Active Transport and Energy Conservation in *Escherichia coli*

by

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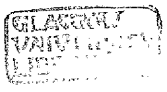

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ABBREVIATIONS

The abbreviations used in this thesis are described in the Biochemical Journal (1975) 145, 1-20, with the following additions:

ABTS	2, 2' azino-di (3-ethyl-benzthiazoline sulphonate-6).
BCIG	5-bromo-4-chloro-3-indolyl-galactoside.
BSA	bovine serum albumin.
CCCP	carbonyl cyanide m-chlorophenyl hydrazone.
MTT	3.(4.5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazdium bromide.
ONPG	orthonitrophenyl galactoside.
PEP	phosphoenol pyruvate.
TDG	thiodigalactoside.
TMG	methyl thio-galactaside.
H^+/O	proton : oxygen ratio; protons ejected per atom O.
m	maintenance requirement for carbon source.
m_{O_2}	maintenance requirement for oxygen.
$m^*_{O_2}$	maintenance requirement for combustion of carbon source used for maintenance.
$P/2e^-$	phosphate : 2 electron ratio; moles ATP synthesised per pair of electrons.
P/O	phosphate : oxygen ratio; moles ATP synthesised per atom O.
Q_{O_2}	specific rate of oxygen utilisation.
μ	specific growth rate.
Y	molar growth yield, for carbon source.
Y_{ATP}	molar growth yield, for ATP.
Y_{O_2}	molar growth yield for oxygen.
Y^G	molar growth yield for carbon source, corrected for maintenance.
Y^{MAX}_{ATP}	molar growth yield for ATP, corrected for maintenance.
$Y^G_{O_2}$	molar growth yield for oxygen, corrected for maintenance.

PREFACE

There are three appendices to this thesis, two of which describe the calculation and manipulation of experimental data presented in the Results section, and one which describes the fast-response pH meter.

To avoid confusion, all figures and tables have been designated 'Figures' and run in four consecutive series; those with no prefix refer to the thesis, with prefix (1.) refer to appendix 1 and similarly (2.) and (3.) for appendices 2 and 3.

When some other part of the thesis is referred to in the text, the section designation e.g. 'Introduction', 'Appendix 1' is given unless it lies within that section of the thesis, when no section designation is given.

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SUMMARY

1. The main aim of this work was to compare the efficiencies of growth of various phenotypes of Escherichia coli. Several strains were grown in arithmetic (Wallace, 1975), chemostat and batch cultures.

Values for maintenance energies were derived in order to calculate maximum growth yields in terms of carbon and oxygen. Efficiencies of ATP synthesis (expressed as $\frac{P}{O}$ ratios) were calculated from yields using the assumption that the energy required for cellular biosynthesis under anaerobic conditions applied to the aerobic cultures. $\frac{P}{O}$ ratios were then compared with $\frac{H^+}{O}$ ratios of the harvested bacteria.

2. The maintenance requirement was lower for arithmetic cultures than for chemostat cultures, suggesting that dropwise addition of nutrient in chemostat culture might uncouple cellular growth from energy supply.

3. E.coli ML30 (which is lac-inducible) had the same maintenance requirement as its lac-constitutive derivative, ML308, when it was grown on a non-inducing carbon source. The gratuitous synthesis and turnover of the lac enzymes therefore make negligible contributions to maintenance.

4. There were at least two classes of reaction responsible for maintenance one of which was independent of temperature while the other was strongly temperature-dependent. The latter had an activation energy similar to that of protein denaturation, suggesting that part of the maintenance requirement may be for macromolecular organisation.

5. Bacteria grown on glucose, glycerol, maltose, galactose or mannitol had $\frac{P}{O}$ ratios of 2, while those grown on lactose, gluconate or glucose 6-phosphate had $\frac{P}{O}$ ratios of 3. The phenotypes with the higher efficiency of energy conservation all transported their carbon sources by a proton-symport mechanism.

6. In pulse-oxidant determinations of $\frac{H^+}{O}$ ratios, cells with a $\frac{P}{O}$ ratio of 3 extruded more protons than those with a $\frac{P}{O}$ ratio of 2. This is an independent confirmation of the conclusion derived from growth yields.

7. Growth on lactose in batch culture or in the chemostat also gave a $\frac{P}{O}$ ratio of 3. High $\frac{P}{O}$ ratios are therefore not peculiar to arithmetic culture.

8. Bacteria which had high $\frac{P}{O}$ ratios had high maintenance coefficients. It is speculated that the additional maintenance requirement may be spent for organisation of a more efficient electron transport chain.

9. A period of logarithmic growth on the proton-symported substrate was required for the extra coupling site to be expressed, since cells which had previously been grown on glycerol with $\frac{P}{O}$ ratio of 2, grew on lactose with a $\frac{P}{O}$ ratio of 2 under carbon limitation in arithmetic culture.

10. *E.coli* ML30 grew on gluconate with a $\frac{P}{O}$ ratio of 3. Therefore, the *lac* enzymes themselves were not required for the higher growth efficiency.

11. When bacteria which had been grown on lactose were starved for 2 h, they lost the additional energy-coupling site as judged by $\frac{H^+}{O}$ ratios. The lac permease also decayed, indicating a possible relationship between the two processes.

12. Cultures of E.coli ML308 grown on either lactose or glycerol had active lac permease, but only the lactose phenotype had its $\frac{H^+}{O}$ ratio enhanced by adding non-metabolisable galactosides. Transport in cells growing on lactose can therefore affect energy conservation.

13. Addition of salt (NaCl, Na_2SO_4) to growth media reduced the $\frac{P}{O}$ ratios of cultures but did not significantly change their $\frac{^{O}H^+}{O}$ ratios. Salt must therefore either decrease the efficiency of the ATPase machinery or have an uncoupling effect on the membrane.

14. Cultures grew on lactose/glucose mixtures with a $\frac{P}{O}$ ratio of 3. Therefore when the additional coupling site was present, all carbon sources were metabolised through the same system with high efficiency.

15. The additional coupling site found in lactose-grown bacteria was not due to energy-conserving transhydrogenase activity nor to an alternative energy-conserving terminal oxidase. In the absence of other experimental data on the composition of the electron transport chain of this phenotype, models are presented to explain the results in terms of novel electron carriers or rearrangement within the membrane of preexisting electron carriers.

16. It is concluded that an additional site of energy conservation is synthesised by E.coli in response to transport of carbon sources which enter the cell via proton symport. This conclusion implies a relationship between active transport and electron transport which have hitherto been assumed to work independently of each other.

INTRODUCTION

Twenty years ago, Cohen and Monod (1957) published their classical paper on bacterial permeases. Although permeation had been recognised as important in the physiology of animal cells, comparitively little attention had been paid to bacteria. The innovative work of Gale (1947) with S.faecalis suggested that amino acids entered the cell by specific transport mechanisms. However, it was difficult to exclude the possibility that cells were non-specifically permeable to the amino acids and retained them by some intracellular binding mechanism (Gale, 1954).

The Paris group, led by Monod, had been studying lactose degradation by β-galactosidase in a 'mutabile' strain of E.coli (Monod et al., 1948), and had discovered a number of substrates which would bind to, but were not hydrolysed by the enzyme. The fortuitous combination of availability of non-metabolisable substrates which apparently entered the cell, and the ease with which mutants of this strain could be obtained led to the discovery and investigation of the 'β-galactoside' or 'lac' permease (Rickenberg et al., 1956). It was found that the lac permease not only catalysed the entry of galactosides into the bacterial cell, but could also concentrate the molecules within the cell. It was acknowledged that this would require expenditure of metabolic energy, but the mechanism of energy coupling to the active transport process remained unclear. Since then, the lac permease has been investigated as a model for active transport in bacteria.

At around the same time and largely from his observations on Micrococcus sp., Mitchell (1957) began to

formulate his 'chemiosmotic' hypothesis on the mechanism of solute transport through bacterial membranes. It developed to compare the transport carrier to an enzyme which was situated vectorially across the membrane. A nutrient in the external medium was the substrate and was delivered by the enzyme, chemically unaltered as the product to the cellular cytoplasm. Active transport was envisaged to occur as a secondary effect, the nutrient being cotransported along with some other bulk flow (Mitchell, 1962), which was thought to be a flux of protons.

The hypothesis was not generally accepted at that time as anything other than a theoretical concept, mainly due to lack of experimental evidence. It was extended (Mitchell, 1961) to include oxidative phosphorylation (another function exclusive to membranes) and for the next decade research focussed on this aspect of energy transduction with mitochondria as the usual experimental system. Meanwhile, research on active transport and the lac permease in particular stumbled from one conflicting model to another. It was not until the early 1970's, when it was shown that transport by the lac permease was linked to proton movements (West, 1970; West & Mitchell, 1972), that the use of bacteria again became fashionable in this context. It is largely as a result of subsequent experiments with bacteria that the 'chemiosmotic hypothesis' of the sixties has become the 'chemiosmotic theory' of 1977.

The revival of interest in energy transduction in bacteria has undoubtedly advanced the understanding of both oxidative phosphorylation and active transport, but these two aspects have rarely been considered together

experimentally, except for occassional acknowledgements that they may share the proton gradient as a common intermediate.

In this work, an attempt was made to estimate the efficiency of energy conservation in growing bacteria and to correlate this with the formation of the proton gradient by the cells. It was found that cells which were growing on a carbon source known to be cotransported with a proton were more efficient at energy conservation and at producing a proton gradient. It would appear that active transport and electron transport which have been considered formerly in isolation, may interact with each other through more than the proton gradient.

1. Transport

Although this thesis is concerned primarily with electron transport and oxidative phosphorylation, it is pertinent to include some review of the current understanding of transport processes, since their mechanisms have some bearing on the interpretation of the results.

Transport has been the subject of some recent reviews (Harold, 1972; Boos, 1974; Simoni & Postma, 1975; Hamilton, 1975; 1977). It is now thought that solute molecules may enter the bacterial cell in at least five ways.

1.1 Transport by simple diffusion

In the pursuit of identifying transport carriers, it is often forgotten that some solutes may enter the cell on their own by simple diffusion. The distributions of lipophilic molecules which have apparently no carrier have been used to measure the magnitude of the transmembrane proton motive force in bacteria (e.g. Griniuviene *et al.*,

1975; Ramos et al., 1976). These molecules are of little physiological significance, but it is likely that some other solutes may enter in a similar way. In particular, a transport carrier has yet to be identified for acetate, although E.coli can grow on it readily. The distribution of acetate has been used extensively in the determination of transmembrane pH gradients of mitochondria and it is likely that molecules such as acetate may enter bacteria by passive diffusion as the uncharged species.

1.2 Transport by facilitated diffusion

Facilitated diffusion is a passive process in which diffusion of the solute is assisted by a membrane protein which binds the solute and translocates it into the cell. Uptake is not coupled to metabolic energy so the process is incapable of concentrating the solute. The glycerol facilitator of E.coli (Richey & Lin, 1972) is the only example well characterised. Facilitated diffusion would appear to be the usual method of permeation of glycerol in bacteria (Lin, 1976).

It may be possible to convert active transport mechanisms to those of facilitated diffusion by 'uncoupling' the supply of energy from transport. This would make teleological sense if the solute were present in excess. However, it appears that the transport system for lactose in E.coli, at least, does not submit to such ideals (Koch, 1971; 1974). Mutants have been isolated which are unable to concentrate solute but which can use the carrier usually capable of active transport as a facilitator (Wilson & Kusch, 1972; West & Wilson, 1973; Rosen, 1973). Although useful in the elucidation of the transport

mechanism, they are of doubtful physiological importance.

Kornberg and Riordan (1976) have shown that galactose may enter E.coli by facilitated diffusion using the product of the umg gene as carrier. This protein is normally involved in the transport and phosphorylation of glucose and its analogues by a phosphotransferase system. A similar result has been obtained for galactose uptake in S. typhimurium (Postma, 1976). These studies have verified an earlier prediction (Tanaka & Lin, 1967) that the enzyme II complex of the phosphotransferase system would catalyse facilitated diffusion in the absence of phosphorylation.

1.3 Transport by phosphotransferase system

Since the initial discovery of a phosphotransferase system by Roseman in 1964, the phosphotransferase (PT) systems of E.coli and S. typhimurium have been studied extensively. Uptake of solute by these systems is accompanied by phosphorylation, with phoshoenol pyruvate donating the phosphoryl group. At least 4 proteins are required to effect translocation and phosphorylation. The identification of the components, the specificities of the enzymes and the elucidation of their mechanisms of action have been well-reviewed recently (Hamilton, 1975; Roseman, 1975; Kornberg & Jones-Mortimer, 1977). In addition to sugars, uptake of AMP in E.coli has been shown to be mediated by a PT system (Yagil & Beacham, 1975).

Current research aims to gain a greater understanding of the control of uptake by the PT systems, which are apparently constitutive, but which may be further induced (Clark & Holms, 1976).

The activity of glucose uptake in vivo is adjusted to variations in growth rate in order to balance the require-

ment for flux through the system (Herbert & Kornberg, 1976), although large changes in the level of expression of the glucose PT system may occur with no effect on growth rate of the culture (Clark & Holms, 1976). Glucose uptake must therefore be subject to 'fine' control. It is likely that availability of phosphoenol pyruvate restricts activity of the PT system and that either pyruvate or acetyl-coA are negative effectors (Kornberg & Jones-Mortimer, 1977).

The uptake of glucose by the PT system can prevent induction of the enzymes for the catabolism of other non-PT substrates e.g. glycerol, lactose, maltose (Saier & Roseman, 1976a). This effect, termed 'inducer exclusion', is due to an interaction of the basal level of permease of the non-PT substrate with the PT enzymes which denies the sugar access to the inside of the cell, thus preventing induction. However, in mutants which have constitutive enzymes for the utilisation of the second substrate glucose can still prevent its utilisation (Boniface & Koch, 1967), even when the second substrate has a PT mechanism such as fructose (Clark & Holms, 1976). A functional enzyme II of the PT system is required for this inhibition. It is possible to obtain mutations in the uptake system of the second sugar which overcome the control mediated by glucose (Amaral & Kornberg, 1975; Saier et al., 1976) suggesting that the second uptake system may have an allosteric site. Mutations in the enzyme I of the system render the cell incapable of taking up both PT sugars and most non-PT sugars (Saier et al., 1976b). A model has been proposed for the inhibition of uptake of other sugars by the PT system (Saier & Stiles, 1975).

The operation of the PT system also regulates the activity of adenyl cyclase (Saier et al., 1976a) to reduce the intracellular level of cAMP. This has a more general effect on the levels of expression of many cellular enzymes ('PT-mediated repression'). Mutants have been isolated which are no longer susceptible to PT-mediated repression (Saier & Roseman, 1976b). They are also defective in the enzymes IIB/III of the system, but it is not known whether either observation is the cause or effect of the other.

Clearly the PT-system is complex, both in its own regulation and in the regulation of other cellular activities. However, it may not be the 'overlord' of uptake as is often supposed; it has been recently reported that the transmembrane electrochemical potential ($\Delta\psi$) can inhibit the uptake of methyl-α-glucoside by the PT system in a cytochrome-deficient mutant (Singh & Bragg, 1976).

1.4 Active transport linked only to hydrolysis of ATP

Activities of some transport systems are drastically reduced by osmotic shock (Heppel et al., 1972) due to the loss of binding proteins from the periplasmic space. This unfortunately renders impossible the use of membrane vesicles (Kaback, 1971) as an experimental approach to investigate the mode of action of these systems.

Berger (1973), using an ATPase-negative mutant of E.coli, showed that the energy requirement for transport of glutamine (a shock-sensitive system) could be satisfied only by ATP or a 'high-energy' compound related to ATP. By contrast, the energy for transport of proline (a shock-

resistant system) came from an 'energised membrane state'. The mutant was incapable of generating an energised membrane state from ATP or of synthesising ATP by electron transport and oxidative phosphorylation. When ATP was supplied solely by glycolysis, it could not transport proline, but could transport glutamine: electron transport could not drive glutamine uptake but could drive proline uptake.

A number of solutes have been identified as having transport systems which are either shock sensitive or use ATP directly. Among them are diaminopimelic acid, arginine, histidine, ornithine (Berger & Heppel, 1974), isoleucine (Kobayashi et al., 1974), glycylglycine (Cowell, 1974), galactose (via mglP; Wilson, 1974a), glutamate (Miner & Frank, 1974), ribose (Curtis, 1974), maltose (Kellerman & Szmelcman, 1974), leucine (Wood, 1975), glycerol-3-phosphate (Silhavy et al., 1976), potassium (Rhoads & Epstein, 1977), and arabinose (via araF; Henderson & Daruwalla, 1977). Of these, transport of maltose, glycerophosphate and galactose have been further investigated.

The periplasmic binding proteins are also important as receptors in the chemotactic response of bacteria (Koshland, 1977). Mutants which lacked the binding protein for maltose (Hazelbauer, 1975) and galactose (Ordal & Adler, 1974) failed to exhibit a chemotactic response to them. However, not all chemotactic receptors are binding proteins. For example, the enzymes II of the mannose and glucose PT systems are responsible for the chemotactic response to these solutes (Adler & Epstein, 1974).

The periplasmic protein for galactose apparently had

two sites which had different affinities for the solute (Boos & Gordon, 1971). It was thought that this reflected the binding affinities for galactose at each side of the membrane. However, it was later shown that the protein had only one site; the second site was an artefact due to an impurity in the radiolabelled galactose (Zukin et al., 1977). The transport and chemotactic functions must therefore share a common binding site.

A protein, glpT, thought to be responsible for the uptake of glycerophosphate was identified from shock fluid of E.coli (Silhavy et al., 1976), but it was difficult to reconcile this function with the stimulation of transport by glycerophosphate in membrane vesicles, when the protein would be lost in their preparation. It is now thought that this protein is not responsible for translocation across the inner membrane of the cell, but may be required to overcome the diffusion barrier which the outer membrane may present to glycerophosphate (Boos et al., 1977). The receptor for phage λ has been identified as an element of the uptake mechanism for maltose (Szelcman et al., 1976). It may form a pore in the lipoprotein of the outer membrane to facilitate the entry of maltose to the periplasmic space. A separate protein, specified by malE is responsible for subsequent translocation of that solute.

These transport systems are apparently more complex in nature and more comprehensive in function than was thought originally. They are incapable of facilitated diffusion, but the site of hydrolysis of the ATP is unidentified. It must presumably be at the cytoplasmic surface of the inner membrane, since the cell is cryptic to nucleotides. The mechanism of this form of transport

is one of the many problems which remains to be solved.

1.5 Active transport linked to cotransport of another species

Free energy must be expended in transporting solutes 'actively' across membranes. In some cases, the free energy is supplied by the hydrolysis of ATP (1.4), but in others the solute may be transported actively against its chemical potential by either fluxing or counterfluxing with another species which is moving down its chemical potential; these systems do not have a periplasmic binding protein and are not sensitive to osmotic shock. Such considerations led Mitchell (1957; 1963) to formulate his chemiosmotic hypothesis of energy transduction. Ions which are cotransported in the same direction as the solute are said to 'symport' with the solute: those which move in the opposite direction to the solute are said to 'antiport' (Mitchell, 1970).

There are many examples of carriers in eukaryotic systems which cotransport solutes with inorganic ions, but evidence for the involvement of inorganic ions in bacterial systems is rather limited. Na^+ and K^+ have been shown to be required for transport of glutamate in E.coli (Halpern et al., 1973) and of α amino isobutyrate in a marine pseudomonad (Drapeau et al., 1966); Na^+ was required for transport of melibiose in S. typhimurium (Stock & Roseman, 1971) and of glutamate in B. licheniformis (Macleod et al., 1973); the transport of citrate in K.aerogenes has been shown to require K^+ (Eagon et al., 1972); Mg^{2+} has been shown to be required for the transport of citrate in B. subtilis (Wilkake et al., 1973). However, it is not known whether the inorganic ions were cotransported with

solute, or required simply as effectors. In addition, inorganic ion/solute symports could be secondary translocations driven by primary H^+/inorganic ion antiports. The existence of Na^+/H^+ and possibly of K^+/H^+ antiports has been demonstrated in E.coli (West & Mitchell, 1974).

The proton is by far the most common ion species involved in cotransport with solutes. Mitchell (1970) has discussed at length the theoretical operation of proton-coupled transport systems.

1.5.1 Proton-coupled active transport

Indirect evidence that transport of ions in bacteria was coupled to cotransport of protons was obtained by Harold & Baarda (1968) who showed that uptake of ions and anaerobic growth of S.faecalis was inhibited by uncouplers which enhance proton conduction. Uptake of thiomethyl galactoside (TMG) by E.coli under anaerobic conditions required a membrane which was impermeable to protons. (Pavlasova & Harold, 1969); active transport was reduced when the membrane was made permeable to protons by uncouplers. These experiments showed that transport could be affected by uncouplers without inhibiting the generation or utilisation of ATP which was produced glycolytically under these conditions. Involvement of ATP as the primary source of energy for transport was therefore unlikely. Until then, it had been thought that ATP was involved directly in active transport (1.5.2).

Uptake of protons was measured by West (1970) who showed that addition of lactose to E.coli resulted in alkalinisation of the medium. This was interpreted as transport powered by proton symport. Proton movement

could be dissociated from solute uptake by a mutation in the lac permease (West & Wilson, 1973). Uptake of TMG by the proton-symporting lac permease was confirmed in non-metabolising cell suspensions of E.coli (West & Mitchell, 1972) and shown to be 'electrogenic' since it involved a net charge displacement. In more quantitative experiments (West & Mitchell, 1973), a proton: lactose stoichiometry of 1:1 was claimed for the uptake of that solute. This stoichiometry is often quoted but was in fact an extrapolation; less than 1 H^+ was observed experimentally to cotransport with each lactose. Earlier Gale and Llewellin (1972) had reported transport stoichiometries for Staph. aureus of 0.65 H^+/aspartate and 0.9 H^+/ glutamate. Harold (1977) has recently listed the transport systems which are thought to work by a chemiosmotic proton-symport mechanism.

Having shown that protons could cotransport with solutes, it was necessary to establish that a 'protonmotive force' existed in bacteria (2.2); was developed by electron transport (2.1), and could power active transport. Many investigations aimed at demonstrating the production of a proton motive force have not estimated it directly, but have used stimulation of transport activity to show that a proton gradient was created. These studies (e.g. Schairer & Haddock, 1972; Cox & Gibson, 1973 for a review) played no part in the elucidation of transport mechanisms.

The evidence that transport could be driven by the protonmotive force was obtained initially with metabolically-resting cells. An artificial potassium gradient, corresponding to the membrane potential component ($\Delta\psi$;

2.2) of the proton motive force was shown to drive active transport of neutral amino acids in S. faecalis (Ashgar et al., 1973). This was confirmed in S. lactis (Kashket & Wilson, 1974) where accumulation of TMG was shown to be related to the magnitude of the protonmotive force.

In Hamilton's laboratory, a theoretical prediction of the chemiosmotic theory of active transport was confirmed experimentally for resting cells of Staph. aureus (Niven et al., 1973; Niven & Hamilton, 1973; 1974). Uptake of lysine, which is positively charged occured electrogenically on its own by a uniport mechanism driven only by the membrane protential component ($\Delta\psi$) of the protonmotive force; uptake of isoleucine, which is neutral, occurred electrogenically by a proton-symport mechanism and responded to both components of the protonmotive force ($\Delta\psi$, ΔpH); uptake of negatively-charged glutamate was electroneutral with a proton-symport mechanism, responding only to the pH component of the protonmotive force.

Harold and Levin (1974) showed that transport of lactate in S. faecalis was electroneutral, and involved a proton-symport mechanism. It was therefore similar to glutamate transport in Staph. aureus. Transport of lactate into the cell could be demonstrated using an artificially-created Δ pH gradient. However, the normal direction of lactate transport during fermentation is out of the cell indicating that the lactate carrier can act passively to the proton-coupling mechanism on either side of the membrane. Functional symmetry has been observed for the lac carrier (Teather et al., 1977).

Recently, the proton-symporting mechanisms of galactose and gluconate transport in Clostridium

pasteuranium have been shown to act respectively in an electrogenic and electroneutral manner (Booth & Morris, 1975). This approach has been extended to galactoside transport in E.coli which was first shown to be driven by the pH component of the protonmotive force (Flagg & Wilson, 1976) and then by both components (Flagg & Wilson, 1977). Entry of galactose will therefore be electrogenic. The observation that transport of 2-keto-3-deoxy-D-gluconate was driven by the pH potential alone implies that transport of this solute occurs electroneutrally (Lagarde & Haddock, 1977).

In the above experiments, metabolically-resting anaerobic cells were used. They do not carry out oxidative phosphorylation, are presumed to have minimal endogenous energy reserves and a negligible transmembrane protonmotive force. The artificially-induced changes in the protonmotive force are not masked or complicated by preexisting potentials. An equal, if not greater, amount of evidence to support the involvement of the protonmotive force in active transport has come from the use of bacterial membrane vesicles (Kaback, 1971) as an experimental system. The vesicles consist essentially of a membrane which includes some transport systems and the apparatus of electron transport which is inactive unless an exogenous energy donor is added. They therefore have a minimal residual protonmotive force in the absence of added energy donors.

Hirata et al., (1973) showed that a membrane potential ($\Delta\psi$) could be developed in E.coli vesicles and could drive proline transport. The work was extended (Hirata et al.,

1974) to show that an artificially imposed membrane potential could drive transport of proline, amino acids or TMG. Hinds and Brodie (1974) showed that uptake of proline was associated with the proton gradient developed by vesicles of Mycobacterium phlei.

These observations, but particularly the demonstration of a proton motive force in vesicles (Hirata et al., 1973) led Kaback (1974) to abandon his respiration-linked model of active transport in vesicles in favour of the chemiosmotic model. His subsequent industrious research programme has done much to confirm the chemiosmotic view of active transport. In particular, it has been shown that uncouplers inhibit transport in vesicles by increasing their proton permeability (Patel et al., 1975) and that the protonmotive force developed by respiration is sufficient to account for the accumulation of solute by active transport (Ramos et al., 1976).

Vesicles can also be made which have the reverse orientation to normal vesicles and to bacterial membranes in vivo. With them, it has been possible to study the active transport of Ca^{2+} into vesicles (equivalent to transport out of cells in vivo; Tsuchiya & Rosen, 1975). It has been shown that the proton gradient is involved in calcium transport by vesicles (Tsuchiya & Rosen, 1976).

1.5.2 Mechanisms for proton-coupled active transport

Before the acceptance of the chemiosmotic view of active transport, it had been assumed that hydrolysis of ATP, or a high energy compound, was coupled to accumulation of solute.

Most investigators used the β-galactoside permease of

E.coli as the model system. The early belief that energy coupling increased the rate of entry of solute (Kepes, 1960) was superceded when kinetic measurements showed that the effect of energy coupling was to decrease the rate of efflux of solute (Winkler & Wilson, 1966; Schachter & Mindlin, 1969; Maloney & Wilson, 1973). This belief was reinforced by the use of energy-uncoupled mutants (Wilson & Kusch, 1972; Wilson et al., 1972). However each study invoked a different conceptual kinetic model to explain its results. In a remarkably systematic study of transport of 2-keto-3-deoxy-D-gluconate in E.coli K12, Lagarde and Stoeber (1975) showed that this system responded to energy coupling by decreasing the affinity of the carrier at the inner membrane; energy coupling had no effect on the influx or efflux rates of carrier which had bound ligand. By contrast, Lancaster et al., (1975) concluded that partial de-energisation affected both influx and efflux of lactose on its carrier. It is often forgotten that the results, if not the interpretation, of these kinetic measurements of active transport are valid and any new model should be compatible with them.

The work of Tanner and coworkers on glucose transport in Chlorella vulgaris (Tanner, 1974, for a review) was the first kinetic investigation of transport to be interpreted along the lines of the chemiosmotic hypothesis. The transport carrier was shown to exist in either a protonated form or an unprotonated form, the former having a higher transport velocity. However, using E.coli, Koch (1971a; 1974) has shown that energy coupling is obligately linked to transport of lactose, even when the solute is entering the cell down its own chemical potential ('downhill').

The effect of uncouplers on downhill transport in energy-starved cells indicated that lactose transport was obligately linked to a proton symport (Cecchini & Koch, 1975), and the energy cost to the cell has been estimated (Purdy & Koch, 1976). Transport systems must therefore differ in being either obligately, or non-obligately linked to their energy supply.

Since his acceptance of the chemiosmotic interpretation of transport in 'his' vesicles, Kaback has used this system to investigate the mechanism of energy coupling to transport. Originally it seemed that the electrical potential developed by vesicles was insufficient to account for observed solute accumulation ratios (Schulinder & Kaback, 1975). Ramos et al., (1976) showed that the transmembrane pH gradient is larger than first thought and that the protonmotive force is sufficient to satisfy the accumulation ratios observed.

In alkaline conditions, the protonmotive force is diminished and is incapable of supporting measured solute accumulation ratios on the basis of transport by a 1 solute : 1 H^+ mechanism. It has been reported that under these conditions the stoichiometry changes to 1 solute : 2 H^+ (Ramos & Kaback, 1977) which is sufficient thermodynamically to account for the accumulation of solute. The mechanism by which this might occur is currently under investigation. During prolonged slow growth in the chemostat, mutants of E.coli were isolated (Collins et al., 1976) which were adventitiously capable of higher accumulation of amino acids. This was thought to occur by a change in the amino acid: H^+ stoichiometry from 1 : 1 to 1 : 2, and eventually to 1 : 4, although

the observed stoichiometries were not integers. The work on this interesting problem has been marred by assuming that the number of protons which cotransport with solute must be an integer value. This is envisaged conceptually as a system of protonation/deprotonation reactions by the carrier. In fact there is no thermodynamic reason for making this assumption. Lagarde (1976) has presented a convincing criticism of this misconception using a non-equilibrium thermodynamic approach. The variance in proton: sugar stoichiometries for the uptake of glucose analogues by Chlorella vulgaris has been studied (Gruneberg & Komor, 1976). Uptake of 1-deoxyglucose has a H^+:sugar stoichiometry of 2:1, but is thought to occur by the carrier cycling within the membrane to pick up a proton each time with a sugar molecule being transported only on every second cycle. Therefore, although the sugar is transported as a 1 carrier:1 sugar:1 H^+ species, the net effect is to increase the H^+:sugar stoichiometry. Future research will no doubt resolve the mechanism of variable proton stoichiometries for solute transport.

Kaback's group, using synthetic fluorescent galactosides, now have some information on the binding of solute to the lac carrier, and on the microenvironment of the lac carrier (Schulinder et al., 1975; Schulinder et al., 1977). The number of binding sites for solute has been shown to increase 10-fold in vesicles in the presence of an energy donor (Rudnick et al., 1976). This suggests an effect of energy coupling at the outside of the membrane, which would disagree with the earlier kinetic models of transport. Harold (1977) has criticised this interpretation by considering the effect of back potentials developed

across the vesicles on the fluorescent properties of the probes. Belaich et al., (1976) found no change in the enthalpy of binding or binding constant of the lac carrier on poisoning whole cells. The effect of energy coupling at the outer membrane may therefore be a property peculiar to membrane vesicles.

The recent reports of solubilisation and isolation of transport carriers which are inactive, but which may be subsequently reconstituted to regain at least some of their original activity (Hirata et al., 1976; Altendorf et al., 1977) are landmarks in the study of bacterial transport mechanisms. It is obvious that great advances have been made recently in the investigation of active transport, but to date no unifying model is available to explain satisfactorily all of the previous observations. Now armed with the powerful tool of reconstitution, the near future should show even greater advances in the understanding of active transport mechanisms.

2. Electron transport and oxidative phosphorylation

The mechanism of coupling of electron transport to oxidative phosphorylation has been subject to discussion since biochemistry was in its infancy (Englehardt, 1930). The early chemical coupling theory of energy transduction (Slater, 1971, for a review) has recently fallen into disfavour, mainly because of the failure to identify the high-energy intermediate which it invoked and to the lack of emphasis which it placed on the integrity of the membrane; the experimental requirement of a closed intact membrane for efficient energy transduction had no explanation in terms of this theory. Alternative hypotheses have

been proposed, based on the requirement for a functionally-intact membrane.

Most notable among the present hypotheses of energy transduction is that of Mitchell (1961; 1966; 1970). Mitchell proposes that the electron carriers are organised vectorially in the membrane like a spiral, so that electron transport generates a gradient of protons across the membrane ('protonmotive force'), the free energy of which drives energy-linked functions. The gradient is created when electrons move down the spiral towards the outside surface of the membrane accompanied formally as an $H^{\bullet}$ species, but return unaccompanied to the inner surface resulting in net translocation of protons from the cytoplasm to the external medium.

Other models also propose some form of vectorial organisation of the membrane. Green (1974) has postulated that charge imbalance within the membrane will occur at distances of greater than 30Å, which is less than the width of the membrane. He proposes, in his paired moving charge model, that electron flow is accompanied within short distances by compensatory movement of cations which return the electrons to the inner surface as the uncharged metal atom thus completing a turn of the spiral. Papa (1976; Papa et al., 1973) has drawn a parallel between proton-linked electron transport and the protonation of haemoglobin. He proposes that a change in the redox level of the metal complexes of the electron-carrier proteins, due to binding of electrons, will through a 'Bohr' effect change the protonation of the proteins. This will result in vectorial proton transfer due to the anisotropic arrangement of the electron carriers. These models only

differ from that of Mitchell in their attempts to explain the molecular mechanism of proton translocation. They all propose obligate coupling of electron transport to oxidative phosphorylation through a proton gradient which is generated between the two aqueous layers separated by the energy-transducing membrane.

Williams (1961; 1974; 1977) has suggested that electron transport results in separation of charge within domains of the membrane. This drives energy-linked functions directly. The proton gradient is not an obligate intermediate in energy transduction, but is given the function of storage of excess free energy, which may be used when electron transport is insufficient for the cellular requirement of energy. Transport, according to Williams, is driven through the proton gradient. A similar proposal to that of Williams has been made by Archibald et al., (1976). A topologically-closed membrane is not required for these hypotheses. Some recent evidence that ATP may be synthesised at an artificial membrane interface (Yaguzhinsky et al., 1976) using enzyme from submitochondrial particles supports Williams' hypothesis.

Current models for energy transduction therefore recognise the importance of the proton gradient as an obligate (Mitchell) or subsidiary (Williams) intermediate and are interpreted on the basis of charge separation effects. Since the 'chemiosmotic' hypothesis of Mitchell is widely accepted as the basis for current research, it is reasonable to use it as the basis for interpretation of results of this thesis.

2.1 Proton-coupled electron transport

The concepts and experimental examinations of the

chemiosmotic hypothesis with respect to electron transport have been adequately discussed in many reviews (e.g. Mitchell, 1966; 1970; Greville, 1969; Harold, 1972; Racker, 1975).

With mitochondria, it was possible to isolate some components of electron transport, to reconstitute them functionally (Green, 1963) and to study their individual reactions in isolation (Ragan, 1976; Papa, 1976). This initially was not possible with bacteria but their well-defined genetics, particularly those of E.coli prompted an alternative approach using mutants or phenotypic variants which lacked various redox carriers (Haddock, 1977, for a review). Now that the reconstitution technique for mitochondria is becoming available for bacteria, the two techniques should complement each other as a powerful tool in the elucidation of the mechanisms of energy transduction.

Sites of energy conservation have been identified in bacteria by comparing the efficiency of coupling of electron transport to energy-linked functions such as transport or the transhydrogenase reaction in mutants, mutants with reconstituted factors, and wild-type organisms (e.g. Haddock & Schairer, 1973; Cox & Gibson, 1974; Bragg & Hou, 1974; Haddock et al., 1974; Poole & Haddock, 1974). These studies have been successful in defining regions of the electron transport chain which are energy-conserving, but invariably energisation of the membrane by electron transport in these systems has been assumed to indicate the presence of a single coupling site. It should be concluded that a single coupling site is only a minimum value. Only when the experimental efficiency of proton

translocation is determined (3.2) can firm conclusions be made on the number of energy-conserving sites in a (reconstituted) region of the respiratory chain.

The chemiosmotic hypothesis requires the anisotropic arrangement of electron carriers within the membrane. Evidence for this is beginning to accumulate for mitochondria (Boxer , 1975; Eytan et al., 1975), but knowledge of bacterial membranes lags far behind. The bacterial membrane is anisotropic in that the ATPase is located at its inner surface. Within the membrane, a transmembrane orientation has been suggested for the nitrate reductase complex of E.coli (Garland et al., 1975a) but apart from this report and an indication that the lipid distribution within the membrane is anisotropic (Rothman & Kennedy, 1977), only indirect evidence is available. If it is assumed that the locations of electron carriers within the membrane are not altered during the preparation of vesicles, it is possible to examine the positions of some respiratory enzymes in normal and inverted vesicles by measuring their accessibilities to electrons from exogenous donors (Harold, 1974; Futai, 1974; Konings, 1974; Boonstra et al., 1975). Care must be taken that the vesicles do not contain a transport system for the exogenous electron donor (Nichols & Hamilton, 1976).

It must be concluded that, whereas electron transport chains of bacteria have been shown to be proton-translocating, the orientation of the component electron carriers is not fully known. Therefore models for electron transport, based on the specific location of the carriers (e.g. Poole & Haddock, 1975; Garland et al., 1975a) may be useful conceptually but must nevertheless be

considered to be speculative.

Recently on the basis of experimental evidence from mitochondria, the chemiosmotic hypothesis of Mitchell (1970) has been revised (Mitchell, 1975; 1976) to incorporate the protonmotive 'Q' cycle. As originally conceived, the electron-transport chain of mitochondria was thought to consist of three loops of electron carriers, each of which translocated 2 protons. Due to the inability to detect a carrier at a suitable redox potential between cytochromes b and c, the observation that ubiquinone works on both sides of cytochrome b (Cox & Gibson, 1974) and that ubiquinone could react with cytochrome c (Wikstrom, 1973), it is postulated that ubiquinone, in various redox forms, cycles within the membrane as a mobile electron carrier. The terminal two proton-translocating loops are thus replaced by a single loop involving ubiquinone and translocating 4 protons. The concept was developed for mitochondria but is equally applicable to bacteria although evidence for its existence in bacteria (Bragg & Hou, 1976) is slight.

The proposed proton-translocation stoichiometry ($2H^+$/loop) has been examined experimentally by measuring phosphate potentials ofrespiring mitochondria (Rosing & Slater, 1972). It was concluded that the theoretical stoichiometry was insufficient to account for the observed phosphate potentials but that a stoichiometry of $3H^+$/loop would suffice. This stoichiometry has been confirmed from observed calcium accumulation ratios (Nicholls, 1977) and from thermodynamic considerations (Van Dam et al., 1977). It has also been shown by pulse-oxidant (Brand et al., 1976)

and steady state determination (Reynafarje et al., 1976) of mitochondrial $\frac{H^+}{O}$ ratios that the higher stoichiometry is valid. The discrepancy between these and earlier values of Mitchell and Moyle (1967b) is accounted for on the basis of phosphate transport accompanied by uptake of protons, which was inhibited in the recent investigations. In a comparison between electron transport in mitochondria and bacteria (Garland & Haddock, 1977) it was concluded that the higher stoichiometry was unlikely in bacteria. This has recently been confirmed for P. denitrificans (John & Whatley, 1977).

2.2 The protonmotive force

The protonmotive force, which is the 'intermediate' in coupling of electron transport to oxidative phosphorylation is defined (Mitchell, 1970) as:-

$\Delta p = \Delta\psi - Z\,\Delta pH$, where Δp = protonmotive force

$\Delta\psi$ = membrane potential

ΔpH = transmembrane pH gradient

Z = conversion factor (pH/mV)

It is only relatively recently that the protonmotive force has been shown to exist in bacteria.

Grinivieve et al., (1975) reported a value of 140 mV in E.coli cells, using an ionic distribution technique, but Collins and Hamilton (1976) using the technique of Mitchell and Moyle (1969) observed a total value of 230 mV, consisting of $\Delta\psi$ of 132 mV and ΔpH of 1.65. This compared favourably with Staph. aureus, where the results were 211 mV, 134 mV, and 1.3 respectively.

Padan et al., (1976), recorded values for E.coli close to those of Grinivieve et al., of 122-129 mV for Δp.

However, if the external pH was varied, the $\Delta\psi$ remained constant at 30 mV but the pH gradient changed from 2.0 at pH 6.0 to zero at pH 7.65. Care must therefore be taken in comparing derived values of the protonmotive force.

The experimental method of manipulating the proton motive force by changing external pH has been used to investigate its relationship to active transport.

With membrane vesicles, oxidising ascorbate the $\Delta\psi$ was found to be constant with changes in external pH but the pH gradient decreased, thereby decreasing the overall protonmotive force (Ramos et al., 1976). These changes have now been related to accumulation ratios for active transport (Ramos & Kaback, 1977; 1.5.2). A study of alkalophilic bacteria, which grow at alkaline pH, and would therefore be presumed to have difficulty generating a pH potential has so far been fruitless (Haddock & Cobley, 1976). Although the magnitude of the protonmotive force has been correlated with accumulation ratios of solute (Kashket & Wilson, 1973; Ramos & Kaback, 1977) no attempt has yet been made at a correlation of Δp with phosphate potential in bacteria.

2.3 Oxidative phosphorylation

Despite convincing evidence that the bacterial ATPase could translocate protons, and was required to generate a protonmotive force for anaerobic transport (Yamomoto et al., 1973) it has been difficult to demonstrate the converse, that ATP could be synthesised by the protonmotive force. The ATPase of E.coli has been purified (Nelson, 1974; Futai & Heppel; 1974) but because of requirements for metals and coupling factors (Abrams et al., 1976)

reconstitution of the five separate components has proved difficult. The reverse reaction, production of a proton gradient by ATP, was demonstrated in E.coli membrane particles, (West & Mitchell, 1974a) but the ATP yield was only 0.58 H^+ translocated/P. Low yields of ATP synthesis for E.coli cells were demonstrated from artificially-induced gradients of protons and membrane potentials (Kashket et al., 1974), but yields were much higher for the anaerobe S. lactis. More recently, a method has been described to load vesicles with ADP, and to obtain vesicle preparations which are free of inverted or 'scrambled' vesicles. ATP is synthesised by them in response to an artificial pH gradient (Tsuchiya & Rosen, 1976a) and to an artificial membrane potential (Tsuchiya & Rosen, 1976b), in a reaction requiring magnesium. Previous low values are attributed to the non-homogeneity of the vesicle preparations, and to the lack of availability of ADP to act as a phosphate acceptor.

The ATPase of cells grown anaerobically with nitrate as acceptor (Hasan & Rosen, 1977) has been shown to be distinct from that synthesised under aerobic conditions, and preliminary evidence suggests that it is closely associated with the nitrate reductase complex. This may indicate a relationship between electron transport and oxidative phosphorylation which at present are thought to be related only by the protonmotive force.

3. Estimation of the efficiency of energy conservation in bacteria

Three approaches have been adopted to evaluate the efficiency of energy conservation in bacteria.

The direct approach (Hempfling, 1970; Knowles & Smith, 1970; Baak & Postma, 1971) involves measurement of the ATP synthesised in response to electron transport in non-growing cells or membrane preparations. In growing cells the ATP would be consumed immediately. The approach is limited particularly by the number of permeable electron donors which are available e.g. cells are cryptic to NADH which acts at the membrane inner surface. This problem may be overcome by using inverted membranes, but they are usually damaged or have essential factors lost during their preparation. Only occasionally with a 'good' preparation are reasonable results obtained. The approach has been justly criticised (van der Beek & Stouthamer, 1973).

The remaining two indirect approaches are the subjects of sections 3.1 and 3.2.

3.1 Estimation of the efficiency of energy conservation from molar growth yields

Since the initial postulation that growth yields of bacteria were proportional to the amount of ATP made available to the cells during growth (Y_{ATP}; Bauchop & Elsden, 1960) it has been possible, with some assumptions, to calculate $\frac{P}{O}$ ratios from growth yields. The approach has been reviewed by Stouthamer and Bettenhaussen (1973), and a modified method for E.coli is presented in Appendix 2 of this thesis. In simplified form, it is possible using growth yield data and a value for Y_{ATP} to calculate the amount of ATP required by the cell. Knowing the amount of ATP supplied by substrate phosphorylation, the deficit is assumed to be supplied by oxidative phosphorylation. Division of this latter amount by the oxygen utilisation

(which is the reciprocal of the oxygen growth yield) gives the $\frac{P}{O}$ ratio.

Some investigators have taken the $\frac{P}{O}$ ratio to be simply the quotient of Y_{ATP} and Y_O (Hadjipetrou et al., 1964; Meyer & Jones, 1973; Neijssel & Tempest, 1975), which neglects to account for the ATP synthesised by substrate-level phosphorylation. In others, in which substrate-level phosphorylation was accounted for (Hadjipetrou & Stouthamer, 1965; Watson, 1970; Stouthamer & Bettenhaussen, 1973; Farmer & Jones, 1976a) no account was taken of the energy production and consumption of the fraction of carbon source which was incorporated. In any case, this fraction is often underestimated (Appendix 2.3). Recently, Hempfling and Mainzer (1975) have calculated $\frac{P}{O}$ ratios for E.coli B by assuming that no ATP is required for synthesis of monomers from carbon source. This is not the case (Appendix 2.2).

The evaluation of $\frac{P}{O}$ ratios from molar growth yields depends critically upon an assumed value for Y_{ATP} for aerobically-growing cells. The value must be assumed, since it is impossible to evaluate Y_{ATP} under aerobic conditions without knowing the $\frac{P}{O}$ ratio. In general, the experimentally-derived Y_{ATP} for anaerobic conditions is assumed to apply for the aerobic case. This is the main contentious assumption of this approach.

A model has been presented (de Kwaadsteniet et al., 1976) which allows determination of Y_{ATP}, Y_{ATP}^{MAX} (3.1.1) and maintenance from measurements made at different growth rates, without requiring an assumed value for Y_{ATP}. However, the values of the constants have such wide

confidence intervals (e.g. Van Verseveld et al., 1976; Stouthamer, 1977) that the model at present is of doubtful significance. Until it is refined or a novel approach is presented, an assumed value of Y_{ATP} will still be required. The next section aims to discuss the evaluation of Y_{ATP} values and the justification for using them in calculations of $\frac{P}{O}$ ratios for E.coli.

3.1.1 Evaluation of Y_{ATP}

Y_{ATP} was initially determined experimentally by Bauchop and Elsden (1960) for fermentations with several species of microorganisms in complex media. They found a mean value of 10.5 g.cells per mol ATP synthesised for the limited range of microorganisms they used. Ten years later, a survey (Forrest & Walker, 1971) of 47 determinations reported since the initial observation showed a mean value of Y_{ATP} of 10.6 ± 1.0 g.mol^{-1}. Some experimental determinations, however, have fallen out with the normal range obtained for fermentations. In particular, low values for Y_{ATP} were observed for Zymomonas sp. (5.9 g.mol^{-1}, McGill & Dawes, 1971; 4.7 g.mol^{-1}, Belaich et al., 1972), whereas high values have been obtained for Lactobacillus casei (20.9 g.mol^{-1}, de Vries et al., 1970), K. aerogenes (16.1, Stouthamer, 1977) and Bdellovibrio bacteriovorus (18.5-25.9 g.mol^{-1}, Rittenberg & Hespel, 1975). This has led to the conclusion (Stouthamer, 1977) that Y_{ATP} may not be the universal biological constant which it is widely assumed to be, but which its original proponents in fact never claimed.

Theoretical approaches to the calculation of Y_{ATP}, most notably by Forrest and Walker (1971) and Stouthamer

(1973), concluded that Y_{ATP}^{MAX} (a value corrected for maintenance energy (Discussion 1), would be of the order of 30 $g.mol^{-1}$. No experimental report has actually achieved this theoretical limit.

Early measurements of growth yields, and therefore of Y_{ATP}, made no allowance of the potential yield which was lost due to maintenance energy (Pirt, 1965). The discrepancy between experimental values of Y_{ATP}, and the theoretically-derived Y_{ATP}^{MAX}, might possibly have been due to this omission. Y_{ATP}^{MAX} may be determined experimentally in anaerobic continuous-culture experiments, when the growth rate is varied. The experimentally-derived values of Y_{ATP}^{MAX}, while higher than the values for Y_{ATP} (24.3 $g.mol^{-1}$ for L. casei, de Vries et al., 1970; 19.9 $g.mol^{-1}$ for K. aerogenes, Stouthamer, 1977), do not approach the theoretical limit. In only two cases have experimental values approached 30 $g.mol^{-1}$. K. aerogenes was grown in an anaerobic tryptophan-limited continuous culture to give Y_{ATP}^{MAX} = 28.4 $g.mol^{-1}$ (Stouthamer & Bettenhaussen, 1973). However it was later reported that the maintenance requirement, which was large, had been overestimated (Stouthamer & Bettenhaussen, 1975). A more conservative estimate was 25.4 $g.mol^{-1}$ (Stouthamer, 1977). In the second report (Stouthamer & Bettenhaussen, 1976), Y_{ATP} for a glucose-limited 'pH-auxostat' of K. aerogenes was found to be 28.5 $g.mol^{-1}$. Except in this isolated instance, experimental values for Y_{ATP}^{MAX} do not approach their theoretical counterparts.

Values for Y_{ATP} also depend on the type of growth media used. Y_{ATP} for S. faecalis (Forrest & Walker, 1965) increased in complex media. The high value already

discussed for Y_{ATP}^{MAX} of L. casei (de Vries et al., 1970) was obtained in complex media; in simple media a value of 10.8 $g.mol^{-1}$ was determined (Brown and van Demark, 1968), Stouthamer & Bettenhaussen (1976) found a dedrease in Y_{ATP}^{MAX} from 19.9 $g.mol^{-1}$ to 14.0 $g.mol^{-1}$ when K. aerogenes was grown anaerobically on glucose in minimal rather than complex media.

The discrepancies between experimental Y_{ATP}^{MAX} values, and between corresponding values for simple and complex media have been partly attributed by Stouthamer (1977) to 'uncoupled' growth (Senez, 1962). Stouthamer suggests that uncoupled growth is more marked in simple than complex media and suggests other factors which may contribute to uncoupling to reduce theoretical values to those observed experimentally. Until experimental evidence for these factors becomes available they must be assumed to be speculative. To this end, the 'energised membrane' has been tentatively identified as a candidate for uncoupling potential energy for growth (Stouthamer & Bettenhaussen, 1977). Comparison of the Y_{ATP}^{MAX} values for an ATPase-negative mutant of E.coli for aerobic growth and its wild-type parent for anaerobic growth indicated that 58% of the cellular energy budget was 'wasted' in maintaining membrane potentials.

At the present time, it must therefore be admitted that the difference between experimental and theoretical values of Y_{ATP}^{MAX} is a puzzle. The postulated 'overflow' metabolism (Neijssel & Tempest, 1975) may account for some of Stouthamer's uncoupled growth, but it is difficult to envisage how this could be a major factor for cells growing at slow growth rates, which are used for

determining Y_{ATP}^{MAX}. Until the contribution of overflow metabolism is assessed properly, it will not be known if the search for further unknown energy functions in bacteria must continue.

From this discussion on the variations of Y_{ATP}^{MAX}, it might appear that it is impossible to employ a single unifying value for Y_{ATP} in the calculation of $\frac{P}{O}$ ratios. This may be true, but for E.coli growing on simple media it is likely that some of the above considerations are irrelevant.

For the experimental determination of Y_{ATP}^{MAX} in anaerobic conditions with complex media, transport and polymerisation of the monomers which form the cellular polymers will be at a maximum: little energy will be expended on the biosynthesis of cell monomers, which are mostly supplied. It seems reasonable to assume that the polymerisation process which make macromolecules under anaerobic conditions will do so with the same efficiency under aerobic conditions. That is, Y_{ATP}^{MAX} (anaerobic) should differ from Y_{ATP}^{MAX} (aerobic) only by the transport requirement for monomers. Stouthamer (1973), by assuming 1 ATP for most solutes transported, has calculated that less than 10% of the total energy requirement of cells is expended on transport. In the light of subsequent studies on transport (1.), which have shown some solutes to be transported with a H^+ (equivalent to $\frac{1}{2}$ ATP) and some not at all, this must now be considered an overestimate. An estimate of 5% for the transport demand would be generous, and would not significantly change the value of Y_{ATP}^{MAX}.

Especially for simple media, great emphasis has been

placed on the different energy requirements for biosynthesis of the cellular monomers from the media components (depending on their identity) and how this may affect Y_{ATP}^{MAX} (Stouthamer, 1973). If the aerobic value of Y_{ATP}^{MAX} is taken to refer strictly to the polymerisation steps (as it does under anaerobic conditions) and a separate account is taken of the energy requirement in converting components of simple media to cellular monomers (as described in Appendix 2), it should be valid to substitute a value of Y_{ATP}^{MAX} obtained under anaerobic conditions for the ATP requirement for polymerisation under aerobic conditions.

The two reported values of Y_{ATP}^{MAX} for anaerobically-grown E.coli (11.2 $g.mol^{-1}$, Stouthamer, 1969; 10.3 $g.mol^{-1}$, Hempfling & Mainzer, 1975) were obtained in simple media, and came close (perhaps fortuitously) to the Elsden value of 10.5 $g.mol^{-1}$, which was assumed in this work (Appendix 2). It is valid to use them, since these anaerobic values were obtained in simple media and cells were grown aerobically during this work in simple media. In addition, the Y_{ATP}^{MAX} values have been corrected for maintenance; the growth yields used with them in the calculation of $\frac{P}{O}$ ratios were also corrected for maintenance, (Discussion 1).

It is therefore with reasonable confidence that, during this work, $\frac{P}{O}$ ratios were calculated from molar growth yields using a value for Y_{ATP}^{MAX} of 10.5 $g.mol^{-1}$.

3.2 Estimation of the efficiency of energy conservation from respiration-linked proton translocation

The pulse-oxidant method developed by Mitchell and Moyle (1965; 1967b) for assessing the energy conservation of mitochondria was adapted successfully for bacteria

(Scholes & Mitchell, 1970; Lawford & Haddock, 1973). Jones (1977) has recently reviewed this technique for assessing bacterial energy conservation.

The method assumes firstly that the chemiosmotic hypothesis of Mitchell (e.g. 1970) is correct and that electron transport results in translocation of protons out of the cell. It further assumes that, for each pair of electrons passing through a redox loop of electron carriers in the membrane, a fixed number of protons (normally 2) will be ejected. Experimentally, a small pulse of oxidant is added to anaerobically resting cells, and the number of protons translocated is determined. The $\frac{H^+}{O}$ ratio is given as the quotient of number of protons translocated by amount of oxidant added.

If it is assumed that the ATPase of the cell operates by proton translocation (West & Mitchell, 1974a), is 100% efficient at recapturing protons, and has a fixed H^+ : ATP stoichiometry, $\frac{H^+}{O}$ ratios may be related to $\frac{P}{O}$ ratios. For equivalences of 2 H^+ per electron-transport loop and 2 H^+ per ATP by the ATPase, the $\frac{H^+}{O}$ ratio should be twice the $\frac{P}{O}$ ratio. H^+ : ATP stoichiometries measured for bacteria have been lower than the assumed value of 2 (2.1).

The simplicity with which $\frac{H^+}{O}$ ratios may be determined has recently made this a popular experimental technique for assessing efficiency of energy conservation.

Proton extrusion was first demonstrated in bacteria with Paracoccus denitrificans (Scholes & Mitchell, 1970). A $\frac{H^+}{O}$ ratio of 8 was obtained for cells oxidising endogenous substrates with oxygen as oxidant. It was concluded (on

the basis of 2 H^+/loop) that electron transport in *Paracoccus* must be similar to that of mitochondria, with 3 energy-conserving segments plus an energy-conserving transhydrogenase segment. For starved cells oxidising exogenous electron donors, the $\frac{H^+}{O}$ ratio of 8 has been confirmed (Lawford *et al.*, 1976) but only for cells in the early phase of exponential growth; cells approaching stationary phase have $\frac{H^+}{O} = 4$. Electrons from glycerol and succinate (presumably FAD-linked) have been shown to be oxidised via 2 segments of the electron transport chain ($\frac{H^+}{O} = 4$), whereas oxidation of ascorbate/TMPD involves only one segment ($\frac{H^+}{O} = 2$; Lawford *et al.*, 1976). Growth of sulphate-limited *P. denitrificans* has been shown to be accompanied by decreased efficiency of energy conservation, as judged by a decreased $\frac{H^+}{O}$ ratio (Meijer *et al.*, 1977). This has been interpreted, on the basis of 3-4 H^+/loop as the loss of one of the two energy-conserving loops for this strain. Clearly, until the number of H^+'s translocated per loop is established, all results of $\frac{H^+}{O}$ experiments must be open to alternative interpretations of this type. Recently, enormous $\frac{H^+}{O}$ ratios in excess of 10 have been measured for *P. denitrificans* (A. H. Stouthamer, personal communication) which would, perhaps, suggest that reconsideration of the H^+/loop stoichiometry of this organism is in order.

E.coli K was used for proton extrusion experiments by Lawford and Haddock (1973) who demonstrated two energy-conserving loops for this strain ($\frac{H^+}{O} = 4$). This was confirmed using another strain, *E.coli* W (Brice *et al.*,

1974). The first energy-conserving segment of an E.coli K12 derivative was shown to be lost during sulphate-limited growth ($\frac{H^+}{O}$ = 2; Poole & Haddock), but this could not be repeated for E.coli W (Farmer & Jones, 1976a). It has been suggested that this may be due to strain differences (Haddock, 1977). $\frac{H^+}{O}$ ratios consistent with 2 energy-conserving loops have also been observed during a study of the action of colicins on E.coli membranes (Gould et al., 1976). Using nitrate as pulse-oxidant , and cells grown anaerobically, it has been shown that E.coli has 2 energy-conserving loops during anaerobic growth with nitrate as acceptor (Garland et al., 1975a). One site of energy conservation may be associated with the transmembrane nitrate reductase complex.

The technique has been used to advantage in Jones' laboratory in a comparitive study of the efficiency of bacterial electron transport (Jones et al., 1975; Jones, 1977). It was shown that $\frac{H^+}{O}$ ratios of bacteria may be correlated with the composition of their electron transport chains. In particular, bacteria lacking cytochrome c (including E.coli) have two energy-cpnserving segments; in other bacteria, the presence of a c-type cytochrome is accompanied by an additional coupling site. Transhydrogenase activities, when present in bacteria, may or may not contribute to energy conservation. It was also possible to demonstrate that one of the terminal branches of the electron transport chain of Azotobacter vinelandii was energy conserving, whereas the other was not (Downs & Jones, 1975).

The pulse-oxidant technique of assessing energy

conservation has distinct advantages in terms of time spent obtaining results, and the ease with which they may be interpreted. However it is often forgotten that cells displaying proton pulses are behaving non-physiologically; normally protons extruded from the cell will be re-captured immediately for energy-linked functions. To base conclusions about the growth efficiency of microorganisms solely on this non-physiological technique may lead to errors. Papa (1976) has discussed several criticisms of this technique for mitochondria but they apply equally to bacteria. In addition, the dogmatic interpretation of results on the basis of tenuous assumptions of stoichiometries of H^+/loop and H^+/ATP may have to be revised in the light of current revelations on the H^+: loop stoichiometry of mitochondria (2.1).

4. Aims of this work

The efficiency of bacterial energy conservation may be inferred by manipulation of molar growth yield data (3.1). The method requires some basic assumptions, most notably a value of Y_{ATP}^{MAX} under aerobic conditions. It is possible to argue that such an assumption is valid (3.1.1), but it is impossible to prove that this is the case.

$\frac{H^+}{O}$ ratios also report on the efficiency of energy conservation (3.2). Their interpretation requires a separate series of assumptions.

It was the aim of this work to estimate the efficiency of energy conservation of E.coli ML308; by the two methods described above. Since the assumptions required for their interpretation are mutually exclusive, the two methods are independent. Agreement of results between the two

approaches would also be strong evidence to validate the assumption of Y_{ATP}^{MAX}.

For cells which were identified as having high efficiencies of energy conservation ($\frac{P}{O} = 3$), an attempt was made to identify the 'effector' and mechanism of this unusual phenotype.

Since this work was commenced, molar growth yields have been compared with $\frac{H^+}{O}$ ratios for several bacterial species (Jones et al., 1975; Jones, 1977).

METHODS

1. Microbiological techniques

1.1 *Escherichia coli* ML308 (ATCC 15224; American Type Culture Collection, Rockville, Maryland, U.S.A.) was used in the majority of experiments. This organism synthesises the *lac* enzymes constitutively.

For some experiments, it was necessary to grow cells without the *lac* enzymes. In these cases strain ML30 (NCIB 10,000; National Collection of Industrial Bacteria, Torry Research Station, Aberdeen), the inducible parent of ML308, was used.

The presence of the glycerol enzymes was required in cells used in proton extrusion experiments. Since the glycerol regulon is normally inducible in wild-type cells (Lin, 1976), a constitutive mutant of ML308 was used. This mutant, designated '51' (Forrest, 1974), synthesises the glycerol enzymes under all circumstances. Therefore, irrespective of phenotype, cells of strain 51 were always able to metabolise glycerol during proton extrusion experiments.

