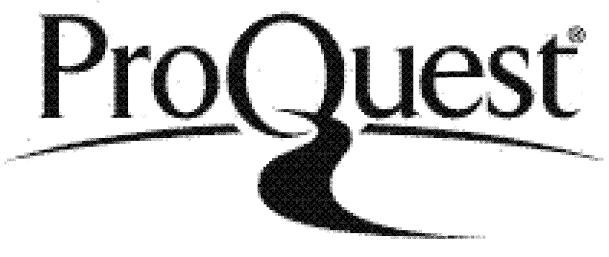
THE UTILISATION OF GLUCONATE BY *ESCHERICHIA COLI* K12

PELIN FAIK B.Sc.

(Department of Biochemistry, University of Leicester)

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

1974



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To Mike and my parents

This work was performed in the Department of Biochemistry, University of Leicester, during the tenure of a Medical Research Council Studentship and a Research Assistantship under a grant awarded to Professor H.L. Kornberg, F.R.S. by the Science Research Council.

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ABSTRACT

Many micro-organisms catablise gluconate via the Entner-Doudoroff pathway. The key enzymes of this pathway are gluconate kinase, 6-phosphogluconate dehydratase (Edd) and 2-keto 3-deoxy 6-phosphogluconate (KDPG) aldolase.

Biochemical and genetic techniques have been used to study gluconate utilisation in Escherichia coli K12. Mutants of the pathway have been isolated, their genetic lesions mapped and their physiological effects studied.

The first step in gluconate utilisation by E. coli is its entry into the cells. It has been established that uptake is an active process induced by growth on gluconate, and appears to be the rate-limiting step in gluconate utilisation. Gluconate thus taken up is then phosphorylated to 6-phosphogluconate by ATP, catalysed by gluconate kinase: mutants completely devoid of this enzyme have not been obtained and it may be that there are two gluconate kinases in E. coli. 6-phosphogluconate can only be metabolised via the Entner-Doudoroff and pentose-phosphate pathways since Edd^- mutants that also lack 6-phosphogluconate dehydrogenase (Gnd^-) do not grow on gluconate but the Entner-Doudoroff pathway plays the predominant role. The importance of the KDPG aldolase that catalyses the cleavage of KDPG to pyruvate and glyceraldehyde 3-phosphate has been studied with mutants devoid of this enzyme. It has been established that KDPG is a very effective competitive inhibitor of 6-phosphogluconate for Gnd.

The enzymes involved in the catabolism of gluconate are inducible. The likely inducer for the uptake system and gluconate kinase(s) is gluconate itself; 6-phosphogluconate probably induces Edd and Kga.

Genetic analysis shows that at least three regions of the E. coli chromosome (at 36, 66 and 85 min) contain genes involved in gluconate utilisation; only some of these genes are linked.

INTRODUCTION

INTRODUCTION

The Entner-Doudoroff pathway of gluconate dissimilation was first described by Entner and Doudoroff (1952) for the oxidative metabolism of glucose in Pseudomonas saccharophila. In this organism, glucose was shown initially to be phosphorylated to glucose 6-phosphate and then to be oxidised to 6-phosphogluconate; this was then catabolized in a further reaction or reactions to yield, finally, pyruvate and glyceraldehyde 3-phosphate. Entner and Doudoroff thought that a rearrangement of 6-phosphogluconate took place prior to the split. A possible compound which could fulfil the role of such an intermediate was 2-keto 3-deoxy 6-phosphogluconate (KDPG) but these authors were unable to demonstrate either the presence of such a compound in reaction mixtures or to prepare the intermediate synthetically. MacGee and Doudoroff (1954) isolated the phosphorylated keto acid, KDPG, by separating two enzyme activities from cell-free extracts of P. saccharophila. They showed that the enzyme 6-phosphogluconate dehydratase converted 6-phosphogluconate to KDPG which could then be converted to pyruvate and glyceraldehyde 3-phosphate by the "splitting enzyme".

A similar mechanism was found in P. fluorescens by Wood and Schwerdt (1954). These authors observed that extracts prepared from this organism grown on glucose were able to catalyse the cleavage of 6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate at a rate ten times faster than the rate of the 6-phosphogluconate

dehydrogenase reaction. They thus suggested that, in this organism, the Entner-Doudoroff pathway may constitute a major pathway of 6-phosphogluconate degradation. Kovachevich and Wood (1955) followed this field of study by separating, purifying and studying the properties of the two key enzymes of the Entner-Doudoroff pathway in P. fluorescens, namely, the 6-phosphogluconate dehydratase and the KDPG aldolase. Meloche and Wood (1964) have studied the mechanism of 6-phosphogluconate dehydratase in the same organism and obtained evidence that this enzyme converts 6-phosphogluconate to the enol form of KDPG and suggested that the ketonisation of the enol was the rate-limiting process, and perhaps the basis of the irreversibility of the reaction.

This route for the catabolism of 6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate, termed the Entner-Doudoroff pathway (see Figure 1), is now known to be involved also in the metabolism of free gluconate.

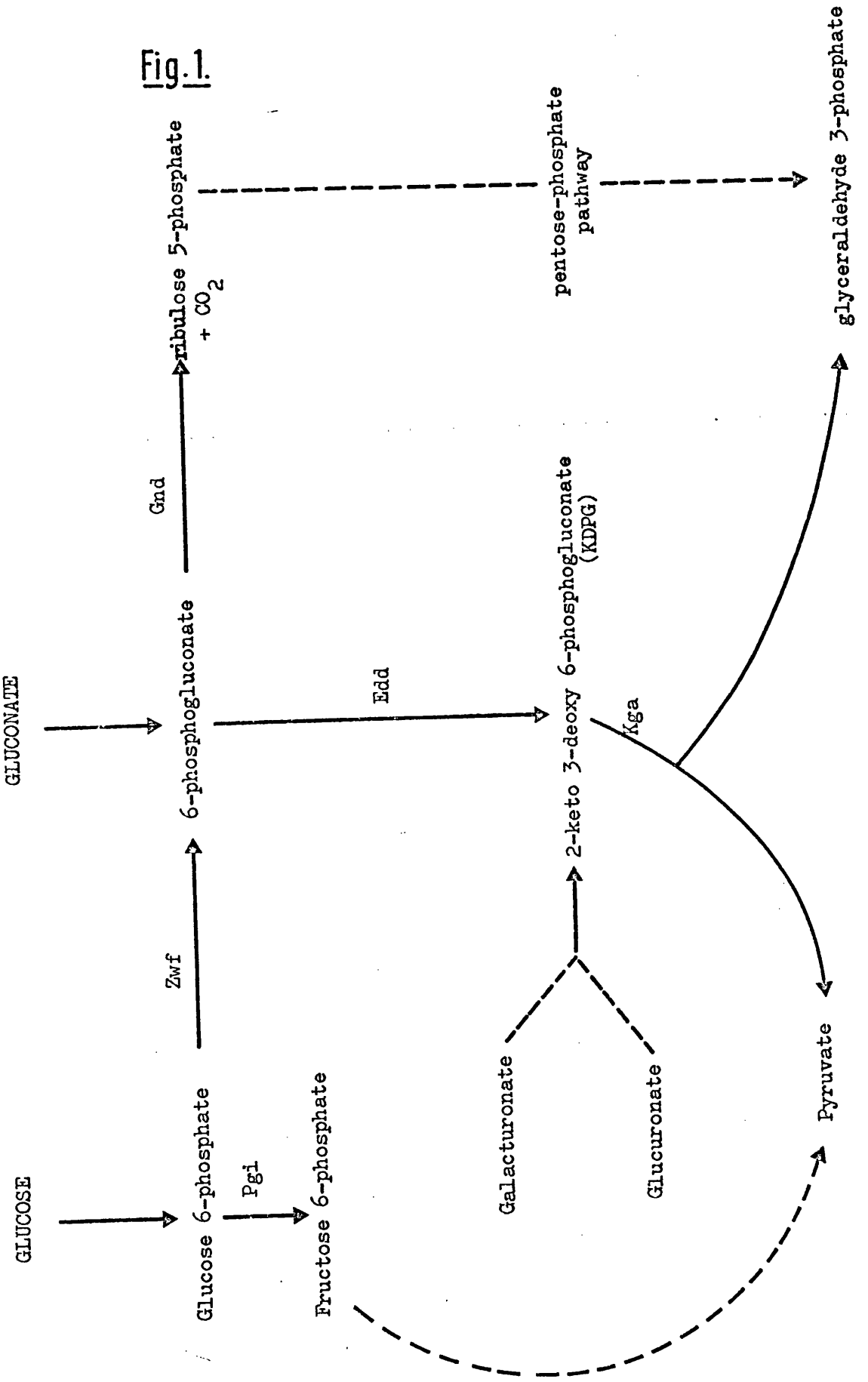
In the 1950's, it was established that gluconate could be formed by the direct oxidation of glucose in moulds and liver. Since it was known that 6-phosphogluconate could be catabolised via the pentose-phosphate pathway, it was of interest to determine whether the first step in the utilisation of gluconate was its phosphorylation to 6-phosphogluconate. S. S. Cohen (1951) found that, in Escherichia coli B, gluconate was first phosphorylated to 6-phosphogluconate by an ATP-dependent kinase. The enzyme catalysing this reaction was called gluconokinase. Although bacterial extracts contained large quantities of hexokinase which was able to catalyse the

FIGURE 1

The reactions of the Entner-Doudoroff and related metabolic pathways.

Abbreviations: Glk = gluconate kinase; Edd = 6-phosphogluconate dehydratase; Kga = KDPG aldolase; Gnd = 6-phosphogluconate dehydrogenase; Zwf = glucose 6-phosphate dehydrogenase; Pgi = phosphoglucose isomerase.

Fig.1.


GLUCOSE
GLUCONATE
Glucose 6-phosphate
Fructose 6-phosphate
6-phosphogluconate
ribulose 5-phosphate
+ CO_2
2-keto 3-deoxy 6-phosphogluconate
(KDPG)
Pgi
Zwf
Gnd
Edd
Kga
Galacturonate
Glucuronate
pentose-phosphate
pathway
glyceraldehyde 3-phosphate
Pyruvate

phosphorylation of a number of hexoses besides glucose, the gluconokinase was absolutely specific for gluconate. The enzyme was found to be inducible, and was preferentially synthesised during adaptation of the cells to growth on gluconate. Since the Entner-Doudoroff pathway had not been discovered at this time, the author suggested that the formation of gluconokinase was the only apparent change involved in the adaptation of E. coli to the utilisation of gluconate. The 6-phosphogluconate formed was then assumed to be utilised exclusively by the pentose-phosphate pathway via the enzyme 6-phosphogluconate dehydrogenase.

Sable and Guarino (1952), using Saccharomyces cerevisiae extracts, also identified an ATP-dependent gluconokinase which was specific for gluconate. It was already known that this organism was able to utilise free gluconate but, since the gluconokinase was a constitutive enzyme, it was suggested that some gluconate was formed during the metabolism of glucose.

Fraenkel and Horecker (1964) showed that a mutant of Salmonella typhimurium impaired in phosphoglucose isomerase activity (Pgi) grew more slowly on glucose, galactose and maltose than did the parental strain. However, on gluconate, glycerol, mannitol and a number of other carbon sources, the growth rate of the mutant was similar to that of the parent strain. Since, in the Enterobacteriaceae, the metabolism of gluconate was considered to proceed via the inducible kinase (which yielded 6-phosphogluconate) and then via the pentose-phosphate pathway (Cohen, 1951), the normal growth of the mutant lacking phosphoglucose isomerase activity on

gluconate suggested that perhaps an alternative route for the metabolism of gluconate was present during growth on gluconate but which did not function during growth on glucose. The results of Fraenkel and Horecker (1964) imply that the capacity of the pentose-phosphate pathway was constant and incapable of adjusting to an increased requirement by the micro-organism, perhaps because one of the reactions of this pathway was rate-limiting. Using gluconate labelled at the C-1 position, and by assaying enzyme activities in cell extracts, Fraenkel and Horecker presented evidence which strongly suggested that the strain of S. typhimurium used in their studies inducibly formed the enzymes of the Entner-Doudoroff pathway. Since the 6-phosphogluconate dehydrogenase (Gnd) activity present in cell extracts was the same in the mutant grown on gluconate or on any other carbon source, the major route of gluconate metabolism in S. typhimurium could not be the pentose-phosphate pathway. These authors concluded that the Entner-Doudoroff pathway must be the major pathway of gluconate metabolism in this organism despite the low activity of the enzyme 6-phosphogluconate dehydratase measured in cell-free extracts. The results also suggest that the mutant strain did not use glucose by the Entner-Doudoroff pathway.

Other workers have also found that, in some bacteria, notably S. typhimurium (Fraenkel and Horecker, 1964), P. natriegens (Eagon and Wang, 1962), Strep. faecalis (Sokatch and Gunsalus, 1957), gluconate but not glucose was metabolised via an inducible Entner-Doudoroff pathway.

However, in E. coli, the situation was far from clear. Two

years before Fraenkel and Horecker's (1964) study of a pgi-mutant of S. typhimurium, de Ley (1962) suggested that, although the dehydratase and the aldolase could be detected in E. coli (Kovachevich and Wood, 1955a, b), these enzymes played no role in the overall metabolism of carbohydrates by this micro-organism. But, six years later, Kersters and de Ley (1968) reviewed the occurrence of the Entner-Doudoroff pathway in bacteria and found that the enzymes Edd and Kga were more widespread than was originally thought. Their results confirm that the pathway is present in P. saccharophila (the organism in which it was first discovered) and show that its component enzymes are also found in a restricted group of Gram-negative, usually strictly aerobic, bacteria from a number of genera. The only known case of an inducible Entner-Doudoroff pathway in a Gram-positive organism is in Strep. faecalis (Sokatch and Gunsalus, 1957). Kersters and de Ley found that, although Edd and Kga were present in E. coli K12, the activity of the dehydratase was very low.

Eisenberg and Dobrogosz (1967), however, obtained evidence that, in the E. coli strain ML30, the Entner-Doudoroff pathway provided a primary route of gluconate metabolism. Their data suggested that gluconate dissimilation was regulated by one or more inducible enzymes.

Fraenkel and Levisohn (1967), using a mutant of E. coli devoid of phosphoglucose isomerase (Pgi) activity, obtained similar results to those obtained with the pgi-mutant of S. typhimurium studied by Fraenkel and Horecker (1964). Using [1-^{14}C]glucose and [1-^{14}C]gluconate, they showed that, in this mutant, glucose was metabolised chiefly via the pentose-phosphate pathway (and perhaps

to a very minor extent via the Entner-Doudoroff pathway) but that gluconate was metabolised both via the Entner-Doudoroff pathway and via the pentose-phosphate pathway. Although, in this mutant, glucose could give rise to glucose 6-phosphate and then to 6-phosphogluconate, this intermediate was unable to be utilised to any great extent by the Entner-Doudoroff pathway. However, when 6-phosphogluconate was derived from gluconate, the enzymes of the Entner-Doudoroff pathway were induced. Enzyme assays confirmed this conclusion. These results contradict those of Loomis and Magasanik (1966) who had obtained data suggesting that, during growth of E. coli K12 on glucose, the enzyme 6-phosphogluconate dehydratase was present in substantial activity thus implying that the Entner-Doudoroff pathway was of major importance both in the metabolism of glucose and of gluconate in E. coli K12. This discrepancy may be in the growth state of the cultures used for enzyme assays: Fraenkel and Levisohn used logarithmic-phase cultures whereas Loomis and Magasanik used stationary-phase cultures.

Zablotny and Fraenkel (1967) further showed the importance of the Entner-Doudoroff pathway in the catabolism of gluconate but not of glucose in E. coli K12 by isolating the first mutant of this pathway: a mutant devoid of 6-phosphogluconate dehydratase activity. This mutant grew at normal rates on glucose and fructose but grew about one third as fast on gluconate as did the parent strain. The doubling time of this mutant on gluconate was approximately the same as that of a mutant of E. coli devoid of phosphoglucose isomerase activity growing on glucose, which suggested that both mutants were

catabolising their substrates via the pentose-phosphate pathway. Zablotny and Fraenkel (1967) concluded that, in E. coli, the Entner-Doudoroff pathway had a major role in the metabolism of gluconate but little, if any, role in the normal metabolism of glucose.

The recent work of Quay et al. (1972) has clarified this situation considerably: in P. saccharophila, and in other pseudomonads (e.g. P. aeruginosa (Stern et al., 1960) and P. lindneri (Dawes et al., 1966)), the Entner-Doudoroff pathway was present during growth of cells on glucose. In other organisms, including E. coli, the enzymes of this pathway were induced by growth in the presence of gluconate but not glucose (Zablotny and Fraenkel, 1967). Quay et al. (1972) isolated mutants of P. fluorescens deficient in the membrane-bound glucose dehydrogenase and showed that such mutants formed the enzymes of the Entner-Doudoroff pathway during growth on gluconate but not glucose. They concluded, therefore, that wild-type cells of P. fluorescens oxidised glucose to gluconate (via the enzyme glucose dehydrogenase) and that it was this latter compound which was the true inducer of the enzymes of the Entner-Doudoroff pathway. Further evidence has been provided by the work of Midgley and Dawes (1973) who have shown that glucose can be taken up by P. aeruginosa by two systems. One of these transported glucose and α-methylglucoside, and the other involved the activity of glucose dehydrogenase which acted extracellularly to produce gluconate from glucose. The gluconate was then transported into the cells. Thus, mutants deficient in this glucose dehydrogenase were only able to transport glucose by the glucose - α-methylglucoside system but were still able to transport gluconate.

In summary, at the time when the present work was initiated, it was known that, in E. coli K12, the metabolism of gluconate involved the following series of reactions: gluconate was phosphorylated to 6-phosphogluconate by the enzyme gluconate kinase. The 6-phosphogluconate thus formed could be metabolised via two pathways:
(i) the Entner-Doudoroff pathway - the enzyme 6-phosphogluconate dehydratase catalysed the formation of KDPG from 6-phosphogluconate; the KDPG was cleaved to pyruvate and glyceraldehyde 3-phosphate by the KDPG aldolase. These C_3-compounds were then able to enter the central routes of metabolism.
(ii) the pentose-phosphate pathway - the 6-phosphogluconate was oxidatively decarboxylated to ribulose 5-phosphate and CO_2. The ribulose 5-phosphate, by a series of reactions involving transaldolase and transketolase, formed fructose 6-phosphate which could then form the C_3-compounds by glycolysis and thus enter the central routes of metabolism.

Zablotny and Fraenkel (1967) had already obtained evidence that the major route of gluconate catabolism in E. coli K12 was via the Entner-Doudoroff pathway; Eisenberg and Dobrogosz (1967) had shown that E. coli was able inducibly to form the enzymes gluconate kinase and 6-phosphogluconate dehydratase; but the data of Kersters and de Ley (1968) showed that wild-type cells of E. coli K12, even after growth on gluconate, contained little (and apparently insufficient) amounts of the 6-phosphogluconate dehydratase.

The purpose of this thesis is to discuss the utilisation of gluconate by E. coli K12. At the time when the study was started, it was important to know firstly, whether the formation of pyruvate and

glyceraldehyde 3-phosphate from 6-phosphogluconate was only due to the activity of Edd and Kga (and, to a limited extent, of Gnd) or, since Kersters and de Ley (1968) had found very low activities of Edd, whether the fragmentation of 6-phosphogluconate involved steps either in addition to, or alternative to, those already known in P. saccharophila. Secondly, was there an uptake system specific for gluconate and, if so, was the gluconate kinase part of it? Thirdly, since the Entner-Doudoroff pathway was known to be inducible, were the genes specifying the synthesis of the enzymes of the pathway linked and, if so, did these genes form an operon?

This thesis attempts to answer these and other questions on gluconate utilisation in E. coli K12. Both biochemical and genetic techniques have been employed: a number of mutants of the pathway have been isolated, their genetic lesions mapped, and the physiological effect of such mutations studied.

The data in this thesis will be presented in the following order:

(i) the properties of mutants defective in KDPG aldolase activity;
(ii) the properties of mutants impaired in gluconate kinase activity;
(iii) the uptake of gluconate by E. coli, and
(iv) the isolation and properties of E. coli mutants impaired in gluconate uptake.

This order of presentation is necessary as the properties of the mutants isolated in the earlier stages of the study are important to the interpretation of data obtained in the later stages.

MATERIALS and METHODS

MATERIALS AND METHODS

Materials

All enzymes, coenzymes and sugar phosphates were obtained from Boehringer GmbH (Mannheim, Germany) and Sigma Ltd. (London, England). Sugars and amino acids were from British Drug Houses Ltd. (Poole, England), sodium gluconate was from Fisons Ltd. (Loughborough, England).

All sugars used were of the D-configuration, unless otherwise stated. Streptomycin sulphate and benzylpenicillin were from Glaxo Ltd. (Greenford, England) and chloramphenicol from Parke-Davis Ltd. (Hounslow, England). Radioactive sodium gluconate was from the Radiochemical Centre (Amersham, England). Ethyl methanesulphonate was purchased from Kodak Ltd. (London, England), triphenyltetrazolium chloride from Sigma Ltd.

Organisms used

The strains of Escherichia coli K12 used in this study are listed in the appropriate sections of the thesis with their genotype and their growth requirements. Abbreviations for the genetic markers are, wherever possible, those listed by Taylor and Trotter (1972). Other genetic lesions are described in the appropriate parts of the text.

Maintenance of organisms

Organisms were maintained by periodic subculture on Difco

nutrient agar slopes supplemented where necessary with appropriate growth factors. The cultures were incubated at 37°C overnight and stored at room temperature. Cultures were routinely checked by streaking onto suitable selective media to ensure that revertants or contaminants were not present.

Growth of organisms

Liquid cultures were obtained by inoculation from the slope either into double-strength Oxoid nutrient broth, or into sterile basal salts medium (Ashworth and Kornberg, 1966) containing the appropriate carbon source at 20 mM, unless otherwise stated, and supplementary growth factors at 80-100 µg/ml. Cultures were grown at 37°C, with shaking, in Erlenmeyer flasks containing not more than two-fifths of their total volume of liquid.

Growth rates were 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 mass by a previously determined factor (Ashworth and Kornberg, 1966); E_{680}nm of 1.0 is equivalent to 0.68 mg.dry mass.ml^{-1}. Cells were centrifuged at room temperature, washed with basal medium, centrifuged again and suspended in warmed medium (containing the carbon source at the concentrations indicated) to an extinction at 680 nm of 0.1 - 0.15. No aseptic precautions were taken for measurements of growth rate (unless otherwise stated) because of the large inocula used and the short duration of these experiments.

For growth on Petri dishes, the basal medium was solidified

by the addition of 1.5% Oxoid No. 1 or Difco agar. Basal salts medium, agar and growth factors (except L-tryptophan) were sterilised by autoclaving for 20 min at 15 $lb.in^{-2}$. Carbon sources were sterilised separately and were mixed at 50°C. Sugar phosphates and streptomycin sulphate were sterilised by membrane filtration.

Nutrient-gluconate-tetrazolium plates were prepared by the method of Zablotny and Fraenkel (1967): double-strength nutrient broth, triphenyl tetrazolium chloride at 50 $\mu g.ml^{-1}$ (and 1.5% Oxoid agar No. 1), were autoclaved together. Gluconate at a concentration of 50 mM was added at 50°C.

Uptake studies

For uptake studies, exponentially growing cultures were harvested, washed with N-free basal medium which had previously been warmed to 25°C, and resuspended in warm N-free medium to a cell density of approximately 0.2 mg dry $mass.ml^{-1}$. The washed cell suspensions were incubated in a shaking water bath at 25°C for 10 min prior to the addition of isotopic material. The final concentration of [U-^{14}C]gluconate normally used in these studies was 5×10^{-5} M (Specific activity = 3.8 $mCi.mmole^{-1}$). Uptake studies were based on the method of Morgan and Kornberg, (1969).

Method 1

After the addition of the labelled substrate, 0.5 ml of the washed cell suspension, removed at known times with an Eppendorff automatic pipette, was filtered through a membrane filter (0.45 μ pore

size) and washed with 10 ml of N-free basal medium (washing buffer) warmed to 25°C. The membrane filters containing the bacteria were dissolved in 5 ml of Bray's fluid (Bray, 1960) and the radioactivity assayed using a Packard Liquid Scintillation Spectrometer, model 4000 or 3385. To calculate the quantity of label taken up by the cell suspension, the radioactivity of 10 µl of the solution of isotopic substrate was assayed. In order to make the conditions of this measurement comparable with those used for other samples, 0.5 ml of filtered cell suspension (which had no isotope added) and 5 ml of Bray's fluid were also added to the vial in addition to the labelled substrate.

Method 2

After the addition of the isotopic material, 0.5 ml of the cell suspension was diluted with 5 ml of the washing buffer and filtered through a membrane filter (0.45 µ pore size). The material on the membrane filter was washed with a further 5 ml of buffer; the radioactivity of this material was assayed as in Method 1.

Kinetic parameters

Rates of uptake of labelled substrates were measured in a final volume of 2.0 ml. Samples (0.5 ml) were removed at 10, 40 and 70 sec after the addition of isotope. For each concentration of substrate, the experiment was performed in duplicate. To determine the quantity of isotope taken up by the cells, 10 µl of each concentration of substrate was counted as described previously. The

background counts which were usually 45 or 75 counts.min^{-1} (depending on which vials were used) were subtracted.

Qualitative differences in the ability of various strains to accumulate radioactive substrates were determined by the film technique described in Chapter IV of the results.

Preparation of cell extracts

The general method for preparing bacterial extracts is given below. Modifications are noted at appropriate places in the text.

All cell cultures were harvested in the exponential growth phase (0.5 - 0.6 mg dry mass.ml^{-1}), unless otherwise stated, by centrifuging at 20,000 g for 5 min at 15°C. The cells were washed with buffer, centrifuged again and suspended at approximately 6 mg dry mass.ml^{-1}. The cell suspensions were disrupted by exposure at 0°C for 30 sec to the output of an M.S.E. 100W ultrasonic oscillator, operating at setting 8. Cell debris was removed by centrifugation at 20,000 g for 15 min at 4°C.

Determination of protein content

The protein content of the cell extracts was determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

ENZYME ASSAYS

All enzyme assays were performed at 25°C.

6-phosphogluconate dehydrogenase (Gnd)

(i) Quantitative assay The washing buffer for the preparation of the cell extracts was 10 mM sodium/potassium phosphate, pH 7.6; the cells were sonicated in the same buffer containing 1 mM $MgCl_2$. Gnd was measured spectrophotometrically in a Unicam SP1800 spectrophotometer. The NADPH formed during the oxidation and decarboxylation of 6-phosphogluconate to ribulose 5-phosphate and CO_2 was measured at 340 nm. The reaction mixture contained in 1.0 ml: 50 µmoles of glycyl glycine buffer, pH 9.5; 5 µmoles of $MgCl_2$; 0.25 µmole of $NADP^+$; cell extract (usually of the order of 50-100 µg of protein). The change in extinction at 340 nm on the addition of 2 µmoles of 6-phosphogluconate, sodium salt, was measured. No reduction of $NADP^+$ was observed before this addition.

(ii) Qualitative Gnd was measured qualitatively as described by Peyru and Fraenkel (1968). In this procedure, the clone to be tested is grown overnight at 37°C in 2.5 ml of nutrient broth. The cells are harvested by centrifugation and resuspended in 10 mM Tris-HCl buffer containing 10 mM $MgCl_2$. The cell suspension is stored in the deep freeze (-20°C) for 24 h. After thawing at room temperature, the suspension is divided into two equal parts. To each part, 0.05 ml of a mixture containing equal portions of Nitro Blue Tetrazolium (Sigma), 2 mg/ml; NADP, 1.4 mg/ml; and chloramphenicol, 1 mg/ml is added. 0.05 ml of gluconate 6-phosphate (10 mg/ml) is then added to one of the tubes. Colour development is estimated visually after 10-30 min at room temperature, the reaction being scored according to the difference between the blank (no substrate added) and the test

(containing the substrate). A positive reaction (Gnd^+) is a violet colour in the complete reaction mixture caused by the substrate-dependent reduction of the dye. This test gave results consistent with the usual enzyme assays performed with crude extracts.

6-phosphogluconate dehydratase (Edd)

Edd was measured spectrophotometrically in a Unicam SP1800 spectrophotometer in two ways: the 2-keto 3-deoxy 6-phosphogluconate (KDPG) formed as a result of the action of the dehydratase was determined by the addition of excess KDPG aldolase (using an extract prepared from the mutant DF 10, which is devoid of Edd activity) which catalysed the cleavage of KDPG to glyceraldehyde 3-phosphate and pyruvate. This latter product was estimated either by the addition of excess lactate dehydrogenase and NADH (method A) or by the addition of phenylhydrazine hydrochloride (method B). Since the Edd activity was the rate-limiting step in this overall reaction, its activity may be determined by following the decrease in extinction at 340 nm due to the removal of NADH (method A) or the increase in extinction at 324 nm as a result of the formation of pyruvate phenylhydrazone. The order of addition of the reagents is important as the dehydratase seems to require substrate activation. The washing buffer was 50 mM phosphate, pH 7.5; the sonicating buffer was the same but also contained 1 mM $MgCl_2$.

METHOD A All extracts were centrifuged at high speed (110,000g) to remove the majority of the NADH oxidase activity. The reaction

mixture contained in 1 ml: 10 μmoles of sodium/potassium phosphate buffer, pH 7.5; 3 μmoles of $MgCl_2$; 0.4% Bovine Serum albumin (Sigma); 5 μmoles of 6-phosphogluconate sodium salt; cell extract (approx. 500 μg of cell protein). This mixture was incubated at room temperature for 2 min. At the end of this period, excess KDPG aldolase [in the form of a cell-free extract prepared from the E. coli mutant DF10 (Edd^-)], crystalline lactate dehydrogenase (25 μg) and 0.1 μmole of NADH were added. A blank cuvette contained all the reagents except 6-phosphogluconate.

METHOD B A mixture containing 25 μmoles of iminazole buffer, pH 6.8; 5 μmoles of $MgCl_2$; 1 μmole of EDTA, and 4 μmoles of phenylhydrazine hydrochloride (Dixon and Kornberg, 1959) was substituted for the NADH and lactate dehydrogenase used in Method A.

In later experiments, purified KDPG aldolase (Kga) was prepared according to the method of Pouysségur and Stoeber (1971): an ammonium sulphate fractionation was performed on an extract prepared from the mutant DF10 grown on nutrient broth plus 10 mM gluconate. The Kga activity was precipitated by ammonium sulphate at 73 -90% saturation. This precipitate was resuspended in distilled water, heated for 4 min at 75°C and centrifuged. The supernatant contained the Kga activity.

METHOD C The third method of measuring dehydratase activity was that of Pouysségur and Stoeber (1972): the sonicating buffer was 50 mM Tris-HCl, pH 7.6, to which fresh $FeSO_4$ and 2-mercaptoethanol were then added to final concentrations of 0.8 and 50 mM respectively.

Owing to the presence of interfering materials in this solution, it was not possible to measure the protein content of the extract by the usual procedure (Lowry et al., 1951). Therefore, when this method was used, specific activity was expressed as enzyme activity.mg dry mass^{-1} of cells.

2-keto 3-deoxy 6-phosphogluconate aldolase (Kga)

Kga activity was measured as the rate of production of pyruvate from the cleavage of KDPG. This was measured either by following the oxidation of NADH in the presence of excess crystalline lactate dehydrogenase, or by trapping the pyruvate as phenylhydrazone. In the first of these procedures, the reaction mixture contained in 1 ml: 10 μmoles of sodium/potassium phosphate buffer, pH 7.5; 3 μmoles of $MgCl_2$; 0.1 μmoles of NADH; 25 μg of crystalline lactate dehydrogenase; cell extract (100-200 μg of protein). The reaction was started by the addition of 2 μmoles of 2-keto 3-deoxy 6-phosphogluconate (KDPG) prepared as described in the Methods to Chapter I. As the extract had been centrifuged for 2-3 h at approx. 110,000 g, only a slight NADH oxidase activity was observed before the addition of KDPG.

The reaction mixture used for the second procedure contained, in 1 ml, 7 μmoles of $MgCl_2$; 25 μmoles of iminazole buffer, pH 6.8; 1 μmole of EDTA; 4 μmoles of phenylhydrazine hydrochloride, and cell extract. The reaction was started by the addition of 2 μmoles of KDPG. The blank cuvette contained all the reagents except KDPG. Pyruvate phenylhydrazone formation was measured as the increase in extinction at 324 nm.

2-keto 3-deoxy 6-phosphogluconate (KDPG)

The assay of KDPG in a given solution is essentially the same as the assay for Kga activity except that Kga prepared from strain DF10 was added in excess and the reaction was started by the addition of the KDPG solution.

Mutagenesis

Ethyl methanesulphonate mutagenesis was performed according to the method of Lin, Lerner and Jorgensen (1962). The mutagenised culture was subject to penicillin treatment (Gorini and Kaufman, 1960) to increase the proportion of mutant cells. The selection media used are described in appropriate parts of the text.

Genetic Procedures

The method of Brice and Kornberg (1967) was used for conjugation experiments. Methods of selection are described in the appropriate Chapters.

Phage P1 kc (laboratory stock) was used for transduction studies (Brice and Kornberg, 1967).

CHAPTER I

CHAPTER I

ISOLATION AND PROPERTIES OF MUTANTS OF E. coli IMPAIRED IN KDPG ALDOLASE (Kga) ACTIVITIES

Methods

Preparation of 2-keto 3-deoxy 6-phosphogluconate (KDPG)

(i) Induction of enzymes of gluconate catabolism in E. coli strain DF1071-2B (Kga^-) KDPG is prepared essentially by the method of Meloche and Wood (1966). To 300 ml of a culture of E. coli, strain DF1071-2B, grown aerobically overnight at 37°C on double-strength 'Oxoid' nutrient broth, were added 6 ml of 0.5 M sodium gluconate and a further 300 ml of double-strength nutrient broth. After shaking at 37°C for 2 h, the cells were harvested by centrifugation at 20,000 g for 15 min, washed once with 0.05 M Tris-HCl buffer, pH 7.5, and resuspended in 10 ml of this buffer. The thick suspension was cooled and the cells disrupted, by exposure at 0°C for 1.5 min to the output of a MSE 100W ultrasonic disintegrator operating at setting 8. Cell debris was removed by centrifugation at 20,000 g for 10 min; more prolonged (2-3 h) centrifugation of the resultant supernatant at 110,000 g yielded a clear solution with low NADH-oxidase activity. The 6-phosphogluconate dehyratase activity of the extract was measured as described in the Methods section.

(ii) Formation of KDPG from gluconate by cell-free extracts of induced strain DF1071-2B 1 ml of a solution containing 30 µmoles of $MgCl_2$ and approx. 550 µmoles of 6-phosphogluconate, trisodium salt,

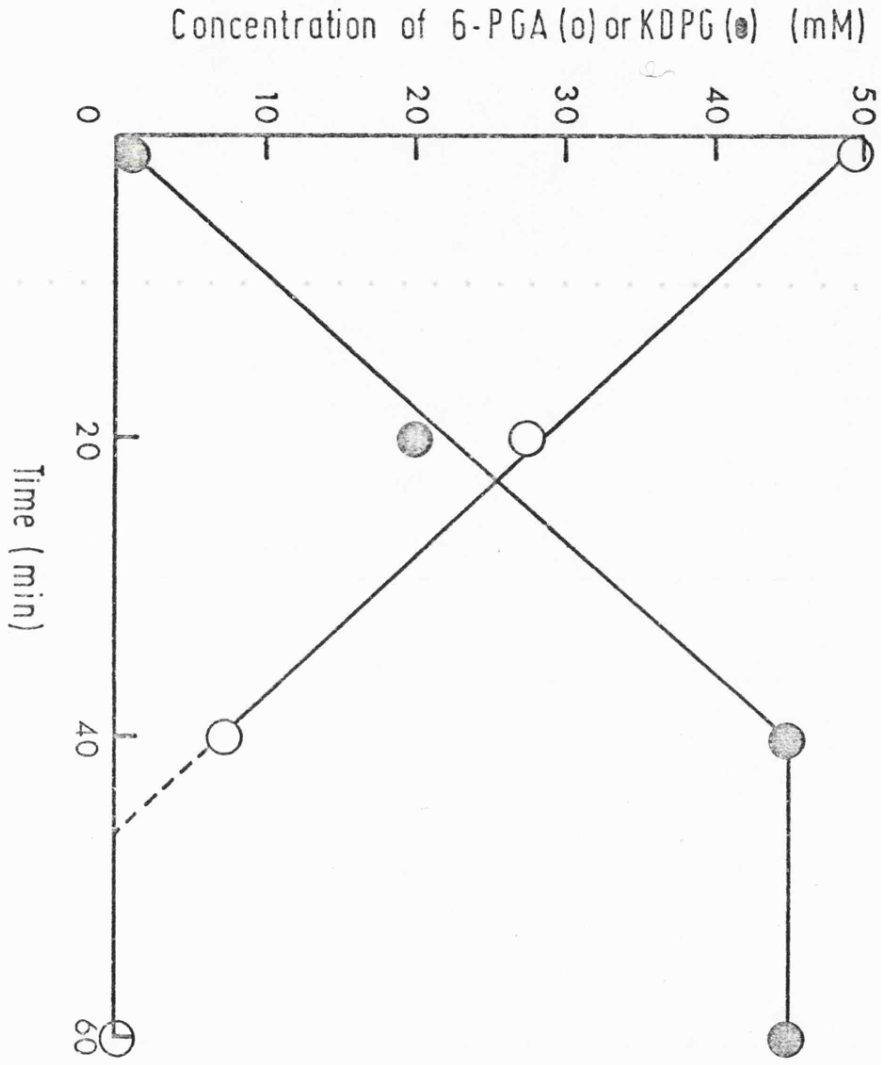
were added to 10 ml of the extract of strain DF1071-2B, induced as described in (i). The mixture was incubated at 37°C and the progress of the reaction was followed by removing samples (0.1 ml) of the reaction mixture at 20 min intervals, adding them to tubes containing 0.9 ml of water in a boiling water bath, and leaving the resultant mixtures at 100°C for 5 min. The 6-phosphogluconate content of the supernatant solutions was measured spectrophotometrically with commercial 6-phosphogluconate dehydrogenase and $NADP^+$ at 340 nm whilst that of 2-keto 3-deoxy 6-phosphogluconate (KDPG) formed was determined (Meloche, Ingram and Wood, 1966) at the same wavelength with commercial lactate dehydrogenase, NADH, and extracts of any Kga^+-strain of E. coli (usually DF10) as a source of KDPG aldolase (Kga). The typical progress plot (Figure 2) shows that there is a quantitative and stoicheiometric transformation of the added 6-phosphogluconate to KDPG. When the 6-phosphogluconate content of the reaction mixture reached zero, 10 ml of a 25% solution of barium acetate was added to the reaction mixture at room temperature. The solution was then brought to pH 3.5 with 6N HCl and the precipitate formed was removed by centrifugation. To the supernatant solution were added 5 volumes of absolute ethanol, and the precipitate was allowed to settle overnight at 4°C. This precipitate was collected by centrifugation, redissolved in 1N HCl, and the pH was adjusted to 3.5 with saturated barium hydroxide. The barium salt of KDPG was precipitated by the addition of 5 volumes of ethanol, recovered by centrifugation, washed once with ethanol, and dried in a vacuum desiccator over anhydrous calcium chloride. The barium salt of KDPG was obtained in 78% yield at a purity of over 90%

FIGURE 2

Conversion of 6-phosphogluconate (○) to KDPG (●) by an extract of DF1071-2B, an *E. coli* mutant devoid of Kga activity

6-phosphogluconate was added to an extract prepared from the strain DF1071-2B grown on nutrient broth plus gluconate. Samples removed at 20 min intervals were assayed for their 6-phosphogluconate content (○) with commercial Gnd and $NADP^+$ at 340 nm and, for the formation of KDPG, by the DF10/LDH/NADH method at the same wavelength.

Fig.2.


Concentration of 6-PGA (o) or KDPG (●) (mM)
0
10
20
30
40
50
Time (min)
20
40
60

as estimated by the DF10 aldolase/LDH/NADH method. The material was converted to its sodium salt by ion-exchange chromatography as follows: 5 ml of water were added to the barium-KDPG and the precipitate was dissolved by the addition of Dowex 50 (hydrogen form). The resin was removed by filtration and the filtrate containing the KDPG (pH about 2) was adjusted to pH 7 with 2N NaOH. The complete removal of the barium was checked by the absence of a precipitate on the addition of a drop of the filtrate to a solution of sulphuric acid. The sodium-KDPG was stored frozen (-20°C) in 1.5 ml batches.

The criterion used for the identification of KDPG was its ability to act specifically as substrate for the KDPG-aldolase, and thus to yield pyruvate as measured both with the NADH / LDH system and with an assay dependent on the formation of the phenylhydrazone, adapted from the procedure of Dixon and Kornberg (1959).

Preparations of KDPG thus made represented an overall yield of approximately 80% of the 6-phosphogluconate originally added and were assayed as between 80-90% pure, based on the weight of barium-KDPG used.

Isolation and Properties of Mutants of *E. coli* impaired in 2-keto 3-deoxy 6-phosphogluconate aldolase (Kga) activities

1. Isolation

KDPG is an intermediate in two types of catabolic route (see Figure 1): both the catabolism of gluconate via the Entner-Doudoroff pathway, and the catabolism of hexuronic acids (Ashwell, Wahba and Hickman, 1960) involve the necessary activity of Kga. In order to

isolate mutants specifically affected in Kga, the starting organism chosen was the mutant DF1071, a derivative of an HfrC strain of E. coli K12 that already lacked 6-phosphogluconate dehydrogenase (Gnd) (Fraenkel, 1968). In this mutant, catabolism of gluconate cannot occur via the pentose-phosphate pathway and therefore must involve the activity of Kga. This organism is further useful as it grows readily in media containing either glucuronate or galacturonate as sole carbon source.

The Gnd^-,Kga^- mutants sought were obtained by treatment of strain DF1071 with ethyl methanesulphonate (Lin, Lerner and Jorgensen, 1962) and selection with penicillin (Gorini and Kaufman, 1960) in the presence of gluconate as sole carbon source. Clones were isolated that grew neither on gluconate nor on galacturonate but had retained the ability to grow on glucose. Of the mutants obtained with these characteristics, one designated DF1071-2B was selected for further study.

