Maintenance energy and molar growth yields of Escherichia coli

by

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Thesis presented for the degree of Doctor of Philosophy

The University of Glasgow

September 1975

ACKNOWLEDGEMENTS

I am indebted to Professor R. M. S. Smellie for the opportunity to study in this Department of Biochemistry, and to the Medical Research Council, for financial support of a Research Studentship from 1972 - 1975.

I wish to thank Dr. W. H. Holms, for his advice, encouragement and help throughout the course of the work, and Dr. C. A. Fewson, Dr. I. D. Hamilton and my colleagues in Laboratory C24 for their criticism and advice at our discussion meetings.

For aid in the solution of numerical problems, I am indebted to Dr. R. Eason, Dr. F. Williams and Mr. J. McG. Bilsland, and for help in the analysis of cell composition, Mrs. W. Harkness.

I would also like to thank Mrs. May Abrahamson who made an excellent job of typing this thesis.

ABBREVIATIONS

The abbreviations used are those recommended by the Biochemical Society (Biochem. J. (1972) 126, 1-19) and those listed below:-

BCIG	5-bromo-4-chloro-indoxyl-β-galactoside
D	dilution rate
E_a	Arrhenius activation energy
IPTG	isopropyl thiogalactoside
m	maintenance coefficient in terms of carbon source
m_{o_2}	maintenance coefficient in terms of O_2
malate	L-malic acid
OAA	oxaloacetic acid
ONPG	o- nitrophenyl β-galactoside
Q substrate	rate of substrate utilisation per unit mass
PEP	phosphoenolpyruvic acid
POPOP	1,4-di-[2-(5-phenyloxazolyl)] benzene
PPO	2,5-diphenyloxazole
R	gas constant
TDG	thiodigalactoside
Y	apparent molar growth yield
Y_G	maximum molar growth yield - carbon source
Y_{GO_2}	maximum molar growth yield - O_2
Y_{ATP}	growth yield per mole ATP

SUMMARY

Maintenance is that fraction of the metabolic activity of growing cells which does not result in the net synthesis of new cell material. The maintenance coefficient (m) is assumed to be constant, and independent of specific growth rate (μ). It can be measured by examining the distribution of carbon to new cells and to energy production at various μ. In the arithmetic growth systems employed in this work μ changes in a predictable way and at the slow growth rates attained (μ = 0.01 - 0.1 h^{-1}) m is a large fraction of the total energy utilisation. These factors made more simple the accurate measurement of m in comparison with methods employed by other workers. Both the maximum molar growth yield (Y_G) and m were readily measured using arithmetic-type continuous culture.

Value of Y_G and Y_{GO_2} were similar to those obtained by other workers using different growth systems. The main conclusions were

(i) anabolic and catabolic processes in carbon-limited were tightly coupled, but coupling broke down under nitrogen limitation;

(ii) using the Bauchop and Elsden Y_{ATP} of 10.5 g/mole, P/O ratios for carbon-limited E. coli were 2 for growth on glucose, malate and glycerol, and 3 for growth on lactose. Addition of 0.5 M sodium chloride to a glycerol-limited culture reduced the P/O ratio to 1.

These P/O ratios seemed reasonable, implying that the previous assumption of the value for Y_{ATP} was probably valid, and hence Y_{ATP} was considerably less than the theoretical maximum of ~30 g/mole.

Maintenance in terms of carbon source and oxygen was measured in the same experiments. Findings were

(i) carbon source used for maintenance was completely oxidised;

(ii) maintenance energy is small in E. coli. m_{ATP} was 1 - 3 mmol (g dry wt)$^{-1}$ h^{-1} at 37^{o};

(iii) at least two components make up the maintenance requirement. One is relatively constant with respect to carbon source and temperature, and the other has a very high temperature coefficient, which is characteristic of the particular carbon source used;

(iv) part of the maintenance requirement may be an osmotic effect. Addition of 0.5 M sodium chloride to a glucose-limited culture at 37^{o} increased m by 44%;

(v) maintenance of the structural organisation of the respiratory apparatus may require energy. The maintenance coefficient of a glycerol-limited culture fell when the P/O ratio decreased;

(vi) m from glycerol-limited chemostat culture was 3× the value of m obtained from glycerol-limited arithmetic-type continuous culture. It was postulated that m may not be constant for all μ, although it may appear to be constant from measurements made over small ranges of μ.

Turnover of enzymes, protein, cell walls and total cell phosphate and carbon were measured in arithmetic-type continuous culture to identify any turnover reactions which may have a similar temperature

coefficient to maintenance. The Arrhenius activation energy of each of these processes was an order of magnitude less than that of the component of maintenance with the high temperature coefficient. However, turnover of macromolecules was not eliminated as a possibility for the maintenance coefficient which remains constant below 30°. The large Arrhenius activation energy of maintenance is of the same order as denaturation. Protein turnover data showed that this component could not be the replacement of denatured protein de novo. It is more likely that it is caused by the disruption and subsequent repair or re-assembly of cell structure, particularly of multienzyme complexes, ribosomes and membrane components.

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INTRODUCTION

Energy substrates consumed by micro-organisms provide energy for growth and non-growth (or maintenance) processes. The conventional energy currency of the cell, ATP, is formed both by substrate level and oxidative phosphorylation under aerobic conditions. The efficiency of the latter process (the P/O ratio) is not known, and can only be calculated if the energetic efficiency of aerobic growth (Y_{ATP}) is assumed to be the same as under anaerobic conditions. Conversely, a value for the aerobic Y_{ATP} cannot be calculated without assuming a value for the P/O ratio. Estimation of the energy requirement under aerobic conditions of the maintenance processes can only be made if the P/O ratio is known.

The aims of the work presented in this thesis were

(i) to resolve the problems caused by the interdependence of Y_{ATP} and the P/O ratio

(ii) to estimate the energy requirement for maintenance; and

(iii) to identify the energy-consuming reactions involved in maintenance

1. Maintenance energy

Energy of maintenance has been established conclusively for higher animals, with the majority of popular interest being centred on growth and maintenance energy of Homo sapiens. In the adult human, health is optimal when the rate of nutrient supplied is equal to the rate at which the nutrient is required for the non-growth processes. Should the rate of nutrient supply exceed this level, growth will occur until a new steady-state (or obese) mass is achieved with a correspondingly higher maintenance requirement, or possibly a less efficient growth yield, due to a change in metabolism. The principal functions of the non-growth, or maintenance, energy are in motility, heat generation and turnover of constituent molecules. This energy accounts for practically all of the energy made available from nutrients during the life of higher animals.

In the plant kingdom, the emphasis is more on growth than on energy for maintenance, although the latter does appear to occur. Kandler (1955) showed in cultures of plant tissues that not all of the assimilated energy was used for growth. Turnover of protein is known to take place in whole plants (Humphrey & Davies, 1975), and in a few unusual cases, so is motility (Setty & Jaffe, 1973).

The existence of maintenance energy in micro-organisms has been suspected since the end of the last century, but it is only within the last 10-15 years that it has been confirmed, and techniques have been available for measurement of the energy used. Duclaux (1898) distinguished between energy for growth and energy for maintenance, then calculated the maintenance coefficient, which is the specific rate of substrate utilisation for maintenance, to be 0.25g sugar g^{-1} h^{-1} for yeast, or 1.4 mmol glucose $g^{-1}.h^{-1}$. This value is remarkably similar to many determinations which have been made in recent years (Figure 69).

From that time, until the early 1960's, the concept but not proof of the existence of maintenance was widely accepted. Theoretically, maintenance was widely predicted (Buchanan & Fulmer, 1928; Rahn, 1932; Netter, 1953; Sugita, 1955; Clifton, 1957), but no real proof had been demonstrated experimentally. Some workers claimed to have evidence consistent with a maintenance requirement, but others claimed to have results in direct contrast. Maze (1902) concluded that maintenance energy was undetectable when he found that the yield of fungus was directly proportional to the quantity of invert sugar or ethanol consumed, with no intercept corresponding to a maintenance requirement. Rottier (1936) found that the linear relationship between growth of Polytoma uvella and acetate concentration did not lead to measurable intercepts. Increases in cell numbers supported by varying substrate concentrations for Glaucoma piriformis by Monod & Tessier (1936), and for Aerobacter aerogenes by Dagley & Hinshelwood (1938) and Lodge & Hinshelwood (1939) led to the same conclusion. The measurement of microbial growth by population counting suffers from a number of shortcomings, principally that an increase in the amount of cell material need not result in a corresponding increase in cell numbers, due to an increase in cell size without division (Mallette, 1969). Probably the most meticulous of the earlier studies was made by Monod (1942). The growth yields, as turbidity, of E. coli and Bacillus subtilis were determined for various concentrations of glucose and sucrose, and the extrapolated graphs of growth yield against concentration were found to pass through the origin. As the measurement of growth by turbidity eliminated the problems of cell size and number, Monod concluded that maintenance energy, if it existed, was an immeasurably small fraction of the total energy used. Monod also restricted the growth rate of cells by changing the rate of agitation of the culture, reasoning that, as cells requiring a longer time to

reach maximum turbidity should use more maintenance energy, the final turbidity of slow-growing cultures should be less than the turbidity attained by freely-growing cultures. No difference was observed in the final turbidities. Dagley, Dawes & Morrison (1951), Battley (1960) and Bauchop & Elsden (1960) gave further examples of experiments where growth of micro-organisms was found to be directly proportional to substrate concentration, with any energy for maintenance being insignificant.

Thus, energy of maintenance could not be detected, although it was thought that this is a reflection of the very low level of maintenance relative to the energy used for growth (Mallette, 1963). "Energy of maintenance" and "endogenous metabolism" must be treated as separate entities, although the two may have many features in common. Strictly speaking, energy of maintenance is that energy which is required to maintain the viability of an organism, whether it is actively growing or not. Endogenous metabolism is the metabolic activity of cells which is not associated with growth, but which may or may not be necessary to preserve the viability of the organism. Continual re-definition of these titles has resulted in corruption of their literal meaning. Maintenance energy is now commonly used to describe the consumption of nutrient or energy in growing cells which does not lead to net synthesis of new cell material. Maintenance energy is thus now used as a synonym for "endogenous metabolism in growing cells". Only when the topic under discussion is viability does maintenance energy retain its original meaning. Endogenous metabolism is now almost exclusively used to describe metabolism which occurs in starving cells.

Much of the earlier work was concerned with maintenance energy for viability rather than maintenance energy in growing cells. The

latter was not able to be conclusively demonstrated, but there are several early instances of observations that an energy supply is necessary to maintain viability of cultures. Freidlin (1928) observed that very low levels of energy sources were inefficient in subculturing bacteria, and Windisch & Nordheim (1957) emphasised the importance of storage compounds in providing energy for the maintenance of viability of starving cells. The role of storage compounds in starvation has been reviewed by Dawes & Ribbons (1964). Nilson (1960) found that the viability of E. coli in cultures fed glucose at a rate equivalent to the maintenance requirement remained constant. McGrew & Mallette (1962), in a more detailed study, added very small quantities of glucose to starving E. coli and found a threshold level, below which the viability of the culture fell as the concentration fell, but no growth occurred, and above which growth took place. These were probably the first occasions maintenance energy had been successfully demonstrated, both in the classical, viability sense, and in the now more usual growth sense.

It can now be seen that, until the early 1960's, maintenance was a process which should occur in growing cells, to repair "chemical wear and tear" (Rahn, 1932), in ion transport (Netter, 1953), because of internal inefficiencies (Sugita, 1955) or in motility (Sherris, Preston & Shoesmith, 1957), but of which it was almost impossible to obtain experimental proof.

The introduction of continuous-flow culture techniques encouraged a breakthrough in the study of maintenance. Variation of microbial growth rates had previously been achieved by considerable alterations to the growth conditions in batch culture. Terroine & Wurmser (1922) attempted to measure maintenance by changing the growth rate using different pH's of growth medium. Monod (1942) attempted the same by

altering the rate of agitation of cultures and by changing the growth temperature. Monod concluded that such fundamental changes in growth conditions would change metabolism in such a way that any effect of maintenance energy on the growth yield would be masked. However, the principle that slow-growing cultures would use proportionally more energy for maintenance than fast-growing cultures under the same growth conditions was essentially correct, and was confirmed by studies of bacterial growth yields in continuous culture. The equations derived by Herbert, Elsworth & Telling (1956) for growth in continuous culture predicted that the molar growth yield (Y) would be constant irrespective of the specific growth rate (μ). Results obtained from continuous culture showed that Y did not remain constant, but decreased as μ fell (Herbert, 1958). The slower growth rates used in continuous culture allowed maintenance to be a larger proportion of the total energy used, sufficient to have a detectable effect on the molar growth yield.

Marr, Nilson & Clark (1963) derived a mathematical expression relating Y with growth rate, which made the assumption that maintenance was a process independent of μ and which depended only on the growth conditions. For any given conditions, the value of the specific maintenance would be constant. Marr et al. used an unusual type of continuous culture, previously described by Jordan & Jacobs (1944), to prove the validity of this expression. The usual flow system in continuous culture is one in which limiting nutrient is supplied to the growth vessel at a constant flow rate, and the volume of culture is held constant by an overflow device. Marr et al. did not incorporate an overflow and so, instead of being held constant, μ progressively declined as the mass of cells in the growth vessel increased. If maintenance were zero, then the rate of increase of cell mass would be expected to be constant. The experimental results

of Marr *et al.* for *E. coli* showed that the rate of increase of cell mass progressively fell in a manner consistent with their assumption that specific maintenance was independent of μ. This type of continuous culture was used during the course of the work described in this thesis to measure maintenance energy in *E. coli*. As the idea in this instance stemmed from previous work in this laboratory concerning arithmetic growth of pre-induced *E. coli* on non-inducing carbon sources, the growth system was termed *arithmetic-type continuous culture*.

The measurement of maintenance energy used by Marr *et al.* was the specific maintenance, with units h^{-1}. While this term is useful, it suffers from the disadvantage that it incorporates the maximum molar growth yield Y_G and hence the maintenance coefficient, expressed in mol substrate (g cells)$^{-1}$ h^{-1}, which is the more useful form for calculations of energy used for maintenance, can not be calculated if values of Y_G are not available.

Schulze & Lipe (1964) derived maintenance coefficients for *E. coli* from continuous culture using the equation

$$Q_{substrate} = m + \frac{\mu}{Y_G}$$

where $Q_{substrate}$ is the specific rate of substrate utilisation, m is the maintenance coefficient and μ is the specific growth rate.

This equation was modified by Pirt (1965) to the one now in most common use:

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_G}$$

where Y is the apparent molar growth yield at specific growth rate μ.

Thus, the maintenance coefficient could be determined from chemostat culture results by a double reciprocal plot of $1/Y$ v $1/\mu$, or at steady state $1/Y$ v $1/D$ where D is the dilution rate. The ordinate intercept is $1/Y_G$ and the gradient of the line is m. Pirt used data from the work of Pirt (1957), Herbert (1958) and Hobson (1965) to confirm the validity of the above equation, and the equation has been used since then to calculate the maintenance coefficients of a wide number of micro-organisms (Figure 69).

In general, the magnitude of maintenance coefficients has been found to be variable, but small relative to the quantity of energy used for growth. It has been shown that the only reason maintenance could not be measured in the earlier batch culture experiments was that it was so small as to be negligible, and undetectable by the methods used. The recent work of Stouthamer & Bettenhaussen (1975) has shown that the maintenance coefficient of A. aerogenes is small, in contrast to a previous observation of a large maintenance coefficient (Stouthamer & Bettenhaussen, 1973) which, if real, would have meant that at a μ of 0.1, 90% of available energy would be consumed by maintenance. The conclusion is that the earlier maintenance coefficient was caused by uncoupling in the tryptophan-limited culture, and Stouthamer & Bettenhaussen emphasise the requirement that maintenance coefficients, to be valid, must be measured in a culture where the energy source is limiting, in order to prevent uncoupling (Rosenberger & Elsden, 1960; Ng, 1969; Nagai et al., 1969; Harrison & Loveless, 1971; Belaich, Belaich & Simonpietri, 1972; Harrison, 1972; Neijssel & Tempest, 1975).

Bauchop & Elsden (1960) rationalised many of the apparent inconsistencies of molar growth yields of micro-organisms by the conversion of molar growth yield data to yields in terms of the

currency of biological energy, ATP. In much the same way, maintenance coefficients can, in many cases, be converted to energy utilisation in terms of ATP (Figure 69). Indeed, in many cases, the maintenance coefficient is only meaningful when expressed as ATP utilisation, as then the production of excretion products can be accounted for (von Meyenberg, 1969; Watson 1970; Stouthamer & Bettenhaussen, 1973 and 1975).

Despite the proliferation in the experimental measurement of maintenance energy since the advent of continuous culture, little more is known about the functions of this energy drain. Indeed, the early proposed functions of maintenance are still as valid as ever, and are still being put forward without any experimental proof. The only direct evidence as to the nature of the maintenance requirement is that addition of sodium chloride to the growth medium of Saccharomyces cerevisiae increases the maintenance coefficient (Watson ,1970), and that increasing the ammonium chloride concentration increases the maintenance coefficient of A. aerogenes (Stouthamer & Bettenhaussen, 1973).

The turnover of RNA (Norris & Koch, 1972; Westover & Jacobson, 1974) and protein (Willetts, 1967b; Pine, 1972) is known in many instances to be independent of μ, which makes them probable candidates for the utilisation of maintenance energy. Phospholipids (Thompson, 1973) and cell walls (Mauck, Chan & Glaser, 1971) are known to turn over and may also utilise maintenance energy in some organisms. Several authors have, without direct evidence, proposed turnover of macromolecules to be a maintenance function in growing cells (Marr et al., 1963; Righelato et al., 1968; Stouthamer & Bettenhaussen, 1973 and 1975).

Increasingly sophisticated knowledge of microbial biochemistry has not, in general, aided the identification of the maintenance processes, although Dalton & Postgate (1969) and Rogers & Stewart (1974) have evidence suggesting that maintenance energy is required for the preservation of the intricate structure of multienzyme complexes. In these two cases involving the nitrogenase of Azotobacter chroococcum and the respiratory chain of Candida parapsilosis respectively, the maintenance coefficient increased when the enzyme complexes were present.

One of the aims of the work presented in this thesis was to measure maintenance energy in E. coli and to investigate the nature of maintenance processes.

2. Molar growth yields and oxidative phosphorylation

As this section is intended to provide an introduction to, rather than a review of, molar growth yields and oxidative phosphorylation, reference to a large volume of the literature will be omitted. There are several adequate reviews dealing with this subject (Stouthamer, 1969; Payne, 1970; Forrest & Walker, 1971; Stouthamer & Bettenhaussen, 1973).

Growth yields of micro-organisms have attracted interest ever since bacteria were first cultured in the laboratory. A number of early examples of findings that bacterial growth is directly proportional to the quantity of nutrient available have been given in the last section (pp. 3 - 4). Many more exist, and indeed the fact that a linear relationship of this sort was found to apply for the concentration of any limiting nutrient led to the development of methods for the estimation of amino acids, purines, pyrimidines and the B group of vitamins. However, not a great deal of meaning was

attached to these yields until Bauchop & Elsden (1960) introduced a new concept to growth yields of micro-organisms - Y_{ATP}, the molar growth yield in terms of g cells produced per mole of ATP made from the energy source.

Bauchop & Elsden made the observation that, in the work of Monod (1942), the dry weight yields of B. subtilis, E. coli and Salmonella typhimurium for any given carbohydrate were approximately equal, and interpreted this to mean that the growth yield of any organism was proportional to the energy produced by the catabolism of the energy source. Earlier, De Moss, Bard & Gunsalus (1951) had observed that the growth yield of Streptococcus faecalis with glucose as energy source was significantly greater than that of Leuconostoc mesenteroides. The catabolic pathway of glucose in L. mesenteroides was found to include xylulose-5-phosphate (Heath et al., 1958) and, instead of 2 ATP/mole of glucose, as formed by the Embden - Meyerhof pathway in S. faecalis, only 1 ATP was produced (Hurwitz, 1958). Bauchop & Elsden proposed that the lower growth yield of L. mesenteroides was due to the operation of the phosphoketolase pathway, which produced less ATP for growth.

The concept that the growth yield of micro-organisms was dependent on energy was not new. Previous work had involved the thermodynamic study of microbial growth (reviewed by Payne, 1970). However, treatment of growth as a purely chemical reaction did not improve the understanding of the process. Obviously, the thermodynamic energy available to S. faecalis and L. mesenteroides from glucose is the same, yet the growth yields differ considerably. Bauchop & Elsden realised that the biochemical conservation of energy in any organism dictates the growth yield obtained from the energy source, rather than the energy potential of the substrate. It should be noted in passing,

however, that thermodynamic studies have shown that microbial growth proceeds according to the second law of thermodynamics. Morowitz (1955) showed that growth of E. coli produced 3.3 times the minimal entropy requirement for the specifications of the second law applied to growth.

Bauchop & Elsden measured the growth yields of S. faecalis, Pseudomonas lindneri, Saccharomyces cerevisiae and Propionibacterium pentosaceum under anaerobic conditions with a number of energy sources, and expressed these yields as g dry wt/mole substrate, rather than the less meaningful units previously used, such as cell number/g substrate or g dry wt/g substrate. As the ATP production was known for each of these organisms and substrates, yields could be expressed as g dry wt/mole ATP produced. They found that the Y_{ATP} was relatively constant, regardless of the organism or energy source used. Values ranged from 8.3 to 12.6 with an average of 10.5. This fundamental idea, that the synthesis of biological material is proportional to the quantity of ATP available for growth, is as valid today as when it was first proposed. Bauchop & Elsden employed complex medium where all the required monomers were present, and the substrate was used solely as a source of energy, with virtually none of the substrate carbon incorporated into cell material. The same principle has been employed where the substrate was a source of both carbon and energy. Provided that the level of incorporation is known, the fraction of carbon source used solely for energy production can be calculated. When this was done, the principle of Y_{ATP} as a biological constant was confirmed to be true for cells growing in simple defined medium (reviewed by Stouthamer, 1969).

Forrest & Walker (1971) pointed out several exceptions which have been found where Y_{ATP} differs considerably from the average value

of 10.5 g/mole obtained by Bauchop & Elsden. The Y_{ATP}'s calculated for Bacteroides amylophilus, Selenomonas ruminantium, 5S (Hobson & Summers, 1967), Actinomyces israelii (Buchanan & Pine, 1967), several Streptococcus species (Moustafa & Collins, 1968) and Lactobacillus casei (de Vries et al., 1970) were considerably in excess of the Bauchop & Elsden value, while those for Zymomonas anaerobia (McGill & Dawes, 1971) and Zymomonas mobilis (Belaich et al., 1972) were considerably lower than 10 g/mole ATP.

Hernandez & Johnson (1967a) and Belaich et al. (1972) pointed out that, unless the energy source is limiting, uncoupling may occur, effectively reducing net ATP production. Low values of Y_{ATP} may be explained in this way. The higher values are more difficult to account for, although Hobson & Summers (1972) have proposed that the Y_{ATP} previously calculated for S. ruminantium was in error because the ATP production had not been fully accounted for. They calculated a new Y_{ATP} in view of new experimental evidence and this value was in good accord with the Bauchop & Elsden value. Hobson & Summers suggest that other anomalous Y_{ATP} values may be due to the same reason.

Thus, in general, the anaerobic growth of bacteria has indicated that Y_{ATP} is relatively constant for the growth of micro-organisms from preformed monomers. Differences from the average Y_{ATP} may be expected if the organism has an unusual composition (Stouthamer, 1973), but for cells of average composition, Y_{ATP} will fall within the range 8.3 - 12.6 g/mole. The spread of Y_{ATP} values found by Bauchop & Elsden may be in part due to slightly different cell compositions, but is also likely to be caused by insufficient knowledge of all of the energy-yielding reactions of the cells used.

While Y_{ATP} has been calculated frequently for anaerobic growth, the same constant has not been determined with any confidence for

respiring cultures. Under anaerobic conditions, all ATP is produced by substrate level phosphorylation, and provided that the metabolic pathway is known, the ATP production can be estimated with confidence. Under aerobic conditions, ATP is produced both by substrate and oxidative phosphorylation. A great deal of effort has been applied to the estimation of the efficiency of oxidative phosphorylation in growing micro-organisms, with only limited success.

Direct measurement of ATP production linked to electron transport is only possible in non-growing cells or membrane preparations, as ATP formed in growing cells is immediately used for synthetic processes. In a few instances, the P/O ratio has been found to be of the same order as found in isolated mitochondria (Knowles & Smith, 1970; Hempfling, 1970; Baak & Postma, 1971). In most other work, the efficiency of oxidative phosphorylation has been found to be significantly less (van der Beek & Stouthamer, 1973), probably due to physical damage to the respiratory apparatus (Mével-Ninio & Yamamoto, 1974).

The proton-motive force, which is the energy potential of a concentration gradient of H^+ across the cell membrane, is known to be part of the mechanism of coupling of electron transport to oxidative phosphorylation (Harold, 1972). Proton extrusion experiments, which measure proton extrusion in response to the passage of electrons to oxygen (the $\rightarrow H^+/O$ ratio), are done routinely to establish the number of coupling sites in bacteria. A $\rightarrow H^+/O$ ratio of 4 has been obtained for E. coli (Lawford & Haddock, 1973; Jones et al., 1975), consistent with a P/2e ratio of 2 if the coupling of re-entry of protons to phosphorylation of ADP is fully efficient. The efficiency of re-entry is not known, however, and so while proton extrusion experiments predict P/O ratios of 2 for glycerol-growing E. coli, they do not

constitute proof of such a high efficiency in growing cells. Estimation of the number of coupling sites can also be done using respiratory inhibitors and artificial electron acceptors and donors (Ishaque, Donawa & Aleem, 1973) but again, the number of coupling sites may be quite different from the P/O ratio in growing cells.

P/O ratios in growing micro-organisms may also be estimated from growth yield data. If one assumes that the Y_{ATP} is the same for both aerobic and anaerobic growth, the total ATP production can be estimated. The extent of substrate phosphorylation can be estimated, and hence the ATP produced by oxidative phosphorylation calculated. Division of this quantity by the oxygen uptake gives the P/O ratio.

While in principle this method appears simple, provided that the necessary growth yields are available, it has only infrequently been used properly. The most common error is to simplify the approach by neglecting the contribution of substrate phosphorylation to total energy production (Hadjipetrou et al., 1964; Meyer & Jones, 1973; Neijssel & Tempest, 1975). The calculations of Hadjipetrou & Stouthamer (1965), Watson (1970) and Stouthamer & Bettenhaussen (1973 and 1975) do take account of substrate phosphorylation, but in no case do they take into account any energy utilised or produced by the fraction of carbon source which is incorporated. Stouthamer (1973) has demonstrated the importance of including this energy when calculating Y_{ATP}. Where the carbon source is glucose, there appears to be little energy gain or loss during the conversion of carbon source to the monomers (Forrest & Walker, 1971) so the results of the calculations mentioned above which ignore this factor are probably quite valid. Calculations which appear later in this thesis for molar growth yields of E. coli take account of as many sources of ATP as possible, which include substrate level phosphorylation and ATP formed

from the conversion of carbon source to monomers.

The basic difficulty remains unresolved, however. Determination of the aerobic Y_{ATP} depends on a knowledge of the P/O ratio, and the only valid method of determining the P/O ratio in growing cells seems to involve assumption of the value of Y_{ATP}. Stouthamer & Bettenhaussen (1975) make mention of a method worked out by the same group, as yet unpublished, which allows calculation of P/O ratios (and hence the aerobic Y_{ATP}) without making the assumption that Y_{ATP} is the anaerobic value. Whether this method requires any fundamental unjustified assumptions remains to be seen.

Insufficient credit has been given to the work of von Meyenberg (1969), which described a method of calculating each of Y_{ATP} and the P/O ratio for S. cerevisiae without a prior assumption of the value of the other. The only assumption which von Meyenberg made was one which is implicit in all other calculations in any case viz. that the Y_{ATP} and the P/O ratio are the same for different values of μ. The method was suitable in this particular case because of the fact that as μ increased, the proportion of glucose fully oxidised decreased, instead of the proportion of energy provided by oxidative phosphorylation being a constant fraction of ATP produced as occurs in most other cases (e.g. Stouthamer & Bettenhaussen, 1975). Essentially, growth at any particular μ was described by an equation in which both the P/O ratio and Y_{ATP} were unknown. The only value of the P/O ratio which allowed Y_{ATP} to remain constant for all μ was found to be 1.1 ± 0.05, for which the corresponding Y_{ATP} was found to be 12.0 ± 0.5 g/mole. This value of Y_{ATP}, within the range of anaerobic values of Y_{ATP} obtained by Bauchop & Elsden, suggests that the method was valid, and that similar calculations should be possible for any organism which has a different metabolic pattern at different μ.

Without doubt the most intractable outstanding problem concerned with the study of molar growth yields is that highlighted by Gunsalus & Shuster (1961), Senez (1962), Forrest & Walker (1971) and Stouthamer (1973) of the discrepancy between experimentally derived values of Y_{ATP} and the yield of cells one would expect when the ATP consumption of all the known energy-utilising reactions of the cell is summed. Forrest & Walker calculated a theoretical Y_{ATP} of 27 g/mole for the synthesis of cells from monomers, compared with the Bauchop & Elsden experimental value of ~10 g/mole. Stouthamer (1973) revised the calculations of Forrest & Walker in the light of the most recent findings on the energy required for polymer synthesis and turnover, and additionally incorporated ATP requirements for transport, monomer synthesis and transhydrogenation. The results of this painstaking study were very similar to all of the others, as the theoretical Y_{ATP} for synthesis of cells from monomers was found to be 31.9 g/mole and, from glucose, 28.8 g/mole.

If these values for the theoretical Y_{ATP} are valid, the conclusion must be drawn that only 1/3 of the energy-utilising reactions of micro-organisms are known, which is a rather humbling admission for a biochemist! Stouthamer & Bettenhaussen (1973) obtained a Y_{ATP}^{max} for A. aerogenes consistent with the theoretical values of Y_{ATP}, but recent work (Stouthamer & Bettenhaussen, 1975) has shown that, as the large maintenance coefficient which led to the extrapolated value of Y_{ATP}^{max} was almost certainly not valid, this Y_{ATP}^{max} is not a real value, and the discrepancy between experimental and theoretical values of Y_{ATP} remains.

A fascinating study of this problem has recently been made with Bdellovibrio bacteriovorus (Rittenberg & Hespell, 1975). This organism is a parasite of E. coli which grows in the periplasmic

space on soluble compounds synthesised by the host, such as amino acids, nucleotides and fatty acids. Taking into account the unusual nature of the monomers available, Rittenberg & Hespell calculated that the expected Y_{ATP} should be 35.6 g/mole. The measured values of Y_{ATP} were 18.5 for a single cycle experiment and 25.9 g/mole for multicycle experiments, much higher than previous measured values for other organisms. The assumption was made that the P/O ratio was 3, and the authors observed that if the P/O ratio was less than this, the Y_{ATP} values would be larger than the theoretical value. While these results are superficially satisfying, the methods used in the experiments were not as well established as methods used in other molar growth yield studies, mainly because of the unusual nature of the organism. Theoretical difficulties also exist. For example, it was shown that nucleotides synthesised by the host were incorporated by B.bacteriovorus. The possibility that, as well as nucleoside monophosphates, nucleoside triphosphates and particularly ATP can diffuse into the periplasmic space is not discussed, and may well have important consequences in the interpretation of the results.

In summary, the main outstanding problems in molar growth yield work are firstly that due to their interdependence, neither the P/O ratio nor the aerobic Y_{ATP} can be easily determined and secondly that experimentally derived values of Y_{ATP} are very much smaller than predicted by purely theoretical considerations.

One of the aims of the work presented in this thesis was to attempt to resolve these problems by the determination of molar growth yields in terms of carbon source and oxygen for E. coli.

1. Microbiological Techniques

1.1 Organisms

The majority of this work was done with Escherichia coli ATCC 15224 (ML308), which has the genetic structure $i^{-}z^{+}y^{+}a^{+}$ for the lac operon. Several experiments required the use of Escherichia coli ATCC 15223 (ML30), which is the wild type strain with genetic structure $i^{+}z^{+}y^{+}a^{+}$. The former strain produces a faulty lac repressor, resulting in the constitutive synthesis of the products of the lac operon (β-galactosidase, β-galactoside permease and thiogalactoside transacetylase). The latter strain can only produce these enzymes in the presence of the inducer, allolactose, a product of the action of β-galactosidase on lactose (Jobe & Bourgeois, 1972).

These strains were obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland, U.S.A.) and characterised by bacteriological tests described (Cowan and Steel, 1965).

1.2 Reconstitution and storage of organisms

The organisms were obtained as lyophilisates in sealed, evacuated glass ampoules. These were opened as recommended (National Collection of Industrial Bacteria Catalogue, Aberdeen) and the cells reconstituted by the addition of a few drops of sterile nutrient broth. The reconstituted culture was inoculated into 10ml of sterile nutrient broth in a 25ml MacCartney bottle and incubated at 37° for 24h, then plated on nutrient agar and incubated at 37° for 24h. A typical clone was picked off, transferred to nutrient broth and grown as before. The nutrient broth cultures were tested for homogeneity both microscopically and by plating out on nutrient agar containing

10µg/ml 5-bromo-4-chloro-indoxyl-β-galactoside (BC1G agar). The strain constitutive for B-galactosidase produces only deep blue clones on BC1G agar, whereas the inducible strain produces clones which are faintly blue in the centre, becoming colourless towards the edges.

A homogeneous nutrient broth culture was used to inoculate 10ml of cooked meat medium in 25ml MacCartney bottles which were again incubated at 37° for 24h. These were then stored at 4° as a long term stock culture.

Every 3 months a cooked meat culture was used to inoculate a number of nutrient broths and these were grown at 37° for 24h, plated on BC1G agar to test for homogeneity then stored at 4°. A fresh nutrient broth was used each month for the preparation of inocula.

1.3 Preparation of specifically trained inocula

Complete defined medium, prepared as described in section 2.5.1, was inoculated with 3 drops of a stock nutrient broth culture and grown on an orbital shaker (Griffin & George Ltd., Wembley, Middlesex) at 37° for 16h. 1ml of this 1st passage was subcultured into 100ml of identical medium and grown under the same conditions for 8h (2nd passage). Within 24h of beginning an experiment, the 2nd passage was subcultured into a 3rd passage and grown as before. This was stored at 4° until required.

1.4 Centrifugation of cells

Cells were routinely harvested by centrifugation at 11,600g for 10 min at 4° in an MSE High Speed 18 refrigerated centrifuge (Measuring and Scientific Equipment Ltd, London). Cells were resuspended in chilled buffer homologous to the growth medium. Where the maintenance of sterility was required, cells were transferred aseptically to a

sterile 50ml polycarbonate centrifuge tube (MSE, London) and resuspended in sterile chilled buffer.

2. Media

2.1 Cooked meat medium

This was prepared from Oxoid dehydrated material. A tablet was soaked in 10ml glass distilled water for 15 min. in a 25ml MacCartney bottle, sterilised by autoclaving at 15 p.s.i. and stored at 4°.

One litre of cooked meat medium contained:

Peptone	10g
'Lab. Lemco' beef extract	10g
Neutralised heart tissue	30g
Sodium chloride	5g
Final pH 7.4	

2.2 Nutrient broth medium

Nutrient broth was prepared from Oxoid dehydrated granules. One litre of nutrient broth contained in distilled water:

'Lab. Lemco' beef extract	1g
Yeast extract	2g
Peptone	5g
Sodium chloride	5g
Final pH 7.4	

Nutrient broth was dispensed 10ml into 25ml MacCartney bottles, sterilised by autoclaving at 15 p.s.i. and stored at 4°.

2.3 Nutrient agar medium

Nutrient agar was prepared using Oxoid dehydrated material 15g of agar was added to 1 litre of nutrient broth medium and dissolved by boiling for 15 min. Agar was sterilised by autoclaving at 121°. and poured into petri dishes in a laminar flow hood (Microflow Ltd., Fleet, Hants) and allowed to solidify. Plates were stored at 4°.

2.4 BC1G agar medium

BC1G was dissolved in dimethyl formamide at a concentration of 2mg/ml and added to nutrient agar to a final concentration of 10µg/ml. The solution was mixed, poured into petri dishes in a laminar flow hood and allowed to solidify. Plates were stored at 4°.

2.5 Defined media

Defined media were prepared by several different procedures. All solutions were dissolved in glass distilled water.

2.5.1 Media for training of inocula

These media were prepared by mixing 3 components:

I PNS medium contained 66.7mM potassium dihydrogen orthophosphate and 16.7mM ammonium sulphate. Where necessary, sodium chloride was added to the required concentration (normally 0.83M for final 0.5M). This solution was adjusted to pH 7.0 with sodium hydroxide and dispensed 60ml into 500ml conical flasks, closed with polystyrene foam bungs and autoclaved at 121°.

II $FeSO_4$ solution contained 0.8mM ferrous sulphate adjusted to pH 2.0 using hydrochloric acid and was sterilised by autoclaving at 121°.

III Carbon source/Mg^{2+} medium contained 1.25mM magnesium sulphate. The concentration of carbon source was one of the following, according to requirements:

glucose	25mM
malate	50mM
lactose	12.5mM
glycerol	50mM

This combined medium was dispensed in 40ml batches before autoclaving at 109°.

Complete defined medium was prepared by adding aseptically 40ml of solution III and 1.25ml of solution II to 60ml of solution I

2.5.2 Media for batch culture growth experiments

This was prepared as 4 separate components:

I P 380ml 42.1mM potassium dihydrogen orthophosphate pH 7.0 sterilised by autoclaving at 121°.

II MgNS contained 40mM magnesium sulphate and 800mM ammonium sulphate sterilised as for solution I.

III $FeSO_4$ 0.8mM ferrous sulphate pH 2.0 sterilised as for solution I.

IV Carbon source was prepared at high concentration and sterilised at 109°.

Minimal salts medium was prepared by adding 5ml each of solutions II and III to solution I and making the final volume to 400ml using the carbon source and sterile distilled water.

The composition of defined medium prepared in this way, and also

as in 2.5.1 was:

KH_2PO_4	40mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	0.5mM
$FeSO_4$	10μM

with NaOH to pH 7.0, and carbon source to the required concentration.

All components were stored at room temperature.

2.5.3 Media for carbon-limiting arithmetic-type continuous culture

In order to support a higher cell density in this culture, it was necessary to increase the concentration of inorganic salts. The solutions described in 2.5.2 were sterilised separately before adding 80ml MgNS and 80ml $FeSO_4$ solutions to a final volume of 1600ml in 40mM phosphate buffer pH 7.0. Thus, the composition of this medium was:

KH_2PO_4	40mM
$(NH_4)_2SO_4$	40mM
$MgSO_4.7H_2O$	2mM
$FeSO_4$	40μM

Carbon source was pumped into the growth flask at a rate of approximately 1ml/h by a variable peristaltic pump (LKB-produkter-AB, S-161 25 Bromma 1, Sweden). The concentration of carbon source was one of the following:

glucose	0.32M
malic acid	0.64M
lactose	0.16M
glycerol	0.64M

Carbon source and phosphate buffer were sterilised in the growth apparatus (see section 6.3.1) by autoclaving at 109°.

2.5.4 Media for nitrogen-limiting arithmetic-type continuous culture

Defined salts medium containing everything required for growth apart from nitrogen source was prepared from the following components:

I P 1280ml 50mM potassium dihydrogen orthophosphate adjusted to pH 7.0 with sodium hydroxide and sterilised by autoclaving at 121°.

II S 1.0M sodium sulphate sterilised by autoclaving at 121°.

III $FeSO_4$ solution as described in section 2.5.1.

IV Mg 0.05M magnesium sulphate sterilised by autoclaving at 121°.

V Carbon source was prepared at high concentration and sterilised by autoclaving at 109°. Concentrations used were:

	glycerol	1.5M
or	glucose	0.5M
or	malate	0.6M

Simple defined medium free of nitrogen source was prepared by mixing 64ml II, 80ml III, 64ml IV and 100ml V to solution I. For malate, the volumes of I and V were 1230ml and 150ml. The composition of the medium was thus:

	KH_2PO_4	40mM
	Na_2SO_4	40mM
	$MgSO_4.7H_2O$	2mM
	$FeSO_4$	40μM
and	glycerol	93.8mM
or	glucose	31.3mM or malate 56.25mM

The nitrogen source, 70mM ammonium sulphate, was pumped into the growth flask at a rate of approximately 1ml/h by a variable peristaltic pump. The $(NH_4)_2SO_4$ solution was sterilised by autoclaving at 121°.

2.5.5 Medium for chemostat culture

This was prepared as 3 separate components:

I P/N 19.6 l 40.8mM potassium dihydrogen orthophosphate pH 7.0 and 10.2mM ammonium sulphate, sterilised by autoclaving at 121°.

II C source/Mg Carbon source was prepared at high concentration in 100mM magnesium sulphate, and sterilised by autoclaving at 109°.

III Chel-metals were prepared as follows: (C.A. Fewson - personal communication) -
In 500ml distilled water were dissolved 50g nitrilotriacetic acid ("chelNTA"), 1.10g $FeSO_4.7H_2O$, 50mg $Na_2MoO_4.2H_2O$, 50mg $MnSO_4$, 50mg $ZnSO_4.7H_2O$, 25mg $CuSO_4.5H_2O$, 25mg $CoCl_2.6H_2O$, 125ml 5M NaOH, to pH 7.0 with 5M HCl and then distilled water to 1 litre. The solution was dispensed 100ml in bottles and sterilised by autoclaving at 109°.

Simple defined medium was prepared by aseptically adding 100ml each of II and III to I. The composition of this medium is the same as described in section 2.5.2 excepting the addition of some trace metals.

3. pH Measurement

All solutions were adjusted to the required pH using either sodium hydroxide or hydrochloric acid while monitoring pH with an EIL 23a or an EIL 2320 pH meter (EIL Ltd., Cambridge). Standard buffer solution was prepared using buffer solution tablets (Burroughs Wellcome & Co. Ltd.). 40ml portions of the buffer standard were autoclaved at 15 p.s.i. then stored at room temperature. Each day a fresh 40ml portion was used for calibration of the pH meters.

The pH of cultures and small volumes was measured using a micro-assembly attached to the EIL 2320 pH meter.

4. Sterilisation

The sterilisation of media was carried out using one of two procedures.

4.1 Autoclaving

Solutions were sterilised in a pressure chamber (Manlove Alliott, Nottingham, England) using steam supplied by a Speedylec-electrode boiler (Bastian & Allen, Harrow, England). The conditions for sterilisation had been determined using thermocouples in the solutions (Fewson, unpublished results). Both the temperature and the time of autoclaving depended on the nature and volume of the solutions being sterilised. Sterilisation was always checked using Browne steriliser control tubes - type one, black spot (Browne Ltd., Leicester, England).

4.2 Filtration

Sterilisation by filtration was carried out using Sterifil filter holders of 250ml capacity fitted with a 0.22µm pore size Millipore

filter (Millipore Corp., Massachussetts, U.S.A.). Once assembled, the unit was sterilised by autoclaving at 121°.

A second procedure for smaller volumes utilised Nalge disposable filter units (0.20μ (Sybron Corp., Rochester, U.S.A.), which were of 100ml capacity and were obtained in a sterile condition.

After filtration, solutions were transferred aseptically to sterile bottles.

5. Glassware

5.1 General glassware

All glassware was cleaned before use either by boiling in 10% v/v nitric acid or by autoclaving in 1% w/v Haemosol solution (Meinecke & Co., Baltimore, U.S.A.). After either treatment all glass was rinsed with tap and distilled water and dried in an oven.

5.2 Pipettes

Pipettes were cleaned by soaking first in 5% v/v propanol and 1% w/v Haemosol (or latterly Kirbychlor disinfectant solution (H. & T. Kirby & Co. Ltd., Mildenhall, Suffolk)), then in 1% Haemosol solution followed by rinsing with tap and deionised water, and drying in an oven. All pipettes were plugged with cotton wool before use.

Pipettes were sterilised by dry heat at 160° for 1¾h either wrapped in paper or in metal canisters. Canister sterilisation was checked by Browne type three steriliser control tubes. Pasteur pipettes were similarly sterilised in canisters.

5.3 Radiochemically contaminated glassware

Contaminated glassware was kept separate, and washed by a different procedure. After rinsing thoroughly with tap water, contaminated glassware was soaked in 1% Haemosol solution, then thoroughly rinsed with tap water. This was repeated twice, using fresh Haemosol solution, before the glass was finally rinsed with distilled water and dried.

Washing of contaminated glassware was confined to an area and a sink allocated for that purpose.

6. Growth

6.1 Batch culture

In batch culture experiments, 400ml of complete defined medium in a 1 litre flat-bottomed pyrex flask fitted with a side arm was inoculated and maintained at the required temperature in the apparatus described by Harvey et al. (1968). The side arm was covered by a Morton culture tube closure (Scientific Products, Evanston, Illinois) and the main neck of the flask sealed by a silicone rubber bung with a glass air inlet tube. Air from the department compressed air supply was passed into the flask at a steady rate, the flow (100-200ml/min) being monitored by a gas flow gauge (G.A. Platon, Croydon, England).

6.2 Chemostat culture

Chemostat culture is a device whereby micro-organisms can be maintained at a single specific growth rate (μ) under a variety of experimental conditions. When the cells have reached steady state, the dilution rate D (which is the quotient of flow rate and culture volume, $\frac{dv}{dt} \cdot \frac{1}{V}$) is identical to μ. All chemostats used were of the carbon-limitation type.

The growth apparatus took the form shown in Figure 1, following the general design described by Baker (1968). The 20l reservoir (Quickfit FV20L; MacFarlane Robson Ltd., Glasgow) of medium was fitted with a top plate (Quickfit MAF 2/2) to allow addition of carbon source/Mg, entry of air through a Microflow filter (Microflow Ltd., Fleet Mill, Minley Road, Fleet, Hants.) and pumping of medium to the growth pot by a Watson-Marlow peristaltic pump Type MHRE 200 (Watson-Marlow Ltd., Falmouth, Cornwall). The flow rate generated by this variable pump was measured by a 10ml pipette assembly, which was fitted with an alarm (Fisons Scientific Apparatus, Loughborough, Leics.) to prevent the feedline accidently being pumped dry. Medium entered the growth pot via a multistage capillary drip assembly in order to prevent back-growth of the culture in the feed-line. The growth pot was a 1 litre fermenting vessel (Quickfit FV1L) fitted with a Quickfit MAF 2/2 top plate. Sterile air was obtained by passing charcoal filtered air from the departmental compressed air supply through a Microflow filter, and was passed into the flask at 100-200 ml/min. One port of the top plate was fitted with a device to receive a sterile plastic syringe (Becton, Dickinson & Co. Ltd., Drogheda, Ireland) thereby allowing inoculation of the culture. A fixed outlet port was incorporated into the wall of the growth pot, through which the overflow of spent medium was pumped using a second Watson-Marlow pump operating at a greater flow rate than the dilution pump. The growth pot contents were mixed and maintained aerobic by agitation with a magnetically coupled stirrer.

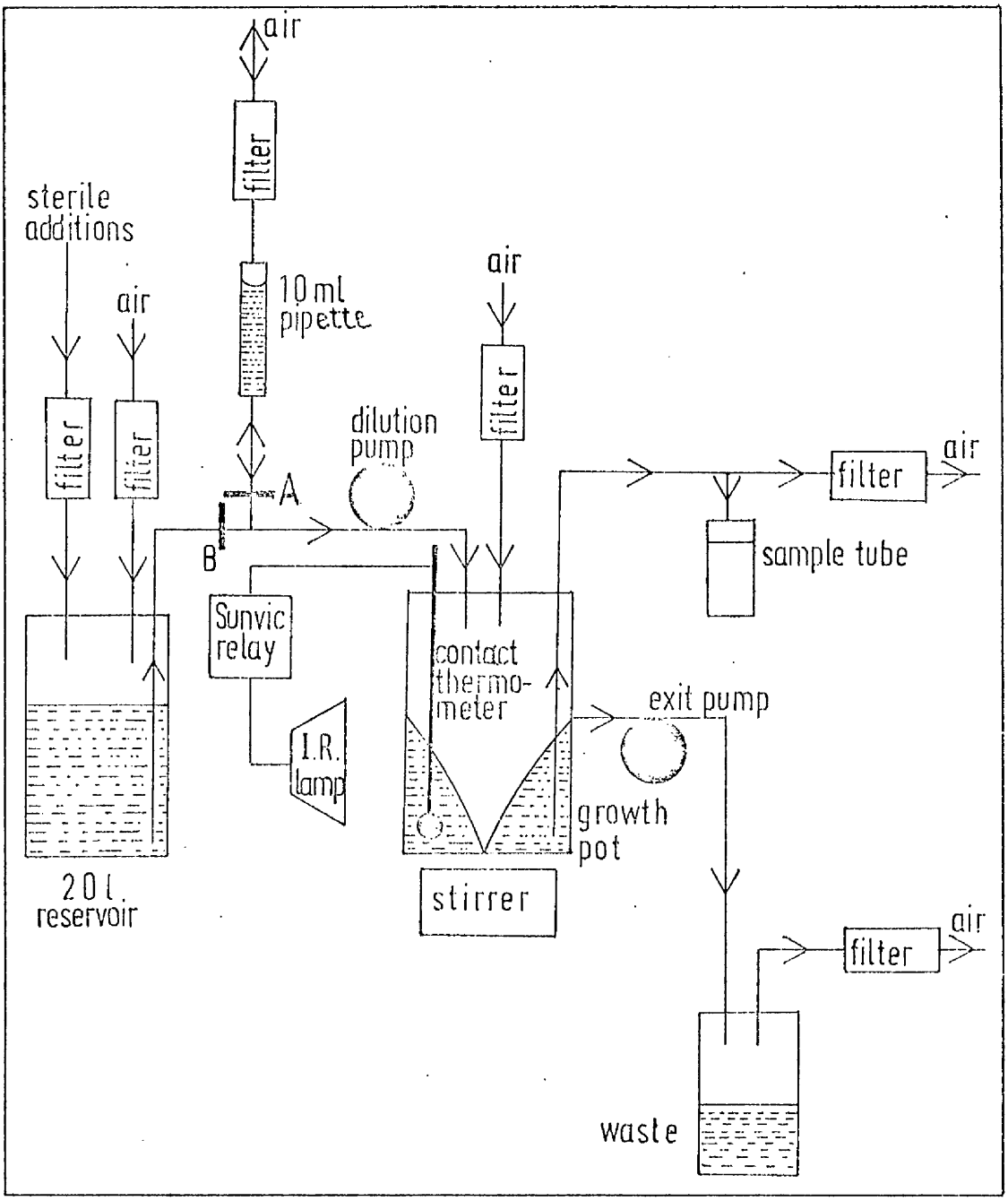
The temperature of the culture was sensed by a fixed contact thermometer (J. C. Cowlishaws, Peary Street, Manchester M4 4JB) which operated a 300W Infra-Red lamp (MacFarlane-Robson Ltd., Glasgow) via a Sunvic electronic relay (AEI type EA4M; J. C. Cowlishaws, Manchester).

Figure 1

Block diagram of chemostat culture growth apparatus

The diagram illustrates the general organisation of the continuous culture growth apparatus described in Methods section 6.2 Clips were provided at the points A and B in the diagram enabling the dilution pump to draw medium either directly from the 20l reservoir or from the 10ml pipette, which served as a flowmeter. The pipette was refilled by opening both clips.

Figure 1


air
filter
sterile additions
air
10 ml pipette
air
filter
filter
filter
dilution pump
A
B
filter
air
sample tube
Sunvic relay
contact thermo-meter
exit pump
I.R. lamp
growth pot
20 l. reservoir
stirrer
filter
air
waste

Provided that both the position of the growth pot and the stirring rate of the culture were constant, the volume of culture remained constant, usually approximately 600ml.

