Control of L-Malate Metabolism in *Escherichia coli* ML308 (ATCC 15224)

by

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List of abbreviations

The following abbreviations are used:

BCIG	5-bromo-4-chloro-indoxyl β galactoside
BSA	bovine serum albumin
IPTG	iso-propyl thiogalactoside
malate	L-malic acid
OAA	oxaloacetic acid
PCA	perchloric acid
PEP	phosphoenolpyruvate
P.S.I.	pounds per square inch
RQ	respiratory quotient
TCA	tricarboxylic acid
U	enzyme unit
Y_{ATP}	yield per mole ATP
Y_G	maximum growth yield
Y_{O_2}	yield per mole oxygen
$Y_{substrate}$	yield per mole substrate

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SUMMARY

Escherichia coli ML308 (ATCC 15224) grows aerobically on L-malate as a sole source of carbon and energy in a medium otherwise containing only inorganic ions. Cells grow immediately on L-malate if previously grown on other dicarboxylic acids, some amino acids or α-ketoglutaric acid but lag before growth on L-malate after growth on glucose, glycerol, pyruvate or acetate. Limitation of growth rate must be due to a factor which is present in cells during growth on the first but not on the second group of carbon sources. The three factors considered were PEP carboxy kinase, malic enzymes and the L-malate transport system.

Acetate stimulates growth rate on L-malate in all circumstances and must, therefore, increase the rate of production of all intermediates. In particular the rate of supply of phosphoenol-pyruvate (PEP) for gluconeogenesis etc. must be increased. Because PEP is made from L-malate by PEP carboxy kinase it follows that this enzyme cannot limit rate of growth on L-malate alone.

The relationship between activities of malic enzymes and growth rate on L-malate was measured directly. Different phenotypes, able to metabolise L-malate at various rates, were found to contain more malic enzyme activity than was expressed during growth on L-malate. Furthermore, malic enzyme activities during adaptation to L-malate do not alter. Consequently, growth rate on L-malate cannot be limited by malic enzyme activities.

Malonate has been reported as a gratuitous inducer of the L-malate transport system. Its presence causes adaptation to L-malate during growth on glycerol, suggesting that transport activity is related to the rate of metabolism of L-malate. The activity of the L-malate transport system, as measured by the rate of pyruvate production, in arsenite inhibited whole cells, increases with adaptation so confirming that the rate of metabolism of L-malate is limited by its transport. The regulation of central metabolism and the characteristics of the enzymes metabolising L-malate are also consistent with the transport mechanism being the process which limits metabolism.

Degree of adaptation was measured as a range of growth rates with corresponding rates of oxygen uptake on L-malate. The relationship between the two permits a maintenance coefficient and Y_G value to be calculated for L-malate growth. Analogous values are also given for growth on acetate. The full interpretation of these data depends on assumptions as to the efficiency (P/O ratio) of oxidative phosphorylation. It seems probable that P/O ratios and maintenance vary with the growth substrate and that when a lower efficiency of oxidative phosphorylation is obtained, a higher level of maintenance is required.

Molar growth yields (as $Y_{c\text{-source}}$ and Y_{O_2}) reflect both P/O ratios and the whole mechanism of biosynthesis. Consideration of the apparent Y_{ATP} for different carbon sources strongly suggests that the P/O ratio varies and depends on the carbon source available to the cells. It is not surprising that the highest P/O ratios are obtained with those carbon sources which support the highest growth rates.

INTRODUCTION

1. Aerobic Growth on a Single Carbon Source

A number of microorganisms including Escherichia coli show considerable nutritional versatility. They are able to grow aerobically in simple salts media with only a single carbon source present as well as in a variety of more complex environments. When growing under aerobic conditions, in a simple salts/single carbon source medium, the cell must overcome the problem of generating both a supply of energy and of intermediates for the synthesis of new cell material from the single carbon source. Both requirements are fulfilled by feeding the carbon source into the amphibolic routes of the cell. During aerobic growth on a single carbon source the amphibolic routes may be considered as glycolysis, the pentose phosphate pathway and the TCA cycle and serve the dual function of supplying the cell with energy from the oxidation of some of the carbon source while the remainder is used to synthesise small molecular weight molecules which the cell can polymerise into macromolecules. It is the end result of all these functions which is observed as the phenomenon of growth.

In all, the amphibolic routes, as defined above, contain 27 compounds of which 11 are used by the cell for biosynthesis. The operation of the amphibolic and various anaplerotic routes ensures that a supply of these 11 compounds is maintained under all nutritional conditions. Since these 11 compounds form the basis of all the

biosynthetic carbon metabolism in the cell, all cellular metabolism from the amphibolic routes is the same irrespective as to the nature of the carbon source used to support growth. Consequently growth can be represented on a simple diagram (Figure I).

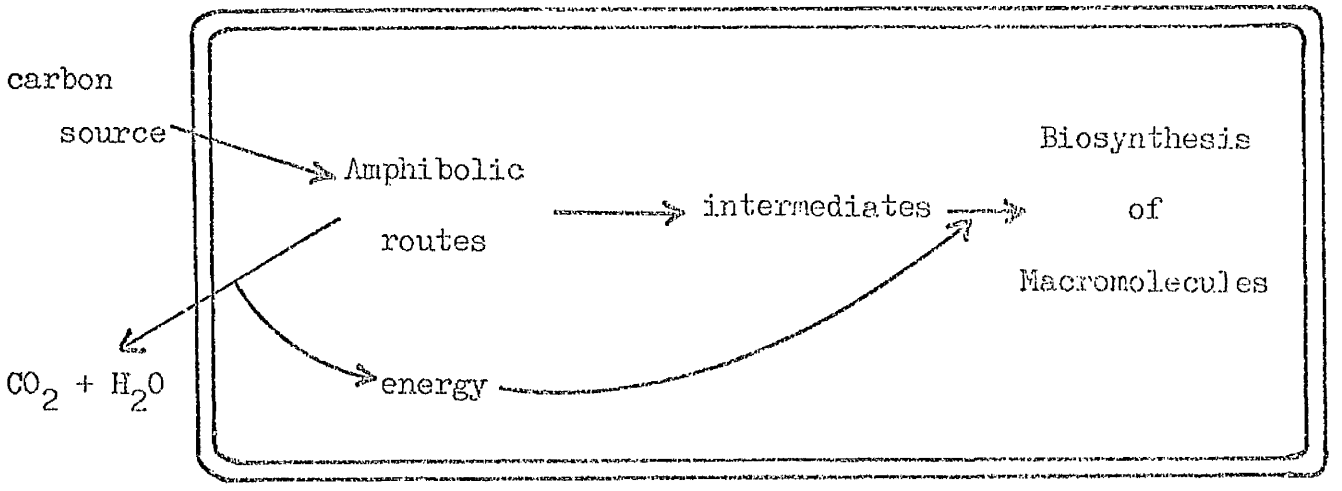


Figure I

The nutritional versatility of the cell depends on the number of carbon sources which can be metabolised to compounds on the amphibolic routes. Many more compounds can be used by the cell as sole carbon sources than are used to synthesise intermediates for biosynthesis. We have shown that E.coli 15224 can grow on at least 58 carbon sources under aerobic conditions in simple salts medium, a number which does not represent the result of an exhaustive survey and many more carbon sources which support growth will exist. To metabolise all these carbon sources and make use of them as growth substrates the cell must be able to synthesise a large number of enzymes, some of which may

only be required to metabolise a single compound.

In view of the complexity required to enable the cell to utilise all these carbon sources and to balance energy production with synthesis of new cell material, it would not be surprising if the cell only incorporated a small proportion of the carbon source carbon into cell material. In fact the cell converts 55-65% of the carbon source into cell material (Siegel & Clifton, 1950). It might also be expected that growth on different carbon sources would lead to the excretion of waste products of cellular metabolism but, except under exceptional circumstances, no more than trace amounts of waste products are excreted (Roberts et al., 1955) and the only quantitatively significant end products of metabolism are cellular material, carbon dioxide and water. The cell makes efficient use of carbon source under all conditions and does so because of the operation of control processes in metabolism.

That control must exist had been known for some time but it is only in the last 15-20 years that real advances have been made in the understanding of control processes and of the molecular mechanisms by which they operate.

Control may be achieved in 3 ways:

a) by controlling the rate of enzyme synthesis.

b) by controlling enzyme activity.

c) by regulation of the supply of carbon source.

Although c) may be regarded as the result of b) it is considered separately because its site of action is markedly different.

In the next section I describe some of the systems whose control has been analysed. I do not however intend to present a comprehensive review but only to survey the types of control which have been observed and, where known, of the mechanisms by which control is achieved at the molecular level.

2. Control of Protein Synthesis

The rate of operation of a metabolic sequence depends directly on the activity of the enzymes of the sequence. One way in which activity can be altered is to control the quantity of enzyme present by regulation of the rate of enzyme synthesis, a mechanism which has been shown to regulate many processes, particularly in catabolic and anabolic sequences.

2.1. Control in the lac operon of E.coli

The lac operon of E.coli has been examined in greater detail than any other regulatory system. The products of the lac operon are β-galactosidase and β-galactoside permease, which are required for the metabolism of lactose, and thiogalactoside transacetylase whose function is unknown. The enzymes are synthesised by the cell when inducer is present in the growth medium (Monod & Cohen, 1952), are always synthesised coordinately (Jacob & Monod, 1961) and as a result of genetic mapping have been shown to be coded for by a single region of the E.coli genome (Jacob & Monod, 1965) so providing support for the operon (Jacob & Monod, 1961), as the controlling element for the synthesis of the lactose metabolising enzymes in E.coli. Considerable additional support for this hypothesis has been obtained over the years and, with some modification, it is accepted today. The control of the lac operon has been reviewed by a number of authors (Beckwith, 1967; Richmond, 1968; Beckwith, 1971).

In the model proposed by Jacob and Monod (1961) the enzymes of the operon are coded for by structural genes in the order shown

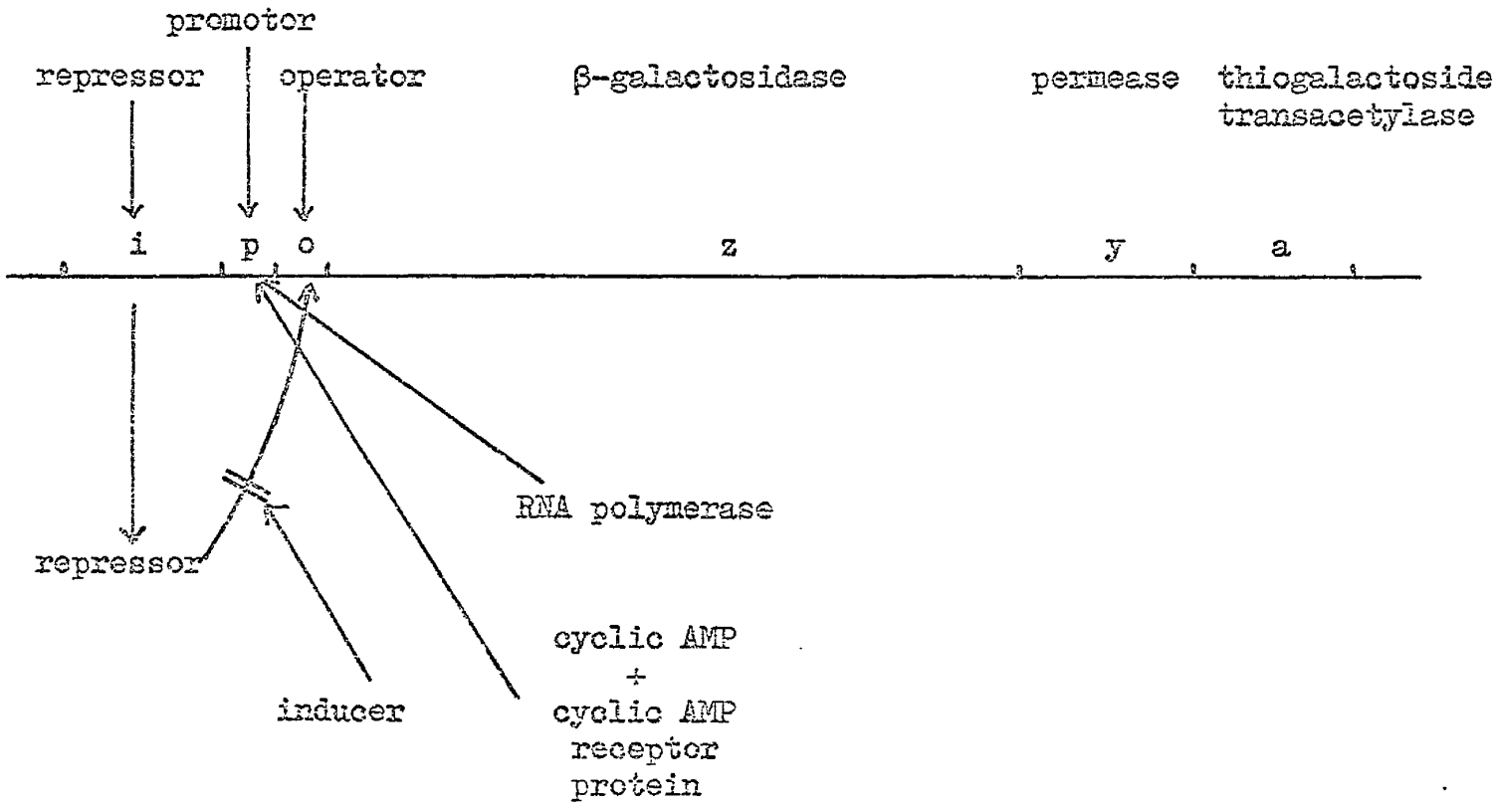


Figure II The lac Operon

(Figure II) and their synthesis is controlled by the operator region. The operator region can exist in two states - one, in which a repressor molecule is bound to the operator and a second, when the operator is free. In the latter case RNA polymerase, which is bound at the promotor site (Chen et al., 1969), can transcribe the lac operon producing lac mRNA and so specify synthesis of the lac enzymes. When the repressor molecule is bound to the operator no transcription can take place. The repressor is synthesised using the i gene as a template and is a protein (Riggs & Bourgeois, 1968) which binds to operator DNA (Riggs et al., 1968). The addition of an inducer such as IPTG to the system results in an interaction between IPTG and the repressor molecule which removes the repressor from the operator region of the DNA (Gilbert & Müller-Hill, 1967; Riggs et al., 1968) allowing transcription to take place. Control at the level of transcription has been demonstrated (Varmus et al., 1970).

Removal of repressor from the operator region is not in itself sufficient to permit transcription but requires an additional interaction with cyclic AMP mediated by cyclic AMP receptor protein (Pastan and Perlman, 1970) which regulates the rate at which RNA polymerase initiates new mRNA synthesis and consequently the rate of synthesis of the enzymes. Catabolite repression (Magasanik, 1961), which is measured as a reduction in the rate of specific enzyme synthesis compared to total protein synthesis, has been explained as a result of the interaction of cyclic AMP with RNA polymerase as described above (Perlman et al., 1969; Silverstone et al., 1969; Jacquet & Kepes, 1969).

The regulation of enzyme synthesis in the lac operon has now been demonstrated in a cell-free system using purified components (de Crombrugghe et al., 1971b) and is the same as the regulation observed in vivo (de Crombrugghe et al., 1971a). Using the cell-free system it was demonstrated that lac mRNA synthesis can be completely controlled, using lac DNA as a template, by lac repressor, RNA polymerase, and cyclic AMP receptor protein (de Crombrugghe et al., 1971b). These authors contend that RNA polymerase synthesises lac mRNA and the initiation of transcription is controlled by binding of either lac repressor to the operator region or cyclic AMP receptor protein to the promotor region. The action of these two latter proteins depends on their interaction with small molecular weight effector molecules (inducer and cyclic AMP respectively).

The lac operon is therefore controlled at the molecular level by the interaction of macromolecules on the DNA template at the operator/promotor site under the influence of intermediates present in the cell.

2.2. Control in the gal operon of E.coli

The gal operon of E.coli has been studied in a similar manner to the lac operon but not in such detail. The enzymes required for the metabolism of galactose are coded for by a single operon (Buttin, 1963a) and are induced by either galactose or fucose (Buttin, 1963b). The sequence of enzymes on the operon (Michaelis & Starlinger, 1967; Shapiro & Adhya, 1969) and the stoichiometry of their synthesis (Wilson & Hogness, 1969) have been studied and are similar in principle to the lac operon. Synthesis is susceptible to catabolite repression

(de Crombrugghe et al., 1969) which can be overcome by addition of cyclic AMP to the culture (Pastan & Perlman, 1970).

The operon has been studied in a cell-free system in less detail than the lac operon, but it has been shown that the rate of synthesis of galactokinase is regulated by cyclic AMP and cyclic AMP receptor protein (Parks et al., 1971) and that control is at the level of transcription (Miller et al., 1971).

Differences do occur between the lac and gal systems with respect to quantitative requirements for cyclic AMP and cyclic AMP receptor protein. Parks et al. (1971) have shown that synthesis from the gal operon continues to a significant extent in the absence of cyclic AMP and cyclic AMP receptor protein. During isolation gal DNA is not entirely separated from phage genes and transcription from the gal operon in the absence of cyclic AMP and cyclic AMP receptor protein may be due to read through into the gal operon from these genes. Parks et al. (1971) have further observed that while guanosine 3'diphosphate 5'diphosphate stimulates lac mRNA synthesis, it inhibits gal mRNA synthesis. Despite these differences it has been concluded that control of the gal operon is very similar to the lac operon (Miller et al., 1971).

Although only these two operons for catabolic enzymes have been studied in detail, a large number of enzyme systems in many micro-organisms have been shown to be subject to induction control, apparently similar in type to the lac operon. Some have been shown to be sensitive to catabolite repression - the repression being overcome

by addition of cyclic AMP to the culture (de Crombrugghe et al., 1969). As a result of these investigations it was proposed that the synthesis of many inducible, catabolic enzyme systems requires cyclic AMP which implies, in view of the more recent cell-free studies, that many catabolic operons require cyclic AMP and cyclic AMP receptor protein to initiate mRNA synthesis.

2.3. Control in the ara operon of E.coli

The ara operon which codes for the enzymes of arabinose degradation, does not resemble the lac operon model in the regulation of enzyme synthesis. The 3 enzymes required for metabolism of arabinose in E.coli are coded for by a single operon whose activity is dependent on the product of another part of the genome, ara C (Gross & Engelsberg, 1959). The operon is induced by L-arabinose and induction is inhibited by D-fucose (Schleif, 1969).

Based on several lines of evidence Engelsberg et al. (1965) proposed that the regulation of the ara operon was under positive control by the product of the ara C gene. Positive control was supported by an analysis of merodiploids in the ara operon (Sheppard & Engelsberg, 1967). Engelsberg et al. (1969) have presented further evidence to show that, in addition to acting as an activator for enzyme synthesis, the ara C gene product, in the absence of L-arabinose, acts as a repressor at a site on the operon distinct from its site of action as an activator and have proposed a model to explain the observed regulation (Figure III). In the model the product of the ara C gene, P1, represses the transcription of the operon by acting at the O region.

The presence of L-arabinose, the inducer, converts the repressor to another form, P2, which acts at the I region as an activator of operon transcription.

Although this system is markedly different from the lac system it is sensitive to catabolite repression which is relieved by cyclic AMP (Katz & Engelsberg, 1971).

The system has been examined in cell-free preparations where arabinose specifically induces the operon and transcription is stimulated by cyclic AMP (Zubay et al., 1971). As with the lac system, synthesis is stimulated by guanosine 3' diphosphate 5' diphosphate.

The operon was examined in greater detail using a second cell-free system (Greenblatt & Schleif, 1971) and has confirmed the model of Engelsberg et al. (1969) for the regulatory pattern observed in vivo. Greenblatt and Schleif showed that the ara C gene product acts as both a positive and a negative controlling element. Arabinose stimulates while D-fucose inhibits enzyme synthesis.

The arabinose system therefore represents an operon whose control is different from the lac and gal systems but which has features in common with these systems. Enzyme synthesis is matched to the requirements of the cell and is controlled by the interaction of regulatory macromolecules with operator DNA, dependent on the presence of small molecular weight molecules in the cell.

2.4. Control of enzyme synthesis in biosynthetic pathways

The specific activity of many biosynthetic enzymes in cells depends on the nature of the environment (Gale, 1943). In these cases

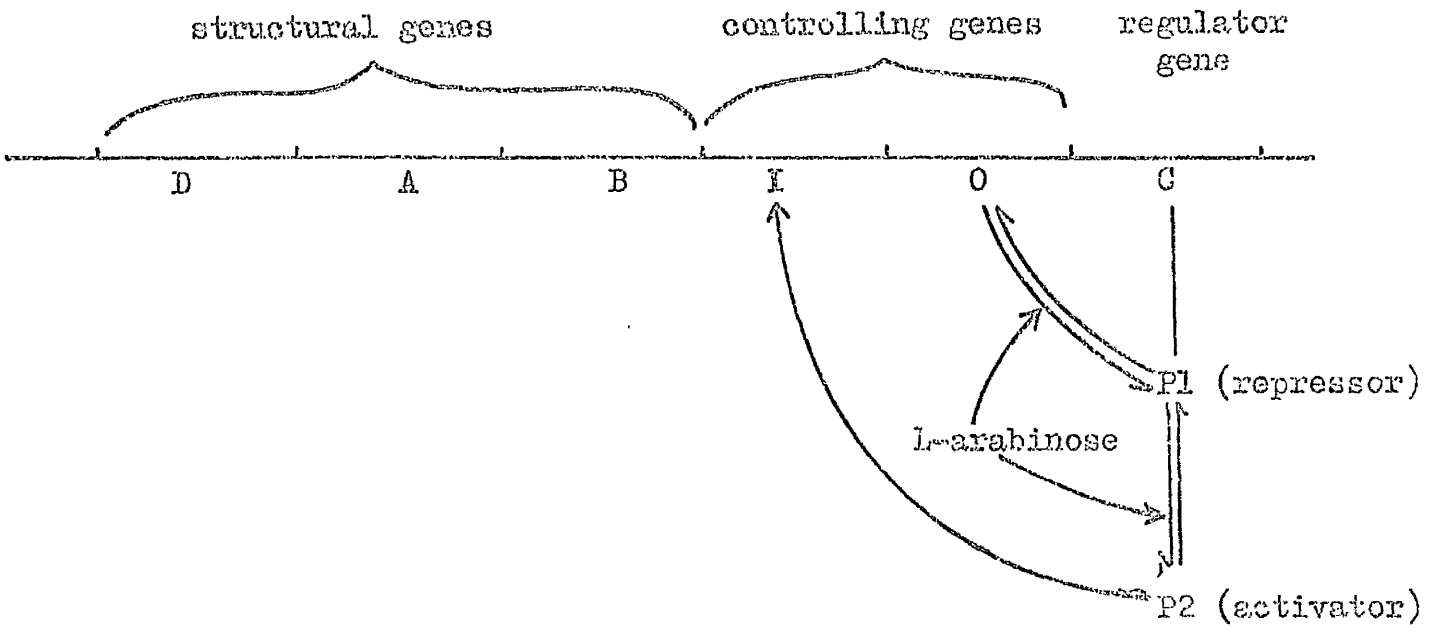


Figure III The ara Operon

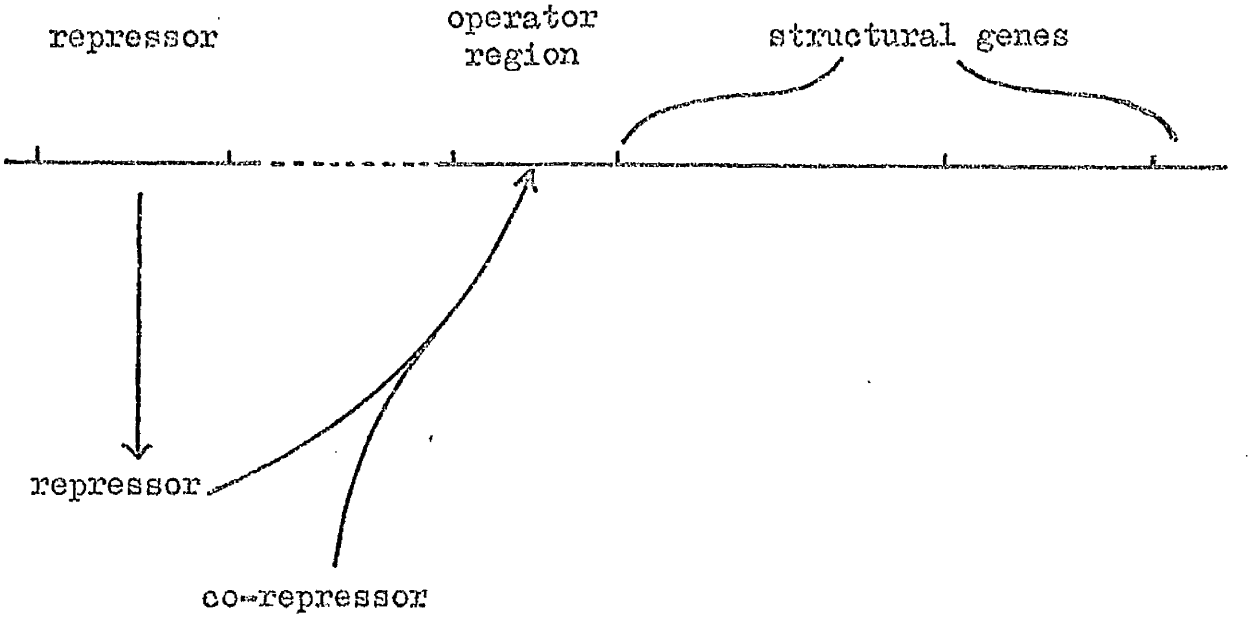


Figure IV A Biosynthetic Operon

the control mechanism is required to fulfil a quite different function from that in catabolic pathways and its mechanism has been selected accordingly. Repression operates only in the presence of the end product of a biosynthetic sequence or of a compound closely related to the end product. It has been shown, for example, that in the presence of tryptophan in the environment the enzymes required for tryptophan biosynthesis are not synthesised (Ames et al., 1967).

An operon model can be constructed which, in general terms, explains the regulation of the synthesis of these enzymes (Figure IV). In this model the regulator gene codes for a protein which has no affinity for the operator region and so does not block mRNA and subsequent enzyme synthesis. The corepressor, which may be the end product of the reaction sequence for which the operon codes, interacts with the repressor molecule and alters its structure, so that its affinity for the operator region increases and it now blocks mRNA and thence enzyme synthesis. Although this model can explain regulation in general terms, it has not been found to apply to any particular system. Umbarger (1969) has observed, with particular reference to amino acid biosynthetic pathways, that whereas the control function is general the mechanism by which it is achieved is not. Indeed each system may be unique in molecular terms. The simplest type, where single feedback repression regulates the synthesis of all the enzymes of a biosynthetic sequence has been observed in the regulation of leucine biosynthesis in Salmonella typhimurium (Burns et al., 1966). Much more complex is the repression of the branched chain amino acid

biosynthetic enzymes in E.coli (Freudlich et al., 1962) where the presence of all the branched chain amino acids are necessary for complete repression. Multiple enzymes which catalyse the same reaction are another complicating factor and in the synthesis of amino acids from aspartate in E.coli 3 aspartokinases exist, each regulated by feedback repression control by a different end product (Truffa-Bachi & Cohen, 1968). There are therefore no apparent restrictions on the patterns of regulation employed by cells.

Umbarger (1969) has considered many of these pathways and one of the few common elements to emerge from the study of regulatory mutants is the role of amino acyl tRNA as the corepressor for many amino acid biosynthetic pathways. In the regulation of the histidine operon several genes, unlinked on the genome, were shown to influence the synthesis of the histidine biosynthetic enzymes (Roth et al., 1966; Anton, 1968) which suggested a much more complex regulatory mechanism than had previously been observed but it has since been shown that many of these mutations are associated with the synthesis of his tRNA and not the synthesis of the histidine biosynthetic enzymes (Roth & Ames, 1966; Silbert et al., 1966). One exception appears to fulfil the role of a regulator protein analogous to the i region in the lac operon. Similar data from other systems (Yaniv & Gros, 1966; Nazario et al., 1971) support the role of amino acyl tRNAs as the corepressors that regulate enzyme synthesis. Hirshfield et al. (1968) have shown, however, that in the arginine biosynthetic operon in E.coli the bulk of the arginine specific amino acyl tRNA is not involved in the repression of the

arginine biosynthetic enzymes. There is, therefore, evidence both for and against the role of amino acyl tRNA's as corepressors in different systems and it is unlikely that the nature of the corepressors in these systems will be finally established until cell-free systems are developed.

The regulation of the synthesis of enzymes in biosynthetic pathways may be even more complex since there have been several reports that the presence of amino acid mixtures in the medium can lead to an alteration in the rate of synthesis of the enzymes required for the biosynthesis of amino acids not present in the mixture (Lee et al., 1966; Nester, 1968; Lavalle & De Hauwer, 1970; Carsiotis et al., 1970; Stubbs & Stubbs, 1971). There may in some of these cases be an element of positive as well as negative control concerned in the regulation of biosynthetic enzyme synthesis.

The analysis of regulation in these systems is, however, not nearly so far advanced as in the lac and gal systems and indeed the level at which regulation is expressed has not been ascertained. Roth et al. (1966) have suggested that control could occur at the level of translation. Although this is not widely supported there is some evidence from other systems to suggest that regulation at the level of translation can occur (Perlman & Pastan, 1969; Yudkin & Moses, 1969). The data depend on the inhibition of mRNA synthesis which is difficult to guarantee as 100% effective. There is however no evidence to suggest that regulation at the level of translation does not play a role in cellular regulation.

3. Regulation of Enzyme Activity

Alterations to the activity of an enzyme is another mechanism used by cells to regulate metabolism. Enzyme activity once present in bacterial cells is not rapidly degraded - protein turnover being of the order of 5%/h (Mandelstam, 1963) - but is diluted out by subsequent cellular growth. Consequently when enzyme synthesis ceases in response to a change in environment, either by removal of inducer or addition of corepressor, enzyme specific activity decreases slowly which could lead to a wasteful use of nutrient unless a mechanism to regulate enzyme activity is present. Regulation is also necessary to maintain balanced levels of intermediates during growth and Atkinson (1969) has observed that if the concentration of intermediates in the cells are measured during growth, the stability of cellular metabolite pools will reflect not the absence but the sensitivity of the cellular control processes.

3.1. Regulation of biosynthetic pathways

Biosynthetic sequences have provided many examples of the regulation of enzyme activity and it was with just such pathways that the first examples of inhibition of enzyme activity by end products of metabolism were observed (Umbarger, 1956; Yates & Pardee, 1956). As with repression many different control mechanisms have been evolved and each regulatory system may be unique in itself. Many reviews have been published on the nature and mechanisms of these control processes (Stadtman, 1966; Atkinson, 1969; Shapiro & Stadtman, 1970; Calvo & Fink, 1971).

3.2. General control of central metabolism

Although metabolic sequences, particularly those leading from the central metabolic or amphibolic pathways have been examined in some detail, the actual regulation of the amphibolic pathways has not received so much attention.

One mechanism by which regulation of the amphibolic pathways can be achieved is by changes in the energy charge in the cell. The concept of the energy charge as a control function has been developed by Atkinson (1966) whose original data applied to the energy metabolism of mammalian cells. Phosphofructokinase in glycolysis (Shen et al., 1968) and isocitrate dehydrogenase in the TCA cycle (Atkinson, 1968) have been shown to be susceptible to regulation by energy charge. Although most of the data come from mammalian systems, bacterial systems contain enzymes which are susceptible to energy charge such as phosphoribosyl pyrophosphate synthetase (Atkinson & Fall , 1967) and citrate synthase (Jangaard et al., 1968) in E.coli. Atkinson (1969) has proposed that energy charge has a control function not only in energy metabolism but also in metabolism in general.

Based on the susceptibility of mammalian, yeast and E.coli citrate synthases to energy charge regulation (Jangaard et al., 1968; Parvin & Atkinson, 1968), Atkinson has suggested that the TCA cycle is regulated by energy charge in these systems. However direct measurement of energy charge in E.coli (Lowry et al., 1971) has shown that, under a variety of growth conditions and growth rates, the energy charge is very stable. Lowry et al. (1971) conclude that large changes

in energy charge are not responsible for metabolic regulation but, that energy charge places the cellular regulatory systems in a position where they are sensitive to changes in other metabolites. In E.coli it is found that NADH also inhibits citrate synthase (Weitzman, 1966) malate dehydrogenase (Sanwal, 1969) and several other enzymes (Sanwal, 1970). Based on these enzyme inhibitions and changes in the intracellular NADH concentration, Sanwal (1970) has proposed that the TCA cycle in E.coli is regulated by the NADH concentration in the cell.

3.3. Regulation by catabolite inhibition

Catabolite inhibition is similar to feedback inhibition but has as its site of action either the transport systems in the cell membrane or the first enzyme of subsequent metabolism (McGinnis & Paigen, 1969). It involves the regulation of the initial steps in one metabolic system by the presence of other nutrients in the medium. Examples in E.coli include the inhibition of lactose utilisation by glucose (McGinnis & Paigen, 1969), the inhibition of maltose and galactoside uptake by glucose (McKinstry & Koch, 1972) and the inhibition of glycerol utilisation by glucose (Edgar et al., 1972). Quite unrelated metabolic systems might similarly interact. Evans et al. (1942) reported that sugars, particularly glucose, inhibit either tryptophan uptake or metabolism in E.coli and hydrogen reduces the utilisation of fructose in a Hydrogenomonas sp. (Blackkolb & Schlegel, 1968). Other examples of catabolite inhibition are listed by Paigen & Williams (1970).

4. Malate Metabolism

The term amphibolic pathways (Davis, 1961) describes the central metabolic routes which fulfil both anabolic and catabolic functions in the cell. Glycolysis, the pentose phosphate pathway and the TCA cycle which form the core of the amphibolic pathways in the cell, are required to different extents under different growth conditions. Regulation has been much more difficult to analyse in this region partially because it is difficult, if not impossible, to obtain cells in a condition where a particular function is absent and partially because of the complexity of the regulatory patterns involved. Despite these disadvantages some progress has been made in the understanding of the regulation of central metabolism.

Malate is used by E.coli as a sole carbon and energy source for growth. It is also a central metabolite of the cell, being involved in several metabolic functions. Malate is a component of the TCA cycle (Krebs & Johnson, 1937) and after transport across the cell membrane is metabolised by the enzymes of the cycle to generate a supply of energy (Ajl, 1958; Kornberg, 1959). malate is also involved in the inter-conversions of C_3 and C_4 compounds in the cell during growth on glucose or acetate (Kornberg, 1965) and is required after oxidation to OAA for synthesis of aspartate and of the aspartate family of amino acids. The regulation of growth on compounds like malate which are directly involved in the amphibolic pathways of the cell has not been rigorously examined despite an understanding of the intracellular metabolism.

In this section I deal specifically with malate metabolism in E.coli and all data refer to that organism unless otherwise stated.

4.1. Transport of malate into the cell

The first evidence for the involvement of a transport system for the metabolism of dicarboxylic acids in E.coli was obtained from the study of the ability of cells to oxidise succinate after previous growth in different media. Halpern et al. (1964) found that, while extracts of glucose grown E.coli contained 35% of the respiratory activity of extracts of succinate grown cells to oxidise succinate, glucose grown whole cells possessed only 9% of the respiratory activity on succinate compared to succinate grown whole cells. The difference they ascribed to a permeability barrier to succinate in glucose grown cells. Their conclusion was supported by Takahashi & Hino (1968a) who showed that aeration of anaerobically grown cells in complex medium resulted in the cells acquiring the ability to oxidise both malate and fumarate without altering the oxidative ability of cell extracts towards these substrates.

The involvement of a single specific system in the transport of dicarboxylic acids was demonstrated by studying mutants of E.coli which were resistant to growth inhibition by 3 fluoromalate (Kay & Kornberg, 1969). Resistant cells are unable to utilise exogenous dicarboxylic acids although they contain all the necessary enzymes and can metabolise endogenous compounds. Further evidence to support the single transport system for dicarboxylic acids was provided by the observation that some cells, with an impaired ability to utilise dicarboxylic acids contain a low level of the transport system (Herbert & Guest, 1971).

4.2. Energy production from malate

The bulk of the energy supply in E.coli under aerobic conditions is produced by operation of the TCA cycle (Krebs & Kornberg, 1957). During growth on malate the TCA cycle and two ancillary reactions are used to produce energy. The substrates required by the cycle are OAA and acetyl CoA. OAA is produced from malate by malate dehydrogenase (E.C. 1.1.1.37) using NAD as a cofactor (Murphey & Kitto, 1969). Acetyl CoA is the product of two decarboxylation reactions. The first is catalysed by malic enzyme (E.C. 1.1.1.40 and E.C. 1.1.1.38) which decarboxylates malate to pyruvate and generates a molecule of reduced nicotinamide cofactor (Katsuki et al., 1967). Pyruvate is subsequently decarboxylated by the pyruvate dehydrogenase complex (E.C. 1.2.4.1) to acetyl CoA - a reaction which produces a further molecule of reduced cofactor (Reed & Willms, 1966).

Acetyl CoA and OAA condense under the action of condensing enzyme or citrate synthase (E.C. 4.1.3.7) to yield citrate (Weitzman, 1969) which is metabolised in the TCA cycle by rearrangement, decarboxylation and oxidation reactions to regenerate one molecule of malate from the two initially metabolised. Reduced cofactors generated during these reactions are reoxidised by electron transport coupled to oxidative phosphorylation to provide the cell with its energy supply (Kornberg, 1959).

Pyruvate carboxylase (E.C. 6.4.1.1.) which can also catalyse the production of pyruvate from OAA has not been detected in E.coli (Kornberg, 1965).

4.3. Intermediates from the TCA cycle

The operation of the TCA cycle is not exclusive to energy generation but also supplies intermediates for biosynthesis. OAA, αketoglutarate, and succinyl CoA are all used to synthesise amino acids, nucleotides and porphyrins. Pyruvate and acetyl CoA are also used for biosynthesis.

4.4. PEP production

During growth on malate the cells require to synthesise PEP from malate using PEP carboxykinase (E.C. 4.1.1.49) (Kornberg, 1966; Hsie & Rickenberg, 1966). The enzyme requires ATP as a source of the phosphate group of PEP (Utter & Kurahashi, 1954). A second enzyme PEP carboxylase (E.C. 4.1.1.31) which interconverts PEP and OAA (Bandurski & Greiner, 1953) does not act in the direction of PEP synthesis (Kornberg, 1965).

PEP is used to synthesise sugars by gluconeogenesis and operation of the pentose phosphate pathway and acts as a substrate for amino acid biosynthesis.

A modification to Figure I can be constructed to describe growth of the cells on malate as a sole carbon and energy source (Figure V).

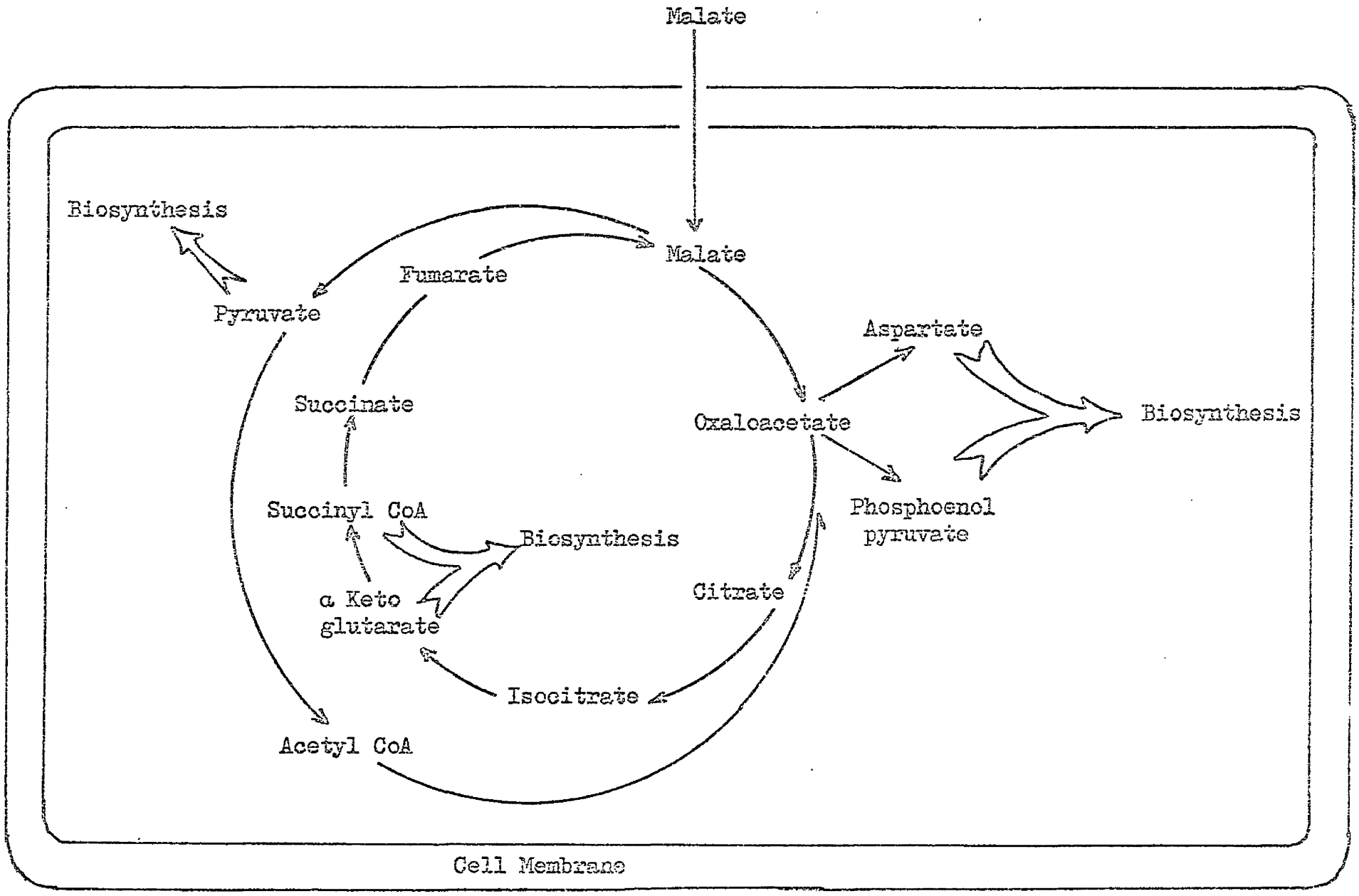


Figure V Growth of E.coli on Malate/Salts Medium

5. Control of Malate Metabolism

As with growth on any medium, growth on malate is regulated by cellular control processes. The enzymes involved in malate metabolism are subject to metabolic regulation but have not been collectively considered under conditions of growth on malate. The metabolism of this region involving malate has been examined in detail during growth on glucose and, to a lesser extent, on acetate and an overall picture of the metabolic controls as they operate under these conditions considered (Kornberg, 1965). Some aspects of the control of this region of metabolism have also been considered by Sanwal (1970a) while reviewing control of central metabolic pathways in general. At no time has the role of transport in malate metabolism been considered with regard to metabolic regulation.

The control in this region is complex and has been investigated by two major methods - the metabolic approach of Kornberg's group and the enzymological approach of the groups of Sanwal and Katsuki. In the next few sections the control of each phase of malate metabolism is considered.

5.1. Control of malate transport

The characterisation of the dicarboxylic acid transport system as a distinct protein was first reported by Kay and Kornberg (1969). By genetic analysis of mutants they showed that a protein required for transport is coded for by a region of the genome which is distinct from the genes coding for other proteins involved in malate metabolism.

The transport system is responsible for the uptake of malate, fumarate, succinate and, to a lesser extent, aspartate (Kay & Kornberg, 1971). The system was reported to be constitutive in the cells (Kay & Kornberg, 1969) but has since been characterised as inducible and to be induced by the presence of dicarboxylic acids in the medium (Kay & Kornberg, 1971). The results of Takahashi and Hino (1968a) support an inducible dicarboxylic acid permease since they observed that, although aeration of anaerobic cells leads to an altered permeability barrier to fumarate, aeration in the presence of glucose does not. Takahashi & Hino did however obtain induction in a complex medium which did not contain dicarboxylic acids.

Takahashi and Hino (1968a) provide evidence to suggest that energy is necessary for fumarate entry into the cells. Kay & Kornberg (1971) confirmed that energy was necessary by showing that uptake of dicarboxylic acids could be inhibited by dinitrophenol and other compounds believed to inhibit energy linked membrane processes but could find no energy source which would enable dicarboxylic acids to be accumulated above medium concentration in membrane vesicles.

No control on the activity of this system is known.

5.2. Control of energy production

Energy, as described in section 4.2. is produced from complete oxidation of malate in the TCA cycle. There are 3 facets of this process which must be considered.

a) OAA production from malate

b) acetyl CoA production from malate

c) operation of the TCA cycle.

5.2.1. OAA production

OAA is produced from malate by the action of malate dehydrogenase. Only one malate dehydrogenase is synthesised in E.coli and is coded for at a genetic locus distinct from both the dicarboxylic acid permease and the other enzymes of the TCA cycle (Courtright & Henning, 1970). Although growth in an aerobic environment produces a higher enzyme specific activity than growth under anaerobic conditions (Takahashi & Hino, 1968b) regulation of enzyme synthesis has not been analysed. The enzyme is inhibited at NADH concentrations which are physiologically significant (Sanwal, 1969).

5.2.2. Control of acetyl CoA production

There is now considerable evidence that malic enzyme catalyses pyruvate production from malate (Kornberg, 1966) and that the enzyme under physiological conditions does not catalyse the reverse reaction.

There are two malic enzymes in E.coli (Katsuki et al., 1967; Takeo, 1969) and the activity of both is regulated by the cell. No definite induction/repression control has been established for these enzymes but some indications of control can be obtained by comparing specific activities after growth in various media. Sanwal and Smando (1969) have suggested that the synthesis of NADP-linked malic enzyme is regulated by catabolite repression while Murai et al. (1971) have shown that it is repressed by glucose, glycerol, pyruvate and acetate and induced by malate with a high specific activity in malate trained cells. Repression is dominant over induction. NAD-linked malic

enzyme is repressed by glucose and induced by malate but in this case induction is dominant over repression (Murai et al., 1971). No changes in specific activities greater than 5-fold have been observed between fully induced and fully repressed levels. All controls operate to reduce malic enzyme synthesis when pyruvate or metabolites linked to pyruvate are present in the medium while the presence of dicarboxylic acids stimulates synthesis.

Enzyme activity is also regulated by conditions in the cell and an increased NADH concentration as is associated with glycolysis (Wright & Sanwal, 1969) will reduce enzyme activity. Both malic enzymes are controlled allosterically. NADP-linked malic enzyme is inhibited by NADH, NADPH, OAA and acetyl CoA (Sanwal & Smando, 1969). NAD-linked malic enzyme is inhibited by ATP and coenzyme A - at a physiologically significant coenzyme A concentration - but the coenzyme A inhibition is overcome by aspartate (Sanwal, 1970b). Sanwal has suggested that the NAD-linked malic enzyme is used by the cell to degrade dicarboxylic acids as it is only active when they are present in the medium. Based on a similar analysis of the regulatory parameters of these two iso-functional enzymes, the energy generating sequence via acetyl CoA and the TCA cycle has been ascribed to NAD-linked malic enzyme while NADP-linked malic enzyme serves to supply pyruvate and acetyl CoA for bio-synthesis (Murai et al., 1971). The data on regulation of enzyme activity also support the pyruvate producing function of the enzymes.

The evidence for the unidirectional activity of malic enzymes comes from analysis of mutants. Mutants of E.coli unable to grow on

glucose unless supplemented with dicarboxylic acids in the medium are found to lack PEP carboxylase (Kornberg, 1965). Malic enzyme is present in these cells but, since the cells do not grow, is unable to synthesise dicarboxylic acids by carbon dioxide fixation. Cooper & Kornberg (1971) have also shown that radioactive carbon dioxide is incorporated into malate to a much greater extent in the presence of PEP than pyruvate because under these conditions the cells utilise PEP synthase and PEP carboxylase to catalyse malate synthesis. PEP synthaseless mutants, although they contain malic enzyme, are unable to carboxylate pyruvate to malate since in the presence of labelled carbon dioxide and pyruvate no label appears in malate. Malic enzyme therefore acts unidirectionally to synthesise pyruvate from malate.

Pyruvate synthesised in this way is decarboxylated to acetyl CoA by pyruvate dehydrogenase - a complex enzyme which is specifically induced by pyruvate (Dietrich & Henning, 1970) and whose activity is stimulated by PEP, AMP and guanosine diphosphate but is inhibited by acetyl CoA (Sanwal, 1970a).

5.2.3. Regulation of the TCA cycle

The regulation of the TCA cycle in E.coli is not fully understood. Gray et al. (1966) have shown that glucose in the environment can repress enzyme synthesis and Takahashi & Hino (1968b) have observed changes in enzyme specific activity during growth under aerobic and anaerobic conditions. Because cells growing under anaerobic conditions do not contain α-ketoglutarate dehydrogenase the TCA cycle can only operate as such under aerobic conditions (Amarasingham & Davis, 1965).

The enzymes are not coordinately induced and do not appear to be grouped in operons although mapping has shown that the genes for citrate synthase, ∝-ketoglutarate dehydrogenase and succinate dehydrogenase are located in one region of the genome (Courtright & Henning, 1970).

Some control of enzyme activity has been observed. Both citrate synthase (Weitzman, 1966) and malate dehydrogenase (Sanwal, 1969) are inhibited by NADH, and, by analogy with other systems where citrate synthase activity regulates the TCA cycle (Krebs, 1969), Sanwal (1970a) has suggested that NADH concentration regulates the TCA cycle in E.coli.

5.3. Regulation of PEP production

The studies of Kornberg (1965) have shown that production of PEP from OAA is catalysed by PEP carboxykinase. The enzyme is induced by the presence of dicarboxylic acids in the medium (Teraoka et al., 1970) and its activity regulated by NADH concentration (Wright & Sanwal, 1969) which is high during glycolysis but lower during growth on succinate (Wright & Sanwal, 1969).

5.4. Metabolism during growth on malate

Although a considerable amount of data has been reported over the last few years the concept of growth on malate described by Kornberg (1965) has not markedly altered. Malate is taken into the cell by an inducible transport system and used to fulfil all growth requirements. Energy is produced by operation of the TCA cycle and during this process intermediates are synthesised. Pyruvate, required for operation of the TCA cycle and for biosynthesis, is exclusively produced from malate by

malic enzyme. PEP, also required for biosynthesis of sugars and amino acids, is produced by PEP carboxykinase.

6. Control of Metabolism

Control processes are vital to the economy of the cell. Repression/induction achieves an economy of protein synthesis; activation/inhibition an economy in the distribution of small molecules. It is the activity of enzymes which regulates the flux of small molecular weight compounds through metabolism and has been shown to be active in the biosynthetic processes of the cell.

Although few examples are available the flux of molecules into metabolism via the catabolic pathways must be regulated. Glycerol metabolism is a system where regulation has been analysed and its role in metabolism evaluated. Glycerol enters the cell by facilitated diffusion and relies, for a flux into the cell, on a low internal glycerol concentration. The first step in glycerol metabolism is phosphorylation, catalysed by glycerokinase which is subject to allosteric inhibition by fructose-1-6-diphosphate (Zwaig & Lin, 1966). Cells growing on glycerol contain a concentration of fructose-1-6-diphosphate sufficient to inhibit glycerokinase by 70% and analysis of regulatory mutants has shown that inhibition operates *in vivo* (Zwaig et al., 1970).

Although this is the only catabolic system where regulation of enzyme activity linked to transport has been established similar mechanisms must exist in other catabolic pathways to regulate intake of substrate. Transport has been involved in the regulation of lactose and maltose metabolism but only as a mechanism of transporting inducer

into the cell (Kepes & Cohen, 1952). A similar mechanism has also been reported by London & Meyer (1970) to regulate the induction by malate of malic enzyme in Streptococcus faecalis. Further regulation of uptake after induction is however required to ensure that the rate of substrate uptake matches the rate of substrate utilisation.

Evidence on the role transport systems play in the regulation of growth has been obtained by Shehata & Marr (1971) and Von Meyerburg (1971). Shehata & Marr (1971) have examined the influence of substrate concentration on growth rate and found that growth rate may be considered as the sum of two limiting enzymic processes. K_m values for these processes are, at least in the case of growth on tryptophan, similar to the K_ms for transport systems and they conclude that the growth rate of the cell is limited by the activity of transport mechanisms which they suggest from additional data is a general phenomenon. Von Meyerburg (1971) isolated a pleiotrophic mutant which has an impaired ability to grow at low concentrations of a range of substrates and showed that the cellular transport systems have decreased affinities for their substrates. The results of Shehata & Marr (1971) and Von Meyerburg (1971) suggest that transport processes limit growth at low concentrations of substrate, because of their affinity for the substrate, and at high substrate concentrations because they are operating at maximum velocity. If this is so, alterations to transport system activities will result in changes in rate of substrate utilisation. Transport systems are therefore likely sites for regulation of growth and of substrate utilisation but little

or no evidence of such control has been reported although catabolite inhibition, where rates of uptake of nutrients are altered, may be the result of the operation of controls in entry systems.

The role of transport systems in the regulation of growth as shown by Shehata & Marr and Von Meyerburg was not obvious when we began this research. Nevertheless we believed that malate metabolism must be regulated at the level of permeation across the cell membrane. When we embarked on this work to analyse the rate of malate metabolism we hoped to establish that regulation can be expressed at the level of permeation of the carbon source into the cell.

