Competitive Utilization of Glucose and Glycerol by *Escherichia coli*

by

Ian S. Forrest

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List of Abbreviations

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

BCIG	5-bromo-4-chloro-indoxyl-β-galactoside
PEP	phosphoenolpyruvate
FDP	fructose-1,6-diphosphate
G6P	glucose-6-phosphate
F6P	fructose-6-phosphate
TCA	trichloracetic acid
PCA	perchloric acid
G6P DH	glucose-6-phosphate dehydrogenase
PTS	phosphotransferase system
cAMP	cyclic adenosine-3',5'-monophosphate
L-α-GP	L-α-glycerophosphate
α-MG	α-methyl-glucoside
CAP	chloramphenicol
FCCP	carbonyl cyanide p-trifluoromethoxyphenyl hydrazone
UV	ultraviolet

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SUMMARY

1) When glycerol is the sole source of carbon and energy in E.coli its utilization is regulated by the level of glycerokinase and the intracellular concentration of fructose-1,6-diphosphate, the negative modifier of glycerokinase activity.

2) E.coli 15224 exhibits diauxic growth on a mixture of glucose and glycerol in simple defined media under aerobic conditions.

3) When cells have been trained to and are growing on glycerol, the addition of glucose represses, almost immediately, the synthesis of glycerokinase. Glycerol utilization falls by about 50% at the time of glucose challenge and continues to decrease for about 50 min, when it ceases altogether, and does not start again until glucose is exhausted. After glucose challenge, the glycerol carbon used continues to enter protein, nucleic acid and lipid with a slight increase in the proportion entering the last. The delay before glycerol utilization is completely shut off suggests that this process depends on some feature of the glucose phenotype which is not present in glycerol trained cells. This is supported by the observation that the addition of glucose to chloramphenicol treated cells depresses the rate of glycerol metabolism but never abolishes it.

4) In the above case total inhibition of glycerol utilization is an example of 'catabolite inhibition' (McGinnis & Paigen, 1969). In this case glycerol metabolism could be regulated by fructose-1,6-diphosphate concentration

which, in turn, could reflect the rate of glucose metabolism. However fructose-1,6-diphosphate inhibits glycerokinase of this strain to a maximum of 85%. Direct measurement shows that the intracellular concentration of fructose-1,6-diphosphate does not change significantly on glucose challenge. The fructose-1,6-diphosphate in cells growing on glycerol permits 31% of the glycerokinase activity to be utilized and, after glucose challenge, at least 26% of glycerokinase activity would be used if fructose-1,6-diphosphate was the only regulator. Thus total inhibition of glycerol metabolism cannot depend on glycerokinase inhibition by fructose-1,6-diphosphate. Glycerokinase is not inhibited by any other metabolite of glucose which has been tested.

5) The rate of free diffusion of glycerol into cells of E.coli is sufficient to account for a substantial proportion of the rate of glycerol utilization in the absence of glucose. Furthermore glucose does not affect the facilitated diffusion of glycerol. Thus glucose does not inhibit glycerol utilization by regulation of the rate of entry of glycerol into the cells.

6) The rate of glycerol utilization, the rate of glucose utilization and the ratios of glycerokinase and glucose PTS are all inter-related. The utilization of glucose and glycerol are mutually inhibitory, the degree of inhibition depending on the relative levels of glycerokinase and glucose PTS. However the rate of glycerol utilization depends on the rate of glucose utilization when the enzyme levels are constant. A model has been proposed in which glycerokinase and glucose PTS compete for a common element.

This has been supported by observations, both in growing cells and washed cell suspensions, and it is speculated that the common element may involve the energy requirement of the first steps of glycerol and glucose utilization.

INTRODUCTION

1. General Considerations

Many bacteria show considerable nutritional versatility. The pseudomonads for instance can grow on well over a hundred different compounds including various hydrocarbons, phenols and aliphatic amides (Mandelstam & McQuillan, 1968). The strain of Escherichia coli (E.coli 15224) used in this laboratory has been shown to utilize 58 compounds as sole sources of carbon and energy when growing in simple salts medium (Hamilton, 1972). This is by no means an exhaustive survey of the versatility of this organism and many more possible carbon and energy sources may exist.

Unlike higher forms of life, bacteria do not have obligatory life cycles and can reproduce continually as vegetative, undifferentiated cells. Bacteria also differ from many differentiated metazoans in that even if they are not actually growing they are poised to do so. However, free life and unicellularity impose a requirement for a high degree of adaptability as changes in the environment cannot be met by homeostasis, since this demands the interaction of specialized cells. Rather, bacteria deal with physical and chemical alterations of their surroundings by exhibiting a different set of metabolic activities. That is, they are capable of existing in a variety of physiological states which can be quite different from one another. In addition, bacteria shift from one such physiological state to another in a rapid and efficient manner.

In view of the strong selective pressures on bacteria to utilize available nutrients as efficiently as possible, and to achieve optimal growth rates while doing so, (Stanier et al, 1971; Koch, 1971) it is to be expected that complex controls of bacterial metabolism exist. It might be imagined that a bacterium must have acquired, through selection in the environment to which it is adapted, two main features of metabolic control: the ability to utilize as efficiently as possible the nutrients normally available in those environments, and the capacity to respond rapidly to environmental changes.

Specific control mechanisms can be sub-divided into three major types: control of enzyme synthesis, control of enzyme activity, and regulation of the supply of carbon into the cell. The latter sub-division may be considered as a special case of the two former divisions. However the cell membrane is such a potentially important locus for control of bacterial metabolism that this is treated separately.

Most studies on the control of metabolic pathways have centred on induction and repression of enzymes concerned with carbohydrate metabolism, especially the lac operon of E.coli (Jacob & Monod, 1961; de Crombrugghe et al, 1971) and on the repression and inhibition of divergent, branched chain pathways of amino-acid biosynthesis in E.coli and Salmonella typhimurium (Cohen, 1965; Datta, 1969).

It is not intended to present an exhaustive survey of the control mechanisms that exist in bacteria. The

following sections merely present a general view of the mechanisms responsible for the control of bacterial metabolism during growth in any environment and during changes in that environment.

2. Enzyme Regulation

Current understanding of the regulation of bacterial enzymes stems directly from the work of Jacob & Monod (1961) and Monod, Changeux & Jacob (1963). They proposed the concept that small molecules may regulate the activities of proteins. These modulations occur at two organisational levels in the cell to control enzyme activity and to regulate gene expression.

2.1. Regulation of gene expression

Studies on regulation of gene expression in bacteria are based on the operon model (Jacob & Monod, 1961) for the control of the lac region of E.coli. The model proposes negative control of gene expression at the level of transcription by means of a cytoplasmic repressor. The repressor is coded by one regulatory gene (i) and binds to another regulatory site (O, operator), contiguous with the structural genes coding for β-galactosidase, β-galactoside permease, and thiogalactoside transacetylase. The presence of the inducer decreases the binding of the repressor to the operator thus allowing transcription and consequently protein synthesis.

Confirmation of this theory was obtained when Gilbert & Müller-Hill (1966) isolated the repressor, a protein containing no detectable nucleic acid (Riggs & Bourgeois, 1968); the repressor binding specifically to the lac operator (Chen et al, 1971) and being released by inducer (Gilbert & Müller-Hill, 1967; Riggs, Suzuki & Bourgeois, 1970).

Transcription of lac operon is not only regulated by

the i-gene product (repressor) but requires the presence of cyclic AMP receptor protein (de Crombrugghe et al, 1971). RNA polymerase is only bound to DNA if cyclic AMP receptor protein is attached to the promoter region (contiguous with O but distal to the structural genes) (Ippen, Miller, Scaife & Beckwith, 1968). Thus regulated, cell-free transcription of lac mRNA proceeds only in the presence of cyclic AMP, required for binding of the receptor protein to DNA, and of inducer, which removes lac repressor from the operator (de Crombrugghe et al, 1971). In the absence of cyclic AMP no substantial synthesis of lac mRNA occurs.

The dominant control in transcription of lac operon is the cyclic AMP concentration as this absolutely determines the binding of RNA polymerase to its substrate. Thereafter transcription only occurs when inducer is present. In whole cells given exogenous inducer the degree of induction of the lac operon will firstly depend on the level of cyclic AMP in the cell and then on the concentration of inducer in the cell.

The gal operon of E.coli has been studied in a similar manner to the lac operon. The enzymes required for metabolism of galactose are coded for by a single operon (Buttin, 1963a) and are induced by either galactose or fucose (Buttin, 1963b). 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).

The specific activity of many biosynthetic enzymes in

cells depends on the nature of the environment (Gale, 1943). It has been shown, for example, that in the presence of tryptophan the enzymes required for tryptophan biosynthesis are not synthesised (Ames et al, 1967). Jacob & Monod (1961) proposed that the repressible synthesis of enzymes in anabolic pathways is probably subject to a control basically similar to that for the lac operon in which a cytoplasmic apo-repressor is activated by the end product of a biosynthetic sequence, or a compound closely related to the end product, to repress transcription. 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 this is achieved is not. 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). 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 three aspartokinases exist, each regulated by feedback repression control by the end product of different branches (Truffa-Bachi & Cohen, 1968).

Over the years the Jacob-Monod model has been used as a basis for an understanding of induction and

repression in a variety of systems. This model has also been extended to include regulons (Maas & Clark, 1964; Cozzarelli et al, 1968) and operons under positive control, the best defined of which is the arabinose system in E.coli (Englesberg et al, 1965; Irr & Englesberg, 1971).

Translation as a further site of control of enzyme synthesis is not excluded by the proof that control occurs at the level of transcription (Cline & Bock, 1966). Miller et al (1971) were unable to rule out translational control in the expression of the gal enzymes. Control at the level of translation has been shown for two enzymes of the arginine pathway (McLellan & Vogel, 1970) by an unknown mechanism.

2.2. 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 about 5%/h (Mandelstam, 1963) but is diluted out by subsequent cellular growth. Consequently when enzyme synthesis ceases in response to a change in environment 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 the stability of intracellular pools during growth reflect the sensitivity rather than the absence, of cellular control processes.

The first modulation of enzyme activity to be identified in bacteria was feedback inhibition of isoleucine biosynthesis in E.coli (Umbarger, 1956). Since then

feedback inhibition has been observed in a large number of anabolic pathways (reviews by Umbarger, 1961; Cohen, 1965; Atkinson, 1969; Datta, 1969). The simplest form of feedback inhibition is seen in a linear pathway, for example histidine biosynthesis, where the first enzyme in the pathway is inhibited by histidine, the end product (Ames, Martin & Garry, 1961). In divergent, branched-chain biosynthetic pathways, more complex controls are found allowing economic use of different branches at different rates.

In addition to simple feedback inhibition by a single metabolite, concerted feedback inhibition, requiring two or more end products to achieve inhibition, and co-operative inhibition, in which inhibition of a common enzyme is greater in the presence of a mixture of end products than in the presence of a single end product, have also been described (Datta, 1969).

Within the generally divergent biosynthetic pathways there are elements of convergence, for example methionine biosynthesis involves the methylation of homocysteine by a specific fraction of the tetrahydrofolate pool. In Neurospora crassa these convergent pathways are synchronised by feedback inhibition controls on both convergent paths and a cross-pathway activation by methyltetrahydrofolate (Selhub, Savin, Sakami & Flavin, 1971).

Regulation of the activity of enzymes of the central metabolic or amphibolic pathways are more complex than that observed for anabolic enzymes (Atkinson, 1969; Sanwal, 1970). One mechanism whereby regulation of the amphibolic pathways can be achieved is by changes in the energy charge in the

cell. The concept of energy charge as a control function has been developed by Atkinson (1969). Although the original data came from mammalian systems, bacterial enzymes such as phosphoribosyl pyrophosphate synthetase (Atkinson & Fall, 1967) and citrate synthase (Jangaard et al, 1968) in E.coli are also susceptible to regulation by energy charge.

Atkinson has suggested that, because of the sensitivity of mammalian, yeast and E.coli citrate synthases to energy charge regulation, 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. Chapman et al (1971) maintains that this stability represents the sensitivity to, and not the absence of, control of enzyme activity by energy charge. 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 whereby they are sensitive to changes in other metabolites. In E.coli it is found that NADH inhibits citrate synthetase (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.

It has also been shown that many sites of allosteric

activation exist in the amphibolic pathways. For example FDP stimulates the activity of pyruvate kinase and pyruvate carboxylase; FDP and PEP stimulate the activity of one of the enzymes of glycogen synthesis; and acetyl coenzyme A activates pyruvate carboxylase (Stanier et al, 1971; Atkinson, 1969).

Regulation of activity of catabolic pathway enzymes is less well documented than regulation of anabolic or amphibolic pathways. Sanwal (1970) quotes several cases in which the activity of the first enzyme of several amino acid degradative pathways is subject to regulation by the energy charge of the cell.

In both anabolic and amphibolic pathways the first step in a reaction sequence is subject to control. In a catabolic pathway the first step may be entry into the cell and certainly a pathway with an active transport system would be most economically controlled at this level. Permeation across the cell membrane is thus potentially an important locus for the control of cellular metabolism.

3. Permeation

A semi-permeable membrane was first postulated because of the behaviour of animal and plant cells under varying osmotic conditions (described in Loewy & Siekewitz, 1969). It is the barrier through which all materials from the external environment must pass in order to enter the cell. Four general types of translocation across the bacterial membrane have been recognised (reviews by Kaback, 1970; Kotyk, 1973) - passive, or free, diffusion; facilitated diffusion; active transport; and group translocation. Bulk transport does not exist in bacteria (Loewy & Siekewitz, 1969). Presumably this is a consequence of the cell wall which encloses the membrane and the high intracellular osmotic pressure which presses the membrane against the wall. However, other mechanisms exist which permit large molecules to penetrate the cell. This is exemplified by transport of DNA during transformation (Stanier et al, 1971).

3.1. Passive diffusion

Stein (1967) has reviewed the nature and extent of passive diffusion across cell membranes and concluded

'a) diffusion within the membrane ... results in a 100 to 1000 fold reduction of transfer rate in comparison with an equivalent thickness (5 nm) of water, however,

b) to enter the membrane, each hydrogen-bonding acceptor or donor group that the permeant makes with the water molecules of the aqueous phase has to be broken, a step which lowers the transfer rate by a further 6 to 10 fold, and,

c) each bare $-CH_2-$ group in the permeant will increase the transfer rate by some twofold.'

The cell wall of the bacterial cell is freely penetrated by most small molecules (Mandelstam & McQuillen, 1968) and consequently the permeability properties of the cell are determined by the membrane alone.

Haest et al (1972) find that the permeability of the cell membrane of E.coli to erythritol depends on the lipid composition of this membrane. The cell membrane varies from being relatively permeable to a permeant like methanol to being highly impermeable to a permeant like lactose.

Examples of passive diffusion in bacteria are not well documented. However, Eagon (1971) has proposed that 2-deoxyglucose enters cells of Pseudomonas aeruginosa by passive diffusion before being further metabolized, but this has been challenged by Midgley (1972).

3.2. Facilitated diffusion

Facilitated diffusion systems operate on an existing chemical or electrochemical gradient of the permeant and lead to the disappearance of the gradient. The permeant has the same chemical identity on both sides of the membrane. These systems require no further input of energy, except for that required to maintain the integrity of the cell membrane (Stein, 1967).

Facilitated diffusion systems are also poorly documented in bacteria, more extensive studies having been done in eukaryotic cells, notably glucose transport in erythrocytes (Wilbrandt & Rosenberg, 1961). Substrate specificity, saturation kinetics, competitive inhibition

by chemical analogues and non-competitive inhibition by protein reactants have been observed. Perhaps the best experimental confirmation of this facilitated diffusion system is the 'counter-transport' of xylose by glucose in erythrocytes reported by Park et al (1956).

3.3. Active transport

Active transport systems have been identified by the accumulation of the permeant inside the cell against a chemical, or electrochemical, gradient, the permeant having the same chemical identity on both sides of the membrane. However, these results have been obtained in non-growing cells and it is not known if accumulation also occurs in cells growing actively on the permeant.

The crucial kinetic feature of an actively transporting system is the different effective binding power of the carrier at each of the two membrane sides, which leads to accumulation of the permeant (Kotyk, 1973). This can be achieved in two principally different ways. (1) By a change in the translocation rate across the membrane. (2) By a change in the affinity of substrate.

In the simplest model describing such energy-driven transport (due to Jacquez, 1964), the change in conformation is obligatorily accompanied by switching over from one membrane side to the other. However this simple model predicts that the steady-state ratio of the intracellular to extracellular concentrations should remain constant over the whole range of extracellular concentrations. This contradicts experimental findings (e.g. Kotyk, 1973).

Rosenberg & Wilbrandt (1963) and Silverman & Goresky (1965) developed models which overcame this difficulty.

They proposed a mechanism in which the carrier exists in two affinity states at both membrane faces and in which they envisage that energy drives the conversion between these two states. This results necessarily in a steady-state situation in which the intracellular concentration of permeant is greater than the extracellular concentration and where the steady-state ratio depends on this concentration.

Although a kinetic model of active transport has been developed the site of energy input is still unclear. It has been concluded by Wong et al (1971) that the dissociation of the carrier-substrate complex on the internal face of the membrane is the energy coupled step for β-galactoside transport in E.coli. This is at variance with the conclusions of Kepes (1971) and Manno & Schachter (1970) who, for the same system, assume the translocation of the carrier from the inner to the outer face of the membrane to be energy coupled.

The site of energy coupling has been defined more clearly for other systems. Energy is required for the interconversion of the two affinity states of the carrier at the inner face of the membrane for monosaccharide transport in the yeast Rhodotorula glutinis (Höfer, 1971) whereas in Chlorella vulgaris the translocation of carrier-substrate complex across the membrane is energy coupled. This latter mechanism has been demonstrated for amino acid transport in various fungal species (Crabeel & Grenson, 1970; Kotyk & Říhová, 1972a; Hunter & Segel, 1971).

The energy sources for active transport processes vary considerably. The immediate source of energy for active transport in animal cells appears to be the sodium

ion concentration gradient (Kotyk, 1973) whereas uptake of amino acids by Saccharomyces cerevisiae is linked to a high-molecular weight polyphosphate in the cell (Kotyk & Říhová, 1972b).

Kaback's group (review Kaback, 1972) have shown the transport of amino acids, some monosaccharides and β-galactosides to be linked to D-lactate oxidation in isolated membrane vesicles of E.coli. West (1970) and West & Mitchell (1972) have shown that the influx of β-galactoside is coupled to a flow of protons, supporting the suggestion (Mitchell, 1963, 1966) that the accumulation of β-galactosides by E.coli is due to the operation of a proton-β-galactoside symporter, analogous to the Na^+-glucose and Na^+-amino acid symporters thought to occur in animal cells. Pavlasova & Harold (1969) support this hypothesis for E.coli with their observations that uncouplers of oxidative phosphorylation, which also collapse the hydrogen ion gradient across the bacterial membrane, prevent the accumulation of β-galactosides by anaerobic cells even although the intracellular ATP concentration is not affected under these conditions.

The protonmotive force has also been implicated as the primary energy source for potassium transport in Streptococcus faecalis (Harold, 1972b) and for phosphate uptake (Jeacocke et al, 1972) and amino acid transport (Niven & Hamilton, 1972) in Staphylococcus aureus.

3.4. Group translocation

Group translocation is a transport system in which the permeant is chemically modified as an integral part of the transport process. The best known and characterised

example of this is the vectorial phosphorylation of sugars by the phosphoenolpyruvate-phosphotransferase system (PEP-PTS) in bacteria (reviewed by Kaback, 1972). In general, it is known to contain a cytoplasmic, heat-stable protein (H Pr) of molecular weight less than 10,000 which is phosphorylated, in the presence of cytoplasmic, Mg^{2+}-activated enzyme (enzyme I), by phosphoenolpyruvate. The phosphorylated heat-stable protein acts as the phosphate donor for the sugar in a reaction catalysed by a membrane-bound, Mg^{2+}-activated enzyme (enzyme II) which, in E.coli, is composed of two protein fractions and a phospholipid (Kundig & Roseman, 1971). HPr and enzyme I are constitutive and common to all sugars whereas a different enzyme II exists for each sugar, specifically catalysing the phosphorylation of that sugar. The PEP-PTS has been found in a wide range of facultative anaerobes, but not in strict aerobes (Romano, Erberhard, Dingle & McDowell, 1970). The range of sugars transported by this system varies in different organisms. For example, lactose is transported by vectorial phosphorylation in Staphylococcus aureus (Hengstenberg, Egan & Morse, 1967) while the possibility that galactosides are accumulated by vectorial phosphorylation in E.coli has been eliminated by Pastan & Perlman (1969) and Barnes & Kaback (1970).

3.5. Regulation of permeation

Feedback inhibition of transport has been recorded. Histidine uptake is subject to inhibition by intracellular histidine (Crabeel & Grenson, 1970) and glucose transport is inhibited by glucose-6-phosphate (Azam & Kotyk, 1969) in the yeast Saccharomyces cerevisiae. Kaback (1970) has found that the phosphoenolpyruvate-phosphotransferase

system in isolated E.coli membranes is subject to inhibition by glucose-1-phosphate and glucose-6-phosphate. Dreyfuss & Pardee (1966) attributed the approach to equilibrium by overshoot during sulphate transport in Salmonella typhimurium to feedback inhibition by 3'-phosphoadenosine-5'-phosphosulphate.

Several different types of transport system have been identified. Very little is known of the actual transport mechanism within the membrane, but some knowledge of the nature and role of the enzymic ancillary systems is being acquired in the more complex transport systems. Classically, biochemistry involves the stripping of a system to its individual components, analysing each component and then reconstituting the system with known units. However, to destroy a membrane is to destroy the basis on which transport depends and the membrane structure requires to be well understood before reconstruction experiments have any validity. Neither facilitated diffusion nor active transport chemically affect the permeant and thus in vitro analysis of the chemical mechanism is difficult. The observer is thus forced to analyse a complex unit in operation with all the inherent difficulties that this entails.

4. Control of Metabolism during Growth on Dual Carbon Sources

4.1. Enzyme repression

The synthesis of inducible enzymes in *E.coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* is repressed in the presence of glucose or glucose-6-phosphate. Enzyme systems affected include those degrading lactose, galactose, glycerol, arabinose, L-tryptophan and D-serine (de Crombrugghe et al, 1969). Similar phenomena are widespread in bacteria (review Paigen & Williams, 1970) and have been termed 'catabolite repression' (Magasanik, 1961). For example Schlegel & Trüper (1966) found that molecular hydrogen represses synthesis of the enzymes of the Entner-Doudoroff pathway in *Hydrogenomonas* and in *P. aeruginosa*, the enzymes of glucose catabolism are subject to repression in the presence of citrate (Hamilton & Dawes, 1960; Hamlin, Ng & Dawes, 1967) and the amidase is subject to repression in the presence of succinate (Clarke & Brammar, 1964).

'Catabolite repression' of the *lac* operon has been exhaustively studied in *E.coli* by de Crombrugghe et al (1971). Repression is mediated by the concentration of cyclic AMP in the cell. Glucose and other compounds that cause 'catabolite repression' decrease the intracellular concentration of cyclic AMP and cause loss of cyclic AMP to the external environment (Makman & Sutherland, 1965). The mechanism of this phenomenon is unclear. Adenyl cyclase activity may be modulated both by altered substrate (ATP) and allosteric effector (pyruvate) concentrations (Tao & Lipmann, 1969) which reflect the carbon source in use.

4.2. Enzyme inhibition

McGinnis & Paigen (1969) demonstrated that glucose inhibited the utilization of other carbohydrates by E.coli. The phenomenon is distinct from 'catabolite repression' in that enzyme activity rather than enzyme synthesis is inhibited. The inhibition has as its site of action the transport systems of the cell membrane or the first enzyme of subsequent metabolism, however the mechanism of inhibition is unknown. Glucose has been shown to inhibit the transport of galactose (Horecker, Thomas & Monod, 1960), galactosides (Kessler & Rickenberg, 1963), maltose (McKinstry & Koch, 1972), tryptophan (Boezi & De Moss, 1961) in E.coli and histidine in Salmonella typhimurium (Ames, 1964). Hydrogen reduces the utilization of fructose in Hydrogenomonas (Blackkolb & Schlegel, 1968) and yeast extract inhibits glucose metabolism in Clostridium tetanomorphum (Anthony & Guest, 1968).

5. Glycerol Metabolism in E.coli

Glycerol and L-α-glycerophosphate (L-α-GP) are dissimilated by E.coli through a converging catabolic pathway (Figure I). Glycerol enters the cell by facilitated diffusion (Sanno, Wilson & Lin, 1968) and is phosphorylated to L-α-GP by an adenosine triphosphate (ATP) dependant reaction catalysed by glycerokinase (Hayashi & Lin, 1965b and 1967). L-α-GP is actively transported into the cell (Hayashi, Koch & Lin, 1964) where the intracellular pool of L-α-GP, derived from both branches of the pathway, provides the common substrate for either the aerobic L-α-GP dehydrogenase (Lin et al, 1962; Weiner & Heppel, 1972) or the anaerobic L-α-GP dehydrogenase (Kistler et al, 1969; Kistler & lin, 1972). Both dehydrogenases are flavin linked and convert L-α-GP to dihydroxyacetone phosphate (Weiner & Heppel, 1972; Kistler & Lin, 1972; Cooper & Anderson, 1970) a metabolite of the central glycolytic pathways.

The gene for glycerol facilitator (glp F) and the gene for glycerokinase (glp K) appear to comprise a single operon (Berman-Kurtz et al, 1971) located at minute 76 (Cozzarelli & Lin, 1966) on the chromosome map described by Taylor and Trotter (1972). The genes for the L-α-GP transport system (glp T) (Cozzarelli et al, 1968) and for the anaerobic L-α-GP dehydrogenase (glp A) (Kistler & Lin, 1971) are located next to each other at minute 45, and perhaps also belong to a single operon. The gene for aerobic L-α-GP dehydrogenase (glp D) is found in a third region at minute 66, adjacent to the glp R locus specifying the repressor which regulates the expression of all the known operons of the glp regulon (Cozzarelli et al, 1968).

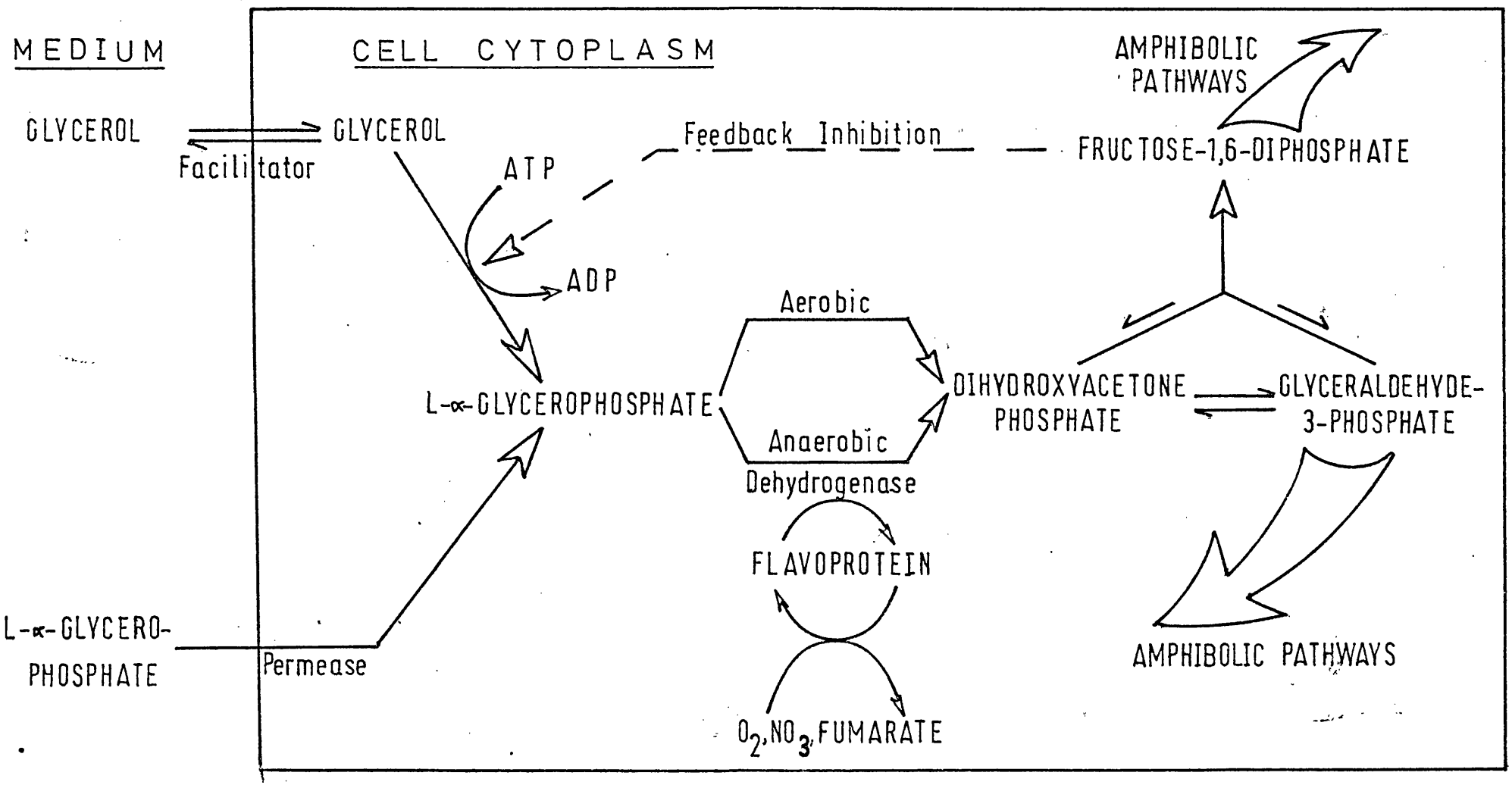


FIGURE I - Growth of E.coli on glycerol or L- α -glycerophosphate

5.1. Control of glycerol metabolism

The inducer of the operons of the glp system is L-α-GP (Hayashi & Lin, 1965a). All three operons require the presence of cyclic AMP for expression (Berman, Zwaig & Lin, 1970) and are subject to catabolite repression by glucose. However, the three units are not repressed equally, aerobic L-α-GP dehydrogenase, L-α-GP transport (and anaerobic L-α-GP dehydrogenase), and glycerokinase (and facilitator protein) showing increasing sensitivity to repression by glucose (Koch, Hayashi & Lin, 1964; Freedberg & Lin, 1973).

A further control on the synthesis of the L-α-GP flavo-dehydrogenases is exerted by the nature of the electron acceptor available (Kistler & Lin, 1971). The level of aerobic L-α-GP dehydrogenase is maximal when oxygen is the exogenous electron acceptor whereas the anaerobic L-α-GP dehydrogenase level is at a maximum when the acceptor is fumarate (Freedberg & Lin, 1973).

On top of these controls the activity of glycerokinase is modulated by feedback inhibition by fructose-1,6-diphosphate (Zwaig & Lin, 1966; Zwaig, Kistler & Lin, 1970).

Competitive utilization of carbon and energy sources by E.coli has been studied in this laboratory for some time. It is hoped that investigation of the control mechanisms involved in competitive situations may lead to a better understanding of the controls involved in regulation of the utilization of a single carbon and energy source.

Preliminary results (Edgar et al, 1972) showed that E.coli 15224 exhibited diauxic growth on a mixture of glycerol and glucose in simple defined medium under aerobic conditions which indicated the enzymes of glycerol catabolism were repressed by glucose. It was also shown that glycerokinase was repressed and that existing enzyme activity was inhibited on glucose challenge to glycerol growing cells. Although glycerokinase was known to be inhibited by FDP the intracellular concentration was not sufficient to be the mechanism of control.

Therefore it was thought to be a good system to examine the interaction of the metabolism of two carbon sources. It was hoped that this might lead to elucidation of the mechanisms controlling carbon flow into the cell and the reasons for selection of such mechanisms. It was also hoped that a mutant constitutive for the enzymes of glycerol catabolism might circumvent repression and allow us to investigate inhibition alone.

METHODS

1. Microbiological Techniques

1.1. Organism

Escherichia coli ATCC 15224 (ML308) was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A. This organism has the genetic structure $i^{-}z^{+}y^{+}a^{+}$ for the lac operon. A defective repressor is produced and consequently the synthesis of the products of the lac operon (β-galactosidase, β-galactoside permease and thiodigalactoside transacetylase) is constitutive. In all other respects it was assumed to be wild type.

A mutant strain of E.coli 15224, constitutive for the synthesis of the products of the glp regulon, was derived (strain glp R^{c}) and used in some experiments.

A further mutant strain was isolated, constitutive for the synthesis of the products of the glp regulon and producing a glycerokinase with impaired sensitivity to inhibition by fructose-1,6-diphosphate (strain 15224 glp $R^{c}K^{i}$).

All strains were characterised by bacteriological tests described (Cowan and 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°C for 24 h, then plated on nutrient agar and incubated at 37°C 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 on nutrient agar containing 5-bromo-4-chloro-indoxyl-β-galactoside (BCIG agar). BCIG is a substrate, but not an inducer, of the products of the lac operon. A blue colour is formed on hydrolysis of BCIG, thus only lac constitutive cells produce blue colonies on BCIG agar, a homogeneous culture producing 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°C for 24 h. These were then stored at 4°C 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°C for 24 h, plated on BCIG agar to test homogeneity then stored at 4°C. A fresh nutrient broth was used each month for the preparation of inocula.

1.3. Preparation of specifically trained inocula

100 ml of complete defined medium 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°C. 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

Figure 1

PREPARATION OF INOCULA

Inocula of E.coli, trained to different substrates, were prepared by growth through 3 passages in 100 ml of minimal salts medium containing the carbon source of 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°C under aerobic conditions on an orbital shaking table for the periods of time shown.

Carbon source	Concentration (mM)	1st passage (h)	2nd passage (h)	3rd passage (h)
Glucose	10	16	7	7
Glycerol	20	16	7	7
Fructose	10	16	7	7
Mannose	10	24	8	8
Ribose	12	16	8	8
Succinate	20	16	8	8

cultures were stored at 4°C. 24 h before an experiment 1 ml of the 2nd passage was subcultured into a 3rd passage, grown as above, and stored overnight at 4°C. 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 11,750 g and 4°C for 10 min, resuspended to the required cell density in chilled 40 mM phosphate buffer, pH 7.1, and stored on ice until required. If actively growing cells were required a portion of the 3rd passage was inoculated into 100 ml of complete defined medium and grown for several hours at 37°C on an orbital shaker. The inoculum was then prepared as described.

1.4. Isolation of the glp R^c mutant

This mutant was isolated essentially by the method of Hayashi, Koch and Lin (1964).

1.4.1. Mutagenesis

A logarithmically growing culture of E.coli 15224 was harvested by centrifugation at 11,750 g and 4°C for 15 min. Approximately 10^9 cells were resuspended in 2 ml, 0.2 M tris-HCl buffer, pH 7.5, containing 0.03 ml of freshly dissolved ethylmethanesulphonate. This suspension was transferred to a test-tube and shaken in a water-bath at 37°C for 2 h, after which it was diluted into 50 ml complete defined glucose medium and grown on an orbital shaker at 37°C for 60 h. This eliminated most of the auxotrophs. 5 samples were treated in this way.

1.4.2. Enrichment

A 1% inoculum of each culture was alternately subcultured between defined glycerol and defined glucose medium. Each subculture was grown at 37°C for 7 h on an orbital shaker. *Glp* constitutive mutants grow immediately on glycerol after growth on glucose, whereas the inducible parent strain does not. Each cycle enriches the mutants by a factor of two.

1.4.3. Identification and Inoculation

Each final culture was diluted so that approximately 100 cells could be plated onto each of 5 tryptone agar plates. Inoculated plates were incubated at 37°C for 48 h. The colonies produced were sprayed with 1 M glycerol in 6 mM chloramphenicol (CAP). The plates were incubated for 2 h at 37°C and then sprayed with 30 mM triphenyltetrazolium chloride in 1 M KH_2PO_4 buffer, pH 7.0. Under these conditions only those colonies which had constitutively produced all the enzymes required for glycerol utilization were able to reduce the tetrazolium dye to a red formazon.

In contrast inducible colonies remained white because CAP prevented enzyme induction. Cells from red colonies were streaked on nutrient agar from which single clones were isolated, inoculated into nutrient broth and grown for 24 h at 37°C. These were stored at 4°C. At least one constitutive mutant was obtained from each set of 5 plates.

1.4.4. Characterisation of mutants

The mutants were shown to have the same range of abilities as *E.coli* 15224 by the following tests:

1) Their ability to ferment glucose, dulcitol and

lactose with production of acid and gas.

2) Their inability to ferment sucrose or citrate.

3) Their lack of urease production.

4) Their ability to form blue colonies on BCIG agar.

1.5. Isolation of glp R^cK^i mutant

1.5.1. Mutagenesis

The mutagenesis was carried out as described in Section 1.4.1. E.coli 15224 was replaced by E.coli 15224 glp R^c as the parent strain. 10 samples of cells were treated.

1.5.2. Enrichment

A 2% inoculum of each culture was subcultured three times into defined glycerol medium. Each subculture was incubated at 37°C for 7 h on an orbital shaker. A glp R^cK^i mutant grows faster than the parent strain on glycerol (Zwaig, Kistler & Lin, 1970).