6.3 Arithmetic-type continuous culture

The apparatus described in this section was used for a type of continuous culture which differs from the chemostat in that μ did not remain constant even although the rate of pumping of the limiting substrate was constant. Since none of the cell material was removed from the flask, and the amount of cell material was continuously increasing, the substrate available per cell continuously decreased, and so the specific growth rate μ progressively decreased. A complete theoretical treatment of this type of culture is made in section 3 of the Development of Analytical Methods section. In that section are derived the equations necessary for the calculation of molar growth yields and maintenance coefficients in terms of carbon source and oxygen.

6.3.1 Maintenance experiments

The growth apparatus took the form shown in Figure 2.

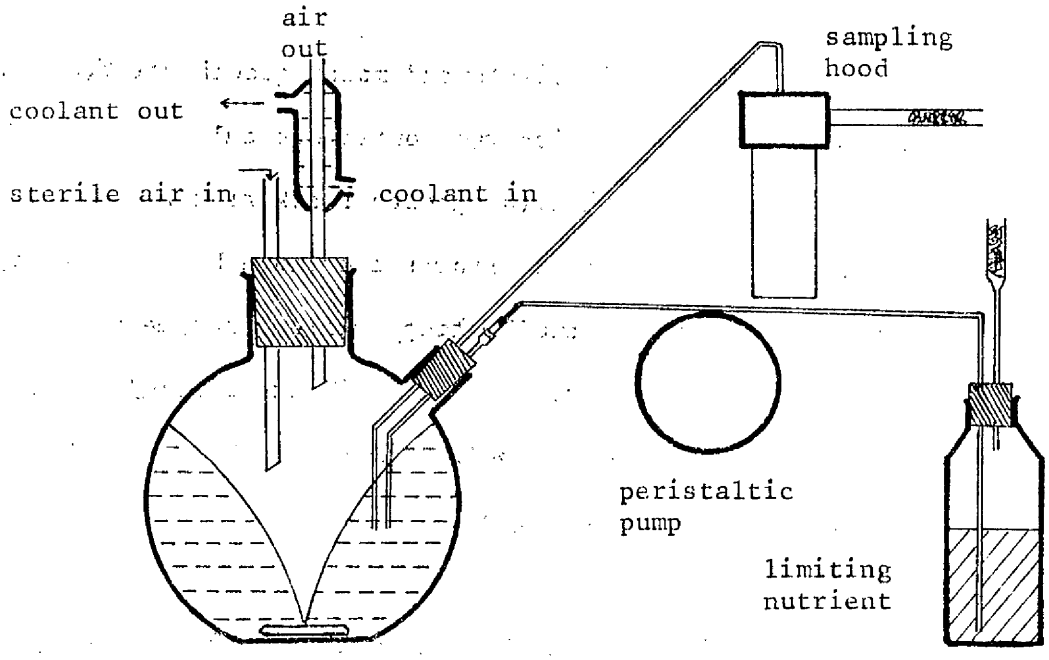
The limiting nutrient was pumped into the growth flask at a flow rate of approximately 1ml/h by an LKB Varioperpex peristaltic pump (LKB-Produkter AB, S-161 25 Bromma - Sweden). The precise flow rate was measured by a 1ml pipette assembly incorporated into the feed-line. Nutrient entered the growth flask through a disposable plastic cannula, of 0.63mm diameter (Portex Ltd., Hythe, Kent, England) which was led through a metal cannula inserted through a silicone rubber bung (Esco (Rubber) Ltd., Gt. Portland Street, London). The junction between plastic and metal cannulae was sealed using Silcoset 153 (I.C.I. Ltd., Stevenston, Ayrshire, Scotland), a silicone rubber

Figure 2 Growth apparatus for maintenance experiments

Maintenance coefficients and molar growth yields were measured using arithmetic-type continuous culture. Limiting nutrient was pumped into the growth flask at a rate of ~1ml/h, and samples were withdrawn from the culture by a sampling hood. The flow rate of limiting nutrient was measured by incorporating a T-junction and 1ml pipette assembly into the feedline in the same way as described in Figure 1. Tubing used was silicone rubber throughout.

The apparatus is fully described in Methods section 6.3.1.

Figure 2


air
out
coolant out
sterile air in
coolant in
sampling
hood
peristaltic
pump
limiting
nutrient

adhesive sealant.

The growth vessel consisted of a 2 litre flat-bottomed pyrex flask containing 1600ml of the required limiting medium and was maintained at the required temperature in the apparatus described by Harvey et al. (1968). The flask was fitted with a short sidearm which was sealed by a silicone rubber bung (Esco (Rubber) Ltd., London) as was the mouth of the flask.

Sterile air filtered by a Microflow filter (Microflow Ltd., Fleet, Hants.) entered the growth flask via glass tubing of diameter 6mm. The gas left the flask through a 7cm water-cooled condenser, then was led to the O_2 and CO_2 analysers. Ice-water was used as coolant, and this was circulated from an insulated reservoir by 3 circulating pumps in series (Universal Scientific, Plashet Road, London E.13). This precaution greatly reduced the effect of water vapour on the analysers.

The culture was sampled using a stainless steel sampling hood (L. H. Engineering Co. Ltd., Slough, Bucks.) into which sterile MacCartney bottles could be inserted. A device was incorporated into the bung in the mouth of the flask to allow replacement of the sample volume with sterile limiting medium.

The frequency of sampling and the sample volume were minimised to reduce perturbation of the culture. The normal sample volume was 7ml, taken at a frequency of 4 samples each 24h, with a minimum interval between samples of 2h. Samples were examined for cell density, pH, and excretion products.

6.3.2 Radiochemical experiments

Turnover experiments using radio-labelled cells were done in a simplified, reduced scale version of the apparatus described in the

last section. No gas analysis was done, and sampling was more frequent, the sample volumes not being replaced.

The limiting nutrient was pumped at a rate of approximately 1ml/h by an LKB Varioperpex pump as before. The growth vessel was a 1 litre flat-bottomed pyrex flask, contained 400ml of the required limiting medium and fitted with a side arm closed by a stainless steel Morton culture tube closure (Scientific Products, Evanston, Illinois).

Samples were removed through the side arm using a plastic pipette (Falcon Plastics, Oxnard, CA93030, U.S.A.) which was rinsed with culture before the sample was removed. Samples were assayed for cell density and radioactivity as described elsewhere.

7. Sampling of cultures

Samples were removed from cultures, chilled in an ice-water slurry and processed for assay of enzymes within 30 min.

Samples removed for the assay of substrates and excretion products were treated by blowing 4ml of culture on to 1ml ice-cold 30% w/v perchloric acid, mixing, then, after standing on ice for 10 min, 3ml of chilled potassium hydroxide was added to return the pH to 7.0. The potassium hydroxide ($\sim$1M) was diluted to the required concentration on the day of each experiment such that 3ml neutralized exactly 1ml of 30% w/v perchloric acid. When the potassium perchlorate had precipitated, the samples were decanted into 15ml centrifuge tubes (Corning Glass Works, New York, U.S.A.) and centrifuged at 11,600g and 4^{o} for 10 min in an MSE 18 refrigerated centrifuge (M.S.E., Crawley, England). The supernates were decanted into plastic vials (M. & H. Plastics (Romford) Ltd., Romford, Essex), frozen, and stored at -10^{o} until assayed.

Dilution of the culture sample was carried out by measuring 5ml volumes by pipette and making up to 25, 50, 100 or 250 ml in a volumetric flask.

8. Measurement of Growth

8.1 Turbidity

Cell density was determined turbidimetrically on samples (5ml) taken from the culture on to 1 drop of formaldehyde solution (40%). The absorbance at 420nm was measured in glass cuvettes (Type 1, 1cm light path) (Ross Scientific Co., Ltd., Hornchurch, England) using an SP800 double beam spectrophotometer (Unicam Instruments Ltd., Cambridge, England) fitted with a Servoscribe potentiometric chart recorder (Smiths Industries Ltd., Wembley, England).

By dilution of an initial cell culture, a calibration curve relating observed turbidity to cell density was drawn (Fig.3). This relationship was found to be linear up to an optical density of 0.2. Above this cell density, internal light scattering caused an increased transmission of light to the collector and therefore a progressive decrease in the optical density/cell density ratio was evident as cell density increased.

The shape of this calibration curve is governed only by the geometry of the spectrophotometer (Koch, 1970). The calibration curve was found to be unchanged after a period of more than two years after the initial calibration.

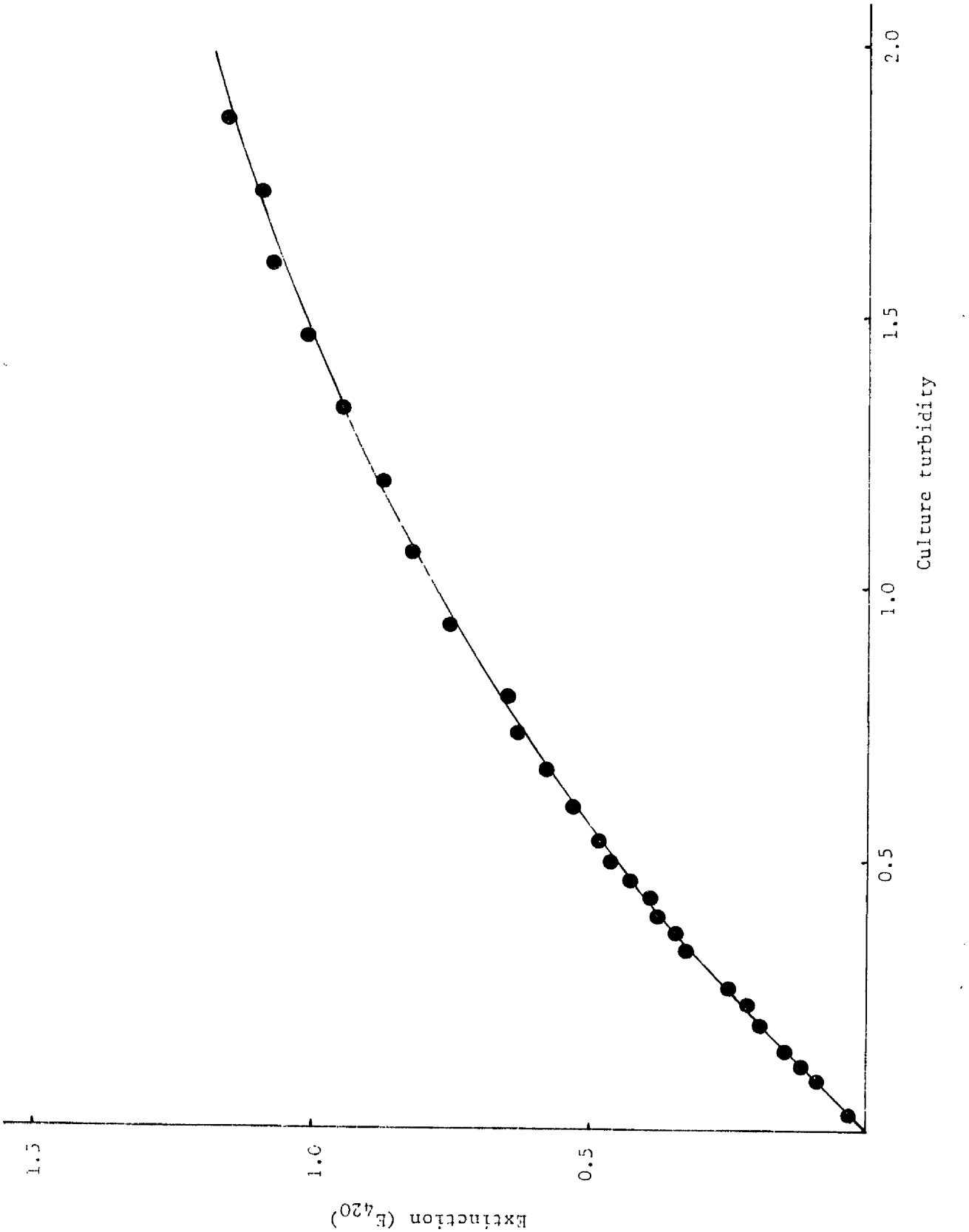
Although this calibration curve was adequate for the determination of turbidity without dilution during a logarithmic growth experiment, it was found to be unsatisfactory for the accurate measurement of turbidities greater than 1, in particular during maintenance experiments and chemostat cultures. In these cases, the sample from the culture was carefully diluted in the appropriate phosphate buffer to an optical density of 0.1-0.2, and read using the maximum possible scale expansion factors of the spectrophotometer. It was found

Figure 3

Turbidity calibration curve

Cultures of a wide range of turbidity were read at 420nm in an SP800 double beam spectrophotometer against air as a blank. Portions of the culture were diluted in 40mM phosphate buffer pH 7.0 to give an E_{420} of less than 0.2 at which level extinction is directly proportional to the density of the suspension. From these data the calibration curve was drawn.

Figure 3



necessary to check the zero of the spectrophotometer frequently. Consistency of results was also improved by ensuring that, for every turbidity reading, the procedure used was absolutely identical, and that one, scrupulously clean, cuvette was used throughout.

8.2 Dry weight

35ml samples for the estimation of dry weight were harvested by centrifuging at 11,600g at 4^{o} for 10 min in 50ml polycarbonate centrifuge tubes (M.S.E., London). The cells were washed twice by resuspending the cells in distilled water and centrifuging as before. The final pellet was resuspended in a minimal volume of distilled water and transferred to a glass vial. The vial and contents were dried in an oven at 105^{o} for 18h, then transferred to a desiccator for 30 min to cool before weighing on a semi-microbalance (Stanton Instruments Ltd., London).

9. Measurement of molar growth yields and maintenance coefficients in chemostat culture

In chemostat culture experiments, the parameters measured were flow rate, culture volume, turbidity and carbon source concentration. In order to determine the dilution rate, the rate of supply of medium was measured, and expressed in ml/h. The volume of culture (ml) was measured at the end of the experiment, and the dilution rate D (h^{-1}) was calculated by dividing the rate of supply by the culture volume. After a minimum of 5 doubling times at any given dilution rate, the turbidity of the culture was measured. Although the culture may not have reached steady state for that particular D, it was found in several experiments that the value of the turbidity did not change significantly after this time. After 5 generations, only 3% of the original cell material will be present in the culture. The concen-

tration of the limiting carbon source (glycerol) was measured for each reservoir used. The molar growth yield Y was calculated by converting turbidity to g dry weight/ml and dividing this quantity by the concentration of glycerol. Y was therefore expressed in g dry weight cells/mole glycerol.

10. Analysis of culture gas phase

The O_2 and CO_2 content of the gas leaving the growth flask was measured during all maintenance experiments by a method similar to that described by Hamilton and Holms (1970). In separate experiments, the effluent gas was examined for hydrogen content using a palladium catalyst, and collecting the resultant water vapour in anhydrous magnesium sulphate.

10.1 O_2 consumption and CO_2 evolution

This method consisted of measuring the O_2 and CO_2 content of the effluent gas in a continuous flow system, and thereby calculating O_2 consumption and CO_2 evolution.

10.1.1 Measurement of gas flow rate

The success of this method of measuring gas exchange depended critically on the measurement of gas flow rate and on the constancy of the air supply, which would ideally be free from pulsing. The flow system finally adopted took the form shown in Fig.4.

Air from the departmental compressed air supply was first passed through a reduction valve (British Industrial Gases Ltd., Enfield, Middlesex) then a Flostat Minor Mark II (G.A. Platon Ltd., Croydon, Surrey) to reduce pressure fluctuations. The air was then filtered through charcoal. A continuous flow meter fitted with a flow control valve (GAP meter; G.A. Platon, Croydon, Syrret) was then

Figure 4

Gas flow system

Gas flow was regulated, stabilised and measured before entering the growth flask, then cooled to remove water vapour before being passed to the gas analysers. The apparatus used is described in Methods section 10.

Figure 4 Gas flow system

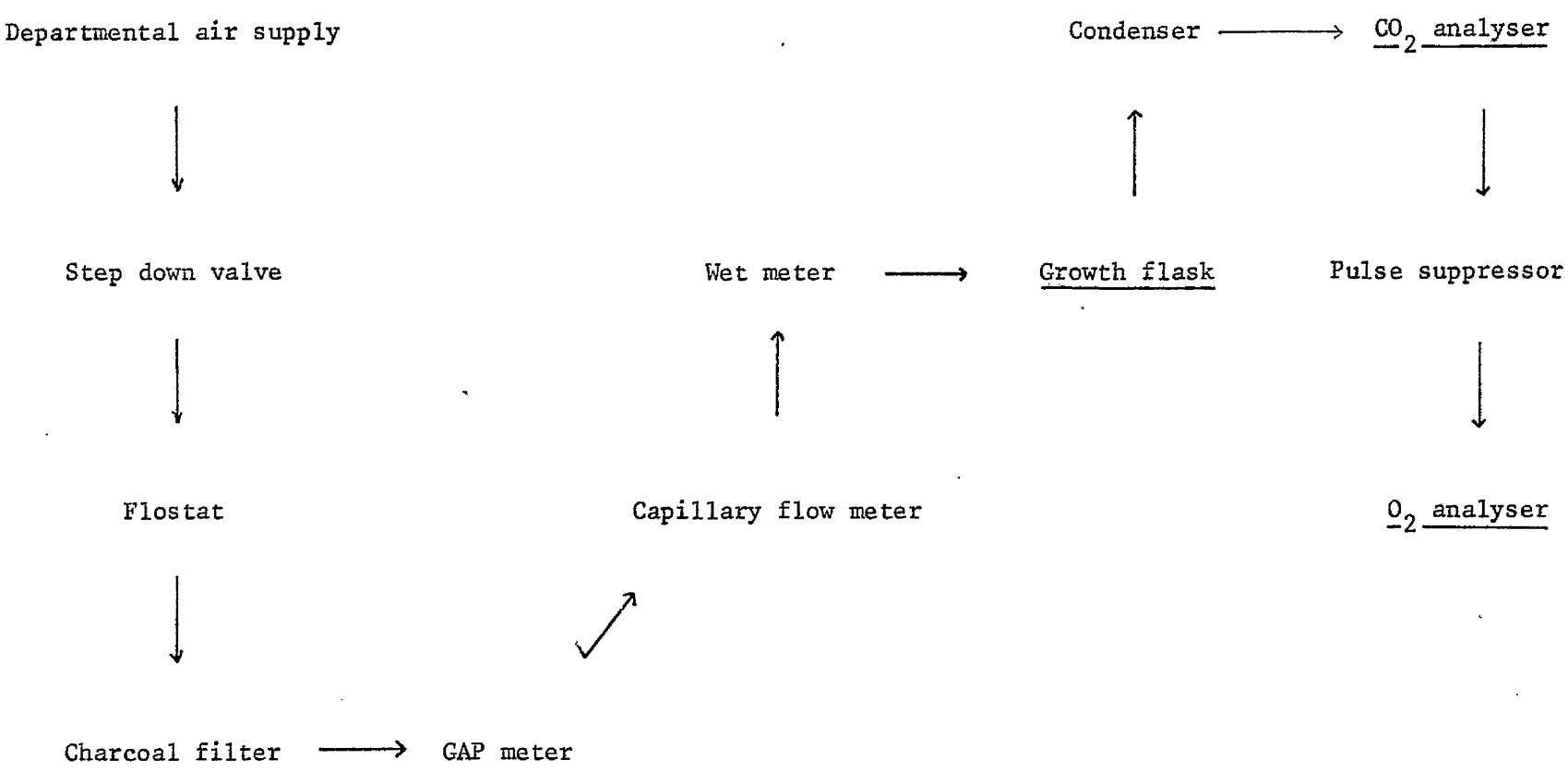


Figure 5 Pulse suppressor

The frame of this twin channel pulse suppressor was made from perspex. The material used for the membrane (shaded areas) on the outside and separating the two chambers was taken from vinyl examination gloves (Travenol Laboratories Ltd., Thetford, Norfolk). The pulse suppressor was inserted into the flow system between the CO_2 and O_2 analysers.

Figure 6 Measurement of air flow by wet meter

At each sampling time during a maintenance experiment, the volume registered on the wet meter was recorded. The entry flow rate in this case was 5.516 1/h.

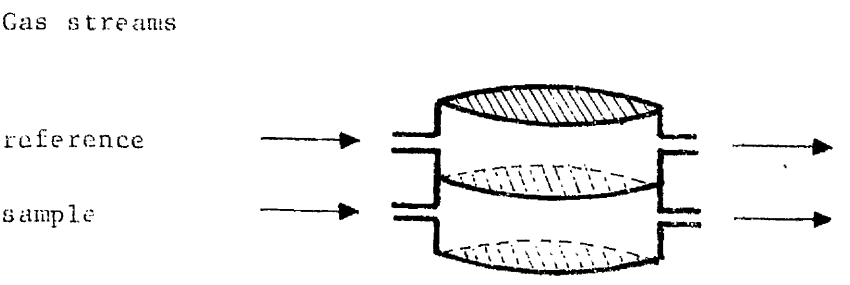


Figure 5

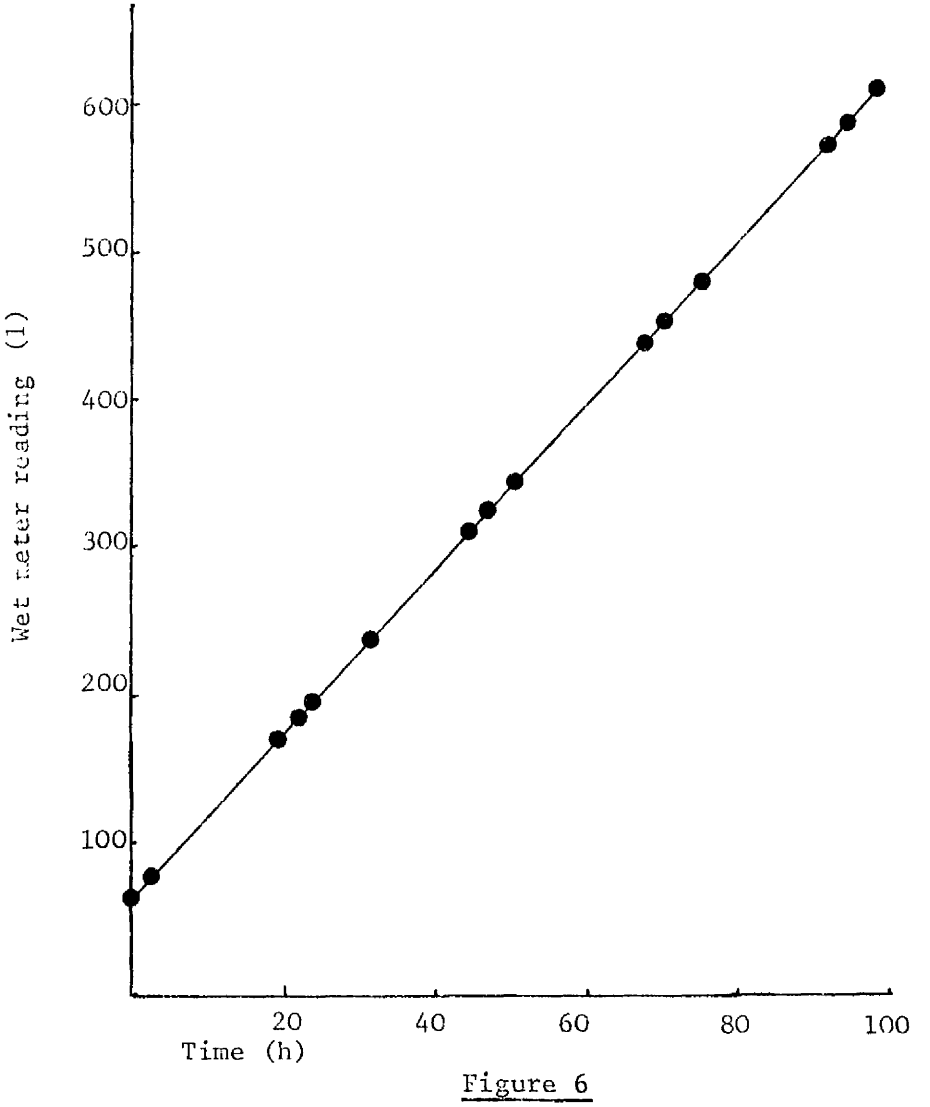


Figure 6

used as a fine control and also for an approximate indication of the flow rate. A more precise measurement of the flow rate was given by a calibrated capillary flow meter (Gallenkamp, London) filled with a light oil (Diala Oil B; Shell, Bishopbriggs, Lanarkshire). The total volume of air passed was registered on a wet type gas meter (Alexander Wright & Co. (Westminster) Ltd., Tooting High Street, London) filled with Diala Oil B. The air temperature was continuously monitored using a thermograph (Cambridge Instruments Co. Ltd., North Finchley, London).

The air was passed into the flask through a Microflow filter at a rate of approximately 100ml/min and the effluent gas was stripped of water vapour by a condenser maintained at 2^{o}. A GAP meter indicated the approximate effluent flow rate, before the gas was passed to an infrared carbon dioxide analyser (MSA Lira 300; Mine Safety Appliances Co. Ltd., Glasgow). Finally, the effluent gas was passed through a pulse suppressor (Figure 5) to the OA 184 oxygen analyser, and then to the atmosphere.

The reference air stream followed a parallel flow system, except that it bypassed the CO_2 analyser. The rate of flow was adjusted to approximately 100ml/min using a capillary flow meter.

Once an experiment had started, no alterations were made to any of the settings. At the time each sample was withdrawn from the growth flask, the readings on both the sample and reference wet meters were recorded. Figure 6 shows the results from one maintenance experiment, which demonstrate the success of the gas flow system.

10.1.2 Measurement of O_2 and CO_2 content of gas

The partial pressure of CO_2 in the effluent gas was measured by an MSA Lira 300 infrared analyser, set to read 0-1% carbon dioxide.

The output from the analyser was connected to a Servoscribe potentiometric chart recorder (Smiths Industries Ltd., Wembley, Middlesex) fitted with a fibre pen (W & W electronic 11-0034; Gilford Instruments Ltd., Morden, Surrey). The analyser was modified to include a thermistor proportional temperature control circuit, which greatly reduced temperature fluctuation in the aluminium block housing the sample and reference cells and detector, thereby reducing oscillations in the output. The instrument could reproducibly measure changes of 0.01% carbon dioxide.

The change in partial pressure of O_2 in the effluent gas compared to the reference flow was measured using an OA184 oxygen analyser, an instrument which measures the paramagnetism of each gas stream, and expresses the output as a difference in partial pressure between the two streams. Thus a 10mV output covers the range 19.96-20.96% oxygen. This was also recorded using a Servoscribe potentiometric chart recorder fitted with a fibre pen.

The operating zero of each analyser was checked periodically during the course of an experiment. The sample gas flow was passed through a flask identical to the growth flask but containing distilled water.

10.1.3 Calculation of O_2 consumption and CO_2 evolution of the culture

The following variables must be known in order to convert O_2 and CO_2 partial pressures of effluent gas to measurements of respiration:-

a - O_2% of effluent gas

b - CO_2% of effluent gas

T - air temperature (^oK)

f - flow rate of air entering flask (ml/min)

p - pH factor (correction for dissolved CO_2)

v - volume of culture (l)

ΔCO_2% min - rate of change of CO_2% in effluent gas

The formula for the calculation of O_2 consumption is then

$$O_2 \text{ uptake} = \left[\frac{20.96}{79.04}(100-b-a) - a\right] \frac{f.273.10^{-2}.79.04}{(100-b-a).T.22.4}$$

$$CO_2 \text{ evolution} = b.\frac{f.273.10^{-2}.79.04}{(100-b-a)\ T.22.4} + p.v.\Delta CO_2\%/\text{min mmoles/min}$$

Thus, the maximum level of gas exchange which can be measured is 40.9 μmol/min each of oxygen and carbon dioxide at 25° at a flow rate of 100ml/min.

10.1.4 Calibration of carbon dioxide analyser

Carbon dioxide partial pressure was measured over the range 0-1%. The measurement depends upon absorption of infra-red radiation by carbon dioxide passing through a flow cell which reduces the carbon dioxide specific radiation reaching the detector. The detector contains carbon dioxide which absorbs the unabsorbed carbon dioxide specific radiation coming through the flow cell and so induces a pressure change which is compared with the pressure of a second detector maintained under standard conditions to give a measure of the carbon dioxide in the sample. Since absorption depends on the total carbon dioxide present in the flow cell an increase in pressure increases the carbon dioxide reading. The instrument can reproducibly

measure changes of 0.01% carbon dioxide.

The equipment was calibrated using two gas mixing pumps (Wösthoff, Bochum, W. Germany) connected in series (Figure 7a), taking the carbon dioxide content of air as the base line. Using this system, the partial pressure of carbon dioxide in the sample gas was varied in steps of 0.1% over the range 0-1%. These values were used to construct a calibration curve (Figure 7b), which was non-linear. Thus, the experimental readings on the chart recorder had to be corrected using the calibration curve.

Some aspects of the use of carbon dioxide analysers and their calibration have been described (Elsworth, 1970).

10.1.5 Calibration of oxygen analyser

The OA184 oxygen analyser is a double channel instrument which can detect changes in oxygen content of 1 part in 2000 (0.01% at 20%). It was at this level of sensitivity that it was used. The measurement depends on the force required to maintain a small hollow dumb-bell in a magnetic field. When surrounded by oxygen, a paramagnetic gas, the dumb-bell rotates in the magnetic field. The magnitude of the feedback current is related to the oxygen partial pressure in the gas. The OA184 contains one of these systems in each channel and compares gas from one channel with that from the second channel.

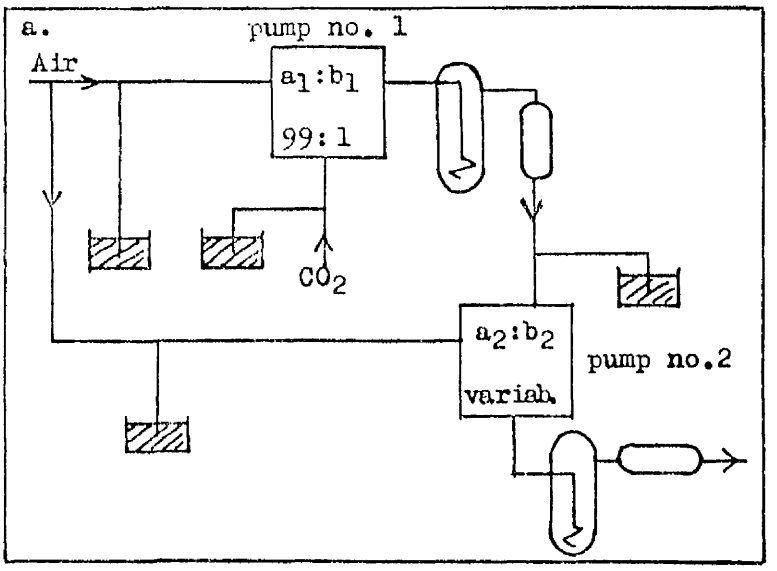
The analyser has been calibrated using gas mixing pumps, and was found to have a linear response over the range 19.96 to 20.96% (Hamilton, 1972).

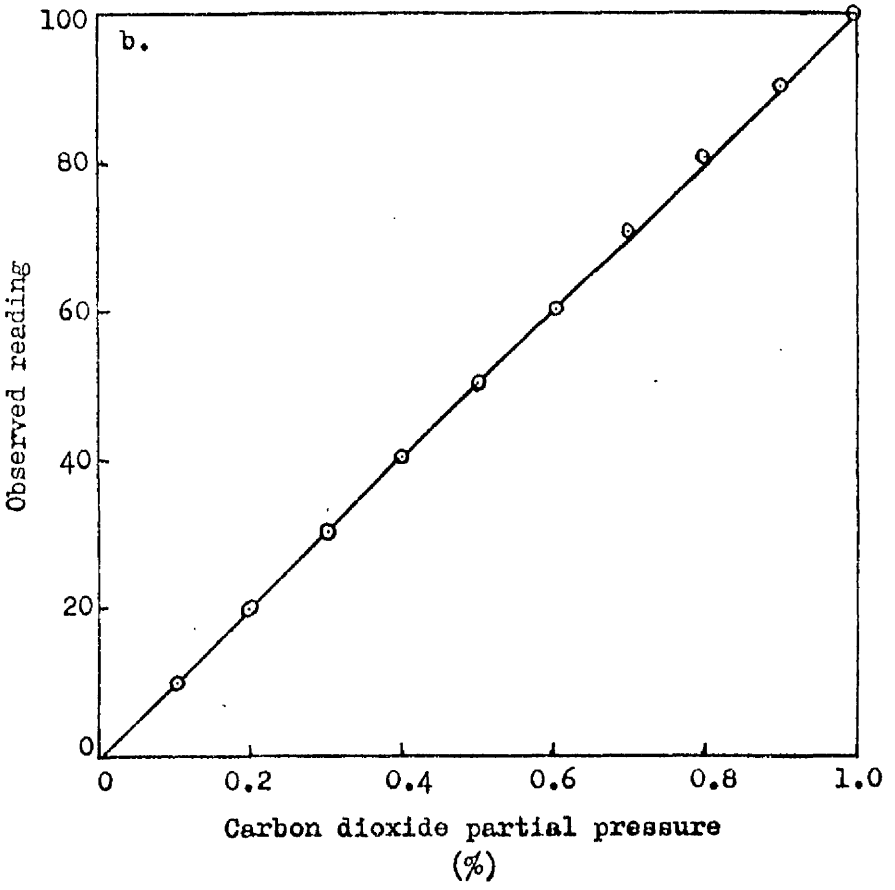
Since the 0-100% scale was also found to be linear, the analyser could be calibrated without the use of gas mixing pumps using

Figure 7

Calibration of carbon dioxide analyser

The first stage of the Wösthoff pumps was used to produce 1% research grade carbon dioxide in air which was further diluted in the second stage to yield gas mixtures within the range 0 - 1% (Figure 7a). A small portion of the effluent mixtures was fed through the gas apparatus and the response of the carbon dioxide analyser recorded. Comparison of these data with the known composition of the mixtures permitted the calibration curve (Figure 7b) to be drawn. Apparatus was calibrated before every occasion of use.


a.
pump no. 1
Air
$a_1:b_1$
99:1
CO_2
$a_2:b_2$
variab.
pump no.2


b.
100
80
60
40
20
0
Observed reading
0
0.2
0.4
0.6
0.8
1.0
Carbon dioxide partial pressure
(%)

oxygen-free nitrogen (OFN) as a zero standard and air as 20.96% oxygen. OFN was passed through both measuring cells at 100ml/min. The reference zero was set to read 0% oxygen, and the sample zero was adjusted to read zero with respect to the reference cell. Air was then passed through both cells at the experimental flow rates. The reference span was adjusted to 20.96% oxygen, and the sample span was again adjusted to read a zero difference with respect to the reference cell. For experimental readings, the analyser was set to read a difference of 0-1% between the measuring cells.

Elsworth (1970) has described some aspects of the design and calibration of oxygen analysers.

10.2 Measurement of H_2 evolution

Hydrogen was measured in the apparatus shown in Figure 8. Air was passed through the growth flask at a rate of 100-200ml/min, and the effluent gas was dried by passing through silica gel. The dry gas was then passed over 'M' catalyst (Engelhard Chemical Division, Cinderford, Glos.) to oxidise any hydrogen gas present to water. The catalyst and glass tubing between the catalyst and the first water vapour trap were heated to a temperature of $>100^{o}$ by heating tape (Electrothermal Engineering Ltd., Neville Road, London E.7) to prevent condensation. The resulting gas was passed through a pre-weighed water trap, consisting of a 1cm diameter glass tube containing $\sim$4g anhydrous magnesium perchlorate. The gas was then passed through a second identical trap. The traps were weighed after a given sampling interval in order to calculate hydrogen production from the weight of water produced. It was found that the second trap did not increase in weight at any time.

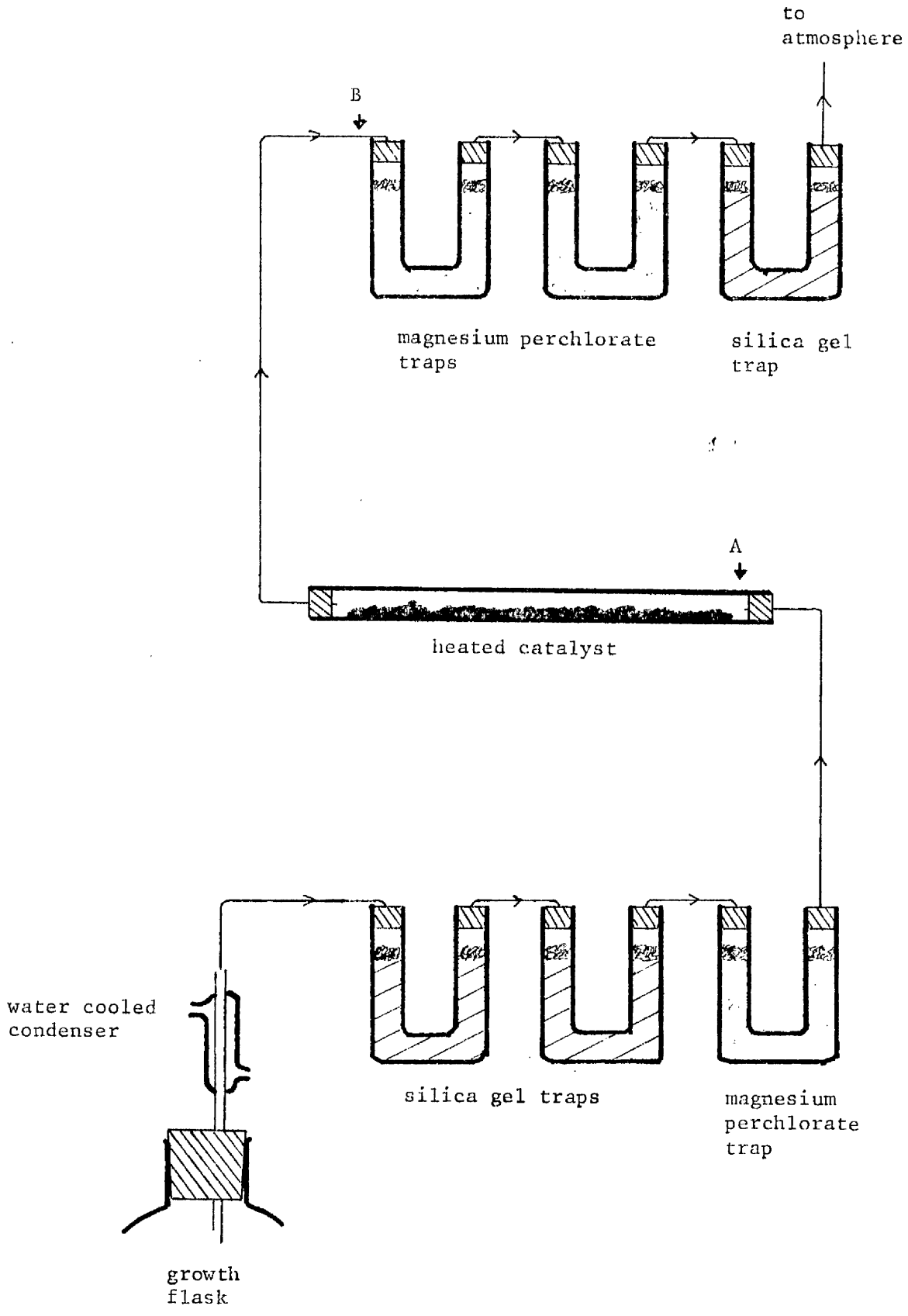
The efficiency of trapping of H_2 was checked by passing

Figure 8

Apparatus for trapping of hydrogen gas

The effluent gas from the growth flask was stripped of water vapour by the condenser and silica gel and magnesium perchlorate traps. Any hydrogen present was oxidised to water by heated 'M' catalyst. The gas was then passed through anhydrous magnesium perchlorate to trap the water formed. The apparatus between pointsA and B was heated to prevent condensation.

Figure 8


to
atmosphere
B
magnesium perchlorate
traps
silica gel
trap
A
heated catalyst
water cooled
condenser
silica gel traps
magnesium
perchlorate
trap
growth
flask

hydrogen gas through a wet type gas meter (Alexander Wright & Co. (Westminster) Ltd., Tooting High Street, London) before it entered the apparatus. Thus, the weight of water trapped can be compared with the hydrogen volume introduced.

11. Analysis of culture medium

11.1 General considerations

Substrate concentrations were measured on samples taken from the culture. Possible excretion products were also assayed for in similar samples. All except the general method for measurement of volatile organic acids were enzymic assays, performed using an SP800 double beam spectrophotometer (Unicam Instruments Ltd., Cambridge, England) fitted with a Servoscribe recorder.

11.2 Treatment of samples

Samples from the culture were treated with perchloric acid as described in section 7. These PCA extracts were thawed and mixed thoroughly immediately before use, then refrozen and stored again at -10^{o}.

11.3 Estimation of glucose

Glucose was assayed using the Boehringer 'GOD-Perid' method which is based on that of Werner et al. (1970). After an incubation time of 25-30 min. at 27^{o} the extinction of the assay decreases. Consequently assays were incubated at 27^{o} for 25 min then read at 660nm. The assay is linear over the range 0-250 nmol glucose/assay.

11.4 Estimation of glycerol

Glycerol was measured by a modification of the commercially

available Boehringer 'neutral fat' method which is based on the method of Eggstein and Kreutz (1966).

The composition of the assay medium was:

triethanolamine	81mM
magnesium sulphate	3.2mM
ATP	0.54mM
PEP	0.17mM
NADH	0.1mM
Lactate dehydrogenase	13µg/ml
pyruvate kinase	7µg/ml
glycerokinase	3µg/ml

The assay was done at pH 7.6 in a total volume of 3ml. All reagents with the exception of glycerokinase were mixed before addition to a portion of sample. The assay was initiated by addition of glycerokinase, incubated at 27^{o} for 60 min and read at 340nm. The assay is linear over the range 0-300 nmol glycerol/assay and 1 mol of glycerol in the assay gives an extinction of 2.07×10^{6}.

11.5 Estimation of acetate

Acetate was assayed by the method described by Boehringer. The composition of the assay was:

triethanolamine	62.5mM
magnesium sulphate	6.7mM
ATP	6.6mM
PEP	3.0mM
NADH	0.25mM
lactate dehydrogenase	13µg/ml
myokinase	13µg/ml
pyruvate kinase	13µg/ml
acetate kinase	33µg/ml

The assay was done at pH 7.4 in a total volume of 3ml. All reagents with the exception of acetate kinase were mixed before addition to a portion of sample. Acetate kinase was added to initiate the assay which was incubated for 1h at 27° and read at 340nm. The assay is linear over the range 0-500 nmol acetate/assay. 1 mol acetate in the assay gives an extinction of 2.07×10^6.

11.6 Estimation of pyruvate

Pyruvate was assayed using a modified standard method (Bücher et al., 1963)

The composition of the assay was:

potassium dihydrogen orthophosphate	30mM
NADH	0.22mM
lactate dehydrogenase	3µg/ml

The assay was done at pH 7.4 in a total volume of 3ml. The assay was initiated by adding a portion of sample to the assay mixture and incubated at 27° for 30 min, then read at 340nm. The assay is linear over the range 0-500 nmol/assay. 1 mol of pyruvate in the assay gives an extinction of 2.07×10^6.

11.7 Estimation of volatile organic acids

Volatile organic acids were assayed by steam distillation and subsequent titration of the distillate. 1ml 10N H_2SO_4 was added to 10ml sample in a Markham distillation unit. The entry port was washed with ∿10ml distilled water. The distillate was titrated against $\frac{N}{100}$ NaOH. 10mM sodium formate and 10mM sodium acetate were used as standards.

11.8 Estimation of methyl glyoxal

Methyl glyoxal was estimated by the method of Klotzch and

Bergmeyer (1963), which involves the formation of S-lactyl glutathione by glyoxalase I from glutathione and methyl glyoxal. The formation of S-lactyl glutathione can be followed by its absorption at 240nm.

The composition of the assay was:

0.1M	potassium phosphate buffer pH 6.8
0.54mM	glutathione
6.7µg/ml	glyoxalase I

in a total volume of 3ml. 1 mol methyl glyoxal in the assay gives an extinction of 3.12×10^6.

11.9 Estimation of lactate

Estimation of L-lactate was carried out by the method described by Boehringer.

The composition of the assay was:

3.0ml	glycine buffer pH 9.0 (22.8g glycine and 20.0ml hydrazine hydrate made up to 500ml in distilled water)	
0.2ml	30mg/ml	NAD^+
0.02ml	5mg/ml	lactate dehydrogenase

The assay was initiated by addition of 0-2 ml sample, and incubated at 27^o for 30 min, then read at 340nm. The assay is linear over the range 0-500 nmol lactate/assay. 1 mol lactate in the assay gives an extinction of 1.81×10^6.

11.10 Estimation of ethanol

Estimation of ethanol was carried out by the method described by Boehringer.

Solution 1 was prepared by dissolving 10g $Na_4P_2O_7.10H_2O$, 2.5g semicarbazide hydrochloride and 0.5g glycine in 250ml distilled water,

adjusting the pH to 8.7 with NaOH, then making up to 300ml with distilled water.

Solution 2 consisted of 60mg NAD^+ dissolved in 5ml distilled water.

Solution 3 was 30mg/ml alcohol dehydrogenase.

3.0ml solution 1, 0.1ml solution 2 and 0.2ml sample were mixed, and the E_{340} in an SP800 double beam spectrophotometer noted, 0.02ml of solution 3 was then added, the mixture incubated at 27^o for 1h and read again at 340nm. The assay is linear over the range 0-500 nmol ethanol/assay. 1 mol ethanol in the assay gives an extinction of 1.90×10^6.

11.11 Estimation of malate

Malate was measured by a modification of a standard method (Hohorst, 1963) in which the oxidation of malate by malate dehydrogenase is coupled to NAD^+ reduction.

The composition of the assay medium was:

Glycine	0.45M
hydrazine	0.18M
EDTA	2.4mM
NAD^+	3.2mM
sodium hydroxide to pH 9.5	
malate dehydrogenase	50µg/assay

The assay was done at pH 9.5 in a total volume of 3ml. Assay was initiated by the addition of NAD^+ and incubated at 27^o for 90 min then read at 340nm. The assay is linear over the range 0-500 nmol malate/assay. 1 mol of malate in the assay gives an extinction of 2.07×10^6.

12. Radiochemical techniques

12.1 Autoradiography

Cells growing on glucose ($\mu = 0.067$) in the apparatus described in section 6.3.2 were pulsed with 2mCi (1 - ^{3}H)-glucose (4.2 Ci/mmol). After 1h, μ having fallen to $\mu = 0.063$, samples from the culture were fixed by heat on glass microscope slides.

Photographic emulsion was applied to the slides in a darkroom using a Kodak safety lamp (Kodak Ltd., London). 5g of Ilford Nuclear Research Emulsion type L4 (Ilford Ltd., Ilford, Essex) was melted at 47° and mixed with 10ml distilled water at the same temperature. The slides were dipped in the emulsion solution, drained, then dried with cold air and stored in a light-tight box containing approximately 1g of silica gel, which was changed after 24h. The box was wrapped in aluminium foil.

After six weeks, the slides were developed in the darkroom using developer D19B, which consisted of:

2.2g	Metol (Kodak Ltd., London)
144g	$Na_2SO_3 . 7H_2O$
8.8g	hydroquinone
48g	anhydrous sodium carbonate
4g	KBr

added sequentially to 1 litre of distilled water.
Slides were developed with D19B for 5 min at 20°, washed once with distilled water, then fixed with 20% Amfix (May & Baker, Dagenham, England). The fixed slides were then rinsed for 2 min under a running cold tap and allowed to dry. It was then possible to view the developed slides by oil-immersion phase microscopy.

12.2 Estimation of turnover by radiochemical means

Turnover of cell components was measured by specifically labelling the inoculum, then measuring the loss of the radioactive isotope by the cells in a medium containing an excess of the appropriate unlabelled compound during the course of a growth experiment.

12.2.1 Preparation of specifically labelled inocula

On the day preceding an experiment, the labelled compound was added to a growing 3rd passage of cell density 180µg dry weight/ml. The culture was then grown to stationary phase by shaking the flask at 37° for 2½h.

When cells were labelled with $^{32}PO_4$, the buffer used instead of phosphate was 40mM Tris H Cl at pH 7.8. 2.5ml 40mM KH_2PO_4 pH 7.0 was also added to the growth flask to provide sufficient phosphate for growth.

The labelled cells were stored at 4° overnight and harvested as described in section 1.4.

12.2.2 Filtration of samples

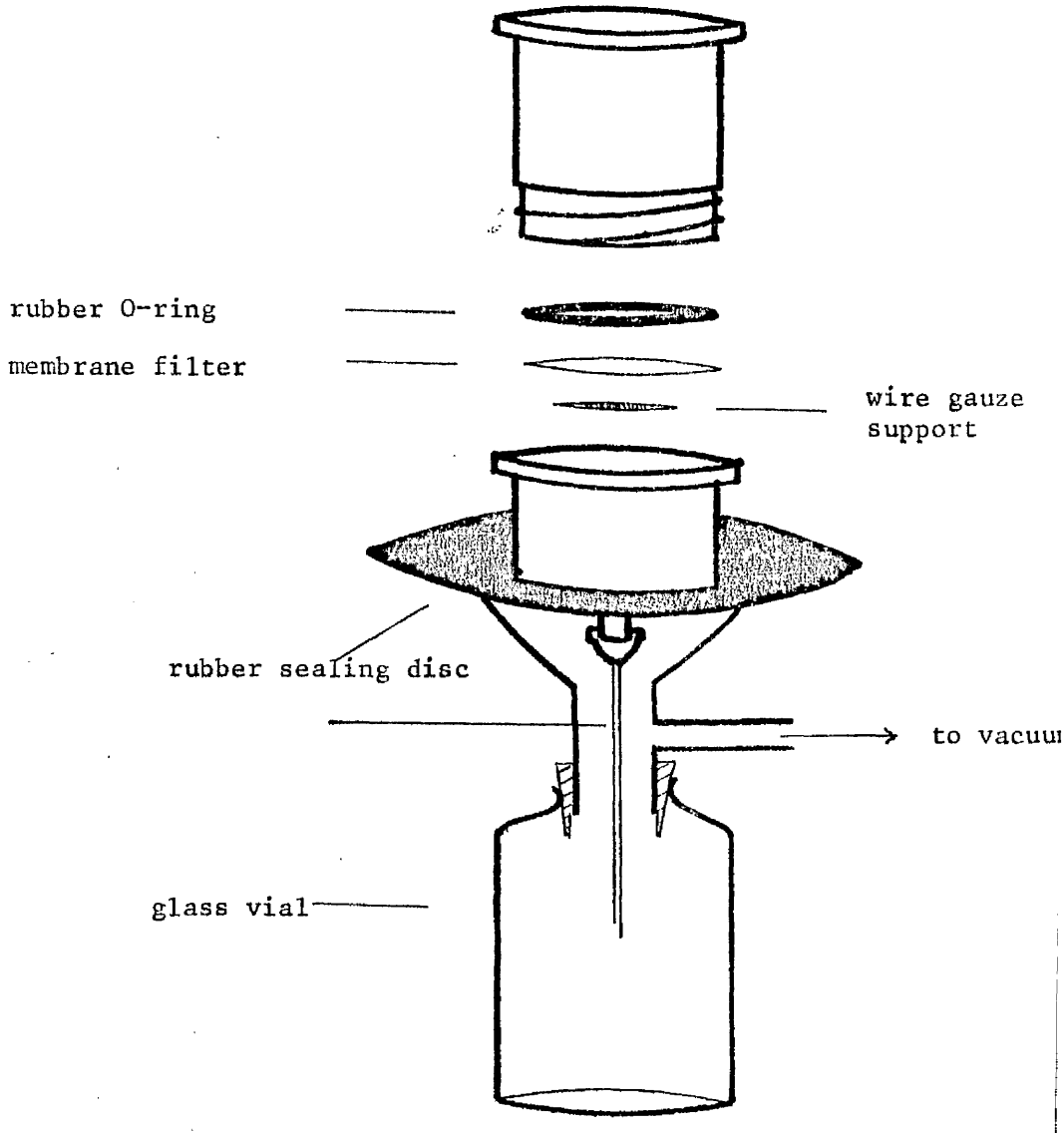
1ml of sample from a culture was immediately passed through the filtration assembly (Figure 9) which consisted of a Gelman filter holder (Gelman Instrument Company, Ann Arbor, Michigan, U.S.A.) fitted with a 0.20µmmembrane filter (Sartorius, Göttingen, West Germany). The vacuum was generated by a Speedivac vacuum pump (Edwards High Vacuum Ltd., Crawley, Sussex). The residue on the filter was immediately washed with 1ml of distilled water at room temperature. The filter was then removed and transferred to a liquid scintillation vial. 200µl of the first filtrate was also transferred to a liquid scintillation vial.

