UTILIZATION OF FRUCTOSE AND FRUCTOSE 1-PHOSPHATE BY

ESCHERICHIA COLI

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A thesis submitted in partial fulfilment of the regulations governing the Ph.D. degree at the University of Leicester.

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PREFACE

I wish to thank my supervisor, Professor H.L. Kornberg, F.R.S., for his invaluable guidance throughout the course of this work.

I also wish to thank Mrs. Janet Smith for expert technical assistance at various phases of this research and Mrs. Joan Skinner for the typing of this thesis.

This work was performed during the tenure of a Science Research Council Studentship.

STATEMENT

The accompanying thesis submitted for the degree of Ph.D. entitled

UTILIZATION OF FRUCTOSE AND FRUCTOSE 1-PHOSPHATE BY *ESCHERICHIA COLI*

is based on work conducted by the author in the Department of Biochemistry of the University of Leicester mainly during the period between October 1968 and September 1971.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

SIGNED: T. Ferenci DATE: 17th Dec. 1971

ABSTRACT

1. The phosphoenolpyruvate (PEP):fructose phosphotransferase system plays a key role in the utilization of fructose by Escherichia coli. Mutants devoid of, or altered in, this system are not only modified in their ability to phosphorylate fructose but are also altered in their ability to take up this hexose from solutions.

2. The major product of fructose phosphorylation in E. coli is fructose 1-phosphate, though fructose 6-phosphate can also be formed by a PEP-dependent mechanism. The relationship between PEP:fructose 1- and PEP:fructose 6-phosphotransferase activities is discussed.

3. PEP-dependent fructose phosphorylation, fructose uptake by whole cells and ATP-dependent fructose 1-phosphate phosphorylation to fructose 1,6-diphosphate are activities that, in E. coli, are induced by fructose. A regulatory mutant that exhibits these activities constitutively has been isolated; its properties indicate that the expression of fructose-induced proteins is controlled as a regulon.

4. The utilization of fructose by E. coli is subject to control at the level of fructose entry into whole cells. Fructose uptake is inhibited by intracellular hexose phosphates or by carbon sources that can readily give rise to hexose phosphates, such as glucose or galactose.

5. Fructose 1-phosphate can serve as a sole carbon source for the growth of E. coli. Fructose 1-phosphate, like other hexose-phosphates such as glucose 6-phosphate and fructose 6-phosphate, is a substrate of an inducible hexose phosphate transport system. However, unlike glucose 6-phosphate, fructose 1-phosphate, when present in the growth medium of E. coli, does not give rise to the induction of this uptake system.

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MATERIALS AND METHODS

TABLE 1 (contd)

(2) Selected by the spontaneously acquired resistance to the inhibitory effect of 2-deoxyglucose on the growth of K2.1t on fructose (see Chapter III).

(3) Selected from K2 after ethyl methanesulphonate mutagenesis and penicillin treatment (for methods, see later in this Chapter) for its inability to grow on glycerol or lactate whilst retaining the ability to grow on glucose (see Chapter I).

(4) Selected by trimethoprim treatment of K2.21No.4 (for method, see later in this Chapter).

(5) Selected from KL16.21 for its spontaneously acquired ability to grow well on fructose 1-phosphate (see Chapter IV).

TABLE 1 (contd)

Strain	Mating type	Genetic Markers*	Source or Reference
K2.21No.4URA	F^-	his,met,trp,pyrE,ptsF21, str	Recombinant from a cross AB2243 x K2.21No.4
AB674	Hfr		E.A. Adelberg
AT2243	Hfr	pyrE,metB	A.L. Taylor
K10	Hfr		D.G. Fraenkel
DF40	Hfr	pgi	D.G. Fraenkel
DF2001	Hfr	zwf	D.G. Fraenkel
KL16	Hfr		B. Low
KL16.11^c.21	Hfr	ptsF21,uhp^c	From KL16.21;(5) spontaneous
KL16.21	Hfr	ptsF21	From KL16 by mutagenesis
KL16.23	Hfr	ptsF23	From KL16 by mutagenesis
R4.1.5^c	Hfr	met,pps,iclR	H.L. Kornberg

* The abbreviations used indicate a requirement for: his = histidine; arg = arginine; thr = threonine; leu = leucine; trp = tryptophan; met = methionine; thy = thymine; ilv = isoleucine + valine; and pyrE = uracil. The absence of the following enzymes is indicated by pgi = phosphoglucose isomerase; zwf = glucose 6-phosphate dehydrogenase; ptsF = PEP:fructose phosphotransferase; ctr = PEP:hexose phosphotransferase; pfk = 6-phosphofructokinase; fdp = fructose 1,6-diphosphatase; pps = PEP synthase, while uhp denotes the loss of the ability to take up hexose phosphates. Mutations in regulatory genes resulting in the constitutive synthesis of the glyoxylate cycle enzymes, the hexose phosphate uptake system and the fructose utilizing enzymes are indicated by iclR, uhp^c and fruR respectively.

(1) Selected by trimethoprim treatment of K2.1.23 (see later in this Chapter)

TABLE 1 Strains of E. coli K12 used in this thesis

Strain	Mating type	Genetic markers*	Source or Reference
K2	F^-	his,arg,thr,leu,trp,str	Brice and Kornberg (1967)
K2.1t	F^-	his,arg,thr,leu,pps,str	Brice and Kornberg (1967)
K2.1.5^c.7	F^-	his,thr,leu,pps,pgi,iclR, str	Kornberg (1970)
K2.1.5^c.7.15	F^-	thr,leu,pps,iclR,pgi,zwf, str	Recombinant from a cross DF2001 x K2.1.5^c.7
K2.1.5^c.15	F^-	met,pps,iclR,zwf,str	Recombinant from a cross R4.1.5^c x K2.1.5^c.7.15
K2.1.5^c.15.16	F^-	pps,iclR,pfk,zwf,str	Kornberg and Smith (1970)
K2.1.5^c.16	F^-	his,arg,thr,leu,pps,iclR, pfk,str	Kornberg and Smith (1970)
K2.1.7	F^-	his,thr,leu,pps,pgi,str	Recombinant from a cross DF40 x K2.1t
K2.1.7.11^c	F^-	his,pgi,pps,uhp^c,str	Recombinant from a cross DF40 x K2.1.11^c
K2.1.10	F^-	met,his,pps,ctr,str	Kornberg (1970)
K2.1.11^c	F^-	his,thr,leu,pps,uhp^c,str	Recombinant from a cross KL16.11^c.21 x K2.1.11i
K2.1.11i	F^-	his,thr,leu,ilv,pps,uhp str	Kornberg and Smith (1969)
K2.1.23	F^-	arg,pps,ptsF23,str	Recombinant from a cross KL16.23 x K2.1t
K2.1.23T	F^-	arg,pps,thy,ptsF23,str	Derived from K2.1.23 (1)
K2.1.DG^{R}D	F^-	his,arg,thr,leu,pps,fruR, str	H.L. Kornberg (2)
K2.19	F^-	his,arg,thr,leu,trp,fdp, str	H.L. Kornberg (3)
K2.21No.4	F^-	his,arg,trp,ptsF21,str	Recombinant from a cross KL16.11^c.21 x K2
K2.21No.4T	F^-	his,arg,trp,thy,ptsF21, str	Derived from K2.21No.4 (4)

MATERIALS AND METHODS

(i) Materials

The enzymes, coenzymes and sugar phosphates used in this work were obtained from Boehringer GmbH (Mannheim, Germany) and Sigma Ltd. (London, England). Sugars, including fructose (glucose-free), and amino acids were from British Drug Houses Ltd. (Poole, England). All sugars used were of the D- configuration. Streptomycin sulphate and benzylpenicillin were from Glaxo Ltd. (Greenford, England) and chloramphenicol from Parke-Davis Ltd. (Hounslow, England). Radioactive sugars and sugar phosphates were from the Radiochemical Centre (Amersham, England). Ethyl methanesulphonate was purchased from Kodak Ltd. (London, England).

Organisms used

The strains of *Escherichia coli* K12 used in this study are presented in Table 1, which shows their genotype and hence also their growth requirements. Abbreviations for the genetic markers are those listed by Taylor (1970) except the fructose lesions *fruR*, *ptsF21* and *ptsF23*, whose selection and properties will be described in the appropriate parts of this thesis. A scheme is presented in Figure 1 showing the areas of carbohydrate metabolism relevant to this work and the enzymic lesions in the strains used in this study. Many of these organisms were gifts from the sources indicated in Table 1.

Maintenance of Organisms

Organisms were maintained by periodic sub-culture on Oxoid nutrient agar slopes supplemented where necessary with appropriate growth factors. The cultures were incubated at 37° overnight and

FIG. 1

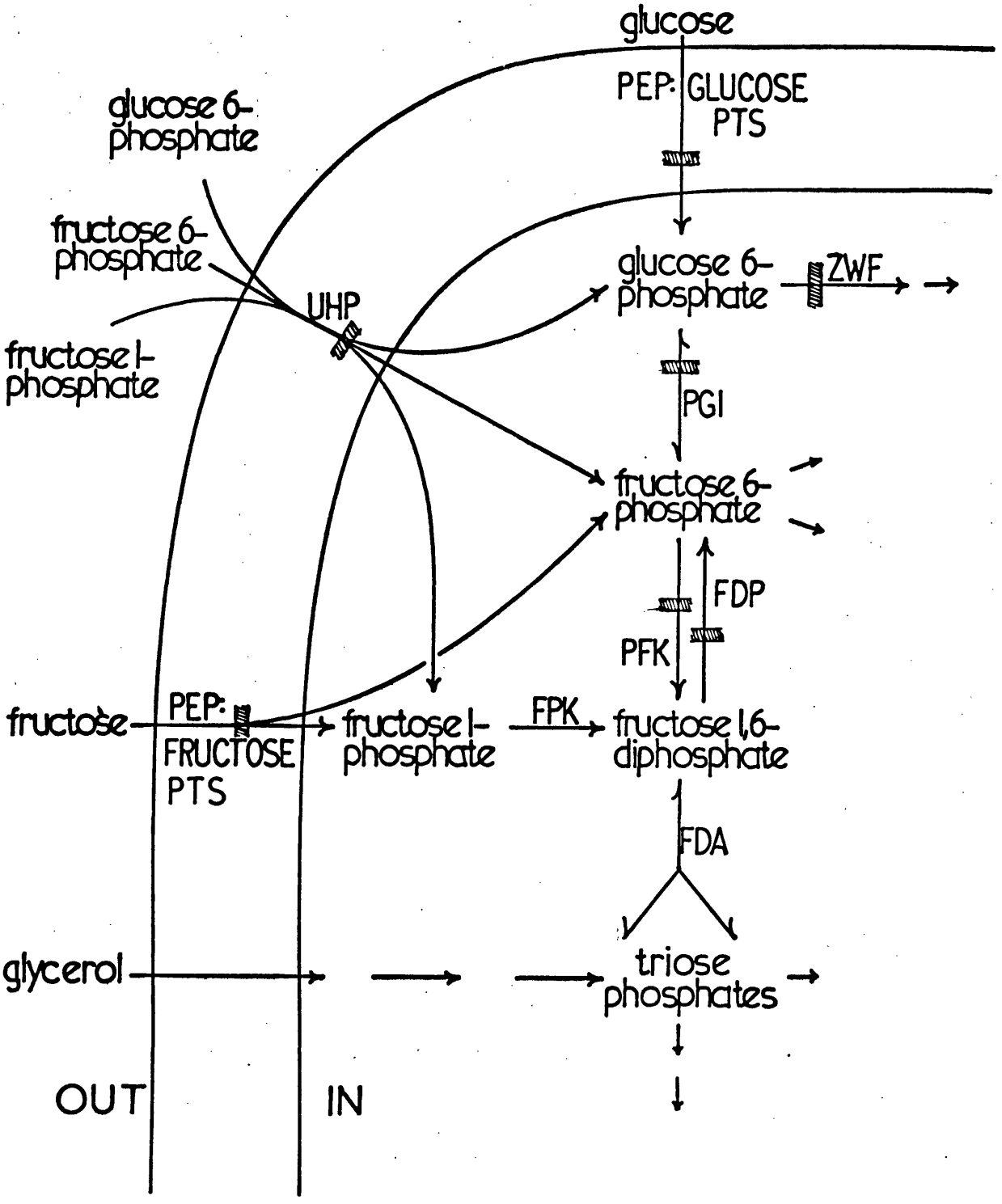
glucose
PEP: GLUCOSE PTS
glucose 6-phosphate
fructose 6-phosphate
UHP
glucose 6-phosphate
ZWF
fructose 1-phosphate
PGI
fructose 6-phosphate
FDP
PFK
fructose
PEP: FRUCTOSE PTS
fructose 1-phosphate
FPK
fructose 1,6-diphosphate
FDA
glycerol
triose phosphates
OUT
IN

FIGURE 1 A scheme for the area of carbohydrate utilization described in this thesis. The hatched bars indicate steps that are missing in the mutants listed in Table 1.

stored at room temperature. Cultures were routinely checked by streaking onto suitable selective media to ensure that revertants or contaminants were not present.

(ii) Growth of organisms

Liquid cultures were obtained by inoculation from the slope into sterile basal salts medium (Ashworth and Kornberg, 1966) containing the appropriate carbon source at 25 mM, unless otherwise stated and supplementary growth factors at 40-100 µg/ml. Cultures were also grown in Oxoid nutrient broth. Incubation was in Erlenmeyer flasks containing not more than two-fifths of their volume as liquid, at 37° in thermostatically maintained reciprocating shakers. For growth curves and uptake studies, exponentially growing cultures were obtained by dilution of overnight cultures in the same medium. For growth in Petri dishes, the basal medium was solidified by the addition of 1% Oxoid No. 1 agar. Basal salts medium, agar, growth factors and carbon sources were sterilized separately by autoclaving for 20 min at 15lb/in^2 and were mixed at 50°. Sugar phosphates and streptomycin sulphate were sterilized by membrane filtration.

For growth curves, the rate of doubling was measured as the change in extinction at 680 nm in a Unicam SP600 spectrophotometer, using 3 ml cuvettes of 1 cm light path. At this wavelength, extinction is related to cell dry weight by a factor previously obtained (Ashworth and Kornberg, 1966): $E_{680nm} = 1.0$ is equivalent to 0.68 mg dry wt/ml. Cells were centrifuged at room temperature, resuspended and washed with basal medium, recentrifuged and resuspended in prewarmed medium containing carbon source at the concentrations indicated to an extinction of 0.1 - 0.15 at 680nm. Growth experiments were not performed aseptically because of the large inocula used.

(iii) Uptake Studies

The method used for studying the uptake of fructose or hexose phosphates entailed measurement of the rate of accumulation by suspensions of bacteria, of isotope from appropriately labelled radioactive substrates. Samples of bacteria were taken at known times by membrane filtration and the radioactivity accumulated in the cells on the filter was determined.

The preparation of washed cell suspensions and the method of sampling were essentially by the techniques of Morgan & Kornberg (1969). For routine uptake studies, the concentration of the ^{14}C-labelled substrate in the medium was 0.1 mM, of specific activity 0.29 μCi/ml, 0.25 μCi/ml and 0.25 μCi/ml for fructose, fructose 6-phosphate and glucose 6-phosphate respectively. The samples of the bacteria on membrane filters were dissolved in 3 ml of Bray's fluid (Bray, 1960) and their radioactivity was assayed in a Packard Model 4000 scintillation spectrometer. The isotope content of a sample of the external uptake medium was always measured under the same conditions; results are expressed as nmoles of substrate taken up per minute per mg dry weight of cells.

For the determination of kinetic parameters, duplicate initial rates of uptake at each concentration of substrate during the first 20 or 30 seconds were measured. These experiments were performed on a smaller scale, with a total volume of 0.4 ml in a test tube shaken at 37° in a water bath. Corrections were applied for the binding of label non-specifically to cells and filters; these blank values were determined at each concentration of substrate. Values obtained were 25 counts per min at 0.1 mM fructose, increasing to 60 counts per min at 5.0 mM fructose, which were obtained by substituting cells kept at 90-100° for 5 min in the normal uptake procedures. Background counts for fructose 6-phosphate and glucose 6-phosphate uptakes ranged from 35 cpm

at 0.1 mM to 45 at 0.3 mM.

Qualitative differences in the ability to accumulate radioactive substrates were also detected in cells growing on agar plates. The level of accumulation of radioactive substrate, as measured by autoradiography, could be used to distinguish between mutants that could or could not take up fructose or between clones that had inducible or constitutive uptake systems for hexose phosphates. It was in this latter role that the technique was most useful. The method involved growing patched clones of the strains under study on agar plates containing growth factors with 5 mM glucose as the major carbon source plus 20 μM [U-^{14}C]-fructose 6-phosphate (0.05 μCi/ml). After overnight growth, the clones were adsorbed onto sterile Whatman No. 40 filter paper. The papers were dried and left in contact with Kodak 'Blue-Brand' X-ray film for 24-48 hours. After development and fixing, the film, as can be seen in Figure 25 , was considerably more blackened by contact with constitutive clones than with the inducible strains.

The rates of fructose (and glucose) incorporation and removal from the medium by exponentially growing cultures of E. coli were also determined by the use of ^{14}C-labelled sugars. Usually, 5 mM concentrations of the sugar were used, of specific activity 0.2 μCi/ml. The amounts of fructose or glucose incorporated or removed from the medium were determined by membrane filtration of samples of growth medium.

(iv) Enzyme Assays

FRUCTOSE PHOSPHORYLATION - (i) Manno(Fructo)Kinase

This enzyme was assayed by the method of Sebastian & Asensio (1967), with their described preparation of cell-free extracts and spectrophotometric assay.

All spectrophotometric assays were performed at room temperature on a Hilger-Gilford modification of a Unicam SP500 recording spectrophotometer.

(ii) Phosphoenolpyruvate-dependent phosphotransferase system (PTS) for fructose phosphorylation

The phosphoenolpyruvate (PEP)-dependent phosphorylation of fructose was assayed in three ways. Total activity was measured by following the formation of [^{14}C]fructose-phosphate from [^{14}C]fructose, (method A), or by the fructose-dependent release of pyruvate, which was the product assayed (method B). The latter was the more convenient, though less sensitive, method and was routinely used. PEP-dependent fructose 6-phosphate formation was assayed by the spectrophotometric determination of fructose 6-phosphate after a stop-assay (method C).

Disruption of cells for the assay of the PTS by the usual techniques of sonic vibration or by passage through a French Pressure Cell leads to a significant loss of phosphorylative ability. Decryptification of the PTS in E. coli either by the freezing and thawing techniques of Ghosh & Ghosh (1968) or the toluene treatment of Gachelin (1969) gives 10-20 times the phosphorylative activity towards fructose obtained by Fraenkel (1968a), who used sonication for the disruption of cells. Both decryptification procedures, which were the techniques used for the disruption of cells, were carried out in 100 mM phosphate buffer, pH 7.5. For freezing and thawing, cell concentrations were 30-40 mg dry wt/ml and for toluene treatment 1-3 mg dry wt/ml.

METHOD A Assays at 37° contained, in a total volume of 0.8 ml, in μmoles: [U-^{14}C]fructose (2.9 μCi/ml), 1.0; PEP, 2.5; magnesium chloride, 0.5; potassium fluoride, 100; potassium phosphate, pH 7.5, 20. 0.1 ml cells, toluenized or frozen and thawed, were added to start the reaction,

which was stopped at known times by the addition of 0.2 ml of 0.5 M unlabelled fructose. After centrifugation to remove cell debris, 0.1 ml of the supernatant solution was analysed for fructose phosphate content after ion exchange chromatography. This was performed using Dowex 1 chloride columns (0.5g Dowex 1, chloride form, Bio-Rad Ag1-X8, 100-200 mesh, column size 2 cm x 0.5 cm) by an adaptation of the procedure described by Phibbs & Eagon (1970). After application of 0.1 ml samples, the columns were washed with 3 x 1 ml of water to remove free sugars. The sugar phosphates were then eluted by washing the columns with 3 x 1 ml of 1M lithium chloride. This latter eluate was collected in a scintillation vial and the sugar phosphates measured by counting in the Packard scintillation spectrometer after addition of 10 ml of Bray's fluid. The radioactivity of 0.1 ml of the unseparated sample was similarly assayed, in 3 ml of 1M lithium chloride and 10 ml of Bray's fluid, so that the results obtained could be quantitated.

METHOD B This complete assay system, incubated at 37°, contained in a final volume of 1 ml, in μmoles: fructose, 1; PEP, 1; potassium fluoride, 100; magnesium chloride, 0.5; sodium arsenite, 10; potassium phosphate, pH 7.5, 20. The reaction was started by adding decryptified cells, and stopped at known intervals by the addition of 0.33 ml of 0.1% (w/v) 2,4 dinitrophenylhydrazine in 2N hydrochloric acid followed by the addition of 1.67 ml of 2.5N sodium hydroxide 15 min later. The pyruvate 2,4 dinitrophenylhydrazone content (Friedemann & Haugen, 1943) of each was obtained after centrifugation to remove cell debris, by measuring the ΔA_{445nm} in an SP600 spectrophotometer (measurements of standard solutions had shown ΔA_{445nm} of 0.7, in cuvettes of 1 cm light path, 3 ml volume, to be equivalent to 0.1 μmole of pyruvate). Each assay was read against a blank, incubated as above with all components of the complete system except fructose. Fructose by itself

at concentrations of 1 mM and below gives negligible reaction in the assay, but at higher concentrations the interference cannot be ignored. METHOD C The complete system contained in 1 ml, in μmoles: fructose, 50; PEP, 1.25; magnesium chloride, 1.0; potassium phosphate pH 7.5, 50. Reaction at 37° was started by the addition of toluene-treated cells and stopped at known times by rapid membrane filtration through Sartorius 0.45μ pore size filters. The filtrates were assayed for glucose 6-phosphate and fructose 6-phosphate; these assays involved the spectrophotometric determination at 340nm of the reduction of NADP in the presence of glucose 6-phosphate dehydrogenase and phosphoglucose isomerase. Contents of this assay, total volume 1 ml: NADP, 0.6 μmole; iminazole-HCl pH 7.2, 100 μmole; crystalline glucose 6-phosphate dehydrogenase, 6.25 μg and phosphoglucose isomerase, 6.25 μg.

Fructose 1-phosphate kinase (1-phosphofructokinase, FPK)

Cell-free extracts were prepared for the assay of this enzyme, after washing harvested cells in 1% (w/v) KCl, by resuspension in buffer pH 7.5 containing 10 mM tris-HCl, 10 M $MgCl_2$ and 2 mM mercapto-ethanol and disruption at 0-4° by exposure for 2 min to the output of a MSE 100W sonicator. Cell debris was removed by centrifugation for 1 hour at 30,000g, which also removed most of the NADH oxidase activity. The enzyme was assayed in a system containing, in a final volume of 1 ml, in μmoles: iminazole-HCl buffer pH 7.2, 67; magnesium chloride, 10; fructose 1-phosphate, 2.5; ATP, 1.25; NADH, 0.15; and also crystalline triosephosphate isomerase + α-glycerolphosphate dehydrogenase, 20 μg and crystalline aldolase, 12.5 μg. Under these conditions, the phosphorylation of 1 μmole of fructose 1-phosphate was taken to be equivalent to the oxidation of 2 μmoles of NADH, which was measured spectrophotometrically as ΔA_{340nm}.

The same assay was used for 6-phosphofructokinase, with 1 mM fructose 6-phosphate substituted for the fructose 1-phosphate in the system above.

Phosphoglucose Isomerase

Cell-free extracts for the assay of this enzyme were prepared as for FPK assays. The reaction was measured at 340nm by following the reduction of NADP in a system containing in 1 ml: 67 µmoles of iminazole-HCl pH 7.2; 10 µmoles of $MgCl_2$; 0.25 µmole of NADP; 6.25 µg of glucose 6-phosphate dehydrogenase and 2.5 µmoles of fructose 6-phosphate.

Fructose 1,6 Diphosphatase

This enzyme was assayed by the method of Fraenkel & Horecker (1965). The preparation of cell-free extracts was, however, modified and cells were sonicated in 50 mM phosphate buffer pH 6.9 which also contained 1 mM EDTA, 1 mM mercaptoethanol and 0.5 mM magnesium chloride. Protein estimation was by the method of Lowry, Rosebrough, Farr & Randall (1951).

(v) GENETIC TECHNIQUES

Mutagenesis Mutants of E. coli were induced with ethyl methanesulphonate (Lin, Lerner and Jorgensen, 1962). To increase the proportion of mutant to parental cells, mutagenised cultures were treated with penicillin (Gorini & Kaufman, 1960). The carbon sources used in the selection of specific mutants are described in the appropriate part of the text.

Bacterial matings in liquid medium Genetic crosses by interrupted conjugation were carried out as described by Brice & Kornberg (1967).

Methods of selection of specific recombinants are described in the appropriate parts of the text.

Transduction Studies Phage P1kc (laboratory stock) was used in these experiments. The maintenance and preparation of P1kc lysates and the procedure for the phage mediated transduction were essentially those used by Brice & Kornberg (1967). The conditions used for selection of transductants are described in Chapter IV.

Selection of Thy^- Mutants These were derived from the mutant K2.21 No. 4 and K2.1.23 (Table 1) by treatment with trimethoprim (Stacey & Simson, 1965).

CHAPTER I - PATHWAY OF FRUCTOSE UTILIZATION IN *E. coli*

(i) Introduction

The metabolism of fructose has been studied for many years, especially in mammalian systems. Its widespread occurrence and ready utilizability made it of interest to early nutritionists, and studies of its intermediary metabolism soon followed. (For reviews of early work, see Racker, 1954; Hers, 1957). Fructose had also long been known to support the growth of bacteria (including E. coli) and fructose fermentation was often used as a diagnostic test in early bacteriology. However, studies of the reactions involved in the utilization of fructose by bacteria are more recent and will be discussed below.

It soon became clear that there were two major possibilities for the initial step in the metabolism of fructose, its phosphorylation at the 1 or 6 hydroxyl group. Conversion to fructose 6-phosphate results in direct entry into the Embden-Meyerhof pathway (Axelrod, 1960) while fructose 1-phosphate has to undergo at least one other reaction before it can enter glycolysis. Both routes are found in animals, though segregated in different tissues; likewise, both pathways are found in bacteria, though usually in different species. The reactions involved will be considered in turn.

Fructose to fructose 6-phosphate The earliest, and perhaps best known, enzyme which catalyses this reaction is the hexokinase (ATP:D-hexose 6-phosphotransferase, EC.2.7.1.1.) found in yeast (Berger et al., 1946; Kunitz and McDonald, 1946). This enzyme, which requires ATP and Mg^{2+} for its activity, shows high affinity for fructose, and also phosphorylates mannose and glucose. Enzymes with similar properties are present in some animal tissues like muscle and brain (Slein et al., 1950) and also in sperm. However, all these enzymes are strongly inhibited by glucose and

so probably do not play an important role in fructose utilization in most tissues. The important exception is in sperm, which use mainly fructolysis to provide energy for their motility (Mann, 1964). Semen contains little glucose but does contain fructose, which is broken down to lactate by the Embden-Meyerhof pathway.

In bacteria, a long-standing problem has been an inability to demonstrate kinases for the phosphorylation of fructose and other hexoses which support growth, what Wood (1966) called "the mystery of the missing kinases". However, ATP-dependent kinases phosphorylating fructose in the 6-position have come to light in a few species (ATP:D-fructose 6-phosphotransferase, EC2.7.1.4); these kinases are usually specific for fructose, in contrast to the hexokinases found in yeast and animals. Doudoroff et al. (1956) found a fructokinase in extracts of Pseudomonas saccharophila and more recently, Phibbs and Eagon (1970) described a fructose-induced fructokinase in P. aeruginosa. Other species found to have this type of kinase include Rhodopseudomonas spheroides (Szymona and Doudoroff, 1960), Alcaligenes faecalis (Domagk and Horecker, 1965), Mycobacterium phlei (Szymona and Szumilo, 1966) and Leuconostoc mesenteroides (Sapico and Anderson, 1967). The enzyme in this last bacterium also phosphorylates D-mannose. Attempts have been made to demonstrate such kinases in the Enterobacteriacae, but the results, on the whole, have been unsatisfactory in accounting for the fast growth rates obtained on fructose. Aerobacter aerogenes, contrary to earlier reports, (Kamel and Anderson, 1966), does have an ATP-dependent fructokinase, but this enzyme is induced by growth on sucrose and not normally during growth on fructose, so the role of this enzyme in exogenous fructose utilization is dubious (Kelker, Hanson and Anderson, 1970). In E. coli a constitutive mannokinase, with some phosphorylative activity towards

FIG.2

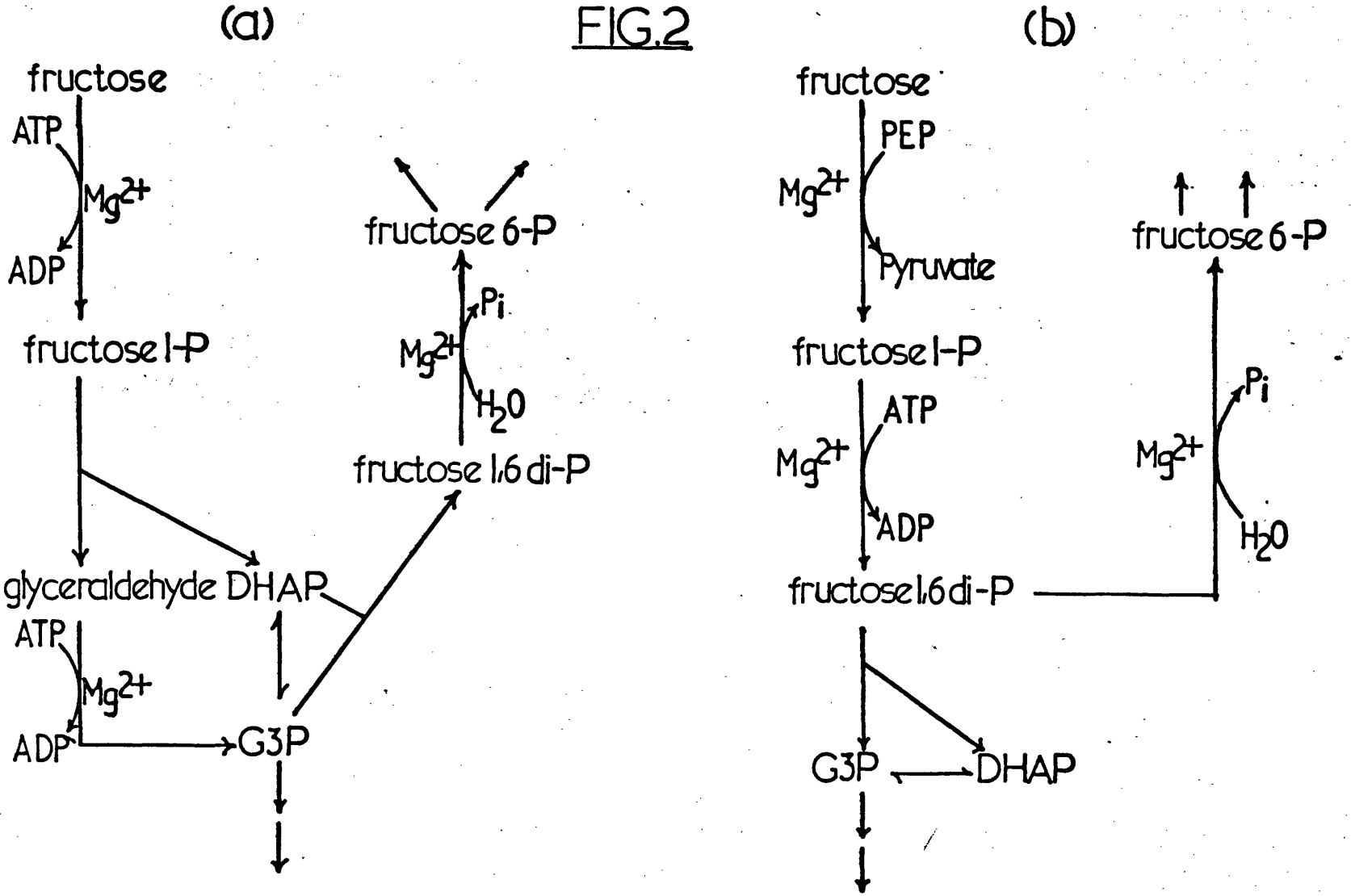
(a)
fructose
ATP
Mg^{2+}
ADP
fructose 1-P
fructose 6-P
Pi
Mg^{2+}
H_2O
fructose 1,6 di-P
glyceraldehyde DHAP
ATP
Mg^{2+}
ADP
G3P
(b)
fructose
PEP
Mg^{2+}
Pyruvate
fructose 1-P
ATP
Mg^{2+}
ADP
fructose 1,6 di-P
fructose 6-P
Pi
Mg^{2+}
H_2O
G3P
DHAP

FIGURE 2 Sequence of reactions in the metabolism of fructose *via* fructose 1-phosphate in (a) mammalian liver and (b) bacteria (see text for references).

fructose, has been identified by Sebastian & Asensio (1967), and the possible role of this enzyme in fructose metabolism is discussed later in this chapter.

Fructose to fructose 1-phosphate Pathways of fructose metabolism thought to proceed via fructose 1-phosphate are presented in Figure 2. In mammalian systems, the quantitatively most important route of fructose utilization is by the sequence of reactions sometimes known as the Hers pathway, which is catalysed by a set of enzymes found in the liver (Hers, 1957; Heinz, Lamprecht & Kirsch, 1968). As indicated in Figure 2a, the first step is catalysed by a ketohexokinase (ATP:D-fructose 1-phosphotransferase EC.2.7.1.3) (Staub & Vestling, 1951; Hers, 1952), which does not phosphorylate glucose. This enzyme is present in greater activity than glucokinase in the liver and probably explains why fructose can be utilized faster than glucose by this organ. Fructose 1-phosphate can be cleaved by an aldolase, aldolase 1B in the nomenclature of Rutter (1964) to dihydroxyacetone phosphate and glyceraldehyde. Both triose moieties derived from fructose can enter glycolysis because glyceraldehyde can be phosphorylated by triokinase to give glyceraldehyde 3-phosphate.

In bacteria, the reactions both for the synthesis and the further metabolism of fructose 1-phosphate have been found to be different, as shown in Figure 2b. The possible significance of fructose 1-phosphate in bacterial metabolism was only recently recognised, with the finding of the enzyme 1-phosphofructokinase (fructose 1-phosphate kinase, ATP:D-fructose 1-phosphate 6-phosphotransferase, abbreviated FPK); in Bacteroides symbiosus by Reeves, Warren & Hsu (1966):

$$\text{Fructose 1-phosphate} + \text{ATP} \xrightarrow{Mg^{2+}} \text{Fructose 1,6-diphosphate} + \text{ADP}.$$

Enzymes with similar activities have been detected in A. aerogenes (Hanson and Anderson, 1966), E. coli (Fraenkel, 1968a), Clostridium pasteurianum (Kotze, 1968), Clostridium thermocellum (Patni and Alexander, 1971) and Bacillus subtilis (Gay, Carayon and Rapoport, 1970). In all these bacteria, the level of activity of this enzyme is greater during growth on fructose than on other carbon sources, which implicates it in fructose metabolism.

The reactions for the formation of fructose 1-phosphate, in most if not all of the bacteria mentioned above, have also been clarified recently and found to involve the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) first described by Kundig, Ghosh and Roseman, (1964):

$$\text{PEP} + \text{HPr} \xrightarrow{\text{Enzyme I, } Mg^{2+}} \text{P - HPr} + \text{Pyruvate}$$

$$\text{P - HPr} + \text{sugar} \xrightarrow{\text{Enzyme II, } Mg^{2+}} \text{P - sugar} + \text{HPr}$$

where HPr is a heat stable protein cofactor, Enzyme I catalyses the PEP-dependent phosphorylation of HPr and Enzyme II catalyses the transfer of phosphate from P-HPr to a specific sugar. The system was initially found to phosphorylate N-acyl-D-mannosamine, glucose, mannose, glucosamine and mannosamine (Kundig, Ghosh and Roseman, 1964) in a number of bacteria but was later also implicated in the transport of sugars in E. coli (Kundig, Kundig, Anderson and Roseman, 1966). This aspect of the PTS as a mediator of sugar uptake will be discussed in more detail in Chapter II.

The full role of the PTS in the initiation of metabolism of sugars became clear through the work of Tanaka and Lin (1967), who showed that three classes of mutation could affect mannitol metabolism in

A. aerogenes. Two of the classes were pleiotropic, that is the mutations affected growth on sorbitol, glucose, mannose, fructose and mannitol: these were shown to be due to the absence of Enzyme I and HPr respectively. The third class was affected only in mannitol metabolism and lacked Enzyme II of the PTS for mannitol (Tanaka, Lerner & Lin, 1967). Numerous cases have been reported of a mutation that renders a bacterium like E. coli unable to metabolize several sugars, including fructose, even though enzymes responsible for their degradation could be demonstrated in extracts; at least in some of these cases the lesion has been shown to be in Enzyme I or HPr of the PTS. (For a list of references, see Anderson & Wood, 1969).

