GENETIC STUDIES OF ALLANTOIN METABOLISM IN *PSEUDOMONAS AERUGINOSA*

By

BEVERLY BOND BRUCE

Bachelor of Science

Louisiana State University

Baton Rouge, Louisiana

1950

Submitted to the faculty of the Graduate School of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 1965

Thesis
1965
B886g
cop. 2

OKLAHOMA
STATE UNIVERSITY
LIBRARY

[illegible] 28 1965

GENETIC STUDIES OF ALLANTOIN METABOLISM

IN *PSEUDOMONAS AERUGINOSA*

Thesis Approved:

Elizabeth T. Gaudy

Thesis Advisor

[signature]

[signature]

[signature]

Dean of the Graduate School

581314

ACKNOWLEDGMENTS

Thanks is extended to Dr. Elizabeth Gaudy for her inspiration, assistance and guidance in directing this study.

The author is indebted to Dr. Glen Bulmer for the procedure for isolation of amino acid mutants, and the F116 phage, which he obtained from Dr. B. W. Holloway, and for the Pseudomonas aeruginosa cultures obtained from the Oklahoma State Public Health Laboratory.

Graditude is extended to my husband and daughters for their patience and encouragement.

TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. MATERIALS AND METHODS. 7

A. Strains of Bacteria. 7

B. Cultivation of Bacteria. 7

C. Treatment with Mutagens. 8

1. Ultraviolet light. 8
2. Ethylmethane sulfonate 8

D. Isolation of mutants 9

E. Tests for carbon source utilization. 9

F. Phage strains. 10

G. Phage titration. 10

H. Phage plate stocks 11

I. Phage isolation. 11

J. Transduction 11

III. EXPERIMENTAL RESULTS 13

A. Mutant Isolation and Selection 13

1. Ethylmethane sulfonate treatment 13
2. Penicillin treatment 13
3. Ultraviolet light as a mutagen 19

B. Mutant Characterization. 24

1. Metabolic pool removal 24
2. Advantage for the wild type. 24
3. Carbon-source utilization. 27

C. Transduction Studies 34

1. Phage plate stock titers 34
2. Quantitative Transduction. 34

Chapter Page

3. Tube method 38
4. Plate spot transduction 40

IV. DISCUSSION. 45

V. SUMMARY 50

A SELECTED BIBLIOGRAPHY 53

LIST OF TABLES

Table Page

I. Growth on Vogel and Bonner minimal salts containing citrate 14

II. Maximum non-lethal concentration of EMS for Pseudomonas aeruginosa. 15

III. Comparison of mutant and prototroph survival in penicillin . 18

IV. Mutation to streptomycin resistance. 23

V. Comparison of methods for removal of the metabolic pool in mutants 25

VI. Growth of mutant and wild type in nutrient broth 26

VII. Carbon-source utilization by strains of PA-1 and mutants of PA-1 from EMS treatment. 28

VIII. Carbon-source utilization by mutants of PA-1 from UV irradiation 30

IX. Growth on glyoxylurea in liquid medium 32

X. Utilization of two-carbon compounds by mutants of group B. . 33

XI. Carbon-source utilization found after irradiation of group B-1 mutants 35

XII. PFU titers of phage F116 plate stocks. 36

XIII. Relation of phage concentration to transduction frequency. . 39

XIV. Transduction between allantoin-negative mutants induced by EMS 42

LIST OF FIGURES

Figure Page

1. Proposed pathways for allantoin degradation 4

2. Survival of cells treated with injectable penicillin G (sodium) 17

3. Survival of wild type and allantoin-negative mutant in allantoin minimal medium containing 50,000 units/ml penicillin G (potassium) 20

4. Ultraviolet survival curve for Pseudomonas aeruginosa 22

5. Wild type recombinants on minimal agar. 41

6. Proposed pathway showing positions of the probable genetic blocks associated with the different groups of mutants . . 48

CHAPTER I

INTRODUCTION

Lower animals, such as the amphibia and fish are known to degrade allantoin (1). Allantoin is formed from uric acid in the catabolism of purines (2). Laskowski (3) described the conversion of allantoin to allantoic acid as catalyzed by the enzyme allantoinase and the cleavage of allantoic acid as requiring the enzyme allantoicase.

The degradation of allantoin by bacteria was first described by Barker (4) in 1943. He isolated a new species of anaerobe, *Streptococcus allantoicus* from San Francisco Bay mud by the use of an allantoin-containing enrichment medium. The fermentation products formed by this organism when grown on allantoin were studied and found to include ammonia, urea, carbon dioxide, acetic, lactic, and oxamic acids and possibly glycolic acid. Barker stated that the anaerobic pathway for allantoin fermentation must be very different from the pathway in aerobic organisms as described by Krebs and Eggleston (5). The aerobic degradation resulted in the formation of almost 2 moles of urea as compared to the anaerobic production of only 0.6 mole urea per mole of allantoin.

Young and Hawkins (6) found that only 3 of 10 common intestinal bacteria tested possessed the ability to remove allantoin from the medium. These were *Escherichia coli*, *Aerobacter aerogenes* and *Proteus vulgaris*. Assuming the pathway to be allantoin ⟶ allantoic acid ⟶ urea + glyoxylic acid ⟶ ammonia + carbon dioxide + oxalic acid, they measured the amount of ammonia present in the *E. coli* culture after all of the

allantoin had disappeared, i.e., 3 days. They found less than the expected amount of ammonia and concluded that this pointed to a more complex scheme for degradation than the then accepted pathway.

Di Carlo et al (7) studied allantoin catabolism in Saccharomyces cerevisiae Hansen, describing an effective method for the preparation of cell-free extracts containing the enzyme allantoinase. They found that decreasing quantities of biotin were necessary for the comparable growth of this organism on the intermediates allantoic acid, urea and glyoxylic acid. They presented these data as evidence that the breakdown of allantoin proceeds through these intermediates in yeast.

Domnas (8), however, studying Saccharomyces cerevisiae and Candida utilis was able to demonstrate both the enzyme allantoinase and the enzyme glyoxylurease in cell-free extracts of both organisms when they had been grown on allantoin or on urea. These yeasts were found to take up and utilize allantoin, allantoic acid and urea from the medium although there was no urease activity present. The author states that allantoin metabolism in both species proceeds through allantoic acid to urea but the urea so formed is metabolized through a system differing from urease.

Campbell (9) working with a species of Pseudomonas, isolated and identified the following intermediates in the aerobic microbial pathway: allantoic acid, glyoxylic and formic acids. He also felt that the anaerobic and aerobic degradation pathways were markedly different, although conceding that the initial hydrolytic steps were probably identical. Using both cell suspensions and cell-free extracts, he proposed the pathway to be allantoin $\longrightarrow$ allantoic acid $\longrightarrow$ glyoxylic acid + urea; glyoxylic acid $\longrightarrow$ formate + carbon dioxide; formate $\longrightarrow$ carbon dioxide and water. Thiamine pyrophosphate and Mg^{++} or Mn^{++} were required for the oxidation

of glyoxylate to formate and carbon dioxide.

More recently, Vogels (10) in his thesis on allantoin metabolism, proposed a pathway in which allantoin is hydrolyzed to allantoate, which then can go to ureidoglycine and hence to ureidoglycolate or, through the mediation of allantoicase, directly to ureidoglycolate. Ureidoglycolate is then cleaved to give glyoxylic acid and urea. There are then, in this scheme, two possible routes from allantoic acid. Vogels' studies were done with both aerobic and anaerobic organisms.

Valentine, Bojanowsky, Gaudy and Wolfe (11) working at the same time but independently of Vogels, stated that allantoic acid is hydrolyzed to yield urea and glyoxylurea. Glyoxylurea is the name proposed by these workers for the compound called ureidoglycolate by Vogels. In this pathway, then, there is but one intermediate between allantoic acid and glyoxylate, i.e., glyoxylurea.

The proposed pathways may be summarized in a composite scheme as shown in Figure 1.

In view of the lack of specific assays for the intermediates of the pathway, it was felt that the grouping of allantoin negative mutants by means of transduction tests might be a useful tool for resolving some of the questions concerning the pathway.

Transduction, first described by Zinder and Lederberg (12) occurs when a phage particle carries a portion of the chromosome of the bacterial cell on which it was propagated to the cell which it subsequently infects (13). This fragment then synapses with the homologous region of the chromosome of the recipient bacterium, resulting, after cross-over, in a recombination of genetic markers of the region involved. It is evident, then, that transduction can be detected only if the donor and the recipient cells differ in genetic constitution. Therefore, transduction

