THE NUTRITION AND PHYSIOLOGY OF

ERWINIA SPECIES

By

CHARLES FRANK PARHAM

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

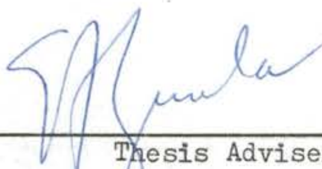
1964

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1967

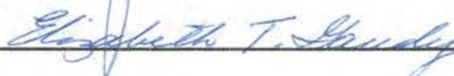
THE NUTRITION AND PHYSIOLOGY OF

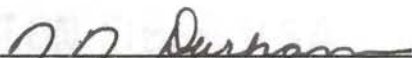
ERWINIA SPECIES

Thesis Approved:



Thesis Adviser





Dean of the Graduate College

OKLAHOMA
STATE UNIVERSITY
LIBRARY

JAN 16 1968

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. E. A. Grula, under whose direction this study was conducted, for continual guidance, assistance, and encouragement.

The author wishes also to acknowledge Dr. Mary Grula for her assistance and time consumed during this study, and to the Department of Microbiology for use of facilities.

Thanks are due to Messrs. Wayne Benson and Roy Hopfer for technical assistance.

Financial support was received from the National Science Foundation through a research grant awarded to Drs. E. A. and Mary Grula (Grant Numbers GB-1532 and GB-4357) and through a training grant awarded to the Department of Microbiology (PHS grant number GM-1102-03).

Special gratitude is also extended to my wife, Beverly, and my parents for encouragement throughout this study.

359707

TABLE OF CONTENTS

Chapter | Page

I. INTRODUCTION 1

II. LITERATURE REVIEW 3

Part A. Possible Mechanisms of Action of D-amino Acids 3
Part B. Glucose Metabolism in *Escherichia coli* 7
Pantoyl Lactone 23

III. MATERIALS AND METHODS 25

Test Organism 25
Media 25
Growth of Cells 26
Procedures for Cell Fractionation 26
Determinations of Dry Weight 27
Techniques for Isotope Labeling 28
Procedures for Determination of Uptake of Labeled Compounds 28
Acid Hydrolysis of Protein Material 29
Preparation of Hydrazone 29
Chromatography and Detection of Amino Acids 30
Quantitation of Amino Acids 30
Chromatography and Detection of Hydrazones 31
Radioautography 31
Conversion of Pantoyl Lactone to Pantoic Acid 31
Procedure for Counting Planchets 32

IV. RESULTS AND DISCUSSION 33

Glucose Utilization in *Erwinia* Species 33
Stimulation of Growth by Pantoyl Lactone 34
Identification of a Glucose Metabolic Product 40
Stimulation of Growth by Pyruvic Acid 40
Stimulation of Growth by Intermediates of the Citric Acid Cycle 43
Summary of Glucose Metabolism 49
Effect of D-serine on the Metabolism of Erwinia Species 51
Effect of D-serine on the Labeling Patterns From Glucose and Aspartic Acid 55
Labeling Pattern of D-serine 55

Chapter | Page

IV. (Continued)

Reversal of the D-serine Effect by Pantoyl Lactone 58
Reversal of the D-serine Effect by Ammonium Chloride 60
Reversal by Glutamic Acid 63
Isotope Uptake Experiments 66
Accumulation of Keto-Acids in the Medium in the Presence and Absence of D-serine 70

V. DISCUSSION AND CONCLUSIONS 75

A SELECTED BIBLIOGRAPHY 79

LIST OF TABLES

Table		Page
I.	*Escherichia coli* and Cultural Characteristics and Relationship of Mutants to Metabolic Blocks in Metabolism	22
II.	Labeling Pattern Obtained Using Glucose-UL-C^{14} in the GA Medium	35
III.	Labeling Patterns With Glucose-UL-C^{14} in the GA Medium With Added Pantoyl Lactone	37
IV.	Stimulation of Growth by Precursors of Pantothenic Acid and Pantothenic Acid in a Glucose-Ammonium Salts Medium	39
V.	Information on Hydrazone Forming Compounds	41
VI.	Stimulation of Growth by Various Citric Acid Cycle Intermediates in the GA Medium	44
VII.	Carbon Dioxide Uptake in Cells of *Erwinia* sp. and *Escherichia coli*	47
VIII.	Labeling Patterns for Carbon Dioxide Fixation in *Erwinia* sp. in the Presence and Absence of Pantoyl Lactone	48
IX.	Normal Labeling Patterns of *Erwinia* sp. After Growth in the Presence of Glucose and Aspartic Acid	53
X.	Amino Acid Labeling Pattern of *Erwinia* sp. After Growth in the Presence of Glucose and Aspartic Acid	54
XI.	Labeling Patterns of *Erwinia* sp. Obtained Using Glucose and Aspartic Acid in the Presence of D-serine	56
XII.	Amino Acid Labeling Patterns of *Erwinia* sp. After Growth in the Presence of Aspartic Acid and Glucose With Addition of D-serine	57

Table | Page

XIII. Incorporation of D-serine-3-C^{14} Into Cellular Amino Acids 59

XIV. Labeling Patterns of Glucose, Aspartic Acid, and D-serine in the Presence of Pantoyl Lactone 61

XV. Labeling Pattern of Glucose, Aspartic Acid, and D-serine After Growth in the Presence of Ammonium Chloride 62

XVI. Labeling Patterns of Aspartic Acid and Glutamic Acid in the Aspartic Acid Medium 64

XVII. Effect of D-serine on the Metabolism of Aspartic Acid and Glutamic Acid in *Erwinia* sp. 65

XVIII. Labeling Patterns of D-serine-3-C^{14} in the Aspartic Acid Medium With and Without Glutamic Present for Reversal 67

XIX. Specific Activities of Cells With Glutamic Acid Reversal of D-serine Inhibition of Growth and Division 68

XX. Specific Activities of Pyruvate Hydrazones Formed in the Aspartic Acid-Glucose Medium 74

LIST OF FIGURES

Figure | Page

1. Embden-Meyerhof Pathway and Citric Acid Cycle for Glucose Metabolism 9

2. Synthesis of Histidine 10

3. Synthesis of the Serine Family of Amino Acids 12

4. Synthesis of L-tyrosine and L-phenylalanine 13

5. Synthesis of the Pyruvic Family of Amino Acids 14

6. The Synthesis of the Glutamic Acid Family of Amino Acids 15

7. The Synthesis of the Aspartic Acid Family of Amino Acids 17

8. The Synthesis of Cellular Fatty Acids 18

9. The Synthesis of Some Phospholipids 20

10. Synthesis of the Nucleic Acid Bases 21

11. Stimulation of Growth by Pantoyl Lactone in the GA Medium 36

12. Stimulation of Growth by Pyruvate (0.127 M) in the Presence and Absence of Glucose 42

13. Biosynthetic Pathways in Erwinia sp. 52

14. Uptake of Aspartic-UL-C^{14} in the Presence and Absence of D-serine 69

15. Uptake of D-serine-3-C^{14} in the Presence and Absence of Glutamic Acid and Alpha-Ketoglutarate in an Aspartic Acid Medium 71

16. Reversal of D-serine Inhibition of Aspartic Acid Uptake by Various Reversing Agents 72

CHAPTER I

INTRODUCTION

Grula (1960, pp. 369-374) reported that cell division in *Erwinia* sp. was partially inhibited by the addition of D-glucose to nutrient broth. It was also reported that *Erwinia* sp. would not grow in a defined medium containing only D-glucose, ammonium, and mineral salts unless an organic source of nitrogen (preferably aspartic acid) was present. Addition of the DL-isomers of serine, methionine, phenylalanine, histidine, threonine, or tryptophan to the L-aspartic acid-glucose medium (no ammonium ions added) resulted in varying amounts of inhibition of division and growth of the organism. Further testing revealed that the D-isomer of each amino acid was causing these inhibitions (Grula, 1960, pp. 375-385).

Although several agents that inhibit cell division in *Erwinia* sp. have been studied in this laboratory, D-serine has received the greatest attention. In a series of publications, it has been reported that D-serine causes the following biochemical lesions: (a) inhibits synthesis of *alpha*-ketopantoic from *alpha*-ketoisovaleric acid and subsequent reduction of *alpha*-ketopantoic acid, (b) inhibits *alpha*-decarboxylation of aspartic acid which would allow formation of *beta*-alanine, (c) inhibits mucopeptide synthesis (Grula and Grula, 1962, pp. 981-988; 1962, pp. 989-997; 1963; 1964; Grula, Smith and Grula, 1965). The authors suggested that the inhibition of cell wall mucopeptide synthesis, leading to a defective cell wall, could result in secondary membrane damages, thus

leading to inhibition of cell division. The importance of the membrane in cell division was inferred from the fact that hypertonic conditions prevented division inhibition from occurring. They also reported that D-serine was incorporated into the cell wall mucopeptide of Erwinia sp., replacing glycine; however, neither pantoyl lactone nor ammonium chloride would stop the incorporation of D-serine.

As a result of these studies, it has been concluded that the cell membrane is probably the key structural entity in the division of Erwinia sp. (Grula and Grula, 1964; Grula, Smith, and Grula, 1965). Since structural alteration of the membrane could result from some defect in the metabolism of carbon in the cell, the utilization of carbon by Erwinia sp. was investigated.

The first problem was simply one of growth since, as stated above, this organism will not grow in a glucose-ammonium salts medium unless supplemented with an organic source of nitrogen (preferably aspartic acid). Once utilization of aspartic acid and glucose was better understood, data were collected relative to utilization of carbon from these and other compounds using conditions that allowed or did not allow cell division.

CHAPTER II

LITERATURE REVIEW

Part A. Possible Mechanisms of Action of D-amino Acids

It has been reported that D-amino acids, and in some cases L-amino acids, can cause inhibition of growth and (or) morphological changes in cells. It appears that the D-isomers of the amino acids generally have more toxic effects than the L-isomers.

Studying the effects of certain D-amino acids, Fox, Fling, and Bollenback (1944), Fling and Fox (1945), Kobayashi, Fling, and Fox (1948), and Yaw and Kakavas (1952) reported inhibition of growth by some of these compounds. Coleman (1959) was the first to report division inhibition, as well as growth inhibition, using a D-amino acid. Using D-glutamic acid to inhibit cell division in *Rhodospirillum rubrum*, he reported that D-glutamic acid inhibited synthesis of nucleic acid, particularly ribonucleic acid. Tuttle and Gest (1960) reported that a variety of morphological changes occurred in *Rhodospirillum rubrum* due to the addition of D-isomers of glutamic acid, isoleucine, alanine, aspartic acid, serine, valine, histidine, lysine, leucine, or methionine. They reported that these D-amino acids caused an *increase* in the amino sugar content of the cell wall of the organism.

Grula (1960, pp. 375-385) reported that the D-isomers of serine, histidine, tryptophan, phenylalanine, threonine, and methionine

inhibited both growth and division in *Erwinia* sp. Division inhibition by D-serine could be prevented by the addition of pantoyl lactone, pantoic acid, *para*-amino-benzoic acid, D or L-alanine, ammonium chloride, or by osmotically protecting the cell (Grula, 1960; Grula and Grula, 1962; 1964).

Maas and Davis (1950) reported that D-serine interfered with the synthesis of pantothenic acid by blocking the condensation of *beta*-alanine and pantoic acid in *Escherichia coli*. Grula and Grula (1962) reported that D-serine did not inhibit the condensation reaction but, rather, inhibited the hydroxymethylation of *alpha*-ketoisovaleric to *alpha*-ketopantoic acid and the further reduction of *alpha*-ketopantoic to pantoic acid in *Erwinia* sp. They also noted that D-serine inhibited the *alpha*-decarboxylation of L-aspartic acid to form *beta*-alanine (Grula and Grula, 1963). These blocks in the synthesis of pantothenic acid were responsible for the lowered levels of pantothenate and coenzyme-A in *Erwinia* sp. These metabolic lesions resulted in accumulation of pyruvic acid since pantothenic acid is an integral part of the coenzyme-A molecule which functions in oxidative-decarboxylation (pyruvic acid oxidizing complex) of pyruvic acid. Durham and Milligan (1961; 1962), using nutritional data alone, suggested that *beta*-alanine synthesis is inhibited by D-serine in a species of *Flavobacterium*.

Grula and Grula (1962, pp. 981-988) further observed that although pantoyl lactone and pantoic acid were able to overcome division inhibition caused by D-serine, they could not restore completely normal pantothenate levels in the cell even though division appeared normal. The addition of pantoyl lactone and *beta*-alanine together did allow normal and even excessive levels of pantothenate to be synthesized

(Grula and Grula, 1963); from this it was inferred that a deficiency of beta-alanine occurred in the presence of D-serine.

It was apparent that the inhibition of pantothenic acid synthesis by D-serine did not account for the inhibition of cell division in Erwinia sp. since compounds such as alanine or ammonium chloride which overcome inhibition of division by D-serine did not restore ability of the cells to synthesize pantothenic acid. These authors have also reported that D-serine interferes with the metabolism of aspartic acid in the cell.

Eisenstadt, Grossman, and Klein (1959) reported that the D-isomer of aspartic acid inhibited protein synthesis in Pseudomonas saccharophilia. The conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) by transamination with L-aspartic acid was impaired when D-aspartic acid was present. This caused a decrease in adenosine triphosphate (ATP) synthesis and a lowering of total energy potential for the cell and thus slowed growth and division. These workers claimed that protein synthesis was inhibited because of an energy deficit in the cell.

Murachi and Tashiro (1958) showed that D-lysine inhibited D-amino acid oxidase by competing with the substrate for the apo-oxidase protein.

Lark and Lark (1959) reported that D-methionine induced the formation of crescent-like protoplasts in Alcaligenes faecalis. The D-methionine effect resembled the action of penicillin in that both compounds caused a decrease in cell wall mucopeptide (Lark and Lark, 1959; 1961, Lark, Bradley and Lark, 1963). Lark (1959) demonstrated that D-methionine would inhibit the incorporation of L-methionine into the

cell wall of Alcaligenes faecalis but not into protein. The author postulated that two intracellular pools were present in the organism. They were the expandable pool which was maintained by the exogenous amino acids and the internal pool which was not directly affected by the exogenous amino acids. They postulated that D-methionine was replacing L-methionine in the expandable pool, but not in the internal pool This would allow the organism to utilize the L-isomer for protein synthesis while depending on the D-isomer of the expandable pool for cell wall synthesis. The idea of two metabolic pools was supported by Kempner and Cowie (1960) using Escherichia coli.

Lark and Lark (1961) further demonstrated that the incorporation of D-methionine into the mucopeptide was chloramphenicol resistant. In another publication, Lark, Bradley, and Lark (1963) reported that the D-methionine incorporated into the cell wall could be released by treating the wall with lysozyme. The D-methionine peptides behaved differently in paper chromatographic systems than those released from normal cells. They observed that D-methionine was neither N- nor C-terminal in the peptide. When the organism was grown in a medium which contained only low concentrations of D-methionine lesions in the cell wall mucopeptide could be observed with the electron microscope.

Neuhaus (1962) isolated an enzyme (D-alanyl-D-alanine synthetase) from Streptococcus faecalis which would substitute D-serine for one of the D-alanine molecules in the dipeptide present in the cell wall mucopeptide. The compound normally formed is D-alanyl-D-alanine. When D-serine was added to the reaction mixture, D-alanyl-D-serine was formed. The D-alanyl-D-serine formed by the enzyme inhibited the action of the enzyme on its normal substrate.

Grula and Grula (1964) reported that D-serine inhibited mucopeptide synthesis 30 to 40 per cent in *Erwinia* sp. The filamentous cells which developed were not osmotically fragile. It has also been shown that D-serine is incorporated into the cell wall mucopeptide and can partially replace glycine present in the cell wall in either *Erwinia* sp. or *Micrococcus lysodeikticus* (Grula, Smith, and Grula, 1965; Whitney and Grula, 1964). An alteration in ratio of the other mucopeptide amino acids was not observed in either organism.

Grula and Grula (1964) have suggested that the inhibition of cell wall synthesis could cause secondary membrane damage to *Erwinia* sp. When the cells are protected from division inhibition with pantoyl lactone or osmotically protective agents, inhibition of mucopeptide synthesis still occurs. Also, neither pantoyl lactone nor ammonium chloride prevent incorporation of D-serine into the cell wall (Grula, Smith, and Grula, 1965). Thus, neither inhibition of mucopeptide synthesis nor incorporation of D-serine in the mucopeptide is, by itself, a sufficient condition for inhibition of division. Membrane damage, occurring as a result of wall damage, is essential. When the membrane is physically protected under hypertonic conditions, division can occur normally.

Part B. Glucose Metabolism in *Escherichia coli*

When glucose is used as sole carbon source, it is a simple matter to account for all the carbon metabolized by the bacteria.

The main flow of carbon used for synthetic reactions is siphoned from compounds formed via the Embden-Meyerhof scheme or from carbon that enters the citric acid cycle (Roberts, Cowie, Abelson, Bolton, and

Britten, 1963, pp. 418-428). These schemes are illustrated in Figure 1. The pentose shunt is also very important and provides five carbon sugars (ribose and deoxyribose) for synthesis of the nucleic acids.

