SUMMARY

1. The glucose and fructose phosphotransferase systems (PTS) of E. coli 15224 are constitutive but increase about 7-fold in the presence of their substrates. However, inoculation of glucose trained cells into fructose medium leads to a rapid increase in fructose PTS activity which overshoots the final value obtained. This overshoot is prevented by chloramphenicol suggesting that it results from de novo synthesis rather than activation of a precursor. The magnitude of the overshoot is directly linked to the degree of catabolite repression existing before transfer into fructose alone and the overshoot does not occur in the presence of exogenous cyclic AMP.

2. Glucose challenge to fructose growing cells eventually results in sequential utilisation of the glucose and fructose since glucose both represses the synthesis of, and inhibits the activity of, the fructose PTS. Near glucose exhaustion fructose PTS synthesis is rapidly induced and the activity overshoots the final value. This allows a short period of co-utilisation explaining the absence of diauxie for E.coli 15224 growing on glucose and fructose.

3. Rates of sugar utilisation in vivo are not directly related to the PTS activities measured in vitro suggesting that, in vivo, the PTS's are under inhibitory control.

4. Glucose inhibition of fructose utilisation requires induction of the glucose PTS. The use of protein-reacting reagents shows that the interaction of glucose with the glucose PTS prevents fructose binding to the fructose PTS and thus inhibits fructose utilisation. Hexose-phosphates may also be implicated in this inhibition of fructose utilisation but the data suggest that they are more likely to be involved in the normal mechanism of controlling carbon flux into the cell.

5. The sequential utilisation of glucose and fructose is thus mediated by control of their respective PTS activities.

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Control of the Sequential Utilisation of Some Sugars by Escherichia coli ATCC 15224

by

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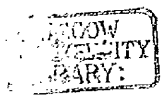

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List of Abbreviations

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

assay buffer	0.2 M NaH_2PO_4 pH 7.2 with 2 mM $MgSO_4$
BCIG	5-bromo-4-chloro-indoxyl-β-galactoside
BES	NN-Bis-(2-hydroxyethyl)-2-amino-ethane sulphonic acid
buffer	40 mM KH_2PO_4 pH 7.0 with 2 mM $MgSO_4$
cyclic AMP	cyclic adenosine 3' 5' monophosphate
F1P	fructose-1-phosphate
F6P	fructose-6-phosphate
G1P	glucose-1-phosphate
G6P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
LDH	lactate dehydrogenase
∝ MG	∝-methyl glucoside
NEM	N-ethyl maleimide
PEP	phosphoenol pyruvate
6PG	6-phospho-gluconate
PGI	phosphoglucose isomerase
PTS	phosphoenol pyruvate phosphotransferase system
TEO	triethanolamine buffer
(μ)	specific growth rate
μ	μm when applied to pore sizes of filters

CONTENTS

INDEX

INTRODUCTION

METHODS

DEVELOPMENT OF ANALYTICAL METHODS

INDEX TO FIGURES

INTRODUCTION

METHODS

DEVELOPMENT OF ANALYTICAL METHODS

RESULTS

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INTRODUCTION

1. Sugar Utilisation

1.1. The choice of alternative substrates

A number of micro-organisms, including Escherichia coli show considerable nutritional versatility. They are able to grow aerobically in simple salts media with only a single carbon source, as well as in a variety of more complex environments. Hamilton (1971) has reported that E.coli will grow on at least 58 carbon sources and there will be others not yet investigated. However, in nature, bacteria grow in mixed cultures on a collection of salts and energy sources and, since survival may depend on the ability to choose the optimum substrate for growth (Paigen & Williams, 1970), it is apparent that these micro-organisms might have a means whereby the entry of substrates can be carefully controlled.

Micro-organisms have a general ability to choose a preferred substrate. For example, Hydrogenomonas preferentially uses certain electron donors. This organism, which can grow autotrophically on a mixture of hydrogen and carbon dioxide, represses adaptation to a variety of organic substrates on exposure to molecular hydrogen (Blackkolb & Schlegel, 1968). Similarly, synthesis of at least three enzymes of the Entner-Doudoroff pathway is repressed by molecular hydrogen (Schlegel & Trüper, 1966, Blackkolb & Schlegel, 1968). Thus hydrogen is the substrate preferred to carbon dioxide. Other examples of such preferences are given by Paigen & Williams (1970).

Pardee (1961) explains the behaviour towards mixed substrates by suggesting that the ability to grow fast is of immense selective advantage to micro-organisms. Holms & Bennett (1971) have shown that E.coli 15224 growing on glucose excretes acetate which is not used

until glucose is exhausted. It is suggested that this process, while apparently wasteful, allows the cells to grow faster than they otherwise could. In the laboratory it has been shown that economy of protein synthesis and strict control of enzyme activity is of selective advantage (Roeptke et al., 1944; Zamenhof & Eichhorn, 1967; Baich & Johnson, 1968). Thus one would expect that bacteria presented with a choice of substrate would, initially, use the one promoting faster growth. This is not the case for Pseudomonas putida growing on mandelate and p-hydroxybenzoate (Higgins & Mandelstam, 1972a); mandelate supports a higher growth rate but the latter is preferentially utilised. Economy of protein synthesis might be the answer since p-hydroxybenzoate requires less metabolic enzymes than mandelate which is higher up the same pathway.

1.2. Diauxie

Diauxie is a special case of the preferred utilisation discussed above and was first examined by Monod (1941, 1942), who showed that bacterial cultures exposed to two carbon sources may use one until it is exhausted from the medium and then undergo a growth lag before starting to utilise the other.

Diauxie depends on two essential conditions. Firstly, adaptation to the less preferred or secondary carbon source is completely prevented in the presence of the preferred substrate. Secondly, the process of adaptation either requires the permease to generate internal inducer or the induced enzymes must first metabolise the carbon and energy supply. Combinations which do meet these requirements often have glucose as a primary substrate and the second substrate is one whose utilisation requires the synthesis of an inducible pathway. This is not, however, a universal rule as, for example, Pseudomonas aeruginosa prefers citrate to glucose and thus

displays "reverse diauxie" (Hamilton & Dawes, 1959). Complete repression of the enzymes in a pathway may be achieved by catabolite repression itself or any mechanism which decreases the internal concentration of inducer.

Growth of E.coli on a mixture of glucose and lactose is one of the most studied examples of diauxie. The cells grow initially on glucose, and lactose is prevented from inducing the lac operon for a number of reasons, but mainly because the levels of permease and β-galactosidase are very low and any ability to transport or metabolise lactose is subject to catabolite inhibition by glucose (McGinnis & Paigen, 1969). However, if any inducer were to be made, synthesis of the lac operon proteins would be effectively prevented by catabolite repression. At glucose exhaustion, there is a lag during which the lac operon is induced before the cells resume growth. In contrast to this system, not all sugar combinations show diauxic growth patterns. Monod (1941, 1942) reported that combinations such as glucose and fructose, glucose and mannose or glucose and mannitol did not show diauxic growth and this may mean that they are either sequentially used without lag or co-utilised. It has been shown (W.H. Holms - personal communication) that E.coli 15224 (constitutive in the lac operon) shows, not diauxic growth, but sequential utilisation when grown on glucose and lactose, implying that catabolite inhibition (McGinnis & Paigen, 1969) is exerted to ensure glucose dominance. Consequently, there is no reason to believe at this time that glucose and fructose, glucose and mannitol, or glucose and mannose are not sequentially utilised in a similar manner, since Roseman (1969) has shown that the systems for the uptake and metabolism of fructose, mannitol and mannose are constitutive. Therefore control mechanisms must exist to ensure glucose dominance

over other sugars.

Control of diauxie or sequential utilisation may be achieved:

a) by controlling the rate of synthesis of the uptake system and subsequent metabolic enzymes.

b) By regulation of the activity of the transport system.

The next section describes some of the systems where the control of enzyme synthesis has been analysed.

2. Control of Protein Synthesis

During the early twentieth century it was reported that glucose prevented the development of various fermentative capacities in bacteria (Berman & Rettger, 1918; Monod, 1942; Gale, 1943). The "glucose effect" was described by Epps & Gale (1942) who demonstrated that repression by glucose was limited to certain enzymes. Monod (1941, 1942) described diauxie and this is now recognised as another manifestation of catabolite repression. Magasanik (1961) has reviewed the basic aspects of the glucose effect and renamed it catabolite repression.

2.1. Catabolite repression

2.1.1. Control of the lac operon of E.coli

The lac operon of E.coli has been examined in more detail than any other regulatory system. The products of the lac operon are β-galactosidase and β-galactoside permease, which are required for the metabolism of lactose, and thiogalactoside transacetylase whose function is unknown. The enzymes are synthesised in the cell when inducer is present in the growth medium (Monod & Cohen, 1952). The inducer was originally thought to be lactose, but recent work (Jobe & Bourgeois, 1973) has shown that lactose is actually an anti-inducer since it binds to the lac repressor and stabilises the repressor/operator complex. The natural inducer of the lac operon is allolactose (1,6-O-β-galactopyranosyl-D-glucose) produced from lactose by β-galactosidase (Jobe & Bourgeois, 1972) in a re-arrangement reaction which converts the 1 to 4 linkage of lactose to a 1 to 6 linkage. The lac enzymes are always co-ordinately synthesised (Jacob & Monod, 1961) and, as a result of genetic mapping, have been shown to be coded for by a single region of the E.coli genome (Jacob & Monod, 1965). This provides support for the operon

(Jacob & Monod, 1961) as the controlling element for the synthesis of the lactose metabolising enzymes in E.coli. Considerable additional support for this hypothesis has been obtained over the years and, with some modification, it is accepted today. The control of the lac operon has been reviewed by a number of authors (Beckwith, 1967; Richmond, 1968; Beckwith, 1971).

In the model proposed by Jacob & Monod (1961) the enzymes of the operon are coded for by structural genes in the order shown (Figure I.1) and their synthesis is controlled by the operator region. This region can exist in two states - one, in which a repressor molecule is bound to the operator and a second, when the operator is free. In the latter case RNA polymerase, which is bound at the promotor site (Chen et al., 1971), can transcribe the lac operon producing lac mRNA and so specify synthesis of the lac enzymes. When the repressor molecule is bound to the operator very little transcription can take place. The repressor is synthesised using the I gene as a template and is a protein (Riggs & Bourgeois, 1968) which binds to operator DNA (Riggs et al., 1968) preventing transcription taking place. Control at the level of transcription has been demonstrated (Varmus et al., 1970).

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

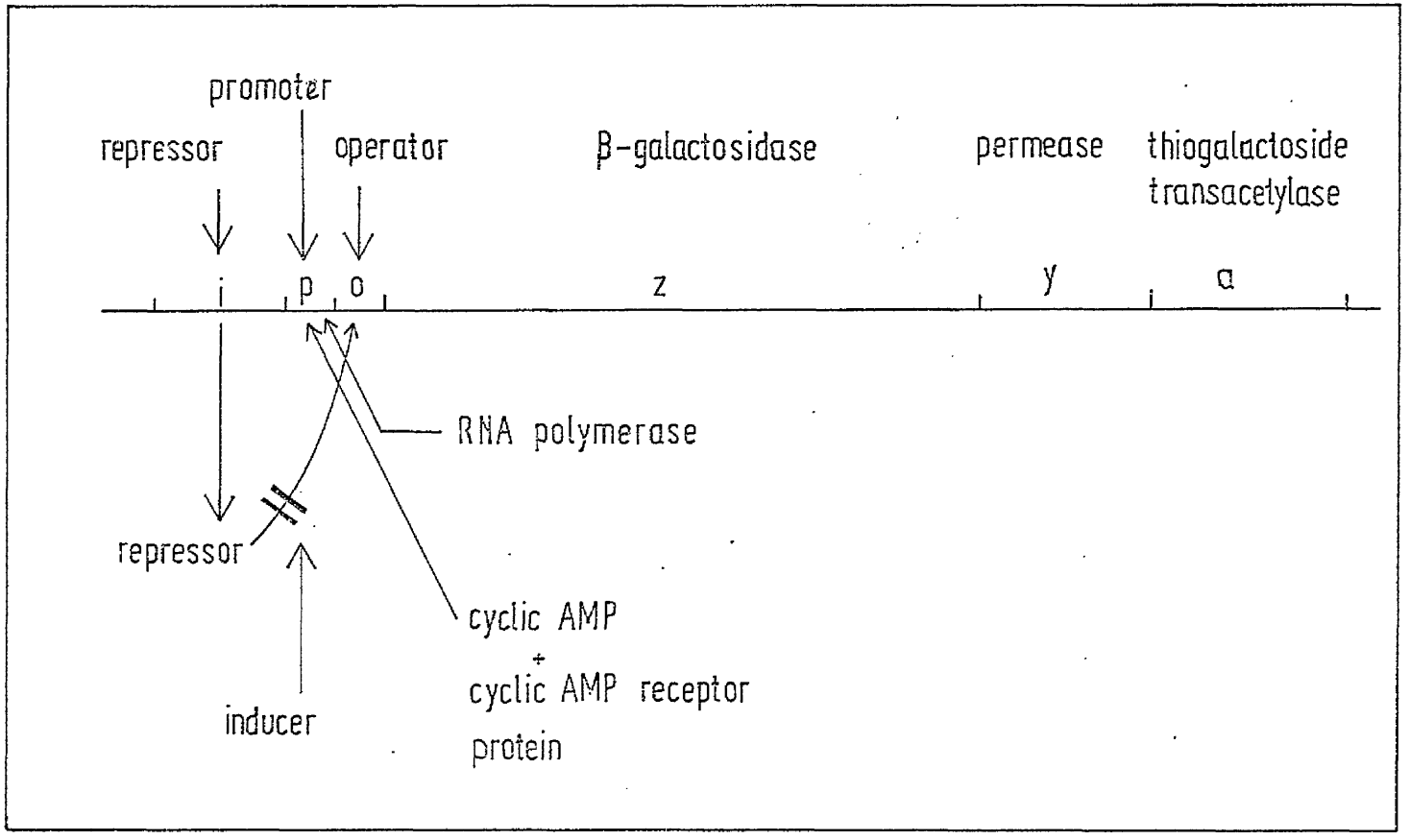


Figure I.1 The lac Operon

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

The lac operon is therefore controlled at the molecular level by the interaction of macromolecules on the DNA template at the operator/promoter site under the influence of intermediates present in the cell. It also provides a means of controlling the transport of lactose and the first step in its metabolism.

2.1.2. Control of the gal operon in E.coli

The gal operon has been studied in a similar manner to the lac operon but not in such detail. The enzymes required for the metabolism of galactose are coded for by a single operon (Buttin,1963a) and induced by either galactose or fucose (Buttin,1963b). The sequence of enzymes on the operon (Michaelis & Starlinger, 1967; Shapiro & Adhya, 1969) and the stoicheiometry of their synthesis (Wilson & Hogness, 1969) have been studied and are similar in principle to the lac operon. Synthesis is susceptible to catabolite repression (de Crombrugghe et al., 1969) which, in a similar manner to the lac operon, can be overcome by the addition of cyclic AMP to the culture (Pastan & Perlman, 1970).

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

Differences do occur between the lac and gal systems with respect to quantitative requirements for cyclic AMP and cyclic AMP receptor protein. Parks et al. (1971) have shown that synthesis from the gal operon continues to a significant extent in the absence of cyclic AMP and cyclic AMP receptor protein and also that, while guanosine 3'diphosphate 5'diphosphate stimulates lac mRNA synthesis, it inhibits gal mRNA synthesis. Despite these differences it has been concluded (Miller et al., 1971) that control of the gal operon is very similar to the lac operon.

2.1.3. Control in the ara operon of E.coli

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

Englesberg et al. (1965) proposed that the regulation of the ara operon was under positive control by the product of the ara C gene. Positive control was supported by an analysis of merodiploids in the ara operon (Sheppard & Englesberg, 1967). Englesberg et al. (1969) have presented further evidence to show that, in addition to acting as an activator for enzyme synthesis, the ara C gene product, in the absence of L-arabinose, acts as a repressor to a site on the operon distinct from its site of action as an activator and have proposed a

model to explain the observed regulation (Figure I.2). In the model the product of the ara C gene, P1, represses the transcription of the operon by acting at the O region. The presence of L-arabinose, the inducer, converts the repressor to another form, P2, which acts at the I region as an activator of operon transcription.

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

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

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

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

2.1.4. The role of cyclic AMP in catabolite repression

The observations by Makman & Sutherland (1965) that cells growing on glucose had low cyclic AMP levels led to postulations

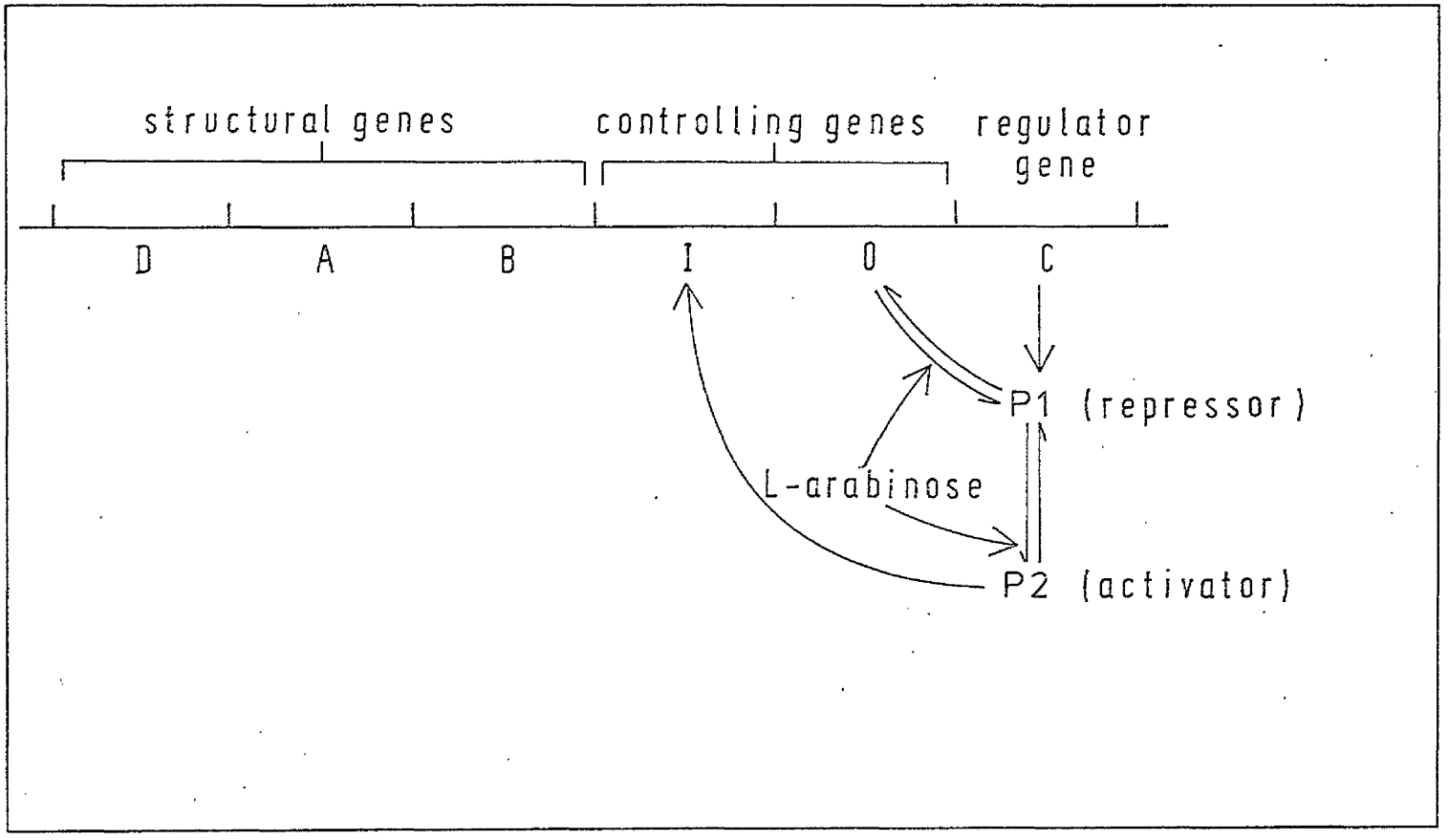


Figure I.2 The ara Operon

that cyclic AMP was involved in the processes of catabolite repression. It was shown (Makman & Sutherland, 1965) that glucose actively stimulated the extrusion of cyclic AMP from the cell and, since work by de Crombrugghe et al. (1969) has shown the requirement of cyclic AMP for regulation of enzyme synthesis, it was thought that depression of intracellular cyclic AMP levels by glucose led to catabolite repression. This hypothesis was substantially strengthened by Pastan & Perlman (1969) who showed that a mutant deficient in glucose enzyme II (EII) of the PTS was resistant to repression by glucose. Furthermore, phosphorylation of the sugar was not required since mutants deficient only in enzyme I (EI) or heat stable protein (HPr) were still sensitive to glucose repression. It was concluded (Pastan & Perlman, 1969) that glucose and glucose EII act in such a way as to lower the intracellular level of cyclic AMP giving rise to catabolite repression; this has been confirmed by Roseman (1972a).

2.2. Transient repression

Transient repression by glucose was first reported for β-galactosidase in certain mutant strains of E.coli (Paigen, 1966; Moses & Prevost, 1966). Paigen (1966) has shown that some strains of E.coli, including ML-strains, are not transiently repressed by glucose and other strains show varying responses to the presence of glucose (Tyler et al., 1967). It has been shown (Moses & Prevost, 1966; Paigen, 1966; Palmer & Moses, 1967; Tyler et al., 1967) that, in transient repression, glucose does not act by excluding the inducer. It is likely that the addition of glucose causes a rapid fall in cyclic AMP concentration which subsequently recovers to some extent (Makman & Sutherland, 1965). Transient repression of the lac operon has also been observed in Salmonella typhimurium (Tyler et al., 1967). Other enzymes are also sensitive to transient repression, notably

tryptophanase (Paigen, 1966), galactokinase (Paigen, 1966) and D-serine deaminase (Moses & Prevost, 1966). Other examples are reviewed by Paigen & Williams (1970).

Originally, both Paigen (1966) and Moses & Prevost (1966) considered transient repression to be a special case of catabolite repression and further work (Perlman & Pastan, 1968; Pastan & Perlman, 1968; Perlman et al., 1969) has shown that the two phenomena are similar. In both cases control is exerted on mRNA synthesis, the same enzymes are sensitive and glucose produces both types of repression.

Nevertheless Tyler et al. (1967) have suggested that the two phenomena may not be identical and genetic evidence has been reported (Perlman & Pastan, 1968; Pastan & Perlman, 1968; Perlman et al., 1969) which suggests the separate identity of catabolite and transient repression. Despite this, Perlman and Pastan (1968) have suggested that catabolite and transient repression are manifestations of the same phenomena since both can be reversed by the addition of cyclic AMP.

2.3. Transient derepression

This is not a well documented phenomenon and appears to have been investigated only by Gorini & Maas (1957), Holzer (1966), Cohn & Horibata (1959) and Okinaka & Dobrogosz (1967a). Cohn & Horibata (1959) showed that, if an aerobic culture of E.coli growing on glucose is subjected to anaerobic shock by transferring it to an environment of nitrogen and carbon dioxide, growth continues after a slight lag but repression of β-galactosidase synthesis is temporarily lost. Transient derepression has been associated with the disappearance of pyruvate from the medium (Okinaka & Dobrogosz, 1967a) but little more is known about this phenomenon at this time.

3. Control of Enzyme Activity

Control by catabolite repression, as described above, enables micro-organisms to exert a long-term control over the protein content of the cell, but this type of control is slow to respond to changes in the environment. Even if synthesis of an enzyme or transport system ceases when it is no longer required it will take at least 6 generations of growth to dilute it out to 1% of the original level. Consequently, a process such as catabolite inhibition, which enables the activity rather than the amount of transport system to be controlled directly, would enable micro-organisms to make a rapid and effective selection between substrates.

3.1. The discovery of catabolite inhibition

The existence of catabolite inhibition as a distinct phenomenon was recognised by Gaudy *et al.* (1963) and Stumm-Zollinger (1966) who showed, respectively, that glucose inhibited the uptake of sorbitol and galactose in mixed cultures trained to these substrates. From this work it became clear that earlier results obtained by Woods (1935), who reported that production of indole from tryptophan by washed cell suspensions of *E.coli* was inhibited by glucose and Evans *et al.* (1942) who confirmed these results, were also examples of catabolite inhibition. Other examples of catabolite inhibition, which is clearly distinct from catabolite repression, are given by Paigen & Williams (1970). These authors conclude that a generalised system of catabolite inhibition exists by which microbial cells choose a preferred carbon source.

3.2. Mechanism of catabolite inhibition

Inhibition may be exerted at the level of entry of substrates into the cell or on the first enzyme that catalyses metabolism of the substrate.

Inhibition of the first enzymes of metabolism has been reported *in vitro* for the β-glucosidase of *Schizophyllum commune* by glucose (Wilson & Niederpruem, 1967) and for the glycerokinase of *E.coli* by fructose-1, 6-diphosphate (Zwaig & Linn, 1966).

The uptake of various sugars is inhibited by glucose and this inhibition will be considered in greater detail being pertinent to this thesis.

3.3. Control of inducer entry

Glucose can inhibit the uptake of sugars such as galactose (Horecker *et al.*, 1960; Adhya & Echols, 1966), β-galactosides (Kepes, 1960; Kessler & Rickenberg, 1963; Koch, 1964; Winkler & Wilson, 1967), maltose (Egan & Morse, 1966) and sucrose (Egan & Morse, 1966). It was reported (Koch, 1964) that prior growth of the cells on glucose was required to inhibit the uptake of other carbohydrates, implying that glucose does not directly inhibit other specific permeases. This has been confirmed by Kornberg (1972).

Data obtained by several workers (Kepes, 1960; Kessler & Rickenberg, 1963; Koch, 1964) suggest that competition occurs between active permeases for a limited quantity of a common factor required for carbohydrate transport. This common factor has been tentatively identified (Tanaka & Lin, 1967; Tanaka *et al.*, 1967) with the phosphotransferase system (PTS), discovered by Kundig *et al.* (1964), since a functional PTS is not present in some pleiotropic mutants (Egan & Morse, 1965; Tanaka & Lin, 1967; Tanaka *et al.*, 1967; Wang & Morse, 1968) and glucose is unable to inhibit sugar uptake. The PTS, to be described in greater detail, contains a heat-stable protein (HPr) which can be phosphorylated to serve as an energy donor to a number of sugar specific enzymes II. Competition for this phosphorylated

HPr may well account at least for the observed glucose inhibition of transport of other PTS-sugars (see Figure I.3). However, Kaback (1969) and Kornberg (1972, 1973a,b) have suggested that hexose-phosphates play a major role in the control by glucose of the uptake of various PTS-sugars.

The actual site or means by which glucose or its metabolites act to inhibit the PTS is at present unknown.

While glucose inhibition of the PTS seems to be relatively well understood, little is known about the way glucose acts to inhibit the uptake of sugars not transported by the PTS (non-PTS-sugars: Figure I.3.). Work by Roseman (1972a) using E.coli has shown that, while the inhibition exerted by glucose on the transport systems for non-PTS-sugars is probably not mediated by competition for metabolic energy, it cannot be ruled out that PTS-sugars uncouple the non-PTS-sugar transport systems from metabolic energy. However, Roseman (1972a) states that the results are consistent with the conclusion that the most important effect exerted by the PTS-sugars is to exclude non-PTS-sugars from the cell thus preventing synthesis of the transport systems normally induced by these non-PTS-sugars. Roseman (1972a) also states that the PTS membrane components, particularly the enzymes II, may in some way regulate the action of the non-PTS-sugar transport systems but concludes that more work is required to clarify all these observations.

It is certain, however, that catabolite inhibition is a quantitatively important factor in establishing glucose dominance over the metabolism and uptake of other sugars. It is particularly important in the intense enzyme repression seen during diauxic growth (McGinnis & Paigen, 1969) since workers such as Silver & Mateles (1969) have shown that catabolite repression alone is not sufficient to cause diauxic growth of E.coli on a mixture of glucose and lactose; catabolite inhibition is required to exclude lactose and prevent

Sugars Transported by the PTS

(Staphylococcus aureus; Escherichia coli)

S.aureus and E.coli

PTS-sugars

Glucose, fructose, mannose (N-acetyl-mannosamine), mannitol, sorbitol, β-glucosides, glucosamine, N-acetylglucosamine

S.aureus PTS-sugars	E.coli non-PTS-sugars *
Lactose, sucrose, galactose, trehalose, melibiose, melezitose, pentose, maltose, glycerol.	Lactose, maltose, melibiose glycerol, pentoses, hexose-P galactose.

* PTS-sugars are taken up by group translocation
non-PTS-sugars by other processes.

Figure I.3 (Reproduced from Roseman, 1972a)

induction. The role of the PTS in inducer exclusion has been confirmed by Saier & Roseman (1970, 1972). They showed that the failure of melibiitol to induce the melibiose operon in the presence of ∝-methyl glucoside was a result of inducer exclusion since the cyclic AMP concentration was not sufficiently low to totally repress enzyme synthesis.

The information examined in this section suggests that the sugar transport systems play a pivotal role in catabolite inhibition in that this inhibition is exerted at the actual transport step. Consequently, the means available to the cell for substrate transport will be examined in the next section.

4. Transport Systems

The historical aspects of transport have been comprehensively reviewed by Cirrillo (1961) and Hokin & Hokin (1963) and need not be further described here. The three main ways by which micro-organisms take up nutrients from their external media will, however, be examined.

4.1. Facilitated diffusion

This mode of transport requires no metabolic energy since it is 'powered' by the concentration gradient across the cell membrane. It differs from simple diffusion in that the substance to be transported combines with a protein on one side and is released on the other side of the membrane in an unchanged form. Facilitated diffusion is the mechanism whereby glucose is transported into red blood cells (Stein, 1967) but this process plays only a minor role in enteric bacteria. The uptake of glycerol (Sanno *et al.*, 1968), carboxylic acid ions (Kornberg & Smith, 1967) and succinate (Kay & Kornberg, 1971) in enteric bacteria appears to be mediated by facilitated diffusion. In contrast, bacilli can actively accumulate succinate against a concentration gradient (Ghei & Kay, 1972).

4.2. Active transport

Active transport is similar to facilitated diffusion except that the solute is moved across the membrane against the concentration gradient by a process which requires the expenditure of metabolic energy. The ability to concentrate a substrate in this manner was initially investigated by Rickenberg *et al.* (1956) who found that a washed suspension of lactose grown cells of *E.coli* would accumulate methyl-(S^{35})-thiogalactoside (TMG) to such an extent that it formed nearly 4% of the total dry weight of the organism. Wild-type cells catalysed this uptake only if they had been previously exposed to lactose or an analogue which induced synthesis of this transport system.

Monod (1956) indentified that the y and z genes of the lac operon exist at different loci and this work led to the discovery of other 'active transport' systems and the use of mutants to investigate the genes that specify either the structure or the rates of synthesis of specific uptake systems. The available information on genetic mapping of this nature has been reviewed by Lin (1970).

The nature of the components of active transport systems has been investigated. Fox & Kennedy (1965) first identified and isolated the lactose permease of E.coli. Further work (Fox et al., 1967; Carter et al., 1968) confirmed that a specific protein capable of binding the lactose analogue β-D-galactosyl-1-thio-β-D-galactoside (TDG) and protecting its -SH groups against attack by N-ethylmaleimide (NEM) was present in the membrane fractions isolated from E.coli, but only if the cells possessed a functional lac operon. The lac permease or M-protein was subsequently purified (Jones & Kennedy, 1969) from cells of E.coli ML30. Similarly, Boos (1972) has identified a product of the mglP gene which specifies the system for the uptake of the galactose analogue methyl-β-D-galactoside which, in contrast to the M-protein, can be removed from the membrane of E.coli by osmotic shock (Neu & Heppel, 1965). This protein (Boos, 1972) has been implicated in the uptake of a variety of amino acids, sugars and inorganic salts (Heppel et al., 1972) but its role in transport has not been clarified.

Other workers have identified the means by which energy is coupled to transport. The uptake and retention of glycine (Kaback, 1960), sugars and sugar-phosphates (Kaback, 1972) has been shown in membrane vesicles in the presence of the appropriate energy source. Although the addition of inhibitors of energy production such as 2,4-dinitrophenol abolishes active transport both in vesicles and in whole cells it has been shown (Pavlasova & Harold, 1969) that there is no necessary

relationship between active transport and the oxidative formation of ATP from ADP and pyrophosphate. Observations such as these (reviewed by Kaback, 1972) have led to the proposal of two fundamentally different models to explain the results.

In one model Kaback (1972) suggests that the flux of electrons from a suitable donor to oxygen causes conformational changes in the electron carriers which can be translated into vectorial binding and release of substrates. In E.coli, Barnes & Kaback (1971a,b) have shown that the oxidation of D-lactate apparently provides the energy for β-galactoside transport, at least in membrane vesicles. Other substrates, whose oxidation proceeds via flavins and cytochrome b to oxygen, can also be used (Kaback, 1972) and several compounds which interfere with electron flow between donor and oxygen also inhibit transport. Kaback (1972) has observed that both D-lactate dehydrogenase activity and the initial rates of solute transport have the same activation energy and postulates that, for each uptake system, there should be an intermediate somewhere between D-lactate dehydrogenase and cytochrome b having a specific binding site for each substrate to be transported. However the exact nature of these intermediates is not yet known.

The alternative model for energy coupling between electron flux and the uptake of substrates has arisen from Mitchell's chemiosmotic hypothesis (reviewed by Mitchell, 1970; Harold, 1972). This hypothesis allows for a protonmotive force to be established by separation of H^+ and OH^- ions on opposing sides of the membrane. Neutral sugars are taken up together with a proton (West & Mitchell, 1972) in what, in Mitchell's terminology, is called a 'symport' process. In the case of lactose the M-protein might be part of the symport system.

It is not yet possible to decide between the two hypotheses

but, as stated by Kornberg (1973b), the weight of evidence would appear to favour Mitchell's hypothesis for the proton-motive force powering the uptake for at least some sugars.

4.3. Group translocation

Group translocation differs from the uptake systems previously described in that the sugar taken up appears in the cell in the form of sugar-phosphate (Rogers & Yu, 1962). The means by which this is achieved was first described by Kundig et al. (1964) who discovered the phosphoenolpyruvate dependent sugar phosphotransferase systems (PTS) in E.coli and Staphylococcus aureus. The PTS, which has been reported in many micro-organisms (for review see Romano et al., 1970), has been found to possess sugar specific components (Enzymes II) whereas the equivalent components in active transport systems have not yet been identified. Consequently it seemed that studies of the control of sugar utilisation would be best carried out using substrates of the PTS. It is probable that, whereas the effects of repression, inhibition or whatever other control mechanisms exist might be studied directly by examining their effects on the sugar specific enzymes II, this is not the case for active transport systems.

The salient features of the PTS are examined in some detail in the next section.

5. The Phosphoenolpyruvate: Sugar Phosphotransferase System (PTS)

The group translocation process that effects the uptake of a number of sugars by mesophilic bacilli (Freeze et al., 1970), thermophilic bacilli (Harris & Kornberg, 1972), Enterobacteriaceae (Romano et al., 1970) and other facultatively anaerobic bacteria (Tanaka et al., 1967; Hanson & Anderson, 1968; Romano et al., 1970) involves at least four components (Roseman, 1969, 1972b).

(i) A small histidine containing, heat-stable protein (HPr),

(ii) phosphoenolpyruvate (PEP),

(iii) enzyme I (EI),

(iv) membrane bound enzymes II (EII) which are sugar specific.

The overall reactions of the PTS are:

$$\text{PEP} + \text{HPr} \xrightarrow{\text{EI}} \text{phospho-HPr} + \text{pyruvate} \quad (1)$$

$$\text{sugar} + \text{phospho-HPr} \xrightarrow{\text{EII}} \text{sugar-phosphate} + \text{HPr} \quad (2)$$

$$\text{net reaction: PEP} + \text{sugar} \xrightarrow{\text{EI, EII}} \text{sugar-phosphate} + \text{pyruvate} \quad (3)$$

The complete reactions are given in Figure I.4 for the glucose and fructose PTS's. Reaction (1) is common to the sugar PTS's described by Kundig et al. (1964) and contains what Roseman (1972b) calls the 'general proteins'; that is, HPr and EI. Reaction (2) depends on which EII complex is present. The properties of the two reactions (1 & 2) will be considered separately.

5.1. The general proteins of the PTS

5.1.1. Enzyme I

EI has been partially purified (Simoni et al., 1968; Hengstenberg et al., 1969; Roseman, 1969) or purified to apparent homogeneity from Salmonella typhimurium, E.coli and Staphylococcus aureus,

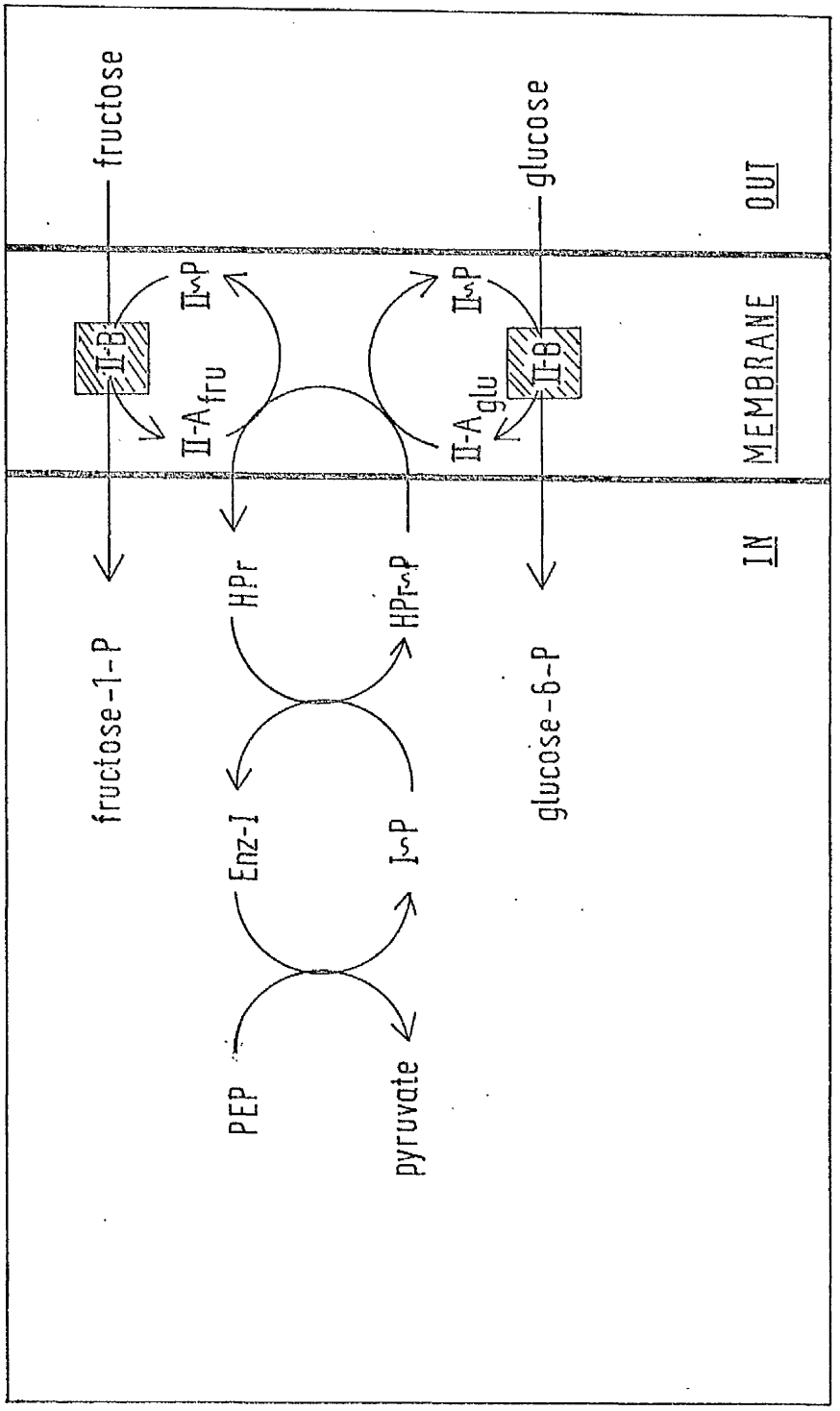


Figure I.4 The Phosphotransferase System

and Bag (1974) has recently obtained a similar preparation from Vibrio cholerae. The molecular weight, as determined by Sephadex gel filtration, is approximately 80,000. This protein is rapidly inactivated by sulphydryl reagents and contains an unknown number of sub-units. Difficulties have been reported (Roseman, 1972a) in studying this purified protein because of its instability. However transfer of a phosphoryl group from PEP to EI and from phospho-EI to HPr has been reported (Simoni et al., 1968: Nakazawa et al., 1971).

5.1.2. Heat stable protein

HPr is a low molecular weight protein of 8,500 - 9,500 depending on the bacterial species from which it has been isolated. HPr has recently been purified to homogeneity from S.typhimurium, E.coli and S.aureus (Anderson et al., 1968, 1971) and from V.cholerae (Bag, 1974). Although two forms of HPr were isolated, one form gave rise to the other by deamination during the initial heat step used in the preparation. Deaminated HPr was not as effective as native HPr as a phosphate carrier and it is now believed that neither form of HPr isolated in previous work (Roseman, 1969) was actually native HPr.

HPr contains no organic phosphate or carbohydrate, no cysteine, tyrosine or tryptophan and 2 mol of histidine per mol protein (Anderson et al., 1968; Roseman, 1969). It is reported (Kundig et al., 1964; Anderson et al., 1968; Roseman, 1969) that approximately 1 mol of P^{32} was transferred from P^{32}-enolpyruvate to 1 mol of HPr and that this phosphoryl residue is bound to histidine in the N-1 position. The energy of this phosphoryl ~HPr bond is approximately 2 x ATP (Anderson et al., 1971). These two components, EI and HPr, are the general proteins of the PTS and are always found in the cytoplasm.

5.2. Sugar specific components of the PTS

At least two sugar-specific proteins are always required for

the transfer of the phosphoryl group from phospho-HPr to a given sugar. In some cases both proteins are in the membrane (II-A, II-B systems) while in some cases one protein is in the membrane (II-B) while the other is found in the cytoplasmic fraction (factor III).

Membrane preparations from E.coli or S.typhimurium grown on any carbon source contain enzymes II capable of transferring phosphate from phospho-HPr to glucose (or glucose analogues such as ∝-methyl glucoside or 2-deoxy glucose), fructose and mannose or N-acetylmannosamine (Kundig & Roseman, 1969; Kundig et al., 1969; Kundig & Roseman, 1971). These constitutive enzymes-II have been fractionated into components (Kundig & Roseman, 1969, 1971) comprising II-A^{glucose}, II-A^{mannose}, II-A^{fructose}, II-B and phosphatidyl-glycerol. Enzyme II-B is a common component of the EII complex and associates with the required II-A's. The lipid requirement is specific for phosphatidyl-glycerol and maximum activity of the EII complex can only be regained if the three components II-A, II-B and phosphatidyl-glycerol are mixed in a specific order (Kundig & Roseman, 1969, 1971).

S.aureus differs from E.coli and S.typhimurium in that lactose is taken up and phosphorylated by the PTS instead of by active transport (Kennedy & Scarborough, 1967; Simoni et al., 1968; Hengstenberg et al., 1968; Anderson et al., 1968; Nakazawa et al., 1971; Simoni et al., 1973). Proteins II-A^{lac}, III^{lac} and II-B have been reported (Nakazawa et al., 1971) for the lactose PTS and a factor III and II-B for mannitol (Simoni et al., 1968; Hengstenberg et al., 1969). Roseman (1972a) has reported that protein II^{lac}, phospho-III^{lac} and lactose form a ternary complex. This is in contrast to E.coli and S.typhimurium systems where a phospho-II and sugar (not lactose) complex is formed.

In contrast to the systems examined above, Rose & Fox (1969)

have isolated a β-glucoside specific EII from S.aureus which does not contain any chemically detectable lipid phosphorus whereas all other EII complexes do (Kundig & Roseman, 1969, 1971). Walter & Anderson (1973) also report that the inducible fructose PTS of Aerobacter aerogenes (as opposed to the constitutive fructose PTS) does not require HPr. This system, which is induced in response to low fructose concentrations, has an absolute requirement for an inducible soluble protein which is distinguishable from HPr both by its size and inducibility (Hanson & Anderson, 1968). The inducible $EII^{fructose}$ can also be distinguished from the constitutive $EII^{fructose}$.

The phosphotransferase components from different organisms are not in all cases completely complementary. For example, HPr from E.coli is only 5 - 10% as effective as the HPr from S.aureus in the S.aureus PTS reactions (Simoni et al., 1968). However Bag (1974) has reported that the HPr, EI and EII's of V.cholerae are completely interchangeable with those of E.coli.

The structure and components of the PTS have been reviewed in more detail by Roseman (1969, 1972a,b).

5.3. Role of the PTS in sugar transport

The role of the PTS in sugar transport was suggested by Kundig et al. (1966) who showed that α-methyl glucoside was accumulated by E.coli as the sugar ester and Winkler (1966) also showed that only α-methyl glucoside-6-phosphate could be detected during the first minute of transport. Heppel (1967) subjected cells of E.coli to osmotic shock and showed that the subsequent loss of HPr reduced the ability of the cells to transport sugar. At this time genetic evidence was obtained for the involvement of the PTS in transport by Tanaka & Lin (1967) in A.aerogenes and Simoni et al. (1967) in S.typhimurium. These authors showed that EI deficient mutants

failed to accumulate or grow on many sugars although the parental and revertant strains did. Ghosh & Ghosh (1968) reported that frozen-thawed cells of E.coli could form 2-deoxy-D-glucose-6-phosphate in the external medium when incubated with 2-deoxy-glucose and PEP. However, fresh cells only accumulated the phosphate ester intracellularly. Since a 2-deoxyglucokinase could not be detected it was suggested (Ghosh & Ghosh, 1968) that the sugar was simultaneously transported and phosphorylated by the PTS; freezing affected the membrane such that the PTS was exposed to the exterior of the cell.

The use of membrane vesicles enabled Kaback (1969) to obtain convincing evidence for the role of the PTS in transport. He showed that PEP was specifically required for the uptake of certain sugars which accumulated almost completely as phosphorylated derivatives. Furthermore, membranes prepared from an E.coli mutant deficient in EI are unable to accumulate sugar under any conditions.

Gachelin (1970) has obtained evidence that facilitated diffusion of ∝-methyl glucoside precedes the phosphorylation of that analogue by E.coli and suggests that the PTS acts as a "trapping system" rather than a means of translocating sugars. These results are not supported by Winkler (1971) who found that, not only do EI and EII mutations abolish the intracellular accumulation of ∝-methyl glucoside-6-phosphate, but entry of glucose into the cell is almost completely prevented such that the intracellular concentration is much lower than the extracellular concentration.

Recent work by Kornberg & Smith (1972) and Kornberg & Miller (1972) using EII mutants has also provided convincing evidence for the involvement of the PTS in transport. This work has been corroborated by Kornberg & Reeves (1972a) who demonstrated that the rates at which intact E.coli take up ∝-methyl glucoside are identical to the rates at which toluenised

cells effect the PEP-dependent phosphorylation of ∝-methyl glucoside. Consequently, it must be concluded that the PTS is responsible for the group translocation of many sugars. The evidence for this has been comprehensively reviewed (Anderson & Wood, 1969; Roseman, 1969, 1972a,b; Lin, 1970) and need not be further discussed at this time.

5.4. Role of the PTS in initiating metabolism

Wood (1966) remarked that it had long been a problem to demonstrate the presence in bacteria of kinases for the phosphorylation of mannitol and fructose. ATP dependent fructokinases have been reported in A.aerogenes (Kelker et al., 1970) and E.coli (Sebastian & Asensio, 1967) but they are either not induced by growth on fructose or are insufficiently active to play a major role in fructose uptake and utilisation. It was subsequently recognised that fructose, like other hexoses, is phosphorylated by the PTS (Hanson & Anderson, 1966, 1968; Sapico et al., 1968; Fraenkel, 1968; Kelker et al., 1970) and that mannitol is similarly phosphorylated (Tanaka et al., 1967). It has also been shown (Fraenkel et al., 1964; Tanaka et al., 1967) that a glucokinase-negative mutant (FR-1) of E.coli is able to grow normally on glucose because glucose enters the cell as glucose-6-phosphate by the action of the PTS (Kundig et al., 1964). In some cases, therefore, it now seems certain that kinases are not essential for the metabolism of exogenously supplied hexoses and hexitols (Anderson & Wood, 1969) but scavenge for free intracellular sugar (Anderson & Wood, 1969).