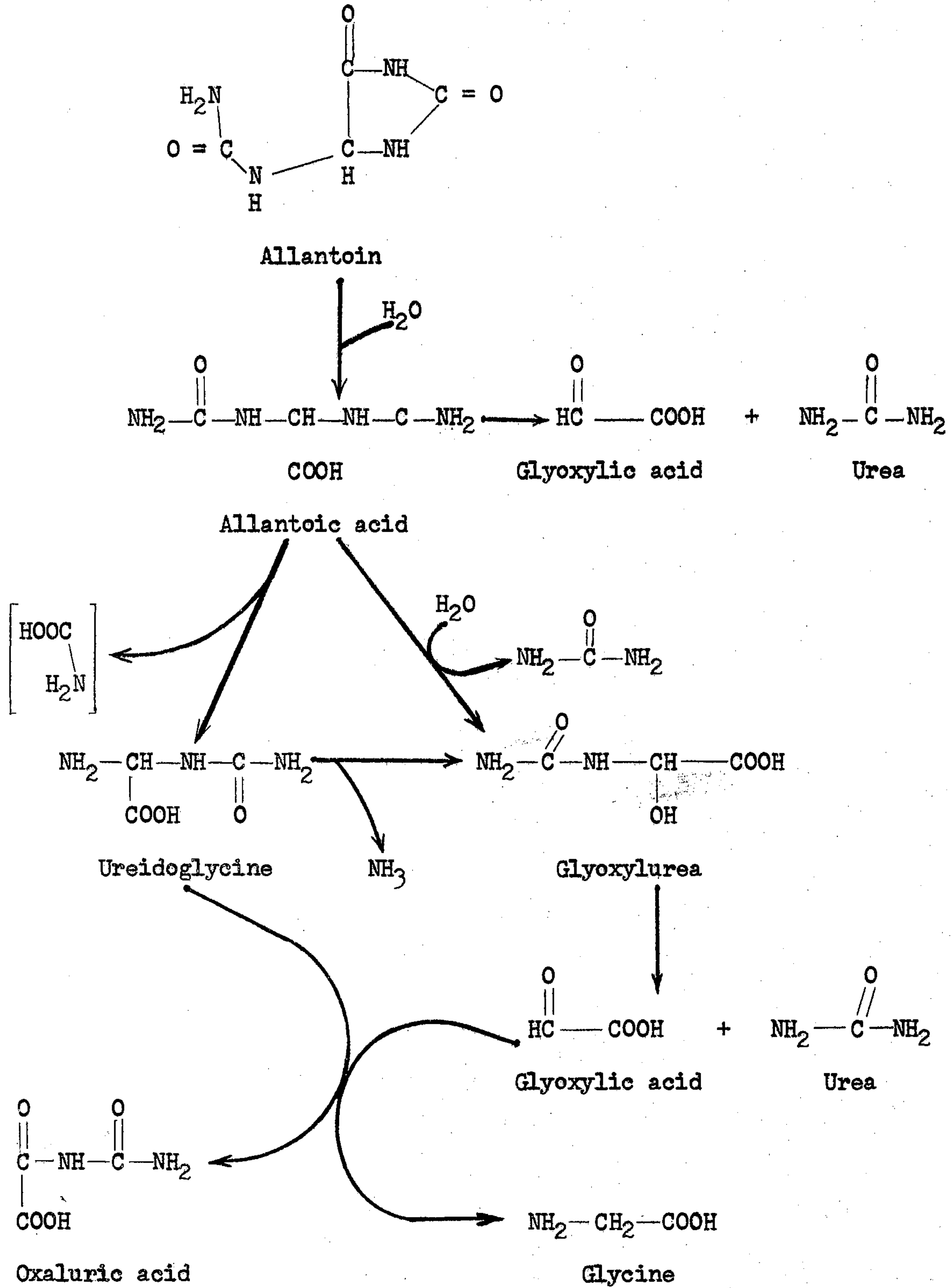

Figure 1

Proposed Pathways for Allantoin Degradation

experiments may be used to determine whether or not two phenotypically similar mutants are genetically identical (14).

The phenomenon of generalized or unrestricted transduction (15) has been employed extensively in work with metabolic pathways. Demerec and Hartman (16) working with tryptophan mutants of Salmonella typhimurium were able to demonstrate by means of transduction tests that four loci controlling tryptophan synthesis were linked and arranged on the chromosome in the same sequence as the reactions they controlled in the synthesis of tryptophan. The same situation was found to hold true in the histidine loci of Salmonella typhimurium by Hartman (17), again using transduction tests.

Yura (18), studying purine-requiring mutants, stated that in all cases where biochemical and genetic studies were made to determine the genetic block present in a mutant, good correspondence was obtained between a group based on transduction tests and a single reaction step in the pathway.

The existence of a generalized transducing system in Pseudomonas aeruginosa was first reported by Loutit (19). He used culture filtrates of this organism and obtained genetic transfer within a single strain.

Using phage F110, Holloway and Monk (20) were able to obtain transduction between the wild type and various auxotrophic mutants of strain 1 of Pseudomonas aeruginosa. These workers also studied transduction using phages B 3 and F 116 with strains 1 and 2 of Pseudomonas aeruginosa (21).

The following work was undertaken to study the aerobic degradation of allantoin with special emphasis on the place occupied by glyoxylurea. It was felt that the best procedure would be to isolate allantoin - negative mutants of Pseudomonas aeruginosa and to separate these mutants

into groups corresponding to the steps of the catabolic pathway for allantoin. By combining the results of transduction tests with biochemical tests, it was felt that further elucidation of the degradative pathway would be obtained.

CHAPTER II

MATERIALS AND METHODS

A. Strains of Bacteria

Strain 1 of P. *aeruginosa* (designated PA-1) was the organism used throughout these studies. This strain was kindly supplied us by B. W. Holloway, University of Melbourne, Australia. Mutants were numbered in the order in which they were isolated. The following notation was used: PA-1-100, PA-1-101, PA-1-102, etc.

B. Cultivation of Bacteria

Since *Pseudomonas* produces ammonia when grown on allantoin, raising the pH sufficiently to limit growth, a highly buffered synthetic medium was developed. This was a modification of Robert's M-9 salts (22) containing (amounts per liter): $Na_2HPO_4 \cdot 7H_2O$, 8.2 gm; KH_2PO_4, 2.7 gm.; $MgSO_4 \cdot 1H_2O$, 0.4 gm; NH_4Cl, 1.0 gm.; $FeSO_4$, 0.1% solution, 0.5 ml; and 2% agar when desired. The pH was 7.0 before autoclaving. When glucose (5 gm/l) was the carbon source, it was autoclaved separately at a concentration of 10 percent (w/v). Allantoin (10 gm/l) was added to the salts in half the water volume and heated to 60°-65° C. to dissolve. Higher temperatures result in the breakdown of allantoin. This was filter-sterilized and added to the autoclaved half containing the agar just before pouring. When the salts and agar were to be autoclaved together, the minimal time and temperature were utilized to avoid discoloration and precipitation of the phosphates.

Vogel and Bonner (23) salts (V and B) were used when treating with the mutagen ethylmethane sulfonate (EMS). This formula was modified by the omission of citric acid. Since the components are to be added in order, the omission altered the solubility of the remaining compounds and a 2x strength stock solution was used.

C. Treatment with Mutagens

1. Ultraviolet light

The UV source used was a 15 watt Sylvania germicidal lamp. This was placed 40 cm. from the cell suspension. 5 ml of a washed cell suspension in saline containing approximately 10^8 cells/ml were irradiated in a petri dish. The suspension was agitated during exposure. The cell suspension was added to an equal volume of nutrient broth and incubated for 4 hours in the dark to allow for phenotypic lag (24).

2. Ethylmethane sulfonate

The procedure for EMS treatment and subsequent isolation of the mutants produced was originated by B. W. Holloway for obtaining amino acid mutants of *Pseudomonas*. The procedure was used without modification in this laboratory to obtain amino acid auxotrophs. However, considerable modification was required for use with mutants in the allantoin pathway. For treatment with EMS, 20 ml of V and B glucose minimal medium was inoculated and grown overnight with aeration. To a 5 ml portion of this was added 0.1 ml (2 drops) EMS. This was incubated 1 hour at 37° C. without shaking. (The EMS was obtained from Eastman Kodak Corp.) 0.5 ml of the treated culture was inoculated into fresh V and B glucose minimal medium. After overnight growth the treatment was repeated. 0.5 ml was

again placed in fresh glucose minimal and incubated overnight. The cells were treated once more and inoculated into fresh medium. After overnight growth, the culture was centrifuged, the cells washed twice with saline and frozen.

D. Isolation of mutants

The frozen cells from either mutagen treatment were resuspended in an equal volume of saline. 0.1 ml of this suspension was inoculated into V and B salts with 1% allantoin containing 50,000 U of penicillin/ml. (The penicillin used was obtained from Calbiochem of Los Angeles as Penicillin G, Potassium, U.S.P.) The penicillin was weighed and added directly to the medium. The culture was incubated overnight on the shaker, then centrifuged, washed once and resuspended in an equal volume of 0.85% saline. 0.1 ml of 0, 10^{-1}, 10^{-2}, and 10^{-3} dilutions in saline were spread with a glass rod on glucose minimal agar plates and incubated at 37° C. The cell suspension was kept at 4° C. until growth could be scored on the glucose plates. The dilution giving between 50 and 100 colonies per plate was noted and 50 plates of glucose minimal agar were inoculated by spreading 0.1 ml of the same dilution on the surface. Incubation was at 37° C. until the colonies were 1-2 mm in diameter. The colonies were then replica-plated by the technique of Lederberg and Lederberg (25) to allantoin and glucose minimal agar plates. Mutants were those which formed colonies on glucose but not on allantoin. These were picked individually from glucose plates and checked for growth on fresh glucose and allantoin minimal agar.

E. Tests for carbon source utilization

The modified Roberts minimal salts were used as the base for the

medium, with intermediates in the pathway acting as carbon source. The salts plus 1.5% agar were poured in 10 ml portions into sterile petri plates. 0.1 ml of a saline cell suspension washed from a fresh nutrient agar slant was added to the agar before pouring. Approximately 50 mg of the compound to be tested was placed on the surface. Incubation was at 37° C. for 72 hours. The following compounds were tested as carbon sources: allantoin (Nutritional Biochemicals or Sigma); allantoic acid as the potassium salt made in this laboratory by the procedure of Young and Conway (26); sodium glyoxylurea, prepared by the method described by Gaudy in 1962 (27); sodium glyoxylate (Nutritional Biochemicals and Sigma); glycine (Nutritional Biochemicals); glycolic acid (Fisher, purified); sodium acetate (Baker Chemical, "Baker Analyzed" Reagent).

F. Phage strains

The bacteriophage used for transduction was F116, a temperate phage isolated by B. W. Holloway and kindly shared with us.

G. Phage titration

The agar used for *Pseudomonas* phage (21) was composed of (amounts/liter) Difco nutrient broth, 8 gm; Difco Yeast extract, 5.0 gm; NaCl, 5.0 gm. Eleven grams of agar per liter was added to the bottom layer; plates contained 30 ml. The top layer was semi-soft with only 6.5 gm agar per liter.

The suspension to be titered was serially diluted in nutrient broth. 0.1 ml of the dilution was added to 2.5 ml of the top phage agar along with 2 drops of a heavy suspension of sensitive bacteria and one drop of 0.1 M $CaCl_2$. This was mixed and poured over the bottom layer. Incubation was for approximately 12 hours. Longer incubation results in spreading of the plaques which makes counting difficult.

H. Phage plate stocks

Plate stocks of phage grown on both mutant and prototrophic strains were prepared by a modification of the technique of Swanstrom and Adams (28) as follows: 0.1 ml of a phage suspension containing approximately 5×10^6 phage particles per ml was plated with the desired cells as described above. After incubation, 5 ml of nutrient broth was added to each plate and allowed to soak for 30 minutes. This was pipetted off and centrifuged to remove the cells. The supernatant was filtered through a Millipore filter (H.A. 0.45 µ pore size). This method yields plate stocks of F116 with a count of 10^{10}-10^{11} PFU/ml.