The significance of the PTS in fructose metabolism in A. aerogenes has been demonstrated directly by Hanson & Anderson (1968), who have also provided strong evidence that the pathway shown in Figure 2b operates in this bacterium (Hanson & Anderson, 1966; Sapico, Hanson, Walter & Anderson, 1968; Kelker, Hanson & Anderson, 1970). Evidence for the occurrence of this pathway in A. aerogenes includes:

(a) the PEP:Fructose PTS, which catalyses the formation of fructose 1-phosphate, can be resolved into four components, one or possibly two of which are induced by fructose. A mutant lacking an inducible component of the PTS exhibits defective growth on fructose, but not on other hexoses.

(b) a fructose-induced FPK can be demonstrated to be present in cell-free extracts of the bacteria; mutants lacking this enzyme are defective in growth on fructose but not other sugars.

(c) mutants lacking 6-phosphofructokinase (EC 2.7.1.11) grow well on fructose but mutants lacking the enzyme fructose 1,6-diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase,,EC 3.1.3.11) do not, as

would be expected from a pathway via fructose 1-phosphate (see Figure 2b).

Fraenkel (1968a) has provided some evidence that this same pathway functions during fructose utilization by E. coli. He demonstrated fructose 1-phosphate formation by the PEP-dependent PTS, and also showed that FPK was present in extracts of E. coli, which indicated that the pathway of fructose utilization is fructose ⟶ fructose 1-phosphate ⟶ fructose 1,6-diphosphate. Also, mutants devoid of 6-phosphofructokinase activity grow well on fructose (Morrissey and Fraenkel, 1968; Kornberg and Smith, 1970), in agreement with this scheme. However, in E. coli, a number of reports have indicated that mutants lacking fructose 1,6-diphosphatase (FDPase) grow at virtually wild-type rates on fructose (Fraenkel and Horecker, 1965; Fraenkel, 1967). This is not in agreement with the suggestions that the pathway of fructose metabolism is solely via fructose 1-phosphate and as Fraenkel (1968a) rightly concludes, "on balance, most of the available data on fructose metabolism in E. coli and A. aerogenes favor the major pathway being via fructose 1-phosphate. But in order to accommodate all the data more mutant analysis and dissection of the enzyme systems will be necessary". It is the purpose of this chapter to give the results of such work and to show that, in E. coli, a pathway via fructose 6-phosphate also operates in the metabolism of fructose.

(ii) Additional evidence for fructose metabolism via fructose 1-phosphate

As already mentioned, Fraenkel (1968a) has provided evidence that the enzymes required for the metabolism of fructose via fructose 1-phosphate are present in E. coli. If this meant that the pathway of

TABLE 2 PEP-dependent phosphotransferase activity in KL16 and KL16.21

Organism	Carbon source for growth	PEP:fructose PTS activity*	PEP:glucose PTS activity**
KL16	Fructose + glycerol	21.0	-
KL16	Glucose	2.5	41.0
KL16.21	Fructose + glycerol	< 1.0	-
KL16.21	Glucose	< 1.0	37.5

The phosphotransferase activity was followed by sugar-dependent pyruvate release (Method B, Materials and Methods).

* nmole fructose phosphorylated x min^{-1} x mg dry weight of $cells^{-1}$.

** nmole glucose phosphorylated x min^{-1} x mg dry weight of $cells^{-1}$.

fructose utilization described in Figure 2b was essential for fructose utilization, it would be expected that mutants could be obtained that were impaired in their growth on fructose, but not on any other carbon source. Such mutants were selected (Materials and Methods) by EMS mutagenesis of KL16 and penicillin treatment using fructose as carbon source. The mutagenised organisms were plated onto glucose-minimal agar plates and replica plated onto fructose-minimal agar plates. The clones that could grow on glucose but not on fructose were picked and purified by single colony isolation. The growth properties of one mutant thus obtained, KL16.21, were further investigated. The mutant grew normally on agar plates and in liquid culture on glucose and other hexoses, gluconate, glycerol, hexose phosphates and hexitols. It did not grow at all on 10 mM, or any other concentration, of fructose but did grow normally on 5 mM fructose 1-phosphate (Figure 21), when previously grown on glucose 6-phosphate as described in Chapter IV.

These growth characteristics of KL16.21 suggest that the lesion in this mutant is in a step (or steps) solely involved in fructose utilization. Assuming that the pathway shown in Figure 2b operates in E. coli, the ability of KL16.21 to grow on fructose 1-phosphate would suggest that it is blocked in the formation of fructose 1-phosphate from fructose, but not in its subsequent metabolism to fructose 1,6-diphosphate. This further pinpointed the lesion to, probably, the PEP:fructose phosphotransferase system. This prediction was tested by assaying in vitro the two functions involved.

Table 2 shows the results of assays of the PEP:fructose PTS, by Method B (Materials and Methods), using frozen and thawed cells. The results indicate that as compared with the parent KL16 grown under the same conditions, the level of PEP-dependent phosphorylation of fructose is very low in KL16.21 grown under the same conditions; it is

TABLE 3 Inducible formation of fructose 1-phosphate kinase in KL16 and KL16.21

Carbon source for growth	Specific activity of FPK* in		Increase in specific activity (-fold)	
	KL16	KL16.21	KL16	KL16.21
Glucose 6-phosphate	14	4	-	-
Fructose 1-phosphate	111	39	8	9
Fructose	138	n.g.	10	n.g.

* nmoles of fructose 1-phosphate phosphorylated x min^{-1} x mg of $protein^{-1}$.

n.g. = no growth, hence this assay could not be done.

also lower than the phosphorylation obtained with KL16 grown under conditions that do not induce the PTS. That the lesion in KL16.21 is in the fructose-specific component (possibly Enzyme II) of the PTS is indicated not only by the finding that the PEP-dependent phosphorylation of glucose by the PTS in KL16.21 (Table 2) is functional, but also by the normal growth on the other carbon sources (including mannitol) which are thought to be activated by the PTS.

The results of assays of FPK activity (Materials and Methods) in cell-free extracts of KL16 and KL16.21 are shown in Table 3. The levels of the enzyme in KL16 and KL16.21 before and after growth on 5 mM fructose 1-phosphate are indicated; in each case there is an 8-10 fold increase in the specific activity of FPK after $1\frac{1}{2}$ generations on this carbon source. However, the activity of FPK in KL16.21 is about a third of that obtained in the wild-type; no higher specific activity than about 40 nmoles of fructose 1-phosphate phosphorylated per min per mg protein have been measured in KL16.21 under any other growth conditions that might induce the enzyme, such as growth on fructose + glycerol. There are a number of possible explanations for this finding; firstly, the lesion in KL16.21 may have a polar effect (Zipser, 1969) on the fructose system, but as yet there is no evidence that the genes specifying the fructose-specific protein of the PEP: fructose PTS and FPK form an operon. Secondly, it is possible that the intracellular level of inducer for FPK (which may be fructose or fructose 1-phosphate) is below the required level for full induction of this enzyme. This may be because during growth on fructose 1-phosphate, which is poorly transported (see Chapter IV), the intracellular pool of the inducer may be low. Similarly, in the case of

KL16.21, the presence of external fructose, which is not phosphorylated (Table 2) or accumulated (see Chapter II) by this organism would not give rise to a level of induction found in wild-type cells during growth in the presence of fructose.

The observation that E. coli mutants unable to utilize free fructose can grow on fructose 1-phosphate as a sole carbon source, and hence use that hexose phosphate without prior dephosphorylation, is by itself good evidence that fructose 1-phosphate plays a role in E. coli metabolism (see Chapter IV). As growth on fructose 1-phosphate is accompanied by induction of FPK, the metabolic role of this enzyme in the utilization of fructose 1-phosphate is indicated. Also, as there is good evidence for the phosphorylation of fructose to fructose 1-phosphate by the PTS (Fraenkel, 1968a) and as the importance of the PTS in fructose metabolism is confirmed by the behaviour of KL16.21, there can be little doubt of the functional significance of the steps:

$$\text{Fructose} \longrightarrow \text{fructose 1-phosphate} \longrightarrow \text{fructose 1,6-diphosphate}$$

during the utilization of fructose by E. coli.

(iii) The role of fructose 1,6-diphosphatase in fructose utilization

It would be expected from the above sequence of reactions that the biosynthesis of carbohydrates such as glycogen, pentoses or cell-wall polysaccharides from fructose would require the formation of fructose 6-phosphate from fructose 1,6-diphosphate and that the step would involve fructose 1,6-diphosphatase (FDPase):

$$\text{Fructose 1,6-diphosphate} + H_2O \xrightarrow{Mg^{2+}} \text{fructose 6-phosphate} + Pi$$

However, although E. coli mutants devoid of FDPase activity do not grow

FIG. 3

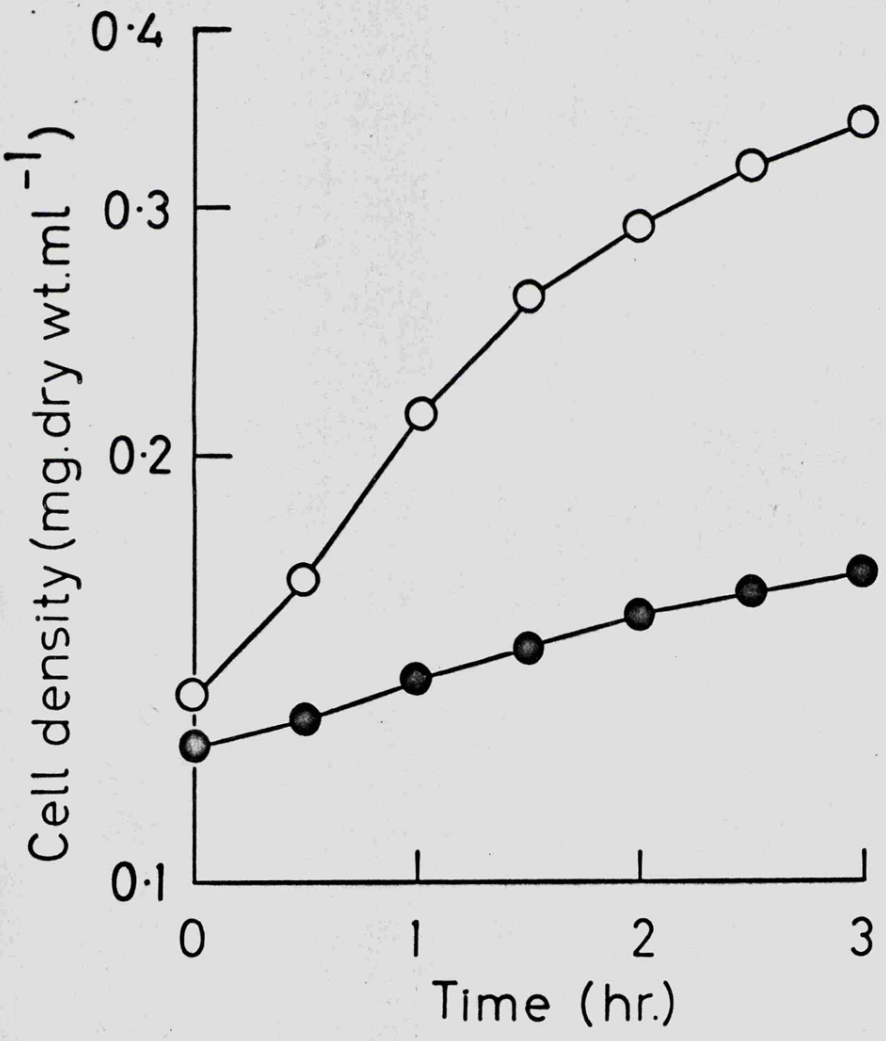

FIGURE 3 Growth of the wild-type strain K2 (○) and of the mutant K2.19 (●), devoid of fructose 1,6-diphosphatase activity, on media containing 5 mM fructose 1-phosphate as sole carbon source.

TABLE 4 Fructose 1,6-diphosphatase activities in K2 and K2.19

Organism	Carbon source for growth	Specific Activity*
K2	Fructose	12
K2	Glycerol	14
K2.19	Fructose	0.6
K2.19	Glycerol	n.g.

* Specific activity of FDPase is here expressed as nmoles of NADP reduced x min^{-1} x mg $protein^{-1}$ in the assay of Fraenkel and Horecker (1965).

n.g. = no growth, hence this assay could not be done .

on substrates such as glycerol, lactate, acetate or C_4-acids, such mutants grow at virtually wild-type rates on fructose (Fraenkel and Horecker, 1965; Fraenkel, 1968a). These growth properties were also confirmed in the mutant K2.19 (Table 1), an independently selected mutant that lacks FDPase activity. This mutant was derived from K2 by EMS mutagenesis and penicillin selection (Materials and Methods) for inability to grow on glycerol or lactate whilst retaining the ability to grow on glucose. The levels of the enzyme in mutants and wild-type, assayed as described in Materials and Methods, are shown in Table 4.

In addition to these usual carbon sources, growth was also checked in liquid cultures on fructose 1-phosphate. K2 behaved as expected when transferred from growth on glucose 6-phosphate to 5 mM fructose 1-phosphate (see Chapter IV): rapid doubling was obtained for more than one generation [Figure 3]. However, when K2.19, which grows normally on glucose 6-phosphate, is transferred to 5 mM fructose 1-phosphate, no such rapid growth can be demonstrated. Thus fructose 6-phosphate could not arise from fructose 1-phosphate in K2.19, although it could in K2: hence FDPase activity is necessary for the utilization of fructose 1-phosphate as carbon source for growth under conditions where it is not necessary for the utilization of fructose. This confirmed <u>in vivo</u> the <u>in vitro</u> findings that, under a variety of conditions, no fructose 6-phosphate formation from fructose 1-phosphate could be detected in cell-free extracts of fructose-grown <u>E. coli</u> (also Fraenkel, 1968a).

In experiments to test whether the growth of K2.19 on fructose 1-phosphate could be stimulated by the addition of fructose

FIG.4

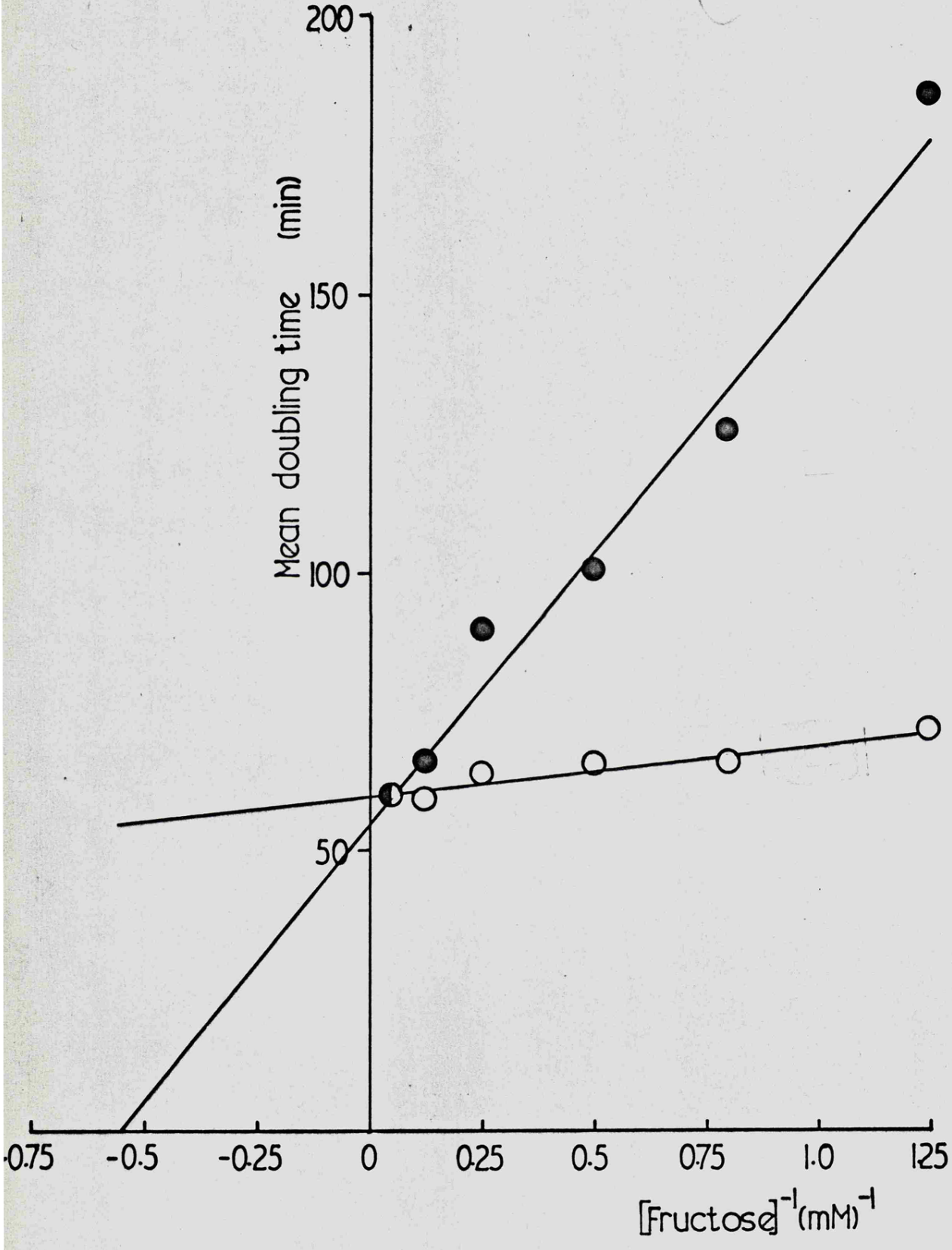
200
150
100
50
Mean doubling time (min)
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1.0
1.25
[Fructose]$^{-1}$ (mM)$^{-1}$

FIGURE 4 Growth of the wild-type strain K2 (O) and of the mutant K2.19 (●), devoid of fructose 1,6-diphosphatase activity, on media containing fructose as sole source of carbon. The data are expressed as the reciprocals of the maximum growth rates attained at various concentrations of fructose, plotted against the reciprocals of those fructose concentrations.

to the growth medium, it was found that low concentrations of fructose did not increase the growth rate. As a consequence of this finding, the growth of K2 and K2.19 was followed at a number of fructose concentrations, in the absence of fructose 1-phosphate. Although the mutant grew on a medium containing 20 mM fructose at a rate indistinguishable from the wild-type parent K2, K2.19 grew considerably slower when media containing lower concentrations of fructose were used. This sharp decrease in growth rate was not exhibited by the parent organism. The patterns are illustrated in Figure 4: when the least mean doubling times for the growth of these organisms on media of various fructose concentrations (i.e. the reciprocals of the maximal growth rates attained) were plotted against the reciprocal of those fructose concentrations, the results obtained were similar to those given by Lineweaver-Burk (Lineweaver and Burk, 1934) plots of enzyme kinetics. As can be seen, the maximal growth rate achievable on fructose ("V_{max}") is not greatly affected by the absence of FDPase; however, half this maximal rate was attained by the mutant only at approximately 1.8 mM fructose ("K_m"), whereas in the wild-type, 0.16 mM sufficed.

That this decreased growth rate at low fructose concentrations was due solely to the loss of FDPase activity in K2.19, was shown in two ways. Firstly, organisms derived from K2.19 by reversion to growth on glycerol were shown to regain simultaneously the ability to grow on low fructose concentrations, tested by growth on 0.5 mM fructose-minimal agar plates. Revertants were obtained by plating approx. 10^9 cells of K2.19 on glycerol-minimal agar plates; of the 25 revertants obtained after 48 hr at 30°, all regained the ability to grow on the low concentrations of fructose.

Secondly, the same pattern was shown also by recombinants of K2.19 obtained from two genetic crosses. The Hfr strain AB674 transfers its genome to recipient F^- cells in the order o-argH-fdp....; strain K10 injects in the reverse order, o-leu-thr-fdp..., the markers being located as shown in the linkage map in Figure 6. Both strains were crossed with the mutant K2.19 and, after periodic interruption of conjugation, recombinants were selected on plates containing (a) glucose as carbon source for arg^+ and thr^+leu^+ respectively, and (b) glycerol as carbon source for fdp^+. In all cases the male phenotype was counterselected by inclusion of streptomycin in these plates. In confirmation of previous findings (Yu, Kaney and Atwood, 1965), the fdp allele was found to enter 6 min from the arg and 6 min from thr,leu markers. All of the 195 fdp^- recombinants tested manifested the phenotype of K2.19: they grew on plates containing 20 mM fructose but failed to grow readily on plates containing 0.5 mM fructose as carbon source. Correspondingly, all of the 75 fdp^+ recombinants grew readily on either concentration of fructose. Hence both genetic approaches indicated that restoration of a single function (i.e. FDPase) results in the ability to grow both on glycerol, and on low concentrations of fructose.

These observations suggested that, at low concentrations of fructose (<2 mM), FDPase plays an important role in the generation of fructose 6-phosphate from the growth substrate, but that at higher fructose concentrations, fructose 6-phosphate can arise by reaction(s) that bypass the diphosphatase. The characteristics of growth on fructose 1-phosphate, however, suggested that fructose 6-phosphate was not formed from fructose 1-phosphate directly and that free fructose

TABLE 5 ATP:D-fructose 6-phosphotransferase activity in E. coli

Organism	Carbon source for growth	Specific Activity*
KL16	Fructose	2.5
KL16	Glycerol	2.9
KL16.21	Glycerol	1.9
KL16.23	Glycerol	4.0
K2	Glycerol	3.2
K2.19	Glycerol	2.8

* Specific activities are expressed as nmoles fructose 6-phosphate formed x min^{-1} x mg $protein^{-1}$ in the assay system of Sebastian and Asensio (1967).

was likely to be the source of the 6-phosphate. Possible reactions for the formation of fructose 6-phosphate from fructose are considered below.

(iv) Fructose phosphorylation to fructose 6-phosphate in E. coli

A number of possible mechanisms can be envisaged for the phosphorylation of fructose at the 6-position in E. coli. These might include phosphorylation by (a) an ATP-linked kinase, (b) the PEP-dependent PTS and (c) a phosphotransferase system using another phosphoryl donor.

(a) The mannokinase already mentioned (Sebastian and Asensio, 1967) is an ATP-Mg^{2+}-dependent kinase which can also phosphorylate glucose and fructose. The results shown in Table 5 confirm that such an activity (towards fructose) can be demonstrated in cell-free extracts of E. coli; the assays were performed as described by Sebastian and Asensio (1967). The levels of phosphorylative activity towards fructose in crude cell-free extracts of E. coli were found, however, to be somewhat lower than those quoted in the literature, with specific activities of about half the published values, under the same conditions of assay. The activity is constitutive and not increased after growth on fructose, unlike the PEP:fructose PTS or FPK, enzyme systems implicated in the utilization of fructose. ATP-dependent phosphorylation of fructose can still be detected in KL16.21, a mutant unable to grow on fructose and also in K2.19, a mutant which is unable to grow normally on less than 2 mM fructose, because of a lack of a supply of fructose 6-phosphate.

(b) The observation that an increase in the external concentration

TABLE 6 PEP:Fructose 6-phosphotransferase activity in E. coli

Organism	Carbon source for growth	Assay system	Specific Activity*
K2.1.5^{c}.7	Fructose	complete**	4.1
"	"	-PEP + ATP†	0.3
"	"	-Mg^{2+}	1.8
"	"	-fructose + fructose 1-phosphate†	0
"	Glucose	complete	1.1
"	Glycerol	"	0.7
K2.19	Fructose	"	2.8
KL16	Fructose + Glycerol	"	2.5
KL16	Glycerol	"	0.6
KL16.21	Fructose + Glycerol	"	0.2
KL16.21	Glycerol	"	0.3

* Specific activity is expressed as nmoles of fructose 6-phosphate formed x min^{-1} x mg dry weight of $cells^{-1}$.

** The complete system is described under Method C (Materials and Methods).

† These additions were made at a concentration of 1 mM.

of fructose stimulated the growth of K2.19 (Figure 4), suggested that the supply of fructose 6-phosphate in this organism is limited not only by a phosphorylation reaction, but also by a possible rate-limiting step at the level of fructose transport. As there is strong evidence (presented in Chapter II) that the uptake of fructose in E. coli is solely mediated by the PEP-dependent PTS, and as this mechanism of uptake involves phosphorylation, the phosphorylation of fructose by the PTS in the absence of a permeability barrier was further investigated. PEP-dependent phosphorylation of fructose to fructose 6-phosphate, at higher concentrations of substrate, was detected by a spectrophotometric assay for fructose 6-phosphate (Method C, Materials and Methods). The activities obtained are presented in Table 6.

Full fructose 6-phosphate-forming activity by the PTS required the presence of low concentrations of Mg^{2+} and was dependent on PEP, and ATP could not effectively replace PEP in the assay system. Also, fructose 1-phosphate could not replace fructose; neither was the fructose 6-phosphate formed indirectly via glucose 6-phosphate, possibly through glucose contamination of the fructose, as cells lacking phosphoglucose isomerase (in mutant $K2.1.5^{c}.7$) were still able to form fructose 6-phosphate. The PEP:fructose 6-PTS is an inducible activity, as 4-5 fold higher specific activities of the system were detected in fructose-grown cells, comparied to glucose- or glycerol-grown cells. PEP-dependent fructose 6-phosphate formation is barely detectable in mutant KL16.21 and the level of activity is not increased under normally inducing conditions in this mutant. This finding, together with the inducibility data, suggest that the PEP:fructose 6-PTS activity is unlikely to be the result of an overlapping specificity of another Enzyme II,

but is a property of the intact PEP:fructose PTS.

The concentration of fructose used in the standard assay for the formation of fructose 6-phosphate was 50 mM; at 1 mM fructose the reaction rate is only about 10% of the activity at the higher concentration. Attempts were made to obtain a measure of the dependence of the reaction rate on fructose concentration, in order to correlate the K_m for fructose in the phosphorylation reaction with the "K_m" for fructose in the growth experiments with K2.19 (Figure 4). Unfortunately, the kinetic parameters obtained for fructose phosphorylation to fructose 6-phosphate were not reproducible from cell preparation to cell preparation. Attempts to stabilize the system by the presence of sulphydryl reagents or metal ions were unsatisfactory. No reason for the variability was found; it may possibly have been due to variable changes in membrane conformation during decryptification.

(c) The properties of other sugar phosphotransferase systems found in bacteria are summarised in a review by Anderson and Wood (1969). An acyl phosphate:hexose 6-phosphotransferase has been demonstrated in extracts of A. aerogenes (Kamel and Anderson, 1966), hence acetyl phosphate was tried as a phosphoryl donor for fructose in E. coli extracts, prepared as for the FPK assays (Materials and Methods). No formation of fructose 6-phosphate was detected by the procedure used, which involved measurement of any fructose 6-phosphate formed by its isomerization to glucose 6-phosphate, and TPN-dependent oxidation to 6-phosphogluconate, using a continuous phosphoglucose isomerase-glucose 6-phosphate dehydrogenase linked assay.

(v) The relationship between the PEP:fructose 1- and fructose 6-

phosphotransferase activities in E. coli: Properties of mutant KL16.23

The growth and phosphorylative activities of E. coli so far presented suggested that the phosphorylation of fructose to fructose 6-phosphate may be of physiological significance in fructose utilization. But as fructose 6-phosphate formation in vivo (in K2.19) and in vitro (by the PEP-dependent PTS) was significant only at high fructose concentrations, it was thought possible that further mutants could be derived that showed a concentration dependence in their growth properties, i.e. no growth on low but growth on high concentrations of fructose. Mutants that lack FPK would be expected to use the pathway via fructose 6-phosphate in their utilization of fructose and hence show such a concentration dependence in their growth on this carbon source. Similarly, if the PEP:fructose 1-PTS and the PEP:fructose 6-PTS were not mediated by the same protein, mutants might be obtained by the same selection procedure that lacked the former, but retained the latter.

After EMS mutagenesis of KL16 and penicillin treatment on 1 mM fructose (see Materials and Methods), mutants that could grow on high but not on low concentrations of fructose were identified by a small colony isolation procedure. This involved plating about 200 cells of the mutagenized culture onto minimal agar plates containing 0.5 mM fructose + 0.5 mM glycerol. After three days incubation at 37°, smaller than average sized colonies were picked and tested for growth on plates containing 0.5 mM fructose, 25 mM fructose and 25 mM glycerol respectively. Clones were picked that grew on glycerol, and hence were not lacking FDPase, but were impaired in their growth on fructose at the higher concentration and did not grow at all on the lower concentrations. The properties of one mutant thus obtained, KL16.23, were investigated

FIG.5

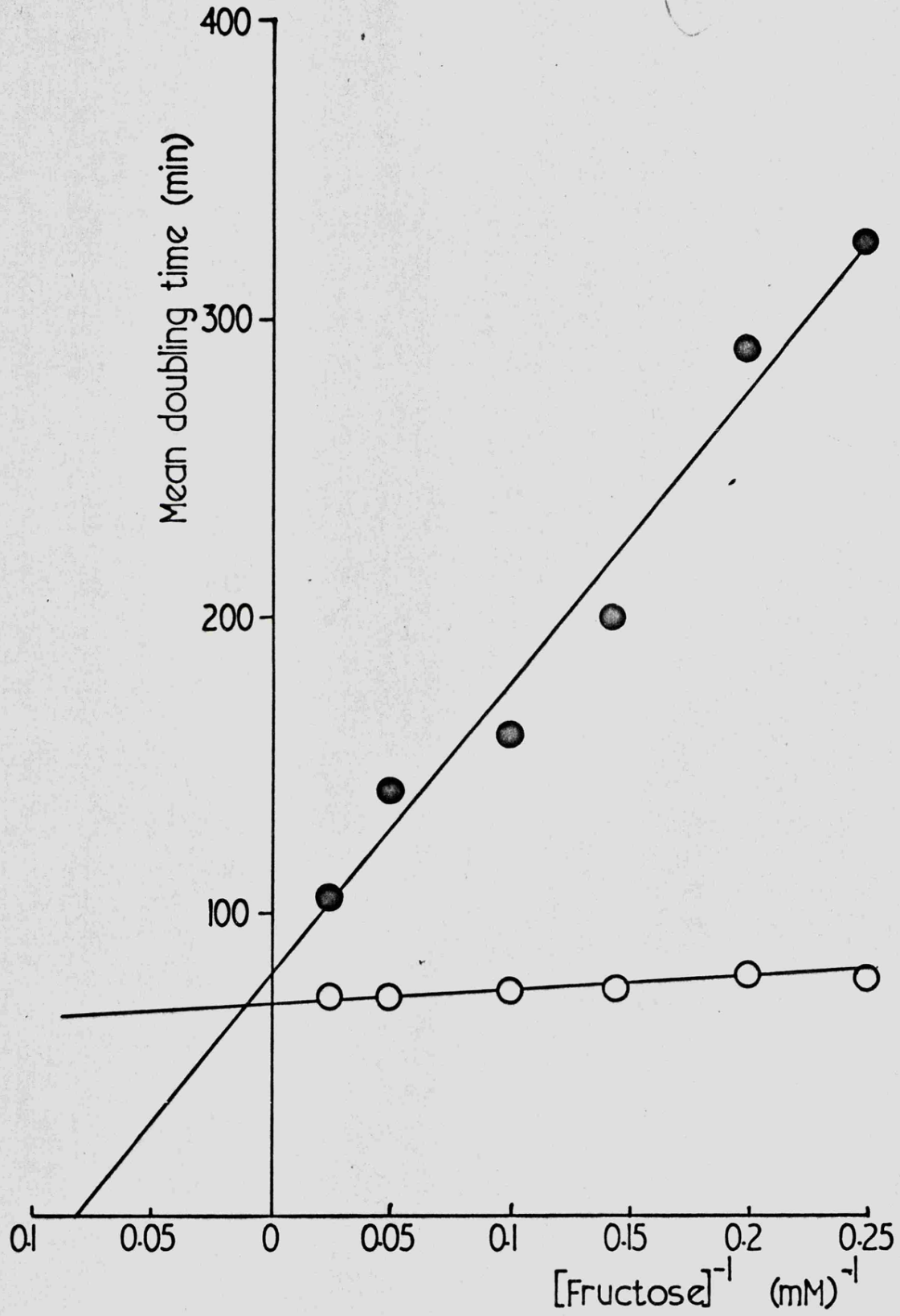
400
300
200
100
Mean doubling time (min)
0.1
0.05
0
0.05
0.1
0.15
0.2
0.25
[Fructose]$^{-1}$ (mM)$^{-1}$

FIGURE 5 Growth of the wild-type strain KL16 (○) and of the mutant KL16.23 (●), on media containing fructose as sole source of carbon. The data are expressed as the reciprocals of the maximum growth rates attained at various concentrations of fructose, plotted against the reciprocals of those fructose concentrations.

TABLE 7 Enzyme levels in fructose-grown KL16.23

Enzyme	Specific Activity
Fructose 1-phosphate kinase	66.0*
PEP:fructose PTS (Assay B)	1.0**

* nmole of fructose 1-phosphate phosphorylated min^{-1} mg $protein^{-1}$.

** nmole of fructose phosphorylated min^{-1} mg dry weight of $cells^{-1}$.

in detail.

KL16.23 exhibits normal growth characteristics on low (1 mM) and high (25 mM) concentration of glucose, glycerol, mannitol and hexose phosphates and is only defective in its growth on fructose as carbon source. This mutant also grows in the usual manner on 5 mM fructose 1-phosphate, when transferred from a culture grown on glucose 6-phosphate, which suggested that the lesion in KL16.23 was not in FPK. This was confirmed by the results of the assays of the enzyme presented in Table 7. The level of FPK in KL16.23 (this time after growth on fructose) is lower than in wild-type KL16; this may be analogous to the finding with KL16.21 and perhaps due to any of the reasons already discussed in connection with that earlier mutant.

The dependence of the growth rate of KL16 and KL16.23 on fructose concentration is illustrated in Figure 5. The results are again plotted as the reciprocals of the maximum measured growth rate (least mean doubling time) at each fructose concentration, versus the reciprocals of these fructose concentrations. As expected, KL16.23 shows a much greater concentration dependence in its growth rate on fructose than does KL16, indeed an even greater dependence than K2.19 (Figure 4). The half-maximal growth rate of KL16.23 is achieved at approximately 12 mM fructose, compared to a value of approximately 0.5 mM obtained for KL16. The maximal growth rate attainable on fructose is, however, not greatly decreased by the lesion, with least mean generation times of approx. 79 min for KL16.23 compared to 71 min for the wild-type.

The ability of this mutant to phosphorylate fructose was investigated. The results of assays of the ATP-dependent phosphorylation

TABLE 8 PEP-dependent fructose 6-phosphate formation in KL16 and KL16.23

Organism	Carbon source for growth	Assay system	Specific activity*
KL16.23	Fructose	complete**	15.5
KL16.23	Fructose	-PEP +ATP	1.1
KL16.23	Glycerol	complete	1.9
KL16	Fructose	complete	5.0

* the specific activity is expressed as nmoles of fructose 6-phosphate formed min^{-1} mg dry weight of $cells^{-1}$

** the complete system is described in Method C (Materials and Methods Section).

of fructose by extracts of KL16.23 are shown in Table 5 and of the PEP-dependent activity in Tables 7 and 8. The manno(fructo)kinase is still present in this mutant, and the level of the enzyme is, if anything, slightly higher. However, a striking difference can be seen in the PEP-dependent phosphorylation of fructose by KL16.23, compared to the wild-type. When phosphorylation at low fructose concentrations (0.5 mM) was measured, no activity was detected in the mutant by the pyruvate release assay (Method B, Materials and Methods) even after KL16.23 was grown on fructose (Table 7). However, when the PEP-dependent formation of fructose 6-phosphate was assayed (at 50 mM fructose) there was at least a three-fold higher level of activity than found in KL16, also grown on fructose (Table 8). The results obtained with KL16.23 show even more clearly the inducible nature of the PEP:fructose 6-PTS and also confirm that ATP is not an effective phosphoryl donor in this system.

The results of the phosphorylation assays explain the requirement for high fructose concentrations for the fast doubling of this mutant, because the lesion in KL16.23 causes an impairment of the phosphorylation of fructose at low concentration of the sugar. Also, as this decrease in the rate of phosphorylation at low concentrations is coupled with an increase in the rate of formation of fructose 6-phosphate at high concentrations of fructose, in KL16.23, it is likely that the PEP:fructose 1-PTS and the PEP:fructose 6-PTS are very closely related, if not identical, systems in E. coli.

The question whether the mutation in KL16.23 results in an alteration in the affinity of the Enzyme II for fructose, or the specificity, or both, is answered by the data in Table 9. This shows

TABLE 9 PEP-dependent fructose phosphorylation by KL16 and KL16.23

Organism	Fructose concentration in assays (mM)	Overall rate* of fructose phosphorylation	Rate of ** fructose 6-phosphate formation	Proportion of fructose phosphorylated to fructose 6-phosphate (%)
KL16	1.0	38.7	0.53	1.4
KL16	50.0	54.5	5.8	11
KL16.23	1.0	1.6	0.96	60
KL16.23	50.0	33.0	15.7	48

* Measured as described in Method A, in the Materials and Methods section.

