ENZYME STUDIES ON ALLANTOIN-NEGATIVE MUTANTS OF *PSEUDOMONAS AERUGINOSA*

By

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ENZYME STUDIES ON ALLANTOIN-NEGATIVE MUTANTS OF PSEUDOMONAS AERUGINOSA

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CHAPTER I

INTRODUCTION

Three different pathways have been proposed for the degradation of allantoin in microorganisms. These pathways differ primarily in the number of reactions involved in the conversion of allantoic acid to glyoxylic acid. Figure 1 shows the portions of the three pathways which are pertinent to the present study.

H.A. Barker (1) first studied the fermentation of allantoin in 1943 with the homofermentative organism Streptococcus allantoicus which he isolated from San Francisco Bay mud using allantoin enrichment medium. He ascertained from fermentative studies the concentrations of the following products in moles per mole of allantoin degraded: ammonia, 2.26; urea, 0.62; oxamic acid, 0.45; carbon dioxide, 1.68; formate, 0.09; acetate, 0.15; glycolate, 0.14; and lactate, 0.01. Oxamic acid was the key product found. S. allantoicus forms both ammonia and urea, but does not contain urease; therefore, Barker concluded that one ureido group is decomposed by a pathway not involving urea. This was confirmed by addition of $^{15}NH_3$ to the medium and the finding that it was not incorporated into oxamic acid (2). The oxamic acid,