I. Phage isolation

A total of 22 temperate phages active on PA-1 were isolated from lysogenic Pseudomonas aeruginosa cultures in this laboratory. The isolation procedure was as follows: a culture was grown overnight in nutrient broth. This was centrifuged at 30,000 rpm for 15 minutes and cells removed from the supernatant by Millipore filtration. The filtrate was diluted 10^{-2} to 10^{-3} and plated with strain PA-1. A single plaque was picked by puncturing with a wire needle, which was then shaken in 2 ml nutrient broth. The suspension was replated and a single plaque pick made again.

J. Transduction

The transduction technique of Murphy and Rosenblum (29) for Staphylococcus aureus was used with slight modification. The plating medium was allantoin minimal agar. 0.1 ml of a saline suspension of cells from a fresh nutrient agar slant was spread on the agar. One drop of a

phage plate stock was then placed on the plate. At least four different plate stocks could be tested on each plate. Growth was scored after 72 hours at 37° C.

CHAPTER III

EXPERIMENTAL RESULTS

A. Mutant Isolation and Selection

1. Ethylmethane sulfonate treatment

It was necessary to use Vogel and Bonner minimal salts (23) when treating with ethylmethane sulfonate (EMS) because the cells did not survive the second treatment in M-9 salts. The reason for this difference is not known. To determine whether PA-1 could utilize the citric acid in the V and B salts formula, growth in unsupplemented salts was compared to growth in salts with added allantoin and glucose. Table I indicates that 67% of the growth attained with allantoin as the carbon source is possible in the unsupplemented salts. This finding prompted the omission of citric acid from the formula since the basal medium must support no growth during the penicillin step. There was no measurable growth on the salts without citrate.

A high concentration of EMS, as compared to other chemical mutagens, may be employed, producing mutation without killing (30). The highest concentration for use with PA-1 may be seen in Table II. The concentration used in all subsequent experiments was 2%.

2. Penicillin treatment

Because Pseudomonas aeruginosa is very resistant to the action of penicillin, difficulty was encountered in finding a type and a brand of

TABLE I

GROWTH ON VOGEL AND BONNER MINIMAL SALTS CONTAINING CITRATE

Carbon Source Added	Optical Density
none	0.375
1% Allantoin	0.555
0.5% Glucose	1.280

Basal medium was V and B minimal salts. Incubation was overnight on a reciprocal shaker at 37°. Optical density was read on a Coleman Jr. Spectrophotometer at 540 mμ.

TABLE II

MAXIMUM NON-LETHAL CONCENTRATION OF EMS FOR
PSEUDOMONAS AERUGINOSA

Percent EMS in 5 ml Medium	Optical Density
0.2	0.789
0.6	0.789
1.0	0.870
2.0	0.817
3.0	0
4.0	0

Cells were treated with EMS for 1 hour at 37° in glucose minimal medium. 0.5 ml of treated cells was inoculated into fresh glucose minimal. Optical density was determined after 24 hours at 37°.

penicillin which could be used in a sufficiently high concentration to be effective. Sutherland (31) reported that Pseudomonas aeruginosa was the most resistant of the 14 gram-negative bacteria he tested to all of the penicillins used. He feels that this is due both to the intrinsic insensitivity of the organism and to the production of penicillinase by these cultures.

Figure 2 is a typical curve obtained using injectable penicillin G sodium with procaine, showing surviving fractions of cells at three concentrations of penicillin. This penicillin was in the form of an insoluble suspension, however, which necessitated extensive washing with accompanying loss of mutants.

A soluble, powdered, form of penicillin G sodium (Nutritional Biochemicals) was tested but, in all concentrations tried, large clumps of cells were formed. These clumps could not be completely resuspended even after repeated washing with saline.

To determine whether penicillin action could be improved by use of a different amount of inoculum, flasks of allantoin minimal medium with penicillin were inoculated with 1 ml of a mutagen-treated suspension containing approximately 2×10^8 cells/ml and with 0.1 ml of the same suspension. The smaller inoculum gave a rate of killing proportional to the time of treatment, while the larger number of cells tended to overcome the killing effect after 10 hours of treatment.

Once allantoin-negative mutants had been isolated, it was possible to make comparison counts to determine penicillin action on prototrophic, growing cells and on mutant, non-growing cells.

Table III gives the results of a representative experiment. The data show imperfect separation of mutant from prototroph. The small proportion of cells which were mutated to loss of the allantoin pathway

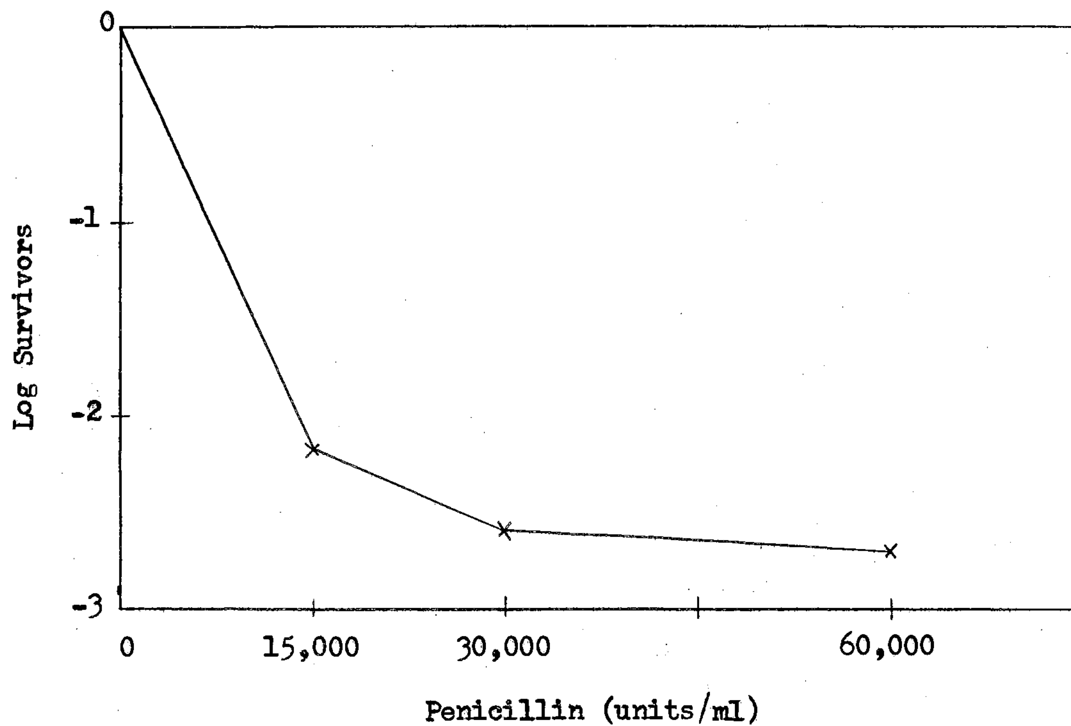

Figure 2

Survival of cells treated with injectable penicillin G (sodium). Cells were incubated in minimal medium containing 1% allantoin and indicated amounts of penicillin at 37° for 18 hours, washed free of penicillin and plated on glucose minimal agar.

TABLE III

COMPARISON OF MUTANT AND PROTOTROPH SURVIVAL IN PENICILLIN

Penicillin Units/ml	PA-1 (cells/ml) Time		PA-1-108 (cells/ml) Time	
	8 hours	22 hours	8 hours	22 hours
0	1.8×10^{8}	-	9.5×10^{6}	-
30,000	3.0×10^{5}	2.8×10^{5}	3.0×10^{6}	2.7×10^{6}
45,000	2.0×10^{5}	3.0×10^{3}	1.3×10^{6}	2.2×10^{5}

Penicillin was penicillin G sodium (Nutritional Biochemicals) in 1% allantoin minimal medium. Surviving counts were determined on glucose minimal agar plates.

made it mandatory to kill almost all of the wild type cells in order to isolate these mutants.

The action of the potassium salt of penicillin from Calbiochem approached more nearly the ideal, and, in addition, gave no difficulty with clumping of the cells. It is not felt, however, that the differences observed were due entirely to the use of the potassium salt rather than the sodium salt. The minimal salts formula contained both potassium and sodium ions in fairly high concentrations so it should not be of great significance whether the sodium or potassium salt form of the penicillin was used. It is much more likely that the more effective action was due to a combination of the difference in brands and the high concentration. Figure 3 shows survival curves for PA-1 and the allantoin-negative mutant PA-1-105.

The antibiotic vancomycin was reported by Jordon (32) to kill growing cells by blocking the synthesis of cell wall mucopeptide. This antibiotic was tested in concentrations of 167, 333 and 831 µg/ml with both PA-1 and mutant cells. Again difficulty was experienced with clumping of the cells. The approximate counts obtained in allantoin minimal medium indicated that the mutant cells (PA-1-108) were being killed at the same rate as the wild type. This mutant was checked at this time for wild type reversion as a possible explanation but was found to have no measurable reversion rate.