Escherichia coli K10 was also used. It was the gift of Dr. P. J. F. Henderson, Department of Biochemistry, University of Cambridge.

All strains were characterised by bacteriological tests described by Cowan and Steel (1965). These were: growth, and fermentation of each of glucose, lactose, sucrose and dulcitol when added to peptone water; growth on Koser's citrate medium; growth, acid production and acetoin fermentation in Voges-Proskauer medium; indole production

and synthesis of urease.

1.2 Reconstitution and storage of organisms

ML strains were obtained as freeze-dried samples in evacuated glass vials, and stored at 4°C. The vials were opened as recommended (National Collection of Industrial Bacteria Catalogue, Aberdeen). The lyophilisate was taken up in a few drops of nutrient broth, transferred to 10 ml of nutrient broth and incubated at 37°C for 24 h.

K10 strains were obtained on agar slopes and stored at 4°C. Some cells were removed from the surface of the agar using a sterilised platinum loop (Macfarlane Robson, Glasgow) and transferred to 10 ml of nutrient broth, which was incubated at 37°C for 24 h.

In both cases, the preliminary broth cultures were plated on nutrient agar. After incubation at 37°C for 24 h, a typical colony was picked off and used to inoculate 10 ml of fresh nutrient broth. Once grown, the broth was checked for homogeneity - both microscopically and by plating on BCIG agar. Lac constitutive strains produce blue colonies on BCIG agar, whereas inducible strains have white colonies. A homogeneous nutrient broth was the inoculum for a cooked meat culture of the organism. It was in this form that long-term liquid cultures were maintained.

Every three months, six fresh nutrient broths were prepared from the cooked meat culture. The homogeneity of these broths was checked microscopically and by plating on BCIG agar. Each month, a fresh nutrient broth was used for preparation of inocula for experiments.

1.3 Preparation of trained inocula for experiments

While stored in nutrient broth cells are dormant. Before being used for growth experiments they were grown in batch culture in medium similar to that to be used for the growth experiment. This process was termed 'training'.

Three drops of a fresh nutrient broth culture were used to inoculate 100 ml of complete medium (3.5.1), which was contained in a 250 ml conical flask. This, 'the first passage', was incubated at 37°C on a rotating shaking table (Griffin and George Ltd., East Kilbride, Scotland) for a period of time which was dependent on both the organism and carbon source (Fig. 1). When fully grown, 1 ml of this culture was used as inoculum for 100 ml of fresh complete medium ('second passage') and incubated at 37°C for the appropriate time (Fig. 1). The 'third passage' was prepared in a similar way, using 1 ml of second passage as inoculum.

Fully-grown third passages were used as inocula in most experiments.

1.4 Centrifugation

To prepare washed cell suspensions, small volumes of cells (less that 250 ml) were centrifuged for 10 min at 4°C and 11,750 g in a MSE '18' centrifuge (MSE Ltd., Crawley, Sussex). For larger volumes, a MSE '6L' centrifuge (MSE Ltd., Crawley, Sussex) equipped with a 6 x 750 ml rotor was used. In this case, cells were centrifuged for 25 min at 4°C and 6,000 g.

When it was required to maintain the sterility of the culture during centrifugation, sterile 50 ml closed polycarbonate tubes were used (MSE Ltd., Crawley, Sussex).

Figure 1 PREPARATION OF INOCULA

The concentrations of carbon sources used for training of inocula are shown in column B. The media (2.5.1) were made by mixing component parts, of which carbon/Mg (solutions H, H^1 in column A; (2-5) was one.

The time required for training of inocula (1.3) depended on the passage number and carbon source. It also depended on the strain - K10 strain took longer ( ) than ML strains to achieve full growth.

carbon source	A concentration in solutions H,H^1. (mmol.l^{-1})	B concentration in flasks for training (mmol.l^{-1})	Passage(h.)		
			1	2	3
Galactose	25	10	16 (36)	7 (12)	7 (12)
Sodium Gluconate (pH7.0)	25	10	17	8	8
Glucose	25	10	16 (24)	7 (10)	7 (10)
Sodium Glucose-6-Phosphate (pH7.0)	25	10	17	7	7
Glycerol	50	20	17 (36)	8 (12)	8 (12)
Lactitol	12.5	5	16	7	7
Lactose	12.5	5	16	7	7
Lactulose	12.5	5	16	7	7
Sodium Malate (pH7.0)	50	20	17	8	8
Maltose	12.5	5	16	7	7
Mannitol	25	10	16	7	7

1.5 Viability

Viability was assessed by a modification of the micro-colony counting technique described by Postgate (1969). It was developed during this work. A full description is given in Development of Methods 3.

2. Sterilisation

Sterilisation of apparatus and media was carried out by one of four procedures.

2.1 Moist heat

Growth apparatus and solutions were sterilised in a pressure chamber (Manlove Alliott, Nottingham, England) using steam generated by a Speedylic electrode boiler (Bastian and Allen, Harrow, England).

At both operating temperatures (109°C, 121°C), the time for sterilisation had been checked for various volumes using thermocouples immersed in the solutions (C. A. Fewson, personal communication). Efficiency of sterilisation was always checked using Browne steriliser control tubes (Type 1, black spot; A. Browne Ltd., Leicester, England).

2.2 Dry heat

Only glass pipettes or pasteur pipettes, and occassionally empty bottles, were sterilised by this method.

Pipettes were either wrapped in Kraft paper or placed in metal canisters, and sterilised by dry heat at 160°C for 1¾ h. Sterilisation was checked by including a Browne steriliser control tube (Type 3; A. Browne Ltd., Leicester, England) in each canister or batch of wrapped pipettes.

2.3 Filtration

Sterilisation by filtration was carried out using

Sterifil filter holders fitted with 0.22 μm pore size Milliport membranes (Millipore Corp., Massachussetts, U.S.A.). Once assembled, the holders were sterilised by autoclaving at 121°C.

For smaller volumes (~100 ml) Nalge disposable filters (0.20 μm, Sybron Corp., Rochester, U.S.A.) were used. These were obtained in the sterile condition. Although sold as disposable items, these filters were washed, resterilised (2.4), and re-used.

In both cases, solutions which had been sterilised were transferred to sterile bottles.

2.4 Ethylene Oxide

Apparatus made of plastic (pippettes and filters) which were formerly used as disposable items were recycled and sterilised by ethylene oxide.

All items were sealed in polythene film and exposed to ethylene oxide (Anprolene) for 12 h in a steriliser box (AN74; H. W. Anderson Products Ltd., Clacton-on-Sea, England). Exposure to the gas was verified by including gas indicator tubes (AN85) with each item. All apparatus was aired for at least 24 h prior to use to remove residual traces of the gas.

3. Media

All media were prepared using glass-distilled water.

3.1 Cooked meat medium

A tablet of dehydrated cooked meat medium (Oxoid CM440) was soaked in 10 ml of distilled water for 15 min in a 25 ml Universal bottle (Macfarlane Robson, Glasgow), sterilised by autoclaving at 121°C and stored at 4°C.

3.2 Nutrient broth

Dehydrated nutrient broth granules (Oxoid CM1) were reconstituted (13 $g.l^{-1}$), dispensed by 10 ml into 25 ml universal bottles, sterilised by autoclaving at 121°C and stored at 4°C.

3.3 Nutrient agar

Oxoid No. 1 agar (3 g) was added to 200 ml nutrient broth solution and sterilised by autoclaving at 121°C. The molten agar was cooled to 60°C in a thermostatted water bath, and poured into petri dishes in a lamina flow hood (Microflow Ltd., Fleet, Hants), which had been swabbed previously with n-propanol. Plates were generally dried overnight at 37°C and then stored at 4°C.

3.4 BCIG nutrient agar

One ml of BCIG (2 mg/ml of dimethyl formamide) was added to 200 ml sterile nutrient agar which had been cooled to 60°C. Plates were poured as for nutrient agar (3.3).

3.5 Defined media

Several types of defined media were used, depending on the culture conditions. Due to incompatability during autoclaving, each medium was prepared as separate components which were mixed aseptically prior to use. The following solutions were used:

Solution A; contained KH_2PO_4 (66.7 $mmol.l^{-1}$) and $(NH_4)_2SO_4$ (16.7 $mmol.l^{-1}$) which were brought to pH 7.0 with NaOH, dispensed in 60 ml amounts into 250 ml conical flasks which were closed with polystyrene foam bungs (A. & J. Beveridge Ltd., Edinburgh), and autoclaved at 121°C.

Solution B; KH_2PO_4 (42.1 $mmol.l^{-1}$; 760 ml), adjusted

to pH 7.0 with NaOH, was placed in a 1 l side arm flask and autoclaved at 121°C.

Solution C; KH_2PO_4 (44.4 mmol.l^{-1}; 1440 ml), adjusted to pH 7.0 with NaOH, was placed in a 2 l side arm flask and autoclaved at 121°C.

Solution D; KH_2PO_4 (40.9 mmol.l^{-1}; 19.55 l), adjusted to pH 7.0 with NaOH was placed in a 20 l reservoir (FV 20L; Macfarlane Robson Ltd., Glasgow). Solution G (100 ml) was added and the combined solution autoclaved at 121°C.

Solution D^1; contained the same KH_2PO_4 content as solution D, but 100 ml solution G^1 was added prior to autoclaving.

Solution E; contained $MgSO_4$ (40 mmol.l^{-1}) and $(NH_4)_2SO_4$ (0.8 mol.l^{-1}).

Solution E^1; contained $MgCl_2$ (40 mmol.l^{-1}) and NH_4Cl (0.8 mol.l^{-1}).

Solution F; contained $FeSO_4$ (0.8 mmol.l^{-1}), adjusted to pH 2.0 with HCl and autoclaved at 121°C.

Solution F^1; contained $FeCl_2$ (0.8 mmol.l^{-1}), adjusted to pH 2.0 with HCl and autoclaved at 121°C.

Solution G; Chel metals, was prepared as follows (C. A. Fewson, personal communication). In 500 ml distilled water, to which was added 125 ml NaOH, were dissolved 50 g nitrilotriacetic acid ("Chel NTA"). The combined solution was brought to pH 7.0 with HCl. 1.1 g (4 mmol) $FeSO_4 . 7 H_2O$, 50 mg (0.21 mmol) $Na_2MoO_4 . 2 H_2O$, 50 mg (0.22 mmol) $MnSO_4$, 50 mg (0.77 mmol) $ZnSO_4 . 7 H_2O$, 25 mg (0.10 mmol) $CuSO_4 . 7 H_2O$, and 25 mg (0.11 mmol) $CoCl_2 . 6 H_2O$ were added and then distilled water to 1 l. The solution was dispensed in 100 ml amounts and autoclaved at 109°C.

Solution G^1; Chel metals - sulphate limitation, was identical in molar composition to solution G, except that chloride salts replaced sulphate salts.

Solution H; carbon source /Mg, contained carbon sources as described in Fig. 1, Col A and $MgSO_4$ (1.25 mmol.l^{-1}). They were dispensed by 40 ml and autoclaved at 109°C.

Solution H^1; carbon source/Mg - sulphate limitation, had the same carbon composition as solutions H, but $MgCl_2$ (1.25 mmol.l^{-1}) replaced $MgSO_4$. They were dispensed in 40 ml amounts and autoclaved at 109°C.

3.5.1 Defined media for training of inocula

40 ml of carbon source/Mg (solutions H) and 1.25 ml solution F were added aseptically to 60 ml solution A. These media therefore contained:

KH_2PO_4 (pH 7.0)	40 mmol.l^{-1}
$(NH_4)_2SO_4$	10 mmol.l^{-1}
$FeSO_4$	0.01 mmol.l^{-1}
$MgSO_4$	0.5 mmol.l^{-1}

+ carbon source, generally containing 60 mg atom carbon.l^{-1}.

3.5.1.1 Defined media for training of inocula - sulphate limitation

Media for sulphate limitation were prepared as in (3.5.1) using the chlorides instead of sulphates. Limiting sulphate was added as sterile K_2SO_4.

3.5.1.2 Defined media for training of inocula - high salt conditions

In addition to the constituents described in (3.5.1), these media contained NaCl (0.5 mol.l^{-1}) or Na_2SO_4 (0.25 mol.l^{-1}).

3.5.2 Defined media for batch culture growth experiments

These media had the same inorganic salts composition as defined media for training of inocula (3.5.1). Solutions E and F (10 ml of each) were added to 760 ml solution B. Carbon source was made up at high concentration, and sterilised either by autoclaving at 109°C or by filtration and added to complete the medium.

3.5.2.1 Defined media for batch culture growth experiments - sulphate limitation

These media contained the same inorganic salts as (3.5.1) except that chlorides were used instead of sulphates. To 760 ml solution B was added 10 ml of each of solution E^1 and of solution F^1. Sterile carbon source was added, together with an appropriate volume of sterile K_2SO_4.

3.5.3 Defined media for arithmetic culture growth experiments

In arithmetic culture, much higher cell densities are achieved than are normally used for batch culture. It was necessary to increase the inorganic salts content of these media to ensure that no accidental salt limitation was imposed.

To 1440 ml solution C was added 80 ml solution E and 80 ml solution F.

These media therefore contained:

KH_2PO_4 (pH 7.0)	40 $mmol.l^{-1}$
$(NH_4)_2SO_4$	40 $mmol.l^{-1}$
$FeSO_4$	0.04 $mmol.l^{-1}$
$MgSO_4$	2.0 $mmol.l^{-1}$

Carbon source was made up separately, sterilised by filtration and placed in the sterile reservoir for carbon source.

The concentration of carbon source depended on its nature:

Galactose	0.32 $mol.l^{-1}$
Gluconic acid	0.32 $mol.l^{-1}$
Glucose	0.32 $mol.l^{-1}$
Glucose 6-phosphoric acid	0.32 $mol.l^{-1}$
Glycerol	0.64 $mol.l^{-1}$
Lactose	0.16 $mol.l^{-1}$
L-Malic acid	0.64 $mol.l^{-1}$
Maltose	0.16 $mol.l^{-1}$
Mannitol	0.32 $mol.l^{-1}$

In experiments where dual carbon sources were used, the total carbon content in the reservoir was 1.92 g.atom carbon.l^{-1}.

3.5.3.1 Defined media for sulphate-limited arithmetic culture experiments

In these experiments, carbon source was present in the growth flask in excess, while limiting K_2SO_4 was pumped from the side reservoir.

To 1340 ml solution C, was added 80 ml solution E^1 and 80 ml solution F^1 and carbon source (100 ml, containing 0.192 g. atom carbon). Sterile K_2SO_4 (5 $mmol.l^{-1}$) was placed in the side reservoir.

3.5.3.2 Defined media for arithmetic culture experiments - high salt conditions

These media were identical to those of (3.5.3) except that either NaCl (0.5 $mol.l^{-1}$) or Na_2SO_4 (0.25 $mol.l^{-1}$) was present in the growth vessel.

3.5.4 Defined media for continuous culture experiments

To 19.65 l solution D was added 250 ml solution E and 100 ml carbon source which had been sterilised by filtration. Carbon source was added to give final concentrations of 30 mg atom carbon.l^{-1}.

i.e. glucose	5 mmol.l^{-1}
glycerol	10 mmol.l^{-1}
lactose	2.5 mmol.l^{-1}

These media were therefore basically of the same inorganic salts composition as defined media for training of inocula (3.5.1) but contained, in addition a selection of trace metals.

3.5.4.1 Defined media for sulphate-limited continuous culture experiments

To 19.65 l solution D^1 was added 250 ml solution E^1 and 100 ml carbon source sterilised by filtration. Sterile K_2SO_4 was added to the required concentration.

3.5.5 Solid defined media

These media had the same composition as defined media for training of inocula (3.5.1) but contained in addition Oxoid No. 1 agar (15 g.l^{-1}).

Generally 120 ml solution A (3.5), and 3 g agar granules were mixed and then autoclaved at 121°C. Once cooled to 60°C, 80 ml carbon source/Mg (Fig. 1, col A) and 2.5 ml solution F (3.5) were added and the molten agar poured into plastic petri dishes in a lamina flow hood (Microflow Ltd., Fleet, Hants). Once dried, plates were stored at 4°C.

4. pH measurement of media and cultures

Solutions were adjusted to the required pH by addition

of acid or alkali. pH was determined using a direct reading pH meter (Model 7010; EIL Ltd., Chertsey, Surrey) connected to a combined glass electrode (No. 224; Probion Ltd., Lesley, Fife).

The pH of cultures was determined by transferring a small volume of culture (normally 4 ml) to a microassembly equipped with microelectrodes (EIL) which were connected to a pH meter with large scale expansion (Model 2320; EIL Ltd., Chertsey, Surrey).

In both cases meters were calibrated daily using a fresh solution of standard buffer, pH 7.0 (B.D.H. Ltd., Poole, Dorset).

5. Glassware

5.1 General glassware

All glassware was cleaned before use by autoclaving in hemosol solution (10 $g.l^{-1}$; Meinecke & Co., Baltimore, U.S.A.). The glass was rinsed with tap water, twice with distilled water and dried in an oven.

5.2 Pipettes

Pipettes were cleaned by soaking in 'Kirbychlor' disinfectant solution (H. & T. Kirby & Co., Ltd., Mildenhall, Suffolk), then in hemosol solution (10 $g.l^{-1}$) followed by rinsing in tap and distilled water, and drying in an oven. All pipettes were plugged with cotton wool before sterilisation.

6. Growth apparatus

6.1 Batch culture

Some experiments were done in batch culture. Complete

defined medium (800 ml), in a 1 l flat-bottom flask equipped with side arm, was inoculated with bacteria and maintained at 37°C in the apparatus of Harvey et al., (1968).

In experiments when gas analysis was not done, the side arm was covered by a Morton culture tube closure (Scientific Products, Evanston, Illinois). Samples could be removed through the side arm by pipette. The open neck of the flask was closed with a silicone bung drilled to accommodate a glass capillary tube. Sterile air (100-200 $ml.min^{-1}$) was supplied to the culture, through this port, from the departmental compressed air supply. Flow was monitored by gas flow guage (G. A. Platon, Crawden, England).

For experiments on which analysis of culture gas was to be done, the side arm was sealed with a small silicone bung through which was inserted a metal canula fitted to a 10 ml plastic syringe (Becton, Dickinson & Co., Ltd., Drogheda, Ireland). Cultures were sampled by rinsing the syringe with culture, then removing a portion to a test tube. The open end of the flask was sealed with a silicone bung drilled to accommodate an air entry port and exit condenser (similar to Fig. 4b).

6.2 Continuous culture

With continuous culture the growth rate of the culture (μ) may be varied over a wide range, whereas in batch culture cells generally grow only at one specific growth rate, the μ_{max}, an intrinsic constant for the particular culture conditions imposed. Some theoretical considerations of continuous culture are discussed in Development of Methods (1), which will explain the

operational difference between the two types of apparatus which were used during this work.

The first system, which is used routinely in many laboratories, was similar in design to that first described by Baker (1968). Variable speed pumps controlled the rate of entry to (and exit from) the culture vessel and different growth rates were achieved by altering the rate of entry of fresh medium to the culture, which was maintained at constant volume.

In the second system, fixed rate pumps were used and growth rate varied by changing the volume of the culture. Because of its relatively low cost and the simplicity of its operation, most of the continuous culture experiments were done using this second system.

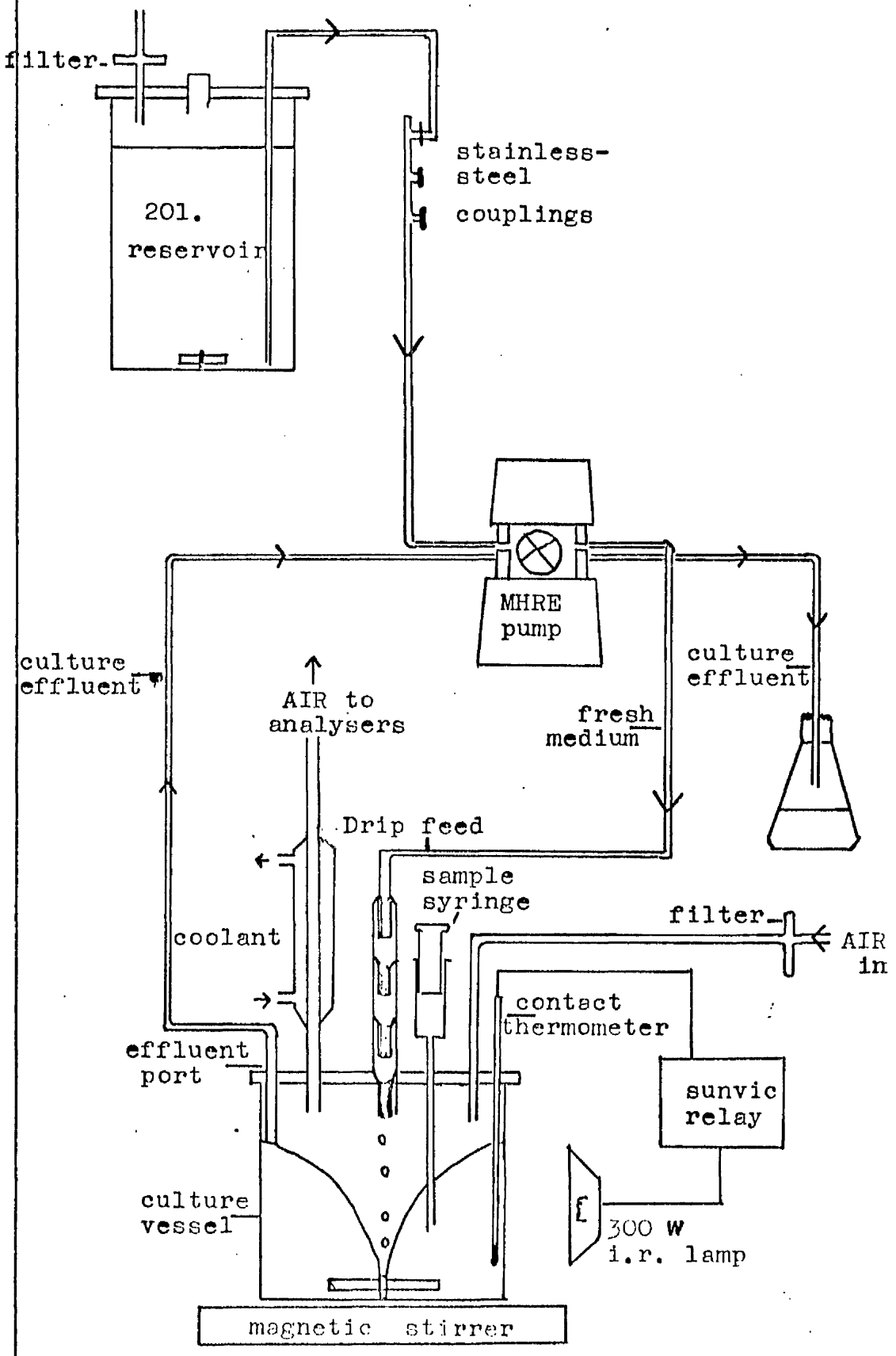
6.2.1 Continuous culture - variable flow, constant volume

This system (Fig. 2) has been described recently by Clark and Holms (1976), but several modifications were necessary to enable gas exchange measurements to be made.

Culture medium was stored in a 20 l reservoir (Quickfit FV 20L; Macfarlane Robson Ltd., Glasgow) fitted with a top plate (Quickfit MAF 2/2) which had ports to allow addition of a carbon source, venting by sterile air and removal of the medium to the growth pot. Incorporated in this feed-line was a system of stainless steel couplings (made in the Departmental Workshop by Mr. N. Harvey) which allowed reservoirs of fresh medium to be added to and empty reservoirs to be removed from the system. The medium was pumped into the growth vessel by a variable speed pump (MHRE 200; Watson-Marlow Ltd., Falmouth, Cornwall), equipped with dual tubing capstan. A multistage drip assembly

Figure 2 CONTINUOUS CULTURE GROWTH APPARATUS - VARIABLE FLOW, CONSTANT VOLUME

The apparatus was similar in principle to that of Baker (1968), but modified to allow gas exchange measurements to be made. It is described in Methods (6.2.1).


filter
20l.
reservoir
stainless-
steel
couplings
MHRE
pump
culture
effluent
AIR to
analysers
fresh
medium
culture
effluent
Drip feed
sample
syringe
coolant
filter
AIR
in
contact
thermometer
effluent
port
sunvic
relay
culture
vessel
300 W
i.r. lamp
magnetic stirrer

prevented back-growth of the culture along the feed-line. The growth vessel was a 0.5 l fermentation pot (Quickfit FV 500F) closed with a five-port top plate (Quickfit MAF 1/75).

Spent medium was removed via a metal canula which was secured to one of the ports of the top plate and could be raised or lowered to give the required volume (usually 210 ml). Removal of medium to the waste reservoir was effected by a large-bore tube placed in the second channel of the MHRE pump.

Entering air was monitored for flow rate by wet meter (A. Wright, Ltd., London), sterilised by a Microflow filter (Microflow Ltd., Fleet, Hants) and passed over the culture. It was removed to the gas analysers via a vertically mounted Leibig condenser (Quickfit Q-U/12) through which cold water was circulated to remove water vapour from the effluent air.

The culture was stirred magnetically on a plinth similar in design to the growth apparatus of Harvey et al., (1968). The temperature of the culture was sensed by a fixed contact thermometer, (37°C; J. C. Colinshaws Ltd., Manchester) which operated a 300 W infra-red lamp (Macfarlane Robson Ltd., Glasgow) via a Sunvic electronic relay (AEI type EA 4M; J. C. Colinshaws, Manchester M4 4JB).

Flow rate of medium into and out of the culture was determined by taking culture effluent into a preweighed conical flask closed by a cotton wool bung. After a measured time, the flask was reweighed and the flow rate calculated by difference. Conversion of medium constituents to cells (0.5 mg/ml dry weight) made an insignificant change to the density of the effluent medium.

Samples of cultures were removed to a plastic syringe (Becton, Dickinson & Co., Ltd., Drogheda, Ireland) via a metal canula which was inserted below the surface of the culture. When the syringe was removed during sampling, sterility was maintained by directing a flame at the canula head until a new sterile syringe could be replaced.

6.2.2 Continuous culture - constant flow, variable volume

The system was developed during this work (Development of Methods (1); Fig. 3b). Cultures were grown in 500 ml conical flasks, stirred magnetically and maintained at 37°C in the apparatus of Harvey et al., (1968). Each flask was sealed with a silicone rubber bung (Esco Rubber Co. Ltd., London) which had been drilled to accommodate a number of ports (Fig. 3a).

Fresh medium was stored as in (6.2.1), and pumped to the growth vessel by a peristaltic 'minipump' (Scientific Industries Ltd., Loughborough, England). Backgrowth was prevented by passing the fresh medium through a series of micropipette tips which served as a functional dripfeed system. Spent medium left the vessel via a stainless steel tube, which could be raised or lowered to alter the culture volume and pumped by another 'minipump' to a preweighed flask. Flow rate was estimated from the rate of increase in mass of the effluent flask (6.2.1).

Air entered the growth vessel via a glass capillary tube. Its rate of entry was monitored by wet meter, (A. Wright Ltd., London) and sterilised by passing through a microflow filter (Microflow Ltd., Fleet, Hants). Air left the vessel via a condenser to remove water vapour and was passed to the gas analysers.

Figure 3 CONTINUOUS CULTURE GROWTH APPARATUS - CONSTANT FLOW, VARIABLE VOLUME

Fig. 3a Each growth vessel was a 500 ml conical flask, closed with a silicone bung which accommodated the various entry/exit ports.

Fig. 3b Several growth flasks were used simultaneously to make more efficient use of the time available. Each growth vessel had separate input and output pumps, but medium came from a common reservoir.

This system is fully described in Methods (6.2.2).

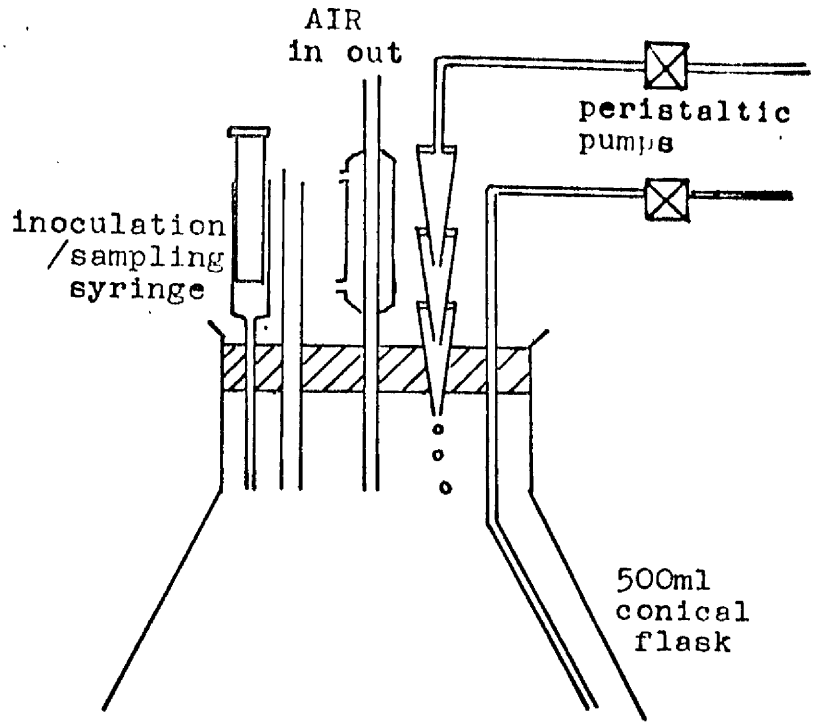
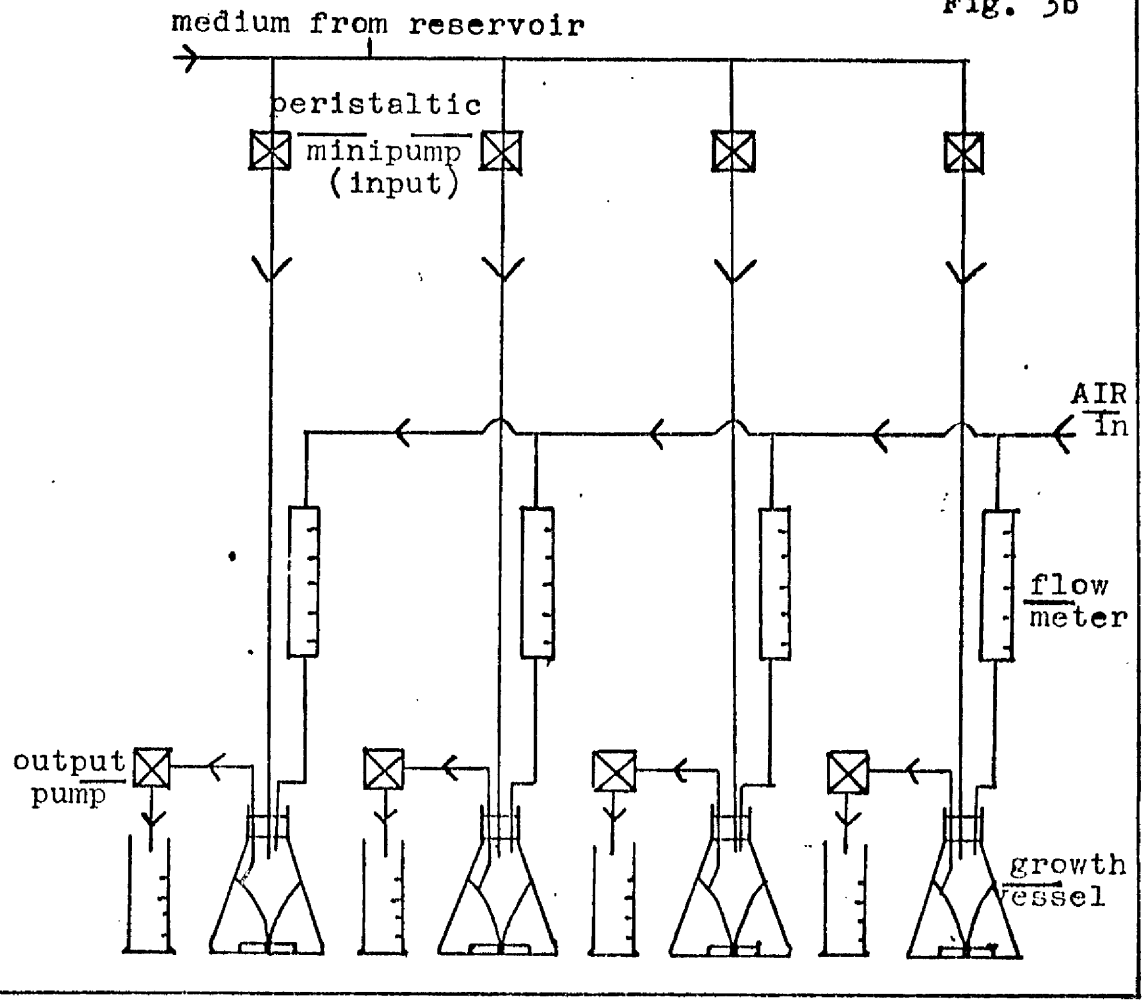

AIR
in out
peristaltic
pumps
inoculation
/sampling
syringe
500ml
conical
flask

Fig. 3a

Fig. 3b


medium from reservoir
peristaltic
minipump
(input)
AIR
in
flow
meter
output
pump
growth
vessel

Samples were removed through a metal canula connected to a piece of silicone tubing which passed into the culture, or by taking a portion of fresh culture effluent (Dev. Methods, 1).

6.3 Arithmetic-type culture

Arithmetic-type culture was done in a modified form (Fig. 4b) of the apparatus used for batch culture experiments (Wallace, 1975).

Cells were grown in a 2 l flat-bottomed pyrex flask with side arm, and were maintained in the apparatus of Harvey et al., (1968). The side port was sealed with a silicone rubber bung which accommodated two stainless steel tubes. Sampling, and inoculation, was done through the first, and limiting nutrient was supplied through the second. The open neck of the flask was sealed with a silicone bung, drilled to accommodate the air inlet tube and output condenser.

Limiting nutrient was stored in a graduated measuring cylinder which, once calibrated, gave a measure of flow rate. The medium was pumped (1 $ml.h^{-1}$) to the growth flask by a Varioperpex pump (Model 12000; LKB Produkter A. B., Sweden) via a small bore nylon intravenous canula (200/100/010; Portex S. A., Berck-sur-Mer, France). At a flow rate of 1 $ml.h^{-1}$, medium moved through the canula at a rate of 0.25 $m.min^{-1}$ - sufficiently fast to prevent back growth.

Air, sterilised by filtration (Microflow filter; Microflow Ltd., Fleet, Hants), was supplied to the vessel from the departmental compressed air supply. Flow rate was monitored by wet meter (A. Wright Ltd., London).

Figure 4 ARITHMETIC CULTURE GROWTH APPARATUS - EQUIPPED FOR GAS ANALYSIS

Fig. 4a The system used to measure gas exchange (Methods, 8.1) was the same, irrespective of culture conditions. Effluent gas from the growth vessel was passed through the CO_2 analyser. Effluent gas from the (uninoculated) reference flask was passed directly to the reference channel of the O_2 analyser.

Fig. 4b The arithmetic-culture growth vessel was a 2 l side-arm flask. Limiting nutrient was supplied (1 $ml.h^{-1}$) from a calibrated measuring cylinder. Culture gas was stripped of water vapour before passing to the analysers.

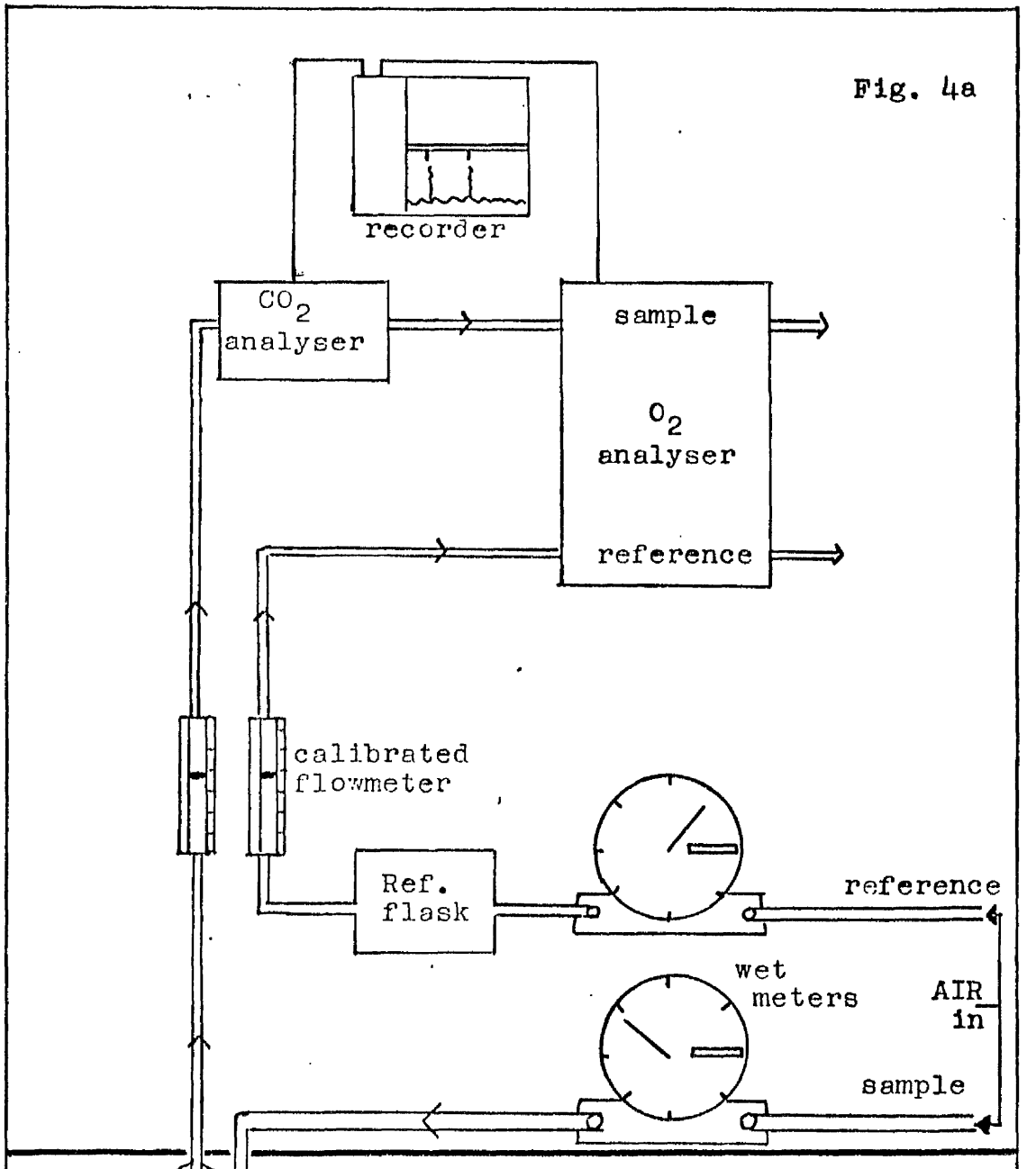

recorder
CO_2 analyser
sample
O_2 analyser
reference
calibrated flowmeter
Ref. flask
reference
wet meters
AIR in
sample

Fig. 4a

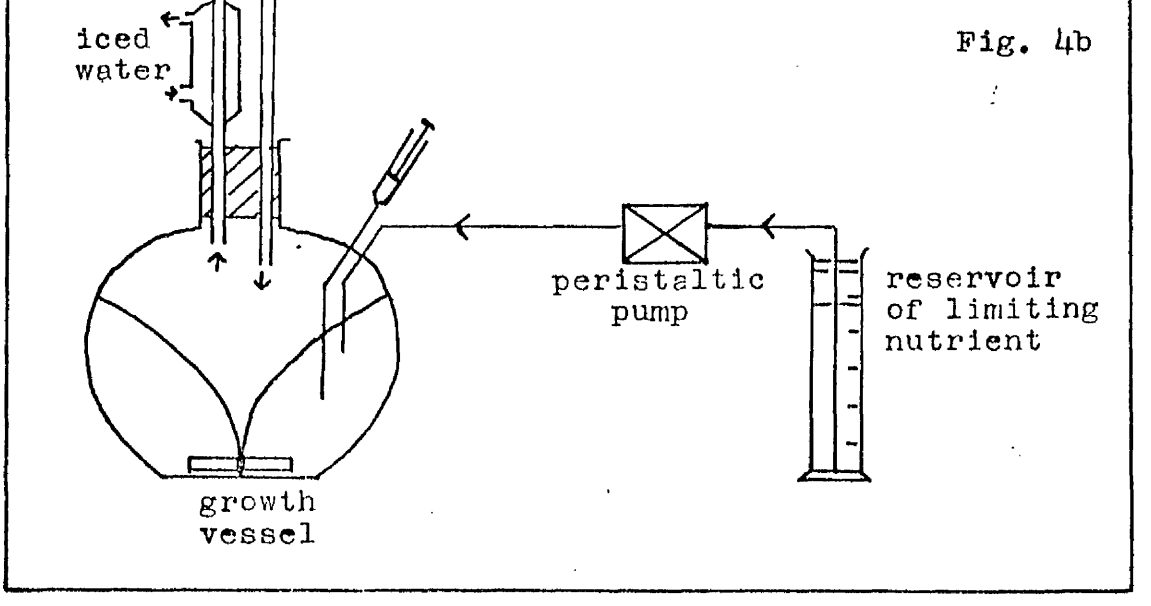

iced water
peristaltic pump
reservoir of limiting nutrient
growth vessel

Fig. 4b

7. Measurement of growth

Growth of bacterial cultures was estimated turbidimetrically. A sample of culture (4 ml) was killed by adding 2 drops of 10% ($^{v}/v$) formalin. The extinction of the suspension (E_{420}) was measured in an SP 800 double beam spectrophotometer (Pye Unicam Ltd., Cambridge, England) fitted with a potentiometric chart recorder (Servoscribe 1s; Belmont Instruments Ltd., Glasgow).

The E_{420} of cells was directly proportional to dry weight in the range $0 \leqslant E_{420} \leqslant 0.35$, but at E_{420} of 0.35 or more, the relationship was not direct. A calibration curve of E_{420} versus dry weight was drawn up for the range E_{420} = 0-1.5 (Fig. 5). This was constructed by taking a culture of known dry weight (Dev. Methods 2.2), making serial dilutions from it, and reading the apparent E_{420}. The curve was shown to be valid for cells of ML and K strains grown on different substrates in batch, arithmetic and continuous culture conditions.

The calibration curve becomes inaccurate at $E_{420} \geqslant 0.5$. Since both arithmetic and continuous cultures supported high cell densities, these cultures were diluted in phosphate buffer (KH_2PO_4, 40 mmol.l^{-1}; pH 7.0) prior to determining the extinction. All dilutions were done using Grade A volumetric pipettes and flasks to obtain a suspension in the linear range of the calibration curve.

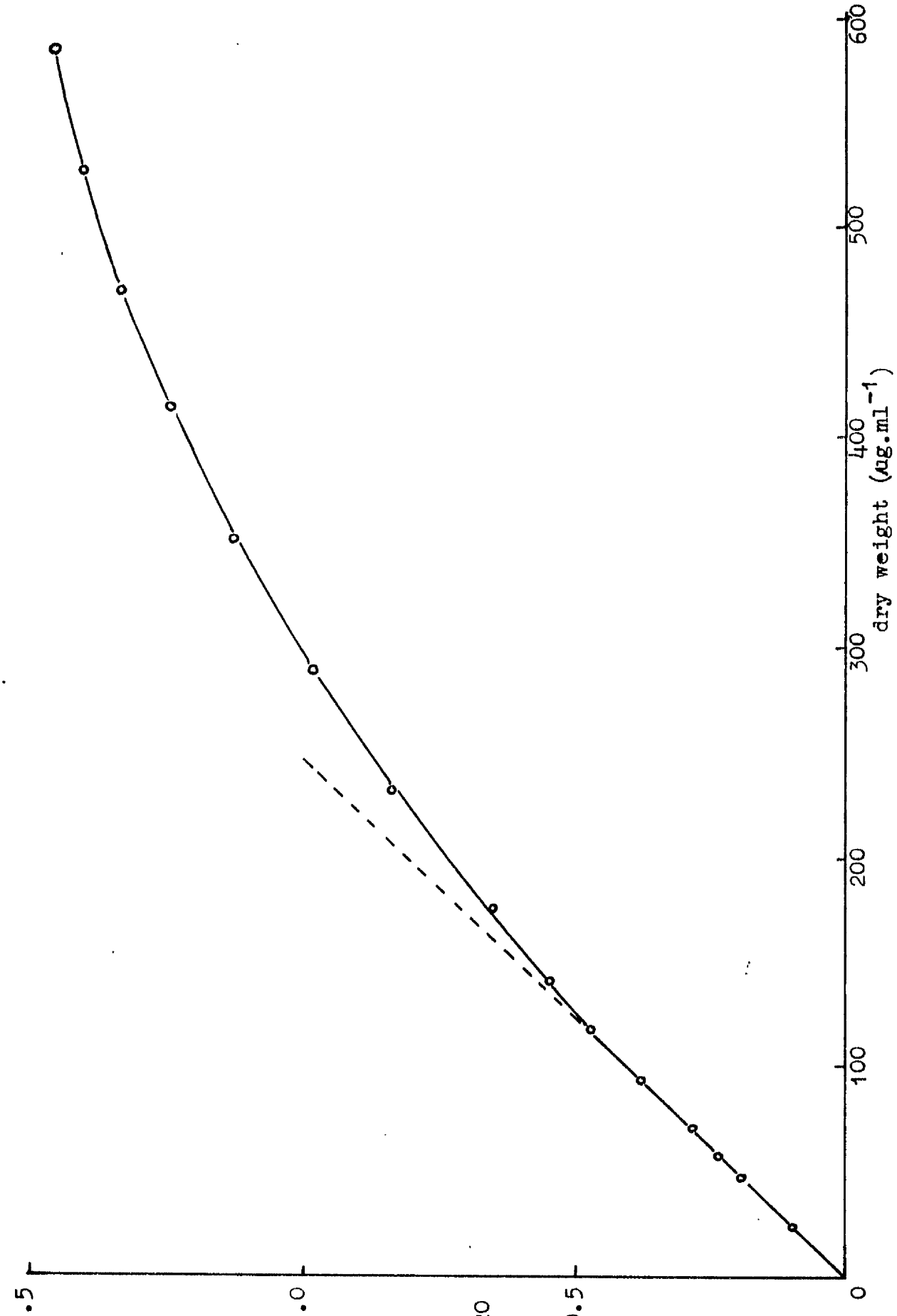
8. Gas exchange measurements

8.1 Method

Analysis of culture gas for oxygen uptake and carbon

Figure 5 TURBIDITY CALIBRATION CURVE

The curve was obtained by taking serial dilutions of a culture of known dry weight (Dev. Methods, 2.2) and reading the E_{420} in an SP 800 spectrophotometer. The curve was linear in the range E_{420} = 0-0.4. It was shown to be valid for all E.coli irrespective of strain or growth condition. Extrapolation of the linear portion of the curve gave the relationship, E_{420} = 1 equivalent to 246 μg. dry weight.ml^{-1}.


1.5
1.0
0.5
0
E_{420}
100
200
300
400
500
600
dry weight ($\mu g.ml^{-1}$)

dioxide production was performed as described by Wallace ((1975) and Fig. 4a).

A constant stream of air was passed over the growing culture. The rate of entry of air to the flask, Fe, was monitored by wet-type gas meters (A. Wright Ltd., London), which had been calibrated previously by volumeter (model 1056; Brooke Instruments Ltd., Cheshire, England). The temperature of the gas passing through each wet meter, T^{o} (^{o}K), was recorded (Thermograph; Cambridge Instruments Ltd., London).

Gas leaving the growth flask was stripped of water vapour as it passed through a small condenser fitted in the bung of the flask. The temperature of the water in the condenser jacket was maintained at $2^{o}C$ by circulating chilled water through the system by small pumps (Eheim 100; Universal Scientific Ltd., London), three of which were connected in series.

The effluent gas was passed first through a flow meter (G. A. Platon, London) to the carbon dioxide analyser (Lira 300; Mine Safety Appliances Ltd., Glasgow). This instrument estimates the partial pressure of CO_2 by infra-red analysis, in the range 0-1%. From the CO_2 analyser the gas passed to the oxygen analyser (Model 1082; Servomex Ltd., Crowborough, England). This is a twin-channel instrument which compares the oxygen content of culture effluent gas with that of air. The measurement depends on the force required to maintain a small hollow dumbbell in a magnetic field. When surrounded by oxygen, a paramagnetic gas, the dumbbell rotates in the magnetic field. Rotation is balanced by a feedback current applied to a torsion wire such that the dumbbell remains stationary

in the magnetic field. The magnitude of the feedback current is related to the oxygen partial pressure in the gas. It was operated in the range 21-20% for O_2 i.e. 0-1% oxygen taken up by the culture. The reference channel of the analyser was supplied with air at a rate equivalent to that supplied to the culture. The outputs from the analysers were monitored continuously and displayed on a twin channel potentiometric recorder (Servoscribe 2s; Belmont Instruments Ltd., Glasgow). Sensitivity for both analysers is better than 0.01%. Calibration of the instruments (8.3; 8.4) was performed before each experiment.

8.2 Calculation of results

Readings from the gas analysers may be quantitated using the equations derived by Hamilton (1972).

These are:-

$$O_2 \text{ uptake} = 20.96 \times \left[\frac{(100-b-a)}{(79.04)} - a \right] \cdot \frac{Fe \times 273 \times 10^{-2} \times 79.04}{(100-b-a) \times T \times 22.4} \quad -(1)$$

$$CO_2 \text{ production} = bx \left[\frac{Fe \times 273 \times 10^{-2} \times 79.04}{(100-b-a) \times T \times 22.4} \right] + (\text{pH factor} \times V \times \Delta CO_2), \quad -(2)$$

Units are mmol/min, where

a = O_2 reading (%)

b = CO_2 reading (%)

Fe = rate of entry gas ($ml.min^{-1}$)

T = ambient temperature (^oK)

pH factor = a constant to account for dissolved CO_2 (Hamilton, 1972)

V = vol of culture (l).

ΔCO_2 = rate of change of CO_2 reading ($\%.min^{-1}$)

For calculation of gas exchange in arithmetic and continuous cultures, the second term in equation (2) was omitted. These cultures exchange gas at, or close to, steady state, so there is no change in CO_2 reading over a period of time. For analysis of gas exchange in batch culture, where there is no steady state, the results were corrected for the effects of the dead space of the system, as suggested by Hamilton (1972).

Cumulative gas exchange by a culture was calculated by integrating the rates over a time period. Cumulative oxygen uptake was necessary to obtain yield values in batch culture; cumulative CO_2 production was required in calculation of carbon balances.

Handling of gas exchange data to obtain yield and maintenance values is complex and is the subject of Appendix 1.

8.3 Calibration of oxygen analyser

The Servomex 1082 has been shown to have a linear response to oxygen in the range 0-100% O_2, and more particularly in the range 19.96-20.96% O_2, (Hamilton, 1972).

Oxygen-free nitrogen was passed through both channels of the instrument. This gave a base line for gas without oxygen. The sample channel was zeroed to the recorder, and the reference channel was balanced against the sample. Air was then passed through both channels at experimental flow rates. The span control of the sample channel was adjusted to 20.96%, the fraction of oxygen in air, and the span of the reference balanced against this. The instrument was then able to compare the oxygen contents of the sample and reference channels, displaying the difference on the

recorder. A 1% difference corresponded to the full scale deflection of the recorder.

8.4 Calibration of carbon dioxide analyser

The Lira 300 will detect CO_2 in the range 0-1%. To calibrate the instrument, it was necessary to have available a selection of gases with known CO_2 contents in this range. This was achieved by using the gas mixing pumps (Wösthoff, Bochum, W. Germany) connected in series (Fig. 6a). The first pump mixed gas in the ratio 99 : 1 for air and CO_2 (D. C. L. Ltd., Glasgow) respectively. The gas leaving this pump, therefore, contained 1% CO_2. The second pump had variable settings, and could mix 1% CO_2 with air in various ratios. A calibration curve was constructed under experimental conditions in the range 0-1% by 0.1% steps (Fig. 6b), taking the CO_2 content of air to be zero.

Neither of the two Liras used during this work had a proportional response to CO_2, but No. 1 was more linear than No. 2. The calibration curves did not change with aging of the analysers.

9. Measurement of substrate concentration

All estimations were performed by enzymic assays at 27°C, and read in an SP 800 double beam spectrophotometer fitted with a potentiometric recorder with air as blank. In every case calibration curves were constructed in the extinction range 0-1. The time of incubation necessary for each assay was determined during preparation of the standard curve. When necessary, samples were diluted to give extinctions in this range.

9.1 Treatment of samples

A 4 ml sample of culture was blown into 1 ml of ice-

Figure 6 CALIBRATION OF CARBON DIOXIDE ANALYSERS

Fig. 6a To calibrate the analysers, it was necessary to have a selection of gases with CO_2 contents in the range 0-1%. CO_2 was mixed with air, in the ratio 1 : 99, by pump no.1. The resulting gas (1% CO_2) was re-mixed with air by pump 2 to give gasses of CO_2 content of 0-1%.

Fig. 6b Calibration curves for carbon dioxide analysers:

No. 1 o —o

No. 2 Δ —Δ

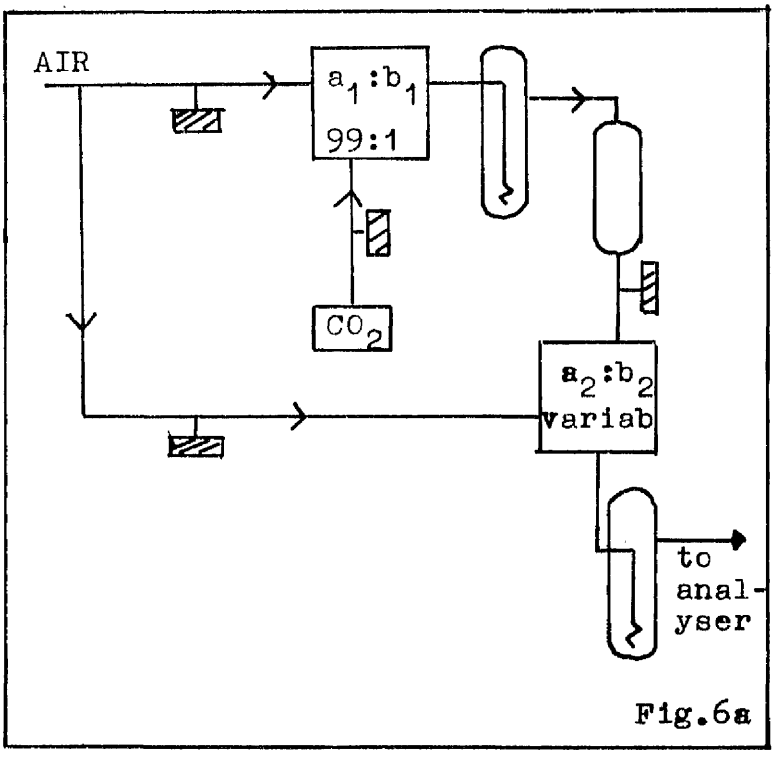

AIR
$a_1:b_1$
99:1
CO_2
$a_2:b_2$
variab
to
anal-
yser

Fig.6a

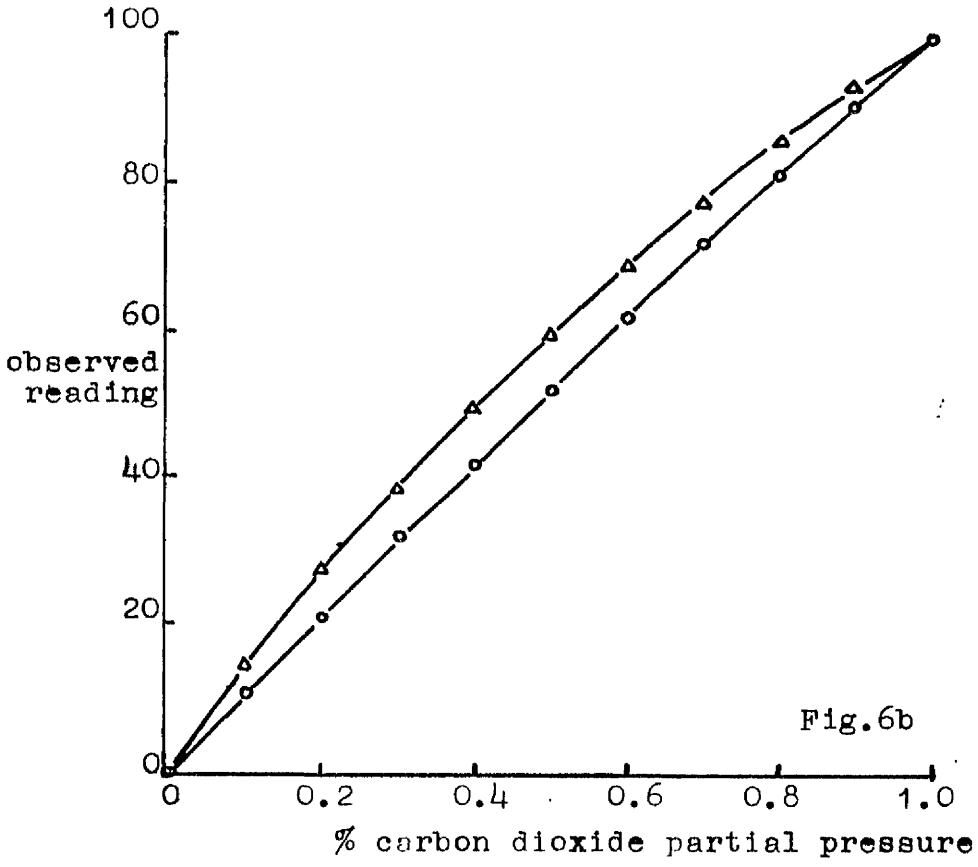

100
80
60
observed
reading
40
20
0
0
0.2
0.4
0.6
0.8
1.0
% carbon dioxide partial pressure

Fig.6b

cold perchloric acid (3 mol.l^{-1}), mixed for 10 s on a vortex mixer (Scientific Industries Ltd., Loughborough, England) and allowed to stand on ice for 10 min. 3 ml of chilled potassium hydroxide ($\sim$1 mol.l^{-1}) was added to return the sample to pH 7.0. (The potassium hydroxide had been diluted previously to ensure accurate neutralisation of the perchloric acid). After a further 10 min, when potassium perchlorate had precipitated, the samples were decanted into 15 ml Corex centrifuge tubes (Gallenkamp Ltd., East Kilbride, Scotland) and centrifuged (11,750 g, 4°C, 10 min) to remove the remainder of the precipitate and cellular debris. The supernates were decanted into glass vials, frozen rapidly in the deep freeze and stored at -18°C.

Each sample was thawed and mixed thoroughly before a portion was removed for assay. The remainder was frozen again and stored at -18°C.

9.2 Estimation of galactose

Two methods of estimating galactose were employed.

9.2.1 Estimation by galactose dehydrogenase

Galactose was oxidised to galacturonate by galactose dehydrogenase (E.C. 1.1.1.48) linked to reduction of NAD to NADH. Kits for this assay were purchased from Boehringer.

The composition of the assay medium was:

Tris HCl, pH 8.6	150 μmol.ml^{-1}
NAD	0.3 μmol.ml^{-1}
galactose dehydrogenase	5 μg.ml^{-1}

The reaction was initiated by mixing 2 ml assay medium with 1 ml of sample and incubated at 27°C for 40 min, then read at 340 nm. E_{340} = 1 was equivalent to 480 nmol.$assay^{-1}$.

9.2.2 Estimation by galactose oxidase

Galactose was oxidised by galactose oxidase (E.C. 1.1.3.9) to galacturonic acid, with concomitant production of H_2O_2. The H_2O_2 reacted with a reduced dye ('perid') to give H_2O and oxidised 'perid', which absorbs as 660 nm. This reaction was mediated by peroxidase (E.C. 1.11.1.7).

The composition of the assay medium was:

NaH_2PO_4, pH 7.0	100 μmol.ml^{-1}
galactose oxidase	250 μg.ml^{-1}
peroxidase	25 μg.ml^{-1}
'perid'	1 mg.ml^{-1}

The reaction was initiated by mixing 2.5 ml assay medium with 0.5 ml of sample and incubated at 27°C for 70 min, then read at 660 nm. E_{660} = 1 corresponded to 150 nmol.assay^{-1}.

9.3 Estimation of glucose

Glucose was estimated by the Boehringer 'GOD-Perid' method which is based on the test of Werner et al., (1970). It is similar in principle to the galactose oxidase method for galactose estimation (9.2.2).