1.5.3. Identification and Isolation

The ability of glycerol to prevent growth and cause cell death of a glp R^cK^i mutant growing on succinate (Zwaig, Kistler & Lin, 1970) provided a means of isolating such a mutant.

Each culture was subcultured (2% inoculum) into defined succinate medium, grown for 16 h at 37°C on an orbital shaker and diluted such that approximately 100 cells could be plated onto each of 5 succinate agar plates. These were incubated at 37°C for 96 h and then replicated onto glycerol agar plates, which were incubated for a further 24 h at 37°C.

Those colonies that had grown on succinate agar but not on glycerol agar were inoculated into nutrient broth and grown for 24 h at 37°C. These were stored at 4°C. Glycerol trained cultures were prepared and the ability of FDP to inhibit glycerokinase activity was measured using assay I (Section 10.2.1.).

One mutant was obtained which produced a glycerokinase exhibiting a reduced sensitivity to FDP inhibition.

1.5.4. Characterisation of the mutant

The mutant was characterised as deriving from E.coli 15224 glp R^c by the tests described in Section 1.4.4. and by the ability to produce the enzymes required for glycerol utilization constitutively.

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°C.

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.

One 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

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

2.3. Nutrient agar medium

Nutrient agar was prepared using Oxoid dehydrated material. Nutrient agar medium was dissolved by boiling for 15 min. Nutrient agar medium had the same

composition as nutrient broth medium with the addition of 15 g agar/1.

Agar was sterilised by autoclaving at 15 p.s.i., poured into petri dishes under ultraviolet (UV) light and allowed to solidify. Plates were stored at 4°C.

2.4. BCIG agar medium

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

2.5. Tryptone agar medium

15 g of agar was added to 1 litre of 0.12 M tris buffer, pH 7.5, containing 500 mg Bacto-tryptone and 250 mg yeast extract. The mixture was sterilised by autoclaving in 250 ml batches at 15 p.s.i. for 10 min (more vigorous heating caused serious decomposition of the nutrients resulting in poor growth of colonies), poured into petri dishes under UV light and allowed to solidify. Plates were stored at 4°C.

2.6. Succinate and glycerol agar medium

15 g of agar was added to 1 litre of 40 mM potassium dihydrogen orthophosphate, pH 7.0, containing 20 mM ammonium sulphate and 15 mM carbon source. The agar was sterilised by autoclaving at 5 p.s.i. 8 mM magnesium sulphate (autoclaved separately at 15 p.s.i.) was added to the molten agar to give a final concentration of 0.5 mM. Molten agar was poured into petri dishes under UV light and allowed to solidify. Plates were stored at 4°C.

2.7. Defined media

Defined medium consisted of:

40 mM potassium dihydrogen phosphate

10 mM ammonium sulphate

0.5 mM magnesium sulphate

10 μM ferrous sulphate

adjusted to pH 7.0 with sodium hydroxide and carbon source added at required concentration.

Defined medium was prepared by two different procedures. All solutions were dissolved in glass-distilled water.

2.7.1. 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 2). 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.

2.7.2. Medium for growth experiments

This was prepared from 4 separate components:

I P 40mM 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 and sterilised at 5 p.s.i.

Minimal salts medium was prepared by addition of 1.25 ml solution II and 1.25 ml solution III to every 97.5 ml of solution I.

2.7.3. Medium for washed cell suspension experiments

Medium for washed cell suspension experiments (CAP buffer) was prepared from minimal salts medium by addition of chloramphenicol (CAP) to a final concentration of 0.3 mM.

2.7.4. Storage

Components of all media, with the exception of carbon sources, were stored at room temperature. Carbon sources were stored at $4^{\circ}C$.

3. pH Measurement

All solutions were adjusted to the required pH using 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 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.

4. Sterilisation

All media was sterilised by one of two procedures.

4.1. Autoclaving

Solutions, and where necessary apparatus, 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 and the time of autoclaving depended on the nature and volume of the solutions being sterilised. The efficiency of sterilisation was always checked using Browne steriliser control tubes - type one, black spot (Browne Ltd, Leicester, England).

4.2. Filtration

Sterilisation by filtration of volumes up to 250 ml was carried out using Sterifil holders fitted with 0.22 μ

pore size Millipore filters (Millipore Corp., Massachussetts, U.S.A.). The unit was sterilised by autoclaving at 15 p.s.i.

Nalge disposable filter units (0.20 μ pore) (Sybron Corp., Rochester, U.S.A.) were used for volumes up to 100 ml and were obtained pre-sterilised. After filtration solutions were transferred aseptically to sterile bottles.

5. Glassware

5.1. General Glassware

All glassware was cleaned 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°C for $1\frac{3}{4}$ h either wrapped in paper or in metal canisters. Canister sterilisation was checked by Browne steriliser control tubes.

6. Conditions for Growth andCell Suspension Experiments

Growth of the organism was always carried out in batch culture.

Cultures were maintained at 37°C in the apparatus of Harvey, Fewson and Holms (1968). Cultures were contained in 500 ml conical flasks, for volumes up to 200 ml, or flat-bottomed, side-arm flasks, for volumes greater than 200 ml. Charcoal filtered air was delivered to the flasks at a rate of 200 ml/min if the culture volume was greater than 200 ml. The flow rate was monitored on gas flow gauges (G.A. Platon, Croydon, England).

7. Measurement of Growth

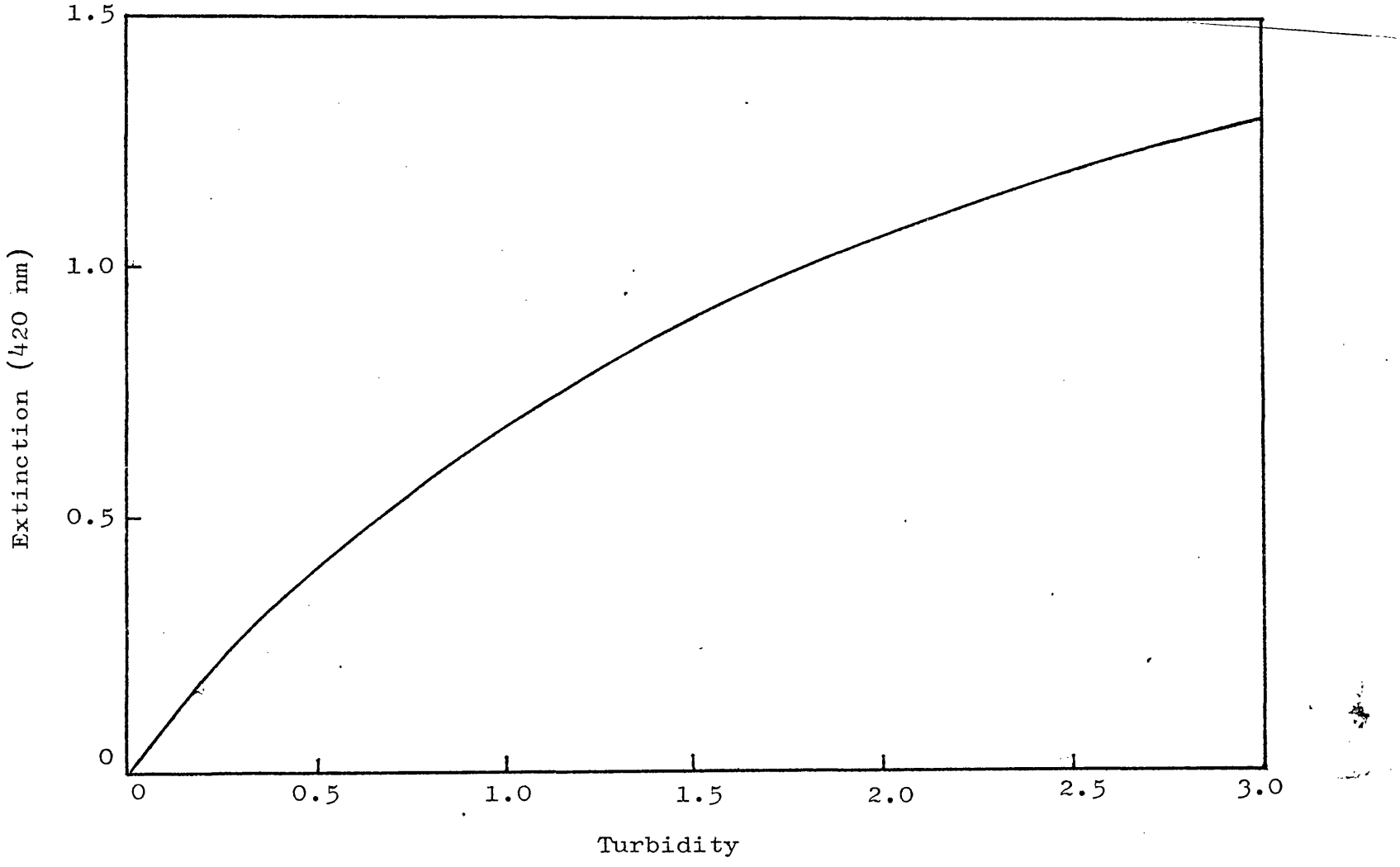
Cell density was determined turbidimetrically on samples (4 ml) taken from cultures into formaldehyde solution (40%, 1 drop). The apparent E_{420} was measured in glass cuvettes (Type 1, 10 mm light path) (Ross Scientific Co.Ltd., Hornchurch, England) using a SP800 double beam spectrophotometer (Unicam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

The absorption due to 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 due to secondary light scattering. A calibration curve of optical density against culture

Figure 2

TURBIDITY CALBIBRATION CURVE

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


1.5
1.0
0.5
0
Extinction (420 nm)
0
0.5
1.0
1.5
2.0
2.5
3.0
Turbidity

turbidity was drawn (Figure 2) enabling 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 and Bennett, 1971).

8. Measurement of Substrate Concentration

8.1. General considerations

Substrate concentrations were measured in samples taken from the culture. Assays were carried out for glycerol, glucose, L-α-glycerophosphate and acetate. All were enzymic assays and all read on an SP800 double beam spectrophotometer fitted with a Servoscribe recorder against air as a blank.

8.2. Treatment of samples

A sample (4 ml) of culture was acidified with ice-cold perchloric acid (PCA) (3M, 1 ml) to give a final concentration of 0.6 M, mixed vigorously and left at 0°C for 10 min. The PCA extract was neutralised with a volume (3 ml) of potassium hydroxide (approximately 1 M), left for a further 10 min and centrifuged in 15 ml centrifuge tubes (Corning Glass Works, New York, U.S.A.) at 11,750 g and 4°C for 10 min in an M.S.E. 18 refrigerated centrifuge (M.S.E., Crawley, England). The supernate was stored in vials at -10°C until assayed. Samples were thawed and thoroughly mixed before a portion was removed for substrate estimation.

8.3. 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 assays medium was:

Triethanolamine	83 mM
Magnesium sulphate	3.3 mM
ATP	0.94 mM
PEP	0.31 mM
NADH	0.17 mM
Lactate dehydrogenase	13 μg/ml
Pyruvate kinase	7 μg/ml
Glycerokinase	13 μg/ml
Assay pH	7.6

Assay was done in total volume of 3 ml. All reagents were mixed before addition to a portion of sample to initiate the assay. The assay was incubated at 27°C for 45 min and read at 340 nm. The assay is linear over the range 0-400 n mol/assay and 1 mol of glycerol in the assay gives a change in extinction of 2.08×10^6. Pyruvate and ADP interfered with this assay. The contribution due to pyruvate and ADP was estimated in parallel assays lacking glycerokinase.

8.4. Estimation of glucose

Glucose was assayed using the Boehringer 'GOD-Perid' method which is based on the method of Werner, Rey and Wielinger (1970).

The assay medium consisted of:

Phosphate buffer	90 mM, pH 7.0
2, 2' azino-di-(3 ethylbenzthiazoline-6-sulphonate)	0.9 mg/ml
Glucose oxidase	160 µg/ml
Peroxidase	18 µg/ml

obtained as a single lyophilised reagent.

The assay was done in a total volume of 5 ml. The assay was initiated by addition of reagent (at 27°C) to a portion of sample, incubated for 30 min at 27°C, and read at 660 nm. The assay was linear over the range 0-600 n mol/assay and 1 mol glucose in the assay gives an extinction of 1.57×10^6.

8.5. 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 total volume of 3 ml. All reagents were mixed before addition to a portion of sample to initiate the assay. The assay was incubated for 90 min at 27°C and read at 340 nm. The assay was linear over the range 0-400 n mol/assay and 1 mol acetate in the assay gives an extinction of 2.08×10^6.

8.6. Estimation of L-α-glycerophosphate

L-α-glycerophosphate was estimated by a modification of the method of Hohorst (1963).

The composition of the assay was:

Glycine buffer	190 mM, pH 9.8
Magnesium chloride	1.8 mM
Hydrazine sulphate	1.7 mM
NAD	0.7 mM
Glycerol-1-phosphate dehydrogenase	33 μg/ml

The assay was done in a total volume of 3 ml. All reagents with the exception of the enzyme were mixed before addition to a portion of sample. The assay was initiated by addition of the enzyme, incubated for 80 min at 27°C and read at 340 nm. The assay is linear over the range 0-400 n mol/assay and 1 mol L-α-glycerophosphate gives an extinction of 2.08×10^6.

8.7. Estimation of carbon dioxide evolution and oxygen consumption

Carbon dioxide evolution and oxygen consumption were estimated as described by Hamilton and Holms (1970).

9. Measurement of Intracellular Metabolites

9.1. General considerations

Estimations of intracellular metabolite concentration were carried out in samples taken from the culture. Assays were carried out for fructose-1, 6-diphosphate (FDP), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P) and adenosine triphosphate (ATP). All were enzymic

assays adapted from the methods of Slein (1963) and Racker (1963).

The assays were linked to the production of NADPH which was measured using a fluorescence spectrophotometer.

9.2. Treatment of samples

The above metabolites were extracted from cells with PCA as described in Section 8.2. Sampling and dilution in PCA took more than 1 but less than 3 s. 70% of the metabolites were extracted within one minute of addition to PCA and extraction was complete by 10 min. All were stable in 0.6 M PCA at 0°C for at least 26 min. The supernates were stored at 0°C and assayed the same day. The metabolites were stable in the neutralised extract at 0°C for up to 3 h.

A portion of culture was filtered through Sartorius membrane filters (25 mm diameter, 0.22 μpore size) (Sartorius-Membranfilter GmbH, Göttingen, W. Germany) and the filtrate used to determine the extracellular concentration of metabolites.

9.3. Assay of intracellular metabolites

9.3.1. Assay of G6P

The composition of the assay was:

Tris buffer	70 mM, pH 8.0
Magnesium chloride	1 mM
Ethylenediaminetetra-acetic acid	0.19 mM
Bovine serum albumin	0.07 mg/ml
NADP	0.05 mM
Glucose-6-phosphate dehydrogenase (G6PDH)	0.25 μg/ml

and 0.5 ml of neutralised cell extract to a final volume of 2 ml. All reagents with the exception of the enzyme were mixed and incubated with cell extract for 5 min at 27.5°C before the initial fluorescence was determined. The enzyme was then added, the assay incubated in the cuvette for 25 min at 27.5°C and the increase in fluorescence determined. A parallel sample without enzyme gives a measure of the increase in blank fluorescence under the conditions of the assay. The level of G6P in the sample was estimated from the difference between the increase of fluorescence in the presence and absence of G6PDH.

9.3.2. Assay of F6P

The composition of the assay was as in Section 9.3.1. with the addition of 5 μg/ml phosphoglucose isomerase (PGI). F6P was estimated from the difference between the increase of fluorescence in the presence and absence of PGI.

9.3.3. Assay of FDP

The composition of the assay was as in Section 9.3.2. with the addition of 5 μg/ml fructose-1, 6-diphosphatase (FDPase). FDP was estimated from the difference between the increase in fluorescence in the presence and absence of FDPase.

9.3.4. Assay of ATP

The composition of the assay was as in Section 9.3.1. with the addition of 2.5 μg/ml hexokinase and 0.02 mM glucose. ATP was estimated from the difference between the increase in fluorescence in the presence and absence of hexokinase. 0.02 mM glucose was included in the assay in the absence of hexokinase to allow for the increase in fluorescence due to glucose: NADP oxidoreductase

activity in the G6PDH preparation. None of the enzymes used contributed significantly to the blank fluorescence. All assays were done in duplicate.

9.4. Apparatus

The fluorescence of NADPH was determined using a Hitachi Perkin-Elmer MPF-2A fluorescence spectrophotometer (Perkin-Elmer Ltd., Beaconsfield, England), thermostatted at 27.5°C and fitted with a linear back-off facility (10 fold full scale deflection). The machine was operated in the ratio mode with settings as follows:

	Wavelength	Slit width	Filter	
			Type	Transmission wavelength
Emmision	465 nm	23 nm	430 nm	430 nm
Excitation	340 nm	30 nm	UVD 25	250-400 nm

Sensitivity at 5 and sample 100% adjust at lowest setting. All assays were carried out in 10 mm rectangular silica cells of low fluorescence as use of vials or test-tubes of glass or plastic led to irreproducible increases in the blank fluorescence. Cells were maintained at 27.5°C in a thermostatted cell holder. The difficulties inherent in the high fluorescence blanks of cell extracts due to FAD and FMN (Lowry et al, 1971) were reduced by use of the 10 fold full scale deflection back-off facility. The machine was calibrated using standard solutions of G6P (mono-sodium salt) or FDP. The assay was linear over the range 0-2.0 n mol/assay. The sensitivity was approximately 2.3 n mol G6P or FDP per 100 fluorescence units (full scale deflection).

9.5. Calculation of intracellular concentration

The intracellular concentration of metabolites was calculated using the relations:

196 μg dry wt. cells/ml culture is equivalent to a culture turbidity of 1.0 at 420 nm (Holms and Bennett, 1971)

and cells containing 2.7 ml intracellular water per g dry wt. (Winkler and Wilson, 1966).

10. Measurement of Glycerokinase Activity

10.1. Preparation and extraction of samples

Samples of exponentially growing cells were harvested at 11,750 g and 4°C for 10 min and resuspended in 5 ml ice-cold tris buffer (20 mM, pH 7.5). A portion was diluted in 40 mM phosphate buffer, pH 7.0, and used for estimation of turbidity.

4.5 ml of the cell suspension was transferred to a small glass vial and subjected to ultrasonic disruption at 0°C using a Soniprobe (Dawe Instruments, London, England) at 3.4 A for 2 min. The disruption time of 2 min consisted of 4 x 0.5 min periods of ultrasonic vibration with 0.5 min intervals to ensure the suspension remained chilled. The experimental set up has been described (Holms & Bennett, 1971).

The extract was centrifuged at 27,000 g and 4°C for 30 min and the supernate stored on ice until assayed the same day.

10.2. Assay of glycerokinase activity

10.2.1. Assay I: This consisted of a spectrophotometric assay for glycerokinase (EC2.7.4.30) adapted from the Boehringer 'neutral fat' method for estimation of glycerol which is based on the method of Eggstein and Kreutz (1966).

250 µl of cell extract were added, using an Oxford pipette (Boehringer, London) to an assay which contained 249 µ mol triethanolamine pH 7.6, 9.9 µ mol magnesium sulphate, 0.6 µ mol NADH, 3.3 µ mol ATP, 0.39 µ mol PEP, 19.8 µg lactate dehydrogenase, 9.9 µg pyruvate kinase and 19.8 µ mol glycerol in a final assay volume of 3 ml. This was placed in the reference beam of an SP800 double beam spectrophotometer maintained at 27°C. Allowance was made for NADH oxidase activity by placing a duplicate assay, with glycerol replaced by water, in the sample beam of the spectrophotometer. The differential rate of oxidation of NADH was measured by extinction at 340 nm. The output of the spectrophotometer was traced on a Servoscribe potentiometric recorder from which the differential rate of change of NADH concentration could be measured. The results were expressed as enzyme units/ml culture. 1 enzyme unit represents the oxidation of 1 mol of nucleotide/min at 27°C under the conditions of the assay.

The specific activity is enzyme units/g cellular dry wt.

10.2.2. Assay II: Glycerokinase was assayed radiochemically by a modification of the method of Thorner and Paulus (1973).

150 µl of cell extract was added, using a Finn pipette (Buckley Membranes Ltd., Amersham, England) to an

assay containing 12.5 μmol triethanolamine pH 7.0, 0.5 μmol magnesium chloride, 0.5 mg gelatine, 0.5 μmol ATP and 1.0 μmol radiolabelled glycerol (0.1 mCi/m mol glycerol-(U)-^{14}C or 1.2 mCi/m mol glycerol-1(3)-^{3}H) in a final volume of 0.5 ml and incubated for 10 min at 25°C. The reaction was terminated by addition of ice-cold 2 M glycerol (0.5 ml), and duplicate 50 μl aliquots were spotted on discs (2.3 cm diameter) of DEAE-cellulose filter paper (Whatman, DE-81)(W. & R. Balston Ltd., England). After at least one minute, papers were immersed in approximately 500 ml of 0.1 M glycerol and washed for 30 min with two more changes of 0.1 M glycerol. Radiolabelled glycerophosphate is retained on the filter papers whereas free glycerol is lost by dilution. Papers were transferred to scintillation vials and counted by liquid scintillation spectrometry. Results of parallel assays carried out in the absence of ATP were used as blanks. The results were expressed as d.p.m. retained/min. The retention of label was linear with respect to time of incubation and volume of extract.

11. Measurement of Glucose Phosphotransferase Activity

Glucose phosphotransferase activity was measured by the method of Clark (1974).

11.1. Preparation of samples

Samples containing about 4 mg dry wt. of exponentially growing cells were harvested at 11,750 g and 4°C for 10 min and resuspended in 11 ml ice-cold buffer (0.2 M sodium dihydrogen phosphate pH 7.2, 2mM magnesium chloride). A portion was diluted in 40 mM phosphate buffer pH 7.0 and used for estimation of turbidity. 100 µl of 4 M benzene in ethanol was added drop-wise to 10 ml of cell suspension during vigorous mixing on a Whirlimixer (Fisons Scientific Apparatus, Loughborough, England). Mixing was continued for 70 s after which benzene was removed from solution by aspiration for 20 min on ice with oxygen-free nitrogen.

11.2. Assay of glucose phosphotransferase system

0.5 ml of benzene treated cell suspension was added, by Oxford pipette, to an assay which contained 150 µ mol sodium dihydrogen phosphate pH 7.2, 1.5 µ mol magnesium chloride, 12 µ mol PEP, 0.63 µ mol NADP, 12 µg glucose-6-phosphate dehydrogenase and 10 µ mol glucose in a final volume of 1.5 ml. The rate of production of NADPH was measured by extinction at 340 nm in a SP800 double beam spectrophotometer at 27°C against 0.5 ml benzene treated cell suspension and 1 ml buffer (0.2 M NaH_2PO_4 pH 7.2, 2 mM $MgCl_2$) as blank. The output from the spectrophotometer was traced on a Servoscribe potentiometric recorder from which the rate of NADPH production could be measured. The results were expressed as enzyme units/g cellular dry wt. where 1 enzyme unit represents the production of 1 µ mol of nucleotide/min at 27°C under the conditions of the assay.

12. Fractionation of Radiolabelled Cells into Protein, Nucleic Acid and Lipid

The factionation procedure was a modification of the method of Roberts, et al. (1957).

A 40 ml sample of culture containing more than 2.5 mg and less than 8 mg dry wt. cells was acidified with ice-cold PCA (10 ml, 3 M), to give a final concentration of 0.6 M, and left on ice for 10 min. A portion was filtered by millipore filtration, washed with ice-cold water (5 ml) and used to determine the total radioactivity in acid insoluble material.

A 30 ml portion was centrifuged at 11,750 g and 4°C for 15 min the precipitate treated as follows. The suspensions were centrifuged between each step under the same conditions as above.

1) Washed (extracted and the supernate discarded) with ice-cold trichloracetic acid (TCA)(10 ml, 0.3 M).

2) Extracted with 13 M ethanol (4 ml), for 30 min at 45°C.

3) Extracted with a solution (4 ml) containing ether (2 ml) and 13 M ethanol (2 ml) for 15 min at 45°C. The supernate from this and the preceding extraction were combined to give an alcohol-ether soluble fraction.

4) Washed with the alcohol-ether solution (4 ml) for 15 min at 45°C.

5) Extracted with 0.3 M TCA (4 ml) for 15 min in a boiling water bath. The supernate gives a hot TCA soluble fraction containing nucleic acid.

6) Washed with 0.3 M TCA (4 ml) for 15 min in a boiling water bath. The precipitate was solubilised with 1 M potassium hydroxide (18 h, room temperature) and is the major protein containing fraction.

The alcohol-ether soluble fraction was further extracted with ether (4 ml), phase separation being achieved by addition of water (6 ml). The resulting alcohol-water fraction was re-extracted with ether (6 ml) and the two ether fractions combined. This procedure yields an alcohol soluble, ether soluble fraction (containing lipid) and an alcohol soluble, ether insoluble fraction (alcohol soluble protein).

Aliquots of the nucleic acid, lipid and protein containing fractions were counted by liquid scintillation spectrometry.

13. Measurement of Permeation Rates

13.1. Entry rate

13.1.1. Estimation of entry rate of permeant

The rate of entry of permeant was estimated using a modification of the method of Sanno, Wilson and Lin (1968) which depends on turbidity changes induced by hyperosmotic solutions of permeant.

Exponentially growing cells were harvested by centrifugation at 11,750 g and 4°C for 10 min. The cells were resuspended in 40 mM phosphate buffer pH 7.0 to give a turbidity of about 2.0. This cell suspension was incubated at 27°C.

2.5 ml of the suspension was placed in a cuvette in a double beam spectrophotometer. A 1 ml disposable syringe (Becton, Dickinson and Co.Ltd., Drogheda, Ireland), connected to the cuvette via a short length of PTFE tubing (1 mm bore) (Polypenco, Welwyn City, England), was used for rapid addition of permeant solution from outside the machine, thus allowing continuous measurement of turbidity. 0.5 ml of a 5 M solution of the permeant was added to the cell suspension in less than 0.3 s. The narrow bore tubing and rate of addition of the permeant solution assured efficient mixing of the permeant and cell suspension.

The rate of change of turbidity was measured at 420 nm on a SP800 double beam spectrophotometer at 27°C against 0.5 ml 40 mM phosphate buffer, pH 7.0, and 2.5 ml cell suspension as blank. The output of the spectrophotometer was traced on a Servoscribe potentiometric recorder, with a chart speed of 2 mm/s. The turbidity changes were not corrected for changes in medium refractive index due to addition of permeant as these changes were expected to be small for the permeants used.

13.1.2. Calculation of rate constant for entry

The rate of entry of permeant has first order kinetics. A graph of the logarithm of $(E_t - E_\infty)$, where E_t is the turbidity at time t and E_∞ is the turbidity of 2.5 ml cell suspension diluted with 0.5 ml, 40 mM phosphate buffer pH 7.0, against time yields a straight line (Figure 3) from which k, the first order rate constant, can be derived.

Figure 3

DETERMINATION OF RATE CONSTANTS FOR PERMEATION

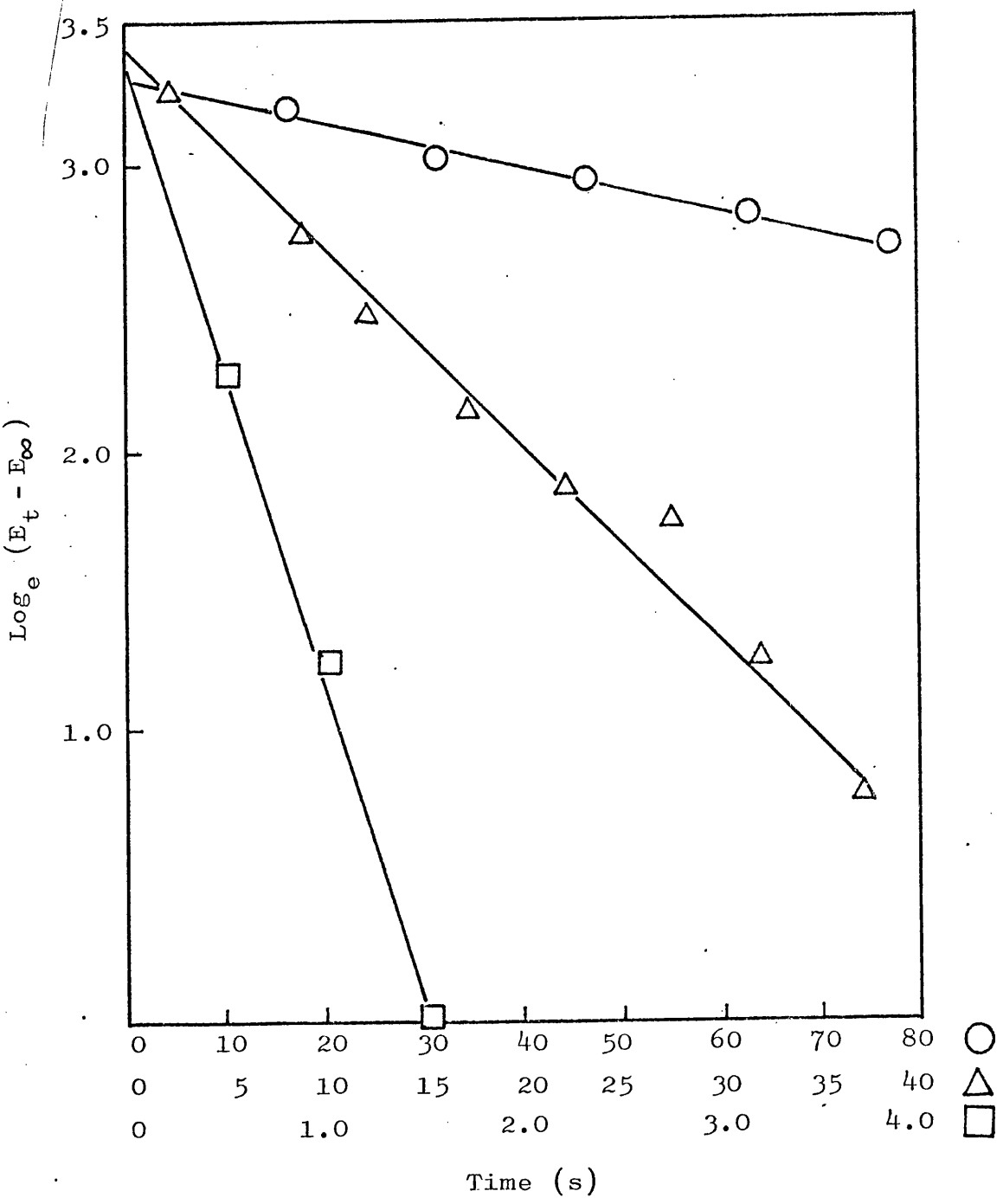
Cells of E.coli 15224 trained to glucose were harvested and resuspended in phosphate buffer (40 mM, pH 7.0) to a turbidity of about 2.0. The rate of change of turbidity on addition of a hyperosmotic solution of the permeant was measured as described in Section 13.1. The decrease in the turbidity with time is shown. The rate constant for permeation was calculated from the gradient of the graph.

Turbidity $(\log_e (E_t - E_\infty))$

○ on addition of erythritol

△ on addition of urea

□ on addition of ethylene glycol


3.5
3.0
2.0
1.0
$Log_e (E_t - E_\infty)$
0 10 20 30 40 50 60 70 80
0 5 10 15 20 25 30 35 40
0 1.0 2.0 3.0 4.0
Time (s)

Rate constants over a 300 fold range (0.0076-2.2 s^{-1}) (Figure 3) could be estimated by this method, the upper limit being determined by the reaction time of the equipment.

13.2. Exit rate

13.2.1. Estimation of exit rate of erythritol

The exit rate of erythritol was estimated radiochemically using a modification of the method of Haest et al. (1972).

A sample of exponentially growing cells containing 80 mg dry wt. was harvested at 11,750 g and 4°C for 10 min, washed with ice-cold CAP buffer, contrifuged and resuspended in 250 µl CAP buffer containing 2.5 µ mol erythritol-^{14}C (1.2 mCi/m mol).

The suspension was incubated for 10 min at 30°C when 25 µl aliquots were transferred to 100 ml conical flasks, containing 25 ml CAP buffer, in a shaking (110 strokes/min, 2.4 cm stroke) water bath at 30°C. 1 ml samples were removed at intervals (by Oxford pipette) and immediately filtered through Sartorious membrane filters (25 mm diameter, 0.22 µ pore size)(Sartorious-Membranfilter, GmbH., Göttingen, W. Germany). The filters were washed 3 times with 1 ml volumes of CAP buffer at 30°C containing 10 mM erythritol, immediately transferred to vials containing scintillation fluid (13 ml) and later counted by liquid scintillation spectrometry.

The apparatus has been described (Cook, 1971).

The amount of radioactivity trapped in intercellular water and by the filter was assumed to be constant and was estimated from a sample taken after equilibration of the

intra- and extracellular concentration of erythritol.

13.2.2. Calculation of rate constant for exit

The rate constant, k, for exit was calculated as in Section 13.1.2. where E_t is the radioactivity on the filter at time t and E_∞ is the radioactivity on the filter after equilibration of the intra- and extracellular concentration of erythritol.

13.3. Calculation of permeability constant

The permeability constant (P) in $cm.s^{-1}$ was calculated using the equation:

$$P(cm.s^{-1}) = k \times \frac{V}{FA} \times 2.7 \times 10^{-7}$$ (Stein, 1967)

where, V is the volume of the cell in μm^3

A is the surface area of the cell in μm^2

and F is the volume of cells associated with unit dry weight of cells

V and A were calculated assuming a cell of E.coli to be a cylinder, with hemispherical ends, of dimensions 1.2 x 3.0 μm (Stanier et al, 1971).

14. Measurement of Cell Volume available to Glycerol in the Presence and Absence of Glucose

The cell volume available to glycerol was estimated by a modification of the volume-distribution technique of Conway and Downey (1950).

A sample of exponentially growing cells containing 100 mg dry wt. was harvested at 11,750 g and $4^{\circ}C$ for 10 min, washed with ice-cold CAP buffer and centrifuged. The centrifuge tube was carefully dried and the pellet resuspended

in 100 µl ice-cold CAP buffer containing 20 µ mol glycerol-1(3)-^{3}H (0.01 mCi/m mol) or 20 µ mol glycerol-1(3)-^{3}H (0.01 mCi/m mol) and 20 µ mol glucose, to give a 17% (w/v) cell suspension.

The suspension was incubated for 1 min at 37°C and a 20 µl aliquot removed by glass-micro-pipette (H.J. Elliot Ltd., Treforest, England) and counted by liquid scintillation spectrometry. The suspension was then centrifuged at 11,750 g and 4°C for 10 min and a 20 µl aliquot of the supernate removed and counted as before. The difference between the amount of radioactivity in the supernate and the cell suspension gives a measure of the cell volume innaccesible to glycerol.

15. Liquid Scintillation Spectrometry

15.1. Liquid scintillation fluid

To 1 l toluene (A.R., British Drug Houses Ltd., Poole, England) was added 300 ml ethanol (fermented, absolute: Burroughs Wellcome & Co., London, England) and 5 g 2,5-diphenyloxazole (puriss; Koch-Light Laboratories, Colnbrook, England). 13 ml portions of this fluid were dispensed by zippette into glass vials (Packard Instruments, Downers Grove, U.S.A.) with disposable plastic caps (Metal Box Co.Ltd., Portslade, England).

Ethanol was present in the scintillation fluid to dissolve water associated with samples studied. 13 ml scintillation fluid dissolved up to 200 µl of aqueous solution (about 0.1 M and at counting temperature (7°C)).

Neither chemiluminescence nor phosphorescence was detected with this scintillation fluid under the conditions used.

15.2. Apparatus

Samples were counted in Philips Liquid Scintillation Analysers (Philips Scientific Equipment, Eindhoven, Netherlands) fitted with calculators to convert c.p.m. to absolute units.

Efficiency was calculated by the channels ratio technique (Wang and Willis, 1965). Wide range efficiency curves for toluene based scintillation fluid were prepared with chloroform as the polar quenching agent. For each curve, ten vials were prepared containing a known quantity of chloroform. The radioactive reference materials used were toluene-^{14}C (Packard Instrument Co.Ltd., Downers Grove, U.S.A.) and n-hexadecane-1,2-^{3}H (The Radiochemical Centre, Amersham, England). The counting efficiency was expressed as a quadratic function of the observed channels ratio. The constants of these quadratic equations were entered into the calculators in the Liquid Scintillation Analysers. The efficiency of counting ^{14}carbon was 84% and tritium 20-24%.

16. Statistical Methods

The parameters of lines or curves of 'best fit' were calculated by the method of least squares. Calculation of parameters and analysis of variance were carried out with the aid of a Canon-Canola 167P programmable, desk-top calculator (Nig-Banda, Banda House, London, England).

