.Microbiol., 58, 277

Hinkle P. & Mitchell P. (1970) J.Bioenerg., 1, 45

Holme T. (1957) Acta Chem.Scand., 11, 763

Horio T., Higashi T., Yamanaka T. & Okunuki K. (1961) J.Biol.,Chem., 236, 944

Hughes D.E. & Wimpenny J.W.T. (1969) Adv.Microb.Physiol., 3, 197

Hyndman L.A., Burris R.H. & Wilson P.W. (1953) J.Bact., 65, 522

Ishikawa S. & Lehninger A.L. (1962) J.Biol.Chem., 237, 2401

Jackson J.B., Crofts A.R. & von Stedingk L-V. (1968) Eur.J.Biochem., 6, 41

Jagendorf A.T. & Uribe E. (1966) Proc.Nat.Acad.Sci.,USA, 55, 170

Jensen H.L. (1965) Amer.Soc.Agron.Monogr., 10, 436

John P. & Whatley F.R. (1970) Biochim.Biophys.Acta, 216, 342

Johnson E.J. & Johnson M.K. (1961) Proc.Soc.Exp.Biol.Med., 108, 728

Jones C.W., Ackrell B.A.C. & Erickson S.K. (1971a) FEBS Letters, 13, 33

Jones C.W., Ackrell B.A.C. & Erickson S.K. (1971b) Biochim.Biophys.Acta, 245, 54

Jones C.W., Ackrell B.A.C. & Erickson S.K. (1972) Biochem.J., 127, 74P

Jones C.W., Brice J.M., Downs A.J. & Drozd J.W. (1975) Eur.J.Biochem.52,265

Jones C.W., Brice J.M., Wright V. & Ackrell B.A.C. (1973) FEBS Letters, 29, 77

Jones C.W. & Redfearn E.R. (1966) Biochim.Biophys.Acta, 113, 467

Jones C.W. & Redfearn E.R. (1967a) Biochim.Biophys.Acta, 143, 340

Jones C.W. & Redfearn E.R. (1967b) Biochim.Biophys.Acta, 143, 354

Jurtshuk P., Bednarz A.J., Zey P. & Denton C.H. (1969) J.Bact., 98, 1120

Kaplan N.O., Colowick S.P., Neufeld E.F. & Ciotti M.M. (1953) J.Biol.Chem., 205, 17

Kashket E.R. & Brodie A.F. (1963) Biochim.Biophys.Acta, 78, 52

Kelly M. (1969) Biochim.Biophys.Acta, 191, 527

Khmel I.A. & Andreeva N.B. (1969) in "Continuous Cultivation of of Microorganisms" (Ed. I. Málek) Czechoslovak Acad.Science, Prague.

Kiesow L. (1964) Proc.Nata.Acad.Sci.,USA, 52, 980

Kihlberg R. (1972) Ann.Rev.Microbiol., 26, 427

Knowles C.J. & Redfearn E.R. (1968) Boichem.Biophys.Acta, 162, 348

Knowles C.J. & Redfearn E.R. (1969) J.Bact., 97, 756

Knowles C.J. & Smith L. (1970) Biochim.Biophys.Acta, 197, 152

Kröger A. & Dadák (1969) Eur.J.Biochem., 11, 328

Lawford H.G. & Haddock B.A. (1973) Biochem.J., 136, 217

Lees H. & Postgate J.R. (1973) J.Gen.Microbiol., 75, 161

Lindsay J.C., Dutton P.L. & Wilson D.F. (1972) Biochemistry, 11, 1937

Lisenkova L.L. & Khmel I.A. (1967) Mikrobiol., 36, 905

Luria S.E. (1960) in "The Bacteria" (Eds. I.C. Gunsalus & R.Y. Stanier Vol. 1, Academic Press, London p.1

L'vov N.P. & Lyubimov V.I. (1965) Izvest.Akad.Nauk.S.S.S.R., Ser.Biol., 30, 250

MacLennan D.G. & Pirt S.J. (1966) J.Gen.Microbiol., 45, 289

McGill D.J. & Dawes E.A. (1971) Biochem.J., 125, 1059

Meyer D.J. & Jones C.W. (1973a) Eur.J.Biochem., 36, 144

Meyer D.J. & Jones C.W. (1973b) FEBS Letters, 33, 101

Mitchell P. (1961) Nature, 191, 144

Mitchell P. (1966) "Chemiosmotic coupling in oxidative and photosynthetic phosphorylation", Glynn Research, Bodmin.

Mitchell P. (1967) Fed.Proc., 26, 1370

Mitchell P. (1968) "Chemiosmotic coupling and energy transduction" Glynn Research, Bodmin.

Mitchell P. (1969) in "The molecular basis of membrant function" (Ed. D.C. Tosteson), Prentice-Hall, Englewood Cliffs, p. 483

Mitchell P. (1970) Symp.Soc.Gen.Microbiol., 20, 121

Mitchell P. (1971) in "Energy transduction in respiration and photosynthesis" (Eds. E. Quagliariello, S. Papa & C.S. Rossi) Adriatica Edritice, Bari, p. 123

Mitchell P. (1972) FEBS Symp., 28, 353

Mitchell P. & Moyle J. (1967a) Biochem.J., 104, 588

Mitchell P. & Moyle J. (1967b) Biochem.J., 105, 1147

Mitchell P. & Moyle J. (1967c) in "Biochemistry of mitochondria" (Eds. E.C. Slater, Z. Kaniuga & L. Wojtczak), Academic Press, New York, p. 53

Mitchell P. & Moyle J. (1968) Eur.J.Biochem., 4, 530

Mitchell P. & Moyle J. (1969) Eur.J.Biochem., 9, 149

Monod J. (1942) Recherches sur la croissance bacteriénnes, Masson, Paris

Mortenson L.E. (1964) Biochim.Biophys.Acta, 81, 473

Mortenson L.E. (1965) in "Non-haeme iron proteins: rôle in energy conversion" (Ed. A. San Pietro) Antioch Press, p. 243

Mortenson L.E. & Wilson P.W. (1954) Arch.Biochem.Biophys., 53, 425

Mortenson L.E. & Wilson P.W. (1955) J.Biol.Chem., 213, 713

Moyle J. & Mitchell P. (1973) FEBS Letters, 30, 317

Murphy P.M. & Koch B.L. (1971) Biochim.Biophys.Acta, 253, 295

Nagai S. & Aiba S. (1972) J.Gen.Microbiol., 73, 531

Nagai S., Nishizawa Y. & Aiba S. (1969) J.Gen.Microbiol., 59, 163

Negelein E. & Gerischer W. (1934) Biochem.Zeitschrift, 268, 1

Neijssel O.M., Hueting S., Crabbendam K.J. & Tempest D.W. (1975) Symp.Soc.Gen.Microbiol.

Niven D.F. & Hamilton W.A. (1974) Biochem.Soc.Trans., 2, 537

Ohnishi T. & Pring M. (1974) in "Dynamics of energy transducing membranes" (Eds. L. Ernster, R.W. Estabrook & E.C. Slater) Elsevier, Amsterdam.

Oppenheim J. & Marcus L. (1970) J.Bact., 101, 286

Orme-Johnson W.H. (1973) Ann.Rev.Biochem., 42, 159

Palmieri F. & Klingenberg M. (1967) Eur.J.Biochem., 1, 439

Payne W.J. (1970) Ann.Rev.Microbiol., 24, 17

Pinchot G.B. (1953) J.Biol.Chem., 205, 65

Pirt S.J. (1965) Proc.Roy.Soc.Lond., 163B, 224

Postgate J.R. (Ed.) (1971) "The Chemistry and biochemistry of nitrogen fixation" Plenum Press, London

Pressman B.C. (1967) in "Methods in Enzymology" Vol.X (Eds. R.W. Estabrook & M.E. Pullman) Academic Press, New York, p. 714

Reid R.A., Mitchell P. & Moyle J. (1966) Nature, 212, 257

Reid R.A. (1970) Biochem.J., 116, 12P

Scholes P. & Mitchell P. (1970) J.Bioenerg., 1, 309

Scholes P., Mitchell P. & Moyle J. (1969) Eur.J.Biochem., 8, 450

Segal I.H. (1968) "Biochemical Calculations" John Wiley, New York

Siegell S.D. & Gaden E.L. (1962) Biotech.Bioeng., 4, 435

Silver W.S. & Postgate J.R. (1973) J.Theor.Biol., 40, 1

Slater E.C. (1953) Nature, 172, 975

Slater E.C. (1967) Eur.J.Biochem., 1, 317

Slater E.C. (1971) Quart.Rev.Biophys., 4, 35

Slater E.C. (1972) in "Mitochondria: biogenesis and bioenergetics" (Eds. S.G. Van den Bergh, P. Borst & E.C. Slater) North Holland, Amsterdam, p. 133

Smalley A.J., Jahrling P. & van Demark P.J. (1968) J.Bact., 96, 1595

Smith L. (1954) Arch.Biochem.Biophys., 50, 299

Stewart W.D.P., Haystead A. & Pearson H.W. (1969) Nature, 224, 226

Stouthamer A.H. (1969) in "Methods in microbiology", Vol. 1 (Eds. J.R. Norris & D.W. Ribbons) Academic Press, New York, p. 629

Stouthamer A.H. (1973) Antonie van Leeuwenhoek, 39, 545

Stouthamer A.H. & Bettenhaussen C. (1972) Antonie van Leeuwenhoek, 38, 81

Stouthamer A.H. & Bettenhaussen C. (1973) Biochim.Biophys.Acta, 301, 53

Stouthamer A.H. & Bettenhaussen C. (1975) Arch.Microbiol., 102, 187

Swank R.T. & Burris R.H. (1969) J.Bact., 98, 311

Temperli A. & Wilson P.W. (1960) Biochem.Zeitschrift, 320, 195

Tempest D.W. & Dicks J.W. (1967) in "Microbial physiology and continuous culture" (Eds. E.O.Powell, C.G.T.Evans, R.E.Strange & D.W.Tempest) H.M.S.O. p.140

Tempest D.W., Herbert D. & Phipps P.J. (1967) Ibid., p.248

Thayer W.S. & Hinkle P.C. (1973) J.Biol.Chem., 248, 5395

Tissières A. (1956) Biochem.J., 64, 582

Tissières A. & Slater E.C. (1955) Nature, 176, 736

Uribe E.G. & Li E. (1973) J.Bioenerg., 4, 435

van den Broek H.W.J., Santema J.S., Wassink J.H. & Veeger C. (1971a) Eur.J.Biochem., 24, 31

van den Broek H.W.J., van Breemen J.F.L., van Bruggen E.F.J. & Veeger C. (1971b) Eur.J.Biochem., 24, 46

van den Broek H.W.J. & Veeger C. (1968) FEBS Letters, 1, 301

van der Beek E.G. & Stouthamer A.H. (1973) Arch.Microbiol., 89, 327

Van Gelder B.F. & Muijsers A.O. (1966) Biochim.Biophys.Acta, 118, 36

Vanneste W.H. & Vanneste M.T. (1965) Biochem.Biophys.Res.Comm., 19, 182

Varley M.E. (Ed.) (1974) in "Energy flow through ecosystems", Unit 1, Open University Press, Milton Keynes, p. 27

Vernon L.P. & Mangum J.H. (1960) Arch.Biochem.Biophys., 90, 103

de Vries W., Kapteyn W.M.C., van der Beek E.G. & Stouthamer A.H. (1970) J.Gen.Microbiol., 63, 333.

Walker D.J. (1968) App.Microbiol., 16, 1672

Webb J.L. (1963) in "Enzyme & Metabolic Inhibitors" Vol. 1, Academic Press, London, p. 153

Weibull C. & Bergström L. (1958) Biochim.Biophys.Acta, 30, 340

West I. & Mitchell P. (1972) J.Bioenerg., 3, 445

West I. & Mitchell P. (1974) FEBS Letters, 40, 1

Whitaker A.M. & Elsden S.R. (1963) J.Gen.Microbiol., 31, xxii

Wilkinson J.F. & Monro A.L.S. (1967) in "Microbial physiology and continuous culture" (Eds. E.O. Powell, C.G.T. Evans, R.E. Strange & D.W. Tempest) H.M.S.O., p. 173

Wilkström M.K.F. (1973) Biochim.Biophys.Acta, 301, 155

Wilkström M.K.F. & Saris N.E.L. (1970) in "Electron transport and energy conservation" (Eds. E. Quagliariello & E.C. Slater) Adriatica Editrice, Bari, p. 77

Williams A.M. & Wilson P.W. (1954) J.Bact., 67, 353

Wilson D.F. (1974) Ann.Rev.Biophys.Bioeng., 3, 203

Wilson D.F. & Dutton P.L. (1970) Arch.Biochem.Biophys., 136, 583

Wilson P.W. (1958) in "Encyclopaedia of plant physiology" (Ed. W. Ruhland), Springer, Berlin. p.9

Wilson P.W., Hull J.F. & Burris R.H. (1943) Proc.Nat.Acad.Sci.,USA, 29 289

Wimpenny J.W.T. (1969) Symp.Soc.Gen.Microbiol., 19, 161

Yates M.G. (1970) J.Gen.Microbiol., 60, 393

Yates M.G. & Daniel R.M. (1970) Biochim.Biophys.Acta, 197, 161

Yates M.G. & Jones C.W. (1974) Adv.Microbial Physiol., 10, 97.

A.J. DOWNS. Ph.D. Thesis. 1975.

RESPIRATION AND ENERGY CONSERVATION IN BACTERIA

Summary of a thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Leicester by A.J.Downs, B.Sc.