7. Molar Growth Yields

The use of microbial growth to measure the concentration of a specific nutrient is a technique which has been used for a considerable period of time. Although simpler techniques have replaced biological assays, the study of the relationship between growth and nutrient concentration has continued. Research has concentrated on the role of the energy yielding substrate in growth. The observations of Monod (1942) established that under aerobic conditions the yield of cells from a culture of bacteria is proportional to the concentration of the energy yielding substrate in the medium. These results which reflect the operation of metabolic controls during growth were the start of an analysis into the relationship between energy production and growth. The most useful data in this regard have been obtained from the study of growth under anaerobic conditions because it was, and indeed still is, impossible to accurately calculate energy production under aerobic conditions. Because of a lack of data from E.coli it is necessary to consider results obtained in other systems. Where data from E.coli are available they are preferentially considered. Molar growth yields and energy yields have been reviewed by a number of authors (Stouthamer, 1969; Forrest, 1969; Payne, 1970; Forrest, 1971).

7.1. Anaerobic yields

Much useful data have been obtained by the study of molar growth yields under anaerobic conditions. Bauchop & Elsden (1960) grew several microorganisms on different media under anaerobic conditions.

From an analysis of the products of the fermentations and a knowledge of the degradative pathways used by the cells they were able to calculate the total ATP production in each culture and to relate it to the growth of the cells to show that for every mole of ATP generated about 10.5 ± 2 g of cellular dry weight is synthesised. In their experiments Bauchop & Elsden grew the cells in a rich medium and energy was largely required for transport and polymer formation. A variety of microorganisms growing on a variety of media have been studied and give similar results. Many are listed by Payne (1970).

E.coli has only been examined under anaerobic conditions to determine a Y_{ATP} on glucose (Hernandez & Johnson, 1967a) and a value of 9.4 was obtained. In their calculation of ATP production Hernandez & Johnson assumed succinate was produced from pyruvate by the action of pyruvate carboxylase, and not from PEP by PEP carboxylase as is the case (Kornberg, 1965), and, as a result, overestimated ATP production. Correction of the data for this error gives a Y_{ATP} = 10.0. Stouthamer (1969) reported a YATP for E.coli on glucose of 11.2 under anaerobic conditions.

7.2. Aerobic yields

Aerobic molar growth yields of facultative anaerobes are much easier to obtain than anaerobic yields. The original data of Monod (1942) were derived from an analysis of aerobic growth. It is, however, difficult to measure oxygen uptake with growth and is impossible to relate oxygen uptake data to ATP production because of the uncertainty of calculating ATP production from oxidative processes. The calculation

of YATP requires not only a Y substrate or Y_{O_2} value but also the efficiency with which the cells couple ATP production to electron transport i.e. a P/O ratio value.

7.2.1. Efficiency of oxidative phosphorylation

In mitochondria the number of high energy phosphate bonds formed during the transfer of $2e^-$ from NADH to oxygen has been found to be three. No such value for the P/O ratio has been established for bacteria.

A number of attempts have been made to assess P/O ratios in bacterial cells using cell-free extracts (Stouthamer, 1962; Cel'man et al., 1967; Mickelson, 1969). All of these approaches gave results suggesting that the efficiency of oxidative phosphorylation in bacteria is much lower than in mitochondria. Although Stouthamer (1962) made out a case for the low P/O ratio which he obtained in cell-free extracts of Gluconobacter liquefaciens being an accurate value for these cells, it seems likely that cell-free extracts have a much lower efficiency of energy coupling because of the breakdown of cell membranes (Stouthamer, 1969). This is supported by comparison of calculated oxidative phosphorylation efficiencies from molar growth yields with cell-free extract estimates (Hadjipetrou et al., 1964; Mickelson, 1972).

Direct measurements of the P/O ratio associated with oxidation of NADH in E.coli B have been made by Hempfling (1970a). In cells which were grown in rich medium, harvested and subjected to a shift from anaerobic to aerobic conditions he measures a P/O ratio of 3. He has further shown (Hempfling, 1970b) that the P/O ratio is susceptible to changes in the growth environment, addition of glucose to the complex

medium reducing the P/O ratio to 0.1 but that the reduced P/O ratio could be due to catabolite repression since addition of cyclic AMP with the glucose results in the P/O ratio returning to 3 (Hempfling & Beeman, 1971). These data of Hempfling of direct P/O ratio measurements show that the P/O ratio is variable and dependent upon environmental conditions.

7.2.2. P/O values from molar growth yields

In the absence of reliable estimates of ATP production during aerobic growth several attempts have been made to calculate P/O ratios from molar growth yield data on the assumption that the Y_{ATP} of 10.5, found under anaerobic conditions, is true under aerobic conditions. The best that can then be achieved is to show that the results are at least self consistent.

The first attempts to calculate P/O ratios considered differences in yield/mol substrate for 2 substrates whose metabolism differed by a single oxidation reaction (Stouthamer, 1962; Gunsalus & Shuster, 1961) but was extended to total substrate oxidation (Chen, 1964; Hernandez & Johnson, 1967b). Hernandez & Johnson's data showed that a P/O ratio of 1.0 during growth of E.coli on a complex glucose medium is consistent with a Y_{ATP} of 8.9.

To maintain a constant Y_{ATP} during aerobic growth on different carbon sources it is necessary to assume that the P/O ratio is not constant and can in some cases have non-integer values (Hadjipetrou et al., 1964; Payne, 1970). A similar approach based on the calculation of the number of electrons available in the substrate for transference

to oxygen has been made (Mayberry et al., 1968).

7.3. Aerobic growth yield values

Although E.coli has not been a preferred choice for molar growth yield determinations, some yield data are available. Yields/mol substrate are only available for growth on glucose (Sedlaczek et al., 1966; Ribbons, 1969; Ng, 1969) and the values are variable with Sedlaczek et al. reporting a $Y_{Glucose}$ of 67.8 and the others values of 90-94.

Molar growth yields on other substrates have not been reported for E.coli. Of relevance are molar growth yields on dicarboxylic acids during aerobic growth. Several microorganisms give a $Y_{substrate}$ of between 38-42 for growth on dicarboxylic acids (Payne, 1970).

An alternative parameter Y_{O_2}, first used by Whitaker & Elsden (1963) can be used under aerobic conditions. Some data are available from growth of E.coli (Figure VI). The data attributed to Stouthamer (1969) he obtained from Whitaker & Elsden (1963). No statement of these data by Whitaker & Elsden (1963) exists.

Substrate	Y_{O_2}	reference
Glucose	40.4	Whitaker & Elsden (1963)
Glucose (minimal)	41.3	Hernandez & Johnson (1967b)
Glucose (complex)	23.9	"
Glutamate	23.4	Stouthamer (1969)
Succinate	22.6	"
Lactate	23.4	"
Acetate	12.4	"

Figure VI Aerobic growth yields of E.coli

7.4. Maintenance requirement of growing cells

All the previous material on molar growth yields has not taken into account any maintenance requirement of the cells during growth. A maintenance requirement was considered negligible from the results of Monod (1942) but with the development of continuous culture techniques, a maintenance requirement was necessary to explain deviations, at low dilution rates, from the original theory (Dawes & Ribbons, 1964).

Defining the maintenance coefficient (m) as the rate of substrate utilisation used solely for maintenance, Pirt (1965) derived an equation to relate maintenance coefficient to molar growth yield and specific growth rate of the cells which can be applied to both batch and continuous culture. Several estimates of maintenance coefficients have been made using this equation (Pirt, 1965; Righeleto et al., 1968; Watson, 1970; de Vries et al., 1970; Carter et al., 1971). Using the data of Marr et al. (1963), Pirt (1965) calculated a maintenance coefficient of 0.07 g glucose/g dry wt./h for E.coli growing at 37° under aerobic conditions. Previously Schultze & Lipe (1964) had found that 0.055 g glucose/g dry wt./h was necessary to fulfil the requirement of E.coli for maintenance.

Although maintenance has been estimated for several micro-organisms, there is little information on the function it fulfils. Marr et al. (1963) have suggested that maintenance is dependent on the growth temperature. Watson (1970) showed that the maintenance requirement of a culture of Saccharomyces cerevisiae depends on the concentration of sodium chloride in the medium and suggests the energy is used to maintain

an electrochemical gradient of Na^+ across the cell membrane. The mechanism of energy expenditure is not known.

7.5. Integration of molar growth yield, P/O ratios and maintenance requirement

Little has been published either on molar growth yields per mole carbon source utilised or per mole oxygen taken up for E.coli growing under aerobic conditions on simple salts medium in the presence of a single carbon source. No attempt has been made to reconcile estimates of molar growth yield with maintenance requirement. Consequently, in view of the more recent attempts to measure P/O ratios and the improvements in instrumentation which we describe, it is of interest to measure molar growth yields under aerobic conditions for E.coli and to try to interpret these with regard being given to both maintenance requirement and P/O ratios.

The uptake of oxygen during growth on malate, a carbon source which utilises only oxidative phosphorylation as a means of producing energy, is a direct measure of energy production and is a suitable system to analyse, under aerobic conditions, the relationships between growth rate, cell yield, energy production and the regulation of cellular metabolism.

METHODS

1. Microbiological Techniques

1.1. Organism

The organism used throughout this work was Escherichia coli ATCC 15224 (ML308) which has the genetic structure $i^{-}z^{+}y^{+}a^{+}$ for the lac operon. Consequently the synthesis of the products of the lac operon (β-galactosidase, β-galactoside permease and thiogalactoside transacetylase) is constitutive because the cell produces a defective repressor. In all other respects it was assumed to be wild type.

The strain was obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland, U.S.A.) and characterised by bacteriological tests described (Cowan & Steel, 1965).

1.2. Reconstitution and storage of organism

The organism was obtained as a lyophilisate in a sealed evacuated glass ampoule which was 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 10 ml of sterile nutrient broth in a 25 ml Maccartney bottle and incubated at 37° for 24 h, then plated on nutrient agar and incubated at 37° for 24 h. A typical clone was picked off, transferred to nutrient broth and grown as before. The nutrient broth culture was checked for homogeneity both microscopically and by plating out on nutrient agar containing 10 μg/ml 5-bromo-4-chloro-indoxyl-β-galactoside (BCIG agar). A cell constitutive for β-galactosidase produces a blue colony on BCIG agar

so a homogeneous culture of lac-constitutive cells produces only blue clones.

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

Every 3 months a cooked meat culture was used to inoculate 6 nutrient broths and these were grown at 37° for 24 h, plated on BCIG 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 (L.H. Engineering Co. Ltd. England) at 37°. This was a 1st passage and 1 ml of it was subcultured into 100 ml of identical medium (2nd passage) and grown under the same conditions. These cultures were stored at 4°. 24 h before an experiment 1 ml of the 2nd passage was subcultured into a 3rd passage and grown as above. This was stored overnight at 4°. The growth time of each passage depended on the carbon source (Figure 1).

To prepare an inoculum, a portion of the 3rd passage was harvested at 11,600 g for 10 min at 4°, resuspended to the required cell density in chilled 40 mM phosphate buffer pH 7.0 and stored on ice until required.

Figure 1. PREPARATION OF INOCULA

Inocula of E.coli 15224, trained to different substrates, were prepared by growth through 3 passages in 100 ml of minimal salts medium containing the carbon source at the indicated concentration. The 1st passage was inoculated with 3 drops of a nutrient broth culture, the 2nd and 3rd passages with 1 ml from the previous passage. All growth was carried out at 37° under aerobic conditions on an orbital shaking table for the periods of time shown.

Carbon Source	Concn. mM	1st Passage h	2nd Passage h	3rd. Passage h
Glucose	10	16	7	7
Glycerol	20	16	7	7
Malate	20	16	8	8
Succinate	20	16	8	8
Fumarate	20	16	8	8
Acetate	30	36	24	24
Pyruvate	24	16	7	7
Gluconate	10	16	7	7
Alanine	24	48	12	12
oketo glutarate	12	120	7	7
Glutamate	12	120	12	12
Aspartate	20	48	12	12

2. Media

2.1. Cooked meat medium

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

One litre of cooked meat medium contained:

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

2.2. Nutrient broth medium

Nutrient broth was prepared from Oxoid dehydrated granules. 1 litre of nutrient broth contained in distilled water

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

Nutrient broth was dispensed 10 ml into 25 ml 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. 15 g of agar was added to 1 litre of nutrient broth medium and dissolved by boiling for 15 min. Agar was sterilised by autoclaving at 15 p.s.i.,

poured into petri dishes under ultraviolet light and allowed to solidify. Plates were stored at 4°.

2.4. BCIG agar medium

BCIG was dissolved in dimethyl formamide at a concentration of 2 mg/ml and added to nutrient agar to a final concentration of 10 µg/ml. Solution was mixed, poured into petri dishes under ultraviolet light and allowed to solidify. Plates were stored at 4°.

2.5. Defined media

Defined media were prepared by two 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 66.7 mM potassium dihydrogen phosphate (9.07 g/l KH_2PO_4) and 16.7 mM ammonium sulphate (2.2 g/l $(NH_4)_2SO_4$) to pH 7.0 using sodium hydroxide. This was dispensed 60 ml into 500 ml conical flasks and sterilised by autoclaving at 15 p.s.i.

II $FeSO_4$ solution contained 0.8 mM ferrous sulphate (0.22 g/l $FeSO_4.7H_2O$) to pH 2.0 using hydrochloric acid and was sterilised by autoclaving at 15 p.s.i.

III Combined carbon source and 1.25 mM magnesium sulphate (0.31 g/l $MgSO_4.7H_2O$). The concentration of carbon source depended on the required final carbon source concentration (Figure 1). This solution was taken to pH 7.0 only if necessary due to the nature of the carbon source and dispensed into

bottles in 40 ml batches before autoclaving at 5 p.s.i.

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

2.5.2. Medium for growth experiments

This was prepared as 4 separate components:

I P 760 ml potassium dihydrogen phosphate (5.44 g/l KH_2PO_4) pH 7.0 sterilised by autoclaving at 15 p.s.i.

II MgNS contained 40 mM magnesium sulphate (9.84 g/l $MgSO_4.7H_2O$) and 800 mM ammonium sulphate (105.6 g/l $(NH_4)_2SO_4$) pH 7.0 - sterilised by autoclaving at 15 p.s.i.

III $FeSO_4$ 0.8 mM ferrous sulphate (0.22 g/l $FeSO_4.7H_2O$) pH 2.0 sterilised by autoclaving at 15 p.s.i.

IV Carbon source prepared at high concentration to pH 7.0 if required and sterilised at 5 p.s.i.

Minimal salts medium was prepared by adding 10 ml each of solutions II and III to solution I and making the final volume to 800 ml using sterile distilled water.

The composition of defined medium made by either method was:

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

with NaOH to pH 7.0

and carbon source at the required concentration.

Components, with the exception of the carbon sources, were stored at room temperature. Carbon sources were stored at 4°.

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 direct reading pH meter (EIL Ltd. Cambridge). Standard buffer solution was prepared using buffer solution tablets (Burroughs Welcome Ltd.). 40 ml portions of the buffer standard were autoclaved at 15 p.s.i. then stored at room temperature. Each day a fresh 40 ml portion was used to calibrate the pH meter.

The pH of cultures was determined using a micro-assembly attached to an EIL 2320 pH meter (EIL, Cambridge). Calibration with buffer tablets was carried out as above.

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 pressure (5 or 15 p.s.i.) 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 250 ml capacity fitted with a 0.22 μ pore size Millipore filter (Millipore Corp. Massachussetts, U.S.A.). Once assembled the unit was sterilised by autoclaving at 15 p.s.i. After filtration solutions were aseptically transferred to sterile bottles.

A second procedure for smaller volumes used Nalge disposable filter units (0.20 μ pore) (Sybron Corp. Rochester, U.S.A.). These had 100 ml 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, then in 1% w/v 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 $1\frac{3}{4}$ h either wrapped in paper or in metal canisters. Canister sterilisation was checked by Browne steriliser control tubes.

6. Growth

Growth of the organism was always carried out in batch culture. 800 ml of complete defined medium contained in a 1 litre flat-bottomed pyrex flask (Figure 2) fitted with a short side arm was inoculated and maintained at 37° in the apparatus described by Harvey et al. (1968) (Plate 1). The side arm was plugged with a silicone rubber bung through which passed a 6" dispensing canulus carrying a disposable syringe. The main neck of the flask was sealed by a silicone rubber bung through which two ports were made. The first was fitted with a short condenser maintained at 2° by a circulating coolant of 10% propanol in water. A peristaltic pump (Glen Creston, Stanmore, England) pulled the coolant through a copper coil immersed in an ice-water slurry then through the condensers before returning the coolant to the reservoir. The second port was the gas inlet. Air from the departmental compressed air supply was filtered using charcoal and passed into the flask at a steady flow rate. The flow rate was monitored on gas flow gauges (G.A. Platon, Croydon, England). The apparatus ensured that the gas phase above the culture was not disturbed by sampling but was fully mixed with the liquid phase so the culture was always truly aerobic.

7. Sampling of Culture

Samples were removed from the culture using the syringe which was rinsed with culture before the sample was removed. Samples of

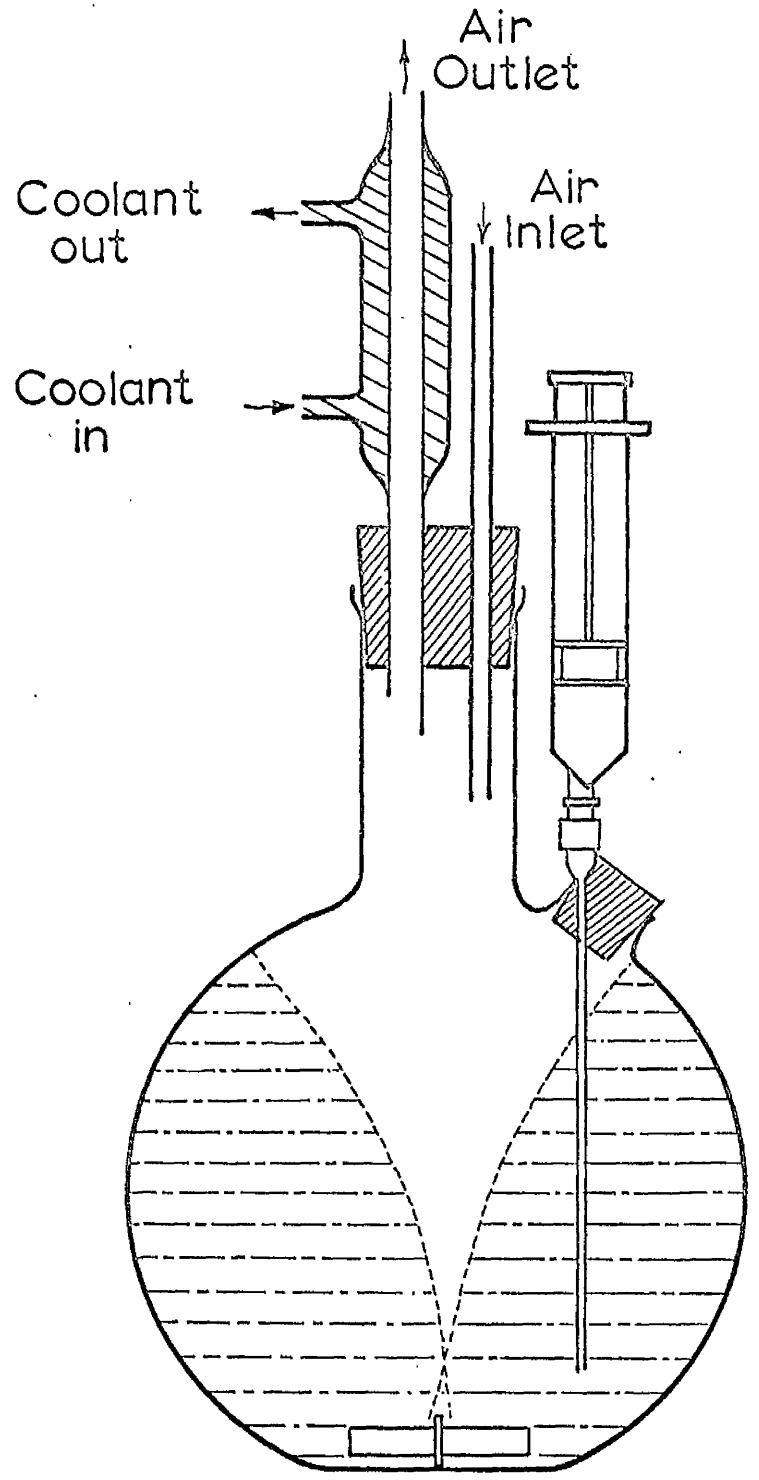

Air
Outlet
Coolant
out
Air
Inlet
Coolant
in

Plate 1

Photograph of apparatus with overlay

Key

1 - recording thermograph

2 - digital clock

3 - oxygen analyser

4 - ratio box

5 - chart recorders

6 - flow controller

7 - carbon dioxide analyser

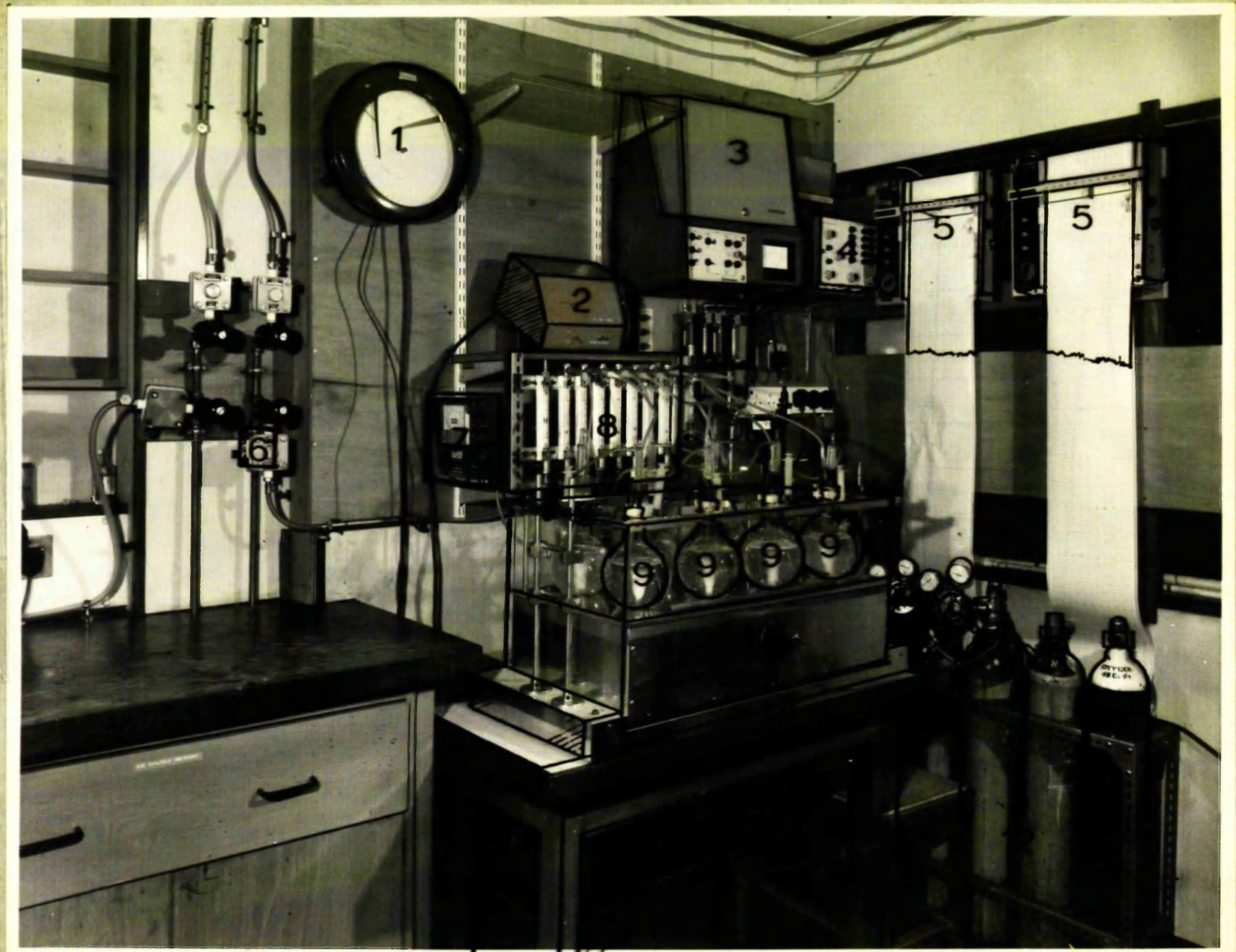
8 - GAP meter assembly

9 - growth flasks

10 - growth apparatus

11 - cooling unit

12 - calibration cylinders


1
3
2
4
5
5
6
8
9
9
9
9

various sizes could be taken depending on the size of the syringe used. Samples were used to measure culture density, culture pH and substrate concentrations.

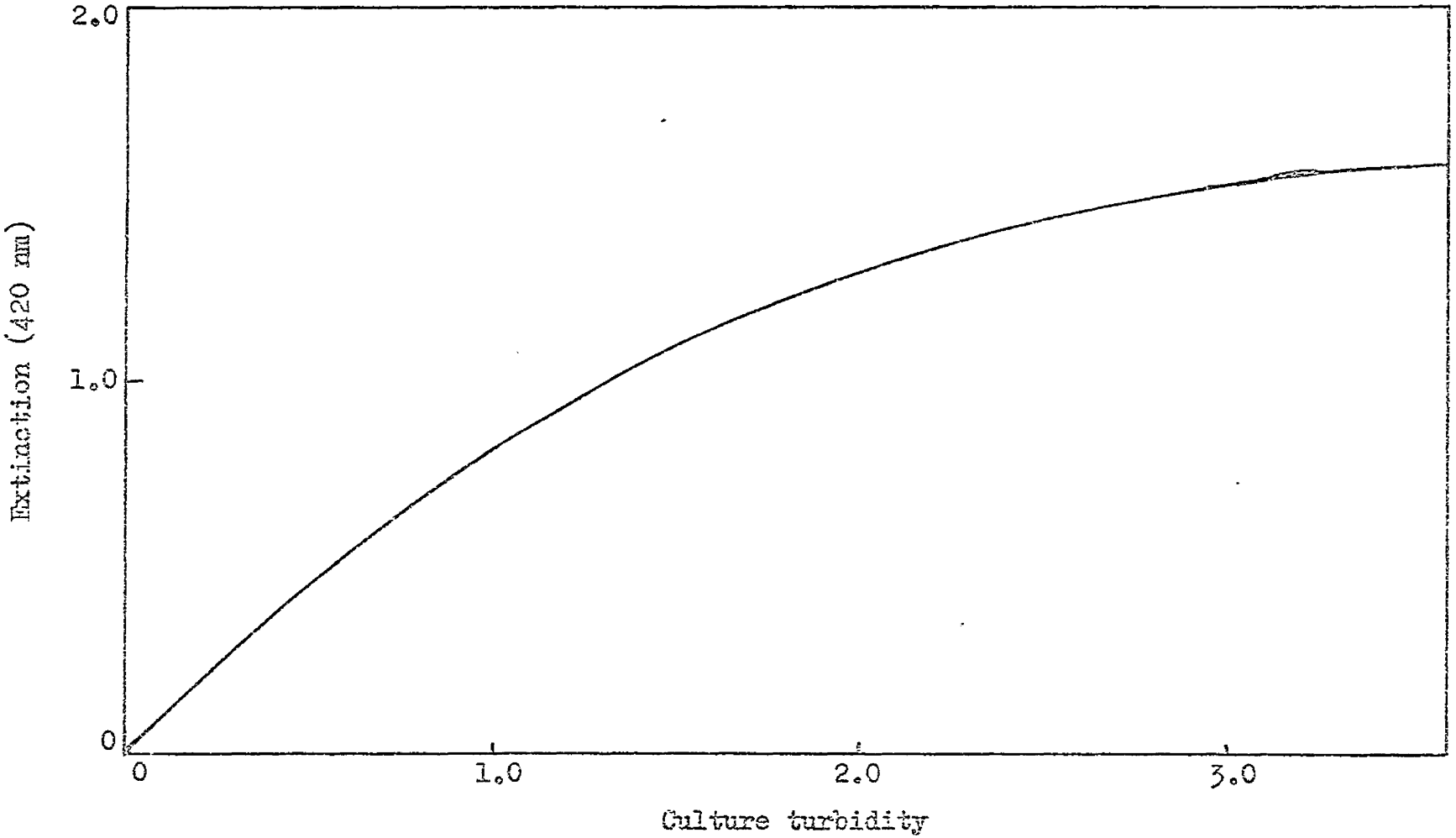
8. Measurement of Growth

Cell density was determined turbidimetrically on samples (4 ml) taken from the culture into formaldehyde solution (40%, 1 drop). The apparent E_{420} was measured in glass cuvettes (Type 1, 10 mM light path) (Ross Scientific Co. Ltd., Hornchurch, England) using a SP800 double beam spectrophotometer (Unicam Instruments Ltd., Cambridge, England) fitted with a Servoscribe potentiometric chart recorder (Smiths Industries Ltd., Wembley, England).

The absorption due to the cells was linear with culture turbidity up to an optical density of 0.2. Above this value the optical density was lower than the true culture turbidity. Consequently a calibration curve was drawn between optical density and culture turbidity (Figure 3) to enable turbidities to be determined without dilution. A culture turbidity of 1.0 at 420 nm is equivalent to a cell density of 196 μg dry weight/ml culture (Holms, unpublished results).

Figure 3 TURBIDITY CALIBRATION CURVE

Cultures of a wide range of turbidity were read at 420 nm in a SP800 double beam spectrophotometer against air as a blank. Portions of the cultures were diluted in 40 mM phosphate buffer pH 7.0 to give 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.


2.0
1.0
0
Extinction (420 nm)
0
1.0
2.0
3.0
Culture turbidity

9. Measurement of Substrate Concentration

9.1. General considerations

Substrate concentrations were measured on samples taken from the culture. Assays were carried out for malate, glucose, glycerol, fumarate, acetate and pyruvate. All with the exception of fumarate were enzymic assays. In every case assays were read on an SP800 double beam spectrophotometer fitted with a servoscribe recorder against air as a blank.

9.2. Treatment of samples

A 4 ml sample taken from the culture was blown onto 1 ml of chilled 30% w/v perchloric acid, mixed and allowed to stand on ice for 10 min, when 3 ml of chilled potassium hydroxide (~1 N) was added to return the sample to the original pH value. When the potassium perchlorate had precipitated the samples were decanted into 15 ml centrifuge tubes (Corning Glass Works, New York, U.S.A.) and centrifuged at 11,600 g and 4^{o} for 10 min in an MSE 18 refrigerated centrifuge (M.S.E., Crawley, England). The supernate was decanted into glass vials and frozen in a mixture of solid carbon dioxide and ethanol then stored at -10^{o} until assayed.

Samples were thawed and thoroughly mixed before a portion was removed for substrate estimation. Samples were then refrozen and stored as above.

9.3. 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	0.45 M
Hydrazine	0.18 M
EDTA	2.4 mM
NAD^+	3.2 mM
sodium hydroxide to pH 9.5	
malate dehydrogenase	50 µg/assay.

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

9.4. Estimation of fumarate

Fumarate was measured directly by absorption at 240 nm (Racker, 1950). The assay was linear over the range 0-800 n mol fumarate/ ml but was usually only used over the range 0-400 n mol/ml. 1 M fumarate gives an extinction of 2.5×10^3.

9.5. 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	81 mM
magnesium sulphate	3.2 mM

ATP	0.54 mM
PEP	0.17 mM
NADH	0.1 mM
Lactate dehydrogenase	13 µg/ml
pyruvate kinase	7 µg/ml
glycerokinase	3 µg/ml
Assay pH	7.6

Assay was done in a total volume of 3 ml. 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° for 60 min and read at 340 nm. The assay is linear over the range 0-300 n mol glycerol/assay and 1 mol of glycerol in the assay gives an extinction of 2.07×10^6.

9.6. Estimation of pyruvate

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

The composition of the assay was:

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

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

9.7. 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 20 min at 27° the extinction of the assay decreases (Figure 4a). Consequently assays were incubated for 20 min at 27° then read at 660 nm. The assay is linear over the range 0-250 n mol glucose/assay (Figure 4b).

9.8. Estimation of acetate

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

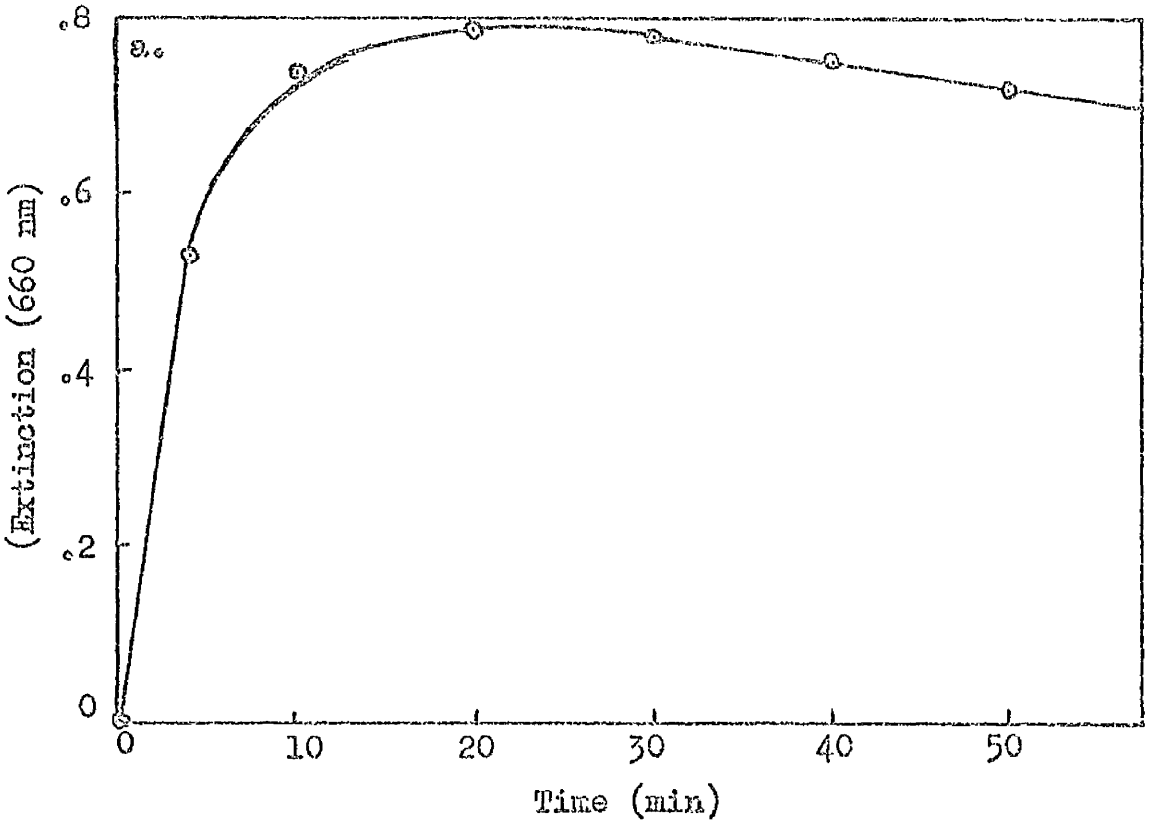
triethanolamine	62.5 mM
magnesium sulphate	6.7 mM
ATP	6.6 mM
PEP	3.0 mM
NADH	0.25 mM
lactate dehydrogenase	13 µg/ml
myokinase	13 µg/ml
pyruvate kinase	13 µg/ml
acetate kinase	33 µg/ml

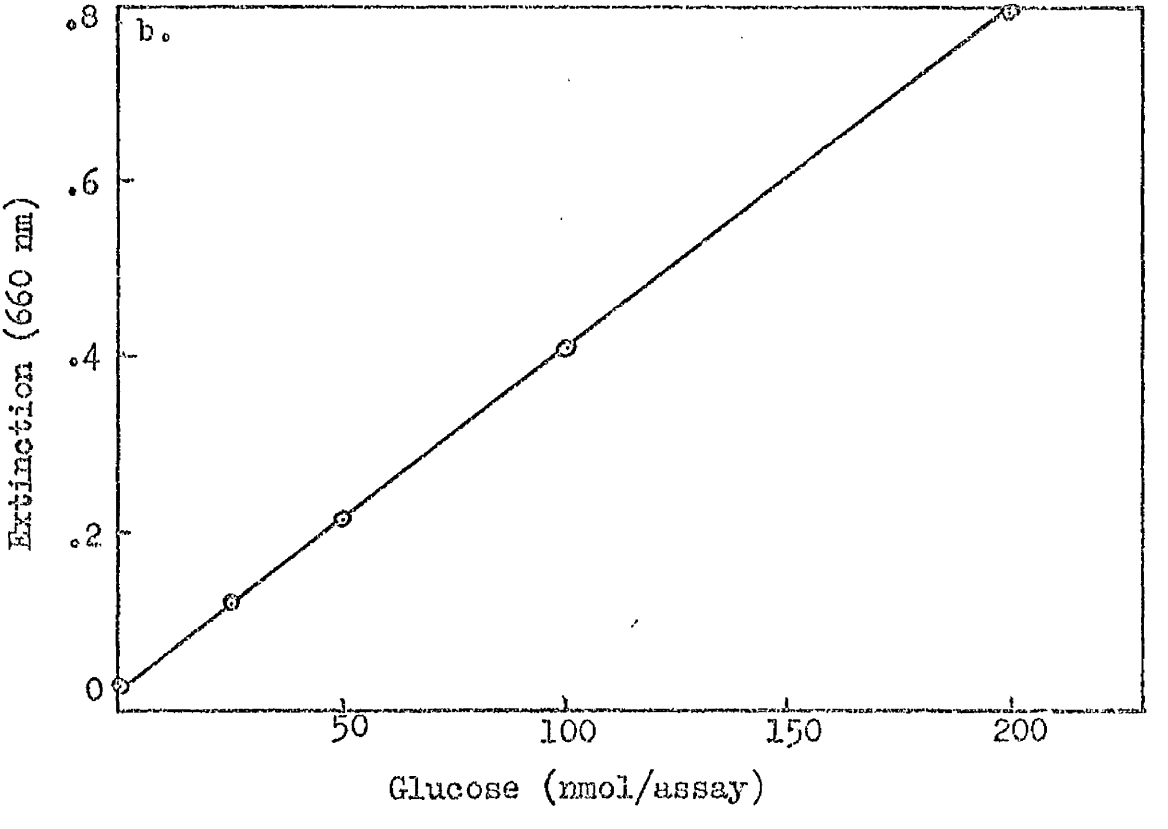
Assay was done at pH 7.4 in a total volume of 3 ml. All reagents with the exception of acetate kinase were mixed before addition to a portion of sample. Acetate kinase was added to initiate the assay which was incubated 1 h at 27° and read at 340 nm. The assay is linear over the range 0-500 n mol acetate/assay.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

Figure 4 ESTIMATION OF GLUCOSE

Samples of glucose containing culture medium were treated as described in section 9.2. A portion, containing 200 nmol glucose in a total volume of 0.5 ml, was taken, 4.5 ml GOD-Perid reagent added and the change in optical density at 660 nm and 27^{o} followed using a SP800 double beam spectrophotometer fitted with a chart recorder. The change in optical density with time is shown (Figure 4a).

Portions of culture, containing different amounts of glucose in 0.5 ml, were taken, 4.5 ml GOD-Perid reagent added and the assay incubated at 27^{o} for exactly 20 min then read as above. A calibration curve was constructed (Figure 4b).


a.
.8
.6
.4
.2
0
(Extinction (660 nm)
0
10
20
30
40
50
Time (min)


b.
.8
.6
.4
.2
0
Extinction (660 nm)
50
100
150
200
Glucose (nmol/assay)

10. Measurement of Gas Exchange

10.1. General considerations

Gas exchange of a culture was measured using the growth flask described in Figure 2 in the apparatus shown on Plate 1. A stream of air was equilibrated with a culture and monitored for partial pressure of oxygen and carbon dioxide. The details of calibration, of development of the method and of the calculation of results are discussed later. A description of the method and its application to batch culture has been published (Hamilton and Holms, 1970), but a less sensitive method has also been described (Ribbons, 1969).

10.2. Gas exchange of a culture of growing bacteria

The method of gas exchange permitted the measurement of both oxygen uptake and carbon dioxide production during growth of a culture of bacteria at a cell density greater than 10 µg dry wt/ml. Simultaneously with the measurement of gas exchange the system allowed samples to be removed from the culture for measurement of cell density, of pH and of substrate concentration in the medium.

Gas entered and left the growth flask at a constant rate of flow. The rate of flow of the gas was set before the start of an experiment and was not altered during the experiment. On leaving the flask water vapour was stripped from the gas by the condenser maintained at 2°. The rate of flow of gas was then measured using a calibrated flow meter (G.A. Platon, Croydon, England). The gas was monitored by infra red analysis for carbon dioxide content over the range 0-1% using a

lira 300 analyser (Minc Safety Appliances, Glasgow) and by paramagnetism for oxygen content over the range 21-20% using an OA184 oxygen analyser (Servomex, Crowborough, England). The output from both gas analysers was continuously recorded on separate servoscribe potentiometric recorders.

The data obtained were gas flow rate (F), partial pressures of oxygen (O_2) and carbon dioxide (CO_2) and the rate of change of carbon dioxide partial pressure (ΔCO_2). These data plus a measurement of temperature (^oA) (T), volume of culture in the growth flask (V) and pH of the culture allowed the gas exchange rates of the growing culture to be determined. It was assumed the pressure in the system was always atmospheric.

The rate of carbon dioxide production was calculated using the equation

$$CO_2 \text{ prod}^n = \frac{CO_2 \times F \times 273 \times 10^4}{T \times 22.4 \times V} + K\Delta CO_2$$

where K is a constant dependent on temperature and pH.

The rate of oxygen uptake was calculated using the equation

$$O_2 \text{ uptake} = \left[\frac{20.960(100-O_2-CO_2)}{79.040} - O_2\right] \frac{F \times 273 \times 10^4}{V \times T \times 22.4}$$

In both cases the results were expressed as n mol/ml culture/min.

10.3. Accumulative gas exchange of a culture of growing bacteria

The gas exchange occurring throughout the growth of a culture was determined from the rates of gas exchange calculated above. This equation applies to both carbon dioxide production and oxygen uptake. If we measured gas exchange as E_1 at time t_1, as E_2 at time t_2 and as

E_n at time t_n then the accumulative gas exchange (A) was given by

$$A = \tfrac{1}{2} \sum_{n=1}^{n=n} (E_n + E_{n-1})(t_n - t_{n-1})$$

The units were n mol/ml culture or μ mol/ml culture.

10.4. Measurement of the gas exchange of cells harvested from another culture

The ability of cells grown on one carbon source to metabolise other compounds was measured as oxygen uptake and carbon dioxide production using the gas exchange equipment with a slightly modified method.

A sample containing approximately 25 mg of cellular dry wt. was taken from a culture, chilled, harvested (4,000 *g*, 4°, 30 min), resuspended in chilled 40 mM phosphate buffer pH 7.0 to a total volume of 30 ml and harvested again (11,600 *g*, 4°, 10 min). The pellet was resuspended in 10 ml chilled 40 mM phosphate buffer pH 7.0 and inoculated into a growth flask containing minimal salts medium and the carbon source whose rate of metabolism was being measured. Carbon dioxide and oxygen content of the gas leaving the flask were measured and the rates of oxygen uptake and of carbon dioxide production calculated as under 10.2. Gas exchange was measured at 10 min intervals over a 1 h period and the rate of gas exchange results extrapolated to zero time to measure the rate of metabolism of the culture at inoculation. Samples were also removed from the flask to determine the rate of growth of the cells on the test metabolite.

11. Measurement of Malic Enzyme Activities

11.1. Preparation of samples

Samples, containing approximately 15 mg dry wt. of cells, were removed from a culture for the assay of malic enzyme activities. These samples were chilled in an ice-water slurry then harvested at 4,000 g and 4° for 30 min in an MSE Mistral 6L refrigerated centrifuge (M.S.E., Crawley, England). Cells were resuspended in chilled 40 mM phosphate buffer pH 7.0 to a volume of 20 ml and harvested at 11,600 g and 4° for 10 min in an M.S.E. 18 refrigerated centrifuge (M.S.E., Crawley, England). The centrifuge tube containing the cell pellet was covered with parafilm and stored at -70° in a deep freeze (Revco, Deerfield, Michigan, U.S.A.).

11.2. Preparation of cell extracts

Within 24 h of harvesting the cells were resuspended to a cell density of approximately 2 mg dry wt/ml in chilled 20 mM phosphate buffer pH 7.0 containing 5 mg/ml BSA and 0.15 M sodium chloride. A portion was diluted in 40 mM phosphate buffer pH 7.0 for measurement of turbidity at 420 nm as described (methods 8). 3 ml of the cell suspension was pipetted into a small glass bottle and the cells disrupted by ultrasonic irradiation at 0° using a Soniprobe (Dawe Instruments, London, England) at 2A for 3 min. The irradiation time of 3 min consisted of 6 x $\frac{1}{2}$ min periods of irradiation with $\frac{1}{2}$ min intervals to ensure that the sample remained chilled. The experimental set up has been described (Holms and Bennett, 1971).

The extract was clarified by centrifugation (22,000 g, 4°, 20 min). The supernate was collected and a portion (2 ml) centrifuged at 125,000 g and 4° for 90 min in a Spinco model L2 ultracentrifuge (Beckman Instruments, Glenrothes). Both supernates were stored on ice until assayed.

11.3. Assay of NADP-linked malic enzyme

The 22,000 g supernate was used to estimate the activity of the NADP-linked malic enzyme (E.C. 1.1.1.40) by a modification of a standard method (Ochoa, 1955a).

250 µl of cell extract and 750 µl of medium (PO_4, 20 mM; BSA, 5 mg/ml; NaCl, 0.15 M; pH 7.0) were added, using an Oxford pipette (Boehringer, London) to an assay which contained 60 µ mol tris pH 7.2 30 µ mol sodium malate, 3 µ mol $NADP^+$, and 0.6 µ mol manganese sulphate in a final assay volume of 3 ml. The rate of production of NADPH was measured by extinction at 340 nm in an SP800 double beam spectrophotometer at 27° against air as a blank. The output of the spectrophotometer was traced on a servoscribe potentiometric recorder from which the rate of change of NADPH concentration could be measured. The results were expressed as enzyme units/ml cell extract where 1 enzyme unit represents the reduction of 1 µ mol of nucleotide/min at 27° under the conditions of the assay.

11.4. Assay of NAD-linked malic enzyme

The 125,000 g supernate was used to estimate the activity of the NAD-linked malic enzyme (E.C. 1.1.1.38) using a modification to a standard method (Ochoa, 1955b). NADH oxidase activity was much reduced

by ultracentrifugation (Katsuki et al., 1967).

250 µl of cell extract and 750 µl of resuspension medium (PO_4, 20 mM; BSA, 5mg/ml; NaCl, 0.15 M; pH 7.0) were added, by Oxford pipette, to an assay containing 60 µ mol tris pH 7.2, 30 µ mol sodium malate, 3 µ mol NAD^+, 0.6 µ mol manganese sulphate and 0.25 µ mol NADH in a final assay volume of 3 ml. The rate of NADH production at 27^o was measured by extinction at 340 nm using a SP800 double beam spectrophotometer fitted with a chart recorder against air as a blank.

The same assay system less sodium malate was used to determine the residual NADH oxidase activity. The difference between NADH production in the presence and absence of malate was used to estimate the total NAD-linked malic enzyme activity in the extract. The results were expressed as enzyme units/ml cell extract where 1 enzyme unit represents the reduction of 1 µ mol of nucleotide/min at 27^o under the assay conditions.

Specific activity of both enzymes is enzyme units/mg cellular dry wt.

The development and verification of both enzyme assays and of the extraction procedure are described in detail on p. 94.

12. Estimation of Pyruvate Production

The ability of cells to decarboxylate malate to pyruvate was determined using a washed cell suspension in the presence of 1 mM sodium arsenite.

A sample of a culture containing 50 mg dry wt. of cells was harvested at 4,000 g and 4° for 30 min. Cells were resuspended in 40 ml of chilled 40 mM phosphate buffer pH 7.0, harvested at 11,600 g and 4° for 10 min and the pellet resuspended in chilled 40 mM phosphate buffer pH 7.0 to a cell density of 2 mg dry wt/ml. A portion was further diluted in 40 mM phosphate buffer pH 7.0 and used to determine cell density by turbidity at 420 nm as described.

Cells were incubated at 37° for 5 min then inoculated to final cell densities ranging from zero to 0.4 mg dry wt/ml into a series of tubes containing at final concentrations:

Potassium dihydrogen phosphate/NaOH	pH 7.0	40 mM
Sodium malate		20 mM
Sodium arsenite		1 mM
Sodium pyruvate		0.4 mM

Tubes were incubated at 37° and mixed and aerated by a stream of air bubbles from a pasteur pipette standing in the tube. Samples were taken from each tube at intervals after inoculation and treated as described under methods (section 9.2.) and stored at -10°.

Simultaneously an identical series of tubes but lacking sodium malate were inoculated, sampled and the samples treated as described above.

Samples were assayed for pyruvate as described in section 9.6. The rate of pyruvate production in each malate containing tube was calculated and plotted against cell density to derive a rate of pyruvate production/mg cellular dry wt. Similarly the rate of pyruvate disappearance in the absence of malate was calculated. The difference between the rates of production in the presence and absence of malate was used to measure the rate of pyruvate production from malate in the cells.

13. Measurement of Maintenance Requirement

An estimate of the maintenance requirement of the cells during growth was made. The estimate was based on the equation relating total energy production and growth of the cells with maintenance requirement (Pirt, 1965).

For all cultures a substrate balance can be constructed:

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

This applies to all substrates including oxygen.

$$\therefore \quad \left(\frac{ds}{dt}\right)_t = \left(\frac{ds}{dt}\right)_m + \left(\frac{ds}{dt}\right)_G$$

$$\therefore \quad \frac{1}{x}\left(\frac{ds}{dt}\right)_t = \frac{1}{x}\left(\frac{ds}{dt}\right)_m + \frac{1}{x}\left(\frac{ds}{dt}\right)_G$$

$$= \frac{1}{x}\left(\frac{ds}{dt}\right)_m + \frac{1}{x}\frac{dx}{dt}\left(\frac{ds}{dx}\right)_G$$

but $\frac{1}{x}\left(\frac{ds}{dt}\right)_t$ = rate of oxygen uptake/g cellular material.

$\frac{1}{x}\left(\frac{ds}{dt}\right)_m$ = maintenance coefficient with units -mol. mass^{-1} time^{-1}.

$\frac{1}{x}\frac{dx}{dt}$ = μ - specific growth rate.

and $\left(\frac{ds}{dx}\right)_G = \frac{1}{Y_G}$ where Y_G = max. molar growth yield/mol oxygen consumed.

so Rate of oxygen uptake/g dry wt. = $m + \frac{\mu}{Y_{G_{O_2}}}$

Graph of rate of oxygen uptake/g dry wt. against specific growth rate gives a line of slope $\frac{1}{Y_{G_{O_2}}}$ and intercept m and can be used to derive values for $Y_{G_{O_2}}$ and m.

$(\frac{ds}{dt})(\frac{ds}{dt})_t$ need not only be applied to oxygen. It can equally be applied to rate of ATP production. In this case values for Y_G and m are related to yield/mol ATP and maintenance coefficient in terms of ATP.

A similar approach to the calculation of maintenance has been made (Carter et al., 1971).

DEVELOPMENT OF ANALYTICAL METHODS

1. Gas Exchange Measurements

1.1. General considerations

The gas exchange of a culture of bacteria was measured using the apparatus shown in Plate 1. The system enabled both rate of oxygen uptake and of carbon dioxide production to be measured. The environment of the culture was maintained and the culture sampled as described on p. 49.

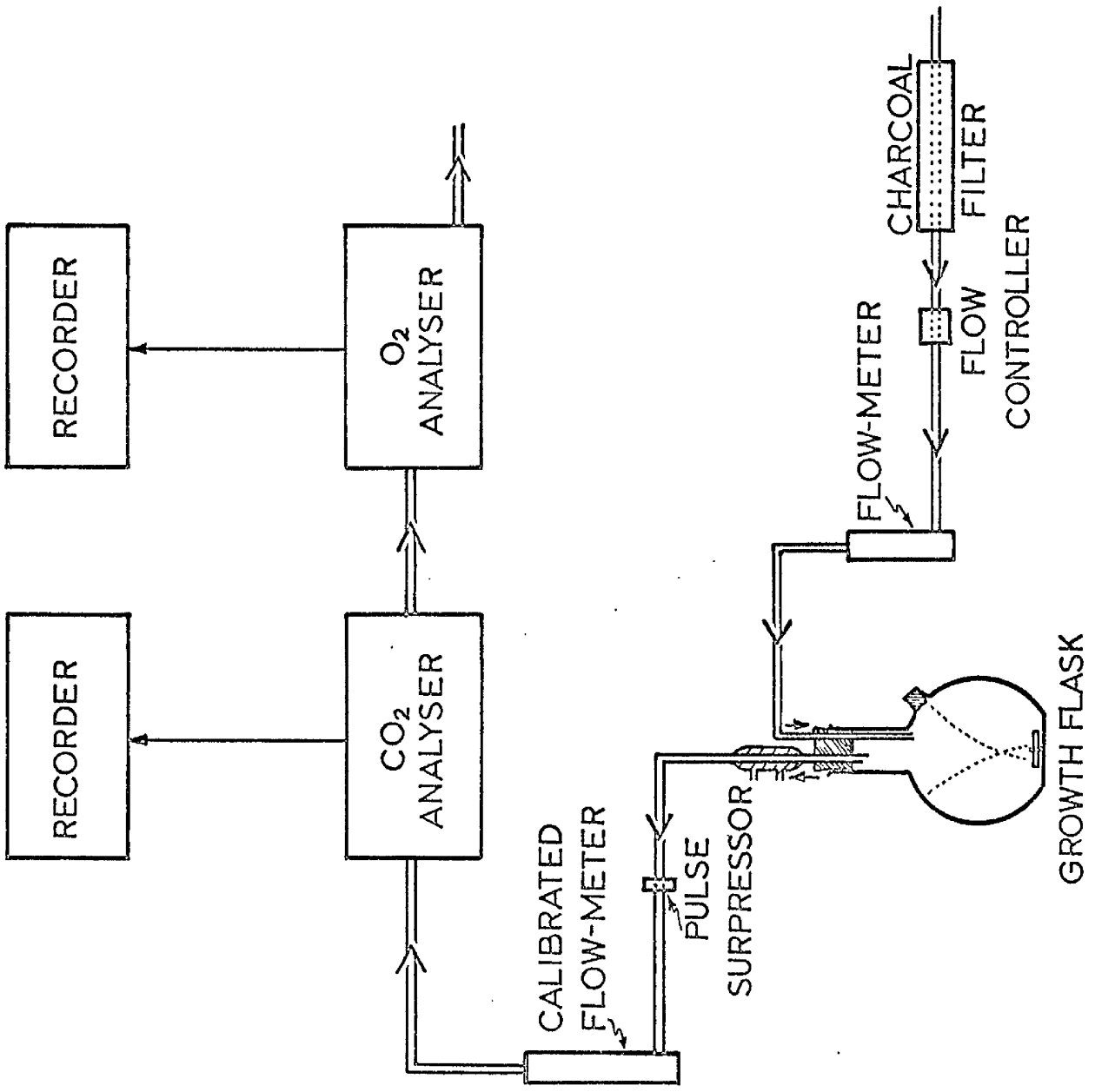
A continuous flow system was used for the measurements (Figure 5). Air, obtained from the departmental compressed air supply, was cleaned by charcoal filtration and monitored for rate of flow before passing into the growth flask. On leaving the growth flask water vapour was reduced by chilling to 2^{o} in a condenser and rate of flow measured in a calibrated GAP-meter (G.A. Platon, Croydon, England). Pulse suppressors could be inserted into the system between the flask and the GAP meter if necessary.

