The Isolation by Insertion Mutagenesis of Auxotrophs of *Escherichia coli* K-12 requiring L-Serine or Glycine.

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DINDIAL RAMOTAR.

ABSTRACT

The Isolation by Insertion Mutagenesis of Auxotrophs of *Escherichia coli* K-12 requiring L-Serine or Glycine.

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Five independent L-serine-requiring auxotrophs were isolated by insertion of Mu : dlac : amp or a derivative thereof into *E.coli* K-12. These mutants have been shown to carry single insertions, all of which map in or near serA at 52 minutes. All these auxotrophs are deficient in the enzyme coded by serA, phosphoglycerate dehydrogenase, the first enzyme of the phosphorylated pathway. Other enzymes of the pathway have not been assayed.

The level of ß. galactosidase synthesized from the lacZ gene of the insertion varies greatly in the five mutants, from a low of 2 units to a high of 2000. This may indicate that the insertions are not all in the same gene.

These mutants have been used to show that the enzyme activity L-serine deaminase (L-SD) is responsible for the in vivo degradation of L-serine. Serine auxotrophs deficient in L-SD require less L-serine per cell mass synthesized; auxotrophs in which L-SD is induced require more L-serine.

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INTRODUCTION:

The intent of this work was to isolate L-serine auxotrophs from *Escherichia coli* K-12 by insertion mutagenesis and to address the following questions:

(1) Is the well characterized phosphorylated pathway (58, 82), the only pathway of L-serine biosynthesis in *E. coli* K-12 ?.

(2) How is the phosphorylated pathway to L-serine regulated ?.

(3) To what extent is L-serine deaminated in glucose-grown *E. coli* K-12?.

In the introduction, five known pathways of L-serine biosynthesis described in microorganisms will be reviewed. In two of these pathways, L-serine is derived from intermediates of glycolysis via either phosphorylated or nonphosphorylated intermediates. In the third it is made from the amino acid threonine. In the fourth pathway L-serine is derived from tricarboxylic acid cycle intermediates. The final pathway begins with one carbon compounds.

What is known about the regulation of the phosphorylated pathway to L-serine in *E. coli* K-12 will be considered next. Then finally some of the characteristics of the enzyme L-serine deaminase (L-SD) will be discussed in relation to L-serine metabolism.

THE PHOSPHORYLATED PATHWAY:

Although considerable information was obtained on the uptake and metabolism of L-serine in *E. coli* K-12 (36) by 1962, the principal biosynthetic pathway to L-serine in *E. coli* K-12,

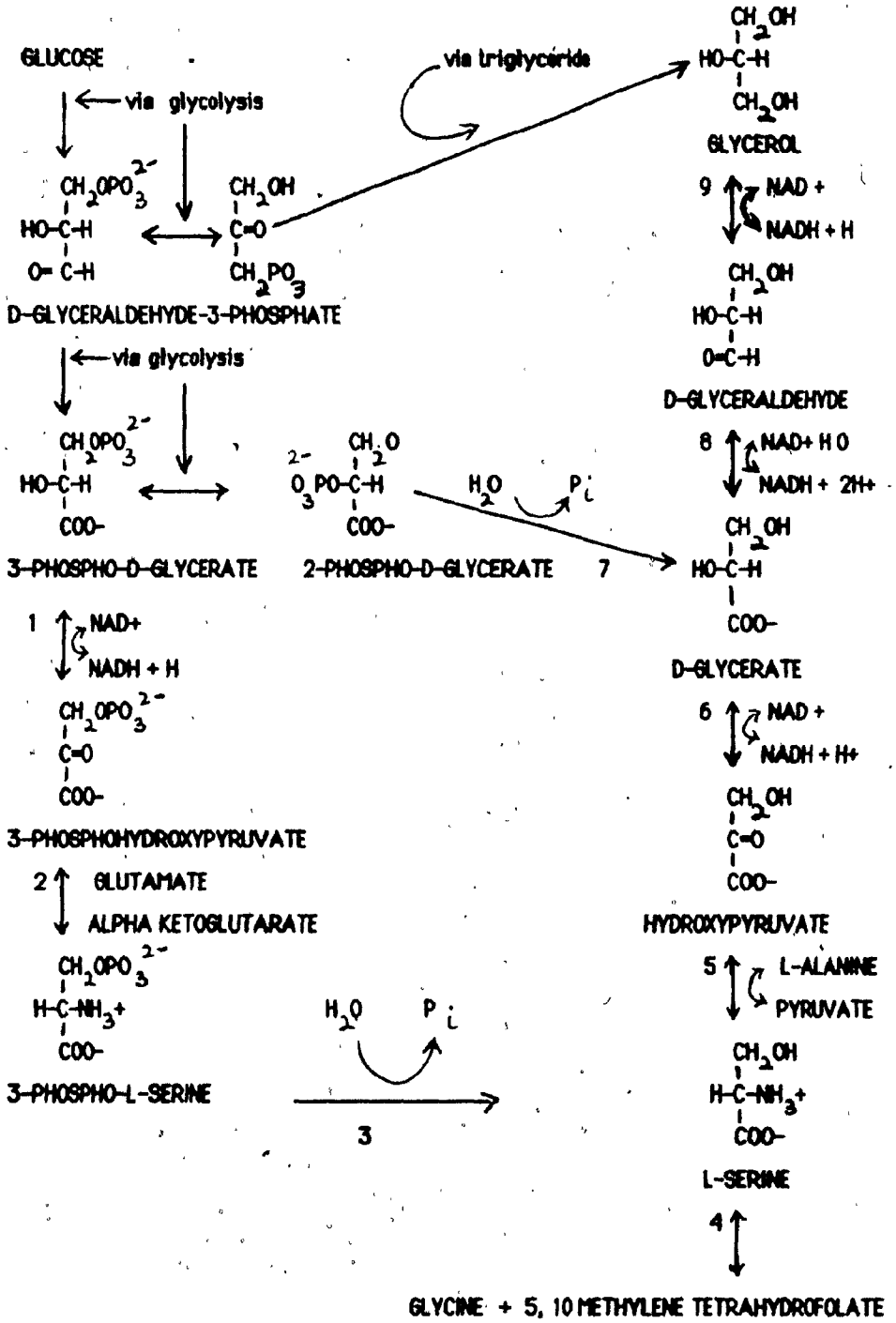
the phosphorylated pathway, which begins with the glycolytic intermediate 3-phosphoglycerate was established by Pizer in 1963 (58) and by Umbarger, Umbarger and Siu, 1963 (82).

The phosphorylated pathway was first shown to exist in mammalian liver (28). It involves the following set of enzyme reactions: 3-phosphoglycerate, an intermediate of the glycolytic pathway, is oxidized by phosphoglycerate dehydrogenase to phosphohydroxypyruvate, which is transaminated by phosphoserine transaminase to phosphoserine using glutamate as a nitrogen source. Finally phosphoserine is dephosphorylated to L-serine by phosphoserine phosphatase (Fig. 1). Microorganisms such as *Salmonella typhimurium*, (83) and *Haemophilus influenzae* (61) also contain enzymes of the phosphorylated pathway.

THE NONPHOSPHORYLATED PATHWAY:

Sallach (71), and Willis and Sallach (86) described the synthesis of L-serine in beef liver via another pathway, namely the nonphosphorylated pathway. This pathway involves the dephosphorylation of the glycolytic intermediates 3- or 2-phosphoglycerate to glycerate. This is followed by a dehydrogenation step to hydroxypyruvate by glycerate dehydrogenase and transamination of hydroxypyruvate by serine-pyruvate transaminase to L-serine. Alanine is used as the nitrogen donor (Fig. 1). This nonphosphorylated pathway to L-serine was also shown in *Acetobacter suboxydans* (68) and in *Pseudomonas* AM1 (34).

FIG. 1: PHOSPHORYLATED AND NONPHOSPHORYLATED PATHWAY TO L-SERINE.



1, 3-phosphoglycerate dehydrogenase; 2, glutamate-phosphoserine transaminase; 3, phosphoserine phosphatase; 4, serinehydroxymethyl transferse; 5, alanine-serine transaminase; 6, D-glycerate dehydrogenase; 7, 2- phosphoglycerate phosphatase; 8, D-glyceraldehyde dehydrogenase; 9, glycerol-3-dehydrogenase

L-SERINE FROM THREONINE:

Dainty et. al., 1970 established that *Clostridium pasteurianum* derives its glycine principally through the cleavage of threonine to acetaldehyde and glycine (Fig. 2). Radioactive tracer experiments showed that all the carbons of glycine were derived from exogenous aspartate or threonine. In the presence of exogenous L-serine, only about half the glycine carbon was derived from radioactive threonine. Dainty et. al. interpreted this observation as indicating that half the glycine molecules were derived from serine by the action of serinehydroxymethyl transferase (SHMT) and the other half from threonine. This interpretation was also based on the fact that threonine aldolase, which cleaves threonine to glycine and acetaldehyde, was found in cells grown on glucose with or without supplements of threonine, L-serine and glycine. These authors suggested that in this organism, *C. pasteurianum*, the sole route of glycine formation from glucose is by way of threonine. In *E. coli*, this is a minor pathway as suggested by Dainty et. al., (17). However, Fraser and Newman (23) showed that *E. coli* K-12 altered by several mutations could use either exogenously supplied or endogenously synthesized threonine as a precusor of glycine. The glycine formed could be readily converted to L-serine by the SHMT enzyme.

L-SERINE-GLYCINE FROM ISOCITRATE:

Biosynthesis of L-serine in *Saccharomyces* involved at least two pathways (67). One was via the phosphorylated pathway, mentioned above. In the second, L-serine was derived from a tricarboxylic acid cycle intermediate, isocitrate, via conversion to glyoxylate and glycine (67).