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1. The characteristics of the respiratory system of the aerobic, nitrogen-fixing bacterium A.vinelandii were very similar during growth on a medium containing combined nitrogen (urea) compared with growth on nitrogen-free medium (nitrogen-fixing conditions; see Yates and Jones,1974). The growth yield of the organism with respect to ATP (Y_{ATP}, g cells. mol ATP equivalents^{-1}) was much higher during growth on combined nitrogen medium than during growth on nitrogen-free medium, probably due to the energy requirements of nitrogen fixation. In spite of its outstanding adaptations for growth in aerobic, nitrogen-deficient environments, A.vinelandii shows a poor ability to modify its respiratory system for efficient growth on a source of combined nitrogen.

2. Measurements of $\rightarrow H^+/O$ quotients (Mitchell,1966) on batch-grown B.megaterium strains D440 and M suggested the presence of two functional proton-translocating loops in the respiratory chains of these organisms. Substrate-loading experiments (Lawford and Haddock, 1973) demonstrated the presence of proton-translocating loops 1&2 in strain D440 and 2&3 in strain M. The involvement of loop 3 in strain D440 was probably precluded by the absence of cytochrome c from the respiratory chain of this organism. Measurements of the in situ respiratory activity of chemostat cultures of B.megaterium allowed calculation of Y_{ATP} and M

(maintenance coefficient, mol ATP equivalents. g cells^{-1}. h^{-1}) during growth under various culture conditions. Y_{ATP} values of 13.1 and 10.4 g cells. mol ATP equivalents^{-1} were obtained for strains D440 and M respectively during glycerol-limited growth. Similar values were obtained during NH_4Cl-limited and oxygen-limited growth. These values are considerably lower than the theoretical maximum value for Y_{ATP} (Forrest and Walker, 1971). Possible reasons for this are discussed.

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Table 1. *Energy conservation properties of E. coli W and K. pneumoniae*

Results are expressed as the average value ±S.E.M. with the number of determinations in parentheses.

Growth conditions	Fraction	Parameter measured	*E. coli* W			*K. pneumoniae*		
Aerobic, exponential	Whole cells	$\rightarrow H^+/O$ ratio (g-equiv. of $H^+ \cdot$ g-atom of O^{-1})	4.00	±0.07	(7)	3.92	±0.22	(7)
		μ (h^{-1})	0.61	±0.02	(7)	1.00	±0.05	(9)
		Q_{O_2} (mol of $O_2 \cdot h^{-1} \cdot$ g of $cell^{-1}$)	0.0106	±0.0006	(5)	0.0145	±0.0010	(9)
		M (mol of ATP equiv. $\cdot h^{-1} \cdot$ g of $cells^{-1}$)	0.0103			0.0006		
		Y_{ATP} (g of cell $\cdot$ mol of ATP equiv.$^{-1}$)	19.0			17.8		
	Respiratory membranes	P/O (NADH)	0.59	±0.09	(3)	0.41	±0.06	(3)
Aerobic, oxygen-limited	Whole cells	$\rightarrow H^+/O$ ratio	2.59	±0.27	(5)	2.97	±0.07	(4)
	Respiratory membranes	P/O (NADH)	0.20	±0.05	(3)	0.21	±0.04	(3)
Anaerobic+nitrate	Whole cells	$\rightarrow H^+$/nitrate ratio	4.20	±0.14	(3)	3.85	±0.48	(4)
Anaerobic+fumarate	Whole cells	$\rightarrow H^+$/fumarate ratio	1.15	±0.16	(4)	1.13	±0.16	(3)

from proton translocation via loops 1 and 2 only (see Mitchell, 1966); the absence of cytochrome *c* from these organisms probably precludes loop 3. The lower $\rightarrow H^+/O$ ratios observed during O_2-limited growth suggest that cytochrome oxidases a_1 and/or *d* react with O_2 in such a way as to bypass loop 2. These conclusions are supported by $\rightarrow H^+$/nitrate ratios of approx. 4 (P/O = 2) for cells cultured anaerobically on glycerol plus nitrate (Table 1; see also Stouthamer & Bettenhausen, 1973) and by the substrate-loading experiments of Lawford & Haddock (1973). The $\rightarrow H^+$/fumarate ratios of approx. 1 (P/O = 0.5; Table 1) which were observed with cells cultured anaerobically on glycerol plus fumarate were lower than expected and may reflect either the inward retranslocation of a proton in association with entry of a fumarate molecule or an altered endogenous substrate composition. Proton translocation with all three electron acceptors was abolished by 15 μM-tetrachlorosalicylanilide.

According to Harrison & Loveless (1971), for energy-limited, aerobic cultures:

$$Q_{O_2} = \frac{\mu}{Y_{ATP} \cdot N} + \frac{M}{N}$$

where Q_{O_2} is the respiratory activity (mol of $O_2 \cdot h^{-1} \cdot g$ of cells^{-1}) and Y_{ATP} is the ATP yield constant (g of cells·mol of ATP^{-1}) or, more precisely, g of cells·mol of ATP equiv.$^{-1}$). By using values of *M* calculated from data of the type contained in Fig. 1(*b*) and values of Q_{O_2} determined for cultures growing exponentially in excess of oxygen (Table 1), *E. coli* and *K. pneumoniae* yield Y_{ATP} values (uncorrected for substrate-level phosphorylation) of 19.0 and 17.8 g of cells·mol of ATP equiv.$^{-1}$ respectively.

Ackrell, B. A. C. & Jones, C. W. (1971*a*) *Eur. J. Biochem.* **20**, 22–28
Ackrell, B. A. C. & Jones, C. W. (1971*b*) *Eur. J. Biochem.* **20**, 29–35
Harrison, D. E. F. & Loveless, J. E. (1971) *J. Gen. Microbiol.* **68**, 35–43
Hughes, D. E. & Wimpenny, J. W. T. (1969) *Advan. Microbial Physiol.* **3**, 197–232
Lawford, H. G. & Haddock, B. A. (1973) *Biochem. J.* **136**, 217–220
Meyer, D. J. & Jones, C. W. (1973) *Eur. J. Biochem.* **36**, 144–151
Mitchell, P. (1966) *Biol. Rev. Cambridge Phil. Soc.* **41**, 445–502
Mitchell, P. & Moyle, J. (1967) *Biochem. J.* **104**, 588–600
Stouthamer, A. H. & Bettenhausen, C. (1973) *Biochim. Biophys. Acta* **301**, 53–70
West, I. & Mitchell, P. (1972) *Bioenergetics* **3**, 445–462
Wimpenny, J. W. T. (1969) in *Microbial Growth* (Meadow, P. & Pirt, S. J., eds.), pp. 161–197, Cambridge University Press, Cambridge

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Energy Conservation in *Bacillus megaterium*

ANDREW J. DOWNS and COLIN W. JONES

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.

Bacillus megaterium is a Gram-positive aerobe whose respiratory system is characterized by the presence of menaquinone (Bishop *et al.*, 1962) cytochrome *b* and cytochrome oxidases aa_3 and *o* (Kroger & Dadak, 1969).

Batch cultures of *B. megaterium* (D440) were grown under high aeration conditions in a glycerol (66mM)–mineral salts medium. Cells were harvested, prepared and assayed for measurement of $\rightarrow H^+/O$ ratios (Mitchell & Moyle, 1967) essentially as described by Meyer & Jones (1973). In the presence of 30mM-potassium thiocyanate, the $\rightarrow H^+/O$ ratio was 4.06±0.10 (S.E.M.; nine determinations).

The organism was also cultured under glycerol-limited conditions in a chemostat of 200ml capacity, similar to that described by Baker (1968), connected to a gaseous O_2 analyser containing a YS14004 Clark electrode (Yellow Springs Instruments Company, Yellow Springs, Ohio, U.S.A.). The analyser was of simple design and worked extremely well for the assay of respiratory activity *in situ*. The cost of the apparatus compared favourably with other equipment containing paramagnetic analysers (e.g. Harrison &

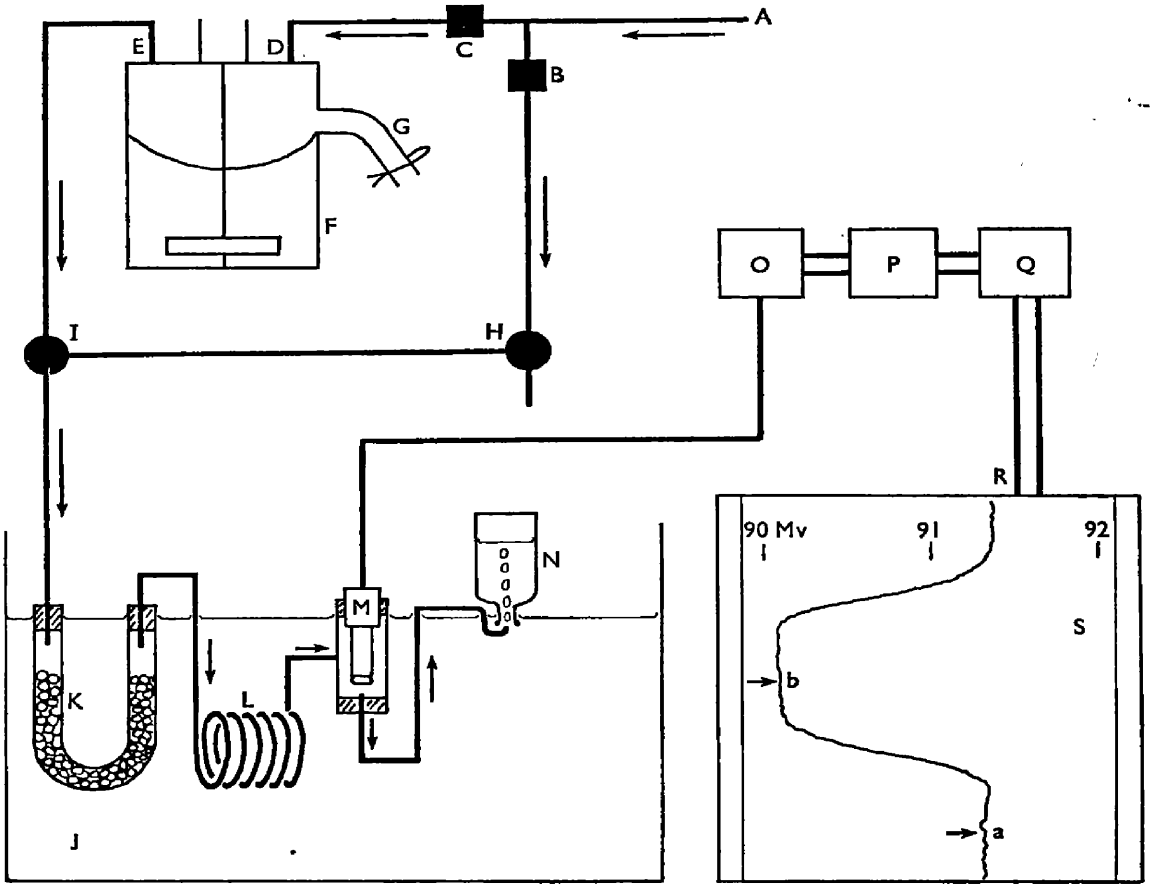

Fig. 1. *Schematic diagram of the equipment used for measuring the respiratory activity of B. megaterium chemostat cultures in situ*

A, air supply; B and C, air-flow regulators; D and E, entry and exit ports for chemostat air; F, chemostat vessel; G, culture overflow clip; H and I, two-way taps; J, constant-temperature-water bath (±0.05°C); K, gas drier (silica gel); L, temperature equilibrator; M, O_2-electrode; N, flow-rate measurer; O and P, amplifiers; Q, back-off circuit; R, chart recorder; S, chart with typical trace (a, taps H and I switched from input to effluent air; b, the reverse); → direction of air flow.

Pirt, 1967; Senior *et al.*, 1972) and Mackereth Oxygen probes (e.g. Harrison & Loveless, 1971).

The Clark electrode measured alternately the O_2 partial pressure of the input and effluent chemostat air (see Fig. 1). Differences in O_2 partial pressure (equivalent to O_2 concentration) could be detected down to $\leqslant 0.04\%$ O_2 concentration by means of an amplifier and a back-off circuit, as described by Hayes *et al.* (1968). During operation of the analyser, however, the air flow rate through the chemostat, determined gravimetrically by displacement of water, was always adjusted so that changes of $\leqslant 0.2\%$ O_2 concentration were realized on the chart recorder. The full-scale deflexion of the recorder corresponded to a difference in O_2 concn. of 0.454% with the back-off voltage set at 90.0mV and the recorder on the 2mV range. The noise level was equivalent to less than 0.015% O_2 concentration.