Fructose is phosphorylated at C-1 by the PTS and, for further metabolism, fructose-1-phosphate kinase (FPK) is required. This enzyme, which converts fructose-1-phosphate to fructose-1, 6-diphosphate, was first found in Bacteroides symbiosus (Reeves et al., 1966) and subsequently in A.aerogenes by Hanson & Anderson (1966, 1968). Mutants of A.aerogenes lacking FPK do not grow on fructose.

Fraenkel (1968) has shown that E.coli inducibly forms FPK when exposed to fructose. Although this work has been confirmed some major differences have emerged (Ferenci & Kornberg, 1971). One such difference concerns the role of fructose as a gluconeogenic growth substrate. The production of fructose-6-phosphate from fructose requires the operation of fructose-1,6-diphosphatase (FDPase). However, although mutants of A.aerogenes devoid of FDPase do fail to grow on fructose as would be expected (Sapico et al., 1968), similar mutants of E.coli grow well on fructose (Fraenkel, 1968; Fraenkel & Horecker, 1965). This apparently anomalous behaviour of E.coli has been resolved by Ferenci & Kornberg (1971) who showed that, at concentrations of fructose greater than 4mM, the fructose PTS would produce both fructose-1-phosphate and fructose-6-phosphate. Tanaka et al. (1967) have also shown that further metabolism of mannitol-1-phosphate requires a mannitol-1-phosphate dehydrogenase. Consequently, it appears that the PTS plays a dual role of simultaneously transporting substrates and initiating their metabolism in the cell. It might be anticipated that controls will be exerted on the PTS by the cell and these will be examined.

5.5. Control of the PTS

5.5.1. Glucose PTS

The glucose PTS appears to be regulated both by induction (Kornberg, 1972) and catabolite inhibition by sugar-phosphates (Kaback, 1969). Kornberg (1972) has shown that UMG^+ mutants (constitutive for the uptake of α-methyl glucoside) of E.coli grown on fructose can take up and phosphorylate α-methyl glucoside. Hence the glucose PTS is constitutive in the usual sense. However the glucose PTS can be further induced by growth on glucose (Kornberg, 1972) and Kornberg & Reeves (1972a) have shown that variations in the level of

induced glucose PTS activity are paralleled by similar variations in the PTS activity measured in toluenised cells. Glucose PTS level is therefore controlled by induction. There is no evidence in the literature that the glucose PTS is controlled by catabolite repression. The activity of the glucose PTS is finely controlled by sugar-phosphates (Kaback, 1969), particularly G1P and G6P, and Kaback (1969) has postulated that G1P plays a central role in the regulation of sugar transport since it selectively inhibits transport of other sugars as well as glucose.

5.5.2. Control of the fructose PTS

The control of the fructose PTS has been examined in some detail by Kornberg (1972, 1973b). Kornberg (1972) has shown that both fructose PTS and fructose-1-phosphate kinase (FPK) are induced by the exposure of *E.coli* to fructose or fructose-1-phosphate. Both Kornberg (1972) and Maas & Clarke (1964) have found evidence to suggest that both enzymes are co-ordinately regulated as a 'regulon' since Kornberg (1972) has shown that derepression of FPK synthesis does not require fructose-1-phosphate to be present. There is no evidence at this time to suggest that the fructose PTS:FPK regulon may not be controlled in a similar fashion to the *lac* or *gal* operons. While there appears to be no evidence that FPK is subjected to inhibitory controls, Kornberg (1972, 1973b) has obtained evidence that fructose PTS activity is subjected to catabolite inhibition. Kornberg (1972, 1973b) suggests that it is essential for glucose to be actively phosphorylated in order to exert its inhibitory effect on fructose uptake and that this may be yet another instance of catabolite inhibition (McGinnis & Paigen, 1969). This inhibition (Kornberg, 1972, 1973b) may also involve competition between glucose and fructose for common components of the PTS (c/f Roseman, 1969). However Kaback (1969) and

Kornberg (1972) have found that hexose-phosphates strongly inhibit fructose PTS activity. Kornberg (1972) concludes that the rate at which fructose can be taken up and used as a source of carbon and energy for E.coli may be at least partly controlled by the level of hexose-phosphates in the cell.

At this time the actual site(s) of glucose inhibition of fructose utilisation in E.coli, be it competition for PTS components, inhibition by hexose-phosphates or any other control mechanism, is unknown. The control of other PTS sugars does not seem to have been investigated in any detail.

6. The Thesis

Monod (1941, 1942) has reported that growth of E.coli on mixtures of glucose and fructose, glucose and mannose, or glucose and mannitol is not diauxic. This means that the substrates are either used sequentially or together. Holms (personal communication), using the gas-exchange technique, obtained preliminary results which suggested that glucose and fructose or glucose and mannitol were in fact used sequentially, even though growth was not diauxic. This led to the suggestion that glucose controlled the PTS activities and that changeover of growth from glucose to the secondary growth substrate was extremely well synchronised. Such control of PTS activities could be investigated by the isolation of mutants with control defects since this would establish the level of control at the genetic level and it might then be possible to establish the nature of the components involved in the control systems.

However, the sugar specific components of the PTS have been identified and can be assayed conveniently and specifically, at least in other strains of E.coli (Ghosh & Ghosh, 1968; Gachelin, 1969). During the initial stages of the work in this thesis, Reeves & Kornberg (1972a,b) confirmed the earlier work and reported a modification of Gachelin's method (1969). We concluded that, if such an assay could be applied to E.coli 15224, it could be used to investigate how glucose affects the level and activity of its own and other sugar PTS's.

To successfully study the dominance of glucose over other PTS's, the PTS's under investigation should be constitutive to avoid the complications of induction lags. Preliminary experiments (Figure R.1) indicated that the PTS's for glucose, fructose, mannose and mannitol were constitutive in that growth of cells trained to any

of the carbon sources studied (Figure R.1) was immediate on these four substrates. In contrast, inoculation into sorbitol of all but sorbitol trained cells gave a considerable lag.

It was decided not to investigate glucose control of the sorbitol PTS since Figure R.2a shows that growth of glucose or sorbitol trained cells on a mixture of glucose and sorbitol is always diauxic. For Figure R.2b approximate growth yields were estimated from the points at which changes in growth rate were observed. Figure R.2b shows that inoculation of glucose trained cells into glucose and mannitol appears to result in the utilisation of the glucose followed by the mannitol. However, inoculation of glycerol or mannitol trained cells into a similar mixture results in some co-utilisation of the glucose and mannitol indicating that the control by glucose of the mannitol PTS depends on the history of the cells. In contrast, Figure R.2b shows that inoculation of glucose, fructose or glycerol trained cells always appears to give the same sequential utilisation of the glucose and fructose. The glucose and mannose situation was not investigated because the commercially available mannose was very impure. Consequently the glucose-fructose system was chosen in which to investigate how glucose dominates the utilisation of some sugars without the necessity for diauxic growth. This system is also convenient in that assays for glucose and fructose are readily available.

The work in this thesis will therefore consist of applying (or developing) assays for the glucose and fructose PTS's and using these, and any other necessary techniques, to establish how glucose dominates fructose metabolism such that sequential utilisation, but not diauxie, is observed.

METHODS

1. Microbiological Techniques

1.1. Organism

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

The strain was obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland, U.S.A.) and characterised by bacteriological tests described by Cowan & Steel, 1955.

1.2. Re-constitution and storage of organism

The organism was obtained as a lyophilisate in a sealed evacuated glass ampoule which was opened as recommended (National Collection of Industrial Bacteria Catalogue, Aberdeen) and the cells reconstituted by the addition of a few drops of sterile nutrient broth. The reconstituted culture was inoculated into 10 ml of sterile nutrient broth in a 25 ml MacCartney bottle and incubated at 37° for 24 h, then plated on nutrient agar and incubated at 37° for 24 h. A typical clone was picked off, transferred to nutrient broth and grown as before. The nutrient broth culture was checked for homogeneity both microscopically and by plating out on nutrient agar containing 10 μg 5-bromo-4-chloro-indoxyl-β-galactoside /ml agar (BCIG agar). A cell constitutive for β-galactosidase produces a blue colony on BCIG agar so a homogeneous culture of lac-constitutive cells produces only blue clones.

A homogeneous nutrient broth culture was used to inoculate

10 ml of cooked meat medium in 25 ml MacCartney bottles which were again incubated at 37° for 24 h. These were then stored at 4° as long term stock cultures.

Every 3 months a cooked meat culture was used to inoculate 6 nutrient broths and these were grown at 37° for 24 h, plated on BCIG agar to test for homogeneity, then stored at 4°. A fresh nutrient broth was used each month for the preparation of inocula.

1.3. Preparation of specifically trained inocula

Complete defined medium, prepared as described in section 2.5.1., was inoculated with 3 drops of a stock nutrient broth culture and grown on an orbital shaker (L.H. Engineering Co. Ltd., England) at 37°. This was a 1st passage and 1 ml of it was subcultured into 100 ml of identical medium (2nd passage) and grown under the same conditions. These cultures were stored at 4°, 24 h before an experiment 1 ml of the 2nd passage was subcultured into a 3rd passage, grown as above and stored overnight at 4°. The growth times were 16 h for the 1st passage and 7 h for the 2nd and 3rd.

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

When an inoculum was required for a continuous culture experiment, a portion of the 3rd passage was aseptically transferred to the growth flask by a sterile syringe.

2. Media

2.1. Cooked meat medium

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

One litre of cooked meat medium contained:

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

2.2. Nutrient broth medium

Nutrient broth was prepared from Oxoid dehydrated granules.

One litre of nutrient broth contained in distilled water:

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

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

2.3. Nutrient agar medium

Nutrient agar was prepared using Oxoid dehydrated granules dissolved in distilled water by boiling for 15 min. The agar was sterilised by autoclaving at 15 p.s.i., poured into petri dishes under ultraviolet light and allowed to solidify. Plates were stored at 4°. The composition of the nutrient agar medium was similar to that reported above (section 2.2.), with the addition of 15 g agar/l.

2.4. BCIG agar medium

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

2.5. Defined media

Defined media were prepared by three different procedures. All components were made up in glass distilled water.

2.5.1. Media for preparation of specifically trained inocula

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 the carbon source was 25 mM for hexoses and hexitols and 50 mM for glycerol and malate. This solution was dispensed into bottles in 40 ml batches before autoclaving at 5 p.s.i.

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

2.5.2. Medium for batch growth experiments

This was prepared as 4 separate components:

I P 760 ml 40 mM 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 $(NH_4)_2SO_4$) sterilised by autoclaving at 15 p.s.i.

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

IV Sugar prepared at high concentration and sterilised by filtration.

Complete defined medium was prepared by adding 10 ml each of solutions II and III and the required volume of solution IV to solution I. The inoculum and distilled water made the final volume up to 800 ml.

2.5.3. Medium for continuous culture experiments

This was prepared as 4 separate components:

I P 19.6 l 40 mM potassium dihydrogen phosphate (5.44 g/l KH_2PO_4) pH 7.0 sterilised by autoclaving at 5 p.s.i. for 60 min.

II MgNS contained 1 M ammonium sulphate (132 g/l $(NH_4)_2SO_4$) and 50 mM magnesium sulphate (12.5 g/l $MgSO_4.7H_2O$). This solution was dispensed 200 ml in bottles and sterilised by autoclaving at 15 p.s.i.

III Chel-metals were prepared as follows (C.A. Fewson - personal communication):
In 500 ml distilled water were dissolved 50 g nitrilotriacetic acid ("chel NTA"), 1.10 g $FeSO_4.7H_2O$, 50 mg $Na_2MoO_4.2H_2O$, 50 mg $MnSO_4$, 50 mg $ZnSO_4.7H_2O$, 25 mg $CuSO_4.5H_2O$, 25 mg $CoCl_2.6H_2O$, 125 ml 5 M NaOH, to pH 7.0 with

5 M HCl and then distilled water to 1 litre.

The solution was dispensed 100 ml in bottles

and sterilised by autoclaving at 5 p.s.i.

IV Carbon source prepared at high concentration

and sterilised by filtration.

The growth medium was prepared by adding 200 ml of solution II, 100 ml of solution III and 100 ml of solution IV to solution I via a Sterifil filter holder of 250 ml capacity fitted with a 0.22 μ pore size Millipore filter (Millipore Corp., Massachussetts, U.S.A.). This arrangement is fully described in section 6.2.1.

The composition of defined medium made by any of the three methods was:

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

with NaOH to pH 7.0

and sugar(s) at required concentration.

Chel-metals were present in the defined medium for continuous culture experiments. Components, with the exception of phosphate, were stored at 4^{o}.

3. pH Measurement

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

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

4. Sterilisation

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

4.1. Autoclaving

Solutions and some 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 pressures (5 or 15 p.s.i.) and the time of autoclaving depended on the nature and volume of the solutions being sterilised. Sterilisation was always checked using Browne steriliser control tubes - type one, black spot (Browne Ltd., Leicester, England).

4.2. Filtration

Sterilisation by filtration was carried out using Sterifil

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

A second procedure for smaller volumes used Nalge disposable filter units (0.2 μ pore) (Sybron Corp., Rochester, U.S.A.). These held 100 ml and were obtained in a sterile condition. After filtration solutions were transferred aseptically to sterile bottles.

5. Glassware

5.1. General glassware

All glassware was cleaned before use either by boiling in 10% v/v nitric acid or by autoclaving in 1% w/v haemosol solution (Meinecke & Co., Baltimore, U.S.A.). After either treatment all glass was rinsed once with tap water, three times with 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 followed by rinsing with tap and deionised water, and drying in an oven. All pipettes were plugged with cotton wool before use.

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

6. Growth

6.1. Batch culture

Some growth experiments were carried out in batch culture. 800 ml of complete defined medium contained in a 1 litre flat-bottomed pyrex flask fitted with a short side arm was inoculated and maintained at 37^{o} in the apparatus described by Harvey et al. (1968). The side arm was closed by a stainless steel cap and the main neck of the flask was sealed by a silicone rubber bung through which a port was made. This was fitted with a glass tube and used as the gas inlet. Air from the departmental compressed air supply was filtered through charcoal and passed into the flask at a steady flow rate. The flow rate was monitored at 100 ml/min on gas flow gauges (G.A. Platon, Croydon, England). The culture was agitated by a magnetically coupled stirrer to ensure that the culture was always aerobic.

6.2. Continuous culture

Growth of the organism was also studied under continuous culture conditions. The practical background to continuous culture is well documented in the literature. A useful selection of references is quoted: Baker (1968), Evans et al. (1970), Garland & Light (1971), Jannash (1965), Postgate (1965), Powell (1965), Tempest (1965).

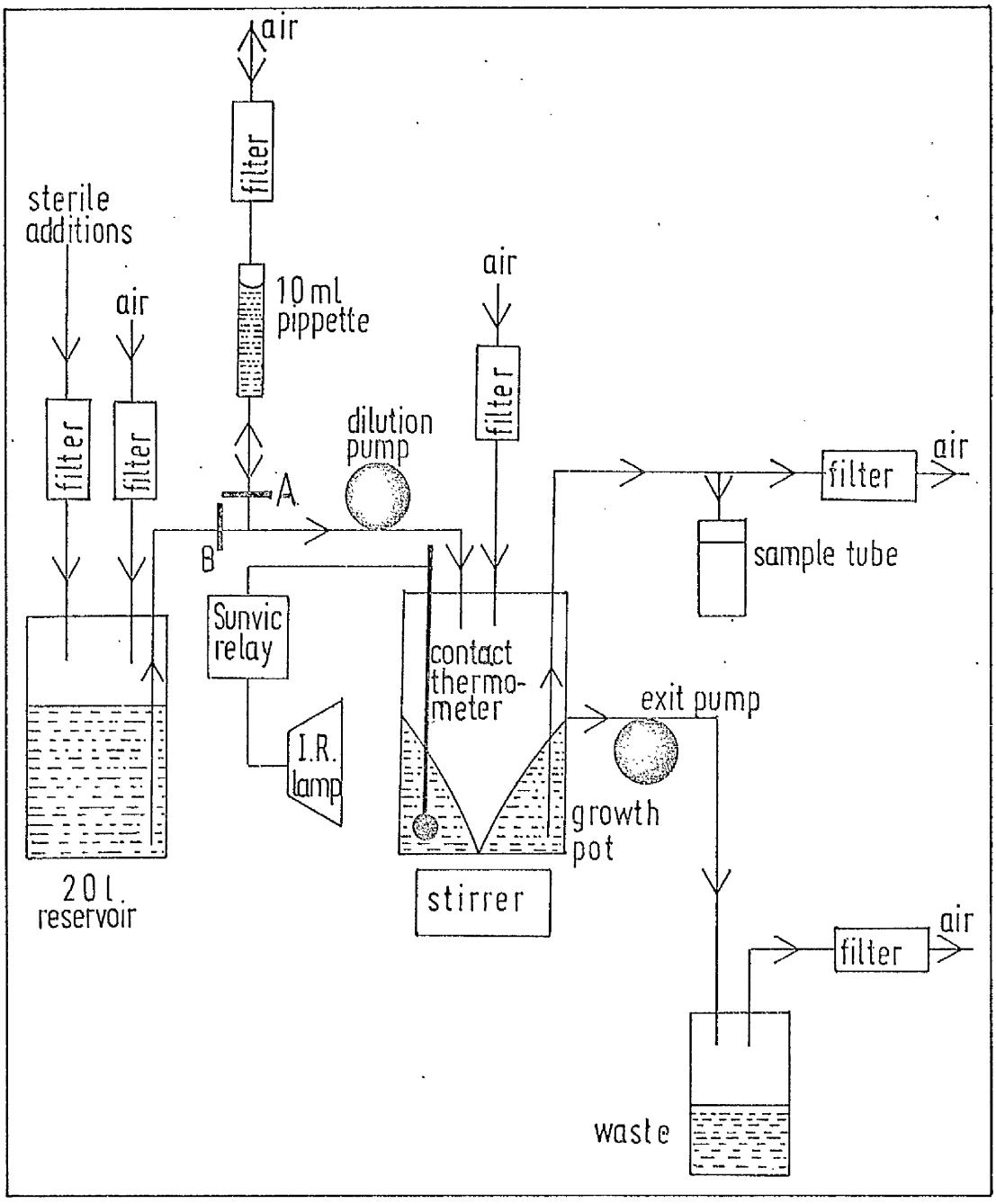
6.2.1. Growth apparatus

The growth apparatus took the form shown in Figure M.1 and follows the general design reported by Baker (1968). The 20 l reservoir (Quickfit FV20L; MacFarlane-Robson Ltd., Glasgow) of growth medium was fitted with a Sterifil filter holder assembly of 250 ml capacity with a 0.22 μ pore size millipore filter to enable sterile additions to be made to the reservoir (C.A. Fewson - personal communication).

Figure M.1

BLOCK DIAGRAM OF CONTINUOUS CULTURE GROWTH APPARATUS

The diagram illustrates the general organisation of the continuous culture growth apparatus described in section 6.2.1. The only variation in this system was to the volume of the growth pot (see sections 6.2.2. and 6.2.3.). Tubing clips were provided at the points A and B in the diagram enabling the dilution pump to draw medium either directly from the 20 l reservoir or from the 10 ml pipette, which served as a flowmeter (see section 6.2.4.). The pipette was refilled by opening both clips.


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

Medium was pumped from the reservoir to the growth pot, via a 10 ml pipette assembly to measure flow rate, by a Watson-Marlow peristaltic pump Type MHRE 200 (Watson-Marlow Ltd., Falmouth, Cornwall). Provision for aseptic changeover of supply reservoirs was incorporated into the feed-line. The flow rate was adjusted as required and the size of growth pot used depended on whether turbidostat (6.2.2.) or chemostat (6.2.3.) experiments were being performed. The top plate (Quickfit MAF 2/2) of the growth pot provided ports for input of sterile air and medium, sampling, inoculation and a contact thermometer. The medium and sterile air entered the growth flask via a device for preventing back-growth of the organism into the feed-line. Sterile air was obtained by passing charcoal filtered air from the departmental supply through a Microflow filter (Microflow Ltd., Fleet Mill, Minley Road, Fleet, Hants.), and was monitored at 100 ml/min. Arrangements were also made for the aseptic withdrawal of samples. Inoculation was performed by a sterile syringe through a self sealing rubber cap (MacFarlane-Robson Ltd., Glasgow). A fixed outlet port was incorporated into the wall of the growth pot in order to maintain a constant volume of culture. The overflow of spent medium and cells was pumped to waste by a second Watson-Marlow pump, which maintained a flow rate greater than that of the dilution pump. The growth pot contents were mixed and maintained aerobic by agitation with a magnetically coupled stirrer.

The temperature of the culture was sensed by a fixed contact thermometer (J.C. Cowlishaws, Peary Street, Manchester, M4 4JB) which operated a 300 W Infra-Red lamp (MacFarlane-Robson Ltd., Glasgow) via a Sunvic electronic relay (AEI type EA4M; J.C. Cowlishaws, Peary Street, Manchester, M4 4JB) to maintain the temperature at 37^{o}.

The growth pot, 20 l reservoir and associated tubing were sterilised together by autoclaving at 5 p.s.i. before use.

6.2.2. Turbidostat

The growth pot was a 2 l fermenting vessel (Quickfit FV2L) containing 1 l of stirred culture. In these experiments the concentrations of all constituents of the growth medium were in excess of those required to maintain growth. Consequently the turbidity of the culture was kept at the required value (E_{420} = 0.5) solely by fine adjustments to the dilution rate to maintain $\mu = \mu$ max (Bryson, 1952).

6.2.3. Chemostat

In this sytem the growth pot was a 1 litre fermenting vessel (Quickfit FV1L) containing 360 ml of stirred culture. The concentrations of all constituents of the medium were in excess of those required to maintain growth, except for the sugar concentration, which was limiting. The specific growth rate (μ) was altered solely by changing the flow rate of the dilution pump and was always less than μ max.

6.2.4. Calibration of Watson-Marlow peristaltic pumps

Before inoculation the growth pot was routinely filled with complete medium and equilibrated to 37°. The speed control of the Watson-Marlow pump was also calibrated in terms of flow rate. For most settings in the range available a linear relationship between the setting and flow rate induced was observed.

6.2.5. Theoretical considerations

Much has been written on the theory of microbial growth in continuous culture since the first principles were expounded by Monod (1950) and by Novic and Szilard (1950). Only a cursory treatment is attempted in this section and the reader is referred elsewhere (Tempest, 1970; Powell, 1965) for a more detailed study.

To describe the behaviour of microbial cultures in a chemostat

it is necessary to consider the effect of dilution rate (D) on the concentration of growth - limiting substrate (s) and of organisms (x) in the culture. Substrate enters the growth vessel at a concentration S_r. For the simplest case when all S_r is consumed for growth, then:

$$\text{Change} = \text{Input} - \text{Consumption}$$

$$\frac{dx}{dt} = DS_r - \frac{\text{Growth}}{\text{Yield}}$$

$$= DS_r - \frac{\mu x}{y}$$

at steady state $\frac{dx}{dt} = 0$

$$S_r = \frac{\mu x}{DY} = \frac{x}{y} \quad (\text{since } \mu = D)$$

(Y = growth yield on growth-limiting substrate)

Since y is known for the sugars used in these experiments (R.J. Wallace - personal communication) then the approximate value of S_r required to maintain a given concentration (x) of cells can be calculated.

7. Sampling of the Culture

7.1. Batch culture

Samples were removed through the side arm of the growth flask using a pipette which was rinsed with culture before the sample was removed.

7.2. Continuous culture

Samples were removed by siphoning off the required volume of culture into a sterile 50 ml polycarbonate centrifuge tube (M.S.E. Ltd., Crawley, Sussex, England) attached to the sampling port (Figure M.1). The tube was removed, capped and a replacement fitted immediately.

Samples derived from either system were used to measure culture density, culture pH, substrate concentration and PTS activity. In addition, samples from the continuous culture system were plated out daily on BCIG agar plates to check the homogeneity of the culture.

8. Measurement of Growth

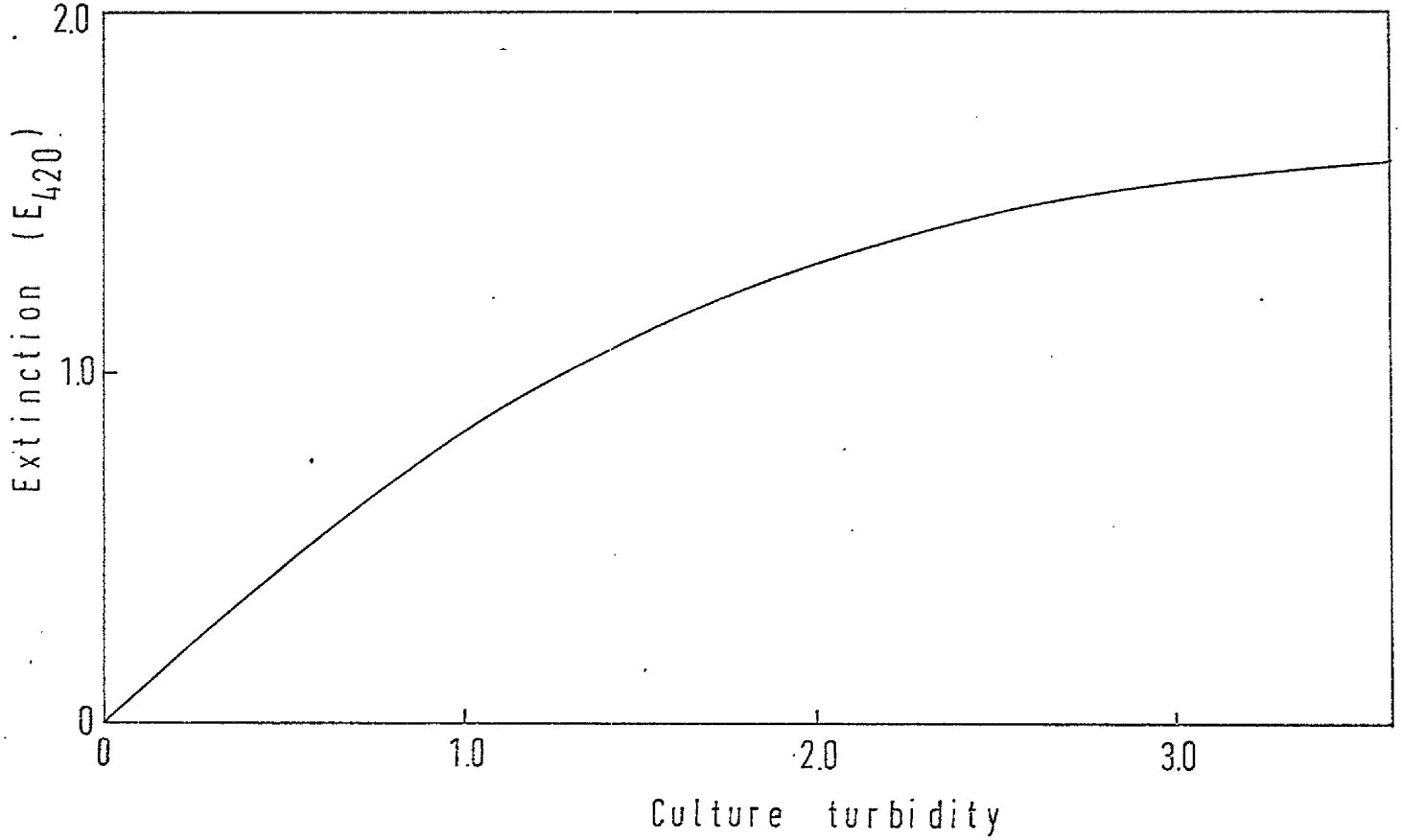
Cell density was determined turbidimetrically on samples (4 ml) taken from the culture into formaldehyde solution (40%, 1 drop). The apparent E_{420} was measured in glass cuvettes (Type 1, 10 mM light path; Ross Scientific Co. Ltd., Hornchurch, England) using an SP800 double beam spectrophotometer (Unicam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

The absorption due to the cells was linear with culture turbidity up to an optical density of 0.2. Above this value the optical density was lower than the true culture turbidity. Consequently a calibration curve was drawn between optical density and culture turbidity (Figure M.2) to enable turbidities to be determined without dilution. A culture turbidity of 1.0 at 420 nm is equivalent to a cell density of 196 μg dry weight/ml culture (Holms et al., 1972). This relationship was found to apply for growth on both glucose and fructose.

Figure M.2

TURBIDITY CALIBRATION CURVE

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


2.0
1.0
0
Extinction (E_{420})
0
1.0
2.0
3.0
Culture turbidity

9. Measurement of Substrate Concentration

9.1. General considerations

Substrate concentrations were measured on samples taken from the culture. Enzymic assays were carried out for glucose, fructose, pyruvate and glucose-6-phosphate. The assays were read on an SP800 double beam spectrophotometer fitted with a Servoscribe chart recorder against air as blank.

9.2. Treatment of samples

A 4 ml sample taken from the culture was blown onto 1 ml of chilled 30% w/v perchloric acid, mixed and allowed to stand on ice for 10 min, when 3 ml of chilled potassium hydroxide ($\sim$1 M) was added to return the sample to pH 7.0. 0.1 M BES was added to the potassium hydroxide to buffer the effects of slight variations in potassium hydroxide concentration (W.H. Holms - personal communication). When the potassium perchlorate had precipitated the samples were decanted into 15 ml centrifuge tubes (Corning Glass Works, New York, U.S.A.) and centrifuged at 37,000 g and 4° for 10 min in an M.S.E. - 18 refrigerated centrifuge (M.S.E. Ltd., Crawley, Sussex, England). The supernate was decanted into 10 ml plastic vials (M. & H. Plastics (Romford) Ltd., Chesham Close, Romford, Essex) and stored at -10° until assayed.

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

9.3. Estimation of glucose

Glucose was originally assayed using the Boehringer 'GOD-Perid' method which is based on that of Werner *et al.* (1970). However the presence of BES in the sample extracts interfered with the assay and the method was rejected.

Glucose was assayed by a modification of the method described by Boehringer for determining glucose-6-phosphate dehydrogenase activity.

The composition of the assay was:

Sodium dihydrogen phosphate	200 mM
magnesium sulphate	2 mM
ATP	0.4 mM
NADP	0.2 mM
hexokinase	3 µg/ml
glucose-6-phosphate dehydrogenase	3 µg/ml

The assay was performed at pH 7.2 in a total volume of 3 ml. All reagents were mixed before addition to a portion of the sample. The assays were incubated at 27^{o} for 60 min and then read at 340 nm. The assay is linear over the range 0-500 n mol glucose/assay. 1 mol glucose in the assay gives an extinction of 2.07×10^{6}.

9.4. Estimation of fructose

Fructose was assayed by a modification of the method described by Boehringer for determining phosphoglucose isomerase activity. The composition of the assay was similar to that in section 9.3., but included 3 µg/ml phosphoglucose isomerase. The assays were again incubated at 27^{o} for 60 min and then read at 340 nm. The assay is linear over the range 0-500 n mol fructose/assay. 1 mol of fructose in the assay gives an extinction of 2.07×10^{6}.

9.5. Estimation of pyruvate

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

The composition of the assay was:

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

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

9.6. Estimation of glucose-6-phosphate

Glucose-6- phosphate was assayed using a modification of the standard Boehringer assay for glucose-6-phosphate dehydrogenase.

The composition of the assay was:

Sodium dihydrogen phosphate	200 mM
magnesium sulphate	2 mM
NADP	0.2 mM
glucose-6-phosphate dehydrogenase	3 µg/ml

The assay was performed at pH 7.2 in a total volume of 3 ml. All reagents were mixed before addition to a portion of the sample. The assays were incubated at 27^{o} for 60 min and then read at 340 nm. The assay is linear over the range 0-500 n mol glucose-6-phosphate/assay. 1 mol of glucose-6-phosphate gives an extinction of 2.07×10^{6}.

10. Spectrophotometric Determination of PTS Activities

10.1. Preparation of cells

Samples, containing approximately 3 mg dry wt. of cells, were removed from a culture for the assay of PTS activities, chilled in an ice-water slurry and then harvested at 11,600 g and 4^{o} for 10 min in an M.S.E.-18 centrifuge. The cells were washed to remove residual sugar by resuspension in chilled buffer to a volume of 20 ml and harvested as above. The centrifuge tube containing the cell pellet was covered with parafilm and stored on ice.

10.2. Decryptification of cells

Within 7 h of harvesting, the pellets were resuspended to a cell density of approximately 200 µg dry wt./ml in 15 ml chilled buffer. 4 ml was removed for measurement of turbidity at 420 nm as described (methods 8). 10 ml of the cell suspension was blown onto 100 µl of 35% (v/v) benzene in ethanol contained in a chilled 15 x 140 mm test-tube and vortexed for 70 sec (Whirlimixer, Scientific Instruments, International Inc. - (U.K.) Ltd.). The tube was returned to the ice-bath and aspirated for 20 min with OFN monitored at 100 ml/min. The cells were then ready for immediate assay.

10.3. Assay of glucose PTS activity

All assays were carried out in plastic cuvettes of 1 cm light path (No. 40/1010; Walter Sarstedt (U.K.) Ltd., 47 Highmere Road, Leicester, LE4 7LZ) in a final volume of 1.5 ml. A mixture, containing 0.375 µ mol NADP, 9 µg glucose-6-phosphate dehydrogenase, 15 µ mol PEP, 500 µl decryptified cell suspension and assay buffer to 1.4 ml, was equilibrated at 27^{o} for 5 min. The assay was then started by the addition of either glucose (3 µ mol, 100 µl) or glucose and fructose (3 µ mol each, in 100 µl) if the presence of fructose was required. Control assays contained similar mixtures but with either PEP or

glucose omitted. The rate of NADPH production at 27°, in the test and control cuvettes, was measured by extinction at 340 nm, using an SP800 double beam spectrophotometer fitted with a Servoscribe chart recorder, against a blank cuvette containing 500 µl decryptified cells and assay buffer to 1.5 ml. The results were expressed as milli-enzyme units/ml decryptified cell suspension where 1 enzyme unit represents the reduction of 1 µ mol of nucleotide/min at 27° under the assay conditions. An alternative method of assay is given in section 10.4.

10.4. Assay of fructose and total PTS activity

All assays were performed in plastic cuvettes of 1 cm light path in a final volume of 1.5 ml. A mixture, containing 0.1 µ mol NADH, 9 µg lactate dehydrogenase, 15 µ mol PEP, 500 µl decryptified cell suspension and assay buffer to 1.4 ml, was equilibrated at 27° for 5 min. The assay was then started by the addition of either fructose (3 µ mol, 100 µl) or a mixture of glucose and fructose (3 µ mol each, in 100 µl). The rate of decrease of extinction concomitant with the oxidation of NADH was measured at 340 nm and 27° for both test and control cuvettes, using an SP800 double beam spectrophotometer fitted with a Servoscribe chart recorder, against a blank cuvette containing 500 µl decryptified cells, 0.1 µ mol NADH and assay buffer to 1.5 ml. The results were expressed as milli-enzyme units/ml decryptified cell suspension where 1 enzyme unit represents the oxidation of 1 µ mol of reduced nucleotide/min at 27° under the assay conditions.

Specific activity of PTS enzymes was expressed as milli-enzyme units (mU)/mg cellular dry wt.

The development and verification of the enzyme assays and decryptification procedure are described in detail in Development section 1.

11. Determination of PTS Activities by Radioactive Assay

11.1. Liquid scintillation spectrometry

11.1.1. Liquid scintillation fluid

To 1 l toluene was added 300 ml ethanol and 5 g 2,5-diphenyloxazole. 13 ml portions of this fluid were dispensed by Zipette (30 ml size) into glass vials (6001008; Packard Instrument, Downers Grove, Illinois, U.S.A.) with disposable plastic caps (22R3; Metal Box Co. Ltd., Plastics Group, Portslade, Sussex).

Ethanol was present in the scintillation fluid to dissolve water associated with samples studied. 13 ml scintillation fluid would dissolve 250 µl of water associated with a damp filter to give a single liquid phase at counting temperature (7^{o}).

11.1.2. Apparatus

Samples were counted on Philips Liquid Scintillation Analysers (PW 4510/00; Philips Scientific Equipment, Eindhoven, Netherlands) fitted with calculators to convert d.p.m. to absolute units.

11.2. Preparation of cells

The samples were prepared exactly as in section 10.1.

11.3. Decryptification of cells

With the exception that the cells were resuspended in either assay buffer or 0.2 M BES, 2 mM $MgSO_4$, pH 7.2. the decryptification procedure was executed exactly as in section 10.2.

11.4. Assay of glucose PTS activity

The assay mixture contained, in a final volume of 1 ml, 500 µl decryptified cells, 1.0 µ Ci D-glucose-C^{14} (U), 2 µ mol glucose and assay buffer or 0.2 M BES, 2 mM $MgSO_4$, pH 7.2 to 0.9 ml. The assay also contained 2 µ mol fructose if required. The mixture was chilled until the reaction was started by the addition of 10 µ mol PEP (100 µl) and incubation at 27^{o} for 15 min. The reaction was terminated

by the addition of 1 ml chilled 2 M glucose solution and the mixture returned to the ice-water slurry. A control assay contained similar mixtures but was terminated at 0 min and chilled throughout the 15 min incubation period. 50 µl portions were removed and spotted onto duplicate 2.3 cm circles of DE81 filter paper (Whatman DE81, MacFarlane-Robson Ltd., Glasgow) which were washed 3 times in 600 ml of 0.1 M glucose solution. The circles were dried and measured for retained radioactivity as described (section 11.1.).

11.5. Assay of fructose PTS activity

The assay mixture contained, in a final volume of 1 ml, 500 µl decryptified cells, 1.0 µ Ci D-fructose-C^{14} (U), 2 µ mol fructose and assay buffer or 0.2 M BES, 2 mM $MgSO_4$, pH 7.2 to 0.9 ml. The assay also contained 2 µ mol glucose if required. The mixture was chilled until the reaction was started by the addition of 10 µ mol PEP (100 µl) and incubation at 27^{o} for 15 min. The reaction was terminated by the addition of 1 ml chilled 2 M fructose solution and the mixture returned to the ice-water slurry. A control assay contained similar mixtures but was terminated at 0 mins and chilled throughout the 15 min incubation period. 50 µl portions were removed and spotted onto duplicate 2.3 cm circles of DE81 filter paper which were washed 3 times in 600 ml 0.1 M fructose solution. The circles were dried and measured for retained radioactivity as described (section 11.1.).

The specific activity of both enzyme systems was expressed as c.p.m./mg dry wt. cellular material.

Justification for this assay system is given in Development 2.

12. Estimation of Glucose and Fructose Utilisation

The ability of cells to utilise glucose and fructose singly and in combination was determined in complete growth medium containing 0.3 mM chloramphenicol (CAP) to inhibit protein synthesis.

A sample of culture containing 20 mg dry wt. of cells was harvested at 11,600 g and 4^{o} for 10 min. Cells were resuspended in 20 ml of chilled 40 mM phosphate pH 7.0, harvested as above and the pellet resuspended in chilled 40 mM phosphate pH 7.0 to a cell density of 2 mg dry wt./ml. A portion was further diluted in 40 mM phosphate buffer pH 7.0 and used to determine cell density by turbidity at 420 nm as described.

Cells were inoculated to a final cell density of 80 µg dry wt./ml into a series of flasks containing at final concentrations:

KH_2PO_4 (NaOH to pH 7.0)		40 mM
$MgSO_4$		0.5 mM
$(NH_4)_2SO_4$		10 mM
$FeSO_4$		10 mM
glucose and/or fructose	each	2 mM
chloramphenicol		0.3 mM

Flasks were incubated at 37^{o} and agitated by magnetically coupled stirrers to provide aeration. Samples were taken from each flask at intervals after inoculation and treated as described (section 9.2.). Samples were subsequently assayed for glucose and fructose as required by the methods described (sections 9.3. and 9.4. respectively). The rate of sugar utilisation in each flask was calculated in terms of µ mole/h/ml. The specific rate of utilisation was expressed as µ mole/h/mg cellular dry wt.

13. Estimation of the Effect of NEM on PTS Activities

This was carried out using a modification of the procedure described by Haguenauer-Tsapis & Kepes (1973).

13.1. Preparation of cells

Cell pellets were obtained as in section 10.1. and resuspended to a cell density of approximately 200 µg dry wt./ml in 20 ml chilled buffer. 4 ml was removed for measurement of turbidity as described.

13.2. NEM treatment

The system contained, in a final volume of 15 ml in a 15 x 140 mm test-tube, 13 ml cell suspension, 1.5 µ mol of glucose and/or fructose and buffer to 14 ml. The mixture was equilibrated at 27° for 10 min when 3 µ mol NEM (in 500 µl) was added. The treatment was terminated 2 min later by the addition of 75 µ mol 2-mercaptoethanol (in 500 µl). Control tubes contained similar mixtures but either the appropriate sugar(s) was omitted or NEM and 2-mercaptoethanol were added simultaneously at 10 min and incubated at 27° for a further 2 min. When treatment was complete, the tubes were chilled in an ice-water slurry.

13.3. Determination of PTS activities

10 ml portions of the chilled suspensions were decryptified (section 10.2.) and assayed for glucose and fructose PTS activities (sections 10.3. and 10.4. respectively).

14. Purification of Sorbitol

The commercially available sorbitol was purified using the method described: Horwitz (1966).

DEVELOPMENT OF ANALYTICAL METHODS

1. Measurement of PTS Activity

1.1. General considerations

It was decided at the outset that since the reactions of the PTS are:

glucose + PEP $\xrightarrow{\text{GPTS}}$ glucose-6-phosphate + pyruvate

fructose + PEP $\xrightarrow{\text{FPTS}}$ fructose-1-phosphate + pyruvate

(or other PTS substrate) (sugar-phosphate)

then glucose PTS activity could be conveniently determined from either G6P or pyruvate production and fructose or other sugar PTS by pyruvate production. It will be seen, too, that if the assay contained both glucose and any other PTS substrate the total PTS activity would be given by pyruvate production, the glucose PTS activity specifically by G6P and the difference (pyruvate - G6P) would be the activity of the other PTS under these conditions. Accordingly one essential requirement of the assay system would be the equivalence between G6P and pyruvate production when studying glucose PTS activity. G6P was to be determined using G6PDH linked to NADP and pyruvate by using LDH linked to NADH. The activities of the PTS's were not expected to be constant. Consequently the assay used for the PTS must permit measurement over the range of activities found, give equivalence of G6P and pyruvate production and allow reproducible results to be obtained. The final requirement for the assays was that they be followed kinetically to enable an instant check on linearity.

1.2. Investigation of existing PTS assays

1.2.1. Ghosh - frozen thawed method

This method (Ghosh & Ghosh, 1968) relies on the fact that freezing cells at -20° for 20 h followed by thawing modifies the cell

membrane such that PTS activity can be determined by addition of sugar and PEP. Cells of E.coli 15224 were treated as described (Ghosh & Ghosh, 1968) and assayed as in methods (section 10.3.) except that only 1.5 μ mol PEP was present and the blank cuvette was omitted. The results gave very low, irreproducible PTS activities (∿10 mU/mg dry wt.) and the method was rejected.

1.2.2. Gachelin - toluenisation method

Cell suspensions of E.coli 15224 trained to glucose were prepared as in methods (section 10.1.) and subjected to treatment with toluene (Gachelin, 1969). The decryptified cells were assayed for glucose PTS as in methods (10.3.) except that only 1.5 μ mol PEP, .75 μ mol glucose were used, the assay buffer was 0.2 M NaH_2PO_4 pH 7.2, with 2 mM $MgCl_2$ and the blank cuvette was omitted. The technique gave irreproducible results of similar activities to those in (1.2.1.). However, Gachelin (1969) used E.coli K12 and the method has been successfully modified (Kornberg & Reeves, 1972a) for other strains. Because of this, and since toluenisation appeared to be a quick and convenient means of decryptification, it was decided to investigate if the method could be modified to decryptify cells of E.coli 15224 and to develop an assay which allowed maximal PTS activity to be observed.

1.3. Toluene induced decryptification and modification of the Gachelin PTS assay system

1.3.1. Effect of vortexing cells with toluene

Cell suspensions of E.coli 15224 trained to glucose were harvested and resuspended to a cell density of approximately 200 μg dry wt./ml in 15 ml chilled buffer. 4 ml was removed for measurement of turbidity at 420 nm as described. 10 ml of cell suspension was blown onto 100 μl of 10% (v/v) toluene in ethanol

(to promote emulsion formation) contained in a 15 x 140 mm test-tube and vortexed for periods ranging from 10 - 90 sec. Glucose PTS activity was determined as described (methods 10.3.) except that 1.5 μ mol PEP was used, with 0.2 M NaH_2PO_4 pH 7.2, and 2 mM $MgCl_2$ as assay buffer and the blank cuvette was omitted. The activity made available by toluenisation seemed to be reasonably constant from 50 - 90 sec treatment and so 60 sec was chosen for future work. The glucose PTS activities obtained were much higher ($\sim$40 mU/mg dry wt.) than formerly observed.

1.3.2. Toluene activity release curve

This was generated using the procedure reported in section 1.3.1. except that the toluene in ethanol concentration varied from 5 - 30% (v/v) and the vortexing time was 60 sec. The activity released peaked sharply at 1 μl toluene/ml cell suspension as reported (Gachelin, 1969) (Figure D.1.) and so 1 μl toluene/ml cell suspension (used as 10% v/v toluene in ethanol) was chosen for future use.