FIG. 2: SERINE FROM THREONINE IN C. PASTEURIANIUM

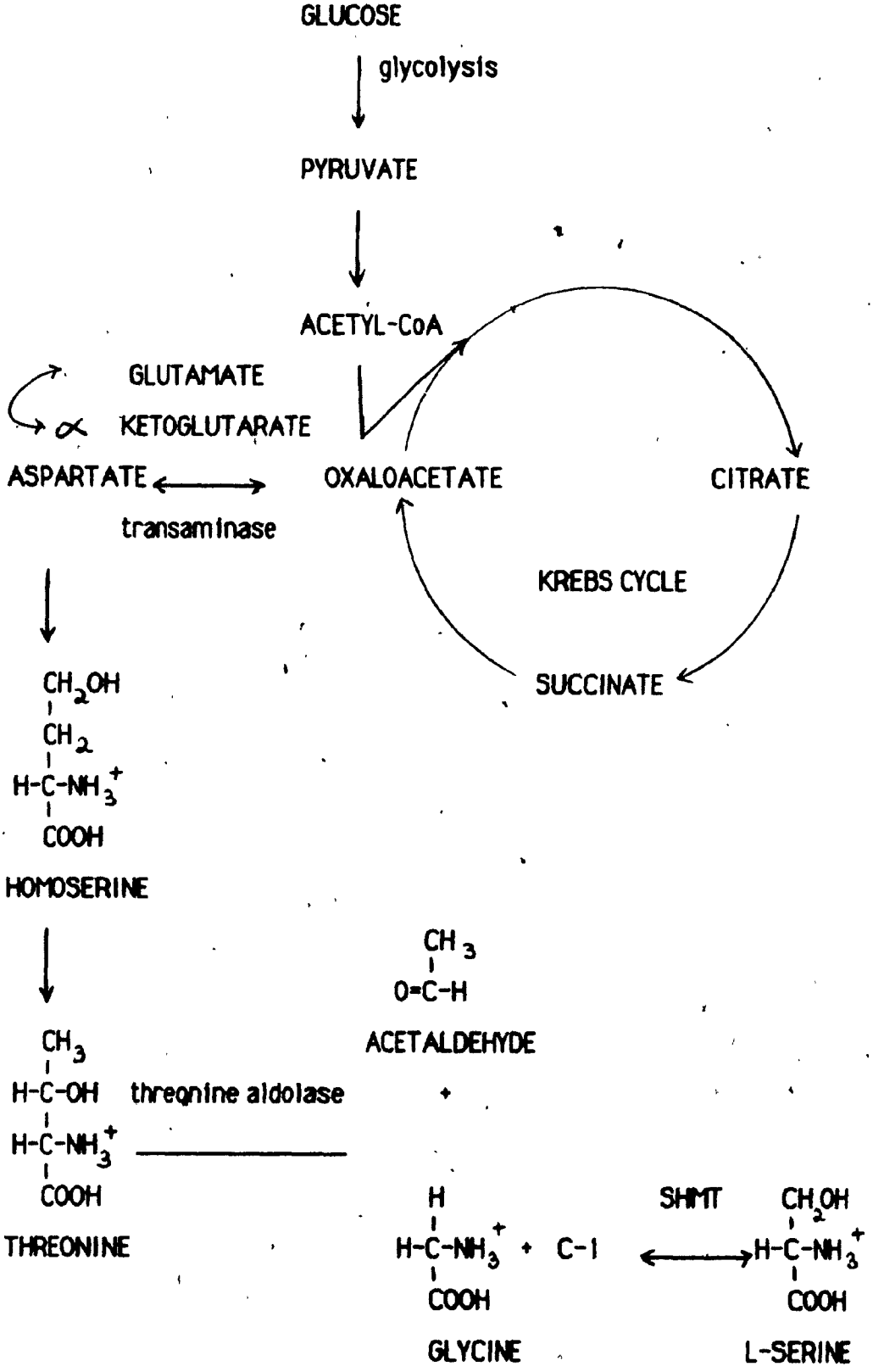
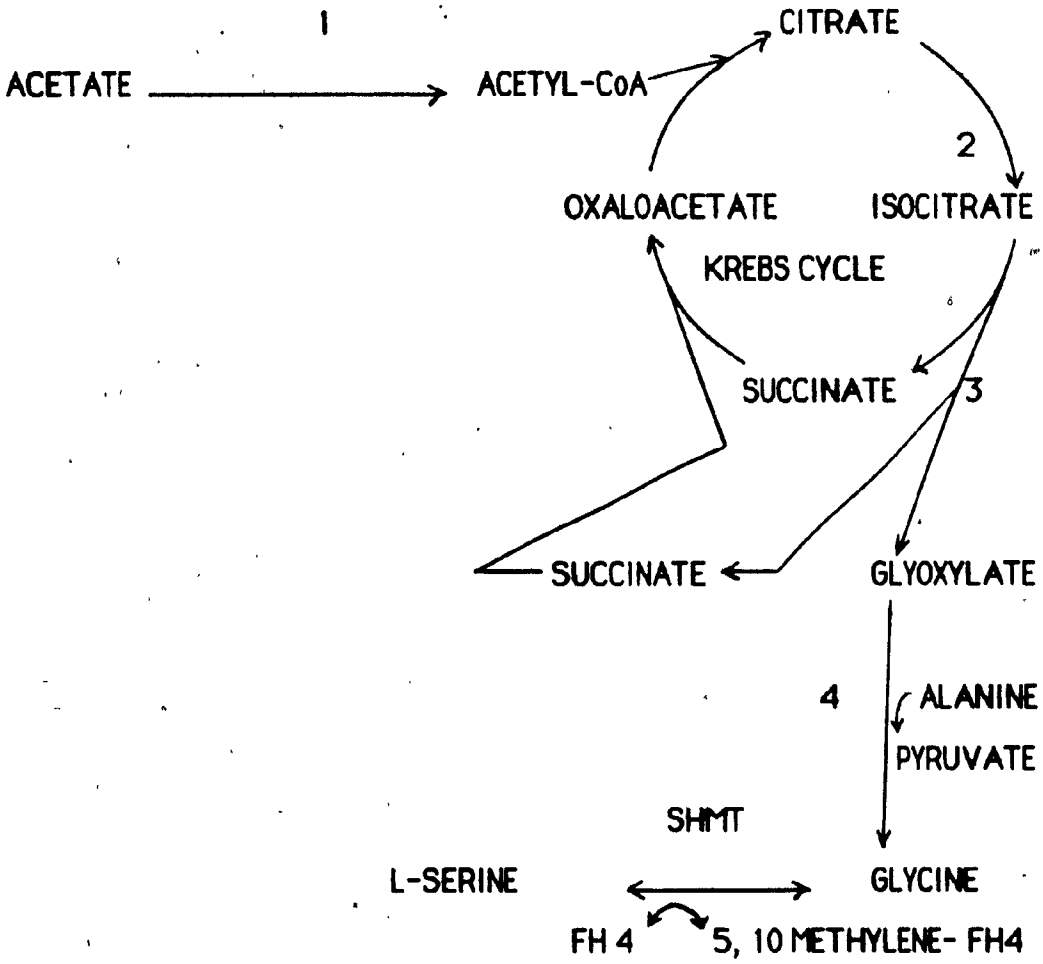
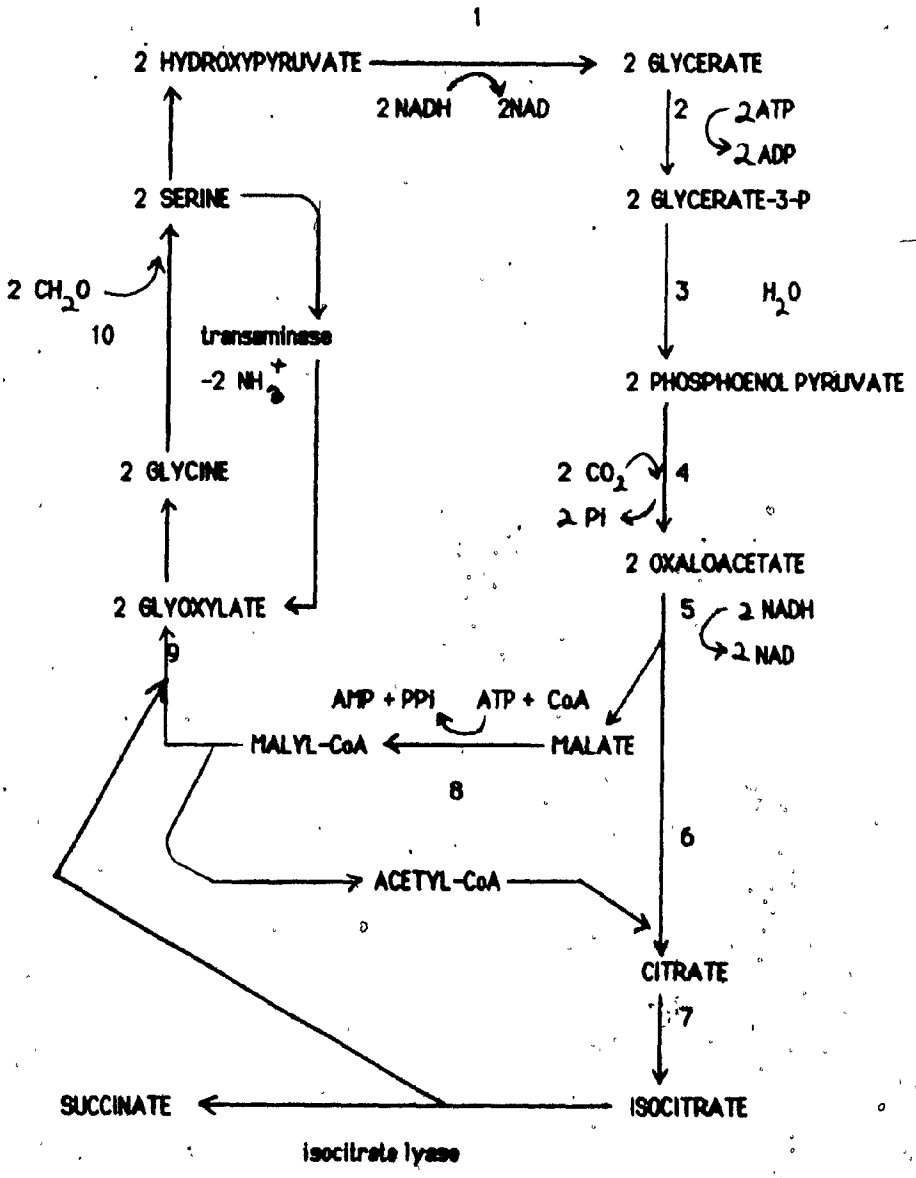


FIG. 3: SERINE AND GLYCINE FROM ISOCITRATE LYASE PATHWAY IN *SACCHAROMYCES*



1, acetyl-CoA synthetase; 2, cis-aconitase; 3, isocitrate lyase;
4, transaminase

FIG. 4: THE SERINE AND ISOCITRATE LYASE PATHWAYS OF C-1 UTILIZERS.



1, hydroxypyruvate reductase; 2, glycerate kinase; 3, mutase and enolase; 4, PEP carboxylase; 5, malate dehydrogenase; 6, citrate synthetase; 7, cis aconitase; 8, malyl-CoA synthetase; 9, malyl-CoA lyase; 10, serinehydroxymethyl transferase.

isocitrate was cleaved by isocitrate lyase to form succinate and glyoxylate. The glyoxylate was subsequently transaminated by L-alanine-glyoxylate transaminase to glycine (Fig. 3). The synthesis of these enzymes, isocitrate lyase and L-alanine-glyoxylate transaminase, was repressed by growth in glucose media. Thus glucose-grown *Saccharomyces* would posses only the phosphorylated pathway to make L-serine, whereas acetate-grown cells would posses both that and the glyoxylate pathway from the tricarboxylic acid cycle. There was no evidence for the existence of the nonphosphorylated pathway in these yeast strains (67).

The derivation of L-serine and glycine from isocitrate via glyoxylate has also been described in *Methylosinus* and *Methylocystis* and a number of facultative C-1 utilizers, which grow on formaldehyde (25). In addition, these C-1 users, when grown on one carbon compounds, can also derive their L-serine or glycine from malate by the L-serine pathway (Fig. 4). In this pathway, malate is converted to malyl-CoA by the enzyme malyl-CoA synthetase. Malyl-CoA subsequently forms glyoxylate catalyzed by malyl-CoA lyase. Glyoxylate is then transaminated to glycine (Fig. 4). Thus C-1 utilizers can make serine and glycine via two routes. The serine pathway by itself is insufficient to supply the cell with oxaloacetate or phosphoenol pyruvate for biosynthetic purposes. This is achieved when the L-serine pathway is combined with the isocitrate lyase pathway, which is the reason for two pathways to L-serine or glycine in these microorganisms (25). In essence, this isocitrate lyase pathway accomplishes the net formation of succinate from carbon dioxide and formaldehyde (Fig. 4). Succinate can form oxaloacetate and thence to phosphoenol pyruvate.

PATHWAYS TO L-SERINE USED BY *E. coli*

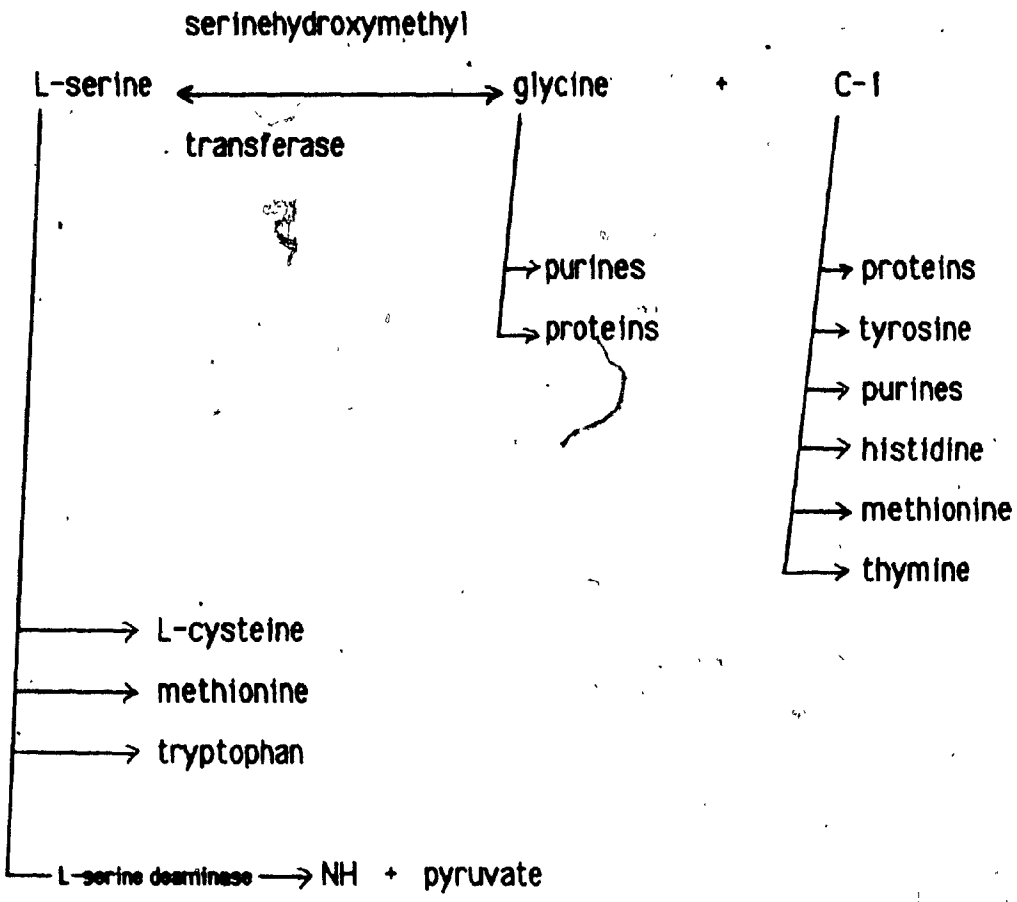
E. coli is considered to have only one pathway of L-serine biosynthesis because auxotrophs requiring L-serine can be isolated in a single step. Thus mutants in which the phosphorylated pathway is interrupted by mutation cannot make L-serine and therefore must not have a functional second (e.g. non-phosphorylated) pathway.

In acetate media, *E. coli* K-12 has the enzyme, isocitrate lyase, that results in glyoxylate formation. However, the glyoxylate is not converted to glycine. Instead malate synthetase catalyzes the condensation of acetyl-CoA with the glyoxylate to yield malate- a reaction analogous to the citrate synthetase reaction. Since *E. coli* K-12 has no direct way to synthesize 3-carbon compounds from acetate such as phosphoenolpyruvate (PEP), it thus employs the two anaplerotic enzymes, isocitrate lyase and malate synthetase, to form malate. Malate will eventually form PEP via malate deyhdrogenase and PEP carboxykinase. There is no evidence that *E. coli* can use this glyoxylate cycle to make glycine.

SOURCE OF GLYCINE AND C-1 UNITS.

L-serine may be converted as an intact molecule to cysteine, tryptophan, and methionine (Fig. 5). L-serine is also incorporated into phospholipids. When it is cleaved by SHMT, L-serine is used in the synthesis of glycine and C-1 units. C-1 units are essential for the synthesis of purines, tyrosine, histidine, methionine, thymine, and N-formylmethionine (the starting amino acid in protein synthesis in bacteria). C-1 units can also be made from the cleavage of glycine by the glycine cleavage enzyme complex (62).

FIG. 5: FATE OF L-SERINE, GLYCINE AND C-1 UNITS IN *E. coli*



In this cleavage, glycine is decarboxylated, deaminated and C-1 unit is released.

Glycine can be made by several reactions; the cleavage of exogenously supplied threonine in *E. coli* K-12 (23); the breakdown of purine (64) and the amination of glyoxylate (43). In *E. coli* K-12 the major pathway for glycine biosynthesis is via L-serine. This conversion of L-serine to glycine was first demonstrated by Shemin (1946), who adminstered serine labeled with ^{15}N in the amino group and ^{13}C in the carboxyl group to rats and guinea pigs (72). The isolated glycine was found to have the same $^{15}N/^{13}C$ ratio as the administered serine indicating that L-serine was converted into glycine by the splitting off of the ß carbon atom. Roberts et. al., (1955) showed with radioactive glucose that neither L-serine nor glycine was labeled in cells grown in the presence of exogenously supplied unlabeled L-serine. Thus glycine is derived from L-serine in *E. coli* K-12 (66).