Amino Acid Synthesis

The synthesis of histidine from glucose is illustrated in Figure 2. The carbon backbone comes from ribose 5-phosphate formed via the pentose shunt (White, Handler, and Smith, 1964, pp. 501-506). This compound may be formed in one of two ways. Glyceraldehyde-3-phosphate from the Embden-Meyerhof pathway may condense with sedoheptulose-7-phosphate from the pentose shunt to form xylulose-5-phosphate and ribose-5-phosphate, or ribose-5-phosphate may come exclusively via the pentose shunt.

The serine family of amino acids includes serine, glycine, and cysteine (Roberts et al., 1963, pp. 280-286). Serine may be formed by one of two pathways. The first and most direct method is the reduction and dephosphorylation of dihydroxyacetone phosphate from the Embden-Meyerhof pathway to form hydroxypyruvic acid, and transamination of this compound to form serine (Sallach, 1956). The second method involves the oxidation of 3-phosphoglyceric acid to 3-phosphohydroxypyruvic acid and subsequent transamination to form 3-phosphoserine (Ichihara and Greenberg, 1957). The 3-phosphoserine may then be used for phosphatide synthesis, or dephosphorylated to form serine (White, Handler, and Smith, 1964, pp. 488-489). Glycine may be formed from L-serine by a loss of the *beta*-carbon of serine. This reaction appears to be reversible, requiring tetrahydrofolic acid as a C_1 accepter. L-Cysteine is formed from L-serine essentially by the substitution of a

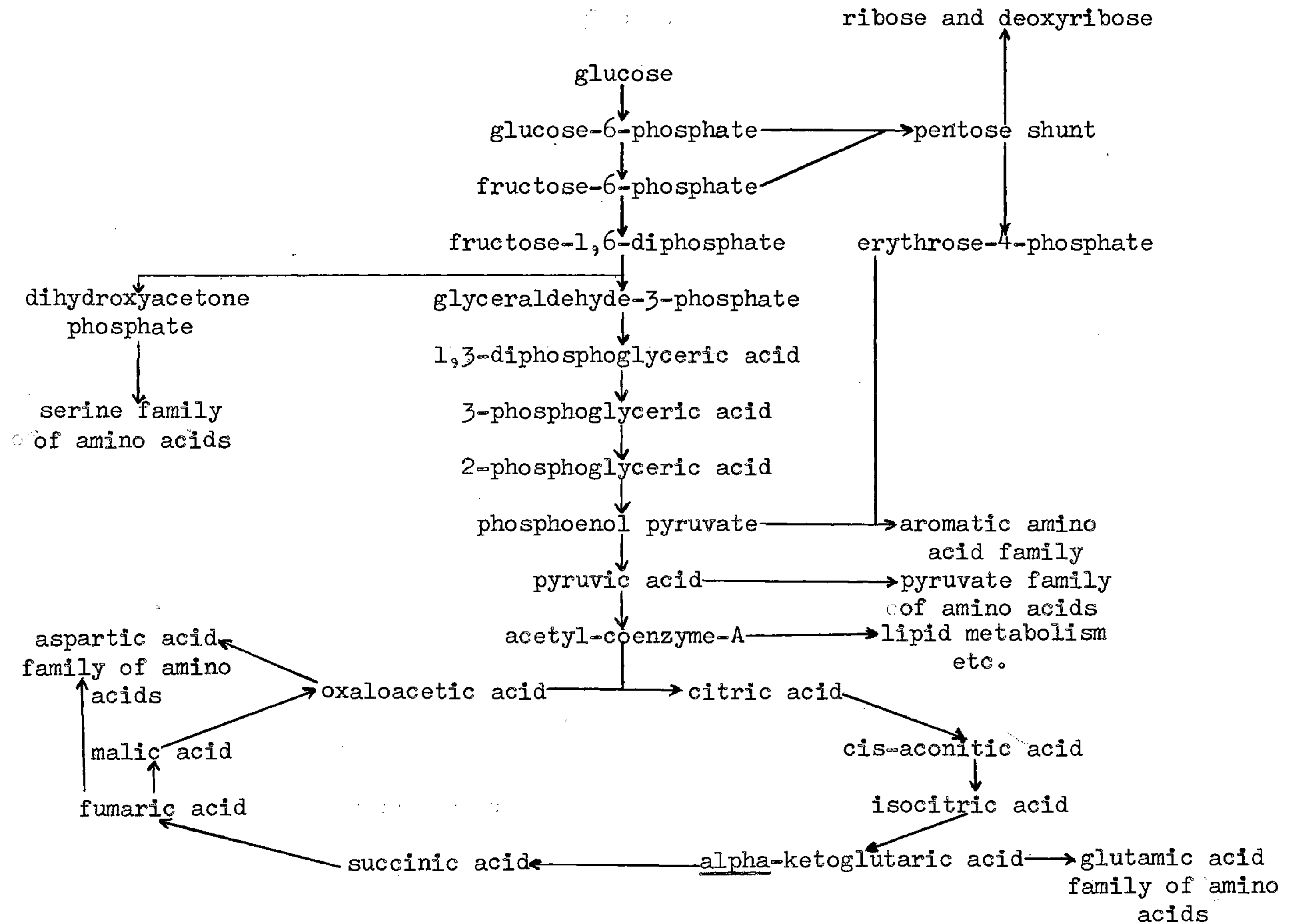


Figure 1. Embden-Meyerhof Pathway and Citric Acid Cycle for Glucose Metabolism

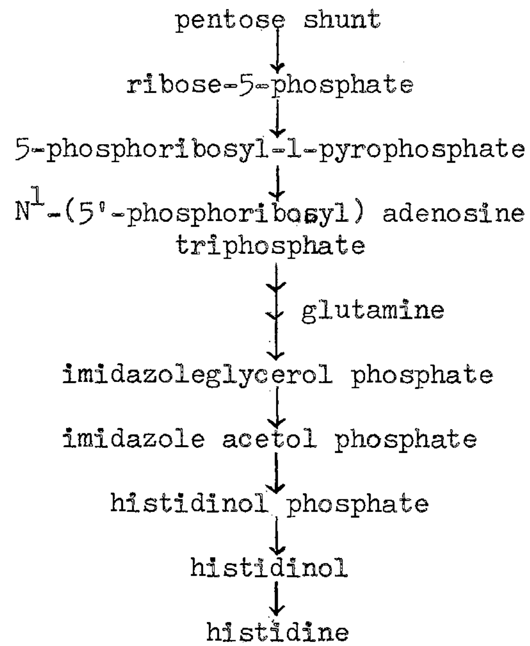


Figure 2. Synthesis of Histidine

sulfhydryl group for the hydroxyl group on the beta-carbon of L-serine. These pathways are illustrated in Figure 3.

The aromatic amino acids, tyrosine, tryptophan, and phenylalanine are formed from two intermediates of glucose metabolism (White, Handler, and Smith, 1964, pp. 522-523). Erythrose-4-phosphate, from the pentose shunt and phosphoenolpyruvate, from the Embden-Meyerhof pathway, condense to form the first intermediate leading to synthesis of aromatic amino acids. These pathways are illustrated in Figure 4.

The precursor molecule for alanine, valine, and leucine synthesis is pyruvic acid (Roberts et al., 1963, pp. 275-280). The synthesis of L-alanine involves the transamination of pyruvic acid or its direct amination. Valine and leucine are formed after the initial condensation of two pyruvate molecules with elimination of carbon dioxide to form the longer chained carbon backbone. These pathways are illustrated in Figure 5.

There are two families of amino acids which originate from the citric acid cycle. These include the glutamic and aspartic acid family of amino acids.

The glutamic family of amino acids are formed from alpha-ketoglutarate, a normal constituent of the citric acid cycle (White, Handler, and Smith, 1964, pp. 495-499). L-Glutamic acid may be formed either by the direct amination of alpha-ketoglutarate or by transamination with aspartic acid or alanine. The direct amination of alpha-ketoglutarate represents one of the most important ammonia utilizing reactions in the cell. Arginine and proline are formed essentially from the carbon backbone of glutamic acid (Roberts et al., 1963, pp. 249-259). These pathways are illustrated in Figure 6.