3. Ultraviolet light as a mutagen

It has been shown by a number of investigators (33) (34) that the effectiveness of different mutagens varies with the marker selected. It was thought that ultraviolet irradiation might prove to be a more effective mutagen than EMS for producing mutations in the allantoin

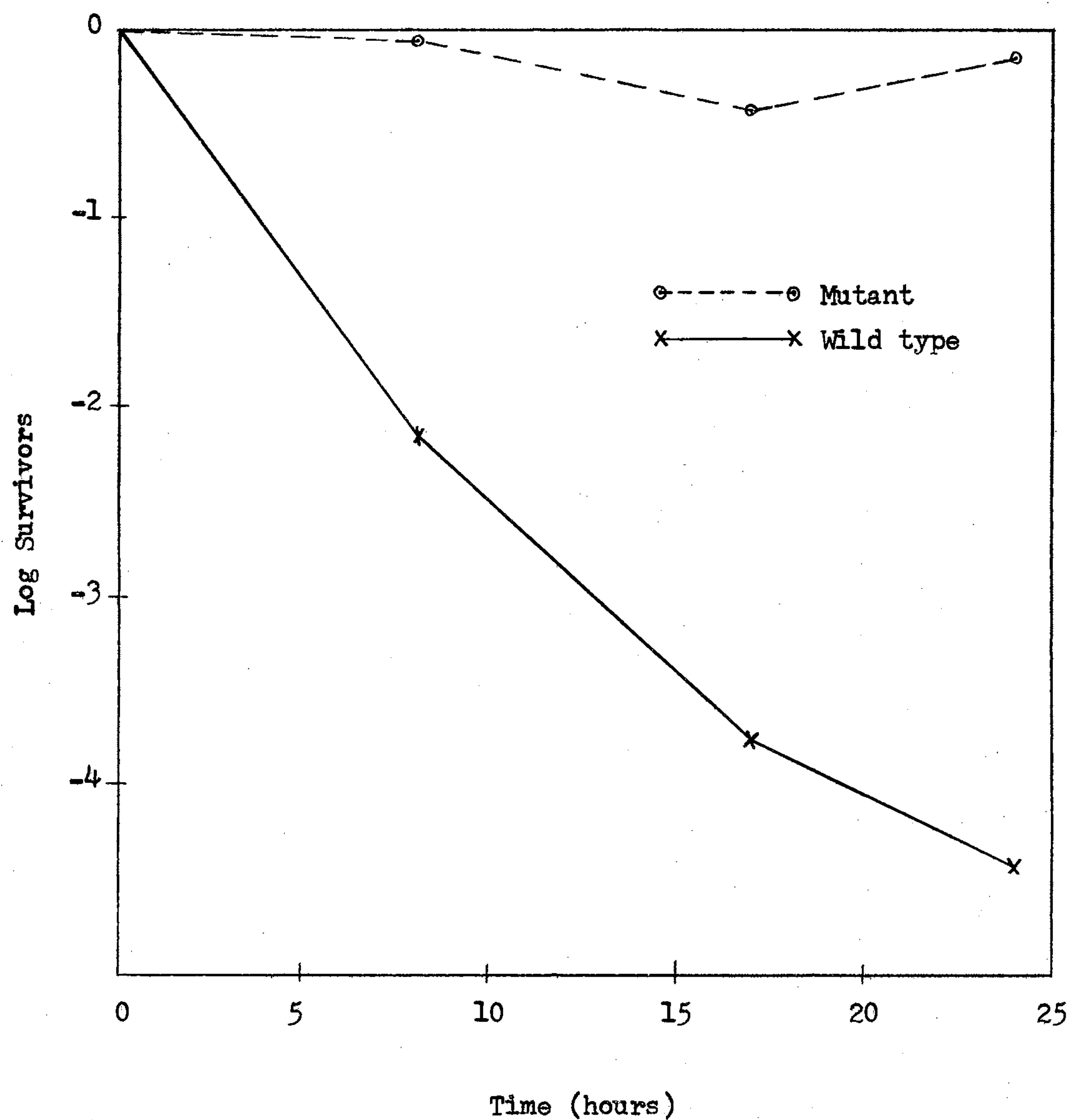

Figure 3

Survival of wild type and allantoin-negative mutant in allantoin minimal medium containing 50,000 units/ml penicillin G (potassium). Cells were incubated at 37°, washed and plated on glucose minimal agar.

pathway. Figure 4 shows the ultraviolet survival curve for PA-1 from which the optimal UV dosage was chosen.

Because streptomycin resistance is an easily selected character, it was used as a measure of the mutation of PA-1 with the selected UV dose. Since streptomycin resistance is a dominant character, it was not necessary to allow for phenotypic lag in selecting for this character. The streptomycin was incorporated into nutrient agar so that the cells could be plated directly on this after irradiation. The effectiveness of UV in increasing the mutation rate is shown in Table IV.

In contrast, when an irradiated, penicillin-treated culture of PA-1 was examined for allantoin-negative mutants by replica plating, none were found on 50 plates. It was concluded that the frequency of the desired mutation with UV was lower than that for streptomycin resistance, or that the relatively inefficient penicillin selection did not allow isolation of mutants produced at low frequency by UV. After it was found that the prototroph was impermeable to both glyoxylurea and glyoxylate, this fact appeared to offer a positive selection technique of efficiency comparable to that for streptomycin resistance. Therefore, ultraviolet irradiation was again employed in the isolation of mutants permeable to these compounds.

The normal irradiation procedure was used, allowing growth for four hours in enriched medium (nutrient broth). The cells were then centrifuged at 30,000 rpm for 15 minutes and resuspended in saline. 0.1 ml of the saline suspension was spread on the surface of 0.5% glyoxylurea agar. Colonies appeared after 48-72 hours incubation. These were all mutants and will be described in detail in a later section.

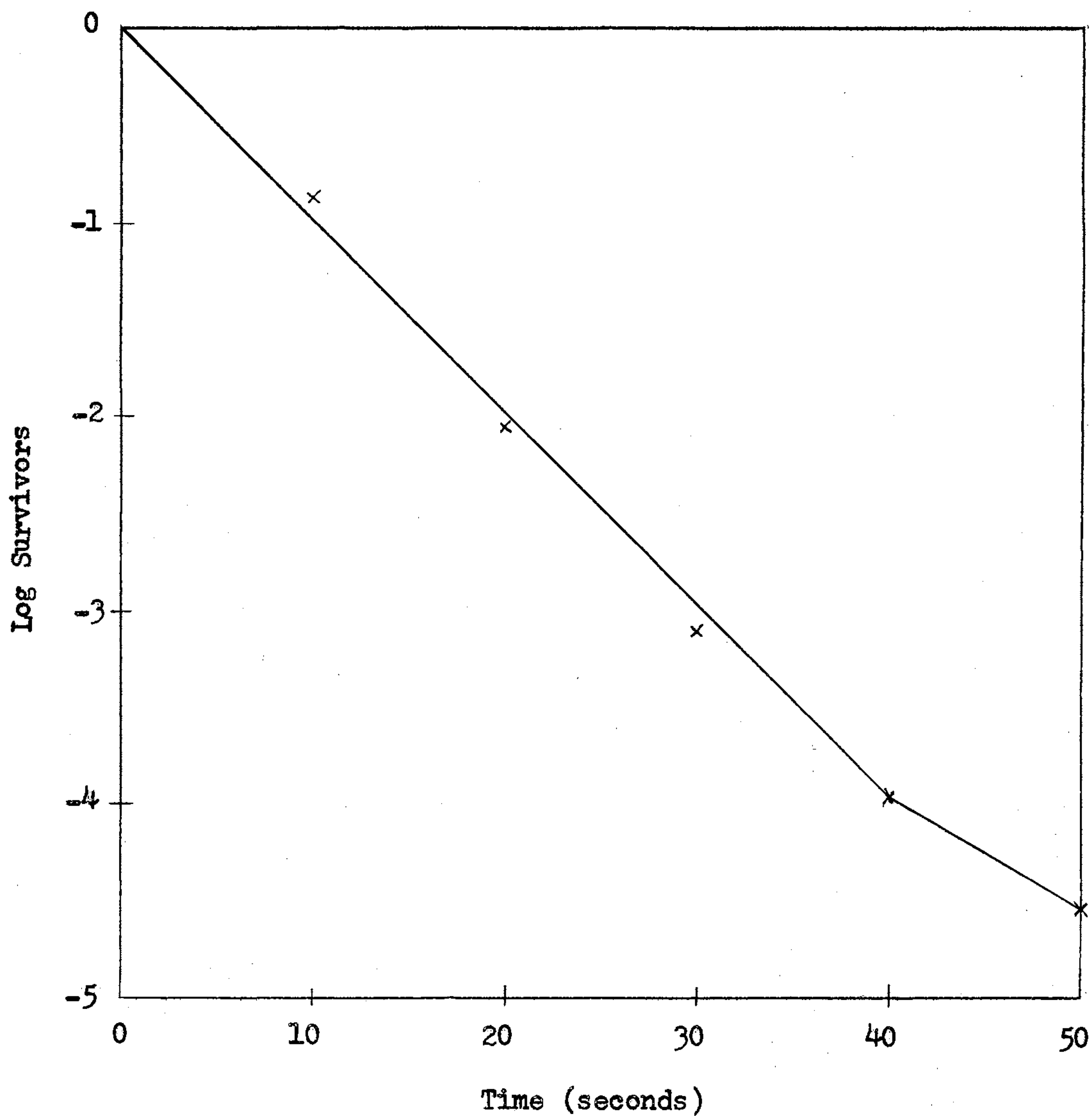

Figure 4

Ultraviolet survival curve for <u>Pseudomonas aeruginosa</u>. 5 ml cells were irradiated with UV source at 40 cm and samples removed at times indicated. Surviving cells were determined by plate counts on nutrient agar.

TABLE IV

MUTATION TO STREPTOMYCIN RESISTANCE

Streptomycin Concentration	Spontaneous Mutant Fraction	UV induced Mutant Fraction	Increase with Irradiation
50 µg/ml.	3.7×10^{-6}	5.3×10^{-3}	1.4×10^{3}
100 µg/ml.	6.5×10^{-7}	1.1×10^{-4}	1.7×10^{2}
200 µg/ml.	All cells were killed in this concentration		

Streptomycin was incorporated into 15 ml nutrient agar. Irradiation was for 20 sec at 40 cm distance. Cells were plated immediately, taking precautions to prevent photoreactivation.

B. Mutant Characterization

1. Metabolic pool removal

In order to determine the proportion of spontaneous reversions to the wild type in a mutant population, it was first necessary to find the best way to remove the metabolic pool. The first method used was to incubate the cells in a medium lacking a carbon and a nitrogen source for four hours. As this was tedious and time-consuming, the simple alternative of washing with saline was used and the results in terms of growth on allantoin minimal medium scored. At the same time, the effect of the age of the cells on the ease of pool removal was determined. Table V is a resume of the results of this experiment. In all later experiments, the cells were washed with saline to eliminate the pool and overnight cultures were used for convenience.

2. Advantage for the wild type

In an attempt to explain the findings listed in Table V for mutant PA-1-108, which prior to this time had no measurable reversion rate, nutrient broth tubes were inoculated with varying ratios of mutant and wild type revertant cells. The purpose was to determine if nutrient broth favored prototrophic growth over the mutant. It should be pointed out that these mutants were selected on the basis of loss of allantoin pathway only, so they should have no other genetic defects.

The favorable conditions for wild type growth at the expense of the mutant resulted in the loss of several mutants. To avoid this, cells were grown in glucose minimal medium whenever possible.