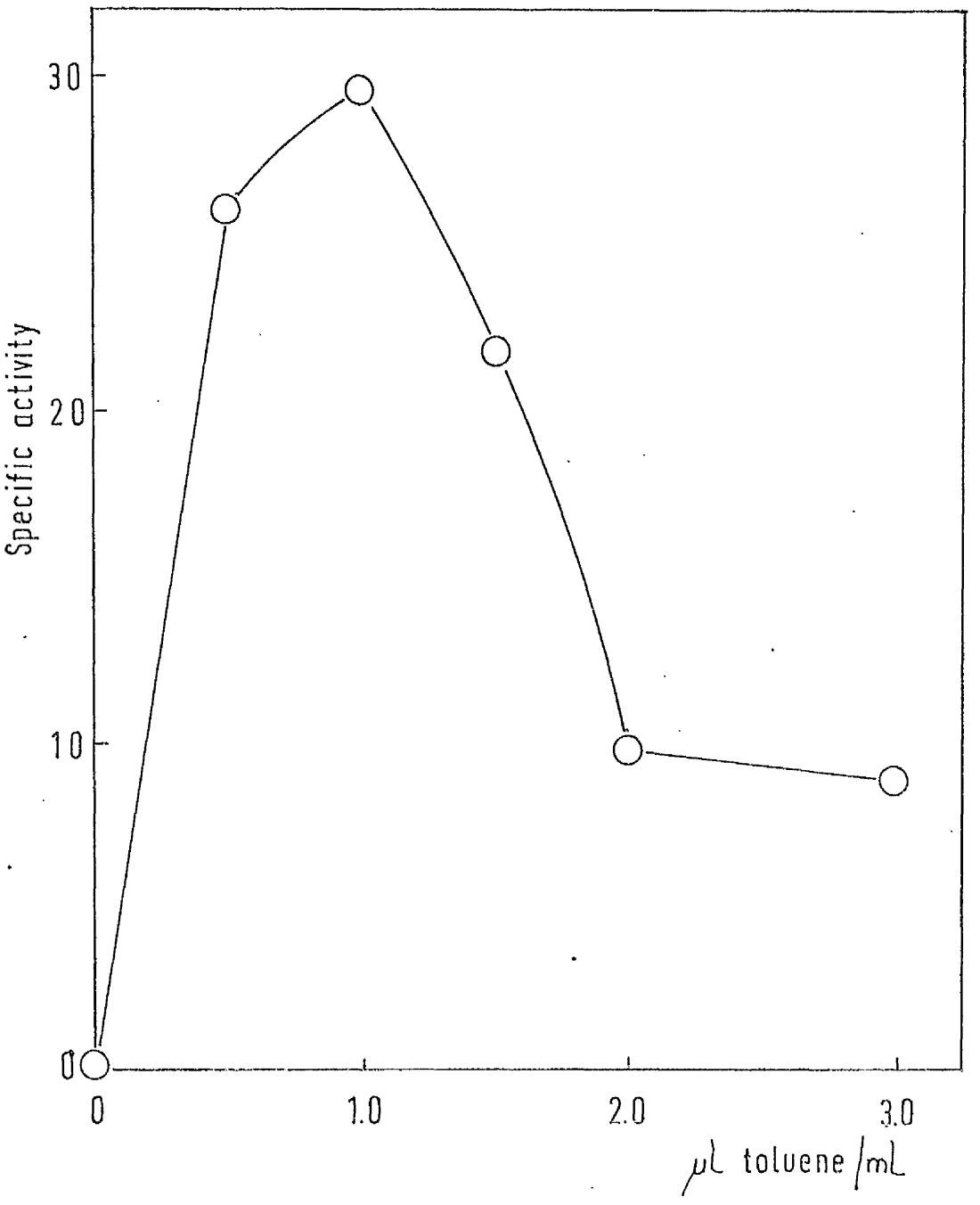
1.3.3. Comparison of PTS activities determined on single and double beam spectrophotometers

Up to this time all PTS assays had been performed using the SP800 as a single beam spectrophotometer with air as blank. The results of assays showed that the activities seemed to decay over a period of 3 h after toluene treatment. However it was reported (Jackson & De Moss, 1965) that toluene at concentrations greater than 0.6 μl toluene/ml cells induced cell lysis. It was therefore suspected that this apparent decay arose because the toluene treated cells became increasingly susceptible to toluene induced lysis at 27^{o}. To minimise the effect the toluenised cells were subsequently stored chilled as recommended (Jackson & De Moss, 1965) until assayed. The effect meant that the observed activity was a function of the production of NADPH and the decrease in turbidity of the cell suspension

Figure D.1

EFFECT OF TOLUENE CONCENTRATION ON THE DECRYPTIFICATION OF THE GLUCOSE PTS IN *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested, washed in chilled buffer and resuspended to a density of 200 µg dry wt./ml. Portions of the suspension were decryptified with the required concentration of toluene in ethanol by vortexing (60 sec). Glucose PTS activities were determined.


30
20
10
0
Specific activity
0
1.0
2.0
3.0
µl toluene /mL

and was therefore less than the actual activity. This was corrected for by reading the assay against a cuvette containing cells only in a double beam spectrophotometer (Figure D.2). For pyruvate assays the cuvette contained cells and also NADH (.1 μ mol) to correct for the slight NADH oxidase activity exhibited by the cells.

1.3.4. Effect of buffers on the assay

Cell suspensions of E.coli 15224 trained to glucose were harvested and resuspended to a cell density of approximately 200 μg dry wt./ml in 15 ml of the chilled buffers described in Figure D.4. 4 ml was removed for measurement of turbidity as described. The cells were decryptified as in 1.3.1. and glucose PTS activity determined as in methods (10.3.) using 1.5 μ mol PEP and the required assay buffers (Figure D.4.). Maximal activity was obtained when the cells were decryptified in 40 mM KH_2PO_4 pH 7.0, with 2 mM $MgSO_4$ (referred to as "buffer") and assayed in 0.2 M NaH_2PO_4 pH 7.2, with 2 mM $MgSO_4$ ("assay buffer"). It will be noticed that $MgSO_4$ has replaced $MgCl_2$ because Cl^- was found to inhibit PTS activity (Figure D.3.) when supplied as NaCl.

1.3.5. Effect of PEP concentration on PTS assays

Use of the modified assay showed that the results were still irreproducible. This was traced partly to using 1.5 μ mol PEP in the assay. The effect of PEP concentration was determined. A reciprocal plot of the data obtained (Figure D.5) gave a Km for PEP of 1.25 mM. This is higher than the value reported by Gachelin (1969). The observed activity was independent of PEP concentration above 10.5 μ mol PEP/assay. 15 μ mol/assay was used for future assays.

1.3.6. Discrepancy between pyruvate and G6P production

1.3.6.1. General considerations

It was observed at this stage that glucose PTS activity when

Figure D.2

COMPARISON OF GLUCOSE PTS ACTIVITIES IN E.coli 15224 AS MEASURED BY SINGLE AND DOUBLE BEAM SPECTROPHOTOMETERS

Glucose trained cells of E.coli 15224 were harvested, washed in chilled buffer, resuspended to a density of 200 µg dry wt./ml and decryptified with toluene. The cell suspension was returned to the ice-bath. Samples were removed at intervals for simultaneous glucose PTS assay using the SP800 as a single beam spectrophotometer (air as blank) and double beam spectrophotometer (cells in assay buffer as blank) to nullify any turbidity changes in the assay cuvette.

△ glucose PTS activity (single beam)

○ glucose PTS activity (double beam)

Figure D.3

INHIBITION OF PTS ACTIVITY IN E.coli 15224 BY Cl^- IONS

Glucose trained cells of E.coli 15224 were harvested, washed in chilled buffer, resuspended to a density of 200 µg dry wt./ml, and decryptified with toluene. Glucose PTS activity was determined in the presence of Cl^- added as NaCl.

Figure D.2

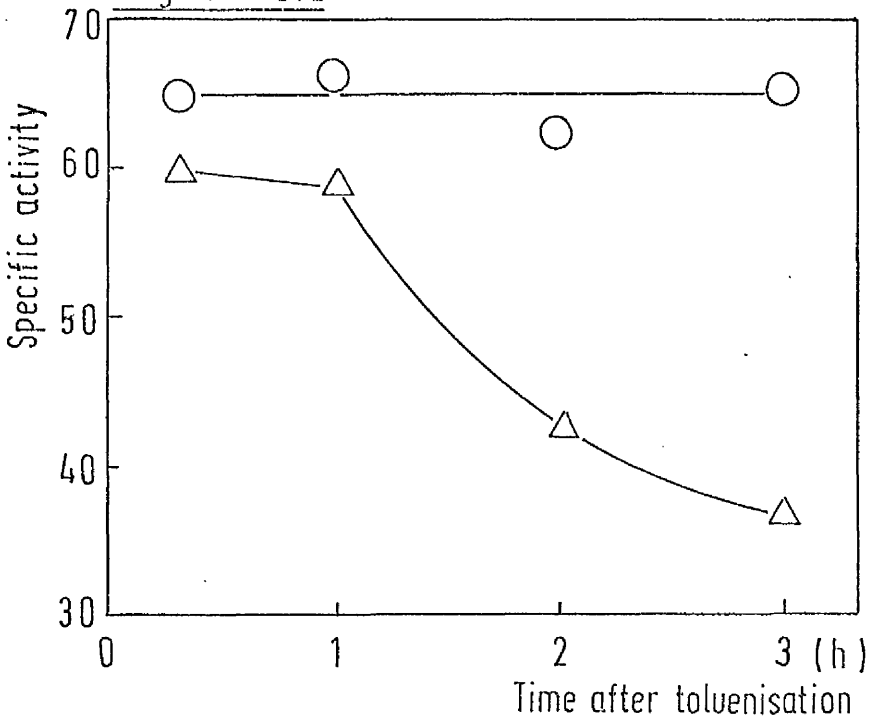


Figure D.3

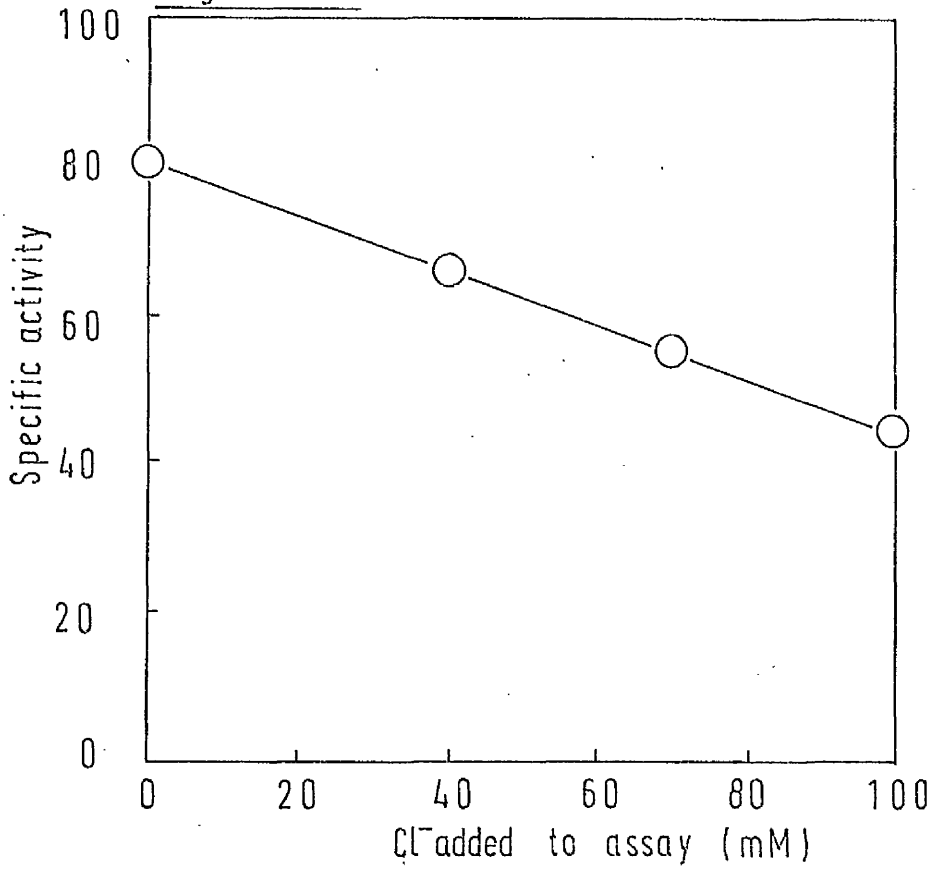


Figure D.4

EFFECT OF VARIOUS BUFFERS ON THE RELEASE AND ASSAY OF GLUCOSE PTS IN E.coli 15224

Glucose trained cells of E.coli 15224 were harvested, washed in chilled buffer and reharvested. The cell pellets were resuspended in the required buffer, decryptified with toluene in ethanol (10% v/v) and assayed, in the corresponding buffers given in the table, for glucose PTS activity.

Cells Resuspended in: *	Glucose PTS assayed in: *	Glucose PTS activity:
40 mM KH_2PO_4 **	40 mM KH_2PO_4	42
	0.2 M NaH_2PO_4	86
	0.4 M NaH_2PO_4	63
0.2 M NaH_2PO_4	0.2 M NaH_2PO_4	34
	0.4 M NaH_2PO_4	25
40 mM KH_2PO_4	0.2 M Tris	55
	0.4 M Tris	70
0.2 M Tris	0.2 M Tris	30
	0.4 M Tris	29
40 mM KH_2PO_4	0.2 M TEO	58
	0.4 M TEO	53
0.2 M TEO	0.2 M TEO	32
	0.4 M TEO	27
40 mM KH_2PO_4	0.2 M BES	37
	0.4 M BES	54
0.2 M BES	0.2 M BES	42
	0.4 M BES	42

* All buffers contain 2 mM $MgSO_4$

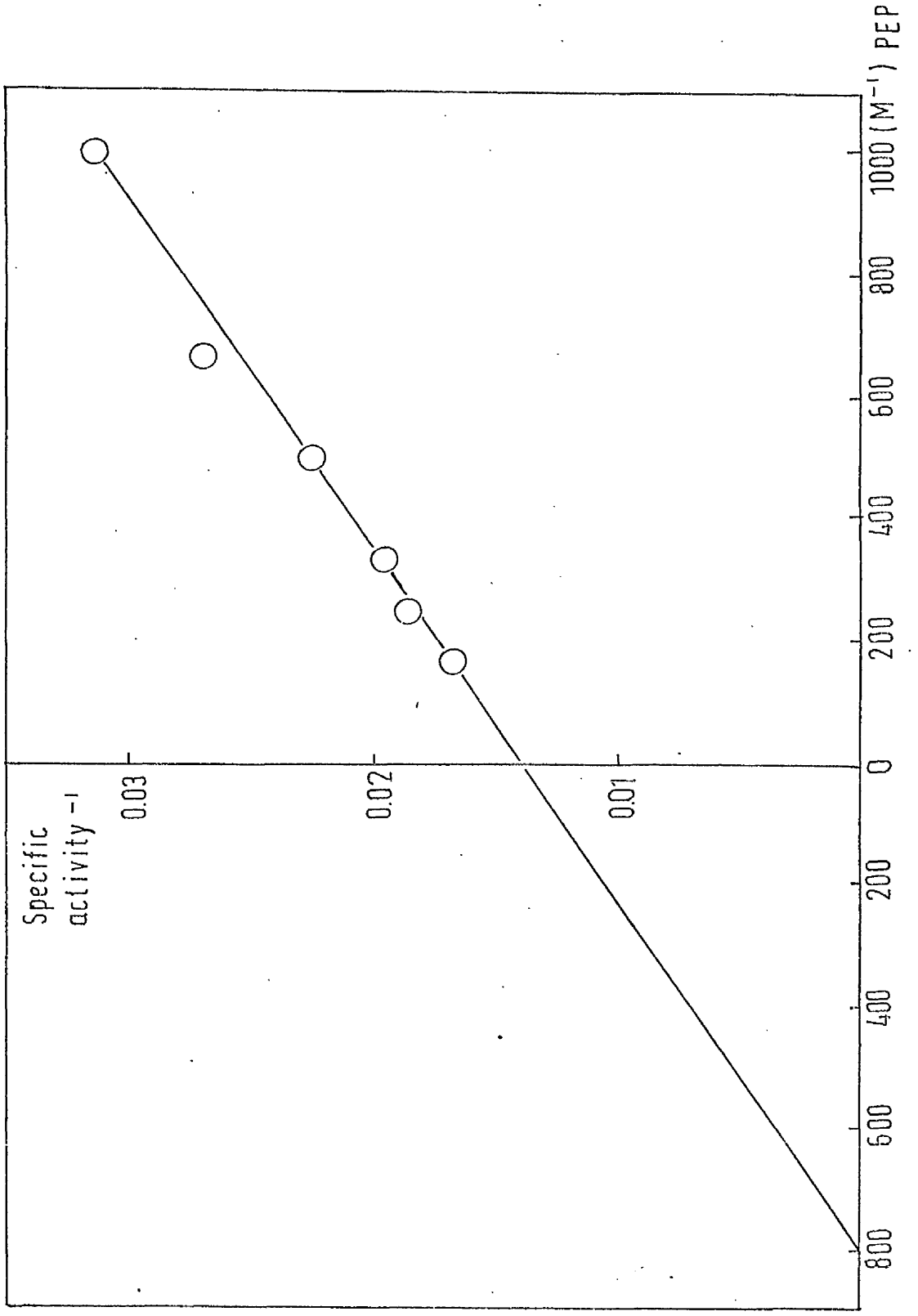
** 40 mM KH_2PO_4 only at pH 7.0. Remaining buffers at pH 7.2.

Figure D.5

DETERMINATION OF THE APPARENT K_m FOR PEP

OF THE GLUCOSE PTS IN *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested, washed in chilled buffer, resuspended to a cell density of 200 µg dry wt./ml and decryptified with (10% v/v) toluene in ethanol by vortexing (60 sec). Glucose PTS activities were determined for the required concentrations of PEP.


Specific activity $^{-1}$
0.03
0.02
0.01
800
600
400
200
0
200
400
600
800
1000 (M^{-1}) PEP

determined by pyruvate production (methods 10.4.) was 30% greater than when determined by G6P (methods 10.3.).

1.3.6.2. Comparison of kinetic and discrete sampling methods for PTS assays

Decryptified cell suspensions of E.coli 15224 trained to glucose were prepared as in methods (10.1.) and section 1.3.1. above. A large scale assay (50 ml) was set up (methods 10.3.), but lacked G6PDH and NADP. The reaction was started by the addition of glucose and samples were removed at intervals and treated as in methods (9.2.). The PCA extracts were subsequently assayed for G6P and pyruvate. Plots of the data (Figure D.6) revealed that the rate of production of pyruvate was 30% greater than that of G6P confirming the results quoted in section (1.3.6.1.).

1.3.6.3. Addition of phosphoglucose isomerase (PGI) to the PTS assay

The addition of 10 µo phosphoglucose isomerase to the assay

1.3.6.3. Addition of phosphoglucose isomerase (PGI) to the PTS

system in methods (10.3.) corrected the discrepancy in the rate of production of pyruvate and G6P suggesting that the G6P produced was being converted to F6P by the cells.

1.3.7. Effect of removal of toluene and ethanol from decryptified cells

1.3.7.1. General considerations

It was felt that, although the activities obtained with the modified assay were higher than those obtained by any other available technique, they were not as high as they might be. Consequently the system was investigated to see if either toluene or ethanol inhibited the PTS.

1.3.7.2. Effect of aeration and aspiration with OFN on decryptified cells

Glucose trained cells of E.coli 15224 were harvested (methods 10.1.) and decryptified as in methods (10.2.) except that toluene (10% v/v) in ethanol was used, vortexed for 60 sec, and the period of aeration, or

Figure D.6

RELATIVE RATES OF PRODUCTION OF GLUCOSE-6-PHOSPHATE AND PYRUVATE BY THE GLUCOSE PTS OF *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested, washed in chilled buffer, resuspended to a density of 200 µg dry wt./ml and decryptified with (10% v/v) toluene in ethanol by vortexing (60 sec). A large scale (50 ml) PTS assay was set up which contained only cells (98 µg dry wt./ml), PEP (10 mM) and glucose (2 mM). At suitable intervals PCA extracts were prepared and glucose-6-phosphate and pyruvate concentrations were subsequently determined.

○ pyruvate concentration

△ glucose-6-phosphate concentration

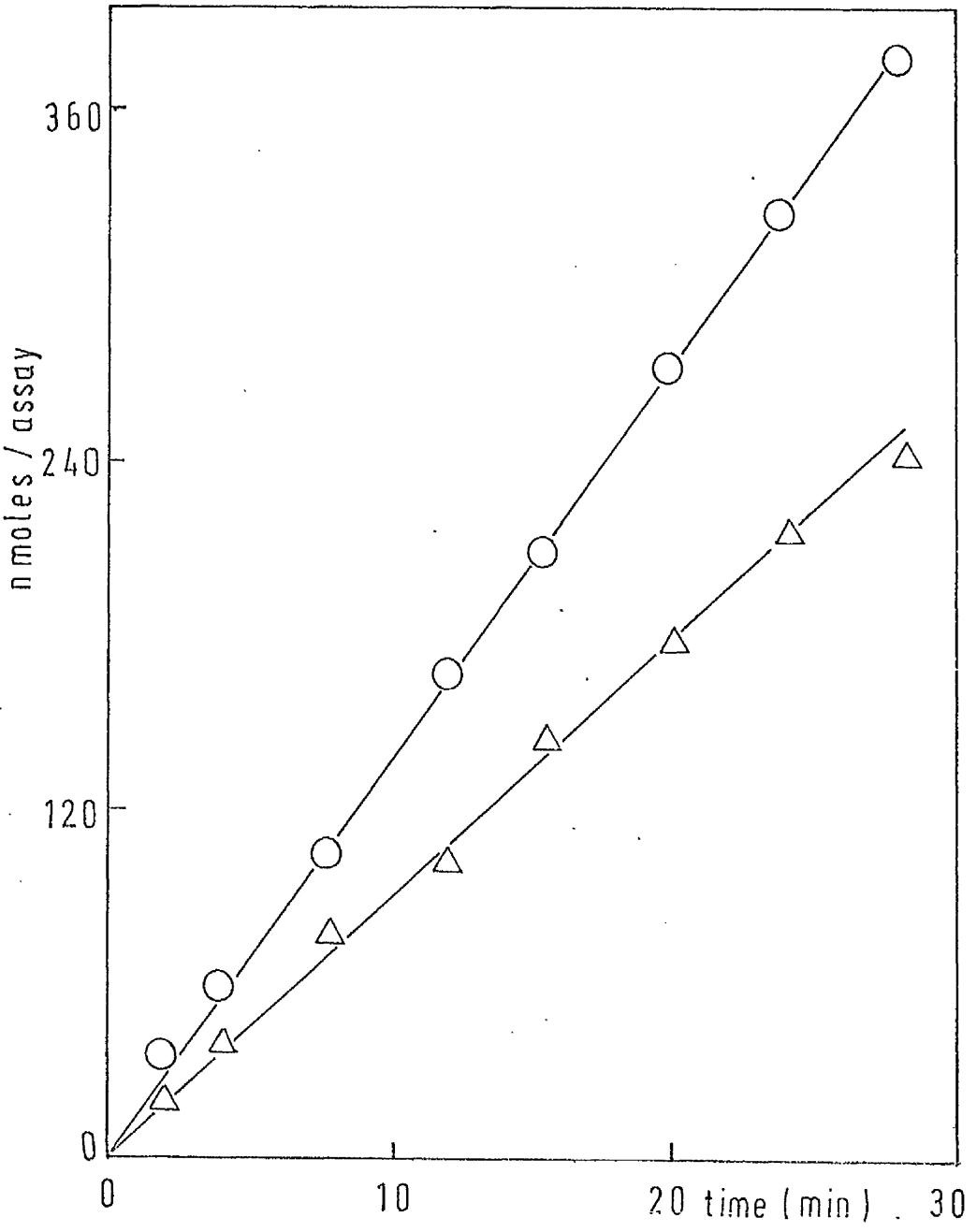

360
240
120
0
n moles / assay
0
10
20 time (min)
30

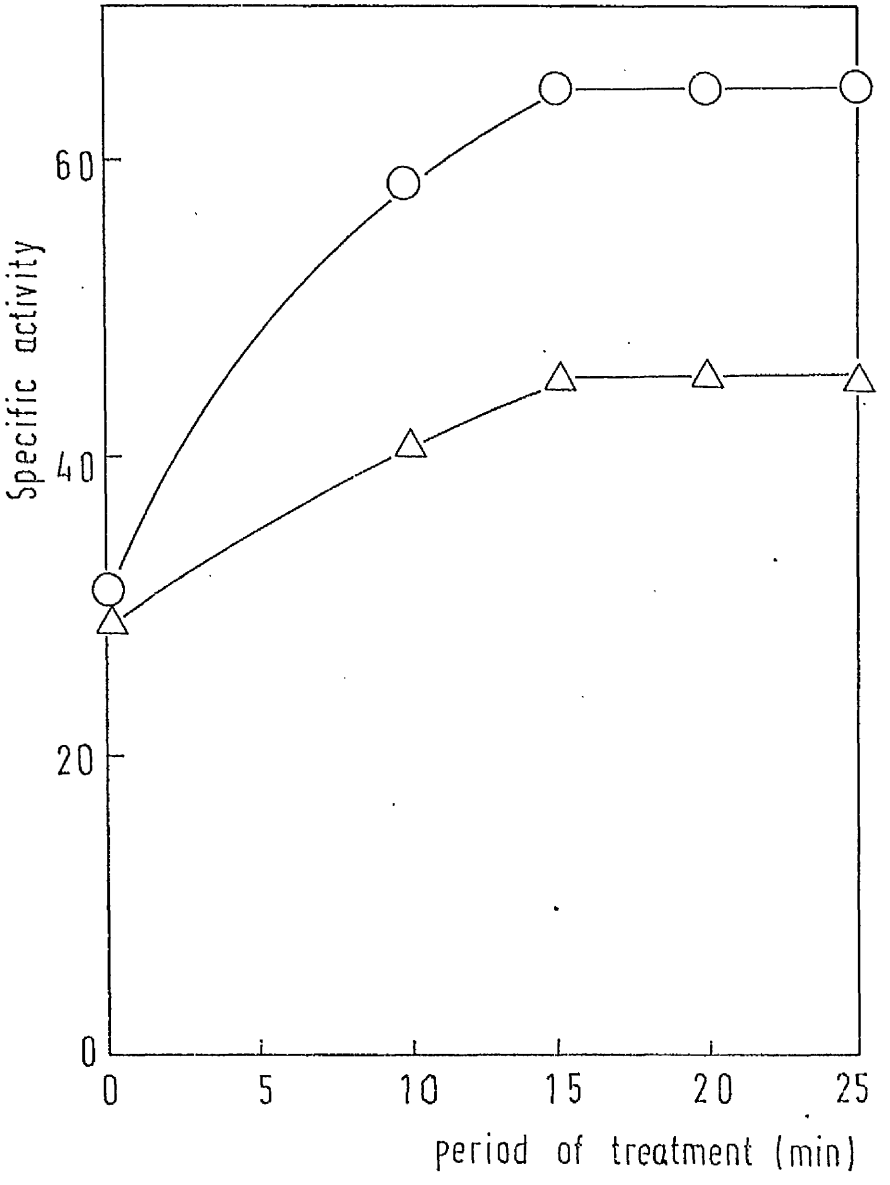
Figure D.7

COMPARISON OF THE EFFECTS OF AERATION AND ASPIRATION WITH OFN ON THE PTS ACTIVITY OF TOLUENISED CELLS OF *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested, washed in chilled buffer, resuspended to a density of 200 µg dry wt./ml and decryptified with (10% v/v) toluene in ethanol by vortexing (60 sec). The suspensions so prepared were returned to the ice-bath and either aerated or aspirated with OFN, both at 100 ml/min. Portions were withdrawn for glucose PTS activity determinations at suitable intervals.

○ aspiration with OFN

△ aeration


Specific activity
60
40
20
0
0
5
10
15
20
25
period of treatment (min)

aspiration with OFN varied from 10 - 40 min. Cells so produced were assayed for glucose PTS (methods 10.3.) with PGI (10 µg) present in the assay (Figure D.7).

A period of aspiration with OFN for 20 min was chosen for future experiments.

1.3.7.3. Effect of aspiration on toluene activity release curve

Glucose trained cells of E.coli 15224 were harvested, decryptified with a range of toluene in ethanol (6 - 12% v/v) concentrations as in methods (10.2.) but vortexed for 60 sec, and assayed (methods 10.3.) for glucose PTS activity with PGI (10 µg) present in the assay. The controls were non-aspirated cells. The results are presented in Figure D.8.

1.3.7.4. Effect of readdition of toluene and ethanol to aspirated cells

Toluene, as toluenised buffer, was added to PTS assays of the cells produced in section (1.3.6.2.). The results (Figure D.9) show that readdition of toluene to its original concentration reduces the PTS activity to its original value and further inhibits the activity of preparations from which toluene had not been removed.

The addition of ethanol (0 - 4.5 µl/assay) to either system had no effect on the PTS activities observed.

1.3.7.5. Effect of aspiration on the requirement for PGI

The production of glucose-6-phosphate and pyruvate is equivalent if toluene is removed by aspiration and extracellular PGI is no longer required in the assay. Furthermore, the addition of PGI to the assay does not affect this equivalence. However, the modified assay procedure reported in 1.4.1. incorporated PGI (10 µg) into the assay since these facts were not obtained until a new assay for the PTS was being investigated (see section 1.5.). Since PGI does not adversely affect the assay the results obtained with this technique are perfectly valid.

Figure D.8

EFFECT OF OFN ASPIRATION ON THE DECRYPTIFICATION OF GLUCOSE PTS BY TOLUENE IN *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested, washed in chilled buffer and resuspended to a cell density of 200 µg dry wt./ml. Portions were decryptified with the required amount of toluene (in ethanol) mixture by vortexing (60 sec). This operation was performed in duplicate for each toluene concentration, one batch being aspirated with OFN (100 ml/min). Glucose PTS activity was determined.

○ glucose PTS activity (aspirated with OFN)

△ glucose PTS activity (non-aspirated cells)

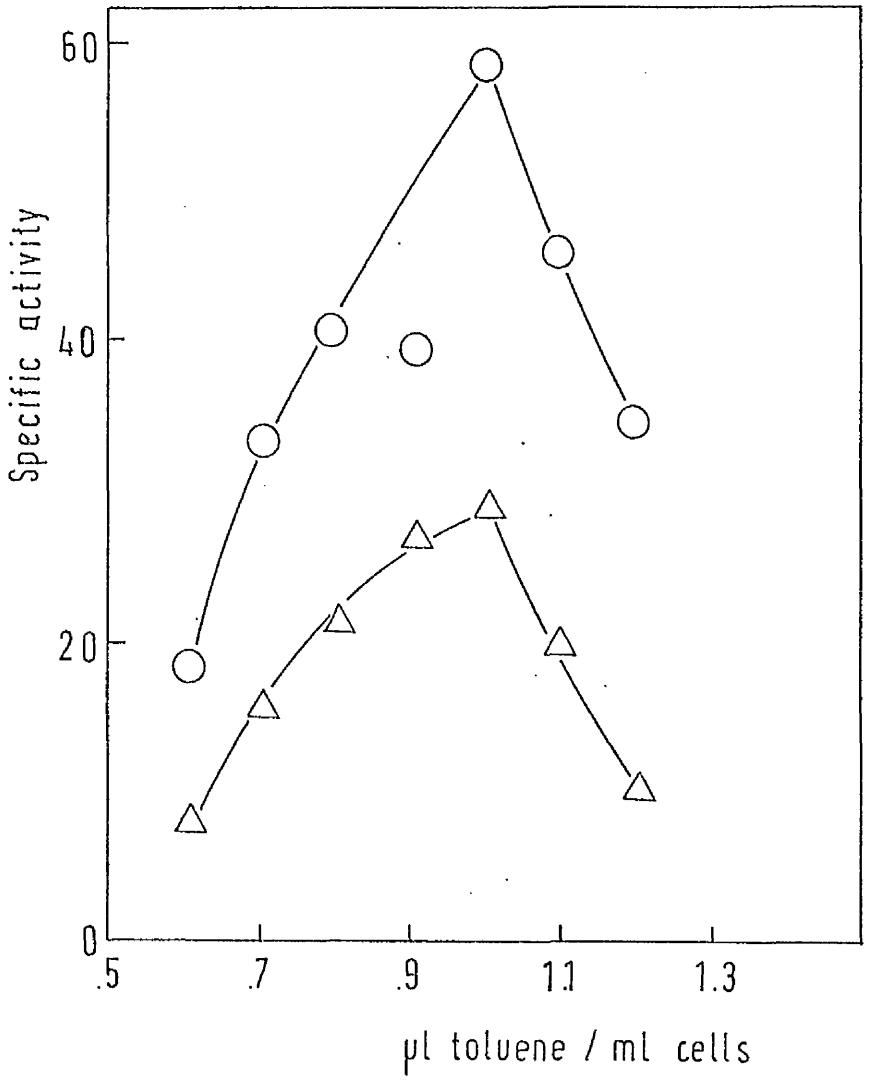

60
40
20
0
Specific activity
.5
.7
.9
1.1
1.3
µl toluene / ml cells

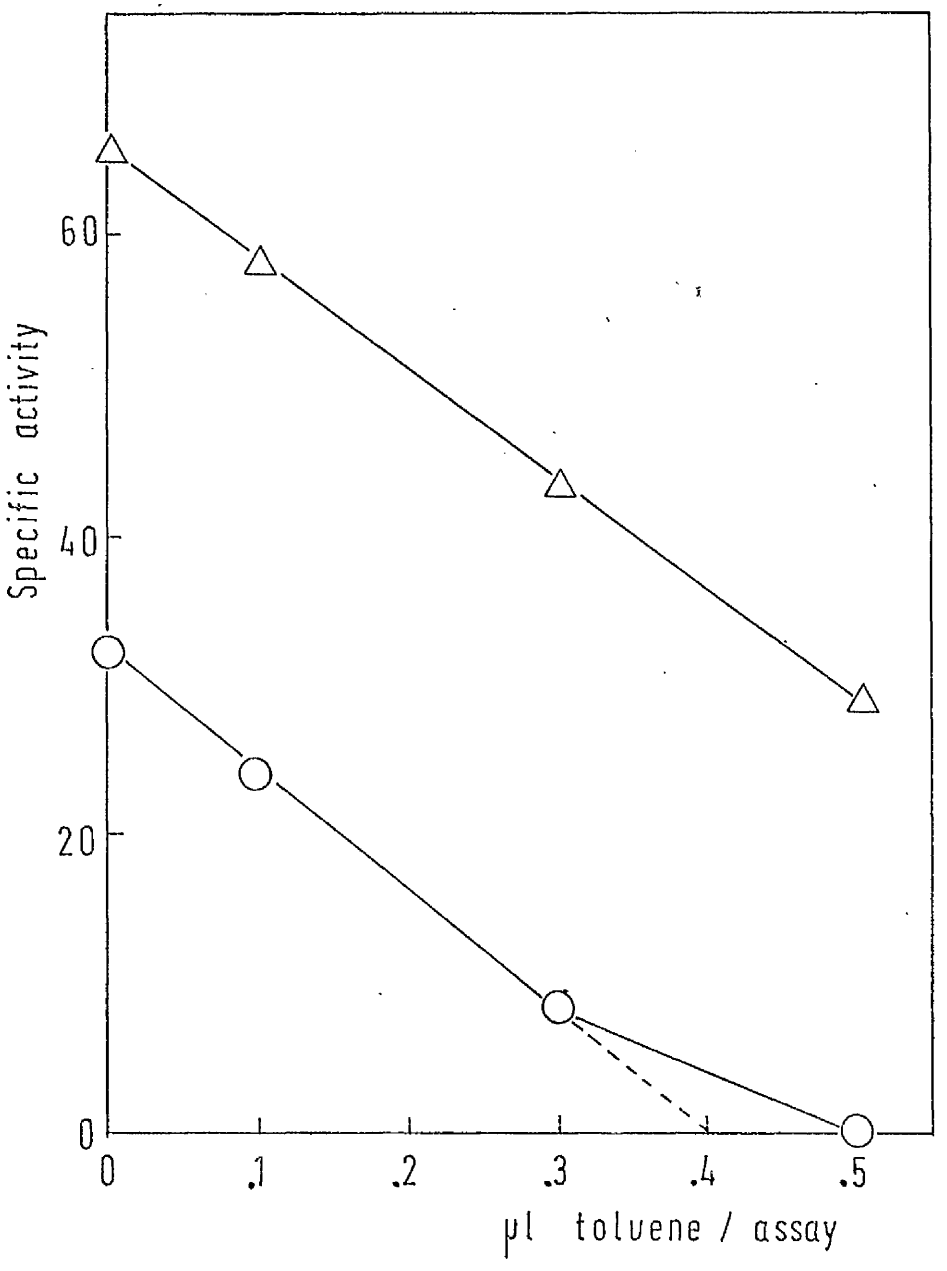
Figure 0.9

TOLUENE INHIBITION OF THE GLUCOSE PTS

Glucose trained cells of E.coli 15224 were harvested, washed in chilled buffer, resuspended to a density of 200 µg dry wt./ml and decryptified with toluene in ethanol (10% v/v) by vortexing (60 sec). One portion of the decryptified cells was then aspirated with OFN. Glucose PTS assays were carried out on both batches of cells and toluene (as toluenised buffer) was added to the assays as required.

○ non-aspirated cells

△ aspirated cells


Specific activity
60
40
20
0
0
.1
.2
.3
.4
.5
µl toluene / assay

1.4. Summary of the modified assay and its use

1.4.1. The modified assay

Samples were prepared (methods 10.1.), decryptified and aspirated as in methods (10.2.) except that 10% (v/v) toluene in ethanol was used vortexed for 60 sec. PTS activities were determined (methods 10.3. and 10.4.) with addition of PGI (10 µg) to the assays.

The control cuvettes for the assays lacking either PEP or sugar never gave observable activity. The fact that the assays lacking added sugar but containing PEP gave no measurable activity justified the adopted washing procedure (methods 10.1.). Substitution of ATP for PEP at the same concentration gave less than 1% of the activity observed with PEP confirming that the assays were detecting only PTS activity. Production of G6P and pyruvate was linear and equivalent for at least 10 min after initiation of the assay.

The assays were directly proportional to the amount of decryptified cells added over the range 0 - 120 µg dry wt. cellular material/assay.

1.4.2. Use of the modified assay

Despite the modifications to the assay the PTS activities obtained were still irreproducible. On a given day assays performed on five identical samples would yield activities with a standard deviation in specific activity of $\pm$ 12%, which was considered excessive for the work envisaged. Also from day to day the absolute PTS activities themselves could vary from 20 - 70 mU/mg dry wt. It was felt that these results emanated from the very acute toluene activity release curve. The results obtained (Figure D.8) suggested that, although the toluene inhibition was removed by aspiration, toluene irreversibly damaged some component of the PTS. Because of these factors, and although the use of toluene was quoted in the literature as being a reproducible means

of decryptifying PTS activities in other strains (Gachelin, 1969; Kornberg & Reeves, 1972a) it appeared that this method was not suitable for the decryptification of E.coli 15224 and it was rejected.

1.5. Use of benzene to decryptify cells of E.coli 15224

1.5.1. General considerations

Previous work had shown that toluene was unsuitable as a reproducible means of decryptifying cells of E.coli 15224. An observation that the use of Aristar toluene always released less PTS activity than Analar toluene prompted an investigation into the reasons for this. The only difference proved to be that Aristar toluene contained less benzene (200 p.p.m.) than Analar toluene (5000 p.p.m.). The use of benzene to decryptify cells of E.coli 15224 was investigated.

1.5.2. Benzene induced decryptification

1.5.2.1. Benzene activity release curve

Cells of E.coli 15224 trained to glucose were harvested (methods 10.1.) and decryptified as in methods (10.2.) except that the benzene concentration in ethanol varied from 20 - 45% (v/v) and the vortexing time was 60 sec. A control batch of cells was not aspirated. Glucose PTS activities were determined for both batches of cells (methods 10.3.). In the case of non-aspirated cells the activity released was not increased above 2.75 µl benzene/ml and fell above 3.5 µl benzene/ml. Removal of benzene by aspiration with OFN gave constant, and higher, activity from 2.75 µl benzene/ml up to the highest level tested (4.5 µl benzene/ml). These results are reported (Figure D.10), and show a marked difference from the results obtained with toluene (Figure D.8). The concentration of 35% (v/v) benzene in ethanol was chosen for future use.

1.5.2.2. Optimum vortexing time

Cells of E.coli 15224 trained to glucose were harvested

Figure D.10

EFFECT OF BENZENE CONCENTRATION ON THE DECRYPTIFICATION OF THE GLUCOSE PTS OF E.coli 15224

Glucose trained cells of E.coli 15224 were harvested in the exponential phase of growth, washed in chilled buffer and resuspended to a density of 200 µg dry wt./ml. Duplicate portions were decryptified with the required concentration of benzene in ethanol by vortexing (60 sec), and one portion was aspirated with OFN (100 ml/min, 20 min) in an ice-bath. Glucose PTS activities were determined.

○ cells aspirated with OFN

△ cells not aspirated with OFN

Figure D.11

BENZENE INHIBITION OF GLUCOSE PTS ACTIVITY IN E.coli 15224

Glucose trained cells of E.coli 15224 were harvested in the exponential phase of growth, washed in chilled buffer and resuspended to a density of 200 µg dry wt./ml. A portion was decryptified with benzene in ethanol (35% v/v) by vortexing (70 sec) and aspirated with OFN (100 ml/min, 20 min). Glucose PTS activity was determined in the presence of the required amount of benzene added as benzenised buffer.

Figure D.10

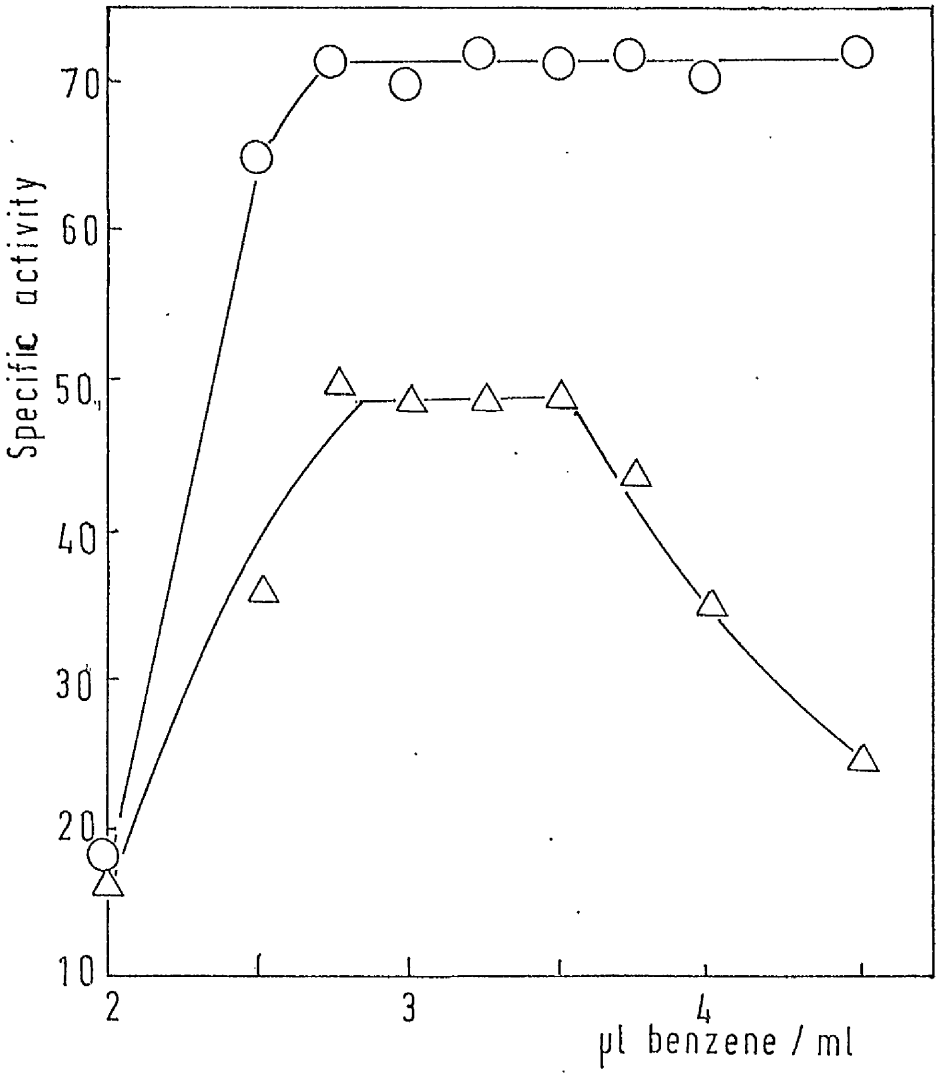
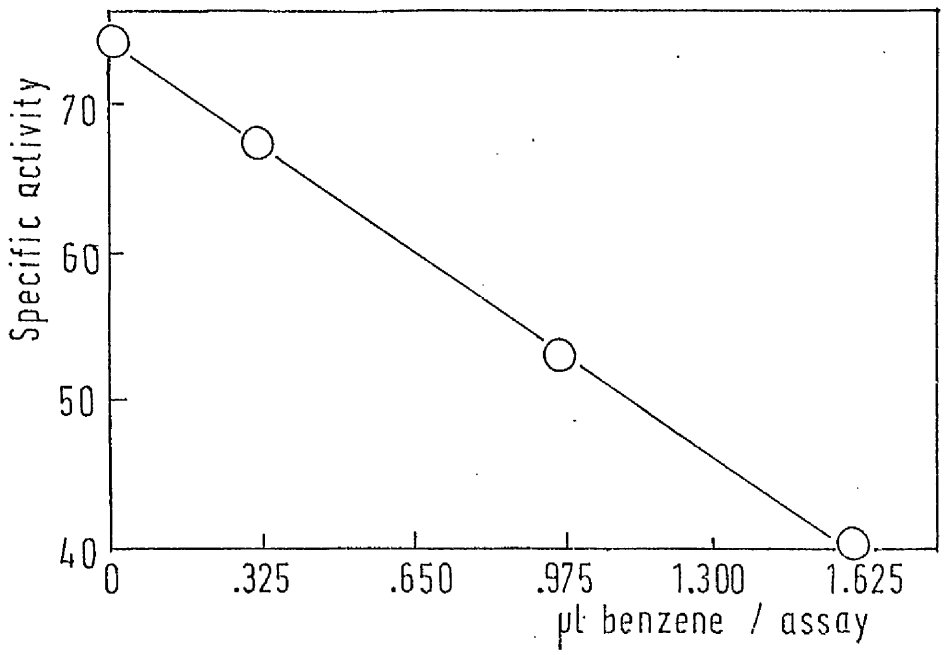


Figure D.11



(methods 10.1.) and decryptified as in methods (10.2.) except that the period of vortexing varied from 10 - 100 sec. The activity released was maximal and constant from 60 - 100 sec, and 70 sec was chosen for future use.

1.5.2.3. Optimum OFN aspiration time

Cells of E.coli 15224 trained to glucose were harvested (methods 10.1.) and decryptified as in methods (10.2.) except that the period of aspiration was varied from 0 - 30 min. The activity released was maximal and constant from 15 - 30 min and 20 min aspiration with OFN was chosen for future use.

1.5.2.4. Benzene inhibition of PTS activity

Cells of E.coli 15224 trained to glucose were harvested (methods 10.1.), decryptified (methods 10.2.) and assayed for glucose PTS activity (methods 10.3.) in the presence of benzene added as benzene treated buffer. The results (Figure D.11) show that benzene inhibits PTS activity in a similar fashion to toluene. The addition of ethanol (3.25 µl/assay) had no effect on the observed PTS activities.

1.5.3. Effect of buffers on the assay

Maximal activity was observed when using the buffer system developed in 1.3.4. where the cells are decryptified in 40 mM KH_2PO_4 pH 7.0, with 2 mM $MgSO_4$ ("buffer") and assayed in 0.2 M NaH_2PO_4 pH 7.2 with 2 mM $MgSO_4$ ("assay buffer").

1.5.4. Effect of PGI on the assay of glucose PTS activity

The production of glucose-6-phosphate and pyruvate was only equivalent when PGI was added to the assay or the benzene removed from the cell suspension by aspiration. Thus this system is similar to the toluene system in its requirements for PGI. However the presence of PGI in the assay of aspirated cell suspensions occasionally results in a lowering of the normal observed activities. Consequently PGI was

not incorporated into the final assay system.

1.5.5. Summary of the modified assay and its use

1.5.5.1. The modified assay

Cell samples were prepared, decryptified and assayed exactly as described in methods (10.). The control cuvettes for these assays lacking either PEP or sugar never gave observable activity. The fact that the assays lacking added sugar but containing PEP gave no measurable activity justified the washing procedure adopted (methods 10.1.). Substitution of ATP for PEP at the same concentration gave less than 1% of the activity observed with PEP confirming that the assays were detecting only PTS activity. Production of G6P and pyruvate was linear and equivalent for at least 10 min after initiation of the assay.

The assays were directly proportional to the amount of decryptified cells added over the range 0 - 120 µg dry wt. cellular material/assay (Figure D.12).