2. Properties of mutant DF1071-2B

The mutant DF1071-2B grew at wild-type rates on glucose, glucose 6-phosphate, lactate and glycerol (Table 1): it had thus suffered no obvious lesions in either the glycolytic or gluconeogenic pathways. However, it not only failed to grow on gluconate or galacturonate, but the addition of either of these materials to cultures growing on nutrient broth or glycerol resulted in a rapid cessation of growth (Figure 3). When cells, whose growth had thus been arrested, were harvested, suspended in 1.0 ml of water, and boiled, the solution obtained was rich in KDPG. Assay of samples of such solutions indicated

TABLE 1

Doubling times of strains K10, DF1071 and DF1071-2B of *E. coli* K12 on various carbon sources

Carbon source	Doubling time (min)		
	K10	DF1071	DF1071-2B
20 mM glucose	75	75	70
20 mM glucose 6-phosphate	63	66	70
20 mM gluconate	75	90	no growth
25 mM glycerol	100	93	105
25 mM lactate	100	100	126

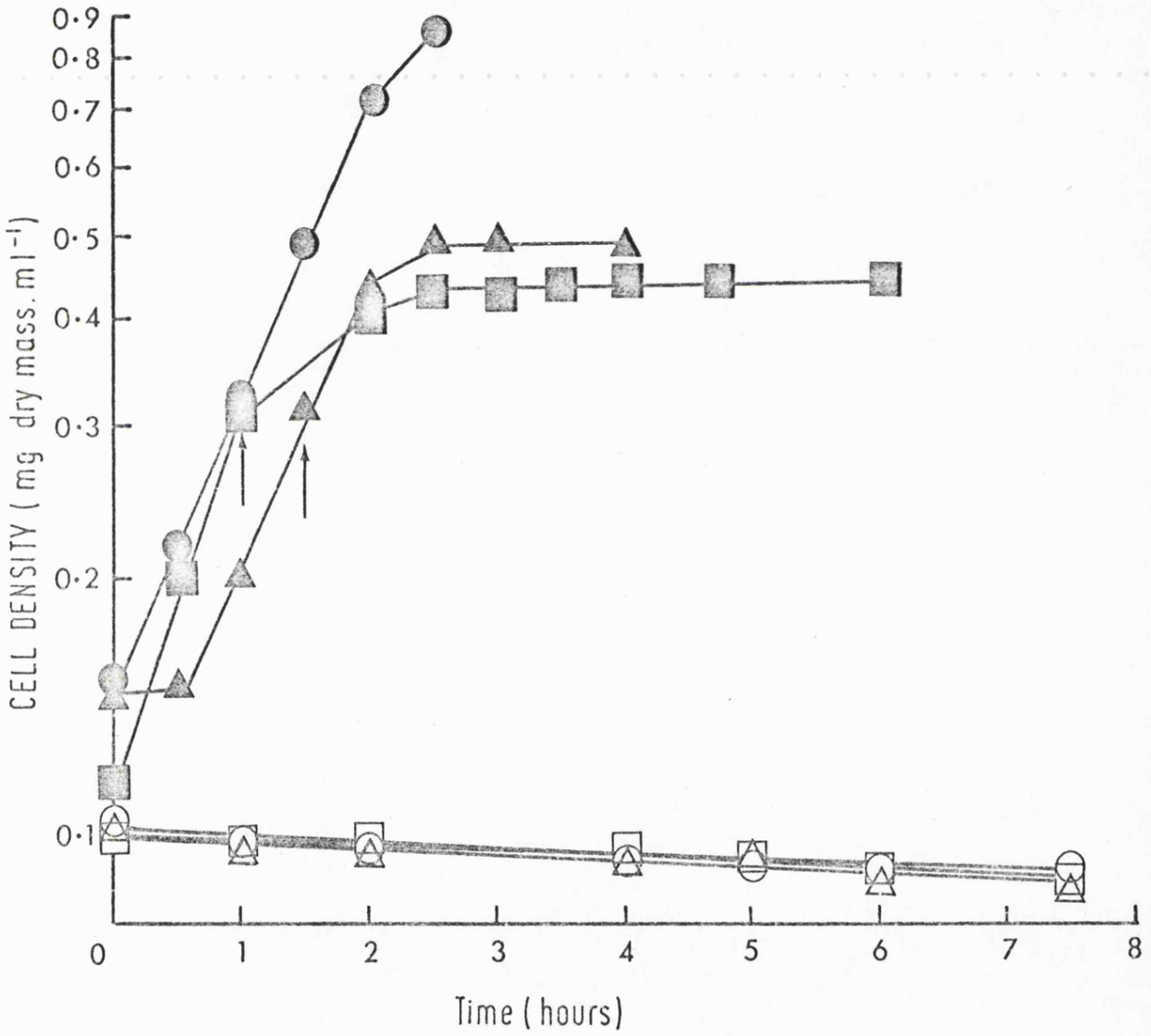
Cultures grown overnight on 25 mM glycerol were harvested and resuspended in minimal medium containing the various carbon sources.

FIGURE 3

Growth properties of E. coli strain DF1071-2B

The organism was grown overnight on minimal medium containing 25 mM glycerol as carbon source. The cells were harvested and resuspended in media containing the following carbon sources: 20 mM galacturonate (□); 20 mM glucuronate (○); 20 mM gluconate (△); double-strength nutrient broth (●); double-strength nutrient broth to which was added at the time indicated (↑) 5 mM gluconate (▲) or 5 mM galacturonate (■).

Fig.3.


CELL DENSITY (mg dry mass. ml⁻¹)
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
7
8
Time (hours)

that 3 h after the addition of 5 mM gluconate, a culture of strain DF1071-2B (18 mg dry weight total) had accumulated 0.26 μmole of KDPG; on the assumption that 1 mg dry mass of cells represents 2.7 μl of cell water (Winkler and Wilson, 1966), this is equivalent to a KDPG concentration in the cell water of approximately 4 mM. The phosphorylated ketohexonic acid was also excreted into the medium: in the 3 h incubation period, the 18 mg dry weight of mutant cells had excreted 18 μmoles of KDPG. The excretion of KDPG by similar mutants has also been reported by Pouysségur and Stoeber (1971).

The absence of Kga activity in strain DF1071-2B was confirmed by direct spectrophotometric assay. For this purpose, the substrate KDPG (which is not commercially available) was prepared as described in the Methods section. It is pertinent to note that the 6-phosphogluconate removed from the incubation mixture by extracts of strain DF1071-2B was converted quantitatively to KDPG (Figure 2); in contrast, the removal of 6-phosphogluconate by extracts of Kga^+ cells is not accompanied by a significant accumulation of KDPG. It is of further interest to note that, although the mutant DF1071-2B lacks both Gnd and Kga activities, growth on minimal media containing glucose or glucose 6-phosphate is at wild-type rates. From the metabolic pathway shown in Figure 1, it will be seen that these two carbon sources can be metabolised either via the Embden-Meyerhof Pathway or be converted to KDPG via glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydratase. Thus, one might expect some KDPG to be formed and, hence, some inhibition of growth, when this organism is grown either on glucose or glucose 6-phosphate as sole carbon source. However,

Zablotny and Fraenkel (1967) have shown that the major pathway for the metabolism of these two carbon sources is the Embden-Meyerhof Pathway. Thus, only a very small amount of KDPG will be formed and the data in Table 1 indicate that this amount is not sufficient to cause a slowing down of growth.

3. Location of the gene specifying Kga activity (kga) on the E. coli chromosome

The organisms used in this study are shown in Table 2. When the streptomycin-sensitive HfrC strain DF1071-2B was crossed with the F^- recipient K2.1t (his,arg,thr,leu,pps,str) (Brice and Kornberg, 1967), streptomycin-resistant recombinants were isolated that had lost specific amino acid requirements. Kga^- clones were detected, as clones whose growth on glycerol or nutrient agar plates was inhibited by gluconate and which did not grow on agar plates containing galacturonate or glucuronate as sole carbon sources, only among recombinants that had lost their requirement for histidine (Table 3). A 30% linkage was observed between kga and his. As expected from the known close linkage of his and gnd (Peyru and Fraenkel, 1968), all the His^+ recombinants tested were also Gnd^-.

In order to investigate further the relationship of the gnd and kga markers, one of the recombinants from this cross (designated K2.1.8.24) was purified by repeated isolation of single colonies. This organism had some of the properties of the female parent (insofar as it was Pps^- and therefore did not grow on lactate as sole carbon source, and was streptomycin-resistant), and had some of the properties

TABLE 2 Organisms used

Strain	Derivation or reference	Genetic markers	Response to streptomycin	Mating type
DF1071	Fraenkel (1968)	gnd	S	HfrC
DF1071-2B	DF1071, EMS galacturonate$^-$	gnd,kga	S	HfrC
DF10	Fraenkel (1968)	edd	S	HfrC
K2.1t	Brice and Kornberg (1967)	his,arg,thr,leu,pps	R	F$^-$
K2.1.8.24	[DF1071-2B x K2.1t] His$^+$	thr,leu,gnd,kga	R	F$^-$
AT2571	Gift from A.T. Taylor (Denver,Col., USA)	-	S	Hfr(o-pps-his)
KL96	B.J. Bachman (Yale University, Conn. USA)	-	S	Hfr(o-gnd-pps)
K2.1.24	[KL96 x K2.1.8.24] slow growth on gluconate	kga,pps,thr,leu	R	F$^-$

TABLE 3

Analysis of recombinants obtained from a cross between DF1071-2B and K2.1t

Donor	Recipient	Selected marker	Number of recombinants scored	Kga^- ($gluconate^-$, $galacturonate^-$)
DF1071-2B	K2.1t	$(Thr.Leu)^+$	70	0
		Arg^+	71	0
		His^+	78	24

of the male parent (insofar as it grew neither on gluconate nor on galacturonate, and its growth on nutrient broth was stopped by the addition of either of these materials; Figure 4). As found with strain DF1071-2B, KDPG accumulated in both intracellular water (to a concentration of about 4 mM) and in the growth medium (25 mg dry weight of cells excreted 19.2 μmoles of KDPG in 3 h). Moreover, extracts of strain K2.1.8.24 contained less than 2% of the Kga activity of strain K2.1t. The specific activity of Kga in extracts of strain K2.1t, induced by growth on nutrient broth + 5 mM gluconate, was 0.46 $\mu mole.min^{-1}.mg\ protein^{-1}$, whereas under the same conditions, extracts of strain K2.1.8.24 contained the enzyme at 0.0075 $\mu mole.min^{-1}.mg\ protein^{-1}$.

Kga^+ recombinants were obtained from genetic crosses between K2.1.8.24 and two Hfr strains of *E. coli*. Strain AT2571 injects its genome in the order o-*trp*-*pps*-*his* (Figure 5). Analysis by periodic interruption of conjugation and selection for the Pps^+ phenotype on solid media containing lactate as sole carbon source, and for Kga^+ on gluconate and on galacturonate plates, showed that Kga^+ recombinants appeared approximately 4 min after the entry of the *pps* allele (Figure 6). Strain KL96, on the other hand, injects its genome in the order o-*gnd*-*his*-*pps*-*trp*: recombinants capable of growth on galacturonate (and hence Kga^+) appeared approximately 4 min before the entry of the *pps* marker (Figure 6). Although the *his* and, therefore, the *gnd*, markers are among the earliest to be transferred by strain KL96, no colonies appeared in a selection for $gluconate^+$ $galacturonate^-$ (i.e. Gnd^+Kga^-) until colonies selected for Kga^+ were

FIGURE 4

Growth properties of the E. coli strain K2.1.8.24 (gnd,pps,kga)

The organism was grown overnight on 25 mM glycerol as carbon source. After harvesting the cells were resuspended in media containing: 20 mM gluconate (△); 20 mM galacturonate (□); 25 mM lactate (◇); or nutrient broth to which 5 mM gluconate (▲) or 5 mM galacturonate (■) was added at the times indicated (↓). L-threonine and L-leucine were present at 80 $\mu g.ml^{-1}$.

Fig 4

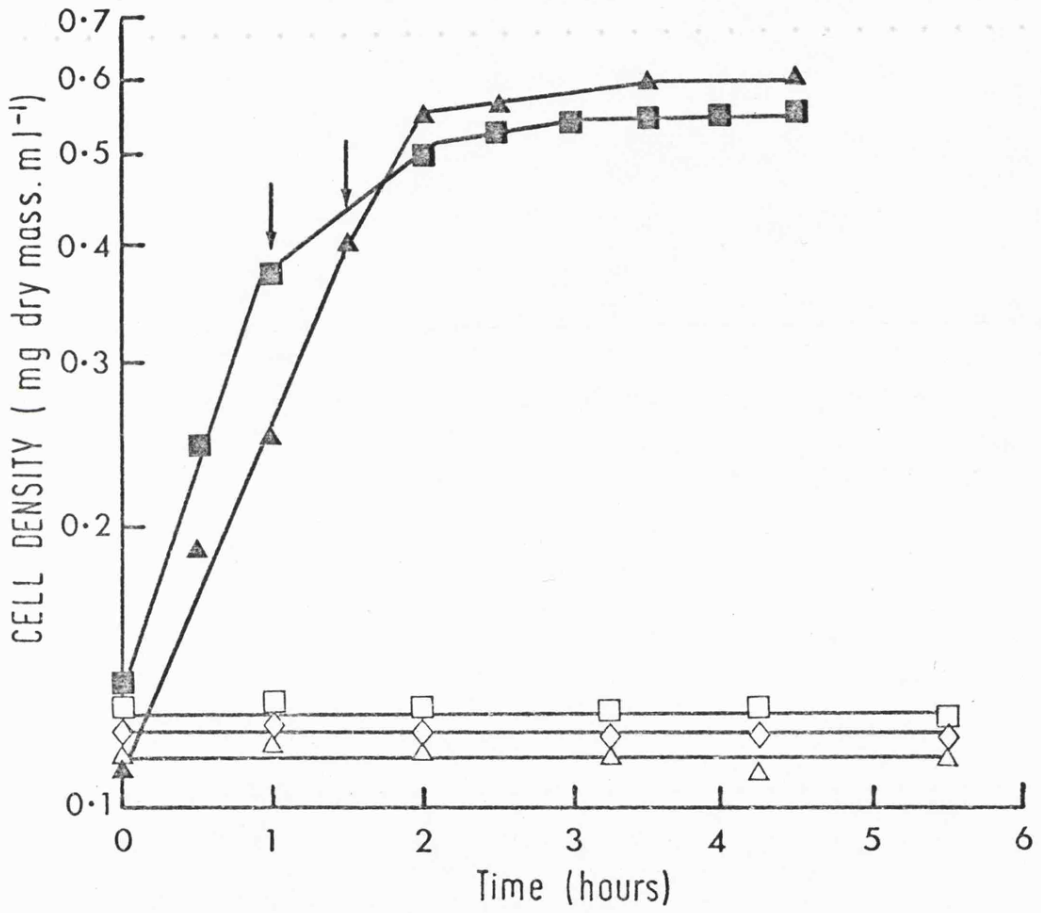

CELL DENSITY (mg dry mass. ml⁻¹)
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
Time (hours)

Fig.5.

Part of the genetic map of E.coli indicating the points of entry and directions of genome transfer of Hfr strains KL96 and AT 2571

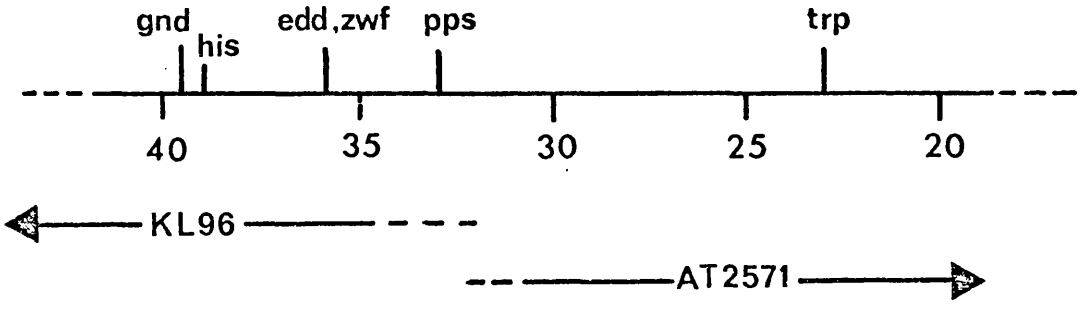
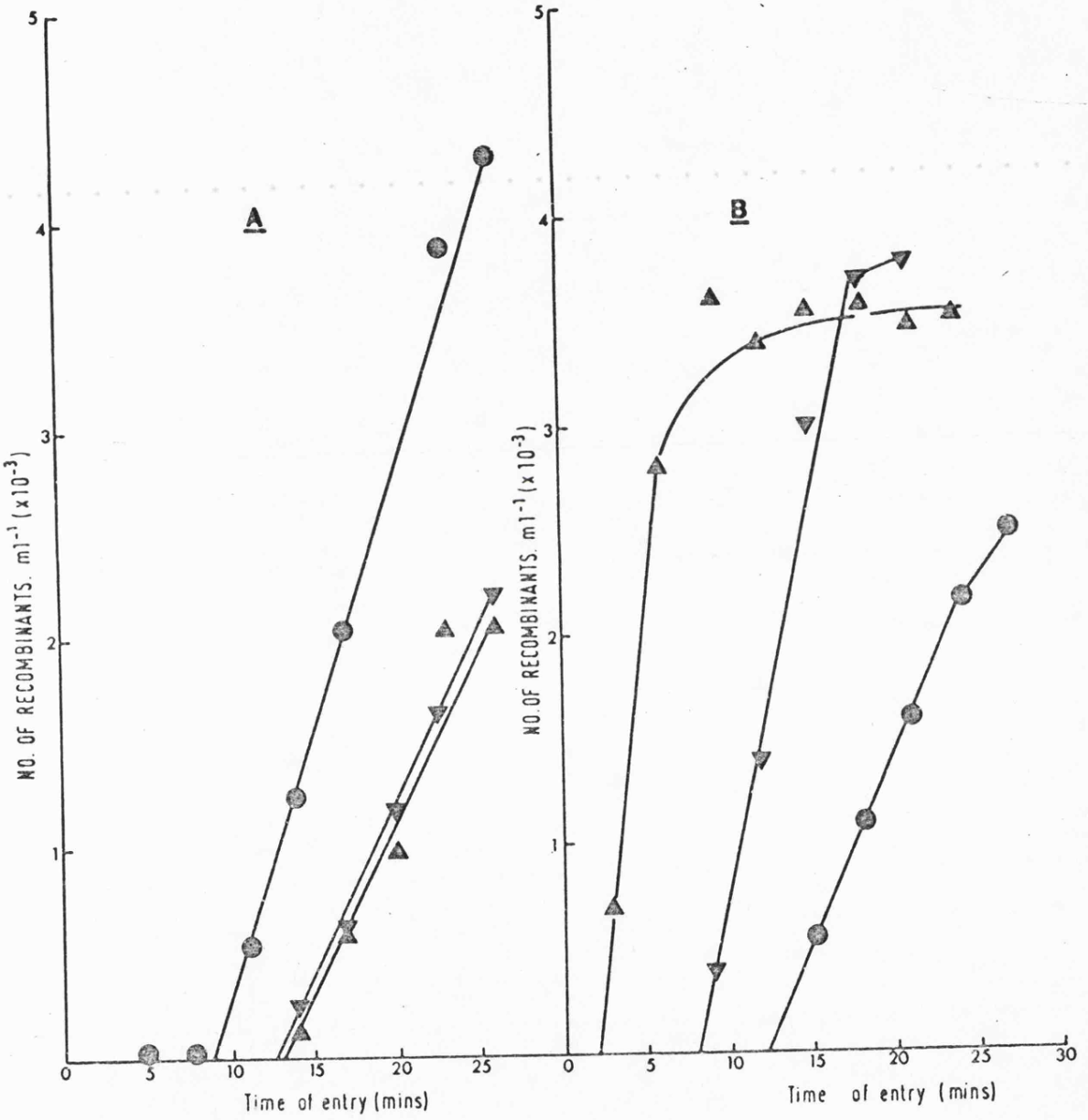


FIGURE 6

The transfer of markers to the F^- recipient K2.1.8.24 (gnd,kga,pps) from the Hfr strain AT2571 (A) and from the Hfr strain KL96 (B).

The conjugation experiments were performed at 37°C and the kinetics of transfer of the markers conferring the ability to grow on lactate (●), gluconate (▲), and galacturonate (▼) measured.

Fig.6.


A
B
NO. OF RECOMBINANTS. ml⁻¹ (x10⁻³)
Time of entry (mins)
0
5
10
15
20
25
30
1
2
3
4
5

already grown. Such growth was not a result of crossfeeding between Kga^+ and Kga^- recombinants because, as can be seen from Figure 6, a large number of Gnd^+,Kga^- recombinants were detected in samples of the mating mixture before any Kga^+ recombinants were obtained. The properties of these Gnd^+,Kga^- recombinants are discussed later in the Chapter.

The conjugation experiments described above place the kga allele very close to the genes that specify 6-phosphogluconate dehydratase (edd) and glucose 6-phosphate dehydrogenase (zwf) activities (Figure 5). This location was confirmed by analysing Kga^+ recombinants for growth on galacturonate. Such recombinants were obtained from a cross between strain DF10, which carries the edd marker (Zablotny and Fraenkel, 1967) but grows on galacturonate and is thus Kga^+, and strain K2.1.24 (one of the Gnd^+,Edd^+,Kga^- recombinants obtained from the cross between strains KL96 and K2.1.8.24 shown in Figure 6). All the 60 Kga^+ recombinants that were tested were found to be Edd^-, as evidenced by their growth as deep red colonies on agar plates containing nutrient broth, gluconate and triphenyl tetrazolium chloride (Zablotny and Fraenkel, 1967).

The location of the kga marker on the E. coli genome was established more precisely by means of phage-mediated transduction. For this purpose, phage P1-kc was grown (Brice and Kornberg, 1967) on the E. coli mutant DF10 (edd) and used to infect strain K2.1.24 (kga). Kga^+ transductants were selected on agar plates containing galacturonate. By testing the 107 transductants thus obtained for the colour of clones produced after growth on nutrient broth : gluconate : tetrazolium media, and by direct assay of extracts of a sample of the clones,

grown on nutrient broth containing 5 mM gluconate, for Edd-activity, it was found that 105 of the Kga^+ transductants had received the edd-marker of the phage donor. This close linkage of edd and kga was confirmed by transducing phage P1-kc grown on strain DF10 (edd), into the original mutant DF1071-2B (gnd,kga): again, Kga^+ transductants were selected on galacturonate. Of 1080 such Kga^+ transductants, 1038 were Edd^- whereas only 42 were Edd^+: however, all 1080 transductants had retained the Gnd^- phenotype which was assayed qualitatively according to the method of Peyru and Fraenkel (1968). These results therefore confirm both that gnd and edd are not sufficiently closely linked to be co-transducible (Zablotny and Fraenkel, 1967) and that two of the enzymes of the Entner-Doudoroff pathway, the 6-phosphogluconate dehydratase and the KDPG aldolase, are specified by genes that are over 95% co-transducible.

4. Properties of Gnd^+,Kga^- organisms

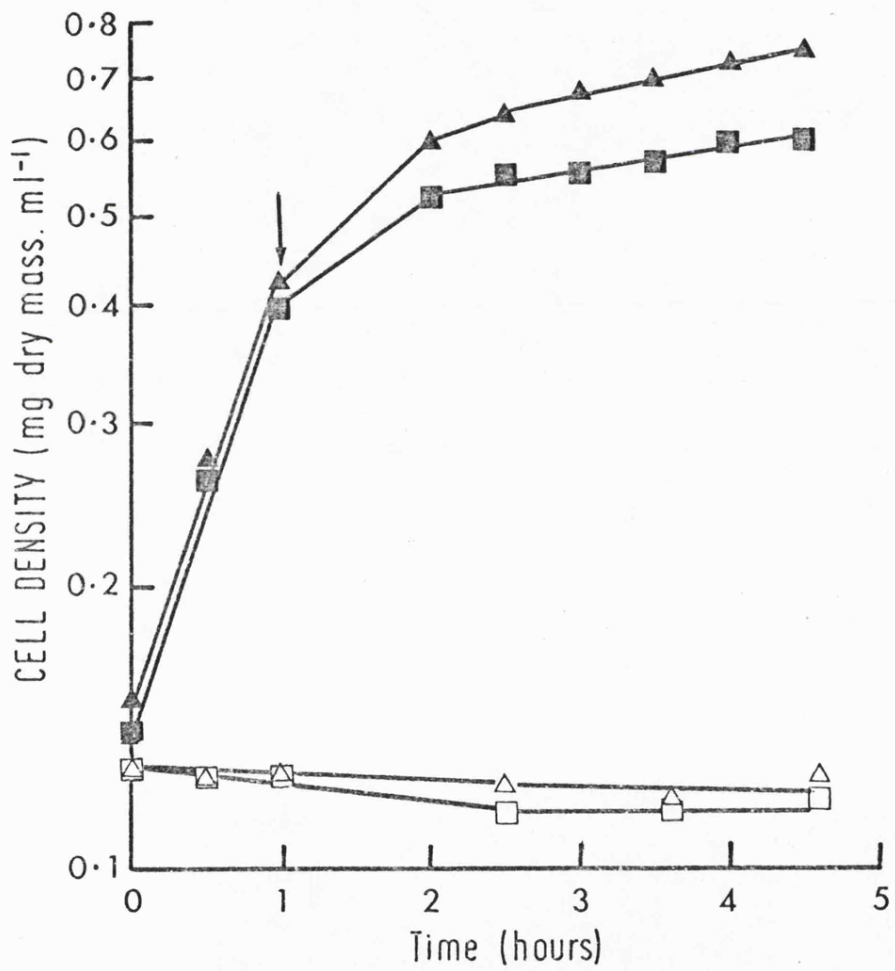
These organisms were obtained as recombinants, in a cross between strains KL96 and K2.1.8.24, as shown in Figure 6. Such organisms grew slowly on gluconate solid media but did not grow on solid media containing either galacturonate or pyruvate as sole carbon sources (therefore, they were Kga^-,Pps^-). Cultures of these organisms grew poorly in gluconate media (mean doubling-time: approx. 8 h); their growth in nutrient broth was markedly slowed down but not stopped by the addition of 5 mM gluconate or 5 mM galacturonate (Figure 7) with consequent accumulation of KDPG in cells and medium. Enzyme assays using cell-free extracts showed that they contained Gnd activity

FIGURE 7

Growth properties of the E. coli strain K2.1.24 (pps,kga)

The organism was grown overnight on 25 mM glycerol as carbon source. After harvesting, the cells were resuspended in media containing 20 mM gluconate (△), 20 mM galacturonate (□); nutrient broth to which 5 mM gluconate (▲) or 5 mM galacturonate (■) was added at the time indicated (↓). L-threonine and L-leucine were present at 80 $\mu g.ml^{-1}$.

Fig. 7.


CELL DENSITY (mg dry mass. ml⁻¹)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
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2
3
4
5
Time (hours)

but were devoid of Kga. Therefore, despite the presence of functional 6-phosphogluconate dehydrogenase, the formation and accumulation of KDPG severely restricts the utilisation of 6-phosphogluconate via the pentose-phosphate shunt. It is predicted from this explanation that Edd^- mutants that were also devoid of Kga activity would not be able to form KDPG from 6-phosphogluconate and would not show this type of inhibition of growth by added gluconate (though they are still strongly inhibited by the addition of galacturonate or glucuronate): experiments fully bear out this prediction.

When a Gnd^+,Kga^- organism is spread onto plates containing gluconate minimal medium, phenotypic revertants are easily obtained which now grow rapidly on gluconate. The mean doubling-time on gluconate (approx. 2 h) is only slightly greater than that observed with wild-type (Gnd^+,Kga^+) organisms but these phenotypic revertants do not grow on galacturonate or glucuronate (and are therefore still Kga^-). Moreover, as with other Kga^- mutants, growth on nutrient broth is arrested by the addition of 5 mM galacturonate with the consequent accumulation of KDPG in cells and medium (Figure 8). Direct enzyme assays showed that these phenotypic revertants to growth on gluconate, which are still Kga^-, have also lost Edd activity. None of the revertants obtained in this way had become Kga^+.

Thus, there appears to be a strong selective pressure in kga mutants to become also edd, perhaps to prevent the formation of even a very small amount of KDPG by the basal level of the dehydratase.

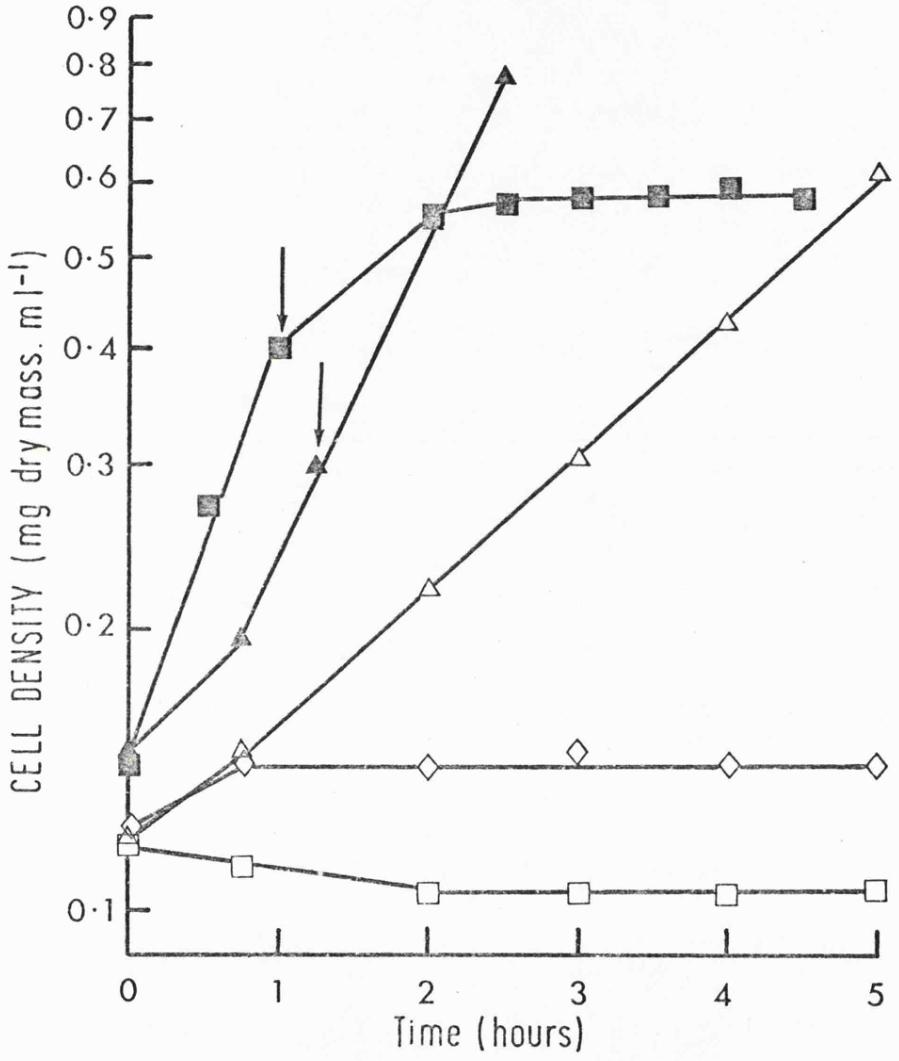
The observation that Gnd^+,Kga^- organisms are still inhibited

FIGURE 8

Growth properties of the *E. coli* strain K2.1.9.24 (edd,kga,pps)

The organism was grown overnight on 25 mM glycerol minimal medium. After harvesting, the cells were resuspended in media containing 20 mM gluconate (△); 20 mM galacturonate (□); 20 mM lactate (◇); nutrient broth to which 5 mM gluconate (▲) or 5 mM galacturonate was added at the time indicated (↓). L-threonine and L-leucine were present at 80 $\mu g.ml^{-1}$.

Fig. 8.


CELL DENSITY (mg dry mass. ml⁻¹)
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
Time (hours)

in their growth on nutrient broth (and other carbon sources, such as glycerol, glucose, succinate) on the addition of gluconate was unexpected. It must be assumed that the formation and accumulation of KDPG is toxic to E. coli cells and leads to growth stasis. The accumulation of many other phosphorylated compounds has long been noted to exert toxic effects (Englesberg and Baron, 1959; Cozzarelli et al., 1965; Böck and Neidhardt, 1966; Ferenci and Kornberg, 1973). A number of hypotheses have been put forward to explain these observations:

(a) The formation of the phosphorylated intermediate may cause a depletion of the ATP or inorganic phosphate pools in the cell and this could result in growth stasis. Bowien and Schlegel (1972), using Hydrogenomonas eutropha, measured the ATP pool in a kga mutant and found that the addition of gluconate led both to the accumulation of KDPG and a dramatic decrease in ATP content: the 'energy charge' (Atkinson, 1968) of the cells decreased from 0.8 to 0.63. These workers suggest that KDPG accumulated intracellularly does not by itself exert toxic effects but growth inhibition is observed because the conversion of gluconate to KDPG acts as an energy sink.

(b) The phosphorylated intermediate may be toxic only when present in high concentrations because it interferes with another enzyme reaction in another pathway by competing with the physiological substrate. This would apply, however, to both phosphorylated and non-phosphorylated compounds. But, as phosphorylated compounds seem to be more deleterious, perhaps this illustrates the importance of phosphorylated compounds in intermediary metabolism and the likelihood that many enzymes have affinity for a phosphate moiety.

As 6-phosphogluconate is able to be metabolised either via the Entner-Doudoroff pathway or via the pentose-phosphate shunt, the accumulation of KDPG in Kga^- mutants must restrict the utilisation of gluconate along the latter pathway. Using strain DF10, which lacks Edd activity (Zablotny and Fraenkel, 1967), the effect of KDPG on gluconate kinase and 6-phosphogluconate dehydrogenase in cell-free extracts was tested (Figure 9). Although KDPG did inhibit the ATP-dependent phosphorylation of gluconate to 6-phosphogluconate to some extent (22% using more than a ten-fold excess of KDPG), the inhibition of 6-phosphogluconate dehydrogenase was much more marked. With equal concentrations of 6-phosphogluconate and KDPG (5×10^{-5} M) a 25-30% inhibition was observed. The inhibition curve is very similar to that obtained by Brown and Wittenberger (1971) for the inhibition of 6-phosphogluconate dehydrogenase from Strep. faecalis by fructose 1,6-diphosphate. The inhibition increases with increasing KDPG to about 70% over the range of KDPG concentrations used, and is competitive. Apparently, KDPG and 6-phosphogluconate are of sufficiently similar structure to compete for the active sites on Gnd (Figure 10). The measured K_i for KDPG of 7.5×10^{-5} M is sufficiently low to compete effectively with the normal substrate 6-phosphogluconate ($K_m = 4 \times 10^{-5}$ M). This effect is the counterpart of the competition of KDPG and 6-phosphogluconate for the Kga described by Pouysségur and Stoeber (1971), though, in that example, both the K_m of the enzyme for KDPG (2×10^{-4}M) and the K_i for 6-phosphogluconate (8×10^{-4} M) are an order of magnitude higher.

FIGURE 9

The effect of KDPG on the gluconate kinase (●) and 6-phosphogluconate dehydrogenase (○) activities of E. coli strain DF10

The rate of NADPH formation from $NADP^+$ by extracts incubated with 4×10^{-5} M 6-phosphogluconate (6-phosphogluconate dehydrogenase) or 4×10^{-5} M gluconate and 4×10^{-5} M ATP in the presence of excess commercial 6-phosphogluconate dehydrogenase (gluconate kinase) was measured spectrophotometrically at 340 nm.

Fig. 9.

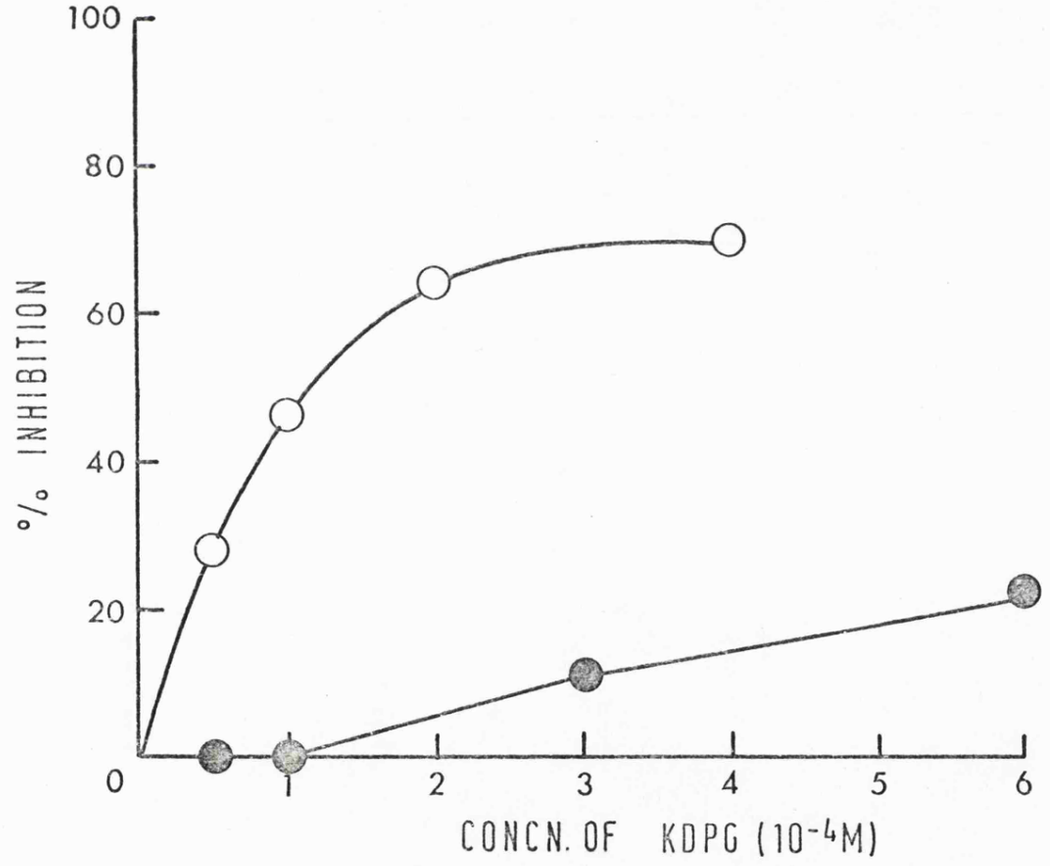


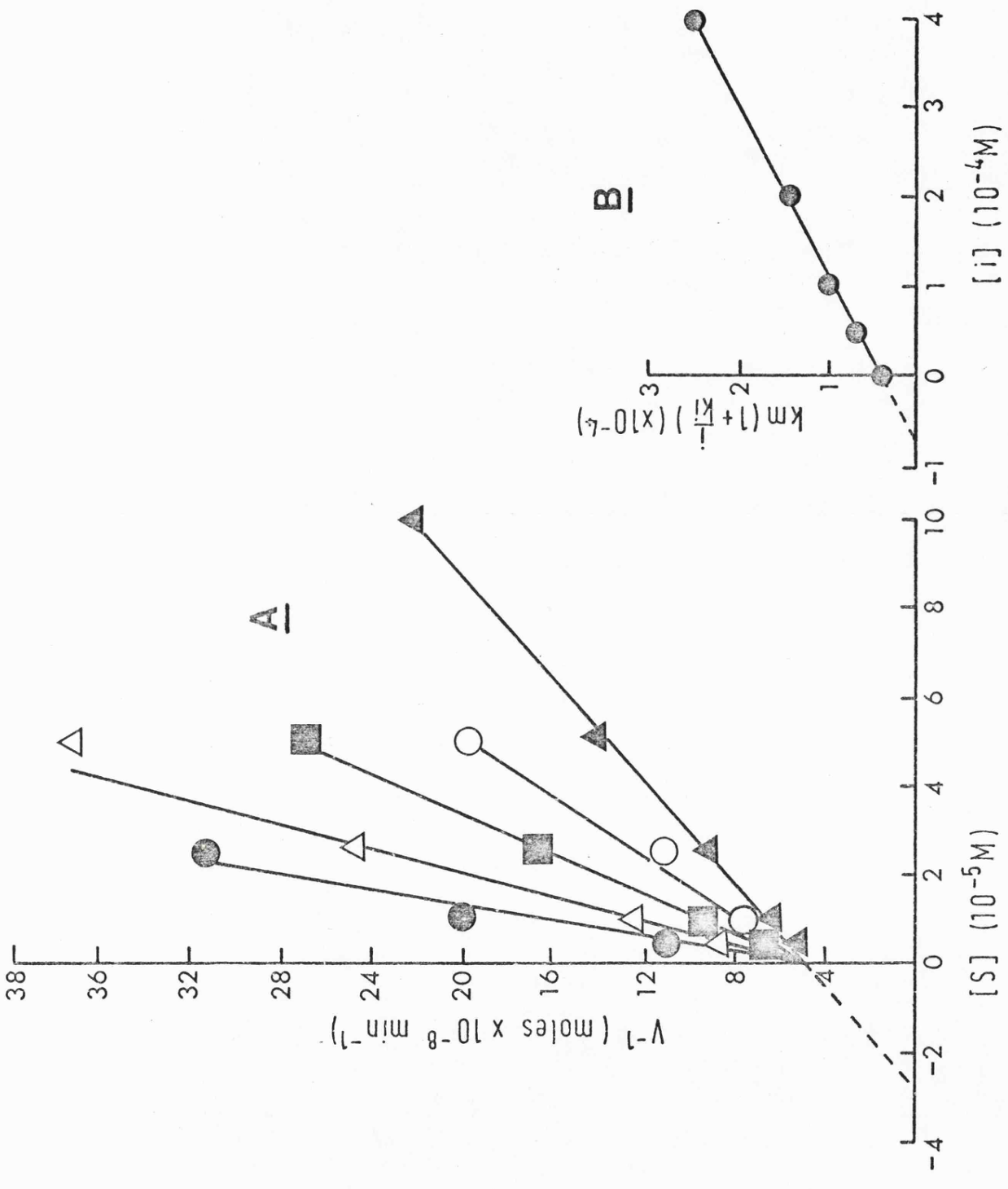
FIGURE 10

Competitive inhibition of the 6-phosphogluconate dehydrogenase activity of E. coli, strain DF10

(A) The rate of $NADP^+$ production from NADP by extracts incubated with various concentrations of 6-phosphogluconate either alone (▲) or in the presence of (○) 50μM; (□) 0.1 mM; (△) 0.2 mM; and (●) 0.4 mM KDPG was measured spectrophotometrically at 340 nm.

(B) The K_i of the inhibition of Gnd by KDPG was calculated from the data of (A).

Fig. 10.



CHAPTER II

CHAPTER II

ISOLATION AND PROPERTIES OF *E. coli* MUTANTS IMPAIRED IN GLUCONATE KINASE ACTIVITY

Introduction

A common method of obtaining mutants of *E. coli* modified in the regulation of carbohydrate metabolism or transport of its substrates is to select for resistance to an inhibitor. Buttin (1963) selected mutants on a medium containing galactose as sole carbon source in the presence of thiomethyl-β-D-galactoside, a non-metabolisable inhibitor of induction of the enzymes of galactose metabolism by galactose; the colonies that developed were mutants constitutive for the galactose enzymes. In a similar way, Englesberg *et al.* (1965) selected mutants constitutive for L-arabinose metabolism on L-arabinose as sole carbon source in the presence of D-fucose. No such non-metabolisable analogue for D-gluconate has been found.

In the previous Chapter, the toxicity of KDPG was discussed. Mutants lacking Kga activity did not grow on gluconate. Phenotypic revertants to growth on agar plates containing gluconate were found still to be Kga^- but had also lost Edd activity. The metabolism of gluconate in these revertants was now solely via the pentose-phosphate pathway. Therefore, in order to isolate mutants of *E. coli* affected in either the uptake of gluconate or in gluconate kinase, it was necessary first to construct an organism which, on the addition of gluconate, would form an intermediate of gluconate catabolism which was toxic. For this purpose, the strain K2.1.5^c.8.9, lacking

6-phosphogluconate dehydratase (Edd) and 6-phosphogluconate dehydrogenase (Gnd) activities, was made. The organisms used in this study are shown in Table 4.

Methods

(i) Gluconate uptake by whole cells Cells were washed with 50 mM potassium phosphate buffer, pH 7.0, and were resuspended in this buffer at 0.2 mg dry mass.ml^{-1}. Chloramphenicol (50 μg.ml^{-1}) was added to inhibit any de novo synthesis of proteins. The suspensions were shaken gently at 25°C. The uptake reaction was started by the addition of sodium [U-^{14}C]gluconate (3.4 Ci.mole^{-1}) to a final concentration of 0.05 mM. At appropriate times, samples (0.5 ml) were withdrawn, diluted into 2.5 ml of 50 mM potassium phosphate buffer, pH 7.0, at room temperature, filtered immediately on HA Millipore filters (0.45 μ pore size) and washed again with 2.5 ml of the same buffer. The filters were then air-dried, dissolved in 5 ml of Bray's (1960) fluid, and the radioactivity assayed with a Packard model 4000 scintillation spectrometer.

(ii) Chromatographic analysis of cell contents Cells induced with gluconate were washed twice with 50 mM potassium phosphate buffer, pH 7.0, and incubated at 1.4 mg dry mass.ml^{-1} with 0.3 mM sodium [U-^{14}C]gluconate. At various times, 1 ml of the suspensions was spun down; the pellet was washed with the phosphate buffer, resuspended in 0.5 ml of distilled water and boiled for 5 min. After centrifugation, the supernatant solution was concentrated to dryness

TABLE 4 Organisms used

Strain	Derivation or reference	Genetic markers	Response to streptomycin	Mating type
DF1070	Fraenkel (1968)	gnd edd	S	HfrC
K2.1t.5^{c}	Kornberg (1967)	his arg thr leu pps iclR	R	F^{-}
K2.1.5^{c}.8.9	(DF1070 x K2.1t.5^{c})His^{+}	arg pps edd gnd iclR	R	F^{-}
R6	K2.1.5^{c}.8.9 resistant to gluconate	arg pps edd gnd iclR	R	F^{-}

under vacuum and the residue was suspended in 0.1 ml of distilled water. Samples (10-30 µl), together with authentic sodium [U-^{14}C]-gluconate and 6-phosphogluconate, were applied to Whatman No. 1 paper and chromatographed overnight in butanol-propionic acid-water (Benson et al. 1950). The radioactive materials on the chromatogram were located by autoradiography with Kodak 'Blue Brand' X-ray film. Phosphate esters were detected by the procedure described by Steinitz (1961).