Since the proportion of prototrophic revertants in a given population was a variable factor, it was necessary to determine this number at

TABLE V

COMPARISON OF METHODS FOR REMOVAL OF THE METABOLIC POOL IN MUTANTS

		Growth of culture used	Count on glucose medium (cells/ml)	Count on allantoin medium (cells/ml)	Fraction growing on allantoin
PA-1-105	4 hrs. in min. salts	Overnight	1.5×10^{9}	0	0
		5-hour	3.5×10^{9}	0	0
	Saline Washing	Overnight	7.8×10^{8}	0	0
		5-hour	1.5×10^{9}	3.4×10^{3}	2.0×10^{-6}
PA-1-108	4 hrs. in min. salts	Overnight	1.4×10^{9}	$>1.0 \times 10^{5}$	$>7.0 \times 10^{-5}$
		5-hour	4.9×10^{9}	$>1.0 \times 10^{5}$	$>2.0 \times 10^{-5}$
	Saline Washing	Overnight	1.1×10^{9}	$>1.0 \times 10^{5}$	$>9.0 \times 10^{-5}$
		5-hour	1.6×10^{9}	$>1.0 \times 10^{5}$	$>6.0 \times 10^{-5}$
PA-1-162	4 hrs. in min. salts	Overnight	8.4×10^{8}	3.3×10^{4}	4.0×10^{-5}
		5-hour	5.4×10^{9}	1.4×10^{5}	2.6×10^{-5}
	Saline Washing	Overnight	9.8×10^{8}	2.3×10^{4}	2.3×10^{-5}
		5-hour	8.0×10^{9}	7.4×10^{4}	9.3×10^{-6}

Original culture was in nutrient broth. Minimal salts is modified M-9 salts with no C or N source. Cells in this medium were on shaker for 4 hours. Centrifugation for saline washing was at 30,000 rpm for 15 min. at room temperature. Saline washing was repeated twice.

TABLE VI

GROWTH OF MUTANT AND WILD TYPE IN NUTRIENT BROTH

Percent Wild Type	
Initial	Final
0	0
50	82
< 10	51
< 1	26

PA-1-140 and a back mutant 140A were grown separately in nutrient broth and inoculated in the ratios shown into fresh nutrient broth. After 8 hours 1 drop of each was placed in fresh nutrient broth and allowed to grow overnight. Counts were made on nutrient agar and allantoin minimal agar.

the time a mutant culture was to be used, e.g. when preparing phage stocks or when using cells for transduction studies.

3. Carbon-source utilization

The mutants were first separated into groups on the basis of growth on the proposed intermediates in the pathway. Ureidoglycine, the intermediate proposed by Vogels (10), is a hypothetical compound, not available for testing. Table VII shows the growth patterns of the mutants isolated from the EMS treatments. A total of 82 mutants were isolated from four different experiments.

That the data obtained with PA-1 are typical of wild-type growth for Pseudomonas aeruginosa is indicated by the correlation of the results obtained with 15 other strains of this organism. [These cultures were obtained through the courtesy of Dr. Glen Bulmer who obtained them from the Oklahoma State Public Health Laboratory].

No growth of any of the wild type strains on glyoxylurea or glyoxylate was found on carbon source plates or in liquid cultures containing 0.5% glyoxylurea or glyoxylate. This must necessarily be due to impermeability of the cells to these compounds, since it is possible to demonstrate glyoxylurease activity using extracts of PA-1 grown on allantoin. This view is further supported by the growth pattern of group A mutants. These were originally selected as allantoin-negative mutants. All of them reverted to allantoin positive but retained the ability to grow on glyoxylurea.

Group C mutants were able to utilize only glyoxylurea. This indicates a double mutation occurring in this group, i.e., the loss of the allantoicase which converts allantoate to glyoxylurea and the mutation to permeability to glyoxylurea.

TABLE VII

CARBON-SOURCE UTILIZATION BY STRAINS OF PA-1 AND MUTANTS OF PA-1 FROM EMS TREATMENT

Group*	Allantoin	Allantoic Acid (Potassium Salt)	Glyoxylurea (Sodium Salt)	Glyoxylic Acid (Sodium Salt)
Wild type	+	+	-	-
A	+	+	+	-
B	-	-	-	-
C	-	-	+	-
D	-	+	-	-

*Strains in groups:

Wild type: PA-1 and 15 other strains of Pseudomonas aeruginosa
A: PA-1-119, 138, 144, 149, 150, 152, 153, 157, 170, 174, 175, 180.
B: PA-1-105, 116, 117, 121, 122, 123, 126, 133, 136, 143, 154, 155, 158, 160, 161, 163, 172, 179, 182, 151.
C: PA-1-120, 125, 127, 130, 131, 134, 135, 137, 140, 141, 142, 146, 156, 159, 162, 165, 166, 178.
D: PA-1-111, 144, 145, 148, 164.

1.5% agar in minimal salts was melted, cooled to 47° and mixed with 0.1 ml of a saline suspension of cells from a fresh slant. This was poured and when hard a few mg. of compound were placed on the surface. Growth was scored after 48-72 hours at 37°.

Group D would seem to have a single mutation, the loss of the allantoinase, which converts allantoin to allantoate.

The action of EMS in alkylating the guanine residues of the cell DNA and splitting off these 7-alkylated residues at neutral pH (35) results in "deletion mutations". The appearance of a double mutation such as in group C is not an unexpected finding under these conditions, particularly since three successive treatments with EMS were used.

Mutants of PA-1, induced by UV irradiation and selected for growth on glyoxylurea or glyoxylate, gave the carbon source utilization pattern shown in Table VIII. A total of 42 mutants from 5 different experiments were isolated.

Brief exposure to UV will produce "point mutations" which should result in a single mutation. One can calculate the frequency of single mutations when selecting for glyoxylurea-positive strains as approximately 4×10^{-4} which is close to that for streptomycin resistance (Table IV). The frequency of double mutations can be calculated as 1.6×10^{-7} so that a double mutation occurs approximately once for every 100 single mutations. Since there are found only 40-45 colonies per isolation plate, there is a high degree of probability that the mutants isolated after UV treatment have but a single mutation.

This is compatible with the data in Table VIII if one notes that glyoxylurea is an unstable compound and will establish a non-enzymatic equilibrium according to the following reaction (27).

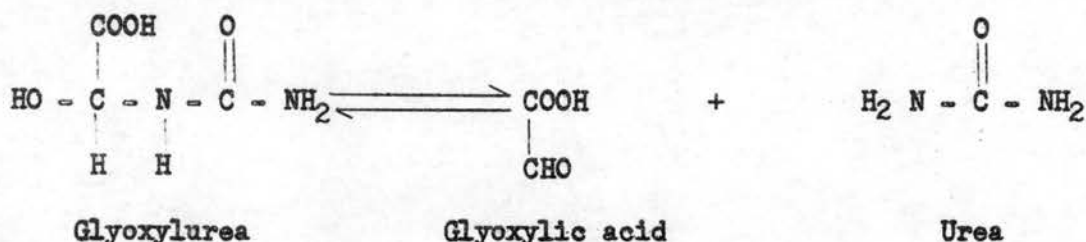

TABLE VIII

CARBON-SOURCE UTILIZATION BY MUTANTS OF PA-1 FROM UV IRRADIATION

Group	Number in Group	Allantoin	Allantoic acid (Potassium salt)	Glyoxylurea (Sodium salt)	Glyoxylic acid (Sodium salt)
1	41	+	+	+	+
2	1	+	+	+	-

The procedure was the same as that described in Table VII.

The equilibrium constant for this reaction as written is 7.6 (27). If the organism is utilizing glyoxylate, the equilibrium will be pulled to the right until almost all of the glyoxylurea is converted to glyoxylate. For this reason, it is impossible to distinguish mutants utilizing glyoxylate only from those utilizing both glyoxylurea and glyoxylate. Mutants which utilize only glyoxylurea are easily distinguished, however.

In order to obtain a better estimate of the amount of growth possible when glyoxylurea was the sole carbon source, liquid cultures were used. Table IX shows the optical density found after 48 hours at 37° in minimal salts with 0.5% glyoxylurea serving as the sole carbon source. It can be seen from these data that growth on glyoxylurea is not abundant even for mutants which are permeable. This may indicate low permeability, or growth on this carbon source may be limited for some other reason.

Since the allantoin pathway involves the utilization of a two-carbon compound for biosynthesis, it is possible that group B (Table VII) mutants could have a genetic defect which blocks utilization of a C_2 compound and hence is unrelated to allantoin degradation. To check these mutants for such a defect, three two-carbon compounds were tested as carbon sources: glycolic acid, glycine and sodium acetate. Table X shows that 11 of the mutants, those of group B-2, were indeed unable to utilize a C_2 compound and hence should no longer be considered as having mutations involving the allantoin pathway.

The wild type and seven mutants, group B-1, were able to grow readily on both glycine and sodium acetate. Glycolic acid would not support growth of any of the cultures tested and, in fact, was inhibitory. Depending upon the interconversions of 2-carbon compounds in this organism, these mutants may be blocked in the direct pathway for allantoin degradation. Since the wild type is impermeable to both glyoxylurea and glyoxylate,

TABLE IX

GROWTH ON GLYOXYLUREA IN LIQUID MEDIUM

Mutant Number	Optical Density
Wild type	0.0327
PA-1-132	0.1805
-152	0.2273
-130	0.2903
-120	0.1135
-135	0.2756
-137	0.1739

Minimal salts with 0.5% glyoxylurea, supplemented by 0.02% yeast extract were inoculated with 0.1 ml of a saline suspension of cells from a fresh slant. Optical Density was read at 540 mμ on a Coleman Jr. spectrophotometer after 48 hours on a shaker at 37°.

TABLE X

UTILIZATION OF TWO-CARBON COMPOUNDS BY MUTANTS OF GROUP B

Group*	Glycolic acid	Glycine	Sodium acetate
B-1	-	+	+
B-2	-	-	-
Wild Type	-	+	+

* Strains in groups:

B-1: PA-1-105, 116, 117, 121, 133, 179, 182.

B-2: PA-1-122, 123, 126, 136, 143, 151, 154, 155, 158, 160, 161, 163, 172.

Wild Type: PA-1

Procedure was the same as is described in Table VII.

the block may be concerned with the formation of either of these compounds.

