THE DERIVATION OF GLYCINE FROM THREONINE IN MUTANT STRAINS OF *E. COLI* K12

Judith Fraser

A Thesis

in

The Department

of

Biological Sciences

Presented in Partial Fulfillment of the Requirement
for the degree of Master of Science at
Concordia University
Montréal, Québed, Canada

April, 1975

THE DERIVATION OF GLYCINE FROM THREONINE IN MUTANT STRAINS OF *E. COLI* K12

Judith Fraser

ABSTRACT

The enzyme serine transhydroxymethylase (STHM) has been considered to be the only enzyme responsible for significant glycine biosynthesis in *E. coli*. Thus strain AT2046 previously shown to lack this enzyme requires exogenous glycine for growth. Mutant strains, (JEV73 and JEV73R) isolated from AT2046 and lacking STHM are shown here to derive their glycine from a new pathway involving endogenously synthesized threonine.

Leucine is shown to be closely involved in the regulation of this pathway for glycine biosynthesis, possibly by increasing threonine conversion to glycine.

A possible interpretation for the metabolism of JEV73 and JEV73R as regulatory mutants of AT2046 is presented. It is suggested that leucine may have a role in the regulation of nitrogen metabolism in the cell.

ACKNOWLEDGEMENTS

This project would not have been possible without the assistance of many people.

Foremost among these is Dr. Newman, who for the past three years has been a source of guidance and encouragement to me. Without this I would never have begun and certainly never have completed this project. It is impossible to express my appreciation to her for all she has done during this time.

I would like to thank Vinod Kapoor, Peter Weyman, Robert Potter and Gerald Baptiste for their helpful assistance in so many ways too numerous to mention. Thanks also to Paula Kat who typed this thesis.

This work was made financially possible by a post-graduate scholarship from the National Research Council of Canada.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In the last twenty years the biosynthetic pathways leading to most of the common L-aminoacids have been elucidated. Most of these amino acids are synthesized by a single pathway made up either of one enzyme or of a series of enzymes. However, in the area of serine-glycine biosynthesis the possibility has arisen that more than one biosynthetic route exists. One pathway of glycine biosynthesis in Escherichia coli, in which serine is converted to glycine by serine transhydroxymethylase (STHM) is already well known. The present investigation was done to determine whether another pathway of glycine biosynthesis might exist in E. coli, to investigate the nature of such a pathway, and to determine the extent and circumstances for its use.

In this introduction the evidence for the presently accepted picture of serine - glycine biosynthesis will be discussed. This discussion will take the form of a review of the evidence for a single pathway from glucose to serine and serine to glycine and a discussion of the nature of this pathway. A survey of studies on the interconversion of serine to glycine will also be included. Work on possible alternative biosynthetic routes to glycine will then be reviewed. This includes considerable amount of work done on the conversion of threonine to glycine by the enzyme threonine aldolase and an account of radioactive

competition experiments indicating that this pathway is used only in the presence of exogenous threonine in E. coli. The work on the possibility of glyoxylate to glycine pathway in certain organisms will be cited and it will be shown that there is no evidence that this pathway functions in glucose grown cells of E. coli. Serine, threonine and glycine are important not only for themselves but as potential one carbon donors. A strain with a variation in serine, glycine and threonine biosynthesis might also be expected to have alterations in one carbon metabolism. Therefore the account will finish with a discussion of the various sources of one carbon units available to the cell. Because much of the study of the sources of one carbon units has depended on the isolation, and occasionally, the degradation of purine molecules, the use of determinations of purine specific activity will be reviewed briefly.

Evidence for a Single Pathway of Glycine Biosynthesis

It is generally considered that in E. coli serine is synthesized from glucose while glycine is synthesized from serine and that this represents the only pathway available for serine and glycine biosynthesis (Equations 1-5).

$$\text{D-Glucose} \longrightarrow \xrightarrow[\text{Pathway}]{\text{Embden-Meyerhof}} \longrightarrow \text{D-3-Phosphoglycerate} \quad (1)$$

$$\text{D-3-Phosphoglycerate} + \text{NAD}^+ \underset{\text{pyruvic acid} + \text{NADH} + \text{H}^+}{\overset{\text{3-Phosphoglycerate dehydrogenase}}{\rightleftharpoons}} \text{3-Phosphohydroxy-} \quad (2)$$

3-Phosphohydroxypyruvic acid + L-Glutamate

$$\xrightleftharpoons{\text{Phosphoserine-glutamate transaminase}} \text{L-phosphoserine} \qquad (3)$$

$$\text{L-Phosphoserine} + H_2O \xrightarrow{\text{phosphoserine phosphatase}} \text{L-serine} + Pi \qquad (4)$$

$$\text{L-Serine} + FH_4 \xrightleftharpoons{\text{Serine transhydroxymethylase}} \text{L-Glycine} + N^5, N^{10}\text{-methylene } FH_4 \qquad (5)$$

This is based on several kinds of evidence. Roepke et al (1944) described a mutant of E. coli with alternative requirements for serine or glycine. These were isolated with the frequency expected of a one step mutation and were shown to revert with the same frequency. Thus a single mutation impaired both serine and glycine biosynthesis. From this it was argued that there could be only one pathway for serine/glycine biosynthesis for if there were a second pathway for glycine biosynthesis, the glycine so formed would provide serine and the mutant phenotype would not be seen.

That a block in serine biosynthesis would result in a requirement for serine or glycine had been indicated by these experiments of Roepke. Later the particular enzymes (listed in the diagram) in the biosynthetic pathway to serine were demonstrated in extracts, and it was shown

that the lack of one of them (3-phosphoglycerate dehydrogenase) would result in a requirement for serine or glycine (Umbarger and Umbarger, 1962). The fact that a mutant, strain AT2046, lacking the enzyme known to convert serine to glycine (STHM) was able to grow on glycine but not on serine provides further evidence for a single pathway of glycine biosynthesis (Piser 1965).

Still further evidence for a single unique pathway of glycine biosynthesis has recently been provided by Stauffer and Brenchly (1974) working with a mutant of Salmonella typhimurium known to have mutations in the genes responsible for the synthesis of the enzymes 3-phosphoglycerate dehydrogenase and serine transhydroxymethylase. The phenotype of this mutant was such that it required both serine and glycine for growth. They conclude that STHM is the only enzyme responsible for the conversion of serine to glycine and no other major pathway of glycine biosynthesis exists.

In view of the available evidence for the existence of a single pathway for serine-glycine biosynthesis, why should we suspect the existence of other pathways? Evidence that another pathway for glycine biosynthesis might function comes from two sources. First, a serine-glycine auxotroph has been described which is capable of growth on sodium formate and the question arises as to its source of serine

or glycine when growing on formate (Newman, 1970). Secondly, the glycine auxotroph previously described, strain AT2046, lacking STHM, can meet its nutritional requirement by being provided not only with glycine but also with threonine. In this case the exogenous threonine is cleaved to glycine, possibly by the enzyme threonine aldolase (Meister, p681). If the cell could use endogenous threonine in this way, it would be able to grow without supplement. The fact that the mutant requires glycine for growth indicates that endogenously formed threonine is not converted to glycine. E. coli clearly possess the genetic information to make the enzymes for this pathway, but this information appears only to be used in the presence of exogenous threonine.

In this thesis, however, mutant strains have been isolated in which glycine can be derived from threonine in the absence of exogenous threonine. Two such strains are described; one, JEV73, in which exogenous leucine is required for the expression of this pathway and one, JEV73R, in which no exogenous inducer need be added.

Nature of the Pathway from Glucose to Serine

The actual enzymes of the pathway for serine biosynthesis in E. coli and S. typhinurium have been described (see previous diagram). Umbarger and Umbarger, (1962) and Umbarger et al (1963), were the first to study in extracts the activities of the three enzymes involved in the conver-

sion of 3-phosphoglycerate to serine both in the prototroph and two serine auxotrophs of S. typhimurium. One of the auxotrophs studied was unable to synthesize 3-phosphoglycerate dehydrogenase while the other was deficient in phosphoserine phosphotase. Similar studies were done in E. coli (Umbarger et al 1963; Piser, 1963; Piser and Potochny, 1964). The fact that strains deficient in a particular enzyme are unable to make serine is evidence that the pathway in which it participates actually provides serine to the cell. Further proof that this pathway is of physiological significance comes from Piser (1963) who showed that in E. coli two of the three enzymes of the pathway, the dehydrogenase and the phosphatase, are inhibited by high concentrations of serine. He found that the first enzyme of the pathway, the dehydrogenase, was more sensitive than the phosphatase, and concluded that this represented end product inhibition, as a control of the functioning of the pathway. These two kinds of evidence; description of enzymes and their absence in serine/glycine auxotrophs and end product inhibition, do strongly indicate that this is the functioning biosynthetic pathway. Finally, the physiological importance of this pathway has been confirmed by identification of the enzymatic step at which the pathway is controlled.

Studies on the Interconversion of Serine and Glycine

Shemin (1946) was the first to show that serine was converted to glycine in rats fed serine labelled with ^{15}N amino and ^{13}C carboxylic groups. The fact that the glycine formed had the same $^{15}N/^{13}C$ ratio as the serine indicated that this was a direct conversion of serine to glycine. That this also occurs in E. coli was shown by Roberts et al (1955) who observed that when the cells were grown on ^{14}C glucose there was incorporation into serine and glycine. However, if glycine was added as a competitor, counts were observed in serine but not in glycine. Serine as a competitor reduced incorporation into both serine and glycine (Table 1).

Competitor Added	Radioactivity of Bacterial Amino Acids Relative to Control	
	Gly.	Ser.
None (control)	100	100
Glycine	0	100
Serine	5	5

Table 1: Metabolism of Radioactive Glucose in E. coli: Effect of supplements on the incorporation of radioactivity into the amino acids (Roberts et al, 1955).

This data indicates that in a prototroph grown with glucose,

glycine is made from serine.

The enzyme (STHM) responsible for the interconversion of serine and glycine has been studied by several investigators. Holland and Meinke (1949) showed that in bacteria tetrahydrofolateacid (FH_4) was required as a cofactor in the reaction. Roberts later (1955) demonstrated that the β carbon of serine was released in its conversion to glycine. It is thought that this β carbon is transferred to the cofactor FH_4 forming N^5N^{10} methylene FH_4. Thus FH_4 acts as a one carbon carrier in the reaction and in this way the enzyme provides not only glycine but one carbon units used in the biosynthesis of various compounds including purines, methionine, histidine and thymidine.

Synthesis of Glycine from Threonine

While there appears to be only one physiologically important route for serine and glycine biosynthesis in E. coli several instances of the derivation of glycine from threonine have been reported in other organisms.

Meltzer and Sprinson (1952) studied the incorporation of ^{15}N and ^{14}C labelled threonine in rats and found that one-fifth to one-third of the threonine was cleaved to give glycine. Previously Braunstein and Vilenkina (1949) had observed in mammalian liver and kidney that threonine was cleaved to give glycine and acetaldehyde. Later the

enzyme responsible for this reaction, threonine aldolase, was described by Karasek and Greenberg (1964). Using purified preparations of the mammalian enzyme they were able to show a requirement for pyridoxal phosphate.

Certain microorganisms have been shown to convert threonine to glycine. Working with the anaerobe Clostridium pasteurianum Dainty and Peel (1970) found that ^{14}C from aspartate was incorporated into threonine, glycine and serine. They interpreted this as indicating that this organism obtains about half of its glycine from serine and the other half from threonine. They assayed and purified the enzyme responsible for the cleavage of threonine in cell free extracts by measuring acetaldehyde formation.

The presence of this enzyme among aerobic bacteria has also been demonstrated. Morris (1969) working with Pseudomonas putida, growing on threonine, has shown that the first step in the utilization of threonine as a carbon and energy source involves the enzyme threonine aldolase. This catabolic form of the enzyme possesses different properties from the biosynthetic enzyme of clostridium. Another aerobe in which this enzyme has been studied was Bacillus subtilis (Willetts and Turner, 1971). They concluded that the characteristics of the enzyme in this organism suggested a catabolic role for it similar to that in Pseudomonas.

The evidence therefore suggests that in anaerobes threonine aldolase functions as part of a major pathway for the formation of glycine while in the aerobes, Pseudomonas and B. subtilis, it functions as the first step in a pathway which enables threonine to be used as a carbon and energy source.

In E. coli the evidence for the formation of glycine from threonine, possibly by threonine aldolase, indicates that this conversion occurs only in the presence of exogenous threonine. Roberts et al (1955) showed that when E. coli is grown with glucose and carbon dioxide as carbon sources, threonine is not a major source of glycine. This is indicated by the fact that $^{14}CO_2$ is heavily incorporated into threonine (via oxaloacetate and aspartate) but only slightly into glycine. However, adding exogenous threonine U^{14}C to the culture resulted in incorporation into glycine, such that fifty per cent of the glycine formed was derived from threonine. Piser and Potochny (1964) working with a serine/glycine auxotroph of E. coli observed slow growth on exogenous threonine. However, when the strain was grown with limiting amounts of serine growth stopped when the serine supply was exhausted, indicating again that endogenous threonine was not capable of supplying enough glycine for further growth. Van Lenten and Simmonds (1965) were able to isolate a derivative of a serine/glycine auxotroph E. coli which would grow on exogenous threonine. When

^{14}C threonine was provided extensive conversion to glycine was seen.

The previous experiments all indicate that exogenously provided threonine is converted to glycine and endogenously formed threonine is not. However, some evidence in the literature indicates that in E. coli some endogenous threonine can be converted to glycine. Simmonds and Miller (1957) described the surprising fact that when a serine/glycine auxotroph was supplied with exogenous serine or glycine, it did not obtain all of its serine and glycine from exogenous sources, but synthesized some five to fifteen percent from glucose (Table 2).

Growth Supplement	Percent of Bacterial Amino Acids Synthesized from Glucose	
	Glycine	Serine
Serine	14	4
Glycine	8	8

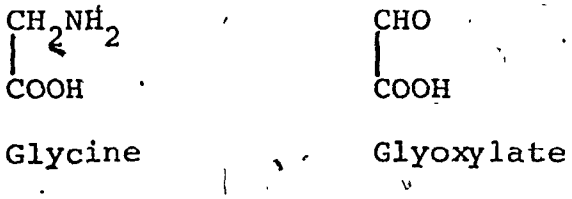
Table 2: Shows the percentage of glycine and serine synthetized from glucose when a serine/glycine auxotroph of E. coli was grown on serine and glycine. (Simmonds and Miller, 1957).

It is clear then that some other pathway of glycine biosynthesis can function under some circumstances and it appeared likely to the investigators that the pathway included

threonine as a precursor of glycine.