(iii) Induction of gluconate kinase, and measurement of 6-phosphogluconate formation To cultures (500 ml) of organisms growing aerobically at 37°C on nutrient broth was added 5 mM sodium gluconate when the cell density had reached 0.3 mg dry mass.ml^{-1}. Samples, of a size sufficient to contain approx 15 mg dry mass of cells, were withdrawn at known times and were centrifuged at 4°C and 20,000 g for 5 min. The 6-phosphogluconate content of samples of these supernatant solutions was assayed by incubating them at 25°C with 50 µmoles of Tris-HCl buffer, pH 8.0, 3 µmoles of $MgCl_2$, 0.25 µmole of $NADP^+$ and 25 µg of crystalline 6-phosphogluconate.dehydrogenase, in a volume of 1 ml, until no further increase in extinction at 340 nm occurred. ΔE_{340}nm of 0.622 was taken to be equivalent to 0.1 µmole of 6-phosphogluconate. This measurement represented the 6-phosphogluconate liberated by the cells into the media.

The cells were taken up in 4 ml of 10 mM potassium phosphate buffer, pH 7.5. A sample (1 ml) of this suspension was removed and was placed in boiling water for 5 min: the 6-phosphogluconate content

of the supernatant obtained from this treatment, which represented that of the cell contents, was assayed as described above. To the remaining 3 ml of cell suspensions were added a further 15 ml of phosphate buffer, the cells were collected by centrifugation, and the resultant pellet of washed cells was taken up in 4 ml of phosphate buffer supplemented with $MgCl_2$ to 1 mM. These suspensions were cooled in ice and exposed to the output of a MSE 100W sonicator for 45 sec; the virtually clear suspensions were centrifuged at 4°C and 20,000 g for 5 min and the gluconate kinase activities of the supernatant solutions thus obtained were assayed as described (Pouysségur and Stoeber, 1972).

(iv) Measurement of gluconate kinase Gluconate kinase was assayed by measuring the reduction of $NADP^+$ at 340 nm in a Unicam SP1800 recording spectrophotometer. The reaction mixture contained in 1.0 ml: 100 µmoles of Tris-HCl buffer, pH 8.0; 5 µmoles of $MgCl_2$; 0.25 µmoles of $NADP^+$; 25 µg of crystalline 6-phosphogluconate dehydrogenase; 2 µmoles of sodium gluconate; cell extract containing 100-200 µg protein. The reaction was started by the addition of 2 µmoles of ATP.

(v) Measurement of 6-phosphogluconate phosphatase activity The possible phosphatase activity of strain R6 was measured in two ways. In one procedure, crude sonic extracts of induced cells were incubated for 20 min at 25°C with 10 mM sodium 6-phosphogluconate in buffers at pH 5.5 (acetate) 7.0 (Tris-HCl) and 9.5 (ethanolamine HCl), with and without 1 mM $MgCl_2$; any phosphate released was measured by

the procedure of Fiske and Subba Row (1925). In the second procedure, a thick suspension (5 mg dry mass.ml^{-1}) of induced R6 was permeabilised by the method of Reeves and Sols (1973) and was incubated at 25°C for 30 min with 2 mM sodium 6-phosphogluconate in 50 mM Tris-HCl, pH 8.0. Samples were withdrawn at zero time and after 15 and 30 min, boiled and their 6-phosphogluconate contents were measured as described. A control incubation mixture contained all ingredients but with boiled cells.

Isolation and properties of mutants lacking 6-phosphogluconate dehydratase (Edd) and 6-phosphogluconate dehydrogenase (Gnd) activities: strain K2.1.5^{c}.8.9

It has been reported that mutants of E. coli lacking Edd activity grow much more slowly on gluconate than do wild-type organisms (155 min doubling-time compared to 56 min; Zablotny and Fraenkel, 1967). Mutants unable to grow on gluconate isolated from these Edd^{-} organisms were found also to lack Gnd activity (Fraenkel, 1968). One such double mutant, DF1070 (HfrC, gnd,edd) was kindly given by Dr. D.G. Fraenkel (Harvard Medical School, Boston, U.S.A.).

Strain K2.1.5^{c}.8.9 of E. coli K12 was prepared by crossing strain DF1070 (which injects its genome counter-clockwise from about minute 10 on the E. coli linkage map with the F^{-} recipient K2.1t.5^{c} (his,arg,thr,leu,pps,iclR,str)) (Kornberg, 1967) and selecting for His^{+} recombinants. Recombinants thus obtained were screened for their inability to grow on gluconate, and their lack of the two enzymes of 6-phosphogluconate metabolism, since it was known (Peyru and Fraenkel,

1968) that the edd and gnd markers were located at respectively minutes 36 and 39, close to his at minute 39. Edd$^-$ recombinants were recognised as forming deep red colonies on nutrient : gluconate : tetrazolium media; they grew slowly on gluconate. The double mutants (edd,gnd) were unable to grow on gluconate. The absence of Edd and Gnd activities in cultures of a number of such recombinants, designated K2.1.5^c.8.9, was confirmed spectrophotometrically.

A consequence of the double dysfunction in strain K2.1.5^c.8.9 (edd,gnd) is that the addition of gluconate to cultures growing on nutrient broth, glycerol or succinate minimal medium leads to the intracellular accumulation of 6-phosphogluconate (Table 5). As discussed previously, such accumulation of phosphorylated compounds often leads to a marked retardation or arrest of the growth of the mutant.

The toxic effects exerted by phosphorylated compounds can be overcome in any one of a number of ways:

1. The phosphorylated intermediate could be degraded as soon as it is formed by, for example, the action of a phosphatase.
2. The toxic compound itself may no longer be made - as discussed in the previous Chapter, mutants of E. coli lacking Kga activity which accumulate KDPG from gluconate, phenotypically revert to growth on gluconate-containing media by acquiring a second mutation at the edd locus. Nikaido (1961) observed that galactose-sensitive mutants of Salmonella (lacking galactose 1-phosphate uridyl transferase) accumulated galactose 1-phosphate on the addition of galactose. Mutants resistant to galactose had lost kinase activity and no longer accumulated this phosphorylated intermediate from galactose.

TABLE 5

Accumulation of 6-phosphogluconate by cultures of strain K2.1.5^{c}.8.9

Carbon source	Concentration of 6-phosphogluconate in cell water (mM)
Nutrient broth	18
Glycerol	10
Succinate	15

The organism was grown overnight on nutrient broth, 20 mM glycerol, and 20 mM succinate. The cells were resuspended in fresh medium and 5 mM gluconate was added when the cultures were growing logarithmically. L-arginine was present at 80 $\mu g.ml^{-1}$. 1.5 h later, samples containing 5 mg dry mass.ml^{-1} of cells were harvested and their 6-phosphogluconate contents assayed as described in the Methods section.

3. A precursor of the toxic intermediate may no longer be made. Englesberg and Baron (1959), using Salmonella typhosa, found that this organism was not only unable to grow on rhamnose, but that the addition of rhamnose resulted in growth inhibition. Resistant mutants lacked rhamnose isomerase and rhamnulose kinase activities.

4. The mutant may revert at the original mutational site to a wild-type genotype and thus no longer form the toxic intermediate.

Using such a rationale, strain K2.1.5^{c}.8.9 (edd,gnd), which accumulates 6-phosphogluconate from gluconate, was used in an attempt to obtain mutants impaired in the activity of the enzyme (gluconate kinase) which catalyses the ATP-dependent phosphorylation of gluconate, or in the uptake system for gluconate.

Isolation of a mutant of K2.1.5^{c}.8.9 able to grow in the presence of gluconate: isolation of mutant R6

When a culture of strain K2.1.5^{c}.8.9 is spread onto agar plates containing minimal medium and a readily utilisable carbon source such as glycerol, no growth is observed if gluconate is also present in the medium. However, after 2-3 days at 37^{o}C, 50-100 colonies appeared (for approximately 10^{8} cells plated). These were picked and tested for their ability to grow on plates containing glycerol plus gluconate and gluconate alone. Of those that grew on the former but not on the latter medium, one colony (designated R6) was purified by repeated isolation of single colonies and was used for subsequent work.

Properties of mutant R6

The mutant R6 which (apart from its tolerance of gluconate) carries the same genetic markers as its parent K2.1.5^{c}.8.9 (F^{-},*edd*, *gnd*,*pps*,*iclR*,*argH*,*str*)) was only slightly retarded, if at all, by the presence of 5 mM gluconate in its rate of growth on nutrient broth, 20 mM glycerol, or on 10 mM glucose or fructose (Table 6). However, it did not grow on gluconate alone nor did extracts of cells that had grown in the presence of gluconate contain 6-phosphogluconate dehydrogenase or 6-phosphogluconate dehydratase activities. The acquisition of the ability of strain R6 to tolerate the presence of gluconate in growth media was therefore not due to a reversion that enabled 6-phosphogluconate to be catabclised; it must have involved a change either in the process whereby gluconate enters the cells, or in the intracellular conversion of gluconate to 6-phosphogluconate.

That the latter is the case is indicated by the data of Figure 11. A washed suspension of a culture of strain R6, grown on nutrient broth in the absence of added gluconate, took up little [^{14}C]gluconate (open triangles); in contrast, a suspension of cells that had grown for approximately one generation on nutrient broth supplemented with 5 mM unlabelled gluconate took up [^{14}C]gluconate rapidly (closed circles), at about the same rate and to about the same extent as did a suspension of the parent organism K2.1.5^{c}.8.9, similarly grown (open circles). This shows that the uptake systems for gluconate are inducible and are present in both types of mutant. However, the fate of the gluconate taken up is abnormal in strain R6; whereas the addition of 5 mM unlabelled sodium gluconate to the

TABLE 6

Growth rates of the mutant R6 on various carbon sources

	Doubling-time (min)
Nutrient broth	45
" " + 5 mM gluconate	54
20 mM glycerol	75
" " + 5 mM gluconate	99
10 mM glucose	150
" " + 5 mM gluconate	180
10 mM fructose	80
" " + 5 mM gluconate	87
10 mM gluconate	no growth

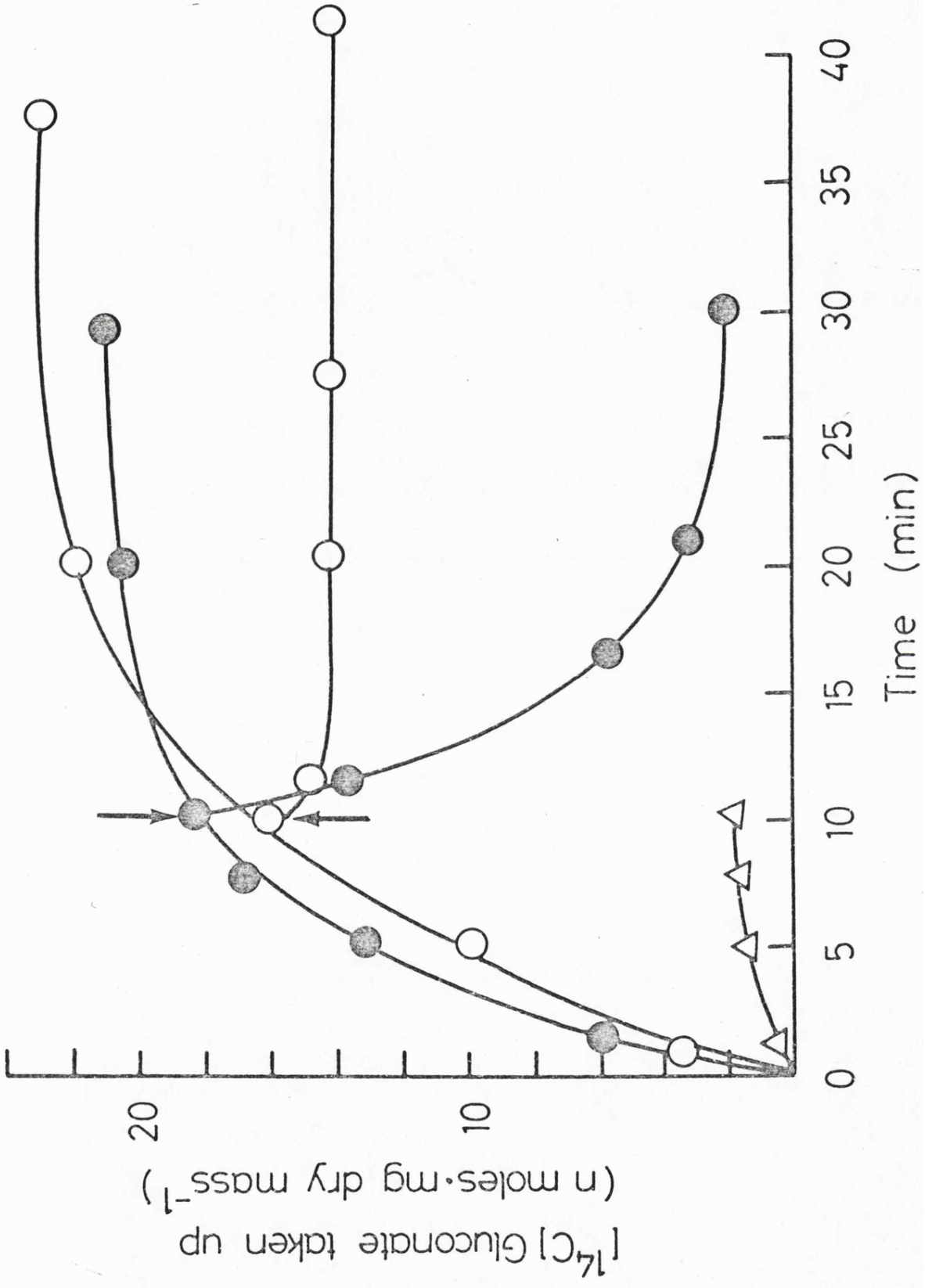
The mutant R6 was grown on 20 mM fructose overnight. The cells were harvested and resuspended in minimal media containing various carbon sources. L-arginine was present at 80 $\mu g.ml^{-1}$.

FIGURE 11

Uptake of [^{14}C]gluconate by washed suspensions of strain K2.1.5^{c}.8.9 and its mutant R6

The time course of uptake of 0.05 mM sodium [^{14}C]gluconate by washed suspensions of nutrient-grown strain R6 (△), and by strain R6 grown on nutrient in the present of 5 mM gluconate (●), was measured as described in the Methods. To a duplicate flask of strain R6 induced for gluconate uptake was added 10 mM sodium [^{12}C]gluconate after 10 min (◐). The experiments with induced cells were repeated with strain K2.1.5^{c}.8.9 (○).

Fig.11.


[14C] Gluconate taken up
(n moles·mg dry mass-1)
20
10
0
0
5
10
15
20
25
30
35
40
Time (min)

suspension of K2.1.5^{c}.8.9, incubated with 0.05 mM [^{14}C]gluconate for 10 min, removed less than 20% of the labelled material taken up, over 80% of the accumulated radioactive material was washed out of the R6 cells. The simplest explanation of these findings is that neither mutant is impaired in the ability to take up [^{14}C]gluconate, but that only strain K2.1.5^{c}.8.9 converts the gluconate taken up to 6-phosphogluconate. Further evidence for this was obtained from three types of experiment.

Firstly, cultures of the two mutants were incubated with sodium [^{14}C]gluconate and the material accumulated was analysed by chromatography (Methods, this Chapter). Over 80% of the labelled material in strain K2.1.5^{c}.8.9 co-chromatographed with authentic 6-phosphogluconate, whereas over 90% of the radioactivity present in R6 cells remained as gluconate (Figure 12). Thus, whole cells of strain R6 did not convert the gluconate taken up into 6-phosphogluconate, whereas the parent organism, K2.1.5^{c}.8.9, did.

This is also shown by the time course of induction of gluconate kinase by cultures of the two strains growing on nutrient broth and on minimal medium containing glycerol as carbon source when 5 mM gluconate was added to them. As can be seen from Figure 13, the nature of the growth substrate seems to exert a considerable influence on the induction of the kinase: both the rate at which the enzyme levels rise after addition of gluconate and the final specific activities achieved, are lower in the presence of glycerol than in that of nutrient broth. Figure 13 also shows that, under both conditions tested, strain R6 formed much less gluconate kinase than

FIGURE 12

Chromatographic analysis of material accumulating in the strain K2.1.5^{c}.8.9 and its mutant, R6

The organisms were grown on nutrient broth plus gluconate and exposed to [^{14}C]gluconate. Samples withdrawn at 0, 10, 20, and 60 min after the addition of labelled substrate were applied to Whatman No. 1 paper together with authentic 6-phosphogluconate and sodium [U-^{14}C]gluconate and chromatographed overnight. The radioactive materials were identified as described in the Methods.

Fig.12.

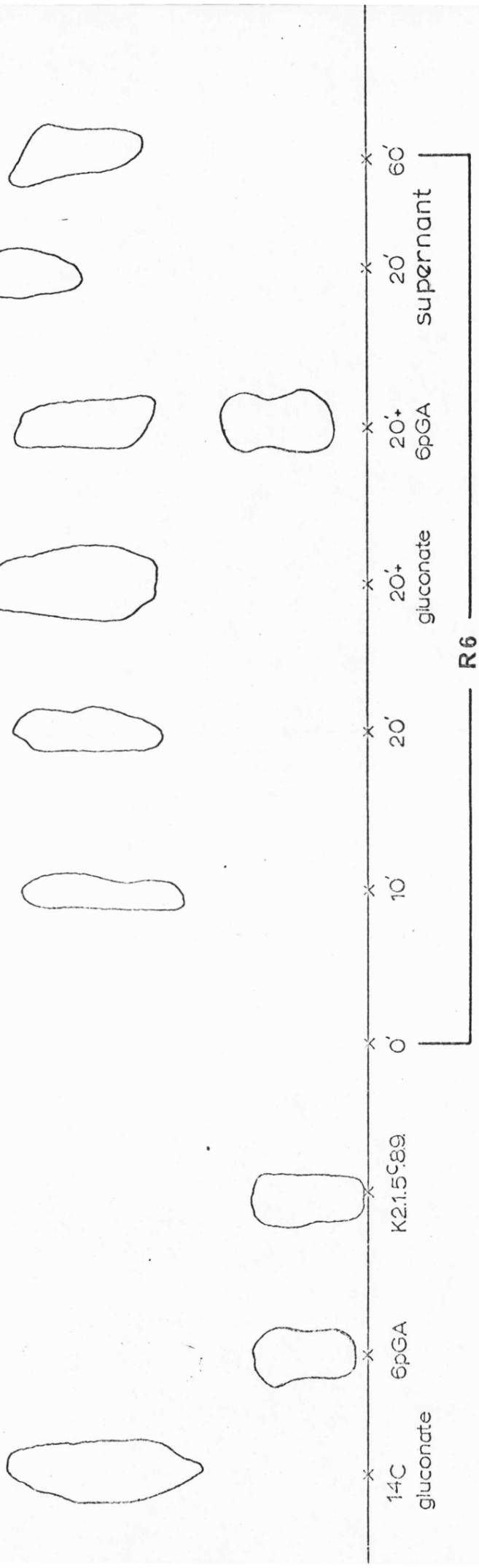
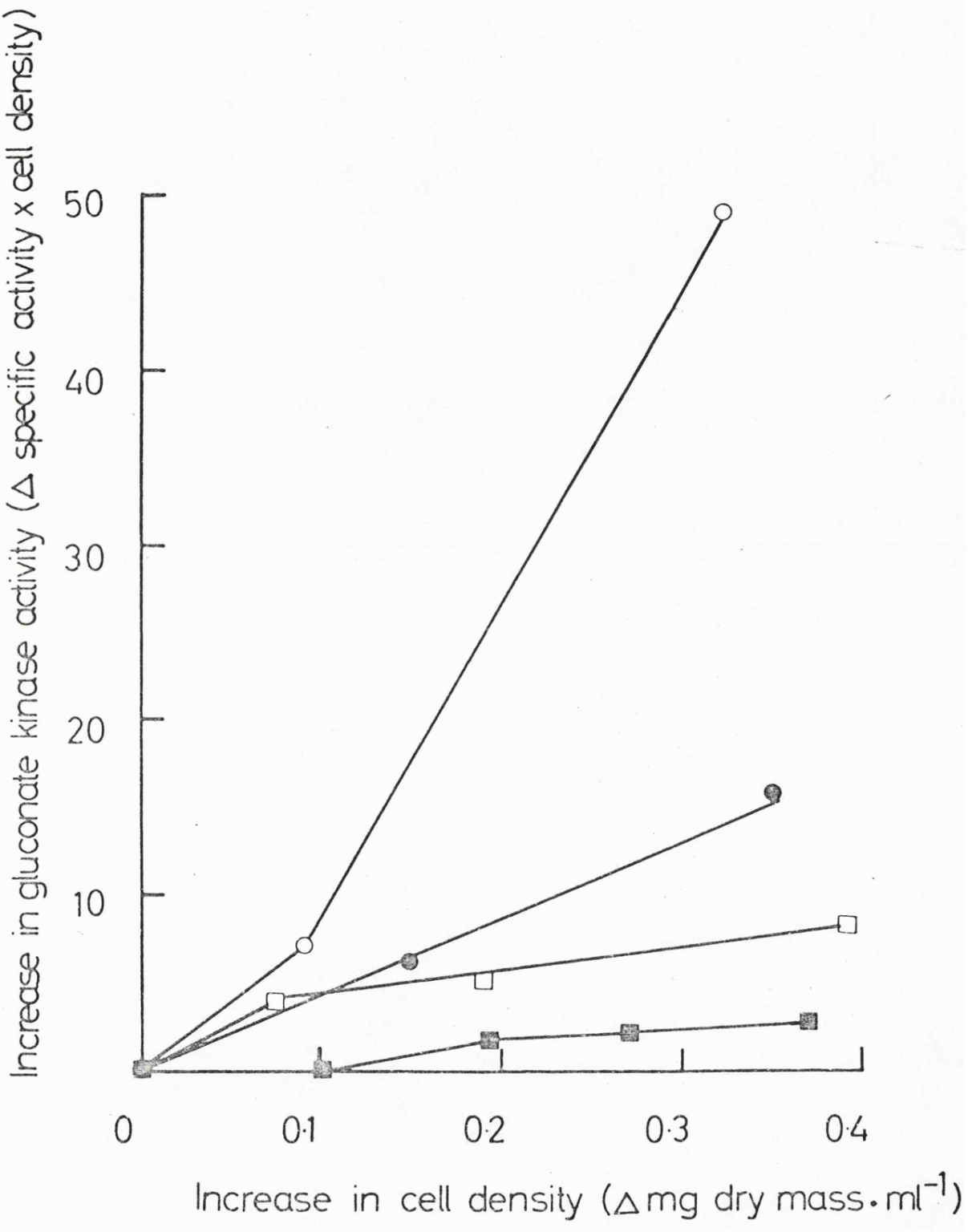

14C gluconate
6pGA
K2.1.5c89
0'
10'
20'
20' + gluconate
20' + 6pGA
20' supernant
60'
R6

FIGURE 13

Induction of gluconate kinase in cultures of strain K2.1.5^{c}.8.9 and its mutant, R6

5 mM sodium gluconate was added at zero time to cultures of strain K2.1.5^{c}.8.9 growing on nutrient broth (○) or glycerol (●), and to cultures of strain R6 growing on nutrient broth (□) or glycerol (■). The cell densities and gluconate kinase activities of samples, taken thereafter, were measured as described in the Methods. The results are plotted to illustrate the differential rates of synthesis of the enzymes (Monod, 1956).

Fig.13.


Increase in gluconate kinase activity (Δ specific activity x cell density)
50
40
30
20
10
0
0·1
0·2
0·3
0·4
Increase in cell density (Δ mg dry mass . ml⁻¹)

did its parent organism. This could suggest that the defect in strain R6 was in the gluconate kinase. Results similar to those of Figure 12 would be expected if the affinity of the gluconate kinase of strain R6 for one of its substrates, either gluconate or ATP was altered. The kinetic parameters of the gluconate kinase formed by strain R6 were, however, identical with those of the enzyme formed by the parent mutant: in both cases, the K_m for gluconate was determined as 0.11 mM and the K_m for Mg-ATP was 0.9 mM (Figures 14, 15).

In a third type of experiment, the net rates of 6-phosphogluconate formation in cultures of the two strains of mutants were measured. When 5 mM gluconate was added to cultures of strain K2.1.5^c.8.9 that were growing either on nutrient broth or on glycerol, 6-phosphogluconate rapidly appeared in the cells and, in 100 min, rose to nearly 18 mM in the presence of nutrient broth and 8 mM in the presence of glycerol (calculated on the assumption that 1 mg dry mass of E. coli represents 2.7 µl of cell water, Winkler and Wilson, 1966) (Figure 16). Surprisingly, significant amounts of 6-phosphogluconate appeared as such also in the suspending medium; the amounts that thus appeared reflected the intracellular levels of this compound (Figure 17). In contrast, the intracellular 6-phosphogluconate content of strain R6 rose to less than 7 mM and 5 mM respectively under these conditions (from an initial level of about 4 mM: this was presumably the 6-phosphogluconate formed via the glucose 6-phosphate dehydrogenase during growth) and only traces of 6-phosphogluconate appeared in the media. This latter observation also shows that the low intracellular levels of 6-phosphogluconate found with strain R6 are not due to an accelerated efflux of 6-phosphogluconate that might have been formed

FIGURE 14

Effect of gluconate concentration on the initial rate of gluconate kinase in strain K2.1.5^{c}.8.9 and its mutant, R6

The initial rates of gluconate kinase activity in crude extracts prepared from induced cultures of strain K2.1.5^{c}.8.9 (○) and strain R6 (●) were measured in the presence of 2 mM ATP and varying concentrations of gluconate by following the reduction of $NADP^{+}$ at 340 nm. The reciprocals of these rates are plotted against the reciprocals of gluconate concentrations.

Fig. 14.

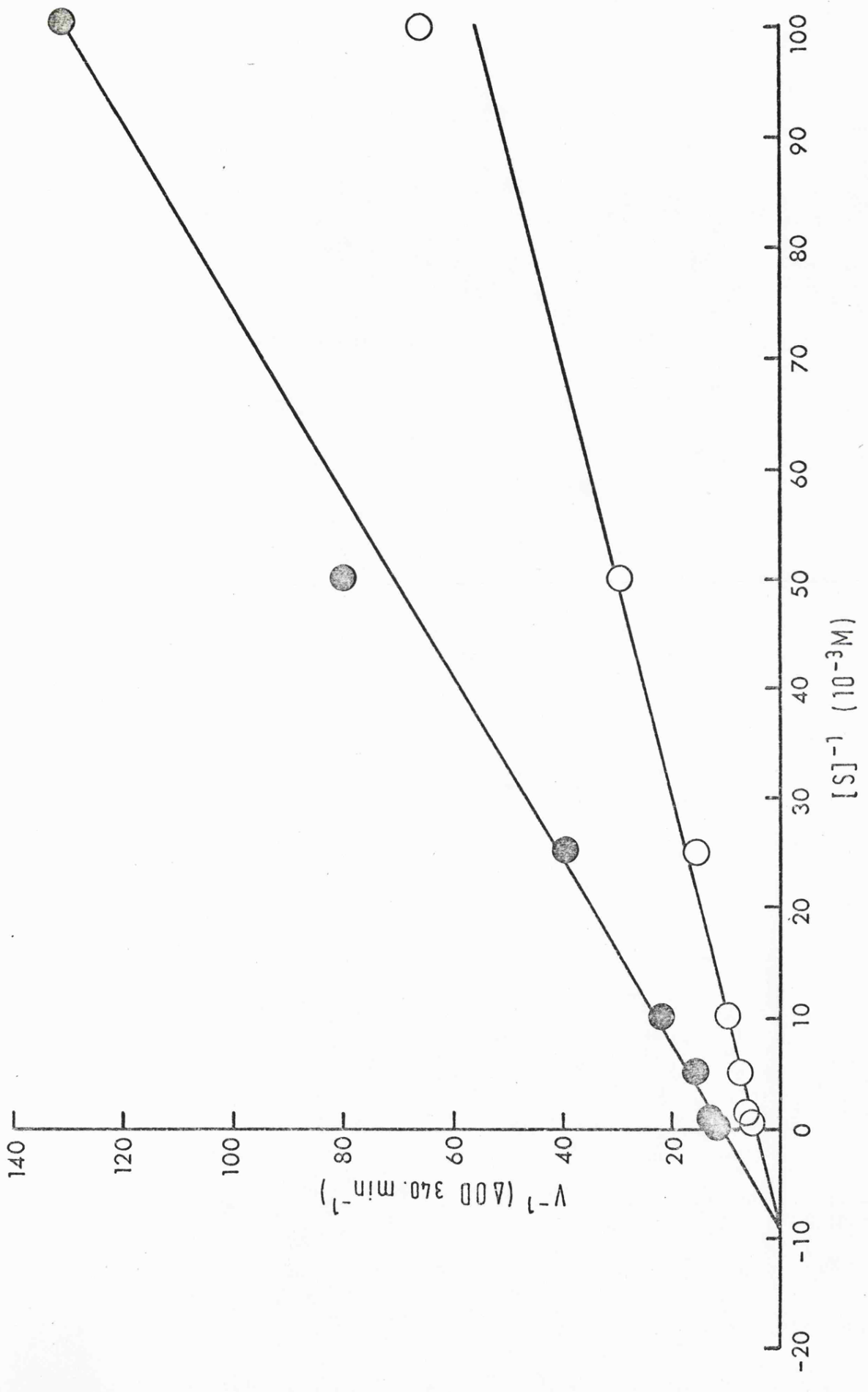

140
120
100
80
60
40
20
V^{-1} (ΔOD 340. min^{-1})
-20
-10
0
10
20
30
40
50
60
70
80
90
100
$[S]^{-1}$ (10^{-3}M)

FIGURE 15

Effect of ATP concentration on the initial rate of gluconate kinase in strain K2.1.5^{c}.8.9. and its mutant R6

The initial rates of gluconate kinase activity in crude extracts prepared from induced cultures of strain K2.1.5^{c}.8.9 (○) and strain R6 (●) were measured in the presence of 2 mM sodium gluconate and varying concentrations of ATP. The reciprocals of these rates are plotted against the reciprocals of ATP concentrations.

Fig.15.

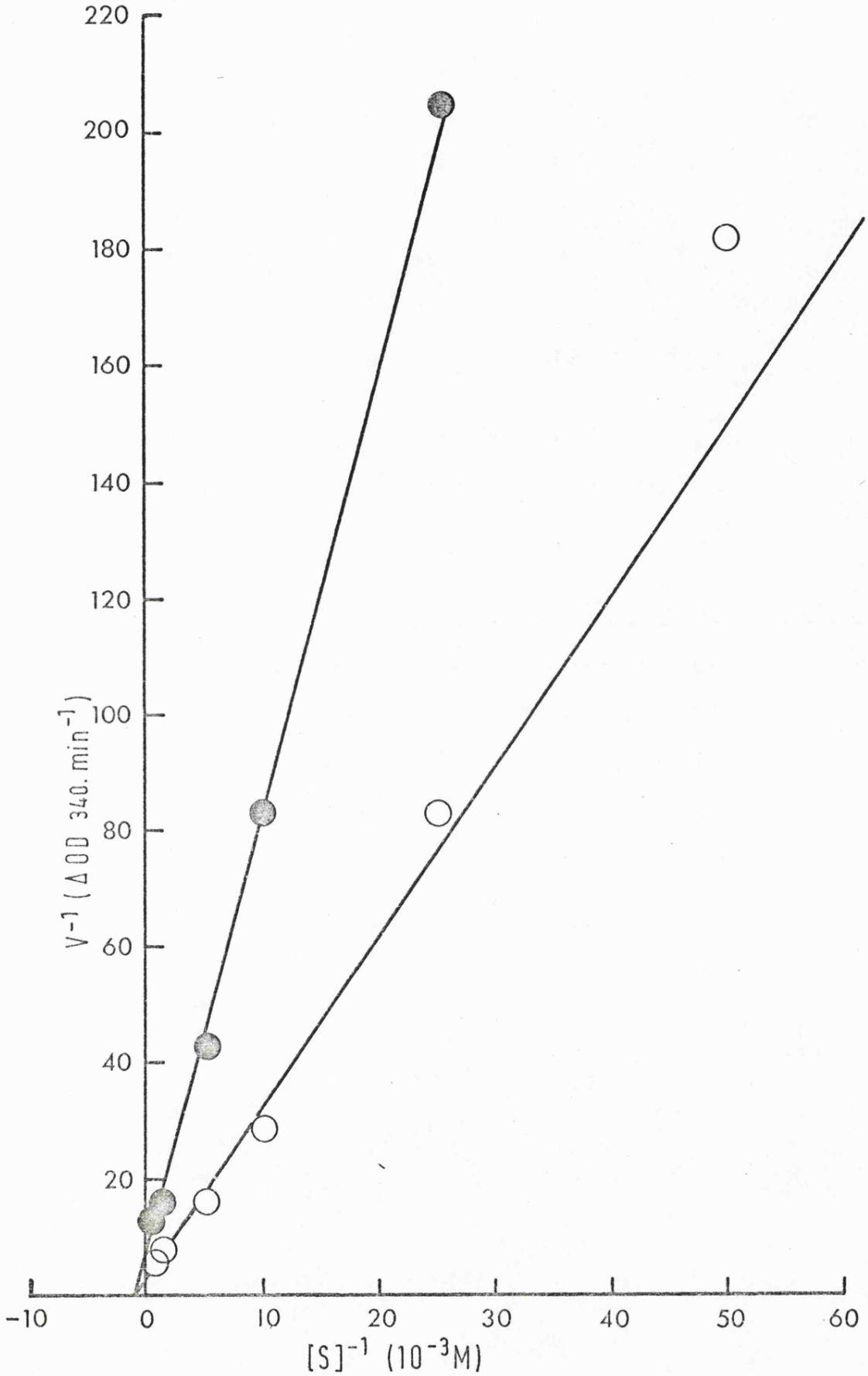
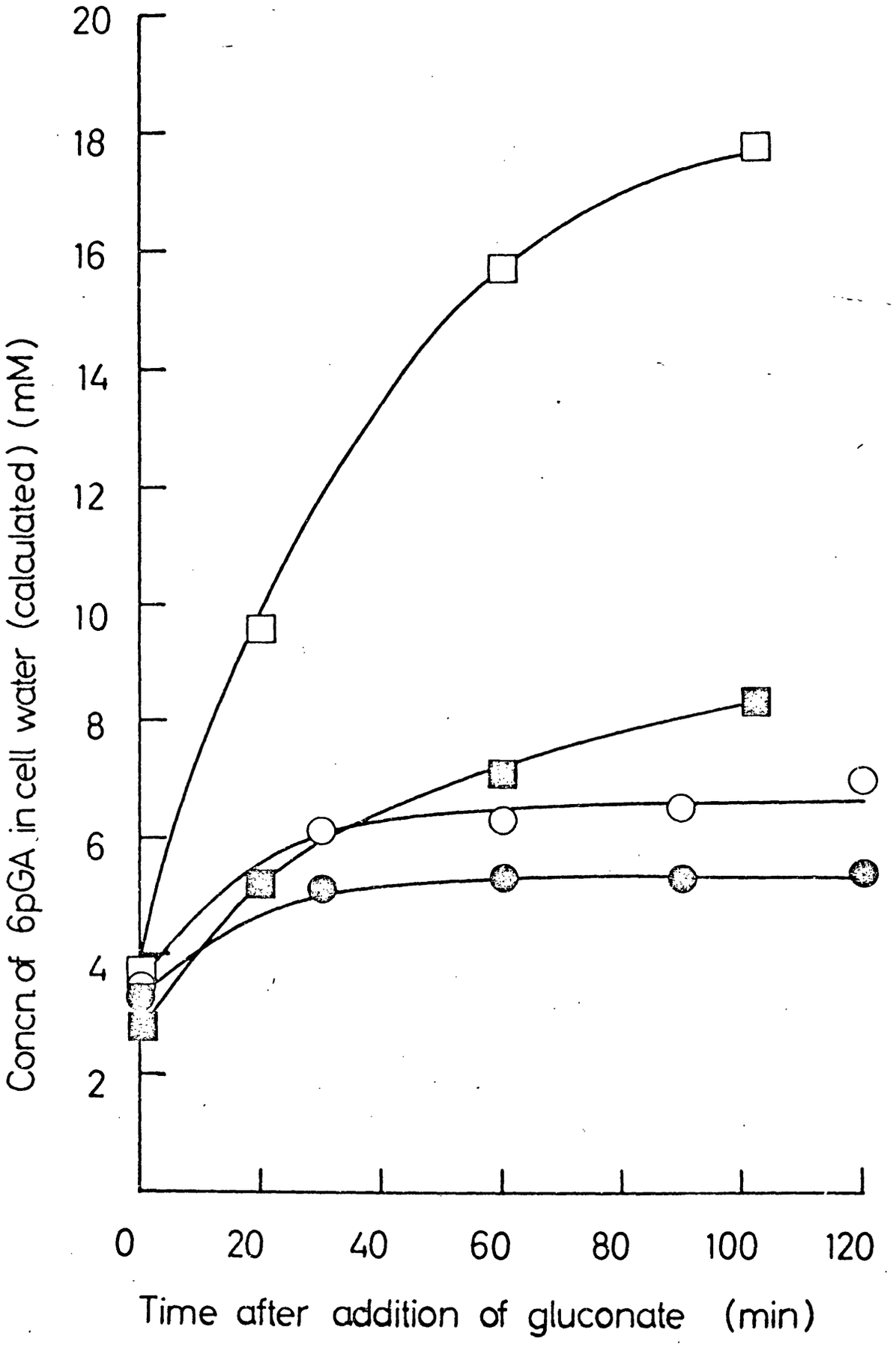

220
200
180
160
140
120
100
80
60
40
20
V⁻¹ (Δ OD 340. min⁻¹)
−10
0
10
20
30
40
50
60
[S]⁻¹ (10⁻³M)

FIGURE 16

Accumulation of 6-phosphogluconate by cultures of strain K2.1.5^{c}.8.9 and its mutant R6

5 mM sodium gluconate was added at zero time to cultures of strain K2.1.5^{c}.8.9 growing on nutrient broth (□) or glycerol (■), and to cultures of strain R6 growing on nutrient broth (○) or glycerol (●). The 6-phosphogluconate content of samples of cells, taken thereafter, was measured as described in the Methods. For the purposes of this calculation, it was assumed that 1 mg dry mass of cells contains 2.7 µl of water.

Fig.16.


20
18
16
14
12
10
8
6
4
2
Concn of 6pGA in cell water (calculated) (mM)
0
20
40
60
80
100
120
Time after addition of gluconate (min)

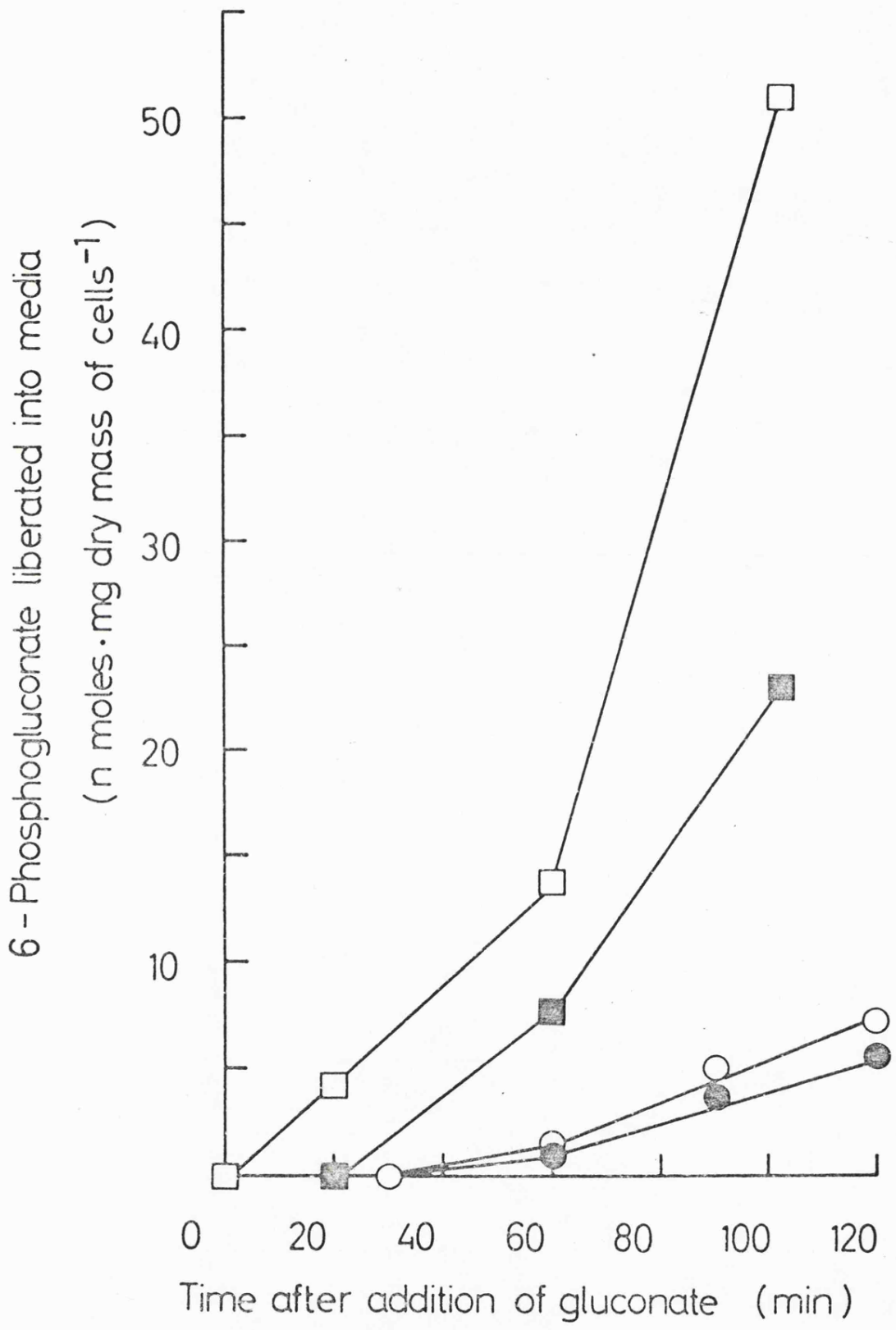
opp. 64

FIGURE 17

Appearance of 6-phosphogluconate in media after addition of gluconate to cultures of strains K2.1.5^{c}.8.9 and R6

5 mM sodium gluconate was added at zero time to cultures of strain K2.1.5^{c}.8.9 growing on nutrient broth (□) or glycerol (■), and to cultures of strain R6 growing on nutrient broth (○) or glycerol (●). Samples were taken thereafter and centrifuged; the 6-phosphogluconate content of the supernatant solutions was assayed as described in the Methods.

Fig.17.


6-Phosphogluconate liberated into media
(n moles·mg dry mass of cells^{-1})
50
40
30
20
10
0
20
40
60
80
100
120
Time after addition of gluconate (min)

at a normal rate.

It was possible that the gluconate kinase of strain R6 was more susceptible to feedback inhibition than the enzyme from the parent strain K2.1.5^{c}.8.9. 6-phosphogluconate was thought to be a possible inhibitor firstly because it was the product of the reaction under study, and, secondly, it was the accumulation of this intermediate which was causing the toxicity. The formation of a small amount of 6-phosphogluconate as a result of the action of the gluconate kinase in strain R6 might be sufficient to inhibit any further activity of the kinase. Such an inhibition may not necessarily be obvious when the enzyme is measured in the standard way: the reduction of $NADP^{+}$ at 340 nm by extracts of *E. coli* on the addition of gluconate, ATP, and commercial 6-phosphogluconate dehydrogenase.

$$\text{gluconate} + \text{ATP} \longrightarrow \text{6-pGA} + \text{ADP}$$

$$NADP^{+} + \text{6-pGA} \xrightarrow[\text{dehydrogenase}]{\text{6-phosphogluconate}} \text{NADPH} + \text{ribulose 5-P} + CO_2$$

Any 6-phosphogluconate formed is removed immediately and therefore inhibition may not be observed.

An alternative method of assaying the enzyme was by a modification of the pyruvate kinase assay of Malcovati and Kornberg (1969):

$$\text{gluconate} + \text{ATP} \xrightarrow[\text{kinase}]{\text{gluconate}} \text{6-pGA} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{Lactate} + \text{NAD}^+$$

The activity of gluconate kinase could then be followed by measuring the rate of oxidation of NADH to NAD^+ at 340 nm. The advantage of this second method for assaying gluconate kinase is that any 6-phosphogluconate formed is not removed. The presence of high NADH oxidase activity in the extracts used required the partial purification of the gluconate kinase: upon ammonium sulphate fractionation, the majority of the gluconate kinase activity was in the fraction which precipitated at 55-70% ammonium sulphate saturation. Yields were usually of the order of 50% of the gluconate kinase activity present in the crude extracts used.