MATERIALS

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

Obtained from Boehringer Corporation, London, were:

NADP

PEP

Triethanolamine

GOD-Perid reagent

Neutral fat assay kit

Lactate dehydrogenase

Glucose-6-phosphate dehydrogenase

Phosphoglucose isomerase

Hexokinase

Glycerol-1-phosphate dehydrogenase

Fructose-1,6-diphosphatase

Obtained from Sigma, London, were:

ATP

BCIG

NADH

Cyclic AMP

G6P

F6P

FDP

NAD

DL- α - glycerophosphate

Ribose

Obtained from the Radiochemical Centre, Amersham, England, were:

Glycerol - (U) - ^{14}C

Glycerol - 1(3) - ^{3}H

Erythritol - (U) - ^{14}C

Hexadecane - 1,2 - ^{3}H (n)

Obtained from Oxoid, London, were:

Cooked meat medium

Nutrient agar medium

Nutrient broth medium

Obtained from Difco Laboratories, East Molesey, England, were:

Gelatine

Bacto-tryptone

Yeast extract

Formaldehyde and thiourea were obtained from May & Baker, Dagenham, England; 1,2,4 butanetriol and ethylmethane sulphonate from Eastman Organic Chemicals, Rochester, U.S.A; glycine from T.J. Sas & Son Ltd., London; bovine serum albumin from Armour Pharmaceutical Co.Ltd., Eastbourne, England; ethylene glycol from Thomson, Skinner & Hamilton Ltd., Glasgow; 2,5 - diphenyloxazole from Koch-Light Laboratories Ltd., Colnbrook, England; ethanol from Burroughs, Wellcome & Co., London; toluene - ^{14}C from Packard Instrument Co.Ltd., Downers Grove, U.S.A; oxygen free nitrogen from the British Oxygen Co.Ltd., Glasgow; tris-HCl from Mann Research Laboratories, New York, U.S.A; chloramphenicol from Parke, Davies & Co., Pontypool, England; mannose from Fluka, Buchs, Switzerland; triphenyltetrazolium chloride from PAL Chemicals Ltd., London; and FCCP was a gift from Dr. G. Lindsay, University of Glasgow.

RESULTS

1. Metabolism of E.coli 15224 Growing on Glycerol

Cells of E.coli 15224 trained to and growing on glycerol were used to provide standard estimates of the rate of metabolism of glycerol achieved under our defined growth conditions. The determinations of these standard estimates are shown in Figures 4 - 6 and are summarised in Figure 7.

1.1. Growth of E.coli 15224 on glycerol

Cells trained to glycerol and inoculated into minimal salts medium containing glycerol grew immediately. Growth was logarithmic over the entire period of growth and stopped due to exhaustion of glycerol (Figure 4). Cell material doubled every 50 min representing a specific growth rate of 0.80 h^{-1} (Figure 7a)

1.2. Substrate utilization during growth of E.coli 15224 on glycerol

E.coli 15224 utilized glycerol logarithmically with growth. The amount of glycerol used was proportional to the amount of cell material synthesised in the culture (Figure 5b). The molar growth yield of cells growing on glycerol was obtained from this line and gave a value of 47.6 g of cellular dry wt./mol substrate utilized (Figure 7a). The yield and the specific growth rate of the cells are related to the rate of substrate utilization by the equation:

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

Figure 4

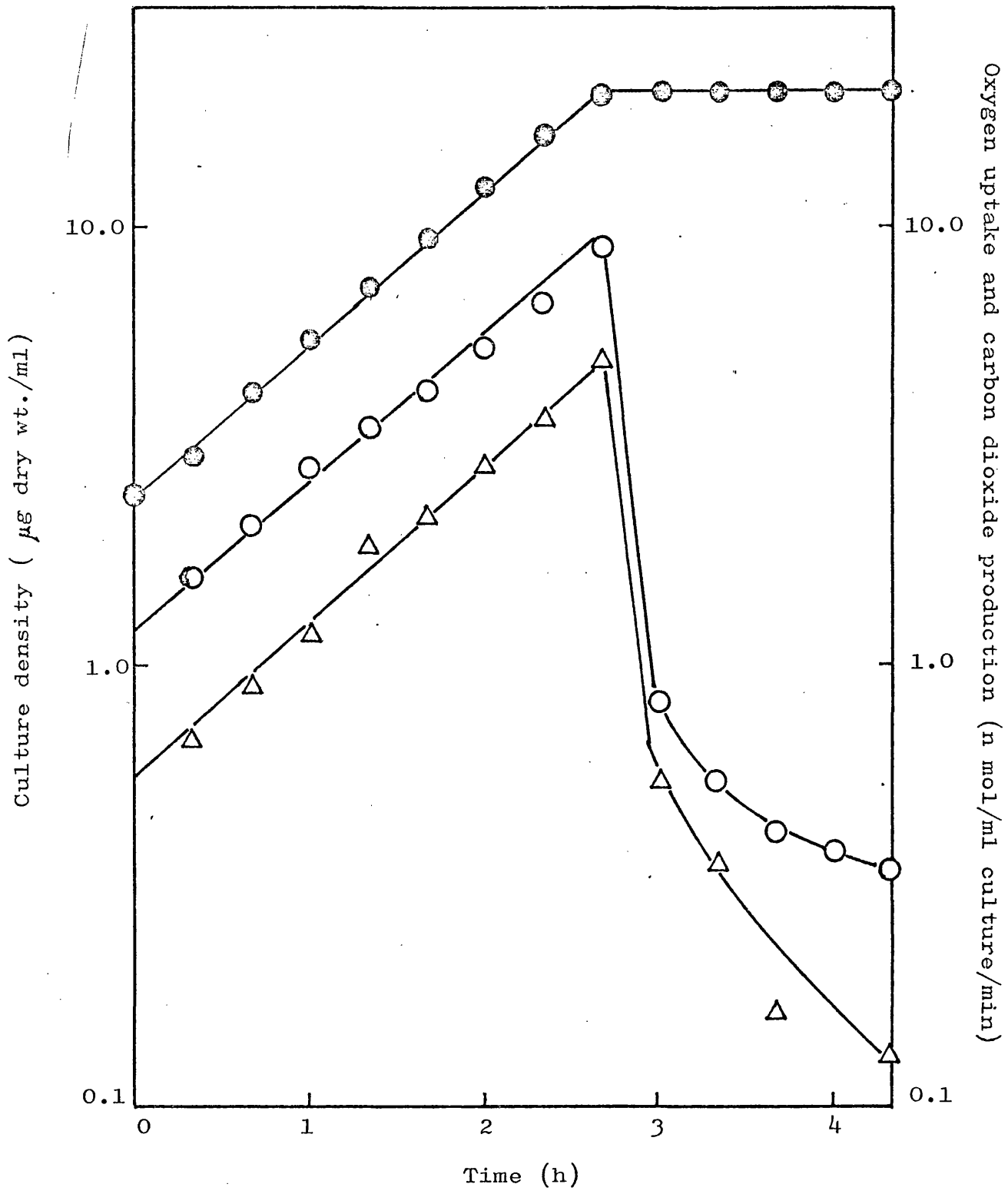
GROWTH AND GAS EXCHANGE OF E.coli 15224 ON GLYCEROL

Cells of E.coli 15224, trained to glycerol were washed and inoculated into minimal salts medium containing 4 mM glycerol. Measurements of growth, oxygen consumption and carbon dioxide production were made.

◉ growth

○ oxygen consumption

△ carbon dioxide production


Culture density (μg dry wt./ml)
Oxygen uptake and carbon dioxide production (n mol/ml culture/min)
10.0
1.0
0.1
0
1
2
3
4
Time (h)

giving a rate of glycerol utilization of 280 μmol/g dry wt./min (Figure 7a).

1.3. Oxygen uptake of E.coli 15224 during growth on glycerol

During growth of E.coli 15224 on glycerol under aerobic conditions the rate of oxygen consumption increased exponentially with growth of the culture decreasing 10 fold within 20 min of exhaustion of glycerol (Figure 4). The rate of oxygen uptake depended on the quantity of cell material present (Figure 5a) and the gradient of this graph provided an estimate of the rate of oxygen uptake. which was 432 μmol/g dry wt./min (Figure 7a).

1.4. Carbon dioxide production of E.coli 15224 during growth on glycerol

Carbon dioxide production increased exponentially with growth and decreased 10 fold within 20 min of exhaustion of glycerol (Figure 4). As with oxygen uptake the rate of carbon dioxide production depended on the quantity of cell material present in the culture (Figure 5a) and was 248 μmol/g dry wt./min (Figure 7a).

1.5. Yield of cells, oxygen uptake and $^{P}/_{O}$ ratio during growth of E.coli 15224 on glycerol

The yield of cells growing on glycerol and the rates of oxygen uptake and glycerol utilization are related to the yield of cells per mol oxygen consumed by the equation:

$$\text{Yield per mol oxygen} = \text{Yield per mol glycerol} \times \frac{\text{rate of glycerol utilization}}{\text{rate of oxygen consumption}}$$

giving a yield of 30.8 g dry wt. cellular material/mol oxygen consumed (Figure 7a).

Figure 5a

GAS EXCHANGE OF E.coli 15224 GROWING ON GLYCEROL

The data for oxygen uptake, carbon dioxide production and growth obtained from the culture described in Figure 4 were used.

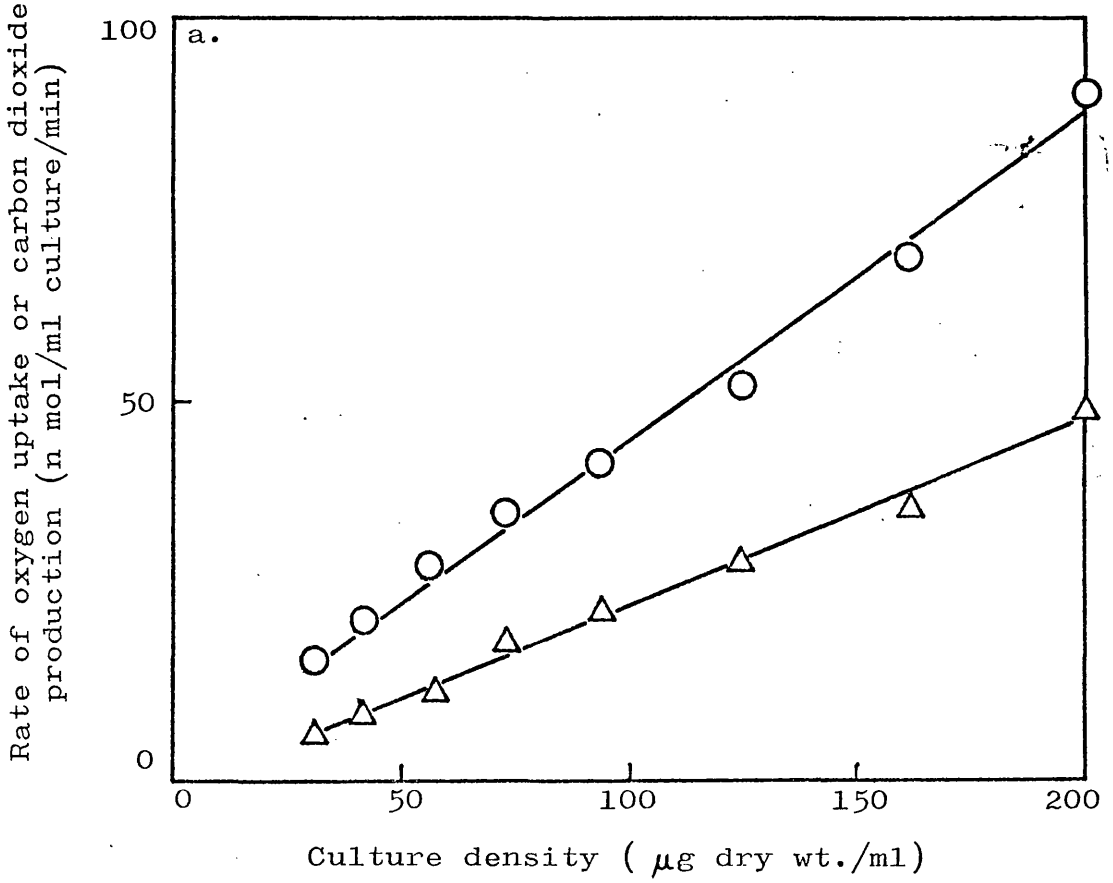
○ oxygen consumption

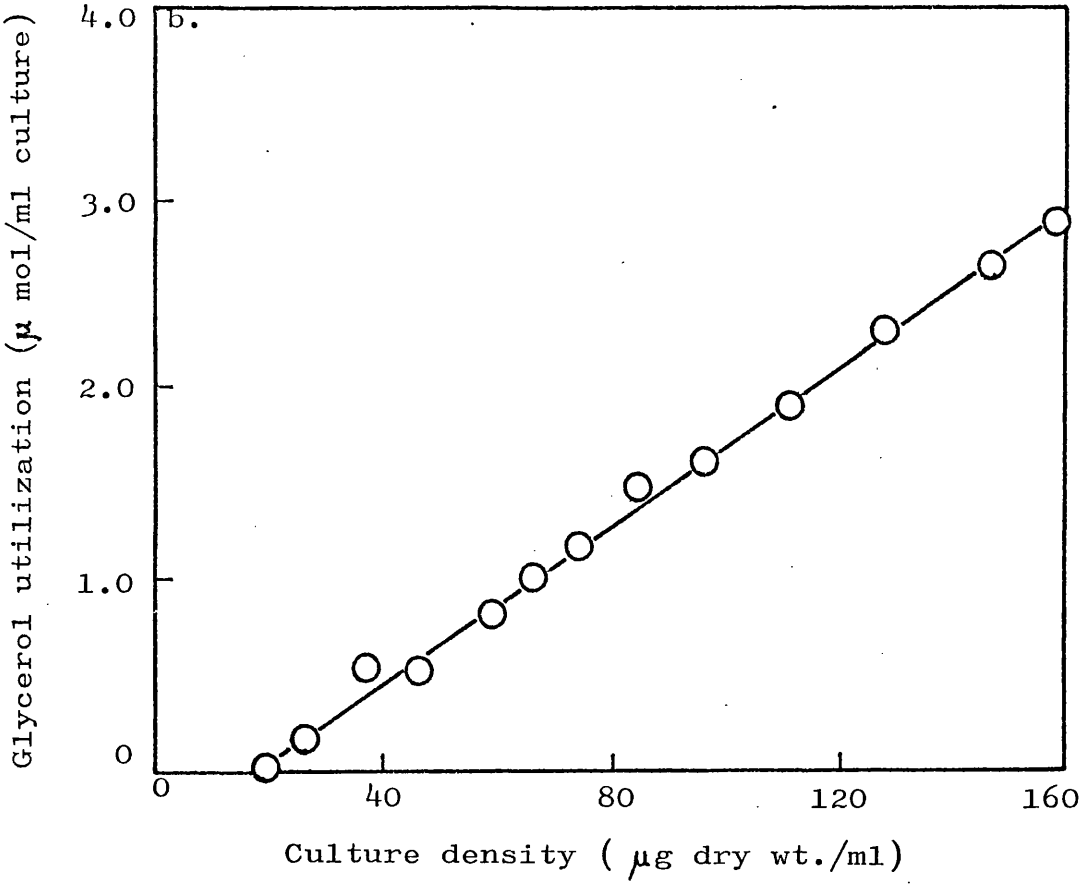
△ carbon dioxide production

Figure 5b

SUBSTRATE UTILIZATION DURING GROWTH OF E.coli 15224 ON GLYCEROL

The concentration of glycerol in the medium was measured during growth of the culture described in Figure 4. Glycerol utilization was plotted against culture density.


a.
Rate of oxygen uptake or carbon dioxide production (n mol/ml culture/min)
100
50
0
0
50
100
150
200
Culture density (μg dry wt./ml)


b.
Glycerol utilization (μ mol/ml culture)
4.0
3.0
2.0
1.0
0
0
40
80
120
160
Culture density (μg dry wt./ml)

The $^{P}/_{O}$ ratio was calculated assuming a yield of 10.5 g dry wt. cellular material/mol ATP (Bauchop & Elsden, 1960) which gives a value of 1.5 for growth on glycerol.

These findings are summarised in Figure 7a. Respiratory quotient (0.57) was calculated from these data.

1.6. Distribution of glycerol carbon during growth of E.coli 15224 on glycerol

The amount of glycerol incorporated into acid insoluble material, protein, nucleic acid and lipid was determined during growth of glycerol trained E.coli 15224 on glycerol-(U)-^{14}C. The amount of glycerol incorporated into each fraction depended on the amount of new cell material that was synthesised (Figure 6). The amount incorporated into each fraction (mol/g dry wt.) was estimated from the gradients of the lines. The results were expressed as a percentage of the amount of glycerol utilized per g dry wt. cellular material. 29% of the glycerol utilized was incorporated into protein, 14% into nucleic acid and 5% into lipid.

The proportion of glycerol carbon released as carbon dioxide (34%) was calculated from the data shown in Figure 4a. The proportion of glycerol incorporated into acid soluble material (19%) was calculated using the equation:

% glycerol incorporated into acid soluble material =

$$100 - (\%\text{ glycerol incorporated into acid insoluble material} + \%\text{ glycerol converted to carbon dioxide})$$

The results are summarised in Figure 7b.

Figure 6

DISTRIBUTION OF GLYCEROL CARBON DURING GROWTH OF E.coli 15224 ON GLYCEROL

Cells of E.coli 15224, trained to glycerol were inoculated into 400 ml minimal salts medium containing 3 mM glycerol -(U)-^{14}C (6.3 μCi/m mol). During growth of the culture samples were taken to measure the concentration of glycerol in the medium and the amount incorporated into acid insoluble material, protein, nucleic acid and lipid. Cell material was fractionated as described (Methods, Section 12). The amount of glycerol incorporated was plotted against the culture density.

Glycerol incorporation into:

○ acid insoluble material

△ protein

□ nucleic acid

◍ lipid

—— glycerol utilized

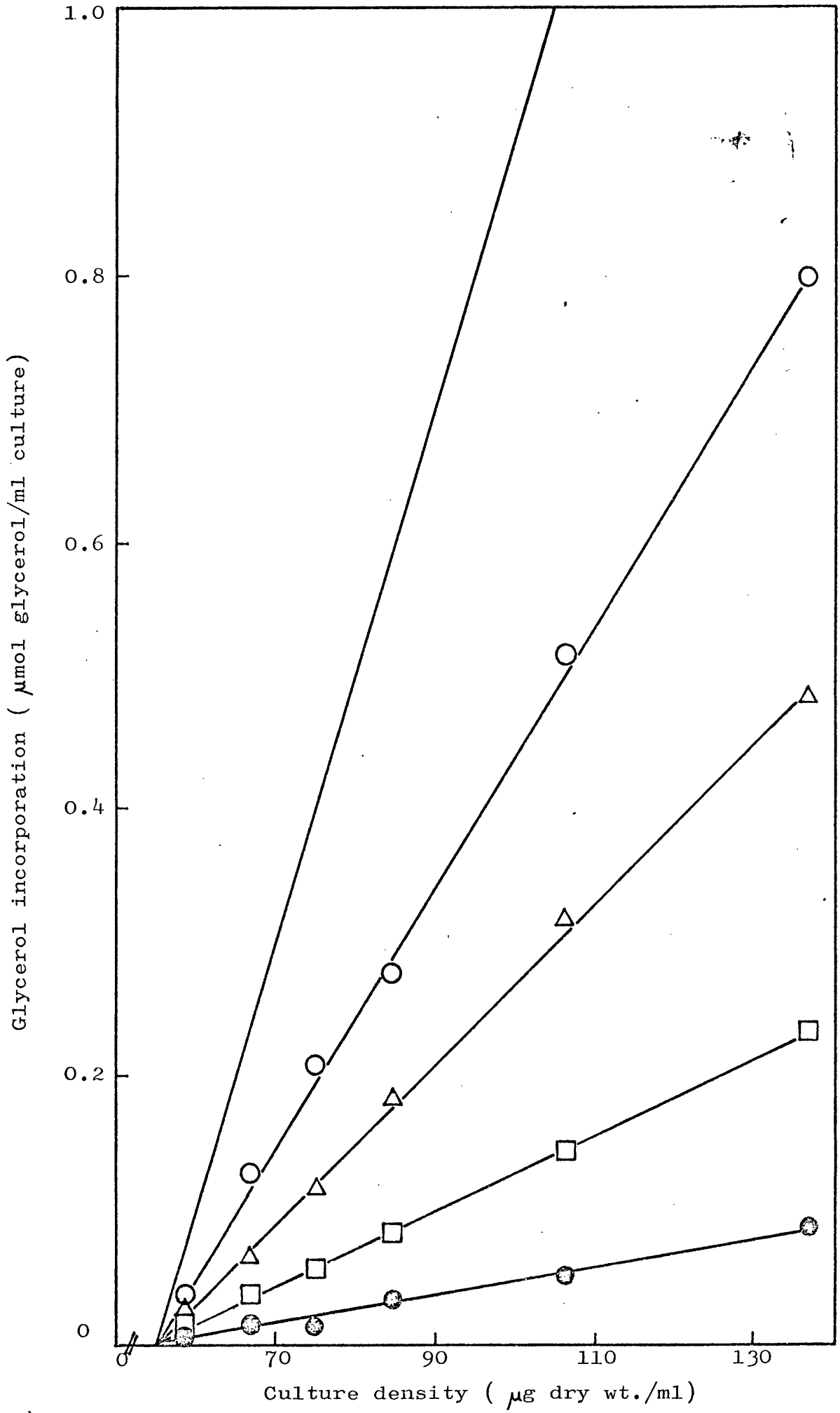

1.0
0.8
0.6
0.4
0.2
0
0
70
90
110
130
Glycerol incorporation (μmol glycerol/ml culture)
Culture density (μg dry wt./ml)

Figure 7

METABOLISM OF E.coli 15224 GROWING ON GLYCEROL

Figure 7a summarises data from Figures 4 and 5 for cells of E.coli 15224 growing on glycerol as sole source of carbon and energy and Figure 7b summarises data for the distribution of glycerol carbon during growth on glycerol. The $^{P}/_{O}$ ratio was calculated as described in text (Section 1.5.).

a.

Specific growth rate (h^{-1})	0.80
Rate of oxygen consumption (μmol/g dry wt./min)	432
Rate of carbon dioxide production (μmol/g dry wt./min)	248
Respiratory quotient	0.57
Yield on glycerol (g dry wt./mol glycerol)	47.6
Yield on oxygen (Y_{O_2}) (g dry wt./mol oxygen)	30.8
Rate of glycerol utilization (μmol/g dry wt./min)	280
$P/_O$ ratio	1.5

b.

Amount of glycerol incorporated (%)	
into acid insoluble material	47
into protein	29
into nucleic acid	14
into lipid	5
into acid soluble material	19
Amount of glycerol converted to carbon dioxide	34

2. Inhibition of Glycerokinase from E.coli by Fructose-1,6-Diphosphate (FDP)

Glycerokinase activity is inhibited allosterically by fructose-1,6-diphosphate (FDP). The inhibition of glycerokinase from E.coli by FDP was studied using assay II (Methods, Section 10.2.2.).

Crude extracts of E.coli were used and the activity of glycerokinase in the presence of various concentrations of FDP (0.25 - 10 mM) was measured.

The results were analysed on the basis of the subunit model of Monod, Wyman and Changeaux (1965). This model assumes that a multi-subunit protein can exist in two states, an active (R) state and an inactive (T) state, an allosteric inhibitor having affinity exclusively for the inactive (T) state. The velocity of the reaction in the presence of inhibitor is related to the inhibitor concentration by the equation:

$$\sqrt[n]{\frac{v_i}{V - v_i}} = \frac{1}{\sqrt[n]{L_\alpha}} \cdot \frac{1}{1 + \frac{i}{K_i^T}}$$

where v_i is the velocity in the presence of inhibitor

V is the maximum velocity at constant substrate concentration and zero inhibitor concentration.

n is the number of binding sites

L_α is the equilibrium constant between the R and T states at constant substrate (α) concentration.

i is the inhibitor concentration and

K_i^T is the dissociation constant between the inhibitor and the T state.

Inhibition by FDP of glycerokinase activity extracted from cells of E.coli 15224 exhibited slight sigmoidal characteristics with total inhibition never being achieved (Figure 8). The value of n when $\sqrt[n]{\frac{v_i}{V - v_i}}$ against $\frac{1}{i}$ (Figure 9) gives a straight line represents the number of FDP binding sites per molecule of glycerokinase. The value of n found was 4 binding sites/ molecule of glycerokinase.

A plot of $\sqrt[4]{\frac{v_i}{V - v_i}}$ versus $\frac{1}{i}$ (Figure 9) yields a straight line with the $\frac{1}{i}$ intercept equal to $-\frac{1}{K_i{}^T}$, the negative of the reciprocal of the dissociation constant for the inhibitor - enzyme (in the T conformation) complex. The dissociation constant for FDP and glycerokinase from E.coli 15224 was 0.34 mM. The maximum inhibition obtainable by FDP (86%) was calculated from the value of $\sqrt[4]{\frac{v_i}{V - v_i}}$ when $\frac{1}{i}$ equals zero. Glycerokinase extracted from E.coli 15224 glp R^c had the same characteristics (Figure 10) as glycerokinase from E.coli 15224. Glycerokinase extracted from E.coli 15224 glp R^cK^i yielded a $K_i{}^T$ for FDP of 1.5 mM and was maximally inhibited by 84% (Figure 10). This mutant glycerokinase is therefore 5 times less sensitive to inhibition by FDP than glycerokinase from E.coli 15224 although the same maximum inhibition can be obtained at high FDP concentrations.

Figure 8

INHIBITION OF GLYCEROKINASE FROM *E.coli* 15224 BY FRUCTOSE-1,6-DIPHOSPHATE (FDP).(I)

Cells of *E.coli* 15224 trained to and growing logarithmically on glycerol were extracted as described (Methods, Section 10). The activity of glycerokinase, in the presence of various concentrations of FDP, was measured by assay II (Methods, Section 10.2.2.) and the fractional velocity (the velocity of the reaction in the presence of FDP, v_i, divide by the velocity of the reaction in the absence of FDP, V) plotted against the inhibitor concentration, i.

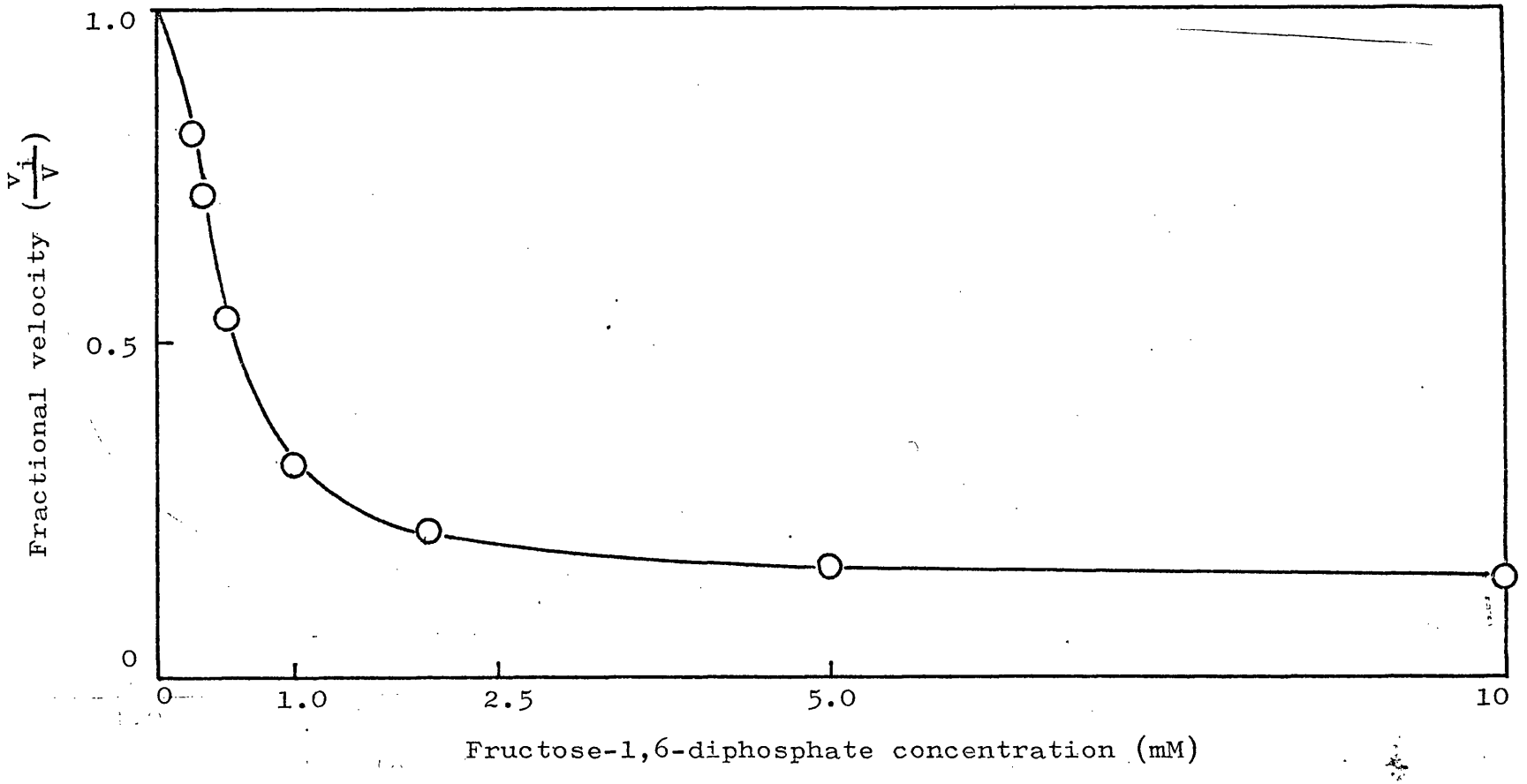

1.0
0.5
0
Fractional velocity ($\frac{v_i}{V}$)
0
1.0
2.5
5.0
10
Fructose-1,6-diphosphate concentration (mM)

Figure 9

INHIBITION OF GLYCEROKINASE FROM E.coli 15224 BY FDP (II)

The data obtained as described in Figure 8 were used to plot $\sqrt[n]{\frac{v_i}{V - v_i}}$ against $\frac{1}{i}$ for various values of n

□ $n = 1$

□ $n = 2$

△ $n = 3$

○ $n = 4$

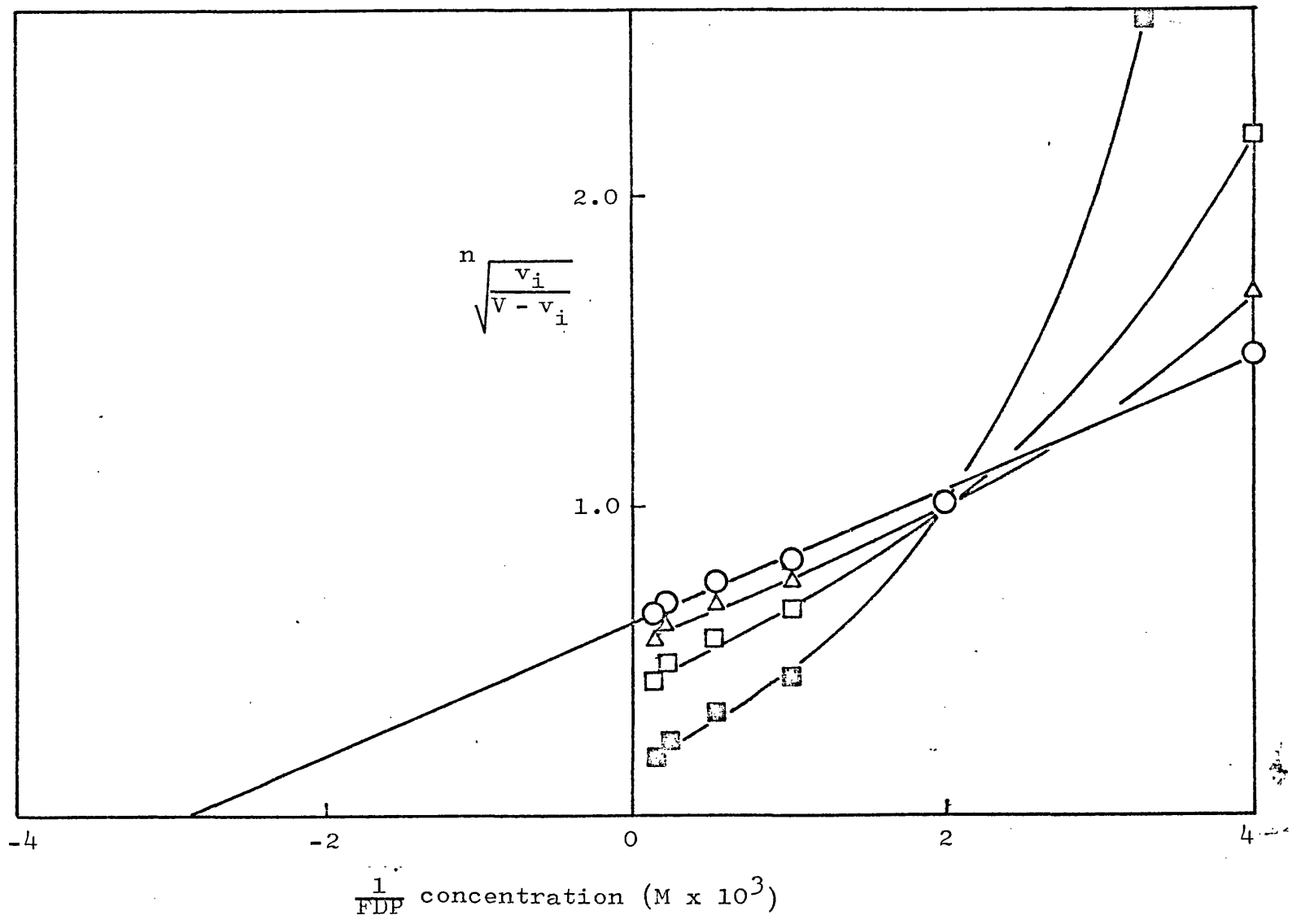

2.0
1.0
$\sqrt[n]{\frac{v_i}{V - v_i}}$
-4
-2
0
2
4
$\frac{1}{FDP}$ concentration (M x 10^3)

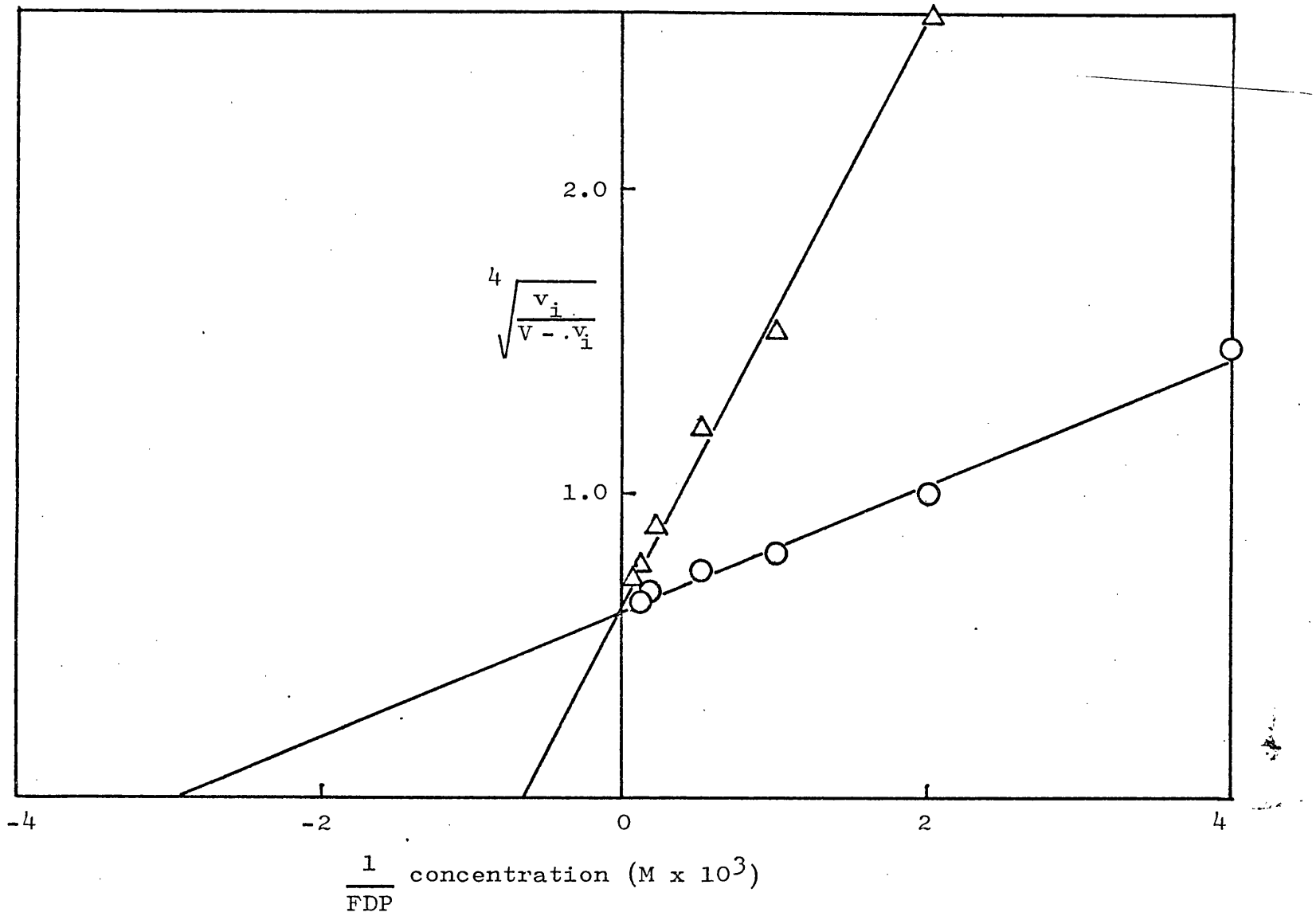
Figure 10

INHIBITION BY FDP OF GLYCEROKINASE FROM *E.coli* 15224 *glp* R^c AND 15224 *glp* R^cK^i

Cells of *E.coli* 15224 *glp* R^c and 15224 *glp* R^cK^i trained to and growing logarithmically on glycerol were extracted as described (Methods, Section 10). The activity of glycerokinase at various FDP concentrations was estimated by assay II and $\sqrt[4]{\frac{v_i}{V - v_i}}$ plotted against $\frac{1}{i}$.