** Measured as described in Method C, in the Materials and Methods section.

Rates are expressed as nmole fructose phosphorylated min^{-1} mg dry weight of $cells^{-1}$.

the total phosphorylation of fructose at 1 mM and 50 mM by toluene-treated cells of KL16 and KL16.23 and the proportion phosphorylated to fructose 6-phosphate at these same concentrations. Total fructose phosphorylation and fructose 6-phosphate formation were determined in the same assay by a combination of assays A (^{14}C-fructose ⟶ ^{14}C-fructose phosphates) and C (fructose ⟶ fructose 6-phosphate), using 1 mM and 50 mM U-^{14}C-labelled fructose (both 2.9 μCi/ml). While in KL16, fructose 6-phosphate formation accounts for less than 2% of the total fructose phosphorylated at 1 mM concentrations of the substrate, and at 50 mM for about 11%, in KL16.23 about 60% of the fructose phosphorylated is converted to fructose 6-phosphate at 1 mM, and about 48% at 50 mM. In confirmation of the earlier results in Tables 7 and 8, the total rate of fructose phosphorylation at the lower concentration in KL16.23 is only about 4% of the wild-type rate, while at the higher concentration, over 60% of the wild-type rate can be demonstrated. Hence both the affinity for fructose and the relative proportion of the products of fructose phosphorylation have been altered in KL16.23.

In the light of the possibility that a single enzyme is responsible for both fructose 1-phosphate and fructose 6-phosphate formation from fructose in E. coli, the properties of the lesion in the mutant KL16.21 were further investigated. As already shown in Tables 2 and 6, this mutant lacks both types of fructose phosphorylation. To show that this was not simply due to a double mutation, revertants of KL16.21 were obtained by plating cells previously grown on glycerol onto 25 mM fructose minimal agar plates. After 48 hr at 37°, seven colonies were picked at random and purified by single colony isolation. Liquid growth studies of these seven revertants were carried out on

low (1 mM) and high (25 mM) concentrations of fructose; it was found that all seven could grow on both concentrations, suggesting that the original, single, mutation caused the total loss of the ability to grow on fructose. A mutation in a single Enzyme II could have this consequence, just as another mutation in the Enzyme II, in KL16.23, could cause an alteration in the proportion of fructose phosphorylated to fructose 1-phosphate and fructose 6-phosphate. However, it cannot be ruled out that the mutation in KL16.21 is in another component of the PTS which is required for fructose phosphorylation to both fructose 1-phosphate and fructose 6-phosphate, such as a common Factor III, which is a sugar-specific component of the PTS required for the phosphorylation of lactose or mannitol in Staphylococcus aureus (Simoni, Smith and Roseman, 1968).

(vi) Genetic analysis of the fructose mutants

The lesions responsible for the loss or alteration in the ability of mutants KL16.21 and KL16.23 to grow on fructose were located to be at approximately 41-43 min on the E. coli linkage map of Taylor (1970). Preliminary analyses of crosses between the Hfr mutants and the F^- strain K2.1t showed that selection of his^+ recombinants resulted in a high proportion of recombinants that carried the fructose lesions as well, suggesting some linkage between the his and the fructose markers. The $fructose^-$ lesions are designated ptsF21 and ptsF23, as found in mutants KL16.21 and KL16.23 respectively.

In order to obtain a more accurate location of the lesions on the linkage map, the mutations had to be transferred to F^- strains, so that time of entry experiments could be performed. In a cross

FIG.6

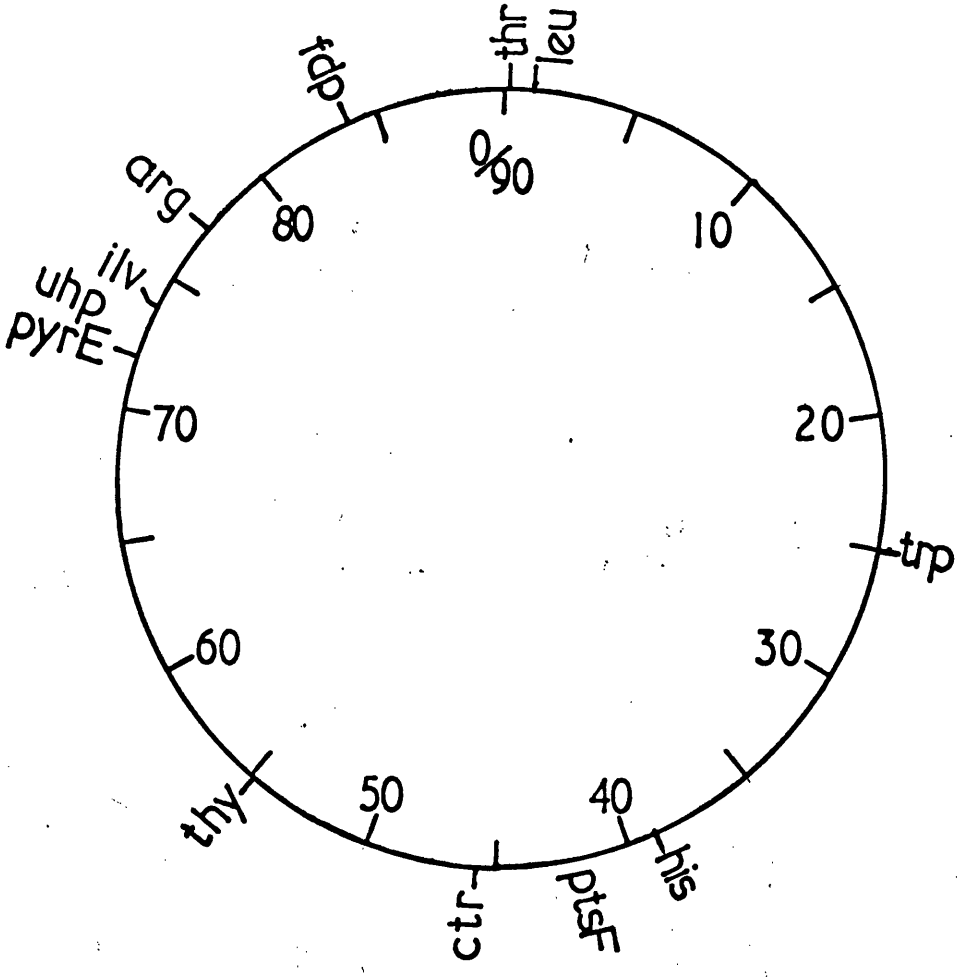
thr
leu
0/90
10
20
trp
30
his
ptsF
ctr
50
40
thy
60
70
pyrE
uhp
ilv
arg
80
tdp

FIGURE 6 The linkage map of *Escherichia coli* K12, adapted from Taylor (1970). The *ptsF* marker is located at 41-43 min on the linkage map, as described in the text.

FIG.7

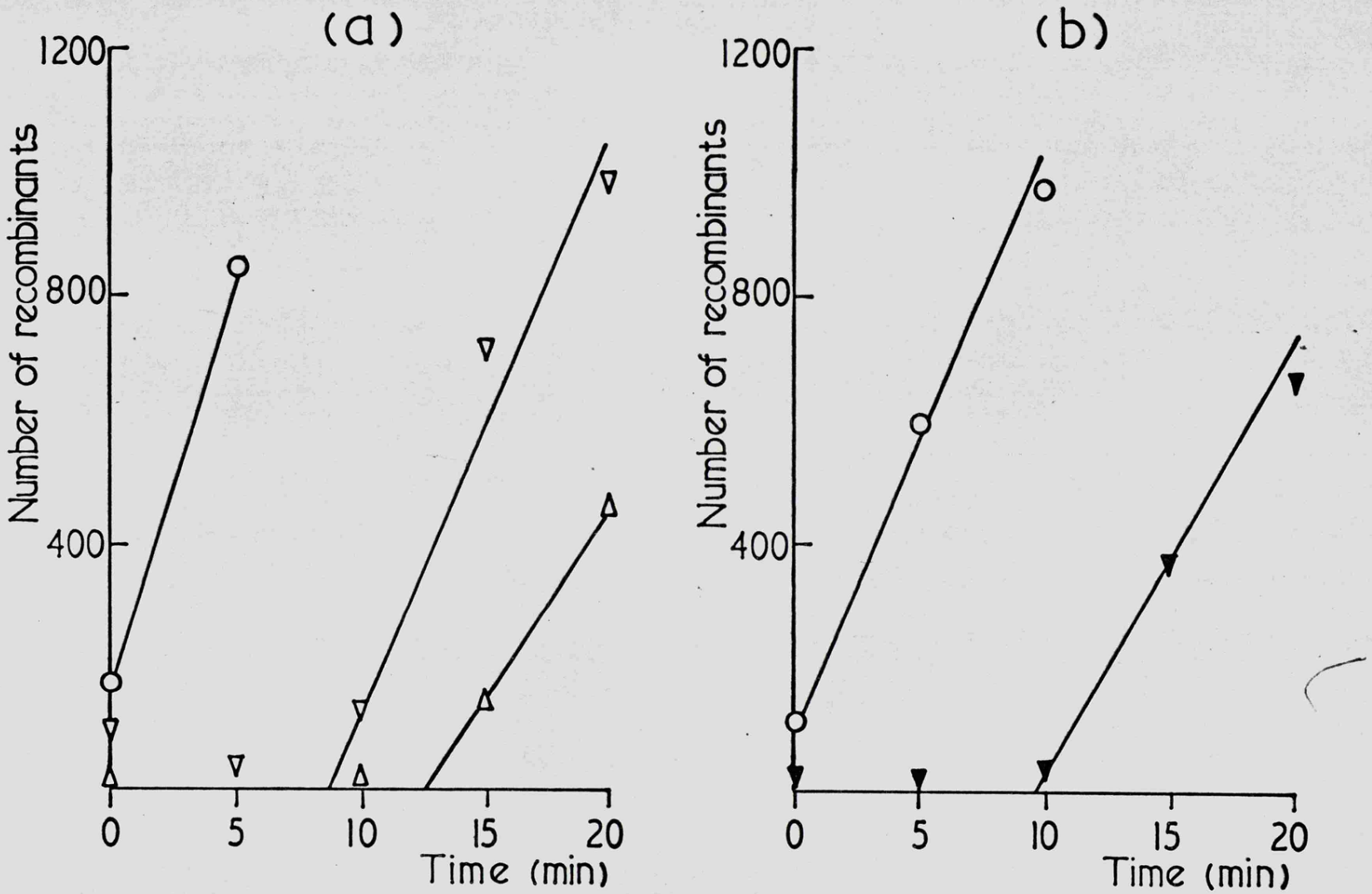

FIGURE 7 Times of entry of the thy (O), his (Δ), ptsF21 (∇) and ptsF23 (▼) genetic markers in interrupted matings between the wild-type Hfr strain KL16 and the F^- strains (a) K2.21 No. 4T or (b) K2.1.23.T.

between Kl16.21 and K2, an *thr*$^+$,*leu*$^+$ recombinant was selected, which had retained the *try*,*arg* and *his* markers but had become fructose$^-$ and hence had gained the *pts*F21 lesion. This recombinant, called K2.21.No.4, was then subjected to trimethoprim treatment (Materials and Methods) to obtain a *thy*$^-$ organism, designated K2.21.No.4 T. This K2.21.No.4T was then used as recipient in an interrupted conjugation experiment with the wild-type Hfr KL16, which transfers its genome in the order *o*-*thy*-*his*..., and the times of entry were obtained for the *thy*$^+$,*his*$^+$ and fructose$^+$ markers; this is illustrated in Figure 7a. As shown, fructose$^+$ enters approximately 10-15 min after *thy*$^+$ but less than 5 min before *his*$^+$. This would locate the *pts*F21 lesion at approximately 41-43 min on the linkage map shown in Figure 6, which is adapted from the one presented by Taylor (1970).

For the mapping of *pts*F23, KL16.23 was crossed with K2.1t and a *his*$^+$ recombinant, which was unable to grow on low concentrations (1 mM) of fructose and hence carried the *pts*F23 lesion, was obtained. This recombinant, called K2.1.23, was treated with trimethoprim (Materials and Methods) to select a thymine$^-$ mutant, designated K2.1.23T. Again, an interrupted mating between this K2.1.23T and the wild-type Hfr KL16, gave times of entry of the *thy* and the *pts*F23 markers, as shown in Figure 7b. This fructose lesion also enters just before 15 min after *thy*$^+$, close to the *pts*F21 mutation already described.

To give a preliminary analysis of the linkage between the *pts*F21 and *pts*F23 lesions, *his*$^+$ and *thy*$^+$ recombinants were obtained from a cross between KL16.23 and K2.21.No.4T. Of each type of recombinant, 50 were checked for their ability to grow on low (1 mM) and high (10 mM) concentrations of fructose. None was obtained that

could grow on the low concentration of fructose though some could grow on the higher concentration. This result indicates a very close linkage between the two ptsF markers, as no recombinants had regained the wild-type ability to grow on fructose, and were both ptsF21$^+$ and ptsF23$^+$. The data are, however, not detailed enough to show whether both lesions are in the same gene, specifying a fructose-specific component of the PEP:fructose PTS.

Linkage data has also been obtained between the ptsF21 mutation and a lesion pleiotropically affecting the utilization of hexoses, carried in strain K2.1.10 (Kornberg, 1970). This latter lesion is of the ctr type (Wong et al., 1969; Epstein et al., 1970), probably causing a loss of enzyme I or HPr activity in the PEP-dependent PTS, and maps at 46 min on the E. coli linkage map. Recombinants that could grow on glucose were selected after a genetic cross between KL16.21 and K2.1.10; about 30% of the recombinants from such a cross grew on all hexoses, and had therefore received ctr$^+$ without also receiving the ptsF21 lesion, and thus had the phenotype of KL16.21. It is therefore likely that the ptsF mutation is distinct from, though fairly closely linked to, the ctr marker, in agreement with the time of entry studies, which would place the ptsF marker approximately 3-5 min from ctr on the linkage map shown in Figure 6.

(vii) DISCUSSION

Two additional lines of investigation have provided evidence for the *in vivo* operation of the sequence fructose → fructose 1-phosphate → fructose 1,6-diphosphate, first postulated to occur in *E. coli* by Fraenkel (1968a). Firstly, the ability of *E. coli* to utilize fructose 1-phosphate, through the induction of the enzyme 1-phosphofructokinase, suggests that the second half of the above pathway can function *in vivo*. Secondly, the fact that a mutation resulting in the loss of an assayable PEP:fructose phosphotransferase system, which has been shown to catalyse the phosphorylation of fructose to fructose 1-phosphate (Fraenkel, 1968a), causes KL16.21 to lose totally the ability to grow only on fructose, argues for the importance of the PTS in fructose utilization. This importance is also reflected in the behaviour of the mutant KL16.23, in which an alteration in the PEP:fructose PTS results in decreased rates of fructose 1-phosphate formation and also results in impaired growth on fructose.

Although the significance of the pathway via fructose 1-phosphate is beyond doubt, evidence in this Chapter suggests that a minor pathway via fructose 6-phosphate also operates in *E. coli* during growth on the external concentrations of fructose normally used in growth experiments. This finding resolves the paradox that *E. coli* mutants devoid of the enzyme fructose 1,6-diphosphatase grow on fructose (Fraenkel and Horecker, 1965; Fraenkel, 1968a) although the major pathway of fructose conversion to fructose 1,6-diphosphate is in essence a gluconeogenic process. The demonstration by Fraenkel (1968a) that fructose 1-phosphate is the only product of fructose phosphorylation by the PTS in *E. coli* is not in conflict with the suggestion that fructose 6-phosphate is

also a product because his assays were carried out at a fructose concentration of 0.04 mM, at which fructose 6-phosphate formation is not detectable. Even at high concentrations of fructose (50 mM), the major pathway of fructose utilization is likely to be via fructose 1-phosphate, as fructose 6-phosphate formation accounts for only about 10% of the assayable PEP-dependent phosphorylation of fructose. This proportion is, however, likely to be enough to account for the ability of mutants lacking FDPase to grow at higher concentrations of fructose.

The role of the manno(fructo)kinase identified by Sebastian and Asensio (1967) is still unclear, though this enzyme is unlikely to be of major importance in the normal metabolism of extracellular fructose. This is mainly because, as the properties of KL16.21 indicate (see also Chapter II), there is no route for the entry of fructose into E. coli other than via the PEP-dependent PTS, and inherent in this mechanism is the phosphorylation of fructose to fructose 1-phosphate or fructose 6-phosphate. Also, the inability of K2.19, a mutant lacking the enzyme fructose 1,6-diphosphatase, to grow on fructose at low external concentrations of the sugar would argue against the physiological significance of the manno(fructo)kinase in the formation of fructose 6-phosphate. The broad specificity, low activity and uninducible nature (by fructose at least) of this enzyme suggest that the function of the manno(fructo)kinase may be more general, that is the rephosphorylation of sugars like mannose or fructose that may have been dephosphorylated after entry via the PTS.

A number of clear differences are discernible between the reactions of fructose utilization in E. coli, as described in this chapter, and in Aerobacter aerogenes, as described in detail by Anderson's

group (reviewed in Wood and Anderson, 1969). The main difference is the lack of a measurable PEP-dependent fructose 6-phosphate-forming system in A. aerogenes; even at high concentrations of fructose the sole product of the PEP:fructose PTS is fructose 1-phosphate (Hanson and Anderson, 1968). This is consistent with the finding that a mutant of A. aerogenes lacking FDPase does not grow on fructose, even when supplied at 28 mM (Sapico et al., 1968), in contrast to the behaviour of mutants of E. coli lacking the same enzyme. A specific ATP:fructose 6-phosphotransferase (fructokinase) activity, induced by growth on sucrose but not exogenous fructose, has been described in A. aerogenes (Kelker, Hanson and Anderson, 1970); as already mentioned, the only fructokinase activity detectable in E. coli is due to the enzyme described by Sebastian and Asensio (1967). No induction of this enzyme by sucrose could be studied as none of the strains of E. coli K12 used in this thesis is able to grow on sucrose, again in contrast to the behaviour of A. aerogenes.

There has been no previous report in the literature that a sugar can be phosphorylated at two positions by the PEP-dependent PTS. So far, phosphorylation at the 6-hydroxyl group has been described for glucose, mannose, glucosamine, mannosamine (Kundig and Roseman, 1966), galactose (Kundig et al., 1965) and at the 1-position for fructose (Hanson and Anderson, 1968; Fraenkel, 1968a) and mannitol (Tanaka, Lerner and Lin, 1967). It is difficult to assess whether in most of these cases phosphorylation was followed at a wide enough range of substrate concentration to rule out some ambiguity in the sites of phosphorylation of the sugars involved. In the case of fructose 1-phosphate formation by the PEP-dependent PTS in E. coli, it is unlikely that the reaction is just a result of an overlapping specificity of

another system that phosphorylates at the 6-position, like the system for glucose. This is because fructose 6-phosphate formation, like all PEP-dependent fructose phosphorylation, is an activity induced solely by growth on fructose; also the behaviour of KL16,21 and KL16.23 make clear that an alteration in the growth properties of these mutants on fructose is accompanied by an alteration in both fructose 1-phosphate and fructose 6-phosphate formation.

The mutations in the PEP:fructose PTS, ptsF21 and ptsF23, have been mapped to be at about 41-43 min on the E. coli linkage map (Taylor, 1970). The lesions in KL16.21 and KL16.23 are closely linked but is is not yet known whether they are in the same gene; they are also within 3-5 min of the genes specifying Enzyme I (ptsI) and HPr (ptsH) of the PTS, which are located at about 46 min on the linkage map (Epstein, Jewett and Fox, 1970).

As already mentioned, and as will be discussed more fully in Chapter II, the PEP-dependent phosphotransferase system is also implicated in the uptake, as well as the phosphorylation of sugars. In this chapter, it has been possible to correlate the phosphorylative properties of the system towards fructose with the growth properties exhibited by mutants of E. coli. The next chapter will try to answer the question whether the uptake properties of E. coli towards fructose can also be correlated with the phosphorylative activity exhibited by the PTS.

CHAPTER II - THE UPTAKE OF FRUCTOSE BY *E.coli*

(i) Introduction

Until recently, the study of sugar uptake processes in E. coli was carried out only on intact cells. This approach yielded a great deal of kinetic data concerning fluxes and intracellular levels of accumulated molecules. Such information gave rise to a number of models of active transport; for the development and the latest version of one such model, of the much-studied β-galactoside uptake system, see Kepes (1971). Also, this whole-cell approach, together with microbial genetic studies, yielded a number of useful operational definitions, like that of the 'permease' (Cohen and Monod, 1957). Biochemical lines of investigation, of seeking information about a system by studying its components, have been hampered, in work on uptake processes, by the loss of any assayable transport activity once cells are disrupted, because, of course, no directional flux can then be measured. This problem was exemplified by the work of Kennedy's group, who isolated the 'M protein', chemically and genetically identifiable as the β-galactoside permease (Fox and Kennedy, 1965; Fox, Carter and Kennedy, 1967; Carter, Fox and Kennedy, 1968). Although this was a notable achievement in membrane protein chemistry, the purification of this protein has not helped to elucidate the interactions involved in β-galactoside transport. How then, is it possible to study the molecular mechanism of biological transport in the absence of an intact cell?

There are at present three active lines of investigation to study this problem, using bacterial systems. The first of these involves study of 'binding proteins', which are removable from Gram-negative bacteria by an osmotic shock procedure (Heppel, 1969). There

have been attempts to correlate the properties of some of these binding proteins, such as the galactose binding protein of E. coli (Boos and Sarvas, 1970; Lengeler et al., 1971), the L-arabinose binding protein of E. coli (Schleif, 1969) and the sulphate binding protein of Salmonella typhimurium (Pardee, 1968) with the properties of the systems responsible for the transport of these substrates. The contribution these studies have made to an understanding of transport processes has recently been reviewed (Kaback, 1970).

Another approach has been that of Kaback's group, who have used membrane vesicles isolated from E. coli to study the uptake of a number of metabolites; this work is also described in detail in Kaback's (1970) review.

The third line of investigation involves work on the PEP-dependent phosphotransferase system (PTS), first described by Kundig, Ghosh and Roseman (1964). The role of the PTS in the initiation of sugar metabolism has already been discussed in Chapter I. Ever since the PTS was first implicated in the uptake, as well as the phosphorylation, of sugars (Kundig, et al., 1966) it has seemed possible that studies of the interactions of its components can provide some insight into the mechanism of sugar transport. The advantage of this system is, of course, that the components of the PTS can be identified by their enzymatic properties in the absence of a permeability barrier.

Roseman (1969) has presented six lines of evidence in favour of the role of the PTS in sugar transport:

1. Addition of purified HPr partially restores sugar transport in osmotically shocked E. coli (Kundig, Kundig, Anderson and Roseman, 1966).

2. Sugars are initially accumulated as sugar phosphates during

transport in whole cells (Rogers and Yu, 1962; Winkler, 1966).

3. Genetic correlations. A pleiotropic mutant that is unable to grow on or transport 11 sugars in Staphylococcus aureus was described by Egan and Morse (1965); this mutant was shown to lack Enzyme I of the PTS (Simoni, Smith and Roseman, 1968). Mutants with similar properties have been described in A. aerogenes (Tanaka and Lin, 1967), Salmonella typhimurium (Simoni et al., 1967) and E. coli (Tanaka, Fraenkel and Lin, 1967; Fox and Wilson, 1968; Wang and Morse, 1968; Kornberg, 1970); in each case the defect could be shown to be associated with mutations in either HPr or Enzyme I of the PTS. Mutations in sugar specific Enzymes II have also been described; in these cases growth and uptake of only one sugar is affected; like mannitol (Tanaka, Lerner and Lin, 1967) or β-glucosides (Fox and Wilson, 1968).

4. Identity between inhibitors of the PTS and uptake systems. Phosphorylation of galactose and TMG (methyl β-thiogalactoside) by membrane preparations is inhibited in the same way (Roseman, 1969) as is the transport of these compounds in intact cells (Adhya and Echols, 1966).

5. Specificities of Fractions II. The pattern of inducibilities of Enzymes II, more properly called Fractions II, and uptake systems for various sugars correspond qualitatively and quantitatively (Roseman, 1969).

6. Sugar uptake in membrane vesicles has been shown to be PEP-dependent and sugars to be accumulated as sugar phosphates (Kaback, 1968). Furthermore, Kaback (1968) showed, with double-labelling techniques, that the sugar phosphate ester accumulated by the vesicles are formed during

transport rather than after penetration into a pool of the free sugar inside the membranes.

The components of the PTS have begun to be characterised. Purifications of Enzyme I and HPr and the constitutive Fraction II of E. coli have been described (Kundig and Roseman, 1971a and b). The Fraction II component of glucose-grown cells can be separated into a number of components that are required for a reconstitution of phosphotransferase activity: II-A, which can be further sub-fractionated into three protein components each of which is required for the sugar-specific phosphorylations of glucose, mannose and fructose respectively and II-B, an aggregating protein required for the phosphorylation of all three sugars when interacting with phosphatidyl glycerol, which forms a complex with II-B in the presence of Mg^{2+} or Ca^{2+}.

All inducible systems so far investigated require additional protein factors for phosphotransferase activity. In S. aureus, cells grown on mannitol or lactose have induced PTS's for the respective sugars and both require cytoplasmic 'Factors III' for full activity (Roseman, 1969). These proteins are distinct for lactose and mannitol and are induced, together with the Fractions II, during growth on the given sugar. In the case of the PEP:fructose PTS in A. aerogenes, a fructose specific protein distinct from Fraction II has been detected (Hanson and Anderson, 1968). This protein, called a 'K_m factor', decreased the Michaelis constant of the PTS for fructose phosphorylation and was postulated to function by increasing the affinity of (a possibly constitutive) Enzyme II for fructose.

As the results of the last Chapter indicated, the phosphorylation of fructose by the PTS in E. coli appears to have two products, fructose

1-phosphate and fructose 6-phosphate. As the agent(s) catalysing these two reactions appear to have different affinities for fructose, and as the "-21" and "-23" mutants described are altered in these steps, it was investigated whether the properties of the fructose uptake system in wild-type and mutant E. coli can be correlated with the phosphorylative activities exhibited by these strains. This Chapter contains the results of such work. Attempts to correlate the phosphorylative and transport aspects of the PTS have been made by Roseman (1969), for the methylgalactoside permease in S. typhimurium, and by Gachelin (1970), for the α-methylglucoside permease in E. coli. On the basis of their results, these authors have postulated conflicting mechanisms for the functioning of the PTS during transport; Roseman (1969) believes that the PTS functions as a group translocation mechanism, with the Enzyme II being both the transporter and the phosphorylating enzyme during sugar transport, while Gachelin (1970) believes that the transport of sugars is a two-step mechanism, with the Enzyme II-mediated phosphorylation preceded by a permeation step, which can be unmasked as a facilitated diffusion mechanism in the absence of phosphorylation.

(ii) Methodology

The technique used for the uptake studies involves the measurement of the accumulation of uniformly labelled [^{14}C]fructose. Although the method of using metabolizable substrates in uptake studies is to some extent unsatisfactory, because any measure of the rate of uptake will be a measure of transport plus the further metabolism of the substrate, this limitation has been at least partly overcome in the

FIG. 8

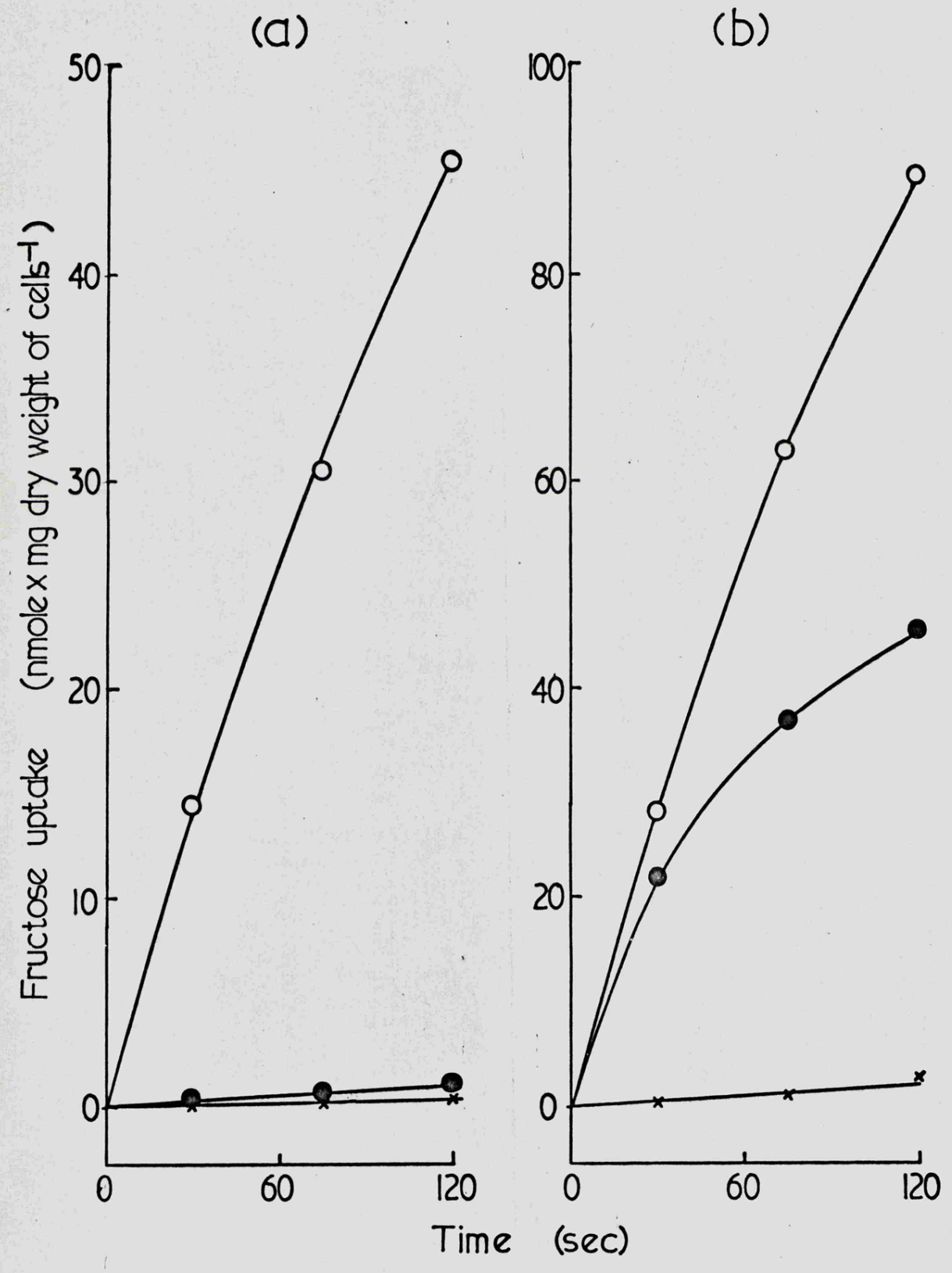
(a)
(b)
50
40
30
20
10
0
100
80
60
40
20
0
0
60
120
0
60
120
Fructose uptake (nmole x mg dry weight of cells^{-1})
Time (sec)

FIGURE 8 The time-course of [U-^{14}C]fructose uptake by washed cell suspensions of wild-type strain KL16 (O) and of mutants KL16.21 (×) and KL16.23 (●). Fructose was supplied at a concentration of (a) 0.1 mM or (b) 5.0 mM. The cells were previously grown on fructose, in the case of KL16 and KL16.23 and on fructose + glycerol, in the case of KL16.21.

present studies by the determination of rates of uptake of fructose over relatively short period of time, during which the entry of fructose is still near linear with respect to time. Figure 8 shows the time course of accumulation during the first two minutes after the addition of radioactive fructose to washed cell suspensions of E. coli; the methods of preparation and sampling are described in the Materials and Methods section. As can be seen, the rate of uptake of fructose at 0.1 mM (Figure 8a) and 5.0 mM (Figure 8b), within the range of concentration of the sugar used in these studies, is almost linear for 20 sec, which is the time interval used in obtaining initial rates for the kinetic studies described in this Chapter. If anything, because of the slight curvature of these time courses, even during the 20 sec intervals, the rates used for the calculation of kinetic parameters are likely to be underestimates of the real initial rates of entry of fructose. The quoted apparent K_m and V_{max} values were obtained by using linear regression analysis (Hewlett-Packard 9100 calculator) of the data presented in the relevant figures. Also, the initial rates of fructose entry are proportional to the cell concentration in the uptake system between 0.2 - 0.6 mg dry wt per ml and all uptake measurements were performed within this range.

(iii) The entry of fructose into KL16, KL16.21 and KL16.23

The alterations in the phosphorylative properties of the PEP:fructose PTS in the mutants KL16.21 and KL16.23 described in Chapter II imply that, if the PTS is involved in both sugar uptake and phosphorylation, these mutants would also be altered in their ability to accumulate fructose. The data in Figure 8 support these predictions.

FIG. 9

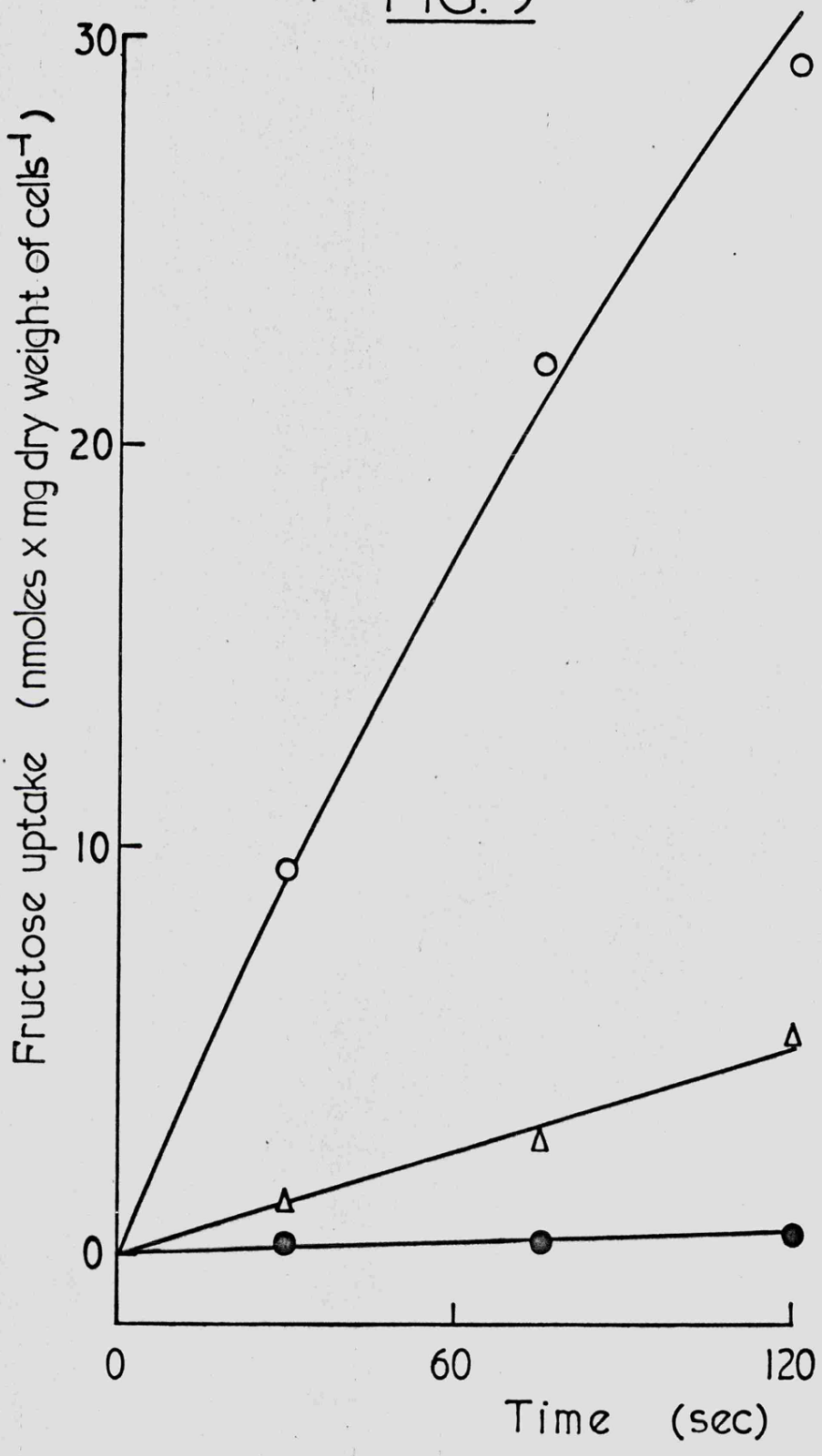
30
20
10
0
Fructose uptake (nmoles x mg dry weight of cells^{-1})
0
60
120
Time (sec)

FIGURE 9 Time-course of 0.1 mM [U-^{14}C]fructose uptake by washed cell suspensions of wild-type KL16, grown on fructose 1-phosphate (O) and glycerol (Δ), and of the mutant KL16.21 (●), grown on fructose 1-phosphate.

KL16.21, which, as demonstrated in Chapter I, is unable to grow on or phosphorylate fructose at low or high concentrations of the sugar, takes up fructose at much lower rates than does the parent KL16 at both the lower and the higher concentrations of fructose. After both were grown on 5 mM fructose 1-phosphate, the mutant KL16.21 has less than 1% of the ability of KL16 to accumulate 0.1 mM fructose; this is less even than the rate obtainable with uninduced wild-type cells (Figure 9).