Figure 9

Simple filtration assembly

The filtration assembly consisted of a Gelman filter holder fitted with a 0.22μmSartorius membrane filter. Suspensions were filtered by vacuum into a glass vial. Clarification of the filtrate was improved by sealing the edges of the membrane filter and O-ring with silicone oil.

Figure 9


rubber O-ring
membrane filter
wire gauze support
rubber sealing disc
to vacuum
glass vial

During the course of these experiments with radio-labelled cells, problems were encountered which were caused by leakage in the filter holders, where a good seal was not always obtained round the edges of the membrane filters. The resultant appearance of even a small quantity of highly labelled cells in a very low specific activity filtrate created serious problems in the interpretation of results. This difficulty was obviated by creating a hydrophobic seal around the edge of the filter and the filter assembly using silicone oil (Scientifica & Cook Electronics Ltd., Acton, London).

12.2.3 Trapping of $^{14}CO_2$

The growth flask was sealed and checked for leaks. Air was passed into the flask at a rate of 100ml/min. The outflowing gas was passed into a liquid scintillation vial containing 10ml phenyl ethylamine scintillation fluid, with the scintillation fluid being blown into a Vigreux column. After each sample, 4ml of scintillation fluid was washed through the Vigreux column into the vial. All of the gas passing out of the Vigreux column was subsequently passed through absolute alcohol to prevent toluene reaching the atmosphere.

12.3 Liquid scintillation spectrometry

12.3.1 Liquid scintillation fluid

To 1 litre toluene was added 300ml ethanol and 5g 2,5-diphenyloxazole (PPO). 14ml portions of this fluid were dispensed by an Oxford Pipettor (Boehringer Mannheim GmbH, Mannheim, Germany) into glass vials (Packard Instruments Ltd., Caversham, Berks.) fitted with disposable plastic caps (Metal Box Co. Ltd., Portslade, England). This concentration of ethanol was sufficient to dissolve 200µl of

aqueous solution.

12.3.2 $^{14}CO_2$ absorption scintillation fluid

Phenylethylamine scintillation fluid as described by Woeller (1961) was prepared by making up

27ml	redistilled phenyl ethylamine
27ml	absolute methanol
500mg	PPO
10mg	POPOP

to 100ml with toluene.

This solution was dispensed in 10ml portions into glass liquid scintillation vials.

After use, the Vigreux columns were washed with 4ml of scintillation fluid which contained all of the above components except phenyl ethylamine, and the fluid was then added to the 10ml volume in the scintillation vials.

12.3.3 Apparatus

Samples were counted in Philips Liquid Scintillation Analysers (Philips Scientific Equipment, Eindhoven, Netherlands) fitted with calculators to convert c.p.m. to absolute units. The efficiency of counting was calculated by the channels ratio technique (Wang & Willis, 1965). Efficiency curves were prepared using chloroform as the polar quenching agent and ^{14}C-toluene and ^{3}H-toluene (both obtained from Packard Instruments Ltd., Caversham, Berks.) as radioactive standards. The counting efficiency was expressed as a quadratic function of the observed channels ratio. This calibration was periodically checked using similar radioactive standards. The constants of these quadratic equations were entered into the calculators in the Liquid Scintillation Analysers.

13. Disruption of cells by sonication

5ml of cell suspension was pipetted into a small glass bottle and irradiated at 0^{o} in a Soniprobe (Dawe Instruments, London) at 3A for 3 min made up of 6 x ½ min periods of irradiation with ½ min rest intervals to ensure that the sample remained chilled. The experimental set up has been described (Holms & Bennett, 1971). The extract was clarified by centrifugation (11,600g, 4^{o}, 15 min) and the supernate was stored on ice until assayed.

Sonicated cell extracts were used for the assay of β-galactosidase, glycerokinase and α-glycerophosphate dehydrogenase.

14. Measurement of enzyme activity

14.1 Assay of β-galactosidase

Samples from cultures were sonicated and assayed for β-galactosidase (EC 3.2.1.23) using a modification of a standard method (Cohn & Monod, 1951).

The composition of the assay was:

0.1M sodium dihydrogen phosphate

5mM magnesium sulphate

3.3mM o-nitropheny1-β-galactoside

in a total volume of 3ml at pH 7.25

The assay was carried out at 27^{o}. ONP production was followed at 420nm in an SP 800 double beam spectrophotometer against air as a blank. The output of the spectrophotometer was traced on a servoscribe potentiometric recorder from which the rate of ONP production could be measured. The results were expressed as enzyme units (U)/ml cell extract where 1 enzyme unit represents the production of 1 μmol ONP/min at 27^{o} under the conditions of the assay.

14.2 Assay of β-galactoside permease

Estimation of β-galactoside permease activity in whole cell suspensions was carried out using a modification of a standard method (Rickenberg et al. 1956). ONPG hydrolysis was measured in cell suspensions in the presence and absence of 5mM thiodigalactoside (TDG), an inhibitor of β-galactoside permease. The difference between the two activities was taken to be the true activity of the permease, eliminating non-specific permeation.

The composition of the assay was:

0.1M	sodium dihydrogen phosphate
5mM	magnesium sulphate
3.3mM	o-nitrophenyl-β-galactoside
5mM	TDG

in a total volume of 3ml, pH 7.25

The activity of the permease was determined in the same way as for β-galactosidase.

14.3 Assay of glycerokinase

Glycerokinase (EC 2.7.4.30) activity in sonicated cell extracts was determined using the commercially available Boehringer 'neutral fat' test combination, replacing the glycerokinase with glycerol and assaying for enzyme activity rather than substrate concentration.

The composition of the assay was:

0.1M	triethanolamine
4mM	magnesium sulphate
0.2mM	NADH
1.1mM	ATP
3.7mM	PEP
13.3µg/ml	lactate dehydrogenase
6.7µg/ml	pyruvate kinase
13.3mM	glycerol

The assay was done at pH 7.6 in a total volume of 3ml. The rate of NADH oxidation was followed at 340nm at 27° in an SP800 double beam spectrophotometer, with the sample cuvette being placed in the reference beam and the blank, containing buffer in place of glycerol, in the sample beam. Enzyme activity was expressed as enzyme units/ml cell extract where 1 enzyme unit represents the oxidation of 1 µmol of nucleotide/min at 27° under the assay conditions.

14.4 Assay of α-glycerophosphate dehydrogenase

L-α-glycerophosphate dehydrogenase (EC.1.1.1.8) in the sonicated extract was assayed by a modification of the method described by Lin et al. (1962). The oxidation of L-α-glycerophosphate by the enzyme is coupled to the reduction of the dye thiazolyl blue (3- (4,5-dimethyl thiazolyl 1-2) 2,5-diphenyl tetrazolium bromide) via the electron carrier phenazine methosulphate. Reduced thiazolyl blue absorbs at 550nm.

The composition of the assay was:

60mM	potassium dihydrogen phosphate
33mM	D,L-α-glycerophosphate
33µg/ml	thiazolyl blue
100µg/ml	phenazine methosulphate
10mM	potassium cyanide

in a total volume of 3ml, pH 7.5. Cyanide was added to the assay to prevent non-specific reduction of thiazolyl blue by cytochromes.

The assay was carried out at 27° in an SP800 double beam spectrophotometer with air as a blank, the output being traced by a servoscribe potentiometric chart recorder. Results were expressed as enzyme units/ml extract, where 1 enzyme unit represents the oxidation of 1 µmol α-glycerophospate/min at 27° under the conditions of the assay.

15. Estimation of elementary content of cells

Cells for elementary analysis were harvested and washed twice, then lyophilised. The resulting powder was analysed using a Perkin-Elmer 240 Elemental Analyser (Perkin-Elmer Ltd., Beaconsfield, Bucks.). Results for each element were expressed as % w/w.

16. Photomicrography

Autoradiography slides prepared as described in section 11.1 were stained by flooding the slides with carbol fuchsin for 2 min and rinsing thoroughly with distilled water. The slides were dried in air. Photomicrographs were taken using an Ortholux microscope fitted with an Orthomat automatic microscope camera (Ernst Leitz GMBH, Wetzlar, Germany). The slides were viewed under oil immersion at maximum magnification. The film used was DIN 18, and the camera settings found to give best resolution were H50% for the object dark/light ratio and setting 'a' for the film speed.

17. Processing of experimental data

Routine calculations, particularly of gas exchange, carbon balance, linear regression and correlation coefficients, which involve the repetitive use of formulae, were performed using a Canon Canola 167P programmable calculator with the mechanics of the calculation stored on programme cards (Nig Banda, Elmbank Street, Glasgow). This electronic calculator was also used for less complex calculations where the programme facility was not required.

DEVELOPMENT OF ANALYTICAL METHODS

1. Gas exchange measurements

1.1 General considerations

Respiration of an arithmetic-type continuous culture was measured by the apparatus shown in Figure 2. The apparatus is described in Methods section 6.3.1, and allows measurement of both oxygen consumption and carbon dioxide evolution in addition to sampling of the medium for the measurement of cell density, substrate and excretion products concentrations, and pH. A continuous flow system was used for respiration measurements. The supply of air, derived from the departmental compressed air supply, was filtered, measured and stabilised as described on p. 39. Gas leaving the flask was substantially stripped of water vapour by chilling to 2^{o}. In this way the effect of water vapour on the gas analysers was minimised. In any case, the relatively small effect of water vapour on the gas analysers' response was corrected for by passing air through an identical, non inoculated flask, chilling the effluent gas in the same way, and recording the zero uptake readings on the analysers. A pulse suppressor, as shown in Figure 5, was found to improve the stability of the oxygen analyser readings when fitted on both the reference and sample gas lines immediately before the analyser.

The carbon dioxide content of the effluent gas was measured using a Lira 300 infra-red analyser (Mine Safety Appliances, Glasgow). The change in oxygen content between the effluent gas and air was measured using an OA184 oxygen analyser (Servomex Controls, Crowborough, England) which measures the oxygen content of sample and reference gas streams and expresses the difference between the two in terms of

partial pressure of O_2. The output of both analysers was continuously recorded on chart recorders (Servoscribe).

The accuracy of the measurements depends on the calibration of the gas analysers, the calibration of the gas monitoring system, and the efficacy of pulse suppression. All equipment was calibrated before use, and no adjustment of any kind was made during the course of an experiment.

1.2 Calibration of capillary flow meters and wet meters

Gas flow was measured in two ways: wet-type gas meters (Alexander Wright & Co. (Westminster) Ltd., Tooting High Street, London) were used to measure the cumulative total of air passed into the growth flask, and capillary flow meters (Gallenkamp, London) were used as an indication of the gas flow rate at any given time. The calibration of both devices was dependent on the use of a Vol-u-meter (Brooks Instruments Ltd., Cheshire, England).

The wet meters were calibrated directly from the Vol-u-meter (Figure 10). Since the conditions of temperature and pressure were identical in both instruments, the calibration of one with the other was absolute. A known volume of air was passed from the Vol-u-meter through the wet meter at a rate of approximately 100ml/min. The volume of oil in the wet meter was adjusted so that the flow of 250ml of air gave one revolution of the gas chamber. Once calibrated, the mean deviation of the wet meter reading was ±0.5%. During the course of an experiment, the air temperature was monitored by a thermograph. No correction was made for changes in atmospheric pressure.

The capillary flow meters were calibrated using the previously calibrated wet meters. Glass capillary tubing was made so that a flow rate of 100ml/min gave a pressure difference of approximately

Figure 10

Calibration of wet meter using a vol-u-meter

The vol-u-meter was filled with air by opening screw clip A, keeping clip B closed. Air was then passed through the wet meter by closing clip A and opening clip B such that the flow rate through the wet meter was approximately 100ml/min.

The level of oil in the wet meter was adjusted until the volume registered on the wet meter corresponded with the volume of air passed from the vol-u-meter.

Figure 11

Calibration of capillary manometer

The flow rate of a stabilised air supply was altered by means of a GAP-meter fitted with a flow control valve. The air stream was passed through the capillary manometer, then a previously calibrated wet meter. The flow rate was measured by timing a given volume change in the wet meter. This flow rate was plotted against the difference between the levels of oil in the two limbs of the manometer.

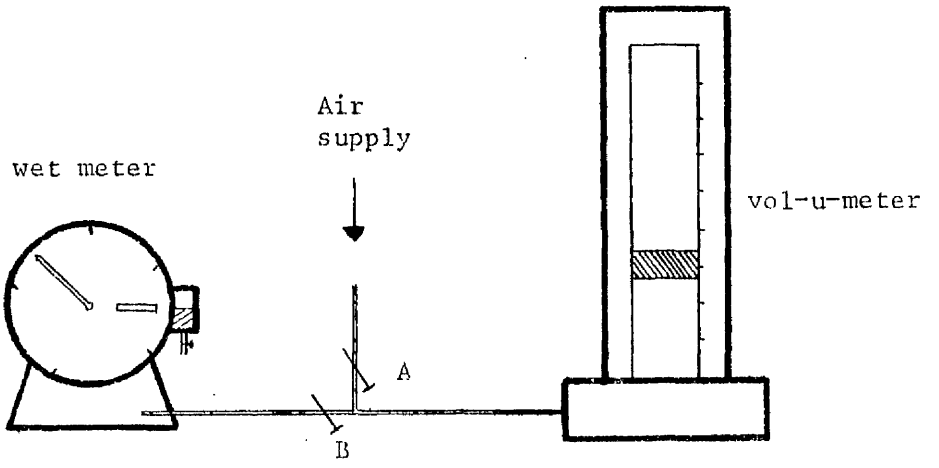


Figure 10

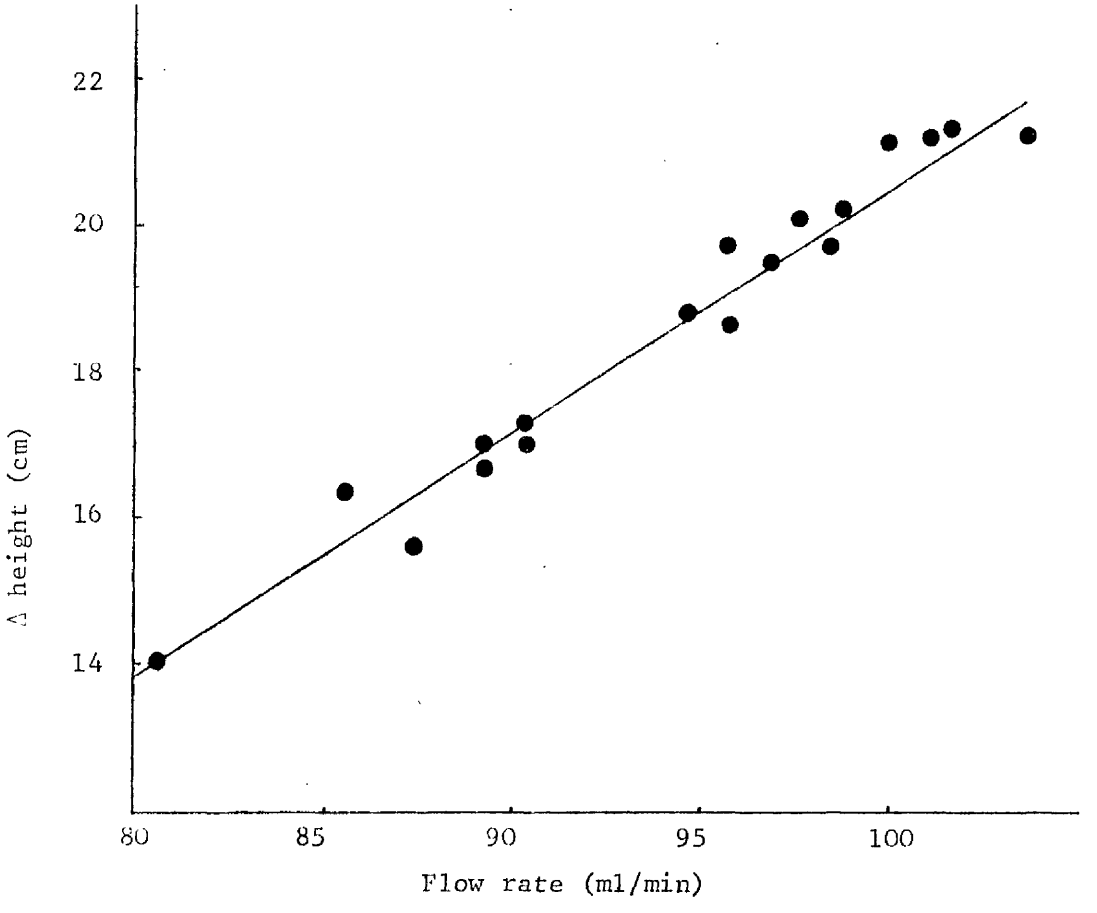


Figure 11

20cm of oil between the two manometer-limbs. Figure 11 shows a typical calibration curve.

The most critical gas flow readings taken during the course of an experiment were those of the wet meters. Figure 6 demonstrates the constancy of the gas flow using this system, and the consequent accuracy of the determination of gas flow rates.

1.3 Calculation of gas exchange data

The gas analysers give readings as changes in the partial pressures of oxygen and carbon dioxide in the effluent gas. These data are then converted to rates of uptake of oxygen and evolution of carbon dioxide in μmoles/min for the culture.

1.3.1 Calculation of oxygen uptake

Let a% be the partial pressure of O_2 in the effluent gas

b% be the partial pressure of CO_2 in the effluent gas

fx be the flow rate of effluent gas /ml/min)

and fe be the flow rate of air into the flask (ml/min)

The N_2 content of air entering the flask = 79.04%

The N_2 content of the effluent gas is the total less O_2 and CO_2 content = 100 - a - b%.

However, the quantity of N_2 in both gas streams is the same

$$\therefore \quad \frac{100 - a - b}{100} \cdot fx = \frac{79.04}{100} \cdot fe$$

$$\text{i.e.} \quad fx = \frac{79.04}{100 - a - b} \cdot fe$$

The flow rate of O_2 into the flask = $\frac{20.96}{100}$ · fe ml/min and the flow rate of O_2 leaving the flask = $\frac{a}{100}$ · fx ml/min

$$\therefore \text{ Oxygen uptake} = \frac{20.96}{100} \cdot fe - \frac{a}{100} \cdot fx \text{ ml/min}$$

$$= \frac{20.96}{100} \cdot fe - \frac{a}{100} \cdot \frac{79.04}{100 - a - b} \cdot fe \text{ ml/min}$$

$$= \left(20.96 - \frac{79.04\,a}{100 - a - b} \right) \frac{fe}{100} \text{ ml/min}$$

$$= \left(\frac{100 - a - b}{79.04} \cdot 20.96 - a\right) \frac{fe}{100} \cdot \frac{79.04}{100 - a - b} \text{ ml/min}$$

$$O_2 \text{ uptake} = \left[\frac{20.96}{79.04}(100 - a - b) - a\right] \frac{79.04 \cdot fe}{100(100 - a - b)} \text{ ml/min}$$

1 mmole of gas occupies 22.4ml at N.T.P., so correcting for T(^{o}K), the equation becomes

$$O_2 \text{ uptake} = \left[\frac{20.96}{79.04}(100 - a - b) - a\right] \frac{79.04 \cdot fe \cdot 273}{100(100 - a - b).T.22.4} \text{ mmoles/min}$$

1.3.2 Calculation of carbon dioxide evolution

The calculation for carbon dioxide evolution is simplified since there is no CO_2 in the gas entering the flask. Thus, for gas of b% CO_2 at a flow rate of fx ml/min

$$CO_2 \text{ evolution} = \frac{b}{100} \cdot fx \text{ ml/min}$$

$$= \frac{b}{100} \cdot \frac{79.04}{(100 - a - b)} \cdot fe \text{ ml/min}$$

Correcting for temperature,

$$CO_2 \text{ evolution} = \frac{b}{100} \frac{79.04}{(100 - a - b)} \cdot fe \frac{273}{22.4\,T} \text{ mmoles/min}$$

Hamilton (1972) has described corrections which should be made in the carbon dioxide calculation for dissolved carbon dioxide and bicarbonate. In the maintenance experiments described later in this thesis, the rate of change of carbon dioxide production is small,

so these corrections are not necessary. Since the gas exchange is virtually in steady state over short time intervals, the correction Hamilton describes for the effect of flask dead space is also unnecessary.

1.4 Difficulties encountered in the use of apparatus

The solutions to some of the main problems arising in the use of the gas analysis equipment have been described by Hamilton (1972). In particular, the electrical problems appear to have been overcome. However, there still existed problems with the measurement and stability of gas flow, with the cooling of the effluent gas, and with the use of servoscribe chart recorders.

Hamilton suggested that the measurement of gas flow using GAP meters may give errors as high as 10%. The introduction of wet type gas meters has reduced this source of error to less than 1%.

The main remaining problem was that of pulsing, which could occasionally cause fluctuations in the oxygen analyser reading of 0.01% O_2. It is thought that this was due to two factors. The first, the inevitable pulsing due to the compressor supplying the air, was largely overcome by the introduction of the stabilisers described in the methods section. The second source of pulsing was due to the nature of the growth apparatus, in particular to the stirring of the culture and also to the condensation of water vapour in the glass tubing through which air entered the flask. The former problem was largely overcome by introducing the pulse suppressor (Figure 5) immediately before the oxygen analyser. Increasing the bore of the glass tubing eliminated the latter. As a result, the maximum pulsing observed was 0.002% O_2.

In earlier experiments involving the use of gas analysis

equipment, the ice-water for the cooling of effluent gas was circulated using a Glen Creston peristaltic pump (Glen Creston, Stanmore, Middlesex). Since this pump often had to operate continuously for 4 days, the frequency of breakdown was high due to wear on the silicone rubber tubing. The reliability of the system was markedly improved by the replacement of the peristaltic pump by three pumps (Universal Scientific, Plashet Road, London E13) in series.

The problem associated with the chart recorders was caused by the slow speed (3cm/h) at which they operated, which resulted in the ink flow system failing, particularly when the output was slightly noisy. This was overcome by changing the standard pen assembly to accommodate fibre pens (W & W electronic 11-0034, Gilford Instruments Ltd., Morden, Surrey) designed to operate at slow speeds.

2. Estimation of bacterial dry weight by turbidity measurement

The measurement of bacterial growth has been reviewed in detail by Mallette (1969), summarizing the different methods of growth measurement and some of the main problems associated with each method. The method of measuring growth throughout the work presented in this thesis was to measure the turbidity of a suspension of bacteria and, by using a conversion factor, convert this to a reading of dry weight per unit volume.

There appears to be no absolute method of determining the dry weight of a culture. Mallette discusses several methods, but comes to no firm conclusion as to which has most merit. It was decided to follow most closely the method used by Hadjipetrou *et al.* (1964). The culture was centrifuged and washed twice with distilled water before drying at 105° for 18h. Two main difficulties were encountered with the weighing procedure. Firstly, the vial and

contents had to be cooled to room temperature before being weighed and, secondly, the dried bacteria were found to be very hygroscopic, thus making rapid weighing essential. Although neither of these problems was solved, reproducible results were obtained by paying close attention to several details. When the vial was removed from the oven, it was kept in a desiccator for 30 min before weighing. To minimize the time taken to weigh each sample, the weights of the balance were set to the estimated weight of the vial and contents before the vial was removed from the desiccator. Silica gel was used as a desiccant inside the balance and was changed frequently. When this was done, the rate of water vapour uptake of the dried bacteria was markedly decreased.

The measurement of turbidity was carried out using a Unicam SP800 double beam spectrophotometer. Koch (1970) has discussed the most desirable qualities required of a spectrophotometer which is used to measure turbidity and the SP800, with a path length of 11.5cm and detector of 44mm diameter, is geometrically satisfactory. The fact that it is a double beam instrument, which compensates for fluctuations and wear in the lamp, also makes the SP800 an instrument suitable for the measurement of turbidity.

Another problem Mallette and Koch discuss is that of the calibration curve required to compensate for internal light scattering. The graph of cell density against observed turbidity for the SP800 using washed cell suspensions of E. coli is shown in Figure 3. This graph is virtually linear up to an optical density of 0.2. The extent of the linear portion of the graph is characteristic of the spectrophotometer used. Coultate and Sundaram (1975) have reported that their calibration curve (at 680nm) is linear up to an optical density of 1, while Koch (1970) found that no calibration curve was linear above

0.4 for any of the instruments he tested.

The calibration graph was used for logarithmic growth experiments and other experiments where precise growth yields were not required. For maintenance experiments, the culture was always diluted in buffer to an optical density of <0.2, as the calibration curve was not sufficiently accurate for higher optical densities. The curve was found to be unchanged for the same instrument over a period of 30 months.

There is some controversy over the relationship between dry weight and turbidity. Koch (1970) found that cells of size range 0.1 - 5μm^3 had virtually the same dry weight/turbidity relationship, that E. coli and Bacillus megaterium had the same relationship and that slow-growing chemostat cultures of E. coli and logarithmic cultures of E. coli also had the same relationship. Dean and Rogers (1967) reported that Aerobacter aerogenes has the same dry weight/turbidity relationship when grown on six different limiting nutrients at a number of different growth rates. On the other hand, Pirt (1965) found that the ratio did depend on the growth rate for the chemostat culture of Aerobacter cloacae, and Hadjipetrou et al. (1964) found that the relationship was 9% larger for stationary Aerobacter aerogenes compared with exponentially growing cells. These inconsistencies may be accounted for by the type of spectrophotometer used to measure turbidity in each case. Koch (1970) comments that it can be expected that cell size will influence the ratio obtained with wide-angle instruments, but that since he used a narrow-angle instrument, differences in cell size did not affect his conversion factor.

The chemical composition of bacteria is known to change according to growth rate, growth temperature and limiting nutrient. As the growth rate decreases, the RNA content changes (Tempest & Meers,

Figure 12

Relationship between dry weight and turbidity

Turbidity and dry weight measurements were performed as described in the Methods section. The relationship between cell dry weight and turbidity was determined for several types of cell phenotype.

Type of cell	C source	µg/ml/O.D. unit	No. of determinations
exponential phase	glucose	231 ± 6.6	10
exponential phase	glycerol	219 ± 4.6	8
exponential phase	lactose	225 ± 3.6	8
stationary phase	glucose	227 ± 14.7	5
C-limitation (μ = 0.14)	glycerol	215 ± 3.0	2
N-limitation (μ = 0.02)	glycerol	225 ± 1.8	6

1968; Coffman et al., 1971) and the protein content decreases (Abbott et al., 1974). Examples of changes in composition according to limiting nutrient are glycogen accumulation and utilisation in E. coli in the presence and absence of a nitrogen source (Ribbons & Dawes, 1963) and changes in respiratory chain components in iron-limited E. coli (Rainnie & Bragg, 1973). The principal effect of temperature changes on E. coli is on the fatty acid composition of the lipid fraction (Marr & Ingraham, 1962).

Taking into account that these changes, which are often substantial, do occur in cell composition, it is perhaps surprising that Koch (1970) finds that the dry weight/turbidity ratio does not change with growth rate or limiting nutrient and that Coultate and Sundaram (1975) do not observe any change in the dry weight/turbidity relationship with changing temperature for Bacillus stearothermophilus.

Holms (unpublished results) has estimated the dry weight/turbidity relationship for E. coli ML308 using a Unicam SP800 spectrophotometer to be 196 µg/ml/O.D. unit. Using the methods described above, the same relationship was determined for different phenotypes of E. coli ML308 (Figure 12). These experiments show that there is no significant difference in the dry weight/turbidity relationship for cells of different types. The average value for the conversion factor obtained from these experiments was 225.0 ± 7.2 µg/ml/O.D. unit, and this is the factor used in the conversion of optical density to dry weight cells/ml throughout the work presented in this thesis.

It was not possible to determine whether this relationship was also true for cells growing in high concentrations of NaCl, since washing of this cell pellet with distilled water resulted in lysis of the cells. It was therefore assumed that the relationship was the same.

Determination of dry weight by this method, although it is standard practice (Mallette, 1969), suffers from a systematic error caused by washing the cells with distilled water. Leaching, particularly of the soluble components of the cells is known to occur (Mallette, 1969). An attempt was made to compare the dry weight of leached and non-leached cells using a radioactive marker which can not enter the bacteria.

Sufficient labelled compound was added to a sample from the culture to give a specific activity of $\sim 10^4$ dpm/ml. The cells were then harvested as before, and as much supernate as possible removed from the centrifuge tube. Dry weight/ml and specific radioactivity were measured in the supernate, so that the dry weight of solute could be related to the specific activity of the solution. The pellet was resuspended in distilled water. A portion was taken for counting, and the remainder transferred to a dry weight vial and dried as before. In this way, it was hoped to estimate the amount of solution, and hence the dry weight of solute, trapped in the pellet. By subtracting the weight of solute trapped from the total dry weight, it should then have been possible to estimate the true dry weight of cells.

^{14}C-citrate, ^{3}H-sucrose and ^{3}H-inulin were used as extracellular markers in these experiments. Results of some typical experiments are shown in Figure 13. The results in general were variable, no matter which marker was used. The calculated weight of trapped medium in the pellet was almost invariably more than, and often double, the wet weight actually measured for the pellet. The possibility that the marker was sticking to the centrifuge tube was eliminated by first treating the tube with an excess of cold marker. This precaution did not affect the anomalous results. It was there-

Figure 13

Estimation of medium trapped in pellet by radioactive labelling of extracellular medium

Labelling of extracellular medium was carried out as described on p.75. The weight of extracellular medium trapped in the pellet was calculated by comparison of the specific activity of the medium and specific activity of the resuspended pellet. The wet weight of the pellet was determined by weighing the centrifuge tube after the supernate had been decanted and the tube drained.

Marker	Calculated weight of medium trapped (mg)	Total wet weight of pellet (mg)
^{14}C-citrate	565	290
^{3}H-sucrose	342	277
^{3}H-inulin	524	294

fore assumed that these markers were preferentially sticking to the cells in the pellet and it was decided not to pursue this problem any further.

In summary, the dry weight/optical density relationship was determined for several different types of cells and was found to be 225 µg/ml/O.D. unit for all types of cell used. Although this value did not take into account the loss of cell material through leaching, this is not a serious drawback since most other published work on molar growth yields suffers from the same shortcoming.

3. Arithmetic-type continuous culture

The experiments of Stouthamer and Bettenhaussen (1973) with tryptophan-limited chemostats of Aerobacter aerogenes suggested that, at the specific growth rates found in batch culture, a large proportion of the energy made available from the carbon source is used for maintenance energy. Other workers had found maintenance coefficients which were very much smaller, and had little or no effect on yields obtained in batch culture as compared with continuous culture (van Uden, 1969). In order to resolve whether maintenance energy caused molar growth yields in batch culture of E. coli to be materially different from values of Y_G, it was necessary to find the maintenance coefficient for E. coli. Stouthamer and Bettenhaussen (1973) also tentatively suggested several possible reasons for maintenance energy, but only had experimental evidence for one of the reasons.

The usual way of measuring maintenance and Y_G is in chemostat culture, where the molar growth yield is determined at steady state for different values of the dilution rate. However, results from chemostat culture are seldom as good as those obtained by Stouthamer and Bettenhaussen, since when the maintenance coefficient is lower, the difference between growth yields at high and low dilution

rates is much smaller. It is not practicable to increase the proportion of total available energy devoted to maintenance in the chemostat by decreasing the dilution rate to much less than $0.1h^{-1}$ because of the danger of mutation and also the time involved.

A method was therefore sought which would overcome the disadvantages of using the chemostat for the measurement of maintenance. Firstly, it should allow the energy devoted to maintenance to be a large proportion of the total energy used even for small maintenance coefficients. Secondly, the method should be fairly rapid, and use simple apparatus, to avoid the time-consuming turnover of reservoirs, tubing etc. involved with chemostat culture. Finally the method should minimise any selective advantage of mutations in the growth vessel at the small values of μ necessary to measure the maintenance coefficient accurately.

During the course of my undergraduate laboratory work, I had several times come across the phenomenon called "arithmetic growth". When the inducible strain of *E. coli* (ML30) is trained to lactose, then grown on a β-galactoside which can be metabolised by the *lac* enzymes but not induce the *lac* operon, cells do not grow exponentially. Instead, the growth rate is limited by the rate at which the β-galactosides can be metabolised by a fixed amount of enzyme, and the graph of cell density against time appears to be linear. Thus, as cell density increases, the specific growth rate must fall. Since this was a rapid, simple method of changing μ which enabled the molar growth yield to be measured throughout, it seemed suitable for the measurement of maintenance. However, the method was severely limited by the number of suitable substrates available. Only phenyl-β-galactoside, lactulose and lactitol fall into the category of metabolisable non-inducers of the *lac* operon. To overcome this, it was

decided to impose an artificial limitation of metabolisable substrate, by adding substrate to the culture at a constant rate, rather than rely on substrate supply being held constant by a limiting enzyme activity. A literature survey revealed that a similar method had been used by Jordan and Jacobs (1944) and by Marr, Nilson & Clark (1963) for the measurement of maintenance.

This arithmetic type of continuous culture does not suffer from the disadvantages of the chemostat. 3-4 days is usually sufficient to determine maximum molar growth yields and maintenance coefficients in terms of both carbon source and oxygen. The approximate range of growth rates covered is 0.002 - 0.1 h^{-1}, at which even a small maintenance requirement will consume a sizeable proportion of available energy. In contrast, a single reading for the apparent molar growth yield in a chemostat with a dilution rate of, say, 0.05 will require nearly 3 days. A number of these readings would have to be taken before the maintenance coefficient could be determined.

The apparatus required is also simple and easily handled. All of the required parameters - specific growth rate, oxygen consumption, cell density and rate of substrate supply - can be readily measured. The danger of mutation is also very much less in arithmetic-type continuous culture than in the chemostat, since, even if a single cell did have a "selective advantage" over the others, it could not conceivably become the predominant organism in the culture as none of the other cells present would be washed out, as they would in the chemostat.

One possible shortcoming of the method may be that at no time does the culture reach a steady state. However, the rate of change of μ is so slow that this may not be important.

The method also lends itself to the measurement of maintenance under different conditions and hence to a better understanding of the processes involved in maintenance.

3.1 Derivation of growth equation - carbon source

For the purpose of these calculations, maintenance energy shall be defined as it has been defined by Stouthamer & Bettenhaussen (1975) as all energy which does not result in the net synthesis of new cell material i.e. 'non-growth' energy (see p4). It is assumed in the calculations, and later confirmed by the results, that the maintenance coefficient (the specific rate of substrate utilisation for maintenance) does not vary with the growth rate of the cells, and is a constant for any given set of conditions. The amount of energy used for maintenance therefore depends solely on the quantity of cells present in the growth flask.

The symbols used are:

$\frac{dS}{dt}$ - total rate of substrate utilisation (µmol/h)

$\frac{ds_M}{dt}$ - rate of substrate utilisation for maintenance (µmol/h)

$\frac{ds_G}{dt}$ - rate of substrate utilisation for growth (µmol/h)

m - maintenance coefficient (µmol/g dry wt./h)

X - mass of cells in flask (g dry wt.)

$\frac{dx}{dt}$ - arithmetic growth rate (g/h)

Y - apparent molar growth yield (g/mole)

Y_G - maximum molar growth yield (g/mole)

µ - specific growth rate (h^{-1})

Making the above assumptions, then

Total rate of substrate utilisation = Rate of substrate utilisation for maintenance + Rate of substrate utilisation for growth

i.e. $\frac{dS}{dt} = \frac{ds_M}{dt} + \frac{ds_G}{dt}$ (1)

Since, by definition, $m = \frac{1}{X} \cdot \frac{ds_M}{dt}$

and $Y_G = \frac{dx}{ds_G} = \frac{dx}{dt} \cdot \frac{dt}{ds_G}$

Equation (1) becomes

$$\frac{dS}{dt} = m.X + \frac{1}{Y_G} \cdot \frac{dx}{dt} \quad (2)$$

Thus, if $\frac{dS}{dt}$ is held constant, and since m and Y_G are constants, equation (2) contains only 2 variables - X and $\frac{dx}{dt}$. $\frac{dS}{dt}$ can be held constant simply by keeping the pumping rate of the peristaltic pump constant. Since X and $\frac{dx}{dt}$ (see p.90) can be measured, then m and Y_G can be determined. As X increases, the proportion of available energy channelled to maintenance also increases, so the proportion available for growth falls, and the arithmetic growth rate $\frac{dx}{dt}$ falls as X increases.

Equation (2) can also be applied to nitrogen limitations where, since the rate of supply of nitrogen sources is constant, the $\frac{dx}{dt}$ remains constant. The two variables under these conditions are $\frac{dS}{dt}$ and X.

It is worth noting that, on dividing equation (2) throughout by $\frac{dx}{dt}$,

$$\frac{dS}{dt} \frac{dt}{dx} = m.X.\frac{dt}{dx} + \frac{1}{Y_G}$$

$$\therefore \quad \frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_G}$$

which is the more common form of the growth equation relating molar growth yields and specific growth rate as derived by Pirt (1965).

3.2 Derivation of growth equation - oxygen

A similar equation can be derived relating oxygen molar growth yields to specific growth rate.

The symbols are the same as before, except that $\frac{dO_2}{dt}$, m_{O_2} and $Y_{G\ O_2}$ are the rate of substrate consumption, the maintenance coefficient and the maximum molar growth yield, all in terms of O_2. Q_{O_2} is the specific rate of oxygen consumption, with the units $\mu mol O_2$/ g dry wt/h.

Equation (2) becomes

$$\frac{dO_2}{dt} = m_{O_2} X + \frac{1}{Y_{G\ O_2}} \cdot \frac{dx}{dt}$$

and dividing throughout by X

$$\frac{1}{X} \frac{dO_2}{dt} = m_{O_2} + \frac{1}{Y_{G\ O_2}} \cdot \frac{1}{X} \cdot \frac{dx}{dt}$$

$$\therefore Q_{O_2} = m_{O_2} + \frac{\mu}{Y_{G\ O_2}} \qquad (3)$$

m_{O_2} and $Y_{G\ O_2}$ are constants, and Q_{O_2} and μ are measurable, so a plot of Q_{O_2} against μ should show a linear relationship with gradient $\frac{1}{Y_{G\ O_2}}$ and intercept m_{O_2}

3.3 Preliminary experiments at high μ carbon limitation

Figure 14 shows the results of an arithmetic-type glycerol limitation experiment. Initially the cells grow exponentially on glycerol, which is being pumped at a rate faster than the cells can

Figure 14

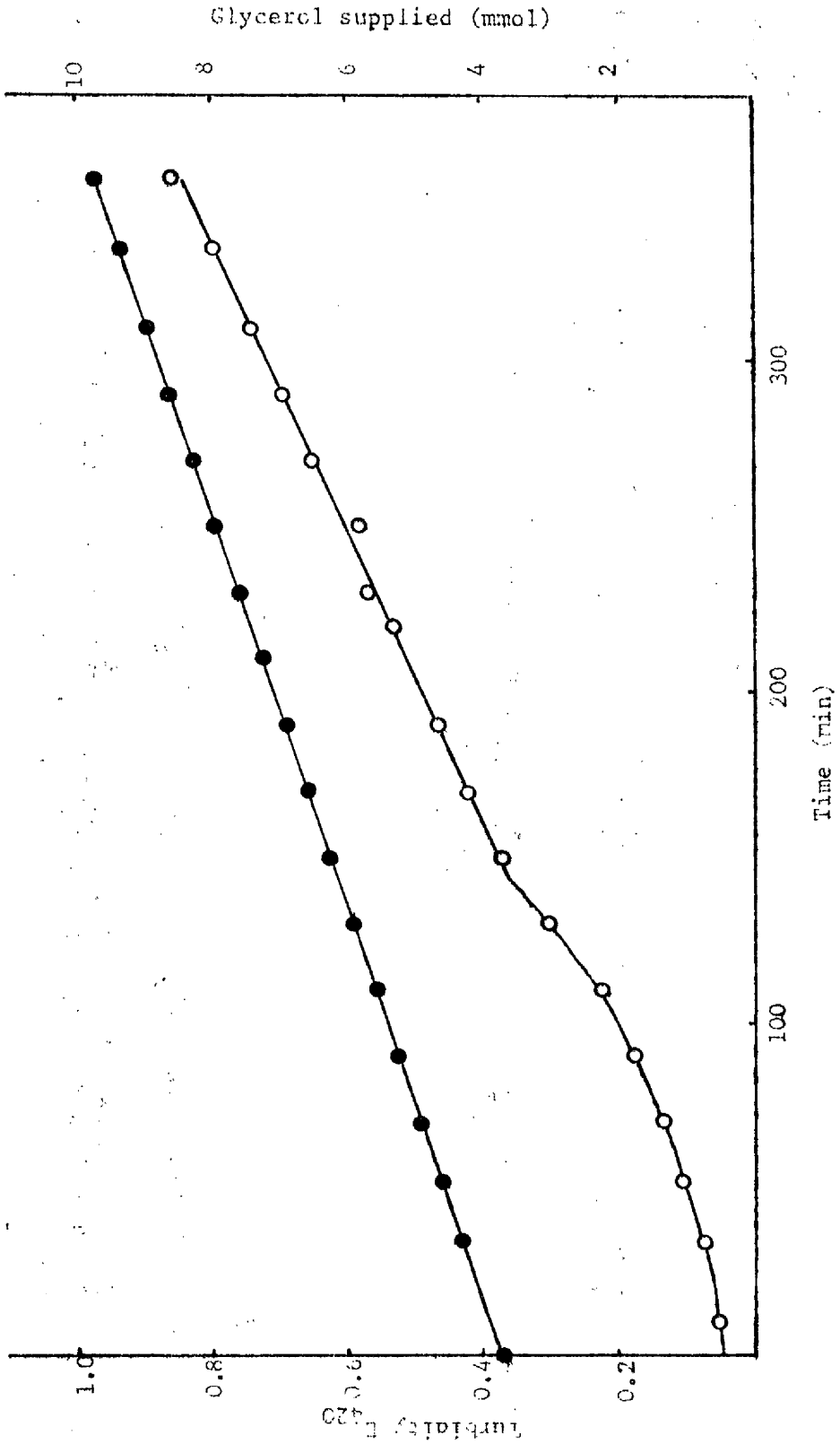
Growth and substrate supply for preliminary arithmetic-type carbon-limited continuous culture

1 M glycerol was pumped at a fixed rate into a culture volume of 1600ml. The Y(glycerol) from this experiment, calculated from the quotient of the two gradients, was 48.5g/mole.

—○— turbidity at 420nm

—●— glycerol supplied (mmol)

Figure 14


Glycerol supplied (mmol)
10
8
6
4
2
Turbidity E_{420}
1.0
0.8
0.6
0.4
0.2
100
200
300
Time (min)

use it. During this phase, glycerol builds up in the medium then as the cell mass increases, the glycerol is used up more quickly, and eventually all of the accumulated glycerol is used up and the cells become carbon-limited. Growth then appears to be linear with respect to time. Since the supply rate of glycerol is also constant, this means that the apparent molar growth yield is constant. According to equation (2), if the maintenance coefficient m is real, then $\frac{dx}{dt}$ should fall as X rises. This is evidently not the case for this experiment. The specific growth rates covered by this experiment were approximately 0.2 - 0.7 h^{-1}. The small value of m did not have any effect on the molar growth yield at these growth rates.

The molar growth yield in this experiment, which was calculated by taking the quotient of the two gradients shown on Figure 14, was found to be 48.5 g/mole, in good agreement with Hamilton (1972). We believe that this type of experiment, where growth rates are sufficiently high to make maintenance energy negligible, may be the most accurate way of determining molar growth yields, rather than the more traditional methods described by Stouthamer (1969). One particular advantage of the system is that no excretion products are found in the culture medium, so the calculation of molar growth yields is simplified. For example, when E. coli ML 308 was grown in batch culture, 0.44 mole of acetate were excreted per mole of glucose utilised. In this type of arithmetic culture, no acetate can be detected in the medium.

3.4 Carbon-limited maintenance experiments

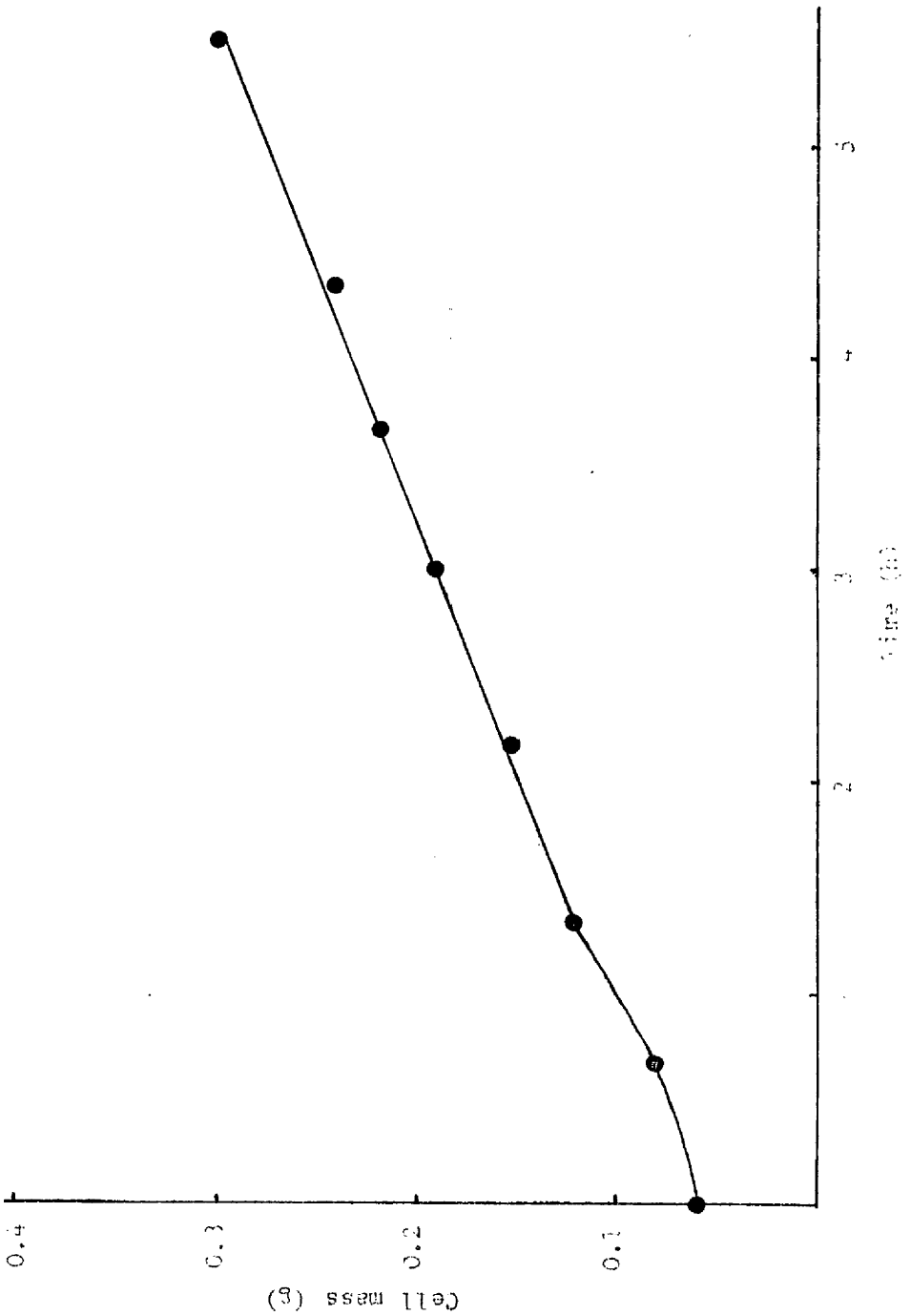
In order to measure the maintenance requirement of cells growing by this type of arithmetic limitation, it was necessary to increase the proportion of available energy used in the non-growth processes. This was achieved by increasing the mass of cells in the

Figure 15

Growth in initial stages of carbon-limited arithmetic-type continuous culture

Growth of cells was limited by a flow of 1.104ml/h 0.40 M glucose. Turbidity at 420nm was converted to dry weight of cells using the relationship between cell density and turbidity of 0.225 mg/ml/O.D. 1.0

Figure 15



flask, thus effectively reducing the specific growth rate.

3.4.1 Typical example of results

Cell density, oxygen uptake, carbon dioxide evolution, substrate supply rate and pH were measured throughout the course of each experiment. In this way, the components $\frac{dS}{dt}$ and X of equation (2) were measured directly. Figure 15 shows the growth curve obtained from the initial stages of a glucose limitation experiment. As before, there is no indication of the arithmetic growth rate $\frac{dx}{dt}$ falling as the mass of cells in the flask increases. However, Figure 16 shows the effect of increasing the cell mass. As the quantity of cells increases with time, the gradient of the growth curve falls. At high cell densities, the proportion of available energy used for maintenance becomes significant, resulting in a decreasing availability of carbon and energy for growth. In terms of equation (2), it can be seen that $\frac{dx}{dt}$ falls as X increases.

In this particular experiment, one of the first to be attempted, the rate of substrate supply was measured by recording the volume change in a 25ml pipette. Figure 17 demonstrates the constancy of the supply rate of substrate delivered by the peristaltic pump.

Equation (3) requires the values of $\frac{dO_2}{dt}$, X and $\frac{dx}{dt}$ for its solution. The method of calculating $\frac{dO_2}{dt}$ has been discussed in section 1, and X is measured directly. Figure 18 shows the relationship of $\frac{dO_2}{dt}$ with time. In order to calculate a value of μ for each value of Q_{O_2} $(\frac{1}{X} \cdot \frac{dO_2}{dt})$ it is again necessary to determine the value of $\frac{dx}{dt}$ for each value of X.

Figure 16

Growth to high cell density in carbon-limited arithmetic-type culture

Cells were grown in a glucose-limited culture. Growth was measured turbidimetrically and converted to cell dry weight. The turbidity at the end of the experiment was 8.28.

This graph is the continuation of that illustrated in Figure 15. The straight line drawn is the initial growth rate $\frac{dx}{dt}$ as determined from Figure 15.