○ glycerokinase activity from *E.coli* 15224 *glp* R^c

△ glycerokinase activity from *E.coli* 15224 *glp* R^cK^i


2.0
1.0
$\sqrt[4]{\frac{v_i}{V - v_i}}$
-4
-2
0
2
4
$\frac{1}{FDP}$ concentration (M x 10^3)

3. Intracellular FDP Concentration and Inhibition of Glycerokinase in Cells of *E.coli* Utilizing Glycerol

The intracellular FDP concentration and the specific activity of glycerokinase were estimated in cells of *E.coli* having different rates of glycerol utilization (Figure 11). The FDP inhibited glycerokinase activity was calculated from the specific activity of glycerokinase and the inhibition expected from the observed intracellular FDP concentration. The inhibition due to FDP was obtained from previous results (Section 2).

The rate of glycerol utilization per unit of enzyme activity (Figure 11, column 4) varied widely (0.4 - 2.4 μmol/min/E.U.) and, although the intracellular FDP concentration (0.1 - 3.3 mM) and the glycerokinase activity (49 - 485 E.U./g dry wt.) also varied considerably, the rate of glycerol utilization per unit of FDP inhibited enzyme activity (Figure 11, last column) predicted from the intracellular FDP concentration was relatively constant (2.0 - 2.4).

Figure 11

GLYCEROL UTILIZATION AND FDP CONCENTRATION IN CELLS OF *E. coli* UTILIZING GLYCEROL

Cells of *E.coli* trained to glycerol or glucose were harvested, washed and inoculated into 800 ml minimal salts medium or 400 ml CAP medium containing 4 mM glycerol. Cells were inoculated into CAP medium to a turbidity of 0.4. The rate of glycerol utilization, the specific activity of glycerokinase and the intracellular concentration of FDP (Methods, Section 9) were measured. The FDP inhibited glycerokinase activity was calculated as described in text (Section 3).

* Minimal salts medium + 4 mM glycerol

† CAP medium + 4 mM glycerol

Cell type	Rate of glycerol utilization (μ mol/min/ g dry wt.)	Glycerokinase activity (E.U./g dry wt.)	Rate of utilization per E.U. (μ mol/min/ E.U.)	Intracellular FDP concentration (mM)	FDP inhibited glycerokinase activity (E.U./g dry wt.)	Rate per FDP inhibited E.U. (μ mol/min/ E.U.)
15224 glycerol trained *	294	485	0.6	1.07 ± 0.14	150	2.0
15224 glp R^cK^i glycerol trained †	250	207	1.2	3.3 ± 0.1	112	2.2
15224 glycerol trained †	198	485	0.4	2.4 ± 0.1	98	2.0
15224 glp R^c glucose trained †	115	49	2.4	0.1 ± 0.1	47	2.4

4. Diauxic Growth of *E.coli* on Glucose and Glycerol

Cells of *E.coli* 15224 trained to glucose and inoculated into minimal salts medium containing glucose and glycerol grew immediately (Figure 12). Growth was logarithmic and continued with a specific growth rate of 0.94 h^{-1} until glucose exhaustion. (Specific growth rate on glucose alone 0.90 h^{-1}). After a short (30 min) diauxic lag the cells grew on glycerol (specific growth rate 0.81 h^{-1}) until growth ceased due to exhaustion of glycerol.

Yields of 47 g dry wt./mol glycerol and 76 g dry wt./mol glucose (76.4 g dry wt./mol on glucose alone) were obtained indicating that no glycerol was used during growth on glucose.

Figure 12

DIAUXIC GROWTH OF E.coli 15224 ON GLUCOSE AND GLYCEROL

Cells of E.coli 15224 trained to glucose were inoculated into 800 ml minimal medium containing 2 mM glucose and 3 mM glycerol. Growth and substrate concentration were measured. Growth of the culture with time is shown. The presence of subtrate in the culture is indicated by the bars at the top of the diagram.

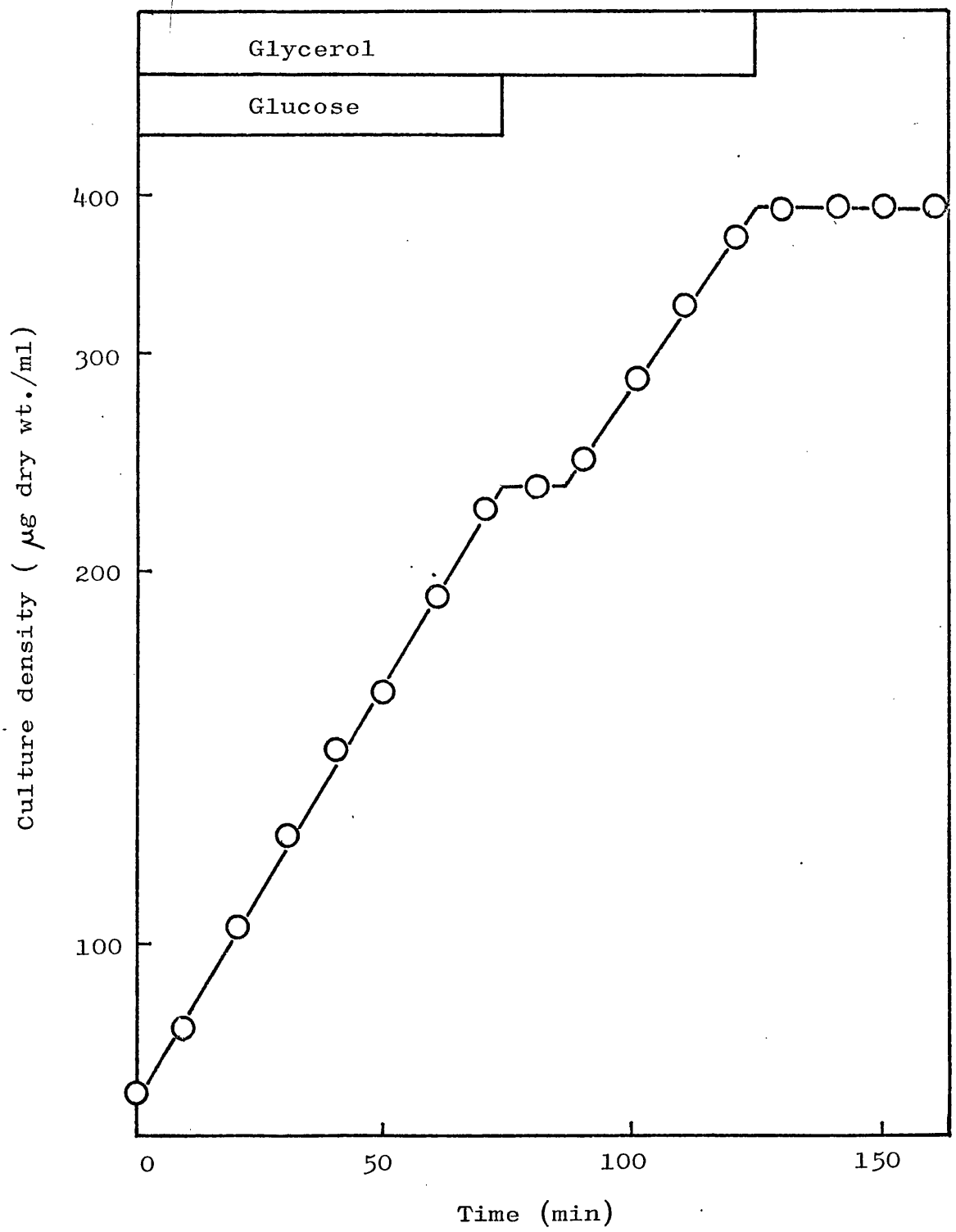

Glycerol
Glucose
400
300
200
100
Culture density (µg dry wt./ml)
0
50
100
150
Time (min)

Figure 13

GROWTH AND SUBSTRATE UTILIZATION ON GLUCOSE CHALLENGE TO *E.coli* 15224 GROWING ON GLYCEROL

Cells of *E.coli* 15224 trained to glycerol were inoculated into 2 flasks (A and B) containing 800 ml minimal salts medium and approximately 4 mM glycerol. 2 mM glucose was added to flask B after 90 min growth. Growth and substrate concentration were measured.

Growth:

— — flask A

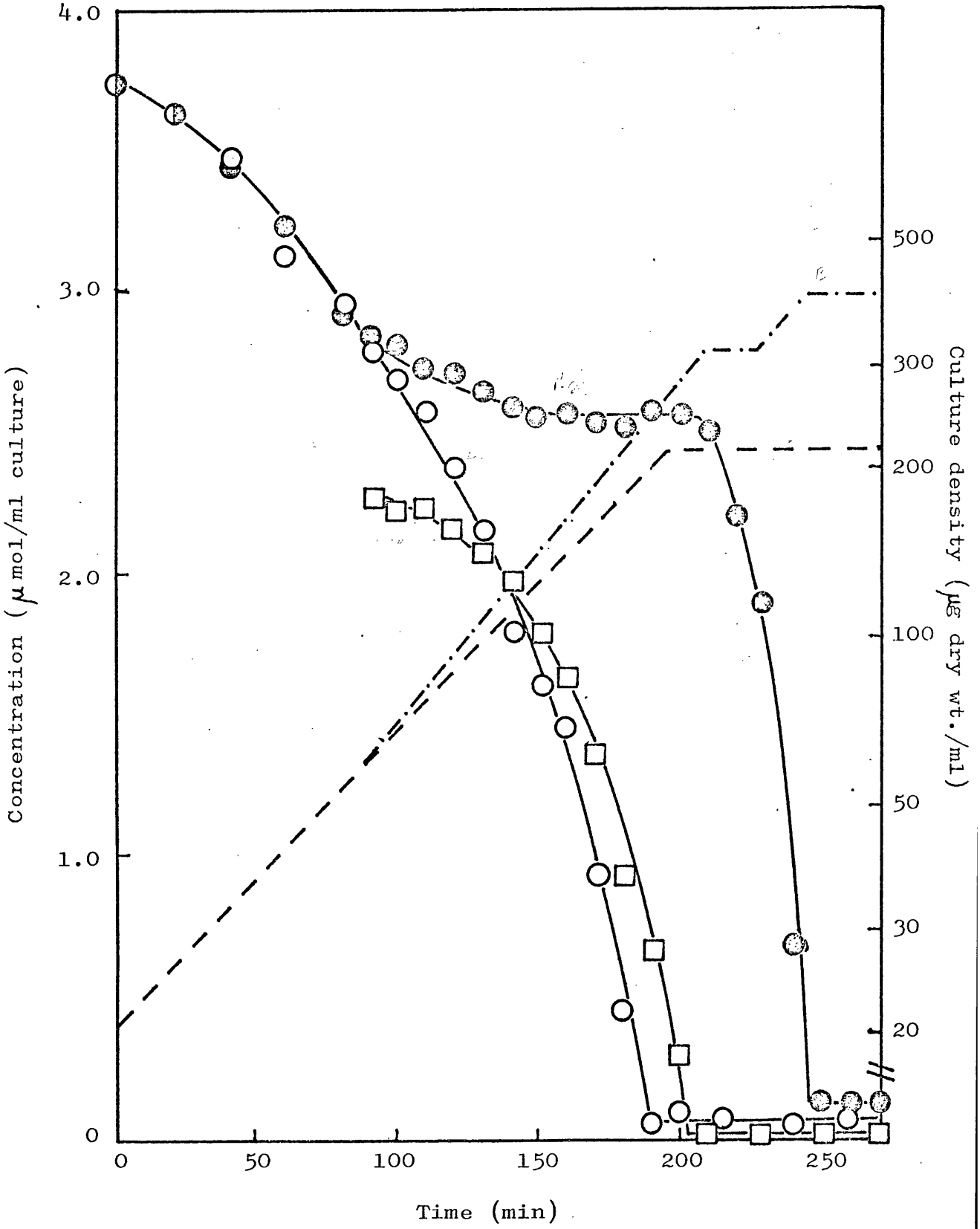
—·— flask B

Substrate concentration:

○ glycerol in flask A

◍ glycerol on glucose challenge to flask B

□ glucose in flask B


4.0
3.0
2.0
1.0
0
0
50
100
150
200
250
Time (min)
Concentration (μmol/ml culture)
500
300
200
100
50
30
20
Culture density (μg dry wt./ml)

5. Metabolism of Glycerol on Glucose Challenge to Cells of E.coli Utilizing Glycerol

The ability of glucose to prevent glycerol utilization was further investigated using cells which contained all the enzymes required for glycerol utilization.

5.1.1. Growth and substrate utilization on glucose challenge to cells of E.coli 15224 growing on glycerol

Cells of E.coli 15224 trained to glycerol were inoculated into minimal salts medium containing glycerol. After a short period (90 min) of growth glucose was added to the culture.

Growth was logarithmic after glucose challenge with a specific growth rate (0.86 h^{-1}) comparable with the specific growth rate on glucose alone (0.90 h^{-1}). The growth rate after glucose exhaustion (0.77 h^{-1}) was similar to the growth rate in the control flask (0.75 h^{-1}).

Substrate estimations made during growth (Figure 13) showed that glycerol utilization fell by 50% at the time of glucose challenge and continued for about 50 min, when it ceased altogether and did not start again until glucose was exhausted.

No pyruvate, acetate or L-α-glycerophosphate was detected in the culture after glucose challenge. Neither pH (5.5 - 7.1) nor the concentration of glucose (1.7 - 4.7 mM) affected the pattern of glycerol utilization on glucose challenge to E.coli 15224 growing on glycerol (data not shown).

5.1.2. Glycerokinase activity on glucose challenge to cells of E.coli 15224 growing on glycerol

During growth of glycerol trained E.coli 15224 on glycerol the level of glycerokinase activity in the culture increased exponentially with growth (Figure 14). The enzyme activity per ml culture depended on the amount of cell material present. The specific activity was 485 enzyme units/g dry wt. cellular material.

Synthesis of glycerokinase ceased almost immediately on glucose challenge to cells growing on glycerol (Figure 14). No loss of activity from the culture was detected for at least 100 min after glucose challenge.

5.1.3. Yield of cells and rate of glycerol utilization

The data for glycerol utilization and growth shown in Figure 13 were used to calculate the yield of cells on glycerol before glucose addition. The total amount of glycerol utilized depended on the amount of new cell material synthesised (Figure 15). The gradient of the line before glucose addition gave a yield of 49.2 g dry wt. cellular material/mol glycerol utilized. The data of Figure 13 were also used to calculate the yield of cells on glycerol after glucose exhaustion. The amount of glycerol utilized depended on the amount of new cell material synthesised after glucose exhaustion (Figure 15). The yield of cells/mol glycerol was 50.8 g dry wt. cellular material, almost identical to the yield before glucose addition.

The rate of glycerol utilization/g dry wt. was calculated from the yield of cells and the specific growth rate as before (Results, Section 1.2.). The rate of glycerol utilization per unit of glycerokinase

Figure 14

GLYCEROKINASE ACTIVITY ON GLUCOSE CHALLENGE TO E.coli 15224 GROWING ON GLYCEROL

Glycerokinase activity was measured during growth of the cultures described in Figure 13. Glycerokinase activity was estimated by assay I (Methods, Section 10.2.1.).

Glycerokinase activity:

○ flask A

◎ flask B

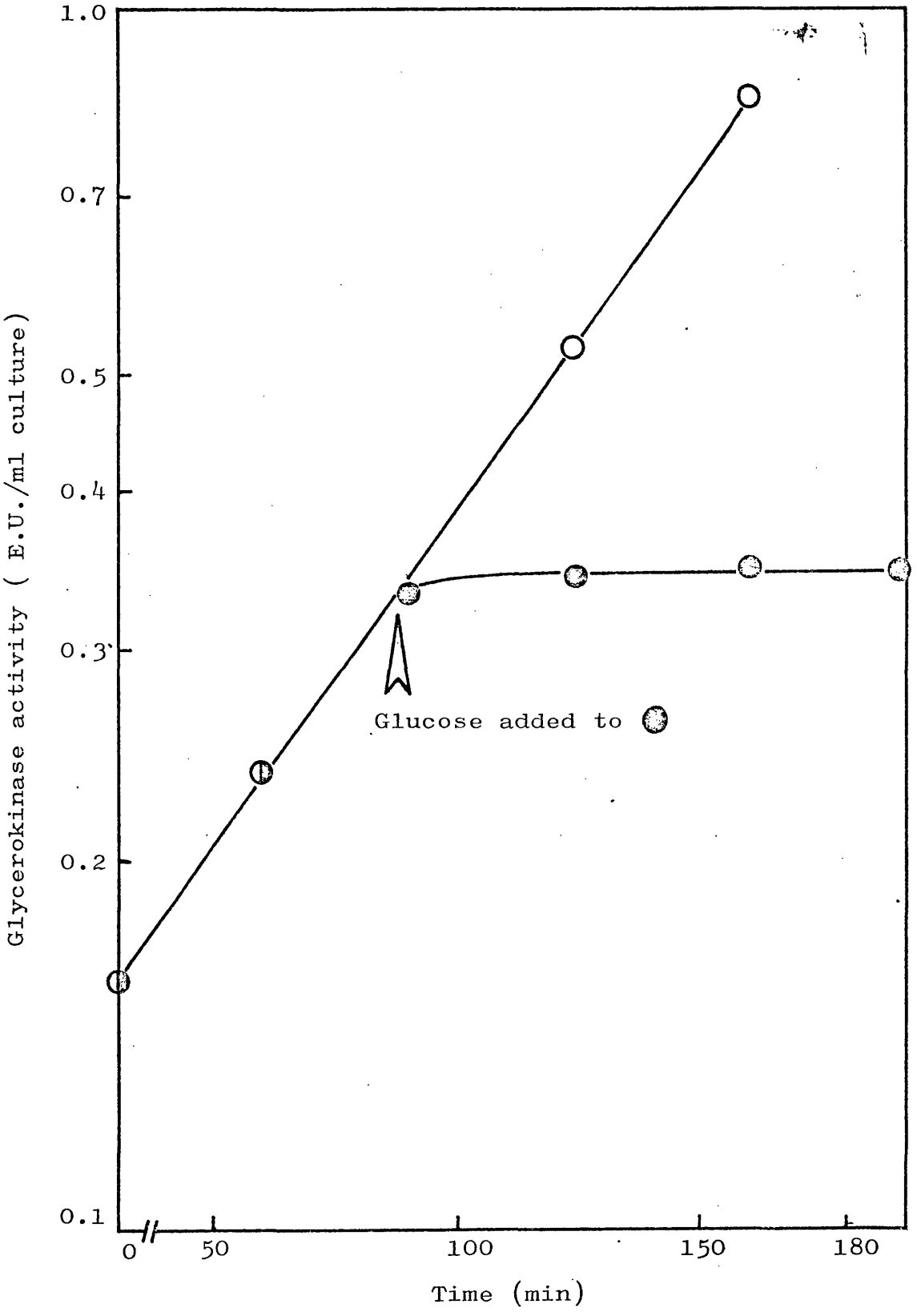

1.0
0.7
0.5
0.4
0.3
0.2
0.1
Glycerokinase activity (E.U./ml culture)
Glucose added to
0
50
100
150
180
Time (min)

Figure 15

SUBSTRATE UTILIZATION ON GLUCOSE CHALLENGE TO *E.coli* 15224 GROWING ON GLYCEROL

The data of Figure 13 were used to plot the glycerol concentration in flask B against culture density. The presence of glucose in the culture is indicated by the bar at the top of the diagram.

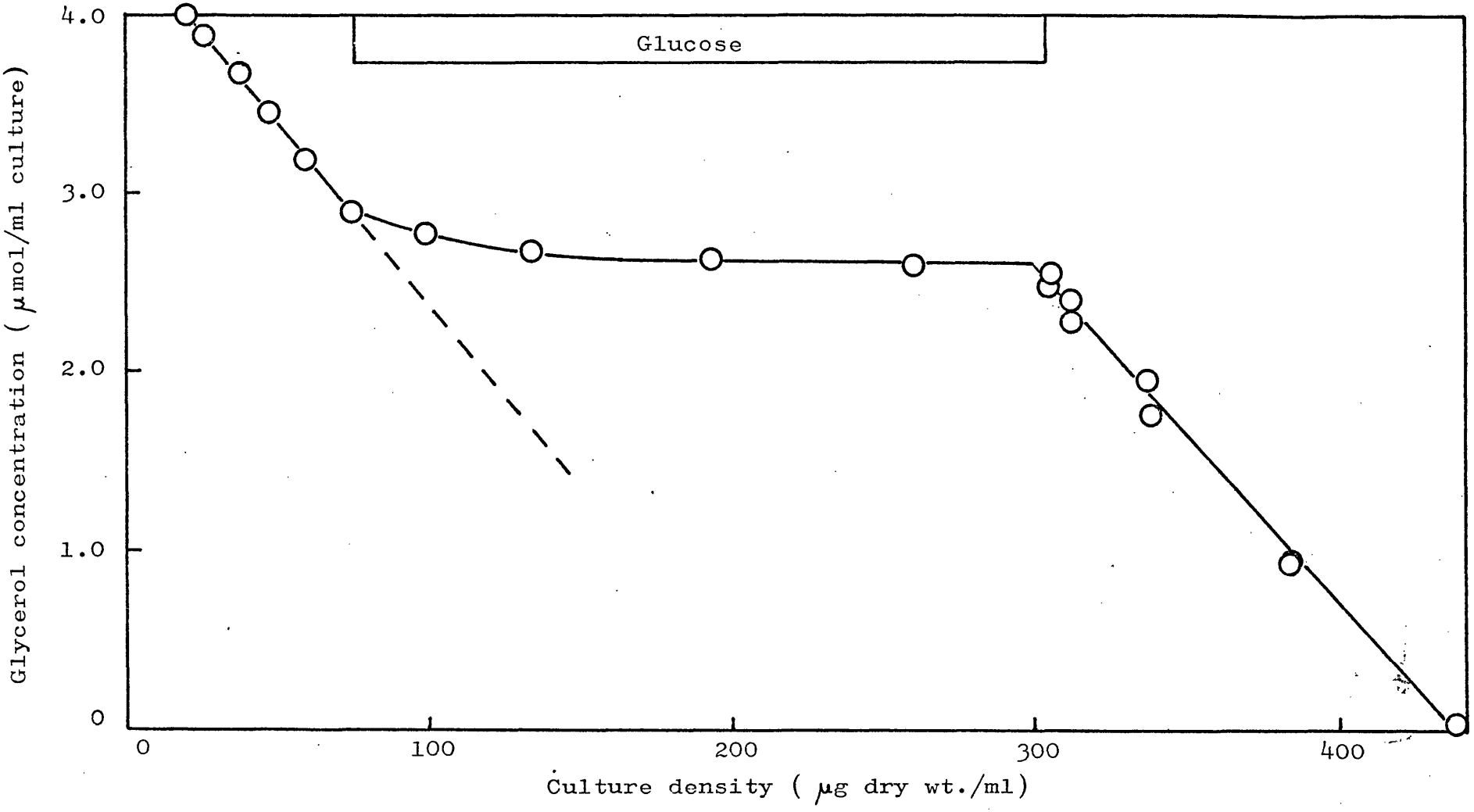

Glucose
4.0
3.0
2.0
1.0
0
0
100
200
300
400
Glycerol concentration (μmol/ml culture)
Culture density (μg dry wt./ml)

Figure 16

GROWTH AND METABOLISM OF *E.coli* 15224 BEFORE ADDITION AND AFTER EXHAUSTION OF GLUCOSE IN A CULTURE CHALLENGED WITH GLUCOSE DURING GROWTH ON GLYCEROL

Figure 16 summarises date from Figures 13 - 15 for growth on glycerol, before glucose addition and after glucose exhaustion.

Time	Yield (g dry wt./mol)	Specific growth rate (h^{-1})	Rate of glycerol utilization (u mol/min/ g dry wt.)	Specific activity of glycerokinase (E.U./g dry wt.)	Rate of utilization per enzyme unit
Before glucose addition	49.2	0.77	262	485	0.54
On exhaustion of glucose	50.8	0.75	246	113	2.18

activity was calculated from the rate of glycerol utilization/g dry wt. and the specific activity of glycerokinase (Enzyme units/g dry wt.). The results are summarised in Figure 16.

The specific growth rate (0.75 h^{-1}) and the rate of glycerol utilization per g dry wt. (246 μmol/min/g dry wt.) after glucose exhaustion were similar to the growth rate (0.77 h^{-1}) and rate of glycerol utilization (262 μmol/min/g dry wt.) before glucose addition. On exhaustion of glucose the rate of glycerol utilization/ unit of glycerokinase activity (2.18 μmol/min/E.U.) was 4 times greater than the rate of glycerol utilization/ enzyme unit before glucose addition (0.54 μmol/min/E.U.) due to the decrease in specific activity of glycerokinase during glucose challenge.

5.1.4. Distribution of glycerol carbon on glucose challenge to cells of E.coli 15224 growing on glycerol

The proportion of glycerol carbon incorporated into acid insoluble material, protein, nucleic acid and lipid was determined after addition of glucose and glycerol-(U)-^{14}C to cells of E.coli 15224 growing on glycerol.

The proportion of glycerol carbon incorporated into acid insoluble material was expressed as a percentage of the total amount of glycerol utilized after glucose challenge (Figure 17a). The amount of glycerol incorporated into protein, nucleic acid and lipid was expressed as a percentage of the amount of glycerol incorporated into acid insoluble material (Figure 17b). After glucose challenge glycerol carbon continued to enter acid insoluble material (approximately 58% incorporation) and to be

Figure 17

DISTRIBUTION OF GLYCEROL CARBON ON GLUCOSE CHALLENGE TO CELLS OF E.coli 15224 GROWING ON GLYCEROL

Cells of E.coli 15224 trained to glycerol were inoculated into 2 flasks containing 400 ml minimal salts medium and 4 mM glycerol. After 90 min growth 10 μCi glycerol-(U)-^{14}C (19.5 mCi/m mol) were added to one flask, which was used as a control, and 2 mM glucose and 10 μCi glycerol-(U)-^{14}C added to the 2nd flask.

Substrate utilization and the amount of glycerol incorporated into acid insoluble material, protein, nucleic acid and lipid were made. Cell material was fractionated as described (Methods, Section 12).

Figure 17a

% Glycerol Incorporated into Acid Insoluble Material

○ on glucose challenge

— — control

Figure 17b

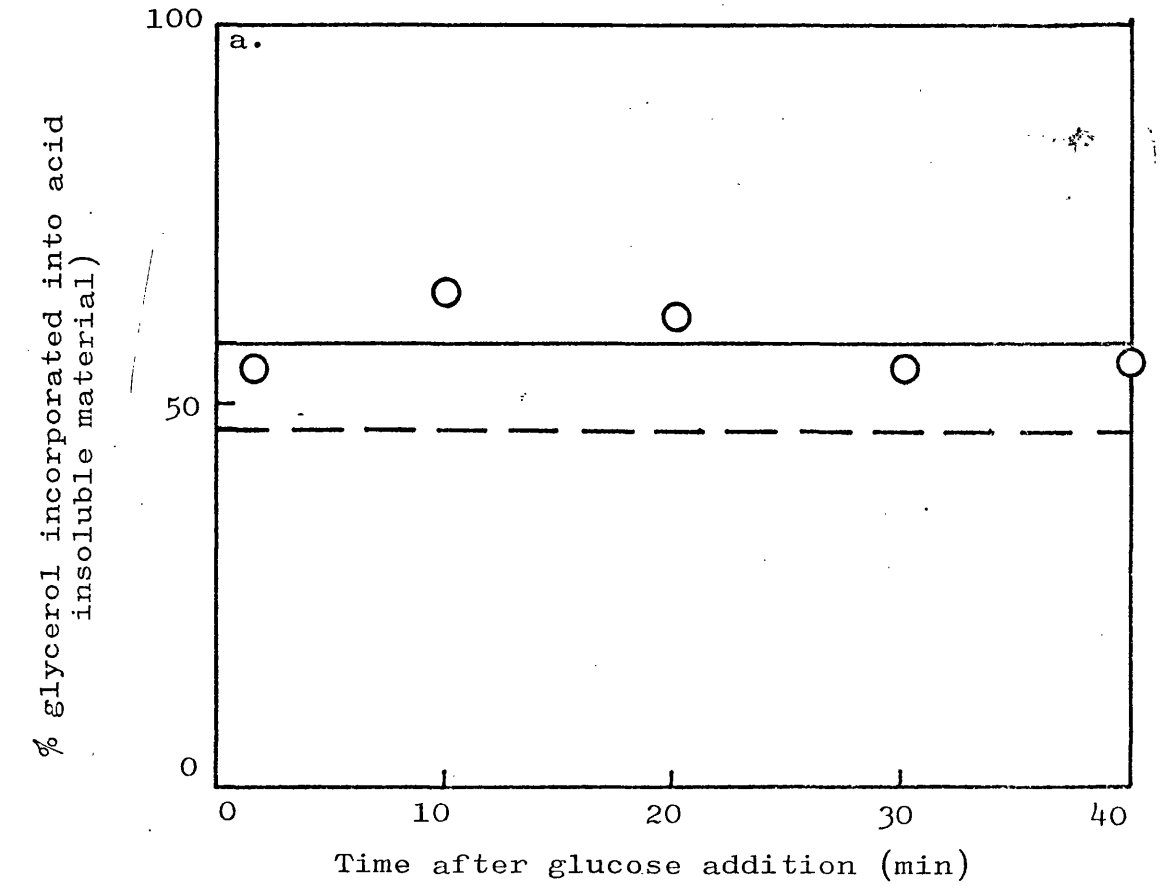
Amount of Glycerol Incorporated on Glucose Challenge

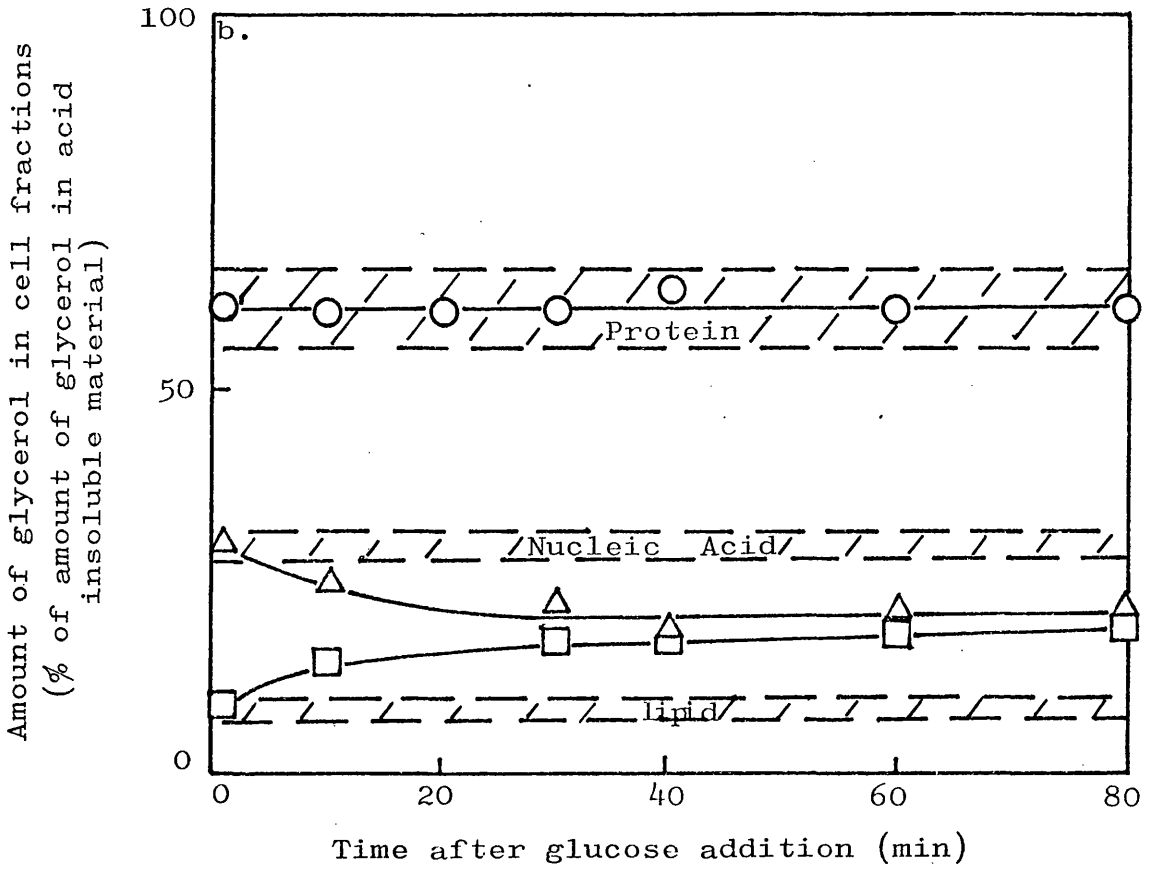
○ into protein

△ into nucleic acid

□ into lipid

The shaded areas show the range of values for the amount of glycerol incorporated into cell fractions in the control.


a.
100
50
0
% glycerol incorporated into acid insoluble material)
0
10
20
30
40
Time after glucose addition (min)


b.
100
50
0
Amount of glycerol in cell fractions (% of amount of glycerol in acid insoluble material)
Protein
Nucleic Acid
lipid
0
20
40
60
80
Time after glucose addition (min)

distributed among protein, nucleic acid and lipid with a relative increase (9 to 19% of glycerol incorporated into acid insoluble material) in the proportion entering lipid and a decrease (30 to 21%) in the proportion entering nucleic acid.

5.2. Rate of glycerol metabolism on glucose challenge to washed cell suspensions of E.coli 15224 glp R^c

Cells with the ability to metabolise glycerol under a variety of circumstances were obtained using a mutant of strain 15224 constitutive for the enzymes of the glycerophosphate (glp) regulon.

The ability of glucose to prevent glycerol metabolism was investigated in washed cell suspensions of cells trained to glycerol or glucose (Figure 18). Glucose trained cells of E.coli 15224 glp R^c utilized glycerol 0.48 times as fast as glycerol trained cells. Glucose inhibited glycerol utilization by only 50% in glycerol trained cells but totally prevented it in glucose trained cells. The ability of glucose to prevent glycerol utilization in glucose trained cells was not affected by the concentration of glycerol (1.7 - 11 mM)(data not shown).

Figure 18

EFFECT OF GLUCOSE ON GLYCEROL UTILIZATION IN *E.coli* 15224 glp R^c

Cells of *E.coli* 15224 glp R^c trained to glucose or glycerol were harvested, washed and inoculated into 200 ml CAP medium containing 3 mM glycerol or 3 mM glycerol and 3 mM glucose to give a turbidity of 0.4. Glycerol utilization was measured.

Cell type	Rate of glycerol utilization (μmol/g dry wt./min)	Rate of glycerol utilization in the presence of glucose	% inhibition
Glycerol trained	242	120	50
Glucose trained	115	0	100

6. Rate of Metabolism of L-α-Glycerophosphate in the Presence of Glucose

L-α-glycerophosphate (L-α-GP) is the product of the first reaction of glycerol metabolism. The effect of glucose on the metabolism of glycerol distal to the formation of L-α-GP could therefore be studied.

6.1. L-α-GP metabolism on glucose challenge to cells of E.coli 15224 growing on L-α-GP

Glycerol trained cells of E.coli 15224 grew immediately when inoculated into L-α-GP medium. Growth was logarithmic after glucose challenge with a specific growth rate of 1.04 h^{-1}, higher than on glucose alone (0.90 h^{-1}). Substrate estimations made during growth (Figure 19a) showed that L-α-GP was co-metabolised with glucose, only a slight reduction in the rate of L-α-GP metabolism being observed which did not increase during utilization of glucose.

6.2. Effect of glucose on the rate of L-α-GP utilization after glucose challenge to cells of E.coli 15224 growing on glycerol

Glycerol utilization by cells of E.coli 15224 has ceased 90 min after glucose challenge to cells growing on glycerol (Figure 13). The effect of glucose on L-α-GP utilization by these cells was investigated in washed cell suspensions (Figure 19b).

Although glucose totally prevented glycerol utilization in these washed cell suspensions the initial rate of L-α-GP utilization was not markedly affected by glucose.

Figure 19a

RATE OF L-α-GLYCEROPHOSPHATE METABOLISM ON GLUCOSE CHALLENGE TO CELLS OF *E.coli* 15224 GROWING ON L-α-GLYCEROPHOSPHATE (L-α-GP)

Cells of *E.coli* 15224 trained to glycerol were harvested, washed and inoculated into 2 flasks (A and B) containing 800 ml minimal salts medium and 8 mM L-α-glycerophosphate. After 90 min growth 2 mM glucose was added to flask B. Growth and substrate utilization were measured.

concentration:

○ flask A

◍ flask B

Growth of the cultures is represented by the bars at the top of the diagram. The presence of glucose in flask B is indicated by the shaded area at the bottom of the diagram.