In contrast, even though KL16.23 shows less than 2% of the accumulative ability of KL16 (Figure 8a) at 0.1 mM fructose, this mutant can have about 80% of the initial rate of uptake of the wild-type at the higher concentration of fructose (Figure 8b). This result correlates quite well with the phosphorylative properties of this mutant: as demonstrated in Chapter I, KL16.23 cannot phosphorylate fructose at low concentrations of the sugar, but can, albeit in an altered manner, at high concentrations. Hence the assumption that the PEP:fructose PTS is responsible for both the accumulation and the phosphorylation of fructose in E. coli seems a valid one.

However, although these experiments indicate a close parallel between fructose phosphorylation and accumulation, they do not, by themselves, prove a link between fructose phosphorylation and transport. This problem is the same as the one mentioned in the Introduction to this Chapter, namely, is the PTS a trapping system (Gachelin, 1970) or the sugar translocator per se (Roseman, 1969)? Gachelin (1970) has shown the presence of a facilitated diffusion mechanism for α-methyl-glucoside in an Enzyme II mutant which lacks active transport and the PEP-dependent phosphorylation of α-methylglucoside, in an attempt to

show that a carrier distinct from Enzyme II operates during the transport of this glucose analogue.

A similar approach was attempted with mutant KL16.21, which is unable to accumulate fructose, or phosphorylate fructose by the mediation of the PTS. The low residual uptake activity in fructose 1-phosphate-grown cells of this mutant was analysed and also found to show an apparent equilibration mechanism for the [^{14}C]fructose used. However, about 60% of intracellular radioactivity, extracted by boiling water treatment of whole cells, consisted of ^{14}C-anionic material, as determined by separation of the intracellular pool on Dowex-formate columns (Winkler, 1966). Hence the apparently equilibrated material in KL16.21 is mostly not free fructose. Also, the proportion of label taken up by KL16.21 was lower than the percentage purity claimed for commercial [U-^{14}C]fructose, so the validity of the results as a measure of fructose uptake in the mutant is questionable. It is therefore uncertain whether there is a facilitated diffusion mechanism operating for fructose, separate from the PTS, in KL16.21.

(iv) The kinetics of fructose uptake

In Chapter I, it was indicated that the concentration at which half-maximal rates of growth were achieved, i.e. the 'K_m' values for growth, by mutants KL16.23 and K2.19 growing on fructose in liquid cultures, were 12 mM and 1.8 mM respectively. These values compare with a 'K_m' for growth of well under 1 mM for the wild-type organisms KL16 and K2. It was therefore investigated whether these 'K_m' values could be correlated with changes in the Michaelis constants

FIG.10

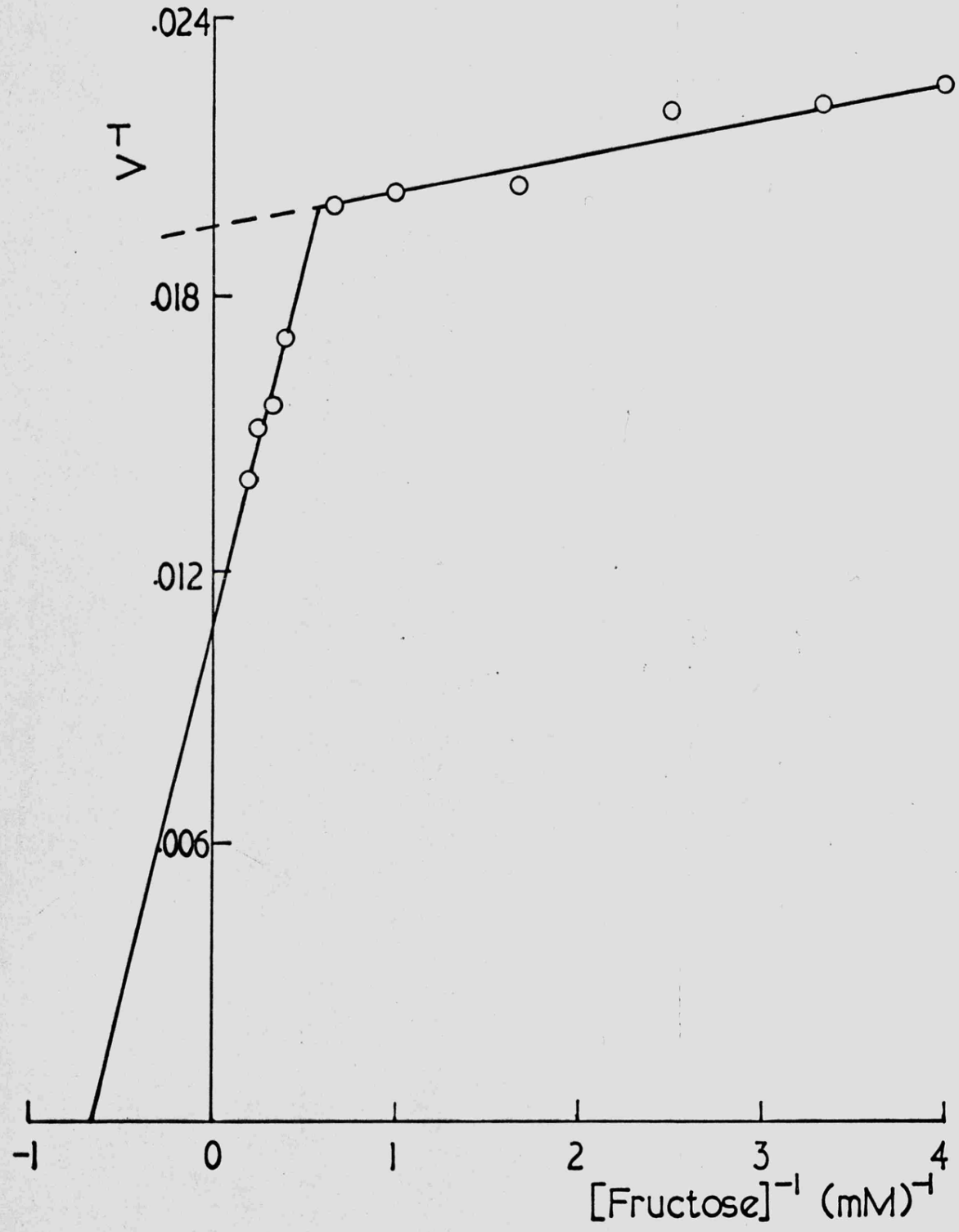

FIGURE 10 The concentration dependence of [U-^{14}C]fructose uptake by washed cell suspensions of the wild-type strain KL16, grown on fructose. The data are expressed as a Lineweaver-Burk plot with the initial rate of uptake, v , being in units of nmoles of fructose taken up per min per mg dry weight of cells.

FIG. 11

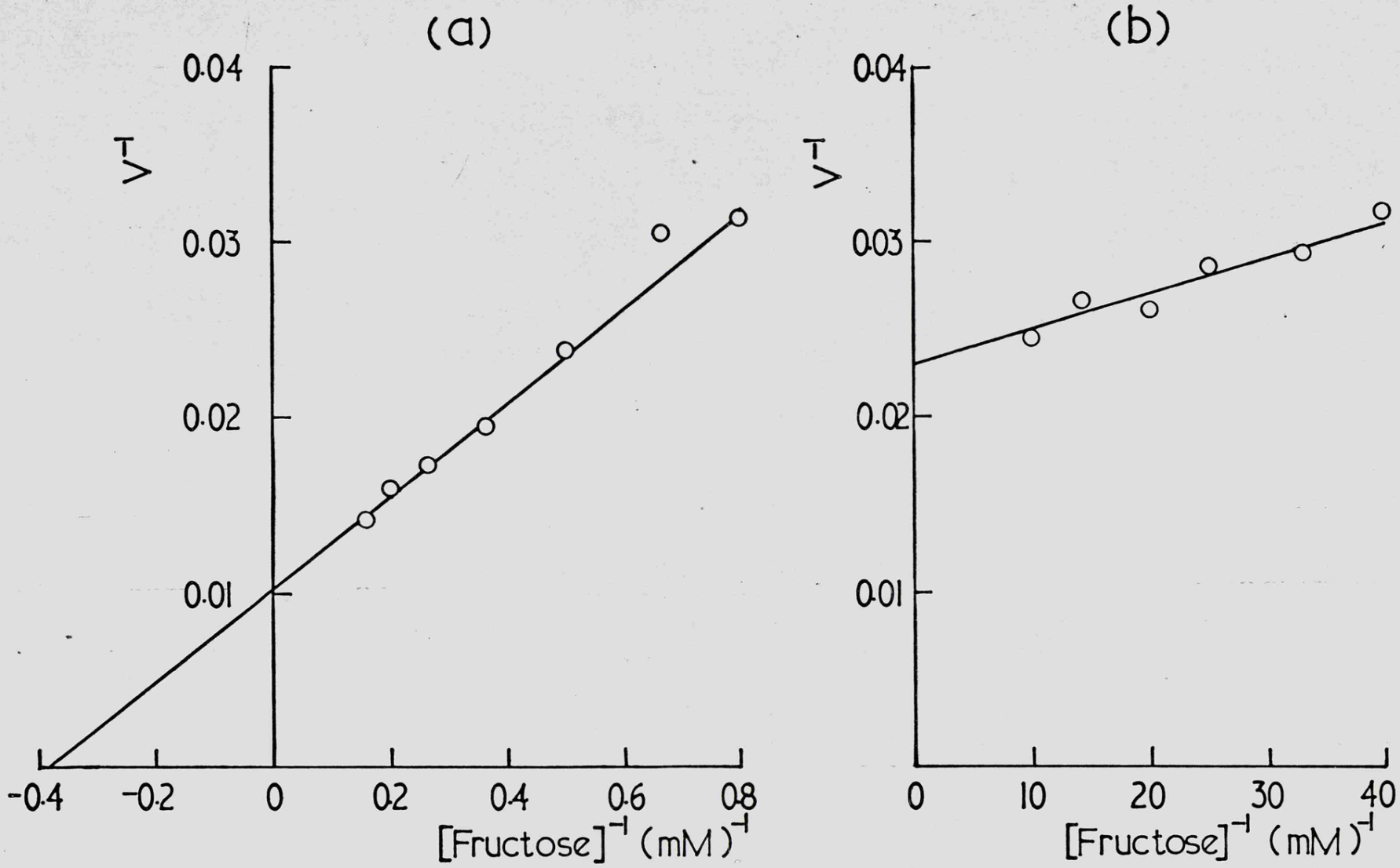
(a)
0.04
0.03
0.02
0.01
V⁻¹
-0.4
-0.2
0
0.2
0.4
0.6
0.8
[Fructose]⁻¹ (mM)⁻¹
(b)
0.04
0.03
0.02
0.01
V⁻¹
0
10
20
30
40
[Fructose]⁻¹ (mM)⁻¹

FIGURE 11 The concentration dependence of [U-^{14}C]fructose uptake by washed cell suspensions of wild-type strain K2, grown on fructose. The data are expressed as Lineweaver-Burk plots, at high, (a) and low, (b), ranges of substrate concentrations. The initial rate of uptake, v , is expressed in units of nmoles of fructose taken up per min per mg dry weight of cells.

for fructose entry into these mutants in washed cell suspensions. The experimental procedures for obtaining the concentration dependence of fructose uptake, presented as Lineweaver-Burk (Lineweaver and Burk, 1934) plots, are described in the Materials and Methods section.

The overall pattern obtained for fructose-grown KL16 is presented in Figure 10. The plot shows a sharp discontinuity but can be divided into two linear components. At higher concentrations of fructose (above approximately 1 mM) an apparent Michaelis constant (K_m) of 1.5 mM and an overall apparent V_{max} of about 90 nmoles of fructose taken up per min per mg dry weight of cells can be obtained; at lower concentrations (below about 1 mM), a component with a much lower apparent K_m, of very approximately 20 μM and with an apparent V_{max} of about 50 nmoles of fructose taken up per min per mg dry weight of cells, can be discerned. Separate determinations of the kinetic parameters of the low and high affinity components, this time in fructose-grown K2, are shown in Figure 11. The 'high affinity' uptake system, measured at low concentrations of fructose, has an apparent K_m of approximately 9 μM and an apparent V_{max} of 43.5 nmoles of fructose taken up per min per mg dry weight of cells. The 'lower affinity' uptake, determined at substrate concentrations of over 1 mM, gives an apparent K_m of 2.6 mM and an overall V_{max} of 97 nmoles of fructose taken up per min per mg dry weight of cells, for the two systems combined.

The pattern of concentration dependence of the rate of uptake of fructose raises the question of whether there are two systems, of widely different affinities, responsible for the uptake of fructose. The evidence derived from the uptake and phosphorylative properties of

FIG.12

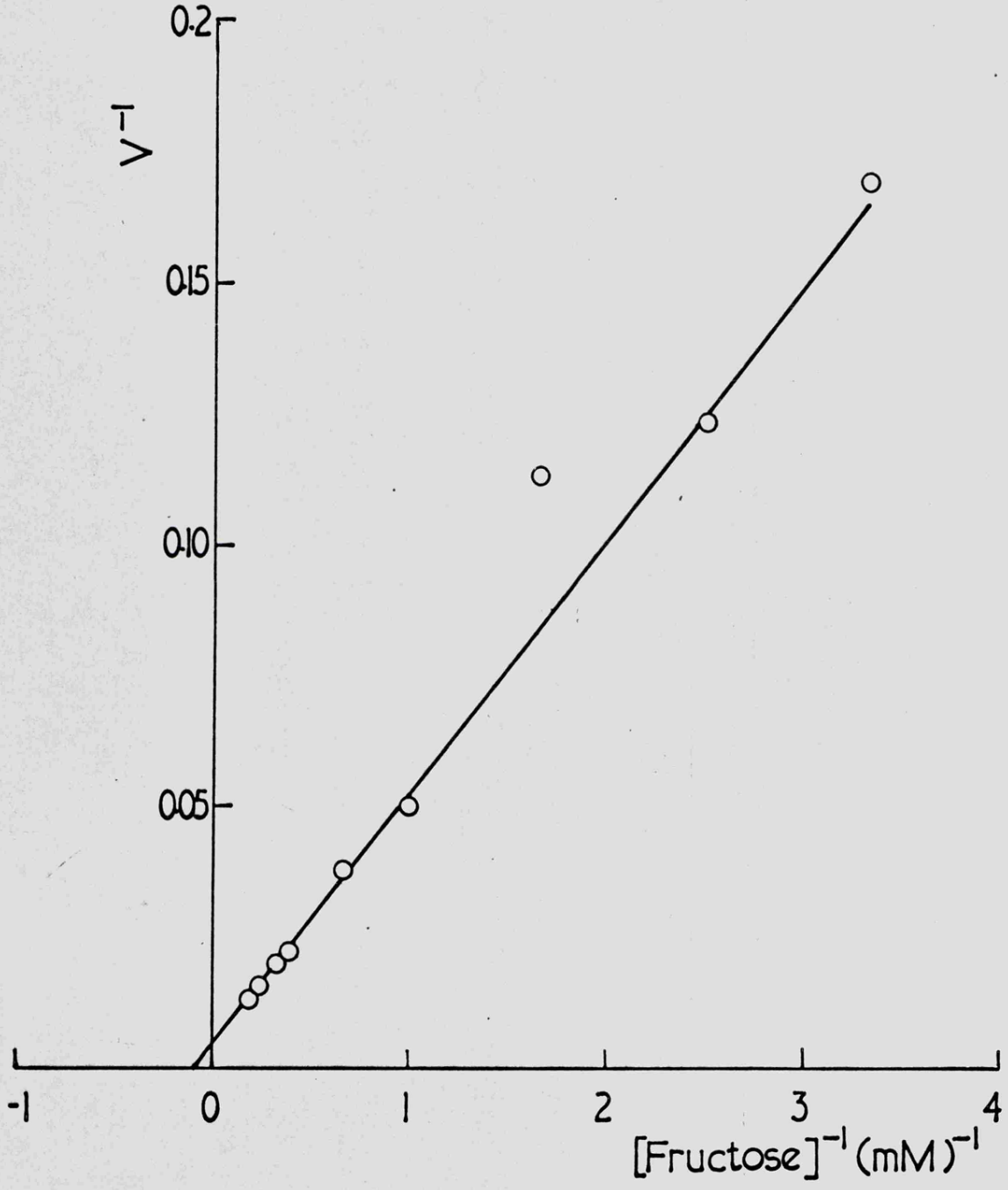

FIGURE 12 The concentration dependence of [U-^{14}C]fructose uptake in washed cell suspensions of the mutant KL16.23 grown on fructose. The data are expressed as a Lineweaver-Burk plot, with the units of the initial rate of uptake, v , being in nmoles of fructose taken up per min per mg dry weight of cells.

the mutant KL16.21 would suggest that if there are two protein-mediated mechanisms for the uptake of fructose, then they must both be components of the PEP:fructose PTS. It has also been shown (see Chapter I) that there are two products of fructose phosphorylation by the PTS, fructose 1-phosphate and fructose 6-phosphate, which could be due to catalysis by two separate sites in the Fractions II for fructose that are also involved in fructose translocation. Moreover, fructose 6-phosphate is formed solely at higher concentrations of fructose and this reaction may then be associated with the 'low affinity' (high K_m) uptake component.

However, it may not be necessary to postulate two parallel mechanisms for fructose phosphorylation/translocation. There are several other possible explanations for biphasic double-reciprocal plots in the kinetics of fructose uptake, as will be discussed at the end of this Chapter, which do not require the presence of more than one enzyme or translocator site. Hence, it cannot be ruled out purely on the kinetic evidence presented that fructose uptake is mediated by a single transport system.

The uptake properties of the mutant KL16.23, which has been shown to have an altered affinity for growth on fructose and also an altered pattern of PEP-dependent fructose phosphorylation (see Chapter I), were further investigated. A Lineweaver-Burk plot of the concentration dependence of rates of fructose uptake by fructose-grown cells of KL16.23, is illustrated in Figure 12. In marked contrast to the pattern shown for wild-type KL16 (Figure 10), the double reciprocal plot is not biphasic, and a single apparent K_m (of 11 mM) and V_{max} (of approximately 250 nmoles of fructose taken up per min per mg dry weight

FIG.13

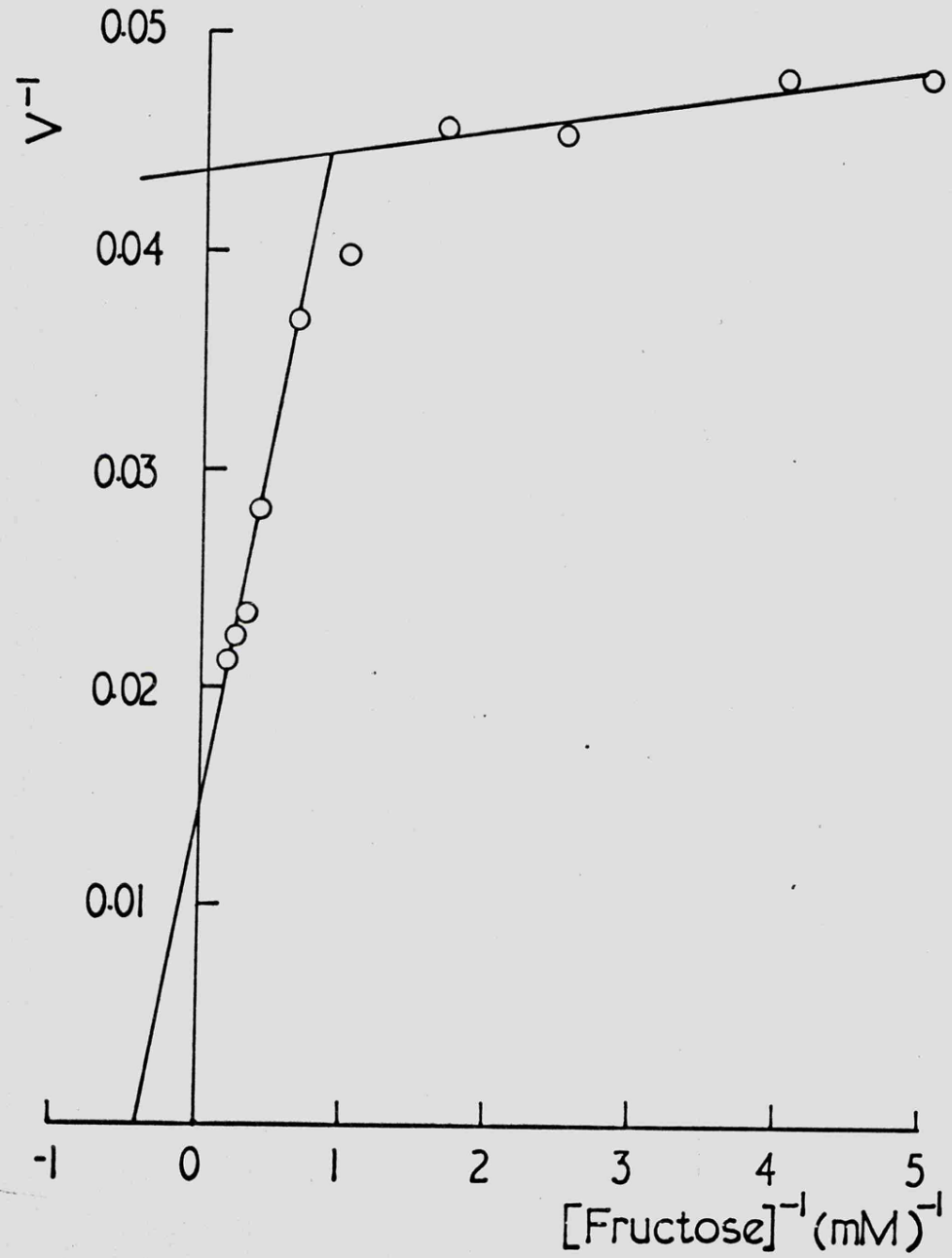

FIGURE 13 The concentration dependence of [U-^{14}C]fructose uptake in washed cell suspensions of fructose-grown K2.19, the mutant devoid of fructose 1,6-diphosphatase activity. The initial rate of uptake, v , is expressed in units of nmoles of fructose taken up per min per mg dry weight of cells.

of cells) can be demonstrated. Although this result could be taken to mean that the 'high affinity' system of a two-component uptake mechanism is absent from this mutant, it is also possible that a single protein is so altered that its activity is modified to give the pattern of activity observed. The pattern of PEP-dependent fructose phosphorylation in KL16.23 (Table 9) also does not distinguish between the two possibilities. Fructose 1-phosphate formation is a lower proportion of the total fructose phosphorylated while fructose 6-phosphate formation is increased in the mutant, which could support either the postulate that a high-affinity system producing solely fructose 1-phosphate is missing, while the low affinity system producing both fructose phosphates is retained, or the postulate that a single site that produces both fructose phosphates at different substrate concentrations has been altered so the balance of phosphorylation is swung in favour of fructose 6-phosphate formation.

The biphasic pattern of kinetics of the uptake of fructose is also exhibited by washed cell suspensions of fructose-grown K2.19 (Figure 13). As indicated in Chapter I, the 'K_m for growth' of this mutant is approximately 1.8 mM (Figure 4); the apparent K_m of the 'low affinity' component for fructose uptake is approximately 2.5 mM, as obtained from Figure 13. There is also a rough correlation between the 'K_m for growth' (of 12 mM) and the apparent K_m for fructose uptake (of 11 mM), measured for the mutant KL16.23 (Figures 5 and 12), which may indicate that the 'K_m for growth' is determined by the affinity of the uptake system for fructose. However, for the wild-type strains KL16 and K2, the 'K_m for growth' values of about 0.5 mM and 0.2 mM respectively, do not correspond to the apparent K_m of either the

'low' or the 'high affinity' components of fructose uptake. This may be at least partly due to the inaccuracy of the 'K_m for growth' values for wild-type cells determined in batch cultures; the rapid utilization of substrate by fast-growing cells introduces large errors into the measurement of growth rates at low substrate concentrations.

From such data as presented in Table 12, it can be calculated that for a wild-type strain growing on 5 mM fructose, with a mean generation time of 60-70 min, the rate of fructose utilization is approximately 70-80 nmoles of fructose removed from the medium per min per mg dry weight of cells. The apparent overall V_{max} for the initial rate of fructose uptake in washed cell suspensions is 90-100 nmoles of fructose taken up per min per mg dry weight of cells and the apparent V_{max} of the 'high affinity' uptake component in wild-type cells is 40-50 nmoles of fructose taken up per min per mg dry weight of cells. These rates of uptake are therefore able to account for the full rate of fructose utilization in growing cultures though perhaps not by the activity of the high affinity uptake component alone. However, such comparisons may be invalid, because the steady-state rates of fructose uptake may not be identical to the initial rates, as measured in washed cell suspensions.

It has already been indicated (Figure 9) that the initial rates of fructose uptake are increased after growth of E. coli on fructose: this will be described more fully in Chapter III. It has also been shown (in Chapter I) that fructose phosphorylation to both fructose phosphates are inducible activities. It was therefore investigated whether differences between the concentration dependence of rates of fructose uptake could be discerned between E. coli grown

FIG. 14

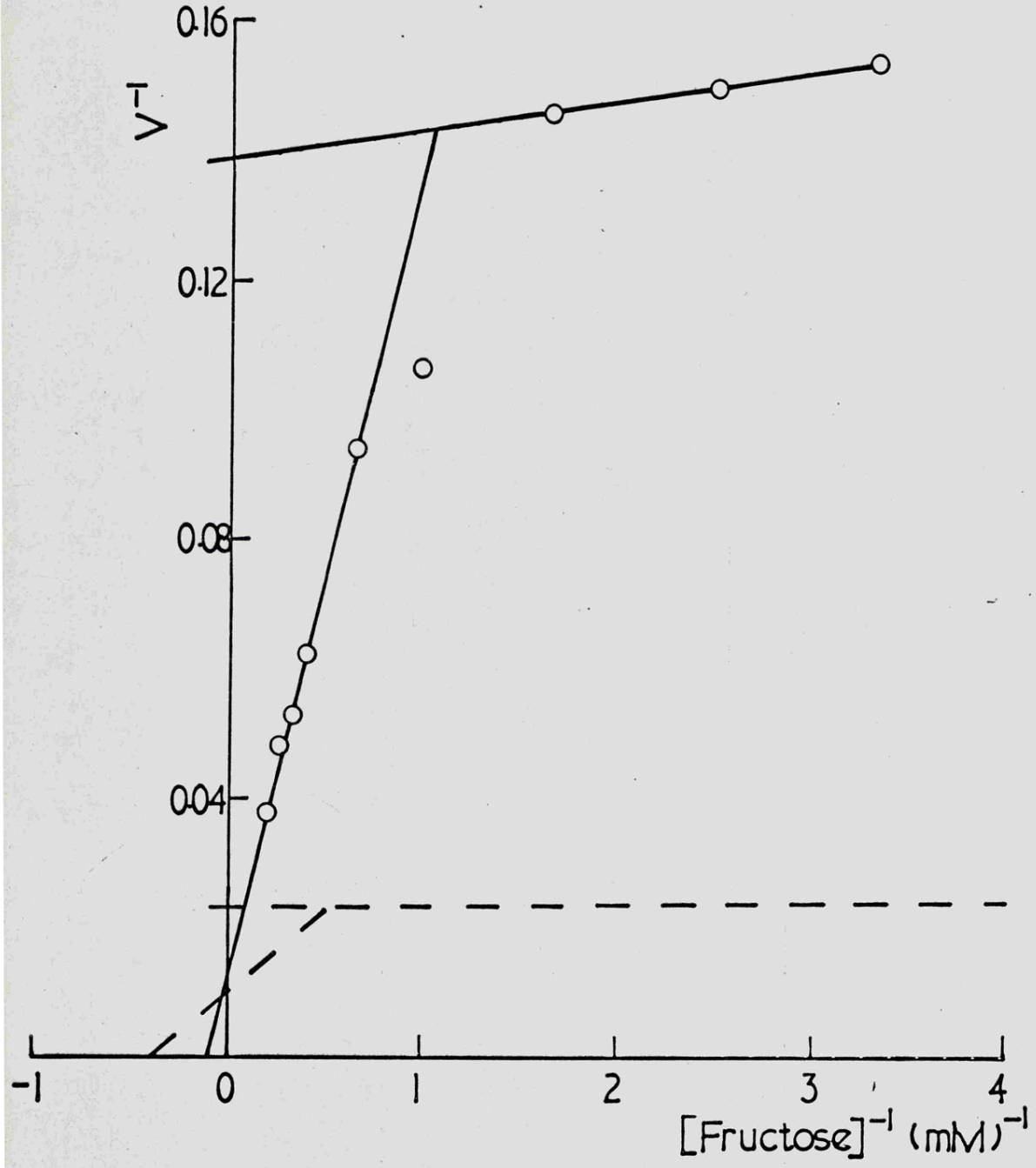

FIGURE 14 The concentration dependence of [U-^{14}C]fructose uptake in washed cell suspensions of glucose-grown, wild-type K2. The dashed line indicates the pattern of concentration dependence of uptake by fructose-grown K2, adapted from the data in Figure 11. The initial rate of uptake, v , is expressed in nmoles of fructose taken up per min per mg dry weight of cells; the data are presented as a Lineweaver-Burk plot.

on fructose and glucose. These are illustrated in Figure 14, with the pattern for fructose-grown K2 taken from the data in Figure 11. By comparison with fructose-grown K2, the apparent V_{max} of the 'high affinity' system is reduced by about 83% in the glucose-grown cells while the V_{max} of the overall rate of fructose uptake is only slightly reduced, to approximately 83 nmoles of fructose taken up per min per mg dry weight of cells. Although the apparent K_m of the 'high affinity' component is of the same order in glucose-grown as in fructose-grown K2, the apparent K_m of the 'low affinity'system is shifted to a higher value, of approximately 10 mM.

If this result represents a genuine shift in the K_m of fructose uptake, then it may be possible to draw a parallel with the pattern of PEP-dependent fructose phosphorylation observed in A. aerogenes. Hanson and Anderson (1968) have shown that upon the induction by fructose of a component of the PTS, called the K_m factor, the K_m for fructose in the phosphorylation reaction is shifted from about 60 mM to below 1 mM. A similar protein may be responsible for lowering the K_m of fructose uptake and phosphorylation by the PTS in E. coli. As shown in Chapter I, fructose phosphorylation is an inducible activity, but it is not known whether the higher specific activity is due to an increased V_{max} or to a decreased K_m of the induced PTS.

(v) DISCUSSION

The evidence presented, including the mutant analysis and the kinetic studies, does not favour the formulation of a concise model for fructose transport in E. coli. However, the high correlation between fructose uptake by whole cells and fructose phosphorylation by the phosphoenolpyruvate-dependent phosphotransferase system, makes it likely that the PTS is involved in both processes, as envisaged by Roseman (1969). The major omission which precludes the building of a convincing molecular model for fructose transport is the lack of knowledge of the number of components required for the functioning of this complex process. Studies involving genetic, or indeed, chemical modification of the phosphotransferase system, as used by Gachelin (1970) in the study of α-methylglucoside transport, cannot conclusively identify both the number and the role of single components of the system, because of the close interactions that are inherent in a membrane-bound, multi-component transport system. Hence, it is not surprising that an alteration in one component of the PEP:fructose PTS, like those observed in mutants KL16.21 and KL16.23, may have an effect on more than one closely related activity associated with the PTS, like phosphorylation to fructose 1-phosphate and fructose 6-phosphate or the apparent two-K_m kinetics of fructose uptake.

Despite these limitations, the information derived from the properties of the mutants provides criteria which any future model would have to satisfy. As already mentioned, such schemes would have to recognise the role of the phosphotransferase system in fructose transport and would also have to explain the mechanism of fructose phosphorylation to fructose 1-phosphate and fructose 6-phosphate.

Superficially, it may be expected that the kinetics of the fructose uptake process may provide fairly rigid criteria for the description of the mechanism of fructose transport. Unfortunately, the patterns of concentration dependence of the initial rates of fructose uptake found in wild-type and mutant cells of E. coli also do not clearly distinguish between a number of possible schemes. There are at least six plausible explanations for the biphasic Lineweaver-Burk plots obtained in the study of the kinetics of fructose uptake by wild-type cells. It is therefore difficult even to decide whether fructose uptake is mediated by one, or more than one, mechanism associated with the fructose-specific phosphotransferase system.

Some of the possible explanations for the biphasic double-reciprocal plots arise out of the inherent limitations of using kinetics to study the uptake of substrates of the phosphotransferase system. The PTS is a multicomponent system, the overall kinetics of which may be complicated by additional rate-limiting factors resulting from interactions with the 'shared' components of the PTS, like HPr or Enzyme I. In addition, the PTS is a two-substrate enzyme system and in the uptake experiments, the intracellular level of PEP is not under control, especially as the fructose used as the substrate can be metabolized; this may also introduce the possibility that not transport, but a subsequent metabolic step, is responsible for the rate-limiting step in the measured rates of fructose uptake. Also, the rates of uptake resulting from free passive diffusion have not been taken into account in the calculated rates of fructose uptake, but the properties of mutant KL16.21 make it likely that the contribution of this factor to the measured rates is fairly low.

As shown in the work of Teipel and Koshland (1969), biphasic Lineweaver-Burk plots can be a feature of the kinetics of certain soluble single enzymes. Conditions for obtaining such plots require that the enzyme must possess more than two substrate binding sites and that the magnitude of the catalytic or binding constant should change during the saturation of the enzyme, which would require ligand-induced co-operative interactions between subunits of the enzyme. At present it is not known whether these conditions can be met by the system involved in fructose transport.

CHAPTER III REGULATION OF THE UTILIZATION OF FRUCTOSE

(i) Introduction

In certain circumstances, the survival of bacteria in nature may depend on the ability to adapt rapidly to the utilization of the optimal carbon source for growth. Probably for this reason, and for reasons of cellular economy, the enzymes involved in the metabolism of carbohydrates by *E. coli* are subject to a number of well-known controls. In this Chapter, three of the regulatory mechanisms found in *E. coli* will be considered, in as much as these affect the utilization of fructose. The controls are: (a) inducible enzyme synthesis, (b) catabolite repression and (c) catabolite inhibition.

(a) Inducible enzyme formation It was known by the turn of the century that the enzymatic properties of micro-organisms depend on the medium on which they have grown (for early references, see Jacob and Monod (1961a)). In the 1930's, Karstrom (1938) recognised the difference between adaptive enzymes, whose formation proceeds only in the presence of their substrates in the medium, and constitutive enzymes, whose formation proceeds regardless of the nature of the medium. Work on the mechanism of the adaptive mechanism, rechristened enzyme induction by Cohn *et al.*(1953), has given rise to a number of important concepts in the study of the genetic regulation of enzyme synthesis, as exemplified by the now classic review of Jacob and Monod (1961a). An extensive and detailed literature relating to a number of inducible (and repressible) systems has accumulated in recent years, and in this Chapter, some of the data on the enzymes involved in fructose utilization will be considered in the light of this knowledge.

(b) Catabolite repression This is the name given by Magasanik (1961) to the also long-known 'glucose effect', first defined by Epps

and Gale (1942). This type of repression, as described in the comprehensive review of Paigen and Williams (1970), is "a reduction in the rate of synthesis of certain enzymes, particularly those of degradative metabolism, in the presence of glucose or other readily metabolized carbon sources". The enzymes of fructose metabolism are subject to this control and it has been reported that adenosine 3',5'-monophosphate (cyclic AMP) overcomes the repression by glucose of the synthesis of Enzyme II for fructose (De Crombrugghe et al., 1969). Mainly through the work of Perlman and Pastan [reviewed in Pastan and Perlman (1970)], it has become clear that glucose catabolite repression is mediated through its effect on the intracellular concentration of cyclic AMP. Indeed, a protein thought to be required for the expression of all inducible operons, is dependent on cyclic AMP for its activity (Zubay, Schwartz and Beckwith, 1970; Emmer et al., 1970).