CONTROL OF L-SERINE BIOSYNTHESIS.

It is generally accepted that the level and activity of enzymes of a biosynthetic pathway vary with the nutritional conditions in which the cells are growing. Thus, if end products of a pathway are present in excess, the cells do not produce de novo the compounds which are already available. This integration of the cell with its environment implies the existence of sensitive mechanisms which control the rate of synthesis of the various molecules. Such controls have been described (31). The two best known control mechanisms are (1) regulation of enzyme synthesis (repression) and (2) regulation of the activity of a given quantity of enzyme (inhibition).

In pathways where these two control mechanism are operating, it is the first enzyme of the pathway that is usually regulated by feedback inhibition. It is possible however that several enzymes concerned with the reaction can have their syntheses repressed. Although many biosynthetic pathways are controlled by both these mechanisms, the L-serine biosynthetic pathway in *E. coli* is apparently only regulated by feedback inhibition. The first enzyme of this pathway, phosphoglycerate dehydrogenase (PGDase), which is well studied, is feedback inhibited about 90 % by low concentrations of L-serine (59, 60, 69, 77, 78, 87).

In cultured human cells, the regulation of L-serine synthesis is somewhat different, in that control is exerted on another enzyme of the pathway. L-serine has no inhibitory effect on the PGDase activity but it inhibits the third enzyme of the pathway, phosphoserine phosphatase (60, 48). The level of phosphoserine phosphatase in cultured human cells was also reported to be repressed in cells grown in the presence of L-serine.

L-serine or glycine, either independently or in combination, do not appear to repress PGDase synthesis in *E. coli* K-12. Thus, the level of PGDase was not altered when an exogenous supply of L-serine was added to glucose-grown cells. However, changing the carbon source used for growth and /or adding to the growth medium amino acids apparently unrelated to L-serine (methionine, isoleucine, threonine and leucine) decreased the amount of enzyme protein (41, 59). Whether the other two enzymes (phosphoserine transaminase and phosphoserine phosphatase) of this pathway are also repressed is not known.

The PGDase level determined on different carbon sources was found to be somewhat related to the generation time i.e. the longer the

generation time, the lower the PGDase level (41). However, there were exceptions i.e. malate and fructose-grown cells had the same generation time but significantly different enzyme levels. Whether it is the transcription or the half-life of the PGDase mRNA that is affected by the growth rate remains unknown.

There is evidence that induction and repression of an enzyme may also be controlled at the translational level, via the amino acyl-tRNA synthetase and possibly the tRNA (70). The addition of L-serine hydroxamate, which competitively inhibits seryl-tRNA synthetase, decreases the amount of charged seryl-tRNA in the cell. However, this lowered amount did not alter the phosphoglycerate dehydrogenase level (41) since PGDase level was neither induced nor repressed. It was concluded that limitation of charged seryl-tRNA exerts no translational control.

It has been reported that L-serine phosphate is an intermediate in the biosynthesis of pyridoxine (19, 20, 73). This may indicate that pyridoxine and L-serine together can cause repression. However, the PGDase enzyme level was the same in extracts from cells grown in minimal media and those grown with an excess of L-serine and pyridoxine. This therefore indicate that in the presence of excess L-serine and pyridoxine, repression of PGDase synthesis does not occur.

CHARACTERISTICS OF L-SERINE DEAMINASE (L-SD) IN *E. coli* K-12.

L-serine deaminase was first described in *E. coli* K-12 by Pardee and Prestidge in 1955. It catalyzes the irreversible conversion of L-serine to pyruvate and ammonia, as assayed in vitro (30, 52).

Although *E. coli* does have a considerable amount of L-SD activity, it has never been shown whether this activity can catalyze a similar conversion in vivo. It is also not known whether L-serine is the only substrate for this activity. Because of its unusual instability, it does not survive purification steps. Thus, one cannot define the range of substrates for this activity.

This enzyme has been extensively characterized in five microbial species (a) *Corynebacterium sp.* (46); (b) *Clostridium acidi-urici* (12); (c) *Arthrobacter globiformis* (24); (d) *Streptomyces rimosus* (79) and (e) *E. coli* K-12 (2, 3, 52).

In *Clostridium acidi-urici*, L-SD is thought to be one of the enzymes of the uric acid fermentation pathway (7). This enzyme is responsible for the utilization of glycine as sole carbon source in *Arthrobacter globiformis* (10) and *Diplococcus glycinophilus* (32). Gluconeogenesis from two-carbon compounds in wheat leaves involves the enzyme L-SD (84). Use of L-SD in photorespiration in plants has also been reported (8). Despite its involvement in other metabolic systems, no obvious physiological role for L-SD in *E. coli* K-12 has been reported.

In microorganisms, this enzyme was reported by many investigators to be quite unstable, whereas L-SD of animal origin is much more stable and can survive purification. An extract of *E. coli* prepared by sonication lost almost 82 % of L-SD activity, when compared to the activity seen in toluene-treated cells. In toluene-treated whole cells, about 60 % of activity was lost in 2 hours at 0 ° C (56). In 1949, Wood and Gunsalus reported a partial purification of L-SD from *E. coli*. During the assay, enzyme activity was totally lost in 5 minutes.

Considerable loss of activity has also been reported in cell free extracts of *S. typhimurium, Bacillus cereus, C. acidi-urici* (7) and *Streptomyces rimosus* (79). Thus the current assay for L-SD in *E. coli* is based on toluenized cells rather than on prepared extracts.

Many inducers of this enzyme were discovered by Newman et. al., (50). These include glycine, leucine, several DNA damaging agents, Luria Broth (rich medium) and a shift in temperature from 37 ° C to 42 ° C. The mechanism by which these inducers act is unknown.

E. coli cannot grow with L-serine as a carbon and energy source (51), but upon induction of L-SD with glycine and leucine, which results in an increase in L-SD activity, growth on L-serine is possible. Mutants without L-SD activity cannot grow on L-serine, glycine and leucine (SGL) media (Newman, in press) but mutants with higher L-SD activity than parent *E. coli* were able to grow with L-serine as a sole carbon and energy source (50). Thus it seems that the failure to grow on L-serine alone was attributed to the insufficient L-SD activity in the uninduced cells. Although serine auxotrophs of *E. coli* K-12 cannot grow on L-serine, in this work they are shown to deaminate L-serine, and to use L-serine deaminase for this reaction.

MATERIALS AND METHODS.

MEDIA:

Strains were grown in a liquid minimal medium containing 0.54 % K_2HPO_4; 1.26 % & KH_2PO_4; 0.2 % $(NH_4)_2SO_4$; 0.2 % $MgSO_4.7H_2O$ and 0.001 % $CaCl_2$ at pH 7.0. All solutions were autoclaved at 15 lbs/in for 20 minutes. The carbon and energy source (glucose) was autoclaved separately and added to a final concentration of 2.0 mg/ml. Other supplements to the growth medium were filter sterilized and were added according to the requirements of the experiment as outlined in the text.

Cultures of all strains were kept by frequent transfer on Luria Broth plates.

Plating experiments were done on minimal medium agar plates consisting of the basic medium described above and 2% agar. Other additions were made according to the requirements of the particular experiments.

MEASUREMENT OF CELL GROWTH.

Growth of cultures was followed in a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Co. Inc, New York, N.Y. Model 800-3) using a number 42 filter. Late exponential phase cultures were subcultured into 250 milliliter Erlenmeyer flasks, fitted with sidearm and containing 20 mls of medium, to an optical density of 20 Klett units. Supplements were added according to the experiment as outlined in the text. Flasks were incubated at 37 ° C on a New Brunswick rotary shaker model G 76 at approximately 180 r.p.m.

Growth was defined as an increase in the turbidity of the culture and is expressed in terms of the apparent doubling time which is the time required for the turbidity to double.

ENZYME ASSAYS.

ß. galactosidase:

This enzyme was assayed by the method described by Miller (45), but using his alternative version: (a) 2 drops of chloroform and 1 drop of 1 % sodium dodecyl sulfate were added instead of the one drop of toluene used (b) After stopping the reaction, the tubes were centrifuged by a desk top centrifuge for 5 minutes and read at 420 nanometers. This centrifugation step eliminates the need to take a second reading at 550 nanometers to account for the cell debris.

Phosphoglycerate dehydrogenase:

Phosphoglycerate dehydrogenase (PGDase) was assayed by the method of McKitrick and Pizer (41) somewhat modified. It uses phosphohydroxypyruvate (HPP) as substrate. Cells were grown in a 1 liter flask containing 200ml of minimal medium with supplements as described in the text. Cells were harvested by centrifugation at 6,000 r.p.m for 10 minutes, pellet was resuspended once with 50 mM potassium phosphate buffer (pH 7.5) and recentrifuged, and the wet weight of the resulting washed pellet was determined. The washed pellet was then frozen in liquid nitrogen for 20 seconds to weakened the cell structure, and it was then resuspended in 5 mls of a buffer (pH 7.5) containing 50 mM potassium phosphate, 1 mM EDTA;
2 mM dithiothreiotol (DTT) and 20 uM L-serine per gram of the original wet weight of the cells.

The fractured cells were sonicated for 10 seconds and allowed to cool in a ice-waterbath for 20-30 seconds. This sonication and cooling step was repeated for another 4-5 times so that the total sonication time is 50-60 seconds. This mild sonication step released PGDase entirely and at the same time it also releases some NADH oxidase into the extract. The extract was then heated at 52 º C for exactly 10 minutes and immediately cooled in an ice-waterbath. This heating step inactivated the NADH oxidase while the activity of PGDase was protected with L-serine in the extract. The extract was centrifuged at 10,000 r.p.m for 25 minutes to remove cell debris. The supernatant 1 was treated with 2 ml of 5 % streptomycin sulfate per gram of original wet weight of the cell with continuous stirring on an ice bath for 15 minutes. The streptomycin treatment removes the nucleic acid. The extract was centrifuged at 8,000 r.p.m for 15 minutes and the supernatant 2 protein was precipitated with 75 % ammonium sulfate $(NH_4)_2SO_4$ by continuous stirring for 45 minutes on ice. The $(NH_4)_2SO_4$ precipitate was centrifuged at 8,000 r.p.m for 15 minutes. The resultant pellet was resuspended in 50 mM potassium phosphate buffer pH 7.5 containing 1 mM EDTA and 2 mM DTT at a density of 1 gram of the original wet weight of the cells per ml.

The assay mixture contained 50 µmoles of Tris-hydrochloride (pH 8.5), 5 µmoles of EDTA, 5 µmoles of DTT, 20 µmoles of Na_2SO_4, 0.1 µmole of NADH and 0.18 µmole of hydroxypyruvic acid phosphate in a total volume of 1 ml.

To start the assay 100 µl of the fractionated extract was added to the assay mixture and the oxidation of NADH was followed at 340 nanometers. The activity of NADH oxidase was determined by

adding 1.9 µmoles of L-serine to the reaction mixture which completely inhibited the PGDase enzyme activity. Thus any further oxidation of NADH was due to the NADH oxidase.

The difference between the inhibited and uninhibited reaction rates was taken to be the activity of the PGDase enzyme. One unit of enzyme activity is defined as the nmole of NADH oxidized per minute at 25 º C. (The modifications that were employed in this assay are the fractionation steps i.e. the precipitation of supernatant 1 with 5 % streptomycin sulfate and supernatant 2 with 75 % $(NH_4)_2SO_4$. Both steps are described in the procedure. In McKitrick's assay supernatant 1 was used directly to assay for PGDase activity).

L-serine deaminase:

.The L-serine deaminase assay is based on the production of pyruvate according to the method of Pardee and Prestidge (56) modified by Isenberg and Newman (30).

PLATE LYSATES

Growth of phage by plate lysates:

All bacteriophage used in this work were grown by the plate lysate method described in Miller (45). Cells were inoculated into 5 ml of Luria Broth (L.B) containing 0.15 ml of 1 % $CaCl_2$ (Ca) and incubated at 37 º C. Overnight grown cells were subcultured by adding 2 drops into fresh L. B Ca (5 ml) and allow to grow at 37 º C until they were in the exponential phase. 0.2 ml of the exponentially growing cells were added to 0.1 ml of P1vir (diluted to approximately 10^6 phage per ml) into small test tubes and allowed to incubate at 37 º C for 20 minutes.

2.5 ml of *R-top agar (kept at 45 º C) were added to each tube and immediately plated on L. B Ca glucose plates. Plates were harvested 24 hours later by adding 2.5 ml L. B Ca on to each plate with swirling at every half hour over the next 3 hours. The liquid portion of each plate was collected in small test tubes containing 0.5 ml chloroform ($CHCl_3$). Tubes were vortexed vigorously for 30 seconds, left at room temperature for 10 minutes and then centrifuged to precipitate the cell debris. The supernatant, which contained the phage, was collected and stored at 0-4 º C in tubes containing 0.5 ml $CHCl_3$.

GENERALIZED TRANSDUCTION MEDIATED BY PHAGE P1

Bacterial strains grown in 5 ml of L. B at 37 º C in small test tubes were centrifuged. The pellet was resuspended in 5 ml of M C buffer (0.1 M $MgSO_4$; 0.005 M $CaCl_2$) and starved for 10 minutes by placing them on a rotor at 30 º C. 0.1 ml of the cells were added to 0.1 ml of various dilutions of the particular phage to be used. Controls contained no phage. The tubes were incubated at 30 º C for 30-45 minutes. 0.2 ml of 1 M sodium citrate was added to each tube to prevent further adsorption of the P1 phage by removing Ca^{2+} ions. 3 ml of @F-top agar kept at 45 º C were added to each tube. The contents of the tube were then plated immediately and incubated at 37 º C or in some experiments at 30 º C, until colonies appeared. Plates used for selection and screening were described in the text.