Figure 16

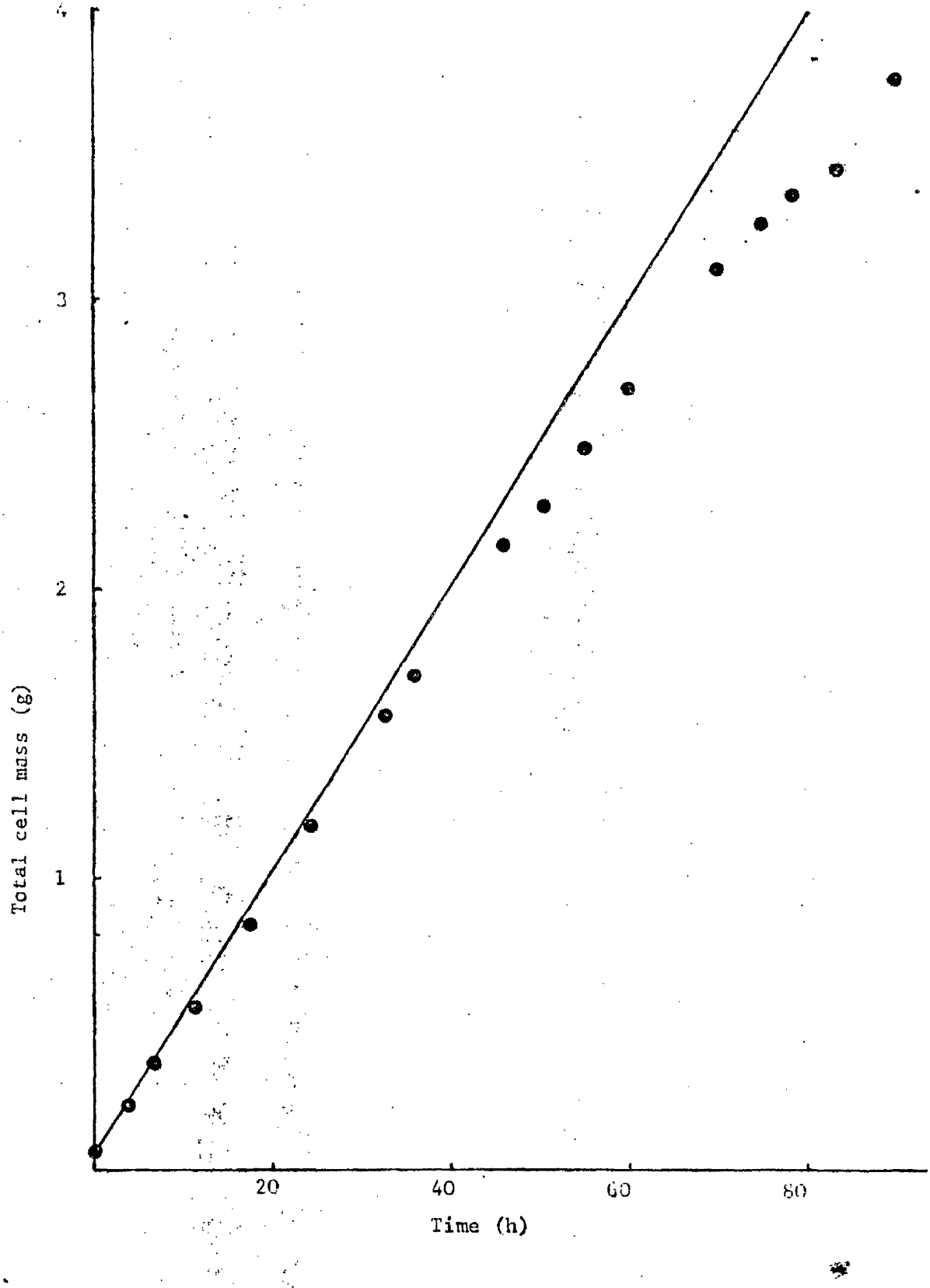


Figure 17

Supply of limiting carbon source delivered by peristaltic pump

0.40 M glucose was delivered by a Varioperpex peristaltic pump. The rate of supply for this experiment, also described by Figures 6, 15 and 16, was 1.104ml/h.

Figure 17

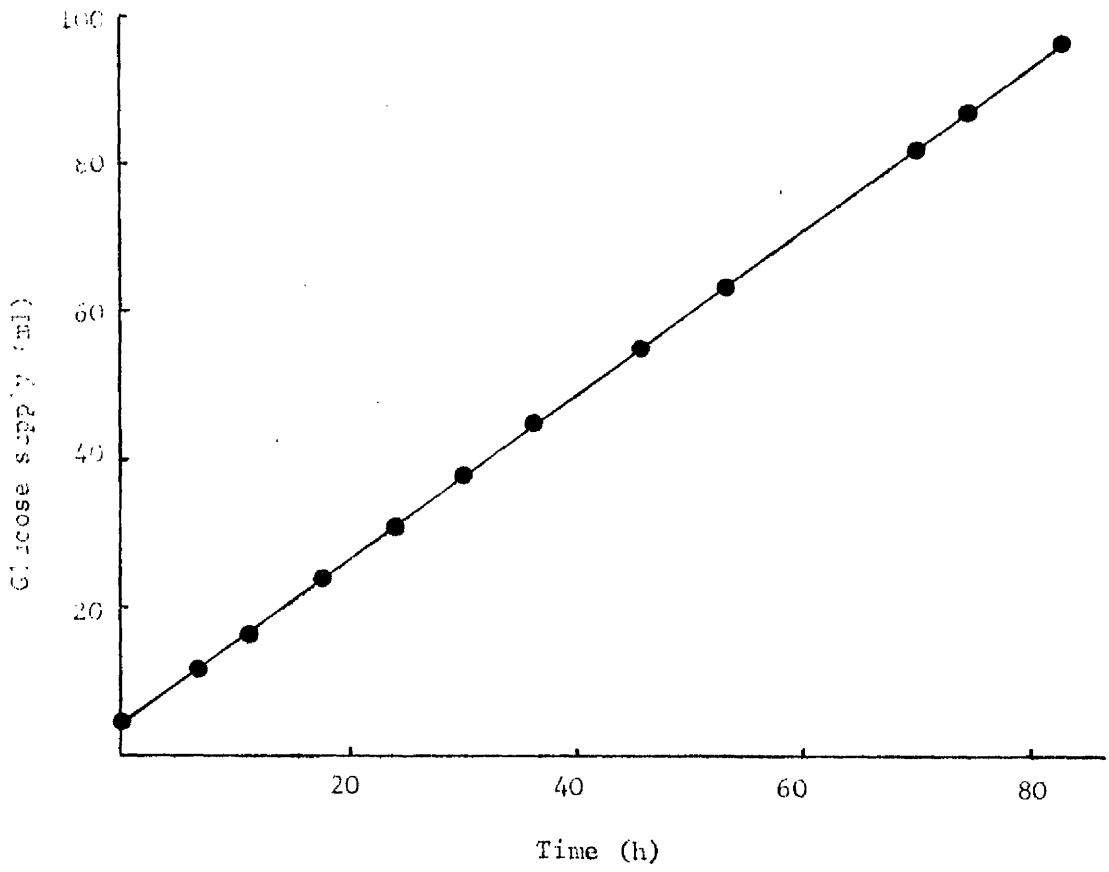


Figure 18

Gas exchange during carbon-limited arithmetic-type continuous culture

O_2 uptake and CO_2 evolution were measured continuously throughout a glucose-limited arithmetic-type continuous culture. Readings on the O_2 and CO_2 analysers were converted from partial pressures to μmol/min (p.68)

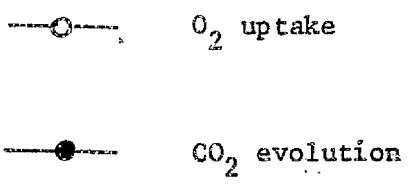
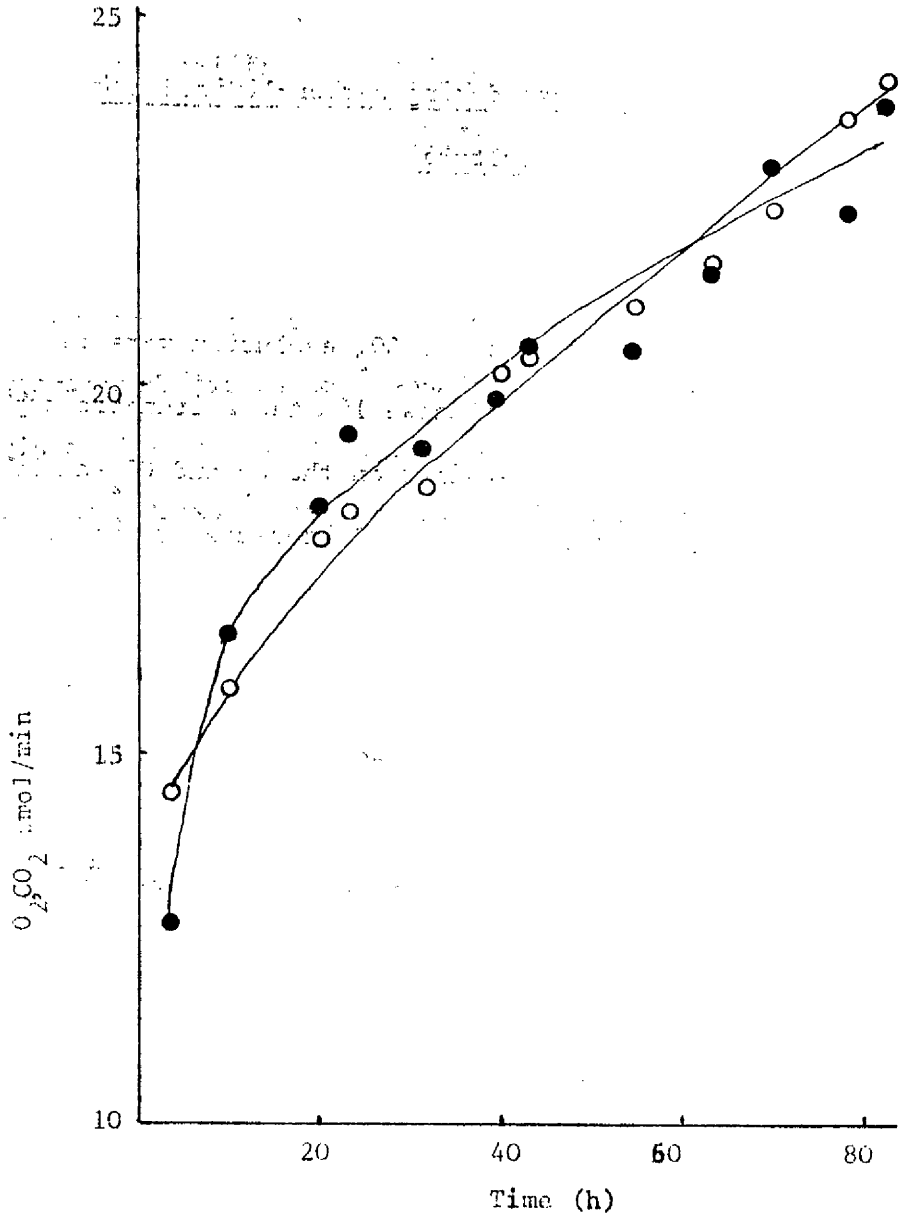


Figure 18



3.4.2 Estimation of $\frac{dx}{dt}$ by curve fitting

$\frac{dx}{dt}$ is the gradient of the tangent to the growth curve X v t at any value of t, and so if the equation of the graph $x = f(t)$ can be determined, $f'(t)$ (i.e. $\frac{dx}{dt}$) can be calculated by differentiation of $x = f(t)$.

Equation (2) can be rearranged:

$$\frac{dS}{dt} = m.X + \frac{1}{Y_G} \cdot \frac{dx}{dt} \qquad (2)$$

$$\therefore \quad \frac{dx}{dt} = Y_G \left(\frac{dS}{dt} - m.X\right) \cdot \qquad (2a)$$

Integrating both sides

$$\frac{dx}{\frac{dS}{dt} - m.X} = Y_G \qquad dt$$

$$\therefore \quad -\frac{1}{m} \cdot \log_e \left(\frac{dS}{dt} - m.X\right) = Y_G \cdot t \qquad (4)$$

Thus, X is now expressed directly in terms of t. In theory, since $\frac{dS}{dt}$ is constant, and X and t are measured variables, it should be possible to calculate m and Y_G directly from equation (4).

Unfortunately facilities were not readily available for the processing of an expression of the complexity of equation (4). However, it was possible to generate best-fitting curves for the graph of X.v.t by computer. The equations generated by this method were of the form

$$X = a + bt + ct^2 + \ldots + z\ t^n$$

Each equation could then be differentiated to give values of $\frac{dx}{dt}$ for each value of t. $\frac{dx}{dt}$ was then plotted against X. Figure 19 shows the results of these calculations for different values of n in the polynomial. None of these graphs is linear, with the deviation from

Figure 19

Generation of $\frac{dx}{dt}$ v X by curve-fitting of X v time

The data for the graph of X v time were fitted to functions

$$X = \sum_{o}^{n} a\, t^{n}$$

for values of n = 2, 3, 4 and 5. The first derivative of the equation was taken, in order to calculate $\frac{dx}{dt}$ for each value of time, and hence also for each value of X. The values of $\frac{dx}{dt}$ so generated were plotted against X.

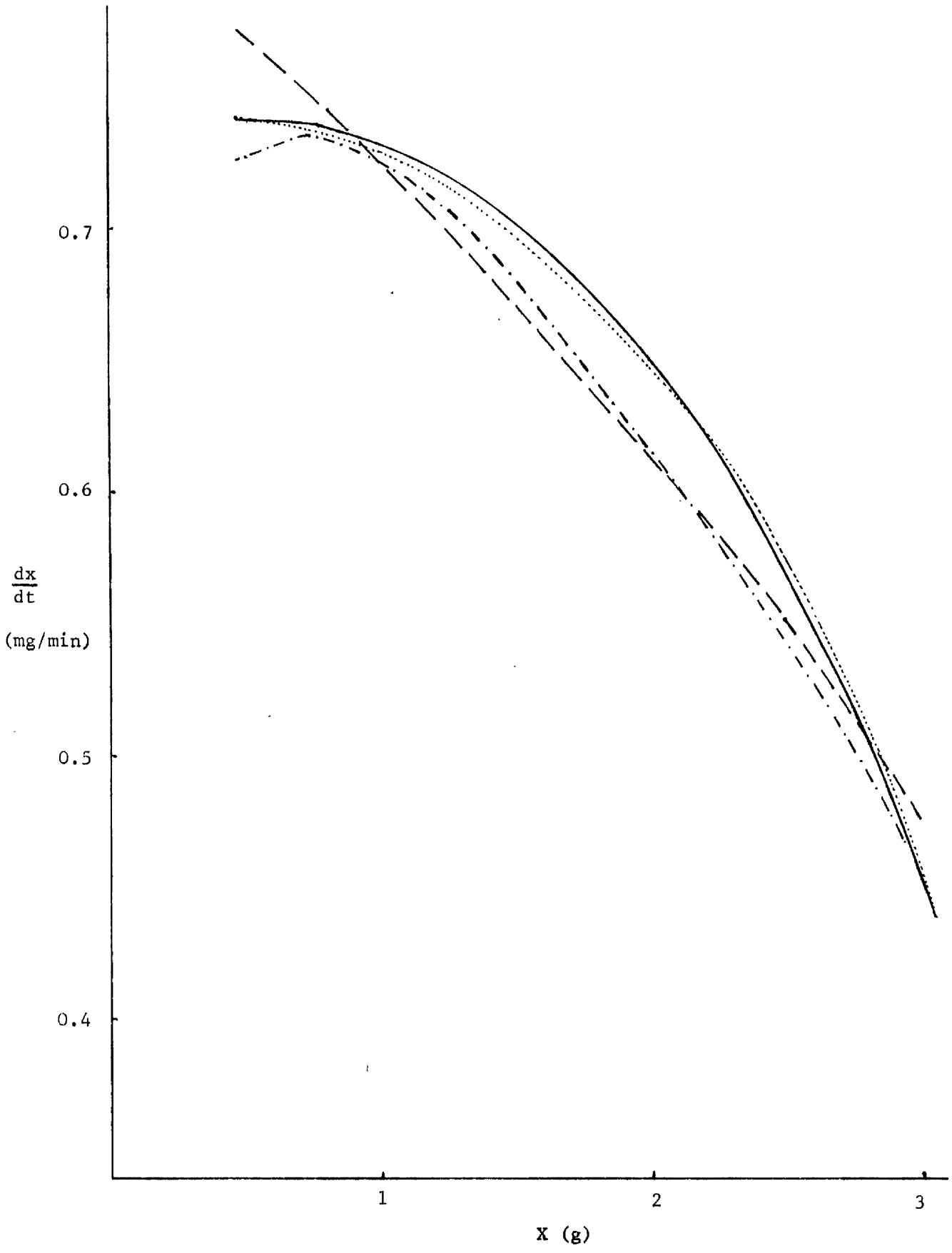
— — — — n = 2

..... n = 3

——— n = 4

—·—·—·— n = 5

Figure 19



linearity increasing with the value of n. If the theory underlying equation (2) is correct, the graph of $\frac{dx}{dt}$ v. X should be linear. It was concluded that curve-fitting introduced additional errors, and that a more direct method of determining $\frac{dx}{dt}$ should be used.

3.4.3 Estimation of $\frac{dx}{dt}$ by carbon balance

A different method of determining $\frac{dx}{dt}$ is by drawing up a carbon balance. Provided that the fate of all of the carbon not being converted to cell material is known and can be measured, the quantity of carbon which is fixed as cell material can be calculated by subtraction. Then, if the carbon content of the cells is known, the rate of synthesis of total cell material can be worked out.

Fortunately, the application of this method to maintenance experiments is relatively simple. The rate of supply of carbon is known. The carbon dioxide evolved is continuously monitored, and no excretion products were found. In addition, an overall carbon balance was carried out for each maintenance experiment performed. It was found that, if all the carbon supplied was entirely accounted for by the sum of carbon dioxide and cell carbon, with no other products formed, a carbon balance was achieved. Over the first 14 maintenance experiments, the carbon recovery as measured by the sum of total carbon dioxide evolved and cell carbon formed was 100.4 ± 5.0%. For these calculations, the carbon content of the cell was assumed to be 44.6% by weight. Also, if a carbon balance was taken over parts of the experiment, rather than over the whole experiment, it was found that all of the carbon entering the flask could be accounted for in terms of cells and CO_2.

For example, the results for the experiment described by Figure 16 are as follows for two arbitrary time intervals:

1200 - 2600 min	CO_2 evolved	=	27.20 mmoles
			27.20 mg atom C
	Cells formed	=	979 mg dry wt
		=	36.39 mg atom C
	Glucose supplied	=	10.30 mmoles
		=	61.80 mg atom C
∴	C recovery	=	102.9%

2600 - 4200 min			
	CO_2 evolved	=	33.66 mmoles
		=	33.66 mg atom C
	Cells formed	=	986 mg dry wt
		=	36.65 mg atom C
	Glucose supplied	=	11.78 mmoles
		=	70.68 mg atom C
∴	C recovery	=	100.5%

This was checked for each maintenance experiment and was found to hold true within experimental error limits in each case. It was therefore possible to estimate the arithmetic growth rate $\frac{dx}{dt}$ by the difference between the rate of substrate carbon supply and the rate of carbon dioxide evolved. The general formula is:

$$\frac{dx}{dt} = \frac{12}{0.446} \left(n. \frac{dS}{dt} - \frac{dCO_2}{dt}\right)$$

where n is the number of carbon atoms per molecule of substrate and $\frac{dCO_2}{dt}$ is the rate of carbon dioxide evolution.

This method of determining the arithmetic growth rate $\frac{dx}{dt}$ was used in all maintenance experiments.

3.4.4 Estimation of molar growth yields and maintenance coefficients

Equation (2a) can be used to determine molar growth yields and maintenance coefficients in terms of the carbon source.

$$\frac{dx}{dt} = Y_G \left(\frac{dS}{dt} - m.X\right) \qquad (2a)$$

$\frac{dx}{dt}$ and X are the variables, so a plot of $\frac{dx}{dt}$ against X should be linear with intercept $Y_G \frac{dS}{dt}$ and gradient $- Y_G.m$. Since $\frac{dS}{dt}$ is known, Y_G and m should be calculable.

Figure 20 shows this plot for the glucose limitation described in section 3.4.1. The best-fitting straight line was determined by the method of least squares (Moroney, 1956). The correlation coefficient in this case is -0.969. The intercept on the $\frac{dx}{dt}$ axis is 703.3 µg/min. Since the value of $\frac{dS}{dt}$ was 7.36 µmol/min, the value of Y_G is $\frac{703.3}{7.36}$ µg/µmol i.e. 95.6 g/mole. The gradient of the line is -56.1 µg g^{-1} min^{-1}. Since Y_G = 95.6 g/mole, then the maintenance coefficient is 0.587 µmol g^{-1} min^{-1} or 35.2 µmol g^{-1} h^{-1}.

Figure 21 shows the relationship between Q_{O_2} and µ as predicted by equation (3).

$$Q_{O_2} = m_{O_2} + \frac{\mu}{Y_{G\,O_2}} \qquad (3)$$

Again, the best-fitting straight line was determined by the least squares method. The correlation coefficient is 0.998. The intercept, which represents m_{O_2}, is 253 µmol g^{-1} h^{-1}. The gradient, which represents $\frac{1}{Y_{G\,O_2}}$, is 22.2 µmolO_2/mg dry wt i.e. $Y_{G\,O_2}$ = 45.1 g/mole O_2.

It can be seen from the values of the correlation coefficients obtained that the use of equations (2a) and (3) is justified. A

Figure 20

Determination of the maintenance coefficient and maximum molar growth yield in terms of carbon source

Equation (2a) predicts a linear relationship between $\frac{dx}{dt}$ and X. The graph of $\frac{dx}{dt}$ against X for the previously described glucose-limited arithmetic-type continuous culture has an intercept which gives a value for Y_G of 95.6 g/mole. The gradient of the graph gives a value of 0.587 μmol g^{-1} min^{-1} for the maintenance coefficient. $\frac{dx}{dt}$ was calculated by the carbon balance method.

Figure 20

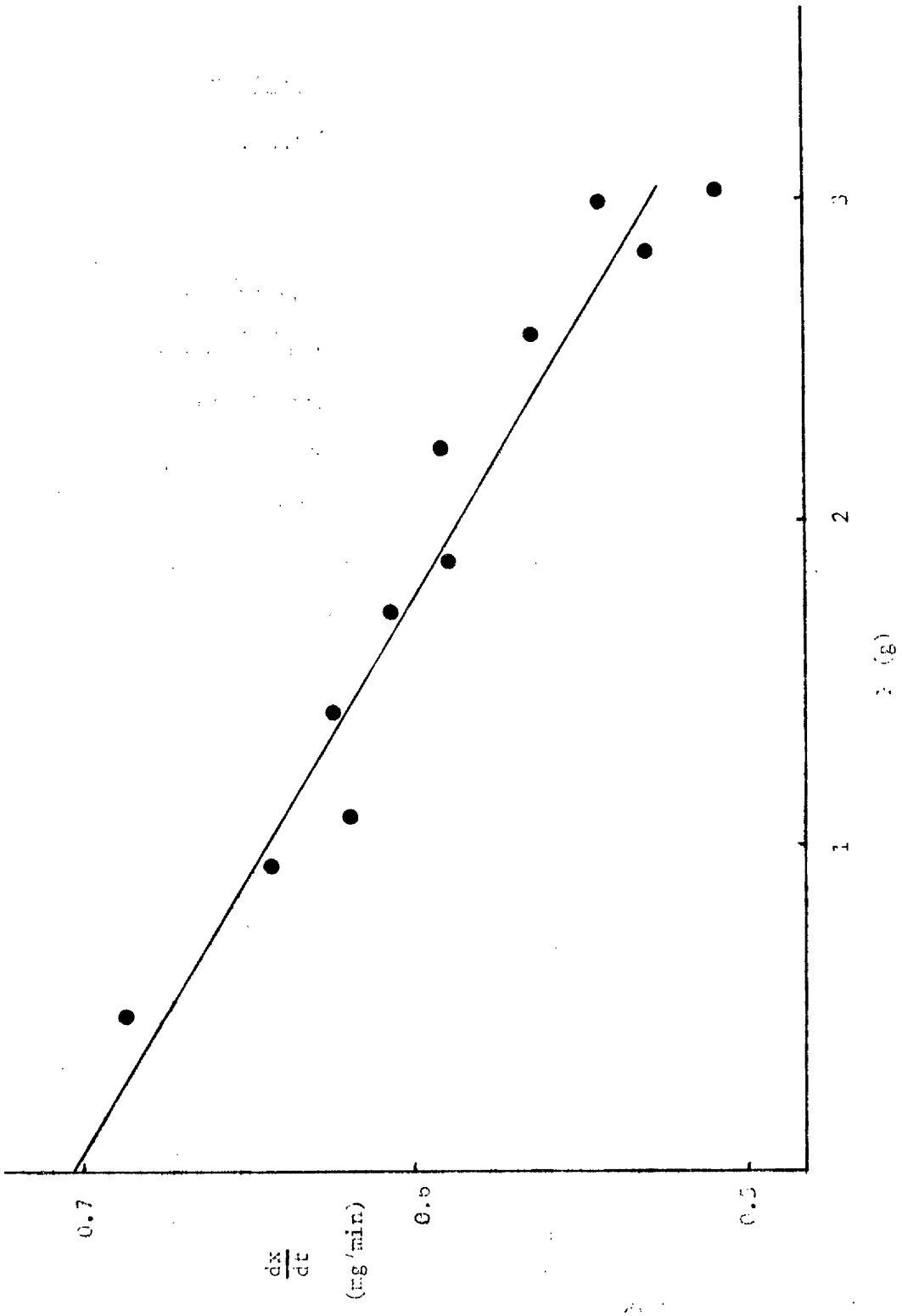
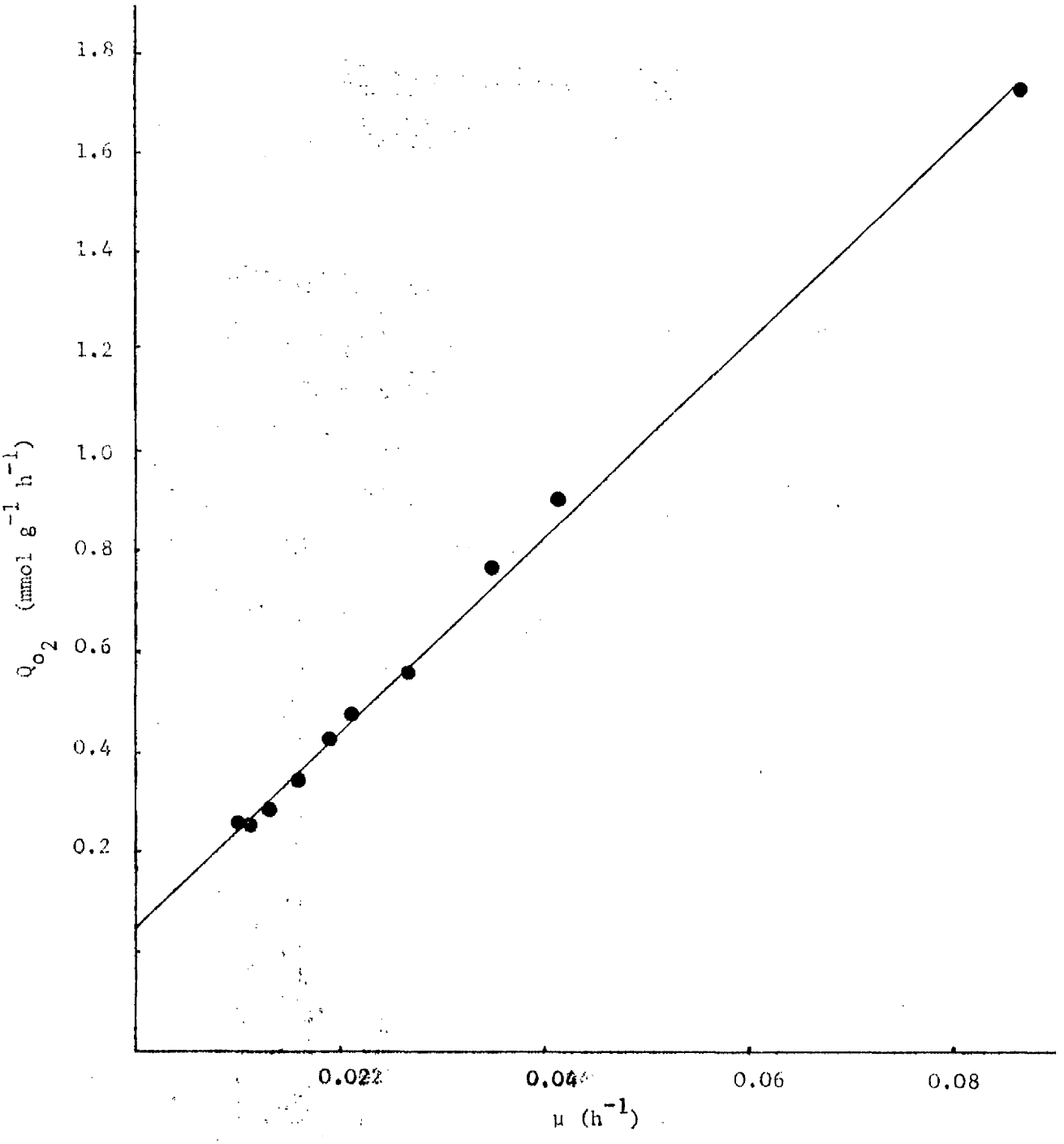


Figure 21

Determination of the maintenance coefficient and maximum molar growth yield in terms of oxygen

Equation (3) predicts a linear relationship between the specific rate of oxygen uptake (Qo_2) and the specific growth rate (μ). The graph of Qo_2 against μ for the previously described glucose-limited arithmetic-type continuous culture has an intercept of 0.252 μmol g^{-1} h^{-1}, which is m_{o_2}. The value of $Y_{G_{O_2}}$, derived from the reciprocal of gradient, is 45.1 g/mole.

Figure 21



complete statistical analysis of the data is complicated by the variance which is present in the parameters on both axes of each graph. If variance were present in the data on only one axis, the treatment would be fairly straightforward. Approximate error limits can be calculated for the intercepts on Figures 20 and 21, making the assumption that there is no error in X and μ respectively.

The standard error of the estimate is

$$Sy = \sigma y \sqrt{1 - r^2}$$

where $\sigma y^2 = \frac{1}{N} \Sigma y_i^2 - (\bar{y}i)^2$

and r = correlation coefficient (Moroney, 1956)

By this method, the intercepts give results

$$Y_G = 95.6 \pm 2.5 \text{ g/mole}$$

$$m_{O_2} = 252 \pm 1.8 \text{ μmol } g^{-1} h^{-1}$$

Calculation of error limits of the constants derived from the gradients would not be justified unless the variance in both axes were taken into account. Indeed, the computed standard error for m_{O_2} must be unrealistic by at least one order of magnitude, since when the experiment was repeated, the value for m_{O_2} was 221 μmol g^{-1} h^{-1} min^{-1}. Obviously, a constant, perhaps systematic error must outweigh statistical fluctuations in each particular experiment.

Due to pressure of time, only three experiments were repeated. The maximum difference between carbon source maintenance coefficients observed in repeat experiments was 11.1%, and the largest difference in repeat values for m_{O_2} was that reported above, which represents a difference of 13.7%.

On the other hand, the error quoted above for Y_G, ± 2.5%, seems to be quite realistic, since repeat experiments gave results within this error range.

4. Estimation of turnover

4.1 Turnover of enzymes

E. coli ML30 is inducible for both the lactose and glycerol regulons. Thus, if *E. coli* ML30 is pre-induced for these regulons, then grown in medium which does not induce the enzymes for lactose and glycerol utilisation, the rate of inactivation of these enzymes can be followed. In all of these experiments, *E. coli* ML30 trained to glycerol in the presence of 0.5mM IPTG was used as inoculum. It was hoped that the rate of inactivation of glycerokinase, α-glycerophosphate dehydrogenase, β-galactosidase and β-galactoside permease would give an indication of the rate of protein turnover, and that the rate of inactivation of these enzymes might vary according to growth conditions in the same way as the maintenance coefficient.

Non-specific permeation of ONPG was found to be a problem in the assay of β-galactoside permease. The total rate of ONPG hydrolysis by whole cells did not undergo the exponential decay found for the other enzymes (Figure 31). However, the difference in activity between whole cells in the presence of, and in the absence of, 5mM TDG was found to give the expected decay of enzyme activity (Figure 31b).

4.2 Turnover of total cell organic material

The rate of loss of label from uniformly ^{14}C-labelled *E. coli* growing in arithmetic-type continuous culture was found to decrease with time. Initially, cells lost label very quickly, with the ½-life for

the decay being of the order of 50 min at 37° (Figure 22). This exponential type of loss did not fall to a zero rate, but to what appeared likely to be a finite, constant rate, which may be the basal or endogenous rate of total carbon turnover. The experimentally measured parameters were rate of loss of $^{14}CO_2$ as a fraction of the total radioactivity of the culture (% h^{-1}) and time. The experimental data were fitted by computer, using the least squares method in order to obtain the best fit, to the general equation

$$k = c_1 + c_2 e^{-c_3 t},$$

where c_1, c_2 and c_3 are constants, and k is the rate of loss of $^{14}CO_2$ at time t. Values of k then tend towards the value c_1 as t increases (Figure 22). This method of curve-fitting was used to calculate values of c_1 (= asymptotic value of k as $t \to \infty$) for the loss of $^{14}CO_2$ for E. coli growing on several substrates at several temperatures.

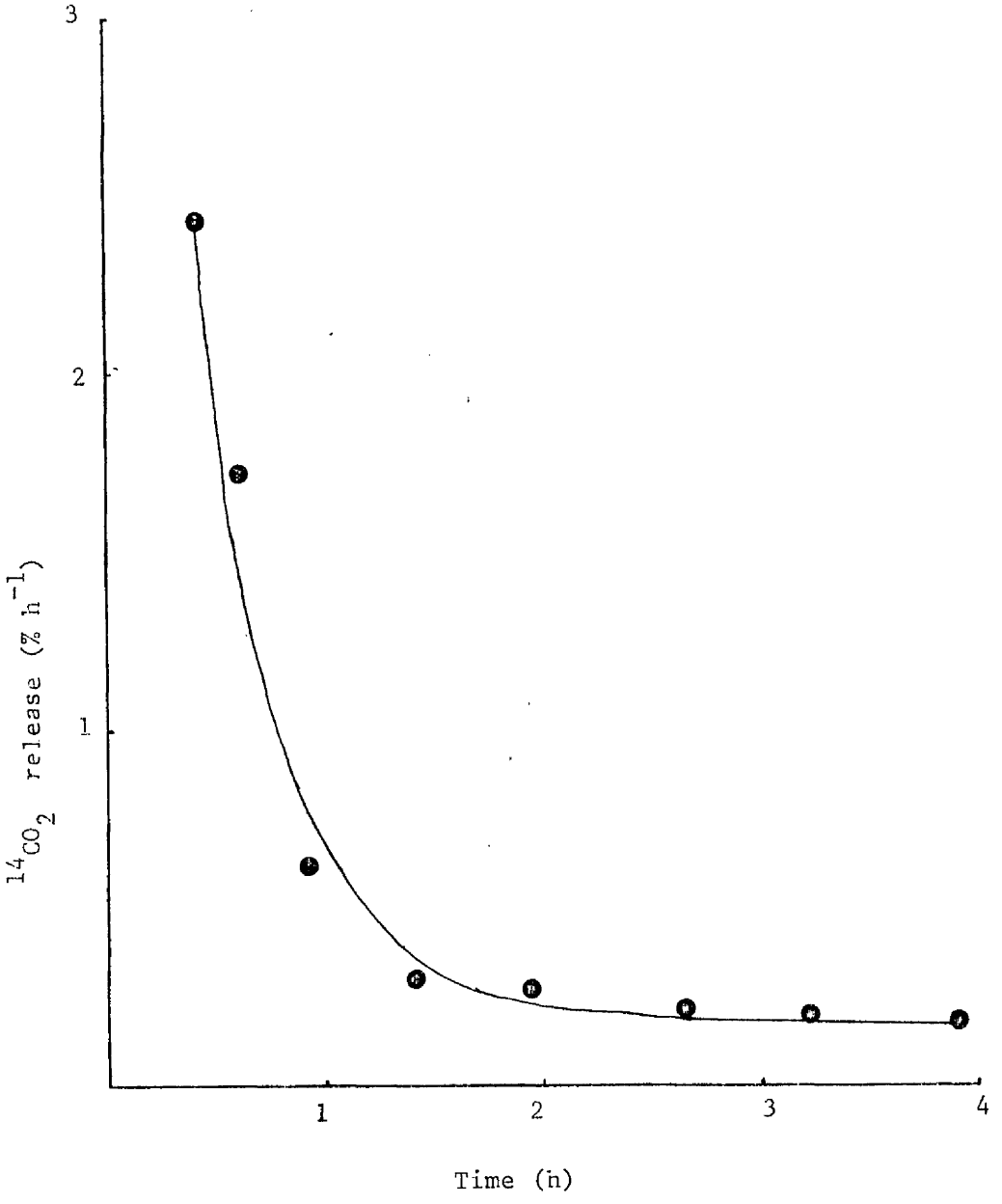
FIGURE 22

Loss of $^{14}CO_2$ from glucose-limited arithmetic-type continuous culture

Uniformly ^{14}C-labelled *E. coli* were inoculated into a glucose-limited arithmetic-type continuous culture at 37°. $^{14}CO_2$ released was trapped and counted. The average rate of $^{14}CO_2$ release, as a percentage of the initial radioactivity in the culture, over the time interval was calculated, and plotted against time elapsed since inoculation.

The best-fitting curve line corresponding to an exponential decay to a constant value was calculated (p. 99) and is drawn on the facing graph. The asymptotic value for the rate of $^{14}CO_2$ loss in this case was 0.179 % h^{-1}.

FIGURE 22



MATERIALS

With the exception of those reagents listed below, all reagents were 'Analar' or the highest purity grade obtainable from British Drug Houses, Poole, England.

Obtained from Sigma, London, were:-
BCIG, Trizma base, NAD, NADH, methyl glyoxal, semicarbazide HCl, TDG, IPTG, D,l-α-glycerophosphate, ONPG, 5,5'-dithiobis-(2-nitrobenzoic acid), acetyl CoA, phenazine methosulphate, diaminopimelic acid, glutathione and glyoxalase I.

Obtained from Boehringer, London, were:-
Myokinase, triethanolamine HCl, acetate kinase, lactate dehydrogenase, blood sugar assay kit, neutral fat assay kit, and malate dehydrogenase.

Obtained from Koch-Light Laboratories Ltd., Colnbrook, England, were:-
Toluene, PPO, POPOP and diaminopimelic acid.

L-(-)-Aepfelsäure (malic acid) was obtained from Fluka, Buchs, Switzerland; Chloroform from Hopkin & Williams, Chadwell Heath, Essex, England; L-histidine and L-isoleucine from T. J. Sas & Son Ltd., London; 40% formaldehyde solution from May & Baker, Dagenham, England; ethanol from Burroughs, Wellcome & Co. Ltd., London; oxygen-free nitrogen from British Oxygen Co. Ltd., Glasgow; carbon dioxide from Distillers Co. Ltd., Glasgow.

All radiochemicals were obtained from the Radiochemical Centre, Amersham, England except standard ^{14}C- and ^{3}H-toluene, which were obtained from Packard Instruments Ltd., Wembley, Middlesex, England.

RESULTS

1. Maintenance energy and molar growth yields

1.1 Chemostat culture

The maintenance coefficient and maximum molar growth yield for *E. coli* in a glycerol-limited chemostat were determined by plotting the reciprocal of the observed molar growth yield against the reciprocal of the dilution rate (Figure 23). From the growth equation derived by Pirt (1965):

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_G}$$

the gradient of the best-fitting straight line gave a value of 307 μmol g^{-1} h^{-1} for the maintenance coefficient. The intercept gave a value of 50.3 g/mole for Y_G. A poor correlation coefficient was found for this line (0.766), which gave a standard error in the estimate of Y_G of 3.0 g/mole.

Gas exchange was not measured for chemostat culture.

1.2 Carbon-limited arithmetic-type continuous culture

A composite table of results of determinations of Y_G, Y_{G_O}, $\underline{m}$, and $\underline{m}_{O_2}$ appears in Figure 24. The column headed $\frac{m_{O_2}}{m_{O_2}^*}$ is the ratio of the measured value of $\underline{m}_{O_2}$ to the predicted value of $\underline{m}_{O_2}$ derived from $\underline{m}$. This predicted value for the maintenance coefficient in terms of O_2 follows the terminology of Nagai and Aiba (1972) who define $\underline{m}_{O_2}^*$ as the oxygen maintenance coefficient which would be expected from the measured value of $\underline{m}$ assuming complete combustion of the carbon source.

A detached copy of the same table may be found inside the rear cover of this thesis.

FIGURE 23

Relationship between apparent molar growth yield and specific growth rate from chemostat culture

E. coli was grown at 37° in a glycerol-limited chemostat as described in Methods section 9. The turbidity of the culture was measured by diluting to an optical density <0.2. The average of at least three determinations was converted to bacterial dry weight/ml using the factor 0.225mg/ml/O.D.10. The glycerol concentration of the feed was measured enzymatically, and the apparent molar growth yield (Y) was obtained by division of the dry wt/ml by glycerol concentration.

The intercept on the 1/Y axis is $^{1}/_{YG}$ and the gradient of the line is *m*.

The best fitting straight line was determined by the least squares method.

FIGURE 23

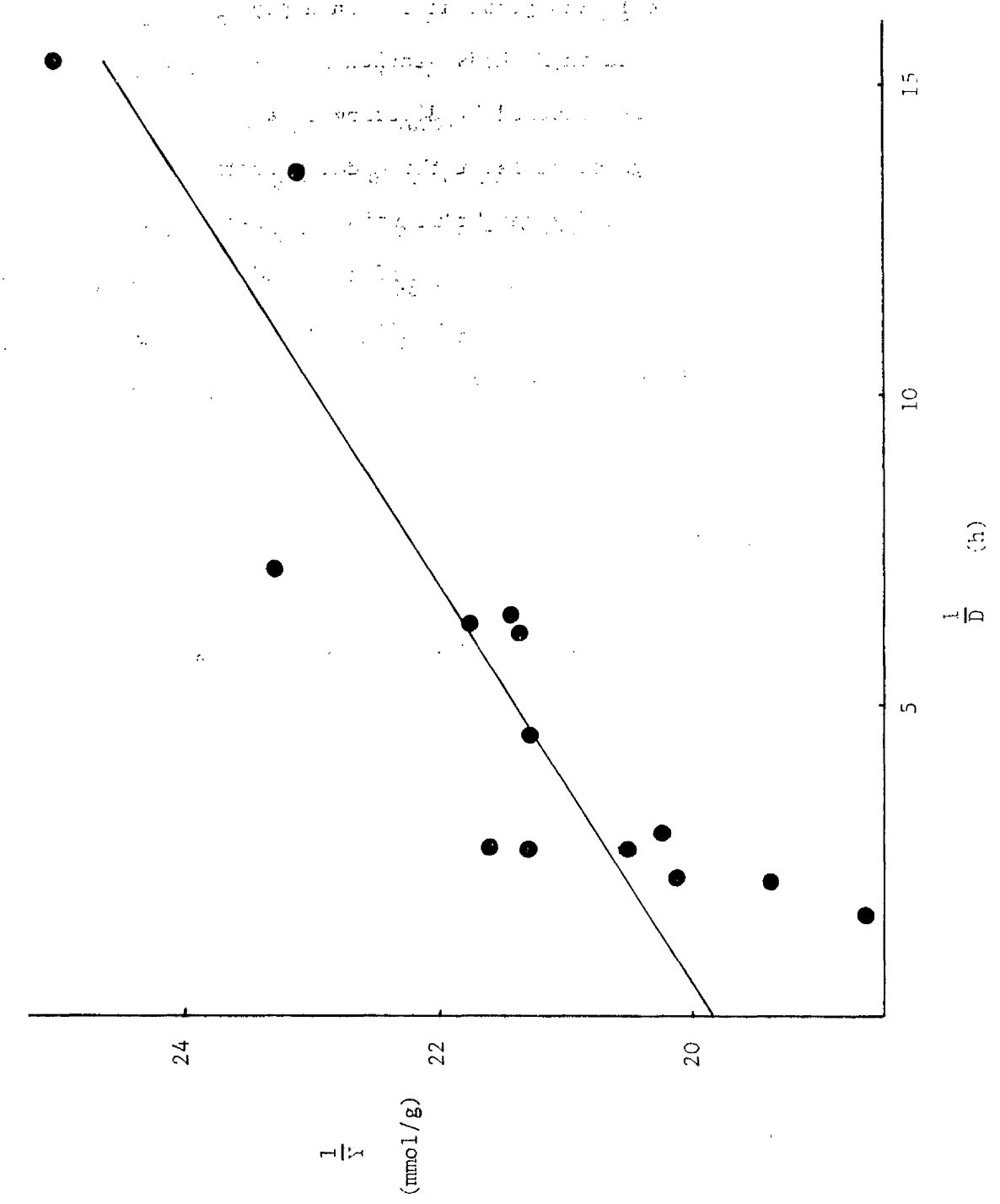


FIGURE 24

Composite table of molar growth yields and maintenance coefficients from all carbon-limited arithmetic-type continuous culture experiments

Carbon	Temp	Concn added NaCl M.	Y_G g/mole	Y_{GO_2} g/mole	m µmol g/h	m_{O_2} µmol g/h	$\frac{m_{O_2}}{m_{O_2}^*}$
Glycerol	40	–	54.7	37.4	232	795	0.98
	37	–	50.8	38.9	109	343	0.90
	37	–	50.6	34.2	97	321	0.94
	30	–	57.3	47.8	38	126	0.95
	25	–	56.8	42.0	40	141	1.01
	37	0.5	47.1	23.4	75	212	0.81
	37	0.5	46.6	26.8	88	337	1.10
	30	0.5	44.9	23.3	53	142	0.76
Glucose	40	–	115.2	67.9	260	1585	1.01
	37	–	95.6	45.1	35	253	1.19
	37	–	94.3	45.5	40	221	0.93
	30	–	103.9	57.2	26	133	0.84
	25	–	111.9	67.6	26	137	0.88
	37	0.25	107.2	54.1	47	291	1.04
	37	0.5	93.2	42.8	54	326	1.01
	40	0.5	102.2	56.9	242	1598	1.10
Malate	40	–	38.9	26.8	473	1394	0.98
	37	–	43.3	33.8	127	419	1.10
	30	–	44.1	38.4	68	197	0.97
Lactose	40	–	210.0	60.9	87	890	0.86
	37	–	212.5	57.4	37	406	0.92
	37	–	224.0	66.6	37	468	1.06
	30	–	217.2	61.8	13	129	0.85
Glycerol in 60mM phosphate buffer (c.f. 40mM)	37	–	55.5	42.0	95	347	1.05
Glycerol chemostat	37	–	50.3	–	309	–	–

FIGURE 24

1.2.1. General observations

The ratio $\frac{m_{O_2}}{m_{O_2}^*}$ is a measure of how close the experimentally derived values for maintenance in terms of carbon source and oxygen approximate to complete combustion of the carbon source used for maintenance. In all cases but one, the ratio lies between 0.8 and 1.2. The majority of these (70%) lie between 0.9 and 1.1. Thus, the results obtained from aerobically-grown carbon-limited arithmetic-type continuous culture confirm that maintenance in E. coli is a process, or processes, which depends on complete combustion of the carbon source rather than its rearrangement into other products.

The response of each of Y_G and m to different conditions is apparently independent of the other, which shows that the requirement for non-growth energy is independent of the efficiency with which energy is utilised for growth. However a common relationship of both quantities with the production, rather than the utilisation, of energy can not be ruled out.

1.2.2. Effect of addition of sodium chloride on maintenance and molar growth yields

The maintenance coefficient of cells growing arithmetically on glucose at 37° was increased by the addition of sodium chloride. 0.25 M sodium chloride increased m by 24% and 0.5 M sodium chloride by 43%. The maintenance coefficients in terms of oxygen increased correspondingly. Neither Y_G nor Y_{GO_2} was radically changed. For growth at 40°, neither m nor m_{O_2} appeared to increase on addition of 0.5 M sodium chloride. However, a change of ~17 μmol glucose g^{-1} h^{-1} (the increase in m at 37° on addition of 0.5 M NaCl) would lie within experimental error limits for the much larger value of m at 40°. A

response of this magnitude may therefore have occurred at 40°, but the method would not be expected to detect the change. The values of Y_G and Y_{GO_2} at 40° fell slightly when 0.5 M sodium chloride was present.

Addition of 0.5 M sodium chloride to glycerol-limited cultures at 37° decreased $\underline{m}$ and $\underline{m}_{O_2}$ slightly, but at 30° increased these maintenance coefficients. More striking differences were observed in the values of Y_{O_2} when salt was present. At both temperatures, a large decrease was found in Y_{O_2} on addition of 0.5 M NaCl. (A decrease of 50% was found at 30°). Y_G was not altered at 37°, but at 30° fell by 20%.

No acetate, pyruvate, lactate, ethanol, methyl glyoxal or volatile organic acids were found in the low-salt medium at the end of an experiment. Neither was any H_2 gas evolved from the culture. Thus the main effect of growth in high salt concentrations appeared to be on maintenance for glucose cultures and the efficiency of oxygen utilisation for glycerol cultures.

The total concentration of the inorganic cations K^+, NH_4^+ and Mg^{2+} was 0.12 M before addition of NaCl.

1.2.3. Effect of temperature on maintenance and molar growth yields

The relationship between temperature and maintenance coefficients for each of glucose-, glycerol-, malate- and lactose-limited cultures appeared to follow the same broad pattern (Figure 25). In the region of 30° and below, $\underline{m}$ and $\underline{m}_{O_2}$ were small and relatively constant, but increased dramatically as temperature increased (Figure 26), $\underline{m}_{O_2}$ was approximately the same for each substrate at 30°, excepting malate, which was 50% larger. (This may reflect the fact that malate is more oxidised than the other three substrates). On the other hand, the increase in

FIGURE 25

Relationship between maintenance coefficient and temperature for glucose-, glycerol-, malate- and lactose-growing cells

Maintenance coefficients are measured in units of μmol substrate g^{-1} h^{-1}. $\underline{m}_{O_2}^{*}$ is the maintenance coefficient for oxygen which would be expected from complete combustion of the carbon source used for maintenance.

Carbon source	Temp.	m	m_{O_2}	m_{O_2}*
glucose	40	260.4	1585	1562
"	37	37.8	237	225
"	30	26.4	133	158
"	25	25.8	137	155
glycerol	40	232.2	795	813
"	37	103.2	332	360
"	30	37.8	126	132
"	25	39.6	141	139
malate	40	473.4	1394	1420
"	37	127.2	419	382
"	30	67.8	197	203
"	25	-	-	-
lactose	40	86.4	890	1037
"	37	36.8	437	442
"	30	12.6	129	151
"	25	-	-	-

FIGURE 25

FIGURE 26

Relationship between m_{O_2} and growth temperature

m_{O_2} for the different combinations of carbon source and temperature was determined by carbon-limited arithmetic-type continuous culture.