Conversion of Glyoxylate to Glycine

Biochemically glyoxylate appears to be closely related to glycine.



Glycine Glyoxylate

The two compounds could be interconverted by a single enzyme, a transaminase. Such an enzyme has been described in mammalian cells but not in E. coli (Meister 1965). Glyoxylate occurs in E. coli as part of the glyoxylate cycle. However, there is no evidence that glyoxylate is converted to glycine in E. coli and indeed glyoxylate is extensively formed in E. coli only when compounds such as acetate are used as a carbon source. In cells grown with glucose the key enzymes of the glyoxylate cycle have been shown to be repressed (Kornberg 1963). In this study glucose was used as a carbon and energy source. Thus it seems very unlikely that this pathway would function under these circumstances.

Sources of One Carbon Units in E. coli

In the conversion of serine to glycine by STHM two products are formed, glycine and one carbon units (C_1). This serves as the major source of C_1 production in the cell. Thus mutants lacking this enzyme must not only have an

alternative source of glycine but also of one carbon units.

One of these sources is exogenous glycine itself, formed by the glycine cleavage pathway worked out in Diplococcus glycinophilus by Sagers and Gunsalus (1961) and Arthrobacter globiformis by Kikuchi (1973). Here carbon one of glycine is released as carbon dioxide while carbon two is transferred to the one carbon carrier, tetrahydrofolic acid. The details of the pathway are not known, however, it is known to be repressed by the intracellular level of one carbon units and induced by glycine (Newman and Magasanik, 1963).

An alternative source of one carbon units has been shown by Newman, Miller and Kapoor (1973) in which serine is the donor by a pathway which does not include STHM. They concluded that this was the only other pathway of one carbon synthesis available to the cell. Thus a mutant lacking STHM, and synthesizing its glycine from threonine, would have to derive its C_1 units from one or other of the above pathways.

Involvement of Glycine and One Carbon Units in Purine Biosynthesis

Studies of one carbon and glycine biosynthesis often rely on the fact that both are precursors of the purine ring and that the specific activity of purines, which is easily determined, can often be used as an indication of the

specific activity of glycine and/or one cabon units, which is not so easily determined. Sources of the various atoms which make up the purine ring are shown in the diagram.

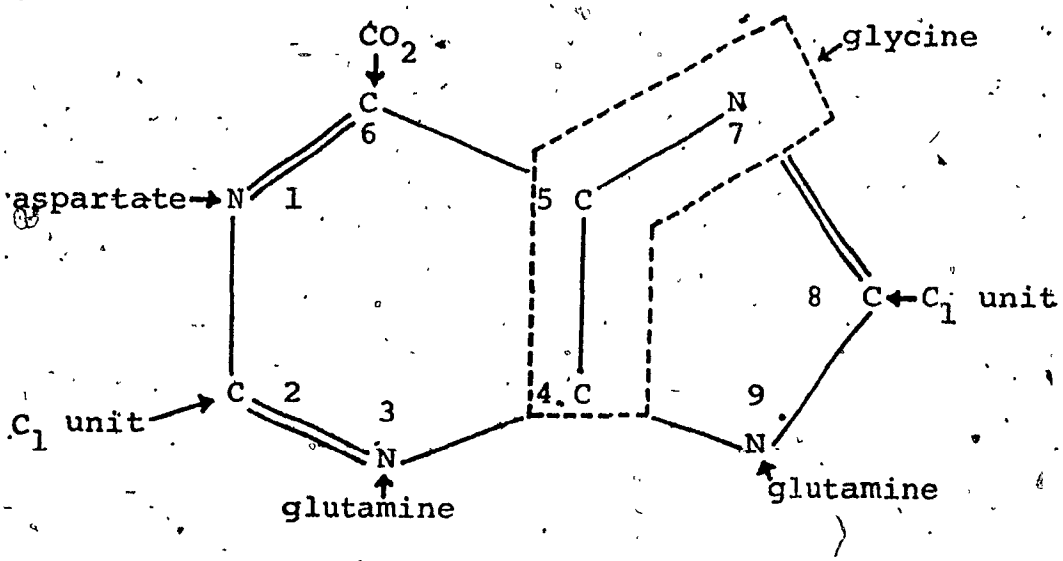


Figure above shows the sources of the various atoms from which the purine ring is synthesized (Meister, 1965). It can be seen that a molecule of glycine is incorporated intact into purines, while one carbon units are used to form carbon two and eight of the ring.

METHODS AND MATERIALS

Growth of Bacteria

Bacteria were grown at 37°C in a gyrotory water bath shaker (New Brunswick Scientific Co., N.J., Model G76) at approximately 180 rpm.

Cells from 8-hour yeast tryptone slant cultures grown at 37°C were inoculated into one-liter Erlenmeyer flasks each containing 100 ml of fresh media and glucose as a carbon and energy source. Auxotrophic strains were grown in media with the required supplement, as outlined in the text.

All strains were grown overnight to the appropriate turbidity required for the particular experiment and auxotrophic strains were then retested to make certain that the culture had not reverted to prototrophy. Growth is defined as an increase in turbidity. This was followed by measuring the turbidity of 2 ml portions of the cultures in a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co. Inc., New York, N.Y., Model 800-3), using a blue, No. 42 filter (spectral range 400-465 mμ).

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% $MgSO_4 \cdot 7H_2O$ and 0.001% $CaCl_2$ at pH 6.4. All solutions were

autoclaved at 15 lb/in^2 for 15 minutes. The carbon and energy source, (glucose) was autoclaved separately and added to a final concentration of 0.2%. Other additions to the growth medium were made according to the requirements of the experiment as outlined in the text.

Cultures of all strains were kept by frequent transfer on slants of yeast tryptone agar containing 1% yeast extract, 1% tryptone, 2% agar and 0.25% K_2HPO_4. Glucose was added to a final concentration of 0.01%.

Plating experiments were done on simple 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 experiment. Glucose was sterilized and added separately to a final concentration of 2%.

Sources of Strains

The representative prototroph used in most experiments involved E. coli K10, a prototrophic F^+ strain of E. coli K12 obtained from A. Garen, Yale University.

Strains HM100, 119 and 129 are serine/glycine auxotrophs obtained from H.E. Umbarger, Perdue University.

Strain BF34 is a double auxotroph obtained from P. Berg, Stanford University, requiring tryptophane and glycine, and shown to be deficient in serine transhydroxymethylase due to a mutation at the gly A locus.

Strain AT2046 is a glycine requiring strain obtained from Dr. L. Pizer. It has also been shown to be STHM deficient, but has not been mapped.

Strains JEV73 and JEV73R are spontaneous mutants isolated from strain AT2046 as described below.

Isolation of Strain JEV73

Strain AT2046 was incubated in medium with glycine (3 µg/ml) and leucine (250 µg/ml). This quantity of glycine was generally found to limit growth resulting in a low turbidity reading of only 50 Klett Units (K.U). However, during these experiments, such a culture was found to result in a high turbidity reading of over 200 K.U. Cells from this culture were investigated further and subcultured into medium with only leucine (250 µg/ml). Single colonies were isolated from dilutions of this culture plated on minimal medium plates supplemented with leucine. These were then retested to assure that they could not grow on unsupplemented medium. A culture originating from one such single colony was used under the name of JEV73.

Isolation of JEV73R

Strain JEV73 was plated at high density on minimal medium plates without further supplement. Colonies were isolated and restreaked for single colonies on minimal medium. A culture originating from one such single colony

was used under the name of JEV73R.

All organisms were maintained in stock on slants of yeast tryptone agar at 4°C. The composition of this and other growth media have been described previously.

Chemical Methods

Nucleic Acids and Proteins

Nucleic acids and proteins were isolated by a Schmidt-Thannhauser fractionation. The nucleic acids were hydrolyzed, purines isolated and their specific activity determined, all according to the methods of Revel and Magasanik (1958). This fractionation involved growing the strain to be investigated under the appropriate growth conditions with a radioactive tracer as described in the results section. These cells were generally harvested at a turbidity reading of about 200 K.U. and extracted by the addition of 5% cold trichloracetic acid. The macromolecules were precipitated and the lipids solubilized by the addition of ethanol. The alcohol insoluble fraction was extracted with hot trichloracetic acid resulting in two fractions. One was the hot trichloracetic acid soluble fraction (containing nucleic acids) and the other the hot trichloracetic acid insoluble fraction (containing proteins). The nucleic acids were then hydrolyzed and the purines precipitated out with silver nitrate. The purine bases were

further separated by paper chromatography (Whatman No. 3) in one dimension (isopropanol:HCl:H_2O::65:19:17). The adenine and guanine separated in this way were eluted in water and their specific activity determined. This is a measure of the number of counts per minute of radioactivity per μ mole of purine extracted.

Radioactivity was determined by counting duplicate samples of 0.1 ml of adenine and guanine in 10 mls of 0.5% 2,5-diphenyloxazole and 0.2% 2-(5-phenyloxazoly)-benzene in toluene in a Nuclear Chicago Unilux II counter. All samples are calculated at the same efficiency of counting. The quantity of purine was determined by their absorption between 200-400 nm in a phosphate buffer at pH 7.2 using a Unicam Sp 800 spectrophotometer.

Proteins separated in the Schmidt-Thannhauser fractionation were washed with alcohol-ether and hydrolyzed in 6N HCl for 18 hours at 118°C. Hydrolysates were dried over $CaCl_2$ in the presence of wet KOH and separated by paper chromatography (Whatman No. 1) in two dimensions, (first: Sec-butyl alcohol:formic acid:H_2O::70:10:20) (Second: phenol: CON.NH_4OH:H_2O::80:0.3:20) as indicated by Roberts et al (1955).

Radioautography of these chromatograms was performed also according to the method of Roberts. Thus chromatograms were exposed to Kodak noscreen Xray films 8" X 10" for 3-6

weeks and developed in the usual manner. Chromatograms were then sprayed with ninhydrin, and a comparison of ninhydrin positive spots was made in order to identify radioactive amino acids.

Enzyme assays

Serine transhydroxymethylase activity was assayed by the method of Taylor and Weissbach, (1965) as modified by Folk and Berg (1970). This assay measures the rate of formation of $H^{14}CHO$ formed from the hydrolytic cleavage of serine $3^{14}C$. The reaction mixtures (0.4 ml) consisted of the following: 0.05 ml of 75 mM potassium phosphate buffer (pH 7.4), 0.05 ml of 25 mM pyridoxal phosphate, 0.08 ml of 10 mM dl-tetrahydrofolic acid, 0.05 ml of 25 mM 2-mercapto-ethanol, 0.07 ml distilled water and 0.05 ml enzyme extract diluted to various concentrations in phosphate buffer with 0.01 M 2-mercaptoethanol. This was then preincubated at 37°C for 5 minutes at which time 0.05 ml of serine $3^{14}C$ was added and the mixture again incubated for 9 minutes at 37°C. The reaction was stopped by adding 0.3 ml of cold 1 M sodium acetate (pH 4.5). The tubes were then chilled in ice water for 5 minutes after which 0.2 ml of 1 M formaldehyde was added followed by 0.03 ml of 0.4 M 5,5-dimethyl-1,3-cyclo-hexanedione (dimedon) in 50% ETOH. (The formaldehyde

equilibrates with the $^{14}CH_2OH$-FH_4 cleaved from serine 3 ^{14}C and is then precipitated with 5,5-dimethyl-1,3-cyclohexanedione). The tubes were then heated for 5 minutes at 95°C and allowed to cool. The precipitates were then collected by vacuum filtration on millipore glass paper filters, washed with 35 ml of cold distilled water, dried and counted to determine the amount of $H^{14}CHO$ formed during the assay.

Threonine deaminase was assayed according to the method of Pardee and Prestige (1955) as modified by Isenberg and Newman (1974). This assay measures the production of α-keto acid (α-ketobutyrate) from L-threonine. The incubation mixture contained 0.3 ml of washed cells (in a phosphate buffer, pH 7.5), 0.02 ml of toluene, and 0.1 ml of the substrate at either 10 or 20 mg/ml. This mixture was incubated for 35 minutes at 37°; 0.9 ml of 2,4 dinitrophenylhydrazine (DNPH) in 4.1% hydrochloric acid was added, and the mixture incubated for a further 20 minutes at room temperature. Following this 1.7 ml of 10% NaOH was added and the absorbance determined with a Klett-Summerson colorimeter No. 54 filter using pyruvate as a standard. Included in this assay was one set of tubes to which no substrate was added. This allowed a measure of endogenous α-ketoacid production. Activity is expressed as the difference between the amounts of keto acid formed with and

without substrate, related to the amount of protein in the cells.

The leucine used for studies of the inhibition of threonine deaminase was purchased from Sigma biochemicals and shown to be essentially isoleucine-free. This was determined according to the method of H.E. Umbarger (Personal communication). Strain 1008, an isoleucine requiring strain, obtained from M. Levinthal (Perdue University) was used in this assay. It was grown with isoleucine to log phase and the cells thoroughly washed. They were then incubated in minimal medium plus 1-^{14}C glycine and the leucine to be tested. Incorporation of 1-^{14}C glycine under these conditions was compared with the incorporation observed of this isotope in the presence of increasing amounts of isoleucine.

Protein determination

Proteins were determined by the method of Lowry et al (1951) using trypsin as a standard.

Chemicals used

Radioactive compounds were purchased from Amersham-Searle. All other chemicals were obtained from Schwartz-Mann, Orangeberg, N.Y. or Sigma, Saint Louis, Missouri.

RESULTS

Various serine glycine auxotrophs of E. coli that use exogenous threonine as a source of glycine were described by Piser (1964, 1965). Preliminary tests in our laboratory showed that strain AT2046 (a glycine requiring strain) could adapt easily to growth with threonine in place of glycine. It could therefore use exogenous, but not endogenous, threonine in this way. This strain thus appeared to provide a good source of material for the study of the conversion of threonine to glycine. The results section of this thesis thus begins with the quantitative aspects of the use of threonine and glycine by strain AT2046. A surprising quantitative relationship between leucine and glycine is described. That a close metabolic relationship between leucine and glycine does exist becomes even more obvious with the isolation of a derivative of AT2046 in which not only threonine but also leucine can replace glycine as sole growth factor. This investigation is taken a step further with the isolation of JEV73R, a derivative of JEV73, which would grow on unsupplemented medium. These new strains JEV73 and JEV73R, are next described and their growth characteristics determined. Tracer studies demonstrating the use of a new pathway for glycine biosynthesis which differs from that used in the prototroph K10 are described. An attempt is made to determine the nature of the physiological changes that have

occurred in the mutation of AT2046 to JEV73 and JEV73R which permits their use of this new pathway.