*R-top agar contained the following in 1 liter of water:- 10 grams of bactotryptone, 1 gram of yeast extract, 8 grams of sodium chloride (NaCl), 8 grams of Bacto-agar, 220 mg of $CaCl_2$ and 1gram of glucose. @F-top agar was made up with the following ingredients in 1 liter of water:- 12 grams of Bacto-agar and 8 grams of NaCl.

Mu INFECTION:

E. coli K-12 were grown in L. B medium containing 10 mM $MgSO_4$ and 5 mM $CaCl_2$. Cells were grown to early stationary phase (approximately 10^9 cells per ml) and adsorbed for 20 minutes at 28-30 ° C with lysates of MH 3821 or CAG 5050 (see Table 1 and text for MH 3821 and CAG 5050 descriptions) that had been diluted to less than 1 phage per cell. To allow expression of the ampicillin (Ap^r) gene carried by Mu, the infected cells were diluted 1:10 with L. B medium and grown at 28-30 °C for a further 30 minutes. Ap^r transductants were selected by plating on L. B Ap plates.

GROWTH YIELD EXPERIMENT:

Overnight grown cells were diluted in saline and 0.1 ml of this diluted culture was added to each flask so as to transfer approximately 100 cells per flask. Flasks were placed on a New Brunswick shaker at 37 or 42 ° C as required by the experiment. Each flask contain 20 ml of glucose minimal medium with further additions as described in the text. All flasks contained 50 ug/ml ampicillin with or without 20 ug/ml of chloramphenicol depending on the strains. The supplements, glycine or L-serine, were provided at a concentration within the range that limits growth. Cultures were monitored until their turbidity was constant for at least 3 hours. Protein was harvested from these cultures by trichloroacetic acid precipitation (T.C.A). The T.C.A precipitates were redissolved in 0.1 N NaOH and the protein content was determined by the method of Lowry et. al. (37).

STRUCTURE OF Mu BACTERIOPHAGE:

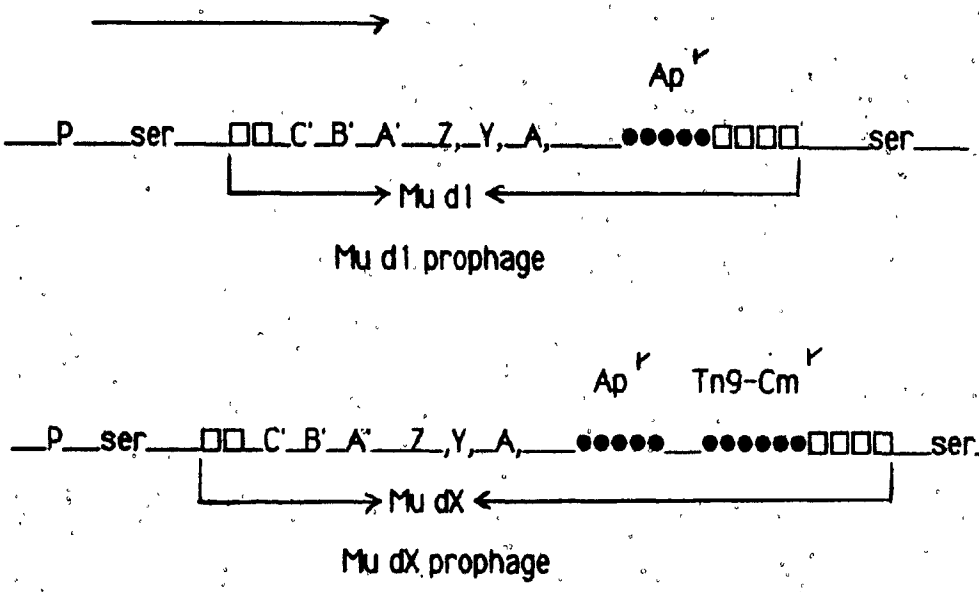
The structure of the bacteriophage derivative Mu d1 (13, 15, 27) is shown in Diagram 1. It was constructed by Casadaban with the following features that facilitate construction of operon fusions:

(1) Phage Mu d1, like phage Mu itself (27), is a translocatable element that can integrate into apparently random sites in *Escherichia coli (E. Coli)* DNA and hence is a nonspecific mutator.

(2) This phage also carries a gene encoding resistance to ampicillin, which facilitates its selection. Mu dX, a derivative of Mu d1, was constructed by Baker et. al.,(4) by the insertion of Tn 9 (chloramphenicol-resistant, cm^r) into the B gene of Mu d1 genome. This Tn 9 insertion in the B gene results in a more stable Mu phage (Mu dX) i.e one with reduced frequency of transposition.

(3) Both Mu d1 and Mu dX carry the structural genes of the lactose operon, but not the promoter, and therefore expression of the lac genes occurs only when the prophage is located in the proper orientation in a transcriptionally active gene. Thus, the level of ß. galactosidase enzyme made by the lacZ gene of Mu d1 or Mu dX can be used as an assay for transcription of the gene carrying the insertion, and for functional and mutational studies of gene regulation.

DIAGRAM 1: SCHEMATIC REPRESENTATION OF Mu d1 AND Mu dX.



____ : *E. coli* DNA segment into which Mu d1 or Mu dX has been inserted.

□□□ : ends of Mu sequence.

●●●● : antibiotic resistance genes in the Mu sequence.

___ : direction of transcription

P : Promoter

C',B'A' : Mu genes

Z,Y,A : lac operon structural genes.

ser : serine biosynthetic gene(s).

This diagram was consolidated from the schematic representations of Mu d1 and Mu dX given by Casadaban and Cohen (13) and by Baker et al. (4) respectively

Results:

The experiments described here involve the isolation by insertion mutagenesis of serine auxotrophs and their physiological and genetic characterization. Serine auxotrophs have been isolated frequently in the past (18-20, 62, 82), and the pathway of L-serine biosynthesis is thought to be reasonably well understood (29, 58, 74, 82). However, certain findings showed that mutational block in the pathway does not give rise to serine auxotroph (53, 73), thus these findings fit less well with the currently accepted biosynthetic pattern which claims the existence of a single biosynthetic route to L-serine in *Escherichia coli*. Therefore it seemed interesting to reinvestigate this problem using insertion mutagenesis whereby the lacZ gene could be put under the control of the serine promoter (s). To do this, the specially constructed insertion sequences Mu dI and Mu dX (described in materials and methods) were used to transduce the parental *Escherichia coli* K-12 strain Cu1008Δlac to antibiotic resistance selecting on plates with ampicillin, and chloramphenicol where appropriate. Single colonies were picked and streaked onto glucose minimal medium (GMM) plates with and without L-serine. Antibiotic-resistant strains which grew on glucose minimal medium plates supplemented with L-serine, but not on unsupplemented media were selected for further study (Table 1).

Characteristics of serine auxotrophs.

A total of six independent auxotrophs were isolated from separate transduction experiments. These six strains carried the following designations DR-4 ; DR-1; DR-2; DR-3; DR-5; and DR-6 (Table 1).

TABLE 1: BACTERIA *E. coli* K-12 STRAINS.

organism	Relevant genotype and comments	source or ref.
Cu1008	ilvA451 prototroph	M. Levinthal
Cu1008Δlac	lac deletion of strain Cu1008	This lab
MH 3821	F- his lys trp malA tsx lac gal rpsL Mu dI(lac Ap^r)	M. Howe
CAG 5050	Mu d X CAM(lac Ap^r) Δ pro lac his met tyr rpsL nal /F' pro lacZ 8305: :Mu cts 62	M. Howe
DR-2, 3, 5, 6	serine requiring mutants. Obtained by the insertion of Mu dI into Cu1008Δlac	This work
DR-1, 4	serine requiring mutants. Obtained by the insertion of Mu dX into Cu1008Δlac	This work
128	thiamine requiring, L-SD deficient	This lab
5198	fda-2, relA1, tonA22, T2 , pit-10, spoT1 (fda-2 is a temperature-sensitive mutation)	●
1321	proA2, aroA2, his-4, thi-1, lacY1, galK2, xyl-5, mtl-1, tsx-36, , supE44?.	●
5076	thrB1000, thi-1, relA1, , spoT1	●
128/DR-1	Serine and thiamine requiring and L-SD deficient. Transductant of DR-1 into 128.	This work
128/DR-4	Serine and thiamine requiring and L-SD deficient. Transductant of DR-4 into 128.	This work
128/DR-5	Serine and thiamine requiring and L-SD deficient. Transductant of DR-5 into 128.	This work

● From B. Bachman, E. coli Genetic Stock Center, Yale University, New Haven, Connecticut.

Though the original screening procedure was designed to isolate L-serine auxotrophs, all mutants could use either L-serine or glycine as their requirement, and were ampicillin resistant Strains DR-4 and DR-1 were also chloramphenicol resistant because they were isolated using the modified insertion element Mu dX (Diagram 1).

The interpretation of physiological experiments using these strains depends on the auxotrophs carrying only one insertion, so that one may be certain that the ß. galactosidase levels measured originate from the same insertion element that causes the mutation. Therefore all six mutants were transduced to serine-independence and shown to lose antibiotic resistance, thus proving that each contained only one insertion sequence.

To do this P1cm phage grown on Cu1008Δlac was used to transduce all serine auxotrophs to serine-independence by selecting on glucose-minimal medium.

Transductants were then screened for ampicillin sensitivity. Table 2 shows the result obtained. For 5 strains 78%-94% of the serine-independent single colonies were ampicillin-sensitive. The fact that loss of antibiotic resistance was coupled to the regaining of serine independence demonstrates that all of these strains contained single insertions and that this was in fact true was strengthened by Smith et al., who showed that strains containing a single insertion give rise to 75% or greater ampicillin-sensitive transductants (75). Since strain DR-6 did not produce serine-independent transductants, this strain was not studied further.

Table 2: Antibiotic resistance of serine-independent transductants.

Strains recipients	donor	# of colonies tested	# of Ap^S colonies
DR 4	Cu1008Δlac	90	81
DR 1	"	35	31
DR-2	"	90	70
DR-6	"	-	-
DR-5	"	110	97
DR-3	"	21	15

Selection was carried out on glucose-minimal medium plates

Table 2 also showed that approximately 6-22 % of the transductants were ampicillin resistant and thus apparently retained the insertion sequence. This may be due to the insertion sequence inserting at another location within the *E. coli* genome during the transduction.

Expression of lacZ gene using the *E. coli* promoter.

The fact that the auxotrophs required L-serine or glycine suggests that the insertion element was located in a gene whose product is involved in the biosynthesis of L-serine. This gene might code for the structure of an enzyme, or it might be involved in regulation. In either case, the absence of this gene product would result in auxotrophy.

The insertion into the gene could be in two directions with respect to the bacterial DNA. If the strain produced ß. galactosidase at all, the insertion must have been made such that lacZ is connected to the promoter of the mutated gene (13-15). In this case, it is a promoter involved in L-serine biosynthesis, and the regulation of the level of ß. galactosidase should be the same as the regulation of the promoter of the L-serine biosynthetic gene. One would therefore expect that whatever regulates the function of the serine promoter in the parent would also regulate lacZ transcription in the serine auxotrophs.

It is well established in *E. coli* and other bacteria that the level of the enzymes of anabolic pathways is regulated by end product repression (31). That is, exogenously supplied end products would repress the synthesis of the anabolic enzymes. Similarly, starvation for the end product would derepress.

Table 3: The level of ß. galactosidase in auxotrophs grown in various media.

Addition	units of ß. galactosidase activity in strains					
	Cu1008 Δlac	DR 4	DR 1	DR-2	DR-5	DR-3
Serine	ND*	252	2	2	1955	10
Serine Leucine	ND	160	2	2	1350	10
Glycine	ND	289	2	2	2500	10
Glycine Leucine	ND	257	2	2	1145	10

ND* not detectable

Experiments were carried out in glucose-minimal medium with antibiotics. All medium contained glucose in excess and (either ampicillin (50 ug/ml) or chloramphenicol (20 ug/ml) and ampicillin (50ug/ml)) and other additions as noted.

L-serine and glycine were added at 900 ug/ml and 300 ug/ml respectively. L-leucine at 50 ug/ml.

Units of ß. galactosidase reported are the average of three or more experiments each using exponential phase cells.

Thus it was interesting to determine how the end products L-serine and glycine affect the expression of ß. galactosidase in the serine-glycine auxotrophs. Surprisingly, L-leucine was also shown to affect the expression of ß. galactosidase.

In Table 3 is shown the level of ß. galactosidase in the five auxotrophs grown in glucose-minimal medium (GMM) supplemented with L-serine, or glycine, in each case with and without L-leucine.

Three of the serine-glycine auxotrophs (DR 1; DR-2 ; and DR-3) showed a very low level of ß. galactosidase activity. Strain DR-5 produced ß. galactosidase at a very high level and strain DR-4 at an intermediate level. This is consistent with the hypothesis that the three strains with low level of ß. galactosidase have their insertion within the same gene, and strains DR-5 and DR-4 have theirs in one (or two) other genes. In this case, these auxotrophs would represent insertion in at least two different genes, which are transcribed at different levels. Both would be crucial to L-serine biosynthesis since a lesion in either leads to serine-glycine auxotrophy. However, it is also possible that these insertions are all in the same gene but at different distances from the promoter. This is made less likely by the fact that the expression of ß. galactosidase varies so greatly.

The level of ß galactosidase for strains DR-5 and DR-4 was higher in glycine grown cells than in those grown with L-serine. As shown later, cells grew more slowly with glycine, and were limited by the rate of entry of glycine to the cell. These cells may therefore be considered to be derepressed for L-serine biosynthetic enzymes Glycine did not alter ß. galactosidase level in the other three mutants.

However these are expressed at such a low level that derepression might not be detectable. Thus glycine had an effect at least on the promoter which expressed DR-5 and DR-4 ß. galactosidase.