The respiratory activity of the bacteria in the chemostat was calculated from the equation:

$$Q_{O_2} = 20.90 - (A/B \times 20.90) \times C/D \times 4.46 \times 10^{-4} \quad (1)$$

where Q_{O_2} = respiratory activity (mol of O_2 consumed $\cdot h^{-1} \cdot$ g dry wt. of bacteria^{-1});

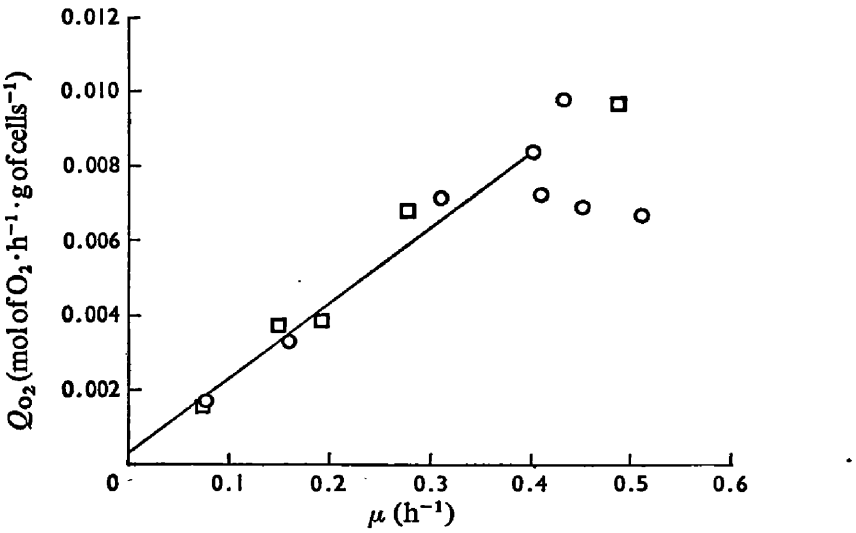

Fig. 2. *The respiratory activity (Q_{O_2}) measured in situ as a function of specific growth rate (μ) of glycerol-limited chemostat cultures of B. megaterium under two aeration conditions*

□, High aeration by means of loose magnetic-follower; ○, extra-high aeration by means of large, captive magnetic-follower. Glycerol concentration of medium supplied to chemostat = 1.3 mM.

A = electrode output due to effluent air (mV); B = electrode output due to input air (mV); C = gas flow rate (litre·h^{-1}); and D = dry wt. of bacteria in chemostat vessel (g). The O_2 concentration of fresh air was taken to be 20.90% (Hayes *et al.*, 1968).

According to Harrison & Loveless (1971) for aerobically growing bacteria whose growth is energy-limited:

$$Q_{O_2} = \mu / Y_{ATP \cdot N} + M/N$$

where μ = specific growth rate (h^{-1}), Y_{ATP} = cell-yield coefficient for ATP = $Y_{ATP}^{max.}$ of Stouthamer & Bettenhausen (1973) (g of cells·mol of ATP equiv.$^{-1}$), N = mol of ATP equiv. produced·mol of O_2 consumed^{-1} (i.e. P/O×2), M = maintenance coefficient (mol of ATP equiv.·g of cells^{-1}·h^{-1}). Fig. 2 shows a graph of Q_{O_2} against μ for glycerol-limited *B. megaterium* growing under two different aeration conditions. The cells were never allowed to become O_2-limited. A straight-line relationship between Q_{O_2} and μ was evident except at fast dilution rates ($\mu > 0.42 h^{-1}$) where there was some deviation ($\mu_{max.} = 0.63 h^{-1}$). From Fig. 2 the slope ($1/Y_{ATP \cdot N}$) was 0.0208 and the intercept (M/N) was 0.00030. Assuming that the $\rightarrow H^+/O$ ratio is equivalent to N, Y_{ATP} is 11.8 g of cells·mol of ATP equiv.$^{-1}$ and M is 0.00122 mol of ATP equiv.·g of cells^{-1}·h^{-1} (both uncorrected for substrate-level phosphorylation).

Difference spectra of isolated respiratory membranes (see Ackrell & Jones, 1971) confirmed the absence of cytochrome *b* and indicated that the concentrations of cytochrome oxidases a_3 and *o* were 0.34 and 0.05 nmol·nmol of cytochrome b^{-1} respectively.

Thus, in spite of the fact that cytochrome a_3 is the major terminal oxidase, $\rightarrow H^+/O$ ratios indicate the presence of only two proton-translocating loops (see Mitchell, 1966); it is not clear at the moment which of loops 0, 1 and 2 are responsible. However, as is the case in *Escherichia coli* and *Klebsiella pneumoniae* (Brice *et al.*, 1974), loop 3 is probably precluded by the absence of cytochrome *c*.

Ackrell, B. A. C. & Jones, C. W. (1971) *Eur. J. Biochem.* **20**, 29–35
Baker, K. (1968) *Lab. Pract.* **17**, 817–824
Bishop, D. H. L., Pandya, K. P. & King, H. K. (1962) *Biochem. J.* **83**, 606–614
Brice, J. M., Law, J. F., Meyer, D. J. & Jones, C. W. (1974) *Biochem. Soc. Trans.* **2**, 523–526
Harrison, D. E. F. & Loveless, J. E. (1971) *J. Gen. Microbiol.* **68**, 35–43
Harrison, D. E. F. & Pirt, S. J. (1967) *J. Gen. Microbiol.* **46**, 193–211

Hayes, D. K., Schechter, M. S., Mensing, E. & Horton, J. (1968) *Anal. Biochem.* **26**, 51–60
Kroger, A. & Dadak, V. (1969) *Eur. J. Biochem.* **11**, 328–340
Meyer, D. J. & Jones, C. W. (1973) *Eur. J. Biochem.* **36**, 144–151
Mitchell, P. (1966) *Biol. Rev. Cambridge Phil. Soc.* **41**, 445–502
Mitchell, P. & Moyle, J. (1967) *Biochem. J.* **104**, 588–600
Senior, P. J., Beech, G. A., Ritchie, G. A. F. & Dawes, E. A. (1972) *Biochem. J.* **128**, 1193–1201
Stouthamer, A. H. & Bettenhausen, C. (1973) *Biochim. Biophys. Acta* **301**, 53–70

Oxidative Phosphorylation in *Hydrogenomonas eutropha* H 16 Grown with and without Iron

JAN W. DROZD and COLIN W. JONES

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.

The obligately aerobic facultative autotroph *Hydrogenomonas eutropha* H 16 was grown heterotrophically (batch or continuous culture) or autotrophically (batch culture) at 30°C. The minimal salts medium (Schlegel *et al.*, 1961) was supplemented with 55mM-fructose (heterotrophic growth) or was under a gas atmosphere of $H_2+O_2+CO_2$ (7:2:1) (autotrophic growth). The chemostat was of 0.5 litre working volume and was based on the design of Baker (1968). The mainly glass and polytetrafluoroethylene construction minimized contamination of the culture or medium by metals. Whole cell $\rightarrow H^+/O$ ratios (see Mitchell & Moyle, 1967), respiratory activities (Q_{O_2}), and growth efficiencies with respect to O_2 (Y_{O_2}) were analysed essentially as described by Meyer & Jones (1973). Respiratory membranes were assayed for cytochrome content essentially as described by Ackrell & Jones (1971).

Heterotrophically grown cells from batch cultures supplemented with iron citrate (20μM-Fe) exhibited $\rightarrow H^+/O$ ratios approaching 8 (equivalent to a P/O ratio of 4, assuming that $\rightarrow H^+/P = 2$) for the oxidation of endogenous substrates or when suspended, after preincubation in approx. 55mM-fructose (Table 1). Cells suspended, after preincubation, in approx. 55mM-succinate exhibited $\rightarrow H^+/O$ ratios approaching 5 (equivalent to a P/O ratio of 2.5). Autotrophically grown cells also exhibited $\rightarrow H^+/O$ ratios approaching 8 for the oxidation of endogenous substrates (Table 1); this is near the value of 7.88 reported by Beatrice & Chappell (1974). A plot of Y_{O_2} (observed)$^{-1}$ against specific growth rate $(\mu)^{-1}$ (see Meyer & Jones, 1973) was a straight line and yielded a Y_{O_2} (true)

Table 1. *$\rightarrow H^+/O$ ratios for H. eutropha H 16 grown with and without added iron*

Each value is given ±S.E.M. with the number of observations in parentheses.

Growth conditions	Substrate added	Extrapolated $\rightarrow H^+/O$ ratio (g-equiv. of $H^+ \cdot$g-atom of O^{-1})
+Fe		
Autotrophic, batch culture	—	7.5 (1)
Heterotrophic, batch culture	—	7.7 ± 0.3 (6)
	Fructose	7.0 (1)
	Succinate	4.7 ± 0.2 (3)
−Fe		
Heterotrophic, batch culture	—	6.2 ± 0.2 (5)
	Fructose	6.1 ± 0.3 (3)
	Succinate	4.1 ± 0.3 (2)
Heterotrophic, continuous culture	—	3.1 ± 0.3 (5)
	Fructose	3.3 ± 0.7 (2)
	Succinate	3.1 (1)

Table 2. *Growth parameters for H. eutropha H 16 grown heterotrophically in batch culture with and without iron*

Growth conditions	Y_{O_2} (true) (g of cells·mol of O_2^{-1})	M (mol of ATP·h^{-1}·g of cells^{-1})	Q_{O_2} (mol of O_2·h^{-1}·g of cells^{-1})
+Fe	100	0.0184	0.0054 ($\mu = 0.21\,h^{-1}$)
−Fe	—	—	0.0116 ($\mu = 0.28\,h^{-1}$)

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Energy Conservation in *Bacillus megaterium*

ANDREW J. DOWNS and COLIN W. JONES

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester

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Abstract. 1. The respiratory chain energy conservation systems of *Bacillus megaterium* strains D440 and M have been investigated following growth in batch and continuous culture. Respiratory membranes from these strains contained cytochromes *b*, aa_3, *o* and *b*, *c*, *a*, *o*, respectively; both readily oxidised NADH but neither showed any pyridine nucleotide transhydrogenase activity.

2. Whole cells of both strains exhibited endogenous $\rightarrow H^+/O$ ratios of approximately 4; when loaded with specific substrates the resultant $\rightarrow H^+/O$ ratios indicated that proton translocating loops 1 and 2 were present in strain D440 and that loops 2 and 3 were present in strain M.

3. *In situ* respiratory activities were measured as a function of dilution rate during growth in continuous culture. True molar growth yields with respect to oxygen (Y_{O_2}) of approximately 50 g cells · mole oxygen^{-1} were obtained for most of the nutrient limitations employed. Average values for Y_{ATP} of 12.7 and 10.8 g cells · mole ATP equivalents^{-1} were subsequently calculated for strains D440 and M respectively.

4. Energy requirements for maintenance purposes were low in energy-limited cultures but were substantially increased when growth was limited by nitrogen source (NH_4^+). Under the latter conditions there is probably a partial uncoupling of energy-conserving and energy-utilising processes leading to energy wastage.

Key words: Respiratory Chain – Energy Conservation – Proton Translocation – Molar Growth Yields – *Bacillus megaterium.*

Earlier studies of the Gram-positive bacterium *Bacillus megaterium* have indicated that it contains a highly active respiratory system of relatively simple composition in which slight interstrain differences in cytochrome content may occur (Weibull and Bergström, 1958; Vernon and Mangum, 1960; Broberg and Smith, 1967; Kröger and Dadák, 1969). The organism grows readily under aerobic conditions in a minimal salts medium supplemented with a simple carbon source and would thus appear to be an excellent system in which to investigate the efficiency of bacterial respiratory chain energy conservation and the energetics of bacterial growth. The former is amenable to investigation through the measurement of respiration-linked proton translocation (Mitchell, 1966) and the latter, most simply, by determination of molar growth yields with respect to molecular oxygen through the measurement of *in situ* respiratory activities of growing cultures (Harrison and Loveless, 1971; Downs and Jones, 1974).

This paper reports the results of such experiments using *B. megaterium* strains D440 and M.

Materials and Methods

Culture of Organisms. Bacillus megaterium strains D440 and M were the gifts of the Medical Research Council Unit for Microbial Systematics, University of Leicester, England, and Dr. C. Weibull, University of Stockholm, Sweden, respectively.

Batch Culture. Organisms were cultured on a minimal salts medium (Sundaram, 1973) supplemented with 66 mM glycerol. 600 ml (high aeration) or 1500 ml (low aeration) batches of medium were inoculated and shaken at 30°C in 2 l baffled flasks at approximately 200 oscillations · min^{-1} in a gyratory incubator. High aeration cultures were harvested in the mid-logarithmic, excess oxygen phase of growth ($A_{680\,nm} \simeq 2$) and low aeration cultures were harvested after overnight growth into oxygen-limitation.

Continuous Culture. Organisms were cultured in a chemostat of 200 ml capacity (Baker, 1968) equipped with temperature control (30°C) and with facilities for measuring the concentration of dissolved oxygen in the culture medium and of gaseous oxygen in the inflowing and effluent air (Downs and Jones, 1974). No pH control was necessary since the low cell densities employed enabled the buffering capacity of the growth medium to maintain the pH at 7.1 ± 0.1. The culture was aerated by means of a captive magnetic flea controlled by a magnetic stirrer. For oxygen-limited growth the culture was stirred slowly and the K_LA (oxygen transfer coefficient) was 4.9×10^{-6} mole $O_2 \cdot hr^{-1} \cdot l^{-1} \cdot mm\,Hg^{-1}$; for non-oxygen-limited growth K_LA was 55.0×10^{-6} mole $O_2 \cdot hr^{-1} \cdot l^{-1} \cdot mm\,Hg^{-1}$ (for determination of K_LA see Harrison and Pirt, 1967). The culture medium was identical to that used for batch culture with the following modifications: *B. megaterium* strain D440, glycerol-limited (7.6 mM glycerol), oxygen-limited (33.0 mM glycerol), ammonium-limited (0.38 mM NH_4Cl); *B. megaterium* strain M, glycerol-limited (5.4 mM glycerol), oxygen-limited (33 mM glycerol), ammonium-limited (0.38 mM NH_4Cl).

Non-Standard Abbreviation. TMPD = N,N,N′,N′-tetramethyl-*p*-phenylenediamine dihydrochloride.