The stream of gas was monitored for carbon dioxide content using a lira 300 carbon dioxide analyser (Mine Safety Appliances, Glasgow) and for oxygen content using an OA184 oxygen analyser (Servomex Controls, Crowborough, England). The output of both analysers was continuously recorded on chart recorders (servoscribe).

The accuracy of the measurements depends on the sensitivity and calibration of the calibrated GAP meter and of the gas analysers. All equipment was calibrated before use.

Figure 5 GAS FLOW DIAGRAM

Air supplied from a compressor was passed into the growth flask at a smooth, constant rate of flow. On leaving the flask water vapour was reduced, rate of gas flow measured and carbon dioxide and oxygen partial pressures monitored.


RECORDER
RECORDER
O_2 ANALYSER
CO_2 ANALYSER
CALIBRATED FLOW-METER
PULSE SURPRESSOR
FLOW-METER
CHARCOAL FILTER
FLOW CONTROLLER
GROWTH FLASK

1.2. Calibration of a GAP meter

All gas flow rates were measured using GAP meters which covered the range 25-500 ml/min. The glass tubes were cleaned by soaking in detergent solution, rinsed copiously with distilled water and dried before assembly. They were fitted so that the tube was in a vertical position and calibrated using a vol-u-meter (model 1056) (Brooks Inst. Ltd., Cheshire, England). The GAP meter was connected in series to the vol-u-meter as shown (Figure 6a) and the rate of flow adjusted to each mark on the GAP meter scale. The vol-u-meter was used to measure the actual rate of gas flow. The GAP meter reading was graphed against the actual gas flow rate to give a GAP meter calibration curve (Figure 6b). Both instruments measure the volume of gas passing in unit time. Since the gas was under identical conditions of temperature and pressure in both the GAP meter and vol-u-meter, the calibration of one with the other was absolute. It was assumed that the temperature and pressure of the gas did not change materially during the short period of each measurement.

All gas measurements were made at atmospheric pressure and room temperature. A manometer next to the flow meter showed that pressure in the flask was not appreciably higher than atmospheric. Room temperature was measured on the day of each experiment.

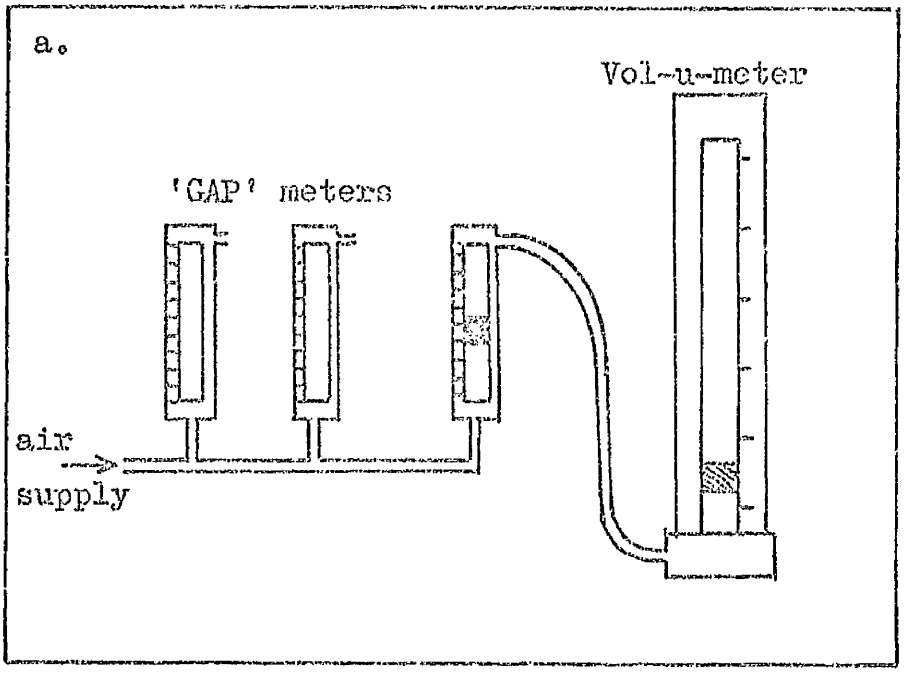
Flow meters and their calibration have been described (Platon, 1970).

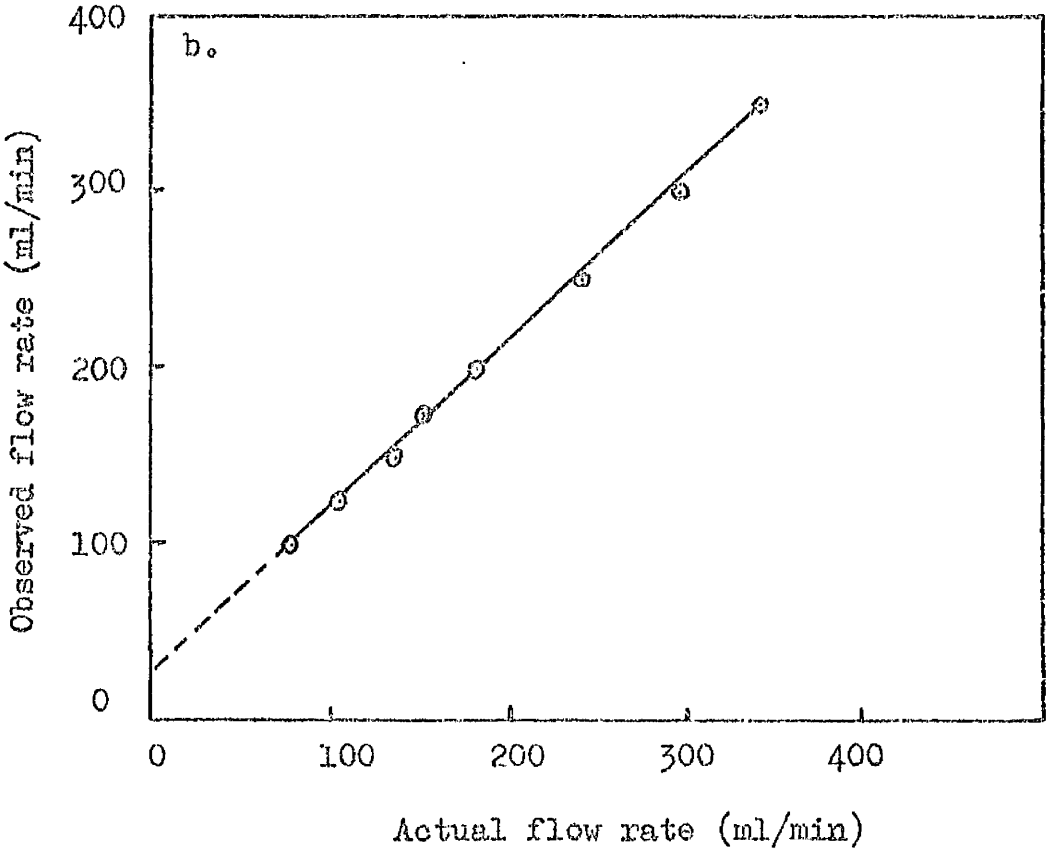
1.3. Calibration of carbon dioxide analyser

Carbon dioxide partial pressure was measured over the range 0 to

Figure 6 CALIBRATION OF GAP METER

A GAP meter was adjusted to a constant flow rate which was measured by collecting a known volume of effluent gas in the vol-u-meter for a measured period of time (Figure 6a). Repetition at flow rates covering the range permitted calibration of individual GAP meters (Figure 6b). It was assumed that the temperature and pressure of the gas was identical in the vol-u-meter and the GAP meter.


a.
Vol-u-meter
'GAP' meters
air
supply


b.
Observed flow rate (ml/min)
400
300
200
100
0
0
100
200
300
400
Actual flow rate (ml/min)

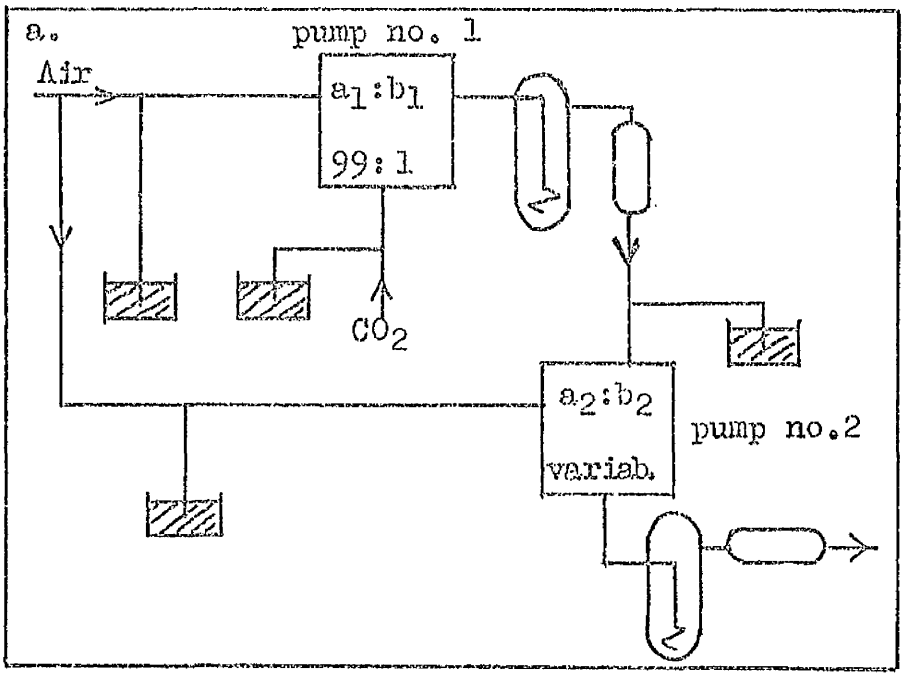
1%. The measurement depends upon absorption of infra-red radiation by carbon dioxide passing through a flow cell which reduces the carbon dioxide specific radiation reaching the detector. The detector contains carbon dioxide which absorbs the unabsorbed carbon dioxide specific radiation coming through 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.

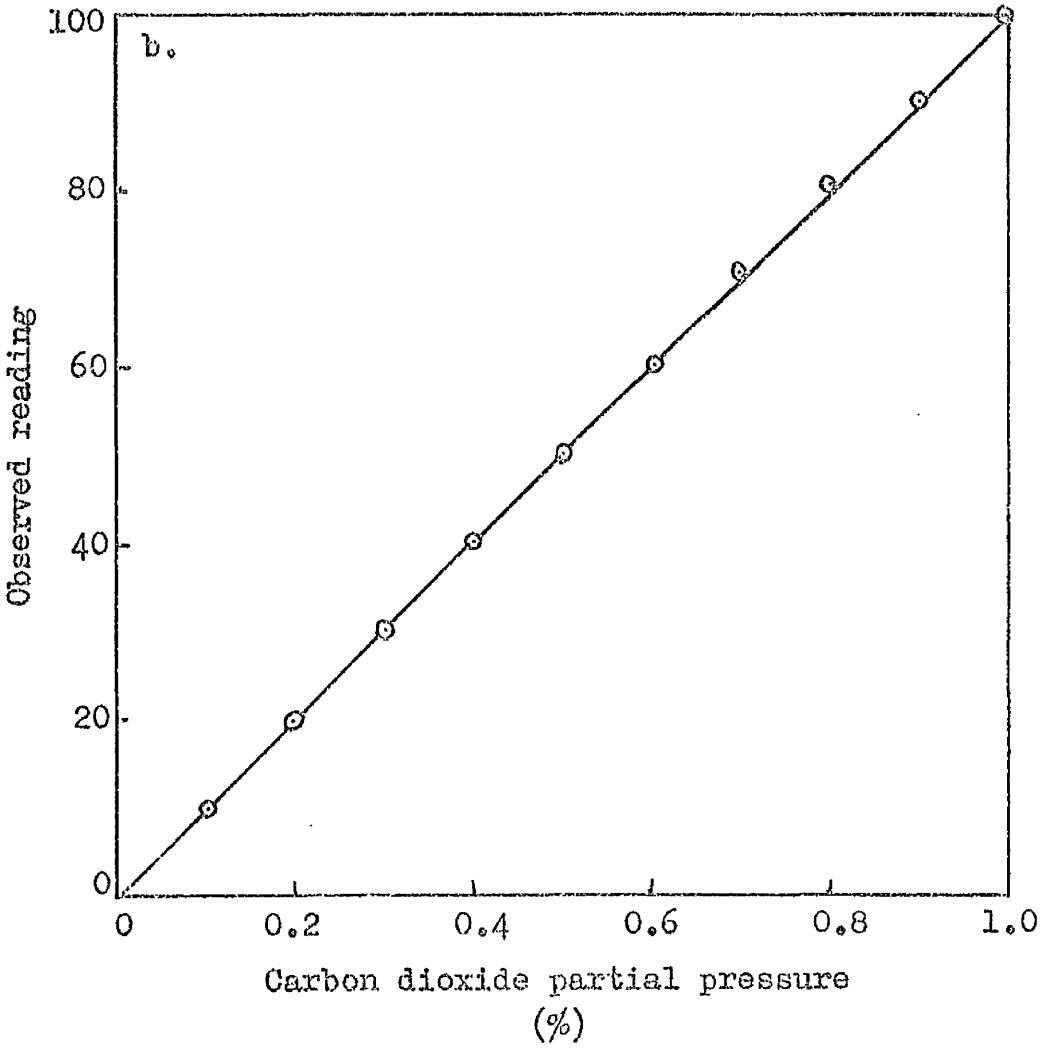
The equipment was calibrated under conditions identical to experimental conditions using two gas mixing pumps (Wösthoff, Bochum, W. Germany) connected in series (Figure 7a) and taking the carbon dioxide content of air as the base line. Using this system the partial pressure of carbon dioxide in the sample gas was varied in steps of 0.1% over the range 0-1%. These values were used to construct a calibration curve (Figure 7b). The calibration was not linear. The gas mixing pumps can be used to calibrate over larger ranges.

A second method of calibration using gas mixtures of known carbon dioxide content between 0 and 1% was used in preliminary experiments. After setting the scale using air and 0.81% carbon dioxide in air, gases of 0.45% and 0.18% carbon dioxide in air were used to check the calibration. This method was not satisfactory because of the difficulty in obtaining mixtures of sufficient accuracy. Some aspects of carbon

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
(%)

dioxide analysers and their calibration have been described (Elsworth, 1970).

1.4. 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. Rotation is balanced by a feedback current applied to a torsion wire such that the dumb-bell remains stationary in the magnetic field. The magnitude of the feedback current is related to the oxygen partial pressure in the gas. The OA184 contains 1 of these systems in each channel and compares gas from one channel with that from the second channel.

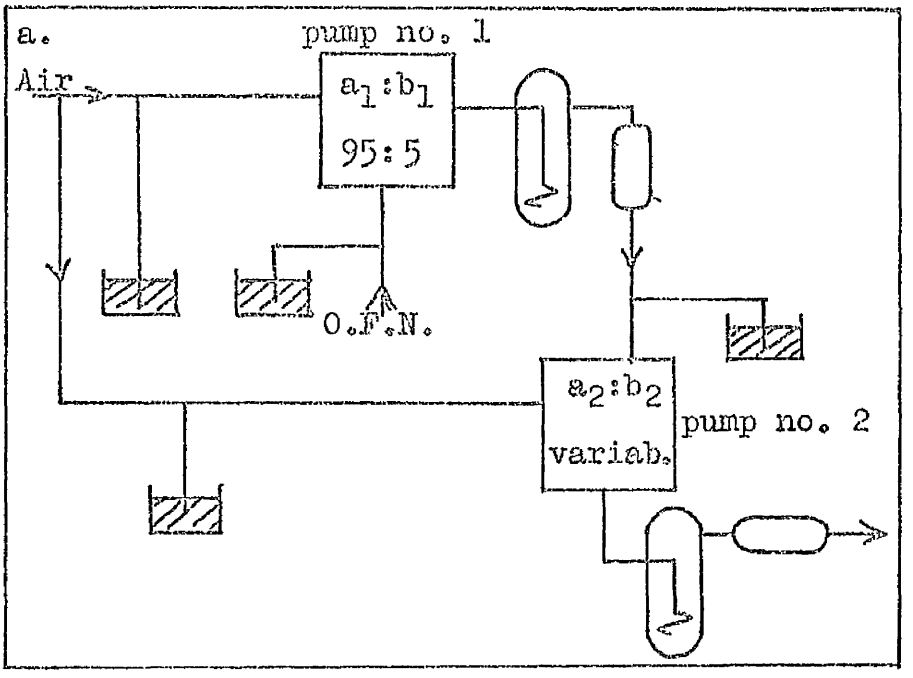
The analyser was calibrated using gas mixing pumps. Using only one pump partial pressures of oxygen from 0 to 100% in 10% steps were produced and showed that both analyser channels had a linear response to the oxygen present in the gas.

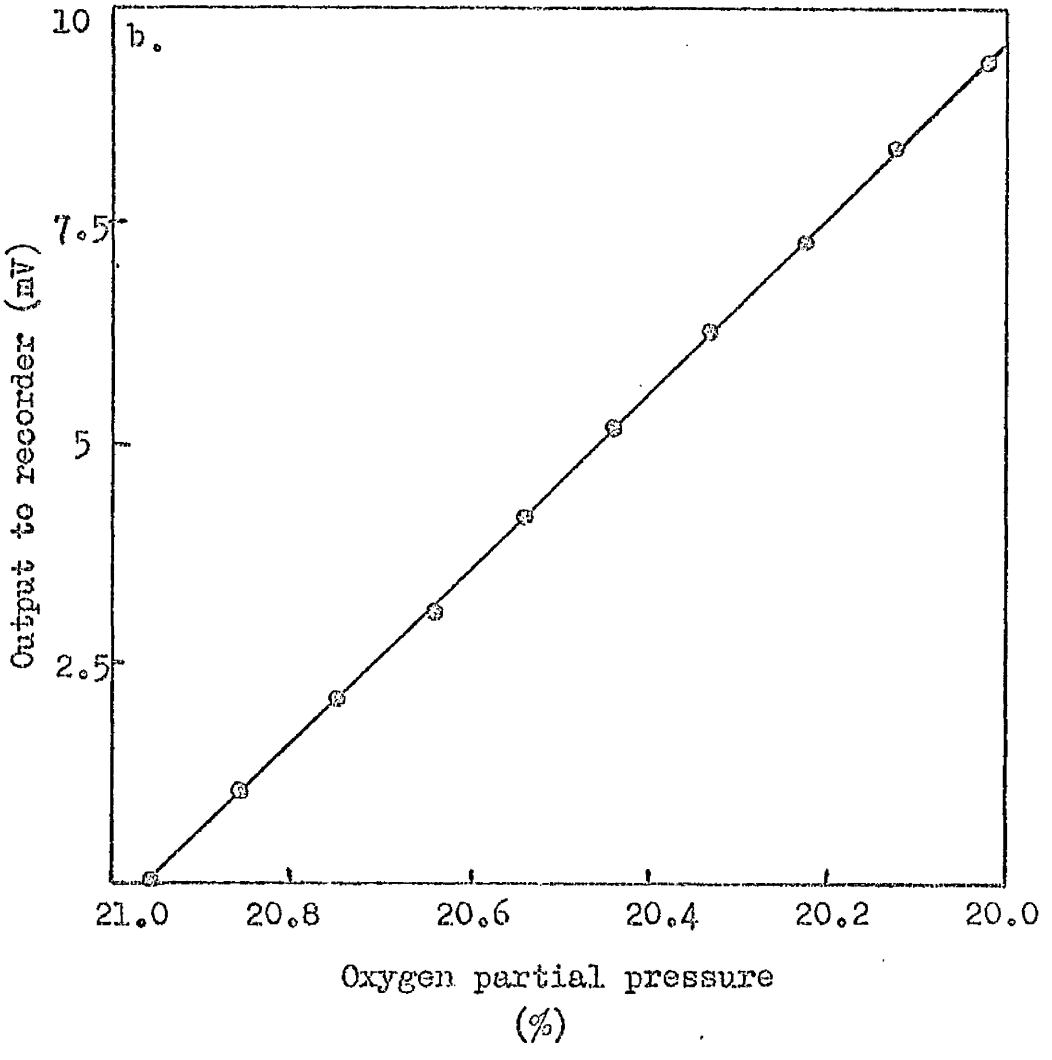
In our experimental conditions air, containing 20.96% oxygen, was used as a standard gas and calibration of one channel was carried out over the range 19.96 to 20.96% oxygen using the second channel at 20.96% oxygen as a reference. To achieve this both gas mixing pumps were used (Figure 8a) to supply gas of known oxygen partial pressure between 19.91% and 20.96% to one channel. Air at 20.96% oxygen was used in the second channel. Care was taken to ensure that gas flow rates were constant at

Figure 8 CALIBRATION OF OXYGEN ANALYSER

The first stage of the Wösthoff pumps was used to dilute air 95 parts in 100 with oxygen free nitrogen and was further diluted in the 2nd stage to yield mixtures within the range 19.91 - 20.96% (Figure 8a). A small portion of the effluent was fed through the gas apparatus and the response of the oxygen analyser recorded. Comparison of these data with the known composition of the mixtures permitted the calibration curve (Figure 8b) to be drawn. Apparatus was calibrated before every occasion of use.

O.F.N. - oxygen free nitrogen


a.
pump no. 1
Air
$a_1 : b_1$
95 : 5
O.F.N.
$a_2 : b_2$
variab.
pump no. 2


b.
10
7.5
5
2.5
Output to recorder (mV)
21.0
20.8
20.6
20.4
20.2
20.0
Oxygen partial pressure
(%)

100 ml/min as these markedly affect the instrument readings. Using these data a calibration curve was drawn to relate analyser output to oxygen partial pressure (Figure 8b). Some aspects of the design and calibration of oxygen analysers have been described (Elsworth, 1970).

1.5. Calculation of data

The analysers only measure gas partial pressures. It is necessary to manipulate these data to obtain results in a more useful form.

1.5.1. Calculation of rate of carbon dioxide production

Carbon dioxide was produced by the cells and appeared as carbon dioxide in the gas phase. The actual volume of carbon dioxide produced is given by the equation

$$\text{Volume} = \frac{CO_2\%}{100} \times \text{flow rate}$$

and is converted to standard gas conditions and to molar units.

$$\text{Gaseous } CO_2 = \frac{CO_2\% \times \text{flow rate}}{100} \times \frac{273}{T^oA \times 22.4}$$

If flow rate is expressed as ml/min

$$\text{Gaseous } CO_2 \text{ prod}^n = \frac{CO_2\% \times \text{flow rate} \times 273 \times 10^4}{T^oA \times 22.4} \text{ n mol/min}$$

This is produced by the whole culture so

$$\text{Gaseous } CO_2 \text{ prod}^n = \frac{CO_2\% \times \text{flow rate} \times 273 \times 10^4}{T^oA \times 22.4 \times \text{culture volume}} \text{ n mol/ml culture/min.}$$

This only measures the carbon dioxide appearing in the gas phase. Considerable quantities of carbon dioxide remain in solution not only as simple dissolved gas but also as bicarbonate.

The actual amount of carbon dioxide dissolved in water is small and depends on the temperature and pressure. If, at 37° and atmospheric

pressure, the gas phase contains 1% carbon dioxide, the aqueous phase will contain 0.253 mM carbon dioxide (Handbook of Chemistry and Physics, 1948).

So carbon dioxide conc^{n} = $CO_2\%$ x 0.253 mM

The amount of bicarbonate in solution depends upon the carbon dioxide concentration and the pH of the solution. These are related by the Henderson-Hasselbalch equation (Umbreit et al., 1957).

$$pH = pK_1 + \log \frac{[HCO_3^-]}{[CO_2]}$$

At a partial pressure of carbon dioxide of 1% and known pH the ratio $\frac{[HCO_3^-]}{[CO_2]}$ can be calculated and the concentration of bicarbonate plus carbon dioxide in solution determined (Figure 9). The effect of pH on total bicarbonate plus carbon dioxide in solution (total solution carbon dioxide) was calculated at each pH and the data used to construct a table (Figure 10). The value obtained at any pH was called the pH factor. The pH factor represents the total solution carbon dioxide as μ mol/ml of culture at 37^{o} and 1 atmosphere pressure when the partial pressure of carbon dioxide in the gas phase is 1%. It also represents the rate of change of total solution carbon dioxide/min under the same conditions when the partial pressure of carbon dioxide in the gas phase is changing at a rate of 1%/min and with the observed rate of change of carbon dioxide partial pressure/min in the gas phase ($\Delta CO_2\%$) gives a measure of the rate of change of total solution carbon dioxide. The rate of carbon dioxide production by the culture is the sum of gaseous and total solution carbon dioxide changes.

Figure 9

pH, DISSOLVED CARBON DIOXIDE AND TOTAL SOLUTION CARBON DIOXIDE

The ratio of bicarbonate to carbon dioxide in solution depends on the pH. Total solution carbon dioxide was calculated using the equation

$$\log \frac{[HCO_3^-]}{[CO_2]} = pH - pK_i$$

where $pK_i = 6.32$ at 37° (Umbreit et al., 1957) and $[CO_2] = 0.253$ mM with 1% carbon dioxide in a gas phase at 37° and atmospheric pressure.

pH	$\log \frac{[HCO_3^-]}{[CO_2]}$	$\frac{[HCO_3^-]}{[CO_2]}$	$\frac{[HCO_3^-]+[CO_2]}{[CO_2]}$	$[HCO_3]+[CO_2]$ mM at 1% CO_2
7.5	1.178	15.07	16.07	4.06
7.4	1.078	11.97	12.97	3.28
7.3	0.978	9.51	10.51	2.66
7.2	0.878	7.55	8.55	2.16
7.1	0.778	6.00	7.00	1.77
7.0	0.678	4.76	5.76	1.45
6.9	0.578	3.78	4.78	1.22
6.8	0.478	3.01	4.01	1.02
6.7	0.378	2.39	3.39	0.86
6.6	0.278	1.90	2.90	0.73
6.5	0.178	1.51	2.51	0.63

Figure 10 TABLE OF pH FACTORS

Calculations, as for Figure 9, were performed for each pH and a table of pH factors constructed. The pH factor is the concentration of total solution carbon dioxide (carbon dioxide + bicarbonate in solution) in μ mol/ml culture at that pH and 37^{o} under atmospheric pressure with 1% partial pressure of carbon dioxide in the gas phase.

pH	.00	.01	.02	.03	.04	.05	.06	.07	.08	.09
7.5	4.060									
7.4	3.280	3.320	3.400	3.480	3.550	3.620	3.705	3.780	3.885	3.990
7.3	2.660	2.710	2.780	2.840	2.890	2.940	3.000	3.060	3.120	3.190
7.2	2.160	2.190	2.230	2.280	2.330	2.390	2.440	2.500	2.545	2.665
7.1	1.770	1.815	1.850	1.885	1.927	1.950	2.005	2.040	2.080	2.110
7.0	1.450	1.480	1.510	1.530	1.565	1.600	1.635	1.675	1.705	1.740
6.9	1.220	1.240	1.260	1.285	1.306	1.330	1.360	1.385	1.400	1.423
6.8	1.010	1.025	1.045	1.065	1.085	1.100	1.130	1.150	1.175	1.200
6.7	.854	.865	.876	.895	.910	.924	.940	.958	.974	.986
6.6	.736	.746	.758	.769	.780	.791	.802	.817	.828	.840
6.5	.638	.645	.656	.664	.672	.682	.696	.706	.714	.726

$$\text{Rate of } CO_2 \text{ prod}^n = \frac{CO_2\% \times \text{flow rate} \times 273 \times 10^4}{T^oA \times 22.4 \times \text{culture volume}} + \text{pH factor} \times \Delta CO_2\% \times 10^3$$

n mol/ml culture/min.

The effect of the solution carbon dioxide on the measurement of culture carbon dioxide production was determined (Figure 11). It is particularly relevant when rapid changes in the rate of carbon dioxide production are taking place.

1.5.2. Calculation of oxygen uptake

The solubility of oxygen in water is low (Handbook of Chemistry and Physics, 1948) so no correction was made for oxygen dissolved in the medium. The partial pressure of oxygen in the incoming gas was not zero and oxygen uptake was calculated on the change in oxygen partial pressure between the incoming and outgoing gas.

$$\text{Vol. of oxygen taken up} = \left(\frac{O_2\% \text{ init.} - O_2\% \text{ final}}{100}\right) \times \text{flow rate}$$

As with carbon dioxide, correction must be made to STP, to molar units and for culture volume.

$$O_2 \text{ uptake} = (O_2\% \text{ init.} - O_2\% \text{ final}) \times \frac{\text{flow rate} \times 273 \times 10^4}{T^oA \times 22.4 \times \text{vol. of culture}}$$

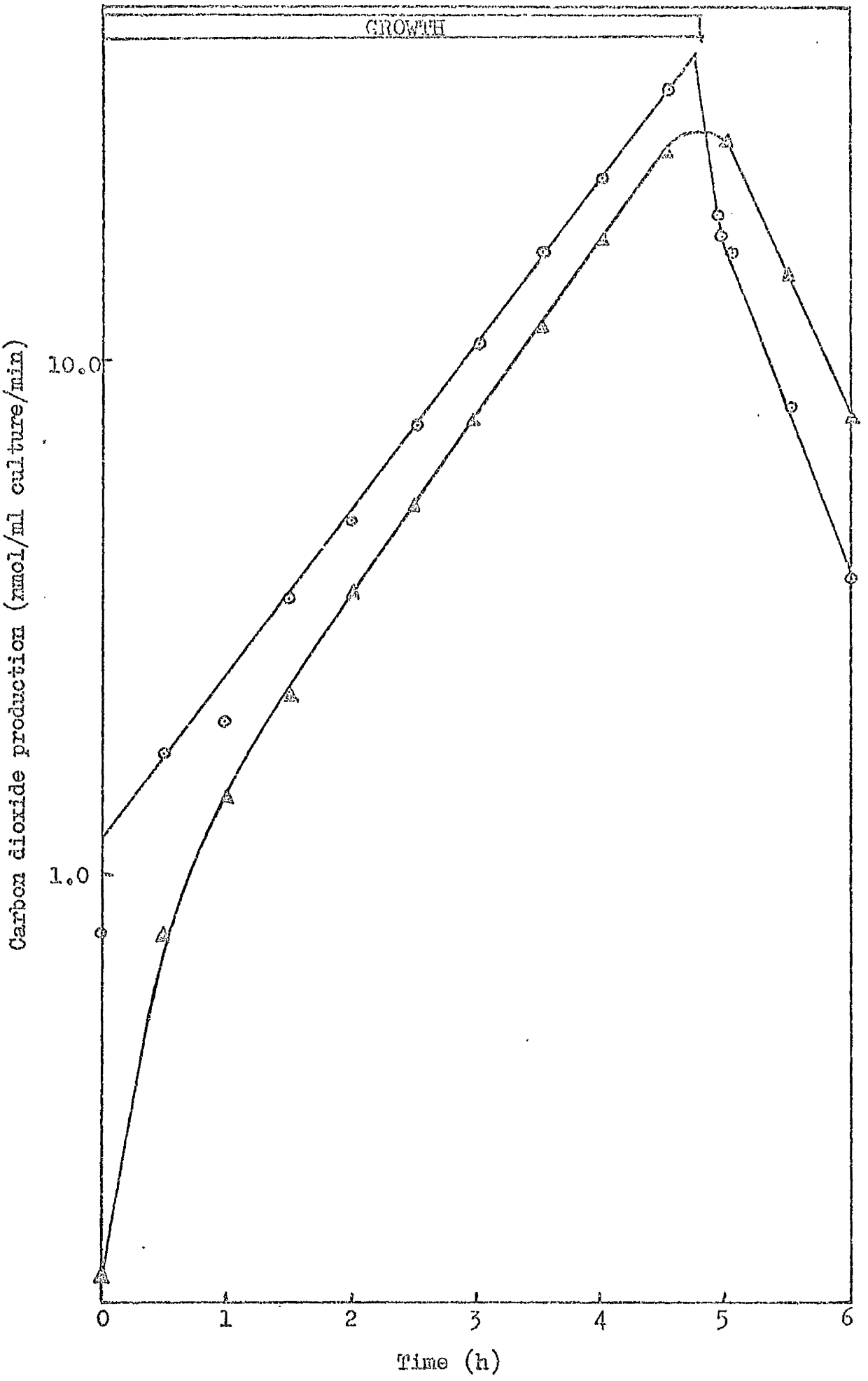
units are n mol/ml/min.

A correction factor was introduced because the volume of gas leaving the flask was not the same as that entering the flask. Addition to or removal from a gas mixture of one specific gas alters the partial pressure of the other gases in the mixture. This applies in this case where oxygen is removed and carbon dioxide added. Calculation corrects for this volume change. Assuming that no oxygen is taken up by the culture, the effect of volume change alone on the oxygen partial pressure

Figure 11

EFFECT OF SOLUTION CORRECTION ON RATE OF CARBON DIOXIDE PRODUCTION

Glycerol trained E.coli 15224 were inoculated into minimal salts medium containing 4 mM glycerol. Growth and carbon dioxide production of the culture were measured. Growth is indicated by the bar at the top of the figure. Carbon dioxide production was calculated using only the carbon dioxide appearing in the gas phase (△—△), and allowing for the carbon dioxide in solution as described in section 1.5.1. (○—○).


GROWTH
Carbon dioxide production (mmol/ml culture/min)
10.0
1.0
0
1
2
3
4
5
6
Time (h)

leaving the flask can be calculated (Pirt & Callow, 1958).

$$\text{Corr } O_2\% \text{ init.} = 20.960 \left(\frac{100 - O_2\% \text{ final} - CO_2\% \text{ final}}{79.040}\right)$$

The difference between Corr $O_2\%$ init. and $O_2\%$ final gives the actual oxygen uptake due to the culture.

$$O_2 \text{ uptake} = \left[20.960 \left(\frac{100 - O_2\% \text{final} - CO_2\% \text{final}}{79.040}\right) - O_2\% \text{final}\right] \frac{\text{flow rate} \times 273 \times 10^4}{T^oA \times 22.4 \times \text{vol. of culture.}}$$

units are n mol/ml culture/min.

The effect of this correction on the oxygen uptake of a culture was determined (Figure 12). It is most important when sudden changes in oxygen uptake are occurring.

1.5.3. Calculation of total gas exchange

The total oxygen taken up or carbon dioxide produced during the growth of the culture was called the accumulative gas exchange and was calculated from the rate of gas exchange data. The calculation is the same for both oxygen uptake and carbon dioxide production and depends on the measurement of the area under the gas exchange curve. As an approximation it was assumed that the area was a trapezium. The nature of the error is seen in Figure 13 and decreases as the time between reading decreases. The accumulative gas exchange taken up between t_1 and $t_2 = \frac{1}{2}(E_2 + E_1)(t_2 - t_1)$ where E_2 and E_1 are rates of gas exchange at time t_2 and t_1 respectively. This calculation is repeated for each time interval and the total accumulated.

$$\text{Accumulative gas exchange} = \frac{1}{2} \sum_{n=1}^{n=n} (E_n + E_{n-1})(t_n - t_{n-1}) \text{ n mol/ml culture}$$

It applies to both oxygen uptake and carbon dioxide production but

Figure 12

EFFECT OF VOLUME CORRECTION ON CALCULATION OF OXYGEN UPTAKE

E.coli 15224 trained to glycerol were inoculated into minimal salts medium containing 4 mM glycerol. Growth and oxygen uptake of the culture were measured. Growth is shown by the bar at the top of the diagram. Oxygen uptake was calculated without volume correction (△—△) and allowing for volume correction as described in section 1.5.2. (○—○).

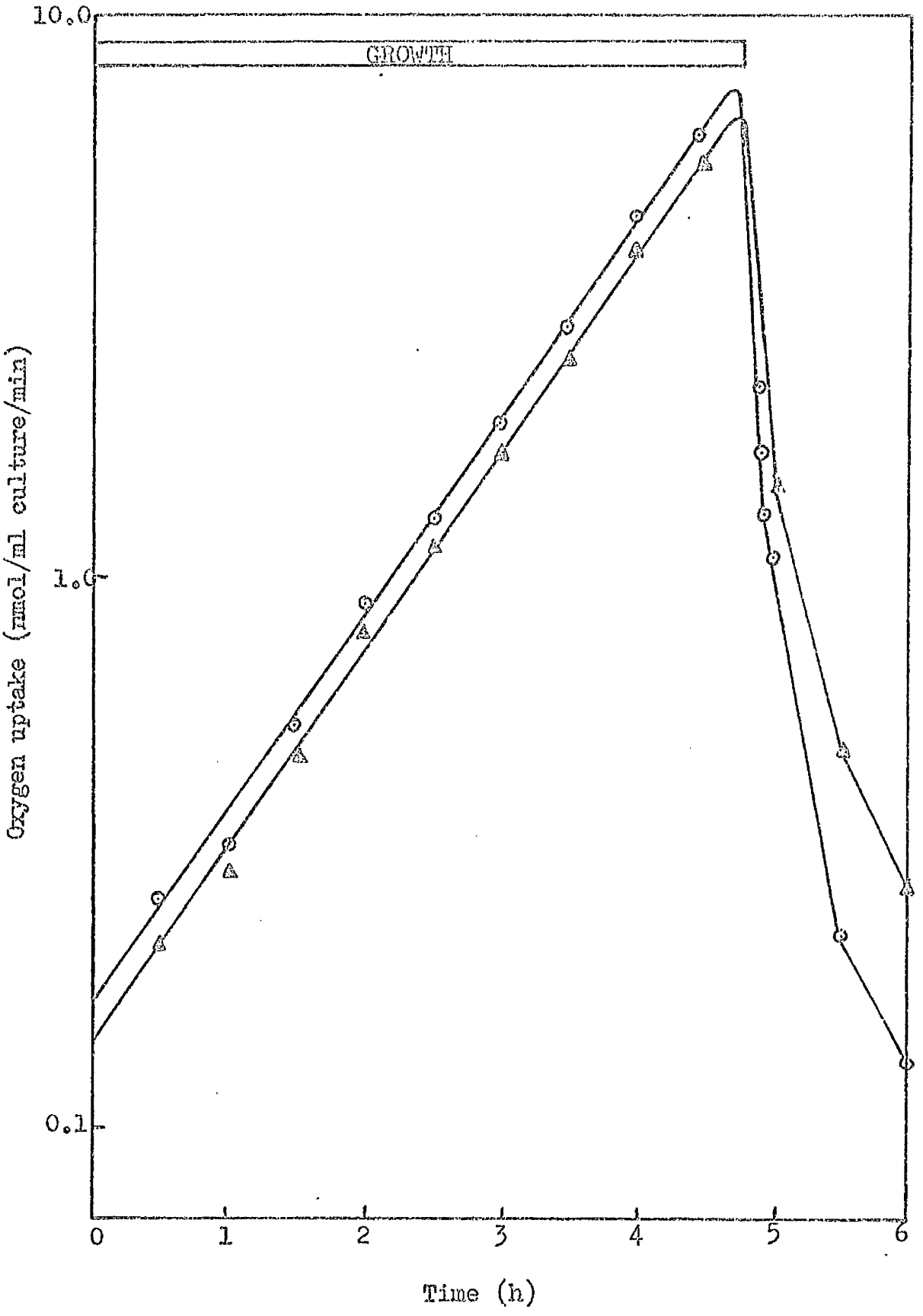
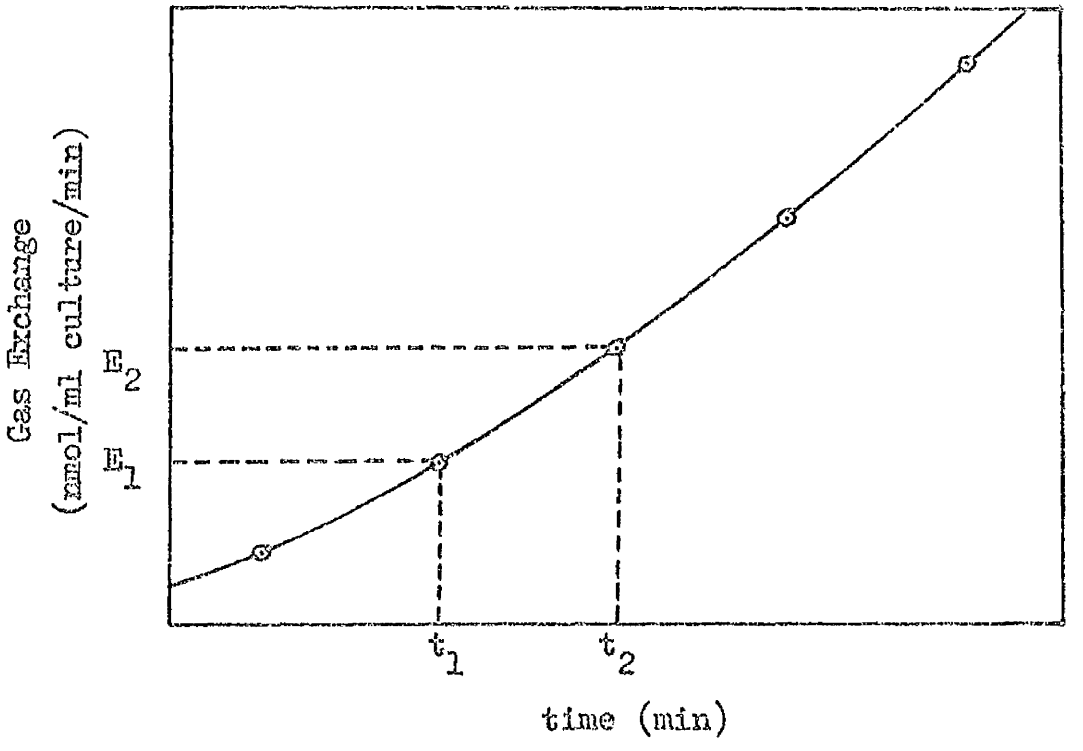

10.0
GROWTH
1.0
0.1
Oxygen uptake (nmol/ml culture/min)
0
1
2
3
4
5
6
Time (h)

Figure 13 CALCULATION OF ACCUMULATIVE GAS EXCHANGE

Accumulative gas exchange is calculated as the area under the rate of gas exchange curve. The method of calculation as shown is an approximation and the nature and size of the error is shown. The size of the error decreases as the time interval between t_1 and t_2 decreases.


Gas Exchange
(mmol/ml culture/min)
E_2
E_1
t_1
t_2
time (min)

requires that the final gas partial pressure is the same as the initial value.

1.6. Effect of flask dead space

The dead space in the flask was defined as that volume of gas in the flask above the culture. It reduces measurement sensitivity and speed of response to changes in gas exchange. The capacity of the dead space varied between 250 and 400 ml during an experiment and affects both rate of gas exchange and accumulative gas exchange measurements. The effect is greater over short periods of time.

1.6.1. Nature of dead space effect

The nature of the effect of dead space was examined by passing oxygen free nitrogen into a flask containing 800 ml medium with an air atmosphere to simulate a sudden change in gas exchange. The partial pressure of oxygen leaving the flask was monitored and decreased over several minutes in a 1st order process of specific half life (Figure 14) which depended on the volume of the dead space and on the rate of gas flow (Figure 15).

1.6.2. Dead space and rate of gas exchange

Rate of gas exchange measurements were usually made over several hours. Half life of the dead space was 2-5 min so under usual growth conditions the magnitude of the dead space effect was small and no correction was made. Correction became necessary when sharp changes in gas exchange occurred such as at the end of growth. Under these circumstances a correction was attempted (Figure 16). Knowing dead space volume and gas flow rate a half life was assessed using Figure 15.

Figure 14

EFFECT OF FLASK DEAD SPACE ON MEASUREMENTS OF GAS EXCHANGE

A change in the rate of gas uptake or production, in a growth flask, is buffered by dilution in the dead space before being measured on the gas analysers. To assess this effect a growth flask containing 800 ml of minimal salts medium (dead space 300 ml) was equilibrated with air at 100 ml/min and the oxygen content of the effluent gas recorded. The air flow was disconnected and replaced by a stream (100 ml/min) of oxygen free nitrogen and the oxygen content measured as before. The data obtained (Figure 14) permit calibration of this dilution rate as a half-life.

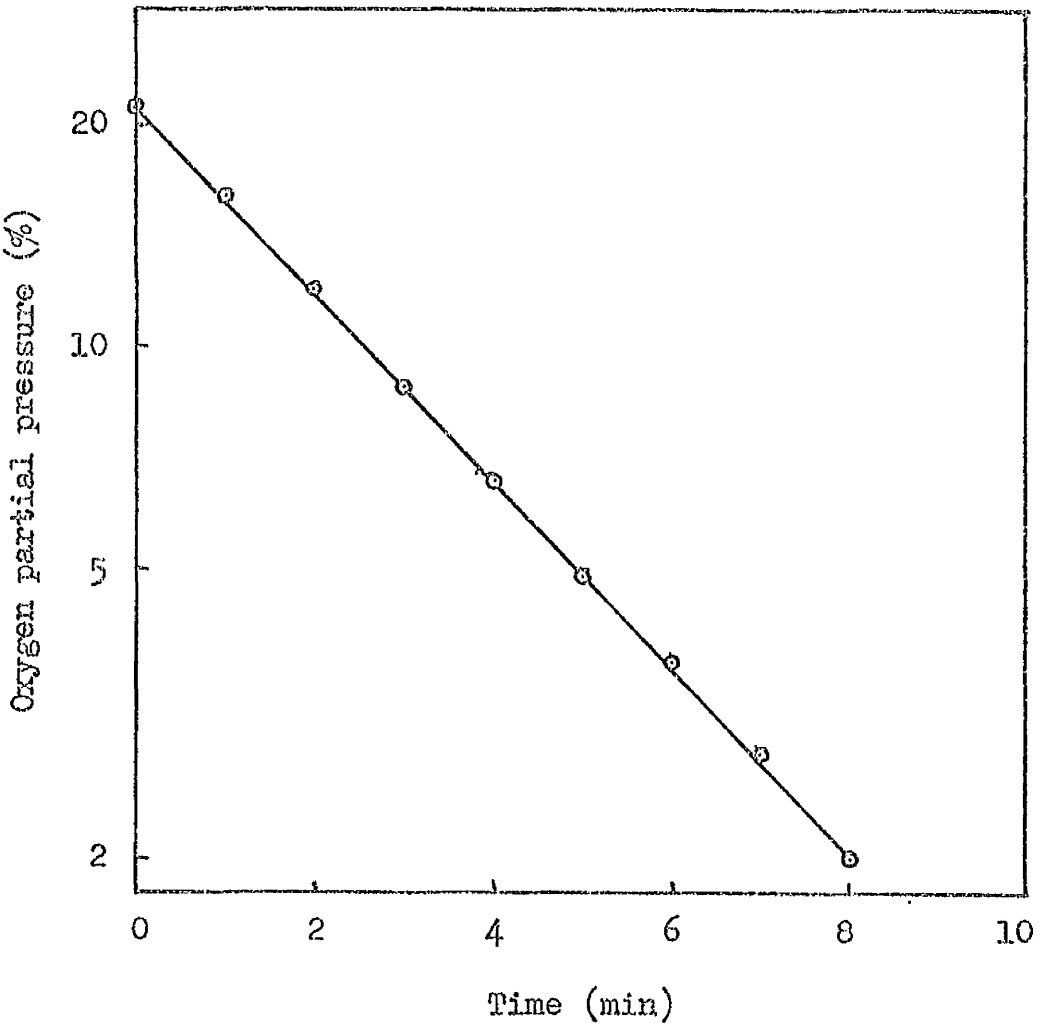

Oxygen partial pressure (%)
20
10
5
2
0
2
4
6
8
10
Time (min)

Figure 15

GAS FLOW RATE, FLASK DEAD SPACE AND HALF-LIFE OF PARTIAL PRESSURE CHANGE

The dilution effect of dead space depends upon its precise volume and the rate of gas flow. The effects of both were measured as described in Figure 14 and its relationships are shown in Figure 15.

o—o 100 ml/min

Δ—Δ 150 ml/min

⊙—⊙ 200 ml/min

▲—▲ 300 ml/min

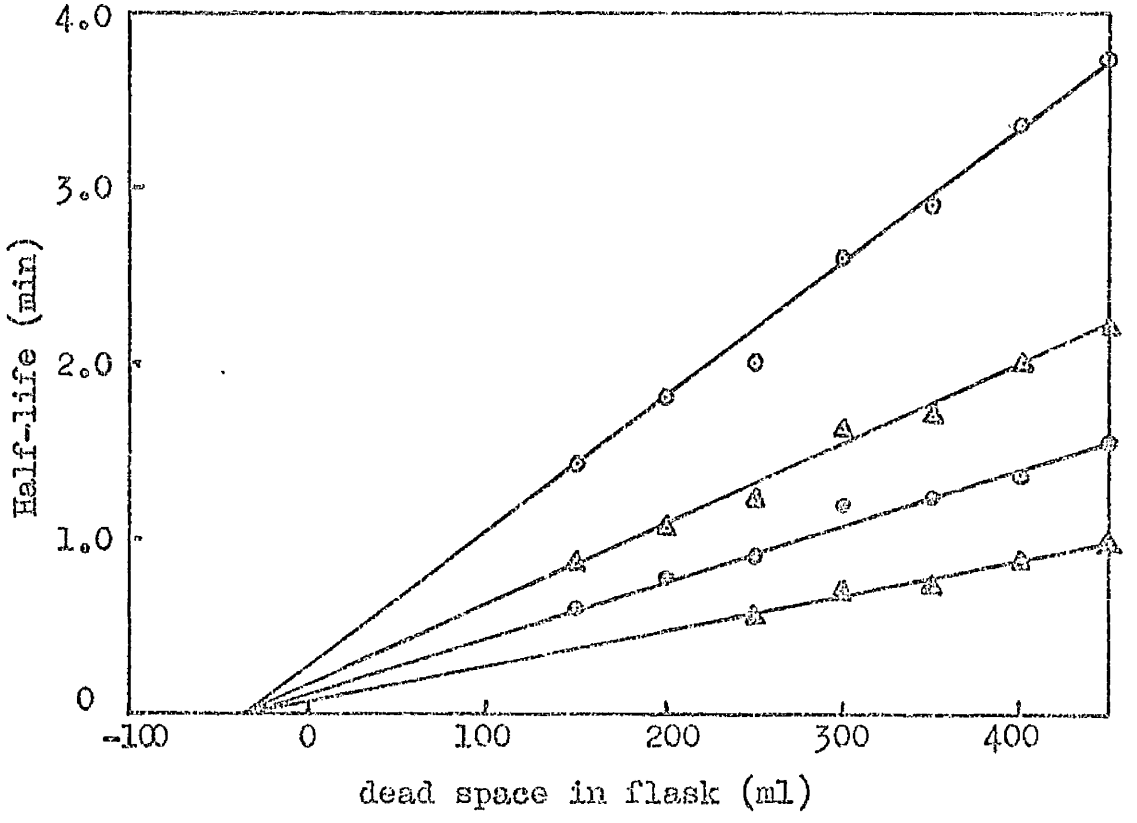
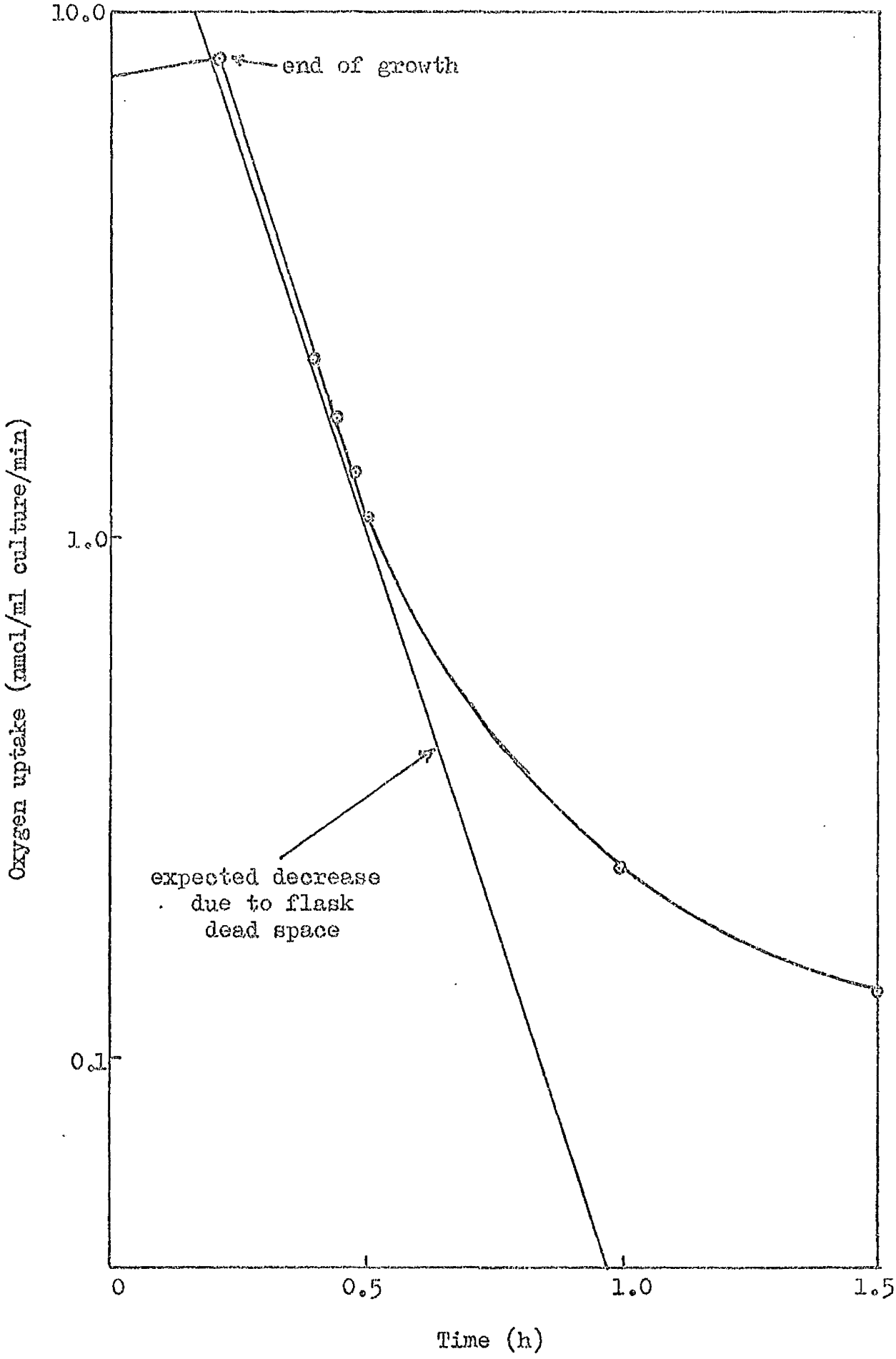

4.0
3.0
2.0
1.0
0
Half-life (min)
-100
0
100
200
300
400
dead space in flask (ml)

Figure 16 CORRECTION FOR FLASK DEAD SPACE

The effect of correction of dead space is only significant when the rate of change of gas exchange is greatest. This is shown in Figure 16 for a culture in glycerol (4 mM) salts medium at the time when the carbon source is exhausted.