The assay composition was:

NaH_2PO_4, pH 7.0	100 μmol.ml^{-1}
glucose oxidase (E.C. 1.1.3.4)	180 μg.ml^{-1}
peroxidase	20 μg.ml^{-1}
'perid'	1 mg.ml^{-1}

The reaction was initiated by mixing 2.5 ml assay medium with 0.5 ml sample, and incubated at 27°C for 25 min, then read at 660 nm. E_{660} = 1 corresponded to 150 nmol.assay^{-1}.

9.4 Estimation of glucose 6-phosphate

Glucose 6-phosphate was estimated by conversion to 6-phosphogluconate by glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49) linked to reduction of NADP to NADPH.

The assay medium contained:

NaH_2PO_4, pH 7.2	200 μmol.ml^{-1}
$MgCl_2$	2 μmol.ml^{-1}
NADP	0.2 μmol.ml^{-1}
glucose 6-phosphate dehydrogenase	3 μg.ml^{-1}

The reaction was initiated by mixing 2.5 ml assay medium with 0.5 ml sample, and incubated at 27°C for 45 min, then read at 340 nm. E_{340} = 1 was equivalent to 480 nmol.assay^{-1}.

9.5 Estimation of glycerol

Glycerol was estimated by conversion to α - glycerophosphate by glycerol kinase (E.C. 2.7.1.30). This requires ATP, which was limiting in the assay, but supplied during conversion of PEP to pyruvate by pyruvate kinase (E.C. 2.7.1.40). Pyruvate production was estimated by conversion to L-lactate by lactate dehydrogenase (E.C. 1.1.1.27) which was linked to production of NAD from NADH.

The composition of the assay medium was:

triethanolamine HCl, pH 7.6	80 μmol.ml^{-1}
$MgSO_4$	3.3 μmol.ml^{-1}
PEP	0.31 μmol.ml^{-1}
ATP	0.94 μmol.ml^{-1}
pyruvate kinase	7 μg.ml^{-1}
lactate dehydrogenase	13 μg.ml^{-1}
glycerol kinase	13 μg.ml^{-1}

2.5 ml assay medium was mixed with 0.5 ml sample to commence the reaction. This was incubated at 27°C for 60 min then read at 340 nm. E_{340} = 1 corresponded to 480 nmol.assay^{-1}.

9.6 Estimation of lactose

Lactose was estimated by two methods.

9.6.1 Estimation of lactose by galactose oxidase

The galactose oxidase preparation (E.C. 1.1.3.9) supplied by Hughes & Hughes Ltd., Romford, Essex was also active with lactose. The incubation medium was identical to that of (9.2.2.).

2.5 ml of incubation medium was mixed with 0.5 ml sample, and incubated at 27°C for 100 min, then read at 660 nm. E_{660} = 1 was equivalent to 150 nmol.assay^{-1}.

9.6.2 Estimation of lactose by hydrolysis to glucose and galactose

Lactose was occasionally estimated by hydrolysis to glucose and galactose, and then estimating either or both of these sugars.

To 1 ml sample, in a stoppered vial, was added 50 µg β-galacosidase (E.C. 3.2.1.23) and 10 µl $MgSO_4$ (0.1 mol.l^{-1}). This was incubated at 37°C for 1 h, by which time all of the lactose was hydrolysed. The sample was then assayed for either monosaccharide (9.2; 9.3).

9.7 Estimation of galactosides

The broad specificity of galactose oxidase (E.C. 1.1.3.9) allowed the estimation of galactosides (particularly lactulose and lactitol) without any hydrolysis to monosaccharides. The method used was identical to (9.6.1).

9.8 Estimation of maltose

Maltose was estimated by first hydrolysing to glucose using α-glucosidase (E.C. 3.2.1.20) and then estimating as for glucose. To 1 ml sample, in a stoppered vial was added 20 μg α-glucosidase. This was incubated at 37°C for 1 h, by which time all of the maltose was hydrolysed. The glucose released was assayed by the 'GOD-perid' method (9.3).

10. Measurement of enzyme activities

All enzyme activities were measured in an SP 800 double beam spectrophotometer equipped with chart recorder. A unit of activity corresponds to 1 μmol of substrate catalysed per min. Specific activities, with the exception of transhydrogenase activity, are expressed as μmol.min^{-1} (mg. dry weight)$^{-1}$. Transhydrogenase has units μmol.min^{-1} (mg. protein)$^{-1}$.

10.1 β-Galactosidase (E.C. 3.2.1.23)

The method of Holms and Robertson (1974) was used. A portion of culture (3.5 ml) was blown on to toluene in ethanol (2% $\frac{0.5ml}{V}$), the suspension was mixed (Vortex mixer: Scientific Industries Ltd., Loughborough, England) for 20 s and incubated at 27°C for 20 min. This process was termed 'toluenisation'.

The assay solution (final volume 3 ml) contained:

NaH_2PO_4, pH 7.25	100 mmol.l^{-1}
$MgSO_4$	0.5 mmol.l^{-1}
Ethanol	125 ml.l^{-1}
ONPG	3.3 mmol.l^{-1}

and cell toluenisate

ΔE_{420} was measured, at either 27°C or 37°C, with air as reference. E_{420} was proportional to ONP concentration in the range E_{420} = 0-1. E_{420} = 1 corresponded to 1130 nmol.assay^{-1}.

10.2 β-Galactoside permease

Permease activity can be affected by the presence of other carbon sources in the assay (Boniface & Koch, 1967), so cells were centrifuged and resuspended in medium without carbon source before the assay. This was not required of arithmetic and continuous culture cells which were grown under carbon limitation.

The assay contained (3 ml):

NaH_2PO_4, pH 7.0	100 mmol.l^{-1}
$MgSO_4$	0.5 mmol.l^{-1}
ONPG	3.3 mmol.l^{-1}
Whole cells	

ΔE_{420} was measured at 27°C or 37°C with cells as reference. The lower pH of the assay (7.0) made the method slightly less sensitive (E_{420} = 1 corresponded to 1220 nmol.assay^{-1}) than that for β-galactosidase (10.1), since the chromogen colour is pH-dependent (Lederberg, 1950).

Results were corrected for non-permease mediated activity. A small volume of neutralised formaldehyde (final concentration 20 mmol.l^{-1}; Cecchini & Koch, 1975) was added to the sample cuvette at about E_{420} = 0.6, and the lower rate (ΔE_{420}) was followed. Formaldehyde is believed to inactivate the carrier protein. Therefore, only non-permease mediated activity is measured when it is present. This was verified by comparing formaldehyde-

inhibited permease activity with activity in the presence of fully-saturating concentrations of thiodigalactoside (5 mmol.l^{-1}), a competitive inhibitor of the system (unpublished results).

10.3 ∝-Glycerosphosphate dehydrogenase

There are several forms of this enzyme present in E.coli, depending on growth conditions. They differ in their specificity for reducing cofactor (Lin, 1976).

10.3.1 NAD-linked ∝-glycerosphosphate dehydrogenase (E.C. 1.1.1.8)

The enzyme was assayed according to the method of Kito and Pizer (1969). Dihydroxyacetone phosphate was used as substrate since the equilibrium favours formation of glycerophosphate.

Cell extracts were prepared by sonication. Cells (5 ml) were sonicated at 3.4A for 6 half-minute intervals (Soniprobe; Dawe Instruments Ltd., London). The cell suspension was contained in a small glass vial, and chilled in an ice/water slurry. The suspension was sonicated and left to cool inalternate half-minutes.

The assay contained (3 ml, air reference):

triethanolamine HCl, pH 7.5	50 mmol.l^{-1}
dithiothreitol	10 mmol.l^{-1}
dihydroxyacetone phosphate	2 mmol.l^{-1}
NADH	0.2 mmol.l^{-1}
sonicated cell extract	

Activity was followed by decrease in E_{340} at 27°C. ΔE_{340} = 1 corresponded to 480 nmol.assay^{-1}. Controls for endogenous NADH oxidase activity were done by omitting dihydroxyacetone phosphate from the assay.

10.3.2 NADP-linked α-glycerophosphate dehydrogenase (E.C. 1.1.1.94)

Preparation of cell extracts and method of assay were identical to (10.3.1) except that NADPH (0.2 mmol.l^{-1}) replaced NADH.

10.3.3 Nicotinamide-independent α-glycerophosphate dehydrogenase (E.C. 1.1.99.5)

The method of Weiner and Heppel (1972), which involves reduction of the dye 3 ((4, 5) dimethyl-thiazolyl-2) 2, 5 diphenyl-tetrazolium bromide (MTT), was used. The purified enzyme from E.coli was shown to contain FAD, which is not covalently bound. Cells were prepared by either toluenising (10.1) or sonicating (10.3.1).

The assay contained (3 ml, air reference):

Tris HCl, pH 7.3	80 mmol.l^{-1}
MTT	30 μg.ml^{-1}
phenazine methosulphate, PMS	60 μg.ml^{-1}
DL α glycerophosphate	50 mmol.l^{-1}
cell extract	

Activity was followed at 570 nm and 27°C. $\Delta E_{570} = 1$ corresponded to 175 nmol.$assay^{-1}$. Results were corrected for endogenous reduction of the dye by doing controls without α-glycerophosphate.

10.4 NAD/NADP transhyolrogenase (E.C. 1.6.1.1)

Oxidative, ATP-linked and non-energy-linked trans-hydrogenase activities of electron-transport particles were determined as described by Houghton et al. (1975).

The particles were prepared as follows: Cells were harvested (11,750 g, 15 min, 4°C) and resuspended to a concentration of 0.5 g wet weight.ml^{-1}. The suspension was brought to pH 7.4 with tris HCl (40 μmol.l^{-1}, pH 8.2)

and sonicated in a glass rosette for 2.5 min at full power (Soniprobe; Dawe Instruments Ltd., London). The suspension was then centrifuged (36,000 g. 15 min, 4°C) to remove cellular debris, and the supernate was recentrifuged (150,000 g. 2 h, 5°C). The pellet was suspended in tris-HCl (40 mmol.l^{-1}, pH 8.2) and used for the assay. Protein was estimated by the method of Lowry et al., (1951), with bovine serum albumin as standard.

11. Polarographic measurement of oxygen uptake

Oxygen uptake was performed measured in an oxygen electrode assembly (Rank Bros., Ltd., Bottisham, Cambridge) and displayed on a potentiometric recorder (Servoscribe 1s; Belmont Instruments Ltd., Glasgow). The electrode vessel was maintained at constant temperature by a circulating water bath.

The electrode was calibrated daily by the method of Robinson and Cooper (1970), taking account of the revised molar absorbtivity of NADH (Moss, 1976). This method is based on calibration by the oxygen activity, not the oxygen concentration, of the medium.

12. Determination of H^+/O ratio

This technique was developed during this work. For theoretical and general considerations, see Development of Methods (4).

12.1 Apparatus

Cells (or spheroplasts) were incubated in a polypropylene vessel (Radiometer, V 524; V. & A. Howe Ltd., London) which was sealed with a plastic plug and drilled to accommodate the electrode, nitrogen gas line and syringe. The medium was stirred magnetically (Radiometer D 4030, V. & A. Howe Ltd., London) and maintained at constant temperature by a

glass water jacket. The medium was kept anaerobic under a nitrogen atmosphere by directing a constant stream of water-saturated oxygen-free nitrogen into the vessel. Changes in pH, in response to added oxygen, were determined using a thin membrane combined glass microelectrode (217.BNC.E7; Probion Ltd., Leslie, Fife), connected to a pH meter with large back-off facility and displayed on a chart recorder (Servoscribe 2s; Belmont Instruments Ltd., Glasgow). The pH meter was similar in concept to that of Light and Garland (1971), and was modified from a design kindly supplied by Dr. W. A. Hamilton, University of Aberdeen. A description of the meter and suppliers of components is included as Appendix 3.

12.2 Method

Cells, harvested by centrifugation, were washed twice in KCl (100 mmol.l^{-1}) and resuspended in KCl (150 mmol.l^{-1}). About 10 mg dry weight were used for each experiment. The incubation medium (pH 7.0, working volume 4 ml) consisted of KCl (150 mmol.l^{-1}), KSCN (50 mmol.l^{-1}), glycerol (1 mmol.l^{-1}) and cells. When spheroplasts were used, sucrose (0.5 mol.l^{-1}) was included in the incubation medium to prevent lysis.

Cells were made anaerobic for at least 10 min before assay. Limiting oxygen was added from a syringe (Hamilton 705-N; V. & A. Howe Ltd., London) as air-saturated KCl and assumed to contain O_2 as calculated by Chappell (1964). The change in medium pH was recorded.

Since each cell suspension had a unique buffering capacity, changes in pH were converted to changes in concentration of H^+ by adding known amounts of anaerobic

HCl (1 mmol.l^{-1}, in KCl (150 mmol.l^{-1})) to the cell suspension after each determination.

The $\frac{H^+}{O}$ ratio was calculated by division of pH response to oxygen by pH response to acid (peak values in each case).

MATERIALS

The suppliers of apparatus have been noted at the relevant part of the Methods and Development of Methods sections.

Fine chemicals, with the exception of those listed below, were 'Anala R' or the highest grade available and were obtained from British Drug Houses, Poole, Dorset.

Obtained from the Boehringer Corporation Ltd., Lewes, Sussex, were:

ABTS ('perid').
galactose dehydrogenase.
β-galactosidase.
glucose 6-phosphate dehydrogenase.
glycerol kinase.
'GOD-Perid' reagent.
lactate dehydrogenase.
NADP.
PEP.
peroxidase.
pyruvate kinase.
sodium glucose 6-phosphate.
triethanolamine.

Obtained from Sigma Ltd., London, were:

ATP.
BCIG.
CCCP.
dihydroxyacetone phosphate.
galactose.
α-glucosidase.
glutathione.

glutathione reductase.

∝glycerophosphate dehydrogenase.

glycyl-glycine.

lysozyme.

MTT.

NAD.

NADH.

Na-∝glycerophosphate.

TDG.

Tris.

BSA was obtained from Armour Ltd., Eastbourne, England; Dithiothreitol from Calbiochem Ltd., London; nitrilotri-acetic acid from Fisons Ltd., Loughborough, Leicestershire; L-malic acid and lactulose from Fluka Ltd., c/o Fluorochem Ltd., Glossop, Derbyshire; toluene from Hopkins & Williams Ltd., Romford, Essex; galactose oxidase from Hughes & Hughes Ltd., Romford, Essex; Lactitol from ICI Ltd., Alderley Park, Cheshire; dulcitol, maltose and mannitol from Thomas Kerfoot Ltd., Vale of Bardsley, Lancashire; ONPG from Koch-Light Ltd., A. & J. Beveridge, Edinburgh; TMG from Mann Research Ltd., New York; formaldehyde from May & Baker Ltd., London; Microbiological media from Oxoid Ltd., Basingstoke, Hants; NADPH from PL Biochemicals Ltd., c/o International Enzymes Ltd., Windsor, Berks; Chloramphenicol from Parke Davis Ltd., London.

Oxygen and oxygen-free nitrogen were obtained from British Oxygen Ltd., Glasgow and CO_2 from Distillers Co., Ltd., Glasgow.

DEVELOPMENT OF METHODS

1. Apparatus for continuous culture, 'constant flow: variable volume'

In continuous culture, the growth rate, μ, of the culture may be varied. Since $\mu = D$, where D is the dilution rate, the growth rate is varied by changing the dilution rate.

Since $D = \frac{1}{V} \cdot \frac{dE}{dt}$ -(1), where

V = culture volume

$\frac{dE}{dt}$ = rate of entry of medium,

the dilution rate may be changed by:

1) varying the entry flow rate, 'variable flow: constant volume',
2) varying the culture volume, 'constant flow: variable volume'.

The apparatus used in the former method (variable flow: constant volume; Fig. 2) has some disadvantages. The variable speed pump has to be of high quality and is expensive, since it is required to provide a range of flow rates of medium to the growth pot, each of which is accurate and constant. Experiments using this system tend to last for long periods of time since the number of individual growth rates necessary to get meaningful results may be more than ten, and a steady state for each must be achieved. This is not only time-consuming but there is a strong possibility of selecting a mutant strain when a culture is grown for prolonged periods (Poole & Haddock, 1975; Collins et al., 1976).

The second system for continuous culture (constant flow: variable volume; Fig. 3) was developed during this work. It is cheaper, quicker and decreased the possibility of selecting a mutant population.

Any continuous culture apparatus requires a pump which will deliver medium at a constant flow rate to ensure constant steady state conditions. Whereas a variable speed pump satisfying this criterion is expensive (e.g. MHRE, Fig. 2), small constant-speed peristaltic pumps are relatively cheap (e.g. 'minipump', Fig. 3). With these pumps the difference in pump rates for two seemingly identical models may be as much as 20%, but each individual pump will deliver medium at a reproducible rate.

When fixed rate pumps are used the growth rate of the culture has to be altered by changing the culture volume. To do this in a predictable manner would require measuring the volume of the stirred culture, which is virtually impossible. It may be done in another way:

rearranging -(1) above, $V = \frac{1}{D} \cdot \frac{dE}{dt}$. Since $\frac{dE}{dt}$ is constant depending on the pump, a predicted growth rate may be obtained by pumping medium into the empty pot for a calculated time period $\frac{(1)}{(D)}$ and setting the effluent tube of the apparatus (Fig. 3a) at that level. For example, to obtain $\mu = 0.5$, medium is pumped for 2 h; for $\mu = 0.3$, medium is pumped for 3.3 h. In practice, the growth rate obtained may be slightly different from that expected, but may be determined accurately by measuring the culture volume at the end of the experiment. The culture volume will be changed if the growth pot is moved slightly or if

there is an alteration to the stirring rate of the culture. To prevent this, each growth vessel was clamped tightly in place and stirring rate checked stroboscopically (Strobe 15K, Griffin & George Ltd., East Kilbride), was held constant throughout each experiment.

Several experiments, all at different growth rates, could be done simultaneously using the same medium source (Fig. 3b), because of the low cost and simplicity of the system. This avoided the long unproductive time intervals required to achieve steady state when experiments are performed consecutively using the same pot. For each dilution rate, at least five but usually ten culture volumes of medium were passed before measurements were made. This was presumed to be sufficient to reach steady state. Once measurements had been made, the growth flask was removed and replaced by another sterile flask which could be operated at a different growth rate. It was inoculated from a fresh culture so that in different experiments measurements were made on cells which had grown for approximately the same number of generations, decreasing the possibility of selecting a mutant. While measurements were made on one flask, the others were approaching steady state, resulting in more efficient use of time and measuring apparatus.

Sampling of the culture, particularly for cell mass or enzyme activity determinations, was often done by collecting some of the culture effluent on to ice rather than removing a portion of culture from the growth vessel. At the flow rates generally employed (2 $ml.min^{-1}$), sample collection took only 3 min and the results were indistinguishable from those where samples were removed from the culture

pot. There is no perturbation of the steady state when samples are taken from the effluent.

2. Growth: evaluation of turbidity/dry weight relationship

2.1 General considerations

The evaluation of growth of bacteria has been discussed at length by Mallette (1969) who concluded that dry weight was perhaps the most meaningful measure of bacterial growth. Earlier, Koch (1961) had suggested that turbidity more probably reflected dry weight than cell number of a culture. Turbidity, then, provides a convenient estimate of dry weight as long as the relationship between the two is known.

The turbidity/dry weight relationship of a bacterial suspension varies with the spectrophotometer being used and with the wavelength. Koch (1971) has discussed the use of various spectrophotometers in this context. During this work, turbidity was estimated in an SP 800 spectrophotometer at 420 nm, with air as reference. This double-beam instrument compensates for any change in light intensity with aging of the light source - an important consideration, since the turbidity/dry weight relationship is non-linear (Fig. 5). The choice of wavelength (420 nm) for turbidity measurements was to avoid changing the wavelength setting of the instrument when performing turbidity and β-galactosidase estimations on the same preparation. It would have been inconvenient to use a longer wavelength which would have made the curve more linear, but less sensitive.

2.2 Method

The turbidity/dry weight relationship was derived as

follows: A culture (usually 2 g. dry weight of cells) was harvested (6,000 g, 20 min, 4°C) in a MSE 6L centrifuge and the pellets were resuspended in 500 ml ice-cold buffer (KH_2PO_4, pH 7.0; 40 mmol.l^{-1}). This was divided into two parts: for dry weight determination and for turbidity measurements respectively.

For the determination of dry weight, 6 x 25 ml of the concentrated culture was centrifuged (11,750 g, 10 min, 4°C) in a MSE 18 centrifuge, resuspended in 25 ml chilled distilled water and recentrifuged (11,750 g, 10 min, 4°C). Each pellet was transferred with ice-cold distilled water to a clean pre-weighed vial and dried at 105°C to constant weight. Weighings were done by the 'rapid weighing' technique (Mallette, 1969) with a Stanton electrical balance. The standard error of the mean for the six vials was within 2% of the mean value.

To construct the turbidity calibration curve, a portion of the concentrated culture was serially diluted in phosphate buffer, and the E_{420} of each dilution was determined (Fig. 5). Grade A volumetric pipettes and flasks were used for all dilutions.

2.3 Discussion

The turbidity calibration curve (Fig. 5) was not linear except in the range E_{420} = 0-0.4. A line extrapolated from the linear portion of the curve gave the relation 246 µg.ml^{-1} for E_{420} = 1, with standard deviation of 3.2 µg.ml^{-1} for seven determinations using different cell phenotypes. The relationship and calibration curve were found to be valid for cells of ML308, ML30 and its mutants, irrespective of culture type or substrate limitation.

They were also valid for K strain.

It is perhaps surprising that all cell types obeyed this universal relationship. Poole and Haddock (1975) found that E.coli cells grown under sulphate-limitation had a morphology distinct from those grown under carbon-limitation. Sulphate-limited cells were longer and narrower (4.6 μ, 0.41 μ) than those grown under carbon-limitation (2.4 μ, 0.6 μ). Koch (1971), from theoretical considerations, has concluded that, "the turbidity of a given dry weight of cells will be nearly independent of size to the first approximation, for cells in the size range 0.1 μ^3 to 5 μ^3". Since E.coli cells fall within this size range, the turbidity/dry weight relationship will be common to all cell types. This has been confirmed (Fig. 5).

The construction of a turbidity/dry weight curve for cells grown in high salt conditions proved impossible because of lysis of cells when transferred from high salt conditions to distilled water. Mager et al. (1956) found that the turbidity of a given bacterial suspension could be changed by up to 140% depending on the tonicity of the medium. However, this result was obtained by taking a bacterial suspension grown at usual tonicity and diluting into hypertonic medium. In the present work, dry weights of cultures grown in high salt conditions were estimated by diluting with isotonic medium and using the standard curve (Fig. 5). Carbon balances of 100%$\pm$2% from gas exchange of these cultures indicated that this was valid.

3. Cell viability

3.1 General considerations

Calculation of growth yields from gas exchange data

assumes that all of the culture is healthy and exchanging gas. Erroneus results would arise if, say, only a fraction of the cell population were alive while gas exchange was measured.

When bacteria are grown for a prolonged period of time, the viability of the culture may decrease, particularly if the culture is growing slowly. The viability of K.aerogenes grown in continuous culture at μ = 0.1 was less than that of faster growing cultures (Tempest et al., 1967). Strange (1968), however, found little decrease in the viabilities of E.coli cultures grown in the range μ = 0.18-0.6 under carbon- and nitrogen-limitations, at least until 50 hours of deprivation. Since, in arithmetic culture, the growth rate may fall to μ = 0.01 or less, it was necessary to determine culture viability at slow growth rates.

If a culture was found to have a good viability, it would be assumed that the majority of cells in it were healthy and equally competent to exchange gas.

3.2 Method

Postgate (1969) has defined viability as, "the capability of multiplying to form two or more progeny in conditions that are 'optimal' for the species and strain of microbe concerned". It was felt that the usual slide culture method of assessing viability (Postgate et al., 1961) was 'suboptimal' for E.coli from arithmetic culture for two reasons. Firstly, the cells had been grown under forced aeration but slide culture involves trapping a small limiting amount of air above the cells to be counted. Secondly, having come from a slow growing culture, it might

take some time for the cells to divide initially - repeated observation of each slide under the light microscope might damage the cells. As an alternative to slide culture, cells were grown aerobically on agar plates:

Small plates (No 122; Sterilin Ltd., Teddington, England) of nutrient agar were prepared as in Methods (2.3). Cells were spread, at a suitable dilution, on the plates which had been preincubated at 30°C. The plates were then further incubated at 30°C rather than 37°C to minimise any termperature effect on maintenance (Fig. 22). Replicate plates were removed at hourly intervals and the cells killed by exposing the surface of the agar to an atmosphere of formaldehyde vapour for 10 min. Plates could be assessed for viability immediately or stored at 4°C until required. Controls with plates of exponentially-growing bacteria confirmed that the formaldehyde treatment was effective in killing the cells.

Observation of the agar surface by phase constrast microscopy allowed a direct estimate of the numbers of live and dead bacteria. A 'live' cell was defined as one forming a microcolony with four or more cells within 3 h; 'activated' cells were collections of two and three cells; single cells were assumed to be 'dead'. At least 500 units were observed for each viability value.

4. Proton extrusion

4.1 General considerations

The pulse oxidant technique for determining the $\frac{H^+}{O}$ ratio of mitochondria (Mitchell & Moyle, 1967b) was adapted successfully for use with E.coli by Lawford and Haddock (1973). The method, which quantitates H^+'s ejected from the

cells and correlates this with a small finite amount of electron transport to oxygen, gives an index of efficiency of energy conservation. It has been applied to other strains (Jones(1977), for a review). However, it is often forgotten that this technique uses cells which are behaving non-physiologically—normally, with growing cells, the H^+'s ejected by electron transport should be immediately recaptured for energy-linked functions (ATP synthesis, transport, etc.,) and will not be released to the external medium. The significance of derived $\frac{H^+}{O}$'s is often over-emphasised.

4.2 Amount of oxygen added

In deriving a $\frac{H^+}{O}$, if oxygen is added in excess of the reductant content of the cells, the value derived will be anomalously low. The usual addition of oxygen (23.5 ng.atom) was the limiting factor in these experiments (Fig. 7). In addition, the quantity of cells usually added (10 mg) had a total respiratory activity (≥ 2 μg.atomO.min^{-1}) sufficient to reduce the oxygen within 0.5-0.8 s.

4.3 Incubation medium

The incubation medium normally used in pulse-oxidant experiments with bacteria (citations of Jones, 1977) is identical to that employed by Mitchell and Moyle for mitochondria (1967b).

It is usual to include glycyl-glycine as the buffer for the incubation medium, but Mitchell and Moyle (1967a) included it solely to decrease the time constant of their glass electrode. Since E.coli may transport glycyl-glycine (Corwell, 1974) and this might interfere with results, the

Figure 7 EFFECT OF AMOUNT OF ADDED OXYGEN ON THE H^+/O RATIO

Fig. 7a Amount of H^+'s pumped from the cells in response to varying amounts of oxygen was plotted, for a glycerol-grown culture of strain ML308 (9.4 mg).

Fig. 7b The data from Fig. 7a were replotted as $\frac{H^+}{O}$ ratio against amount of oxygen added.

Normally 23.5 ng. atom oxygen was added during proton extrusion experiments.

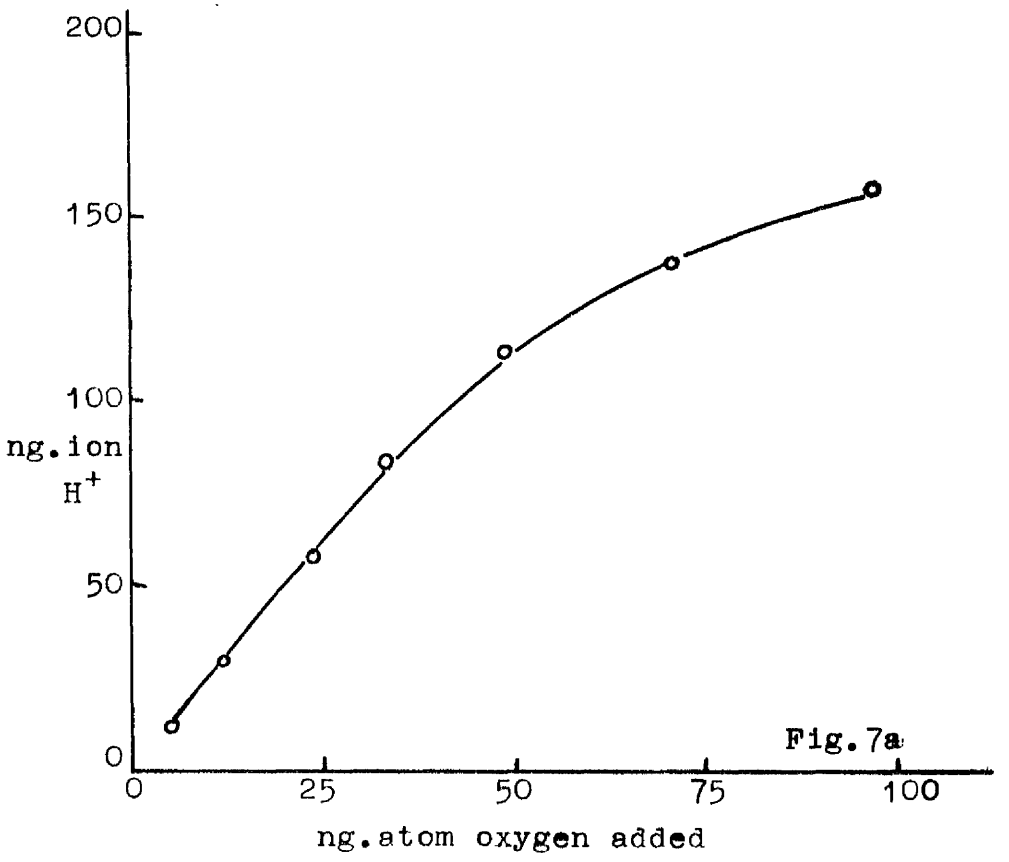

200
150
100
ng.ion
H^+
50
0
0
25
50
75
100
ng.atom oxygen added

Fig. 7a

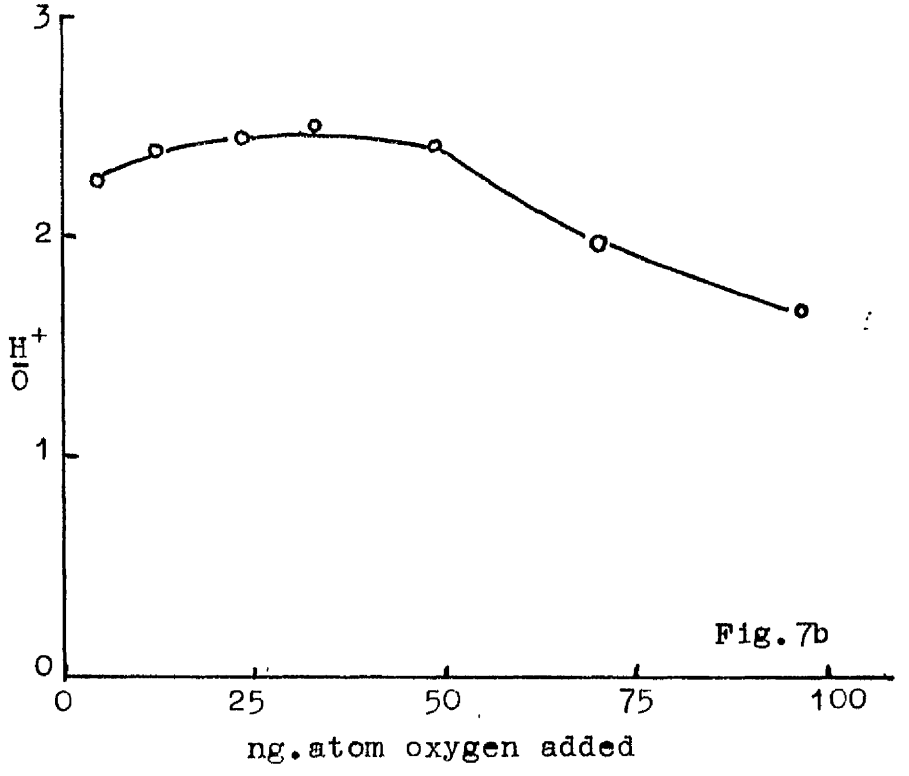

3
2
$\frac{H^+}{O}$
1
0
0
25
50
75
100
ng.atom oxygen added

Fig. 7b

dipeptide was omitted from the incubation medium with no change in the time constant of the measuring system (unpublished experiment). Its omission decreased the buffering capacity of the incubation medium (Fig. 8a) but the titration curve was, surprisingly, still linear in the pH range used. When cells were added to incubation medium without glycyl-glycine the buffering capacity increased (Fig. 8b) presumably from exposed proteins on the cell's surface. The sensitivity of the system to changes in $[H^+]$ was increased when glycyl-glycine was omitted, but the titration curve was non-linear (Fig. 8b). This was not a problem since acid standards were included with each cell suspension.

Omitting KSCN from the incubation medium resulted in smaller $\frac{H^+}{O}$ values and no characteristic decay of the peak of H^+ extrusion (Fig. 9b). This is consistent with SCN^- fulfilling a requirement for a permeant (an) ion to collapse the membrane potential in the system.

Omitting KCl from the medium slowed the rate of appearance of H^+'s from the cells (Fig. 9c). It was not due to an effect on the electrode since the acid calibration pulse did not have the same shape, but may be due to the high level of KSCN in the preparation (150 mmol.l^{-1}). It is also possible that Cl^-, as well as SCN^-, may be required as a permeant ion in the system.

Omitting glycerol from the medium resulted in no proton translocation. The cells used in these experiments had been grown at slow growth rates in arithmetic culture. They were energy-starved and required an exogenous carbon source to provide reducing power for electron transport.

Figure 8 EFFECT OF OMITTING GLYCYL-GLYCINE FROM INCUBATION MEDIUM FOR PROTON EXTRUSION EXPERIMENTS

Fig. 8a Titration curves were obtained in the range pH 6.0-7.2 for incubation medium, with and without glycyl-glycine:

o—o KCl (150 mmol.l^{-1}), KSCN (50 mmol.l^{-1}),

Δ—Δ KCl (150 mmol.l^{-1}), KSCN (50 mmol.l^{-1}), glycyl-glycine (1.5 mmol.l^{-1}).

Fig. 8b Titration curves were obtained for the media of Fig. 8a, but in the presence of cells (10.8 mg of glycerol-grown ML308).

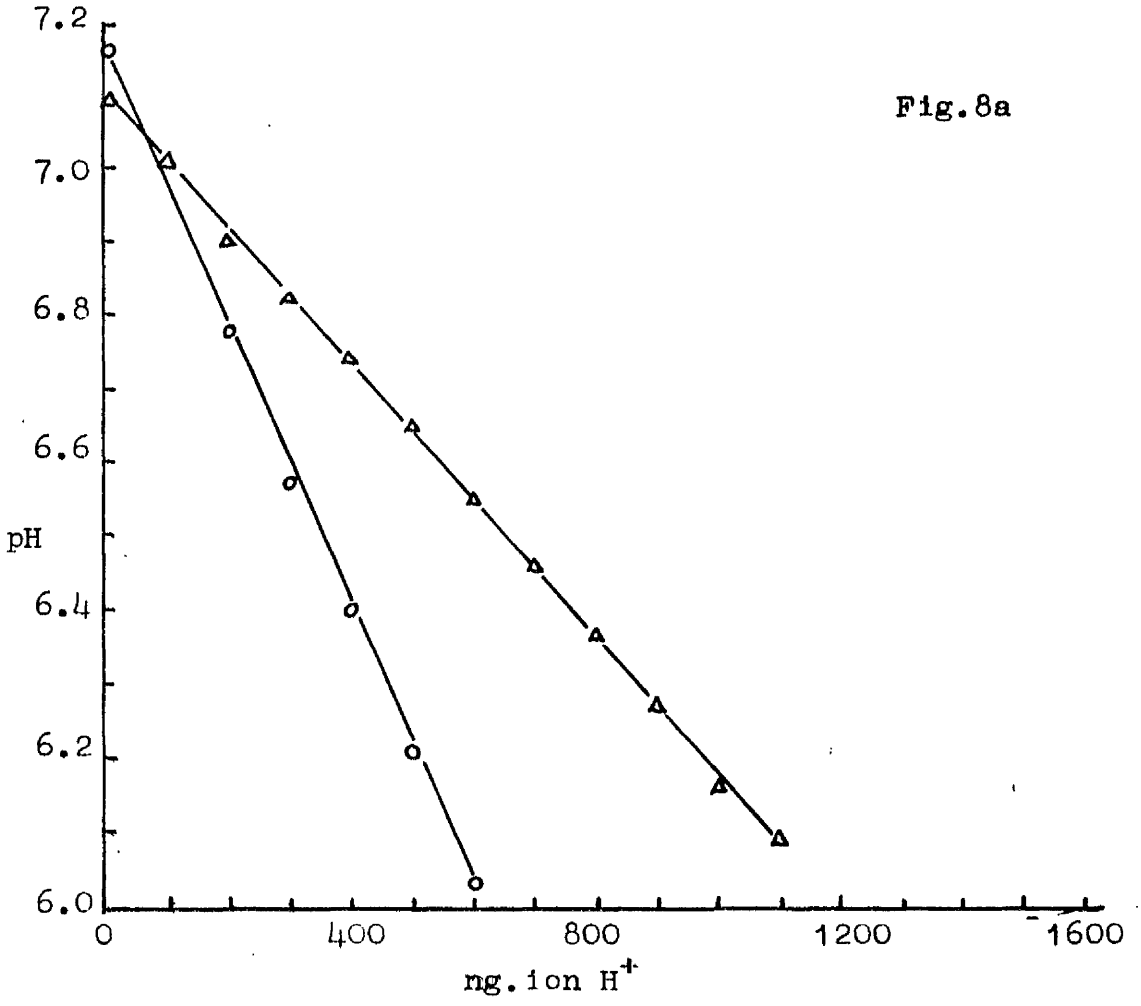

7.2
7.0
6.8
6.6
pH
6.4
6.2
6.0
0
400
800
1200
1600
ng. ion H+

Fig.8a

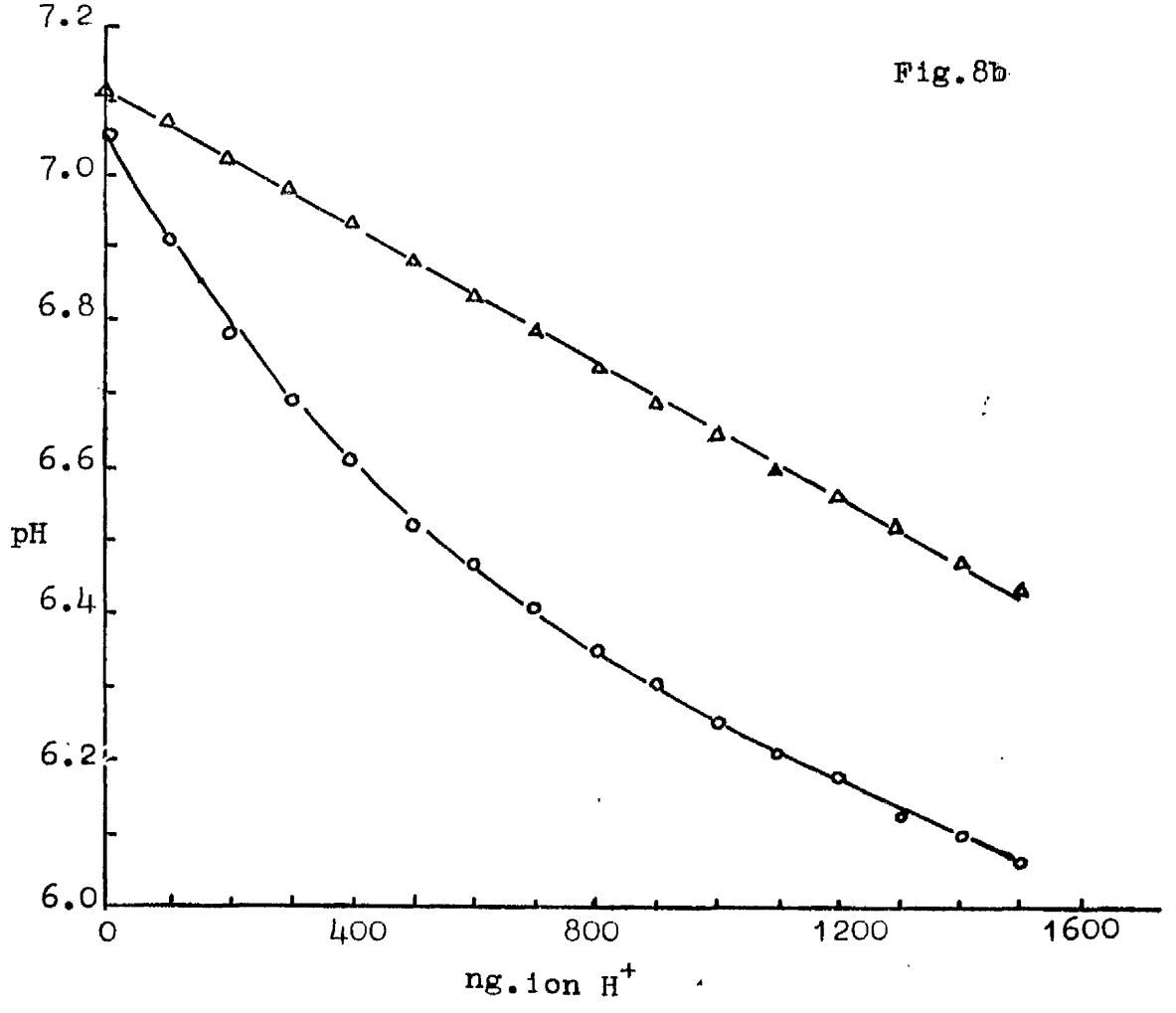

7.2
7.0
6.8
6.6
pH
6.4
6.2
6.0
0
400
800
1200
1600
ng. ion H+

Fig.8b

Figure 9 EFFECT OF OMITTING OTHER COMPONENTS OF THE INCUBATION MEDIUM FOR PROTON EXTRUSION EXPERIMENTS

Oxygen - pulse and acid - calibration H^+ pulses were obtained for lactose-grown '51' cells in the following media:

Fig. 9a KCl (150 mmol.l^{-1}), KSCN (50 mmol.l^{-1}), glycerol (1 mmol.l^{-1}).

Fig. 9b KCl (150 mmol.l^{-1}), glycerol (1 mmol.l^{-1}).

Fig. 9c KSCN (150 mmol.l^{-1}), glycerol (1 mmol.l^{-1}).

Derived $\frac{H^+}{O}$ ratios are shown beside each oxygen-induced pulse.

Fig.9a

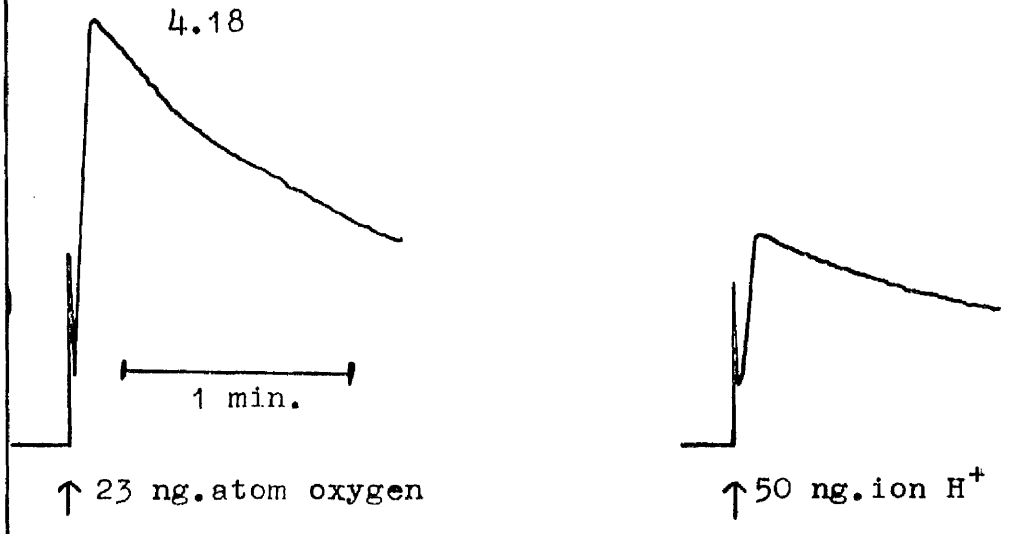


Fig.9b

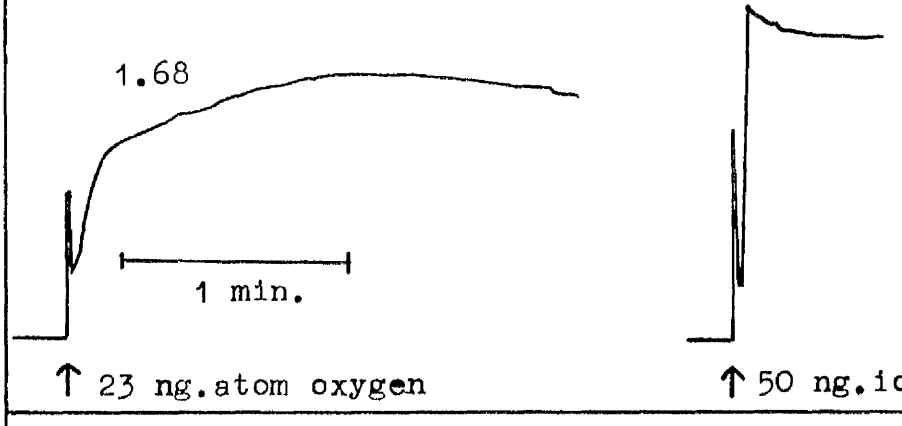
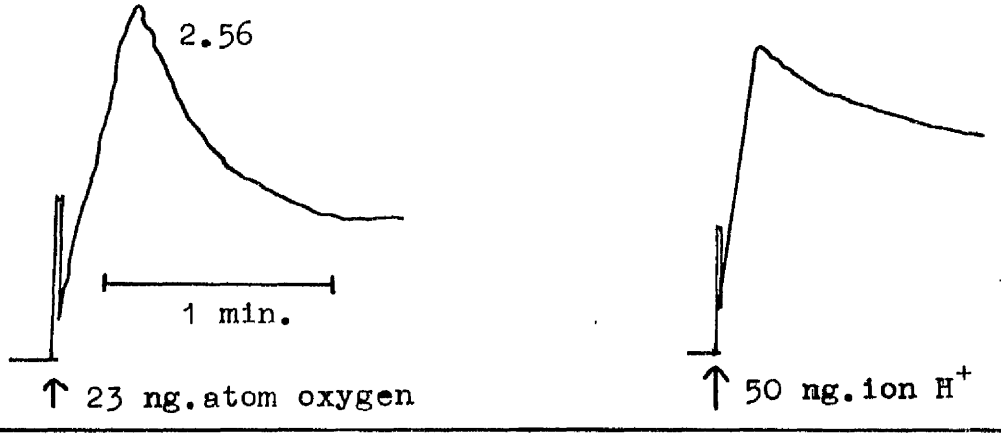


Fig.9c



In summary, all of the components of the 'traditional' incubation medium for proton extrusion experiments with mitochondria (Mitchell & Moyle, 1976b) were required for experiments with E.coli, except glycyl-glycine which was omitted. An exogenous source of reducing power (glycerol) was also included since the cells had low endogenous energy reserves.

4.4 Processing of results

On addition of a small amount of oxygen to an anaerobic cell suspension of pulse of H^+'s is extruded from the cells. This decays with time, presumably as H^+'s are readsorbed by the cells either by leakage or in energy-linked functions. Since the initial rate of decay may be rapid, and the 'dead time' of the instruments may be longer than this, it has been usual to extrapolate the decay curve back to the point when the oxidant would be used up (Mitchell & Moyle, 1976b; Lawford & Haddock, 1973).

The arithmetic culture cells used for proton translocations in the present work show rapid decays of both oxidant-induced and acid-calibration pulses. Although the pulses have different heights, they have decays which are apparently first-order and of equal rate (Fig. 10). Extrapolations of the curves will, therefore, have little effect on the results, so only 'base-peak' changes were used in the calculation of $\frac{H^+}{O}$ ratios.

There will only be a significant increase in the apparent $\frac{H^+}{O}$ ratio when the rate of decay of the acid-calibration pulse is significantly less than the oxidant-induced pulse. This must be the case for most preparations (e.g. Lawford et al., 1976; Meijer et al., 1977), but does

Figure 10 EFFECT OF EXTRAPOLATION OF DECAY CURVE ON DERIVED H^+/O RATIO

Pulse oxidant (Fig. 10a) and acid calibration (Fig. 10b) pulses were obtained for lactose-grown '51' cells (9.7 mg). The decay portion of each curve was plotted semi-logarithmically.

$\frac{H^+}{O}$ ratio, as calculated by deviation of peak height from base line for both pulses, was 3.91.

$\frac{H^+}{O}$ ratio could be calculated by extrapolation of the decay curve back to the time of entry of oxygen or acid (↓). Since the decays for both the pulse oxidant and acid calibration pulses were of equal rate, there was no significant change in the derived $\frac{H^+}{O}$ ratio (3.93).

Fig.10a

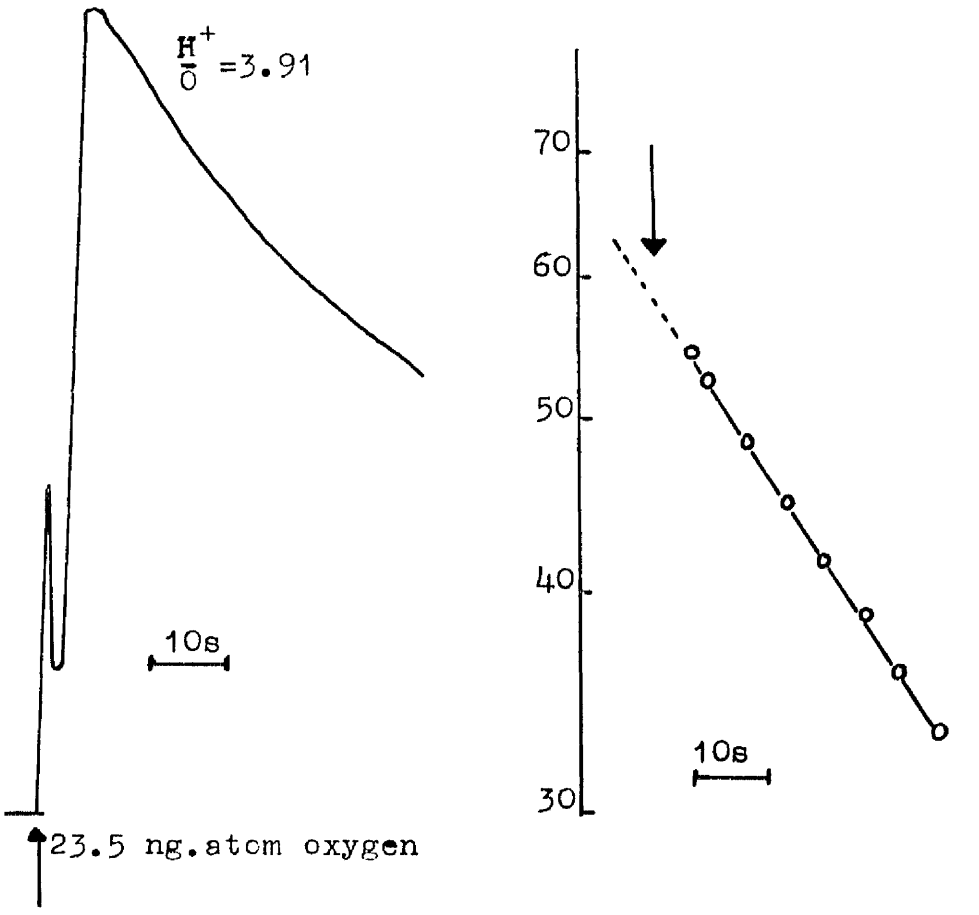
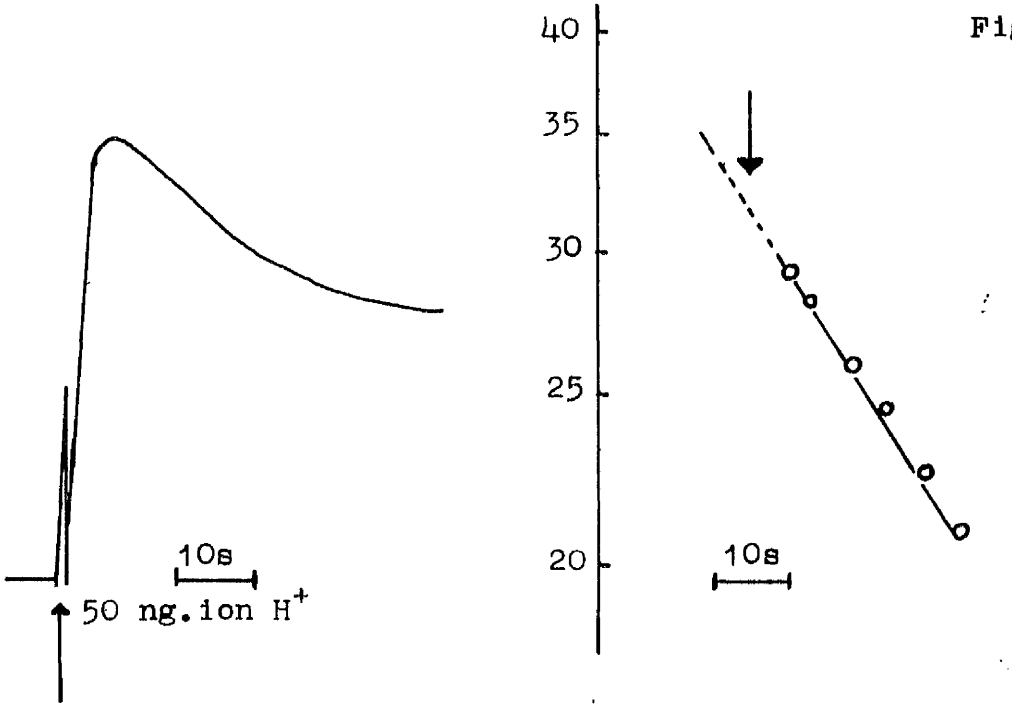


Fig.10b



not apply to arithmetic-culture cells.

4.5 Proton permeability

Protons pumped to the outside of the cytoplasmic membrane by the electron transport carriers (as is envisaged by the chemiosmotic hypothesis) must, in E.coli, pass into the periplasmic space, and in the growing cell be readsorbed during energy-linked reactions. However, during proton extrusion experiments, it is a change in pH of the external medium which is sensed by the glass electrode. To cause a pH change, protons must cross the outer cell membrane from the periplasmic space to the external medium or, conversely, there must be a flux of anion(s) from the external medium to the periplasmic space. If the outer membrane posed a permeability barrier to any ion (as it does for the antibiotic valinomycin), or the electro-chemical activity of protons in the periplasmic space were different from that in the incubation medium as a consequence of the buffering capacity of the periplasmic proteins, the pH change reported by the electrode would not be an accurate measure of the number of protons translocated during electron transport. In addition, the acid-calibration pulse would only refer to ion movements which took place outside the outer membrane.

The method, therefore, assumes that the external medium and periplasmic space behave as one continuous phase. Spheroplasts were used to test this hypothesis (Fig. 11). Since they have no outer osmotic barrier, protons pumped during electron transport will pass directly into the external medium to be sensed by the glass electrode.

$\frac{H^+}{O}$ ratios for spheroplasts were similar to those of whole cells (Fig. 11), indicating that in whole cells the

Figure 11 COMPARISON OF DERIVED H^+/O RATIOS FOR CELLS AND SPHEROPLASTS OF E.coli

$\frac{H^+}{O}$ ratios were determined for whole cells and spheroplasts of '51' cells grown on either glycerol or lactose.

Spheroplasts were prepared according to Witholt et al. (1976a). Sucrose (0.3 $mol.l^{-1}$) was included in the incubation medium for proton extrusion experiments with spheroplasts to prevent lysis.

Results are shown as mean $\pm$ standard deviation with number of determinations in parenthesis.

Growth substrate	$\frac{H^+}{O}$	
	Whole cells	Spheroplasts
glycerol	2.46 ± 0.02 (14)	2.44 ± 0.07 (4)
lactose	4.08 ± 0.05 (11)	3.96 ± 0.09 (5)

periplasmic space and external medium behave as one continuous phase with respect to protons. In general, $\frac{H^+}{O}$ ratios of lactose-trained spheroplasts were slightly lower than those for the corresponding cells, whereas $\frac{H^+}{O}$ ratios for cells and spheroplasts grown on glycerol were virtually indistinguishable. This suggests that lactose-trained cells are more fragile during the preparation of spheroplasts.

5. Formation of spheroplasts from arithmetic-culture cells

Kaback's method (1971) for the preparation of bacterial membrane vesicles involves formation of spheroplasts using lysozyme to break down the cell walls. Whereas his method was ideal for exponentially-growing cells, arithmetic-culture cells were not sensitive to lysosyme under his conditions (Fig. 12a). Osborn et al., (1972) had reported that some E.coli preparations which were resistant to lysosyme could be made sensitive by a modification of Kaback's method. However, this approach was only marginally better than Kaback's for arithmetic-culture cells (Fig. 12b). It seemed, therefore, that arithmetic-culture cells closely resembled stationary-phase cells, which are totally resistant to lysosyme using either method (unpublished work).

Witholt et al., (1976a), investigating membranes of stationary-phase E.coli, had to devise an osmotic shock technique to produce spheroplasts. This method was shown to be equally good for exponentially-growing and arithmetic-culture cells (Fig. 12c).

The change in membrane morphology on going from logarithmic to stationary (and 'arithmetic') phase, which denies access by lysosyme to the cell wall, is not well characterised. It has been suggested that a 'tougher'

Figure 12 FORMATION OF SPHEROPLASTS OF E.coli

The ability of lysosyme to render a suspension of strain ML308 (glycerol-grown) osmotically fragile was followed by decrease in E_{420} of that suspension with time, for cells grown exponentially ($\Delta - \Delta$) and from arithmetic culture ($o - o$).

Fig. 12a Method of Kaback (1971).

Fig. 12 b Method of Osborn et al. (1972).

Fig. 12c Method of Witholt et al. (1976a).