Whole Cell Assays. → *H^+/O ratios:* → H^+/O ratios (g ion H^+ translocated · g atom O consumed^{-1}) of whole cell suspensions were assayed at 30° C after the method of Mitchell and Moyle (1967) as described by Jones *et al.* (1975). Optimum assay conditions for *B. megaterium* were as follows: cell density 6 mg · ml^{-1}, pH 6.6–6.8, 150 mM KSCN – 140 mM KCl – 1 mM Tris/HCl, 0.5 mg · ml^{-1} carbonic anhydrase. To determine the → H^+/O ratios of cells loaded with specific substrates starved cells were first prepared by growing overnight in high aeration batch culture in medium containing reduced concentrations of carbon source (13.0 mM glycerol or 5.8 mM sodium succinate). This procedure was necessary because the conventional method introduced by Lawford and Haddock (1973) yielded insufficiently starved cells. The cells thus produced were then prepared by standard procedures (Jones *et al.*, 1975) and subsequently incubated with 20 mM glycerol or glutamate, 10 mM succinate or malate or 12 mM ascorbate *plus* 7.6 mM TMPD prior to measurement of respiration linked proton translocation as described above.

Respiratory activity (QO_2); *harvested cells:* Respiratory activities of whole cells ± added substrates were assayed at 30° C using a Clark oxygen electrode as described previously (Jones *et al.*, 1975).

In situ chemostat cultures: In situ respiratory activities of chemostat cultures were determined over a range of dilution rates by analysis of the inflowing and effluent air using a Clark oxygen electrode mounted in the air stream (for full details see Downs and Jones, 1974). The activities were fully corrected for deviation from standard temperature, for the influence of water vapour pressure and for the influence of the medium flow rate on the measured air flow rate.

Respiratory Membrane Assays. Respiratory membranes were prepared by the method of Meyer and Jones (1973) and assayed for cytochrome content and respiratory activitiy as described by Ackrell and Jones (1971).

Results

Respiratory membranes prepared from high aeration batch cultures of *B. megaterium* D440 harvested during the late logarithmic phase of growth contained cytochromes *b*, *aa*$_3$ and *o*, of which reduced cytochromes a_3 and *o* combined with carbon monoxide (to yield a wide band with a peak at 425 nm) and hence probably act as terminal oxidases (Fig. 1a and b, Table 1). As expected by analogy with other bacteria (Ackrell and Jones, 1971), the total cytochrome content of these membranes increased considerably when oxygen became the growth-limiting nutrient but the relative concentrations of the individual cytochromes were essentially unaltered. In contrast, respiratory membranes prepared from logarithmic phase cultures of strain M contained cytochromes *b*, *c*, *a*, and *o*. No cytochrome a_3 could be detected in dithionite-reduced + CO *minus* dithionite-reduced difference spectra, where the symmetrical band with a peak at 413 nm and a trough at 430 nm reflected solely cytochrome *o*. However, there was clear evidence from the dithionite-reduced *minus* oxidised spectrum for the presence of cytochrome *a* (603 nm) and for a high concentration of cytochrome *c* (Fig. 2a and b,

Table 1. Oxidase activities and cytochrome content of respiratory membranes prepared from *B. megaterium* strains D440 and M. *B. megaterium* was grown in batch culture at high or low aeration on a glycerol-minimal salts medium. Respiratory membranes were prepared and assayed as described in the "Materials and Methods" section

Strain	Growth conditions	Oxidase activities					Cytochrome concentration							
		NADH	NADPH	NADPH + NAD^+	Malate	Asc-TMPD	*b*	*c*	*a*	a_3	*o*	a_3	*o*	a_3/a ratio
		ngatom O · min^{-1} · mg protein^{-1}					nmoles · g protein^{-1}					% total oxidase		
D440	High aeration excess oxygen	200	0	0	43	104	128	–	42	69	33	68.6	31.4	1.64
	Low aeration; oxygen limited	152	0	0	30	257	211	–	68	120	57	67.8	32.2	1.77
M	High aeration excess oxygen	47	29	29	20	1085	422	700	159	–	167		100	
	Low aeration; oxygen limited	62	62	34	116	1483	581	1032	175	–	341		100	

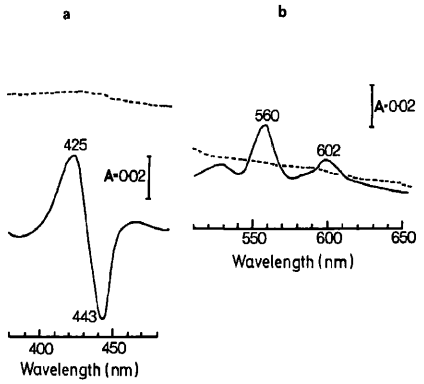

Fig. 1a and b. Difference spectra of respiratory membranes prepared from *B. megaterium* strain D440 grown in high aeration batch culture. (a) Reduced *minus* reduced (——), reduced + CO *minus* reduced (——). (b) Oxidised *minus* oxidised (----), reduced *minus* oxidised (——). The reductant was sodium dithionite; where appropriate, samples were exposed to a steady stream of carbon monoxide for 2 min. 8.3 mg protein · ml^{-1}

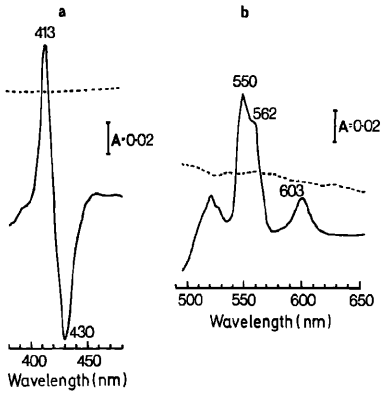

Fig. 2a and b. Difference spectra of respiratory membranes prepared from *B. megaterium* strain M grown in high aeration batch culture. (a) Reduced *minus* reduced (----), reduced + CO *minus* reduced (——), 3.2 mg protein · ml^{-1}. (b) Oxidised *minus* oxidised (----), reduced *minus* oxidised (——), 6.4 mg protein · ml^{-1}. The reductant was sodium dithionite; where appropriate, samples were exposed to a steady stream of carbon monoxide for 2 min

Table 1). The cytochrome content of strain M also increased under conditions of oxygen-limitation, but the concentrations of cytochromes *b*, *c* and *o* increased considerably more than cytochrome *a*. During both excess-oxygen and oxygen-limited growth, the cytochrome content of respiratory membranes from strain M were considerably higher, on a protein basis, than similar membranes from strain D440.

Respiratory membranes prepared from *B. megaterium* D440 cultured under conditions of excess- or limiting-oxygen readily oxidised NADH but showed no NADPH oxidase activity ($\pm$ NAD^+); slight oxidase activity was observed with succinate, malate and α-glycerophosphate (Table 1). Membranes prepared from strain M cultured under excess-oxygen conditions showed similar properties and, in addition, oxidised NADPH at a significant rate. However, membranes from oxygen-limited cells of strain M oxidised malate at a considerably faster rate than NADH, presumably via a particulate flavin-linked malate oxidase. Examination of the high speed supernatant fractions prepared from these two strains indicated the presence of soluble NAD^+-linked malate and glutamate dehydrogenases under all growth conditions. Neither strain exhibited any particulate or soluble pyridine nucleotide transhydrogenase activities (NADPH → NAD^+) as evidenced by the failure of NAD^+ to stimulate NADPH oxidation.

A detailed investigation of the enzyme kinetics of ascorbate-TMPD oxidation confirmed the data in Table 1, *viz.* that respiratory membranes from strain M oxidised reduced TMPD at a considerably faster rate than did membranes from strain D440 (V_{max} 1230 *cf.* 130 ngatom O · min^{-1} · mg $protein^{-1}$) and further indicated that they also exhibited a significantly greater affinity for the reducing substrate (K_m 0.104 *cf.* 0.439 mM TMPD). Since ascorbate-TMPD donates electrons preferentially to endogenous cytochrome *c*, these results reflect the higher concentrations of cytochrome *c* and cytochrome oxidase in strain M *cf.* D440 (see Table 1).

Washed whole cell suspensions of *B. megaterium* strains D440 and M exhibited → H^+/O ratios of approximately 4 for the oxidation of endogenous substrates (Table 2). Following starvation, the → H^+/O ratio of strain D440 decreased to 2.83; this value was further decreased by the addition of the flavin-linked substrates glycerol (as a source of α-glycerophosphate) or succinate to the anaerobic suspension, but was significantly stimulated by the addition of either malate or glutamate, both of which are oxidised via NAD^+. In contrast, the endogenous → H^+/O ratio of strain M was not decreased by starvation and was not significantly altered as a result of loading with either glycerol, succinate (even after growth on succinate), malate or glutamate, although the oxidation of ascorbate-TMPD yielded significantly lower values. Unfortunately, the stimulation of respiratory activity which resulted from loading starved cells of either strain with exogenous substrates was not particularly dramatic in most instances and this was probably responsible for the relatively small changes in the → H^+/O ratios. When corrections were made to the → H^+/O ratios for the contribution of residual endogenous respiration to the total respiratory activity of substrate-loaded cells, the → $H^+/O_{substrate}$ ratios clearly indicated that strain D440 the oxidation of NAD^+-linked substrates caused the outward translocation of approximately 4 protons per oxygen atom reduced compared with

Table 2. $\rightarrow H^+/O$ ratios of *B. megaterium* strains D440 and M respiring endogenous and added substrates. Whole cell suspensions of starved and unstarved cultures of *B. megaterium* were prepared and assayed for respiration-linked proton translocation ($\rightarrow H^+/O$ ratios) and respiratory activity (QO_2) as described in the "Materials and Methods" section. $\rightarrow H^+/O_{substrate}$ ratios were calculated by correcting for the contribution to total proton translocation of that linked to endogenous respiration; in several experiments (∓) the stimulation of QO_2 following the addition of substrate was insufficient to allow an accurate value of $\rightarrow H^+/O_{substrate}$ to be calculated. Values quoted are the average ± the standard error of the mean with the number of determinations in brackets

Strain	Carbon source for growth	State of cells	Substrate added	QO_2 ($\mu l\ O_2 \cdot hr^{-1} \cdot mg\ cells^{-1}$)	$\rightarrow H^+/O$ (equiv. $H^+ \cdot gatom\ O^{-1}$)	$\rightarrow H^+/O_{substrate}$
D440	Glycerol	Unstarved	–	171.0 ± 6.0 (5)	3.95 ± 0.15 (5)	
		Starved	–	23.3 ± 1.8 (5)	2.83 ± 0.19 (5)	
			L-Malate	31.6 ± 3.0 (5)	3.19 ± 0.27 (5)	4.20
			Glutamate	36.6 ± 2.0 (5)	3.24 ± 0.16 (5)	3.96
			Glycerol	56.4 ± 8.5 (5)	2.66 ± 0.23 (5)	2.54
			Succinate	27.8 ± 1.1 (5)	2.56 ± 0.32 (5)	∓
M	Glycerol	Unstarved	–	105.0 ± 5.0 (3)	4.19 ± 0.18 (3)	
		Starved	–	16.2 ± 2.0 (4)	4.23 ± 0.08 (4)	
			L-Malate	17.5 ± 2.0 (4)	4.23 ± 0.08 (4)	∓
			Glutamate	24.6 ± 1.5 (4)	3.98 ± 0.13 (4)	3.50
			Glycerol	71.2 ± 4.1 (4)	4.23 ± 0.16 (4)	4.23
			Succinate	22.0 ± 0.8 (4)	4.05 ± 0.11 (4)	3.55
			Ascorbate-TMPD	340.0 ± 21.0 (4)	3.19 ± 0.01 (4)	3.13
	Succinate	Starved	–	19.2 ± 1.2 (4)	3.71 ± 0.19 (4)	
			Succinate	82.1 ± 4.1 (4)	3.69 ± 0.17 (4)	3.68

approximately 2 for the oxidation of flavin-linked substrates. In contrast, strain M exhibited $\rightarrow H^+/O_{substrate}$ ratios of approximately 4 for the oxidation of both NAD^+- and flavin-linked substrates and a significantly lower value for the oxidation of ascorbate-TMPD. These results strongly suggest the presence of proton translocating loops 1 and 2 in strain D440, *cf.* loops 2 and 3 in strain M. They further indicate that NADH is the major oxidisable endogenous substrate in unstarved cells of strain D440, but that after starvation this role is taken over by a mixture of NADH and reduced flavin; this may also be true for strain M but the absence of loop 1 does not allow such a change to be manifested via a change in the efficiency of proton ejection.

When *B. megaterium* was grown in batch culture under oxygen-limited conditions, endogenous $\rightarrow H^+/O$ ratios of 3.14 ± 0.21 (6) and 4.25 ± 0.20 (3) were obtained for strains D440 and M respectively. The low value obtained with D440 compared with the same strain grown under conditions of excess-oxygen suggested either the partial loss of a proton translocating loop or a change in the nature of the endogenous substrate pool from predominantly NAD^+-linked to a mixture of NAD^+- and flavin-linked substrates. Unfortunately, these cells were not amenable to substrate loading experiments of the type described above and hence these possibilities could not be tested directly. However, as discussed below, the evidence of molar growth yield experiments support the former possibility.

Growth of these two strains of *B. megaterium* in continuous culture was readily achieved under conditions of either glycerol-, ammonium- or oxygen-limitation. Under all conditions culture wash-out occurred at $D = 0.60\ hr^{-1}$ for strain D440 and at $D = 0.16\ hr^{-1}$ for strain M, values which were commensurate with the different logarithmic growth rates of these two strains in batch culture. The cytochrome patterns of respiratory membranes prepared from cells grown under glycerol- or ammonium-limitation were virtually identical, both qualitatively and quantitatively, to those from cells harvested in the logarithmic, excess-oxygen phase of batch growth. In addition, respiratory membranes prepared from oxygen-limited batch and continuous cultures also contained very similar cytochrome contents, *viz.* cytochromes *a*, aa_3, *o* in strain D440 and *b*, *c*, *a*, *o* in strain M. No significant differences in the respiratory activities of isolated membranes were observed on changing from batch to continuous culture.