I. The Ability of Serine/Glycine and Glycine Mutants to Grow on Threonine.

Piser and Potochny (1964) and Piser (1965) had previously reported that serine/glycine auxotrophs normally grown with glucose and glycine, could grow slowly with glucose and threonine. To determine whether the isolate of AT2046 used in our laboratory showed the same characteristics, the strain was grown with glycine 100 µg/ml and subcultured into medium containing 2 µg/ml with and without threonine (250 µg/ml). The glycine alone allowed only limited growth (44 K.U.); however, when threonine was also provided, the culture grew to over 200 K.U. AT2046 grown with threonine, in this manner, could then be subcultured in medium supplemented with only threonine and this would result in extensive growth. It was noted, however, that some subclones of this mutant were unable to grow with threonine even when preadapted in the usual manner. This indicates that these subclones lack either the information to synthesize the enzyme(s) needed for glycine biosynthesis or the ability to express such information on subculture.

To see whether other serine/glycine or glycine auxotrophs might have the same characteristics strains HM100, HM119, HM129 and BF34 were tested in the same way. None of the strains tested, including the other STHM deficient strain BF34, could adapt to the use of threonine as a source of glycine.

II. Use of Threonine By AT2046

Mutants with a metabolic block in a pathway are unable to synthesize the end product of that pathway. Thus no growth occurs without the addition of this growth factor and the extent of growth will be limited, within a certain range, by the amount present in the medium. When a mutant is grown with a concentration of nutrilite within a limiting range, the amount of cell material formed is proportional to the amount of nutrilite provided. Generally mutants grown with the required supplement, at limiting concentrations, will show a linear response to increasing concentrations of this required supplement. However, AT2046 growing on glycine has been reported (Newman, et al 1974) to show such a response only at low (0 to 0.2 µmoles per ml) glycine levels. At higher levels total growth of the culture was considerably lower than would be anticipated from the results at lower levels of glycine. Thus, glycine is used in such a way that low levels are used more efficiently than higher levels.

It was of interest to determine whether threonine was used in a similar way.

To test this, strain AT2046 was preadapted to growth on threonine as previously described and subcultured into media with increasing concentrations of threonine, between 0 and 1.6 μmoles/ml. The turbidity was measured until two readings taken three hours apart were constant. The cultures were then plated on minimal medium plates with and without threonine to ensure that none had reverted to prototrophy during the experiment. It can be seen from Figure 2 that at higher levels of threonine (above 0.83 μmoles/ml) total growth of the culture is not proportional to the amount of threonine added, while at lower concentrations a linear response is observed. Newman (1974) observed that this mutant growing on glycine requires 0.13 μmoles/ml of glycine to reach a turbidity of 90 K.U. However, it can be seen that when threonine is used as a source of glycine in AT2046 a much higher level of threonine (0.5 μmoles/ml) is required to reach the same turbidity.

III. A Relationship Between Glycine and Leucine in Strain AT2046

An auxotroph will not grow in the absence of its required growth factor. When this growth factor is supplied growth will be proportional to its concentration. However, in some cases a second growth factor is found which does not

support growth when provided alone but does increase the amount of cell material formed from a given amount of the first factor. This phenomenon is called sparing and indicates a close metabolic relationship between these two substances. Thus, it is often helpful in pathway studies to determine if a limiting nutrile is spared by the addition of some other factor. In strain AT2046 it was found that leucine spared its requirement for glycine. This was observed by growing AT2046 in minimal medium supplemented with 100 µg/ml glycine and subculturing in triplicate into medium supplemented with 3 µg/ml glycine with and without leucine 100 µg/ml. The turbidity was measured until two readings taken three hours apart were the same to ensure that the cultures had stopped growing and that the total growth observed was therefore the maximum growth possible. From Table 3, it can be seen that cultures supplemented with 3 µg/ml glycine showed limited growth, an average of 44 Klett units, while those in which 100 µg/ml leucine was also added showed an average of 111 Klett units. Thus with the addition of leucine the mutant was able to grow to a turbidity that was 2.5 times greater than that observed for the same amount of glycine without leucine. To ensure that this observation was not an artefact of the turbidity readings the protein content of the cultures was measured and it was found that indeed the increase observed in Klett units

Sparing of Glycine by Leucine in AT2046

	Supplement (µg/ml)		Turbidity (K.U.)	Protein (µg/ml)
	Glycine	Leucine		
(A)	3	-	43,45,44 average 44	23,26,26 average 25
(B)	3	100	110,125,98 average 111	57,72,56 average 61.6
	Gly Leu/Gly		2.5	2.4

Table 3: Sparing of glycine by leucine in strain AT2046. Cells were precultured in minimal medium with glycine and subcultured in triplicate into (A) minimal medium supplemented with 3 µg/ml glycine and (B) minimal medium supplemented with 3 µg/ml glycine and 100 µg/ml leucine. The average turbidity in Klett units and protein measured in µg/ml of culture is shown above.

correlated with a similar increase in protein content. Thus 2.4 times more protein was synthesized in the presence of leucine than in its absence; however, leucine alone did not support the growth of this mutant. Thus the addition of leucine permitted increased growth with glycine, but leucine could not replace glycine entirely. This sparing of glycine by leucine indicated a close metabolic relationship between these two amino acids which was surprising since no basis for such a relationship has been indicated in the biochemical literature.

IV. Isolation and Characterization of Mutant Derivatives of AT2046.

That a relationship does indeed exist between leucine and glycine in metabolism became even more obvious with the isolation of a derivative of AT2046, strain JEV73, which required leucine or glycine or threonine for growth (see Methods section). Thus in this strain leucine no longer spared but could replace glycine as sole growth factor. To determine whether amino acids other than threonine, glycine and leucine would support growth, JEV73 was grown in minimal medium supplemented with 25 µg/ml of each of the following amino acids tested separately: valine, proline, isoleucine plus valine, tryptophane, alanine, arginine, phenylalanine, glutamic acid, lysine, histidine, tyrosine, isoleucine, aspartic acid and methionine. None of these amino acids

tested supported growth. Several other compounds tested also failed to support growth. These included Biotin (0.21 µg/ml), known to be a cofactor in several enzymatic reactions, xanthine (20 µg/ml), a purine precursor and often reported to spare glycine and sodium formate (500 µg/ml), known to support the growth of certain serine/glycine auxotrophs of E. coli (Newman, 1970).

When describing a new mutant dose response curves to its various nutritional requirements are generally performed as was described for AT2046. An attempt was made to determine the dose response of strain JEV73 to its various growth supplements. However, this strain was found to revert to prototrophy at such a high frequency that it was impossible to perform the standard dose response curves. It was possible, however, to determine the lowest amount of each supplement which would ensure growth to an extent unlimited by the supplement without the accumulation of revertants. This was found to be 150 µg/ml of threonine or 50 µg/ml of glycine or 5 µg/ml leucine. This can be compared with strain AT2046 in which 250 µg/ml of threonine or 75 µg/ml glycine are required. The finding that JEV73 would grow in medium supplemented with very low amounts of leucine (5 µg/ml) but not grown in unsupplemented minimal medium was most surprising, especially in view of the fact that the strain is not a leucine auxotroph and can make its own leucine. This seemed

to indicate that leucine, provided in such low quantities, was not acting directly as a precursor of another compound but allowed the activation of a metabolic pathway to glycine which was not used in the absence of leucine. Further evidence to support this conclusion was indicated by experiments in which the ability of JEV73 to make transitions between threonine, glycine and leucine was tested. From Figure 2, it can be seen that cells precultured in glycine to log phase, collected by centrifugation and washed, could then be transferred to leucine or threonine and growth would continue without any appreciable lag. In the same way cells pregrown with leucine would adapt to growth with either glycine or threonine. However, it is clear (Fig. 3) that when pregrown on threonine these cells could adapt to growth with glycine but were unable to adapt to growth with leucine. This lack of adaptation does not appear to result from a permeability problem with respect to leucine, nor from the lack of some cofactor since neither the addition of yeast extract (50 µg/ml) or increasing the quantity of leucine in the culture, up to 300 µg/ml, would enable these cells to begin growth (Table 4).

Thus it appears that JEV73 when growing with leucine obtains its glycine by a leucine dependent pathway the synthesis of the enzymes being repressed by threonine. Thus cells grown with threonine would have none of the required

Figure 2: Response of strain AT2046 to increasing amounts of threonine. AT2046 was precultured in minimal medium supplemented with 0.04 µmoles/ml glycine and 2.09 µmoles/ml threonine. This was then sub-cultured into minimal medium supplemented with increasing concentrations of threonine from 0.0 to 1.6 µmoles/ml. Growth was followed until a constant turbidity was obtained in all flasks. The average turbidity for each concentration studied is plotted on the next page.

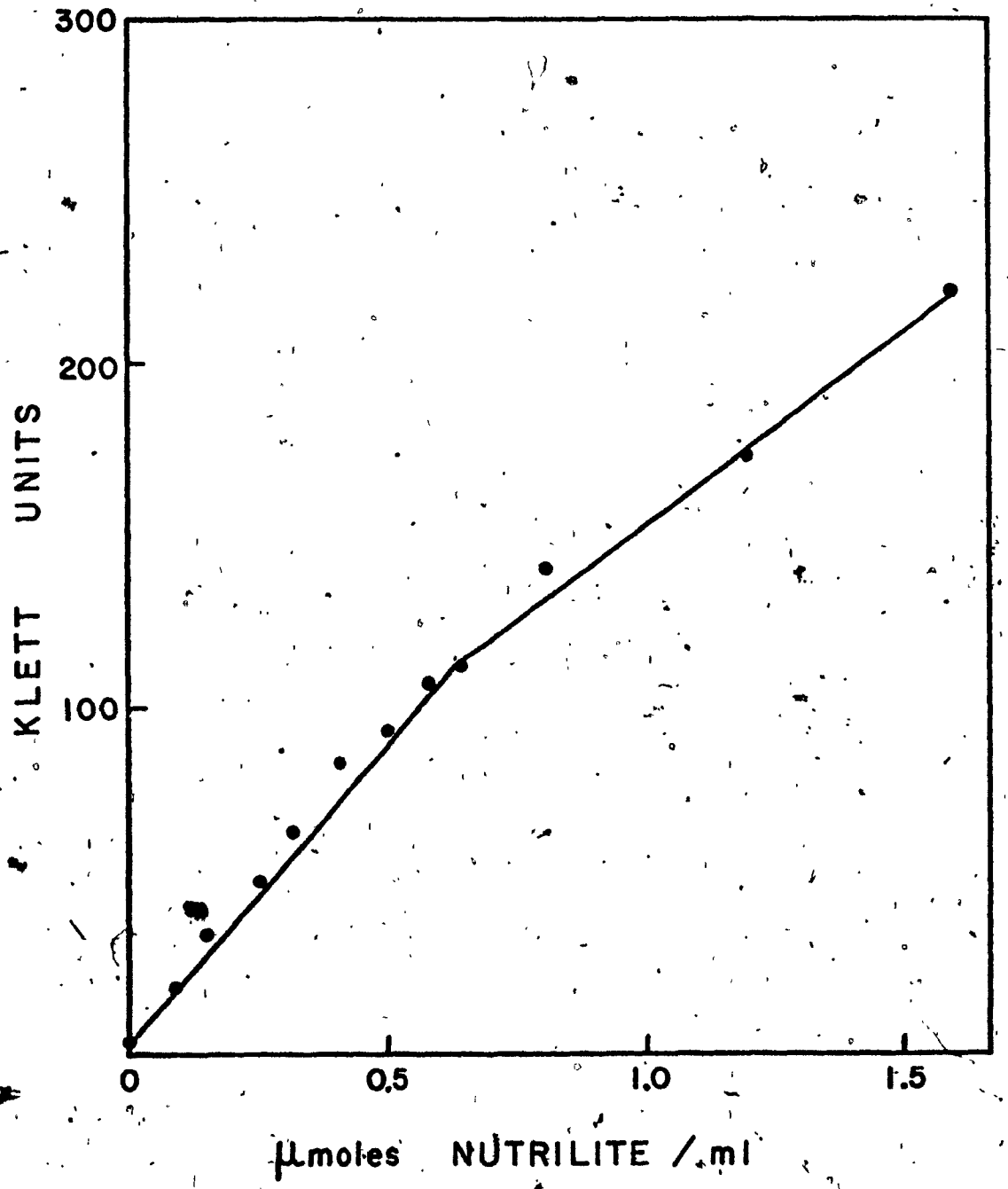
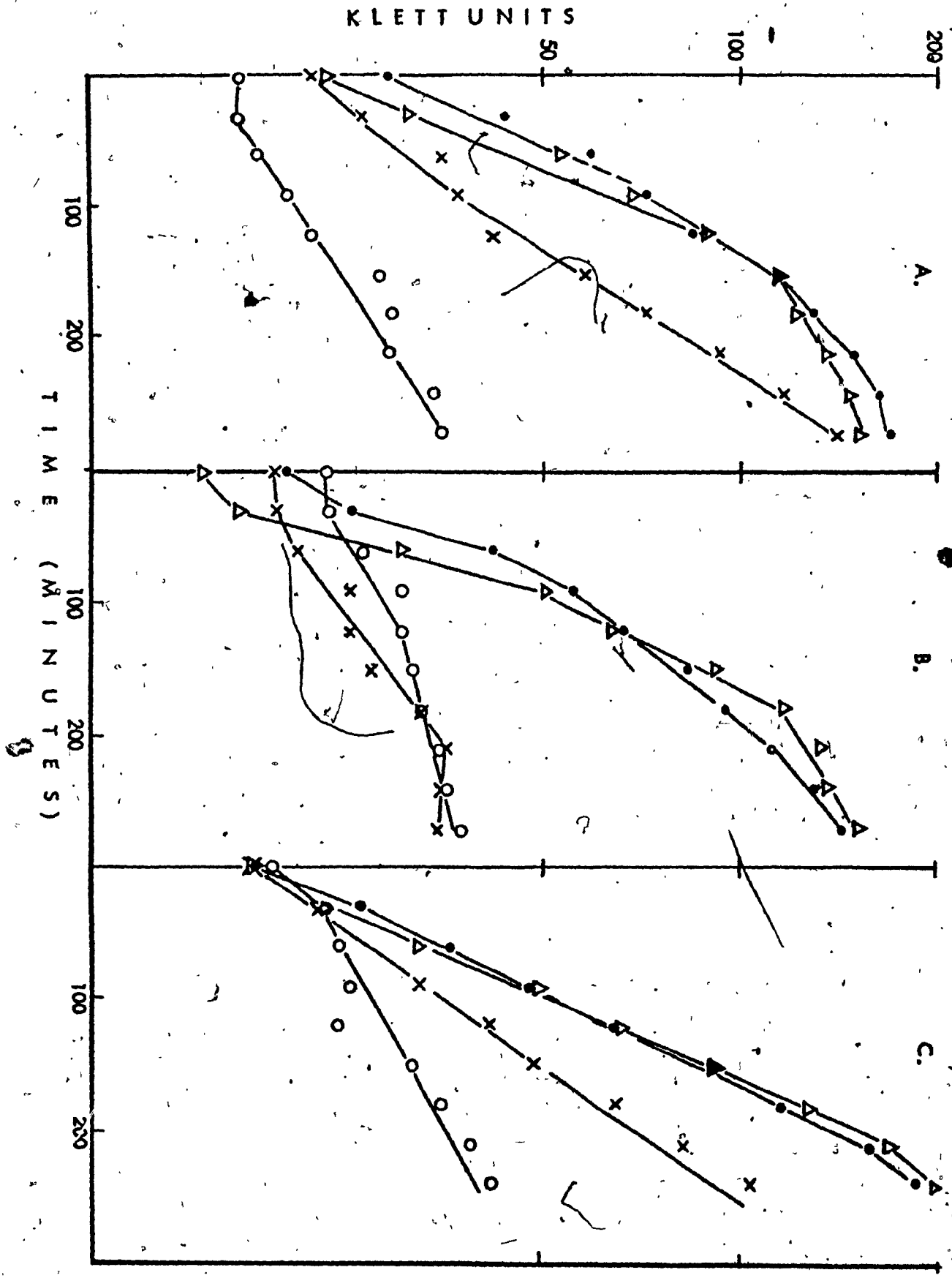

300
200
100
KLETT UNITS
0
0.5
1.0
1.5
µmoles NUTRILITE /.ml

Figure 3: Growth response of E. coli strain JEV73 previously grown on glycine (a), threonine (b) and leucine (c). The cells were collected in log phase, centrifuged and suspended in media supplemented with: Δ——Δ, glycine; ●——●, threonine; x——x, leucine and in non-supplemented medium o——o.


