THE GENETIC CONTROL OF ANAPLEROTIC REACTIONS

IN ESCHERICHIA COLI

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

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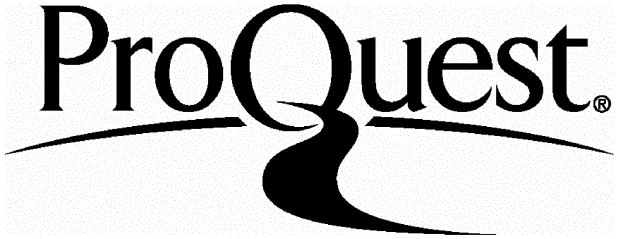

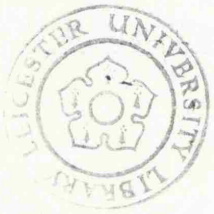

FORWARD

This work was performed in the Department of Biochemistry, the University of Leicester, during the tenure of a Science Research Council Studentship.

I should like to express my gratitude to Professor H.L. Kornberg, F.R.S., for his constant encouragement and guidance, without which much of this work would not have been possible; and also to Professor R.H. Pritchard for advice on many of the genetical procedures used herein.

My thanks are also due to Miss J.M. Farr who spent many evenings assisting me in these experiments and also in preparing media.

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ABSTRACT

Three independent mutants induced with ethylmethane-sulphonate and selected for their inability to grow on acetate, were shown by enzymic assays in comparison with their wild-type parents and revertants to be devoid of isocitrate lyase. Genetic mapping by interrupted conjugation, recombination analysis and phage transduction showed each of these lesions to lie within the same gene locus situated at 78.3 min on the *E. coli* linkage map in the order *metA*, *icl*, *pgi*. Furthermore, the occurrence of Acetate$^+$ recombinants from intragenic crosses between these mutants demonstrated their heteroallelism and located their sites within the *icl* locus in the order *metA*, *icl-1*, *icl-2*, *icl-3*.

Mutants lacking a second anaplerotic enzyme, PEP-synthase, were also obtained through E.M.S. mutagenesis but were selected for their inability to grow on lactate, pyruvate or alanine. Using the previously described mapping techniques the structural gene specifying this enzyme was shown to lie in close proximity to the *aroD* marker at 32.5 min on the *E. coli* linkage map.

These latter mutants were then employed in the isolation of spontaneously occurring *icl* regulatory mutants (*iclR*$^-$), which, by their constitutive production of isocitrate lyase, permitted the growth of these strains on lactate but neither on pyruvate nor acetate plus pyruvate. By interrupted mating and three-point

transduction, the *iclR*$^-$ lesions within two such independent mutants were mapped and shown to lie adjacent to the *icl* structural gene in the order *metA*, *icl*, *iclR*. Furthermore, "cis-trans" tests employing a merodiploid of this region demonstrated the trans-dominance of *iclR*$^+$ over *iclR*$^-$ and showed this lesion to mark the site of an *icl* regulator locus.

These results together with the location of the glyoxylate cycle malate synthase (*masA*) in the position *masA*, *icl* *iclR* (Vanderwinkel & de Vlieghere, 1968) and the demonstration of the co-ordinate formation (Kornberg, 1961, 1966) and de-repression (Vanderwinkel, Liard, Ramos & Wiame, 1963) of these enzymes provide both structural and biochemical evidence for the occurrence of a glyoxylate cycle operon.

GENERAL INTRODUCTION

The glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957, 1958) has long been established as a functional anaplerotic route in the growth of E. coli on acetate (Kornberg, 1959; Kornberg & Elsden, 1961) and its key enzymes shown to be inducibly formed (Kornberg, Phizackerley & Sadler, 1960; Kornberg, 1961, 1966). Biochemical investigations into the manner by which the action of this cycle is regulated revealed that it is subject to both "fine" and "coarse" controls, exerted on the key enzyme isocitrate lyase. The work of Kornberg (1963, 1965, 1966) demonstrated that the synthesis of this enzyme was repressed by the C_3-acids pyruvate and phosphoenolpyruvate, and it was further shown (Ashworth & Kornberg, 1963; Kornberg, 1966) that these compounds also exerted a "fine" control on this enzyme through inhibition of its activity, non-competitive with isocitrate.

However, with the exception of the experiments by Kornberg & Smith (1966) (described in Chapter I) on the genomic location of a mutation resulting in the temperature-sensitive synthesis of isocitrate lyase, this system had not been subjected to genetic analysis when this work began. The purpose of this study was, therefore, to investigate the genetic basis of the glyoxylate cycle and its control.

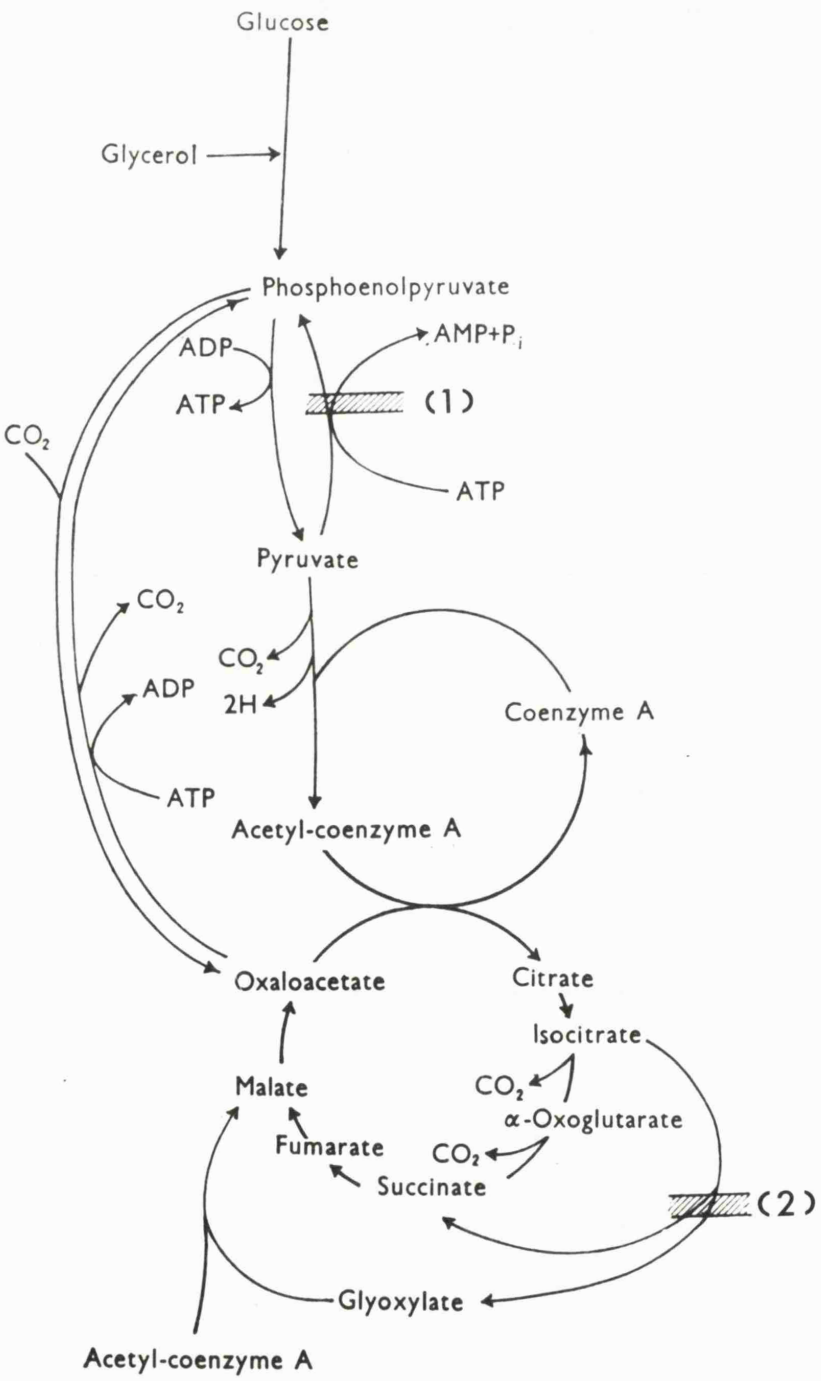


FIGURE 1

Enzymic dysfunctions in mutants of E. coli used in this work. The relationship of the metabolic lesions to the central pathways of metabolism is indicated by the hatched bars. (1) Phosphoenolpyruvate synthase; (2) isocitrate lyase. From Kornberg 1966b.

PART I

The location of the "structural" gene specifying isocitrate lyase

PART I

INTRODUCTION

The first indication of the location of a gene on the *E. coli* chromosome, the mutation of which led to an alteration of the glyoxylate cycle, came from the work of Kornberg & Smith (1966), who described the properties of a mutant of *E. coli* K12 which synthesised isocitrate lyase normally at 30°C but ceased to form the enzyme at 37°C. The demonstration that the enzyme from this strain was considerably more heat-sensitive than that from its wild-type parent led these workers to conclude that the mutation had affected the structure of the enzyme and hence that the location of the marker specifying the altered enzyme, approximately 2 min from the *argHBCE* cluster of markers, was the location of the *icl* structural gene on the *E. coli* genome.

The purpose of the first section of this thesis is to present evidence for the more accurate location of the structural gene specifying this enzyme as determined with a number of independently obtained mutants.

MATERIALS AND METHODS

Organisms

The strains of *E. coli* K12 used in this chapter are listed in table 1. Of these, the following were obtained as gifts: AT 2572 and AB 1911 - from Professor E. A. Adelberg (Yale University Medical School); PA505 - from Professor F. Jacob (Institute Pasteur, Paris); DF11 - from Dr. D. G. Fraenkel (Harvard Medical School); R3, R4 and G6 - from Professor R. H. Pritchard (University of Leicester).

TABLE 1

Strains of *E. coli* K12 used in part I of this thesis

Strain	Parent(s)	Genetic markers	Response to Streptomycin	Mating type
AT 2572	-	-	S	Hfr
R3	-	*thr*,*leu*	S	Hfr
R4-5	R4	*metBF*, *icl-1*	S	Hfr
G6	-	*his*, *thy*	S	Hfr
G6-5	G6	*his*, *thy*, *icl-2*	S	Hfr
AB1911	-	*metBF*, *argHBCE*	S	F^-
AB1911-5	AB1911	*metBF*, *argHBCE*, *icl-3*	S	F^-
K8	AB1911	*metBF*, *argHBCE*	R	F^-
K8-5^{CBB}	AB1911-5	*metBF*, *argHBCE*, *icl-3*	R	F^-
K8-5*m*	G6-5 x K8	*metBF*, *icl-2*	R	F^-
K8-5*a*	G6-5 x K8	*argHBCE*, *icl-2*	R	F^-
DF11	-	*metA*, *pgi*	R	F^-
PA505	-	*metA*, *argHBCE*, *his*, *proA*	R	F^-

Abbreviated symbols indicate a requirement for: *met* = methionine; *arg* = arginine; *his* = histidine; *pro* = proline; *thy* = thymine. The inability to form phosphoglucose isomerase and isocitrate lyase is indicated by the abbreviations *pgi* and *icl* respectively. Numbers following genetic symbols designate independent mutations at different loci. Sensitivity to streptomycine is indicated by *str*. The use of symbols is in general accord with the convention suggested by Demerce, Adelberg, Clark & Hartman (1966).

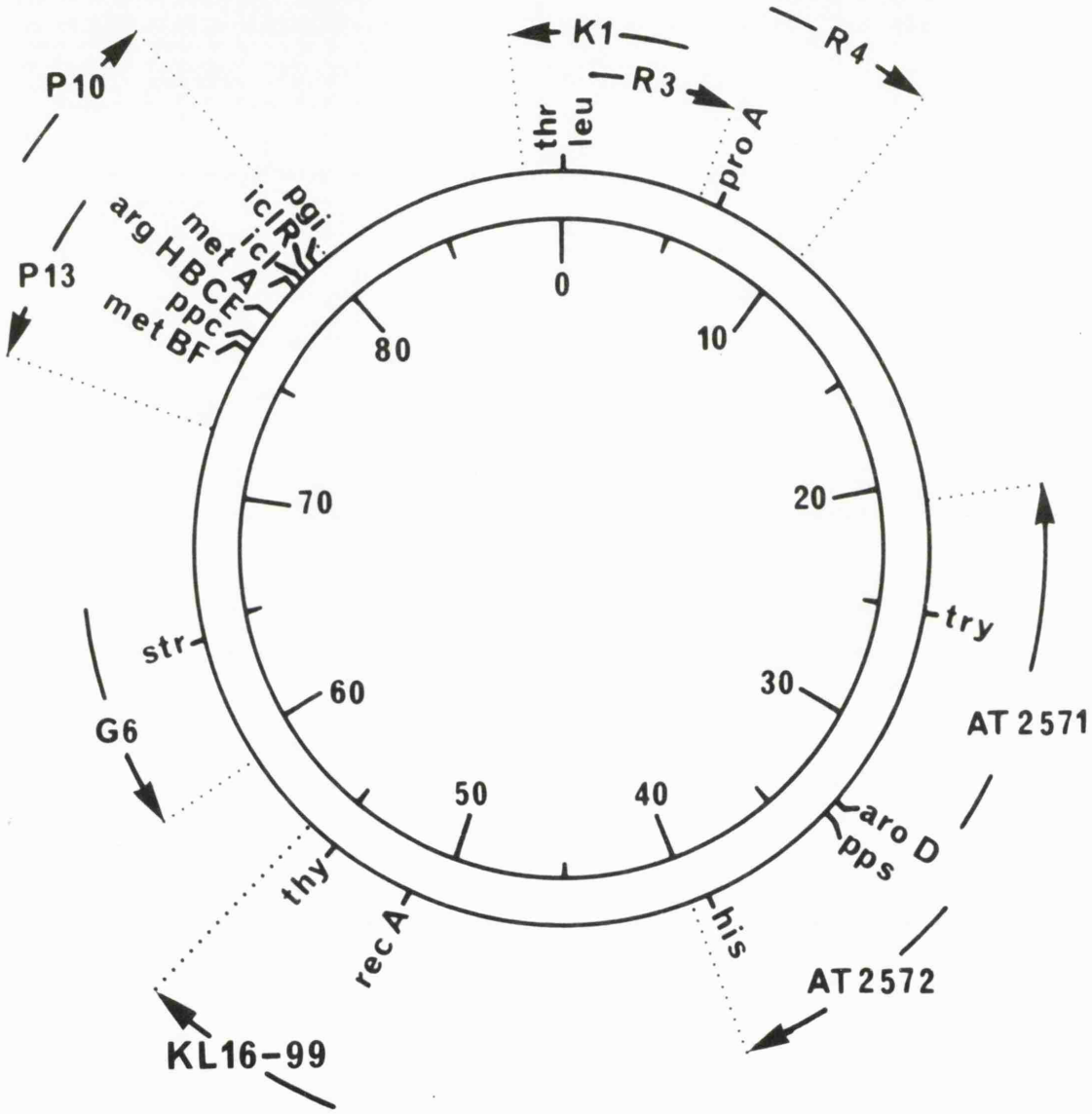


FIGURE 2

The genetic map of E. coli (adapted from Taylor & Trotter, 1967). The map is numbered inside the circle at 10 min intervals. On the outside of the circle are shown the locations of genetic markers referred to in this thesis. The bold arrows indicate the points of origin and direction of genome transfer of the genetic donor strains used in this work.

Maintenance and growth of organisms

Single colony isolates of each strain were stored at room temperature, after overnight growth at 37°C, on Oxoid nutrient agar slopes. These were supplemented where necessary with L-methionine and thymine (40 µg/ml) and thiamine (0.1 µg/ml). Stock cultures of the organisms were subcultured periodically.

For growth in liquid media a loopful of organisms was suspended in the sterile synthetic ("basal") growth medium described by Ashworth & Kornberg (1966), with carbon source as stated, or in Oxoid nutrient broth. The "basal" medium was supplemented with the appropriate growth factors in final concentrations of: L-arginine 100 µg/ml; L-methionine, L-threonine, L-leucine, L-histidine, L-proline 40 µg/ml; and thymine 40 µg/ml.

In order to facilitate aerobic growth conditions, the volumes of cultures were not allowed to exceed 2/5ths the volume of the container.

Cultures were shaken at 37°C on either a New Brunswick "Gyrotory" shaker or a Gallenkamp waterbath shaker. Cell growth was determined by measurements with a Unicam SP600 spectrophotometer of the light absorbed at 680 mµ. These readings were translated from previously calibrated curves into either the dry weight of bacterial cell mass or cell numbers, per ml of suspension.

Solid growth medium Agar plates were prepared either by the

addition of 1.5% (w/v) Oxoid Ionagar No. 2 to the "basal" growth medium or from 2.8% (w/v) Oxoid nutrient agar. Glucose tetrazolium plates consisted of 1% (w/v) D(+)-glucose and 0.005% 2,3,5, triphenyl tetrazolium chloride in addition to the nutrient agar medium.

For the preparation of bacteriophage lysates, the nutrient agar was supplemented with $CaCl_2$ and $MgCl_2$ at final concentrations of 2.5 and 10 mM respectively. This material will be referred to as 'Z agar' henceforth.

Preparation of cell free extracts Samples of bacterial cultures containing 4 - 5 mg dry wt of cells were centrifuged at 15 x g for 5 min, washed with and resuspended in a buffer at pH 7.5 containing 10 mM-sodium/potassium phosphate, 10 mM-$MgCl_2$ and 1 mM-ethylene diamine tetraacetate. The resultant suspension, cooled in melting ice, was then exposed for 2 min to the output of a M.S.E. 60W ultrasonic oscillator operating at 1.5 amps. Cell debris was removed by centrifuging at 15000 x g for 5 min at 4°C, the supernatant solutions were decanted and kept in ice.

Assay of isocitrate lyase activity The enzyme was assayed spectrophotometrically by a modification of the procedure described by Dixon & Kornberg (1959). Silica cuvettes (1.5 ml vol. 1 cm light path) were set up containing in a 1.0 ml final volume: 0.2 ml of R_{im} mixture, 2 µmoles of D isocitrate(dipotassium hydrogen salt)

(added last to the test cell only), cell-free extract and water. The R_{im} was a mixture of 11.52 mg of phenylhydrazine hydrochloride in 1.0 ml of 0.5 mM-iminazole buffer, pH 6.8; 1.0 ml of 0.1 mM-$MgCl_2$; 0.2 ml of 0.1 M-EDTA, pH 6.8 and 1.8 ml of H_2O. The increase in adsorption at 324 mμ, due to the formation of glyoxylate phenylhydrazone, was measured continuously using an "Optica" recording spectrophotometer. The observed rate of increase of $E_{324m\mu}$ is linear with respect to time after an initial lag of 1-3 min, depending on the activity of the sample. Under these conditions the formation of 1 μmole of glyoxylate phenylhydrazone is accompanied by $\Delta E_{324m\mu}$ of 16.8 O.D. units. Activities, calculated from the linear portion of the graph, were therefore expressed as μmoles of glyoxylate (phenylhydrazone) formed per hour per mg of protein. The assay was stoicheometric for isocitrate lyase activity provided the $\Delta E_{324m\mu}$ was not greater than 0.3 units/min.

Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Mutagenesis Two methods of mutagenesis were used:

(i) Ethylmethane sulphonate (EMS) method Icl-mutants were induced with EMS as described by Lin, Lerner & Jorgensen (1962) and selected with penicillin (Gorini & Kaufman, 1960) for their inability to utilise acetate as a sole carbon source. These mutants were detected by replica-plating colonies growing on a non-selective medium

(such as glucose) onto acetate plates. Single colony isolates of the presence of these presumptive mutants were then checked for/their original growth grown markers. Cultures of such mutants/in nutrient broth were disrupted and assayed for isocitrate lyase activity as described.

(ii) Manganous chloride method This technique is a modification of that described by Demerec & Hanson (1951) and was used specifically for the mutation of streptomycin sensitive organisms to streptomycin resistance, as in the cases of AB1911 and AB1911-5^{CBB}. Ten ml of a broth-grown culture, containing approx. 0.7 mg dry wt of cells per ml, were harvested at 15,000 xg for 5 min at 15°C, washed in 10 ml of 0.25 mM-NaCl and finally resuspended in 1.0 ml of 0.4 % $MnCl_2$. The suspension was incubated for 1 hr at 37°C, when 0.1 ml samples were spread directly onto agar plates containing nutrient agar supplemented with streptomycin at 50 µg/ml. The plates were incubated at 37°C for 24 - 36 hr, by which time a number of streptomycin-resistant colonies had appeared; these colonies were picked, purified by single colony isolation, tested for their original growth markers, and maintained on agar slopes.

Interrupted mating Technique Overnight broth cultures of the donor (Hfr) and recipient strains (F^-) were diluted with fresh broth and grown at 37°C to about 3 x 10^8 (Hfr) and 7.5 x 10^8 (F^-) cells/ml. Sufficient of these cultures was mixed together to give final concentrations of 1 x 10^8 Hfr and 5 x 10^8 F^- cells/ml and the mixture

was agitated gently at 37°C for 3-5 min to permit the formation of specific mating pairs. After this period, further pair-formation was reduced by diluting the mixture 1 : 100 into a 25 mM-glucose minimal medium standing at 37°C. At predetermined intervals 2.0 ml samples were withdrawn and agitated violently for 3 min on a Griffin flask shaker to separate the mating pairs. Duplicate 0.1 ml samples of this "blended" mixture were then plated immediately onto each of the selective media, which were supplemented with 0.1 ml of nutrient broth/plate. Plates were incubated for 24 - 48 hrs at 37°C. The parent donor cells were eliminated by inclusion in the selective medium of streptomycin sulphate (50µg/ml) and where possible, the omission of necessary growth factors. Unmated receipient cells were eliminated by the omission from the selective media of the appropriate growth factors. Control samples of 0.1 ml of the unmated Hfr and F^- cultures were also plated onto each of the selective media. The introduction of a wild type allele for isocitrate lyase was scored as the ability to grow on medium containing 50 mM-acetate as carbon source.

This mating method is a modification of the "pulse mating" technique reported by de Haan & Gross (1962) and Taylor & Thoman (1964). The cells were diluted into a glucose minimal medium after the initial attachment period, since it was reported (de Haan & Gross, 1962) that the stability of pairing in some Hfr strains is markedly reduced in a broth medium. However, these authors also state that the efficiency of recombinant formation in zygotes derived from broth-grown cells is some 2-5 times greater in the presence of broth. Consequently, a small quantity of broth was added to the selective plates before use.

Bacteriophage-mediated transduction

Preparation of Lysates An overnight broth culture of the donor bacterial strain was grown at 37°C to a cell density of about 1×10^9 cells/ml. To this was added sterile $CaCl_2$ and $MgCl_2$ in final concentrations of 2.5 mM and 10 mM respectively. Three ml of this suspension was mixed with about 3×10^7 phage P1 kc particles from a strong stock lysate and incubated for 20 min at 37°C to allow phage adsorption. The suspension was then mixed with 17 ml of melted soft (0.6%) agar at 46°C, and 3.0 ml samples were immediately plated onto each of 6 'Z' agar plates. The plates were incubated at 37°C for 6 hr. At the end of this period, 2.0 ml of eluting fluid (0.8 % NaCl, 0.8% peptone, saturated with $CHCl_3$) were added to each plate and together with the soft agar layer, scraped into an homogenising tube. The agar was homogenised for 2 min in order to release the phage progeny therein. The homogenate was then centrifuged at 15,000 xg for 5 min at 4°C and the supernatant fluid decanted, saturated with $CHCl_3$ and stored at 4°C.

Titration of Lysates A suitable bacterial indicator strain in stationary phase was mixed with $CaCl_2$ and $MgCl_2$ as above. Lysate dilutions of 10^{-6} and 10^{-7} were made into nutrient broth and duplicate 0.1 ml samples of each mixed with 0.1 ml of indicator strain. The suspensions were incubated at 37°C for 5 min after which 3.0 ml of melted soft agar was added to each one and the mixtures plated onto

'Z agar'. The plates were incubated at 37°C overnight and scored for phage plaques. The strength of the lysate was expressed as the average numbers of plaque-forming particles per ml of undiluted lysate solution. Lysates obtained in this manner contained about $1 \times 10^{10} - 1 \times 10^{12}$ plaque-forming particles per ml of solution.

Transduction Twenty ml of the recipient bacterial strain grown in broth to a density of $5 \times 10^{8} - 1 \times 10^{9}$ cells/ml were harvested at 15,000 xg for 5 min at 15°C and resuspended in 2.2 ml of 'Z broth' (nutrient broth supplemented with $CaCl_2$ and $MgCl_2$ as in 'Z' agar). Two ml of this concentrate was then mixed in a 100 ml flask with 10-12 ml of the appropriate P1 kc lysate, which had previously been aerated for 20 min at 37°C to remove the chloroform. The mixture was incubated without agitation for 30 min at 37°C after which the cells were harvested as before and resuspended in 1.0 ml of nutrient broth. From this, appropriate dilutions were made and 0.1 ml samples spread onto the selective media. Control samples (0.1 ml) of both the phage lysate and the non-infected recipient strain were also plated onto these media. The plates were incubated at 37°C for 24 - 48 hr. The prototrophic transductants from each selection were then replica-plated onto media selective for the other donor alleles ~~loci~~ in the cross. Co-transduction frequencies were then expressed either as the percentages of these secondarily selected alleles present in the originally selected population or as decimal fractions of a unitary scale.

Chemicals Ethylmethane sulphonate was obtained from Eastmann Chemicals Ltd.; Potassium dihydrogen D isocitrate was a gift from Dr. H.B. Vickery (Connecticut Agricultural Experiment Station, New Haven, Conn, U.S.A.); Streptomycin sulphate and penicillin (crystalline benzyl penicillin) were purchased from The Glaxo Laboratories, Greenford. All other chemicals were purchased through the British Drug Houses Ltd., Poole, Dorset.

RESULTS

Isolation and characterisation of icl-Mutants

A lesion in the glyoxylate cycle does not impair the ability of the cell to grow at the expense of compounds metabolised via the glycolytic sequence and the tricarboxylic acid cycle, such as glucose or glycerol; neither does it affect growth on C_3-acids, which are metabolised via a different anaplerotic pathway from that used in acetate metabolism. Consequently, mutants were selected by their inability to grow at the expense of acetate, whilst retaining the ability to utilise glucose, lactate and succinate as sole carbon sources.

In this manner, two independent icl-mutants were obtained. Strain G6-5 was derived from the Hfr strain G6 and AB 1911-5, and its streptomycin-resistant derivative K8-5^{CBB}, were derived from the F^- strain AB1911. These mutants, together with the R4-5 strain isolated by Kornberg & Smith (1966), were used in subsequent experiments. The principal growth characteristics of these three strains, in comparison with that of their wild-type parents, are summarised in Table 2.

Isocitrate lyase activities in extracts of organisms used

After initial selection, the prospective mutants were grown in nutrient broth and assayed for their isocitrate lyase content in

comparison with that of their parent strains, as described in the "Methods" section of this chapter.

The results obtained, together with the activities of other strains, used in this chapter, are listed in Table 3: they show that extracts of these mutants were indeed devoid of isocitrate lyase activity.

Isolation of revertants from icl-mutants R4-5 and K8-5^{CBB}

Further evidence that the inability of these mutants to grow at the expense of acetate was a consequence of their lack of isocitrate lyase activity, was obtained through the isolation on acetate of revertants of the strains R4-5 and K8-5^{CBB}. Thick suspensions of these strains from broth-grown cultures were spread onto 50 mM-acetate plates supplemented with the appropriate growth factors. The plates were incubated at 30°C for 5-6 days after which a number of revertant clones appeared. Samples of these colonies from each strain were then re-isolated on acetate medium and checked for the presence of their characteristic growth markers to exclude the possibility that the organisms thus isolated were contaminants.