With the exception of *B. megaterium* D440 grown under conditions of oxygen-limitation, both strains of this organism exhibited endogenous $\rightarrow H^+/O$ ratios of approximately 4 over a wide range of dilution rates (Fig. 3a and b). Oxygen-limited cultures of strain D440 were exceptional in that the endogenous $\rightarrow H^+/O$ ratios decreased with decreasing dilution rate. Thus,

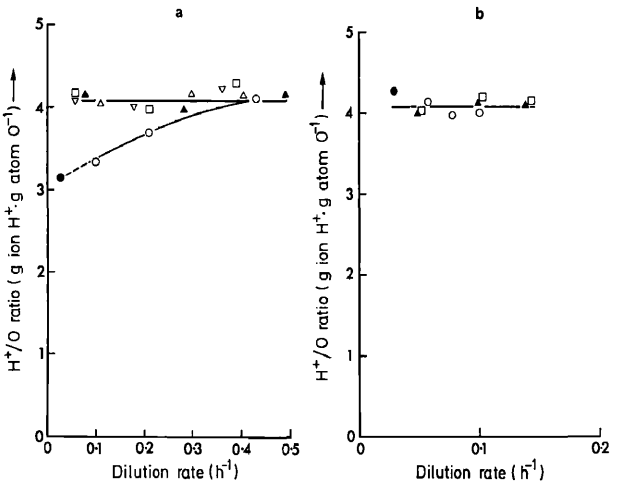

Fig. 3a and b. The variation in the endogenous $\rightarrow H^+/O$ ratio of *B. megaterium* whole cells as a function of dilution rate. $\rightarrow H^+/O$ ratios for the oxidation of endogenous substrates were assayed as described in the "Materials and Methods" section. (a) Strain D440. (b) Strain M. Glycerol-limited (▲); glycerol-limited, pH 7.65 (△); glycerol-limited, pH 5.95 (▽); ammonium-limited (□); and oxygen-limited (○) continuous cultures; oxygen-limited batch culture, $D = \mu$ (●)

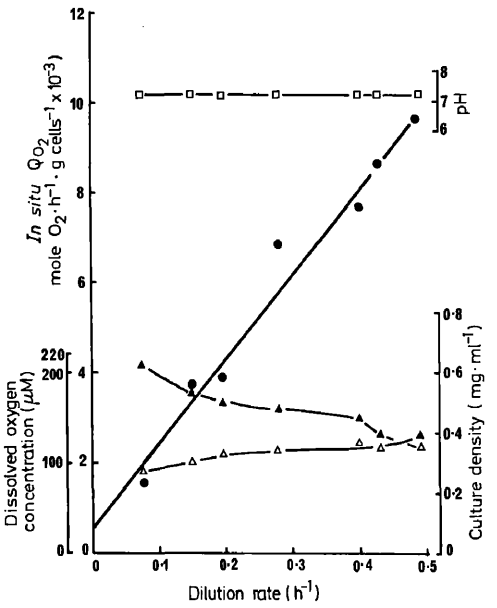

Fig. 4. Glycerol-limited continuous culture of *B. megaterium* strain D440. *In situ* QO_2 (●); dissolved oxygen concentration (▲), pH (□) and culture density (△) were assayed as described in the "Materials and Methods" section. The plot of *in situ* QO_2 *versus* dilution rate was fitted to the experimental points by linear regression analysis using a Hewlett-Packard 1600B bench-top computer; correlation coefficient 0.98

although an endogenous $\rightarrow H^+/O$ ratio of 4.10 was obtained with a high dilution rate culture ($D = 0.43\ hr^{-1}$) this decreased to 3.37 at a dilution rate of $0.10\ hr^{-1}$. These results from continuous culture thus agree well with the $\rightarrow H^+/O$ ratio of 3.14 reported above for the oxidation of endogenous substrates by batch cultures of strain D440 growing slowly ($\mu = 0.03\ hr^{-1}$) under oxygen-limited conditions.

Harrison and Loveless (1971) have reported that for an energy-limited aerobic culture

$$QO_2 = \frac{\mu}{Y_{ATP} \cdot N} + \frac{M}{N}$$

where QO_2 is the *in situ* respiratory activity of the growing culture (mole O_2 consumed · hr^{-1} · g cells^{-1}), μ is the specific growth rate (hr^{-1}; equivalent to the dilution rate, D, of a continuous culture), Y_{ATP} is the true molar growth yield with respect to ATP equivalents (g cells · mole ATP equivalents consumed^{-1}; equivalent to the Y_{ATP}^{max} of Stouthamer and Bettenhausen, 1973), N is the yield of ATP equivalents with respect to oxygen consumption (mole ATP equivalents · mole O_2 consumed^{-1}) and M is the maintenance coefficient (mole ATP equivalents consumed · hr^{-1} · g cells^{-1}). Thus a plot of *in situ* QO_2 *versus* dilution rate should yield a straight line of intercept M/N ($\equiv M_{O_2}$, the maintenance respiration) and of slope $1/Y_{ATP} \cdot N$ ($\equiv 1/Y_{O_2}$, the true molar growth yield on oxygen). In order to ascertain if this relationship holds for *B. megaterium* strains D440 and M, these organisms were grown in continuous culture in a chemostat fitted with an oxygen analyser and the *in situ* respiratory activities of these cultures were assayed over a range of dilution rates under various nutrient limitations. In addition the pH, cell density and dissolved oxygen concentration of the cultures were also determined. It was observed that both strains exhibited straight line plots of *in situ* QO_2 *versus* dilution rate and from the slopes of these plots Y_{O_2} ($\equiv Y_{ATP} \cdot N$) values of approximately 50 g cells · mole O_2 consumed^{-1} were obtained for all cultures except oxygen-limited strain D440 (Figs. 4–7, Table 3).

In *B. megaterium* strain D440 the complete catabolism of 1 mole of glycerol yields 5 moles of reduced pyridine nucleotide (*via* glyceraldehyde 3 phosphate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase), 2 moles of reduced flavin (*via* succinate dehydrogenase and α-glycerophosphate dehydrogenase) and 3 moles of ATP by substrate level phosphorylation (*via* phosphoglycerate kinase, pyruvate kinase and succinyl thiokinase) although one of the latter is consumed by glycerol kinase. Since this strain exhibits proton translocating loops commensurate with the presence of phosphorylation sites I and II, the total ATP yield is 14 moles per 3.5 moles of oxygen consumed (≡ 1 mole glycerol completely

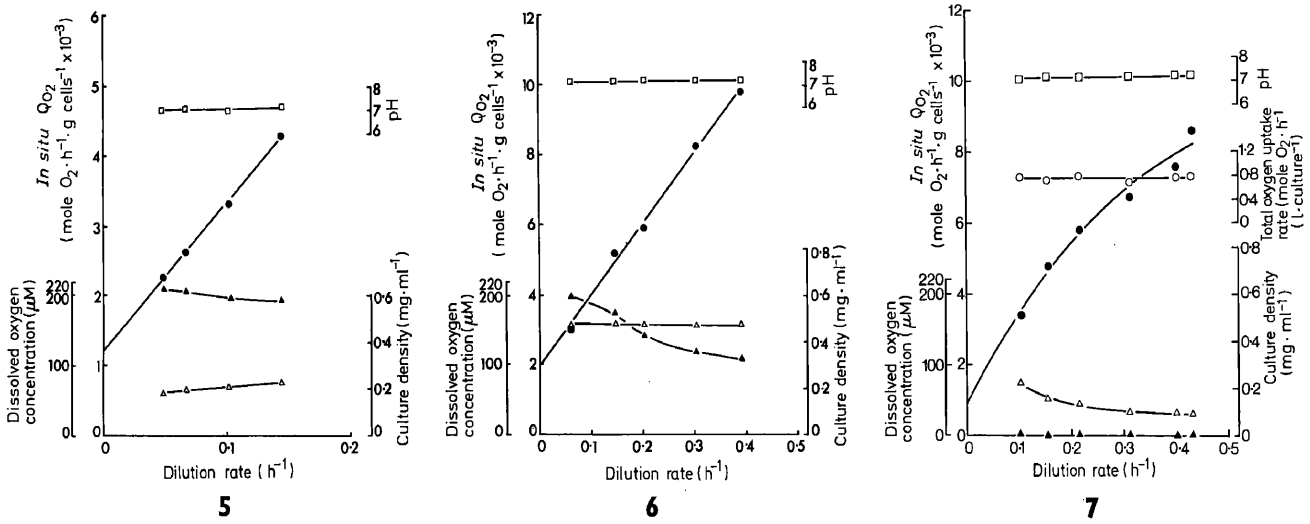

Fig. 5. Glycerol-limited continuous culture of *B. megaterium* strain M. *In situ* QO_2 (●), pH (□), dissolved oxygen concentration (▲) and culture density (△) were assayed as described in the "Materials and Methods" section. Correlation coefficient for *in situ* QO_2 *versus* dilution rate, 0.99

Fig. 6. Ammonium-limited continuous culture of *B. megaterium* strain D440. *In situ* QO_2 (●), pH (□), dissolved oxygen concentration (▲) and culture density (△) were assayed as described in the "Materials and Methods" section. Correlation coefficient for *in situ* QO_2 *versus* dilution rate, 0.99

Fig. 7. Oxygen-limited continuous culture of *B. megaterium* strain D440. *In situ* QO_2 (●), total oxygen uptake rate (○), pH (□), dissolved oxygen concentration (▲) and culture density (△) were assayed as described in the "Materials and Methods" section

oxidised) and thus N is calculated to be 4.00. On the other hand, in strain M, which exhibits proton translocation commensurate with phosphorylation sites II and III, the total ATP yield is 16 and N is 4.57 (the oxidation of malate predominantly by a flavin-linked dehydrogenase rather than an NAD^+-linked dehydrogenase in oxygen-limited cells does not affect the value of N since site I is apparently absent). Y_{ATP} values of approximately 13 g cells · mole ATP equivalents^{-1} were thus obtained for glycerol- and ammonium-limited cultures of strain D440 and of approximately 11 g cells · mole ATP equivalents^{-1} for glycerol-, ammonium- or oxygen-limited cultures of strain M. Slightly acid (pH 5.95) or alkaline (pH 7.65) growth conditions did not significantly affect the molar growth yields with respect to either oxygen or ATP of glycerol-limited strain D440 (Table 3).

The plots of *in situ* QO_2 *versus* dilution rate also yielded a positive intercept on the ordinate axis for each growth condition, thus indicating the presence of maintenance respiration ($M/N \equiv M_{O_2}$). For glycerol-limited cultures of strain D440, M_{O_2} was 0.60 mmole $O_2 \cdot hr^{-1} \cdot g$ cells^{-1} which was equivalent to a maintenance coefficient (M) of 2.40 mmole ATP equivalents · $hr^{-1} \cdot g$ cells^{-1} (Fig. 4, Table 3). The value for the maintenance coefficient was slightly higher for glycerol- and oxygen-limited cultures of strain M (Fig. 5, Table 3) but, most strikingly, it was very much increased for both strains under conditions of ammonium-limitation (Fig. 6, Table 3), *viz.* 7.90 and 12.20 mmole ATP equivalents · $hr^{-1} \cdot g$ cells^{-1} for strains D440 and M respectively.

For oxygen-limited cultures of *B. megaterium* strain D440 (Fig. 7, Table 3) the plot of *in situ* QO_2 *versus* dilution rate yielded rather unexpected results, *viz.* a slight curve indicative of an increasing value for Y_{O_2} with increasing dilution rate. If the maintenance coefficient is assumed to be the same as for glycerol-limited cells (2.40 mmole ATP equivalents · $hr^{-1} \cdot g$ cells^{-1}), as was the case for oxygen- and glycerol-limited cultures of strain M, then at dilution rates of 0.43, 0.22 and 0.11 hr^{-1} Y_{O_2} can be calculated from the above equation to be 53.8, 43.0 and 41.2 g cells · mole O_2 consumed^{-1} respectively. It was clear from the data shown in Fig. 3a that the efficiency of proton translocation (and hence of oxidative phosphorylation) by this strain also increased with increasing dilution rate under oxygen-limited conditions. Thus, it is possible that although the value of Y_{O_2} varies, the value of Y_{ATP} may remain constant (since $Y_{O_2} = Y_{ATP} \cdot N$). Indeed, using values for N of 4.00, 3.77 and 3.54 respectively for the above dilution rates (calculated from the → H^+/O ratios in Fig. 3a) an average value for Y_{ATP} of 12.2 ± 0.6 (3) g cells · mole ATP equivalents^{-1} can be calculated. It should be noted that with oxygen-limited cultures, unlike those limited by glycerol or ammonia, the absolute respiratory activity (mole $O_2 \cdot hr^{-1} \cdot$ l culture^{-1}) remained constant and, as a result, the cell density decreased sharply with increasing dilution rate.