KLETT UNITS
50
100
200
TIME (MINUTES)
100
200
A.
B.
C.

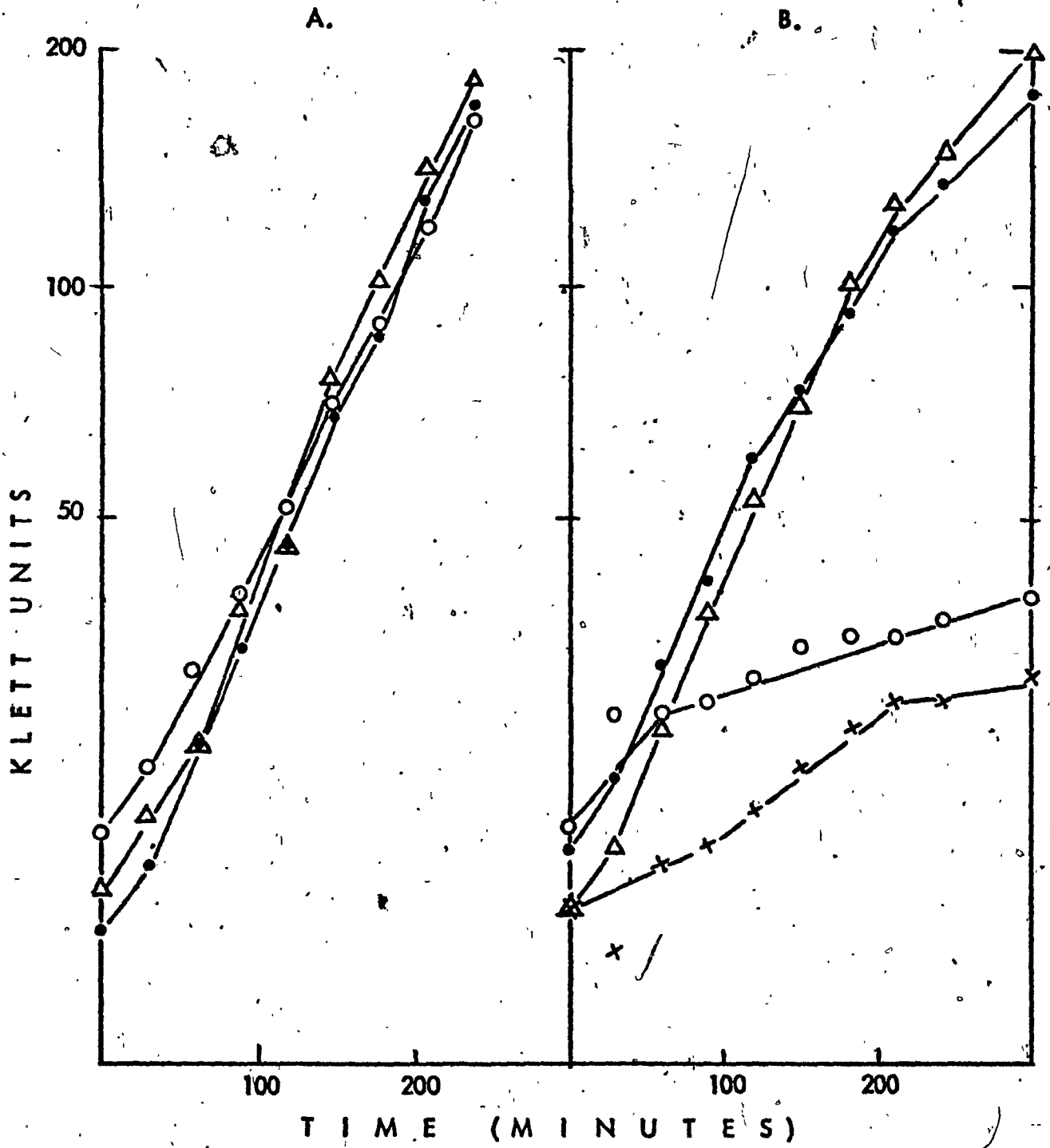
Supplement (µg/ml)	OD (KU) after 330 mins.	% Increase in OD	Apparent No. of generations
0	37	37.8	<1
Threonine (100)	167	88.0	3
Glycine (25)	170	91.1	3 1/2
Leucine (10)	34	44.1	<1
Leu(10) Yeast Extract (50)	51	40.3	<1
Leu(10) Yeast Extract (50)	27	59.2	1
Leucine (20)	34	44.1	<1
Leucine (50)	23	43.4	<1
Leucine (100)	38	57.8	1
Leucine (300)	31	1.6	1

Table 4: Growth response of E. coli [redacted]in JEV73 previously grown on minimal medium supplemented with threonine (100 µg/ml). The cells were collected in log phase, centrifuged and suspended in media containing the various supplements indicated above. The OD after 330 mins, the per cent increase and apparent number of generations resulting are shown above.

enzymes and when transferred to leucine would be starved for glycine and unable to grow.

JEV73 reverts to prototrophy much more frequently than its parent AT2046. If this reversion consisted in the restoration of STHM activity, it would seem that the strains would do this with the same ease. The fact that they do not indicates that when JEV73 reverts to prototrophy it does so by establishing a new pathway of glycine synthesis independent of leucine. To examine this idea further JEV73 was plated on minimal medium (without mutagenesis) and a typical revertant isolated. Thus this strain, JEV73R, would grow in unsupplemented minimal medium. If JEV73R uses the same pathway as JEV73, the difference being only in its leucine-independence in JEV73R, then the same repression by growth in threonine might be seen in JEV73R as in JEV73. To test this JEV73R was pregrown in minimal medium supplemented with 500 µg/ml threonine, to log phase and transferred, as described previously, to media containing threonine or glycine or leucine or unsupplemented minimal medium. These cells were observed to adapt easily to growth on threonine or glycine but could not adapt to leucine or unsupplemented minimal medium. By contrast, when preadapted to growth on minimal medium these cells could adapt without difficulty to growth on leucine or glycine or threonine (Fig. 4). It can thus be

Figure 4: Growth response of E. coli strain JEV73R previously grown on minimal medium (a) and minimal medium supplemented with threonine (b). The cells were collected in log phase, centrifuged and suspended in media supplemented with: Δ——Δ, glycine; ●——●, threonine; x——x, leucine and in non-supplemented medium o——o.


A.
B.
200
100
50
KLETT UNITS
100
200
100
200
TIME (MINUTES)

concluded that in JEV73R glycine is derived from the same pathway as it is in JEV73.

V. STHM Levels in JEV73 and JEV73R.

It appears from the preceding experiments that strain JEV73 is supplied with glycine by a pathway, which is activated by low amounts of leucine, the enzymes of which are repressed by growth on threonine. In JEV73R this pathway can provide glycine whether or not leucine is present (that is, the strain can grow as a prototroph) but the enzymes can still be repressed by growth on threonine. As stated previously AT2046, lacking STHM, seldom reverts to prototrophy and JEV73 has a high reversion rate it therefore appears unlikely that the mutation in JEV73, which results in prototrophy, would be simply the restoration of STHM activity. If this was so the reversion rate would be the same for both AT2046 and JEV73.

To test this more directly STHM was assayed both in JEV73 and JEV73R and the levels compared with those in the prototroph K10. These strains were grown in minimal medium supplemented with 10 µg/ml leucine to log phase, JEV73 was tested to ensure that it had not reverted and STHM activity was assayed. From Table 5 it can be seen that in JEV73 and JEV73R there was no detectable activity while in K10 the usual enzymatic level was observed (Miller and Newman, 1974).

STHM Activity in Strains of E. coli K12

Strain	μmoles $H^{14}CHO$ Formed Per Hour Per mg. Protein
JEV73	0.01
JEV73R	0.01
K10	1.01

Table 5: Strains of E. coli indicated above were grown in minimal medium supplemented with leucine (10 μg/ml) to log phase, extracts of these cultures were prepared and STHM activity measured.

It is clear therefore that in JEV73 and JEV73R glycine is supplied by an alternative pathway which does not involve STHM and in this way differs radically from the prototroph K10.

VI. Source of Glycine Carbons in Strains JEV73, JEV73R and AT2046.

This alternative pathway for glycine biosynthesis which does not involve STHM is repressed by growth on threonine and this suggests that it involves the conversion of aspartate to threonine, followed by its cleavage to glycine, perhaps by the enzyme threonine aldolase. To determine what pathways were being used by the three mutant strains lacking STHM they were grown with various ^{14}C-labelled precursors and the extent of incorporation of ^{14}C into purines and amino acids was determined. The extent of incorporation into purines was used as a quantitative estimate of the extent of incorporation into glycine and one carbon units. The proteins from these experiments were also hydrolyzed and chromatographed. The distribution of ^{14}C into the various amino acids was determined by visual examination of radioautographs.

VI. (a) The Metabolism of Threonine by Strain AT2046.

AT2046 was grown with threonine U^{14}C (specific activity 1.4 X 10^{4}). The adenine and guanine showed an average specific activity of 1.4 X 10^{4} and 1.45 X 10^{4} cpm/μmole respectively (Table 6, line 1). This indicates that four purine carbons are labelled from threonine and suggests that AT2046 growing on threonine makes all its glycine from threonine and all its one carbon units from glycine derived in this way.

If this pathway is physiologically significant it might be expected to be inhibited by high concentrations of glycine in the cell. To test this AT2046 was grown with threonine U^{14}C plus non radioactive glycine (500 μg/ml). In this case the specific activity of the adenine and guanine showed a specific activity of 0.1 X 10^{4} and 0.06 X 10^{4} cpm/μmole respectively (Table 6, line 2). Thus the addition of glycine decreased conversion of threonine to glycine. Further evidence to support this pattern of incorporation from threonine was found in the hydrolyzed proteins extracted from these cells. When this strain was grown with threonine only both threonine and glycine were found to be radioactive while in the presence of non-radioactive glycine, protein glycine was only slightly labelled threonine still being extensively labelled. As expected threonine carbons

Source of Purine Carbons in Strains of E. coli K12 Using Threonine or Leucine or Aspartate as a Radioactive Tracer

	Strain	Radioactive Supplement cpm/μmole	Non-Radioactive Supplement μg/ml	Specific Activity Adenine cpm/μmole	Specific Activity Guanine cpm/μmole	Average No. of Purine Carbons Labelled
(1)	AT2046	$U^{14}C$ Threonine (1.4×10^4)	---	1.40×10^4	1.45×10^4	4
(2)	AT2046	$U^{14}C$ Threonine (1.4×10^4)	Gly (500)	0.10×10^4	0.06×10^4	0.22
(3)	JEV73	$U^{14}C$ Leucine (1.16×10^4)	---	0.01×10^4	0.008×10^4	<0.2
(4)	JEV73	$U^{14}C$ Aspartic Acid (0.83×10^4)	Leu (10)	0.51×10^4	0.58×10^4	2.7
(5)	JEV73R	$U^{14}C$ Aspartic Acid (0.83×10^4)	Leu (10)	0.30×10^4	0.33×10^4	1.5
(6)	K10	$U^{14}C$ Aspartic Acid (0.83×10^4)	Leu (10)	0.04×10^4	0.04×10^4	<0.2

Table 6: Strains of E. coli shown above were grown in minimal medium with various radioactive and non-radioactive supplements. The purines were extracted and their specific activities determined. The average number of these purine carbons, as determined from the ratio of the average specific activity of purine isolated to the specific activity per carbon of the threonine or leucine or aspartate supplied, is indicated.

were found distributed to the other metabolically related amino acids, isoleucine and to a lesser extent aspartate and glutamate.

VI. (b) The Metabolism of Leucine by Strain JEV73.

As previously mentioned all evidence indicated that at the very low concentrations of leucine required to support growth of JEV73 it was very unlikely that leucine itself was acting as a precursor of glycine or one carbon units in the cell. However, to confirm this strain JEV73 was grown with 50 µg/ml leucine (specific activity 1.16×10^4 cpm/µmole). No significant incorporation of leucine carbons into either adenine or guanine was found and no amino acid other than leucine itself was found to be labelled (Table 6, line 3). Thus leucine is required by this strain for growth but does not act as a precursor of glycine or one carbon units or any other amino acid of the cell.