10.0
1.0
0.1
end of growth
expected decrease due to flask dead space
Oxygen uptake (nmol/ml culture/min)
0
0.5
1.0
1.5
Time (h)

The gas exchange at time zero was assumed to decrease in a 1st order process with that half life. The difference between this half life process and the actual data was taken as the actual gas exchange. The procedure is subject to quite large errors because it measures a small difference between 2 large numbers.

1.6.3. Dead space and accumulative gas exchange

The dead space has a significant effect if accumulative gas exchange is measured over a short period of time or if the final partial pressure of the gas leaving the flask has not returned to its zero time value. To assess this the gas was considered as a two phase system - one part being gas which had entered the flask with one composition and left with a different composition and the second being gas in the flask at zero time whose composition had altered.

The 1st part is the fraction already described for accumulative gas exchange (section 1.5.3.) and is assessed as

$$\tfrac{1}{2}\sum_{n=1}^{n=n}(E_n + E_{n-1})(t_n - t_{n-1})$$

The second part for oxygen uptake depends on dead space volume and change in gas composition. It is assessed as

$$\frac{(\text{Corr. } O_2\%_n - \text{Corr. } O_2\%_o) \times \text{dead space} \times 10^4 \times 273}{22.4 \times \text{vol. in flask} \times T^oA}$$

The total oxygen uptake is

$$\tfrac{1}{2}\sum_{n=1}^{n=n}(E_n+E_{n-1})(t_n-t_{n-1}) + \frac{(\text{Corr.}O_2\%_n - \text{Corr.}O_2\%_o) \times \text{dead space} \times 10^4 \times 273}{22.4 \times \text{vol. in flask} \times T^oA}$$

units are n mol/ml culture.

For carbon dioxide production a similar situation exists but change in solution carbon dioxide must also be considered.

The total carbon dioxide production is

$$\frac{1}{2}\sum_{n=1}^{n=n}(E_n+E_{n-1})(t_n-t_{n-1})+(CO_2\%_n - CO_2\%_o)\left(\frac{\text{dead space x273x10}^4}{22.4 \text{ x volume x } T^oA} + \text{pH factor x10}^3\right)$$

Using these formulae accumulative gas exchange over a short period of time can be estimated.

1.7. Difficulties encountered in setting up the gas exchange apparatus

While setting up the apparatus for measurement of gas exchange several technical problems arose. These were associated, essentially, with the oxygen analyser and have been overcome with, in some cases, a reduction in apparatus sensitivity. The oxygen analyser is very sensitive to fluctuations in electrical supply, to the operation of other pieces of equipment in the vicinity and to pulses in the sample gas flow system. As a result of these factors a considerable noise problem in the analyser output was reducing the sensitivity of the analyser.

The major difficulties associated with electricity supply were overcome by installing a special circuit of screened cable from the fuse box which is exclusively used for the oxygen analyser, and greatly reduces both the electrical supply noise and the noise caused by many other pieces of equipment - presumably by reducing feedback through a common electrical circuit.

A cooling unit used to provide the supply of 10% propanol to the condensers caused noise on the output whenever the coolant circuit cut in. Whether this was due to vibrational or electrical noise is not known but the cooling unit was replaced by a pump and ice-water slurry with a considerable improvement in the output signal. Further pick-up of this

type was reduced by using screened output leads.

The sensitivity and suppressed zero of the analyser also created problems. If dry air is supplied to the analyser reference channel and air from a growth flask to the sample channel there is a difference of over 1% in the readings even in the absence of oxygen uptake which is due to saturation of the sample gas with water vapour. Water vapour also condenses in the tubing causing blockages to flow. This problem was overcome by passing both sample and reference gas through growth flasks then removing 90% of the water vapour in the effluent gas by using condensers maintained at 2°.

The method of mixing the growth flask forces the gas to leave the flask as a series of pulses and not as a steady flow causing analyser output to fluctuate and appearing as noise on the output trace. It was only slightly reduced by the use of pulse suppressors but was greatly reduced by connecting a capacitor (200 μF) across the terminals of the recorder. Although this remedy increases the analyser response time the reduction in noise in the output signal makes the system advantageous overall.

The other major difficulty is associated with the measurement of gas flow rate. All gas flow rate measurements were made using calibrated GAP meters but recent calibrations suggest these were not as accurate as was believed. GAP meter calibrations were always carried out after a fresh GAP meter had been set up and allowed to equilibrate to gas flow overnight and were assumed to be constant. Calibrations done on several consecutive days showed a gradual decrease in the flow

rate required to give the same GAP meter reading. The reason for this is not known but it may be due to dirt on the float or glass tube. No visible evidence of this was seen. It is fortunate in retrospect that calibrated flow meters were frequently changed for cleaning and consequently recalibrated. The error involved in flow rate measurement is the greatest in the gas exchange estimating system and may be as great as 10% although no accurate data are available.

Other problems associated with sampling of the culture and equipment calibration have already been examined in the appropriate sections.

2. Malic Enzyme Activity

2.1. General considerations

Malic enzyme activity was measured in extracts of cells of E.coli 15224. The activities of both NAD- and NADP-linked malic enzymes were expected to be low and variable. Consequently the assays developed for these enzyme activities permitted maximal activity to be measured and gave a measurement of activity related to the enzyme present in the cells.

2.2. NADP-linked malic enzyme (E.C. 1.1.1.40)

2.2.1. Assay conditions

A cell suspension of E.coli 15224, trained to malate, was subjected to ultrasonic irradiation to provide an extract which could be used to determine the best conditions for assay of malic enzyme activity.

Specific activity in the assay was highest at pH 7.2 in tris or glycylglycine buffer. Tris (pH 7.2) was used at a concentration of 20 mM. The buffer concentration was not critical.

The enzyme required the addition of both malate and NADP to the assay and, in the absence of either, no activity was detected. Maximal specific activity was obtained with 10 mM malate and 1 mM NADP although at higher concentrations the activity was reduced. Reciprocal plots of the data obtained at low concentrations gave a Km for malate of 4.2×10^{-3} M and for $NADP^+$ of 3.8×10^{-5} M. Similar values have been reported (Sanwal and Smando, 1968).

The enzyme required 0.2 mM Mn^{++} for maximal activity but was not inactive in the absence of added Mn^{++}, presumably due to trace amounts of Mn^{++} or Mg^{++} in the extracts. Concentrations higher than 0.2 mM caused inhibition.

As a result of these studies the conditions of assay were established as shown on p. 63. Similar conditions have been described (Ochoa, 1955a; Sanwal and Smando, 1968; Takeo, 1969; Murai et al., 1971). No NADPH oxidase or NADP/NAD transhydrogenase activities were detected.

2.2.2. Extraction of enzyme from cells

The procedure chosen to extract the enzyme from the cells was ultrasonic irradiation. Conditions which resulted in enzyme stability and maximal release from the cells were investigated.

2.2.2.1. Stability of the enzyme in extracts

Extracts of malate trained cells were prepared by ultrasonic irradiation of cells which had been resuspended in a variety of media. The final resuspension conditions chosen (p. 62) were identical to those used by Holms and Bennett (1970) to measure isocitrate dehydrogenase activity and resulted in an extract of high specific activity which showed no measurable loss of enzyme activity over a 5 h period. Omission of either sodium chloride or BSA from the resuspension medium resulted in an extract of low activity which lost between 5 and 15% of that activity over a 1 h period at 0^{o}. Replacing phosphate buffer by either tris or glycylglycine buffer at the same pH and concentration also resulted in an extract of low activity which could lose as much as 50% of that activity in 1 h at 0^{o}.

Enzyme activity over a 10-fold range was proportional to the amount of extract added. Centrifugation of the extract at 22,000 *g* and 4° for 20 min did not alter the activity.

2.2.2.2. Disruption of the cells

Extracts were prepared by ultrasonic irradiation of 3 ml aliquots of cells in resuspension medium contained in a glass bottle maintained at 0° as described (Holms and Bennett, 1971). Ultrasonic irradiation at a power output of 2A over a 3 min period (6 x ½ min) was sufficient to obtain maximal release of enzyme from the cells. Irradiation for longer periods of time up to 7 min (14 x ½ min) did not result in any loss of activity.

Release of enzyme from the cells, after a 3 min period of irradiation, was proportional (over a 20-fold range) to cell density.

2.3. NAD-linked malic enzyme (E.C. 1.1.1.38)

2.3.1. Assay conditions

Using identical extraction conditions as with the NADP-linked enzyme, but with the additional step of ultracentrifugation at 125,000 *g* and 4° for 90 min, an extract was prepared for assay of NAD-linked malic enzyme activity.

The conditions used to assay NADP-linked malic enzyme, but with NAD in place of NADP, were initially used to assay the NAD-linked malic enzyme activity.

Despite ultracentrifugation, there was still some residual NADH oxidase activity in the extracts which was measured in a similar assay mixture in which malate was replaced by NADH (0.05 mM). Because NADH

at a concentration of 0.05 mM had no effect on the measurement of the NAD-linked malic enzyme activity it was also added to the NAD-linked malic enzyme assay.

Under the assay conditions as described on p. 64, the activity of both enzymes measured was proportional to the amount of extract added. Total NAD-linked malic enzyme activity was calculated as the sum of apparent NAD-linked malic enzyme activity and the NADH oxidase activity. Total activity was proportional to the amount of cell extract added over a 15-fold range of enzyme concentration.

2.3.2. Preparation of cell extracts

Extracts prepared for assay of NADP-linked malic enzyme activity, as described on p. 96, then spun at 125,000 g and 4° for 90 min, were found to be suitable for the assay of NAD-linked malic enzyme activity.

In the extracts, chilled at 0°, the NAD-linked malic enzyme activity showed no loss of activity over a period of 4 h. 3 min of ultrasonic irradiation was sufficient to give maximal release of enzyme from the cells, and although longer periods of irradiation, up to 7 min, did not result in a loss of NAD-linked malic enzyme activity, the amount of NADH oxidase remaining in the 125,000 g supernate increased with the period of irradiation.

In extracts prepared as above, enzyme activity was proportional to the density of cells disrupted over a 10-fold range.

2.3.3. Effect of ultracentrifugation on activity

Ultracentrifugation was used to remove NADH oxidase activity from the cell extracts (Katsuki et al., 1967) but also sedimented some of

the malic enzyme activity. The loss of activity was examined quantitatively by centrifuging identical extracts at different g and measuring NADH oxidase and NAD-linked malic enzyme activities in the supernates. The specific activity of both enzymes in the supernates decreased with increasing g. After centrifugation at 125,000 g and 4° for 90 min over 90% of the oxidase activity but only 8% of the NAD-linked malic enzyme activity had been lost. Consequently ultracentrifugation, as used to sediment NADH oxidase activity, did not result in a loss of malic enzyme activity to any great extent.

2.4. Storage of malic enzyme activities

Cells could not always be assayed immediately after harvesting and the stability of malic enzyme activities in cell pellets was investigated. Cell pellets were prepared from logarithmically growing cells and stored at 0° and at -70°.

At 0° no enzyme activity was lost over a period of 6 h but longer storage resulted in a loss of activity of both NADP and NAD-linked malic enzymes.

At -70° no NADP-linked malic enzyme activity was lost over a 20 day period. NAD-linked malic enzyme activity was not so stable and lost activity to the extent of 5% over a period of 6 days.

2.5. Summary

The methods used to measure malic enzyme activities were established. Activity measured in the assays was proportional to the amount of enzyme added and to the enzyme present in the cells prior to ultrasonic irradiation. The assays therefore provide an accurate

measure of enzyme activity irrespective of the quantity of enzyme present either in the extract or in the cell suspension.

3. Pyruvate Production in Inhibited Washed Cell Suspensions

The rate of production of pyruvate from malate was measured in washed cell suspensions. Cells were harvested and resuspended in chilled 40 mM phosphate buffer before inoculation into the assay solutions (methods 12, p. 65).

The rate of utilisation of pyruvate by a washed cell suspension of E.coli 15224 was much greater than the rate of pyruvate production from malate. Consequently the rate of pyruvate utilisation was inhibited with sodium arsenite, which reduces pyruvate dehydrogenase activity and so the rate of pyruvate decarboxylation. The effect of different concentrations of sodium arsenite on pyruvate utilisation in a washed cell suspension of malate trained E.coli over a period of 45 min was examined (Figure 17a). From these data the rate of pyruvate disappearance in the washed cell suspension could be calculated and graphed against arsenite concentration (Figure 17b). 1 mM arsenite gave a 95% inhibition of the rate of pyruvate metabolism.

The inhibition of pyruvate utilisation by arsenite was independent of the nature of the cells - malate, glucose and glycerol trained cells being affected similarly by different concentrations of arsenite (Figure 18).

The rate of pyruvate utilisation by a cell suspension in the presence of 1 mM arsenite was independent of the concentration of pyruvate in the medium over the range 0.2 mM to 1 mM pyruvate (Figure 19).

The rate of production of pyruvate from malate was measured in

Figure 17

ARSENITE INHIBITION OF PYRUVATE UTILISATION

Malate trained cells of E.coli 15224 were harvested from the logarithmic phase by centrifugation at 11,600 g and 4° for 10 min and resuspended in chilled 40 mM phosphate buffer pH 7.0 to a cell density of 1 mg dry wt./ml. The cell suspension was incubated for 5 min at 37° and 4 ml added to tubes containing 640 μ moles phosphate buffer pH 7.0, 20 μ moles sodium pyruvate and different amounts of sodium arsenite in a total volume of 16 ml. Samples were removed from the tubes at intervals over 45 min, treated as described on p. 54, pyruvate concentration determined using the method on p. 56 and graphed against time (Figure 17a).

○—○ control

◎—◎ 1 mM Arsenite

△—△ 2 mM Arsenite

▲—▲ 4 mM Arsenite

▣—▣ 10 mM Arsenite

Rate of pyruvate utilisation was calculated from the slopes of these lines and graphed against arsenite concentration (Figure 17b).

a.
Pyruvate concn. (mM)
1.0
.8
.6
.4
.2
0
0
15
30
45
Time (min)

b.
Rate of pyruvate utilization (nmol/mg dry wt./min)
300
200
100
0
0
2
4
6
8
10
Arsenite concn. (mM)

Figure 18

ARSENITE INHIBITION IN DIFFERENT PHENOTYPES

Cells trained to glucose, glycerol and malate were prepared and inoculated into buffered medium containing pyruvate and different concentrations of arsenite as described for Figure 17.

The rate of pyruvate utilisation in each of the cultures was measured as described for Figure 17. The activity remaining in the presence of arsenite was expressed as a percentage of the uninhibited rate of pyruvate utilisation.

Arsenite Conc^{n}. (mM)	Malate grown cells activity (nmol/mg dry wt./min)	(%)	Glycerol grown cells activity (nmol/mg dry wt./min)	(%)	Glucose grown cells activity (nmol/mg dry wt./min)	(%)
0	299	100	244	100	177	100
1	13.1	4.4	10.8	4.5	8.7	5.0
2	5.0	1.7	6.0	2.5	2.2	1.2
4	3.0	1.0	3.2	1.3	1.6	0.9
10	1.1	.4	1.6	.6	0	0

arsenite inhibited systems as the sum of pyruvate produced from malate and pyruvate utilised in the absence of malate. The rate at which malate was decarboxylated to pyruvate was also independent of the concentration of pyruvate in the medium (Figure 20).

Figure 19

PYRUVATE CONCENTRATION AND RATE OF UTILISATION OF PYRUVATE

A cell suspension of logarithmic phase, malate trained cells at a cell density of 1 mg dry wt./ml in chilled 40 mM phosphate buffer pH 7.0, was incubated 5 min at 37° and 4 ml added to tubes containing 640 µ mol phosphate buffer pH 7.0, 20 µ mol sodium arsenite and different quantities of pyruvate in a total volume of 16 ml. Samples were taken, treated and assayed for pyruvate in the medium as described for Figure 28. Pyruvate concentration was plotted against time.

Figure 20

PYRUVATE CONCENTRATION AND RATE OF PYRUVATE PRODUCTION FROM MALATE

4 ml of the cell suspension described for Figure 29 was added, after 5 min incubation at 37°, to tubes containing 400 µ mol sodium malate in addition to phosphate buffer pH 7.0, arsenite and different concentrations of pyruvate in a total volume of 16 ml. Changes in pyruvate concentration were again measured over a 45 min period as described for Figure 18 and plotted against time of incubation.

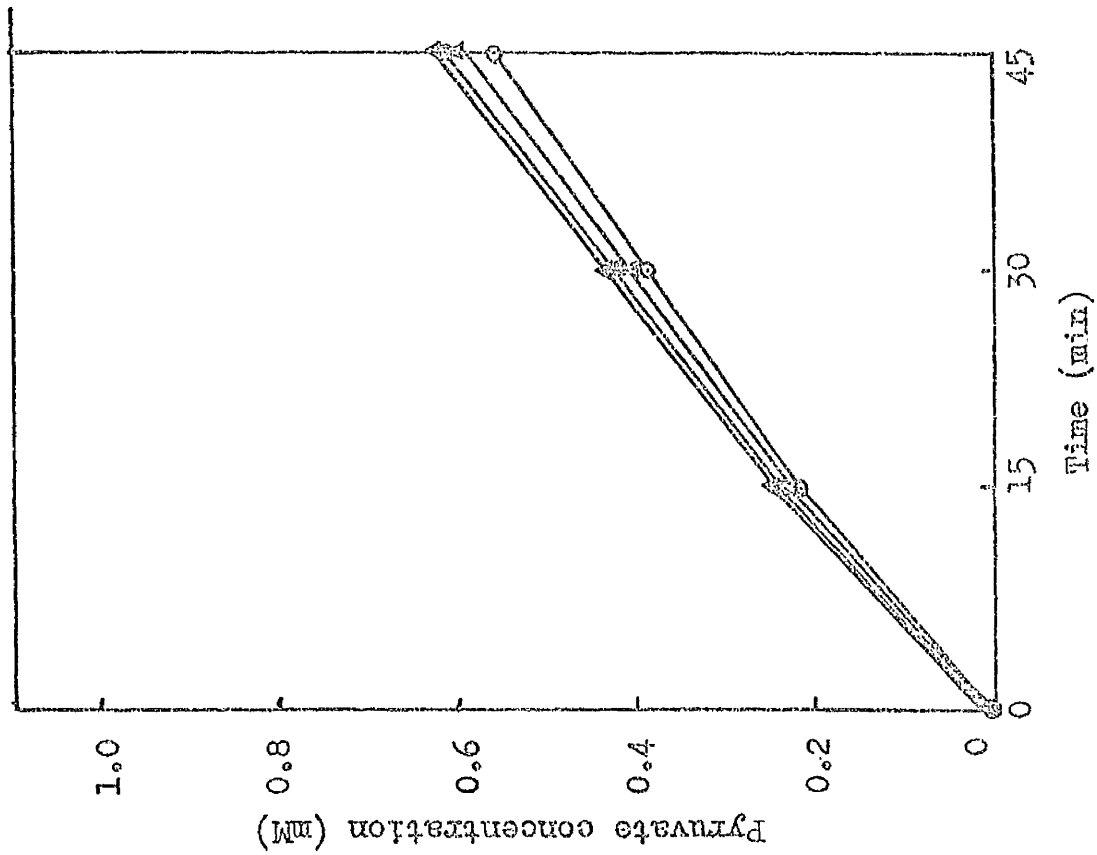


Figure 20

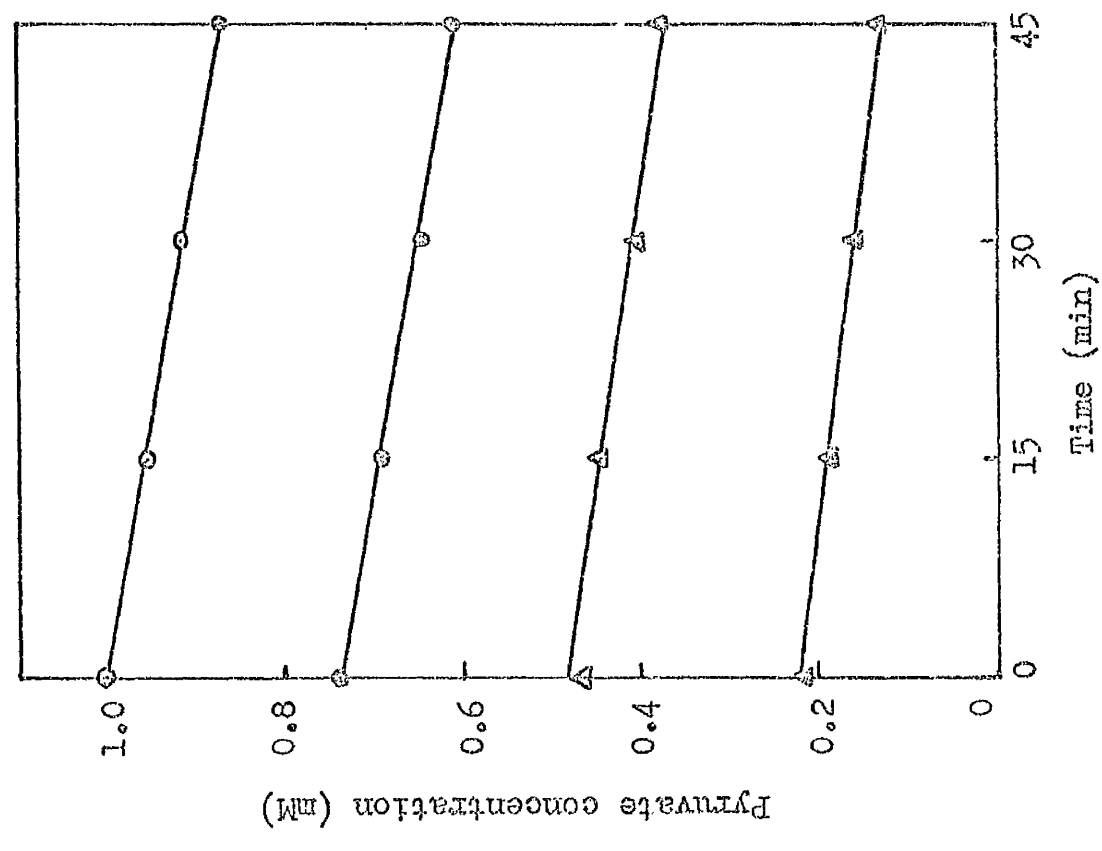


Figure 19

MATERIALS

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

Obtained from Boehringer Corpn., London, were:

NADP,

P.E.P.,

sodium fumarate,

triethanolamine,

acetate kinase,

GOD-Perid reagent,

glycerokinase,

lactate dehydrogenase,

malate dehydrogenase,

myokinase,

pyruvate kinase.

Obtained from Sigma, London, were:

ATP,

BCIG,

NAD,

NADH,

oxaloacetic acid (OAA),

sodium pyruvate,

glycylglycine.

Obtained from T.J. SAS, London, were:

L-glutamate,

L-alanine,

L-aspartate.

Obtained from Fluka, Buchs, Switzerland, were:

α-ketoglutarate,

L-malic acid.

Obtained from Oxoid, London, were:

cooked meat medium,

nutrient broth medium,

nutrient agar medium.

Dimethyl formamide was obtained from Koch Light Labs., Colnbrook, England; Sodium-D-Gluconate from Phanstiehl Laboratories, Illinois, U.S.A.; formaldehyde from May & Baker, Dagenham, England; B.S.A. from Armour Pharmaceuticals Ltd., Eastbourne, England; and tris base and tris HCl from Mann Research Laboratories, New York, U.S.A.

All solutions were prepared using glass distilled water.

High purity carbon dioxide (Research Grade) was obtained from Cambrian Chemicals, Croydon, England; Medical grade carbon dioxide from Distillers Co. Ltd., Glasgow; and air/carbon dioxide mixtures from Air Products Ltd., Baillieston, Glasgow. All other gases were obtained from British Oxygen Co. Ltd., Polmadie, Glasgow.

RESULTS

1. Dicarboxylic Acids as a Group

1.1. with respect to other cellular metabolism

The dicarboxylic acids succinate, fumarate and malate are metabolically linked by the enzymes of the tricarboxylic acid cycle. The grouping of these dicarboxylic acids as a unit with respect to the rest of metabolism was first established. Cells were grown on substrates (Figure 21) chosen to provide different patterns of carbon flow through the central metabolic pathways, and inoculated into a variety of media. The results (Figure 22) showed that, with the exception of alanine, cells trained to substrates which required synthesis of dicarboxylic acids from glycolytic intermediates had difficulty growing on dicarboxylic acids. On the other hand cells trained to compounds, with the exception of acetate, that required a flow of carbon from dicarboxylic acids to glycolytic intermediates grew immediately on dicarboxylic acids. In all cases the results obtained were independent of the dicarboxylic acid used to support growth.

1.2. with respect of other dicarboxylic acids

As far as growth was concerned the cells were unable to distinguish between individual dicarboxylic acids. Cells trained to one dicarboxylic acid grew on the other dicarboxylic acids at a growth rate typical of cells fully trained to that dicarboxylic acid (Figure 22). However, in media containing both malate and fumarate the cells

Figure 21 CHART OF CENTRAL METABOLIC SEQUENCES

Compounds underlined were used to prepare inocula and as sole carbon sources for the growth of the cells shown in Figure 22.

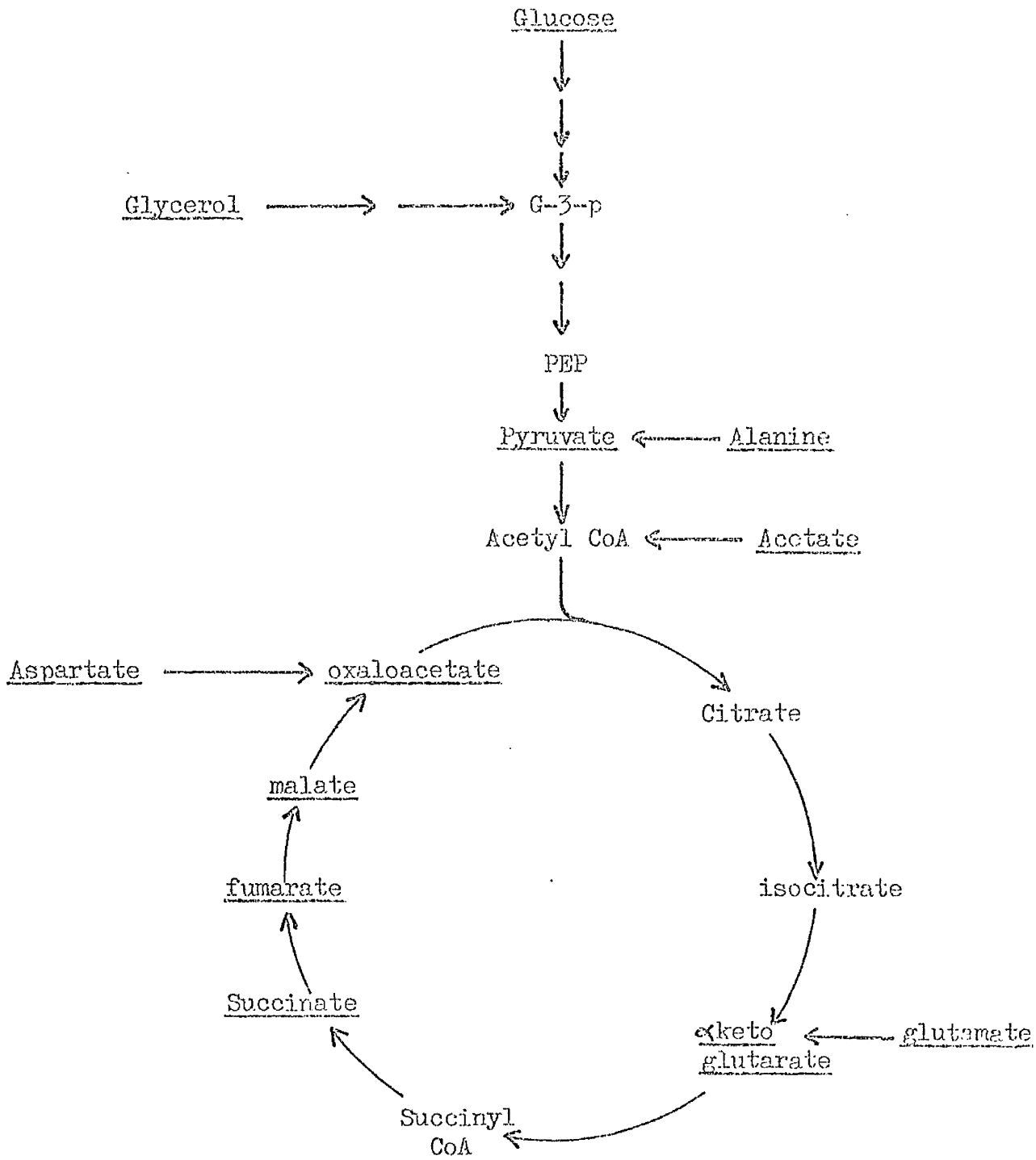

Glucose
Glycerol
G-3-p
PEP
Pyruvate
Alanine
Acetyl CoA
Acetate
Aspartate
oxaloacetate
Citrate
malate
fumarate
isocitrate
Succinate
α keto glutarate
glutamate
Succinyl CoA

Figure 22

GROWTH OF *E.coli* 15224 IN DIFFERENT MEDIA

Cells of *E.coli* 15224 trained to the carbon source shown in column 1 were washed and inoculated into 160 ml of minimal salts medium containing single carbon sources at the concentrations shown at the top of the table.

Growth of the cells was measured over a 10 h period and specific growth rate calculated.

( ) lag (h) before growth begins.

Oxaloacetate was not used as a training substrate because of its decomposition in growth medium.

gluc	glucose	Asp	aspartate
glyc	glycerol	αKG	αketoglutarate
succ	succinate	Glu	glutamate
fum	fumarate	acet	acetate
mal	malate	pyr	pyruvate
OAA	oxaloacetate	Ala	alanine

previous growth substrate	Carbon source used to support growth											
	gluc	glyc	succ	fum	mal	OAA	Asp	αKG	Glu	acet	pyr	Ala
Concn. mM	3	6	6	6	6	6	6	4.5	4.5	12	9	9
gluc	.88	.72(0.5)	(>10)	(>10)	(>10)	.72	(>10)	(>10)	(>10)	.10	.59	(>10)
glyc	.88	.72	.36(6)	.18(6)	.34(4)	.72	(>10)	(>10)	(>10)	.12(4)	.63	(>10)
succ	.88	.69(0.5)	.50	.55	.58	.90	.12	(>10)	(>10)	.30(2)	.65	(>10)
fum	.88	.69(0.5)	.50	.55	.58	.82	.11	(>10)	(>10)	.32(2)	.63	(>10)
mal	.88	.69(0.5)	.55	.57	.58	.82	.12	(>10)	(>10)	.21(1)	.65	(>10)
Asp	.88	.69(0.5)	.52	.53	.58	.80	.18	(>10)	(>10)	.27(1)	.62	(>10)
αKG	.88	.67(0.5)	.57	.57	.58	.73	.17	.61	(>10)	.42(1)	.59	(>10)
Glu	.77	.59(0.5)	.46	.48	.53	.74	.34	(>10)	.35	.25(1)	.50	(>10)
acet	.88	.69(0.5)	.51(5)	.47(3)	.48(4)	.74	(>10)	(>10)	(>10)	.21	.58	(>10)
pyr	.88	.69(0.5)	.35(5)	.48(3)	.48(3)	.87	(>10)	(>10)	(>10)	.27	.69	(>10)
Ala	.88	.69(0.5)	.50	.48	.50	.77	.15	(>10)	(>10)	.25(1)	.62	.28

could distinguish between them but the rate of utilisation of each compound was independent of the previous history of the culture. Both fumarate and malate trained cells used 1.7 parts of fumarate to 1 part of malate (Figure 23).

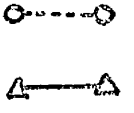
Subsequent experiments used malate as a typical and representative compound for the study of dicarboxylic acid metabolism.

Figure 23

UTILISATION OF DICARBOXYLIC ACIDS FROM A MIXTURE

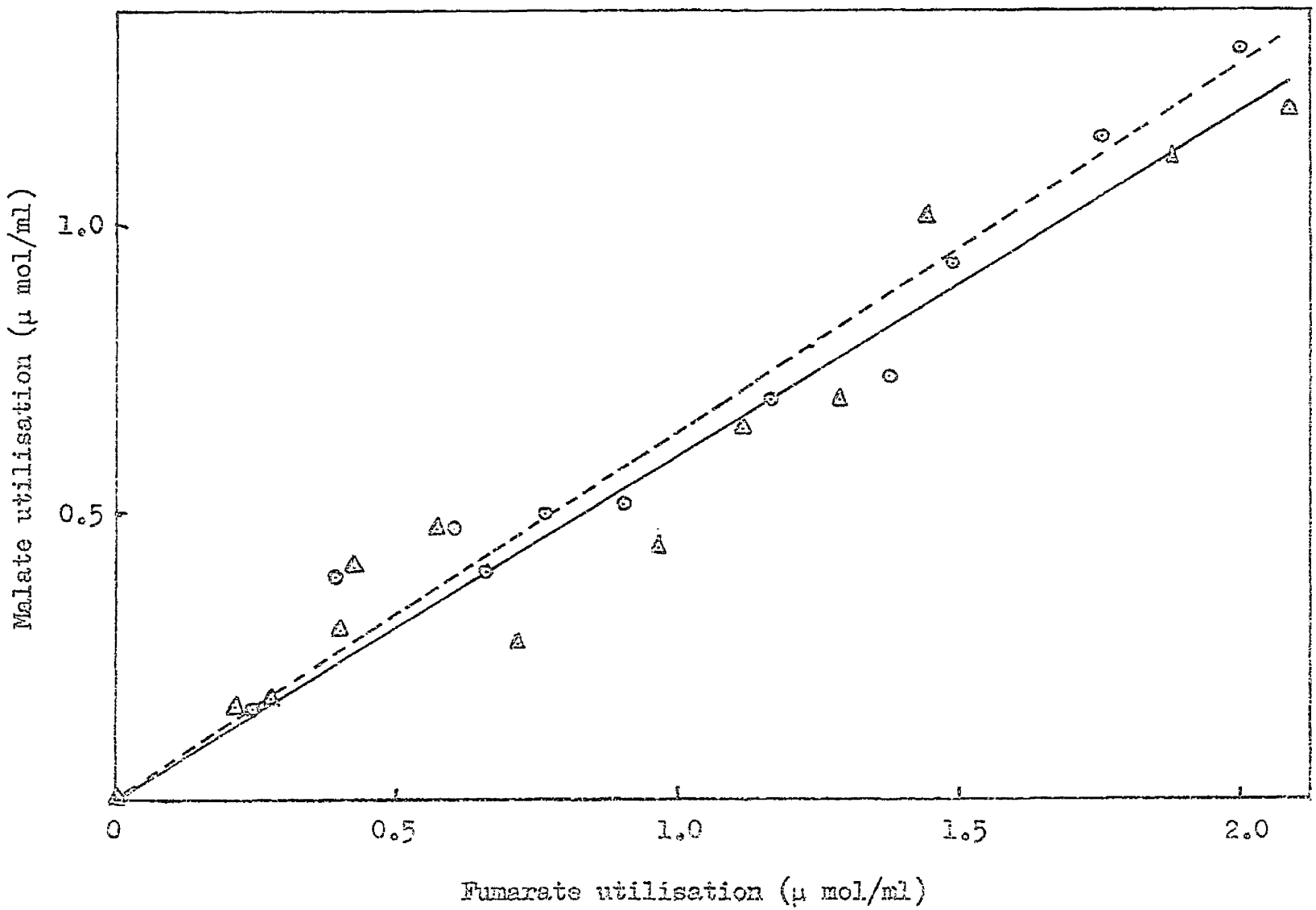
BY *E.coli* 15224

Cells of *E.coli* 15224 trained to either malate or fumarate were washed and inoculated into minimal salts medium containing both malate and fumarate at 4 mM. Utilisation of both substrates by the cells was measured.



malate trained cells

fumarate trained cells


Malate utilisation (μ mol/ml)
1.0
0.5
0
0.5
1.0
1.5
2.0
Fumarate utilisation (μ mol./ml)

2. Metabolism of E.coli 15224 growing on malate

Throughout this thesis cells of E.coli 15224 trained to malate are used to provide standard estimates of the rate of metabolism of malate which can be achieved under our defined growth conditions. The determination of these standard estimates are shown in Figures 24-26 and are summarised in Figure 27.

2.1. Growth of E.coli 15224 on malate

Cells trained to malate and inoculated into minimal salts medium containing malate grew immediately. Growth was logarithmic over the entire period of growth and stopped due to exhaustion of malate (Figure 24). Cell material doubled every 69 min representing a specific growth rate of 0.60 h^{-1} (Figure 27).

2.2. Substrate utilisation during growth of E.coli 15224 on malate

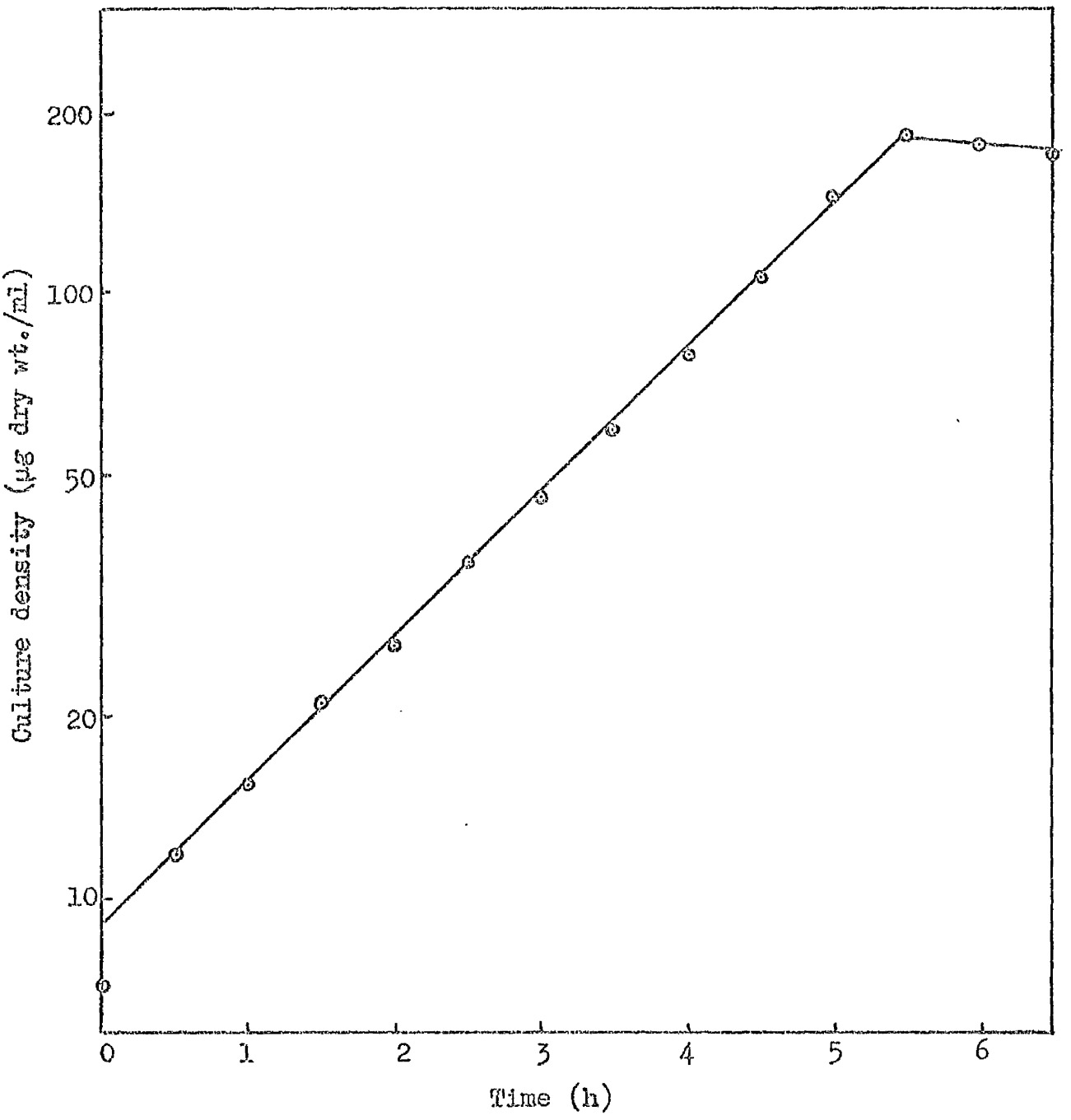
E.coli 15224 utilised malate logarithmically with growth. The amount of malate utilised was proportional to the amount of cell material synthesised in the culture (Figure 26a). The molar growth yield of cells growing on malate was obtained from this line and gave a value of 41.0 g of cellular dry wt./mol substrate utilised (Figure 27). The yield and the specific growth rate of the cells are related to the rate of substrate utilisation by the equation

$$\text{Rate of substrate utilisation} = \frac{\text{specific growth rate}}{\text{yield}} \times \frac{10^6}{60}$$

giving a rate of malate utilisation of 240 μ mol/g dry wt./min (Figure 27).

Figure 24 GROWTH OF *E.coli* 15224 ON MALATE

Cells of *E.coli* 15224, trained to malate were washed and inoculated into minimal salts medium containing 4 mM malate. Growth of the culture was measured.


200
100
50
20
10
Culture density (μg dry wt./ml)
0
1
2
3
4
5
6
Time (h)

2.3. Oxygen uptake of E.coli 15224 during growth on malate

During growth of E.coli 15224 on malate under aerobic conditions rate of oxygen uptake increased exponentially with the growth of the culture and depended on the quantity of cell material present (Figure 25a). The slope of this graph provided an estimate of the rate of oxygen uptake which was 310 μ mol/g dry wt./min (Figure 27).

2.4. Carbon dioxide production of E.coli 15224 during growth on malate

Carbon dioxide was produced by the culture and production increased exponentially with growth. As with oxygen uptake the rate of carbon dioxide production depended on the quantity of cell material present in the culture (Figure 25b) and was 480 μ mol/g dry wt./min (Figure 27).

2.5. Yield of cells and oxygen uptake during growth of E.coli 15224 on malate

The oxygen uptake data shown in Figure 25a were used to calculate the accumulative oxygen uptake of the culture during growth. The total amount of oxygen taken up by the culture depended on the amount of new cell material that was synthesised (Figure 26b). The slope of the line gave a yield/mol oxygen taken up during growth on malate of 33.3 g cellular dry wt. (Figure 27).

These findings are summarised in Figure 27. Respiratory quotient (1.55) and carbon incorporation (41.0%) were calculated from these data (Figure 27).

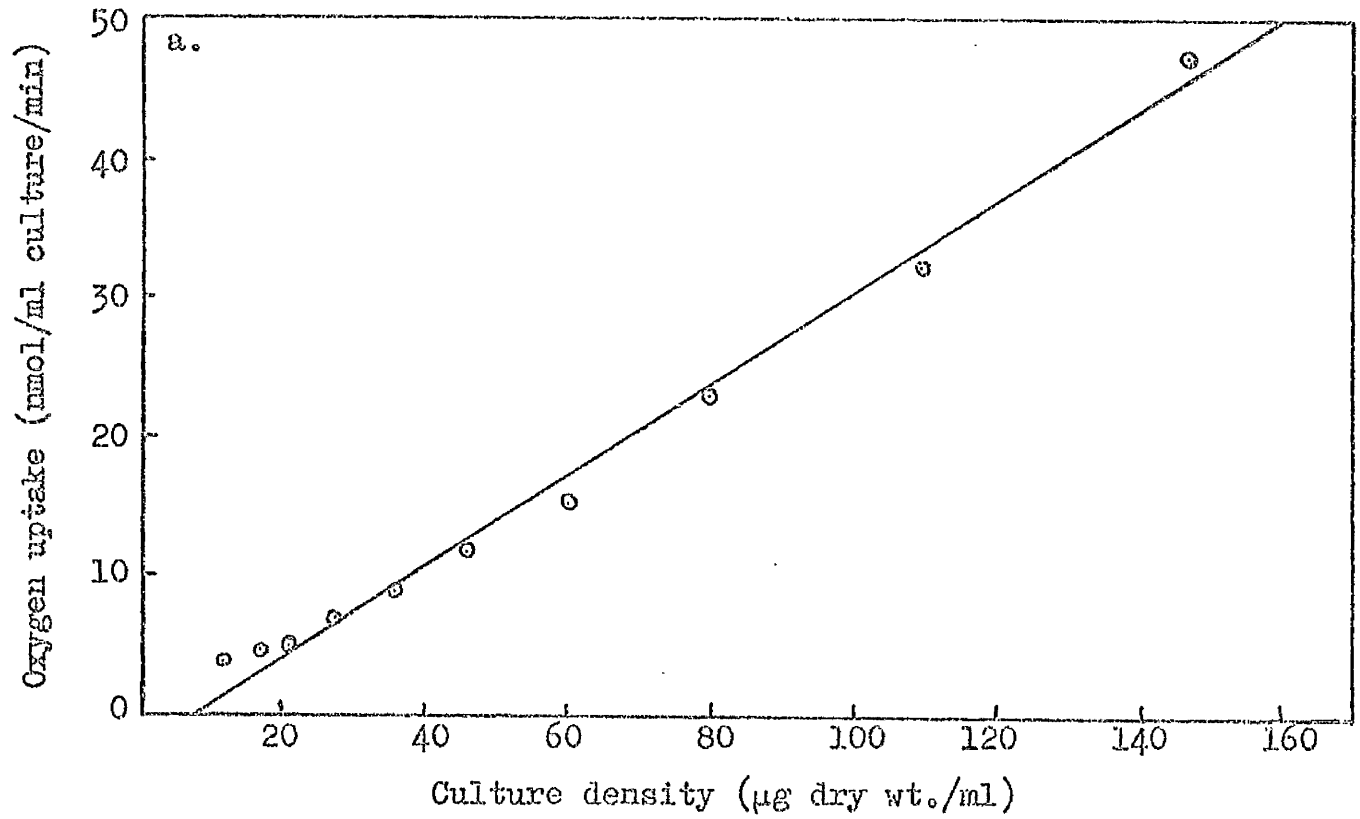
Figure 25

GAS EXCHANGE OF *E.coli* 15224 GROWING ON MALATE

Oxygen uptake and carbon dioxide production were measured during growth of the culture described in Figure 24.

Figure 25a oxygen uptake

Figure 25b carbon dioxide production


a.
Oxygen uptake (nmol/ml culture/min)
0
10
20
30
40
50
20
40
60
80
100
120
140
160
Culture density (µg dry wt./ml)

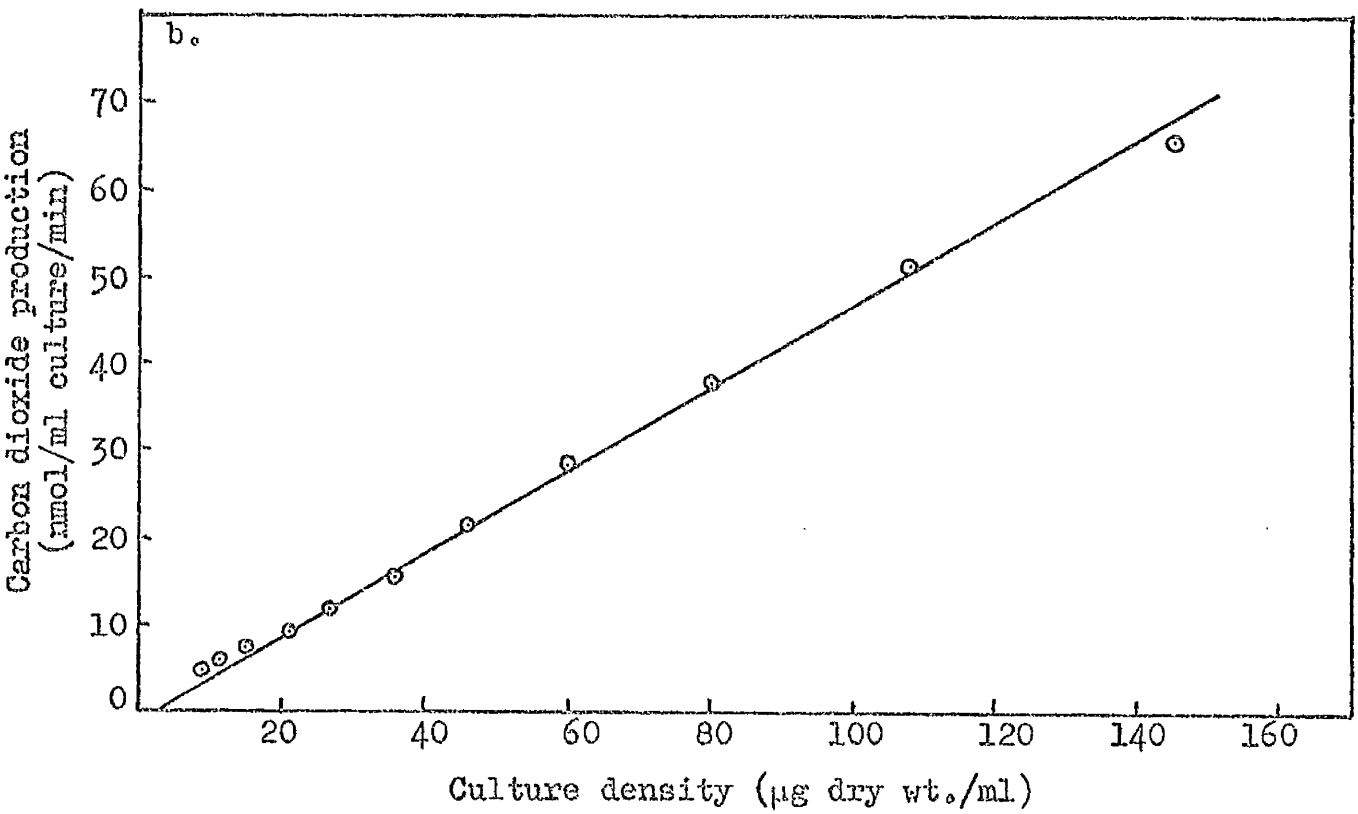
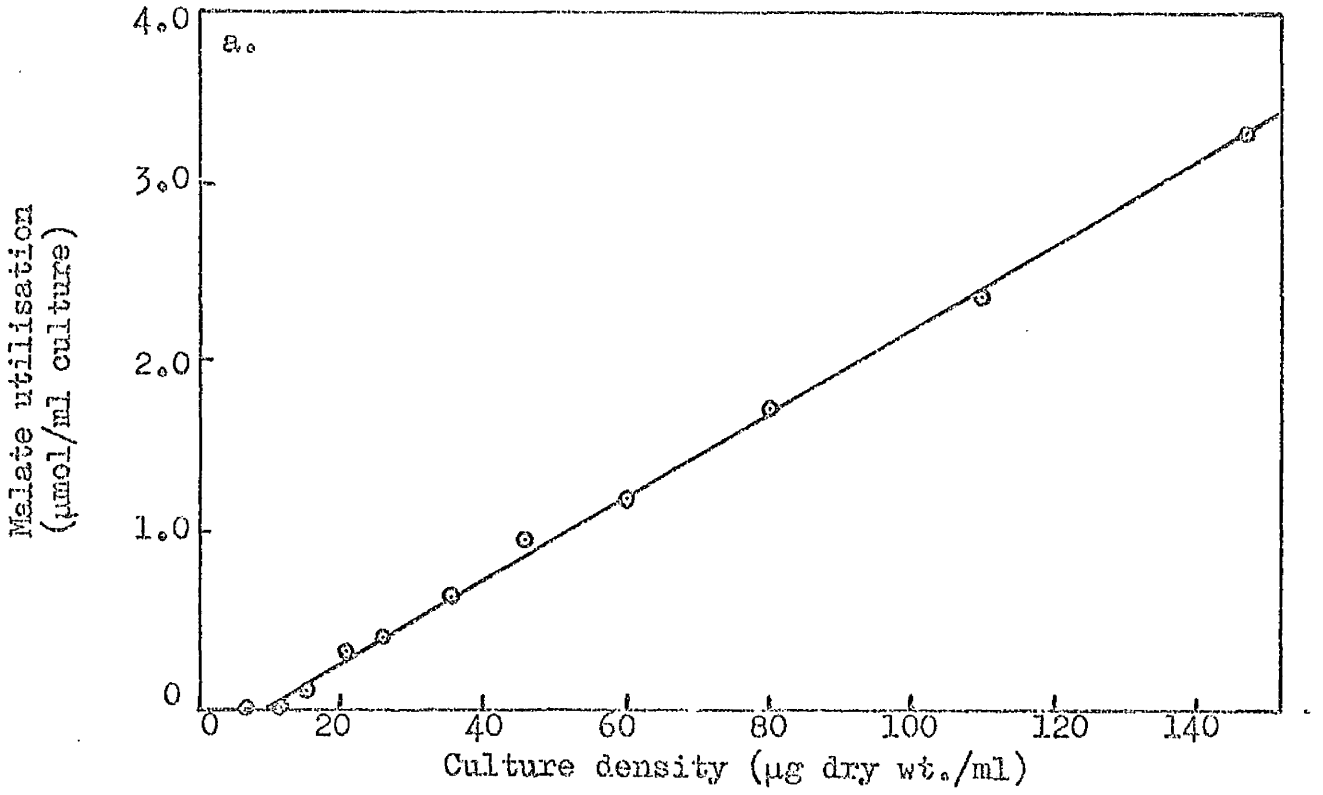

b.
Carbon dioxide production (nmol/ml culture/min)
0
10
20
30
40
50
60
70
20
40
60
80
100
120
140
160
Culture density (µg dry wt./ml)

Figure 26

SUBSTRATE UTILISATION AND ACCUMULATIVE OXYGEN UPTAKE DURING GROWTH OF *E.coli* 15224 ON MALATE

During growth of the culture shown in Figure 24 samples were taken to measure the concentration of malate in the medium. Malate utilisation was plotted against culture density (Figure 26a).