Broth-grown cultures of the revertants from each strain were then assayed for their isocitrate lyase content. As shown in Table 3 each clone tested was found to have regained isocitrate lyase activity although the specific activities of the enzyme differed from those observed with wild-type counterparts of the revertants.

TABLE 2

Growth characteristics of mutants R4-5, G6-5 and K8-5^{CBB}

Carbon Source	* Growth at 37°C	
	wild-type	mutant
25 mM-Glucose	+	+
25 mM-Glycerol	+	+
25 mM-Lactate	+	+
25 mM-Pyruvate	+	+
50 mM-Acetate	+	-
25 mM-Acetate + 10 mM-Pyruvate	+	+
25 mM-Succinate	+	+
25 mM-Glutamate	-	-
25 mM-Aspartate	±	±

* Incubation for 24 - 48 hours.

TABLE 3

Isocitrate lyase activities in extracts of strains used in Part I of this thesis.

Strain	specific activity of isocitrate lyase
R4	4.1
R4-5	<0.1
G6	14.3
G6-5	-
K8	1.13
K8-5a	-
K8-5m	-
K8-5^{CBB}	<0.1
P10	1.05
AT2572	5.55
PA505	1.86
DF11	1.47
R4-5 Ac^{+} revertants	0.26 - 0.27
K8-5^{CBB} Ac^{+} revertants	4.0 - 5.9
Transductants:	
R4-5 ⟶ PA505	0.1
AB1911-5 ⟶ PA505	0.1 - 0.2
AT2572 ⟶ K8-5	4.3 - 5.2
G6-5 ⟶ DF11	-

Specific activities are expressed as μmoles of glyoxylate (phenylhydrazone) formed per hour per mg of protein.

Location of *icl*-markers

A. By Interrupted conjugation

Although the induction of the *icl-2* lesion in the Hfr strain G6-5 permitted its transfer to a variety of F^- strains, since its presence resulted in the inability of the cell to grow at the expense of acetate, its entry into recombinants could not be selected for directly and its location could only be determined by analysing the *icl* genotypes of recombinants prototrophic in various marker genes. However, the findings of Kornberg & Smith (1966) that a structural gene mutation specifying the temperature-sensitive synthesis of isocitrate lyase was located at approximately 78 min on the linkage map of *E. coli* strongly suggested that the *icl-2* allele might lie in a similar position, in which case the preparation of an F^- recombinant carrying both the *icl-2* lesion and a marker gene such as *argHBCE*$^-$ would permit, its accurate location by direct selection (on acetate) for the entry of the wild-type *icl* allele.

In order to prepare such a recombinant, the Hfr strain G6-5 (*his*, *thy*, *icl-2*, *str*) in early logarithmic phase, was mated in broth with a ten-fold excess of the recipient strain K8 (*argHBCE*, *metBF*). After 45 min incubation at 37°C, the cells were diluted ten-fold with dilute basal medium and the mating interrupted as described under "Methods". Samples of 0.1 ml were spread onto 25 mM-glucose media selective for Arg^+ and Met^+ recombinants and the

resulting colonies were then replica-plated onto 50 mM-acetate plates to select organisms which had also received the icl-2 marker. Two useful classes of recombinants were thus isolated: those of the genotype icl-2, argHBCE designated K8-5a and those icl-2, metBF designated K8-5m.

Each of these icl-recombinant strains was mated (as described under "Methods") with the Hfr strain P10 (thr, leu, str), which transfers its genome in the order malB - metA - argHBCE ... ilv as shown in Figure 2. Samples of the mating mixture were withdrawn and interrupted at 3 min intervals over a period of 20 min and plated onto media selective for Acetate$^+$ and either Arg$^+$ or Met$^+$ recombinants as appropriate.

In the cross P10 x K8-5a (Fig. 3), the wild-type allele for isocitrate lyase, conferring the ability to grow on 50 mM-acetate, was transferred to the receipient after 3 min (+ 4 min pairing time) of mating. This was followed one minute later by the wild-type allele of the argHBCE cluster, selected by growth on 25 mM-glucose minimal medium. On mating P10 with the F$^-$ strain K8-5m (Fig. 4), the wild-type allele for isocitrate lyase was again transferred after 3 min (+ 4 min pairing time) of mating. The wild-type allele of the metBF gene followed 2 min later. These results were consistent with the gene order icl-2, argHBCE, metBF and located the gene at approximately 78 min on the chromosome map of E. coli (Taylor & Thoman, 1964). They were also in good accordance with the location reported for the temperature sensitive synthesis lesion

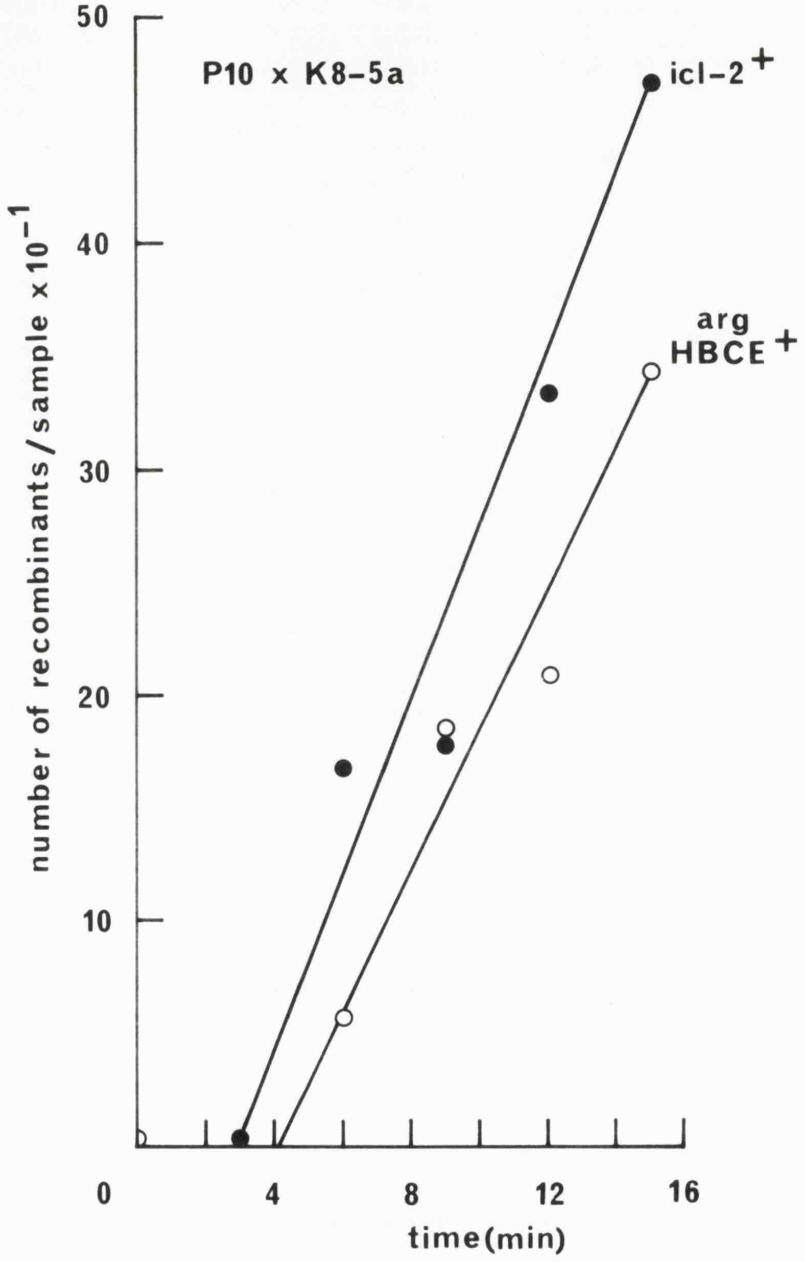


FIGURE 3

Kinetics of transfer at 37°C of the wild-type alleles for argHBCE and icl-2 from the donor strain P10 to K8-5a. The times plotted on the abscissa represent intervals between the dilution of the mating pairs and the interruption of mating.

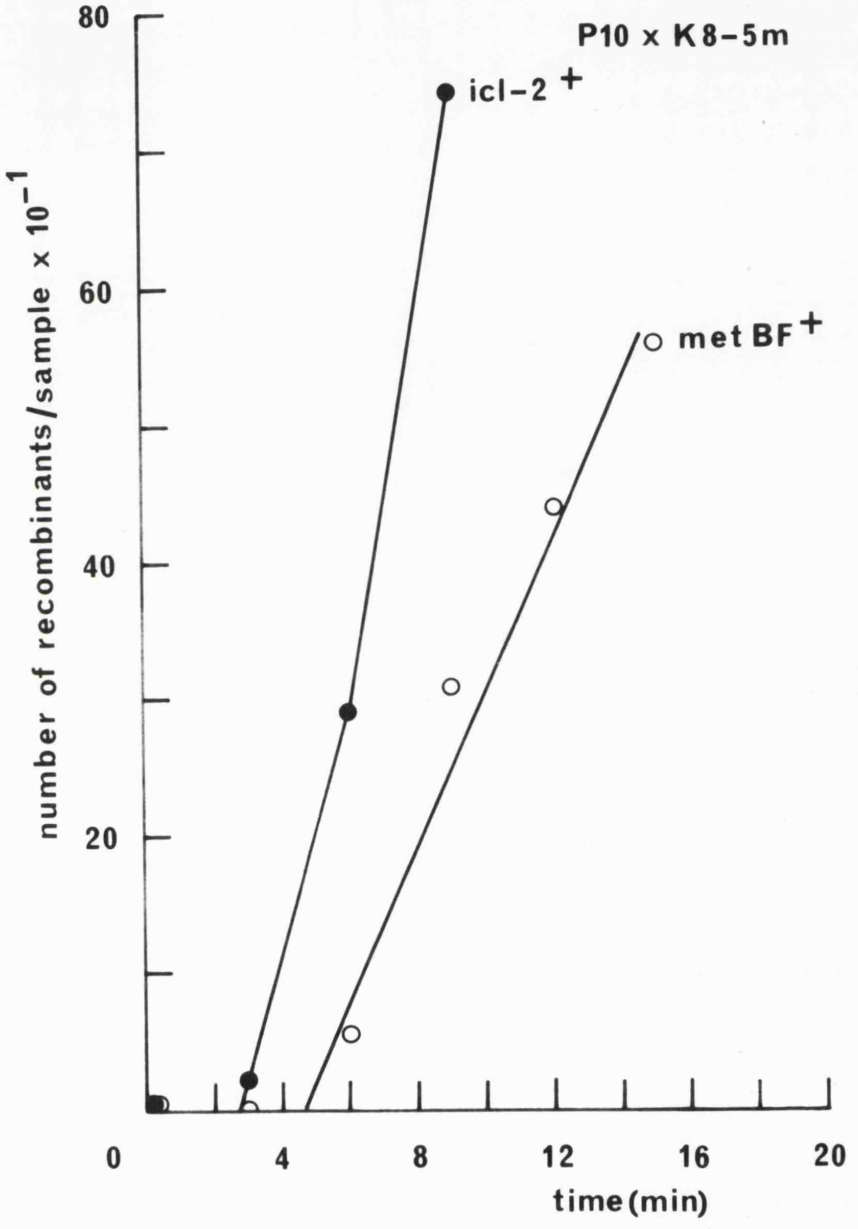


FIGURE 4

The kinetics of transfer at 37°C of the wild-type metBF and icl-2 alleles from the donor strain P10 to K8-5m. The times plotted on the abscissa represent the intervals between the dilution of the mating pairs and the interruption of mating.

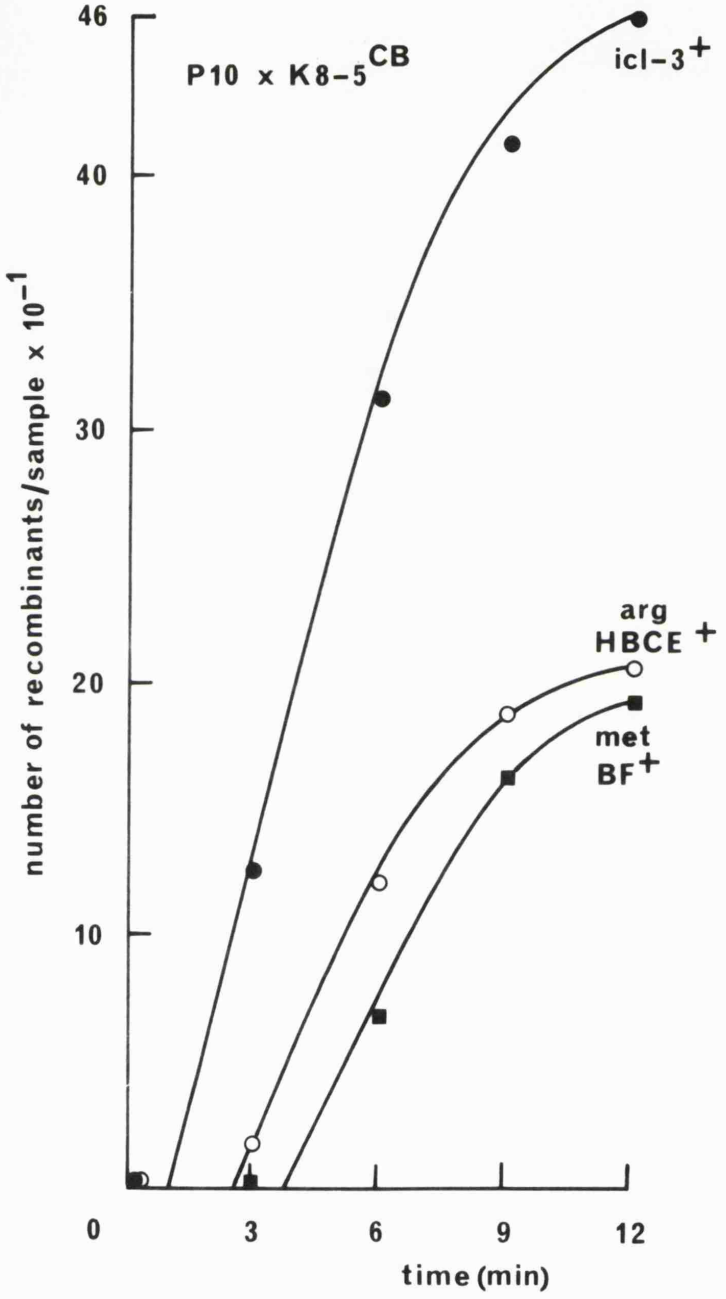


FIGURE 5

Kinetics of transfer at 37°C of the wild-type alleles argHBCE, metBF and icl-3 from Hfr P10 to recipient strain K8-5^{CBB}. The times on the abscissa represent the intervals between the dilution of the mating pairs and the interruption of mating.

by Kornberg & Smith (1966).

(ii) K8-5^{CBB} lesion (icl-3) The closely similar chromosomal positions of the TSS and temperature non-sensitive icl-2 lesions, were reflected in experiments employing a third independent icl-mutant, K8-5^{CBB}.

This lesion was induced with ethylmethansulphonate in the F$^-$ strain AB1911 (argHBCE, metBF, str), in which it could be mapped directly, with respect to the arginine and methionine markers used for the previous mappings. An icl-mutant thus obtained was purified and designated AB1911^{CBB}. Before use this strain was converted to streptomycin resistance by manganous chloride mutagenesis and renamed K8-5^{CBB}.

When strain K8-5^{CBB} was mated with the Hfr strain P10, the wild-type allele specifying isocitrate lyase entered the recombinants approximately 1-1½ min before that of the argHBCE cluster and 2-2½ min before the metBF$^+$ marker (Figure 5). The entry of the markers metBF$^+$ and argHBCE was observed as growth on 25 mM-glucose minimal medium plus either L-arginine or L-methionine as appropriate.

These results obtained were also in good accordance with the gene order icl, argHBCE,metBF. They further showed that the icl-3 lesion lay close to the position reported for icl-1 (TSS) and icl-2.

B. Determination of the recombination frequency between the genes

icl-3, argHBCE and metBF

The order of the genes icl-3, argHBCE and metBF obtained through the interrupted mating between P10 and K8-5^{CBB} was substantiated by the determination of their recombination frequencies from a cross between K8-5^{CBB} and the Hfr strain R3 (thr, leu, str) (Figure 2). The strains were mated as described under "Methods" and the culture was allowed to stand for 35 min at 37°C in a 25 mM-glucose medium supplemented with L-arginine, L-methionine, L-threonine and L-leucine. The mating was then interrupted and samples were plated onto a 25 mM-glucose medium selective for Met$^+$ recombinants. Such recombinants were then replica-plated onto media selective for the Arg$^+$ and Acetate$^+$ alleles in order to analyse their resultant genotypes (Table 4). Since the most distal gene in this cross was shown by interrupted mating to be metBF, a primary selection for the wild-type metBF allele ensured that the resultant ~~recombinants~~ zygotes had all received the wild-type alleles for argHBCE and icl-3 and thus that their genotypes were a valid reflection of the recombination frequencies between these genes and not in part the result of the termination of chromosome transfer between markers.

In these results presented in Table 4, the recombination frequency between the genes argHBCE and icl-3 is represented by the percentage of Met$^+$, Arg$^+$, Acetate$^-$ recombinants in the total

population

$$\text{or} \quad \frac{72}{308} \times \frac{100}{1} = 23.2\%.$$

The recombination frequency between argHBCE and metBF is indicated by the frequency of Met^+, Arg^-, $Acetate^-$ recombinants:

$$\frac{48}{308} \times \frac{100}{1} \quad \text{or } 15.6\%$$

and the recombination frequency between metBF and icl-3 is represented by the total of the above two classes:

$$\frac{120}{308} \times \frac{100}{1} \quad \text{or } 39\%.$$

These results thus show the distance between metBF and icl-3 to be equal to the sum of the distances between metBF - argHBCE, and argHBCE - icl-3 and support the previously indicated order metBF, argHBCE, icl-3.

C. Cotransduction of the icl-2 and icl-3 markers with metBF and argHBCE

A more precise location of the independent lesions of strains G6-5 and $K8\text{-}5^{CBB}$ was achieved by phage P1kc mediated

transduction. The icl-2 marker was mapped in relation to argHBCE and metBF in the recombinant strains K8-5a (argHBCE, icl-2) and K8-5m (metBF, icl-2) both these strains having received the icl-2 marker from G6-5. The icl-3 lesion of K8-5^{CBB} was also mapped in relation to these markers, both of which it carried on its genome.

Each of the three strains was infected as described under ...

(continued on page 27)

TABLE 4

Recombination analysis results from cross R3 x K8-5^{CBB}

Selected genotypic marker	Unselected genotypic markers			
metBF$^+$	*argHBCE*$^+$, *icl*$^+$	*argHBCE*$^+$, *icl*$^-$	*argHBCE*$^-$, *icl*$^-$	*argHBCE*$^-$, *icl*$^+$
308	188	72	48	0

"Methods" with phage P1kc grown on the wild-type donor strain AT2572 (str). After transduction, dilutions were plated onto 25 mM-glucose media selective for either Arg^+ or Met^+ transductants and onto 50 mM-acetate plates, selective for transductants possessing the wild-type isocitrate lyase alleles. The resultant transductants from each selection were then replica-plated onto media selective for the other gene(s) in the cross to determine their relative degress of cotransduction.

(i) Position of the icl-2 marker In the transduction AT 2572/K8-5a (Figure 8, Table 5), 16.5% of the $Acetate^+$ transductants had also received the Arg^+ allele and conversely 13.5% of Arg^+ transductants were also $Acetate^+$. This represents a mean cotransduction value between icl-2 and argHBCE of 15%. In the transduction AT2572/K8-5m (Table 6, Figure 8), 2.4% of the $Acetate^+$ colonies were also Met^+ and 1.1% of the Met^+ transductants were also $Acetate^+$, representing a mean value of 1.75% cotransduction between icl-2 and metBF.

TABLE 5

Cotransduction values between icl-2 and argHBCE from the transduction AT2572/K8-5a

Number of Transductants			% Cotransduction
Selected genotypic marker	Unselected genotypic marker		
icl$^+$	argHBCE		
	+	-	
594	100	494	16.5
argHBCE$^+$	icl		
	+	-	
1978	261	1717	13.5
		Average	15

TABLE 6

Co transduction values between icl-2 and metBF from the transduction AT2572/K8-5m

Number of transductants			% Cotransduction
Selected genotypic marker	Unselected genotypic marker		
icl+	metBF		
	+	-	
463	11	452	2.4
metBF+	icl		
	+	-	
389	4	384	1.1
		Average	1.7%

(ii) Position of icl-3 marker. Using strain K8-5^{CBB} it was possible to perform a direct 3-point transduction between the markers argHBCE, metBF and icl-3 (Table 7, Figure 8).

Colonies selected as Acetate$^+$ showed 13.5% cotransduction with argHBCE and 1.8% cotransduction with metBF. The frequency of colonies containing all wild-type alleles (Arg$^+$, Met$^+$, Acetate$^+$) was also 1.8%, suggesting that icl and metBF were the outside markers in this cross.

Those transductants selected originally at metBF$^+$ exhibited 40% cotransduction with argHBCE and 2.7% cotransduction with icl. Again the frequency of Arg$^+$, Met$^+$, Acetate$^+$ transductants was similar to the cotransduction frequency between icl and metBF at 2.7%.

Analysis of the Arg$^+$ selection showed 24.5% cotransduction between argHBCE and metBF and 9% cotransduction between the argHBCE and icl markers. The frequency of 3-point transductants (Arg$^+$, Met$^+$, Acetate$^+$) in this selection was 0.45% - some 1.4% of the Arg$^+$, Met$^+$, and Arg$^+$, Acetate$^+$ colonies. This would be expected when selecting for the middle marker in the cross since transducing fragments carrying argHBCE$^+$ would carry icl$^+$ or metBF$^+$ in their respective frequencies, and would carry both markers in the product of these frequencies.

Thus: The chance of an argHBCE$^+$ transducing fragment also carrying icl$^+$ = 9%

The chance of an argHBCE$^+$ transducing fragment also carrying metBF$^+$ = 24.5%

The chance of an *argHBCE*$^+$ fragment carrying both *icl*$^+$ and *metBF*$^+$

$$= 24.5\% \text{ of } 9\% = 2.2\%$$

$$2.2\% \text{ of the selected Arg}^+ \text{ colonies} = \frac{281}{100} \times \frac{2.2}{1} = 6$$

Observed number of such 3-point transductants = 4

In order to determine the validity of the Acetate$^+$ selection, a random sample of such transductants was grown in broth and assayed for isocitrate lyase activity, as described under "Methods." The enzyme was found to be present in all cases at specific activities between 4.3 and 5.2 μmoles of glyoxylate formed/hr/mg protein, levels similar to that found in the wild-type donor strain AT2572 (Table 3).

These results establish the gene order *icl*, *argHBCE*, *metBF* and the average cotranduction frequencies between the genes as being:

icl - *argHBCE*	11%
icl - *metBF*	2.2%
argHBCE - *metBF*	32%

TABLE 7

Cotransduction values between icl-3, argHBCE and metBF from the transduction AT2572/K8-5^{CBB}

Selected genotypic marker	Unselected genotypic markers				% Co-transduction	
icl$^+$	arg$^+$, met$^+$	arg$^+$, met$^-$	arg$^-$, met$^-$	arg$^-$, met$^+$	icl-arg	icl-met
838	15	99	724	-	13.5	1.8
arg$^+$	icl$^+$, met$^+$	icl$^+$, met$^-$	icl$^-$, met$^+$	icl$^-$, met$^-$	arg-icl	arg-met
837	4	75	202	556	9	24.5
met$^+$	arg$^+$, icl$^+$	arg$^+$, icl$^-$	arg$^-$, icl$^-$	arg$^-$, icl$^+$	met-arg	met-icl
508	14	192	302	-	40	2.7

D. Cotransduction of icl-1 and icl-3 with metA

The close similarity in mapping positions of the three independent icl- lesions, and their distance from the argHBCE cluster suggested that they might be cotransducible with the metA marker, which is situated at 78.2 min on the linkage map of E. coli (Taylor & Dunham Trotter, 1967).

This was tested by infecting the F^- strain PA505 (metA, argHBCE, his, proA) with lysates prepared from the donor strains R4-5 (metBF, icl-1, str) and AB1911-5^{CBB} (the str parent of strain K8-5^{CBB}) (argHBCE, metBF, icl-3, str).

Samples of 0.1 ml were plated onto a 25 mM-glucose medium selective for Met^+ transductants and the resultant colonies were replica-plated onto 50 mM-acetate plates supplemented with L-proline, L-histidine and L-arginine, to determine the degree of cotransduction between metA and the icl alleles.

(i) Position of icl-3 with respect of metA Of 1006 colonies scored as Met^+, 990 (97.5%) (Figure 8) had also received the icl-3 allele and thus did not grow on acetate; 16 (2%) still retained the ability to grow at the expense of acetate.

This result was checked by assaying a random sample of broth-grown $Acetate^-$ transductants from this cross. Activities were of the order of 0.1 - 0.2 μmoles of glyoxylate formed/hr/mg protein, characteristic of the K8-5^{CBB} (icl-3) allele (Table 3).

(ii) Position of icl-1 with respect to metA In this experiment 706 colonies were selected as Met^+ and of these 699 (99%) (Figure 8) had also received the icl^- allele of R4-5. The remaining 7 colonies (1%) still retained the ability to grow on acetate.

The validity of this result was checked in two ways:

1) Through the assay of broth-grown $Acetate^-$ transductants - colonies thus assayed showed isocitrate lyase activities of less than 0.2 μmoles of glyoxylate formed/hr/mg protein, which is the specific activity of the donor R4-5 (icl-1) mutant when grown at 37°C (Table 3).

2) After incubation at 37°C with the results shown above, the plates containing acetate as carbon source were incubated at 30°C for 24 hours. At the end of this period the 699 colonies which did not grow at 37°C and had been scored as icl^- had all grown, which showed the presence of the icl-1 allele which specifies a temperature-sensitive synthesis of isocitrate lyase (Kornberg & Smith, 1966). This was confirmed by growing a transductant of this genotype in nutrient broth at 30°C and at 40°C: whereas isocitrate lyase activity approached the value found in the wild-type parent R4 at the lower temperature, the enzyme was not detected in the transductant at 40°C.

These results might be subject to an error of approximately 1-2% of their total, due to the presence of the metBF marker on the genome of both donor strains: if the gene order were icl, metA ...

metBF, then about 1-2% of the potential/Acetate$^-$, Met$^+$ transductants would also have received the metBF$^-$ allele from the donor strain and would consequently have retained the phenotype Met$^-$. Such transductants would have escaped selection in the first instance. However, since this reduction applies to both transductions the relative cotransduction frequencies would remain unchanged.

E. Location of the icl-2 marker with respect to the metA and pgi markers

Though it was established in the previous experiment that at least two of the independent icl$^-$ alleles (icl-1 and icl-3) were highly cotransducible with metA, the results did not show on which side of the metA marker these alleles were situated. It was not feasible to determine this with the PA505 [metA, argHBCE, his, proA] receipient for the following reasons:

(1) Such an experiment necessitates a three-point transduction and the only remaining marker which could be thus used, argHBCE, was also carried on the genome of the donor strain AB1911-5.

(2) Owing to the close proximity of the icl and metA markers a conclusive result would require, besides the measurement of transduction frequencies, a full analysis of the arg, met and icl genotypes of the transductants. As the argHBCE marker was only 11% - 15% cotransducible with the metA marker, the most critical class of transductants - those including argHBCE and the middle marker only

- would be very few, amounting to 1% of 11-15% of total colonies.