Table 3. The energetics of *B. megaterium* strains D440 and M during growth in continuous culture. *B. megaterium* was grown in continuous culture at pH 7.1 ± 0.1 (unless otherwise stated) under various nutrient limitations and at a series of dilution rates as described in the "Materials and Methods" section. Values of $Y_{ATP} \cdot N (\equiv Y_{O_2})$ and $M/N (\equiv M_{O_2})$ were obtained from slope and intercept respectively of *in situ* QO_2 *versus* dilution rate plots of the type shown in Figs. 4–7. $\rightarrow H^+/O$ ratios were expressed as for Table 2; values for N were calculated as described in the text. Y_{ATP} (average) values were calculated only from cultures grown at pH 7.1 ± 0.1

Strain	Growth-limiting nutrient	$Y_{ATP} \cdot N (\equiv Y_{O_2})$ (g cells · mole O_2^{-1})	$M/N (\equiv M_{O_2})$ (mole $O_2 \cdot hr^{-1}$ · g cells^{-1})	$\rightarrow H^+/O$ (equiv. H^+ · gatom O^{-1})	N (mole ATP equiv. · mole O_2^{-1})	Y_{ATP} (g cells · mole ATP equivs^{-1})	Y_{ATP} (average)	M (mole ATP equiv. hr^{-1} · g cells^{-1})
D440	Glycerol	53.8	0.00060	4.09 ± 0.05 (3)	4.00	13.5	–	0.00240
	Glycerol (pH 7.65)	54.0	0.00068	4.12 ± 0.03 (3)	4.00	13.5	–	0.00272
	Glycerol (pH 5.95)	50.0	0.00069	4.10 ± 0.05 (3)	4.00	12.5	–	0.00276
	Ammonium	49.2	0.00198	4.15 ± 0.08 (3)	4.00	12.3	–	0.00790
	Oxygen	Variable (≤ 53.8)		Variable (≤ 4.10)	Variable (≤ 4.00)	12.2	12.7	–
M	Glycerol	47.6	0.00123	4.06 ± 0.03 (3)	4.57	10.4	–	0.00562
	Ammonium	52.0	0.00267	4.11 ± 0.03 (3)	4.57	11.4	–	0.01220
	Oxygen	48.9	0.00096	4.03 ± 0.04 (3)	4.57	10.7	10.8	0.00439

No significant concentrations of acetate could be detected by gas-liquid chromatography in the spent growth media from any of the continuous cultures employed in this study, thus indicating that incomplete oxidation of glycerol did not occur to any significant extent. Neither of these two strains of *B. megaterium* were able to ferment glycerol under anaerobic conditions.

Discussion

Respiratory membranes from *B. megaterium* strains D440 and M clearly exhibit different cytochrome patterns. In terms of a possible relationship between respiratory chain composition and the efficiency of proton translocation (the latter being used as a measure of the efficiency of energy conservation under strictly defined conditions), strain D440 can be classified with several other bacilli as a conventional Group I organism according to the classification scheme of Jones *et al.* (1975). It thus contains a basic respiratory system, unsupplemented with either pyridine nucleotide transhydrogenase or cytochrome *c*, and exhibits proton translocating loops I and II. On the other hand, strain M, which contains cytochrome *c* but no transhydrogenase, is classified as a Group II organism and might therefore be expected to exhibit proton translocating loops I, II and III. From the $\rightarrow H^+/O$ ratios which were observed after loading cells with specific substrate this strain clearly contains loops II and III, but not loop I. This unexpected result implies, in terms of the chemiosmotic hypothesis of oxidative phosphorylation (Mitchell, 1966) that the hydrogen and electron carriers of the NADH dehydrogenase are not looped across the respiratory membrane and hence do not translocate two protons outwards per electron pair transferred. Clearly this situation is not totally restricted to strain M; under oxygen-limited conditions and particularly at slow growth rates, strain D440 also appears partially to lose the ability to translocate protons at the level of loop I. With neither strain does there appear to be any obvious physiological advantage to be gained through the complete or partial loss of an energy coupling site.

The other rather curious property of the respiratory system of strain M is the apparent lack of cytochrome a_3 (as evidenced by the failure to detect spectrally any binding of carbon monoxide by an *a*-type cytochrome) in spite of the obvious presence of cytochrome *a*. Clearly the absence of cytochrome α_3 does not hinder physiological respiratory activity since the latter is considerably lower than that observed with the artificial electron donor ascorbate-TMPD (217 e · sec^{-1} · molecule o^{-1}; Table 1) and is well within the electron

transferring capacity of cytochrome *o* from other sources (Jones, 1973; Meyer and Jones, unpublished data).

The energetics of bacterial growth are most easily investigated using anaerobic cultures. If the ATP yield from the fermentation of the chosen carbon source is known, then Y_{ATP} can easily be calculated from the measured molar growth yield with respect to carbon (Bauchop and Elsden, 1960). The accuracy of this value for Y_{ATP} can be improved by using continuous cultures where account may be taken of the effect of specific growth rate (≡ dilution rate). Stouthamer and Bettenhausen (1975) have recently used this approach to calculate a Y_{ATP} of 14.0 g cells · mole ATP (equivalents)$^{-1}$ for *Aerobacter aerogenes* growing anaerobically under glucose-limited conditions.

The unequivocal determination of Y_{ATP} for aerobic cultures is considerably more difficult since it requires prior knowledge of the efficiency of energy conservation by oxidative phosphorylation as well as by substrate level phosphorylation. However, with the advent of techniques to measure the respiration-linked proton translocation (Mitchell, 1966) of cells loaded with specific substrates (Lawford and Haddock, 1973; Drozd and Jones, 1974), independent determination of the numbers of energy coupling sites is greatly simplified and calculation of the total efficiency of energy conservation becomes possible. If true molar yields are then measured with respect to molecular oxygen rather than carbon source, to obviate the necessity to correct for carbon assimilated into cell material, Y_{ATP} can be readily calculated. Using this approach we report average Y_{ATP} values of 12.7 and 10.8 g cells · mole ATP equivalents^{-1} for *B. megaterium* strains D440 and M growing under a variety of nutrient limitations. These values are clearly considerably lower than the theoretical maximum value for Y_{ATP} of 28.8–31.9 g cells · mole ATP equivalents^{-1} predicted by Gunsalus and Shuster (1961), Forrest and Walker (1971) and Stouthamer (1973). Since the biosynthesis of monomers is considered to consume a negligible amount of energy, at least with glucose as carbon source, growth rate-dependent utilisation of energy must occur for processes other than biosynthesis, e.g. nutrient transport, movement, maintenance of osmotic balances or macromolecular turnover (Stouthamer and Bettenhausen, 1975).

Growth of either strain of *B. megaterium* under non-energy-limited conditions (*i.e.* when the concentration of ammonium ions rather than glycerol or oxygen limits growth) clearly had no effect on the value of Y_{ATP} but considerably increased the "maintenance coefficient". Under such conditions there is obviously a high, basal rate of energy utilisation which is independent of growth and which encompasses both the true maintenance coefficient and also a wastage process; the latter is presumably at a minimum under conditions of energy-limitation (*i.e.* in carbon- or oxygen-limited cultures). An apparent partial uncoupling of energy-conserving from energy-utilising processes when growth is limited by the nitrogen source (Senez, 1962) has previously been reported for *Desulphovibrio desulphuricans* (Le Gall and Senez, 1960) and *A. aerogenes* (Pichinoty, 1960), but neither of these studies differentiated between an effect on Y_{ATP} or "maintenance coefficient". Very recently Stouthamer and Bettenhausen (1975) have reported that a culture of *A. aerogenes* in which tryptophan was used as a limiting nitrogen source also exhibits a high "maintenance coefficient", but the situation here is complicated by the fact that the organism exhibits an extraordinary high Y_{ATP}.

Clearly the nature and mechanism of the energy wastage process which appears to be associated with growth under non-energy-limited conditions is worthy of further experimental attention.

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References

Ackrell, B. A. C., Jones, C. W.: The respiratory system of *Azotobacter vinelandii*. 2. Oxygen effects. Europ. J. Biochem. **20**, 29–35 (1971)

Baker, K.: Low cost continuous culture apparatus. Lab. Pract. **17**, 817–824 (1968)

Bauchop, T., Elsden, S. R.: The growth of microorganisms in relation to their energy supply. J. gen. Microbiol. **23**, 457–469 (1960)

Broberg, P. L., Smith, L.: The cytochrome system of *Bacillus megaterium* KM. The presence and some properties of two CO-binding cytochromes. Biochim. biophys. Acta (Amst.) **131**, 479–489 (1967)

Downs, A. J., Jones, C. W.: Energy conservation in *Bacillus megaterium*. Biochem. Soc. Trans. **2**, 526–529 (1974)

Drozd, J. W., Jones, C. W.: Oxidative phosphorylation in *Hydrogenomonas eutropha* H 16 grown with and without iron. Biochem. Soc. Trans. **2**, 529–531 (1974)

Forrest, W. W., Walker, D. J.: The generation and utilisation of energy during growth. Advanc. Microbial Physiol. **5**, 213–274 (1971)

Le Gall, J., Senez, J. C.: Influence de la fixation de l'azote sur la croissance de *Desulphovibrio desulphuricans*. C.R. **250**, 404–406 (1960)

Gunsalus, I. C., Shuster, C. W.: Energy yielding metabolism in bacteria, pp. 1–58. In: The bacteria, I. C. Gunsalus, R. Y. Stanier, eds., vol. 2. New York-London: Academic Press 1961

Harrison, D. E. F., Loveless, J. E.: The effect of growth conditions on respiratory activity and growth efficiency in facultative anaerobes grown in chemostat culture. J. gen. Microbiol. **68**, 35–43 (1971)

Harrison, D. E. F., Pirt, S. J.: The influence of dissolved oxygen concentration on the respiration and glucose metabolism of *Klebsiella aerogenes* during growth. J. gen. Microbiol. **46**, 193–211 (1967)

Jones, C. W.: The inhibition of *Azotobacter vinelandii* terminal oxidase by cyanide. FEBS Letters **36**, 347–350 (1973)
Jones, C. W., Brice, J. M., Downs, A. J., Drozd, J. W.: Bacterial respiration-linked proton translocation and its relationship to respiratory chain composition. Europ. J. Biochem. **52**, 265–271 (1975)
Kröger, A., Dadák, V.: On the role of quinones in bacterial electron transport. The respiratory system of *Bacillus megaterium*. Europ. J. Biochem. **11**, 328–340 (1969)
Lawford, H. G., Haddock, B. A.: Respiration-driven proton translocation in *Escherichia coli*. Biochem. J. **136**, 217–220 (1973)
Meyer, D. J., Jones, C. W.: Oxidative phosphorylation in bacteria which contain different cytochrome oxidases. Europ. J. Biochem. **36**, 144–151 (1973)
Mitchell, P.: Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41**, 445–502 (1966)
Mitchell, P., Moyle, J.: Respiration-driven proton translocation in rat liver mitochondria. Biochem. J. **105**, 1147–1162 (1967)
Pichinoty, F.: Reduction assimilative du nitrate par les cultures aérobies d'*Aerobacter aerogenes*. Influence de la nutrition azotée sur la croissance. Folia microbiol. (Praha) **5**, 165–170 (1960)
Senez, J. C.: Some considerations on the energetics of bacterial growth. Bact. Rev. **26**, 95–107 (1962)
Stouthamer, A. H.: A theoretical study on the amount of ATP required for synthesis of microbial cell material. Antonie v. Leeuwenhoek **39**, 545–565 (1973)
Stouthamer, A. H., Bettenhausen, C.: Utilisation of energy for growth and maintenance in continuous and batch cultures of microorganisms. Biochim. biophys. Acta (Amst.) **301**, 53–70 (1973)
Stouthamer, A. H., Bettenhausen, C.: Determination of the efficiency of oxidative phosphorylation in continuous cultures of *Aerobacter aerogenes*. Arch. Microbiol. **102**, 187–192 (1975)
Sundaram, T. K.: Physiological role of pyruvate carboxylase in a thermophilic Bacillus. J. Bact. **113**, 549–557 (1973)
Vernon, L. P., Mangum, J. H.: Cytochromes of *Bacillus megaterium* and *Bacillus subtilis*. Arch. Biochem. Biophys. **90**, 103–104 (1960)
Weibull, C., Bergström, L.: The chemical nature of the cytoplasmic membrane and cell wall of *Bacillus megaterium*, strain M. Biochim. biophys. Acta (Amst.) **30**, 340–351 (1958)

Dr. Colin W. Jones
Department of Biochemistry, University of Leicester
Leicester LE1 7RH, England

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LEICESTER UNIVERSITY

Bacterial Respiration-Linked Proton Translocation and Its Relationship to Respiratory-Chain Composition

Colin W. JONES, Joan M. BRICE, Andrew J. DOWNS, and Jan W. DROZD

Department of Biochemistry, School of Biological Sciences, University of Leicester

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1. The relationship between respiratory chain composition and the efficiency of respiration-linked proton translocation was studied in nine bacterial species of widely differing taxonomic and ecological status.

2. All the bacteria investigated contained respiratory chain dehydrogenases, ubiquinone and/or menaquinone, cytochrome *b* and cytochrome oxidase aa_3 and/or *o*. In addition, some of these organisms also contained pyridine nucleotide transhydrogenase and/or cytochrome *c*.

3. $\rightarrow H^+/O$ ratios of whole cell suspensions oxidising endogenous substrates were in the approximate range 4–8 mol H^+ translocated per g-atom oxygen consumed. It was concluded from the observed $\rightarrow H^+/O$ ratios of cells loaded with specific substrates that proton-translocating loops 1 and 2 were present in all of the organisms investigated, but that loops 0 and 3 were dependent upon the presence of pyridine nucleotide transhydrogenase and cytochrome *c* respectively.

4. The wide range in energy conservation efficiency which was observed in these organisms is discussed in relation to their respiratory chain composition and natural habitat.

Bacterial respiratory systems are very similar in many respects to those present in the mitochondria of higher organisms. Both are membrane-bound, can contain similar types of respiratory carriers and are capable of conserving energy in a form which subsequently can be used either for ATP synthesis or for driving a variety of energy-requiring processes [1–3]. Recent measurements of bacterial growth efficiencies with respect to molecular oxygen [4–6], of respiration-linked proton translocation by whole bacteria [4,7–10] and of ATP synthesis by isolated respiratory membrane preparations [11–13] have indicated that the efficiency of oxidative phosphorylation in intact bacteria may equal that of mammalian mitochondria.