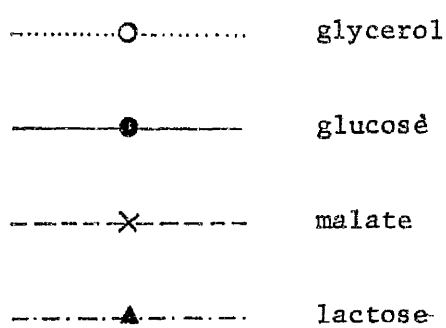
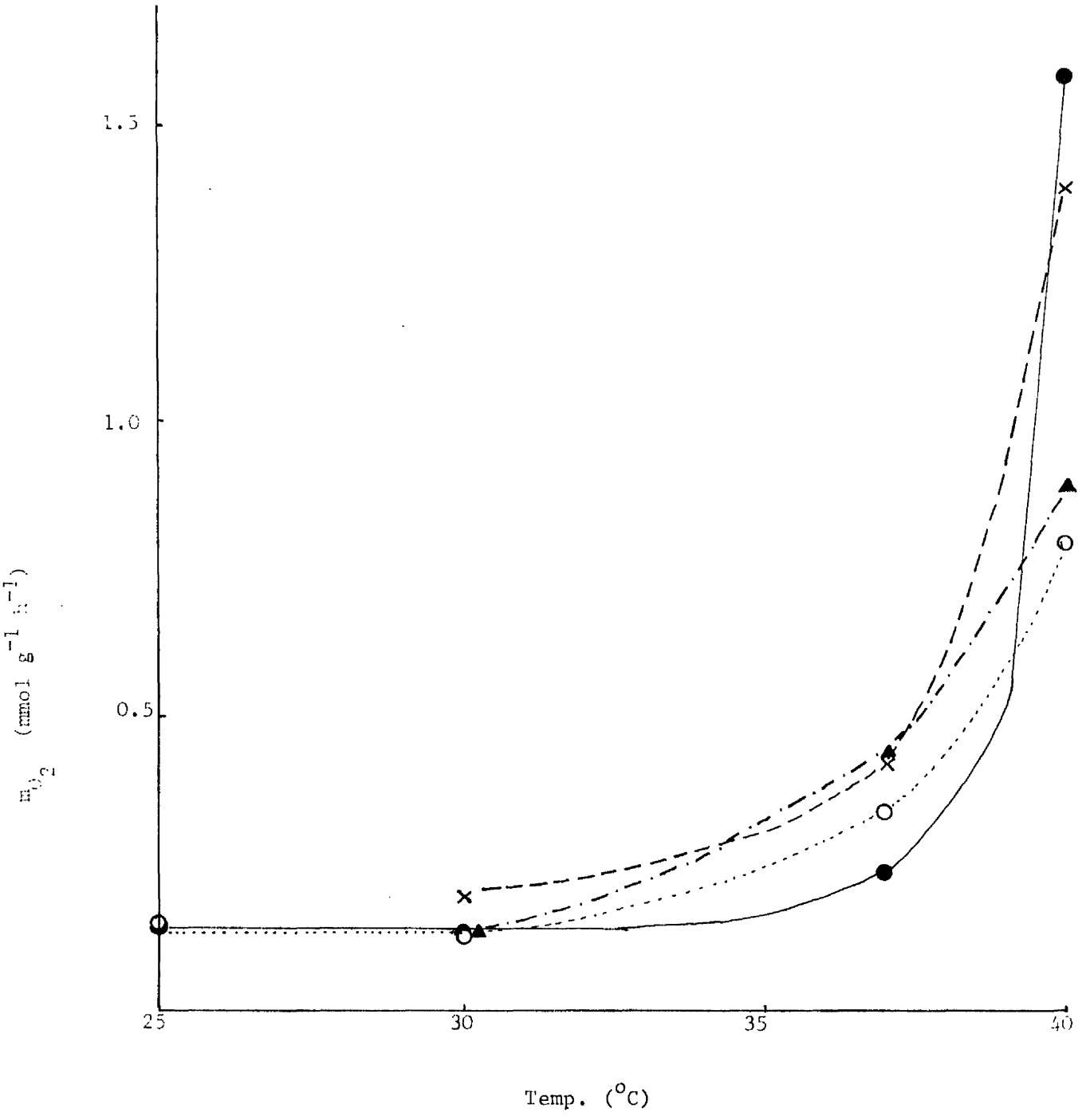


FIGURE 26



maintenance at 37° and 40° was different for each substrate. Arrhenius plots of these data were not linear (Figure 27). This would be expected if more than one component were contributing to the observed maintenance coefficient.

The shape of the graphs for glucose and glycerol maintenance coefficients in Figure 26 may suggest that $\underline{m}_{O_2}$ is relatively constant below 30°. If this assumption is made, and it is also assumed that it is a single different component of maintenance which increases so markedly between 37° and 40°, the activation energy of this second process can be determined. The Arrhenius equation becomes

$$\log_{10} \Delta \underline{m}_{O_2} = \text{constant} - \frac{E_a}{2.3RT}$$

where $\Delta \underline{m}_{O_2}$ is the difference between the $\underline{m}_{O_2}$ at 40° or 37° and the $\underline{m}_{O_2}$ at 30°.

E_a is the activation energy

R is the gas constant

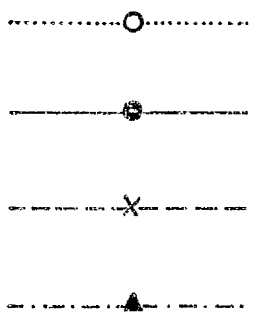
and T is the absolute temperature

Thus the value of E_a for the second process can be determined using the values of $\underline{m}_{O_2}$ measured at 37° and 40°. The E_a's obtained for cells growing on glucose, lactose, glycerol and malate were 169, 58, 78 and 108 kcal/mole respectively. Since only two values were used for each of these determinations and since the assumption made that one component remains constant at all temperatures used may not be completely valid, these E_a's are only very approximate. It is informative, however, that these E_a's are on average almost an order of magnitude larger than E_a's for most enzyme catalysed reactions (Dixon and Webb, 1964).

FIGURE 27

Arrhenius plots of m_{O_2}

The data shown in Figures 25 and 26 were plotted as $\log_{10}(m_{O_2})$ against the reciprocal of absolute temperature.



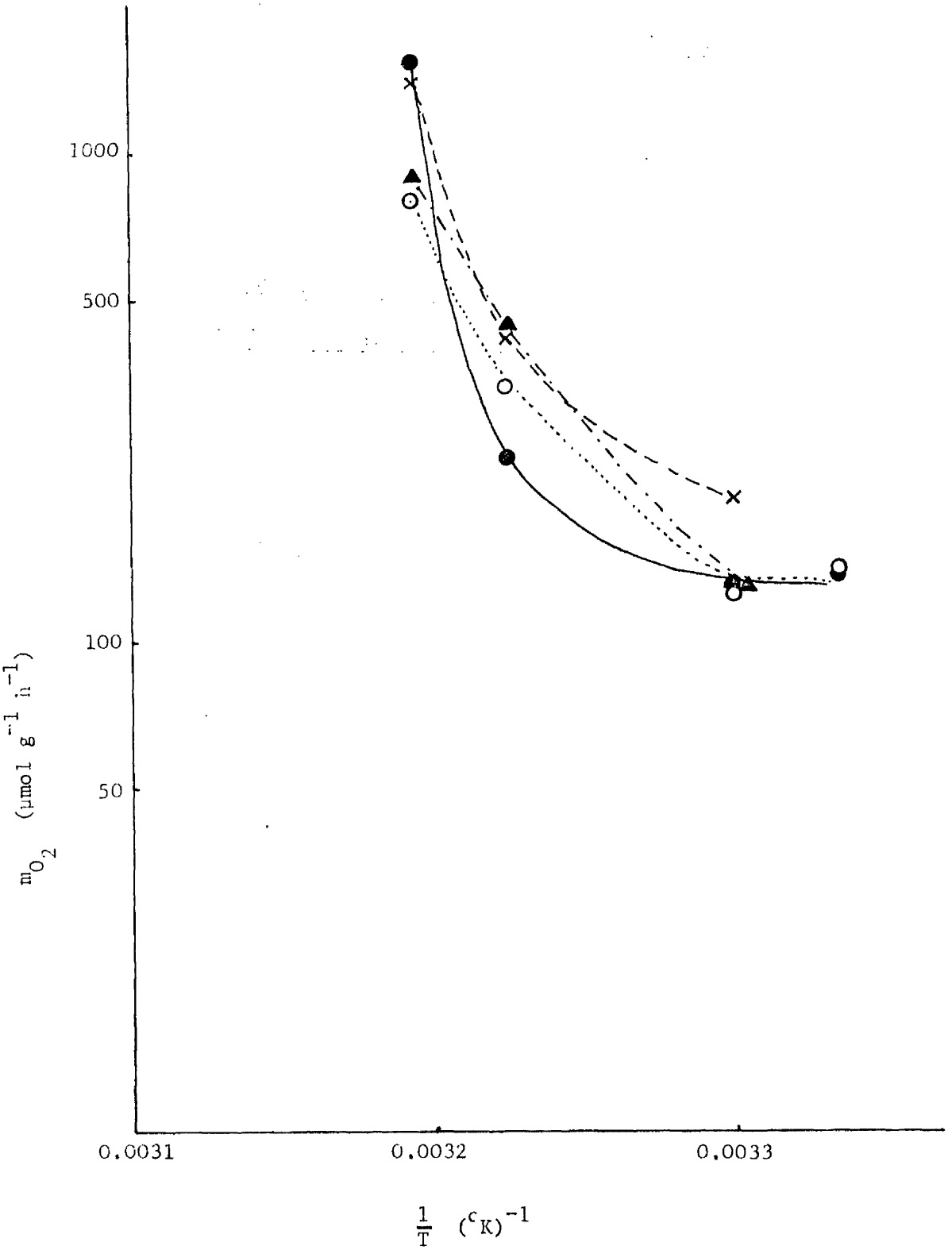
glycerol

glucose

malate

lactose

FIGURE 27



It is interesting that the optimal growth temperature of this organism is 37°, which appears to be at the transition phase in the graph of maintenance coefficient against growth temperature.

The relationship between growth temperature and Y_G (Figure 28) and Y_{GO_2} (Figure 29) was different for each carbon source. Although no consistent pattern can be seen, the fact that cells have their minimum Y_G for glucose and glycerol at 37°, the optimal growth temperature, is of interest. The relationship of Y_G with temperature was the same as Y_{GO_2} with all four substrates.

1.2.4. Effect of growth in 60mM phosphate medium on maintenance and molar growth yields

An increase in the concentration of phosphate in a glycerol-limited culture at 37° resulted in an increase of 17% in both Y_G and Y_{GO_2}. Values of m and m_{O_2} were unchanged by the change in phosphate concentration.

2. Elementary content of E. coli

Elements measured were carbon, nitrogen, hydrogen, phosphorus and sulphur. A direct method for oxygen analysis was not available.

The elementary content of lyophilised E. coli is shown in Figure 30. These values are an average for cells grown at 37° on glucose, glycerol, malate and lactose, for cells growing in a glycerol-limited chemostat at different temperatures and at different dilution rates at 37°. No significant difference was found for any element for these different types of cells. It should be noted that this type of elementary analysis was not possible for cells growing in high concentrations of salt, since the washing procedure resulted in lysis of the cells.

FIGURE 28

Relationship between Y_G and growth temperature

Values of Y_G were determined from carbon-limited arithmetic-type continuous culture at different growth temperatures by the method described in Development of Analytical Methods section 3.

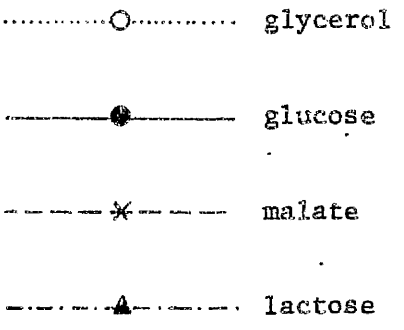


FIGURE 28

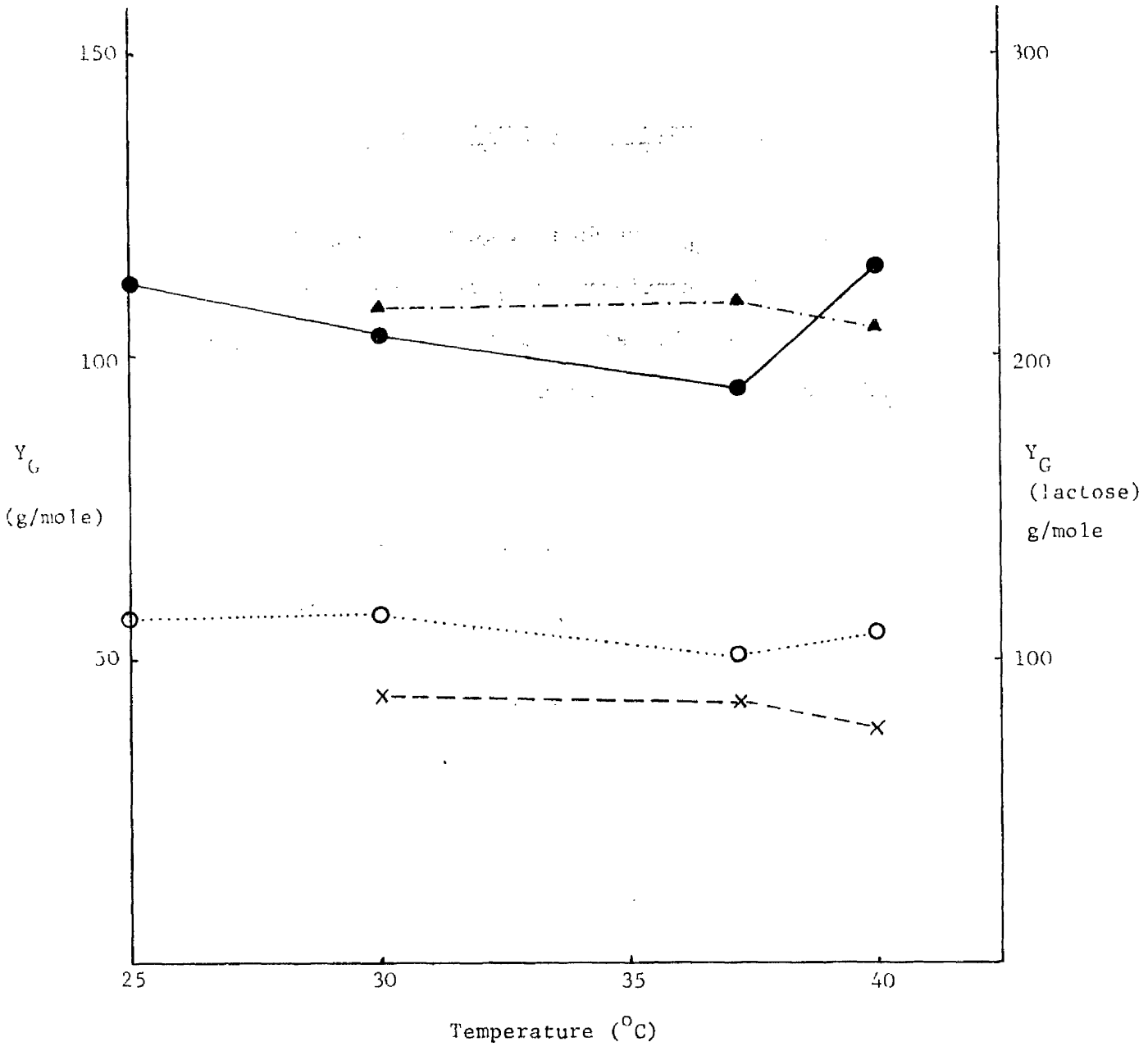

150
100
50
300
200
100
Y_G
(g/mole)
Y_G
(lactose)
g/mole
25
30
35
40
Temperature (°C)

FIGURE 29

Relationship between Y_{GO_2} and growth temperature

Values of Y_{GO_2} were determined from carbon-limited arithmetic-type continuous culture at different growth temperatures, by the method described in Development of Analytical Methods, section 3.

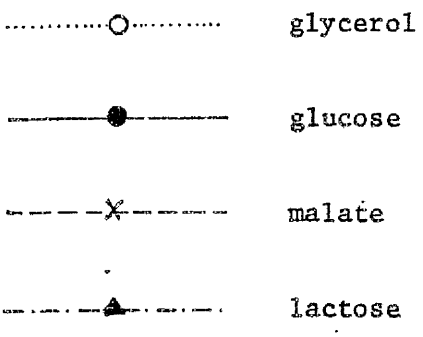


FIGURE 29

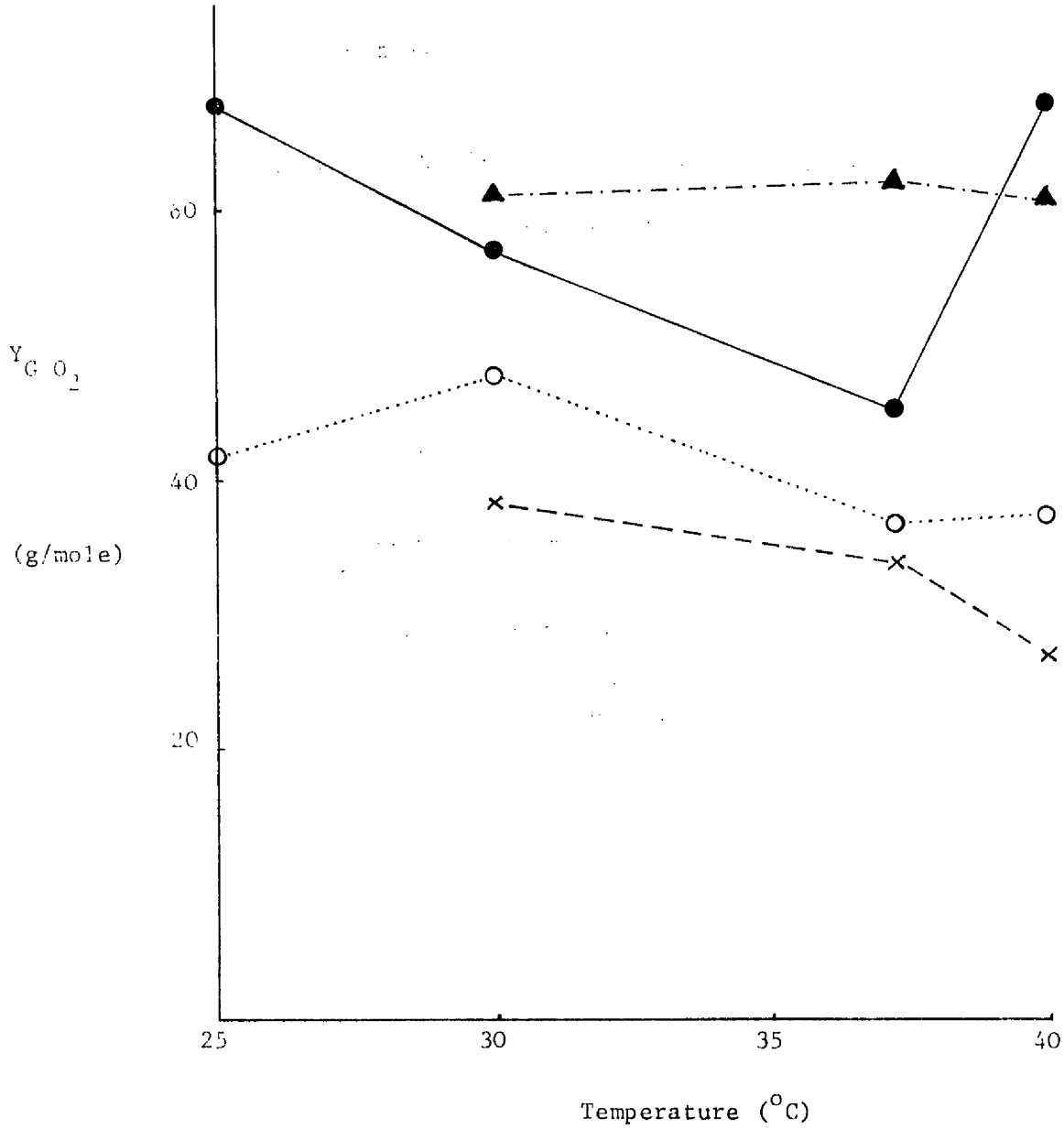


FIGURE 30

Elementary analysis of lyophilised E. coli

The average carbon, nitrogen, hydrogen, phosphorus and sulphur content of lyophilised cells was estimated. These estimates include data for cells grown on glucose, glycerol, malate and lactose at 37° in arithmetic-type continuous culture, cells grown on glycerol in chemostat culture at different temperatures and cells grown on glycerol at different μ at 37° in chemostat culture. In no case did the content of carbon, hydrogen or nitrogen differ significantly from the mean.

Element		% w/w	S.E.M. % w/w	g atoms/ 100g cells	S.E.M. %
carbon	(15)	44.57	2.20	3.71	4.9
hydrogen	(15)	6.65	0.34	6.65	5.1
nitrogen	(15)	12.59	1.11	0.90	8.8
phosphorus	(2)	3.49	0.73	0.11	21.1
sulphur	(2)	2.17	0.86	0.07	39.9

The figure in brackets is the number of determinations

* 3. Autoradiography: incorporation of a pulse of ^{14}C-glucose into individual cells growing in a glucose-limited arithmetic-type continuous culture

Cells growing at 37° under glucose limitation at a μ of approximately 0.067 were pulsed with ^{14}C-glucose as described on p. 54. The cells had been growing under glucose limitation for 24h prior to addition of label.

Examination of slides showed that there were two distinct types of stained material present (Plate 1). The vast majority of stained areas appeared to derive from burst cells, and have several grains over the stained area. The reason for the cells having burst during preparation of the slide for autoradiography is not known. A small number of rods appear to survive the treatment (1.2% of the total stained objects) and are similar to the rod-like shape of cells before treatment (Plate 2). No grains were observed over any of the rods on autoradiography slides.

A count was made of the number of grains appearing over the stained areas thought to be burst cells, and the distribution over 476 total stained areas was as follows:

No. of grains	% total
0	1.6
1 - 3	60.6
4 - 6	32.9
> 6	4.9

No stained area was observed which had more than 10 grains clearly visible over the stained area. Thus, the distribution of labelling of cells seems to indicate that all cells in carbon-limited arithmetic-type continuous culture are taking up the carbon source at approximately

* See Addendum on p. 210.

PLATE 1

Photomicrograph of autoradiography of arithmetically-growing cells pulsed with ^{3}H-glucose

A glucose-limited arithmetic-type continuous culture was pulsed with ^{3}H-glucose, sampled and autoradiography slides were prepared (p.54). After developing, photomicrographs were prepared (p.62). Plate 1 shows a typical field. Most of the stained areas are fairly disperse, and have grains over the stained area. The rod in the centre (indicated by arrow) is one of the unlabelled rods as described on p.115.

PLATE 2

Arithmetically-growing E. coli

Photomicrographs were prepared of a sample from a glucose-limited arithmetic-type continuous culture. The cells were fixed by heat and stained with carbol fuchsin.

Total magnification on Plates 1 and 2 is 1400×

PLATE 1

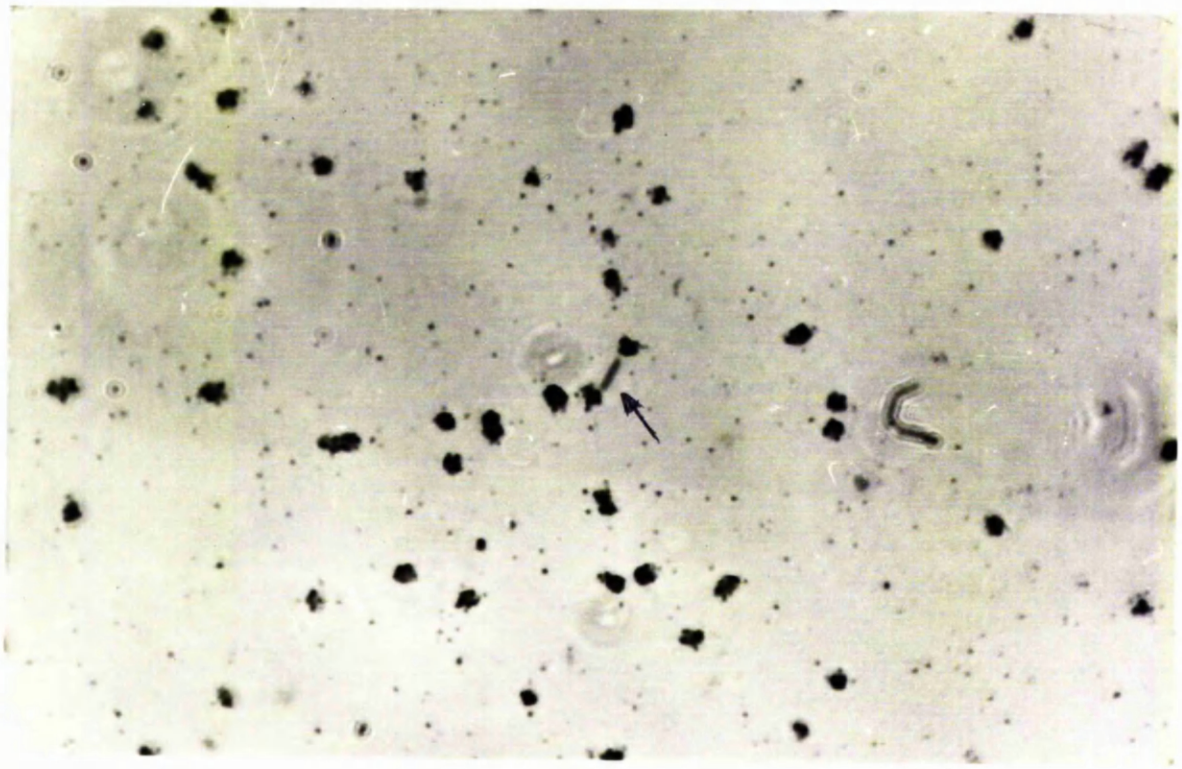
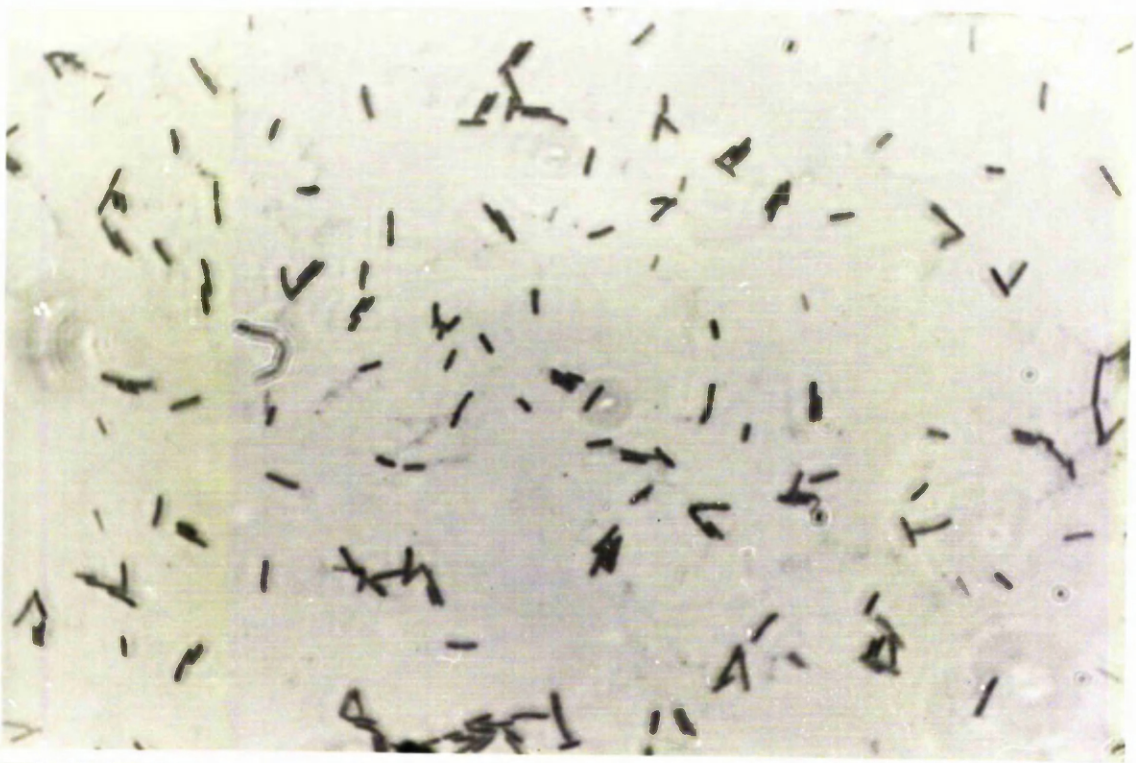


PLATE 2



the same rate, rather than some cells growing exponentially with the remainder stationary or dead. 1.2% of the cells, however, appear to be in a different category. These neither burst during the preparation of slides for autoradiography nor incorporate any labelled glucose.

4. Turnover of enzymes

The decay of each of β-galactosidase, β-galactoside permease, glycerokinase and α-glycerophosphate dehydrogenase at 37° in a glucose-limited arithmetic-type continuous culture occurs at a different rate (Figure 31).

Figure 32 summarizes the results of similar experiments at different temperatures. Each enzyme is inactivated at a different rate for each temperature, and moreover, the relationship between the rate of inactivation and temperature is quite different for each enzyme. For these reasons it was concluded that the study of inactivation of individual enzymes would not help relate the rate of protein turnover to the maintenance coefficient.

A few experiments in addition to those described by Figure 32 were performed. These are summarized in the following table. All of these experiments were done for glucose cultures at 37°. The abbreviations used are the same as for Figure 32.

Type of growth	Loss of activity $\%.h^{-1}$			
	G_z	G_p	GK	GPD
C-lim. arith.	6.2	26.9	6.8	17.9
N-lim. arith.	5.3	30.9	3.8	26.9
logarithmic	0.9	-	0.9	-
0.5 M NaCl) C-lim. arith.)	-	24.4	-	-

FIGURE 31 Decay of β-galactosidase, β-galactoside permease, glycerokinase and α-glycerophosphate dehydrogenase activities at 37°

E. coli ML30 was pre-induced for the lactose and glycerol regulons and inoculated to a cell density of 120 μg/ml in a glucose-limited arithmetic-type continuous culture. Initial activities of β-galactosidase (G_z), glycerokinase (GK), α-glycerophosphate dehydrogenase (GPD) and TDG-sensitive *lac* permease (Gp) were 1820, 107, 6.4 and 67 mU/ml respectively. The growth rate was ~27.5 μg ml^{-1} h^{-1}.

Activity/ml measured at timed intervals after inoculation were expressed as a percentage of the initial activity and plotted on an exponential scale against time.

FIGURE 31a

		% h^{-1} decay calculated
——●——	glycerokinase	6.8
—·—·—▲—·—·—	α-glycerophosphate dehydrogenase	17.9
·····○·····	β-galactosidase	6.2

FIGURE 31b

	TDG-sensitive *lac* permease	26.9

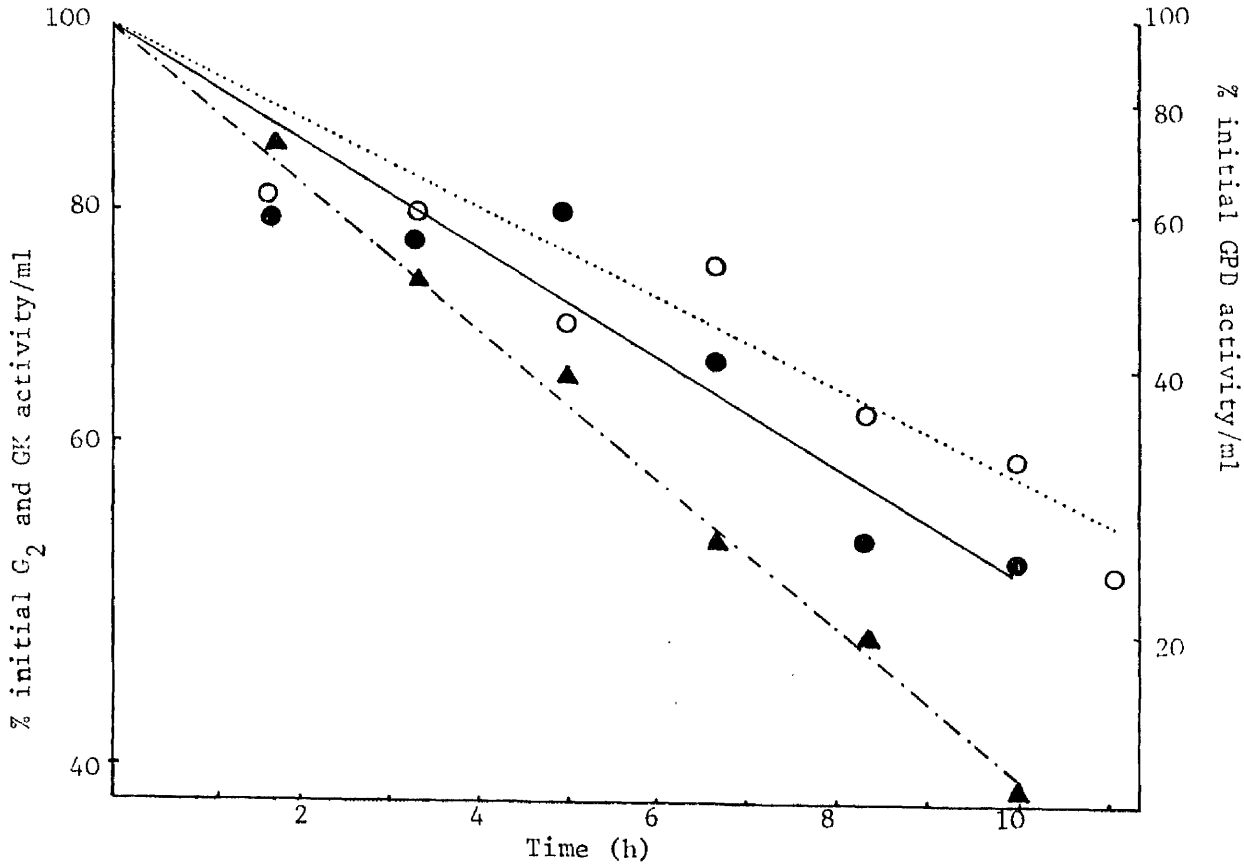


FIGURE 31a

FIGURE 31b

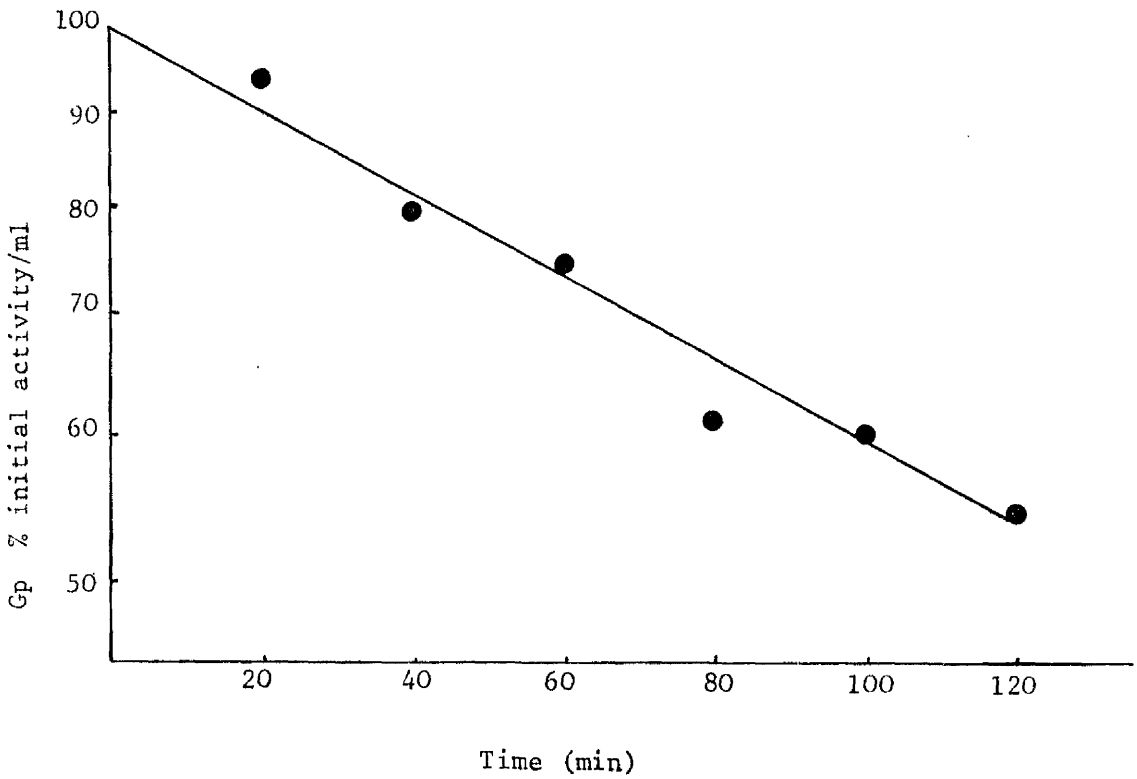


FIGURE 32

Comparison of rates of decay of β-galactosidase, β-galactoside permease, glycerokinase and α-glycerophosphate dehydrogenase at several temperatures

Experiments similar to those described by Figure 31 were performed at several temperatures. In all cases, the cells were inoculated into glucose-limited arithmetic-type continuous culture.

Loss of activity is expressed as % loss/h

G_z - β-galactosidase

Gp - β-galactoside permease

GK - glycerokinase

GPD - α-glycerophosphate dehydrogenase

Temp.	Loss of activity % h^{-1}			
	G_z	Gp	GK	GPD
40	5.8	28.5	13.1	17.9
37	6.2	26.9	6.8	17.9
34	-	24.8	-	-
30	1.6	11.5	4.0	16.6

The rate of inactivation of enzymes in exponentially-growing cultures is less than in either carbon- or nitrogen-limited arithmetic-type continuous cultures. It is perhaps surprising that N-limitation does not affect turnover of all the enzymes, as Pine (1973a) has implicated NH_4^+ as a control factor for protein turnover in E. coli.

5. Turnover of specifically-labelled cells

5.1 Use of amino acids to measure protein turnover

5.1.1 Labelling of cells with L-histidine

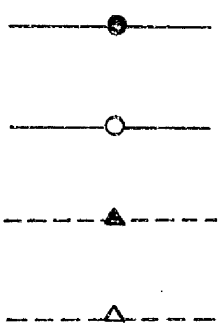
The most important criteria in the choice of an amino acid label were firstly that cells could take up small quantities of high specific activity amino acid into protein and secondly that cells could not use the amino acid as a source of carbon and energy for growth.

Two amino acids which met these criteria were L-histidine and L-isoleucine. Neither of these amino acids participates directly in the synthesis of polymers other than protein. When E. coli was presented with either of these amino acids as sole source of carbon and energy, no growth was observed after 72h, yet uptake of small quantities of high specific activity tritiated compound was found to be rapid when cells were growing on glycerol (Figure 33). The presence of a large excess of unlabelled histidine prevented uptake of label over a period of 5h, confirming that histidine was a suitable label for protein turnover experiments (Figure 34).

FIGURE 33

Uptake of ^{3}H-histidine and ^{3}H-isoleucine by glycerol-growing E. coli

Glycerol-trained E. coli were inoculated into a glycerol batch culture to a cell density of 10 µg/ml at 37°. At zero time, 5µCi ^{3}H-histidine and 10µCi ^{3}H-isoleucine were added to the culture, and the radioactivity in the cells and in the extracellular medium was estimated as described in Methods section 12.2 The first sample was taken within 10s of the labelled compound being added to the culture.



^{3}H-his in cells

^{3}H-his in medium

^{3}H-ile in cells

^{3}H-ile in medium

FIGURE 33

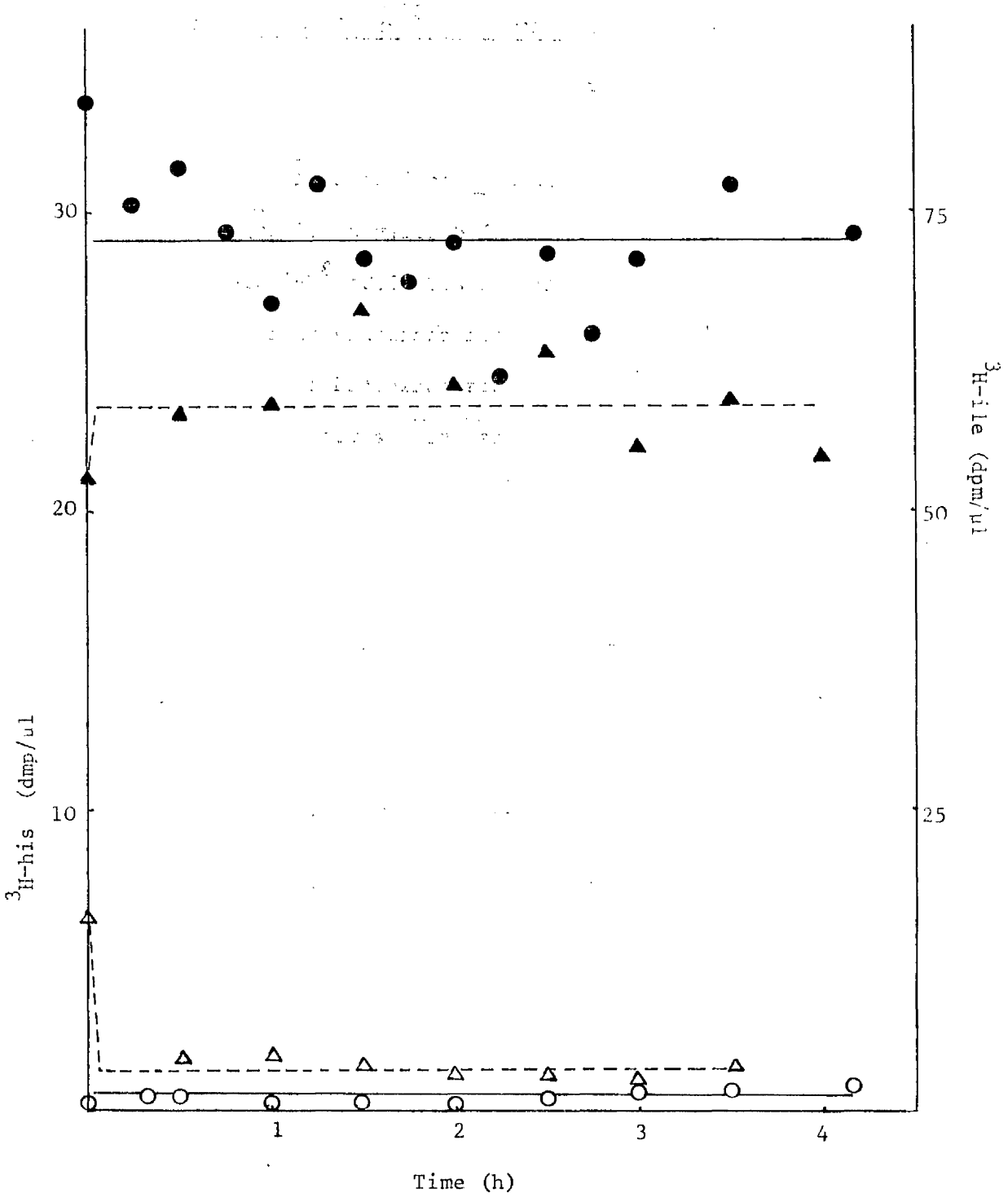


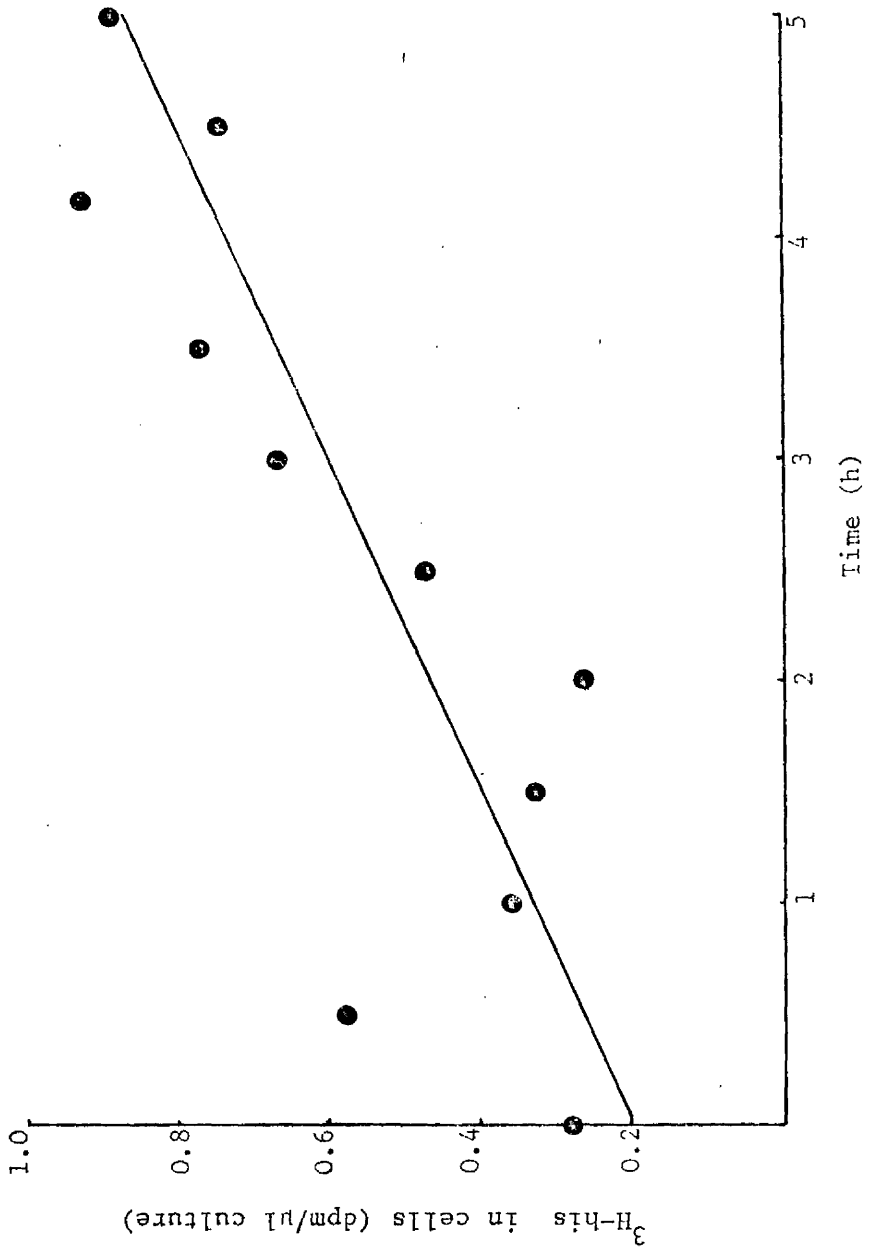
FIGURE 34

Uptake of ^{3}H-histidine in the presence of excess unlabelled histidine

~5μCi ^{3}H-L-histidine were added to a glycerol-limited arithmetic-type continuous culture growing at 37°, which already contained 0.2 mg/ml unlabelled histidine.

The specific activity of the filtrate was 22.6 dpm/μl, so the rate of uptake of ^{3}H-histidine from the medium into cells retained by the filter was small (0.6 % h^{-1})

FIGURE 34



5.1.2 Loss of ^{3}H-L-histidine from pre-labelled cells at 37° in different concentrations of cold histidine

E. coli was labelled with ^{3}H-histidine and inoculated into a glycerol-limited arithmetic-type continuous culture. Loss of label was measured as described on p. 55. It was found that increasing the concentration of cold histidine decreased the rate of loss of label from cells (Figure 35).

No label appeared in the filtrate when cold amino acid was absent from the growth medium. Loss of label was 1.48, 3.01 and 4.49% h^{-1} at concentrations of ^{3}H-histidine of 1.0, 0.2 and 0.03 mg/ml respectively. The apparent inhibition of protein turnover by increasing concentrations of histidine may reflect the role of individual amino acids in the regulation of protein turnover (Pine, 1973b). 0.2 mg/ml was selected as the most suitable concentration for use in subsequent experiments, since this is in the range of concentrations used by other workers (for review see Pine, 1972).

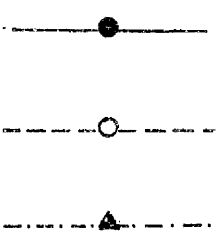
5.1.3. Effect of temperature on the loss of ^{3}H-L-histidine from pre-labelled cells

As temperature of growth increased, the rates of loss of ^{3}H-histidine from cells in glucose- and glycerol-limited arithmetic-type continuous cultures increased (Figures 36 and 37). It is immediately obvious on comparison of the rates of protein turnover and the maintenance coefficients at each temperature (Figure 38) that protein turnover varies with temperature in a way unrelated to the effect of temperature on the maintenance coefficient. Arrhenius plots of the protein turnover data confirm this conclusion (Figure 39). The E_a's for protein turnover are 21 and 27 kcal/mole for glucose- and glycerol-limited cells, compared with 169 and 78 kcal/mole for the maintenance coefficients.

FIGURE 35

Loss of ^{3}H-histidine from pre-labelled cells in the presence of different concentrations of unlabelled histidine

E. coli labelled with ^{3}H-histidine were inoculated into glycerol-limited arithmetic-type continuous cultures at 37° containing different concentrations of histidine. The total specific activity of cells at the beginning of each experiment was 37.0 dmp/µl. Samples were filtered and the filtrate counted.



1 mg/ml L-histidine

0.2 mg/ml L-histidine

0.05 mg/ml L-histidine

FIGURE 35

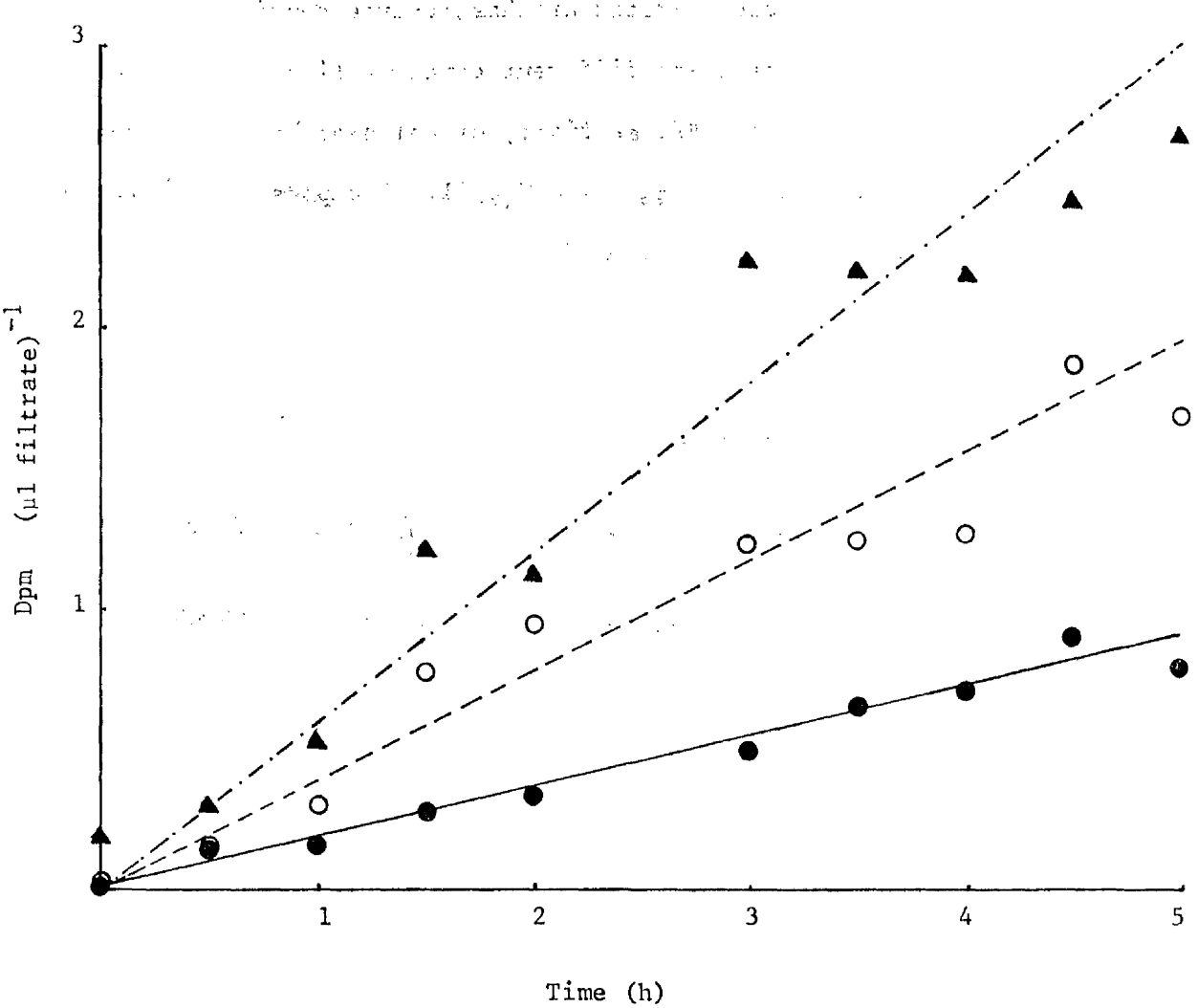


FIGURE 36

Effect of growth temperature on loss of ^{3}H-histidine from pre-labelled cells in a glucose-limited arithmetic-type continuous culture

^{3}H-histidine labelled *E. coli* were inoculated into glucose-limited arithmetic-type continuous culture at 40°, 37° and 30°, containing 0.2 mg/ml unlabelled L-histidine. The specific activity at zero time in each case was 21.6 dpm/µl culture. The rate of appearance of radioactivity in the extracellular medium was determined by counting the specific activity of the filtrate from samples removed from the cultures.