Figure 18 shows the activity of gluconate kinase remaining when the concentration of 6-phosphogluconate included in the reaction mixture is varied. As can be seen, although 6-phosphogluconate inhibits the gluconate kinase present in strain R6, K2.1.5^c.8.9 and K2.1t (a wild-type strain included for comparison), firstly a high concentration of 6-phosphogluconate is required to observe inhibition of activity (5-10 mM produces a 50% inhibition of activity), and, secondly, the inhibition profiles are similar enough not to provide an explanation of the tolerance of strain R6 to gluconate in terms of

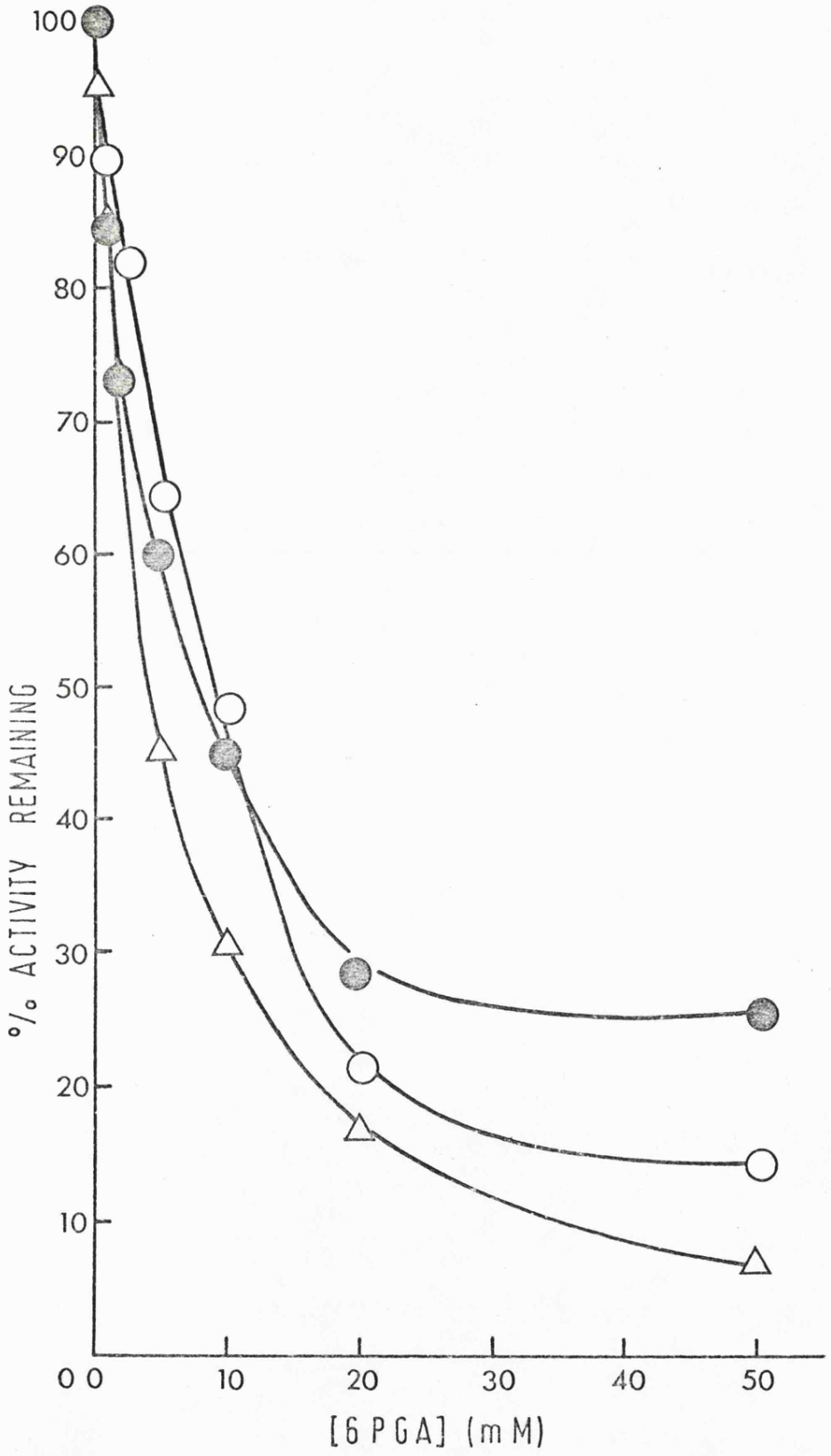
opp. 67

FIGURE 18

Effect of 6-phosphogluconate on the activity of the gluconate kinase from the E. coli strains K2.1t, K2.1.5^{c}.8.9 and R6

Ammonium sulphate fractionations were performed on crude extracts prepared from cells grown on nutrient broth plus gluconate. The gluconate kinase activities were measured in the strains K2.1t (Δ), K2.1.5^{c}.8.9 (O), and R6 (●), and in the presence of 1, 2, 5, 10, 20 and 50 mM 6-phosphogluconate by a modification of the pyruvate kinase assay.

Fig.18.


100
90
80
70
60
50
40
30
20
10
0 0
10
20
30
40
50
% ACTIVITY REMAINING
[6 P G A] (m M)

product inhibition by 6-phosphogluconate of the gluconate kinase present in this strain.

It is conceivable that the 6-phosphogluconate formed from the gluconate taken up by strain R6 might be rapidly hydrolysed again to gluconate which might be retained. This was tested for in two ways: firstly, crude cell-free extracts of strain R6 were obtained from cells induced on nutrient broth containing 10 mM gluconate and tested for phosphatase activity specific to 6-phosphogluconate according to the procedure of Fiske and Subba Row (1925). The results were negative. Secondly, cells of strain R6 were made permeable to external 6-phosphogluconate by the procedure of Reeves and Sols (1973). Table 7 shows that there is no appreciable loss of 6-phosphogluconate upon incubation with cells treated in this way or with boiled cells. Although there is no direct evidence against the view that 6-phosphogluconate is formed from gluconate in strain R6, but is hydrolysed again to gluconate, the results obtained show that any such phosphatase activity is far too low to explain the observed tolerance of strain R6 to gluconate in its media. It is thus likely that strain R6 differs in its ability to convert the gluconate that has been taken up by the cells to 6-phosphogluconate. Since it takes up labelled gluconate at a normal rate and retains it predominantly as such (Figure 11), strain R6 is very well suited to a study of the kinetics of the uptake process. This will be discussed in Chapter III.

The observation that both the rate of induction and the final level of gluconate kinase in cultures of strain K2.1.5^{c}.8.9 grown on glycerol in the presence of gluconate are lower than in

TABLE 7

6-phosphogluconate phosphatase activity in washed cell suspensions of strain R6

Time of sampling (min)	Unboiled	Boiled
	nmoles of 6-pGA present	
0	93.2	91
15	86	88.5
30	90	91
60	90	87

0.1 µmole of 6-phosphogluconate was added to a washed cell suspension of strain R6 grown on nutrient broth plus gluconate and made permeable to 6-phosphogluconate.

Samples removed from the mixture incubating at 37°C at 0, 15, 30, and 60 min after the addition of 6-phosphogluconate were assayed for their 6-phosphogluconate content. A control experiment with boiled cells was performed.

TABLE 8

Specific activities of gluconate kinase in strain K2.1.5^{c}.8.9

Carbon source plus 5 mM gluconate	*Specific activity of gluconate kinase
Nutrient broth	43
Glycerol	8.3
Fructose	101
G 6-P	93.5
Galacturonate	48.4
Malate	38.7
Ribose	35

* Specific activity = nmoles.min^{-1}.mg protein^{-1}

The reduction of $NADP^{+}$ was measured at 340 nm by extracts of strain K2.1.5^{c}.8.9 prepared from cultures previously grown on different carbon sources and induced with 5 mM gluconate for 1.5 generations.

similar cells grown on nutrient broth plus gluconate (Figure 13) led to an investigation of the effect of other carbon sources on the induction of gluconate kinase in this organism. Table 8, which shows the specific activities of gluconate kinase obtained when strain K2.1.5^{c}.8.9 is grown on different carbon sources and induced with 5 mM gluconate for 1½ generations, suggests that the effect is specific for glycerol. Since cells grown on fructose or glucose 6-phosphate and induced with gluconate contain high activities of gluconate kinase, it is unlikely that the triosephosphates, dihydroxyacetone phosphate or glyceraldehyde 3-phosphate are responsible for this effect. The only compounds which are specific to glycerol metabolism and could fulfil such a role are glycerol itself, or α-glycerophosphate. Further studies are required to establish more precisely the nature of this interesting effect since, in general, glycerol exerts little catabolite repression on the enzymes of carbohydrate metabolism (Magasanik, 1961).

(vi) Location of the lesion in the mutant R6 on the *E. coli* chromosome The strain R6 was originally recognised by virtue of its tolerance to the presence of gluconate on agar plates containing glycerol as carbon source. Initial attempts to map the lesion present in strain R6 involved the restoration of sensitivity to gluconate on glycerol plates using various Hfr strains of *E. coli*. Such studies placed the gene coding for the tolerance of strain R6 to gluconate in the vicinity of the *arg*(H-E) area on

the *E. coli* chromosome.

If the mutation in strain R6 does, in fact, lead to an impairment of gluconate kinase activity, then one would expect the strain R6 in which Edd and Gnd activities have been restored to be unable to grow on gluconate minimal media. Experiments bear out this prediction: strain R6 Edd^+ Gnd^+ is unable to grow normally on media containing gluconate as sole carbon source. This observation enabled the gene coding for the mutation in strain R6 to be located more precisely. It has been found that a gene 14% cotransducible with the gene coding for the enzyme fructose diphosphatase (*fdp*; 85 min) when transferred to an organism which grows normally on gluconate minimal media, leads to an inability of this organism to grow on agar plates containing gluconate as sole carbon source. But these same transductants grow in liquid media containing gluconate as carbon source, suggesting that the original R6 mutant, in becoming tolerant to gluconate, has acquired more than one mutation. Investigations in this area are being continued.

CHAPTER III

CHAPTER III

THE UPTAKE OF GLUCONATE

The first step in the microbial utilisation of a nutrient is its passage from the external medium into the cell. There are three main ways in which this is achieved in E. coli (Kornberg, 1973a,b).

1. Facilitated Diffusion The main features of this system are that the substance is equilibrated across the cell membrane by means of specific membrane carriers; no metabolic energy is required and the substance is chemically unchanged initially inside the cell. Glycerol (Sanno et al., 1968), pyruvate (Kornberg and Smith, 1967), and acetate (Brown and Kornberg, to be published), all appear to enter the cell by this system.

2. Active transport This is an energy-dependent process and the substance taken up can be accumulated to much higher concentrations than is present in the external medium. Again, the transported material appears chemically unchanged inside the cell. One consequence of the linkage of transport to the expenditure of metabolic energy is that agents that uncouple oxidative phosphorylation also abolish transport. Active transport is used by E. coli for the uptake of amino acids and a number of carbohydrates. For example, lactose (Cohen and Rickenberg, 1955; Rickenberg et al., 1956), melibiose (Prestige and Pardee, 1965), maltose (Doudoroff et al., 1949) Wiesmeyer and Cohn, 1960), galactose (Rotman, 1959; Rotman et al., 1968), arabinose (Novotny and Englesberg, 1966) and hexose phosphates (Winkler, 1966;

Kornberg and Smith, 1969; Dietz, 1972), are all taken up by E. coli by an active transport mechanism.

3. Group translocation This type of uptake mechanism differs from those mentioned above in that the substance appears inside the cell as its phosphorylated derivative. A phosphotransferase system for the uptake of certain carbohydrates was first described by Kundig, Ghosh and Roseman (1964). The required energy is supplied by the high energy compound phosphoenolpyruvate. Consequently, the non-availability of phosphoenolpyruvate results in the inhibition of transport.

The uptake of gluconate

The first step in the utilisation of gluconate by E. coli is thus its entry from the medium into the cells. Since mutants devoid of components of the phosphotransferase system (Kundig et al., 1964) grow readily on gluconate, this system plays no necessary role in gluconate transport.

The entry of gluconate, taken up as such by the cells, into metabolic pathways requires the action of a kinase that catalyses its intracellular conversion to 6-phosphogluconate with concomitant formation of ADP from ATP. The uptake process and the phosphorylation step that follows it, are catalysed by highly specific proteins that are inducibly formed after exposure of E. coli to gluconate.

Studies of the kinetics and other parameters that affect the uptake of carbohydrates by E. coli are usually performed by exposing suspensions of suitably induced cells to isotopically labelled substrate

and measuring the appearance of isotope in the cells after rapid filtration. However, data obtained in this way measure not only the uptake process, but the net accumulation of isotopic products as a result of the uptake of the substrate, the efflux of the substrate into the medium, the incorporation into cell components of derivatives of that substrate and the loss of isotopic material through its oxidation.

Two techniques have been employed to overcome these difficulties. Where a non-catabolizable substrate analogue is available, that can be taken up by induced cells but cannot be converted to cell components, a valid measure of the kinetics of the uptake process can be readily obtained. Rickenberg et al. (1956) used methyl[^{35}S]thiogalactoside as such an analogue of lactose. Where a non-catabolizable substrate analogue is not available, it may be possible to isolate a mutant that lacks the second step in substrate utilisation. Horecker et al. (1960) used a mutant devoid of galactokinase activity in their studies of galactose uptake. They found that the initial rate at which isotopically-labelled galactose was taken up by the mutant was identical with that at which it was taken up by the wild-type cells, although galactose accumulated only in the mutant. This led the authors to suggest that permeation is the rate-limiting step in galactose utilisation. Similarly, Rickenberg et al. (1956) studied the rate of hydrolysis of the lactose analogue ortho-nitrophenylgalactoside (ONPG) by intact lactose-grown E. coli and by such cells after they had been treated with toluene (where the cell membrane had been disrupted). They found that, under

the latter conditions, the rate of ortho-nitrophenyl formation is increased by at least a factor of ten and therefore suggests that the passage of ONPG across the membrane was the rate-limiting step in the overall rate of hydrolysis of that substrate.

In the previous Chapter, the properties of the mutant R6 were discussed. This organism is unimpaired in its ability to take up inducibly [^{14}C]gluconate but is greatly impaired in its ability to convert the gluconate thus taken up to 6-phosphogluconate. It is therefore analogous to the galactokinaseless mutant described by Horecker *et al.* (1960) and was used to study the kinetic and energetic parameters that affect the uptake of gluconate in *E. coli*. Evidence is presented in this Chapter that the kinetic parameters measured with R6 are not significantly different from those determined with wild-type cells. As gluconate does not accumulate in wild-type cells, it can be concluded that the active transport of gluconate is also the rate-limiting step in gluconate utilisation.

METHODS

Cultures were grown as described under Materials and Methods and induced for gluconate metabolism by the addition of 10 mM sodium gluconate to growing cells. Additional methods are described below.

Osmotic shock

Cells harvested in the logarithmic growth phase (0.5 - 0.65 mg dry mass.ml^{-1}) were subjected to the osmotic cold-shock procedure described by Heppel (1969). After three washings with 30 mM Tris-HCl

buffer, pH 7.3, the cells were gently agitated for 15 min with 80 vol of the same buffer containing 20% (w/v) sucrose and 0.1 mM EDTA, collected by centrifugation, and 'cold-shocked'. This procedure involved the rapid dispersion of the cells by vigorous stirring in 80 vol of ice-cold 0.1 mM $MgCl_2$. After centrifugation, the supernatant (the shock fluid) was removed and the cells were resuspended in 50 mM potassium phosphate buffer, pH 7.0, at a concentration of 2 mg dry mass. ml^{-1} and used within 2 h of the cold-shock treatment.

Preparation of membrane vesicles

The strain R6 was grown on nutrient broth in the presence of 10 mM sodium gluconate. Spheroplasts were prepared by the lysozyme-EDTA method described by Kaback (1971) with only minor alterations: potassium EDTA, pH 7.0, was added to a final concentration of 5 mM, after 15 min lysozyme (Sigma; crystalline) was added to a final concentration of 50 µg/ml and the suspension was incubated for 30 min at room temperature. Membrane vesicles were stored in 0.1 M potassium phosphate buffer, pH 6.6, at a concentration of 4-6 $mg.ml^{-1}$ under liquid nitrogen. They were thawed at room temperature when required.

Gluconate uptake by whole cells and vesicles

Gluconate uptake by whole cells was measured as described in the previous section.

Vesicles were incubated for 15 min at 25°C with 100 mM potassium phosphate buffer, pH 6.6, containing 10 mM $MgSO_4$. At the end of this period, the appropriate electron donor was added to a final

concentration of 20 mM and the reaction was started immediately by the addition of sodium [6-^{14}C] or [U-^{14}C]gluconate, to 0.05 mM final concentration. 0.1 ml samples were withdrawn, washed twice with 2.5 ml of 0.1 M LiCl on HA millipore filters (0.45 μ pore size) and their radioactivity assayed.

Assay of gluconate efflux from whole cells

Cells were allowed to accumulate labelled gluconate in a final volume of 1 ml, containing 0.5 ml of cells (2 mg dry mass.ml^{-1}) and 0.5 ml of 0.5 mM sodium [6-^{14}C]gluconate. When the radioactivity of the cells had reached a steady state, the cells were spun down for 1 min at 16,000 g in an Eppendorf 3200 Microfuge centrifuge. The pellet was quickly resuspended in 80 ml of 50 mM potassium phosphate buffer, pH 7.0, which had been warmed to 25°C. 10 ml samples were withdrawn at measured times and filtered; the filters were washed with 2.5 ml of the same buffer, air dried, and the radioactivity assayed.

Measurement of oxygen uptake

Rates of oxygen uptake by cell suspensions were measured with a Clark electrode at 25°C as described by Estabrook (1967).

A The uptake of gluconate by whole cells of strain R6

1. Inducibility of the uptake system

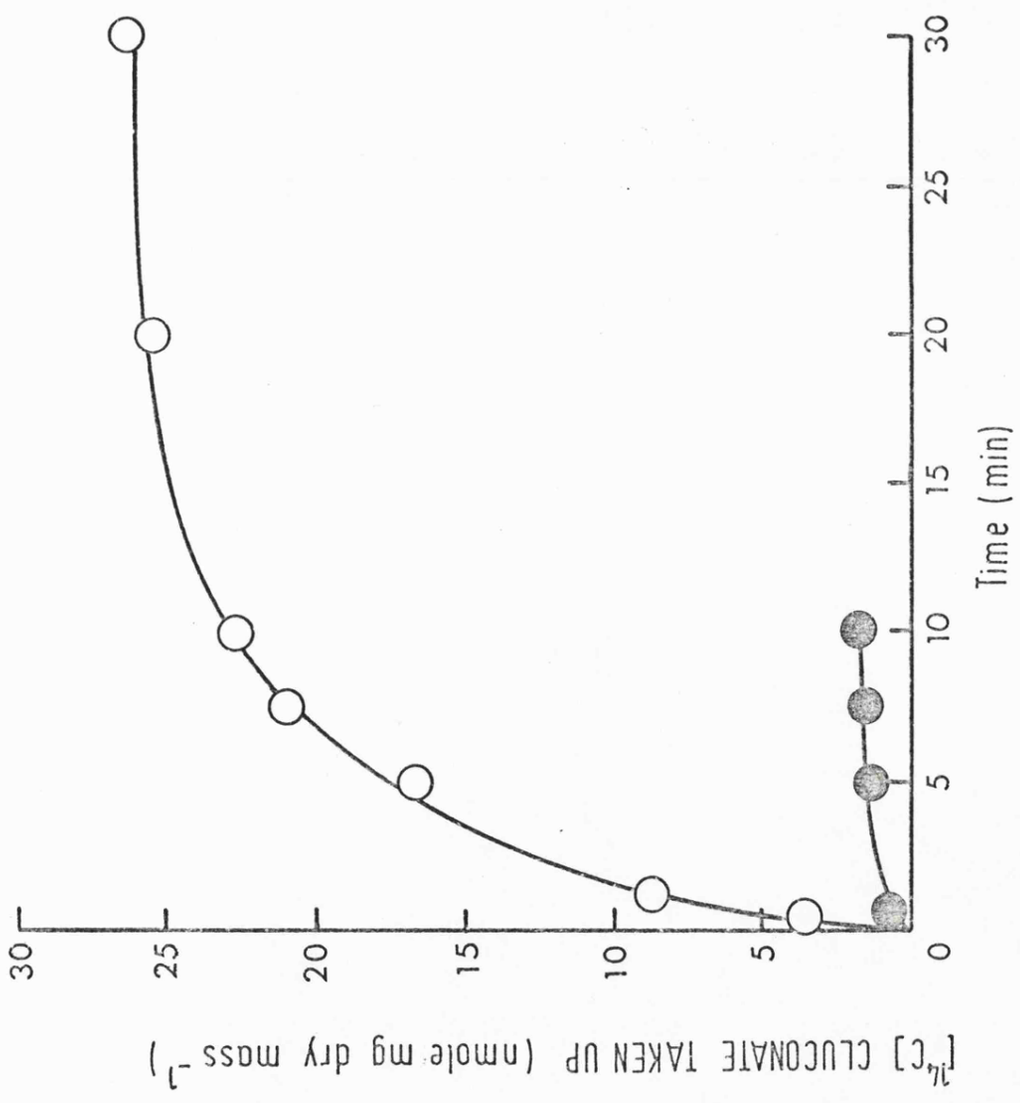
The uptake of labelled gluconate by induced and uninduced cell suspensions of strain R6 was measured. Figure 19 shows that both the initial rate of uptake of labelled gluconate and its steady state level are much higher in cells which have been previously exposed to gluconate. It can be seen from Figure 19 that, in the

FIGURE 19

Uptake of [^{14}C]gluconate by washed suspensions of strain R6

The time-course of uptake of 0.05 mM sodium [U-^{14}C]gluconate by washed suspensions of strain R6 grown on nutrient broth (●) and on nutrient broth in the presence of 5 mM gluconate (○).

Fig.19.


[14C] GLUCONATE TAKEN UP (nmole mg dry mass -1)
30
25
20
15
10
5
0
5
10
15
20
25
30
Time (min)

steady state, strain R6 takes up over 25 nmoles of gluconate.mg dry mass^{-1} when incubated with gluconate at 50 μM. Assuming that 1 mg dry mass of E. coli cells represents the dry mass of cells that contained 2.7 μl of water, this corresponds to a concentration of over 8 mM gluconate in the cell water. The uptake and retention of gluconate to such an extent (over one hundred and fifty-fold) must require the expenditure of metabolic energy.

The induction process appears to be highly specific. Of the compounds tested - glucoheptonate, 3-deoxy 3-fluorogluconate, 2-keto gluconate, xylonate, gulonate, galactonate, gluconolactone, glucuronate, galacturonate, and gluconate - only gluconate (10 mM) induced the uptake system to any significant extent.

2. Kinetics of gluconate uptake

The initial rates at which 0.05 mM sodium [^{14}C]gluconate was taken up by washed suspensions of strain R6 (that had been induced to form the uptake system) were measured over the range 1 - 1000 μM. As shown in the inset of Figure 20, the rates increased with increasing gluconate concentration up to about 60 μM, after which the uptake system appeared to be virtually saturated. The K_m of this process is about 10 μM and the V_{max} about 25 nmoles.min^{-1}.mg dry mass^{-1}.

3. Specificity of the uptake system

The uptake system is highly specific for gluconate (Table 9); the inclusion of the sodium salts of all available hexonic, pentonic, and hexuronic acids, and of 2-ketogluconic, 5-ketogluconic, 2-keto 3-deoxygluconic, and glucaric, acids at 1 mM did not reduce significantly

FIGURE 20

Effect of gluconate concentration on the initial rates of gluconate uptake by strain R6

Initial rates of uptake (V = nmoles.min^{-1}.mg dry mass^{-1}) were calculated from the amounts of [^{14}C]gluconate taken up by washed suspensions of induced strain R6 10, 25 and 40 sec after addition of labelled gluconate, and are plotted in the inset figure against the gluconate concentration added ([S] = μM). The reciprocal of these rates (X10) are plotted on the larger figure.

Fig. 20.

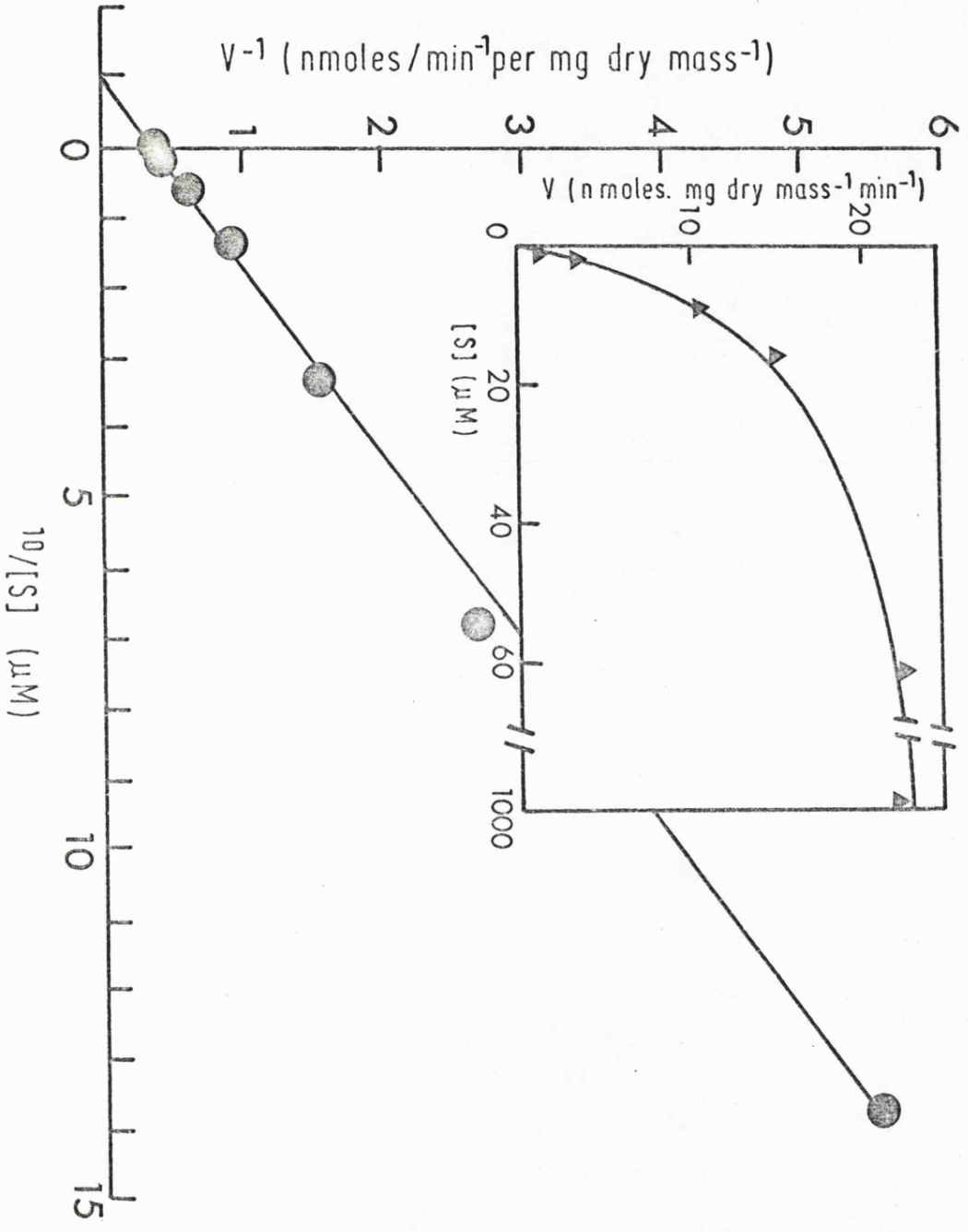

V⁻¹ (nmoles/min⁻¹per mg dry mass⁻¹)
0
1
2
3
4
5
6
V (n moles. mg dry mass⁻¹ min⁻¹)
0
10
20
[S] (μM)
0
20
40
60
1000
10/[S] (μM)
0
5
10
15

TABLE 9 Specificity of the uptake system for gluconate in the strain R6

Substrate tested	% of control
glucoheptonate (potassium)	110
3-deoxy 3-fluorogluconate (potassium)	70
2-ketogluconate "	107
5-ketogluconate "	102
2-keto 3-deoxygluconate "	116
galacturonate "	100
glucuronate "	97
glucarate "	105
xylonate "	145
ribonate "	107
gulonate "	105
galactonate "	98
sorbitol	107
glucose 6-phosphate	100
6-phosphogluconate	105
galactonolactone	120
D-gulono-γ-lactone	108
ribonolactone	100
gluconolactone	14
gluconate	9.7
glucose	105
fructose	108
ribose	102
water)	105
) duplicate	
water)	100

The uptake of sodium [^{14}C]gluconate (0.05 mM) by washed suspensions of strain R6 grown on nutrient broth plus 10 mM gluconate in the presence of various substrates. The amount of labelled material taken up by the suspensions 15 min after the addition of [^{14}C]-gluconate is expressed as a percentage of the control.

the rate at which strain R6 took up 50 μM sodium [^{14}C]gluconate; only 1 mM 3-deoxy 3-fluorogluconate inhibited (30%). The 86% inhibition observed with gluconolactone could be the result of its spontaneous hydrolysis to gluconate. The stimulation of gluconate uptake by xylonate cannot be explained at present since it is not known whether xylonate actually enters the cell or whether it causes a change in the cell membrane which in turn allows more gluconate to be taken up by the cell.

4. Effect of inhibitors on gluconate uptake

Since gluconate is concentrated over one hundred-fold by cell suspensions of strain R6 and retained as free gluconate within the cells (see Chapter II), the uptake and retention of gluconate must require the expenditure of metabolic energy. The reduction of the rate of gluconate uptake by the exclusion of oxygen (Table 10), the addition of amytal [which is known to inhibit a flavoprotein between D-lactic dehydrogenase and cytochrome b_1 (Cox *et al.*, 1970)], and cyanide (which blocks the terminal oxidase) and the inhibition by a variety of agents which uncouple respiration from oxidative phosphorylation (2,4-dinitrophenol, carbonyl cyanide-m-chlorophenylhydrazone) indicate that this energy for gluconate uptake is derived from the activity of the respiratory chain. The inhibition observed with the sulphydryl reagent p-chloromercuribenzoate, and its reversal by β-mercaptoethanol, further indicate that a sulphydryl group is associated with the site that recognises gluconate or with the proteins involved in energetic coupling.

Azide inhibits the terminal oxidase, similarly to cyanide,

TABLE 10

Effect of inhibitors on gluconate uptake by strain R6

Inhibitor used	Concentration added (mM)	Inhibition (%)
Sodium azide	1.0	50
	10	93
Potassium cyanide	1.0	60
	10	76
Sodium arsenate	1.0	12
	10	30
2,4-Dinitrophenol	0.05	30
	0.25	90
	2.5	96
Carbonyl cyanide-m-chloro-phenylhydrazone*	0.001	79
	0.01	98
Amytal*	0.1	21
	1.0	34
Anaerobic conditions	-	55
p-Chloromercuribenzoate	0.1	80
β-Mercaptoethanol	1.0	0
p-Chloromercuribenzoate + β-mercaptoethanol	0.1 + 1.0	12
Ethanol	-	0

The inhibitors, dissolved in water (or, where marked *, in ethanol) were incubated for 15 min at 25°C with the cells before addition of 0.05mM-sodium [^{14}C]gluconate. The amounts of labelled gluconate taken up in a further 15 min were measured as described in the Methods.

but rather inefficiently. At high concentrations (> 1 mM) it probably acts as an uncoupler, too. The presence of cyanide (at 10 mM) causes almost complete inhibition of gluconate accumulation thus providing further evidence that the uptake of gluconate by whole cell suspensions of E. coli strain R6 is linked to respiration.

5. Effect of pH on gluconate uptake

The initial rates of uptake of 0.05 mM gluconate by washed suspensions of strain R6, that had been induced for gluconate uptake, were not greatly affected by changes in pH over the range 6-8 (Figure 21): a 40% increase in the initial rate of gluconate uptake was observed at approximately pH 7.4. These results differ from those obtained by Robin and Kepes (1973) using an apparently similar mutant. Using the same concentration of gluconate (0.05 mM), these authors observed that there was very little change in the initial rates of gluconate uptake between the pHs 5.5 and 6.5 but above this (pH 7.0 to pH 8.0), a six-fold decrease in the rates of gluconate uptake was observed. The reason for this discrepancy is not clear at present - the mutant isolated by Robin and Kepes may have different properties from the mutant R6. Further physiological and genetic studies of the two mutants will help clarify this difference.

6. Effect of temperature on gluconate uptake and the effect of uncoupling agents on gluconate efflux

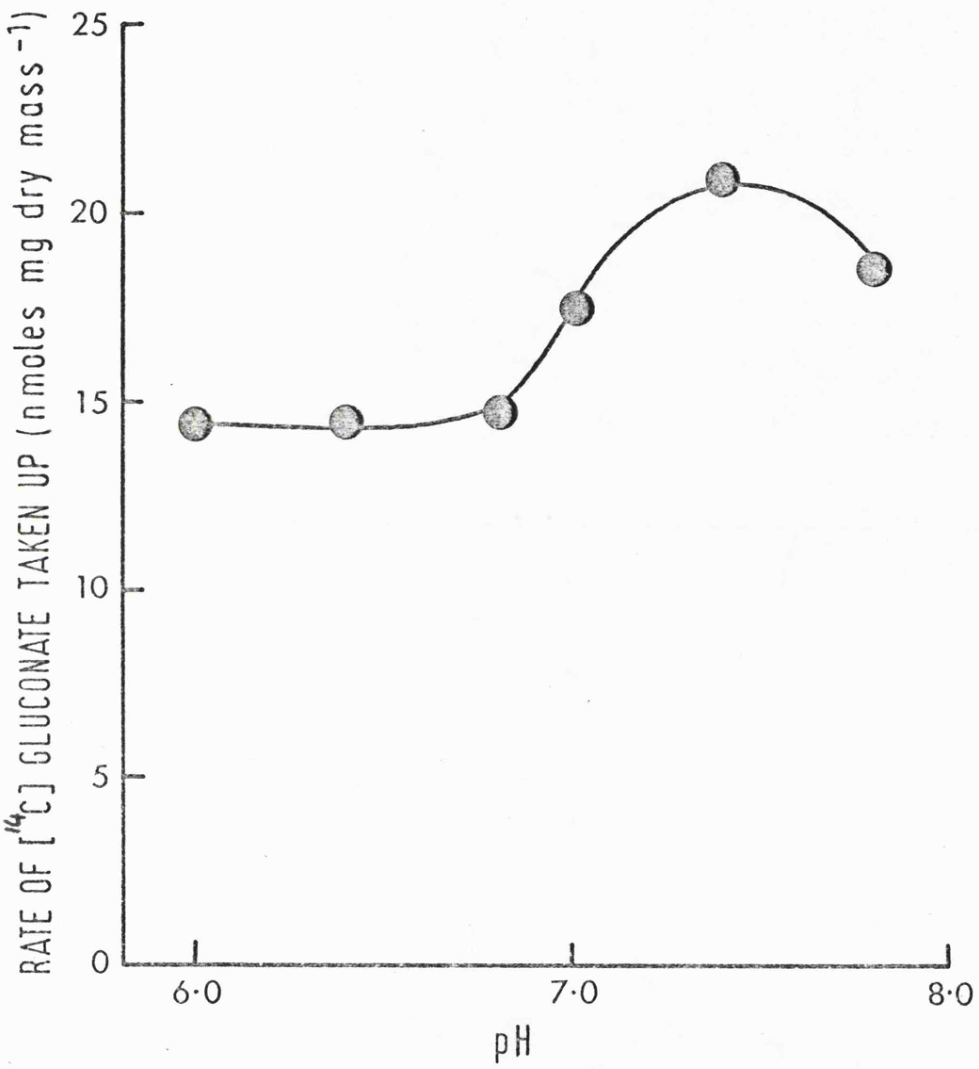
At the pH (7.0) used in these studies, the initial rate of gluconate uptake by strain R6 was very low when measured at 0°C and

FIGURE 21

pH dependence of gluconate uptake in induced cells of E. coli strain R6

The uptake of 0.05 mM [U-^{14}C]gluconate was measured in washed cell suspensions of strain R6 at different pHs of 50 mM phosphate buffer by removing 0.5 ml samples at 15, 45, 75, 120 and 300 sec and assaying their radioactivity. Initial rates of uptake are plotted against pH.

Fig. 21.


RATE OF [^{14}C] GLUCONATE TAKEN UP (n moles mg dry mass^{-1})
25
20
15
10
5
0
6·0
7·0
8·0
pH

rose rapidly with increase in temperature to a peak at about 35°C. Above this temperature, the rate decreased (Figure 22). There was a concomitant rise in the steady-state levels of gluconate maintained by the cells between 0°C and 35°C, and a sharp decline thereafter(Figure 23). The behaviour shown in Figure 22 is characteristic of an active transport process, which exhibits a temperature coefficient much greater ($Q_{10} \geqslant 2.0$) than would passive diffusion, the rate of which is proportional to the absolute temperature. The sharp decrease above 35°C in the quantities of gluconate retained by strain R6 can be only partially accounted for by an increased rate of gluconate efflux from the cells, suggesting an alteration in some energy-dependent process involved in gluconate retention. This view is supported by measurement of the effect of 2,4-dinitrophenol on gluconate efflux (Figure 24). In the absence of this proton-conducting agent (Harold, 1972), a culture of R6 preloaded with [^{14}C]gluconate lost gluconate at only 0.04 nmoles.min^{-1}.mg dry mass^{-1} when diluted into a large volume of 50 mM potassium phosphate buffer without change in pH or temperature. As discussed previously (Chapter II), the addition of [^{12}C]gluconate to cell suspensions of strain R6, which had accumulated labelled gluconate, greatly accelerated this exit reaction: the dilution of cellular radioactivity observed reflects the rapid uptake of the unlabelled gluconate and the consequent displacement of the labelled material already present. The efflux reaction involved in this displacement could be a passive diffusion since it exhibits the small temperature coefficient expected of such a process (Figure 22) and could involve the activity of a mobile carrier protein (Kepes, 1973). However, no such exchange can account for the

FIGURE 22

Temperature dependence of uptake (●) and efflux (■) of gluconate by suspensions of strain R6

Uptake: Flasks containing a suspension of induced strain R6 in 50 mM phosphate buffer, pH 7.0, were incubated for 5 min at different temperatures before the addition of 0.05 mM sodium [^{14}C]gluconate. Samples were removed at 10, 60, 120 sec after the addition of labelled gluconate, washed with buffer at the same temperature, and their radioactivity assayed.

Efflux: Induced cells of strain R6 were allowed to accumulate sodium [^{14}C]gluconate at 25°C, rapidly centrifuged and resuspended in 80 ml of 50 mM phosphate buffer, pH 7.0, at the same temperature. Samples (10 ml) were withdrawn at measured times and their radioactivity assayed.

Fig.22.

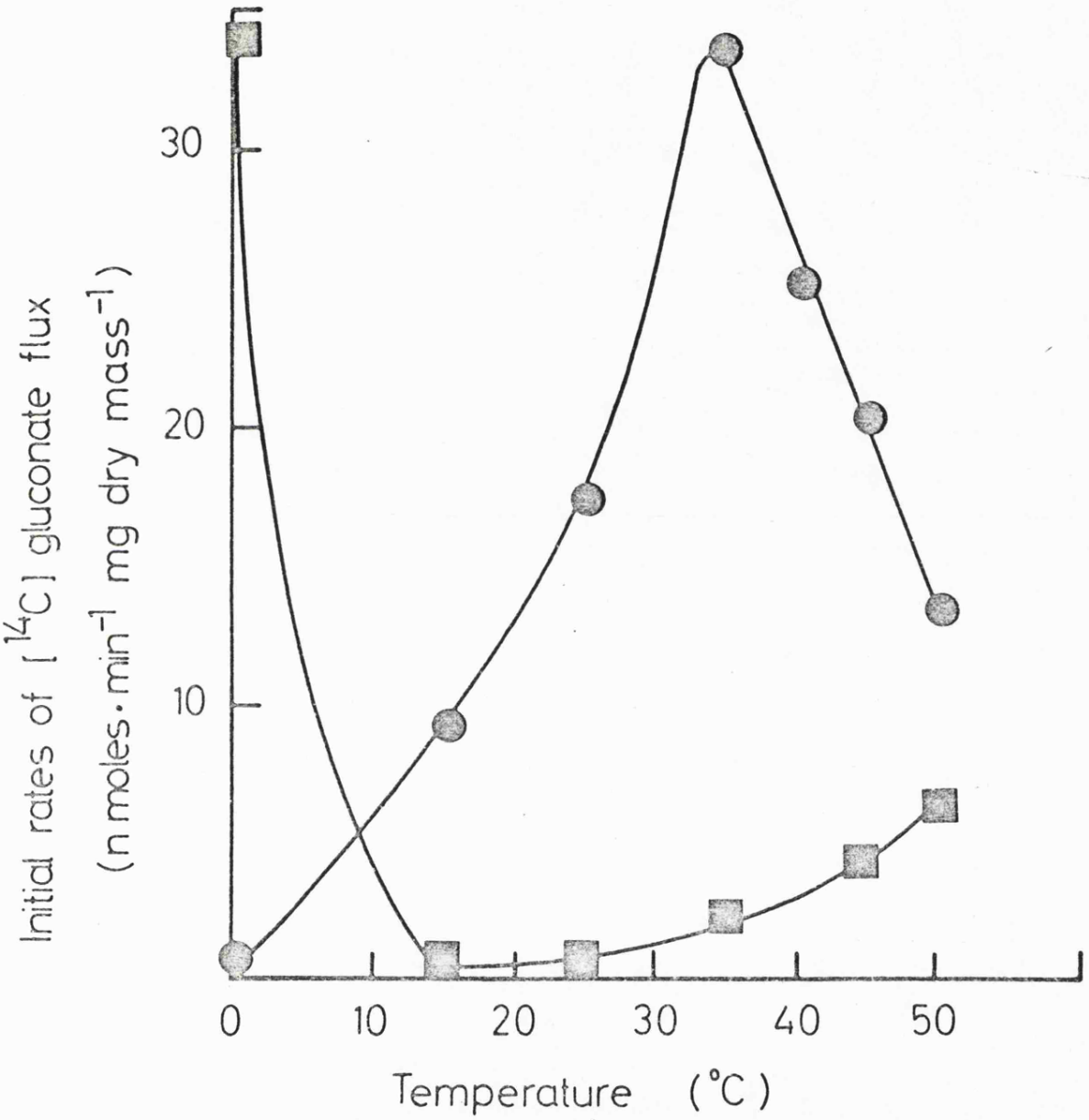

Initial rates of [^{14}C] gluconate flux
(n moles · min^{-1} mg dry mass^{-1})
30
20
10
0
10
20
30
40
50
Temperature (°C)

FIGURE 23

Effect of temperature on the time-course of gluconate uptake by strain R6

Flasks containing 50 mM phosphate buffer, pH 7.0, were incubated for 10 min at 0°C (■), 15°C (□), 25°C (○), 35°C (▲), 40°C (◑), 45°C (△) and 50°C (◒) before the addition of a suspension of induced strain R6. Sodium [^{14}C]gluconate (0.05 mM) was added 2 min later. Samples taken at times indicated were washed with buffer at room temperature and their radioactivity assayed.

Fig.23.