VI. (c) The Metabolism of Aspartate by Strain JEV73.

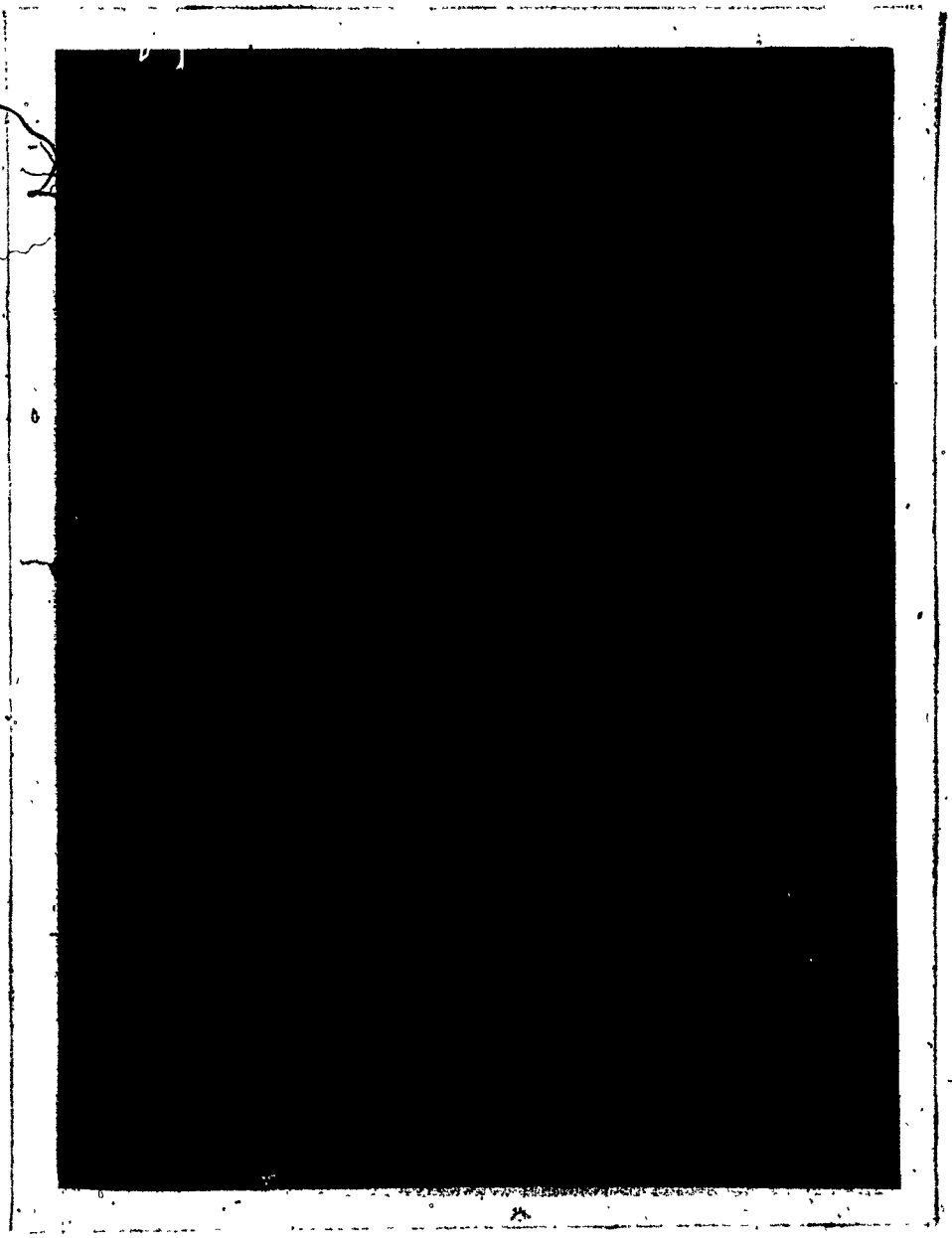
If, as it appears, leucine is activating an alternative pathway for glycine biosynthesis and if this pathway involves the conversion of aspartate to threonine followed by the cleavage of threonine to glycine, it should be possible to show the incorporation of aspartate carbons into the purines. To test this strain JEV73 was grown

with aspartate $U^{14}C$, 100 µg/ml (specific activity 0.83 X 10^4 cpm/µmole) and leucine 10 µg/ml. The adenine and guanine showed an average specific activity of 0.51 X 10^4 and 0.58 X 10^4 cpm/µmole respectively (Table 6, line 4). This indicates that 2.7 purine carbons are labelled from aspartate. An examination of the radioautograms showed that aspartate carbons were incorporated into aspartate, the other metabolically related amino acids arginine, lysine, glutamate, isoleucine, threonine and also into glycine but serine was not labelled (see figure 5).

VI. (d) The Metabolism of Aspartate by JEV73R.

To see if this pathway functioned in the same way in JEV73R, this strain was similarly grown with aspartate $U^{14}C$, 100 µg/ml (specific activity 0.83 X 10^4 cpm/ µmole). The ademine and guanine showed a specific activity of 0.30 X 10^4 and 0.33 X 10^4 cpm/µmole respectively (Table 6, line 5). This is lower than that observed for JEV73 however, it should be noted that both JEV73 and JEV73R are able to make aspartate de novo from glucose. Therefore once the added aspartate has been metabolized the cell will then begin synthesizing aspartate from glucose thus reducing the aspartate and purine specific activity. It may be that the two strains differ in the extent to which they synthesize

Figure 5: Shows a typical radioautogram of a protein hydrolysate extracted from strain JEV73, grown with the radioactive tracer aspartate $U^{14}C$. Heavy incorporation can be observed of aspartate carbons into arginine, lysine, glutamate, threonine and glycine but not into serine.



aspartate de novo under our experimental conditions. In any case an examination of the radioautograms from this experiment revealed the same amino acid labelling pattern as described for JEV73.

VI. (e) The Metabolism of Aspartate by the Prototroph K10.

To be sure that the incorporation pattern from aspartate seen in JEV73 and JEV73R was related to the phenotype, the same experiment was performed with the prototroph K10. Thus this strain was grown with aspartate U ^{14}C, 100 µg/ml (specific activity 0.83 X 10^4 cpm/µmole). No significant incorporation into purines was found. The adenine specific activity was found to be 0.04 X 10^4 and the guanine to be 0.04 X 10^4 (Table 6, line 6). When compared with the adenine and guanine specific activities observed for JEV73 and JEV73R this is reduced approximately by a factor of approximately 12.5 and represents an incorporation of less than 0.2 number of purine carbons labelled. An examination of the radioautograms for K10 also led to the conclusion that aspartate is not converted to glycine to any significant extent in the prototrophic strain. The same labelling pattern described for JEV73 and JEV73R was observed, with the exception that glycine was not labelled (see Figure 6).

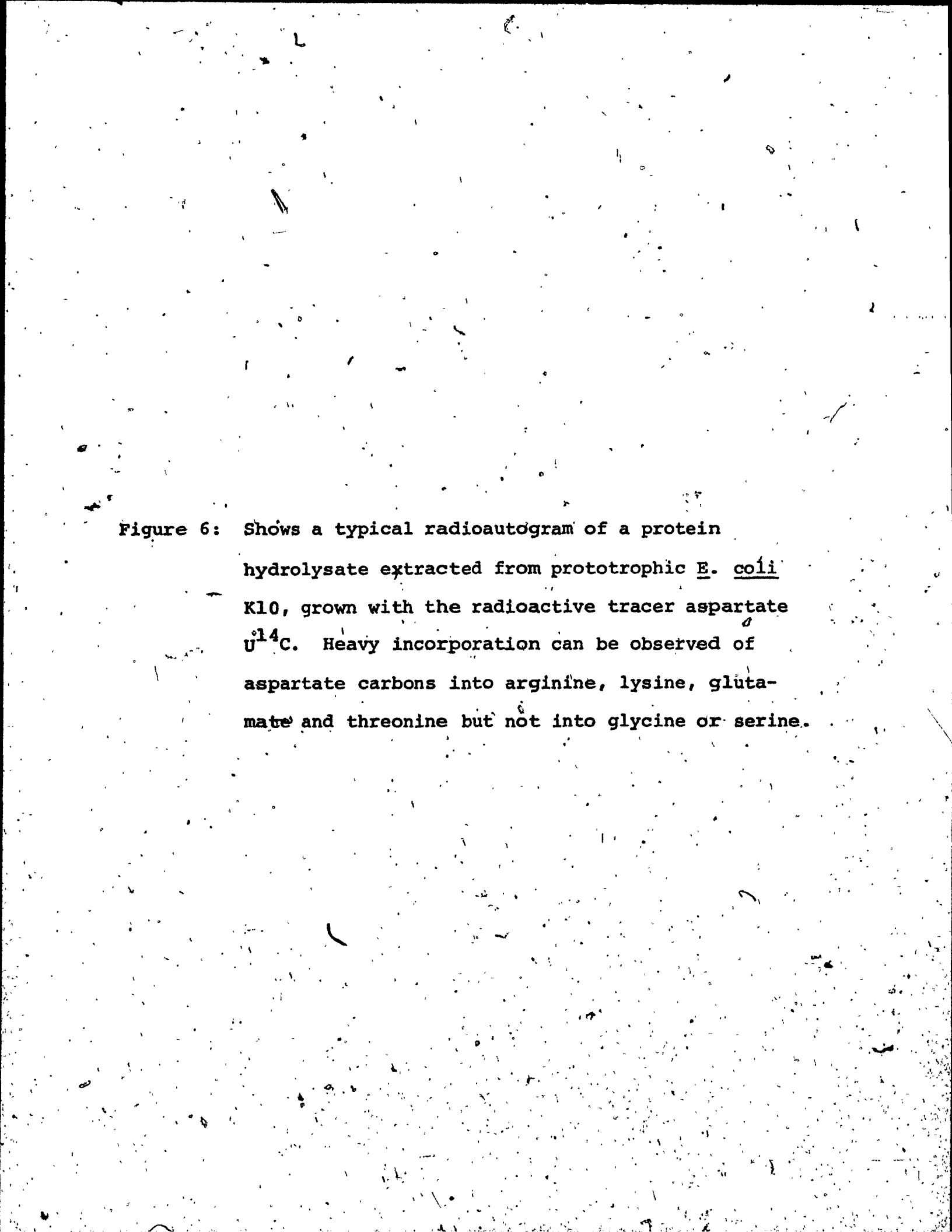
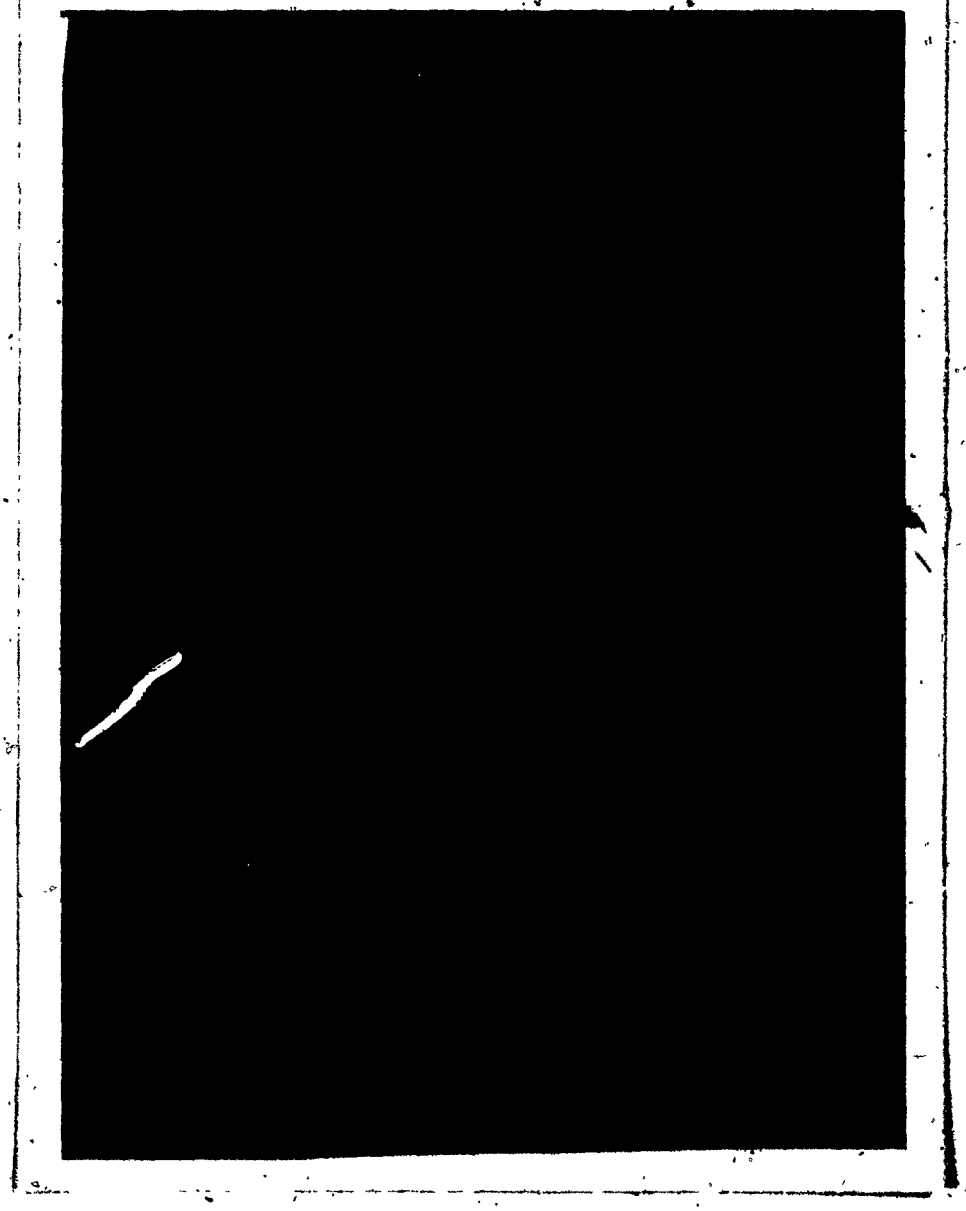


Figure 6: Shows a typical radioautogram of a protein hydrolysate extracted from prototrophic E. coli K10, grown with the radioactive tracer aspartate U-^{14}C. Heavy incorporation can be observed of aspartate carbons into arginine, lysine, glutamate and threonine but not into glycine or serine.



VII. Incorporation of Serine Carbons Into JEV73, JEV73R and K10.

As described the major pathway for glycine biosynthesis in prototrophic E. coli involves serine as the precursor of glycine, serine being converted to glycine by the enzyme STHM. Thus JEV73 and JEV73R, shown previously to lack STHM and convert aspartate to glycine, should metabolize serine differently from the prototroph K10, which has STHM activity and does not convert aspartate to glycine (to any extent).