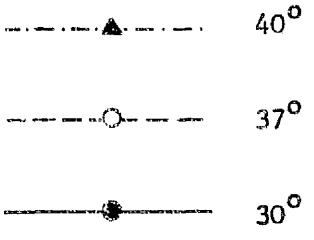


FIGURE 36

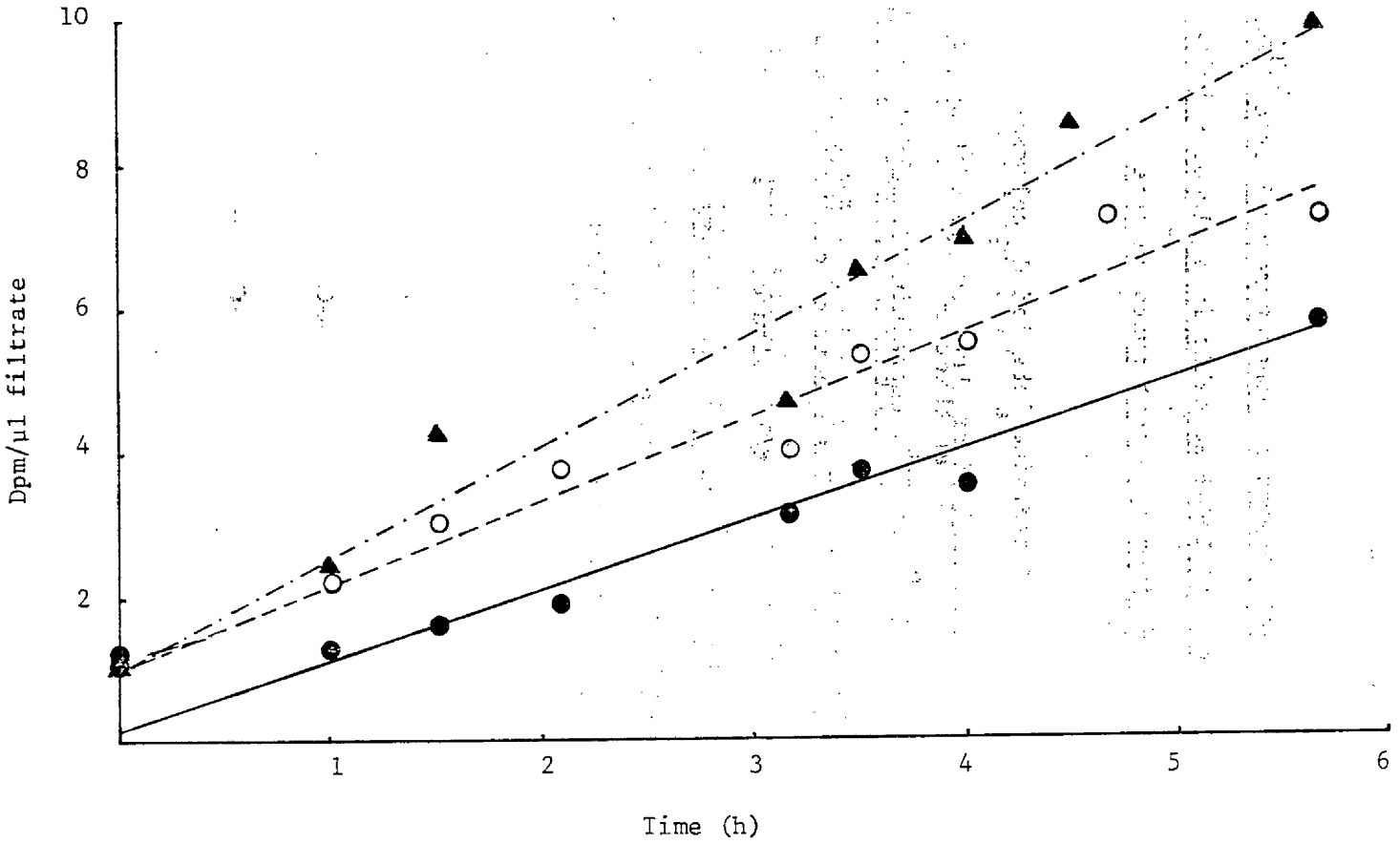
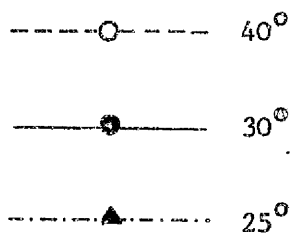


FIGURE 37

Effect of growth temperature on loss of ^{3}H-histidine from pre-labelled cells in a glycerol-limited arithmetic-type continuous culture

^{3}H-histidine labelled *E. coli* were inoculated into glycerol-limited arithmetic-type continuous cultures at 40°, 30° and 25°, containing 0.2 mg/ml unlabelled L-histidine. The specific activity at zero time in each case was 28.5 dpm/µl culture. The rate of appearance of radioactivity in the extracellular medium was determined by counting the specific activity of the filtrate from samples removed from the cultures.



The rate of loss of ^{3}H-histidine at 37° is described by Figure 35.

FIGURE 37

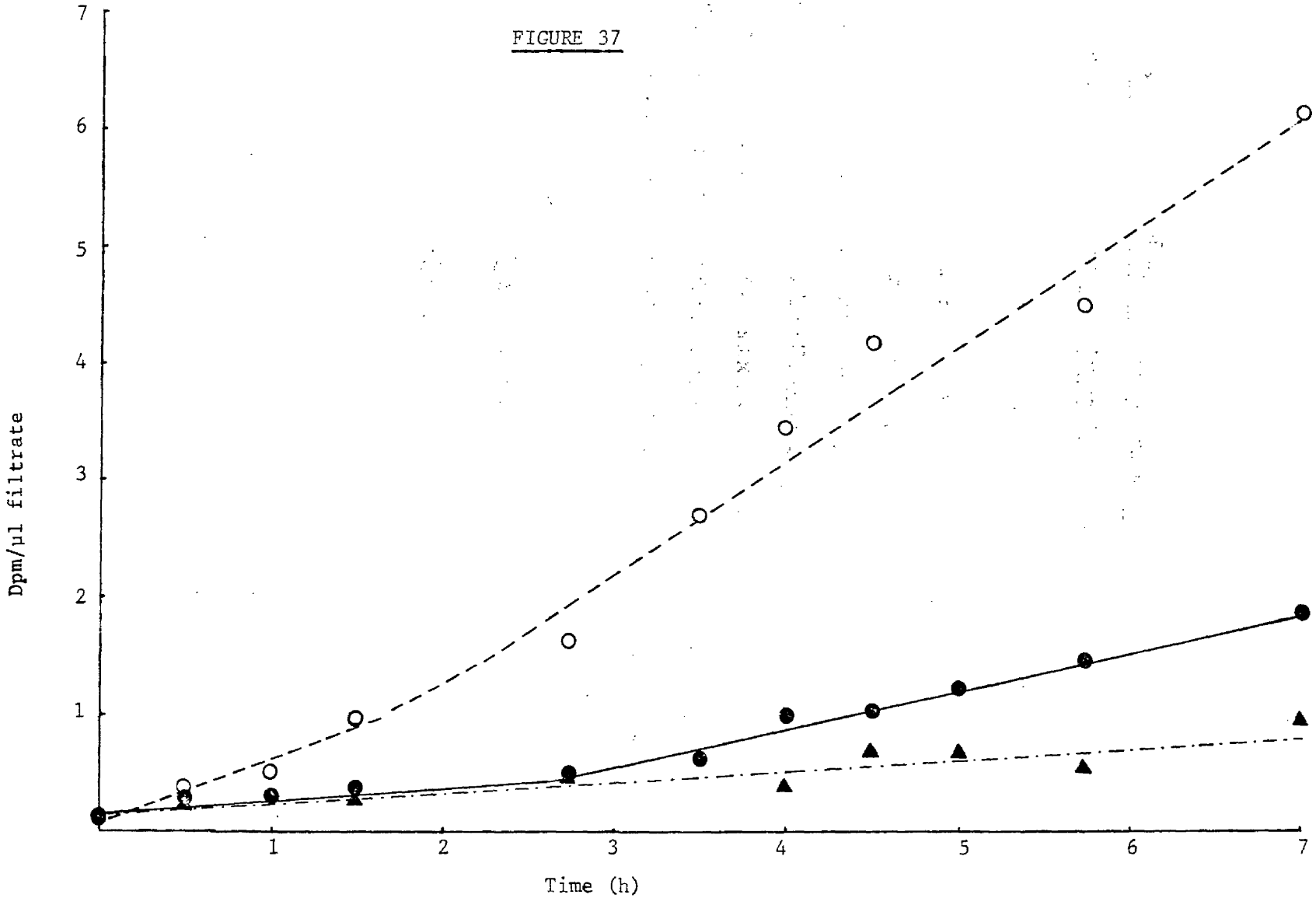


FIGURE 38

Comparison of rates of protein turnover and maintenance coefficients at different temperatures

Protein turnover was measured by the loss of ^{3}H-L-histidine from pre-labelled cells in the presence of 0.2 mg/ml cold L-histidine. The rate of protein turnover is expressed as % h^{-1}.

Maintenance coefficients are expressed as μmol g^{-1} h^{-1}.

Carbon source	Temp.	Protein turnover	m
glucose	40	3.97	260
"	37	2.28	38
"	30	1.26	26
glycerol	40	3.74	232
"	37	3.01	103
"	30	1.15	38
"	25	0.41	40

FIGURE 39

Arrhenius plots for protein turnover

The results of the experiments described by Figure 36-38 were plotted in order to find the Arrhenius activation energy E_a from the general equation relating the rate of any reaction with temperature:

$$\log_{10} k = c - \frac{E_a}{2.3\,RT}$$

where k is the rate constant (% h^{-1} turnover), c is a constant, R is the gas constant and T is the absolute temperature.

The gradients obtained were used to calculate E_a's for protein turnover in glucose-limited E. coli of 21 kcal/mole and in glycerol-limited cultures, 27 kcal/mole

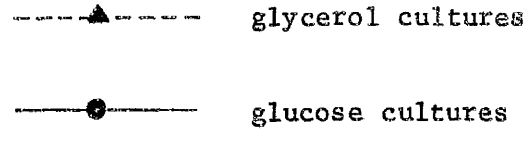
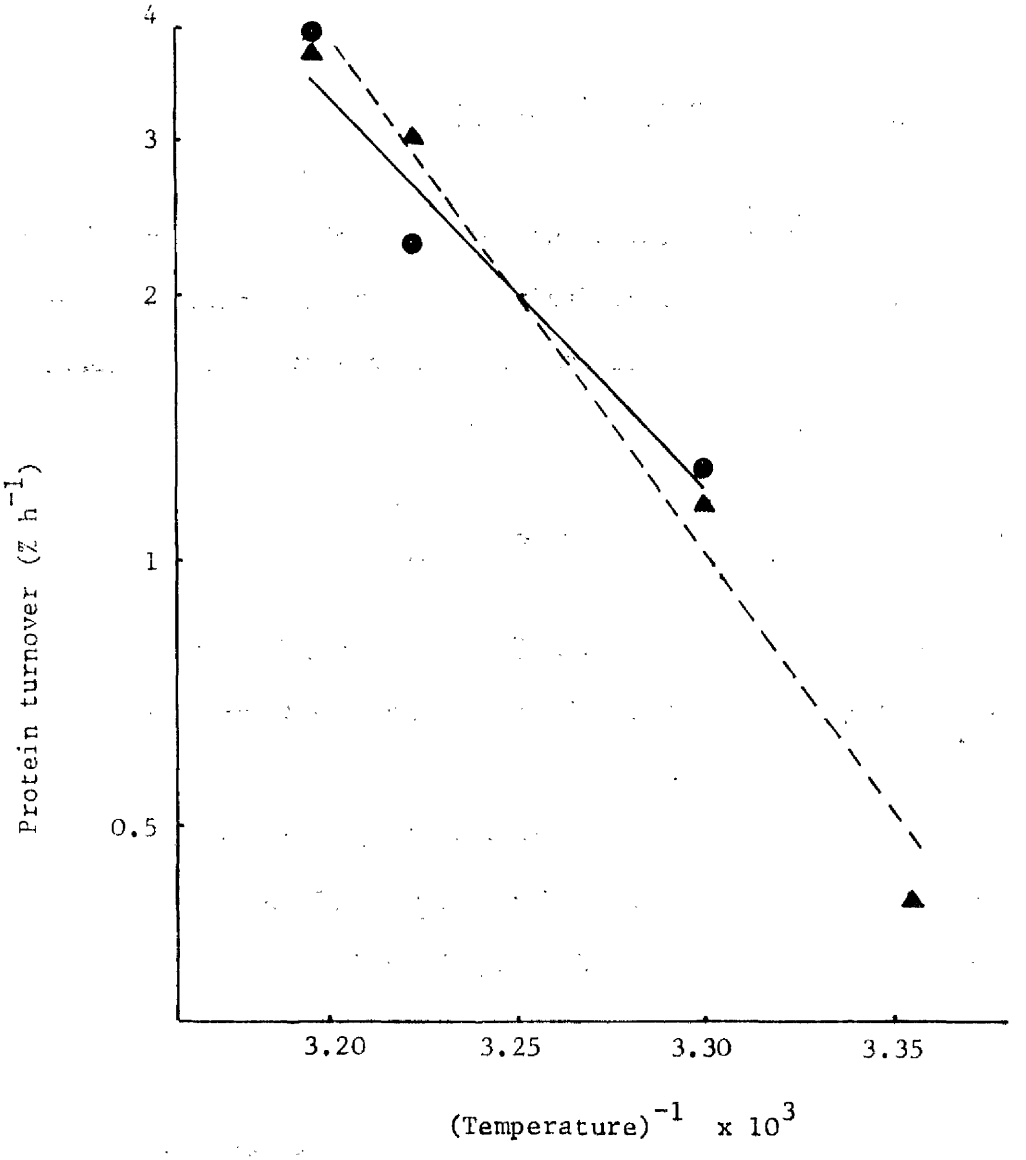


FIGURE 39


4
3
2
1
0.5
Protein turnover (% h^{-1})
3.20
3.25
3.30
3.35
(Temperature)$^{-1}$ x 10^{3}

Addition of 0.5 M sodium chloride to the growth medium decreased the rate of turnover from 2.28 to 1.04% h^{-1} at 37° and from 3.97 to 1.09% h^{-1} at 40° in glucose-limited culture

5.1.4 Loss of ^{3}H-L-histidine from cells grown in batch and carbon-limited arithmetic-type continuous culture

Two batch cultures were done for ^{3}H-histidine-labelled cells at 40°. One was done in the medium used for arithmetic-type continuous culture and the other in conventional batch culture salts medium. Both contained 20mM glycerol and 0.2 mg/ml histidine. Loss of label was 2.19% h^{-1} for the former and 2.14% h^{-1} for the latter culture. The ammonia content of the former medium was 4 × the content of the latter. The ammonium ion has been implicated as a regulator of protein turnover in E. coli by Pine (1973a), yet the difference between turnover rates in these cultures was insignificant. These values compare with the loss of label of 3.74% h^{-1} at the same temperature for glycerol-limited cells in arithmetic-type continuous culture.

5.1.5 Protein turnover as measured by ^{3}H-L-histidine loss and ^{3}H-L-isoleucine loss from pre-labelled cells

^{3}H-isoleucine satisfied the same criteria as ^{3}H-histidine for its use as a marker in the measurement of protein turnover. The uptake of small quantities of high specific activity compound was rapid and isoleucine could not be used as a source of carbon and energy except for direct incorporation into protein as the amino acid.

All experiments in this section were for cells growing at 37° in glycerol-limited arithmetic-type continuous culture. The rates of loss of labelled histidine and isoleucine were compared, and the regulatory effect of histidine on protein turnover investigated (Figure 40).

FIGURE 40

Comparison of rates of protein turnover as measured by ^{3}H-L-histidine and ^{3}H-L-isoleucine labelling

Pre-labelled cells were inoculated into glycerol-limited arithmetic-type continuous cultures at 37°.

Loss of label into the medium is expressed as % h^{-1} of the original radioactivity in the cells.

Label in cells	Conc^{n} cold L-his (µg/ml)	Conc^{n} cold L-ile (µg/ml)	Loss of label % h^{-1}
^{3}H-his	1000	-	1.48
^{3}H-his	200	-	3.01
^{3}H-his	50	-	4.49
^{3}H-ile	-	200	1.01
^{3}H-ile	50	200	1.43
^{3}H-ile	200	200	0.81

Protein turnover as measured by histidine labelling appeared to be 3 × as large as measured by isoleucine turnover. 50 µg/ml histidine stimulated isoleucine turnover, but 200 µg/ml inhibited isoleucine turnover. The effect of isoleucine on protein turnover measured by histidine turnover was not investigated.

5.2 Use of ^{3}H-diaminopimelate to measure cell wall turnover

Cells labelled with (DL + meso)-2, 6-diamino [G - ^{3}H] pimelic acid dihydrochloride were grown in glycerol-limited arithmetic-type continuous culture in the presence of 2mM L-lysine and 1mM diaminopimelate. Loss of label was found to be of the same order as the rate of loss of amino acid label (Figure 41).

The relationship of ^{3}H-diaminopimelate loss to temperature was different from the temperature dependence of the maintenance coefficient (Figure 42). An Arrhenius plot gave an approximate E_a of 9.2 kcal/mole (Figure 43), much less than the E_a for the glycerol maintenance coefficient of 78 kcal/mole.

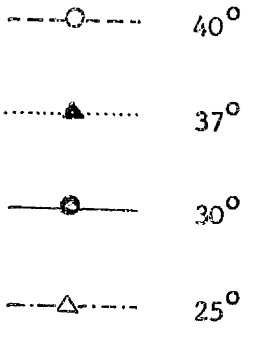
5.3 Loss of ^{32}P from pre-labelled cells in glycerol-limited arithmetic-type continuous culture

The rate of loss of label from ^{32}P-labelled cells was found to be linear with respect to time at each temperature, and was found to increase with temperature (Figure 44). The rate of loss of ^{32}P was of the same order as the rate of loss of labelled amino acids and diaminopimelate. The relationship of the rate of loss to growth temperature again appeared to be different from the same relationship for the maintenance coefficient (Figure 45). The E_a obtained from the Arrhenius plot (Figure 46) was 14.6 kcal/mole, which is of the same order of magnitude as protein and cell wall turnover, but very different

FIGURE 41

Loss of ^{3}H-diaminopimelate from pre-labelled cells in glycerol-limited arithmetic-type continuous culture at different temperatures

^{3}H-diaminopimelate labelled *E. coli* were inoculated into glycerol-limited arithmetic-type continuous cultures at 40°, 37°, 30° and 25°, containing 2mM L-lysine and 1mM diaminopimelate. The initial specific activity of the culture in each case was 4.50 dpm/µl for the 40° culture, 4.85 dpm/µl for the 37° culture, 4.20 dpm/µl for the 30° culture and 5.68 dpm/µl for the 25° culture.



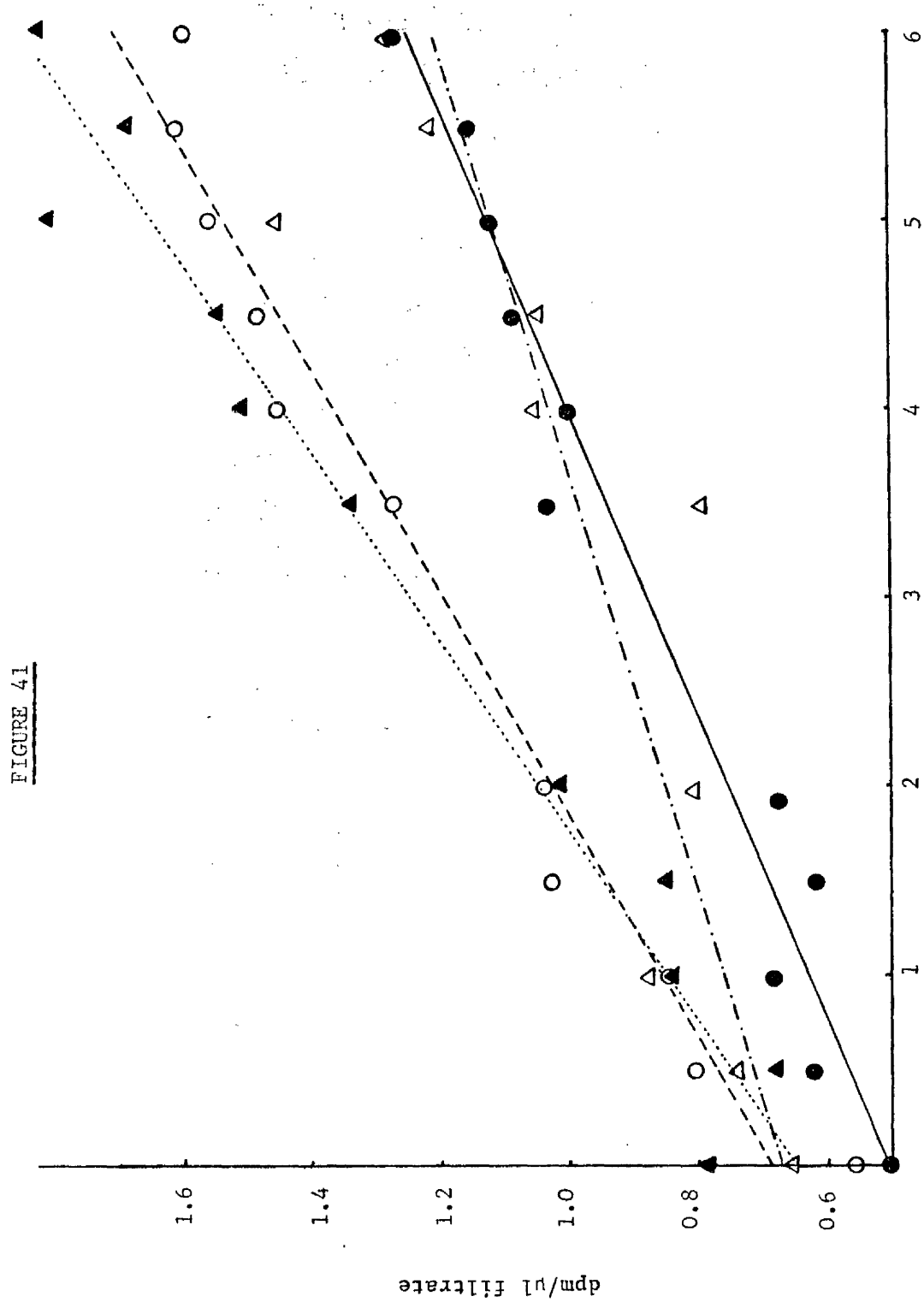


FIGURE 41

FIGURE 42

Comparison of ^{3}H-diaminopimelate turnover and maintenance coefficients at different temperatures

The rate of loss of ^{3}H-diaminopimelate (^{3}H-DAP) at different temperatures was determined from the graphs in Figure 41 and expressed in units % h^{-1}. Maintenance coefficients in terms of glycerol utilisation were also determined at these temperatures (Figure 24).

Temperature (°C)	^{3}H-DAP loss (% h^{-1})	m (Glycerol) (μmol g^{-1} h^{-1})
40	5.73	232
37	3.90	103
30	2.80	38
25	1.62	40

FIGURE 43

Arrhenius plot of ^{3}H-diaminopimelate turnover

The data from Figure 42 were plotted according to the Arrhenius equation:

$$\log_{10} k = c - \frac{E_a}{2.3RT} \qquad \text{(Figure 39)}$$

The activation energy (E_a) was calculated from the gradient, and found to be 9.2 kcal/mole.

FIGURE 43

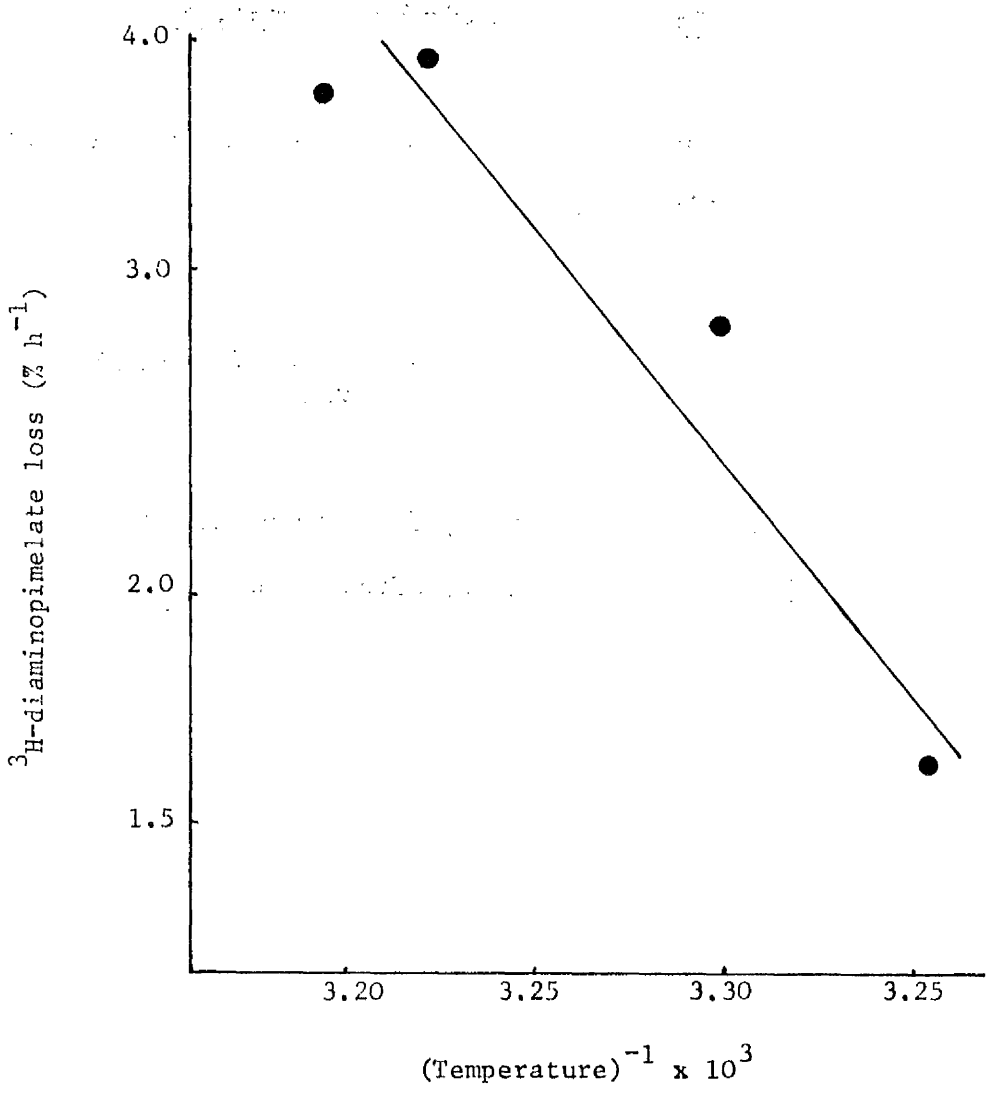

4.0
3.0
2.0
1.5
^{3}H-diaminopimelate loss (% h^{-1})
3.20
3.25
3.30
3.25
(Temperature)$^{-1}$ x 10^3

FIGURE 44

Loss of ^{32}P from pre-labelled cells in glycerol-limited arithmetic-type continuous culture at different temperatures

^{32}P-labelled *E. coli* were inoculated into glycerol-limited arithmetic-type continuous cultures at 40°, 37°, 30° and 25°, in normal 40mM phosphate buffer. The initial specific activity of the culture in each case was 18.6 dpm/µl for the 40° culture, 18.1 dpm/µl for the 37° culture, 17.5 dpm/µl for the 30° culture and 21.9 dpm/µl for the 25° culture.

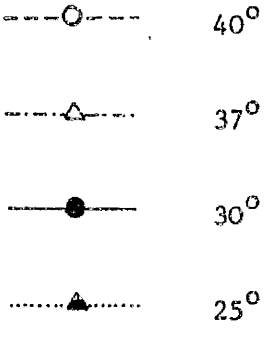


FIGURE 44

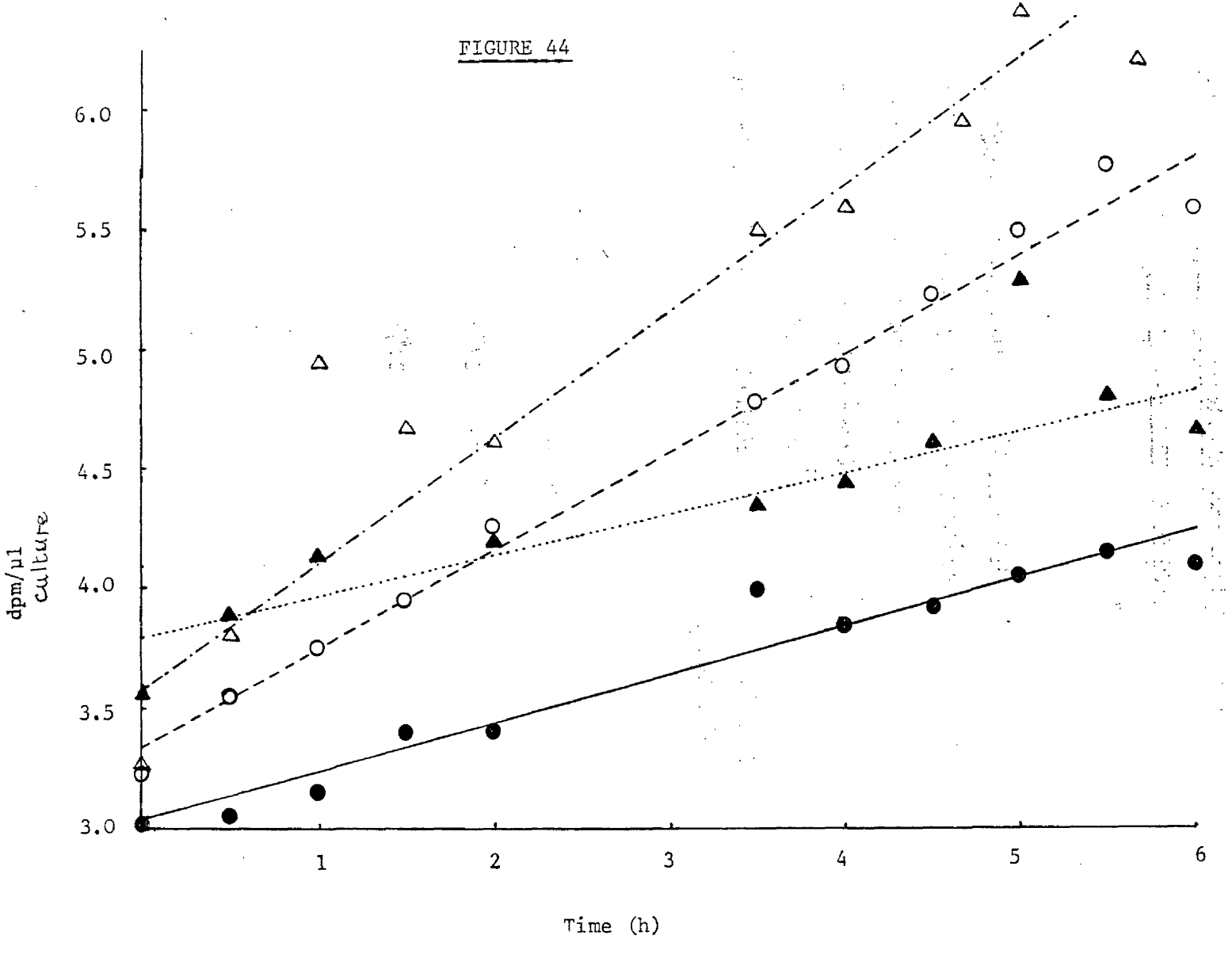


FIGURE 45

Comparison of ^{32}P turnover and maintenance coefficients at different temperatures

The rate of loss of ^{32}P at different temperatures was determined from the graphs in Figure 44 and expressed in units % h^{-1}. Maintenance coefficients in terms of glycerol utilisation were also determined at these temperatures (Figure 24)

Temperature (°C)	^{32}P loss (% h^{-1})	$\underline{m}$ (glycerol) (µmol g^{-1} h^{-1})
40	2.44	232
37	2.96	103
30	1.39	38
25	0.94	40

FIGURE 46

Arrhenius plot of ^{32}P turnover

The data from Figure 45 were plotted according to the Arrhenius equation:

$$\log_{10} k = c - \frac{E_a}{2.3RT} \qquad \text{(Figure 39)}$$

The activation energy (E_a) for ^{32}P loss was calculated from the gradient, and found to be 14.6 kcal/mole.

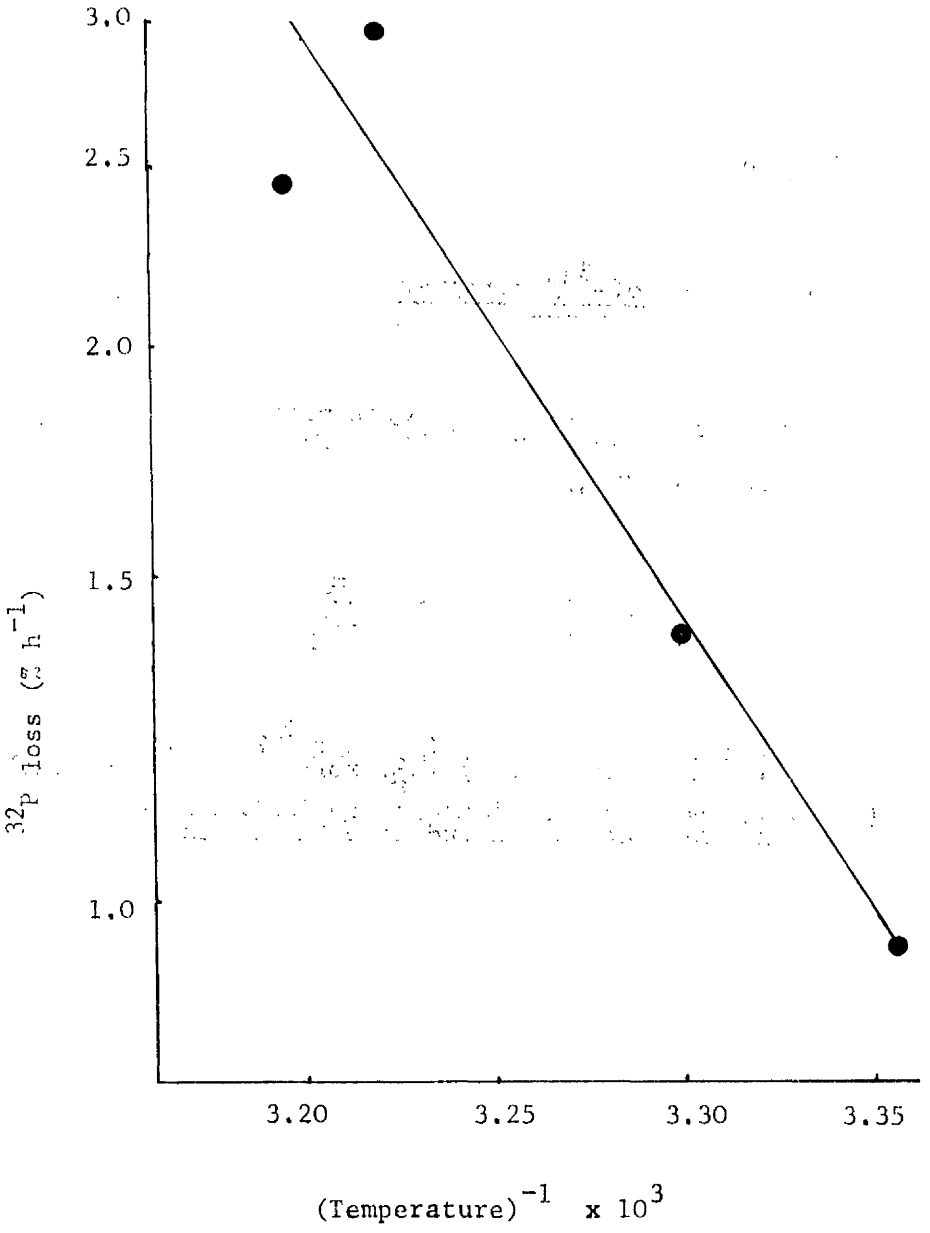


FIGURE 46

from the E_a for the maintenance coefficient.

5.4 Loss of $^{14}CO_2$ from uniformly-labelled cells

The loss of $^{14}CO_2$ with respect to time from uniformly labelled cells was measured for growth in glucose-, glycerol-, malate- and lactose-limited arithmetic-type continuous cultures of E. coli at 40°, 37° and 30°. By the method of curve-fitting illustrated in Figure 22, whereby the value for the "constant" rate of ^{14}C turnover is estimated, the rate of $^{14}CO_2$ release which occurred endogenously was calculated for each carbon source and temperature (Figure 47), and expressed as % original cell ^{14}C content/h. The value for lactose-growing cells at 40° was negative, which is clearly not possible. The best-fitting exponential plot had a poor correlation coefficient. As the other plots in several cases also had poor correlation coefficients, the errors involved in the determination of these constants must be fairly large. A better method of making this estimation would be to make the measurements over a considerably longer time interval, and to determine the basal level experimentally, rather than by a curve-fitting procedure. The loss of label at each temperature was different for each carbon source, and the relationship between that loss and growth temperature was also different for each carbon source. However, this may have been due to the large errors involved in the estimation of the rate of turnover. In any case, the rate of turnover as measured by total ^{14}C turnover did not alter with temperature in the same way as maintenance (Figure 24).

6. Growth of E. coli in nitrogen-limited arithmetic-type continuous culture

With these experiments, it was hoped to compare molar growth yields and maintenance coefficients in carbon- and nitrogen-limited

FIGURE 47

Loss of $^{14}CO_2$ from uniformly labelled cells at different temperatures

$^{14}CO_2$ leaving the growth flask was trapped and counted. The rate of loss of $^{14}CO_2$ was found to decay exponentially (Figure 22) to a constant value. This value was determined by a curve-fitting procedure (p.98) for growth of uniformly labelled *E. coli* at each temperature.

Results are expressed as a percentage of label present in the cells at the beginning of the experiment.

Substrate	Rate of $^{14}CO_2$ loss (% h^{-1})		
	40°	37°	30°
glucose	0.45	0.18	0.18
lactose	-0.25	0.23	0.15
glycerol	0.70	0.28	0.14
malate	0.50	0.06	0.15

cultures in a way similar to the work of Stouthamer and Bettenhaussen (1973 and 1975) with Aerobacter aerogenes.

6.1 Nitrogen-limited glycerol arithmetic-type continuous culture

Glycerol-trained E. coli were inoculated into medium containing all growth requirements except the nitrogen source. 70mM ammonium sulphate was pumped into the flask at a rate of 0.95 ml/h.

After an initial period of adaptation, cell growth was linear with respect to time i.e. $\frac{dx}{dt}$ was constant (Figure 48). As the experiment progressed, the rate of disappearance of glycerol increased i.e. $\frac{dS}{dt}$ increased (Figure 48).

Equation (2) (p. 80) is

$$\frac{dS}{dt} = m.X + \frac{1}{Y_G} \cdot \frac{dx}{dt}$$

Thus if $\frac{dx}{dt}$ is constant, then a graph of $\frac{dS}{dt}$ against X will give the maintenance coefficient under nitrogen limitation, and the intercept will allow calculation of Y_G.

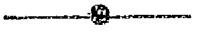
The best-fitting quadratic equation was found for the glycerol utilisation graph. (This is the line which is drawn in Figure 48). The gradient at any point could then be found by differentiation. By this method, a graph of $\frac{dS}{dt}$ against X was drawn (Figure 49). The gradient gave a value of m of 176 μmol g^{-1} h^{-1} and the intercept gave 38.7 g/mole for Y_G. These values compare with 103 μmol g^{-1} h^{-1} and 47.5 g/mole for carbon-limited growth.

The above equation can equally well be applied to oxygen uptake (Figure 50). The graph of $\frac{dO_2}{dt}$ against X was not linear. After an initial pulse of respiration, the rate of oxygen uptake fell. For

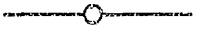
FIGURE 48

Growth and glycerol utilisation of a nitrogen-limited arithmetic-type continuous culture growing on glycerol at 37°

The rate of supply of ammonium sulphate was 66.28 µmol/h. Cell density was measured by turbidity at 420nm and converted to dry weight by the factor 0.225mg ml^{-1} $(O.D.\ unit)^{-1}$. Glycerol was assayed enzymatically for PCA extracts of samples from the culture. Both quantities were converted to totals for the whole culture. The molar growth yield from this experiment was 186.6 g/g atom N.



cell mass



glycerol in culture medium

FIGURE 48

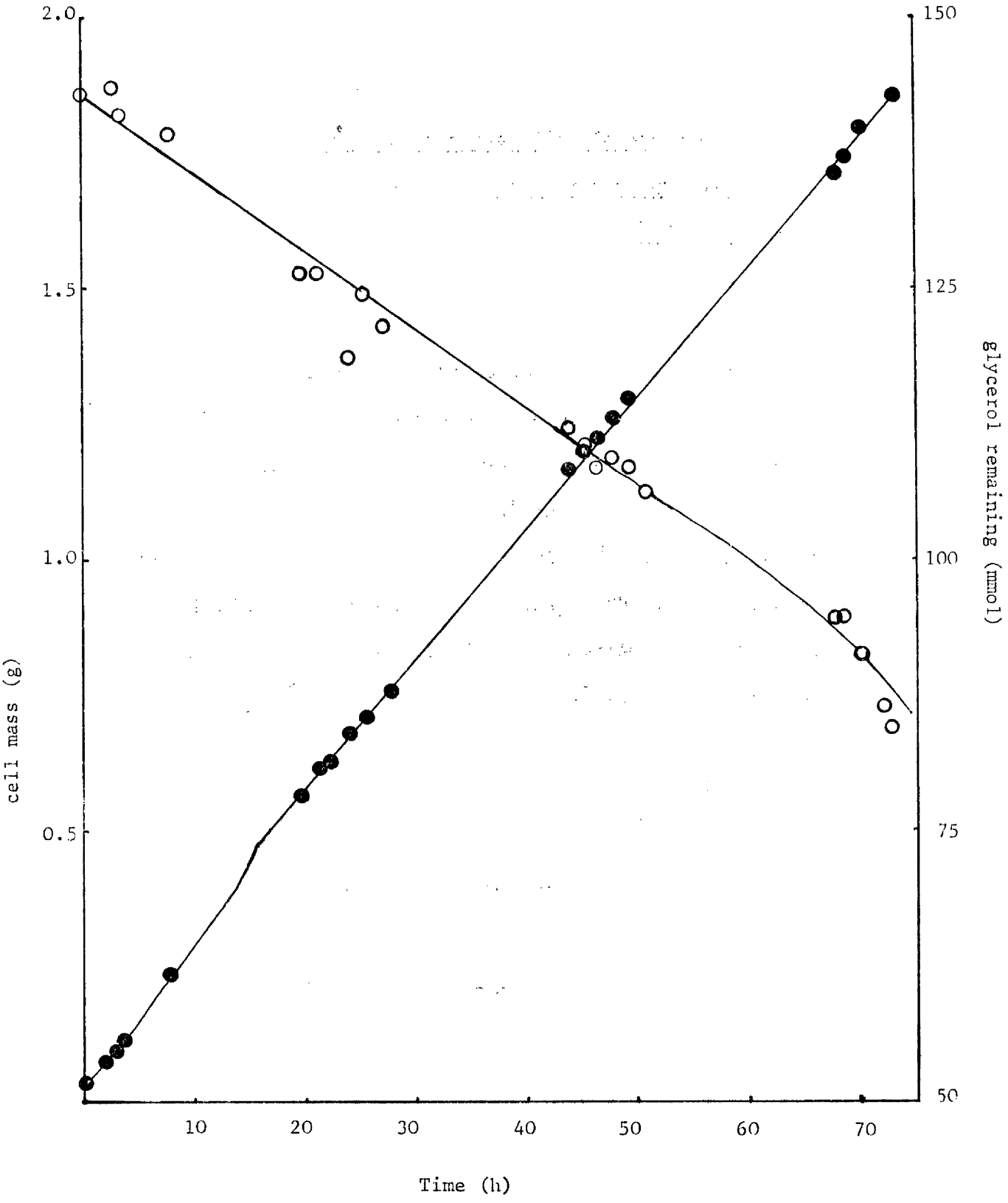

2.0
1.5
1.0
0.5
cell mass (g)
150
125
100
75
50
glycerol remaining (mmol)
10
20
30
40
50
60
70
Time (h)

FIGURE 49

Determination of m (glycerol) and Y_G (glycerol) from nitrogen-limited arithmetic-type continuous culture

The best-fitting quadratic curve was found for the glycerol utilisation described by Figure 48. Thus, the gradient $\frac{dS}{dt}$ could be calculated for any time, and plotted against X, the cell mass, according to the equation

$$\frac{dS}{dt} = m X + \frac{1}{Y_G} \frac{dx}{dt}$$

The gradient of the graph of $\frac{dS}{dt}$ against X is therefore the maintenance coefficient, in this case 0.179 mmol g^{-1} h^{-1}. The intercept allows calculation of Y_G, since $\frac{dx}{dt}$ can be calculated from Figure 48. Y_G in this case was found to be 38.7 g/mole.

FIGURE 49

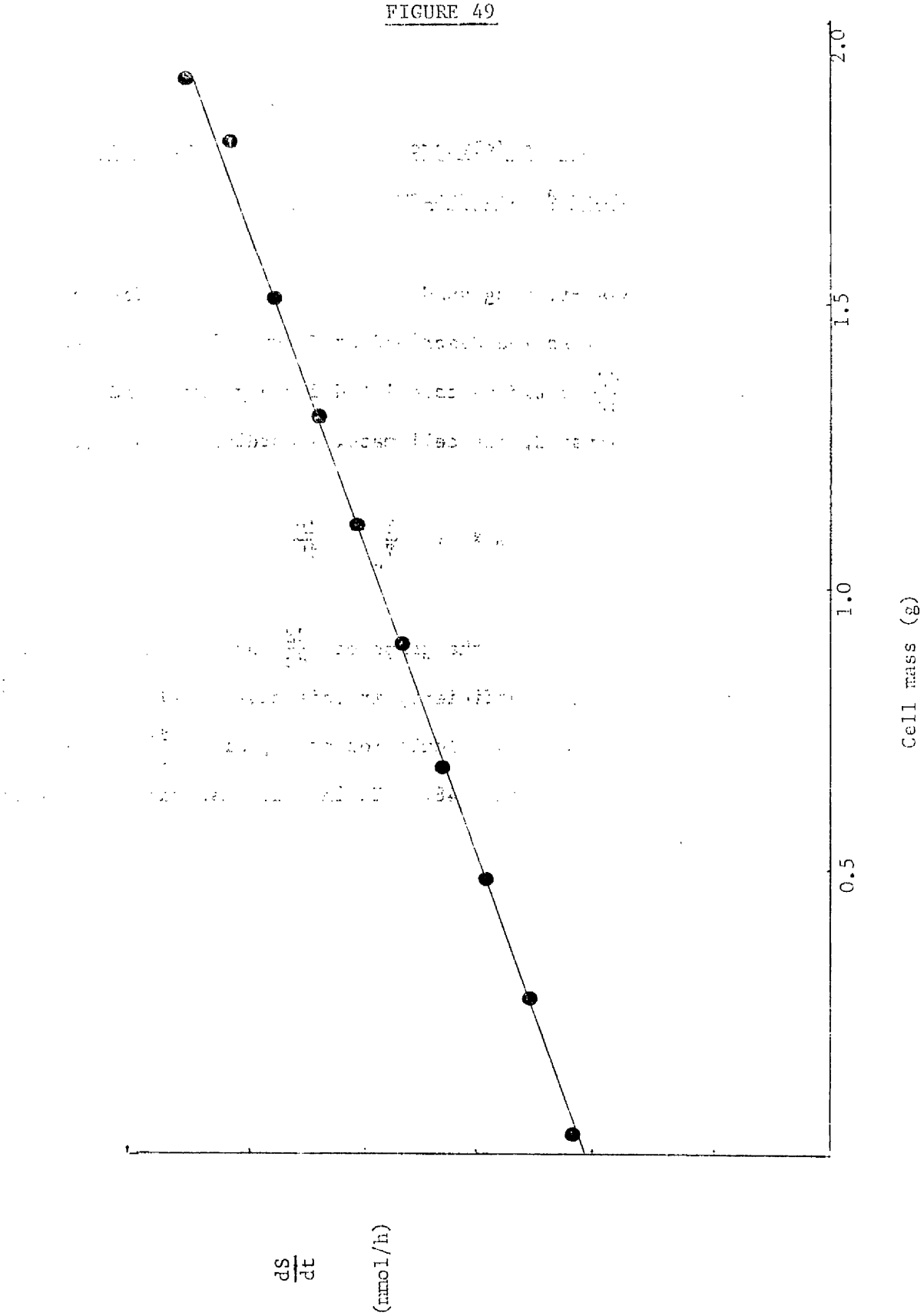


FIGURE 50

Graph of $\frac{dO_2}{dt}$ v X for nitrogen-limited arithmetic-type continuous culture

Oxygen uptake was monitored throughout the nitrogen-limited arithmetic-type growth experiment, and the plot of $\frac{dO_2}{dt}$ against the cell mass (X) should be linear if the equation

$$\frac{dO_2}{dt} = m_{O_2} X + \frac{1}{Y_{GO_2}} \frac{dx}{dt}$$

holds for nitrogen-limited culture.

Two straight lines are drawn. The first is the initial gradient, calculated from the best-fitting straight line for the points between cell mass 0.3g and 0.7g. The second is the best-fitting straight line for the points above a cell mass of 0.7g.