(c) Catabolite inhibition This is the name given to a mechanism whereby glucose inhibits the activity, rather than (or as well as) the formation, of enzymes involved in the utilization of other carbohydrates (McGinnis and Paigen, 1969). These authors have shown that glucose is able to inhibit the metabolism of lactose, galactose, maltose, mannose, xylose, arabinose and glycerol and that only glucose, and to a lesser extent, glucose 6-phosphate, act as inhibitors. They have also shown that the site of inhibition, in the case of galactose metabolism, was at the level of entry into the cell or at the first enzyme, namely galactokinase. Evidence to be presented in this Chapter shows that a comparable mechanism affects the utilization of fructose by E. coli; other examples of this

FIG. 15

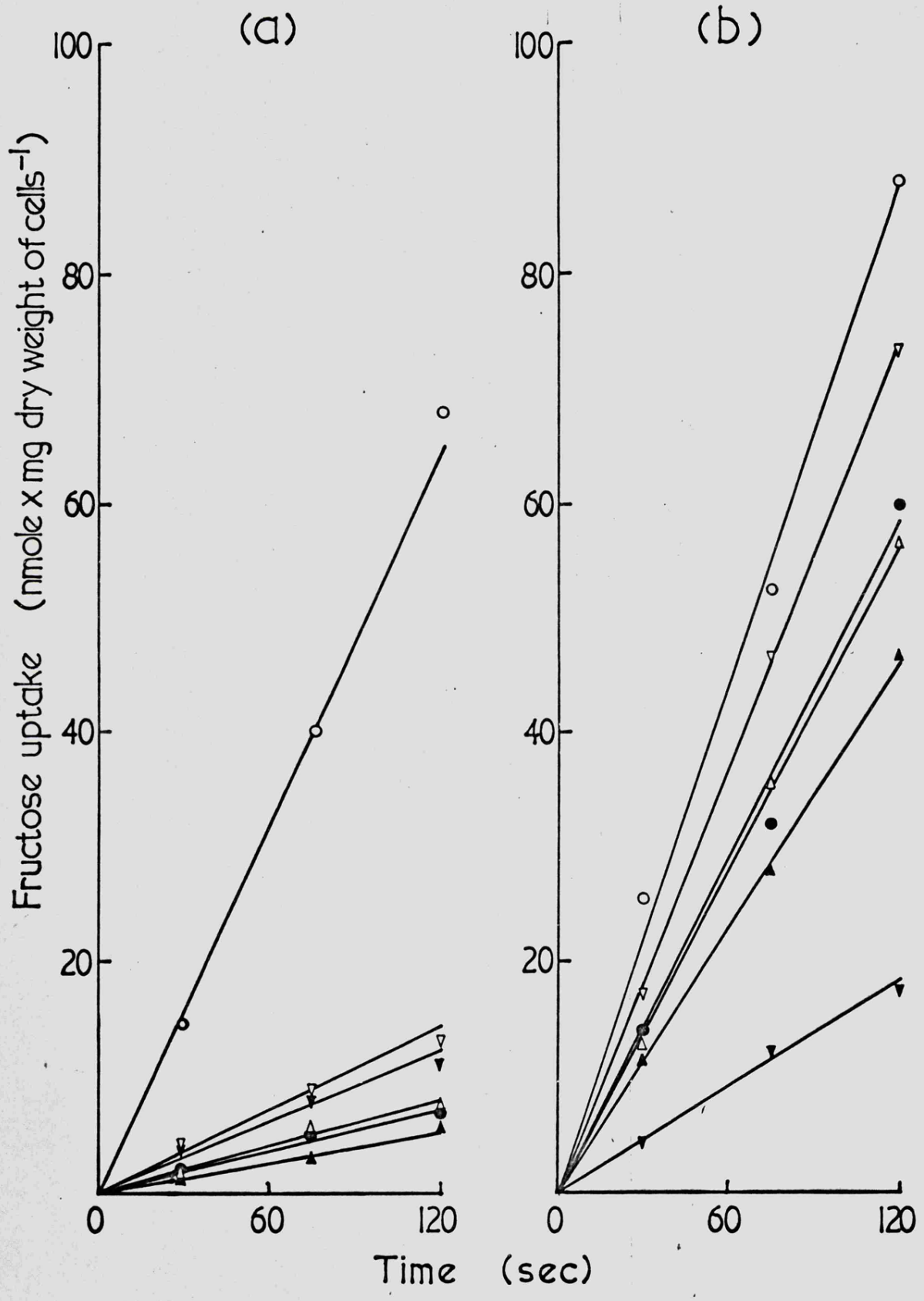
(a)
(b)
100
80
60
40
20
0
60
120
Fructose uptake (nmole x mg dry weight of cells^{-1})
Time (sec)

FIGURE 15 Time-course of 0.1 mM [U-^{14}C]fructose uptake by washed cell suspensions of (a) strain K2.1t or (b) strain K2.1t.DG^{R}D, after the cells were grown on fructose (○), lactose (▽), mannitol (▼), glycerol (●), glucose (△) or gluconate (▲).

TABLE 10 Effect of carbon source for growth on fructose 1-phosphate kinase activity in K2.1t and K2.1t.DG^{R}D

Carbon source for growth	Specific activity* of FPK in K2.1t	K2.1t.DG^{R}D
Fructose	136	278
Glucose	8.0	96.0
Glycerol	4.0	321
Gluconate	8.0	111
Mannitol	4.0	66.3
Lactose	13.0	350

* nmoles of fructose 1-phosphate phosphorylated x min^{-1} x mg protein^{-1} in the assay described in the Materials and Methods section.

regulatory mechanism are quoted in the review by Paigen and Williams (1970).

(ii) The level of fructose-induced enzymes in E. coli

The results presented in Chapter I indicated that the enzyme activities induced during growth on fructose are: fructose phosphorylation, catalysed by the PEP:fructose PTS (Tables 2 and 6) and fructose 1-phosphate phosphorylation to fructose 1,6-diphosphate, catalysed by the enzyme fructose 1-phosphate kinase or 1-phospho-fructokinase (FPK) (Table 3). In addition, the uptake of fructose, when assayed at low concentrations (0.1 mM) of the sugar, is an inducible property of E. coli (Figure 9), as would be expected from the observations that the PTS is responsible for fructose accumulation (Chapter II). Figure 15a illustrates the time course of fructose uptake by washed cell suspensions of strain K2.1t (see Table 1), after growth on a range of carbon sources. The rate of fructose entry is 4-8fold higher after K2.1t is grown on fructose than after growth on the other carbon sources tested. Table 10 shows specific activities of the enzyme FPK after growth of K2.1t on the same set of carbon sources. The level of activity of this enzyme is also highest after K2.1t was grown on fructose, but in contrast to fructose uptake, the rate of fructose 1-phosphate phosphorylation is increased 10-20 fold above the uninduced level.

Of the carbon sources tested, only fructose 1-phosphate (besides fructose) causes high levels of induction of the above activities. The specific activity of FPK, after KL16 was grown on fructose 1-phosphate, is shown in Table 3 and the rate of uptake of fructose,

similarly after grown on fructose 1-phosphate in Figure 9. Full induction of these activities is not quite achieved after doubling on fructose 1-phosphate, perhaps because the intracellular concentration of inducer, which may be fructose 1-phosphate itself, is limited by the rate of entry of fructose 1-phosphate via the hexose phosphate transport system (see Chapter IV). An additional complication is that low concentrations of free fructose can be detected in growth media containing fructose 1-phosphate (Chapter IV), which may itself cause some induction of the fructose-specific enzymes. However, in the mutant KL16.21, extracellular fructose cannot be utilized and this hexose is therefore unlikely to contribute to the induction of the enzyme FPK, which is still observed after KL16.21 is grown on fructose 1-phosphate (Table 3).

Evidence that the synthesis of the fructose-specific component(s) of the PTS and of the enzyme FPK are regulated together comes from the properties of a mutant designated K2.1t.DG^{R}D. This mutant was derived from K2.1t (Table 1) by a modification of the procedure used by Kessler and Rickenberg (1964) for the isolation of mutants constitutive for β-galactosidase synthesis. The growth on fructose of the parent organism, K2.1t, is powerfully inhibited by 2-deoxyglucose; K2.1t.DG^{R}D was selected for its spontaneously acquired ability to grow on 5 mM fructose in the presence of 5 mM 2-deoxyglucose. Mutants selected for their resistance to the inhibition of fructose utilization by 2-deoxyglucose may be altered in one of a number of ways, including (a) alterations in the uptake system for glucose, of which 2-deoxyglucose is an analogue, (b) modifications in the glucose repression mechanism, such as would be found in general catabolite repression resistant mutants, and (c) changes in the regulation of the

synthesis of fructose-specific enzymes.

The first possibility, (a), can be discounted for mutant K2.1t.DG^{R}D, because this mutant can still take up α-methylglucoside, another analogue of glucose, and can still grow on glucose as carbon source at wild-type rates. Other mutants can be derived that are altered in their glucose uptake properties by acquiring 2-deoxyglucose resistance (Kornberg, 1972). The second possibility, (b), was unlikely in the case of K2.1t.DG^{R}D because this mutant has been shown to have wild-type levels of the enzyme β-galactosidase, assayed by the method of Pardee et al. (1959), after growth on lactose and after growth in the presence of glucose, and of isocitrate lyase, as assayed by the method of Kornberg (1963), after growth on fructose or glucose. Finally, the possibility that the alterations in mutant K2.1t.DG^{R}D results in constitutive synthesis of the enzyme FPK and the fructose-induced components of the fructose uptake system were tested; the levels of activity of these two functions are presented in Table 10 and Figure 15b, after the growth of K2.1t.DG^{R}D on a range of carbon sources. The results indicate that both fructose uptake and the enzyme FPK are expressed constitutively in K2.1t.DG^{R}D. Over twice the specific activity of FPK can be detected in cell-free extracts of K2.1t.DG^{R}D after growth on fructose, glycerol or lactose, than can be observed in extracts of K2.1t, even after the wild-type cells were grown on fructose (Table 10). Lower levels of FPK can be detected in K2.1t.DG^{R}D after growth on more repressive carbon sources like glucose and gluconate, but these levels are still at least 10-fold higher than can be found in extracts of wild-type cells grown on glucose or gluconate.

The rates of fructose uptake by K2.1t.DG^{R}D show approximately

the same pattern as FPK levels; uptake is highest after growth on fructose and lower, in descending order, after growth on lactose, glycerol, glucose, gluconate and mannitol. The reason for the low levels of both uptake and FPK after growth on mannitol is unclear, but the observation that the enzyme levels can be repressed in this constitutive mutant confirms that the enzymes of fructose utilization are still subject to catabolite repression.

A preliminary investigation into the location of the lesion resulting in constitutivity suggests that the regulatory gene altered in K2.1t.DG^{R}D is located between the *his* marker and the point of entry of the Hfr strain KL16, which is near the *thy* marker on the *E. coli* linkage map (Figure 6; Taylor, 1970). From a cross between the mutants KL16.21 and K2.1t.DG^{R}D (see Table 1), *his*$^{+}$ recombinants were selected. Of six tested by *in vitro* assay of the enzyme FPK, in extracts of cells grown under non-inducing conditions (on glycerol), four *his*$^{+}$ recombinants had regained wild-type inducibility of the enzyme and hence had received the wild-type regulatory gene from KL16.21. Of these four, two were fructose^{+} and hence *pts*F21^{+}, and two were fructose^{-} and had therefore received the *pts*F21 lesion from KL16.21. Of the two *his*$^{+}$ recombinants which were still constitutive for FPK, one was fructose^{+} and one was fructose^{-}. Hence this data suggests that the regulatory gene for the fructose enzymes and the gene coding for a fructose-specific component of the PTS are not very closely linked, but in the same area on the *E. coli* linkage map (Taylor, 1970).

(iii) *Catabolite repression of fructose-induced enzymes*

The results of De Crombrugghe *et al.* (1969) have shown

FIG.16

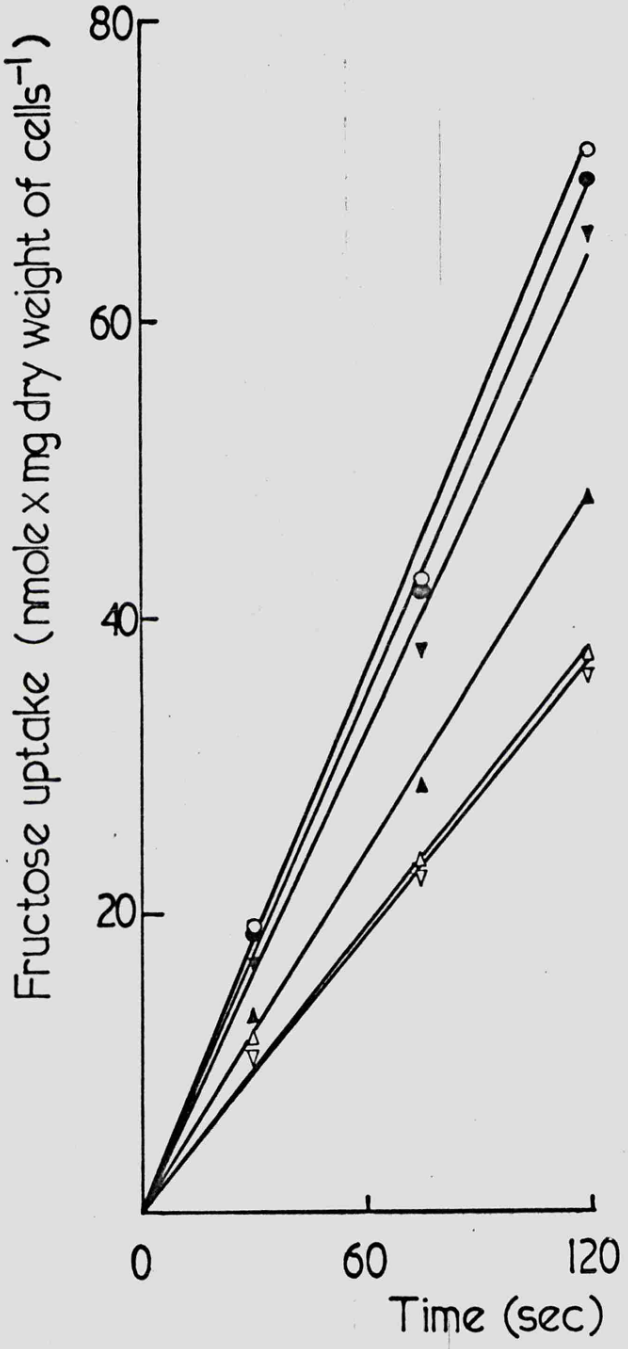
Fructose uptake (nmole x mg dry weight of cells^{-1})
80
60
40
20
0
60
120
Time (sec)

FIGURE 16 Time course of 0.1 mM [U-^{14}C]fructose uptake by washed cell suspensions of strain K2.1t. The cells for this experiment were grown on fructose, harvested, and grown for two doublings on the following carbon sources, all supplied at a concentration of 10 mM; fructose (○), fructose plus glycerol (●), fructose plus mannitol (▼), fructose plus gluconate (▲), fructose plus glucose (Δ) or fructose plus fructose 6-phosphate (∇).

TABLE 11 Repression of fructose 1-phosphate kinase activity in K2.1t

Carbon source for growth*	Specific activity**
Fructose	125
Fructose + Glucose	34.3
Fructose + Glycerol	122
Fructose + Gluconate	55.0
Fructose + Mannitol	94.0
Fructose + Fructose 6-phosphate	44.5

* Cells pre-grown on fructose were transferred to growth for exactly two generations on the mixtures of the carbon sources indicated, all supplied at 10 mM.

** nmoles of fructose 1-phosphate phosphorylated x min^{-1} x mg $protein^{-1}$ in the assay described in the Materials and Methods section.

that the fructose-induced Enzyme II of the PEP-dependent PTS is found at approximately 50% of the fully induced level when E. coli is grown in the presence of fructose plus glucose. The results in Table 11 and Figure 16 show that the expression of the enzyme FPK, as well as fructose uptake activity, are repressed by growth in the presence of the other carbon sources indicated, including glucose. In these experiments K2.1t was grown into exponential phase on fructose, and transferred for exactly two generations to media containing fructose plus the other carbon sources. The usual repression pattern obtained, with least repression by glycerol and mannitol and most by gluconate, glucose and fructose 6-phosphate. Approximately the same pattern can be discerned in the enzyme levels of the constitutive mutant K2.1t.DG^{R}D when growing solely on these various carbon sources (Table 10), with one exception. This occurs after growth on (or in the presence of) mannitol, which does not appear to repress the wild-type but does repress the constitutive. The probable explanation for this is that fructose itself represses and/or inhibits mannitol metabolism, and this effect would only be noticed in the experiments with the wild-type K2.1t, which was grown in the presence of both carbon sources. The lack of effect of, for example, glycerol, may also be due to an inhibition of glycerol utilization by fructose, in which case no repression by glycerol would occur.

As first described by Monod (1942), E. coli exposed to two carbon sources may preferentially utilise one until it is exhausted from the growth medium, and then undergo a growth lag before starting to utilise the other. The presence of this diauxic lag is due to

FIG.17

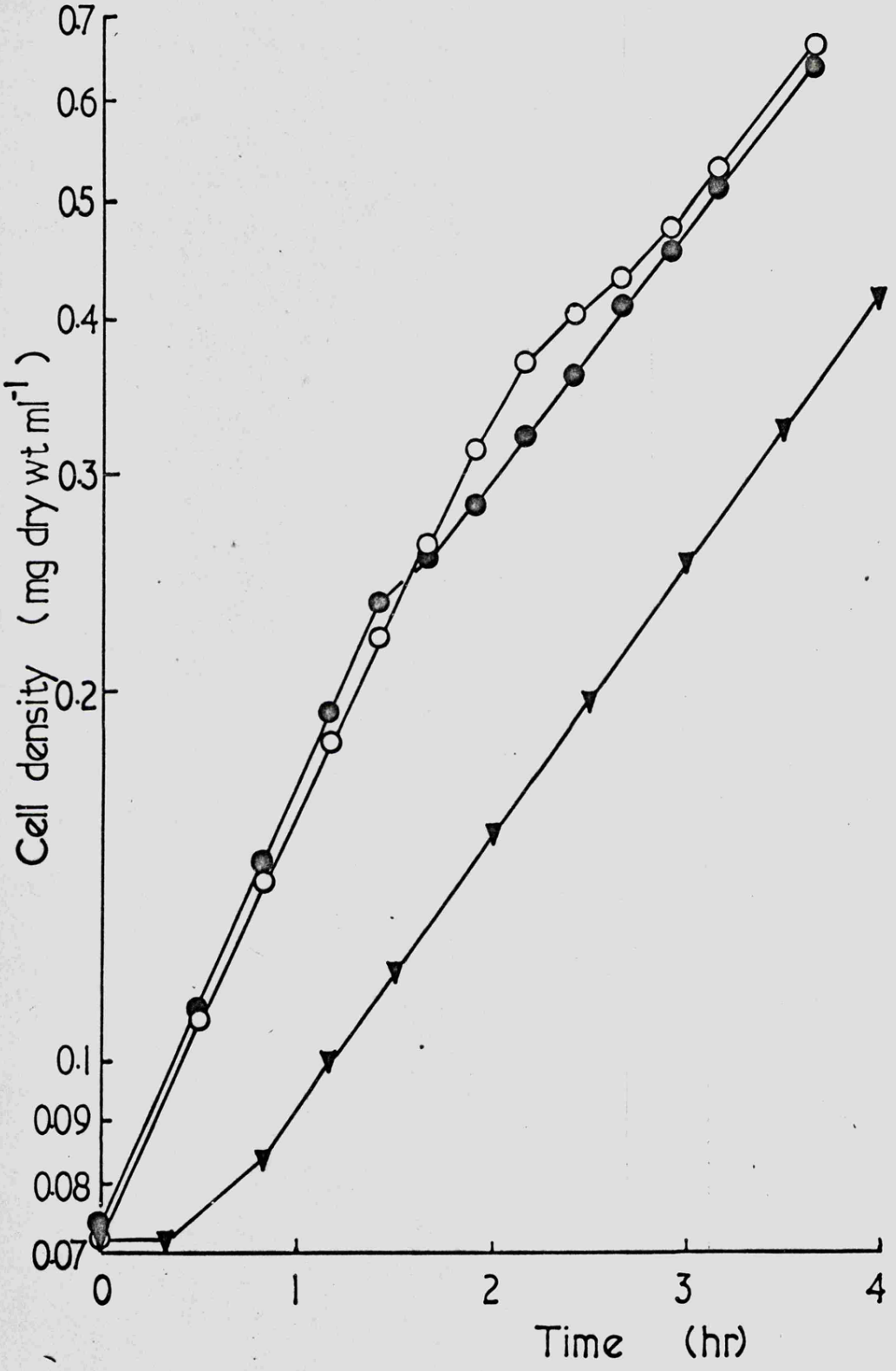
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.09
0.08
0.07
Cell density (mg dry wt ml^{-1})
0
1
2
3
4
Time (hr)

FIGURE 17 Growth of the wild-type strain KL16 on 10 mM fructose (▼), and on a mixture of 1 mM glucose + 10 mM fructose (●), or 2 mM glucose + 10 mM fructose (○). The inocula for this experiment were grown on glucose.

TABLE 12 Inhibition of fructose utilization by glucose in K2.1t

Carbon source(s) at 5 mM	Rates of: Fructose Utilization*		Glucose Utilization*	
	Removal from medium	Incorporation	Removal from medium	Incorporation
fructose	7.3	3.1	-	-
fructose + glucose	1.1	0.43	5.1	2.0

* Rates of utilization are expressed as μmole of sugar removed from the growth medium or incorporated into cell material x mg dry weight increase in cell mass^{-1}, determined as described in the Materials and Methods section.

adaptation to the ability to grow on the less preferred carbon source and does not occur if the enzymic adaptation to the second carbon source can proceed during growth on the first carbon source (see Paigen and Williams, 1970). Glucose is usually used as the primary substrate because it can inhibit the uptake of the second substrate or, less immediately, because it represses the induction of the enzymes required for the utilization of a number of carbon sources. Figure 17 illustrates the growth pattern obtained when KL16, previously grown on glucose, is transferred to 1 and 2 mM glucose plus 10 mM fructose, or 10 mM fructose alone. In the mixed media, a very short lag can be observed between the growth of KL16 on glucose and the growth on fructose, at the characteristic, somewhat slower, growth rate. This brief diauxic lag is compatible with the results that the enzymes of fructose utilization are only partially inducible, but are repressed by glucose and, in particular, as shown in the next section, the entry of fructose into E. coli (and hence the inducer action of fructose) is inhibited by glucose. Monod (1942) did not observe a glucose-fructose diauxie, but this may have been due to differences between the strains of E. coli used.

(iv) Glucose inhibition of fructose utilization

Glucose is known to inhibit the utilization of a number of carbon sources that can support the growth of E. coli (McGinnis and Paigen, 1969), as described in the Introduction to this Chapter. The results in Table 12 demonstrate that glucose also inhibits the utilization of fructose, when present in equimolar concentrations (5 mM) with fructose in the growth medium of K2.1t, with cells that

FIG. 18

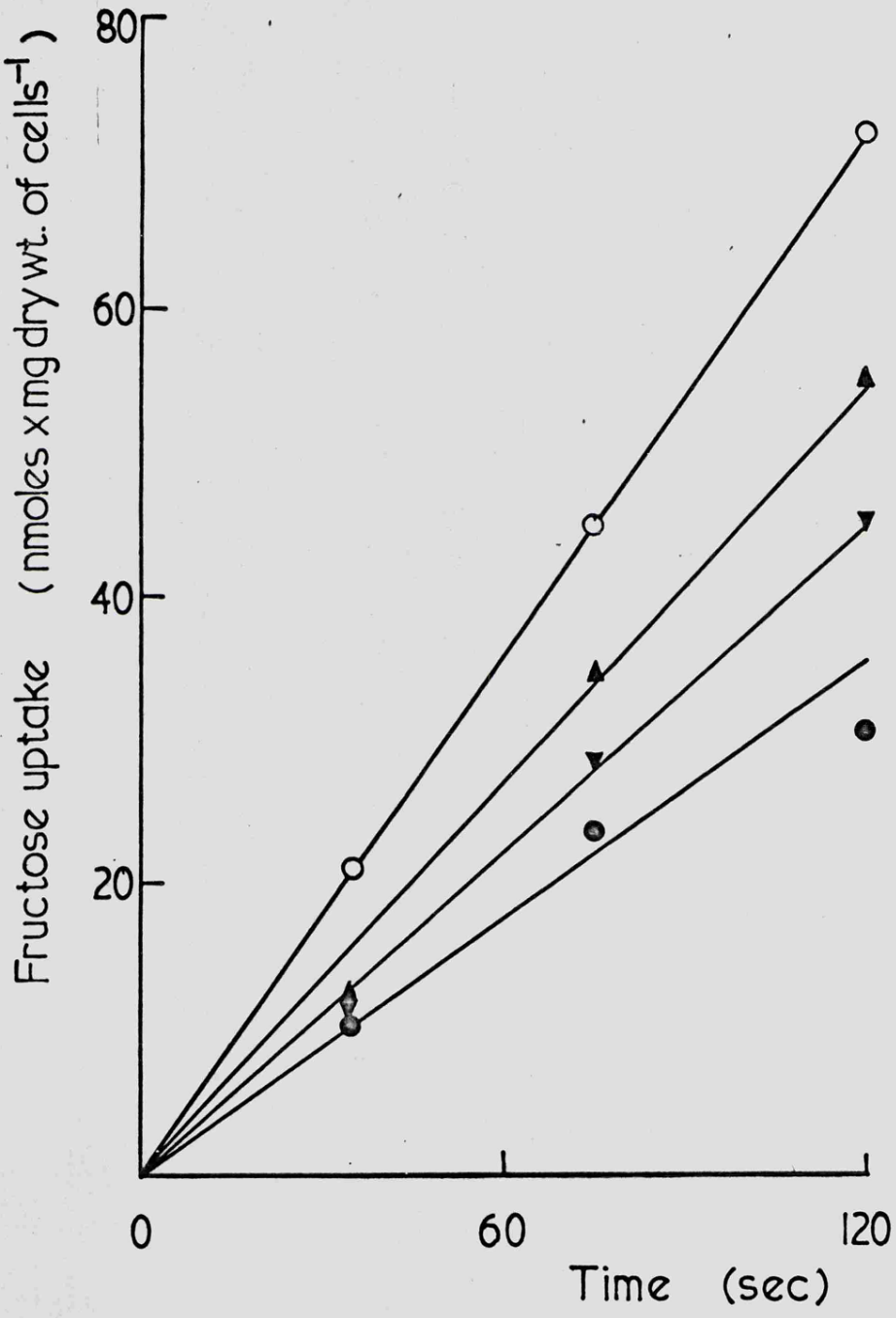
Fructose uptake (nmoles x mg dry wt. of cells^{-1})
80
60
40
20
0
60
120
Time (sec)

FIGURE 18 Inhibition of fructose uptake by glucose, 2-deoxyglucose and α-methyl-glucoside in washed cell suspensions of strain KL16. 0.1 mM [U-^{14}C]fructose uptake is shown in the absence of additions (○), and in the presence of 1 mM glucose (●), 1 mM α-methyl-glucoside (▼) or 1 mM 2-deoxyglucose (▲), which were added 1 min before the addition of the [U-^{14}C]fructose.

TABLE 13 The inhibition of 0.1 mM fructose uptake by glucose in mutants of E. coli

Organism	Rates of fructose uptake * - glucose	+ 1 mM glucose	% inhibition
K2.1t	28	9.3	67
K2.1.5^{c}.7	29	7.6	74
K2.1.5^{c}.15	31	12	61
K2.1.5^{c}.16	30	2.1	93
K2.1.5^{c}.7.15	20	1.6	92
K2.1.5^{c}.15.16	19	0.58	97

* Rates of uptake are expressed as nmoles of fructose taken up x min^{-1} x mg dry weight of $cells^{-1}$. These rates were determined in washed suspensions of fructose-grown cells, and in the appropriate experiments, 1 mM glucose was added 1 min before the ^{14}C-labelled fructose.

had been pre-grown on fructose as sole carbon source. Both the rate of removal from the medium and the rate of incorporation of fructose into cell material, as determined by the use of ^{14}C-labelled fructose, are lower when glucose is present. This shows, as is evident from Table 12, that glucose is utilized in preference to fructose even by cells that had not previously been exposed to glucose. This preferential utilization occurs without detectable lag: it is an inhibition, not a repression, phenomenon that is involved here.

Glucose can also be shown to inhibit the initial rate of the uptake of 0.1 mM fructose in washed cell suspensions. As shown in Figure 18, glucose and, to a lesser extent, the glucose analogues 2-deoxyglucose and α-methyl D-glucoside, inhibit fructose uptake. In order to find out whether this inhibition was extra- or intracellular, determinations of the inhibition of fructose uptake by glucose were performed on mutants blocked in the further metabolism of glucose; if the inhibition was extracellular or was due to free glucose, there should have been no effect on the inhibition of fructose uptake by glucose in these mutants. The results of such experiments are shown in Table 13. The mutants used lacked the enzymes: phosphoglucose isomerase = PGI ($K2.1.5^c.7$), glucose 6-phosphate dehydrogenase = ZWF ($K2.1.5^c.15$), 6-phosphofructokinase = PFK ($K2.1.5^c.16$) and combinations of these lesions, as in $K2.1.5^c.7.15$, which is PGI^-, ZWF^-, or $K2.1.5^c.15.16$, which lacks ZWF and PFK. These strains are described in Table 1 and the location of the blocks in this area of carbohydrate metabolism of E. coli in Figure 1.

It is apparent from the results that lesions that would be expected to increase the intracellular levels of hexose phosphates,

FIG. 19

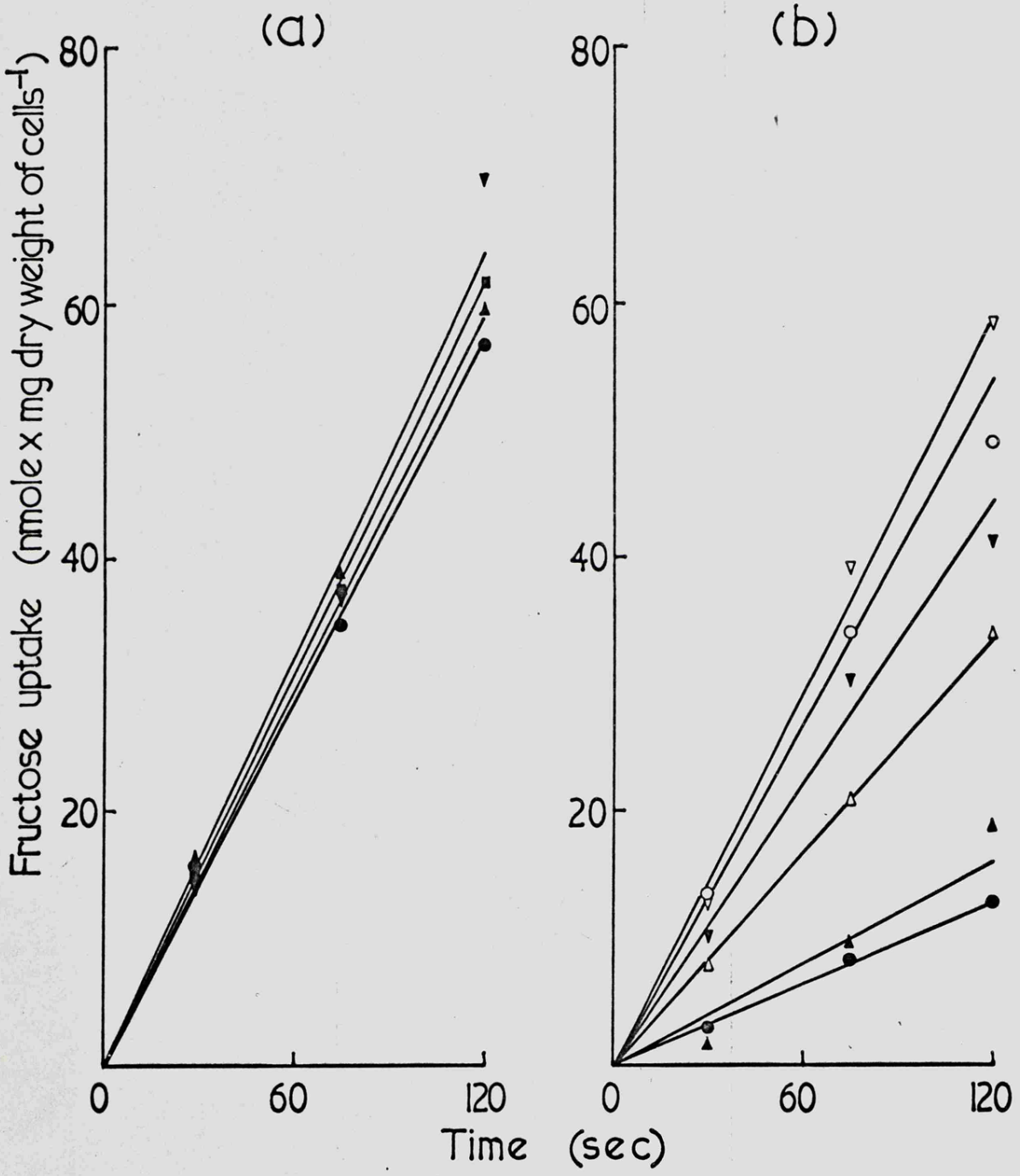
(a)
(b)
Fructose uptake (nmole x mg dry weight of cells^{-1})
80
60
40
20
0
60
120
Time (sec)

FIGURE 19 The effect of growth conditions on the inhibition of 0.1 mM [U-^{14}C]fructose uptake by galactose, mannitol or gluconate in washed cell suspensions of K2.1t.

(a) Fructose uptake by fructose-grown K2.1t, in the absence of inhibitor (■), and in the presence of 1 mM galactose (●), 1 mM mannitol (▼) or 1 mM gluconate (▲).

(b) Fructose uptake by K2.1t pre-grown on fructose, but transferred to grow for one generation in media containing 10 mM fructose plus 10 mM galactose, mannitol or gluconate. Fructose uptake is shown in the absence (open symbols) and presence (closed symbols) of 1 mM galactose (○,●), 1 mM mannitol (▽,▼) or 1 mM gluconate (△,▲), by cells grown in the presence of the respective carbon source.

In each case, the inhibitor was added 1 min before the [^{14}C]fructose.

such as blocks in PGI, PGI + ZWF, PFK and PFK + ZWF, give greater inhibitions by glucose of fructose uptake than are observed in wild-type cells. However, only wild-type level of inhibition is observed in the mutant lacking ZWF alone, but the route via this enzyme is a relatively minor one in the normal metabolism of glucose (Fraenkel, 1968b). Hence, it is likely that a product or products of glucose metabolism are at least partially responsible for the inhibition of fructose uptake. These results also show that the observed inhibition is not just a dilution effect on the labelled fructose by the product(s) of glucose metabolism, as mutants blocked in PGI + ZWF or PFK + ZWF are unable to utilize glucose for growth, but glucose even more strongly inhibits the uptake of fructose in these organisms.

It would be expected that other carbon sources likely to give rise to intracellular hexose phosphates could inhibit the uptake of fructose, if hexose phosphates are indeed the inhibitors. In washed cell suspensions of K2.1t grown on fructose, no or slight inhibition of fructose uptake can be demonstrated by 1 mM galactose, mannitol or gluconate, as illustrated in Figure 19. However, in cells which had been transferred to growth on 10 mM fructose plus 10 mM of these other carbon sources for approximately one generation, about 27% inhibition of fructose uptake can be shown by mannitol, 75% by galactose and 50% by gluconate. The quantitative differences in the levels of inhibition are probably due to the different extents to which the systems responsible for the uptake of galactose, mannitol and ~~glucose 6-phosphate~~ gluconate are induced under the conditions used, and hence the extent to which they are able to give rise to intracellular hexose phosphates. These results again indicate the intracellular nature of the inhibition of fructose uptake.

TABLE 14 The inhibition of 0.1 mM fructose uptake by hexose phosphates in strain K2.1.7.11[c]

Additions to uptake medium*	Initial rate of fructose uptake**	% inhibition
None	26	0
Glucose 6-phosphate (1 mM)	7.8	70
Fructose 6-phosphate (1 mM)	4.2	84
Glucose 1-phosphate (5 mM)	11	58
Fructose 1-phosphate (5 mM)	5.7	78

* Additions were made 1 min before the ^{14}C-labelled fructose.

** Initial rates were measured in washed cell suspensions (Materials and Methods) of fructose-grown cells and are expressed as nmoles of fructose taken up x min^{-1} x mg dry weight of $cells^{-1}$.

Kaback (1969) has shown that hexose phosphates, and especially glucose 1-phosphate, can inhibit the entry of glucose and fructose into isolated membrane vesicles of E. coli. Glucose 1-phosphate was found to be the best inhibitor of the uptake of fructose into membrane vesicles, while glucose 6-phosphate gave almost no inhibition and fructose 6-phosphate and fructose 1-phosphate gave intermediate levels of inhibition. These sugar phosphates were tested for their ability to inhibit the uptake of fructose by intact cells in washed cell suspensions. These experiments were facilitated by the construction of the mutant K2.1.7.11^{c} (Table 1), which had high levels of hexose phosphate uptake ability, even after growth on fructose, because of the presence of the uhp^{c} allele (see Chapter IV for detailed properties of mutants constitutive for hexose phosphate uptake). The initial rates of fructose uptake by K2.1.7.11^{c} which also lacks PGI, are shown in Table 14, in the absence and presence of the four hexose phosphates. Fructose 6-phosphate and glucose 6-phosphate were added at 1 mM while fructose 1-phosphate and glucose 1-phosphate were supplied at 5 mM, because the 1-phosphates are poorer substrates of the hexose phosphate transport system (see Chapter IV). As shown, fructose 6-phosphate and fructose 1-phosphate give about 80% inhibition of fructose uptake, glucose 6-phosphate about 70% and glucose 1-phosphate about 60%. Because of the uncertainty of the intracellular levels of the hexose phosphates, however, it is difficult to assign quantitative significance to these results and they may not be incompatible with the findings of Kaback (1969). Despite the presence of the PGI block, all four hexose phosphates are either inhibitors, or can give rise to inhibitors, of the uptake of fructose.