The oxygen uptake data of Figure 25a were used to calculate accumulative oxygen uptake which was graphed against culture density (Figure 26b).


a.
Malate utilisation (μmol/ml culture)
4.0
3.0
2.0
1.0
0
0
20
40
60
80
100
120
140
Culture density (μg dry wt./ml)

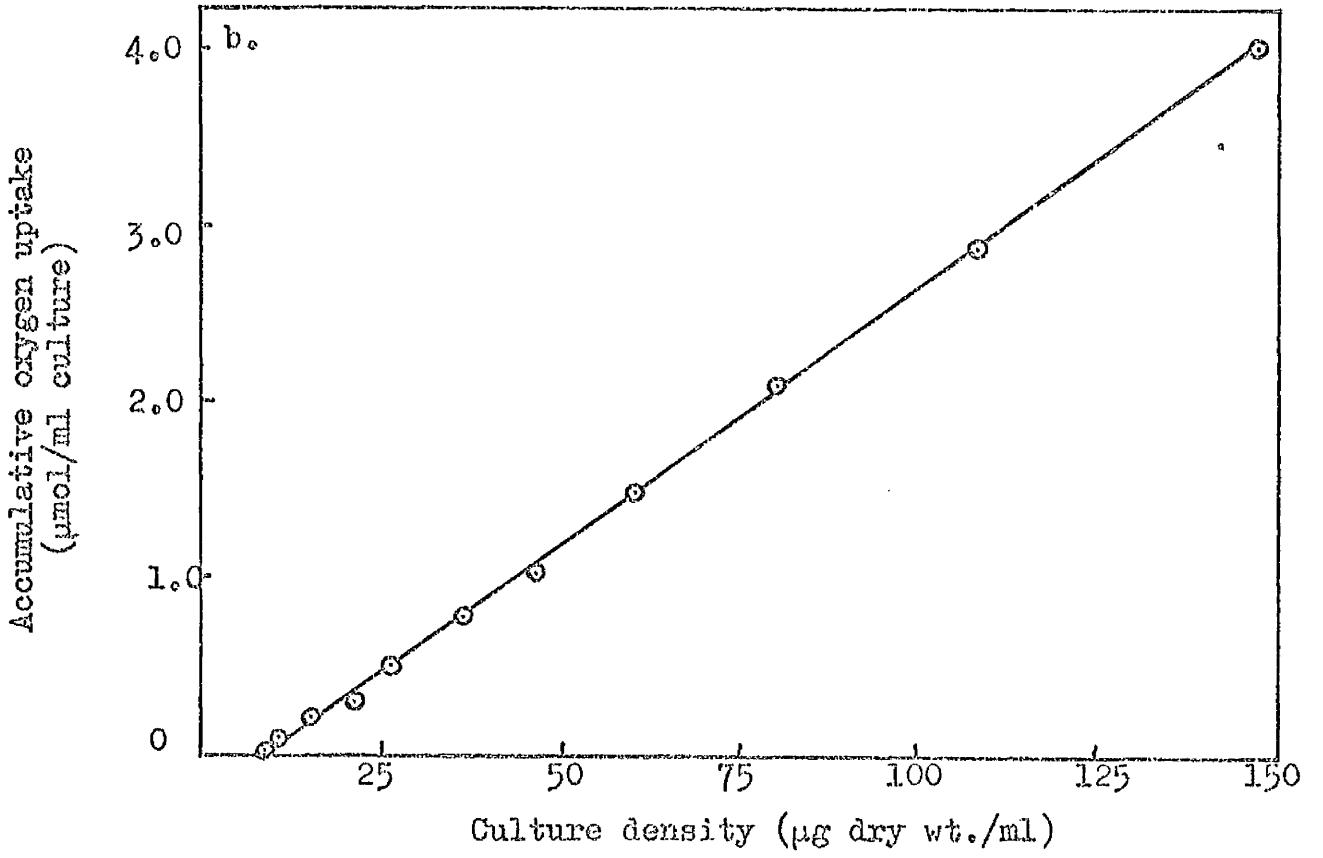

b.
Accumulative oxygen uptake (μmol/ml culture)
4.0
3.0
2.0
1.0
0
25
50
75
100
125
150
Culture density (μg dry wt./ml)

Figure 27

GROWTH AND METABOLISM OF *E.coli* 15224 IN MALATE MEDIUM

Figure summarises data from Figures 24 to 26 for cells of *E.coli* 15224 growing on malate as a sole source of carbon and energy.

Rate of malate utilisation, respiratory quotient and percentage of carbon incorporated were calculated from these data.

Growth rate (h^{-1})	0.60
Rate of oxygen uptake (µmol/g dry wt./min)	310
Rate of carbon dioxide prodn. (µmol/g dry wt./min)	480
Respiratory quotient	1.55
yield/mol malate (g dry wt.)	41.0
yield/mol oxygen (g dry wt.)	33.3
Rate of malate utilisation (µmol/g dry wt./min)	240
% carbon incorporated	41.0

3. Growth, metabolism and Adaptation of E.coli 15224 growing on glycerol/malate medium

The statement that cells of E.coli 15224 trained to glycerol did not grow immediately on malate (Figure 22) was an oversimplification since these cells did grow on malate but slowly and with an accelerating rate of growth over a period of several hours (Figure 28). Glycerol trained cells grew immediately and logarithmically on glycerol. The presence of malate in the medium in addition to glycerol stimulated the growth rate compared to the glycerol containing culture and resulted in a second phase of growth slower than the first (Figure 28).

3.1. Growth and substrate utilisation during growth of E.coli 15224 on glycerol/malate medium

The effect of the presence of malate in the medium on the growth of cells trained to and growing on glycerol was striking and was examined more fully by setting up 2 x 800 ml cultures of minimal salts medium containing either 2 mM glycerol and 6 mM malate or 2 mM glycerol alone. The second flask was used as a control (Figure 31). Measurements of growth, gas exchange, and substrate utilisation were made.

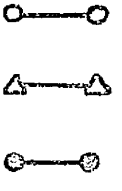
As shown previously (Figure 28) growth of glycerol trained cells on a mixture of glycerol and malate produced a biphasic growth pattern (Figure 29a). Substrate estimations made during growth (Figure 29a) showed that glycerol was present in the culture during the 1st phase of growth when the cells were growing at a specific growth rate of 0.91 h^{-1} which was higher than the specific growth rate of 0.77 h^{-1} for

Figure 28

GROWTH OF GLYCEROL TRAINED *E.coli* 15224 IN GLYCEROL AND MALATE CONTAINING MEDIA

Cells of *E.coli* 15224 trained to glycerol were washed and inoculated into minimal salts medium containing either glycerol, or malate or a mixture of both substrates.

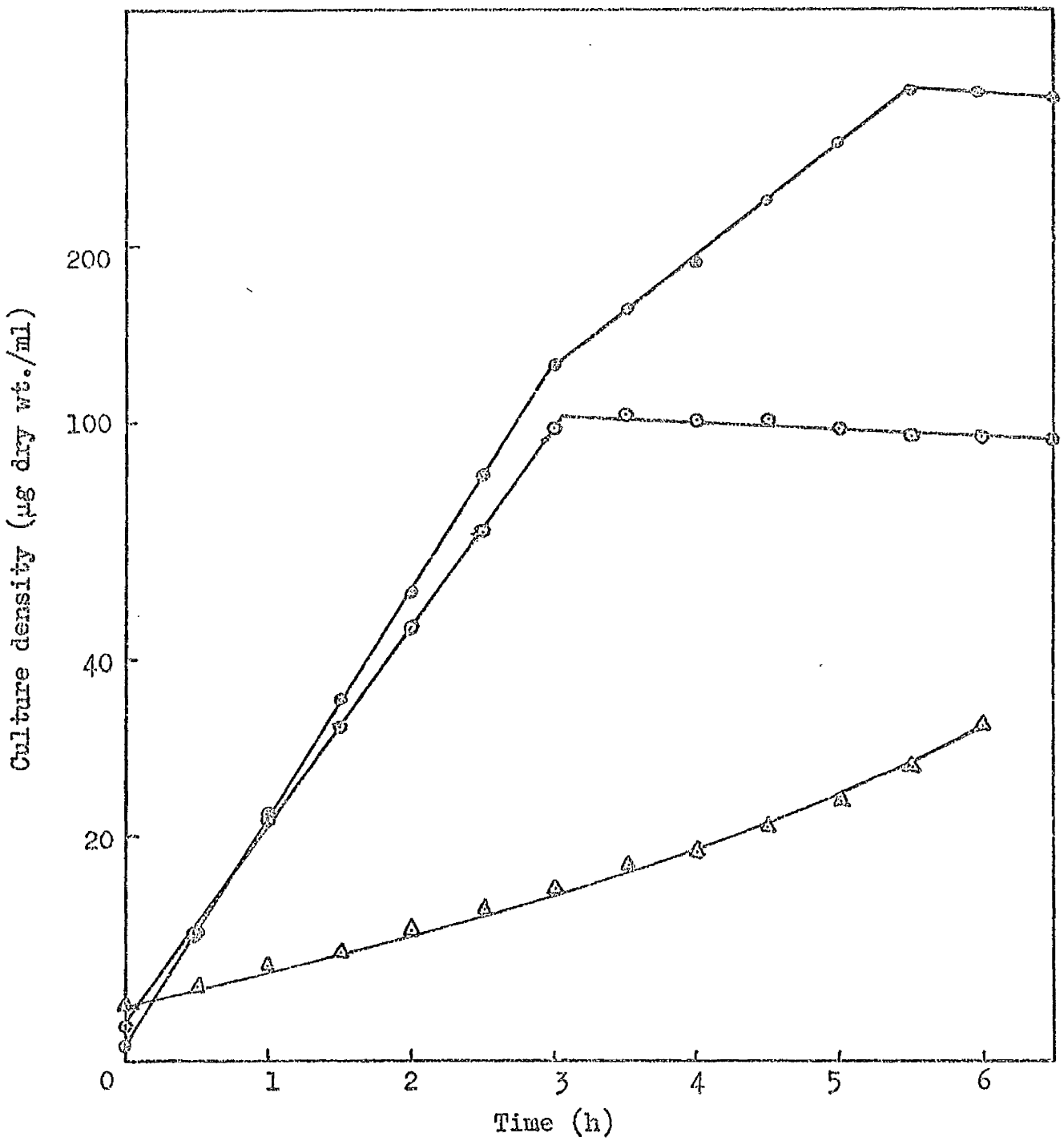
Growth of the cells was measured:



2 mM Glycerol

6 mM Malate

2 mM Glycerol + 6 mM Malate


Culture density (µg dry wt./ml)
200
100
40
20
0
1
2
3
4
5
6
Time (h)

the cells growing on glycerol alone (Figure 31). The fall in growth rate coincided with exhaustion of glycerol from the medium. During the 1st phase of growth malate was used as a substrate simultaneously with the glycerol - during the 2nd phase of growth, malate was the sole substrate remaining in the culture medium.

3.2. Gas exchange during glycerol/malate growth

Gas exchange of the culture varied considerably during growth (Figure 29b). The rate of oxygen uptake per cell remained constant during the 1st phase of growth but fell markedly upon exhaustion of the glycerol. It then increased while the cells metabolised malate during the 2nd phase of growth. The rate of oxygen uptake during the 1st phase of growth was higher than that of the glycerol control culture (Figure 31).

The rate of carbon dioxide production did not follow the same pattern. Carbon dioxide production increased while the cells were growing on the substrate mixture and was reflected in the increasing value for the respiratory quotient with growth (Figure 29c). Upon glycerol exhaustion carbon dioxide production fell although not to the same extent as oxygen uptake, resulting in a marked change in the respiratory quotient (Figure 29c).

3.3. Yield of cells during glycerol/malate growth

The yield of cells/mol substrate during these growth phases was calculated from Figure 30a. During the 1st phase the yield of cells/mol glycerol was 74.5 g cellular dry wt. which represented a marked increase over the control yield (49.3 g) (Figure 31). During this phase 1.3 mM

Figure 29

GROWTH AND METABOLISM OF *E.coli* 15224 IN GLYCEROL/MALATE MEDIUM

Cells of *E.coli* 15224 trained to glycerol were washed and inoculated into minimal salts medium containing 2 mM glycerol and 6 mM malate.

Growth, oxygen uptake, carbon dioxide production and substrate concentration were all measured.

Fig. 29a

Growth

Glycerol concentration

Malate concentration

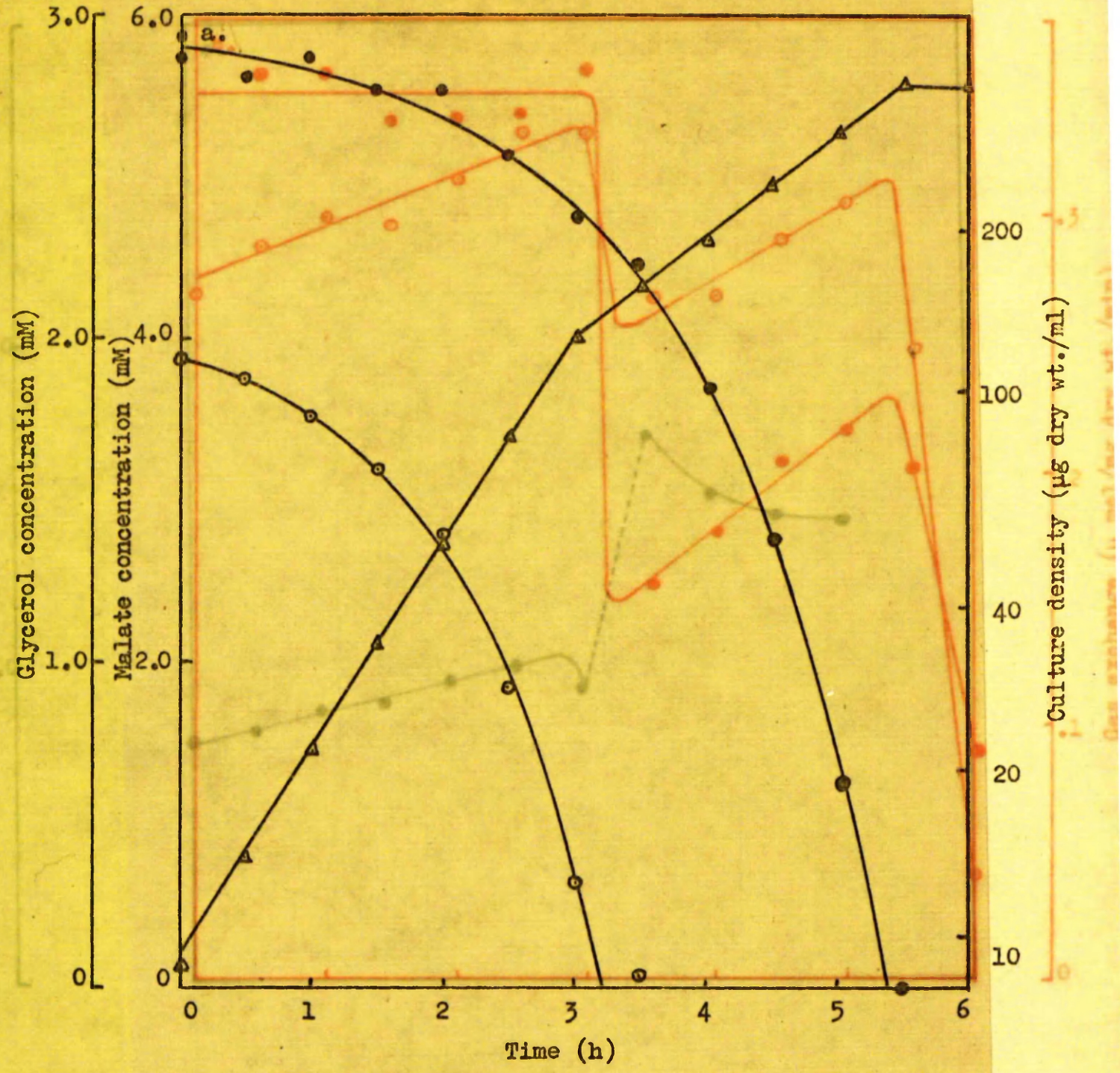
Fig. 29b 1st overlay Gas exchange

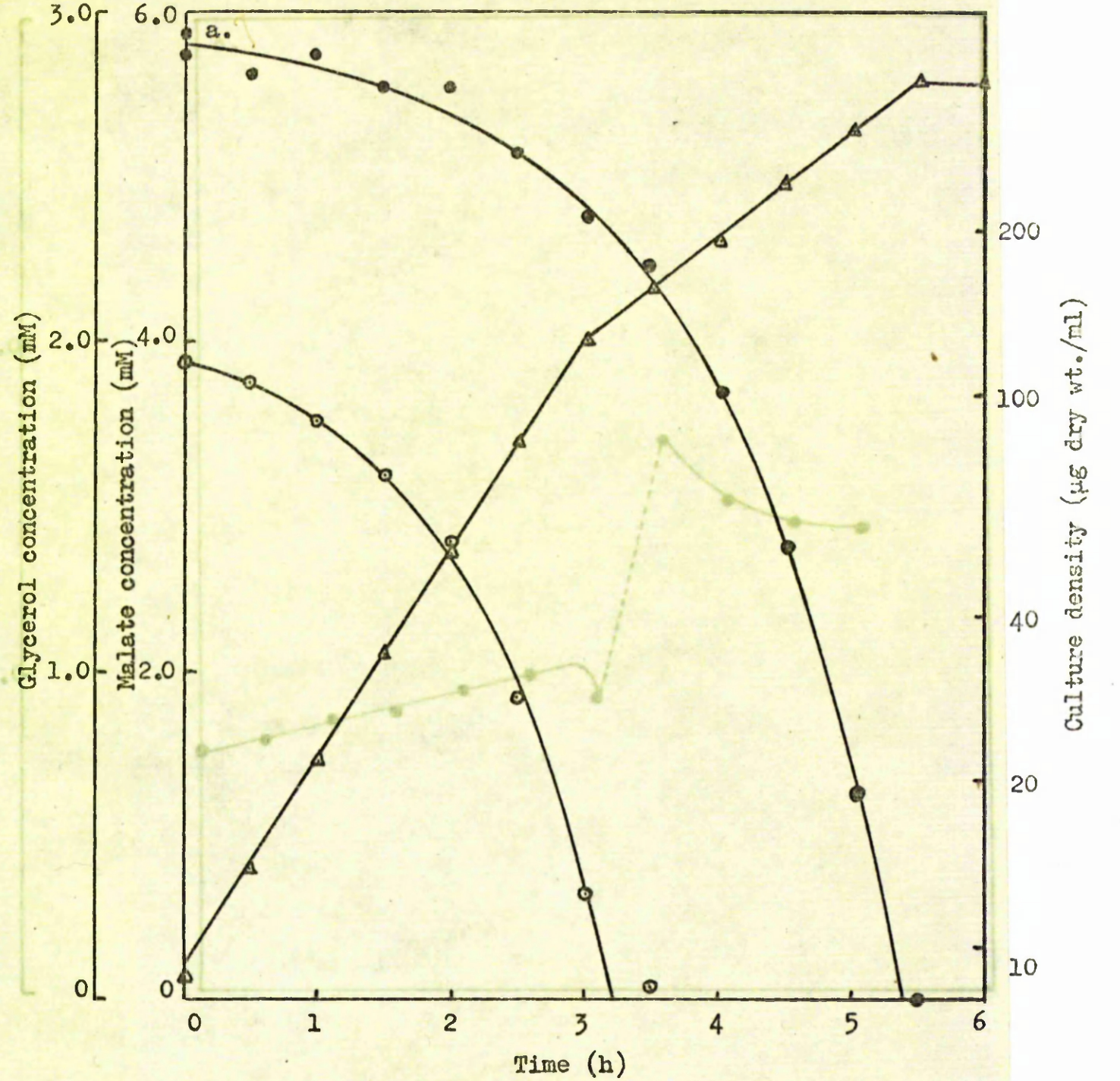
Carbon dioxide production

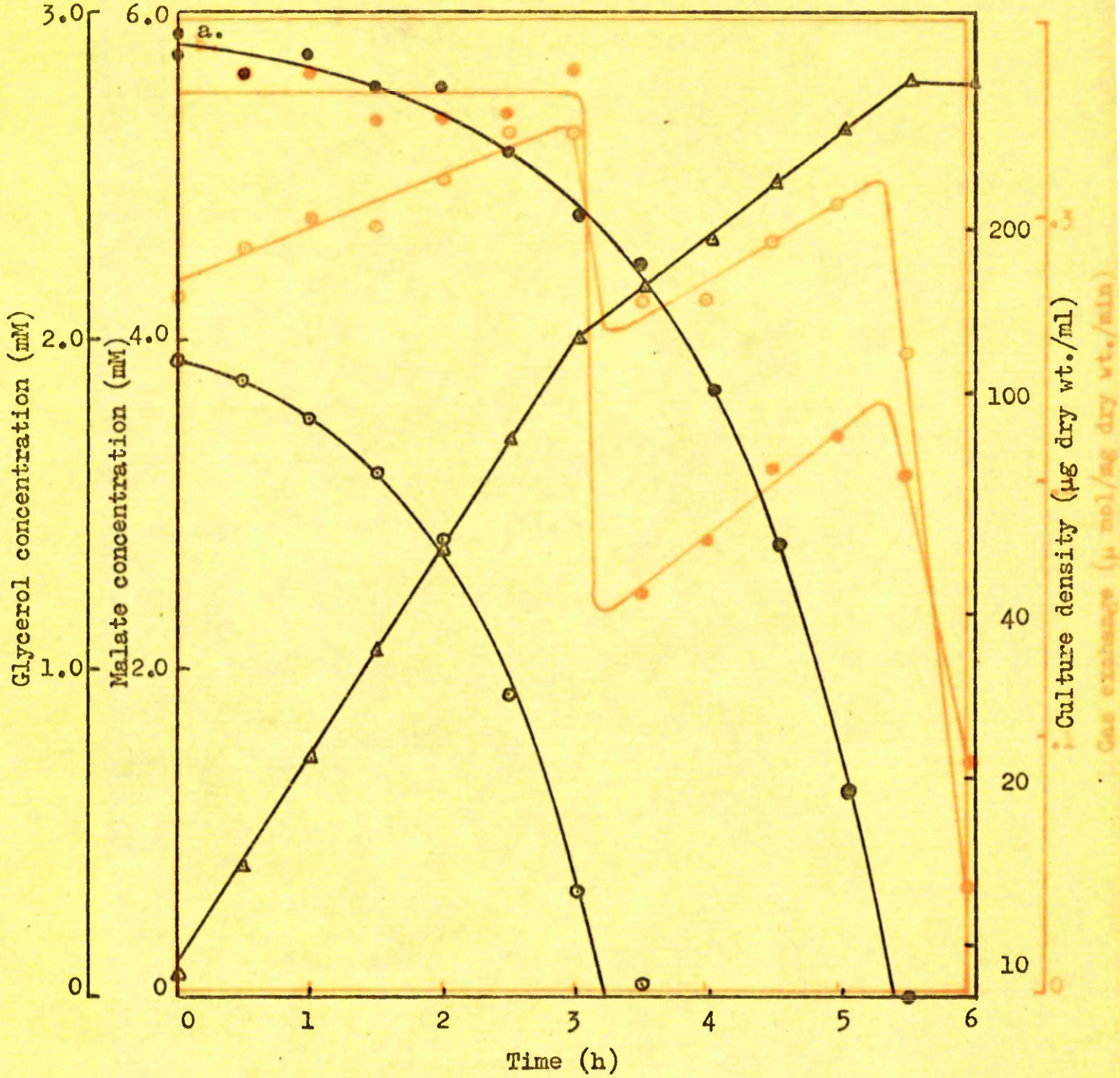
Oxygen uptake

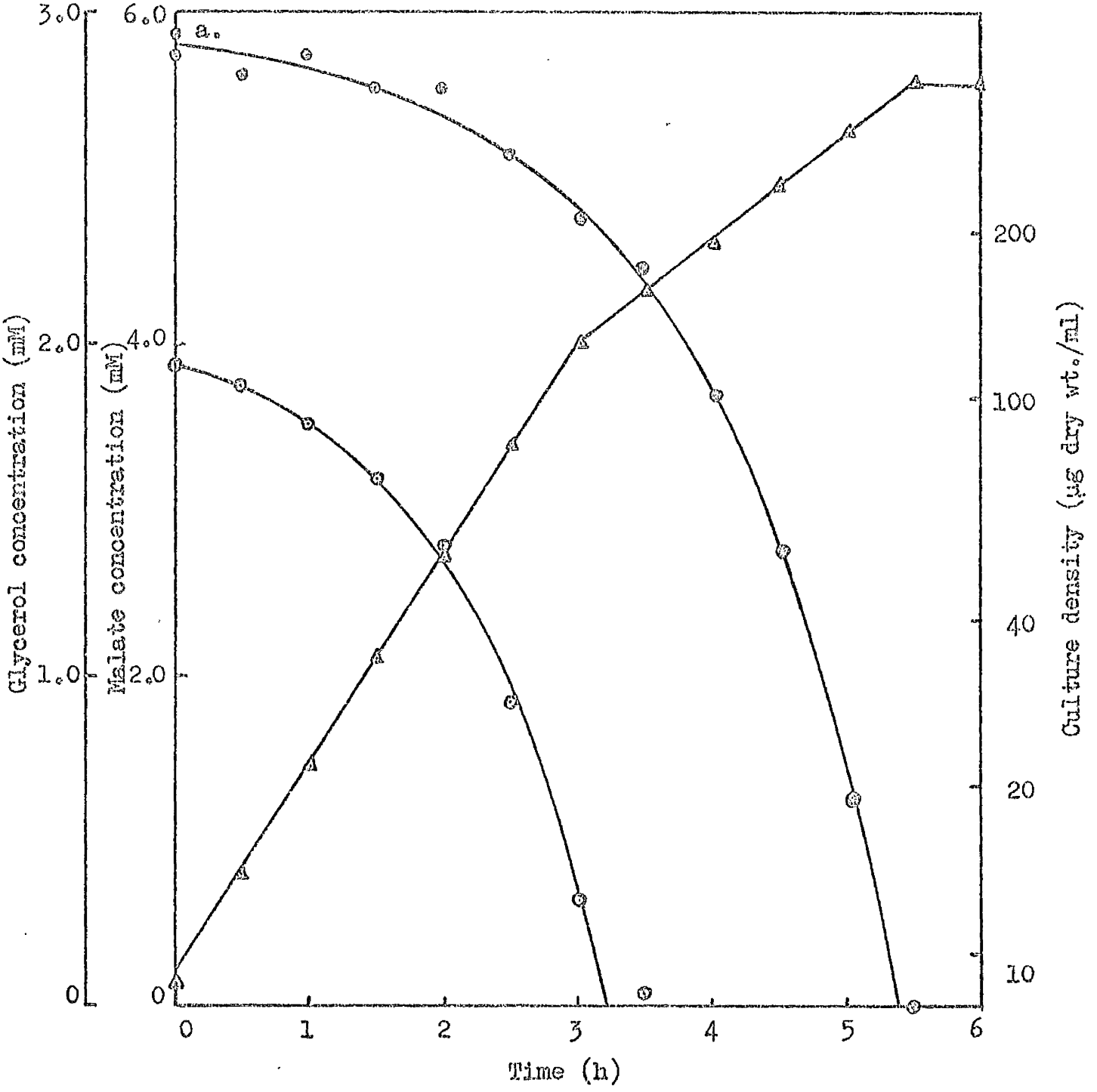
Fig. 29c 2nd overlay

Respiratory quotient


a.
Glycerol concentration (mM)
Malate concentration (mM)
Culture density (µg dry wt./ml)
Time (h)
3.0
2.0
1.0
0
6.0
4.0
2.0
0
200
100
40
20
10
0
1
2
3
4
5
6


a.
Glycerol concentration (mM)
Malate concentration (mM)
Culture density (µg dry wt./ml)
Time (h)
3.0
2.0
1.0
0
6.0
4.0
2.0
0
200
100
40
20
10
0
1
2
3
4
5
6


a.
Glycerol concentration (mM)
Malate concentration (mM)
Culture density (μg dry wt./ml)
Time (h)
3.0
2.0
1.0
0
6.0
4.0
2.0
0
200
100
40
20
10
0
1
2
3
4
5
6


a.
Glycerol concentration (mM)
Malate concentration (mM)
Culture density (μg dry wt./ml)
Time (h)
3.0
2.0
1.0
0
6.0
4.0
2.0
0
200
100
40
20
10
0
1
2
3
4
5
6

malate was metabolised. Yield/mol malate during the 2nd phase of growth was 45 g cellular dry wt.

The yield/mol oxygen did not vary greatly during growth. During the 1st phase it was 41.6 g cellular dry wt./mol and during the 2nd phase was 38 g/mol. The break in slope was difficult to assess but occurred close to glycerol exhaustion (Figure 30b).

A comparison of the metabolic values derived for the glycerol/malate culture (Figure 29a-c) with the glycerol control culture is shown in Figure 31.

Figure 30

YIELD OF *E.coli* 15224 GROWING IN GLYCEROL/MALATE MEDIUM

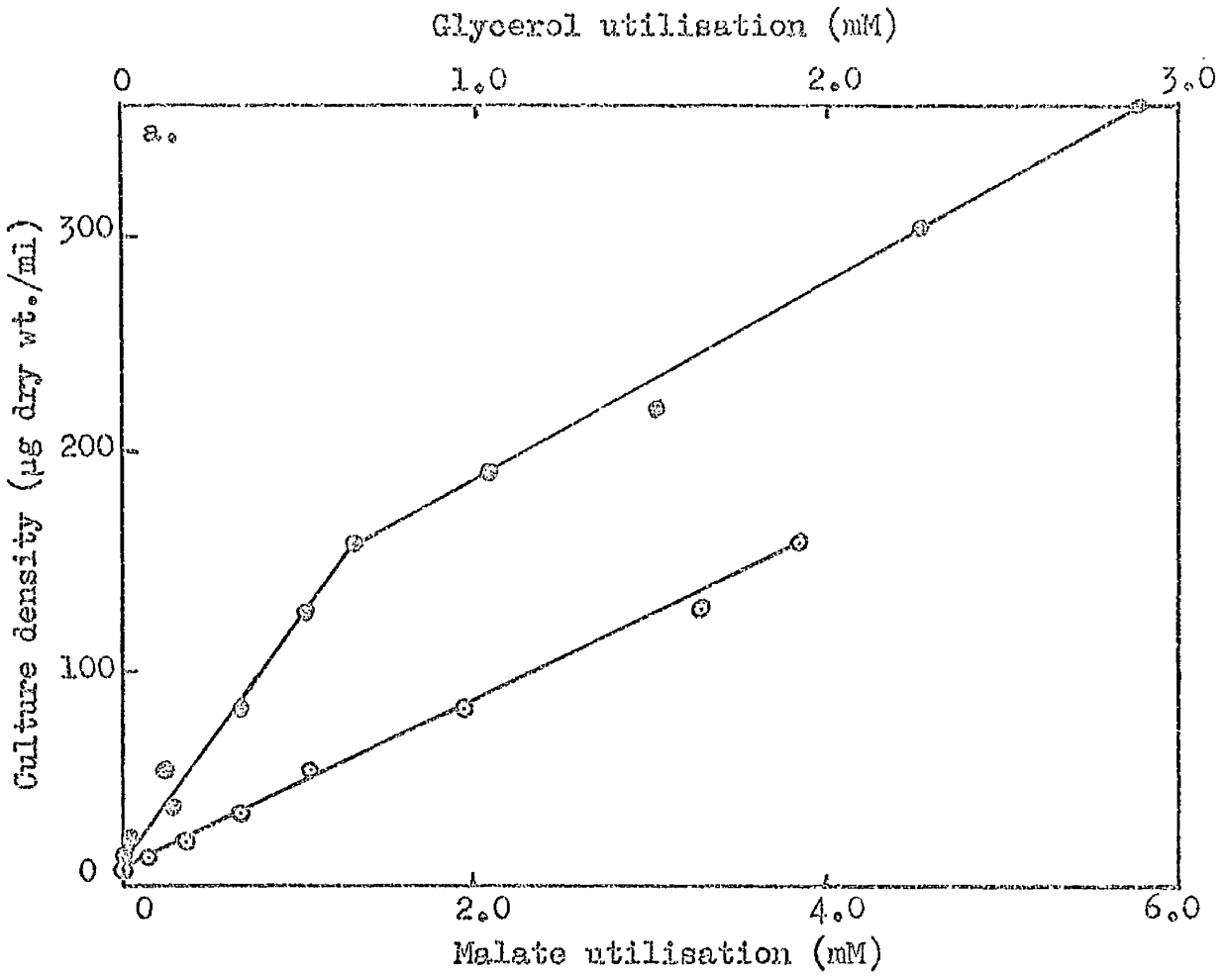
Accumulative oxygen uptake, calculated from the data of Figure 29b, and substrate concentration were graphed against cell density to determine growth yields.

Figure 30a yield/mol carbon source

o—o glycerol

⊙—⊙ malate

Figure 30b yield/mol oxygen


Glycerol utilisation (mM)
0
1.0
2.0
3.0
a.
300
200
100
0
Culture density (µg dry wt./ml)
0
2.0
4.0
6.0
Malate utilisation (mM)

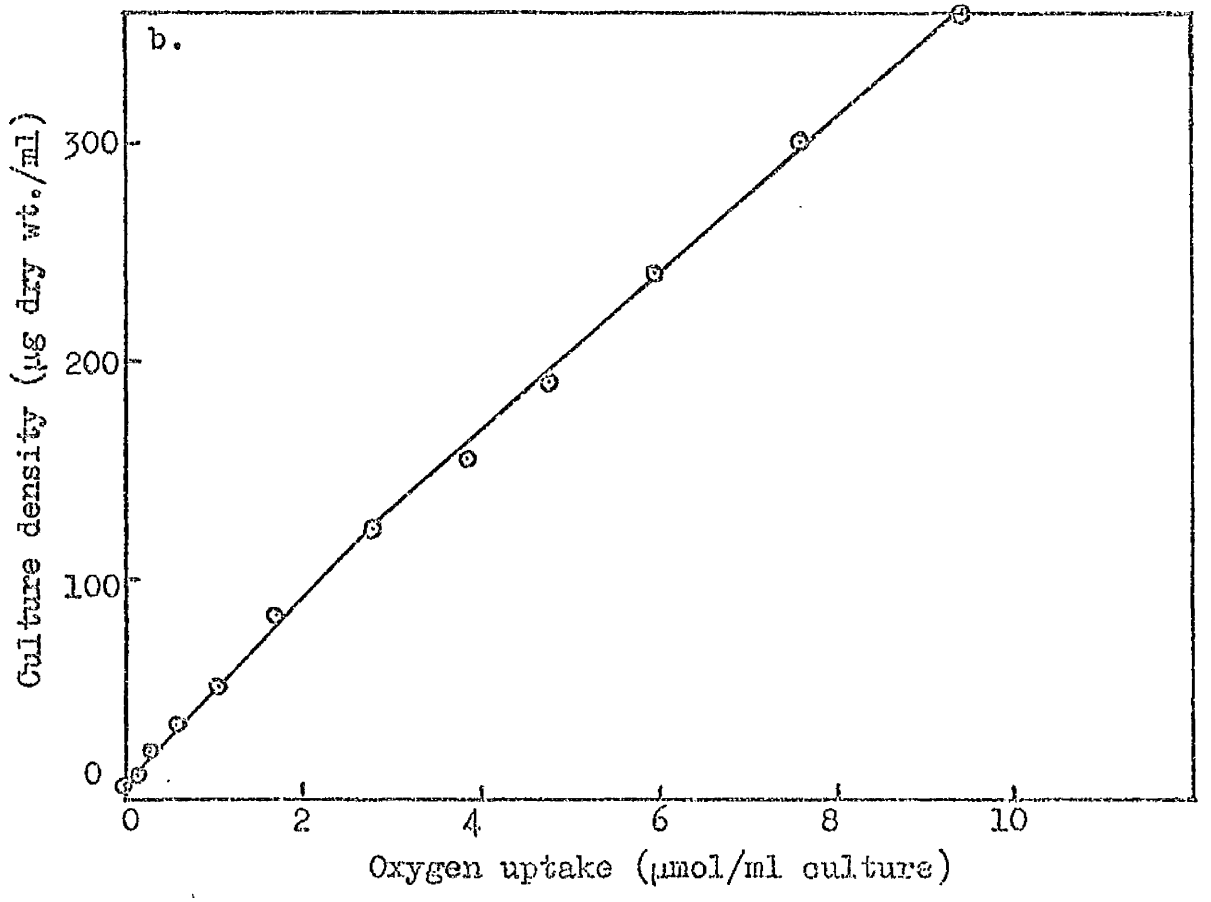

b.
300
200
100
0
Culture density (µg dry wt./ml)
0
2
4
6
8
10
Oxygen uptake (µmol/ml culture)

Figure 31

COMPARISON OF GROWTH OF *E.coli* 15224 ON GLYCEROL WITH GROWTH ON GLYCEROL/MALATE

Cells of *E.coli* 15224 trained to glycerol were harvested, resuspended in chilled 40 mM phosphate buffer pH 7.0 and inoculated into minimal salts medium containing 2 mM glycerol. Growth (Figure 28), gas exchange and substrate utilisation are shown, as glycerol control culture and compared with the corresponding data, from Figures 29 and 30, for the glycerol/malate culture.

	Glycerol Control Culture	Glycerol/Malate Culture
specific gr. rate (h^{-1})	0.77	0.91
Rate of oxygen uptake (μmol/g dry wt./min)	310	350
Rate of carbon dioxide production (μmol/g dry wt./min)	200	280 - 340
respiratory quotient	0.66	0.74 - 1.00
yield/mol O_2 utilised(g dry wt.)	42.2	41.6
yield/mol glycerol utilised(g dry wt.)	49.3	74.5

4. Growth and Metabolism on Malate after Growth on glycerol/malate medium

To examine the effect of glycerol/malate growth on subsequent metabolism of malate a slightly different system was used. Glycerol trained cells were grown on glycerol (1 mM) to increase cell density and make gas exchange greater and consequently measurement more accurate. Within 30 min of glycerol exhaustion, substrate containing 4 mM malate and variable concentrations of glycerol (Figure 33) was added back to the cultures. Growth and gas exchange were followed throughout and the growth rate and rate of gas exchange of the cells calculated at the point of glycerol exhaustion as shown for 1 mM glycerol (Figure 32a and 32b).

The values for growth and gas exchange on malate were calculated for each different glycerol concentration and showed that the growth rate and the rate of gas exchange of the cells on malate increased with increasing duration of growth in glycerol/malate medium (Figure 33).

Figure 32

ADAPTATION, TO A MALATE UTILISING PHENOTYPE, OF *E.coli* 15224 IN GLYCEROL/MALATE MEDIUM

Cells of *E.coli* 15224 trained to glycerol were inoculated into minimal salts medium containing 1 mM glycerol and allowed to grow till the glycerol was exhausted. Within 30 min of glycerol exhaustion 1 mM glycerol and 4 mM malate were added simultaneously to the culture. Growth and gas exchange were followed throughout.

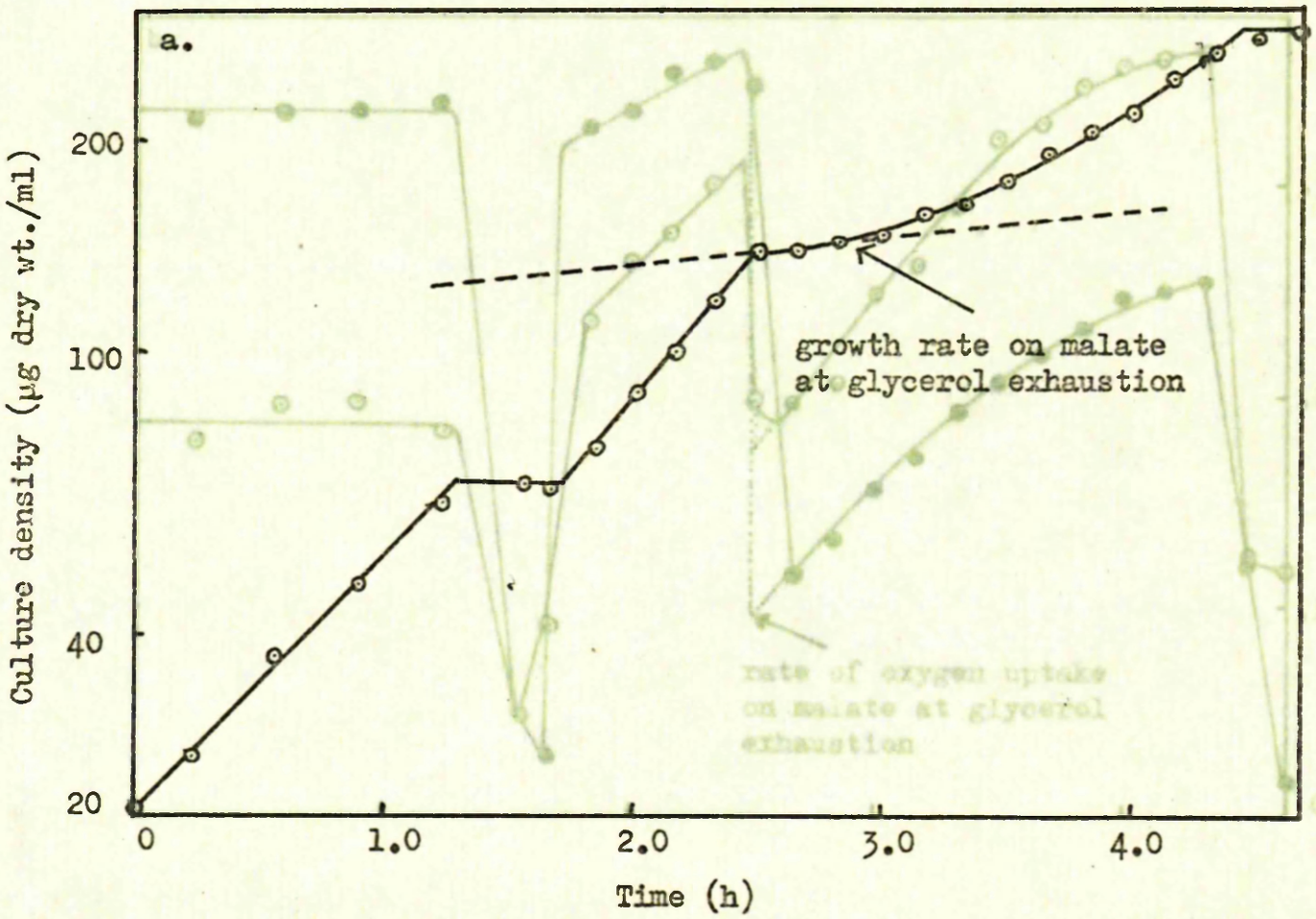
Fig. 32a ○—○ Growth

Fig. 32b overlay

●—● Oxygen uptake

○—○ Carbon dioxide production

The method of evaluating rate of growth and of gas exchange of both oxygen and carbon dioxide at the point of exhaustion of glycerol is shown.


a.
200
100
40
20
Culture density (µg dry wt./ml)
0
1.0
2.0
3.0
4.0
Time (h)
growth rate on malate
at glycerol exhaustion

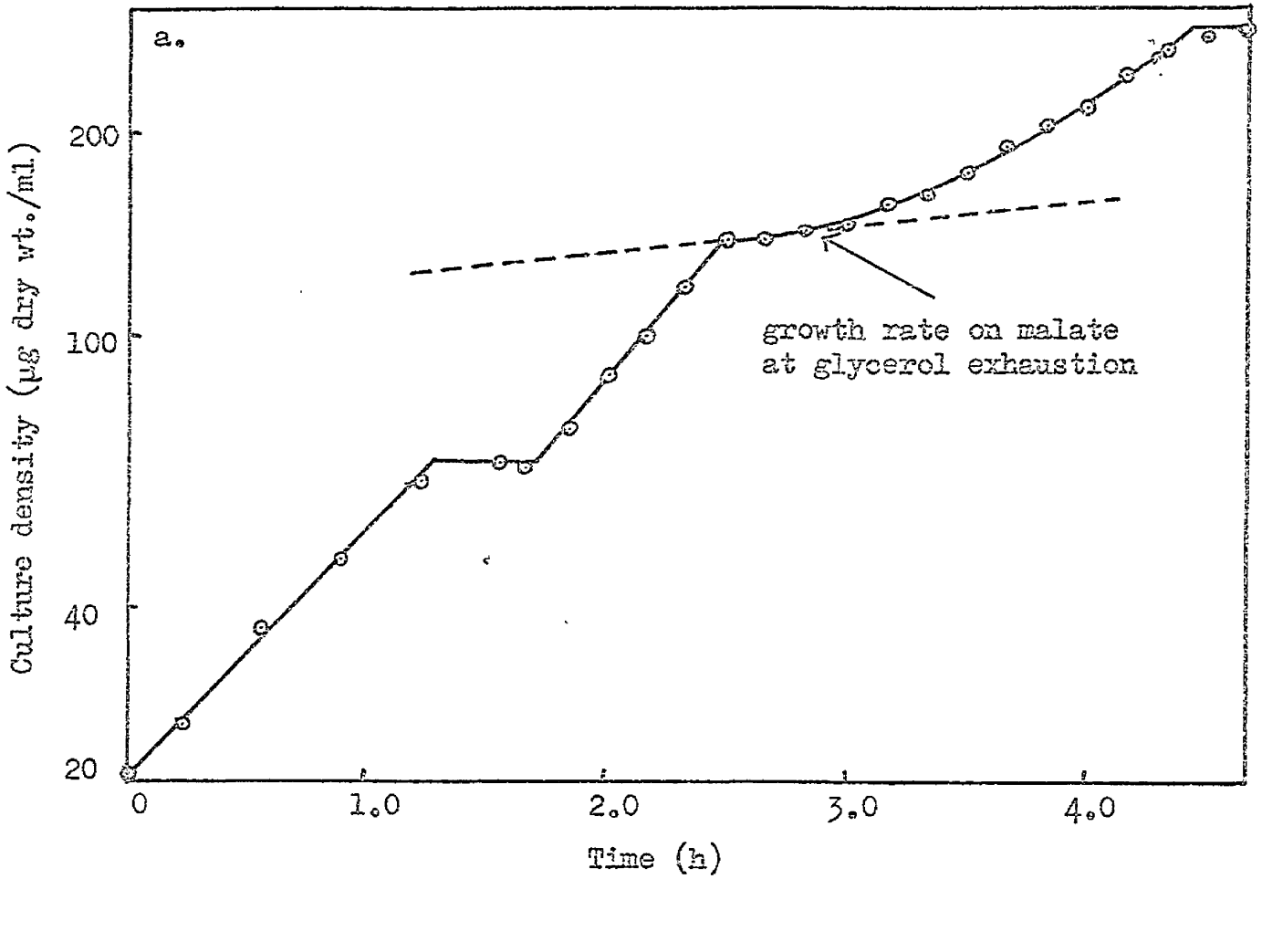

a.
200
100
40
20
Culture density (µg dry wt./ml)
0
1.0
2.0
3.0
4.0
Time (h)
growth rate on malate
at glycerol exhaustion

Figure 33

EFFECT OF GLYCEROL CONCENTRATION ON THE ADAPTATION, TO A MALATE UTILISING PHENOTYPE, OF E.coli 15224 GROWING IN GLYCEROL/MALATE MIXTURES

E.coli 15224 was prepared and grown as described in Figure 32. The data from Figure 32, and from other similar cultures to which different glycerol concentrations were added, simultaneously with malate, were used to determine the effect of glycerol concentration upon subsequent malate growth.

Concn. of glycerol in growth medium (mM)	Growth rate on malate at glycerol exhaustion μ (h^{-1})	Rate of oxygen uptake on malate at glycerol exhaustion (μmol/g dry wt./min)	Rate of carbon dioxide production on malate at glycerol exhaustion (μmol/g dry wt./min)
2.0	.33	180	310
1.0	.23	130	220
0.5	.17	90	160
0	.10	50	90

5. Adaptation to Malate of E.coli 15224 in glucose/malate mixtures

E.coli 15224 did not grow on malate after previous growth on glucose. The cells did grow on glucose with a specific growth rate of 0.85 h^{-1} (Figure 34). Addition of malate to a culture growing on glucose resulted in a growth curve with 3 distinct phases of growth (Figure 34).

5.1. Growth of E.coli 15224 on glucose

A culture of glucose trained E.coli 15224 inoculated into minimal salts medium containing glucose (1 mM) grew immediately and logarithmically (Figure 35a). Oxygen uptake measurements made during growth showed two distinct phases - the larger coinciding with the period of growth of the cells on the glucose but with a second smaller peak about 3-5 h after the growth of the culture had finished (Figure 35a). Carbon dioxide production measurement (not shown) gave similar results. Substrate estimation showed that glucose disappeared from the culture at the same time as growth ceased and that during growth, acetate appeared in the medium reaching a maximum concentration of 0.68 mM at the end of growth. Acetate then slowly disappeared from the culture over a period of several hours. The maximum rate of acetate disappearance occurred between 3 and 5 h after the end of growth (Figure 35b).

5.2. Effect of malate upon glucose growth

The presence of both malate and glucose in the medium before inoculation with glucose trained cells had no effect on the specific

Figure 34

GROWTH OF GLUCOSE TRAINED *E.coli* 15224 IN GLUCOSE AND MALATE CONTAINING MEDIA

Cells of *E.coli* 15224 trained to glucose were washed and inoculated into minimal salts medium containing the following substrates:

○—○ glucose (1 mM)

◎—◎ glucose (1 mM) + malate (6 mM)

△—△ malate (6 mM)

Growth of the cells was measured.