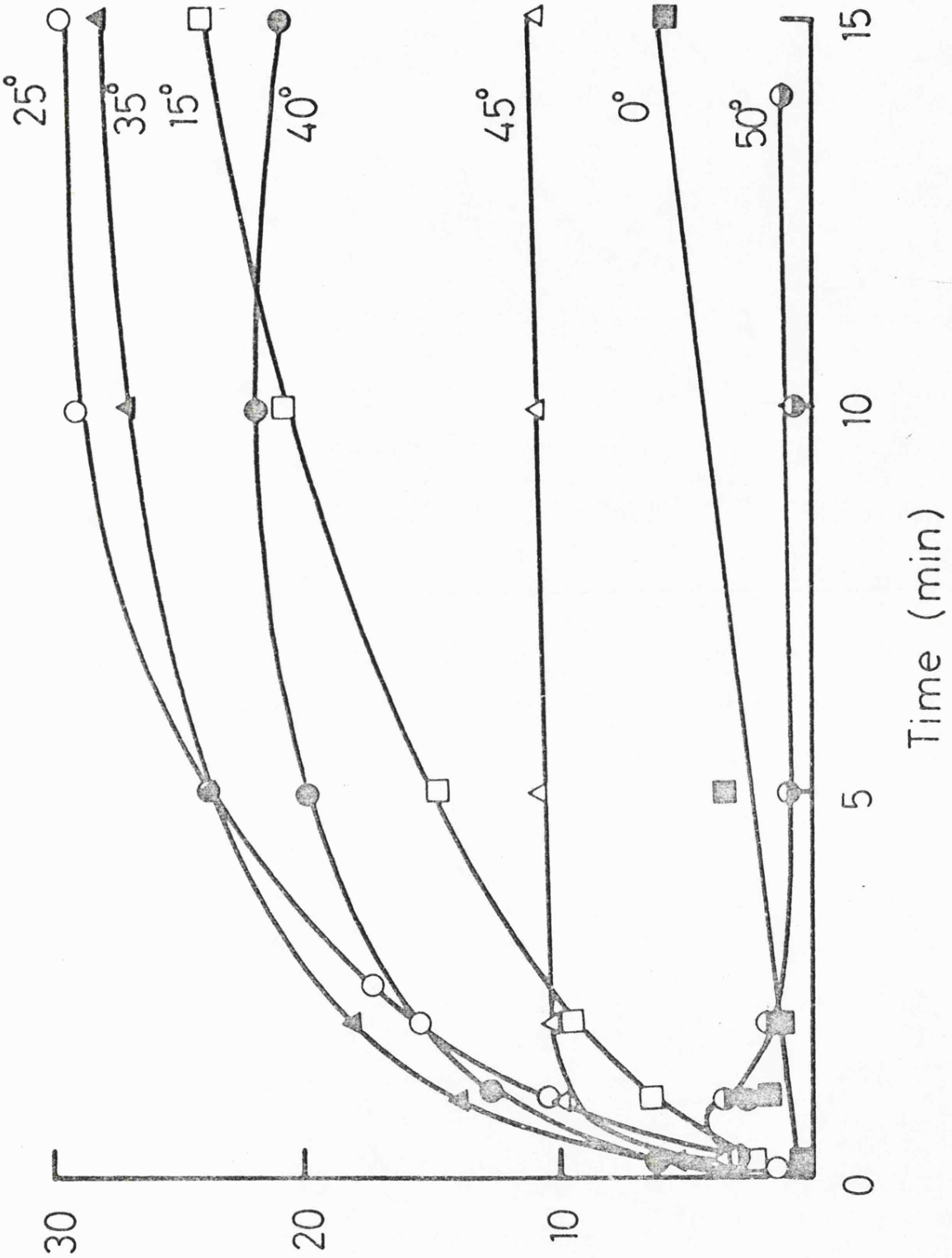
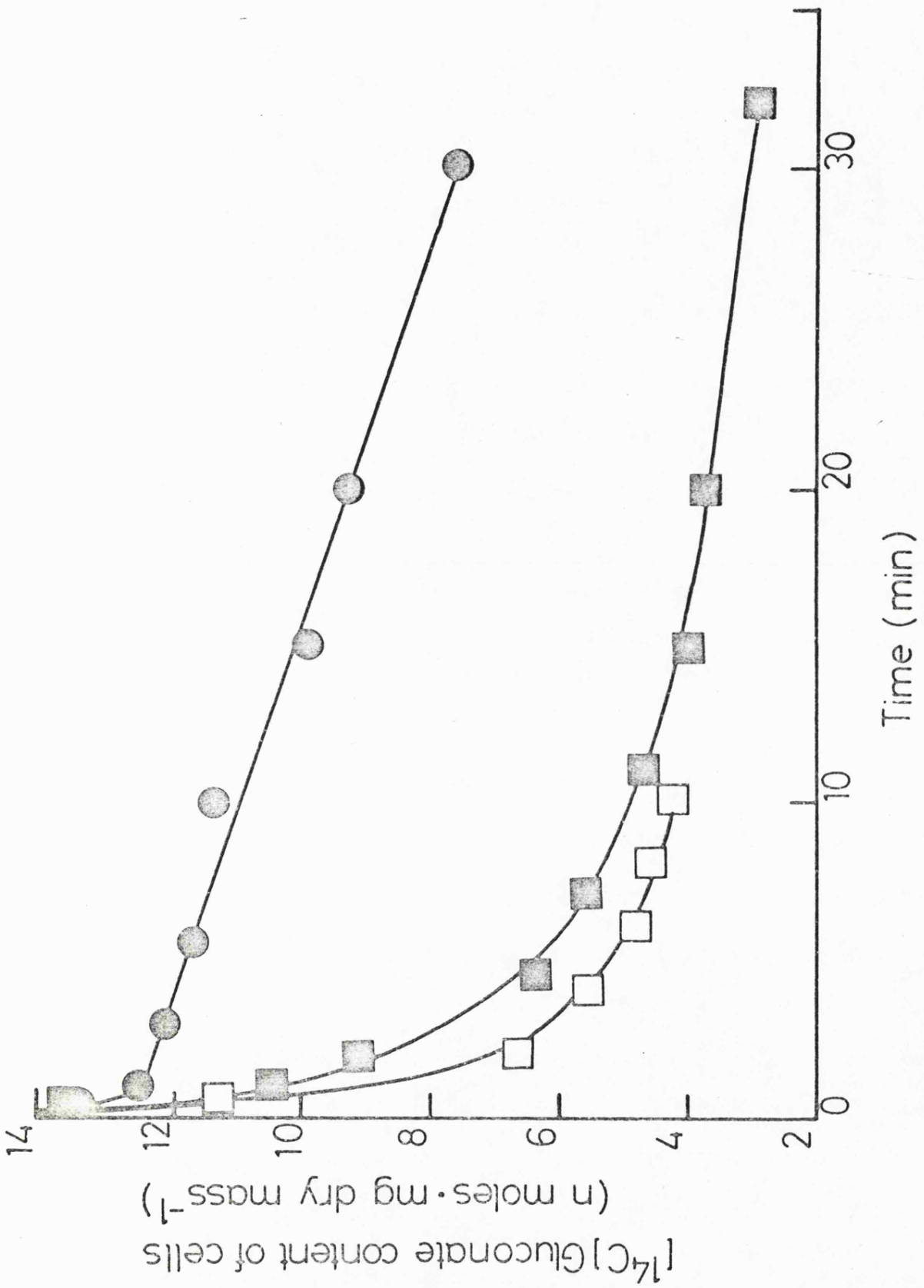

25°
35°
15°
40°
45°
0°
50°
30
20
10
0
5
10
15
Time (min)
[14C] Gluconate taken up
(n moles·mg dry mass-1)

FIGURE 24

Time-course of gluconate efflux from strain R6

Cells were allowed to accumulate [^{14}C]gluconate and diluted in a large volume of 50 mM potassium phosphate buffer, pH 7.0, alone (⊖) or in buffer that also contained 5 mM sodium [^{12}C]gluconate (▣), or 0.25 mM dinitrophenol (□).

Fig.24.


[14C] Gluconate content of cells
(n moles · mg dry mass-1)
14
12
10
8
6
4
2
0
10
20
30
Time (min)

stimulation of gluconate efflux observed after addition of 2,4-dinitrophenol: presumably, some energy-requiring process must operate to retain the gluconate taken up, possibly by reducing the affinity of the gluconate 'carrier' for the substrate present internally (Koch, 1964) or by providing the power for a recapture process at the cell surface (Halpern, 1967).

7. Binding proteins

When cells of Gram-negative bacteria are subjected to an osmotic shock treatment (Neu and Heppel, 1965; Nossal and Heppel, 1966; Heppel, 1967, 1969), they release certain proteins from the periplasmic space which bind specific substances. Concomitant with the loss of these proteins, certain specific transport systems become impaired. These 'binding proteins' have been proposed as components of the transport systems for a variety of amino acids, carbohydrates, and ions (for review, see Lin, 1971). Elucidation of the function of binding proteins has involved restoration of transport activity upon addition of the purified protein to shocked cells together with attempts to identify the structural genes for the various binding proteins.

Many laboratories report lack of success in attempts to restore the transport activity of shocked cells by addition of purified proteins back to such shocked cells (Pardee, 1968; Piperno and Oxender, 1966); however, Anraku (1968) has reported some success for the galactose system. Most evidence linking the binding proteins with transport is indirect. The EDTA treatment followed by osmotic shock not only releases the binding protein but it can cause the loss

of a variety of essential small molecules, some of which may be necessary for energy production (review, Oxender, 1972).

Genetic studies have implicated the binding proteins in transport, but few laboratories have been able to identify the structural genes for the various binding proteins.

In order to investigate whether such binding proteins played any part in the uptake of gluconate, cultures of both wild-type E. coli K12 and of the mutant R6 that had been induced for gluconate uptake were osmotically shocked. In conditions where at least 95% of the shocked cells remained viable (Anraku and Heppel, 1967), there was a decrease by 75-80% in the initial rates of gluconate uptake, which could be partially restored by the addition of concentrated shock fluid (Figure 25). However, this partial restoration could also be achieved by the addition of shock fluid derived from cells that had not been induced for gluconate uptake (Table 11). The material appeared to reside in some component of the fluid that was stable to boiling for 10 min, and was lost when shock fluid from induced cells was dialysed. Shocked cells prepared from E. coli strain R6 behaved in qualitatively the same way as the wild-type cells that had undergone similar treatment (Table 11). Concomitant with the loss of transport activity, there was a parallel decrease in cellular respiration upon cold-shock treatment of both wild-type and E. coli strain R6. It seems unlikely that a gluconate-specific binding protein was released in the shock fluid. Attempts by equilibrium dialysis to detect a protein in the concentrated shock fluid that would bind labelled gluconate were unsuccessful. It is more likely that the osmotic cold-shock procedure causes the loss of

FIGURE 25

Effect of cold osmotic shock treatment on the uptake of [^{14}C]gluconate by wild-type E. coli K12

Time-course of the uptake of 0.05 mM sodium [^{14}C]gluconate by washed cell suspensions of induced cells of strain K10 before (○) and after (●) osmotic shock treatment; and upon the addition of shock fluid (2 mg.ml^{-1} final) to the shocked cells (▲).

Fig. 25.

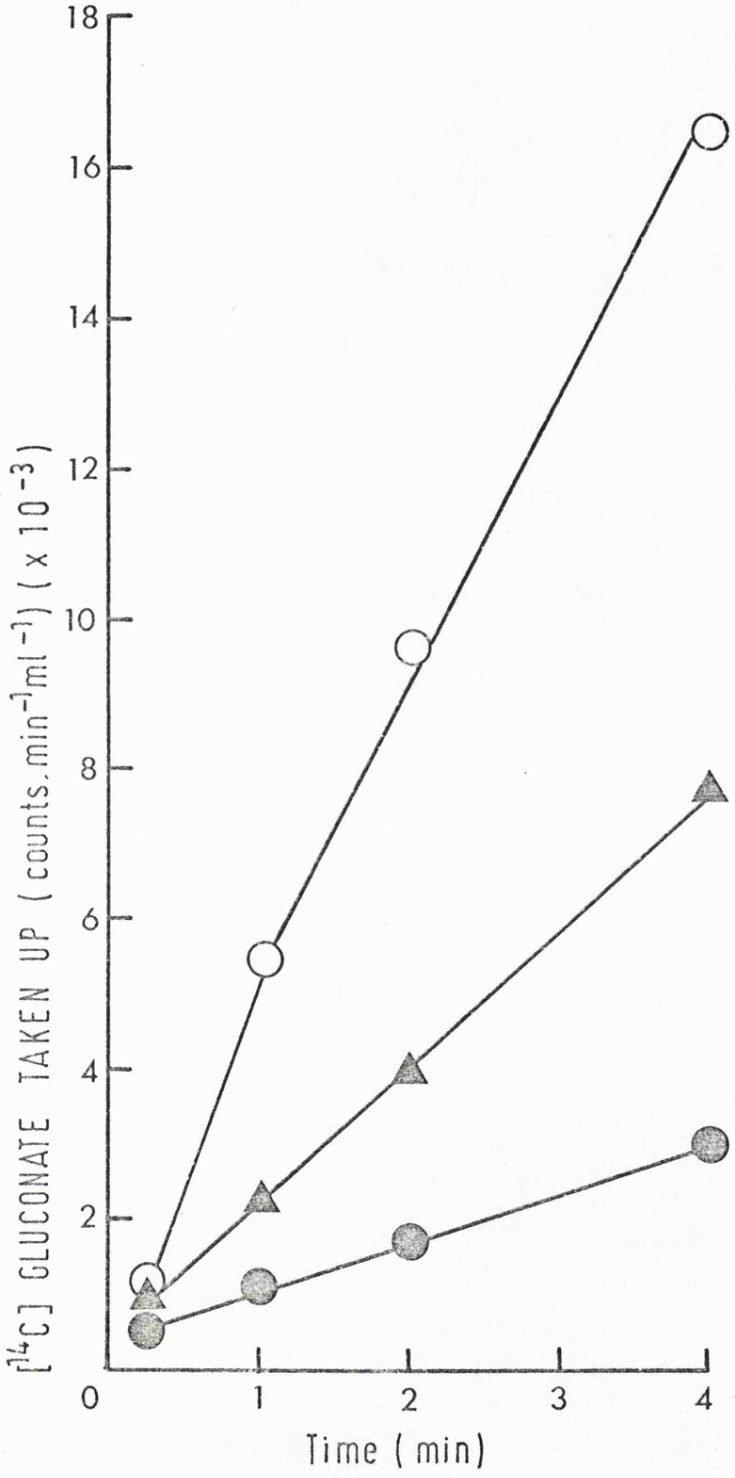

18
16
14
12
10
8
6
4
2
0
1
2
3
4
Time (min)
[14C] GLUCONATE TAKEN UP (counts.min-1ml-1) (x 10-3)

TABLE 11

Effect of cold osmotic shock treatment on the uptake of gluconate and of oxygen by wild-type E. coli K12 and strain R6

	Relative rates (%) of gluconate uptake	oxygen uptake
	WILD-TYPE E. coli K12	
Cells	100 (100)	100 (100)
Shocked cells	26 (20)	20 (16)
+ fluid	62 (54)	20 (16)
+ boiled fluid	78	-
+ fluid from non-induced cells	99	-
	STRAIN R6	
Cells	100	100
Shocked cells	23 (26)	23 (23)
+ fluid	32	-

( ) independent determinations

- not done

Gluconate uptake and respiration were measured within the 2 h following the EDTA-osmotic shock treatment. Cells were grown on nutrient broth supplemented with 5 mM sodium gluconate as inducer. For measurement of oxygen uptake, nutrient broth was used as substrate. Shock fluid (0.6 mg protein.ml^{-1}) was concentrated under vacuum about 40-fold and added at a final concentration of 2 mg protein.ml^{-1}.

small molecules and this is responsible for the impairment of the energy metabolism of these cells.

B Gluconate uptake by membrane vesicles prepared from strain R6

In common with other carbohydrates actively taken up by E. coli (for review, see Kaback, 1970; Kornberg, 1973), gluconate can be taken up and retained against a concentration gradient by membrane vesicles prepared from induced cells, provided a suitable source of metabolic energy is also supplied (Figure 26). The optimum source of thismetabolic energy appears to differ with different carbohydrates. The ability of a substance to be oxidised by vesicle preparations does not reflect its ability to support transport. Vesicles of E. coli oxidise succinate, NADH, D-lactate and L-lactate in that order of preference (Barnes and Kaback, 1971). However, the uptake of gluconate by membrane vesicles prepared from strain R6 was stimulated nearly 30-fold by the presence of ascorbate and phenazine methosulphate (PMS), ten-fold by L-lactate, 3-4-fold by D-lactate and not at all by NADH. This preference for L- over D-lactate differs from the preference for D-lactate exhibited by E. coli vesicles taking up galactosides, and some amino acids (Kaback, 1970, 1972). With ascorbate + PMS as electron donor, vesicles concentrated external 0.05 mM gluconate over one hundred-fold, to about the same level as had been observed with whole cells (Figure 19). Furthermore, and as also observed with whole cells, the addition of [^{12}C]gluconate to vesicles that had taken up [^{14}C]gluconate rapidly displaced over 90% of the radioactive material from them, in 2 min. (Figure 27).

FIGURE 26

Time course of uptake of gluconate by membrane vesicles from strain R6

The uptake of 0.05 mM sodium [^{14}C]gluconate by vesicles (0.4 mg protein.ml^{-1} of buffer) was measured, as described in the Methods, in the absence of added electron donors (●) and in the presence of D-lactate (□), of L-lactate (■) and of ascorbate + phenazine methosulphate (▲).

Fig. 26.

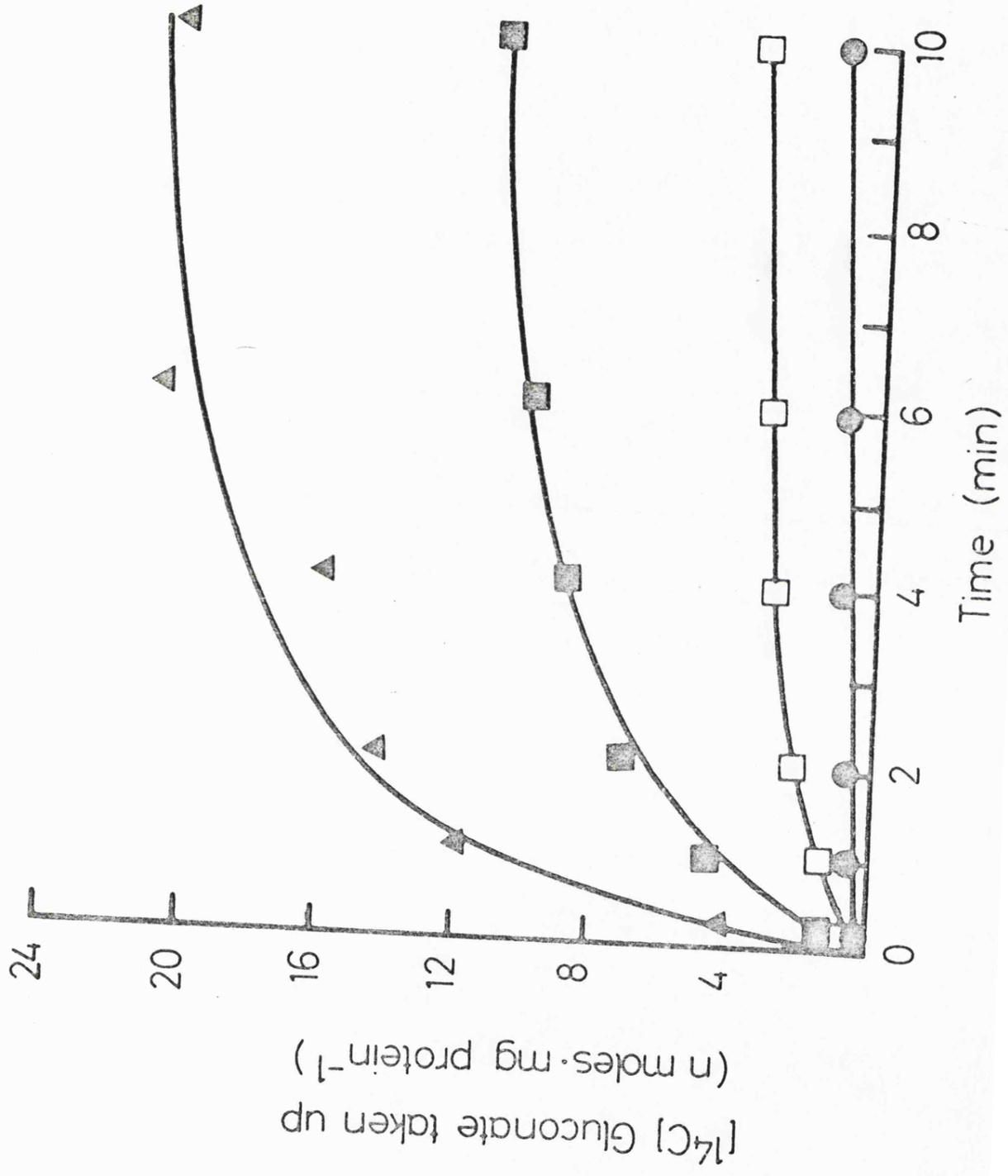
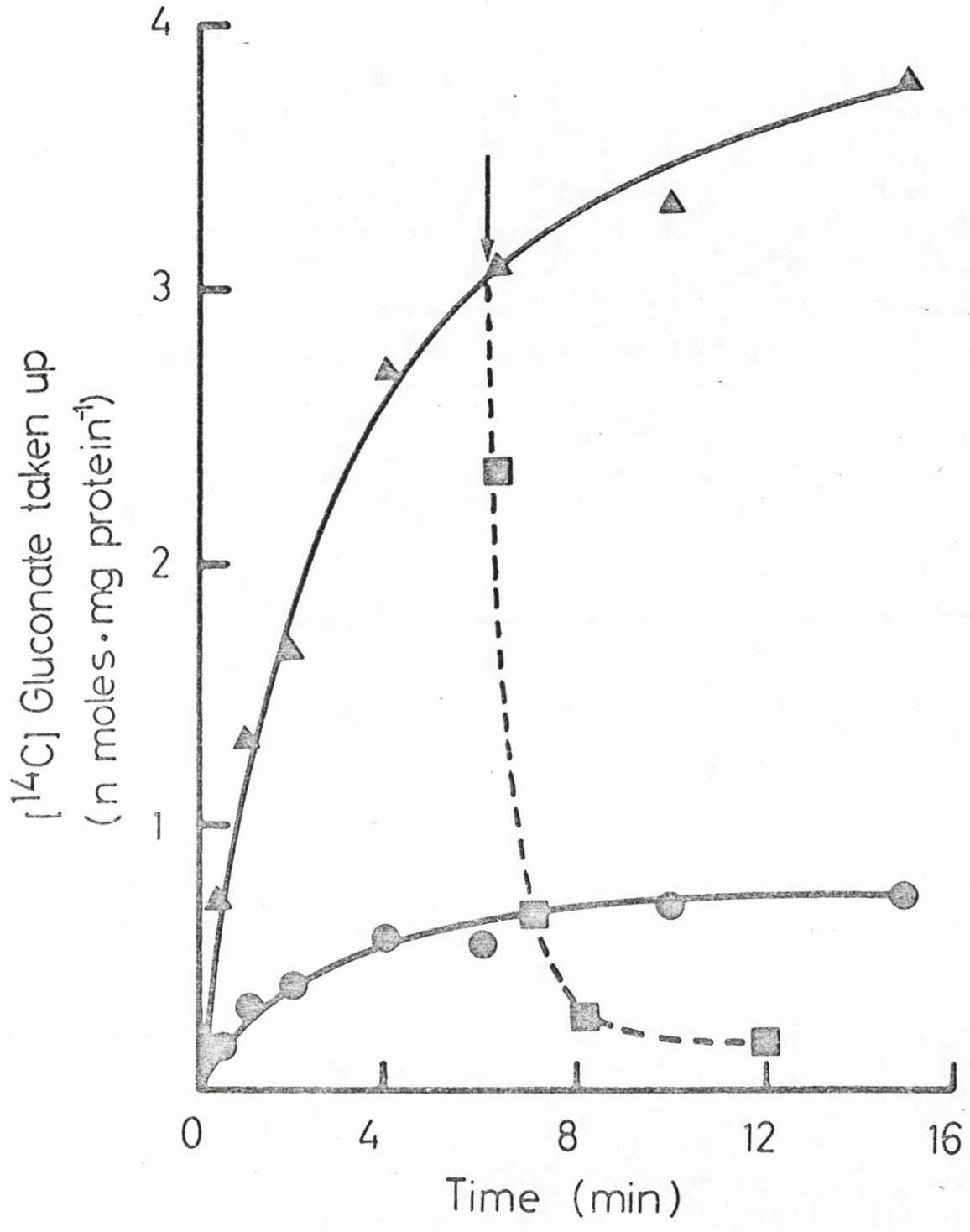

[14C] Gluconate taken up
(n moles · mg protein⁻¹)
24
20
16
12
8
4
0
2
4
6
8
10
Time (min)

FIGURE 27

Time course of uptake of gluconate by membrane vesicles from strain R6

The uptake of 0.05 mM sodium [^{14}C]gluconate by membrane vesicles was measured in the absence of added electron donors (○) and in the presence of D-lactate (▲). At the time indicated by an arrow, 5 mM sodium [^{12}C]gluconate was added to a portion of the latter flask (■).

Fig.27.


4
3
2
1
0
4
8
12
16
Time (min)
[14C] Gluconate taken up
(n moles • mg protein-1)

Stimulation of gluconate uptake was observed also with succinate or α-glycerophosphate as electron donors, but these materials were less effective than ascorbate + PMS, or L-lactate. NADH, which is the preferred electron donor for the uptake of some carbohydrates by B. subtilis membrane vesicles (Matin and Konings, 1973), did not stimulate gluconate uptake by vesicles prepared from E. coli strain R6 (Table 12). Using L-lactate as electron donor, the K_m for gluconate uptake was 5×10^{-5} M (Figure 28) which is of the same order of magnitude as that found for uptake by whole cells.

As noted with whole cells, the uptake of gluconate by membrane vesicles is strongly inhibited by lack of oxygen, electron transfer inhibitors and uncoupling agents (Table 13); the lack of effect by arsenate is consistent with the view (Klein and Boyer, 1972) that ATP or other high-energy phosphate compounds are not directly involved in the transport process.

Vesicles appear to be devoid of binding proteins (Kaback, 1972), which are easily lost by the osmotic shock treatment necessary for their preparation. The observation that vesicles prepared from strain R6 concentrate gluconate to the same order of magnitude as do whole cell suspensions of that organism suggests that, if there is, in fact, a binding protein for gluconate, then this does not play an indispensible role in the translocation of gluconate across the plasma membrane.

Although the precise nature of the coupling of respiration to transport is still at issue, the close parallel between the kinetic and energetic parameters that characterise the uptake of gluconate by whole cells and membrane vesicles support the use of the latter type of experimental material for studies of active transport and show that no indispensible components of the uptake system have been lost in the preparation of the membrane vesicles.

TABLE 12

Effect of various energy sources on the gluconate uptake by membrane vesicles from strain R6

Energy source added	Concentration added (mM)	[^{14}C]gluconate taken up (nmoles. mg protein^{-1})
none	-	1
D-lactate	20	3
L-lactate	20	10
Ascorbate	20	1.5
PMS	0.2	1
Ascorbate + PMS	20 + 0.2	27
Succinate	20	3
DL-α-glycerophosphate	20	3
NADH	20	1.5
NADH + PMS	20 + 0.2	1.5
NADPH	20	1.5
Phosphoenolpyruvate	20	1
ATP	10	1.5

Samples (0.1 ml) containing membranes (0.2 mg protein) from strain R6 were incubated in 100 mM phosphate buffer, pH 6.6, containing 10 mM $MgSO_4$, and the amounts of isotopic material taken up in 15 min from 0.05 mM sodium [^{14}C]gluconate were measured.

FIGURE 28

Effect of gluconate concentration on the initial rates of gluconate uptake by membrane vesicles prepared from strain R6

Initial rates of uptake (v = nmoles.min^{-1}.mg protein^{-1}) were calculated from the amounts of [^{14}C]gluconate taken up by membrane vesicles prepared from induced cultures of strain R6 10, 25, and 40 sec after the addition of labelled gluconate and are plotted against gluconate concentration ([S] = μM), (A) - The reciprocals of these rates are plotted in (B).

Fig. 28.

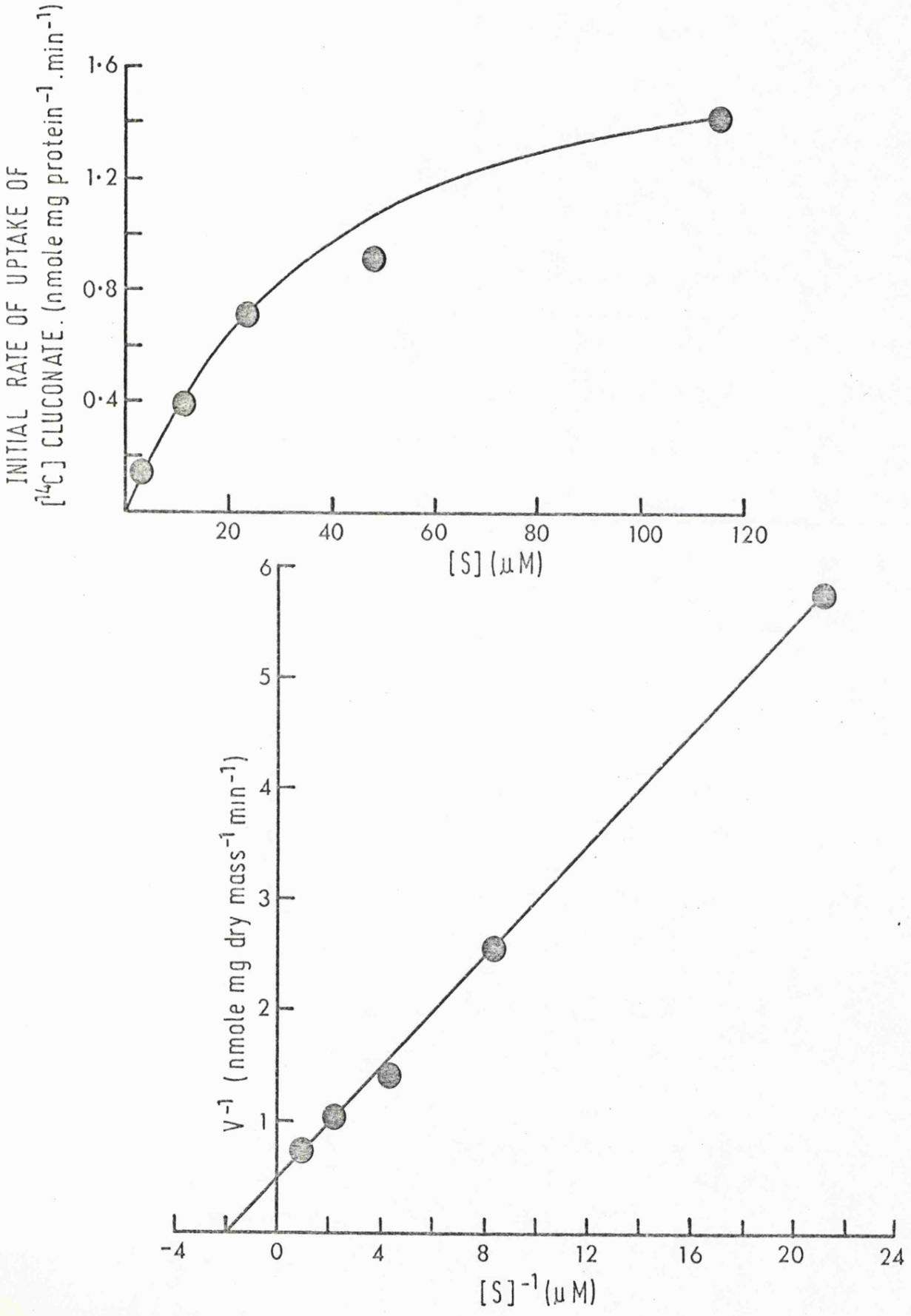

INITIAL RATE OF UPTAKE OF [14C] CLUCONATE. (nmole mg protein-1 .min-1)
1·6
1·2
0·8
0·4
20
40
60
80
100
120
[S] (μM)
6
5
4
3
2
1
V-1 (nmole mg dry mass-1 min-1)
-4
0
4
8
12
16
20
24
[S]-1 (μM)

TABLE 13

Effect of inhibitors on gluconate uptake by membrane vesicles prepared from strain R6

Inhibitor used	Concentration added (mM)	Inhibition %
none	-	0
Ethanol	-	0
Potassium cyanide	1	0
	10	79
Sodium azide	10	0
2,4-dinitrophenol	0.25	97
Carbonyl cyanide-m-chlorophenyl hydrazone*	0.01	97
Amytal*	1	80
p-Chloromercuribenzoate	0.1	94

The inhibitors, dissolved in water (or, where marked *, in ethanol) were incubated for 15 min at 25°C with the membrane vesicles before addition of 0.05 mM sodium [^{14}C]gluconate and 20 mM L-lactate. The amounts of labelled gluconate taken up in a further 15 min were measured as described in the experimental section.

CHAPTER IV

CHAPTER IV

ISOLATION AND PROPERTIES OF MUTANTS AFFECTED IN GLUCONATE UPTAKE

Evidence presented in Chapter III shows that the uptake of gluconate is catalysed by an inducible process that, in vesicles prepared from suitably induced cells of E. coli, can be coupled to the oxidation of L- and D-lactate (see also Kaback, 1970).

The purpose of this Chapter is to describe the isolation and properties of mutants of E. coli affected in gluconate uptake.

It is not clear at present whether such mutants are impaired in one uptake system only and can be induced to form a second, apparently equally specific uptake system (as has been suggested by de Zwaig et al., 1972, 1973), or whether the defect is in the induction of the 'normal' uptake system.

1. Isolation of mutants affected in gluconate uptake

The addition of gluconate [or of other substances such as glucuronate or galacturonate, that are also metabolically converted to 2-keto 3-deoxy 6-phosphogluconate (KDPG)] to mutants devoid of Kga (the aldolase that catalyses the cleavage of KDPG to pyruvate and glyceraldehyde 3-phosphate) leads to the accumulation of KDPG and consequent growth stasis (see Chapter I; Fradkin and Fraenkel, 1971; Pouysségur, 1971). For the same reason, such kdgA mutants do not grow on solid media containing a normally utilisable carbon source if gluconate, galacturonate or glucuronate are also present. However, when approximately 10^8 cells of the kdgA mutant A314 (Table 14)

TABLE 14

Strain	Derivation or reference	Genetic markers *	Response to streptomycin	Mating type
P4X	Pouysségur and Stoeber, (1972)	metB	S	Hfr
A314	" "	metB, kdgA	S	Hfr
PF1	A314	metB, usgA	S	Hfr
A1201	PA309	arg(EH),trp,xyl,malA,gal,kdgA	R	F^-
Δ70	[PA309 x P10-dctl] Pouysségur, (1972); Kay and Kornberg, (1969)	metB,trp,his,arg(HCBE),thr,leu, malA,Δ[dct-kdgK]	R	F^-

* Where possible, the abbreviations are those listed by Taylor and Trotter (1972). In addition, usg indicates the inability to take up gluconate, (this Chapter); kdgA and kdgK specify, respectively, the structural genes for phospho-2-keto 3-deoxygluconate aldolase and 2-keto 3-deoxygluconate kinase; Δ[dct-kdgK] a deletion extending from the dct to the kdgK markers on the E. coli genome.

were spread on plates containing 20 mM glycerol and 5 mM gluconate and were incubated at 30°C or 37°C for 2-3 days, approximately 10^2 colonies appeared that were now resistant to the presence of gluconate but were still devoid of Kga activity, as shown by their inability to grow on hexuronates as sole carbon source. Moreover, although these organisms were resistant to the presence of gluconate, they were still sensitive to the presence of hexuronates: growth on glycerol was arrested by the addition of 5 mM glucuronate or galacturonate, but growth was unaffected by the addition of 5 mM gluconate. The change undergone by strain A314 in acquiring this resistance to gluconate thus involved a mutation that prevented the formation of the (still toxic) KDPG from gluconate in the medium.

There are a number of ways in which the pathway from external gluconate to intracellular KDPG via 6-phosphogluconate can be interupted. The mutant obtained could be affected in the uptake of gluconate, or the phosphorylation of gluconate to 6-phosphogluconate, or in the following step of gluconate utilisation - the dehydration of 6-phosphogluconate to KDPG. A mutation leading to the inability to synthesise any one, or a combination,of these enzymes would lead to a reduction in the amount of intracellular KDPG formed, when such cells are exposed to gluconate.

A mutant lacking dehydratase activity can be readily distinguished from the former two types of mutant since it is able to grow on gluconate as sole carbon source via the pentose-phosphate pathway, although with a doubling-time approximately three times that of a wild-type organism (Zablotny and Fraenkel, 1967). Mutants

affected in gluconate uptake can be distinguished from those affected in gluconate kinase activity by an adaptation of the technique first described by Zwaig and Lin (1966) and subsequently used for the detection of mutants impaired in the uptake of α-methyl glucoside (Kornberg, 1972) or constitutive for the uptake of hexose phosphates (Ferenci et al., 1971). In this procedure, mutants are screened for their ability to take up [^{14}C]gluconate during their growth on plates of nutrient agar containing low concentrations of the isotopic material (10 μM; 0.033 μCi.ml^{-1}). Single colonies grown on such plates are adsorbed onto Whatman No. 40 filter paper, and this paper is air dried and left in contact with Kodak 'Blue Brand' X-ray film overnight. Only clones that take up the labelled gluconate blacken the film. One clone which did not blacken the film under these conditions was purified by single colony isolation and used for further studies.

As all the enzymes involved in gluconate uptake and the Entner-Dourdoroff pathway of gluconate utilisation by E. coli are inducible (although the KDPG aldolase is present at a high basal level) and as only gluconate has been found to induce all these enzymes, physiological studies on the mutant were assumed to require cultures induced by the addition of gluconate to the medium. Since this mutant is still devoid of Kga activity, and therefore its growth on any utilisable carbon source might still be inhibited to some extent by the presence of gluconate, it was desirable to convert it to kdgA$^+$ by phage P1-mediated transduction (Brice and Kornberg, 1967). The kdgA$^+$ derivative thus obtained was designated PF1 and used in subsequent studies.

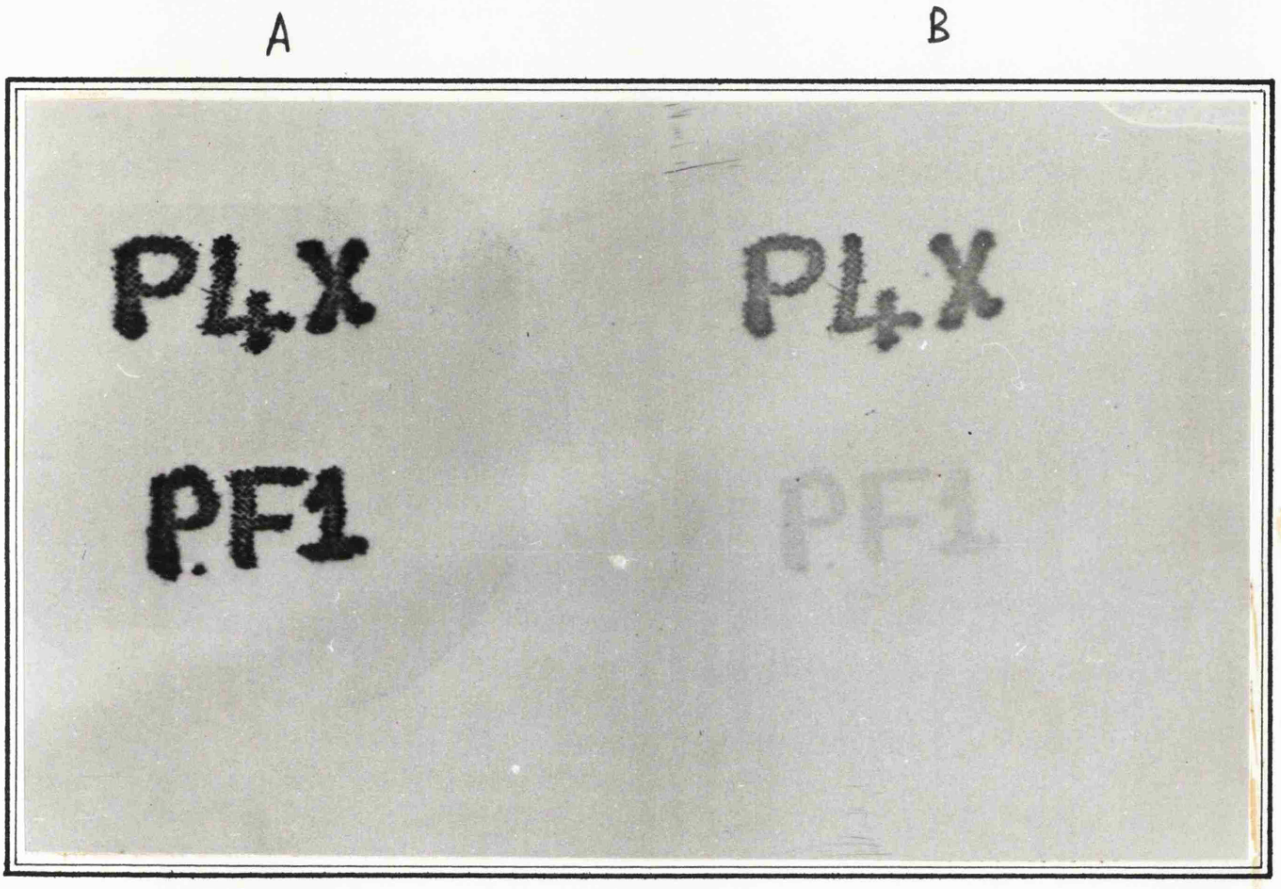
2. Properties of the mutant PF1

Two lines of evidence suggest that the mutant PF1 is defective in its ability to take up gluconate from the medium. Firstly, cells previously grown on nutrient agar plates containing 10 μM [^{14}C]gluconate do not blacken Kodak 'Blue Brand' X-ray film, although cells which have been grown on nutrient agar plates containing 10 μM [^{14}C]α-methylglucoside do (Figure 29). Secondly, when incubated with 0.1 mM [U-^{14}C]gluconate, washed cell suspensions of the mutant PF1 which have previously been grown on nutrient broth containing 5 mM sodium gluconate for at least three generations take up less than 10% of the isotope taken up by similarly grown cells of the wild-type P4X or of the *kdgA* parent A314 from which it was derived (Figure 30). Similar results are obtained with the mutant PF1 grown on 20 mM glycerol plus 10 mM gluconate for many generations (overnight).

(1) Growth properties of the mutant PF1

The mutant PF1 grew at wild-type rates on fructose, glucose, glycerol, xylose and galacturonate (Table 15) but cultures of PF1 took 7 h for one doubling when transferred to 20 mM gluconate as sole carbon source (Figure 31). However, a progressive acceleration of growth was observed throughout this period until about 24 h after transfer when the growth rate of the mutant PF1 on gluconate approached that of the wild-type organism P4X (Table 15 and Figure 31). That this is not a consequence of selecting a further mutant or a reversion to wild-type can be shown in two ways: firstly, when a loopful of cells of the mutant PF1, which were thus grown on 20 mM gluconate, were grown

FIGURE 29 Autoradiography of strains P4X and PF1



The organisms were grown on nutrient agar plates containing 0.1 mM [^{14}C]α-methylglucoside (A) and 10 μM [^{14}C]gluconate (B).

FIGURE 30

Uptake of 0.1 mM sodium [^{14}C]gluconate by washed suspensions of strains P4X (●), A314 (△), and PF1 (○)

The organisms were grown overnight on media containing nutrient broth plus 10 mM gluconate as carbon source.

Fig. 30.

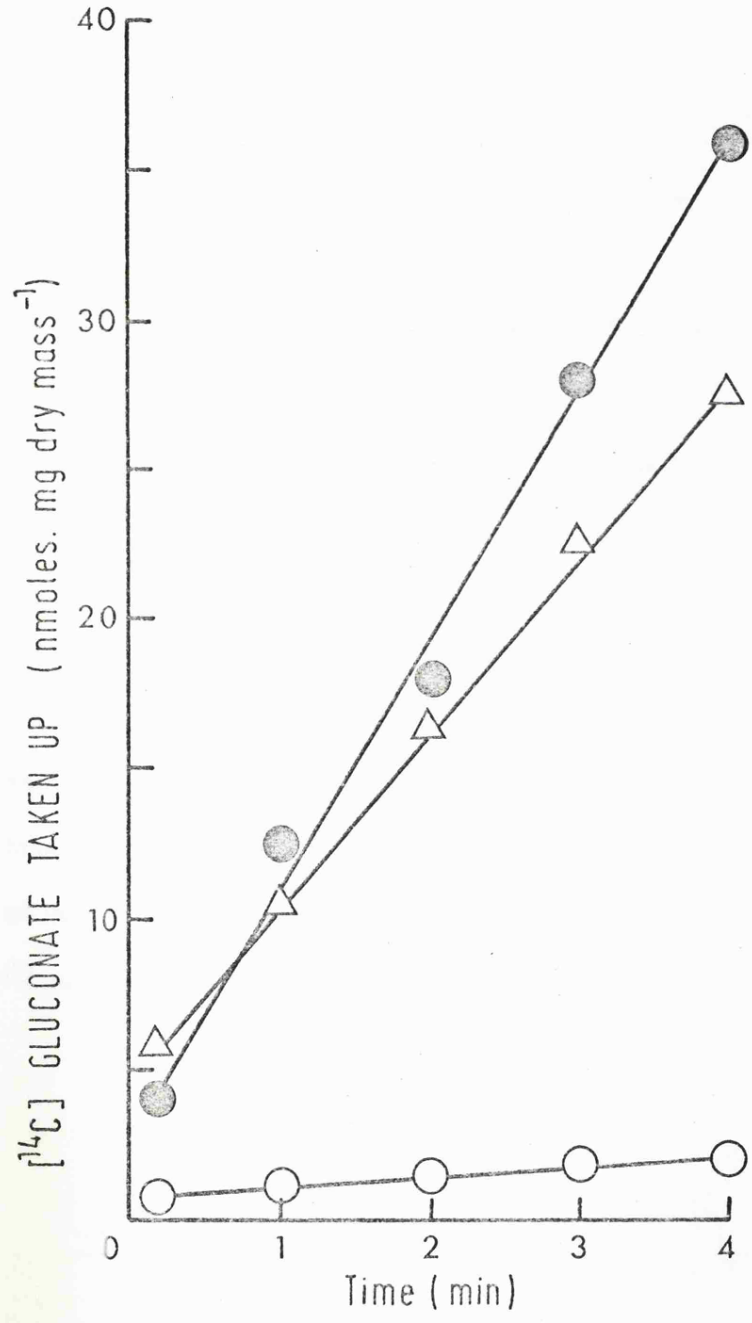

40
30
20
10
0
1
2
3
4
Time (min)
[14C] GLUCONATE TAKEN UP (nmoles. mg dry mass-1)

TABLE 15

Doubling times of the mutant PF1 and the wild-type organism P4X on various carbon sources

Carbon source	Doubling time (min)	
	P4X	PF1
fructose	90	90
glucose	60	60
glycerol	105	102
xylose	81	84
galacturonate	75	78
gluconate	60	420
24 h after transfer	60	60

The organisms were grown overnight on media containing 20 mM fructose as carbon source; they were harvested the next morning, and transferred to minimal media containing the appropriate carbon source at a final concentration of 20 mM. L-methionine was present at 80 $\mu g.ml^{-1}$.