VIII.(a) The Metabolism of Serine by Strain JEV73

JEV73 was grown with serine U^{14}C, 100 µg/ml (specific activity 0.51 X 104 cpm/µmole) and leucine. The adenine and guanine showed an average specific activity of 0.22 X 10^4 and 0.25 X 10^4 cpm/µmole (Table 7, line 1). This indicates that an average of 1.3 purine carbons were labelled from serine. This was surprising as JEV73 had been shown to lack STHM activity. Therefore in an effort to determine how serine carbon was incorporated into the purines JEV73 was grown with serine 3^{14}C, 100 µg/ml (specific activity 1.0 X 10^4 cpm/µmole). The adenine and guanine specific activity were both shown to be 0.2 X 10^4 cpm/µmole which indicates that less than one purine carbon (0.2) was labelled from serine (Table 7, line 2). When the radioautograms from these two experiments were examined the labelling pattern for the various amino acids

Source of Purine Carbons in Strains of E. coli K12 Using Serine as a Radioactive Tracer

	Strain	Radioactive Supplement cpm/μmole	Non-Radioactive Supplement μg/ml	Specific Activity Adenine cpm/μmole	Specific Activity Guanine cpm/μmole	Average No. of Purine Carbons Labelled
(1)	JEV73	Serine $U^{14}C$ (0.51×10^4)	Leucine (10)	0.22×10^4	0.25×10^4	1.3
(2)	JEV73	Serine $3^{14}C$ (1.0×10^4)	Leucine (10)	0.20×10^4	0.22×10^4	0.2
(3)	JEV73R	Serine $U^{14}C$ (0.64×10^4)	Leucine (10)	0.17×10^4	0.12×10^4	0.7
(4)	K10	Serine $U^{14}C$ (0.64×10^4)	Leucine (10)	0.73×10^4	0.72×10^4	3.47

Table 7: Strains of E. coli shown above were grwon in minimal medium with various radioactive and non-radioactive supplements. The purines were extracted and their specific activities determined. The average number of these purine carbons, as determined from the ratio of the specific activity of purine isolated to the specific activity per carbon of the serine supplied is indicated.

were the same. In both labelling was observed in serine, the metabolically related amino acids, glutamate, arginine, lysine, aspartate, threonine and glycine. However, glycine was labelled to a much lesser extent than serine. The observation that glycine was labelled even when serine $3^{14}C$ was used indicated that serine was not being converted to glycine by STHM. The mode of action of STHM is well known and it is the third carbon of serine which is cleaved in its conversion to glycine. Thus if STHM activity was still present in JEV73, when serine $3^{14}C$ is used no labelling should have been observed in glycine. In JEV73 serine must be extensively deaminated to pyruvate since alanine which is derived from pyruvate is labelled. This pyruvate must then enter the Krebs cycle since glutamate, arginine and aspartate which are ultimately formed from Krebs cycle intermediates are also labelled, and be converted to glycine via threonine.

VII. (b) <u>The Metabolism of Serine By Strain JEV73R</u>

JEV73R was grown with serine $U^{14}C$, 100 µg/ml (specific activity 0.64×10^4 cpm/µmole). The adenine and guanine were found to have a specific activity of 0.17×10^4 and 0.12×10^4 cpm/µmoles and this indicates that less than one carbon was labelled from serine (Table 7, line 3). This can be compared with JEV73 in which 1.3 carbons from serine were found to be incorporated into purines. As indicated

previously JEV73R showed a lower purine specific activity when aspartate was used as a radioactive tracer, than that observed for JEV73. If this result represents a greater dilution of the aspartate pool, due to de novo synthesis from glucose, in JEV73R as compared to JEV73 and if serine is indeed being converted to glycine via the Krebs cycle, aspartate and threonine, one would again expect to observe reduced incorporation of serine into purines in JEV73R as compared with JEV73. When the radioautogram was observed the pattern of incorporation was similar to that of JEV73 grown with serine $U^{14}C$, that is serine, alanine, glutamate, arginine, lysine, aspartate and threonine were labelled while incorporation into glycine was only slight.

VII (c) The Metabolism of Serine by the Prototroph K10

Again K10 was grown for comparison with serine $U^{14}C$, 100 µg/ml (specific activity 0.64×10^4). The adenine and guanine extracted were shown to have a specific activity of 0.73×10^4 and 0.72×10^4 cpm/µmole respectively (Table 7, line 4). This indicates that 3.4 purine carbons were labelled from serine and that prototrophic E. coli, unlike JEV73 and JEV73R, obtained almost all of its glycine and one carbon units from serine. The radioautograms from this experiment indicate that serine and glycine are labelled heavily and to about the same extent, while glutamate, alanine, aspartate and threonine were much less heavily labelled. This pattern indicates

that even in K10 serine is extensively metabolized to several amino acids, for example aspartate, which are formed from Krebs cycle intermediates.

VIII. Source of Glycine in Strain HM100.

HM100 is a serine/glycine auxotroph which is not only able to grow on serine and glycine but also on sodium formate. Newman, et al (1970) have shown that when growing on formate these formate carbons are not incorporated into either glycine or serine. The preceding results of this thesis suggested the possibility that HM100 could derive at least its glycine from aspartate via threonine. To determine if in strain HM100 the same pathway was activated, not by leucine (as in strain JEV73) but by formate, this strain was grown with aspartate U ^{14}C, 100 µg/ml (specific activity 0.83 X 10^4 cpm/µmole) and formate and the purines extracted. The adenine and guanine showed a specific activity of 0.05 X 10^4 and 0.05 X 10^4 cpm/µmole (Table 8). This indicates that there is no significant conversion of aspartate to purine. The radioautograms supported this pattern of incorporation and indeed no label was observed in serine or glycine. However, aspartate was being metabolized in the usual way since aspartate, threonine, glutamate and arginine were all labelled. Thus the source of glycine in strain HM100 growing on formate remains unclear, but is in any case different from JEV73 growing

Source of Purine Carbons in Strain HM100 Using Aspartate as a Radioactive Tracer

Strain	Radioactive Supplement cpm/μmole	Non-Radioactive Supplement μg/ml	Specific Activity Adenine cpm/μmole	Specific Activity Guanine cpm/μmole	Average No. of Purine Carbons Labelled
HM100	Aspartate Acid $U^{14}C$ (0.83×10^4)	NA Formate 500	0.05×10^4	0.05×10^4	0.2

Table 8: *E. coli* strain HM100 was grown on NA Formate with aspartate $U^{14}C$ is the radioactive supplement. The purines were extracted and their specific activities determined. The average number of purine carbons, as determined from the ratio of the average specific activity of purine isolated to the specific activity per carbon of the aspartate supplied, is indicated.

on leucine.

IX. Regulatory Defect in JEV73 and JEV73R.

From the previous experiments involving incorporation of ^{14}C labelled precursors of glycine into purines and amino acids it is evident that the sources of glycine in AT2046, JEV73 and JEV73R differ from that of the prototroph K10. The former strains all can derive glycine from threonine while K10 obtains its glycine from serine. Strain AT2046 has only the one alternative source of glycine, i.e. exogenous threonine. Strain JEV73 can derive glycine from aspartate as well as threonine but it can do this only in the presence of leucine. Strain JEV73R uses the same pathway as JEV73 but it does not require exogenous leucine in order for the pathway to function.

Leucine has been shown to shift the pattern of metabolism of even E. coli K10. In the presence of leucine more threonine is converted to glycine. Thus when E. coli was grown with threonine ^{14}C, a much greater amount of ^{14}C glycine was detected when leucine ^{12}C was provided simultaneously (see Appendix I). This allows the formulation of the following hypothesis. If the mutation which distinguishes JEV73 from AT2046 is a regulatory one which results in an increase in the intracellular threonine pool, it is possible that the presence of leucine would then

result in enough threonine being converted to glycine to support growth of this glycine auxotroph. In JEV73R this conversion would occur without the presence of leucine, the mutation in JEV73R resulting in the synthesis of the enzyme(s) converting threonine to glycine being constitutive rather than leucine dependent. As will be discussed later there are two enzymes, one homoserine kinase which converts aspartic semialdehyde to homoserine and the other, threonine deaminase, which converts threonine to α-ketobutyrate, in which a change in regulation might produce the mutant phenotype. Thus if the threonine deaminase in JEV73 and JEV73R was more sensitive to feedback inhibition by leucine than the threonine deaminase of AT2046 or had a lower level of activity than that measured in AT2046 or prototrophic E. coli, less threonine might be converted to isoleucine and the threonine pool in the cell would increase. To investigate this the activity of threonine deaminase was measured (Table 9). No significant difference in enzyme level was seen. The responses of the enzyme to variations in substrate (L-threonine) and inhibitor (L-isoleucine, L-leucine) concentrations was also compared in the three strains. No significant difference was seen (Table 10:A, B, C). It is therefore unlikely that the mutations which created the phenotypes of JEV73 and JEV73R were involved with threonine deaminase formation.

Threonine Deaminase Activity in Strains of E. coli K12

Strain	µmole Pyruvate formed/30 mins/mg protein
AT2046	0.61
JEV73	0.63
JEV73R	0.55
K10	0.55

Table 9: Strains of E. coli indicated above were grown in minimal medium supplemented with glycine (100 µg/ml) to log phase. Threonine deaminase activity was measured in toluenized cells of these cultures.

Effect of Threonine Concentration on Threonine Deaminase Activity in Strains of E. coli K12

Threonine Concentration X 10^{-2}M	% Activity Strains		
(A)	AT2046	JEV73	JEV73R
5	100	100	100
4.2	100	98	100
3.1	94	93	100
2.1	94	94	100
1.6	86	91	109
1.2	89	88	89
0.8	59	70	66
0.4	15	32	39
0.2	3	11	5

Table 10: (A), (B), (C): Strains of E. coli indicated in the above tables were grown in minimal medium supplemented with glycine (100 µg/ml) to log phase. Threonine deaminase activity was measured at various substrate and inhibitor concentrations.

Effect of Isoleucine on Threonine Deaminase Activity in Strains of E. coli K12.

Isoleucine Concentration X 10^{-4}M (B)	% Activity Strains AT2046	JEV73	JEV73R
0	100	100	100
0.16	108	90	109
0.32	89	99	106
0.63	81	87	88
1.25	41	41	43
2.5	4	6	15
5	4	2	3
100	4	2	8

Effect of Leucine on Threonine Deaminase Activity in Strains of E. coli K12

Leucine Concentration X 10^{-2}M (C)	% Activity Strains AT2046	JEV73	JEV73R
0	100	100	100
0.4	97	97	106
0.8	99	93	86
1.2	63	68	74
1.6	36	46	34
2.0	23	26	24
3.0	9	0.5	13
4.3	1	6	5

X. Enzyme Assay of Threonine Aldolase.

An attempt was made to assay the enzyme threonine aldolase, known to convert threonine to glycine in certain organisms, in strain AT2046 growing on threonine. The assay attempted had been used by H. Yamada, et al (1971) in their study of this enzyme in various bacteria and yeasts and it involved using a sensitive colorimetric method to measure aldehyde formation. Several enzyme assays of this type were done, using strain AT2046 and the amount of product formed from threonine was found to be only 0.01 μmoles/mg protein. This level was observed to be five times higher in cells grown with threonine and leucine. Induction by leucine is known to be a characteristic of the enzyme threonine deaminase which converts threonine to the keto acid, α-ketobutyrate (Isenberg and Newman, 1974). This result suggested that perhaps this assay was not specific for threonine aldolase and possibly this colorimetric procedure was not specific enough to distinguish between aldehyde and keto acid (the product of threonine deaminase activity) formation in assay mixtures. It was decided instead to measure glycine formation from threonine which would constitute a more rigorous test for the presence of this enzyme. Extensive efforts, all unsuccessful, were then made to measure glycine formation from threonine in assay mixtures using

radioactive threonine as substrate. Assays were done under a variety of conditions and at several ph values in an effort to establish the presence of this enzyme. Further attempts to assay this enzyme have since been made and significant glycine formation from threonine *in vitro* has not yet been shown to occur (G. Batiste, personal communication).

That glycine formation from aspartate and threonine does in fact occur *in vivo* has beendefinitely established by the radioactive tracer experiments reported here. However, the conditions necessary to assay for the enzyme responsible for formation of glycine from threonine in *E. coli* have still not been established, despite extensive efforts.

DISCUSSION

The phenomenon described in this thesis consists of the following: Starting with an STHM deficient glycine requiring strain, we were able to isolate a variety of mutants which could derive their glycine in novel ways. Strains using exogenous threonine as a source of glycine could be easily isolated. From these a strain using leucine to support growth was obtained (JEV73) and from that it was possible to isolate a prototroph (JEV73R). These strains were shown to derive their glycine from endogenously synthesized threonine.

This discussion consists of an account of the various factors which might influence the derivation of glycine from exogenous and endogenous threonine. One possible model for the metabolism of JEV73, explained in terms of deregulation of threonine biosynthesis is presented in detail. It is suggested that the role of leucine, in supporting growth of JEV73, is not specific to this mutant but is a general phenomenon in E. coli. This aspect is discussed in terms of the regulation of nitrogen metabolism of the cell.

I. Variations in the Ability of Certain Auxotrophic Strains of E. coli to Use Exogenous Threonine as a Source of Glycine.

The auxotrophic strains used in this study varied considerably in their ability to use exogenous threonine to satisfy their nutritional requirement. Most of the serine/glycine and glycine auxotrophs studied proved unable to use exogenous threonine. Thus strains HM100, 119 and 129 as well as BF34 were all unable to grow on threonine-supplemented medium, while strain AT2046 and its derivative JEV73 were able to use exogenous threonine. Strain BF34 and AT2046 both have the same enzymatic deficiency, that is the lack of STHM, however, they differ in their ability to use exogenous threonine as a source of glycine. Therefore the absence of this enzyme, which results in the elimination of serine as a source of glycine, does not appear to be a factor in the ability of E. coli to use exogenous threonine as an alternative source of glycine. Even within the same strain (AT2046) the ability to use exogenous threonine is variable, some subclones of strain AT2046 being unable to use exogenous threonine to satisfy their nutritional requirements.