(3) Furthermore, if the icl-1 mutant, R4-5 were used as the donor strain, these percentages might be reduced by a further 1-2% of their total owing to the cotransduction of the metBF⁻ allele from the donor.

For these reasons it was decided to use the marker pgi, specifying an allele of phosphoglucose isomerase (Fraenkel, 1967) which is situated at 78.7 min on the chromosome map described by Taylor & Trotter (1968), as a third marker in crosses designed to establish the position of the icl gene with respect to metA. Since the pgi marker is nearer to the metA, icl region of the chromosome than is the argHBCE marker, the proportion of critical transductant colonies should be correspondingly enlarged.

An organism suitable for this type of experiment is the mutant DF11 isolated by Fraenkel (1967), which carries both metA and pgi markers. Colonies lacking phosphoglucose isomerase activity may be recognised after growth on nutrient agar medium containing 1% glucose and the redox indicator 2, 3, 5, triphenyl tetrazolium chloride (0.05%). At acid pH values this compound is colourless but at pH 7-14 it is red. Any organism capable of fermenting glucose will, during growth on this carbon source, manufacture acids such as lactic, acetic and succinic acids: their production would cause the colony to remain colourless. However, pgi mutants, unable to utilise glucose further than glucose 6-phosphate will not produce acid and will thus be coloured

red in the presence of the indicator.

As a preliminary experiment the Hfr strain G6-5 (*icl-2,str*) was mated, as previously described, with the F^- strain DF11 (*metA,pgi*). After 4 min pairing time, the mating mixture was diluted 100-fold into a 25 mM-fructose medium supplemented with methionine at 40 µg/ml and incubated without agitation at 37°C. The mating was interrupted at the times shown in Figure 6 and duplicate samples of 0.1 ml were plated onto 25 mM-fructose agar plates containing streptomycin sulphate (50 µg/ml) in order to select for the transfer to the recipient of the *metA*$^+$ allele. Such recombinants were then replica plated onto both 50 mM-acetate and 1% glucose tetrazolium plates to select for the entry of the *icl-2* and *pgi*$^+$ alleles respectively.

From the results of this cross (Figure 6) it appeared that the genes were transferred closely together in the order *metA*, *icl-2*, *pgi* after approximately 16 min [+ 4 min pairing time] of mating and this order was substantiated through the results obtained from the following three-point transduction between these strains. The recipient organism DF11 [*metA*, *pgi*] was infected with phage *P1kc* previously cultured on the mutant G6-5 (*icl2,str*) and appropriate dilutions were plated onto 25 mM-fructose agar plates selective for Met$^+$ transductants. The resultant colonies were then replica-plated onto 50 mM-acetate and onto 1% glucose/tetrazolium plates, to determine their *icl* and *pgi* genotypes. As in the interrupted mating experiment between these strains, the cotransduction results

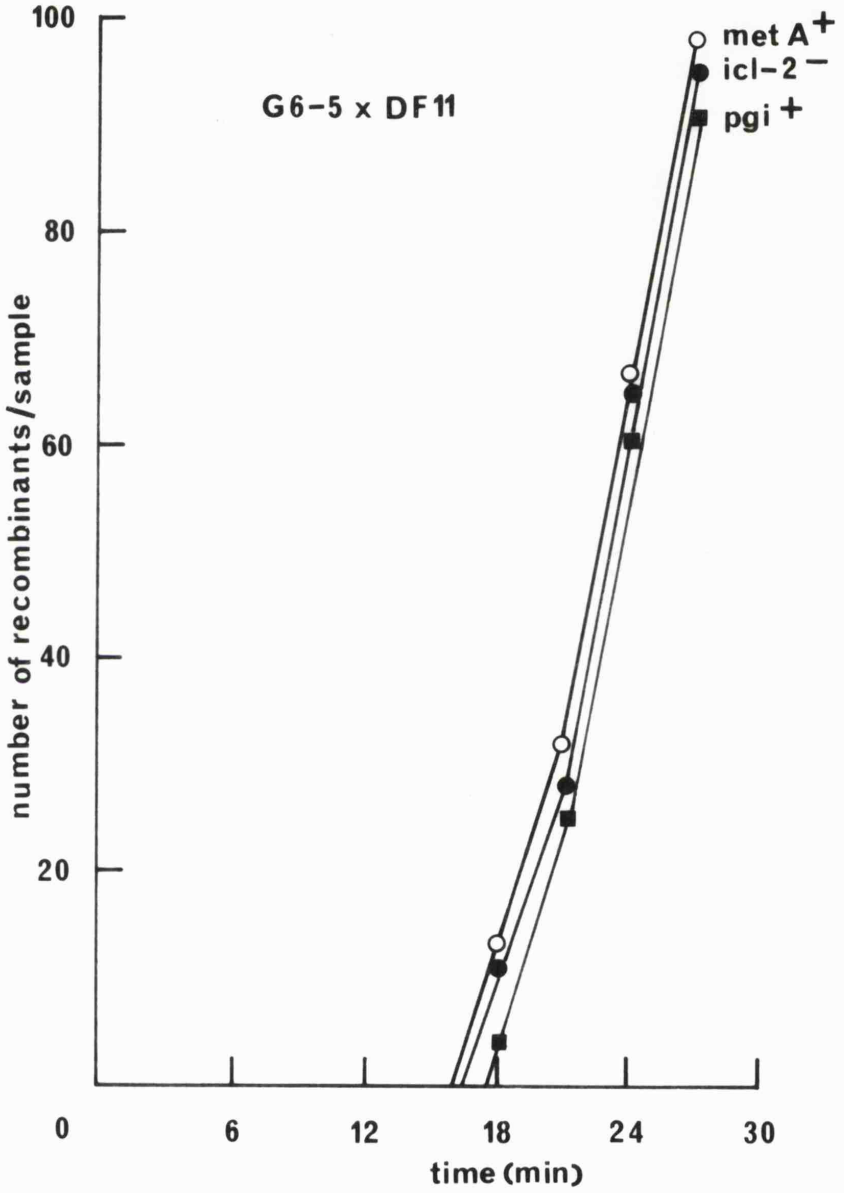


FIGURE 6

The kinetics of transfer at 37°C of the wild-type alleles of metA, icl-2 and phosphoglucose isomerase from Hfr G6-5 to DF11. The times plotted on the abscissa represent the intervals between the dilution of the mating pairs and the interruption of mating.

(Tables 8a and 8b) are an analysis of the colonies resulting from a Met^+ selection, since this was the only direct selection possible in this cross.

Of a total of 391 Met^+ transductants 335 (86%) had also received the icl-2 allele and hence did not grow on acetate, and 157 (35%) the pgi^+ marker. Though these results demonstrated that icl-2 lay closer to metA than did pgi, they did not permit the direct calculation of the cotransduction frequency between icl-2 and pgi necessary to order the genes in this manner. However, an analysis of the genotypes of the resultant transductants (Table 8b) suggested the order metA, icl-2, pgi for the following reasons:

Genotype of Donor	[icl-2 $metA^+$ pgi^+]	[$metA^+$ icl-2 pgi^+]
Genotype of Recipient	$icl\text{-}2^+$ $metA^-$ pgi^-	$metA^-$ $icl\text{-}2^+$ pgi^-
	(A)	(B)

Two possible orders of the genes metA icl-2 and pgi

(i) If the gene order were as in (A) above, a selection for the middle marker ($metA^+$) should result in greater numbers of transductants possessing either icl-2 or pgi^+ alone (icl-2, $metA^+$,

pgi$^-$ or icl-2$^+$, metA$^+$, pgi$^+$) than those possessing both these genes (icl-2, metA$^+$, pgi$^+$). This was not so: the most numerous class of cotransductants were those which had received the metA$^+$ and icl-2 alleles (metA$^+$, icl-2, pgi$^-$) (198) and the second most numerous those colonies in which all three genes had been cotranduced (metA$^+$, icl-2, pgi$^+$) (137). No colonies showing transduction between metA and pgi alone were detected.

(ii) Though in all cases of cotransduction between metA and pgi the icl-2 allele had also been cotransduced, in only 41% of the cotransduction events between metA and icl-2 was the pgi$^+$ allele also transferred.

These results therefore, in conjunction with those of the previously described interrupted mating, indicate that metA and pgi were the outside markers in this cross and suggest the gene order metA, icl-2, pgi.

In view of these facts the cotransduction frequency between icl-2 and pgi was calculated as the percentage of three-point transductants (137) within the total met$^+$ Acetate$^+$ population (335), which gives a minimum value of 41%.

TABLE 8a

Three-point transduction results from the cross between G6-5 and DF11 (recipient)

Number of transductants					% Cotransduction	
Selected genotypic marker	Unselected genotypic markers					
metA$^+$	*icl-2*		*pgi*		*metA-pgi*	35
	+	-	+	-		
					metA-icl-2	86
391	56	335	137	254		
					[*icl-2-pgi*	41 approx.]

TABLE 8b

Genotype analysis of transductants from the cross between G6-5 and DF11 (recipient).

Genotype	Number of transductants
metA$^+$ *icl-2* *pgi*$^+$	137
metA$^+$ *icl-2* *pgi*$^-$	198
metA$^+$ *icl-2*$^+$ *pgi*	56

F. Fine structure mapping within the icl locus

Although the foregoing experiments demonstrated that the three independent icl mutations lay within the same gene locus, the results obtained from these crosses were not sufficient to determine whether or not they were separate mutations and if so their respective positions within the locus. These points were investigated by a series of three-point transductions between the following combinations of the three mutant strains,

R4-5 (argHBCE$^+$, icl-1) (donor) and K8-5a (argHBCE$^-$, icl-2) (recipient)

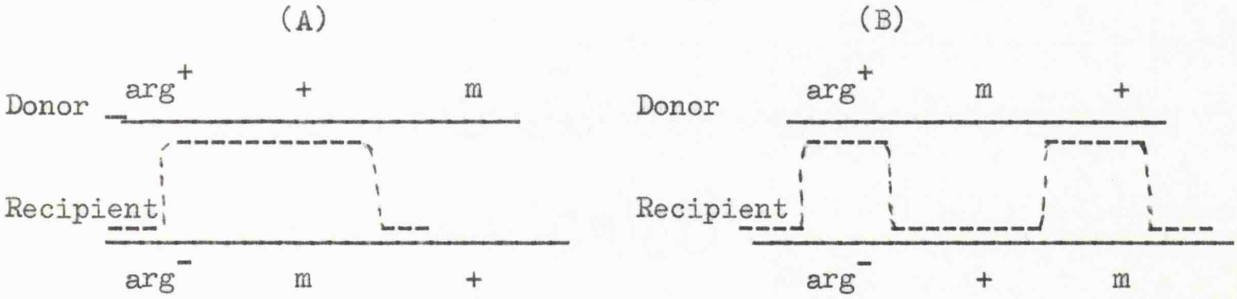
R4-5 (argHBCE$^+$, icl-1) (donor) and K8-5^{CBB}(argHBCE$^-$, icl-3) (recipient)

G6-5 (argHBCE$^+$, icl-2) (donor) and K8-5^{CBB}(argHBCE$^-$, icl-3) (recipient)

selecting initially in each case for Arg$^+$ transductants and scoring this sample, by replica-plating onto appropriately supplemented 50 mM-acetate plates, for any icl$^+$ recombinants. Although owing to the distance between argHBCE and the icl locus, this method probably reduced the resultant number of icl$^+$ recombinants from each transduction, the use of argHBCE as a primary selection marker both reduced the possibility of selecting Acetate$^+$ revertants of the recipient strains, and provided a known number of transductant progeny from which to calculate recombination frequencies between the icl mutations. Control samples of each recipient at cell densities similar to the experimental cultures were also plated onto the

appropriate media in order to correct the totals for reversion to the wild-type phenotype in either character, and where possible the Acetate$^+$ recombinants were also tested for the presence of their non-selected growth markers to avoid the inclusion of contaminants in the totals.

However, although the frequency of Acetate$^+$ recombinants in the above transductions depended upon the distance between the mutant sites, the relative positions of the icl mutations in respect to the argHBCE gene would also influence this frequency in the following way:



If as in (A) the mutant allele carried by the donor strain was the most distal to the argHBCE gene, two crossovers would be required to produce an Arg$^+$ Acetate$^+$ recombinant. Alternatively, if the donor mutation is proximal to the arg gene as in (B), then four crossovers would be necessary to produce a similar organism. Since the frequency of double crossovers would be higher than that of quadruple crossovers, recombination frequencies obtained through a cross of type (A) would be correspondingly greater per unit distance than those

obtained through a cross of type (B). Therefore it would not be valid to use a comparison of the recombination frequencies derived from type (A) and (B) crosses as criteria by which to order these mutations. However, as shown in Table 9, the nature of the three transductions performed in this experiment were such that irrespective of the six possible orders of the three mutant sites, the cross involving the two outside sites and a cross between one of these and the middle site were always of the same type, thus enabling valid comparisons to be made between these recombination frequencies and permitting the identification of the correct order of the three mutations.

The experimental results obtained from these transductions, shown in Table 10, demonstrate the separate identity of the three lesions and indicate that the recombination frequency between icl-1 and icl-3 (1.38%) was greater than that between icl-1 and icl-2 (1.13%) or icl-2 and icl-3 (0.24%) and is approximately equal to the sum of these two latter frequencies.

The application of these results to the six possible orders shown in Table 9 indicated the correct order to be either icl-1, icl-2, icl-3 or the reverse order icl-3, icl-2, icl-1, for the following reasons:

(i) If the order were icl-2, icl-1, icl-3 (Table 9, order 3) then since they both result from a type (B) cross it would be valid to assume that the recombination frequency between the outside sites icl-2 and icl-3 would be greater than the frequency between the sites icl-1 and icl-3. This was not so. For similar reasons the

TABLE 9

The six possible orders of the mutant sites icl-1, icl-2, icl-3 and consequent types of cross resulting from the three transductions performed in this experiment.

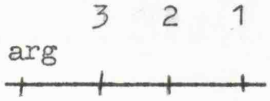
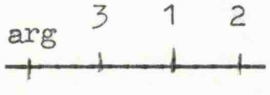
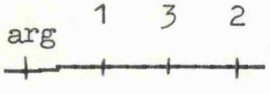
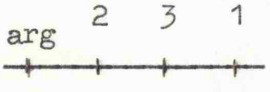
Possible order of sites		CROSSES PERFORMED					
		Donor	Recipient	Donor	Recipient	Donor	Recipient
		icl-1	icl-2	icl-1	icl-3	icl-2	icl-3
		Class of Cross					
1		B		B		B	
2		A		A		A	
3		A		B		B	
4		B		A		A	
5		B		B		A	
6		A		A		B	

TABLE 10

Results obtained from the intragenic crosses between the independent mutations icl-1, icl-2, icl-3

Transduction		Numberof colonies selected		Recombination frequen cy between icl alleles
Donor	Recipient	Arg^+	Arg^+ Ac^+	
arg^+ icl-1	arg^- icl-2	886	10	1.13
arg^+ icl-1	arg^- icl-3	941	13	1.38
arg^+ icl-2	arg^- icl-3	833	2	0.24

reverse order icl-3, icl-1, icl-2 (Table 9, order 4) must also be incorrect.

(ii) Likewise, the order could not be either icl-1, icl-3, icl-2 (Table 9, order 5) or the reverse order icl-2, icl-3, icl-1 (Table 9, order 6) since in both these cases the recombination frequency between the outside markers icl-1 and icl-2 should be greater than that between icl-1 and icl-3. Again this was not so.

(iii) However, with regard to the order icl-1, icl-2, icl-3 (Table 9, orders 1 and 2) since all three crosses would be of a similar type and therefore their resultant recombination frequencies valid for comparison, it would be expected that the recombination frequency between the outside markers icl-1 and icl-3 would be greater than those between either icl-1 and icl-2 or icl-2 and icl-3, and would be approximately equal to the sum of these frequencies. The experimental results thus agree entirely with these premises and demonstrate the order to be one of these two alternatives and since the co transduction frequency between metA and icl-1 is higher than that between metA and icl-3, the order metA, icl-1, icl-2, icl-3 is tentatively advanced as being the correct one.

That the recombinants selected as Acetate$^+$ had indeed regained isocitrate lyase activity was demonstrated by assaying sonic extracts of broth-grown samples from each population. Recombinants from the transduction between R4-5 (icl-1) and K8-5^{CBB} icl-3 exhibited isocitrate lyase specific activities of 1.8 - 2.5 as opposed to 0.2 observed with the parental and non-recombinant

strains. Similarly, Acetate$^+$ recombinants from the transduction between R4-5 (*icl-1*) and K8-5a (*icl-2*) contained isocitrate lyase at a specific activity of 1.34 whereas no isocitrate lyase activity was detected in extracts of the parent and non-recombinant transductants, and the Acetate$^+$ recombinants from the transduction between G6-5 (*icl-2*) and K8-5^{CBB} (icl-3) contained isocitrate lyase at the very high specific activity of 14.2 - 15.2, characteristic of the G6 strain of *E. coli* (Kornberg, 1967).

From these and previous results, the three *icl* markers may be ordered with respect to one another and to the *metA* marker as shown in Figure 7, a compounded transduction map of the *icl* region of the *E. coli* chromosome being shown in Figure 8. The results here presented are in good agreement with those reported for different *icl* mutants by Vanderwinkle & De Vlieghere (1968).

SUMMARY

(1) Two independent *icl* mutants (*icl-2* and *icl-3*) were obtained through ethylmethane sulphonate treatment and penicillin selection for their ability to grow at the expense of acetate, whilst retaining the ability to grow on glucose, succinate or lactate as sole carbon sources.

(2) Enzyme assays of these mutants and their parent strains, together with those of revertans, recombinants and transductants

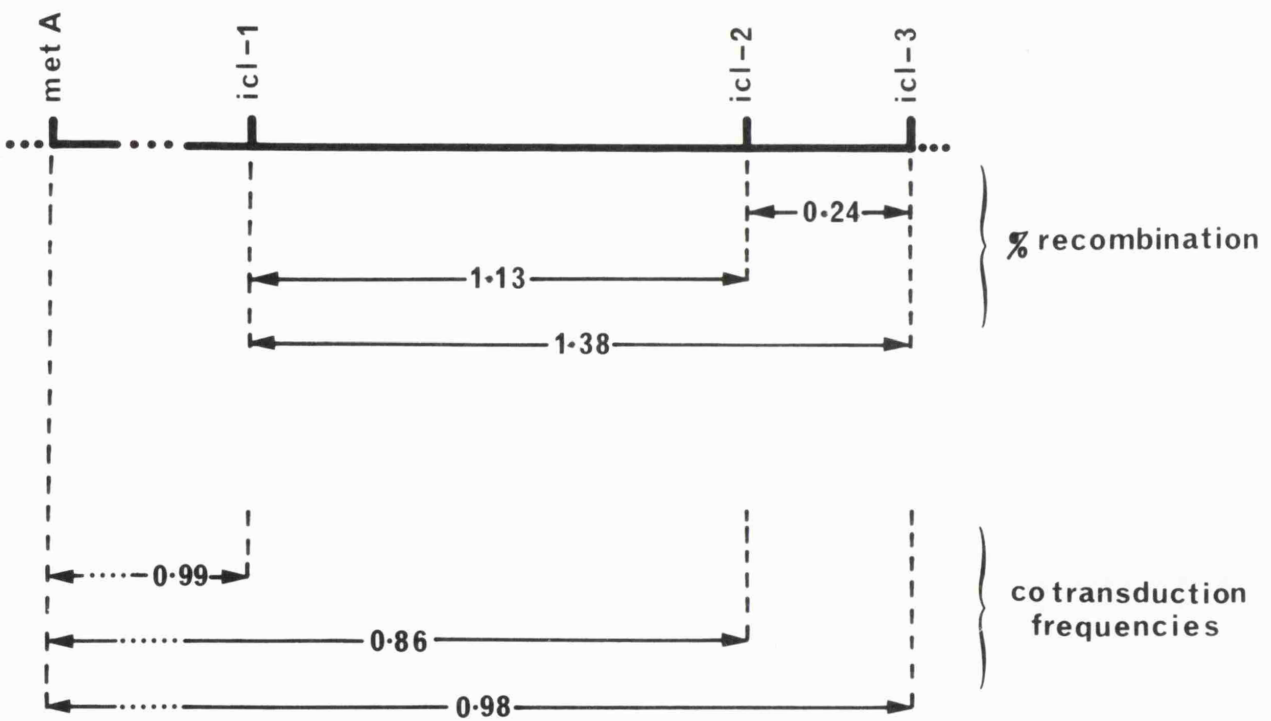


FIGURE 7

The relative order and linkage between the independent mutations icl-1, icl-2 and icl-3, and the metA marker, as determined by intragenic recombination and cotransduction experiments. (Not drawn to scale).

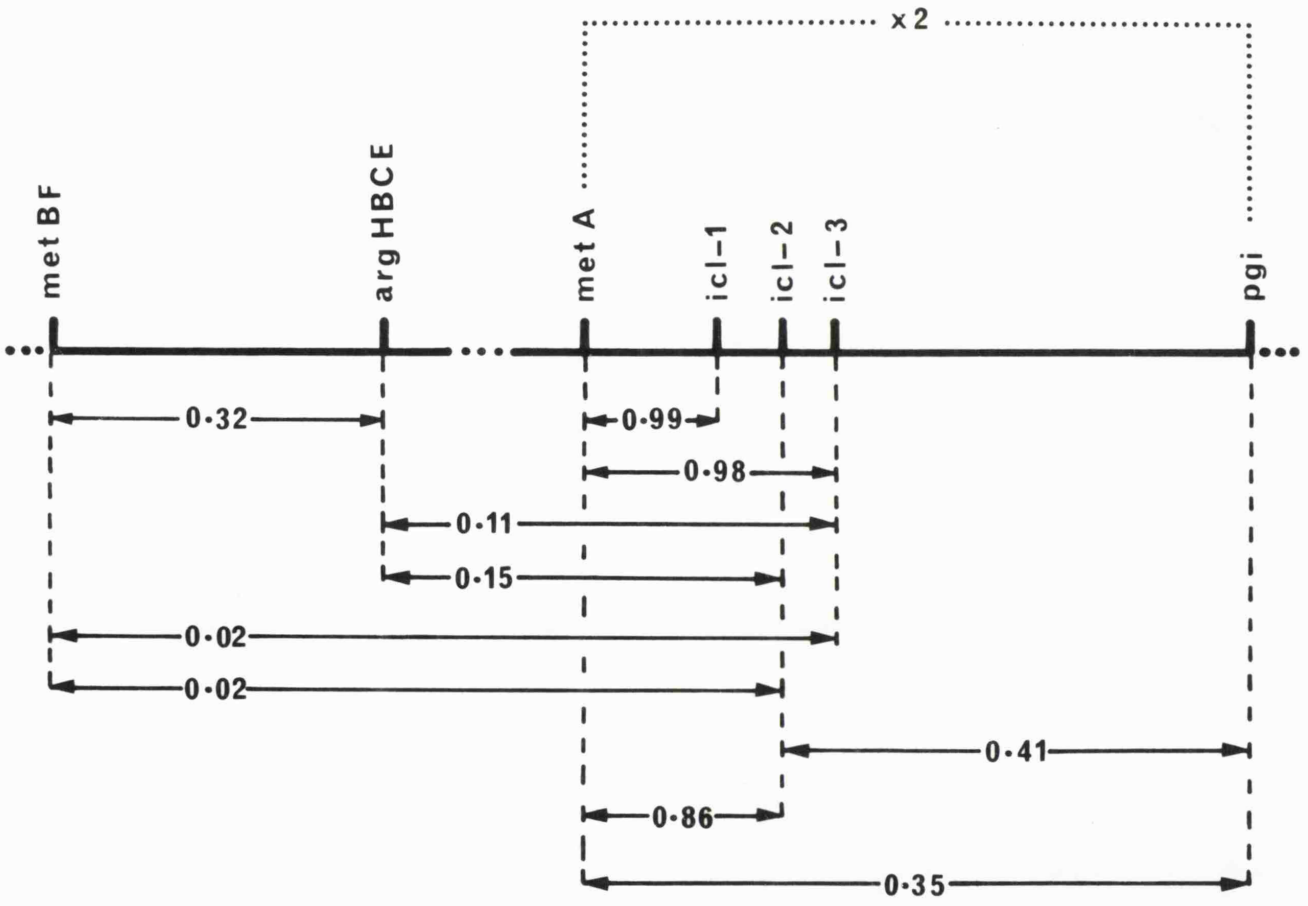


FIGURE 8

A compounded genetic map of the icl region of the E. coli chromosome. Cotransduction frequencies are expressed as decimal fractions of a unitary scale. (Not drawn to scale).

selected for their renewed ability to utilise acetate for growth, indicated that the failure to grow on acetate was due to lack of isocitrate lyase activity.

(3) Experiments using the techniques of interrupted mating and phage-mediated transduction showed these two markers to lie in positions close to a gene specifying the temperature-sensitive synthesis of isocitrate lyase (icl-1) (Kornberg & Smith, 1966).

(4) Phage cotransduction experiments with mutants each carrying one of the three markers revealed that all were highly cotransducible with the metA marker. The final order as determined for one of these lesions (icl-2), was argHBCE, metA, icl-2, pgi.

(5) Intragenic crosses between each of the 3 independent mutants show that though closely linked, they are all separate point mutations and lie in the order metA, icl-1, icl-2, icl-3. It is, however, not yet known whether they lie in separate functional units of the icl locus specifying enzyme subunits.

PART II

The location of a regulatory gene governing the synthesis of isocitrate lyase

PART II

INTRODUCTION

The isolation of a mutant dysfunctional in a regulatory gene necessitates a completely different experimental approach from that used in the selection of "structural" gene mutations.

"Structural" mutants are unable to specify a particular enzyme: they may be isolated by selecting organisms which either require a particular growth factor or which cannot grow on a substrate, the utilisation of which necessitates the action of a particular enzyme. On the other hand, a regulatory mutation results in the inability of the cell to control the production of a particular enzyme or enzymes during growth on a wide range of substrates. It may thus be that regulatory mutants will be phenotypically indistinguishable from the wild-type organism. Any selection procedure for such mutants must therefore utilise the fact that in these mutants the enzyme is produced constitutively (or as with i^s mutants, not at all) and employ conditions whereby this would confer a selective advantage on these cells.

In the isolation of an isocitrate lyase regulator mutant such conditions were obtained by using a strain of *E. coli* K12 which was already lacking in one anaplerotic enzyme - phosphoenol-pyruvate synthase (PEP-synthase). This enzyme, isolated by Cooper & Kornberg (1965) from extracts of *E. coli* B catalyses the formation of phosphoenolpyruvate from pyruvate and ATP and has been shown to be solely responsible for this reaction under physiological

conditions.

$$CH_3.CO.CO_2H + ATP \longrightarrow CH_2:C(CO_2H).PO_3H_2 + AMP + Pi$$

Since it has also been demonstrated (Ashworth & Kornberg, 1966) that the "in vivo" formation of C_4 acids from C_3 precursors occurs solely through the carboxylation of PEP, catalysed by PEP-carboxylase, this enzyme and PEP-synthase co-operate to form an essential anaplerotic route in the growth of cells on C_3 acids such as pyruvate or lactate.

Mutants that lack PEP-synthase are thus unable to grow at the expense of C_3 acids, though they grow normally on precursors of PEP (Such as glucose or glycerol) and on intermediates of the tricarboxylic acid cycle. Such mutants are also unable to manufacture their C_4 compounds via the glyoxylate cycle, by the oxidation of pyruvate to acetate, since high intracellular levels of pyruvate inhibit both the synthesis and activity of isocitrate lyase (Kornberg, 1966; 1967).