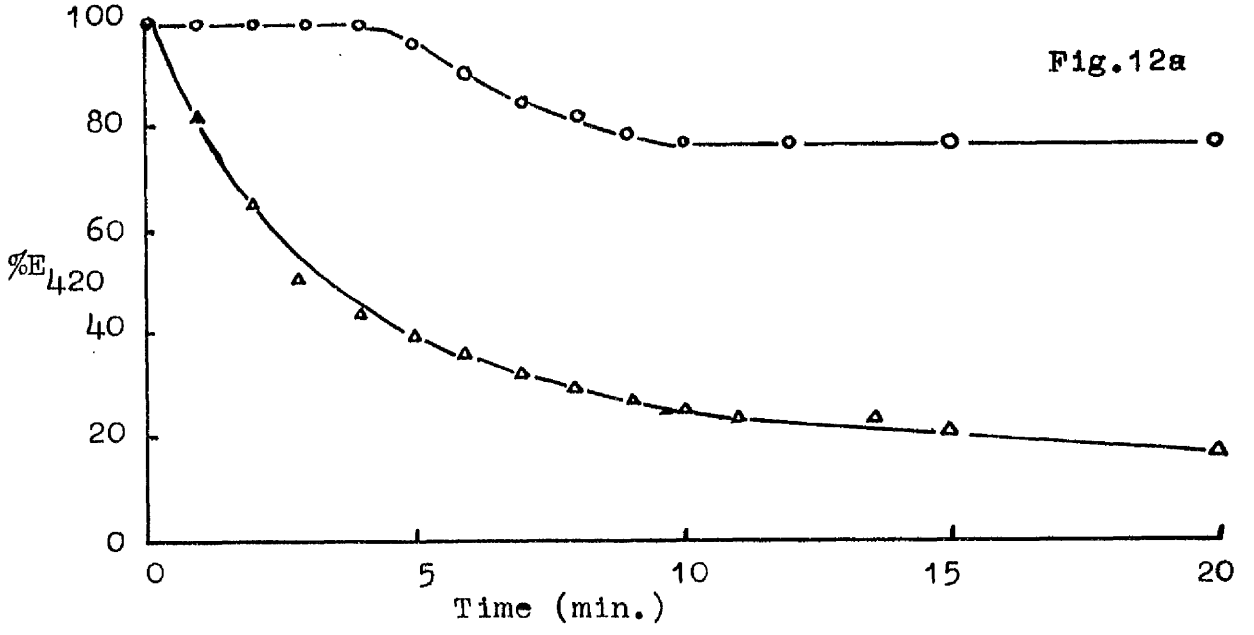


Fig.12a

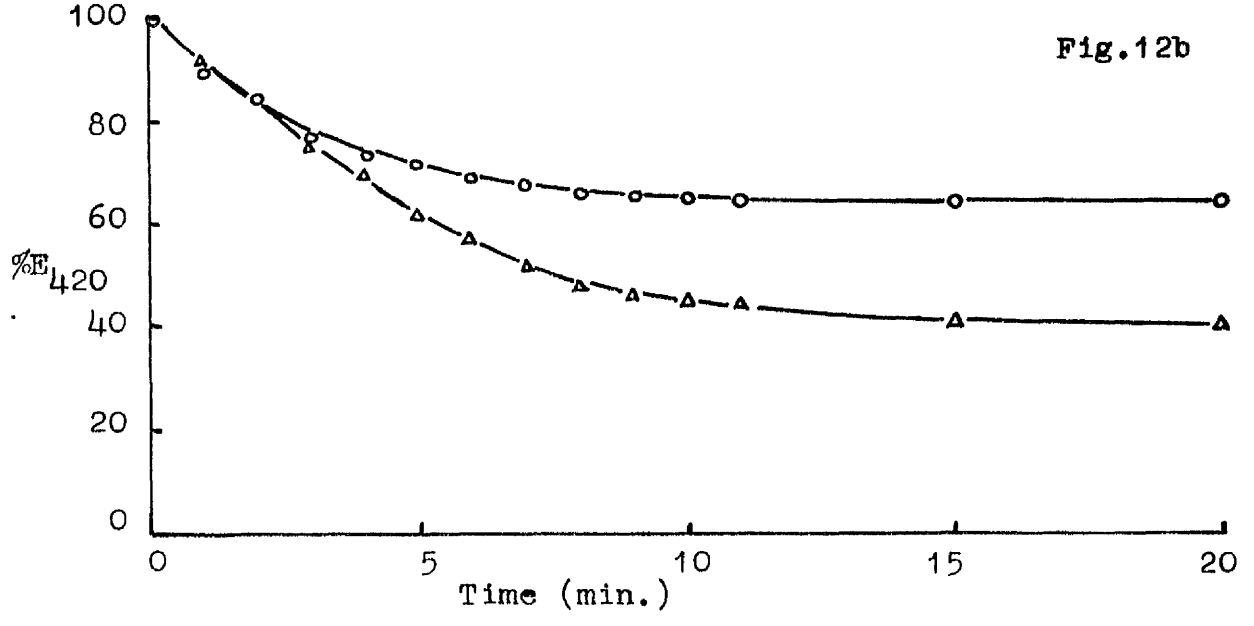


Fig.12b

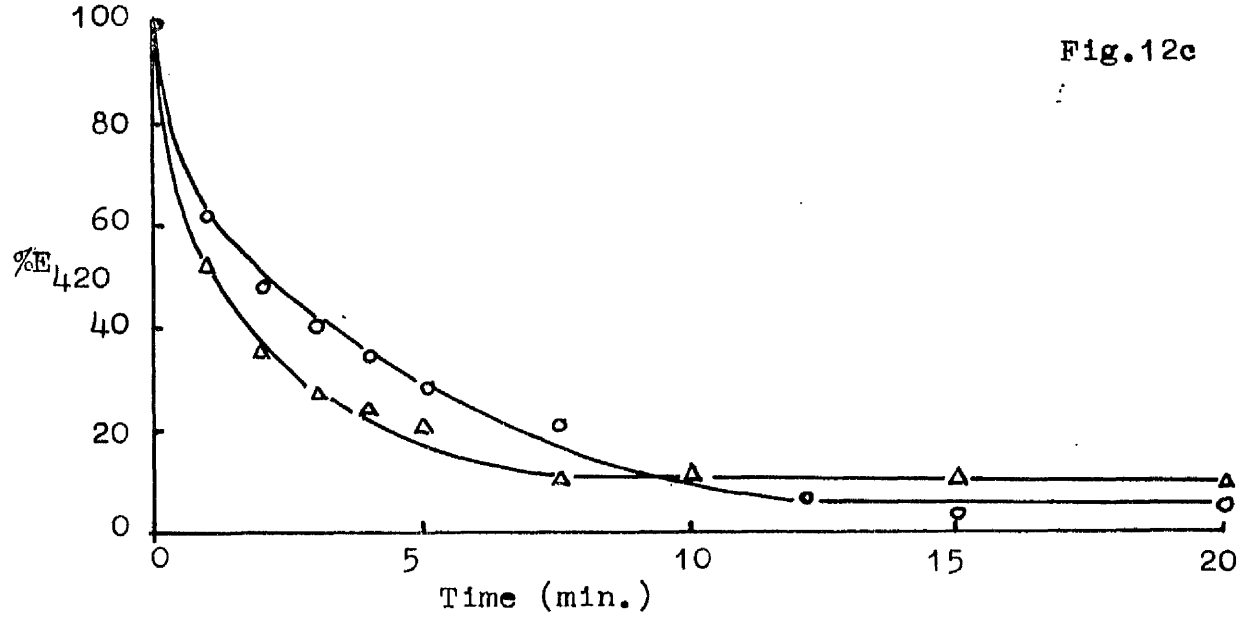


Fig.12c

outer membrane may play a role in protecting stationary-phase cells and that transient changes in the membrane structure caused by osmotic shock allow the lysosyme to enter (Witholt *et al.*, 1976b). Recently osmotic shock has been shown to be important in protoplast formation in *E.coli* (Weiss, 1976).

The method of Witholt *et al.*, (1976a) was, therefore, used in the preparation of all spheroplasts. Spheroplast formation was judged complete when most cells were shown to be spheres by phase contrast microscopy, and when lysis curves (followed in the spectrophotometer) showed no further change in extinction. Spheroplasts were stored on ice, and used within 3 h of preparation. If kept for longer than 5 h, the spheroplasts began to lyse, as judged by increased buffering capacity (from the internal cellular proteins) in the $\frac{H^+}{O}$ apparatus.

1. Growth in arithmetic culture

1.1 Growth on glucose and lactose

1.1.1 Growth on glucose

Glucose-trained cells of E.coli ML308 were grown at 37°C in arithmetic culture. Growth and gas exchange measurements were processed (Appendix 1) to give maintenance and growth yield values (Fig. 13a).

The glycerol-constitutive mutant of ML308 (designated '51') was used in proton translocation experiments. When grown on glucose in arithmetic culture, it had maintenance and yield values which were experimentally indistinguishable from ML308 (Fig. 13a).

$m^*_{O_2}$, a calculated value for complete oxidation of carbon source used for maintenance (m), was close to the experimentally-derived value (m_{O_2}). This verifies that carbon for maintenance was being used for energy functions.

1.1.2 Growth on lactose

Both ML308 and 51 behaved similarly when grown on lactose in arithmetic culture, with m_{O_2} consistent with complete oxidation of carbon source used for maintenance (Fig. 13 b).

1.1.3 Comparison between growth on glucose and on lactose

For convenience of comparison, carbon yields (Y^G) and maintenance coefficients (m) of Fig. 13 were expressed in terms of g.atom carbon for each substrate (Fig. 14).

Oxygen growth yields ($Y^G_{O_2}$) were higher for lactose-grown than glucose-grown cells, and this was reflected in

Figure 13 YIELD AND MAINTENANCE VALUES FOR E.coli GROWN ON GLUCOSE AND LACTOSE

ML308, or its glycerol-constitutive derivative (51) were grown in arithmetic culture at 37°C. Yield and maintenance values were calculated from gas and growth data, as described in Appendix 1. $m^*_{O_2}$ is the amount of oxygen calculated to completely oxidise the amount of carbon source used for maintenance.

Fig. 13a Growth on glucose

Fig. 13b Growth on lactose

cell type	growth substrate	$g \cdot mol^{-1}$		$\mu mol \cdot g^{-1} \cdot h^{-1}$		
		Y^G	$Y^G_{O_2}$	m	m_{O_2}	$m^*_{O_2}$
308	glucose	95	45	35	220	210
51	glucose	96	45	38	217	228

Fig. 13a

cell type	growth substrate	$g \cdot mol^{-1}$		$\mu mol \cdot g^{-1} \cdot h^{-1}$		
		Y^G	$Y^G_{O_2}$	m	m_{O_2}	$m^*_{O_2}$
308	lactose	224	63	37	406	444
51	lactose	228	62	41	465	492

Fig. 13b

Figure 14 COMPARISON BETWEEN GROWTH ON GLUCOSE AND ON LACTOSE

For ease of comparison, the carbon yield (Y^G) and maintenance coefficients (m) of Fig. 13 were expressed in terms of g.atom carbon.

cell type	growth substrate	g.gatom C^{-1} Y^G	g.mol^{-1} $Y^G_{O_2}$	µg.atom $g^{-1}.h^{-1}$ m	µmol.$g^{-1}.h^{-1}$ m_{O_2}
308	glucose	15.8	45	210	220
51	glucose	16.0	45	228	217
308	lactose	18.7	63	444	406
51	lactose	19.0	62	492	465

the carbon yield values (Y^G).

Lactose-grown cultures showed around twice the maintenance requirement of those grown on glucose.

1.1.4 Growth on mixtures of glucose and lactose

Unlike the situation in logarithmically-growing cultures, when glucose metabolism dominates that of lactose (Holms, 1968), in arithmetic culture both carbon sources are used simultaneously when a mixture is supplied.

When cultures which had been trained to either glucose or lactose were supplied with and grew on a mixture of an equal proportion (g.atom carbon) of glucose and lactose, they showed yield values which were characteristic of growth on lactose alone (Fig. 15a). The maintenance coefficients also suggested that although 50% of the carbon was entering the cells as glucose, the entire carbon supply was being used as if it were lactose (cf. Fig. 14). In this situation the lactose 'phenotype' was dominant over that of glucose.

When performing experiments with single substrates it was the normal procedure to inoculate at low cell density and switch on the carbon supply pump simultaneously. Initially the culture grew exponentially, but after a few hours when carbon source became limiting growth became arithmetic. This practice was continued in the mixed substrate experiments described in Fig. 15a. Since during the initial exponential growth phase glucose is used in preference to lactose, cells would have been growing logarithmically on lactose before entering arithmetic growth phase (Fig. 16a). For this reason it was perhaps not surprising that inocula of both glucose and lactose

Figure 15 GROWTH ON MIXTURES OF GLUCOSE AND LACTOSE

Carbon was supplied to arithmetic cultures of ML308 growing at 37°C as mixtures of glucose and lactose. The entering medium always contained the same carbon content (1.92 g.atom.l^{-1}). Yield and maintenance values (Appendix 1) were calculated.

Fig. 15a Effect of type of inoculum on 50% mixtures of lactose and glucose.

Fig. 15b Effect of decreasing lactose content on growth on lactose/glucose mixtures.

Fig. 15c Effect of size of inoculum on growth on lactose/glucose mixtures.

% carbon supplied as glucose	lactose	phenotype of inoculum	g.gatom C^{-1} Y^G	g.mol^{-1} $Y^G_{O_2}$	µg.atom C g^{-1}.h^{-1} m	µmol g^{-1}.h^{-1} m_{O_2}
50	50	lactose	18.8	59	452	442
50	50	glucose	18.8	62	468	454

Fig. 15a

% carbon supplied as glucose	lactose	phenotype of inoculum	g.gatom C^{-1} Y^G	g.mol^{-1} $Y^G_{O_2}$	µg.atom C g^{-1}.h^{-1} m	µmol g^{-1}.h^{-1} m_{O_2}
75	25	lactose	18.3	63.5	402	383
95	5	lactose	18.8	63.7	390	368
100	0	lactose	16.8	47.0	204	192

Fig. 15b

% carbon supplied as glucose	lactose	phenotype of inoculum	g.gatom C^{-1} Y^G	g.mol^{-1} $Y^G_{O_2}$	µg.atom C g^{-1}.h^{-1}	µmol g^{-1}.h^{-1}
50	50	glycerol	16.1	45	252	220
0	100	glycerol	16.7	44	246	232

Fig. 15c

Figure 16 OXYGEN UPTAKE OF E.coli ML308 WHEN INOCULATED INTO GLUCOSE/LACTOSE MIXTURES, AND GROWN IN ARITHMETIC CULTURE

Fig. 16a Oxygen uptake of glucose-trained E.coli ML308 when inoculated into glucose/lactose mixture (50% of each) and grown in arithmetic culture.

Fig. 16b Oxygen uptake of glycerol-trained E.coli ML308, when inoculated at high cell density into defined medium minus carbon source, and grown on limiting glucose/lactose mixture (50% of each).

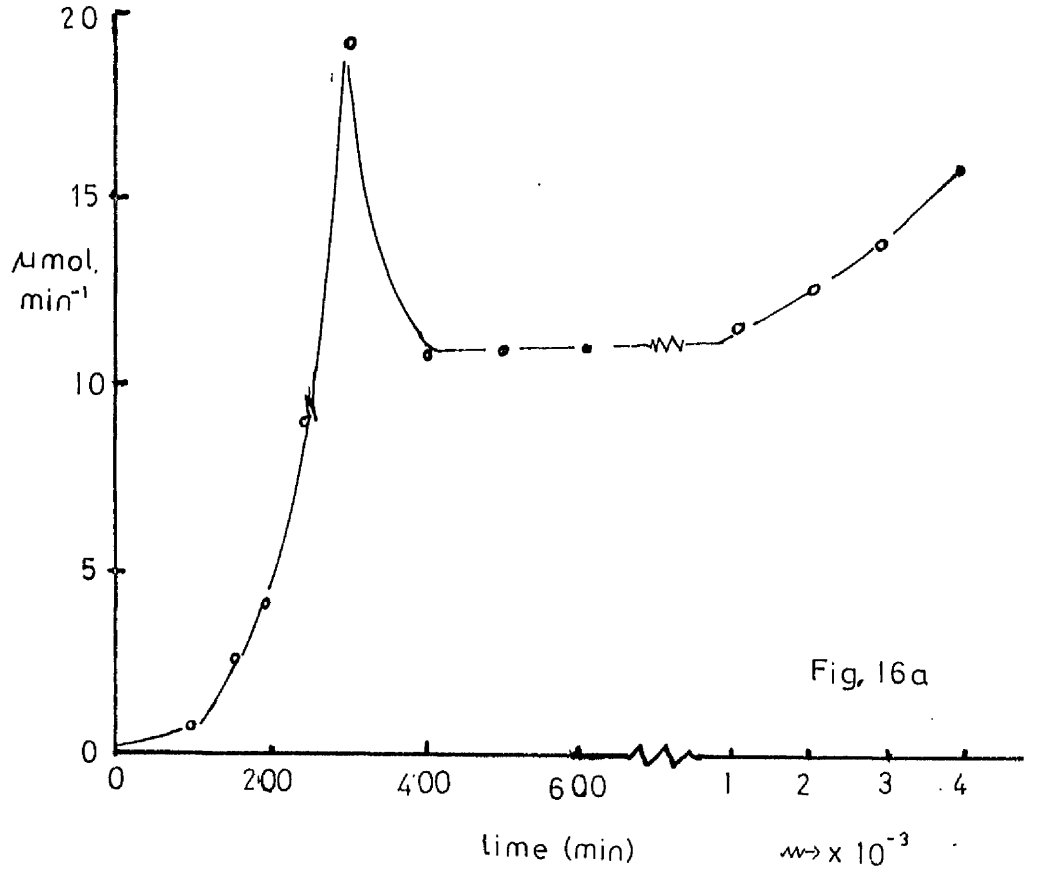


Fig. 16a

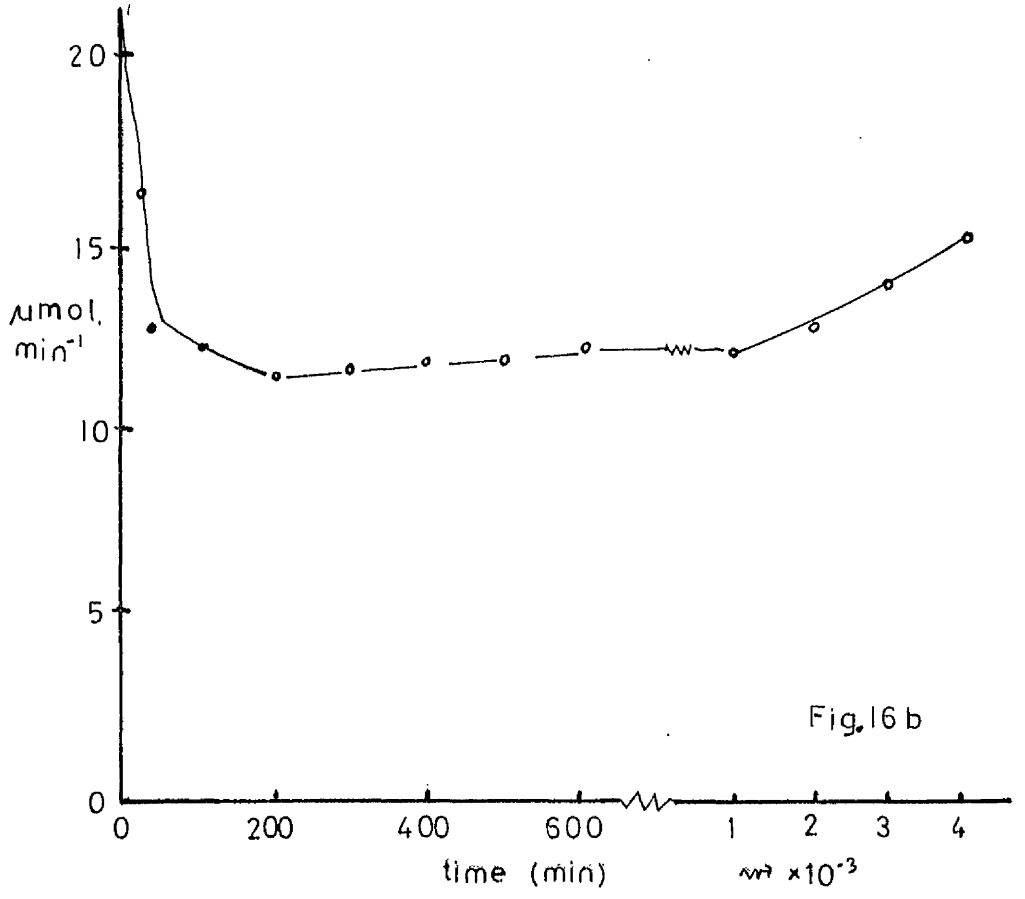


Fig. 16 b

phenotypes displayed virtually identical yield and maintenance constants when grown on a glucose/lactose mixture (Fig. 15a).

To prevent the initial exponential growth phase, cells were inoculated into the growth vessel such that the respiratory and growth potentials of the culture were in excess of that possible at the rate of substrate supply to the culture (Fig. 16b). (There was no carbon source in the growth vessel at inoculation). The cells, which were glycerol-trained, grew on the glucose/lactose mixture to give yield and maintenance values similar to those when grown on glucose alone (Fig. 15c). The growth of the glucose-trained inoculum on the substrate mixture with high yield and maintenance values (Fig. 15a) was most probably due to the period of exponential growth on lactose prior to entering arithmetic growth phase which made it indistinguishable from the lactose-trained inoculum.

The high yield and maintenance values characteristic of growth on lactose were still observed when the proportion of lactose entering the cells was reduced to 25% and even to 5% of the total carbon supply (Fig. 15b). This was not due to an intrinsic property of the inocula (lactose-grown) in these experiments since the same inoculum showed yield and maintenance values characteristic of glucose when grown on that substrate alone. Apparently, only a small proportion of carbon entering the cell as lactose was sufficient to maintain the phenotype characteristic of lactose.

1.2 Growth on glycerol, malate and their mixtures with lactose

1.2.1 Growth on glycerol

The glycerol-constitutive mutant of ML308, (51), grew on glycerol at 37°C in arithmetic culture with the yield and maintenance values shown in Fig. 17a.

1.2.2 Growth on glycerol/lactose mixtures

The strain '51' is constitutive in the enzymes for both glycerol and lactose dissimilation. It can therefore grow immediately on either substrate irrespective of the carbon source used for training. Yield and maintenance values were obtained for growth on glycerol/lactose mixtures with both glycerol and lactose inocula (Fig. 17b). The data of Fig. 17a are included in the same units, for comparison.

It is difficult to assign meaningful yield values to growth on glycerol/lactose mixtures since the two substrates exist at different oxidation levels and are catabolised by different pathways. However, comparison of the results of Fig. 17b shows that growth of the lactose-trained inoculum on the mixture was more efficient than that of the glycerol-trained inoculum. In turn, growth of the glycerol-trained inoculum on the mixture was more efficient than on glycerol alone, but this neglects the oxidation level of the carbon supplied.

Maintenance values did not show any recognisable pattern.

1.2.3 Growth on malate

Yield and maintenance values for ML308 trained to and growing in arithmetic culture on malate are shown in Fig. 18a.

Figure 17 GROWTH ON GLYCEROL AND GLYCEROL/LACTOSE MIXTURE

Strain 51, which is constitutive in the glycerol and lactose enzymes, was grown in arithmetic culture at 37°C on glycerol alone or mixtures containing an equal proportion of glycerol and lactose.

Fig. 17a Growth of glycerol-trained culture on glycerol alone.

Fig. 17b Effect of inoculum phenotype on growth and maintenance with glycerol/lactose mixtures.

There was no initial exponential growth phase in these experiments; cells entered arithmetic growth phase immediately.

growth substrate	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$		
	Y^G	$Y^G_{O_2}$	m	m_{O_2}	$m^*_{O_2}$
glycerol	50	37	100	340	350

Fig. 17a

% carbon supplied as glycerol	% carbon supplied as lactose	phenotype of inoculum	$g.gatom\ C^{-1}$	$g.mol^{-1}$	$\mu g.atom\ C\ g^{-1}.h^{-1}$	$\mu mol\ g^{-1}.h^{-1}$
			Y^G	$Y^G_{O_2}$	m	m_{O_2}
100	0	glycerol	16.7	37	300	340
50	50	glycerol	17.6	47	308	293
50	50	lactose	21.2	72	319	354

Fig. 17b

Figure 18 GROWTH ON MALATE AND MALATE/LACTOSE MIXTURE

Fig. 18a Yield and maintenance values for growth of malate-trained ML308 on malate at 37°C.

Fig. 18b Yield and maintenance values for growth of lactose-trained ML308 on a mixture of malate and lactose. For comparison, the results of Fig. 18a are included in the same units.

growth substrate	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$		
	Y^G	$Y^G_{O_2}$	m	m_{O_2}	$m^*_{O_2}$
malate	45	36	92	267	276

Fig. 18a

% carbon supplied as malate	% carbon supplied as lactose	phenotype of inoculum	$g.gatom\ C^{-1}$ Y^G	$g.mol^{-1}$ $Y^G_{O_2}$	$\mu g.atom\ C\ g^{-1}.h^{-1}$ m	$\mu mol\ g^{-1}.h^{-1}$ m_{O_2}
100	0	malate	11.3	36	368	267
62.5	37.5	lactose	14.3	60.6	390	321

Fig. 18b

1.2.4 Growth on malate/lactose mixtures

Growth of ML308 on a malate/lactose mixture was more efficient than growth on malate alone (Fig. 18b). Maintenance values for growth on the mixture were also slightly higher than for growth on malate alone.

1.3 Growth on other carbon sources

Maintenance and yield coefficients were measured for some other carbon sources (Fig. 19). The substrates used fell into four categories according to their mode of transport (Hamilton, 1975; Introduction 1); glycerol enters by facilitated diffusion; glucose and mannitol by PEP-phosphotransferase; lactose, gluconate and glucose 6-phosphate by chemismotic-type active transport; and maltose and galactose by active transport involving a periplasmic binding protein and powered by ATP hydrolysis. There was a significant increase in the oxygen growth yield ($Y^G_{O_2}$) for those substrates known to enter the cell by a chemiosmotic mechanism (lactose, gluconate, glucose 6-phosphate). This was accompanied by higher maintenance values - most easily compared by considering the m_{o_2} values.

1.4 Growth of other strains in arithmetic culture

1.4.1 E.coli ML30

All of the experiments described in Figs. 13-19 used strain ML308 or its glycerol-constitutive derivative, '51'. These strains are constitutive for the lac enzymes and will always synthesise β-galactosidase and the lac permease, irrespective of the carbon source used for growth. Since there seemed to be some relationship between active

Figure 19 GROWTH AND MAINTENANCE VALUES OF E.coli ML308 FOR SINGLE SUBSTRATES AT 37°C.

This composite table includes some values described in previous figures. The substrates used fall into 4 categories, according to their mechanisms of transport (Results 1.3). The cells had been previously trained to the carbon source used for growth.

carbon source	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$	
	Y^G	$Y^G_{O_2}$	m	m_{O_2}
glucose	96	45	38	217
mannitol	107	44	38	231
glycerol	50	37	100	340
lactose	228	62	41	465
gluconate	102	71	51	304
glucose 6-phosphate	113	72	97	455
maltose	204	48	20	198
galactose	98	44	44	225

transport and high oxygen growth yield (Fig. 19; 1.3), and since the presence of the lac permease in these cells may have been responsible, control experiments were done with ML30, the inducible parent of ML308.

ML30 grew on lactose in arithmetic culture with yield and maintenance values (Fig. 20a) which corresponded well to those of ML308. A high oxygen growth yield ($Y^G_{O_2}$) occured during growth on lactose when the lac enzymes were required to be induced.

When ML30 was grown on gluconate, the results (Fig. 20b) were indistinguishable from those of ML308 grown on gluconate. There was no synthesis of lac enzymes when ML30 was grown on gluconate.

1.4.2 E.coli K10

In a preliminary trial, E.coli K10 was grown in arithmetic culture to determine yield and maintenance values (Fig. 21). This strain is the wild-type of several mutants altered in the ability to accumulate galactose (Henderson et al., 1977).

Whereas yield for E.coli K10 grown on glucose and lactose (Fig. 21) corresponded to these for E.coli ML (Fig. 19), the K strain showed a higher oxygen growth yield ($Y^G_{O_2}$) when grown on galactose than did ML strain. Maintenance values of K strain were around twice those of ML strain, with maintenance for K strain grown on lactose and galactose higher than for growth on glucose.

It was intended to grow some of the galactose transport mutants of Henderson et al. (1977) in arithmetic culture but they, unlike the wild-type, are auxotrophic for histidine and thymine. Calculation of growth and gas

Figure 20 GROWTH AND MAINTENANCE VALUES OF E.coli ML30 AT 37°C

Yield and maintenance values were calculated (Appendix 1) for growth of E.coli ML30 in arithmetic culture at 37°C.

Fig. 20a Growth on lactose of lactose-trained ML30.

Fig. 20b Growth on gluconate of gluconate-trained ML30.

The corresponding values for E.coli ML308 are included, for comparison.

strain	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$	
	Y^G	$Y^G_{O_2}$	m	m_{O_2}
ML 30	208	62	31	363
ML 308	228	62	41	465

Fig. 20a

strain	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$	
	Y^G	$Y^G_{O_2}$	m	m_{O_2}
ML 30	103	71	56	322
ML 308	102	71	51	304

Fig. 20b

Figure 21 GROWTH AND MAINTENANCE VALUES OF E.coli K10 AT 37°C

Yield and maintenance values were calculated (Appendix 1) for E.coli K10, grown in arithmetic culture at 37°C. Cells had been previously trained to the carbon source.

carbon source	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$	
	Y^G	$Y^G_{O_2}$	m	m_{O_2}
galactose	107	66	135	763
glucose	102	43	106	597
lactose	216	65	65	758

exchange data to give yield and maintenance values for arithmetic culture (Appendix 1) requires that there is a finite entry of carbon to the culture, which can be used for growth and energy without an additional nutrient. Attempts to 'cure' the nutrient requirements were fruitless. This was resolved when it was learnt that the mutants contain a his marker which is a deletion, (M. C. Jones-Mortimer, personal communication). It is hoped to continue this approach when mutants are obtained which have been cured of the deletion by transduction.

1.5 Effect of culture conditions on growth in arithmetic culture

1.5.1 Effect of growth temperature

Growth yield and maintenance values were obtained for ML308 growing on glycerol in arithmetic culture at different temperatures in the range 25-42°C (Fig. 22a). The temperature-dependence of $Y^G_{O_2}$ (and Y^G) was biphasic, with a minimum value at 37°C, the usual growth temperature of the organism. m_{O_2} (and m) was not dependent on temperature until 33.5°C, after which it increased sharply. Assuming that the constant component of maintenance ($T < 33.5°C$) is also present at higher temperatures, and that only two components exist, it was possible to obtain a value for the second (temperature-dependent) component by difference. The apparent Arrhenius activation energy of the second component was obtained (Fig. 22b) using the equation:

$$\ln \Delta m_{O_2} = \text{constant} - \frac{Ea}{RT}, \text{ where}$$

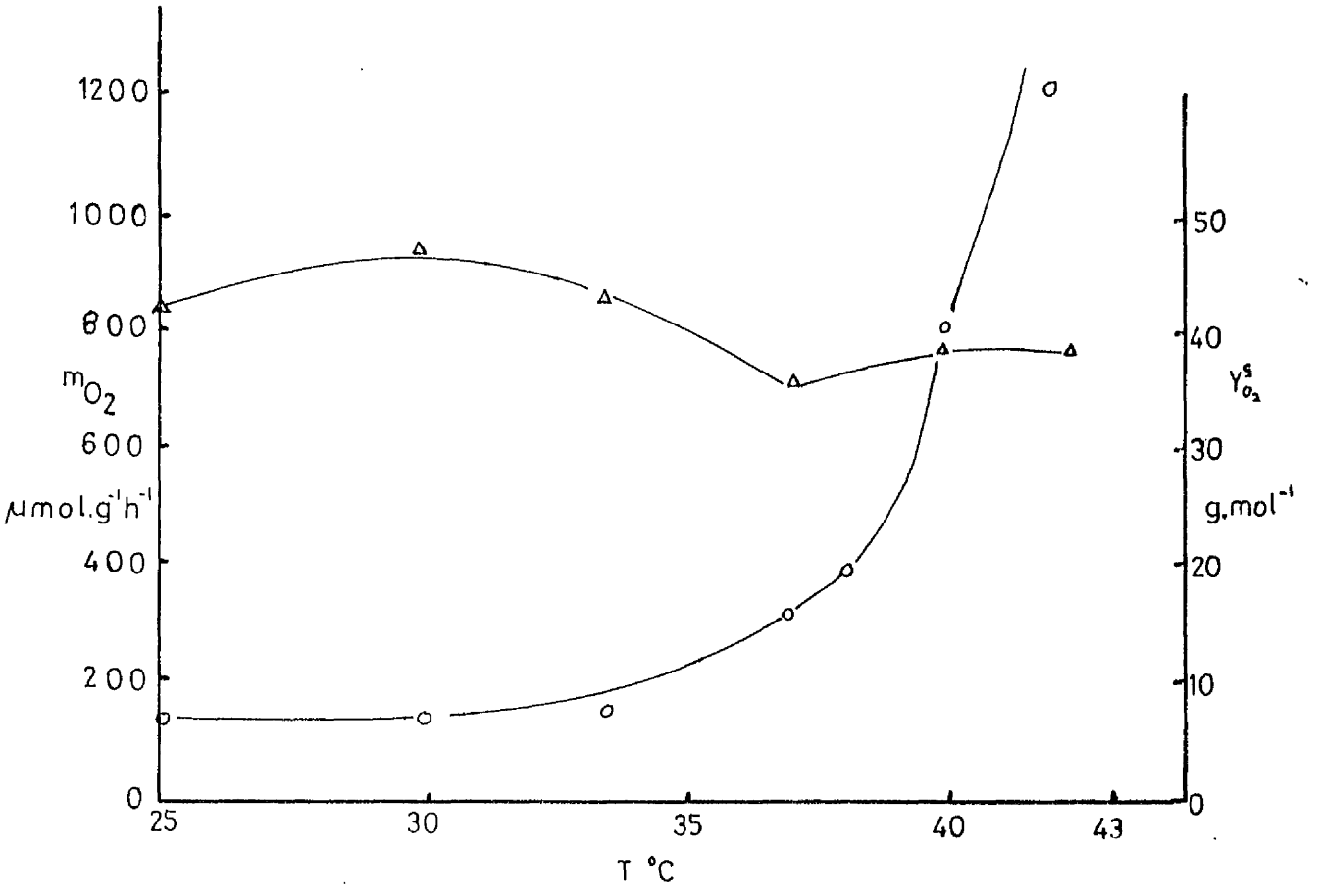
Ea is the activation energy

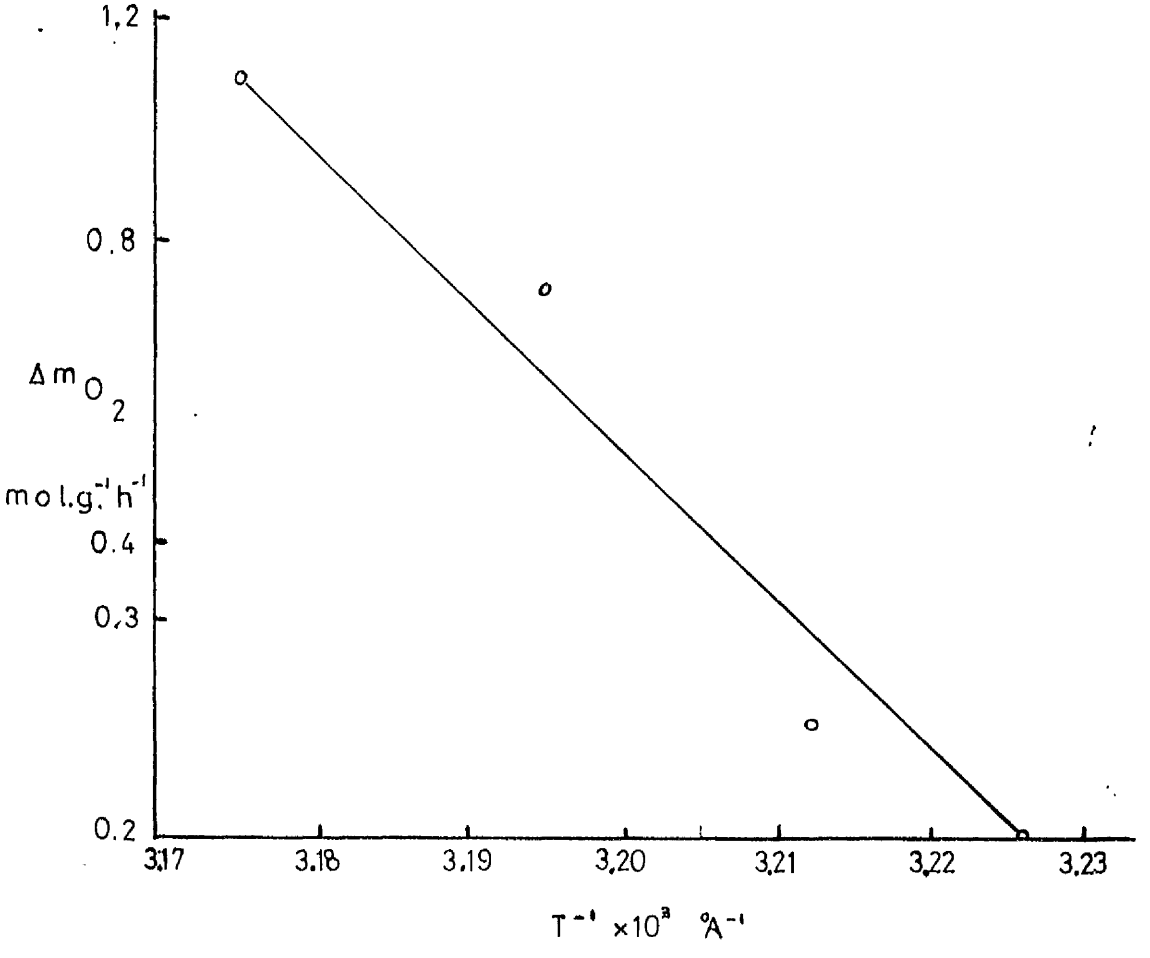
R is the gas constant

Figure 22 EFFECT OF GROWTH TEMPERATURE ON MAINTENANCE AND GROWTH YIELDS OF *E.coli* ML308

Fig. 22a *E.coli* ML308 (glycerol-trained) was grown in arithmetic culture on glycerol at various temperatures. The oxygen requirement for maintenance (m_{O_2}, o—o) and growth yield ($Y^G_{O_2}$ Δ—Δ) as calculated in Appendix 1, were plotted against temperature. The values in terms of carbon (m, Y^G) followed the same pattern. There are apparently two components (at least) of maintenance - one which is constant and one which rises sharply at T > 33.5°C. Y^{O2}_G is a minimum at 37°C.

Fig. 22b The second (temperature-dependent) component of maintenance was plotted semi-logarithmically against the reciprocal of the absolute temperature to obtain the Arrhenius activation energy (Results 1.5.1). The increment in maintenance due to the temperature-dependent component (Δm_{O_2}) was calculated by subtracting the value of the constant component (133 μmol.g.$^{-1}$h.$^{-1}$) from the values (m) of Fig. 22a. The slope of the line gave E_a = 294 kJ.mol.$^{-1}$.


1200
1000
800
600
400
200
0
m_{O_2}
µmol.g⁻¹h⁻¹
50
40
30
20
10
0
$Y^s_{O_2}$
g.mol⁻¹
25
30
35
40
43
T °C


1,2
0.8
0.4
0.3
0.2
Δm_{O_2}
mol.g⁻¹h⁻¹
3.17
3.18
3.19
3.20
3.21
3.22
3.23
T⁻¹ ×10³ °A⁻¹

T is the absolute temperature

Δm_{O_2} is the calculated value of the second component of m_{O_2}.

The activation energy for glycerol-grown cultures was 294 kJ..mol.$^{-1}$.

1.5.2 Effect of salt

Including NaCl (0.5 mol.l^{-1}) in the growth medium of a glycerol culture had little effect on the Y^G, but decreased the $Y^G_{O_2}$ by 35% (Fig. 23). Maintenance coefficients (m, m_{O_2}) were decreased by 25% of the values for growth on glycerol under normal salt conditions.

For growth on lactose in the presence of NaCl (0.5 mol.l^{-1}) both the carbon (Y^G) and oxygen ($Y^G_{O_2}$) growth yields were decreased by 31% and 57% respectively. Under these conditions, the maintenance requirement is the smallest measured during this work.

When Na_2SO_4 (0.25 mol.l^{-1}) replaced NaCl in the growth medium, there was little change in the yield coefficients; they remained at the lower values of the previous paragraph. However, the maintenance requirement increased to around half of that found under normal conditions. Because SO_4 is a divalent ion, the osmolarity of this medium was less than that when NaCl was added.

1.5.3 Sulphate-limited arithmetic culture

Previous experiments had been of carbon-limited arithmetic culture, when the growth vessel contained all the necessary nutrients except carbon which was pumped to the culture at a slow rate (Methods 6.3). For sulphate-limited experiments, the growth vessel contained nutrients including carbon, but without sulphate which was supplied

Figure 23 EFFECT OF SALT ON MAINTENANCE AND GROWTH YIELDS OF E.coli ML

E.coli ML, trained in high salt conditions (Methods 2.5.1.2) was grown in arithmetic culture at 37°C under similar high salt conditions (Methods 2.5.3.2). Maintenance and yield values were calculated as described (Appendix 1). Values for cells grown under normal conditions of osmolarity (Fig. 19) are included for comparison.

salt	concentration ($mol.l^{-1}$)	carbon source	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$	
			Y^G	$Y^G_{O_2}$	m	m_{O_2}
nil	-	glycerol	50	37	100	340
NaCl	0.5	glycerol	47	24	76	272
nil	-	lactose	228	62	41	465
NaCl	0.5	lactose	157	28	7	75
Na_2SO_4	0.25	lactose	153	26	20	270

at a slow rate. It proved impossible to grow cells in this monomer because the excess carbon source in the vessel was oxidised without any new cells being formed.

1.6 Viability of arithmetic cultures

Around 2% of the cell population of a glycerol arithmetic culture (μ = 0.028) at 30°C and 37°C were unable to divide (Fig. 24a). When the carbon supply was stopped starvation did not increase the proportion of dead cells in the culture. At 40°C, and more particularly at 42°C, greater proportions of the culture were unable to divide within 3 h. However, as the starvation time progressed, there was an increase in viabilities of those cultures (Fig. 24a), with fewer dead cells.

Cells grown at 30°C and 37°C were equally competent to form microcolonies ($<$ 4 cells) after 3 h at 30°C, with around 75% of the cell population falling into this category. At 40°C and 42°C fewer cells were able to form microcolonies, and this capability decreased on starvation.

Cells which divided once, but did not undergo a second division were termed 'activated' (Development of Methods 3.2). Their proportions may be calculated by adding the respective values in Figs. 24a and b and taking the difference from 100. The proportion of 'activated' cells increased with starvation for cells grown at 40°C and 42°C, but was constant for cells grown at 37°C and 30°C.

Figure 24 VIABILITY OF ARITHMETIC CULTURES

The viabilities of glycerol arithmetic cultures, grown at different temperatures, were assessed on agar plates at 30°C (Development of Methods, 3.2). The carbon supply to each culture was stopped at μ = 0.028 (mean generation time of 25 h) and cells were removed every 2 h for the next 10 h (0-10 h of starvation).

Fig. 24a Numbers (%) of cells which were dead (single) after 3 h incubation at 30°C, on removal from the growth vessel after 0-10 h starvation.

Fig. 24b Numbers (%) of cells which formed micro-colonies (> 4 cells) after 3 h incubation at 30°C on removal from the growth vessel after 0-10 h starvation.

growth temperature	h of starvation					
	0	2	4	6	8	10
30°C	2	14	1	1	0	0
37°C	2	0	1	10	0	7
40°C	3	48	3	26	5	3
42°C	47	32	6	10	12	18

Fig. 24a

growth temperature	h of starvation					
	0	2	4	6	8	10
30°C	72	17	95	25	68	34
37°C	79	72	72	27	59	13
40°C	56	2	52	5	14	1
42°C	23	10	21	11	21	2

Fig. 24b

2. Growth in continuous culture

2.1 Growth on glucose

When E.coli ML308 was grown on glucose at 37°C in continuous culture, the maximum molar growth yield (Y^G) was found to be 94 $g.mol^{-1}$ with maintenance coefficient (m) of 460 $\mu mol.g.^{-1}h^{-1}$ (Fig. 25). Values for yield and maintenance in terms of oxygen were not determined, since no gas exchange measurements were made on this culture.

2.2 Growth on lactose

Yield and maintenance values in terms of both carbon and oxygen were obtained for E.coli ML308 growing on lactose at 37°C in continuous culture (Fig. 26). The yield values were similar to those obtained in arithmetic culture, but the maintenance coefficients were higher than the corresponding values derived during arithmetic culture.

2.3 Growth on glycerol

When glycerol was the carbon source for continuous culture of strain ML308, the yield values were similar to those obtained during arithmetic culture on glycerol, but maintenance coefficients were about three times higher for growth on glycerol in continuous culture (Fig. 27).

2.4 Sulphate-limited continuous culture

E.coli ML308 was grown in continuous culture (Methods 6.2.2) at 37°C with excess carbon source (either lactose or glycerol) and limiting sulphate (50 $\mu mol.l^{-1}$; Methods 2.5.4.1). The yield and maintenance values determined are summarised in Fig. 28, together with the values obtained during carbon-limitation experiments.

Yield and maintenance values for sulphate-limited

Figure 25 GROWTH OF E.coli ML308 ON GLUCOSE IN CONTINUOUS CULTURE

E.coli ML308 was grown on glucose at 37°C in continuous culture (Methods 6.2.1).

The growth yield, Y, was determined at different growth rates, μ, and plotted according to Pirt (1965). The intercept $(Y^G)^{-1}$ gives Y^G = 94 g.mol^{-1}, with gradient equal to the maintenance coefficient, m, of 460 μmol.g.$^{-1}$h.$^{-1}$.

Oxygen growth yield and maintenance values could not be determined, since no gas analysis was done for this experiment. For the calculation of growth yields, it was assumed that no excretion products were formed. No glucose was found in the culture effluent at any growth rate.

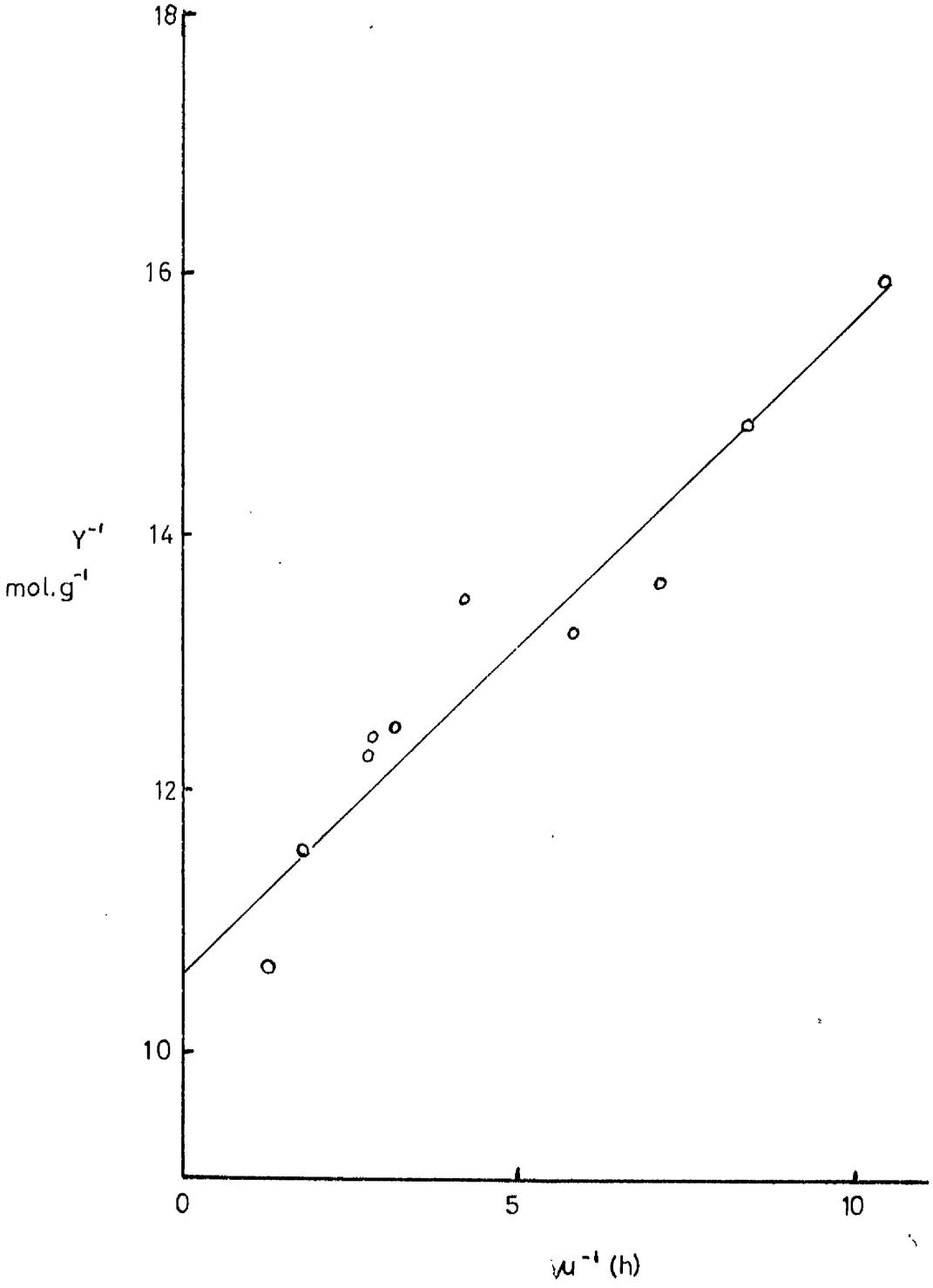

18
16
14
12
10
γ^{-1}
mol.g^{-1}
0
5
10
μ^{-1} (h)

Figure 26 GROWTH OF E.coli ML308 ON LACTOSE IN CONTINUOUS CULTURE

E.coli was grown on lactose at 37°C in continuous culture (Methods 6.2.2). Growth and gas exchange were measured at different growth rates. A carbon balance indicated that all of the lactose supplied to the culture was connected to cells and CO_2. No lactose was found in the culture effluent.

Fig. 26a Carbon yield and maintenance during growth on lactose.

The growth data was plotted according to Pirt (1965). The intercept $(Y^G)^{-1}$ gave $Y^G = 226$ g.mol.$^{-1}$. The gradient of the curve gave $m = 330$ μmol.g.$^{-1}$h.$^{-1}$.

Fig. 26b Oxygen yield and maintenance during growth on lactose.

The specific rate of oxygen utilisation (Q_{O_2}) was plotted against growth rate (μ).

The intercept gave $m_{O_2} = 3.6$ mmol.g.$^{-1}$h.$^{-1}$. The gradient of the curve corresponded to $(Y^G_{O_2})^{-1}$ giving $Y^G_{O_2} = 74.3$ g.mol^{-1}.

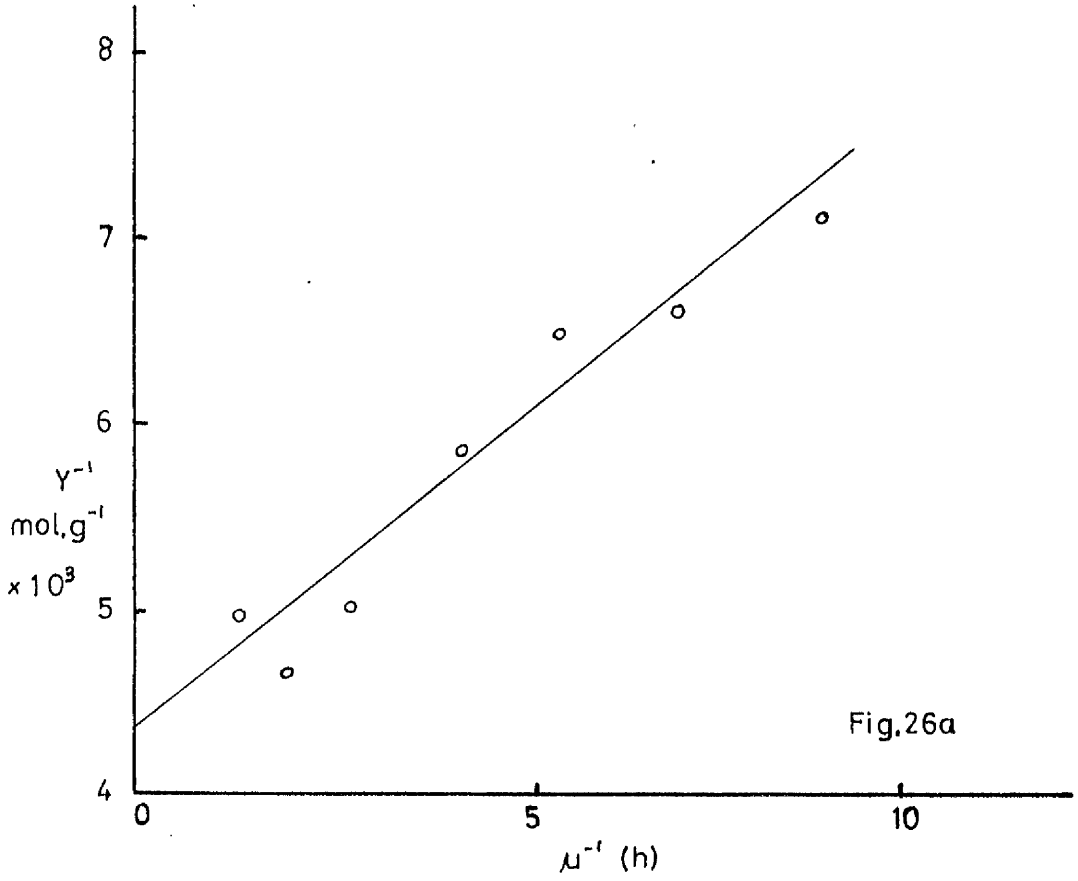


Fig.26a

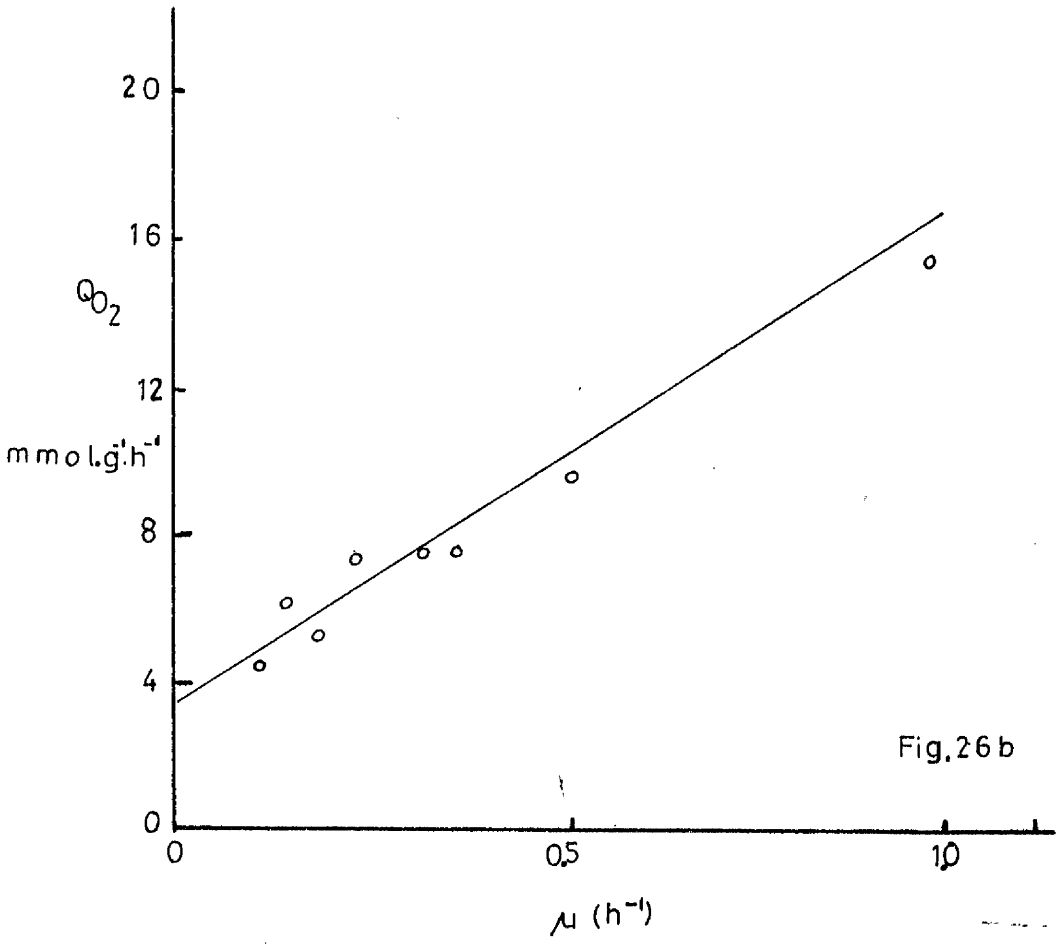


Fig.26b

Figure 27 GROWTH OF E.coli ML308 ON GLYCEROL IN CONTINUOUS CULTURE

E.coli was grown on glycerol at 37°C in continuous culture (Methods 6.2.2). Growth and gas exchange measurements were made at different growth rates. A trace of glycerol was found in the culture effluent at μ = 0.608. Otherwise, all of the glycerol was consumed. A carbon balance indicated conversion of carbon completely to cells and CO_2.

Fig. 27a Carbon yield and maintenance coefficient. Growth yields, determined at different growth rates, were plotted according to Pirt (1965). The intercept $(Y^G)^{-1}$ gave Y^G = 54 g.mol.$^{-1}$. The gradient of the curve gave m = 333 μmol.g.$^{-1}$h.$^{-1}$.

Fig. 27b Oxygen yield and maintenance coefficient. The specific rate of oxygen utilisation (Q_{O_2}) was plotted against growth rate. The intercept gave m_{O_2} = 1.62 mmol.g.$^{-1}$h.$^{-1}$. The gradient of the curve corresponded to $(Y^G_{O_2})^{-1}$, giving $Y^G_{O_2}$ = 40.3 g.mol.$^{-1}$.

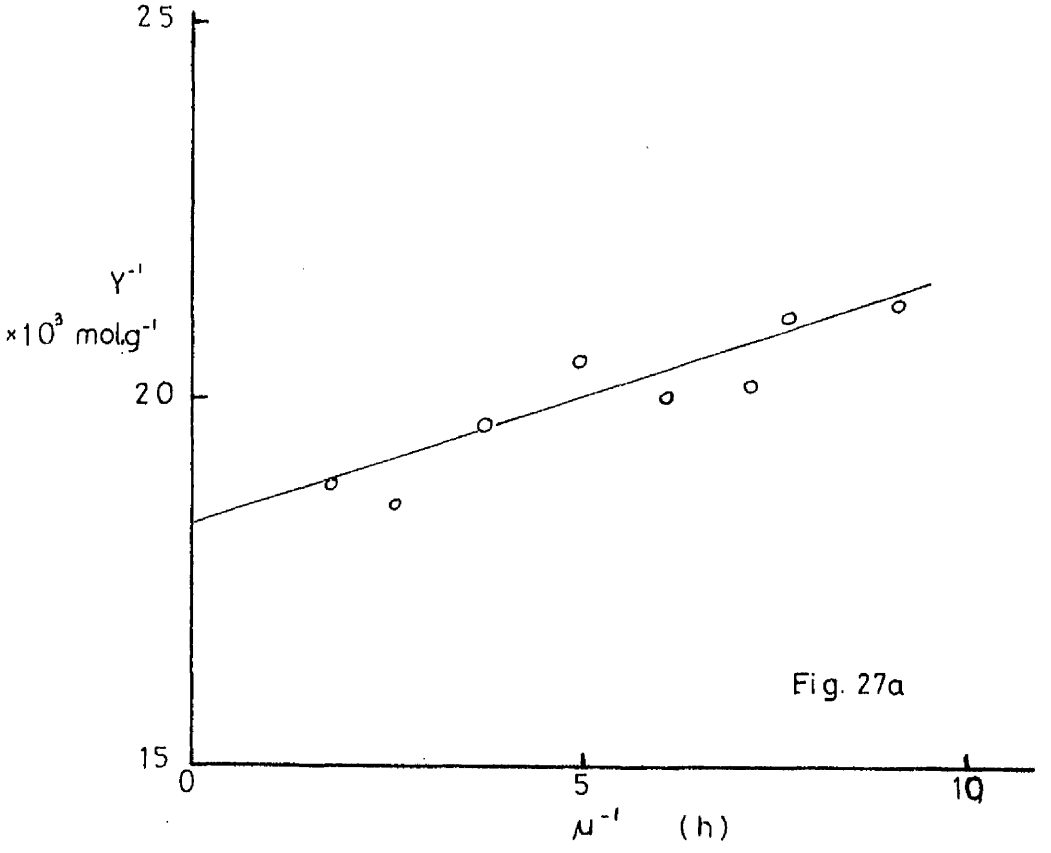


Fig. 27a

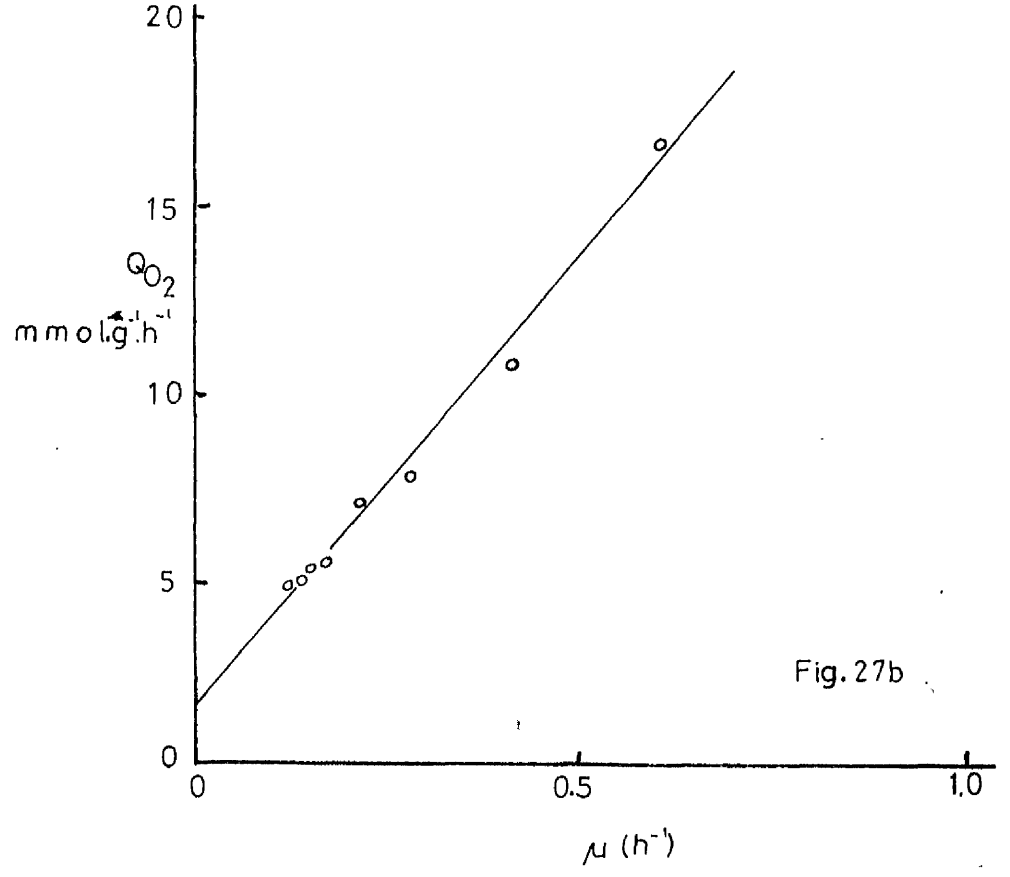


Fig. 27b

Figure 28 GROWTH OF E.coli ML308 IN CONTINUOUS CULTURE

This is a summary table of growth yields and maintenance coefficients determined for E.coli ML308 growing in continuous culture at 37°C on glucose, lactose and glycerol (Figs. 25-27). Values are also included for continuous cultures on lactose and glycerol, where the entering concentration of sulphate was reduced to 50 μmol.l.$^{-1}$.

carbon source	limitation	$g.mol^{-1}$		$\mu mol.g^{-1}.h^{-1}$	
		Y^G	$Y^G_{O_2}$	m	m_{O_2}
glucose	carbon	94	-	460	-
lactose	carbon	226	74	330	3600
lactose	sulphate	221	69	315	3560
glycerol	carbon	54	40	333	1620
glycerol	sulphate	55	38.5	480	1600

cultures were experimentally indistinguishable from the corresponding carbon-limited cultures.