Figure 19b

EFFECT OF GLUCOSE ON THE RATE OF GLYCEROL OR L-α-GP UTILIZATION AFTER GLUCOSE CHALLENGE TO CELLS OF *E.coli* 15224 GROWING ON GLYCEROL

Cells of *E.coli* trained to glycerol were inoculated into 800 ml minimal salts medium containing 4 mM glycerol. 2 mM glucose was added after 90 min growth. After a further 90 min the cells were harvested, washed and inoculated into 400 ml CAP medium containing 6 mM DL-α-GP, 3 mM glycerol or either of these substrates and 3 mM glucose. Substrate utilization was measured.

L-α-GP utilization:

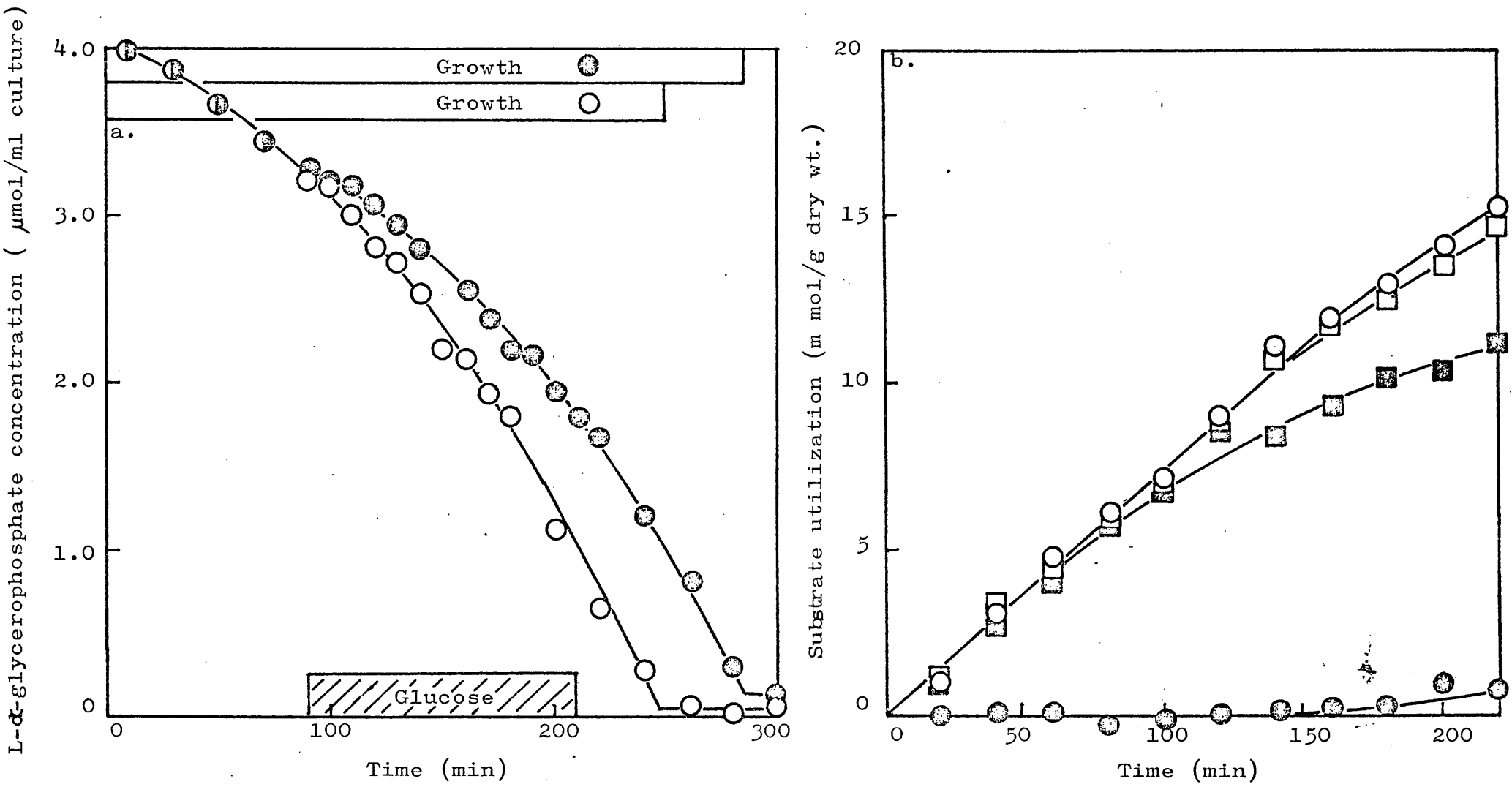
□ alone

▩ + glucose

Glycerol utilization:

○ glycerol alone

● glycerol + glucose


L-α-glycerophosphate concentration (μmol/ml culture)
4.0
3.0
2.0
1.0
0
Growth
Growth
a.
Glucose
0
100
200
300
Time (min)
Substrate utilization (m mol/g dry wt.)
20
15
10
5
0
b.
0
50
100
150
200
Time (min)

7. Intracellular FDP Concentration and Inhibition of Glycerokinase on Glucose Challenge to Cells of E.coli Utilizing Glycerol

7.1. Intracellular FDP concentration on glucose challenge to E.coli 15224 growing on glycerol

Cells of E.coli 15224 trained to and growing on glycerol were challenged with glucose.

Substrate estimations showed that the rate of glycerol utilization/enzyme unit (Figure 20) was reduced by about 50% on glucose challenge and continued to decrease until utilization ceased about 60 min later. Estimations of intracellular FDP concentration (Figure 20) showed that the concentration increased slightly from 1.07 ± 0.14 mM to about 1.2 ± 0.05 mM on glucose challenge and to a maximum of 1.4 ± 0.2 mM 120 min after glucose challenge. A change of 0.93 to 1.6 mM FDP represents a maximum decrease in glycerokinase activity of only 28% due to FDP inhibition.

7.2. Changes in intracellular FDP concentration on glucose challenge to E.coli utilizing glycerol

The effect of glucose challenge on the rate of glycerol utilization and the intracellular FDP concentration in E.coli 15224 glp R^c and 15224 glp R^cK^i was investigated in washed cell suspensions. The results are summarised in Figure 21. The change in intracellular FDP concentration that occurs on addition of glucose to washed cell suspensions utilizing glycerol was only significant in E.coli 15224 glp R^cK^i cells trained to glycerol. The

Figure 20

INTRACELLULAR FDP CONCENTRATION ON GLUCOSE CHALLENGE TO E.coli 15224 GROWING ON GLYCEROL

Cells of E.coli 15224 trained to glycerol were inoculated into 400 ml minimal salts medium, containing 20 mM glycerol, to give a turbidity of 0.3. After 90 min growth (turbidity 1.0) 15 mM glucose was added. Growth, substrate utilization and intracellular FDP concentration were measured.

□ rate of glycerol utilization

○ intracellular FDP concentration before glucose challenge

● intracellular FDP concentration after glucose challenge

The bars denote the standard deviation.

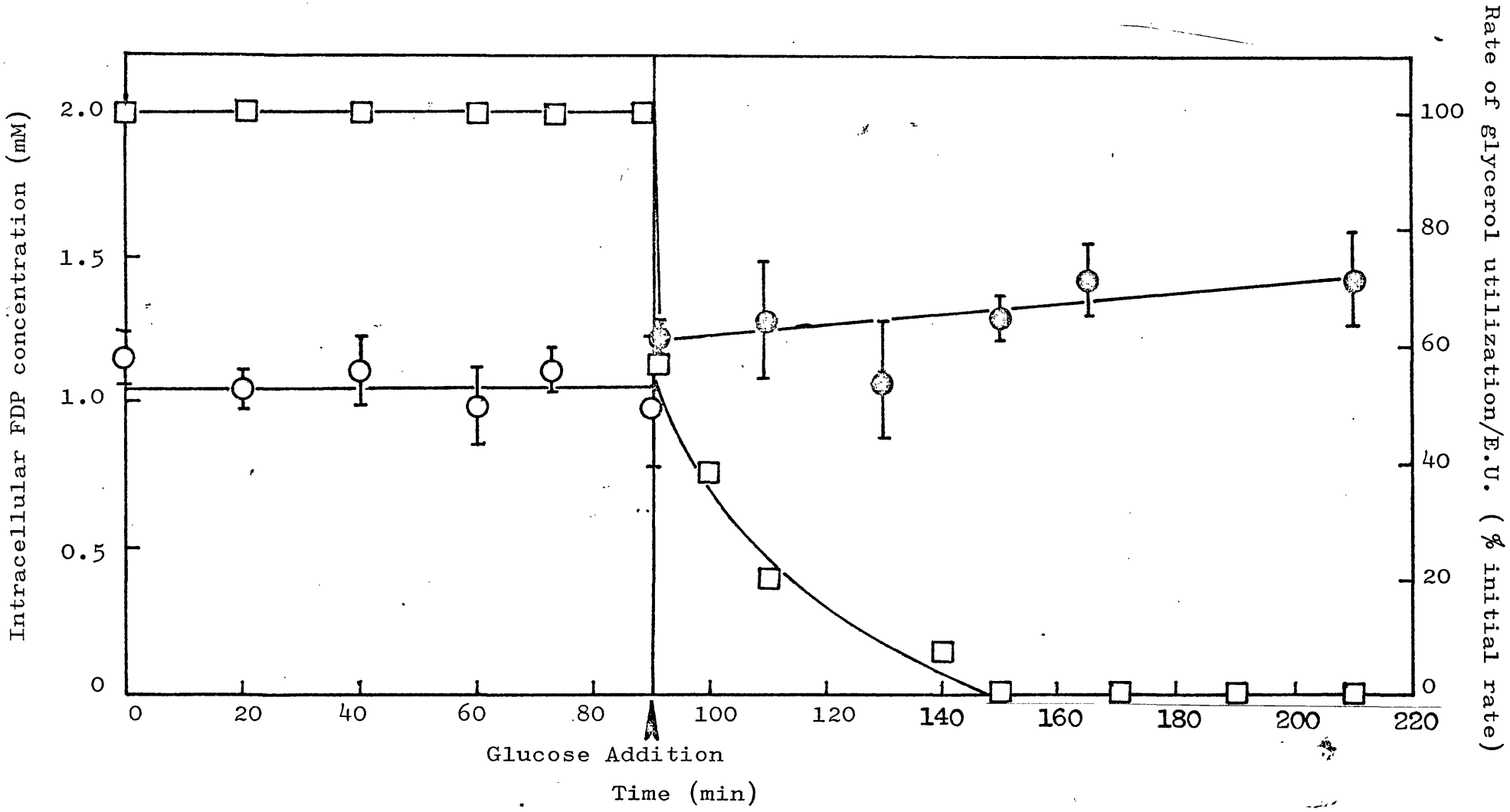

Intracellular FDP concentration (mM)
2.0
1.5
1.0
0.5
0
0
20
40
60
80
100
120
140
160
180
200
220
Glucose Addition
Time (min)
Rate of glycerol utilization/E.U. (% initial rate)
100
80
60
40
20
0

Figure 21

INTRACELLULAR FDP CONCENTRATION IN WASHED CELL SUSPENSIONS OF E.coli

Cells of E.coli 15224 glp R^c and 15224 glp R^cK^i trained to glycerol or glucose were harvested, washed and inoculated into 100 ml CAP medium containing 4 mM glycerol or 4 mM glycerol and 3 mM glucose. Cells were inoculated to a turbidity of 1.0. Substrate utilization was measured. The intracellular FDP concentration was estimated in duplicate samples of cells withdrawn after 60 min.

The change in glycerokinase activity due to the change in FDP concentration was calculated from previous results (Section 5).

	Training carbohydrate	% inhibition of glycerol utilization by glucose	Intracellular FDP concentration (mM)		Decrease in G.K. activity due to change in FDP (%)
			Glycerol	Glycerol + glucose	
15224 glp R^c	glucose	100	0.1 ± 0.1*	0 ± 0.1 *	-5
	glycerol	52	2.4 ± 0.1	2.7 ± 0.7	5
15224 glp R^cK^i	glucose	72	4.1 ± 0.2	4.0 ± 0.1	-2
	glycerol	48	3.3 ± 0.1	4.0 ± 0.1	9

* Triplicate samples

Figure 22

EFFECT OF FDP ON ACTIVITY OF GLYCEROKINASE FROM CELLS OF *E.coli* GROWING ON GLYCEROL, GLUCOSE OR BOTH

Glycerokinase was extracted from cells of *E.coli* 15224 *glp* R^c growing on glucose or glycerol, and was extracted from cells of *E.coli* 15224 before and 90 min after glucose challenge to cells growing on glycerol. The ability of FDP to inhibit glycerokinase activity in these extracts was tested.

Cell type	Carbon source	FDP concentration (mM)	Glycerokinase activity (%)	Assay
15224 glp R^c	glycerol	0	100	II
		0.5	64 ± 1	II
		5.0	15 ± 1	II
	glucose	0	100	II
		0.5	64 ± 3	II
		5.0	17 ± 2	II
15224	glycerol	0	100	I
		5.0	43	I
	glycerol +	0	100	I
	glucose	5.0	51	I

change in glycerokinase activity expected due to changes in FDP concentration was less than 10% in all cases, whereas glucose inhibited glycerol utilization by more than 48% (48 - 100% inhibition) in all cases.

7.3. FDP sensitivity of glycerokinase from E.coli growing on different carbon sources

7.3.1. FDP inhibition of glycerokinase from E.coli 15224 on glucose challenge to cells growing on glycerol

The ability of FDP to inhibit glycerokinase was determined (assay I, Methods 10.2.1.) before and 90 min after glucose challenge to cells of E.coli 15224 growing on glycerol (Figure 22). No difference in sensitivity to FDP inhibition was found.

7.3.2. FDP inhibition of glycerokinase from E.coli 15224 glp R^c trained to and growing on glycerol or glucose

The ability of FDP to inhibit glycerokinase activity extracted from cells of E.coli 15224 glp R^c growing on glycerol or glucose was determined (Figure 22) using assay II (Methods, 10.2.2). No difference in sensitivity to inhibition by 0.5 or 5 mM FDP was found between glycerol and glucose growing cells.

8. Intracellular Metabolite Concentrations on Glucose Challenge to Cells of E.coli Utilizing Glucose

8.1. Intracellular metabolite concentrations on glucose challenge to cells of E.coli 15224 growing on glycerol

Cells of E.coli 15224 trained to glycerol and inoculated into glycerol minimal salts medium were challenged with glucose after 90 min growth.

Substrate estimations showed that the rate of glycerol utilization (Figure 23) fell by about 50% on glucose challenge and continued to decrease until utilization ceased after 60 min. The intracellular ATP concentration (Figure 23) showed little change on glucose challenge to glycerol growing cells and was constant before (3.72 mM) and after (3.54 mM) glucose challenge. Estimations of F6P concentration (Figure 23) also showed little change on glucose challenge and was constant before (0.18 mM) and after (0.25 mM) challenge.

Estimations of the intracellular G6P concentration (Figure 23) showed a large increase in concentration from 0.50 mM to 3.20 mM on glucose challange, falling to a constant level (2.32 mM) within 20 min of glucose challenge.

8.2. Changes in intracellular metabolite concentrations on glucose challenge to E.coli utilizing glycerol

The effect of glucose challenge on the rate of glycerol utilization and the intracellular concentration of G6P, F6P and ATP in E.coli 15224 glp R^c and 15224 glp R^cK^i was investigated in washed cell suspensions. The results are summarised in Figure 24. Neither the

Figure 23

INTRACELLULAR CONCENTRATION OF GLUCOSE-6-PHOSPHATE (G6P), FRUCTOSE-6-PHOSPHATE (F6P) AND ATP ON GLUCOSE CHALLENGE TO CELLS OF E.coli 15224 GROWING ON GLYCEROL

Cells of E.coli 15224 trained to glycerol were inoculated into 400 ml minimal salts medium, containing 20 mM glycerol, to give a turbidity of 0.3. After 90 min growth 15 mM glucose was added. Growth, substrate utilization and the intracellular concentrations of G6P, F6P and ATP were measured.

□ rate of glycerol utilization

▩ intracellular ATP concentration

○ intracellular F6P concentration

△ intracellular G6P concentration

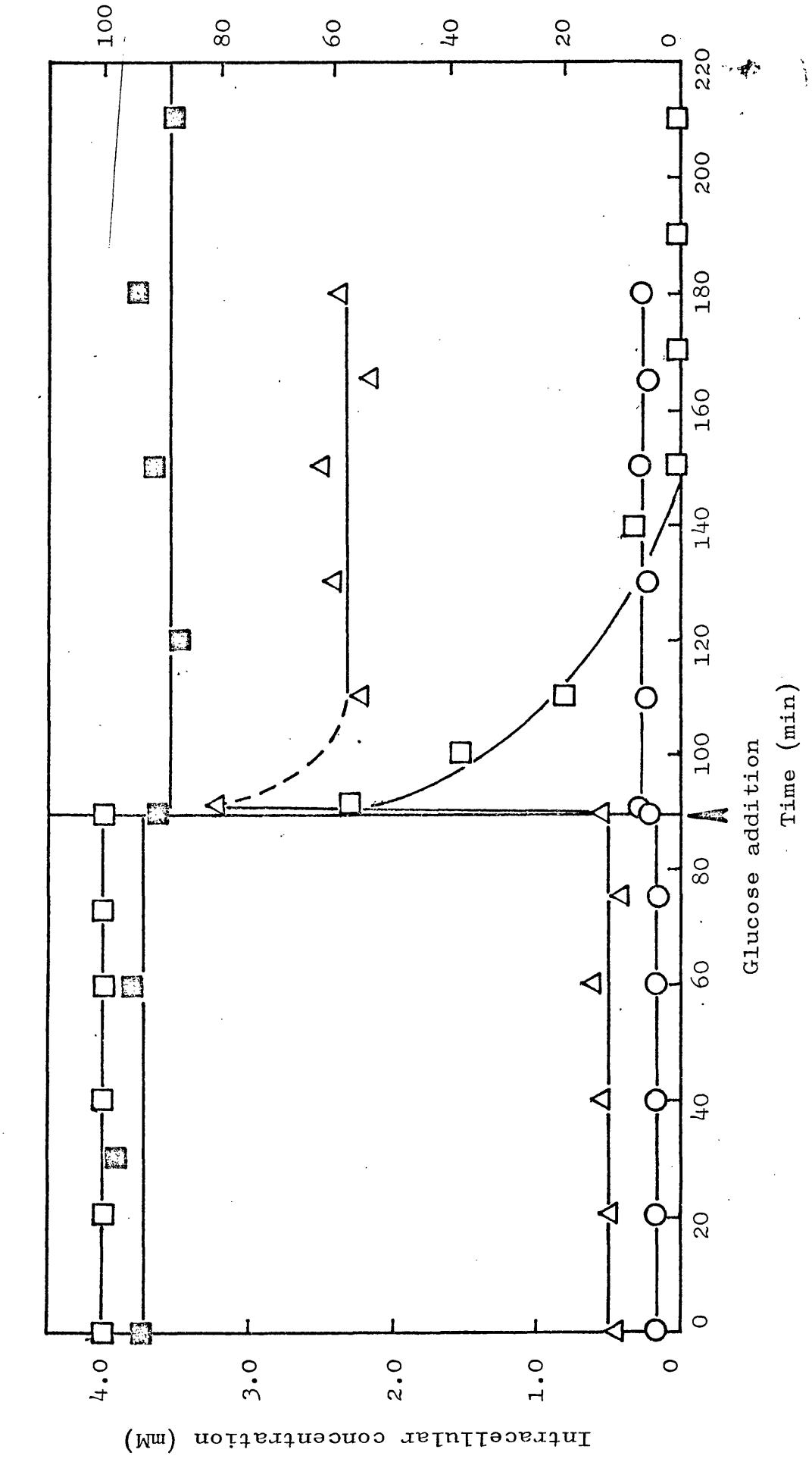

Rate of glycerol utilization/E.U. (% initial rate)
100
80
60
40
20
0
220
200
180
160
140
120
100
80
60
40
20
0
Glucose addition
Time (min)
4.0
3.0
2.0
1.0
0
Intracellular concentration (mM)

Figure 24

INTRACELLULAR CONCENTRATION OF G6P, F6P AND ATP IN WASHED CELL SUSPENSION OF E.coli

Cells of E.coli 15224 glp R^c and 15224 glp R^cK^i trained to glycerol and glucose were harvested, washed and inoculated into 100 ml CAP medium containing 4 mM glycerol or 4 mM glycerol and 3 mM glucose. Cells were inoculated to a turbidity of 1.0. Substrate utilization was measured. The intracellular metabolite concentrations were estimated in duplicate samples withdrawn after 60 min.

Cell type	Training carbohydrate	% inhibition of glycerol utilization by glucose	Intracellular concentration (mM)							
			Glycerol				Glycerol + Glucose			
			FDP	G6P	F6P	ATP	FDP	G6P	F6P	ATP
15224 glp R^c	glycerol	52	0.1 ± 0.1	1.4 ± 0.4	-	-	0 ± 0.1	6.4 ± 0.5	-	-
	glucose	100	2.4 ± 0.1	1.5 ± 0.3	0.7	5.6 ± 0.6	2.7 ± 0.7	6.7 ± 0.2	0.9	9.6 ± 0
15224 glp R^cK^i	glycerol	48	4.1 ± 0.2	1.2 ± 0.3	-	-	4.0 ± 0.1	1.6 ± 0.3	-	-
	glucose	72	3.3 ± 0.1	0.8 ± 0.2	-	-	4.0 ± 0.1	2.3 ± 0.4	-	-

intracellular F6P concentration (0.7 - 0.9 mM) nor the intracellular ATP concentration (5.6 ± 0.6 - 9.6 ± 0.2 mM) increased to a large extent on glucose challenge to glucose trained cells of E.coli 15224 glp R^c utilizing glycerol, but large increases in intracellular G6P concentration on glucose challenge were observed in most cases.

9. Permeation of Small Molecular Weight Compounds into E.coli

9.1. Permeation of E.coli 15224 trained to glucose

The permeability constants for ethylene glycol, 1,3 propanediol, thiourea, urea, 1,2,4 butanetriol, erythritol and glycerol were estimated as described (Methods, Sections 13.1.,13.2.).

The logarithm of $PM^{\frac{1}{2}}$ (where P is the permeability constant and M is the molecular weight) was plotted against N, the number of potential hydrogen bond forming groups in the permeant (Figure 25). The line of 'best fit' was calculated as described (Methods, Section 16). No point was greater than 0.64 log units, that is a factor of 4.3, from the straight line. The values of $PM^{\frac{1}{2}}$ observed for entry of glycerol into cells of E.coli 15224 trained to glucose lay within 0.64 log units of the straight line.

A number of parameters describing the characteristics of permeation through membranes from E.coli 15224 trained to glucose were calculated from the following equations:

From Figure 25, $\log PM^{\frac{1}{2}}_{max} = \log PM^{\frac{1}{2}}$ when N = 0

reduction in permeation rate/pair of hydrogen bonds = antilog gradient

free energy change ($\Delta F^{\ddagger}$) per hydrogen bond = gradient/RT

From the permeation constants for erythritol at 27°C (entry rate) and 30°C (exit rate, Methods, Section 13.2.),

activation energy (A) for erythritol =

$$- \frac{2.303\ R\ \log_{10} {}^{P_1}/_{P_2}}{\frac{1}{T_1} - \frac{1}{T_2}}$$

Figure 25

PERMEABILITY OF MEMBRANES FROM E.coli 15224 TO SMALL MOLECULAR WEIGHT COMPOUNDS (I)

Cells of E.coli 15224 trained to and growing exponentially on glucose were used. Permeability constants (P) were estimated at 27°C as described (Methods, Section 13). The number of potential hydrogen bond forming groups (N) in the permeant were obtained from Stein (1967). The logarithm of $PM^{\frac{1}{2}}$ (where M is the molecular weight of the permeant) was plotted against N.

1	-	glycerol	5	-	1,2,4-butanetriol
2	-	ethylene glycol	6	-	erythritol
3	-	urea	6a	-	erythritol (exit rate, 30°C)
4	-	thiourea	7	-	1,3-propanediol

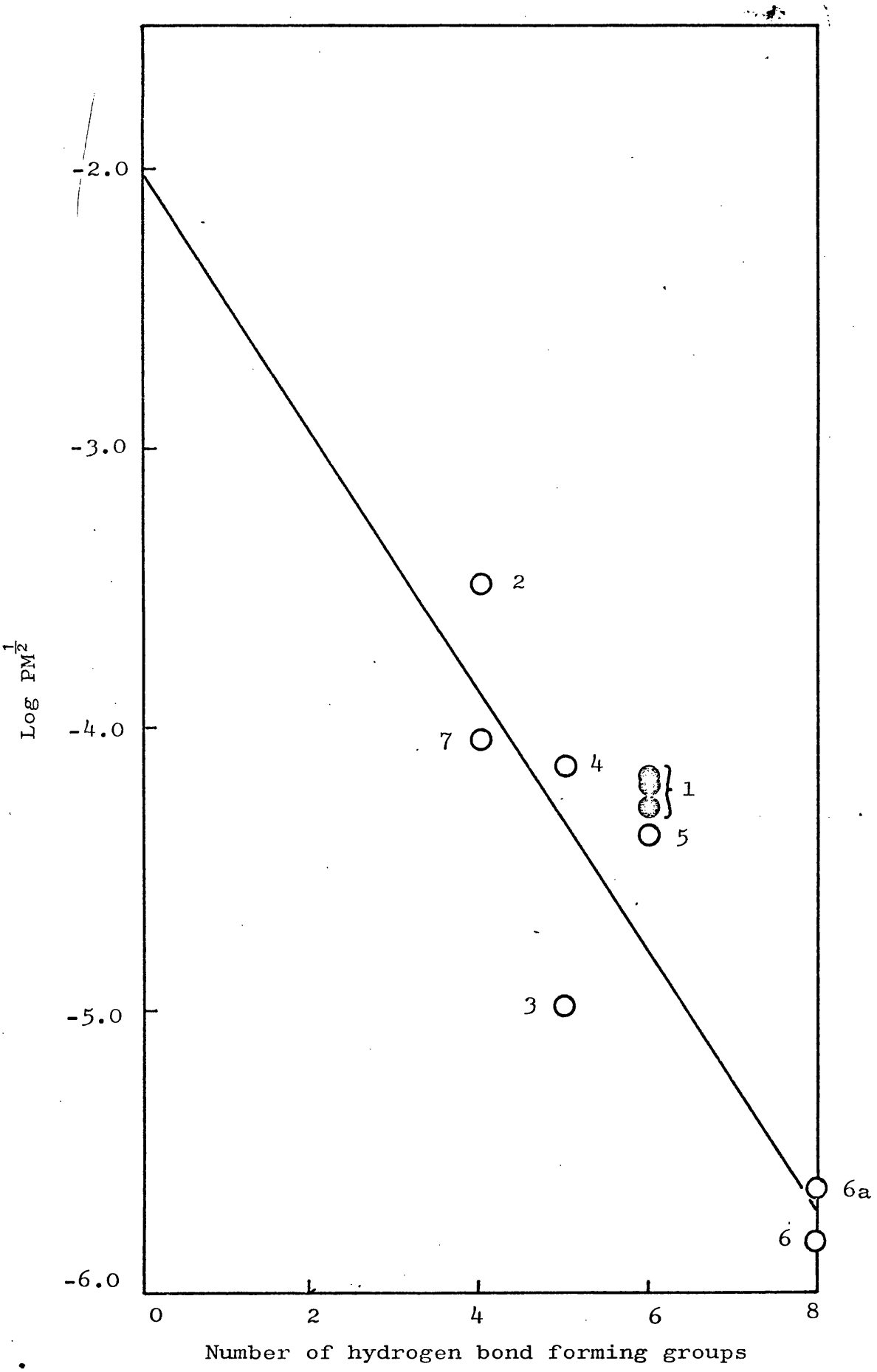

-2.0
-3.0
-4.0
-5.0
-6.0
Log PM$^{\frac{1}{2}}$
0
2
4
6
8
Number of hydrogen bond forming groups
2
7
4
1
5
3
6a
6

Figure 26

PERMEABILITY OF MEMBRANES FROM E.coli 15224 TO SMALL MOLECULAR WEIGHT COMPOUNDS (II)

Figure 26 summarises a number of parameters describing the characteristics of permeation through membranes from glucose trained E.coli 15224. Calculations of these parameters is described in the text (Section 7.1.).

$PM^{\frac{1}{2}}_{max}$ $(cm.s^{-1} mol^{\frac{1}{2}})$	$0.010 \begin{matrix} + 0.058 \\ - 0.005 \end{matrix}$
Reduction in permeation per pair of hydrogen bonds	$8.3 \begin{matrix} + 4.2 \\ - 1.1 \end{matrix}$
Activation energy for erythritol (Kcal/mol)	26.8
Enthalpy change for erythritol (Kcal/mol)	26.2
Enthalpy change per hydrogen bond (Kcal/mol)	3.3
Free energy change per hydrogen bond (Kcal/mol)	$0.77 \begin{matrix} + 0.16 \\ - 0.05 \end{matrix}$
Entropy change per hydrogen bond (e.u./mol)	$8.5 \begin{matrix} + 0.1 \\ - 0.3 \end{matrix}$

enthalpy change ($\Delta H^{\ddagger}$) for erythritol = A - RT

enthalpy change/hydrogen bond = $\frac{A - RT}{N \text{ for erythritol}}$

entropy change ($\Delta S^{\ddagger}$)/hydrogen bond = $\frac{\Delta H^{\ddagger} - \Delta F^{\ddagger}}{T}$

where R is the gas constant, and P_1, P_2 are the permeability constants at temperatures T_1 and T_2 (^{o}K). The results are summarised in Figure 26.

9.2. Rate of glycerol utilization and permeation of glycerol in E.coli

The rate of permeation per unit of concentration is related to the rate constant (Methods, Section 13.1.1.) by the equation:

Rate of permeation (m mol/min/g dry wt./mM) =

$k\ (s^{-1})$ x 0.162

The rate of permeation of glycerol into E.coli 15224 trained to glucose (Figure 27a) was 0.050 $\pm$ 0.005 m mol/min/g dry wt./mM and was unaffected by the presence of glucose (10 mM). This represents approximately 20% of the rate of glycerol utilization in glycerol growing cells (0.28 m mol/min/g dry wt.). A concentration of about 5 mM glycerol would thus be sufficient to produce a permeation rate equivalent to the rate of glycerol utilization during growth on glycerol and 2.5 mM glycerol a rate equivalent to the rate of glycerol utilization in washed cell suspensions of glucose trained 15224 glp R^c (0.12 m mol/min/g dry wt.).

The permeation rate of glycerol in glycerol trained E.coli 15224 and glucose trained 15224 glp R^c was an order of magnitude higher than the rate of glycerol permeation

Figure 27a

PERMEATION RATE OF GLYCEROL IN E.coli

Cells of E.coli 15224 or 15224 glp R^c trained to and growing on glucose or glycerol were used. The rate constants for permeation of glycerol were estimated, in the presence and absence of glucose (10 mM).

The rate of glycerol utilization in glycerol growing cells of E.coli 15224 and in washed cell suspensions of glucose trained E.coli 15224 glp R^c were obtained from previous results.

Figure 27b

CELL WATER AVAILABLE TO GLYCEROL IN E.coli 15224 glp R^c

Cells of E.coli 15224 glp R^c trained to and growing on glucose were used. The cell water available to glycerol was estimated as described (Methods, Section 14). The cells were harvested and inoculated into 200 ml CAP medium containing 3 mM glycerol or 3 mM glycerol and 3 mM glucose. The rate of glycerol utilization was measured and the inhibition calculated from the decrease in rate in the presence of glucose.

a. Cell type	10 mM Glucose	Permeation rate (m mol/g dry wt./min/mM)	Concentration of glycerol to give required rate of utilization (mM)	
			Growing cells	Washed cell suspension
Glucose trained 15224	-	0.050 ± 0.005	5.5 ± 0.6	2.4 ± 0.2
	+	0.053	5.3	2.3
Glycerol trained 15224	-	> 0.56	< 0.5	< 0.2
	+	> 0.56	< 0.5	< 0.2
Glucose trained 15224 glp R^c	-	0.32 ± 0.13	-	-
	+	0.28	-	-

Rate of glycerol utilization in glycerol growing cells	0.28 m mol/g dry wt./min
Rate of glycerol utilization in washed cell suspensions of glucose trained 15224 glp R^c	0.12 m mol/g dry wt./min

b. Carbon source	% inhibition of glycerol utilization by glucose	Total cell water (ml/g dry wt.)	Glycerol available water (ml/g dry wt.)	% of total cell water
Glycerol	0	2.70	2.10	78
Glucose + Glycerol	100	2.70	1.97	73

in glucose trained cells of 15224 (Figure 27a). The permeation rate of glycerol was not affected by the presence of glucose (10 mM).

The permeation rate of glycerol into cells of E.coli 15224 glp R^c growing aerobically on glucose was estimated on samples within 45 s of their removal from the culture. The permeation rate was the same as for the cell suspension of 15224 glp R^c (Figure 27) and was not affected by the presence of glucose.

9.3. Permeation by glycerol of cell water in E.coli 15224 glp R^c

The ability of glucose to prevent glycerol permeation of cell water was investigated in E.coli 15224 glp R^c trained to glucose. The cells were suspended in medium containing glycerol-(U)-^{14}C (33 mM) or glucose (33 mM) and glycerol-(U)-^{14}C (33 mM) and incubated for 1 min at 37°C. The time required for the concentration difference of glycerol across the cell membrane to be reduced by half in these cells was about 0.4 s at 27°C (0.69/rate constant, Methods, Section 13.1.2.), thus 60 s at 37°C was sufficient for equilibration of the intra- and extracellular concentration of glycerol.

No difference was found in the proportion of cell water penetrated by glycerol in the presence (73%) and absence (78%) of glucose (Figure 27b).

10. Adaptation of Cells During Glucose Challenge to E.coli Utilizing Glycerol

The ability of glucose to inhibit glycerol utilization on glucose challenge to cells of E.coli 15224 trained to and growing on glycerol increased with time (Figure 13). The changes taking place in the cell during this period of continued glycerol utilization in the presence of glucose were investigated further.

10.1. Substrate utilization in chloramphenicol (CAP) treated cultures after glucose challenge to cells of E.coli 15224 growing on glycerol

Cells of E.coli 15224 trained to glycerol were inoculated into minimal salts medium containing glycerol. After a short period of growth (90 min) cultures were challenged with glucose.

Glucose challenge was followed immediately, or after various time intervals, by addition of CAP. The addition of CAP to the cultures stopped growth immediately but did not prevent glycerol or glucose utilization.

Substrate estimations (Figure 28a) showed that glucose depressed the rate of glycerol utilization (by 42%) but never abolished it when CAP was added at the time of glucose challenge. The rate of glycerol utilization in the presence of glucose decreased (Figure 28b) as the time between glucose challenge and CAP addition was lengthened, until glucose totally prevented glycerol utilization 60 min after glucose challenge. The inhibition was reversible and glycerol utilization was restored immediately on exhaustion of glucose.