1.5.5.2. Use of the modified assay

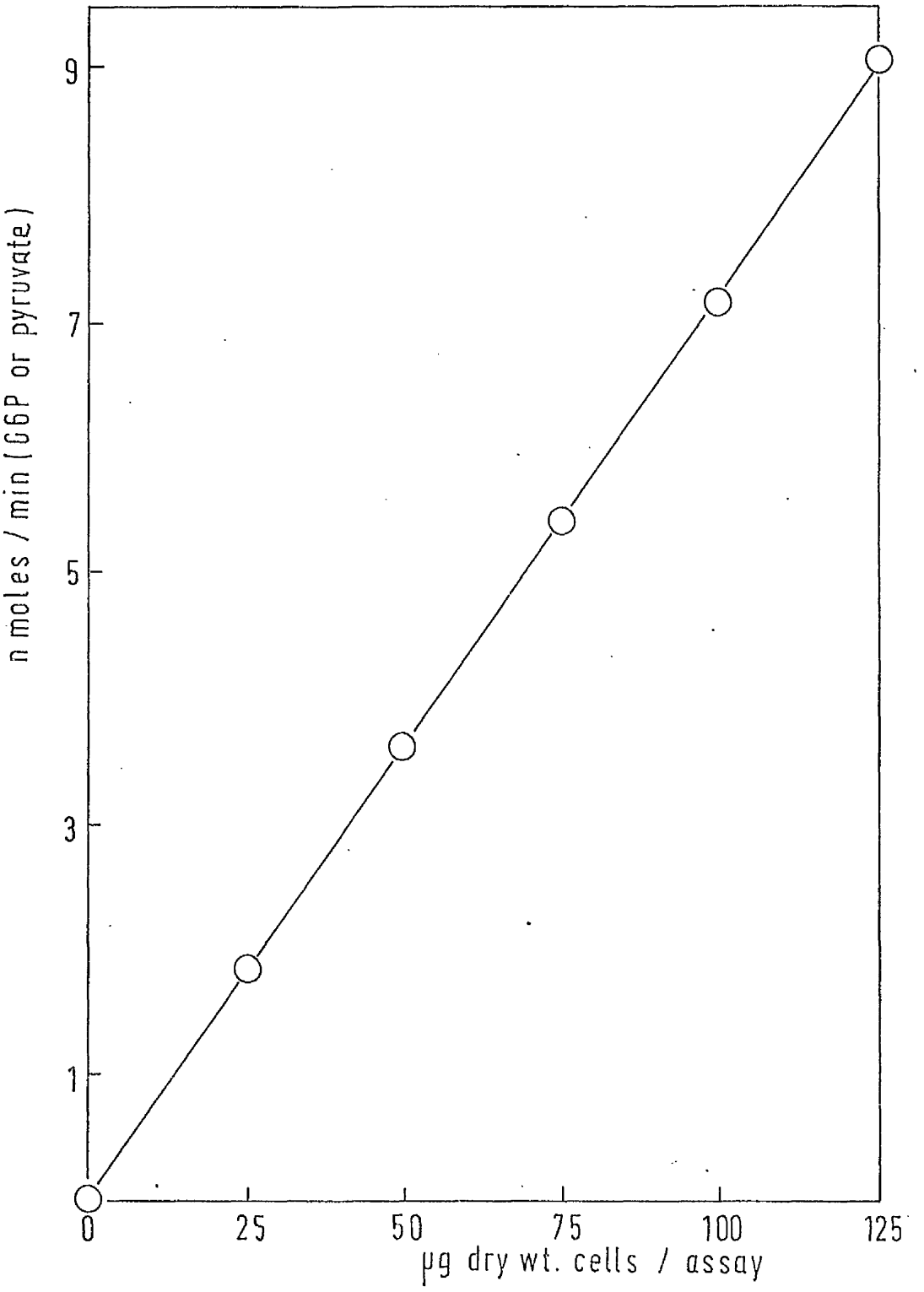
The adoption of benzene and the modifications to the assay system described led to a reproducible method of measuring PTS activities. On a given day, treatment of five identical samples would yield results with a standard deviation in specific activity of only $\pm$ 3%. Also, from day to day, the absolute activities remained constant within a few percent and so this assay system was used instead of the existing toluene decryptification and assay methods.

The reproducibility of this system, in which the mechanism of benzene induced decryptification is unknown, is felt to stem from the fact that benzene does not appear to irreversibly damage the PTS system in the same way as toluene. This can be seen by comparing the aspirated activity release curves of benzene and toluene (Figures D.10 and D.8).

Figure D.12

CORRELATION BETWEEN CELL MATERIAL ADDED AND GLUCOSE PTS ACTIVITY IN BENZENE DECRYPTIFIED CELLS OF *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested in the exponential phase of growth, washed in chilled buffer and resuspended to a density of 250 µg dry wt./ml. The cells were decryptified with (35% v/v) benzene in ethanol by vortexing (70 sec) and aspirated with OFN (100 ml/min, 20 min). Glucose PTS activity was determined in terms of both glucose-6-phosphate and pyruvate production for varying amounts of decryptified cells.


n moles / min (G6P or pyruvate)
9
7
5
3
1
0
25
50
75
100
125
µg dry wt. cells / assay

1.5.5.3. The involvement of PGI in the assay systems

Extracellular PGI is required in the assay system for equivalence of G6P and pyruvate production if neither benzene nor toluene are removed by aspiration. However extracellular PGI is not required if toluene and benzene are removed. This is difficult to explain since intracellular PGI activity is similar in both the presence and absence of benzene or toluene suggesting that the requirement for extracellular PGI in non-aspirated cells is independent of intracellular PGI activity. However, both toluene and benzene decryptified cells behave identically in their requirement for extracellular PGI and, since PGI was not required in the final assay, the inability to explain the involvement of extracellular PGI was not considered to be a drawback to the modified assay.

2. Development of Radioactive Assays for PTS Activities

2.1. General considerations

An assay was required in which PTS activities could be measured under a variety of conditions without modifying the end products of the PTS reactions. Such an assay could then be compared to the spectrophotometric assay in which the end products are modified (G6P → 6-phosphogluconate, pyruvate → lactate). Consequently the assay was in no way required to be quantitative; a simple qualitative comparison would suffice.

It was decided to measure glucose PTS activity by following the conversion of D-glucose-C^{14} (U) to the 6-phosphate and D-fructose-C^{14} (U) to the 1-phosphate. The radioactive phosphates could then be bound to DE81 filter paper along with any other negatively charged ions present, and the unchanged C^{14} glucose or C^{14} fructose removed by thorough washing. The amount of bound radioactivity would be proportional to the activity of the PTS. Therefore, the only requirement for the assay was that it be linear throughout the required period of measurement (15 min). This would mean that the degree of retention of radioactivity and subsequent loss by washing was constant and need not be determined in absolute terms.

2.2. Development of the assays

For reasonable efficiency when counting, it was decided that the 15 min sample should contain about 1000 c.p.m. By calculation this meant that in 1 ml final volume the assay should contain 1.0 μ Ci of either C^{14} glucose or C^{14} fructose. These were present at a final concentration of 2 μ mol. A convenient means of terminating the assay was to add 2 M (1 ml) glucose or fructose and chill. The resulting 1:1000 dilution of the C^{14} sugar and the subsequent chilling

would effectively terminate the reaction. PEP was to be present at 10 μ mol, similar to the spectrophotometric assay. The cells were to be resuspended and decryptified in assay buffer to prevent variation in ionic strength from sample to sample.

2.3. Determination of the linearity of the assays

2.3.1. Assay of glucose PTS activity

Glucose trained cells of E.coli 15224 were harvested (methods 10.1.) and decryptified as in methods (11.3.). Glucose PTS activity was determined on several samples as in methods (11.4.) except the reactions were terminated at different intervals between 0 - 16 mins. The data were plotted (Figure D.13) and the production of D-glucose-C^{14} (U)-6-phosphate appeared to be linear.

2.3.2. Assay of fructose PTS activity

Fructose trained cells of E.coli 15224 were harvested (methods 10.1.) and decryptified as in methods (11.3.). Fructose PTS activity was determined on several samples (methods 11.4.) except the reactions were terminated at different intervals between 0 - 16 min. The data were plotted (Figure D.14) and the production of D-fructose-C^{14}(U)-1-phosphate appeared to be linear.

2.4. Summary

The results obtained showed that the assays were linear up to 15 min, satisfying the requirements stated (2.1.). Consequently only 0 and 15 min samples were required in future. That the washing procedure (to remove unchanged C^{14}glucose and C^{14}fructose) was adequate was seen from the very low counts given by the controls.

The results obtained from either assay were expressed in terms of c.p.m./mg dry wt. cellular material and 8000 c.p.m./mg dry wt. was approximately equivalent to a specific activity of 80 mU/mg dry wt. as determined by spectrophotometric assay (methods 10.).

Figure D.13

LINEARITY OF PRODUCTION OF D-GLUCOSE-C^{14}(U)-6-PHOSPHATE BY GLUCOSE PTS OF E.coli 15224

Glucose trained cells of E.coli 15224 were harvested, washed in chilled buffer and decryptified. Glucose PTS activity was determined on several samples, but the reactions were terminated at different intervals between 0 - 16 min, by following the conversion of D-glucose-C^{14}(U) to C^{14}glucose-6-phosphate.

○ retained C^{14}glucose-6-phosphate (c.p.m.)

Figure D.14

LINEARITY OF PRODUCTION OF D-FRUCTOSE-C^{14}(U)-1-PHOSPHATE BY FRUCTOSE PTS OF E.coli 15224

Fructose trained cells of E.coli 15224 were harvested, washed in chilled buffer and decryptified. Fructose PTS activity was determined on several samples, but the reactions were terminated at different intervals between 0 - 16 min, by following the conversion of D-fructose-C^{14}(U) to C^{14}fructose-1-phosphate.

○ retained C^{14}fructose-1-phosphate (c.p.m.)

Figure D.13

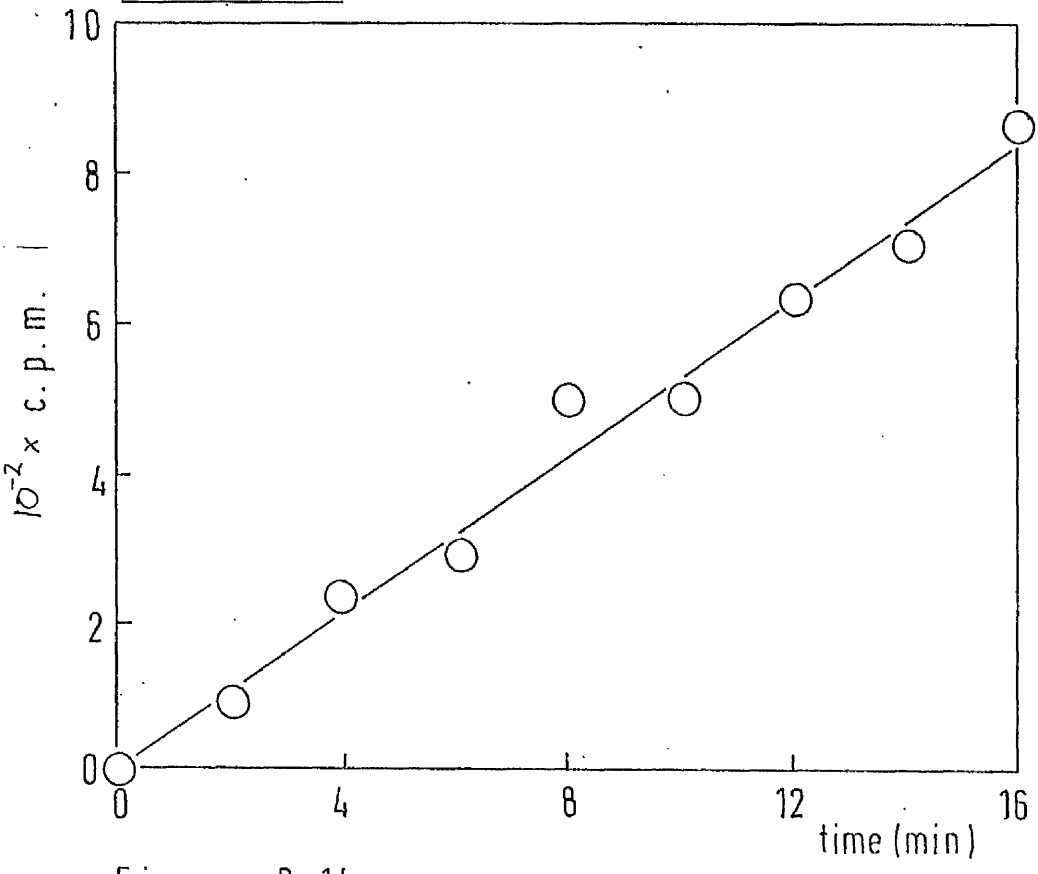
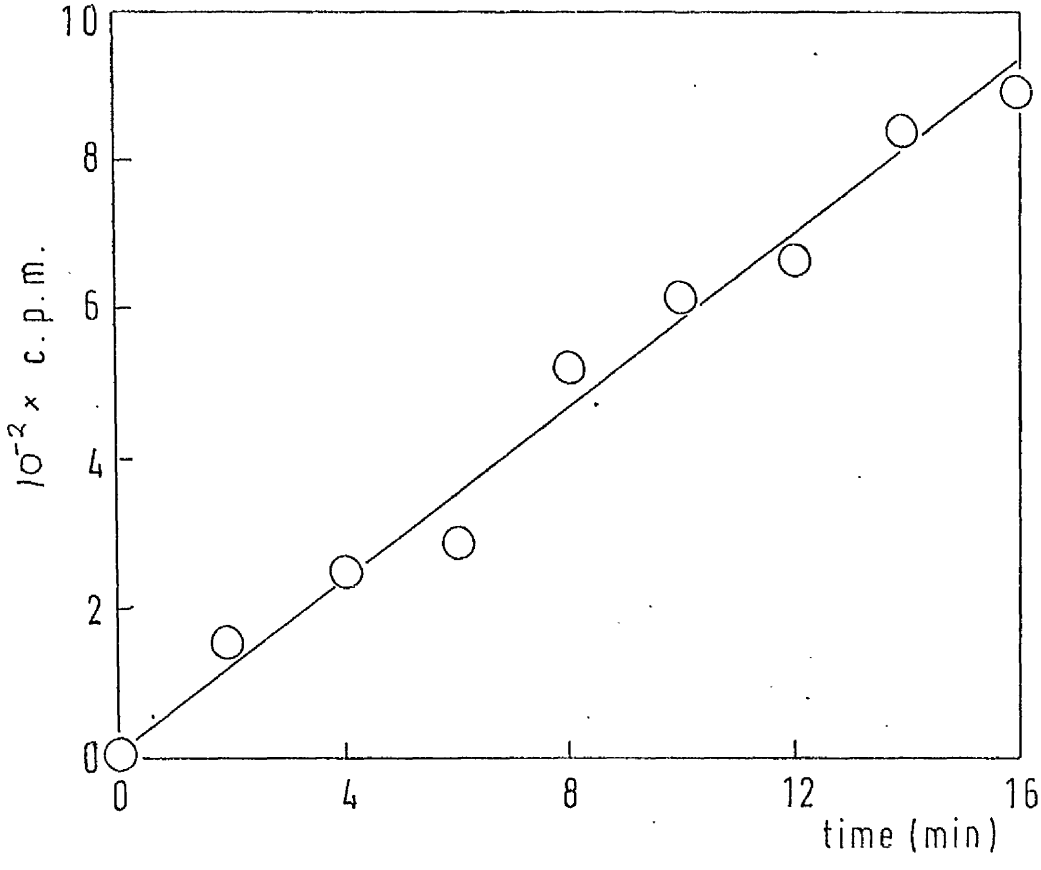


Figure D.14



MATERIALS

With the exception of those reagents listed below, all reagents were 'Analar' or the highest purity grade (apart from fructose (reagent grade) and one batch of 'Aristar' Toluene) obtainable from British Drug Houses, Poole, England.

Obtained from Boehringer Corporation, London, were:

NADP,

PEP,

triethanolamine,

GOD-Perid reagent,

lactate dehydrogenase,

glucose-6-phosphate dehydrogenase,

phosphoglucose isomerase,

hexokinase.

Obtained from Sigma, London, were:

ATP,

BCIG,

NADH,

cyclic adenosine 3' 5' monophosphate - free acid,

glucose-6-phosphate,

glucose-1-phosphate,

fructose-6-phosphate,

fructose-1,6-diphosphate,

6-phosphogluconate.

Obtained from Radiochemicals Ltd., Amersham, England, were:

D-glucose-C^{14}(U),

D-fructose-C^{14}(U).

Obtained from Oxoid, London, were:

cooked meat medium,

nutrient broth medium,

nutrient agar medium.

Mannitol and sorbitol were obtained from T. Kerfoot & Co. Ltd., Vale of Bardsley, Lancashire, England; malic acid and mannose from Fluka, Buchs, Switzerland; chloramphenicol from Parke, Davies & Co., Pontypool, Mon., U.K.; formaldehyde from May & Baker, Dagenham, England; and Tris HCl from Mann Research Laboratories, New York, U.S.A.

All solutions were prepared using glass distilled water.

Oxygen free nitrogen (OFN) was obtained from British Oxygen Co. Ltd., Polmadie, Glasgow.

RESULTS

1. Preliminary Experiments

1.1. Response of glucose and fructose PTS activities to changes of growth media

Fructose or glucose trained cells of E.coli 15224 were inoculated into both glucose salts and fructose salts media and the response of the glucose and fructose PTS activities determined.

Results for fructose trained cells are shown in Figure R.3 and those for glucose trained cells in Figure R.4. Both glucose and fructose PTS activities responded to a change in growth medium. However fructose PTS activity appeared more rapidly (Figure R.4) than glucose PTS activity (Figure R.3) and overshot the final value. Cells inoculated into their original medium maintained constant the initial PTS activities. In all cases, growth of the cells was immediate, without lag.

1.2. Effect of glucose challenge to a fructose culture of E.coli 15224

Figure R.5 shows the effect of glucose addition (challenge) to a culture of E.coli 15224 actively growing on fructose. At the point of glucose challenge the growth rate was stimulated, fructose PTS activity decreased and glucose PTS activity increased. Fructose utilisation slowed down, eventually ceased and did not recommence until glucose was nearly exhausted. At or near the point of glucose exhaustion there was a marked increase in fructose PTS activity which overshot the final value, glucose PTS activity decreased and the growth rate returned to the original value.

Figure R.6 shows the effect of fructose challenge to a culture of E.coli 15224 actively growing on glucose. The addition had little effect on either fructose or glucose PTS activities and

Figure R.1

GROWTH OF *E.coli* 15224 ON VARIOUS SUBSTRATES

Cells of *E.coli* 15224 trained to the substrates described in the table were harvested, resuspended in chilled buffer and inoculated into media containing the substrates shown. Growth was followed and the specific growth rates calculated.

( ) : lag. (min) before growth begins

— : data not obtained.

growth substrate / training substrate	glucose	fructose	mannose	mannitol	sorbitol
glucose	.93 (0)	.73 (0)	.41 (0)	.94 (0)	.59 (80)
fructose	.93 (0)	.73 (0)	—	.94 (<10)	.59 (80)
mannose	.93 (0)	—	0.41 (0)	—	—
mannitol	.93 (0)	.73 (0)	—	.94 (0)	—
sorbitol	.93 (0)	.73 (0)	—	.94 (0)	.59 (0)
glycerol	.93 (0)	.73 (<5)	.41 (0)	.94 (<5)	.59 (70)
malate	.93 (0)	.74 (0)	.32 (0)	.93 (0)	.59 (60)

Figure R.2

GROWTH OF E.coli 15224 ON VARIOUS COMBINATIONS OF SUGARS

Figure R.2a

Cells of E.coli 15224 trained to either glucose or sorbitol were harvested, resuspended in chilled buffer and inoculated into a mixture of glucose (.75 mM), sorbitol (1.5 mM) and salts.

Growth was measured at intervals.

◍ glucose trained cells

○ sorbitol trained cells

Figure R.2b

Cells of E.coli 15224 trained to each of glucose, mannitol, glycerol and fructose were harvested and resuspended in chilled buffer. Glucose, mannitol or glycerol trained cells were inoculated into a mixture of glucose (.75 mM), mannitol (1.5 mM) and salts. Glucose, glycerol or fructose trained cells were similarly inoculated into a mixture of glucose (.75 mM), fructose (1.5 mM) and salts.

Growth was measured at intervals.

△ glucose cells into glucose + mannitol

□ glucose, glycerol or fructose cells into glucose + fructose

⧋ mannitol or glycerol cells into glucose + mannitol

↓ change in specific growth rate

Figure R. 2a

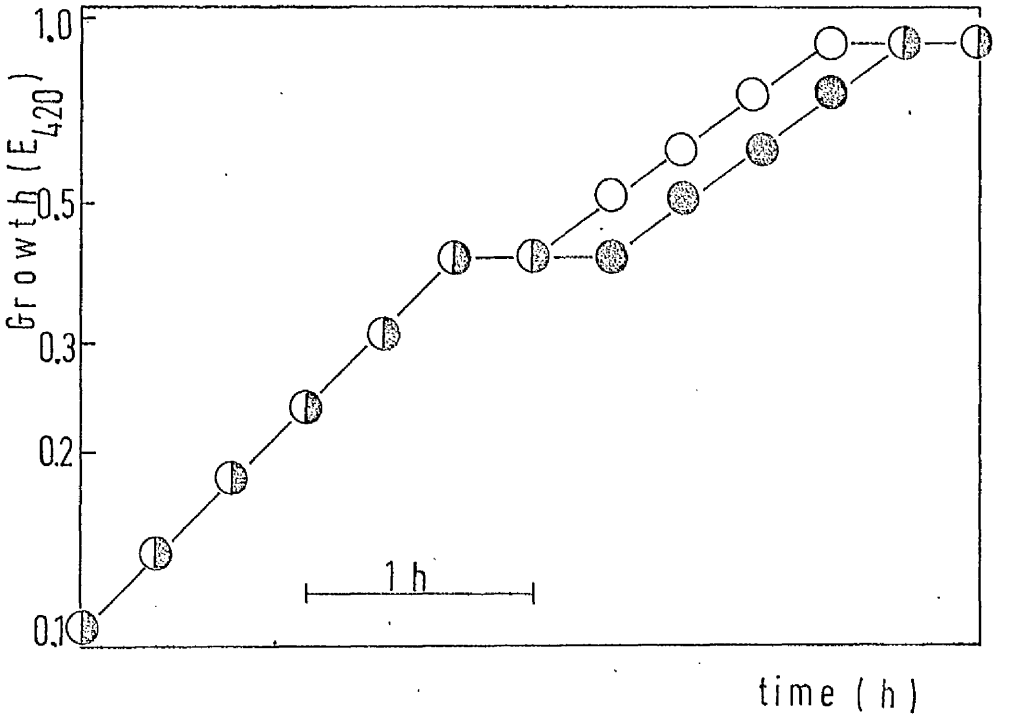


Figure R. 2b

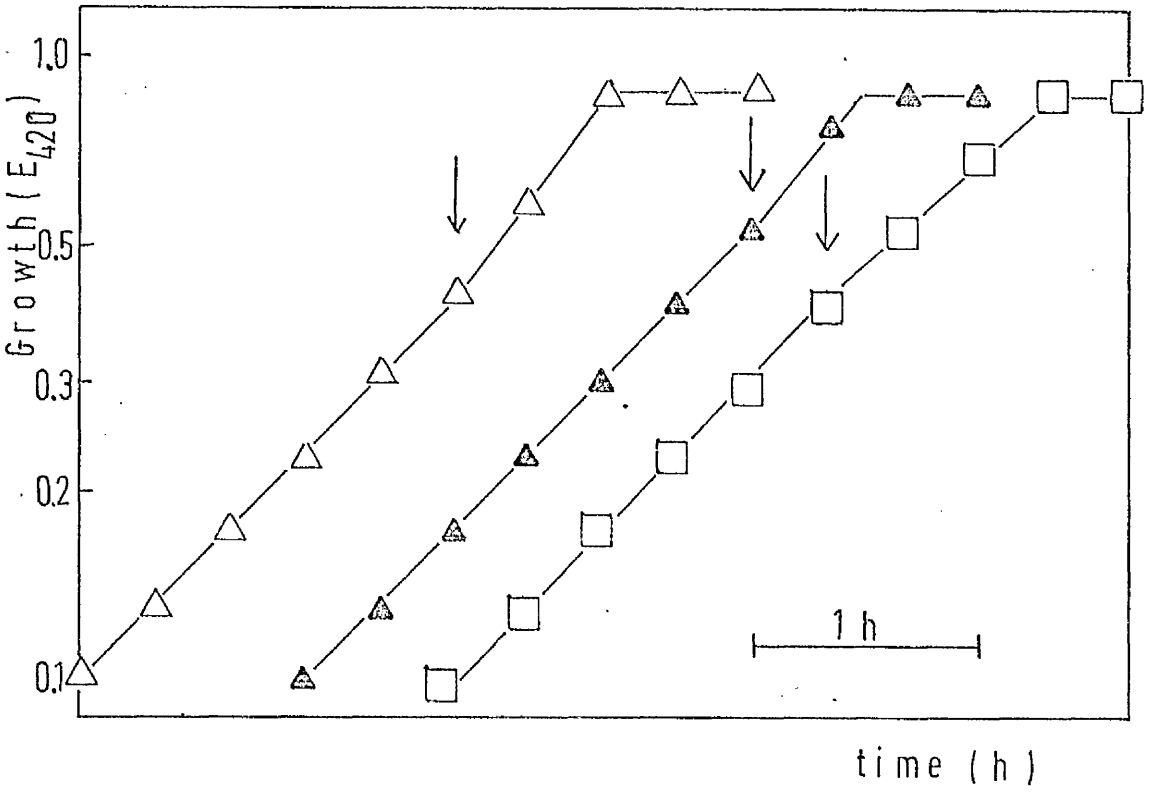


Figure R.3

RESPONSES OF THE PTS IN E.coli 15224

TO CHANGES IN GROWTH MEDIA.(A)

Cells of E.coli 15224 trained to fructose were harvested, resuspended in chilled buffer and inoculated into either glucose or fructose salts media.

Growth, fructose PTS and glucose PTS activities were measured at intervals.

□ growth (as turbidity) on glucose

■ growth (as turbidity) on fructose

○ glucose PTS (glucose salts)

▲ fructose PTS (glucose salts)

● glucose PTS (fructose salts)

△ fructose PTS (fructose salts)

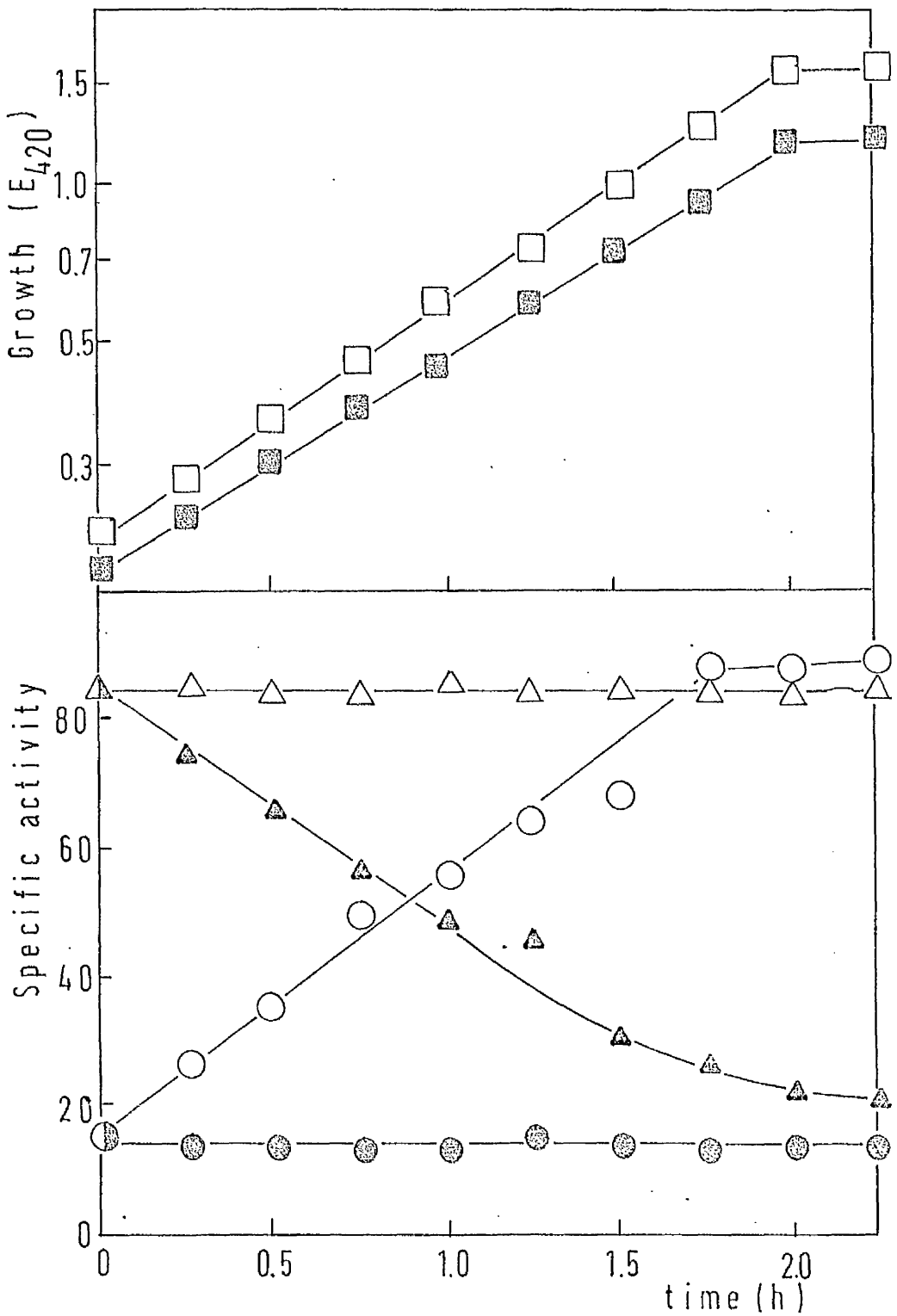

Growth (E_{420})
1.5
1.0
0.7
0.5
0.3
Specific activity
80
60
40
20
0
0
0.5
1.0
1.5
2.0
time (h)

Figure R.4

RESPONSES OF THE PTS IN E.coli 15224

TO CHANGES IN GROWTH MEDIA.(8)

Cells of E.coli 15224 trained to glucose were harvested, resuspended in chilled buffer and inoculated into glucose salts and fructose salts media.

Growth, fructose PTS and glucose PTS activities were measured at intervals.

□ growth (as turbidity) on fructose

▩ growth (as turbidity) on glucose

○ glucose PTS (fructose salts)

△ fructose PTS (fructose salts)

◎ glucose PTS (glucose salts)

⧋ fructose PTS (glucose salts)

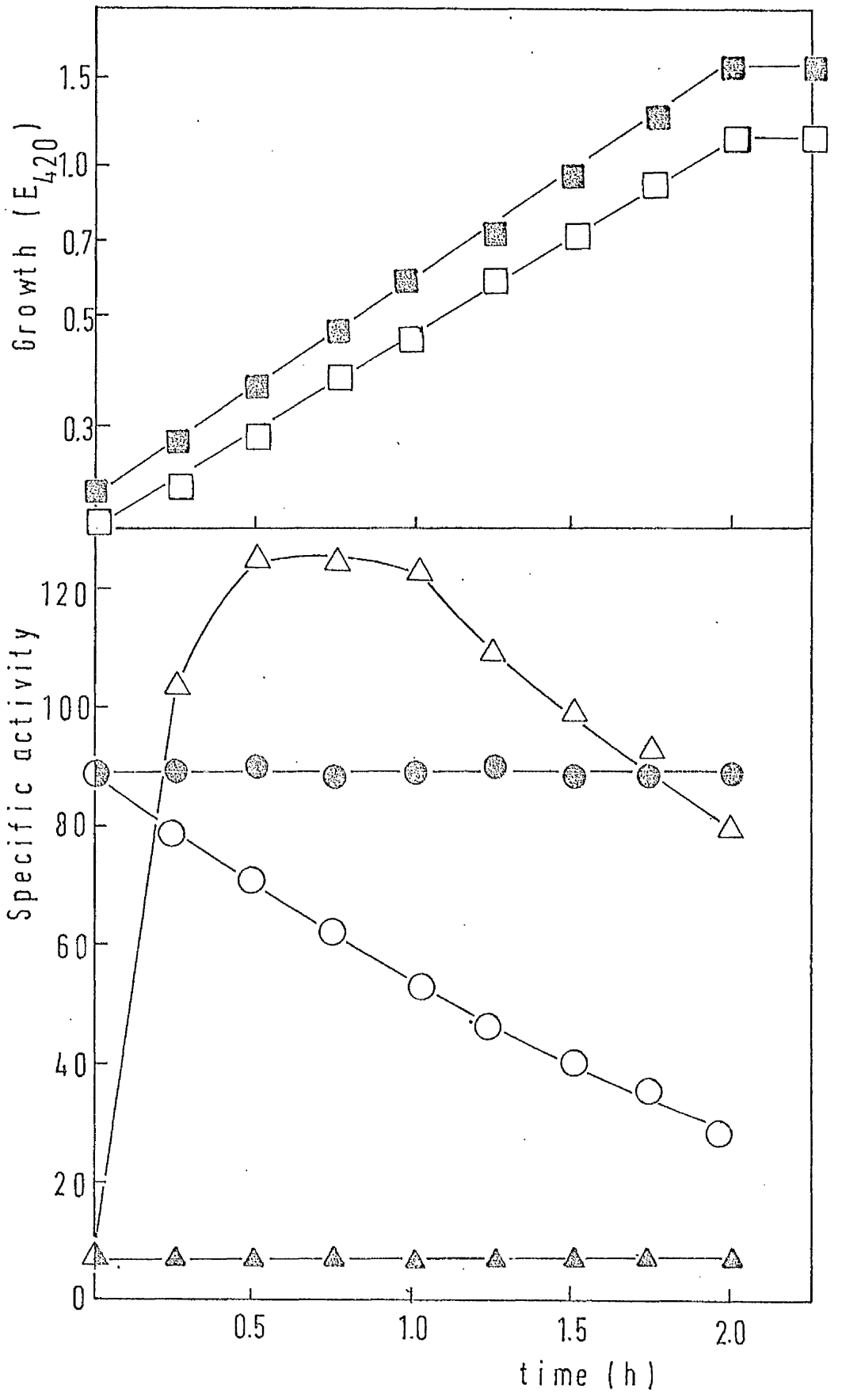

Growth (E_{420})
1.5
1.0
0.7
0.5
0.3
Specific activity
120
100
80
60
40
20
0
0.5
1.0
1.5
2.0
time (h)

Figure R.5

EFFECTS OF GLUCOSE CHALLENGE ON E.coli 15224

GROWING ON FRUCTOSE

Cells of E.coli 15224 trained to fructose were harvested, resuspended in chilled buffer, inoculated into fructose salts medium, and subsequently challenged with glucose.

Growth, substrate concentrations, glucose PTS and fructose PTS were measured at intervals.

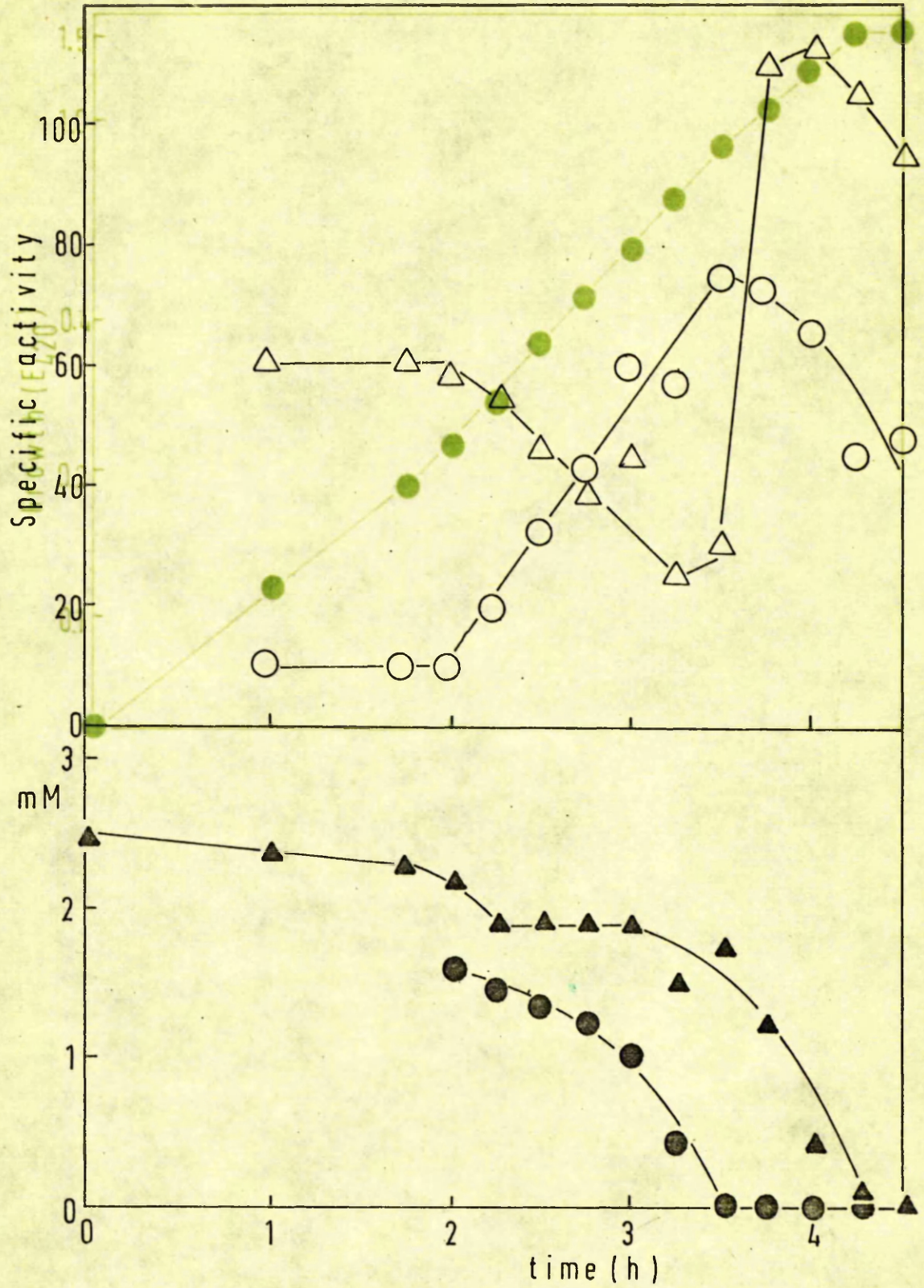
○ glucose PTS activity

△ fructose PTS activity

glucose concentration

fructose concentration

growth (as turbidity)


Specific activity
100
80
60
40
20
0
mM
3
2
1
0
0
1
2
3
4
time (h)

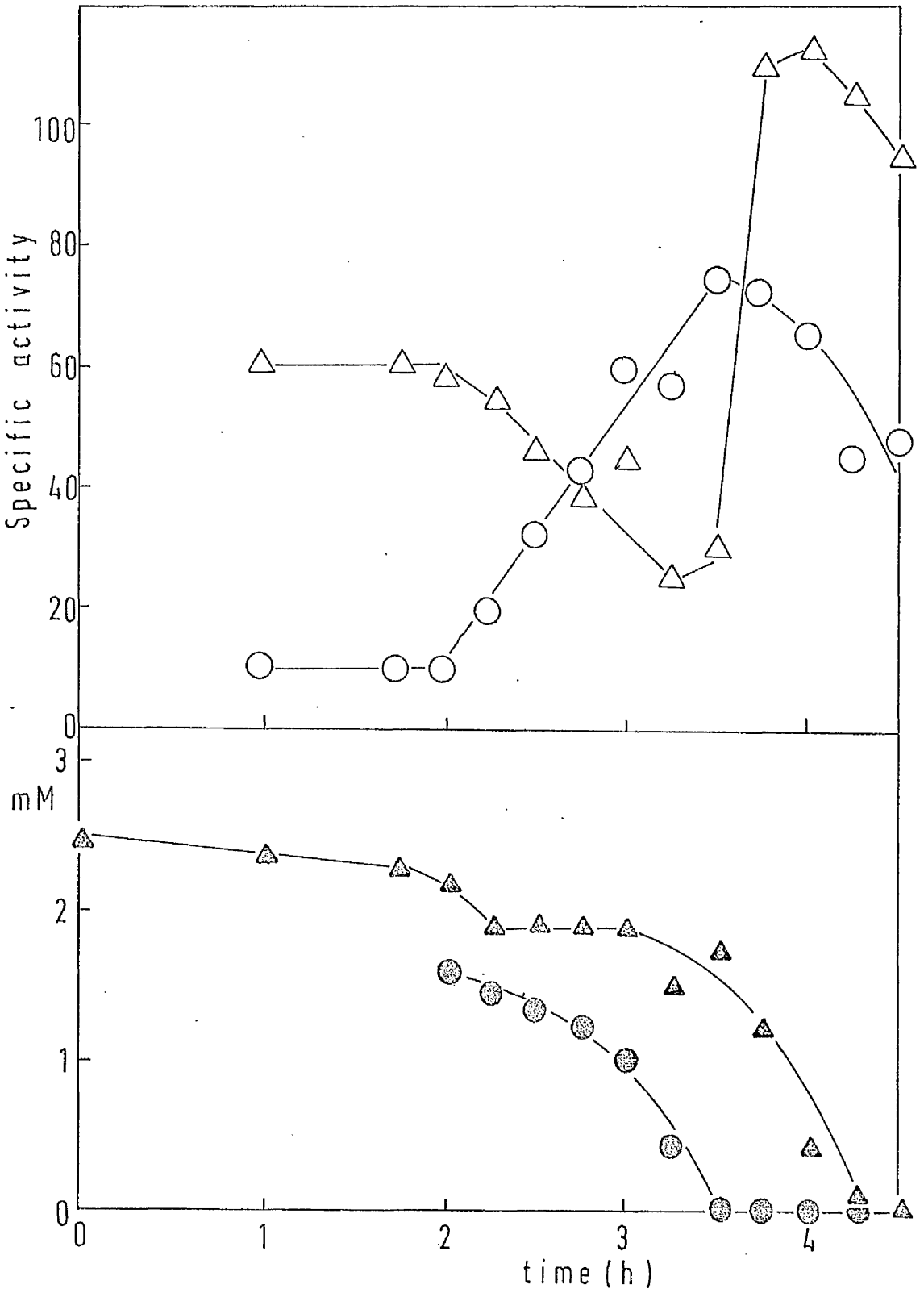

Specific activity
100
80
60
40
20
0
3
mM
2
1
0
0
1
2
3
4
time (h)

Figure R.6

EFFECT OF FRUCTOSE CHALLENGE ON *E.coli* 15224

GROWING ON GLUCOSE

Cells of *E.coli* 15224 trained to glucose were harvested, resuspended in chilled buffer, inoculated into glucose salts medium and subsequently challenged with fructose.

Growth, substrate concentrations, glucose PTS and fructose PTS were measured at intervals.

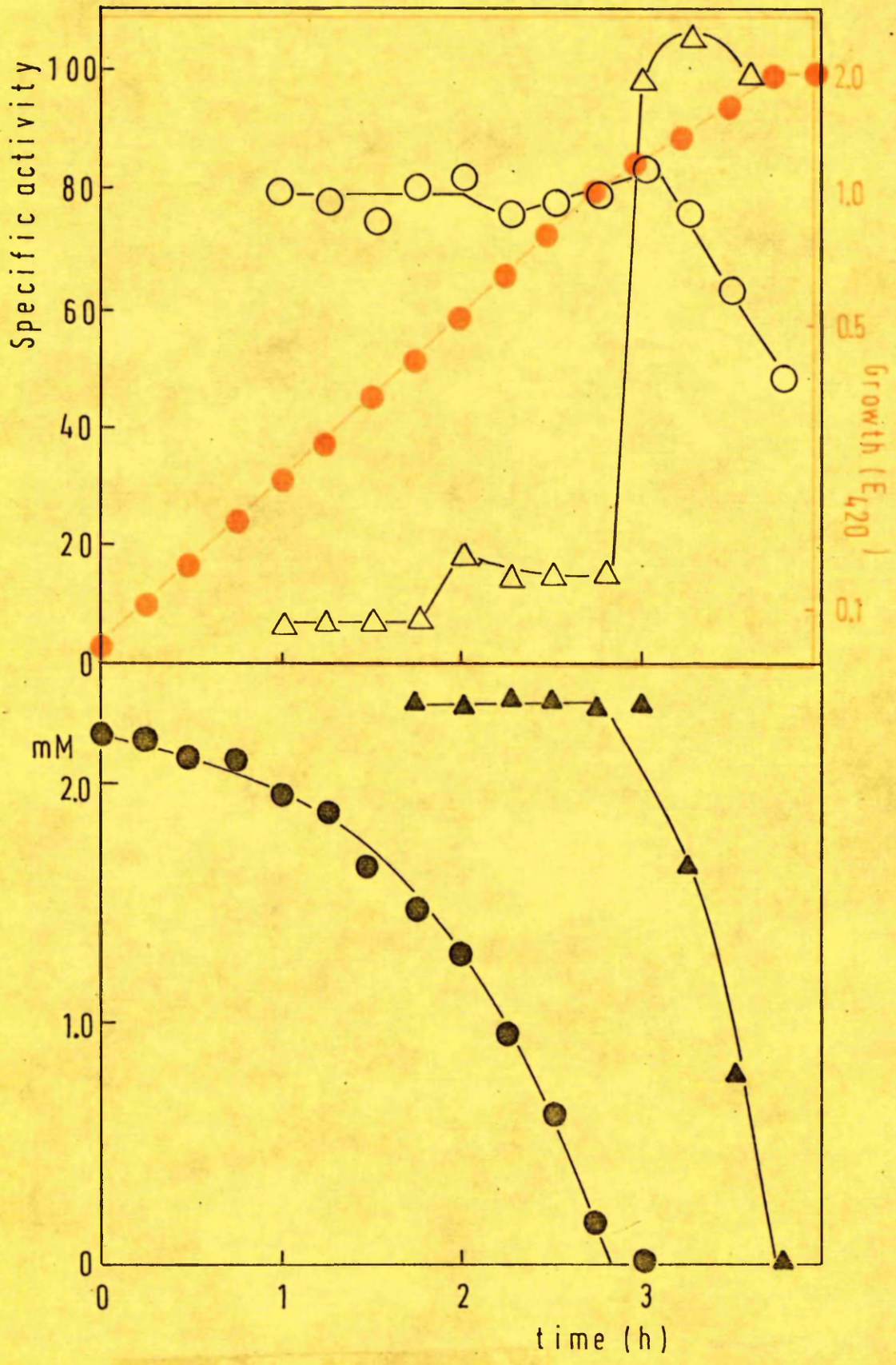
○ glucose PTS activity

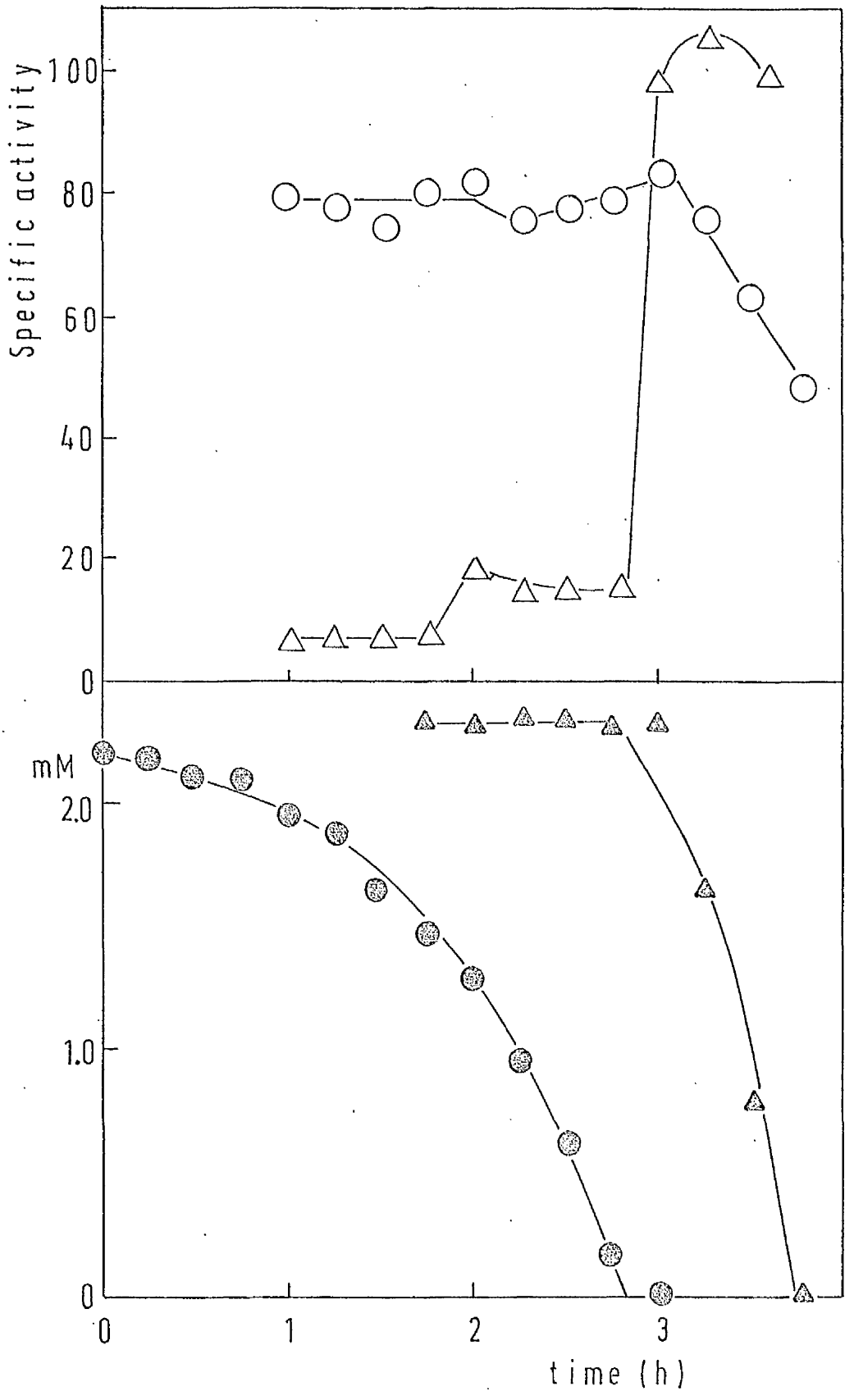
△ fructose PTS activity

◍ glucose concentration

⚠ fructose concentration

○ growth (as turbidity)


Specific activity
100
80
60
40
20
0
2.0
1.0
0.5
0.1
Growth (E_{420})
mM
2.0
1.0
0
0
1
2
3
time (h)


Specific activity
100
80
60
40
20
0
mM
2.0
1.0
0
0
1
2
3
time (h)

apparently no fructose was utilised until glucose was nearly exhausted. At or near glucose exhaustion the fructose PTS overshoot was observed and glucose PTS activity declined.