If, however, thick suspensions of mutants lacking PEP-synthase activity are spread onto lactate agar plates, further mutants appear after about five days incubation at 37°C. These are of two types. A small number are "~~back mutations~~ revertants" which have now regained PEP-synthase activity. The remainder, however, still lack PEP-synthase activity: they now grow on lactate due to a spontaneous secondary mutation which has abolished the ability to

regulate the synthesis of isocitrate lyase, and form this enzyme into specific activities 5 - 30 times those observed with wild-type organisms. The uncontrolled production of these excessive amounts of isocitrate lyase enables the organisms to grow on lactate (which is oxidised to pyruvate and thence acetate) via the glyoxylate cycle.

Thus by using mutants devoid of PEP-synthase, conditions are obtained which facilitate the selection of spontaneously occurring isocitrate lyase regulatory mutants with great sensitivity.

In this manner, isocitrate lyase regulatory mutants were isolated in two strains of E. coli K12: R4-1 and AT2572-1. These mutants were subsequently used in all isocitratelyase regulator experiments.

Providing that both strains involved contained the PEP-synthase lesion, this selection procedure could be used with impunity in matings and transductions designed to locate the isocitrate lyase regulator gene on the E. coli genome. However, if in crosses involving wild-type Hfr strains, the PEP-synthase lesion was inadvertently replaced by its wild-type allele, the selection procedure would become invalid, due to the renewed ability of the recombinants to manufacture PEP-synthase and hence to metabolise C_3-acids by the normal anaplerotic route. This would result in the erroneous location of the isocitrate lyase regulator gene. In order that this might be avoided, it was imperative to know the exact chromosomal location of the PEP-synthase gene, before attempting to map the isocitrate lyase regulator gene.

CHAPTER I

The location of the structural gene specifying PEP-synthase

MATERIALS AND METHODS

Organisms used

The strains of Escherichia coli K12 used in this work and not already described in Part I of this thesis are listed in Table 11 below. Some of these organisms were gifts, gratefully received, from the following: K1 - from Professor R. H. Pritchard (University of Leicester); K2 - from Professor R. H. Walmsley (University of Pennsylvania); AT2572 and AT2571 - from Professor E.A. Adelberg (Yale University Medical School); AB1359 - from Professor A. L. Taylor (University of Colorado Medical School).

The abbreviations used to denote the genetic markers carried by these strains are also listed under Table 11 unless previously explained. With the exception of icl, they are those listed by Taylor & Trotter (1967) and are used in accordance with the convention suggested by Demerec, Adelberg, Clark & Hartman (1966).

TABLE 11

Strains of *E. coli* K12 used in the location of the PEP-synthase gene

Strain	Parent strain(s)	Mating type	Response to streptomycin	Genetic markers
K1	-	Hfr	S	metBF, thy
K1-1	K1	Hfr	S	metBF, thy, pps
AT2571	-	Hfr	S	
AT2572	-	Hfr	S	
AT2572-1	AT2572	Hfr	S	pps
K2	-	F^-	R	argHBCE, thr, leu, try, his
K2-1t	K1-1 x K2	F^-	R	argHBCE, thr, leu, his, pps
K2-5B	G6-5 x K2	F^-	R	argHBCE, try, his, icl
K2-1A-5B	K2-5B	F^-	R	argHBCE, try, his, icl, pps
K2-1B-5B	K2-5B	F^-	R	argHBCE, try, his, icl, pps
AB1359	-	F	S	argHBCE, his, proA, aroD
K6	AB1359	F^-	R	argHBCE, his, proA, aroD
K6-1	AT2572-1 x K6	F^-	R	argHBCE, proA, aroD, pps

The abbreviation aroD (dehydroquinase) is used to represent a requirement for shikimic acid, and pps, the inability to form PEP-synthase. The remaining abbreviations are previously explained in the "Materials and Methods" section of Part I of this thesis. The points of origin and direction of genome transfer of the Hfr strains listed above are shown in Figure 2.

Storage and Growth of Organisms

Cells were grown and maintained in stock form, in the manner previously described in Part I of this thesis.

Growth Media

The basal growth medium was as previously stated, except when 25 mM-L-alanine was used as a carbon source. In this case the nitrogen source (NH_4Cl) was omitted. Sodium pyruvate (sterilised by Millipore filtration) and sodium lactate were used as carbon sources at final concentrations of 20 mM and 25 mM respectively. When necessary the growth media were supplemented with the appropriate amino acids at the final concentrations described in the first part of this thesis and with shikimic acid at a final concentration of 40 µg/ml. Bacteriophage P1kc lysates were prepared and titred on the media described by Glover (1962).

Mutagenesis and Selection procedure

Mutant strains, lacking in PEP-synthase activity were obtained by treating wild-type cultures with ethylmethanesulphonate (Lin, Lerner & Jorgensen, 1962) and selecting with penicillin (Gorini & Kauffman, 1960) for the inability to grow at the expense of C_3 acids such as lactate, L-alanine or pyruvate. The surviving cells were plated onto 25 mM-glucose medium and the resulting colonies replica-plated onto lactate or pyruvate agar plates. Colonies growing on the former but not on the latter medium were further

investigated by direct enzymic assay.

Preparation of cell-free extracts and enzymic assay of PEP-synthase activity

Broth-grown cultures were harvested at 15°C and resuspended in 10 mM-Tris-HCl pH 7.4, 1 mM-$MgCl_2$ buffer to a density of about 10 mg dry wt/ml. These suspensions, cooled in melting ice, were disrupted by exposure for 2 min to the output of a M.S.E. 60W ultrasonic oscillator operating at 1.5 amp and the cell debris removed by centrifugation at 15-20°C for 5 min at 20,000 xg. The enzyme was then precipitated from the extract by the addition of 6 mg of protamine sulphate/20 mg of supernatant protein; the suspension was stirred for 10 min and the precipitated material collected by centrifugation at 15°C for 10 min at 20,000 xg. The PEP-synthase activity was finally brought back into solution by stirring this precipitate with an amount of 0.25 M-sodium/potassium phosphate buffer pH 6.8, equivalent to one quarter of the volume of the previous supernatant.

Such concentrates were then assayed for their ability to catalyse the ATP-dependent removal of pyruvate from solutions thereof in the presence of magnesium chloride, ATP and TRIS-hydrochloride buffer pH 8.0, in the manner described by Cooper & Kornberg (1967). In order to avoid needlessly small numbers, the specific activity of the enzyme was expressed as μmoles of pyruvate removed per mg of protein per hour rather than the customary per minute.

Interrupted mating technique

Though similar in general principle to the interrupted mating technique described in Part I of this thesis, the method employed in the location of the pps gene was not of the "pulse mating" type. The donor and recipient cells once mixed in broth were allowed to remain in this medium, without further dilution, for the duration of the experiment and samples of the mating mixture were diluted 10-fold with sterile basal growth medium immediately prior to the interruption of conjugation.

The results obtained with this method were less satisfactory than those derived by the "pulse mating" technique, since the presence of varying number of "background" recombinants in many early mating samples often rendered the times of gene entry ambiguous. The "pulse mating" technique, which greatly eradicated this "background" was therefore adopted in the other sections of this thesis.

Phage P1kc-mediated transduction

Phage lysates were prepared by the confluent lysis of donor bacterial strains in soft agar as described by Glover (1962). For transduction purposes, the broth-grown recipient strain was resuspended at a density of approximately 2 x 10^9 cells per ml in 50 mM-sodium/potassium phosphate buffer pH 7.5, supplemented with 1 mM-$CaCl_2$. After incubation with the phage as described

in the previous "Materials and Methods" section, the cells were harvested and resuspended at the same density in 50 mM-sodium/potassium phosphate buffer pH 7.5 containing 0.5% (w/v) sodium citrate to chelate any calcium ions carried over in the pellet. Samples were then plated onto each of the selective media as previously described.

Chemicals

Sodium pyruvate was purchased from C. F. Boehringer und Soehne, Mannheim, Germany.

Shikimic acid and L-alanine were obtained from the British Drug House, Poole, Dorset; and Difco 'Bacto' Agar from Difco Laboratories, Detroit 1, Michigan, U.S.A.

All other chemicals were obtained from the sources listed in Part I of this thesis.

RESULTS

Characterisation of mutants of E. coli K12 devoid of PEP-synthase

The growth characteristics of two pps-mutants K1-1 and AT2572-1 derived as described in "Methods", are listed in Table 12. Unlike their respective parent strains, both mutants were unable to grow on media containing C_3-acids such as pyruvate, lactate or alanine as sole carbon sources; though they grew readily if these media were further supplemented with utilisable intermediates of the tricarboxylic acid cycle. These facts suggested that the enzymic dysfunction involved the anaplerotic pathway from pyruvate, rendering such mutants unable to effect from C_3 acids (Kornberg, 1966a), the net synthesis of tricarboxylic acid cycle intermediates necessary for growth. Since these mutants also grew readily on glycerol, acetate or succinate, it follows that the tricarboxylic acid and glyoxylate cycles, the pathway of glycolysis and the pathway of gluconeogenesis from phosphoenolpyruvate functioned normally. Such growth pattern would be expected from mutants unable to convert pyruvate to phosphoenolpyruvate and is similar to that observed by Cooper & Kornberg (1965, 1967) in mutants of E. coli B devoid of PEP-synthase.

Pps-mutants are also distinguished by the fact that their growth on acetate as a sole carbon source is greatly inhibited by pyruvate (Kornberg, 1966b), (Figure 9, Table 10). This effect is due to the inhibition by pyruvate of the first enzyme of the glyoxylate

FIGURE 9

Effect of pyruvate on the growth of *E. coli*, K12 mutant K1-1 (*pps*). A starting culture was grown overnight at 37°C in medium containing 50 mM-acetate, methionine (40 ug/ml.), thymine (40 ug/ml.) and the ammonia-salts mixture described by Ashworth & Kornberg (1966). The organisms were transferred to four flasks containing fresh medium of the same composition and were allowed to continue to grow at 37°C. To two of these flasks (□, ■) pyruvate was added, at the arrow marked 'p' to a final concentration of 10 mM; one of these flasks (■) subsequently received 10 mM-glyoxylate (at the arrow marked 'g') and the other (□) received 5 mM-aspartate (at the arrow marked 'a'). A third flask (●) received 5 mM-pyruvate at the arrow marked 'p' but no further additions were made: the decrease in the pyruvate content (▲) of this flask is plotted in the inset of the figure. All coincident points are designated as (⊙).

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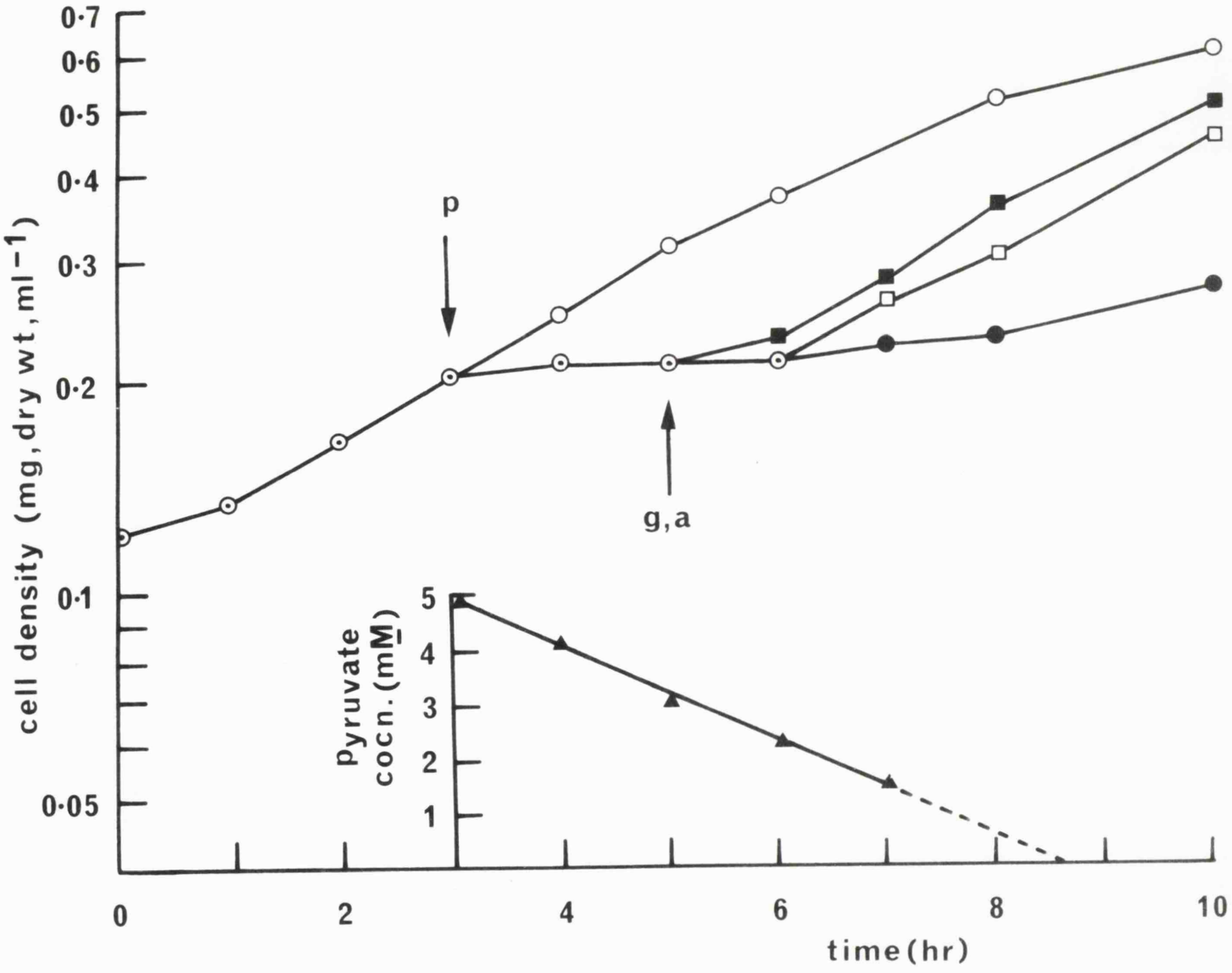
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0·05
cell density (mg, dry wt, ml⁻¹)
p
g,a
Pyruvate cocn. (mM)
5
4
3
2
1
0
2
4
6
8
10
time (hr)

TABLE 12

Growth patterns of Escherichia coli K12 and of its mutants devoid of phosphoenolpyruvate synthase activity.

Substrate	Growth after 18-24 hr incubation at 37°	
	Wild-type	pps-mutants
25 mM-Glucose	++	++
25 mM-Glycerol	++	++
25 mM-Lactate	++	-
20 mM-Pyruvate	++	-
25 mM-L-Alanine	++	-
50 mM-Acetate	++	++
25 mM-Acetate + 10 mM-Pyruvate	++	-
25 mM-Succinate	++	++
25 mM-Glutamate	-	-
25 mM-Aspartate	±	±
20 mM-Lactate + 2 mM-Glutamate	++	++
20 mM-Lactate + 2 mM-Aspartate	++	++

cycle, isocitrate lyase with the concomitant reduction in the supply of tricarboxylic acid cycle intermediates from acetate. Since in these mutants such intermediates cannot be manufactured from pyruvate, the rate of growth is also greatly reduced. In accordance with this explanation growth is resumed upon the addition of the product of the isocitrate lyase cleavage reaction, glyoxylate; or of utilisable intermediates of the tricarboxylic acid cycle; or upon the eventual oxidation of the pyruvate to acetate.

However, the most direct characterisation of pps-mutants rests on the absence of PEP-synthase activity in sonic extracts of the organism. As shown in Table 13, both parent Hfr strains, K1 and AT 2572, contained PEP-synthase at specific activities of 4-5; however, the mutant strains derived from them and those derived from strain K2-5 B are devoid of such activity. Similar enzyme deficiencies are also shown in extracts from pps-recombinants of these mutants, selected by the procedure described under "Methods".

Preliminary location of the pps-gene

The initial location of the pps gene was achieved by mating the Hfr mutant K1-1 (metBF, thy, pps, str) (Figure 2), with the F-strain K2 (thr, leu, try, his, argHBCE). At predetermined intervals samples of the mating mixture were "blended" and plated onto four sets of 25 mM-glucose minimal agar, selective respectively

TABLE 13

PEP-Synthase activity of extracts of organisms used.

The activity of PEP-synthase of extracts of the organisms, grown in 'Oxoid' nutrient broth at 37°C to 0.3 to 0.5 mg dry wt/ml, was assayed as described under "Methods".

Organism used	specific activity of PEP-synthase
K1	4,9
K1-1	0
AT2572	4.1
AT2572-1	0
AT2571	5.0
K2	6.3
K2-1t	0
K2-1A-5B	0
K2-1C-5B	0
K6	5.8
K6-1	0
recombinants from [AT2571 x K6-1]	6.3 - 7.1
aroD$^+$, *pps*$^+$ -phage transductants from the cross AT2572/K6-1	6.3 - 6.8

for Threonine and Leucine$^+$, Tryptophan$^+$, Histidine$^+$ and Arginine$^+$ recombinants. The resulting colonies were then tested, by replica-plating onto appropriately supplemented 25 mM-Lactate agar plates, for their ability to use C_3 acids as a sole carbon source. By these means it was found that a large proportion of the recombinants which had received the wild-type alleles for threonine, leucine, tryptophan and histidine; and a smaller percentage which had received only the threonine, leucine and tryptophan alleles, had also lost the ability to grow at the expense of lactate, implying that the pps-marker was situated between the genes specifying tryptophan and histidine.

In attempting to obtain a more precise chromosomal location for the pps-marker it was clearly preferable to use direct selection procedures rather than a replica plating technique. For this purpose an F^- recombinant of the genotype pps, thr, leu, his, argHBCE was isolated from the above cross and designated K2-1t. This recombinant was then mated with the Hfr strain K1 (metBF, thy, str). The mating was interrupted periodically and 0.1 ml samples were plated in duplicate onto 25 mM-glucose agar medium selective for histidine prototrophs and onto 25 mM-lactate and 25 mM-L-alanine plates selective for pps$^+$ recombinants. As shown in Figure 10 the wild-type allele specifying PEP-synthase entered the recombinants after approximately 33 min of mating, some 6-7 min before that of the wild-type allele for histidine. Furthermore, since the introduction of the pps$^+$ allele restores the ability of the cell

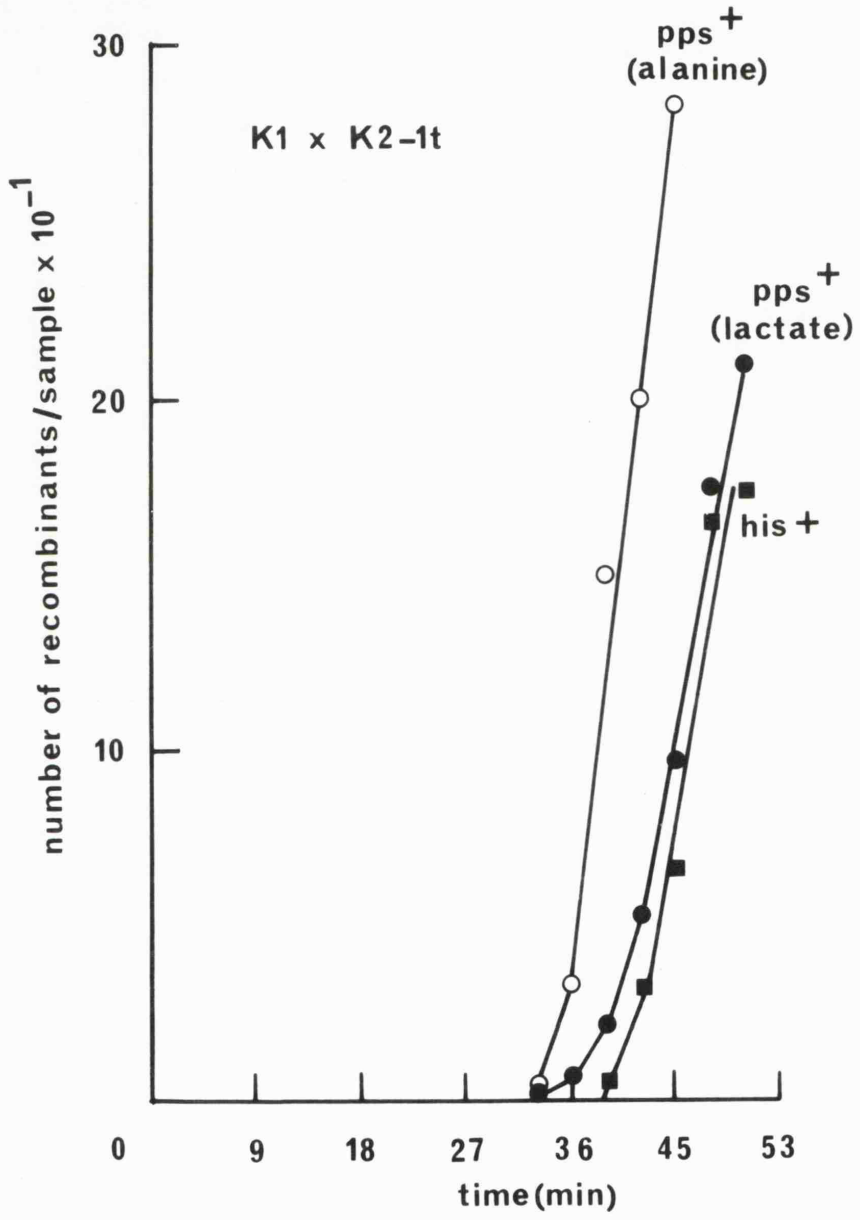

FIGURE 10

Kinetics of transfer at 37°C of the gene permitting growth on alanine or lactate, and of the his allele from K1 to K2-1t. The times plotted on the abscissa are the intervals (min) between mixing the mating cultures and the mechanical interruption of mating.

to grow at the expense of both alanine and lactate, these results confirm the essential anaplerotic role of PEP-synthase during growth on all C_3 acids, as postulated by Cooper & Kornberg (1965, 1967).

Genetic transfer of the pps allele from Hfr strain AT2572 to pps- mutants also devoid of isocitrate lyase activity

Two considerations prompted an alternative approach to the mapping of the pps gene. Firstly, there was a delay of more than 30 min before the pps gene was transferred to recipient organisms by the Hfr strain K1 employed hitherto; with consequent reduction in the efficiency of genetic transfer, and it was clearly preferable to use an Hfr strain which injects its genome into recipient strains at a site closer to the pps marker. Secondly, the main interest in the PEP-synthase lesion is that its presence in the cell facilitates the selection, on lactate media, of isocitrate lyase regulatory mutants. The phenotypes of such mutants could not be readily distinguished from pps^+ organisms and their occurence by reversion in experiments designed to locate the pps gene would lead to erroneous results.

The first consideration was met by the use of Hfr strain AT2572 (str) which transfers its genome in a counter-clockwise direction from a point immediately prior to the his marker (Figure 2). The second point was countered by the preparation of two independently isolated pps mutants from icl-parents. In order to obtain a

suitable parent organism lacking isocitrate lyase, the F^- strain K2 (thr, leu, try, his, argHBCE), was mated with the Hfr strain G6-5 (icl-2, his, thy, str) (Figure 2). From the resulting recombinants, one of the genotype try, his, argHBCE, icl-2 was isolated and named K2-5B. This recombinant, after treatment with ethylmethanesulphonate and selection with penicillin as described under"Methods", yielded the two independently obtained pps-mutants K2-1A-5B and K2-1C-5B (try, his, argHBCE, icl-2,pps). In consequence of their inabilities to form isocitrate lyase and PEP-synthase, neither of these mutants grew on acetate or lactate; however, they grew normally on media containing glucose, glycerol or succinate as a carbon source.

Both mutants were then crossed with the Hfr strain AT2572 (str). After the mechanical interruption of mating, 0.1 ml samples were spread in duplicate onto 25 mM-glucose medium selective for His^+ recombinants and onto 25 mM-lactate, 20 mM-pyruvate and 25 mM-L-alanine media (supplemented with histidine) to select for the transfer of the wild-type pps allele.

In the cross between AT2572 and K2-1A-5B (Figure 11), the wild-type histidine allele entered the recombinants approximately 6 min before the normal PEP-synthase allele, after some 6 min of mating. Similar results were obtained from the cross between AT2572 and K2-1C-5B (Figure 12), the normal his allele entering the recombinants after about 7 min of mating time whilst the ability to grow at the expense of lactate was not transferred until 14 min had elapsed. As also shown in Figure 12, the acquisition of the

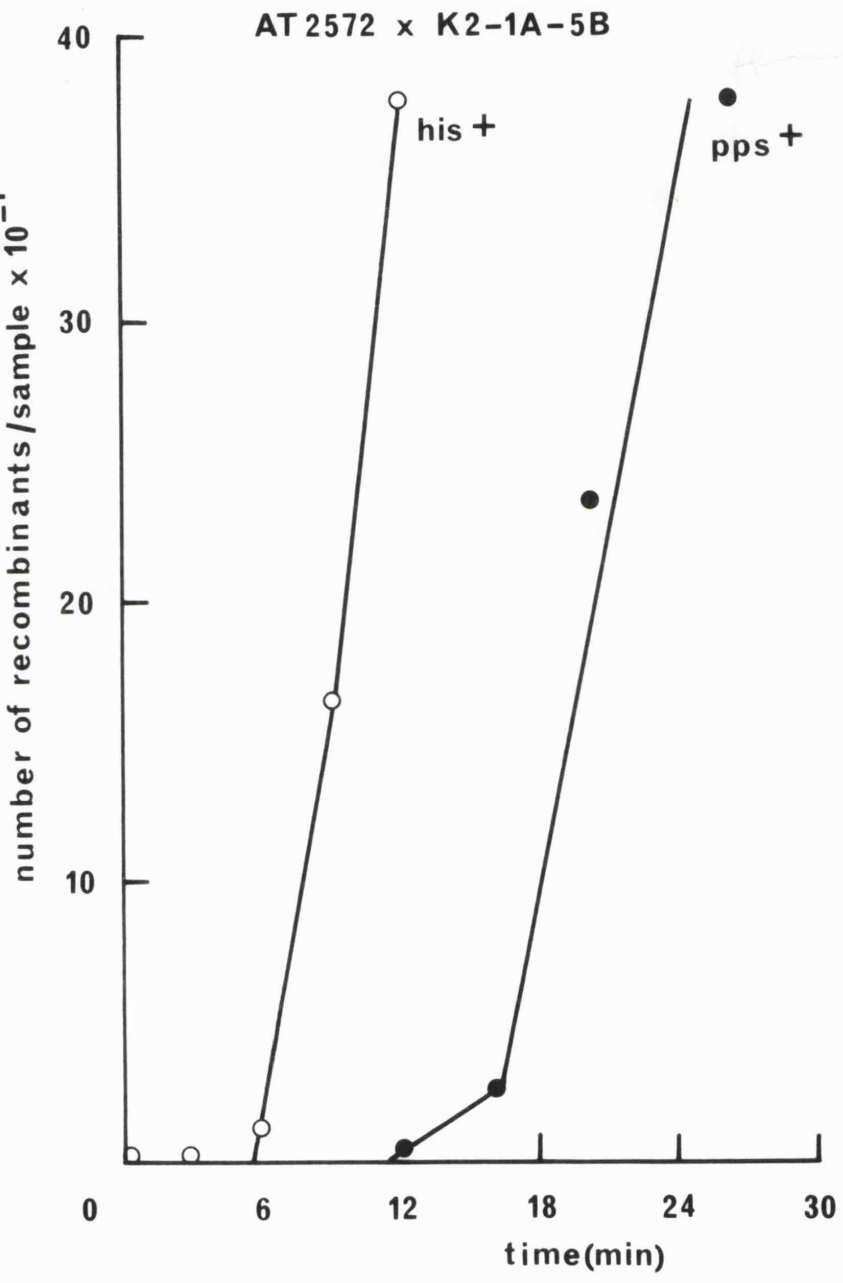

FIGURE 11

Kinetics of transfer at 37°C of the his and pps alleles, from AT2572 to K2-1 A-5B. The times plotted on the abscissa are the intervals (min) between mixing the mating cultures and the mechanical interruption of mating.