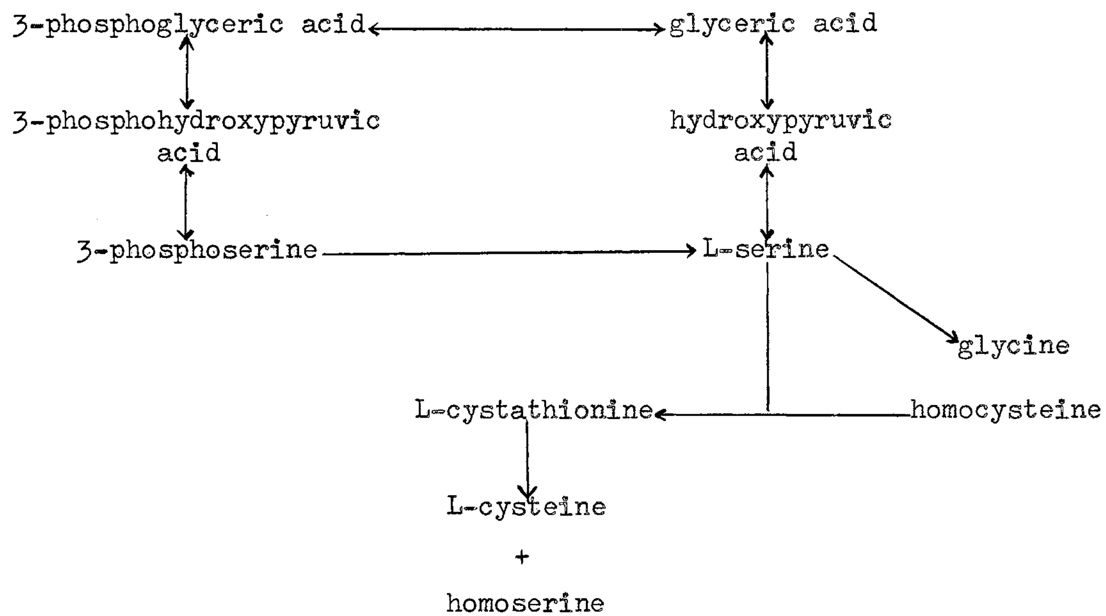


Figure 3. Synthesis of the Serine Family of Amino Acids

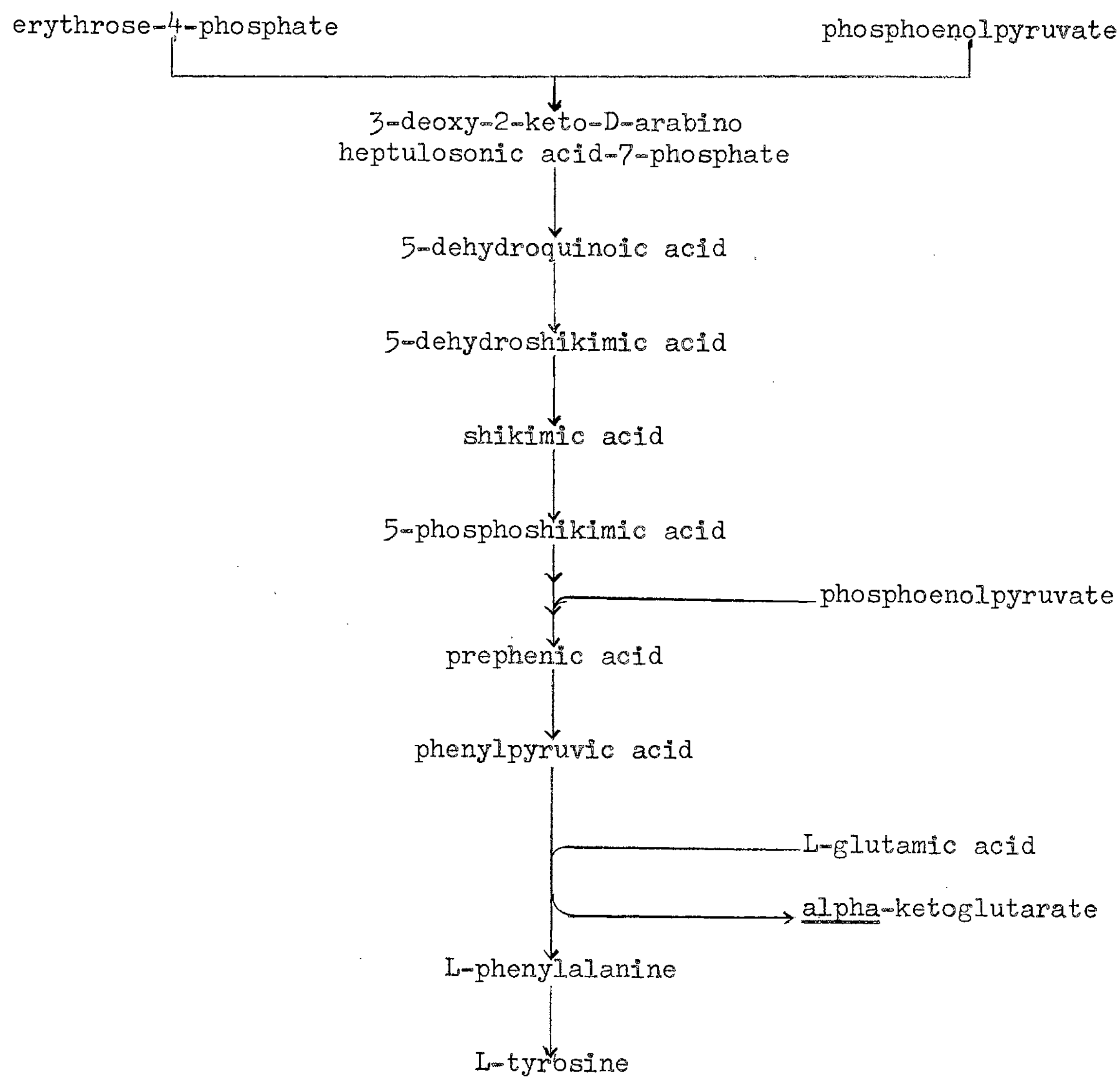


Figure 4. Synthesis of L-tyrosine and L-phenylalanine

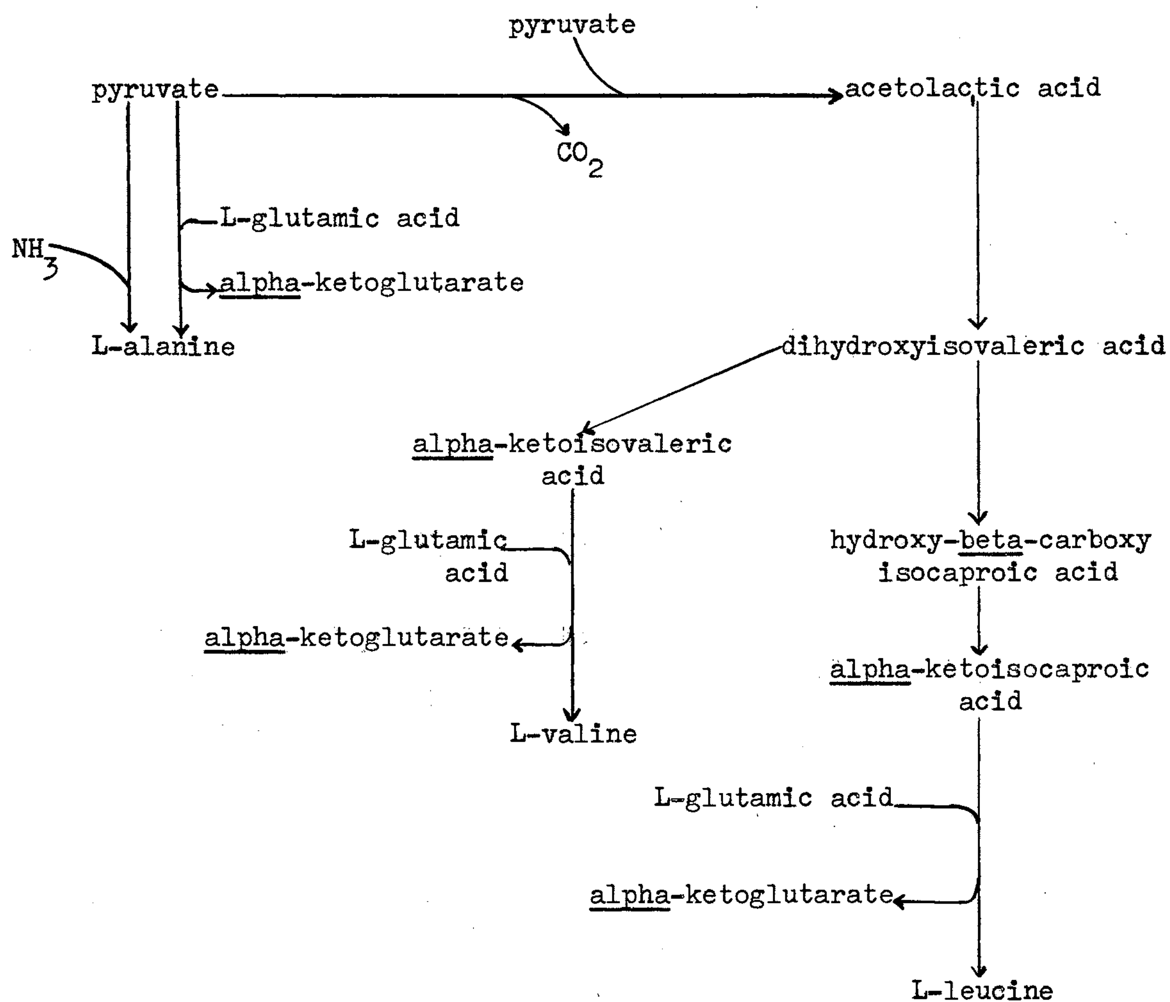


Figure 5. Synthesis of the Pyruvic Family of Amino Acids

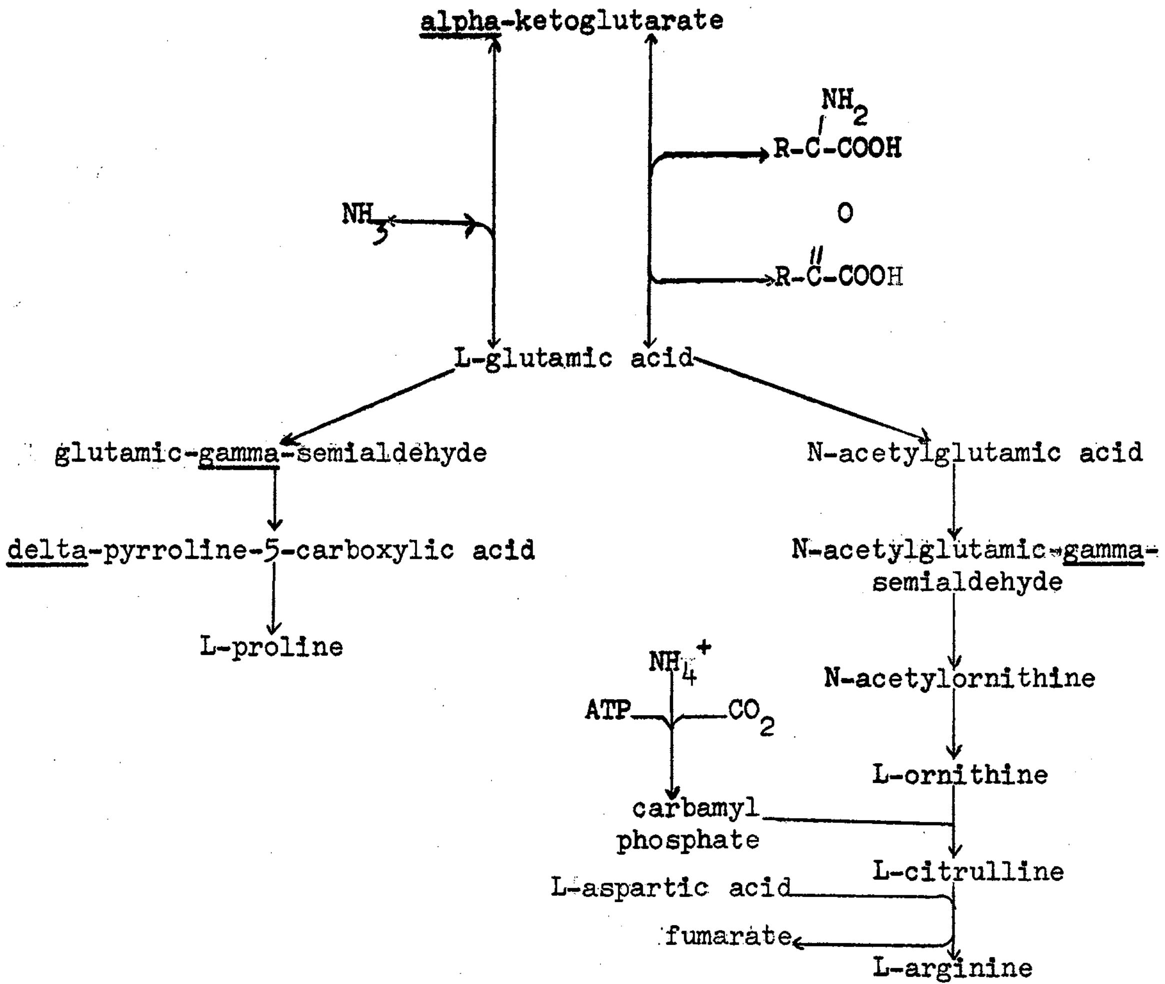


Figure 6. The Synthesis of the Glutamic Acid Family of Amino Acids

The aspartic acid family of amino acids originates from either oxaloacetic acid or fumaric acid (White, Handler, and Smith, 1964, pp. 497-503). L-Aspartic acid may be formed from oxaloacetic acid by transamination, usually with glutamic acid. This is the most direct route for synthesis and is the predominant pathway. The other pathway involves the direct amination of fumaric acid. Other members of the aspartic acid family, including lysine, methionine, threonine, isoleucine, and diaminopimelic acid, are formed from the carbon backbone of aspartic acid (Roberts et al., 1963, pp. 259-268). These pathways are illustrated in Figure 7.

Lipid Material

Most of the cellular lipid of bacteria may be found in the cell membrane and consists primarily of phospholipids (O'Leary, 1962). The lipids generally contain low amounts of carbohydrate, and are high in inositol and glycerol containing lipids, while ethanolamine, choline, and serine are the most common nitrogen-containing phospholipids.

The major synthesis of the fatty acids for lipid material originates from acetyl coenzyme-A (White, Handler, and Smith, 1964, pp. 452-480). One acetyl coenzyme-A molecule and a carbon dioxide molecule condense to form malonyl-coenzyme-A. The malonyl-coenzyme-A molecule is then condensed with an acetyl-coenzyme-A molecule in the presence of reduced pyridine dinucleotide and adenosine triphosphate to form acyl-coenzyme-A with the release of carbon dioxide. The molecule then condenses with more acetyl-coenzyme-A molecules to form the fatty acids. This scheme is illustrated in Figure 8.

The glycerol backbone for phosphatide synthesis is formed from

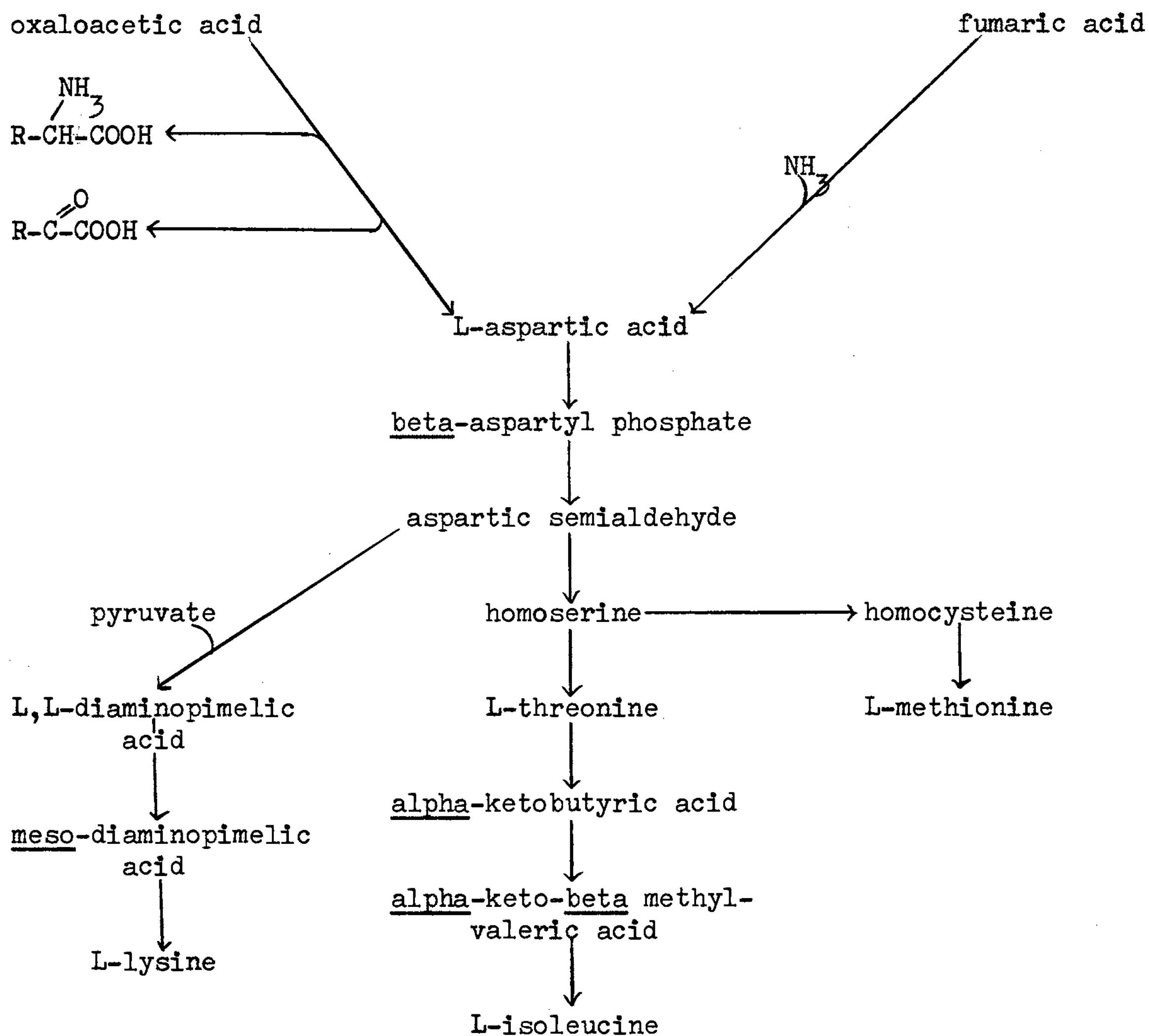


Figure 7. The Synthesis of the Aspartic Acid Family of Amino Acids

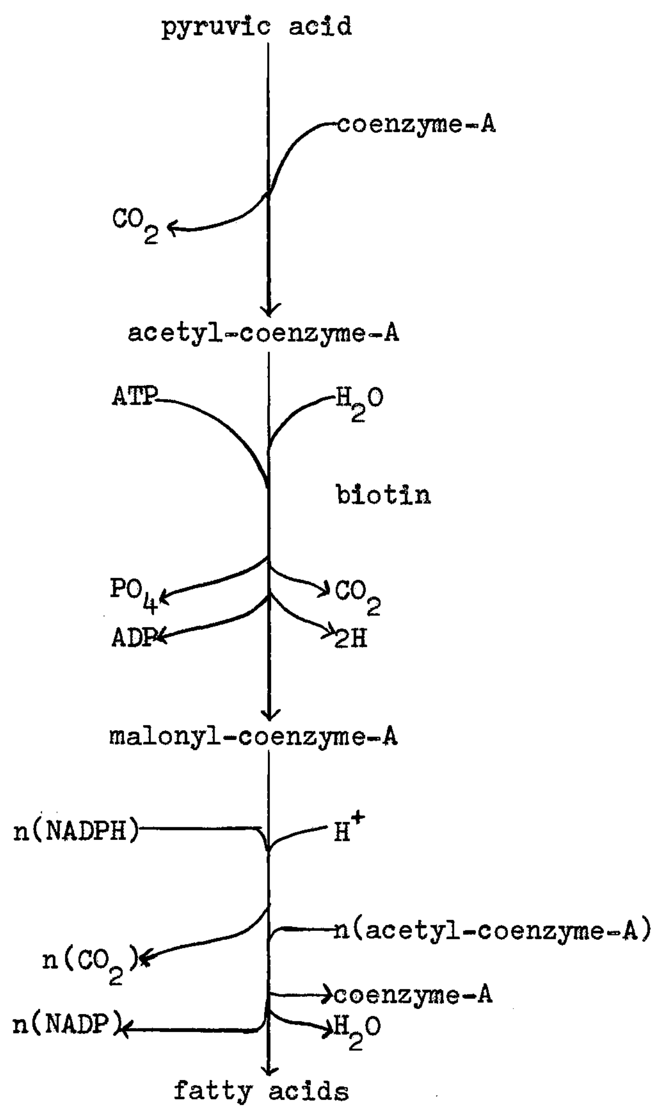


Figure 8. The Synthesis of Cellular Fatty Acids

L-alpha-glycerol-phosphate (White, Handler, and Smith, 1964, pp. 452-480). This compound is condensed with two molecules of fatty acid to form L-alpha-phosphatidic acid. This compound then reacts with cytidine triphosphate to form cytidine diphosphate diglyceride. This compound can then react with L-serine to form phosphatidyl serine or glycerol to yield phosphatides. Phosphatidyl serine may be decarboxylated to form phosphatidyl ethanolamine which in turn can add three methyl groups to yield phosphatidyl choline. These reactions are illustrated in Figure 9.

Nucleic Acid Synthesis

The three components of nucleic acid are a pentose (ribose or deoxyribose) purine or pyrimidine bases and phosphate (Roberts et al., 1964, pp. 282-317). The pentose shunt provides the sugar component for all nucleic acid synthesis. Ribose may be used for ribonucleic acid synthesis or transformed to deoxyribose for deoxyribonucleic acid synthesis.

The purines and pyrimidines derive their carbon primarily from glycine and L-aspartic acid respectively (Roberts et al., 1963, pp. 287-307). Formate and carbon dioxide also contribute to the carbon of the purines and pyrimidines. The general schemes for the synthesis of the nucleic acid bases are presented in Figure 10.

Kornberg (1966), studying various mutants of *Escherichia coli*, has reported that the presence or absence of various enzyme systems in the organism can be deduced from the growth responses obtained in different defined media. The author's findings are presented in Table I. One can determine whether an organism is deficient in a particular enzyme system