2. Investigation of the Fructose PTS Overshoot

2.1. Requirement for inducer

Figure R.7 shows the effect of fructose addition to glucose trained cells of E.coli 15224 grown into, and maintained in, stationary phase. Fructose PTS activity did not increase until fructose was added. It was seen that the magnitude of the overshoot decreased as time progressed and also that the specific activity of the glucose PTS fell and then increased in stationary phase.

Figure R.8a shows that synthesis of the fructose PTS was immediate and continuous and was prevented (Figure R.9) by the presence of chloramphenicol. The exceptional nature of the fructose PTS overshoot is seen by reference to the differential plot in Figure R.8b. Figure R.8a also shows that when the fructose PTS reached a specific activity of 55 mU/mg dry wt. glucose PTS also began to increase until 18 min when both glucose and fructose PTS activities declined.

2.2 Use of continuous culture (turbidostat)

Figure R.10 shows the effect of glucose addition to a fructose turbidostat of E.coli 15224 in which fructose salts were supplied in excess of the growth requirement throughout the experiment. The concentration of glucose (3.3 mM) was in excess of that required to maintain growth on glucose alone (1.3 mM), and caused a decrease in fructose PTS activity, increased glucose PTS activity, prevented any detectable fructose utilisation and stimulated growth (from 0.73 to 0.91 h^{-1}). When glucose was removed the fructose PTS overshoot occurred, μ decreased (to 0.73 h^{-1}) and the PTS activities stabilised at new levels. This experiment was repeated with glucose added at 1.6 mM and a similar pattern of results was observed.

In subsequent experiments, glucose was added to the fructose

Figure R.7

EFFECT OF FRUCTOSE ADDITION TO *E.coli* 15224

GROWN INTO STATIONARY PHASE ON GLUCOSE

Cells of *E.coli* 15224 trained to glucose were harvested, resuspended in chilled buffer and inoculated into 3 flasks containing glucose (2mM) salts medium. The cells were grown into stationary phase and fructose was added to each of the flasks A, B and C at the intervals indicated in the figure.

Glucose PTS and fructose PTS activities were measured at intervals.

◐ glucose PTS activity

△ fructose PTS activity

↓ addition of fructose

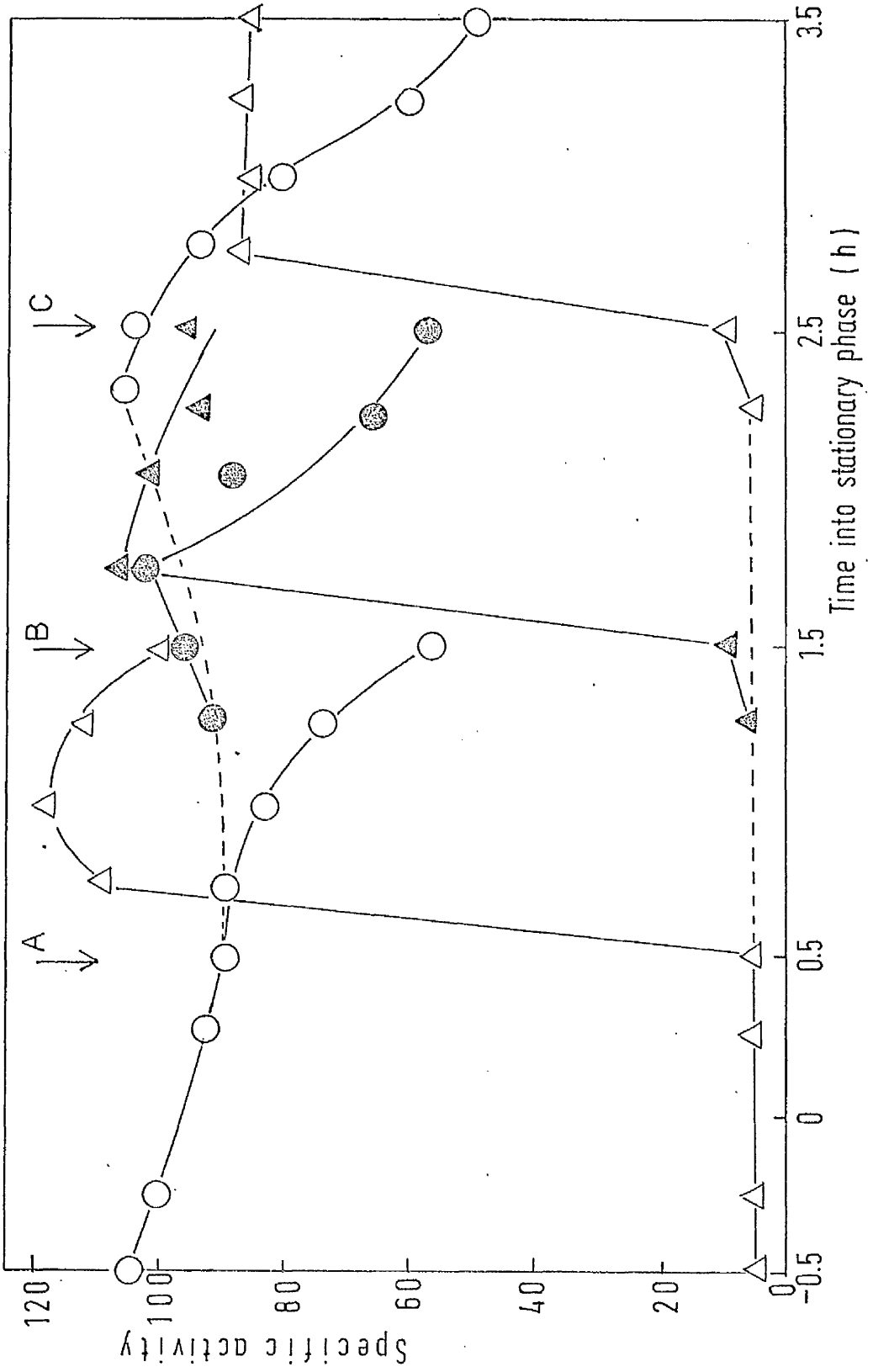

Specific activity
120
100
80
60
40
20
0
-0.5
0
0.5
1.5
2.5
3.5
Time into stationary phase (h)
A
B
C

Figure R.8

CHANGE IN PTS OF *E.coli* 15224 ON TRANSFER FROM GLUCOSE TO FRUCTOSE SALTS MEDIUM

Glucose trained cells of *E.coli* 15224 were harvested, resuspended in chilled buffer and inoculated into glucose salts medium. The cells were subsequently re-harvested in exponential phase, washed, resuspended in chilled buffer and inoculated into fructose salts. At intervals samples were removed and blown into ice-cold buffer containing CAP (final concentration 0.3 mM) to stop protein synthesis. The chilled suspensions were harvested, and washed once in chilled buffer to remove substrate and CAP. Growth, glucose PTS and fructose PTS activities were determined.

Figure R.8a

○ glucose PTS activity

△ fructose PTS activity

Figure R.8b

This figure shows a differential plot of the fructose PTS synthesis shown in Figure R.8a.

Figure R. 8a

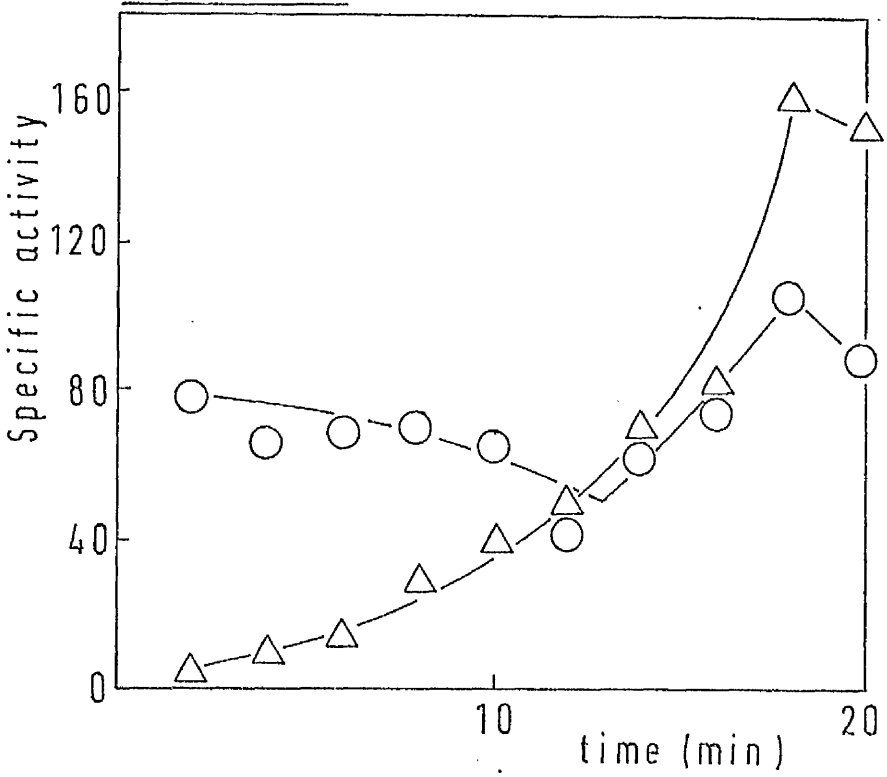


Figure R. 8b

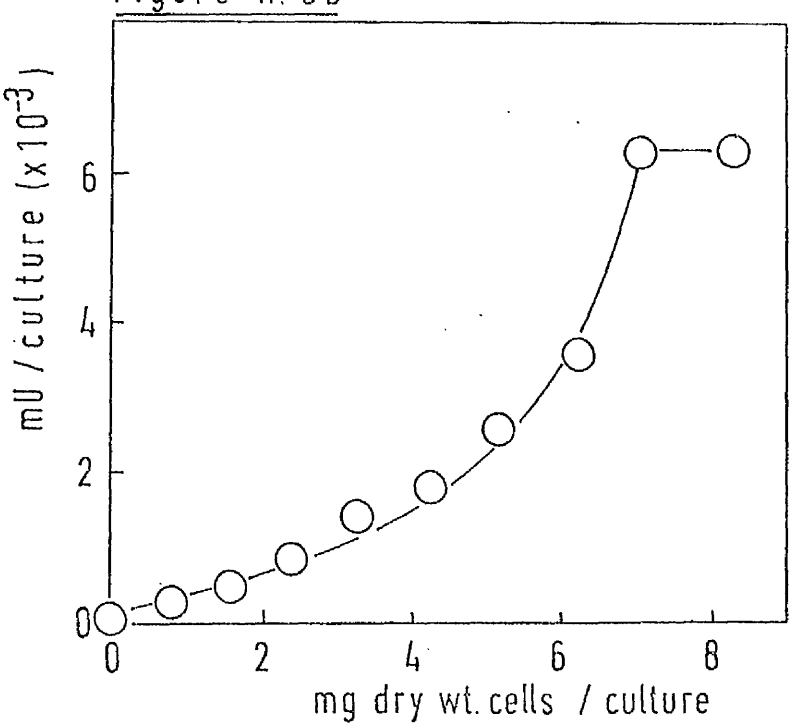


Figure R.9

EFFECT OF CHLORAMPHENICOL ON

FRUCTOSE PTS SYNTHESIS IN *E.coli* 15224

Glucose trained cells of *E.coli* 15224 were harvested, resuspended in chilled buffer and inoculated into glucose salts medium. The cells were subsequently re-harvested in exponential phase, washed, resuspended in chilled buffer and inoculated into flasks containing either fructose salts or fructose salts + CAP (0.3 mM) media.

Glucose PTS and fructose PTS were measured at intervals.

○ glucose PTS activity (fructose salts)

△ fructose PTS activity (fructose salts)

◍ glucose PTS activity (fructose salts + CAP)

◬ fructose PTS activity (fructose salts + CAP)

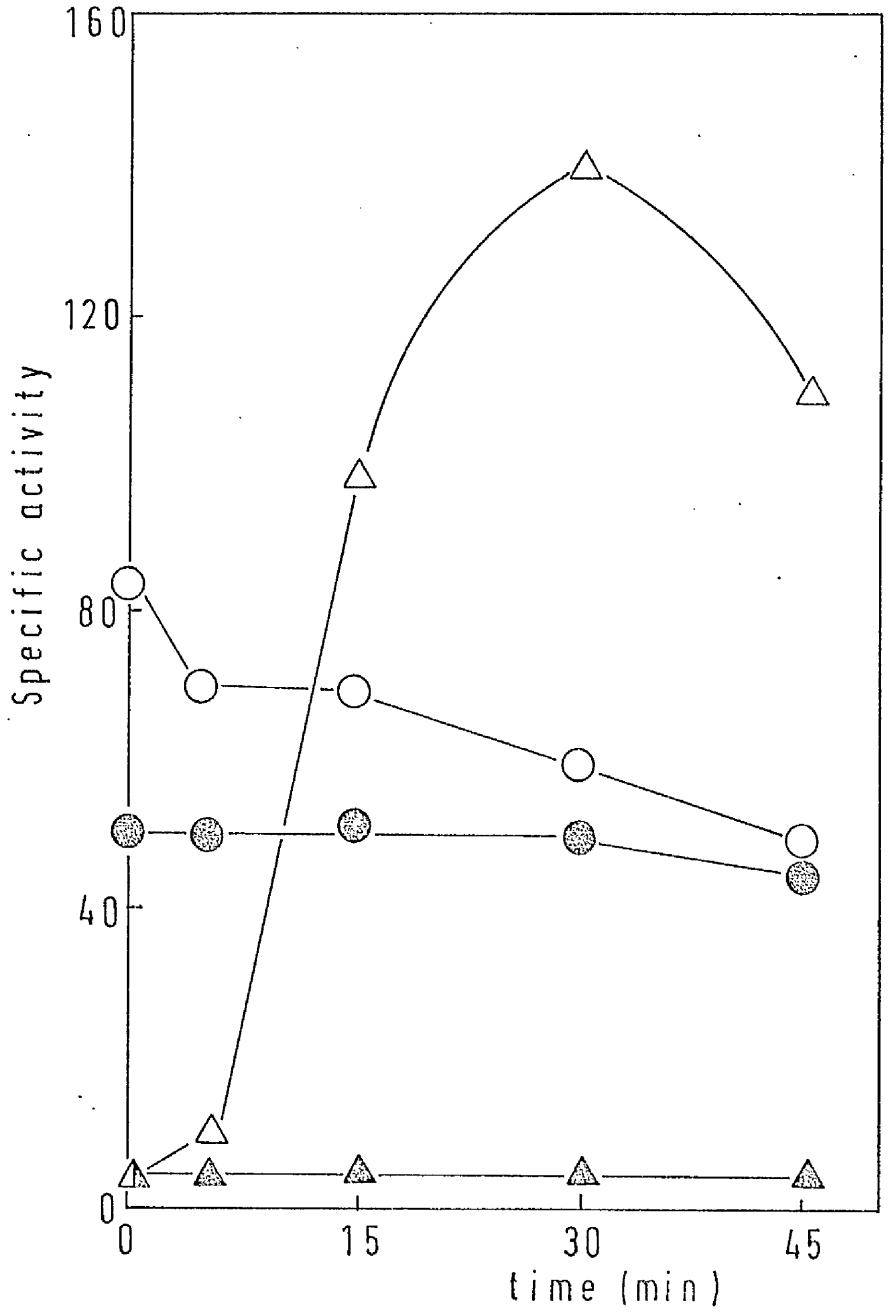

160
120
80
40
0
Specific activity
0
15
30
45
time (min)

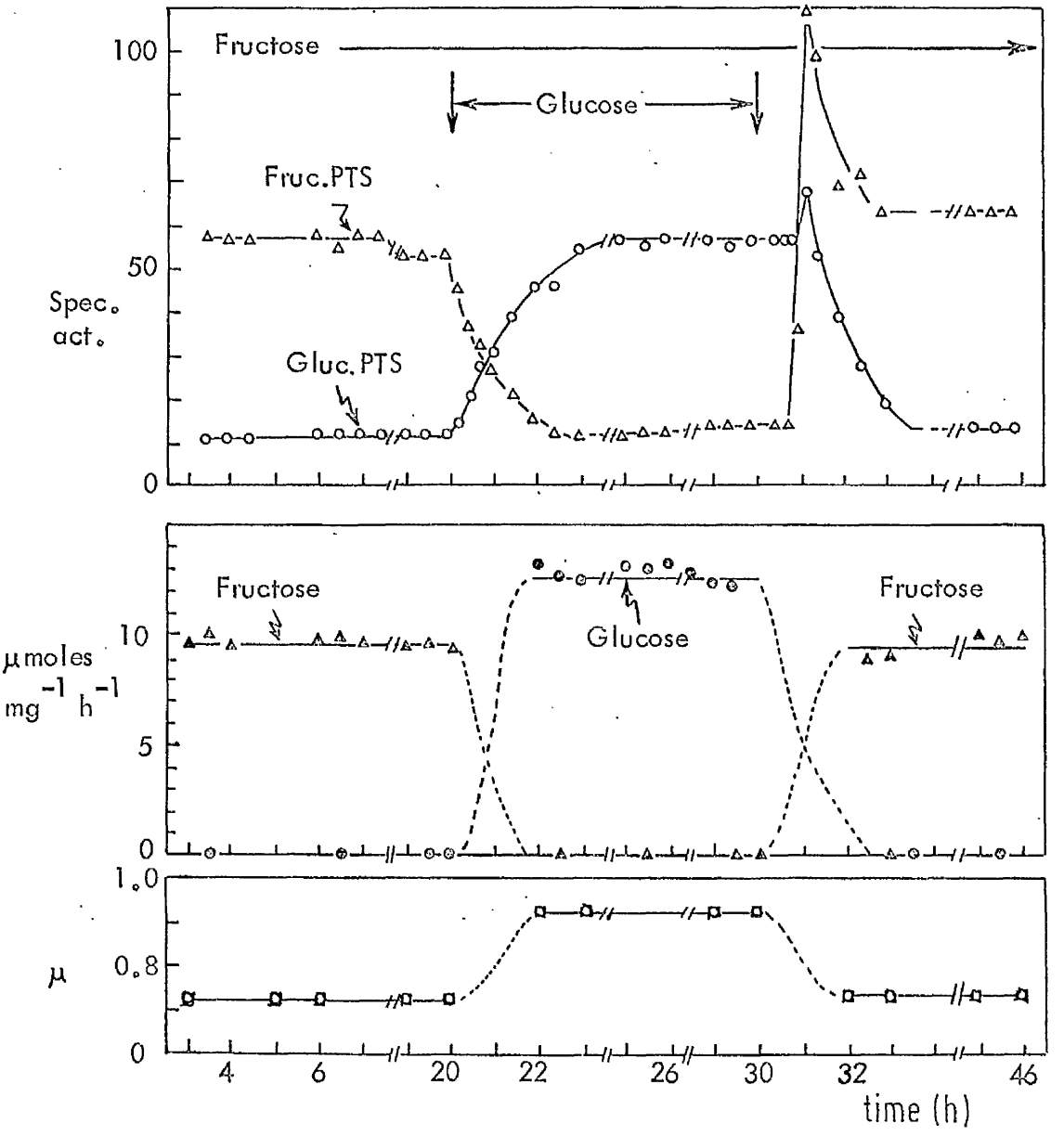
Figure R.10

EFFECT OF GLUCOSE ADDITION TO A FRUCTOSE TURBIDOSTAT OF *E.coli* 15224. (A)

The growth flask was prefilled with the growth medium (3.2 mM fructose salts) and aseptically inoculated with fructose trained cells of *E.coli* 15224. Growth was followed and when the required turbidity (E_{420} = 0.5) was achieved, the dilution pump was started and the turbidity maintained at 0.5 ($\mu = \mu$ max). Subsequently the growth medium was changed (3.2 mM fructose, 3.3 mM glucose and salts) for a suitable period before reverting to the original medium.

Growth, glucose PTS and fructose PTS activities, substrate utilisation and μ were determined at intervals.

- ○ glucose PTS activity
- △ fructose PTS activity
- □ μ (h^{-1}), specific growth rate
- ◎ glucose utilisation (q : μ moles/h/mg dry wt.)
- ▲ fructose utilisation


Fructose
Glucose
Fruc.PTS
Gluc.PTS
Spec. act.
100
50
0
Fructose
Glucose
Fructose
µmoles mg⁻¹ h⁻¹
10
5
0
µ
1.0
0.8
0
4
6
20
22
26
30
32
46
time (h)

turbidostat described above at either 60% (Figure R.11) or 30% (Figure R.12) of the concentration required to maintain growth on glucose alone. In both experiments, all the glucose was used together with some fructose and growth was stimulated (from 0.73 to 0.93 h^{-1}). The resulting PTS activities and the magnitude of the fructose PTS overshoot when glucose was removed from the feed, varied with the rate of glucose (and consequently fructose) utilisation. The specific growth rate also fell (to 0.73 h^{-1}) on glucose removal and the PTS activities eventually stabilised at new levels.

Figure R.13 shows the results obtained when glucose was added to the fructose turbidostat described above at less than 10% of the concentration required to maintain growth solely on glucose. All the glucose was used together with some fructose, glucose and fructose PTS activities responded as shown in Figure R.13 and growth was stimulated (from 0.69 to 0.83 h^{-1}). No fructose PTS overshoot occurred on removal of glucose from the feed, although μ decreased (to 0.73 h^{-1}), the PTS activities readjusted and the rate of fructose utilisation was slightly stimulated.

Figure R.14 shows the effect of fructose addition to a glucose turbidostat of E.coli 15224 in which glucose salts were supplied in excess of the growth requirements throughout the experiment. The concentration of fructose (3.2 mM), which was in excess of the requirement to maintain growth solely on fructose (1.2 mM), stimulated to a small extent both fructose PTS activity and the rate of glucose utilisation but μ remained unchanged (0.89 h^{-1}). Glucose PTS activity decreased but, as far as could be determined, no fructose was utilised by the cells. On removal of fructose, the PTS activities readjusted accordingly and a further slight increase in the rate of glucose utilisation and increase in growth (to 0.9 h^{-1}) was observed.

Figure R.11

EFFECT OF GLUCOSE ADDITION TO A FRUCTOSE TURBIDOSTAT OF *E.coli* 15224. (B)

The growth flask was prefilled with growth medium (3.2 mM fructose and salts) and aseptically inoculated with fructose trained cells of *E.coli* 15224. Growth was followed. When the required turbidity (E_{420} = 0.5) was achieved the dilution pump was started to maintain the turbidity at 0.5 ($\mu = \mu$ max). Subsequently the growth medium was changed (3.2 mM fructose, 0.8 mM glucose and salts) for a period before reverting to the original medium.

Growth, glucose PTS and fructose PTS activities, substrate utilisation and μ were determined at intervals.

- ○ glucose PTS activity
- △ fructose PTS activity
- □ μ (h^{-1}), specific growth rate
- ⊛ glucose utilisation (q : μ moles/h/mg dry wt.)
- ▲ fructose utilisation

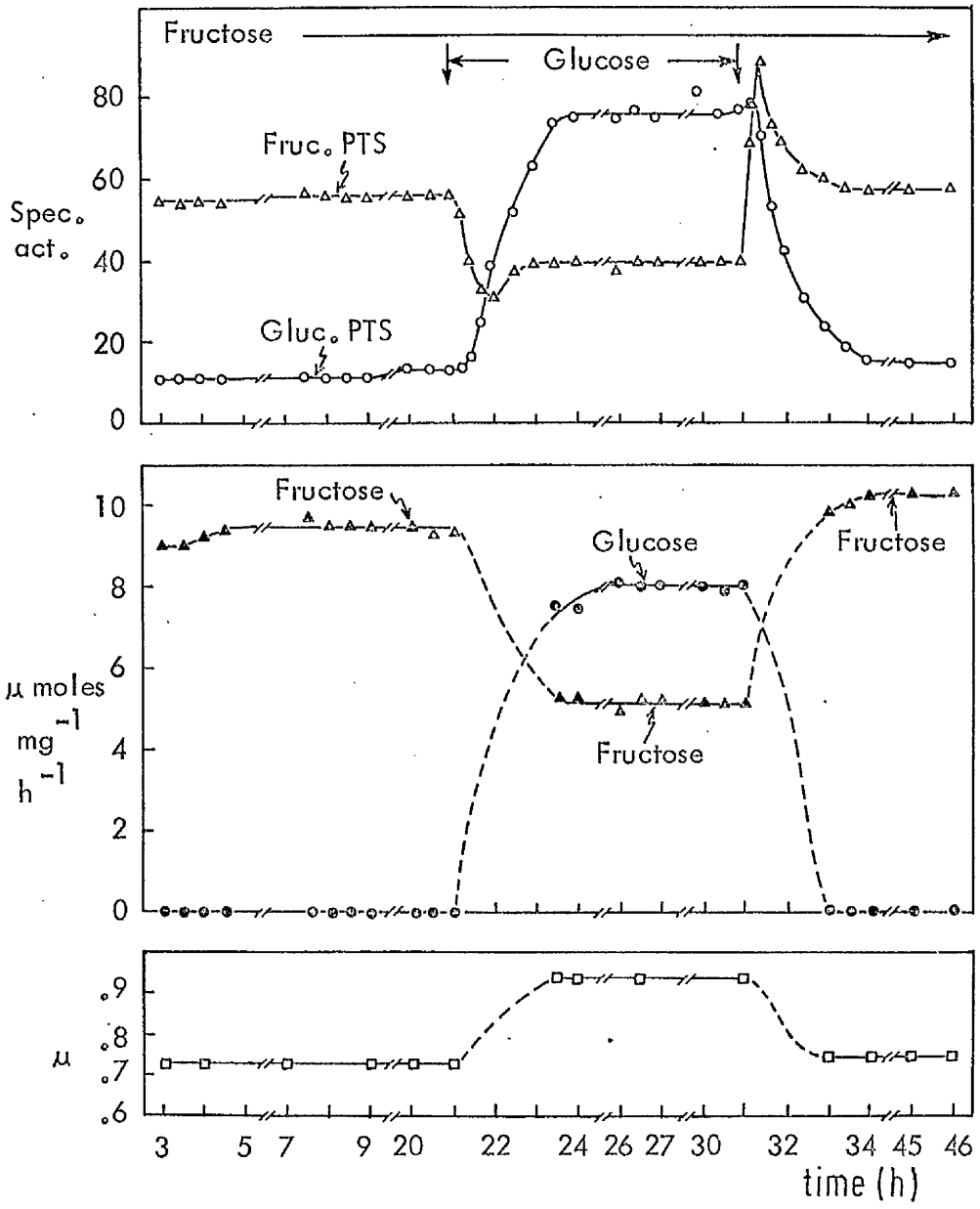

Fructose
Glucose
Fruc. PTS
Gluc. PTS
Spec. act.
80
60
40
20
0
Fructose
Glucose
Fructose
Fructose
μ moles mg⁻¹ h⁻¹
10
8
6
4
2
0
μ
.9
.8
.7
.6
3
5
7
9
20
22
24
26
27
30
32
34
45
46
time (h)

Figure R.12

EFFECT OF GLUCOSE ADDITION TO A FRUCTOSE TURBIDOSTAT OF *E.coli* 15224. (C)

The growth flask was prefilled with growth medium (3.2 mM fructose and salts) and aseptically inoculated with fructose trained cells of *E.coli* 15224. Growth was followed. When the required turbidity (ε_{420} = 0.5) was achieved the dilution pump was started and the turbidity maintained at 0.5 ($\mu = \mu$ max). Subsequently the growth medium was changed (3.2 mM fructose, 0.4 mM glucose and salts) for a suitable period before reversion to the original medium.

Growth, glucose PTS and fructose activities, substrate utilisation and u were determined at intervals.

- ○ glucose PTS activity
- △ fructose PTS activity
- □ μ (h^{-1}), specific growth rate
- ◎ glucose utilisation ($q : \mu$ moles/h/mg dry wt.)
- ▲ fructose utilisation

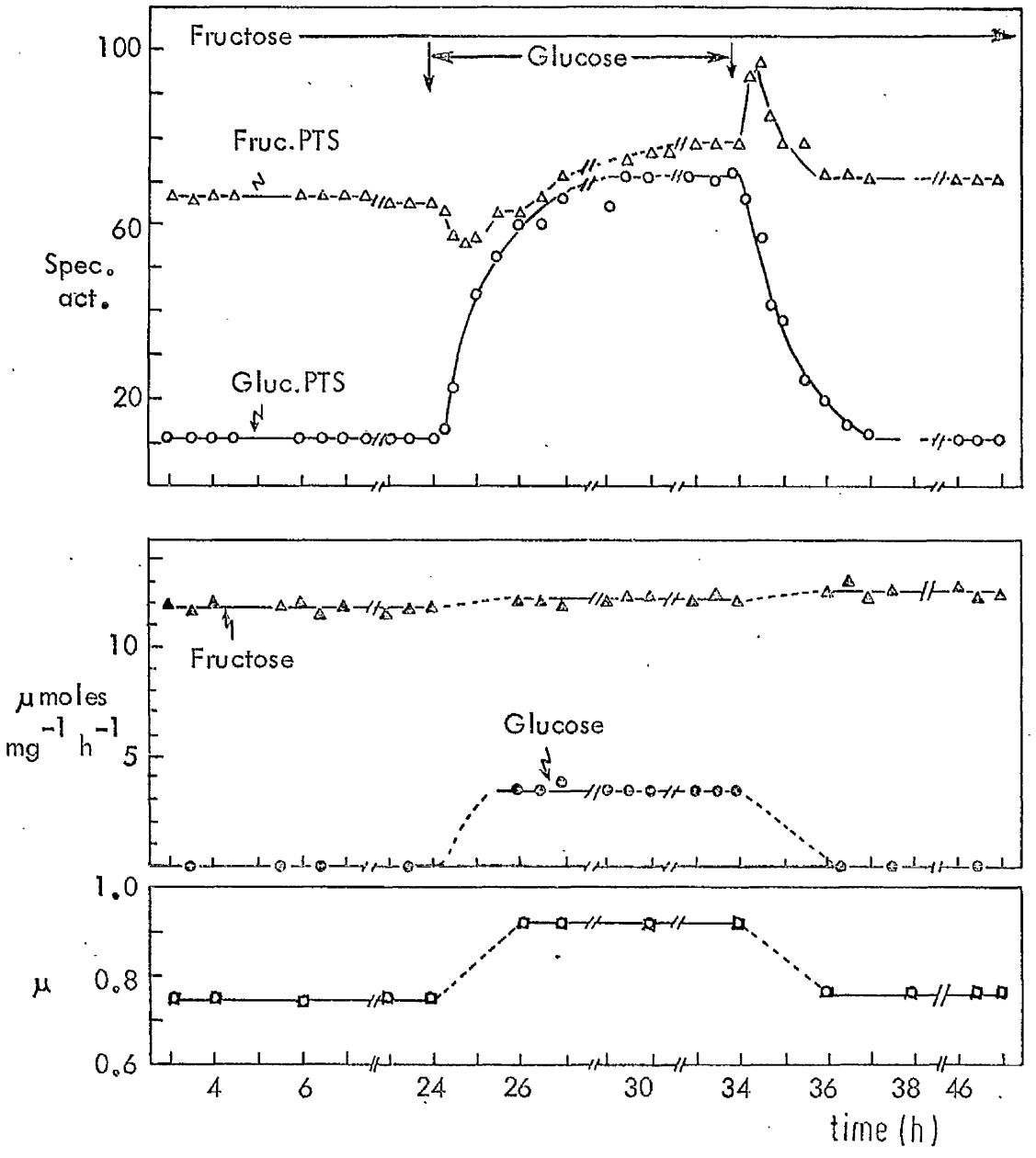

Fructose
Glucose
Fruc.PTS
Gluc.PTS
100
60
20
Spec.
act.
Fructose
Glucose
10
5
μmoles
mg⁻¹ h⁻¹
1.0
0.8
0.6
μ
4
6
24
26
30
34
36
38
46
time (h)

Figure R.13

EFFECT OF GLUCOSE ADDITION TO A FRUCTOSE TURBIDOSTAT OF *E.coli* 15224. (C)

The growth flask was prefilled with growth medium (3.2 mM fructose and salts) and aseptically inoculated with fructose trained cells of *E.coli* 15224. Growth was followed until the required turbidity (E_{420} = 0.5) was achieved when the dilution pump was started to maintain the turbidity at 0.5 ($\mu = \mu$ max). Subsequently the growth was changed (3.2 mM fructose, 0.1 mM glucose and salts) for a suitable period before reverting to the original medium.

Growth, glucose PTS and fructose PTS activities, substrate utilisation and μ were determined at intervals.

- ○ glucose PTS activity
- △ fructose PTS activity
- □ μ (h^{-1}), specific growth rate
- ◉ glucose utilisation (q : μ moles/h/mg dry wt.)
- ▲ fructose utilisation

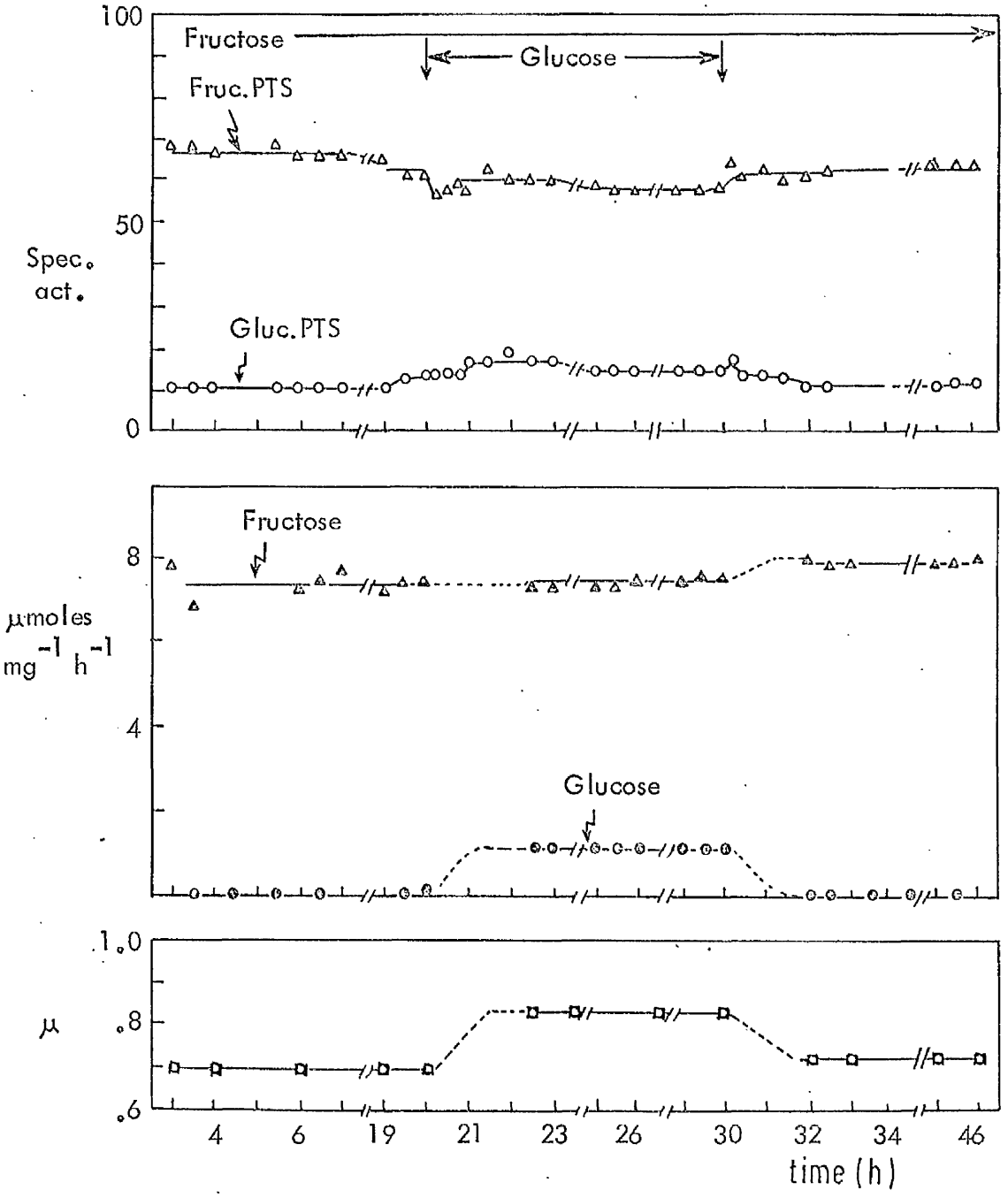

100
Fructose
Glucose
Fruc.PTS
50
Spec.
act.
Gluc.PTS
0
Fructose
8
µmoles
mg-1 h-1
4
Glucose
1.0
µ
.8
.6
4
6
19
21
23
26
30
32
34
46
time (h)

Figure R.14

EFFECT OF THE ADDITION OF FRUCTOSE TO A GLUCOSE TURBIDOSTAT OF *E.coli* 15224

The growth flask was prefilled with growth medium (3.3 mM glucose and salts) and aseptically inoculated with glucose trained cells of *E.coli* 15224. Growth was followed. When the required turbidity (E_{420} = 0.5) was achieved the dilution pump was started to maintain the turbidity at 0.5 ($\mu = \mu$ max). Subsequently the growth medium was changed (3.3 mM glucose, 3.2 mM fructose and salts) for a suitable period before reverting to the original medium.

Growth, glucose and fructose PTS activities, substrate utilisation and μ were determined at intervals.

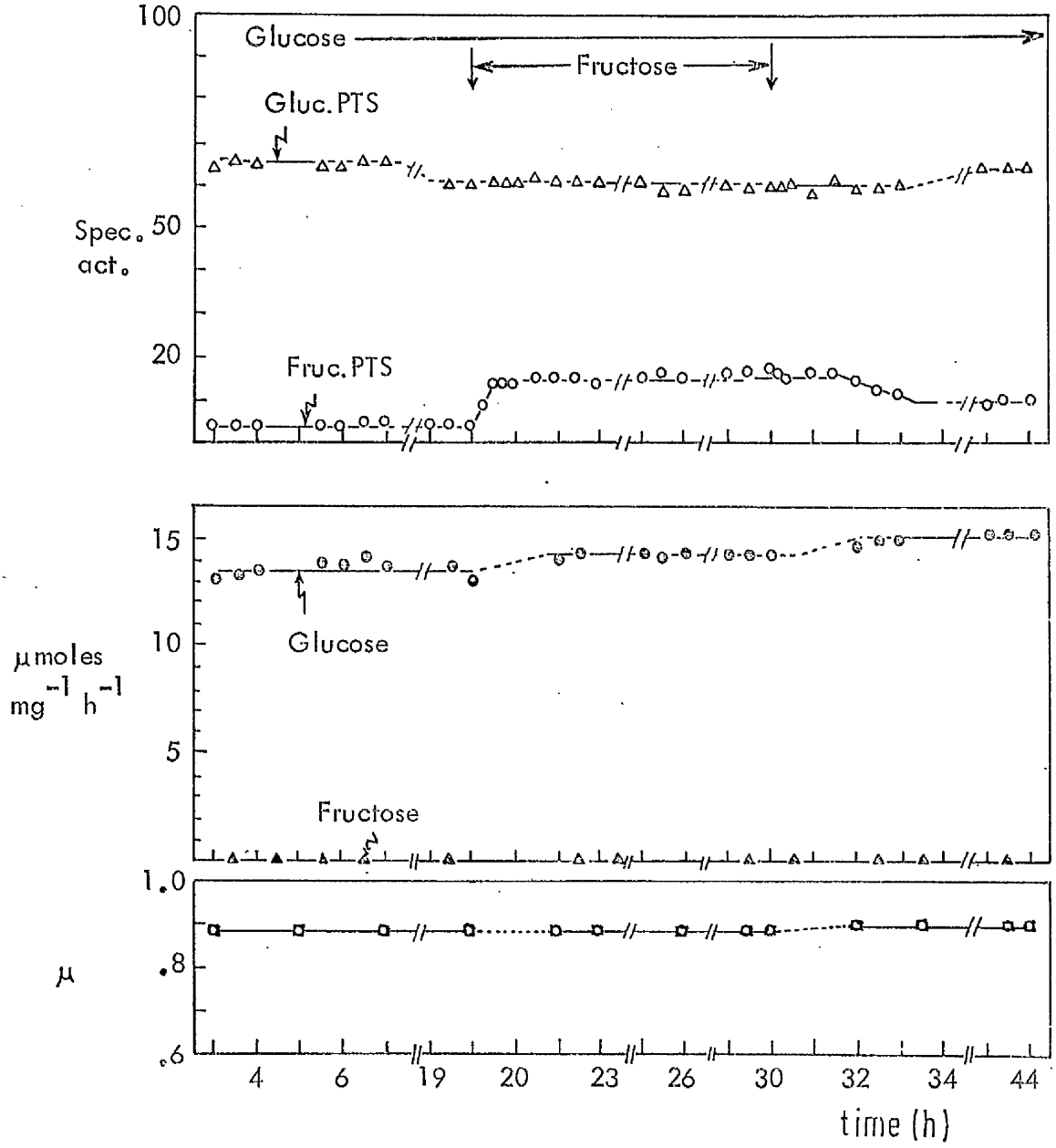
△ glucose PTS activity

○ fructose PTS activity

□ μ (h^{-1}), specific growth rate

◎ glucose utilisation (q : μ moles/h/mg dry wt.)

△ fructose utilisation


100
Glucose
Fructose
Gluc. PTS
Spec. act.
50
20
Fruc. PTS
15
10
5
μmoles mg⁻¹ h⁻¹
Glucose
Fructose
1.0
.8
.6
μ
4
6
19
20
23
26
30
32
34
44
time (h)

2.3. Effect of batch growth conditions on the fructose PTS overshoot

Figure R.15 shows the effect of transferring glycerol trained cells of E.coli 15224, which are not catabolite repressed (Holms & Robertson, 1973), to fructose salts medium. The fructose PTS overshoot did not occur. This may have been because the cells had never been grown on glucose. Figure R.16 shows the effect of inoculating cells trained to glucose in the presence of cyclic AMP into fructose salts medium. Again the fructose PTS overshoot did not occur.

Figures R.17a & R.17b show the effect of glucose challenge to cultures of E.coli 15224 actively growing on fructose in the presence and absence of cyclic AMP respectively. Glucose challenge stimulated growth less in the presence of ($0.83\ h^{-1}$) than in the absence of ($0.93\ h^{-1}$) cyclic AMP. Glucose challenge in the presence of cyclic AMP caused an increase in glucose PTS activity, a decrease in fructose PTS activity and fructose utilisation slowed down, eventually ceased (after 60 min) and did not recommence until glucose was nearly exhausted. At or near glucose exhaustion fructose PTS activity increased (from 40 to 80 mU/mg dry wt.) but did not overshoot the final value. Glucose PTS activity declined having achieved a specific activity of 100 mU/mg dry wt. Glucose challenge in the absence of cyclic AMP (Figure R.17b) caused an increase in glucose PTS activity, a decrease in fructose PTS activity and fructose utilisation slowed down, eventually ceased (after 15 min) and did not recommence until 30 min before glucose exhaustion. At or near glucose exhaustion fructose PTS activity increased rapidly (from 20 - 120 mU/mg dry wt.) and overshot the final value (100 mU/mg dry wt.) reached in this experiment. Glucose PTS activity declined having achieved a specific activity of 60 mU/mg dry wt.

Figure R.18 gathers together some of the results dealing with the fructose PTS overshoot to simplify comparison of the data.

Figure R.15

CHANGE IN PTS OF *E.coli* 15224

ON TRANSFER FROM GLYCEROL TO FRUCTOSE SALTS MEDIUM

Glycerol trained cells of *E.coli* 15224 were harvested, resuspended in chilled buffer and inoculated into glycerol salts medium. The cells were subsequently reharvested in the exponential phase of growth, resuspended in chilled buffer and inoculated into fructose salts medium.

Growth, glucose PTS and fructose PTS activities were determined at intervals.

○ glucose PTS activity

△ fructose PTS activity

◍ growth (as turbidity)

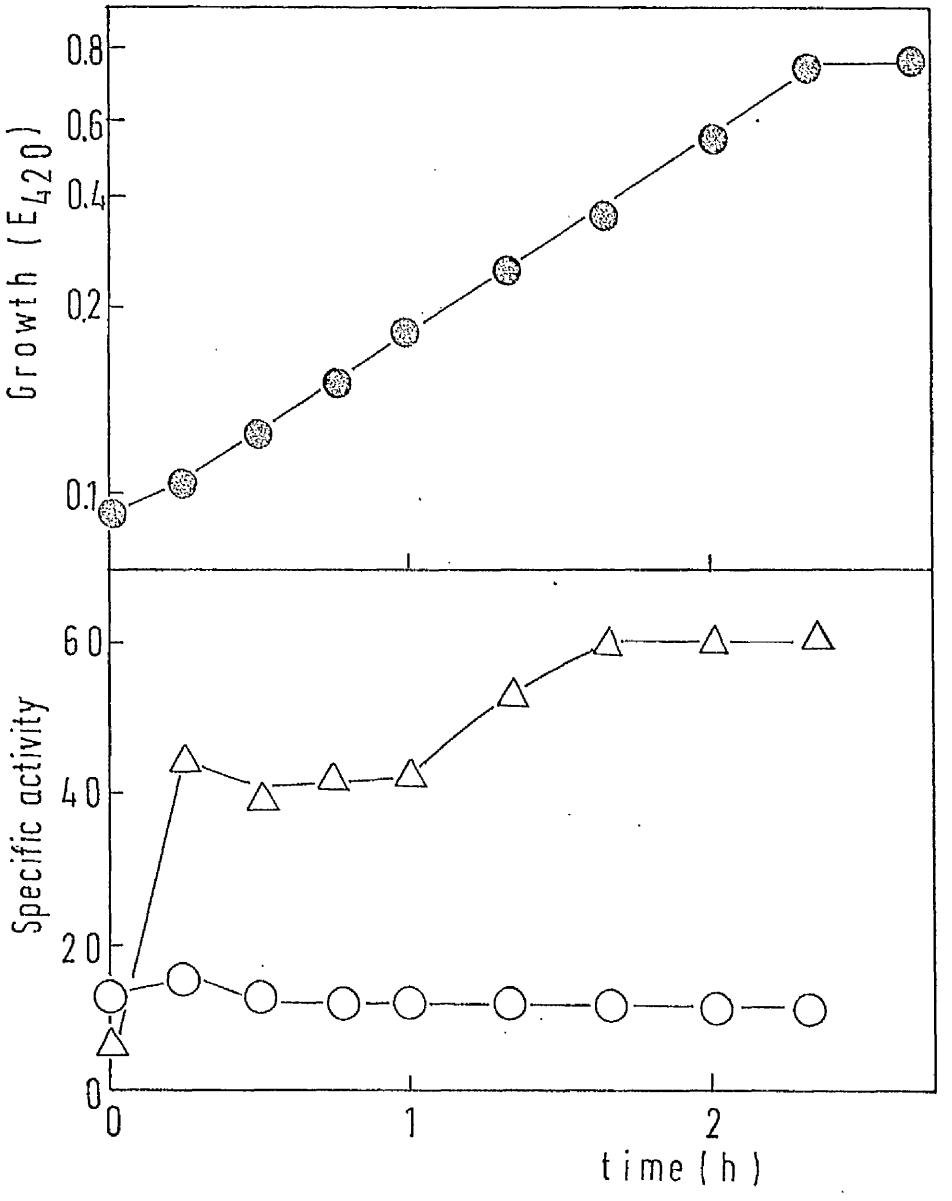

Growth (E_{420})
0.8
0.6
0.4
0.2
0.1
Specific activity
60
40
20
0
0
1
2
time (h)

Figure R.16

CHANGE IN PTS OF E.coli 15224 ON TRANSFER FROM GLUCOSE-CYCLIC AMP TO FRUCTOSE SALTS MEDIUM

Glucose trained cells of E.coli 15224 were harvested, resuspended in chilled buffer and inoculated into glucose salts medium containing cyclic AMP (5 mM). The cells were harvested in exponential phase, resuspended in chilled buffer and inoculated into fructose salts medium.