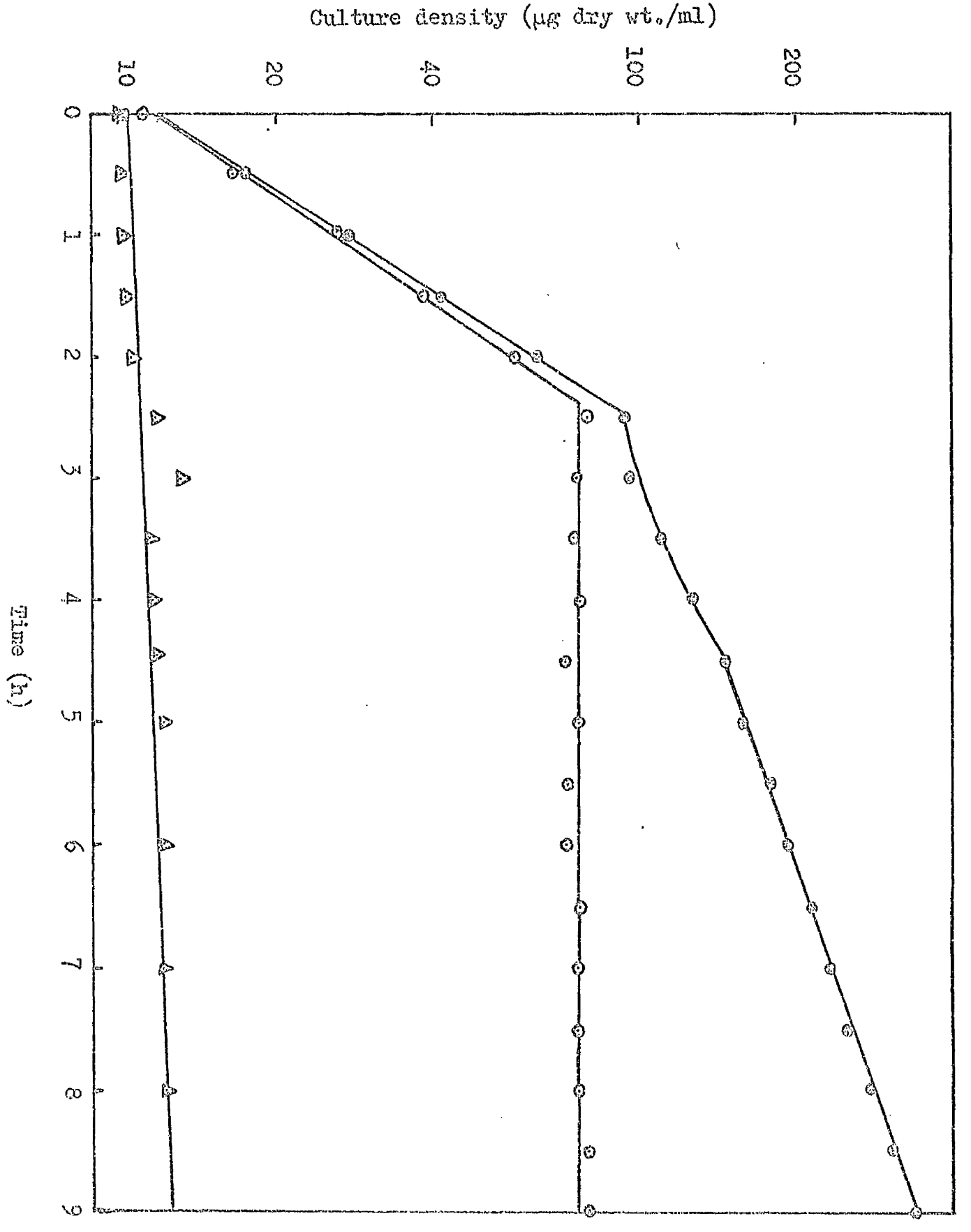

Culture density (µg dry wt./ml)
10
20
40
100
200
0
1
2
3
4
5
6
7
8
9
Time (h)

Figure 35

GROWTH AND METABOLISM OF GLUCOSE TRAINED *E.coli* 15224 ON GLUCOSE

Cells of *E.coli* 15224 trained to glucose were washed and inoculated into minimal salts medium containing 1 mM glucose.

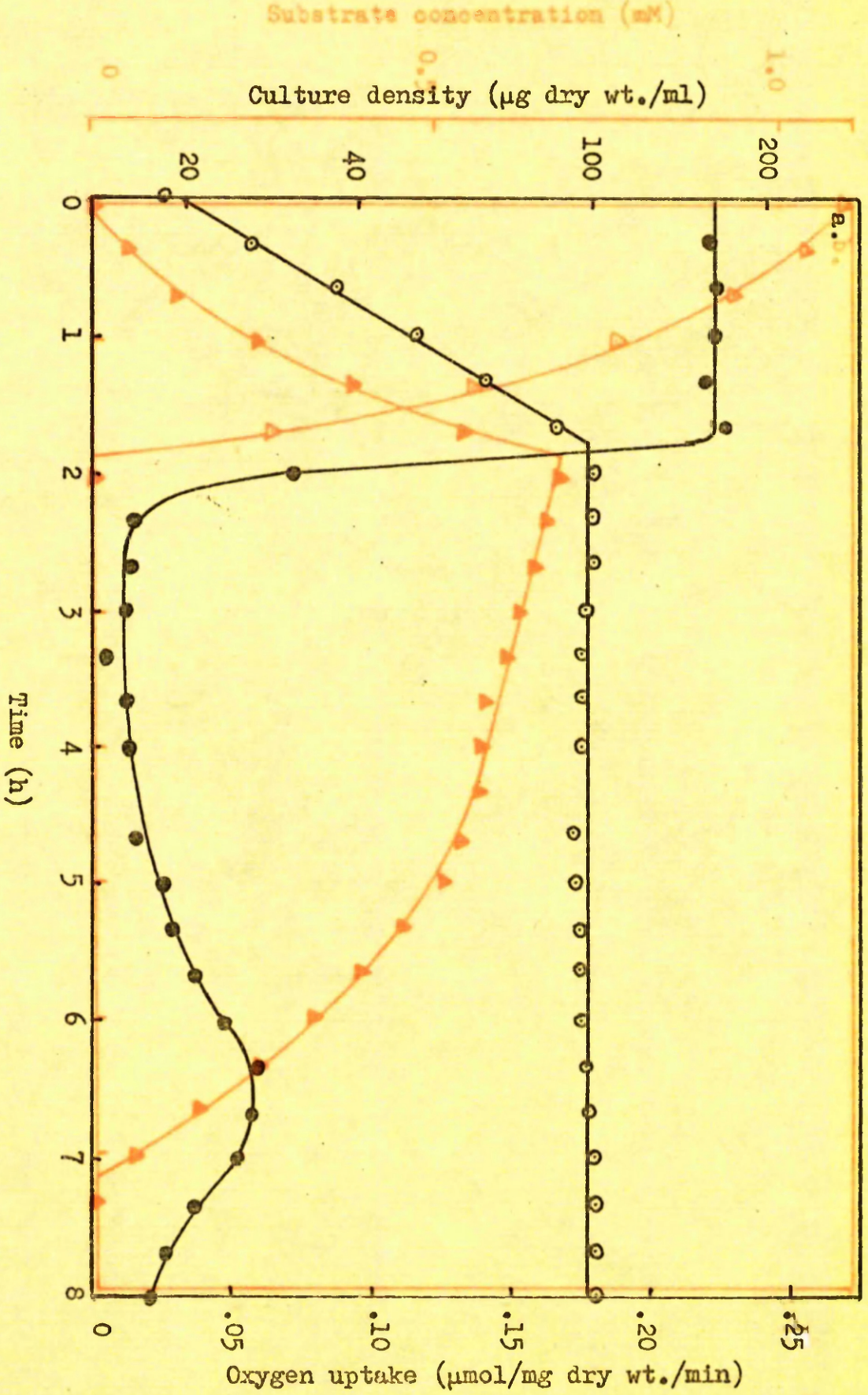
Growth, gas exchange and substrate concentrations were measured.

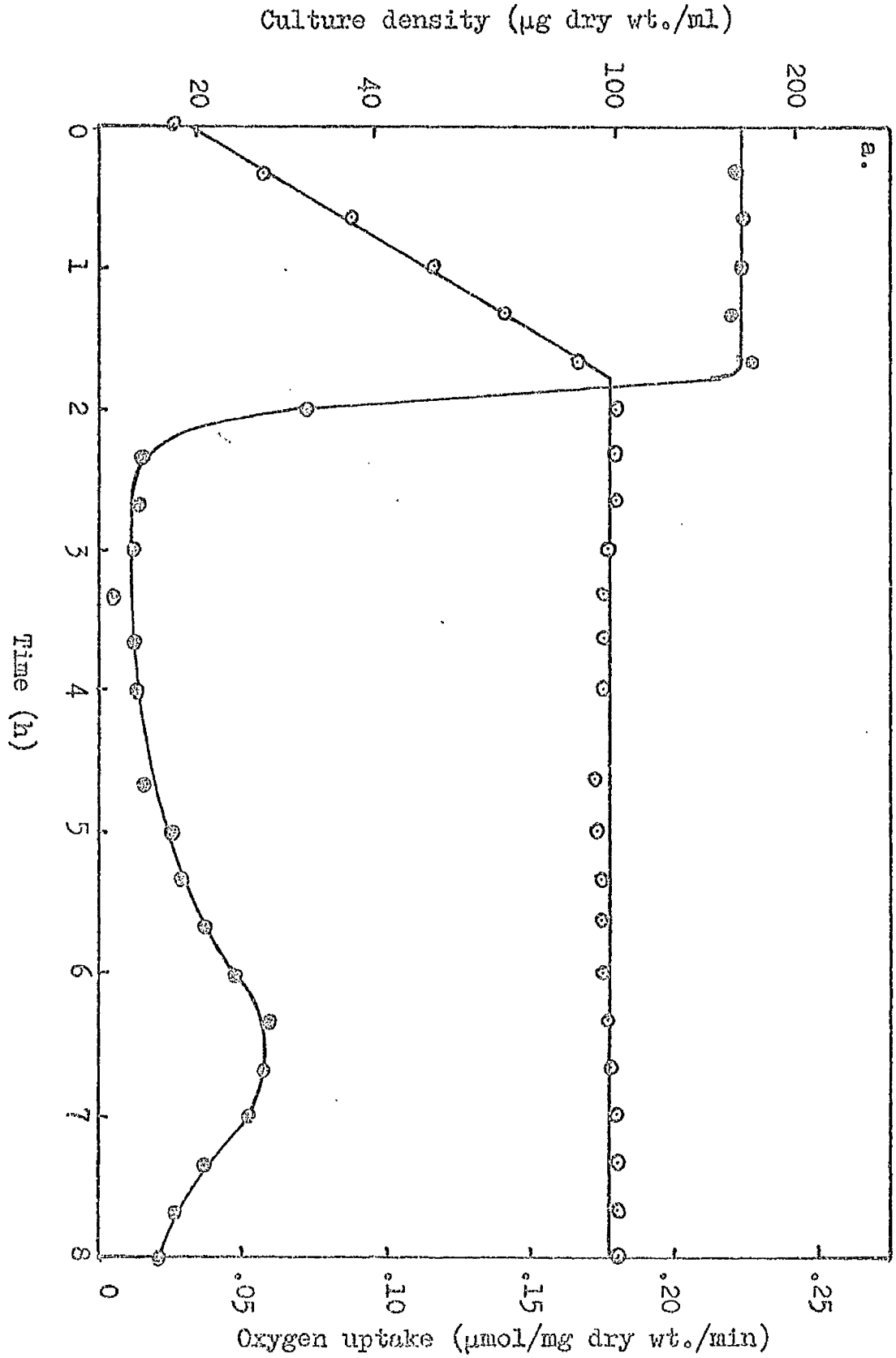
Figure 35a — Growth

Oxygen uptake

Figure 35b — Glucose concentration

Acetate concentration


Substrate concentration (mM)
0
0.5
1.0
Culture density (μg dry wt./ml)
20
40
100
200
a.
0
1
2
3
4
5
6
7
8
Time (h)
0
.05
.10
.15
.20
.25
Oxygen uptake (μmol/mg dry wt./min)


Culture density (µg dry wt./ml)
20
40
100
200
a.
0
1
2
3
4
5
6
7
8
Time (h)
0
.05
.10
.15
.20
.25
Oxygen uptake (µmol/mg dry wt./min)

growth rate of the cells compared to the glucose control but stimulated by 7% the rate of oxygen uptake (Figure 36a). During the 1st period of growth little malate was used and acetate continued to appear in the medium. The acetate concentration at glucose exhaustion was 0.64 mM (Figure 36b).

After exhaustion of glucose from the medium the cells continued to grow but with an accelerating rate of growth accompanied by an increasing rate of oxygen uptake while both acetate and malate were present in the medium (Figure 36a). Growth rate decreased when acetate disappeared from the medium and was accompanied by a fall in the rate of oxygen uptake.

The 3rd and final phase of growth was logarithmic and utilised only malate as a growth substrate. During the final 2 h of growth, the rate of oxygen uptake slowly increased. Growth finally halted when malate was exhausted.

5.3. Effect of malate addition after glucose exhaustion

In this situation the growth, oxygen uptake and acetate excretion before malate addition (Figure 37) are a repetition of the control (Figure 35). After addition of malate, rate of growth and of oxygen uptake (Figure 37a) increased and the concentration of acetate in the medium fell (Figure 37b). The increased rate of growth and of oxygen uptake continued for just under 2 h then both decreased simultaneously (Figure 37a), coinciding with the disappearance of acetate from the medium (Figure 37b). After this point malate was the only substrate present. Growth was slow, corresponding to a low but increasing rate

Figure 36

GROWTH AND METABOLISM OF GLUCOSE TRAINED *E.coli* 15224 ON GLUCOSE IN THE PRESENCE OF MALATE

Cells of *E.coli* 15224 trained to glucose were washed and inoculated into minimal salts medium containing 1 mM glucose and 6 mM malate.

Growth, gas exchange and substrate concentrations were measured.

Figure 36a


Growth

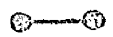
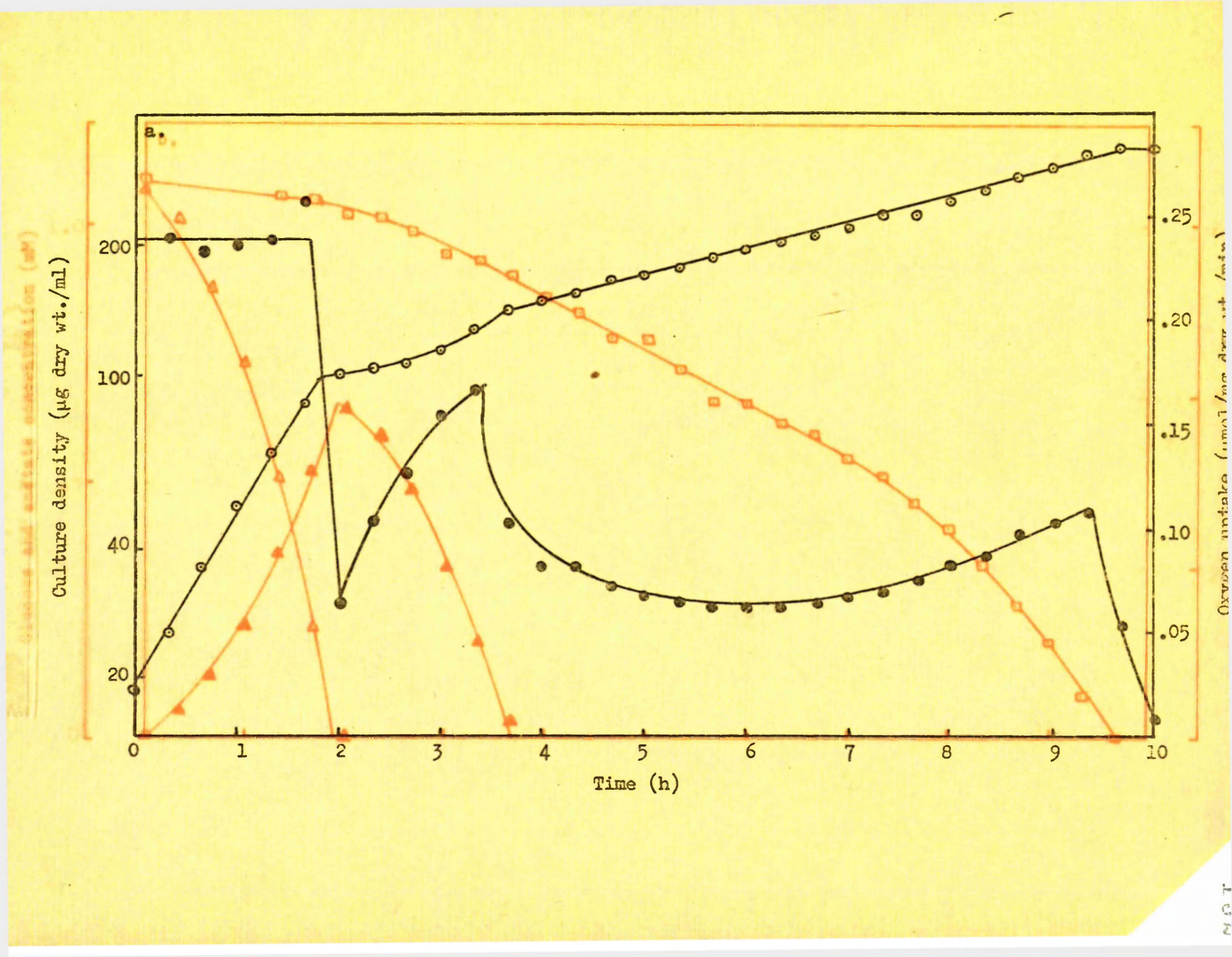

Oxygen uptake

Figure 36b


Glucose concentration


Acetate concentration


Malate concentration


a.
b.
Culture density (µg dry wt./ml)
200
100
40
20
0
1
2
3
4
5
6
7
8
9
10
Time (h)
.25
.20
.15
.10
.05

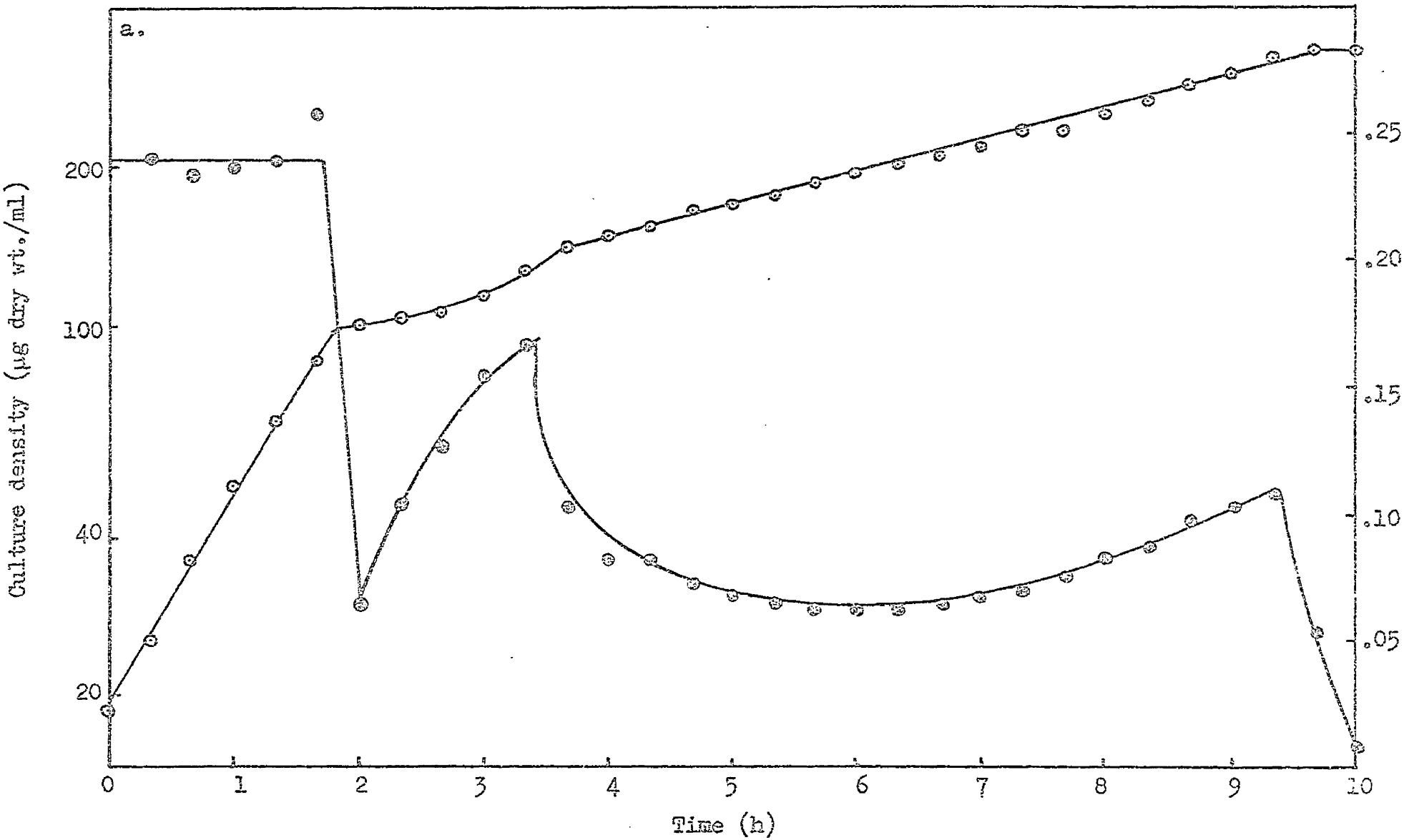

a.
Culture density (µg dry wt./ml)
200
100
40
20
0
1
2
3
4
5
6
7
8
9
10
Time (h)
.25
.20
.15
.10
.05

Figure 37

GROWTH AND METABOLISM OF GLUCOSE TRAINED *E.coli* 15224 WITH MALATE ADDED AT GLUCOSE EXHAUSTION

Cells of *E.coli* 15224 trained to glucose were washed and inoculated into minimal salts medium containing 1 mM glucose. Upon exhaustion of glucose from the medium malate was added to a final concentration of 6 mM.

Growth, gas exchange and substrate concentrations were followed throughout.

Figure 37a

Growth

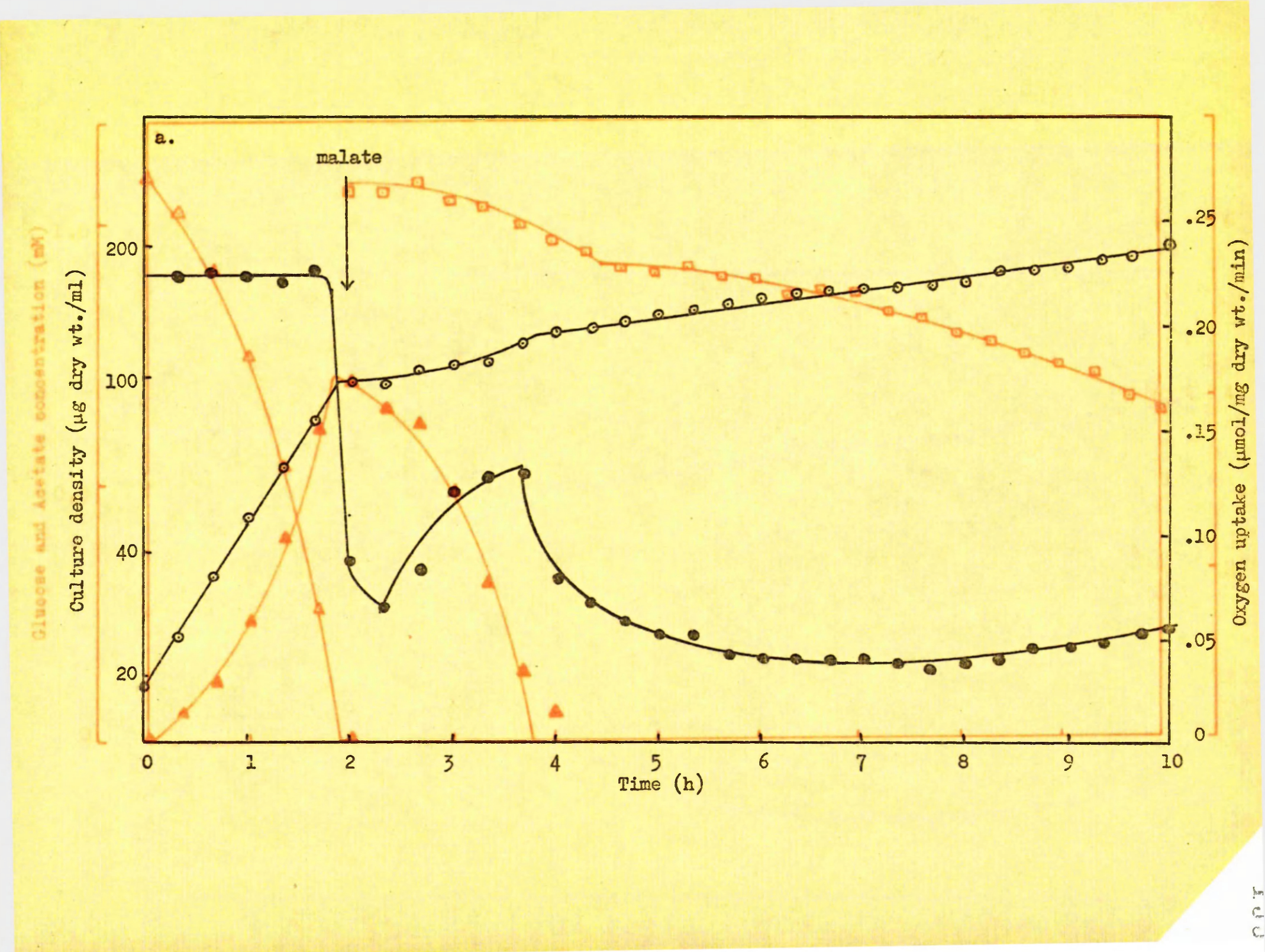
Oxygen uptake

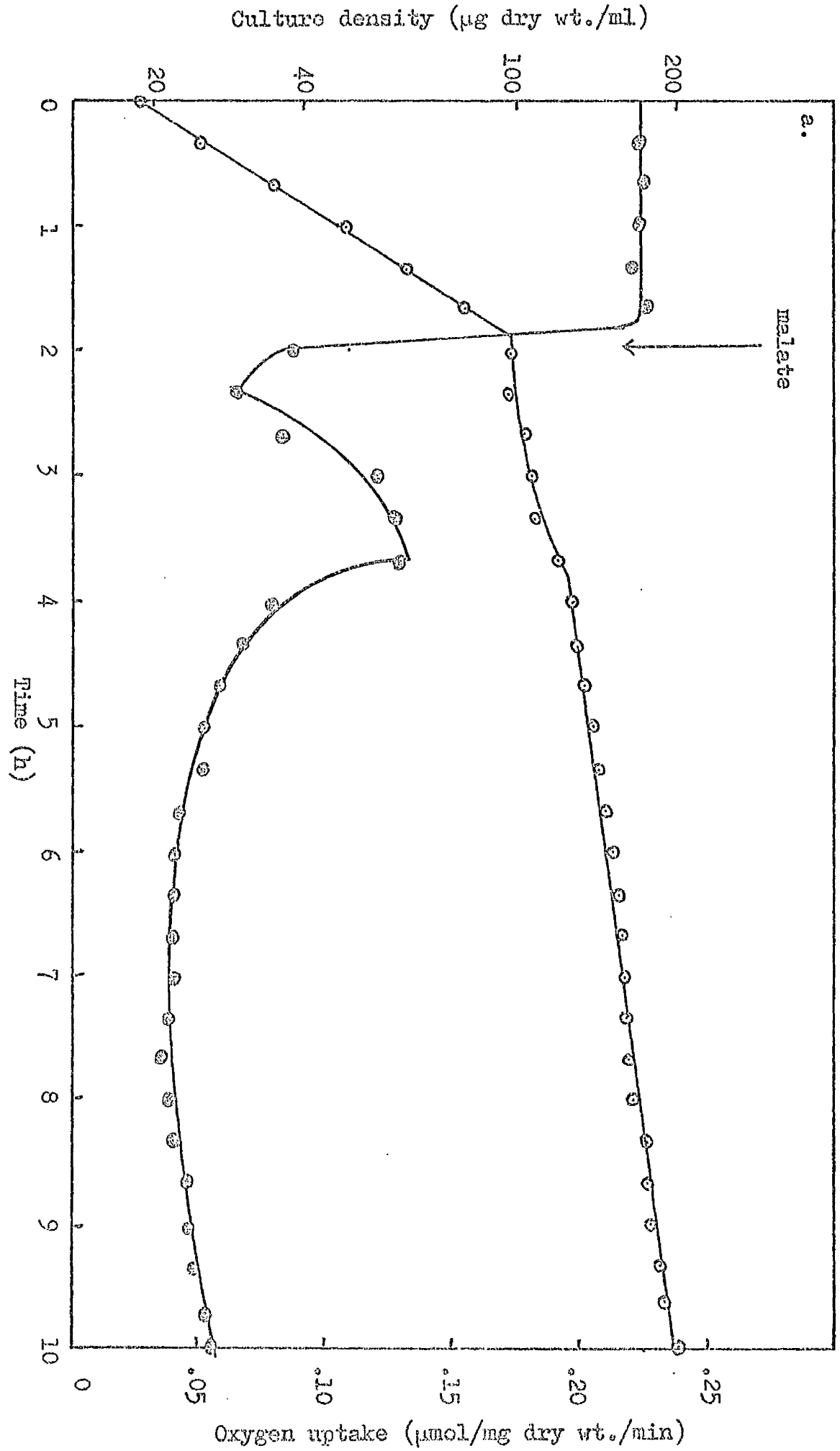
Figure 37b

Glucose concentration

Acetate concentration

Malate concentration


a.
malate
Culture density (µg dry wt./ml)
Glucose and Acetate concentration (mM)
Oxygen uptake (µmol/mg dry wt./min)
Time (h)
200
100
40
20
0
1
2
3
4
5
6
7
8
9
10
.25
.20
.15
.10
.05
0


Culture density (µg dry wt./ml)
20
40
100
200
a.
malate
Time (h)
0
1
2
3
4
5
6
7
8
9
10
0
·05
·10
·15
·20
·25
Oxygen uptake (µmol/mg dry wt./min)

of oxygen uptake (Figure 37a). After 10 h of growth malate had not completely disappeared from the medium. The result is thus similar to that obtained when malate and glucose are present at the time of inoculation except that rate of malate utilisation is somewhat slower.

5.4. Malate utilisation by glucose trained E.coli 15224

The results showing the effect of malate upon glucose trained cells all contained a period of growth when malate was metabolised with a second substrate. In the experiment shown in Figure 38 malate was added to the culture after acetate had been exhausted so that no dual substrate growth occurred.

The results showed that, in the absence of dual substrate growth, the rate of growth of the cells on malate (Figure 38a) and the rate of oxygen uptake on malate (Figure 38b) were low. At the same time the rate of disappearance of malate from the medium was slow (Figure 38c).

5.5. Effect of glucose and acetate in addition to malate on the adaptation of E.coli 15224 to malate

A comparison of the rate of oxygen uptake data from Figures 36, 37 and 38 where malate was added at different times to glucose trained cells growing on glucose shows that the ability of the cells to metabolise malate, as measured by oxygen uptake, was related to growth in the presence of malate.

The ability of glucose trained cells to metabolise malate was very low at 15 μ mol oxygen/g dry wt./min (Figure 38b) but increased to 40 μ mol oxygen/g dry wt./min if malate was added while acetate was still present in the culture medium (Figure 37a). If malate was added

Figure 38

GROWTH AND METABOLISM OF GLUCOSE TRAINED *E.coli* 15224 WITH MALATE ADDED AT ACETATE EXHAUSTION

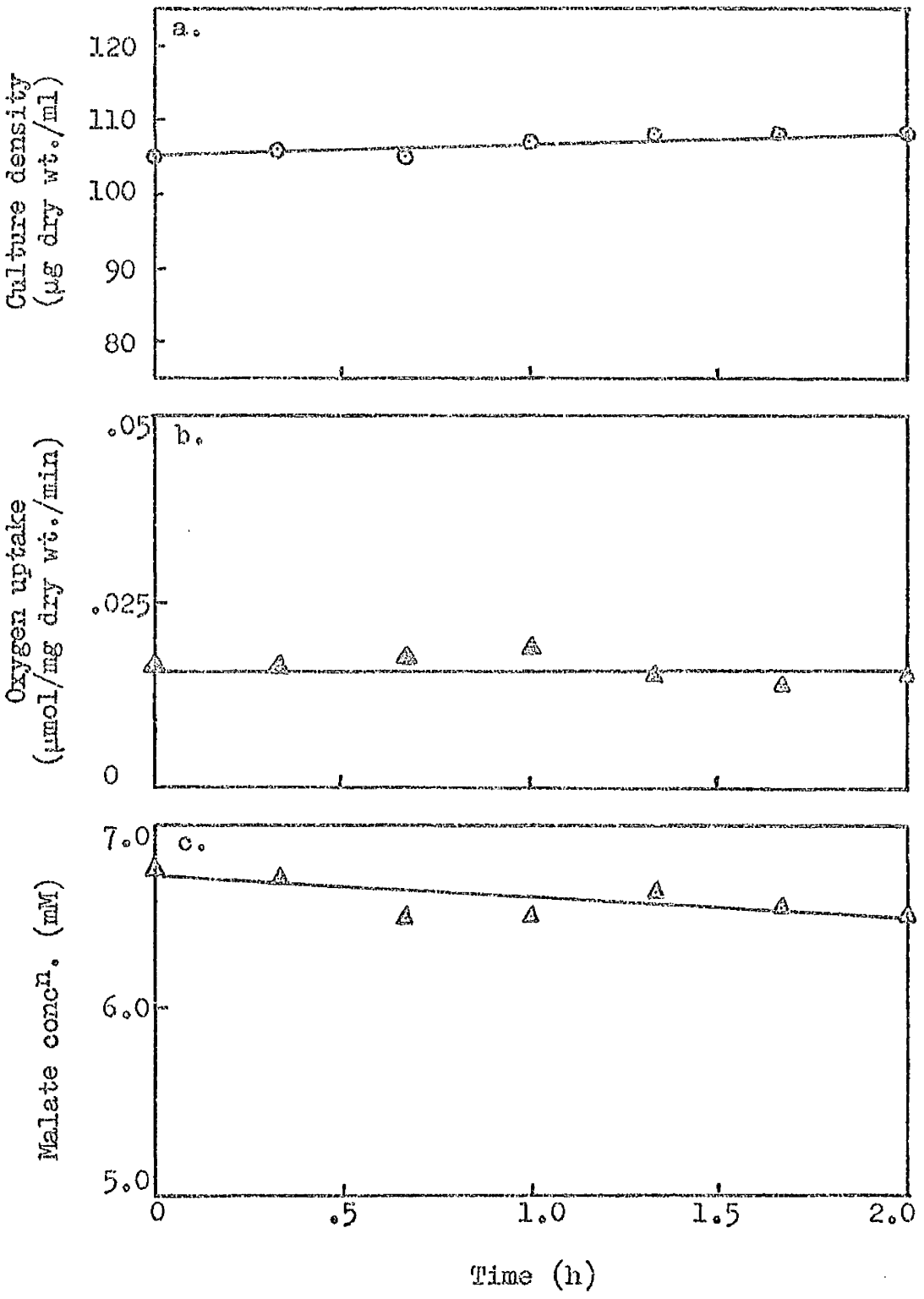
Glucose trained *E.coli* 15224 were washed and inoculated into minimal salts medium containing 1 mM glucose. Upon exhaustion of acetate from the medium, malate, to a final concentration of 6 mM, was added.

Growth, gas exchange and substrate concentration were measured throughout but are only shown from the point of malate addition.

Figure 38a ○—○ Growth

Figure 38b △—△ Oxygen uptake

Figure 38c △—△ Substrate concentration


a.
120
110
100
90
80
Culture density
(μg dry wt./ml)
b.
.05
.025
0
Oxygen uptake
(μmol/mg dry wt./min)
c.
7.0
6.0
5.0
Malate concn. (mM)
0
.5
1.0
1.5
2.0
Time (h)

while glucose was present, then the rate at which malate was metabolised was further increased to 60 μ mol oxygen/g dry wt./min (Figure 36a). Furthermore the peak rate of oxygen uptake observed for metabolism of the acetate/malate mixture increased from 130 μ mol/g dry wt./min to 165 μ mol/g dry wt./min if malate was present when the cells were metabolising glucose (Figures 37a and 36a).

6. Acetate Stimulation of Growth on Malate

Figures 36 and 37 show that both the growth rate and the rate of oxygen uptake of cells growing on malate were stimulated by the presence of acetate in the culture. Stimulation of growth by acetate was a general effect observed with all cells growing on malate.

6.1. Malate trained cells and acetate stimulation

Cells trained to malate when inoculated into malate based medium grew immediately and logarithmically at a specific growth rate of 0.64 h^{-1}. Addition of acetate to the medium resulted in a higher specific growth rate of 0.77 h^{-1} (Figure 39) which was maintained until malate was exhausted from the medium.

6.2. Glycerol trained cells and acetate stimulation

The pattern of growth of glycerol trained cells on malate has already been shown (Figure 28) to be slow, with an accelerating growth rate and was repeated here (Figure 40). In the presence of both acetate and malate in the medium the rate of growth of glycerol trained cells was higher than in malate medium. The growth rate on acetate/malate medium accelerated over a period of 2 h then stabilised at a specific growth rate of 0.69 h^{-1}. Although markedly stimulated in their rate of growth on malate by the addition of acetate to the medium, the cells were not able to use acetate in the absence of malate as shown by the very slow growth rate of the cells on acetate (Figure 40).

6.3. Glycerol/malate cells and acetate stimulation

Figure 29 shows that the ability of glycerol trained cells to

Figure 39

ACETATE STIMULATION OF GROWTH OF MALATE TRAINED

E.coli 15224 ON MALATE

Malate trained E.coli 15224 were washed and inoculated into minimal salts medium containing 6 mM malate. 90 min after inoculation acetate, to a final concentration of 8 mM, was added. Growth was measured.

○——○ 6 mM malate

⊙——⊙ 6 mM malate + 8 mM acetate

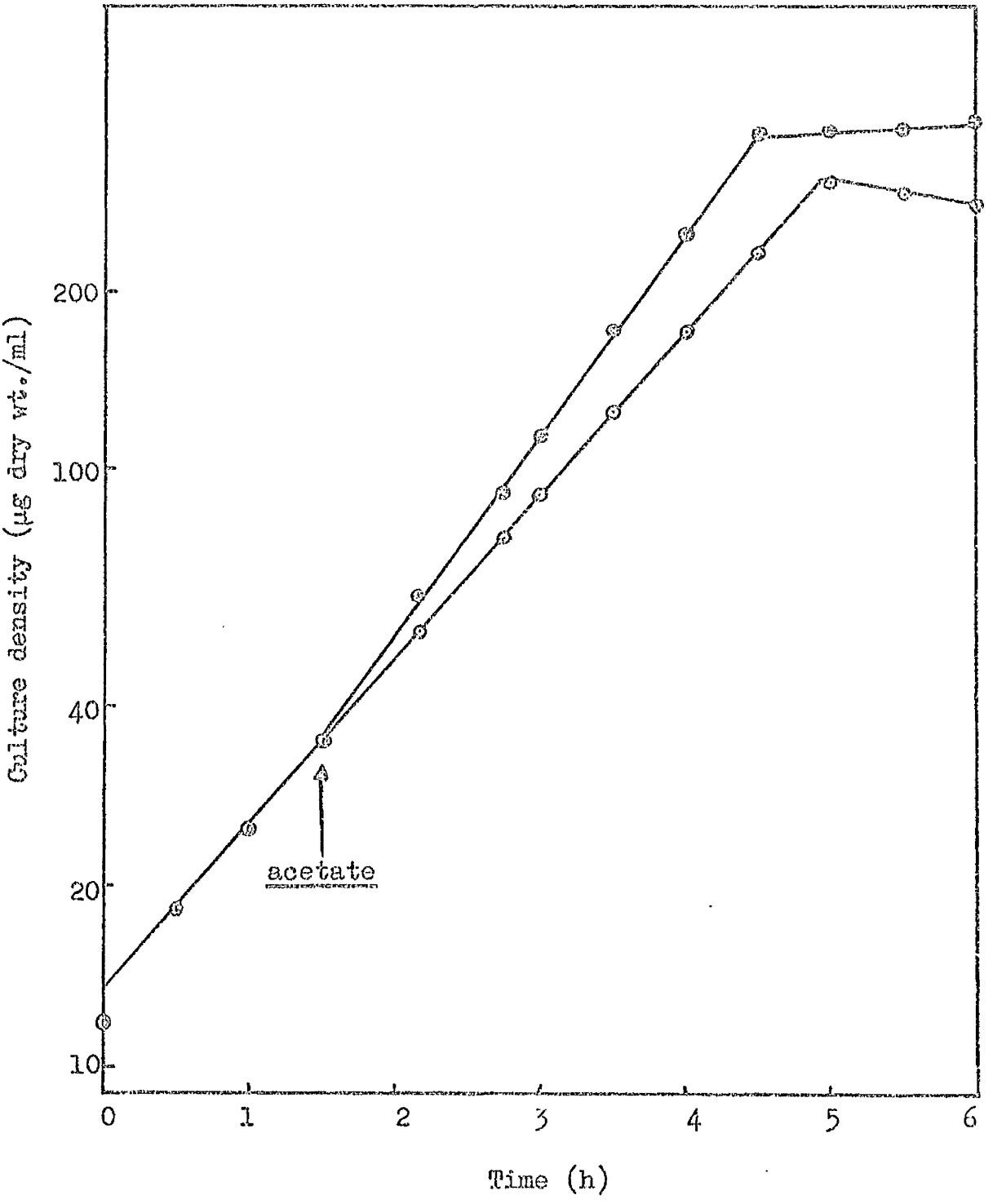

Culture density (µg dry wt./ml)
200
100
40
20
10
acetate
0
1
2
3
4
5
6
Time (h)

Figure 40

ACETATE STIMULATION OF THE GROWTH OF GLYCEROL TRAINED *E.coli* 15224 ON MALATE

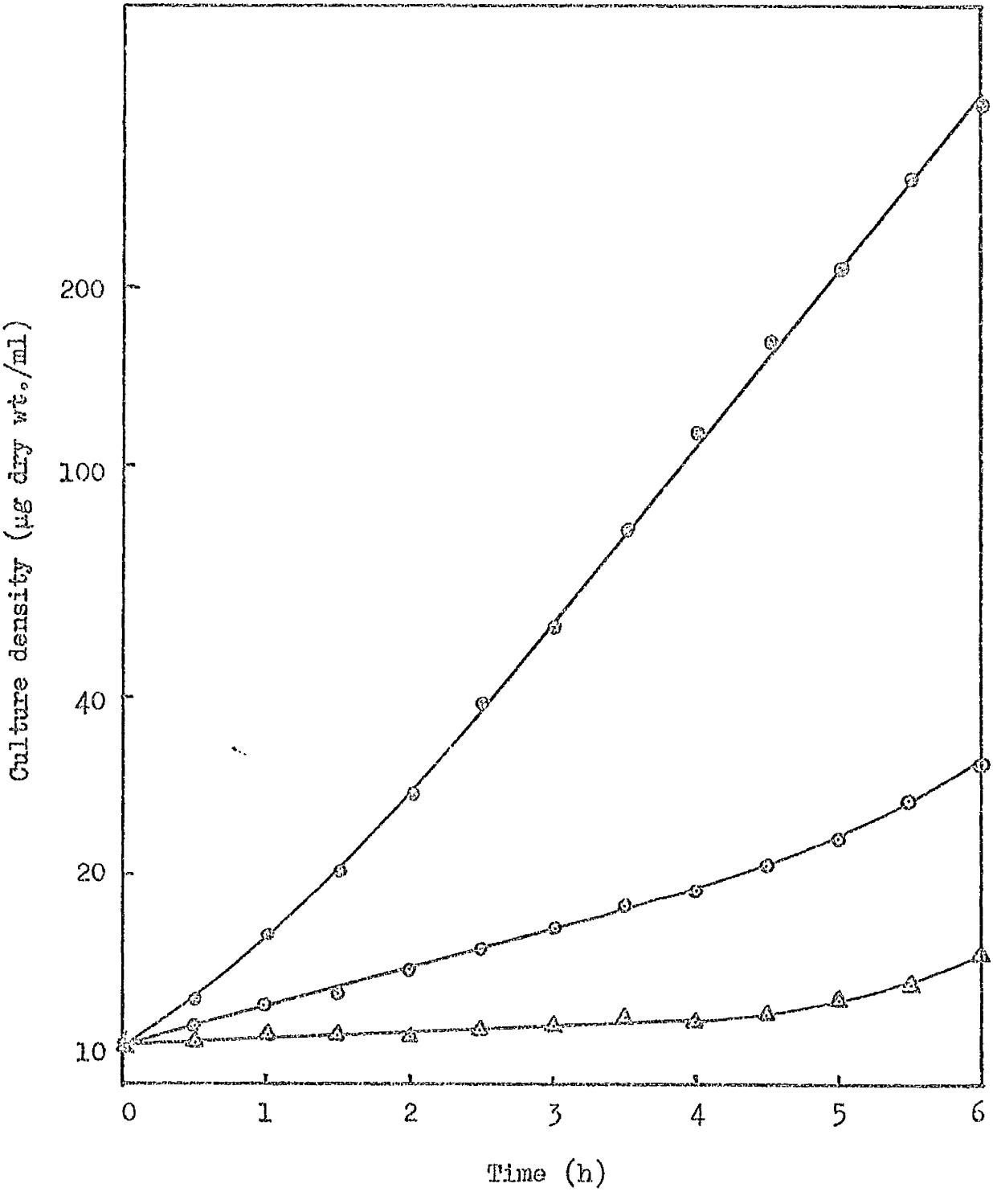
Cells of *E.coli* 15224, trained to glycerol, were washed and inoculated into minimal salts medium containing the following substrates:

○—○ 8 mM malate

△—△ 8 mM acetate

⊙—⊙ 8 mM malate + 8 mM acetate

Growth was measured.


Culture density (μg dry wt./ml)
200
100
40
20
10
0
1
2
3
4
5
6
Time (h)

utilise malate was increased by growth in a mixture of glycerol and malate. The rate of growth and the rate of oxygen uptake on malate, after glycerol/malate growth, was immediately stimulated by addition of acetate to the culture (Figure 41). The data available after acetate exhaustion do not permit assessment of a change in growth rate but the rate of oxygen uptake decreased then stabilised at a value higher than that on malate in the control culture.

6.4. Glucose cells and acetate stimulation

Stimulation of growth of glucose trained cells on malate has been measured in two ways (Figures 42 and 43). Glucose trained cells, after overnight storage at 4°, grew slowly on malate (Figure 42). They grew at a much higher growth rate if acetate was also present in the medium. The cells were however able to make use of acetate as a growth substrate and grow faster on it than on malate. Growth rate on the malate/acetate mixture was higher than growth rate on either of the substrates alone.

Cells grown on glucose to the point of exhaustion of acetate before malate was presented to them represent the cells least able to metabolise malate that have been obtained. These cells did not grow at all on malate over a period of 4 h (Figure 43a) and only took up very small quantities of oxygen (20 μ mol/g dry wt./min). Addition of a small quantity of acetate (0.5 mM) to such a culture produced a marked stimulation in growth rate (Figure 43a) and in the rate of oxygen uptake of the cells (Figure 43b) but stimulation only lasted while acetate was present in the medium. Upon exhaustion of acetate

Figure 41

ACETATE STIMULATION OF GROWTH ON MALATE OF *E.coli* 15224

AFTER GROWTH ON A MIXTURE OF GLYCEROL AND MALATE

Cells of *E.coli* 15224, trained to glycerol, were washed and inoculated into minimal salts medium containing 2 mM glycerol and 6 mM malate. On exhaustion of the glycerol, 0.5 mM acetate was added to the culture as indicated. Growth, substrate and oxygen uptake were measured throughout.

Figure 41a Growth

o—o glycerol/malate

●—● glycerol/malate + acetate

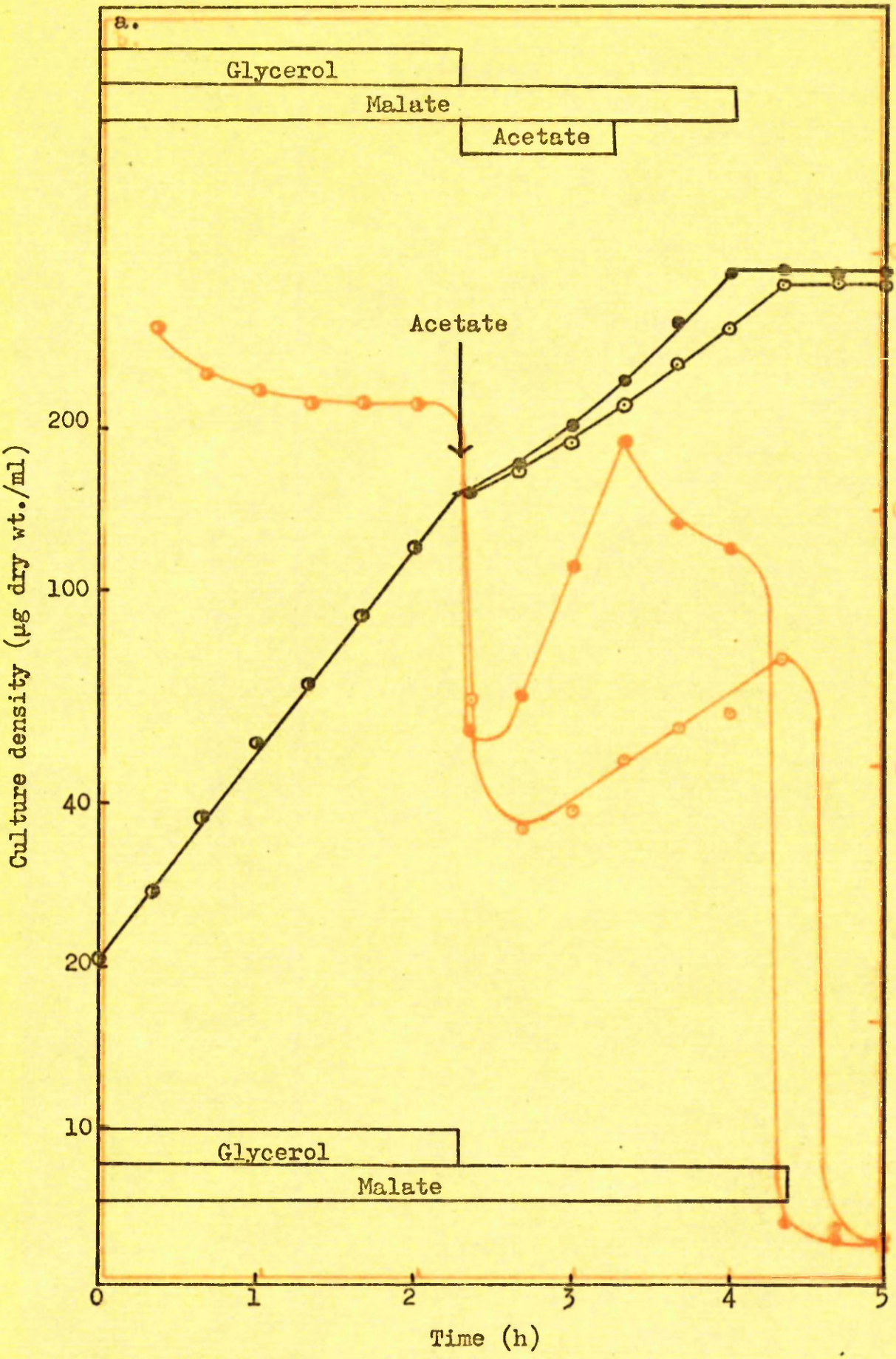
Presence of substrate in the cultures is indicated by the bars at top and bottom of the diagram.

Figure 41b Overlay

Oxygen uptake

o—o glycerol/malate

●—● glycerol/malate + 0.5 mM acetate


a.
Glycerol
Malate
Acetate
Acetate
Glycerol
Malate
Culture density (µg dry wt./ml)
Oxygen uptake (µ mol/mg dry wt./min)
Time (h)
200
100
40
20
10
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5

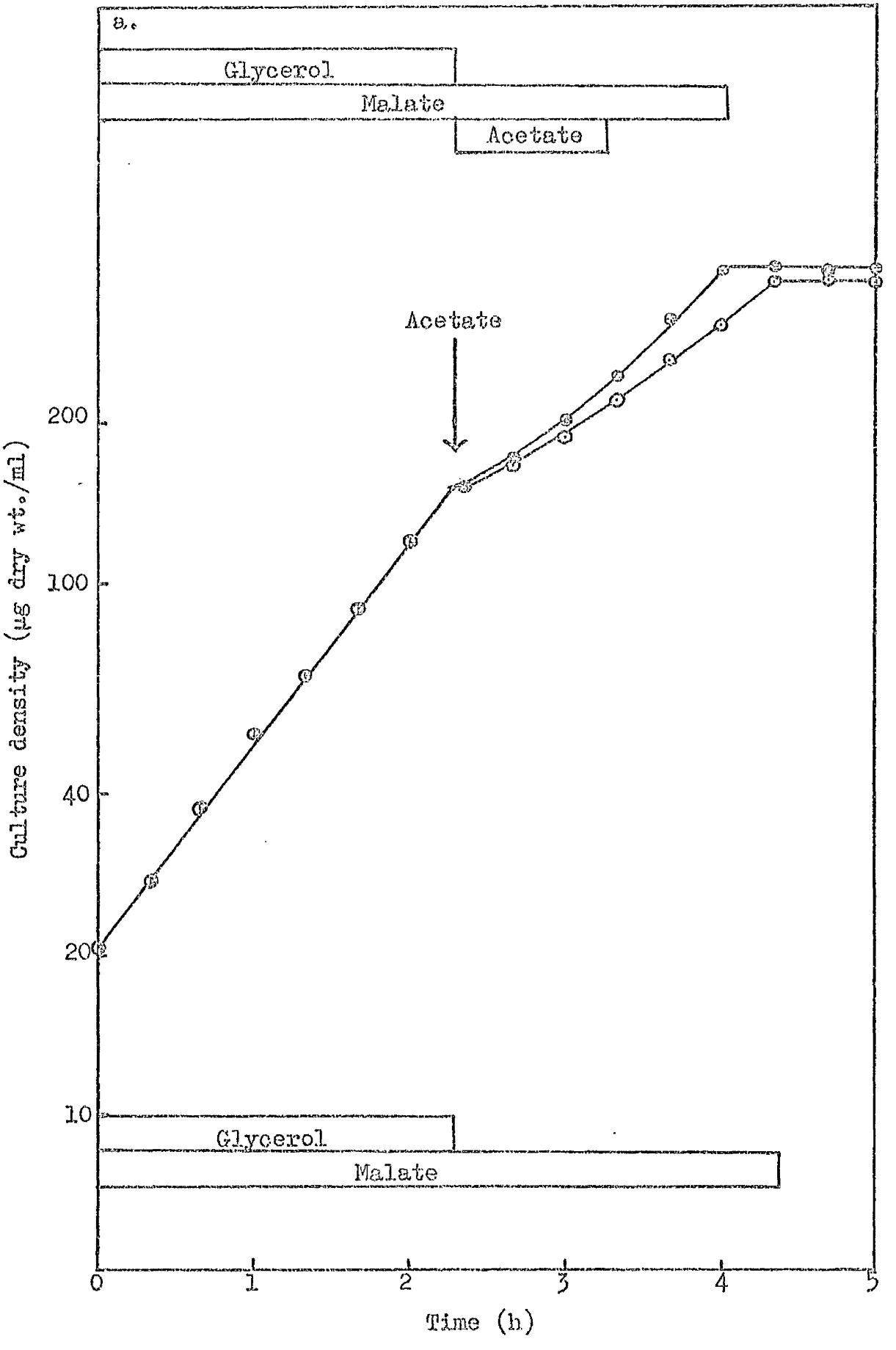

a.
Glycerol
Malate
Acetate
Acetate
200
100
40
20
10
Culture density (µg dry wt./ml)
Glycerol
Malate
0
1
2
3
4
5
Time (h)

Figure 42

STIMULATION BY ACETATE OF GROWTH ON MALATE OF

E.coli 15224 TRAINED TO GLUCOSE

Glucose trained *E.coli* 15224 were harvested, resuspended and inoculated into minimal salts medium containing the following substrates:

8 mM malate

8 mM acetate

8 mM malate + 8 mM acetate

Growth was measured.