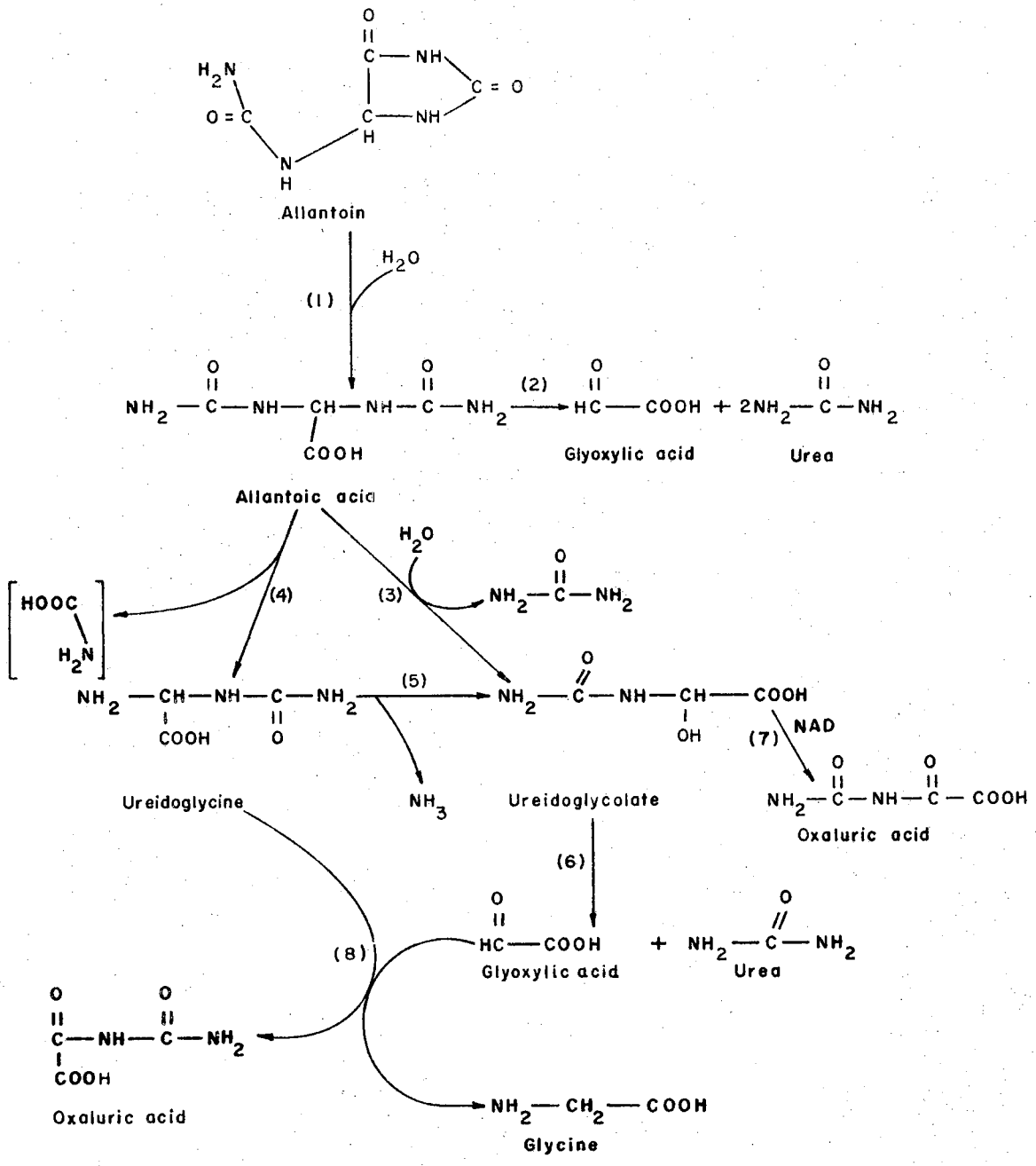


FIGURE 1

PROPOSED PATHWAYS FOR ALLANTOIN DEGRADATION

therefore, had to be formed directly from allantoic acid. Via the aerobic pathway accepted for animals and plants (14) two moles of urea are produced per mole of allantoin (reactions 1 and 2, Figure 1) rather than the 0.6 moles formed in the anaerobic pathway. Because of this difference Barker stated that the two pathways must be quite different.

Of 10 common intestinal bacteria, Young and Hawkins (26) found that only Escherichia coli, Aerobacter aerogenes, and Proteus vulgaris rapidly removed allantoin from a complex growth medium. It was also concluded from the amount of ammonia produced that the pathway was not as simple as had previously been believed.

In Saccharomyces cerevisiae Hansen, DiCarlo, et al (8) found that biotin was required for maximum growth. The requirement for biotin decreased (in order) for growth on allantoin, allantoic acid, and urea, which suggested that the conversion of allantoin into urea is through allantoic acid. The reason for the biotin requirement is not clear. These data implicate a pathway of allantoin degradation having this order of intermediates in yeast.

With a species of Pseudomonas, Campbell (5) found, in manometric studies, that a three to four hour lag period occurred before oxygen uptake or carbon dioxide production from allantoin was observed, when the organism had been grown on nutrient broth or glucose. However, if the organism was exposed to allantoin and manometric assays then conducted, no lag period was observed. He concluded that the

lag period was due to adaptive formation of enzymes in the pathway. With reference to allantoin degradation there was little difference in allantoin- and allantoic acid-grown cells, but with glyoxylic acid-grown cells a long lag period occurred before allantoin was oxidized, even though glyoxylic acid and formic acid were oxidized immediately. With formate-grown cells formic acid was degraded at once, but a period of adaptation was required for allantoin, allantoic acid, or glyoxylic acid degradation. Campbell concluded that the aerobic and anaerobic pathways were different, but that the first steps were probably identical. The reaction sequence he proposed was: allantoin ⟶ allantoic acid ⟶ glyoxylic acid + urea; glyoxylic acid ⟶ CO_2 + HCOOH (requiring thiamine pyrophosphate and magnesium or manganese ions); HCOOH ⟶ CO_2 + H_2O. These include reactions 1 and 2 in Figure 1.

In their studies on the fermentation of allantoin in S. allantoicus, Valentine and Wolfe (21) showed that oxamic acid was produced from carbamyl oxamic acid by oxamic transcarbamylase. The carbamyl phosphate also derived from this reaction is decomposed to carbon dioxide and ammonia with the formation of ATP. In this sequence the intermediates proposed were allantoin, allantoic acid, ureidoglycolate, carbamyl oxamate, and oxamate (reactions 1, 3, and 7, Figure 1). As Barker had proposed, there is a decomposition of one ureido group in the pathway before the production of the two-carbon moiety. After the allantoicase step, the

pathway becomes divergent into an energy-yielding sequence (reaction 7) and a biosynthetic pathway (beginning from reaction 6). Because of this division, either two moles of urea and one mole of glyoxylate are formed per mole of allantoic acid or one mole each of urea, oxamate, ammonia, and carbon dioxide. The products formed depend upon the route of degradation of ureidoglycolate.