Growth, glucose PTS and fructose PTS activities were determined at intervals.

○ glucose PTS activity

△ fructose PTS activity

⊛ growth (as turbidity)

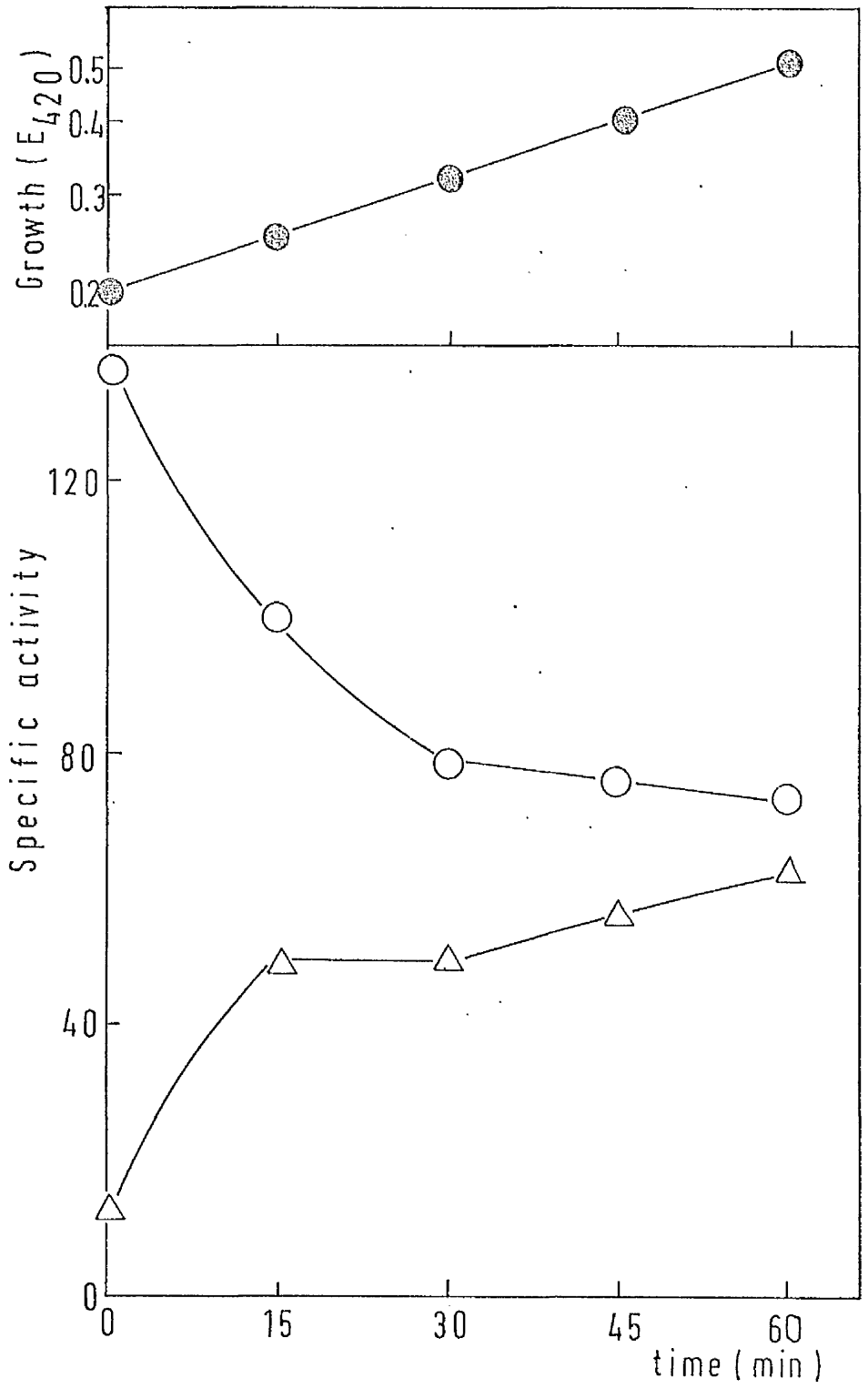

Growth (E_{420})
0.5
0.4
0.3
0.2
Specific activity
120
80
40
0
0
15
30
45
60
time (min)

Figure R.17a

GLUCOSE CHALLENGE ON *E.coli* 15224

GROWING ON FRUCTOSE-CYCLIC AMP SALTS

Cells of *E.coli* 15224 trained to fructose in the presence of cyclic AMP (5 mM) were harvested, resuspended in chilled buffer, inoculated into fructose salts medium containing cyclic AMP (5 mM) and subsequently challenged with glucose.

Growth, substrate concentration, glucose PTS and fructose PTS activity were determined at intervals.

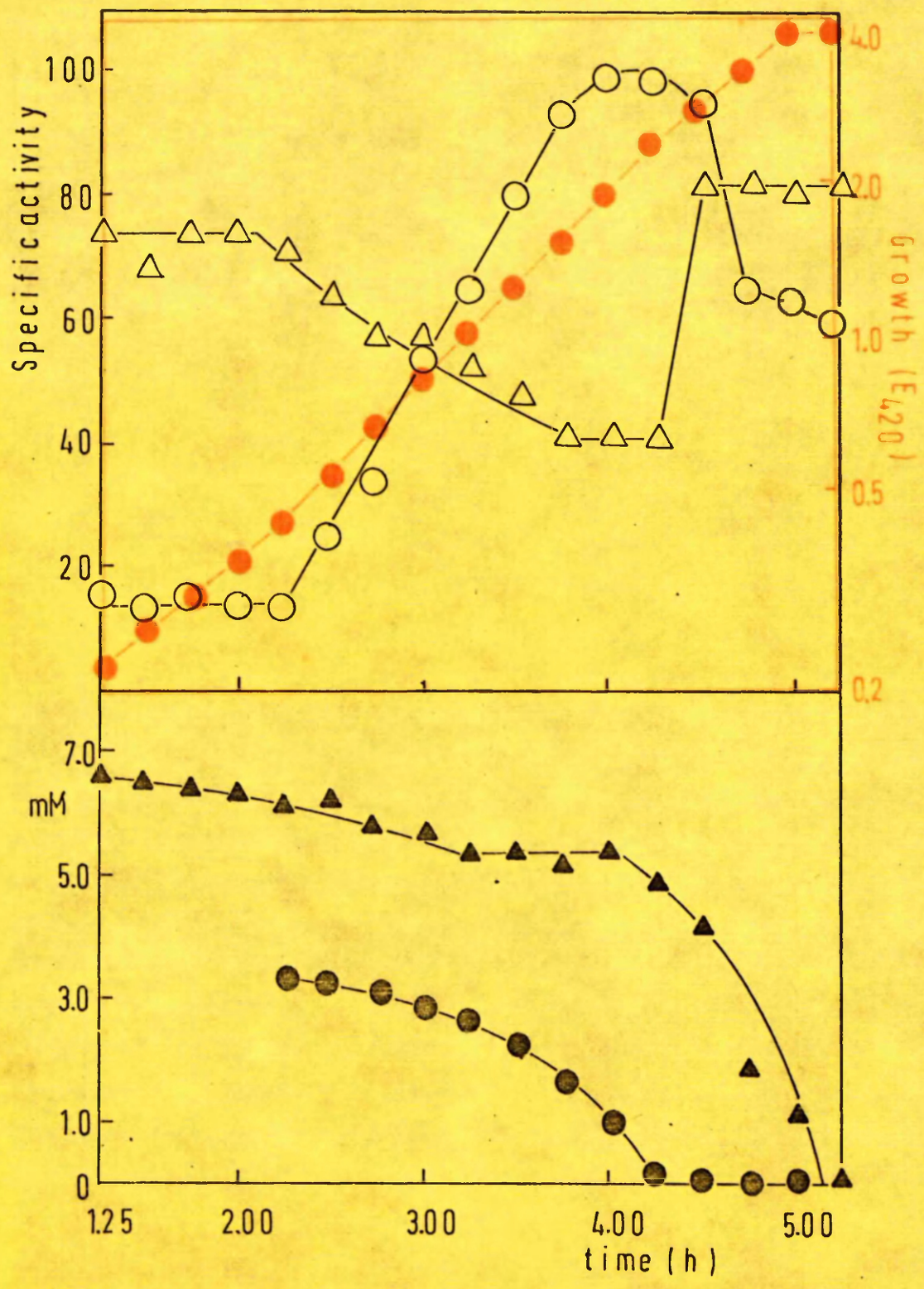
○ glucose PTS activity

△ fructose PTS activity

◍ glucose concentration

◬ fructose concentration

◯ growth (as turbidity)


Specific activity
100
80
60
40
20
Growth (E_{420})
4.0
2.0
1.0
0.5
0.2
7.0
mM
5.0
3.0
1.0
0
1.25
2.00
3.00
4.00
5.00
time (h)

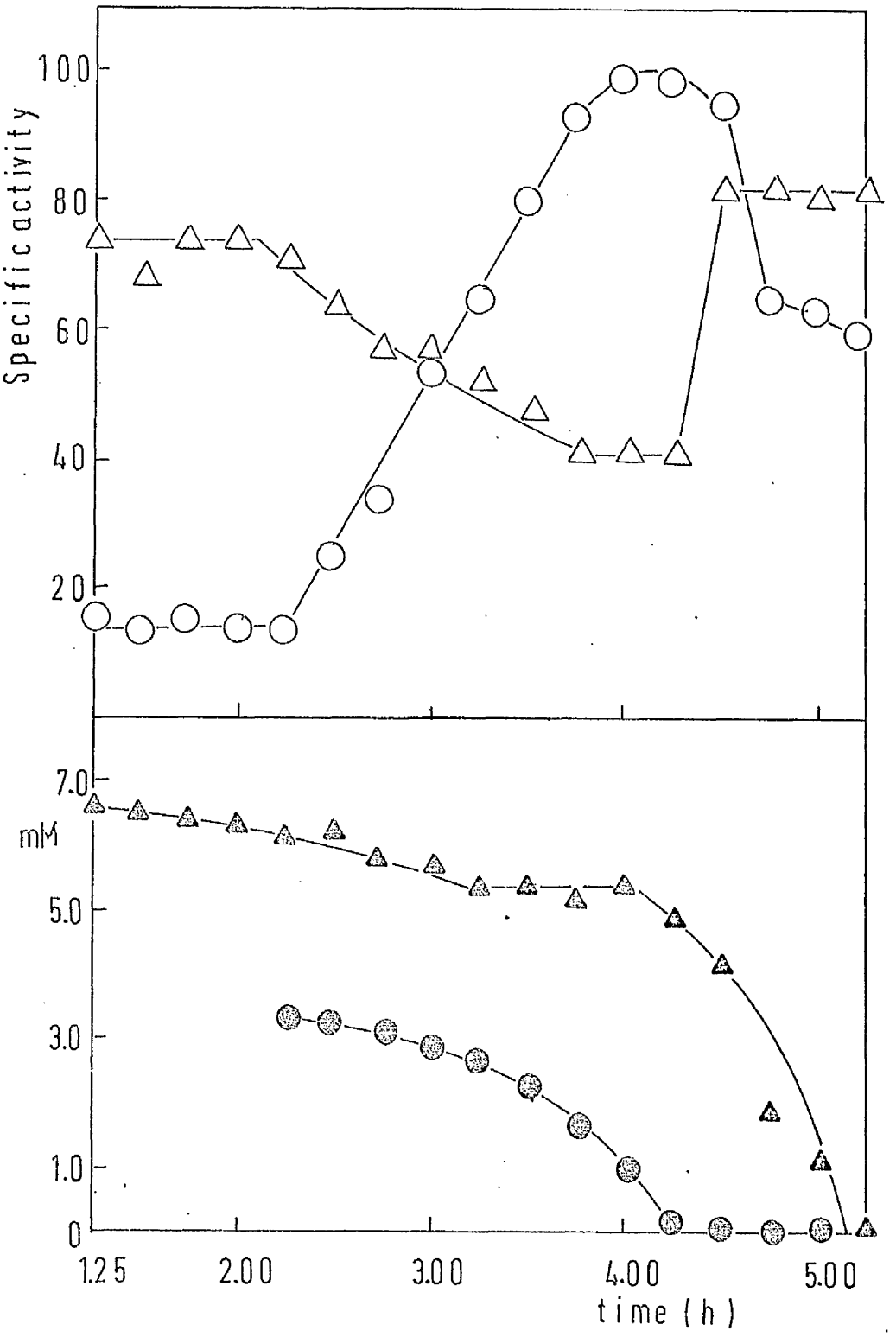

Specific activity
100
80
60
40
20
7.0
mM
5.0
3.0
1.0
0
1.25
2.00
3.00
4.00
5.00
time (h)

Figure R.17b

GLUCOSE CHALLENGE ON *E.coli* 15224

GROWING ON FRUCTOSE SALTS

Cells of *E.coli* trained to fructose in the presence of cyclic AMP (5 mM) were harvested, resuspended in chilled buffer, inoculated into fructose salts medium and subsequently challenged with glucose.

Growth, substrate concentration, glucose PTS and fructose PTS activity were determined at intervals.

The experiments reported in Figure R.17a and R.17b were performed simultaneously.

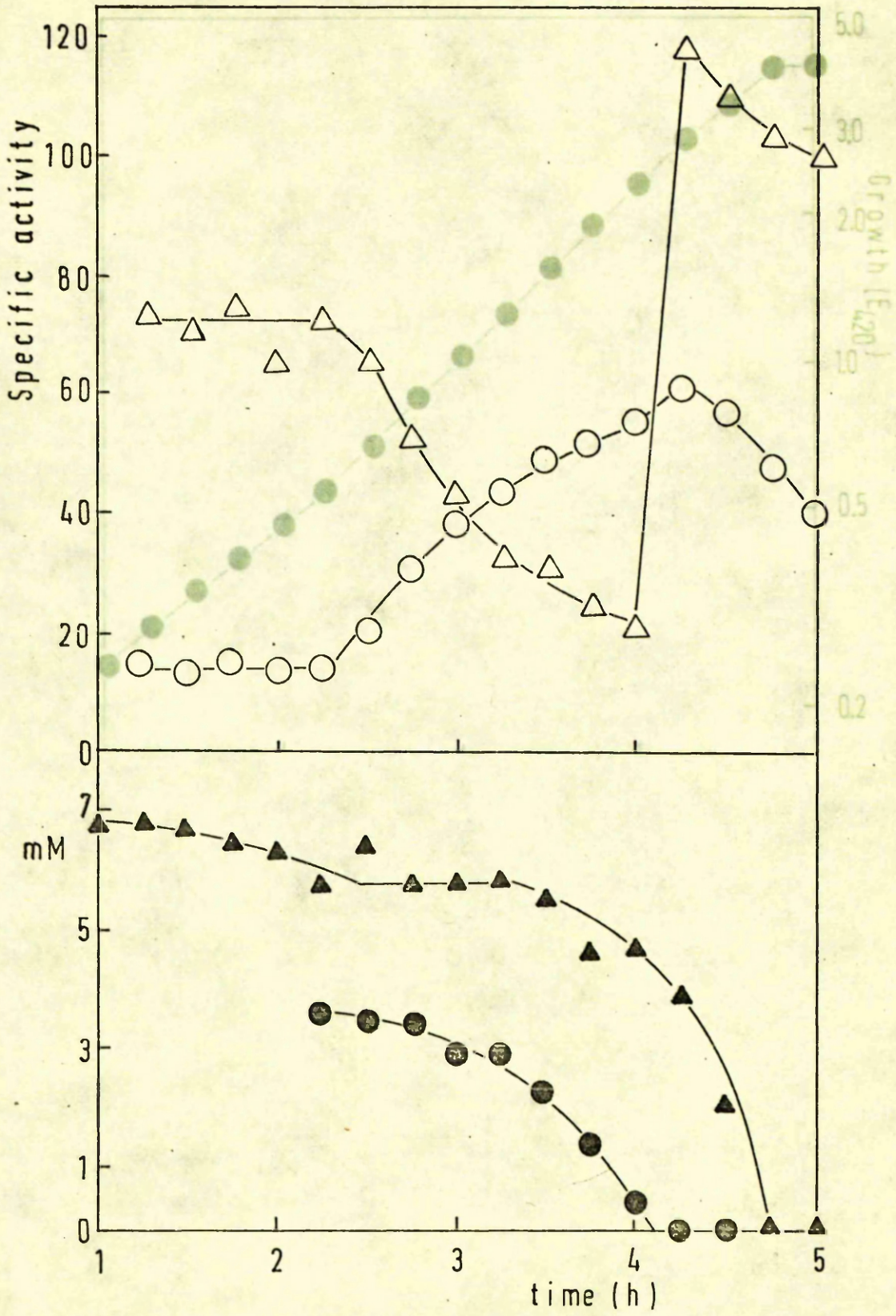
○ glucose PTS activity

△ fructose PTS activity

 glucose concentration

 fructose concentration

● growth (as turbidity)


Specific activity
120
100
80
60
40
20
0
5.0
3.0
2.0
1.0
0.5
0.2
Growth (E_{420})
mM
7
5
3
1
0
1
2
3
4
5
time (h)

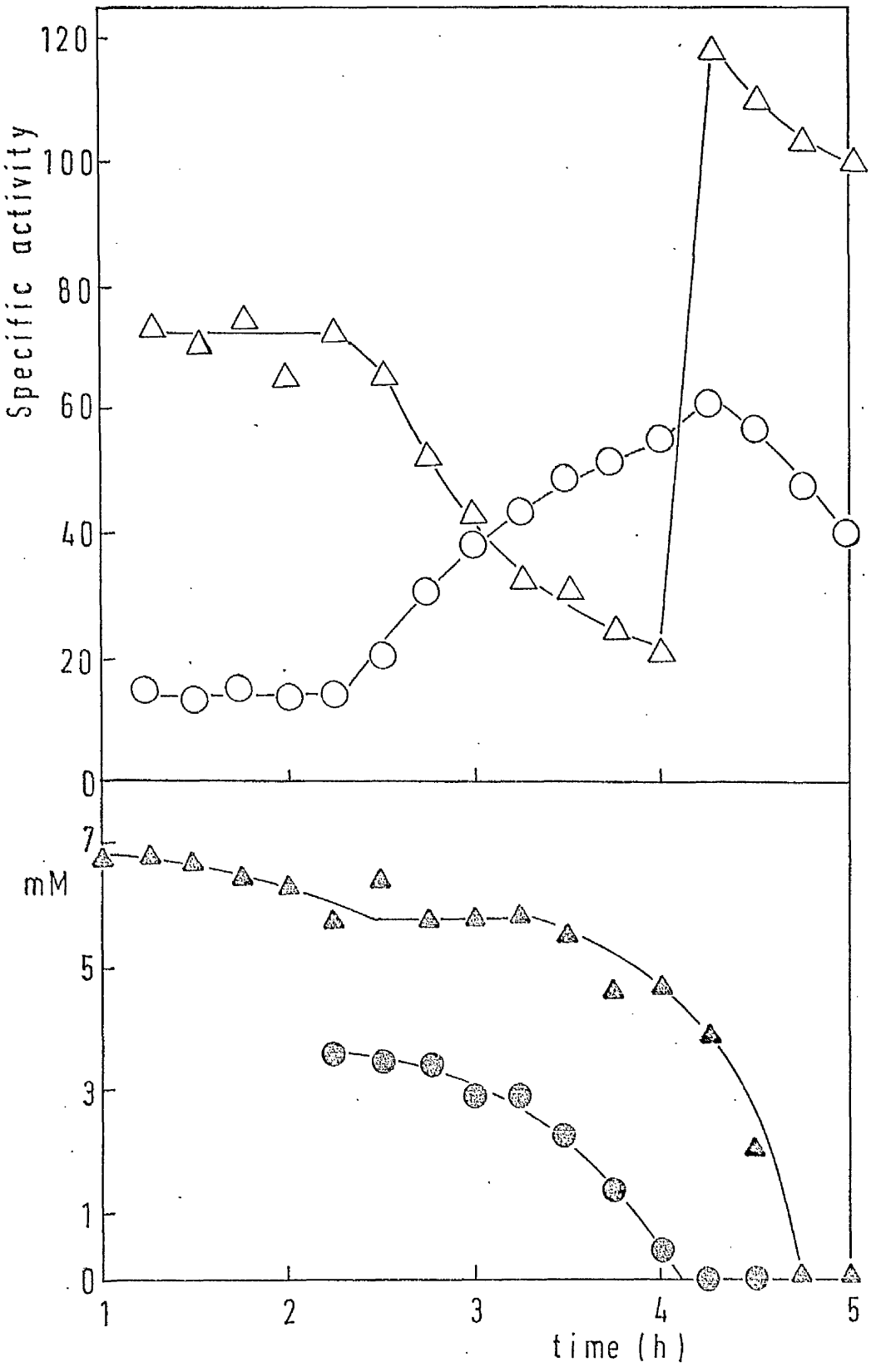

Specific activity
120
100
80
60
40
20
0
mM
7
5
3
1
0
1
2
3
4
5
time (h)

Figure R.18

RESPONSE OF FRUCTOSE PTS OF E.coli 15224

TO CHANGES IN GROWTH MEDIUM

This Figure contains data already reported, showing response of the fructose PTS to changes in growth medium and simplifies correlation of the results.

○ glucose cells into fructose* (Figure R.4)

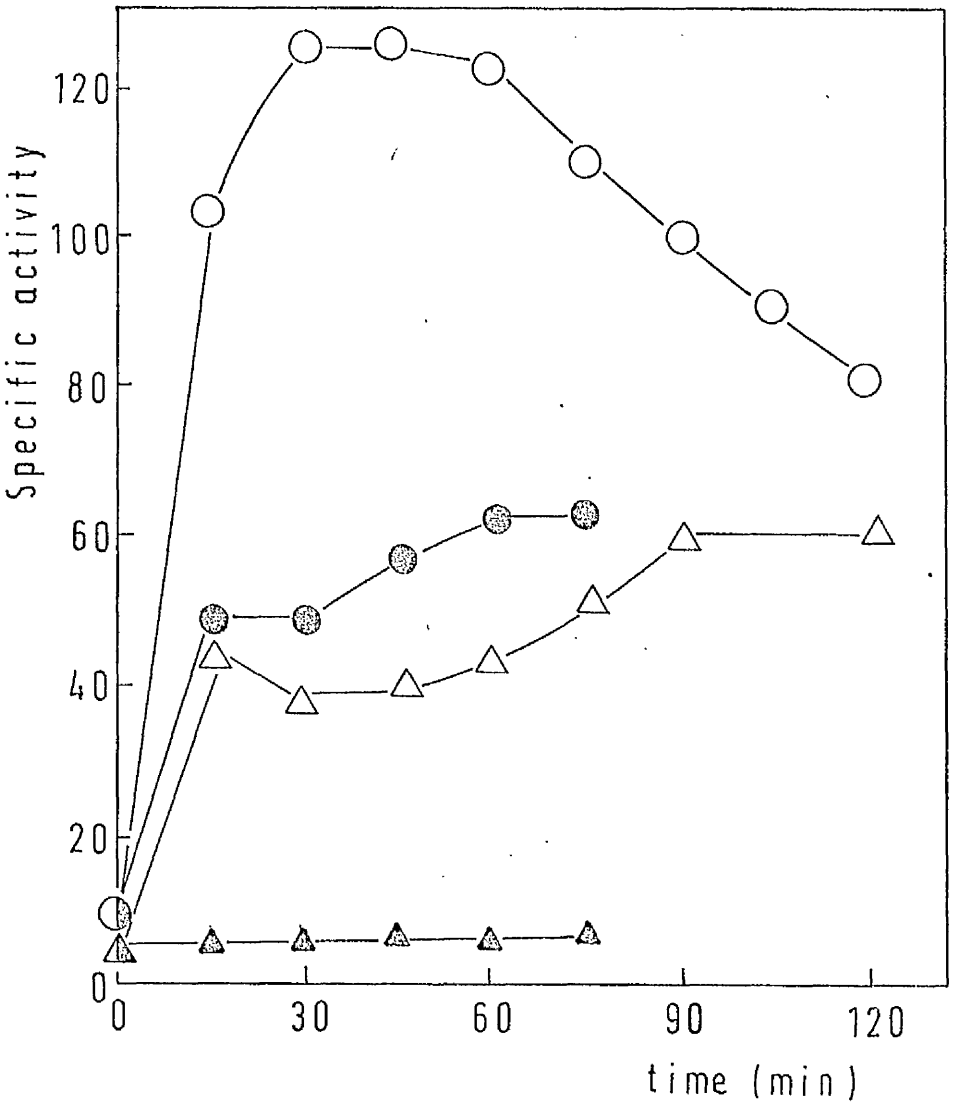
△ glycerol cells into fructose** (Figure R.15)

◎ glucose-cyclic AMP** cells into fructose (Figure R.16)

▲ glucose cells into fructose + CAP (Figure R.9)

* severely catabolite repressed

** not severely catabolite repressed


Specific activity
120
100
80
60
40
20
0
0
30
60
90
120
time (min)

3. Investigations into the Control of Fructose PTS Activity by Glucose

3.1. Chemostat studies

3.1.1. Fructose chemostat

Figure R.19 shows the effect of growing E.coli 15224 in a fructose chemostat. The initial glucose and fructose PTS activities were similar to those obtained in a fructose batch culture. A decrease in substrate utilisation caused by a decrease in μ resulted in a 4-fold increase in fructose PTS activity and an increase in glucose PTS activity. A further decrease in μ stimulated only glucose PTS activity. Return to the original μ and rate of utilisation did not restore the PTS activities to their initial values; for the same rate of fructose utilisation the final fructose PTS activity was 3 times greater than the initial fructose PTS activity showing that the rate of fructose utilisation does not depend on fructose PTS activity in this situation.

3.1.2. Glucose chemostat

Figure R.20 shows the effect of growing cells of E.coli 15224 in a glucose chemostat. The initial glucose and fructose PTS activities were similar to those obtained in a glucose batch culture. In contrast to the fructose chemostat, the initial decrease in μ caused a 4-fold decrease in glucose PTS activity which was unaffected by the subsequent decrease in μ. Return to the original μ and rate of utilisation only restored the glucose PTS activity to 30% of its initial value again showing that rate of utilisation does not reflect PTS activity in this situation. Fructose PTS activity remained low and unchanged throughout the experiment.

3.1.3. A glucose-fructose chemostat

Figure R.21 shows the effect of growing E.coli 15224 in a

Figure R.19

GROWTH OF E.coli 15224 IN A FRUCTOSE CHEMOSTAT

The growth flask was prefilled with growth medium (1.2 mM fructose and salts) and aseptically inoculated with fructose trained cells of E.coli 15224. Growth was followed. Shortly before the calculated stationary phase turbidity was achieved the dilution pump was started and the required μ obtained.

Growth, glucose PTS and fructose PTS activities, fructose utilisation and μ were measured at intervals.

○ glucose PTS activity

△ fructose PTS activity

□ μ (h^{-1}), specific growth rate

▲ fructose utilisation (q : μ moles/h/mg dry wt.)

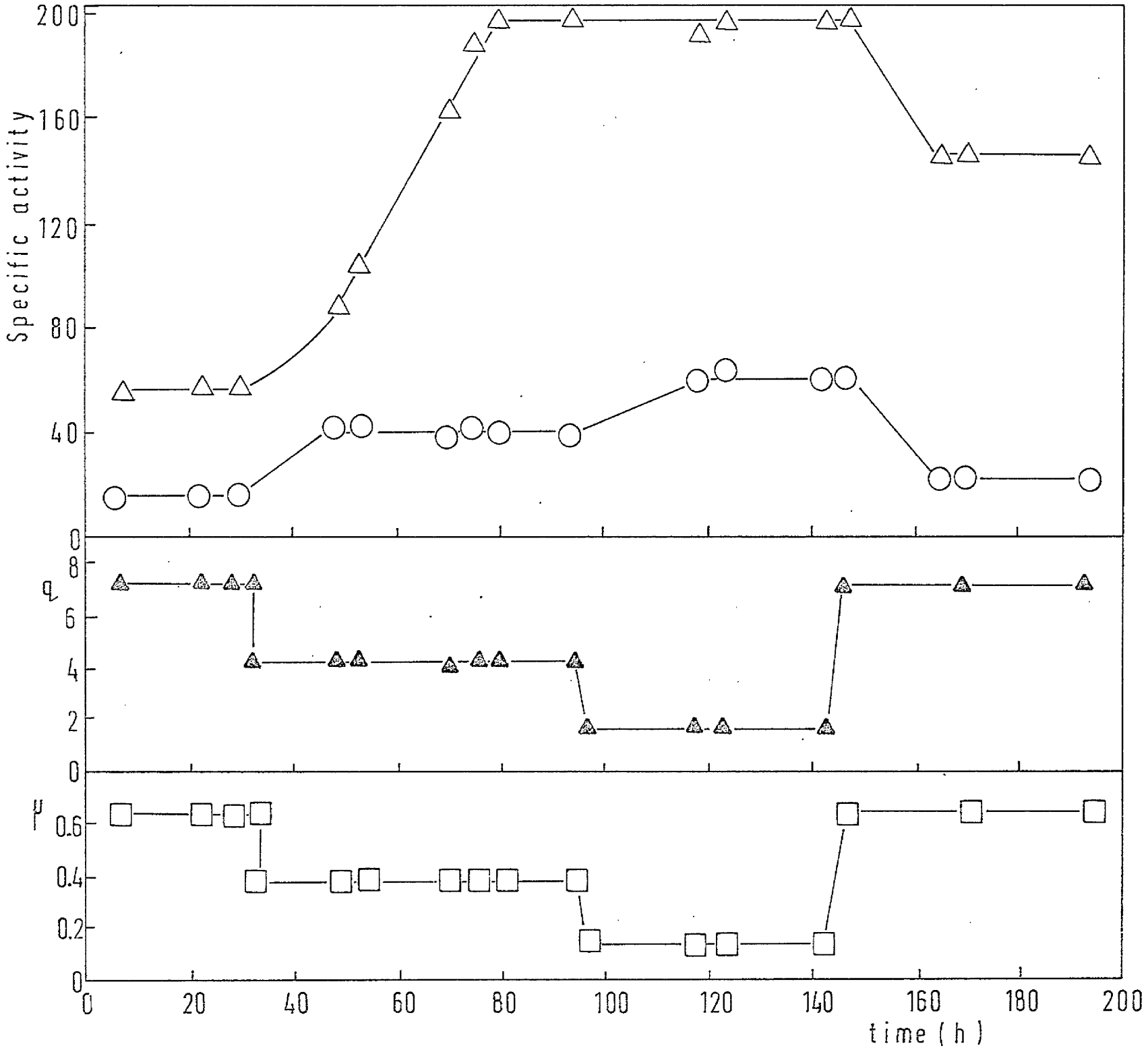

Specific activity
200
160
120
80
40
0
q
8
6
4
2
0
μ
0.6
0.4
0.2
0
0
20
40
60
80
100
120
140
160
180
200
time (h)

Figure R.20

GROWTH OF E.coli 15224 IN A GLUCOSE CHEMOSTAT

The growth flask was prefilled with the growth medium (1.3 mM glucose and salts) and aseptically inoculated with glucose trained cells of E.coli 15224. Growth was followed. Shortly before the calculated stationary phase value was achieved the dilution pump was started and the required μ obtained.

Growth, glucose PTS and fructose PTS activities, glucose utilisation and μ were determined at intervals.

○ glucose PTS activity

△ fructose PTS activity

□ μ (h^{-1}), specific growth rate

◍ glucose utilisation (q : μ moles/h/mg dry wt.)

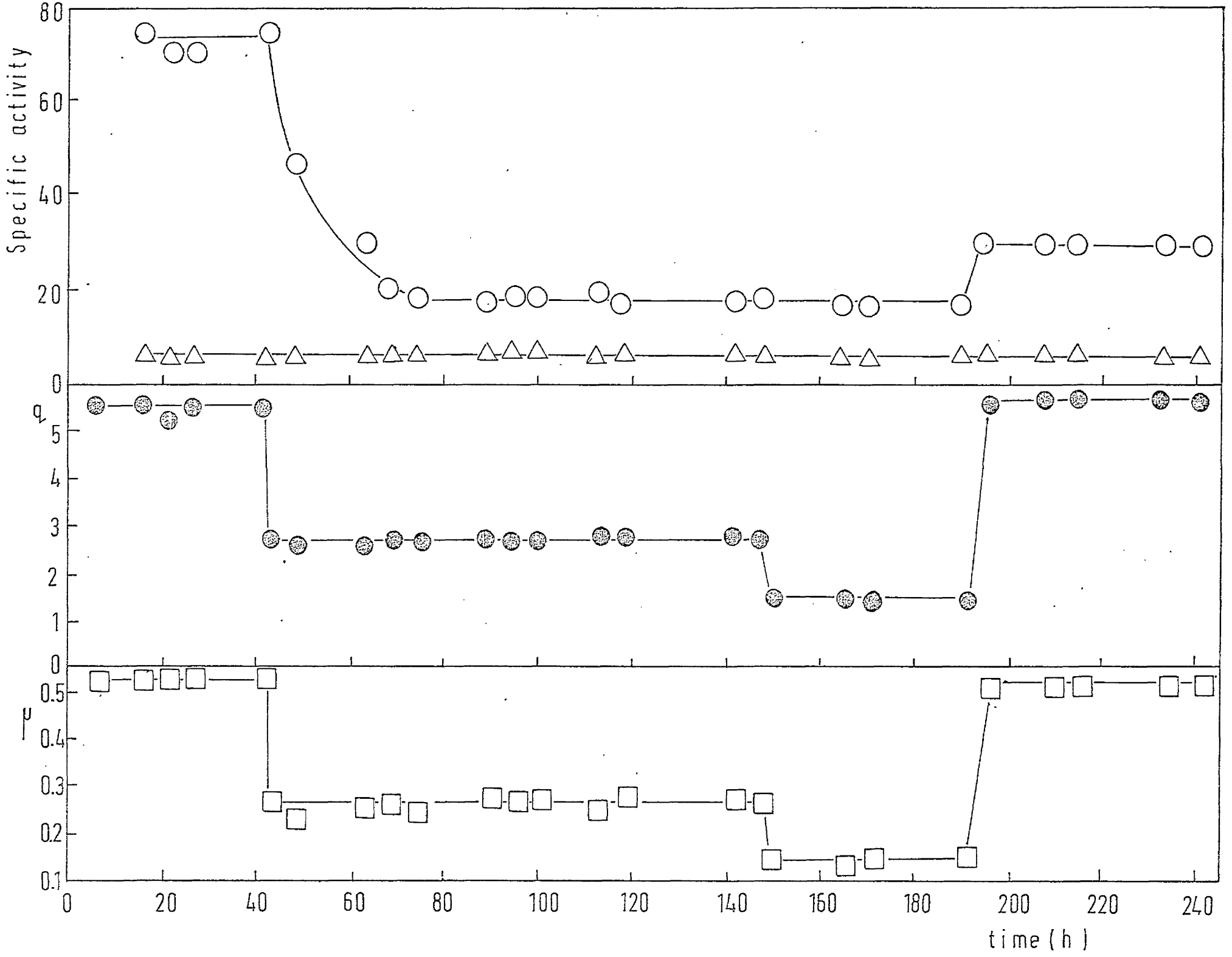

Specific activity
80
60
40
20
0
q
5
4
3
2
1
0
r
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
120
140
160
180
200
220
240
time (h)

Figure R.21

GROWTH OF *E.coli* 15224 IN A GLUCOSE-FRUCTOSE CHEMOSTAT

The growth flask was prefilled with the growth medium (0.6 mM fructose, 0.6 mM glucose and salts) and aseptically inoculated with fructose trained cells of *E.coli* 15224. Growth was followed. Shortly before the calculated stationary phase value was achieved the dilution pump was started and the required μ obtained.

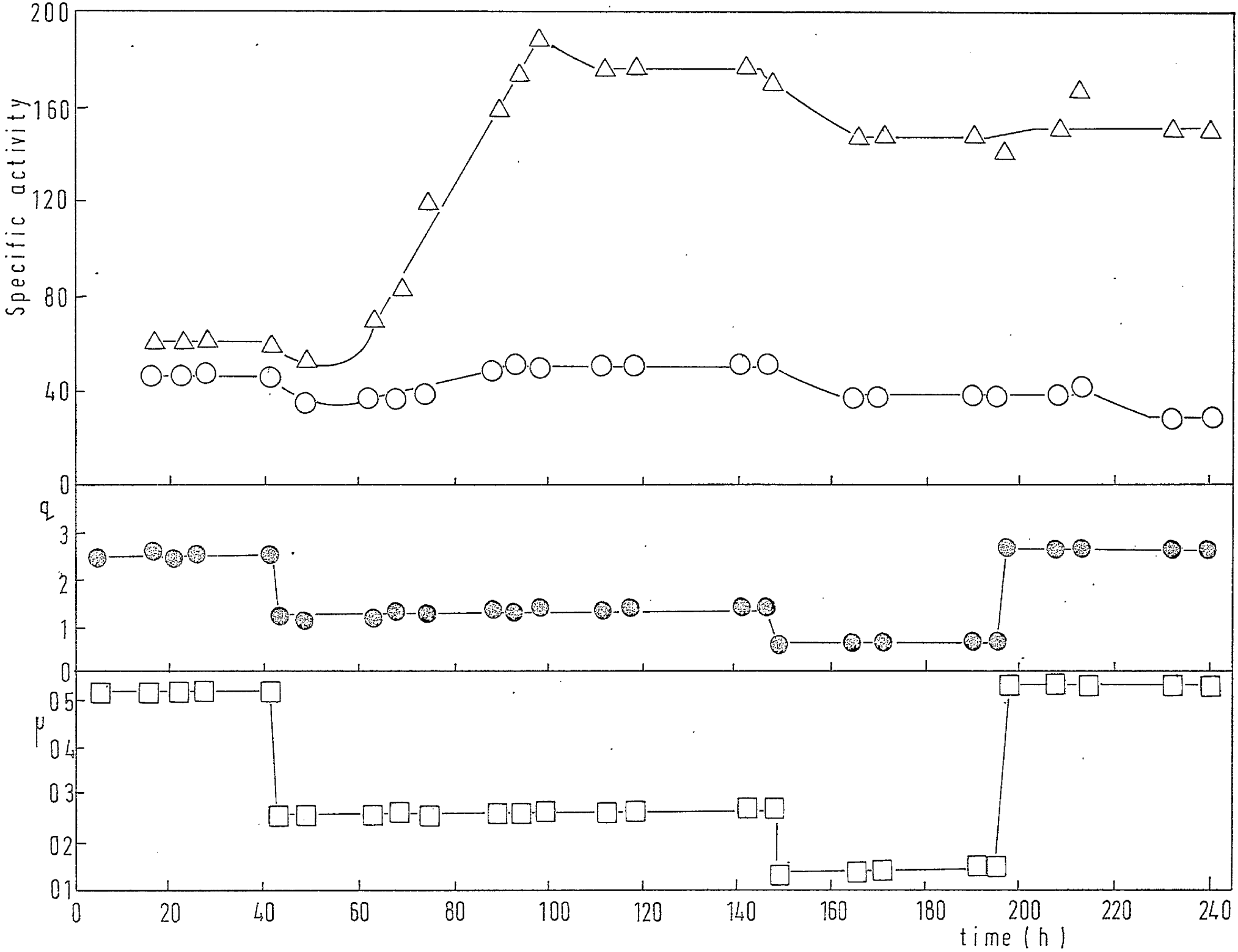
Growth, glucose PTS and fructose PTS activities, glucose and fructose utilisation and μ were determined at intervals.

○ glucose PTS activity

△ fructose PTS activity

□ μ (h^{-1}), specific growth rate

⊗ glucose and fructose utilisation
(q : μ moles/h/mg dry wt.)


Specific activity
200
160
120
80
40
0
q
3
2
1
0
μ
0.5
0.4
0.3
0.2
0.1
0
20
40
60
80
100
120
140
160
180
200
220
240
time (h)

glucose-fructose chemostat. The initial decrease in μ resulted in a 4-fold stimulation of the fructose PTS activity similar to the fructose chemostat (Figure R.19) but, in contrast to the glucose chemostat, glucose PTS activity was substantially unaffected. A subsequent decrease in μ lowered both glucose and fructose PTS activities but not in proportion to the decrease in rates of utilisation. Return to the original μ did not restore the PTS activities to their original values; for the same rates of utilisation glucose PTS had only 50% of the original activity while fructose PTS had 3 times its initial activity - a distinct lack of dependence of PTS activities on the rates of utilisation (or vice-versa).

No substrate was detectable in any of these chemostats as determined by the methods described.

3.2. Response of glucose PTS to glucose when fructose PTS is low

Figure R.22 shows the effect of inoculating glycerol trained cells of E.coli 15224 into glucose. In contrast to the results obtained for inoculation of fructose trained cells into glucose (Figure R.3) the glucose PTS activity lagged before increasing slowly to achieve a much lower final specific activity. Despite the lower glucose PTS activities obtained, the cells grew with the expected specific growth rate (0.93 h^{-1}).

3.3. Glucose inhibition of fructose PTS activity

Figure R.23a shows the effect of glucose challenge on E.coli 15224 actively growing on fructose. Figure R.23b shows glucose inhibition of fructose PTS activity in decryptified cells originally removed from the culture at intervals after glucose challenge. The results show that, while glucose appeared to completely inhibit fructose utilisation 30 min after challenge (Figure R.23a), glucose did not totally inhibit fructose utilisation in decryptified cells derived from

Figure R.22

CHANGE IN GLUCOSE PTS OF *E.coli* 15224

ON TRANSFER FROM GLYCEROL TO GLUCOSE SALTS MEDIUM

Glycerol trained cells of *E.coli* 15224 were harvested, resuspended in chilled buffer and inoculated into glucose salts medium. Growth, fructose PTS and glucose PTS activities were measured at intervals.

○ glucose PTS activity

△ fructose PTS activity

◍ growth as turbidity

---- equivalent glucose PTS activity for fructose trained cells inoculated into glucose salts medium.

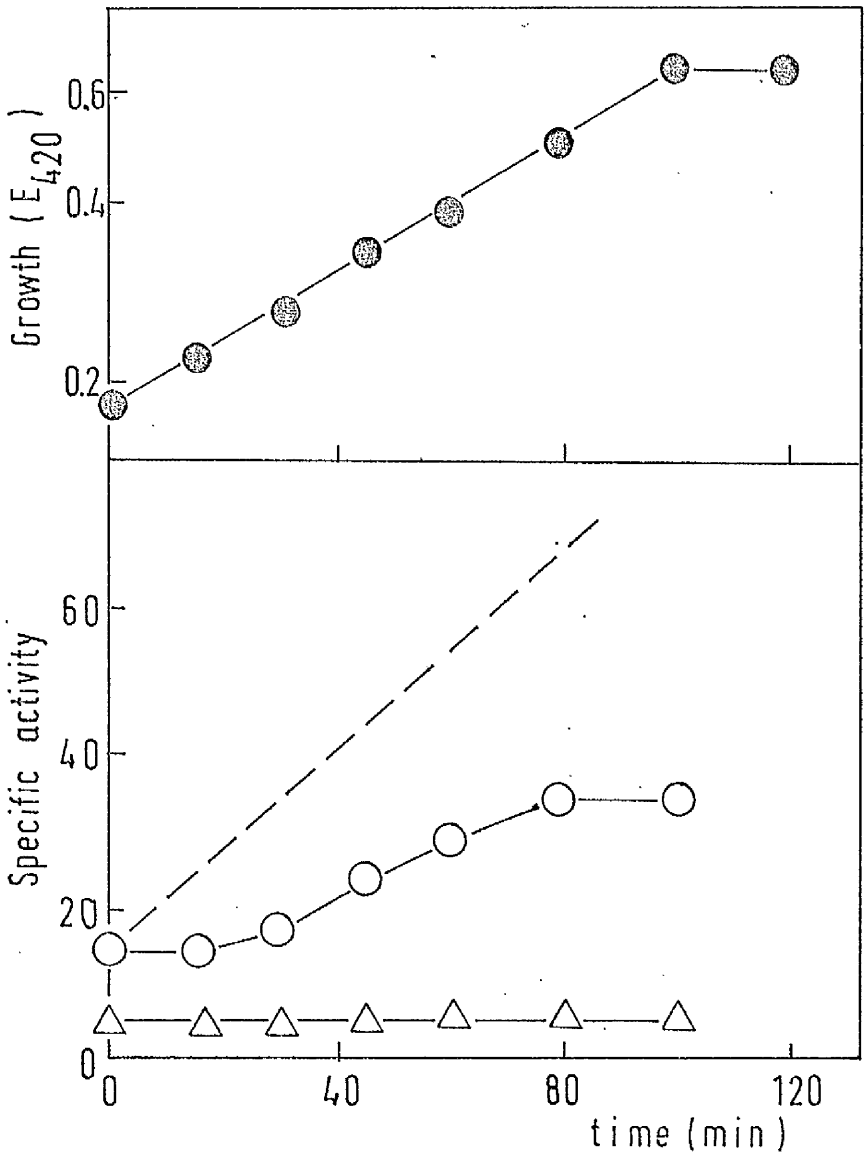

Growth (E_{420})
0.6
0.4
0.2
Specific activity
60
40
20
0
0
40
80
120
time (min)

Figure R.23

GLUCOSE INHIBITION OF FRUCTOSE PTS ACTIVITY

IN DECRYPTIFIED CELLS OF E.coli 15224

Fructose trained cells of E.coli 15224 were harvested, resuspended in chilled buffer, inoculated into fructose salts medium, and subsequently challenged with glucose.

Growth and substrate concentrations were determined at intervals. At the times marked (↓) samples were removed for measurement of glucose PTS activities (in the presence of glucose and glucose-fructose) and also fructose PTS activities (in the presence of fructose and glucose-fructose).