There is some preliminary evidence which suggests that this efficiency may vary between different organisms and within the same organism during growth under different conditions [4,5,14,15]. Obviously, such variations might reflect the diversity of respiratory carrier composition (and hence molecular organisation) which exists in bacterial systems. In order to test this hypothesis we have investigated the respiratory systems of nine species of bacteria which were selected to cover a wide range of natural habitats and to represent a wide spectrum of respiratory chain composition. The results of these investigations confirm that the efficiency of oxidative phosphorylation varies between these different organisms and is related to differences in their respiratory chain composition.

Enzymes. NADH dehydrogenase (EC 1.6.99.3); NADPH dehydrogenase (EC 1.6.99.1); pyridine nucleotide transhydrogenase (EC 1.6.1.1).

MATERIALS AND METHODS

Organisms

The following organisms were used during this study: *Acinetobacter lwoffi* 4B, *Bacillus megaterium* D440, *Bacillus subtilis* D473, *Klebsiella pneumoniae* NCTC 5055 (all of which were the gift of the Medical Research Council Microbial Systematics Unit, University of Leicester); *Escherichia coli* W and *Pseudomonas ovalis* Chester (which were kindly donated by Professor H. L. Kornberg, F.R.S.); *Hydrogenomonas eutropha* H16 (= *Alcaligenes eutrophus* H16; ATCC 17699, which was the gift of Professor H. G. Schlegel); *Bacillus licheniformis* A5.

Table 1. *Cytochrome composition of respiratory membranes from selected bacteria*
Respiratory membranes were prepared and assayed for cytochromes as described in Materials and Methods. Where no value is quoted in the table, none of that particular cytochrome could be detected in the difference spectra

Organism	Cytochromes						
	b (net)	*c*	*a*	a_3	*o*	a_3	*o*
	nmol/mg protein					% total oxidase	
E. coli	0.117				0.086		100
K. pneumoniae	0.033				0.040		100
B. megaterium	0.128		0.042	0.069	0.033	67.7	32.3
B. licheniformis	0.064		0.092	0.113	0.058	66.1	33.9
B. subtilis	0.131		0.043	0.080	0.022	78.4	21.6
M. lysodeikticus	0.206	0.230	0.062	0.102	0.042	70.8	29.2
A. lwoffi	0.077				0.040		100
Ps. ovalis Chester	0.073	0.041			0.007		100
H. eutropha	0.290	0.550	0.034	0.56	0.070	44.4	55.6

Culture Conditions

B. licheniformis and *Micrococcus lysodeikticus* were grown on nutrient broth (Oxoid) 26 g/l, supplemented with 30 mM glycerol. *E. coli*, *K. pneumoniae* and *Ps. ovalis* Chester were grown on a minimal salts medium [16] supplemented with 30 mM glycerol, and *A. lwoffi* was grown on the same salts medium supplemented with 30 mM succinate. *B. megaterium* and *B. subtilis* were grown on a slightly different minimal salts medium [17] supplemented with 30 mM glycerol and *H. eutropha* was grown heterotrophically on the minimal salts medium of Schlegel *et al.* [18] supplemented with 55 mM fructose. All organisms were cultured at 30 °C in 600 ml growth medium in 2-l baffled flasks oscillating at 135 rev./min.

Cultures were harvested during the logarithmic phase of growth under excess oxygen conditions (dissolved oxygen concentration ≥ 20 μM) as determined from previously measured growth and dissolved oxygen concentration curves.

→ H^+/O Quotients

Whole cell suspensions were prepared and assayed for → H^+/O quotients (expressed as mol H^+ translocated/mol oxygen consumed, note that the elementary entities under consideration are ions for H^+ and atoms for oxygen; see [19]) as described previously [4] except that during the assay procedure the potassium thiocyanate concentration was 150 mM, the cell density was approximately 7 mg dry wt/ml and oxygen was added as air-saturated, 140 mM potassium chloride. Where indicated, cells were starved of endogenous substrates by resuspending in a minimal salts solution lacking nitrogen and carbon (final absorbance at 680 nm $\simeq$ 0.5) and shaking for 1–2 h at 30 °C under the same conditions as for growth. Starved cells were prepared and assayed for → H^+/O ratios in the same way as unstarved cells.

Whole Cell Respiratory Activities

Respiratory activities (q_{O_2}) of washed, whole cell suspensions were determined at 30 °C in a Y.S.I. 4004 Clarke electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) in the presence of 25 mM sodium/potassium phosphate buffer, pH 7.4 and either endogenous or added substrates (1.5–5.0 mM).

Preparation and Assay of Respiratory Membranes

Non-phosphorylating respiratory membranes were prepared as described previously [4] and, unless stated, were the standard membrane preparations used for the work described in this paper. Phosphorylating respiratory membranes were prepared from *A. lwoffi* and *Ps. ovalis* Chester by the method of Ackrell and Jones [13].

Energy conservation efficiencies (P/O ratios), cytochrome concentrations and oxygen uptake rates of respiratory membranes were assayed as described previously [13, 20]. Protein was determined by the modified biuret method [21].

RESULTS

The data in Table 1 indicate that three of the nine organisms selected for study contained a relatively simple cytochrome system, *viz.* cytochromes *b* and *o*, during logarithmic growth under excess oxygen conditions (*i.e. E. coli*, *K. pneumoniae* and *A. lwoffi*). In

Table 2. *Reduced pyridine nucleotide oxidase and transhydrogenase activities of respiratory membranes prepared from selected bacteria*
Respiratory membranes were prepared and assayed for oxygen uptake at 30 °C and pH 7.4 as described in Materials and Methods

Organism	Respiratory activity			Relative activities	
	NADH	NADPH	NADPH + NAD^+	$\frac{NADPH}{NADH}$	$\frac{NADPH + NAD^+}{NADH}$
	µg-atom O × min^{-1} × mg $protein^{-1}$				
E. coli	0.116	0.030	0.031	0.26	0.27
K. pneumoniae	0.232	0.065	0.063	0.28	0.27
B. megaterium	0.200	0	0	0	0
B. licheniformis	0.091	0.029	0.029	0.32	0.32
B. subtilis	0.040	0.028	0.030	0.70	0.75
M. lysodeikticus	0.060	0.028	0.023	0.47	0.41
A. lwoffi	0.143	0.025	0.123	0.18	0.86
Ps. ovalis Chester	0.116	0.018	0.121	0.16	1.04
H. eutropha	0.030	0.004	0.025	0.13	0.83

the remaining organisms this pattern was supplemented by the presence of cytochrome oxidase aa_3 (*B. megaterium*, *B. licheniformis* and *B. subtilis*) or cytochrome *c* (*Ps. ovalis* Chester) or both cytochromes (*M. lysodeikticus* and *H. eutropha*). Cytochrome *o* was quantitatively the minor oxidase in all but one of the five organisms in which cytochrome oxidases aa_3 and *o* occurred together.

Investigation of the electron transfer activities of respiratory membranes prepared from each organism (Table 2) revealed that all (with the exception of those prepared from *B. subtilis*) were capable of oxidising NADH at a substantially faster rate than NADPH. However, the addition of NAD^+ (1 mM) dramatically increased the rate of NADPH oxidation by respiratory membranes from *A. lwoffi*, *Ps. ovalis* Chester and *H. eutropha*. This result indicated the presence in these three organisms of an active, membrane-bound NADPH → NAD^+ transhydrogenase capable of catalysing the formation of NADH at a rate sufficient virtually to saturate NADH dehydrogenase (and hence NADH oxidase).

These initial studies thus confirmed that the nine selected organisms exhibited a wide range of respiratory chain composition (Table 3), the latter varying both in absolute terms (*e.g.* the presence or absence of transhydrogenase and/or cytochrome *c*) and also in relative terms (*e.g.* in the precise identities of the cytochrome oxidase and quinone components; see also [22–24]). These organisms thus allowed us to test the hypothesis that the efficiency of bacterial oxidative phosphorylation is directly related to respiratory chain composition.

Measurement of the whole cell → H^+/O ratios of these nine organisms during the oxidation of endogenous substrates (Table 3) yielded values within the approximate range 4–8 mol H^+ translocated/mol O consumed. Organisms which were classified in group I on the basis of their lack of both transhydrogenase and cytochrome *c* (*i.e. E. coli*, *K. pneumoniae*, *B. megaterium*, *B. licheniformis* and *B. subtilis*) exhibited → H^+/O ratios of approximately 4 for the oxidation of endogenous substrates. Assuming an → H^+/P ratio of 2 mol H^+/mol phosphate esterified [25], these results indicate the presence of only two proton-translocating loops (energy-coupling sites) in the respiratory systems of these organisms. Clearly, therefore, the substitution of menaquinone for ubiquinone (both are hydrogen carriers) and the virtual replacement of cytochrome oxidase *o* by oxidase aa_3 (both are electron carriers) in some of these group I organisms has no significant effect on the efficiency of respiration-linked proton translocation.

The presence of either cytochrome *c* (*M. lysodeikticus*, group II) or transhydrogenase (*A. lwoffi*, group III) in addition to the basic respiratory chain of group I organisms caused the endogenous → H^+/O ratios to significantly increase towards 6 (equivalent to three coupling sites; see also [26]). The addition of both cytochrome *c* and transhydrogenase to the basic respiratory chain (*Ps. ovalis* Chester and *H. eutropha*, group IV) further increased the endogenous → H^+/O ratios into the range 6–8 (equivalent to 3–4 coupling sites).

Since 'endogenous substrates' of ill-defined composition were used as the source of reducing power in all of the above measurements of → H^+/O ratios, an exact determination of both the number and the location of energy-coupling sites clearly required that the nature of the oxidisable substrate be more precisely defined. To this end, we carried out a number of substrate-loading experiments of the type which

Table 3. *→ H^+/O ratios for the oxidation of endogenous substrates by whole cells of selected bacteria*
→ H^+/O ratios were assayed at optimum potassium thiocyanate concentrations as described in Materials and Methods. Organisms are classified into groups I–IV on the basis of their respiratory chain composition as determined from Tables 1 and 2, from [22–24], and from personal observation. Abbreviations: Th = transhydrogenase; Ndh = NADH dehydrogenase; MK = menaquinone; Q = quinone. → H^+/O ratios are expressed as the average ± S.E.M. with the number of determinations in brackets

Group	Organism	Respiratory systems							→ H^+/O (endogenous substrates)
									mol H^+/mol O
I	*E. coli*		Ndh	Q	*b*		*o*		4.00 ± 0.07 (7)
	K. pneumoniae		Ndh	Q	*b*		*o*		3.92 ± 0.22 (7)
	B. megaterium		Ndh	MK	*b*		(*o*)	aa_3	4.06 ± 0.10 (9)
	B. licheniformis		Ndh	MK	*b*		(*o*)	aa_3	3.89 ± 0.09 (11)
	B. subtilis		Ndh	MK	*b*		(*o*)	aa_3	3.97 ± 0.16 (3)
II	*M. lysodeikticus*		Ndh	MK	*b*	*c*	(*o*)	aa_3	5.02 ± 0.22 (4)
III	*A. lwoffi*	Th	Ndh	Q	*b*		*o*		5.69 ± 0.15 (4)
IV	*Ps. ovalis* Chester	Th	Ndh	Q	*b*	*c*	*o*		6.56 ± 0.18 (9)
	H. eutropha	Th	Ndh	Q	*b*	*c*	*o*	aa_3	7.70 ± 0.30 (6)

Table 4. *→ H^+/O ratios and respiratory activities of whole cells following starvation and exposure to selected substrates*
Cells were starved, loaded with selected substrates and assayed for → H^+/O ratios and respiratory activities (qO_2) as described in Materials and Methods. Values quoted as the average ± S.E.M. with the number of determinations in brackets. $Ph(NMe_2)_2$, tetramethyl-*p*-phenylenediamine

Cells	Substrate	Immediate electron donor	*A. lwoffi*		*Ps. ovalis* Chester	
			→ H^+/O	qO_2	→ H^+/O	qO_2
			mol H^+/mol O	µl $O_2 \times h^{-1} \times mg^{-1}$	mol H^+/mol O	µl $O_2 \times h^{-1} \times mg^{-1}$
Unstarved	endogenous		5.69 ± 0.15 (4)		6.56 ± 0.18 (9)	
Starved	endogenous		4.40 ± 0.23 (5)	20 ± 2 (5)	5.90 ± 0.31 (4)	37 ± 7 (7)
	+ glutamate	NADPH + NADH	5.44 ± 0.08 (3)	89 ± 18 (4)		
		NADH			5.82 ± 0.15 (5)	104 ± 12 (7)
	+ malate	NADH	4.49 ± 0.26 (4)	249 ± 10 (4)		
	+ glycerol	reduced flavin			3.91 ± 0.28 (3)	95 ± 6 (4)
	+ ascorbate	reduced cytochrome *c*				
	− $Phe(NMe_2)_2$				2.55 ± 0.15 (3)	329 ± 42 (4)

were recently used to show the presence of two energy-coupling sites in *E. coli* [10], *i.e.* cells were starved of endogenous substrates, loaded with substrates linked to $NAD(P)^+$, flavin or cytochrome *c* and finally assayed for → H^+/O ratios and respiratory activities (q_{O_2}). Results with the group I organism *B. megaterium* [27] (A. J. Downs and C. W. Jones, unpublished data) clearly indicated that, as with *E. coli* [10], the endogenous → H^+/O ratios of approximately 4 resulted from the activity of proton-translocating loops (energy-coupling sites) 1 and 2 and thus showed that NADH was the major oxidisable endogenous substrate in unstarved cells.