Figure 28

SUBSTRATE UTILIZATION IN CAP TREATED CULTURES AFTER GLUCOSE CHALLENGE TO CELLS OF *E.coli* 15224 GROWING ON GLYCEROL

Figure 28a

Cells of *E.coli* 15224 trained to glycerol were inoculated into 4 flasks containing 800 ml minimal salts medium and 4 mM glycerol. Growth and substrate concentration were measured. After 90 min growth additions were made as follows:

Glycerol concentration on addition of:

○ no additions

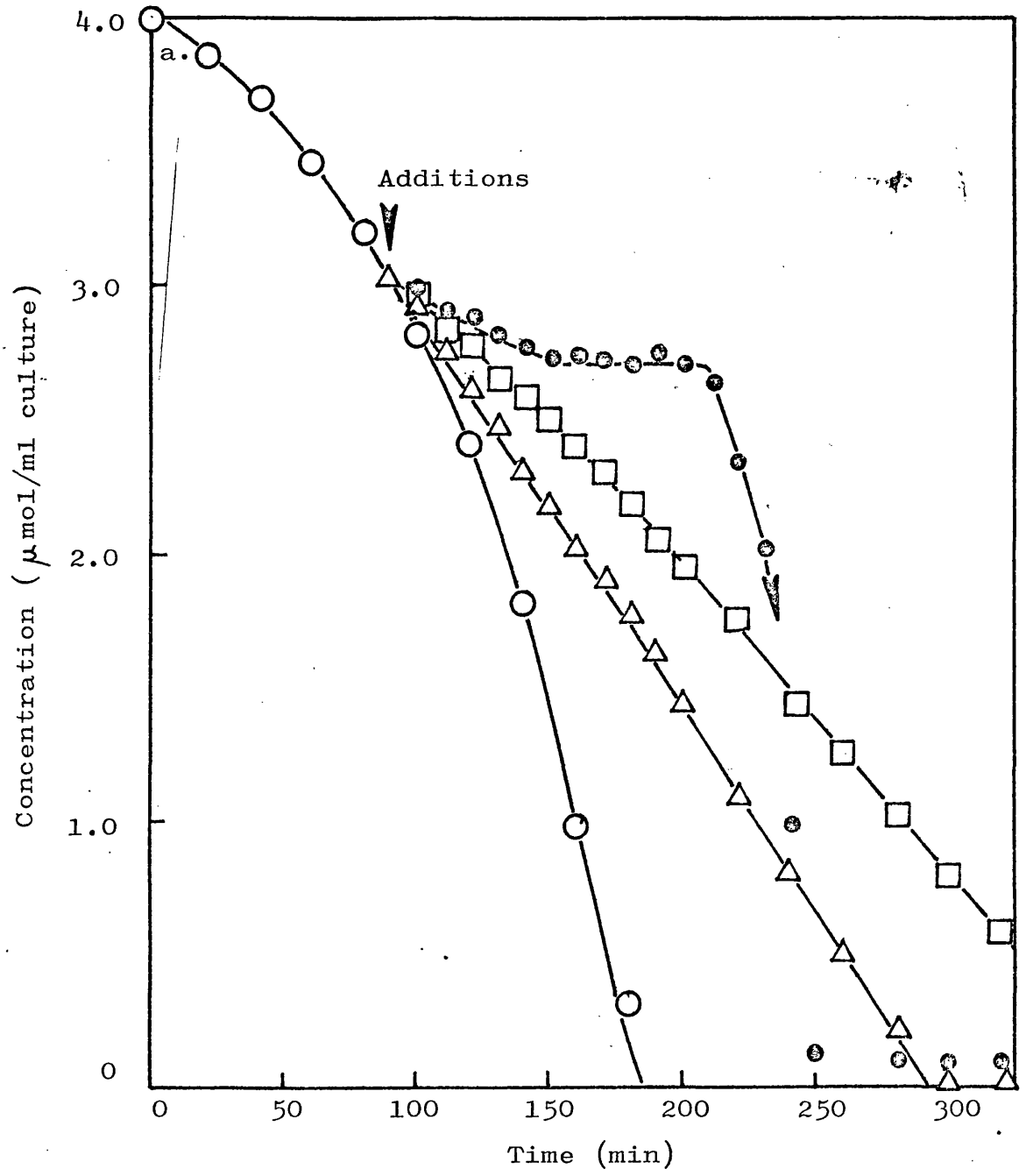
△ 0.3 mM CAP

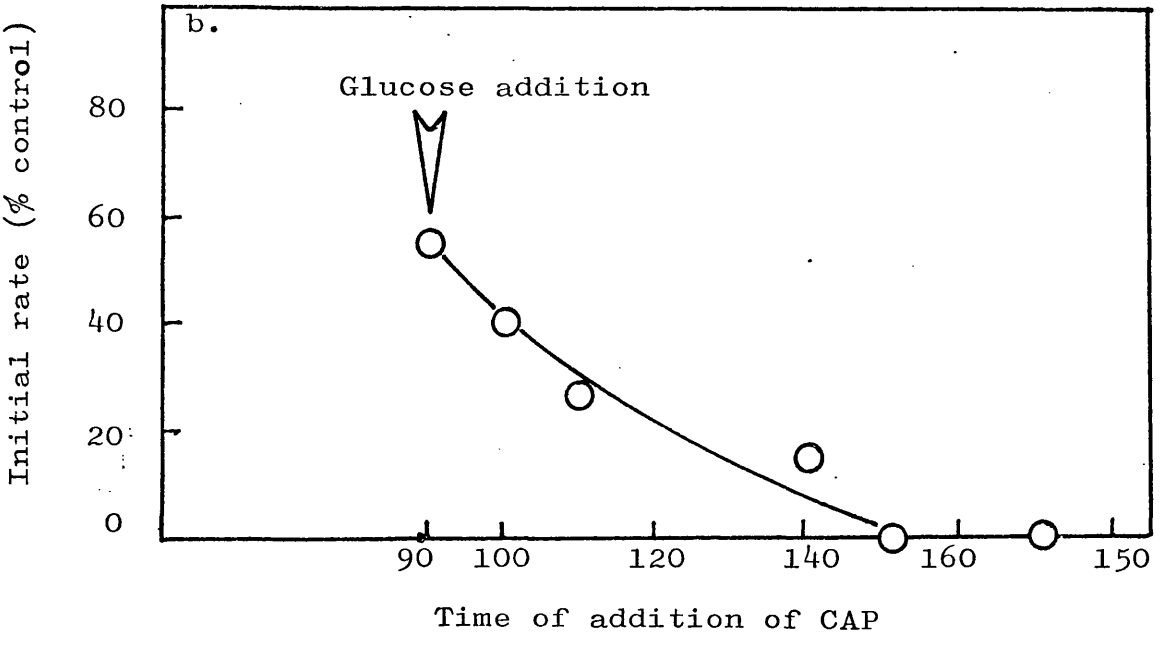
□ 0.3 mM CAP + 2 mM glucose

◎ 2 mM glucose

Figure 28b

Cells of *E.coli* 15224 trained to glycerol were inoculated into 7 flasks containing 800 ml minimal salts medium and 4 mM glycerol. After 90 min growth 0.3 mM CAP was added to one flask which was used as a control. 2 mM glucose was added to the remaining flasks followed by 0.3 mM CAP at 0, 10, 20, 50, 60 and 80 min after glucose challenge. The concentration of glycerol in the flasks was measured and the rate of glycerol utilization, as a percentage of the control, plotted against time.


a.
Additions
Concentration (μmol/ml culture)
4.0
3.0
2.0
1.0
0
0
50
100
150
200
250
300
Time (min)


b.
Glucose addition
Initial rate (% control)
80
60
40
20
0
90
100
120
140
160
150
Time of addition of CAP

10.2. Growth on glycerol in the presence of α-methylglucoside after glucose challenge to cells of E.coli 15224 growing on glycerol

The ability of α-methylglucoside to prevent growth of E.coli 15224 on glycerol was investigated after glucose challenge to cells growing on glycerol.

Growth measurements (Figure 29) showed that the ability of α-methylglucoside to prevent growth on glycerol increased as the time between glucose challenge and harvesting of cells was lengthened. No loss in ability to grow on glycerol alone was observed.

Growth did not occur on glycerol in the presence of α-methylglucoside in cells harvested 60 min after glucose challenge. Cells harvested 30 min after glucose challenge showed (Figure 29) an increasing growth rate on glycerol in the presence of α-methylglucoside.

10.3. Enzyme activity and inhibition of glycerol utilization by glucose in washed cell suspensions of E.coli 15224 glp R^c

The glucose phosphotransferase system (PTS) is the first step in the pathway for dissimilation of glucose in E.coli. It mediates both transport and phosphorylation and thus its activity determines the rate of utilization of glucose. The level of the glucose PTS was investigated in washed cell suspensions of E.coli 15224 glp R^c.

Cells of E.coli 15224 glp R^c were trained to glycerol and glucose and the ability of glucose to prevent glycerol utilization in washed cell suspensions investigated. Measurements were made of the level of glucose PTS and glycerokinase. The results are summarised in Figure 30a.

Figure 29

GROWTH ON GLYCEROL IN THE PRESENCE OF α-METHYLGLUCOSIDE AFTER GLUCOSE CHALLENGE TO CELLS OF E.coli 15224 GROWING ON GLYCEROL

Cells of E.coli 15224 trained to glycerol were inoculated into minimal salts medium containing 4 mM glycerol. After 90 min growth 2 mM glucose was added to the culture. Samples were harvested at 0, 30 and 60 min after glucose challenge, washed and inoculated into minimal salts medium containing 4 mM glycerol and 2 mM α-methylglucoside. Measurements of growth were made.

Growth on glycerol of all cells ●

Growth on glycerol + α-methylglucoside of cells harvested after glucose challenge;

○ 0 min after challenge

□ 30 min after challenge

△ 60 min after challenge

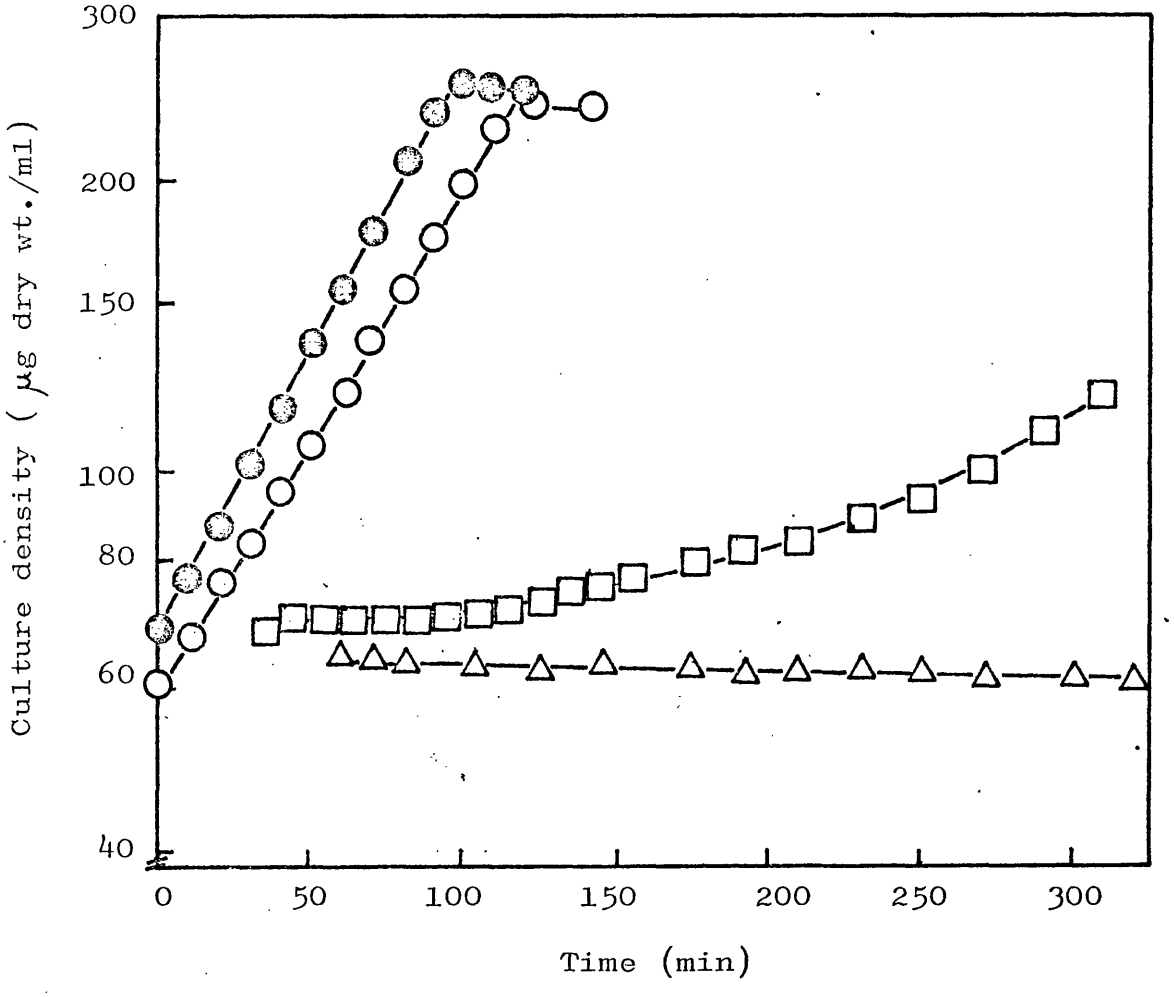

300
200
150
100
80
60
40
Culture density (μg dry wt./ml)
0
50
100
150
200
250
300
Time (min)

Figure 30

GLUCOSE PTS, GLYCEROKINASE AND INHIBITION OF GLYCEROL UTILIZATION BY GLUCOSE IN WASHED CELL SUSPENSIONS OF *E.coli* 15224 glp R^c

Cells of *E.coli* 15224 glp R^c were trained to a number of carbon sources, harvested, washed and inoculated into 200 ml CAP medium containing 3 mM glycerol or 3 mM glycerol and 3 mM glucose. Cells were inoculated to a turbidity of 0.4. Substrate utilization, glucose PTS (Methods, Section 11) and glycerokinase were measured.

Figure 30a

Specific activity of glucose PTS and glycerokinase.

Figure 30b

Relation between glycerol inhibition and specific activity of glucose PTS.

Figure 30c

Relation between glycerol inhibition and specific activity of glycerokinase.

Cells trained to	% inhibition of glycerol utilization by glucose	Specific activity (E.U./g dry wt.)	
		glucose PTS	glycerokinase
a.			
Glycerol	57	21	485
Glucose	100	96	49
b.			
Glycerol (CAP treated)	33	11	485
Glycerol	57	21	485
Glucose/cAMP (7 h growth)	70	200	515
c.			
Glucose/cAMP (5 h growth)	46	100	543
Glucose	100	96	49
Mannose	16	38	795
Ribose	39	19	640
Glycerol	57	21	485
Fructose	71	37	354

Glucose inhibited glycerol utilization to a greater extent in glucose trained cells (100% inhibition) than in glycerol trained cells (57% inhibition). This paralleled an increase in the specific activity of the glucose PTS from 21 E.U./g dry wt. in glycerol trained cells to 96 E.U./g dry wt. in glucose trained cells.

There was also, however, a concomitant decrease in the specific activity of glycerokinase from 485 E.U./g dry wt. in glycerol trained cells to 49 E.U./g dry wt. in glucose trained cells.

10.4. Glucose PTS, glycerokinase and inhibition of glycerol utilization by glucose in washed cell suspensions of E.coli 15224 glp R^c

To discriminate between the effect of increased glucose PTS and decreased glycerokinase, cells of E.coli 15224 glp R^c were trained to a number of carbon sources. Measurements of the ability of glucose to prevent glycerol utilization and the specific activity of glucose PTS and glycerokinase were made.

10.4.1. Glycerol inhibition by glucose and glucose PTS level

Glucose inhibited glycerol utilization by 33%, 57% and 70% in cells which contained glucose PTS at specific activities of 11, 21 and 200 E.U./g dry wt. respectively and a constant level of glycerokinase (Figure 30b).

10.4.2. Glycerol inhibition by glucose and glycerokinase level

A negative correlation (Figure 30c) between the level of glycerokinase and the ability of glucose to inhibit glycerol utilization at constant glucose PTS level was obtained. For example cells grown on glucose/

cAMP (5 mM) for 5 h (Figure 30c) contained the same level of glucose PTS as glucose trained cells, but an increase in the ability of glucose to prevent glycerol utilization (46 - 100% inhibition) corresponded to a 10 fold reduction in the level of glycerokinase (543 - 49 E.U./g dry wt.).

11. Effect of Glycerol on Glucose Utilization in Washed Cell Suspensions of E.coli

The effect of glycerol on glucose utilization was examined in washed cell suspensions of E.coli 15224 glp R^c trained to glycerol or glucose.

Glucose estimations (Figure 31a) showed that glycerol inhibited the initial rate of glucose utilization by 50% in cells trained to glycerol but did not affect glucose utilization in glucose trained cells. For comparison the effect of glucose on glycerol utilization is shown (Figure 31b). Glucose inhibited glycerol utilization by 50% in glycerol trained cells and totally prevented it in glucose trained cells.

Glycerol also inhibited glucose utilization by about 50% in washed cell suspensions of E.coli 15224 trained to glycerol.

Glycerol totally prevents glucose utilization in glycerol trained E.coli 15224 glp R^c after 3 h treatment

Figure 31

MUTUAL INHIBITION OF GLUCOSE AND GLYCEROL METABOLISM IN WASHED CELL SUSPENSIONS OF E.coli 15224 glp R^c

Cells of E.coli 15224 glp R^c trained to glycerol or glucose were inoculated into 200 ml CAP medium containing 3 mM glycerol, 3 mM glucose or both. Cells were inoculated to a turbidity of 0.4. Measurements of substrate utilization were made.

Figure 31a

Glucose utilization:

○ in glycerol trained cells

◎ in the presence of glycerol, glycerol trained cells

□ in glucose trained cells

▣ in the presence of glycerol, glucose trained cells

Figure 31b

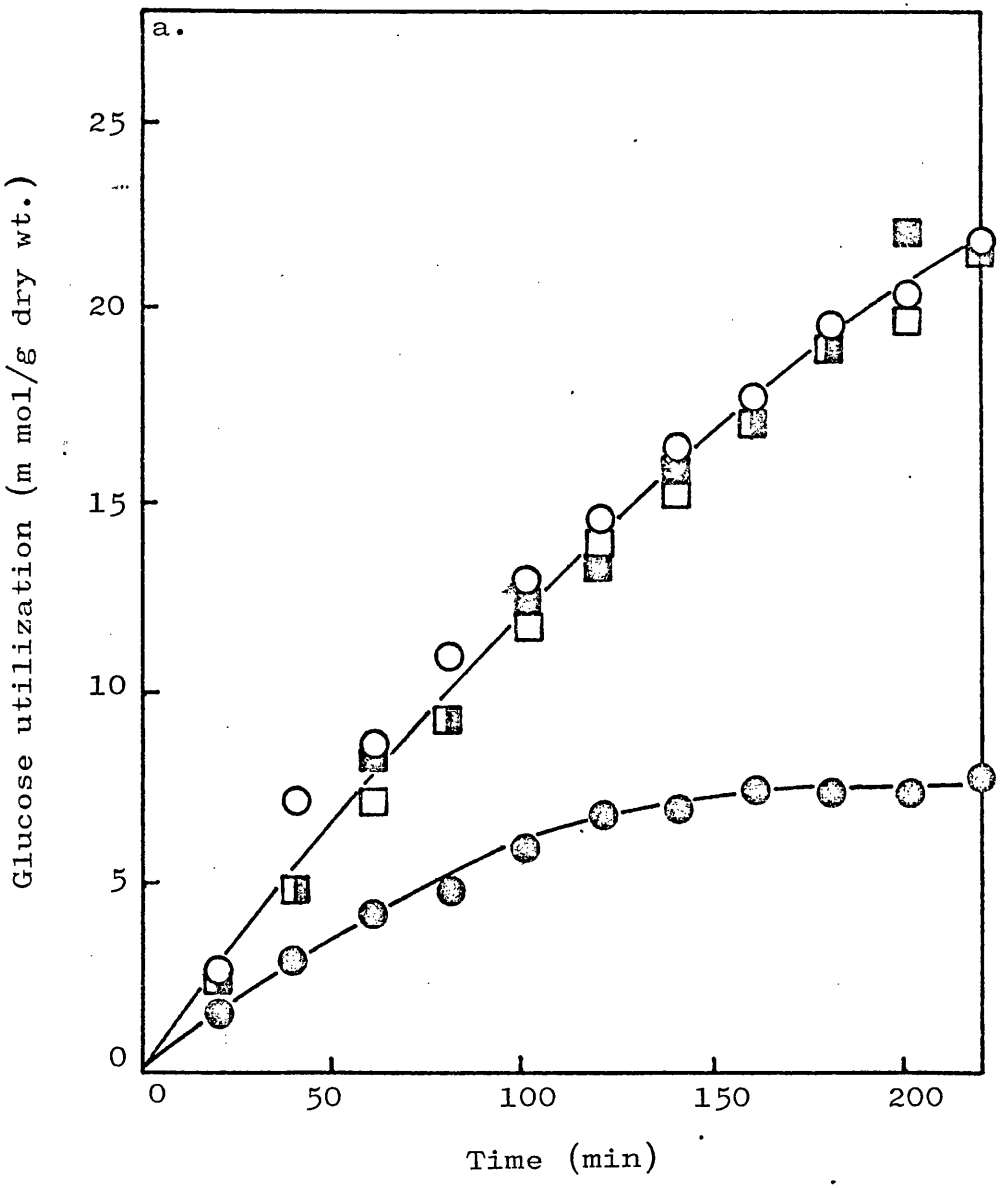
Glycerol utilization:

○ in glycerol trained cells

◎ in the presence of glucose, glycerol trained cells

□ in glucose trained cells

▣ in the presence of glucose, glucose trained cells


a.
Glucose utilization (m mol/g dry wt.)
0
5
10
15
20
25
0
50
100
150
200
Time (min)

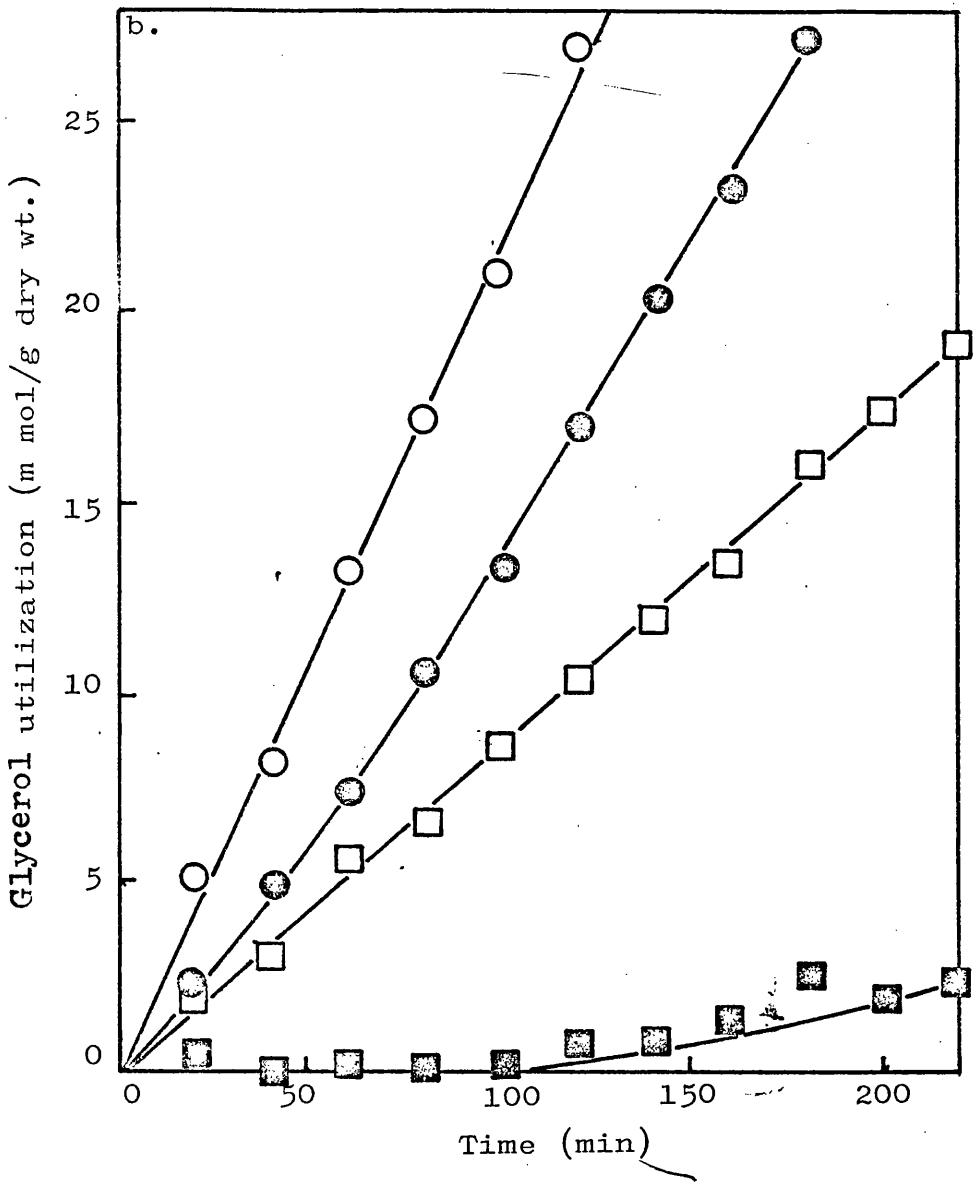

b.
Glycerol utilization (m mol/g dry wt.)
0
5
10
15
20
25
0
50
100
150
200
Time (min)

Figure 32

GLUCOSE PTS, GLYCEROKINASE AND INHIBITION OF GLUCOSE UTILIZATION BY GLYCEROL IN WASHED CELL SUSPENSIONS OF E.coli 15224 glp R^c

Measurements of substrate utilization, glucose PTS and glycerokinase in washed cell suspensions of E.coli 15224 glp R^c described in Figure 30 were made.

Cells trained to	% inhibition of glucose utilization by glycerol	Specific activity (E.U./g dry wt.)	
		glucose PTS	glycerokinase
Glucose/cAMP (5 h growth)	37	100	543
Glucose	0	96	49
Mannose	62	38	795
Fructose	23	37	354

with CAP (Figure 31a) even although glucose is still utilized in the absence of glycerol. The level of glycerokinase in the washed cell suspension remains constant during this period, whereas the glucose PTS level decreases by half in 120 min (data not shown). The decrease in the rate of glucose utilization in the presence of glycerol (Figure 31a) corresponds to an increasing rate of glycerol utilization in the presence of glucose (Figure 31b). Thus the decrease in ability of glucose to prevent glycerol utilization, and the increase in ability of glycerol to prevent glucose utilization, parallels the decrease in the level of the glucose PTS.

However the ability of glycerol to inhibit glucose utilization not only depends on the level of the glucose PTS but, when this is constant, depends on the level of glycerokinase (Figure 32).

12. Rates of Glycerol and Glucose Utilization in a Washed Cell Suspension of E.coli 15224 glp R^c Trained to Glucose

The relation between the rate of glucose utilization and rate of glycerol utilization at constant enzyme level was investigated in a washed cell suspension of E.coli 15224 glp R^c trained to glucose. A diagram of the apparatus is shown in Figure 33. Glucose was supplied to the washed cell suspension at a rate less than the rate of metabolism of glucose when excess glucose was present in the medium.

Estimations of glycerol utilization (Figure 34a) showed that the rate of glycerol utilization decreased as the rate of glucose utilization increased. No excess glucose was detected during the course of the experiment indicating that all the glucose supplied was being metabolised.

The 'best fit' curve for glycerol utilization was calculated for a linear, an exponential and a quadratic function of time. $\Sigma\delta^2/_n$ was calculated for each function (where δ is difference between the calculated and the observed value at time t and n is the number of points). The values were:

Linear	:-	0.00246 (for 2 straight lines)
Exponential	:-	0.0140
Quadratic	:-	0.00123

The 'best fit' is obtained when $\Sigma\delta^2/_n$ is at a minimum.

Figure 33

DIAGRAM OF APPARATUS USED FOR THE DETERMINATION OF THE RELATION BETWEEN THE RATES OF GLYCEROL AND GLUCOSE UTILIZATION

The rate of addition of glucose to the culture vessel was increased by delivery of an increasingly concentrated solution of glucose at a constant rate. The rate of addition was at all times less than the rate of glucose utilization by the cells in the presence of excess glucose.

A linear increase in glucose concentration was obtained by addition of a small volume (12.7 ml) of a concentrated (2 M) glucose solution, at a constant rate (5.9 ml/h) to a mixing chamber containing a large volume of water (about 300 ml). The concentration of glucose in the mixing chamber was estimated and was found to increase linearly from 0 - 70 mM during the experiment.

Constant rates of addition were obtained using a peristaltic pump (Technicon Auto Analyzer Proportioning Pump, Technicon Instruments Co.Ltd., London, England.).

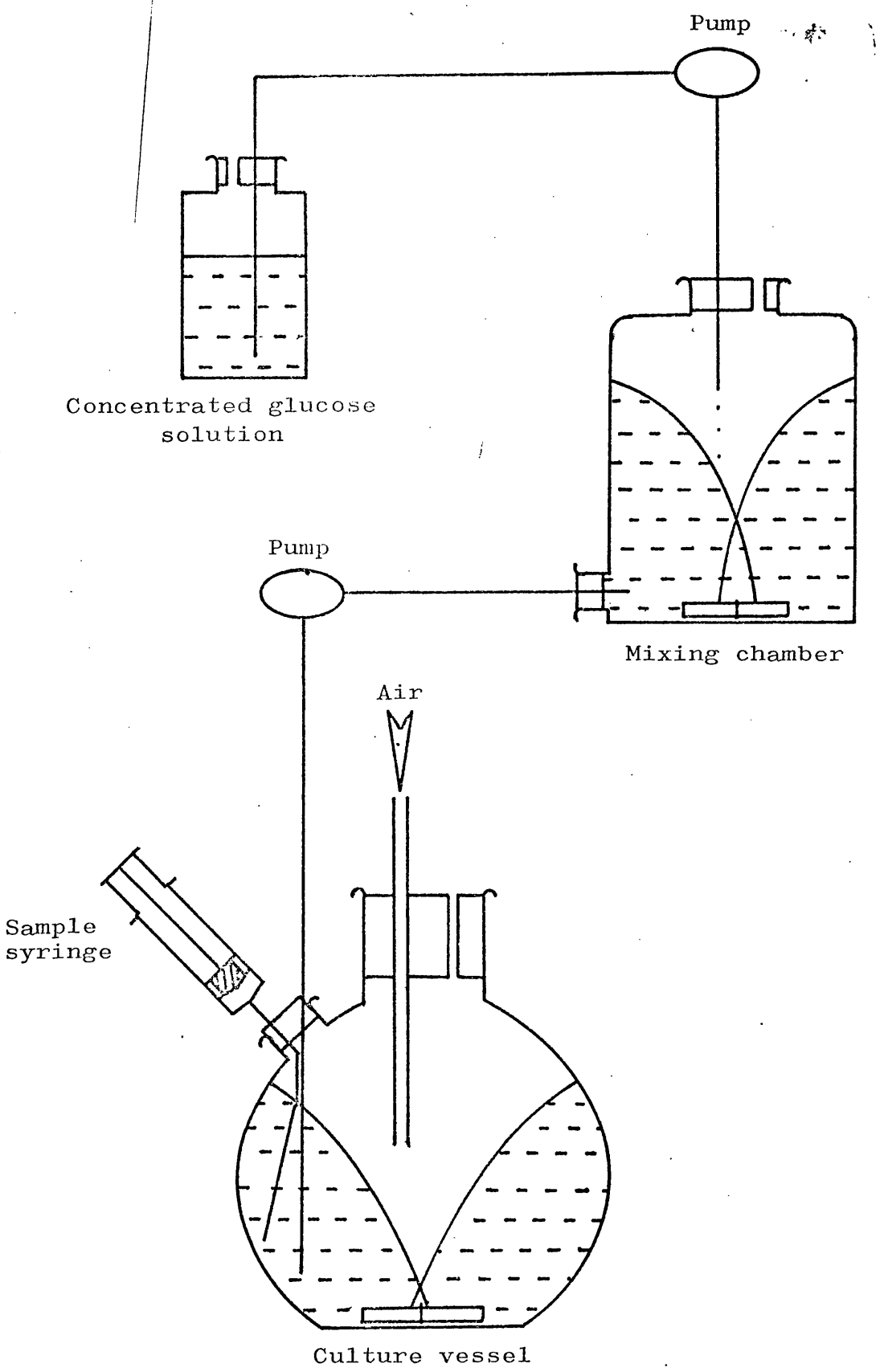

Pump
Concentrated glucose solution
Pump
Mixing chamber
Air
Sample syringe
Culture vessel

Figure 34a

Cells of E.coli 15224 glp R^c trained to glucose were harvested, washed and inoculated into 1.8 l CAP medium containing glycerol (1.5 mM). The rate of glucc utilization was limited by the rate of addition of glucc to the cell suspension. The rate of glucose addition was increased as described (Figure 33).

Samples were withdrawn using a disposable syringe and used to estimate substrate concentrations. Measurements of glycerol utilization, oxygen consumption and carbon dioxide production were made.

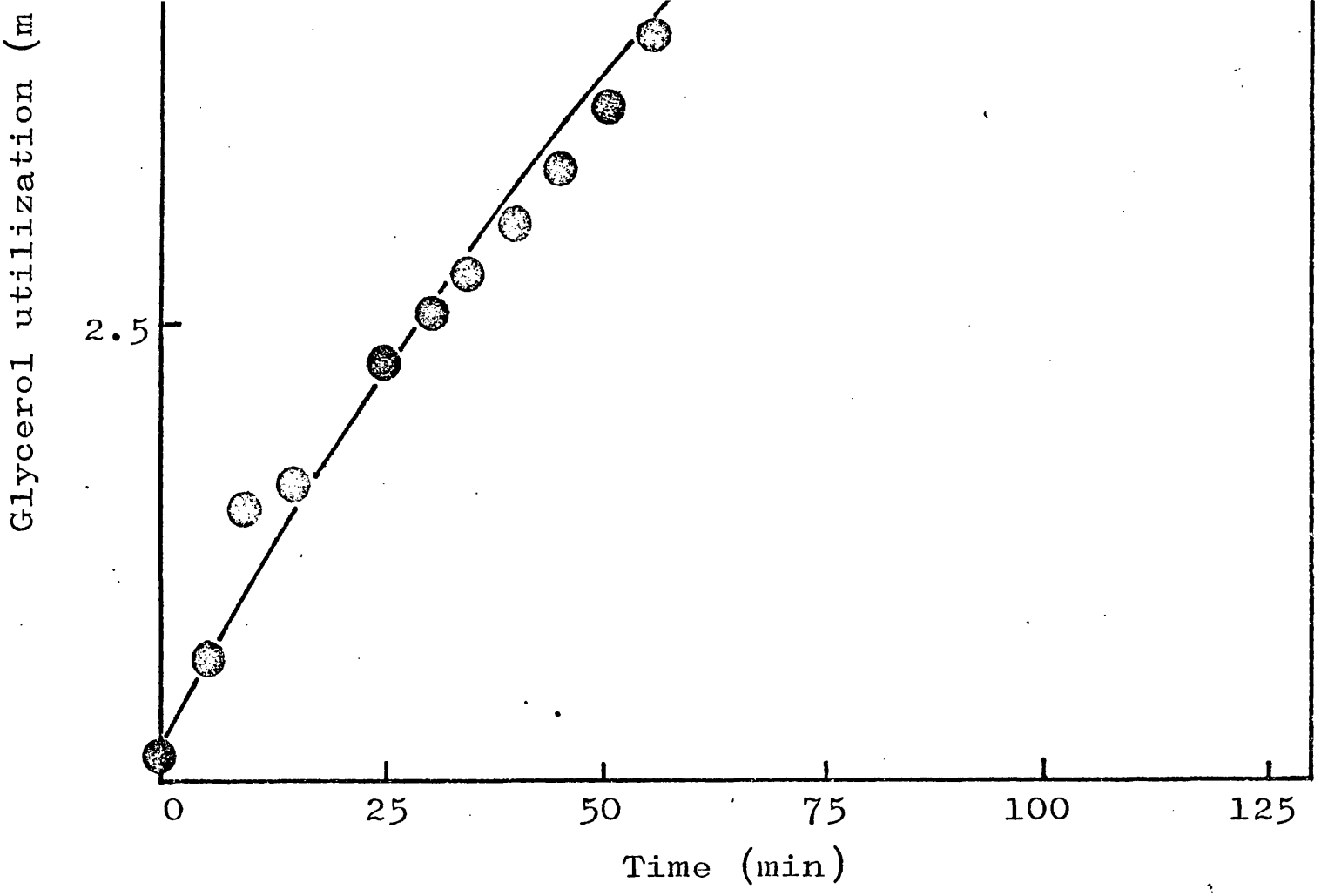
○ glycerol utilization

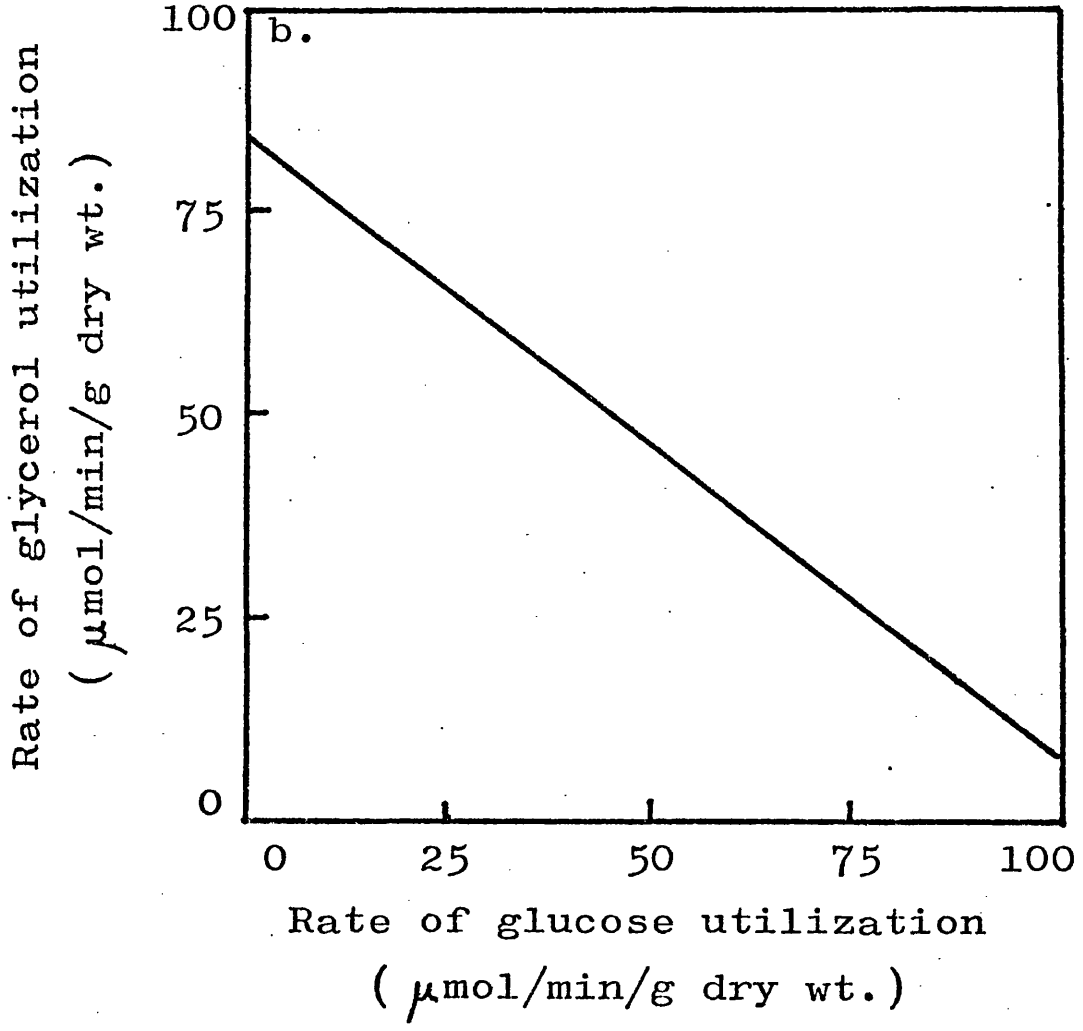
□ oxygen consumption

△ carbon dioxide production

Figure 34b

The rate of glycerol utilization at any time was plotted against the rate of glucose utilization at that time.