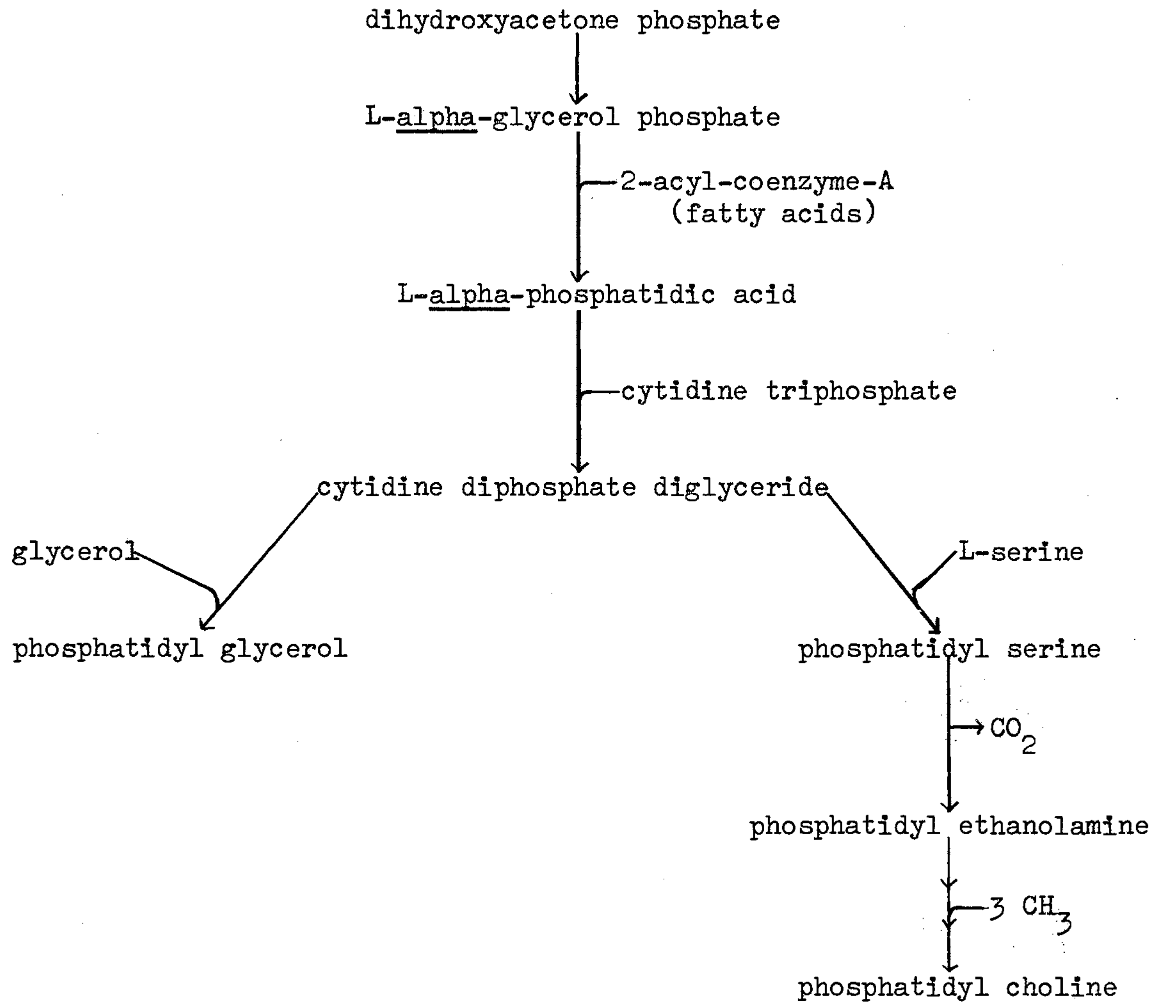


Figure 9. The Synthesis of Some Phospholipids

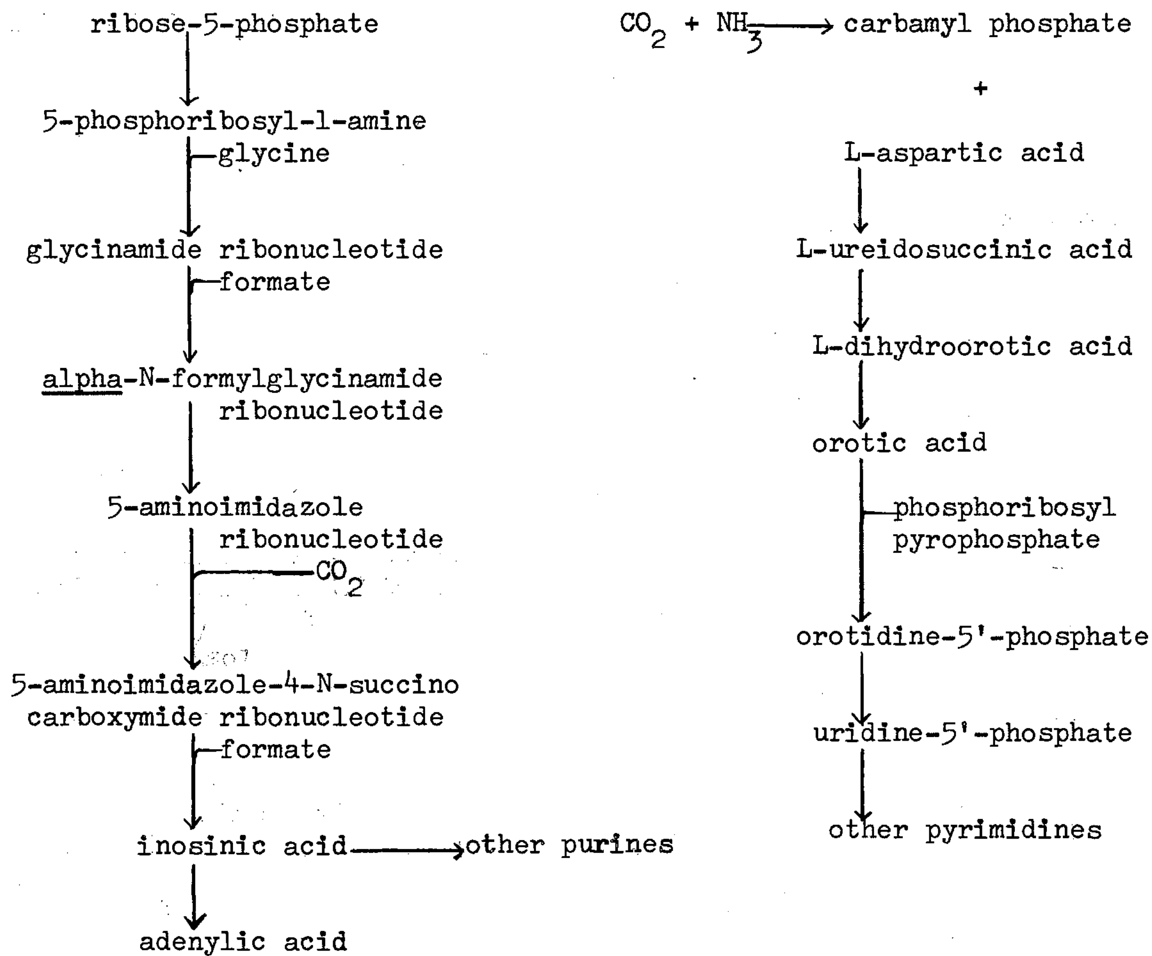


Figure 10. Synthesis of the Nucleic Acid Bases

TABLE I

ESCHERICHIA COLI AND CULTURAL CHARACTERISTICS AND RELATIONSHIP OF MUTANTS TO METABOLIC BLOCKS IN METABOLISM

Enzymic dysfunction	Special growth characteristics
None	None
Lacks citrate synthase	Requires glutamate
Lacks first enzyme of pyruvic dehydrogenase complex	Requires acetate
Lacks isocitrate lyase	Does not grow on acetate
Lacks phosphoenol pyruvate carboxylase	Does not grow on glucose unless medium is supplemented with tricarboxylic acid cycle intermediates; grows on acetate
Lacks phosphoenolpyruvate synthase	Does not grow on pyruvate, grows on glucose or acetate
Lacks phosphoenolpyruvate synthase and phosphoenolpyruvate carboxylase	Does not grow on pyruvate or glucose unless media supplemented with tricarboxylic acid cyle intermediates; grows on acetate
Lacks phosphoenolpyruvate synthase and first enzyme of pyruvic-dehydrogenase complex	Requires acetate
Lacks phosphoenol pyruvate synthase and is constitutive for isocitrate lyase	Grows on pyruvate

by nutritional analysis. A combination of nutritional and isotope tracer data aids in determining the biochemical basis for an organism's ability or inability to utilize a particular carbon source.

Pantoyl Lactone

Several reports have been made of pantoyl lactone as a reverser of cell division inhibition but very little is known of its mode of action (Grula and Grula, 1962, pp. 981-988); Adler and Hardigree, 1964; 1965, pp. 223-226; 1965, pp. 92-102). Grula and Grula (1962, pp. 981-988) have reported that the addition of pantoyl lactone or pantoic acid does not restore normal pantothenate levels in the presence of D-serine. It will, however, allow growth and division of the cell.

Whitney and Grula (1964) reported that D-serine was incorporated and partially replaced glycine in the mucopeptide of *M. lysodeikticus* Grula, Smith, and Grula (1965) reported also that D-serine would enter and partially replace the glycine in the mucopeptide of *Erwinia* sp. They found when pantoyl lactone was added to reverse division inhibition in *Erwinia* sp. no significant decrease in D-serine incorporation into the mucopeptide was noted. This would indicate that reversal of incorporation of the D-amino acid into the wall is not the mechanism of reversal of cell division inhibition by pantoyl lactone. Majerus, Alberts, and Vagelos (1965) reported treatment of the acyl carrier protein with pronase released pantoyl lactone. When the acyl carrier protein is treated with 3N NaOH, a phosphorylated compound is released. If this compound is treated with pronase, it releases pantoyl lactone. They identified the compound as 4'-phosphopantothenic acid. This is

the prosthetic group of the acyl carrier protein and represents a new function for pantoyl lactone.

CHAPTER III

MATERIALS AND METHODS

Test Organism

The organism used throughout this study was a stock culture of a species of *Erwinia*. The biochemical and morphological characteristics most nearly resemble those described for *Erwinia carotovora* in the 7th edition of *Bergey's Manual of Determinative Bacteriology* (Grula, 1960). The organism is gram-negative, motile, and rod-shaped. Optimum temperature for growth is approximately 25 C and acid but no gas is produced from glucose, lactose, and maltose.

Stock cultures were maintained on nutrient agar containing 0.5 per cent sodium chloride with and without 1.0 per cent glucose. Transfers were made daily alternating media with and without glucose. To insure purity, the culture was periodically streaked on Petri plates containing nutrient agar.

Media

The basal medium used in this study contained the following per 100 ml: DL-aspartic acid (300 mg), D-glucose (300 mg), KH_2PO_4 (136 mg), K_2HPO_4 (174 mg) and $MgSO_4$ (30 mg). The following were added as trace mineral salts: H_3BO_3 (0.5 µg), $CaCO_3$ (10.0 µg), $CuSO_4 \cdot 5H_2O$ (1.0 µg), $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (50 µg), KI (1.0 µg), $MnSO_4 : H_2O$ (2.0 µg), MoO3

(1.0 μg), and $ZnSO_4 \cdot 7H_2O$ (5.0 μg). When desired, D-serine (to 0.026 M), pantoyl lactone (to 0.0115 M), and glutamic acid (to 0.0284 M) were added. All solutions were adjusted to pH 6.8 to 7.0 prior to addition to the medium. Glucose, D-serine, and pantoyl lactone were sterilized by filtration. Phosphates, aspartic acid, magnesium sulfate, and mineral salts were sterilized by autoclaving for 10 minutes at 240 F.

Growth of Cells

Nutrient agar slants containing 0.5 per cent sodium chloride were inoculated from stock cultures and incubated 24 hours at 25 C. The cells were suspended in sterile 0.85 per cent saline and washed two times with saline by centrifugation. Cells from one nutrient agar slant were then resuspended in saline to an optical density equivalent to 0.03 at 540 mμ in 12 mm by 75 mm tubes (Coleman Junior Spectrophotometer). One drop of this suspension was used to inoculate 5.0 ml of medium. Twenty-five ml volumes of medium were incubated at 25 C on a Dubnoff metabolic shaking incubator in 50 ml Erlenmeyer flasks. Volumes of 5.0 ml were incubated in 20 mm by 150 mm test tubes at 25 C on a rotary shaker at 120 revolutions per minute. Volumes of 100 ml were incubated at 25 C in 250 ml Erlenmeyer flasks on the rotary shaker. Cells were generally harvested by centrifugation after 14 hours incubation.

Procedures for Cell Fractionation

After growth in a particular medium, cells were harvested and washed two times with 0.85 per cent sodium chloride. The cells were then fractionated by one of two methods. The first method is as

follows: The cells were suspended in water and heated at 100 C for twenty minutes to remove the soluble portion of the cellular material. The extract was centrifuged and the residue was resuspended in 95 per cent ethanol to remove the lipid material. The residue was then washed and hydrolyzed. This technique was used when cellular protein was of greatest interest.

The extraction procedure of Roberts, Cowie, Abelson, Bolton, and Britten (1963, pp. 13-30) were used if more exact data relating to the different fractions of the cell was desired. The cell pools were extracted from the cells with 5 per cent trichloroacetic acid at 4 C for 30 minutes. After centrifugation any residual trichloroacetic acid was wiped from the walls of the tube and the lipid was extracted from the cell with 75 per cent ethanol at 40 C for 30 minutes. Any residual lipid material was then removed from the pellet by extracting with a mixture of 75 per cent ethanol and ether 1:1 at 40 C for 15 minutes. After centrifugation, the nucleic acid was removed from the residue by boiling at 100 C in 5 per cent trichloroacetic acid for 30 minutes. The residue was then washed five times with distilled water and the protein mucopeptide residue was hydrolyzed.

Determinations of Dry Weight

The relationship between dry weight and absorbancy at 540 mμ on a Coleman Junior Spectrophotometer was determined for cells grown in an aspartic acid medium. Cells which had grown for 14 hours were washed three times with 0.85 per cent saline. Serial dilutions of the cell suspension were made and the absorbancy of each sample was measured. Aliquots were pipetted into preweighed aluminum dishes and dried to a

constant weight at 100 C in a dry air oven. Results were plotted as μg dry weight of cells versus absorbancy at 540 mμ.

Techniques for Isotope Labeling

Cells were grown in 25 ml of medium with the desired additions for 14 hours. At that time, the desired labeled compound was added such that the final amount was 0.05 μC per ml of medium. Incubation was continued for various time intervals from 5 minutes to 1.5 hours. The radioactive compound was always diluted with non-labeled carrier in the medium to insure correct concentrations during growth. Immediately before harvest, the absorbancy of the cells was measured at 540 mμ using a Coleman Junior Spectrophotometer. The cells were harvested and fractionated and the radioactivity of the fractions determined. Results are expressed as counts per minute per mg dry weight of cells.

Procedures for Determination of Uptake of Labeled Compounds

Cells were grown in the desired medium at 25 C, harvested, and placed in fresh medium for two hours incubation. The various additions were made and allowed to equilibrate for 5 minutes. The labeled compound was added and 0.3 ml samples of the culture were collected by filtration at various time intervals beginning with 30 seconds. Immediately after the cells were collected on the membrane filter, they were washed three times with a mineral salts solution (1 ml amounts) at the concentration used in the basal medium. The filter pads were dried at 100 C in a warm air oven. One-half ml of 1,4-dioxane was added to the dried sample to be analyzed and the material was allowed to

dissolve. After the sample had dissolved 9.5 ml of the counting fluid, prepared by adding 4.0 g diphenyl-oxazole and 50 mg of 1,4-bis-2 (phenyloxoyl) benzene to 1 liter of 1,4-di-oxane, was added. The samples were counted in a Nuclear of Chicago Scintillation counter. Results are expressed as counts per minute per mg dry weight of cells.

Acid Hydrolysis of Protein Material

Amino acid hydrolysis was performed by placing 0.3 to 2.0 ml of a protein sample in an 8 mm by 100 mm test tube. An equal volume of 12N hydrochloric acid was added and the tube sealed *in vacuo*. Hydrolysis was allowed to proceed for 18 hours at 100 C.

Preparation of Hydrazone

One ml of the material to be analyzed was mixed with 0.2 ml of 2/3 N H_2SO_4, 2.0 ml of 4 per cent tungstic acid, and 3.0 ml of 0.2 per cent 2,4-dinitrophenylhydrazine in 2N HCL (Strassman, Shatton, and Weinhouse, 1960). The reaction was allowed to proceed for 20 minutes without shaking. The reaction mixture was then extracted with ether until the lower layer was colorless. The ether extract was dried under a stream of warm air. The dried residue was redissolved in a mixture of chloroform and 1N NH_4OH (3:1). The NH_4OH layer was removed and the pH lowered to 1.0 with 6N H_2SO_4. This layer was then extracted with ether and the ether fraction dried under a stream of warm air. The residue was then ready to be spotted for chromatography or to be reduced with hydrogen to form the corresponding amino acids. The amino acid is formed by the reduction of the hydrazone with hydrogen, in an

acetic acid solution with platinum black as the catalyst (Kun and Hernandez, 1956).

Chromatography and Detection of Amino Acids

Paper chromatograms for amino acid analysis were developed in the two-dimensional systems of Redfield (1953) and Roberts, Cowie, Abelson, Bolton and Britten (1963, p. 40). Thin layer chromatograms were developed in the two-dimensional system of Heathcote and Jones (1965). Whatman No. 1 filter paper was used for the papergrams and MN-300 cellulose was used for the thin layer plates. Samples of 10 to 200 μl were spotted under a stream of warm air. Amino acids were detected by spraying with a solution of 0.5 per cent ninhydrin in 95 per cent acetone containing 5 per cent water (v/v). After spraying, the chromatograms were heated at 100 C for 3 minutes. Amino acids appeared as blue, yellow, or reddish-brown spots on a white background.

Quantitation of Amino Acid

Amino acids were quantitated according to the procedure of Giri, Radhakrishan, and Vaidyanathan (1952). Protein samples were hydrolyzed and chromatographed on thin layer chromatograms as described previously. The chromatograms were sprayed with 0.5 per cent ninhydrin in 95 per cent ethanol and heated at 65 C for 30 minutes. The ninhydrin-positive spots were scraped off and placed in a test tube. Four ml of 75 per cent ethanol containing 0.05 mg of copper sulfate per ml were added. The tubes were allowed to stand approximately 5 minutes with frequent shaking. The supernatant fluid was decanted and absorbancy measured at

540 mu. The concentrations of amino acids were then read from a standard curve plotted for each amino acid.

Chromatography and Detection of Hydrazones

The dried hydrazone material was taken up in 0.5N NH_4OH and spotted on 8 by 8 inch sheets of Whatman No. 1 filter paper. The sheets were then developed in either 1-butanol; 0.5N NH_4OH; ethanol (70:30:10); or 1-butanol saturated with water (Bassett and Harper, 1958). The hydrazones appear as yellow spots on a white background. When observed under ultraviolet light, the hydrazones appear dark on a light background. If the paper is sprayed with 3N NaOH, hydrazones will turn a chocolate brown.

Radioautography

Radioautography was performed by placing paper chromatograms or thin layer chromatograms next to Blue Brand medical x-ray film and allowing them to stand 1 to 4 weeks at 25 C. Previous work in this laboratory has shown that this method allows detection of 100 counts per minute of a C^{14}-labeled compound in 16 days.

Conversion of Pantoyl Lactone to Pantoic Acid

Since pantoyl lactone but not pantoic acid is available commercially, it was necessary to hydrolyze pantoyl lactone in order to obtain pantoic acid. This was done by heating 2.0 g of pantoyl lactone in 20 ml distilled water containing 0.56 g of NaOH at 100 C for 10 minutes (Grula and Grula, 1962, pp. 981-988). The solution was pH 6.8

to 7.2 after cooling with no adjusting. The solution was maintained at pH 7.2 to prevent reconversion to the lactone form.

Procedure for Counting Planchets

Samples to be analyzed were plated at infinite thinness and counted using a Picker automatic gas-flow planchet counter operated windowless. A counting efficiency of approximately 40 per cent was obtained with this unit. Samples were plated on plain stainless steel planchets. All planchets were discarded after one use.

CHAPTER IV

RESULTS AND DISCUSSION

Glucose Utilization in *Erwinia* Species

Grula (1960) reported that growth will not occur when *Erwinia* sp. is cultured in a glucose-ammonium salts (GA) medium. Two possibilities may be considered to explain inability of the organism to grow under these conditions: (1) Glucose may be unable to enter the cell or (2) a block exists in the metabolism of glucose. The first possibility has been eliminated since Dr. Mary Grula, using a Warburg apparatus, has shown that cells of *Erwinia* sp. immediately oxidize glucose (unpublished data). It, therefore, appeared that the organism possesses a block at some point in the metabolism of the glucose molecule.