Table 3 also shows that the addition of L-leucine to the growth medium decreased the synthesis of ß. galactosidase by approximately 30% in strain DR-5 and 35% in strain DR-4. This indicates that the transcription of the gene(s) involved was regulated by L-leucine.

This L-leucine effect was not seen in the other strains. However again the levels of ß. galactosidase were so low that even a 100% increase or 50% decrease with either glycine or L-leucine would not have been detected.

Expression of lacZ unaffected by L-serine concentration.

It was interesting to determine whether the concentration of L-serine affects the expression of lacZ in strain DR-5. To do this strain DR-5 was grown in glucose-minimal media with various concentrations of L-serine and subscultured in the same medium to determine ß. galactosidase levels.

Table 3A shows that variation in L-serine concentration did not affect the expression of ß. galactosidase in strain DR-5.

Table 3A: ß. GALACTOSIDASE AS A FUNCTION OF L-SERINE CONCENTRATION IN STRAIN DR-5

L-serine concentration ug/ml	units of ß. galactosidase
100	2032
200	1920
400	2042
500	2008
600	1950
900	2006

Units of ß. galactosidase reported are the average of two experiments, each using exponential phase cells.

Map Location of Insertion Sequences.

In order to see if the insertions were in any of the known L-serine biosynthetic genes, the auxotrophs were tested for linkage of their mutations to loci known to be linked near to serA, B and C. This approach made use of the following CGSC strains: 5198; 5076; and 1321 (Table 1). Strain 5198 fda has a point mutation in the fda gene which codes for the enzyme fructose diphosphate aldolase and maps 0.4 minutes away from serA. The mutation makes the strain temperature sensitive (9); it grows at 28 º C but not at 37 º C. Similarly strain 5076 is threonine-requiring due to a mutation near serB at 100 minutes (81), and strain 1321 is aromatic amino acid-requiring (80) due to a mutation near serC at 20 minutes.

P1cm phage were grown on strains 5198, 5076 and 1321 and used to transduce the serine auxotrophs to serine independence. Transductants were screened for the relevant phenotype. Details and results of the experiments are summarized in Table 4, 4A and 5.

Among the 175 -208 transductants (Table 4) isolated after transduction with phage grown on strain 5076, none required threonine. Similarly no requirement for aromatic amino acids was seen in the 112-210 transductants isolated from strain 1321 (Table 4A). This suggested strongly that the insertion was neither at the serB nor at the serC locus in any of the serine auxotrophs.

The fact that 54-75% of the serine independent colonies were temperature sensitive (Table 5), unequivocally proved that the fda- locus of 5198 was cotransduced with the gene carring the insertions and were near or within serA in all of the five serine auxotrophs.

Table 4: An analysis of linkage of insertions to genes located near known serine genes: thr

Recipients	Scoring media		
	GMM Thr (A)	GMM (B)	% B/A
DR-4	183	183	100
DR-1	208	208	100
DR-2	200	200	100
DR-5	190	190	100
DR-3	175	175	100

Using strain 5076 as donor, transductants were isolated on glucose-minimal medium plates supplemented with threonine 50 ug/ml and tested on glucose-minimal medium with (column A) and without (column B) supplement.

Table. 4A: An analysis of linkage of insertions to genes located near known serine genes: aroA

Recipients	Scoring media		%B/A
	GMMAAA* (A)	GMM (B)	
DR-4	163	163	100
DR-1	199	199	100
DR-2	210	210	100
DR-5	200	200	100
DR-3	112	112	100

A.A.A* abbreviates Phenylalanine (25ug/ml); Tyrosine (25ug/ml); and Tryptophan (25ug/ml).

Using strain 1321 as donor, transductants were isolated on glucose minimal plates supplemented with A.A.A* and tested on glucose minimal medium with (column A) and without (column B) supplement.

Table 5: Linkage of insertions to fda gene.

Recipient	Transductants able to grow at 28 ° C (A)	Transductants able to grow at 37 ° C (B)	% Temperature sensitive
DR-4	138	51	63
DR-1	322	149	54
DR-2	381	125	67
DR-5	231	59	75
DR-3	106	34	68

Using strain 5198 as donor, transductants were isolated on glucose-minimal medium at 28 ° C and tested on the same medium at 28 ° C (column A) and at 37 ° C (column B).

Enzymatic Defects in Serine-Glycine Auxotrophs.

It is well established that the phosphorylated pathway of L-serine biosynthesis in *E. coli* consists of three enzymes coded by genes serA, B,C (58, 82). A mutation in any of the three known genes should give rise to a serine auxotroph. However the fact that all mutations were linked to serA suggest that all should affect the serA gene product, phosphoglycerate dehydrogenase. Thus it was interesting to determine phosphoglycerate dehydrogenase level.

It is clear from the results in Table 6 that each of the serine auxotrophs grown with L-serine had less than 10% of phosphoglycerate dehydrogenase (PGDase) activity as compared to the parent strain CU1008Δlac, also grown with L-serine. Since the Mu dI or Mu dX totally inactivates the host gene product, the 10% of PGDase activity seen could be due to some reversion to prototrophy. Nonetheless, these auxotrophs either have the insertion within the structural gene serA which makes PGDase enzyme or in a regulatory gene which is tightly linked to serA and regulates its expression.

One cannot argue that the decrease in PGDase activity seen in the auxotrophs was due to repression of PGDase synthesis by L-serine because L-serine did not in fact repress the synthesis either in the parent studied here (Table 6) or in other strains of *E. coli* (41).

When equal amounts of parent and mutant extracts were mixed together, the resultant PGDase activity corresponded to one half the sum of the extracts measured separately. Thus it can be concluded that the serine auxotrophs did not make an inhibitor which interferes with the PGDase assay.

TABLE 6 : PHOSPHOGLYCERATE DEHYDROGENASE (PDGase) ACTIVITY IN SERINE AUXOTROPHS AND THEIR PARENT STRAIN.

Strains	supplements	Specific Activity of PGDase@
Cu1008Δlac	none	28.80
Cu1008Δlac	serine	30.10
DR-1	serine	0.72
DR-2	serine	2.20
DR-3	serine	1.80
DR-4	serine	1.80
DR-5	serine	0.83
DR-5	glycine	3.91
Cu1008Δlac	leucine 50ug/ml	10.80
Cu1008Δlac	leucine 250ug/ml	2.80
Cu1008Δlac	serine+ leucine 250ug/ml	2.50
Cu1008Δlac plus DR-5	serine	14.50

@ Specific Activities were expressed as nanomoles of NADH oxidized per minute per milligram of protein, and represent the average of determinations on at least two different extracts.

All strains were grown in glucose-minimal medium with the indicated supplements. L-serine was added at 600 ug/ml and glycine at 300 ug/ml. Ampicillin (50 ug/ml) was also added to the auxotrophs growth medium.

Since the lacZ gene, which is under the expression of a promoter involved in L-serine biosynthesis, was found to be regulated by L-leucine in strains DR-4 and DR-5 (Table 3), one would expect that PGDase activity would be similarly decrease by L-leucine. In fact CU1008Δlac grown with L-leucine 250ug/ml showed less than 10% of the activity seen in cells grown in minimal medium (Table 6). However with a lower concentration of L-leucine the PGDase activity was not as greatly decreased (33% activity at 50ug/ml).

Now the auxotroph with PGDase activity of 3.91 required L-serine or glycine for growth. If this requirement was due to PGDase deficiency, then a strain with still lower activity (2.8) should also require addition of L-serine or glycine. The fact that L-leucine grown cells do not require L-serine may indicate that they have another pathway for L-serine biosynthesis.

EFFECT OF CARBON SOURCE ON ß. GALACTOSIDASE (lacZ EXPRESSION) LEVEL.

It has been reported that growth on carbon sources other than glucose affected the level of PGDase (41). Cells grown with carbon sources that resulted in a slower growth rate had lower PGDase levels than glucose-grown, faster growing cells. If this were true, ß. galactosidase expression from the serA promoter should also vary with the carbon source. I therefore examined the level of ß. galactosidase in strain DR-5 grown with a variety of carbon sources (Table 7).

TABLE 7 : β GALACTOSIDASE AS A FUNCTION OF CARBON SOURCE IN STRAIN DR-5.

Carbon sources	Units of β galactosidase
glucose	1955
fructose	1942
ribose	1691
arabinose	1657
maltose	1934
pyruvate	1712
glycerol	1768
succinate	1418

The cells were grown at 37 ° C with the stated carbon sources at 2 mg/ml, serine at 900ug/ml with ampicillin (50 ug/ml).

The data represent averages of two or more determinations.

Surprisingly, these results are different from those reported by McKitrick (41). Variation in carbon sources had no effect on ß. galactosidase level (Table 7), even those that resulted in slower growth rate such as pyruvate, glycerol, maltose and fructose. However, succinate-grown cells showed a somewhat lower level of ß. galactosidase.

GROWTH RATE DETERMINATION

The serine-glycine auxotrophs all grew more slowly in glucose minimal medium supplemented with glycine than in medium supplemented with L-serine. Although all the auxotrophs generally grew slowly in glycine, the growth rate was actually determined only for strain DR-5.

This rate was determined from overnight cultures of strain DR -5, grown in the same medium and subcultured to a turbidity of 20 Klett Units. The turbidity was measured every 30 minutes throughout the exponential phase and plotted on semi-log graph paper against time.

The time required for the turbidity to double was defined as the apparent doubling time (a.d.t.). Table 8 shows the a.d.t. in minutes for strain DR-5 at 37 º C grown in four different media.

TABLE 8 : THE GROWTH RATE OF SERINE-GLYCINE AUXOTROPH STRAIN DR-5.

glucose minimal medium plus supplements	apparent doubling time (minutes).
serine	65
serine + leucine	65
glycine	92
glycine + leucine	92

The concentration of L-serine and glycine given were non-limiting 900 and 300 ug/ml respectively. L-leucine was added at 50 ug/ml.

The numbers reported here were the averages of two or more experiments.

The results in Table 8 indicate that L-leucine did not alter the growth rate. However the strain did in fact grow more slowly in glucose minimal medium supplemented with glycine than in that with L-serine. The slower growth rate with glycine might be explained in various ways. Three reactions might be rate limiting for growth on glycine: glycine uptake, glycine cleavage or conversion of glycine to L-serine.

The fact that the rate of growth of this strain DR-5 was found to be related to the concentration of glycine (Table 9) indicates that glycine uptake was rate limiting. Thus the apparent doubling time (a.d.t.) for a glycine concentration of 100 ug/ml was 102 minutes as compared to 72 minutes for 1000 ug/ml (Table 9). With glycine 2000 ug/ml the a.d.t. was 65 minutes. Thus the rate of entry of glycine was rate limiting. A similar finding was reported for another strain of *E. coli* K-12 by Miller and Newman (44).

On the other hand, the growth rate for strain DR-5 was not found to be dependent on the concentration of L-serine (Table 9). Cells grown with 100 ug/ml of L-serine had the same doubling time of approximately 65 minutes as 1000 ug/ml. 2000 ug/ml of L-serine does not permit growth of either the parent or the mutants.

THE EFFECT OF L-LEUCINE ON L-SERINE AND GLYCINE UTILIZATION.

While the growth rate was not affected by the addition of L-leucine (Table 8), the yield per unit L-serine was drastically decreased (Fig. 6). This was first indicated in experiments in which the growth rate and final turbidity were determined for strain DR-5 grown on limiting L-serine (200 µg/ml) or glycine (145 µg/ml), with and without L-leucine (Fig. 6).

TABLE 9: GROWTH RATE AS A FUNCTION OF L-SERINE AND GLYCINE CONCENTRATION FOR STRAIN DR-5.

supplements	concentration ug/ml	apparent doubling time (minutes)
serine	100	61
serine	200	68
serine	300	65
serine	800	64
serine	1000	65
glycine	80	108
glycine	100	102
glycine	200	99
glycine	300	92
glycine	1000	72
glycine	2000	65

All medium contain glucose in excess and ampicillin 50 ug/ml.

Numbers reported were the averages of two or more experiments.

FIG. 6: GROWTH RATE AND YIELD AS A FUNCTION OF AMINO ACID NUTRITION.