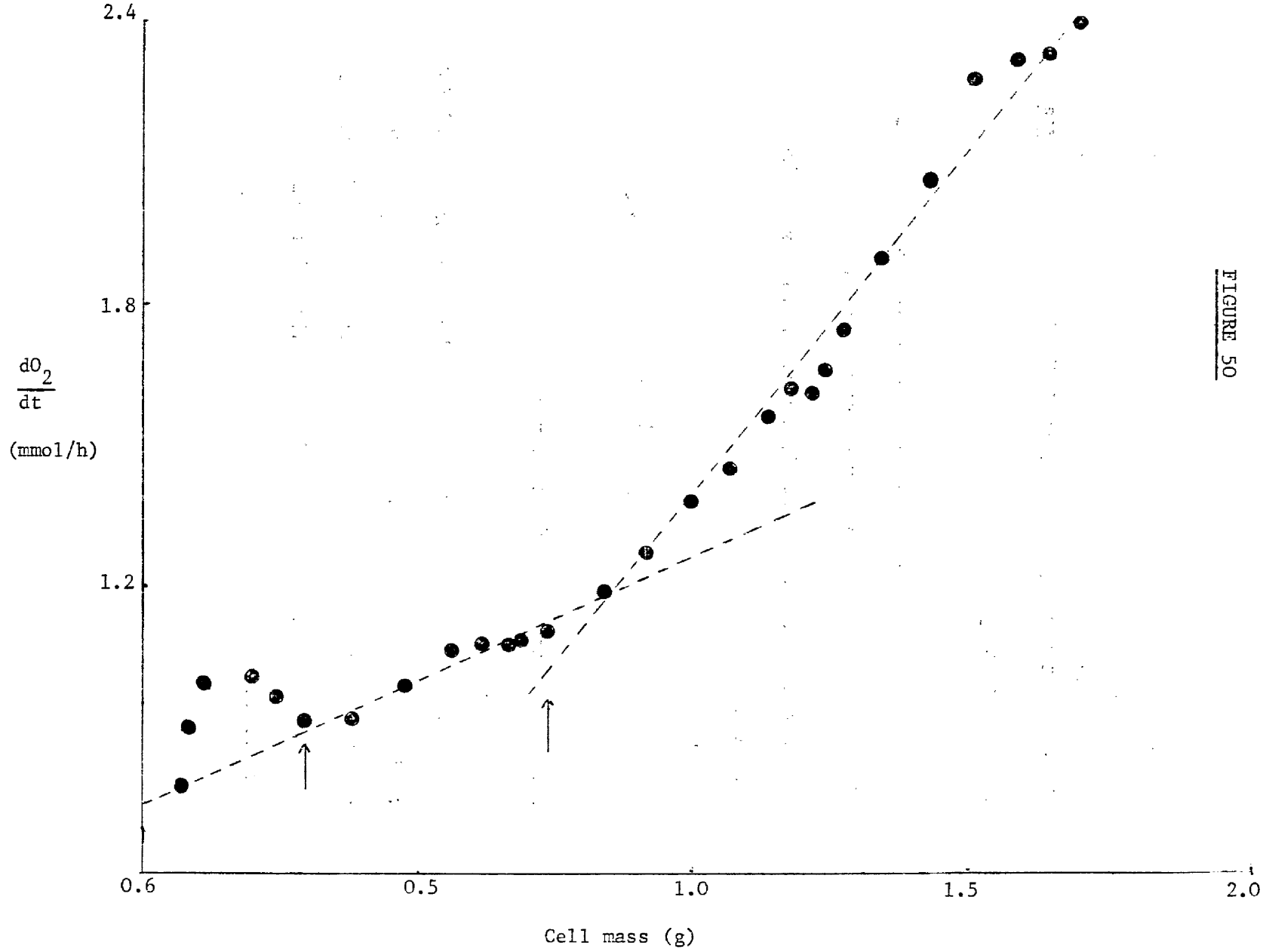


FIGURE 50

the remainder of the experiment, the rate of increase of oxygen uptake accelerated. Two straight lines have been drawn on Figure 50. One is the straight line approximating to the initial rate of increase of oxygen uptake relative to cell mass. The second is the best-fitting straight line through the points after X = 700mg.

The first line gives values of 520 $\mu mol.g^{-1}.h^{-1}$ for $\underline{m}_{O_2}$ and 32.1 g/mole for Y_{GO_2}, both of which are consistent with the same parameters in terms of glycerol.

The intercept of the second line on the $\frac{dO_2}{dt}$ axis is a negative value, very close to the origin (- 12 µmol/h). Thus the Y_{GO_2} calculated from this line was very large and negative (- 1.8 kg/mole O_2). The gradient of this line gave a value of 1430 $\mu mol\ g^{-1}\ h^{-1}$ for $\underline{m}_{O_2}$. Measurements of the rate of carbon dioxide evolution permitted a carbon balance to be drawn up. The carbon recovery after the initial period of high respiration was constant at 79% of glycerol metabolised being recovered as cells and CO_2. Only traces (maximum 0.07mM) of acetate were found in the growth medium. Other products of metabolism were not assayed for. E. coli grown on glycerol under nitrogen limitation therefore appears to have a lower efficiency of conversion of glycerol to cell material than under carbon limitation. The coupling of oxygen uptake to growth appears to break down.

2. Nitrogen-limited malate arithmetic-type continuous culture

This experiment was carried out in the same way as the glycerol nitrogen-limited culture. 50mM-ammonium sulphate was pumped into the culture at a rate of 1.50ml/h.

Growth was again linear with time after an initial period of

adaptation, and the rate of disappearance of malate from the medium increased as the cell mass increased (Figure 51). The graph of $\frac{dS}{dt}$ against X was linear (Figure 52) giving values of 62.2 g/mole for Y_G and 655 µmol g^{-1} h^{-1} for $\underline{m}$, compared with values of 43.3 g/mole and 127 µmol g^{-1} h^{-1} for carbon-limited growth.

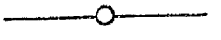
After an initial period of high respiratory activity, the rate of increase of oxygen uptake accelerated until the rate of increase of oxygen uptake became almost independent of the cell mass (Figure 53). The approximate values for Y_{GO_2} and $\underline{m}_{O_2}$ from the initial stage of the curve were 34.9 g/mole and 900 µmol g^{-1} h^{-1}.

Only 75% of malate carbon utilised could be accounted for by cell material and carbon dioxide. No assays were done for possible excretion products.

FIGURE 51

Growth and malate utilisation of a nitrogen-limited arithmetic-type continuous culture growing on malate at 37°

The rate of supply of ammonium sulphate was 74.90 µmol/h. Cell density was measured by turbidity at 420nm and malate concentration was assayed enzymatically for PCA extracts of samples from the culture. Both quantities were converted to totals for the whole culture. The molar growth yield from this experiment was 137.3 g/g atom N. 90 mmol malate were added to the growth flask at the beginning of the experiment.



cell mass

malate in culture medium

FIGURE 51

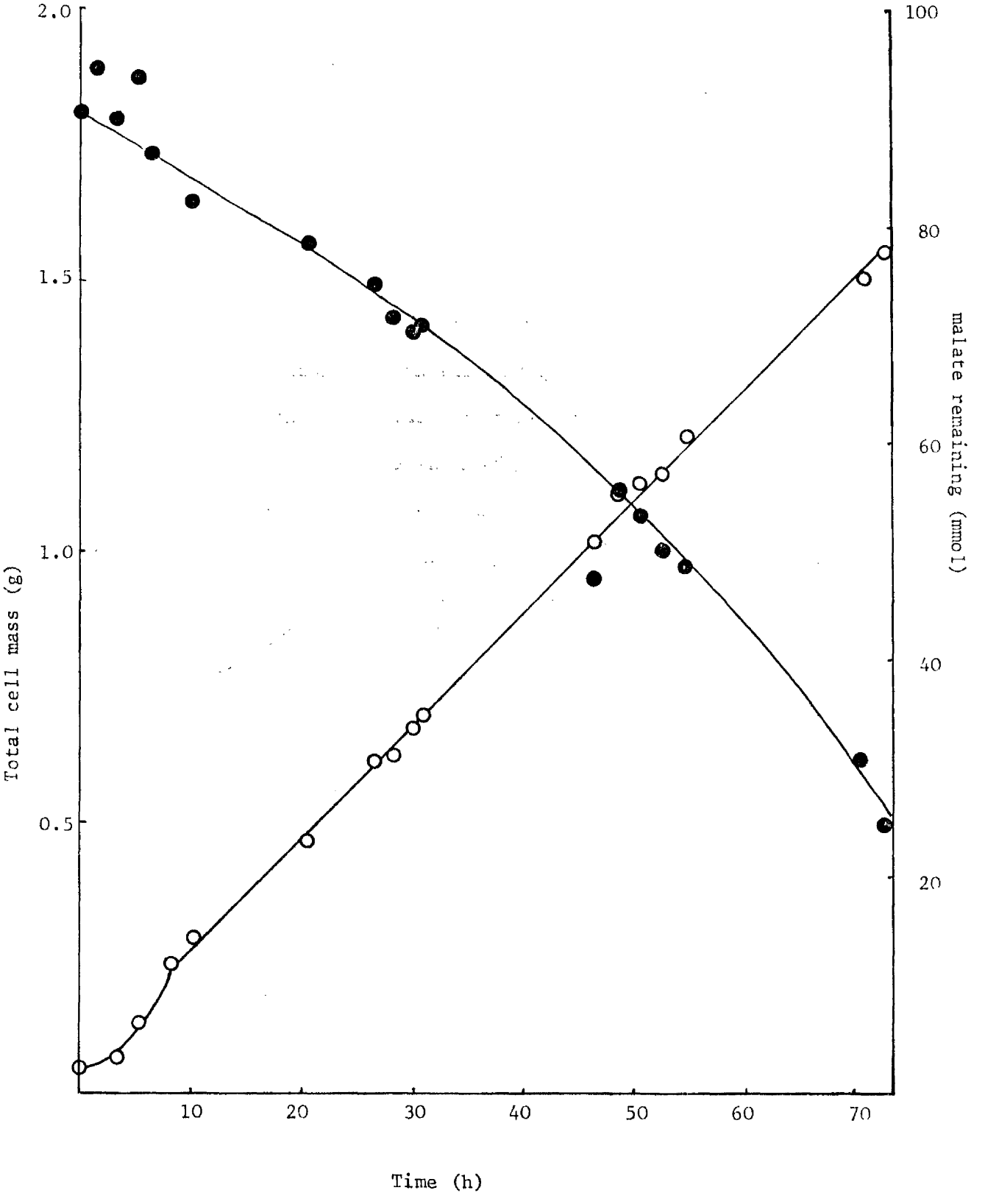

2.0
1.5
1.0
0.5
Total cell mass (g)
100
80
60
40
20
malate remaining (mmol)
10
20
30
40
50
60
70
Time (h)

FIGURE 52

Determination of m (malate) and Y_G (malate) from nitrogen-limited arithmetic-type continuous culture

The graph of malate utilisation (Figure 51) was fitted to a quadratic equation and the rate of malate utilisation estimated from the gradient at each time. The plot of rate of malate utilisation against cell mass allowed calculation of the maintenance coefficient and molar growth yield in terms of malate under nitrogen limitation, using the equation

$$\frac{dS}{dt} = mX + \frac{1}{Y_G} \frac{dx}{dt}$$

Thus, the gradient of the graph is a measure of m and the intercept is $\frac{1}{Y_G}$. The values calculated for these constants were 0.655 mmol g^{-1} h^{-1} for m and 62.2 g/mole for Y_G.

FIGURE 52

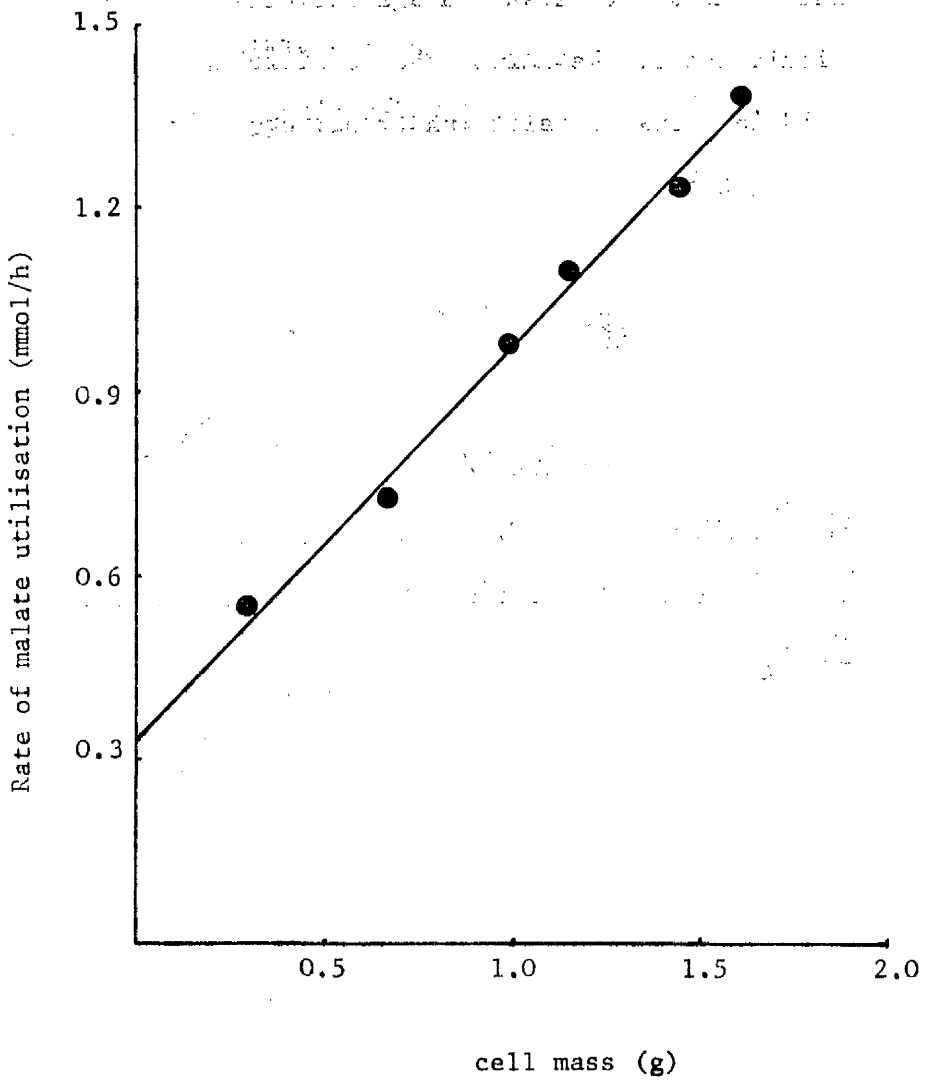

1.5
1.2
0.9
0.6
0.3
Rate of malate utilisation (mmol/h)
0.5
1.0
1.5
2.0
cell mass (g)

FIGURE 53

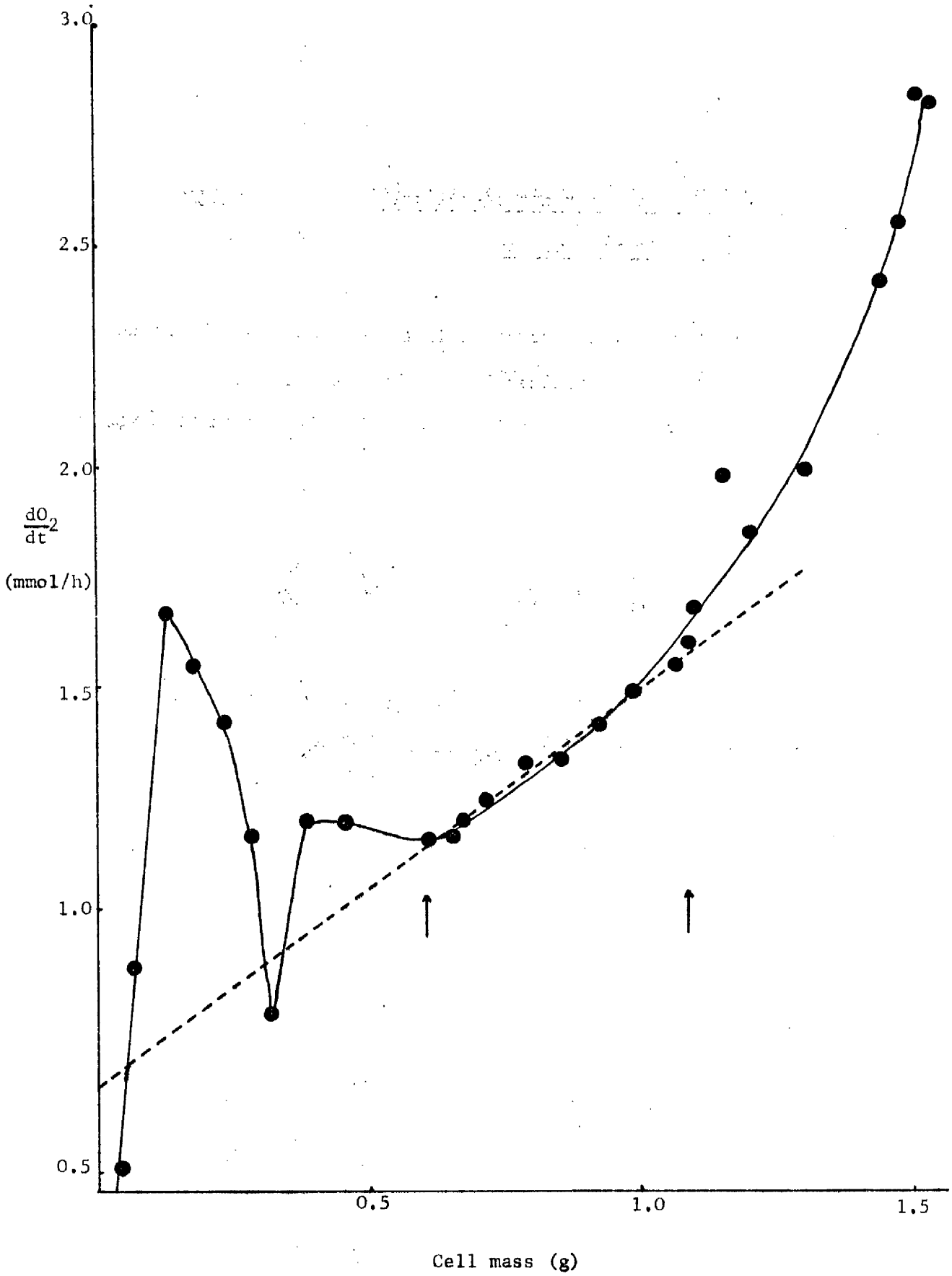
Graph of $\frac{dO_2}{dt}$ v X for nitrogen-limited arithmetic-type continuous culture - malate

The best fitting straight line was drawn for the points between the arrows indicated, allowing estimation of Y_{GO_2} and $\underline{m}_{O_2}$ for growth on malate under nitrogen limitation from the equation

$$\frac{dO_2}{dt} = m_{O_2} X + \frac{1}{Y_{GO_2}} \frac{dx}{dt}$$

The gradient of this line is $\underline{m}_{O_2}$ (0.90 mmol g^{-1} h^{-1}) and, from the intercept, Y_{GO_2} is 34.9 g/mole.

FIGURE 53



DISCUSSION

1. Molar growth yields and oxidative phosphorylation

1.1 General considerations

Maximum molar growth yields (Y_G and Y_{GO_2}) were obtained from carbon-limited arithmetic-type continuous culture (Figure 24). Marr et al. (1963) grew E. coli in a similar growth system, but no molar growth yields were calculated or can be calculated from their published results. All other molar growth yields for E. coli have been obtained from batch or chemostat culture.

It should be emphasised that the determination of these arithmetic type culture growth yields depended critically on the values of $\frac{dx}{dt}$. $\frac{dx}{dt}$ was calculated assuming that

(i) all carbon supplied can be accounted for in terms of cells and carbon dioxide

(ii) the carbon content of cells is 44.6% by weight for all types of cells used

(iii) the turbidity/dry weight relationship is also constant at 225 $\mu g/ml/E_{420}$ unit

While each of these has been found to be true within limits of experimental error, a combination of small errors may lead to quite large differences in $\frac{dx}{dt}$ and hence Y_G and Y_{GO_2}. Over and above these possible systematic errors, the standard error of each estimate of Y_G determined from any experiment assuming (i), (ii) and (iii) to be true is ± 2.5%. When the variance in each of (i), (ii) and (iii) are taken into account, the standard error of each calculated value of Y_G becomes ± 7.3%.

A comparison of the molar growth yields obtained in this work and by other workers reveals a large range of values for some substrates (Figure 54).

Y_G (glucose) was similar to other workers apart from Harrison & Loveless (1971) and Hamilton (1972), both of whom pointed out that considerable quantities of carbon were excreted into the medium. The values of Y_G would therefore be expected to be lower. Y_{GO_2} (glucose) was slightly higher than obtained by others. Again, the incomplete oxidation of glucose in the experiments of Harrison & Loveless and Hamilton makes comparison difficult.

Comparison of Y_G (glycerol) is also complicated by lack of information, although Hamilton (1972) obtained a similar value, with no carbon appearing in the medium. The value of Y_{GO_2} (glycerol) was different from other published values, but fell within the same range. Values of yields for growth on malate were only slightly different from those obtained by Hamilton using batch culture.

As far as can be seen, therefore, the growth yields obtained from carbon-limited arithmetic-type continuous culture are comparable with those obtained by other workers with E. coli, at least in those cases where the carbon source is completely oxidised to CO_2.

Comparison of these molar growth yields for E. coli with those obtained for other organisms is again often hampered by poor carbon balance data. A good example is the list of molar growth yields for A. aerogenes published by Hadjipetrou et al. (1964), who showed that in several cases, only about 80% of carbon was recovered. Any comparison with data of this sort would be meaningless. On the other hand, the molar growth yields obtained for Klebsiella (formerly Aerobacter) aerogenes

FIGURE 54

Comparison of molar growth yields of E. coli obtained by different workers

The molar growth yields obtained for E. coli growing in carbon-limited arithmetic-type continuous culture were those described in the Results section for growth at 37°.

Reference		Carbon source	Y (g/mole)	Y_{O_2} (g/mole)
This work		glucose	95.0	45.3
Schulze & Lipe	(1964)	glucose	90.0	41.2
Ng	(1969)	glucose	90.0	-
Ribbons	(1969)	glucose	94.0	-
Hamilton	(1972)	glucose	76.4	54.0
Harrison & Loveless	(1971)	glucose	66.6	27.3
Whitaker & Elsden	(1963)	glucose	-	40.4
This work		glycerol	50.7	36.6
Hamilton	(1972)	glycerol	49.3	43.0
Poole & Haddock	(1974)	glycerol	32.2	23.8
Meyer & Jones	(1973)	glycerol	-	30.6
This work		malate	43.3	33.8
Hamilton	(1972)	malate	40.8	35.0

by Neijssel and Tempest (1975) in a slow-growing chemostat can probably be meaningfully compared with the results for E. coli in arithmetic culture. Values of Y_{GO_2} are the same for both organisms growing on glucose or glycerol, but Y_G is 17% greater on glucose and 21% greater on glycerol for E. coli. These organisms have almost identical anaerobic yields on glucose (Stouthamer, 1969), so it seems likely that the reason for aerobic yields of E. coli being greater than those of K. aerogenes may be that the efficiency of oxidative phosphorylation is greater in the former organism. Proton extrusion experiments of the type reported for E. coli by Poole & Haddock (1974), if also done for K. aerogenes, might show a difference in the number of coupling sites between the two organisms. Alternatively, it may be that the efficiency of coupling at each site is lower in K. aerogenes than it is in E. coli.

Direct comparisons of molar growth yields should be possible, both for any organism growing on a number of substrates and for different organisms, if the yields can be expressed in terms of ATP. Bauchop & Elsden (1960) made this comparison for several organisms growing anaerobically, and concluded that the energy, as ATP, required to synthesise a given quantity of cells from pre-formed monomers was approximately the same for any organism. This energy requirement gave on average a Y_{ATP} of 10.5 g/mole ATP. Since this publication, several exceptions to this general rule have been found, but E. coli seems to have a Y_{ATP} similar to most other micro-organisms (for review, see Stouthamer, 1969). If Y_{ATP} for the synthesis of cells from pre-formed monomers is assumed to be the same under aerobic conditions as it is anaerobically, then an estimate can be made of the efficiency of oxidative phosphorylation in growing cells. A good example of this sort of calculation is the determination of the P/2e ratio for nitrate

respiration in A. aerogenes by Stouthamer & Bettenhaussen (1973).

There is no real justification for the assumption that Y_{ATP} is the same under both aerobic and anaerobic conditions. The best indication that this may be the case comes from the work of von Meyenberg (1969) with aerobic chemostat culture of Saccharomyces cerevisiae. Working on the assumption that Y_{ATP} and P/O should be constant regardless of the specific growth rate (μ), von Meyenberg utilised the changing oxidation pattern at different μ to predict that the only values of Y_{ATP} and P/O which could be constant for all μ were 12 g/mole and 1.1 respectively. This value of 12 g/mole for Y_{ATP} is sufficiently close to the Bauchop & Elsden value to suggest that Y_{ATP} may indeed be the same aerobically and anaerobically. It should be noted that the work of von Meyenberg makes the tacit assumption that no net energy is produced by the conversion of glucose to the cell's monomers, which was also proposed by Bauchop & Elsden (1960) and Forrest & Walker (1971). Stouthamer & Bettenhaussen (1975) refer to work by de Kwadsteniet et al. (in preparation) which appears to confirm that Y_{ATP} is the same under aerobic and anaerobic conditions. No details of the method were mentioned. The assumption that Y_{ATP} is 10.5 g/mole for E. coli will be made in the following calculations, which are intended to provide an estimate of the efficiency of oxidative phosphorylation in E. coli growing at 37^{o}.

Since the limiting carbon source is used as a source of carbon as well as energy, the energy production must be derived mainly from that fraction of carbon source which is not incorporated into cells. It should be noted that the Bauchop & Elsden value of 10.5 g/mole for Y_{ATP} applies only to the synthesis of cells from preformed monomers. The energy required to convert the carbon which is incorporated to these

monomers must also be taken into account when cells grow on a simple medium containing only carbon source and inorganic salts. Under aerobic conditions 'energy' should include, in addition to ATP, reduced nicotinamide and flavin nucleotides.

The monomer composition of arithmetically-grown E. coli was not determined. The amino acid, nucleotide and triglyceride composition of the cells was taken to be the same as determined by Morowitz (1968). Assumptions made for the nature of bacterial lipid were the same as made by Stouthamer (1973), principally that lipid consists of phosphatidylethanolamine with two C16 fatty acids. The validity of these assumptions will be discussed in the light of the results of the calculations.

1.2 Energy production from the synthesis of monomers

The energy produced from the conversion of glucose, lactose, glycerol and malate to amino acids, nucleotides, fatty acids, α-glycerophosphate and glucose-6-phosphate is different in each case. The calculation of energy made available will be divided into two sections, one concerned with the energy involved with conversion of carbon source to the amphibolic precursor of the monomer (the compound in the Embden-Meyerhof pathway or tricarboxylic acid cycle from which the monomer is directly derived) and the other with the energy requirement for the conversion of these amphibolic precursors to the monomers. The latter calculation is the same for each type of cell, since cell composition is assumed to be identical in each case. On the other hand, the energy change for the conversion of carbon source to amphibolic precursors depends on the carbon source.

1.2.1 Energy production from carbon source → amphibolic precursors

The amphibolic precursors from which each monomer is derived, and the cell content of the monomer were listed (Figure 55). From these data it was possible to estimate the flow of carbon from each amphibolic compound for the synthesis of 1g of cells (Figure 56).

The flow of carbon to each of these amphibolic precursors was mapped (Figures 57, 58 and 59) in order to estimate the quantity of carbon passing through each energy-yielding or energy-utilising reaction. It was assumed for the purposes of this calculation that all of the precursors were accounted for by the eight compounds listed (Figure 56). It was also assumed that the PEP used in the phosphorylative transport of glucose by the phosphotransferase system was equivalent to 1 ATP, and that the equivalent of 1 ATP was consumed for each molecule of lactose transported into the cell.

The energy, as ATP and 2H, utilised and produced by the conversion of each of glucose, lactose, glycerol and malate to the amphibolic precursors was summed (Figure 60).

1.2.2 Energy required for conversion of amphibolic precursors → monomers

The ATP and 2H produced and utilised during the formation of the monomers from the amphibolic precursors was calculated (Figures 61 and 62). The sum of the energy requirement of these processes is therefore (56.39 + 41.70) = 98.09 mol x 10^{-4} ATP and (99.63 + 45.77) = 145.40 mol x 10^{-4} 2H in order to synthesis the monomers of 1g of cells according to the composition derived by Morowitz (1968). This quantity of energy is the same for cells growing on each carbon source, as it was assumed that the cell composition is the same in each case.

FIGURE 55

Monomer composition of E. coli and amphibolic precursors of monomers

The monomer composition of bacterial cells was determined by Morowitz (1968). Amphibolic precursors of monomers are those compounds in the Embden-Meyerhof pathway and tricarboxylic acid cycle from which the carbon atoms of the monomer are derived.

Abbreviations used are:

G6P	glucose-6-phosphate
G3P	glyceraldehyde-3-phosphate
3PG	3-phosphoglycerate
PEP	phosphoenolpyruvate
pyr	pyruvate
acCoA	acetyl coenzyme A
αKG	α-ketoglutarate
OAA	oxaloacetate
dTMP	thymidine 5'-phosphate
UMP	uridine 5'-phosphate
(d)CMP	(deoxy) cytidine 5'-phosphate
(d)GMP	(deoxy) guanosine 5'-phosphate
(d)AMP	(deoxy) adenosine 5'-phosphate
C16FA	palmitate

Monomer	Amount mol x 10^{-4}/g cells	Amphibolic precursor	Monomer	Amount mol x 10^{-4}/g	Amphibolic precursor
ala	4.54	pyr	dTMP	1.39	OAA
arg	2.52	αKG	+UMP		G6P
asp	2.01	OAA			
asn	1.01	OAA	dCMP	1.39	OAA
cys	1.01	3PG	+CMP		G6P
glu	3.53	αKG			
gln	2.01	αKG	dGMP	1.39	OAA
gly	4.03	3PG	+GMP		3PG
his	0.50	G6P			G6P
ile	2.52	(OAA (pyr	dAMP	1.39	OAA
leu	4.03	(2 x pyr (acCoA	+AMP		3PG G6P
lys	4.03	(OAA (pyr	C16 FA	2.80	8 x acCoA
met	2.01	(3PG (OAA	αGP	1.40	G3P
phe	1.51	(G6P (2 x PEP	glucose	10.26	G6P
pro	2.52	αKG			
ser	3.02	3PG			
thr	2.52	OAA			
trp	0.50	(2 x G6P (PEP			
tyr	1.01	(G6P (2 x PEP			
val	3.02	2 x pyr			

FIGURE 56

Flow of carbon from amphibolic precursors necessary for biosynthesis of E. coli

Data from Figure 55 were used to predict the flow of carbon required through the 8 main amphibolic precursors for the synthesis of 1g of cells. Abbreviations used are the same as for Figure 55.

Amphibolic precursor	Quantity necessary for growth mol x 10^{-4}/g cells
G6P	19.84
G3P	1.40
3PG	12.85
PEP	5.54
pyr	25.19
acCoA	26.43
αKG	10.58
OAA	19.66

FIGURE 57

Conversion of glucose to the amphibolic precursors

The data of Figure 56 show the amphibolic precursors necessary for the biosynthesis of 1g of cells. The flow of metabolites from glucose necessary to provide these quantities of precursors can then be summed for each energy-yielding or -requiring reaction. The figure adjacent to each arrow is the quantity of substrate metabolised in that reaction during the conversion of glucose to the monomers. The units are mol x 10^{-4}/g cells.

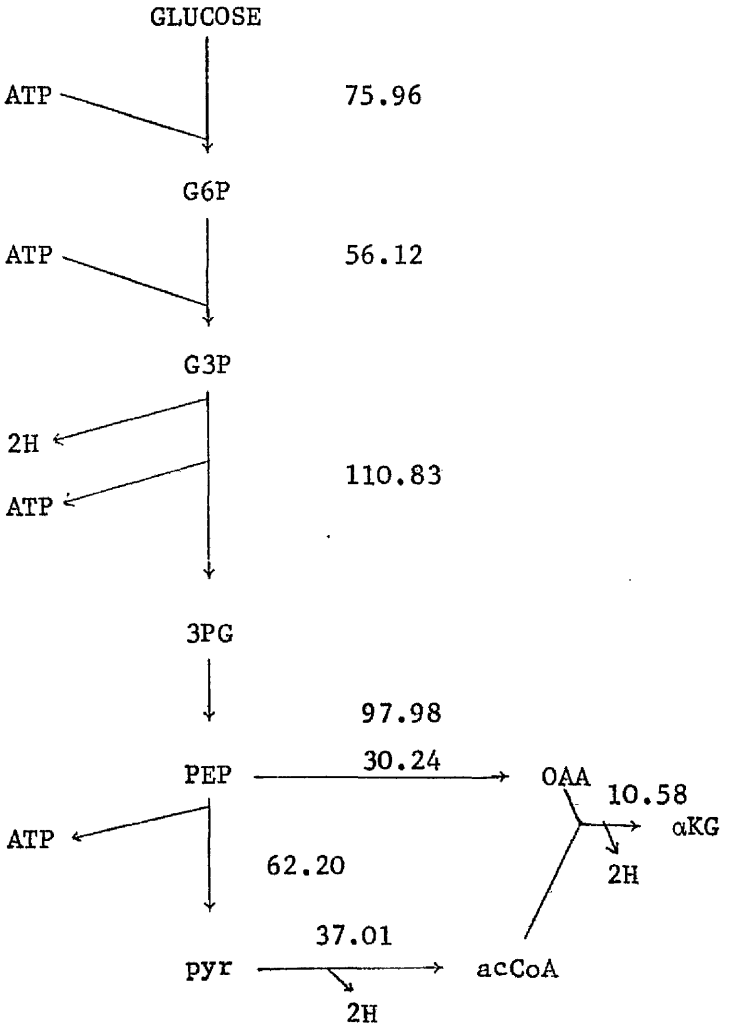


FIGURE 58

Conversion of lactose and glycerol to the amphibolic precursors

The conversion of lactose and glycerol to the amphibolic precursors proceeds mainly according to the chart worked out for glucose (Figure 57), apart from the early reactions. The figures adjacent to the arrows are again in mol x 10^{-4}/ g cells.

It is assumed that 1 ATP is used for the transport of 1 molecule of lactose into the cell. Glycerol enters the cell by a facilitated diffusion mechanism, and so no energy is required for its uptake.

FIGURE 58a

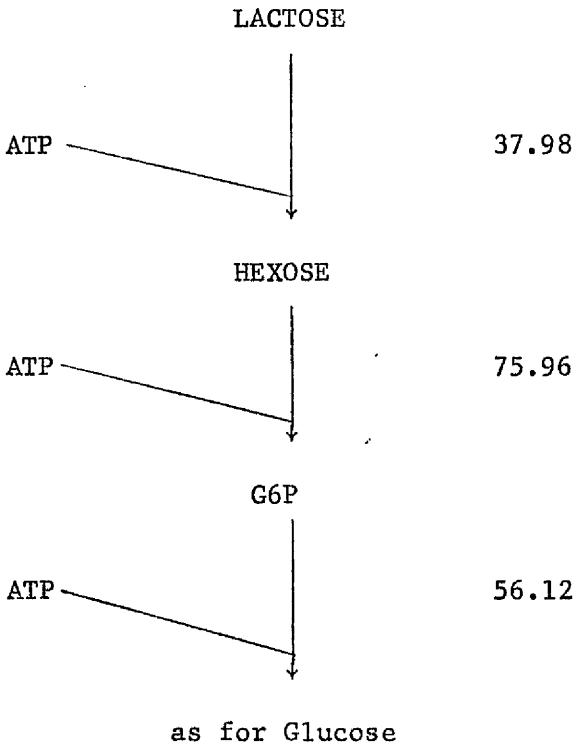


FIGURE 58b

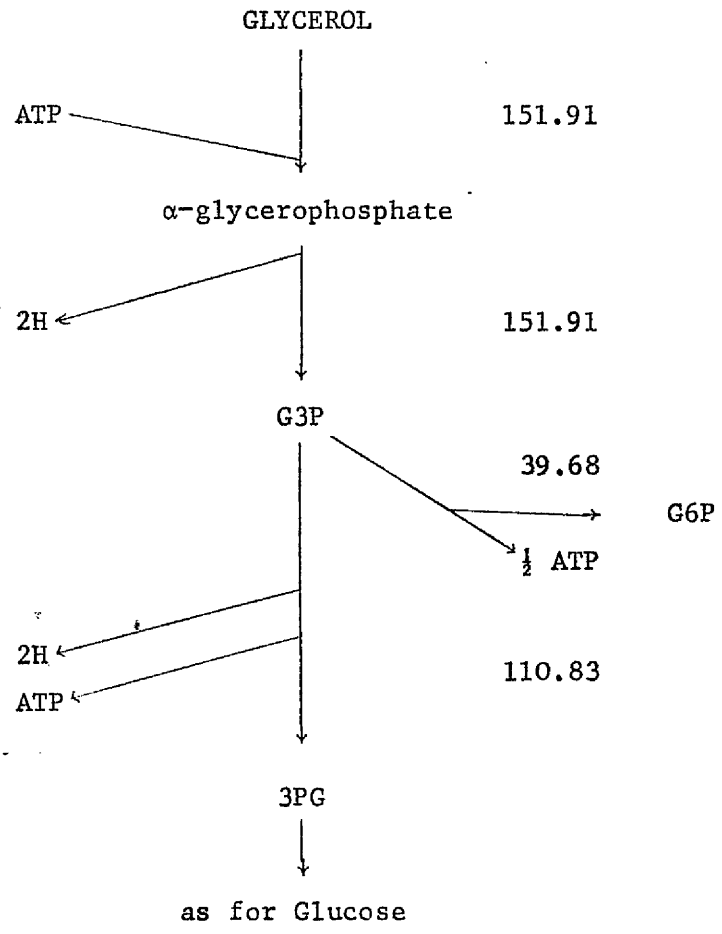


FIGURE 59

Conversion of malate to the amphibolic precursors

When malate enters E. coli, it is metabolised by three enzymes. PEP carboxykinase converts malate to PEP, and requires ATP as a source of the phosphate group (Kornberg, 1966). Malic enzyme provides pyruvate from malate (Katsuki et al., 1967) in order to provide acetyl CoA in the condensation with oxaloacetate to form citrate. Malate dehydrogenase oxidises malate to oxaloacetate.

At this stage it is assumed that no energy is required for malate transport.

The figures are again in units mol x 10^{-4}/g cells.

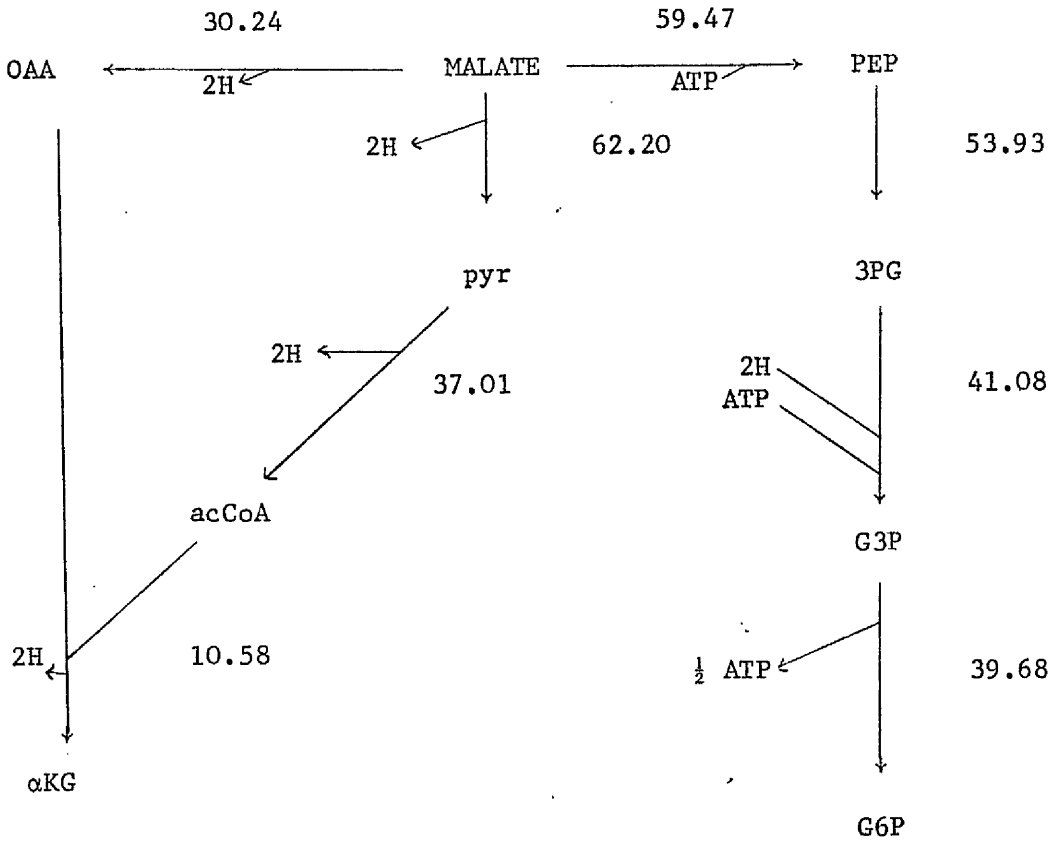

30.24
59.47
OAA
2H
MALATE
ATP
PEP
2H
62.20
53.93
pyr
3PG
2H
37.01
2H
ATP
41.08
acCoA
G3P
2H
10.58
½ ATP
39.68
αKG
G6P

FIGURE 60

Energy made available by conversion of carbon source → amphibolic precursors

The ATP and 2H utilised and produced during the distribution of carbon to the amphibolic precursors required for the synthesis of 1g of cells (Figures 57, 58 and 59) was summed.

Carbon Source	ATP produced (mol x 10^{-4}/g)	2H produced mol x 10^{-4}/g
glucose	40.95	158.42
lactose	2.97	158.42
glycerol	40.96	310.33
malate	-80.71	98.95

FIGURE 61

Energy required for conversion of amphibolic precursors to amino acids

The amino acid content of Morowitz (1968) is again used. Several assumptions have been made in these calculations:-

(i) all NH_3 enters cells by the action of glutamate dehydrogenase, and so for each $-NH_2$ passed on by transamination from glutamate, one 2H is required for its regeneration

(ii) where ATP is hydrolysed to AMP and pyrophosphate, 2 ATP equivalents are used

(iii) 2ATP are used in the synthesis of carbamyl phosphate

(iv) succinyl CoA added and subsequently released as succinate costs the cell 1 ATP

(v) serine used in tryptophan synthetase and released as triose phosphate makes available 1 ATP and 1 2H by regeneration of serine.

Amino acid	Amount mol x 10^{-4}/g	ATP mol/mol	2H mol/mol	ATP mol x 10^{-4}/g	2H mol x 10^{-4}/g
ala	4.54	0	1	0	4.54
arg	2.52	5	4	12.60	10.08
asp	2.01	0	1	0	2.01
asn	1.01	2	1	2.02	1.01
cys	1.01	3	4	3.03	4.04
glu	3.53	0	1	0	3.53
gln	2.01	1	1	2.01	2.01
gly	4.03	0	0	0	0
his	0.50	4	-1	2.00	- 0.50
ile	2.52	2	5	5.04	12.50
leu	4.03	0	1	0	4.03
lys	4.03	2	4	8.06	16.12
met	2.01	5	7	10.05	14.07
phe	1.51	1	2	1.51	3.02
pro	2.52	1	3	2.52	7.56
ser	3.02	0	0	0	0
thr	2.52	2	3	5.04	7.56
trp	0.50	3	2	1.50	1.00
tyr	1.01	1	1	1.01	1.01
val	3.02	0	2	0	6.04
				56.39	99.63

FIGURE 62

Energy required for conversion of amphibolic precursors to nucleotides, fatty acids and α-glycerophosphate

The monomer content of E. coli was assumed to be the same as published by Morowitz (1968).

Assumptions made are included in the legend to Figure 61. In addition, no distinction was made between flavin and nicotinamide nucleotides

Monomer	Amount mol x 10^{-4} /g	ATP mol/mol	2H mol/mol	ATP mol x 10^{-4} /g	2H mol x 10^{-4} /g
AMP	1.15	9	0	10.35	0
dAMP	0.24	9	1	2.16	0.24
GMP	1.15	10	-1	11.50	- 1.15
dGMP	0.24	10	0	2.40	0
CMP	1.15	5	0	5.75	0
dCMP	0.24	5	1	0.70	0.24
UMP	1.15	4	0	4.60	0
dTMP	0.24	6	1	1.44	0.24
C16FA	2.80	1	16	2.80	44.80
αGP	1.40	0	1	0	1.40
				41.70	45.77

1.2.3 Total energy involved in conversion of carbon source → cell monomers

The energy produced and utilised by the conversion of carbon sources to the cell monomers, as calculated in the last two sections, was summed (Figure 63). ATP is required in each case, but is very much greater for the conversion of malate to the monomers than it is for glucose, as observed by Stouthamer (1973). The relatively larger production of reducing power from glycerol suggests that, under conditions where oxidative phosphorylation provides ATP, there will be a net gain of ATP from the conversion of glycerol to the monomers, while the quantity of reduced nucleotides produced by the other three carbon sources will not provide sufficient ATP to balance the ATP requirement for monomer synthesis.

1.3 Energy derived from proportion of carbon source fully oxidised

The quantities of carbon source utilised for the synthesis of the carbon skeletons of the monomers have been calculated (Figures 57, 58 and 59). The total carbon source utilisation is defined by Y_G, and so the total amount of carbon source required to provide both carbon and energy for the synthesis of 1g of cells is given by $\frac{1}{Y_G}$. Thus, the difference between the total carbon source used and the carbon source used for the provision of the monomers represents the proportion used entirely for energy production.

All carbon source fully oxidised was assumed to be metabolised via the Embden-Meyerhof pathway and tricarboxylic acid cycle, and hence the energy produced by the oxidation of this fraction was calculated (Figure 64).

FIGURE 63

Total energy involved in the conversion of carbon source → cell monomers

The energy made available during the conversion of carbon source to amphibolic precursors (Figure 60) and during the synthesis of monomers from those amphibolic precursors (Section 1.2.2) was summed.

Carbon Source	ATP produced (mol x 10^{-4}/g)	2H produced (mol x 10^{-4}/g)
glucose	- 57.14	13.02
lactose	- 95.12	13.02
glycerol	- 57.14	164.93
malate	-178.80	- 46.45

FIGURE 64

Energy made available from fraction of carbon source completely oxidised

The quantities of carbon source used for the provision of energy had been calculated from the monomer composition of Morowitz (1968) (Figures 57, 58 and 59). The total carbon source utilisation was calculated from Y_G (Figure 24). The difference between these totals represents the fraction of carbon source fully oxidised, and this was assumed to be oxidised through the Embden-Meyerhof pathway and tricarboxylic acid cycle.

1 equivalent of ATP was assumed to be released by the conversion of succinyl CoA to succinate by succinyl thiokinase.

FIGURE 64

Carbon Source	Y_G g/mole	Total utilisation mol x 10^{-4} /g	Utilisation for monomers mol x 10^{-4} /g	C source fully oxidised mol x 10^{-4}/g	ATP production mol/mol	2H production mol/mol	ATP production mol x 10^{-4} /g	2H production mol x 10^{-4} /g
glucose	95.0	105.3	76.0	29.3	4	12	117.2	351.6
lactose	218.3	45.8	38.0	7.8	7	24	54.6	187.2
glycerol	50.7	197.2	151.9	45.3	2	7	90.6	317.1
malate	43.3	230.9	151.9	79.0	1	6	79.0	474.0

1.4 Calculation of P/2e and P/O ratios

The total energy made available during growth on glucose, lactose, glycerol and malate was calculated by adding the totals calculated in the previous two sections (Figure 65).

The assumption was made that the energy required for the synthesis of cells from pre-formed monomers is the same under aerobic and anaerobic conditions and that this energy is defined by Y_{ATP} = 10.5g/mol ATP (Bauchop & Elsden, 1960). The ATP required in the synthesis of 1g of cells was therefore $\frac{1}{10.5}$ mol ATP.

The amount of ATP made available by oxidative phosphorylation is the difference between the total ATP known to be required for growth and the total ATP made available by substrate level phosphorylation (Figure 65). Division of this quantity by the amount of 2H calculated to be made available (Figures 63 and 64) gave the P/2e ratio. Division of the same quantity by the known oxygen uptake ($\frac{2}{Y_{GO_2}}$) (Figure 24) gave the P/O ratio (Figure 65).

Two broad conclusions can be made from these calculations. Firstly, the P/O ratios are in each case less than the P/2e, implying that the calculation of the 2H made available did not take into account all of the 2H produced. This may mean that cells are more oxidised than predicted by Morowitz. As a carbon balance was drawn up for each experiment, it is unlikely that any oxidised carbon compound is excreted which could account for the difference. It is possible, however, that an oxidised inorganic compound may build up in the medium. In any case, it seems probable that P/O ratios, which require experimental measurement of oxygen consumption, have more merit than the P/2e ratios calculated.

FIGURE 65 Calculation of P/2e and P/O ratios

The total ATP produced by oxidative phosphorylation was calculated by subtracting the ATP produced by substrate level phosphorylation (B and C) from the total quantity of ATP required for cell synthesis (A). The P/2e ratio was calculated by division of this quantity by the total reducing power made available (F). The P/O ratio was calculated by dividing the same quantity by the observed O uptake (G).

FIGURE 65

	A	B	C	E	F			G	
Carbon Source	ATP required mol x 10^{-4} /g	ATP from complete oxidation mol x 10^{-4} /g	ATP from synthesis of monomers mol x 10^{-4} /g	ATP from oxidative phosphorylation (A-B-C)	Total 2H produced mol x 10^{-4} /g	P/2e $(\frac{E}{F})$	Y_{GO_2} g/mole	O uptake g atom x 10^{-4} /g	P/O $(\frac{E}{G})$
glucose	952.4	117.2	- 57.1	892.3	364.6	2.45	45.3	441.5	2.02
lactose	952.4	54.6	- 95.1	992.9	200.2	4.96	62.0	322.6	3.08
glycerol	952.4	90.6	- 57.1	918.9	482.0	1.91	36.6	546.4	1.68
malate	952.4	79.0	-178.8	1052.2	427.5	2.46	33.8	591.7	1.78

Secondly, the P/O ratios suggest that during growth of E. coli on glucose, glycerol and malate, two coupling sites of oxidative phosphorylation operate, while three sites operate during growth on lactose.

Some commonly quoted P/O ratios for various micro-organisms are not, in fact, ratios which indicate the efficiency of oxidative phosphorylation, since they are calculated simply by dividing the Y_O by Y_{ATP}, thus taking no account of ATP produced by substrate phosphorylation (Hadjipetrou et al., 1964; Meyer & Jones, 1973; Neijssel & Tempest, 1975). The importance of taking into account energy production from that fraction of substrate incorporated into cell material has been stressed by Stouthamer (1973), yet has been ignored in most published work on microbial growth in simple salts/carbon source medium. Both of these factors have been taken into account in this work. In many cases, the end product is similar. For example, Stouthamer (1969) calculated the P/O ratio for E. coli from growth yields without taking either of these factors into account to be 2.0. This is consistent with the calculations done in this study (Figure 65).

The work of Hadjipetrou et al. (1964) with A. aerogenes indicated that the P/O ratio might be different for growth on different substrates, but suffered from both of the above limitations. More recently, the same group has shown that chemostat cultures of A. aerogenes have a P/O ratio of 1.40 under glucose-limitation, but only 0.40 under sulphate limitation (Stouthamer & Bettenhaussen, 1975), using calculations which include substrate phosphorylation by the fraction of substrate fully oxidised, but do not include ATP generated during the conversion of carbon source to monomers.

Direct measurement of the P/O ratio in intact whole cells of E. coli reported by Hempfling (1970) indicated a value of 3.0. However, attempts to repeat these experiments have been unsuccessful. The P/O ratios measured have been very much smaller (van der Beek & Stouthamer, 1973), in common with those measured for other bacteria (Smith, 1968). Similarly, P/O ratios in membrane preparations of bacteria are found to be low. Possible reasons for this are discussed by Mevél-Ninio and Yamamoto (1974), principally that the respiratory system suffers physical damage during preparation of membrane vesicles. However, this type of experiment can still be used to demonstrate differences in numbers of coupling sites. Hertzberg & Hinkle (1974) found a P/O ratio 0.62 with NADH and 0.38 with α-glycerophosphate for inverted membrane vesicles of E. coli, consistent with there being three coupling sites used by NADH and two by the oxidation of α-glycerophosphate.