The organism was grown in GA medium for sixteen hours with D-glucose-UL-C^{14} added as a tracer. At the end of this time, a slight increase in turbidity may occur, depending on inoculum size, but the cells do not exhibit observable lysis. The cells were harvested and extracted to obtain the protein-mucopeptide fraction, as described previously. After hydrolysis and chromatography, amino acids were analyzed by radioautography. Using this approach, it was observed that good labeling occurs in the serine, histidine, and aromatic amino acid families; a very light labeling pattern is present in the pyruvate family. No carbon from glucose could be located in the aspartic or glutamic acid

families of amino acids. These data are presented in Table II.

These data further confirm that Erwinia sp. can take glucose into the cell and in addition demonstrate that the Embden-Meyerhof pathway is operating at least to the phosphoenolpyruvate (PEP) stage since glucose carbon is present in the serine and aromatic families in appreciable amounts. The pentose shunt must also be operating since histidine, which requires prior synthesis of ribose-5-phosphate, is synthesized. Since the pyruvate family is poorly labeled and since no glucose carbon enters the aspartic or glutamic acid families, two blocks involving glucose utilization appear possible. (1) This organism has difficulty in synthesizing pyruvic acid from PEP or, (2) if pyruvate is formed, entry of carbon into the citric acid cycle is completely blocked. Poor labeling into the pyruvate family indicates that pyruvic acid synthesis is probably the key reaction that is affected.

Stimulation of Growth by Pantoyl Lactone

Because lysis of the cells could occur in the GA medium and in that way limit any possible increase in cell mass, pantoyl lactone was added to the medium in the presence and absence of glucose to protect the cell membrane. Pantoyl lactone will allow growth, but only when glucose is present in the medium (Figure 11).

To determine whether pantoyl lactone was entering the carbon pool of the cell, or instead, allowing the utilization of glucose, cells were grown in the GA medium with glucose-UL-C^{14} in the presence of pantoyl lactone. Data presented in Table III reveal that pantoyl lactone is allowing complete utilization of glucose carbon since label from glucose now appears in all families of amino acids. These data do not rule out

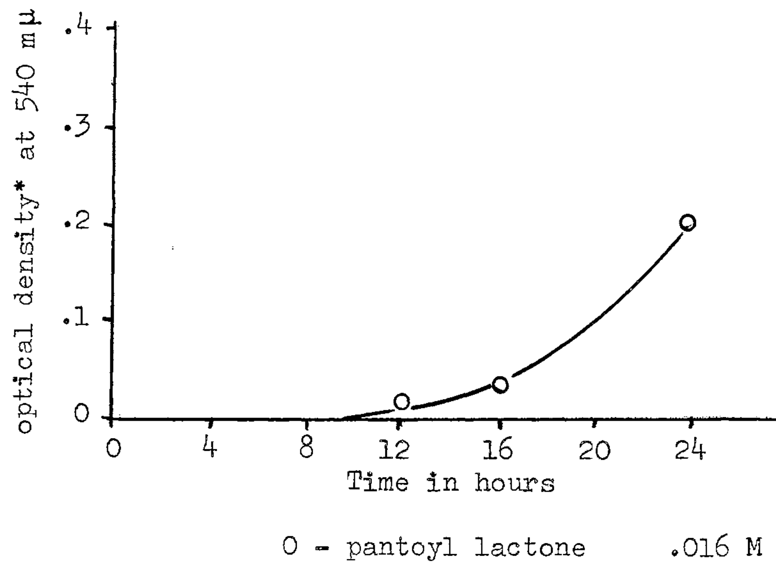
TABLE II

LABELING PATTERN OBTAINED USING GLUCOSE-UL-C^{14} IN THE GA MEDIUM

Amino acid*	Estimated Amount of Label
histidine	++
serine	+++
glycine	+++
tyrosine	+++
phenylalanine	+++
alanine	+
valine	+
leucine	+
aspartic acid	-
lysine	-
methionine	-
threonine	-
isoleucine	-
diaminopimelic acid	-
glutamic acid	-
arginine	-
proline	-

*Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label



O - pantoyl lactone .016 M

*Optical density on the spectronic - 20.

Figure 11. Stimulation of Growth by Pantoyl Lactone in the GA Medium

TABLE III

LABELING PATTERNS WITH GLUCOSE-UL-C^{14} IN THE GA MEDIUM WITH ADDED PANTOYL LACTONE

Amino acid*	Estimated Amount of Label
histidine	+++
serine	+++
glycine	+++
tyrosine	+++
phenylalanine	+++
alanine	+++
valine	+++
leucine	+++
aspartic acid	++
lysine	++
methionine	+
threonine	+
isoleucine	+
diaminopimelic acid	+
glutamic acid	++
arginine	++
proline	+

*Tryptophan and cysteine were not detected since hydrolysis under these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

the possibility that carbon from pantoyl lactone is directly contributing to growth, however, it was known at this time (Dr. Mary Grula, unpublished data) that addition of pantothenic acid in catalytic amounts to the GA medium also allowed growth to occur. Therefore, it appeared that the organism would not grow in the GA medium because the cells could not synthesize the pyruvic acid needed for synthesis of pantothenic acid and ultimately, coenzyme-A.

Because of the leads developed to this point, intermediates in the biosynthetic pathway of pantothenic acid were tested to determine whether they could stimulate growth in a manner similar to pantoyl lactone (Table IV). In all cases, growth was not obtained when glucose was omitted from the medium. Since valine, pantoic acid, and pantothenate all stimulate growth to some extent, it appears that pantoyl lactone allows growth by permitting synthesis of pantothenic acid which, in turn, makes possible the synthesis of coenzyme-A. Inability of the organism to synthesize needed pantothenic acid is not due to a lesion in synthesis of the beta-alanine portion of the molecule since addition of this compound does not allow growth to the extent obtainable when the pantoic acid portion of the molecule is added. Lack of growth response to alpha-ketoisovaleric acid is probably due to lack of entry into the cell by this compound since its amination product (valine) permits good growth. Thus, it appears that synthesis of coenzyme-A, once accomplished, could cause any small amount of pyruvate formed to enter the citric acid cycle and allow growth of the organism in a GA medium, although at a slow rate as shown in Figure 11. Data to be considered later will deal with the aspect of slow growth rate.

TABLE IV

STIMULATION OF GROWTH BY PRECURSORS OF PANTOTHENIC ACID AND PANTOTHENIC ACID IN A GLUCOSE-AMMONIUM SALTS MEDIUM

Compounds added for growth stimulation	Optical density at 540 mμ at 24 hours
pantoyl lactone*	.18
pantoyl lactone* + *beta*-alanine*	.18
pantoic acid*	.125
beta-alanine*	.07
pantoic acid* + *beta*-alanine*	.17
L-valine**	.195
alpha-ketoisovaleric acid**	0.00
pantothenic acid***	.29
glucose only	0.01

Concentration

*0.016 M in the medium

**0.017 M in the medium

***0.0001 M in the medium

Readings taken on the Coleman jr. Spectrophotometer

Identification of a Glucose Metabolic Product

The writer found that a keto-acid of some type was accumulating from cellular metabolism in the GA medium since the compound reacted with 2,4-dinitrophenylhydrazine yielding a hydrazone derivative. This derivative was identified as pyruvic acid hydrazone on the basis of its chromatographic mobility and reductive amination to alanine. However, if lack of pyruvate kinase is the biochemical lesion existing in this organism the product accumulating should be PEP rather than pyruvic acid. The point now to be established was whether or not PEP could undergo destruction during hydrazone preparation and, thus, be identified as pyruvic acid. A carbonyl group for reaction with 2,4-dinitrophenylhydrazine does not exist in PEP; however, the phosphate group is very reactive and should be labile to the acid conditions required for hydrazone formation.

When hydrazones were prepared from PEP, pyruvate, and the compound present in the expended growth medium, all three hydrazones behaved similarly in this paper's chromatographic system (Table V). When the three compounds were reduced with hydrogen in the presence of platinum black to form the corresponding amino acid, all three of the hydrazone derivatives formed alanine. This indicates that the organism could be piling up PEP instead of pyruvic acid.

Stimulation of Growth by Pyruvic Acid

Since all data presented to this point indicated that the organism had difficulty synthesizing pyruvic acid, it was nutritionally tested. Data are given in Figure 12. Pyruvic acid will allow growth either in

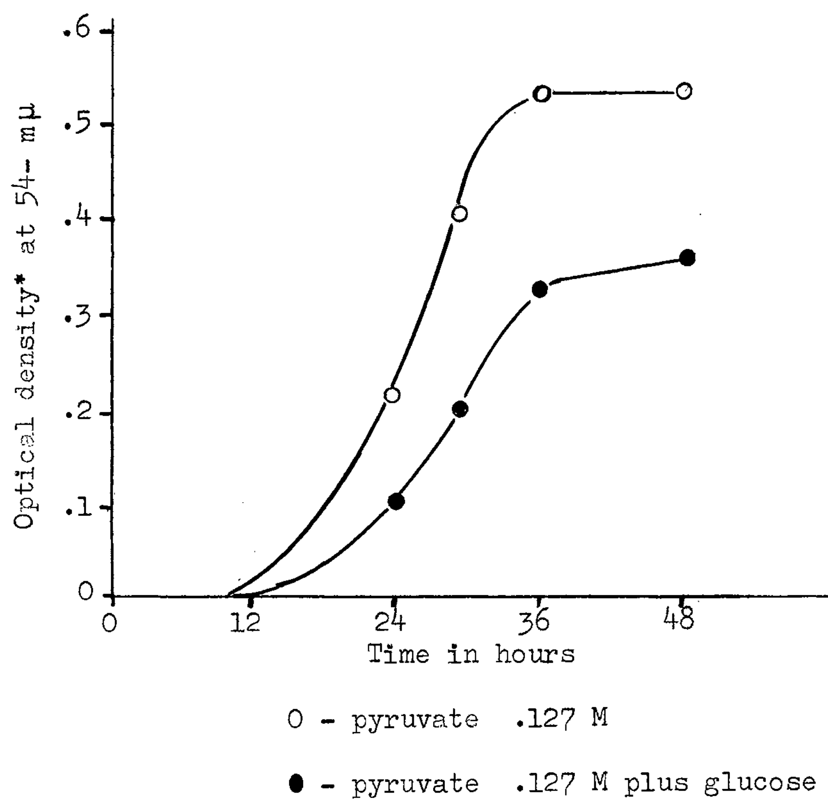
TABLE V

INFORMATION ON HYDRAZONE FORMING COMPOUNDS

Compound	Rf value*	Amino acid formed**
phosphoenolpyruvate hydrazone	.43	alanine
pyruvic acid hydrazone	.43	alanine
medium hydrazone	.44	alanine

*Solvent system: 1-butanol, 0.5M NH_4OH, ethanol (70:20:10).

**Solvent system: Redfield's two dimensional system described previously.



O - pyruvate .127 M

● - pyruvate .127 M plus glucose

*Optical density on the spectronic - 20

Figure 12. Stimulation of Growth by Pyruvate (0.127 M) in the Presence and Absence of Glucose

the presence or absence of glucose; however, a relatively long lag period is noted and total amount of growth is limited. These data indicate that Erwinia sp. probably cannot synthesize pyruvic acid from glucose or PEP; however, it can carry out the reverse reaction making glucose or PEP from pyruvate.

Stimulation of Growth by Intermediates of the Citric Acid Cycle

Grula (1960, pp. 375-385) reported that organic nitrogen, preferably aspartic acid, was required for growth of Erwinia sp. in the GA medium. Our results using either pyruvic acid, pantoyl lactone, or pantothenic acid indicate that the problem is not one involving nitrogen; rather, it involves inability of Erwinia sp. to synthesize needed carbon intermediates (pyruvic acid) from glucose. Even though pyruvate, pantoyl lactone, or pantothenic acid allowed growth (pyruvate in the absence of glucose), we were still not satisfied because of the lengthy lag period and decreased growth yields obtained at the end of 36 hour incubation periods (with aspartic acid present, growth is rapid and quite abundant after 15 hours incubation). The lengthy lag period and final low growth yields indicated that, even though pyruvate was supplied as a growth substrate, the organism might also have difficulty in carbon dioxide fixation reactions emanating from pyruvate or PEP necessary to replenish citric acid cycle intermediates normally drained off for biosynthetic reactions. Therefore, the writer tested for ability of Erwinia sp. to grow in the presence of various intermediates of the citric acid cycle. These data are presented in Table VI. Erwinia sp. grows rapidly and abundantly in the presence of citric, isocitric,

TABLE VI

STIMULATION OF GROWTH BY VARIOUS CITRIC ACID CYCLE INTERMEDIATES IN THE GA MEDIUM

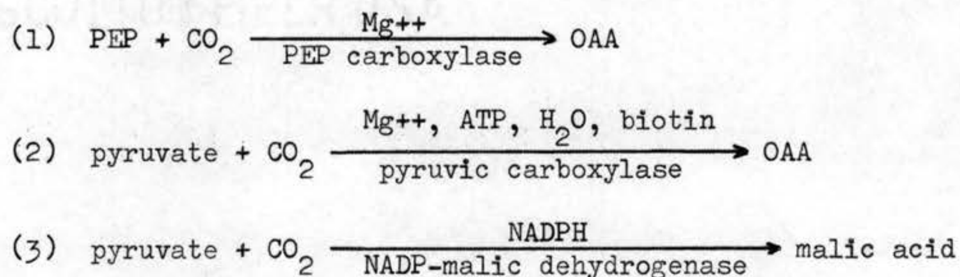
Intermediate added*	Optical density at 540 mμ** 16 hours + glucose	16 hours − glucose	24 hours + glucose	24 hours − glucose	36 hours + glucose	36 hours − glucose
pyruvic acid	0.20	0.30	0.40	0.45	0.50	0.55
acetate	0.00	0.00	0.00	0.00	0.00	0.00
citric acid	0.82	0.50	1.00	0.90	1.20	1.10
isocitric acid	0.86	0.00	1.10	0.00	1.20	0.00
alpha-ketoglutarate	0.00	0.00	0.00	0.00	0.01	0.00
succinic acid	0.00	0.00	0.00	0.00	0.00	0.00
fumaric acid	0.015	0.00	0.05	0.01	0.07	0.01
malic acid	1.30	0.95	1.50	1.20	1.60	1.50
oxaloacetic acid	0.07	0.07	0.32	0.25	0.75	0.60
glyoxalate	0.00	0.00	0.00	0.00	0.00	0.00
aspartic acid	1.10	0.85	1.40	1.10	1.50	1.40
control	0.00	0.00	0.00	0.00	0.00	0.00

*Intermediates added to a concentration of 0.0225 M in the medium.

**Optical density on Spectronic 20.

or malic acid. Growth obtained using malic acid as the carbon source is faster and more abundant than that obtained when aspartic acid is the carbon source (either in the presence or absence of glucose). Growth in the presence of oxaloacetic acid (OAA) is poor compared to malic acid, but slightly better than that obtained when pyruvate is present. This is attributed to the lability of OAA and its probable conversion (spontaneous decarboxylation) to pyruvic acid in the growth medium. Lack of, or poor growth in the presence of alpha-ketoglutarate, succinate, or fumarate can be attributed to poor penetration by these compounds into the cell (Dr. Mary Grula has obtained data using the Warburg apparatus which indicates poor or nonexistent oxidation of these compounds by whole cell suspensions).

The excellent growth obtained in the presence of malic acid appears to confirm the belief that the organism has difficulty in forming this compound (or OAA) from pyruvate or PEP via carbon dioxide fixation. Reactions involving carbon dioxide fixation are as follows:



$$(1)\quad \text{PEP} + CO_2 \xrightarrow[\text{PEP carboxylase}]{\text{Mg++}} \text{OAA}$$

$$(2)\quad \text{pyruvate} + CO_2 \xrightarrow[\text{pyruvic carboxylase}]{\text{Mg++, ATP, } H_2O\text{, biotin}} \text{OAA}$$

$$(3)\quad \text{pyruvate} + CO_2 \xrightarrow[\text{NADP-malic dehydrogenase}]{\text{NADPH}} \text{malic acid}$$

To obtain conclusive data relative to the ability of *Erwinia* sp. to fix carbon dioxide into malate or OAA, cells were cultured in the GA medium in the presence of $C^{14}O_2$ ($NaHC^{14}O_3$). Analysis was based on total incorporation compared to *Escherichia coli* grown under the same conditions and on the appearance of radioactivity in aspartic or other amino acids. Pantoyl lactone was added to duplicate flasks to determine if it

might aid carbon dioxide fixation. Data are presented in Table VII. Erwinia fixes carbon dioxide 49 per cent as well as E. coli. These data indicate that although Erwinia sp. can fix carbon dioxide, the system is only one-half as efficient as in E. coli. The addition of pantoyl lactone did not greatly aid carbon dioxide fixation in either organism, although some stimulation was evident.

Although fixation of carbon dioxide into Erwinia sp. is only one-half as efficient as fixation that occurred using E. coli, it should be remembered that Erwinia sp. has difficulty in forming pyruvic acid when cultured in the GA medium. Because of this metabolic lesion, most carbon dioxide would have to be fixed into PEP rather than into the small amount of pyruvic acid that is synthesized. Fixation into either product should result in synthesis of OAA or malic acid which should replenish the citric acid cycle and allow growth of the organism to at least one-half the amount obtainable using E. coli. This does not occur. Therefore, our data were unsatisfactory and actually in conflict with all theory developed to this point.