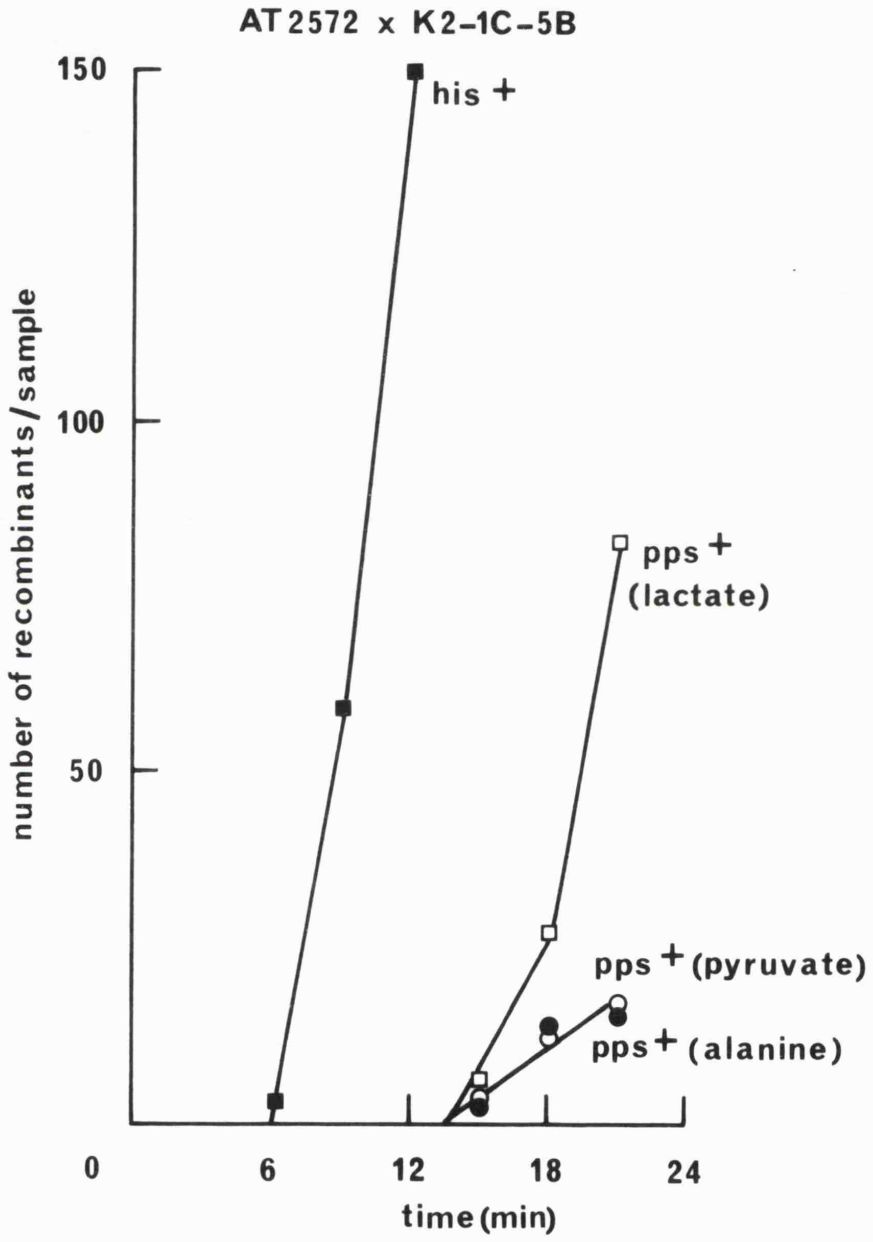

FIGURE 12

Kinetics of transfer at 37°C of the his allele, and the gene permitting growth on lactate, pyruvate or alanine from AT2572 to K2-1 C-5B. The procedures used were as recorded in the legend to figure 11.

ability to grow on both alanine and pyruvate coincided with that for growth on lactate. Although fewer recombinants were obtained on these media the coincidence of their entry curves confirms (as also indicated by Figure 10) that the same enzymic dysfunction prevents growth on any of these C_3 acids.

Genetic mapping of the pps allele in relation to the aroD marker.

Preparation of pps-mutant K6-1

The results obtained with the three independent pps-mutants derived from strains K1 and K2 indicated that the pps gene lay close to the chromosomal position ascribed by Taylor & Thoman (1964) to the aroD marker. Since a mapping in relation to this marker would result in a more precise location of the PEP-synthase gene, a mutant bearing both the pps-allele and the aroD marker was obtained for this purpose as follows:

The aroD bearing F^- strain AB 1359 (his, argHBCE,proA, aroD, str) was first converted to streptomycin resistance by incubating a thick cell suspension on nutrient agar plates containing 100 µg of streptomycin per ml agar, at 37°C. The colonies appearing after some 48 hr incubation were re-isolated and tested for both their characteristic growth markers and their ability to grow in the presence or absence of streptomycin. Of these, one colony resistant to and not dependent upon streptomycin for growth was kept as a stock strain and designated K6.

Strain K6 was then mated in broth for 30 min with Hfr strain AT2572-1 (pps, str) (Tables, 12,13). After interruption the mating, samples were plated onto 25 mM-glucose agar medium supplemented with arginine, proline, shikimate and streptomycin, in order to select for His^+, streptomycin resistant recombinants. By replica-plating such colonies onto suitably supplemented glucose and lactate media, a recombinant was isolated which still carried the aroD marker (and thus required shikimate for growth) and which now also failed to grow on lactate. Assays of cell-free extracts of this recombinant (Table 13) showed it to be devoid of PEP-synthase activity; it was therefore designated K6-1 and used in subsequent experiments.

Results of interrupted matings between Hfr strains AT2571 and AT2572 and the F^- strain K6-1

The relative positions of the pps and aroD alleles carried by strain K6-1 were determined by interrupted matings with the Hfr strains AT2571 (str) (Figure 13) and AT2572 (str) (Figure 14), both of which inject their genome from sites near the aroD marker, though in different directions (Figure 2).

In both cases, after interruption of the mating, samples were plated onto glucose agar medium selective for Aro^+ recombinants and onto lactate agar selective for the entry of the wild-type pps allele. The results of each mating show that the two genes were

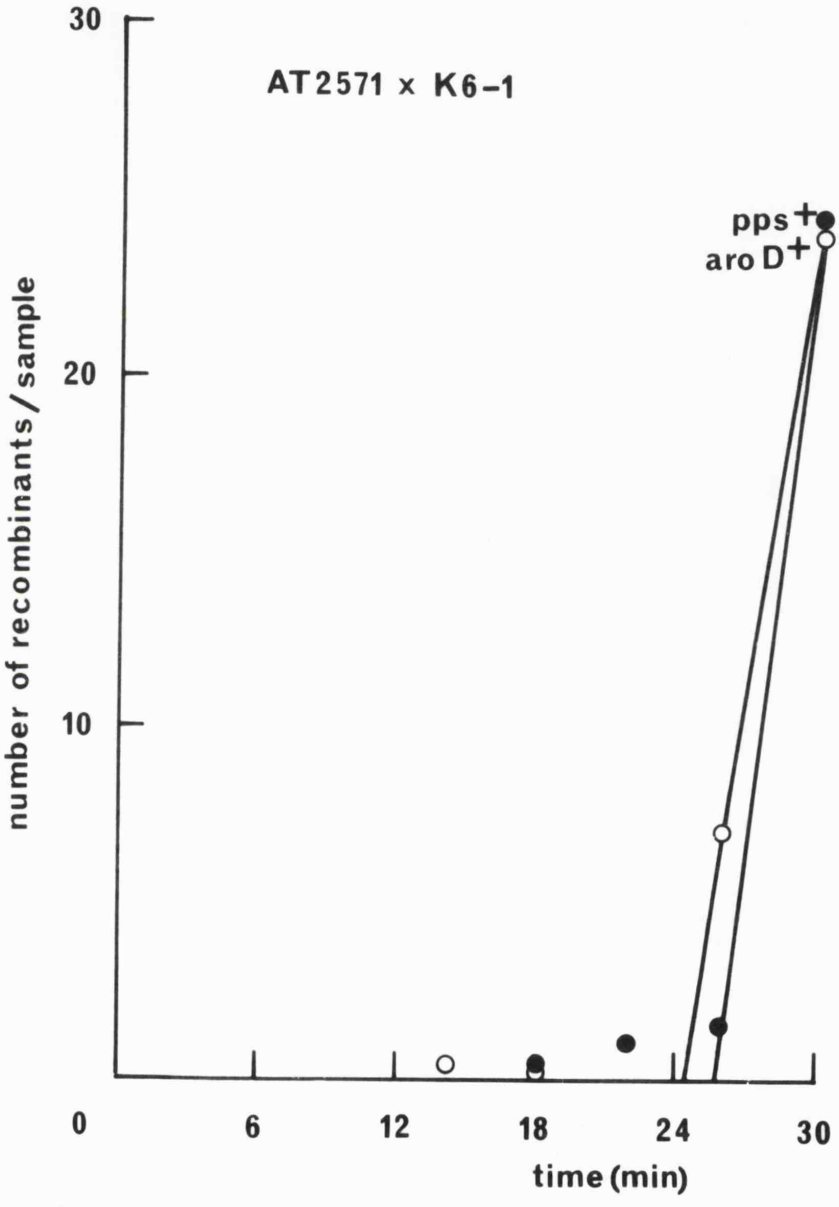

FIGURE 13

Kinetics of transfer at 37°C of the pps and aro-D alleles from AT2571 to K6-1. The times plotted on the abscissa are the intervals (min) between mixing the mating cultures and the mechanical interruption of mating.

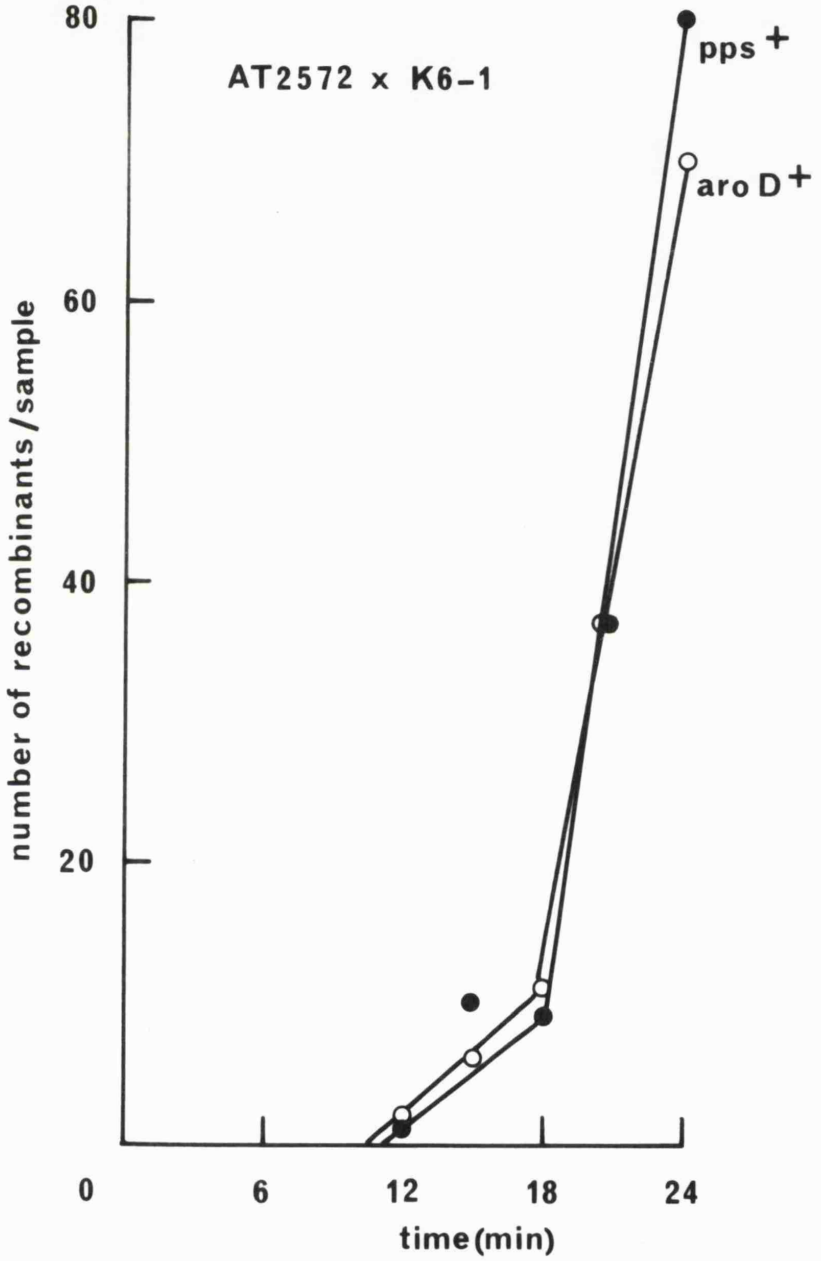

FIGURE 14

Kinetics of transfer at 37°C of the pps and aro-D alleles from AT2572 to K6-1. The procedures used were similar to those recorded in the legend to figure 13.

transferred in close proximity in positions consistent with the gene order his, pps, aroD.

Phage P1kc-mediated transduction of the aroD and pps alleles

The virtual coincidence in the time of entry of the pps and aroD markers suggested that they might be cotransducible through an appropriate phage. This was confirmed by infecting strain K6-1, in the manner previously described in the Methods section, with a phage P1kc lysate cultured on the wild-type Hfr strain AT2572. After transduction 0.1 ml samples of the recipient were plated onto 25 mM-glucose agar selective for aroD$^+$ transductants and onto 25 mM-lactate agar supplemented with shikimate in order to select for pps$^+$ transductants. The colonies arising on these plates after 24-48 hr incubation at 37° were replica plated onto both the alternative and the identical media. After 24 hr incubation at 37°C, these replicas were examined in comparison with the original plates and the incidence of cotransduction scored as the number of colonies from each selection which now grew on both types of media. This conclusion was confirmed by the growth of a number of such presumed cotransductants in liquid media containing lactate but no shikimate, and assay of the PEP-synthase activity in sonic extracts of these organisms grown on nutrient broth. As may be seen in

Table 13, such clones contained the enzyme at specific activities ranging from 6.3 to 6.8, confirming the presence on their genome of the wild-type allele specifying PEP-synthase.

Of a total of 1959 colonies selected as *aroD*$^+$ transductants, 1603 (82%) were found also to have received the wild-type *pps* allele. Likewise of 1454 colonies selected as having received the *pps*$^+$ allele, 1380 (95%) were found also to have lost their requirement for shikimate. In all, of a total of 3413 colonies transduced, 2983 had received both the *pps* and *aroD* markers, an average cotransduction frequency of 88.5%. This high degree of linkage supports the view that the two genetic markers are located in close proximity to each other on the *E. coli* genome, though in the absence of a third genetic marker their order with respect to the *his* marker as implied by interrupted conjugation could not be confirmed.

SUMMARY

1. Four independently isolated *pps*-mutants were obtained through ethylmethanesulphonate treatement and selection with penicillin for their inability to grow at the expense of the C_3 acids lactate, alanine or pyruvate. Two of these, Hfr strains K1-1 and AT2572-1, were used in the preparation of the F$^-$ *pps*-recombinants K2-1t and K6-1 respectively; the remaining two, K2-1A-5B and K2-1C-5B, had been induced in F$^-$ mutants devoid of isocitrate lyase activity.

2. Although selected on different C_3 carbon sources, all these mutants exhibited similar phenotypic growth patterns, failing to grow at the expense of either lactate, pyruvate or alanine, but retaining the ability to grow at the expense of glucose, glycerol or succinate.

3. Ultrasonic extracts of these mutants and their *pps*$^-$ recombinants, together with those recombinants and transductants selected for their renewed ability to grow at the expense of these substrates is due to the inability to form PEP-synthase, confirming the physiological role postulated by Cooper & Kornberg (1965, 1967) for PEP-synthase during the growth of *E. coli* on C_3 acids.

4. By interrupted mating experiments the PEP-synthase "structural" gene was shown to lie close to the *aroD* marker at 33 min on the *E. coli* linkage map, in the probable order *his*, *pps*, *aroD*.

5. The results of a phage *P1kc*-mediated transduction between the *pps* and *aroD* genes confirmed their close proximity, though in the absence of a third genetic marker in the cross the order of these genes implied by interrupted mating experiments could not be substantiated.

Chapter II

The location of the isocitrate lyase regulator gene

MATERIALS AND METHODS

Organisms

Those strains of *E. coli* K12 used in the work described in this chapter and not already cited are listed in Table 14. Strains of KLF10 and KL16-99 were gifts from Dr. B. Low, Yale University Medical School, and Strain P13 from Professor F. Jacob.

Acridine Orange Curing

An inoculum of approximately 5×10^3 cells from an overnight standing culture in nutrient broth at pH 7.0, was added to 2.0 ml of nutrient broth, adjusted to pH 7.8 (Bastarrachea & Willetts, 1968) containing acridine orange at a final concentration of 100 µg/ml. The container was covered with aluminium foil to exclude light and incubated with agitation at 37°C for 20-24 hours. The treated cells were then serially diluted in nutrient broth, pH 7.0, and a range of the dilutions was plated directly onto selective media.

All other materials and methods used in the location of the isocitrate lyase regulator gene were those already stated in the "Materials and Methods" section relating to the location of the structural gene for isocitrate lyase.

Chemicals

Acridine Orange was purchased from the British Drug Houses Ltd., Poole, Dorset.

TABLE 14

Strains of E. coli K12 used in the location of the isocitrate lyase regulator gene

Opposite page 80

The following abbreviations indicate the absence of ppc = phosphoenolpyruvate carboxylase, iclR⁻ = regulation of isocitrate lyase, recA1 = the ability to undergo genetic recombination. The remaining abbreviations are previously explained under "Materials and Methods" in Part I of this thesis.

TABLE 14

Strain	Parent(s)	Genetic Markers	Mating Type	Response to streptomycin
P13	-	-	Hfr	S
AT2572-1-5^{c}	AT2572-1	*pps*, *iclR*$^{-}$	Hfr	S
R4-1-5^{c}	R4-1	*metBF*, *pps*, *iclR*$^{-}$	Hfr	S
KL16-99	-	*recA1*	Hfr	S
KLF10/JC1553		*metB*$^{+}$, *ppc*$^{+}$, *iclR*$^{+}$,/*metB*$^{-}$, *argG*$^{-}$, *leu*$^{-}$, *his*$^{-}$, *recA1*	F'	S
KLF10/recA1	KLF10/JC1553 x K2-1-5^{c}recA1	*metB*$^{+}$, *ppc*$^{+}$, *iclR*$^{+}$/*recA1*, *metB*$^{-}$, *ppc*$^{-}$, *iclR*$^{-}$, *thr*$^{-}$, *leu*$^{-}$, *pps*$^{-}$	F'	R
K2-1-2-5^{c}	K1-1-2-5^{c} x K2-1t	*thr*, *leu*, *pps*, *his*, *metBF*, *ppc*, *iclR*$^{-}$	F^{-}	R
K2-1t-5^{c}	R-1-5^{c} x K2-1t	*argHBCE*, *iclR*$^{-}$, *pps*, *his*	F^{-}	R
K2-1-2-5^{c}recA1	KL16-99 x K2-1-2-5^{c}	*thr*, *leu*, *pps*, *recA1*, *metBF*, *ppc*, *iclR*$^{-}$	F^{-}	R
DF11-1	DF11 his	*metA*, *pgi*, *pps*	F^{-}	R
PA505-1	PA505	*metA*, *argHBCE*, *proA*, *pps*	F^{-}	R
PA505-1-5	PA505-1	*metA*, *argHBCE*, *proA*, *pps*, *icl-4*	F^{-}	R
ma7-5^{c}		*metA*, *argHBCE*, *iclR*$^{-}$, *pps*	F^{-}	R

RESULTS

Isolation and Characterisation of iclR⁻ mutants

The two iclR⁻ mutants used in this work, AT2572-1-5^{c} and R4-1-5^{c}, were, as previously described, spontaneous mutations selected through their ability to permit the growth of pps⁻ organisms on lactate agar plates at 30°C. Such colonies, though distinct from pps⁻ mutants, were now phenotypically indistinguishable on this carbon source from pps⁺ revertants (Table 15) since the constitutive production of isocitrate lyase permits the formation of C_4 compounds from lactate via the glyoxylate cycle and replaces the normal anaplerotic route through the carboxylation of PEP, rendered dysfunctional in these organisms by the pps⁻ lesion.

They could, however, be distinguished from wild-type or pps⁺ revertant strains both phenotypically through their inability to grow on pyruvate or acetate + pyruvate plates (Table 15) and enzymatically through the assay of their PEP-synthase and isocitrate lyase.levels. As may be seen in Table 16, the basal levels of PEP-synthase and isocitrate lyase in these two strains are indicated by their wild-type ancestors AT2572 and R4. In the pps⁻ strains AT2572-1 and R4-1 the levels of isocitrate lyase remain unchanged, though the loss of PEP-synthase activity now prevents growth on C_3 acids as sole carbon source. However, whereas pps⁺ revertants of these strains would be expected to regain the ability to grow on lactate through the recovery (to varying extents) of PEP-synthase

activity, the *iclR*$^-$ derivatives still lack this enzyme but are able to grow on lactate through their constitutive production of from 2.5 - 10 times the basal quantity of isocitrate lyase found in their wild-type ancestors.

TABLE 15

Growth patterns of *iclR*⁻ mutants AT2572-1-5^{c} and R4-1-5^{c} in comparison with those of their wild-type and *pps*⁻ ancestors

Carbon source	Growth after 24 hr incubation at 37°C		
	wild-type	*pps*⁻	[*pps*⁻] *iclR*⁻
25 mM-Glucose	++	++	++
25 mM-Glycerol	++	++	++
25 mM-Succinate	++	++	++
25 mM-Lactate	++	-	+
20 mM-Pyruvate	++	-	-
50 mM-Acetate	+	+	+
25 mM-Acetate plus 10 mM-Pyruvate	++	-	-
50 mM-Acetate plus 10 mM-Succinate	++	++	++

TABLE 16

Specific activities of isocitrate lyase and PEP-synthase in the iclR⁻ mutants AT2572-1-5^c and R4-1-5^c in comparison with those of their respective wild-type and pps⁻ ancestors. All cultures were grown overnight in nutrient broth at 37°C to approximately 0.5 mg dry wt/ml and assayed as described under the appropriate "Methods" sections

Strain	PEP-synthase	isocitrate-lyase
AT2572	4.1	5.5
AT2572-1	<0.1	5.5
AT2572-1-5^c	<0.1	12
R4	3.8	4.1
R4-1	<0.1	4.1
R4-1-5^c	<0.1	44

Mapping of the icl regulator gene

(A) By interrupted mating

The regulator site was initially mapped by crossing the Hfr strain R4-1-5^{c} (metBF, thy, pps, iclR^{-}, str) (Figure 2) with the F^{-} strain K2-1t (thr, leu, pps, his, argHBCE). The mating process was interrupted at 5 minute intervals for 40 minutes and samples were plated onto media selective for Lactate^{+}, Threonine^{+} and Leucine^{+}, and Arginine^{+} recombinants. The gene conferring ability to grow at the expense of lactate was transferred to the recombinants after some 20 minutes of mating and was situated between the genes thr and leu and argHBCE, approximately 2 min from argHBCE (Figure 15); a site corresponding closely to that determined for the isocitrate lyase "structural" gene.

That those recombinants which now grew at the expense of lactate did so due to the inheritance from R4.1.5^{c} of the dysfunctional regulator gene (iclR^{-}) was shown by comparison of their isocitrate lyase activities with those of the parent strains and Lactate^{-} recombinants from this cross after growth on nutrient broth at 37^{o}C. The Lactate^{-} recombinants and the constitutive parent Hfr strain R4-1-5^{c} contained the enzyme at specific activities of 42 - 44.3, whereas those recombinants of the phenotype Thr^{+}, Leu^{+} Lactate^{-}, together with inducible recipient strain K2-1t contained the enzyme at a specific activity of only 1.9. The results obtained from this preliminary location were

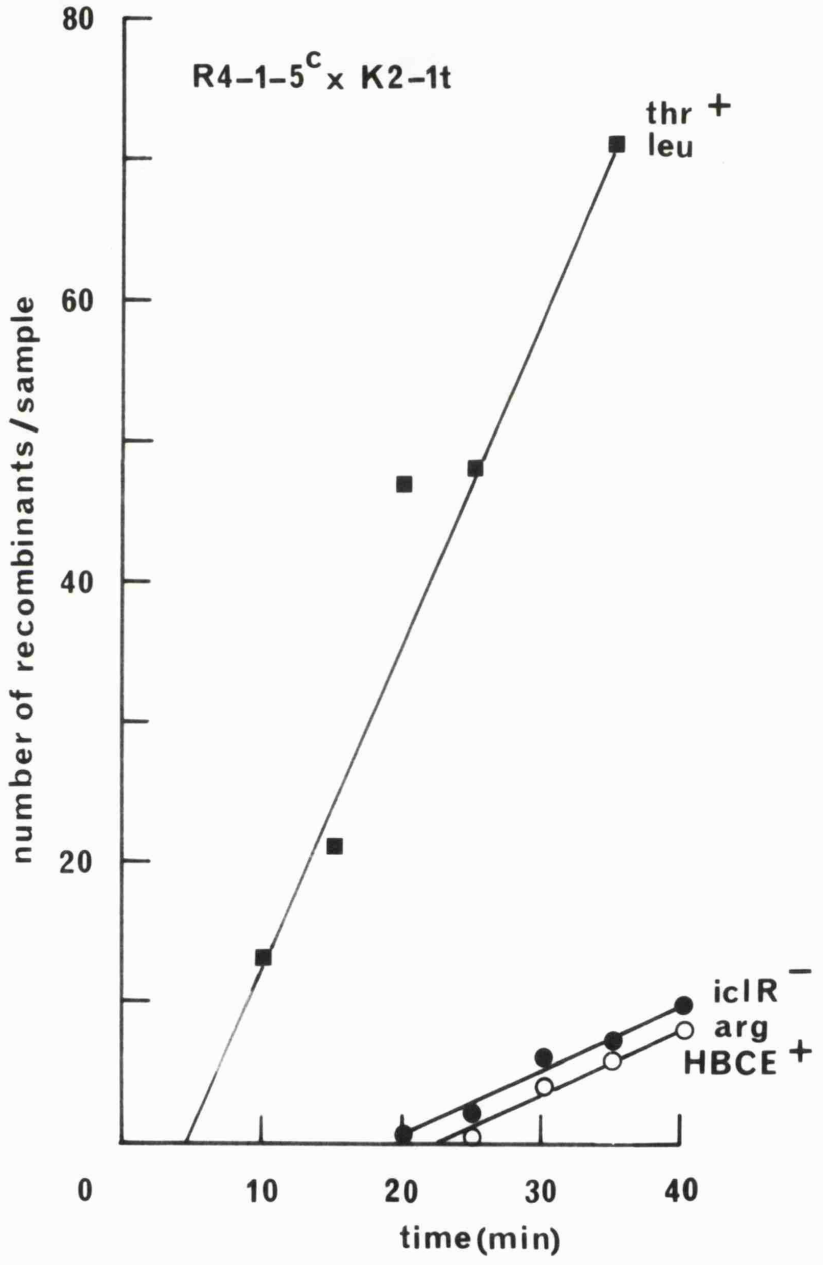

FIGURE 15

Kinetics of transfer at 37°C of the wild-type alleles for thr, leu, argHBCE and iclR^{-} from Hfr R4-1-5^{c} to the recipient K2-1t. The times plotted on the abscissa represent intervals between dilution of the mating pairs and the interruption of mating.