Domnas (9), in his work with extracts of S. cerevisiae and Candida (Torula) utilis, found no urease, but found both allantoicase and glyoxylurease(ureidoglycolate synthetase). The amount of enzyme formed apparently was affected by the nitrogen source upon which the organism was grown. If the yeasts were grown on acetyl-urea or allantoin, the amount of glyoxylic acid formed from allantoic acid was quite significant. If the yeasts were grown on urea, no allantoicase was present. Although both species of yeasts contained ureidoglycolate synthetase, there seemed to be no pathway for degradation of urea through oxamic acid analogous to that described by Barker (2) and Valentine and Wolfe (21) in S. allantoicus.

G.D. Vogels (22) agrees with the degradation of allantoin to allantoic acid, but he proposes that from allantoate ureidoglycine is formed; this is then converted to ureidoglycolate. Alternatively, allantoicase can form ureidoglycolate directly from allantoate. He has named the enzyme, which mediates the step from allantoate to ureidoglycine, allantoate amidohydrolase. Vogels states that

solutions of this enzyme are very stable with little loss of activity after several weeks of storage at 2° C. A peculiar characteristic of this enzyme is that following neutralization to pH 8.5, which is the pH optimum, of an acidified preparation of crude cell-free extracts of S. allantoicus there is an increase in activity. Vogels reported a six-fold increase in activity after acidification below pH 4.5. In the pH range of 4.2-1.75 the amount of activation was the same. Maximum activation occurred within 30 seconds and upon longer acidification the activation diminished. The enzyme is inactivated in the pH range 5.0 to 7.5. The process of activation is proposed to be an intramolecular rearrangement rather than cleavage of a masking group. There was no speculation as to physiological function.

As a result of these studies, Vogels has proposed an alternate pathway of allantoate degradation via ureidoglycine in S. allantoicus (18,19,22,23). In determining the route of degradation a quantitative measure of ammonia and/or urea was made. In the first pathway, catalyzed by allantoate amidohydrolase and ureidoglycine aminohydrolase, ureidoglycolate is formed via ureidoglycine together with two moles of ammonia (reactions 4 and 5). In the second pathway, catalyzed by allantoicase, ureidoglycolate and one mole of urea are formed (reaction 3). Organisms reported by Vogels to utilize this pathway of allantoin degradation are Pseudomonas acidovorans, Arthrobacter allantoicus, Streptococcus allantoicus, Escherichia coli, and E. coli

var. acidilactici.

Upon incubation of a cell-free extract of Pseudomonas aeruginosa with sodium allantoate, no ammonia was formed in the absence of urease. This indicates that the second pathway is operable. Similar results were obtained using P. fluorescens, Penicillium notatum, and Pen. citreo-viride. Ureidoglycolate was always the first product of allantoate degradation in these organisms. Evidence for the presence of allantoicase was obtained by the determination of one mole of urea formed from one mole allantoate. Since no ammonia was detected, the allantoate degradation by these urease-negative organisms was not brought about by the allantoate amidohydrolase-ureidoglycine aminohydrolase reaction sequence.

All the work that has been described above was based on chemical analyses of reaction mixtures. Bruce (3) obtained proof by genetic studies of the allantoin pathway sequence as proposed by Valentine, Bojanowski, Gaudy, and Wolfe (20). Mutants of Pseudomonas aeruginosa were produced by the use of ultraviolet light and ethylmethane sulfonate with the purpose of finding mutants which were blocked at different steps in the pathway. Growth on intermediates of the pathway was the first method used to separate the mutants into groups which corresponded to the steps in the proposed degradative pathway for allantoin. Then transduction studies were completed to facilitate grouping. These data will be reviewed in some detail since the present

studies used mutants isolated by Bruce and were designed to obtain chemical data which could be correlated with the genetic data of Bruce. Only data for mutants involved in the present study will be considered. Mutant PA-1-402 was isolated and characterized more recently by R. Meganathan using N-methyl-N'-nitro-N-nitrosoguanidine and is included hereafter with the appropriate group.

The carbon source data in Table I show that the wild type strain would not grow on ureidoglycolate or glyoxylate. Since ureidoglycolate synthetase activity is found in cells grown on allantoin, the cells must hence be impermeable to these two compounds. This was further supported by EMS-treated groups of cells which, when isolated, were allantoin-negative but were able to grow on ureidoglycolate.

PA-1-105, -117, and -182 grew on none of the four carbon sources. The mutants PA-1-120 and PA-1-166 utilized only ureidoglycolate, which seemed to show the presence of a double mutation, i.e., loss of allantoicase and mutation to ureidoglycolate permeability. Those mutants with an apparent single mutation, the loss of allantoinase, include PA-1-111, -144, -145, -164, and -402.