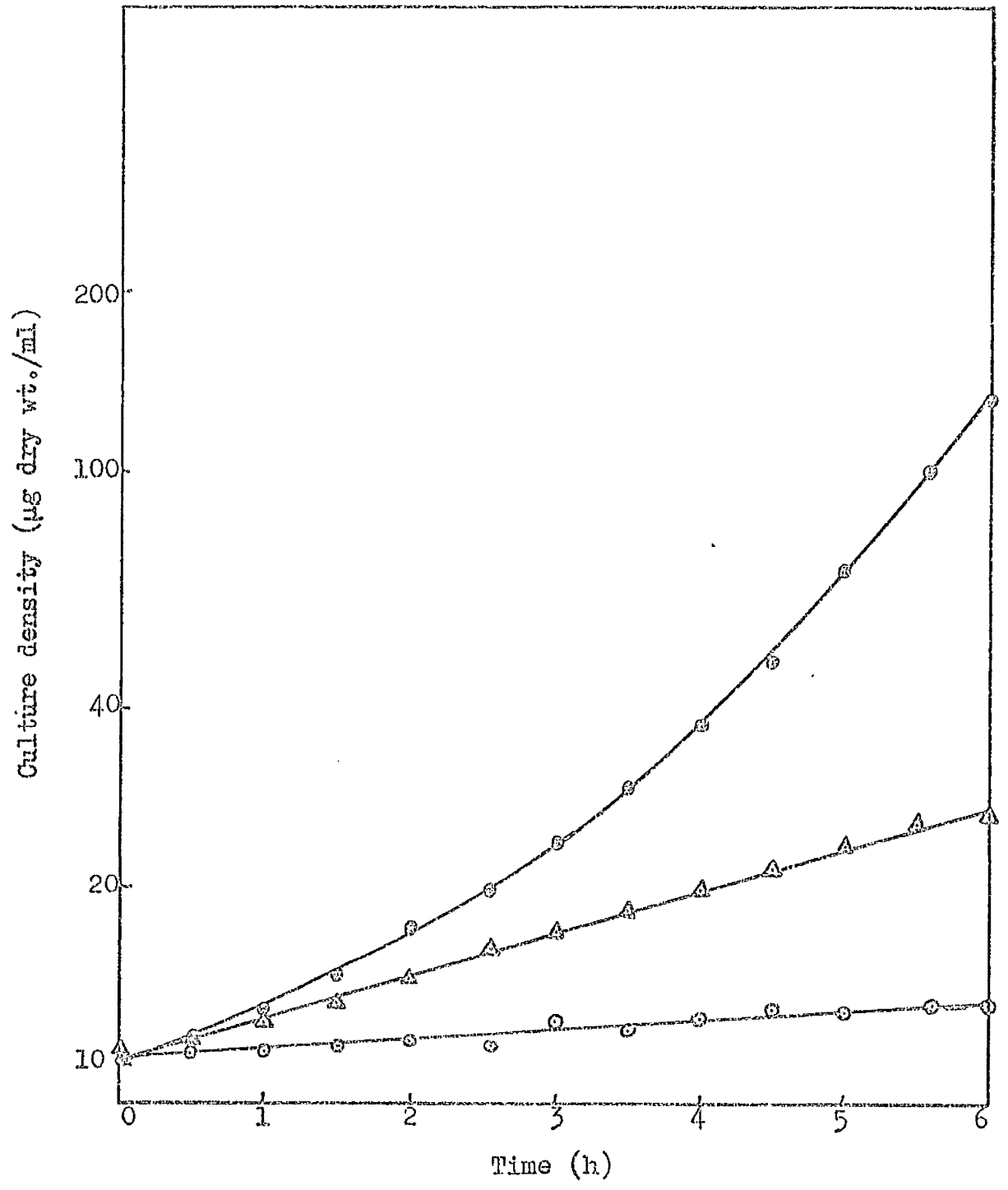

Culture density (µg dry wt./ml)
200
100
40
20
10
0
1
2
3
4
5
6
Time (h)

Figure 43

STIMULATION BY ACETATE OF GROWTH ON MALATE OF *E.coli* 15224 AFTER GROWTH ON GLUCOSE

Cells of *E.coli* 15224 trained to glucose were washed and inoculated into minimal salts medium containing 1 mM glucose and allowed to grow until all acetate had been metabolised. 6 mM malate, followed at 1 h by 0.5 mM acetate, was then added and growth, oxygen uptake and substrate concentrations measured.

Figure 43a Growth

o—o 6 mM malate

●—● 6 mM malate + 0.5 mM acetate

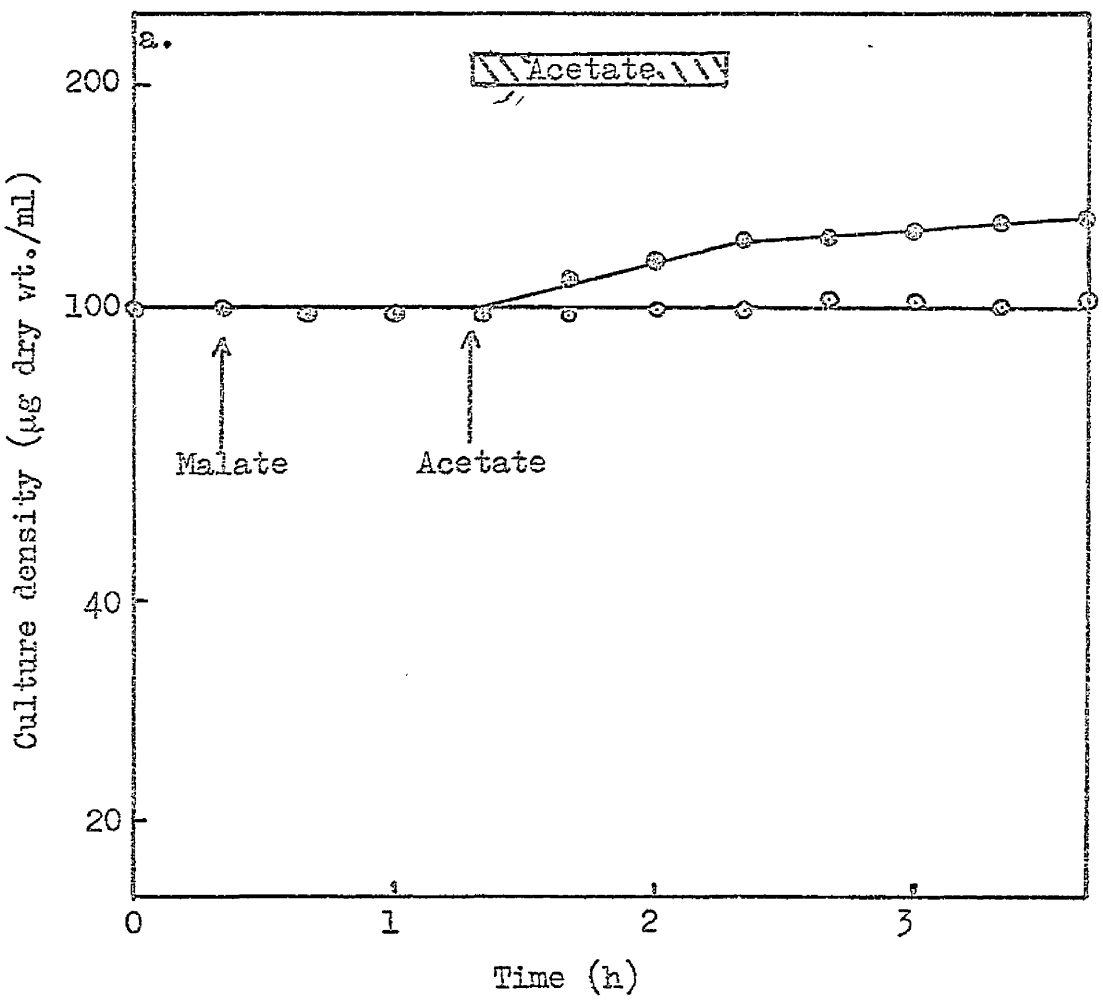
Shaded area shows time acetate was present.

Figure 43b Oxygen uptake

o—o 6 mM malate

●—● 6 mM malate + 0.5 mM acetate

Culture density (µg dry wt./ml)
200
100
40
20
Time (h)
0
1
2
3
Malate
Acetate
Acetate
a.


a.
Acetate
200
100
40
20
Culture density (µg dry wt./ml)
Malate
Acetate
0
1
2
3
Time (h)

both rate of growth and rate of oxygen uptake of the cells fell to a much lower value. Oxygen uptake fell from 130 μ mol/g dry wt./min to 40 μ mol/g dry wt./min. The final values of growth rate and rate of oxygen uptake of the cells on malate were higher than the values obtained before acetate addition.

7. Growth on Acetate/Malate Mixtures and Adaptation to Malate

Some of the data from previous figures (41 and 43) suggest that growth on mixtures of acetate and malate permits the cell to increase its capacity to metabolise malate. This was examined in more detail.

E.coli 15224 with little or no capacity to metabolise malate was obtained by growth on glucose until acetate had been metabolised (Figure 38). If malate was added to these cells no growth occurred. Addition of acetate allowed growth to proceed (Figure 43) and the amount of growth depended on the concentration of acetate added. After the acetate was exhausted the cells continued to grow on malate at a rate of growth which was also dependent on the concentration of acetate added to the culture (Figure 44).

A similar effect was observed if cells trained to acetate were inoculated into minimal salts medium containing mixtures of acetate and malate. Adaptation to the ability to utilise malate occurred if acetate was present at the same time as malate. The extent to which adaptation took place depended on the concentration of acetate in the medium and was indicated by the specific growth rate and the rate of gas exchange, of both oxygen and carbon dioxide, of the cells on malate after exhaustion of acetate (Figure 45).

Figure 44

STIMULATION BY ACETATE/MALATE GROWTH OF THE GROWTH RATE ON MALATE OF GLUCOSE TRAINED *E.coli* 15224

Cells of *E.coli* 15224 were grown on glucose as described for Figure 43. Malate (6 mM), followed 1 h later by acetate at the concentrations shown, was added. Growth of the cultures was measured and the growth rates on malate, after acetate exhaustion, calculated.

Concn. of acetate added to culture (mM)	Growth rate on malate μ (h^{-1})
0	.021
.25	.040
.50	.054
.75	.097
1.00	.112
2.00	.230

Figure 45

ADAPTATION OF E.coli 15224 TO A MALATE UTILISING PHENOTYPE DURING GROWTH ON A MIXTURE OF MALATE AND ACETATE

Cells of E.coli 15224 trained to acetate were washed and inoculated into minimal salts medium containing malate (6 mM) and variable concentrations of acetate as in the figure. Growth and gas exchange of the cultures were measured and the growth rates and the rates of oxygen uptake of the cells on exhaustion of acetate calculated as for Figure 32.

Concn. of acetate in diauxic medium (mM)	Growth rate of malate at exhaustion of acetate μ (h^{-1})	Rate of oxygen uptake on (carbon dioxide prodn.) malate at exhaustion of acetate (µmol/g dry wt./min)
0	0.022	43 (28)
0.25	0.08	65 (60)
0.50	0.22	105 (160)

8. Malic Enzyme Activity in E.coli 15224

Two malic enzymes are involved in malate metabolism in E.coli and both activities have been determined under different conditions.

8.1. Malic enzymes and cell phenotype

Phenotypes of E.coli 15224 which exhibited different abilities to utilise malate were grown in the presence of relatively high substrate concentrations for periods from 4 to 7 h. Samples, taken during logarithmic growth of these cultures, were used to determine the activities of malic enzymes in the cells and to measure the cells ability to metabolise malate using growth rate and rate of oxygen uptake on malate as an assay. Not all parameters were constant throughout logarithmic growth. The range of specific activity of NAD-linked malic enzyme during growth on glucose is shown (Figure 46). 4-5 h after inoculation of the acetate culture the growth rate increased. The effect of increased growth rate on acetate on the rate of malate metabolism and malic enzyme specific activity is shown (Figures 46 and 47a).

In no case is the rate of malate utilisation dependent on the level of the malic enzymes in the cells (Figure 46).

8.2. Malic enzyme activities and adaptation to malate during glycerol/malate growth

The metabolism of cells trained to glycerol and inoculated into a mixture of glycerol and malate has already been described (Figure 29). In the experiment in Figure 47d, high concentrations of substrates

Figure 46

MALIC ENZYMES, GROWTH RATE AND RATE OF GAS EXCHANGE OF *E.coli* 15224 ON MALATE

Cells of *E.coli* 15224 trained to the substrates shown in column 1, were washed and inoculated into 2 litres of minimal salts medium containing their homologous substrates at the indicated concentrations.

Growth of the cultures was measured.

At intervals samples were taken, harvested at 4000 g and 4° for 30 min, washed in chilled 40 mM phosphate buffer pH 7.0 and harvested in 2 portions at 11,600 g for 10 min at 4°. One of the resultant pellets was stored at -70° for determination of malic enzyme activities, the other was resuspended in 40 mM phosphate buffer pH 7.0 for inoculation into minimal salts medium containing 6 mM malate. Growth rate and rate of gas exchange of the cells on malate medium were measured.

	NADP-linked malic enzyme (10^3U/mg dry wt.)	NAD-linked malic enzyme (10^3U/mg dry wt.)	Oxygen uptake in malate med. (µmol/g dry wt./min)	Carbon dioxide production (µmol/g dry wt./min)	Specific growth rate in malate med. µ (h^{-1})
malate (20 mM)	50.0	25.0	324	500	0.63
glycerol (20 mM)	40.0	18.0	75	60	0.12
glucose (10 mM)	20.0	12.7-18.3	45	30	0
acetate (30 mM) (1-4 h)	21.0	13.0	35	20	0
acetate (30 mM) (5-7 h)	32.5	21.0	140	100	0.17

(both 20 mM) were used to ensure that neither substrate was exhausted during the period of sampling. Samples were taken during growth to assay malate utilising ability, as measured by growth rate and rate of oxygen uptake on malate, and showed that the ability of the cells to metabolise malate increased with growth. The activities of the two malic enzymes did not vary greatly and indeed fell a little from the inoculum activity during the growth of the culture.

8.3. Malic enzyme activities and adaptation to malate during glucose/malate growth

Growth of glucose trained cells on a glucose/malate mixture is another system which has been examined (Figure 36) but in contrast to glycerol/malate growth did not result in the cells acquiring, to a large extent, the ability to metabolise malate. This was confirmed during growth in the presence of higher substrate concentrations (Glucose, 10 mM; Malate, 20 mM) where rate of growth and of oxygen uptake of the cells on malate increased only 50% during 5 generations of glucose/malate growth (Figure 47c).

In this medium the rate of synthesis of the NAD-linked malic enzyme was high at 0.037 U/mg dry wt. of cells, immediately after inoculation but gradually decreased with growth to a value of 0.019 U/mg dry wt. of cells. These changes in rate of synthesis were reflected by changes in the specific activity of the enzyme during growth (Figure 47c). An increased rate of synthesis of the NADP-linked malic enzyme was maintained throughout growth.

Figure 47

ADAPTATIC'' OF *E.coli* 15224 TO A MALATE UTILISING PHENOTYPE

Cells of *E.coli* 15224 were washed and inoculated into minimal salts medium with substrates as indicated below. Growth was measured and samples taken at intervals, during logarithmic growth, for estimation of malic enzyme activities, rate of growth and rate of gas exchange on malate as described for Figure 46.

○—○ NAD--linked malic enzyme

□—□ NADP-linked malic enzyme

△—△ Rate of oxygen uptake on malate

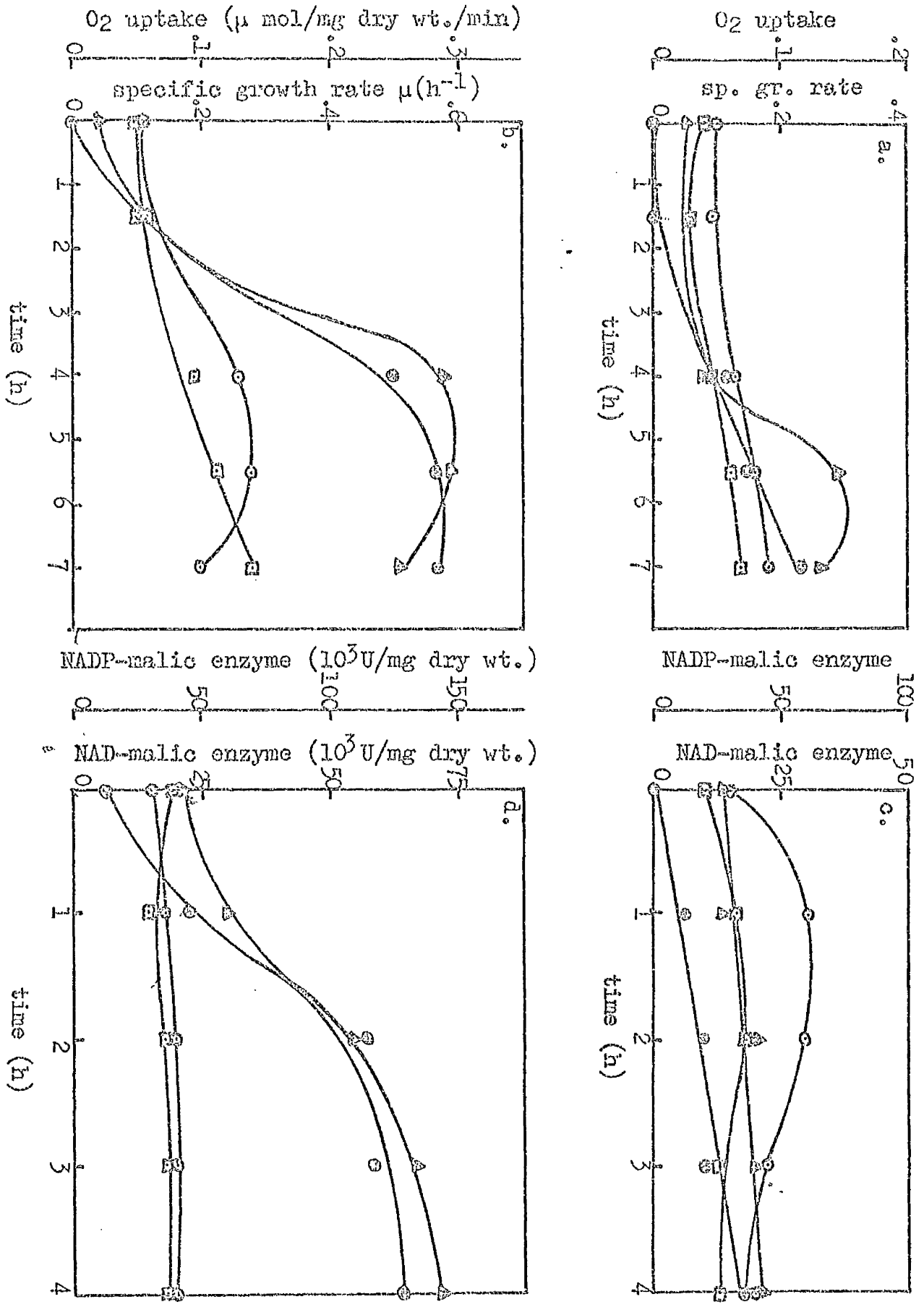
⊙—⊙ Specific growth rate on malate

Figure 47a Acetate trained cells in 30 mM acetate

b Acetate trained cells in 30 mM acetate/20 mM malate

c Glucose trained cells in 10 mM glucose/20 mM malate

d Glycerol trained cells in 20 mM glycerol/20 mM malate


O2 uptake (μ mol/mg dry wt./min)
specific growth rate μ(h⁻¹)
time (h)
b.
NADP-malic enzyme (10³U/mg dry wt.)
NAD-malic enzyme (10³U/mg dry wt.)
d.
O2 uptake
sp. gr. rate
a.
NADP-malic enzyme
NAD-malic enzyme
c.

8.4. Malic enzyme activities and adaptation to malate during acetate/malate growth

Growth in an acetate/malate mixture of acetate trained cells has also been studied as a malate adapting system (Figure 45). A similar result was obtained if cells were grown in a mixture of higher concentrations of acetate (30 mM) and malate (20 mM) and malate utilising ability measured after harvesting. As cells grew in acetate/malate medium their rate of growth and of oxygen uptake on malate increased, showing an increased ability to metabolise malate (Figure 47b). The increase in oxygen uptake observed was 15-fold over the inoculum level.

Malic enzyme activities in the culture also increased over the period of growth but only by 2.5-fold. The increase was approximately the same for both enzymes.

9. Adaptation to Malate Utilisation During Glycerol/Maleic Acid Growth

Glycerol trained cells of *E.coli* 15224 grew in medium containing glycerol (20 mM) and maleic acid (20 mM). The presence of maleic acid neither stimulated nor inhibited the growth of the cells on glycerol. Samples taken during the growth of the culture showed that the cells ability to metabolise malate increased with growth (Figure 48) although little increase was observed during the first generation.

There was no alteration in the activities of the malic enzymes in the culture during growth.

9.1. Purity of maleic acid

The purity of the maleic acid was determined with respect to other dicarboxylic acids which might have been present by measuring the oxygen uptake of malate trained cells inoculated into medium containing 20 mM maleic acid. After inoculation a pulse of oxygen uptake was obtained which was due to the metabolism of metabolisable impurities in the maleic acid. The total quantity of oxygen taken up was proportional to maleic acid concentration and consequently to the concentration of the impurity. The system was calibrated by adding small quantities of malate to the cells after the initial pulse had passed and comparing the total oxygen uptake due to the impurity with that of the standard malate additions. Assuming all the impurity was malate the concentration in 20 mM maleic acid was 0.08 mM which was only 0.4%. If 0.08 mM metabolisable dicarboxylic acid was present it would be metabolised early in growth and would not affect the subsequent growth and adaptation of the cells to malate.

Figure 48

ADAPTATION OF *E.coli* 15224 TO A MALATE UTILISING PHENOTYPE IN GLYCEROL/MALEIC ACID MEDIUM

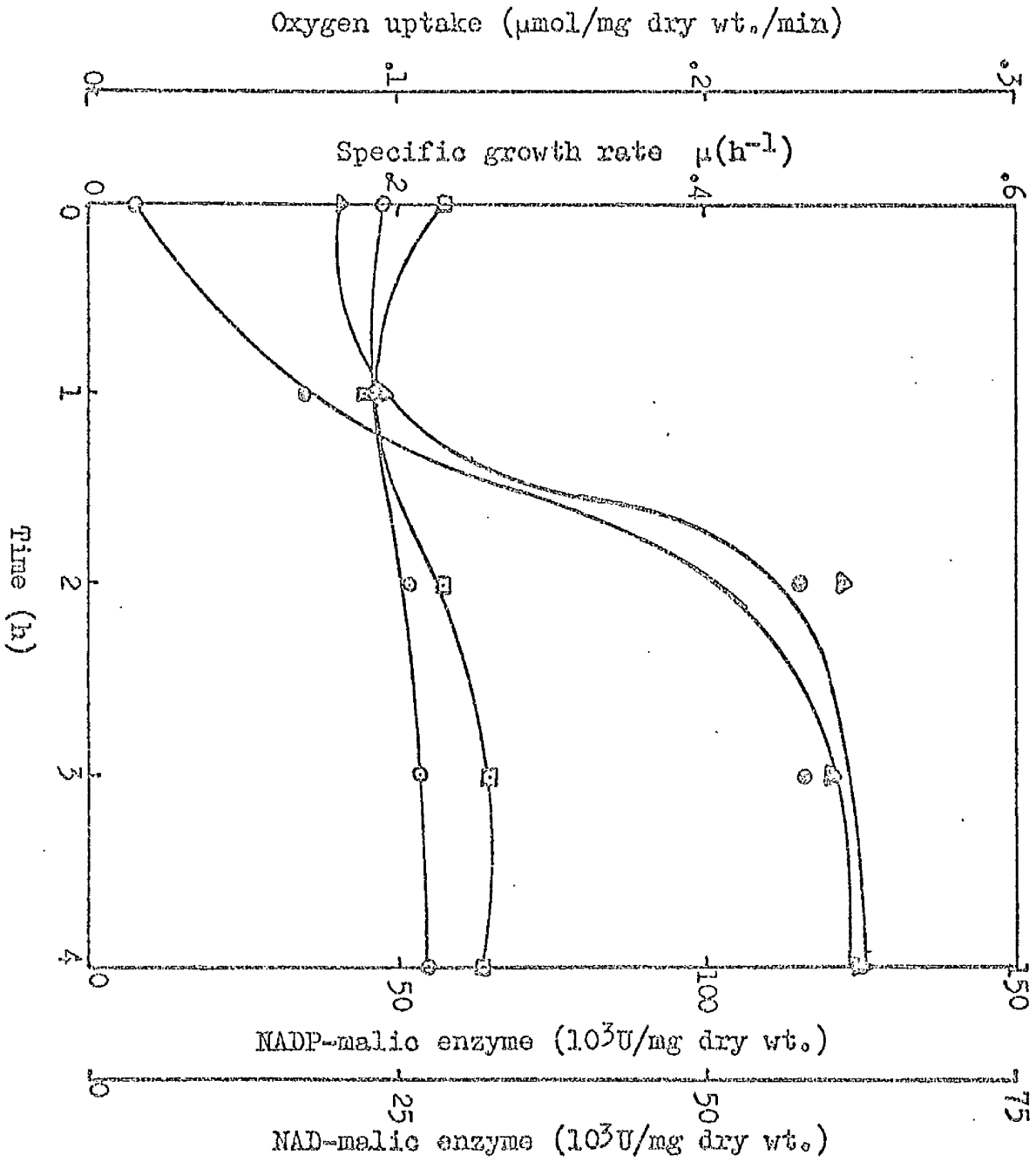
Glycerol trained *E.coli* 15224 were washed and inoculated into minimal salts medium containing 20 mM glycerol and 20 mM maleic acid. Growth of the culture was measured and samples, taken at intervals, were treated as described in Figure 46.

NAD-linked malic enzyme

NADP-linked malic enzyme

Oxygen uptake on malate

Specific growth rate on malate


Oxygen uptake (μmol/mg dry wt./min)
0
·1
·2
·3
Specific growth rate μ(h⁻¹)
0
·2
·4
·6
Time (h)
0
1
2
3
4
0
50
100
150
NADP-malic enzyme (10³U/mg dry wt.)
0
25
50
75
NAD-malic enzyme (10³U/mg dry wt.)

10. Malate Decarboxylation Activity During Adaptation

Glycerol trained cells inoculated into minimal salts medium containing 20 mM glycerol and either 20 mM malate or 20 mM maleic acid increased their ability to metabolise malate with growth (Figures 47c and 48). During the growth of these cultures the ability of the cells to decarboxylate extracellular malate to pyruvate was also markedly increased. After 4 h growth in glycerol/malate medium the cells ability to decarboxylate extracellular malate increased over 20-fold while the ability to utilise pyruvate only increased by 1.15-fold (Figure 49). Specific activities of the malic enzymes did not vary to the same extent - NADP-linked malic enzyme activity fell by 10% and NAD-linked malic enzyme activity rose by 30%.

A very similar picture was obtained for the cells growing on the glycerol/maleic acid medium where the ability to decarboxylate extracellular malate was increased nearly 35-fold during growth. Relatively small changes occurred either in the cells ability to utilise pyruvate or the specific activities of the malic enzymes in the cell (Figure 49).

Malate trained cells possessed pyruvate utilising ability and malic enzyme specific activities not markedly different from glycerol trained cells but had a much larger capacity to decarboxylate extracellular malate than did glycerol trained cells. After growth in either glycerol/malate or glycerol/maleic acid media the cells possessed almost 80% of the ability of malate trained cells to decarboxylate extracellular malate.

Figure 49

ADAPTATION OF E.coli 15224 TO A MALATE UTILISING PHENOTYPE AND MALATE DECARBOXYLATION ACTIVITY

Glycerol trained E.coli 15224 were washed and inoculated into minimal salts medium containing 20 mM glycerol and either, 20 mM malate or 20 mM maleic acid. Growth was measured and samples taken at intervals were harvested and used to measure malic enzyme activities as described for Figure 46. Pellets were also resuspended in chilled 40 mM phosphate buffer pH 7.0 and used to measure decarboxylation activity by the rate of pyruvate production from malate in the presence of 1mM arsenite. The rate of pyruvate utilisation was also measured in the presence of the inhibitor.

E.coli 15224 trained to and growing on malate containing medium was similarly treated to give comparable values for cells fully adapted to malate.

Nature of cells	pyruvate utilisation (nmol/mg/min)	pyruvate production (nmol/mg/min)	NADP malic enzyme (10^3U/mg dry wt.)	NAD malic enzyme (10^3U/mg dry wt.)
Glycerol grown cells	10.0	2.0	41.5	18.0
glycerol/malate (2h)	9.4	35.0	36.0	23.5
glycerol/malate (4h)	12.0	47.0	37.5	23.5
malate adapted cells	14.0	63.0	49.5	23.5
Glycerol grown cells	10.0	1.5	41.5	18.0
glycerol/maleate (2h)	10.0	14.0	45.6	27.0
glycerol/maleate (4h)	11.2	50.5	45.2	23.0
malate adapted cells	14.0	63.0	49.5	23.5

11. Growth Yields and Energy Production

11.1. Molar growth yields

Molar growth yields were obtained from growth experiments where cells were grown in media containing sufficient carbon source to permit a change in cell density of approx. 300 µg dry wt./ml. The carbon source was utilised by the cells both to provide energy for growth and to synthesise new cell material. The yields are expressed as both g of cellular dry wt. synthesised per mole carbon source metabolised and per mole oxygen taken up (Figure 50). Oxygen uptake data were not available for all substrates but were the only data available for acetate.

11.2. ATP production during growth of E.coli 15224

Both substrate and oxidative phosphorylation contribute to ATP production. In Figure 51 the production of ATP by oxidative phosphorylation was assessed on the assumption that each atom of oxygen taken up corresponded to production of 3 molecules of ATP (P/O = 3).

ATP production by substrate phosphorylation depends on the substrate and the mechanism by which it is metabolised and is shown for each substrate in Figure 51. It was assumed that no net ATP production occurred due to substrate phosphorylation in the TCA cycle. Energy produced from succinyl CoA only compensating for the lower energy status of the reduced product of succinate dehydrogenase. There is therefore zero estimated substrate phosphorylation during growth on malate, succinate and acetate.

Figure 50 MOLAR GROWTH YIELDS OF E.coli 15224

Cells of E.coli 15224, trained to the substrates shown were washed and inoculated into minimal salts medium containing their homologous substrate. The growth and oxygen uptake of the cells were measured.

Using initial substrate concentrations and final observed cell density, the quantity of cell material synthesised/mol substrate metabolised was calculated. In some cases samples were taken during growth and assayed for substrate concentration. Graph of changes in substrate concentration against turbidity gave a yield measurement. Both methods gave good agreement.

Accumulative gas exchange was calculated for each culture and graphed against culture density to give a yield/mol oxygen taken up.

Growth substrate	Molar growth yield g cellular dry wt. /mol substrate	/mol oxygen
Glucose	76.4	54.0
Glycerol	49.3	43.0
Gluconate	59.2	49.0
Malate	40.8	35.0
Fumarate	40.4	-
Acetate 30 mM		19.5
6 mM		15.5
Oxaloacetate	29.2	-
aketoglutarate	49.0	-
Succinate	39.5	28.0
Pyruvate	28.7	-

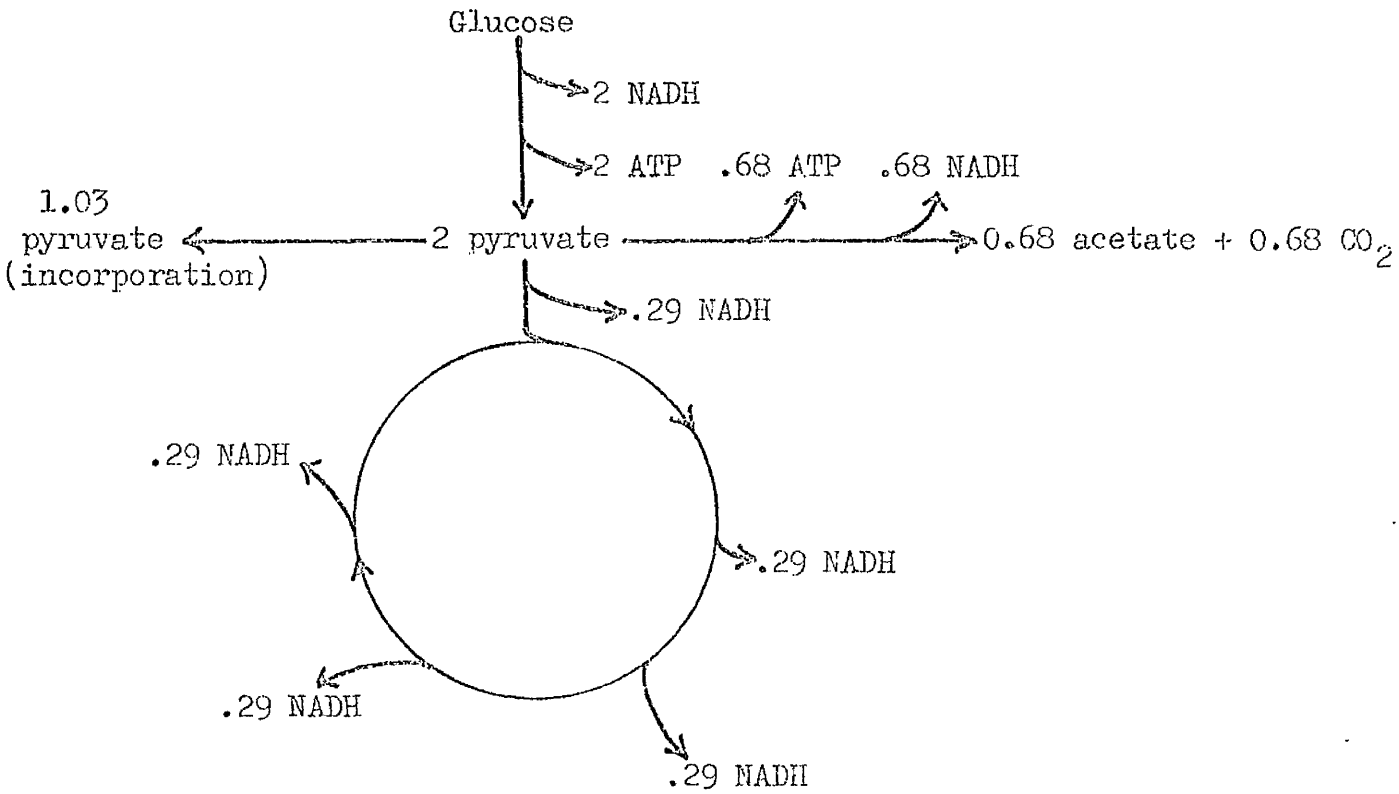
Substrate phosphorylation does contribute to ATP production during growth on glucose, glycerol and gluconate. To assess substrate phosphorylation certain assumptions had to be made. In the method used here to evaluate substrate phosphorylation during glucose growth the assumptions were:

a) 51.5% glucose carbon is incorporated into cell material which can be considered as a polymerised form of pyruvic acid.

b) glucose is metabolised by glycolysis and the TCA cycle.

c) metabolism of 1 mol of glucose gives rise to 0.68 mol of acetate (Figure 35a).

With these 3 assumptions the following simplified scheme was constructed:



By this scheme metabolism of 1 mol of glucose gives 2.68 mol of ATP, by

substrate phosphorylation, and 4.13 mol of NADH equivalent to 2.07 mol of oxygen. There is therefore 1.3 mol of ATP produced by substrate phosphorylation for every mol of oxygen taken up and this fact was used to assess substrate phosphorylation of cells growing on glucose (Figure 51).

To calculate substrate phosphorylation for cells growing on glycerol the following assumptions were made:

a) 66.4% of glycerol carbon is incorporated into cell material and can be considered as polypyruvate.

b) glycerol is metabolised by glycolysis and the TCA cycle.

A scheme similar to that for glucose was constructed for growth on glycerol which indicated that 0.55 mol of ATP were produced by substrate phosphorylation for every mol of oxygen consumed.

The assumptions made to calculate substrate level ATP production on gluconate were:

a) 40% of carbon is incorporated into cell material and can be considered as polypyruvate.

b) gluconate is metabolised by Entner Doudoroff and the TCA cycle.

c) 1 mol of glutamate is produced for every 4 mol of gluconate degraded (P.M. Bennett, personal communication).

With these assumptions 0.32 mol of ATP are produced by substrate phosphorylation for every mol of oxygen consumed (Figure 51).

Total ATP production during growth was calculated by adding substrate and oxidative phosphorylation.

The production of ATP by oxidative phosphorylation depends on the

Figure 51

ATP PRODUCTION DURING GROWTH OF *E.coli* 15224

Growth and oxygen uptake data were obtained as described for Figure 50.

ATP production by oxidative phosphorylation was calculated assuming a P:O = 3. ATP production by substrate phosphorylation was calculated as described in the text.

Growth substrate	Specific growth rate μ (h^{-1})	Rate of oxygen uptake (µmol/g dry wt./min)	Rate of ATP production by ox. phos. (µmol/g dry wt./min)	Estimated rate of ATP production by sub. phos. (µmol/mg dry wt./min)	Total ATP production (µmol/mg dry wt./min)
Glucose	.90	274	1644	356	2000
Glycerol	.75	298	1788	164	1952
Gluconate	.85	357	2142	114	2256
Malate	.57	311	1866	0	0
Succinate	.32	204	1224	0	1224
Acetate (30 mM)	.36	345	2070	0	2070
Acetate (6 mM)	.19	166	996	0	996

ox. phos. - oxidative phosphorylation

sub. phos. - substrate phosphorylation

Figure 52

EFFICIENCY OF ENERGY TRAPPING, ATP PRODUCTION AND YIELD/MOL ATP

Growth and oxygen uptake data were obtained from Figure 51. Total ATP production was calculated using the P:O ratio values shown and substrate phosphorylation values from Figure 51. Yields/mol ATP were calculated for each P:O ratio value.

Growth substrate	Specific growth rate μ (h^{-1})	Total ATP production (µmol/g dry wt./min)			Yield g/mol ATP		
		$P/_O$=1	$P/_O$=2	$P/_O$=3	$P/_O$=1	$P/_O$=2	$P/_O$=3
Glucose	0.90	904	1450	2000	16.6	10.3	7.5
Glycerol	0.75	760	1356	1952	16.5	9.2	6.4
Gluconate	0.85	828	1542	2256	17.1	9.2	6.3
Malate	0.57	622	1244	1866	15.3	7.6	5.1
Succinate	0.32	408	816	1224	13.1	6.6	4.4
Acetate (30 mM)	0.36	690	1380	2070	8.7	4.4	2.9
Acetate (6 mM)	0.19	332	664	996	9.6	4.8	3.2

quantity of ATP produced for every oxygen taken up (P/O ratio) which need not be 3. Total ATP production at P/O ratios of 1, 2 and 3 were calculated. The yield of cells/mol ATP produced was calculated from these data (Figure 52).

11.3. Maintenance requirement of E.coli 15224 growing on malate

During growth, the bulk of the energy generated by the cell is used to drive the synthesis of new cell material. Energy not used for biosynthesis has been termed maintenance energy. Its magnitude can be assessed using cells growing at different rates under identical conditions. Cells growing on malate can grow at a variety of growth rates depending on the state of adaptation of the cells to malate. The degree of adaptation was controlled by growing cells either in mixtures of glycerol and malate (Figure 29) or glucose and malate (Figure 36) and the range of growth rates and rates of oxygen uptake used to assess maintenance requirement on malate (Figure 53).

As described in methods (section 13 ; p. 67) the equation of this line is

$$\text{Rate of oxygen uptake} = m + \frac{\mu}{Y_{G_{O_2}}}$$

so both maintenance coefficient (m) and $Y_{G_{O_2}}$ on malate can be evaluated.

Maintenance coefficient on malate = 15 μ mol Oxygen/g dry wt./min.

Y_G = 40.0 g dry wt./mol oxygen.

11.4. Maintenance requirement of E.coli 15224 on acetate

Growth on acetate was not simple and distinct periods existed during the growth of the culture where the cells were growing

Figure 53

MAINTENANCE REQUIREMENT OF E.coli 15224 GROWING ON MALATE

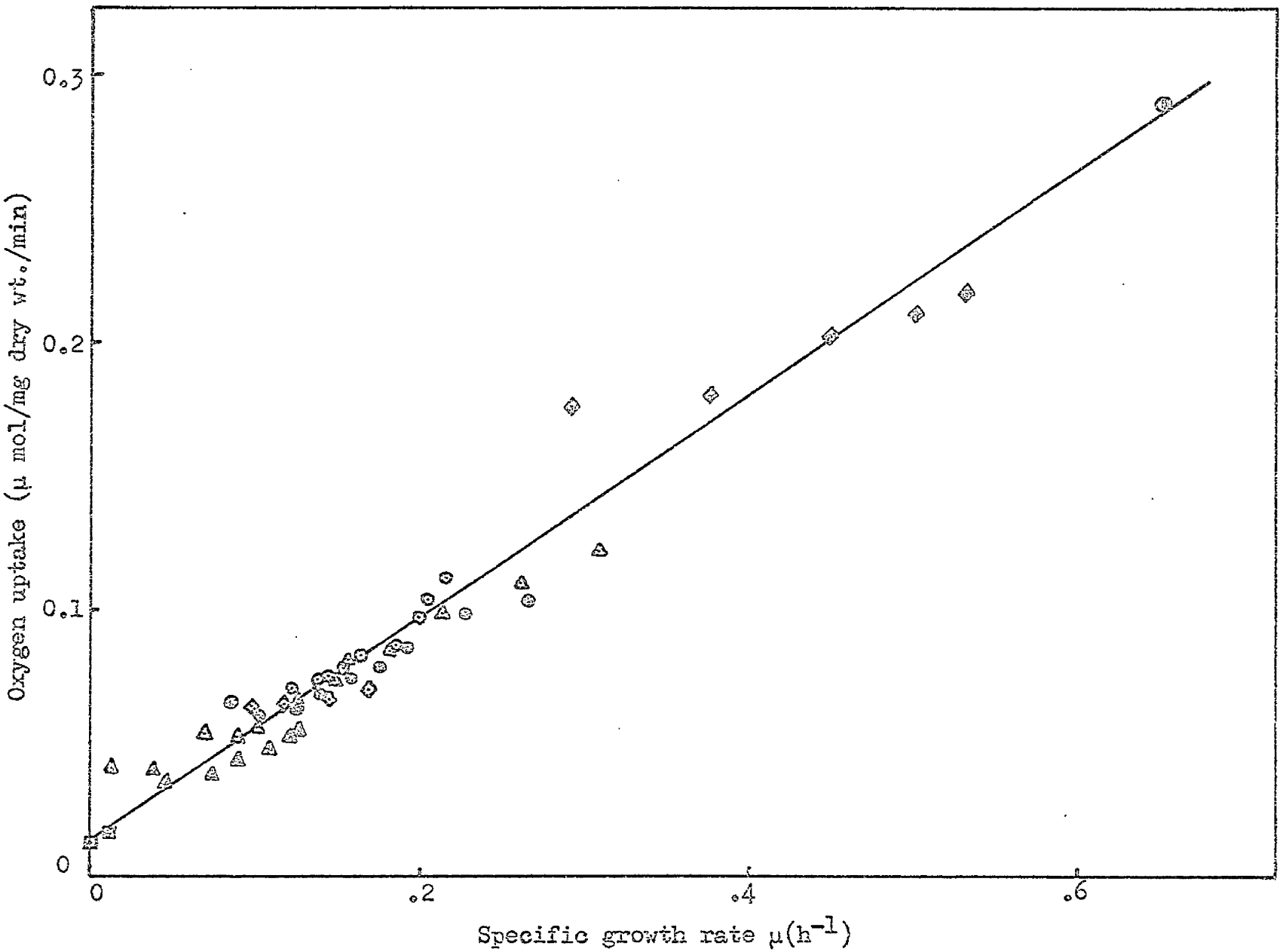
Preparation and growth of E.coli 15224 was carried out as described for Figure 29 using several concentrations of glycerol. Growth and oxygen uptake of the cells on malate after glycerol exhaustion were measured. Specific growth rate and rate of oxygen uptake of the culture at different times during growth on malate alone were calculated. Similar experiments were done using glucose in place of glycerol. Once again rate of growth and rate of oxygen uptake of the cells on malate, after glucose and acetate exhaustion, were calculated.

Growth rate was graphed against rate of oxygen uptake.

The concentrations of glucose and glycerol used are shown below. Malate concentration was 6 mM.

▣ glycerol trained cells	□ glucose trained cells
⊙ 0.5 mM glycerol	△ 0.25 mM glucose
△ 1.0 mM glycerol	◇ 0.5 mM glucose
◈ 2.0 mM glycerol	○ 1.0 mM glucose

◎ malate trained cells


0.3
0.2
0.1
0
Oxygen uptake (μ mol/mg dry wt./min)
0
.2
.4
.6
Specific growth rate μ(h⁻¹)

logarithmically at different growth rates. On low concentrations of acetate growth appeared to be arithmetic so many rates of growth and oxygen uptake existed which permitted the maintenance coefficient on acetate to be calculated (Figure 54).

Maintenance coefficient on acetate = 50 μ mol oxygen/g dry wt./min

$Y_{G_{O_2}}$ = 22.6 g dry wt./mol oxygen.

11.5. Correlation between efficiency of energy trapping and yield of E.coli 15224

Maintenance and P/O ratios are both important in assessing the efficiency of utilisation of both substrate and ATP by the cells. Using the maintenance data of Figure 53 and the ATP production data of Figure 52 a general graph was drawn relating rate of ATP production to growth rate (Figure 55).

The slope of the line (p. 67) represents a value of $Y_{G_{ATP}} = 8.9$.

Figure 54

MAINTENANCE REQUIREMENT DURING ACETATE GROWTH OF *E.coli* 15224

E.coli 15224 trained to acetate were washed and inoculated in minimal salts medium containing either 6 mM acetate or 30 mM acetate.

Growth and oxygen uptake were measured and the specific growth rate and rate of oxygen uptake at different points during growth calculated.

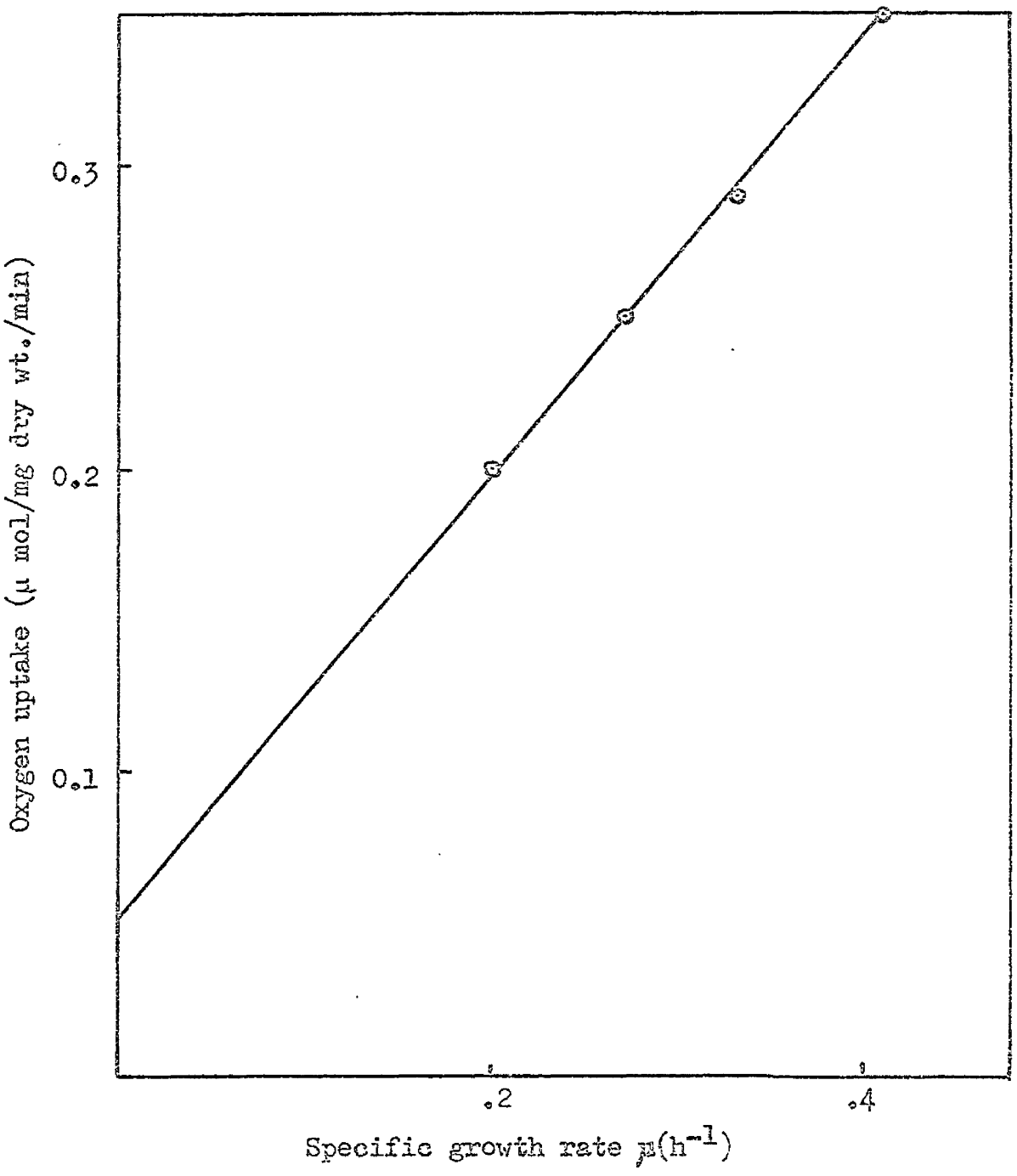

0.3
0.2
0.1
Oxygen uptake (μ mol/mg dry wt./min)
.2
.4
Specific growth rate μ(h⁻¹)

Figure 55

EFFICIENCY OF ENERGY TRAPPING AND Y_{ATP} VALUES OF E.coli 15224 GROWING ON DIFFERENT SUBSTRATES

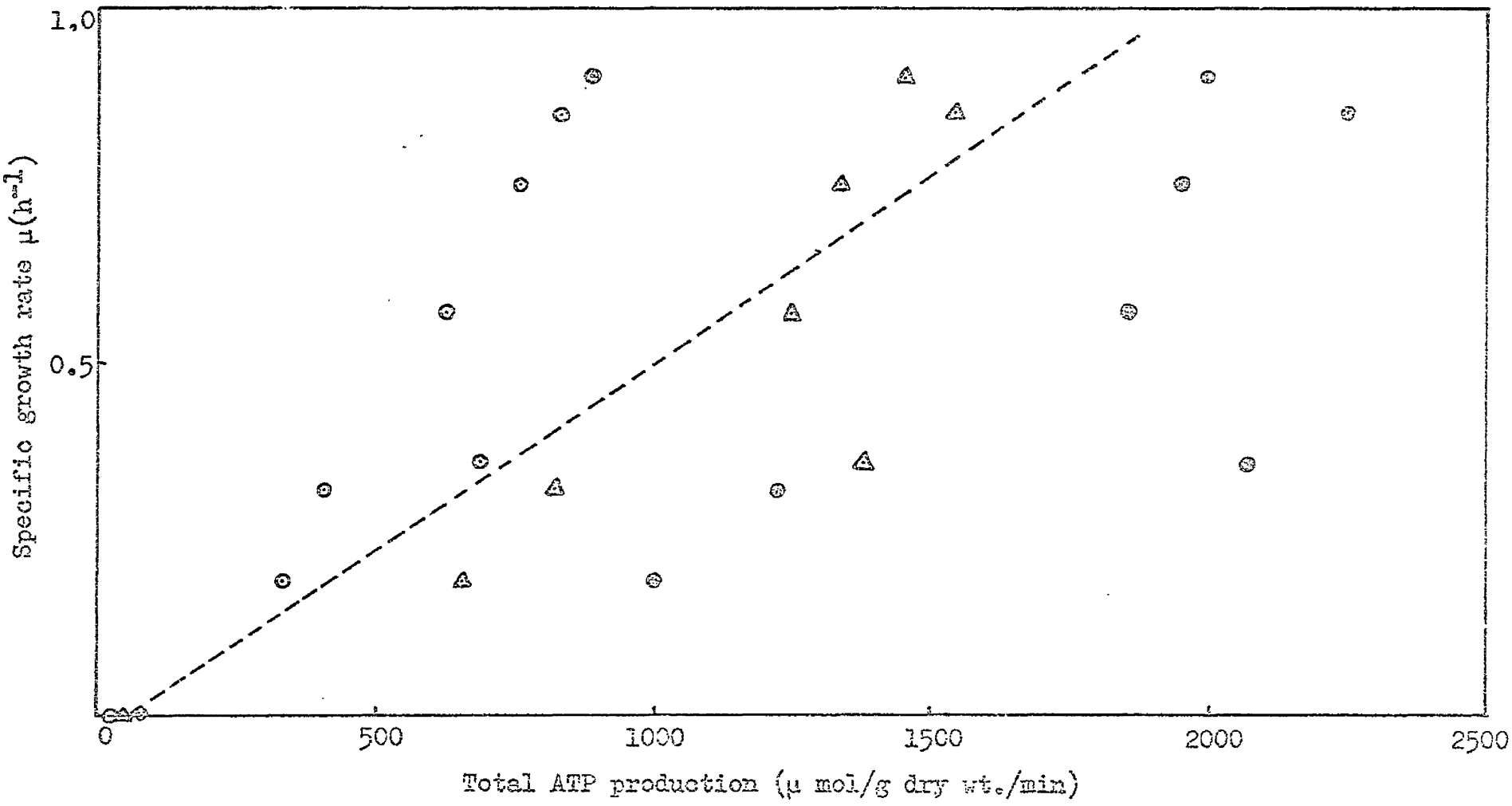
This figure plots the data from Figure 52 of rate of ATP production against growth rate at each P:O ratio. The data for each substrate are plotted.