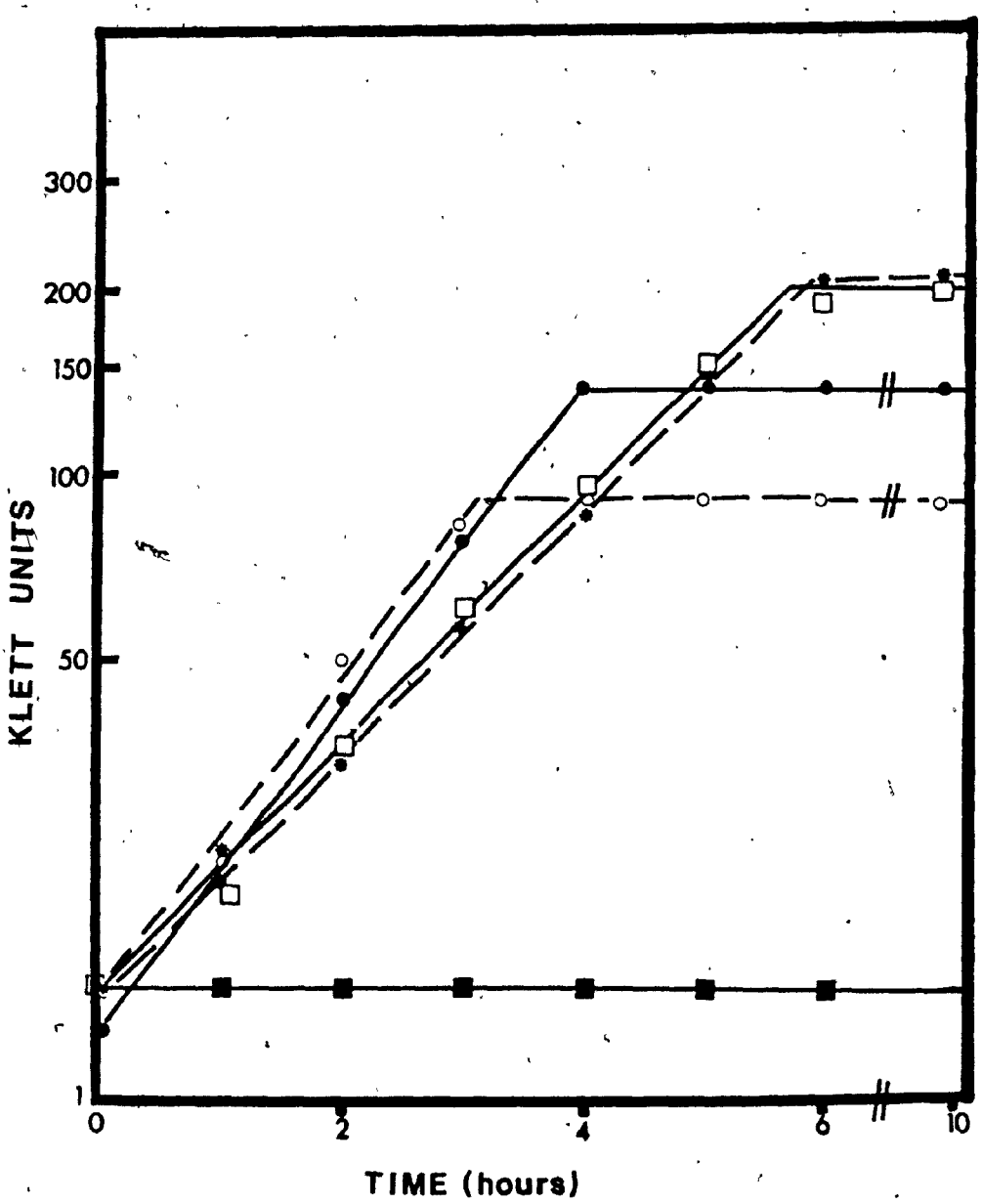
(*) glycine plus L-leucine (50µg/ml)

(□) glycine

(0) L-serine plus L-leucine (50µg/ml)

(●) L-serine

(■) no supplementation


300
200
150
100
50
1
KLETT UNITS
0
2
4
6
10
TIME (hours)

The growth rate on glycine was slower than that on L-serine (as seen earlier) but the yield per µmole was higher on glycine The yield on glycine was unaffected by the addition of L-leucine; the yield on L-serine was considerably decreased. The fact that the yield on L-serine is lower per µmole than that on glycine is surprising.

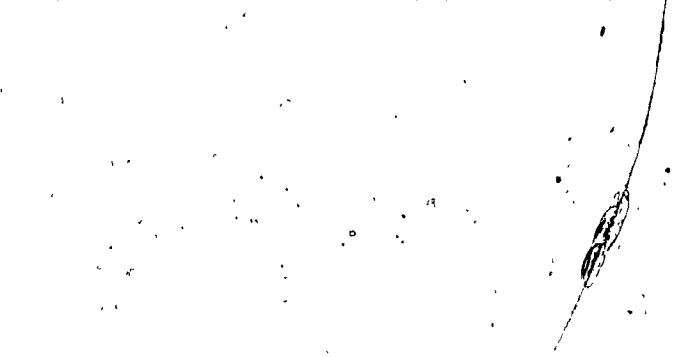
In order to make one molecule of L-serine, the cell has to use two molecules of glycine. Since a µmole of glycine provides less carbon, and since 2 glycine are needed to make one L-serine, it would seem likely that cells grown with equimolar L-serine or glycine would make more cell material from L-serine. The fact that they do not indicates that L-serine is used less efficiently.

YIELD AT STATIONARY PHASE AS A FUNCTION OF L-SERINE AND GLYCINE CONCENTRATION.

To examine this in more detail, strain DR-5 was subcultured into flasks containing various concentrations of L-serine or glycine The cultures were allowed to grow to stationary phase as judged by the constancy of the turbidity readings over a period of 3-4 hours and the final turbidity plotted as a function of L-serine or glycine concentration (Fig. 7).

Fig. 7 clearly illustrates that the optical density (O.D) at the stationary phase for cells grown with glycine was always higher than that of cells grown with equimolar L-serine. This is of course only true for growth-limiting concentrations of L-serine and glycine. Although 4 mM glycine was a non-limiting concentration for glycine-grown cells, 4 mM L-serine was limiting for L-serine-grown cells

FIG. 7: THE EFFECT OF L-LEUCINE ON YIELD.



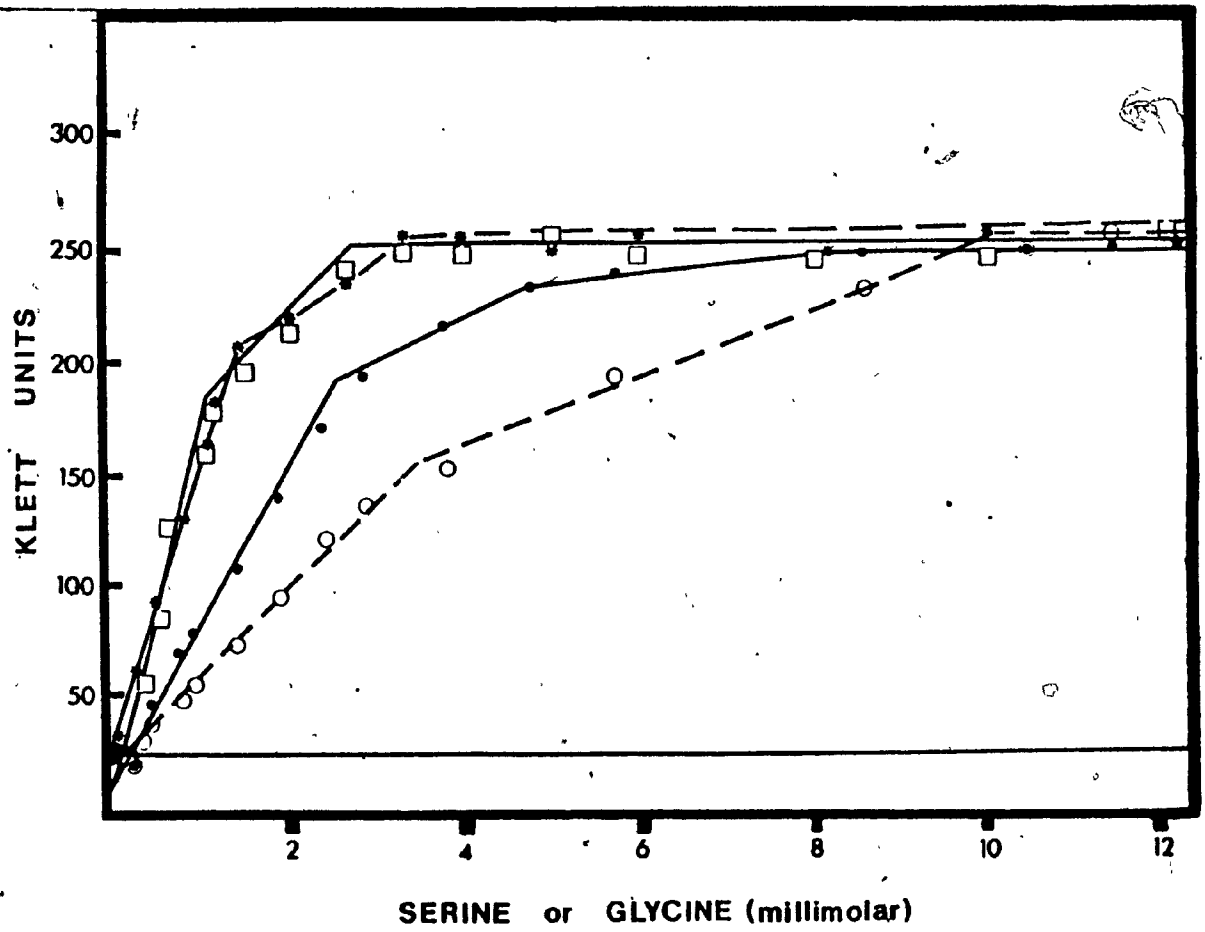
(*) glycine plus L-leucine (50µg/ml)

(□) glycine

(0) L-serine plus L-leucine (50µg/ml)

(●) L-serine

(■) no supplementation


300
250
200
150
100
50
KLETT UNITS
2
4
6
8
10
12
SERINE or GLYCINE (millimolar)

The fact that yield on L-serine was low suggested that L-serine could be used for making things other than those usually considered to be derived from L-serine or glycine. This disparity was increased by L-leucine, an inducer of L-serine deaminase. It seems likely then that L-serine-grown cells convert some of their L-serine to pyruvate and therefore have less L-serine for biosynthetic purposes. Cells grown with L-leucine would then convert even more L-serine to pyruvate.

L-SERINE DEAMINASE (L-SD) ACTIVITY IN SERINE AUXOTROPHS.

To determine the level of L-SD in the mutants, cells were grown in various growth conditions, and the level of L-SD determined (Table 10). L-SD was present at quite high levels in all three mutants studied as compared to the level seen in the parent. Still higher levels were seen either on addition of L-leucine or after a shift in temperature from 37 º C to 42 º C, whether in L-serine- or in glycine-grown cells (Table 10).

TABLE 10 : L-SERINE DEAMINASE (L-SD) ACTIVITY UNDER DIFFERENT CONDITIONS.

	L-SD ACTIVITY					
strains	serine 37 º C	serine leucine 37 º C	serine 42 º C	glycine 37 º C	glycine leucine 37 º C	glycine 42 º C
DR-5	0.120	0.204	0.230	0.120	0.264	0.200
DR-4	0.120	0.210	0.170	0 110	0.234	0.220
DR-1	0.104	0.331	0.231	0 140	0.281	0 304
CuΔlac	0. 056					

Cells were grown in glucose-minimal medium with the additions noted and assayed in exponential phase for L-SD.

Activity is reported as µmoles of pyruvate made by 0.3 ml of a 100 Klett units suspension (Klett 520 filter) in 35 minutes in the standard assay.

The values given are the averages of two or three experiments.

GROWTH YIELD AS AFFECTED BY GROWTH CONDITIONS INDUCING L-SD.

If L-serine is used inefficiently when L-SD is present, then conditions which increase L-SD should decrease the yield per unit L-serine. To test this, the yield per unit L-serine was determined in all 3 auxotrophs in the presence and absence of L-leucine 50ug/ml at 37 º C and for strain DR-5 in the absence of L-leucine at 42 º C. The yield of glycine grown cells, under the above conditions, was also determined for strain DR 5. Table 11 shows the result.

It is clear from these data that an increase in L-SD activity causes a reduction in the yield per unit L-serine. Increase in L-SD activity did not decrease the yield per unit glycine, shown only for strain DR-5 (Table 11).

TABLE 11 : GROWTH YIELD UNDER DIFFERENT CONDITIONS FOR 3 AUXOTROPHS.

	YIELD					
strains	serine 37º C	serine leucine 37 º C	serine 42º C	glycine 37º C	glycine leucine 37º C	glycine 42º C
DR-5	280	140	150	195	195	150
DR-4	310	170				
DR-1	300	180				

Yield is reported as micrograms of protein per ml of culture. The values given are the average of two experiments. Supplements were provided at the following concentrations: L-serine 300 ug/ml, glycine 80 ug/ml and L-leucine 50 ug/ml.

YIELDS OF AUXOTROPHS WITH DEFICIENT L-SD ACTIVITY.

If an increase in L-SD activity decreased the yield, then a decrease in L-SD activity might increase it. To test this, strains were constructed which contained both a mutation leading to serine auxotrophy, and a mutation causing a deficiency in L-SD. To do this, strain 128 (Table 1) deficient in L-SD was transduced with phage grown on the various auxotrophs and antibiotic resistant derivatives isolated. The transductants were then screened for antibiotic resistant serine auxotrophs. Three strains so isolated (128/DR 5, 128/DR-4 and 128/DR-1) were all serine-glycine-requiring and deficient in L-SD activity (Table 12)

Table 13 shows that the double mutants had a higher yield per unit L-serine than the L-SD-containing auxotrophs under the same conditions of growth. Furthermore L-leucine did not lower the yield for these double mutants. Indeed L-leucine slightly increased the yield (Table 13).

TABLE 12 : L-SD ACTIVITY IN DOUBLE MUTANTS.

strains	L-SD ACTIVITY serine at 37 º C	serine + Leucine at 37 º C
128/DR-5	0.020	0.013
128/DR-4	0.013	0.021
128/DR-1	0.013	0.013

Experiment was carried out and reported as in Table 10.

TABLE 13 : YIELD PER UNIT L-SERINE FOR DOUBLE MUTANTS.

	YIELD	
strains	serine at 37 ° C	serine + leucine at 37 ° C
128/DR-5	430	490
128/DR-4	400	430
128/DR-1	380	480

Experiment was carried out and reported as in Table 11.

DISCUSSION

The experiments presented here consist of the isolation and characterization of several serine-requiring auxotrophs of *E. coli* K-12. Serine auxotrophs have been frequently described in the past (18-20, 62, 82). However the present strains were isolated using insertion mutagenesis with lacZ-carrying derivatives of the bacteriophage Mu. This has the advantage of totally inactivating the host gene product and substituting ß. galactosidase for it, thus facilitating studies of its regulation.

In this work I have localized the mutations on the *E. coli* K-12 linkage map and shown that they all result in a loss of the first enzyme of L-serine biosynthesis. Factors influencing expression of the inserted ß. galactosidase have been studied, and L-leucine has been shown to exert a strong though unexpected effect. The auxotrophs have further been used to assess the role of L-serine deaminase (L-SD) in the in vivo deamination of L-serine.

The genetic studies located all five mutations unequivocally at 62 minutes, presumably in serA. Mutants have been described previously at 3 loci, serA at 62.7 minutes, serB at 100 minutes, and serC at 20 minutes (58, 82, 81, 83, 18-20). Markers near serB and serC showed no linkage with the present mutations. However fda at 63.1 minutes showed 53-75 % linkage.