3. Growth in batch culture

3.1 Growth on lactose

E.coli ML308 grew immediately and exponentially on lactose in batch culture with doubling time (MGT) of 47 min (Fig. 29). Oxygen uptake and carbon dioxide production also increased exponentially with time. The rate of substrate utilisation increased with increasing mass of the culture (Fig. 29).

It was possible to calculate both carbon and oxygen growth yields from these data. The carbon growth yield, Y, was obtained from the gradient of a plot of cell mass against substrate utilisation (Fig. 30a), giving $Y = 196\ g.mol^{-1}$. The cumulative gas exchange of the culture (O_2 uptake and CO_2 output) was calculated by integrating the rates of gas exchange over the time course of the experiment. The gradient of a graph of cell mass against cumulative oxygen uptake gave the oxygen growth yield, Y_{O_2}, equal to $58.8\ g.mol^{-1}$ (Fig. 30b). The carbon balance prepared from the substrate utilisation, growth and cumulative CO_2 production data indicated that all of the lactose used by the culture could be accounted for as cells or CO_2.

3.2 Growth on galactose

E.coli ML308 was grown on galactose in batch culture. Growth, gas exchange and substrate utilisation were followed with time (Fig. 31). The cells grew immediately and exponentially on the galactose with MGT = 44 min. After

Figure 29 GROWTH, GAS EXCHANGE AND SUBSTRATE UTILISATION FOR *E.coli* ML308 GROWING ON LACTOSE IN BATCH CULTURE

E.coli ML308 was grown in batch culture (Methods 6.1) on lactose at 37°C. Growth (Methods 7), gas exchange (Methods 8) and subtrate utilisation (Methods 9) were followed with time.

o—o growth.

△—△ oxygen uptake.

□—□ carbon dioxide production.

●—● lactose in culture medium.

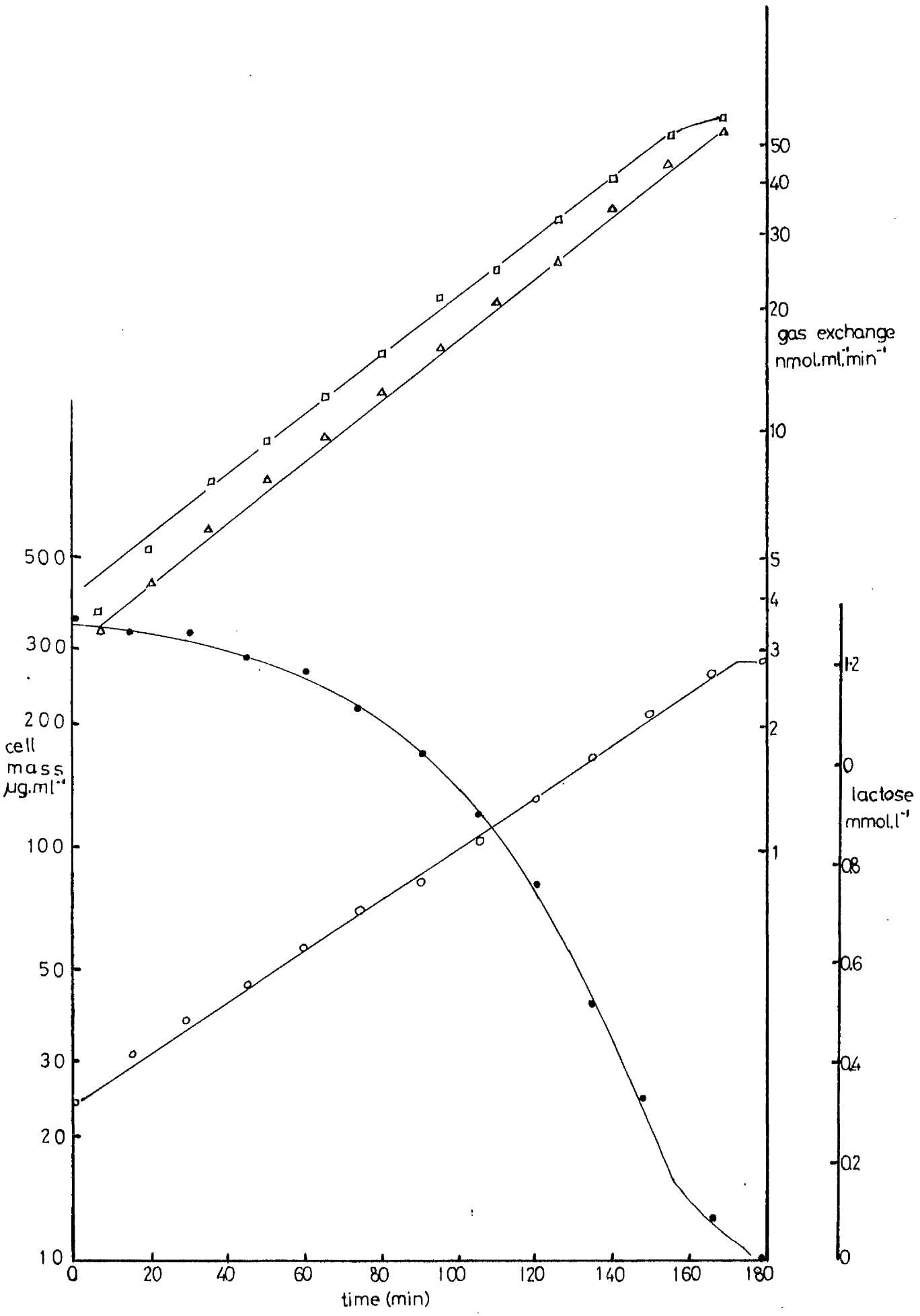

gas exchange
nmol.ml⁻¹.min⁻¹
cell
mass
µg.ml⁻¹
lactose
mmol.l⁻¹
time (min)
0
20
40
60
80
100
120
140
160
180
10
20
30
50
100
200
300
500
1
2
3
4
5
10
20
30
40
50
0
0.2
0.4
0.6
0.8
1.0
1.2

Figure 30 CARBON AND OXYGEN YIELD DURING GROWTH OF E.coli ML308 ON LACTOSE

Fig. 30a Carbon Growth Yield.

Cell mass was plotted against substrate remaining for the experiment described in Fig. 29. The carbon growth yield, given by the negative of the gradient of the curve, was $Y = 196\ g.mol.^{-1}$.

Fig. 30b Oxygen Growth Yield.

Cell mass was plotted against cumulative oxygen uptake (Methods 8.2) for the experiment described in Fig. 29.

The gradient of the curve was the oxygen growth yield, $Y_{O_2} = 58.8\ g.mol.^{-1}$.

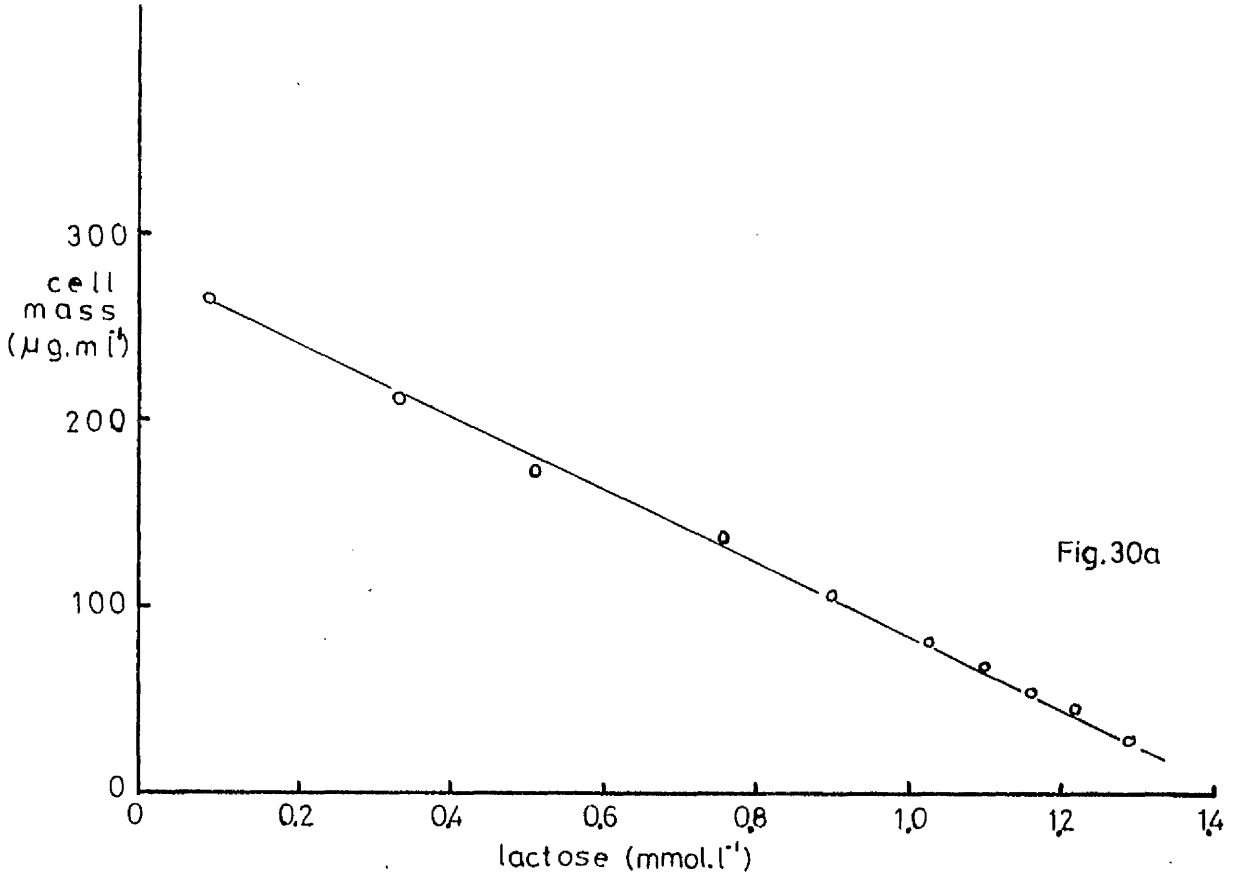


Fig.30a

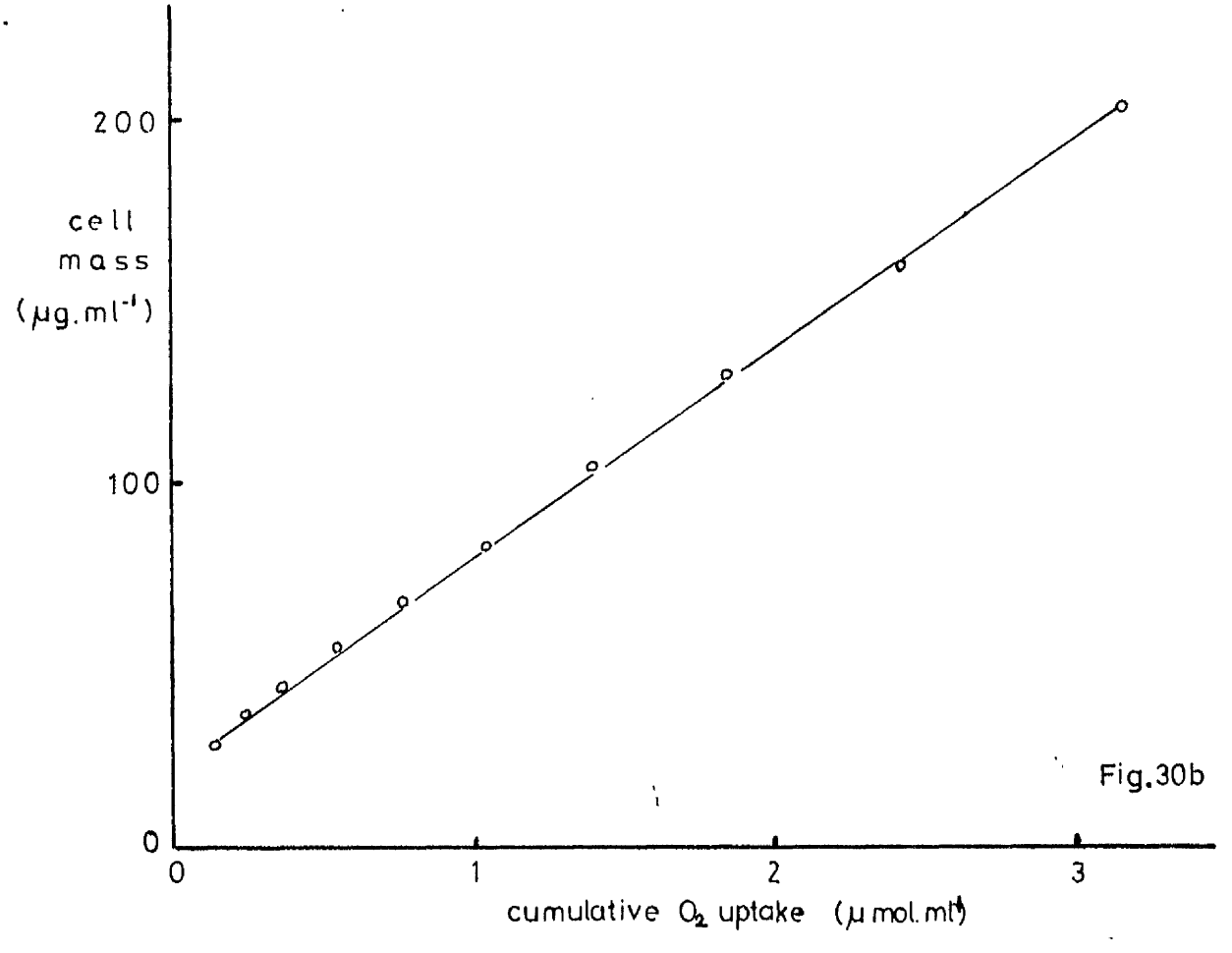


Fig.30b

Figure 31 GROWTH, OXYGEN UPTAKE AND SUBSTRATE UTILISATION FOR E.coli ML308 GROWING ON GALACTOSE IN BATCH CULTURE

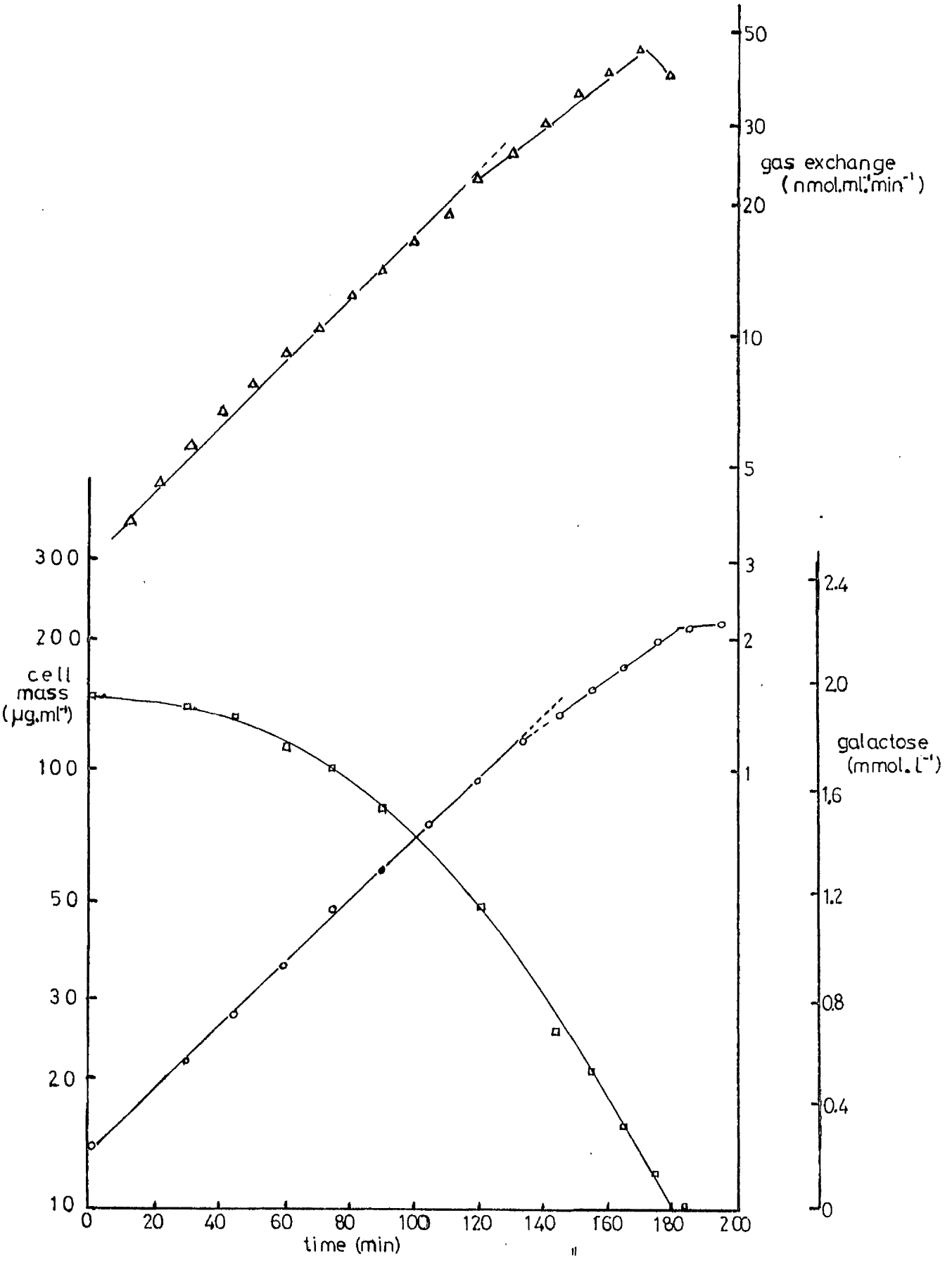
E.coli ML308 was grown in batch culture on galactose at 37°C. Growth, gas exchange and substrate utilisation were followed with time.

o—o growth.

Δ—Δ oxygen uptake.

□—□ substrate utilisation.

Production of carbon dioxide was virtually identical to the rate of oxygen uptake, so is omitted from the figure for clarity.


gas exchange
(nmol.ml⁻¹.min⁻¹)
50
30
20
10
5
3
2
1
cell
mass
(µg.ml⁻¹)
300
200
100
50
30
20
10
galactose
(mmol.l⁻¹)
2.4
2.0
1.6
1.2
0.8
0.4
0
0
20
40
60
80
100
120
140
160
180
200
time (min)

about 130 min, the growth rate fell (MGT = 58 min) and continued at the slower rate, until growth ceased. Oxygen uptake and carbon dioxide production (which is not shown) were exponential but biphasic. The transition to slower rates occured at the same time as that for growth and gas exchange. The rate of galactose utilisation increased as cell mass increased.

The data of Fig. 31 were used to calculate carbon and oxygen growth yields. The carbon growth yield, Y, was found to be 98 $g.mol.^{-1}$ (Fig. 32a). The curve for oxygen growth yield (Fig. 32 b) was found to be biphasic. The initial gradient, corresponding to the period of faster growth (MGT = 44 min), gave Y_{O_2} = 65 g.mol; the second slower growth phase (MGT = 58 min) had Y_{O_2} = 48 $g.mol.^{-1}$. Cumulative CO_2 production showed that all of the galactose used by the culture was converted to either cells or CO_2.

3.3 Growth on other carbon sources

The yields (Y, Y_{O_2}) and MGT's for growth in batch culture of E.coli ML308 on some other carbon sources are summarised in Fig. 33.

Growth on lactulose and lactitol, which are lactose analogues, was accompanied by the high oxygen growth yield typical of growth on lactose. Values for growth on lactose, and the two phases of galactose growth are included for comparison. In all experiments, carbon balances showed that utilisation of carbon source could be accounted for by the formation of new cells and CO_2.

3.4 Growth on sulphate-depleted media

The sulphate content of defined media was eliminated by replacing the sulphate inorganic salts by their

Figure 32 CARBON AND OXYGEN YIELD DURING GROWTH ON GALACTOSE

Fig. 32a Carbon Growth Yield.

Cell mass was plotted against substrate remaining for the experiment described in Fig. 31.

The gradient of the curve corresponds to the negative of the carbon growth yield, Y, equal to 98 $g.mol.^{-1}$.

Fig. 32b Oxygen Growth Yield.

Cell mass was plotted against the cumulative oxygen uptake for the experiment described in Fig. 31.

The gradient of the curve corresponds to the oxygen growth yield, Y_{O_2}. The curve had two distinct portions: the first was steep and gave Y_{O_2} = 65 $g.mol.^{-1}$; the second was less steep and gave Y_{O_2} = 48 $g.mol.^{-1}$.

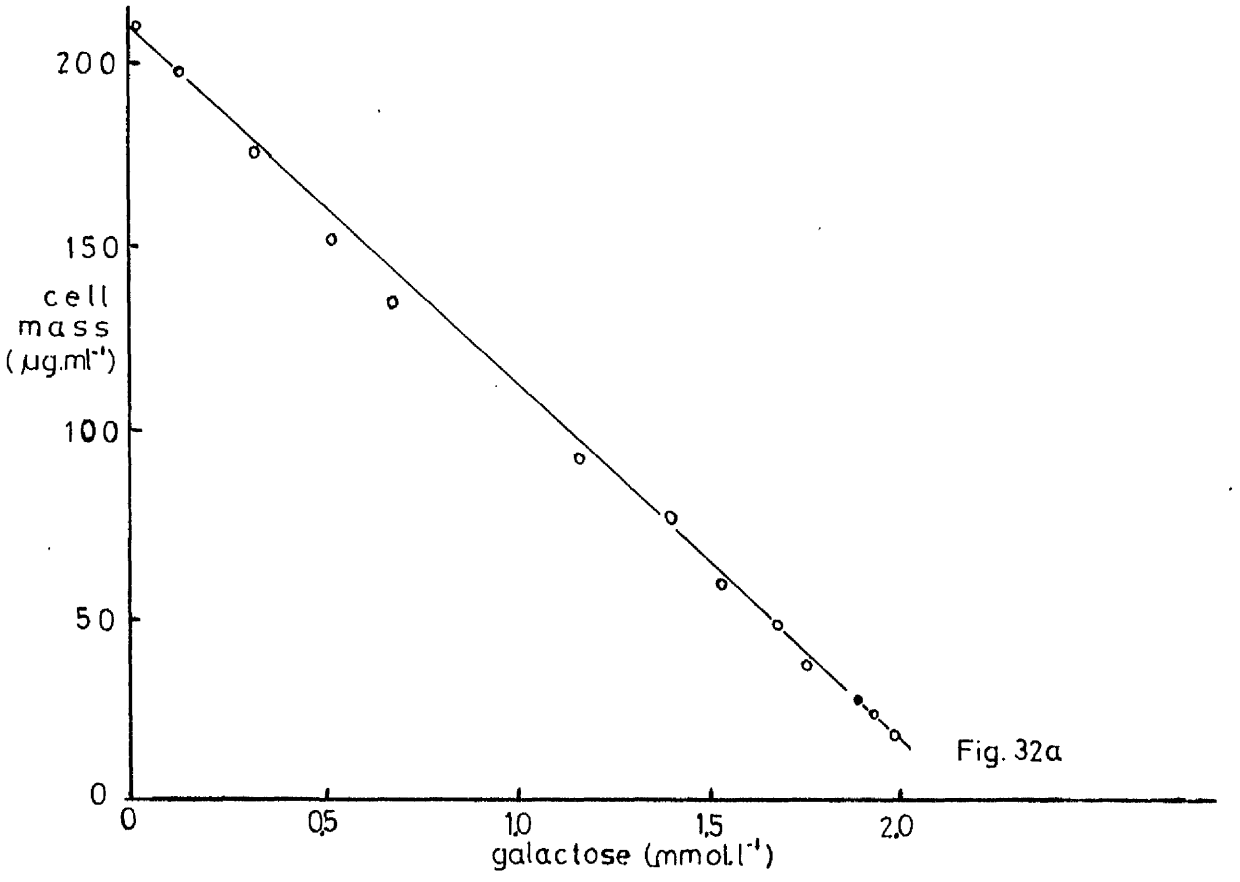


Fig. 32a

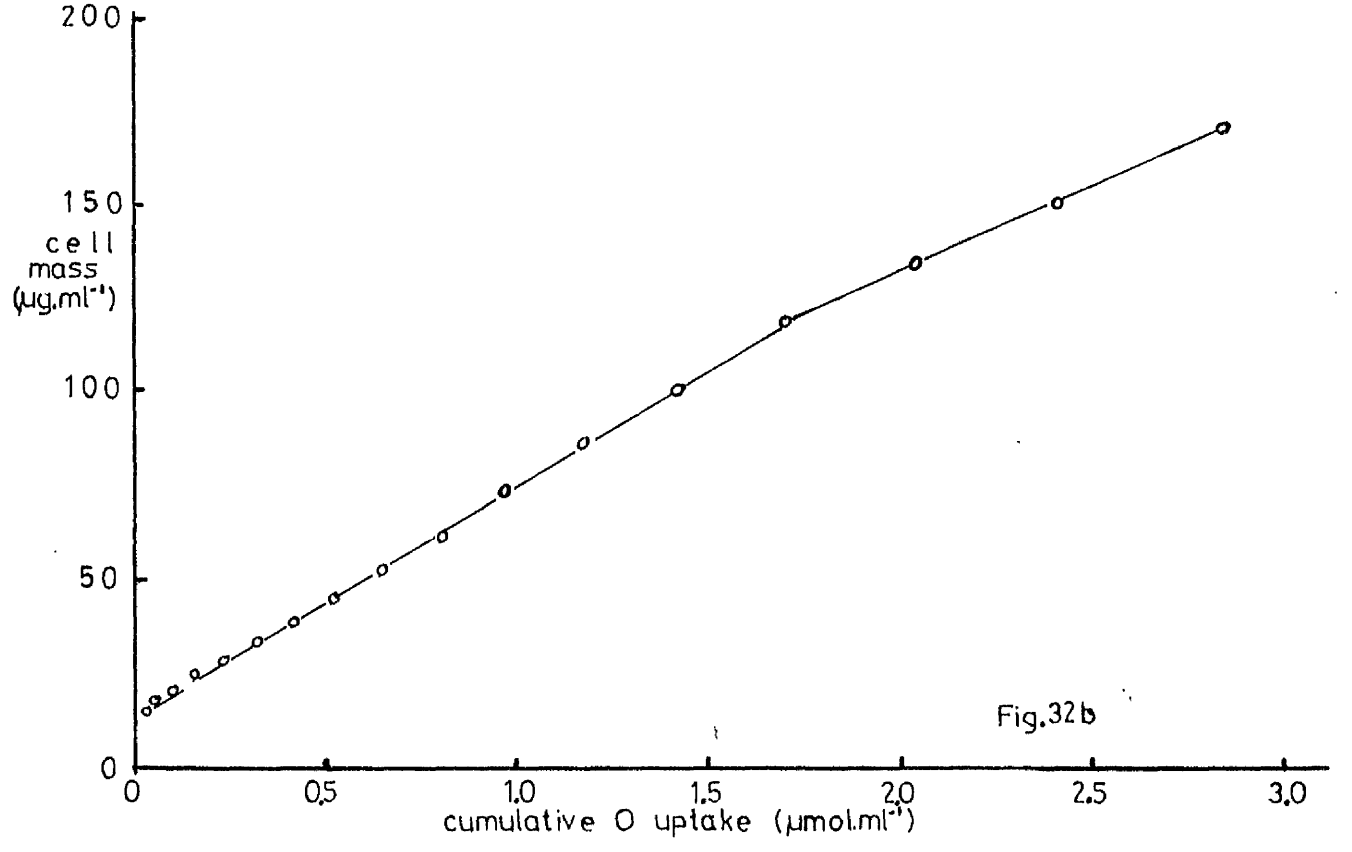


Fig.32b

Figure 33 YIELD VALUES FOR E.coli ML308 GROWING IN BATCH CULTURE

This is a summary table of growth rates (as mean generation times MGT) and carbon and oxygen yield values obtained for growth of E.coli ML308 in batch culture at 37°C.

carbon source	MGT (min)	$g.mol^{-1}$	
		Y	Y_{O_2}
lactose	47	196	59
lactulose	51	204	63
lactitol	56	212	60
glycerol	59	48.5	36
maltose	54	184	47
galactose phase 1	44	98	65
galactose phase 2	58	98	48

chlorides. Sterile sulphate (as K_2SO_4) was added to the desired concentration.

3.4.1 Growth on lactose

Cells of strain ML308 were grown in batch culture on lactose in sulphate-depleted media. They had been trained previously on lactose with limiting sulphate. There was no effect on growth rate or yields of cells under these conditions (Fig. 34a). However there was initially a slow non-exponential phase of growth for about 2 h (c.f. Fig. 35) before the cells achieved the growth rate described in Fig. 34a. This was in contrast to sulphate-sufficient cells which grew immediately on lactose (Fig. 29).

3.4.2 Growth on glycerol

When E.coli, trained on glycerol in the presence of low levels of sulphate, (50 μmol.l^{-1}), was inoculated into fresh media containing glycerol in which the sulphate levels ranged from 5 μmol.l^{-1} to 10 mmol.l^{-1}, the growth rate and apparent yield of the cells depended on the amount of sulphate which had been added (Fig. 35).

Growth apparently ceased in experiments containing sulphate at 5-40 μmol.l^{-1} due to exhaustion of the sulphate, since the growth medium could still support growth when fresh (sulphate-sufficient) cells and sulphate (10 mmol.l^{-1}) was added. Cessation of growth in the experiment containing normal levels of sulphate (10 mmol.l^{-1}) was due to exhaustion of the carbon source, but the identity of the limiting factor in the experiment containing sulphate at 50 mmol.l^{-1} was not clear. It was possible to calculate an apparent yield in terms of sulphate Y_S equal to 7,600 g.mol^{-1}, from the results of this experiment.

Figure 34 GROWTH AND YIELDS OF E.coli ML308 IN BATCH CULTURE, WITH REDUCED LEVELS OF SULPHATE

Fig. 34a Growth on Lactose.

The concentration of sulphate present in the medium for batch culture was reduced from the normal level ($\sim$10 mmol.l^{-1}) to the level stated. Growth, gas exchange and substrate utilisation were followed with time. Growth yields were calculated at each sulphate concentration, together with the growth rate of the culture (expressed as mean generation time).

For each experiment the inocula were trained at the same level of sulphate as was present at the beginning of the growth experiment.

Fig. 34b Growth on Glycerol.

The concentration of sulphate was reduced from the normal level ($\sim$10 mmol.l^{-1}) to 50 μmol.l^{-1}.

Growth, gas exchange and substrate utilisation were followed with time. Growth yields and growth rate (expressed as mean generation time) were calculated from these data.

The concentration of sulphate used for training of inocula was identical to that used for the growth experiments.

$\mu mol.l^{-1}$ sulphate concentration	min	$g.mol^{-1}$	
	MGT	Y	Y_{O_2}
10.5×10^3	47	196	59
250	48	187	57
50	47	193	59
15	46	186	59

Fig.34a

$\mu mol.l^{-1}$ sulphate concentration	min	$g.mol^{-1}$	
	MGT	Y	Y_{O_2}
10.5×10^3	59	48.5	36
50	80	43.5	29

Fig.34b

Figure 35 GROWTH OF E.coli ML308 ON GLYCEROL IN BATCH CULTURE, WITH DIFFERENT CONCENTRATIONS OF SULPHATE

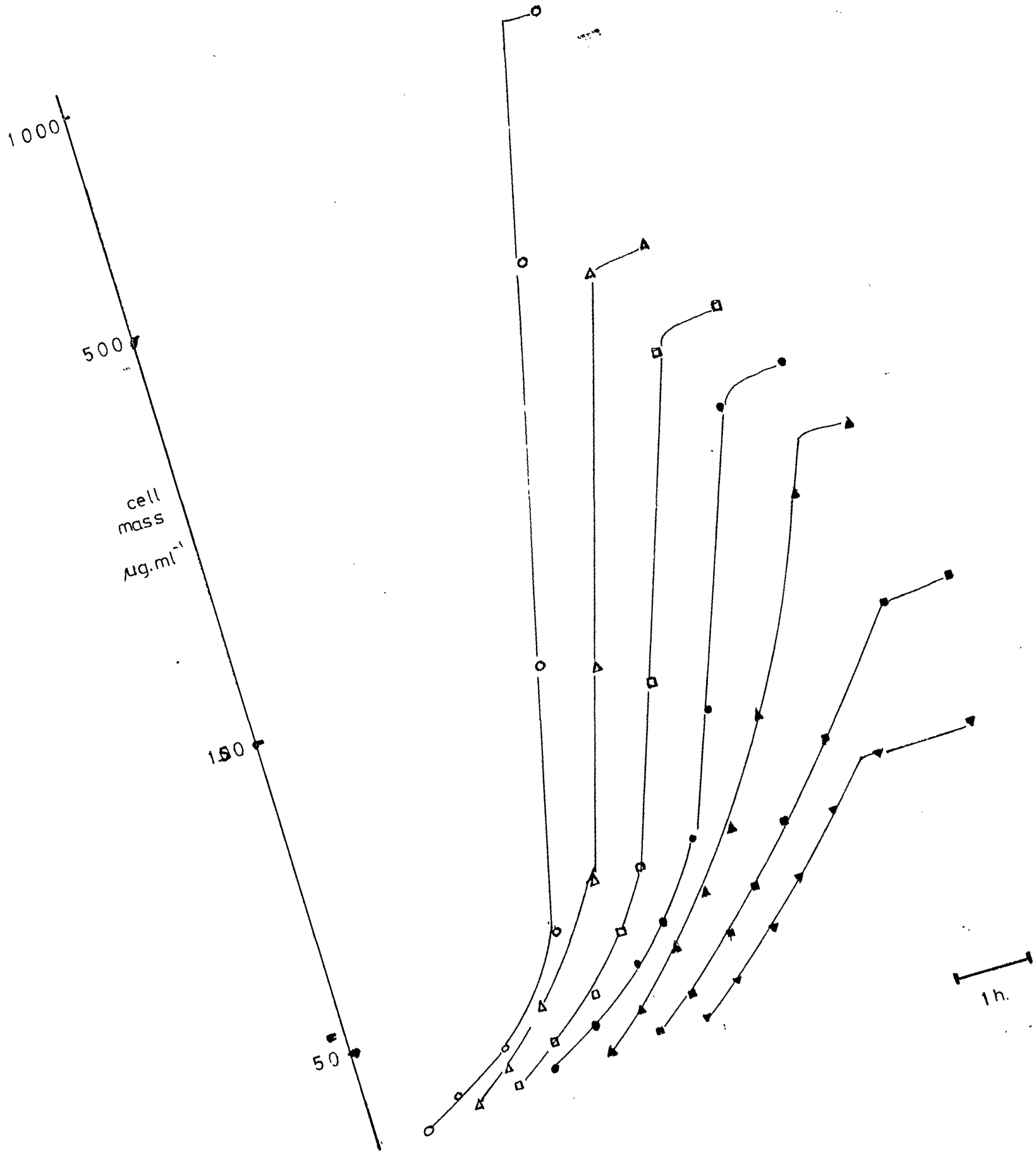
E.coli ML308, trained on glycerol in the presence of reduced sulphate (50 μmol.l^{-1}) was washed and inoculated into fresh glycerol/salts media (Methods 3.5.2.1) which contained different levels of sulphate. Growth was followed until it ceased. The starting concentration of glycerol was 20 mmol.l^{-1} in all experiments.

Sulphate was added at:

o —o	10 mmol.l^{-1}.
▲ —▲	50 μmol.l^{-1}.
□ —□	40 μmol.l^{-1}.
● —●	30 μmol.l^{-1}.
▲ —▲	20 μmol.l^{-1}.
■ —■	10 μmol.l^{-1}.
▼ —▼	5 μmol.l^{-1}.

↑ denotes time of inoculation for each experiment.

No glycerol was present at the end of growth for experiments with sulphate at 10 mmol.l^{-1} and 50 μmol.l^{-1}. In the other experiments, glycerol was present, as judged by the ability of the growth medium to support further growth when inoculated with a fresh (sulphate-sufficient) culture and sulphate to 10 mmol.l^{-1}.


1000
500
150
50
cell mass
µg.ml⁻¹
1 h.

Growth, gas exchange and substrate utilisation were followed in a more rigorous experiment for E.coli ML308 growing on glycerol with sulphate added to a concentration of 50 μmol.l^{-1}. The cells grew initially at a non-exponential rate (c.f. Fig. 35) but, after about 2 h, grew exponentially with MGT of 80 min (Fig. 34b). This was considerably slower than the rate for sulphate-sufficient cells. There was also a significant reduction in the carbon and oxygen growth yields. The carbon balance showed that no excretion products were formed during the experiment.

4. H^+/O ratios for arithmetic-culture cells

4.1 Effect of cell phenotype on H^+/O ratio

Arithmetic-culture cells fell into one of two classes when their $\frac{H^+}{O}$ ratios were determined with glycerol as source of reducing power for electron transport.

Glycerol-trained cells were typical of the first class. They had $\frac{H^+}{O}$ ratios which were about 2.45 (Fig. 36a). The second class, of which lactose-trained cells were a typical example, showed higher $\frac{H^+}{O}$ ratios of about 4.1 (Fig. 36b). Proton extrusion was in response to the oxygen added (as air-saturated KCl) since no change in medium pH occurred if aliquots of nitrogen-saturated KCl were added to the cell suspension. Addition of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP; 4 nmol in 10 μl ethanol) to the cell suspension abolished the proton-extrusion pulse in response to oxygen (Fig. 36). When malate, succinate, pyruvate, or lactate replaced glycerol in the incubation medium, no proton extrusion

Figure 36 H^+/O RATIOS FOR E.coli GROWN ON GLYCEROL AND LACTOSE

E.coli strain 51 was grown at 37°C in arithmetic culture on either glycerol or lactose. The number of protons leaving the cells in response to a limited amount of oxygen added as air-saturated KCl were determined to allow calculation of the $\frac{H^+}{O}$ ratio (Methods 12).

The derived $\frac{H^+}{O}$ ratios are shown beside the oxygen-induced pulses.

Controls were done with nitrogen-saturated KCl and with oxygen pulses after addition of uncoupler (CCCP; 1 μmol.l^{-1}).

Fig. 36a $\frac{H^+}{O}$ ratio of glycerol-trained cells (8.2 mg).

Fig. 36b $\frac{H^+}{O}$ ratio of lactose-trained cells (11.8 mg).

↑ 50 µl air-saturated KCl added.

↑ 50 ng.ion H^+ added.

↑ 50 µl nitrogen-saturated KCl added.

↑ CCCP added to 1 μmol.l^{-1} final concentration.

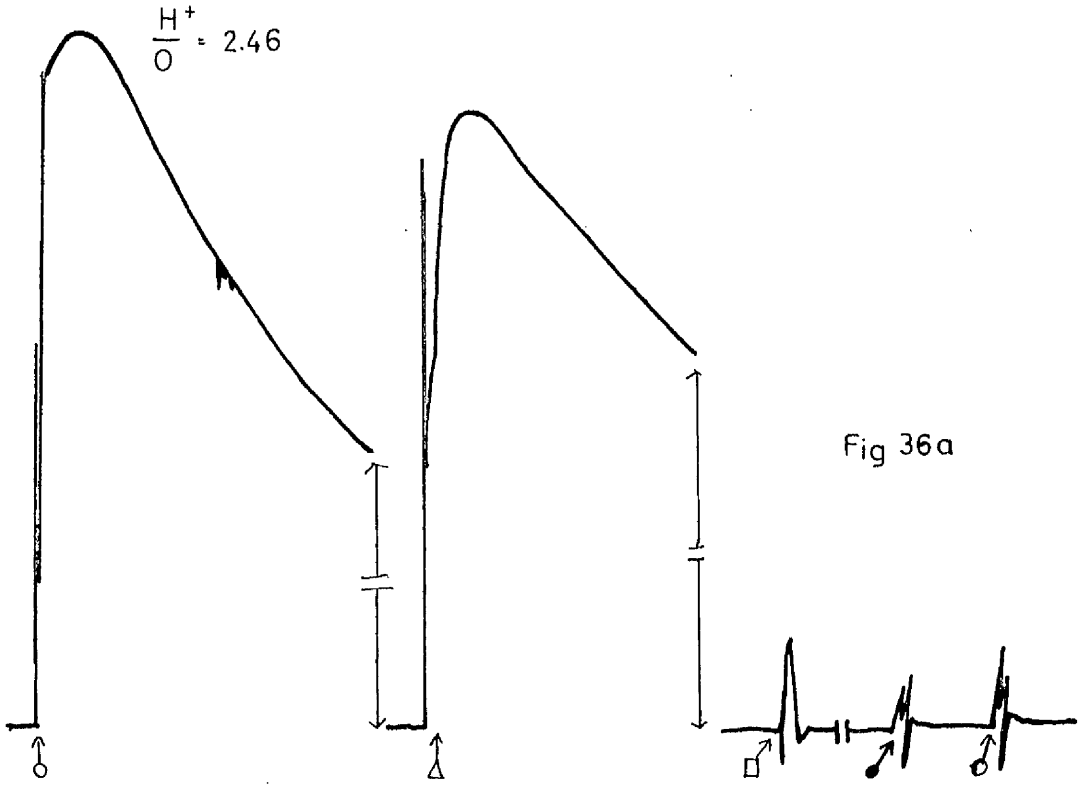


Fig 36a

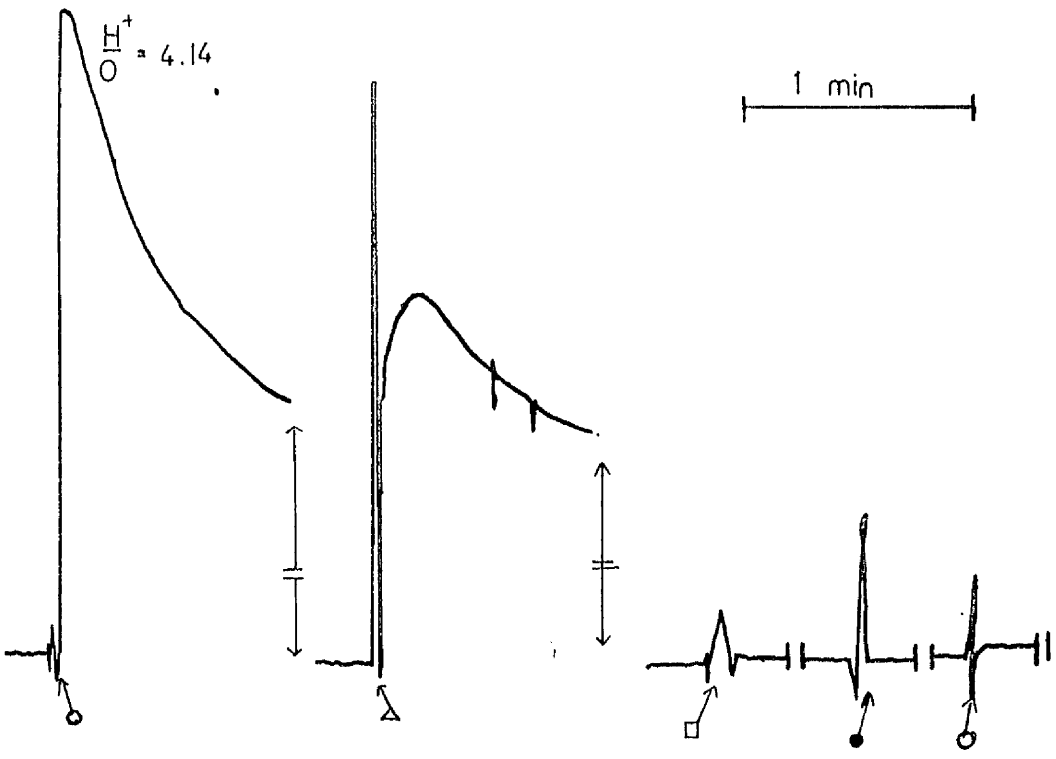


Fig. 36 b

from the cells was observed.

The $\frac{H^+}{O}$ ratios determined for several cell phenotypes, including glycerol and lactose, are summarised in Fig. 37. Values did not appear to change with the age of the culture i.e. how long it had been maintained as an arithmetic culture.

4.2 Effect of incubation temperature on H^+/O ratio

Normally, proton extrusion experiments (and enzyme assays) are done at 27°C. However, since the cells had been grown at 37°C and gas analysis was done on the growing cells, some proton extrusion experiments were done at 37°C to allow comparison of energy conservation estimated from yields by gas analysis, and from $\frac{H^+}{O}$ ratios.

Lactose-trained cells had higher $\frac{H^+}{O}$ ratios than glycerol-trained cells when assayed at 37°C (Fig. 38a).

4.3 H^+/O ratios of cells grown in high salt conditions

Cells grown on glycerol or lactose in the presence of NaCl (0.5 $mol.l^{-1}$) had $\frac{H^+}{O}$ ratios which were slightly lower than the values obtained for cells under named growth conditions (Fig. 38b), but lactose cells still had higher $\frac{H^+}{O}$ values than the corresponding glycerol cells.

4.4 Effect of a starvation period on H^+/O ratio

Exponential-phase cells are usually shaken for 2 h in complete medium without carbon source prior to assay of $\frac{H^+}{O}$ ratio to deplete the cells of endogenous energy reserves (Lawford & Haddock, 1973). This was not usually done with arithmetic-culture cells since they had been grown at slow growth rates and were presumed to be energy-starved. The

Figure 37 H^+/O RATIOS FOR E.coli ML

This is a summary table of $\frac{H^+}{O}$ ratios determined for cells of E.coli strain 51 grown in arithmetic culture at $37^{o}C$.

Results are expressed as mean $\pm$ standard deviation, with number of determinations in parenthesis.

carbon source for growth	H^+/O ratio
glucose	2.42 ± 0.03 (9)
glycerol	2.46 ± 0.02 (14)
lactose	4.08 ± 0.05 (11)
maltose	2.45 ± 0.06 (4)
mannitol	2.51 ± 0.04 (4)
galactose	2.46 ± 0.02 (4)
gluconate	4.11 ± 0.05 (4)

Figure 38a EFFECT OF INCUBATION TEMPERATURE ON H^+/O RATIO

Cells of strain 51 which had been grown on either glycerol or lactose were incubated at 27°C and 37°C in order to investigate the effect of assay temperature on derived $\frac{H^+}{O}$.

Figure 38b EFFECT OF GROWING CELLS IN HIGH SALT CONDITIONS ON H^+/O RATIO

Cells of strain 51 were grown in high salt conditions (growth medium supplemented NaCl; 0.5 $mol.l^{-1}$). $\frac{H^+}{O}$ ratios were determined, and compared with values for cells grown in normal salt conditons. Sucrose (0.8 $mol.l^{-1}$) was added to the incubation medium for determination of $\frac{H^+}{O}$ ratios to prevent lysis of cells grown in high salt conditions.

Figure 38c EFFECT OF STARVATION OF CELLS ON H^+/O

The effect of a 2 h starvation period (staking at 37°C in growth medium minus carbon source) was investigated in cells of strain 51 which had been grown on either lactose or glycerol. This is the usual method of depleting logarithmic cells of endogenous energy reserves before estimation of $\frac{H^+}{O}$ ratio.

carbon source for growth	Temp. °C	H^+/O
lactose	27	4.11
lactose	37	4.06
glycerol	27	2.43
glycerol	37	2.45

Fig. 38a

carbon source for growth	H^+/O normal salt	H^+/O high salt
lactose	4.08	3.78
glycerol	2.43	2.34

Fig. 38b

carbon source for growth	H^+/O untreated	H^+/O starved
lactose	4.10	2.53
glycerol	2.44	2.42

Fig.38c

effect of a starvation period on $\frac{H^+}{O}$ ratios of arithmetic-culture cells was investigated.

A period of starvation before assay had no effect on the $\frac{H^+}{O}$ ratio of glycerol cells, but reduced the $\frac{H^+}{O}$ ratio of lactose-trained cells to that typical of a glycerol culture (Fig. 38c).

4.5 Effect of addition of galactosides on H^+/O ratio

The addition of an anaerobic solution of thiomethyl galactoside (TMG, a substrate for the lac permease but not β-galactosidase) enhanced the $\frac{H^+}{O}$ ratio of lactose-trained cells (Fig. 39a). Thiodigaloctiside (TDG) a substrate with the same enzyme specificity as TMG) also enhanced the $\frac{H^+}{O}$ ratio of lactose cells, but the effect was not so great (Fig. 39b). Glycerol-trained cells, which are still constitutive in the lac enzymes, showed no enhancement of their typical $\frac{H^+}{O}$ ratio when either TMG (Fig. 39c) or TDG (Fig. 39d) was added to the cell suspension.

5. Enzyme activities

5.1 lac enzymes

5.1.1 Activity of lac enzymes in continuous and arithmetic culture

The specific activity of β-galactosidase varied with growth rate, when cells of strain 51 were grown on lactose in continuous culture (Fig. 40a). Activity was a maximum for cells grown at $\mu = 0.2$. The β-galactosidase activities of cells grown in arithmetic culture compared well with those of cells grown in continuous culture at similar

Figure 39 ADDITION OF GALACTOSIDES TO CELLS DURING PROTON EXTRUSION EXPERIMENTS

Cells of strain 51, which had been grown either on lactose or glycerol, were incubated anaerobically and had their $\frac{H^+}{O}$ ratios determined every 5 min. TMG or TDG were added as anaerobic suspensions (50 µl of 0.4 mol.l^{-1}). $\frac{H^+}{O}$ ratios were determined after addition of the galactoside.

Fig. 39a Addition of TMG to lactose-trained cells.

Fig. 39b Addition of TDG to lactose-trained cells.

Fig. 39c Addition of TMG to glycerol-trained cells.

Fig. 39d Addition of TDG to glycerol-trained cells.

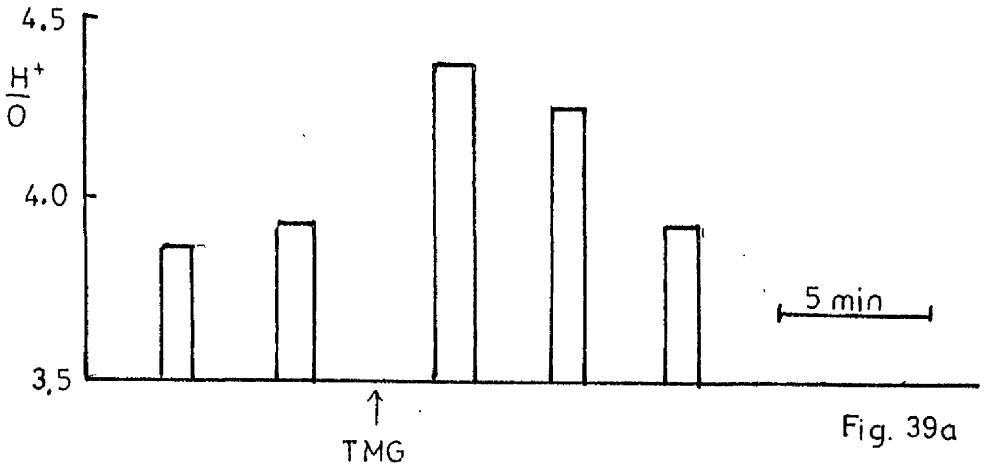


Fig. 39a

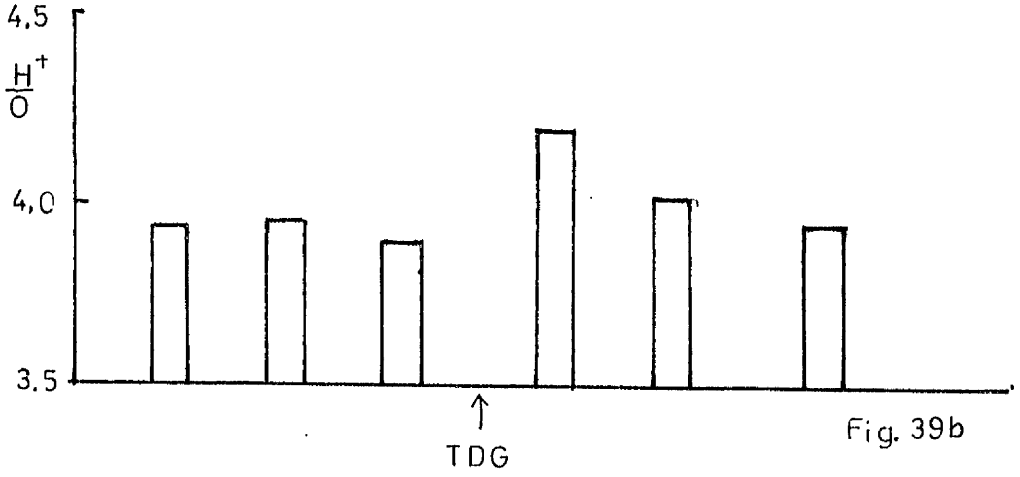


Fig. 39b

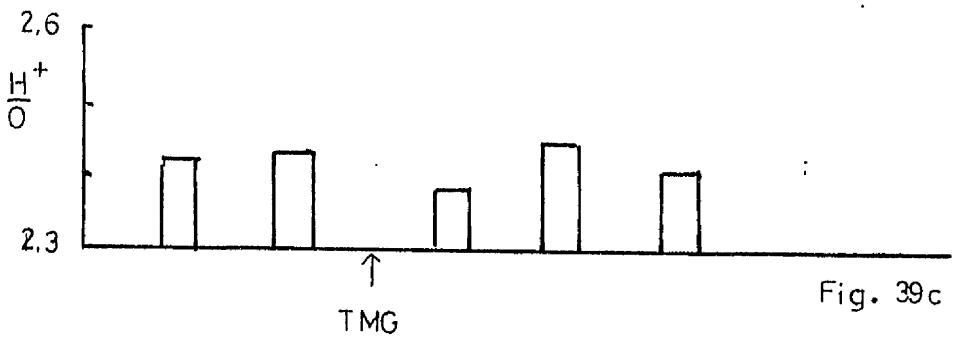


Fig. 39c

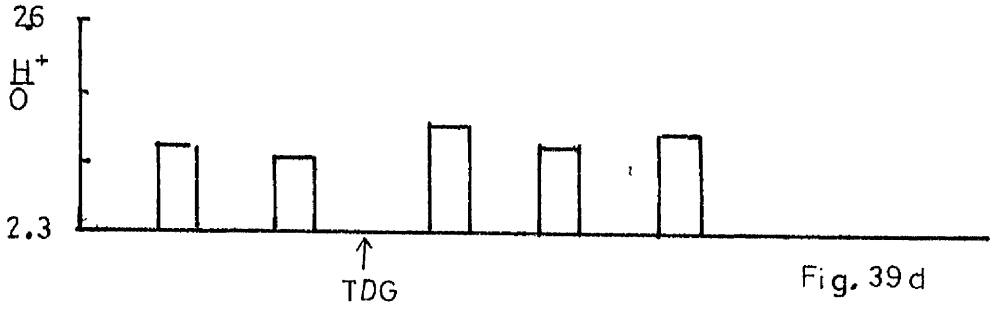


Fig. 39d

Figure 40 EFFECT OF GROWTH RATE ON EXPRESSION OF lac ENZYMES OF E.coli ML DURING GROWTH ON LACTOSE

Activities of β-galactosidase and lac permease were measured (Methods 10) for cells of E.coli strain 51 which had been grown at varying growth rates in continuous- or arithmetic-culture.

o-o cells from chemostat culture.

□-□ cells from arithmetic culture.

Fig. 40a Activity of β-galactosidase.

Fig. 40b Activity of lac permease.

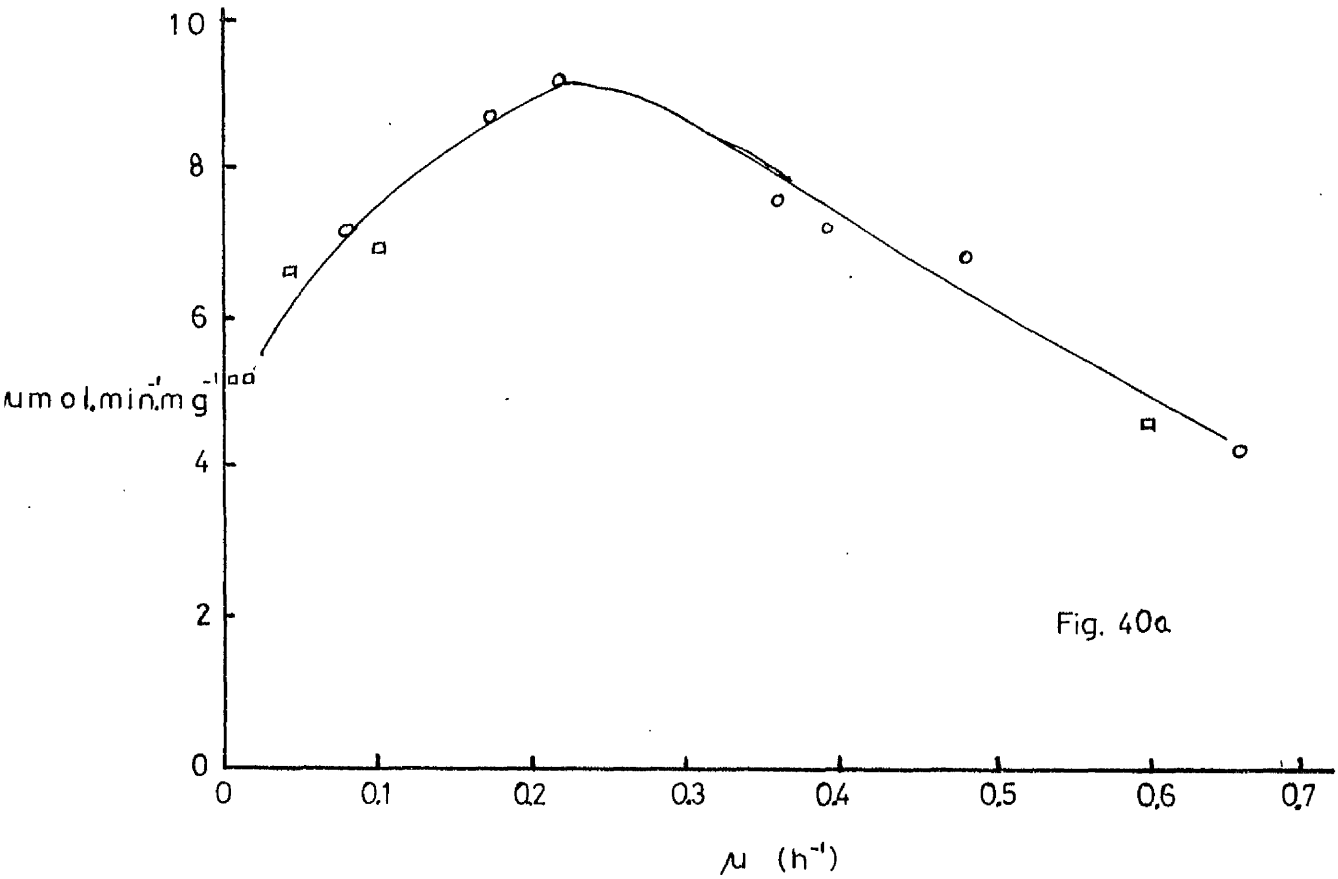


Fig. 40a

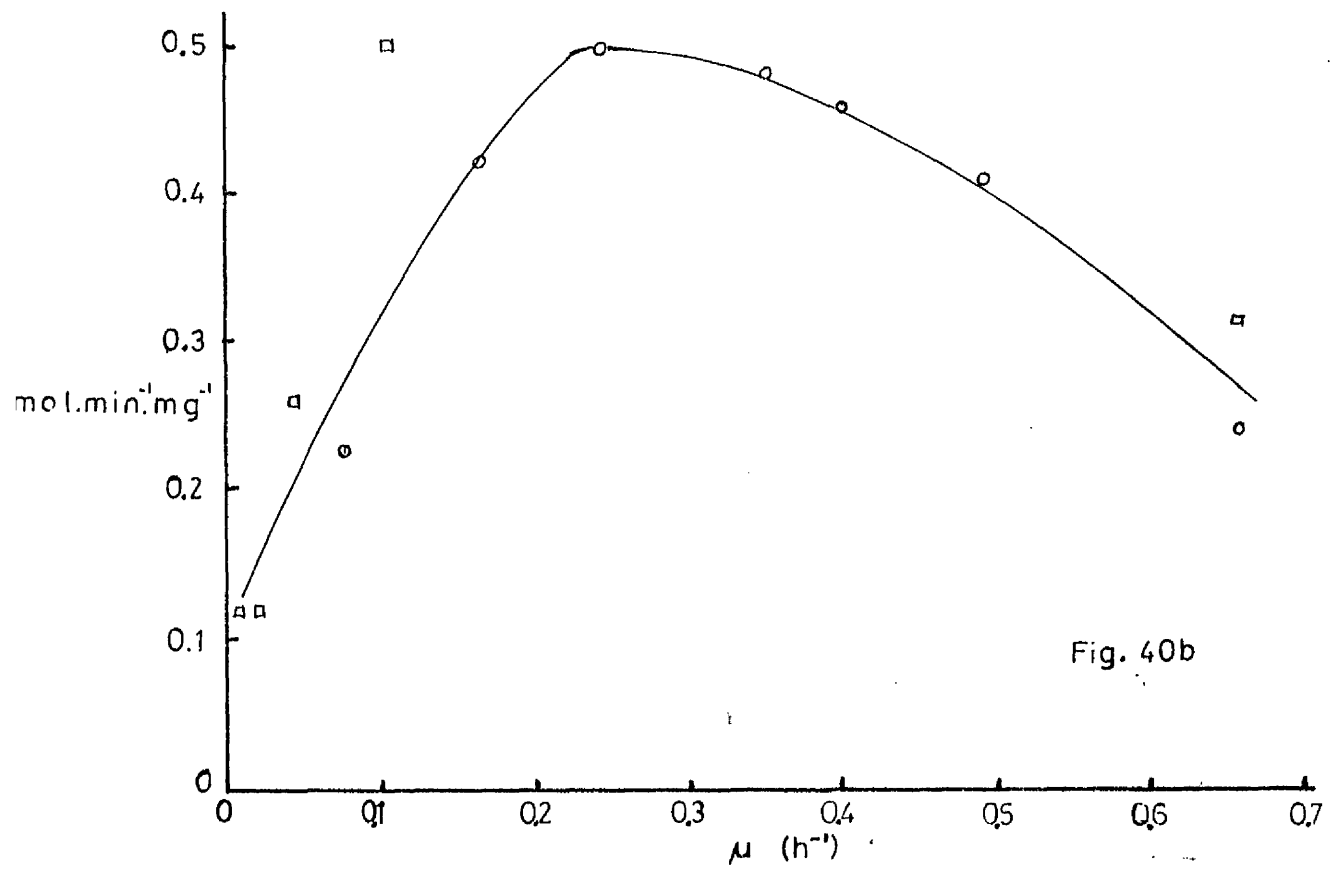


Fig. 40b

growth rates. Enzyme activity at slow growth rates ($\mu < 0.02$) in arithmetic culture was virtually constant at ~ 5 Eu.mg^{-1}, which was slightly greater than the activity present in exponentially-growing cells.