In order to test this hypothesis, all seven mutants were subjected to UV irradiation. This should produce an additional single mutation and shed some light on the position of the original mutation or mutations. These mutants were selected by plating on 0.5% glyoxylurea agar after irradiation. Approximately twenty mutants of each strain were isolated and of these 10 to 15 from each original strain were picked for further study. With one or two exceptions, all of the mutants of a particular strain gave a consistent carbon source utilization pattern. These results are shown in Table XI. Mutant PA-1-116 gave no mutants which were glyoxylurea-positive with this selection technique.

C. Transduction Studies

1. Phage plate stock titers

Table XII lists the plaque forming titers of the plate stocks propagated on mutants. These plate stocks were used in all of the transduction tests between mutants and were prepared by the method previously described. These titers are unusually high for a temperate phage but phage infecting *Pseudomonas aeruginosa* were found to have many unusual features.

2. Quantitative Transduction

It was the original plan to do quantitative transduction studies of the mutants using F116 phage. It soon became evident, however, that a quantitative system was not feasible. The difficulty appeared to be in the absorption of the phage to the cells. Free phage counts (plated on sensitive cells) done before and after incubation with cells in the

TABLE XI

CARBON-SOURCE UTILIZATION FOUND AFTER IRRADIATION OF GROUP B-1 MUTANTS

Group*	Allantoin	Allantoic Acid (Potassium Salt)	Glyoxylurea (Sodium Salt)	Glyoxylic Acid (Sodium Salt)
I	-	-	+	+
II	-	+	+	+
III	-	trace	+	-

*Strains in groups:

I: PA-1-105

II: PA-1-117, 121, 182

III: PA-1-133, 179

Procedure was the same as that described in Table VII.

TABLE XII

PFU TITERS OF PHAGE F116 PLATE STOCKS

Mutant Number	PFU/ml x 10^{10}	Mutant Number	PFU/ml x 10^{10}
PA-1-105	3.2	-148	0.58
-111	2.4	-149	1.7
-116	7.2	-151	1.9
-117	4.4	-154	7.7
-120	6.0	-155	1.1
-121	1.7	-156	1.3
-123	3.3	-158	1.6
-125	4.9	-159	3.8
-126	2.3	-160	1.1
-127	18.0	-161	8.2
-130	14.0	-162	0.74
-131	1.3	-164	0.20
-133	6.0	-165	4.3
-134	1.5	-166	3.2
-135	4.2	-170	7.1
-137	4.4	-172	1.6
-140	1.4	-174	1.9
-142	5.5	-178	3.2
-143	0.4	-179	7.4
-145	0.29	-180	2.5
-146	0.54	-182	2.3

Plate stocks were titered for PFU on PA-1 cells by the agar layer method. Plaques were counted at 12 hours.

transduction mixture showed no measurable change. Holloway (21) stated that he found 25% adsorption with phage B 3 and 60% with phage F116 after 30 minutes adsorption time. Evidently the PA-1 phages adsorb slowly in all cases. Adams (36) states that adsorption of less than 20% in the available time cannot be measured accurately with the present phage assay methods.

It is possible that the higher adsorption rate for F116 found by Holloway was due to a required factor found as a trace impurity in Australian brands of media but not present in the products used in this study.

The basic transduction procedure was described by Holloway (21). This procedure was modified by the following alterations in an attempt to improve adsorption and transduction efficiency:

1. Ca^{++} as 0.5 M $CaCl_2$ was added to the reaction mixture.
2. Log phase cells and stationary phase cells were used.
3. Cells were kept chilled except when in the transduction mixture.
4. Yeast extract was added to the transduction mixture.
5. The multiplicity of the phage with respect to the cells was varied from 0.1 to 5.
6. Glucose was added to the cells at the time of plating.
7. A water suspension of phage was used.
8. Adsorption time was varied from 10 to 30 minutes.
9. NaCl was added to the reaction mixture.

None of the above modifications resulted in an effective quantitative transduction system.

At the same time, several of the temperate phages isolated from Pseudomonas aeruginosa cultures in the laboratory were tested in the quantitative system. None gave better results than F116.

3. Tube method

Because the adsorption time appeared to be of an extraordinary length, a method of transduction was tried in which the cells were allowed to grow up in the presence of the phage. A tube containing 5 ml of nutrient broth was inoculated with 0.5 ml of a phage plate stock containing approximately 10^{10} phage per ml and 0.5 ml of a saline suspension of cells from a fresh nutrient agar slant. This was put on the shaker for overnight growth. The cells were then centrifuged and washed with saline. 0.1 ml samples of the resuspended cells were spread on allantoin plates for numbers of wild type colonies. The suspension was then diluted and plated on glucose minimal agar for total cell count. Table XIII gives the results of an experiment to determine the optimum number of phage which could be added without significant killing effect on the cells, while, at the same time giving maximum transduction.

The transduction rate fell off rapidly as the number of phage particles decreased. The growth of the cells was not affected by the highest phage concentrations.

While this method resulted in transduction, it appeared to have several possible disadvantages. Because the cells were allowed to grow for so long, any reversions of the mutant to wild type could give falsely positive results even though the control was negative. The reversion rate of these mutants is often so variable that the validity of the results obtained with the tube method might be open to question. Another factor considered in evaluating the results obtained by this transduction method was the fact that even one wild type cell produced by a rare intraallelic recombination, which occurred early, could give a strongly positive result. Because of these considerations, it was decided to employ

TABLE XIII

RELATION OF PHAGE CONCENTRATION TO TRANSDUCTION FREQUENCY

Phage Added	Transduction Frequency
None	0
1.6×10^{10}	2.9×10^{-5}
1.6×10^{9}	1.1×10^{-5}
1.6×10^{8}	1.7×10^{-6}
1.6×10^{7}	7.9×10^{-9}

0.5 ml of phage plate stock containing the given number of PFU was added to 5 ml nutrient broth. 0.1 ml of a saline suspension from a fresh agar slant of PA-1-105 was added. The mixture was grown overnight on the shaker, washed with saline and plated on glucose and allantoin minimal agar for counts.

a method which embodied internal controls.

4. Plate spot transduction

The transduction method described by Murphy and Rosenblum (29) for *Staphylococcus aureus* was tried and adopted as a means of genetic grouping. This method had the advantage of allowing any reversions to prototrophy to be counted on the same plate as the transduced colonies. The colonies formed by transduced cells were normal, wild type growth within the circle of the phage drop as is shown in the photograph in Figure 5. Mutant PA-1-182 (group B) was the recipient. Donors 135, 137, 140, 142, and 146 are all members of group C and do recombine with the recipient.

That the colonies found were the result of transduction and not transformation was demonstrated by incubating the phage suspension prepared from PA-1 with deoxyribonuclease (10 µg/ml) for 15 minutes at 37° prior to testing (37). No decrease in the number of recombinant colonies was found in seven different transductions.

Data are reported as number of colonies, but this is not a quantitative procedure in which the numbers of colonies is considered in the grouping of mutants. Table XIV shows the groupings found when crosses in both directions were done on all of the mutants isolated after EMS treatment. Mutants 111 and 144 form one genetic group since they do not recombine with each other. This correlates with the fact that both grow on allantoic acid. The remainder of the mutants utilizing allantoate, 144, 145, and 164, recombine with the first group and with all other mutants tested but not with each other. These two groups are outlined by dashed lines in Table XIV.

The mutants listed as "Not suitable for use as a recipient" are those which show a consistently high reversion rate making the detection

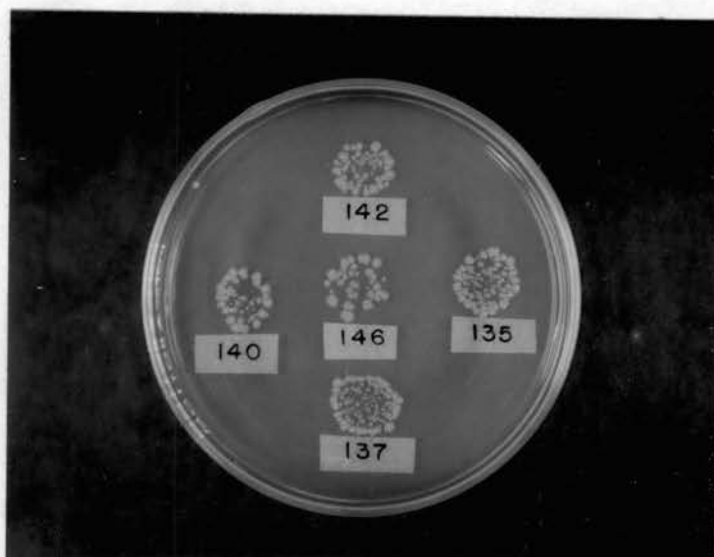

Figure 5

Wild type recombinants on allantoin minimal agar. Plates were spread with allantoin-negative mutant PA-1-182. Numbers on the plate are those of the mutant on which each phage was grown. All donors belong to group C and grow on glyoxylurea.