The results which were obtained with more complex organisms of groups III and IV are shown in Table 4. Starvation of the group III organism *A. lwoffi* caused the → H^+/O ratio of cells oxidising endogenous substrates to decrease from 5.69 to 4.40 and the latter was not significantly altered after loading with malate, which is oxidised predominantly *via* NAD^+ in this organism. However, when starved cells were loaded with glutamate (which readily caused the reduction of both NAD^+ and $NADP^+$ on addition to cell-free extracts) the → H^+/O ratio increased to 5.44. Clearly, the availability of both NADH and NADPH for oxidation (the latter initially *via* transhydrogenase) increased the efficiency of respiration-linked proton translocation over that observed for the oxidation of NADH alone. The results thus support the conclusion that proton translocating loops 0 (transhydrogenase), 1 and 2 are active in *A. lwoffi*. The endogenous → H^+/O ratio of 5.69 for unstarved cells suggests that the major

oxidisable endogenous substrate prior to starvation was probably NADPH and that, following starvation ($\rightarrow H^+/O = 4.40$), NADPH was largely replaced by NADH.

Starvation of the group IV organism *Ps. ovalis* Chester caused the endogenous $\rightarrow H^+/O$ ratio to decrease from 6.56 to 5.90. When subsequently loaded with glutamate (NAD^+-linked), glycerol (flavin-linked *via* glycerol-3-phosphate dehydrogenase) or ascorbate/tetramethyl-*p*-phenylenediamine (to reduce endogenous cytochrome *c*) these cells yielded $\rightarrow H^+/O$ ratios of 5.82, 3.91 and 2.55 respectively and showed clearly the presence of three proton-translocating loops between NADH and molecular oxygen (*i.e.* loops 1, 2 and 3). Attempts to demonstrate directly the presence of a fourth loop (transhydrogenase, loop 0) by loading starved cells with the $NADP^+$-linked substrate isocitrate were doomed by the failure of these glycerol-grown cells to oxidise isocitrate at a significant rate. However, if cells were exposed to 1 mM isocitrate for 1 h during the later stages of the starvation procedure they acquired the ability to oxidise isocitrate rapidly and when loaded with this substrate yielded an $\rightarrow H^+/O$ ratio of 6.41 ± 0.17 ($n = 5$) compared with a ratio of 5.93 ± 0.04 ($n = 3$) when loaded with glutamate. The $\rightarrow H^+/O$ ratio of 6.56 which was observed for the oxidation of endogenous substrates by unstarved cells of *Ps. ovalis* Chester, suggested the partial use of a fourth loop as might be expected if NADPH was a minor component of the endogenous, reduced pyridine nucleotide pool. Following starvation, the endogenous $\rightarrow H^+/O$ ratio decreased to 5.90, thus suggesting that NADH was now the sole oxidisable substrate.

The presence of proton translocating loops 0, 1 and 2 in *A. lwoffi* and 0, 1, 2 and 3 in *Ps. ovalis* Chester (and also in *H. eutropha* [24]) was further investigated by measuring P/O ratios of phosphorylating respiratory membranes isolated from these organisms (Table 5). P/O ratios for the oxidation of NADPH + NAD^+, although low, were significantly higher than for the oxidation of NADH, thus implying some slight ATP synthesis concomitant with transhydrogenase activity. The P/O ratios which were observed for the oxidation of the flavin-linked substrates succinate (*A. lwoffi*) and malate (*Ps. ovalis* Chester) were lower than those observed with NADH alone. These results confirmed energy coupling at site 1 in these organisms and further suggested that sites 2 and/or 3 were also present. Slight ATP synthesis concomitant with the rapid oxidation of ascorbate/tetramethyl-*p*-phenylenediamine (*via* cytochrome *c*) was obtained with *Ps. ovalis* Chester respiratory membranes and thus confirmed the loop 3 proton translocation which was observed with whole cells (Table 4). P/O ratios of isolated respiratory membranes are invariably low *cf.* whole cells, probably due to heterogeneous sidedness and structural imperfections within the vesicle population; the particularly low efficiency of energy conservation at site III (see also [13]) is probably caused by the ability of tetramethyl-*p*-phenylene diamine (H_2) to penetrate the membranes and donate electrons to all respiratory chains, whereas ADP can only react with "inside-out" vesicles. Respiratory membranes from *A. lwoffi* failed to oxidise ascorbate/tetramethyl-*p*-phenylene diamine at a significant rate, probably due to the absence of cytochrome *c* from this organism.

Table 5. *P/O ratios of respiratory membranes prepared from* A. lwoffi *and* Ps. ovalis *Chester*
Phosphorylating respiratory membranes were prepared and assayed for ATP synthesis concomitant with oxygen uptake as described in Materials and Methods. P/O ratios are expressed as the average ± S.E.M. with the number of determinations in brackets. n.s., no significant oxygen uptake. $Ph(NMe_2)_2$, tetramethyl-*p*-phenylenediamine

Substrate	P/O ratios	
	A. lwoffi	*Ps. ovalis* Chester
	mol ATP/mol O	
NADPH	n.s.	n.s.
NADPH + NAD^+	0.57 ± 0.04 (3)	0.41 ± 0.03 (7)
NADH	0.46 ± 0.06 (3)	0.31 ± 0.01 (7)
Malate		0.22 ± 0.04 (4)
Succinate	0.29 ± 0.04 (3)	
Ascorbate-$Ph(NMe_2)_2$		0.22 ± 0.00 (3)

DISCUSSION

The results described in this paper show that in the nine species of bacteria investigated the efficiency of respiratory chain proton translocation ranges from 4 to 8 mol H^+/g-atom of oxygen consumed.

It is clear that the different patterns of respiratory chain composition which are exhibited by these organisms are responsible for the wide variations in proton translocation efficiency. Those organisms in which both pyridine nucleotide transhydrogenase and cytochrome *c* are absent (group I) exhibit only two proton-translocating loops. The precise identity of the quinone component appears to be unimportant, a conclusion which is perfectly compatible with the basic tenets of the chemiosmotic hypothesis of oxidative phosphorylation [2, 28] since both menaquinone and ubiquone can be similarly reduced (± 2H) and are thus capable of functioning as essentially interchangeable hydrogen carriers in loop 2.

If cytochrome *c* is present (as in organisms of groups II and IV) proton translocation at loop 3 becomes apparent. This is similar to the situation in the mitochondrial respiratory chains of higher organisms where cytochrome *c* appears immediately to precede cytochrome oxidase and thus to act as the electron carrier of loop 3 [2]. Where cytochrome *c* is absent (as in organisms of group I and III) loop 3 is, of course, also absent and cytochrome oxidase probably receives electrons directly from cytochrome *b*.

It is clear from the results described above, that when an active pyridine nucleotide transhydrogenase comprises part of a membrane-bound bacterial respiratory system (as in organisms of groups III and IV) proton translocation at loop 0 can be detected *via* the increased efficiency of respiration-linked proton translocation in whole cells. Proton translocation concomitant with NADPH → NAD^+ transhydrogenase activity ($\rightarrow H^+/2e = 2$) has previously been invoked to explain endogenous $\rightarrow H^+/O$ ratios of approximately 8 for whole cells of *Micrococcus denitrificans* (*i.e.* transhydrogenase plus the three loops of NADH oxidase) [7]. This stoichiometry was unequivocably confirmed for mammalian mitochondria following the addition of acetoacetate (as an oxidant for NADH) to mitochondria loaded with isocitrate (as a reductant for $NADP^+$; see [29]). Energy conservation concomitant with NADPH → NAD^+ transhydrogenase activity has been detected in submitochondrial particles derived from beef heart mitochondria by direct measurement of ATP synthesis [30] or movement of synthetic anions [31]; slight ATP synthesis concomitant with transhydrogenase activity in isolated respiratory membranes from *A. lwoffi* and *Ps. ovalis* Chester is also reported in this paper. It is likely that the latter is only transient and occurs when the [NADPH]/[$NADP^+$] and [NAD^+]/[NADH] ratios are particularly high, as during the initial stages of these experiments. The standard redox potentials (E_0') of these two couples are so close that during the balanced growth of these organisms the pyridine nucleotide transhydrogenase probably serves mainly to catalyse the energy-dependent reduction of $NADP^+$ rather than the energy-conserving oxidation of NADPH. It is probable that only loops 1, 2 and 3 (where present) contribute to the build up of the proton-motive force which is required to drive ATP synthesis *via* the inwardly-directed proton translocating ATPase.

It seems likely that the differences in respiratory chain composition (and hence in the efficiency of energy conservation) between the various organisms used in this study in part reflect their physiology. Organisms in group I, which appear to carry out energy conservation with low efficiency, comprise either facultative anaerobes (*e.g. E. coli, K. pneumoniae, B. licheniformis*) or aerobic members of the *Bacillaceae* originally isolated from relatively rich environments such as food or decomposing organic matter (*e.g. B. megaterium, B. subtilis*; see [32]). Thus, these organisms either may employ fermentation instead of respiratory chain phosphorylation as a means of energy conservation or at least can afford to utilise their relatively abundant natural sources of reducing power with poor efficiency. At the other extreme the group IV organisms *Ps. ovalis* Chester and *H. eutropha*, which exhibit high efficiencies of oxidative phosphorylation, are obligate aerobes originally isolated from relatively poor environments such as soil or water [32, 33]. These organisms therefore appear to conserve as efficiently as possible the energy available from their naturally meagre supply of reducing power.

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REFERENCES

1. Harold, F. M. (1972) *Bacteriol. Rev. 36*, 172–230.
2. Mitchell, P. (1972) *Biomembranes: Molecular Arrangements and Transport Mechanisms* (I. L. M. van Deenan, J. C. Riermersma & J. M. Tager, eds) pp. 353–370, North-Holland, Amsterdam.
3. Slater, E. C. (1972) *Mitochondria: Biogenesis and Bioenergetics* (S. G. Van den Bergh, P. Borst & E. C. Slater, eds) pp. 133–146, North-Holland, Amsterdam.
4. Meyer, D. J. & Jones, C. W. (1973) *Eur. J. Biochem. 36*, 144–151.
5. Harrison, D. E. F. & Loveless, J. E. (1971) *J. Gen. Microbiol. 68*, 35–43.
6. Stouthamer, A. H. & Bettenhausen, C. (1973) *Biochim. Biophys. Acta, 301*, 53–70.
7. Scholes, P. B. & Mitchell, P. (1970) *J. Bioenergetics, 1*, 309–323.
8. West, I. & Mitchell, P. (1972) *J. Bioenergetics, 3*, 445–462.
9. Jeacocke, R. E., Niven, D. F. & Hamilton, J. A. (1972) *Biochem. J. 127*, 57P.
10. Lawford, H. G. & Haddock, B. A. (1973) *Biochem. J. 136*, 217–220.
11. Aleem, M. I. H. (1968) *Biochim. Biophys. Acta, 162*, 338–347.
12. Keisow, L. (1964) *Proc. Natl Acad. Sci. U.S.A. 52*, 980–988.
13. Ackrell, B. A. C. & Jones, C. W. (1971) *Eur. J. Biochem. 20*, 22–28.
14. Harrison, D. E. F. & Maitra, P. K. (1969) *Biochem. J. 112*, 647–656.
15. Brice, J. M., Law, J. F., Meyer, D. J. & Jones, C. W. (1974) *Biochem. Soc. Trans. 2*, 523–526.
16. Ashworth, J. M. & Kornberg, H. L. (1966) *Proc. R. Soc. Lond. B. Biol. Sci. 165*, 179–188.
17. Gary, N. D. & Bard, R. C. (1952) *J. Bacteriol. 64*, 501–512.
18. Schlegel, H. G., Kaltwasser, H. & Gottschalk, G. (1961) *Arch. Mikrobiol. 38*, 209–222.
19. Mitchell, P. & Moyle, J. (1967) *Biochem. J. 104*, 588–600.

20. Ackrell, B. A. C. & Jones, C. W. (1971) *Eur. J. Biochem. 20*, 29–35.
21. Gornall, A. G., Bardawill, C. S. & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.
22. Bishop, D. H. L., Pandya, K. P. & King, H. K. (1962) *Biochem. J. 83*, 606–614.
23. Francis, M. J. O., Hughes, D. E., Kornberg, H. L. & Phizackerly, P. J. R. (1963) *Biochem. J. 89*, 430–438.
24. Drozd, J. & Jones, C. W. (1974) *Biochem. Soc. Trans. 2*, 529–531.
25. Mitchell, P. & Moyle, J. (1968) *Eur. J. Biochem. 4*, 530–539.
26. Beatrice, M. C. & Chappell, J. B. (1973) *Biochem. Soc. Trans. 1*, 125–127.
27. Downs, A. J. & Jones, C. W. (1974) *Biochem. Soc. Trans. 2*, 526–529.
28. Mitchell, P. (1966) *Biol. Rev. 41*, 445–502.
29. Moyle, J. & Mitchell, P. (1973) *Biochem. J. 132*, 571–585.
30. Van de Stadt, R. J., Nieuwenhuis, F. J. R. M. & Van Dam, K. (1971) *Biochim. Biophys. Acta, 234*, 173–176.
31. Skulachev, V. P. (1971) *Curr. Top. Bioenergetics, 4*, 127–190.
32. Bread, R. S., Murray, E. G. D. & Smith, N. R. (1957) *Bergey's Manual of Determinative Bacteriology*, Ballière, Tindall and Cox, London.
33. Gottschalk, G., Eberhardt, U. & Schlegel, H. G. (1964) *Arch. Mikrobiol. 48*, 95–108.

C. W. Jones, J. M. Brice, A. J. Downs, and J. W. Drozd, Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester, Great Britain LE1 7RH