The specific activity of lac permease was also a maximum at $\mu = 0.2$, but decreased sharply at slower growth rates (Fig. 40b). This was a consequence of an increase in non-specific uptake of ONPG at slow growth rates. The activities of cells grown in arithmetic culture were comparable to continuous-culture cells at similar growth rates, but remained constant at $\sim$0.1 Eu.mg^{-1} when cells were grown at $\mu < 0.01$.

Activities of the lac enzymes were also measured for cells of strain 51 (lac constitutive) which were grown in identical conditions to Fig. 40, but on glycerol. Both β-galactosidase and lac permease activities increased with decreasing growth rate, to a maximum at around $\mu = 0.25$, then decreased sharply at slower growth rates. The activities of the lac enzymes of cells growing on glycerol at $\mu < 0.02$ in arithmetic culture were about 50% of those growing on lactose.

5.1.2 Decay of lac permease on starvation, and reactivation by glycerol

The activity of the lac permease decreased with time when lactose-trained logarithmic cells were harvested and stirred without carbon source at 37°C (Fig. 41). The decrease in activity was proportional to time, but greater when cells were stirred in phosphate buffer alone, rather than in defined medium minus carbon source. Addition of glycerol to the starved culture rapidly reactivated the

Figure 41 DECAY OF lac PERMEASE ACTIVITY ON STARVATION, AND REACTIVATION OF ACTIVITY BY GLYCEROL

Logarithmic cultures of E.coli strain 51 which had been grown on lactose were harvested, and resuspended in buffer which contained

(1) o—o	KH_2PO_4, pH 7.0	40 $mmol.l^{-1}$.
	$(NH_4)_2\ SO_4$	10 $mmol.l^{-1}$.
	$MgSO_4$	0.5 $mmol.l^{-1}$.
	$FeSO_4$	10 $\mu mol.l^{-1}$.
(2) □—□	KH_2PO_4, pH 7.0	40 $mmol.l^{-1}$.

The cultures were incubated at 37°C, stirred at normal rates for batch growth experiments and supplied with air at 100 $ml.min^{-1}$. The activity of the lac permease, at 37°C, corrected for non-specific uptake, was estimated (Methods 10.2) with time.

Fig. 41a At ↓, glycerol was added to the cultures.

Fig. 41b At ↓, glycerol plus chloramphenicol (0.3 $mmol.l^{-1}$) was added to the cultures.

The culture which was resuspended in buffer (1), and to which glycerol alone was added, was the only one which grew in the latter part of the experiment.

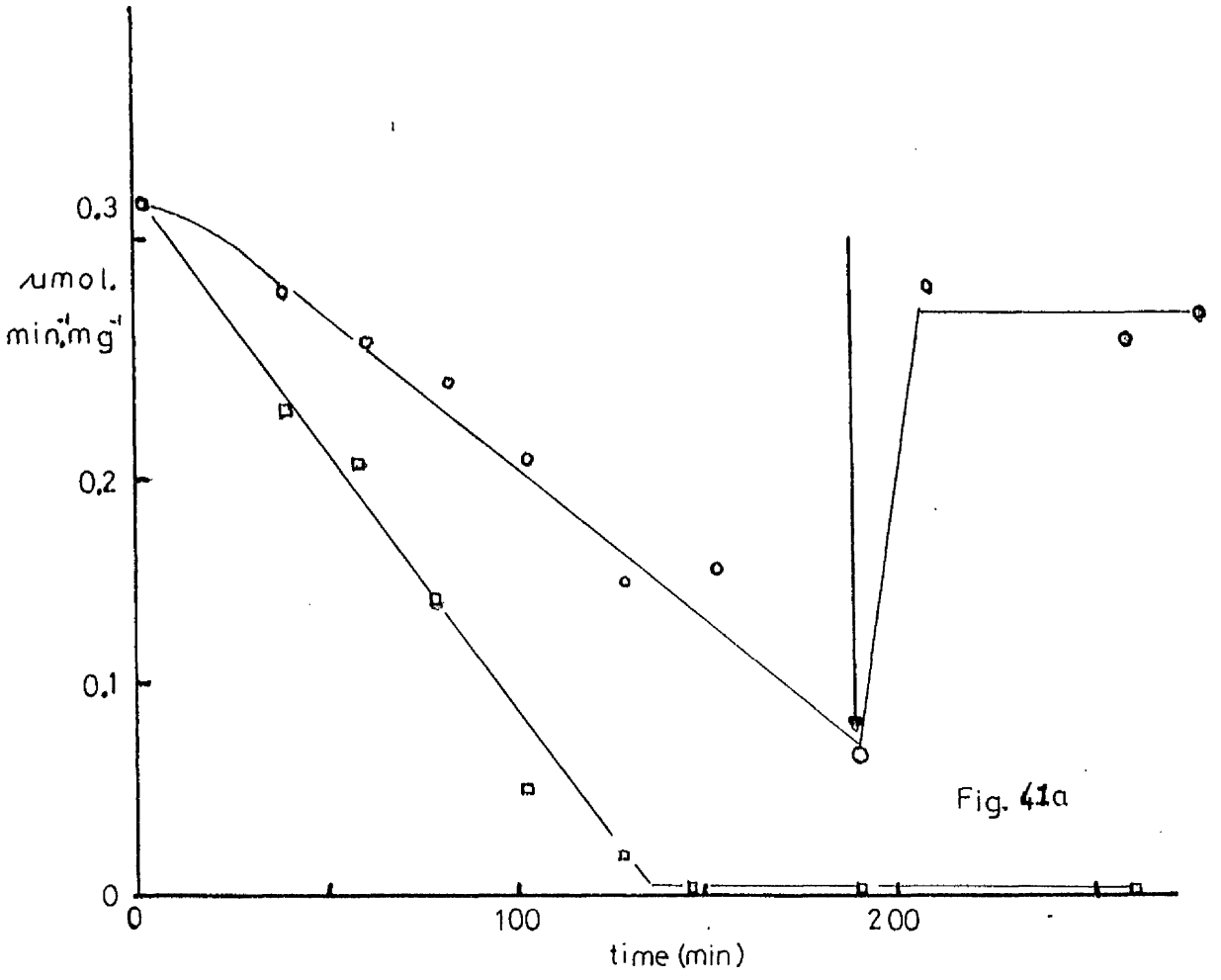


Fig. 41a

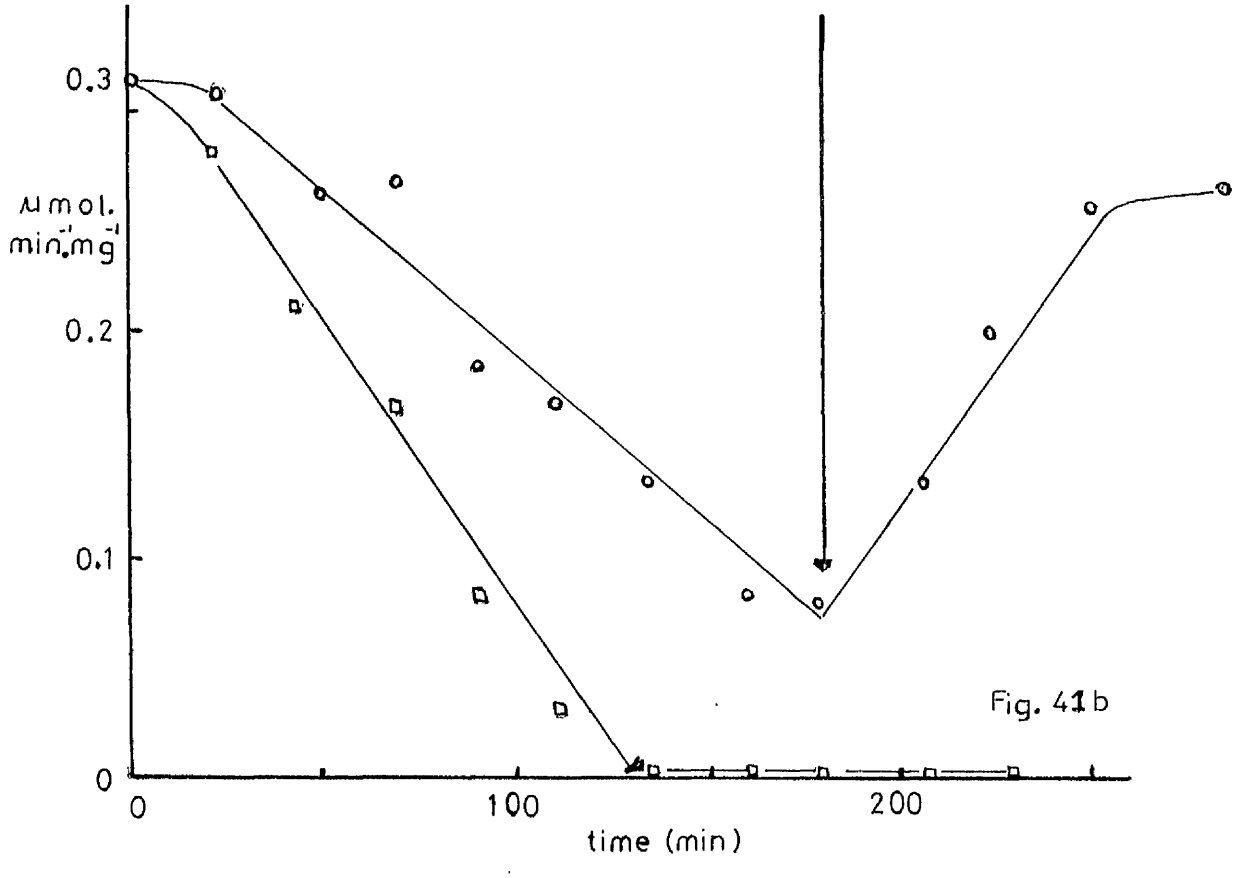


Fig. 41b

lac permease activity, to 85% of its activity at harvesting, for cells starved in defined medium, but not for cells starved in phosphate buffer alone (Fig. 41a).

When chloramphenicol + glycerol was added to a culture starved in defined medium, the permease activity was still reactivated (Fig. 41b) but at a slower rate than when chloromphenicol was omitted. The cells did not grow.

When cells were stirred without carbon source in a nitrogen atmosphere the decay of activity of the lac permease was similar to that in an air atmosphere, but glycerol failed to reactivate the activity.

5.2 ∝ Glycerophosphate dehydrogenase

The activity of ∝glycerophosphate dehydrogenase in cell extracts of E.coli strain 51 was negligible when coupled to nicotinamide cofactors (Fig. 42a). Activity could be measured in the presence of the dye MTT, which was used by Weiner and Heppel (1972) in the assay for flavin-linked enzyme (E.C. 1.1.99.5).

When a toluenised cell extract replaced the sonicated extract in the assay, the flavin-linked activity was enhanced, and endogenous activity linked to the dye was decreased. No nicotinamide-linked activity was detected in toluenised extracts.

5.3 Transhydrogenase

The transhydrogenase activities of electron-transport particles prepared from arithmetic-culture cells of strain 51 were comparable, when the cells were grown on either lactose or glycerol (Fig. 42b).

5.4 Cytochrome oxidase

The cytochrome oxidase activities of cells of strain

Figure 42a COFACTOR SPECIFICITY OF ∝GLYCEROPHOSPHATE DEHYDROGENASE DURING AEROBIC GROWTH OF E.coli strain 51

The activity of ∝glycerophosphate dehydrogenase, which is a constitutive enzyme in E.coli 51 was estimated in sonicated cell extracts as described in Methods 10.3. The activity was estimated at 27°C with the following cofactors:

NAD/NADH - E.C. 1.1.1.8

NADP/NADPH - E.C. 1.1.1.94

Flavin - E.C. 1.1.99.5 (in the assay, coupled to reduction of the dye MTT)

In each case the activities in the presence of substrate were corrected for endogenous activities.

* toluenised cell extract replaced sonicated extract.

Figure 42b TRANSHYDROGENASE ACTIVITIES OF E.coli IN ARITHMETIC CULTURE

The oxidative, ATP-linked and non-energy linked activities of electron-transport particles were determined at 27°C as described in Methods 10. The particles were prepared from cells of strain 51, which had been grown on lactose or glycerol in arithmetic culture.

Units are in nmol $\begin{cases} \text{NADPH} \\ \text{NADH} \end{cases}$ formed min^{-1} $(mg\ protein)^{-1}$.

Formation of NADPH was confirmed by a reduction in E_{340} when glutathione/glutathione reductase was added.

cofactor	endogenous activity $\mu mol.g^{-1}.h^{-1}$	activity + substrate $\mu mol.g^{-1}.h^{-1}$
NAD	3.5	4.2
NADP	3.4	5.3
flavin	27.5	69.2
flavin *	8.50	105

Fig. 42a

activity	glycerol cells	lactose cells
oxidative NADPH formed	5.3	8.7
ATP-linked NADPH formed	19.4	22.6
non-energy linked NADH formed	33	40

Fig. 42b

51 (measured indirectly by oxygen uptake with glycerol as exogenous carbon source) differed in their sensitivity to cyanide, depending on culture condition and carbon source used for growth (Fig. 43).

Arithmetic-culture cells which had been grown on lactose or glycerol were more resistant to cyanide than the comparable logarithmic cultures (Fig. 43a, b). Glucose-grown arithmetic cells were less resistant to cyanide than logarithmic cells, but the effect was marginal (Fig. 43c). The sensitivity of stationary-phase lactose cells was intermediate between that of logarithmic and arithmetic cultures (Fig. 43b).

It was possible, from the cyanide inhibition kinetics, to calculate an apparent Ki for cyanide of the cytochrome oxidase of each cell type (Fig. 44). Lactose-trained cells, irrespective of growth condition showed higher oxygen uptakes in the oxygen electrode than glycerol- or glucose-trained cells (Fig. 44).

Figure 43 INHIBITION BY CYANIDE OF CYTOCHROME OXIDASE ACTIVITY IN E.coli strain 51

Cytrochrome oxidase activities were estimated at 37°C from rates of oxygen uptake (Methods 11) for washed cell suspensions of strain 51, when glycerol (1 mmol.l^{-1}) was added. Activities were also measured in the presence of KCN. Results were plotted according to Dixon and Webb (1964) as $\frac{I_0}{I}$ versus CN , where

I_0 = uninhibited rate.

I = rate in presence of cyanide.

This plot has gradient equivalent to $(Ki)^{-1}$ for cyanide.

o-o Cells harvested during exponential growth phase.

□-□ Cells harvested from arithmetic culture.

Δ-Δ Cells harvested from stationary phase.

Fig. 43a lactose-grown cells.

Fig. 43b glycerol-grown cells.

Fig. 43c glucose-grown cells.

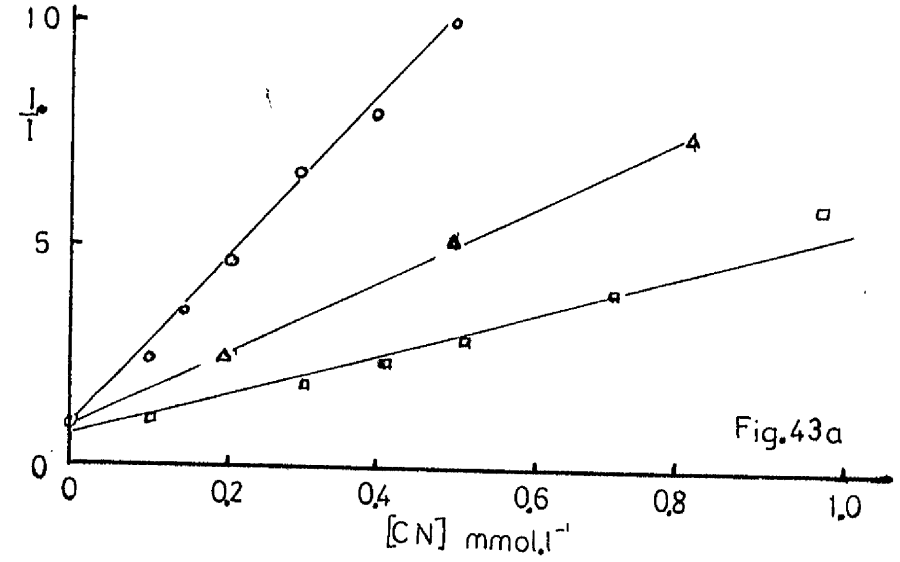


Fig.43a

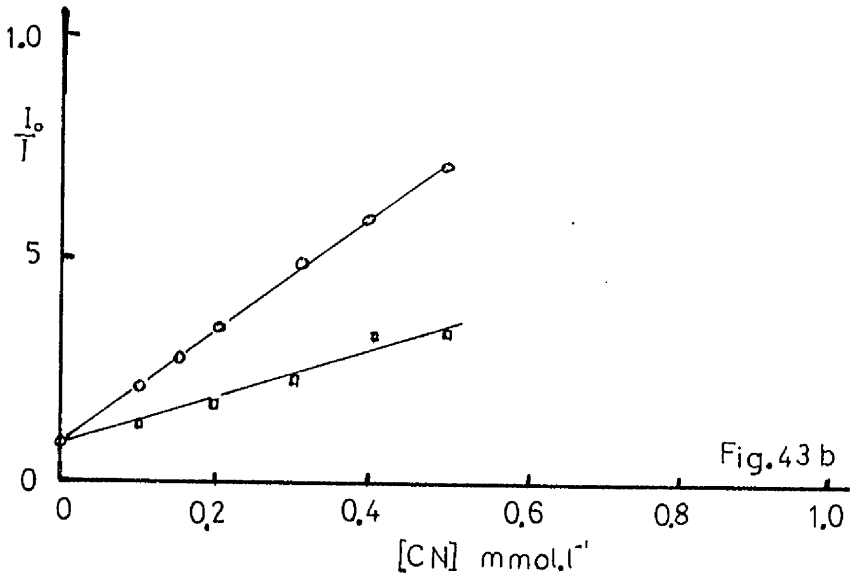


Fig.43b

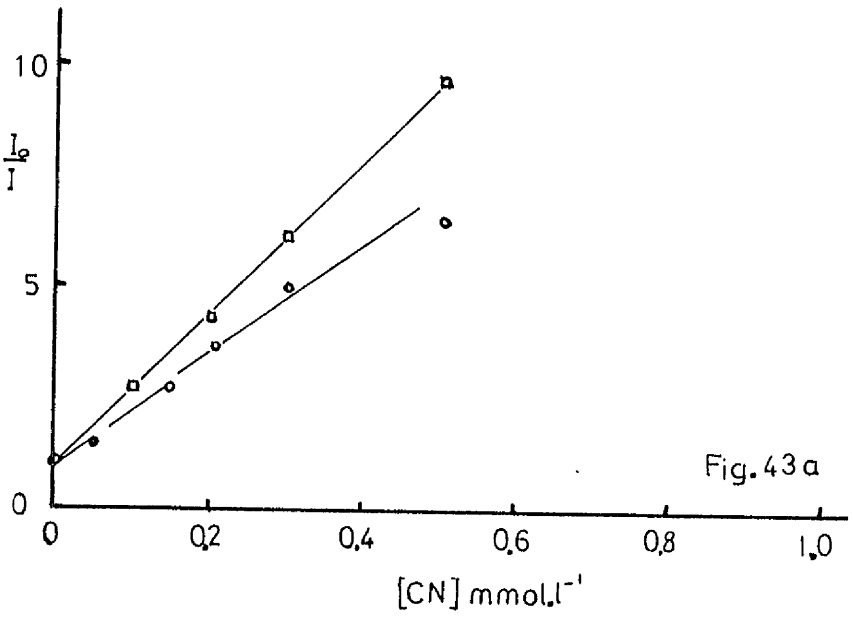


Fig.43a

Figure 44 Ki (cyanide) and RATES OF RESPIRATION OF E.coli strain 51 GROWN IN ARITHMETIC AND BATCH CULTURE

The apparent Ki (cyanide) for cytochrome oxidase activity of different phenotypes of strain 51 was determined from the reciprocal of the gradient of a Dixon plot (Fig. 43), with varying concentrations of potassium cyanide.

Uninhibited rates of oxygen uptake (equivalent to I_0 in Fig. 43) are also included.

carbon source	growth phase	K_i (CN) $\mu mol.l^{-1}$	uninhibited oxygen uptake $\mu mol.min^{-1}.mg^{-1}$
lactose	logarithmic	54	93.2
	arithmetic	240	87.4
	stationary	122	71.8
glycerol	logarithmic	66	43.6
	arithmetic	113	27.2
glucose	logarithmic	68	33.9
	arithmetic	54	31.8

DISCUSSION

1. Maintenance energy

The energy produced by the bacterial cell is required for two purposes: some is expended on growth of the micro-organism, and some on 'maintenance energy'. Maintenance functions are thought to be necessary to preserve the integrity of the cell and to retain its capability for growth, but they do not contribute to growth. This drain of energy results in lower yields than would be found if maintenance functions did not exist. Since one of the aims of this work was to use growth yields to evaluate energy flow during cellular growth, it was necessary to consider the energy which was required for maintenance. Growth yields (Y, Y_{O_2}) could then be expressed as 'maximum' growth yields (Y^G, $Y^G_{O_2}$): that is, yields which would be achieved if there was no maintenance and all of the energy made available to the cell were used for growth.

1.1 Calculation of maintenance energy and m_{ATP}

In the determination of maintenance energy for arithmetic- (Appendix 1) and continuous cultures (e.g. Fig. 25), it was assumed that maintenance was independent of growth rate (Pirt, 1965), since plots of Y^{-1} versus μ^{-1}, and Q_{O_2} versus μ, were linear.

Non-linear plots have been found (Hobson, 1965; Hempfling & Vishniac, 1967; Meyer & Jones, 1973), especially when the limitation has been a nutrient other than the carbon source (Rosenberger & Elsden, 1960; Belaich & Senez, 1965; Stouthamer & Bettenhausen, 1973; Farmer & Jones, 1976a) indicating that, in these cases, maintenance energy

was some function of μ.

Neijssel and Tempest (1976a) obtained linear double reciprocal plots (Y^{-1}, μ^{-1}) for growth of K.aerogenes in continuous culture but pointed out that this did not necessarily mean that maintenance energy was independent of μ. They claimed that maintenance energy was linearly-dependent on μ (which is mathematically compatible with linear double reciprocal plots), on the experimental evidence of changes in the intracellular concentrations of osmotically-active molecules and of endogenous metabolism which accompanied changes in growth rate. An unstated corollary of their argument is that: if maintenance is dependent on μ and hence cannot be quantified by the method of Pirt (1965), growth yields cannot be used to evaluate energy flow during growth.

It was necessary, therefore, to establish that the values for maximum growth yields obtained experimentally and used in the calculations of energy flow (Appendix 2), were reasonable and corrected for maintenance.

The maintenance requirements for arithmetic cultures and for continuous cultures were found to be different (Figs. 19, 28). It might also be expected that any growth-rate dependence of maintenance would be different, which would lead to different estimates of the maximum growth yield. Since maximum growth yields were found to be similar, it seems reasonable to conclude that, for E.coli, maintenance is independent of μ, but will depend on the culture condition imposed (1.4). In addition, maximum growth yields (Y^G) for glycerol, obtained by arithmetic- and continuous cultures were virtually indistinguishable from the growth yield (Y) obtained in

batch culture. This is also true of glucose, if the acetate excreted during batch culture is accounted for (unpublished experiment). During batch culture the specific growth rate at which Y is measured is around $\mu = 1$. The maximum growth yield, Y^G, refers to an extrapolation of the yields measured in the range $\mu = 0\text{-}1$ to the point where $\mu = \infty$. That the yields are virtually indistinguishable at $\mu = 1$ (Y) and $\mu = \infty$ (Y^G) suggests that if maintenance is linearly dependent on μ the dependence is so slight as to be ignored. It is therefore possible to estimate energy flow from maximum growth yields. The special case of lactose is discussed later (2.1).

The energy for maintenance functions will be derived from combustion of the carbon source used for growth. Nagai and Aiba (1972) introduced the term $m^*_{O_2}$ to check if this were true. It is simply the amount of oxygen calculated to be required for total oxidation of the carbon source used for maintenance. For example, the combustion of glucose requires 6 molecules of oxygen, so $m^*_{O_2} = 6 \times m_{glucose}$. The close similarity between calculated values of $m^*_{O_2}$ and experimentally-derived values of m_{O_2} (e.g. Fig. 13) confirmed that the carbon required for maintenance was totally oxidised and therefore used for energy supply.

The efficiency with which energy released from the oxidation of carbon source for maintenance purposes is trapped will depend on the bacterial species, the catabolic pathways and the efficiency of oxidative phosphorylation. Since maintenance is completely attributed to energy-linked processes, which will involve hydrolysis of ATP, it seemed

Figure 45 CALCULATION OF MAINTENANCE COEFFICIENTS IN TERMS OF ATP

The maintenance coefficients in terms of carbon source consumed (Fig. 19) were expressed in terms of ATP (m_{ATP}) as follows:

Col. A: maintenance coefficient in terms of carbon source.

Col. B: ATP produced by substrate-level phosphorylation during oxidation of carbon source used for maintenance ($mol.mol^{-1}$).

Col. C: ATP produced by substrate-level phosphorylation during oxidation of carbon source ($\mu mol.g.^{-1}h^{-1}$; Cols. A x B).

Col. D: Reducing equivalents generated during oxidation of carbon source ($mol.mol^{-1}$) used for maintenance. For phenotypes with 2 coupling sites electrons from FADH were assumed to pass through 1 site; and 2 sites for cells which had normally 3 coupling sites for oxidation of NADH.

Col. E: $\frac{P}{O}$ ratios have been calculated for each phenotype (Fig. 2.12).

Col. F: ATP produced by oxidative phosphorylation during oxidation of carbon source used for maintenance was calculated as the product of Cols. A, D & E.

Col. G: m_{ATP} was calculated as the sum of Cols. C & F.

growth substrate	A m	B ATP by sub. phosph^{n}	C	D red equiv.	E	F ATP by oxphos	G m_{ATP}
	μmol $g^{-1}.h^{-1}$	mol. mol^{-1}	μmol. $g^{-1}.h^{-1}$	mol. mol^{-1}	P/O	mmol.$g^{-1}.h^{-1}$	
glycerol	100	2	200	4/1/2	2.06	1.24	1.44
glucose	38	4	152	8/2/2	2.02	0.84	0.99
mannitol	38	4	152	9/2/2	2.08	0.95	1.10
lactose	41	8	328	16/4/4	2.98	2.77	3.10
gluconate	51	2	102	6/2/2	3.57	1.70	1.80
glucose 6-phosphate	97	5	485	8/2/2	3.16	3.47	3.96
maltose	20	7	140	16/4/4	2.20	0.97	1.11
galactose	44	3	132	8/2/2	2.23	1.08	1.21

reasonable to estimate maintenance not only in terms of consumption of carbon source and oxygen, but also in terms of ATP consumed by maintenance processes, m_{ATP} (e.g. Brown & van Demark, 1968). Comparison of maintenance coefficients of different organisms in terms of m_{ATP} rather than m or m_{O_2} is certainly more valid. It is possible that one organism consuming a low level of substrate (m, m_{O_2}) for maintenance but with a high efficiency of oxidative phosphorylation will be expending more energy on maintenance (m_{ATP}) than another with higher m, m_{O_2} but less efficient energy conservation. The maintenance coefficients (m) for several experiments (Figs. 19, 21) were calculated in terms of ATP (Fig. 45), taking account of the efficiencies of oxidative phosphorylation derived in Appendix 2.

1.2 Maintenance energy and growth substrate

The maintenance requirement depended on the identity of the carbon source which limited growth (Fig. 19). This is hardly surprising. Maintenance has been found to be higher when the limiting nutrient was one other than the carbon source (e.g. Meyer & Jones 1973; Stouthamer & Bettenhaussen 1975; Wallace, 1975; Neijssel & Tempest, 1976; Meijer et al., 1977) but few comparative studies have been made during carbon-limited growth (Hempfling & Mainzer, 1975; Wallace, 1975).

Maintenance was small ($m_{ATP} \doteqdot 1.0$ mmol.g.$^{-1}$h^{-1}; Fig. 45) when growth was limited by glucose or mannitol, which both enter the cell by phosphotransferase mechanisms (Kornberg & Jones-Mortimer, 1977; Solomon & Lin, 1972). Low values were also found for growth on maltose and galactose, which in arithmetic culture are assumed to enter

the cell by ATP-linked active transport processes (Simoni & Postma, 1975). Glycerol cells, where the carbon source enters the cell by facilitated diffusion (Lin, 1976) had an intermediate maintenance level, whereas maintenance was high for growth on gluconate, lactose and glucose 6-phosphate, all of which enter the cell via H^+-symport transport mechanisms (Robin & Kepes, 1973; West & Mitchell, 1973; Essenberg & Kornberg, 1975). Hempfling and Mainzer (1975), in their comparitive study with E.coli B, found m_{O_2} increased in the order: glucose, galactose, mannitol, glycerol, succinate, acetate. Succinate is known to be taken up by a H^+-symport mechanism (Gutowski & Rosenberg, 1975).

There is a good correlation between maintenance and method of transport, with the highest values for maintenance occurring when carbon source is transported actively into the cell, using a proton gradient as energy source.

Some arithmetic cultures were grown on a mixture of two carbon sources. In these cultures, maintenance coefficients corresponded to the value for one of the two substrates rather than the mean of the two. For example, the high maintenance coefficient characteristic of lactose cultures predominated on mixtures when glucose was 50% and even 95% of the carbon entering the culture (Fig. 15b). When lactose was no longer fed to the culture (100% glucose), the maintenance coefficient fell to that characteristic of growth on glucose (Fig. 15b). It was, however, possible to grow cells on a lactose/glucose mixture or lactose alone with maintenance coefficient characteristic of glucose alone (Fig. 15c) by using an inoculum which was still lac

constitutive but which had been trained to glycerol and inoculated at high cell density (Fig. 16b). This culture also showed an oxygen growth yield which was characteristic of growth on glucose alone.

The maintenance coefficients for growth on glycerol and malate alone were not sufficiently different from those for growth on lactose to decide which phenotype was dominant in glycerol/lactose (Fig. 17) and malate/lactose mixtures (Fig. 18).

In summary, maintenance of E.coli ML has been found to depend on the carbon source. This presumably reflects the range of individual maintenance functions which vary with phenotype and which contribute to the overall maintenance requirement. One governing factor appears to be transport mechanism, since cells which take up carbon source by proton-linked transport have higher maintenance requirements. In mixtures of lactose and glucose, the maintenance coefficient is characteristic of growth on lactose alone. The lactose phenotype dominates the glucose phenotype in this respect. When a glycerol-trained inoculum was grown immediately under severe carbon limitation on lactose alone, or a glucose/lactose mixture, the less efficient glucose phenotype appeared to predominate. Apparently a period of exponential growth was required for the lactose phenotype to become expressed. The high maintenance coefficients of cells growing on carbon sources which are transported by proton symport were accompanied by high oxygen growth yields and high energy conservation.

1.3 Maintenance energy and bacterial strain

The maintenance energies of E.coli ML308 were lower

than those of E.coli K10 when grown in identical conditions on the same substrate (Fig. 21). There must be different contributions, depending on strain, from some of the individual processes of which maintenance is the sum. This suggests that isolated similarities between derived maintenance values for different strains may be fortuitous e.g., the maintenance requirement during growth on succinate of E.coli B (Hempfling & Mainzer, 1975) and E.coli W (Meyer & Jones, 1973).

Maintenance was similar for E.coli ML30 and E.coli ML308 growing on lactose (Fig. 20a, Fig. 19), when β-galactosidase was induced in the former strain, and synthesised constitutively in the latter. When E.coli ML30 was grown on gluconate, β-galactosidase was not induced but maintenance was still similar (Fig. 20b) to that when β-galactosidase was synthesised gratuitously by ML308. This indicates that gratuitous synthesis and turnover of β-galactosidase in ML308 makes a negligible contribution to maintenance.

Large differences in maintenance requirement have been reported depending on the species of microorganism. These have ranged from 712 mmol.g.$^{-1}$h^{-1} for Debaromyces subglobosus growing on phenol (Wase & Hough, 1966) to 0.2 mmol.g.$^{-1}$h^{-1} for S.cervisiae growing on glucose (Watson, 1970). Comparison of maintenance between species is always difficult unless values are quoted in terms of ATP (1.1). The estimates of maintenance energy in arithmetic culture obtained during this work, m_{ATP} = 1-4 mmol.g.$^{-1}$h^{-1}, are among some of the lowest recorded to date for prokaryotes. Maintenance energy is

known to be lower in simple rather than complex medium (Stouthamer & Bettenhaussen, 1975), but the low values obtained may be related to the technique of culture (1.4). If maintenance in arithmetic culture reflects the maintenance requirement of cultures growing exponentially (i.e. m independent of μ), a small fraction of the total substrate utilisation will be expended on maintenance. For example, the specific rate of glucose utilisation during exponential growth (MGT = 45 min) is $\frac{\mu}{Y} = \frac{0.92}{96} = 9.58$ mmol.g.$^{-1}$h.$^{-1}$. The maintenance requirement, 38 μmol.g.$^{-1}$h^{-1} forms only 0.4% of the total substrate utilisation of the culture. The inability of Monod (1942) and Ng (1969) to detect changes in yields of E.coli when grown at normal temperatures in batch culture at different growth rates suggests that maintenance is low for the organism growing exponentially. By contrast, healthy adult mammals use their entire nutrient uptake for maintenance functions; the mammal feeds but does not grow.

1.4 Maintenance energy and cultivation technique

Maintenance was higher in continuous culture than in arithmetic culture (compare Fig. 28 with Fig. 19). This was in contrast to the work of Palumbo and Witter (1969), who found no difference in maintenance between the two culture techniques for growth of Pseudomonas fluorescens. The specific rate of oxygen utilisation (Q_{O_2}) was higher in continuous culture than arithmetic culture at growth rates common to both, indicating that there was a real difference in the amount of carbon source being used for maintenance. The reason for this is not clear. One possibility was the different methods of adding carbon

source to the growth vessel: in arithmetic culture substrate was fed continuously to the vessel through a small bore canula (Methods, 6.3), whereas an intermittent dripfeed system was used for continuous culture (Methods, 6.2). The discontinuous growth pattern which likely exists when limiting nutrient is fed intermittently could lead to 'uncoupled growth' of the culture (Stouthamer, 1977). This would be expressed as a higher maintenance requirement. The 'uncoupling' effect would have to be the same at all growth rates (or linearly dependent on growth rate) to give linear plots (e.g. Fig. 26). Neijssel and Tempest (1976b) have reported an increased maintenance requirement for K.aerogenes when substrate is pulsed into the culture. It would be interesting to feed limiting nutrient to a continuous culture with the canula system normally used for arithmetic culture, to see if the maintenance requirement could be reduced.

1.5 Maintenance energy and temperature

The maintenance requirement of a glycerol culture was dependent on the temperature of cultivation (Fig. 22a), but only at temperatures in excess of 33.5°C. At lower temperatures, maintenance was virtually constant. Monod (1942) reported decreased yields of E.coli when grown in batch culture at 40°C and attributed this to a temperature-dependent increase of maintenance, but other possibilities existed (decrease in Y_{ATP}^{MAX}; decrease in efficiency of oxidative phosphorylation). Although other studies had reported an increase in maintenance with temperature (Senez, 1962; Marr et al., 1963; Ng, 1969), the alternatives were not eliminated. In more rigorous

studies, (Wallace, 1975; Mainzer & Hempfling, 1976; Farmer & Jones, 1976b), it was confirmed that the decreased growth yields at higher temperature were due to maintenance.

The relationship between temperature and maintenance is apparently dependent on the strain of the organism. Farmer and Jones (1976b) found that maintenance in E.coli W increased continuously with temperature, and calculated an apparent Arrhenius activation value (E_a) of 98 $kJ.mol^{-1}$ for the process. Wallace (1975) found that maintenance of E.coli ML308, grown in arithmetic culture, was constant at temperatures below 30°C, but increased sharply at higher temperatures. He calculated E_a's of 710, 244, 328 and 705 $kJ.mol^{-1}$ for growth on glucose, lactose, glycerol and malate, but these values were based on the increase in maintenance between only two temperatures (37°C and 40°C). The E_a of 294 $kJ.mol^{-1}$ for the glycerol culture over an extended temperature range (Fig. 22b) confirms his findings. Mainzer and Hempfling (1976) found a peculiar dependence of maintenance on temperature for E.coli B growing on glucose. Maintenance increased from 17.5°C to a first maximum at 32°C, then decreased to a minimum at 37°C, thereafter increasing sharply to 40°C. They did not calculate activation energies, but a value of around 420 $kJ.mol^{-1}$ may be calculated for the change in maintenance between 37°C and 40°C.

Arrhenius activation energies of enzyme-catalysed reactions are seldom greater than 100 $kJ.mol^{-1}$. This suggests that enzymes are not involved in the process(es) contributing to the sharp increase in maintenance which

occurs at temperatures in excess of 33.5°C.

A high activation energy has been reported for protein denaturation (Stearn, 1949), which is of the same order as the experimental value for activation energy of maintenance. It is possible that the temperature-dependent component of maintenance is connected with denaturation of macromolecules, and is expended in maintaining the structural integrity of the cell. Cells which have higher oxygen growth yields and more efficient energy coupling (Fig. 19; Hempfling & Mainzer, 1975) have higher maintenance. This indicates that energy may be expended in maintaining the juxtaposition of electron carriers in the membrane in such a way that energy coupling remains more efficient. When the maximum oxygen growth yield of glucose-grown E.coli B was reduced at 32°C (interpreted as a change in $\frac{P}{O}$ ratio from 2 to 1), there was a sharp decrease in the maintenance requirement of the culture (Mainzer & Hempfling, 1976) which would explain the peculiar biphasic response of maintenance to temperature in this strain. Rogers and Stewart (1974) found that the maintenance requirement of S.cerevisiae decreased when grown micro-aerobically, when the respiratory apparatus was not fully synthesised. Aiking et al.(1977), with the same strain, found that the number of coupling sites was reduced by one on K^+-limitation, but did not include their determinations for maintenance.

It is therefore possible to speculate on the identity of maintenance processes in E.coli ML308. Below 33.5°C, enzyme-catalysed reactions which have low activation energies, such as turnover and maintenance of salt gradients, will largely account for maintenance. At

higher temperatures an additional component with high activation energy forms an increasing part of the maintenance requirement. This appears to be associated with the degree of organisation of the respiratory apparatus within the cell membrane. On the basis of published experiments, it appears that E.coli B resembles E.coli ML308 in its temperature dependence of maintenance, whereas E.coli W does not show the second (high E_a) component of maintenance, even when grown at 40°C.

1.6 Maintenance energy in high salt conditions

The maintenance requirement of E.coli ML308 was reduced when grown on lactose or glycerol in the presence of high concentrations of salt (Fig. 23). This was in constrast to previous studies (Watson, 1970; Stouthamer & Bettenhaussen, 1973), where maintenance increased with a higher osmolarity of the growth medium. However, Recheilt and Baumann (1974) and Neijssel and Tempest (1976b) observed no decrease in Y^G with increased salt, so it was possible that the decrease in maintenance observed during this work was related to the decrease in maximum growth yields, and perhaps of energy coupling, which accompanied them (Fig. 23). The osmolarity of the cytoplasm of E.coli normally exceeds that of the growth medium, since sucrose has to be added to the incubation media for spheroplasts to prevent lysis (Fig. 11). A decrease in the maintenance requirement might be expected if the osmolarity of the growth media were increased, thus reducing the osmotic difference across the membrane. This would be particularly true if the ions used to increase the tonicity of the growth media (Na^+, Cl^-, SO_4^{2-}) were impermeable, or had

their permeabilities controlled.

1.7 Maintenance energy and viability

The ability of cells taken from arithmetic culture to grow and divide decreased as the growth temperature of the culture was increased (Fig. 24). In this limited experiment it was impossible to quantitate change in viability to change in temperature. However, it appeared that, at elevated temperatures (40°C, 42°C), most starving cells could divide only once, whereas at lower temperatures a large proportion of the starving cells could form microcolonies. During growth it is likely that more energy will have to be expended at higher temperatures to retain the capability to grow, but insufficient data are available to establish a definite relationship between maintenance and viability.

2. Yields

Molar growth yields in terms of carbon (Y) and oxygen (Y_{O_2}) were measured in batch culture. In the two systems where growth rate was varied (arithmetic and continuous cultures), it was possible to correct for maintenance effects on yields (1.), to obtain the 'maximum' molar growth yields (Y^G, $Y^G_{O_2}$), i.e. values which would be obtained if no energy were used for maintenance. No correction for maintenance could be made for yields obtained in batch culture. In some instances, particularly in experiments with mixed carbon sources, it was more convenient to quote carbon yields in terms of carbon atoms (g.cells (g.atom carbon)$^{-1}$), rather than in molar terms which would be meaningless.

2.1 Yield and carbon source

All of the carbon sources used for growth experiments fell into two distinct classes: those which supported growth with high oxygen growth yields, of the order of 60 $g.mol^{-1}$ e.g. lactose, and those which had lower yields, of the order of 40-45 $g.mol^{-1}$ e.g. glucose. It has been calculated that the former class in which carbon source is transported by a H^{+}-symport mechanism (Mitchell, 1970) had more efficient energy conservation (Appendix 2; Fig. 2.14). These cells also had higher carbon growth yields, and higher maintenance which was discussed earlier (1.2).

The growth yields for most carbon sources did not vary with cultivation technique, indicating that maintenance was normally low during batch culture (1.4).

Maltose and lactose were exceptions, since they had lower yields in batch culture (Y) than in arithmetic- or continuous culture (Y^{G}), although the oxygen yields did not show such a large difference. In both arithmetic- and continuous culture, the rate of entry of carbon source into the cells is limited artificially by the input pump, whereas in batch culture the cells themselves regulate their uptake of carbon source, which is in excess. Energy expended when the rates of cellular catabolism and anabolism do not balance, or in regulating uptake to overcome this, fall under the definition of maintenance, but have been termed 'slip' (Neijssel & Tempest, 1976c). 'Slip' reactions may be responsible for the decreased yields during growth on lactose and maltose in batch culture. Recently, it has been shown that the yield from lactose in batch culture may be increased to that of continuous culture when thiodigalactoside (TDG), a non-

metabolisable competitive inhibitor of lactose transport is added to the growth medium (W. H. Holms, personal communication). It is envisaged that TDG may reduce the flux of lactose into the cell by binding to the carrier protein, thereby reducing the energy normally expended on this control function.

Cells grown in batch culture on the lactose analogues lactulose and lactitol had high oxygen growth yields similar to batch growth on lactose (Fig. 33). No comparison with maximum yields was possible since these compounds were difficult to obtain as pure samples in the quantities required for continuous- or arithmetic culture. It is possible that the yields obtained in batch culture were less than the maximum yields which could be achieved.

Lactitol showed the highest batch yield of the three galactosides used (Fig. 33). This may be because catabolism of this analogue produces one more reducing equivalent than lactose or lactulose and this will be made available to the cell during growth. Mannitol, which contains an extra reducing equivalent relative to glucose, had a higher growth yield (Y^G) than glucose (Fig. 19). Comparative yields between carbon sources which differ by a reducing equivalent have been used to attempt to evaluate $\frac{P}{O}$ ratios (Smalley et al., 1968).

The $\frac{P}{O}$ ratios for growth on mannitol and glucose (2.08, 2.02; Fig. 2.12) which were calculated from their respective yields are in close agreement. This suggests that the value assumed for Y_{ATP} (10.5 $g.mol^{-1}$) is valid.

Growth on galactose in batch culture was unique in that there were two distinct phases (Fig. 31). The first

(MGT = 44 min) had a high oxygen growth yield (Fig. 32b), whereas the second slower phase of growth (MGT = 58 min) had a lower yield (Fig. 32b), similar to that for growth on galactose in arithmetic culture (Fig. 19). No difference could be detected in carbon growth yield for the two phases (Fig. 32a).

Henderson et al. (1977) have used biochemical and genetic techniques to show that at least two galactose transport systems are present in E.coli K10. One system, specified by the gal P locus, transports galactose and other sugars by a proton-symport mechanism (Mitchell, 1970). The other system, of which the mgl P locus is a component, does not involve cotransport of protons but is thought to have a mechanism which involves binding of substrate to a periplasmic protein prior to transport, the energy for which is supplied by ATP. The Km of the mgl P protein for galactose (0.5 μM) is much lower than that of the gal P system ($>$ 150 μM; Henderson & Daruwalla, 1977). Although there is little difference in galactose uptake rates between a mgl P^+, gal P strain and mgl P, gal P^+ strain (Henderson et al., 1977), the former strain grows slower (MGT = 4½ h) than the latter (MGT = 2½ h) (unpublished experiment) suggesting that the mgl P system has a lower transport velocity in vivo. Wilson (1974) studied the two galactose transport systems in E.coli K12. He concluded that there was some mutual interaction between the systems and that the lower affinity (gal P) system was responsible for the major fraction of galactose uptake during normal growth.

If the two systems are also present in E.coli ML308 and have kinetic properties similar to the corresponding

systems in E.coli K10, the growth pattern (Fig. 31) could be explained as follows: In the initial faster phase of growth the gal P system was responsible for the major fraction of galactose uptake. This phase, in common with growth on carbon sources which are transported by proton-symport mechanism (Fig. 19), was accompanied by a high oxygen growth yield. Later, as galactose was removed from the medium ($\sim$800 μM), the mgl P system, which is not a proton-symporter, became the primary system for galactose transport. Having a lower K_m, it would be more suited to transport galactose as the carbon source became limiting, but the growth rate of the culture was reduced since this system has a lower velocity of transport. During the second phase of growth the Y_{O_2} was 48 $g.mol^{-1}$; close to the value for arithmetic culture when galactose was limiting.

Although the explanation is speculative, the data fit this interpretation. A simple test of this hypothesis would be to compare $\frac{H^+}{O}$ ratios for cells in each phase of growth. However, logarithmic cells require a period of starvation prior to determination of $\frac{H^+}{O}$ ratio and this reduces $\frac{H^+}{O}$ values of cells with high efficiency of energy conservation to the level found normally for cells with two coupling sites (Fig. 38b). The maximum growth yields for E.coli K10 grown on lactose and glucose in arithmetic culture (Fig. 21) compared well with the respective yields for E.coli ML308 (Fig. 19). This was not true for galactose: E.coli K10 had high growth yields (similar to lactose) for growth on galactose in arithmetic culture, whereas E.coli ML308 had low yields (similar to glucose).

The results may be best explained by suggesting that the mgl P system is primarily responsible for uptake of limiting galactose in arithmetic culture of E.coli ML, but that the gal P system is dominant over the mgl P system (Wilson, 1974) for E.coli K, even when galactose is limiting.

In batch culture, when two carbon sources are present, the utilisation of one carbon source usually dominates the other. However in arithmetic culture when cells are limited severely by availability of carbon they will always metabolise two carbon sources when supplied with a mixture. Cells grew on mixtures of glucose and lactose in arithmetic culture with growth yields which were characteristic of lactose alone, even when lactose was as little as 5% of the substrate utilisation of the culture (Fig. 15).

When growing on a glucose/lactose mixture, the glucose was taken into the cell by the phosphotransferase system which phosphorylated the sugar to glucose 6-phosphate; the lactose was hydrolysed inside the cell to glucose and galactose which were phosphorylated by intracellular kinases. To obtain high yields characteristic of lactose alone on the mixture, all of the glucose being taken up from the medium must have been metabolised as if it were carbon derived from lactose. This means that a cell which is 'set up' to grow with higher efficiency takes all the carbon which enters the cell and oxidises it with higher efficiency. It does not discriminate carbon from lactose, whose uptake appears to be the phenotypic reason for the higher growth efficiency.

Glycerol-trained cells which had been grown immediately

under carbon limitation grew on lactose or a lactose/ glucose mixture with the lower yield usually found with glucose alone (Fig. 15c). To grow with the higher efficiency, cells apparently required an exponential phase of growth on lactose prior to becoming limited by carbon source; it was not possible for cells to acquire the capability for more efficient growth on lactose while carbon-limited. This seems reasonable since reorganisation of, or synthesis of new components for an electron transport chain which is more efficient may initially require expenditure of energy which is not available under conditions of carbon limitation; sustaining a more efficient respiratory system which was operational during exponential growth may require less energy (the energy of maintenance), which may still be possible under conditions of carbon limitation.

Recently, Long et al. (1977), have reported some experiments which are worth discussing in this context. They took a strain of E.coli which was constitutive in the lac enzymes and had been grown on glycerol, and measured the heat evolution when the permease substrate TMG was added. They found that the energy expended by the cells (as heat evolved) was 13 times greater than could be accounted for as energy expended in transport. It is possible that this additional energy was spent at altering the topography of the electron transport chain to enhance the efficiency of energy conservation which was low, since the cells had been grown on glycerol.

It was difficult to compare yields between lactose and its mixtures with each of glycerol and malate since these compounds enter central metabolism of the cell at

different points (in contrast to glucose and lactose). However, mixtures did give high yields. In particular, the comparative growth yield for the lactose/glycerol mixture was higher than for that of lactose alone. When carbon is supplied at the levels of both lactose and glycerol, energy need not be expended in converting lactose to the level of glycerol (for lipid synthesis) or glycerol to the level of lactose (for the synthesis of cellular polysaccharide). This saving is expressed as a higher growth yield.

In summary, high growth yields were found irrespective of culture technique for growth on single carbon sources which were transported into the cell via a proton-symport system, and for mixtures of carbon sources if one component was being transported by such a mechanism. The transport system had not only to be present but had to be operating to give the higher growth efficiency. It was not possible for the cell to 'Shift up' to a higher growth efficiency if it was shifted to growth on a H^+ symport substrate under severe carbon-limitation. It is concluded that it is the flux of carbon source through the H^+-symport system which is responsible for the higher energy conservation, but a period of nutrient excess is required to make the alterations to the electron transport chain. Once made, more maintenance energy is spent in maintaining the more efficient topography.

2.2 Yield during sulphate limitation

When the sulphate concentration of the growth media was reduced from the usual level (10.5 $mmol.l^{-1}$) to a low level (15 $\mu mol.l^{-1}$), there was no effect on the growth

rate or yields (Y, Y_{O_2}) of E.coli ML308 growing in batch culture on lactose (Fig. 34a). There was no change in Y^G, $Y^G_{O_2}$ when the same strain was grown on lactose or glycerol in continuous culture with sulphate at 50 μmol.l^{-1} (Fig. 28). However, there was a reduction in growth rate and yields (Y, Y_{O_2}) when the same strain was grown on glycerol in batch culture at the same level of sulphate (Fig. 34b). Growth and growth rate on glycerol in batch culture were dependent on sulphate concentration at levels below 50 μmol.l^{-1} (Fig. 35).

Continuous culture of Candida utilis under sulphate- and iron-limitation produced yeast with mitochondria which lacked the first site of energy conservation (Haddock & Garland, 1971; Light & Garland, 1971), presumably due to loss of an iron/sulphur protein of the respiratory chain. Poole and Haddock (1975) found that growth in continuous culture (μ = 0.16) of a low sulphate-requiring strain of E.coli K12 became limited by sulphate at less than 300 μmol.l^{-1}. This was accompanied by a reduction in cell yield with respect to carbon source (glycerol) and a decrease in $\frac{H^+}{O}$ ratio for sulphate-limited cells, interpreted as a loss of the coupling site for energy conservation between NADH and flavin. Farmer and Jones (1976a) repeated these experiments with E.coli W, but expanded the study to a range of growth rates since it was possible that the earlier results for yield with K12 were due to some effect of sulphate limitation on maintenance, which had not been accounted for. They found no change in yield (Y^G, $Y^G_{O_2}$) or $\frac{H^+}{O}$ ratio when E.coli W was grown in continuous culture under sulphate limitation.

The decreased yield of K.aerogenes obtained in sulphate-limited continuous culture (Stouthamer & Bettenhaussen, 1975) was interpreted as a loss in energy conservation, and a recent study (Meijer et al., 1977) of P.denitrificans has shown a decreased yield and reduction of $\frac{H^+}{O}$ ratio for sulphate-limited cells.

The difference in effect of sulphate limitation on E.coli K12 and E.coli W has been attributed to strain differences (Haddock, 1977). The behaviour of E.coli ML on sulphate limitation does not resemble either of the other strains: there is a decreased yield for sulphate-limited batch growth on glycerol, but not in continuous culture. Sulphate-depleted growth on lactose showed no decrease in yield for either batch- or continuous culture. A more rigorous analysis would be required to make firm conclusions, but growth on lactose has a lower requirement for sulphate than growth on glycerol. There was no firm evidence that lactose cells were sulphate-limited concentrations as low as 15 μmol.l^{-1}, whereas growth yields were much reduced for glycerol cells at this level of sulphate (Fig. 35). The difference between cultivation technique for sulphate-limited growth on glycerol may prove to be enlightening: the medium for batch culture (Methods, 2.5.2.1) lacked the trace metal supplements used in continuous culture (Methods, 2.5.4.1). Of these trace metals, Mn^{2+} has been omitted in experiments which show low yields on sulphate limitation (Haddock & Garland, 1971; Poole & Haddock, 1975; batch culture, this work), whereas it has been present when no change in yield has been detected (Farmer & Jones, 1976a; continuous culture, this work). This suggests that the presence of Mn^{2+} may be

an important factor in energy conservation during sulphate-limited growth. Molybdenum and selenium have been shown to be trace requirements for the proper functioning of nitrate reductase and formate dehydrogenase in anaerobic electron transport chains (Enoch & Lester, 1972).

2.3 Yield and temperature

Many studies of effect of temperature on yield have been made with batch culture (Monod, 1942; Harder & Veldkamp, 1967; Ng, 1969; Coultate & Sundarum, 1975). Although yields have been shown to decrease with increasing temperature no account was, or could, be taken of the contribution of maintenance energy to reduction in growth yield at higher temperatures.

When the effect of temperature on yield has been investigated in continuous culture, the results have varied with each study. Farmer and Jones (1976b) found that growth yields (Y^G, $Y^G_{O_2}$) of *E.coli* W were a maximum at 30°C which is the usual temperature for growth of the strain. The yield ($Y^G_{O_2}$) for growth of *E.coli* B on glucose was constant with increasing temperature until it fell to a second lower value at temperatures in excess of 32.5°C. This was interpreted as loss of a functional coupling site (Mainzer & Hempfling, 1976). The second less-efficient phase of growth had a minimum yield at 37°C. The pattern for *E.coli* ML308 was similar to *E.coli* B in that there was a minimum at 37°C, but there was no rapid change at 32.5°C (Fig. 22). The growth yield minimum at 37°C is surprising since this is the normal temperature for growth of the organism in its natural environment.

It is not clear whether the changes in maximum growth yield were a result of parallel changes in Y_{ATP}^{MAX}, or due to some other effect. Mainzer and Hempfling (1976) found that Y_{ATP}^{MAX} for anaerobic growth of glucose increased slightly as the temperature of growth was lowered from 37°C to 25°C. This would explain the increase in yield at temperatures below 37°C. The effect of temperature on Y_{ATP}^{MAX} was not studied at higher temperatures. The biphasic response of yield to temperature suggests that there are two classes of reaction operating within the cell: one which has increased efficiency with increasing temperature, the other which has decreased efficiency with increasing temperature. Although the normal growth temperature of the organism coincides with the minimum growth yield corrected for maintenance, which is puzzling, it must be remembered that in the natural environment it will be a combination of efficiency of growth rate, actual yield and viability which will be important. At 40°C the growth rate and Y^G would be higher, which would be advantageous. However the effect of temperature on maintenance (1.5) and the decrease in viability at 40°C (1.7) would more than offset the advantages.

2.4 Yield in high salt conditions

The maximum growth yields of *E.coli* ML decreased when salt was added to the culture medium (Fig. 23). This could be a consequence of a decrease in Y_{ATP}^{MAX}, or energy conservation, or both. Although no study of effect of concentration of salt in growth medium on Y_{ATP}^{MAX} has been made for *E.coli*, Watson (1970) found no change in Y_{ATP} when yeast was grown in high salt conditions and Reichelt

and Baumann (1974) found an increase in yield of Vibrio cholereae when grown with added salt. If Y_{ATP} does not change, this would suggest that the low yields are a consequence of decreased energy conservation. There was no significant change in $\frac{H^+}{O}$ ratios of cells grown on glycerol in high salt conditions (Fig. 38b), although lactose cells, which are apparently more fragile (Dev. Methods, 5), did show some decrease in $\frac{H^+}{O}$ ratio when grown in high salt conditions. Glycerol, which was used as exogenous source of reducing power in $\frac{H^+}{O}$ experiments, supplies electrons to the electron transport chain mainly at the level of flavin. These experiments therefore did not report an energy conservation between NADH and flavin, i.e. siteI. It is possible that cells grown in high salt conditions had lower yields due to loss of energy conservation at siteI. However, it is more probable that the effect was due to an increase in the passive permeability of the membrane to protons, when high levels of salt were present. The $\frac{P}{O}$ ratios (calculated by the method of Appendix 2) for growth on glycerol and lactose in high salt conditions were: 1.19, 1.26 respectively (calculations not presented). This suggests that the extra energy conservation for lactose cells (compare $\frac{H^+}{O}$ ratios of Fig. 38b) was being lost by a weak 'uncoupling' effect of salt on the membrane.