TABLE XIV

TRANSDUCTION BETWEEN ALLANTOIN-NEGATIVE MUTANTS INDUCED BY EMS

	RECIPIENT																											
DONOR	111	144	145	148	164	121	117	182	105	133	179	120	125	130	131	134	135	137	140	142	146	156	159	162	165	166	178	116
111	0	0	13		5	—		27	5	4	4	3	11	2	1		5	2	8	2	2	5	3	5	1	1	5	2
144	0	0	—		0	—		TMC	12	5	4	—	—	—	—		—	—	—	—	—	—	—	—	—	—	—	9
145	73	21	0		0	—		4	1	1	5	14	2	3	2		0	1	4	1	1	4	2	1	2	2	2	1
148	90	5	1		0	—		4	4	7	2	2	—	1	6		2	—	—	2	2	—	—	—	—	—	—	4
164	81	1	0		0	—		1	4	14	7	1	3	5	3		2	2	16	1	2	10	3	7	2	10	2	4
121	75	68	—	NOT SUITABLE FOR USE AS A RECIPIENT	120	3	NOT SUITABLE FOR USE AS A RECIPIENT	0	3	2	7	0	6	28	1	NOT SUITABLE FOR USE AS A RECIPIENT	2	2	—	—	14	3	1	2	2	1	—	3
117	10	6	—		23	1		1	17	14	5	2	2	11	4		2	2	—	—	17	4	1	4	9	5	—	4
182	TMC	TMC	—		150	2		0	8	5	5	6	25	25	5		2	4	—	—	30	4	13	5	1	10	—	6
105	17	26	18		34	4		24	0	0	0	0	2	0	0		0	0	—	—	2	0	0	0	0	0	—	3
133	60	90	30		150	5		30	1	0	0	1	0	1	0		3	3	—	—	0	0	0	0	0	0	—	5
179	TMC	TMC	TMC		150	15		50	0	0	0	0	3	5	0		0	0	—	—	3	0	0	0	0	0	—	9
120	—	—	15		35	—		39	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	1	0	0	2
125	50	—	30		41	—		38	0	0	25	0	0	0	0		0	0	0	0	1	0	0	0	0	0	0	4
130	88	—	16		3	—		TMC	0	0	0	0	0	0	0		0	0	0	0	0	0	1	0	4	0	0	1
131	112	—	12		1	—		11	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	2
134	99	—	TMC		TMC	—		21	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	1	1	2	4
135	221	—	18		4	—		42	0	0	0	0	1	0	0		0	0	1	0	0	0	0	0	0	0	0	3
137	49	—	48		TMC	—		23	0	0	1	0	0	0	0		0	0	0	0	0	0	0	0	0	0	0	2
140	76	—	TMC		TMC	—		26	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	1	5
142	53	—	9		1	—		18	0	0	0	0	0	0	1		1	0	0	0	0	0	0	0	0	0	0	1
146	92	—	11		2	—		TMC	0	9	15	0	0	0	0		0	0	0	0	0	0	0	0	0	0	1	3
156	32	—	36		33	—		31	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	1	0	1	3
159	106	—	100		TMC	—		39	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	1	1
162	166	—	TMC		TMC	—		43	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	3	3
165	78	—	40		10	—		18	0	1	0	0	0	0	0		0	0	1	0	0	0	0	0	0	0	1	5
166	79	—	166		50	—		36	0	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	0	1	2
178	36	—	12		27	—		TMC	1	0	0	0	3	2	4		4	3	2	14	4	4	3	8	5	2	3	11
116	40	TMC	50		130	42		TMC	11	11	TMC	9	15	10	4		14	4	—	26	6	9	3	13	9	14	TMC	0
PA-1	49	40	50		50	30		45	6	5	10	4	18	3	2		8	12	17	8	12	5	5	4	16	10	20	1

TMC = Too many colonies to count.

of transduced colonies impossible.

Mutants 120, 125, 130, 131, 134, 135, 137, 140, 142, 146, 156, 159, 162, 165, 166, and 178 are all found in group C (Table VII) and are all able to utilize glyoxylurea. These mutants are, in addition, permeable to glyoxylurea. Numbers 105, 133, and 179 are members of group B-1 (Table X). After irradiation these mutants were found to be permeable to glyoxylurea. The assumption can therefore be made that the original genetic block, induced by EMS treatment, was at the conversion of allantoic acid to glyoxylurea (allantoicase). These did not gain the permeability to glyoxylurea originally that group C mutants did. On the basis of transduction, these mutants can all be placed in a single group, validating the conclusion as to original blocks which was based on carbon source utilization. This group is indicated by a dashed line in Table XIV. The presence of only one genetic group in all of the mutants which are able to grow on glyoxylurea, is evidence for the direct conversion of allantoic acid to glyoxylurea as proposed by Gaudy (27). If one or more intermediates were involved, as in the pathway proposed by Vogels (10), there should be at least two genetically different groups of mutants utilizing glyoxylurea.

Mutant 178, a member of group C on the basis of carbon source utilization, did not recombine with other members of this group when used as a recipient. However, when 178 was the donor, transduced colonies were found with almost all of the other mutants, regardless of the carbon source utilization pattern. The reason for this behavior is not known. PA-1-116 was found to recombine with all other mutants both as a donor and as a recipient, and therefore appears to have a genetic defect at a site different from any other mutant isolated.

Noting that the wild type was impermeable to glyoxylurea while the

mutants of group C and those isolated after irradiation of PA-1 were readily permeable to this compound, attempts were made to transduce the character of permeability to glyoxylurea into the wild type. In addition to the 16 members of group C, 2 of the mutants of PA-1 isolated after UV irradiation were used as donors. The plate spot transduction technique was used with selection on 0.5% glyoxylurea agar. No transduced colonies were found in any of the crosses. It would appear from this that the character of permeability is not transducible.

CHAPTER IV

DISCUSSION

There appears to be very little doubt that the compound glyoxylurea is present in the aerobic microbial degradation pathway for allantoin. The prototroph, PA-1, while not permeable to glyoxylurea, did possess a very active glyoxylurease when grown on allantoin, as shown in cell-free extracts. The finding of the 18 mutants (group C, Table VII) which were able to utilize glyoxylurea on the carbon source plates, while not utilizing glyoxylate, is evidence that the compound is sufficiently stable to be tested in this manner. The utilization of glyoxylurea in the absence of glyoxylate utilization was found again upon irradiation of mutants PA-1-105, 133, and 179. The utilization of glyoxylurea but not glyoxylate was confirmed by growth in liquid media. Since the reverse situation, the utilization of glyoxylate but not glyoxylurea, was not found, one must accept the fact that the non-enzymatic breakdown of glyoxylurea proceeds at a rate rapid enough to allow the organism to grow on the glyoxylate formed. There was not a noticeable delay in the growth on glyoxylurea with those utilizing both, so the possibility of growth on both compounds cannot be ruled out. It must be stated again that assay for the enzyme glyoxylurease is the only reliable basis for the identification of a mutant growing only on glyoxylate. The technique of inducing this enzyme in allantoin-negative mutants will require further work.

The genetic data obtained in the transduction tests give evidence for the presence of one and only one intermediate between allantoic acid and glyoxylic acid and urea. Those mutants which grew only on glyoxylurea

fell into one genetic group. Mutants which acquired permeability to glyoxylurea, e.g. those found after UV irradiation of PA-1, while remaining allantoin-positive, represent only an alteration in permeability, not a defect in enzyme. Those which show growth on both glyoxylurea and glyoxylate should be further characterized by enzyme assay in order to check groupings based on transduction tests; for allantoin-positive mutants transduction cannot be used.

The altered permeability to the intermediates in the pathway found after mutagen treatment can best be explained on the basis of a genetically controlled enzyme, mediating transport into the cell. Since UV irradiation of the prototroph will result in mutants able to grow on glyoxylurea, the permeability to this compound must be genetically controlled. There is much evidence that there may be a multifunctional permease for the intermediates of the allantoin pathway in Pseudomonas aeruginosa. The irradiation of the mutants originally isolated after EMS treatment, gave some very interesting results in terms of carbon source utilization. PA-1-105 had a genetic block at the enzyme allantoicase from the original EMS treatments. The UV irradiation altered the permeability of this mutant so that growth was found on glyoxylurea and glyoxylate. The enzyme block remained, however. This is confirmed by the transduction tests, which place PA-1-105 in the same group as the mutants of group C (Table VII), those with the defect of the allantoicase enzyme. Mutants PA-1-133, and 179 are also placed in this group by transduction tests and by the carbon source utilization pattern after irradiation. The trace of growth on allantoate (Table XI) with these two mutants may be due to the fact that these two mutants are "leaky".

The second group of mutants, PA-1-117, 121, 182 can possibly be grouped with the mutants of group D (Table VII) based on growth on

allantoic acid after irradiation. This is the first evidence of alteration in permeability to allantoate. The mutants listed in group D were originally classified together on the basis of carbon source utilization. However, as shown in Table XIV, transduction tests indicated that there were two sub-groups within group D. Since all grew well on allantoate, the most probable explanation for the finding of two genetically distinct groups is that one group lacks the permease for allantoin while the other lacks the enzyme for converting allantoin to allantoic acid. If this assumption is correct, then the finding of positive transduction indicates that it is possible to transfer the ability to form at least one permease by transduction.

The mutants which were unable to utilize a two-carbon compound (Table X) are considered to have a genetic defect not directly involved in allantoin metabolism and have not been studied further.

The data discussed above may be summarized as shown in Figure 6. Group D is shown as having two blocks, one applicable to each sub-group. Numbers for mutants whose positions have not been definitely confirmed are enclosed in parentheses.

The position of mutant PA-1-116 was assigned by elimination and is quite tentative since neither transduction tests, nor carbon source utilization, nor UV irradiation have placed it definitely. The positions shown for the other groups and single mutants have been confirmed by transduction tests and by carbon source utilization.

Because there is a problem of adsorption with F116 phage and *Pseudomonas aeruginosa*, the transduction procedure used was ideal in that it allowed a long contact between phage and cells but did not present the problem of falsely positive tests that was present in the tube method. Another advantage of the use of this technique is the fact

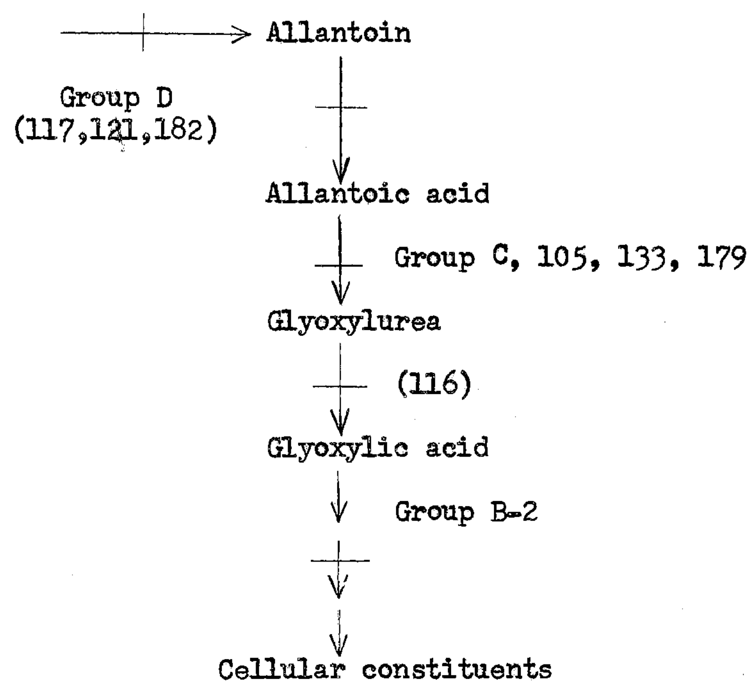

Figure 6

Proposed pathway showing positions of the probable genetic blocks associated with the different groups of mutants.

that the number of revertants, a variable factor, could be read directly on the transduction plate. In practice, it was found that a plate with even a moderate number of revertants was not accurate as a transduction test.