Fixation of carbon dioxide in the GA medium by E. coli results in heavy and equal labeling into all amino acids of the aspartic and glutamic acid families (Roberts et al., 1963, pp. 95-112). When radioautograms of the protein-mucopeptide amino acids labeled in the carbon dioxide fixation experiment were observed, it was very clear that Erwinia sp. fixed the great bulk of $C^{14}O_2$ into carbamyl phosphate, since arginine was by far the most heavily labeled compound (Table VIII). Although the aspartic and glutamic acid families of amino acids were labeled, the amount of labeling was low. Since it was known that PEP is formed (heavy labeling from glucose into the aromatic amino acids),

TABLE VII

CARBON DIOXIDE UPTAKE IN CELLS OF *ERWINIA* SP. AND *ESCHERICHIA COLI*

Organism	Specific Activity*
Erwinia sp.	2490
Escherichia coli	5130
Erwinia sp. plus pantoyl lactone	2950
Escherichia coli plus pantoyl lactone	6150

*Counts per minute per mg cells dry weight.

TABLE VIII

LABELING PATTERNS FOR CARBON DIOXIDE FIXATION IN *ERWINIA* SP. IN THE PRESENCE AND ABSENCE OF PANTOYL LACTONE

Amino acid*	Estimated Amount of Label no pantoyl lactone	 plus pantoyl lactone
histidine	-	-
serine	-	-
glycine	-	-
phenylalanine	-	-
tyrosine	-	-
alanine	-	-
valine	-	-
leucine	-	-
glutamic acid	++	++
proline	++	++
arginine	++++	++++
aspartic acid	++	++
methionine	++	+
threonine	++	++
isoleucine	++	++
lysine	++	++
diaminopimelic acid	+	-

*Tryptophan and cysteine were not detected since hydrolysis under these conditions destroys the amino acids.

- - no label
- + just detectable
- ++ light label
- +++ moderate label
- ++++ heavy label

these data indicated that Erwinia sp. does have a sluggish mechanism for fixing carbon dioxide into PEP, so sluggish that it is apparently not sufficient to replenish the carbon drained from the citric acid cycle for biosynthetic reactions. Apparently, the cells are better adapted to fix carbon dioxide into pyruvic acid since growth is possible when pyruvic acid is present (with or without glucose). Carbamyl phosphate synthesis in Erwinia sp. is extremely efficient, but, unfortunately, carbon dioxide fixed into arginine is poorly utilized by the cell for priming the citric acid cycle. Data to support the latter statement have been obtained by feeding radioactive arginine to Erwinia sp. and following utilization of carbon. Carbon from arginine is not cycled since arginine is the only amino acid that is labeled in the cellular protein-mucopeptide fraction after an exposure time of one and one-half hours when the cells are grown in the presence of aspartic acid. Also, Erwinia sp. will not grow in the GA medium when supplemented with arginine.

Summary of Glucose Metabolism

The inability of Erwinia sp. to grow in the GA medium is due to at least two blocks in the metabolism of the glucose molecule. Radioautography data have shown that glucose carbon reaches PEP probably via the Embden-Meyerhof pathway. Further synthetic reactions appear to be very sluggish. Phosphoenolpyruvic carboxylase and pyruvic kinase appear to be blocked. Although pyruvic kinase functions, it is at too slow a rate to provide carbon either for pantothenate synthesis or synthesis of citric acid cycle intermediates. This was demonstrated by the ability of the organism to grow in the GA medium when pantothenate, or its

precursors (pantoic acid and pantoyl lactone), pyruvic acid, or some intermediates of the citric acid cycle were added to stimulate growth. Further evidence that a block exists at the PEP carboxylase and pyruvic kinase steps was obtained by demonstrating the possible accumulation of PEP in the expended growth medium.

The stimulation of growth by the citric acid cycle intermediates indicates that *Erwinia* sp. has difficulty forming these acids in the GA medium. This is further evidence that the organism has difficulty fixing carbon dioxide to PEP or pyruvate. When *Erwinia* sp. was checked for ability to fix carbon dioxide, it assimilated it only 49 per cent as effectively as *E. coli*. This carbon dioxide fixation was primarily into carbamyl phosphate used for arginine synthesis. The lack of carbon dioxide fixation into malate or OAA is evident since these compounds, if present, not only stimulate growth, but if synthesized from glucose would cause heavy labeling of aspartic acid in the cell. Therefore, there must be a complete block in PEP carboxylase. The inability to form the citric acid cycle intermediates from pyruvate is due to the sluggish pyruvic kinase and also a lack of pantothenic acid synthesis by the cell.

Erwinia sp. will grow on pyruvate, aspartate, OAA, malate, and citrate as sole carbon sources. Since PEP carboxylase and pyruvic kinase are blocked or very sluggish in the organism there must be some alternate route providing carbon for synthesis of Embden-Meyerhof intermediates from pyruvic acid. Kornberg (1965) reported that *E. coli* was capable of converting pyruvic acid to PEP without involvement of pyruvic kinase. The author called the enzyme phosphoenolpyruvic synthase. This enzyme must be operating in *Erwinia* sp. and its activity

would allow any molecule which could reach pyruvic acid to enter the Embden-Meyerhof pathway. The proposed model for the pathways of carbon utilization in Erwinia sp. is presented in Figure 13.

Effect of D-serine on the Metabolism of Erwinia Species

After the metabolic blocks involving glucose metabolism were better understood, the effect of D-serine on the utilization of various carbon sources was tested. It was first necessary to determine the normal responses of the organism to two major carbon sources. The organism was grown for fourteen hours in an aspartic acid-glucose medium (aspartic carbon was utilized since radioactive malic acid could not be purchased) and then divided into two portions. Glucose-UL-C^{14} was added to one flask and aspartic-UL-C^{14} to the other. The cells were then incubated in the presence of label for 1.5 hours, harvested and fractionated as described in the materials and methods section. The specific activities of the cell pool, lipid, nucleic acid, and protein-mucopeptide fractions were determined after planchet counting (Table IX).

Aspartic acid and glucose are both extensively incorporated into the cell. Glucose labels lipid and nucleic acid material to a greater extent than aspartic acid. This is probably because glucose contributes relatively large amounts of carbon to lipids, carbohydrates, pentoses, and purines in addition to the proteins of the cell. The protein-mucopeptide fraction is well labeled by both aspartic acid and glucose.

After hydrolysis and chromatography, amino acid labeling patterns were analyzed using radioautography (Table X). Glucose contributes carbon to the amino acids of the histidine, serine and aromatic

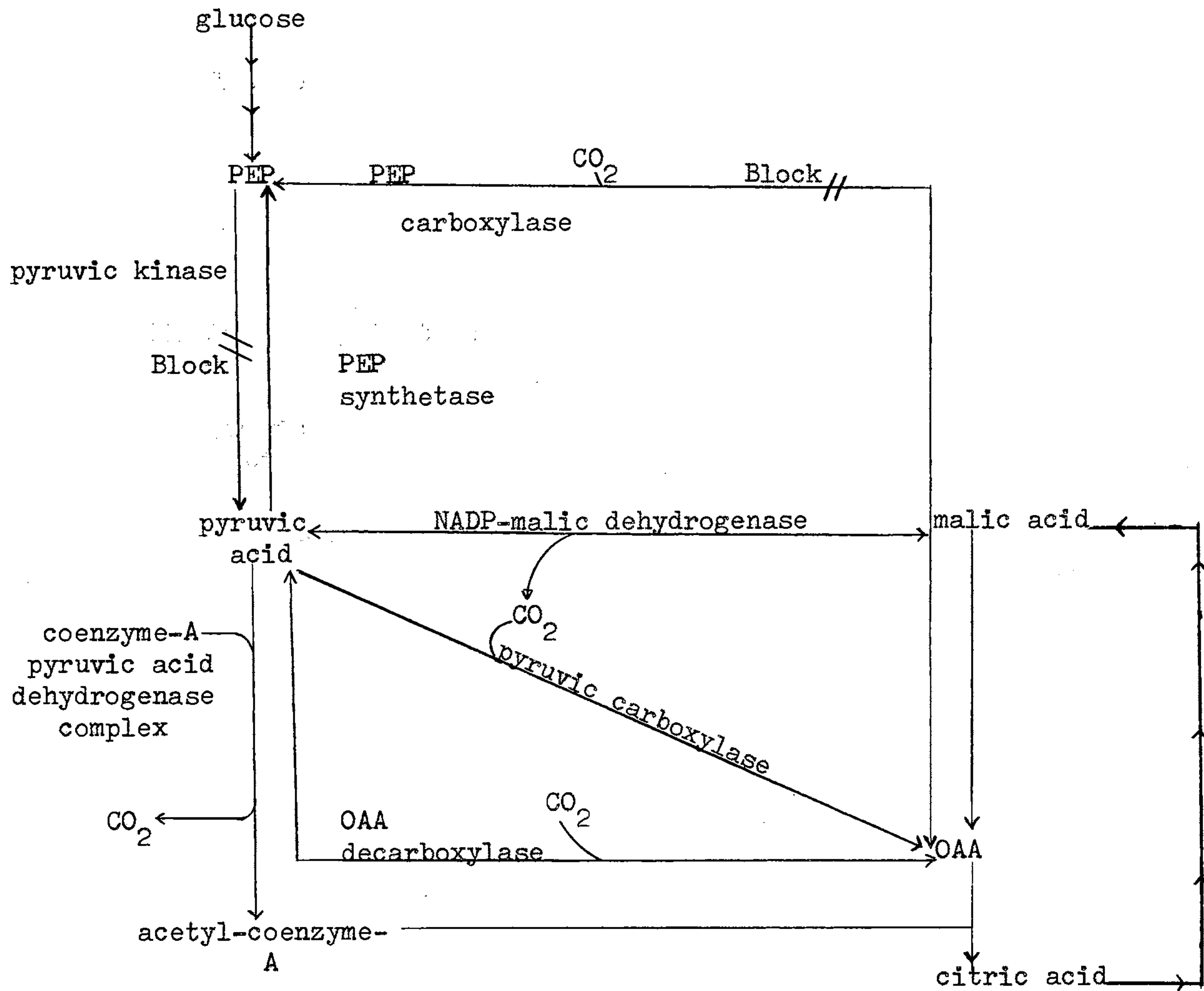


Figure 13. Biosynthetic Pathways in <u>Erwinia</u> sp.

TABLE IX

NORMAL LABELING PATTERNS OF *ERWINIA* SP. AFTER GROWTH IN THE PRESENCE OF GLUCOSE AND ASPARTIC ACID

Fraction		Specific activity*	
		Glucose-UL-C^{14}	Aspartic acid-UL-C^{14}
cold trichloroacetic acid (cell pool)		137	35
ethanol, 75% (lipid-protein)		700	501
ethanol, 75%, ether (1:1) (lipid)		365	40
hot trichloroacetic acid (nucleic acid)		226	93
protein-mucopeptide		1,780	1,695
	Total	3,208	2,364

*Counts per min per mg cells dry wt.

TABLE X

AMINO ACID LABELING PATTERN OF ERWINIA SP. AFTER GROWTH IN THE PRESENCE OF GLUCOSE AND ASPARTIC ACID*

Amino acid**	Labeling Compound Glucose-UL-C^{14}	Aspartic-UL-C^{14}
histidine	++++	+
serine	++++	+
glycine	++++	+
phenylalanine	++++	-
tyrosine	++++	-
alanine	+++	++
valine	+++	++
leucine	+++	++
glutamic acid	+	++++
proline	+	++++
arginine	+	++++
aspartic acid	-	++++
methionine	-	++++
threonine	-	++++
isoleucine	-	++++
lysine	-	++++
diaminopimelic acid	-	++++

*Cells were grown in the aspartic acid-glucose salts medium.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

families, while both aspartic acid and glucose contribute carbon to the pyruvate family of amino acids. Aspartic acid apparently provides all the carbon for the aspartic and glutamic acid families of amino acids.

Effect of D-serine on the Labeling Patterns From Glucose and Aspartic Acid

D-Serine was added to the medium to study its effects on utilization of aspartic acid and glucose (Table XI). The level of D-serine which was used inhibited both growth and division of the cell. These data show that although glucose and aspartic acid labeling in all fractions of the cell, except lipid from aspartic acid, are reduced by D-serine, the greatest effect is in the labeling of the protein-mucopeptide fraction of the cells grown in the presence of labeled aspartic acid. After hydrolysis and chromatography the amino acids were analyzed by radioautography (Table XII). These data reveal that D-serine does not have a marked qualitative effect on metabolism of glucose or aspartic acid; however, there is a very sizable quantitative effect on utilization of aspartic acid. The labeling pattern obtained from aspartic acid into the protein-mucopeptide fraction thus appeared to be an excellent method for studying the effects of D-serine and, possibly, those compounds which reverse the action of D-serine.

Labeling Pattern of D-serine

To obtain the labeling pattern for D-serine, cells were grown in the presence of cold D-serine for fourteen hours in the GA medium, after which they were incubated for an additional 1.5 hours in the presence of added D-serine-3-C^{14}. Chromatography of the hydrolyzed

TABLE XI

LABELING PATTERNS OF *ERWINIA* SP. OBTAINED USING GLUCOSE AND ASPARTIC ACID IN THE PRESENCE OF D-SERINE

Fraction	Specific Activity Glucose-UL-C^{14}	Specific Activity Aspartic-UL-C^{14}
cold trichloroacetic acid (cell pool)	50	85
ethanol, 75% (lipid-protein)	478	214
ethanol, 75%, ether (1:1) (lipid)	222	69
hot trichloroacetic acid (nucleic acid)	120	63
protein-mucopeptide	983	515
Total	1,853	946

*Counts per min per mg cells dry wt.

TABLE XII

AMINO ACID LABELING PATTERNS OF ERWINIA SP. AFTER GROWTH IN THE PRESENCE OF ASPARTIC ACID AND GLUCOSE WITH ADDITION OF D-SERINE*

Amino acid**	Labeling Compound	
	Glucose-UL-C^{14}	Aspartic-UL-C^{14}
histidine	++++	-
serine	++++	-
glycine	++++	-
phenylalanine	++++	-
tyrosine	++++	-
alanine	+++	+
valine	+++	+
leucine	+++	+
glutamic acid	+	++
proline	+	++
arginine	+	++
aspartic acid	-	+
methionine	-	+
threonine	-	+
isoleucine	-	+
lysine	+	+
diaminopimelic acid	-	+

*Cells were grown in the aspartic acid-glucose medium with D-serine present

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- \- no label
- \+ just detectable
- ++ light label
- +++ moderate label
- ++++ heavy label

protein-mucopeptide fraction was accomplished after fractionation and amino acids analyzed for radioactivity by radioautography (Table XIII). D-Serine labeled serine, histidine, and alanine in the protein-mucopeptide fraction. The label in alanine is due to contamination by alanine of the D-serine. Grula, Smith, and Grula (1965) reported that D-serine replaces glycine present in the mucopeptide. Therefore, the label in serine is probably present only in the mucopeptide. The label in histidine is probably derived from the number three carbon (β-carbon) of serine. When serine is converted to glycine, the β-carbon is removed and transferred by tetrahydrofolic acid as a C_1 moiety to be used in purine synthesis. Carbon present in the purine ring can be utilized for synthesis of the imadazole ring of histidine. The nucleic acid fraction of D-serine-labeled cells has a high specific activity indicating that the β-carbon can be used for purine synthesis. Thus, in labeling the histidine, serine must also be converted to glycine. Therefore, D-serine carbon is utilized for the synthesis of glycine, histidine, and purines. Also, D-serine can be incorporated intact into the mucopeptide portion of the cell wall. It should be noted that D-serine does not contribute carbon to either the aspartic or glutamic acid family of amino acids; therefore, the quantitative change in utilization of carbon from aspartic acid in the presence of D-serine cannot be attributed to isotopic dilution (Table XII).

Reversal of the D-serine Effect by Pantoyl Lactone

The inhibition of growth and division in _Erwinia_ sp. by D-serine can be reversed using several compounds; for example, pantoyl lactone and ammonium chloride. When pantoyl lactone is added to an aspartic

TABLE XIII

INCORPORATION OF D-SERINE-3-C^{14} INTO CELLULAR AMINO ACIDS*

Amino acid*	Amount of Label
histidine	++
serine	+++
glycine	-
phenylalanine	-
tyrosine	-
alanine	++
valine	-
leucine	-
glutamic acid	-
proline	-
arginine	-
aspartic acid	-
methionine	-
threonine	-
isoleucine	-
lysine	-
diaminopimelic acid	-

*Cells were grown in the aspartic acid-glucose medium with D-serine present.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

acid-glucose-D-serine medium, division occurs in what appears to be a normal manner. Since the writer knew the labeling patterns that could be expected from glucose and aspartic acid in the presence or absence of D-serine as well as the labeling pattern of D-serine, effects by pantoyl lactone on the patterns in the presence of D-serine were studied. Data are given in Table XIV. Pantoyl lactone has no marked effect on the qualitative labeling patterns of aspartic acid or D-serine. It does, however, affect utilization of glucose carbon for synthesis of serine and glycine since these amino acids are labeled very extensively in the presence of pantoyl lactone. Also, pantoyl lactone caused the synthesis of at least three compounds from glucose that could not be identified by these procedures. The qualitative and quantitative changes occurring in the metabolism of glucose as a result of growth in the presence of pantoyl lactone were not further studied since new compounds were involved and they were present in low amounts. Regardless of the importance of these unknown compounds in the division process, pantoyl lactone does have its effects primarily on the metabolism of glucose since the amounts of glucose carbon shunted to the synthesis of serine and glycine are very striking.