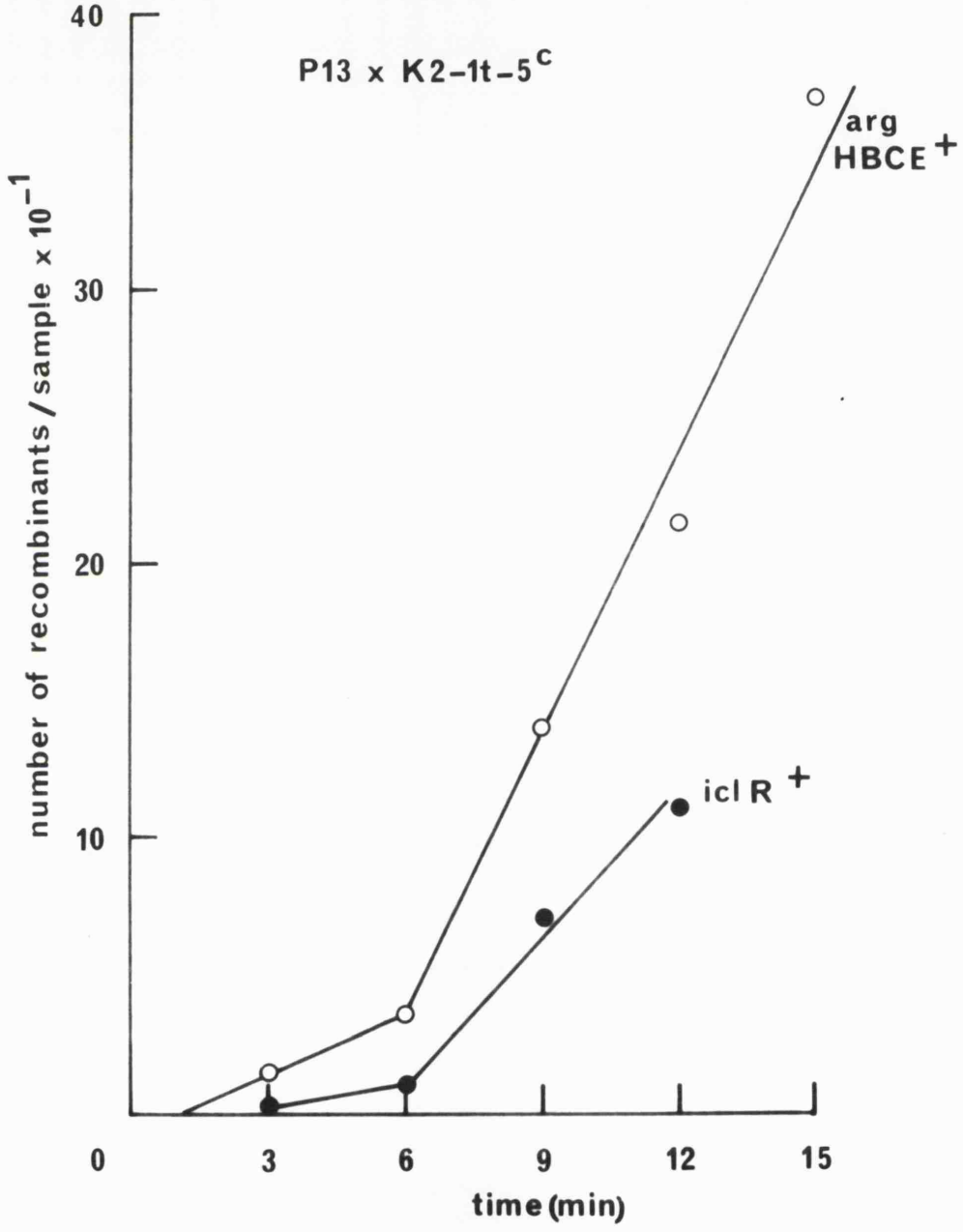

FIGURE 16

Kinetics of transfer at 37°C of the wild-type alleles for argHBCE and the icl regulator gene from Hfr P13 to the recipient K2-1t-5^{c}. The times plotted on the abscissa represent intervals between dilution of the mating culture and the interruption of mating.

substantiated in the following manner through a second interrupted mating experiment: An F^- recombinant of the genotype his, argHBCE, pps, iclR$^-$, isolated from the previous cross and named K2-1t-5^c, was mated with the wild-type Hfr P13 (Figure 2), which injects its genome in the order ilv, metBF, argHBCE. The mating was interrupted at 3 min intervals and samples plated onto 25 mM-glucose/histidine medium in order to follow the transfer of the argHBCE$^+$ allele. The Arg$^+$ recombinants so obtained were then replica-plated onto 25 mM-lactate media to determine the point in the cross, relative to the argHBCE marker, at which the ability of K2-1t-5^c to grow on lactate was abolished by the transfer of the wild-type regulatory gene from Hfr P13. As shown in Figure 16, this transfer occurred approximately 1½ - 2 min after that of the argHBCE$^+$ allele, a position consistent with the location determined from the cross between R4-1-5^c and K2-1t, and some 45 min before the expected transfer of the pps$^+$ allele by this strain.

B. Three-point transduction experiments

The position of the iclR gene implied by the previously described interrupted mating results was confirmed directly by three-point transduction experiments; first by locating it within the same intimate chromosomal segment in the icl structural gene and secondly by determining its position within this segment in respect to those of the icl structural gene and the metA marker. For three-point transduction experiments involving the metA marker the

Hfr strain AT2572-1-5^{c} (*pps*, *iclR*$^{-}$, *str*), carrying an independently isolated *icl* regulator mutation was used as the donor strain. Though independent, this mutant was isolated under similar conditions to the R4-1-5^{c} strain and manifests similar phenotypic characteristics (Tables 15 and 16). This change was necessary since all R4 derivatives, although *metA*$^{+}$, carry the *metBF*$^{-}$ marker. This is known (Part I of this thesis) to be cotransducible with the *icl* and *metA* markers, and in selections for the latter marker would consequently result in the loss of some *metA*$^{+}$ transductants with a possible alteration in the transduction frequency between *metA* and *iclR*.

(i) Mapping of the regulator gene in respect to the markers *metA* and *pgi* The most suitable recipient strain for this transduction was the F^{-} strain DF11 (*metA*, *pgi*). However,in order to facilitate the expression of the *iclR*$^{-}$ allele in transductants growing on lactate, it was necessary that the recipient strain should also contain the *pps*$^{-}$ allele. This requirement was fulfilled by mating the Hfr strain AT2572-1 (*pps*, *str*) with a mutant of DF11 auxotrophic for histidine - DF11 his (*metA*, *pgi*, *his*). The His^{+} recombinants from this cross were screened for the presence of the *pps*$^{-}$ allele by replica plating onto lactate medium. One such colony which was unable to grow at the expense of lactate and still retained the markers *metA* and *pgi* was isolated as a suitable recipient strain and named DF11-1.

This organism was then infected as described under "Methods"

with phage P1kc previously cultured on the donor strain AT2572-1-5^{c} (pps, iclR^{-}). After transduction dilutions of the suspension were plated onto 25 mM-glucose medium selective for Met^{+} transductants. The resulting colonies were then replica-plated onto 25 mM-lactate and 1% glucose tetrazolium plates in order to determine their iclR and pgi genotypes.

The results of this experiment, summarised in Table 17 and Figure 17, show that of a total of 693 metA^{+} transductants 633 (91%) had also received the iclR^{-} gene and hence now grew on lactate, and 310 (45%) the pgi^{+} marker. It was not possible from this selection to calculate directly the cotransduction frequency between iclR^{-} and pgi^{+} and thus order the three genes in this way. However, analysis of the genotypes of the transductants (Table 15) suggested the gene order to be metA - iclR - pgi for the following reasons:

(1) In every case of cotransduction between metA^{+} and pgi^{+} the iclR^{-} gene was also cotransduced, whereas only 45% of the metA^{+}, iclR^{-} cotransductants were also pgi^{+}. This strongly suggests that iclR^{-} was the middle marker in the cross.

(2) The most numerous class of transductants were cotransductants between metA^{+} and iclR^{-} and the second most numerous those in which all three genes had been cotransduced.

This fact again suggested that metA was an outside marker in the cross, for if it were the middle marker the two most numerous classes of transductants resulting from this selection should have been those carrying metA^{+} and iclR^{-} or pgi^{+} alone; whilst the three-point

transductants would be expected to occur only in numbers equivalent to the product of these frequencies.

(3) The smallest class of colonies were those in which only the *metA*$^+$ gene had been transduced. According to the results of this experiment *metA* is 55% non-cotransducible with *pgi* and 9% non-cotransducible with *iclR*. If *metA* were the middle marker in this cross then the frequency of such single point transductants should be equivalent to the product of the non-cotransduction frequencies between *metA* and *iclR* and *metA* and *pgi*.

i.e. The chance of *metA*$^+$ being transduced without *pgi*$^+$ = 100% - 45% = 55%

The chance of *metA*$^+$ being transduced without *iclR*$^-$ = 100% - 91% = 9%

∴ the chance of *metA*$^+$ being transduced without either of these markers = 9 % of 45% of the total colonies,

$= \frac{693}{100} \times (\frac{45}{1} \times \frac{9}{1}) =$ 34 colonies

However, the observed number of such single point transductants was 60, suggesting that these colonies were more likely to be the result of the solitary transduction of an outside marker.

In view of these facts the cotransduction frequency between *iclR* and *metA* was calculated as the percentage of three-point transductants within the total *metA*$^+$ *iclR*$^-$ population

$$= \frac{310}{633} \times \frac{100}{1} = 49\%$$

This figure however, must be regarded as a minimum value

since the originally selected marker - *metA*$^+$ - was only 91% cotransducible with *iclR*$^-$ and in selecting primarily for transductants possessing *metA*$^+$ the number of *iclR*$^-$ and *pgi*$^+$ cotransductants obtained would be accordingly reduced.

These results thus showed *iclR* to be between *metA* and *pgi*, in a position virtually identical with that of the *icl* structural gene.

(ii) *Location of iclR in relation to the icl structural gene and the metA marker* In view of the apparent juxtaposition of the *icl* structural and regulator genes and the fact that cotransduction frequencies are subject to small experimental variations, the individual cotransduction frequencies between *metA* and *icl*, and *metA* and *iclR* were clearly insufficient evidence upon which to order these three genes in respect to one another. A conclusive result therefore necessitated analysis of the genotypes of the transductants resulting from a three-factor cross between these markers.

This was accomplished through a transduction between AT2572-1-5^c, which bore the markers *metA*$^+$, *icl*$^+$ and *iclR*$^-$ and the recipient strain PA505-1-5, which carried the contrasting markers *metA*$^-$, *icl-4* and *iclR*$^+$ and the *pps*$^-$ gene necessary for the expression of the *iclR*$^-$ gene in transductants. The *icl-4* marker was an independent mutation induced directly in strain PA505-1 by ethylmethanesulphonate mutagenesis, since the proximity of *metA* and *icl* prevented the introduction of a previously mapped *icl* lesion without the abolition of the *metA*$^-$ marker carried by this strain.

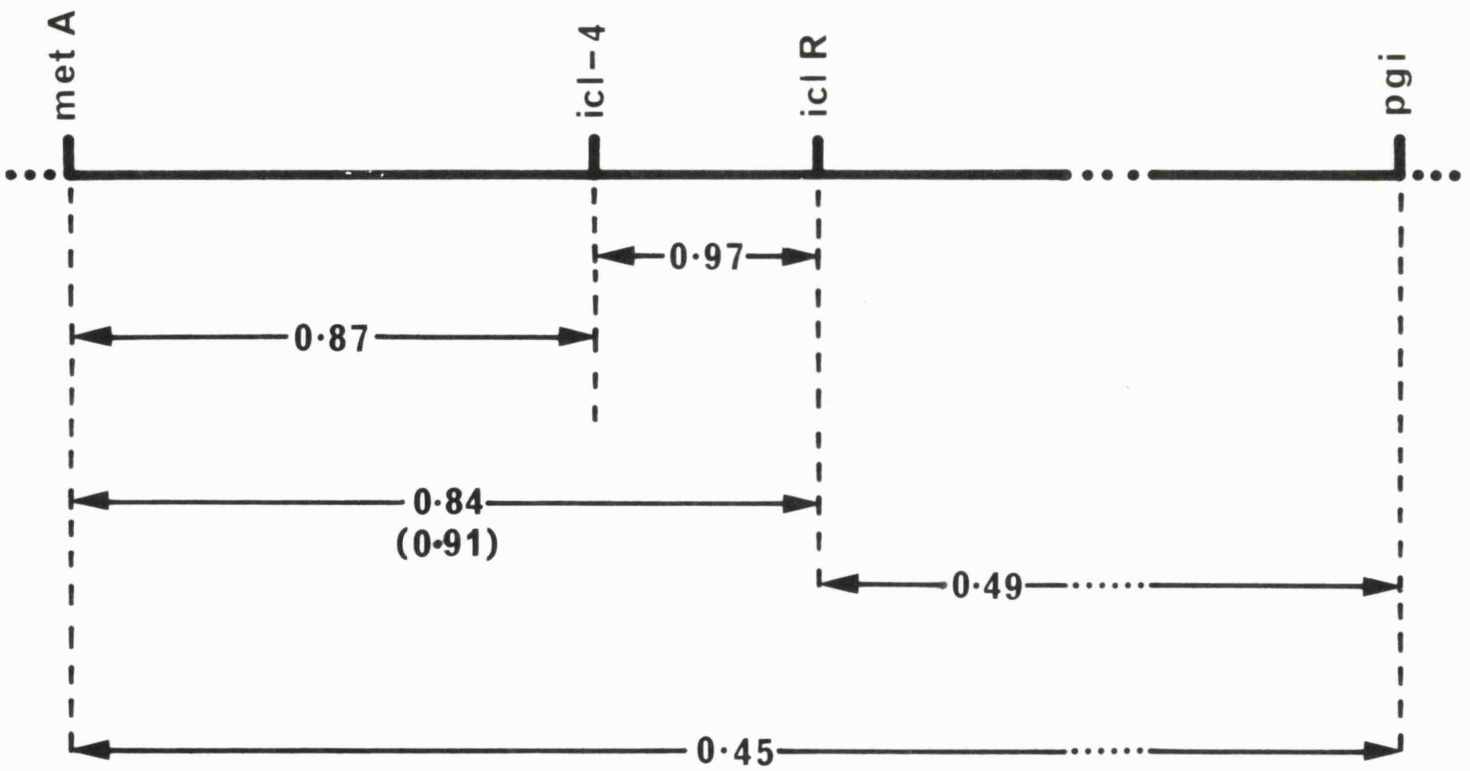

FIGURE 17

The relative order and linkage between the genes metA, icl-4, iclR and pgi as determined by three-point cotransduction experiments.

The PA505-1-5 recipient was infected with a phage P1kc lysate previously cultured on the donor strain AT2572-1-5^{c} and after incubation and resuspension as previously described, dilutions were plated onto media selective for Met^{+} transductants. The resulting colonies were then tested by replica-plating for their ability to grow on 50 mM-acetate and 25 mM-lactate as sole carbon sources.

If the gene order were metA, iclR, icl, then since the iclR^{-} lesion cannot be expressed on lactate without the presence of an intact icl structural gene, only the three-point transductants in the population would be able to grow at the expense of lactate and consequently all colonies of the phenotype Met^{+} , Acetate^{+} would also be lactate^{+}. However, if the gene order were metA, icl, iclR, then since cotransduction between metA^{+} and icl^{+} should not necessarily involve the simultaneous transduction of iclR^{-} , a small percentage of the Met^{+}, Acetate^{+} colonies would remain inducible (iclR^{+}) and consequently unable to grow at the expense of lactate. As shown in Table 18 and Figure 17, of a total of 1124 transductants selected initially as Met^{+}, 971 (87%) also grew at the expense of acetate. Of these latter colonies 939 (84%) also grew on lactate, indicating that they had received the iclR^{-} gene, whilst 32 remained inducible (iclR^{+}) and were therefore unable to utilise this carbon source for growth. The validity of these results was confirmed by comparing the isocitrate lyase activities in broth-grown cultures of both Lactate^{+} and Lactate^{-} transductants with that of the constitutive donor strain AT2572-1-5^{c}. Both the parent donor strain and the Lactate^{+}

TABLE 18

Three-point transduction results from the cross between AT2572-1-5^{c} and PA505-1-5

Number and Genotype of transductants			% Cotransduction		
Selected genetic marker	Unselected genetic markers		metA-icl	metA-iclR	icl-iclR
metA^{+} 1124	icl^{+} iclR^{-}	939	87	84	97
	icl^{+} iclR^{+}	32			
	icl^{-} [iclR^{+}]	53			

transductants contained the enzyme at specific activities of 12.8 - 28, whilst the Lactate$^-$ transductants contained the enzyme at specific activity of only 1.97.

These results demonstrated that the gene metA and icl could be cotransduced without simultaneously cotransducing iclR, and together with the results of the previous experiments established the gene order as being metA, icl, iclR.

Although the definite position of icl-4 in respect to the other icl markers used in this thesis has not been determined, it is cotransducible with metA in similar frequency to the icl-2 marker and for this reason is tentatively included between the icl-1 and icl-2 markers in the compounded genetic map of this region shown in Figure 18.

C. Identification of the nature of the icl regulatory lesion

In view of the juxtaposition of the icl structural and regulatory genes, the constitutivity conferred by the iclR$^-$ lesion might have been due to a mutation within either a regulator gene or an operator locus. Since both i$^-$ and o^c mutations exhibit the constitutive phenotype in the normal haploid cell, it was necessary to perform a "cis-trans" test using an iclR$^+$, iclR$^-$ merodiploid strain in order to distinguish between these two possibilities.

A major problem encountered in the construction of such organisms is the maintenance of a stable partial diploid state, since the spontaneous integration of the merogenote into the recipient

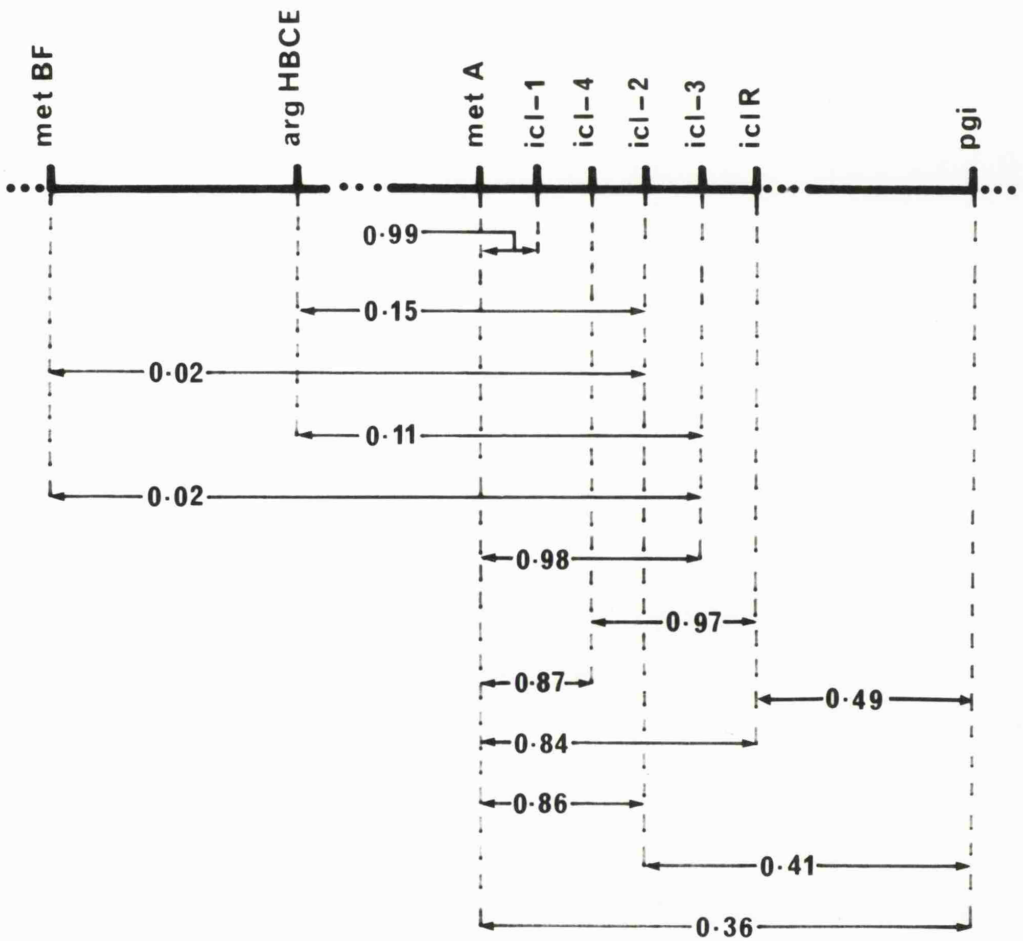

FIGURE 18

A genetic map of the icl region of the E. coli chromosome showing the suggested positions of the icl structural and regulatory mutations used in this work. The distances are expressed as cotransduction frequencies.

genome would result in haploidy and yield misleading results. This problem was countered by the use of recipient cells carrying the recA1 lesion (Low, 1968). This mutation greatly reduces the ability of the recipient cell to form conjugational recombinants with donor genetic material, but does not affect its ability to form mating pairs and receive the donor chromosome (Clark & Margulies, 1965). RecA1 recipients are therefore readily converted to merodiploids by the injection of the appropriate chomosomal region through a suitable F' or Hfr strain.

In order to prepare a suitable recA1 recipient the F^- strain K2-1-2-5^c (metBF, ppc, iclR$^-$, thr, leu, pps, his) was mated for 30 min without interruption with the Hfr strain KL16-99 (thi, recA1, str), which injects the recA1 lesion as an early marker as shown in Figure 2. After interruption of the mating samples were plated onto 25 mM-succinate media selective for His^+ recombinants and the resulting colonies were screened for the presence of the recA1 lesion by the determination of their resistance to ultra-violet irradiation (Clark & Margulies, 1965). Each recombinant was streaked onto two nutrient plates, one of which was exposed to an output of 6 erg/sec/mm^2 from a lamp at 40 cm distance for periods of 20-80 sec prior to incubation at 37^{o}C. Those recombinants killed by exposure of more than 20 sec were scored as recA1 and their counterparts from the unirradiated plate were purified by single colony isolation for further use. One such colony which now carried

the markers recA1, metBF, thr, leu, ppc, pps and which contained isocitrate lyase at the specific activity of 42.4 after growth on succinate (and was thus also iclR$^-$) was chosen as a suitable recipient and designated K2.1.2.5^c - recA1.

This strain was then mated with the F' strain KLF10/JC1553 (icl$^+$, iclR$^+$, ppc$^+$, metB$^+$/metB$^-$, argE$^-$, leu$^-$, his$^-$, recA1) in order to convert it to an iclR$^+$/iclR$^-$ merodiploid through the injection of the KLF10 merogenote (o, iclR$^+$, icl$^+$, ppc$^+$, metB$^+$). The mating was interrupted after 30 min and serially diluted samples were plated onto 25 mM-glucose agar supplemented with methionine, threonine, leucine and streptomycin in order to select for K-2-1-2-5^c - recA1 colonies which had received the ppc$^+$ marker. Fifty such colonies were then further tested for inheritance of the iclR$^+$ gene by replica-plating onto 25 mM-lactate agar: all failed to grow, indicating that they were now iclR$^+$. This was confirmed by assaying the isocitrate lyase content of four of these colonies after growth on 25 mM-glucose; the specific activities were 0.1 - 0.45.

These colonies were thus shown to have received the ppc$^+$ and iclR$^+$ genes from KLF10/JC1553, presumably due to the transfer of the KLF10 merogenote. One such clone, designated KLF10/recA1, was then submitted to further tests to determine more accurately its genetic constitution.

(i) Test for the presence of the F factor If KLF10/recA1 had received the ppc$^+$ and iclR$^+$ genes through the transfer

of the KLF10 merogenote, then it should also have received the F factor responsible for the mobility of this episome and hence should now possess the ability to form recombinants in a cross with a known F^- strain. In order to test this premise KLF10/recA1 was mated with the F^- strain ma7-5^c (metA$^-$, argHBCE$^-$, iclR$^-$). The mating was interrupted after 30 min and serially diluted samples were plated onto 25 mM-glucose media selective for Met^+ and Arg^+ recombinants. After 24 hr incubation at 37^oC the plates which had received 0.1 ml of the 1000-fold diluted mating culture supported an average of 100 cols per plate, indicating high efficiency of transfer of the genes metA and argHBCE. Assays of randomly selected recombinants grown in glucose media revealed isocitrate lyase specific activities of 0.25 - 0.30, in comparison with that of 16.5 exhibited by the F^- parent ma7-5^c, indicating that iclR$^+$ had been simultaneously transferred with metA$^+$ and argHBCE$^+$. These results thus demonstrate the male phenotype of KLF10/recA1.

(ii) Test for merodiploidy Although the ability to transfer the genes iclR$^+$, metA$^+$ and argHBCE$^+$ indicated that KLF10/recA1 contained the KLF10 F-merogenote segment, it did not determine whether this chromosomal segment was present in KLF10/recA1 as a free merogenote (F') or whether it had become integrated into the main chromosome (as in the Hfr state). Since it is known (Hirota, 1960) that F-merogenotes may by removed from F-merodiploids by treatment with acridine orange (apparently through the preferential inhibition of F replication [Hirota, 1960; Jacob, Brenner and Cuzin, 1963; Stouthamer,

de Haan and Bulten, 1963; Bastarrachea and Willetts, 1968]) it was possible, by using this technique and selecting for the abolition of the ppc$^+$ marker and the re-expression of the iclR$^-$ marker, to determine whether KLF10/recA1 was F' or Hfr.

An inoculum of approximately 5 x 10^3 cells of KLF/10 recA1 was added to 2.0 ml of nutrient broth pH 7.8 containing 100 µg/ml of acridine orange and incubated as described under "Methods". After "curing" serially diluted samples were plated onto both 25 mM-succinate and 25 mM-lactate agar supplemented with methionine, threonine and leucine and incubated at 37°C for 24-48 hr.