There was a possibility that, since the allantoin pathway involves the utilization of glyoxylate, which is a two-carbon compound, the mutation in strains of the first group, which did not grow on any of the four carbon sources, occurred in another pathway. To test this hypothesis, growth on several two-carbon compounds, glycolic acid,

TABLE I

CARBON SOURCE UTILIZATION BY PA-1 AND MUTANTS OF PA-1[1]

ORGANISM	ALLANTOIN	ALLANTOIC ACID (POTASSIUM SALT)	UREIDOGLYCOLATE (SODIUM SALT)	GLYOXYLIC ACID (SODIUM SALT)
Wild Type	+	+	-	-
105 117 182	-	-	-	-
120 166	-	-	+	-
111 144 145 164 402	-	+	-	-
301	-	-	+	+
117-2 182-1	-	+	+	+

[1]Data obtained from Bruce.

glycine, and sodium acetate, was tried. All three mutants, PA-1-105, -117, and -182 showed the same growth pattern as the wild type, i.e., growth on glycine or acetate but not on glycolate.

It may be remembered that, since the wild type is impermeable to both ureidoglycolate and glyoxylate, the block may be in the formation of either of these compounds. Ultraviolet irradiation was employed to produce a single further mutation and possibly elucidate the location of the previously produced mutation. Mutants were selected for ability to grow on ureidoglycolate, i.e., specifically for a mutation affecting permeability to this compound. All mutations produced from a single parent mutant gave identical growth patterns. These are shown in Table II.

Next the genetic studies by transduction were made. Only the data for the mutants which are important in the enzyme study will be shown. From Table III the following information was gathered. Mutants PA-1-120 and -166 were placed in the same group since they can both utilize ureidoglycolate and are permeable to it, but are presumably blocked at the allantoicase step. These two mutants seem to have become permeable to ureidoglycolate. Mutant PA-1-105 is a member of the same group since after UV irradiation it was found to be permeable to ureidoglycolate and grew on this compound but not on allantoin or allantoate. It can therefore be assumed that the original mutation occurred at the conversion of allantoic acid to ureidoglycolate, i.e.,

TABLE II

CARBON SOURCE UTILIZATION FOUND AFTER IRRADIATION[1]

STRAINS	ALLANTOIN	ALLANTOIC ACID (POTASSIUM SALT)	UREIDOGLYCOLATE (SODIUM SALT)	GLYOXYLIC ACID (SODIUM SALT)
105	-	-	+	+
117, 182	-	+	+	+

[1]Data obtained from Bruce.

TABLE III

TRANSDUCTION BETWEEN ALLANTOIN-NEGATIVE MUTANTS[1]

	Recipient									
Donor	111	144	402	145	164	105	120	166	182	117
111	0	0	0	13	5	5	3	1	27	
144	0	0			0				TMC	
402			0			TMC	TMC			
145	73	21		0	0	3	14	2	0	
164	81	1	1	0	0	4	1	10	1	
105	17	26	26	18	34	0	0	0	24	
120			106	15	35	0	0	0	39	
166	79			166	50	0	0	0	36	
182	TMC	TMC		TMC	150	8	6	10	0	
117	10	6		10	23	17	2	5	1	
PA-1	49	40	22	50	50	6	4	10	45	

Not suitable for use as recipient

[1]Data obtained from Bruce.

at the allantoicase step. With the transduction tests, PA-1-105, -120, and -166 can be placed in the same group, thus validating the carbon source results. PA-1-117 and -182 were not placed with certainty by transduction.

According to carbon source data, the mutants PA-1-111, -144, -145, -164, and -402 grow only on allantoic acid. However, by transduction data this group was divided into two smaller groups, one containing PA-1-111, -144, and -402 and a second group containing PA-1-145 and -164. It was surmised that one of these groups lacked the permease for allantoin and the other group lacked allantoinase. Either loss would give identical results for carbon source utilization.

Since this group was so small we can conclude that transduction is a sufficiently sensitive system to detect differences in mutations. Therefore, Bruce concluded that there is only one enzyme between allantoic acid and ureidoglycolate in P. aeruginosa, since there were so many mutants obtained with blocks at this step, yet only one genetic group was found. Trijbels and Vogels have recently confirmed this conclusion using biochemical data (19).