The map distance between fda and serA is known to be 0.4 minute, which corresponds to a linkage of 51 %. Therefore the observed linkage is consistent with the present mutants being located in serA. This could be further tested with complementation tests, but this was considered unnecessary for the present study.

Though all the insertions are in the same region, they do not have similar amounts of ß. galactosidase. Two of the mutants had a great deal of activity (1860 and 231 units). The other three had much lower but still detectable levels (2, 2, and 10 units).

There are a number of ways of accounting for this difference in ß. galactosidase levels. According to some hypotheses, all the insertions would be in the same gene; According to others they would be in two or more genes. I will review first the various hypotheses which attempt to account for different levels of ß. galactosidase expressed from different insertions in the same gene. Then I will discuss at some length a possible model, that in which there are two genes both at 62 minutes. However this cannot be proved on the evidence presently available.

SECTION 1

HYPOTHESES FOR VARIATION OF ß. GALACTOSIDASE WITH INSERTIONS WITHIN ONE GENE:

(1:a) POINT OF INSERTION INTO THE GENE:

Different insertions into a single gene are known to give different values of ß. galactosidase, even though all are clearly controlled by the same promoter. Thus in the case of fusions to tyrR gene, [which is involved in aromatic amino acid biosynthesis and transport] (11), ß. galactosidase levels of 44, and 106 units were described in two mutants.

This was also observed with lacZ fused to argA, a gene involved in arginine biosynthesis in *E. coli* K-12 (21). The level of ß. galactosidase reported for two mutants were 2470 and 341 units.

In a similar study, Goldie et. al., 1982 described the fusion of Mu dI to glnA, the structural gene for glutamine synthetase in *Klebsiella aerogenes*. They found a considerable variation (15,600-403 U/mg protein) in the specific activity of ß galactosidase of eight mutants studied (26). Because all were regulated similarly in different growth conditions, they concluded that all were transcribed from the same promoter.

Similarly in *Salmonella typhimurium*, Maloy et. al., studied the regulation of proline utilization by using Mu dI fusion. These authors showed that four mutants, each with an insertion in putA, expressed ß. galactosidase at different levels, 319; 162; 208; and 369 units (38).

The first three authors ascribed this variation of ß. galactosidase to the distance of the insertion from the promoter. The fourth author gave no explanation. Although this hypothesis may be correct, the authors provided no evidence to support it.

(1:b) POLARITY EFFECT

Aksoy et al. 1984, in their study of trp-lac fusion system, introduced a frame shift mutation early in trpB and studied its effect on the transcription and translation of lacZ fused to trpA (1). Their results showed that the frame shift mutation reduced the ß. galactosidase expression to 7 % of the control value (316 vs 4,545 units). The authors showed that the decreased level of ß galactosidase expression was due primarily to translational defects rather than transcriptional, since the amount of the lacZ mRNA made by the frame shift mutant was virtually the same as the control. This suggests that in the work described here,

the auxotrophs with low levels of ß. galactosidase might have lacZ in a different reading frame. However, it should be noted that the extent of variation (10-fold) seen by the trp-lac fusion was less than that seen in this work (150-fold) Thus frame shift mutation alone cannot explain the great variation in ß. galactosidase levels seen here.

Casadaban, 1976 suggested that the insertion might sometimes be placed in such a way as to form a nonsense codon at the fusion joint, thereby decreasing translation of the lacZ mRNA (14, 15) He isolated different ara-lacZ fusion strains, all of which produced different amounts of ß. galactosidase (26 fold variation) Those with the highest levels of ß galactosidase were assumed to show the least interference with translation through the fusion joint (14, 15). Reznikoff et. al. investigated this hypothesis as an explanation for low levels of ß. galactosidase in trp-lac fusions, but considered it unlikely since a nonsense suppressor did not increase ß galactosidase levels

(1:c) HYBRID PROTEIN:

Reznikoff et. al. demonstrated variations of 60 fold in ß. galactosidase in their trp-lac fusions (65). They attributed this to translational difficulties rather than transcriptional ones since lacZ mRNA was greatly in excess of the amount expected from the amount of ß. galactosidase seen. They considered the possibility that certain fusion mutants might make a less stable ß. galactosidase than other mutants. They discarded that hypothesis because sodium dodecyl sulfate (SDS)-acrylamide gel analysis of a crude extract from each fusion strain showed a single band at the position of ß. galactosidase and the intensity of the band was approximately proportional to the level of

ß. galactosidase in the strains. This excluded the existence of a hybrid protein. Moreover, the structure of Mu d1 or Mu dX does not permit the formation of hybrid protein (diagram 1). Whether the sequences of Mu prior to lacZ (diagram 1) have any strong effect on transcription and or translation cannot be ruled out.

(1:d) INEFFICIENT TRANSLATION OF lacZ:

Reznikoff et. al. finally settled on the hypothesis that the mutants differed in the frequency of initiation of translation at the start of lacZ. They suggested that the trp codons fused to the start of the lacZ gene might result in formation of a mRNA with decreased ability to bind ribosomes. These authors did not investigate this possibility further but indicate that this model could be tested by directly isolating the trp-lac mRNA and assaying its ability to bind ribosomes, and comparing this with the binding of ribosomes to normal lacZ mRNA.

(1:e) DIRECTION OF THE INSERTION WITHIN THE GENE.

In a recent personal communication, Casadaban explains that the Mu d 1 or Mu dX insertion element could be placed in the wrong orientation within serA in order to be read from the serA promoter. As a result of this orientation, there will be little or no ß. galactosidase expression. At this time there is no evidence to support this hypothesis. Further work needs to be done in order to see whether this hypothesis explains the low levels of ß. galactosidase seen in this work.

SECTION 2

A 2-GENE HYPOTHESIS THAT CAN ACCOUNT FOR VARIATION OF ß. GALACTOSIDASE.

(2:a) A COMPARISON OF ß. GALACTOSIDASE PRODUCTION IN FUSIONS TO REGULATORY AND STRUCTURAL GENES.

It is clear that there is a great variation in ß. galactosidase levels in fusion strains, even in cases where all insertions are likely to be in the same gene. The cases described show variations from 24-60 fold. Still others showed less variation (1, 5, 75, 68). However in the work described here a variation of over 150-fold was seen. This suggests that insertion into a single gene cannot explain all the variation and it therefore seems that the insertions studied here may be in different genes.

This is made even more likely by the fact that the low level of expression was low enough to be consistent with insertion in a regulatory gene. Evidence for this comes from the work of McFall et. al., (39) who not only described insertions into the regulatory and structural genes of D-serine deaminase but also identified the regulatory gene product. ß. galactosidase expressed from the regulatory gene promoter had an activity of 7.2 U/mg protein whereas ß. galactosidase expressed from the structural gene promoter had an activity of 1960. This 272-fold variation between ß. galactosidase inserted into these two genes is clearly higher than that seen for the insertions reported to be in a single gene.

If the present insertions were in fact in different genes, it seems reasonable to assume that the three auxotrophs with low expression of β. galactosidase were in one gene and the two others (strains DR-5 and DR-4) in a second. Each of these genes would have its own promoter.

Mutations in either of the two putative genes would result in auxotrophy, and a lack of phosphoglycerate dehydrogenase. One might then assume that one of the genes was the structural gene, and the other be a regulatory gene. Previous isolates of serA mutants could be at either or both loci (58, 83). While phosphoglycerate dehydrogenase has been shown to be decreased in all auxotrophs, the other two enzymes of L-serine biosynthesis have not been assayed. It is possible that either class of mutants (or both) would show decreased synthesis of the other enzymes as well.

A possible model for the control of the expression of the serA locus then is that there are two genes, a structural gene serA and a regulatory gene serR. Since insertions in serR inactivate it, resulting in a loss of enzyme activity, serR would code for a positive regulatory protein. The serR protein would then attach to the promoter of serA and turn on transcription of serA mRNA (possibly unwinding the double stranded DNA and facilitating RNA polymerase binding). According to this model, lesions in either serR or serA would prevent production of phosphoglycerate dehydrogenase.

This model can explain the decrease in ß. galactosidase level seen with L-leucine as seen in strain DR-5 and DR-4 if the serR protein also interacts with L-leucine. L-leucine might then induce a new conformation of the serR protein that might bind to serA promoter less efficiently, thus decreasing transcription.

(2:b) RELATIONSHIP OF serR TO serB AND serC:

Since no attempt was made to assay the enzymes coded by the serB and serC loci, one cannot say whether serR controls these two loci also. However, if serR acted as a positive regulator for serB or serC as described above for serA, then one would imagine that a strain with a mutation in the structural gene serA (DR-4 and DR-5) would produce the other two enzymes, whereas strains (DR-1; DR-2 and DR-3) with a mutation in serR would have none of the three enzymes of the pathway. Umbarger (1964) and Dempsey (1969), described mutants of *E. coli* K-12 which lacked the serA gene product (PGDase) but showed the other two enzyme activities. This may indicate that their strains had mutations in the structural gene.

REASONS FOR NOT ASSAYING serB AND serC GENE PRODUCT:

The assay of the other two enzyme is a more major job which did not fall within the scope of this work. It involves synthesizing L-serine-phosphate as a substrate. This would then be used to determined either release of inorganic phosphate (enz. 3) or conversion of alphaketoglutarate to glutamate (enz. 2) by coupling to PGDase (enz. 1). This would be an interesting line for further enquiry.

SECTION 3

WHY ARE ALL THE INSERTIONS IN serA ?

One would normally expect to find auxotrophs with insertions in any of the three genes of the pathway. However all the insertions isolated in this work proved to be at serA. serC mutants have been described previously but require pyridoxine as well as L-serine. Since pyridoxine has not been added to the screening plates, mutants that grew on L-serine plus pyridoxine but not with L-serine alone would not have been isolated.

Mutants at serB are less well known in *E. coli* K-12. Indeed only one has been described, by Mckitrick (41). It was a strain obtained from S. Glover. It lacked L-serine phosphatase and was a leaky mutant. If such mutants are generally leaky, perhaps because other phosphatases can substitute for the serB encoded phosphatase, then these mutants too would have been missed in the screening used here.

SECTION 4

FACTORS INVOLVED IN THE REGULATION OF PGDase SYNTHESIS

(4:a) EXOGENOUSLY ADDED L-SERINE DOES NOT REPRESS PGDase SYNTHESIS.

Pizer and Potochny have reported that PGDase synthesis is not repressed by growth with L-serine. They stated, (without quoting data) that the specific activity of PGDase in cells grown without L-serine was the same as that of cells grown in the presence of L-serine, the same report was made by Umbarger and Umbarger (82). Further, McKitrick and Pizer 1980 reported activity for PGDase of 16.6 and 17.0 U/mg of protein in the presence and absence of L-serine respectively.

The results shown here are consistent with this in that exogenously provided L-serine did not repress the enzyme. However evidence of derepression in mutants grown with glycine was obtained. Mutant cells grown with glycine instead of L-serine showed a significantly higher level of β. galactosidase. These cells were growing at a considerable reduced rate limited by availability of glycine and therefore also of L-serine. Therefore the level of transcription of serA probably is controlled to some extent by the level of L-serine and/or glycine and/or their metabolite (s) in the cell.

While the level of the enzyme does not seem to be altered by the presence of L-serine, activity of the enzyme was regulated closely. The earlier work with competition studies showed that the presence of L-serine in the medium inhibited synthesis of L-serine and glycine from glucose or fructose by more than 90% (66). This was correlated with the demonstration of inhibition of PGDase activity by L-serine (59, 60, 69, 77, 78, 87) and indeed the present assay incorporates this as part of the estimate of enzyme activity. That is, PGDase activity is taken to be that part of phosphohydroxypyruvate-dependent NADH oxidation which can be inhibited by L-serine.

(4:b) EFFECT OF CARBON SOURCE ON PGDase SYNTHESIS

McKitrick and Pizer (41) reported the finding that cells grown on carbon sources other than glucose had lowered PGDase enzyme levels. Carbon sources that supported a slower growth rate had lower PGDase levels. However, malate- and fructose-grown cells had the same growth rate but significantly different enzyme levels. These authors concluded that the growth rate has an influence on PGDase levels.

In the present experiments no significant decrease in ß. galactosidase level was observed even when in media where the cells grew more slowly (e.g. glycerol, pyruvate, maltose and fructose as carbon source). Only cells grown with succinate showed a somewhat decreased level. These result do not fit in with the explanation given by McKitrick et. al. Possibly PGDase synthesis was repressed to varying degrees during growth on gluconeogenic substrates. This would therefore be consistent with the present finding that succinate decreased ß. galactosidase level. Alternatively, ß. galactosidase messages or the protein itself are more stable than that of PGDase or its message, the more so if stability were a function of the carbon source or growth rate.

(4:C) EFFECT OF L-LEUCINE ON PGDase SYNTHESIS

Even though L-serine and various carbon sources did not repress PGDase synthesis, there is some evidence that other compounds did. A decrease of approximately 31% in cells grown with L-threonine, L-methione, L-leucine, DL-isoleucine and glucose was reported (59). In this work (Table 6) L-leucine alone at a high level (250 ug/ml) decreased PGDase activity in the parent strain more than 90 %, indeed to below the level seen in auxotrophs. A lesser decrease (31 %) was seen with 50 ug/ml of L-leucine. One would expect a corresponding effect of L-leucine on ß. galactosidase in the auxotrophs. However L-leucine at 50 ug/ml or 250 ug/ml decreased ß. galactosidase only 30 %. This may indicate that normal functioning of the promoter is altered by the insertion element. Even so, it is obvious that L-leucine has a profound effect on the synthesis of this enzyme.