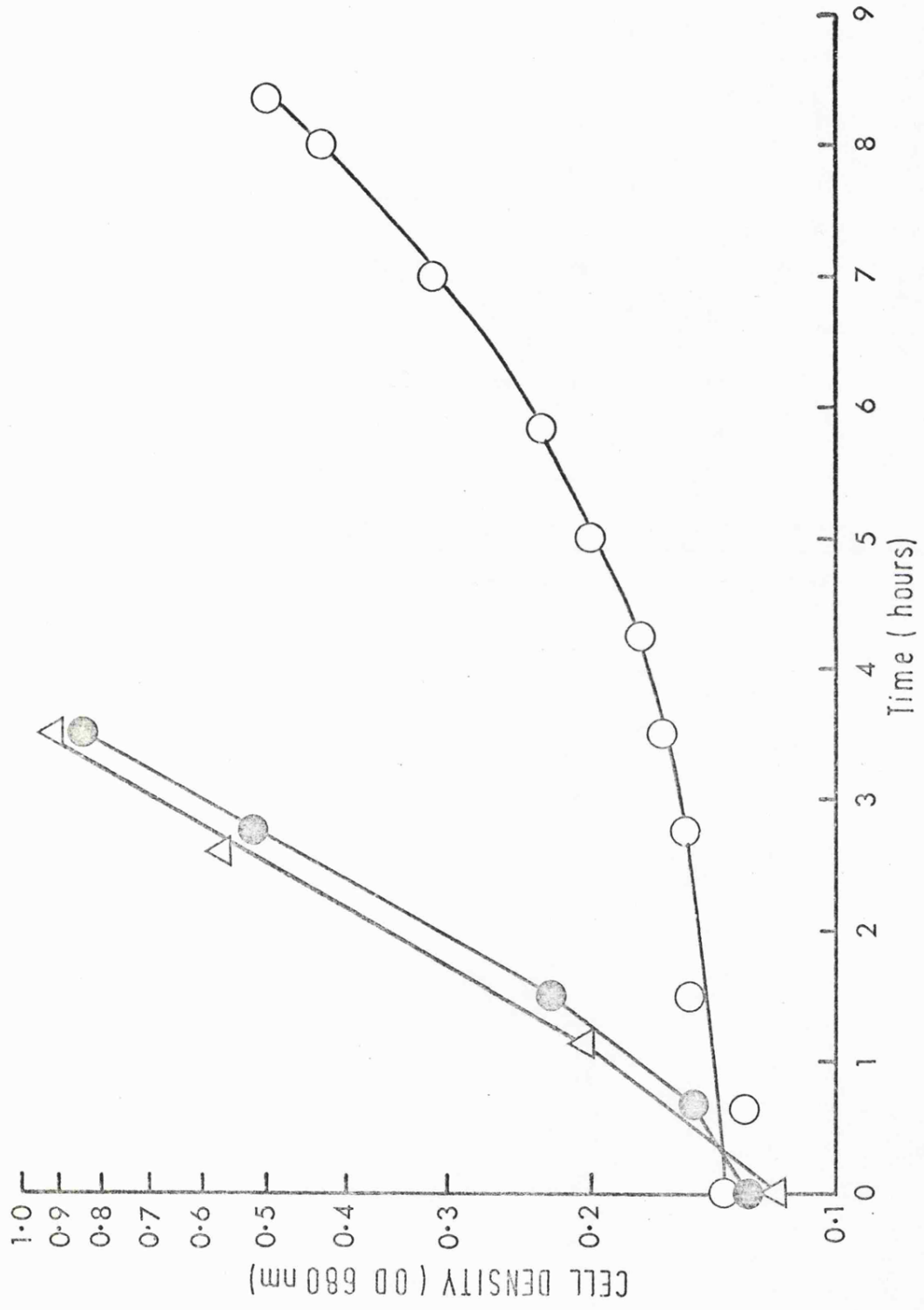
FIGURE 31

Growth of strains P4X (●) and PF1 (○) on transfer to media containing 20 mM gluconate as sole carbon source

The organisms were grown overnight on media containing 20 mM fructose as carbon source. Growth was followed by measuring the optical density of the suspensions at 680 nm. L-methionine was present at 80 $\mu g.ml^{-1}$.

(△) shows the growth rate of the strain PF1 after growth on media containing gluconate (20 mM) for approximately 24 h.

Fig.31.


CELL DENSITY (OD 680 nm)
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
7
8
9
Time (hours)

on 20 mM fructose overnight, harvested and transferred to fresh medium containing 20 mM gluconate, a similar lag and gradual acceleration of growth was observed. Secondly, when such cells which have been induced for growth on gluconate were streaked onto a nutrient agar plate, replica plated onto similar plates containing 10 μM [U-^{14}C]gluconate and tested by the film technique described previously, they did not blacken Kodak 'Blue Brand' X-ray film although (Figure 29) the wild-type cells did. Clearly, the delayed but ultimately virtually normal growth on gluconate was a difficultly inducible and reversible phenomenon, and not a mutational event.

(2) Uptake of gluconate by cell suspensions of the mutant PF1

The observed lag and gradual acceleration of growth of the mutant PF1 when transferred to media containing 20 mM gluconate as sole carbon source is associated with the induction of an uptake system for gluconate (Figure 32). Preliminary studies indicated that the kinetic properties of this uptake system were different from those of the uptake system normally present in wild-type E. coli, such as the strain P4X from which PF1 was derived. Whereas washed suspensions of wild-type cells of E. coli took up [U-^{14}C]gluconate from solutions in the micromolar range (apparent K_m 1-2 x 10^{-5} M) suspensions of gluconate-grown PF1 required much higher gluconate concentrations for uptake at significant rates and the apparent K_m of this process was of the order of 0.1 mM (Figure 33).

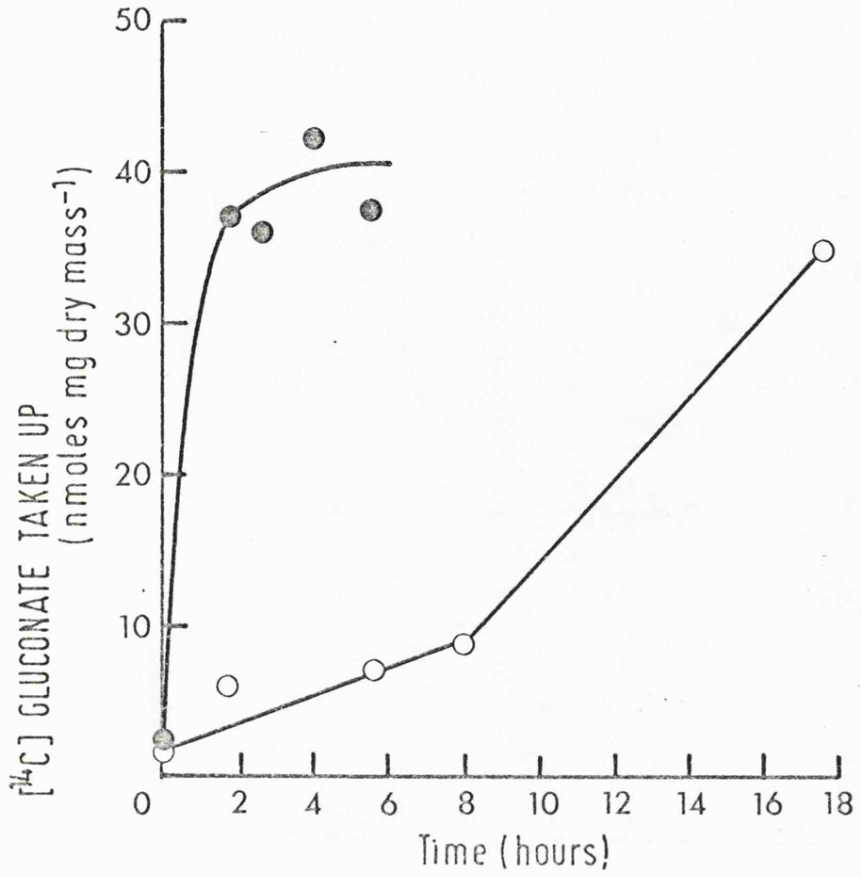
Further investigations with a number of other mutants of gluconate metabolism which are apparently not affected in gluconate

FIGURE 32

Induction of the uptake system for gluconate in cultures of strain P4X (●) and the mutant PF1 (○)

The organisms were grown on 20 mM fructose overnight. The cultures were harvested, washed with minimal medium and resuspended in media containing 10 mM gluconate as carbon source. L-methionine was present at 80 $\mu g.ml^{-1}$. The cell densities and the uptake of 0.1 mM [^{14}C]gluconate were measured in samples taken at known times after transfer to gluconate minimal media.

Fig.32.


[14C] GLUCONATE TAKEN UP
(nmoles mg dry mass-1)
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
Time (hours)

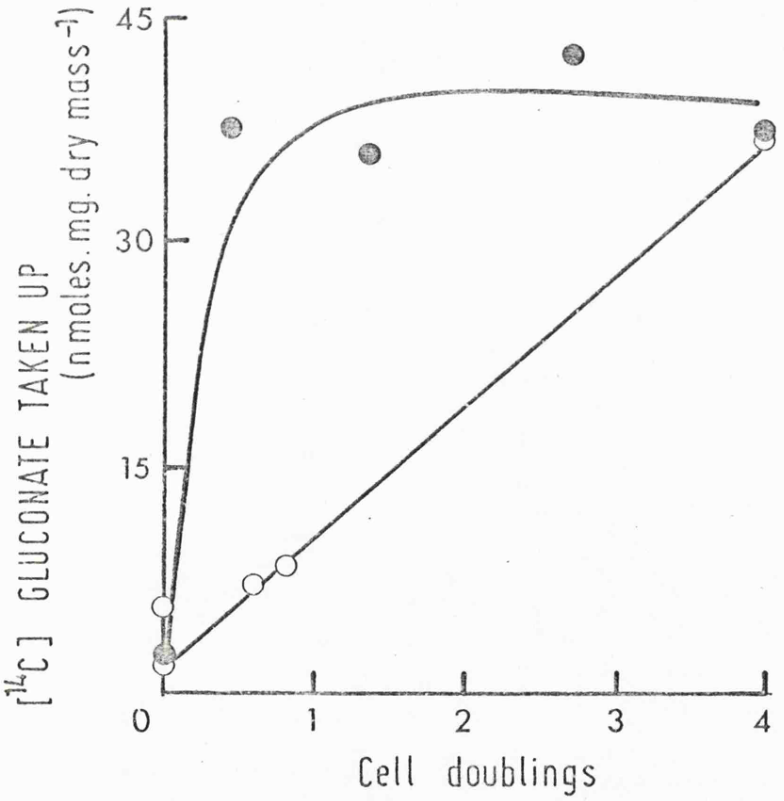
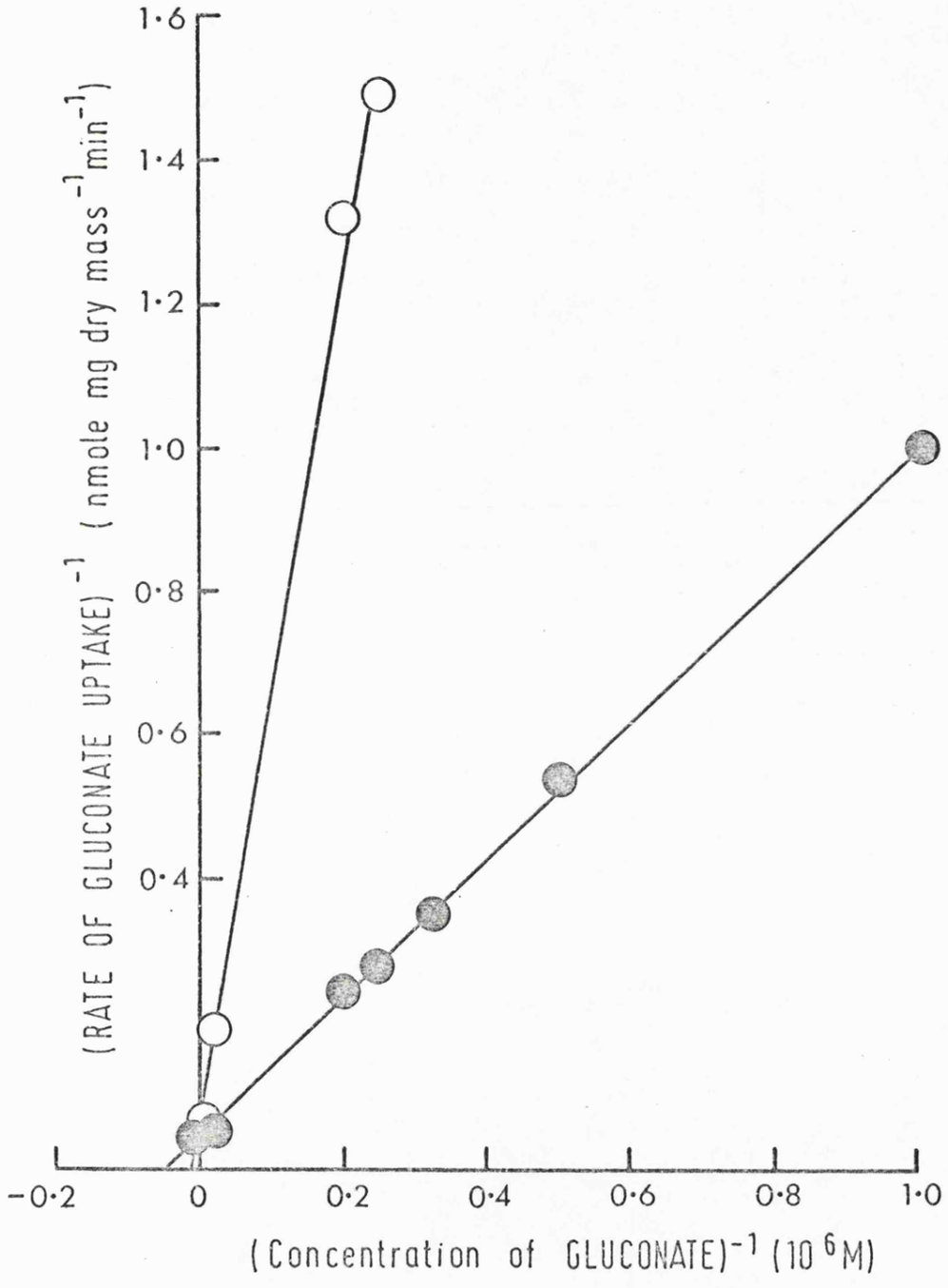

[14C] GLUCONATE TAKEN UP
(nmoles.mg.dry mass-1)
45
30
15
0
1
2
3
4
Cell doublings

FIGURE 33

Effect of gluconate concentration on the rates of gluconate uptake by washed suspensions of gluconate-grown E. coli strains P4X and PF1

The rates of uptake were calculated from the amounts of [^{14}C] incorporated by washed suspensions of cells (0.34 mg dry wt) in the first 30 sec of incubation with the isotopic material; the reciprocals of these rates are plotted against the reciprocals of the gluconate concentration at those points.

Fig.33.


1·6
1·4
1·2
1·0
0·8
0·6
0·4
(RATE OF GLUCONATE UPTAKE)$^{-1}$ (nmole mg dry mass^{-1}min^{-1})
−0·2
0
0·2
0·4
0·6
0·8
1·0
(Concentration of GLUCONATE)$^{-1}$ (10^{6}M)

uptake now suggest that these measurements are incorrect, and that the uptake system induced in the mutant PF1 grown on gluconate has approximately the same K_m for gluconate as do suspensions of similarly grown wild-type cells. The difference in the apparent K_m values which had been previously observed may have resulted from difficulty encountered in attempting to measure the amount of isotope taken up by suspensions of the mutant PF1. At low concentrations of gluconate, the rate of uptake observed was very low but significantly large amounts of isotopic material were bound to the membrane filters even at very early times after the addition of the isotope. For such reasons, the method for the measurement of isotopic material taken up by cell suspensions has been modified (see Experimental, method 2). The sample is now diluted 10-fold with the washing buffer prior to filtering. This dilutes the isotope present in the medium and minimizes both the binding of isotopic material to the membrane filter and the further uptake of the isotope on the membrane filter.

The uptake system induced in the mutant PF1 grown on 20 mM gluconate, as is that present in its parent organism, is highly specific for gluconate. Only unlabelled gluconate competes to any extent and xylonate appears to stimulate this uptake system. Qualitatively similar results have been obtained with the mutant R6 which is impaired in gluconate kinase activity (Chapter II) and with the E.coli strain P4X.

(3) Levels of the enzymes of gluconate catabolism in the mutant PF1

In order to determine whether one or more parameters of

gluconate metabolism were affected in the mutant PF1, the levels of the component enzymes were measured in crude extracts prepared from induced and non-induced cells as described in Materials and Methods.

Table 16 shows that the wild-type strain P4X forms little gluconate kinase and 6-phosphogluconate dehydratase in the absence of gluconate but that these enzymes are synthesised during growth on glycerol in the presence of gluconate, or on gluconate alone. The mutant PF1 forms only the 6-phosphogluconate dehydratase in normal amounts under these conditions: although the gluconate kinase is induced, it has consistently been observed that its specific activity does not exceed 50% of that observed with extracts prepared from wild-type cells even after prolonged growth on gluconate. Table 16 also shows that the ratio of the levels of the enzymes gluconate kinase and KDPG aldolase in the mutant PF1 and the wild-type organism (P4X), after growth on medium containing glycerol plus gluconate or on gluconate alone, vary by approximately a factor of two. The levels of the enzyme 6-phosphogluconate dehydrogenase do not differ greatly when either the wild-type or the mutant PF1 cells are grown in the presence or absence of gluconate.

These results suggest that the mutant PF1 is greatly affected in its ability to induce an uptake system for gluconate. The observation that the induced levels of the enzymes gluconate kinase and KDPG aldolase are approximately 50% of that of the wild-type organism grown under similar conditions indicates that, in the mutant PF1, the activities of these enzymes are affected co-ordinately. That the level of the 6-phosphogluconate dehydratase is apparently not affected in this way may be due to the difficulties encountered in measuring the activity of this enzyme accurately.

TABLE 16 Effect of growth substrates on intracellular levels of enzymes of gluconate catabolism

Carbon source for growth	Organism	Specific activity* of: Gluconate kinase	6-phospho gluconate dehydratase**	Phospho 2-keto 3-deoxygluconate aldolase	6-phospho-gluconate dehydrogenase
20 mM Glycerol	P4X	< 1	20	250	78
	PF1	< 1	27	290	90
20 mM Glycerol + 5 mM gluconate	P4X	110	150	780	95
	PF1	50	110	500	86
20 mM Gluconate	P4X	130	300	1100	120
	PF1	70	330	680	70

The methods used for assay of these enzymes are as described by Pouysségur and Stoeber (1972)

* nmoles.min^{-1}.mg protein^{-1}

** Calculated from dry wt of cells used

(4) Induction of gluconate kinase in the mutant PF1

The lag period of at least 4 h in the growth of PF1 on gluconate is associated with the induction of an uptake system for gluconate. In Chapter II, the properties of the mutant R6 were described; in this organism the uptake system for gluconate is induced normally but the gluconate kinase appears to be impaired. This suggested that a functional gluconate kinase was not necessary for the induction of a normal uptake system. It was of interest, therefore, to determine whether the defect in the mutant PF1 was solely the rate of induction of an uptake system for gluconate or whether the defect also affected the rate of induction of the gluconate kinase.

Figure 34 shows that, in the wild-type (E. coli strain P4X), the uptake system for gluconate and the gluconate kinase are induced co-ordinately. However, in the mutant PF1, the gluconate kinase is induced to high levels much earlier than the uptake system is induced to any significant extent. The gluconate kinase in this mutant almost reaches its fully induced level (which is, as mentioned previously, only 50% of that obtained with wild-type cells) by the end of the lag period whereas the uptake system at this time is only just beginning to be induced.

These results suggest that, in the mutant PF1, the activity of the uptake system is not co-ordinately linked to that of the gluconate kinase.

(5) Repression of the uptake system in the mutant PF1

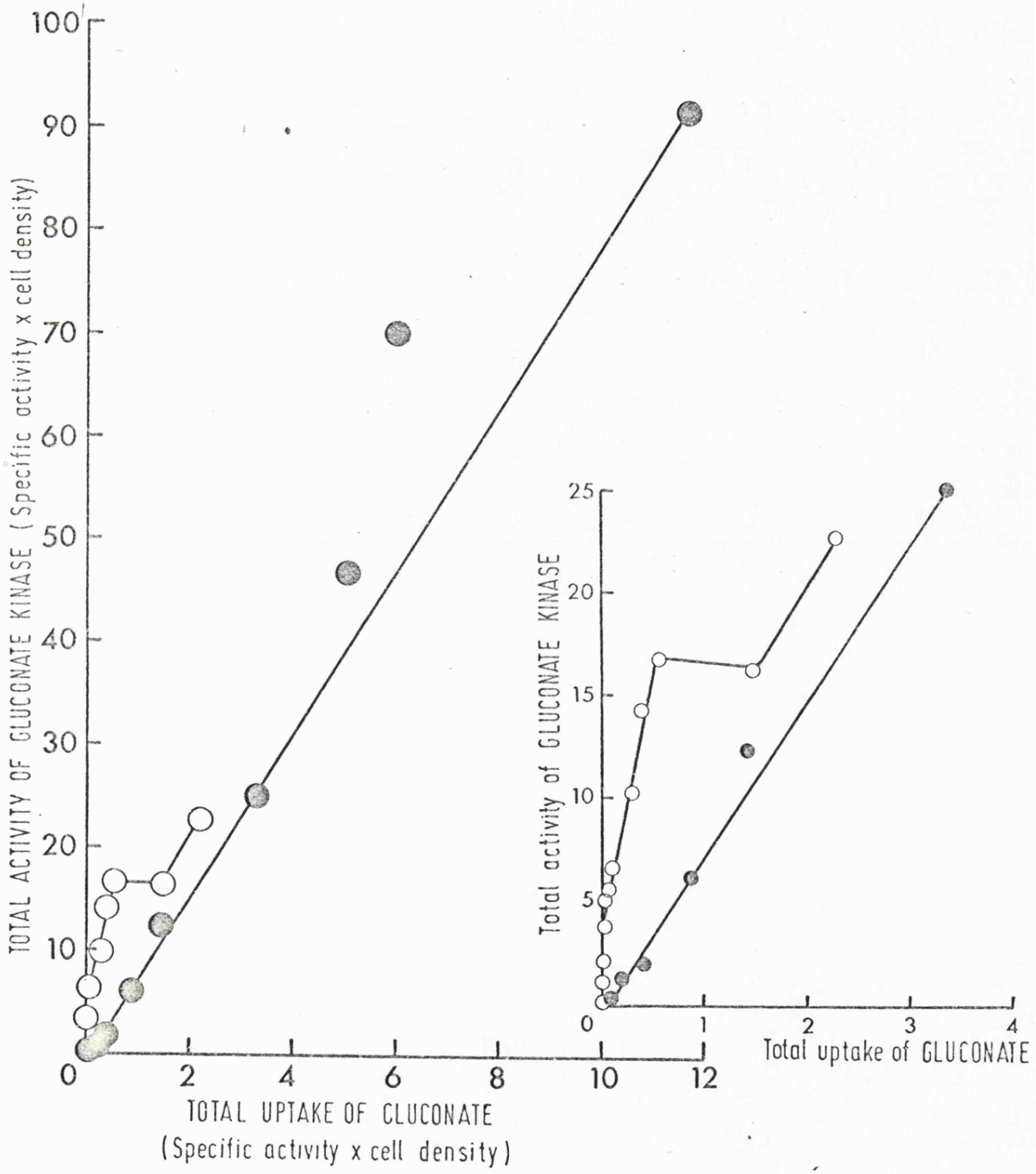
Data presented earlier in this Chapter show that when the

FIGURE 34

Induction of the uptake system for gluconate and gluconate kinase in cultures of strains P4X (●) and PF1 (○)

The organisms were grown on 20 mM fructose overnight. The cultures were harvested, washed with minimal medium and resuspended in media containing 20 mM gluconate as carbon source. L-methionine was present at 80 $\mu g.ml^{-1}$. Cell densities, uptake of 0.1 mM [^{14}C]gluconate and gluconate kinase activities were measured in samples taken at known times after transfer to gluconate minimal media.

Fig.34.


TOTAL ACTIVITY OF GLUCONATE KINASE (Specific activity x cell density)
TOTAL UPTAKE OF GLUCONATE
(Specific activity x cell density)
Total activity of GLUCONATE KINASE
Total uptake of GLUCONATE

mutant PF1 is transferred to growth on gluconate, a lag of 4 h or more is observed during which an uptake system for gluconate is gradually induced. If the mutant PF1 is grown on media containing 20 mM glycerol plus 10 mM gluconate, either for one generation or overnight (conditions under which the gluconate kinase, 6-phosphogluconate dehydratase and 2-keto 3-deoxy 6-phosphogluconate aldolase are induced), the uptake system for gluconate is not induced to any great extent (Figure 35).

This suggests that the uptake system which is induced in the mutant PF1 during prolonged growth on gluconate is either highly repressed or that the mutant PF1 is indeed defective in the induction of an uptake system for gluconate. Data are presented in Table 17, which attempt to distinguish between these two possibilities and show that the uptake system for gluconate induced in the mutant PF1 after previous growth on gluconate appears to be no more susceptible to catabolite repression than that of the wild-type organism grown under the same conditions.

It is clear that the mutant PF1 is defective in its ability to induce an uptake system for gluconate. This uptake system is only induced after prolonged growth on gluconate: when grown on either nutrient broth containing 10 mM gluconate or glycerol (20 mM) plus gluconate (10 mM), this uptake system is not induced. It was of interest, therefore, to determine whether the uptake system once induced in the mutant PF1, after growth on gluconate, was maintained when such cells were transferred to nutrient broth containing 10 mM gluconate. Figure 36 shows that, under such conditions, the induction of the uptake system is maintained in the wild-type organism

opp. 120

FIGURE 35

Uptake of 0.1 mM sodium [^{14}C] gluconate by washed suspensions of strains P4X and PF1

Strain P4X was grown overnight on media containing 20 mM glycerol plus 10 mM gluconate (Θ). Strain PF1 was grown overnight on media containing 20 mM glycerol plus 10mM gluconate (○); 20 mM glycerol (△); 20 mM glycerol followed by the addition of 10 mM gluconate for one generation (▣).

Fig. 35.

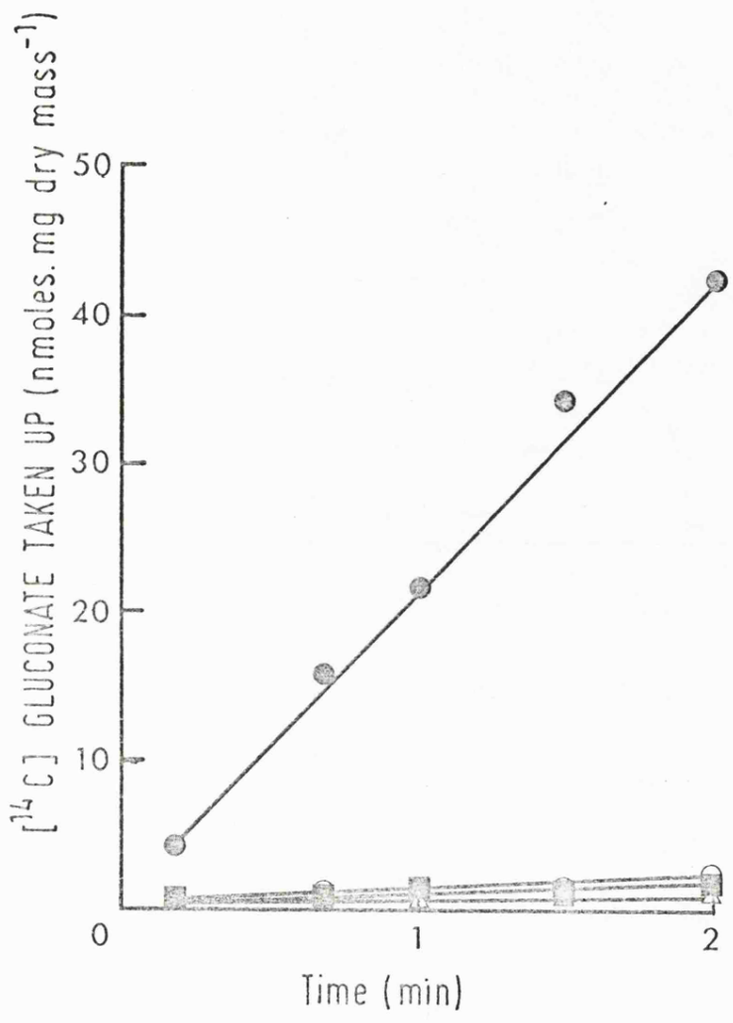

[14 C] GLUCONATE TAKEN UP (nmoles. mg dry mass-1)
50
40
30
20
10
0
1
2
Time (min)

TABLE 17

Levels of the uptake system in the mutant PF1 and the wild-type strain P4X

(*) Carbon source	Level of gluconate uptake system (%) P4X	PF1
20 mM gluconate	100	100
Nutrient broth + 10 mM gluconate	67	47
20 mM glycerol + 10 mM gluconate	88	103
10 mM glucose + 10 mM gluconate	40	38
10 mM glucose + 10 mM gluconate + 5 mM 3'5' cAMP	102	94

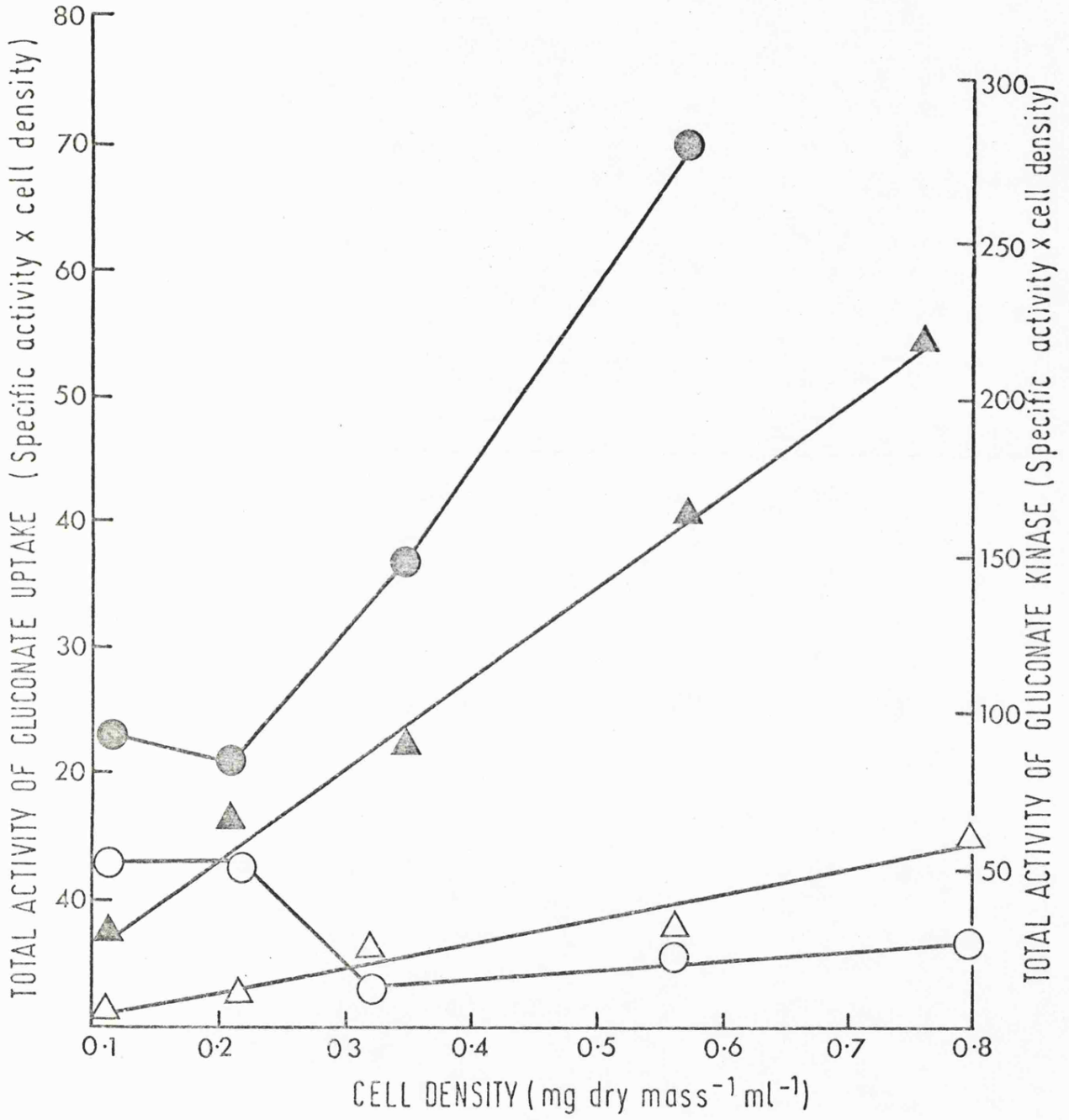
The organisms were grown overnight on media containing 20 mM gluconate as sole carbon source. Next morning, the cells were harvested and transferred to various carbon sources (*) for at least 3 generations.

FIGURE 36

Levels of the uptake system for gluconate and gluconate kinase in the strains P4X and PF1

The organisms were grown on 20 mM gluconate for 36 h. The cultures were harvested and washed with minimal medium. The cell densities, levels of the uptake system for gluconate in strains P4X (●) and PF1 (○), and gluconate kinase [P4X (▲), PF1 (△)], were measured at known times after transfer to nutrient broth containing 20 mM gluconate.

Fig.36.


TOTAL ACTIVITY OF GLUCONATE UPTAKE (Specific activity x cell density)
TOTAL ACTIVITY OF GLUCONATE KINASE (Specific activity x cell density)
CELL DENSITY (mg dry mass⁻¹ ml⁻¹)
80
70
60
50
40
30
20
40
300
250
200
150
100
50
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8

P4X but not in the mutant PF1. The induction of the gluconate kinase, however, is still maintained in the mutant PF1 but to a somewhat lower level than in P4X.

(6) Genetic location of the defect in the mutant PF1

For convenience, the genetic marker specifying the defective uptake system for gluconate will be designated usgA. It was located on the E. coli linkage map with the aid of the organisms shown in Table 14.

The Hfr strain PF1 (which injects its genome counter-clockwise in the order o-thr-argH; Figure 37) was crossed with an F^- recipient A1201 and recombinants were selected for the loss of auxotrophic markers or for the utilisation of appropriate sugars (Figure 37, Table 18). These were then tested for their ability to take up [U-^{14}C]gluconate by the film technique. As can be seen from Table 18A, out of 50 recombinants able to grow on xylose, 18 had acquired the $UsgA^-$ phenotype whereas 58 out of 76 recombinants selected for growth on maltose had done so.

Analysis of the recombinants selected for their ability to utilise maltose (Table 18B), showed that of those recombinants which acquired the ability to utilise both maltose and xylose, 80% had also acquired the $UsgA^-$ phenotype indicating that the location of the usgA marker was in the malA region.

This location was confirmed by phage P1kc mediated transduction. Phage P1-kc, grown on a recombinant obtained from the above cross, which carried the usgA and xyl markers, was used to infect a recipient (Δ70) carrying malA and a deletion of the dct allele

Fig.37.

The genetic map of the Ecoli chromosome indicating the positions of the relevant markers and the point of entry of the Hfr strain PFI.

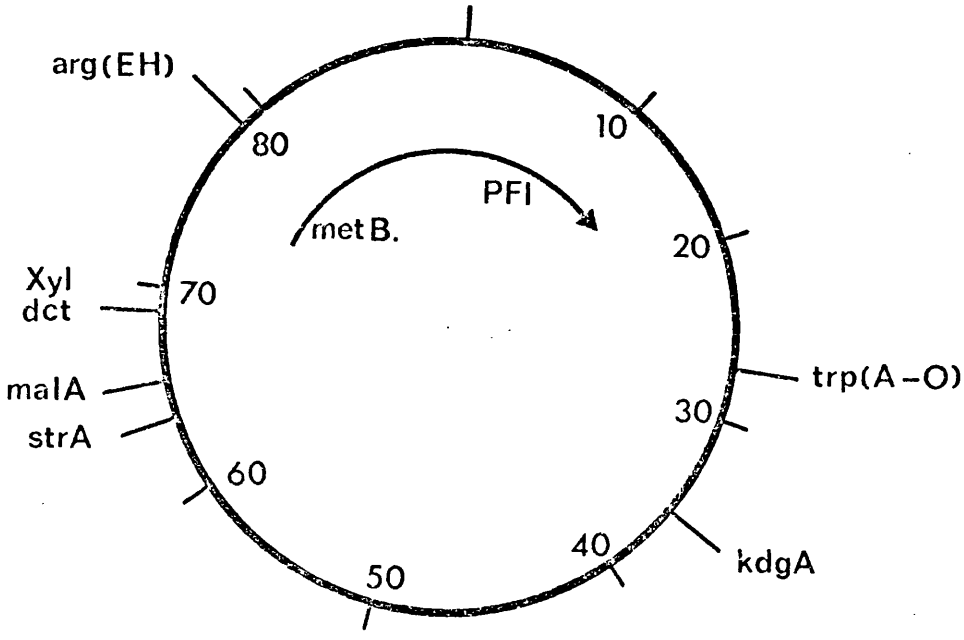


TABLE 18

A Analysis of recombinants obtained from a cross between the Hfr strain PF1 and the recipient A1201

Selected marker	Number of recombinants tested	No. which have acquired the UsgA⁻ phenotype	% which have acquired the UsgA⁻ phenotype
arg	60	5	8
xyl	50	18	36
malA	73	58	80
tryp	72	3	4

B Analysis of recombinants from the above cross which have acquired the ability to utilise maltose

Unselected marker	% which have acquired the UsgA⁻ phenotype
xyl	82.5
str	59
arg	64

C Analysis of the transductants obtained with phage P1 carrying the usgA allele and the recipient Δ70

Selected marker	Number of transductants tested	No. which have acquired the UsgA⁻ phenotype
malA	100	26
dct	210	0

(Kay and Kornberg, 1969) known to be 30% cotransducible with *xyl* (Pouysségur, 1972). As shown in Table 18C, of the transductants which were phenotypically Maltose$^+$, 25% were impaired in gluconate uptake; in contrast, none of the 210 transductants selected for growth on C_4-acids (and hence *dct*$^+$) was affected in gluconate uptake. It is thus probable that the uptake system for gluconate specified by the *usgA* allele is located at about 66 min on the *E. coli* linkage map, close to the *malA* marker.

DISCUSSION

DISCUSSION

The uptake and phosphorylation of gluconate

There are three main ways by which carbohydrates can be taken up by E. coli: group translocation, active transport and facilitated diffusion. It has been demonstrated that the uptake of gluconate is an active transport process, induced by growing the cells on gluconate. Studies of the factors that affect the uptake of carbohydrates by E. coli, which involve exposure of induced cells to isotopically labelled substrate and depend on measurements of the amounts of isotope that appear in the cells, measure not only the uptake process per se but also the incorporation of metabolic derivatives of the substrate into cell components. In order to measure the uptake process only, it would be desirable to use either the isotopically labelled substrate under conditions where it is not metabolised, or an analogue of the substrate which can be taken up by the cells but cannot be metabolised further. Since no such analogue of gluconate had been found, it was decided to obtain mutants impaired in the next step of gluconate utilisation, namely a mutant impaired in gluconate kinase activity. The E. coli strain R6, the properties of which have been discussed in Chapter III, is such a mutant and its isolation has made possible a study of the kinetics of the uptake process with washed whole cell suspensions: the apparent K_m for the uptake of gluconate is of less than 50 μM and the measured V_{max} is approximately 25 nmoles of gluconate.min^{-1}. mg dry mass^{-1}. These parameters are very similar to those found

with wild-type cells, which suggests that the uptake process is the rate limiting step in gluconate utilisation and that, provided measurements are made over a brief period of time, kinetic measurements of gluconate uptake may be made even with wild-type strains.

A third method of obtaining information on the active transport of a nutrient is to measure its uptake by membrane vesicles, where very little (if any) further metabolism of the substrate can take place. It has been demonstrated that membrane vesicles, prepared from appropriately induced cells, are able to transport gluconate in the presence of various electron donors at rates, and to an extent, similar to those obtained with washed cell suspensions, thus showing that no indispensable components of the uptake process have been lost in the preparation of membrane vesicles. The data presented suggest that L-lactate is a better source of energy than D-lactate which appears to be at variance with Kaback's view that E. coli vesicles are optimally energized by D-lactate. However, Kaback (1974) pointed out that the optimum source of energy does depend on the system under study. The slight effect of arsenate on the uptake process is consistent with the view that the uptake of gluconate is an active process not directly linked to the energy available from ATP; but that some inhibition of uptake is observed in the presence of arsenate may reflect that ATP hydrolysis is able to act as an alternative energy source.

The precise mechanism of active transport is not clear at present. Evidence is accumulating (for review, see Kaback, 1974)

that the active transport of some carbohydrates may be coupled to the movement of protons. The only published data on the gluconate system is that of Robin and Kepes (1973) who have studied the ion balance during gluconate uptake. They suggest that gluconate is transported in one of three ways: as undissociated gluconic acid; as negatively charged gluconate ion transported with an H^+ ion; or that there is the countertransport of a gluconate ion against an OH^- ion.

When gluconate has entered the cell, gluconate kinase catalyses its phosphorylation to 6-phosphogluconate with concomitant cleavage of ATP to ADP. Attempts to obtain mutants of E. coli with no detectable gluconate kinase activity have proved difficult: it may be, as reported by Hung et al. (1970) in a very brief abstract, that there are two gluconate kinases in E. coli. According to these workers, the two kinases differ in their heat stability, substrate affinity and genetic control; one gluconate kinase is said to be synthesised during anaerobic growth on gluconate but both kinases are present during aerobic growth on this substrate, since extracts prepared from aerobically grown cultures show biphasic thermal denaturation patterns. The K_m for gluconate of the aerobic gluconate kinase is reported to be 3×10^{-4} M and that for the anaerobic gluconate kinase, which is more stable to thermal denaturation at 49°C 7.5×10^{-5} M. As no publications have appeared containing details of these experiments, it is difficult to assess their significance; furthermore, we have been unable to obtain biphasic thermal denaturation curves for the gluconate kinase activity from aerobically-grown E. coli and work currently underway in this laboratory, by

Dr. B. Bächi, challenges Hung et al.'s interpretation.