A study of the possible factors determining the use of exogenous threonine as a source of glycine was carried out by Van Lenten and Simmonds (1965). They described a similar phenomenon beginning with a serine/glycine auxotroph (strain SG), which could not use threonine, they obtained a substrain (strain S GT) which could use

threonine as a growth factor. They tried to determine the basic biochemical differences existing between these two closely related strains which would enable one to grow on threonine while the other could not. They examined three explanations of this problem: (a) that the ability of strain SGT to grow on exogenous threonine might be due to a greater ability by this strain to convert threonine to glycine when compared with SG unable to use exogenous threonine; (b) that the inability of strain SG to grow on threonine was due to the rapid destruction (deamination) of glycine, formed from threonine, so that the glycine level in the cell was never high enough to support the growth of this strain; and (c) that the inability of strain SG to grow on threonine was due to a high level of threonine deaminase which results in so much threonine being destroyed (deaminated) that the amount left to be converted to glycine is insufficient to allow growth. The investigators excluded explanations (b) and (c), and showed that while both strains can convert threonine to glycine, strain SGT does so at a much greater rate. Therefore even in two closely related strains of E. coli, the ability to synthesize the enzyme(s) of this pathway can differ to the extent that synthesis rate becomes the determining factor in their ability to grow on threonine-supplemented medium. Thus it seems possible that those

subclones of AT2046, which are capable of growth on threonine have the ability to induce the enzyme(s) of threonine to glycine pathway when grown with threonine. This allows them to form sufficient quantities of glycine to support their growth. However, the subclones of this strain, unable to grow with threonine, are either no longer able to synthesize the enzyme(s) of this pathway to levels which result in the adequate formation of glycine or possibly have lost the ability to synthesize this enzyme(s). That the enzyme(s) is most likely inducible by threonine in some strains of E. coli is indicated by the fact that, as described previously, AT2046 after growth in glycine must be adapted to growth on threonine. During the adaptation period sufficient quantities of glycine are provided to allow protein synthesis to occur, thus presumably allowing the production of the enzyme(s) needed for the use of threonine. Cultures pregrown with glycine and inoculated directly into a threonine medium rarely grow, indicating that enzymatic levels necessary for the synthesis of enough glycine to support growth are not constituitively synthesized. This is in contrast to Clostridium pasteurianum where Dainty and Peel (1970) demonstrated the presence of the enzyme (threonine aldolase), which is able to convert threonine to glycine in cells grown with and without threonine. The level of activity was found to be in the same range under both conditions.

Thus, the ability of E. coli to use the threonine to glycine pathway in the presence of exogenous threonine does not appear to be widespread. When this ability does exist it may be related to the inducibility of the enzyme(s) of the pathway by threonine.

II. The Quantitative Aspects of the Use of Exogenous Threonine by Glycine Requiring Auxotrophs of E. coli

Newman et al (1974) studying the quantitative aspects of the use of glycine by strain AT2046 considered that at low glycine concentrations the glycine provided was used only for glycine itself and not as a source of one carbon units, these being synthesized from serine by a new pathway not involving STHM. Thus at these low concentrations a linear dose response curve is obtained to glycine. At higher concentrations this curve is no longer linear and under these conditions they considered that the glycine provided was used as a source of glycine and one carbon units. It has been shown here that the dose response curve with threonine is similar to that with glycine. When threonine is used at high concentrations as the source of glycine by strain AT2046 a non-linear dose response is again obtained. At these levels ^{14}C from theonine serves as precursor of both glycine and one carbon units. Since at low threonine concentrations the dose response is linear, strain AT2046 may

have the ability to use the new one carbon pathway under these conditions.

Strain AT2046 seems to require a good deal more threonine than glycine to produce a given amount of cell material. Thus an optical density of 36 Klett units is reached with 0.16 µmoles/ml of threonine and an optical density of 90 Klett units is reached with 0.13 µmoles/ml of glycine. Threonine must of course also serve as a source of protein, threonine and isoleucine and this presumably accounts for the greater amount of threonine needed in this mutant.

III. A Survey of the Ability of Strains of E. coli to Use Endogenous Threonine as a Source of Glycine.

The ability to use endogenous threonine as a source of glycine has never been found to occur in E. coli to any significant extent. Roberts et al (1955) found that prototrophic E. coli could convert exogenous ^{14}C threonine but not exogenous ^{14}C aspartate to glycine (Table 11).

Tracer	Relative Specific Radioactivity of Amino Acids		
	Aspartic Acid	Threonine	Glycine
^{14}C threonine	0	1.0	0.5
^{14}C aspartic acid	1.0	1.0	0.05-0.1
^{14}C glycine	0	0	1.0

Table: 11. The interrelationship of threonine-glycine metabolism in E. coli. The incorporation of radioactive precursors into specific amino acids. (Roberts et al, 1955).

They interpreted this to mean that endogenous threonine synthesized from the aspartate provided is not converted to glycine. The prototroph K10 studied here behaves similarly to Roberts strain in that no significant incorporation of aspartate carbons into purines was found. However, it is clear that the glycine mutants JEV73, growing on leucine and JEV73R, growing on leucine or minimal medium do convert endogenous threonine, synthesized from the exogenous aspartate provided, to glycine. Thus these mutants differ significantly from the wild type in their ability to use endogenous threonine as a source of glycine for this pathway. Some other strains of E. coli also have this ability to a limited extent. Simmonds and Miller (1957), demonstrated that a serine/glycine auxotroph of E. coli (strain SG)

was capable of forming five to fifteen per cent of its glycine de novo from glucose. In their later study Van Lenten and Simmonds (1965) found that the threonine utilizing substrain SGT, when grown with glycine, required less glycine than strain SG to support the same amount of growth. The investigators considered that strain SGT can make some of its glycine de novo from glucose in the medium, probably by the alternative biosynthetic pathway: glucose -----> threonine -----> glycine, but that this was not sufficient, by itself, to support rapid growth. Pizer and Potochny (1965) also considered that endogenous threonine could not support rapid growth of a serine/glycine auxotroph of E. coli. They considered that a normal strain of E. coli maintains its endogenous threonine pool at too low a level to permit diversion of threonine to glycine. Only in the presence of exogenous threonine would threonine be concentrated in the cell to the point where conversion to glycine would take place. Roberts (1955, Page 265) on the basis of $^{14}C\ O_2$ incorporation studies also reached the conclusion that "..... there can be no sizable pool of metabolically produced threonine in the E. coli cell." This was based on the evidence that $^{14}CO_2$ was incorporated extensively into aspartate and threonine and very little into glycine. Since even very low concentrations (0.4 µg/ml) of exogenous ^{14}C threonine were found to be converted to glycine it

would seem that the pool of threonine must be even smaller than that formed with very low concentrations of exogenous threonine.

Therefore, it is very probable that the biosynthesis of threonine in the cell is regulated in such a way that the threonine pool is normally maintained at a comparably low level, it serving as a precursor of isoleucine. This is probably achieved by a high sensitivity feedback of threonine on its own biosynthesis. This type of control would then constitute a major factor in determining whether or not endogenous threonine was converted to glycine. Thus Pizer and Potochny (1965) considered it likely that strains, in which the pathway for the biosynthesis of threonine (from aspartate) was controlled so as to allow a larger endogenous threonine pool, would be capable of utilizing this threonine as a source of glycine. A regulatory defect which would result in either an increase in the biosynthesis of threonine or a decrease in its breakdown would lead to a higher level of intracellular threonine. This kind of regulatory alteration could then be one of the factors responsible for the ability of certain E. coli mutants to use endogenous threonine as a source of glycine.

IV. The Factors Which Permit the Use of Endogenous Threonine as a Source of Glycine.

From the preceding discussion, it would seem that

endogenous threonine could be used as a source of glycine if two conditions were met: (a) that the intracellular threonine pool is sufficiently large; and (b) that the strain can make a sufficient quantity of the enzyme(s) required to convert threonine to glycine. Considering the strains studied here, strain AT2046 must have the second ability at least when provided with exogenous threonine but it must not make enough threonine endogenously to support growth (or perhaps to induce enzyme synthesis). Strains JEV73 and JEV73R are derived directly from AT2046 and therefore have the ability to synthesize the threonine to glycine enzymes. However, they are able to use not only exogenous but endogenous threonine as a source of glycine. If a mutation in JEV73 and JEV73R has resulted in an alteration in the cells regulatory mechanism so as to create a larger intracellular threonine pool, these strains would now have both of the conditions necessary to ensure the use of endogenous threonine as a source of glycine. There are two sites at which a change in regulation might result in a larger intracellular threonine pool. One of these which might involve a change in the sensitivity to inhibitors or level of activity of threonine deaminase, which would result in a decrease in threonine degradation, in JEV73 and JEV73R as compared with the parent strain AT2046 or the prototroph K10 has already been discounted. The other site might involve

homoserine kinase which converts aspartic semialdehyde to homoserine. A change in regulation here would result in an increase in threonine biosynthesis. Evidence for this type of regulatory defect comes from the observation that when JEV73 and JEV73R were precultured with threonine they were found to adapt easily to further growth with threonine or glycine but were unable to adapt to growth with leucine, in the case of JEV73, or to leucine or minimal medium when JEV73R is considered. JEV73 growing with leucine and JEV73R growing with leucine or minimal medium must form glycine from endogenously formed threonine. Initial growth with threonine must in some way result in this endogenous threonine supply being limited to the extent that it is unable to supply the glycine required for growth of these mutants. Thus the functioning of this pathway is important as a source of endogenously formed threonine. Preculture with threonine makes it impossible for the cell to use this route once threonine is withdrawn. It would seem that growth with threonine decreases the quantity of enzyme(s) forming endogenous threonine to the extent that these enzyme(s) no longer suffice to produce threonine in a quantity permitting conversion to glycine.

Threonine is synthesized in the cell from aspartate by a series of reactions shown in Table 12.

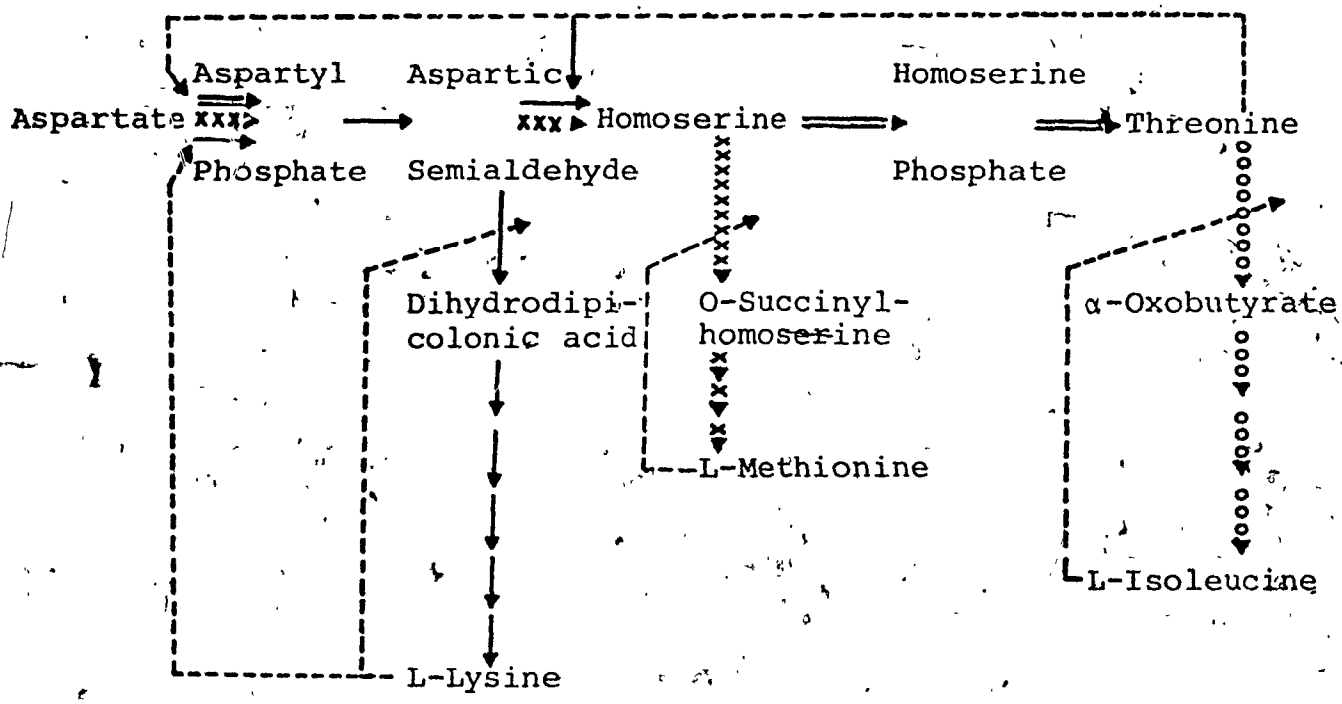


Table 12. The systems of regulation in E. coli K12 for the biosynthesis of the amino acids derived from aspartate (Patte et al, 1967).

The regulation of this pathway by threonine has been shown to occur at two levels. Patte et al (1967) state that in E. coli K12 the reaction by which aspartate is converted to aspartyl phosphate is catalyzed by three distinct aspartokinases each being repressed by one of the end products; lysine or methionine or threonine (plus isoleucine). Only two of these are subject to feedback inhibition (as shown in Table 12) one by threonine and one by lysine. The reaction by

which aspartate semialdehyde is converted to homoserine is catalyzed by two homoserine dehydrogenases one being repressed by growth on methionine and the other repressed by growth on threonine. The threonine repressible homoserine dehydrogenase isoenzyme is also subject to feedback inhibition by threonine. Exogenous threonine then would be expected to inhibit one of the three aspartokinases and to repress its synthesis, and to inhibit one of the two homoserine dehydrogenases and repress its synthesis. However, even in the presence of threonine two aspartokinases and one homoserine dehydrogenases should be active and homoserine must be made. The first step in the conversion of homoserine to threonine is catalyzed by homoserine kinase, an enzyme whose synthesis is repressed by threonine but whose activity is not greatly affected by threonine (Thèze et al. 1974). In the presence of threonine therefore, synthesis of this enzyme would decrease, but any enzyme present might still be active.

Using this as a base, what mutations could account for an increase in the threonine pool? Suppose that aspartokinase/homoserine dehydrogenase, both of these being carried on the same polypeptide chain, became insensitive to feedback regulation. They would produce increasing amounts of homoserine and this could be converted to threonine because threonine is a poor feedback inhibitor of homoserine

kinase. However, if the cell had been grown on threonine the homoserine kinase level would be much repressed and while homoserine might accumulate, it would not be converted to threonine.

Other alternatives such as a loss of repressibility of aspartokinase homoserine dehydrogenase or of homoserine kinase do not seem to account for all aspects of the phenotypes seen.

Thus when JEV73 is grown with leucine an aspartokinase/homoserine dehydrogenase, no longer subject to feedback inhibition by threonine, produces sufficient homoserine and this is converted to threonine via homoserine kinase. When JEV73 is grown with threonine, synthesis of the homoserine kinase enzyme stops and adaptation to leucine is made difficult.

The assumption made then is that JEV73 and JEV73R show the same defect in the regulation of threonine biosynthesis and thus both have an increased threonine pool. A further assumption which will be defended in the following section is that this does not itself suffice to permit conversion of threonine to glycine. The enzyme(s) involved are induced by exogenous leucine and therefore JEV73 grows in the presence of leucine being able to form a lot of threonine and to convert it to glycine. The mutation which differentiates JEV73R from JEV73 would then be a mutation

to constitutivity, that is leucine independence, of the threonine to glycine conversion.