Figure R.23a

▩ growth (turbidity)

◍ glucose concentration

△ fructose concentration

Figure R. 23a

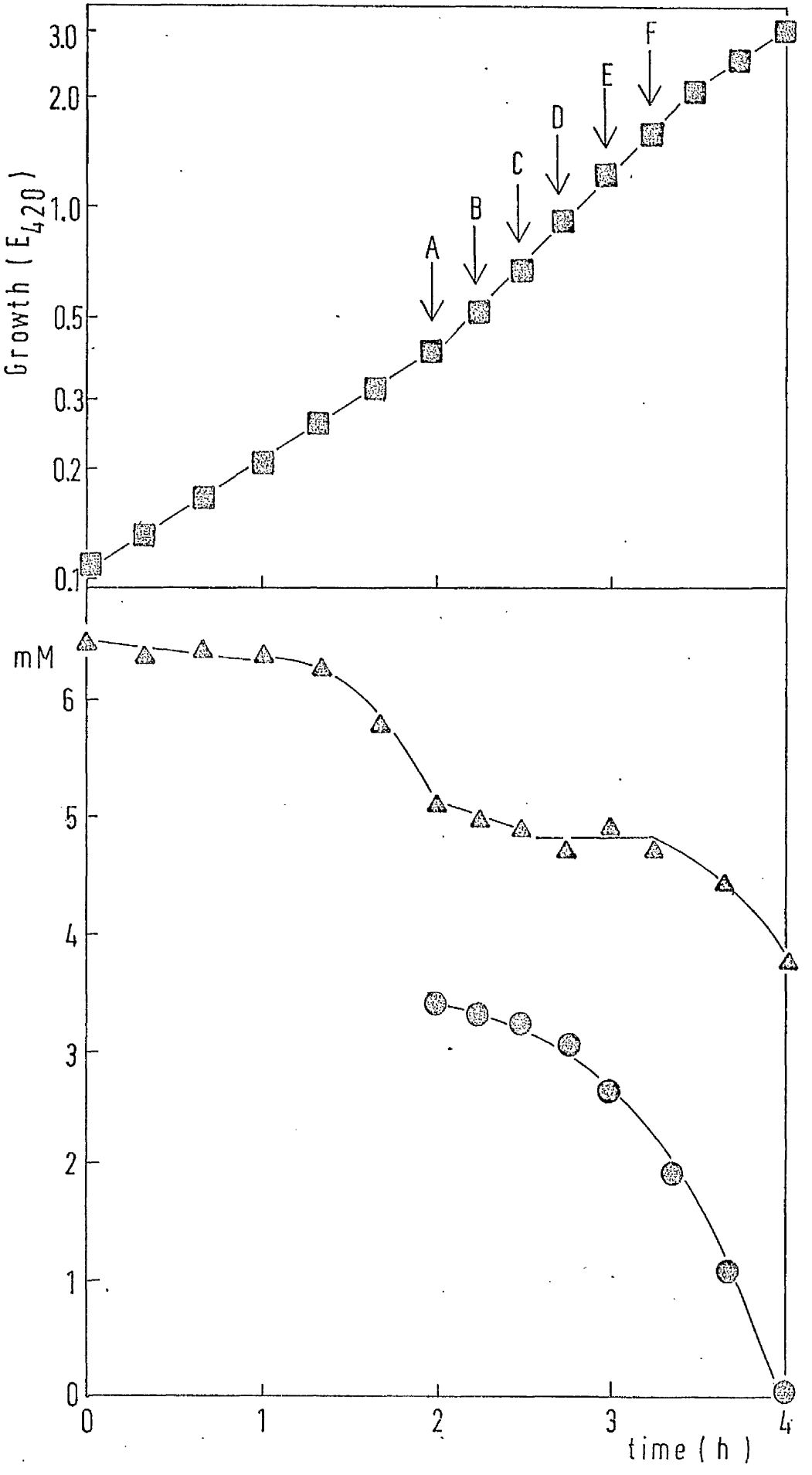


Figure R.23

GLUCOSE INHIBITION OF FRUCTOSE PTS ACTIVITY

IN DECRYPTIFIED CELLS OF E.coli 15224

Figure R.23b

PTS activities were determined on cells removed from the culture at points A – F.

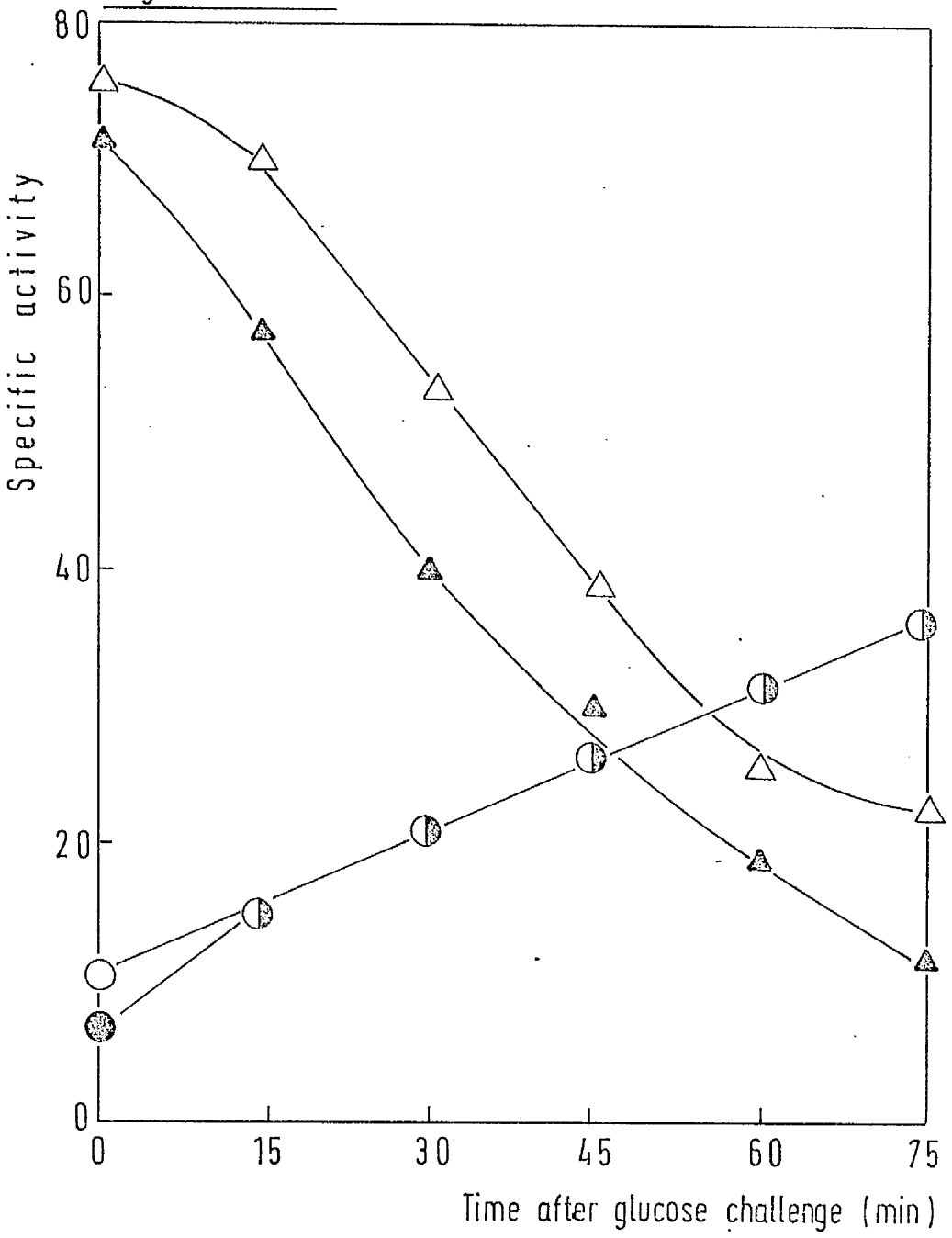
○ glucose PTS activity (determined on glucose only)

△ fructose PTS activity (determined on fructose only)

⊛ glucose PTS activity (determined on glucose–fructose)

⧋ fructose PTS activity (determined on glucose–fructose)

Figure R.23b


80
60
40
20
0
Specific activity
0
15
30
45
60
75
Time after glucose challenge (min)

the challenge situation. However, while glucose inhibited fructose PTS activity to an increasing extent in decryptified cells, only initially did fructose inhibit glucose PTS activity.

Figure R.24 shows the inhibition of glucose and fructose PTS activities by some sugar-phosphates in decryptified cells of E.coli 15224.

3.4 Effects of PTS levels on rates of utilisation

Figure R.25 shows the rates of utilisation of glucose and fructose, singly and in combination, for cells of E.coli 15224 having different levels of glucose and fructose PTS activities. The cells were produced under the conditions described in the table and not derived from a challenge situation. The general trend of the table shows that rates of utilisation do not appear to depend on PTS activities. Glucose completely inhibited fructose utilisation only when fructose PTS activity was low; appreciable levels of fructose PTS resulted in co-utilisation.

Figure R.26b shows the rates of utilisation of glucose and fructose, singly and in combination, for cells of E.coli 15224 harvested at intervals after glucose challenge to a fructose growing culture. Figure R.26a shows the ability of glucose to inhibit fructose PTS activity in the challenged cells. When measured on glucose only, glucose PTS activity constantly increased after glucose challenge (Figure R.26a) but glucose utilisation did not follow a similar pattern (Figure R.26b). However, when measured on fructose only, fructose PTS activity and fructose utilisation decreased in a similar manner. When determined in the presence of both glucose and fructose, fructose PTS activity was never completely inhibited by glucose at any time sampled after challenge, but fructose utilisation was totally inhibited 45 min after challenge. In contrast, only initially did fructose inhibit glucose PTS activity and its inhibition of glucose utilisation decreased as time progressed.

Figure R.24

INHIBITION OF GLUCOSE AND FRUCTOSE PTS ACTIVITY

IN E.coli 15224 BY SOME SUGAR-PHOSPHATES

Cells of E.coli 15224 trained to either glucose or fructose were harvested in the exponential phase of growth and assayed for glucose PTS and fructose PTS activities, respectively. The assays were also performed in the presence of the sugar-phosphates (5mM) (7.5 μ mol/assay) detailed in the table, added to the assay 1 min before addition of substrate.

cells trained to:	assayed in the presence of:	specific activity:	% inhibition
glucose; glucose PTS activity determined	glucose	81	0
	glucose + G1P	63	22
	glucose + G6P	65	20
	glucose + F6P	68	16
	glucose + FDP	46	44
	glucose + 6PG	46	44
fructose; fructose PTS activity determined	fructose	71	0
	fructose + G1P	65	9
	fructose + G6P	65	9
	fructose + F6P	72	0
	fructose + FDP	22	69
	fructose + 6PG	35	51

G6P = glucose-6-phosphate; G1P = glucose-1-phosphate

F6P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate

6PG = 6-phosphogluconate

Figure R.25

EFFECT OF GLUCOSE AND FRUCTOSE PTS ACTIVITIES ON THE UTILISATION OF THEIR RESPECTIVE SUBSTRATES BY *E.coli* 15224

Cells of *E.coli* 15224 trained to the substrates described in the table were harvested in the exponential phase of growth and assayed, both for glucose PTS and fructose PTS activities and the ability to utilise glucose and fructose singly and in combination as described (methods 12). Cells trained to glucose-cyclic AMP were also harvested, inoculated into fructose salts, reharvested after 15 or 60 min growth (full details, Figure R.16) and assayed as described above.

			*Utilisation of:			Inhibition of Utilisation:	
cells trained to:	glucose PTS activity	fructose PTS activity	glucose alone	fructose alone	**glucose + fructose	glucose by fructose (%)	fructose by glucose (%)
glucose	62	4	6.5	1.2	6.5/0	0	100
fructose	9	62	5.8	5.3	2.3/5.3	57	0
glycerol	9	3	3.4	0.8	3.6/0	0	100
glucose-cyclic AMP.	129	12	6.0	2.5	6.1/0	0	100
glucose-cyclic AMP; inoculated into fructose and harvested at 15 min	101	49	6.4	5.3	5/2.4	30	22
glucose-cyclic AMP; inoculated into fructose and harvested at 60 min	61	62	5.7	6.4	4.2/3.4	25	47

*expressed as μ moles/h/mg dry wt. cellular material

**utilisation of glucose and fructose in the presence of both substrates

Figure R.26

COMPARISON OF SUGAR UTILISATION AND PTS ACTIVITIES FOR GLUCOSE CHALLENGED CELLS OF *E.coli* 15224

Fructose trained cells of *E.coli* 15224 were harvested, resuspended in chilled buffer, inoculated into fructose salts medium and subsequently challenged with glucose.

Growth and substrate concentrations were determined at intervals and are reported in Figure R.23a. At the times marked (↓) samples were removed and assayed for glucose PTS activities (in the presence of glucose and glucose-fructose), fructose PTS activities (in the presence of fructose and glucose-fructose) and also the ability to utilise glucose and fructose, singly and in combination as described (methods 12).

Figure R.26a

- ○ glucose PTS activity (determined on glucose only)
- △ fructose PTS activity (determined on fructose only)
- ◎ glucose PTS activity (determined on glucose + fructose)
- ◬ fructose PTS activity (determined on glucose + fructose)

Figure R.26b

(q : μ moles/h/mg dry wt.)

- ○ glucose utilisation (determined on glucose only)
- △ fructose utilisation (determined on fructose only)
- ◎ glucose utilisation (determined on glucose + fructose)
- ◬ fructose utilisation (determined on glucose + fructose)

Figure R. 26a

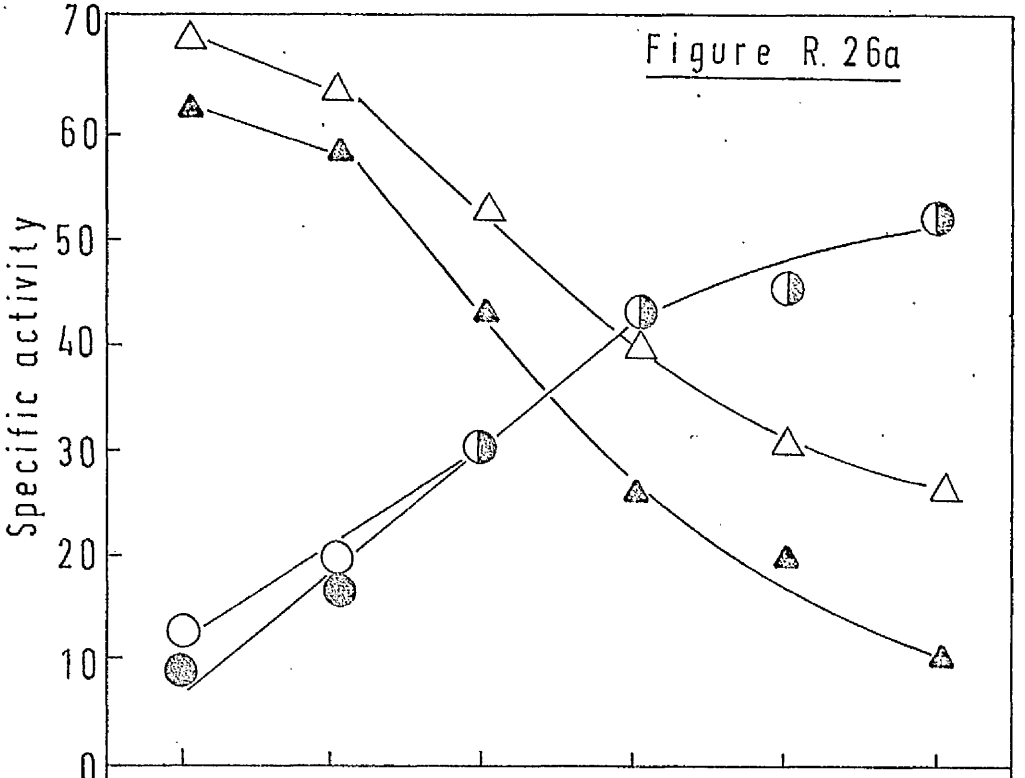
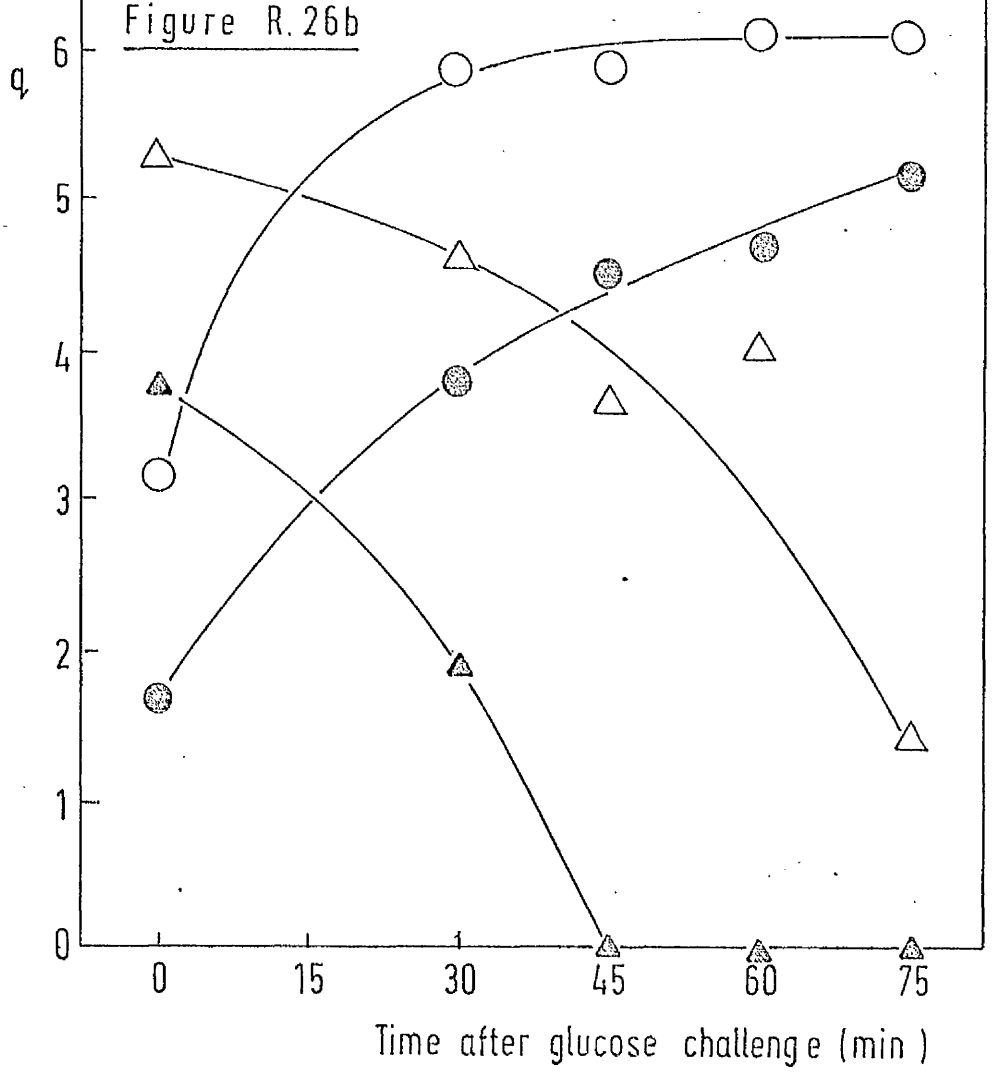


Figure R. 26b



3.5. Use of the radioactive PTS assay

The failure to observe a total inhibition by glucose on fructose PTS activity in decryptified cells might have resulted from removal of the PTS end-products (G6P and pyruvate by the enzymes of the spectrophotometric assay. Figure R.27 shows glucose and fructose PTS activities as determined by radioactive assay, for cells of E.coli 15224 harvested at intervals after glucose challenge on a fructose growing culture. The results do not differ significantly from those obtained by conventional spectrophotometric assay (Figure R.23b). However, if the radioactive assays were performed in the absence of phosphate (Figure R.28) then glucose did appear to totally inhibit fructose PTS activity.

3.6. Effect of the protein reagent NEM on PTS activity

Figure R.29 shows the effect of NEM on glucose and fructose PTS activities, for various phenotypes of E.coli 15224, in the presence or absence of glucose, fructose or glucose and fructose. Glucose sensitised the glucose PTS to attack by NEM in all phenotypes tested, since glucose PTS activities were substantially decreased, but had little effect on fructose PTS activity. Fructose sensitises the fructose PTS of cells trained to fructose and glucose in the presence of cyclic AMP, to NEM attack. Fructose also sensitises the glucose PTS of these cells but to a lesser extent than glucose. When both glucose and fructose were present, glucose appeared to protect the fructose PTS from attack by NEM in both glucose and glucose and cyclic AMP trained cells, since fructose PTS was not reduced to the value obtained for fructose alone. This protection was not observed for fructose trained cells. No protection of the glucose PTS by fructose was observed.

Figure R.30 shows the effect of NEM on glucose and fructose

Figure R.27

GLUCOSE INHIBITION OF FRUCTOSE PTS ACTIVITY

IN DECRYPTIFIED CELLS OF *E.coli* 15224

DETERMINED BY RADIOACTIVE ASSAY

Fructose trained cells of *E.coli* 15224 were harvested, resuspended in chilled buffer, inoculated into fructose salts medium and subsequently challenged with glucose.

Growth and substrate concentrations were measured at intervals and are reported in Figure R.23a. Samples were removed between 2 - 3 hours for determination of PTS activities using the radioactive assay (methods 11). Conventional spectrophotometric PTS assays were also performed as controls and are reported (Figure R.23b).

○ glucose PTS activity (determined on glucose only)

△ fructose PTS activity (determined on fructose only)

◎ glucose PTS activity (determined on glucose and fructose)

⚠ fructose PTS activity (determined on glucose and fructose)

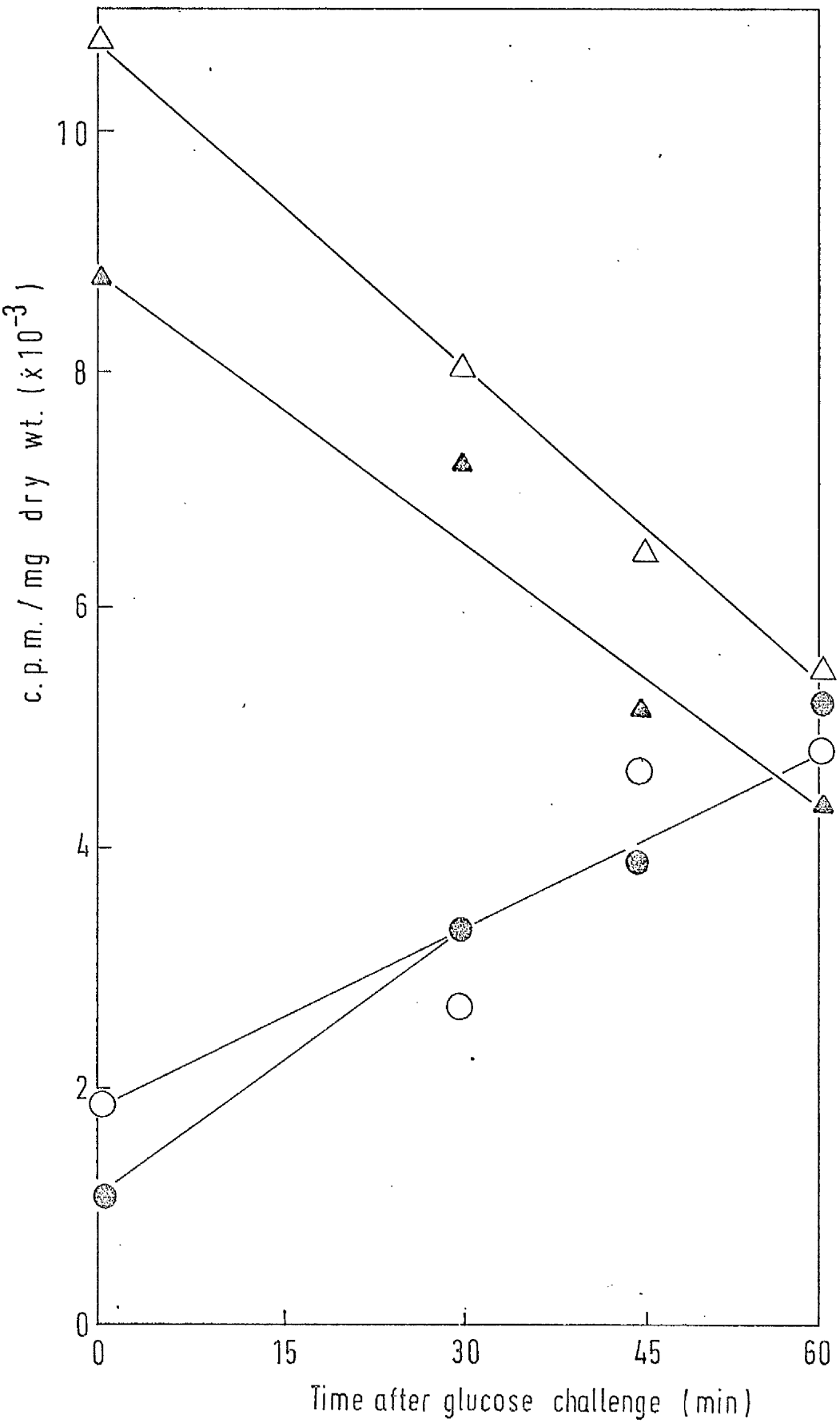

c.p.m. / mg dry wt. (x10^{-3})
0
2
4
6
8
10
0
15
30
45
60
Time after glucose challenge (min)

Figure R.28

GLUCOSE INHIBITION OF FRUCTOSE PTS ACTIVITY

IN DECRYPTIFIED CELLS OF E.coli 15224

IN THE ABSENCE OF PHOSPHATE

Fructose trained cells of E.coli 15224 were harvested, resuspended in chilled buffer, inoculated into fructose salts medium and subsequently challenged with glucose.

Growth and substrate concentrations were measured at intervals and are reported in Figure R.23a. Samples were removed between 2 - 3 hours for determination of glucose PTS and fructose PTS activities using the radioactive assay described (methods 11), except that assay buffer was substituted for by 0.2 M BES pH 7.2, 2 mM $MgSO_4$ buffer. Conventional spectrophotometric PTS assays were also performed as controls and are reported in Figure R.23b.

○ glucose PTS activity (determined on glucose only)

△ fructose PTS activity (determined on fructose only)

◍ glucose PTS activity (determined on glucose + fructose)

◬ fructose PTS activity (determined on glucose + fructose)

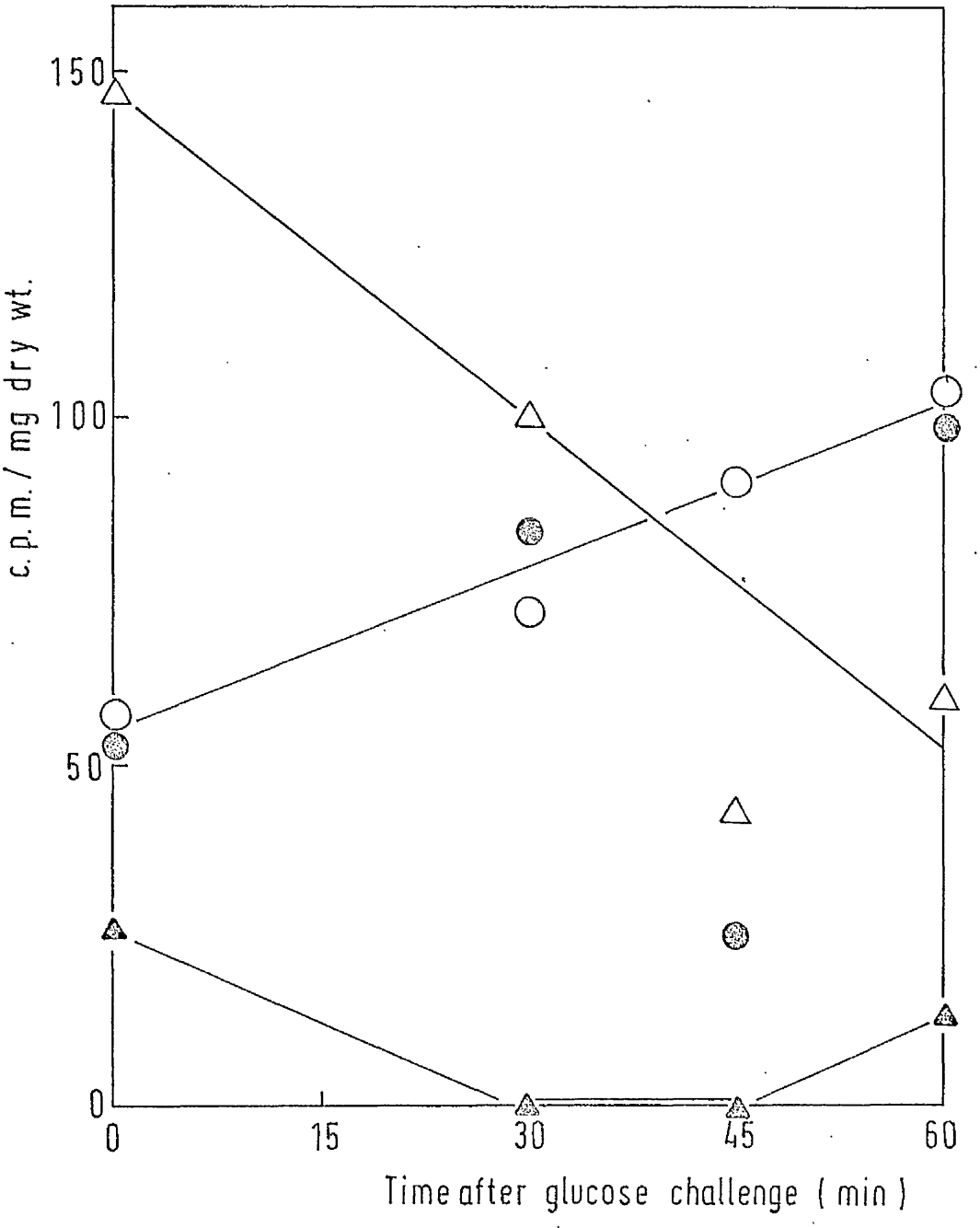

150
100
50
0
c.p.m. / mg dry wt.
0
15
30
45
60
Time after glucose challenge (min)

Figure R.29

EFFECT OF NEM ON THE GLUCOSE AND FRUCTOSE PTS ACTIVITIES OF E.coli 15224

Cells of E.coli 15224 trained to glucose and fructose were harvested in the exponential phase of growth. The effects of NEM on glucose and fructose PTS activities in the presence and absence of their respective substrates, as well as with glucose-fructose together were determined as described (methods 13). Cells trained to glucose-cyclic AMP were also harvested, resuspended in chilled buffer, inoculated into fructose salts medium and reharvested after 45 min. The effect of NEM was determined as above.

cell type: / treatment with:	glucose trained		fructose trained		trained to glucose & c AMP into fructose	
	*GPTS	+FPTS	GPTS	FPTS	GPTS	FPTS
----	71	5	13	60	63	49
NEM only or NEM & 2ME++ together	88	11	14	78	85	73
NEM & glucose	16	11	10	76	45	71
NEM & fructose	78	3	11	24	61	24
NEM, glucose & fructose	18	12	10	26	40	59

*GPTS : glucose PTS activity

+FPTS : fructose PTS activity

++2ME : 2-mercaptoethanol

Figure R.30

EFFECT OF NEM ON GLUCOSE AND FRUCTOSE PTS ACTIVITIES OF GLUCOSE CHALLENGED CELLS OF *E.coli* 15224

Cells of *E.coli* 15224 trained fructose were harvested, washed in chilled buffer and inoculated into fructose salts medium. When the cells were subsequently challenged with glucose, samples were taken at intervals for determination of the effect of NEM on glucose and fructose PTS activities in the presence and absence of their respective substrates, as well as with glucose-fructose together. Growth and substrate concentrations were measured at intervals throughout the experiment and are reported (Figure 23a).

	glucose PTS activity					fructose PTS activity				
min after glucose challenge: / treatment with:	0	30	45	60	76	0	30	45	60	75
——	10	30	49	55	64	75	56	45	30	25
NEM only or NEM & 2ME* together	15	40	53	61	73	81	63	49	40	36
NEM & glucose	11	10	10	10	11	75	56	47	40	37
NEM & fructose	10	14	24	34	43	26	16	14	12	10
NEM, glucose & fructose	10	19	9	10	10	26	25	22	22	21

*2ME : 2-mercaptoethanol

PTS activities measured at intervals after fructose growing cells of E.coli 15224 were challenged with glucose. As in the previous experiment (Figure R.29) glucose and fructose sensitised their own PTS's to attack by NEM. Fructose sensitises glucose PTS to NEM attack, though to a lesser extent than glucose. When both glucose and fructose were present, glucose initially (0 min) did not protect the fructose PTS but subsequent protection was observed. It can be seen, too, that with NEM and fructose alone the fructose PTS activities for the 30 - 75 min samples are essentially constant and that with NEM, fructose and glucose the activities are again essentially constant but higher. No protection of the glucose PTS by fructose was observed.

4. Supplementary Results

Figure R.31 shows the effect of glycerokinase activity on the rates of glycerol utilisation in *E.coli* 15224/*glp* R^c. The methods and results quoted in this figure are personal communications (I.S. Forrest, Ph.D. Thesis, Glasgow, 1974). The general trend of the table shows that the rate of glycerol utilisation does not appear to depend on glycerokinase activity.

Figure R.31

EFFECT OF GLYCEROKINASE ACTIVITY ON

GLYCEROL UTILISATION IN *E.coli* 15224/*glp* R^C

Cells of *E.coli* 15224/*glp* R^C were trained to different carbon sources to obtain a wide range of glycerokinase activities. Portions were harvested and assayed both for glycerokinase activity and the ability to utilise glycerol in the presence of chloramphenicol.

The methods and results quoted are personal communications (I.S. Forrest, Ph.D. Thesis, Glasgow, 1974).

glycerokinase activity U/mg dry wt.	glycerol utilisation μ moles/h/mg dry wt.
7.9	12.8
6.4	12.8
4.8	15.5
3.8	10.1
3.5	12.8
3	10.9
1.94	10.2
1.16	9.8
1.04	8.8
.485	7.5

DISCUSSION

1. General Considerations

It is appropriate at this point to justify certain terms used in the text. Tempest (1970) has rightly criticised terms such as "mid-log phase" and "stationary-phase cells" by saying that they engender a complete misunderstanding of the real situation. However the terms "exponential phase" and "stationary-phase" have been used throughout this thesis since it is felt that, not only do they serve as a convenient shorthand for the respective statements: "cells growing and dividing in an exponential fashion" and "cells which have exhausted some essential nutrient and are thus unable to further divide," but they are readily understood by the majority of microbiologists. Since the work of Lodge & Hinshelwood (1943) and Hinshelwood & Lodge (1944) the term "training" may be ambiguous unless clearly defined. For the work in this thesis the term training refers to growth of the organism on the required carbon source, for a maximum of 25 generations, in order that the enzymes required for transport and metabolism of the carbon source may be fully induced. This process is efficacious as Figures R.3 and R.4 show that the enzymes for the transport and metabolism of fructose and glucose are fully induced. Inoculation of fructose trained cells into fructose (Figure R.3) does not lead to an increase in fructose PTS activity; similarly, inoculation of glucose trained cells into glucose (Figure R.4) does not increase glucose PTS activity. In addition, growth on the respective carbon sources is immediate.

2. Response of the PTS Activities to Changes in Media

It is the intention in this thesis to investigate whether or not the sequential utilisation of glucose and fructose is reflected in their PTS activities and in the control of these activities by factors such as repression and inhibition. Accordingly it is essential to determine if the glucose and fructose PTS's are constitutive, or respond to the presence of their substrates, or both.

In Figure R.3, when fructose trained cells are inoculated into glucose, growth of the cells is immediate without lag even though glucose PTS activity is only 15 mU/mg dry wt. As growth proceeds, glucose PTS activity increases and fructose PTS synthesis is repressed. Fructose trained cells inoculated into fructose (Figure R.3) maintain constant the original PTS activities. Also, in Figure R.3, the same specific growth rate (0.93 h^{-1}) is maintained on glucose for the range of glucose PTS activities 15 - 85 mU/mg dry wt. Since fructose trained cells possess a low activity of glucose PTS which permits immediate growth on glucose, the glucose PTS can be said to be constitutive, but further inducible by glucose, when defined in these terms. Kornberg & Reeves (1972b) observed essentially similar results when strains B11 and K2 were transferred from sodium gluconate to glucose. Figure R.4 shows that inoculation of glucose-trained cells into fructose permits immediate growth on fructose even with the initially low level of fructose PTS activity and so the fructose PTS may also be said to be constitutive when defined in these terms. The fructose PTS activity increases rapidly (Figure R.4) and overshoots the final observed value. This effect is comparable to a similar result observed by Gorini & Maas (1957) for ornithine transcarbamylase; the activity of this enzyme overshoots the final observed value when cells of arginine grown *E.coli* W are inoculated into minimal medium + lactate. The same

specific growth rate (0.73 h^{-1}) is maintained over the range of fructose PTS activities measured (Figure R.4). It must be concluded from this, and the similar effect observed for the glucose PTS, that substrate is utilised more efficiently at low PTS activities than at high though the significance of this is not clear at this time. The rate of fructose PTS synthesis (Figure R.4) is much greater than that of the glucose PTS (Figure R.3) corresponding to the results obtained by Kornberg & Reeves (1972b) for E.coli B11. Gratuitous induction of the glucose PTS by fructose was not observed in Figure R.4 though it was (Kornberg & Reeves, 1972b) for E.coli K2 and F⁻0144. The fructose PTS overshoot was not observed by Kornberg & Reeves (1972b) but this will be discussed later.

It was concluded from these investigations that, while glucose and fructose PTS's are constitutive (as defined above), both respond to the presence of their respective substrates.

It is necessary to determine at this point if, and under what conditions, glucose will dominate fructose metabolism. Figure R.5 shows the effect of glucose challenge on a fructose growing culture. At the point of challenge fructose PTS synthesis is repressed, glucose PTS synthesis is induced and the growth rate immediately stimulated. This stimulation can probably be explained: growth on fructose results in the formation of fructose-1-phosphate by the fructose PTS (Hanson & Anderson, 1966; Kelker et al., 1970; Sapico et al., 1968; Fraenkel, 1968; Hanson & Anderson, 1968) which is converted to fructose-1,6-diphosphate by an inducible kinase (Fraenkel, 1968). Fructose is thus a gluconeogenic growth substrate and it would be expected that the formation of, for example, glucosamine moieties of cell wall components would require the formation of fructose-6-phosphate from the diphosphate by fructose-1,6-diphosphatase (FDP-ase). However, FDP-ase negative mutants of E.coli

grow well on fructose but this has been resolved (Ferenci & Kornberg, 1971; Kornberg, 1972) since the fructose PTS gives both fructose-6-phosphate and fructose-1-phosphate at high fructose (> 4 mM) levels. Consequently the addition of glucose has a sparing effect on the need for the rate limiting FDP-ase step.

The addition of glucose (Figure R.5) completely inhibits fructose utilisation after 15 - 30 min; the effect is inhibition because fructose PTS activity is still high (55 mU/mg dry wt.) when fructose utilisation ceases. This inhibition persists until glucose is nearly exhausted. A similar inhibition of fructose utilisation by ∝-methyl glucoside and 3-deoxy-3-fluoroglucose has been observed (Kornberg, 1973a) in E.coli cells which have high glucose and fructose PTS activities. The glucose PTS has been shown to be directly involved in the inhibition of uptake of non-PTS-sugars such as galactose (Asensio et al., 1963; Tanaka et al., 1967) since EI mutants of the PTS (Tanaka et al., 1967) are unable to phosphorylate glucose and glucose cannot inhibit the uptake of other non-PTS-sugars. Kornberg (1972) also concludes that synthesis of the glucose PTS is required for glucose to inhibit fructose uptake by E.coli. The results in Figure R.5 differ from those reported by Mateles et al. (1967) where glucose challenge on fructose growing E.coli did not affect the rate of fructose utilisation. Bag (1974) reports that, when Vibrio cholerae is inoculated into glucose and fructose, glucose totally inhibits fructose utilisation until glucose is nearly exhausted. However, growth of V.cholerae on glucose and fructose is diauxic (Bag, 1974) and so the results are not comparable to those obtained in Figure R.5. Figure R.5 shows that, at or near glucose exhaustion, the fructose PTS increases rapidly and overshoots the final observed value. In the absence of glucose, the glucose PTS is no longer induced and its activity declines.

Figure R.6 shows that addition of fructose to a glucose growing culture results in a slight increase in fructose PTS activity and a slight fall in glucose PTS activity which recovers. Silver & Mateles (1969) suggest that the degree of catabolite repression in E.coli growing on a lactose-glucose mixture is insufficient to prevent induction of the lac operon and therefore glucose must inhibit the entry of lactose into the cell. Saier & Roseman (1970, 1972) have shown directly that ∝-methyl glucoside depresses internal levels of melibiitol, an inducer of the melibiose operon and that failure to induce cannot be attributed to lack of cyclic AMP. If this is so for E.coli 15224, then in the experiment previously discussed (Figure R.6) glucose must inhibit the entry of fructose into the cell, although the slight degree of induction observed suggests that a limited amount is not excluded. Again, at or near glucose exhaustion (Figure R.6), fructose utilisation commences and a rapid induction of the fructose PTS activity is seen which overshoots the final activity.

The fructose PTS overshoot is significant in that it may be responsible for the absence of diauxie when E.coli is growing on glucose and fructose (Monod, 1942). Such a rapid appearance of the fructose PTS near glucose exhaustion would probably enable the cell to overcome any remaining glucose inhibition so that the two substrates are co-utilised for a short period before glucose is finally exhausted.

From these experiments it was concluded that fructose and glucose are used sequentially because glucose both represses synthesis of the fructose PTS and inhibits fructose entry into the cell. The presence of glucose results in induction of glucose PTS synthesis and this step is required before glucose can inhibit fructose utilisation. Growth of the cells in the presence of glucose and fructose leads, at glucose exhaustion, to a rapid increase in fructose PTS activity which overshoots

the final value. At glucose exhaustion the glucose PTS is no longer induced and the activity is diluted out by growth. Therefore, sequential utilisation of glucose and fructose involves the operation of the controls of induction, repression and inhibition on the PTS's as described above in a somewhat similar fashion to the glucose lactose situation (McGinnis & Paigen, 1969). However, the absence of diauxie for glucose and fructose requires explanation and so the fructose PTS overshoot must be investigated further. As discussed in the introduction, the site at which glucose acts to inhibit fructose utilisation is not known for certain and this must also be examined in more detail.

3. Investigation of the Fructose PTS Overshoot

3.1. Preliminary investigations

There are at least two potential explanations for the fructose PTS overshoot. It could be the result of activation of a precursor inactive in the presence of glucose or some other property connected with prior growth on glucose. Figure R.7 shows the effect of growing glucose trained cells of E.coli 15224 into stationary phase. In the absence of fructose no increase in fructose PTS is observed but the glucose PTS activity falls and then increases. It is known that cells growing on glucose excrete acetate (Britten,1954) and once glucose is exhausted the cells adapt to this excreted acetate (Ashworth & Kornberg, 1964; Holms & Bennett, 1971). Consequently, adaptation to acetate might also result in recovery of that fraction of glucose PTS lost. No explanation as to how this is achieved can be attempted at this time. However, isocitrate dehydrogenase activity follows a similar pattern to that of the glucose PTS but the reasons for this behaviour are known (Holms & Bennett, 1971). The addition of fructose (Figure R.7) 0.5 h into stationary phase results in the usual fructose PTS overshoot but subsequent additions result in smaller overshoots whose magnitudes decrease with time. A transient increase in glucose PTS activity also occurs on addition of fructose 1.25 h into stationary phase. The results (Figure R.7) suggest that fructose is required for induction of fructose PTS synthesis leading to the overshoot. The magnitude of this overshoot decreases with time so it is also possible that (a) fructose is required to activate a precursor previously synthesised in the presence of glucose which is turned over and lost in stationary phase or (b) some other process is responsible for the observed results.

Before attempting to distinguish between (a) and (b) it is essential to determine exactly how the fructose PTS responds to a change

from glucose to fructose salts media. Figure R.8a shows that inoculation of glucose trained E.coli 15224 into fructose salts leads to a continuous appearance of the fructose PTS which reaches a maximum of 160 mU/mg dry wt. before declining, and the differential plot (Figure R.8b) emphasises the unusual nature of the fructose PTS synthesis under these conditions. When the fructose PTS activity reaches 55 mU/mg dry wt. the glucose PTS activity also increases to 100 mU/mg dry wt. before it declines and we call this effect the "transient glucose PTS increase". This effect is not always observed since samples are usually taken at 15 min intervals. Kornberg & Reeves (1972b) have observed that the glucose PTS may be gratuitously induced to a constant activity by growth of E.coli K2 on fructose and have designated this effect by the misleading name "fructose-kick". However, in the experiment in Figure R.8a, the glucose PTS activity responds only transiently to the presence of fructose and thus differs from the "fructose-kick" (Kornberg & Reeves, 1972b) described above. The significance of the transient glucose PTS increase is not clear at this time.

A subsequent experiment (Figure R.9) shows that the presence of chloramphenicol, an inhibitor of protein synthesis (Gale & Folkes, 1953; Wisseman et al., 1954; Brock, 1961; Allison et al.,1962) prevents the fructose PTS overshoot occurring on transfer of glucose trained cells to fructose and chloramphenicol. This suggests, but does not prove, that synthesis of the fructose PTS in the overshoot situation is de novo rather than activation of a precursor; an enzyme, induced by fructose, may be required for the activation step. The disparity between the initial glucose PTS activities (Figure R.9) in glucose and glucose + chloramphenicol requires comment since the same inoculum was used in both cases. It has been observed by Allison et al. (1962), Makman &

Sutherland (1965) and in the work performed in developing the PTS assay for this thesis, that the effect of chloramphenicol is not solely bacteriostatic. It is found, contrary to the results reported by Kornberg & Reeves (1972b) for other strains, that PTS activities decrease in cells of E.coli 15224 treated with chloramphenicol while untreated cells maintain essentially constant activities for at least 7 hours. On this basis, the unavoidable delay between harvesting and assay of PTS activity probably accounts for the observed difference in glucose PTS activities.

It was concluded from these experiments that the fructose PTS overshoot does not occur in the absence of fructose and, from the work using chloramphenicol, is more likely to result from de novo synthesis rather than activation of a precursor. This needs to be confirmed.

3.2. Use of continuous culture (turbidostat)

3.2.1. General considerations

It was felt that, at this stage, no further insight could be gained into the fructose PTS overshoot or glucose inhibition of fructose utilisation by use of batch culture since all observed effects of glucose challenge are transitory and hence not easily studied. Other workers (Herbert, 1961a; Hamlin et al., 1967; Harrison & Maitra, 1969) have reported similar problems arising from the ability of micro-organisms to adapt quickly to sudden changes in their environment. Tempest (1970) concludes that it is impossible to study the physiology of growing micro-organisms without the use of an "open" (Herbert, 1961b) or continuous culture system.

In order to simulate the processes occurring in batch culture it would be necessary to maintain the total sugar concentration in excess of the growth requirement. This could be achieved by use of a turbidostat (Bryson, 1952) or some form of salt limitation (eg NH_4^+, K^+, Mg^{2+}, PO_4^{3-}, SO_4^{2-}

in a chemostat. It has been reported that nitrogen limitation leads to severe catabolite repression (Wainwright & Neville, 1956; Mandelstam, 1961, 1962; Ng & Dawes, 1973) and may also result in uncoupling of oxidative phosphorylation in E.coli 15224 (R.J. Wallace - personal communication). It was felt unwise to place any external restraints on cell growth since most of the results discussed earlier were observed in the log phase of growth. Thus a turbidostat design of continuous culture was chosen to further investigate the observed effects. Dean (1967) reached a similar conclusion when comparing the growth of Aerobacter aerogenes in batch culture and a turbidostat.

3.2.2. Use of the turbidostat

From the reasoning given above a fructose turbidostat was set up in which fructose was always fed at 3.2 mM. The turbidity was maintained constant (E_{420} = 0.5) and 1.2 mM fructose was utilised for growth. Consequently the cells grew in the presence of a 2 mM excess of fructose. The essential salts and trace elements were also fed in excess and glucose could be added as required. If the cells are to grow solely on glucose at constant turbidity (E_{420} = 0.5) then 1.3 mM glucose will be utilised for growth.