Reversal of the D-serine Effect by Ammonium Chloride

Effects of ammonium chloride on the utilization of carbon from aspartic acid, glucose, and D-serine are presented in Table XV. The effects of ammonium chloride are very striking and this compound allows what appears to be normal utilization of aspartic acid. It does not alter metabolism of D-serine since label is still present in serine and histidine. Since D-serine does not qualitatively alter glucose

TABLE XIV

LABELING PATTERNS OF GLUCOSE, ASPARTIC ACID, AND D-SERINE IN THE PRESENCE OF PANTOYL LACTONE*

Amino acid**	Labeling Compound		
	Glucose-UL-C^{14}	Aspartic-UL-C^{14}	D-Serine-3-C^{14}
histidine	(+)?	+	++
serine	++++	+	++
glycine	++++	+	-
phenylalanine	++	-	-
tyrosine	++	-	-
alanine	+	++	++
valine	+	++	+
leucine	+	++	+
glutamic acid	+	++	-
proline	-	++	-
arginine	-	?	-
aspartic acid	+	+	-
methionine	-	-	-
threonine	-	+	-
isoleucine	-	+	-
lysine	-	+	-
diaminopimelic acid	?	-	?

*Medium contains glucose, aspartic acid, pantoyl lactone and D-serine.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

TABLE XV

LABELING PATTERN OF GLUCOSE, ASPARTIC ACID, AND D-SERINE AFTER GROWTH IN THE PRESENCE OF AMMONIUM CHLORIDE

		Labeling Compound	
Amino acid**	Glucose-UL-C^{14}	Aspartic-UL-C^{14}	D-serine-3-C^{14}
histidine	+++	+	++
serine	++	++	++
glycine	++	++	-
phenylalanine	++++	-	-
tyrosine	++++	-	-
alanine	++	++	++
valine	++	++	-
leucine	++	++	-
glutamic acid	+	++++	-
proline	+	++++	-
arginine	+	++++	-
aspartic acid	-	++++	-
methionine	-	++++	-
threonine	-	++++	-
isoleucine	-	++++	-
lysine	+	++++	-
diaminopimelic acid	-?	+	?

*Medium contains glucose, aspartic acid, ammonium chloride, and D-serine.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

metabolism (Table XII), ammonium chloride would not be expected to have an effect on glucose utilization. Therefore, the net effect of growth in the presence of ammonium chloride is to allow what appears to be normal utilization of aspartic acid. It should be remembered that the most obvious effect of D-serine on these cells is to inhibit utilization of aspartic acid (Table XII).

Reversal by Glutamic Acid

Grula and Grula (unpublished data) have observed that added glutamic acid will reverse the effects of D-serine on growth and division in a medium containing aspartic acid as the sole source of carbon, nitrogen, and energy. Before effects on carbon utilization by glutamic acid could be studied, it was necessary to establish carbon flow patterns for a glutamic-aspartic acid medium. Experiments were carried out as previously described; i.e., cells were grown in the desired medium for approximately fourteen hours after which either radioactive aspartate or glutamate (both uniformly labeled) were added and incubation continued for an additional 1.5 hours. Normal labeling patterns are given in Table XVI. These data were very striking and revealed that glutamic acid carbon flows very poorly into both the citric acid cycle and the Embden-Meyerhof pathway. They further revealed that glutamate carbon is used almost exclusively for synthesis of glutamic acid and its two family members (proline and arginine), thus greatly diluting flow of aspartic acid carbon into these compounds.

When the experiment was duplicated employing D-serine in the different media, the patterns shown in Table XVII were obtained. It is clearly evident that D-serine does not alter utilization of carbon from

TABLE XVI

LABELING PATTERNS OF ASPARTIC ACID AND GLUTAMIC ACID IN THE ASPARTIC ACID MEDIUM*

Amino acid**	Medium and Labeling Compounds		
	Aspartic-UL-C^{14} only	Cold glutamic acid*** + aspartic-UL*C^{14}	Cold aspartic acid + glutamic-UL-C^{14}
histidine	++++	++++	+
serine	++++	++++	+
glycine	++++	++++	+
phenylalanine	++++	++++	+
tyrosine	++++	++++	+
alanine	++++	++++	+
valine	++++	++++	+
leucine	++++	++++	+
glutamic acid	++++	+	++++
proline	++++	+	++++
arginine	++++	+	++++
aspartic acid	++++	++++	+
methionine	++++	++++	+
threonine	++++	++++	+
isoleucine	++++	++++	+
lysine	++++	++++	+
diaminopimelic acid	++++	++++	+

*Cells were grown in the aspartic acid medium with glutamic acid added.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

***0.0288 M added to medium.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

TABLE XVII

EFFECT OF D-SERINE ON THE METABOLISM OF ASPARTIC ACID AND GLUTAMIC ACID IN *ERWINIA* SP.*

Amino Acid**	Aspartic-UL-C^{14}	Aspartic-UL-C^{14} + D-serine and glutamic acid	Aspartic acid + D-serine + glutamic-UL-C^{14}
histidine	++	++++	++
serine	-	++++	+
glycine	-	++++	+
phenylalanine	++	++++	+
tyrosine	++	++++	+
alanine	++	++++	+
valine	++	++++	+
leucine	++	++++	+
glutamic acid	++	+	++++
proline	++	+	++++
arginine	++	+	++++
aspartic acid	+	++++	+
methionine	+	++++	+
threonine	+	++++	+
isoleucine	+	++++	+
lysine	+	++++	+
diaminopimelic acid	+	++++	+

*Cells were grown in the aspartic acid medium with D-serine and glutamic acid added.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

- no label
+ just detectable
++ light label
+++ moderate label
++++ heavy label

glutamic acid in contrast to its effects on aspartic acid carbon. Further, it is obvious that glutamic acid allows what appears to be normal utilization of aspartic acid. Data given in Table XVIII reveal that glutamic acid does not qualitatively alter utilization of D-serine; however, data shown in Table XIX supplement data given in Tables XVII and XVIII revealing profound quantitative effects by glutamic acid on aspartic acid and D-serine utilization by cells of _Erwinia_ sp.

Since glutamic acid was aiding utilization of aspartic acid while inhibiting utilization of D-serine, it appeared that glutamic acid or a compound formed from it was necessary for aspartic acid utilization. The efficient utilization of aspartic acid carbon, in turn, could greatly dilute flow and, thus, utilization of carbon from D-serine. It was also possible that glutamic acid was exerting its effects by an action involving entry of aspartic acid and D-serine into the cells (increasing aspartic acid and decreasing D-serine entry).

Isotope Uptake Experiments

Since it was possible that glutamic acid was reversing inhibition of growth and division by the action on cell permeases, the effect of glutamic acid and several other reversers of cell division inhibition on the uptake of aspartic acid and D-serine was studied. It was first necessary to determine if D-serine inhibited the uptake of aspartic acid into growing cells. Data given in Figure 14 show that D-serine is blocking entry of aspartic acid into the cell. Therefore, the first effect of D-serine on metabolism is the inhibition of uptake of aspartic acid.

Since glutamic acid appeared to be inhibiting, the uptake of

TABLE XVIII

LABELING PATTERNS OF D-SERINE-3-C^{14} IN THE ASPARTIC ACID MEDIUM WITH AND WITHOUT GLUTAMIC PRESENT FOR REVERSAL*

Amino acid**	Aspartic acid + D-serine-3-C^{14}	Aspartic acid + D-serine-3-C^{14} + glutamic acid
histidine	+	+
serine	++	+
glycine	+	-
phenylalanine	-	-
tyrosine	-	-
alanine	++	+
valine	-	-
leucine	-	-
glutamic acid	-	-
proline	-	-
arginine	-	-
aspartic acid	-	-
methionine	-	-
threonine	-	-
isoleucine	-	-
lysine	-	-
diaminopimelic acid	-	-

*Cells were grown in the aspartic acid medium with D-serine with and without glutamic acid.

**Tryptophan and cysteine were not detected since hydrolysis using these conditions destroys the amino acids.

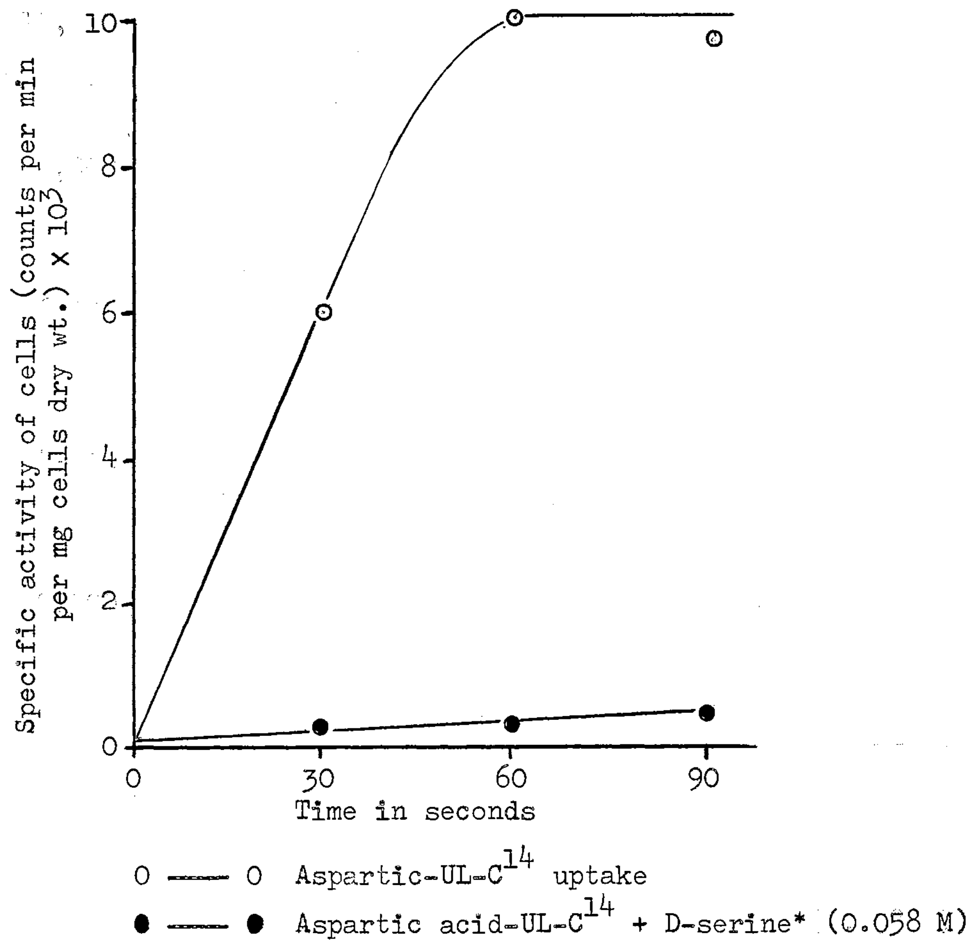
- \- no label
- \+ just detectable
- ++ light label
- +++ moderate label
- ++++ heavy label

TABLE XIX

SPECIFIC ACTIVITIES OF CELLS WITH GLUTAMIC ACID REVERSAL OF D-SERINE INHIBITION OF GROWTH AND DIVISION

Medium	Specific activity of cells*
aspartic-UL-C^{14} + D-serine	3935
aspartic acid + D-serine-3-C^{14}	3218
aspartic-UL-C^{14} + D-serine + glutamic acid	5835
aspartic acid + D-serine-3-C^{14} + glutamic acid	1091

*Counts per min per mg cells dry wt.



*Cells were grown in the aspartic acid medium for fourteen hours, harvested, resuspended in fresh aspartic acid medium and cold D-serine was added one minute before labeled aspartic acid was added.

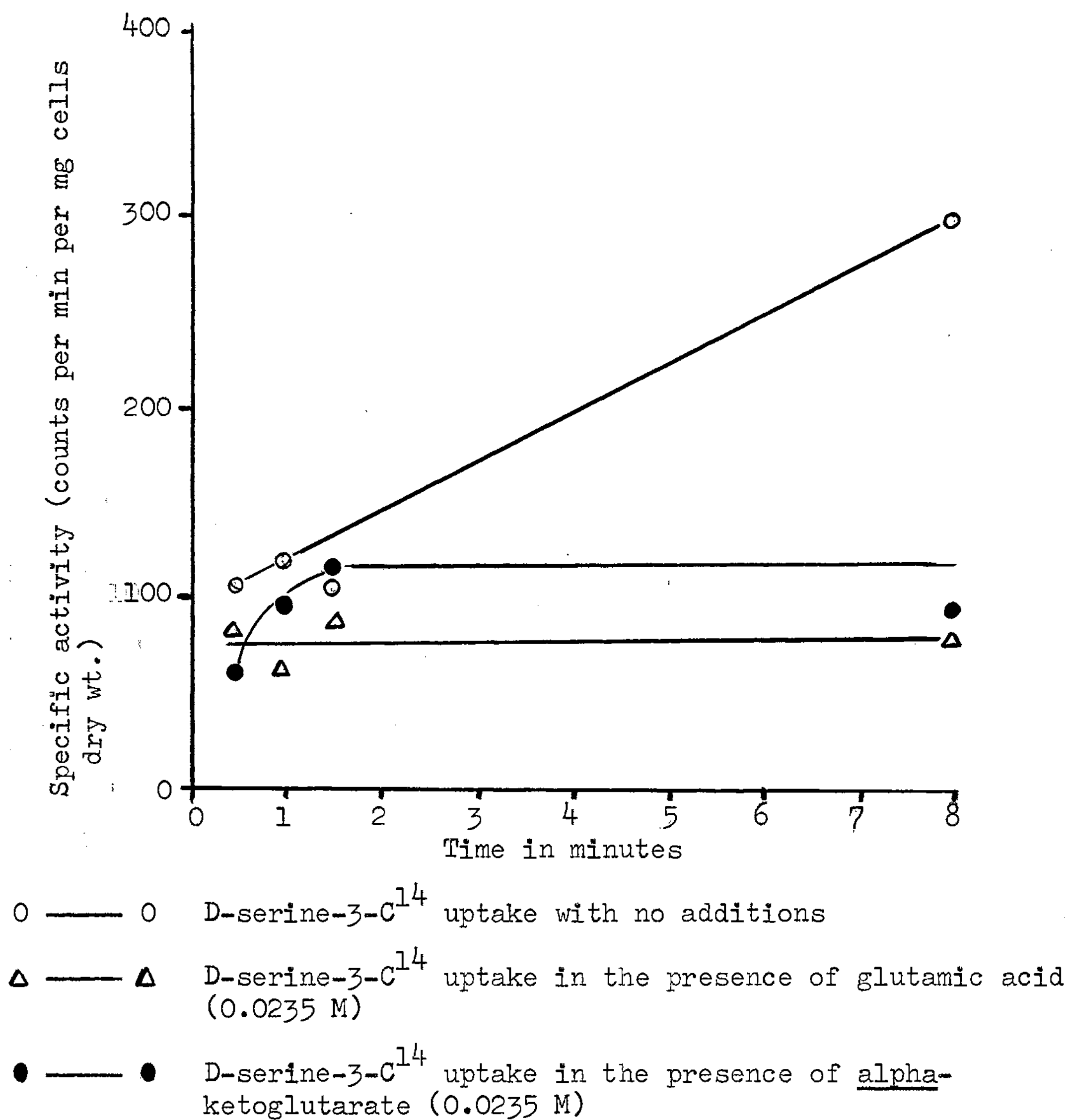
Figure 14. Uptake of Aspartic-UL-C^{14} in the Presence and Absence of D-serine*

D-serine, the effect of glutamic acid and a related compound, *alpha*-ketoglutarate, were tested for ability to inhibit the uptake of D-serine (Figure 15). Both glutamic acid and *alpha*-ketoglutarate inhibit uptake of D-serine.

Various reversers of the D-serine inhibition of growth and division were then tested in the system to determine whether the inhibition of aspartic acid uptake was reversed in their presence (Figure 16). The only compound which stimulated the uptake of aspartic acid was glutamic acid. Spermine, spermidine, and pantoyl lactone, which allow division to occur do not stimulate the uptake of aspartic acid by the cell. Grula and Grula (1964) suggested that pantoyl lactone allowed division to occur by somehow protecting the membrane from secondary damage. These data support the hypothesis that pantoyl lactone, as well as spermine and spermidine, protect the membrane rather than relieving some block in carbon utilization within the cell.