Glycerol utilization (m
2.5
0
25
50
75
100
125
Time (min)


b.
100
75
50
25
0
Rate of glycerol utilization
(μmol/min/g dry wt.)
0
25
50
75
100
Rate of glucose utilization
(μmol/min/g dry wt.)

This analysis indicated that the curve corresponded to a quadratic function of time. This quadratic function was used to calculate the rate of glycerol utilization at any time. The graph of the rate of glycerol utilization against the rate of glucose utilization (Figure 34b) gives a straight line. Duplicate experiments give values of -0.78 and -0.80 for the gradient of this line.

The rate of oxygen consumption was constant (103 μmol/min/g dry wt.), and the rate of carbon dioxide production increased at a constant rate (from 58 - 107 μmol/min/g dry wt.) during the course of the experiment.

13. Enzyme Level and Rate of Substrate Utilization in Washed Cell Suspensions of *E.coli* 15224 glp R^c

The relation between the rate of substrate utilization and enzyme specific activity was investigated in washed cell suspensions.

Neither the rate of glucose utilization (Figure 35a) nor the rate of glycerol utilization (Figure 35b) depended on the specific activity of their respective enzymes, a 20 fold increase (40 - 800 E.U./g dry wt.) in the specific activity of glycerokinase corresponding to a 2 fold increase (115 - 210 μmol/min/g dry wt.) in the rate of glycerol utilization.

14. Glycerol Utilization, Glucose Utilization and the Specific Activities of Glycerokinase and Glucose PTS on Glucose Challenge to *E.coli* 15224 Growing on Glycerol

Previous results suggest that the rate of glycerol utilization in the presence of glucose depends both on the specific activity of glucose PTS and of glycerokinase.

Glycerol utilization, glucose utilization and the specific activities of glycerokinase and glucose PTS were measured in cells of *E.coli* 15224 treated with CAP at various times after glucose challenge to glycerol growing cells.

The rate of glycerol utilization (μmol/min/g dry wt.) by these cultures was plotted against the ratio of the enzyme specific activities (Figure 36a). The rate of glycerol utilization decreased as the ratio glycerokinase/glucose PTS decreased.

Figure 35

ENZYME LEVEL AND RATE OF SUBSTRATE UTILIZATION IN WASHED CELL SUSPENSIONS OF E.coli 15224

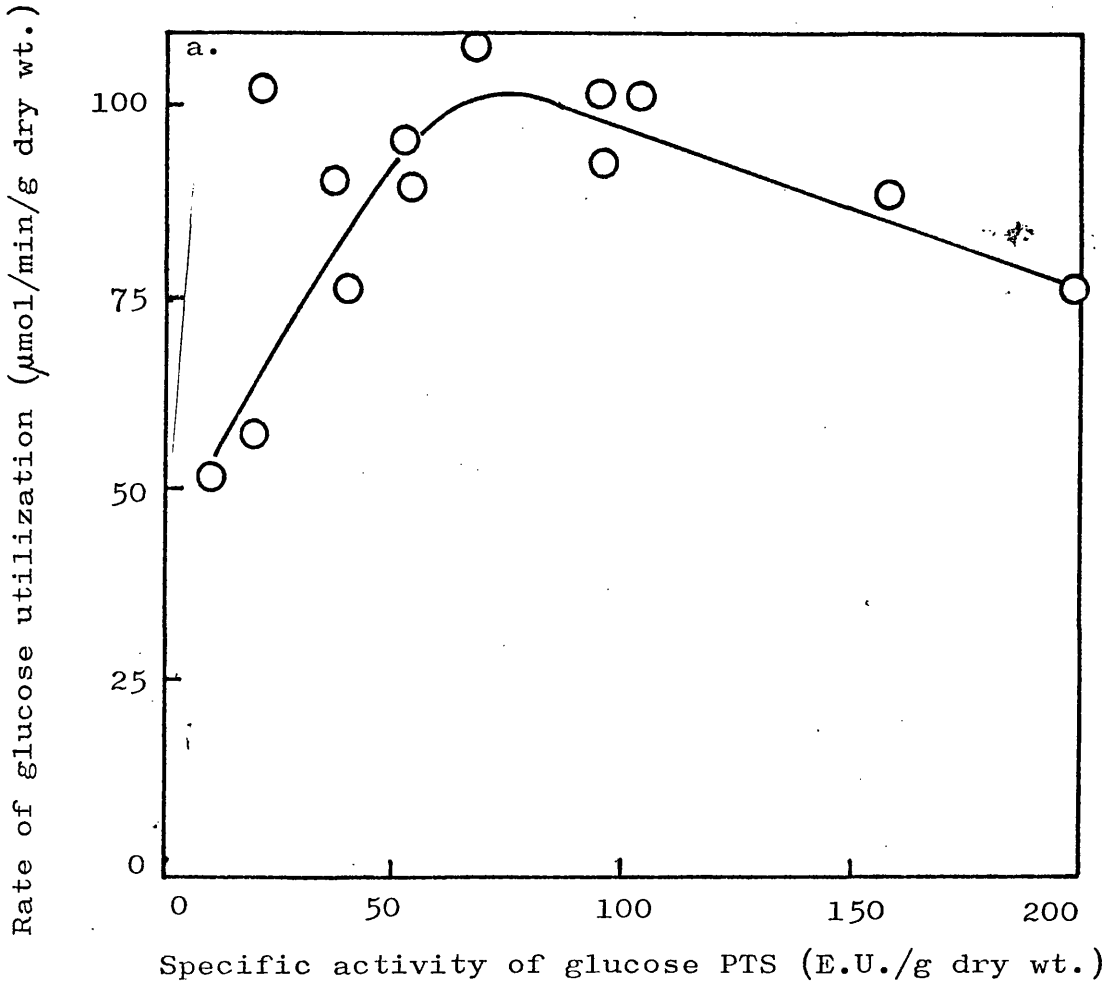
Cells of E.coli 15224 glp R^c trained to various carbon sources were inoculated into 200 ml CAP medium containing 3 mM glycerol or 3 mM glucose. Cells were inoculated to a turbidity of 0.4. Measurements of substrate utilization and the specific activities of glucose PTS and glycerokinase were made.

Figure 35a

Rate of glucose utilization and specific activity of glucose PTS.

Figure 35b

Rate of glycerol utilization and specific activity of glycerokinase.


a.
Rate of glucose utilization (μmol/min/g dry wt.)
100
75
50
25
0
0
50
100
150
200
Specific activity of glucose PTS (E.U./g dry wt.)

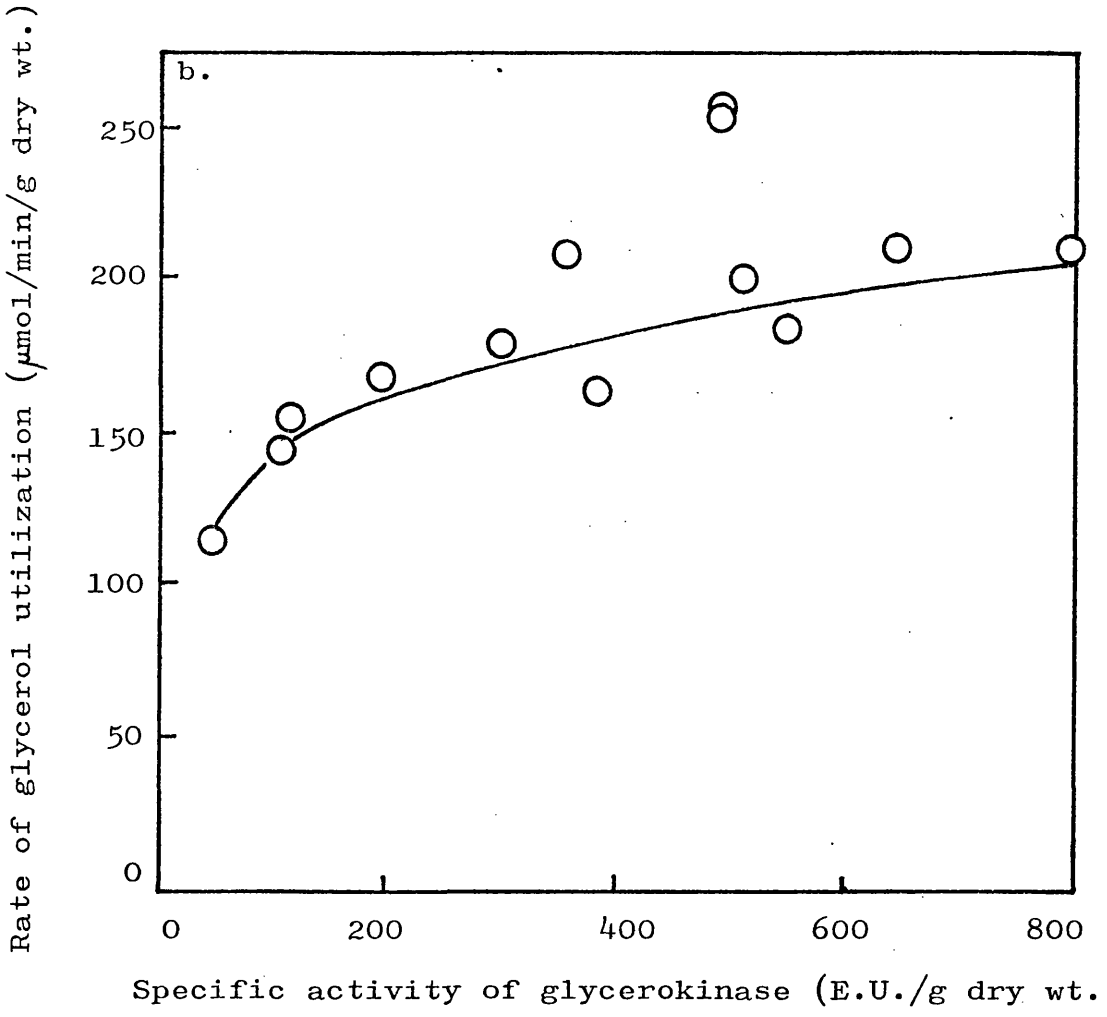
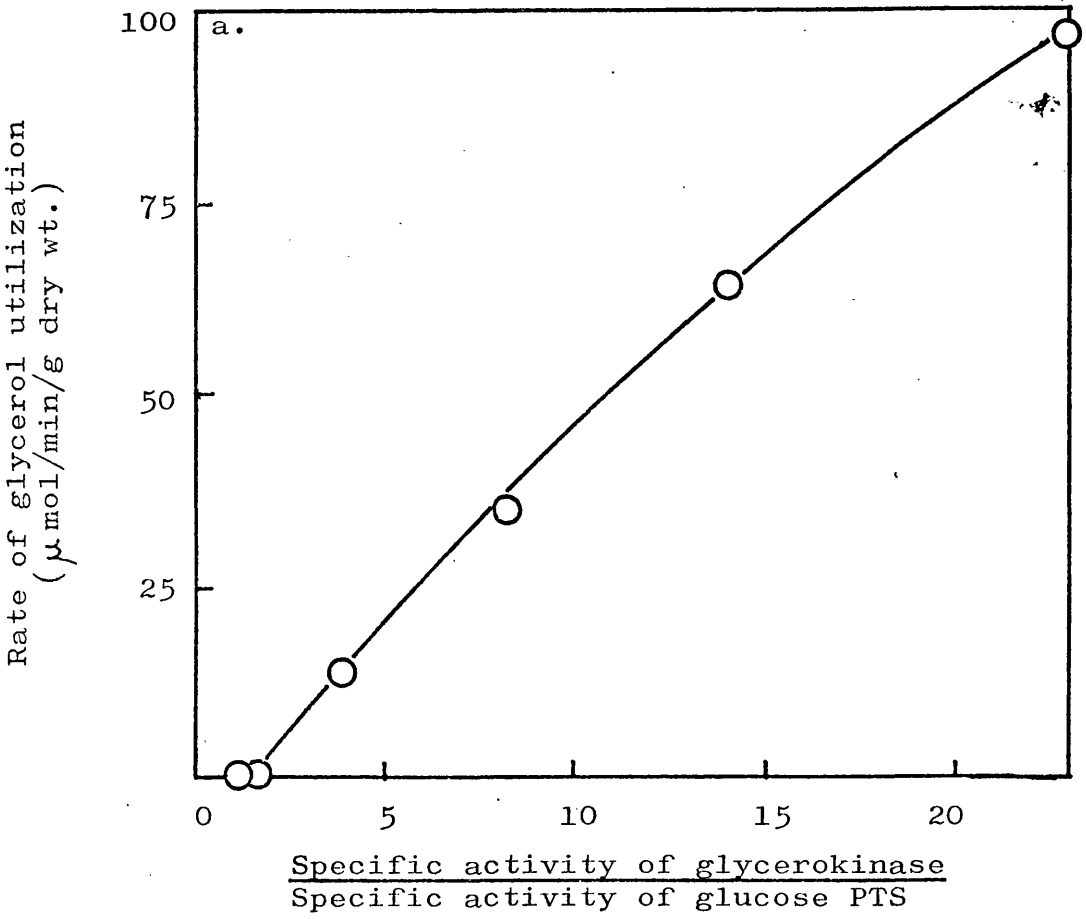
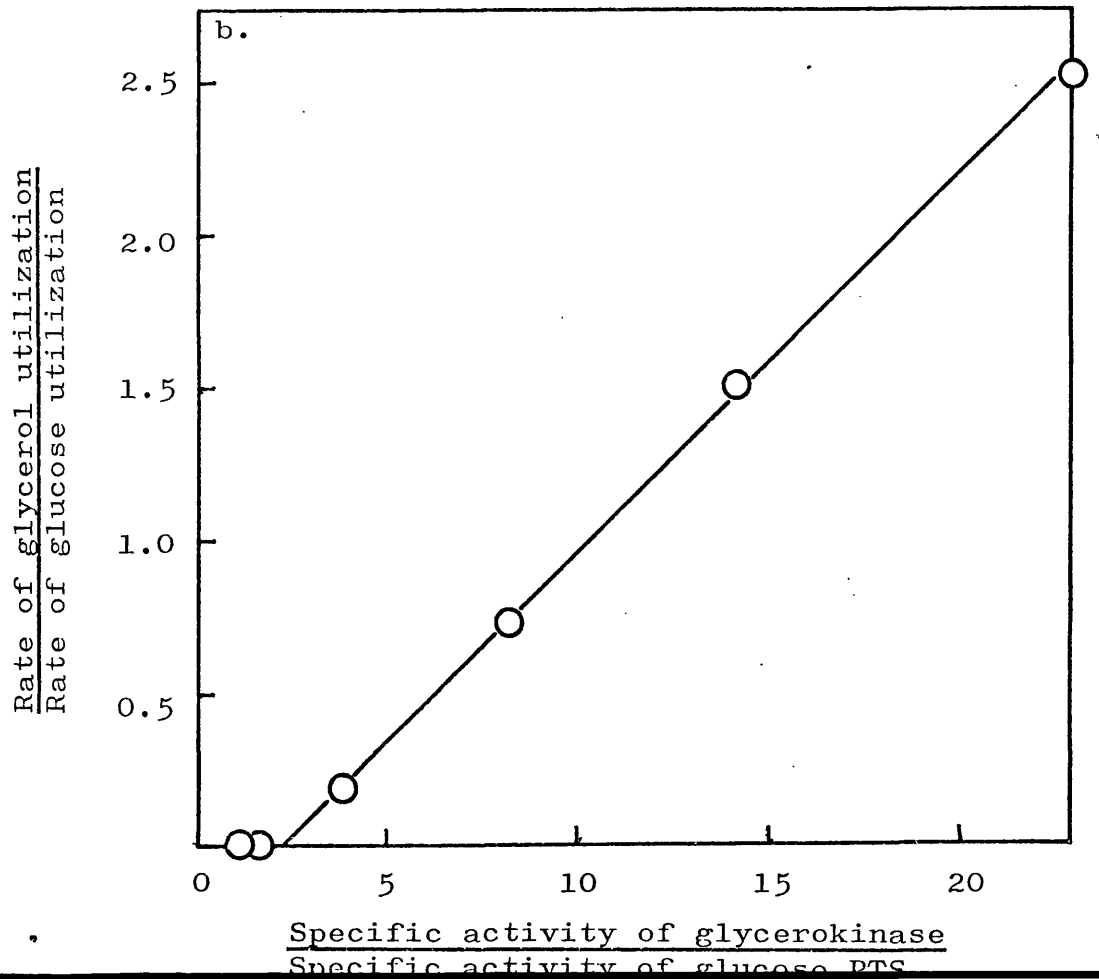

b.
Rate of glycerol utilization (μmol/min/g dry wt.)
250
200
150
100
50
0
0
200
400
600
800
Specific activity of glycerokinase (E.U./g dry wt.)

Figure 36

GLYCEROL UTILIZATION, GLUCOSE UTILIZATION AND THE SPECIFIC ACTIVITIES OF GLYCEROKINASE AND GLUCOSE PTS ON GLUCOSE CHALLENGE TO E.coli 15224 GROWING ON GLYCEROL

Glycerol utilization, glucose utilization and the specific activities of glucose PTS and glycerol PTS were measured in the cultures described in Figure 28b. The rate of glycerol utilization (Figure 36a) and the ratio of the rate of glycerol utilization and the rate of glucose utilization (Figure 36b) were plotted against the ratio of the enzyme specific activities.


a.
100
75
50
25
0
5
10
15
20
Rate of glycerol utilization
(μmol/min/g dry wt.)
Specific activity of glycerokinase
Specific activity of glucose PTS


b.
2.5
2.0
1.5
1.0
0.5
0
5
10
15
20
Rate of glycerol utilization
Rate of glucose utilization
Specific activity of glycerokinase
Specific activity of glucose PTS

The ratio of the rate of glycerol utilization to the rate of glucose utilization plotted against the ratio of the enzyme specific activities (Figure 36b) gives a straight line with a positive gradient.

15. Effect of an Uncoupling Agent on Glucose and Glycerol Utilization of *E.coli* 15224 glp R^c

The effect of an uncoupling agent (FCCP) on the rate of utilization of glycerol or glucose was studied in washed cell suspensions of *E.coli* 15224 glp R^c

The results, summarised in Figure 37, are compared with the effect of glucose on glycerol utilization and the effect of glycerol on glucose utilization.

FCCP partially inhibited (75% inhibition) glycerol utilization in glycerol trained cells and totally prevented glycerol utilization in glucose trained cells. FCCP inhibited glucose utilization by 70% in glycerol trained cells but by only 37% in glucose trained cells.

Figure 37

CARBONYL CYANIDE p-TRIFLUOROMETHOXYPHENYL HYDRAZONE (FCCP) INHIBITION AND MUTUAL INHIBITION OF GLUCOSE AND GLYCEROL UTILIZATION IN WASHED CELL SUSPENSIONS OF E.coli 15224 glp R^c

Cells of E.coli 15224 glp R^c trained to glucose or glycerol were harvested, washed and inoculated into CAP medium to a turbidity of 0.4. 3 mM glycerol or 3 mM glucose in the presence or absence of 20 μM FCCP, and 3 mM glycerol plus 3 mM glucose were added to washed cell suspensions. Measurements of substrate utilization were made.

Cells trained to	Rate of glycerol utilization (μmol/min/g dry wt.)			Rate of glucose utilization (μmol/min/g dry wt.)		
	Control	+FCCP	%	Control	+FCCP	%
Glycerol	192	47	25	100	30	30
Glucose	116	0	0	92	58	63
	Control	+Glucose	%	Control	+Glycerol	%
Glycerol	250	116	47	100	50	50
Glucose	116	0	0	92	92	100

DISCUSSION

1. Aerobic Growth on Glycerol

1.1. General aspects

Cells of E.coli 15224 trained to glycerol grow immediately on inoculation into minimal salts medium containing glycerol as sole source of carbon and energy. Growth is exponential with time (Figure 4) and is a linear function of substrate utilization (Figure 5b). On exhaustion of the defined carbon source growth ceases. Oxygen consumption and carbon dioxide production are proportional to the amount of cell material present (Figure 5a). 47% of the glycerol utilized is incorporated into the macromolecules (Figure 6, 7b) of the cell of which 62% is comprised of protein, 30% of nucleic acid and 11% of lipid (Figure 17b). 34% of glycerol carbon is lost as carbon dioxide and the remainder comprises the acid soluble pools. The value of the molar growth yield of oxygen derived from this culture (Figure 7a) can be used to calculate the $^{P}/_{O}$ ratio for growth of E.coli on glycerol. A growth yield (Y_{ATP}) of 10.5 $\pm$ 2.1 g dry wt./mol ATP has been observed by Bauchop & Elsden (1960) for anaerobic growth of Streptococcus faecalis, Saccharomyces cerevisiae and P.lindneri. Determination of Y_{ATP} for aerobic growth has proved much more difficult; results for different organisms are sometimes difficult to interpret and occasionally evidence is conflicting (Stouthamer, 1969; Forrest & Walker, 1971). The observations of Hempfling (1970) that $^{P}/_{O}$ ratios in E.coli vary (0.2 - 3.3) under conditions of varying catabolite repression has added

another difficulty to this sort of observation. Nevertheless, if the Y_{ATP} is constant for all bacteria, a comparison of the Y_{O_2} and the Y_{ATP} shows a $^{P}/_{O}$ ratio of 1.5 (Figure 7a) for E.coli 15224 growing aerobically on glycerol. No data are available in the literature for the $^{P}/_{O}$ ratio of E.coli growing on glycerol, however a value of 1.5 has been reported for Aerobacter aerogenes (Stouthamer, 1969).

1.2. Regulation of glycerol utilization

Zwaig and Lin (1966) reported that glycerokinase is inhibited by FDP and it was further postulated (Zwaig, Kistler & Lin, 1970) that feedback inhibition of glycerokinase by FDP limited the rate of glycerol metabolism during growth in simple salts medium containing glycerol. Inhibition of glycerokinase from E.coli 15224 by FDP was therefore investigated. FDP inhibited glycerokinase activity in sonicated extracts of E.coli to a maximum of 85%, had a dissociation constant of 0.34 mM and an $i_{0.5}$ (the inhibitor concentration at half-maximum inhibition) of 0.45 mM (Figures 8, 9). Although Zwaig & Lin (1966) did not report less than 100% inhibition, the results agree well with those for purified glycerokinase, from E.coli K12, obtained by Thorner & Paulus (1973) who found a maximum inhibition of 80% and an $i_{0.5}$ of 0.5 mM.

Glycerokinase extracted from E.coli 15224 glp R^cK^i is maximally inhibited by FDP to 85% with a dissociation constant of 1.5 mM (Figure 10). The mutation to K^i is therefore most probably due to a minor change in amino acid composition close to the binding site for FDP which reduces the affinity of the enzyme for FDP 5 fold.

Although glycerokinase from E.coli is inhibited by FDP, under no circumstances can FDP totally prevent glycerokinase activity. Before the role of FDP in the control of glycerol utilization can be elucidated further, the concentration of FDP in the cell must first be estimated.

1.2.1. Intracellular FDP concentration

1.2.1.1. Validity of intracellular metabolite estimations

Intracellular metabolites were measured by enzyme linkage to NADP, the reduced form of which was estimated by fluorescence spectrophotometry. This technique enables measurements of concentrations less than 10^{-6} M with a signal to noise ratio greater than 100.

It is important in any estimation of intracellular metabolite concentration that quenching of enzyme activity should be as rapid as possible to avoid spurious changes in pool sizes induced by experimental manipulation. Maitra (Harrison & Maitra, 1969) stated that it takes less than 0.5 s for PCA to inactivate the enzyme system in yeast. Presumably inactivation will take no longer for E.coli. Even if all the enzymes are not destroyed almost immediately it is unlikely that they will have much activity at the low temperature ($0^{\circ}C$) and pH of the acid extract.

It takes less than 3 s to transfer a sample from the culture to PCA. During this period of time the composition of the external environment of the cells will stay essentially constant, the most rapid change being in the dissolved oxygen concentration. The solubility of oxygen is 220 n mol/ml at $37^{\circ}C$ and 0.2 atmosphere pressure

(equivalent to 1 atmosphere pressure of air)(Hogman et al, 1959). According to Kempner (1937) the rate of respiration in E.coli at 37°C falls off below an oxygen concentration of 8.2 μM which means that 96% of the oxygen in solution can be utilized before the respiration rate of cells will be affected. The maximum rate of oxygen consumption encountered is 432 n mol/min/ml culture for a culture at a turbidity of 5.0 and utilizing glycerol. Thus respiration of these cells would only be affected if sampling takes longer than 30 s. For most samples the rate of oxygen consumption is less, and changes in dissolved oxygen concentration will not affect metabolism in the 3 s taken for sampling the cultures. Thus intracellular metabolite concentrations should remain constant during sampling.

I therefore suggest that the sampling and extraction procedures are such that the estimated level of FDP in the extract will reflect the intracellular concentration in the cell at the time of sampling. Estimation of the concentration of FDP in the cell depends on the determination of the amount of cell water. Winkler & Wilson (1966) estimated cell water using inulin. This compound does not penetrate cell walls and thus does not allow for the volume of water associated with cell wall and periplasmic space.

Stanier et al (1971) quote that most Gram negative bacteria have a cell wall 10 - 30 nm thick. Assuming E.coli to be a cylinder of dimensions 1.2 x 3.0 μm (Stanier et al, 1971) with hemispherical ends and to have a cell wall 30 nm thick, it can be calculated that 11% of the total water of the cell is associated with the cell wall. All

intracellular concentrations calculated on the basis of the results of Winkler & Wilson (1966) will therefore be slightly lower (probably less than 11%) than the actual concentration in the cell but will be accurate relative to each other. Moses & Sharp (1972) reported that a large number of metabolites are excreted by cells of E.coli into the extracellular medium. Their results indicate that di-and tri-phosphorylated compounds are retained within the cell membrane whereas monophosphorylated compounds appear in the extracellular medium. In particular we find (data not shown) that although some metabolites are excreted by E.coli utilizing glycerol, glucose or both no FDP is detectable in the extracellular medium.

1.2.1.2. Intracellular FDP concentration and rate of glycerol utilization

It has been postulated (Zwaig, Kistler & Lin, 1970) that the intracellular concentration of FDP controls the rate of substrate utilization in cells utilizing glycerol as the sole source of carbon and energy.

The rate of glycerol utilization will be directly proportional to the level of glycerokinase in the cell (that is the rate of glycerol utilization/enzyme unit will be constant) if, and only if, no inhibition of enzyme activity occurs. The rate of glycerol utilization/enzyme unit varies widely (0.4 - 2.4 μmol/min/E.U.)(Figure 11) unless inhibition of glycerokinase activity due to the intracellular FDP concentration is taken into account, when an approximately constant value is obtained (2.0 - 2.4 μmol/min/E.U.).

These results lead to a circular argument in which if we assume that the measured intracellular FDP concentrations are accurate we can lend support to the hypothesis that the FDP concentration regulates the rate of glycerol utilization, and if we assume that the rate of glycerol utilization is regulated by the intracellular FDP concentration we can substantiate our arguments that the measured intracellular concentrations of FDP reflect the true concentrations in the cells.

Our discussion of the method of measuring intracellular concentrations leads us to believe that the estimated concentration reflected the true concentration of the metabolite in the cells. Thus the results shown in Figure 11 substantiate the hypothesis that the intracellular FDP concentration regulates the rate of metabolism in cells utilizing glycerol as the sole source of carbon and energy.

At first sight it is perhaps surprising to note that in glycerol growing cells, with an intracellular FDP concentration of 1.07 ± 0.14 mM (Figure 11), glycerokinase activity is inhibited by about 70%. Koch (1971) argues that in its natural ecosystem, an average doubling time for E.coli of once or twice a day is all that is permitted by the volume of, and flow through, the intestine and because of the power of 'Malthusian' growth, much of its life must be spent under conditions of chronic starvation. From this fact and the consideration that natural selection must act almost purely in favour of rapid growth for an organism in such an ecological niche, E.coli must be highly efficient in utilizing nutrients. High levels of

glycerokinase favour the efficient use of low concentration of glycerol. However, any sudden increase in glycerol concentration, and hence rate of glycerol utilization, leads to formation of methylglyoxal and cell death (Freedberg et al, 1971; Zwaig et al, 1970) unless glycerokinase activity is subject to feedback inhibition. Thus inhibition of glycerokinase by FDP may have evolved as a protective mechanism to enable E.coli to cope with a 'feast and famine' existence.

2. Inhibition of Glycerol Utilization by Glucose

2.1. General considerations

Growth in any environment containing a number of potential carbon and energy sources requires the operation of numerous enzymic reactions some of which must be susceptible to control, thus permitting the overall rate of metabolism, and the rate of metabolism of individual carbon and energy sources, to be regulated. The essence of these controls is illustrated in the phenomenon of diauxie (Monod, 1947) where, when cells are presented with two carbon sources, one substrate is utilized exclusively until exhaustion before growth begins on the second substrate.

The control mechanisms of induction and repression operating during diauxic growth have been well characterised (Richmond, 1968; Paigen & Williams, 1970) but constitute relatively insensitive mechanisms for control of substrate utilization, requiring many generations before a new steady state can be established. A more sensitive and rapid control of enzyme activity ('catabolite inhibition') has been described for E.coli by McGinnis & Paigen (1969), Gaudy et al (1963) and Stumm-Zollinger (1966), for Hydrogenomonas by Blackkolb and Schlegel (1968) and for Clostridium tetanomorphum by Anthony & Guest (1968). 'Catabolite inhibition' describes the ability of one carbon source to inhibit, or prevent, the utilization of a second carbon source by cells containing the enzymes necessary for catabolism of that carbon source.

Growth of E.coli on a mixture of glucose and glycerol is diauxic (Figure 12), glucose being utilized first until exhaustion before growth on glycerol commences. Glycerol

utilization is inhibited by glucose and this is clearly illustrated on glucose challenge to cells of E.coli 15224 growing on glycerol (Figure 13). Glucose reduces and prevents glycerol utilization in these cells even although all the enzymes required for glycerol catabolism are present. The inhibition is reversible, and the full rate of glycerol utilization is restored after exhaustion of glucose (Figures 15, 16).

It is perhaps surprising that the rate of glycerol utilization per unit mass of cells is the same before addition, as after exhaustion of glucose, even although the level of glycerokinase has decreased 4 fold (Figures 15, 16). However, this can be accounted for by changes in the concentration of the intracellular pool of FDP when glucose is exhausted and cells enter diauxic lag (Figure 13). A decrease in the intracellular concentration of FDP leads to an increase in the activity of glycerokinase thus maintaining the rate of glycerol utilization per unit mass of cells constant. These results illustrate the efficiency of high glycerokinase activity linked to feedback inhibition as a regulatory mechanism maintaining an adequate flow of glycerol carbon into the cell.

The power of glucose to dominate glycerol metabolism is further illustrated in a mutant strain of E.coli constitutive for the enzymes of glycerol utilization (E.coli 15224 glp R^c). Glucose totally prevents the utilization of glycerol by cells of this mutant trained to glucose despite the retention of ability to metabolize glycerol in the absence of glucose (Figure 18).

It was proposed by McGinnis & Paigen (1969) that the site of 'catabolite inhibition' was either at the first metabolic conversion or at the level of entry of substrate into the cell.

The first catabolic reaction in glycerol utilization is phosphorylation to L-α-glycerophosphate (L-α-GP) by glycerokinase. Glucose does not prevent L-α-GP utilization on glucose challenge to cells of E.coli 15224 growing on L-α-GP (Figure 19a), nor does it affect the rate of L-α-GP utilization in washed cell suspensions of E.coli 15224 (Figure 19b) when, under similar circumstances, glycerol utilization is totally inhibited by glucose. These results indicate that the site of inhibition of glycerol utilization is proximal to the convergence of the catabolic pathways of L-α-GP and glycerol. It follows that the site of inhibition is at the level of glycerokinase activity.

This conclusion can be deduced from the specificity of the method used for estimation of glycerol. The assay is specific for glycerol and thus measurement of the rate of glycerol utilization is in effect a measurement of the rate of conversion of glycerol to L-α-GP by glycerokinase. The possibility of regulation at the level of entry of glycerol into the cell is not eliminated by identification of glycerokinase as the regulatory site for glucose inhibition of glycerol utilization. Glucose has been shown to inhibit the entry of a number of compounds, notably galactose (Horecker, Thomas & Monod, 1960), galactosides (Kessler & Rickenberg, 1963), histidine (Ames, 1964), tryptophan (Boezi & De Moss, 1961), maltose and sucrose (Egan & Morse, 1966).

The activity of glycerokinase can be regulated either by decreasing the availability of substrate, for example by limiting the rate of permeation of glycerol, or by increasing the intracellular concentration of some metabolite of glucose which inhibits glycerokinase.

Having established the factors which could account for the inhibition of glycerol utilization by glucose, experiments were designed to determine which of these factors mediated this inhibition.

2.2. Intracellular metabolite concentrations

2.2.1. Intracellular FDP concentration on glucose challenge

FDP is the only known feedback inhibitor of glycerokinase in *E.coli* (glucose does not affect enzyme activity)(Zwaig & Lin, 1966; Thorner & Paulus, 1973). Zwaig, Kistler & Lin (1970) suggest that as glucose gives rise to FDP, glucose metabolism can suppress phosphorylation of glycerol and thus prevent glycerol utilization.

It has been shown (Figures 8, 9; Thorner & Paulus, 1973) that FDP inhibits glycerokinase to a maximum of 85%. It has also been shown that the intracellular concentration of FDP in glycerol growing cells is 1.07 ± 0.14 mM (Figure 11) resulting in a 70% inhibition of glycerokinase activity. It is thus evident that even if the addition of glucose to glycerol growing cells resulted in a 10 fold increase in the intracellular FDP concentration the rate of glycerol utilization could be reduced by no more than half.

The intracellular FDP concentration was therefore determined after glucose challenge to cells growing on or utilizing glycerol to elucidate the role played by FDP in the inhibition of glycerol utilization by glucose.

On glucose challenge to cells of E.coli 15224 growing on glycerol the level of glycerokinase in the culture remains constant (Figure 14) but the rate of glycerol utilization/enzyme unit decreases to zero. This will only occur if there is a progressive increase in the inhibition of glycerokinase activity. The intracellular FDP concentration increases slightly (Figure 20) from 1.07 $\pm$ 0.14 mM before glucose challenge to about 1.3 mM when glycerol utilization has ceased. Even 120 min after glucose challenge, when glycerol utilization has been totally prevented for 60 min, the intracellular concentration of FDP is only 1.4 $\pm$ 0.2 mM. Thus the increase in FDP concentration (from a minimum of 0.93 mM to a maximum of 1.6 mM) would only lead to a decrease in glycerokinase activity from 34% to 25% (Figure 8), a decrease of 28%.

The above results are confirmed in washed cell suspensions. The intracellular concentration of FDP does not change markedly (Figure 21) on glucose challenge to cells utilizing glycerol, glycerokinase activity changing by less than 10% in all cases. Glucose inhibits glycerol utilization by more than 48% (48 - 100%) in every case. It is perhaps surprising that the intracellular FDP concentration does not change on glucose challenge even when the concentration of FDP during glycerol utilization is very low (0.1 mM in glucose trained cells of E.coli 15224 glp R^c, Figure 21). These results were obtained both for duplicate samples assayed at the same time in the same way and using the same reagents as for the rest of the samples and for a repetition of the

experiment on a different day. The results therefore appear to be a true reflection of the intracellular concentration of FDP, implying that the FDP concentration is determined, at least to some extent, by the pre-history of the cells rather than by the nature, or the rate of utilization, of the carbon source.

Lowry et al (1971) and Moses & Sharp (1972) have also shown that, in a variety of strains of E.coli, there is little difference in the intracellular FDP concentration between glycerol growing and glucose growing cells.