The possible site(s) of regulation of the utilization of fructose were sought by studying the effects of hexose phosphates on the decryptified PEP:fructose PTS and on the enzyme FPK, in crude cell-free extracts. Less than 5% inhibition of the FPK activity in cell-free extracts of fructose-grown K2.1.7 was elicited by 2 mM concentrations of glucose 6-phosphate, glucose 1-phosphate and 6-phosphogluconate, when present in the usual assay system. Inhibition by fructose 6-phosphate could not be determined in this assay because of 6-phosphofructokinase and mannitol 1-phosphate dehydrogenase activities in the crude extracts. More surprisingly, when glucose 6-phosphate, glucose 1-phosphate, 6-phosphogluconate, fructose 6-phosphate and fructose 1-phosphate (all at 2.5 mM) were tested for their inhibitory effect in the pyruvate release assay for PEP:fructose PTS activity (Method B, Materials and Methods) in frozen-thawed extracts of fructose-grown K2.1.7, no significant inhibition was detected. Similarly, in the more specific assay involving measurements of the rate of conversion of [^{14}C]fructose to [^{14}C]fructose-phosphate (Method A, Materials and Methods), again on K2.1.7, less than 5% inhibition of PEP:fructose PTS activity were found with 1 mM glucose 1-phosphate, glucose 6-phosphate or 6-phosphogluconate.

(v) DISCUSSION

The proteins of the system responsible for the uptake of fructose are expressed at a relatively high level, whatever the carbon source for growth. There is only a 4-8 fold increase in uptake rates after growth on fructose; this allows for rapid adaptation to the ability to utilize fructose whenever it is present in the growth medium of *E. coli*. This semi-constitutive nature of the fructose uptake system would put it intermediate between uptake systems that are constitutive, like the one for α-methylglucoside, and those that are fully inducible, like the system for β-galactosides, which can be induced 100-fold (Cohen and Monod, 1957). The enzyme fructose 1-phosphate kinase can be induced over a greater range of activity (10-20 fold) than can fructose uptake; however, the properties of a mutant constitutive for both fructose uptake and fructose 1-phosphate kinase suggests that expression of both activities is controlled together, either as a regulon (Maas and Clark, 1964) or as an operon (Jacob and Monod, 1961b). No mutants lacking fructose 1-phosphate kinase have been described for *E. coli*, so there is no genetic information concerning the location of the gene for this enzyme and whether it is close to the gene(s) for the component(s) of the PEP fructose phosphotransferase system mapped in Chapter I.

Preliminary evidence also indicates that the regulatory gene altered in a mutant constitutive for the fructose enzymes, and the gene specifying a fructose-specific component of the phosphotransferase system, are located in the same region of *E. coli* linkage map, but are unlikely to be closely linked and hence form part of an operon.

In confirmation of the findings of De Crombrugghe *et al.* (1969),

the fructose uptake system and, in addition, the enzyme fructose 1-phosphate kinase, are subject to catabolite repression. Again, the expression of the enzyme fructose 1-phosphate kinase appears to be under greater regulatory control than expression of the components of the uptake system; for instance, repressive conditions which can cause about 70% lowering of fructose 1-phosphate kinase levels only results in a 45% decrease in rates of fructose uptake. This may imply that different promoter regions (Silverstone et al., 1969) are involved in the expression of these proteins.

The glucose inhibition of fructose utilization described in this Chapter has, on the surface, a number of properties in common with the mechanism of catabolite inhibition, as described by McGinnis and Paigen (1969). The inhibitory effect can be demonstrated to affect fructose uptake, and is elicited by glucose and glucose 6-phosphate. According to McGinnis and Paigen (1969), catabolite inhibition regulates at an early phase in the utilization of galactose and lactose just as glucose inhibition seems to regulate fructose uptake. However, the uptake of fructose can be inhibited not only by glucose and glucose 6-phosphate, but by other carbon sources, which can inhibit after the induction of their respective enzyme systems, suggesting the general intracellular nature of the effect. The mutants with metabolic blocks in the enzymes of carbohydrate metabolism indicate that the accumulation of hexose phosphates is one of the major, if not the sole, cause of the inhibition of fructose uptake.

The exact site of inhibition is still unclear. The evidence presented does not suggest that the activity of the enzyme fructose 1-phosphate kinase is regulated by most hexose phosphates; also, in A. aerogenes the enzyme has been purified and its activity was found

not to be modified by effectors such as mannose 6-phosphate, glucose 6-phosphate or glucose 1-phosphate, but to be competitively inhibited by fructose 6-phosphate, with a K_i of 1 mM (Sapico and Anderson, 1969). Fructose 6-phosphate is unlikely to be the sole inhibitor of fructose uptake in E. coli, however, because high levels of inhibition of fructose uptake by glucose can still be observed in mutants in which fructose 6-phosphate cannot be formed from glucose.

The probable site of regulation of fructose utilization is at the level of fructose translocation through the membrane. Although no inhibition of fructose phosphorylation by hexose phosphates could be demonstrated in decryptified cell preparations, inhibition of fructose uptake can be observed in whole cells and also in membrane vesicles, isolated from E. coli by Kaback (1969), in which sugar translocation has been shown to be PEP-dependent (Kaback, 1968). It is possible either that fructose translocation, rather than just phosphorylation, is required for the inhibition to be demonstrable, or that decryptification destroys the regulatory site(s) of the phosphotransferase system.

In the case of glucose at least, inhibition of fructose uptake and utilization may be due in part also to competition for the common components of the PTS, as proposed by Roseman (1969).

CHAPTER IV GROWTH ON FRUCTOSE 1-PHOSPHATE

(i) Introduction

It has been generally recognised in the past that cells exhibit a selective permeability towards compounds, especially charged molecules. In the absence of specific transport systems, cell membranes tend to be impermeable to such cations as Na^+ and K^+ and to organic anions such as nucleotides and sugar phosphates. The recognition of selective permeability mechanisms in bacteria occurred in the late 1940's and early 1950's, culminating in the work of Cohen and Monod (1957; also for early references in this field). Their ideas led to the now prevalent belief that transport across bacterial membranes is mediated by stereospecific, and sometimes inducible, proteins. However, it was wrongly held till as late as 1960, that E. coli was likely to be impermeable to intact glucose 6-phosphate (Horecker, Thomas and Monod, 1960), despite the early findings that certain 'ferments' were able to utilize exogenous glucose 6-phosphate (Warburg and Christian, 1933). Also, Roberts and Wolffe (1951) found that E. coli growing in the presence of fructose 6-phosphate incorporated the phosphate from fructose 6-phosphate into nucleic acids without an intermediary dephosphorylation of fructose 6-phosphate, suggesting the entry of intact fructose 6-phosphate into the cells.

The first direct demonstration of the utilization of a phosphorylated carbon source, without prior hydrolysis, was that mutants of E. coli K10 lacking glycerol kinase, which could not grow on glycerol, could still grow on L-α-glycerophosphate (Lin et al., 1962). Soon after, Fraenkel, Falcoz-Kelly and Horecker (1964) showed that glucose 6-phosphate can be utilized for growth in mutants of E. coli unable to utilize free glucose, which suggested that the phosphate ester entered

intact. The presence of an inducible uptake system for hexose 6-phosphates in E. coli was demonstrated by Winkler (1966). He also isolated mutants that could grow on mannose, glucose or fructose but could not grow on the corresponding hexose 6-phosphates. The inducibility of glucose 6-phosphate uptake in E. coli B was confirmed by Pogell et al. (1966) who further showed that glucose 1-phosphate can be taken up in glucose 6-phosphate-grown cells and that fructose 1-phosphate, among a number of phosphorylated compounds, is an inhibitor of glucose 6-phosphate uptake.

More recent work on the hexose phosphate transport system has been aimed at elucidating the mechanism of genetic regulation of this system, with suggestions that glucose 6-phosphate is the true inducer of the system and acts as such only when supplied exogenously (Heppel, 1969; Winkler, 1970). The implications of these suggestions are discussed below. A genetic approach to this problem has been initiated by Kornberg and Smith (1969), who have located a lesion resulting in the loss of hexose phosphate uptake ability at about 72 min on the E. coli linkage map (Taylor, 1970).

Although it has now been demonstrated that E. coli can grow on a variety of sugar phosphates, it was important for the purposes of the present work to be able to demonstrate that fructose 1-phosphate could also be utilized as sole carbon source by E. coli. The previous Chapters have shown how much this finding has helped in the clarification of the role of fructose 1-phosphate in fructose utilization. It is the purpose of this Chapter to describe the characteristics of the growth of E. coli on fructose 1-phosphate and the properties of the hexose phosphate system this phenomenon helped to elucidate.

FIG. 20

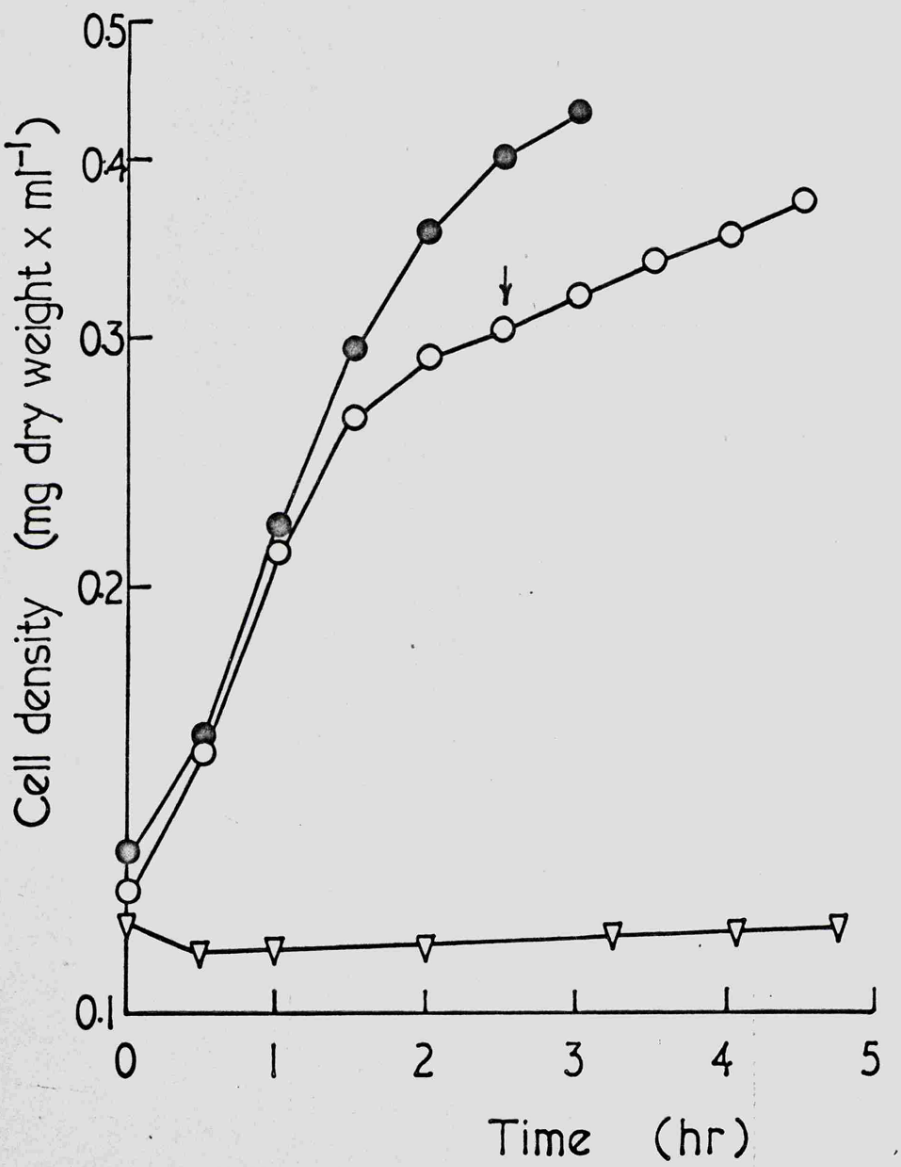

FIGURE 20 Growth of the wild-type strain KL16 on fructose 1-phosphate. Cells growing exponentially on glucose 6-phosphate were harvested and resuspended in media containing 10 mM fructose 1-phosphate (●) and 5 mM fructose 1-phosphate (○) as sole source of carbon. At the time arrowed, an additional 2.5 mM fructose 1-phosphate was added to the latter culture. KL16 pre-grown on glucose and transferred to 5 mM fructose 1-phosphate is also shown (▽).

(ii) Growth pattern on fructose 1-phosphate

The ability of E. coli to grow on fructose 1-phosphate is an inducible characteristic and can be demonstrated only if cells are transferred from cultures growing on a hexose 6-phosphate. Growth on fructose 1-phosphate does not occur at a fast rate if E. coli is pre-grown on glucose, fructose, glycerol or any other non-phosphorylated carbon source tested. This growth pattern is illustrated in Figure 20, which shows the growth of wild-type KL16 on fructose 1-phosphate in liquid cultures. There is no rapid growth on fructose 1-phosphate by cells pregrown on glucose, but KL16 adapted to growth on glucose 6-phosphate as carbon source continues doubling rapidly for 1-1½ generations after transfer to fructose 1-phosphate, supplied at 5 mM concentration and slightly longer on this substrate at 10 mM. The growth then slows to a rate probably due to the utilization of free fructose in the medium and this rate is not greatly increased by the addition of further fructose 1-phosphate, as indicated in Figure 20. This unusual growth pattern suggests that the utilization of fructose 1-phosphate may proceed via the hexose phosphate transport system, as the property is dependent on the state of induction of this system. Also, as growth continues exponentially for about only 1 generation after transfer to fructose 1-phosphate, it would seem likely that the presence of fructose 1-phosphate by itself is not sufficient to maintain the transport system in the fully induced state. The experiments set out below tend to confirm that fructose 1-phosphate is a substrate, but not an inducer, of the hexose phosphate transport system.

To show that intact fructose 1-phosphate and not fructose obtained by dephosphorylation is utilized in growth experiments, a mutant

FIG. 21

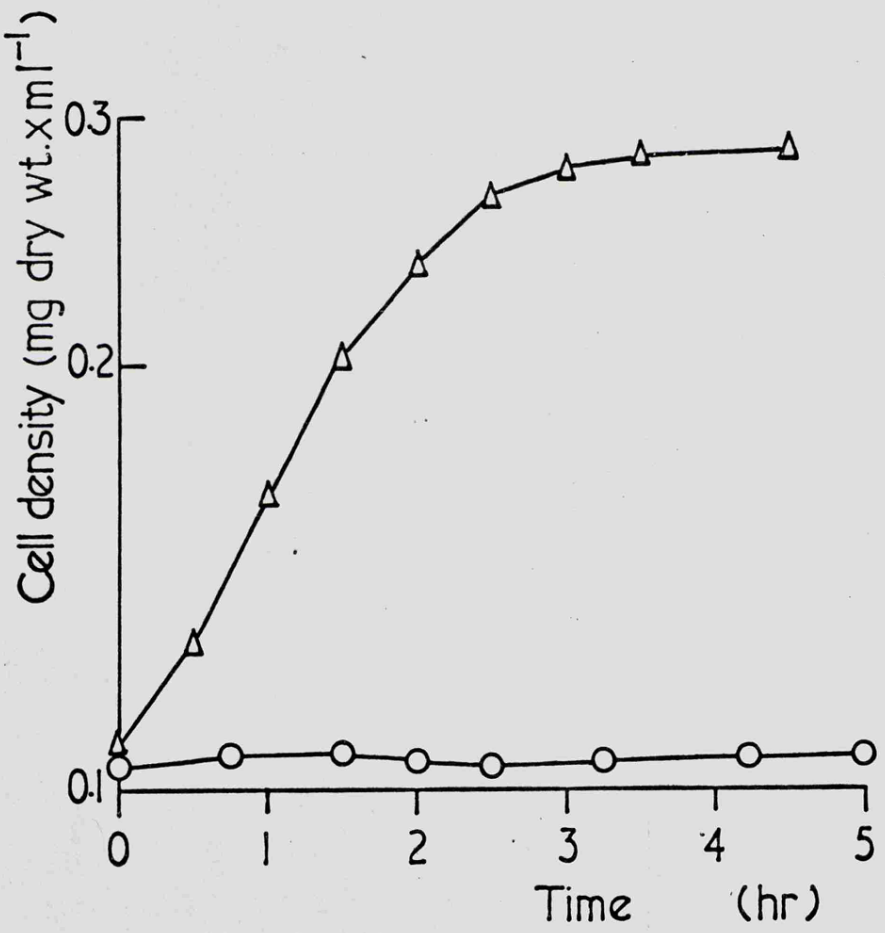

FIGURE 21 The growth of strain KL16.21 on fructose and fructose 1-phosphate. Cells growing exponentially on glucose 6-phosphate were harvested and transferred to media containing 5 mM fructose 1-phosphate (Δ) or 5 mM fructose (O) as sole carbon source.

FIG. 22

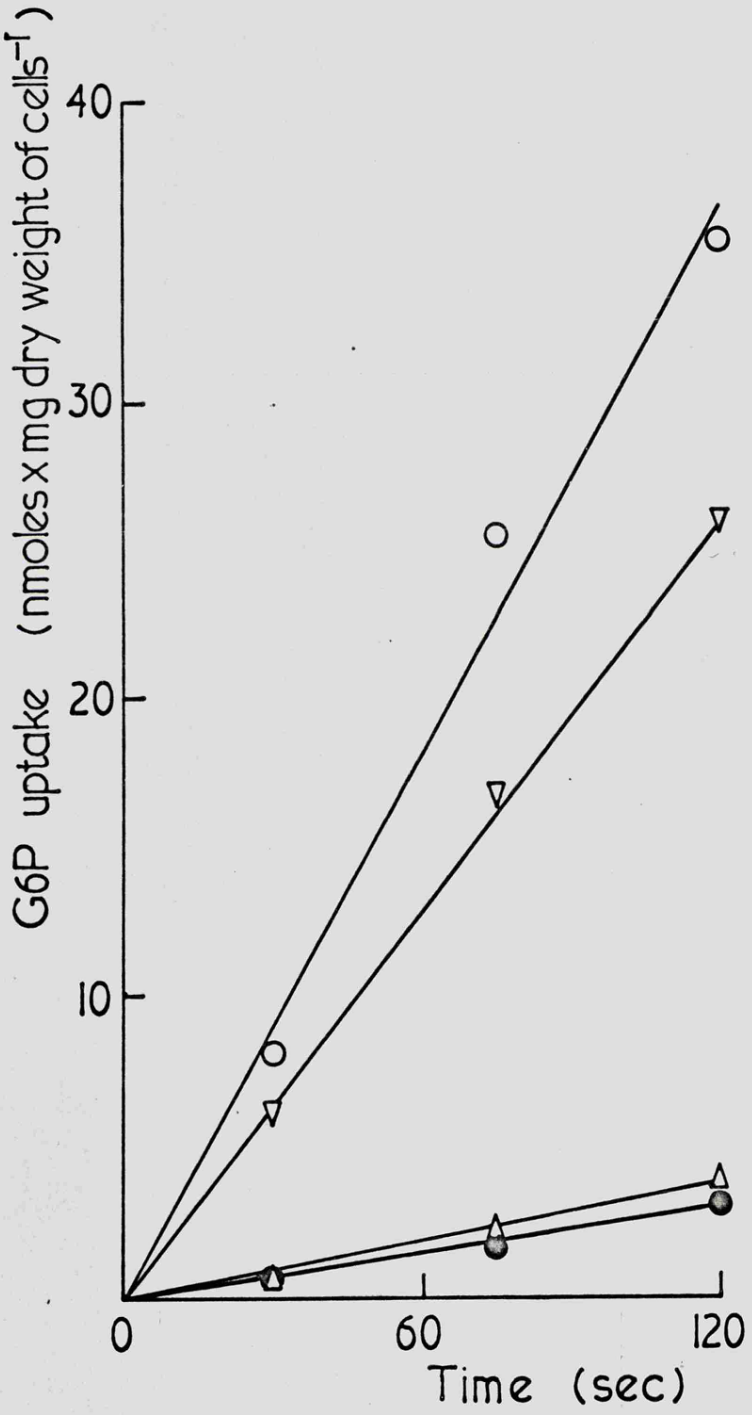
G6P uptake (nmoles x mg dry weight of cells^{-1})
40
30
20
10
0
60
120
Time (sec)

FIGURE 22 Time-course of 0.1 mM [U-^{14}C]glucose 6-phosphate uptake by glucose 6-phosphate-grown KL16, in the presence of added: nothing (O), 1 mM fructose 1-phosphate (∇) and 1 mM fructose 6-phosphate (Δ). The inhibitors were added 1 min before the glucose 6-phosphate.

The uptake of glucose 6-phosphate, after the growth of KL16 on fructose 1-phosphate, is also indicated (●).

unable to grow on free fructose was tested for growth on fructose 1-phosphate. This mutant, KL16.21, as shown in Figure 21, is unable to grow on 5 mM fructose, but is still able to grow on 5 mM fructose 1-phosphate when transferred to a medium containing this substrate, after prior growth on glucose 6-phosphate. The growth rate obtained is almost as good as with KL16, but after the rapid phase, growth ceases completely. This agrees with the suggestion made previously, that the slow rate observed with the wild-type organism after this point is due to the utilization of free fructose (produced probably through hydrolysis of fructose 1-phosphate) in the medium. The presence of 0.2 - 0.3 mM fructose in the growth medium of KL16.21 has also been detected by the diphenylamine assay of Ashwell (1957), after fructose 1-phosphate had been removed by treatment with excess ion exchange resin (Dowex-1 Cl^- form).

As have been described more fully in Chapters I and II, the lesion in KL16.21 is a deficiency in the uptake and phosphorylation system for fructose, which would not be expected to result in the impairment of intracellular metabolism of fructose 1-phosphate. This proceeds by the induction of the enzyme fructose 1-phosphate kinase (FPK) during growth on fructose 1-phosphate. This too has been shown in Chapter I, which deals with the reactions of fructose utilization.

(iii) Uptake of hexose phosphates

The link between the hexose phosphate transport system and growth on fructose 1-phosphate is further strengthened by the data in Figure 22. The results confirm in KL16 the finding of Pogell *et al.* (1966), that, in *E. coli* B, both the 1- and 6-phosphate esters of fructose

FIG. 23

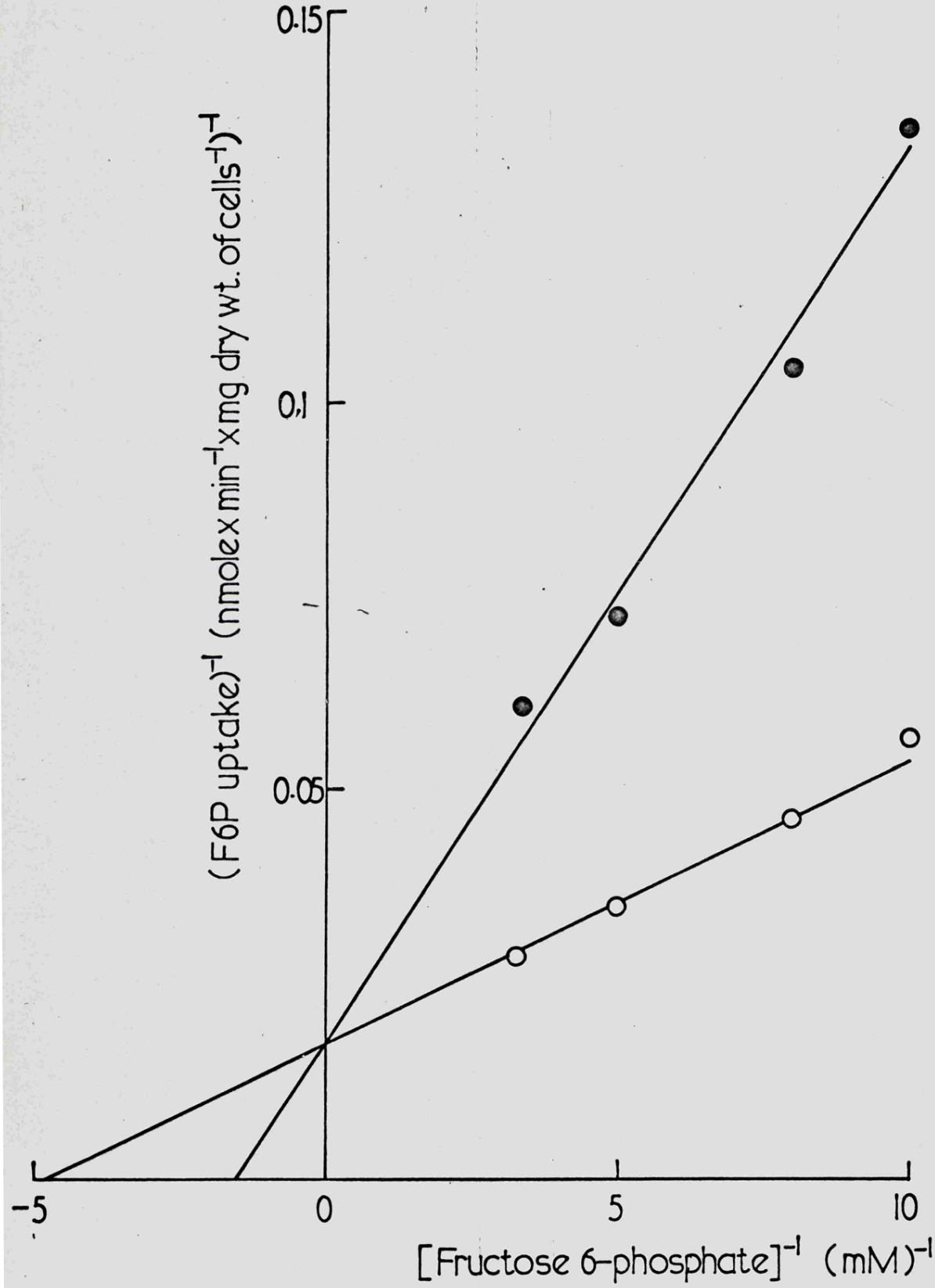
0.15
0.1
0.05
(F6P uptake)$^{-1}$ (nmole x min^{-1} x mg dry wt. of cells^{-1})$^{-1}$
-5
0
5
10
[Fructose 6-phosphate]$^{-1}$ (mM)$^{-1}$

FIGURE 23 Lineweaver-Burk plots for the concentration dependence of the initial rates of [^{14}C]labelled fructose 6-phosphate uptake by glucose 6-phosphate-grown KL16.21 in washed cell suspensions, in the absence (O) and presence (●) of 2.5 mM fructose 1-phosphate.

inhibitors of the initial rate of uptake of glucose 6-phosphate: this latter substrate is transported via the hexose phosphate transport system (Winkler, 1966). However, the 1-phosphate is a poorer inhibitor of the uptake of 0.1 mM glucose 6-phosphate than is fructose 6-phosphate at the same concentration (1 mM) but this does suggest that both fructose phosphates have an affinity for the hexose phosphate transport system. The data in Figure 22 do not show in what manner fructose 1-phosphate inhibits the uptake of glucose 6-phosphate. If fructose 1-phosphate is also a substrate of the hexose-phosphate transport system, it should be a competititve inhibitor of the uptake of both fructose 6- and glucose 6-phosphate. Figure 23 shows the Lineweaver-Burk plots obtained when the initial rates of uptake of fructose 6-phosphate are measured at the concentrations indicated in the presence and absence of 2.5 mM fructose 1-phosphate. It is found that the presence of 1-phosphate does not affect the V_{max} for fructose 6-phosphate uptake but increases the concentration of the 6-phosphate required to produce half the maximal rate of its uptake; that is the Michaelis constant (K_m) for fructose 6-phosphate is shifted to a new, higher value (K_p). This indeed indicates that fructose 1-phosphate is a competitive inhibitor of fructose 6-phosphate uptake; it is also possible to calculate a value for the inhibitor constant (K_i) of fructose 1-phosphate for the hexose phosphate transport system from the equation $K_p = K_m (1 + \frac{i}{K_i})$ (Dixon and Webb, 1957). With E. coli strain KL16, the K_i for fructose 1-phosphate is approximately 1.3 mM, as against the K_m for fructose 6-phosphate of 0.21 mM and for glucose 6-phosphate (obtained independently) of 0.20 mM. These values confirm the earlier suggestion that fructose 1-phosphate has a much lower affinity for the hexose phosphate transport system than do fructose 6-phosphate and glucose 6-phosphate. The K_m

FIG. 24

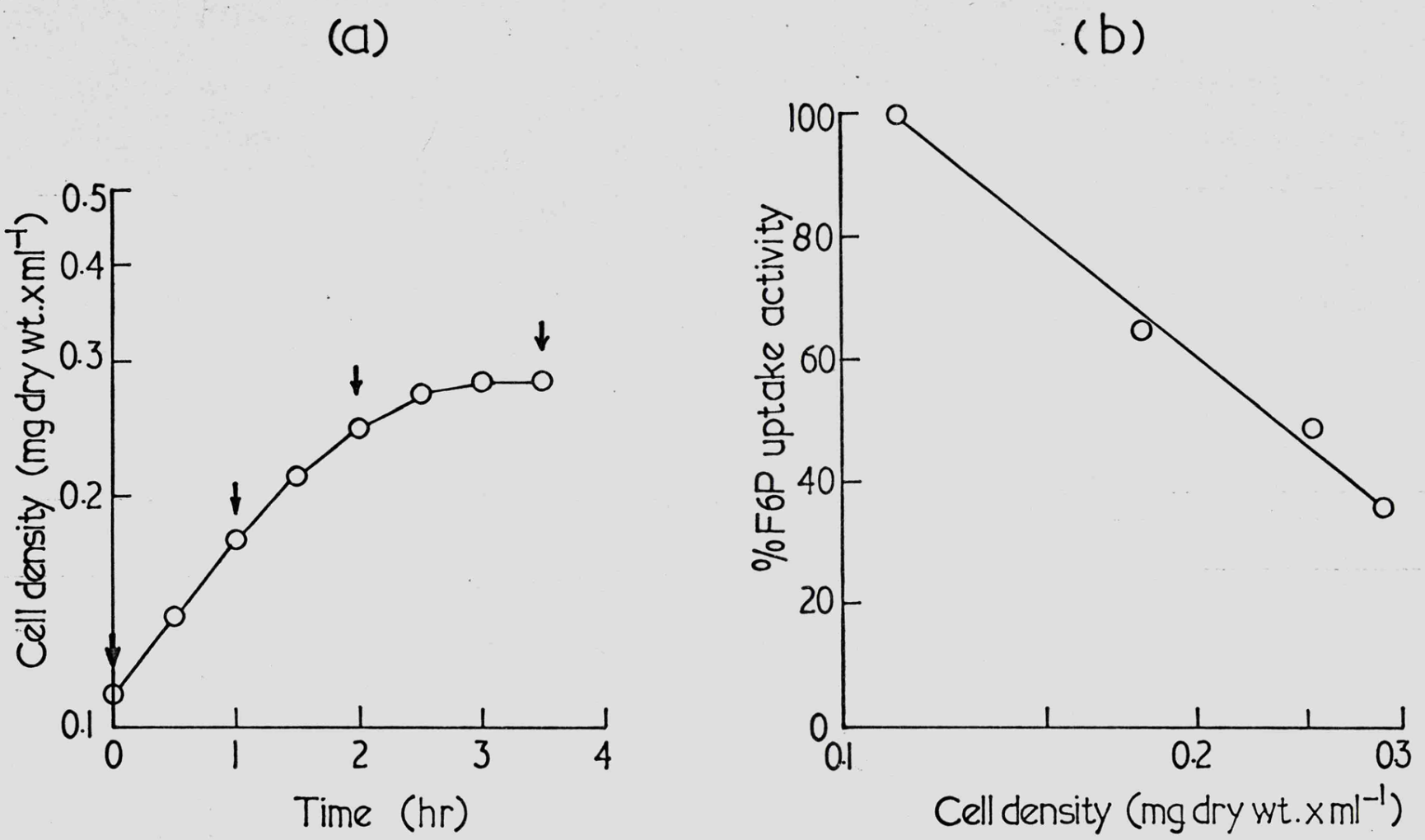
(a)
Cell density (mg dry wt. x ml⁻¹)
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
Time (hr)
(b)
% F6P uptake activity
100
80
60
40
20
0
0.1
0.2
0.3
Cell density (mg dry wt. x ml⁻¹)

FIGURE 24

(a) Growth of strain KL16.21 on 5 mM fructose 1-phosphate as sole carbon source, after transfer from growth on glucose 6-phosphate. At the times indicated, samples of the culture were taken, and the ability of the cells to take up [U-^{14}C]-fructose 6-phosphate was measured in washed cell suspensions.

(b) The decrease in fructose 6-phosphate uptake ability during growth on fructose 1-phosphate, with the uptake activity at zero time in the growth curve taken as 100%.

values for the uptake of these two hexose phosphates are somewhat lower than the values of 0.5 mM and 0.4 mM obtained by Winkler (1966) with strain ML308, and the 0.27 mM for glucose 6-phosphate reported by Pogell et al. in E. coli B.

It has already been stated that the hexose phosphate transport system is inducible, and Figure 22 shows the rate of 0.1 mM glucose 6-phosphate uptake by washed cells that had been grown on glucose 6-phosphate. This rate is seen to be greatly reduced after the glucose 6-phosphate-grown cells had been permitted to continue to grow on fructose 1-phosphate; this reduction in uptake ability is probably one of the causes of the cessation of growth on fructose 1-phosphate after 1-1½ generations. If, as Figure 22 indicates, fructose 1-phosphate is not an inducer of the hexose phosphate transport system, it would be expected that the ability to take up hexose phosphates would be diluted out during growth on fructose 1-phosphate. The pattern of dilution out of the ability to take up glucose 6-phosphate is shown in Figure 24 as a differential rate, the measure introduced by Monod et al.(1952) in the study of induced enzyme synthesis. Taking the fully induced rate of uptake as 100%, it can be seen that approximately 49% of the activity is lost during one doubling, as against a theoretical 50% that would be expected during growth in the absence of an inducer of the system. This result confirms that fructose 1-phosphate is not an inducer for the hexose phosphate uptake system. It is also apparent that, by the time the rate of growth on fructose 1-phosphate slows markedly, over 60% of the uptake ability has been lost. This finding, together with the low affinity of the transport system for fructose 1-phosphate, probably explains the cessation of fast growth on fructose 1-phosphate shown in Figure 20.

(iv) Selection of mutants constitutive for hexose phosphate transport

The growth properties described in the previous section suggested a simple selection procedure for obtaining mutants constitutive for hexose phosphate transport. As fructose 1-phosphate supports the growth of E. coli only when the transport system is near fully induced, spontaneous mutants ought to be found which could grow exponentially on fructose 1-phosphate, either by acquiring high phosphatase activity and so being able to grow on the free fructose released, or by having the hexose phosphate transport system present under normally uninducing conditions. The phenotype of this latter mutation should then be expressed not only as an ability to grow exponentially on fructose 1-phosphate, but also as an ability to take up fructose 6-phosphate and glucose 6-phosphate constitutively.

The method of selection for such mutants simply involved plating about 10^9 cells of the required type on agar plates containing 2 mM fructose 1-phosphate as sole carbon source. The plates were incubated at 37° for 48-72 hours; colonies that appeared were picked, streaked for single colonies, and tested for original growth markers, if any.