ROLE OF L-LEUCINE IN *E. coli* METABOLISM

L-leucine lies at the end of a biosynthetic pathway and does not serve as a precursor for any amino acids. However it does have several effects on the synthesis and breakdown of amino acids. L-leucine induces the enzyme, threonine dehydrogenase which cleaves threonine to form glycine (16, 23, 63). It also induces L-serine deaminase which deaminates L-serine to pyruvate and ammonia (30, 56), and it is further considered to induce glycine cleavage to C-1 units (23). The catabolism of these amino acids induced by L-leucine, therefore results in an increased ammonia pool. L-leucine also affects assimilation reactions such as the assimilation of ammonia into glutamate using glutamate dehydrogenase (88). It was suggested that L-leucine acted as a signal to increase catabolism of some amino acids when in excess and to assimilate the ammonia produced (88). When the ammonia pool is increased by catabolic reactions, perhaps the ammonia contributed by L-serine will be shut off indirectly but not completely by L-leucine and this occurs at the level of repression of PGDase synthesis.

SECTION 5

EVIDENCE FOR A SECOND PATHWAY TO L-SERINE.

(5:a) EFFECT OF L-LEUCINE INDICATES A SECOND PATHWAY.

The parent strain from which the auxotrophs were isolated showed an activity for phosphoglycerate dehydrogenase of 28.8 units per mg protein. The auxotrophs showed considerably less (3.91 units per mg protein). It seems likely that this is the reason why they were auxotrophs, though they may be missing other enzymes as well.

The finding that the parent strain grown with L-leucine (250 ug/ml) has a PGDase level of 2.8 (Table 6) is not consistent with the preceding data. If the strain with 3.8 units per mg protein is an auxotroph, then the parent strain grown with leucine should also require L-serine and it does not. L-leucine is not a precusor of L-serine (53) and does not support growth of the auxotrophs. This then implies that the deficiency of PGDase is not the primary cause of auxotrophy in these mutants, or that L-leucine induces a new pathway of L-serine biosynthesis.

(5:b) SECOND PATHWAY INDICATED BY A STUDY OF serC REVERTANTS.

There have been various suggestions of the existence of a second pathway of L-serine biosynthesis. One of these was based on a study of serC mutants. Auxotrophs with a double requirement for L-serine and pyridoxine (or hydroxypyruvate) have been isolated in both strains K-12 and B of *E. Coli*. These have been attributed to a lesion in serC which encodes the second enzyme of the phosphorylated pathway, phosphoserine-oxoglutarate transaminase (73). In *E. coli* K-12 but not *E. coli* B, it was possible to isolate partial revertants from the serC

phenotype, i. e. strains which require L-serine but not pyridoxine. From these ser - pdx + strains, it was possible to isolate serine-independent strains. The surprise was that these did not regain phosphoserine-oxoglutarate transaminase activity. The authors concluded from this that there must be another pathway other than the phosphorylated pathway to make L-serine. It is of course also possible that the revertants made an unstable enzyme which did not survive extraction methods.

(5:c) ANOTHER PATHWAY INDICATED BY STUDIES OF FORMATE-USING AUXOTROPHS.

Existence of a second pathway was indicated in a study of mutants with an alternative requirement for L-serine, glycine, or formate. Auxotrophs requiring L-serine or glycine were grown with glucose and formate as a substitute for the amino acids. Since radioactive formate was not a precusor of either L-serine or glycine, these must have been synthesized de novo from glucose. Then formate must have induced a second pathway or restored the mutated one (53).

DEGRADATION OF L-SERINE:

The auxotrophs grew more slowly in glucose-minimal medium supplemented with glycine than in medium supplemented with L-serine. The concentrations of glycine (300 ug/ml) and L-serine (800 ug/ml) were shown to be in excess of the cells nutritional requirement, which excludes the possibility that the difference in growth rate was due to a limited supply of one of the amino acids in the medium.

The growth rate was shown to be proportional to the exogenous glycine concentration at concentrations well above the limiting concentration. Thus, the apparent doubling time was 92 minutes at 300 ug/ml, 72 minutes at 1000 ug/ml and 65 minutes for 2000 ug/ml of glycine. This proportionality was not seen with L-serine.

The slower growth rate on glycine can be explained in any of the following ways:

(1) The rate at which glycine is transported into the cell is a function of the external glycine concentration.

(2) The rate of provision of C-1 unit from the cleavage of glycine by the glycine cleavage enzyme complex (GCV) may be rate limiting (see equation 1).

(3) The rate of formation of L-serine by the L-serine hydroxmethyl transferase enzyme (SHMT) from glycine and C-1 unit may be rate limiting (see equation 2).

Glycine <----> CO2 + NH4 + 5,10 Methylene THF --------(1)

Glycine + 5,10 Methylene THF <----> L-serine ---------(2)

It is unlikely that glycine cleavage is rate limiting because addition of glycine actually induced glycine cleavage so that the cells would not be limited by the rate of formation of C-1 units (42, 57). However this induction could be proportional to the external glycine concentration, and even an induced level might be limiting. Moreover, it was not shown by these authors that the increase in GCV enzyme complex activity measured in vitro corresponded to an increase activity in vivo.

It cannot be that glycine repressed SHMT synthesis, thus limiting the rate of L-serine formation, which would ultimately increase the apparent doubling time, because the level of SHMT has reported to be only slightly affected by the addition of glycine to the growth medium of a serine-glycine auxotroph of *E. coli* K-12 (44). Growth in glycine at 100 ug/ml had no effect on SHMT enzyme activity. At 3000 ug/ml the SHMT activity was repressed to 43% (44). However, even though the SHMT activity was repressed, the apparent doubling time was decreased to 65 minutes with this concentration (3000 ug/ml) as compared to 100 minutes for 100 ug/ml (44). This slow growth rate on glycine was attributed to the limited capacity of *E. coli* K-12 to transport glycine as determined by uptake experiments (44). This explanation presumably holds true also for this present study, since the serine-glycine auxotrophs studied here were also derived from *E. coli* K-12.

L-SERINE DEGRADATION.

Although the growth rate for glycine and L-serine grown cells was unaffected by L-leucine (Fig 6), the yield per unit L-serine but not glycine was drastically decreased. This decrease in yield with serine-grown cells was first observed when the auxotroph grown with glycine had a higher turbidity at the stationary phase than those grown with the same molar concentration of L-serine (Fig. 6). The concentration of glycine and L-serine was growth-limiting in both cases. This was surprising since a umole of glycine provides less carbon than a umole of L-serine and 2 molecules of glycine are needed to make 1 molecule of L-serine. Thus it was expected that the yield would be lower with glycine than with L-serine grown cells, but in fact the

reverse was seen (Fig. 6 and 7). This indicated that L-serine was used less efficiently than glycine, particularly in the presence of L-leucine.

It has been reported that *E. coli* K-12 strain grown in glucose-minimal medium makes a considerable amount of an enzyme activity, L-serine deaminase (L-SD), which catalyzes the conversion of L-serine to pyruvate and ammonia (30, 52, 56). Since pyruvate does not support growth of L-serine/glycine auxotrophs, this enzyme activity must be irreversible. If L-serine were then extensively deaminated by this enzyme, the cell would have less L-serine available to supply its requirement for L-serine, glycine and their various derivatives. Further, since L-SD activity is induced by a variety of treatments (50, 52, 56), one would suppose that the yield of an auxotroph grown with limiting L-serine would vary inversely with the level of L-SD. It is because of the deamination of L-serine by the enzyme L-SD to a compound (pyruvate) which is not in equilibrium with L-serine, that studies were pursued to investigate the role of this inducible enzyme in the metabolism of L-serine.

The three serine-glycine auxotrophs studied showed an approximately two-fold increase in L-SD activity when grown with L-leucine and or shifted from 37º C to 42º C, whether grown with L-serine or glycine (Table 10). It should be noted here that the level of L-SD is about the same for cells grown with glycine and with L-serine (Table 10). Since L-serine is not an inducer of this enzyme (but glycine is), this may mean that the mutants, grown with L-serine, maintain a higher internal glycine pool than the parent.

If this enzyme were responsible for degradation of L-serine, yields ought to be decreased at 42 º C , or at 37 º C with leucine, since more L-SD was made in these conditions. It is clear from the results in Table 10 and 11 that an increase in L-SD activity corresponded to a reduction in the yield per unit L-serine. The inducing conditions used decreased the yield by about 40-50 %. This data is consistent with that observed in Fig. 7 for the range of L-serine shown to be limiting. The turbidity at the stationary phase was lower with L-leucine than in its absence.

That L-SD is the activity responsible for the inefficient use of L-serine was clearly shown by the use of double mutants. Double mutants were constructed which were serine-glycine requiring and deficient in L-SD activity (Table 12). When the same concentration of L-serine (300 ug/ml) was given to these double mutants as to the serine-glycine auxotrophs, they demonstrated a higher yield per unit L-serine both under the inducing and non-inducing conditions (Table 13). It is not clear why all the double mutants studied had a slightly higher yield per unit L-serine in the presence of L-leucine than in its absence (Table 13), but it is clear that in the absence of L-SD, L-serine was not deaminated. This is, in fact, the first evidence that L-SD deaminates L-serine <u>in vivo</u>.

From these data, (Table 11 and 13), it can be inferred that even when grown without inducers, the serine-glycine auxotrophs had enough L-SD enzyme activity to deaminate L-serine, since in the double mutants, the yield per unit L-serine was higher than in the auxotrophs which had L-SD activity.

The increase in L-SD activity did not decrease the yield per unit glycine under the three growth conditions examined (only shown for strain DR-5 Fig. 7 and Table 11). This can be explained by the fact that the rate of entry of glycine was limiting. Thus the intracellular pool size of L-serine would always be low in glycine-grown cells. Because of the high K_M of L-SD for L-serine (52), it is likely that L-SD would not deaminate L-serine derived from glycine.

With a high concentration of glycine, the growth rate was decreased to 65 minutes, suggesting that the intracellular pool of L-serine is probably high. However, even though L-SD would deaminate L-serine in this condition, one could not see a decrease in yield because there would always be an excess of glycine to provide more L-serine.

It appears then that L-SD was responsible for the unexpectedly low yield seen with L-serine in vivo. From the findings reported here, it was not possible to assess the physiological role of L-SD in *E.coli* K-12. The L-serine biosynthetic pathway is not regulated at the level of repression of enzyme synthesis by its end product. The activity of the first enzyme of the pathway (PGDase) is 90 % inhibited by end product inhibition as reported by Pizer et. al. (59, 60). Since inhibition is not 100% the cell might accumulates more L-serine than it can use, and this may be toxic (40). L-SD might then be a detoxifying enzyme.

Further, it can also be speculated that at times of stress for ammonia in the cell, L-SD can deaminate L-serine to produce the ammonia needed for amino acid biosynthesis since it was reported that the parent *E. coli* strain Cu1008 can use L-serine as a nitrogen source (54).

SUMMARY

In this work L-serine-requring auxotrophs of *E. coli* K-12 were isolated and their physiological and genetic characteristics described. Although L-serine-requiring auxotrophs were isolated previously (18-20, 62, 82), the approach taken here was different in that these auxotrophs were isolated by insertion mutagenesis using the Mu dI and Mu dX bacteriophage.

The auxotrophs studied were all L-serine- or glycine-requiring. Each was shown to contain a single insertion and to map at serA, which encodes the first enzyme of the L-serine biosynthesis pathway, phosphoglycerate dehydrogenase.

The ß. galactosidase level expressed from the inserted element showed a variation of over 150-fold among the five auxotrophs (2-2000 units). This variation is probably too great to be consistent with the variation of ß. galactosidase level (24-60 fold) reported to be expressed from insertion elements within any one gene (5, 11, 21, 26, 38, 75, 85). Because of the extent of variation seen in these serine auxotrophs, I postulated the existence of two genes at the serA locus.

Addition of L-leucine to the growth media decreased the synthesis of ß. galactosidase by approximately 30 % in strain DR-5 and 35 % in strain DR-4. Whether L-leucine had such an effect on the expression of ß. galactosidase in the other three strains (DR-1, DR-2 and DR-3) could not be determined because of their low levels of ß. galactosidase (2-10 units).

The fact that all mutations were linked to serA suggested that all should affect the serA gene product, PGDase. Indeed the level of PGDase in all the auxotrophs was less than 10 % of that seen in the

parent strain, making it likely that the insertions are within the structural gene serA or in a regulatory gene which is tightly linked to serA and regulates its expression.

PGDase level was also affected by L-leucine. With 250 ug/ml of L-leucine the PGDase level was 2.8 units/mg of protein in the parent. This low level caused by L-leucine is lower than that seen in the serine auxotroph with 3.9 units/mg of protein. If the L-serine or glycine requirement in the auxotrophs is a result of a decreased PGDase level, then the parent strain grown with L-leucine (250 ug/ml) should also require L-serine. The fact that it does not suggests that *E. coli* K-12 may have another pathway for L-serine biosynthesis.

The auxotrophs have been shown to grow much more slowly in glucose-minimal medium supplemented with glycine than in medium supplemented with L-serine. L-leucine did not alter the growth rate. Further, the growth rate for glycine grown-cells was shown to be dependent on the glycine concentration. Thus the apparent doubling time was 102 mins with 100 ug/ml, 92 mins with 300 ug/ml and 65 mins with 2000 ug/ml of glycine. For serine grown-cells, the apparent doubling time was independent of L-serine concentration. The slower growth rate on glycine was attributed to the limited capacity of these auxotrophs to transport glycine into the cell.

Although the growth rate for glycine and L-serine grown-cells was unaffected by L-leucine, the yield per unit L-serine but not glycine was drastically decreased. This decrease in yield seen on L-serine was a result of the activity of the inducible enzyme L-serine deaminase (L-SD), which deaminates L-serine irreversible to form NH_4^+ and pyruvate.

I have shown that an increase in L-SD activity lowered the yield per unit L-serine by 40-50 %. If an increase in L-SD decreased the yield, then a decrease in L-SD activity might increase it. Mutants deficient in L-SD activity were shown to have higher yield per unit L-serine than L-SD containing auxotrophs under the same conditions of growth. This is in fact the first evidence that L-SD deaminates L-serine <u>in vivo</u>.

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