When glucose is added at 3.3 mM (Figure R.10) in excess of the growth requirement for sole growth on glucose (1.3 mM) the synthesis of the fructose PTS is repressed and glucose PTS is induced. The fructose PTS is also transiently repressed since the decrease in PTS activity is exponential (i.e. dilution by wash out and no synthesis) until the activity falls to about 25 mU/mg dry wt. when the new rate of synthesis is achieved to maintain the specific activity at 12 mU/mg dry wt. Transient repression was first shown by Paigen (1966) and Moses & Prevost (1966) but, in contrast to the results obtained (Figure R.10) the ML-strains (E.coli 15224 is ML308) were not reported to show transient

repression (Paigen, 1966). In Figure R.10 the period of transient repression is followed by catabolite repression in which the fructose PTS is maintained at 12 mU/mg dry wt. but fructose is not used. It was not certain at this time whether the low fructose PTS activity observed resulted from the glucose PTS non-specifically phosphorylating fructose at about 20% of the glucose rate. Figure R.22 shows that this is not the case. Inoculation of glycerol trained cells of E.coli into glucose leads to an increase in glucose PTS activity but not fructose PTS. If glucose PTS does non-specifically phosphorylate fructose then the fructose PTS activity ought to increase with increasing glucose PTS activity. Figure R.22 shows that it does not.

The pattern of changeover of glucose and fructose PTS activities (Figure R.10) on going from fructose to fructose + excess glucose is by no means unique to enteric bacteria. This pattern of co-ordinate induction and repression has been shown by Higgins & Mandelstam (1972a) in Pseudomonas putida; in a nitrogen limited chemostat benzoylformate decarboxylase decreases and p-hydroxybenzoate hydroxylase increases on changing from mandelate to mandelate + p-hydroxybenzoate. Pardee (1961) suggests that the ability to grow fast is of selective advantage to micro-organisms and it has been shown in the laboratory that strict control of enzyme activity and economy of protein synthesis gives this advantage (Roeptke et al., 1964; Zamenhof & Eichhorn, 1967; Baich & Johnson, 1968). Figure R.10 shows that glucose challenge to the fructose turbidostat stimulates the growth rate (0.73 to 0.9 h^{-1}; equivalent to batch rates) as expected and glucose is preferentially utilised in agreement with the suggestion of Pardee (1961). A similar effect was observed for Pseudomonas oxalaticus (Blackmore & Quayle, 1968) but not for P.putida growing on mandelate and p-hydroxybenzoate (Higgins & Mandelstam, 1972a). Here the latter supports a lower growth

rate but is preferentially utilised and it seems that economy of protein synthesis is the answer since the latter requires less metabolic enzymes. On this basis glucose may also be preferentially utilised to fructose since fructose uptake and metabolism requires the two enzymes fructose EII and fructose-1-phosphate kinase (Fraenkel, 1968) to be induced in contrast to only glucose EII for glucose.

After glucose addition (Figure R.10), the fructose and glucose PTS activities stabilise and no fructose utilisation can be detected. Removal of glucose from the feed leads to the fructose PTS overshoot as observed in batch culture (Figure R.5) before the PTS activities readjust to approximately the original values. The transient glucose PTS increase is also observed. From the peak activities reached the glucose and fructose PTS activities fall exponentially suggesting that no synthesis occurs for about 45 min and 30 min respectively, before synthesis recommences to maintain the specific activities at constant levels. Similar results to these were obtained when glucose was added to the fructose chemostat at 1.6 mM, in excessof the growth requirement (1.3 mM).

It was concluded from these experiments that the presence of glucose in the medium at a concentration greater than that required to maintain growth on glucose alone will repress fructose PTS synthesis down to the constitutive level and prevent any detectable fructose utilisation. The initial addition of glucose leads to transient repression of fructose PTS synthesis. Removal of this excess of glucose from the feed results in the fructose PTS overshoot and the transient glucose PTS increase, exactly as in batch culture experiments.

When glucose is fed at 0.8 mM (Figure R.11) the fructose PTS is not so severely catabolite repressed and stabilises at 40 mU/mg dry wt. Glucose PTS activity also reaches a higher value (75 mU/mg dry wt.) than

in the previous experiments. Under these conditions all the glucose supplied is used together with some fructose to give a total sugar utilisation of about 13 μ mol/h/mg dry wt. - a similar value to that obtained for growth on glucose alone (Figure R.10), suggesting that the total flow of carbon into the cell is closely controlled. Glucose fed at 0.4 mM (Figure R.12) results in a fructose PTS activity of about 70 mU/mg dry wt., a glucose PTS of about 70 mU/mg dry wt. and is all used together with some fructose. The total rate of sugar utilisation is about 15 μ mol/h/mg dry wt. In the first of these two experiments (Figure R.11) the response of the fructose PTS to the presence of glucose shows that there is an initial transient repression lasting 15 - 30 min and the activity undershoots the final value. There are insufficient values for the second experiment (Figure R.12) to compare observed with theoretical curves. When glucose is supplied at concentrations less than that required to maintain growth solely on glucose (Figures R.11 - 12), halving the glucose feed rate (from 0.8 to 0.4 mM) doubles the fructose PTS activity (from 40 - 80 mU/mg dry wt.) while the glucose PTS is essentially unaffected. These observations accord with the idea of regulation of the fructose PTS level by induction with fructose (or its metabolites; Kornberg, 1972) and repression by glucose as described (Pastan & Perlman, 1969) and suggest that the intracellular level of cyclic AMP is determined by the rate of glucose utilisation. Mateles *et al.* (1967) observed somewhat similar results for a chemostat of *E.coli* growing on glucose and fructose at high dilution rates; they did not determine PTS enzyme activities but suggested that the activities would reflect the rates of utilisation. Such competitions between inducer and repressor are well documented for several inducible enzyme systems. Examples are the mandelate pathway of *Pseudomonas fluorescens* (Stanier *et al.*, 1965; Mandelstam & Jakoby, 1965; Stevenson & Mandelstam,

1965), the amidase of P.aeruginosa (Clarke & Brammar, 1964) and the control of glucose metabolism in P.aeruginosa by citrate (Ng & Dawes, 1973). In P.aeruginosa glucose utilisation is dominated by citrate (Hamilton & Dawes, 1959, 1960, 1961; Hamlin et al., 1967; Ng & Dawes, 1973) and, although glucose is actively transported into the cell (Midgley & Dawes, 1973) its entry does not involve the PTS (Romano et al., 1970; Phibbs & Egan, 1970; Midgley & Dawes, 1973). Mateles et al. (1967) report that, in contrast to E.coli, fructose utilisation in the presence of glucose shows a marked 'on-off' effect for P.fluorescens below or above a dilution rate of 0.25 h^{-1} respectively. This may suggest that the results obtained with species lacking the PTS cannot be extrapolated to, or compared with, species possessing the PTS, but, as discussed above, competition for inducer and repressor seems to be a property of many species of bacteria. In Figures R.11 & R.12 the addition of glucose stimulates the growth rate up to that for glucose alone probably for the reasons already given in section 1. When the 0.8 mM glucose feed is removed (Figure R.11), the fructose PTS overshoot occurs, but the magnitude is much reduced (40 - 90 mU/mg dry wt.), before falling to the original activity. The transient glucose PTS increase does not occur and both fructose and glucose PTS activities fall exponentially for about 30 and 60 min respectively before synthesis recommences to maintain the activities at the final levels. When the 0.4 mM glucose feed is removed (Figure R.12) the fructose PTS overshoot is very small, no transient glucose PTS increase occurs and glucose PTS activity falls exponentially until the specific activity reaches 25 mU/mg dry wt. However, the fructose PTS does not fall exponentially on removal of the glucose feed suggesting that synthesis continues but at a lower rate. It is difficult to explain the stimulation in fructose utilisation on removing the glucose feed since fructose utilisation does

not return to its original rate. This may be a consequence of selection in continuous culture for cells having the ability to grow faster on fructose. Cells removed from this, and all other continuous cultures, for plating on BCIG agar gave blue colonies suggesting that the culture was not contaminated.

Glucose added at 0.1 mM (Figure R.13) has somewhat different effects. The fructose PTS undershoots the new value suggesting very brief transient repression and glucose PTS activity increases slightly. The growth rate is only stimulated from 0.7 to 0.83 h^{-1} with 0.1 mM glucose (Figure R.13) and this substantiates the explanation given in section 1. for the "sparing effect" of glucose on fructose metabolism. The degree to which glucose "spares" the need for the FDP-ase reaction will depend on the rate of glucose utilisation and this premise is borne out in these experiments. Removal of the glucose (Figure R.13) gives neither a transient glucose PTS increase nor a fructose PTS overshoot and the growth rate falls to 0.73 h^{-1}. As in the previous experiment, fructose utilisation is slightly stimulated on glucose removal.

It was concluded at this stage that addition of glucose to a fructose turbidostat at concentrations less than that required to maintain growth on glucose alone results in utilisation of all the glucose together with some fructose. Furthermore, the fructose PTS activity varies inversely with the rate of glucose utilisation whereas the magnitude of the fructose PTS overshoot varies directly with glucose utilisation.

The experiment in Figure R.14 serves as a control for the fructose turbidostat experiments. Fructose addition to a glucose turbidostat caused a slight fall in glucose PTS, a slight increase in fructose PTS and no stimulation of growth; results analogous to those obtained in batch culture. Since Silver & Mateles (1969) suggest that

catabolite repression alone is not enough to prevent synthesis of the lac operon in E.coli, glucose must be inhibiting (McGinnis & Paigen, 1969) fructose entry into the cell. However, as discussed for batch culture, sufficient must enter to give the observed induction, although no detectable utilisation occurs. It may also be that, in this system, the inducer and repressor interact and repression can be at least partly reversed at high inducer concentrations. This has been observed by Higgins & Mandelstam (1972a) in P.putida where the inhibition by p-hydroxybenzoate of benzoylformate decarboxylase is partially alleviated by high concentrations of mandelate. Removal of fructose (Figure R.14) has little effect except that it seems to stimulate the rate of glucose utilisation. This latter phenomenon, and its converse, has been observed before and probably involves selection for cells having the ability to grow more rapidly on these substrates.

The results obtained in the turbidostat experiments will initially be explained in terms of the fructose PTS overshoot.

Growth of E.coli in the presence of glucose leads to a depletion of intracellular cyclic AMP (Makman & Sutherland, 1965; de Crombrugghe et al., 1969; Tyler & Magasanik, 1970; del Campo et al., 1970; Aboud & Berger, 1971a,b). Makman & Sutherland (1965) and Hempfling & Beeman (1971) have shown that the intracellular cyclic AMP concentration varies inversely with the rate of glucose utilisation. The results presented in Figures R.10 - 14 show that fructose PTS activity varies inversely with the rate of glucose utilisation and therefore the fructose PTS activity is inversely proportional to the degree of catabolite repression. Figures R.10 - 14 show that the magnitude of the fructose PTS overshoot decreases with decreasing glucose utilisation and it seems that there is a connection between the degree of catabolite repression and the fructose PTS overshoot.

Makman & Sutherland (1965) have shown that near glucose exhaustion in a glucose growing culture the intracellular cyclic AMP concentration increases rapidly from 1×10^{-5} to 7.5×10^{-5} mM before falling to about 1.4×10^{-5} mM. These events take place over about 1.5 h and a graph of cyclic AMP concentration against time (Makman & Sutherland, 1965) somewhat resembles the fructose PTS overshoot curve (Figure R.10). It is feasible that a decrease in catabolite repression resulting from a decreased rate of glucose utilisation will reduce the magnitude of the cyclic AMP response curve (Makman & Sutherland, 1965). If this is so, then results obtained in Figures R.10 - 14 might be explained on this basis since a decrease in glucose utilisation leads to a decrease in fructose PTS overshoot. (The apparent stimulation of fructose PTS activity (Figure R.12) on addition of 0.4 mM glucose probably only results from disturbing the original steady state since the final value obtained (76 mU/mg dry wt.) falls well within the normal range of fructose PTS activities observed for growth on fructose alone.)

It was concluded from this work that, for cells growing on different mixtures of glucose and fructose, fructose PTS activity is inversely proportional to the degree of catabolite repression. Also there seems to be a direct relationship between the fructose PTS overshoot and the degree of catabolite repression but this needs to be confirmed.

3.3. Investigations in batch culture

The conclusions reached in the previous section were further tested in batch culture. In Figure R.15 glycerol trained cells of E.coli 15224, which are not catabolite repressed (Holms & Robertson, 1973) were inoculated into fructose salts. Figure R.15 shows that, although the fructose PTS rose rapidly (c/f Kornberg & Reeves, 1972b)

to 40 mU/mg dry wt. and subsequently increased, there was no real suggestion of a fructose PTS overshoot. The rapid rise in fructose PTS activity (Figure R.15) is followed by no synthesis for about 30 min meaning that there is either an initial burst of synthesis or that synchronous synthesis of the fructose PTS occurs. It could be argued that cells which had previously demonstrated the fructose PTS overshoot had, at some stage, been grown on glucose and therefore might possess an inactive precursor synthesised during growth on glucose. Figure R.16 shows the results of inoculating cells of E.coli 15224 trained to glucose in the presence of cyclic AMP. These cells are substantially derepressed (de Crombrugghe et al., 1969) and, although fructose PTS activity again increased rapidly there was no suggestion of a fructose PTS overshoot (glucose PTS is high in these cells (Figure R.16) showing that glucose partially represses synthesis of its own PTS). These results are in accord with the hypothesis that the level of catabolite repression and the size of the fructose PTS overshoot are directly linked. It was concluded that the initial batch experiment (Figure R.5) must be repeated with glucose challenge to fructose cells in the presence and absence of cyclic AMP. It has been shown (de Crombrugghe et al., 1969) that cyclic AMP overcomes glucose repression during growth on glucose and fructose by at least 80% meaning that the cells, although challenged by glucose, would be substantially derepressed.

Figure R.17a shows the effect of glucose challenge in the presence of cyclic AMP. At glucose challenge, glucose PTS is induced and synthesised at a much faster rate than in the absence of cyclic AMP (Figure R.17b) showing that the cells in Figure R.17a are not severely repressed. Glucose (Figure R.17a) eventually inhibits fructose utilisation (McGinnis & Paigen, 1969) which accounts for the decrease

in fructose PTS synthesis as these cells are not severely catabolite repressed. In the presence of cyclic AMP growth is only stimulated to 0.83 h^{-1} (Figure R.17a) but in the absence of cyclic AMP is stimulated to 0.91 h^{-1} (Figure R.17b). This difference is explained on the basis that many enzymes, including the lac enzymes in this strain (Holms & Robertson, 1973), which are normally repressed during growth on glucose, will now be synthesised in the presence of cyclic AMP. Therefore a proportion of the cells' metabolism will now be diverted to the synthesis of unnecessary enzymes resulting in the slower growth rate observed (Figure R.17a). At, or very near to, glucose exhaustion (Figure R.17a) the fructose PTS activity increases rapidly but does not overshoot the final observed activity. In contrast, glucose challenge in the absence of cyclic AMP (Figure R.17b) which, but for the higher substrate concentrations, is a repeat of the original experiment (Figure R.5) severely represses the fructose PTS (Figure R.17b) and a large fructose PTS overshoot at or near glucose exhaustion is observed. The transient glucose PTS increase is also seen. Although the fructose PTS overshoot does not occur in the presence of cyclic AMP (Figure R.17a), the relatively high fructose PTS near glucose exhaustion (c/f Figure R.17b) probably accounts for the lack of diauxie.

It seems from these experiments that the fructose PTS overshoot is much more likely to result from transient derepression of the cells occurring at or very near to glucose exhaustion than from activation of a precursor previously inactive in the presence of glucose. Transient derepression has been described in the Introduction. Cohn & Horibata (1959) have shown that the period of transient derepression following respiratory shock lasts for about 1 generation; similar to the results observed for the system under investigation. Activation of a previously inactive precursor can be ruled out since, whereas the effect is observed

when cells trained to glucose (Figure R.18) and cells grown in the presence of glucose and fructose (Figures R.5 and R.17b) are transferred to fructose alone, the fructose PTS overshoot is not observed if these cells are derepressed by the addition of cyclic AMP (Figures R.16 and R.17a respectively). Figure R.18 clearly shows the effect of catabolite repression on the fructose PTS overshoot, and it is concluded that the fructose PTS overshoot results from the transient derepression of synthesis occurring near glucose exhaustion. The occurrence of transient derepression will explain two other observations. Firstly, transient derepression, occurring as it does just before glucose exhaustion (Makman & Sutherland, 1965) will allow a burst of rapid glucose induced synthesis of the glucose PTS just before glucose is finally exhausted, giving rise to the transient glucose PTS increase observed. Therefore this transient glucose PTS increase differs from the "fructose-kick" described by Kornberg & Reeves (1972b). Secondly, a rapid reversal of this transient derepression caused by the fall in cyclic AMP levels (Makman & Sutherland, 1965) would effectively reduce the rate of fructose PTS synthesis causing the rapid decrease in activity back to the final level (Figure R.5).

Kornberg & Reeves (1972b) have obtained results with E.coli B11 that differ from the results obtained in this thesis. Transfer of gluconate grown cells of E.coli B11, which are severely catabolite repressed (Okinaka & Dobrogosz, 1967b), into fructose salts medium does not result in the fructose PTS overshoot (Kornberg & Reeves, 1972b). It is reported (Makman & Sutherland, 1965) that incubation of repressed cells in carbon free medium at 37^{o} leads to an increase in intracellular cyclic AMP concentration which does not occur if the cells are chilled. Kornberg & Reeves (1972b) harvest and wash their cells at 15^{o} whereas the similar procedures in this thesis are performed at less than 4^{o}.

This may account for the apparent discrepancy but it could be simply a strain difference.

It was concluded from the experiments discussed in this section that the fructose PTS overshoot results from transient derepression of fructose PTS synthesis occurring when the severe catabolite repression caused by growth on glucose is released. This conclusion explains why glucose trained cells transferred to fructose medium (Figure R.4) and glucose challenged cells (Figure R.5) demonstrate the fructose PTS overshoot. This conclusion would be confirmed by determining the intracellular cyclic AMP levels when the PTS overshoot occurs but this was not done.

It can be concluded from the results discussed up to this point that a number of factors are involved in the control of sequential utilisation of glucose and fructose. When fructose growing cells are challenged with glucose, synthesis of the fructose PTS is repressed and the glucose PTS is induced. Repression in itself is not sufficient to ensure glucose dominance over fructose utilisation. Dominance is ensured since glucose inhibits fructose utilisation until glucose is nearly exhausted. Near glucose exhaustion the degree of catabolite repression decreases enabling synthesis of the fructose PTS to be induced and the activity increases rapidly and overshoots the final observed value. This enables the fructose PTS to overcome the remaining glucose inhibition such that fructose utilisation can commence. Thus, while the controls of induction, repression and inhibition are responsible for the sequential utilisation of glucose and fructose, the fructose PTS overshoot accounts for the lack of diauxie during growth of E.coli 15224 on glucose and fructose.

4. Relationship of PTS Activity to Rates of Utilisation

4.1. General considerations

The factors regulating synthesis and activity of intracellular enzymes are well known. Rate-limiting enzymes of the metabolic pathways have a 'fine' control exerted by allosteric effectors to stimulate or inhibit their activities. In addition, some of these rate-limiting enzymes are synthesised to any extent only when required. Although these processes of control are essential for regulating the flow of metabolites into the cell it has been shown (Rickenberg et al., 1956; Cohen & Monod, 1957; Horecker et al., 1960; Lin, 1970) that permeation is the initial rate limiting step. Kornberg & Reeves (1972a) showed that the observations made by these earlier workers apply just as much to the PTS since the rate of uptake of α-methyl glucoside by intact cells corresponds closely to its rate of phosphorylation by the PTS.

4.2. Relationship of rates of utilisation to PTS activity as determined by the chemostat

4.2.1. General considerations

It has already been established by turbidostat experiments that the induced level of fructose PTS in cells of E.coli 15224 growing on mixtures of glucose and fructose is determined by the rate of utilisation of glucose. Having established this relationship it was decided to investigate if there is also a relationship between the rate of substrate utilisation and the resulting PTS activity (or vice-versa).

Control of the rate of utilisation is only possible in some form of chemostat since limitations to utilisation in batch culture would not result in equilibrium enzyme levels being achieved (Tempest, 1970). Growth could either be carbon or salt limited but, as noted earlier, nitrogen limitation leads to severe catabolite repression and so carbon limitation was used.

4.2.2. Chemostat investigation of utilisation and PTS activity

Figure R.19 shows the results obtained for growth of E.coli 15224 in a fructose chemostat. Initially the chemostat was operated at a specific growth rate of 0.63 h^{-1}, fairly close to μ_{max} (0.73 h^{-1}) and it is seen that the cells maintain the activity of the inoculum. Decreasing the growth rate (to 0.36 h^{-1}) leads to a slow increase in fructose PTS activity and an increase in glucose PTS activity suggesting that the glucose PTS is being gratuitously induced (Kornberg & Reeves, 1972b). The high fructose PTS activity observed (196 mU/mg dry wt.) cannot easily be explained. Release of catabolite repression is not probable in the sense that cells of E.coli 15224 grown on fructose and cyclic AMP (Figure R.17a) have similar activities to those grown on fructose alone (Figures R.4, R.5 & R.17b). However Vogel (1957) has said that the synthesis of all enzymes present in growing organisms are regulated by the level of intracellular pools of metabolites (see also Umbarger, 1963; Kornberg, 1965, 1970; Sanwal, 1970); these pools are large in batch culture and small in the chemostat. Hence, not only will the enzyme contents in the two be different (Vogel, 1957) but the responses to changes in environments will also differ. On this basis the fructose PTS level may well be determined by factors other than catabolite repression. The results (Figure R.19) suggest that the fructose PTS is synthesised in response to the very low level of fructose existing in the medium. It has been shown (Sapico et al., 1968; Hanson & Anderson, 1968) that in Acrobacter acregenes a fourth protein component was required for fructose phosphorylation by the PTS. However, this component was required for phosphorylation only at low fructose concentrations and it was postulated (Hanson & Anderson, 1968) that it increased the affinity of the fructose enzyme II for fructose. This factor was subsequently shown to replace HPr and was induced only

when required for this high affinity fructose PTS (Walter & Anderson, 1973) but this does not alter the fact that an increased fructose PTS activity is observed at low concentration. It is difficult to predict if the appearance of such a factor would result in higher activities (Figure R.19) as measured by the assay described but the possibility of such an occurrence should be considered. A subsequent decrease in growth rate (to 0.12 h^{-1}) only stimulated glucose PTS activity (Figure R.19). While it is possible that this is gratuitous induction of the glucose PTS by fructose it is also possible that a trace of glucose in the fructose feed might be sufficient to cause induction; the latter is unlikely as a decrease in the rate of glucose feed would not be likely to lead to greater induction since there appears to be no catabolite repression in these cells. Return to the original feed rate does not restore the PTS activities to their original values. The results suggest that the rate of fructose utilisation does not depend on fructose PTS activity. Initially, for a rate of utilisation of 7.2 μ mol/h/mg dry wt. the PTS activity is 58 mU/mg dry wt. At the end of the experiment the PTS activity is 132 mU/mg dry wt. for the same rate of utilisation. Examples of this kind of hysteresis are not uncommon in chemostat work. Higgins & Mandelstam (1972b) report an hysteresis effect for the enzyme benzoylformate decarboxylase in cells of P.putida growing on various concentrations of D,L-mandelate in a chemostat. Tempest (1970) has shown that if the dilution rate of a culture of A.aerogenes is progressively increased to a value approaching the critical dilution rate and then lowered to the initial value the changes in steady-state macromolecular composition and metabolic activity of the organisms show hysteresis. Silver & Matèles (1969) have also shown a similar effect of dilution rate on aspartase activity in E.coli. If a factor is synthesised (Figure R.19) which increases the affinity of the fructose PTS and this

affects the assay as discussed earlier then it would explain the observed hysteresis. This factor would probably continue to be synthesised on return to the initial dilution rate since the concentration of fructose in the medium is still very low.

The effect of varying the rate of glucose utilisation was studied in the chemostat (Figure R.20). The initial specific growth rate (0.5 h^{-1}) maintains glucose PTS activity at a level similar to that observed in batch culture. Halving the dilution rate results in a 4-fold decrease in glucose PTS activity but a subsequent halving of the dilution rate has no effect. Return to the original dilution rate does not return the glucose PTS to its original activity suggesting that the rate of glucose utilisation does not depend on glucose PTS activity. Initially, for a rate of utilisation of 5.4 μ mol/h/mg dry wt. the PTS activity is 74 mU/mg dry wt. At the end of the run the glucose PTS activity is only 30 mU/mg dry wt.; an inverse effect to the results obtained with the fructose chemostat.

Figure R.21 shows the results obtained with a glucose-fructose chemostat. The initial dilution rate (0.5 h^{-1}) maintains PTS activities at values which cannot be compared to any previously obtained in batch culture (Figure R.21). Halving this rate results in stimulation of fructose PTS activity similar to that observed in the fructose chemostat (Figure R.19) but the activity slightly overshoots the final steady state value (Figure R.21). Such overshoots on change of dilution rate have been predicted theoretically for cell mass and substrate concentration (Powell, 1967) and observed experimentally by Mateles et al. (1965). Overshoots caused by derepression have been shown for enzyme activity (Gorini & Maas, 1957; Holzer, 1966). Hamlin et al. (1967) have observed such overshoots for glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities when altering the ratio of

glucose:citrate concentrations for P.aeruginosa growing in a nitrogen limited chemostat. Figure R.21 shows that, in contrast to the results obtained in the glucose chemostat (Figure R.20) for the same decrease in specific growth rate (0.54 to 0.26 h^{-1}), the glucose PTS activity is essentially unaffected. A subsequent decrease in growth rate (Figure R.21) lowers both glucose and fructose PTS activity but not in proportion to the decrease in utilisation. Return to the original dilution rate does not affect the fructose PTS but decreases the glucose PTS further. Again the rates of utilisation of fructose and glucose do not appear to depend on their respective PTS activities.

Glucose PTS activity does not fall in the glucose-fructose chemostat (Figure R.21) for the initial decrease in growth rate (0.54 to 0.26 h^{-1}) whereas there is a 4-fold decrease in glucose PTS activity in the glucose chemostat (Figure R.20). This discrepancy led to the hypothesis that the glucose PTS was synthesised in response to the presence of fructose PTS activity. Figure R.22 shows the effect of inoculating glycerol trained cells of E.coli 15224 into glucose salts. Glycerol trained cells possess both low glucose and low fructose PTS activities and, on inoculation into glucose salts, the cells grow immediately with the normal specific growth rate (0.91 h^{-1}) but the glucose PTS lags for 15 min before increasing slowly to about 34 mU/mg dry wt. This is in complete contrast to the response of the glucose PTS on inoculating fructose trained cells into glucose (shown in Figure R.22). It is also noteworthy that a glucose PTS activity of 15 mU/mg dry wt. will support growth at the maximal rate (Figure R.22) suggesting the PTS is more efficient at low levels - a fact noted earlier. This may be true since mannitol cells (data not presented) possess low mannitol PTS activity (40 mU/mg dry wt.) when fully induced, but the cells grow at a specific growth rate of 0.94 h^{-1}. It was concluded

from the experiment reported in Figure R.22 that both the rate of glucose PTS synthesis and the final activity achieved on transfer of cells of E.coli 15224 into glucose depends on the past history of the cells; cells with high fructose PTS and low glucose PTS leads to synthesis of glucose PTS at a faster rate than for glycerol cells with both low glucose and fructose PTS activity. Thus the hypothesis that the glucose PTS seems to be synthesised in response to the presence of the fructose PTS would seem to be correct. This effect may result from fructose trained cells having higher levels of the general PTS proteins since it might be essential to maintain a stoicheiometric ratio between the enzymes II and the general proteins for the purposes of control. This hypothesis cannot be tested until a means of quantitatively isolating the PTS components from the cell is developed but it would account for the rapid synthesis of glucose PTS observed for fructose cells as a means of obtaining this stoicheiometric ratio.

It was concluded from the work performed with the chemostat that there appears to be no direct relationship between PTS activity in vitro and rates of utilisation of glucose and fructose in vivo for E.coli 15224. This contrasts to work by Kornberg & Reeves (1972a), who did find a direct relationship between rates of uptake and PTS activity for other strains, and Hamlin et al. (1967) who found that glucose utilisation in P.aeruginosa depends on glucose-6-phosphate dehydrogenase activity (or vice-versa). However, Hamlin et al. (1967) reported that the activity of hexokinase, the first enzyme in glucose metabolism (c/f glucose PTS) did not always correspond to the rates of glucose utilisation observed. This apparent lack of dependence of utilisation and PTS activity will be further investigated along with studies on the glucose inhibition of fructose utilisation (section 5).

5. Glucose Inhibition of Fructose Utilisation

5.1. Preliminary investigations

Figure R.25 shows the effect of glucose and fructose PTS activities on the rates of utilisation of their respective substrates. It is seen that glucose is used at essentially similar rates for cells having glucose PTS activities in the range of 9 - 101 mU/mg dry wt. Glycerol trained cells (Figure R.25) differ slightly in that a lower rate of utilisation is given by an activity of 9 mU/mg dry wt. It was concluded at this stage that, although Kornberg & Reeves (1972a) had obtained a relationship (for other strains) between α-methyl glucoside uptake by intact cells and PTS-dependent phosphorylation in toluene-treated cells, no such relationship is observed between glucose utilisation and glucose PTS activity in E.coli 15224. Forrest (1974) has also observed that the rate of glycerol utilisation does not appear to depend on glycerokinase activity in E.coli 15224/glp R^c (Figure R.31); glycerokinase is the pacemaker for glycerol dissimilation (Zwaig et al., 1970) and has a role equivalent to the PTS in sugar transport in the sense that glycerokinase is the first phosphorylating enzyme in the glycerol pathway. Hamlin et al. (1967) found that the activity of hexokinase, which is the first enzyme in the glucose metabolic pathway of P.aeruginosa (c/f glycerokinase in glycerol metabolism), did not always depend on the rates of glucose utilisation for certain growth conditions in a chemostat. Thus the rate of utilisation of carbon sources is independent of total measured activity of what are probably the rate-limiting steps in vivo. The conclusion must be that the flux of carbon into the amphibolic pathways is controlled by inhibition of these key enzyme systems.

Figure R.25 shows that fructose utilisation does appear to depend on fructose PTS activity since increasing rates of utilisation

are generally reflected in increasing PTS activities. The very low rate of fructose utilisation in glycerol cells (Figure R.25) may explain the very slight lag sometimes observed when glycerol trained cells are inoculated into fructose since fructose PTS synthesis may be required before growth can occur. However, the fructose PTS activity increases rapidly (Figure R.15) and so only a slight lag is observed.

When measuring utilisation of glucose and fructose in the presence of both these substrates (Figure R.25) glucose completely inhibits fructose utilisation when fructose PTS activities are very low ($\sim$12 mU/mg dry wt.) and glucose PTS activity falls in the range 9 - 62 mU/mg dry wt.

When fructose PTS is high (62 mU/mg dry wt.) and glucose PTS low (9 mU/mg dry wt.) glucose does not affect fructose utilisation at all (Figure R.25). Under these conditions fructose does inhibit glucose utilisation but the reason for this is not clear at this time. It was concluded from these observations that the glucose PTS needs to be induced before glucose will inhibit fructose utilisation. This is in accord with similar results obtained by Kornberg (1972).

Figure R.25 shows that, when both glucose and fructose PTS activities are high, glucose inhibits fructose utilisation by 50%. This contrasts with results discussed earlier (Figure R.5) where glucose challenge eventually results in complete inhibition of fructose utilisation even when fructose PTS activity is high. However the results (Figure R.25) are similar to those obtained by Kornberg (1972) who observed that glucose inhibits fructose utilisation by 70% in fructose grown E.coli K2.1t. This strain, when grown on fructose, has high levels of both glucose and fructose PTS activity (Kornberg, 1972). The reasons for the apparent discrepancy between the two experiments (R.5 and R.25) are investigated.

Fructose growing cells were challenged with glucose (Figure R.26a,b) and then studied for the ability to utilise glucose and fructose, singly and in combination. After glucose challenge, glucose PTS activity increases constantly throughout the experiment (Figure R.26a) in contrast to the ability to utilise glucose (Figure R.26b) which increases rapidly to reach an essentially constant rate after 30 min. This confirms that the rate of glucose utilisation does not depend on glucose PTS activity, as determined in benzene treated cells. The decrease in fructose PTS also produced a fall in utilisation and this dependence was noted earlier. Figure R.26b shows that, for cells removed at intervals after glucose challenge, glucose will completely inhibit fructose utilisation 45 min after challenge. This corresponds fairly well to results observed in Figure R.23a where fructose utilisation in the culture is inhibited after about 30 min. Figure R.26b shows that glucose totally inhibits fructoseutilisation when the PTS activities (Figure R.26a) are both about 43 mU/mg dry wt. This contrasts sharply with results in Figure R.25 where, with equivalent glucose and fructose PTS activities (60 mU/mg dry wt.), glucose is unable to completely inhibit fructose utilisation. It was concluded that there is a difference between cells grown on single carbon sources and those derived from the challenge situation. The procedure by which the high fructose and glucose PTS activities in Figure R.25 were derived may be responsible for this. Growth of cells of E.coli on cyclic AMP may totally alter the enzyme content of the cell; in particular it may lead to higher levels of the constitutive proteins (EI and HPr) of the PTS and this may account for the discrepancies. Quantitative estimation of the EI and HPr levels in these cells is required to confirm or refute this hypothesis. Fructose (Figure R.26b) also inhibits glucose utilisation, though to a decreasing extent as glucose and fructose PTS

activities alter (Figure R.26a) and this is in accord with the results in Figure R.25 where high fructose PTS and low glucose PTS activities enable fructose to inhibit glucose utilisation. Since increasing glucose PTS activity (Figure R.26a) leads to an increasing inhibition of fructose utilisation by glucose (Figure R.26b) it was concluded that the glucose PTS needs to be induced before glucose inhibition occurs. It may be that fall in fructose PTS activity is an equal factor in this process but Kornberg (1972) has also obtained convincing evidence that glucose PTS induction is an essential process in glucose inhibition.

Several conclusions can be drawn from this section. Firstly, studies on the rates of utilisation and PTS activities, both in the chemostat and in batch culture, show that PTS activities determined in vitro bear little relationship to the actual PTS activities (as measured by rates of utilisation) manifest in vivo when the PTS is under feed-back control. From this it must be concluded that the PTS activities assayed in vitro are a measure of the potential "machinery" available to cell for sugar transport. At very low PTS activities that potential must be fully exploited and this explains the observations made earlier that sugars seem to be utilised more efficiently at low PTS activities. When the PTS activities are high, only a fraction of the system is used since normal feed-back control is exerted by the cell to control the rate of carbon flux through the cell. However, in the competitive situation, for glucose challenge on a fructose growing culture, glucose appears to directly inhibit the fructose PTS and so this glucose inhibition (equivalent to catabolite inhibition, McGinnis & Paigen, 1969) is possibly quite different from the feed-back control discussed above. Secondly, glucose PTS requires to be induced before glucose can inhibit fructose utilisation and thirdly, there is a

difference between cells derived from the challenge situation and cells where high PTS activities have been generated in the presence of cyclic AMP. This may result from induction of higher levels of the general PTS proteins but this is not known.

5.2. Inhibitory control by hexose-phosphates

There is a marked discrepancy between glucose inhibition of fructose utilisation (Figure R.26b) and glucose inhibition of fructose PTS activity (Figure R.26a) but, 75 min after glucose challenge, there is an approximately 60% inhibition by glucose of fructose PTS activity (Figure R.26a). This means by which this is effected was investigated.

Kornberg (1972) has obtained convincing evidence, particularly by use of mutant strains of E.coli, that hexose-phosphates have an important role in the control of fructose PTS activity and hence over the rate of fructose utilisation. These proposals (Kornberg, 1972) are in accord with a similar suggestion advanced by Pardee & Palmer (1973) when discussing the implication of transport systems in the control of metabolic rates.

Figure R.24 shows the effect of some hexose-phosphates on glucose and fructose PTS activities. For this experiment (Figure R.24) glucose PTS activity was determined by pyruvate production (c/f fructose PTS) so that the effects of G6P could be determined. G1P, G6P and F6P inhibit glucose PTS activity to a greater extent than fructose PTS activity. The results contrast with those obtained by Kornberg (1972) where these hexose-phosphates exert a strong inhibitory effect (about 75%) on fructose PTS activity in intact cells which are constitutive for the uptake of hexose-phosphates. The failure to observe such a severe inhibition (Figure R.24) may be a result of the benzene decryptification procedure. This will be investigated later. Both FDP and 6PG (Figure R.24) inhibit strongly and affect the

fructose PTS to a greater extent than the glucose PTS. FDP, which exerts a 25% greater inhibition on the fructose PTS is known to play a vital role in regulating metabolic processes. FDP and 6PG may therefore have a role in establishing glucose dominance over fructose utilisation. It does not seem reasonable that G1P, G6P or F6P, which inhibit the glucose PTS more than the fructose PTS, will play a part in establishing glucose dominance since the glucose PTS would always be more inhibited and so they may be responsible for generally controlling the uptake of any PTS-sugar. The results obtained by Kornberg (1972) and in this thesis (Figure R.24) show that G1P is not the most effective of the inhibitors in contrast to Kaback (1969) who showed that G1P is the most effective inhibitor of PTS dependent uptake of glucose and fructose by membrane vesicles.

In considering the inhibition caused by hexose-phosphates it must be remembered that benzene treated cells contain a functional phosphoglucose isomerase and so added F6P would lead to production of G6P and vice-versa. However, the hexose-phosphates are initially present at 5 mM and added only 1 min before initiating the assay so that the amount of isomerisation occurring should not be significant, at least while the initial enzyme rates are determined.

It was concluded at this point that inhibition by G1P, G6P and F6P is not likely to play a role in establishing glucose dominance. What is likely, however, is that these hexose-phosphates have a role in vivo of controlling the rate of flux of carbon into the cell (i.e. normal feedback-control). Kornberg & Reeves (1972a) have shown that, in other strains of E.coli, the rate of uptake of ∝MG depends directly on the glucose PTS activity which suggests that the PTS may be the first rate-limiting step in the metabolism of PTS-sugars. This would be in accord with similar suggestions made by Pardee & Palmer (1973).

Consequently, inhibition by hexose-phosphates of PTS activity will enable a fine control of the passage of carbon into the cell. FDP and 6PG may be involved in establishing glucose dominance since they, and particularly FDP, exert a strong inhibition on fructose utilisation. However, it is unlikely that hexose-phosphate inhibition accounts solely for the total glucose dominance observed.

The failure to observe a complete inhibition of fructose PTS activity in the assay led to the speculation that the assay system was at fault in that the enzymes and co-factors added to the assay, or the products of the assay system (6-phosphogluconate and lactate), were adversely affecting the results. Figure R.27 shows that taking cells from the challenge situation and assaying for glucose inhibition or fructose PTS activity by the radioactive assay method (to which no enzymes or co-factors are added) does not give substantially different results to the activities determined spectrophotometrically (Figures R.23b or R.26a). Radioactive assay of the PTS activities in the absence of phosphate buffer (Figure R.28) does show a marked inhibition by glucose of the fructose PTS suggesting that the concentration of phosphate in the assay system ($\sim$ 0.2 M) is masking any inhibitory effects by, say, the hexose-phosphates. However the observed activities (Figure R.28) are very low and it is not known how significant these results are, since spectrophotometric assays in the presence of BES buffer also gave very low PTS activities and the results are not felt to be reliable. Bag (1974) has observed that glucose will inhibit the fructose PTS of V.cholerae by 90% in the presence of phosphate buffer and so it is unlikely that phosphate is masking the inhibitory effects.

It was concluded from the results discussed above; that inhibition by hexose-phosphates, particularly FDP and 6PG, is probably involved in establishing glucose dominance over fructose utilisation;

that the failure to observe a total inhibition of fructose PTS activity is not a fault of the spectrophotometric assay; that the interrelationship of PTS activity and the presence of phosphate needs to be further investigated and that other hexose-phosphates, particularly G1P, G6P and F6P have a general role in controlling metabolism by exerting feed-back inhibition on the PTS to limit the passage of carbon into the cell.

6. Investigation of the Site of Glucose Inhibition

While it was concluded that hexose-phosphates may, at least in part, account for the glucose dominance, the complete inhibition of fructose utilisation by glucose *in vivo* (Figure R.5) still needs to be explained. It was concluded in an earlier section that induction of the glucose PTS is a necessary factor in establishing glucose dominance and this led to the speculation that control mechanisms other than hexose-phosphate inhibition were involved.

The idea that, because of the lack of structural homology in the PTS (i.e. different sugar specific enzymes II), glucose inhibits at the level of phosphate transfer from HPr to EII (Kundig *et al.*, 1964) was supported by McGinnis & Paigen (1969) who suggest that preferential utilisation by the glucose PTS of a limited supply of HPr would account for the observed inhibitory effect of glucose.

It has been reported (Kundig & Roseman, 1971; Negrel *et al.*, 1973; Simoni *et al.*, 1973) that glucose transport in *E.coli* by the PTS could be inactivated by thiol reagents such as NEM but that the localisation of essential thiol in this multienzyme system remained controversial. According to Kundig & Roseman (1971) and Negrel *et al.* (1973) only EI has essential thiol but Gachelin (1970) showed that the target of NEM in *E.coli* was EII and in *S.aureus* both EI and EII are sensitive (Simoni *et al.*, 1973). HPr was ruled out because of the absence of cysteine in its amino acid sequence (Roseman, 1969). Haguenauer-Tsapis & Kepes (1973) report that the sensitivity of *E.coli* K12 to inactivation by NEM and 1-fluoro-2,4-dinitrobenzene is strongly variable with the functional state of the transport system and that the main target of the inactivating agents is the membrane bound component. They conclude (Haguenauer-Tsapis & Kepes, 1973) that the EII exists in two different states which can be qualified as the

energised and non-energised state; the energised state is the phosphorylated EII. It was shown that the inactivation of ∝-methyl glucoside transport in E.coli by NEM was strongly enhanced by the presence of glucose and ∝-methyl glucoside (Haguenauer-Tsapis & Kepes, 1973) and also by NaF, an inhibitor of PEP synthesis (Haguenauer-Tsapis & Kepes, 1972). It was subsequently concluded that the de-energised state (EII) only is sensitive to attack and that this sensitisation effect is specific. Glucose, for example, only sensitises the glucose PTS (Haguenauer-Tsapis & Kepes, 1973) and not the D-glucuronic acid transport system (Haguenauer-Tsapis & Kepes, 1973; Abendano & Kepes, 1973) and the converse also applies. Observations of similar sensitisations have been observed for mitochondrial translocators (Leblanc & Clauser, 1972), for red blood cell membrane sugar carriers (Edwards, 1973) and for the coupling factor in chloroplasts (McCarthy & Fagan, 1973) suggesting that sensitisation by the substrate is as wide spread as protection by the substrate (eg. Fox & Kennedy, 1965; Kepes, 1960).

Extension of this work to show fructose sensitisation of the fructose PTS would mean that this procedure could be used to study the site of action of glucose inhibition. The essential keys to these studies are firstly, that inhibition of energy supply sensitises the PTS to NEM attack (Haguenauer-Tsapis & Kepes, 1973) and so, if glucose inhibits the transfer of high energy phosphate from HPr to fructose EII this should sensitise the fructose PTS to NEM attack. Secondly, if glucose acts to prevent binding of the fructose to its EII this should leave the EII in its energised state such that it will not be inactivated to the same extent as for fructose alone.

Figure R.29 shows the effect of combinations of glucose, fructose and NEM on the fructose PTS. Controls for the system are

treatment with NEM, or pre-complexed NEM and 2-mercaptoethanol in the absence of substrate. Both controls give the same results and this means that no sensitisation occurs in the absence of substrate. Also the activities are higher than those samples not so treated. We attribute this to a protective effect of 2-mercaptoethanol on the PTS during the decryptification and aspiration procedures. This explains why aspiration with OFN gives higher activities than aeration (see Development 1.) since the essential thiol is probably not oxidised to the same extent. Figure R.29 shows that fructose sensitises both the fructose PTS and, to a lesser extent, the glucose PTS, to attack by NEM. It is possible that fructose may inhibit energy transfer to the glucose EII since, in Figure R.25, fructose does inhibit glucose utilisation when fructose PTS is high and glucose PTS is low, but the significance of this phenomenon is not known at this time. It was concluded from this that the method of Haguenauer-Tsapis & Kepes (1973) can be extended to the fructose PTS as this is sensitised by fructose to NEM attack.

The results in Figure R.29 for glucose, fructose and glucose-cyclic AMP trained cells show that glucose and NEM inactivate the glucose PTS but have no effect on fructose PTS activity. These results would seem to be at variance with the suggestion (Kundig et al., 1964) that glucose acts to inhibit transfer of phosphate from HPr to fructose EII since this effect is not observed here. Fructose trained cells treated with NEM in the presence of both glucose and fructose (Figure R.29) give similar PTS activities to those obtained with the single substrates. However, glucose and glucose-cyclic AMP trained cells (with high glucose PTS activity) treated similarly show that, while glucose PTS activity is essentially unchanged from that obtained on glucose alone, the fructose PTS activity is much higher than the

comparative value obtained on fructose alone. These results suggest that glucose, in the presence of its induced PTS, acts allosterically to prevent fructose binding (and thus de-energising the phosphorylated fructose EII) rather than inhibiting energy transfer. This is further substantiated by studying the results obtained (Figure R.29) with glucose-cyclic AMP trained cells which possess both high glucose and high fructose PTS activities. Since Haguenauer-Tsapis & Kepes (1973) have shown that inhibition of energy supply sensitises the PTS to attack, if glucose did prevent transfer of high energy phosphate to the fructose EII this should certainly be manifest under conditions where these cells are presented with both glucose and fructose (Figure R.29). The presence of fructose would de-energise the existing fructose EII and, if glucose inhibits further energy transfer, the fructose EII would not be re-energised. In consequence, the fructose EII should be much more sensitive to NEM attack than in the presence of fructose alone. Figure R.29 shows that less sensitisation occurs and therefore glucose does not control fructose utilisation by inhibiting energy transfer. It was felt the results (Figure R.29) suggest that glucose inhibits fructose utilisation by preventing binding to fructose EII but these results need to be confirmed for cells in the challenge situation. Figure R.30 shows such results. Glucose and NEM affect the glucose PTS such that, in every instance, the glucose PTS is lowered to the same level ($\sim$10 mU/mg dry wt.), but the fructose PTS is scarcely affected. Fructose and NEM inactivate the fructose PTS and, apart from the zero time sample (26 mU/mg dry wt.), the remaining activities are lowered to a similar level (16 - 12 mU/mg dry wt.). The combination of glucose, fructose and NEM (Figure R.30) affects glucose PTS activity in a similar fashion to glucose alone. However fructose PTS activities now fall in the range 26 - 22 mU/mg dry wt.

showing that the presence of glucose protects the fructose PTS from NEM attack. The zero time sample is not protected and therefore synthesis of the glucose PTS is essential in mediating this protection (c/f Kornberg, 1972). This means that there is an interaction between the two PTS's (indeed it was suggested earlier that glucose PTS seems to be synthesised in response to the presence of the fructose PTS) which is not mediated by competition for HPr. It is significant that in Figure R.30, fructose and NEM lower the fructose PTS to an essentially constant activity while glucose, fructose and NEM lower it to a constant higher activity. These results are interpreted as implying that, 30 min after glucose challenge, glucose and the glucose PTS will interact to inhibit the fructose PTS activity down to a constant activity regardless of the actual activity of the PTS.

It was concluded from this work that glucose inhibits fructose utilisation by inducing synthesis of the glucose PTS. Glucose and the glucose PTS then interact to inhibit fructose PTS activity. This, plus the inhibition by FDP and 6PG probably accounts in total for the ability of glucose to totally inhibit fructose utilisation 30 min after glucose challenge on a fructose growing culture and while fructose PTS activity is still high.

The final conclusion is that glucose challenge on a fructose growing culture results in sequential utilisation of the glucose and then the fructose because glucose both represses the synthesis of, and interacts with glucose PTS to inhibit the activity of, the fructose PTS. By these means glucose will dominate fructose utilisation about 30 min after challenge. However, near glucose exhaustion synthesis of the fructose PTS is induced and the activity increases rapidly enabling the fructose PTS to overcome any remaining glucose

inhibition and the two substrates are then co-utilised for a short time. This explains why glucose and fructose are sequentially utilised by E.coli 15224 but without a diauxic growth pattern. Consequently, the sequential utilisation of glucose and fructose is mediated by control of their PTS's.

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