The technique of first grouping the mutants by carbon-source utilization patterns before transduction was attempted was very helpful. In general, results obtained by the two methods correlated quite well, indicating that the pathway as proposed is probably essentially correct. Another technique which was very effective was the UV irradiation of multisite mutants formed by EMS treatment. The two techniques combined with transduction studies should be very effective in resolving all of the remaining questions concerning the pathway for allantoin catabolism in bacteria.

CHAPTER V

SUMMARY

The aerobic degradation of allantoin was studied in *Pseudomonas aeruginosa* with special emphasis placed on the glyoxylurea step in the pathway proposed by Valentine, et al (11). The parent organism, strain 1 of *Pseudomonas aeruginosa*, was treated with the mutagen ethylmethane sulfonate or ultraviolet light. A procedure for the isolation of allantoin-negative mutants, after mutagen treatment, was designed using penicillin screening and the Lederberg replica plating technique (25). Since PA-1 is relatively insensitive to penicillin action, a comparison of brands and survival rates is included. An ultraviolet survival curve for this organism was also done.

The allantoin-negative mutants were first characterized by growth patterns using the available intermediates of the pathway as carbon sources. The intermediates tested were: allantoin, allantoic acid, glyoxylurea and glyoxylic acid. The wild type was found to be impermeable to glyoxylurea and glyoxylate. Mutants were isolated which were able to grow on these compounds. Use was made of this difference in permeability in designing an isolation procedure to select mutants able to grow on glyoxylurea and/or glyoxylate.

The grouping obtained by carbon source utilization was as follows:

1. Group D - those able to utilize allantoic acid. These mutants could be blocked at the allantoinase-catalyzed conversion of allantoin to allantoic acid or in allantoin uptake.

2. Group C - those able to utilize glyoxylurea. These must be blocked at the allantoicase-catalyzed cleavage of allantoic acid to yield glyoxylurea and urea.
3. Group B - those growing on none of the intermediates tested. These were further subdivided on the basis of the ability to utilize a two-carbon compound. The group found unable to utilize glycine or sodium acetate was considered to have genetic defects not concerned with allantoin degradation. The seven mutants which were able to grow on a two-carbon compound were irradiated and tested again for carbon-source utilization. Three were found to be able to grow on allantoate and three on glyoxylurea. One, PA-1-116, did not yield mutants which were glyoxylurea-positive (which was the basis of selection).

Transduction tests were used to confirm the groupings obtained by carbon source utilization. Phage F116 was the transducing phage. The "plate spot" transduction method of Murphy and Rosenblum (29) was chosen for this study.

Group D mutants were found to form two genetically distinct groups by transduction tests. It was concluded that one group probably lacks a permease and the other lacks allantoinase. The specific defect for each group has not been identified.

Group C consisted of only one genetic group, indicating that there is only one step in the formation of glyoxylurea from allantoic acid. No evidence was found for a second intermediate as proposed by Vogels (10).

Group B separated into two genetically distinct groups. The seven mutants able to grow on glycine and sodium acetate were separated into three groups. The mutants which utilized glyoxylurea after UV irradiation were grouped by transduction with group C. Those which utilized allantoate

after irradiation, probably fit into group D. PA-1-116 recombined with all of the mutants of groups B, C, and D.

The aerobic pathway for allantoin is concluded to be allantoin ⟶ allantoic acid ⟶ urea + glyoxylurea ⟶ urea + glyoxylic acid ⟶ ⟶ cellular constituents. Thus, the initial reactions of allantoin degradation are the same in the aerobe, Pseudomonas aeruginosa, and the anaerobe, Streptococcus allantoicus.

A SELECTED BIBLIOGRAPHY

1. Sumner, J.B., and G. F. Somers. 1953. Chemistry and methods of enzymes, 3rd ed., p. 148. Academic Press, Inc., New York.

2. Mahler, H.R., H. M. Baum and G. Hubscher. 1956. Enzymatic oxidation of urate. Science 124:705-708.

3. Laskowski, M. 1951. Allantoinase and allantoicase, p. 946-950. In J.B. Sumner and K. Myrback (editors), The enzymes, Vol. 1, Part 2. Academic Press, Inc., New York.

4. Barker, H.A. 1943. Streptococcus allantoicus and the fermentation of allantoin. J. Bacteriol. 46:251-259.

5. Krebs, H.A., and L. V. Eggleston. 1939. Bacterial urea formation. Enzymologia 7:310-320.

6. Young, E.G. and N.W. Hawkins. 1944. The decomposition of allantoin by intestinal bacteria. J. Bacteriol. 47:351-353.

7. Di Carlo, F.J., A.S. Schultz and A.M. Kent. 1953. The mechanism of allantoin catabolism by yeast. Arch. Biochem. Biophys. 44:468-474.

8. Domnas, A. 1962. Amide metabolism in yeasts. J. Biochem. 52:149-154.

9. Campbell, L.L. 1954. The mechanism of allantoin degradation by a Pseudomonas. J. Bacteriol. 68:598-603.

10. Vogels, G.D. 1963. On the microbial metabolism of allantoin. Ph.D. Thesis, University of Delft, Delft, Holland.

11. Valentine, R.C., R. Bojanowski, E. Gaudy and R.S. Wolfe. 1962. Mechanism of the allantoin fermentation. J. Biol. Chem. 237:2271-2277.

12. Zinder, N.D. and J. Lederberg. 1952. Genetic exchange in Salmonella. J. Bacteriol. 64:679-699.

13. Demerec, M. and Z. E. Demerec. 1956. Analysis of linkage relationships in Salmonella by transduction techniques. Brookhaven Symposia Biol. No. 8:75-84.

14. Demerec, M., I. Blomstrand and Z. E. Demerec. 1955. Evidence of complex loci in Salmonella. Proc. Nat. Acad. Sci., U.S. 41:359-364.

15. Hayes, W. 1964. The genetics of bacteria and their viruses, p. 530. John Wiley and Sons, Inc. New York.

16. Demerec, M. and Z. Hartman. 1956. Tryptophan mutants in *Salmonella typhimurium*. Genetic studies with bacteria. Carnegie Inst. Wash. Pub. No. 612:5-17.

17. Hartman, P.E. 1956. Linked loci in the control of consecutive steps in the primary pathway of histidine synthesis in *Salmonella typhimurium*. Genetic studies with bacteria. Carnegie Inst. Wash. Pub. No. 612:35-61.

18. Yura, T. 1956. Evidence of non-identical alleles in purine-requiring mutants of *Salmonella typhimurium*. Genetic studies with bacteria. Carnegie Inst. Wash. Pub. No. 612:35-61.

19. Loutit, J. S. 1958. A transduction-like process within a single strain of *Pseudomonas aeruginosa*. J. Gen. Microbiol. 18:315-319.

20. Holloway, B. W. and M. Monk. 1959. Transduction in *Pseudomonas aeruginosa*. Nature 184:1426-1427.

21. Holloway, B. W., M. Monk, L. Hodgins and B. Fargie. 1962. Effects of radiation on transduction in *Pseudomonas aeruginosa*. Virology 18:80-94.

22. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britten. 1957. Studies of biosynthesis in *Escherichia coli*, 2nd printing. Carnegie Inst. Wash. Pub. 607:5.

23. Vogel, H.J. and D. M. Bonner. 1956. Acetyl ornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. 218:97-106.

24. Davis, B. D. 1950. The isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc. Nat. Acad. Sci. U.S. 35: 1-10.

25. Lederberg, J. and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399-405.

26. Young, E.G. and C. F. Conway. 1942. On the estimation of allantoin by the Rimini-Schryver reaction. J. Biol. Chem. 142:839-853.

27. Gaudy, E. T. 1962. Glyoxylurease, a new enzyme in allantoin degradation. Ph.D. Thesis, University of Illinois, Urbana.

28. Swanstrom, M. and M. H. Adams. 1951. Agar layer method for production of high titer phage stocks. Proc. Soc. Exptl. Biol. Med. 78:372-375.

29. Murphy, W. H. and E. D. Rosenblum. 1964. Selective medium for carbohydrate-utilizing transductants of *Staphylococcus aureus*. J. Bacteriol. 87:1189-1201.

30. Loveless, A. and S. Howarth. 1959. Mutation of bacteria at high levels of survival by ethylmethane sulfonate. Nature. 184:1780-1782.

31. Sutherland, R. 1964. The nature of the insensitivity of Gram-negative bacteria towards penicillins. J. Gen. Microbiol. 34:85-98.

32. Jordan, D. C. 1961. Effect of vancomycin on the synthesis of the cell wall mucopeptide of Staphylococcus aureus. Biochem. Biophys. Res. Commun. 6:167-170.

33. Glover, S. W. 1956. A comparative study of induced reversions in Escherichia coli. Genetic studies with bacteria. Carnegie Inst. Wash. Pub. No. 612:121-136.

34. Kaplan, R. W., H. Beckmann and W. Rüger. 1963. Different 'spectra' of mutant types by extracellular treatment of phage Kappa with differing mutagens. Nature. 199:932-933.

35. Lawley, P. D. and P. Brooks. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem. J. 89:127-144.

36. Adams, M. H. 1959. Bacteriophages, p. 468. Interscience Publishers, Inc., New York.

37. Takashaki, I. 1961. Genetic transduction in Bacillus subtilis. Biochem. Biophys. Res. Commun. 5:171-175.

VITA

BEVERLY BOND BRUCE

Candidate for the Degree of

Master of Science

Thesis: GENETIC STUDIES OF ALLANTOIN METABOLISM IN *PSEUDOMONAS AERUGINOSA*

Major Field: Microbiology

Biographical:

Personal Data: Born at New Orleans, Louisiana on April 3, 1929, daughter of Howard T. and Inez A. Bond; married Rufus E. Bruce in 1951; mother of three daughters.

Education: Graduated from Behrman High School in New Orleans, Louisiana in 1946; received the Bachelor of Science degree from Louisiana State University in 1950 with a major in pre-medical; received medical technician certification from Charity Hospital of Louisiana at New Orleans in 1951; completed requirements for the Master of Science degree in January, 1965.

Professional experience: Worked as a medical technician in hospitals in: New Orleans, Louisiana; Jackson, Mississippi; Shreveport, Louisiana; Monroe, Louisiana; and Stillwater, Oklahoma.