 P:O ratio = 1

 P:O ratio = 2

 P:O ratio = 3

The line shown was drawn on the assumption that during growth on acetate P:O = 1.0 (Figure 54) and during growth on malate P:O = 2.0 (Figure 53).


1.0
0.5
0
500
1000
1500
2000
2500
Specific growth rate μ(h⁻¹)
Total ATP production (μ mol/g dry wt./min)

DISCUSSION

1. Limitation During Growth on Malate

1.1. General considerations

Growth in any environment requires the operation of a large number of enzymic reactions some of which must be susceptible to control and thus permit the overall rate of metabolism to be regulated. It is, however, not known to what extent different cellular processes limit growth on different carbon sources. Identification of the limiting reaction allows a specific assay to be devised and the effect of metabolites on the synthesis and activity of the limiting factor to be studied. In the lac operon in E.coli analysis of the control mechanism was only possible once a specific assay to measure β-galactosidase activity was available. Similar assays were also available for other studied systems. We have obtained evidence to suggest that transport of malate is the reaction regulating overall malate metabolism in E.coli 15224 and describe an assay which measures specifically the activity of the transport system.

Although the bulk of the data presented were obtained during growth on malate and, consequently, are only directly applicable to growth on that compound, preliminary experiments suggest that the regulation observed for malate applies equally to other dicarboxylic acids. The establishment of the dicarboxylic acids as a group was achieved by presenting cells, grown in media containing one carbon source, with an alternative carbon source and measuring growth (Figures 21 and 22). Of

particular relevance are the growth rates obtained when cells were inoculated into dicarboxylic acid containing media. Although some of the results, such as growth of alanine trained cells on dicarboxylic acids, are not readily explicable, it was observed in every case that, if cells could grow on one dicarboxylic acid then they could grow on the others. In some cases, such as after previous growth on glycerol or pyruvate, growth rate is lower but again was observed with all dicarboxylic acids (Figure 22). While these results establish nothing about either the rate or regulation of dicarboxylic acid metabolism, they show that the cells respond to different dicarboxylic acids in an identical manner and show both that malate may be regarded as a typical dicarboxylic acid and that the factor limiting growth is common to the metabolism of malate, fumarate and succinate.

It was essential before further analysis of the system was attempted to determine the nature of the reactions which can limit growth on malate. Consideration of cells of different phenotype and the ease with which they can grow on malate simplified the problem. Cells trained to glucose or glycerol are unable to metabolise malate without a period of adaptation (Figures 34 and 28). Glucose trained cells can grow on glucose at a specific growth rate of 0.88 (Figure 35) and glycerol trained cells on glycerol at a specific growth rate of 0.77 (Figure 31). Malate trained cells grow on malate at a specific growth rate of 0.60 (Figure 27) so it follows that the rate of synthesis of cell material and, consequently, the rate of utilisation of intermediates is higher during growth on either glucose or glycerol than

during growth on malate. Since intermediates, in all 3 cases, are synthesised from the metabolites of the amphibolic routes, both glucose and glycerol trained cells can grow on malate if provided with a sufficient flow of carbon into the amphibolic routes. The cells do not grow (Figures 34 and 28) so growth must be limited by the specific reactions involved either in feeding malate into the amphibolic routes or in the amphibolic routes themselves. These limiting reactions must be specific to metabolism of malate but have no function when cells are growing on either glucose or glycerol. There are only 3 such reactions.

Firstly, the cells require a supply of malate inside the cell which is produced by the activity of the dicarboxylic acid transport system. In the absence of the transport system cells are unable to take up labelled dicarboxylic acid which only diffuses slowly into the cell (Kay & Kornberg, 1969). On the assumption that intracellularly produced dicarboxylic acids cannot diffuse out of the cell into the medium during growth on other carbon sources, the cells have no requirement for the dicarboxylic acid transport system unless they are provided with an exogenous supply of dicarboxylic acids. No data are available on the activity of the system which transports malate. Kay & Kornberg (1969) report a fumarate uptake rate of 8 μ mol/g cellular dry wt./min which must represent only a fraction of the total activity since calculation of the rate of malate uptake necessary to support growth is 240 μ mol/g dry wt./min (Figure 27). The activity of the transport system must be at least as great if it is the only means by which malate can enter the cell.

Secondly, the cells require a supply of PEP. During growth on carbon sources such as glucose and glycerol, which do not allow immediate growth on malate, PEP is synthesised in glycolysis and dicarboxylic acid synthesis is achieved by the irreversible PEP carboxylase (Kornberg, 1965). During growth on dicarboxylic acids, PEP synthesis is by PEP carboxykinase (Kornberg, 1965) which could limit growth on malate.

Thirdly, the cells require a supply of pyruvate which is derived from malate by malic enzyme. No definite function has been ascribed to malic enzyme but during growth on malate it is required to decarboxylate malate to pyruvate to allow energy production in the TCA cycle. It may also be needed during growth on many carbon sources to generate a supply of NADPH (Young et al., 1964) and acetyl CoA (Jacobson et al., 1966) for fatty acid biosynthesis but the cellular requirement during growth on malate will be greater than the requirement during growth on other carbon sources. Malic enzyme could therefore limit growth on malate.

An indication of the activity required of the transport system has been shown to be of the order of 240 μ mol malate transported/g cellular dry wt./min (Figure 27). Similar estimates, although of greatly reduced accuracy, can be obtained for the other metabolic reactions which could limit growth on malate. Malic enzyme activity can be estimated from oxygen uptake and malate oxidation data. Oxygen uptake on malate is 310 μ mol/g dry wt./min (Figure 27) corresponding to a maximum oxidation rate of malate of 103 μ mol/g dry wt./min which, since all

malate oxidised must be metabolised by malic enzyme, must account for a part of malic enzyme activity. Malic enzyme also supplies intermediates for biosynthesis so total activity in the cells will be greater than 103 μ mol malate decarboxylated/g dry wt./min. No more than the residual malate can be metabolised by malate dehydrogenase and PEP carboxykinase so these activities must be less than 137 μ mol substrate transformed/g dry wt./min. Although there are considerable errors involved in these estimates they show that neither malic enzyme nor PEP carboxykinase is required to the same extent as the transport system.

Having established the factors which could limit the rate of metabolism of malate, and obtained estimates of their required activity, experiments were devised to attempt to assess the activity of each of these systems in the cells and to establish which influenced the rate of malate metabolism.

Glucose and glycerol trained cells both display low capacities to metabolise malate (Figure 46) but respond quite differently when presented with a mixture of malate and their homologous substrate in the growth medium. Growth of glycerol trained cells on a mixture of glycerol and malate allows adaptation to a malate metabolising phenotype (Figure 29) - growth of glucose trained cells on a glucose/malate mixture allows very little adaptation to take place (Figure 36). Both systems provide information on the reaction limiting malate metabolism.

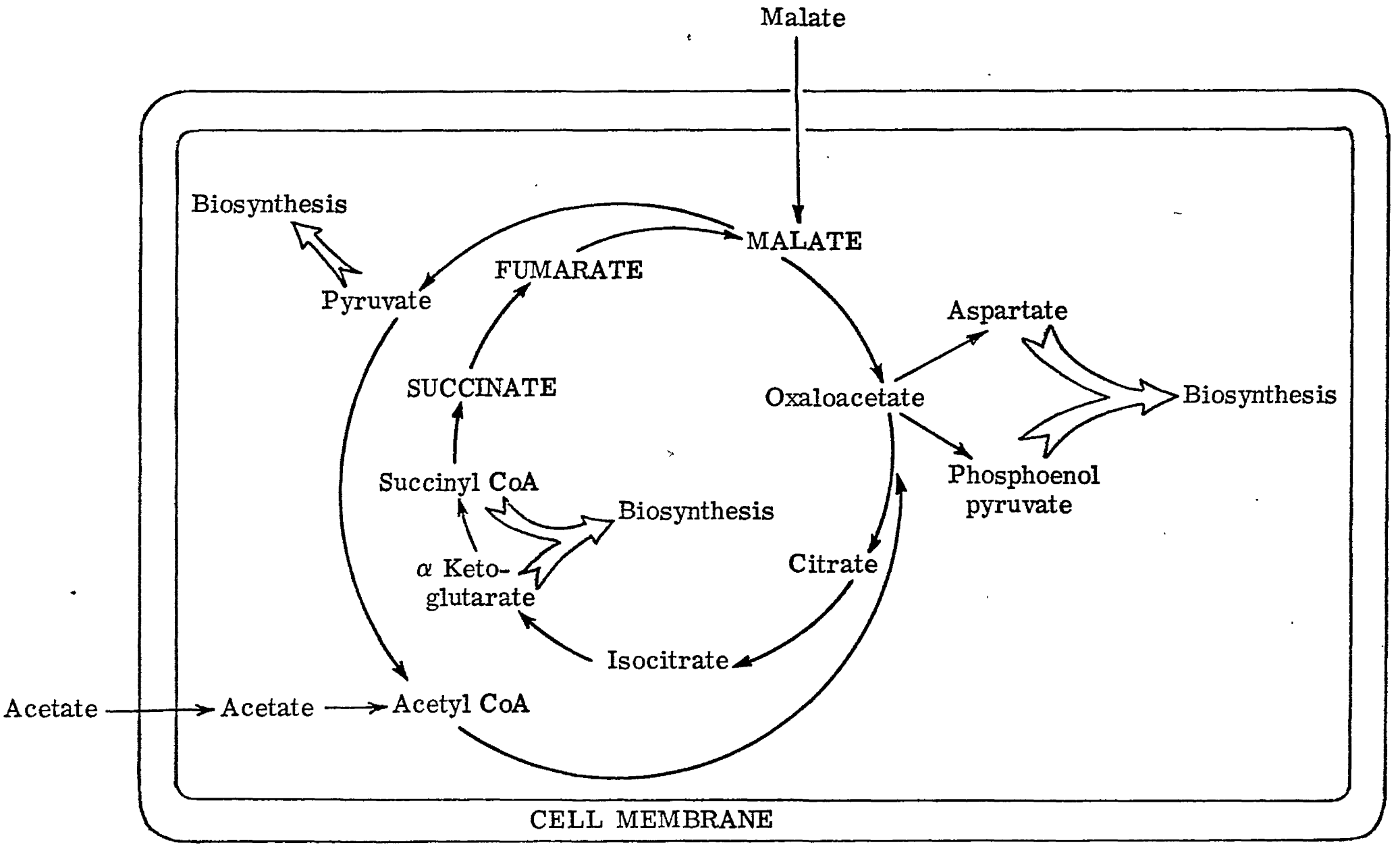
1.2. PEP carboxykinase

The influence of PEP carboxykinase activity on the rate of

metabolism of malate can be determined by examination of the growth of E.coli in media containing either glucose alone or glucose/malate mixtures. During growth on glucose, acetate is excreted into the medium (Figure 35) (Holms & Bennett, 1971). The presence of malate in the medium does not affect acetate excretion while glucose is present but, after glucose exhaustion, malate and acetate are used concurrently to support growth (Figure 36b). Since the rate of growth on malate alone is low when acetate is exhausted (Figure 36a), it follows that the activity of the enzyme limiting growth on malate alone must be low.

Acetate stimulates the rate of growth of the cells on malate (Figure 36a). Consider, however, the metabolism of acetate in E.coli (Figure 56). After uptake into the cell acetate is metabolised in the TCA cycle. The glyoxylate bypass, which is active when cells are growing on acetate (Kornberg, 1965), does not operate when malate is also present in the medium (Holms & Bennett, 1971). So all acetate, not incorporated as acetyl CoA or glutamate, is metabolised by the TCA cycle and generates, in addition to energy, one molecule of malate for every molecule of OAA required for its metabolism. There is no other mechanism present by which the cells can catabolise acetate.

The acetate stimulation of growth on malate (Figure 36a) implies an increased supply of all metabolites from the amphibolic routes including those synthesised from PEP. Acetate, however, cannot affect the production of PEP in the cell by any means other than through malate and PEP carboxykinase (Figure 56). An increased rate of growth on acetate/malate means that more PEP is being synthesised from malate


Malate
Biosynthesis
MALATE
FUMARATE
Pyruvate
Aspartate
SUCCINATE
Oxaloacetate
Biosynthesis
Succinyl CoA
Phosphoenol
pyruvate
Biosynthesis
α Keto-
glutarate
Citrate
Isocitrate
Acetate
Acetate
Acetyl CoA
CELL MEMBRANE

so it follows that the cell has a greater enzymic capacity to synthesise PEP from malate than it expresses when growing on malate alone and that PEP carboxykinase cannot be a limiting factor during growth on malate.

There are several other theoretically possible mechanisms by which the cell could make an increased supply of PEP available during growth on acetate/malate mixtures. PEP could be generated by the reversal of PEP carboxylase which is present in both glucose and glycerol trained cells (Teraoka et al., 1970). However, since mutants devoid of PEP carboxykinase activity are unable to grow on dicarboxylic acids (Kornberg, 1965), the PEP carboxylase reaction is irreversible and cannot operate to provide an increased supply of PEP during growth on an acetate/malate mixture.

PEP could be generated from pyruvate by the action of PEP synthase. PEP synthase is only made when pyruvate, or 3 carbon compounds which are metabolised through pyruvate, are present in the medium (Cooper & Kornberg, 1967). As with PEP carboxylase, if PEP synthase can synthesise PEP when cells are growing on dicarboxylic acids then PEP carboxykinaseless mutants would be able to grow on dicarboxylic acids. Clearly, PEP synthesis by either PEP carboxylase or PEP synthase cannot explain the increased growth rate observed during growth on an acetate/malate mixture.

PEP could be generated by blocking the activity of pyruvate kinase and consequently making more PEP available. Consider however the conditions under which pyruvate kinase is active in cells growing on malate. Glucose grown E.coli contain 2 pyruvate kinases, one of which

requires fructose-1-6-diphosphate for activity (Malcovati & Kornberg, 1969), and a second which is activated by AMP and is active when the energy pools in the cell are low (Sanwal, 1970a). During growth on succinate, and probably also on malate, the pool of fructose-1-6-diphosphate is low (Lowry et al., 1971). Pyruvate production from PEP will only be catalysed by the AMP activated enzyme. The presence of acetate, which stimulates oxygen uptake (Figure 36a) and consequently ATP production, decreases the pool of AMP in the cells, inhibits pyruvate kinase and so reduces the conversion of PEP to pyruvate and increases PEP availability. It infers that the primary cause of low growth rate is a poor supply of pyruvate for the generation of energy and not the supply of PEP. PEP is available but cannot be used for biosynthesis because pyruvate, and consequently energy production, is limiting growth.

There is therefore no means by which the rate of growth of cells on a mixture of acetate and malate can be higher than the rate of growth on malate alone if PEP production limits growth. It follows that PEP production by PEP carboxykinase or by any other known means does not limit growth on malate.

Since the stimulation of growth on malate by acetate allows PEP carboxykinase to be excluded as a factor limiting growth, a survey was carried out using cells growing on malate after previous growth in different media. In all cases acetate stimulates the rate of growth of the cells. The growth rates of cells trained to malate, with maximum capacity to utilise malate (Figure 39), and cells, which had

been grown on glucose to exhaustion of acetate before malate was presented to them, with minimum capacity to utilise malate (Figure 43) are stimulated. Similarly stimulated are the growth rates of a number of cells of intermediate malate utilising phenotype (Figures 40, 41, 42) showing that at no time is the rate of metabolism of malate limited by the activity of PEP carboxykinase.

The conclusion that PEP carboxykinase does not limit growth on malate is supported by the data of Teraoka et al.(1970) who have measured PEP carboxykinase activity in cells of E.coli W. The activities they observe in extracts of glucose and glycerol trained cells are 23% and 77% respectively of the activity in malate trained cells. If comparable enzyme activities are present in E.coli 15224, where rates of metabolism of malate in glucose and glycerol trained cells are only 14% and 25% of the rate in malate trained cells (Figure 46) it follows that there is more PEP carboxykinase activity in glucose and glycerol trained cells than is expressed when these cells are growing on malate.

Unfortunately the stimulation of malate growth by acetate does not allow any other reactions to be excluded. Pyruvate synthesis depends on the activity of malic enzyme but the available pyruvate pool can be increased by the presence of acetate in the culture medium because pyruvate is no longer needed to generate energy in the TCA cycle when acetyl CoA from an alternative source is available. Similarly malate availability, which depends on the activity of the transport system, can be increased by acetate because less malate need

be decarboxylated to pyruvate to generate energy.

1.3. Malic enzyme

Unfortunately no growth experiments can be devised to differentiate between limitation of malate metabolism due to malate transport and malic enzyme activity so direct measurement of these activities are necessary.

Measurement of the transport system, as discussed later, is difficult so malic enzyme activity in the cells was estimated in cell-free extracts and compared with the activities in malate adapted cells. Initially enzyme was assayed in glucose, glycerol, acetate and malate trained cells as these possess different abilities to metabolise malate as shown by measurement of growth and oxygen uptake on malate as a sole carbon source (Figure 46). Extracts prepared from these cells contain both malic enzymes in sufficient quantity to allow a much faster rate of malate metabolism than is observed (Figure 46). For example glycerol trained cells growing on glycerol contain between 70 and 80% of both malic enzyme activities compared to malate trained cells but, on transfer to malate containing medium, take up only 23% of the oxygen and grow at 19% of the growth rate of malate trained cells. Cells growing on glucose and acetate give results of a similar nature and, although variations in enzyme specific activities during growth are observed, no correlation between specific activity and rate of malate metabolism is found (Figures 46 and 47a). On the assumption that both malic enzyme activities are equivalent and that all oxidation of malate is achieved *via* malic enzymes, calculation shows

that there is sufficient malic enzyme present in glycerol trained cells to permit 335% of the observed oxygen uptake. Glucose and acetate trained cells contain sufficient malic enzyme to permit 335% and 409% respectively of the observed oxygen uptake. It follows that malic enzyme does not limit malate utilisation in glucose, glycerol or acetate trained phenotypes. Similar malic enzyme activities have been reported in these same phenotypes in another strain of E.coli by Murai et al. (1971).

None of these cells show changes in their ability to metabolise malate due to the presence of malate in the medium. The system chosen to analyse changes in activity during adaptation was growth on a glycerol/malate mixture because in these circumstances the cells utilise both substrates simultaneously and increase their capacity to metabolise malate.

In the mixed substrate medium, the cells grow at a higher growth rate (Figures 28, 29a) and with a higher rate of oxygen uptake (Figure 29b) than do cells growing on glycerol alone (Figure 31). Calculation from yield/mol glycerol and growth rate data shows that the rate of glycerol utilisation in mixed substrate medium is less than in glycerol medium (204 and 260 μ mol/g dry wt./min respectively) so the increased growth rate observed in the dual substrate medium must be due to utilisation of malate and is confirmed by direct measurement of malate concentration in the medium (Figure 29a).

The evidence for a proportionately increasing use of malate during glycerol/malate growth is not so convincing and depends on the

variation of the respiratory quotient (RQ) during growth (Figure 29c). The RQ depends both on the nature of the carbon source used for growth and on the incorporation of the carbon source into cell material. Clifton and Logan (1939) measured an RQ of 0.75 for the utilisation of glycerol by E.coli in a washed cell suspension compared with a theoretical value of 0.86 for complete glycerol oxidation. On fumarate the RQ was 1.5 and not the theoretically predicted value of 1.33. They showed that deviations from theoretical RQ values are due to incorporation, presumably into glycogen, of carbon source and that in the presence of o-dinitrophenol, which abolishes incorporation, the RQs give the expected theoretical values. Conditions of measurement in washed cell suspensions give less incorporation than is observed with growing cells and RQ values will deviate even more from expected values if incorporation is increased. The observed RQ of 0.66 during growth on glycerol (Figure 31) and of 1.55 during growth on malate (Figure 27) are therefore not abnormal. Changes in RQ can be due to changes in incorporation as well as changes in substrate utilisation but as shown in Figure 30a the incorporation of both glycerol and malate into cell material is constant so variation of RQ during growth is solely due to changes in the proportion of the substrates utilised. The RQ during growth on glycerol/malate medium rises from 0.7 to 0.9 (Figure 29c) showing an increasing proportion of malate being used to support growth. There is one flaw to this argument which is that although total incorporation from all carbon sources should remain constant the yield/mol glycerol should increase as the proportion of

malate utilised increases. Changes in yield/mol substrate during glycerol/malate growth are not observed (Figure 30a) but if malate utilisation is continuously changing the method of molar growth yield measurement used may be insufficiently sensitive to detect the changes. The yield/mol malate (Figure 30a) and growth rate of the culture (Figure 31) require a rate of malate utilisation of 125 μ mol/g dry wt./min which is in excess of the ability of glycerol trained cells to metabolise malate (a value of ~40-50 μ mol/g dry wt./min is obtained from the data in Figures 33 and 46). There must therefore be some doubt as to the rates of glycerol and malate utilisation during glycerol/malate growth but there is no doubt that the ability of the cells to metabolise malate increases with growth in glycerol/malate medium (Figures 33, 47d). The increasing rate of malate metabolism must reflect changes in the specific activity of the system limiting malate metabolism in the cells.

During growth on glycerol/malate medium the ability of the cells to metabolise malate as measured by oxygen uptake increases by 350% (Figure 47d). The specific activity of both malic enzymes remains constant (Figure 47d) throughout adaptation. If malic enzyme is limiting growth the rate of oxygen uptake will be directly related to the specific activity of the enzymes which, clearly, is not the case here. The cells growing on glycerol contain sufficient malic enzymes to allow a rate of oxygen uptake of 285 μ mol/g dry wt./min (Figure 47d) so the malic enzymes are functioning at only 28% capacity when malate is being metabolised by glycerol trained cells.

Although there is sufficient total malic enzyme activity present in extracts of cells grown on glycerol to permit a faster rate of malate metabolism than is observed, there is no evidence to suggest that all of the malic enzyme in these cells is active. The cell may contain neither a NADPH oxidase nor a NADP/NAD transhydrogenase system and as a result be unable to metabolise NADPH. With no NADP in the oxidised form NADP-linked malic enzyme will be inactive and only NAD-linked malic enzyme available to metabolise malate. If NADPH cannot be metabolised it follows that growth on malate requires the synthesis of either NADPH oxidase or NADP/NAD transhydrogenase activity and adaptation to malate in glycerol/malate medium is due to an increase in one of these activities. The absence of detectable levels of both NADPH oxidase and NADP/NAD transhydrogenase activities in malate trained cells (p. 95) does not deny their existence. Clearly the induction of any system required for malate metabolism but not directly involved in that metabolism can limit growth on malate and means that it is not the presence of malate but the metabolism of malate that allows adaptation to occur. Gratuitous induction of the malate limiting system enables the influence of malate metabolism to be distinguished from the effect of malate in the medium.

Maleic acid is a gratuitous inducer of the dicarboxylic acid transport system (Kay & Kornberg, 1971). The presence of maleic acid in a culture of cells growing on glycerol results in adaptation to the malate utilising phenotype (Figure 48). No changes in malic enzyme activities are observed. It follows that neither the activity of malic

enzyme nor the metabolism of malate can limit growth on malate.

A limitation by malic enzyme during growth on malate has been excluded by comparing cells of different malate utilising ability with malate trained cells. It is impossible by this means to determine if malic enzyme limits the growth of malate trained cells on malate. The adaptation of cells in glycerol/malate medium (Figure 47d) however results in a final rate of oxygen uptake of 285 μ mol/g dry wt./min with a total malic enzyme specific activity of 60 U/g dry wt. so the rate of oxygen uptake is 4.75 μ mol/U malic enzyme. Malate trained cells (Figure 46) show an oxygen uptake of 325 μ mol/g dry wt./min and possess a total malic enzyme specific activity of 75 U/g dry wt. giving an oxygen uptake of 4.33 μ mol/U malic enzyme which suggests that malic enzyme is not working at full capacity during the growth of malate trained cells on malate.

1.4. Dicarboxylic acid transport system

It follows from exclusion of other possibilities that growth must be limited by the activity of the dicarboxylic acid transport system. The involvement of the transport system in malate limitation was inferred from two previous pieces of evidence. Firstly, cells growing on acetate do not grow on malate without a period of adaptation (Figure 22). Since cells growing on acetate must synthesise PEP, pyruvate and OAA through malate they must possess the ability to metabolise endogenously supplied dicarboxylic acids. Lack of growth on malate could be due to a low activity of pyruvate dehydrogenase. Although cells of E.coli K12 contain a low level of pyruvate

dehydrogenase, when growing on acetate, the activity is 50% of that found in malate trained cells (Dietrich & Henning, 1970) and should allow a higher rate of malate metabolism than is observed (Figures 45 and 46). The only other system required, which is not required during growth on acetate, is the dicarboxylic acid transport system. Secondly, cells trained to either malate or fumarate and inoculated into a mixture of these two compounds metabolise both simultaneously in the ratio 1.7 parts fumarate to 1.0 part malate (Figure 23) which means that the system limiting the growth of the cells acts at a stage when malate and fumarate can be distinguished. The dicarboxylic acid transport system can distinguish between malate and fumarate and has a greater ability to transport the latter (Kay & Kornberg, 1969).

The evidence that growth on malate is limited by the transport system has so far been negative. Direct measurement of the transport system would provide some positive evidence. Essentially there are two methods by which transport systems have been measured. The uptake of substrate into the cells (Kay & Kornberg, 1969) or the disappearance of substrate from the medium (Takahashi & Hino, 1968a) can be measured. Both methods have disadvantages because they do not separate the activity of the uptake reaction from subsequent cellular metabolism. The alternative method is to measure the pool of transport system substrate inside the cell using a labelled, non-metabolisable substrate for the transport system (Cohen & Monod, 1957). In the case of malate uptake, non-metabolisable substrates are available but are not accumulated above medium concentration by the cells (Kay & Kornberg,

1971). Activity has to be measured by the rate at which equilibration inside the cell is achieved and is complicated by the trapping of extracellular fluid between the cells (Cook, 1971).

Although these methods could have been used to measure transport activity during adaptation we decided to measure transport activity by the rate of entry of substrate into the cell. Malate, after entry, can be metabolised by decarboxylation to pyruvate. If further metabolism of pyruvate is inhibited by arsenite (Dawes & Foster, 1956) the rate of production of pyruvate in the cells can be measured. Provided malic enzyme activity is in excess of the activity of the transport system then the rate of pyruvate production will measure the throughput of the malate transport system.

Although malic enzyme is in excess in cells growing on malate, it may not be in excess in washed cell suspensions in the presence of 1mM arsenite. The required throughput of the transport system in fully adapted cells is greater than that of malic enzyme and arsenite has been shown to inhibit the latter in some systems (Webb, 1966). Consequently it is essential to ensure that malic enzyme does not restrict pyruvate production before pyruvate production can be related to the throughput of the transport system.

On the assumption that both malic enzyme activities are equivalent, the total malic enzyme activity in malate trained cells is 73 U/g cellular dry wt. (Figure 49) and is equivalent to a rate of decarboxylation of malate of 73 μ mol/g dry wt./min. In malate trained cells the rate of pyruvate production in washed cell suspensions is 63 μ mol/g dry wt./min

(Figure 49). If, in this case with maximal permease activity, pyruvate production is limited by malic enzyme activity then the maximum rate of pyruvate production that can be achieved without malic enzyme becoming limiting is 86% of the total malic enzyme specific activity. In all cases, in which the rate of pyruvate production is less than 86% of the total malic enzyme activity, pyruvate production will measure the throughput of the transport system. At no time in any assay, other than in malate trained cells, does the rate of pyruvate production exceed 75% of the total malic enzyme activity and, usually, is much less (Figure 49). It follows that the rate of pyruvate production in all cases is limited by malate transport and therefore measures transport activity.

Using the rate of pyruvate production as an assay, transport system activity was measured in cells which were adapting to malate utilisation under two conditions. Cells, previously grown on glycerol contain very low permease activity at inoculation into glycerol/malate medium but activity increases 17-fold over a period of growth of 2 h and again over a further 2 h growth period (Figure 49). Although the ability of these cells to metabolise malate was not measured, other than by pyruvate production, the results in Figures 33 and 47d show that during growth on glycerol/malate mixtures the ability of cells to metabolise malate increases in a similar manner to the increase in transport system activity. There is therefore a correlation between permease activity and the rate at which malate is metabolised by the cells.

Similar observations, made on cells growing in a glycerol/maleic

acid mixture, show an increased transport activity over a period of 4 h (Figure 49) which can be related to adaptation to a malate utilising phenotype during growth in glycerol/maleic acid medium (Figure 48).

There is therefore a relationship between the observed transport activity and the rate at which the cells metabolise malate which confirms that the rate of growth of the cells during adaptation to malate is limited by the activity of the malate transport system.

2. Control of Malate Metabolism

2.1. General considerations

Malate is intimately involved in the central metabolic pathways of the cell. To achieve balanced growth on malate some control must be exercised over the rate at which malate is metabolised. An analysis of the regulation of the region of metabolism in which malate is involved, explains why it would be an advantage to the cell to express regulation on the rate of entry of malate into the cell.

In many systems, based on the inhibition of citrate synthase by ATP, it has been proposed that energy charge regulates the TCA cycle (Atkinson, 1966). In mammalian systems the regulation of the TCA cycle by citrate synthase and consequently by energy charge has been firmly established (Krebs, 1969). In E.coli, however, citrate synthase is not inhibited by ATP and regulation is dependent on the NADH concentration (Sanwal, 1970a). Any change in the NADH concentration results in an altered rate of operation of the TCA cycle and leads to an altered growth efficiency unless metabolism accommodates the change. Malate is in equilibrium with OAA through the NAD-linked malate dehydrogenase, so any change in malate concentration with respect to OAA results in a pressure to change the NADH concentration. Balance is maintained by NADH inhibition of malate dehydrogenase (Sanwal, 1969) but the mechanism will be strained if the cell is growing in an environment with a high extracellular malate concentration, unless control over transport is effective.

The regulation of growth and the characteristics of the enzymes metabolising malate must be consistent with growth limited by transport which can be investigated by asking two questions.

1) How is the activity of the malate transport system controlled by the cell?

2) Are the known regulatory characteristics of intracellular enzymes consistent with malate transport as a growth limiting function?

2.2. Regulation of malate transport system activity

2.2.1. Regulation of synthesis

Since metabolism of malate is limited by the activity of the malate transport system, it follows that the rate of malate metabolism, as measured by oxygen uptake, is a measure of the throughput of the malate transport system and oxygen uptake of the cells under identical conditions reflects the activity of the malate transport system.

2.2.1.1. Repression

The activity of the transport system found in cells depends on the conditions of growth. Growth on glucose or acetate represses the synthesis of the transport system more severely than growth on glycerol (Figure 46). The low level found in glucose and acetate grown cells may be due to either a high concentration of corepressor or a low concentration of inducer in cells of these phenotypes. The mechanism cannot be catabolite repression which is lower during growth on acetate than on glucose (Hsie & Rickenberg, 1967). Repression by acetate, which is present in the medium of cells growing on glucose or acetate, is equally unlikely since repression continues in glucose/malate medium

(Figure 47c), when acetate is at a low concentration, but is relieved in acetate/malate medium (Figure 47b). Acetyl CoA is another compound which may be at equivalent concentrations during growth on glucose or acetate although there is no evidence to support this postulate. Addition of malate to cells growing on glucose would have no effect on the acetyl CoA concentration since acetate continues to be excreted by these cells (Figure 36b). Malate added to cells utilising acetate however results in an increased rate of oxygen uptake (Figure 36a) over acetate alone (Figure 35a) indicating an increased rate of operation of the TCA cycle and a possible fall in the acetyl CoA pool in the cells. Since the presence of malate in a culture growing on acetate causes induction (Figure 45), repression by acetyl CoA is consistent with the observations. However acetyl CoA in cells acts as an indicator of a dicarboxylic acid requirement. It stimulates PEP carboxylase to divert PEP from glycolysis to generate OAA when OAA is in short supply (Canovas & Kornberg, 1965) and inhibits NADP-linked malic enzyme to conserve supplies of dicarboxylic acids (Sanwal & Smando, 1969). It seems unlikely that acetyl CoA would be used to repress the synthesis of the malate transport system when it acts as a signal for the cell to synthesise dicarboxylic acids.

There are no other obvious compounds which are likely to both repress synthesis and be at comparable intracellular concentrations during growth on glucose or acetate so repression must be due to the cells containing a low concentration of inducer. Since Lowry et al. (1971) have shown that both malate and aspartate are at equivalent concentrations

during growth on glucose or acetate this interpretation is possible. Unfortunately no data are available for cells growing on glycerol for comparative purposes. Glucose could be particularly dominant in the presence of inducer because of catabolite repression.

2.2.1.2. Induction

Although there does not appear to be a specific repressor acting on the synthesis of the malate transport system, there does appear to be specific induction as shown by growth on glycerol/malate (Figures 47d and 49) and acetate/malate mixtures (Figure 47b). Induction is most clearly shown by the response of the cells growing on glycerol to the presence of maleic acid in the medium (Figures 48 and 49). Since maleic acid is not metabolised (Kay & Kornberg, 1971) it does not alter cellular metabolism and must cause its effect by its presence in the medium. There are three mechanisms by which induction could occur.

Induction could be caused by the presence of inducer in the extracellular medium as reported by Winkler (1971) for the induction of glucose-6-phosphate permease by glucose-6-phosphate. The system has two characteristic features. Induction is related to the concentration of inducer added to the medium and begins on addition of inducer exhibiting no autocatalytic properties. On the first count no data are available but on the second, the data show that induction of the transport system in glycerol/malate (Figure 47d), acetate/malate (Figure 47b) and glycerol/maleic acid (Figure 48) mixtures is autocatalytic with little or no induction taking place during the first generation of growth in the presence of inducer.

Since the cells contain an intracellular concentration of malate of approximately 1.8 mM during growth on a variety of substrates [calculated from data of Lowry et al. (1971) assuming 2.7 µg cell water is associated with each µg cellular dry wt. (Winkler & Wilson, 1966)] it is possible that the transport system is not induced but synthesised continuously and incorporated in an active form in the membrane only in the presence of substrate in the medium. This mechanism of regulation will be unrelated to cellular metabolism and consequently not liable to the repression observed during growth on glucose/malate (Figure 47c). Furthermore the system should respond immediately to the presence of inducer in the medium which is inconsistent with the observed auto-catalytic induction (Figures 47b, 47d, 48).

It seems most likely that induction is due to an intracellular concentration of inducer which is generated by the activity of the transport system.

2.2.1.3. Nature of inducer

Induction of the transport system occurs in a variety of media which have only dicarboxylic acid metabolism in common (Figure 22). Experiments of this type do not allow the inducing molecule to be identified other than to say that it is associated with dicarboxylic acid metabolism. Lowry et al. (1971) have shown that, during growth on succinate, cells contain a 4-fold higher pool of malate than during growth on a variety of other media. However, these cells are growing in medium containing a very high succinate concentration which may affect the internal malate concentration. If this is so, then during

growth on 6 mM succinate, which induces (Figure 22), the intracellular malate concentration may not be higher than that existing under non-inducing growth conditions. The inhibition of malate dehydrogenase by NADH (Sanwal, 1969) will ensure an altered malate/OAA ratio if dicarboxylic acids are present in excess, and alterations in the ratio of two compounds (Atkinson, 1968b) is a mechanism which could regulate transport system synthesis.

While it is possible that dicarboxylic acid levels in cells do cause induction, a strong case can be made to suggest that aspartate, a compound metabolically linked to dicarboxylic acids, acts as an inducer. Aspartate is important in regulating the metabolism of dicarboxylic acids in the cell. It inhibits PEP carboxylase (Nishikido et al., 1965) so allowing the cell to conserve glycolytic intermediates if dicarboxylic acids are already available; it stimulates NAD-linked malic enzyme by overcoming Coenzyme A inhibition (Sanwal, 1970b) and so acts as an indicator of a surplus of dicarboxylic acids; it acts as a nutritional repressor of PEP carboxylase and as a nutritional inducer of PEP carboxykinase, in both cases as effectively as malate (Teraoka et al., 1970); it is a substrate for the transport system (Kay & Kornberg, 1971) and is at low concentrations (0.4 - 0.6 mM) during growth in glucose and acetate containing media (Lowry et al., 1971) in which the cells contain low levels of transport system activity (Figure 46). Courtright and Henning (1970) have also proposed, from analysis of malate dehydrogenaseless mutants, that during anaerobic growth of E.coli on glucose, succinate is produced from OAA via aspartate and

fumarate and not via malate. Aspartate is therefore directly involved in the metabolism of dicarboxylic acids, regulates the production and degradation of dicarboxylic acids, and acts as a signal of the presence of excess dicarboxylic acids in the cell. It would therefore be a suitable compound to act as inducer of the synthesis of the malate transport system.

There are two other lines of evidence which support this hypothesis. Cells trained to alanine grow on dicarboxylic acids without lag (Figure 22) and so contain considerable levels of the malate transport system. Consider however the metabolism of alanine. Loss of the amino group generates pyruvate, a compound which does not induce and whose metabolism does not cause induction (Figure 22). The carbon metabolism of alanine cannot produce induction. Details of the metabolism of the amino group are not known but glutamate, although it is synthesised by direct amination of α-ketoglutarate (Halpern & Umbarger, 1960), is degraded by transamination with OAA producing aspartate. The amino group is released by action of aspartase (Marcus & Halpern, 1969). By analogy, alanine may be degraded by transamination with aspartate, or with glutamate and thence to aspartate, with the amino group being lost by action of aspartase. As a result the nitrogen metabolism of alanine will cause the cells to contain a pool of aspartate which could induce the synthesis of the malate transport system.

Takahashi and Hino (1968a) have also observed adaptation in the absence of dicarboxylic acids but in the presence of amino acids. In this case induction could be caused by aspartate.

While none of these arguments can provide definite evidence for any specific compound as an inducer, aspartate has the advantage that it is not directly involved in the energy metabolism of the cell. Furthermore, many amino acids are accumulated by cells above the level in the medium (Piperno & Oxender, 1968). If this is true for aspartate it requires the cells to contain a second uptake system for aspartate but will permit much greater fluctuations in the aspartate pool than in dicarboxylic acid pools and so enable greater sensitivity to be developed in the regulation of synthesis of the malate transport system.

2.2.2. Regulation of activity

Regulation of the activity of the transport mechanism is also essential to maintain balanced uptake of nutrient during growth. Takahashi and Hino (1968a) and Kay and Kornberg (1971) have shown that metabolic energy is required for activity of the transport system and if energy production is blocked transport is markedly reduced. Kay and Kornberg (1971) have also shown that malate is not accumulated in the cell against a concentration gradient. Energy status may have a regulatory function in this system to ensure that cells do not obtain more malate than they can metabolise. Lowry et al. (1971) report that the rate of disappearance of succinate from the medium increases after addition of glucose to the medium. Incorporation, however, also increases and insufficient data are available to allow a rate of succinate uptake per cell in the presence of glucose to be calculated. Murai et al. (1971) on the other hand have found that addition of glucose to cells growing on malate represses synthesis of NAD-linked

malic enzyme. Repression is not due to catabolite repression, since it is overcome by a 4-fold increase in malate concentration in the medium, but could be due to a lower pool of inducer in the cells because of a reduced rate of uptake. There is therefore some evidence to suggest that regulation of activity of the malate uptake system exists.

There are two factors involved in regulation of this region of metabolism - energy charge and NADH concentration.

Energy is involved but whether it has a catalytic or regulatory function is not known. Measurement of energy charge has shown that it is stable during growth of E.coli on different substrates (Lowry et al., 1971) but stability does not invalidate its possible function as a regulatory parameter.

NADH concentration has been shown to regulate the activity of many enzymes involved in malate metabolism and it would be economical to use the same effector to regulate malate uptake and so couple uptake directly to metabolism. Since metabolism of glucose results in a high NADH/NAD ratio (Wright & Sanwal, 1969) inhibition of malate transport by NADH would constitute a catabolite inhibition mechanism which would reinforce the observed dominance of glucose metabolism over malate metabolism (Figure 47c). Lower NADH pools during metabolism of glycerol or acetate would permit entry and so allow induction when malate is added to the medium (Figure 47d and 47b). While regulation by NADH is not inconsistent with observations it is pointless to speculate further on this point in the absence of firm data.

2.3. Integration of malate transport activity with cellular metabolism during growth on malate

If the rate of metabolism of malate is restricted by malate uptake it means that the cells obtain variable supplies of malate depending on their degree of adaptation. Consequently regulation of enzyme activity is essential to direct the flow of carbon into each metabolic route. As far as malate utilisation is concerned, only three enzymes are directly involved in metabolism - two malic enzymes, which generate pyruvate, and malate dehydrogenase, which generates OAA and subsequently PEP. Under conditions of substrate limitation, effective competition regulated by metabolic effectors can only be achieved if the enzymes have comparable affinities for substrate or their affinities are altered by interaction with effectors. Sanwal (1970a) has observed that the distribution of PEP into pyruvate and OAA is achieved by the action of pyruvate kinase of K_m for PEP of 3.5 mM and PEP carboxylase of K_m for PEP of 10 mM which would result in the PEP carboxylase being virtually inactive. The presence of acetyl CoA reduces the K_m of PEP carboxylase from 10 mM to 1 mM so enabling OAA to be synthesised as necessary.

NAD-linked malic enzyme and malate dehydrogenase have similar affinities for malate [K_m = 1.0 mM (Sanwal, 1970b) and 0.7mM (Sanwal, 1969) respectively]. NADP-linked malic enzyme has a K_m for malate of 4 mM (Development of methods p. 94) (Sanwal & Smando, 1969) which can reduce to 1.5 mM depending on environmental conditions (Sanwal & Smando, 1969). The K_m values are directly comparable and ensure that effective control of carbon distribution is achieved by the specific metabolic controls

irrespective as to the rate of supply of malate into the cell. The nature of the intracellular enzymes metabolising malate is compatible with growth of the cells being limited by the rate of malate uptake.

This conclusion is confirmed by an examination of growth on malate at different rates where growth rate is dependent on the rate of supply of malate into the cell (Figure 53). The pool of malate inside the cell will be very low at low growth rates, nevertheless the efficiency with which the cells utilise energy, derived from malate, above a threshold value, to synthesise new cell material is constant over the entire observed range of growth rates, which can only be achieved if metabolic controls are effective in distributing substrate carbon through metabolism even at low substrate concentrations.

3. Energy Metabolism of Cells

3.1. General considerations

When attempting to analyse growth and its relationship to energy production at different growth rates it is essential to ensure that the metabolism, and consequently the energy production, of the cells is not changing with the growth rate. The advantage of using a chemostat for studying the effect of changes in growth rate is that it is possible to maintain cells in a steady state of balanced growth at different growth rates under identical conditions for long periods of time. Elsden (1967) has drawn attention to the fact that balanced growth in this context is bad use of terminology and that it would be better to consider growth in the chemostat as being limited by a particular nutritional requirement. Altered growth rates are the result of altered rates of supply of the required nutrient. While it is impossible to achieve a steady state during batch culture it is possible to obtain cells whose growth is limited by a particular nutritional requirement. If that requirement is the operation of a transport mechanism then the system has similarities to the chemostat although, in this case, the limitation of nutrient supply is regulated by the cell membrane and not by the dilution rate. Changes in the activity of the transport system would be akin to changes in the dilution rate and would result in a culture whose growth rate had been altered by an alteration in the rate of supply of limiting nutrient to the cells.

In the system described here, growth is limited by the passage of malate into the cell and may be regarded as an approximation to a chemostat whose growth rate depends on the rate of supply of malate to the cells. It must be realised that there exists neither steady state nor balanced growth and observations made at an instant in time are taken as equivalent to dilution rates.

Nevertheless, although the system can only be regarded as an approximation to a chemostat, it can be used to provide cells whose metabolism is identical but for the operation of a single limiting reaction and changes in growth rate can be directly related to rate of nutrient supply in the absence of alterations to metabolism.

Accepting that growth on malate, where transport is limiting, is a suitable system to examine the relationship between rate of growth and substrate utilisation, I intend to consider my data in this regard and with regard to other substrates only briefly because I feel that although the results are of some interest they are insufficient, both in quantity and undetermined statistical reliability to justify a more detailed analysis.

3.2. Growth yields and maintenance requirement on malate and acetate

The results in Figure 53 show that a fraction of the cell's energy supply is used for maintenance and, based on the not unreasonable assumptions that under constant growth conditions both energy coupling and maintenance requirement are independent of the growth rate, that energy produced in excess of maintenance requirement is used to

synthesise new cell material at constant efficiency.

Similar conclusions can be derived from growth on acetate although in this case the limitation by transport and accuracy of the results are not so well established as for malate (Figure 54).

Maximum molar growth yield/mol oxygen ($Y_{G_{O_2}}$) on these two substrates derived from Figures 53 and 54 are markedly different being 40 g/mol oxygen during growth on malate and 22.9 g/mol oxygen during growth on acetate. It follows that, if Y_{ATP} is constant, then the coupling of energy production from electron transport, although it may be constant during growth on one substrate, is not identical when cells are growing on different substrates. On the assumption that $Y_{ATP} = 10.5$ for growth of the cells on all substrates (Bauchop & Elsden, 1960) calculation derives a P/O ratio of 1.9 during growth on malate and 1.09 during growth on acetate.

Because of the low variability of cell composition and metabolism, it was anticipated that the maintenance requirement of the cells during growth on different substrates would be constant but clearly this is not true. Even allowing for the different P/O ratios the maintenance coefficient during growth on malate is 57 μ mol ATP/g dry wt./min and during growth on acetate is 113 μ mol ATP/g dry wt./min. Maintenance therefore depends on the growth substrate.

Several processes have been proposed to explain the function fulfilled by maintenance energy. Marr et al. (1963) suggested protein turnover but also discussed results of Berger which, because of a low temperature coefficient, suggested diffusion could account for the

maintenance requirement. Watson (1970) proposed that the establishment of ion gradients across the cell membrane, particularly of Na^+ in Saccharomyces cerevisiae, may be the explanation. None of these suggestions has been definitely established and none can explain the altered maintenance requirement observed between cells growing on malate and acetate. The metabolism of cells growing on malate and acetate is almost identical. The only obvious difference is the P/O ratios or efficiency of energy coupling in the cells.

Rosenberger & Elsden (1960) observed uncoupling during anaerobic growth of Streptococcus faecalis in tryptophan limited continuous culture in the presence of excess energy source. The rate of energy source catabolism is high and they concluded that ATP is being produced but not used by the cells. Direct confirmation of uncoupling as the action of an ATPase and not lack of ATP synthesis was obtained by measurement of the ATP pool. Forrest & Walker (1965) observed in washed cell suspensions of Streptococcus faecalis that glucose metabolism in the absence of growth was accompanied by a high ATP pool. In Streptococcus faecalis energy is generated by substrate level phosphorylation and its production is directly involved in the chemical reactions of energy source catabolism.

During growth of E.coli under aerobic conditions the bulk of the energy is produced by oxidative phosphorylation which can be uncoupled from electron transport (Hempfling, 1970b). Consequently a lower net P/O ratio could be due either to a less efficient ATP production or to the presence of a system to hydrolyse ATP produced in excess of cellular

requirement. Irrespective as to the mechanism employed by the cells to regulate the apparent P/O ratio during growth, the process results in a release of free energy associated with electron transport. Cells trained to acetate (P/O = 1.09) will release more free energy during electron transport than cells trained to malate (P/O = 1.90). There is therefore an increased maintenance requirement in cells with an increased release of free energy associated with electron transport.

The release of free energy could cause damage to the cell structure and repair or resynthesis of these structures will be necessary. Goldberg (1972) has reported that a system exists in E.coli to degrade incorrect or damaged protein molecules and has suggested that the largest source of damaged molecules is likely to be the denaturation of enzymes inside the cell. Goldberg has further observed that the process involved in the degradation of these proteins requires a supply of metabolic energy. It is not inconceivable that a decreased efficiency of energy coupling could increase the denaturation of enzymes inside the cell and that the energy required to repair or replace denatured proteins contributes to the maintenance requirement of the cell. The corollary of this hypothesis is that cells, growing under conditions where P/O ratios are high and uncoupling is small, should have decreased requirements for maintenance. Results, still to be discussed, suggest glucose in batch culture may fall into this category.

3.3. Growth yields and P/O ratios

There is evidence from growth yield measurements that P/O ratios vary with the carbon source used to support growth (Hadjipetrou et al.,

1964; Payne, 1971). Yields per mol oxygen for growth on a variety of substrates, some of which have contributions to their energy production from both substrate and oxidative phosphorylation, are shown in Figure 50. Cells growing on glucose with an assumed P/O ratio of 3 obtain 14% of their total energy production by substrate phosphorylation (Figure 51) and this fraction increases with decreasing P/O ratio (Figure 52). Since P/O ratios have not been established it is not possible to correct for substrate phosphorylation without assuming a P/O ratio. Figure 51 calculates energy production for cells assuming a P/O ratio of 3 while in Figure 52, P/O ratios at different integer values are used to calculate both rates of energy production and corresponding Y_{ATP} values. No obvious correlation was found.

On the basis that the rate of energy production should be related to the rate of synthesis of cell material or the specific growth rate, Figure 55 was constructed. The slope of the line shown gives a measure of Y_{ATP} during growth on all substrates, assuming it is constant, and has a value of 8.9 g which is not surprising since the line was drawn using acetate and malate data where P/O ratios of 1.09 and 1.9 were calculated as consistent with Y_{ATP} of 10.5. Maintenance, which may vary with the P/O ratio, cannot be accurately estimated but the value for malate was taken as a reasonable average.

Several interpretations can be put on to Figure 55. Any line gives a constant Y_{ATP} value. The one drawn assumes that maintenance requirement is low (4% of total energy production on malate) and that P/O ratios vary. Under these conditions cells growing on glucose have

a P/O value close to 3 similar to that reported by Hadjipetrou et al. (1964) for Aerobacter aerogenes. This interpretation is the most likely.

A P/O ratio which is independent of the growth substrate only gives a reasonable line at P/O ratios of 1 or 2. A P/O ratio of 1 gives a Y_{ATP} of 21.7 but suggests that maintenance requirement is high, constituting 31% of total energy production during growth on malate. A P/O ratio of 2 gives a $Y_{ATP} = 10.7$, which is close to the accepted value of approximately 10.5 for anaerobic growth, but still has associated with it a high requirement for maintenance.

The results show the importance of determining P/O ratios in cells under a variety of growth conditions before any real progress in the rationalisation of aerobic molar growth yields can be achieved. In the absence of assumptions about the P/O ratio the data of Figure 55 are at best, indicative and at worst, useless.

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Maleate has been reported as a gratuitous inducer of the L-malate transport system. Its presence causes adaptation to L-malate during growth on glycerol, suggesting that transport activity is related to the rate of metabolism of L-malate. The activity of the L-malate transport system, as measured by the rate of pyruvate production, in arsenite inhibited whole cells, increases with adaptation so confirming that the rate of metabolism of L-malate is limited by its transport. The regulation of central metabolism and the characteristics of the enzymes metabolising L-malate are also consistent with the transport mechanism being the process which limits metabolism.

Degree of adaptation was measured as a range of growth rates with corresponding rates of oxygen uptake on L-malate. The relationship between the two permits a maintenance coefficient and Y_Q value to be calculated for L-malate growth. Analogous values are also given for growth on acetate. The full interpretation of these data depends on assumptions as to the efficiency (P/O ratio) of oxidative phosphorylation. It seems probable that P/O ratios and maintenance vary with the growth substrate and that when a lower efficiency of oxidative phosphorylation is obtained, a higher level of maintenance is required.

Molar growth yields (as $Y_{C\text{-source}}$ and Y_{O_2}) reflect both P/O ratios and the whole mechanism of biosynthesis. Consideration of the apparent Y_{ATP} for different carbon sources strongly suggests that the P/O ratio varies and depends on the carbon source available to the cells. It is not surprising that the highest P/O ratios are obtained with those carbon sources which support the highest growth rates.

SUMMARY

Escherichia coli ML308 (ATCC 15224) grows aerobically on L-malate as a sole source of carbon and energy in a medium otherwise containing only inorganic ions. Cells grow immediately on L-malate if previously grown on other dicarboxylic acids, some amino acids or αketoglutaric acid but lag before growth on L-malate after growth on glucose, glycerol, pyruvate or acetate. Limitation of growth rate must be due to a factor which is present in cells during growth on the first but not on the second group of carbon sources. The three factors considered were PEP carboxy kinase, malic enzymes and the L-malate transport system.

Acetate stimulates growth rate on L-malate in all circumstances and must, therefore, increase the rate of production of all intermediates. In particular the rate of supply of phosphoenol-pyruvate (PEP) for glucogenesis etc. must be increased. Because PEP is made from L-malate by PEP carboxy kinase it follows that this enzyme cannot limit rate of growth on L-malate alone.

The relationship between activities of malic enzymes and growth rate on L-malate was measured directly. Different phenotypes, able to metabolise L-malate at various rates, were found to contain more malic enzyme activity than was expressed during growth on L-malate. Furthermore, malic enzyme activities during adaptation to L-malate do not alter. Consequently, growth rate on L-malate cannot be limited by malic enzyme activities.