3. H^+/O ratios

The $\frac{H^+}{O}$ ratios for glycerol cells of E.coli ML determined during this work (2.4b; Fig. 36a) were lower than the integer values (4.00) determined in Jones'

laboratory (Brice et al., 1974; Jones et al., 1975) for glycerol-trained cells of E.coli W using endogenous reductant. Arithmetic-culture cells showed no proton extrusion unless exogenous glycerol was added, presumably because they had low endogenous levels of reducing power during growth in arithmetic culture. In E.coli, glycerol is metabolised via glycerokinase, ∝ glycerophosphate dehydrogenase (which for aerobic growth is flavin-linked; Fig. 42a), and then by the Embden-Meyerhof pathway. Under the anaerobic conditions of the $\frac{H^+}{O}$ assay, the amount of glycerol metabolised will most likely be limited by the pool size and turnover of $FADH_2$ which is available to ∝ glycerophosphate dehydrogenase; dihydroxyacetone phosphate produced as a result will be rapidly metabolised by the Embden-Meyerhof pathway, especially if the NADH/NAD equilibrium is low - an assumption supported by the absence of proton extrusion when glycerol is omitted. Metabolism of glycerol will be stoichiometrically related to production of $FADH_2$, and will cease when the major part of the $FAD(H_2)$ pool available to ∝ glycerophosphate dehydrogenase exists as $FADH_2$. If the $FAD(H_2)$ pool is small in comparison to that of NAD(H), which is likely, the NADH produced as a result of this small amount of glycerol utilisation will make little difference to the NADH/NAD equilibrium of the larger NAD(H) pool. Consequently, the majority of the reducing power available for electron transport from glycerol will be $FADH_2$, under these conditions.

Electron transport from $FADH_2$ to oxygen has been shown for mitochondria (Mitchell & Moyle, 1965) and E.coli (Poole & Haddock, 1975) to involve fewer energy-conserving

'loops' than electron transport from NADH to oxygen. It is not therefore surprising that $\frac{H^+}{O}$ ratios for arithmetic-culture cells using exogenous glycerol are smaller than the values obtained by Jones and co-workers for proton translocation from endogenous reductant, which will be mainly NADH. Lawford and Haddock (1973) found that the $\frac{H^+}{O}$ ratio of glycerol-grown cells of E.coli K decreased from 3.53 for endogenous reductant to 2.36 when glycerol was added. This was presumably due to a greater contribution of electrons from $FADH_2$ to electron transport in the latter case. Farmer and Jones (1976a) attributed the decrease in $\frac{H^+}{O}$ ratio of oxygen-limited cells to a shift in favour of flavin utilisation for electron transport, when no exogenous carbon source was supplied in the assay.

Arithmetic-culture cells only showed proton extrusion in response to oxygen when exogenous glycerol was added in the assay: malate, succinate, pyruvate and lactate could not substitute for glycerol. This was unusual, since these compounds were used in other laboratories as exogenous carbon sources during H^+-translocation experiments with E.coli (e.g. Poole & Haddock, 1975; Farmer & Jones, 1976a). The reason for the anomaly was not clear. It was not due to absence of enzymes to metabolise the compounds, since each gave measurable uptakes of oxygen which were 30% or less than the uptake in the presence of glycerol (unpublished experiment).

It may be that, for arithmetic-culture cells under anaerobic conditions, transport of charged compounds such as malate created a chemical potential of H^+'s which

opposed further uptake. The logarithmic cells which have been used by other workers have usually been starved for 2 h before the assay. This procedure renders the cell permeable to valinomycin (Lawford & Haddock, 1973), and to lysozyme (unpublished experiment), and reduces the $\frac{H^+}{O}$ ratio of cells trained to lactose (Fig. 38c), so it may also increase the passive proton permeability of the membrane to allow sufficient charged substrate to be taken up to provide reductant for electron transport with the logarithmic cells.

Another possibility was that the pulse of protons emitted from the arithmetic-culture cells in response to oxygen was used immediately for the transport of the charged substrate, so that no change was observed in the pH of the external medium. The rate of uptake of the charged substrate would have to have exceeded that of electron transport, which was unlikely.

A search was made for an alternative to glycerol for proton extrusion experiments, but no other non-fermentable, uncharged carbon source which was not transported concurrently with a proton could be found.

$\frac{H^+}{O}$ ratios of cells grown on glucose, maltose, mannitol and galactose were similar to those of glycerol cells (Fig. 37). This was consistent with the presence of two energy-conserving loops of the electron transport chain (Poole & Haddock, 1975), if 2 H^+ were translocated per loop.

$\frac{H^+}{O}$ ratios of cells grown on lactose and gluconate were higher than those of the other cell types (Fig. 37).

The results were consistent with the presence of an extra loop of the electron transport chain during growth on these carbon sources (but see 4., for a fuller treatment).

A value close to 6 was reported (Essenberg & Kornberg, 1975) for the $\frac{H^+}{O}$ ratio of an E.coli K12 strain which was constitutive for the transport of glucose 6-phosphate. Apart from this, and a report of $\frac{H^+}{O} = 6$ for E.coli W (Meyer & Jones, 1973) which was subsequently disclaimed (Brice et al., 1974), only 2 energy-conserving loops have been reported for E.coli ($\frac{H^+}{O} = 4$, at a maximum; see Jones, 1977, for a review). To date, there has been no report for $\frac{H^+}{O}$ ratios of E.coli grown on gluconate or lactose. However, if cells with high energy conservation were subjected to a starvation period before the assay of $\frac{H^+}{O}$ ratio (which is the usual procedure with logarithmic cells), the value decreased from that consistent with 3 coupling sites to that usual for cells with 2 sites (Fig. 38c). The high value for $\frac{H^+}{O}$ ratio reported by Essenberg and Kornberg (1975) used cells which were not starved and it is possible that some of the other values of $\frac{H^+}{O}$ ratios reported for E.coli have been lowered as a result of the starvation procedure.

Since cultures with higher energy conservation have an increased maintenance requirement (Fig. 19), some of which may be concerned with organ-isation of the cell at the supramolecular level (1.2), it is tempting to suggest that the topology of the electron transport chain is altered during the starvation procedure in a way which reduces its capability to translocate protons to the outside

of the cell; the more efficient topology would normally be preserved during growth at the cost of a higher maintenance requirement.

The activity of the lac permease of logarithmically-grown cells decayed with time when cells were shaken without carbon source (Fig. 41). The permease activity of arithmetic-culture cells also decayed with time (result not shown). This effect had been observed previously (Rickenberg et al., 1956), and attributed to autooxidation of the permease carrier (Kennedy, 1970). The previous studies which measured total uptake activity had a first-order decay of permease with time: in the present study the total uptake activity had a first-order decay (results not shown), but carrier-mediated activity (total activity corrected for non-specific uptake) had a zero-order decay (Fig. 41).

It is unlikely that the decay kinetics for auto-oxidation of the carrier would be zero order. In addition, the same decay kinetics were observed for cells shaken in a nitrogen atmosphere (unpublished experiment), where the rate of autoxidation of the carrier might be expected to be decreased. It is more likely that the decrease in transport activity was due to the depletion of the transmembrane gradient of protons which drives transport via the lac permease. Verification of the hypothesis could be obtained by measuring the protonmotive force present across the cell membrane (e.g. Collins & Hamilton, 1976) during the starvation period. It has been calculated that because of the reverse potential generated, proton-linked transport could only occur for less than a second

in cells which have been totally depleted of energy (Cecchini & Koch, 1975).

Permease activity could be reactivated by adding glycerol (Fig. 41a), the metabolism of which would re-establish the transmembrane proton gradient. Addition of glycerol did not reactivate permease activity when cells were shaken in phosphate buffer alone (Fig. 41a). It is possible that some inorganic component of the growth medium was required for the reactivation process. Oxygen uptake was observed when cells were resuspended in phosphate buffer alone and placed in the oxygen electrode with glycerol (unpublished experiment), indicating that the result was not due to inability to metabolise glycerol when the inorganic salts were omitted. Glycerol metabolism was responsible for the reactivation, since there was no change in permease activity when glycerol was added to a culture stirred under a nitrogen atmosphere, when glycerol could not be metabolised (unpublished experiment). Protein synthesis *de novo* was not required for reactivation, since chloramphenicol did not prevent reactivation by glycerol (Fig. 41b).

It appears that electron transport and the *lac* permease may interact with each other. A decrease in the protonmotive force due to shaking under starvation conditions reduced the activity of the *lac* permease (Fig. 41) and lactose cells which had a low permease activity after a starvation period had a reduced efficiency of electron transport which creates the protonmotive force (Fig. 38c).

Addition of TMG or TDG to lactose-grown cells transiently increased the $\frac{H^+}{O}$ ratio (Fig. 39a, b). A H^+

will accompany the transport of each molecule of TMG and TDG into the cell (West & Mitchell, 1973). If some of the H^+-pulse usually sensed by the pH electrode were dissipated for transport this would be expected to reduce rather than increase the $\frac{H^+}{O}$ ratio. It is possible that the proton-linked flux of galactosides reduced any residual membrane potential which was present in the cells and dissipated the back potential created by proton efflux during the pulse, enhancing the number of protons extruded. This effect was noted when a trace of uncoupler was added to cells of P. denitrificans (Lawford et al., 1976). However, the increase in $\frac{H^+}{O}$ ratio was not observed on addition of galactosides to glycerol-trained cells which were constitutive for the lac permease (Fig. 39c, d) and would be subject to the same uncoupling effect, if it existed. The effect, therefore, was a property only of lactose-trained cells. TMG was more effective than TDG in stimulating the $\frac{H^+}{O}$ ratio. The rate of influx of TMG by the permease (Vmax) is 7 times that of TDG (Cohen & Kepes, 1962), so the enhancement may be related to rate of transport. The increase in $\frac{H^+}{O}$ ratio was only transient; values returned to their former levels after 10 min. By this time, at steady state, influx of galactoside would be balanced by efflux. The enhancement effect was only observed immediately after addition of the compound when influx would greatly exceed rate of efflux. Addition of a second aliquot of galactoside caused a second transient increase in $\frac{H^+}{O}$ ratio (data not shown). It would therefore appear that a net influx of galactoside occuring concurrently

with electron transport stimulated the efficiency of the latter, but the effect was confined to lactose-trained cells.

$\frac{H^+}{O}$ ratios of lactose-trained and glycerol-trained cells did not change if experiments were conducted at 37°C instead of 27°C (Fig. 38a), although the decays of the proton pulses were faster at the higher temperature. Mitchell and Moyle (1965) found an increase in $\frac{H^+}{O}$ ratio of mitochondria incubated with succinate and assayed at 5°C. They attributed this to a reduction in the activity of the 'succinate/OH antiporter' at the lower temperature, but the effect has been attributed recently to inhibition of phosphate transport (Reynafarje et al., 1976). The difference between $\frac{H^+}{O}$ ratio of glycerol and lactose cells was not due to some effect of temperature peculiar to cell phenotype.

Cells which had been grown in high salt conditions had $\frac{H^+}{O}$ ratios which were slightly lower than those of cells grown under normal salt conditions (Fig. 38b). This may reflect a degree of fragility in the respiratory apparatus of the former cells. The results do not confirm the loss of two coupling sites for growth on lactose and one site for growth on glycerol which were indicated from the decrease in molar growth yields (Fig. 23). $\frac{P}{O}$ ratios derived from growth yields measure the overall efficiency of utilisation of energy produced by oxidative phosphorylation; $\frac{H^+}{O}$ ratios only measure the efficiency of production of the proton gradient which is used to

synthesise ATP. The results suggest that growth in high salt conditions does not affect the efficiency of electron transport, but does affect the utilisation of energy produced by electron transport.

4. Active transport and energy conservation in E.coli

4.1 Comparison of P/O ratios and H^+/O ratios

The derivation of $\frac{P}{O}$ ratios from molar growth yields depends critically upon a value assumed for Y_{ATP}^{MAX} (Appendix 2). The $\frac{P}{O}$ ratios calculated (Fig. 2.14) from growth yields obtained during this work (Fig. 19) fell into two categories: those which had $\frac{P}{O} = 3$, and those which had $\frac{P}{O} = 2$. Without a definite experimental value for Y_{ATP}^{MAX} for aerobic growth, which at present is considered impossible to obtain, the results could also be interpreted as a difference of a factor of $\frac{3}{2}$ for the Y_{ATP}'s of cells in each class with all cell types having $\frac{P}{O}$ ratios which were similar. It is possible to argue, but impossible to prove that a 50% difference in energy requirement is unlikely for the synthesis of cellular polymers from their monomers (Y_{ATP}^{-1}; Appendix 2.1), especially when all the cell types were grown in the same energy-starved arithmetic-culture conditions.

The experimental determination of $\frac{H^+}{O}$ ratios provides a means of verifying the derived $\frac{P}{O}$ ratios. Cells which had $\frac{P}{O}$ ratios of 3, as calculated from growth yields (Fig. 2.14), had higher $\frac{H^+}{O}$ ratios (Fig. 37) than cells which had calculated $\frac{P}{O}$ ratios of 2.

It is true that certain assumptions must be made in the interpretation of $\frac{H^+}{O}$ ratios, and that assumptions are necessary for the calculation of $\frac{P}{O}$ ratios, but they are mutually exclusive. Therefore, it was energy conservation and not Y_{ATP}^{MAX} which varied by a factor of $\frac{3}{2}$, depending on cell phenotype.

4.2 Growth conditions necessary for high energy conservation

From the results obtained during this work, it is clear that E.coli cells grow with higher energy conservation on carbon sources which enter the cell by active transport systems which have a proton-symport mechanism. The high energy conservation was dominant over phenotypes with lower efficiency, since the total uptake of carbon by the culture was utilised with high efficiency when mixtures were supplied which had only a small fraction of the carbon in a form which was taken up by a proton-symport system.

Cells must grow logarithmically on a proton-symported substrate in order to gain the third site of energy conservation, but can maintain this phenotype during growth in arithmetic culture under carbon limitation. During this work, inocula with high energy conservation were prepared by growing cells at μ_{max} in batch culture on a proton-symported carbon source. Shifting from growth on glycerol ($\frac{P}{O} = 2$) to growth on lactose under immediate carbon limitation did not allow synthesis of the extra site during growth on lactose. The possibility that the extra site during growth on lactose. The possibility that growth in continuous culture at or close to μ_{max}

might also allow the acquisition of an extra coupling site has not been investigated.

4.3 'Effector' of high energy conservation

Lactose- and glycerol-trained cells were used as examples of cells which had respectively high and low energy conservation. The bacterial strain used, '51', was constitutive for the lac and glycerol enzymes. Therefore, the high energy conservation during growth on lactose was not due to the presence or absence of the lac or glycerol enzymes.

During the 4-day period usual for growth in arithmetic culture, the growth rate of the culture fell from its initial value of μ_{max} ($\doteq$ 1) to at most μ = 0.01, but constant values for $\frac{P}{O}$ ratios were derived over intervals of this period. Higher growth efficiency was therefore not due to some effect of growth rate which was peculiar to some phenotypes. Aiking et al., (1977) reported a change in energy conservation of S. cerevisiae at $\mu < 0.2$ in continuous culture under potassium limitation. However Farmer and Jones (1976a) found no change in efficiency of energy conservation with growth rate of E.coli W which were carbon-limited. This is consistent with the results of carbon-limited cultures reported here. The level of expression of cellular enzymes changes with growth rate (Fig. 40; Macleod et al., 1975). It is likely that the levels of components of the electron-transport chain will also fluctuate with growth rate (Aiking et al., 1977) but this does not apparently alter the efficiency of energy conservation in E.coli. The electron-transport carriers of E.coli B have been shown to be subject to repression

mediated by glucose (Hempfling, 1970) which may be reversed by cyclic AMP (Hempfling & Beeman, 1971). This has prompted an investigation of the involvement of catabolite repression in the synthesis of membrane carriers (Daoud & Haddock, 1976). A study of the dependence of growth rate on expression of electron carriers might be enlightening. As growth rate changes, some electron carriers will have lower rates of expression than others. The information derived might indicate the stoichiometrical relationship which each component has with its neighbours to preserve the efficiency of operation of the electron transport chain.

From the experimental evidence available, it seems reasonable to deduce that the high energy conservation of some cell phenotypes may be related to the flux of substrate through the proton-symport transport system. The lac permease was present gratuitously in all cell types, since the strains were lac constitutive, but only when it or another proton-symporting transport system was involved actively in uptake was the higher energy conservation observed. The high energy conservation was labile, since it disappeared under starvation conditions (Fig. 38c). Inward flux of TMG and TDG enhanced the $\frac{H^+}{O}$ ratio of lactose-grown cells, but not of glycerol-grown cells (Fig. 39). The evidence available would therefore point to some interaction between the transport system and that of electron transport which is dependent on the former being active catalytically. A more extensive investigation would have to be made to enlarge upon this speculative conclusion.

4.4 Proposed mechanisms for high energy conservation

We have shown that phenotypes dependent on proton-symported substrates have a higher level of energy conservation than phenotypes where other mechanisms are used to bring the carbon source across the membrane. While these observations are interesting in themselves, it is important to relate them to the current models of electron transport and energy conservation.

According to the hypothesis of Mitchell (1966), electron transport is coupled to synthesis of ATP by a proton gradient. The anisotropic arrangement of electron carriers in the membrane (Introduction 2.1) results in protons being extruded to the outside of the cell as electrons are passed from reducing equivalents to terminal acceptor (oxygen, for aerobic growth). The free energy trapped as the proton gradient is used to synthesise ATP as protons return to the cytoplasm via the ATPase.

Among the other current hypotheses for mechanisms of energy transduction (Introduction 2), Williams has argued that protons do not leave the membrane at steady state (i.e. proton extrusion is an artefact), but charge separation within the membrane is still required as source of free energy. Williams further argues that a proton gradient may be synthesised from energy conserved by charge separation but that the proton gradient is not an obligate intermediate in energy transduction. However, at present there is little evidence to support his hypothesis, and ample evidence to suggest that at least active transport is obligately linked to the proton gradient (Introduction 1.5), which Williams would not dispute (Williams, 1977).

From work with mitochondria, it was deduced that 2 H^+

were extruded for each 'redox' loop of the electron transport chain and that the stoichiometry of the ATPase was 1 ATP : 2 H^+ (Mitchell & Moyle, 1973). This meant that the proton stoichiometry ($\frac{H^+}{O}$) would be twice the phosphate stoichiometry ($\frac{P}{(O)}$), with respect to oxygen. The 'redox loop' stoichiometry for mitochondria is currently subject to dispute (Introduction 2.1), but will be assumed initially in this section.

Higher energy conservation can occur as the result of two changes: the number of H^+'s extruded as electrons pass down the electron transport chain can increase, or the H^+ : ATP stoichiometry of the ATPase can decrease. It is impossible to distinguish these effects using $\frac{P}{O}$ ratios derived from growth yields. However, $\frac{H^+}{O}$ ratios only measure the efficiency of electron transport and suggest that the former was the case. The question remains: how can the electron transport chain become more efficient?

Normally, E.coli is thought to have two sites of energy conservation (Haddock & Jones, 1977; for a review). The most efficient bacteria, e.g. P. denitrificans (Scholes & Mitchell, 1970) have an energy-conserving loop associated with NAD/NADP transhydrogenase activity, and three loops in the electron transport chain from NADH to oxygen, two of which are between FADH and oxygen. The transhydrogenase activities of lactose-grown cells of E.coli were similar to those of glycerol-grown cells (Fig. 42b), indicating that a change in transhydrogenase activity was not responsible for the increased energy conservation in the former cells. The redox midpoint potentials of the NADPH/NADP and NADH/NAD couples differ

by only 4 mV, which make its operation unlikely under physiological conditions (Jones, 1977). Csonka and Fraenkels (1977) have also concluded that transhydrogenase activity plays a minor role in production of NADPH by E.coli. In any case, an extra coupling site associated with transhydrogenase would not have increased the $\frac{H^+}{O}$ ratio of lactose cells when FADH/NADH was the source of reductant (3; Fig. 37). It is more likely that the additional loop is associated with electron transport below the level of transhydrogenase.

Poole and Haddock (1975) found that cells of E.coli K which were grown on glycerol excess, with sulphate limitation lost the energy-conserving loop associated with electron transport between the levels of NADH and FADH, and attributed this to loss of an iron-sulphur centre. Attempts were made during this work at sulphate-limited growth of E.coli ML on lactose (Fig. 34a). This was to examine the possibility that an iron-sulphur protein might be involved in the mechanism of higher conservation efficiency, and might be lost on sulphate limitation giving some idea of its location in the electron transport chain. No loss of energy conservation was observed, but it is difficult to draw conclusions since it is doubtful if the culture was in fact sulphate-limited.

The inability of arithmetic-culture cells to use substrates during proton-extrusion experiments which supply electrons solely at the level of NADH (e.g. malate) or FADH (e.g. succinate) rendered impossible a systematic study (e.g. Lawford & Haddock, 1973; Poole & Haddock, 1975; Jones et al., 1975) of the relative positions of the redox loops in their electron transport chains. However

the increase in $\frac{H^+}{O}$ ratio for lactose cells, when the reductant was likely to be mainly FADH (3; Fig. 37) suggests that the additional loop for electron transport lies between FADH and oxygen. The alternative would be a second loop between the levels of NADH and FADH, but the midpoint potential difference between the two of 270 mV makes the presence of a second loop unlikely. This segment of the electron transport chain appears to be common to all bacteria (Jones, 1977).

The conventional electron transport chain for glycerol-grown E.coli has two redox loops, one of which lies between FADH and oxygen and is thought to involve passage of electrons from coenzyme Q to a cytochrome b and cytochrome o (Poole & Haddock, 1975; Fig. 46a). The cytochrome o has been observed to be kinetically-competent to act as a terminal oxidase for electron transport (Haddock et al., 1976). For glycerol-grown cells, it has a redox midpoint potential of +220 mV, giving a potential difference of about 250 mV between FADH and cytochrome o (P. B. Garland, personal communication). For the same reasoning as applied to the NADH/$FADH_2$ segment, it would be difficult to envisage an additional (second) redox loop between FADH and this cytochrome o species.

It is possible that the high energy conservation during growth on lactose and other substrates is due to the synthesis and operation of a novel electron transport chain. Phenotypic variations in respiratory chain composition have been noted for many bacteria, including E.coli (Haddock, 1977, for a review). An additional segment could arise in lactose cells 'distal' to cytochrome o (+220 mV) and before oxygen (+800 mV), but

such a variation would be completely unprecedented.

Work in Jones' laboratory (Jones et al., 1975; Jones, 1977) has correlated high efficiency of energy conservation $\left(\frac{P}{O} = 3\right)$ with bacterial species which have c-type cytochromes in their respiratory chains. No cytochrome of the c-type was observed in glycerol-grown cells of E.coli K, which had $\frac{P}{O} = 2$ (Haddock et al., 1976). Unfortunately, there has been no report to date of cytochrome spectra for E.coli cells grown on carbon sources such as lactose. The apparatus required for such a study (dual-wavelength spectrophotometer which can operate at low temperature, with stopped-flow function) is only available in a few laboratories since it is intricate and expensive. An investigation of lactose cells using this approach would be a good proposal for future research.

E.coli certainly possesses the genetic information necessary for synthesis of c-type cytochromes since they have been detected in anaerobically-grown cells (Gray et al., 1963; Haddock et al., 1976). A cytochrome c, with E_o = 150 mV, which was kinetically distinct from the anaerobic species was detected in extracts of aerobically-grown E.coli (Barrett & Sinclair, 1967). This would be a candidate for an electron carrier in a more efficient respiratory chain, but was only synthesised in media containing a high sulphur content. That cells grew on lactose with high efficiency in sulphate-depleted media suggests that this cytochrome, if it were present in ML strain, was not involved in the mechanism of higher efficiency. It remains to be seen whether lactose-grown

cells of E.coli ML have a novel cytochrome of the c-type.

Branching of the respiratory chain has been observed for many bacteria (White & Sinclair, 1971; Haddock & Jones, 1977; Jones, 1977; for reviews). For Azotobacter vinelandii, different branch pathways are associated with different efficiencies of energy conservation (Downs & Jones, 1975). Branched chains have been detected by differences in affinities of the terminal oxidases of the branches for oxygen and cyanide. The usual terminal oxidase in aerobically-grown E.coli, cytochrome o, was highly sensitive to inhibition by cyanide (Pudek & Bragg, 1974). During growth in the presence of cyanide a more cyanide-resistant terminal oxidase, cytochrome d, was synthesised (Ashcroft & Haddock, 1975). The inhibition by cyanide of the terminal oxidases were measured for E.coli cells grown on lactose, glycerol and glucose and in arithmetic- and batch culture (Figs. 43, 44). The oxidases of lactose-grown and glycerol-grown batch culture cells had affinities for cyanide which corresponded to that of cytochrome o (Ki = 75 μM; Ashcroft & Haddock, 1975), but cells grown in arithmetic culture had oxidase inhibition kinetics for cyanide which corresponded to cytochrome d (Ki = 200 μM). Interestingly, glucose-grown cells had the same inhibition kinetics which corresponded to cytochrome o irrespective of the culture technique. Bacteria have been forced to use cytochrome d rather than cytochrome o as a terminal oxidase by decreasing the oxygen tension in the culture media (Arima & Oka, 1965; Jones, 1973; Weston et al., 1974; Haddock et al., 1976). The oxygen tension of the culture media during growth in arithmetic culture was close to that for batch culture (R. J. Wallace,

personal communication). The slow growth rates of arithmetic culture may be responsible for the shift to cytochrome d as terminal oxidase. In previous studies, it may have been the decrease in growth rate of the culture which accompanied the reduction in oxygen tension, and not the change in oxygen tension itself, which was responsible for the shift. The results with lactose and glycerol phenotypes of strain ML confirm the observation of Pudek and Bragg (1975) that alternative respiratory pathways exist in E.coli, but the terminal oxidase is not unique to any cell phenotype. This does not eliminate the possibility that an alternative, more efficient branch could be present in cells growing on lactose which could use a cytochrome of either the o- or d-type, depending on culture conditions.

Mitchell (1975; 1976) has recently modified his chemiosmotic hypothesis by introducing the concept of the protonmotive 'Q' cycle. This accounts for the lack of experimental evidence for a hydrogen carrier in the third loop of the mitochondrial electron transport chain and for the peculiar kinetic behaviour of mitochondrial b-type cytochromes. At present, there is little experimental evidence to support the operation of the Q cycle in bacteria, but recently Singh and Bragg (1976) have obtained evidence to suggest that the Q cycle may operate in E.coli. The protonmotive Q cycle would readily explain branching of the respiratory chain at the level of co-enzyme Q (Garland et al., 1975). It would therefore be worthy of consideration when examining apportionment and kinetics of electron transfer between branches terminating in cytochromes o and d in aerobically-grown E.coli (e.g. Pudek & Bragg, 1975; Haddock et al., 1976; this work).

Theoretical pathways for electron transfer incorporating the Q cycle have been applied to bacteria which have respiratory chains similar to mitochondria, in that they have 3 energy-conserving segments and cytochrome c (Garland et al., 1975; Fig. 46b) and to bacteria, such as E.coli which lack cytochrome c and have only 2 segments (Fig. 46c). In order to preserve the experimentally-observed stoichiometry of proton translocation in the latter case, it is necessary to presume that the cytochrome oxidase is situated on the external surface of the membrane. This is in contrast to the model without the Q cycle for electron transport of E.coli with 2 segments (Fig. 46a) which requires that the terminal oxidase be situated on the inner surface of the membrane. By shifting the location of the terminal oxidase to the inner surface of the membrane, it is possible theoretically to account for a high efficiency of energy conservation (Fig. 46d) with the same cytochrome complement as exists when two segments are observed.

The change in energy conservation by the electron transport chain from lower $\left\{\frac{H^+}{O} = 6\right\}$ efficiency, without the synthesis of a novel c-type cytochrome, can therefore be envisaged as occuring in two ways: 1) The electron transport chain with terminal oxidase fixed at the surface of the inner membrane could alter its proton-translocation stoichiometry by operating without $\left\{\frac{H^+}{O} = 4\right\}$ or with $\left\{\frac{H^+}{O} = 6\right\}$ the Q cycle. 2) If the use of the Q cycle is obligatory, different stoichiometries would be obtained by shifting the oxygen-binding site of the terminal oxidase from the outside $\left\{\frac{H^+}{O} = 4\right\}$ to the inside of the

Figure 46 THEORETICAL ELECTRON PATHWAYS IN BACTERIAL MEMBRANES

Fig. 46a *E.coli*; 2 coupling sites, without 'Q' cycle (Poole & Haddock, 1975).

Fig. 46b Bacteria with cytochrome c e.g. *P. denitrificans*; 3 coupling sites, with 'Q' cycle (Garland *et al.*, 1975).

Fig. 46c *E.coli*; 2 coupling sites, with 'Q' cycle (Garland *et al.*, 1975).

Fig. 46d *E.coli*; 3 coupling sites, with 'Q' cycle (this work).

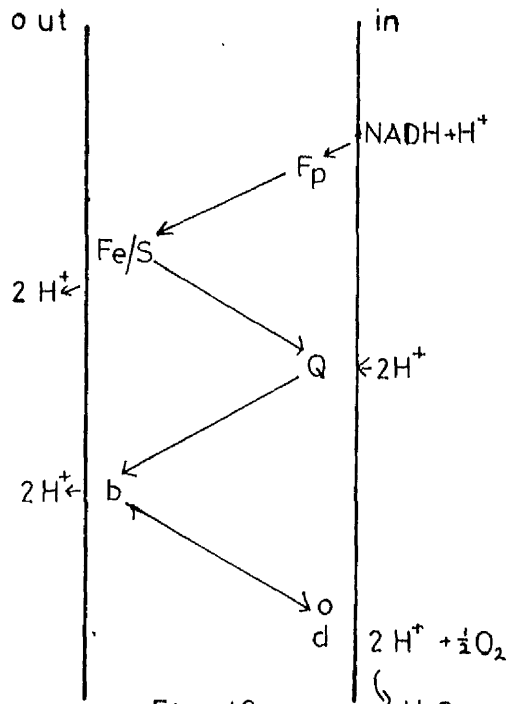


Fig. 46a

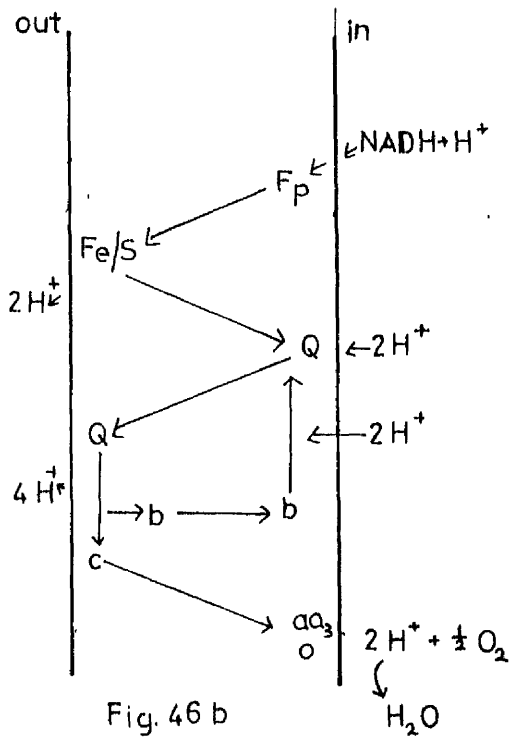


Fig. 46 b

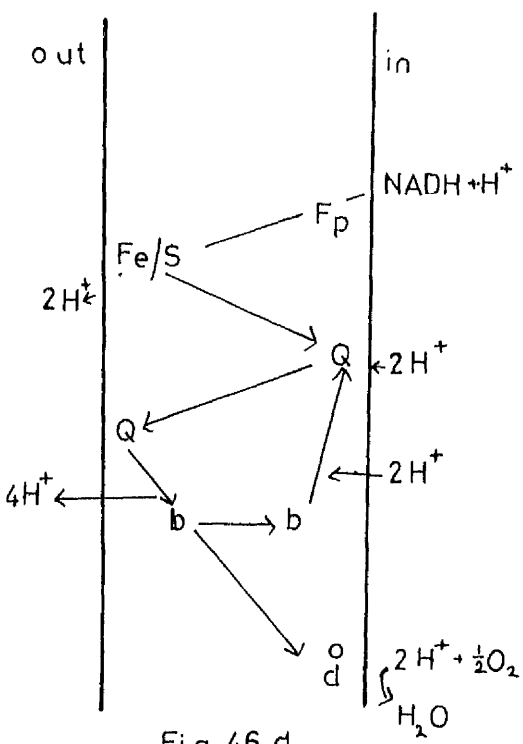


Fig. 46 d

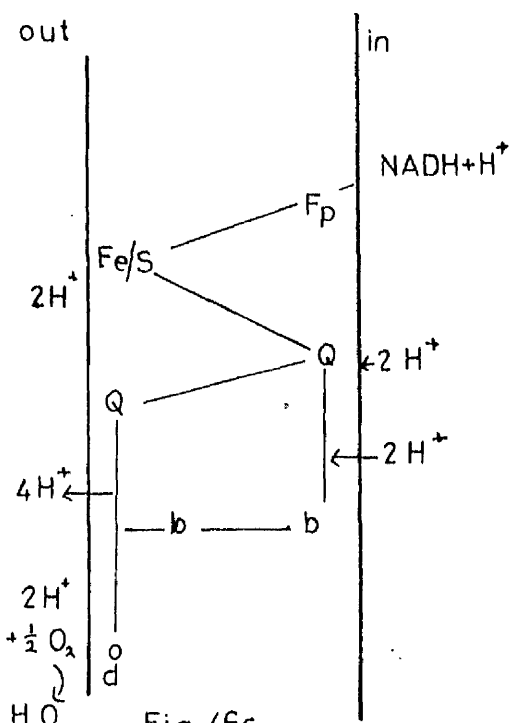


Fig. 46c

membrane. A direct test of this second possibility would be to determine the location of cytochrome oxidase on the membranes of different cell phenotypes. Preliminary experiments of this type have been done for the nitrate reductase of E.coli (Boxer & Clegg, 1975), but no result is available for the cytochrome oxidase of E.coli. Some terminal oxidases have been found to span the membrane (Boxer & Clegg, 1975; Eytan et al., 1975). If this were the case for the terminal oxidase of E.coli, a switch of the active site for oxygen from the outer membrane surface to the inner surface would formally increase the efficiency of energy conservation.

The results presented in this thesis form no more than a preliminary investigation of the relationship between energy coupling and active transport in E.coli. The observation was made, using growth and gas analysis data, of high efficiency of energy coupling when transporting carbon sources by proton-symport mechanisms, and was confirmed independently in proton-extrusion experiments. An attempt has been made, using simple techniques, to gain an insight into the composition of the electron transport chains of cells growing on carbon sources such as lactose. The mechanism whereby the efficiency of energy conservation of E.coli may be enhanced, whether by synthesis of a novel cytochrome c-containing pathway, or by use of the proton-motive Q cycle remains open to further investigation. It is thought to be the unidirectional flux of substrate through the proton-symporting active transport system which triggers the enhanced efficiency of energy conservation (4.3). This could occur by some mutual interaction

of the transport system with the electron transport chain. In this context, it is interesting that the redox model of Kaback (Kaback & Hong, 1973), now refuted, had experimental evidence that the lac carrier of E.coli vesicles was closely related with cytochrome b, which would be a component of the Q cycle (Fig. 46d).

Future work would attempt to identify and locate the electron carriers within the membrane, and investigate possible ways in which electron transport and active transport could interact with each other at the molecular level.

APPENDIX 1

The use of arithmetic culture to obtain yield and maintenance values

1.1 Introduction

Maintenance energy is the fraction of energy produced by the cell which does not result in the synthesis of new cell material. Pirt (1965) formalised the method of evaluating the fraction of substrate utilisation which was used for maintenance. By assuming that the maintenance requirement of a culture would be independent of its growth rate, he derived the relation:

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y^G} \quad - (1)$$

where Y = growth yield ($g.mol^{-1}$)

Y^G = max growth yield ($g.mol^{-1}$)

m = maintenance requirement ($mol.g.^{-1}h^{-1}$)

μ = specific growth rate (h^{-1})

Therefore as the rate of substrate supply to the culture is varied (varying μ), the apparent growth yield (Y) will change. A plot of Y^{-1} against μ^{-1} will have gradient m and intercept $(Y^G)^{-1}$. The linearity of experimentally-derived double reciprocal plots (Y^{-1}, μ^{-1}) led Pirt to believe that his assumption (that maintenance was independent of growth rate) was justified. This assumption has been justly criticised by Tempest and Neijssel (1976), who claim that a linear double reciprocal plot may also mean that maintenance is linearly dependent on growth rate (see also Discussion, 1.1).

It is normal practice to evaluate Y^G and m by growing cells in continuous culture (Methods 6.2.1) over a range

of growth rates and expressing the results as double reciprocal plots. However the use of continuous culture in this context has many disadvantages:

When the maintenance coefficient, m, is small there will be little change in Y over a wide range of growth rates, μ (Equation (1)). Results will therefore tend to be in-accurate.

Each experimental point requires that the cells be in steady state. For μ = 0.05 (culture volume 600 ml) this would take over a week, making the technique costly in time and in culture medium.

The frequent replenishment of culture medium which is required for long runs increases the possibility of contaminating the culture.

Growth of a culture for long extended periods can lead to selection of mutants, which will complicate the results.

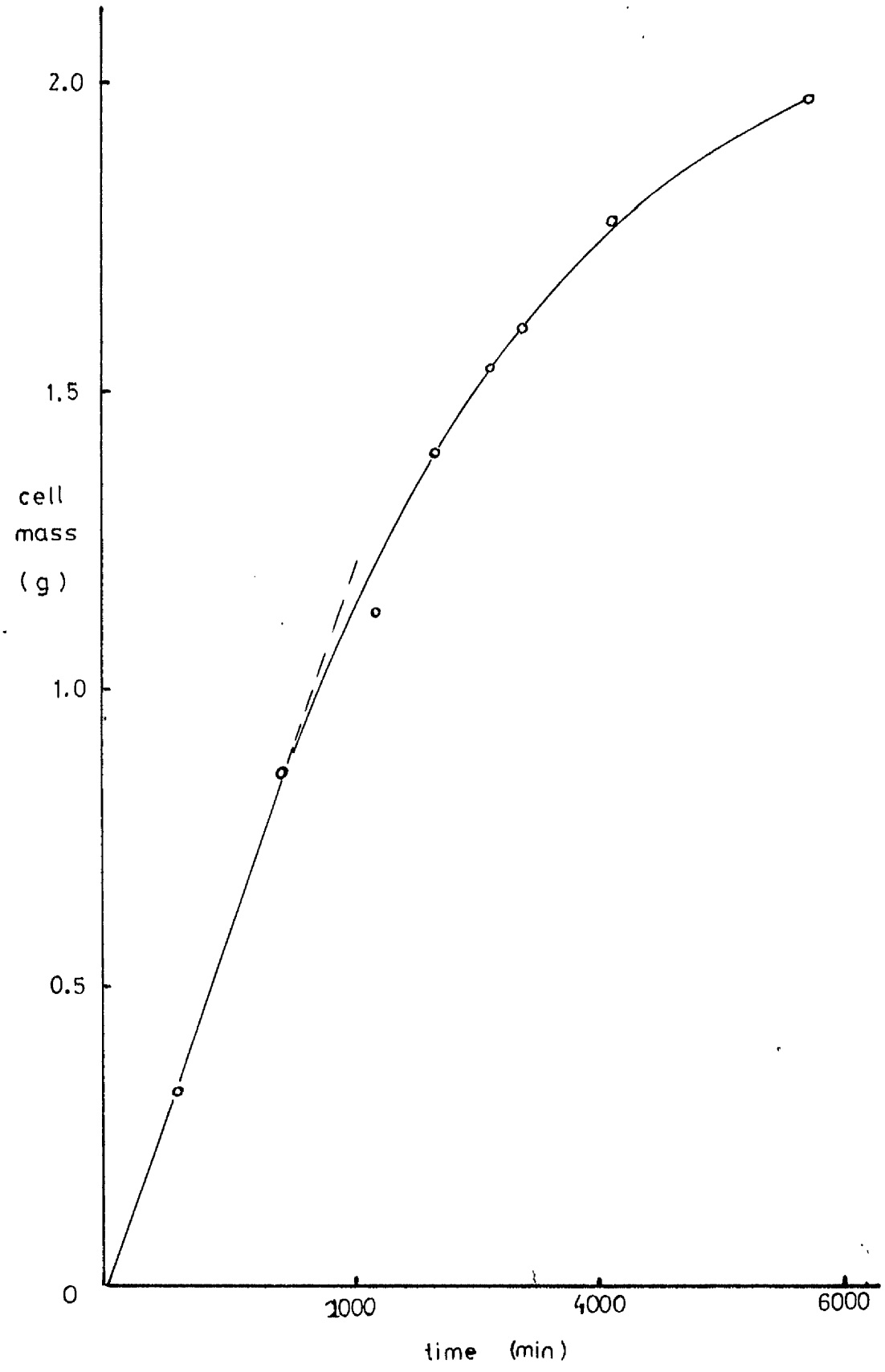
Arithmetic culture was used to avoid these drawbacks.

1.2 Method

In arithmetic culture, carbon is fed at a fixed rate to a culture grown and maintained in the apparatus of Methods 6.3. Initially, maintenance is small relative to the amount of energy going to growth so that cell growth appears to be linear with time (Fig. 1.1). In fact, the deviation from linearity falls within the experimental error of measurement. As the total mass of the culture increases, the maintenance requirement of the culture also increases. This higher demand for maintenance energy can only be satisfied (from the fixed carbon input to the system) by decreasing the proportion of energy made

Figure 1.1 GROWTH OF E.coli '51' ON LACTOSE IN ARITHMETIC CULTURE

E.coli '51' was grown on lactose in arithmetic culture (Methods 6.3). Growth (Methods 7) was followed with time.


2.0
1.5
1.0
0.5
0
cell
mass
(g)
1000
4000
6000
time (min)

available for growth, thus decreasing the rate of increase in cell mass (Fig. 1.1). Analysis of the experimental values for carbon input, cell mass, and gas exchange allow calculation of the yield and maintenance values.

The method of calculation, which has been described previously (Wallace, 1975) is summarised here for completeness.

1.2.1 Calculation of carbon yield and maintenance values

Equation (1), $\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y^G}$, may be rewritten.

Since $Y^G = \frac{dx}{dt} \cdot \frac{dt}{ds}$ (where $\frac{dx}{dt}$ = rate of cell mass synthesis,

$\frac{ds}{dt}$ = rate of substrate utilisation)

and $\mu = \frac{dx}{dt} \cdot \frac{1}{X}$ (where X = cell mass),

it follows that

$$\frac{ds}{dt} \cdot \frac{dt}{dx} = m.X.\frac{dt}{dx} + \frac{1}{Y^G} \quad -(2)$$

Multiplying (2) by $\frac{dx}{dt}$, $$\frac{ds}{dt} = m.X + \frac{dx}{dt} \cdot \frac{1}{Y^G} \quad -(3)$$

Rearranging (3), $$\frac{dx}{dt} = -Y^G.m.X + Y^G \frac{ds}{dt} \quad -(4)$$

In equation (4), $\frac{dx}{dt}$ and X are variables, and $\frac{ds}{dt}$ is held constant experimentally (Methods 6.3) and is known. If Y^G and m are constants, they can be determined from the intercept and gradient of a plot of $\frac{dx}{dt}$ against X (Fig. 1.2).

X may be determined experimentally (Dev. Methods, 2),

Figure 1.2 CARBON YIELD AND MAINTENANCE VALUES

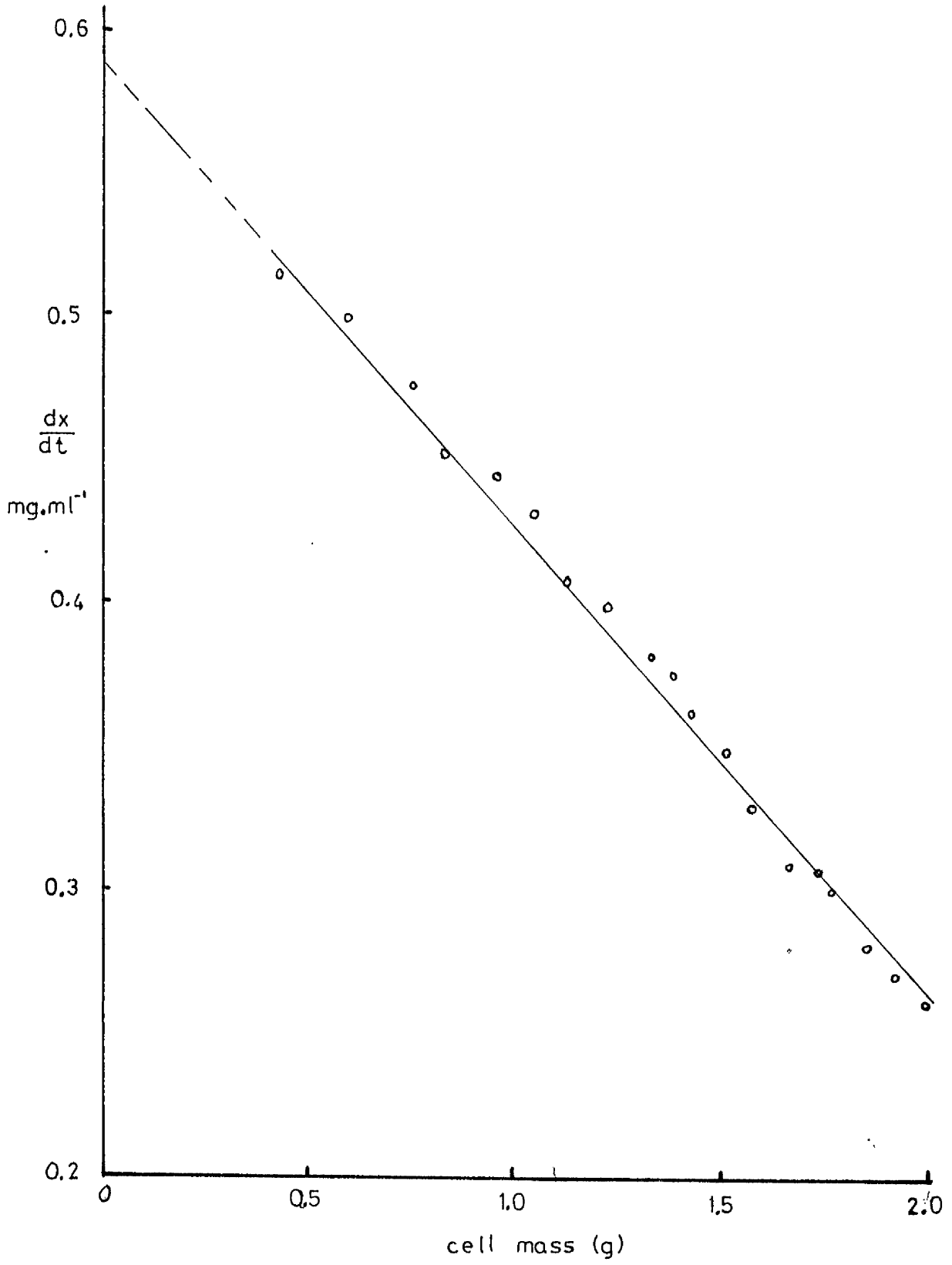
In arithmetic culture, the relation:

$\frac{dx}{dt} = -Y^G.m.X + Y^G \circ \frac{ds}{dt}$ holds, (Equation 4, (1.2.1)).

A plot of $\frac{dx}{dt}$ against X will have intercept = $Y^G \circ \frac{ds}{dt}$, and

gradient = $-Y^G.m$.

From gas exchange measurements obtained during the growth of E.coli '51' on lactose in arithmetic culture (Fig. 1.1), $\frac{dx}{dt}$ could be calculated (1.2.1), for any given cell mass (Xg). The curve was linear (r = 0.996) with intercept of 0.589 $mg.min^{-1}$. Since $\frac{ds}{dt}$ was 2.583 $\mu mol.min^{-1}$, this gave $Y^G = \frac{0.589}{2.583} \times 10^3 = 228$ $g.mol^{-1}$. The gradient of the line was -156 $\mu g.g.^{-1}min^{-1}$. Since $Y^G = 228$ $g.mol^{-1}$, this gave $m = \frac{156}{228} = 0.684$ $\mu mol.g.^{-1}min^{-1}$ or 41 $\mu mol.g.^{-1}h^{-1}$.


0.6
0.5
dx/dt
mg.ml⁻¹
0.4
0.3
0.2
0
0.5
1.0
1.5
2.0
cell mass (g)

and $\frac{dx}{dt}$ may be calculated from a differential carbon balance:

Carbon entering the growth vessel $\frac{(ds)}{(dt)}$ goes to either cells $\frac{(dx)}{(dt)}$ or CO_2 $\frac{(dCO_2)}{(dt)}$ i.e. $\frac{ds}{dt} = \frac{dx}{dt} + \frac{dCO_2}{dt}$.

CO_2 production is monitored (Methods, 8), therefore $\frac{dx}{dt}$ can be calculated by difference of $\frac{dCO_2}{dt}$ from $\frac{ds}{dt}$, knowing that the carbon content of cells is 44.6% by weight (Wallace, 1975). Total carbon balances in all experiments were close to 100%, indicating that no excretion products were found in arithmetic culture.

1.2.2 Calculation of oxygen yield and maintenance values

Unlike carbon utilisation, the rate of oxygen utilisation is not constant with time. Equation (1) may be written in terms of oxygen, (5).

$$\frac{1}{Y_{O_2}} = \frac{m_{O_2}}{\mu} + \frac{1}{Y^G_{O_2}} \quad -(5)$$

Multiplying by μ, $\frac{\mu}{Y_{O_2}} = m_{O_2} + \frac{\mu}{Y^G_{O_2}}$

Since $\frac{\mu}{Y_{O_2}} = Q_{O_2}$ (the specific rate of oxygen utilisation), a plot of Q_{O_2} against μ will give $\frac{1}{Y^G_{O_2}}$ as gradient and m_{O_2} as intercept, if maintenance is independent of growth rate (Fig. 1.3). Q_{O_2} may be determined by dividing the rate of oxygen uptake, $\frac{dO_2}{dt}$, (Methods, 8) by the cell mass (Dev. Methods, 2). μ is determined by

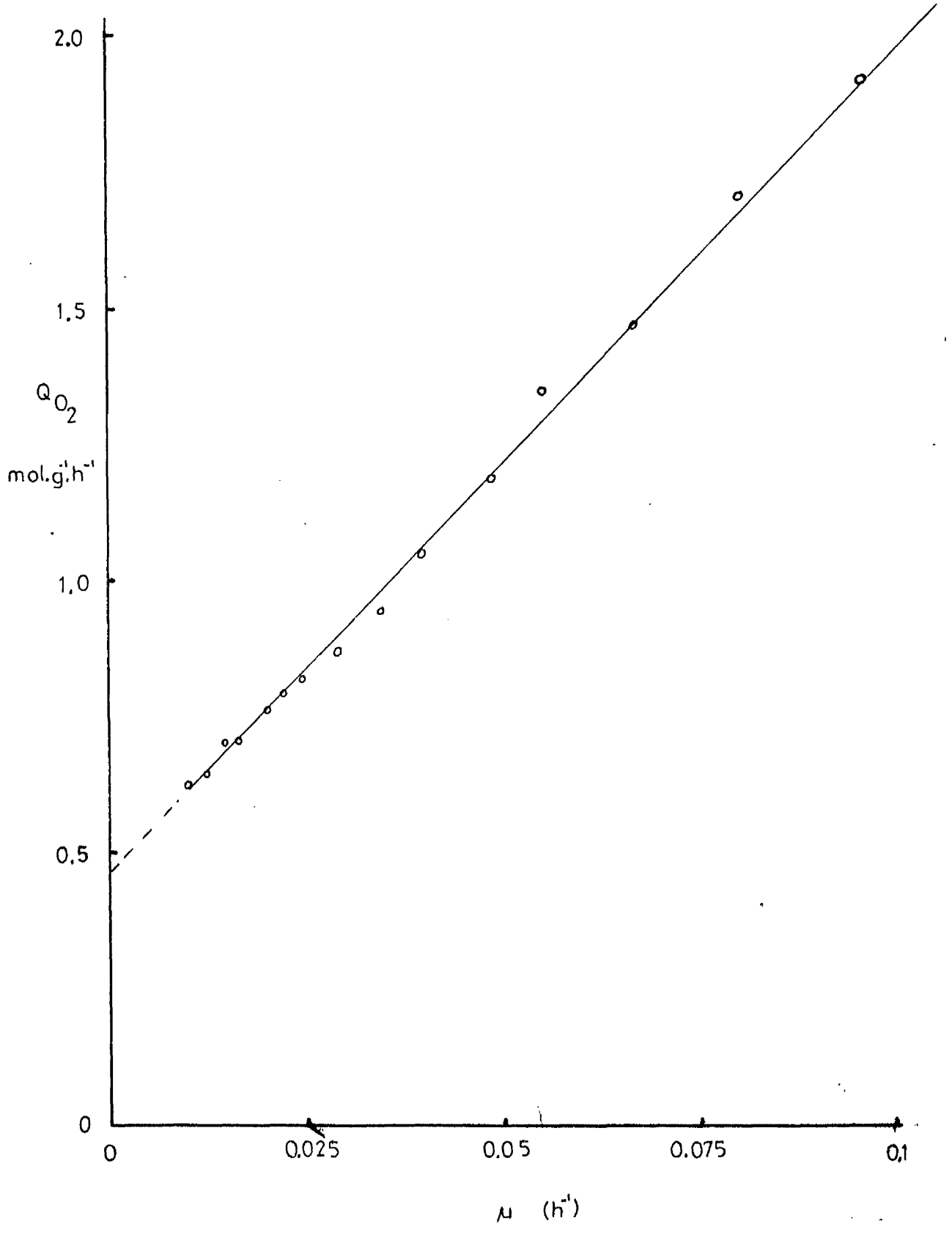
Figure 1.3 OXYGEN YIELD AND MAINTENANCE VALUES

The specific rate of oxygen utilisation, Q_{O_2}, was plotted against growth rate, μ, (1.2.2), for E.coli '51' growing on lactose in arithmetic culture (Fig. 1.1).

Maintenance and yield values can be obtained using Equation (6) of (1.2.2).

$$Q_{O_2} = m_{O_2} + \frac{\mu}{Y^G_{O_2}} .$$

The curve was linear ($r = 0.984$) with intercept, m_{O_2}, = 465 μmol.g.$^{-1}$h^{-1}. The gradient of the curve was 16.13 μmol.g^{-1}. This corresponded to $(Y^G_{O_2})^{-1}$, giving $Y^G_{O_2}$ as 62 g.mol^{-1}.


2.0
1.5
Q_{O_2}
mol.g⁻¹.h⁻¹
1.0
0.5
0
0
0.025
0.05
0.075
0.1
μ (h⁻¹)

dividing $\frac{dx}{dt}$ (1.2.1) by the cell mass.

1.3 Discussion

As cells grow in arithmetic culture, the maintenance requirement of the culture changes from a small to a large fraction of the (limiting) carbon supplied. This contrasts with continuous culture where maintenance energy is only a small fraction of the substrate utilisation of the culture. For example in the specimen calculation for arithmetic culture (1.2), the derived maintenance coefficients were: $m = 41\ \mu mol.g^{-1}.h^{-1}$ (Fig. 1.2), $m_{O_2} = 465\ \mu mol.g^{-1}.h^{-1}$ (Fig. 1.3) with carbon supplied to the culture at a rate of 2.583 μmol lactose.min^{-1} (155 μmol.h^{-1}). Early in the experiment at t = 200 min, cell mass was 0.1 g (Fig. 1.1), so that only 0.1 x 41 = 4.1 μmol lactose.h^{-1} was used for maintenance. This represents $\frac{4.1}{155}$ = 2.6% of the lactose entering the flask. Later (t = 5000 min, cell mass = 1.92 g), maintenance will form $\frac{1.92 \times 41}{155}$ = 51% of the substrate utilisation by the culture. Since the maintenance oxygen requirement, m_{O_2}, is consistent with the oxidation of the carbon requirement for maintenance, m (i.e. $m_{O_2} = 12\ m$), the increase in carbon used for maintenance will be accompanied by an increase in oxygen uptake. This, together with CO_2 output, is readily measured (Methods, 8). The large change in maintenance requirement of the culture therefore affords more accurate determination of the maintenance coefficients and of the maximum growth yields.

Each experiment is complete within 4 days (Fig. 1.1), which is much less than is required of conventional continuous culture especially at the growth rates used ($\mu < 0.1$). The short duration of the experiment and the fact that no cells are pumped from the flask minimise the possibility of enriching mutants, which would interfere with results.

However, since no cells are pumped from the flask, it is impossible to study the effects of changes in nutrient limitation - cells which are synthesised during the first few hours of the experiment are still present and contributing to measurements at the end. If a change in nutrient limitation were made midway through an experiment, cells synthesised under the initial limitation would be at least 50% of the cell material exchanging gas at the end. This problem did not arise for the experiments done during this work, since they were all of the carbon-limitation type.

APPENDIX 2

THE CALCULATION OF P/O RATIOS FROM MOLAR GROWTH YIELDS

2.1 General considerations

Because bacteria are cryptic to adenine nucleotides, it has proved impossible to measure $\frac{P}{O}$ ratios of bacteria directly using the technique which was developed for mitochondria. An alternative approach is to derive $\frac{P}{O}$ ratios indirectly from molar growth yields.

The energy required by the cell, in terms of ATP, to take preformed monomers supplied in the growth medium and to polymerise them into the structural macromolecules which constitute the cell is constant for any given cellular composition, and is given by $(Y_{ATP})^{-1}$.

Y_{ATP} is the yield of cells obtained from preformed monomers per mol of ATP made available during growth. It may be determined experimentally during anaerobic growth in complex media which are assumed to contain all the monomers required for growth. A single carbon source is added and fermented by the microorganism through defined pathways to produce a known amount of ATP. The majority of the ATP produced during the fermentation will be used in the polymerisation of the nutrients which are supplied: little ATP will be required for the biosynthesis of the precursors which form cells. Bauchop and Elsden (1960) found a mean value of 10.5 g cells synthesised per mol of ATP for a selection of microorganisms grown under these conditions (see Introduction 3.1, for a discussion of Y_{ATP}).

The macromolecules of cells must still be polymerised from monomers during aerobic growth in simple medium on a single carbon source. The energy requirement will be the same as that for polymerisation of monomers under anaerobic conditions, $(Y_{ATP})^{-1}$. However, there are other factors which contribute to the ATP requirement during aerobic growth: the carbon skeletons of the monomers must be synthesised from the single carbon source via the amphibolic and anabolic pathways which rearrange the carbon atoms of the carbon source into the monomers, producing and consuming ATP and NADPH and producing NADH. Some of the carbon source is also combusted to CO_2, and the oxidation of reducing equivalents thus generated yields ATP. The efficiency with which the apparatus for oxidative phosphorylation traps the free energy released from electrons as they pass from reducing equivalents to oxygen is expressed as the $\frac{P}{O}$ ratio.

$\frac{P}{O}$ ratios may be derived from the maximum molar growth yields (Y^G, $Y^G_{O_2}$), a value for Y_{ATP}, and the composition of cells expressed in terms of the monomers which, when polymerised, will form the cellular macromolecules.

The utilisation of carbon source by the cells (equivalent to the reciprocal of the growth yield, Y^G) may be followed through the metabolic pathways which carbon atoms take to form new cells and CO_2.

1) The composition of E.coli has been analysed in terms of its monomer components (Morowitz, 1968). The fraction of carbon source utilised for synthesis of these monomers and the net utilisation of ATP and reducing equivalents

involved may be calculated.

2) The net production of reducing equivalents and of ATP by substrate phosphorylation may also be calculated from the remaining fraction of carbon source which is oxidised to CO_2.

3) The amount of ATP consumed in the polymerisation of monomers to the macromolecules of the cell under aerobic conditions is assumed to be equivalent to the value derived for anaerobic growth, $(Y_{ATP})^{-1}$.

4) The amount of ATP produced by oxidative phosphorylation is the amount by which the requirement for ATP $(Y_{ATP})^{-1}$ exceeds the net production and utilisation at the substrate level.

5) The $\frac{P}{O}$ ratio is calculated by dividing the ATP synthesised during oxidative phosphorylation by the oxygen utilisation $(Y^{G}_{O_2})^{-1}$.

6) The $\frac{P}{2e}$ ratio may also be calculated as the ATP synthesised by oxidative phosphorylation, divided by the number of reducing equivalents made available from the utilisation of carbon source.

The approach was used by Stouthamer (1973) to obtain a theoretical value for Y^{MAX}_{ATP} for cells growing in complex medium, and by Wallace (1975) for growth of E.coli. The calculations are modified here in an attempt to account for the energy requirement of transport, and extended to include the wider range of single carbon sources used during this work.