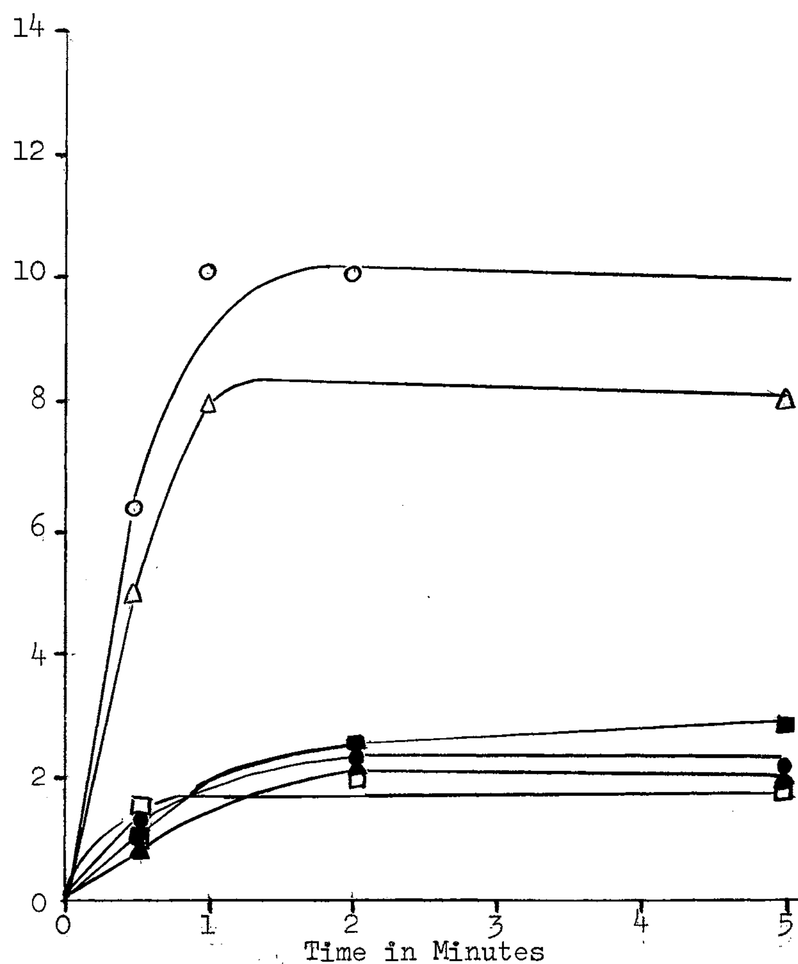
Accumulation of Keto-Acids in the Medium in the Presence and Absence of D-serine

Grula and Grula (1962, pp. 989-997) reported that D-serine caused the accumulation of pyruvic acid when cells are grown in the presence of D-serine. They attributed this accumulation to the inhibition in synthesis of pantothenic acid and coenzyme-A by D-serine. It had never been determined which carbon source (glucose or aspartic acid) was contributing most of the carbon for synthesis of the accumulated pyruvic acid. Since it is known that both compounds contribute carbon to pyruvic acid, and since it has been postulated that D-serine greatly interferes with utilization of aspartic acid, tests were conducted to



*Cells were grown for 14 hours in the aspartic acid medium, harvested, and resuspended in fresh aspartic acid medium and allowed to equilibrate for two hours. The glutamic acid and alpha-ketoglutarate were added five minutes prior to the addition of the hot D-serine.

Figure 15. Uptake of D-serine-3-C^{14} in the Presence and Absence of Glutamic Acid and Alpha-Ketoglutarate in an Aspartic Acid Medium*



O —— O Aspartic-UL-C^{14} acid uptake

● —— ● Aspartic-UL-C^{14} acid + D-serine

△ —— △ Aspartic-UL-C^{14} acid + D-serine + glutamic acid

▲ —— ▲ Aspartic-UL-C^{14} acid + D-serine + pantoyl lactone

□ —— □ Aspartic-UL-C^{14} acid + D-serine + spermine (.006 M)

■ —— ■ Aspartic-UL-C^{14} acid + D-serine + spermidine (.012 M)

*Cells were grown in the aspartic acid medium, harvested, and resuspended in fresh aspartic acid medium. D-serine (0.058 M) was added with the other reversers of cell division and allowed to equilibrate one hour before labeled aspartic acid was added.

Figure 16. Reversal of D-serine Inhibition of Aspartic Acid Uptake by Various Reversing Agents*

determine: (a) the relative amounts of carbon contributed by glucose and aspartic acid in the basic medium; (b) the effect of D-serine on this ratio; and (c) the effect of pantoyl lactone on any change produced by D-serine. These tests were to be done by studying differential labeling of pyruvate hydrazones obtained under the various conditions. The specific activity of these pyruvate hydrazones are presented in Table XX. Although D-serine increased the amount of material reacting with the reagent from both glucose and aspartic acid, the metabolism of aspartic acid was altered to a much greater extent. The addition of pantoyl lactone and ammonium chloride to the medium reduces the amount of reactive compound formed from both aspartic acid and glucose. These data constitute further evidence that D-serine is interfering with utilization of aspartic acid.

TABLE XX

SPECIFIC ACTIVITIES OF PYRUVATE HYDRAZONES FORMED IN THE ASPARTIC ACID-GLUCOSE MEDIUM

Additions	Specific activity of pyruvate hydrazone*	
	Glucose-UL-C^{14}	Aspartic-UL-C^{14}
None	240	383
D-serine	1,840	23,050
D-serine and pantoyl lactone	1,720	5,525
D-serine and NH_4Cl	860	1,700

*Counts per min per mg cells dry wt.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Data presented in this thesis relate to two problem areas involving the species of *Erwinia*: (1) Inability of the organism to grow in a GA medium and (2) inhibition of growth and cell division by D-serine and reversal of this inhibition by specific agents.

The key reaction that is difficult for *Erwinia* sp. to perform is synthesis of pyruvic acid from glucose. Lack of this metabolic reaction results in inability to synthesize sufficient pantothenic acid (and coenzyme-A) to prime the citric acid cycle with needed acetylated coenzyme-A. Although the cycle can be primed with acetylated coenzyme-A molecules by addition to the medium of pyruvate, pantothenate, pantoyl lactone, or pantoate, the growth that results is still unsatisfactory in terms of lengthy lag time and insufficient total yield. Further examination of the problem revealed that carbon dioxide fixation via PEP carboxylase is probably nonexistent and even when pyruvic acid is present for fixation of carbon dioxide, rate of fixation is not conducive to rapid or extensive growth. The writer's nutritional and chemical experiments constitute proof that carbon dioxide is not fixed to PEP and is poorly fixed to pyruvic acid. Experiments utilizing $C^{14}O_2$ are more direct proof for our conclusions.

Utilizing information gained from these experiments, vexing questions relative to growth, lack of growth, and slow or diminutive growth

can be satisfactorily answered.

Additional data reported in this thesis shed more light on inhibition of the cell division process by D-serine in this organism. A key reaction that has been focused involves the extremely poor entry of aspartic acid into the cell in the presence of D-serine. The inhibition of entry caused by D-serine definitely aids in explaining inhibition of growth by this compound. However, the discovery of this phenomenon makes it difficult in the absence of direct enzymatic proof to ascertain if endogenous utilization of aspartic acid or one of its metabolic intermediates is also inhibited by D-serine. Therefore, whether the key to lack of cell division activity is intimately associated with endogenous utilization of aspartic acid remains a somewhat shady problem area.

In experiments utilizing glutamic acid and ammonium chloride for reversal of cell division inhibition, it was observed that these compounds permit normal utilization of aspartic acid at least with regard to synthesis of amino acids. Although stimulation of endogenous utilization of aspartic acid by these compounds was not studied, it was conclusively demonstrated that addition of glutamic acid to the growth medium resulted in near normal entry of aspartic acid in the presence of D-serine. Unfortunately, glutamic acid also inhibits entry of D-serine; therefore, it must be further postulated that endogenous levels of D-serine are reduced by glutamic acid and might, therefore, be too low to exert any "normal" inhibition of utilization of aspartic acid. For these reasons, a strong defensive position relative to possible lack of inhibition in utilization of aspartic acid by D-serine under these conditions cannot be maintained. A strong position can be taken only to inhibition of entry of aspartic acid by D-serine.

Several agents (spermine, spermidine, and pantoyl lactone) that allow cell division in the presence of D-serine do not overcome inhibition of entry of aspartic acid by D-serine. Division activity that occurs in the presence of these compounds must be stimulated in a manner different from that which occurs in the presence of glutamic acid since growth also remains largely inhibited. Lack of growth would be expected if a major carbon source could not enter the cell. If normal entry of aspartic acid and subsequent utilization is the major prerequisite for cell division, then compounds that reverse division inhibition caused by D-serine should always allow near normal entry of aspartic acid; otherwise, the endogenous levels of aspartic acid will remain low as in a division inhibited system. Under such circumstances, the cells should not divide.

At this writing, the data can be correlated in the following way. Inhibition in entry of aspartic acid by D-serine causes starvation for all compounds arising from carbon intermediates in the citric acid cycle since glucose carbon is poorly utilized either via pyruvate and its subsequent oxidative-decarboxylation to acetyl coenzyme-A or via fixation of carbon dioxide to PEP or pyruvate. The net result is certainly a lack of potential energy yielding reactions, but also, two major amino acids (diaminopimelic and glutamic acid) for fabrication of wall mucopeptide will be in short supply.

The paucity of these amino acids will result in partial inhibition of mucopeptide formation which, in turn, could result in secondary membrane damage unless the membrane is physically protected. A malfunctional cell membrane will lead to lack of invagination activity by this structure, a necessary prerequisite for initiation of the cell division

process (Grula, Smith, and Grula, 1965).

Although more data are offered in this thesis concerning effects of D-serine, the theory that evolves still supports a hypothesis already presented; i.e., D-serine inhibits cell division by causing inhibition in synthesis of the cell wall mucopeptide which, in turn, results in secondary physical damage to the cell membrane (Grula and Grula, 1964).

A SELECTED BIBLIOGRAPHY

Adler, H. I., and A. A. Hardigree. 1964. Analysis of a gene controlling cell division and sensitivity to radiation in Escherichia coli. J. Bacteriol. 87:720-726.

Adler, H. I., and A. A. Hardigree. 1965. Growth and division of filamentous forms of Escherichia coli. J. Bacteriol. 90:223-226.

Adler, H. I., and A. A. Hardigree. 1965. Post-irradiation, growth, division, and recovery in bacteria. Radiation Res. 25:92-102.

Bassett, E. W., and W. J. Harper. 1958. Isolation and identification of acidic and neutral carbonyl compounds in different varieties of cheese. J. Dairy Sci. 41:1206-1217.

Coleman, G. S. 1959. The effect of DL-glutamic acid on the growth of Rhodospirillum rubrum. Biochim. Biophys. Acta. 31:55-65.

Durham, N. N., and R. Milligan. 1961. Reversal of the D-serine inhibition of growth and division in a Flavobacterium. Biochem. Biophys. Res. Commun. 5:144-147.

Durham, N. N., and R. Milligan. 1962. A mechanism of growth inhibition by D-serine in a Flavobacterium. Biochem. Biophys. Res. Commun. 7:342-345.

Eisenstadt, J. M., L. Grossman, and H. P. Klein. 1959. Inhibition of protein synthesis by D-aspartate and a possible site of its action. Biochim. Biophys. Acta. 36:292-294.

Fling, M., and S. W. Fox. 1945. Antipodal specificity in the inhibition of growth of Lactobacillus arabionosus by amino acids. J. Biol. Chem. 160:329-336.

Fox, S. W., M. Fling, and G. N. Bollenback. 1944. Inhibition of bacterial growth by D-leucine. J. Biol. Chem. 155:465-468.

Giri, K. V., A. N. Radhakrishnan, and C. S. Vaidyanathan. 1952. A simple paper chromatographic method for the study of transamination reactions. Nature. 170:1025-1026.

Grula, E. A. 1960. Cell division in a species of Erwinia. I. Initial observations relating to nutritional dependency. J. Bacteriol. 80:369-374.

Grula, E. A. 1960. Cell division in a species of Erwinia. II. Inhibition of division by D-amino acids. J. Bacteriol. 80:375-385.

Grula, E. A., and M. M. Grula. 1962. Cell division in a species of Erwinia. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin, and ultraviolet light. J. Bacteriol. 83:981-988.

Grula, E. A., and M. M. Grula. 1963. Inhibition in synthesis of beta-alanine by D-serine. Biochim. Biophys. Acta. 74:776-778.

Grula, E. A., and M. M. Grula. 1964. Cell division in a species of Erwinia. VII. Amino sugar content of dividing and nondividing cells. Biochem. Biophys. Res. Commun. 17:341-346.

Grula, M. M., and E. A. Grula. 1962. Cell division in a species of Erwinia. IV. Metabolic blocks in pantothenate biosynthesis and their relationship to inhibition of cell division. J. Bacteriol. 83:989-997.

Grula, E. A., G. L. Smith, and M. M. Grula. 1965. Cell division in a species of Erwinia. VIII. Amino acid composition of the mucopeptide in dividing and nondividing cells. Can. J. of Microbiol. 11:605-610.

Heathcote, J. G., and K. Jones. 1965. The rapid resolution of complex mixtures of the naturally occurring amino acids by two-dimensional thin-layer chromatography on cellulose powder. Biochem. J. 97:15.

Ichihara, A., and D. M. Greenberg. 1957. Further studies on the pathway of serine formation from carbohydrate. J. Biol. Chem. 224: 331-340.

Kempner, E. S., and D. B. Cowie. 1960. Metabolic pools and the utilization of amino acid analogs for protein synthesis. Biochim. Biophys. Acta. 42:401-408.

Kobayashi, Y., M. Fling, and S. W. Fox. 1948. Antipodal specificity in the inhibition of growth of Escherichia coli by amino acids. J. Biol. Chem. 174:391-398.

Kornberg, H. L. 1966. The role and control of the glyoxylate cycle in Escherichia coli. Biochem. J. 99:1-11.

Kornberg, H. L., and R. A. Cooper. 1965. Net formation of phosphoenolpyruvate from pyruvate by Escherichia coli. Biochim. Biophys. Acta. 104:618-620.

Kun, E., and M. G. Hernandez. 1956. The oxidation of tartaric acid by an enzyme system of mitochondria. J. Biol. Chem. 218:201-211.

Lark, C., and K. G. Lark. 1959. The effects of D-amino acids on Alcaligenes fecalis. Can. J. Microbiol. 5:369-379.

Lark, K. G. 1959. Isotopic competition between D- and L- methionine in Alcaligenes fecalis. Can. J. Microbiol. 5:381-394.

Lark, C., D. Bradley, and K. G. Lark. 1963. Further studies on the incorporation of D-methionine into the bacterial cell wall. Its incorporation into the R-layer and the structural consequences. Biochim. Biophys. Acta. 78:278-288.

Lark, C., and K. G. Lark. 1961. Studies on the mechanism by which D-amino acids block cell wall synthesis. Biochim. Biophys. Acta. 49:308-322.

Maas, W. K., and B. D. Davis. 1950. Pantothenate studies. I. Interference by D-serine and L-aspartic acid with pantothenate synthesis in Escherichia coli. J. Bacteriol. 60:733-745.

Majerus, P. W., A. W. Alberts, and P. R. Vagelos. 1965. Acyl carrier protein IV. The identification of 4-phosphopantetheine as the prosthetic group of the acyl carrier protein. Proc. Nat. Acad. Sci. 53:410-417.

Murachi, T., and M. Tashiro. 1958. The inhibition of D-amino acid oxidase by D-lysine. Biochim. Biophys. Acta. 29:645-646.

Neuhaus, F. C. 1962. The enzymatic synthesis of D-alanyl-D-alanine. I. Purification and properties of D-alanyl-D-alanine synthetase. J. Biol. Chem. 237:778-786.

O'Leary, W. M. 1962. The fatty acids of bacteria. Bacteriol. Rev. 26:421-447.

Redfield, R. R. 1953. Two-dimensional paper chromatographic systems with high resolving power for amino acids. Biochim. Biophys. Acta. 10:344-345.

Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. N. Britten. 1963. Studies of biosynthesis in Escherichia coli. Carnegie Institute of Washington Publication 607. Washington, D. C.

Sallach, H. J. 1956. Formation of serine from hydroxypyruvate and L-alanine. J. Biol. Chem. 223:1101-1108.

Strassman, M., J. B. Shatton, and S. Weinhouse. 1960. Conversion of alpha-acetolactic acid to the valine precursor, alpha-beta-dihydroxyisovaleric acid. J. Biol. Chem. 235:700-705.

Tuttle, A. L., and H. Gest. 1960. Induction of morphological aberrations in Rhodospirillum rubrum by D-amino acids. J. Bacteriol. 79:213-216.

White, A., P. Handler, and E. L. Smith. 1964. Principles of Biochemistry. The Blakiston division of the McGraw-Hill Book Co., New York, N. Y.

Whitney, J. G., and E. A. Grula. 1964. Incorporation of D-serine into the cell wall mucopeptide of Micrococcus lysodeikticus. Biochem. Biophys. Res. Commun. 14:375-381.

Yaw, K. E., and J. C. Kakavas. 1952. Studies on the effects of D-amino acids on Brucella abortus. J. Bacteriol. 63:263-268.

VITA

Charles Frank Parham

Candidate for the Degree of

Master of Science

Thesis: THE NUTRITION AND PHYSIOLOGY OF ERWINIA SPECIES

Major Field: Microbiology

Biographical:

Personal Data: Born at Alexandria, Louisiana, November 17, 1942, son of Shelby and Frances Parham; married to Beverly Keck on August 15, 1964.

Education: Graduated from Enid High School, Enid, Oklahoma, in 1960. Received the Bachelor of Science degree with a major in Microbioloby from Oklahoma State University, Stillwater, Oklahoma, May, 1964. Completed requirements for the Master of Science degree in October, 1966.

Experience: Graduate Research Assistant, and Graduate Teaching Assistant, Department of Microbiology, Oklahoma State University, 1964 to 1966.