At present, we have no evidence for two uptake systems for gluconate in E. coli. de Zwaig and colleagues (de Zwaig et al., 1973) isolated a mutant (M2) after nitrosoguanidine treatment whose aerobic growth shows a lag of 5 h after transfer to gluconate minimal media. When the various components of the gluconate system were assayed, these authors observed that the mutant M2 showed a lowered activity of gluconate transport, gluconate kinase and 6-phosphogluconate dehydratase. However, inspection of the data reported by de Zwaig et al. (1973) indicates that the reported activities of the components of the gluconate system which were measured are not, in fact, very different from activities observed with extracts of wild-type organisms. The greatest difference recorded was a 50% decrease in the level of the 6-phosphogluconate dehydratase. Since it is difficult to measure the activity of this enzyme, anyway, these results may not be very significant.

Although the mutant M2 appears to have similar properties to the mutant PF1 described in Chapter IV (the genes coding for both lesions map in the malA region on the E. coli chromosome), there is one major difference: revertants isolated from these two organisms have different properties. All of 20 independent revertants of the mutant PF1, which now grew normally on gluconate, were still inducible for gluconate uptake, measured at a concentration of external gluconate characteristic of a system with an apparent K_m of 2×10^{-5} M. The revertants isolated by Zwaig et al. (1973) from the mutant M2, however, are all constitutive for a low affinity transport system (K_m for gluconate: 5×10^{-4} M), the gluconate kinase (they do not report

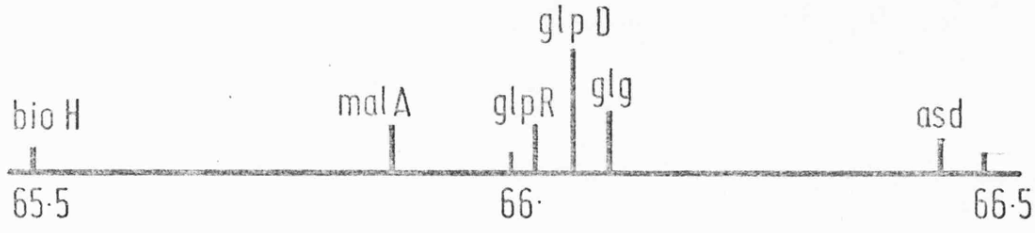
which one) and the 6-phosphogluconate dehydratase. Their data suggest that these revertants have acquired a second mutation which maps near asd (67 min on the E. coli linkage map). The high affinity transport system (K_m for gluconate: 2×10^{-5} M) which was expressed in the mutant M2 is no longer expressed in the revertants.

The study of the mutant M2 led de Zwaig and colleagues to analyse a number of organisms with known deletions in the malA region, in an attempt to obtain information on the functions specific for the gluconate system coded for by genes in this region of the E. coli chromosome. They have concluded that genes affecting a low affinity transport system for gluconate and a gluconate kinase activity are located near asd (Figure 38), a regulatory gene very close to asd exerts negative regulation since its loss leads to Edd constitutivity; and finally, a gene or genes affecting the transport of gluconate at lower concentrations (the high affinity transport system) is located between the genes bioH and malA.

I have not attempted a fine structure genetic analysis of the gluconate system and thus am not in a position to comment on the results of Zwaig and colleagues. The only organism available at present which was originally thought to possess a low affinity uptake system for gluconate (the mutant PF1) has since been shown to possess an uptake system with a similar affinity for gluconate ($K_m \leqslant 50$ µM) as that present in wild-type cells. The organism PF1 is impaired in its ability to induce normally an uptake system for gluconate. The gene coding for the lesion is also cotransducible with malA (Faik and Kornberg, 1973). Furthermore, our colleague, Dr. B. Bächi, has recently obtained evidence that a gene coding for a mutation which

Fig 38

Part of the genetic map of E.coli K12 indicating the markers in the mal A region.



results in the constitutive synthesis of the uptake system, the gluconate kinase and the 6-phosphogluconate dehydratase, also maps in the malA region.

6-phosphogluconate dehydratase (Edd)

The enzyme 6-phosphogluconate dehydratase (Edd) catalyses the dehydration of 6-phosphogluconate to KDPG. There is no published evidence for more than one species of Edd. The gene coding for Edd activity has been identified as a structural gene and has been located at 36 min on the E. coli genetic map: it is 97% cotransducible with the gene coding for glucose 6-phosphate dehydrogenase activity (Zwf) (Peyru and Fraenkel, 1968).

The enzyme from P. fluorescens has been purified by Kovachevich and Wood (1955a) but there are no published data on its purification from E. coli. We have found that the enzyme from E. coli is very unstable: it appears to require substrate activation and is inactivated upon dilution. Our results suggest that the addition of 6-phosphogluconate, bovine serum albumin and Mg^{2+} help to prevent the inactivation of the enzyme. Although various methods to stabilise the enzyme have been tested, under no condition has it proved possible to preserve the activity of the 6-phosphogluconate dehydratase overnight in crude extracts and, therefore, we have not obtained further information on this enzyme.

KDPG aldolase

The enzyme KDPG aldolase catalyses the cleavage of KDPG to

pyruvate and glyceraldehyde 3-phosphate. Pouysségur and Stoeber (1971a) have purified extracts obtained from cells grown on gluconate, galacturonate and glucuronate by DEAE-Sephadex chromatography; data obtained from thermal denaturation studies on such extracts led them to propose that the KDPG-aldolase activity is in a single homogeneous protein.

As shown in Chapter I, the gene specifying the enzyme KDPG aldolase is highly cotransducible (96%) with that specifying 6-phosphogluconate dehydratase activity (Faik et al., 1971; Fradkin and Fraenkel, 1971; Pouysségur and Stoeber, 1971b).

It has been demonstrated that organisms devoid of this enzyme grow very poorly on gluconate minimal media (doubling time > 8h) and are unable to grow on either galacturonate or glucuronate. The addition of gluconate to a growing culture of such a mutant leads to a rapid slowing down of growth. KDPG is accumulated within the cells to high concentrations and is excreted into the medium. These unexpected observations with organisms possessing 6-phosphogluconate dehydrogenase (Gnd) activity suggested that in such mutants (devoid of Kga activity) the pentose phosphate pathway may not be functioning normally. It has indeed been observed that KDPG is a very effective competitive inhibitor of 6-phosphogluconate for the active sites on 6-phosphogluconate dehydrogenase (Chapter I).

Induction of the enzymes of gluconate catabolism

The enzymes involved in the catabolism of gluconate, that is, the uptake system, the gluconate kinase(s), 6-phosphogluconate

dehydratase and KDPG aldolase, are inducible by growing E. coli on gluconate minimal medium.

Gluconate appears to be the only compound which will induce the uptake system. The inducer is not necessarily gluconate itself but may be a compound readily formed from gluconate [for instance, lactose is able to induce the lactose operon but the true inducer has been shown to be allo-lactose (Jobe and Bourgeois, 1972)]. Although the mutant PF1 is impaired in its ability to induce an uptake system for gluconate normally, the small amounts of gluconate taken up during the lag period appear to be sufficient to induce the gluconate kinase, albeit at a lower level than is present in the wild-type strain P4X. Thus in this mutant, the induction of an uptake system for gluconate can be dissociated from the induction of the gluconate kinase(s). The converse situation has also been found - the mutant R6 is able to induce an uptake system for gluconate normally but not the gluconate kinase(s).

Since 6-phosphogluconate cannot be taken up by E. coli, there is no direct way of showing whether this compound is able to induce the gluconate kinase(s) (and/or 6-phosphogluconate dehydratase and the KDPG aldolase). Kornberg and Soutar (1973), using an organism which lacks PEP-synthase, glucose 6-phosphate isomerase and 6-phosphogluconate dehydrogenase, have shown that where glucose 6-phosphate can only be metabolised via the Entner-Doudoroff pathway, the 6-phosphogluconate dehydratase can be induced. Under these same conditions, neither the uptake system for gluconate, nor the gluconate kinase(s) are induced. This would suggest that 6-phosphogluconate derived from glucose 6-phosphate is not an inducer of the

gluconate kinase but it is able to induce the 6-phosphogluconate dehydratase. The level of the KDPG aldolase was not tested in these experiments since high basal levels of this enzyme are always present in cells.

The KDPG aldolase can be induced 3-4 fold by growing cells on gluconate or the hexuronates, galacturonate or glucuronate (Pouysségur and Stoeber, 1971a). These workers have also shown (Pouysségur and Stoeber, 1972) that in an organism devoid of 6-phosphogluconate dehydratase activity, gluconate is still able to increase the basal levels of the aldolase by a factor of 3-4 to the induced level. Their results indicate that the inducer for the KDPG aldolase is probably 6-phosphogluconate. During growth on hexuronates, the inducer for the aldolase may be 2-keto 3-deoxy gluconate (KDG).

In summary, it may be proposed that the likely inducer for the uptake system for gluconate and for the gluconate kinase(s), is gluconate itself, and 6-phosphogluconate is probably the inducer for the 6-phosphogluconate dehydratase and the KDPG aldolase.

Genetic control of the Entner-Doudoroff Pathway

As all the enzymes of the Entner-Doudoroff pathway are induced by growing cells on gluconate, one of the aims of this work was to establish whether the genes controlling the synthesis of these enzymes are controlled as an operon.

The data presented clearly show that at least three regions of the chromosome contain genes involved in gluconate utilisation in *E. coli*. Firstly, *edd* and *kga* (later designated *kdgA* by Pouysségur and Stoeber (1972) are highly cotransducible and map at approximately

36 min on the *E. coli* genetic map. Although it appears likely that 6-phosphogluconate may be the true inducer of 6-phosphogluconate dehydratase and KDPG aldolase, the genes involved are not regulated as an operon: the level of Edd in non-induced cells is extremely low whereas there is a high basal level of the KDPG aldolase. The two enzymes are, however, induced by growing the cells on gluconate minimal media. Available evidence suggests that these are the structural genes for the synthesis of these enzymes (Pouysségur and Stoeber, 1972b). Secondly, a region of the chromosome near *malA* (65.7 min) seems to be concerned with the uptake system(s) for gluconate and perhaps a gluconate kinase. The mutant PF1 is unable to induce normally an uptake system for gluconate, but the activities of the remaining enzymes of the Entner-Doudoroff pathway all appear to be affected co-ordinately. These results suggest that, in this region of the chromosome, there are genes coding for the ability of *E. coli* to induce an uptake system for gluconate and perhaps a gene or genes for the control of the gluconate kinase, 6-phosphogluconate dehydratase and the KDPG aldolase. Zwaig *et al.* (1973) have shown that a mutation in this region can lead to a derepression of the synthesis of the uptake system for gluconate, the gluconate kinase and the 6-phosphogluconate dehydratase; the level of the KDPG aldolase in such organisms was not measured. Thirdly, a region of the chromosome close to the gene coding for fructose diphosphatase activity is somehow involved in the utilisation of gluconate in *E. coli* (strain R6 has a mutation in a gene located in this area). The function of this gene is not understood at present.

It may be concluded that, firstly, in *E. coli* gluconate is

only metabolised by the Entner-Doudoroff and pentose-phosphate pathways since mutants lacking 6-phosphogluconate dehydratase and 6-phosphogluconate dehydrogenase are unable to grow at all on gluconate. Secondly, there is an uptake system specific for gluconate which is not functionally linked to the kinase. The uptake of gluconate is an active transport process induced by growing the cells in the presence of gluconate. Finally, although all the enzymes of the Entner-Doudoroff pathway are induced by growing the cells on gluconate, only some of the genes specifying the synthesis of these enzymes are linked and may form an operon.

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ISOLATION AND PROPERTIES OF *E. COLI* MUTANTS AFFECTED IN GLUCONATE UPTAKE

Pelin FAIK and H.L. KORNBERG

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, England

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1. Introduction

Enteric bacteria convert external gluconate to intermediates of central metabolic pathways by a process that involves at least four steps. Gluconate must first be taken up by the cells. There is evidence that this uptake is catalysed by an inducible process that, in vesicles prepared from *E. coli*, can be coupled to the oxidation of D-lactate [1]; it has also been briefly reported [2] that this process can be catalysed by two distinct transport systems that differ in their affinity for gluconate. Gluconate kinase (EC 2.7.1.12) catalyses the phosphorylation of the gluconate thus taken up with concomitant conversion of ATP to ADP [3]; it has been reported [2,4] that *E. coli* also contain two gluconate kinases. 6-Phosphogluconate thus formed can then be catabolized via pentose phosphates, and via the Entner–Doudoroff pathway [5] which has been shown [6] to be of predominant importance for gluconate dissimilation by *E. coli*.

Although the isolation and properties of mutants impaired in one or more component steps of 6-phosphogluconate catabolism have been described [7–9], mutants impaired in the uptake and phosphorylation of gluconate have been reported only in brief abstracts [2,4]. It is the main purpose of the present paper to remedy this deficiency. A simple procedure is described for the isolation, recognition and analysis of mutants that are impaired in one gluconate uptake system although they can be induced to form a second: we propose to designate the genetic marker specifying this missing uptake system for gluconate *usgA*. These mutants also form gluconate kinase to a somewhat lower specific activity than do wild-type cells: it is possible that the process used for their isolation favours the selection of polar mutants or of organisms carrying deletions of *usgA* and one of the two postulated gluconate kinases.

2. Experimental

The organisms used in this work are listed in table 1. The procedures used for their growth, for measurement of the rates of uptake of labelled substrates by washed cell suspensions, and for genetic analyses, were as previously described [10–12]. Methods for the isolation of mutants impaired in gluconate utilization are reported in the following section.

3. Results and discussion

3.1. *Isolation of mutants*

The addition of gluconate (or of other substances, such as glucuronate or galacturonate, that are also metabolically converted to phospho-2-keto-3-deoxygluconate) to mutants devoid of the aldolase that catalyses the cleavage of phospho-2-keto-3-deoxygluconate to pyruvate and glyceraldehyde 3-phosphate [13], leads to the accumulation of the phosphorylated ketoacid and consequent growth stasis [8,9,14]. For the same reason, such *kdgA* mutants do not grow on solid media if these contain gluconate as well as another, normally utilizable, carbon source. However, when such plates, of media containing 20 mM glycerol and 5 mM gluconate, were inoculated with ca. 10^8 cells of *kdgA*-mutant A314 (table 1)

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Table 1
Organisms used in this study.

Strain	Derivation or reference	Genetic markers*	Response to strepto-mycin	Mating type
P4X	[21]	*metB*	S	Hfr
A314	[21]	*metB, kdgA*	S	Hfr
PF1	A314	*metB, usgA*	S	Hfr
A1201	PA309	*arg(EH), trp, xyl, malA, gal, kdgA*	R	F^-
Δ70	[PA309 × P10-*dct1*] [19, 20]	*metB, trp, his, arg(HCBE) thr, leu, malA,* Δ[*dct–kdgK*]	R	F^-

* Where possible, the abbreviations used are those listed by Taylor [18]. In addition, *usg* indicates the inability to take up gluconate described in this paper; *kdgA* and *kdgK* specify, respectively, the structural genes for phospho-2-keto-3-deoxygluconate aldolase and 2-keto-3-deoxygluconate kinase; Δ[*dct–kdgK*] a deletion extending from the *dct* to the *kdgK* markers on the *E. coli* genome.

and were incubated at 30° or 37° for 2–3 days, approx. 10^2 colonies appeared that were now resistant to the presence of gluconate but were still devoid of phospho-2-keto-3-deoxygluconate aldolase activity, as shown by their inability to grow on hexuronates as sole carbon source. Indeed, these organisms resistant to the presence of gluconate were still sensitive to the presence of hexuronates: growth on 20 mM glycerol was arrested by the addition of 5 mM glucuronate or galacturonate although it was unaffected by the addition of 5 mM gluconate. The change undergone by strain A314 in acquiring this resistance to gluconate thus involved a mutation that prevented the formation of the (still toxic) phospho-2-keto-3-deoxygluconate from gluconate in the medium.

There are a number of ways in which the pathway from external gluconate to intracellular phospho-2-keto-3-deoxygluconate via 6-phosphogluconate, can be interrupted; mutants affected in one or more of these steps can be readily recognized by an adaptation of the technique first described by Zwaig and Lin [15] and subsequently used for the detection of mutants impaired in the uptake of methyl α-glucoside [16] or constitutive for the uptake of hexose phosphates [17]. In this procedure, mutants are screened for their ability to take up [^{14}C]gluconate during their growth on plates of nutrient agar that also contain the isotopic material in low concentration (≤10 μM). By the use of this technique, many colonies were obtained from strain A314 that did not take up [^{14}C]gluconate under these conditions. One of these was converted to *kdgA*$^+$ by phage P1-mediated transduction [8] and the resultant organism, designated PF1, was used for further study. (A culture of strain PF1 has been deposited with the *E. coli* Genetic Stock Center, Department of Microbiology, Yale University Medical School, New Haven, Conn., USA).

3.2. *Properties of the mutant PF1*

As expected from the method used for its recognition, the mutant PF1 was markedly impaired in its ability to take up [^{14}C]gluconate. When incubated with 0.1 mM sodium [U-^{14}C]gluconate at 30°, a washed suspension of the mutant, that had been grown for many generations on a medium containing 20 mM glycerol and 10 mM sodium gluconate, took up less than 10% of the isotope that was taken up by a similarly-treated suspension of the wild-type *E. coli* (fig. 1). Since this experimental procedure measures both the entry of labelled gluconate into the cells and its incorporation into stable cell components, it was possible that this impairment of uptake was associated with a dysfunction of transport, of gluconate kinase, or both.

The presence of a lesion in strain PF1 that affects more than one parameter of gluconate utilization is indicated by the growth properties of the mutant, and by the specific activities at which enzymes of

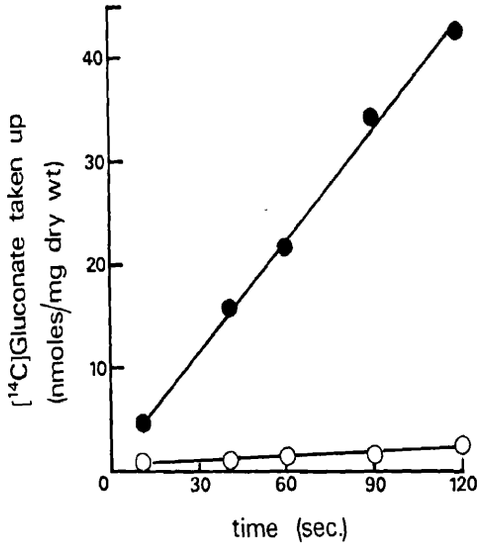

Fig. 1. Uptake of 0.1 mM sodium [U-^{14}C]gluconate by washed suspensions of *E. coli* strains P4X (•) and PF1 (○). The organisms had been grown overnight on media containing 20 mM glycerol plus 10 mM gluconate as carbon source.

gluconate catabolism are formed during growth. Although, like wild-type *E. coli*, the mutant PF1 grew readily on glucose, glycerol, glucuronate or on a variety of gluconeogenic substrates, cultures of strain PF1 required over 7 hr for one cell doubling to occur when the organism was transferred to aerobic growth at 37° with gluconate as sole carbon source. However, a progressive acceleration of growth rate was observed throughout this period, which continued until, after a day or more, the doubling time of the cells had approached that of wild-type *E. coli*. This was not the consequence of selecting a further mutant, or a revertant to wild-type, but was associated with the induction of a gluconate uptake system with properties different from those normally predominating in wild-type *E. coli* grown on gluconate (fig. 2). In contrast to such suspensions of wild-type cells, which readily take up [^{14}C]gluconate from solutions in the micromolar range (apparent $K_m = 1 \times 10^{-5}$ M), suspensions of gluconate-grown PF1 require much higher gluconate concentrations for uptake at significant rates, and the apparent K_m of this process is of the order of 0.1 mM.

The slow adaptation to an alternative and apparently more concentration-dependent mode of gluconate uptake is indicated also by the induction of enzymes of gluconate catabolism (table 2). Whereas the wild-

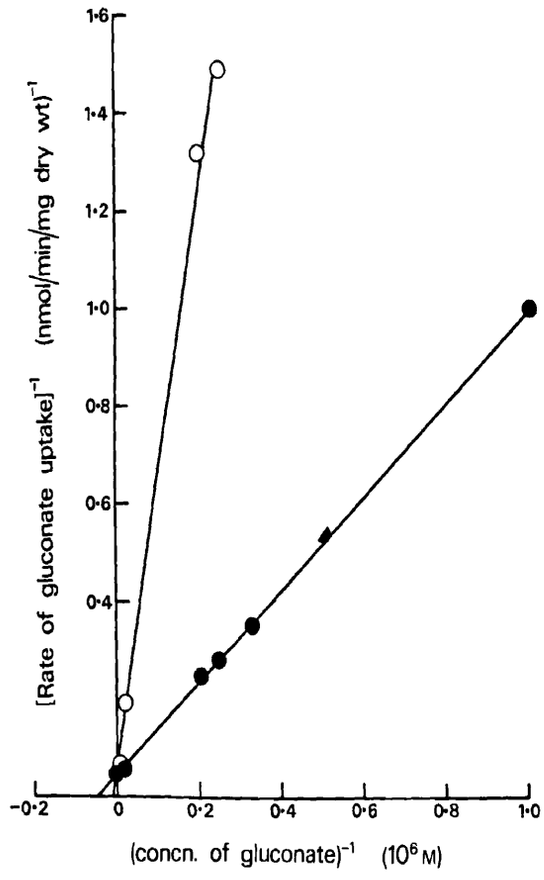

Fig. 2. Effect of gluconate concentration on the rates of gluconate uptake by washed suspensions of gluconate-grown *E. coli* strains P4X (•) and PF1 (○). The rates of uptake were calculated from the amounts of ^{14}C incorporated by washed suspensions of cells (0.34 mg dry wt) in the first 30 sec of incubation with the isotopic material; the reciprocals of these rates are plotted against the reciprocals of the gluconate concentration at those points.

type strain P4X, which forms little gluconate kinase and 6-phosphogluconate dehydratase in the absence of gluconate, inducibly synthesizes both enzymes during growth on glycerol in the presence of gluconate, or on gluconate alone, the mutant PF1 forms only the dehydratase in normal amounts under these conditions: although gluconate kinase activity is induced to high levels, its specific activity does not exceed half that observed with wild-type cells, even after prolonged growth on gluconate. On the other hand, the variations with growth substrate in the levels of other enzymes of gluconate catabolism are not grossly dissimilar in mutant and wild-type cells. These observations support the view [2,4] that *E. coli* contains two alleles that specify transport systems, and two

Table 2
Effect of growth substrates on intracellular levels of enzymes of gluconate catabolism.

Carbon source for growth	Organism	Specific activity* of: Gluconate kinase	6-Phospho-gluconate dehydratase**	Phospho 2-keto 3-deoxygluconate aldolase	6-Phospho-gluconate dehydrogenase
20 mM Glycerol	P4X	<1	20	250	78
	PF1	<1	27	290	90
20 mM Glycerol plus 5 mM gluconate	P4X	110	150	780	95
	PF1	50	110	500	86
20 mM Gluconate	P4X	130	300	1100	120
	PF1	70	330	680	70

The methods used for assay of these enzymes are as described by Pouysségur and Stoeber [21].

* nmoles·min^{-1}·mg^{-1} of protein.
** Calculated from dry wt. of cells used.

that specify kinase activity; it would appear that mutant PF1 is impaired in a region of the genome that governs the formation both of one (high-affinity, low K_m) uptake system and one form of gluconate kinase. This pleiotropic lesion might be associated with a polar mutation or a small deletion: the extraordinarily low reversion rate of mutant PF1 favours the latter.

3.3. *Location of the* usgA-*marker on the E. coli genome*

The Hfr-strain PF1 was crossed with a recipient strain A1201, recombinants were selected for the utilization of appropriate sugars or for the loss of auxotrophic markers, and these were tested for gluconate uptake by the film technique. It was found that, of 50 recombinants able to grow on xylose, 18 had acquired the UsgA$^-$-phenotype, whereas 58 out of 76 recombinants selected for growth on maltose had done so. Analysis of one *usgA*-recombinant still unable to utilize maltose or xylose supported the location of the *usgA*-marker between those specifying response to streptomycin (64 min on the linkage map [18]) and the ability to grow on xylose (70 min), and close to the *malA*-marker (66 min). This location was confirmed by transduction in which phage P1, grown on a recombinant carrying the *usgA* and *xyl* markers, infected a recipient carrying *malA* and a deletion known to be 30% co-transducible with *xyl* [19] of the *dct*-allele [20]. Of the transductants phenotypically Maltose$^+$, about a quarter were impaired in gluconate uptake; in contrast, none of the 210 transductants selected for growth on C_4-acids (and hence *dct*$^+$) were affected in gluconate uptake. It is thus probable that the high-affinity uptake system for gluconate specified by the *usgA*-allele is located at about 66 min on the *E. coli* linkage map, close to the *malA* marker. It is also evident that the properties of mutant PF1, its ability apparently inducibly to form a second (low-affinity) uptake system for gluconate, and the genetic location of the *usgA*-marker, are so closely similar to those briefly reported by de Zwaig et al. [2] as to make it likely that they are mutants of the same system.

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Utilization of Gluconate by *Escherichia coli*

UPTAKE OF D-GLUCONATE BY A MUTANT IMPAIRED IN GLUCONATE KINASE ACTIVITY AND BY MEMBRANE VESICLES DERIVED THEREFROM*

By J. M. POUYSSÉGUR,† PELIN FAIK and H. L. KORNBERG
Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, U.K.

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1. From *Escherichia coli* strain K2.1.5°.8.9, which is devoid of 6-phosphogluconate dehydrogenase (*gnd*) and 6-phosphogluconate dehydratase (*edd*) activities, a mutant R6 was isolated that was tolerant to gluconate though still *edd*$^-$, *gnd*$^-$. 2. Measurements of the fate of labelled gluconate, of the conversion of gluconate into 6-phosphogluconate, and of the induction of gluconate kinase by the two organisms show that, although both inducibly form a gluconate-transport system, strain R6 is impaired in its ability to convert the gluconate thus taken up into 6-phosphogluconate; it was therefore used for study of the kinetics and energetics of gluconate uptake. 3. Suspensions of strain R6 induced for gluconate uptake took up this substrate via a 'high affinity' transport process, with K_m about 10μM and $V_{max.}$ about 25nmol/min per mg dry mass; a 'low affinity' system demonstrated to occur in certain *E. coli* mutants was not induced under the conditions used in this work. 4. The uptake of gluconate was inhibited by lack of oxygen and by inhibitors of electron transport; such inhibitors also promoted the efflux of gluconate taken up. 5. Membrane vesicles prepared from strain R6 also manifested these properties when incubated with suitable electron donors, at rates similar to those observed with whole cells. 6. The results indicate that the active transport of gluconate into the cells is the rate-limiting step in gluconate utilization by *E. coli*, and that the mechanism of this process can be validly studied with membrane vesicles.

The first step in the utilization of gluconate by *Escherichia coli* is its entry from the medium into the cells. Since mutants devoid of components of the phosphotransferase system (Kundig *et al.*, 1964) grow readily on gluconate, this step is not accompanied by the obligatory phosphoenolpyruvate-dependent phosphorylation of the substrate that accompanies the uptake of some carbohydrates (for review, see Roseman, 1972; Kaback, 1972; Kornberg, 1973). The entry of gluconate, taken up as such by the cells, into metabolic pathways requires the action of a kinase that catalyses its intracellular conversion into 6-phosphogluconate with concomitant formation of ADP from ATP. The uptake process, and the phosphorylation step that follows it, are catalysed by highly specific proteins that are inducibly formed after exposure of *E. coli* to gluconate. Although a mutation in a common regulatory gene may derepress both the enzymes that effect these steps, and also the enzyme catalysing the subsequent dehydration of 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate (3-deoxy-6-phospho-L-*glycero*-2-hexulosonate) (de Zwaig *et al.*, 1973; Zwaig *et al.*, 1973), this latter enzyme can be induced without inducing the uptake system or the kinase (Kornberg & Soutar, 1973).

Studies of the kinetic and other parameters that affect the uptake of carbohydrates by *E. coli* are usually performed by exposing suspensions of cells, suitably induced, to some isotopically labelled substrate and then measuring the appearance of label in the cells, which are removed from their medium by rapid filtration. However, data thus obtained may not measure only the uptake process: what is measured is the sum of the amounts of labelled materials that have been taken up and the progressively increasing amounts of labelled products that have been converted into cell components. Moreover, with increasing times of incubation, the loss of labelled material through its oxidation may also progressively distort the situation.

Two main means have been used to overcome these difficulties. Where a non-catabolizable substrate analogue is available, which can be taken up by induced cells but cannot be converted into cell components, a valid measure of the kinetics of the uptake process can be readily obtained. By using methyl [^{35}S]thiogalactoside as such an analogue of lactose, Rickenberg *et al.* (1956) were the first to use

* This paper is dedicated to Professor O. Hoffmann-Ostenhof on the occasion of his 60th birthday (18 October 1974).

† Present address: Unité de Virologie, Institut National de la Santé et de la Recherche Medicale, 1 Place du Professeur Joseph Renaut, 69-Lyon (8e), France.

this approach; their conspicuous success laid the foundation for much subsequent work. Where a non-catabolizable substrate analogue is not available, it may be possible to isolate a mutant that lacks the second step in substrate utilization. This approach was first used by Horecker *et al.* (1960) in their studies of galactose uptake by an *E. coli* mutant devoid of galactokinase activity. They found that the initial rate at which labelled galactose was taken up by the mutant was identical with that at which it was taken up by wild-type cells, although galactose accumulated only in the former organism. This led Horecker *et al.* (1960) to suggest that permeation is the rate-limiting step in galactose utilization.

The present paper reports the isolation of a mutant, R6, of *E. coli* K12, that is unimpaired in its ability inducibly to take up [^{14}C]gluconate but is greatly impaired in its ability to convert the gluconate thus taken up into 6-phosphogluconate. This mutant is therefore analogous to the galactokinase-less mutant described by Horecker *et al.* (1960). It was used for a study of the kinetic and energetic parameters that affect the uptake of gluconate by *E. coli*. The kinetic parameters measured with the mutant impaired in gluconate kinase activity are not significantly different from those determined with wild-type cells. Because gluconate does not accumulate in wild-type cells, it thus appears that the active transport of gluconate is also the rate-limiting step in gluconate utilization. The active transport of a variety of amino acids and carbohydrates can also be demonstrated with membrane vesicles prepared from appropriately induced cells (Kaback, 1960, 1970, 1972); the rate of gluconate uptake and the energetics of this process were therefore studied also with such membrane vesicles. It is evident that such preparations can take up gluconate, by a process dependent on a flux of electrons and protons, at rates and to the extent observed with whole cells.

Just before submission of this paper, a brief report appeared (Robin & Kepes, 1973) of measurements of gluconate uptake with an *E. coli* mutant deficient in gluconate kinase, derived from the DF1070 mutant of *E. coli* strain K10 (Fraenkel, 1968*a*) Although the *E. coli* K12 mutant R6 described in our present paper was derived in part from the same strain DF1070 as used by Robin & Kepes (1973), there appear to be some differences in the behaviour of the two mutants. Comparison of the two mutants will require further study, and particularly will require their genetic analysis.

Experimental

Organisms used

Strain K2.1.5^c.8.9. of *E. coli* K12 was prepared by crossing the Hfr C strain DF 1070, devoid of 6-phosphogluconate dehydratase (*edd*) and of 6-phosphogluconate dehydrogenase (*gnd*) (Fraenkel, 1968*a*) with the F$^-$ strain K2.1t.5^c (*his*, *arg*, *thr*, *leu*, *pps*, *iclR*, *str*; Kornberg, 1970*a*), selecting recombinants for His$^+$ and screening such recombinants for their inability to grow on gluconate and their lack of the two enzymes of 6-phosphogluconate metabolism. The isolation of the mutant R6, impaired in gluconate kinase activity, is described in the Results section. Cultures of strains K2.1.5^c.8.9 and R6 have been deposited with the *E. coli* Genetics Stock Center, Department of Microbiology, Yale University, New Haven, Conn. 06520, U.S.A.

The conditions used for the aerobic growth of the organisms, the preparation of ultrasonic extracts and the assay of enzymes of gluconate metabolism were as described previously (Pouysségur & Stoeber, 1972; Kornberg & Soutar, 1973). Cells were induced by the addition of 5mM-sodium gluconate to cultures growing on either nutrient broth or minimal media containing salts (Ashworth & Kornberg, 1966) and 20mM-glycerol as carbon source.

Osmotic shock

Cells harvested in the exponential growth phase (0.5–0.65mg dry mass/ml) were shocked by the method of Heppel (1969). After three washings with 30mM-Tris–HCl buffer, pH7.3, the cells were gently agitated for 15min with 80vol. of the same buffer containing 20% (w/v) sucrose and 0.1mM-EDTA, collected by centrifugation, and cold-shocked in 80vol. of ice-cold 0.1mM-$MgCl_2$. After centrifugation, the cells were resuspended in 50mM-potassium phosphate buffer, pH7.0, at a concentration of 2mg dry mass/ml and used within 2h of the cold-shock treatment.

Preparation of membrane vesicles

The strain R6 was grown on nutrient broth in the presence of 10mM-sodium gluconate. Spheroplasts and membrane vesicles were prepared by the lysozyme–EDTA method described by Kaback (1971). Membranes were stored in 0.1M-potassium phosphate buffer, pH6.6, at a concentration of 4–6mg/ml under liquid N_2. They were thawed at room temperature when required.

Gluconate uptake by whole cells and vesicles

Cells were washed with 50mM-potassium phosphate buffer, pH7.0, and were resuspended in this buffer at 0.2mg dry mass/ml; chloramphenicol (50μg/ml) was added to inhibit any synthesis of proteins *de novo*. The suspensions were shaken gently at 25°C. The uptake reaction was started by the addition of sodium [6-^{14}C]gluconate (3.4Ci/mol) to a final

concentration of 0.05mM. At appropriate times, samples (0.5ml) were withdrawn, diluted into 2.5ml of 50mM-potassium phosphate buffer, pH7.0, at room temperature, filtered immediately on HA Millipore filters (0.45μm pore size) and washed again with 2.5ml of the same buffer. The filters were then air-dried, dissolved in 5ml of Bray's (1960) fluid, and the radioactivity was assayed with a Packard model 4000 liquid-scintillation spectrometer.

Vesicles were incubated for 15min at 25°C with 100mM-potassium phosphate buffer, pH6.6, containing 10mM-$MgSO_4$. At the end of this period the appropriate electron donor was added to a final concentration of 20mM and the reaction was started immediately thereafter by the addition of sodium [6-^{14}C]gluconate, to 0.05mM final concentration. Samples (0.1ml) were withdrawn, washed twice with 2.5ml of 0.1M-LiCl on HA Millipore filters, and their radioactivity was assayed as described above.

Assay of gluconate efflux from whole cells

Cells were allowed to accumulate labelled gluconate in a final volume of 1ml, containing 0.5ml of cells (2mg/ml) and 0.5ml of 500μM-sodium [6-^{14}C]gluconate. When the radioactivity of the cells had reached a steady state, the cells were spun down for 1min at 16000*g* in an Eppendorf 3200 Microfuge centrifuge. The pellet was quickly resuspended in 80ml of 50mM-potassium phosphate buffer, pH7.0, which had been warmed to 25°C. Samples (10ml) were withdrawn at measured times and were filtered; the filters were washed with 2.5ml of the phosphate buffer, air-dried, and the radioactivity was assayed.

Chromatographic analysis of cell contents

Cells induced with gluconate were washed twice with 50mM-potassium phosphate buffer, pH7.0, and incubated at 1.4mg dry mass/ml with 0.3mM-sodium [6-^{14}C]gluconate. At various times, 1ml of the suspensions was spun down; the pellet was washed with the phosphate buffer, resuspended in 0.5ml of water and boiled for 5min. After centrifugation, the supernatant solution was concentrated to dryness under vacuum and the residue was resuspended in 0.1ml of water. Samples (10–30μl), together with authentic sodium [^{14}C]gluconate and 6-phosphogluconate, were applied to Whatman no. 1 paper and chromatographed overnight in the butanol–propionic acid–water solvents of Benson *et al.* (1950). The radioactive materials on the chromatogram were located by radioautography with Kodak Blue Brand X-ray film. Phosphate esters were detected by the procedure described by Steinitz (1961).

Induction of gluconate kinase and measurement of 6-phosphogluconate formation

To cultures (500ml) of organisms growing aerobically at 37°C on nutrient broth was added 5mM-sodium gluconate when the cell density had reached 0.3mg dry mass/ml. Samples, of a size sufficient to contain approx. 15mg dry mass of cells, were withdrawn at known times (see Fig. 2) and were centrifuged at 4°C and 20000*g* for 5min. The 6-phosphogluconate content of samples of these supernatant solutions was assayed by incubating them at 25°C with 50μmol of Tris–HCl buffer, pH8.0, 3μmol of $MgCl_2$, 0.25μmol of $NADP^+$ and 25μg of crystalline 6-phosphogluconate dehydrogenase, in a volume of 1ml, until no further increase in extinction at 340nm occurred. A ΔE_{340} of 0.622 was taken to be equivalent to 0.1μmol of 6-phosphogluconate. This measurement represented the 6-phosphogluconate liberated by the cells into the media.

The cells were taken up in 4ml of 10mM-potassium phosphate buffer, pH7.5. A sample (1ml) of this suspension was removed and was placed in boiling water for 5min: the 6-phosphogluconate content of the supernatant obtained from this treatment, which represented that of the cell contents, was assayed as described above. To the remaining 3ml of cell suspensions was added a further 15ml of the phosphate buffer, the cells were collected by centrifugation, and the resultant pellet of washed cells was taken up in 4ml of the phosphate buffer supplemented with $MgCl_2$ to 1mM. These suspensions were cooled in ice and exposed to the output of a MSE 100W sonicator for 45s; the virtually clear suspensions were centrifuged at 4°C and 20000*g* for 5min and the gluconate kinase activities of the supernatant solutions thus obtained were assayed as described (Pouysségur & Stoeber, 1972).

Measurement of 6-phosphogluconate phosphatase activity

The possible phosphatase activity of strain R6 was measured in two ways. In one procedure, crude sonic extracts of induced cells were incubated for 20min at 25°C with 10mM-sodium 6-phosphogluconate in buffers at pH5.5 (acetate), 7.0 (Tris–HCl) and 9.5 (ethanolamine–HCl), with and without 1mM-$MgCl_2$; any phosphate released was measured by the procedure of Fiske & SubbaRow (1925). In the second procedure, a thick suspension (5mg dry mass/ml) of induced strain R6 was rendered permeable by the method of Reeves & Sols (1973) and was incubated at 25°C for 30min with 2mM-sodium 6-phosphogluconate in 50mM-Tris–HCl, pH8.0. Samples were withdrawn at zero time and after 15 and 30min, boiled and their 6-phosphogluconate contents were

measured as described above. A control incubation mixture contained all ingredients but boiled cells.

Measurement of oxygen uptake

Rates of oxygen uptake by cell suspensions were measured with a Clark electrode at 25°C, as described by Estabrook (1967).

Chemicals

Sodium [6-^{14}C]- and [U-^{14}C]-gluconate were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Lithium salts of D(−)- and L-lactic acid were obtained from Sigma (London) Chemical Co. Ltd., Kingston on Thames, Surrey, U.K. Crystalline 6-phosphogluconate dehydrogenase (yeast) was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. D-Xylonate and 3-deoxy-3-fluoro-D-gluconate (calcium salts) were generous gifts from Dr. M. McKillen (Dublin) and Professor N. F. Taylor (Windsor, Ont.) respectively. All other chemicals were analytical-grade reagents of commercial origin.

Results and Discussion

Isolation of E. coli mutant R6

The mutant K2.1.5^c.8.9 of *E. coli* K12 (Kornberg, 1970*b*) lacks the ability to form the enzymes that catalyse the oxidation and the dehydration of 6-phosphogluconate, 6-phosphogluconate dehydrogenase (*gnd*, EC 1.1.1.43) and 6-phosphogluconate dehydratase (*edd*, EC 4.2.1.12). A consequence of this double dysfunction (see Scheme 1 of Kornberg & Soutar, 1973) is that the addition of gluconate to cultures of this mutant leads to the intracellular accumulation of 6-phosphogluconate. As has been noted previously with other phosphorylated compounds (Kurahashi & Wahba, 1958; Englesberg & Baron, 1959; Nikaido, 1961; Englesberg *et al.*, 1962; Cozzarelli *et al.*, 1965; Böck & Neidhardt, 1966; Fraenkel, 1968*b*; Kelker *et al.*, 1970; Gay & Rapoport, 1970; Pouysségur & Stoeber, 1971; Kornberg, 1972; Solomon & Lin, 1972; Ferenci & Kornberg, 1973; Faik & Kornberg, 1973), this accumulation exerts a toxic (though not lethal) effect and growth of the mutant is speedily arrested. By the same token, cultures of the mutant strain K2.1.5^c.8.9 spread on agar plates containing minimal medium and a readily utilizable carbon source (such as succinate or glycerol) do not grow if the media also contain gluconate. However, after 2–3 days at 37°C, some 50–100 colonies appeared from about 10^8 cells thus plated. They were picked and tested for their ability to grow on plates containing glycerol plus gluconate, and gluconate alone. Of those that grew on the former but not on the latter medium, one colony (designated R6) was further purified by repeated isolation of single colonies and was used for subsequent work.

The mutant R6 which (apart from its tolerance of gluconate) carries the same genetic markers as does its parent K2.1.5^c.8.9 (F$^-$, *edd, gnd, pps, iclR, argH, str*), was only slightly retarded by the presence of 5mM-gluconate in its rate of growth on nutrient broth, on 20mM-glycerol, or on 10mM-glucose, fructose or succinate; however, it did not grow on gluconate alone, nor did extracts of cells that had grown in the presence of gluconate contain 6-phosphogluconate dehydrogenase or 6-phosphogluconate dehydratase activities. The acquisition of the ability of strain R6 to tolerate the presence of gluconate in growth media was therefore not due to a reversion that enabled 6-phosphogluconate to be catabolized; it must have involved a change either in the process whereby gluconate enters the cells, or in the intracellular conversion of gluconate into 6-phosphogluconate.

That the latter is the case is indicated by the data of Fig. 1. A washed suspension of a culture of strain R6, grown on nutrient broth in the absence of added gluconate, took up little ^{14}C when incubated with [^{14}C]gluconate (open triangles); in contrast, a suspension of cells that had grown for approximately one generation on nutrient broth supplemented with 5mM-unlabelled gluconate took up [^{14}C]gluconate rapidly (closed circles), at about the same rate and to about the same extent as did a suspension of the parent organism K2.1.5^c.8.9, similarly grown (open circles).

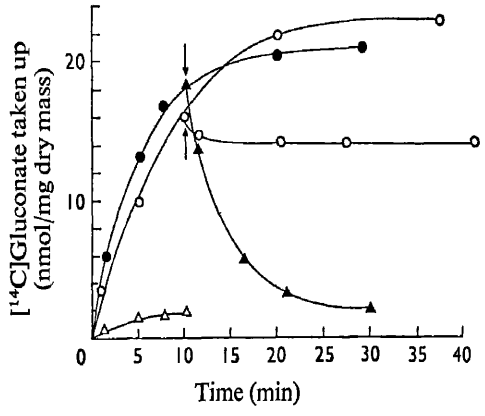

Fig. 1. *Uptake of* [^{14}C]*gluconate by washed suspensions of strains K*2.1.5^c.8.9 *and its mutant R6*

The time-course of uptake of 0.05mM-sodium [^{14}C]gluconate by washed suspensions of nutrient-grown strain R6 (△), and by strain R6 grown on nutrient in the presence of 5mM-gluconate (●), was measured as described in the Experimental section. To a duplicate flask of strain R6 induced for gluconate uptake was added 10mM-sodium [^{12}C]gluconate after 10min (▲). The experiments with induced cells were repeated with strain K2.1.5^c.8.9 (○). Arrows show where [^{12}C]gluconate was added.

This shows that the uptake system(s) for gluconate are inducible, and are present in both types of mutant. However, the fate of the gluconate taken up is abnormal in strain R6: whereas the addition of 5mM-unlabelled sodium gluconate to the suspension of K2.1.5^c.8.9, incubated with 0.05mM-[^{14}C]gluconate for 10min, removed from the cells less than 20% of the labelled material taken up, over 80% of the accumulated ^{14}C was washed out of the R6 cells. The simplest explanation of these findings is that neither type of mutant is impaired in the uptake of [^{14}C]gluconate, but that only strain K2.1.5^c.8.9 converts the gluconate taken up into 6-phosphogluconate. This view is supported by three types of experiment. In the first, cultures of the two mutants were incubated with sodium [^{14}C]gluconate and the material accumulated by the cells was analysed by chromatography. Over 80% of the labelled contents of strain K2.1.5^c.8.9 were found to co-chromatograph with authentic 6-phosphogluconate, whereas over 90% of the radioactivity present in R6 cells remained as gluconate. Clearly, strain R6 did not effect the net conversion into 6-phosphogluconate of gluconate that had entered the cells, which was evident in its parent organism.