Those colonies which had arisen on the lactate medium and had thus been selected as iclR$^-$ were then replica-plated onto similarly supplemented 25 mM-glucose medium in order to determine whether a return to the iclR$^-$ phenotype was also accompanied by the loss of the ppc$^+$ marker. As shown in Table 19, of 995 iclR$^-$ colonies thus selected, 962 (96.7%) failed to grow on glucose, indicating that they had also lost the ppc$^+$ gene. Since it was not possible to select directly for the elimination of the ppc$^+$ marker a sample of acridine orange-treated cells was first plated onto non-selective succinate medium and the resulting colonies then tested for the elimination of the ppc$^+$ marker and the simultaneous re-expression of the iclR$^-$ gene by replica-plating onto glucose and lactate media respectively. As also shown in Table 19, of 856 colonies tested, 789 (92.3%) had lost the ppc$^+$ marker and simultaneously regained the

TABLE 19

Resultant genotypes after the "curing" of F'KLF10/recA1 by acridine orange treatment

Carbon Source	Total Colonies	Selected Marker	Unselected markers			
			Genotype	$\underline{ppc}^-$ $\underline{iclR}^-$	$\underline{ppc}^+$ $\underline{iclR}^-$	$\underline{ppc}^-\underline{iclR}^+$	$\underline{ppc}^+$ $\underline{iclR}^+$
			Phenotype	glucose$^-$ lactate$^+$	glucose$^+$ lactate$^+$	glucose$^-$ lactate$^-$	glucose$^+$ lactate$^-$
25 mM-Succinate	856	-		789	28	34	4
25 mM-Lactate	995	$\underline{iclR}^-$		962	33		

iclR$^-$ phenotype (glucose$^-$ lactate$^+$), 34 colonies (3.9%) had lost the ppc$^+$ marker but remained inducible (glucose$^-$ lactate$^-$), 28 colonies (3.2%) had retained the ppc$^+$ marker but regained expression of the iclR$^-$ gene (glucose$^+$ lactate$^+$) and 4 colonies (0.46%) were cured for neither marker (glucose$^+$ lactate$^-$). These results were confirmed by assaying the isocitrate lyase levels in samples of "cured" (glucose$^{\pm}$ lactate$^+$) and uncured (glucose$^{\pm}$ lactate$^-$) colonies after growth on nutrient broth at 37^{o}C. The "cured" colonies contained isocitrate lyase at specific activities of 10.6 - 13.0 in contrast with the specific activities of 1.57 - 1.73 exhibited by the uncured cells.

The collective results of the foregoing two experiments demonstrate that the genes iclR, icl, ppc, metA and argHBCE are grouped together with the F-factor as an F-merogenote segment separate from the main chromosome of KLF10/recA1 and that this organism is thus an F-merodiploid spanning the chromosomal region malB-metB.

Thus the isocitrate lyase activities recorded for the strains KLF10/JC1553, K2.1.2.5^{c}-recA1 and KLF10/recA1 constitute the results of a valid "cis-trans"test. From these results shown in Table 20 it may be seen that the individual specific activities of isocitrate lyase within succinate-grown cultures of KLF10/JC1553 and K2-1-2-5^{c}-recA1 were 0.29 and 42.4 respectively. However, when the wild-type regulatory gene contained on the KLF10 merogenote was introduced into K2-1-2-5^{c}-recA1, so forming the merodiploid strain

TABLE 20

Isocitrate lyase activities of the F^- and F' strains used in the previously described cis-trans test and acridine orange curing experiments

Strain	Mating Type	Source	Specific activity of isocitrate lyase	Carbon source
K2-1-2-5^c-recA1	F^-	-	42.4	25 mM-Succinate
KLF10/JC1553	F'	-	0.29	25 mM-Succinate
KLF10/recA1	F'	KLF10/JC1553 x K2-1-2-5^c-recA1	0.1 - 0.45	25 mM-Glucose
KLF10/recA1	F^-,	<u>iclR</u>$^-$ product from A.O. curing of KLF10/recA1	10.6 - 13.0	Nutrient broth
KLF10/recA1	F^-	<u>iclR</u>$^+$ product from A.O. curing of KLF10/recA1	1.57 - 1.76	Nutrient broth

KLF10/recA1, the isocitrate lyase activity of this strain after growth on glucose fell to the inducible level of 0.1 - 0.45, demonstrating the "trans" dominance of *iclR*$^{+}$ over *iclR*$^{-}$. These implications were also supported by the re-expression of the *iclR*$^{-}$ allele within KLF10/recA1 after acridine orange curing, resulting in constitutive enzyme activities of 10.6 - 13.0.

It is therefore probable that the regulatory gene for isocitrate lyase acts through the production of a diffusable cytoplasmic repressor molecule and by analogy with mutants of the *lac* operon (Jacob & Monod, 1961) that *iclR*-mutants are of the *i*$^{-}$ type rather than the *o*^{c}.

SUMMARY

1. The incubation of thick suspensions of two *pps*$^-$ Hfr strains, AT2572-1 and R4-1, on lactate plates at 30°C yielded two "revertant" strains (AT2572-1-5^c and R4-1-5^c) able to utilise this substrate for growth. Assays of the PEP-synthase and isocitrate lyase activities of these strains, together with those of their *pps*$^-$ parents and wild-type ancestors, indicated that their renewed ability to grow at the expense of lactate though not pyruvate or acetate plus pyruvate was due not to a recovery of PEP-synthase activity but to a spontaneous secondary mutation which had abolished the regulation of isocitrate lyase synthesis, resulting in the constitutive production of this enzyme and permitting the formation of C_4 compounds from lactate via the glyoxylate cycle.

2. Interrupted conjugation experiments employing the mutant Hfr strain R4-1-5^c showed that in this organism the site of the regulatory mutation (*iclR*$^-$) lay close to the position previously ascribed to the *icl* "structural" gene.

3. Phage *P1kc*-mediated transductions employing the phenotypically similar *iclR*$^-$ mutant AT2572-1-5^c, reflected the chromosomal position established for the *iclR*$^-$ lesion of R4-1-5^c and located the regulatory lesion with respect to the *icl* "structural" gene in the order *metA*, *icl*, *iclR*, *pgi*.

4. A "cis-trans" test using a *recA1* merodiploid spanning chromosomal region *metB* to *malB* demonstrated that (i) the *iclR*$^-$ mutation was recessive to its wild-type allele and (ii) that this

wild-type regulatory gene was capable of repressing the synthesis of isocitrate lyase from a "trans" position on the F merogenote. Therefore, in accordance with the operon theory of genetic regulation, the *iclR*$^-$ mutation isolated in the strains AT2572-1-5^c and R4-1-5^c is held to mark the site of an *icl* regulator gene.

DISCUSSION

DISCUSSION

The growth characteristics of the three mutants R4-5, G6-5 and K8-5^{CBB} are those which might be expected from organisms defective in the anaplerotic pathway effecting growth on acetate. Their ability to grow on glucose, glycerol, succinate and C_3-acids such as lactate and pyruvate shows that in these organisms the pathways of glycolysis, the tricarboxylic acid cycle, gluconeogenesis from PEP and the anaplerotic route providing C_4 compounds from C_3-acids function normally; whilst their growth on acetate plus pyruvate demonstrates that their inability to grow on acetate as a sole carbon source is due to the inability to form C_4 compounds from this substrate via the glyoxylate cycle. The virtual absence of isocitrate lyase in cell-free extracts of these mutants when compared with those of their wild-type parents, and the reappearance of the enzyme (albeit in different specific activities from wild-type cells) in revertants of these strains isolated for their renewed ability to grow on acetate further demonstrates that the inability of the three mutants to grow at the expense of this carbon source is due to their inability to form isocitrate lyase.

Since one of the objects of this work was to present evidence for the accurate location of the genes specifying the glyoxylate cycle enzymes, these three independent *icl*-mutants were then mapped by interrupted mating, recombination analysis and

transduction experiments in relation to a variety of genetic markers.

The results of interrupted cojugation experiments employing the mutations icl-2 and icl-3 show that they both lie within 1-1½ min from argHBCE and 2-2½ min from metBF, in positions closely similar to that of icl-1, which has been previously shown by Kornberg & Smith (1966) to be situated at approximately 78 min on the E. coli linkage map. These locations are supported by the findings that both icl-2 and icl-3 are 11-15% cotransducible with argHBCE and 1.7 - 2.2% cotransducible with metBF, since these values correspond well with the range of frequencies shown by Taylor & Trotter (1967) to be representative of genes situated 1 min and 1.5-1.8 min apart respectively.

However, the most direct evidence for the location of these mutations is the fact that each one is highly cotransducible (in frequencies of 86-99%) with the metA marker situated at 78.2 min on the linkage map of E. coli. Such results indicate that all three mutations lie within the same gene locus, which has been shown by three-point transduction to lie between metA and pgi at 78.3 min on the E. coli linkage map.

The occurrence of icl^+ recombinants from 3-point transductions between these icl-mutants and argHBCE demonstrates that although the mutations are situated within the same gene locus they are not homoallelic, and enables their sites within the locus to be ordered in relation to one another. In view of the results obtained and with reference to their cotransduction frequencies with metA and argHBCE

it is suggested that the three lesions lie in the order *metA*, *icl-1*, *icl-2*, *icl-3*, although since these frequencies appear to be the products of quadruple crossovers involving the chromosomal region *argHBCE* -*icl* as well as the respective *icl* mutations, they cannot be considered true estimates of the distances between these latter sites.

The location of the PEP-synthase "structural" gene is based on the results obtained with four independent mutants, some of which had been selected for their inability to grow on lactate and others for their inability to utilise either pyruvate or alanine. Irrespective of their method of isolation the phenotypes of all these mutants are identical and suggest that they are lacking in an enzyme involved in the anaplerotic pathway effecting growth from pyruvate. This is confirmed by the absence in all these strains of PEP-synthase activity.

The similar phenotypic properties of these strains are reflected in the results of interrupted mating experiments which show that the *pps*$^-$ lesion in each organism lies within the same gene locus, situated close to the *aroD* marker at 32.5 min on the *E. coli* linkage map, apparently in the order *his*, *pps*, *aroD*. The close proximity of these two latter genes is also shown by their high degree of cotransduction, but in the absence of a third cotransducible genetic marker the previously suggested order cannot be confirmed by this technique.

Since two of these independent mutants also contain *icl* structural gene mutations, the possibility that their renewed ability to grow on lactate is due to the constitutive production of isocitrate lyase resulting from a spontaneous mutation in the *icl* regulator gene rather than the introduction of the wild-type *pps* allele can thus be discounted.

The two *iclR*$^-$ strains AT2572-1-5^c and R4-1-5^c are, as previously described, secondary mutants obtained by incubating suspensions of *pps*$^-$ strains on lactate plates at 30°C. Although by virtue of their ability to grow on lactate these mutants are phenotypically indistinguishable on this carbon source from *pps*$^+$ revertants, they can be distinguished both enzymatically, through their constitute production of isocitrate lyase and absence of PEP-synthase, and by their inability to grow on either pyruvate or acetate plus pyruvate plates. It has been shown by Kornberg (1967) that, although pyruvate no longer represses the synthesis of isocitrate lyase in such mutants, it still exerts a "fine" control through the inhibition of enzyme activity and this failure of *iclR*$^-$ mutants to grow in the presence of pyruvate accords with that explanation. On lactate media, however, the interposition of an enzymic step in the formation of pyruvate, coupled with its removal via acetate and the glyoxylate cycle, apparently prevents the accumulation of this compound in concentrations sufficiently high to inhibit growth.

The results obtained from interrupted conjugation and

transduction experiments employing the two independent iclR$^-$ mutants are in good agreement and show this locus to lie adjacent to the icl structural gene in the order metA, icl, iclR, pgi.

Experiments performed with a ppc$^+$, iclR$^+$/ ppc$^-$, iclR$^-$ merodiploid strain show that whereas the transfer of the iclR$^+$ bearing merogenote effects a reduction of isocitrate lyase activity in the KLF10/recA1 merodiploid from the constitutive level of 43.4 µmoles to 0.1 - 0.45 µmoles its elimination by acridine orange treatment results in a return to the constitutive specific activity of 10.6 - 13.0.

Such results which are analogous with those obtained in experiments with the i$^-$ gene of the β-galactosidase operon (Jacob & Monod, 1961) demonstrate the trans-dominance of the iclR$^+$ over iclR$^-$ and show that the regulation of the synthesis of isocitrate lyase is controlled through the action of a diffusible repressor molecule specified by the adjacent iclR regulator locus.

The structural gene specifying the second enzyme of the glyoxylate cycle, malate synthase (masA), has recently been located by Vanderwinkel & de Vlieghere (1968) and shown also to lie in close proximity to the icl gene in the order metA, masA, icl, iclR. By thermal denaturation in the presence of 10 mM-oxalate at 61°C (Falmagne, Vanderwinkel & Wiame, 1965)these workers were also able to determine the levels of this enzyme in a mixture of two such isoenzymatic proteins and demonstrate that it is synthesized in similar relative amounts to that of isocitrate lyase during growth on a variety of carbon sources.

These facts together with the demonstrations of the co-ordinate formation of the glyoxylate cycle enzymes (Kornberg, 1961, 1966), and their parallel derepression within a constitutive strain (Vanderwinkel, Liard, Ramos & Wiame, 1963) provide both genetic and biochemical evidence for the view that the genes of the glyoxylate cycle comprise an operon which is regulated through the intracellular levels of C_3 compounds such as pyruvate and phosphoenolpyruvate.

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Brice C.B. & Kornberg H.L., Journal of Bacteriology, 96 No. 6, p.2185, 1968.

These publications are enclosed in a folder in the rear cover of this thesis.

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of Leicester, entitled "The Genetic Control of Anaplerotic Reactions in *Escherichia coli*" is the result of work done mainly by me during the period of registration for the above degree.

(C. B. BRICE)

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Genetic Control of Isocitrate Lyase Activity in *Escherichia coli*

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Genetic Control of Isocitrate Lyase Activity in *Escherichia coli*

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Isocitrate lyase (EC 4.1.3.1) plays a necessary role in the growth of *Escherichia coli* on acetate as sole carbon source (1). Pyruvate and phosphoenolpyruvate both inhibit the activity and repress the synthesis of this enzyme (5). Mutants in which the synthesis of isocitrate lyase is no longer subject to control by these C_3-compounds, and which thus form isocitrate lyase constitutively, have been obtained as secondary mutants from parent organisms which lack the ability either to carboxylate phosphoenolpyruvate (9) or to form phosphoenolpyruvate from pyruvate (5). It is the purpose of this communication to report the location on the genome of *E. coli* K-12 of genes which specify the structure (*icl*) and constitutivity (*iclR*) of isocitrate lyase.

After treatment of a variety of strains of *E. coli* with ethylmethane sulfonate (7) and selection with penicillin (4) for inability to grow on acetate, four independently isolated mutants were affected in their ability to form isocitrate lyase; the genotypes of these organisms are listed in Table 1. By periodic interruptions of conjugation, it was established that all the *icl* alleles lay about 2 min from the *argHBCE* gene cluster, indistinguishably close to the *metA* marker and about 1 min from *pgi* (3); all *icl* mutants were thus mapped at 78 min on the linkage map of Taylor and Trotter (8). The genes affected were located more precisely, with reference to the *metBF*, *argHBCE*, *metA*, and *pgi* markers, by phage *P1*kc-mediated transduction. All *icl* mutants were cotransducible with well over 80% frequency with *metA* (Fig. 1), which is in good agreement with the findings of Vanderwinkel and de Vlieghere (10). The occurrence of icl^+ recombinants from genetic crosses among different icl^- mutants (which differed also in their *met* or *arg* alleles) showed that these mutants were not identical. Thus, of 886 arg^+ transductants obtained from the cross *icl-1* × *icl-2*, 10 were icl^+; of 941 arg^+ from *icl-1* × *icl-3*, 13 were icl^+; and of 833 arg^+ from *icl-2* × *icl-3*, 2 were icl^+. Thus, these results also support the order of these three *icl* mutations given in Fig. 1; *icl-4* has not yet been located to this degree of precision.

Similar procedures were used for the location of the marker which conferred constitutivity of isocitrate lyase synthesis on a variety of mutants. Such *iclR* mutants were obtained from pps^- parents and were recognized by their ability to grow on lactate, but neither on pyruvate nor on acetate plus pyruvate as carbon sources; that isocitrate lyase was formed constitutively was confirmed by enzymatic assay of sonic extracts of such *iclR* mutants (6).

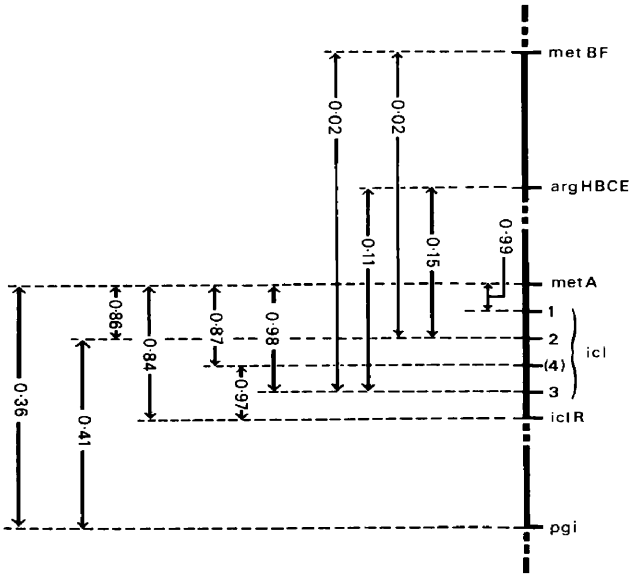

FIG. 1. *Genetic map of the icl region of the E. coli chromosome. The distances between markers are expressed as cotransduction frequencies.*

Like the *aceD* mutation, which confers constitutivity of glyoxylate cycle enzymes on *E. coli* (2), the *iclR* marker was closely linked both to *metA* and *icl*. To determine its position relative to *metA* and *icl*, the *iclR* and *metA* alleles were transduced from AT 2572-1-5° (*iclR*, *pps*) into the recipient PA 505-1-5 (*metA*, *icl-4*, *pps*). Of 1,124 $metA^+$ transductants, 971 were also icl^+: of these latter, 939 had received both the *icl* and *iclR* markers, whereas 32 transductants formed

TABLE 1. *Characteristics of icl and iclR mutants*[a]

Strain	Derived from	Genetic markers	Response to streptomycin	Mating type
R4		*metBF*	S	Hfr
R4-5	R4	*metBF, icl-1*	S	Hfr
G6		*his, thy*	S	Hfr
G6-5	G6	*his, thy, icl-2*	S	Hfr
K8	AB 1911	*argHBCE, metBF*	R	F^-
K8-5a	[G6-5 × K8]	*arg, icl-2*	R	F^-
K8-5m	[G6-5 × K8]	*metBF, icl-2*	R	F^-
K8-5CB	K8	*argHBCE, metBF, icl-3*	R	F^-
AT 2572-1	AT 2572	*pps*	S	Hfr
AT 2572-1-5[c]	AT 2572-1	*pps, iclR*	S	Hfr
DF 11		*metA, pgi*	R	F^-
PA 505		*metA, argHBCE, pro, his*	R	F^-
PA 505-1	[AT 2572-1 × PA 505]	*metA, argHBCE, pps, pro*	R	F^-
PA 505-1-5	PA 505-1	*metA, argHBCE, pps, pro, icl-4*	R	F^-

[a] The symbols used indicate a requirement for *met* = methionine, *his* = histidine, *thy* = thymine. *arg* = arginine, *pro* = proline, or the absence of *icl* = isocitrate lyase, *iclR* = regulation of isocitrate lyase, *pps* = phosphoenolpyruvate synthase, *pgi* = phosphoglucose isomerase. R and S denote resistance and sensitivity to streptomycin; Hfr and F^- denote male (donor) and female (recipient) mating types.

isocitrate lyase inducibly. This phenomenon shows that the *metA* and *icl* markers can be cotransduced at high frequency, without simultaneously transducing *iclR*, and supports the order *metA . . . icl . . . iclR*.

Since the gene which specifies the second enzyme of the glyoxylate cycle, malate synthase A (*masA*), is also cotransducible with *metA* and *icl* at high frequency (the order being *metA . . . masA . . . icl*; 10), it is tempting to conclude that the structural and regulator genes of the glyoxylate cycle form an operon.

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Location of a gene specifying phosphopyruvate synthase activity on the genome of *Escherichia coli*, K12

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A gene which specifies the activity of phosphopyruvate synthase has been located on the genome of *Escherichia coli*. By interrupted mating of a variety of genetic donor (Hfr) and recipient (F^-) strains of *Escherichia coli*, K12, which carried alleles of the phosphopyruvate synthase (*pps*) gene, it was established that the *pps* gene is close to the *aro-D* marker. This was confirmed by phage-mediated transduction of these genes, which were shown to exhibit a high degree of linkage.

Introduction

Among the evidence cited by Cooper & Kornberg (1965, 1967) for the physiological role of phosphopyruvate synthase (*PEP*-synthase), the enzyme which catalyses the net formation of phosphopyruvate, *AMP* and inorganic phosphate from pyruvate and *ATP*, was the observation that mutants of *Escherichia coli*, strain B, could be obtained which differed from their wild-type parents in being unable to utilize C_3-acids (such as pyruvate, alanine or lactate) as sole carbon source, although such mutants were not impaired in their ability to grow upon glucose, glycerol, acetate or utilizable intermediates of the tricarboxylic acid cycle. Extracts of such mutants were found to be devoid of *PEP*-synthase activity.

It is the purpose of the present paper to report the isolation of a variety of similar mutants of *Escherichia coli*, K12, and their use in the location on the bacterial genome of a gene which specifies *PEP*-synthase activity. A preliminary report of the substance of this work is given in the preceding paper.

Materials and Methods

Organisms used

The strains of *E. coli*, K12 used are listed in table 1. Many of these organisms were obtained as gifts from the following: K1—from Professor R. H. Pritchard (University of Leicester); AT2571 and AT2572 from Professor E. A. Adelberg (Yale University Medical School); AB1359—from Professor A. L. Taylor (University of Colorado Medical School); K2—from Professor R. H. Walmsley (University of Pennsylvania).

Of each organism, single colonies were isolated at 37 °C on Oxoid nutrient agar plates, supplemented where appropriate with thymine (40 μg/ml.), and were transferred to slopes of the same medium. After overnight growth at 37 °C, the slopes were stored at room temperature. The organisms were subcultured at 2 to 3 weekly intervals. For growth in liquid media the organisms were inoculated either into Oxoid nutrient broth or into the defined media described by Ashworth

by the selection procedure used, such mutants were unable to grow on media containing C_3-acids (such as alanine, lactate or pyruvate) as sole carbon sources: however, they grew readily if these media were also supplemented with utilizable intermediates of the tricarboxylic acid cycle. This observation already suggested that the enzymic dysfunction in such mutants involved the anaplerotic pathway

TABLE 2. GROWTH PATTERNS OF *ESCHERICHIA COLI*, K12 AND OF ITS MUTANTS DEVOID OF PHOSPHOPYRUVATE SYNTHASE ACTIVITY

	growth at 37 °C after 18 h of	
substrate	wild type	*pps* mutants
glucose	+ +	+ +
glycerol	+ +	+ +
lactate	+ +	−
pyruvate	+ +	−
alanine	+ +	−
acetate	+ +	+ +
acetate + pyruvate	+ +	−
succinate	+ +	+ +
glutamate	−	−
aspartate	±	±
lactate + glutamate	+ +	+ +
lactate + aspartate	+ +	+ +

(Kornberg 1966*a*) from pyruvate, and hence that such mutants (though able to oxidize pyruvate) were unable to effect the net synthesis, from pyruvate, of the intermediates of the tricarboxylic acid cycle which are required for cellular syntheses. Since these mutants (as shown in table 2) also grew readily on glycerol, acetate or succinate, it follows that the tricarboxylic acid and glyoxylate cycles, the pathway of glycolysis, and the pathway of gluconeogenesis from phosphopyruvate, functioned normally. As previously reported for similar mutants of *E. coli*, B (Cooper & Kornberg 1965, 1967), the observed growth pattern was that expected from mutants devoid of the ability to catalyse the net formation of phosphopyruvate from pyruvate.

A second characteristic of mutants devoid of *PEP*-synthase activity is that their growth on acetate as sole carbon source is markedly inhibited by the addition of pyruvate (Kornberg 1966*b*). This effect, which is illustrated in figure 1, is associated with the inhibition by pyruvate of isocitrate lyase, the first enzyme of the glyoxylate cycle. In consequence of this inhibition of the activity of this anaplerotic enzyme, the rate of net formation of tricarboxylic acid cycle intermediates from acetate is markedly reduced: since, in the absence of *PEP*-synthase, this lack of tricarboxylic acid cycle intermediates cannot be made good from pyruvate, the rate of growth is also sharply reduced. As expected from this explanation, the mutants resume more rapid growth when the added pyruvate has been oxidized; moreover, the addition of glyoxylate, or of utilizable intermediates of the tricarboxylic acid cycle, overcomes the inhibitory effect of pyruvate.

The third, and most direct, test for the absence of *PEP*-synthase activity from the mutants is the assay of the ability of ultrasonic extracts of the organisms

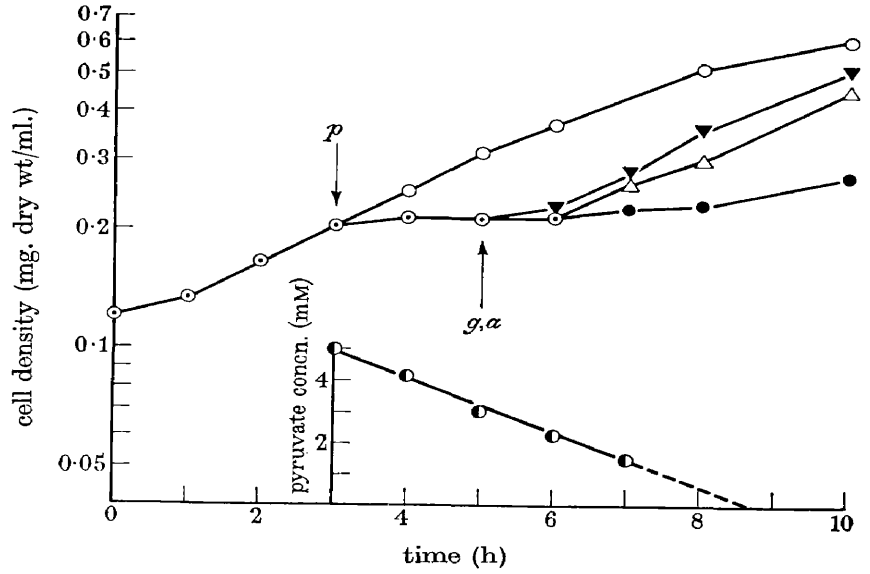

FIGURE 1. Effect of pyruvate on the growth of the *E. coli*, K 12 mutant K 1-1 (*pps*). A starting culture was grown overnight at 37 °C in medium containing 50 mM-acetate, methionine (40 μg/ml.), thymine (40 μg/ml.) and the ammonia-salts mixture described by Ashworth & Kornberg (1966). The organisms were transferred to four flasks containing fresh medium of the same composition and were allowed to continue to grow at 37 °C. To two of these flasks (△, ▼) pyruvate was added, at the arrow marked '*p*' to a final concentration of 10 mM; one of these flasks (▼) subsequently received 10 mM-glyoxylate (at the arrow marked '*g*') and the other (△) received 5 mM-aspartate (at the arrow marked '*a*'). A third flask (●) received 5 mM-pyruvate at the arrow marked '*p*' but no further additions were made: the decrease in the pyruvate content (◐) of this flask is plotted in the inset of the figure. All coincident points are designated as (⊙).