V. The Role of Leucine in the Glycine Metabolism of Mutant Strains of E. coli.

Leucine has been shown to have several effects on the metabolism of E. coli. One of these, directly related to the preceding discussion on regulation, is its ability to stimulate the conversion of threonine to glycine (Jane Hardwick, see Appendix I). Thus it was shown that when a prototroph of E. coli, strain LR1, was grown with threonine ^{14}C and leucine ^{12}C was also present more threonine carbons were converted to glycine than in the absence of leucine. However, when the same strain was grown with aspartate ^{14}C the presence of leucine ^{12}C did not result in a higher level of incorporation of these aspartate carbons into glycine. Thus it appears that even in prototrophic E. coli, able to synthesize STHM and form glycine from serine, the effect of leucine is to stimulate glycine synthesis from threonine. This observation provides a possible explanation for the finding that the glycine auxotroph strain AT2046 showed an increase in final optical density units when grown with glycine and leucine as compared with glycine. If leucine increases conversion of threonine to glycine the provision of leucine might be the equivalent of

the provision of more glycine, and this would then increase the total growth on the glycine provided, as was seen. Thus in strain AT2046 leucine stimulates the conversion of even endogenously formed threonine to glycine and must also do so in strain JEV73 which is derived from AT2046. However, if JEV73 has already an increased threonine pool, the additional effect of leucine might suffice to produce enough glycine for growth. The effect of leucine would supply only enough glycine to spare in AT2046, the threonine pool being so much smaller. This would explain the finding that leucine is not a precursor of glycine and not incorporated into protein glycine or purines.

The exact point at which leucine affects the metabolism of threonine in E. coli or the exact mechanism by which leucine acts can only be speculative at this time. However, the observation that leucine is no longer able to substitute for glycine and act as sole growth factor for strain JEV73 when the enzymes of the pathway from aspartate to threonine have been repressed, suggests that the role of leucine is not related to the synthesis of the enzymes of this pathway. In addition it has already been observed that leucine does not appear to act by inducing the enzymes of this pathway since the presence of leucine was not found to stimulate the incorporation of aspartate carbons into purines in strain LR1. Therefore it seems likely that the action of leucine may be on the enzyme(s) of the pathway

from threonine to glycine. The low levels at which leucine acts together with the fact that it was not found to be the precursor of glycine or one carbon units suggests a "co-factor" type of action for leucine in this area of metabolism. Other evidence will be cited later showing that leucine has been found to act in other areas of metabolism in a similar capacity.

VI. The Effects of Leucine in the Glycine Metabolism of E. coli.

Leucine is known to have several effects on the glycine metabolism of E. coli, other than the one discussed; its ability to replace glycine and act as sole growth factor for certain glycine auxotrophs. This is especially true under conditions where no nitrogen source is added to the medium and glycine itself is then used as the nitrogen source by E. coli. To do this glycine has to be metabolized by the following reactions.

(1) Glycine $\xrightarrow[\text{Pathway}]{\text{Glycine cleavage}}$ C_1

(2) Glycine + C_1 $\xrightarrow{\text{STHM}}$ Serine

(3) Serine $\xrightarrow[\text{Deaminase}]{\text{Serine}}$ Pyruvate + Ammonia

Thus by this pathway glycine is converted to serine which is then deaminated to form pyruvate, this reaction yielding ammonia (a source of nitrogen) to be used by the cell. When glycine is used as the nitrogen source in this way and leucine is also present it is found that the rate as well as the extent of growth of prototrophic E. coli is increased (See Appendix II). The mechanism of action of leucine in supporting the growth of the glycine auxotroph, strain JEV73, was previously discussed and was shown to involve surprisingly low levels of leucine. Again we find that when leucine is present at levels as low as 5 µg/ml and glycine is used as the nitrogen source the rate of growth of prototrophic E. coli K10 is increased suggesting that in these two instances the mechanism of action for leucine may be similar.

A recent report by McGivan et al (1973) finds that leucine has a profound effect on the nitrogen metabolism in rat liver mitochondria. Thus it is found that leucine complexes with the enzyme glutamate dehydrogenase, responsible for the synthesis of glutamate from α-ketoglutarate and ammonia by the following reaction:

$$\alpha\text{-Ketoglutarate} + NH_4^+ + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{L-glutamate} + NAD^+$$

McGivan et al (1973) state that at low ammonia concentrations this reaction proceeds in the direction of glutamate synthesis very slowly however when low concentrations of leucine (5mM) are also present this stimulates the reaction such that glutamate synthesis is greatly increased. In this case leucine actually complexes with the enzyme to influence the direction of the reaction and stimulate the assimilation of ammonia into organic form.

An important source of this ammonia required for glutamate synthesis comes from deamination reactions in the cell. Two enzymes involved in such deaminations are serine deaminase (previously mentioned as a key enzyme in the use of glycine as a nitrogen source) and threonine deaminase both of which have been shown to be induced by leucine in E. coli (Isenberg and Newman, 1974). Thus perhaps under conditions where the cell has difficulty obtaining ammonia, as would occur when glycine is used as the nitrogen source, leucine may act to induce certain deamination reactions and perhaps favour the storage of the ammonia they produce, in the form of glutamate, to be used later in amino acid biosynthesis.

In summary leucine may act as a major regulatory signal in the cell, to increase the scavenging of nitrogen from excess organic nitrogen containing compounds (that is, deamination) and also increase the incorporation of ammonia

into nitrogen storage compounds like glutamate. The effects described in this thesis would then be coincidental results of the interplay between regulation by leucine and differences in certain mutants. Thus in strain JEV73 the actual mutation may have nothing to do with leucine metabolism. Leucine would increase threonine to glycine conversion in all strains. However, in strain JEV73 this would make the difference between growth and no growth.

VII. Source of Glycine in Strain HM100.

From all available evidence it has always been considered that the biosynthesis of glycine proceeds via the two pathways previously discussed. However, as early as 1955 Roberts et al reported the possibility that a third pathway for glycine synthesis exists. They observed that E. coli K12 grown on glucose and $^{14}CO_2$ incorporated ^{14}C into glycine and that the precursor of this glycine is not threonine or serine. They estimated that when glucose is used as the carbon and energy source about ten percent of the glycine may be derived by this pathway.

Strain HM100, a serine/glycine auxotroph, can grow on sodium formate. Its source of serine and glycine under these circumstances is not known, and it was suggested that the presence of formate restores a pathway of serine

biosynthesis (Newman 1970). The possibility that threonine acts as a source of glycine (and serine) has been tested and excluded in this thesis. The problem of the origin of glycine (and serine) in strain HM100 therefore remains unsolved.

REFERENCES

1. Braunstein, A.E., and Vilenkina, G.Y. 1949. Enzymic formation of glycine from serine, threonine and other hydroxyamino acids in animal tissue. Doklady. Akad. Nauk. S.S.S.R. 66: 243-246.

2. Dainty, R.H., and J.L. Peel. 1970. Biosynthesis of amino acids in Clostridium pasteurianum. Biochem. J. 117: 573-584.

3. Dainty, R.H., 1970. Purification and properties of threonine aldolase from Clostridium pasteurianum. Biochem. J. 117: 585-592.

4. Folk, W.R., and P. Be[illegible] 1970. Isolation and partial characterization of Escherichia coli mutants with altered glycl transfer ribonucleic acid synthetases. J. Bacteriol. 102: 193-203.

5. Holland, B.R., and W.W. Meinke. 1949. The serine requirement of Streptococcus R as a function of the basal medium. J. Biol. Chem. 178(1): 7-21.

6. Isenberg, S., and E.B. Newman. 1974. Studies on L-serine deaminase in Escherichia coli K12. J. Bacteriol. 118: 53-58.

7. Kerasek, M.A., and D.M. Greenberg. 1957. Properties of threonine aldolase. J. Biol. Chem. 227: 191-205.

8. Kikuchi, G. 1973. The glycine cleavage system; composition, reaction mechanism and physiological significance. Mol. Cell. Biochem. 1: 169-187.

9. Kornberg, H.L. 1963. The role of acetate in isocitrate lyase induction. Biochim. Biophys. Acta. 73: 517-519.

10. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

11. Meister, A. 1965. Biochemistry of Amino Acids; 2nd ed. Vol. 2. Academic Press, New York. p. 681.

12. Meltzer, H.L. and D.B. Sprinson. 1952. The synthesis of 4-C^{14}, N^{15}-L-threonine and a study of its metabolism. J. Biol. Chem. 197: 461-474.

13. McGivan, J.D., N. Bradford, M. Crompton and J.B. Chappell. 1973. Effect of L-leucine on the nitrogen metabolism of isolated rat liver mitochondria. Biochemical Journal, 134: 209-215.

14. Miller, B.A., and E.B. Newman. 1974. Control of serine transhydroxymethylase synthesis in Escherichia coli K12. 20: 41-47.

15. Morris, J.G. 1969. Utilization of L-threonine by a Pseudomonad: a catabolic role for L-threonine aldolase. Biochemical Journal, 115: 603-605.

16. Newman, E.B., and B. Magasanik. 1963. The relation of serine-glycine metabolism to the formation of single-carbon units. Biochim. Biophys. Acta. 78: 437-448.

17. Newman, E.B. 1970. Metabolism of serine and glycine in E. coli K12. I. The role of formate in the metabolism of serine-glycine auxotrophs. Canadian Journal of Microbiology. 16: 933-940.

18. Newman, E.B., B. Miller and V. Kapoor. 1974. Biosynthesis of single-carbon units in Escherichia coli K12. Biochim. Biophys. Acta. 338: 529-539.

19. Pardee, A.B., and L.S. Prestidge. 1955. Induced formation of serine and threonine deaminases by Escherichia coli. J. Bacteriol. 70: 667-674.

20. Patte, J.C., G. LeBras and G.N. Cohen. 1967. Regulation by methionine of the synthesis of a third aspartokinase and a second homoserine dehydrogenase in Escherichia coli K12. Biochim. Biophys. Acta. 136: 245-257.

21. Pizer, L.I. 1963. The pathway and control of serine synthesis in E. coli. J. Biol. Chem. 238: 3934-3944.

22. Pizer, L.I., and M.L. Potochny. 1964. Nutritional and regulatory aspects of serine metabolism in Escherichia coli. J. Bacteriol. 88: 611-619.

23. Pizer, L.I. 1965. Glycine synthesis and metabolism in E. coli. Journal of Bacteriology. 89: 1145-1150.

24. Revel, H.R.B. and B. Magasanik. 1958. Utilization of the imidazole carbon-2 of histidine for the biosynthesis of purines in bacteria. J. Biol. Chem. 233: 439-443.

25. Roberts, R.B., P.H. Abelson, D.B. Cowie, E.T. Bolton, and R.J. Britten. 1955. Studies of biosynthesis in Escherichia coli. Carnegie Inst. Wash. Publ. 607.

26. Roepke, R.R., R.L. Libby and M.H. Small. 1944. Mutation or variation of E. coli with respect to growth requirements. J. Bacteriol. 49: 401-412.

27. Sagers, R.D., and L.C. Gunsalus. 1961. Intermediary metabolism of Diplococcus glycinophilus. L-glycine cleavage and one-carbon interconversion. J. Bacteriol. 81: 541-549.

28. Shemin, D. 1946. The biological conversion of L-serine to glycine. J. Biol. Chem. 162: 297-307.

29. Simmonds, S., and Miller, D.A. 1957. Metabolism of glycine and serine in E. coli. J. Bacteriol. 74: 775-783.

30. Stauffer, G.V. and J.E. Brenchley. 1974. Evidence for the involvement of serine transhydroxymethylase in serine and glycine interconversion in Salmonella typhimurium. Genetics. 77: 185-198.

31. Taylor, R.T., and H. Weissbach. 1965. Radioactive assay for serine transhydroxymethylase. Anal. Biochem. 13: 80-84.

32. Thèze, J., L. Kleidman, and I. Saint Girons. 1974. Homoserine kinase from Escherichia coli K12: properties, inhibition by L-threonine, and regulation of biosynthesis. Journal of Bacteriology. 118: 577-581.

33. Umbarger, H.E. 1974. Personal communication.

34. Umbarger, H.E., and M.A. Umbarger. 1962. The biosynthetic pathway of serine in Salmonella typhimurium. Biochim. Biophys. Acta. 62: 193-195.

35. Umbarger, H.E., M.A. Umbarger and P.M.L. Siu. 1963. Biosynthesis of serine in E. coli. J. Bacteriol. 85: 1431-1439.

36. Van Lenten, E.J., and S. Simmonds. 1965. Metabolic relations between L-threonine and glycine in Escherichia coli. J. Biol. Chem. 240: 3361-3371.

37. Willetts, A.J., and J.M. Turner. 1971. L-threonine acetaldehyde-lyase in a strain of Bacillus subtilis. Biochim. Biophys. Acta. 252: 105-110.

38. Yamada, H., H. Kumagai, T. Nagate and H. Yoshida. 1971. Formation of threonine aldolase by bacteria and yeasts. Agricultural Biol. Chem. 35: 1340-1345.

The Derivation of Purines in *E. coli* Strain LR1 Grown With Various Radioactive Precursors.

Radioactive Nutrilite	S.A. cpm/μmole	C_{12} competitor	Purine S.A. adenine	guanine	ave	# Carbons Derived From C_{14}
Aspartate-U-C^{14}	0.8	---	.04	.02	.03	<0.1
Aspartate U-C^{14}	0.8	Leucine	.05	.07	.06	∿0.1
Threonine-U-C^{14}	1.4	---	.09	.02	.05	∿0.1
Threonine-U-C^{14}	1.4	Leucine	.6	.4	.5	1.5

Appendix 1: *E. coli* strain LR1 was grown in minimal medium with various radioactive and non-radioactive supplements. The purines were extracted and their specific activities determined. The average number of these purine carbons, as determined from the ratio of the specific activity of purine isolated to the specific activity of the radioactive supplement supplied is indicated. These experiments were done by Jane Hardwick and the results are included here by her kind permission.

(A) Effect of leucine on growth rate of K10 grown in minimal medium-nitrogen.

Nitrogen Source	Growth Rate Apparent Doubling Time (mins.)
Glycine	360
Glycine, Leucine	180
Leucine	no growth

(B) Effect of leucine on the extent of growth of K10 grown in minimal medium-nitrogen.

Supplement (µg/ml)		Total Nitrogen (µmoles NH_4/ml)	Turbidity (K.U.)	Protein (µg/ml)
Glycine	Leucine			
100	-	1.3	77	127
100	100	2.1	143	199
gly	leu/gly	-	1.8	1.5

Appendix II: Tables A and B show the increase in the rate and extent of growth observed by E. coli K10 when glycine is used as the nitrogen source and leucine is also present. These experiments were done by E. Newman and V. Kapoor. The results are included here by their kind permission.