Similar work, designed to determine the number of coupling sites, has utilised metabolic inhibitors and energy poisons (Ishaque, Donawa & Aleem, 1973). Recent interest in the relationship of the protonmotive force to oxidative phosphorylation has resulted in techniques where the efficiency of proton extrusion has been measured in E. coli. The number of coupling sites has been found to be a maximum of two for E. coli grown on glycerol (Poole & Haddock, 1974), which falls to one under sulphate limitation, similar to the findings of Stouthamer & Bettenhaussen (1975) from growth yield data of A. aerogenes. Jones et al. (1975) report a $\rightarrow H^+/O$ ratio of 4 for E. coli (i.e. a P/O ratio of 2), despite a previous report (Meyer & Jones, 1973) of 3 coupling sites in E. coli.

These results only apply to the extrusion of protons. If the coupling to oxidative phosphorylation of the re-entry of the protons into the cell is not fully efficient, the P/O ratio in vivo will be

different from the number of coupling sites, as found by Meyer & Jones (1973). The only way the efficiency of oxidative phosphorylation in terms of ATP production in growing cells can be assessed is from growth yield data.

The data in Figure 65 suggests that, especially for glucose and lactose, the coupling at each site is quite efficient, as the values calculated are fairly close to integers. The relatively lower value of the P/O ratio in glycerol-growing cells may be connected with the finding of Hertzberg & Hinkle (1974) that one coupling site fewer operates when the electron donor is α-glycerophosphate rather than NADH. Since a large proportion of the 2H made available is derived from α-glycerophosphate oxidation, this would make an appreciable difference to the calculated value of the coupling efficiency. Assuming that the P/O ratio for α-glycerophosphate oxidation is 1.0, the P/O ratio for the remainder of oxidative phosphorylation can be calculated. As Y_G (glycerol) = 50.7 g/mole, then $\frac{10^4}{50.7}$ = 197.2 mol x 10^{-4} glycerol/g cells must pass through this step. The ATP made available by the remainder of oxidative phosphorylation is therefore (918.9 - 197.2) = 721.7 mol x 10^{-4}/g cells. The O uptake for the remainder will be (546.4 - 197.2) = 349.2 g.atom x 10^{-4}/g cells, and so the P/O ratio of the remainder is $\frac{721.7}{349.2}$ = 2.07, close to the value for glucose.

Similarly, the P/O ratio for growth on malate was considerably less than 2.0. No account was taken in the calculation of any energy which may be used in the transport of malate across the cell membrane. If it is assumed that 1 ATP is hydrolysed for each molecule taken up, then, as the uptake is $(\frac{10^4}{Y_G})$ = $(\frac{10^4}{43.3})$ = 230.9 mol x 10^{-4} malate/g cells, the ATP required from oxidative phosphorylation will be (1052.2 + 230.9) = 1283.1 mol x 10^{-4}/g cells (Figure 65). As the O uptake is 591.7 g.atom

x 10^{-4}/g cells, the P/O ratio is $\frac{1283.1}{591.7} = \underline{2.17}$, again fairly close to an integral value. No similar correction was necessary for glycerol permeation, as no energy is required for facilitated diffusion.

The most probable reason for the difference between the calculated P/O ratios and integral numbers is the assumption that Y_{ATP} = 10.5g/mole. For example, for a P/O ratio of 2.00 for growth on glucose, Y_{ATP} would have to be 10.6g/mole in the calculations. Bauchop & Elsden (1960) found a range in values of Y_{ATP} between different organisms of 8.3 to 12.6g/molATP, so a Y_{ATP} of 10.6 for E. coli is well within this range. It may be that the P/O ratios are in fact integral, with a Y_{ATP} of 10.6g/mole being the real value.

Lactose transport involves the symport of protons (West & Mitchell, 1973) in a way very similar to the involvement of the proton-motive force in oxidative phosphorylation (Harold, 1972). The appearance of an extra site of oxidative phosphorylation in lactose-growing E. coli may perhaps be in some way a consequence of the function of the permease as a proton carrier. It would be of great interest to compare the $\rightarrow H^+/O$ ratios for glucose- and lactose-grown E. coli.

The growth yields of E. coli on glycerol at 37° in the presence of 0.5 M sodium chloride (Figure 24) are of interest, since Y_{GO_2} falls considerably while Y_G remains similar to the value under low salt growth conditions. When the P/O ratio was calculated in the same way as before, the value obtained was 1.11. Thus, the presence of 0.5 M sodium chloride appears to destroy one coupling site. As this P/O ratio is not less than the integer, as was found under low salt conditions, it is reasonable to conclude that the coupling site which is lost is the one between NADH oxidase and ubiquinone. The coupling

site for the oxidation of both α-glycerophosphate and NADH will therefore be the same one, beyond ubiquinone.

No similar change in the number of coupling sites can occur for growth on glucose in high salt concentrations as Y_{GO_2} (glucose) is similar under high and low salt conditions.

In summary, growth yield data for E. coli indicate a P/O ratio of 2 for growth on glucose, glycerol and malate and a P/O ratio of 3 for growth on lactose. Addition to the growth medium of 0.5 M sodium chloride causes the loss of one coupling site during growth on glycerol, but not during growth on glucose.

1.5 Calculation of expected oxygen yields from cell composition data

The elementary composition determined for E. coli (Figure 30) is similar to that found by Shiloach & Bauer (1975) for E. coli and by Fewson (personal communication) for Acinetobacter calcoaceticus:

	% W/W		
Element	This work	Shiloach & Bauer	Fewson
C	44.6	44.1	47.5
H	6.7	7.1	6.7
N	12.6	12.7	12.0
P	3.5	2.9	2.1
S	2.2	0.6	1.7

Fewson also estimated that the total content of inorganic ions was 1.1%

It is possible from these data to calculate the expected Y_{GO_2}. The oxygen in cells, CO_2 and H_2O can be estimated. This must be equivalent to the total oxygen entering the system. Since the oxygen

content of the growth substrates is known, then the uptake of molecular O_2 necessary to balance the oxygen in the products can be calculated by subtraction.

Assuming the inorganic ion content of the cell to be the same for E. coli as for A. calcoaceticus, the total cell material accounted for by the above table is 70.7%, compared with 68.5% for the estimations by Shiloach & Bauer and 71.1% for A. calcoaceticus. The oxygen content of E. coli was therefore assumed to be 29.3%.

The fate of all of the oxygen entering the growth system is cell material, CO_2 and H_2O. The oxygen in cells is 29.3% of the cell mass. The carbon appearing in CO_2 is estimated from the difference between the total carbon entering the system and the carbon appearing as cell material. This carbon balance has been proved (p. 93). Oxygen appearing in CO_2 is therefore 2 x CO_2. The oxygen released as H_2O is similarly estimated from the difference in hydrogen content of substrate and cells (Figure 66).

Oxygen entering the growth system consists of carbon source, phosphate and sulphate as well as molecular oxygen. This oxygen uptake is offset by the uptake of hydrogen atoms in ammonia (Figure 67). The difference between the oxygen known to be necessary for the formation of cells, CO_2 and H_2O and the oxygen entering the system as carbon source, phosphate and sulphate is equivalent to the uptake of molecular O_2, and so the expected Y_o can be calculated (Figure 67).

In each case, the Y_o calculated by this method corresponds reasonably well with the measured Y_o. Thus, the observed discrepancy between the reducing equivalents calculated to be made available and the observed oxygen uptake is due to an error in the estimation of

FIGURE 66

Total oxygen requirement

All of the oxygen entering the cells is accounted for in cells, CO_2 and H_2O. Since the elementary content of both the carbon source and cells is known, this quantity of oxygen can be estimated. The results are expressed in g atoms/cells produced by 1 mole of carbon source (i.e. g atoms/Y_G).

The elementary content of lactose is expressed as the sum of the content of glucose and galactose, the products of the hydrolysis of lactose by β-galactosidase.

FIGURE 66

Substrate	Content of substrate (g atom/mole)			Content of cells g atom/100g			Y_G g/mole	Content of cells g atom/Y_G			Δ content substrate-cells		O required g atoms		ΣO cells + CO_2 + H_2O g atoms
	C	H	O	C	H	O		C	H	O	C	H	CO_2	H_2O	
glucose	6	12	6	3.71	6.65	1.83	95.0	3.52	6.32	1.74	2.48	5.68	4.96	2.84	9.54
lactose	12	24	12	3.71	6.65	1.83	218.3	8.10	14.52	3.99	3.90	9.48	7.80	4.74	16.53
glycerol	3	8	3	3.71	6.65	1.83	50.7	1.88	3.37	0.93	1.12	4.63	2.24	2.32	5.49
malate	4	6	5	3.71	6.65	1.83	43.3	1.61	2.88	0.79	2.39	3.12	4.78	1.56	7.13

FIGURE 67

Total substrate oxygen and calculated oxygen yields

All oxygen atoms used by E. coli consist of those entering as carbon source, phosphate, sulphate and molecular O_2. Since the total oxygen requirement is known (Figure 66), and oxygen entering as carbon source, phosphate and sulphate can be estimated, the expected Y_o can be calculated, and compared with the measured oxygen yield.

3 O atoms are assumed to enter with P, and 4 O atoms with S.

The hydrogen entering the cell as ammonia (3H for every N) must be taken into account, and subtracted from the total oxygen entering as H_2O. Thus, the number of H atoms entering as NH_3 is halved before subtraction.

The cell content of P, S and N are shown in Figure 30.

All figures are calculated with respect to 1 mole of carbon source and lactose is taken as the sum of its hydrolytic products (glucose and galactose).

FIGURE 67

Carbon Source	C Source O g atom /mole	phosphate O g atom /mole	sulphate O g atom /mole	ammonia H g atom /mole	ΣO in g atom /mole	ΣO out g atom /mole	ΔΣO (gas) g atom /mole	Y_G g/mole	Calculated Y_O g/mole	Measured Y_O g/mole
glucose	6	0.31	0.27	2.57	5.30	9.54	4.24	95.0	22.4	22.7
lactose	12	0.72	0.61	5.89	10.38	16.53	6.15	218.3	35.5	31.0
glycerol	3	0.17	0.14	1.37	2.62	5.49	2.87	50.7	17.7	18.3
malate	5	0.14	0.12	1.17	4.67	7.13	2.46	43.3	17.6	16.9

reducing equivalents and not to an error in the oxygen yield measured. The values for P/O ratios are consequently more meaningful than the P/2e values (Figure 65).

The most probable explanation of the difference between expected and observed oxygen uptake is that some of the assumptions made concerning the monomer composition of the cell are wrong, or that the monomer composition determined by Morowitz (1968) is wrong or incomplete.

2. Influence of growth conditions on molar growth yields

The influence of high concentrations of sodium chloride on growth has been discussed in the last section, with the conclusion that the P/O ratio was changed for cells growing on glycerol, but not for cells growing in high salt on glucose. The Y_{GO_2} in the glycerol case was quite different under low and high salt concentrations, whereas the Y_{GO_2} for glucose was similar in each case (Figure 24). The Y_G on both glucose and glycerol was slightly decreased, in contrast to the findings of Watson (1970) and Reichelt & Baumann (1974) who found that the addition of NaCl to the growth medium did not alter Y_G for several micro-organisms. The composition of E. coli may have been altered by the addition of salt, causing the decreased Y_G, but it was not possible to obtain an elementary analysis of the cells, and a complete biochemical analysis was not possible in the time available. The reduced P/O ratio for glycerol would in any case cause a decreased Y_G.

The effect of changing growth temperature on growth yields was also different from other published work. For glucose and glycerol, the values of Y_G and Y_{GO_2} were at a minimum at 37^{o}, but the pattern was different for growth on malate and lactose (Figures 28 and 29).

Shiloach & Bauer (1975) reported that the growth yields of E. coli on glucose at different temperatures were identical, but the yields were in the region of 80g/mole, less than measured by arithmetic-type continuous culture, and the medium was supplemented with autolysed yeast powder, so a direct comparison is not valid. Harrison & Loveless (1971) observed that the growth yield of E. coli in a glucose-limited chemostat was unaffected by changes in temperature. The influence of temperature on bacterial growth has been reviewed by Forrest (1967), who concludes that, in batch culture, the molar growth yield depends on the balance between catabolism and anabolism at each temperature. Thus, if the catabolic rate is greater than the rate at which cells can be synthesised at a particular temperature, the growth yield will be less than it would be if the two processes were balanced. The work of Senez (1962) with A. aerogenes shows that if the pattern of catabolism changes at different temperatures, the molar growth yield will be different at different temperatures. As the rate of catabolism is always limiting in carbon-limited arithmetic-type continuous culture, the type of uncoupling described by Forrest can not occur. Also, since no catabolic products are found at any temperature, and the oxygen uptake per mole carbon source is relatively constant (from Figure 24), it is unlikely that the pattern of catabolism is altered at different temperatures. The growth systems used in other studies of the influence of temperature on growth yields (Ng, 1969; Forrest, 1969; Senez, 1962) suffered from the fact that the fate of all the carbon entering the growth system was not known.

The cell composition is known to be different at different temperatures. The most marked change is in the fatty acid content. As temperature falls, the chain length of fatty acids tends to become less and the content of unsaturated fatty acids rises in order to

maintain fluidity of the membrane (Marr & Ingraham, 1962). Such changes would influence the energy requirement for formation of monomers, and perhaps even change Y_{ATP}, resulting in altered molar growth yields. The fact that the Y_G's of cells growing on different substrates do not vary in the same way (Figures 28 and 29) suggest that, although the elementary content of the cells may be the same, the biochemical composition of cells growing on different substrates may be different.

The molar growth yields obtained from nitrogen-limited culture (Figures 48-53) are difficult to interpret. As a carbon balance was not successful, and the pattern of metabolism was not determined, the yields can not be usefully employed to find, for example, the P/O ratio. However, both Y_G and the initial Y_{GO_2} for glycerol fell by a similar proportion, yet Y_G for malate increased by 44% while the Y_{GO_2} remained the same as under carbon limitation. The result for glycerol is consistent with the work of Rosenberger & Elsden (1960), Nagai et al. (1969), Belaich et al. (1972), Harrison (1972), Neijssel & Tempest (1975) and many others, as it is generally found that if the culture is limited by a nutrient other than the energy source, unbalanced growth, or uncoupling, occurs, which causes the molar growth yield to fall. Belaich et al. (1972) suggest that under these conditions ATP production must be greater than the potential for its utilisation for growth, and so the ATP formed is hydrolysed to ADP by an enzyme whose function it is to control the energy charge of the cell. Hence ATP production is less efficient and the molar growth yield falls. Chance (1959) also postulated this sort of enzymic control mechanism for yeast.

The Y_G on malate, however, was found to rise from 43.3g/mole to 62.2g/mole, with no change in the initial Y_{GO_2} under nitrogen limitation. This anomalously high value for Y_G may be a consequence of the growth conditions being non-ideal, with the application of the growth equation in this case being unjustified. Otherwise, the implication is that, in some way, either Y_{ATP} or the P/O ratio must have been increased.

The P/O ratio is known to change under different limiting conditions. Stouthamer & Bettenhaussen (1975) found different P/O ratios under carbon-, nitrogen- and sulphate limitation for A. aerogenes, Downie & Garland (1973) found a change in the $\rightarrow H^+/O$ ratios between glycerol- and sulphate-limited yeast and Rainnie & Bragg (1973) observed uncoupling in iron-limited chemostats of E. coli. Nitrogen limitation in general causes a decrease in the Y_G. The reason for this observed increase in Y_G for malate, if real, may be an increase in the P/O ratio caused by nitrogen limitation.

3. Maintenance coefficients

Maintenance energy in E. coli was measured in carbon-limited arithmetic-type continuous culture for cells growing on glucose, lactose, glycerol and malate (Figure 24). Maintenance coefficients were calculated from experimental data and expressed in mmol C source g^{-1}. h^{-1} and mmol O_2 g^{-1} h^{-1}. From the values of Y_G and Y_{GO_2} obtained from the same experiments, P/O ratios were calculated (Figure 65). The maintenance coefficient in terms of ATP (m_{ATP}) was calculated from m assuming that all of the carbon source was completely oxidised via the Embden-Meyerhof pathway and tricarboxylic acid cycle, and that the reducing power generated was converted to ATP with the efficiency

defined by the calculated value for the P/O ratio (Figure 68).

$\underline{m}_{O2}$* was introduced by Nagai & Aiba (1972), and represents the maintenance requirement in terms of O_2 which would be expected from the complete combustion of the carbon source used for maintenance. Thus $\underline{m}_{O_2}$* = 6 x $\underline{m}$ (glucose). $\underline{m}_{O_2}$* can then be compared with measured values of $\underline{m}_{O_2}$. It would be expected from the concept of maintenance that the carbon source used for maintenance should be used entirely for the production of energy. The validity of this idea is borne out by comparison of $\underline{m}_{O_2}$ and $\underline{m}_{O_2}$* (Figure 24). In all cases $\underline{m}_{O_2}$ and $\underline{m}_{O_2}$* are very similar.

Maintenance energy has also been measured by arithmetic-type continuous culture by Marr, Nilson & Clark (1963) and by Palumbo & Witter (1969). Marr et al. measured the specific rate of maintenance for glucose-limited E. coli PS and E. coli ML30, but as no values of Y_G were given, calculation of the maintenance coefficient is not possible. If it is assumed that Y_G was the same as for E. coli ML308, i.e. 95.0g/mole, then estimates of maintenance coefficients in terms of glucose are 0.30 mmol g^{-1}, h^{-1} for E. coli PS and 0.19 mmol g^{-1} h^{-1} for E. coli ML30, compared with 0.04 mmol g^{-1}, h^{-1} measured for E. coli ML308 in this work (Figure 24). The errors involved in the measurement of these maintenance coefficients are relatively small, yet the values obtained differ significantly, which is perhaps surprising as E. coli ML30 is the parent strain of E. coli ML308. It is not, however, unusual to find quite different maintenance coefficients obtained by different workers for the same or closely related organisms (Figure 69). Palumbo & Witter found a value for $\underline{m}$ (glucose) of 1.39 mmol g^{-1} h^{-1} for Pseudomonas fluorescens using the same method. Significantly,

FIGURE 68

Calculation of m_{ATP}

The maintenance coefficient in terms of ATP was calculated from m using the P/O ratios calculated in Figure 65, assuming that all of the carbon source devoted to maintenance was fully oxidised by the Embden-Meyerhof pathway and the tricarboxylic acid cycle.

FIGURE 68

Carbon Source	m mmol g^{-1} h^{-1}	Substrate phosphorylation ATP mol/mol	2H mol/mol	P/O	Oxidative phosphorylation ATP mol/mol	Total ATP mol/mol	m_{ATP} mmol g^{-1} h^{-1}
glucose	0.038	4	12	2.02	24.2	28.2	1.07
lactose	0.037	7	24	3.08	73.9	80.9	2.99
glycerol	0.013	2	7	1.68	11.8	13.8	1.42
malate	0.127	1	6	1.78	10.7	11.7	1.48
glycerol[a]	0.307	2	7	1.68	11.8	13.8	4.27

a from chemostat culture

Palumbo & Witter found that the value of m derived from chemostat culture was very similar, whereas in this work m (glycerol) from arithmetic-type culture was 0.103 mmol g^{-1} h^{-1} while the value from chemostat culture was 0.307 mmol g^{-1} h^{-1} (Figure 23). Even allowing for the relatively large errors involved in the measurement of m from chemostat results, the difference between the two values must be real. Whether this reflects an intrinsic physiological difference between cells growing in arithmetic-type and chemostat cultures, perhaps due to the very small specific growth rates employed in arithmetic-type culture, or the difference between the two is a function of the method of measurement, is unclear. Hempfling & Vishniac (1967) found that, in chemostat culture of Thiobacillus neapolitans, the maintenance coefficient at low μ was $\frac{1}{2}$ of that at high μ. The same may be true of E. coli. Values of Y_G from each type of culture are virtually the same (50.7 and 50.3g/mole glycerol for arithmetic-type and chemostat culture respectively (Figure 24), which suggests that the difference in the values of m is due to a decreased requirement for maintenance energy in arithmetic-type culture rather than a poorer efficiency of energy production in chemostat culture.

A number of different methods have been used to measure maintenance. Chemostat culture is most widely used. Arithmetic-type culture is only a modification of the chemostat. Several batch culture techniques have also been employed. The work of Jones and co-workers with various micro-organisms uses oxygen-limited batch cultures (Meyer & Jones, 1973; Downie & Jones, 1974; Brice et al., 1974; Drozd & Jones, 1974). The quality of the maintenance results obtained by this method is not good, and in addition, the limitation

FIGURE 69

Published maintenance coefficients

Maintenance coefficients determined by other workers were converted, if necessary, to units of mmol g^{-1} h^{-1}. Where values of m_{ATP} were not quoted, an estimation has been made from similar results for E. coli in this work. These estimated values of m_{ATP} are in parentheses. The data are listed in approximate order of magnitude.

1. Wase & Hough, 1966
2. Meyer & Jones, 1973
3. Stouthamer & Bettenhaussen, 1973
4. Palumbo & Witter, 1969
5. Nagai & Aiba, 1972
6. Abbott, Laskin & McCoy, 1974
7. Drozd & Jones, 1974
8. Hempfling & Vishniac, 1967
9. Pirt, 1965
10. van Uden, 1968
11. Brice et al., 1974
12. Harrison & Loveless, 1971
13. Schulze & Lipe, 1964
14. Marr et al., 1963
15. Hamilton, 1972
16. Stouthamer & Bettenhaussen, 1975
17. Dalton & Postgate, 1969
18. Righelato et al., 1968
19. Carter et al., 1971
20. de Vries et al., 1970
21. Rogers & Stewart, 1974
22. Downie & Jones, 1974
23. Watson, 1970

Reference	Temp ^{o}C	Organism	Carbon Source	m mmol $g^{-1}h^{-1}$	m_{O_2} mmol $g^{-1}h^{-1}$	m_{ATP} mmol $g^{-1}h^{-1}$
1	30	Debaromyces subglobosus	phenol	712	-	-
2	30	Xanthomonas hyacinthi	succinate	-	-	44.4
3	30	A. aerogenes	glucose	-	-	38.7
4	30	Pseudomonas fluorescens	glucose	1.39	-	(38)
2	30	B. subtilis	succinate	-	-	26.4
5	30	Azotobacter vinelandii	glucose	0.8	5.5	(22)
6	35	A. calcoaceticus	ethanol	2.4	-	(21)
7	30	Hydrogenomonas eutropha	fructose	-	-	18.4
8	30	Thiobacillus neapolitanus	CO_2	-	-	16.5
9	37	A. cloacae	glucose	0.52	-	(14)
10	30	A. aerogenes	glycerol	0.85	3.29	(11)
11	30	E. coli	glycerol	-	-	10.3
12	30	K. aerogenes	glucose	-	1.4	8.8
13	30	E. coli	glucose	0.31	0.50	(8)
14	30	E. coli PS	glucose	0.30	-	(8)
15	37	E. coli	acetate	-	3.0	6.8
16	30	A. aerogenes	glucose	-	-	6.8
17	30	Azotobacter chroococcum	mannitol	0.22	-	(6)
14	30	E. coli ML30	glucose	0.19	-	(5)
2	30	Acetobacter T71	succinate	-	-	4.5
This work	37	E. coli - chemostat	glycerol	0.31	-	4.3
2	30	Acinetobacter lwoffi	succinate	-	-	4.1
15	37	E. coli	malate	-	0.90	3.4
18	25	Penicillium chrysogenum	glucose	0.12	0.74	(3)
19	30	Aspergillus nidulaus	glucose	0.10	0.55	(3)
This work	37	E. coli	lactose	0.04	0.44	3.0
20	30	Lactobacillus casei	glucose	-	-	1.5
This work	37	E. coli	malate	0.13	0.42	1.5
This work	37	E. coli	glycerol	0.10	0.33	1.4
21	30	Candida parapsilosis	glucose	0.05	-	1.3
22	-	Bacillus megaterium	glycerol	-	-	1.2
21	30	S. cerevisiae	glucose	0.07	-	1.1
This work	37	E. coli	glucose	0.04	0.24	1.1
11	30	K. pneumoniae	glycerol	-	0.24	0.6
23	20	S. cerevisiae	glucose	0.2	-	0.5

of growth by oxygen may cause changes in metabolism depending on the pO_2 (Harrison & Loveless, 1971). The oxygen growth yields obtained by Jones are not consistent with those obtained by other workers, which casts further doubt as to the validity of the maintenance coefficients obtained. Hamilton (1972) derived maintenance coefficients for E. coli from cultures where the specific growth rate in batch culture was dependent on the degree of adaptation of cells to malate. Fewson (unpublished results) has measured the maintenance requirement of Acinetobacter calcoaceticus by comparing the batch culture growth yields and specific growth rates for growth with various sources of nitrogen.

The spread of values obtained for maintenance coefficients covers two orders of magnitude (Figure 69). These range from the work of Wase & Hough (1966) with Debaromyces subglobosus where at $\mu < 0.1$ "endogenous metabolism tended to outstrip the rate at which phenol was supplied", to that of Ng (1969) who found that the molar growth yield of E. coli between $\mu = 0.2$ and $\mu = 0.85$ at 30° was virtually unchanged. The values of maintenance energy obtained in this work fall among the lower values published. Many of the higher values are likely to be unusual cases. The possible action of phenol as an uncoupler cannot be ignored when considering the results obtained by Wase & Hough (1966). The high values of m_{ATP} obtained for Xanthomonas hyacinthi and Bacillus subtilis by Meyer & Jones (1973) were obtained from the unusual oxygen-limited batch cultures. Stouthamer & Bettenhaussen (1975) have recently reviewed the high value of m_{ATP} found for A. aerogenes in their 1973 work, and concluded that this value is high because the culture was limited by tryptophan, not the carbon source. When they measured the m_{ATP} for carbon-limited

culture, they found it fell to 6.8 mmol g^{-1} h^{-1}, which is among the more commonly found values of m_{ATP}. The value of 0.8 mmol glucose g^{-1} h^{-1} for Azotobacter vinelandii was also obtained from oxygen-limited culture (Nagai & Aiba 1972), which casts doubts on the validity of this high value. This leaves the m obtained by Palumbo & Witter (1969) for P. fluorescens alone among the highest values. The molar growth yield of P. fluorescens was also found to be exceptionally high (120g/mol glucose) and it may be that the two observations are connected.

In general, therefore, m_{ATP} is less than 20 mmol g^{-1} h^{-1} and usually less than 10 mmol g^{-1} h^{-1} for most organisms, and for E. coli ML308 growing exponentially on glucose, the maintenance requirement is so small that it utilises <0.5% of the available energy, according to the maintenance coefficient obtained from arithmetic-type culture, in good agreement with the early work of Monod (1942), who was not able to measure a maintenance requirement for E. coli.

The values of m_{ATP} calculated for E. coli (Figure 68) depend on the P/O ratios previously calculated (Figure 65). It has been pointed out that the P/O ratio for growth on glycerol would be 2.0 if the value of Y_{ATP} were 9.8 instead of 10.5g/mole. Even if integral P/O ratios were used in the calculation of m_{ATP}, no great difference would be seen in m_{ATP}. The relative position of the m_{ATP}'s obtained in this work would be unchanged.

The non-linear graphs obtained from nitrogen-limited arithmetic-type continuous culture (Figures 50 and 53) confirm the findings of Stouthamer & Bettenhaussen (1975), Rosenberger & Elsden (1960), Belaich & Senez (1965) and many others that limitation by a nutrient

other than the energy source causes a breakdown of the balance between catabolism and anabolism, or uncoupling. The shape of the maintenance graphs for oxygen utilisation under nitrogen limitation in addition suggests that the efficiency of energy production coupled to O_2 consumption progressively tends to zero, which is consistent with electron transport and oxidative phosphorylation becoming uncoupled. Respiratory control apparently breaks down under nitrogen limitation, from the situation of tight coupling for the energy-limited cells at the beginning of the experiment to zero coupling towards the end of the experiment. As the uncoupling is progressive, not immediate, it seems likely that the reason cells become uncoupled is that new cells formed under nitrogen limitation do not possess a coupling factor, or the coupling factor made is inoperative.

4. Influence of growth conditions on, and possible nature of, maintenance energy

Despite the wide use of chemostat culture, and the substantial number of determinations of the maintenance coefficient for various organisms, there is little information about the nature of non-growth processes which require the energy, provided by the carbon source, allocated to maintenance. An attempt has been made in this work to identify these energy-requiring processe.

4.1 Influence of salt concentration

The most positive finding concerning the influence of salt concentration was made by Watson (1970). Addition to the growth medium of 1.0 M NaCl increased the maintenance coefficient of Saccharomyces cerevisiae in terms of glucose from 0.2 to 2.0 mmol g^{-1} h^{-1}. Due to incomplete oxidation of glucose under high salt

conditions, this was only equivalent to a rise in m_{ATP} from 0.52 to 2.20 mmol g^{-1} h^{-1}. Addition to the growth medium of 0.25 M and 0.50 M NaCl in this work (Figure 24) increased the glucose maintenance coefficient from 0.038 mmol g^{-1} h^{-1} to 0.047 and 0.054 mmol g^{-1} h^{-1} for E. coli. Thus, in both cases, addition of sodium chloride in high concentration increased the maintenance coefficient considerably. Stouthamer & Bettenhaussen (1973) found that the maintenance coefficient of A. aerogenes was strongly dependent on the concentration of ammonium chloride in the medium. A twofold increase in NH_4Cl concentration caused a 30% increase in the maintenance coefficient, with the higher concentration of NH_4Cl being 75mM. However, the effect of the NH_4^+ ion in the regulation of the cell's metabolic processes cannot be ignored.

The fact that an increase in the total salts concentration from 0.12 M to 0.62 M only increased the maintenance coefficient of E. coli by 40% (Figure 24) suggests that although part of the maintenance requirement is due to salt, a large proportion, probably the majority, is used for processes unrelated to the salt effect. There is no way of telling whether the salt effect is a drain of energy simply by a sodium pump in the membrane for the maintenance of the correct ionic composition of the cell, or if the NaCl is stimulating an intercellular non-growth process. Addition of NaCl appeared to decrease the rate of protein turnover (p. 129) but this may have been because it interfered with transport of L-histidine, causing poor equilibration of intra- and extracellular pools.

Tempest & Meers (1968) found that increasing NaCl concentrations at $\mu = 0.3$ decreased the molar growth yield of A. aerogenes, but it is unclear if this was a change in Y_G or an increase in the maintenance

requirement. Some evidence was presented which suggested that high concentrations of NaCl interfere with the uptake of magnesium ions. This also may be a reason for the increased m for growth in high [NaCl] (Figure 24).

The increase in maintenance coefficient in the presence of high salt concentrations was not observed for growth on glycerol at 37°, although a slight increase was observed at 30° (Figure 24). The values of Y_G suggest that one coupling site of oxidative phosphorylation is lost when cells grow on glycerol at high salt concentrations at 37° (p.172). It may not be merely coincidental that the maintenance coefficient falls rather than rises in the presence of high salt concentrations (Figure 24). On the other hand, as Y_{GO_2} is greatly reduced by the presence of salt, the P/O ratio at 30° must fall, yet the maintenance coefficient rises in high concentrations of salt. Until the effect of high salt concentrations is studied for growth on other carbon sources, no firm conclusion can be made. It seems likely, however, that glycerol is a special case and, in general, unless NaCl affects metabolism, increasing the salt concentration will cause an increase in the maintenance coefficient. Rogers & Stewart (1974) found that yeast grown micro-aerobically had a lower maintenance coefficient than cells grown under fully aerobic conditions, and proposed that, as micro-aerobically grown cells do not possess the full complement of respiratory apparatus, part of the function of maintenance energy is to maintain the structural organisation of the respiratory chain. The decrease in the maintenance coefficient, together with the apparent loss of a coupling site for growth of E. coli on glycerol at 37° in 0.5 M NaCl (p.172) may be a manifestation of the same phenomenon.

4.2 Influence of growth temperature on maintenance energy and turnover

Several workers have suggested that turnover of macromolecules in growing cells may be responsible for the loss of energy described as maintenance. Turnover of cell walls is essential for the maintenance of viability of B. subtilis (Mauck et al., 1971) and P. chrysogenum (Righelato et al., 1968). The turnover of protein and RNA has been implicated in maintenance for other micro-organisms (Marr et al., 1963; Pirt, 1965; Stouthamer & Bettenhaussen, 1973 and 1975), although no direct proof has been obtained. Turnover of the lipid fraction is also known to occur in bacteria (Thompson, 1973). If any of these processes was independent of the growth rate of cells, then it would be expected that the energy necessary for them would be a part of the maintenance requirement.

The energy necessary for protein turnover seemed to be of the correct order of magnitude. Willetts (1965) showed that protein turnover in growing E. coli was 0.6% h^{-1}. From Figure 61, the amino acid content of cells is 47.85×10^{-4} mol amino acids/g cells. Thus $0.6 \times 47.85 \times 10^{-6}$ mol amino acids per hour are incorporated as a direct result of protein turnover in 1g cells. As 4 ATP are required for the incorporation of each amino acid into protein (Lucas - Lenard & Lipmann, 1971), the energy requirement for protein turnover is $4 \times 0.6 \times 47.85$ μmol ATP g^{-1} h^{-1} = 115 μmol g^{-1} h^{-1}. This compares with a maintenance requirement of ~1 mmol ATP g^{-1} h^{-1}. Protein turnover therefore seemed to be a likely candidate for one of the components of maintenance. The effect of growth temperature on maintenance has not been previously studied in any depth. Marr et al. (1963) suggested from arithmetic-type culture results that the temperature coefficient of E. coli was high, and Harrison & Loveless

(1971) proposed that their molar growth yields of E. coli at different temperatures were consistent with this observation. On the other hand, Marr et al. discussed results of Berger who found a low temperature coefficient for E. coli (6 kcal/mole) and suggested that this E_a was a consequence of maintenance being the leakage of intracellular pools across the cell membrane. Palumbo & Witter (1969) found a similar value for P. fluorescens (8.4 kcal/mole). The activation energies found for maintenance energy in E. coli (Figures 25-27) were an order of magnitude greater, at least for the component which increased enormously between 30° and 40°. Below 30°, the values seemed to be fairly constant and similar for different substrates, judging by $\underline{m}_{O_2}$ values (Figure 26). This finding has important consequences for the comparison of maintenance coefficients of the same and different organisms in Figure 69. If each organism has a different temperature dependence, or if the cells are grown at different tempertures, then direct comparison can not be valid.

The effect of 0.5 M sodium chloride on the maintenance coefficient for glucose was no greater at 40° than at 37° (Figure 24), suggesting that the increase in the maintenance coefficient between 37° and 40° was independent of the salt effect.

Protein turnover was measured at different temperatures in an attempt to correlate this and the maintenance coefficient. The turnover of individual enzymes and its relationship to temperature was investigated (Figure 32). The turnover rate of all of these enzymes at 37° was greater than overall rates of turnover measured in growing cells (Mandelstam, 1958; Willetts, 1967a; Pine, 1972). As each of the enzymes was redundant under the growth conditions used, it may be that their rate of breakdown was higher than would normally be

the case if the enzymes were necessary for the metabolism of the carbon source. Similar accelerated breakdown of non-functional protein has been observed (Goldberg & Dyce, 1974). It is possible that, when enzymes are unnecesssary or inactive, they join the fast-turnover component of protein turnover, which represents only ~5% of total cell protein (Willetts, 1967b; Nath & Koch, 1971; Pine 1973a). Thus, the decay of any of these enzymes is unlikely to be representative of protein turnover as a whole. In addition, each had a different relationship to temperature also found by Willetts (1967a) and Fensom & Pirt (1975), none of which was similar to the effect of temperature on the maintenance coefficient (Figure 25).

Most reported measurements of protein turnover have employed labelling of cells with a ^{14}C- or ^{3}H- labelled radioactive amino acid, followed by a chase during growth on a medium containing an excess of cold amino acid. Due to various theoretical difficulties (Mandelstam, 1963), it is unlikely that the absolute measurement of protein turnover is possible by this method. This deficiency has been recognised, and two recent publications describe methods which involve the labelling of protein by $^{3}H_2O$ and $H_2{}^{18}O$ (Humphrey & Davies, 1975; Fensom & Pirt, 1975) thus overcoming the problems of pool sizes, equilibration of pools etc. inherent in previous methods, and also the effect described in this thesis (Figure 40) of possible regulation of protein turnover by the cold amino acid necessary to chase out released labelled amino acid. Similar control of protein turnover by individual amino acids has been found by Pine in E. coli (1973b).

Despite these drawbacks, it was decided to use the convenient traditional method of protein turnover, reasoning that if conditions were kept the same at each temperature, then the measurements made

would be internally consistent, and so a valid determination of the relationship of protein turnover to temperature should be possible.

With histidine as label, the rates of turnover at 37° for glucose- and glycerol-growing cells were 2.28 and 3.01% h^{-1} (Figure 35) equivalent to an energy requirement in each case of 0.44 and 0.58 mmol ATP g^{-1} h^{-1}, compared with maintenance coefficients of 1.00 and 1.33 mmol g^{-1} h^{-1}. Comparison of maintenance coefficients and protein turnover rates (Figure 68) at different temperatures shows that protein turnover is not responsible for the large increase in maintenance coefficient at 40° relative to 37° (Figure 38). The Arrhenius activation energy constants for glucose-growing cells were 21 and 169 kcal/mole for protein turnover and maintenance energy, and the same constants for glycerol-growing cells were 27 and 78 kcal/mole (Figure 39). In addition, $\underline{m}$ (glycerol) is virtually the same at 30° and 25° (Figure 24) while the rate of protein turnover falls from 1.15% h^{-1} to 0.41% h^{-1} (Figure 37).

Thus, although the results show that the rate of protein turnover is independent of μ (Figure 35) as found by Willetts (1967b), it does not contribute greatly to the maintenance energy requirements, especially at the higher temperatures.

Turnover of cell walls has been found to be very small in E. coli (Mauck, Chan & Glaser, 1971; Higgins & Shockman, 1971). In contrast to these findings, cells in carbon-limited arithmetic-type culture lost labelled DAP at 3.90% h^{-1} (Figure 41). The Arrhenius activation energy for this process was again much smaller than the activation energy for maintenance energy (Figures 42 and 43) (9.2 kcal/mole compared with 78 kcal/mole) but of the same order as activation energies

of enzyme-catalysed reactions (Dixon & Webb, 1964).

Labelling of cells with $^{32}PO_4^{3-}$ would give labelling principally of nucleic acids and phospholipids, both of which are known to undergo extensive turnover. The rate of loss of ^{32}P from cells (Figure 44) was found to be of the same order as the rates of protein and cell wall turnover. No attempt was made to determine the source of the label released into the medium, but in any case the Arrhenius activation energy was too low (15 kcal/mole) (Figure 46) for the process to be identified with the large increase in maintenance energy between 30° and 40° (Figure 45).

The experiments which measured the loss of $^{14}CO_2$ from pre-labelled cells may be of little value. The reason for the apparent exponential decay to a constant value (Figure 22) is not clear. It is possible that two sources of $^{14}CO_2$ existed, one of which was metabolised quickly, and was not associated with maintenance, and the other which was associated with maintenance and turned over slowly. However, the poor quality of the results obtained when this turnover was related to growth temperature (Figure 47) do not permit any meaningful conclusions. A more informative method of tackling this problem would be to label cells uniformly, then follow the loss of label from isolated fractions of cells such as lipid, protein and nucleic acid, and to determine the effect of growth temperature on the loss of label from each of these classes of compound.

In summary, therefore, it can be said that although cell polymers do turn over during growth, the energy used by these processes does not have the same relationship to temperature as maintenance energy. This observation does not preclude the possibility that turnover of macromolecules is a maintenance process, but rather that, if it is, it

does not cause the large rise in the requirement for maintenance energy between 30^{o} and 40^{o}.

* 5. Assessment of viability from autoradiography

Arithmetic-type continuous culture is a good growth system for the study of maintenance. However, very little is known about the nature of cells grown in this way. The system is unusual in that the specific growth rate of the cells is extremely low, much lower than used in most chemostat culture, and the cells' physiological condition may be closer to that of starving cells than to cells growing at more usual specific growth rates.

It is widely accepted that starving cells have a protein turnover rate considerably greater than exponentially growing cells. It was found, both from the study of the decay of enzyme activities and from radiochemical labelling studies, that protein turnover in carbon-limited arithmetic-type continuous culture is considerably greater than in exponentially growing cultures (p.129). This is consistent with carbon-limited arithmetic-type cultures being similar to stationary cells. Investigation of storage carbohydrate and RNA content would also be expected to show levels similar to starving cells, if this were the case.

The main problem with this type of culture was expected to be with the "health" of the cells. The maintenance coefficients obtained were less than had been obtained for E. coli by other workers, and less than was obtained for chemostat culture, with higher values of μ, for the same organism (Figure 69). If only a fraction of the cells in arithmetic-type continuous culture was viable, then it is reasonable to suppose that only the same fraction would be consuming energy for

* See Addendum on p. 210.

maintenance. This might have accounted for the unusually low maintenance coefficients.

Viable counting is a measure of the viability of cells when transferred to the medium in which they are being counted. It is not a measure of the cells' metabolic activity in the growing culture (Postgate, 1969). A viable count of cells in arithmetic-type continuous culture would therefore give little information as to the metabolic activity of individual cells in the culture. It was decided that the best method of assessing viability was to determine the fraction of cells assimilating substrate in situ. This was done by making the carbon supply radioactive for a short time and determining the distribution of label among the cells by autoradiography. Grains were found to be evenly distributed among stained areas, which is good evidence that all of the cells in the culture were equally capable of incorporating the carbon source supplied (Plate 1) and so suggests that the maintenance coefficient measured is the maintenance requirement for all of the cells in the culture, and not only a fraction of them.

Autoradiography also showed that there are apparently two populations of cells within the culture. The vast majority take up, and incorporate substrate. For some unknown reason, they also lyse during the preparation of autoradiography slides. 1.2% of stained areas on the slides remained rod-like, however. Not a single grain was detected over these rods, which means that they were not incorporating substrate. It is possible that the reason they did not lyse during the preparation of autoradiography slides was that they were already leaky or punctured. If this was the case, incorporation of substrate would not be observed. However, the fact that none of the

rods was labelled does not eliminate the possibility that the cells were still catabolically active, despite their failure to anabolise.

Cells growing exponentially on glucose have a μ of 0.88 h^{-1} (Hamilton, 1972). The Y_G is 95g/mole. Thus, the rate of uptake of glucose is $\frac{0.88}{95}$ = 9.3 mmol g^{-1} h^{-1}. If 1.2% of the cells in arithmetic culture were catabolising at the same rate as in batch culture, the rate of glucose wastage would be 0.11 mmol g^{-1} h^{-1}, compared with the maintenance coefficient of 0.04 mmol g^{-1} h^{-1}. From this calculation it is obvious that, if this class of cell was catabolising substrate at a similar rate to the rate of catabolism in batch culture, then the energy drain would be of the same order as maintenance.

While such an explanation of maintenance seems mathematically feasible, it is difficult to explain why these non-incorporating cells should take up substrate at more than 10 × the rate at which the other cells in the culture take it up. It therefore seems unlikely that this class of cell is the cause of the drain of energy to "maintenance". If the cells were taking up substrate at the same rate as other cells in the culture, then the energy drain would be minimal compared with maintenance energy. Nevertheless, a similar labelling experiment has been done at 40° in order to find if the number of non-incorporating rods increases to the same extent as the maintenance coefficient. The results should soon be available.

6. Conclusions - maintenance energy

Carbon-limited arithmetic-type continuous culture proved to be a convenient, and fairly adaptable, method of measuring the maintenance coefficient. It was shown directly that addition of salt to E. coli

caused an increase in the maintenance requirement, but that the salt effect was only sufficiently large to account for a fraction of the energy used for maintenance. Indirect experiments, designed to show any correlation between temperature and maintenance or any of the processes likely to be responsible for maintenance, were not successful in proving any of the suggested candidates to be definite drains of maintenance energy. Although turnover of macromolecules is to some extent independent of μ, no proof of any kind was obtained for this being a major cause of maintenance energy, particularly at 37^{o} and 40^{o}.

One commonly proposed candidate for the utilisation of maintenance energy is motility (Weibull, 1960). While this must certainly be the case for highly motile organisms, as E. coli ML308 is non-motile, it is unlikely that any energy is used for that purpose in this organism. Marr et al. (1963) proposed that maintenance energy would be required to transport back into the cell solutes lost by diffusion through the cell membrane. However, the activation energy of the diffusion process (~5 kcal/mole) is quite different for the activation energy obtained for the maintenance coefficient.

It has been proposed earlier in this discussion (p.189) that a part of maintenance energy may be involved in the preservation of the respiratory apparatus. A reasonable, more general, postulate would be that energy is required for maintenance of the various degrees of organisation within the cell. Organisation in E. coli ranges from the molecular level to the overall structure of the cell. The individual protein units of multienzyme complexes such as the phosphotransferase system (Kundig & Roseman, 1971) and the pyruvate dehydrogenase complex (Koike, Reed & Carroll, 1963) are ordered in a way analogous to the respiratory apparatus, and presumably must require energy for the

maintenance of the organisation necessary for their controlled activity. The maintenance coefficient of A. chroococcum is very much larger when the cells use molecular nitrogen rather than ammonia as source of nitrogen (Dalton & Postage, 1969). The reason for this twenty-five fold increase is believed to be the protection of the nitrogenase complex, which is only necessary when cells fix N_2. On a larger scale, energy may be essential for the organisation of cell structure. An example of this might be the occurrence of the cell's genetic material as a distinct nuclear body, rather than a form more dispersed throughout the cytoplasm. In the same vein, an interesting suggestion has been made that, as cells growing at low μ spend a longer period undergoing division, this may have a connection with maintenance energy (Dawes & Ribbons, 1964). Energy is likely to be used in cell division. If the energy is required to produce an energised state, then it seems probable than the longer the cell spends in that state, the greater will be the energy requirement.

The idea that maintenance energy is used to organise the cell's constituent parts is an attractive one, particularly as the Arrhenius activation energy constants for maintenance (Figure 27) are of the same order of magnitude as those for denaturation (Dixon & Webb, 1964), rather than enzyme activity. Similar activation energies would be obtained for physical damage to cells. Regrowth is a widely accepted phenomenon which leads to "population turnover" in starving cells (Dawes & Ribbons, 1964). Growing cells can therefore use dead cells' macromolecules as nutrients, leading to no net formation of new cell material, but obviously requiring energy to synthesise new polymers. If cell death, and thus the rate of re-growth, is a result of physical damage, then population turnover might be responsible for an appreciable

proportion of maintenance energy. The main criticism of this theory is that such re-growth would not be distinguishable from cell turnover as measured by labelling experiments. It was found that turnover did not have the same relationship to temperature as maintenance (Figures 38, 42 and 45) so presumably re-growth can not be responsible for the majority of the maintenance coefficient.

Marr et al. (1963) considered that active transport may be one of the functions of maintenance energy, but found that addition of 1mM methylthiogalactoside (TMG) to the growth medium did not increase the maintenance coefficient of E. coli. TMG is a non-metabolisable β-galactoside, so Marr et al. reasoned that energy required to maintain the transmembrane concentration would increase the maintenance requirement. This finding is not surprising, however, as it has been found that energy is required only in the formation of the transmembrane gradient, but not in its maintenance (Holms, unpublished observations). It may be the case that energy may be required for the maintenance of other transmembrane gradients, particularly of K^+ and Na^+ ions.

Until now, consideration of the role played by turnover in maintenance has been confined to the turnover of macromolecules. Extensive turnover of acid-soluble pools is also known to occur, particularly of high-energy compounds. Turnover of the ATP pool in micro-organisms is rapid, complete turnover occurring within a matter of seconds (Forrest, 1965; Harrison & Maitra, 1969). Much of this turnover must be a consequence of the ATP utilised for growth being regenerated from ADP and AMP, and so will not contribute to maintenance energy, but it has also been suggested that a portion of the ATP formed is dissipated non-productively in order to regulate the energy charge.

Chance (1959) concluded that some of the ATP synthesised in yeast mitochondria was immediately dephosphorylated, perhaps by reversal of the ATP-synthesising system. The regeneration of ADP was proposed to be the reason for some ATP breakdown in Streptococcus faecalis (Rosenberger & Elsden, 1960). Lazdunski & Belaich (1972) predicted a similar ATPase function in regulation of the ATP pool in Zymomonas mobilis. The reversible nature of the membrane ATPase in vitro is well known (Evans, 1969). If the function of this ATPase in vivo is both for the synthesis of ATP at low intracellular ATP pool levels and for the formation of ADP at high ATP pool levels, then it must constitute a drain of maintenance energy. The size of the ATP pool in micro-organisms is constant, irrespective of the specific growth rate (Forrest, 1965; Harrison & Maitra, 1969) although uncoupling causes the ATP pool to rise (Forrest, 1965; Lazdunski & Belaich, 1972). Measurement of the rate of ATP pool turnover at different specific growth rates may show that part of the turnover is not associated with growth, in much the same way as measurement of molar growth yields at different values of μ showed that part of the substrate taken up is not used for growth.

A regulatory ATPase may not be the only enzyme which non-productively hydrolyses ATP, or other high energy compounds. The pair of ribosomal proteins L7 and L12 have a GTPase activity which can be uncoupled from ribosome function (Möller, 1974). Patterson & Gillespie (1972) found that initiation of polypeptide synthesis was particularly sensitive to a slightly elevated temperature in E. coli. A connection between this thermal sensitivity and the relationship of the maintenance coefficient to temperature is possible.

Complementary pairs of enzymes are also likely to contribute to non-productive ATP turnover. Glutamine synthetase forms glutamine from glutamate and NH_3, and glutaminase catalyses the hydrolysis of glutamine back to glutamate. If the synthesis, hydrolysis and re-synthesis of glutamine was cyclic, then the ATP used would not be contributing to growth, and so would be a drain of maintenance energy. Other similar pairs of enzymes might be phosphofructokinase and fructose diphosphatase, asparagine synthetase and asparaginase, and hexokinase and glucose-6-phosphatase. Indeed, the presence of alkaline phosphatase in E. coli may cause reversal of many kinase reactions. It would be of interest to compare maintenance coefficients of wild type and alkaline phosphatase negative mutants of E. coli.

Maintenance energy in growing cells and endogenous metabolism in starving cells may involve many of the same metabolic processes. The importance of endogenous metabolism in the survival of micro-organisms has been stressed (Mallette, 1963). Whether maintenance energy in growing cells is a necessary inefficiency so that micro-organisms can immediately produce energy by endogenous metabolism on depletion of nutrients, or whether maintenance energy provides a function essential for growth is uncertain. More detailed knowledge of the processes involved in maintenance should provide a starting point in an attempt to answer this question. The possibility that a "maintenance-less" mutant could be selected for, and its survival characteristics compared with the wild type, is an interesting idea.

The final conclusion must be that, although arithmetic-type continuous culture has enabled precise measurement of maintenance coefficients of E. coli, the identification of the cellular reactions utilising maintenance energy is far from being complete.

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ADDENDUM

Autoradiography

(see pages 117, 196 and 197)

Experiments subsequent to those reported on pp. 115-117 showed that unlabelled rod-shaped bacteria present in autoradiographs (Plate 1) were not derived from arithmetic-type cultures. When photographic emulsion was applied to a blank slide, the rod-shaped bacteria were again visible after developing. Hence the intact bacteria in the earlier autoradiographs were not derived from the culture, but from the emulsion. Discussion of the possible role of these cells in maintenance (p 197) is therefore irrelevant.