When one such clone derived from KL16 on fructose 1-phosphate plates was tested for glucose 6-phosphate uptake ability, it could take up glucose 6-phosphate, apparently constitutively. These cells, grown on glucose, had five times the usual fully-induced rate of glucose 6-phosphate uptake, but this rate was inhibited by glucose, unlike normal glucose 6-phosphate uptake. It was thus likely that in these cells, the ^{14}C-label derived from ^{14}C-glucose 6-phosphate was entering via the constitutive glucose uptake system and that the mutation selected

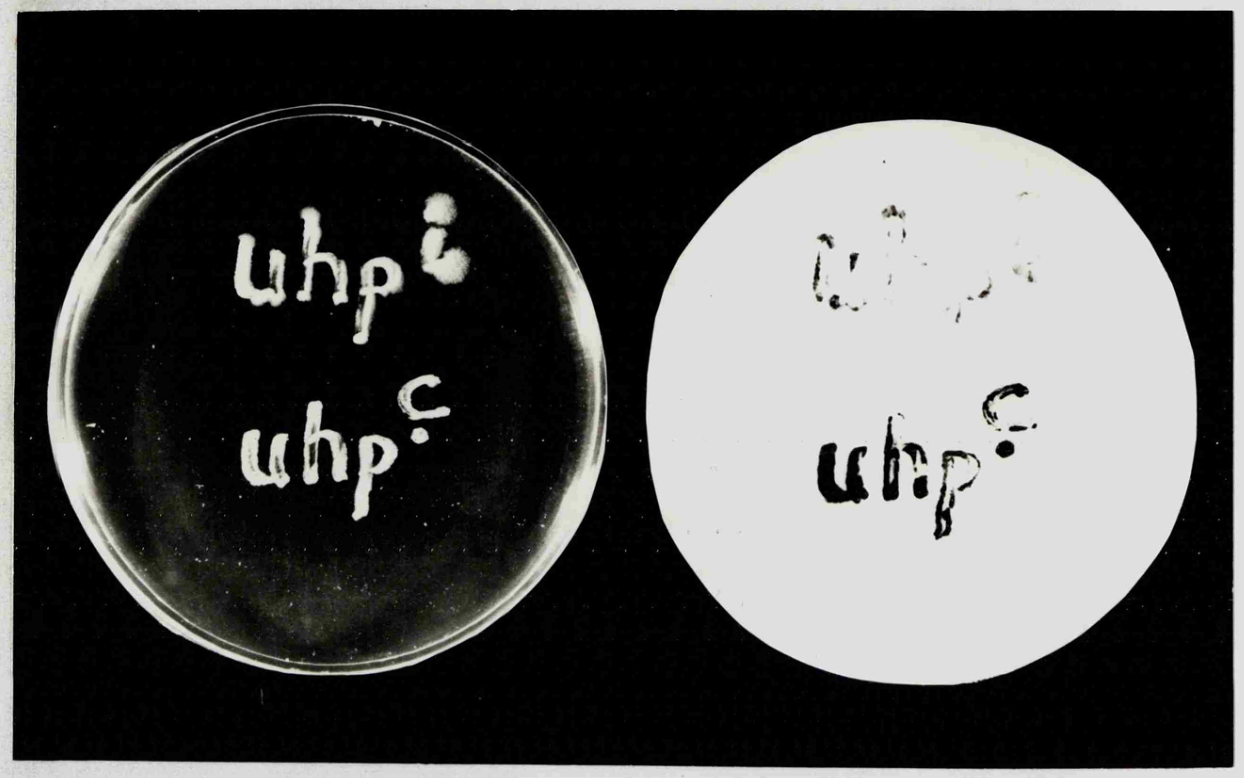

FIGURE 25 Identification of clones inducible (uhp^i) and constitutive (uhp^c) for the uptake of hexose phosphates.

Left: two uracil$^+$-transductants (see text) after growth of the organisms on agar plates containing 5 mM glucose plus 20 μM ^{14}C-fructose 6-phosphate.

Right: blackening of X-ray film after exposure to these organisms adsorbed on filter paper. For experimental details, see the Materials and Methods section.

for by growth on fructose 1-phosphate resulted in a derepression of a dephosphorylation reaction of hexose phosphates.

To decrease the likelihood of phosphatase-type mutations, KL16.21 was substituted in the procedure. As already mentioned, this mutant is unable to utilize fructose so there would be no selective advantage in it being able to dephosphorylate fructose 1-phosphate. Colonies derived from KL16.21, picked from fructose 1-phosphate plates, were tested for growth on fructose and fructose 1-phosphate. About half the colonies were revertants to fructose positivity (presumably in the fructose formed from fructose 1-phosphate in the plates after two days at 37°) and were unable to grow on plates containing fructose 1-phosphate overnight. All other spontaneous mutants of KL16.21 tested were still unable to grow on fructose but could now grow on plates containing fructose 1-phosphate overnight. The frequency of this type of mutation is approximately 1 in 10^8 of cells plated.

The identification of this latter type of mutation was greatly facilitated by the film technique, practical details of which are in the Materials and Methods section. The principle of the method is that wild-type E. coli with an inducible transport system is unable to accumulate low concentrations (20 μM) of fructose 6-phosphate when colonies are grown on a substrate such as glucose (25 mM) as the major carbon source. When ^{14}C-labelled fructose 6-phosphate is used, the difference between inducible and constitutive clones can be distinguished, as shown in Figure 25, by the darkness of the X-ray film after autoradiography. Constitutives, designated uhp^c, appear much darker owing to the label from fructose 6-phosphate that has been incorporated into the cells.

FIG. 26

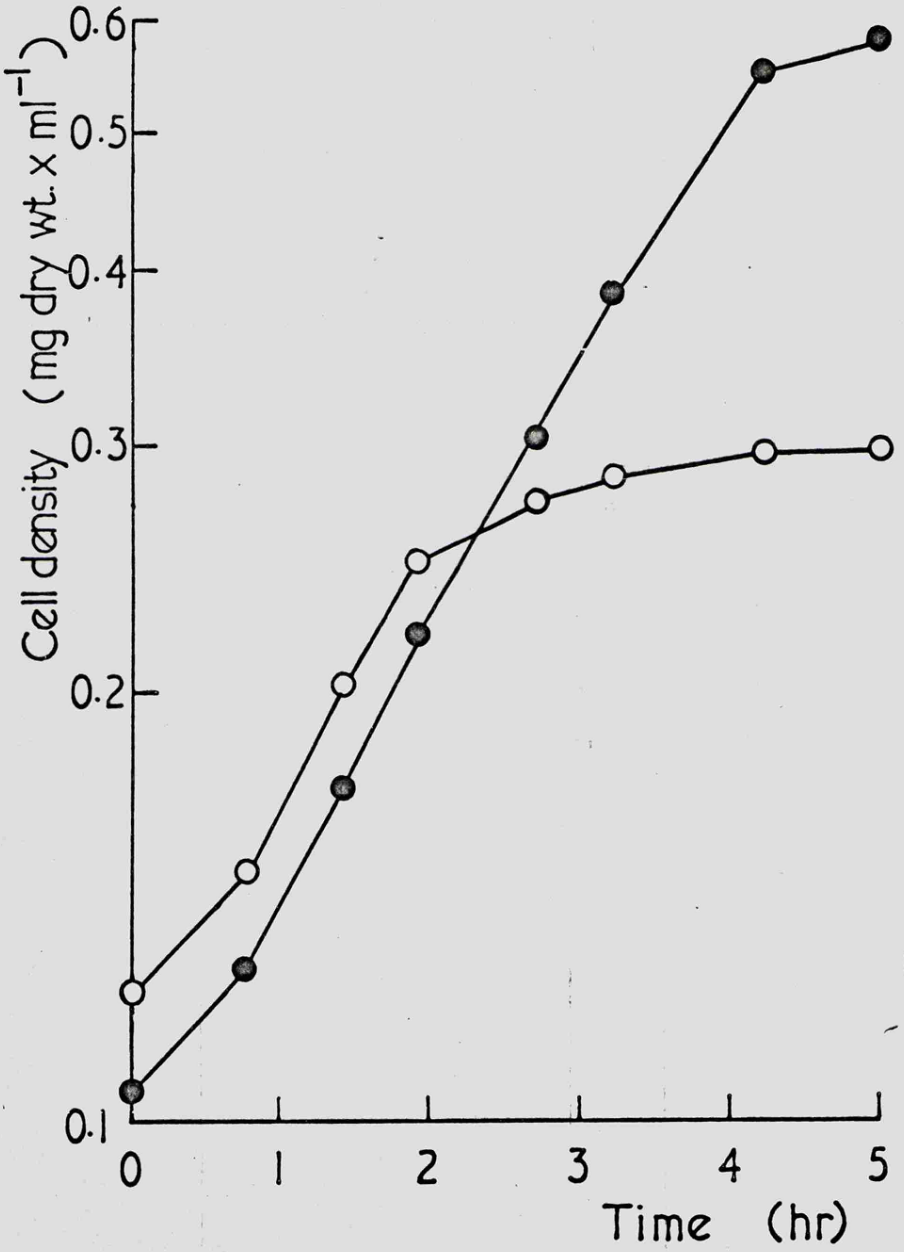

FIGURE 26 Growth of KL16.21 (O) and KL16.11^{c}.21 (●) on 5 mM fructose 1-phosphate as sole carbon source, after transfer from growth on glucose 6-phosphate.

FIG.27

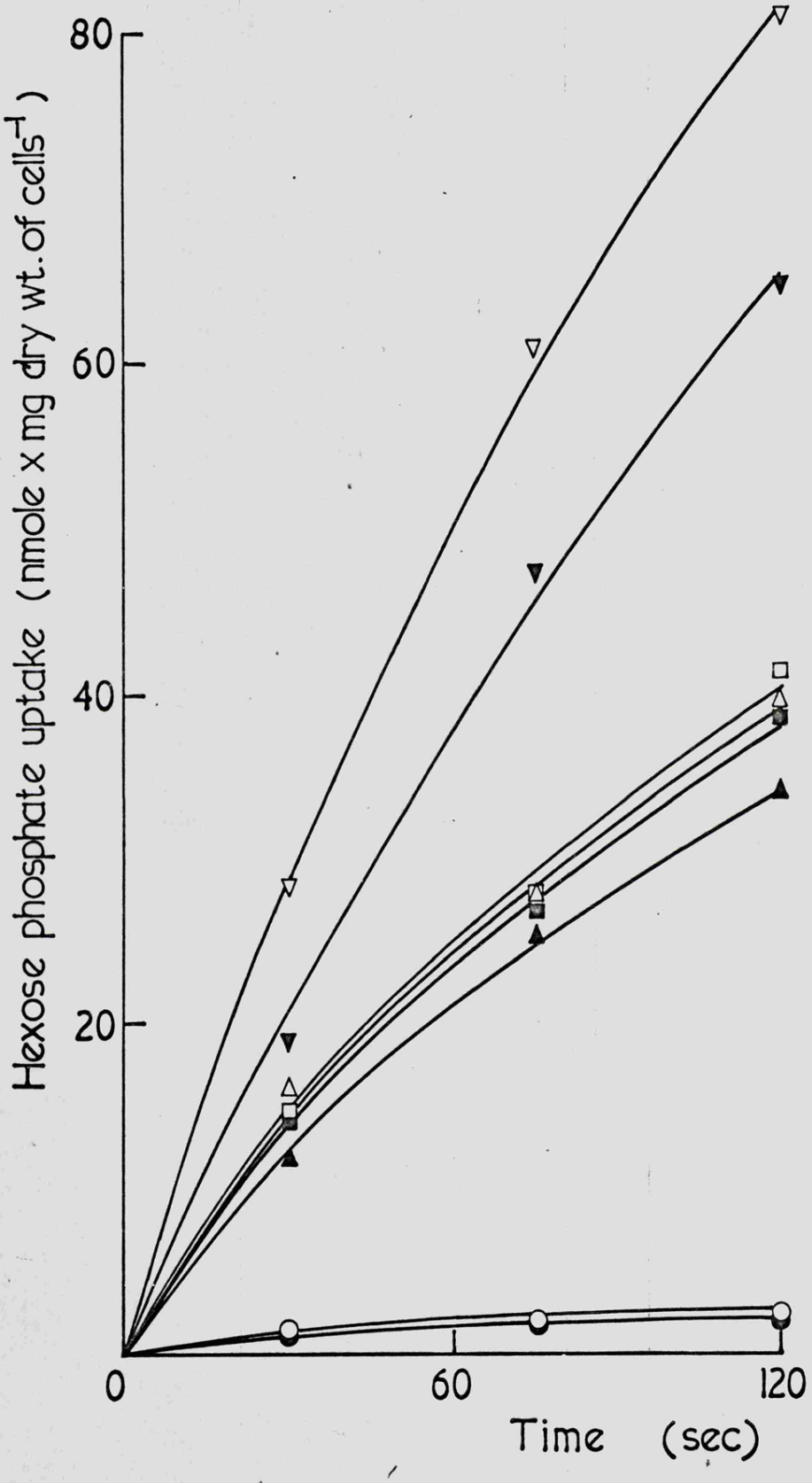
Hexose phosphate uptake (nmole x mg dry wt. of cells^{-1})
80
60
40
20
0
60
120
Time (sec)

FIGURE 27 Time-course of 0.1 mM [^{14}C]glucose 6-phosphate (open symbols) and 0.1 mM [^{14}C]fructose 6-phosphate (closed symbols) uptake by washed cell suspensions of strain KL16.21 and the constitutive strain KL16.11^{c}.21.

KL16.21 was grown on glycerol (○ , ●) or glucose 6-phosphate (△ , ▲) and KL16.11^{c}.21 was also grown on glycerol (▽ , ▼) or glucose 6-phosphate (□ , ■).

This technique is very useful when a large number of clones have to be identified, as in the genetic experiments described later in this Chapter. Similar procedures have been used previously by Zwaig and Lin (1966) in the selection of catabolite repression resistant mutants of E. coli and by Kashket and Wilson (1969) to select mutants with an increased level of phosphorylation of thiomethyl-β-galactoside.

(v) Properties of a mutant constitutive for hexose phosphate transport

The behaviour of one mutant, selected from KL16.21 by its ability to grow readily on plates containing fructose 1-phosphate, was investigated in detail: this mutant was designated KL16.11^{c}.21. Its growth properties on 5 mM fructose 1-phosphate are shown in Figure 26 and, as would be expected, there are two changes in the pattern compared with KL16.21. Firstly, previous growth on glucose 6-phosphate is not required for the growth of the mutant on fructose 1-phosphate, as glucose-grown cells grow readily on this substrate. Secondly, growth of KL16.11^{c}.21 is near-exponential until the 5 mM fructose 1-phosphate is exhausted, which again differs from the pattern observed with KL16.21. It seemed likely that these changes are associated with a change in the induction pattern of the hexose phosphate transport system and this was tested by measuring the uptake of fructose 6- and glucose 6-phosphate in KL16.11^{c}.21. As shown in Figure 27, suspensions of glycerol-grown KL16.11^{c}.21 can take up either hexose 6-phosphate, while the parent KL16.21 grown under the same conditions cannot. The rate of uptake of hexose phosphates by KL16.11^{c}.21 grown on glycerol is approximately twice that observed with suspensions of this mutant grown on glucose or glucose

6-phosphate; this latter rate is about the same as is obtained with fully induced KL16.21 grown on glucose 6-phosphate.

These properties of the mutant KL16.11^{c}.21 confirm the relationship between fructose 1-phosphate and the hexose phosphate transport system indicated by the earlier inhibition and induction data; in wild-type E. coli like KL16, fructose 1-phosphate is a substrate but not an inducer of the hexose phosphate transport system. A mutation to "constitutive" growth on fructose 1-phosphate is accompanied by a change to constitutivity in hexose phosphate transport.

(vi) Genetic control of hexose phosphate uptake

The isolation of a mutant constitutive for hexose phosphate uptake opened up a number of possibilities in the study of the genetic regulation of this system. Firstly, the location of the lesion on the E. coli linkage map (Taylor, 1970) could be determined and its relationship to the uhp allele already mapped (Kornberg and Smith, 1969) could be analysed. Secondly, the nature of the mutation could throw light on the mechanism of regulation of this inducible transport system. This could be partially achieved by studying the properties of merodiploids carrying wild-type and mutant alleles of the regulatory gene of the hexose transport uptake system; this kind of work has been initiated. Also the extent to which catabolite repression (for a review, see Paigen and Williams, 1970) plays a part in the regulation of this system could be studied in this mutant; the first indication that it may be involved came from the data in Figure 27 which shows that the rate of uptake of glucose 6- and fructose 6-phosphate is higher in glycerol-grown KL16.11^{c}.21 than it is in cells grown on glucose 6-phosphate.

Mapping of the uhp^c lesion Close linkage of the uhp^c and uhp mutationswas initially indicated by a mating involving the Hfr strain KL16.11^c.21 and the F^- strain K2.1t.11i. This recipient strain carried the uhp^- mutation isolated by Kornberg and Smith (1969) and also required isoleucine and valine for growth; recombinants were selected for the ability to grow on glucose in the absence of isoleucine and valine. The recombinants were checked for their hexose phosphate uptake characteristics by the autoradiographic technique and all were found either still unable to utilize glucose 6-phosphate (and were hence UHP^-) or to have acquired the constitutive phenotype (UHP^c). This suggested a very high degree of linkage between the uhp^c and uhp markers.

Co-transducibility of uhp^c and pyrE The close linkage of the uhp and uhp^c markers made it likely that uhp^c is also closely linked to pyrE, a marker specifying orotidylic acid pyrophosphorylase, which is located at about 72 min on the E. coli linkage map (Taylor, 1970). To confirm this, phage P1-kc were grown on KL16.11^c.21 and were used to infect K2.21 No. 4,URA, which was constructed by bringing in the pyrE marker from AT2243, an Hfr strain (see Table 1), into the F^- recipient K2.21 No. 4(ARG^-,URA^+) and selecting for ARG^+URA^-; this recombinant did not grow in the absence of uracil. $Uracil^+$ transductants were selected and of the 182 picked, 93 had remained inducible for the hexose phosphate uptake system but 89 had acquired the uhp^c allele; Figure 25 shows the difference in blackening of X-ray film by two of the $uracil^+$ transductants. This distribution is very similar to the degree of linkage between uhp and pyrE markers (Kornberg and Smith, 1969) and confirm that the uhp^c mutation maps at about 72 min on the E.coli

FIG. 28

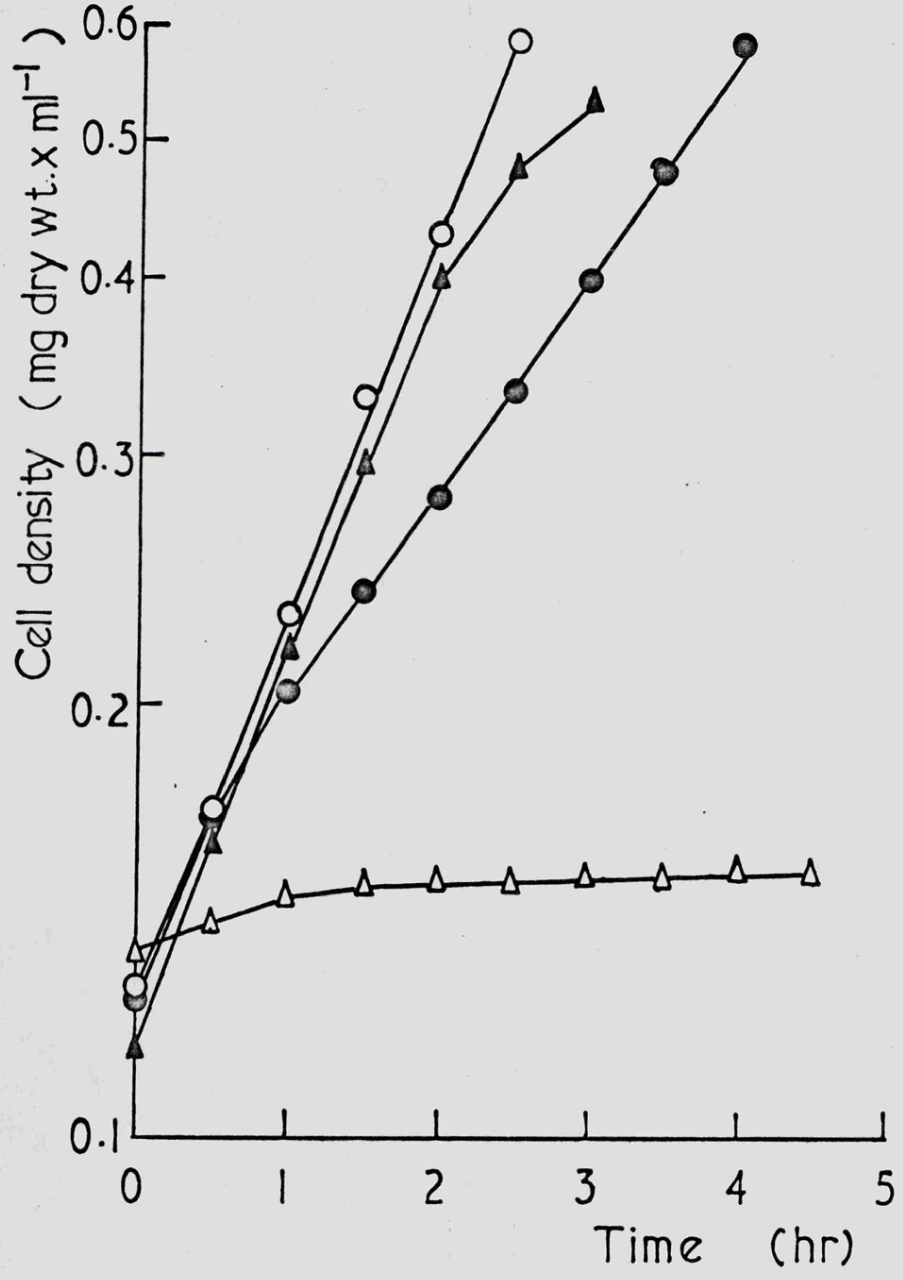

FIGURE 28 Growth of inducible (open symbols) and constitutive (closed symbols) transductants on 5 mM glucose 6-phosphate (○ , ●) or 5 mM fructose 1-phosphate (△ , ▲) as sole carbon sources. Inocula for this experiment were grown on glucose.

linkage map, as indicated in Figure 6. It is not yet known whether the uhp lesion is in a structural gene required for hexose phosphate transport and it therefore remains to be determined whether this system behaves as an operon (Jacob and Monod, 1961b).

The parallel relationship between constitutivity in hexose phosphate uptake and the ability to grow exponentially on fructose 1-phosphate was also exhibited by transductants. Those transductants that took up [^{14}C]fructose 6-phosphate constitutively (Figure 25) were also able to grow exponentially on fructose 1-phosphate when pre-grown on glucose (Figure 28). Conversely, transductants that were inducible for hexose phosphate uptake were unable to grow on fructose 1-phosphate after transfer from glucose (Figure 28).

Catabolite repression of the hexose phosphate transport system Catabolite repression is a general effect on the synthesis of most inducible enzyme systems, elicited by the presence of glucose, or other readily utilizable carbon sources during the growth of E. coli (for a review of the phenomenon, see Paigen and Williams, 1970). Glucose 6-phosphate is itself a strong catabolite repressor (Hsie and Rickenberg, 1967). It was therefore interesting to observe that in KL16.11^{c}.21, the hexose phosphate uptake system is expressed less well in growth media containing glucose 6-phosphate, than when growing on a less repressive carbon source like glycerol (Figure 27). There is approximately a two-fold difference between the rates of fructose 6-phosphate uptake measureable in KL16.11^{c}.21 after growth on glycerol and after growth on glucose or glucose 6-phosphate. This latter repressed level is approximately equivalent to the rate of uptake observed in KL16.21

FIG. 29

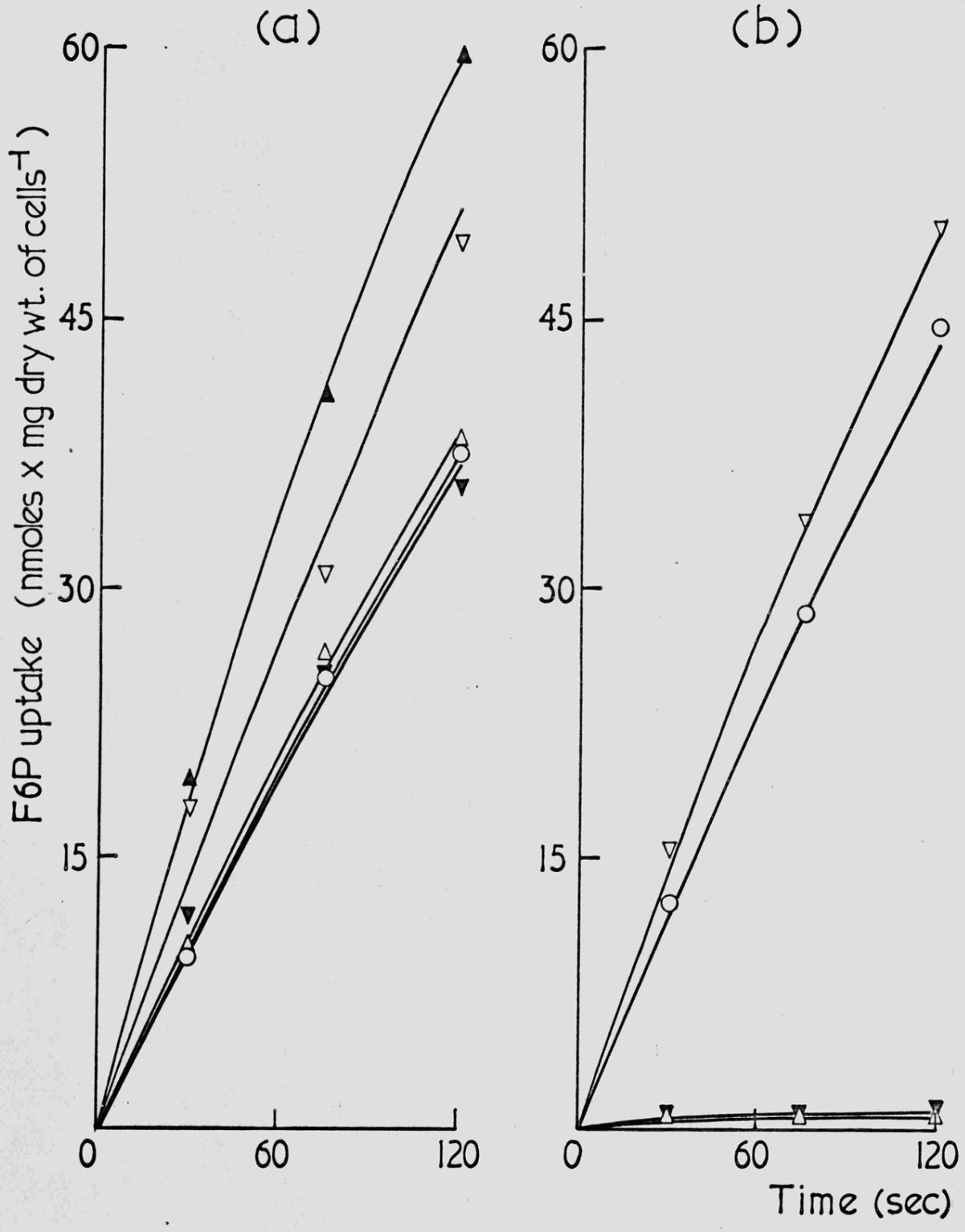
(a)
(b)
60
45
30
15
0
60
120
F6P uptake (nmoles x mg dry wt. of cells^{-1})
Time (sec)

FIGURE 29 The uptake of 0.1 mM [U-^{14}C]fructose 6-phosphate by washed cell suspensions of:

(a) KL16.11^{c}.21, previously grown on glucose (Δ), or glucose 6-phosphate (○), or after transfer, for 90 min, from media containing 10 mM glucose to media containing 10mM glucose plus 5 mM cyclic AMP (▲), or from 10 mM glucose 6-phosphate to 10 mM glucose 6-phosphate plus 5 mM cyclic AMP (▽), or from 10 mM glucose 6-phosphate to 10 mM glucose 6-phosphate plus 5 mM cyclic AMP plus 60 μg/ml chloramphenicol (▼).

(b) KL16.21, previously grown on glucose (Δ) or after transfer, for 90 min, from media containing 10 mM glucose to media containing 10 mM glucose 6-phosphate (○), or from glucose to 10 mM glucose 6-phosphate plus 5 mM cyclic AMP (▽) or from glucose to 10 mM glucose 6-phosphate plus 60 μg/ml chloramphenicol (▼).

when fully induced after growth on glucose 6-phosphate; it is therefore likely that in wild-type cells, the normal level of expression of the hexose phosphate uptake system is governed by a balance between induction by glucose 6-phosphate and the catabolite repression caused by growth on glucose 6-phosphate.

As described in the Introduction to Chapter III, the work of Pastan and Perlman (1970) indicates that cyclic adenosine 3',5'-monophosphate (cyclic AMP) overcomes the glucose repression of a number of inducible enzymes and proteins and that the level of expression of these systems is likely to be regulated by the intracellular concentration of cyclic AMP. It was therefore investigated whether the addition of cyclic AMP to the growth media of KL16.21 and KL16.11^{c}.21 could increase the measured rate of hexose phosphate uptake by these organisms. Figure 29a illustrates the rate of uptake by KL16.11^{c}.21 grown on glucose, glucose 6-phosphate and on these two carbon sources in the presence of 5 mM cyclic AMP for 90 min. For the data in Figure 29b, KL16.21 was pre-grown on glucose and transferred, for 90 min, into media containing glucose 6-phosphate, glucose 6-phosphate plus cyclic AMP and glucose 6-phosphate plus chloramphenicol (60 μg/ml). As shown, in all cases, the induction of the hexose phosphate transport system was enhanced by the presence of cyclic AMP, and these increases due to induction or de-repression were inhibited by chloramphenicol. These results indicate that, as would be expected, the induction of the hexose phosphate uptake system requires protein synthesis and that the level of expression of this system is at least partially dependent on the presence of cyclic AMP in E. coli.

(vii) DISCUSSION

The ability of E. coli to grow on fructose 1-phosphate has highlighted a number of properties of the hexose phosphate transport system.

(a) Broad specificity In addition to fructose 6-phosphate, glucose 6-phosphate and mannose 6-phosphate (Winkler, 1966) and 2-deoxyglucose 6-phosphate (Dietz and Heppel, 1971a), the substrates of this system are also the 1-phosphates, fructose 1-phosphate and glucose 1-phosphate (Pogell et al., 1966; Dietz and Heppel, 1971c). Evidence that fructose 1-phosphate is a substrate of the hexose phosphate transport system comes from (i) the inducible nature of growth on fructose 1-phosphate, (ii) inhibition studies by fructose 1-phosphate of fructose 6-phosphate uptake and (iii) the ability to select a mutant constitutive for hexose phosphate transport by a spontaneous mutation to exponential growth on fructose 1-phosphate. Evidence that glucose 1-phosphate is also a substrate of the transport system comes from the work of Pogell et al. (1966) who demonstrated that ^{14}C-labelled glucose 1-phosphate is readily taken up by glucose 6-phosphate-grown E. coli B. More recently, Dietz and Heppel (1971c) have confirmed this; furthermore, they have isolated mutants constitutive for hexose phosphate transport from mutants defective in glucose utilization by the same rationale as used in our selection procedure, that is by selecting for spontaneous growth on glucose 1-phosphate. From their work, glucose 1-phosphate also appears to be a substrate, but not an inducer, of the hexose phosphate transport system.

(b) Inducibility Winkler (1970) has found that glucose 6-phosphate is the only physiological inducer for the hexose phosphate transport

system in E. coli. Fructose 6-phosphate is only an inducer when it can be converted to glucose 6-phosphate intracellularly and does not induce the system in strains lacking the enzyme phosphoglucose isomerase. Glucose 1-phosphate (Dietz and Heppel, 1971a) or fructose 1-phosphate (evidence in this Chapter) are also not inducers for the system but 2-deoxyglucose 6-phosphate is an effective analogue for the true inducer (Dietz and Heppel, 1971a).

The difference between the wide spectrum of substrates and the narrow range of inducers for this system indicates that different recognition sites are involved in the functions of transport and induction. This may be important, in that induction by external, but not intracellular, glucose 6-phosphate has been postulated for this system (Heppel, 1969; Winkler, 1970; Dietz and Heppel, 1971b). The main evidence against cytoplasmic induction for the hexose phosphate transport system comes from work using mutants lacking phosphoglucose isomerase (PGI) and glucose 6-phosphate dehydrogenase (ZWF) (see Figure 1), which accumulate up to 60 mM glucose 6-phosphate in the presence of external glucose (Heppel, 1969; Winkler, 1970). Under these conditions of high internal glucose 6-phosphate concentration, the hexose phosphate transport system is not expressed, but if external glucose 6-phosphate is added, the transport system is then induced. A similar pattern is obtained when the non-metabolizable analogue 2-deoxyglucose accumulates in wild-type cells as 2-deoxyglucose 6-phosphate; low concentrations of external 2-deoxyglucose 6-phosphate induce the transport system while the accumulated sugar phosphate does not (Dietz and Heppel, 1971b). However, these high internal levels of sugar phosphate may affect this

system by catabolite repression; the results in this chapter obtained with the constitutive mutant indicate that lower transport activities are obtained during growth on repressing carbon sources, even without intracellular accumulation of sugar phosphates. Furthermore, induction of the transport system is accompanied by efflux of the glucose 6-phosphate pool into the medium (Dietz and Heppel, 1971b) which could have the effect of lowering the level of repression of the system.

Mechanisms for explaining the difference between extracellular and intracellular glucose 6-phosphate in the induction of the hexose phosphate transport system are difficult to visualize. Compartmentation may be invoked (Winkler, 1970) or perhaps a membrane associated induction-repression system or possibly an enzymatic conversion of glucose 6-phosphate to a true inducer (Heppel, 1969).

(c) Genetics Analysis by conjugation and phage-mediated transduction establishes *uhp*^{c}, the gene mutated in KL16.11^{c}.21 to give constitutivity in hexose phosphate uptake, to be closely linked to the *uhp* gene already mapped (Kornberg and Smith, 1969). The *uhp*^{c} gene is also closely linked to *pyrE* and is located at about 72 min on the *E. coli* linkage map (Taylor, 1970). It is not yet known how the *uhp* and the *uhp*^{c} genes are functionally related.

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PUBLICATIONS

The following publications contain parts of the work presented in this thesis:

Ferenci T. and Kornberg H.L. (1971). FEBS Lett., 13, 127.

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ABSTRACT

1. The phosphoenolpyruvate (PEP):fructose phosphotransferase system plays a key role in the utilization of fructose by Escherichia coli. Mutants devoid of, or altered in, this system are not only modified in their ability to phosphorylate fructose but are also altered in their ability to take up this hexose from solutions.

2. The major product of fructose phosphorylation in E. coli is fructose 1-phosphate, though fructose 6-phosphate can also be formed by a PEP-dependent mechanism. The relationship between PEP:fructose 1- and PEP:fructose 6-phosphotransferase activities is discussed.

3. PEP-dependent fructose phosphorylation, fructose uptake by whole cells and ATP-dependent fructose 1-phosphate phosphorylation to fructose 1,6-diphosphate are activities that, in E. coli, are induced by fructose. A regulatory mutant that exhibits these activities constitutively has been isolated; its properties indicate that the expression of fructose-induced proteins is controlled as a regulon.

4. The utilization of fructose by E. coli is subject to control at the level of fructose entry into whole cells. Fructose uptake is inhibited by intracellular hexose phosphates or by carbon sources that can readily give rise to hexose phosphates, such as glucose or galactose.

5. Fructose 1-phosphate can serve as a sole carbon source for the growth of E. coli. Fructose 1-phosphate, like other hexose-phosphates such as glucose 6-phosphate and fructose 6-phosphate, is a substrate of an inducible hexose phosphate transport system. However, unlike glucose 6-phosphate, fructose 1-phosphate, when present in the growth medium of E. coli, does not give rise to the induction of this uptake system.

PATHWAY OF FRUCTOSE UTILIZATION BY *ESCHERICHIA COLI*

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1. Introduction

Escherichia coli grows readily on fructose as sole source of carbon. It was long assumed that the manner in which this sugar is utilized is analogous to that by which glucose enters the main metabolic pathways of the cells; this would imply the occurrence of an initial enzymatic phosphorylation by ATP of fructose to fructose-6-phosphate and ADP, followed by a second phosphorylation to fructose-1,6-diphosphate. Enzymes catalysing both these reactions have been demonstrated to be present *E. coli* [1, 2]. However, recent studies in several laboratories have cast doubt on this view, and have suggested that fructose is initially phosphorylated to fructose-1-phosphate (reaction 1) with concomitant conversion of phospho*enol*pyruvate (PEP) to pyruvate. Fructose-1-phosphate is then further phosphorylated to fructose-1,6-diphosphate, through the agency of an ATP-linked fructose-1-phosphate kinase (reaction 2) distinct from the action of the phosphosfructokinase that catalyses the phosphorylation of fructose-6-phosphate to fructose-1,6-diphosphate.

$$\text{fructose} + \text{PEP} \rightarrow \text{fructose-1-phosphate} + \text{pyruvate} \quad (1)$$

$$\text{fructose-1-phosphate} + \text{ATP} \rightarrow \text{fructose-1,6-diphosphate} + \text{ADP} \quad (2)$$

The main evidence for this alternative route of fructose utilization may be summarized as follows:

(i) although a fructo(manno) kinase, which catalyses the conversion of fructose to fructose-6-phosphate with concomitant cleavage of ATP to ADP, has been isolated from *E. coli* [1], its activity is low and appears to be insufficient to account for the rate of fructose utilization by cells growing on this hexose;

(ii) *E. coli* mutants devoid of phosphofructokinase activity, which do not grow on glucose, grow at virtually wild-type rates on fructose [2, 3];

(iii) the pathway summarized in reactions (1) and (2) has been shown to occur in another member of the Enterobacteriaceae, *Aerobacter aerogenes* [4];

(iv) the product of the PEP-linked phosphotransferase reaction [5] with fructose as substrate has been identified as fructose-1-phosphate [6];

(v) *E. coli* inducibly forms fructose-1-phosphate kinase when exposed to fructose [6].

Thus, as Fraenkel [6] rightly concludes, "on balance, most of the available data on fructose metabolism in *E. coli* and *A. aerogenes* favor the *major* pathway being via fructose-1-phosphate. But, in order to accommodate all the data more mutant analysis and dissection of the enzymes will be necessary". It is the main purpose of this paper to describe the properties of a mutant of *E. coli* that provide direct evidence for the occurrence of this pathway.