2.2 Method

The pathways which carbon atoms take, during growth,

to form new cell material and CO_2 were identified by referring to metabolic pathways described in standard texts (Mandelstam & McQuillen, 1973: Dagley & Nicholson, 1970). The carbon flow and production/consumption of ATP were estimated at each level of intermediary metabolism and compounded to calculate the ATP made available by substrate phosphorylation. For consistency, production of ATP was designated '-', and consumption of ATP was designated '+'. ATP produced by oxidative phosphorylation was calculated by difference from Y_{ATP}^{MAX} (assumed to be 10.5 $g.mol.^{-1}$) and related to experimentally-derived oxygen uptake to give the $\frac{P}{O}$ ratio. The production/consumption of reducing equivalents during growth was also estimated to give derived $\frac{P}{2e}$ ratios.

2.2.1 Conversion of carbon source to cell monomers

2.2.1.1 Conversion of amphibolic precursors to monomers

The cell composition, in terms of monomers, was taken to be that of Morowitz (1968), shown in Fig. 2.1, col. A. The amounts of the eight amphibolic precursors necessary to give the monomer composition according to Morowitz were estimated by referring to standard texts (Fig. 2.2). Production and consumption of ATP (Fig. 2.1, col. D), and reducing equivalents (Fig. 2.3) were estimated and summed for the conversion of amphibolic precursors to cell monomers. These series of reactions consumed ATP (113.04×10^{-4} $mol.g^{-1}$) and NADPH (137.67×10^{-4} $mol.g^{-1}$), but produced NADH (31.25×10^{-4} $mol.g^{-1}$).

2.2.1.2 Conversion of growth substrate to amphibolic precursors

The amphibolic precursors (Fig. 2.2) are produced by

catabolism of the carbon source used for growth. The schemes of reactions which carbon atoms must pass through to form the amphibolic precursors are shown for each growth substrate used in arithmetic culture (Figs. 2.4-2.8).

The production/consumption of ATP and reducing equivalents during conversion of carbon source to amphibolic precursors were calculated (Fig. 2.9, cols. A).

2.2.1.3 Summary: conversion of carbon source to cell monomers

The involvement of ATP and reducing equivalents in the conversion of carbon source to cell monomers was calculated (Fig. 2.9, cols. C) as the sums of the contributions determined in (2.2.1.1) and (2.2.1.2).

There was a net consumption of ATP and NADPH, with production of NADH, for the conversion of each growth substrate to cell monomers. Conversion of glycerol to cell monomers also produced $FADH_2$.

2.2.2 Contribution from the fraction of carbon utilisation which is fully oxidised

The amount of each carbon source available for complete oxidation was calculated as the difference between the total utilisation of carbon source and the requirement for carbon source by biosynthesis (Fig. 2.10).

The production of ATP and reduced nucleotides from this fraction was calculated, for complete oxidation of the carbon source (Fig. 2.11).

2.2.3 Calculation of P/O ratio

The ATP produced by oxidative phosphorylation (Fig. 2.12, col. D) was calculated as the quantity necessary to meet the demand for biosynthesis, accounting

for ATP production and consumption from other sources (Fig. 2.12, cols. A-C).

The $\frac{P}{O}$ ratio could then be calculated as the ATP produced by oxidative phosphorylation, divided by the experimentally-derived oxygen uptake.

2.2.4 Calculation of P/2e ratio

It is possible to calculate $\frac{P}{2e}$ ratios from the data collected during calculation of the $\frac{P}{O}$ ratios.

The amounts of $FADH_2$ and NADH produced, and of NADPH consumed, during growth were calculated (Fig. 2.13, cols. C). A single 'normalised' value for the amount of reducing power available for electron transport (Fig. 2.13, col. D) was calculated in terms of NADH. It was assumed that NADH and NADPH were energetically equivalent, and that electrons from $FADH_2$ passed through one energy-conserving segment of the electron transport chain when the $\frac{P}{O}$ was 2 and two segments when the $\frac{P}{O}$ was 3. The $\frac{P}{2e}$ ratio (Fig. 2.13, col. F) was derived by dividing the ATP made available by oxidative phosphorylation by the net amount of reducing equivalents produced during metabolism.

2.3 Results

The derived $\frac{P}{O}$ ratios for growth of E.coli are summarised in Fig. 2.14, together with the calculated values for the $\frac{P}{2e}$ ratios.

In general, $\frac{P}{2e}$ ratios were higher than the corresponding $\frac{P}{O}$ ratios.

Figure 2.1 ATP INVOLVEMENT IN CONVERSION OF AMPHIBOLIC PRECURSORS TO MONOMERS

Col. A: The monomer composition of E.coli was taken to be that of Morowitz (1968).

Col. B: The amphibolic precursors, from which the monomers are derived, were identified by referring to standard texts (2.2). Abbreviations are those of Fig. 2.2

Col. C: The ATP involved in the conversion of each precursor to monomer was identified by referring to standard texts (2.2).

Col. D: The ATP requirement for the synthesis of the monomer composition of E.coli was calculated as the product of Cols. A and C.

It was assumed that:

(a) when ATP is hydrolysed to AMP and P.Pi, 2 ATP equivalents are used.

(b) the synthesis of carbomyl phosphate requires 2 ATP's.

(c) succinyl coA entering a metabolic sequence, to be subsequently released as succinate, costs the cell 1 ATP equivalent. This also applies to acetyl—coA/acetate.

(d) the use of the 'activated' forms of the precursors (G6P, α glycerophosphate) for carbohydrate and lipid biosynthesis saves the cell 1 ATP equivalent.

(e) in tryptophan biosynthesis, where serine is used and triose phosphate is produced, the regeneration of serine produces 1 ATP.

monomer	A composition $mol \times 10^{-4} \cdot g^{-1}$ cells	B precursors	C ATP $mol \cdot mol^{-1}$	D ATP requirement $mol \times 10^{-4} \cdot g^{-1}$
alanine	4.54	PYR	0	0
arginine	2.52	KG	6	15.12
aspartate	2.01	OAA	0	0
asparagine	1.01	OAA	2	2.02
cysteine	3.02	PG	4	12.08
glutamate	3.53	KG	0	0
glutamine	2.01	KG	1	2.01
glycine	4.03	PG	0	0
histidine	0.50	G6P	5	2.50
isoleucine	2.52	OAA,PYR	2	5.04
leucine	4.03	2PYR,ACCOA	0	0
lysine	4.03	OAA,PYR	2	8.06
methionine	2.01	PG,(CYS-PYR)	2	4.02
phenylalanine	1.51	G6P,2PEP	1	1.51
proline	2.52	KG	1	2.52
serine	3.02	PG	0	0
threonine	2.52	OAA	2	5.04
tryptophan	0.50	2G6P,PEP	4	2.00
tyrosine	1.01	G6P,2PEP	1	1.01
valine	3.02	2PYR	0	0
AMP	1.15	G6P,PG	9	10.35
dAMP	0.24	G6P,PG	9	2.16
GMP	1.15	G6P,PG	10	11.50
dGMP	0.24	G6P,PG	10	2.40
CMP	1.15	OAA,G6P	5	5.75
dCMP	0.24	OAA,G6P	5	1.20
UMP	1.15	OAA,G6P	4	4.60
dTMP	0.24	OAA,G6P	6	1.44
C16 fatty acid	2.80	8ACCOA	8	22.40
glycerophosphate	1.40	G3P	-1	-1.40
carbohydrate (as glucose)	10.26	G6P	-1	-10.26

Figure 2.2 AMOUNTS OF AMPHIBOLIC PRECURSORS NECESSARY TO MAKE CELLS

The monomer composition of 1 g. cells (Morowitz, 1968; Fig. 2.1, col. A) may be synthesised from eight amphibolic precursors. The amounts of each precursor necessary to make 1 g. cells were calculated by summing the amount of each monomer (Fig. 2.1, col. A), which arose from the amphibolic precursors (Fig. 2.1, col. B).

It was assumed that the sulphur atom of methionine came from cysteine. Therefore, one cysteine was consumed and one pyruvate produced for each methionine synthesised.

amphibolic precursor	abbreviation	requirement for biosynthesis molx10^{-4} g^{-1} cells
acetyl-coA	ACCOA	26.43
glucose 6-phosphate	G6P	19.84
glyceraldehyde 3-phosphate	G3P	1.40
∝ketoglutarate	KG	10.58
oxalacetate	OAA	19.66
phosphoenolpyruvate	PEP	5.54
3-phosphoglycerate	PG	14.86
pyruvate	PYR	23.18

Figure 2.3 INVOLVEMENT OF REDUCING EQUIVALENTS DURING CONVERSION OF AMPHIBOLIC PRECURSORS TO MONOMERS

Col. A: The monomer composition of E.coli was taken to be that of Morowitz (1968).

Col. B: The consumption of NADH during the synthesis of each monomer from its precursor was identified by referring to standard texts (2.2).

Col. C: The consumption of NADH for conversion of amphibolic precursors to the monomer composition of E.coli was calculated as the product of Cols. A and B.

Col. D: The consumption of NADPH during the synthesis of each monomer from its precursor was identified by referring to standard texts (2.2).

Col. E: The consumption of NADPH for conversion of amphibolic precursors to the monomer composition of E.coli was calculated as the product of cols. A and D.

It was assumed that:

(a) NH_3 enters cellular metabolism via glutamate dehydrogenase (E.C. 1.1.1.4), which is NADP-linked. Transamination reactions will cost one equivalent of NADPH.

(b) in purine and arginine biosynthesis, where an 'N' group is donated from aspartate (producing fumarate), one equivalent of NADH is produced and one equivalent of NADPH is consumed in conversion of the fumarate back to aspartate.

(c) in tryptrophan biosynthesis, where serine is used and triose phosphate is produced, the regeneration of serine produces two equivalents of NADH, and consumes one equivalent of NADPH.

	A monomers molx10^{-4} g^{-1} cells	B NADH consumed mol.mol^{-1}	C NADH molx10^{-4} g^{-1} cells	D NADPH consumed mol.mol^{-1}	E NADPH molx10^{-4} g^{-1} cells
alanine	4.54	0	0	1	4.54
arginine	2.52	-1	-2.52	4	10.08
aspartate	2.01	0	0	1	2.01
asparagine	1.01	0	0	1	1.01
cysteine	3.02	-1	-3.02	5	15.10
glutamate	3.53	0	0	1	3.53
glutamine	2.01	0	0	1	2.01
glycine	4.03	-1	-4.03	1	4.03
histidine	0.50	-3	-1.50	0	0
isoleucine	2.52	0	0	5	12.60
leucine	4.03	-1	-4.03	2	8.06
lysine	4.03	0	0	4	16.12
methionine	2.01	0	0	3	6.03
phenylalanine	1.51	0	0	-2	-3.02
proline	2.52	0	0	3	7.56
serine	3.02	-1	-3.02	1	3.02
threonine	2.52	0	0	3	7.56
tryptophan	0.50	-2	-1.0	-4	-2.00
tyrosine	1.01	-1	-2.02	-2	-2.02
valine	3.02	0	0	2	6.04
AMP	1.15	-3	-3.45	1	1.15
dAMP	0.24	-3	-0.72	2	0.48
GMP	1.15	-3	-3.45	0	0
dGMP	0.24	-3	-0.72	1	0.24
CMP	1.15	-1	-1.15	-1	-1.15
dCMP	0.24	-1	-0.24	0	0
UMP	1.15	-1	-1.15	-1	-1.15
dTMP	0.24	-1	-0.24	1	0.24
C16 fatty acid	2.80	0	0	14	39.20
glycerophosphate	1.40	0	0	1	1.40
carbohydrate (as glucose)	10.26	0	0	0	0

Figure 2.4 CONVERSION OF GLUCOSE TO AMPHIBOLIC PRECURSORS

The amounts of the amphibolic precursors necessary to form 1 g. cells had been calculated previously (Fig. 2.2). It was assumed that glucose was catabolised to the amphibolic precursors by the Embden-Meyerhof pathway. The amount of carbon (from glucose) passing through each stage of central metabolism was calculated, and the production/consumption of ATP and reducing equivalents was assessed.

It was assumed that PEP consumed in the transport of glucose by the phosphotransferase system was equivalent to ATP.

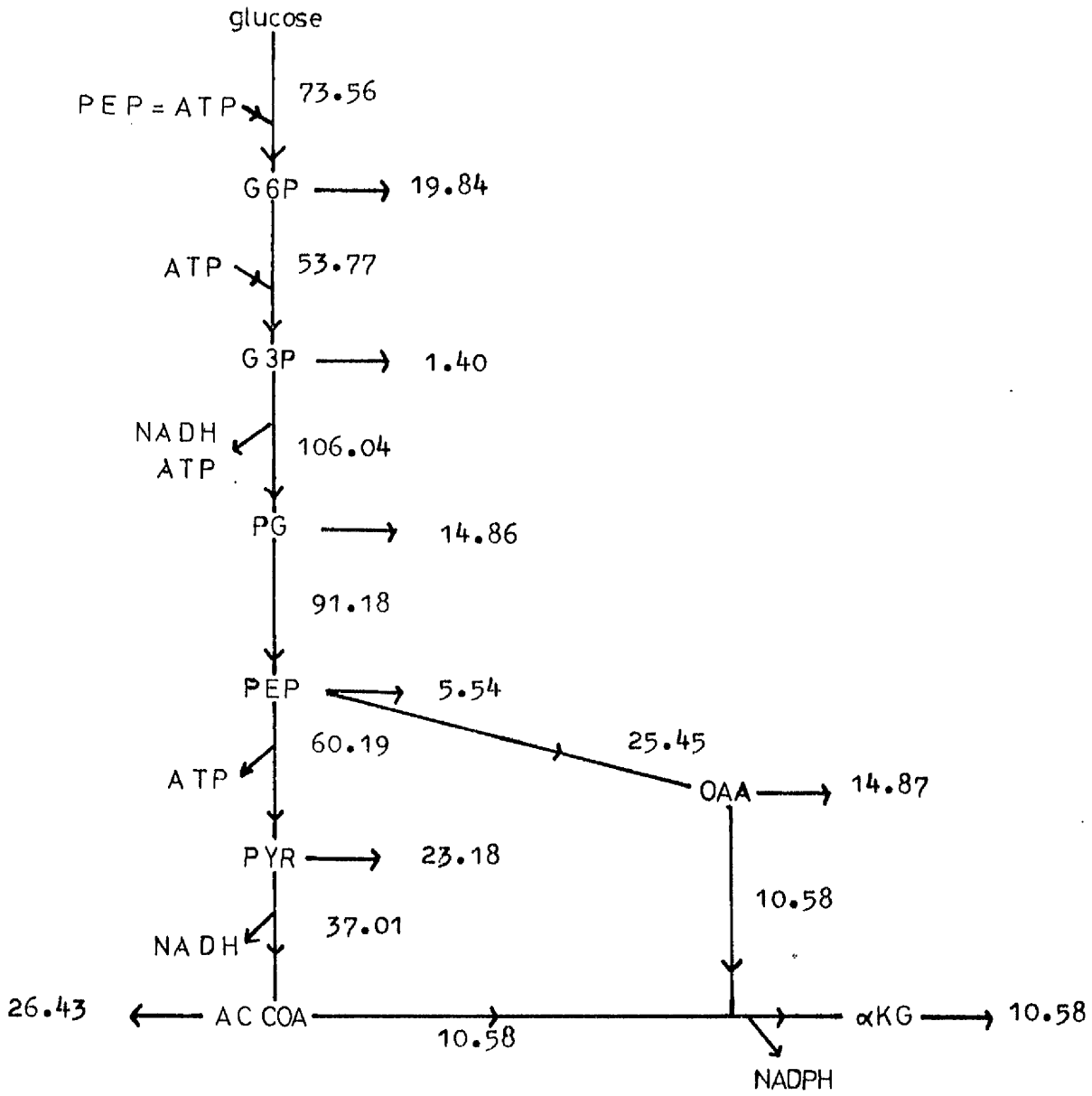

glucose
PEP = ATP
73.56
G6P
19.84
ATP
53.77
G3P
1.40
NADH
ATP
106.04
PG
14.86
91.18
PEP
5.54
60.19
25.45
ATP
OAA
14.87
PYR
23.18
10.58
NADH
37.01
26.43
AC COA
10.58
αKG
10.58
NADPH

Figure 2.5a CONVERSION OF GLUCOSE 6-PHOSPHATE TO AMPHIBOLIC PRECURSORS

Glucose 6-phosphate was assumed to be catabolised by the Embden-Meyerhof pathway. The pattern of catabolism was similar to that of glucose (Fig. 2.4), except in the early stages when glucose 6-phosphate is transported into the cell by a proton-symport mechanism (Essenberg & Kornberg, 1975). This was assumed to consume 1 H^+ per glucose 6-phosphate transported, and to be equivalent to 1/6 NADH, since the cells had $\frac{P}{O} = 3$.

Figure 2.5b CONVERSION OF GLYCEROL TO AMPHIBOLIC PRECURSORS

Glycerol was assumed to be catabolised to α glycerophosphate and subsequently, in a FAD-linked reaction (Results 5.2), to triose phosphate. The requirement for G6P was supplied from triosephosphate by gluconeogenesis; the remainder was metabolised from triose phosphate as for glucose.

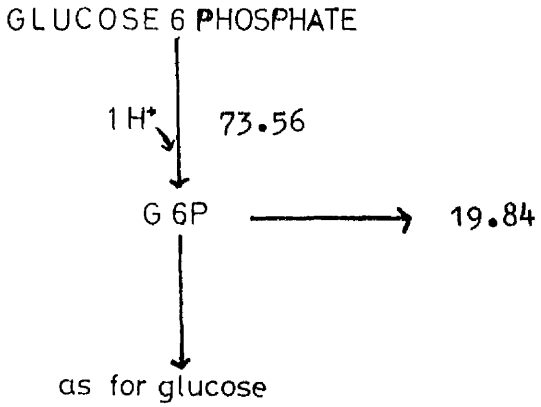


Fig. 2.5 a

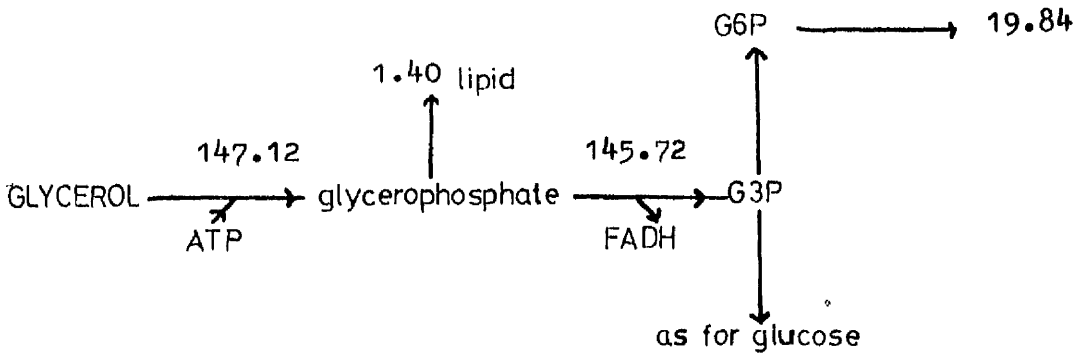


Fig. 2.5b

Figure 2.6a CONVERSION OF MANNITOL TO AMPHIBOLIC PRECURSORS

It was assumed that mannitol was taken up by the cell by a phosphotransferase system, and that mannitol 1-phosphate was dehydrogenated to fructose 6-phosphate (Solomon & Lin, 1972). Fructose 6-phosphate was metabolised by the Embden-Meyerhof pathway (Fig. 2.4).

The PEP consumed by the phosphotransferase system was assumed to be equivalent to ATP.

Figure 2.6b CONVERSION OF LACTOSE TO AMPHIBOLIC PRECURSORS

It was assumed that lactose was taken up by a proton-symport system (West & Mitchell, 1972), involving 1 H^+ per lactose transported. The lactose released by β-galactosidase was assumed to be metabolised by the Embden-Meyerhof pathway (Fig. 2.4).

The H^+ consumed in the transport of each lactose was assumed to be equivalent to $\frac{1}{6}$ NADH, since these cells had $\frac{P}{O} = 3$.

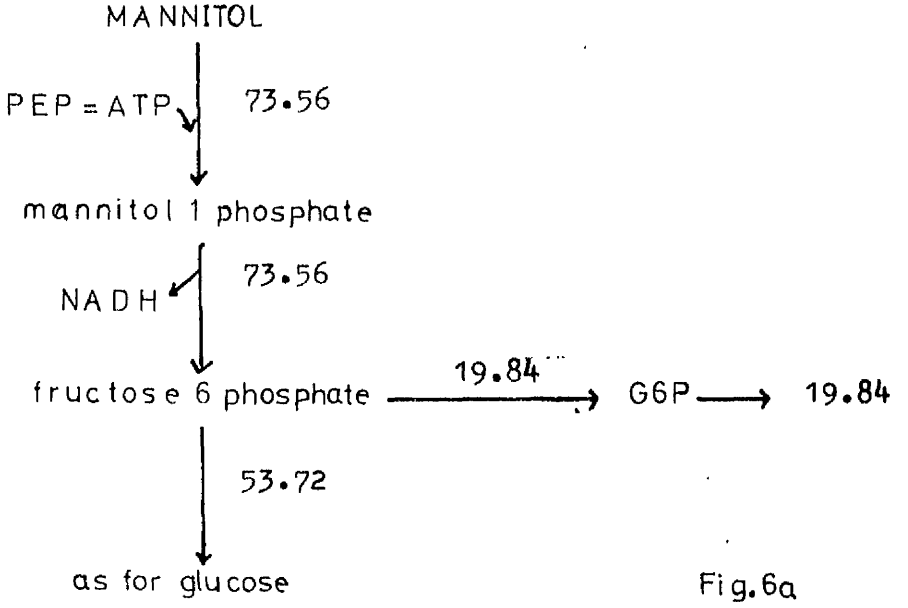

MANNITOL
PEP = ATP
73.56
mannitol 1 phosphate
NADH
73.56
fructose 6 phosphate
19.84
G6P
19.84
53.72
as for glucose

Fig.6a

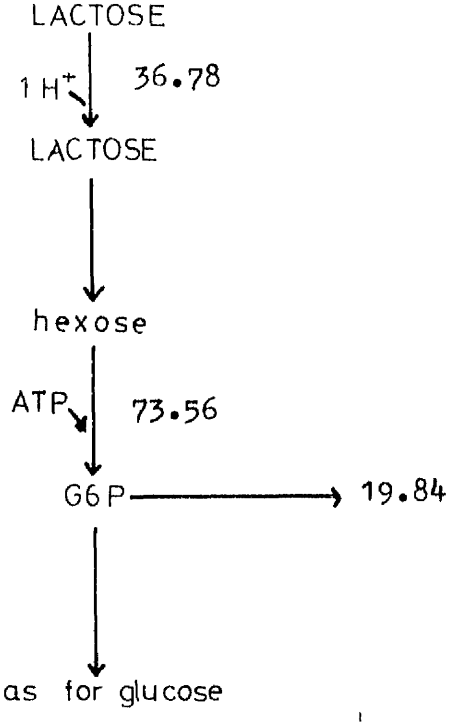

LACTOSE
1 H+
36.78
LACTOSE
hexose
ATP
73.56
G6P
19.84
as for glucose

Fig.6b

Figure 2.7a CONVERSION OF MALTOSE TO AMPHIBOLIC PRECURSORS

It was assumed that maltose transport involved a periplasmic binding protein system, coupled to ATP hydrolysis (Boos, 1974).

The glucose released by α glucosidase was assumed to be catabolised by the Embden-Meyerhof pathway.

Figure 2.7b CONVERSION OF GALACTOSE TO AMPHIBOLIC PRECURSORS

It was assumed that the mglP transport system (Henderson, et al., 1977) would be operating during growth on galactose in arithmetic culture. This system involves a periplasmic binding protein, coupled to ATP hydrolysis.

Galactose 1-phosphate was assumed to be catabolised via the Embden-Meyerhof pathway.

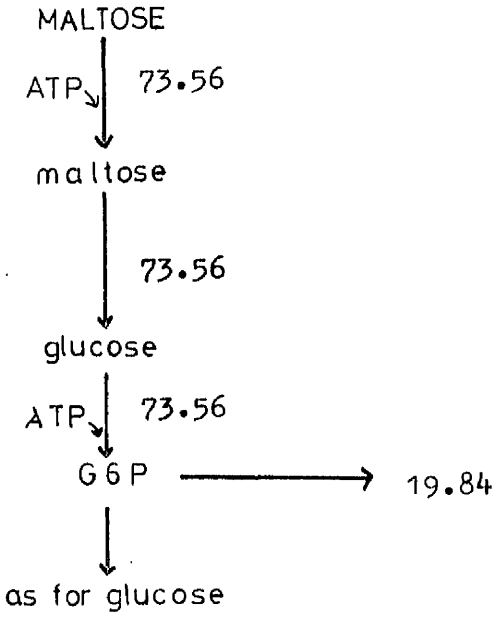

MALTOSE
ATP
73.56
maltose
73.56
glucose
ATP
73.56
G6P
19.84
as for glucose

Fig. 7a

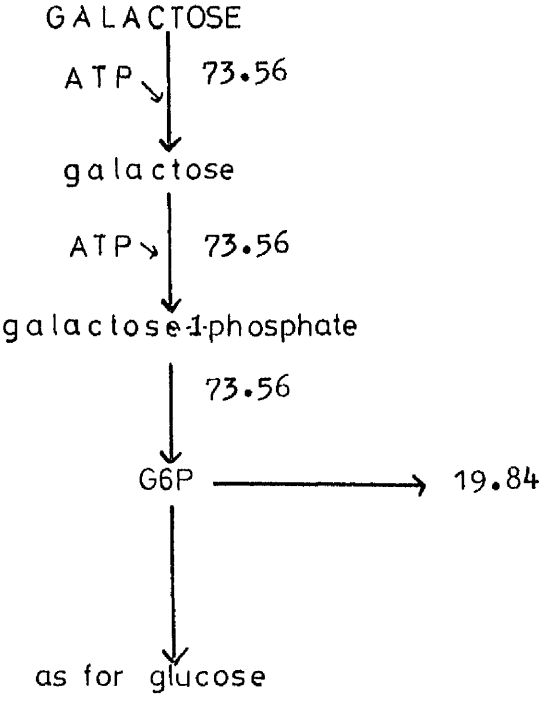

GALACTOSE
ATP
73.56
galactose
ATP
73.56
galactose-1-phosphate
73.56
G6P
19.84
as for glucose

Fig. 7b

Figure 2.8 CONVERSION OF GLUCONATE TO AMPHIBOLIC PRECURSORS

Gluconate was assumed to be transported by a proton-symport system (Robin & Kepes, 1973) with a stiochiometry of 1 H^+: gluconate transported. Once inside the cell, gluconate was assumed to be catabolised by the Entner-Doudoroff pathway (Kornberg & Soutar, 1973).

The use of 6-phosphogluconate as precursor in place of G6P reduced the production of NADPH by 19.84×10^{-4} mol. $(g.cells)^{-1}$.

It was assumed that each H^+ used in transport of gluconate was equivalent to $\frac{1}{6}$ NADH, since gluconate cells had a $\frac{P}{O}$ ratio of 3.

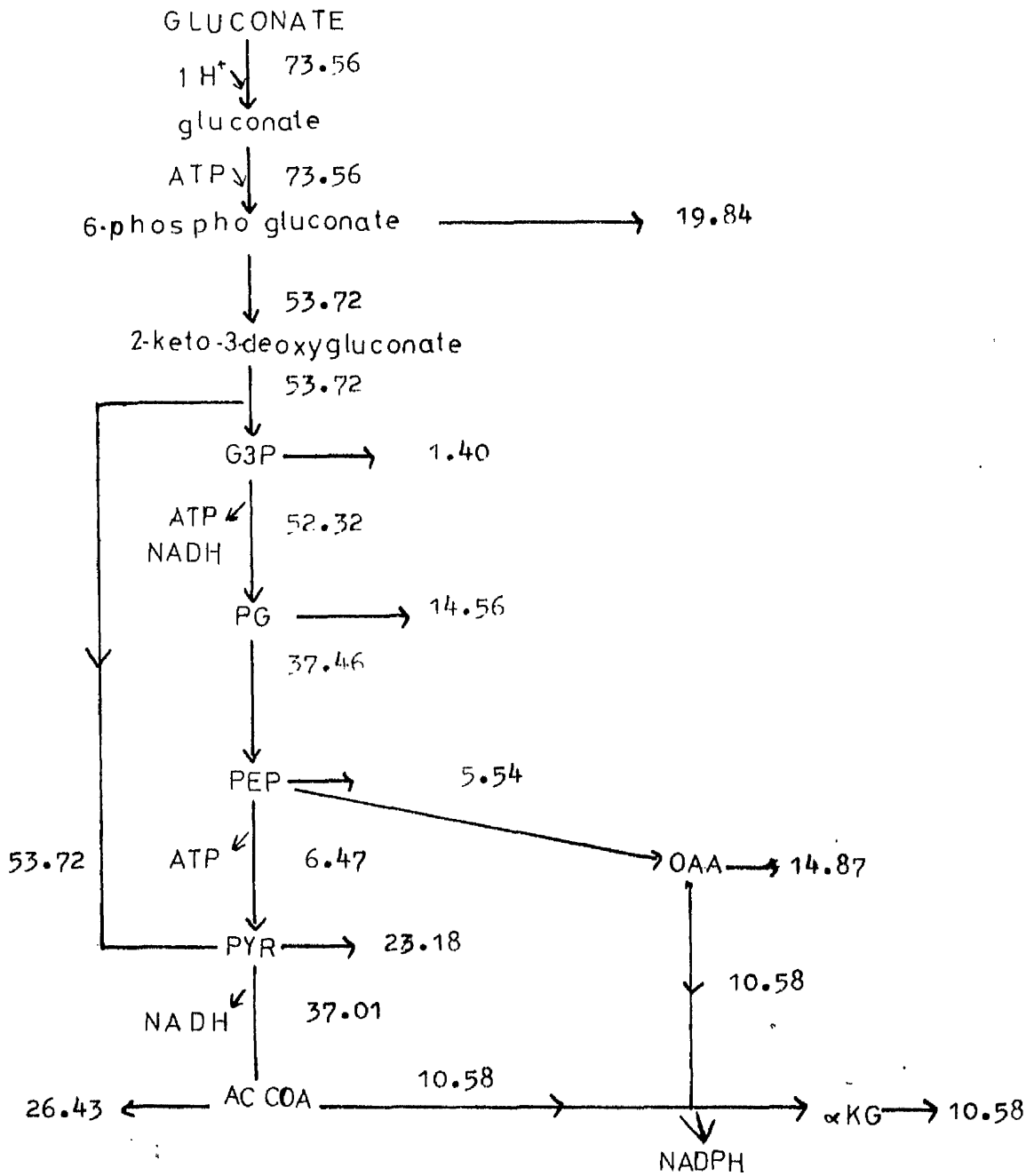

GLUCONATE
1 H+
73.56
gluconate
ATP
73.56
6-phospho gluconate
19.84
53.72
2-keto-3-deoxygluconate
53.72
G3P
1.40
ATP
NADH
52.32
PG
14.56
37.46
PEP
5.54
53.72
ATP
6.47
OAA
14.87
PYR
23.18
10.58
NADH
37.01
AC COA
10.58
26.43
∝KG
10.58
NADPH

Figure 2.9 CONVERSION OF CARBON SOURCE TO CELL MONOMERS

Cols. A: The involvement of ATP and reducing equivalents in the conversion of carbon source to amphibolic precursors depended on the growth substrate (Figs. 2.4-2.8).

Cols. B: The involvement of ATP and reducing equivalents in the conversion of amphibolic precursors to cell monomers was assumed to be constant (Figs. 2.1 and 2.3), irrespective of growth substrate.

Cols. C: The consumption of ATP, NADH, NADPH and FADH during conversion of carbon source to cell monomers was calculated by summing the respective values in cols. A and B.

All quantities are in the units: mol x 10^{-4} $(g.cells)^{-1}$.

carbon source	A carbon source → amphibolic precursors			B amphibolic precursors → cell monomers			C carbon source → cell monomers		
	ATP	NADH	NADPH	ATP	NADH	NADPH	ATP	NADH	NADPH
glucose	-39.3	-143.1	-10.6	113.0	-31.3	137.8	73.7	-174.3	127.1
glucose 6-phosphate	-112.5	-130.8	-10.6	113.0	-31.3	137.8	0.5	-162.0	127.1
glycerol	-60.2	-143.1	-10.6	113.0	-31.3	137.8	52.9	-174.3	127.1
mannitol	-38.9	-216.6	-10.6	113.0	-31.3	137.8	74.1	-247.9	127.1
lactose	-38.9	-136.9	-10.6	113.0	-31.3	137.8	74.1	-168.6	127.1
maltose	-2.2	-143.1	-10.6	113.0	-31.3	137.8	110.9	-174.3	127.1
galactose	34.6	-143.1	-10.6	113.0	-31.3	137.8	147.7	-174.3	127.1
gluconate	14.8	-77.1	9.3	113.0	-31.3	137.8	127.8	-108.3	146.9

Figure 2.10 THE FRACTION OF CARBON UTILISATION WHICH IS COMPLETELY OXIDISED

Col. A: The amount of carbon source required for synthesis of cell monomers was taken to be the quantity of growth substrate passing through the initial reaction in each of Figs. 2.4-2.8.

Col. B: The total utilisation of growth substrate by the cells was given by the reciprocal of the molar growth yield (Y^G; Fig. 19).

Col. C: The amount of carbon source available for complete oxidation was calculated as the difference of col. A from col. B.

Quantities are expressed as mol x 10^{-4} $(g.cells)^{-1}$.

carbon source	A C source for biosynthesis	B total C source utilisation $(Y^G)^{-1}$	C C source available for complete combustion
glucose	73.6	105.3	31.7
glucose 6-phosphate	73.6	88.2	14.6
glycerol	147.1	200.0	52.9
mannitol	73.6	93.5	19.9
lactose	36.8	43.9	7.1
maltose	36.8	49.0	12.2
galactose	73.6	102.1	28.5
gluconate	73.6	98.0	24.5

Figure 2.11 PRODUCTION OF ATP AND REDUCING EQUIVALENTS FROM THE FRACTION OF CARBON SOURCE WHICH IS FULLY OXIDISED

Col. A: The amounts of each carbon source which were fully oxidised had been determined previously (Fig. 2.10, col. C).

Col. B: The numbers of equivalents of ATP and reduced nucleotides produced from the oxidation of each equivalent of carbon source are shown in parentheses.
The production of ATP and reduced nucleotides was calculated, knowing the amount of carbon source available for complete combustion.

It was assumed that:

(a) All carbon sources, except gluconate, were catabolised by the Embden-Meyerhof pathway and TCA cycle.

(b) Gluconate was catabolised by the Entner-Doudoroff pathway (Kornberg & Soutar, 1973) and TCA cycle.

(c) GTP produced in the succinyl thiokinase reaction was equivalent to ATP.

(d) The H^+ required for transport of each gluconate, lactose or glucose 6-phosphate molecule was equivalent to $\frac{1}{6}$ NADH, since cells of those phenotypes had three energy-conserving sites.

Units are mol x 10^{-4} $(g.cells)^{-1}$.

carbon source	A carbon utilisation for complete combustion	B production of ATP and reducing equivalents during combustion of carbon source			
		ATP	NADH	NADPH	FADH
glucose	31.7	-127.0 (4)	-253.9 (8)	-68.5 (2)	-63.5 (2)
glucose 6-phosphate	14.6	-73.1 (5)	-114.5 (78)	-29.2 (2)	-29.2 (2)
glycerol	52.9	-105.8 (2)	-211.5 (4)	-52.9 (1)	-105.8 (2)
mannitol	19.9	-79.6 (4)	-179.0 (9)	-39.8 (2)	-39.8 (2)
lactose	7.1	-56.6 (8)	-111.9 (158)	-28.3 (4)	-28.3 (4)
maltose	12.2	-85.6 (7)	-195.7 (16)	-48.9 (4)	-48.9 (4)
galactose	28.5	-85.6 (3)	-228.3 (8)	-57.1 (2)	-57.1 (2)
gluconate	24.5	-73.4 (3)	-167.2 (68)	-48.9 (2)	-48.9 (2)

Figure 2.12 CALCULATION OF P/O RATIO

Col. A: The amount of ATP required to synthesise cells from their monomers was given as $(Y_{ATP}^{MAX})^{-1}$.

Col. B: The ATP consumed during conversion of carbon source to monomers had been calculated previously (Fig. 2.9, Col. C).

Col. C: The ATP produced during complete oxidation of carbon source had been calculated previously (Fig. 2.11).

Col. D: The ATP produced by oxidative phosphorylation to meet the cellular demand was calculated from the sums of Cols. A, B and C.

Col. E: The oxygen utilisation was the reciprocal of the experimental oxygen growth yield (Fig. 19).

Col. F: The $\frac{P}{O}$ ratio was calculated as the quotient of Cols. D and E.

It was assumed that:

(a) Y_{ATP}^{MAX} was constant (10.5 $g.mol^{-1}$), irrespective of growth substrate.

(b) There was a complete balance between ATP requirement for growth and ATP production i.e. there was no 'spillage' of ATP such as substrate cycles or 'slip' (Neijssel & Tempest, 1975).

Units for Cols. A-E are mol x 10^{-4} $(g.cells)^{-1}$.

carbon source	A ATP consumed monomers cells $\frac{1}{Y}_{ATP}^{MAX}$	B ATP consumed carbon source monomers	C ATP consumed oxidation of carbon source	D ATP produced by oxphos	E oxygen utilisation $\frac{1}{Y}_{O}^{G}$	F P/O
glucose	952.4	73.7	-127.0	-899.2	444.4	2.02
glucose 6-phosphate	952.4	0.54	-73.1	-879.8	277.7	3.16
glycerol	952.4	52.9	-105.8	-899.5	540.5	1.67
mannitol	952.4	74.1	-79.6	-947.0	454.0	2.08
lactose	952.4	74.1	-56.6	-969.9	325.1	2.98
maltose	952.4	110.9	-85.6	-977.7	444.1	2.20
galactose	952.4	147.7	-85.6	-1014.4	454.6	2.23
gluconate	952.4	127.8	-73.4	-1006.8	281.7	3.57

Figure 2.13 CALCULATION OF P/2E RATIOS

Cols. A: The involvement of reduced nucleotides during conversion of carbon source to cell monomers had been calculated previously (Fig. 2.9, Cols. C).

Cols. B: The production of reduced nucleotides during the combustion of the fraction of carbon utilisation which was fully oxidised had been calculated previously (Fig. 2.11, Cols. B).

Cols. C: The total involvement of reduced nucleotides during growth was calculated as the sum of Cols. A and B.

Col. D: A single value was derived for the production of reducing equivalents during growth. It was assumed 1 FADH = 0.5 NADM in most cases, with 1 FADH = 0.67 NADH for cells with high $\frac{P}{O}$ ratios

Col. E: The ATP produced by oxidative phosphorylation had been calculated previously (Fig. 2.12, Col. D).

Col. F: The $\frac{P}{2e}$ ratio was calculated as the quotient of Cols. E and D.

Units for Cols. A-E are mol x 10^{-4} $(g.cells)^{-1}$.

carbon source	A Reducing equivalents carbon source → monomers		B combustion of carbon source		C Total		D	E	F
	NADH (FADH)	NADPH	NADH (FADH)	NADPH	NADH (FADH)	NADPH		ox-phos	P/2e
glucose	-174.3	127.1	-253.9 (-63.5)	-63.5	-428.2 (-63.5)	63.6	-396.4	899.2	2.26
glucose 6-phosphate	-162.0	127.1	-114.5 (-29.2)	-29.2	-276.5 (-29.2)	97.8	-198.0	879.8	4.44
glycerol	-174.3 (-145.7)	127.1	-211.5 (-105.8)	-52.9	-385.8 (-251.5)	74.2	-437.3	899.5	2.06
mannitol	-247.9	127.1	-179.0 (-39.8)	-39.8	-426.9 (-39.8)	83.7	-363.1	946.9	2.61
lactose	-168.6	127.1	-111.9 (-28.3)	-28.3	-280.5 (-28.3)	98.9	-200.3	969.9	4.84
maltose	-174.3	127.1	-195.7 (-48.9)	-48.9	-370.0 (-48.9)	78.2	-316.3	977.7	3.09
galactose	-174.3	127.1	-228.3 (-57.1)	-57.1	-402.6 (-57.1)	70.0	-361.1	1014	2.80
gluconate	-108.3	146.9	-167.2 (-48.9)	-48.9	-275.5 (-48.9)	98.0	-209.7	1007	4.80

Figure 2.14 P/O RATIOS AND P/2e RATIOS FOR E.coli

The $\frac{P}{O}$ ratios and $\frac{P}{2e}$ ratios, calculated in this appendix, are summarised, for E.coli growing on a variety of carbon sources in arithmetic culture.

carbon source	P/O	P/2e
glucose	2.02	2.26
glucose 6-phosphate	3.16	4.44
glycerol	1.67	2.06
mannitol	2.08	2.61
lactose	2.98	4.84
maltose	2.20	3.09
galactose	2.23	2.80
gluconate	3.57	4.80

2.4 Discussion

The calculation of $\frac{P}{O}$ ratios by this method (2.2) requires that the monomer composition of E.coli, grown in arithmetic culture, is independent of the growth substrate, and that this composition is equivalent to that determined by Morowitz (1968) for logarithmically-grown cells. The elemental analysis for cells of the composition of Morowitz can be predicted, (Fig. 2.15). It is comparable to that derived experimentally by Wallace (1975) for arithmetic-culture cells (Fig. 2.15), indicating that the two cell types are most likely similar in monomer composition.

The second major assumption required for the calculation is that $Y_{ATP}^{MAX} = 10.5$ g.mol^{-1}, and is constant irrespective of growth substrate. Its validity is discussed elsewhere (Introduction, 3.1).

The fraction of total carbon utilisation which was completely oxidised has been calculated for each growth substrate (Fig. 2.10). The values derived by this method differ considerably from the usual assumed value for carbon incorporation. For example, it was calculated for glucose that 73.56×10^{-4} mol. of substrate was passed to the amphibolic pathways for the synthesis of 1 g. cells (Fig. 2.10), i.e. $73.56 \times 10^{-4} \times Y^{G}$ moles of glucose were used for synthesis of cell material per mole of glucose used ($Y^{G} = 95$ g.mol^{-1}). This represented 69.9% of the glucose used, so only 30.1% was available for complete combustion. The more usual method of calculation would be from the carbon content (44.6%, Fig. 2.15) and Y^{G}: incorporation = carbon content x $Y^{G} = 0.446 \times 95 = 42.4$ g.mol^{-1}

$= 3.53$ g. atom carbon.mol^{-1}.

Since glucose contains 6 carbon atoms, incorporation = $\frac{3.53}{6} \times 100 = 58.8\%$. Incorporation would be 58.8%, with 41.2% available for complete combustion.

A greater proportion of total carbon utilisation is therefore destined for cellular synthesis than is normally assumed. Failure to take account of this difference would alter results of experiments designed to follow isotope labelling patterns during cell synthesis, if the carbon source were not labelled uniformly.

The $\frac{P}{2e}$ ratios calculated are higher than the corresponding $\frac{P}{O}$ ratios (Fig. 2.14) and are not close to integer values. This suggests that not all of the reducing equivalents made available during growth have been accounted for, i.e. there may be an oxidised product which has not been determined. The oxygen content of arithmetic-culture cells compares favourably with that for the data of Morowitz (Fig. 2.15) suggesting that it is not arithmetic culture cells themselves which are more oxidised. The difference can not be attributed to excretion of an oxidised carbon compound during growth, since carbon balances were proved for those experiments. It may be that an oxidised inorganic compound in the growth medium could account for the production of the extra reducing equivalents.

It is possible from the elemental composition of the cells (Fig. 2.15) to calculate an expected oxygen yield for growth on each carbon source (Figs. 2.16-2.18). This indicates that $\frac{P}{O}$ ratios derived from oxygen yields are

Figure 2.15 ELEMENTAL COMPOSITION OF E.coli

The elemental content of E.coli, had been determined by Wallace (1975) for cells grown in arithmetic culture. Results are expressed as mean $\pm$ standard deviation, with number of determinations in parenthesis. Oxygen content was determined by difference from 100%, taking account of the inorganic content of cells (1.7%).

The monomer composition of exponentially-grown E.coli, as determined by Morowitz (1968) could be used to predict an elemental composition for these cells. 97.5% of the cellular contents could be accounted for in this calculation. Including the inorganic composition (1.7%; Tempest et al., 1966) brought the composition accounted for close to 100%.

element	% w/w	
	Wallace (1975)	Data of Morowitz (1968)
carbon	44.57 ± 2.20 (15)	47.3
hydrogen	6.65 ± 0.34 (15)	6.7
nitrogen	12.59 ± 1.11 (15)	11.7
phosphorus	3.49 ± 0.73 (2)	2.15
sulphur	2.17 ± 0.86 (2)	0.96
oxygen	28.83	28.6

Figure 2.16 CALCULATION OF EXPECTED OXYGEN GROWTH YIELDS: CARBON AND HYDROGEN PRODUCED DURING GROWTH

Cols. A: The elemental analysis of E.coli (Wallace, 1975; Fig. 2.15) in terms of carbon, hydrogen and oxygen is expressed in the units g.atom element. $(100\ g.cells)^{-1}$.

Cols. B: The molar growth yield for each substrate was determined experimentally (Fig. 19).

Cols. C: The carbon, hydrogen and oxygen contents of E.coli are expressed in the units g.atom $(mol\ substrate)^{-1}$ by taking the product of Cols. A and B, and dividing by 10^2.

Cols. D: The carbon, hydrogen and oxygen contents of each substrate are expressed in the units $g.atom.mol^{-1}$. Lactose and maltose were assumed to be their hydrolysis products (i.e. 2 x hexose).

Col. E: The amounts of carbon and hydrogen which were lost during growth were calculated as the difference of Cols. C from D.

carbon source	A			B	C			D			E	
	C	H	O		C	H	O	C	H	O	C	H
glucose	3.71	6.65	1.83	96	3.56	6.38	1.76	6	12	6	2.44	5.62
glucose 6-phosphate	3.71	6.65	1.83	113	4.19	7.51	2.07	6	11	5	1.81	3.49
glycerol	3.71	6.65	1.83	50	1.86	3.33	0.91	3	8	3	1.14	4.67
mannitol	3.71	6.65	1.83	107	3.96	7.11	1.96	6	14	6	2.04	6.89
lactose	3.71	6.65	1.83	228	8.46	5.16	4.17	2	24	2	3.54	8.84
maltose	3.71	6.65	1.83	204	7.57	3.57	3.73	2	24	2	4.43	0.43
galactose	3.71	6.65	1.83	98	3.64	6.52	1.79	6	12	6	2.36	5.48
gluconate	3.71	6.65	1.83	102	3.78	6.78	1.87	6	12	7	2.22	5.22

Figure 2.17 CALCULATION OF EXPECTED OXYGEN GROWTH YIELDS: OXYGEN PRODUCED DURING GROWTH

Cols. A: The carbon and hydrogen which was lost from cells during growth had been calculated previously (Fig. 2.16, Cols. E).

Cols. B: The oxygen accompanying the loss of carbon (as CO_2) and hydrogen (as H_2O) was calculated by referring to Cols. A.

Col. C: The oxygen content of cells had been determined previously (Fig. 2.16, Col. C).

Col. D: The oxygen output of the culture during growth was the sum of the oxygen content of cells (Col. C) and the oxygen leaving as CO_2 and H_2O (Cols. B).

All units are in $g.atom.mol^{-1}$.

carbon source	A elemental content of substrate cells		B oxygen lost as		C oxygen content of cells	D oxygen output of culture
	C	H	CO_2	H_2O		
glucose	2.44	5.62	4.88	2.81	1.76	9.45
glucose 6-phosphate	1.81	3.49	3.62	1.25	2.07	6.94
glycerol	1.14	4.67	2.28	2.34	0.91	5.53
mannitol	2.04	6.89	4.08	3.44	1.96	9.48
lactose	3.54	6.84	7.08	4.42	4.17	15.67
maltose	4.43	10.43	8.86	5.22	3.73	17.61
galactose	2.36	5.48	4.72	2.74	1.79	9.25
gluconate	2.22	5.22	4.44	2.61	1.87	8.92

Figure 2.18 CALCULATION OF EXPECTED OXYGEN GROWTH YIELDS: OXYGEN ENTERING CELLS, AND CALCULATED Y_O^G

Cols. A: Oxygen entered cells as carbon source and as phosphate and sulphate. The phosphorus and sulphur contents had been determined (Fig. 2.15). Three atoms of oxygen were assumed to enter with phosphorus and four with sulphur. Therefore the oxygen involvement could be calculated.

For example, for glucose:

P content = 3.49% = $\frac{3.49}{31}$

= 0.11 g.atom. $(100\ g.cells)^{-1}$.

= $0.11 \times Y^G \times 10^{-2}$

= $0.11 \times 95 \times 10^{-2}$

= 0.105 $g.atom.mol^{-1}$.

Three oxygen atoms accompany phosphorus

$\therefore$ 0.32 $g.atom.O.mol^{-1}$ enter with phosphorus.

Col. B: Hydrogen entered cells as NH_3. The number of hydrogens entering in this way was calculated as above.

Col. C: The total oxygen entering cells was the sum of that contained in the carbon source, phosphate and sulphate (Cols. A) minus half the hydrogen entering with NH_3 (Col. B).

Col. D: The oxygen appearing as cells, CO_2 and water had been calculated previously (Fig. 2.17, Col. D).

Col. E: The molecular oxygen taken up was calculated

as the difference between Cols. D and E.

Col. F: The molar growth yield was determined experimentally (Fig. 19).

Col. G: The calculated oxygen growth yield was the quotient of Cols. F and E.

Col. H: The experimental oxygen growth yield (Fig. 19) is included for comparison.

The units for Cols. A-E are mol. x 10^{-4} $(g.cells)^{-1}$.

	A			B	C	D	E	F	G	H
									$g.mol^{-1}$	
	O	PO_4	SO_4	NH_3	Σ in	Σ out	Σ net	Y^G	calc Y^G_O	exp Y^G_O
glucose	6	0.32	0.27	2.59	5.30	9.45	4.15	96	23.1	22.5
glucose 6-phosphate	5	0.37	0.32	3.05	4.17	6.94	2.78	113	40.7	36
glycerol	3	0.17	0.14	1.35	2.61	5.53	2.92	50	17.1	18.5
mannitol	6	0.35	0.30	2.89	5.20	9.48	4.27	107	25.0	22
lactose	11	0.75	0.64	6.16	10.31	15.87	5.36	228	42.5	31.0
maltose	12	0.67	0.57	5.51	10.49	17.61	7.13	204	28.6	22.5
galactose	6	0.32	0.27	2.65	5.26	9.25	3.99	98	24.5	22
gluconate	7	0.34	0.29	2.75	6.26	8.92	2.66	102	37.5	36

probably more valid than the $\frac{P}{2e}$ ratios, which are calculated in abstract terms.

Some of the $\frac{P}{O}$ ratios calculated by this method (Fig. 2.14) are close to integer values e.g. glucose, mannitol = 2; lactose, glucose 6-phosphate = 3; but others (gluconate, maltose and galactose) are higher than integers.

Gluconate is unique among the carbon sources studied in that it is assumed to be catabolised by the Entner-Doudoroff pathway (Fig. 2.8). The pentose phosphate pathway exists as an alternative for gluconate utilisation, but work with mutants deranged in each pathway suggested that the Entner-Doudoroff pathway is the preferred route for gluconate catabolism (Fraenkel, 1968). The $\frac{P}{O}$ ratio, calculated from growth yields, for growth on gluconate via the pentose phosphate pathway alone would be 3.76 (unpublished calculation). The Entner-Doudoroff pathway is one of the poorest energy-yielding catabolic pathways, since 50% of the gluconate carbon is converted to pyruvate with no energy production. It may be that several pathways are used for gluconate catabolism in arithmetic culture, and if they were more energetically favourable, the $\frac{P}{O}$ ratio of 3.48 would be reduced. From the results, it is assumed that the $\frac{P}{O}$ ratio for growth on gluconate approximates to 3.

Galactose and maltose have $\frac{P}{O}$ ratios of around 2.2. They are both assumed to be transported into the cell by uptake systems which involve binding of substrate to a periplasmic protein, and hydrolysis of ATP as the source

of free energy for active transport. For the calculation of $\frac{P}{O}$ ratios, it was assumed that one equivalent of ATP was used per equivalent of substrate transported (Fig. 2.7). This stoichiometry is speculative, since the mechanism of action of these systems is poorly understood. The inability of ATPase - negative mutants to transport substrate (e.g. Wilson, 1974) showed that there is a definite requirement for ATP during transport but the ATP/substrate stoichiometries have never been determined because of technical difficulties. If it is assumed that no ATP is expended during transport, $\frac{P}{O}$ ratios of 2.00 and 2.09 may be calculated for galactose and maltose respectively. These will be lower limits for the derived $\frac{P}{O}$ ratios.

The $\frac{P}{O}$ ratio for glycerol (1.67) is the lowest observed. A large fraction (45%) of the reducing power available for electron transport during growth on glycerol is $FADH_2$, since all of the carbon entering central metabolism has to pass through a flavin-linked reduction (α-glycerophosphate dehydrogenase). Electrons entering the electron transport chain from $FADH_2$ pass through only one of the two potential energy-conserving segments which are present during growth on glycerol (Poole & Haddock, 1975). Therefore, the energy conserved from electrons supplied from $FADH_2$ will be 50% of that possible when NADH is the electron donor. This will lower the apparent $\frac{P}{O}$ ratio. A correction may be made as follows:

total carbon utilisation = $(Y^G)^{-1}$ = 200 x 10^{-4} mol.g^{-1} cells.

i.e. 200 x 10^{-4} mol $FADH_2$ produced. (g. cells)$^{-1}$.

ATP production from oxphos =

899.20 mol x 10^{-4}. (g. cells)$^{-1}$ (Fig. 2.12)

$\Rightarrow$ 899.20 -200 = 699.20 mol x 10^{-4} (g. cells)$^{-1}$ made available exclusive of $FADH_2$.

Total oxygen utilisation = 540 g.atom x 10^{-4}. (g. cells)$^{-1}$ of which 200 g.atom is used in $FADH_2$ oxidation and (540 - 200) = 340 g.atom for the balance, $\therefore$ $\frac{P}{O}$ for the remainder of oxidative phosphorylation = $\frac{699.20}{340}$ = 2.06. Therefore, accounting for the large amount of $FADH_2$ produced, the $\frac{P}{O}$ for growth on glycerol is 2.06.

In summary, the method allows calculation of $\frac{P}{O}$ ratios from molar growth yields. The values derived fall close to integer values, with $\frac{P}{O}$ = 2 for growth on glucose, glycerol, mannitol, maltose and galactose and with $\frac{P}{O}$ = 3 for growth on lactose, glucose 6-phosphate and gluconate.

APPENDIX 3

FAST RESPONSE pH METER

The pH meter used for the determination of $\frac{H^+}{O}$ ratios must be capable of 'backing off' the potential present normally across the pH electrode at pH 7.0, and of measuring rapidly (< 1 s) and accurately a small change in pH due to protons extruded by the cells in response to added oxygen.

A meter satisfying these criteria was constructed in the Departmental workshop by Mr. A. Brown to a design supplied by Dr. W. A. Hamilton, University of Aberdeen. It was necessary to alter the values of some of the components of Dr. Hamilton's circuit to increase the 'back off' facility of the instrument. The modified circuit diagram is shown in Fig. 3.1. The performance of the meter when connected to a recorder is described in Methods, 12.

Component List and Specifications

R1	2.7K	VR1	10K, 25T	C1	1 μF
R2	36K	VR2	1K, 25T	C2	0.22 μF
R3	100K	VR3	10K, 10T		
R4	200K	VR4	10K, 10T	D1	BZY 88 (12V)
R5	900	VR5	25K, 25T	D2	BZY 88 (12V)
R6	100	VR6	100K, 10T		
R7	1.35M				

IC's E70; E77

Meter 50-0-50 μA

Power Supply ± 15V at 100 mA

S1 single pole switch, panel mounted

S2, S3 dipole switches, panel mounted

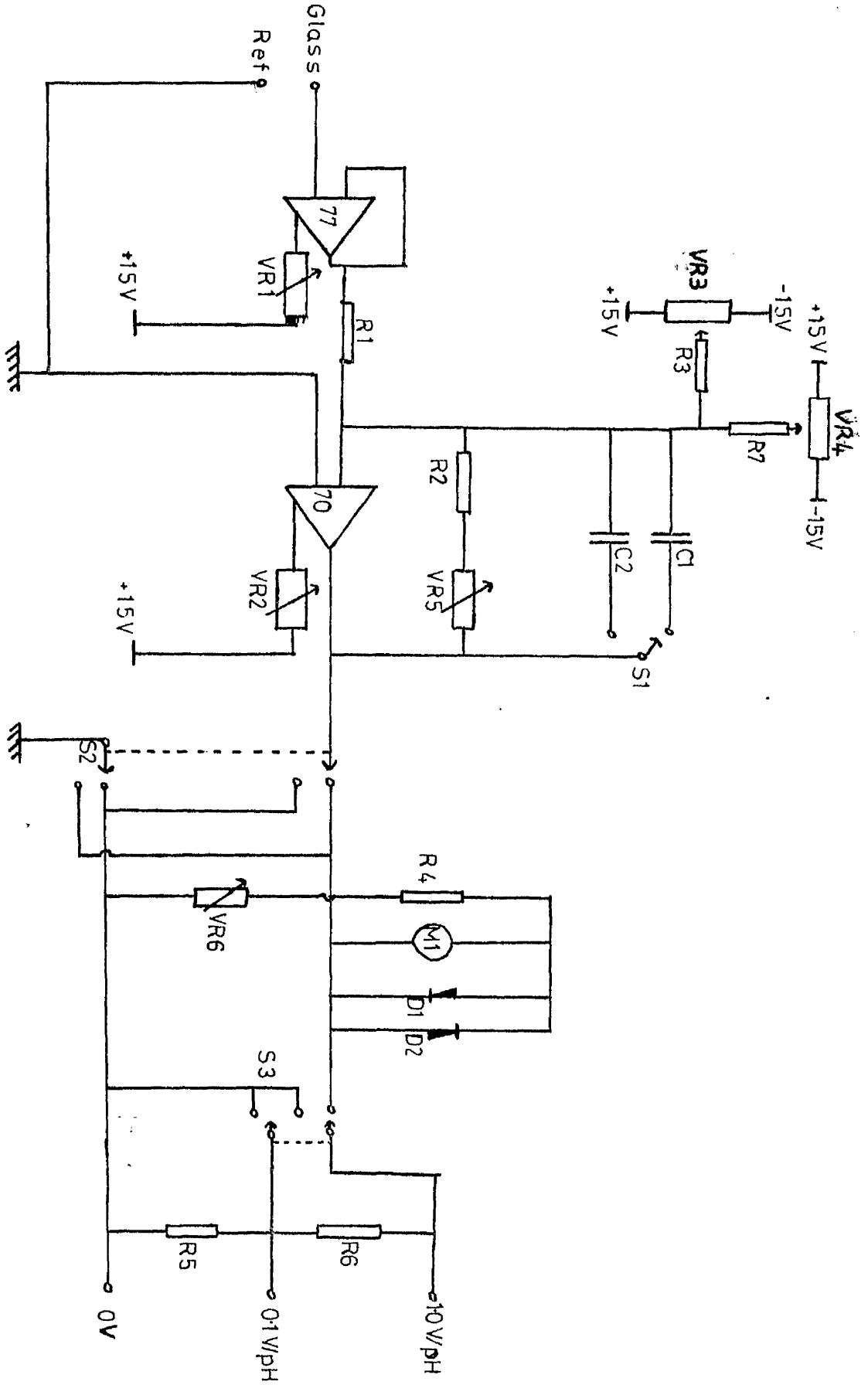
BNC socket, and 4 mm output sockets.

Figure 3.1 CIRCUIT DIAGRAM OF FAST-RESPONSE pH METER

The values of the components shown in the circuit diagram are listed in Appendix 3.

The components had the following functions:

VR1	offset null E77
VR2	offset null E70
VR3	Zero coarse adjust
VR4	Zero fine adjust
VR5	reset gain
VR6	meter gain
C1, C2	frequency response
S1	response change-over
S2	meter polarity
S3	recorder short.


Glass
Ref
77
70
VR1
VR2
VR3
VR4
VR5
VR6
R1
R2
R3
R4
R5
R6
R7
C1
C2
S1
S2
S3
M1
D1
D2
+15V
-15V
0V
0·1V/pH
1·0V/pH

SUPPLIERS

IC's: E70A; E77C; Computing Techniques Ltd., Brokers Road, Billinghurst, Sussex.

Power Supply: DP5-25 and mating socket SK-C4; Ancorn Ltd., Devonshire Street, Cheltenham.

All other components, including instrument case and meter; Radiospares.

Setting up procedure

With a 1M resistor across the input:

Trim, VR1 to give zero at the output

VR3 and VR4 to give zero at their wipers

VR2 to give zero at the output

VR5 to give a net gain of 16.7 : 1

VR6 to calibrate the meter.

If difficulty is experienced, connect the input of E77 to OV and continue as before with no resistor.

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