TABLE 3. PHOSPHOPYRUVATE SYNTHASE ACTIVITY OF EXTRACTS OF ORGANISMS USED

The activity of phosphopyruvate synthase of extracts of the organisms, grown in 'Oxoid' nutrient broth at 37°C to 0·3 to 0·5 mg dry wt/ml., was assayed as described under Methods

organism used	specific activity of *PEP*-synthase
K1	4·9
K1-1	0
AT 2572	4·1
AT 2572-1	0
AT 2571	5·0
K2	6·3
K2-1t	0
K2-1A-5B	0
K2-1C-5B	0
K6	5·8
K6-1	0
recombinants from [AT 2571 × K6-1]	6·3–7·1
aro-D$^+$, *pps*$^+$-phage tranductants from K6-1	6·3–6·8

(suitably concentrated to permit of the ready measurement of low activities: see the Methods section) to promote the removal of pyruvate from solutions thereof in the presence of magnesium chloride, *ATP* and *TRIS*-hydrochloride buffer at pH 8·0 (Cooper & Kornberg 1967). As shown in table 3, both the Hfr strains K 1 and AT 2572 contained *PEP*-synthase at specific activities of 4 or 5, but the presumed *pps* mutants K 1-1 and AT 2572-1, derived from these organisms, contained less than 1 % of the *PEP*-synthase activities of their parents.

Isolation of pps *mutant K* 2-1 *t of* E. coli, *K* 12

In order to locate on the genome of *E. coli* the gene which specificed *PEP*-synthase activity, the mutant K 1-1 (Hfr, *pps*, *met*, *thy*, *str*) was mated with the F⁻ recipient K 2 (*thr*, *leu*, *try*, *his*, *arg*) and, after mechanical interruption of the mating, recombinants were selected on plates containing glucose, streptomycin, and the amino acids required by K 2 but with appropriate components omitted. Such recombinants were tested, after replica-plating on media containing lactate, streptomycin, and all the appropriate amino acids, for their ability to use the C_3-acid as carbon source By this test, it was found that a large proportion of the recombinants which no longer required histidine for growth, and a lesser proportion of those which still required histidine but not tryptophan, had also lost the ability to grow on lactate. The *pps* gene had thus, presumably, entered after that specifying the tryptophan requirement but before that specifying the biosynthesis of histidine: the location of these genes on the chromosome of *E. coli*, K 12 is indicated in figure 2.

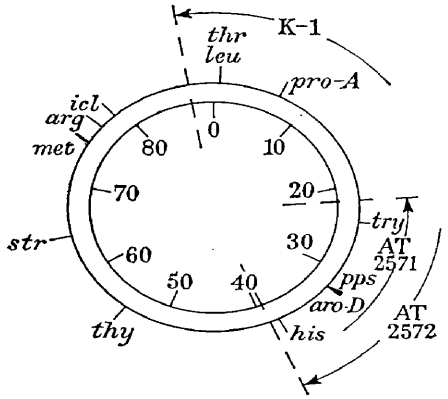

FIGURE 2. Genetic map of *Escherichia coli*. This map [adapted from Taylor & Thoman (1964)] is numbered inside the circle at intervals of 10 min. On the outside of the circle are shown the locations of genetic markers referred to in the text. The bold arrows indicate the points of origin and direction of genome transfer of the genetic donor strains used in this work.

After purification of a number of such *pps* recombinants, by single-colony isolation, one designated K 2-1 t was kept and used for subsequent experiments. It had the genotype *pps*, *thr*, *leu*, *his*, *arg*; phenotypically, this mutant was resistant to streptomycin and manifested the characteristics recorded in tables 2 and 3 and figure 1.

Genetic transfer of pps *allele from K* 1 *to K* 2-1*t*

That the *pps* gene was located on the chromosome close to the *his* marker was shown by analysis of the genetic cross K 1 × K 2-1 t, which is illustrated in figure 3. Mating was interrupted mechanically, and the number of recombinants which had either regained the ability to grow on alanine or lactate as carbon sources, or had lost the requirement for histidine, was plotted against the time of mating. The results obtained show that the normal *pps* allele entered 6 to 7 min before the *his* marker. Since the introduction of this allele into K 2-1 t restored the ability to grow both on lactate and on alanine, these results confirm the role postulated for *PEP*-synthase in the growth of *E. coli* on C_3-acids (Cooper & Kornberg 1965, 1967).

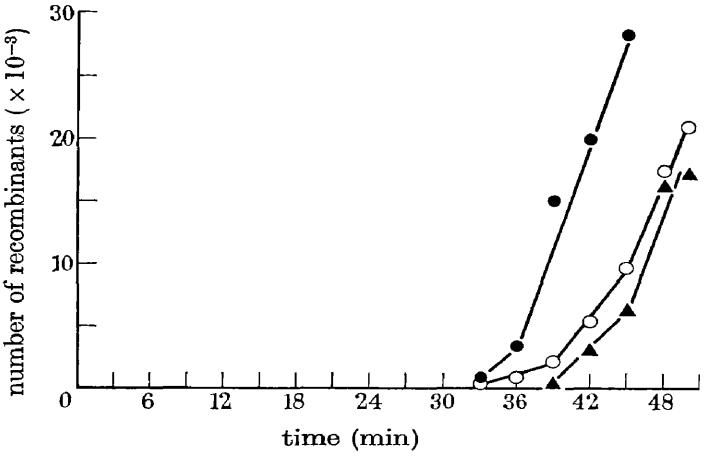

FIGURE 3. Kinetics of transfer at 37 °C of the gene(s) permitting growth on alanine (●) or lactate (○), and of the *his* allele (▲), from K 1 to K 2-1 t. The parental strains for the cross were K 1: Hfr, *met*, *thy*, *str*; K 2: F⁻, *pps*, *thr*, *leu*, *his*, *arg*. The times plotted on the abscissa are the intervals (min) between mixing the mating cultures and the mechanical interruption of mating.

Genetic transfer of pps *allele from AT* 2572 *to* pps *mutants also devoid of isocitrate lyase activity*

Two considerations prompted an alternative approach to the mapping of the *pps* gene. In the first place, there was a delay of more than half an hour before the *pps* gene was transferred to recipient organisms by the Hfr strain K 1 employed hitherto, with consequent reduction in the efficiency of genetic transfer, and it was clearly preferable to use an Hfr strain which injects its genome into recipient *E. coli* at a site closer to the *pps* marker. Secondly, *pps* mutants of *E. coli* have been described in which further genetic alterations, specifying either the regulation of synthesis of enzymes of the glyoxylate cycle (Kornberg 1966*b*) or the enzymic mechanism(s) involved in the utilization of exogenous pyruvate and, in consequence, affecting the activity of enzymes of the glyoxylate cycle (H. L. Kornberg, to be published), permit growth on C_3-acids to occur despite absence of *PEP*-synthase activity. The phenotype of such mutants could not be readily distinguished from wild-type organisms, and might lead to erroneous location of the *pps* marker.

The first of these points was met by the use of the Hfr strain AT2572 (*str*), which, as indicated in figure 2, transfers its genome in a counter-clockwise direction from a point immediately prior to the *his* marker. The second point was met by the preparation and isolation of two independently obtained *pps* mutants, K2-1A-5B and K2-1C-5B, which were derived from K2-5B, a derivative of K2 which had previously undergone a mutation in the *icl* structural gene. These mutants, which were streptomycin-resistant and required tryptophan, histidine and arginine for growth, grew neither on acetate (in consequence of the absence of isocitrate lyase activity) nor on lactate, alanine or pyruvate (in consequence of the absence of *PEP*-synthase activity: see table 3); however, both mutants grew readily on glucose, glycerol or succinate as carbon sources.

When the mutant K2-1A-5B was mated with the genetic donor organism AT2572, and recombinants were selected for the loss of the histidine requirement and/or the ability to grow on lactate, it was observed that the normal *his* allele entered approximately 6 min after the beginning of the experiment and approximately 6 min before the *pps* marker. This is illustrated by figure 4.

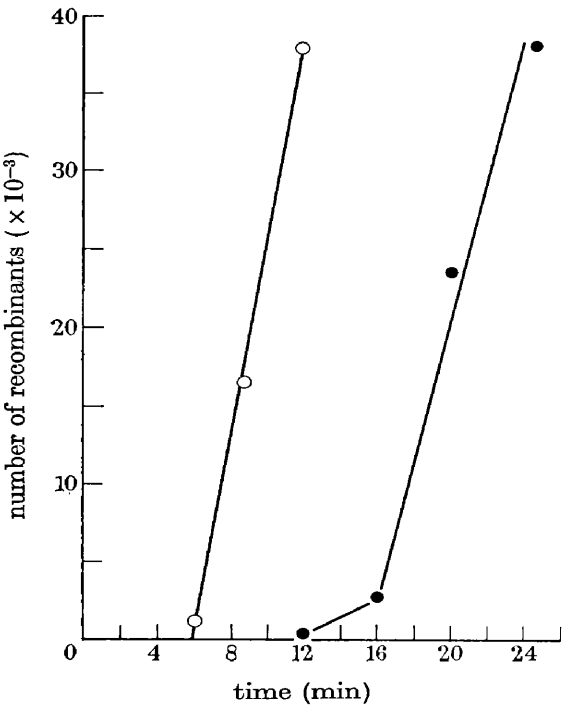

FIGURE 4. Kinetics of transfer at 37 °C of the *his* (○) and *pps* (●) alleles, from AT2572 to K2-1A-5B. The parental strains for the cross were AT2572: Hfr, *str*; K2-1A-5B: F⁻, *pps, icl, try, his, arg*. The times plotted on the abscissa are the intervals (min) between mixing the mating cultures and the mechanical interruption of mating.

Similar results were obtained when K2-1C-5B was used as genetic recipient. Again, the normal *his* allele entered at about 7 min and the ability to grow on lactate appeared to be regained after about 14 min. As shown in figure 5, the acquisition of the ability to grow on lactate coincided with that for growth on

pyruvate or alanine as carbon sources. Although the slopes of the lines relating the duration of mating to the number of recombinants obtained with pyruvate or alanine differed somewhat from the slope obtained with lactate, the coincidence of their intercepts with the time axis confirms (as also indicated by figure 3) that the same enzymic dysfunction prevents growth on any of these three C_3-acids.

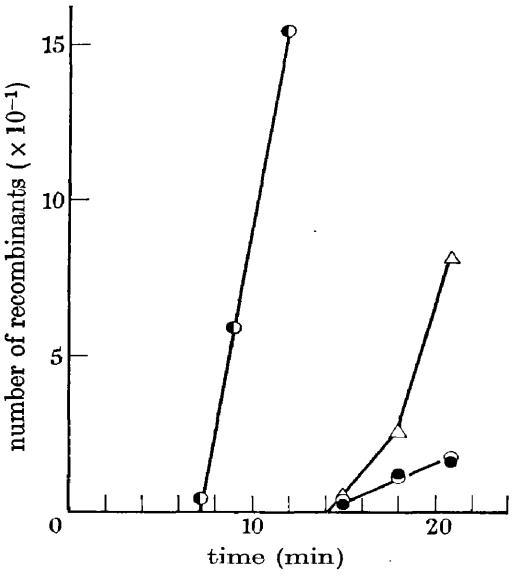

FIGURE 5. Kinetics of transfer at 37 °C of the *his* (◐) allele, and the gene(s) permitting growth on lactate (△), pyruvate (○) or alanine (●) from AT2572 to K2-1C-5B. The genotype of the organisms and the procedures used were as recorded in the legend to figure 4.

Preparation and use of pps *mutant K*6-1

The results obtained with mutants of K2 indicated that the gene which specified *PEP*-synthase activity lay close to the position ascribed by Taylor & Thoman (1964) to the *aro-D* marker. This made it desirable to obtain a mutant bearing the *aro-D* marker which also lacked *PEP*-synthase activity. Such a mutant was obtained as follows.

The Hfr strain AT2572 was subjected to treatment with ethyl methanesulphonate, and a *pps* mutant *AT*2572–1 was obtained therefrom after penicillin selection: as shown in table 3, this mutant was indeed devoid of *PEP*-synthase activity.

A thick suspension of the recipient strain of *E. coli*, *AB*1359, which (among other markers) carried the *str* and *aro-D* alleles was smeared on to nutrient-agar plates which also contained streptomycin at 100 μg/ml. of medium. The few colonies which grew on these plates after 2 days at 37 °C were picked and purified by single-colony isolation. One of these colonies, which grew with equal facility in the presence or absence of streptomycin but which still carried the *his* and *aro-D* markers, was kept as stock culture for future use and was designated K6. When mated with the donor strain AT2572-1 (*pps*), recombinants were obtained which had lost the histidine requirement of the parental K6, which still carried the *aro-D* marker (and thus required shikimate for growth) but which did not grow

when lactate was used as carbon source. As shown in table 3, analysis of an extract of one such recombinant (designated K 6-1) showed this organism to be devoid of *PEP*-synthase activity: this mutant was therefore used in subsequent experiments.

When K 6-1 (*aro-D*, *pps*) was mated with AT 2572, and recombinants were selected for streptomycin-resistance and their ability to grow either on lactate in the presence of shikimate, or on glucose in the absence of shikimate, the rate of appearance of such recombinants, illustrated by figure 6, was virtually identical for either the *pps* or *aro-D* markers and both genes entered after about 12 min.

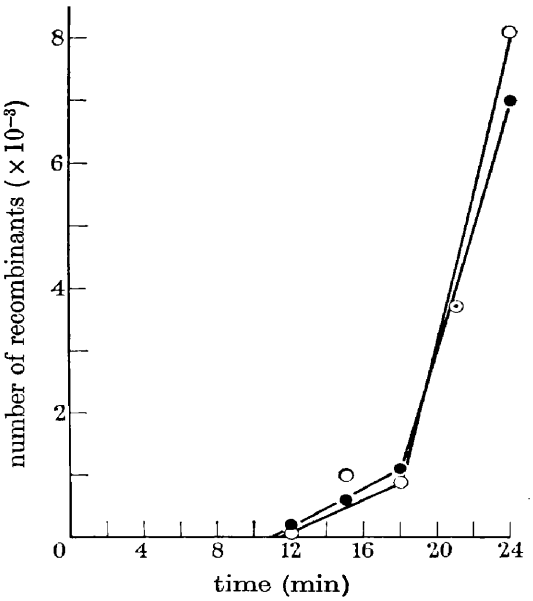

FIGURE 6. Kinetics of transfer at 37 °C of the *pps* (○) and *aro-D* (●) alleles from AT 2572 to K 6-1. The parental strains for the cross were AT 2572: Hfr, *str*; K 6-1: F⁻, *pps*, *aro-D*, *pro-A*, *arg*. The times plotted on the abscissa are the intervals (min) between mixing the mating cultures and the mechanical interruption of mating.

A similar conclusion was reached when the Hfr strain AT 2571, which transfers its genome in the direction *try- aro-D- his* (as indicated in figure 2), was used as genetic donor. The *aro-D* allele was transferred about 25 min after the beginning of the experiment, and the *pps* allele entered shortly thereafter: as shown in figure 7, the rates of entry of these two alleles also suggested that they were located on the chromosome of *E. coli*, K 12 in close proximity. As shown also in table 3, recombinants which grew on lactate indeed contained *PEP*-synthase activity.

Phage transduction of the aro-D *and* pps *alleles*

The virtual coincidence in the time of entry of the *pps* and *aro-D* markers suggested that these alleles might be sufficiently closely linked to be co-transducible by an appropriate phage. This was confirmed as follows.

The recipient organism K 6-1 (*pps*, *aro-D*) which had been grown on Oxoid nutrient broth at 37 °C was suspended at a density of about 2×10^9 cells/ml. in a

buffer at pH 7·5, containing 50 mM-sodium potassium phosphate and 1 mM-calcium chloride. Infective lysate, prepared as described in the Methods section, was added at a multiplicity of 0·5, 0·75 and 1·0 phage particles/bacterium. The mixtures were incubated at 37 °C for 30 min without agitation and were centrifuged at 20000 *g* for 5 min at 15 °C. The resultant pellet was suspended in a buffer at pH 7·5, containing 50 mM-sodium potassium phosphate and 0·5 % (w/v) sodium citrate. Samples of 0·1 ml. were plated on selective media (either containing

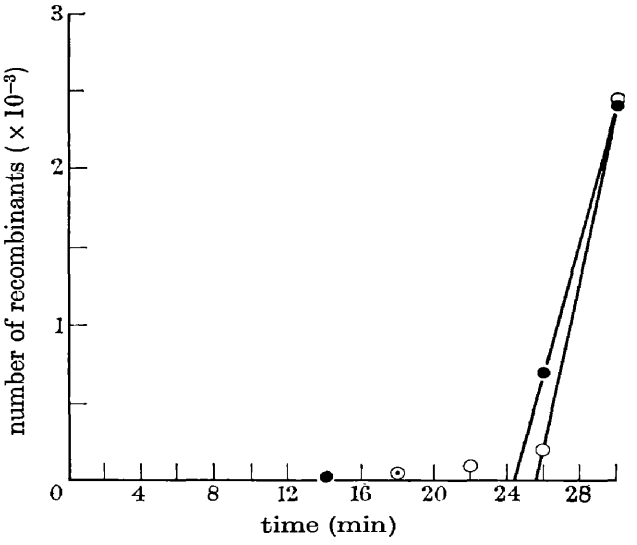

FIGURE 7. Kinetics of transfer at 37 °C of the *pps* (○) and *aro-D* (●) alleles from AT2571 to K6-1. The genotype of the organisms, and the procedures, used were similar to those recorded in the legend to figure 6.

lactate or pyruvate as carbon source and supplemented with shikimate, or containing glucose as carbon source but no shikimate). The plates were examined after 48 h at 37 °C and the number of colonies on them was recorded. The colonies on each plate were then replica-plated on the alternative medium and on the identical medium; where growth on both media occurred, both the normal alleles of the *pps* and *aro-D* genes had presumably been introduced. This conclusion was confirmed by the growth of a number of such presumed co-transductants on liquid media containing lactate but no shikimate, and assay of the *PEP*-synthase activity of extracts of the organisms thus grown. As shown in table 3, the normal *pps* allele had indeed been introduced by the phage.

Of a total of 1959 colonies examined which had received the normal *aro-D* allele, 1603 (82 %) were found also to have received the normal *pps* gene. Likewise, of 1454 colonies recognized as having regained the gene specifying *PEP*-synthase activity, 1380 (95 %) were found also to have lost their requirement for shikimate. Hence of a total of 3413 colonies transduced, 2983 had received both *pps* and *aro-D* alleles. This high degree of linkage supports the view that the two genetic markers are located on the genome of *E. coli* in close proximity.

Discussion

The results reported in this paper were obtained with four independently isolated *pps*-mutants of *Escherichia coli*, K 12. Two of these (K1-1 and AT2572-1) were mutants of Hfr strains, and gave rise to F⁻ *pps*-recombinants K2-1t and K6-1 respectively; the other two (designated K2-1A-5B and K2-1C-5B) had been induced in F⁻ mutants devoid of isocitrate lyase activity. Although some of these mutants had been selected for inability to grow on lactate as carbon source, and others for inability to utilize either pyruvate or alanine, their phenotype was the same. All grew readily on glucose, glycerol or succinate; those not devoid of isocitrate lyase activity also grew readily on acetate. However, none grew on media containing alanine, lactate or pyruvate as carbon source, and ultrasonic extracts of all these mutants were devoid of *PEP*-synthase activity.

By genetic mapping, the *pps* gene was shown to lie within the same segment of the bacterial genome of all these mutants. Although the more precise location, achieved by the phage-mediated co-transduction of the *pps* and *aro-D* markers, applies strictly only to the K6-1 mutant bearing both these alleles, there is no evidence that more than one gene specifies *PEP*-synthase activity.

These results confirm the physiological role postulated by Cooper & Kornberg (1965, 1967) for *PEP*-synthase in the growth of *Escherichia coli* on C_3-acids, and locate the gene which specifies the synthesis of this enzyme on the bacterial genome.

We thank the donors of organisms used (listed under Methods) for their generosity. We are particularly grateful to Professor R. H. Pritchard for helpful discussions and advice on genetical procedures. This work was performed during the tenure by C. B. B. of a Studentship of the Science Research Council, and was supported by that Council through Grant B/SR/652.

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PRELIMINARY NOTES

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Location on the chromosome of *Escherichia coli* of a gene specifying phosphopyruvate synthase activity

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Location on the chromosome of *Escherichia coli* of a gene specifying phosphopyruvate synthase activity

When Enterobacteriacae grow upon C_3-acids, such as pyruvate or lactate, as sole carbon source, the net formation of phosphoenolpyruvate necessary for the biosynthesis of cell components, and for the anaplerotic maintenance of the tricarboxylic acid cycle[1], has been shown[2] to be effected *via* the agency of phosphopyruvate synthase, which catalyses Reaction 1.

$$\text{Pyruvate} + \text{ATP} \rightleftharpoons \text{phosphopyruvate} + \text{AMP} + P_i \qquad (1)$$

As part of the evidence for the physiological role of this enzyme, COOPER AND KORNBERG[2] described the isolation of mutants of *Escherichia coli* which, whilst retaining the ability to grow normally on glucose, acetate or utilizable intermediates of the tricarboxylic acid cycle, no longer grew when C_3-acids were supplied as sole carbon source: such mutants also lacked phosphopyruvate synthase activity. It is the purpose of this communication to report the location of a gene specifying this enzyme on the bacterial chromosome.

The derivatives of *E. coli* K12 used in this work are listed in Table I. The points of origin and direction of genome transfer by the Hfr strains used, and the location of appropriate markers on the chromosome, are indicated in Fig. 1.

When the Hfr-H strain K1 was mated with the *pps*$^-$-F$^-$ strain K2-1t, the gene restoring ability to grow on lactate, pyruvate or alanine entered the recipient after 32–33 min of mating, which was about 6–7 min before the entry of the *his*$^+$ marker (Table II). A more precise location of the *pps* gene was achieved by mating three independently obtained F$^-$-*pps*$^-$ mutants with the Hfr strains AT2571 and AT2572, which inject their genome in opposite directions (Fig. 1). Again, the *pps*$^+$ gene entered 6–7 min from the *his*$^+$ marker, which, according to the genetic map published by TAYLOR AND THOMAN[3], suggested that the *pps* gene was situated close to the *aro-D* marker. This suggestion was confirmed in two ways. Interrupted mating of AT2571 and AT2572 with mutant K6-A, which carried both the *aro-D*$^-$ and *pps*$^-$ markers, showed these markers to be virtually indistinguishable by measurement of time of entry: the distance separating these genes was found to be less than 1 min (Table II). Transduction of K_6-A with a virulent mutant of bacteriophage P1-*kc* (ref. 5), which had been grown on wild-type *E. coli*, gave rise to transductants selected for *aro-D*$^+$, or *pps*$^+$, or both. When the transductants selected for *aro-D*$^+$ only, and those selected for *pps*$^+$ only, were streaked onto plates containing lactate as carbon source but none of the amino acids required by organisms carrying the *aro-D*$^-$ marker, approx. 82 % of the *aro-D*$^+$ colonies, and approx. 95 % of the *pps*$^+$ colonies, were found to be both *pps*$^+$ and *aro-D*$^+$. It can thus be concluded that a gene specifying phosphopyruvate synthase activity is located on the chromosome in close proximity to the *aro-D* marker.

Abbreviations: *pps*$^-$, lack of phosphopyruvate synthase activity; *icl*$^-$, lack of isocitrate lyase activity (*cf.* ref. 4). Most of the abbreviations used for genetic markers are those listed by TAYLOR AND THOMAN[3].

TABLE I

DERIVATIVES OF *E. coli* K12 USED

Organism	*Mating type*	*Genetic markers*
K1	Hfr	str^S; met^-; thy^-
AT2571	Hfr	str^S; thi^-
AT2572	Hfr	str^S; thi^-
K2-1t	F$^-$	str^R; thr^-; leu^-; his^-; arg^-; pps^-
K2-1A-5B	F$^-$	str^R; try^-; his^-; arg^-; icl^-; pps^-
K2-1C-5B	F$^-$	str^R; try^-; his^-; arg^-; icl^-; pps^-
K6-A	F$^-$	str^R; $pro\text{-}A2^-$; $aro\text{-}D^-$; $arg\text{-}A^-$; thi^-; pps^-

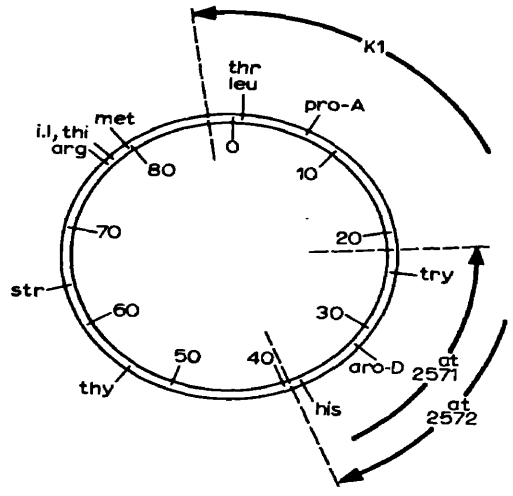

Fig. 1. Genetic map of *E. coli* (adapted from TAYLOR AND THOMAN[3]). The map is numbered (inside the circle) at 10-min intervals and (outside the circle) shows the location of markers referred to in the text. The arrows indicate the point of origin, and direction of genome transfer, of the Hfr strains used.

TABLE II

ENTRY OF GENETIC MARKERS IN INTERRUPTED MATINGS

Hfr strain	*F$^-$ strain*	*Time of entry (min) of*			*Distance (min) between genetic markers*
		pps$^+$	*his$^+$*	*aro-D$^+$*	
K1	K2-1t	32–33	39	—	6–7
AT2572	K2-1A-5B	12–13	6	—	6–7
AT2572	K2-1C-5B	12–13	6	—	6–7
AT2572	K2-1t	12–13	6	—	6–7
AT2571	K2-1t	24–25	31–32	—	6–8
AT2572	K6-A	16	—	16	<1
AT2571	K6-A	25	—	24–25	<1

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SUMMARY

Three independent mutants induced with ethylmethane-sulphonate and selected for their inability to grow on acetate, were shown by enzymic assays in comparison with their wild-type parents and revertants to be devoid of isocitrate lyase. Genetic mapping by interrupted conjugation, recombination analysis and phage transduction showed each of these lesions to lie within the same gene locus situated at 78.3 min on the *E. coli* linkage map in the order *metA*, *icl*, *pgi*. Furthermore, the occurrence of Acetate$^+$ recombinants from intragenic crosses between these mutants demonstrated their heteroallelism and located their sites within the *icl* locus in the order *metA*, *icl-1*, *icl-2*, *icl-3*.

Mutants lacking a second anaplerotic enzyme, PEP-synthase, were also obtained through EMS mutagenesis but were selected for their inability to grow on lactate, pyruvate or alanine. Using the previously described mapping techniques the structural gene specifying this enzyme was shown to lie in close proximity to the *aroD* marker at 32.5 min on the *E. coli* linkage map.

These latter mutants were then employed in the isolation of spontaneously occurring *icl* regulatory mutants (*iclR*$^-$), which, by their constitutive production of isocitrate lyase, permitted the growth of these strains on lactate but neither on pyruvate nor acetate plus pyruvate. By interrupted mating and three-point

transduction, the *iclR*$^-$ lesions within two such independent mutants were mapped and shown to lie adjacent to the *icl* structural gene in the order *metA*, *icl*, *iclR*. Furthermore, "cis-trans" tests employing a merodiploid of this region demonstrated the trans-dominance of *iclR*$^+$ over *iclR*$^-$ and showed this lesion to mark the site of an *icl* regulator locus.

These results, together with the location of the glyoxylate cycle malate synthase (*masA*) in the position *masA*, *icl*, *iclR* (Vanderwinkel & de Vlieghere, 1968) and the demonstration of the co-ordinate formation (Kornberg, 1961, 1966) and de-repression (Vanderwinkel, Liard, Ramos & Wiame, 1963) of these enzymes provide both structural and biochemical evidence for the occurrence of a glyoxylate cycle operon.

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