2. Experimental

The mutant KL 16–21 was derived from the Hfr strain KL 16 of *E. coli* K12, by treatment with ethyl methane sulphonate [7] and was selected with penicilin [8] for its inability to grow on medium containing salts [9] and fructose as carbon source, whilst retaining the ability to grow normally on similar medium containing glucose as carbon source. The mutant was purified by repeated single-colony isolation; it grew at wild-type rates also on glucose-6-phosphate, fructose-6-phosphate, gluconate and glycerol. The organism was stored on slopes of Oxoid

nutrient agar; liquid cultures were grown at 37° aerobically on a Gallenkamp incubator shaker. Growth was measured as $\Delta A_{680\,nm}$; under our conditions $\Delta A_{680nm} = 1.0$ was found to be equivalent to an increase of 0.68 mg dry wt of cells ml^{-1} of culture

2.1. *Enzyme assays*

The activity of the phosphotransferase system that catalyses reaction (1) was measured as the fructose-dependent release of pyruvate from PEP concomitant with the enzymic phosphorylation of fructose to fructose-1-phosphate. Cultures of *E. coli* were harvested, washed in 1% (w/v) KCl and suspended at 30–40 mg dry wt/ml in 0.1 M potassium phosphate buffer pH 7.5. The suspension was stored at −20° overnight and thawed at room temperature [10]. The assay systems, incubated at 37° for 0, 3 and 6 min, respectively, contained in a final volume of 1.0 ml (μmoles): fructose 1; PEP 1; potassium phosphate pH 7.5, 20; sodium arsenite 10; potassium fluoride 100; magnesium chloride 0.5. The reaction was started by the addition of thawed cells (1–2 mg dry wt) and stopped by the addition of 0.33 ml of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 N HCl. The pyruvate 2,4-dinitrophenylhydrazone content was measured [11] after centrifugation to remove cell debris. Each assay was read against a blank, incubated as above with all components of the complete system except fructose, which by itself gives negligible reaction.

For assays of fructose-1-phosphate kinase activity, cultures of *E. coli* were harvested from appropriate growth media, washed in 1% (w/v) KCl and suspended, at 1–5 mg dry wt ml^{-1}, in buffer, pH 7.5, containing 10 mM tris, 10 mM $MgCl_2$ and 2 mM mercaptoethanol The cells were disrupted at 0° by exposure for 2 min to the output of a MSE 100W sonicator and cell debris was removed by centrifugation for 1hr at 30,000 *g*. The enzyme was assayed in a system containing in a final vol. of 1 ml, a mixture of (μmoles): imidazole buffer, pH 7.2, 67; magnesium chloride 10; fructose-1-phosphate 2.5; ATP 1.25; NADH 0.15; and crystalline aldolase 12,5 μg; triosephosphate isomerase + α-glycerolphosphate dehydrogenase, 20 μg. Under these conditions, the phosphorylation of 1 μmole of fructose-1-phosphate is taken to be equivalent to the oxidation of 2 μmoles of NADH, which is measured as ΔA_{340nm}. All measurements were corrected for the small residual NADH-oxidase activity of the sonic extracts.

3. Results and discussion

Since the mutant KL 16–21 grew at wild-type rates on glucose, glucose-6-phosphate and fructose-6-phosphate, its inability to utilize fructose indicated a dysfunction in either the formation of fructose-1-phosphate (reaction 1) or its phosphorylation to fructose-1,6-diphosphate (reaction 2). The experiments illustrated by fig. 1 and table 1 rule out the latter possibility.

Studies with wild-type cells showed [12] that, although fructose-1-phosphate did not normally support the growth of *E. coli*, it could serve to a limited extent as a carbon source for the growth of cells that had been previously grown on hexose phosphates. Under these conditions, the bacteria inducibly form a transport system that specifically effects the uptake of hexose phosphates [13–15]: fructose-1-phosphate is a substrate transported by this system, but is not an inducer of it. As expected from this property, cultures of the wild-type KL16, grown on medium containing 10 mM glucose-6-phosphate as sole carbon source, grew rapidly for about 1 generation when the harvested cells were transferred to medium containing

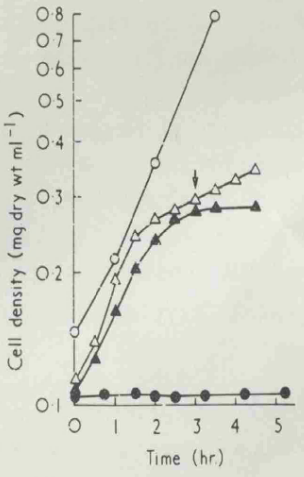

Fig. 1. Growth of KL 16 (open symbols) and its mutant KL 16–21 (closed symbols), previously grown on 10 mM glucose-6-phosphate, after transfer to 5 mM fructose (○, ●) or 5 mM fructose-1-phosphate (△, ▲) as carbon sources. A further portion of fructose-1-phosphate, sufficient to raise its concentration in the medium by 2.5 mM, was added at the arrow.

Table 1
Inducible formation of fructose-1-phosphate kinase in KL 16 and KL 16–21.

Growth substrate	Specific activity* of fructose 1-phosphate kinase in KL 16	Increase (fold)	KL 16–21	Increase (fold)
Glucose-6-phosphate	14	–	4	–
Fructose-1-phosphate	111	8	39	9
Fructose	138	10	n.g.	n.g.

* Specific activity = nmoles of fructose-1-phosphate phosphorylated. min^{-1} mg of $protein^{-1}$.
n.g. = no growth on fructose; hence this assay could not be done..

5 mM fructose-1-phosphate; thereafter, the rate of growth decreased sharply and was not increased by further addition of fructose-1-phosphate (fig. 1). The slow growth now observed was presumably due to the slow hydrolysis of fructose-1-phosphate in the medium, to yield fructose which, as shown in fig. 1, is an excellent growth substrate for KL 16. Cultures of the mutant KL 16–21, previously grown on glucose-6-phosphate, also grew rapidly on fructose-1-phosphate for about 1 generation: thereafter, growth ceased, as any fructose produced from the hydrolysis of fructose-1-phosphate could not be utilized by this mutant (fig. 1).

That this rapid growth on fructose-1-phosphate involved the activity of fructose-1-phosphate kinase is shown in table 1. The specific activity of this enzyme in extracts of cells grown on glucose-6-phosphate was low, but was 8–9 fold higher in extracts of cells which had been allowed to grow for one generation on fructose-1-phosphate. The similar induction of fructose-1-phosphate kinase after growth of the wild-type KL 16 for 1 generation on fructose is further evidence [6] for the role of this enzyme in fructose metabolism.

Two main findings indicate that the inability of the mutant KL 16–21 to grow upon fructose is associated with a lesion in the complex system [4, 5] that effects the formation of fructose-1-phosphate. This system contains at least three components: a histidine-containing protein (HPr), phosphorylated by PEP in the presence of an enzyme (enzyme I: ref. 5) to yield HPr-P, and a second enzyme (Enzyme II) presumably specific for the transfer of the phosphate from HPr-P to the 1-position of fructose. Mutants defective in the formation of HPr or enzyme I are pleiotropic, and cannot utilize a variety of sugars including fructose [16]; a mutant devoid only of the ability to utilize fructose might be expected to lack enzyme II activity. This is borne out by the results of a genetic cross between the mutant KL 16–21 and a recipient pleiotropically affected in hexose utilization [17]. About 30% of the recombinants from such a cross grew on all hexoses, and had therefore received the functional allele specifying HPr and/or enzyme I without also receiving the defective allele for fructose utilization; the remaining recombinants grew on glucose but not upon fructose and thus had the phenotype of KL 16–21. It is therefore likely that KL 16–21 which is not impaired in either HPr or enzyme I, lacks enzyme II; it is also likely that the gene specifying enzyme II is fairly closely linked to the gene specifying the pleiotropic defect. Evidence to this effect is provided also by measurements of the time of entry of the marker for fructose utilitzation, from the wild-type KL 16 which injects its genome in the order *O-thy-his*, into a F^- mutant K2.1.21, which carried the defective allele specific for fructose utilization and also required thymine and histidine for growth. The results showed that the ability to grow upon fructose was acquired by the recipient approx. 13 min after the entry of *thy* and approx. 3 min before *his*; this would place the marker for enzyme II of the fructose system at about 42 min on the *E. coli* linkage map [18], and about 4 min from the marker specifying the pleiotropic defect in sugar utilization [19].

A dysfunction of enzyme II should have two consequences. In the first place, mutants thus defective should not be able to effect the phosphorylation of fructose (reaction 1). This was found to be so: whereas frozen and thawed suspensions of the wild-type KL 16, which had been grown on glycerol plus fructose, catalysed the PEP-dependent formation of 21 nmoles of fructose-1-phosphate from fructose min^{-1} mg dry

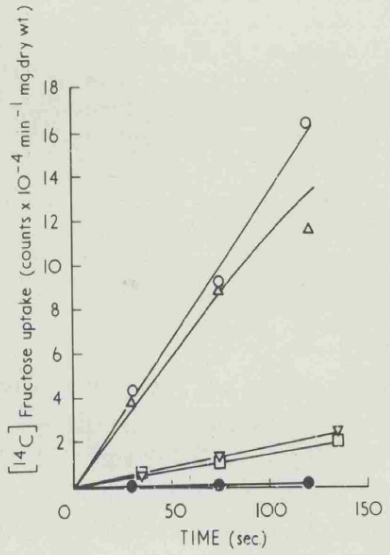

Fig. 2. Uptake of U-^{14}C-fructose by KL 16 (open symbols) and its mutant KL 16–21 (closed symbols). Cultures of KL16 had been grown on fructose (○), fructose-1-phosphate (△), glucose (▽) or glycerol (□); those of KL 16–21 had been grown on fructose-1-phosphate (●). The uptake of ^{14}C-fructose (0.1 mM; 0.29 μCi ml^{-1}) by washed suspensions of cells (0.3 mg dry wt ml^{-1}) thus grown, was measured as previously described [21].

wt^{-1} of cells, suspensions of KL 16–21, similarly grown, phosphorylated less than 1 nmole of fructose under these conditions. Secondly, the phosphorylation reaction (1) has been shown to be necessarily associated with uptake of hexoses by *E. coli* (for review, see [20]). It would thus be expected that a mutant lacking enzyme II activity would be impaired in the uptake of labelled fructose. This is shown in fig. 2. Whereas washed suspensions of the wild-type KL16 inducibly formed a system effecting the uptake and incorporation of ^{14}C-fructose after growth on either glycerol plus fructose or on fructose-1-phosphate, suspensions of KL 16–21 were grossly impaired in this uptake, even when the mutant had been grown on those carbon sources that acted as good inducers in the wild-type.

The results thus show that the growth of *E. coli* on fructose necessitates the occurrence of the pathway summarized in reactions (1) and (2). The mutant KL 16–21 here described probably lacks the enzyme II component of reaction (1); the gene specifying this defect is located about 4 min from that specifying the HPr and/or enzyme I components of this reaction [19].

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ROLE OF FRUCTOSE-1,6-DIPHOSPHATASE IN FRUCTOSE UTILIZATION BY *ESCHERICHIA COLI*

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1. Introduction

The major pathway for the utilization of fructose by *Escherichia coli* involves the formation of fructose-1-phosphate (reaction 1), catalysed by the phosphopyruvate phosphotransferase (PT) system [1], followed by an ATP-dependent phosphorylation of this product (reaction 2) to fructose-1,6-diphosphate [1, 2].

fructose + PEP → fructose-1-phosphate + pyruvate (1)

fructose-1-phosphate + ATP → fructose-1,6-diphosphate + ADP (2)

It would be expected from this sequence that the biosynthesis of carbohydrates, such as glycogen, pentoses or the *N*-acetylglucosamine moieties of cell walls, would require the formation of fructose-6-phosphate from fructose-1,6-diphosphate, and that this step would involve fructose-1,6-diphosphatase (reaction 3)

fructose-1,6-diphosphate + H_2O → fructose 6-phosphate + Pi (3)

However, although *E. coli* mutants devoid of fructose diphosphatase activity do not grow on substrates such as glycerol, lactate, acetate, or C_4-acids, such mutants grow at virtually wild-type rates on fructose [1, 3]. This suggests that either fructose, or the fructose-1-phosphate derived from it, can yield fructose-6-phosphate directly and in a manner that by-passes reaction (3).

It is the purpose of this paper to present evidence that reaction(s) associated with the PT-system can effect the formation of fructose-6-phosphate from fructose at the high concentrations normally used in media containing fructose as carbon source for growth, and that any direct formation of fructose-6-phosphate from fructose-1-phosphate does not occur at a significant rate.

2. Experimental

The strains of *E. coli* used in this work are listed in table 1. Procedures for the growth of organisms and for the preparation of extracts have been previously published [2]. Fructose diphosphatase activity was assayed by the method of Fraenkel and Horecker [1]. Genetic procedures were as previously described [4]. The assay of the fructose–PT-system is described in table 3.

3. Results and discussion

Although the mutant K2-19, which is devoid of fructose diphosphatase activity (table 2), grew on media containing 20 mM fructose at a rate indistinguishable from its wild-type parent, K2, this growth was considerably slower when media containing lower concentrations of fructose were used: the striking diminution of growth rate observed was not exhibited by the parent organism. When the least mean doubling times for the growth of these organisms on

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Table 1
Strains of *Escherichia coli* used in this work.

Strain	Genetic markers*	Mating type	Source
K2	*his,arg,thr,leu,trp,str*	F^-	[4]
K2-19	*his,arg,thr,leu,trp,fdp,str*	F^-	EMS**-mutant of K2
K2.1.5^c,7	*his,thr,leu,pgi,iclR,str*	F^-	[5]
K10	–	Hfr	D. Fraenkel
AB674	–	Hfr	E.A. Adelberg
KL16	–	Hfr	B. Low
KL16-21	pts_F	Hfr	[2]

* The abbreviations used indicate a requirement for *his* = histidine, *arg* = arginine, *thr* = threonine, *leu* = leucine, *trp* = tryptophan; *fdp* = absence of fructose diphosphatase; *pgi* = absence of phosphoglucoseisomerase, *iclR* = constitutive formation of glyoxylate cycle enzymes, pts_F = absence of enzyme II for fructose phosphorylation via phosphopyruvate phosphotransferase system, *str* = resistance to streptomycin.

** EMS = ethylmethanesulphonate. Mutagenesis [6] and selection with penicillin [7] for inability to grow on glycerol or lactate, whilst retaining ability to grow on glucose, was by the general procedures previously described [4].

Table 2
Fructose diphosphatase activity of *E. coli* strains used.

Organism	C source for growth	Specific activity of fructose diphosphatase*
K2	fructose	12
K2	glycerol	14
K2-19	fructose	< 0.6
K2-19	glycerol	no growth

* Specific activity is here expressed as nmoles of NADP reduced × min^{-1} × mg $protein^{-1}$ in the assay of Fraenkel and Horecker [3].

media of various fructose concentrations, i.e. the reciprocals of the maximal growth rates attained, were plotted against the reciprocal of these fructose concentrations, the results obtained were similar to those given by Lineweaver-Burk plots of enzyme kinetics (fig. 1). As will be apparent from this figure, the maximal growth rate achievable on fructose ("V_{max}") is not greatly affected by the absence of fructose diphosphatase; however, half this maximal rate ("K_m") was attained by the mutant only at approx. 1.8 mM fructose, whereas in the wild-type organism approx. 0.16 mM sufficed.

This behaviour was shown also by recombinants of K2-19 obtained from two genetic crosses. The Hfr strain AB674 transfers its genome to recipient (F^-) cells in the order *O–argH–fdp*.....; strain K10 injects in the reverse order, *O–leu–thr–fdp*..... .

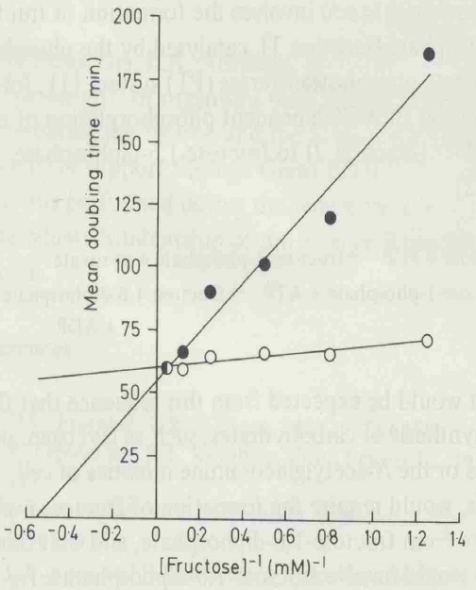

Fig. 1. Growth of the wild-type of *Escherichia coli* K2 (○) and of its mutant K2-19 (●) devoid of fructose diphosphatase activity, on media containing fructose as sole carbon source. The data are expressed as the reciprocals of the maximum growth rates attained at 37° at various concentrations of fructose, plotted against the reciprocals of those fructose concentrations.

Both strains were crossed with the mutant K2-19, and, after periodic interruption of conjugation, recombinants were selected on plates containing (a) glucose as carbon source for arg^+ and thr^+leu^+ respectively, and (b) glycerol as carbon source for fdp^+. In all cases, the male phenotype was counterselected by inclusion

Table 3
Fructose 6-phosphate formation from 50 mM-fructose by toluene-treated *E. coli.*

Organisms	C source for growth	Assay system*	Rate of fructose-6-phosphate formation**	Activity (%)
K2.1.5^{c}.7	fructose	complete	4.1	100
		−PEP, + ATP	0.3	7
		$-Mg^{2+}$	1.8	45
		−fructose, + F1P	0	0
K2.1.5^{c}.7	glucose	complete	1.1	26
K2.1.5^{c}.7	glycerol	complete	0.7	17
K2-19	fructose	complete	2.8	–
KL16	fructose + glycerol	complete	2.5	–
KL16.21	fructose + glycerol	complete	< 0.6	–

* The complete system contained in 1 ml, in μmoles: $MgCl_2$ 1, PEP 1.25, fructose 50, potassium phosphate pH 7.5 50, and toluene-treated cells [11], 1–1.5 mg dry wt. Where indicated, ATP (1 μmole) and fructose-1-phosphate (1 μmole) replaced the PEP and fructose respectively. After 20 min at 37°, the reactions were stopped by millipore filtration and samples (0.5 ml) of the filtrates were assayed for glucose-6-phosphate and fructose-6-phosphate: these assays involved the spectrophotometric determination at 340 nm of the reduction of NADP (0.6 μmole) in the presence of iminazole pH 7.2 100 μmoles, and crystalline glucose-6-phosphate dehydrogenase 6.25 μg and (for fructose-6-phosphate assay) phosphoglucoseisomerase 6.25 μg.

** nmoles of fructose-6-phosphate formed × min^{-1} × mg dry wt of $cells^{-1}$.

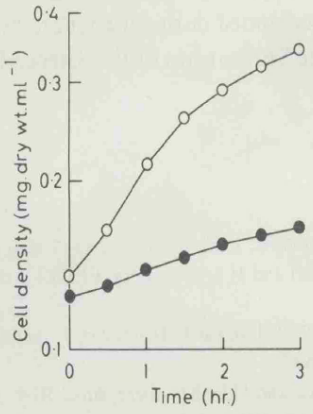

Fig. 2. Growth of the wild-type strain of *Escherichia coli*, K2 (○) and of the mutant K2-19 (●), devoid of fructose diphosphatase activity, on media containing 5 mM fructose-1-phosphate as sole carbon source. Inocula for these experiments were grown on glucose-6-phosphate [2].

of streptomycin in these plates. In confirmation of previous findings [8], the *fdp*$^{+}$-allele was found to enter 6 min from the *arg* and 6 min from the *thr,leu* markers. All of the 195 *fdp*$^{-}$-recombinants tested manifested the phenotype of K2-19: they grew on plates containing 20 mM fructose but failed to grow readily on plates containing 0.5 mM fructose as carbon source. In contrast, all of the 75 *fdp*$^{+}$-recombinants grew readily on either concentration of fructose.

These observations suggested that, at low concentrations of fructose (< 2 mM), fructose diphosphatase plays an important role in the generation of fructose-6-phosphate from the growth substrate but that, at higher fructose concentrations, fructose-6-phosphate can arise by reaction(s) that by-pass the diphosphatase, either directly or from fructose-1-phosphate.

The latter possibility is rendered unlikely by two findings. Firstly, and in confirmation of the work of Fraenkel [1], we fail to find any in vitro conversion of fructose-1-phosphate to fructose-6-phosphate, by cell-free extracts of fructose-grown *E. coli*. Secondly, the in vivo occurrence of such a conversion is ruled out by the inability of K2-19 to grow on fructose-1-phosphate (fig. 2). As was previously reported [2], wild-type strains of *E. coli* can grow on fructose-1-phosphate as sole carbon source if the system that effects the uptake of hexose phosphates is first induced by prior growth on glucose-6-phosphate. Strain K2 manifested this behaviour: when cultures of this organism were transferred from glucose-6-phosphate

to a medium containing 5 mM fructose-1-phosphate, this latter compound served as a good growth substrate for more than 1 doubling. However, although the mutant K2-19, devoid of fructose diphosphatase, also grew readily on glucose-6-phosphate, cells thus grown did not utilize fructose-1-phosphate. Thus, fructose-6-phosphate could not arise from fructose-1-phosphate in K2-19, although it could in K2: hence, fructose diphosphatase activity is necessary for the utilization of fructose-1-phosphate as carbon source for growth, under conditions where it is not necessary for the utilization of fructose.

A direct formation of fructose-6-phosphate from fructose could be catalysed either by an ATP-linked kinase, such as the fructo(manno)kinase known [9] to be present in *E. coli*, or by the PT-system. Our evidence favours the latter for two main reasons.

In the first place, *E. coli* mutants devoid of enzyme I of the PT-system [10] or of the enzyme II specific for fructose [2] do not grow on fructose, nor do suspensions of such mutants take up ^{14}C-labelled fructose, even when this is supplied at high concentrations. This implies that the PT-system is necessarily involved in fructose utilization, and that any process of transport of fructose via any other system is of negligible significance. It is thus probable that the growth of K2-19 on fructose necessarily involves the operation of the PT-system.

Secondly, and more directly, we show (table 3) that toluene-treated *E. coli* [11] can effect the net formation of fructose-6-phosphate from added fructose at high concentrations of the sugar; this process requires Mg^{2+} and PEP (the phosphate-donor in the PT-system) and does not occur if ATP replaces PEP. In the complete system, which contained 50 mM fructose, both fructose-1-phosphate and fructose-6-phosphate were formed; when low concentrations of fructose were used, such as the 0.04 mM employed by Fraenkel [1] to demonstrate fructose-1-phosphate formation via the PT-system, fructose-6-phosphate formation was not detected.

The possibility that small amounts of glucose, present in the fructose solutions used, might have given rise to glucose-6-phosphate and then to fructose-6-phosphate was ruled out by demonstrating this process with a mutant K2.1.5^c.7 that lacked phosphoglucoseisomerase activity: toluene-treated suspensions of the fructose-grown cells formed approx. 4 nmoles of fructose-6-phosphate $\times$ min^{-1} $\times$ mg dry wt^{-1}, and this rate was maintained linearly over at least 20 min. Suspensions of the mutant grown on glycerol or glucose catalysed this reaction at only 17–26% of this rate, which further argues for its specific, and probably inducible, nature. As expected, mutants that lacked the enzyme II component of the fructose PT-system [2] failed to form either the 1- or 6-phosphate ester.

The results here presented resolve the paradox [1, 3] that *E. coli* mutants devoid of fructose diphosphatase grow on fructose although the major pathway of fructose conversion to fructose diphosphate is, in essence, a gluconeogenic process. It remains to be investigated why similar mutants of *Aerobacter aerogenes* [12] behave differently.

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ISOLATION AND PROPERTIES OF A REGULATORY MUTANT IN THE HEXOSE PHOSPHATE TRANSPORT SYSTEM OF *ESCHERICHIA COLI*

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1. Introduction

Escherichia coli can utilize a variety of hexose phosphates without first hydrolysing them to the free sugars [1]. The system that effects the transport of these esters into the cells is inducible [1, 2] and common to them all: growth in the presence of glucose-6-phosphate, or fructose-6-phosphate, or mannose-6-phosphate, elicits the formation of the uptake system [3], and mutants that lack the ability to take up one of these hexose phosphates also lack the ability to take up the others [3, 4]. The gene specifying this uptake system, designated *uhp*, is cotransducible with *pyrE* and is thus located at about 72 min [4] on the *E. coli* linkage map [5].

We recently observed [6] that *E. coli* can grow on fructose-1-phosphate as sole carbon source. However, rapid growth occurred only if the cells were previously exposed to hexose-6-phosphates; the rate of growth on fructose-1-phosphate by cells thus induced decreased sharply after 1–1½ doublings. This suggested that fructose-1-phosphate could be transported by the hexose phosphate uptake system but was not an inducer of that system. We now confirm this interpretation, and utilize it to select mutants that grow readily on fructose-1-phosphate without prior exposure to hexose-6-phosphates, and whose rate of growth on this substrate remains constant for many doublings. Such mutants form the uptake system for hexose phosphates constitutively; their genotype is designated *uhp*^{c}. Analysis by conjugation and by phage-mediated transduction, establishes *uhp*^{c} to be closely linked to *uhp* and to be also cotransducible with *pyrE*; this raises the possibility that the uptake of hexose phosphates by *E. coli* may be regulated as an operon [7].

2. Experimental

The procedures used for the growth of *E. coli*, for measurement of the rates of uptake of labelled substrates by washed cell suspensions, and for genetic procedures, were as previously described [6, 8, 9]. Methods for the isolation and detection of *uhp*^{c}-mutants are discussed in the following section.

3. Results and discussion

The observation [6] that an *E. coli* mutant devoid of the ability to grow upon fructose can grow upon fructose-1-phosphate shows that this phosphorylated compound can enter *E. coli* without prior hydrolysis to fructose. However, this ability to utilize fructose-1-phosphate is manifested only by cultures that have been exposed previously to substances that induce the uptake system for hexose-6-phosphates (UHP), and persists for only about 1–1½ doublings on fructose-1-phosphate [6]. This implies that fructose-1-phosphate is not an inducer of the UHP-system but can be transported by it. The results shown in fig. 1 support this view. Washed suspensions of the wild-type strain KL 16, which had been grown on glucose-6-phosphate as sole carbon source, rapidly incorporate isotope when exposed to 0.1 mM ^{14}C-glucose-6-phosphate, as expected [3, 4] from a transport system common to all hexose-6-phosphates. This incorporation is sharply reduced if unlabelled hexose-6-phosphates, such as the fructose-6-phosphate shown in fig. 1, are also added. Although unlabelled fructose-1-phosphate also dilutes the uptake of ^{14}C-glucose-6-phosphate, it does so less effectively than does fruc-

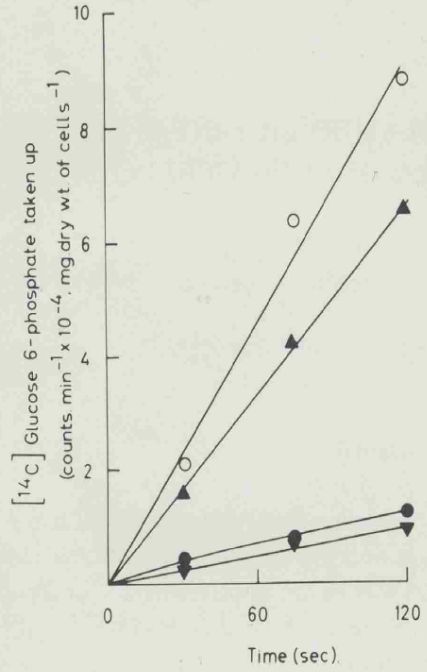

Fig. 1. Uptake of ^{14}C-glucose-6-phosphate by the wild-type *E. coli* strain KL 16. Washed suspensions (0.3 mg dry wt/ml) of the organism grown on glucose-6-phosphate were incubated with 0.1 mM ^{14}C-glucose-6-phosphate (0.3 μCi/ml) alone (○) or in the presence of 1 mM fructose-1-phosphate (▲) or fructose-6-phosphate (▼). The uptake of ^{14}C-glucose-6-phosphate by cells grown for approx. 1½ doublings on fructose-1-phosphate is shown as ●.

tose-6-phosphate: this indicates that fructose-1-phosphate is a substrate, but a poor one, for the UHP system. Growth on fructose-1-phosphate is not sufficient to maintain the induction of this uptake system and, as shown in fig. 1, the ability of the cells to take up ^{14}C-glucose-6-phosphate had decreased to a low level by the time that rapid growth on fructose-1-phosphate also ceased. Measurements (not shown) of the rates of ^{14}C-glucose-6-phosphate uptake by cultures transferred from growth on glucose-6-phosphate to growth on fructose-1-phosphate confirm that the UHP-system is indeed 'diluted out' under these conditions.

Since fructose-1-phosphate is not an inducer of the UHP-system, it would be predicted that mutants able to grow on this substance for more than 1–1½ doublings would differ from their parents in one of two ways: they would either be able to effect the rapid hydrolysis of the phosphate ester [10], and thus in effect grow on the fructose produced thereby, or be derepressed for the UHP-system. A mutant of the latter type, KL 16-11^{c}-21, was isolated from the Hfr strain KL 16-21: this strain is not capable of utilizing free fructose [6]; selection of mutants of the latter type would thus be favoured. KL 16-11^{c}-21 was obtained by spreading cultures of KL 16-21 on minimal salts–agar plates containing 2 mM fructose-1-phosphate as carbon source: the colonies that appeared after 48–72 hr at 37° were picked and purified by single-colony isolation. In liquid media, such clones grew rapidly on fructose-1-phosphate, although they had not been previously exposed to inducers of the UPH-system, and growth continued for many doublings; they did not grow on fructose.

That this ability constitutively to utilize fructose-1-phosphate was associated with an altered regulation

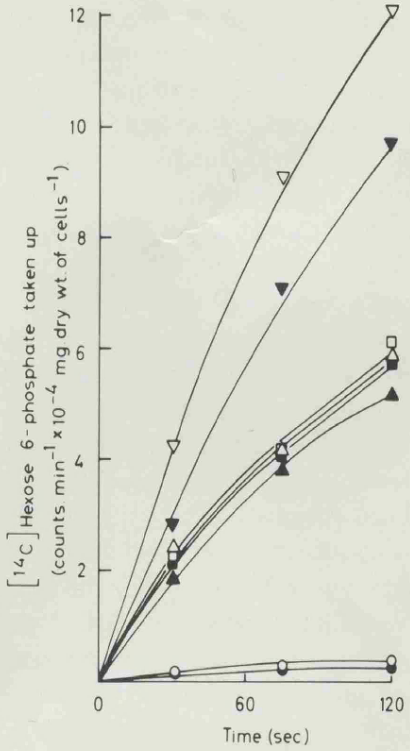

Fig. 2. Uptake of ^{14}C-glucose-6-phosphate (open symbols) and ^{14}C-fructose-6-phosphate (closed symbols) by suspensions of KL 16-21 grown on glycerol (○, ●) or glucose-6-phosphate (△, ▲) and by suspensions of KL 16-11^{c}-21 grown on glycerol (▽, ▼) or glucose-6-phosphate (□, ■).

of the UHP-system was demonstrated in three ways. KL 16-11^{c}-21 and its parent KL 16-21 were grown on minimal salts–agar plates containing 5 mM glucose and 20 μM ^{14}C-fructose-6-phosphate (0.05 μCi/ml), the colonies were adsorbed onto Whatman no. 40 filter paper, and this paper left in contact with Kodak 'Blue-Brand' X-ray film for 1–2 days [11]. The film was considerably more blackened by contact with the KL 16-11^{c}-21 than with its parent KL 16-21 colonies; this indicated that the former mutant could take up labelled fructose-6-phosphate under conditions where the latter could not, and, hence, that the UHP-system was now derepressed. This was confirmed by direct measurement of the ability of washed suspensions of the two organisms to take up ^{14}C-glucose-6-phosphate and ^{14}C-fructose-6-phosphate. As shown in fig. 2, KL 16-21 took up either of these substances when the UHP-system was induced by prior growth on glucose-6-phosphate, but not when the cells had grown on glycerol. In constrast, KL 16-11^{c}-21 took up ^{14}C-glucose-6-phosphate very rapidly, and ^{14}C-fructose-6-phosphate slightly less rapidly, even after growth on glycerol: indeed, these rates of uptake were considerably higher than those observed with either strain after growth on glucose-6-phosphate.

The third procedure involved transfer of the *uhp*^{c}-allele from KL 16-11^{c}-21 to suitable recipients. When the Hfr strain KL 16-11^{c}-21 was incubated with a F^{-} strain K2.1t.11i., which carried the UHP^{-}-allele (and was thus devoid of the ability to take up hexose phosphates) and which required isoleucine and valine for growth [4], all the recombinants selected for the ability to grow on glucose in the absence of isoleucine and valine were found (by the film technique) to be either still unable to utilize glucose-6-phosphate (UHP^{-}) or to have acquired the *uhp*^{c}-character: this indicated a very high degree of linkage between *uhp* and *uhp*^{c}. This was also indicated by the results of phage-mediated transfer of the *uhp*^{c}-marker. For this, phage P1-kc were grown [8, 9] on KL 16-11^{c}-21 and were used to infect a strain of *E. coli* which was unable to utilize free fructose and which carried the *pyrE*-marker: in consequence of this latter lesion, it required uracil for growth. Transductants were selected on minimal salts–agar plates containing glucose but no uracil. Tests, by the film technique, showed that of 182 uracil^{+}-transductants, 93 had remained inducible for the UHP-system but 89 had acquired the *uhp*^{c}-allele (fig. 3). This distribution is very similar to the degree of linkage between *uhp* and *pyrE* [4]. Moreover, when such UHP-inducible and UHP-constitutive transductants were grown on glucose and transferred to media containing fructose-1-phosphate, the latter transductants grew rapidly and virtually without lag, whereas the former did not. As expected, both types of transductant grew on glucose-6-phosphate (fig. 4).

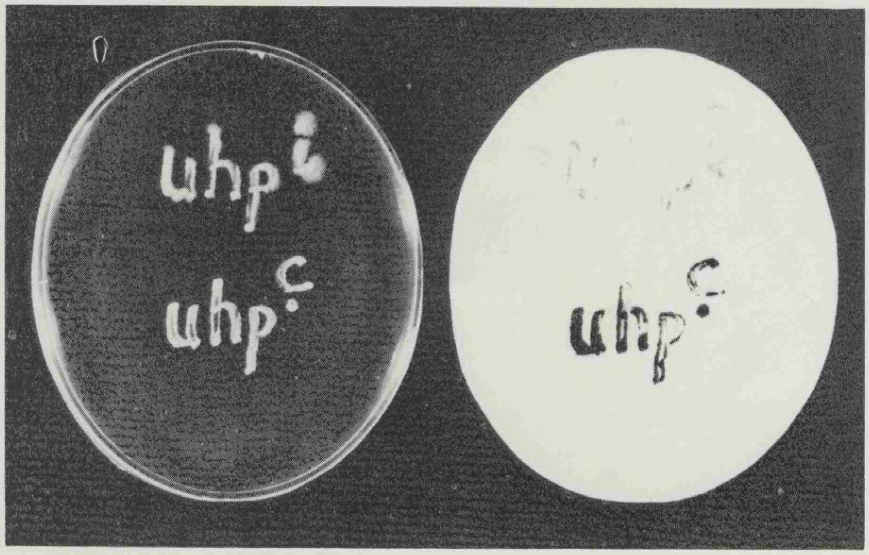

Fig. 3. Identification of uracil^{+}-transductants inducible (*uhp*^{i}) and constitutive (*uhp*^{c}) for the uptake of hexose phosphates. *Left*: organisms after growth on 5 mM glucose plus 20 μM ^{14}C-fructose-6-phosphate; *right*: blackening of X-ray film after exposure to these organisms absorbed on filter paper. For experimental details, see text.

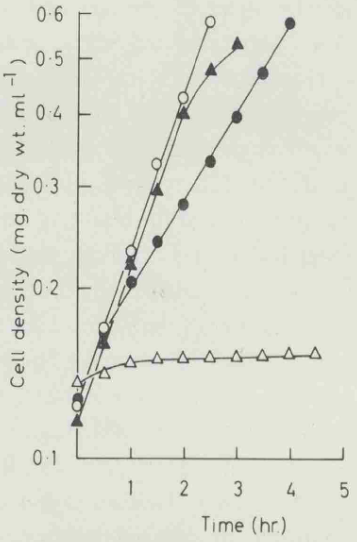

Fig. 4. Growth of UHP-inducible (open symbols) and UHP-constitutive (closed symbols) transductants on 5 mM glucose-6-phosphate (○, ●) or 5 mM fructose-1-phosphate (△, ▲) as carbon sources.

The results reported in this paper thus show that mutants selected for rapid growth on fructose-1-phosphate are also derepressed in their ability to take up hexose phosphates; conversely, recombinants or transductants selected for their constitutivity of hexose phosphate uptake are also able to grow rapidly and continuously on fructose-1-phosphate. Besides elucidating the manner in which fructose-1-phosphate enters *E. coli*, these findings provide a means for isolating mutants with altered regulation of hexose phosphate transport. Such mutants will be useful for investigating the mechanism of induction of the UHP-system [10, 12].

The results further show that the ability to effect the uptake of hexose phosphates, and the regulation of that ability, are specified by closely linked genetic markers. It is not yet known whether this system behaves as an operon.

Acknowledgements

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