It follows from the results presented that the intracellular concentration of FDP cannot account for the inhibition of glycerol metabolism on glucose challenge to cells utilizing glycerol unless the sensitivity of glycerokinase to FDP is affected by the pre-history of the cells. FDP inhibits glycerokinase activity extracted from E.coli 15224 to the same extent before and 90 min after glucose challenge to glycerol growing cells, and to the same extent in cells of E.coli 15224 glp R^c trained to glycerol or glucose (Figure 22).

Thus although FDP inhibition of glycerokinase constitutes a regulatory mechanism controlling the flux of glycerol into the cell when this compound is utilized as the sole source of carbon and energy it does not mediate the ability of glucose to inhibit, and prevent, glycerol utilization when both these substrates are presented to the cell.

2.2.2. Intracellular G6P & F6P concentration

Two criteria must be satisfied if changes in the intracellular concentration of a metabolite of glucose

are to be considered as the mechanism by which glycerol utilization is inhibited by glucose.

i) The intracellular concentration of the metabolite must increase on glucose challenge to cells utilizing glycerol and must increase progressively after glucose challenge to growing cells

and ii) the ability of the metabolite to inhibit glycerokinase activity in cell extracts must be demonstrated.

The intracellular F6P concentration shows little change on glucose challenge either to cells of E.coli 15224 growing on glycerol (0.18 mM - 0.26 mM, Figure 23) or to cells of E.coli glp R^c utilizing glycerol (0.7 - 0.9 mM, Figure 24). The intracellular concentration of G6P increases 6 fold (0.5 - 3.2 mM, Figure 23) on glucose challenge to cells of E.coli 15224 growing on glycerol, before falling to a constant value (2.32 mM) within 20 min of challenge. The intracellular concentration of G6P also increases (Figure 24) on glucose challenge to washed cell suspensions of E.coli 15224 glp R^c.

Lowry et al (1971) have shown that, for E.coli K12, the concentration of G6P increases 5 fold on glucose challenge to glycerol growing cells. Their method gives values of the intracellular G6P concentration of 0.16 mM rising to 0.85 mM on glucose challenge. These values were obtained from extracts of cells collected on millipore filters. The filtration step required 30 - 60 s for completion and during this time the external environment of the cell must be undergoing considerable changes, especially in the dissolved oxygen concentration, which may affect the intracellular concentration of metabolites.

However in the results presented (Figures 23, 24) no account was taken of the concentration of G6P in the extracellular fluid. Moses & Sharp (1972) have estimated that approximately 50% of the total G6P in a culture of E.coli is present in the external medium. We have found a value of about 70% (data not shown) for cells utilizing glucose or glycerol. If this is taken into account our results and those of Lowry et al (1971) are in good agreement.

The intracellular concentration of both G6P and F6P change rapidly on glucose challenge to cells growing on glycerol (Figure 23) and a new steady state is achieved at least 40 min before glycerol utilization ceases.

Neither G6P nor F6P show any ability to inhibit glycerokinase activity in cell extracts (data not shown) or in purified glycerokinase preparations (Zwaig & Lin, 1966; Thorner & Paulus, 1973).

Thus neither G6P nor F6P satisfy the criteria for consideration as the mechanism by which glucose inhibits glycerol utilization although the increase in G6P concentration on glucose challenge partially fulfils these requirements.

2.3. Inhibition by limitation of substrate availability (ATP, glycerol)

2.3.1. Limitation of intracellular ATP

Glycerokinase has 2 K_m's for ATP, 0.1 mM and 0.5 mM (Thorner & Paulus, 1973). If the intracellular concentration of ATP is to mediate glucose inhibition of glycerol utilization, the ATP concentration must be in the region of these K_m's and must decrease on glucose challenge. The intracellular ATP concentration does not change

significantly on glucose challenge to cells of E.coli 15224 growing on glycerol (Figure 23) and increases on glucose challenge to cells of E.coli 15224 glp R^c utilizing glycerol (Figure 24). The concentration of ATP in glycerol trained cells is 3.7 mM, more than 7 fold the value of the K_m and thus substantial changes in intracellular concentration are required if glycerokinase activity is to be affected.

However it is not ATP itself but the magnesium ATP complex that is the true phosphate donor for glycerokinase. Only the total ATP concentration and not that of the Mg^{2+} ATP complex is estimated by the method described. Sols & Marco (1970) stated that the intracellular concentration of free Mg^{2+} ions depends to a large extent on the concentration of potential chelating agents, probably the most important being citrate. If the concentration of citrate or some other chelating agent increases on glucose challenge to cells utilizing glycerol this may affect the intracellular Mg^{2+} ATP concentration and hence glycerokinase activity. However the concentration of citrate in glucose growing cells has been estimated (Lowry et al, 1971) to be in the region of 10 mM and the total concentration of Mg^{2+} ions to be about 150 mM (Nelson & Kennedy, 1971). Thus, if only the concentration of citrate is taken into account, the amount of free Mg^{2+} ions appear to be sufficient to totally saturate all the intracellular ATP. However the amount of Mg^{2+} bound to protein, and by other chelating agents, cannot be accounted for and limitation of Mg^{2+} ATP as a means of regulating glycerokinase activity remains a possibility until further information is available.

2.3.2. Limitation of glycerol availability

2.3.2.1. General considerations

The rate of conversion of glycerol to L-α-GP by glycerokinase may be regulated by the rate of entry of glycerol into the cell.

Glucose does not exclude glycerol from the intracellular water space in E.coli 15224 glp R^c (Figure 27b) when glycerol utilization is completely prevented. Although this result suggests that glucose does not create a total permeability barrier to glycerol, it is the rate of entry of glycerol into the cells that is important if glycerokinase activity is to be regulated by this means.

2.3.2.2. Permeation rate of glycerol

Sanno, Wilson and Lin (1968) report that glycerol enters the cell by a facilitated diffusion system induced during growth on glycerol. The rate of free diffusion of glycerol was therefore measured in cells which did not contain this system. The permeation rate was estimated in cells of E.coli 15224 trained to and growing on glucose, which contain only basal levels of glycerol enzymes, and was assumed to represent the rate of free diffusion of glycerol. Several lines of evidence support this assumption.

i) The permeability constants for free diffusion of a number of compounds were estimated in E.coli 15224 trained to and growing on glucose. When these values are related to the molecular weight and the number of potential hydrogen bond forming groups of the permeant (Figure 25), the rate of free diffusion of any compound into these cells can be predicted within a factor of 5 (Stein, 1967). The

measured values for the permeability constant of glycerol (Figure 25) all lie within a factor of 4.3 of that predicted from its molecular weight and number of potential hydrogen bond forming groups. Thus glycerol appears to enter these cells by free diffusion.

No data are available in the literature for calculating parameters of permeation of small molecular weight compounds into cells of E.coli. However the values of $PM^{\frac{1}{2}}_{max}$ $(0.010 \begin{smallmatrix} + 0.058 \\ - 0.005 \end{smallmatrix})$, the reduction in permeation rate/pair of hydrogen bonds $(8.3 \begin{smallmatrix} + 4.2 \\ - 1.1 \end{smallmatrix})$ and the free energy change $\Delta F^{\ddagger}$/hydrogen bond $(0.77 \begin{smallmatrix} + 0.16 \\ - 0.05 \end{smallmatrix})$ (Figure 26) calculated from the data of Figure 25 are consistent with those for the alga Chara ceratophylla ($PM^{\frac{1}{2}}_{max} = 0.009$ cm.s^{-1} $mol^{\frac{1}{2}}$; reduction in permeation rate/pair of hydrogen bonds = 9.1, $\Delta F^{\ddagger}$/hydrogen bond = 0.8 Kcal/mol) calculated by Stein (1967). The value of $PM^{\frac{1}{2}}_{max}$ for E.coli membranes is an order of magnitude less than that for Bovine erythrocytes and the internodal cells of the alga Tollypellopsis stelligera, but is of the same order as that for unfertilized eggs of the sea urchin Arbacia punctulata and for 'erythrocytes' of Phascolosoma (Stein, 1967).

The possibility cannot be excluded that some of the compounds used to determine free diffusion, for example ethylene glycol or 1,2,4-butanetriol, may act as analogues of glycerol thus leading to a false picture of diffusion.

Data for diffusion into cells of E.coli were obtained using a high concentration of permeant (800 mM). It is feasible that diffusion at low concentration of permeant (less than 10 mM) may have different characteristics. Measurement of permeation rates at low concentrations

requires a radiochemical method. Unfortunately the exit rate of glycerol from E.coli 15224 cannot be estimated by this method as the intra- and extracellular concentration reaches equilibrium extremely rapidly (less than 10 s). At least 10 s is required for filtration and washing of samples, with a further 10 s required for transfer to scintillation fluid, before a second sample can be filtered. However the exit rate of erythritol is measurable by this technique. The rate constant for entry of 800 mM erythritol at 27°C is 0.0076 and for 10 mM erythritol at 30°C is 0.0093. These values are very close, the slight discrepancy probably being due to the 3 degree difference in temperature. Support for the view that the difference is due to temperature is obtained from the values calculated (Results, Section 7.1.) for the enthalpy change ($\Delta H^{\ddagger}$)/hydrogen bond (3.3 Kcal/mol) and for the entropy change ($\Delta S^{\ddagger}$)/hydrogen bond ($8.5 \begin{smallmatrix} + 0.1 \\ - 0.3 \end{smallmatrix}$ e.u./mol)(Figure 26). These values agree well with the enthalpy change/hydrogen bond (2.3 - 5.1 Kcal/mol) calculated for diffusion by Stein (1967) and for the increase in entropy due to formation of two kinetic units from a single unit (8 e.u./mol) by Lumry (1959).

It is concluded, therefore, that it is valid to assume that there is no difference between the permeability constants for free diffusion at high and low concentration of permeant.

ii) Alemohammad & Knowles (1974) have reported that the rate of glycerol permeation into cells of E.coli K12 trained to glucose is proportional to the applied glycerol concentration suggesting that glycerol enters these cells by free diffusion. The rate constant for entry of glycerol into glucose trained cells of E.coli K12

($0.40\ s^{-1}$) is in close agreement with that observed for glucose trained E.coli 15224 ($0.31\ s^{-1}$).

iii) If the permeability constant is expressed in terms of m mol/g dry wt./min per unit of concentration, the rate of free diffusion of glycerol can be calculated for any applied glycerol concentration. The rate of glycerol diffusion required for growth on glycerol is 0.28 m mol/g dry wt./min. The rate of diffusion of glycerol into glucose trained cells of E.coli 15224 was estimated to be 0.050 ± 0.005 m mol/g dry wt./min/mM. (Figure 27a), which suggests that the rate of free diffusion should become limiting below a glycerol concentration of about 5 mM. Richey & Lin (1972) isolated a mutant of E.coli K12 lacking the protein which mediates facilitated diffusion of glycerol. Thus glycerol only enters these cells by free diffusion. Richey & Lin (1972) show that the growth rate of this mutant is limited by the diffusion rate of glycerol only at concentrations less than 5 mM. Thus the estimated permeability constant for E.coli can be correlated with the rate of free diffusion of glycerol.

It is therefore concluded that glycerol permeates cells of E.coli 15224 trained to and growing on glucose by free diffusion alone and that measured permeability constants can be used to predict the rate of entry of glycerol into cells.

2.3.2.3. Permeation rate of glycerol and the ability of glucose to inhibit glycerol utilization

For glucose to prevent glycerol utilization by inhibition of facilitated diffusion, the rate of entry of glycerol by free diffusion must be very much less than the

rate of glycerol utilization in the absence of glucose. Conversely if the rate of entry by free diffusion is of the same order of magnitude as the rate of glycerol utilization, glucose cannot prevent glycerol utilization even if facilitated diffusion is inhibited.

The permeation constant for free diffusion of glycerol into cells of E.coli 15224 trained to and growing on glucose is 0.050 $\pm$ 0.005 m mol/g dry wt./min/mM (Figure 27a). Thus for 3 mM glycerol, the concentration used in most experiments, the rate of diffusion of glycerol (0.15 m mol/g dry wt./min) is greater than that required for utilization of glycerol by washed cell suspensions of glucose trained E.coli 15224 glp R^c (0.12 m mol/g dry wt./min), and is 55% of the rate of glycerol utilization during growth (0.28 m mol/g dry wt./min).

It therefore follows that glucose cannot prevent glycerol utilization at the level of glycerol permeation by inhibition of facilitated diffusion: The permeation rates of glycerol into cells of glycerol trained E.coli 15224 or glucose trained 15224 glp R^c are an order of magnitude greater than that into glucose trained 15224, and neither permeation rate is affected by the presence of 10 mM glucose (Figure 27a) even although glycerol utilization is completely prevented by glucose in glucose trained E.coli 15224 glp R^c. These cells are essentially anaerobic during estimation of permeation rates, and may not reflect the conditions found in aerobic cultures. However glucose does not affect the permeation rate of glycerol estimated in samples of cells of E.coli 15224 glp R^c trained to and growing aerobically on glucose

(Results, Section 9.2.). The permeation rate was estimated within 45 s of sampling. The cells were essentially aerobic during this period of time as the rate of oxygen consumption was such that aerobic metabolism continued for at least 80 s after sampling. However the possibility that the lack of ability of glucose to affect glycerol permeation is due to a large concentration ratio (80:1) cannot be excluded.

If glucose affects facilitated diffusion and the rate of free diffusion of glycerol is only limiting below 5 mM glycerol (Figure 27a; Richey & Lin, 1972) then the external concentration of glycerol will affect the ability of glucose to prevent glycerol utilization in washed cell suspensions of glucose trained E.coli 15224 glp R^c. The glycerol concentration (1.7 - 11 mM) does not affect the ability of glucose to prevent glycerol utilization (Results, Section 3.2.).

Thus not only is the rate of free diffusion of glycerol sufficient to account for the rate of glycerol utilization in the absence of glucose but glucose does not affect facilitated diffusion of glycerol. It follows that glucose does not inhibit glycerol utilization under any circumstances by regulation of the rate of entry of glycerol into the cells.

2.4. Conclusion

In conclusion, therefore, neither substrate limitation (limited permeation or reduced ATP) nor feedback inhibition by some catabolite of glucose metabolism (FDP, G6P, F6P) can explain the ability of glucose to inhibit and prevent glycerol utilization.

3. Adaptation of Cells During Glucose Challenge to *E.coli* Utilizing Glycerol

Total inhibition of glycerol utilization takes time to be established (Figure 13) after glucose challenge to cells of *E.coli* 15224 growing on glycerol. The glycerol utilized during this period is distributed throughout cell material and is both incorporated and converted to carbon dioxide (Figure 17). This time interval before cessation of glycerol utilization suggests that protein synthesis or cell growth may be important. Glucose inhibits glycerol utilization by about 50% in cells of *E.coli* 15224 treated with an inhibitor of protein synthesis, chloramphenicol (CAP), at the time of glucose challenge, but never abolishes it (Figure 28a).

Inhibition of glycerol utilization by glucose progressively increases as the time interval between glucose challenge and CAP addition increases, until glycerol utilization is totally prevented 60 min after challenge (Figure 28b). The inhibition is reversible and glycerol is utilized immediately on exhaustion of glucose. It is concluded that protein synthesis is therefore required before glucose can prevent glycerol utilization. However this may be an increase in a specific protein or may be due to dilution of glycerokinase activity due to the general protein synthesis required for growth.

Only a few enzymes are required for growth on glucose that are not also required for growth on glycerol. They are phosphofructokinase, and the glucose phosphotransferase system. It is not known whether the level of phosphofructokinase changes between glycerol and

glucose growing cells. However, glucose does not prevent glycerol utilization in mannose or ribose trained cells of E.coli 15224 glp R^c (Figure 30c) where glycerol utilization is totally prevented in glucose trained cells. Phosphofructokinase is required for growth on mannose and ribose as well as for growth on glucose. The level of this enzyme would therefore be expected to be similar in all cases and an increase in glucose inhibition of glycerol utilization could not be explained by changes in phosphofructokinase level.

The glucose phosphotransferase system (PTS) is the first step in the pathway of dissimilation of glucose. It mediates both transport and phosphorylation and thus its activity determines the rate of utilization of glucose. α-methylglucoside (α-MG) is an analogue of glucose which is transported and phosphorylated by the glucose PTS but is not metabolised further. During glucose challenge to cells of E.coli 15224 growing on glycerol, the ability of α-MG to prevent growth on glycerol (Figure 29) increases. α-MG does not significantly affect growth of cells of E.coli trained to and growing on glycerol. However 60 min after glucose challenge cells, although still able to grow on glycerol alone, do not grow in the presence of α-MG. McKinstry & Koch (1972) have shown that the ability of cells of E.coli K12 to transport α-MG increases 4 fold between glycerol and glucose trained cells. It follows that, on glucose challenge, the ability of cells to transport glucose increases as the ability of glucose to prevent glycerol utilization increases and, as α-MG progressively inhibits growth on glycerol, this inhibition

is mediated by operation of the glucose PTS. However α-MG may not be a realistic analogue of the glucose as this compound is not further metabolised after phosphorylation and can lead to a very rapid decrease in the intracellular concentration of ATP (data not shown) on addition to growing cultures, whereas glucose maintains a constant ATP level (Figure 23).

Glucose totally prevents glycerol utilization in washed cell suspensions of E.coli 15224 glp R^c trained to glucose but only inhibits it by 57% in glycerol trained cells. Direct measurement of the level of the glucose PTS (Figure 30a) confirms that glucose trained cells possess higher levels (5 times) of activity than glycerol trained cells. It would be misleading to conclude that the increase in the level of the glucose PTS thus mediates the increase in the ability of glucose to prevent glycerol utilization as there is also a concomitant decrease (10 fold) in the level of glycerokinase between glycerol and glucose trained cells (Figure 30a).

3.1. Glycerokinase or glucose PTS ?

Having demonstrated that an increase in the glucose PTS occurs on glucose challenge to cells of E.coli growing on glycerol it is pertinent to attempt to discriminate whether the increase in glucose PTS or decrease in glycerokinase is the important factor in the inhibition of glycerol utilization by glucose.

Cells of E.coli 15224 glp R^c trained to different carbon sources exhibit different levels of glycerokinase and glucose PTS which can be correlated with the ability of glucose to inhibit glycerol utilization. When the

level of glycerokinase is constant, glucose inhibition of glycerol utilization increases as the level of the glucose PTS increases (Figure 30b) whereas at constant glucose PTS level a negative correlation between glucose inhibition of glycerol utilization and the level of glycerokinase exists (Figure 30c).

It is therefore concluded that both the specific activity of the glucose PTS and of glycerokinase are important factors in determining the ability of glucose to inhibit glycerol utilization.

3.2. Effect of glycerol on glucose utilization

Although the discussion has been limited to the ability of glucose to inhibit glycerol utilization, it is important to note that glycerol inhibits glucose utilization, which is regulated by the activity of glucose PTS, under certain circumstances (Figure 31a). Glucose inhibits glycerol utilization by about 50% in glycerol trained cells and totally prevents it in glucose trained cells, whereas glycerol does not affect glucose utilization in glucose trained cells but inhibits it by 50% in glycerol trained cells. That is, glucose prevents glycerol utilization when cells contain high glucose PTS levels and low glycerokinase levels; and glycerol inhibits glucose utilization when cells contain low glucose PTS and high glycerokinase levels. Inhibition of α-MG uptake by, glycerol, in glycerol trained cells, has also been reported (Kessler & Rickenberg, 1963).

Glycerol totally prevents glucose utilization in glycerol trained *E.coli* 15224 *glp* R^c after 3 h treatment with CAP (Figure 31a) even although glucose is still

utilized in the absence of glycerol. The level of glycerokinase in the washed cell suspension remains constant during this period, whereas the glucose PTS level decreases by half in 120 min. The decrease in the rate of glucose utilization in the presence of glycerol (Figure 31a) corresponds to an increasing rate of glycerol utilization in the presence of glucose (Figure 31b). It follows that the decrease in ability of glucose to prevent glycerol utilization, and the increase in ability of glycerol to prevent glucose utilization, corresponds to the decrease in the level of the glucose PTS. The ability of glycerol to inhibit glucose utilization not only depends on the level of the glucose PTS but, when this is constant, depends on the level of glycerokinase (Figure 32).

It is concluded that glycerol and glucose mutually inhibit the utilization of each other, the extent of this inhibition depending on the relative amounts of the glucose PTS and of glycerokinase.

4. The Model

4.1. General considerations

It has been stated that glucose could regulate glycerokinase activity either by increasing the intracellular concentration of some metabolite of glucose which inhibits glycerokinase, or by limitation of the availability of substrate. It has been shown that the intracellular concentration of FDP (Figures 20, 21) does not change significantly on glucose challenge to cells of E.coli utilizing glycerol and that FDP could not totally prevent glycerol utilization by inhibition of glycerokinase activity (Figures 8 - 10) even if this were not so. It has also been shown that although the level of G6P increases on glucose challenge to cells utilizing glycerol (Figures 23, 24) neither the intracellular concentration of G6P nor that of F6P could explain glucose inhibition of glycerol utilization. The possibility that another feedback inhibitor of glycerokinase exists has been made unlikely by a fruitless but exhaustive search. All the intermediates of the glycolytic pathway (Zwaig & Lin, 1966), tricarboxylic acid cycle intermediates and their related amino acids, pyruvate, phosphoenolpyruvate, cyclic AMP, formate, lactate, acetyl phosphate, as well as acetyl coenzyme A, nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide, all at a concentration of 1 mM, have been shown to be ineffective in altering the activity of crystalline glycerokinase (Berman & Lin, 1971).

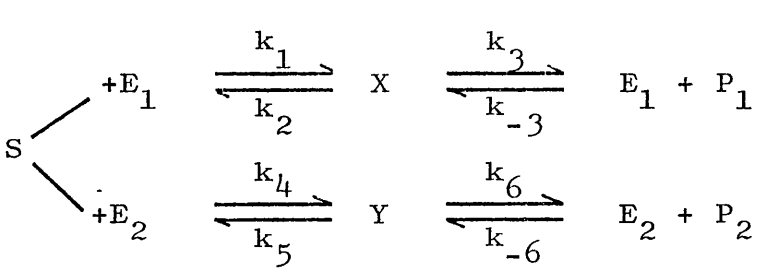
Glucose has been shown to inhibit the transport of a number of compounds, notably galactose (Horecker, Thomas & Monod, 1960), galactosides (Kessler & Rickenberg, 1963),

histidine (Ames, 1964), tryptophan (Boezi & De Moss, 1961), maltose and sucrose (Egan & Morse, 1966). The results obtained for permeation of glycerol (Figures 25 - 27) clearly show that glucose cannot and does not inhibit or prevent glycerol utilization by this means, at least under the conditions where permeation was measured.

The effect of glucose on the transport of galactose was explained in terms of a 'common carrier' by Horecker et al (1960). This was later extended to include galactosides by Winkler & Wilson (1967) and has most recently been restated for the interaction of maltose and lactose transport with that of glucose by McKinstry & Koch (1972). It is not feasible to seek to explain the effect of glucose on glycerol utilization in terms of a 'common carrier' as it has been shown that glycerol utilization is not controlled at the level of permeation. However the mutual inhibition of glycerol and glucose utilization (Figure 31) and the dependence of this on the relative amounts of glycerokinase and glucose PTS (Figures 30, 32) suggest that either some inhibitor of glycerokinase increases and some inhibitor of glucose PTS decreases as the ratio of glucose PTS to glycerokinase increases (and vice-versa) or that glycerol and glucose utilization share some common element. The possibility that an inhibitor of glycerokinase activity exists and increases on glucose challenge to glycerol utilizing cells has already been ruled out. Consequently the former alternative is untenable.

4.2. Statement of the model

If we assume that glycerokinase (E_1) and glucose PTS (E_2) share a common substrate (S), we can propose a model for the system as follows:



where P_1, P_2 are the products of the respective reactions; X, Y are the enzyme substrate complexes; and k_1, k_2 . . . are the rate constants of the reactions.

This model involves no statement as to the identity of the common substrate and would apply equally well if this substrate was a small molecule, a protein cofactor, a lipid component or even spaces in the phospholipid complex of the membrane.

If we consider only the case in which no product is present (at time t = 0 or if product is being removed on formation, the normal condition in growing bacteria) then p_1, p_2 = 0 and the overall velocity (v_o) of the reaction is given by the equation:

$$v_o = -\frac{ds}{dt} = \frac{dp_1}{dt} + \frac{dp_2}{dt} = v_1 + v_2$$

where s, p_1, p_2 are the concentrations of S, P_1, P_2 respectively.

The kinetic equations for this model are:

$$-\frac{ds}{dt} = k_1 s.e_1 + k_4 s.e_2 - k_2 x - k_5 y$$

$$\frac{dx}{dt} = k_1 s.e_1 - (k_2 + k_3)\, x$$

$$\frac{dy}{dt} = k_4 s.e_2 - (k_5 + k_6)\, y$$

$$\frac{dp_1}{dt} = k_3 x$$

$$\frac{dp_2}{dt} = k_6 y$$

and the conservation equations are:

$$s_o = s + x + y$$

$$e_{1_o} = e_1 + x$$

$$e_{2_o} = e_2 + y$$

where k_1, k_2 . . . are the rate constants; small letters (e_1, s, etc.) are the concentrations of the species described in the model; s_o, e_{1_o} and e_{2_o} are the total concentrations of substrate and enzymes in the system and s, e_1 and e_2 are the concentrations of free substrate and enzymes.

If we consider the Briggs-Haldane steady-state model, when $\frac{dx}{dt} = \frac{dy}{dt} = 0$; and that e_{1_o}, $e_{2_o} \gg$ s, when $e_1 + x \longrightarrow e_{1_o}$ and $e_2 + y \longrightarrow e_{2_o}$, then the velocity of the individual reactions are given by the equations:

$$v_1 = \frac{k_3 s_o e_{1_o} K_{m_2}}{(e_{1_o} + K_{m_1})(e_{2_o} + K_{m_2}) - e_{1_o} e_{2_o}}$$

and v_2 $\dfrac{k_6 s_o e_{2_o} K_{m_1}}{(e_{1_o} + K_{m_1})(e_{2_o} + K_{m_2}) - e_{1_o} e_{2_o}}$

where K_{m_1}, K_{m_2} are the Michaelis constants for E_1 and E_2 and are given by the equations:

$$K_{m_1} = \frac{k_2 + k_3}{k_1} , \quad K_{m_2} = \frac{k_5 + k_6}{k_4}$$

These equations for reaction velocity are analogous to those obtained for competitive inhibition, when two substrates compete for a common enzyme. The results are more usefully expressed in the form:

$$\frac{v_1}{v_2} = \frac{k_3}{k_6} \cdot \frac{K_{m_2}}{K_{m_1}} \cdot \frac{e_{1_o}}{e_{2_o}}$$

when, as k_3, k_6, K_{m_2} and K_{m_1} are constants, the ratio of the reaction velocities are proportional to the ratio of the enzyme activities.

4.3. Experimental support for the model

The proposed model predicts that the ratio of the rate of glycerol to glucose utilization will be proportional to the ratio of glycerokinase to glucose PTS in situations where both these carbon and energy sources are present. Figure 36b shows that a straight line is obtained when the appropriate ratios are plotted after glucose challenge to cells of E.coli 15224 growing on glycerol whereas if the rate of glucose utilization is not taken into account (Figure 36a) a straight line is not obtained. Significant changes in the intracellular FDP concentration would cause an upward deviation from the straight line shown in Figure 36b. However Clark (1974)(personal communication) has stated that the glucose PTS may also be inhibited by FDP and thus changes in the intracellular concentration of this metabolite would affect both enzyme activities and the enzyme ratio would remain the same. As the effect due to

changes in the intracellular FDP concentration is unknown this has been ignored. Only a rough correlation (correlation coefficient 0.8705; 12 degrees of freedom - results not shown) is obtained for E.coli 15224 glp R^c trained to different carbon sources and this was not considered satisfactory. It is possible that this lack of adequate correlation is due to differences in the physiology of the cells when trained to a variety of different carbon sources. The proposed relationship will hold if, and only if, the K_m's for the enzymes are constant, no matter to which carbohydrate the cells are trained. Kessler & Rickenberg (1963) have shown that this may not be the case for the glucose PTS as the K_m for ∝-methyl glucoside uptake (transported by glucose PTS) depends on the nature of the training carbon source.

However from the conservation equations we obtain:

$$s_o - s = (e_{1_o} - e_1) + (e_{2_o} - e_2)$$

which reduces to

$$s_o - s = e_{1_o} + e_{2_o} \quad \text{if } e_{1_o}, e_{2_o} \gg s_o$$

If we include the rate equations for formation of product we get:

$$s_o - s = \frac{v_1}{k_3} + \frac{v_2}{k_6}$$

Combining these two equations we obtain the relation:

$$v_1 = k_3 (e_{1_o} + e_{2_o}) - \frac{k_3}{k_6} v_2$$

That is, the model predicts that if the enzyme level is kept constant, the rate of glycerol utilization will be proportional to the rate of glucose utilization, with a negative correlation. This condition was obtained by

treatment of glucose trained cells of E.coli 15224 glp R^c with chloramphenicol, an antibiotic that prevents protein synthesis, and thus the enzyme levels remain constant over a short period of time. The rate of glycerol utilization was estimated while increasing the rate of glucose utilization over this time interval (Figures 33, 34). The rate of glycerol utilization decreases linearly as the rate of glucose utilization increases (Figure 34b), the gradient of this line (0.8) giving the ratio of the rate constants $(\frac{k_3}{k_6})$ which, if Michaelis-Menten kinetics are assumed, determine the rate of glycerol or glucose utilization.

The model is also consistent with the results of Kessler & Rickenberg (1963) who have shown that glycerol competitively inhibits the uptake of α-methyl glucoside in E.coli. If the concentration of glycerol in the medium falls to the level of the K_m of glycerokinase for this substrate, then the rate of glycerol utilization will decrease, resulting in a concomitant increase in the rate of glucose utilization. That is, the mutual inhibition of glycerol and glucose utilization will have the characteristics of classical competitive inhibition of two substrates for one enzyme.

The results obtained are consistent with the existence of, and competition for, an element common to the first steps of glycerol and glucose utilization. For the model to be valid it is necessary that the concentration of the hypothetical common element be much less than the concentration of the enzymes. This can be achieved if the rate of production of this common element is less than the potential maximum rate of utilization of the element. In this case the rate of production of the common element

will determine the overall rate of carbon source utilization.

It must be emphasised that these results apply only to the utilization of glucose and glycerol when both substrates are present in the medium and not to the utilization of a single carbon source. Under these circumstances neither the rate of glucose utilization nor the rate of glycerol utilization vary to the same extent as the respective enzyme activities (Figure 35). This implies that both enzymes are subject to feedback inhibition, that for glycerokinase being FDP and perhaps G6P, glucose-1-phosphate (Kaback, 1970) or FDP (Clark, 1974)(personal communication) for the glucose PTS.

4.4. Nature of the common element

The nature of the common element in glycerol and glucose utilization is obscure. It has been shown that glycerol utilization cannot be controlled by glucose at the level of permeation (which eliminates a 'common carrier protein') nor can it be controlled by changes in the concentration of the intracellular pool of a metabolite (in particular FDP). The site of regulation has been shown to be glycerokinase and thus it must be concluded that regulation of the supply of phosphate donor determines the activity of this enzyme when glycerol and glucose are utilized competitively.

The only common link between the first steps of glucose and glycerol metabolism (and glucose and the metabolism of other compounds whose utilization it inhibits) is the requirement for energy or the requirement for Mg^{2+}. The latter alternative seems improbable as the total internal magnesium concentration in E.coli is about 150 mM (Nelson &

Kennedy, 1971), however no information is available concerning the relative concentrations of free and bound Mg^{2+} and this may be a possibility however unlikely. Thus we are forced on two accounts to conclude that glucose and glycerol compete for a common energy supply. This immediately raises the problem of the *in vitro* energy requirements of the first enzymes of glycerol and glucose catabolism. Crystalline glycerokinase has been shown (Thorner & Paulus, 1973) to specifically use Mg^{2+} ATP as a phosphate donor, whereas *in vitro* studies of the glucose phosphotransferase system have shown that phosphoenolpyruvate is the donor. However an uncoupler of oxidative phosphorylation, FCCP, inhibits the utilization of glucose and glycerol (Figure 37). This is usually taken to indicate an energy requirement for utilization (or uptake) (e.g. Boezi & De Moss, 1961; Pavlasova & Harold, 1969) but a more important consideration is that glucose and glycerol utilization are both subject to inhibition. Furthermore this inhibition depends on the training carbohydrate, and hence correlates with the amount of glycerokinase or glucose PTS present. These results suggest that, *in vivo*, both these enzymes may utilize the same, or easily interconvertible, energy sources.

Circumstantial evidence, obtained from Figure 34a, supports the hypothesis that the rate of energy production satisfies some of the criteria of the model. As has been previously shown, the model predicts that

$$s_o - s = (e_{1_o} - e_1) + (e_{2_o} - e_2)$$

and if $e_{1_o}, e_{2_o} \gg s_o$ then $e_{1_o} - e_1 \longrightarrow e_{1_o}$, $e_{2_o} - e_2 \longrightarrow e_{2_o}$

and $s_o - s \longrightarrow s_o$, and thus $s_o = e_{1_o} + e_{2_o}$.

That is, when the enzyme levels are constant, s_o must be constant for the model to hold. When the rate of glucose utilization by glucose trained E.coli 15224 glp R^c treated with chloramphenicol is increased, a concomitant decrease in the rate of glycerol utilization occurs but the rate of oxygen consumption remains constant (Figure 34a). This implies that the rate of energy production, whether in the form of a hydrogen ion gradient or ATP, remains constant. However the rate of production of the latter energy source will only remain constant if the $^P/_O$ ratio remains constant during the period of the experiment. The observations of Hempfling (1970) that $^P/_O$ ratios in E.coli vary under different conditions of catabolite repression cast some doubt on this assumption. These $^P/_O$ ratios were observed in cells trained to, or growing on different carbon sources and therefore may not apply to the utilization of a pair of carbon sources by cells trained to one of them.

The primary energy source for glycerokinase and glucose PTS in vivo may therefore be the same source, or easily interconvertible sources. The model still holds if one or more energy sources are interconvertible by equilibrium, or near equilibrium, reactions, provided that the rate of production of these energy sources is rate limiting. However the potential rate of energy production in washed cell suspensions is greater than the actual rate of production (cf. oxygen consumption of growing cells with washed cell suspensions, Figures 7, 34). Thus general energy production cannot be rate limiting and limitation must depend on a specialized energy source or on a cofactor (protein or otherwise) required for the coupling of energy to the enzymes.

Some energy sources, for example a hydrogen ion gradient across the cell membrane and ATP (Harold, 1972a), are easily interconvertible. However the energy source cannot be intracellular ATP as this is high (37 mM, Figure 23) and does not change on glucose challenge to cells growing on glycerol. Glycerokinase is soluble when cells are extracted by sonication but it is possible that this procedure destroys a loose association of the enzyme with the membrane. If this is the case it is also feasible that cytoplasmic ATP is not the direct phosphate donor for glycerokinase but that the phosphate donor is produced at a limiting rate in the membrane. This could be ATP produced by oxidative phosphorylation, the phosphorylated low molecular weight protein of the phosphotransferase system, a lipid or protein factor required for coupling of ATP production to the hydrogen ion gradient or one required for direct coupling of the enzymes to either the hydrogen ion gradient or ATP production.

In E.coli the metabolism of glycerol alone is regulated by the interaction of fructose-1,6-diphosphate and glycerokinase. In mixtures glucose represses synthesis of glycerol enzymes and diauxic growth results. When glucose is added to cells growing on glycerol, glycerokinase is immediately repressed and the activity of the existing enzyme inhibited by 50%. As growth continues inhibition of glycerol utilization increases to 100%. The dominance of glucose over glycerol (even in glp

constitutive mutants) does not depend on the regulation of glycerokinase by intracellular metabolites or on permeation of glycerol into the cell.

Before dominance is complete, flow of the two substrates are mutually inhibitory and partition between the two routes depends on the relative levels of glycerokinase and glucose PTS. At constant enzyme levels the flow of glycerol is regulated by the rate of glucose utilization. The data are consistent with the proposition that the two systems compete for an element common to each which may be some interconvertible energy donors.

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observed inhibition of glycerokinase by fructose-1,6-diphosp

vitro with direct measurements of the intracellular fructose-

diphosphate and glycerokinase content.

2) Glucose represses synthesis of glycerol enzymes and diauxic

observed in mixtures of glucose and glycerol. When glucose

to cells growing on glycerol, glycerokinase is immediately r

and the utilisation of glycerol is halved. As growth contin

inhibition of glycerol utilization increases to 100%. After

challenge, glycerol carbon used continues to enter protein,

acid and lipid with little change in the proportion entering

Glycerol utilization only starts again when glucose is exhau

3) The dominance of glucose over glycerol utilization (even in

constitutive for the enzymes of glycerol utilization) does n

on regulation of glycerokinase activity by the intracellular

tion of fructose-1,6-diphosphate, nor on inhibition by any o

metabolite of glucose that has been tested. Furthermore dom

does not depend on regulation of permeation of glycerol into

The flows of glucose and glycerol are mutually inhibit

partition between the two routes depends on the relative lev

glycerokinase and the glucose phosphotransferase system. At

enzyme levels the flow of glycerol is regulated by the rate

utilization. The data are consistent with the proposition t

two enzyme systems compete for an element common to each whi

a pool of interconvertible energy donors.