This is shown also by measurements of the differential rates of synthesis (Monod, 1956) of gluconate kinase by cultures of the two strains, growing on either nutrient broth or minimal medium containing glycerol as carbon source, when 5mM-gluconate was added to them (Fig. 2). It is apparent that the nature of the growth substrate exerts a considerable influence on the induction of the kinase: the rates at which the enzyme activities rise after addition of gluconate are lower in the presence of glycerol than in that of nutrient broth. Since, in general, glycerol exerts little catabolite repression on enzymes of carbohydrate metabolism (Magasanik, 1961), it is possible that this repressive effect is a physiological 'feedback' associated with the metabolic role of gluconate kinases. In the Entner–Doudoroff pathway, which forms the predominant route for gluconate catabolism in *E. coli* (Zablotny & Fraenkel, 1967), gluconate kinase initiates a sequence of reactions that results in the cleavage of the C_6 compound 2-keto-3-deoxy-6-phosphogluconate to pyruvate and glyceraldehyde 3-phosphate. Since this triose phosphate is interconvertible with the C_3 product of glycerol catabolism dihydroxyacetone phosphate, the repression of gluconate kinase synthesis observed during growth on glycerol may be a further example of a well-known phenomenon. It is also apparent from Fig. 2 that, under both conditions tested, strain R6 formed much less gluconate kinase than did its parent organism. The kinetic parameters of the gluconate kinase formed by strain R6 were, however, identical with those of the enzyme formed by the parent mutant: in both cases, the K_m for gluconate was determined as 0.11mM, and the K_m for Mg-ATP was 0.9mM.

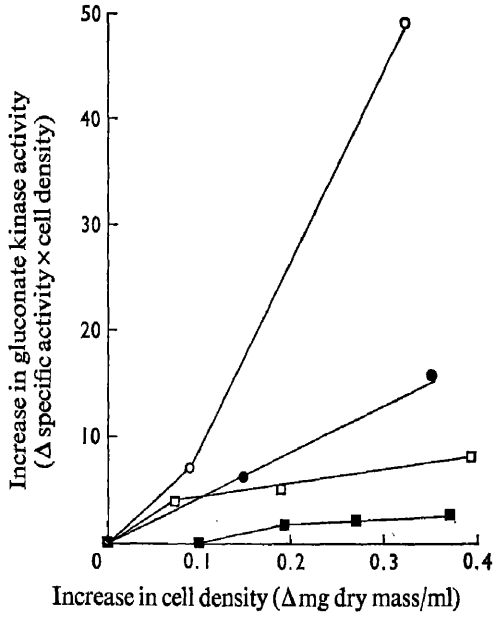

Fig. 2. *Induction of gluconate kinase in cultures of strain K2.1.5^c.8.9 and its mutant R6*

Sodium gluconate (5mM) was added at zero time to cultures of strain K2.1.5^c.8.9 growing on nutrient broth (○) or glycerol (●), and to cultures of strain R6 growing on nutrient broth (□) or glycerol (■). The cell densities and gluconate kinase activities of samples, taken thereafter, were measured as described in the Experimental section. The results are plotted to illustrate the differential rates of synthesis of the enzyme (Monod, 1956).

In a third experiment, the net rates of 6-phosphogluconate formation in cultures of the two strains of mutants were measured. When 5mM-gluconate was added to cultures of strain K2.1.5^c.8.9 that were growing either on nutrient broth or on glycerol, 6-phosphogluconate rapidly appeared in the cells and, in 100min, increased to nearly 18mM in the presence of nutrient broth, and 8mM in the presence of glycerol (calculated on the assumption that 1mg dry mass of *E. coli* K12 contains 3μl of cell water; see Roberts *et al.*, 1955; Winkler & Wilson, 1966) (Fig. 3). Surprisingly, significant amounts of 6-phosphogluconate appeared as such also in the suspending medium; the amounts that thus appeared reflected the intracellular concentrations of this compound (Fig. 4). In contrast, the intracellular 6-phosphogluconate content of strain R6 increased to less than 7mM and 5mM respectively under these conditions (from an initial value of about 4mM) and only traces of 6-phosphogluconate appeared in the media. This latter observation also shows that the low intracellular concentrations of 6-phosphogluconate found with strain R6 are not due to an accelerated efflux of 6-phosphogluconate that might have been formed at a normal rate.

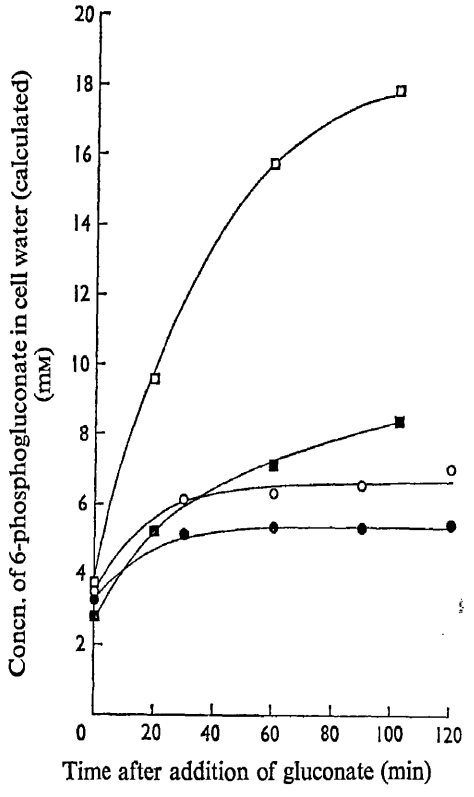

Fig. 3. *Accumulation of 6-phosphogluconate by cultures of strain K2.1.5°.8.9 and its mutant R6*

Sodium gluconate (5mM) was added at zero time to cultures of strain K2.1.5°.8.9 growing on nutrient broth (□) or glycerol (■), and to cultures of strain R6 growing on nutrient broth (○) or glycerol (●). The 6-phosphogluconate content of samples of cells, taken thereafter, was measured as described in the Experimental section. For the purposes of this calculation, it was assumed that 1 mg dry mass of cells contains 3 μl of water.

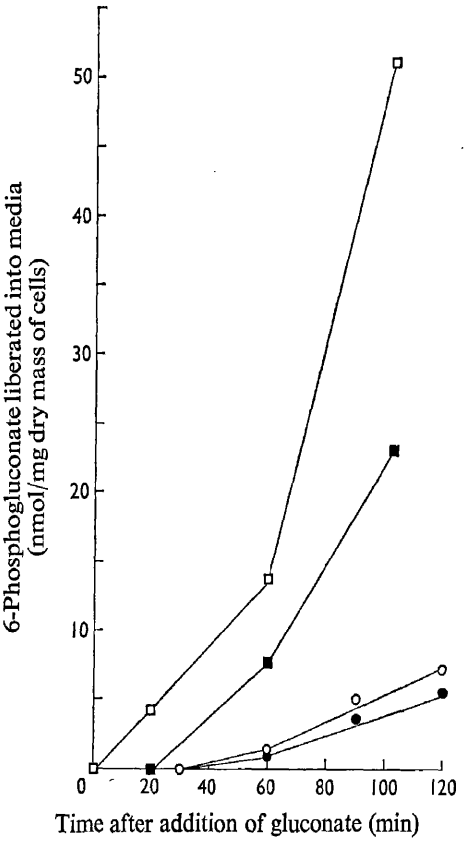

Fig. 4. *Appearance of 6-phosphogluconate in media after addition of gluconate to cultures of strains K2.1.5°.8.9 and R6*

Sodium gluconate (5mM) was added at zero time to cultures of strain K2.1.5°.8.9 growing on nutrient broth (□) or glycerol (■), and to cultures of strain R6 growing on nutrient broth (○) or glycerol (●). Samples were taken thereafter and centrifuged; the 6-phosphogluconate content of the supernatant solutions was assayed as described in the Experimental section.

It is conceivable that the 6-phosphogluconate formed from the gluconate taken up by strain R6 might be rapidly hydrolysed again to gluconate, which might be retained. Although there is no direct evidence against this, we have failed to find any increased rate of hydrolysis of 6-phosphogluconate by extracts of strain R6 or by suspensions of this organism made permeable to external 6-phosphogluconate by the procedure of Reeves & Sols (1973). It is thus likely that strain R6 indeed differs from its parent organism in its ability enzymically to convert into 6-phosphogluconate the gluconate that has been taken up by the cells. Since, in consequence, it takes up labelled gluconate at a normal rate and retains it predominantly as such (Fig. 1), this organism is admirably suited to a study of the kinetics of the uptake process. The experiments with whole cells reported in the next section were therefore done with this mutant.

Uptake of gluconate by strain R6

The initial rates at which 0.05mM-sodium [^{14}C]-gluconate was taken up by washed suspensions of strain R6 (that had been induced to form the uptake system) were measured over the range 1–1000μM. As shown in the inset of Fig. 5, the rates increased with increasing gluconate concentration up to about 60μM, after which the uptake system appeared to be virtually saturated. The K_m of this process is about 10μM and the $V_{max.}$ about 25nmol/min per mg dry mass, values which are identical with the parameters previously determined in wild-type *E. coli* (Faik & Kornberg, 1973). This is particularly significant, since a mutant of *E. coli* has been described (Faik

& Kornberg, 1973) that is capable of forming a gluconate-uptake system with low affinity (K_m above 100μM) for gluconate and that lacks the high-affinity system. Since no increases in the initial rates of gluconate uptake by strain R6 were observed at gluconate concentrations between 60 and 1000μM, it is safe to conclude that the low-affinity uptake system was not induced under the conditions used with strain R6 and that the properties measured here are indeed those of the high-affinity system.

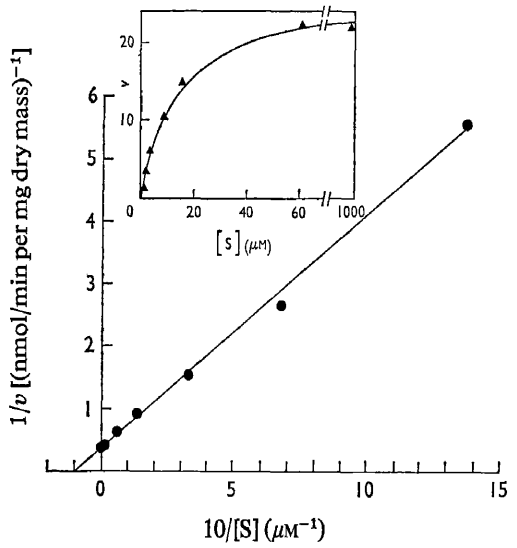

Fig. 5. *Effect of gluconate concentration on the initial rates of gluconate uptake by strain R6*

Initial rates of uptake (v = nmol/min per mg dry mass) were calculated from the amounts of [^{14}C]gluconate taken up by washed suspensions of induced strain R6 10, 25 and 40s after addition of labelled gluconate, and are plotted in the inset figure against the gluconate concentration added ([S] = μM). The reciprocals of these rates (×10) are plotted on the larger figure.

The uptake system is highly specific for gluconate: the inclusion of the sodium salts of all available hexonic, pentonic and hexuronic acids, and of 2-oxogluconic acid, 5-oxogluconic acid, 2-oxo-3-deoxygluconic (3-deoxy-L-*glycero*-2-hexolusonic) acid and glucaric acid at 1mM did not decrease significantly the rate at which strain R6 took up 50μM-sodium [^{14}C]gluconate; only 1mM-3-deoxy-3-fluorogluconate inhibited by 50%. A similar specificity appears to be exhibited by the induction process: only the addition of gluconate itself to cultures of R6 growing on glycerol or nutrient broth induced the uptake system.

It is apparent from Fig. 1 that, in the steady state, strain R6 takes up over 20nmol of gluconate/mg dry mass when incubated with that substance at 50μM: this corresponds to a concentration of over 6mM-gluconate in the cell water. Clearly the uptake and retention of gluconate to this extent (over 100-fold) must require the expenditure of metabolic energy. The decreased rate of uptake found under anaerobic conditions, and the strong inhibition exerted by amytal, cyanide, and a variety of agents known to interfere with energy conservation (Table 1), indicate that this energy for gluconate uptake is derived from the activity of the respiratory chain. The inhibition observed with *p*-chloromercuribenzoate and its reversal by 2-mercaptoethanol further indicate that a

Table 1. *Comparison of the effect of inhibitors on gluconate uptake by intact cells and membrane vesicles prepared from strain R6*

The inhibitors, dissolved in water (or, where marked *, in ethanol) were incubated for 15min at 25°C with the cells and membrane vesicles before addition of 0.05mM-sodium [^{14}C]gluconate and 0.05mM-sodium [^{14}C]gluconate and 20mM-L-lactate respectively. The amounts of labelled gluconate taken up in a further 15min were measured as described in the Experimental section. —, not tested.

Inhibitor used	Concn. added (mM)	Inhibition (%)	
		Cells	Vesicles
NaN_3	1.0	50	—
	10	93	0
KCN	1.0	60	0
	10	76	79
Sodium arsenate	1.0	12	—
	10	30	0
2,4-Dinitrophenol	0.05	30	—
	0.25	90	97
Carbonyl cyanide *m*-chlorophenylhydrazone*	0.001	79	—
	0.01	98	97
Amytal*	0.1	21	80
Anaerobic conditions	—	55	—
p-Chloromercuribenzoate	0.1	80	94
β-Mercaptoethanol	1.0	0	—
p-Chloromercuribenzoate + β-mercaptoethanol	0.1+1.0	12	—
Ethanol	—	0	0

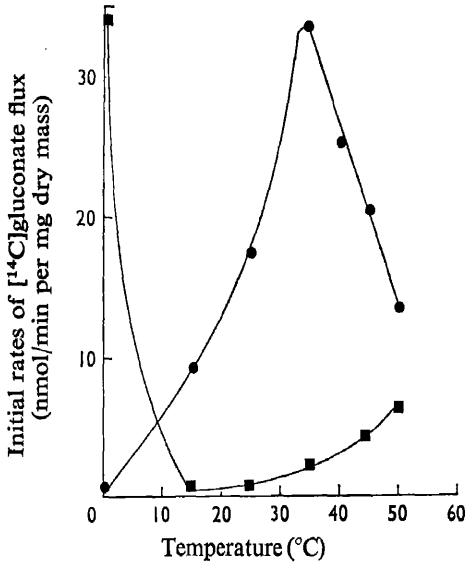

Fig. 6. *Temperature dependence of uptake* (●) *and efflux* (■) *of gluconate by suspensions of strain R6*

For conditions see the text.

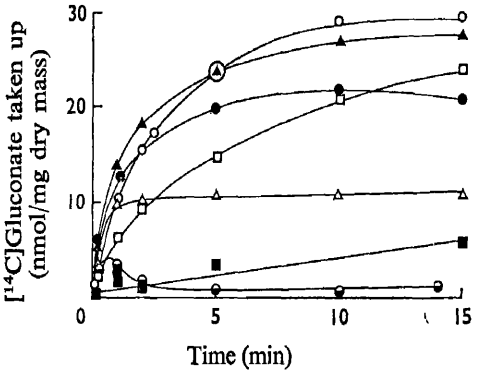

Fig. 7. *Effect of temperature on the time-course of gluconate uptake by strain R6*

Flasks containing 50mM-potassium phosphate buffer, pH7.0, were incubated for 10min at 0°C (■), 15°C (□), 25°C (○), 35°C (▲), 40°C (●), 45°C (△) and 50°C (◒) before addition of a suspension of induced strain R6. Sodium [^{14}C]gluconate (0.05mM) was added 2min thereafter. Samples were taken at times indicated and washed at room temperature; for details see the Experimental section.

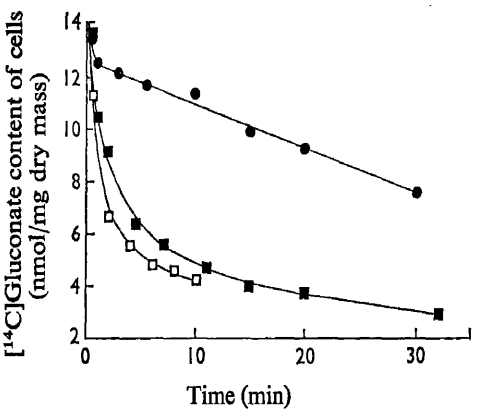

Fig. 8. *Time-course of gluconate efflux from strain R6*

Cells were allowed to accumulate [^{14}C]gluconate as described in the text and diluted in a large volume of 50mM-potassium phosphate buffer, pH7.0, alone (●) or in buffer that also contained 5mM-sodium [^{12}C]gluconate (■) or 0.25mM-dinitrophenol (□).

thiol group may be associated with the site that recognizes gluconate, or with the energetic coupling. The initial rates of uptake of 0.05mM-gluconate by washed suspensions of strain R6 that had been grown on nutrient broth plus gluconate at 37°C were not greatly affected by changes in pH over the range 6–8; this behaviour thus differs from that of the apparently similar mutant briefly reported by Robin & Kepes (1973). At the pH of 7.0 usually used in our work, the rate of gluconate uptake by strain R6 was very low when measured at 0°C and increased rapidly with increase in temperature to a peak at about 35°C, above which it declined (Fig. 6). There was a concomitant rise in the steady-state concentrations of gluconate maintained by the cells between 0°C and 35°C, and a sharp decline thereafter (Fig. 7). The behaviour shown in Fig. 6 is characteristic of an active-transport process, which exhibits a temperature coefficient much greater ($Q_{10}>2$) than would passive diffusion, the rate of which is proportional to the absolute temperature.

The sharp decrease above 35°C in the quantities of gluconate retained by strain R6 can be only partially accounted for by an increased rate of gluconate efflux from the cells. That an alteration in some energy-dependent process in gluconate retention might be involved is supported by measurement of the effect of 2,4-dinitrophenol on gluconate efflux (Fig. 8). In the absence of this proton-conducting agent (Harold, 1972) a culture of strain R6 that had accumulated [^{14}C]gluconate lost gluconate at only 0.04 nmol/min per mg dry mass when diluted into a large volume of 50mM-potassium phosphate buffer without change in pH or temperature. As shown also in Fig. 1, the addition of [^{12}C]gluconate greatly accelerated this exit reaction: clearly the dilution of cellular radioactivity observed reflected the rapid uptake of the unlabelled gluconate and the consequent displacement of the labelled material already present. The efflux reaction involved in this exchange could be a passive diffusion, since it exhibits the small temperature coefficient expected of such a process (Fig. 6), and could involve the activity of a mobile carrier protein (Kepes, 1973). However, no such exchange can account for the

stimulation of gluconate efflux observed after addition of 2,4-dinitrophenol: presumably some energy-requiring process must operate to retain the gluconate taken up, possibly by decreasing the affinity of the gluconate 'carrier' for the substrate present internally (Koch, 1964) or by providing the power for a re-capture process at the cell surface (Halpern, 1967).

The uptake of a number of carbohydrates by *E. coli* appears to involve 'binding proteins' (for review, see Lin, 1971), which can be liberated from the periplasmic space by EDTA treatment followed by osmotic shock (Heppel, 1969). In order to investigate whether such 'binding proteins' played any part in the uptake of gluconate, cultures of wild-type *E. coli* K12 were osmotically shocked. In conditions where at least 95% of the shocked cells remained viable (Anraku & Heppel, 1967) there was a decrease, by 75–80%, in the initial rates of gluconate uptake, which could be partially restored by addition of the concentrated shock fluid (Table 2). However, this partial restoration of uptake could also be achieved by the addition of shock fluid derived from cells that had not been induced for gluconate uptake. It appeared to reside in some components of the fluids that were stable to boiling for 10min, and was lost when shock fluid from induced cells was dialysed. It is therefore unlikely that a gluconate-specific 'binding protein' was released in the shock fluid; indeed, our attempts by equilibrium dialysis to detect a protein in concentrated shock fluids that would bind labelled gluconate were unsuccessful. Rather it is likely that the osmotic cold-shock procedure impairs the energy metabolism of the cells, perhaps by the loss of small molecules: this is also indicated by the major decrease in cellular respiration upon cold-shock treatment (Table 2).

Table 2. *Effect of cold osmotic-shock treatment on the uptake of gluconate and of oxygen by wild-type E. coli K12*

Gluconate uptake and respiration were measured within the 2h after the EDTA–osmotic-shock treatment. Cells were grown on nutrient broth supplemented with 5mM-sodium gluconate as inducer. For measurements of oxygen uptake, nutrient broth was used as substrate. Shock fluid (0.6mg of protein/ml) was concentrated under vacuum about 40-fold and added at a final concentration of 2mg of protein/ml.

	Relative rates (%) of	
	Gluconate uptake	Oxygen uptake
Experiment 1.		
Cells	100	100
Shocked cells	20	16
Shocked cells+fluid	54	16
Experiment 2.		
Cells	100	100
Shocked cells	26	20
Plus fluid	62	20
Plus boiled fluid	78	—
Plus fluid from non-induced cells	99	—

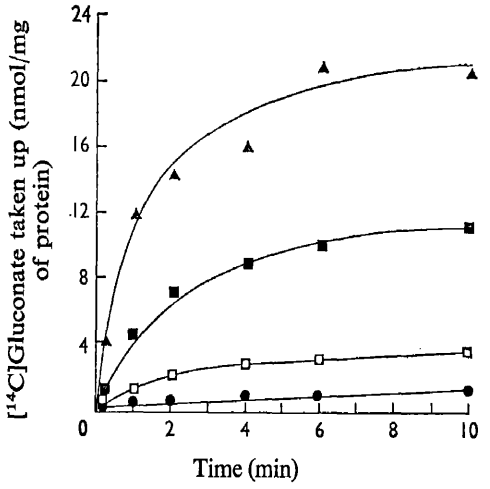

Fig. 9. *Effect of electron donors on the uptake of gluconate by membrane vesicles from strain R6*

The uptake of 0.05mM-sodium [^{14}C]gluconate by vesicles (0.4mg of protein/ml of buffer) was measured, as described in the Experimental section, in the absence of added electron donors (●) and in the presence of D-lactate (□), of L-lactate (■) and of ascorbate+phenazine methosulphate (▲).

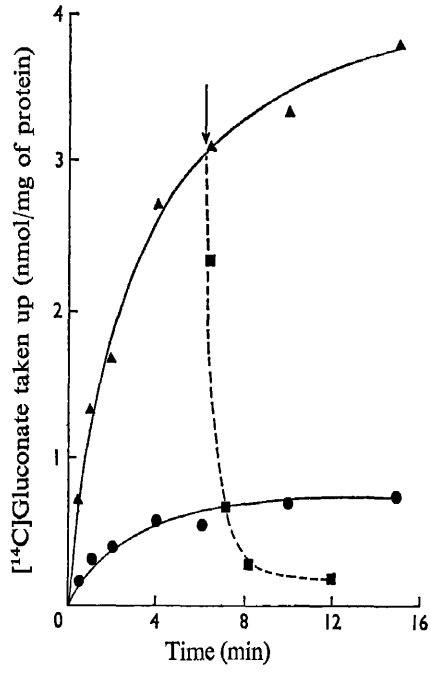

Fig. 10. *Time-course of uptake of gluconate by membrane vesicles from strain R6*

The uptake of 0.05mM-sodium [^{14}C]gluconate by membrane vesicles was measured in the absence of added donors (●) and in the presence of D-lactate (▲). At the time indicated by an arrow, 5mM-sodium [^{12}C]gluconate was added to a portion of the latter (■).

Gluconate uptake by membrane vesicles

In common with other carbohydrates actively taken up by *E. coli* (for review, see Kaback, 1970; Kornberg, 1973), gluconate can be taken up and retained against a concentration gradient by membrane vesicles prepared from induced cells, provided that a suitable source of metabolic energy is also supplied (Fig. 9). The optimum source of this metabolic energy appears to differ with different carbohydrates. Thus the uptake of gluconate by membrane vesicles prepared from strain R6 was stimulated nearly 30-fold by the presence of ascorbate and phenazine methosulphate, tenfold by L-lactate, and only 3–4-fold by D-lactate; this preference for L- over D-lactate differs from the preference for D-lactate exhibited by *E. coli* vesicles taking up, e.g., galactosides (Kaback, 1970, 1972). With ascorbate+phenazine methosulphate as electron donor, vesicles concentrated external 0.05mM-gluconate over 100-fold, to the approx. 6mM concentration that had also been observed with whole cells (Fig. 1). Further, and also as observed with whole cells (Figs. 1 and 8), the addition of [^{12}C]gluconate to vesicles that had taken up [^{14}C]gluconate rapidly displaced from them over 90% of the radioactive material, in 2min (Fig. 10).

Stimulation of gluconate uptake was observed also with succinate or α-glycerophosphate as electron donors, but these materials were less effective than ascorbate+phenazine methosulphate or L-lactate; NADH, which is the preferred electron donor for the uptake of some carbohydrates by *Bacillus subtilis* membrane vesicles (Matin & Konings, 1973), did not stimulate gluconate uptake by vesicles prepared from *E. coli* strain R6 (Table 3). With L-lactate as electron donor, the K_m for gluconate uptake was 5×10^{-5}M, which is of the same order of magnitude as that found for uptake by whole cells.

As noted with whole cells, the uptake of gluconate by membrane vesicles is strongly inhibited by inhibitors of electron transfer, and the pattern of inhibition observed is similar to that observed with whole cells (Table 1). In both cases, the uptake process is powerfully inhibited by proton-conducting agents, such as carbonyl cyanide *m*-chlorophenylhydrazone and 2,4-dinitrophenol; the lack of effect by arsenate is consistent with the view (Klein & Boyer, 1972) that ATP or other high-energy phosphate compounds are not directly involved in the transport process.

The close parallel between the kinetic and energetic parameters that characterize the uptake of gluconate by whole cells and that by membrane vesicles support the use of the latter type of experimental material for studies of active transport, and also show that no indispensible components of the uptake process have been lost in the preparation of membrane vesicles. Comparison of the rates of gluconate uptake by suspensions of induced strain R6, its parent, and its grandparent organisms also confirm that, provided measurements are made over brief periods after addition of a labelled substrate, the measured rates of appearance of label in the cells are very close to the 'true' rates at which the substrate is taken up, and that this uptake process is thus the rate-limiting step in gluconate utilization. This leads to the comforting conclusion that, although mutants devoid of the first catabolic enzyme subsequent to the uptake step provide a powerful and unambiguous means for investigating that uptake process, they are not essential for determining its kinetic parameters.

Table 3. *Effect of various energy sources on the gluconate uptake by membrane vesicles from strain R6*

Samples (0.1 ml) containing membranes (0.2 mg of protein) from strain R6 were incubated, and the amounts of label taken up in 15min from 0.05mM-sodium [^{14}C]gluconate were measured, as described in the Experimental section.

Energy source added	Concn. added (mM)	[^{14}C]Gluconate taken up (nmol/mg of protein)
None	—	1
D-Lactate	20	3
L-Lactate	20	10
Ascorbate	20	1.5
Phenazine methosulphate	0.2	1
Ascorbate+phenazine methosulphate	20+0.2	27
Succinate	30	3
DL-α-Glycerophosphate	20	3
NADH	20	1.5
NADH+phenazine methosulphate	20+0.2	1.5
NADPH	20	1.5
Phosphoenolpyruvate	20	1
ATP	10	1.5

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ISOLATION AND PROPERTIES OF *ESCHERICHIA COLI* MUTANTS DEFECTIVE IN 2-KETO 3-DEOXY 6-PHOSPHOGLUCONATE ALDOLASE ACTIVITY

Pelin FAIK, H.L. KORNBERG and E. McEVOY-BOWE*

Department of Biochemistry, School of Biological Sciences, University of Leicester, Leicester LE1 7RH, England

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1. Introduction

Many microbial species [1] effect the entry of gluconate into the central routes of metabolism via the Entner-Doudoroff pathway [2]. The component enzymes of this route are: (i) gluconate kinase (GLK), which catalyses the phosphorylation of gluconate to 6-phosphogluconate; (ii) 6-phosphogluconate dehydrase (EDD), which catalyses the removal of the elements of water from its substrate to yield 2-keto 3-deoxy 6-phosphogluconate (KDPG); and (iii) KDPG aldolase (KGA), which effects the aldol cleavage of KDGP to glyceraldehyde 3-phosphate and pyruvate. This sequence of reactions is summarized in fig. 1.

Two main lines of evidence illustrate the role that this pathway plays in gluconate utilization by *Escherichia coli*. Although 6-phosphogluconate can be catabolized via the pentose-phosphate shunt, mutants devoid of 6-phosphogluconate dehydrogenase (GND) are impaired to only a slight extent in the rate of their growth on gluconate [3]; in contrast, GND^+ mutants that lack the dehydrase (EDD^-) grow very slowly on gluconate [4]. Moreover, whereas the specific activities of GND in *E. coli* extracts appear to be independent of the nature of the carbon source used for the growth of the organism, EDD is present in only trace amounts unless its synthesis is induced by exposure of *E. coli* to gluconate [5].

Peyru and Fraenkel [6] have located on the genome of *E. coli* the genes that specify the synthesis of GND (*gnd*) and EDD (*edd*): they are not closely linked, and are situated on the linkage map [7] at, respectively, 39 min (co-transducible with the *his* locus) and 35 min. Since EDD is a component enzyme of the Entner-Doudoroff pathway but GND is not, it is of interest to determine whether the genes that specify the synthesis of other enzymes of that pathway, and of the possible regulator genes that determine the inducibility of these enzymes, are linked to either of these markers. We therefore describe the isolation and properties of *E. coli* mutants devoid of KGA activity, and report that this marker (*kga*) is so closely linked to that specifying EDD activity as to be over 95% co-transducible.

2. Experimental

2.1. *Isolation of kga mutants*

KDPG is an intermediate in two types of catabolic route (fig. 1): both the catabolism of gluconate via the Entner-Doudoroff pathway, and the catabolism of hexuronic acids [8], involve the necessary activity of KGA. In order to isolate mutants specifically affected in *kga*, therefore, the starting organism chosen was the mutant DF1071, a derivative of an Hfr C strain of *E. coli* K12 that already lacked GND [9]. In this mutant, the catabolism of gluconate had perforce to involve KGA; moreover, this organism grew readily in media containing either glucuronate or galacturonate as sole carbon source.

The *gnd,kga*-mutants sought were isolated by treatment of DF1071 with ethylmethane sulphonate [10] and selection with penicillin [11] for clones that grew neither on gluconate nor on galacturonate, but that retained the ability to grow on glucose. (The composition of media, and the biochemical and genetic proced-

* Present address: Department of Biochemistry, University of Singapore Medical School, Singapore 3.

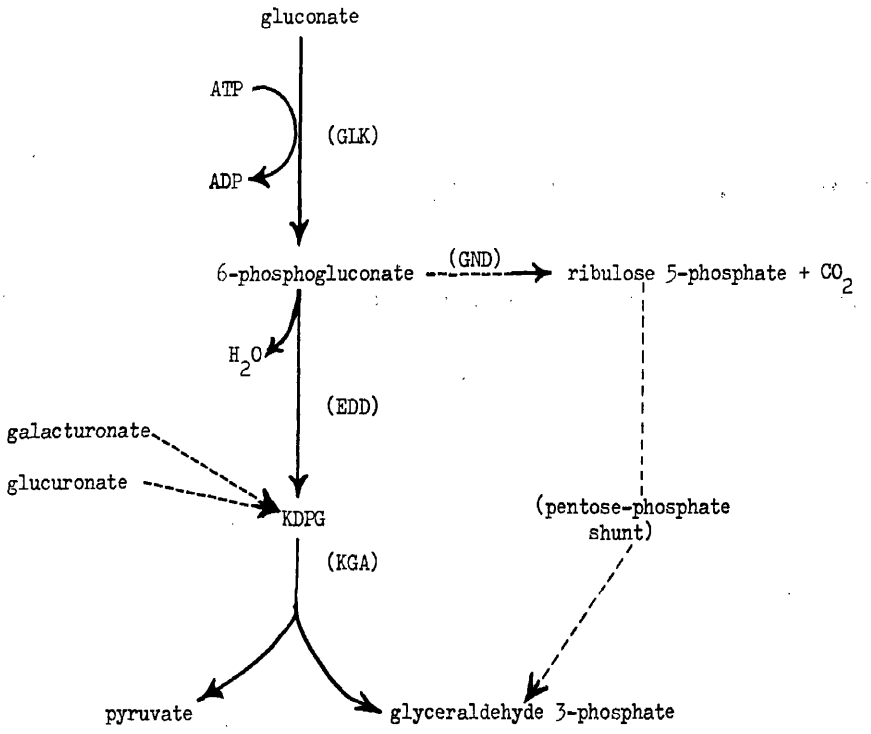

Fig. 1. Reactions of the Entner-Doudoroff pathway are indicated by bold arrows: related pathways mentioned in the text by broken arrows.

ures employed, have been previously described [12]). Of the mutants obtained with these characteristics, one designated DF1071-2B, was selected for further study.

3. Results and discussion

The above mutant grew at wild-type rates on glucose, glucose 6-phosphate, lactate, and on acetate: it had thus suffered no obvious lesions in either glycolytic or glucogenic pathways. However, it not only failed to grow on gluconate or on galacturonate, but the addition of either of these materials to cultures growing aerobically in nutrient broth resulted in a speedy cessation of growth. When cells, whose growth had been thus arrested, were harvested, suspended in a little water, and boiled, the solution obtained was rich in KDPG. Assay [13] of samples of such solutions indicated that, 3 hr after the addition of 5 mM gluconate, a culture of DF1071-2B (18 mg dry wt total) had accumulated 0.26 μmole of KDPG; this represents a KDPG concentration in the cell water of approx. 4 mM. The phosphorylated ketohexonic acid was also excreted as such into the medium [14]: in this experiment, the 18 mg dry wt of mutant cells had excreted over 18 μmoles of KDPG.

The absence, from DF1071-2B, of KGA activity was confirmed by direct spectrophotometric assay. For this purpose, the substrate KDPG (which is not commercially available), is readily prepared as follows: to 300 ml of a culture of DF1071-2B, grown overnight on double-strength "Oxoid" nutrient broth, with shaking at 37°, are added 6 ml of 0.5 M sodium gluconate and a further 300 ml of double-strength nutrient broth. After further shaking at 37° for 2 hr, the cells are harvested by centrifugation, washed once with 50 mM Tris-HCl pH 7.5, and are resuspended in 10 ml of this buffer. The thick suspension is cooled and the cells disrupted, by exposure for 1.5 min to the output of a MSE 100W ultrasonic disintegrator. Cell debris is removed by centrifugation at 20,000 *g* for 10 min; more prolonged (2–3 hr) centrifugation of the resultant suspension, at approx. 110,000 *g*, yields a clear solution with only low $NADH_2$ oxidase activity. To it (10 ml) is added 1 ml of a solution containing 30 μmoles of $MgCl_2$ and approx. 550 μmoles of 6-phosphogluconate, Na_3-salt. The mixture is incubated at 37°, samples (0.1 ml) being removed at 20 min inter-

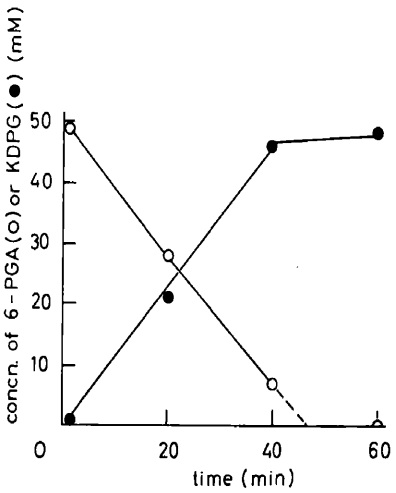

Fig. 2. Conversion of 6-phosphogluconate (○) to KDPG (●) by an extract of DF1071-2B, an *E. coli* mutant devoid of KGA activity. For details, see text.

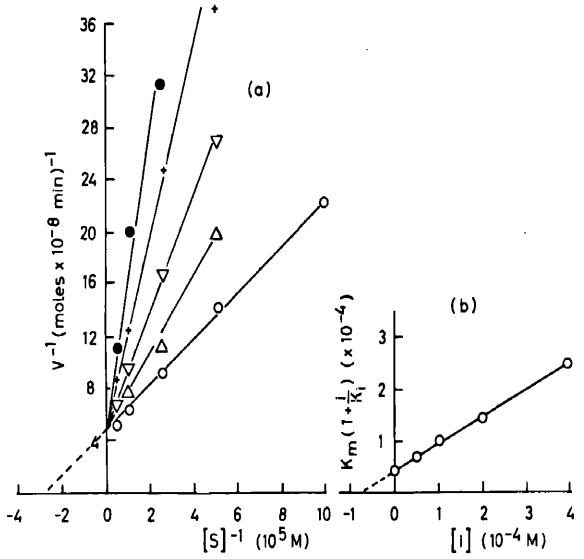

Fig. 3. Competitive inhibition of the 6-phosphogluconate dehydrogenase activity of *E. coli*, strain DF10. (a) The rate of $NADPH_2$ production from NADP by extracts incubated with various concentrations of 6-phosphogluconate either alone (○) or in the presence of (△) 50 μM, (▽) 0.1 mM, (+) 0.2 mM, and (●) 0.4 mM KDPG, was measured spectrophotometrically at 340 nm. (b) The K_i of the inhibition of GND by KDPG was calculated from the data of (a).

vals to assess the progress of the reaction. Such samples are pipetted into 0.9 ml of water and are boiled for 5 min; the 6-phosphogluconate content is assayed spectrophotometrically with commercial GND and NADP at 340 nm, whilst that of KDPG formed can be readily determined [13] at the same wavelength with commercial lactate dehydrogenase, $NADH_2$, and extracts of any strain of *E. coli* (centrifuged at high speed to reduce $NADH_2$ oxidase activity) as source of KGA. The typical progress plot, given in fig. 2, shows that there is a quantitative and stoicheiometric transformation of the added 6-phosphogluconate to KDPG: although extracts of KGA^+-cells also remove 6-phosphogluconate under these conditions, no such accumulation of KDPG occurs. The data of fig. 2 thus also provide evidence for the absence of KGA from the mutant DF1071-2B. The KDPG formed can be easily isolated as the barium salt and purified in good yield [15].

When the HfrC-strain DF1071-2B, which is sensitive to streptomycin, was crossed with the F^- recipient K2.1t, (*his*, *arg*, *thr*, *leu*, *pps*, *str*, i.e. which requires histidine, arginine, threonine and leucine for growth, is devoid of PEP-synthase activity, and is resistant to streptomycin, [12]), and streptomycin-resistant recombinants were selected that had lost specific amino acid requirements, KGA^--clones were detected only among recombinants that had lost their requirement for histidine. As expected from the known [6] close linkage of *his* and *gnd*, all the HIS^+ recombinants tested were also GND^-.

In order to investigate further the relationship of the *gnd* and *kga* markers, one of the recombinants (designated K2.1.8.24) was purified by repeated isolation of single colonies. This organism had the properties of the female parent, in so far as it was PPS^- and streptomycin-resistant, and of the male parent, in so far as it grew neither on gluconate nor on galacturonate, and its growth on nutrient broth was arrested by the addition of either of these materials: as found with DF1071-2B, KDPG accumulated in both intracellular water and in the growth medium. Moreover, whereas the KGA activity [13] of extracts of K2.1t sufficed to form more than 0.5 μmole of pyruvate from KDPG/min/mg of protein, similar extracts of K2.1.8.24 contained less than 1% of this activity.

KGA^+ recombinants were obtained from genetic crosses between K2.1.8.24 and two Hfr strains of *E. coli*. Strain AT2571 injects its genome in the order *o...trp...pps...his...*: analysis by period interruption of conjugation showed that KGA^+ recombinants appeared approx. 4 min after the entry of the *pps* allele. Strain KL96, on the other hand, injects its genome in the order *o...his, gnd...pps...trp...*: recombinants capable

of growth on galacturonate (and hence KGA^+) appeared approx. 4 min before entry of the *pps*-marker. Surprisingly, although the *his* (and hence the *gnd*) markers are among the earliest to be transferred by KL96, no colonies appeared in a selection for gluconate$^+$ galacturonate$^-$ (i.e. GND^+, KGA^-) until colonies selected for KGA^+ were already fully grown. When these tardy gluconate$^+$ colonies were isolated and tested, it was found that their growth in gluconate media was very poor (mean doubling time approx. 8 hr); that their growth in nutrient broth was arrested by the addition of gluconate, with consequent accumulation of KDPG in cells and medium; and that cell-free extracts were GND^+ and KGA^-. It was thus apparent that, despite the restoration of 6-phosphogluconate dehydrogenase activity, the formation and accumulation of KDPG from gluconate severely restricts the utilization of gluconate via the pentose-phosphate shunt. This explanation is supported by the properties of mutants devoid both of EDD and KGA activities. Such mutants grow on gluconate, since KDPG is not formed from that substrate [14]; however, the addition of galacturonate speedily arrests their growth.

KDPG and 6-phosphogluconate are of sufficiently similar structure to compete for the active sites on GND: the measured K_i for KDPG (7.5×10^{-5} M; fig. 3) is sufficiently low to compete effectively with the normal substrate, 6-phosphogluconate ($K_m = 4 \times 10^{-5}$; cf. [16]). This effect is the counterpart of the competition of KDPG and 6-phosphogluconate for the KDPG-aldolase, described by Pouysségur and Stoeber [14], though in that example both the K_m of the enzyme for KDPG (2×10^{-4} M) and the K_i for 6-phosphogluconate (8×10^{-4} M) are ten times higher.

The location of the *kga* marker on the *E. coli* genome was established more precisely by means of phage-mediated transduction. For this purpose, phage P1-kc was grown [12] on the *E. coli* mutant DF10, which is known to lack EDD activity [4] but grows on galacturonate and is thus KGA^+. Infection of strain K2.1.24 (one of the GND^+, EDD^+, KGA^- recombinants obtained from the cross of KL96 and K2.1.8.24) and selection on agar plates containing galacturonate (for KGA^+) yielded 107 transductants; tests with nutrient broth:gluconate:tetrazolium media [4] and direct assay of EDD activity in extracts of clones grown on nutrient broth + 5 mM gluconate, showed that 105 of these were EDD^-. This close linkage of *edd* and *kga* was confirmed by transducing phage P1-kc, grown on strain DF10 (*edd*), into the orginal mutant DF1071-2B (*gnd, kga*): again, KGA^+ transductants were selected on galacturonate. Of 1080 such KGA^+ transductants tested, 1036 were EDD^- whereas only 42 were EDD^+: however, all 1080 transductants had retained the GND^- phenotype. These results therefore confirm both that *gnd* and *edd* are not sufficiently closely linked to be co-transducible [4] and that two of the enzymes of the Entner-Doudoroff pathway, the dehydrase and the aldolase, are specified by genes that are over 95% co-transducible.

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THE UTILISATION OF GLUCONATE BY *ESCHERICHIA COLI* K12

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PELIN FAIK B.Sc.

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ABSTRACT

Many micro-organisms catablise gluconate via the Entner-Doudoroff pathway. The key enzymes of this pathway are gluconate kinase, 6-phosphogluconate dehydratase (Edd) and 2-keto 3-deoxy 6-phosphogluconate (KDPG) aldolase.

Biochemical and genetic techniques have been used to study gluconate utilisation in Escherichia coli K12. Mutants of the pathway have been isolated, their genetic lesions mapped and their physiological effects studied.

The first step in gluconate utilisation by E. coli is its entry into the cells. It has been established that uptake is an active process induced by growth on gluconate, and appears to be the rate-limiting step in gluconate utilisation. Gluconate thus taken up is then phosphorylated to 6-phosphogluconate by ATP, catalysed by gluconate kinase: mutants completely devoid of this enzyme have not been obtained and it may be that there are two gluconate kinases in E. coli. 6-phosphogluconate can only be metabolised via the Entner-Doudoroff and pentose-phosphate pathways since Edd^- mutants that also lack 6-phosphogluconate dehydrogenase (Gnd^-) do not grow on gluconate but the Entner-Doudoroff pathway plays the predominant role. The importance of the KDPG aldolase that catalyses the cleavage of KDPG to pyruvate and glyceraldehyde 3-phosphate has been studied with mutants devoid of this enzyme. It has been established that KDPG is a very effective competitive inhibitor of 6-phosphogluconate for Gnd.

The enzymes involved in the catabolism of gluconate are inducible. The likely inducer for the uptake system and gluconate kinase(s) is gluconate itself; 6-phosphogluconate probably induces Edd and Kga.

Genetic analysis shows that at least three regions of the E. coli chromosome (at 36, 66 and 85 min) contain genes involved in gluconate utilisation; only some of these genes are linked.