PA-1-117 and -182 pose another problem since they were grouped together on the basis of the carbon source data but transduction studies did not confirm this. This is not unexpected since negative carbon source data provide little information. Neither of the two mutants grew on any of the intermediates, but further data point to a permeability

defect in these mutants, since after irradiation PA-1-117 and -182 grew on allantoic acid.

Some of the mutants resisted all efforts by the previous methods to place them in particular groups.

A large group of mutants have been isolated which have mutations at various steps in the allantoin degradative pathway and genetic studies have been completed. It was the purpose of this study to verify these genetic results by enzyme methods and thereby obtain additional proof that the pathway occurs as proposed by Valentine, et al (20), Since allantoin utilization is inducible, it was also anticipated that studies of enzyme induction in allantoin-negative mutants would provide some information relative to control mechanisms in P. aeruginosa.

To aid in later interpretation of the data obtained in this research, various proposed control mechanisms will be briefly discussed.

The operon model proposed by Jacob and Monod (13) for degradative pathways may be briefly described as follows: (1) Messenger RNA (mRNA) carries information from the structural gene where it is produced to a ribosome where a polypeptide is made from this message. (2) Synthesis of mRNA can be initiated only at particular locations on the DNA strand called the operator. A single operator may control the production of mRNA from several adjacent structural genes. This group of genes controlled by a single operator is called an operon. (3) The regulator gene can produce a

repressor which associates reversibly with a specific operator to halt all synthesis of proteins controlled by that operator. (4) These repressors are able to react with inducer molecules, thereby inactivating the repressor and allowing transcription of the operon and protein synthesis to occur.

Demerec (7) in his work with Salmonella typhimurium employed the phage as a vector to measure recombination of two genetic markers. The markers will recombine if they are located on the same transducing fragment which is estimated to be of a length one-hundredth that of a chromosome. A definition of a gene cluster is given here as a group of two or more genes controlling related functions, located in the same transducing fragment, and, as far as has been determined, including no unrelated loci within the group. Demerec found 72.4% of the gene loci arranged in clusters.

In the research carried out by Fargie and Holloway (10) P. aeruginosa was determined by transduction to contain only one clear example of linked loci. Their method consisted of comparing the frequency of transduction by phage grown on the parent with that grown on a mutant; the parent gives maximum transduction. If the two frequencies were similar, then it was assumed that the two mutations were not closely linked, but if a reduction in number of wild type colonies resulted when the phage was propagated on another mutant, cotransduction had occurred and the markers were assumed to be linked. No biochemical data relevant to control of

enzyme formation have been obtained in P. aeruginosa.

Stevenson and Mandelstam (16) have suggested in their study of mandelate degradation in P. fluorescens that the term "operon" be reserved for those genes that are known to be linked genetically; whereas, the term "regulon" refers to those genes, also regulated as a unit, but not known to be linked. Certain steps in the mandelate pathway are induced and repressed simultaneously, but no evidence is available concerning gene linkage since no system of genetic exchange has been found for this organism. There are three regulons in this pathway and the end products of each repress the formation of the enzymes of the first regulon. Because the first regulon is controlled independently by several metabolites, it is said to be "multi-sensitive."

CHAPTER II

MATERIALS AND METHODS

A. Strains of Bacteria

PA-1, which is strain 1 of Pseudomonas aeruginosa, was the wild type used in these studies; it was originally obtained from B.W. Holloway, University of Melbourne, Melbourne, Australia. The mutants used were acquired from Beverly Bruce and had been numbered in the order of isolation as PA-1-100, PA-1-101, PA-1-102, etc. Mutant PA-1-402 was isolated and characterized by transduction and carbon source utilization by R. Meganathan.

B. Cultivation of Bacteria

Since ammonia is produced when P. aeruginosa is grown on allantoin, the medium must be highly buffered. The components of this minimal salts medium are as follows (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; distilled water; and 2% agar when required. Before autoclaving the pH was 7.0. Glucose, when used, was prepared as a 10% (w/v) solution, autoclaved separately, and added aseptically to the medium to a final concentration of 5 gm/l. Allantoin was used at a concentration of 5 gm/l. It was

added to half the amount of the minimal salts solution and heated to 60° C until dissolved; a higher temperature resulted in allantoin breakdown. The allantoin solution was passed through a Millipore filter (HA, 0.45 μ pore size) and added to the autoclaved portion, which contained the agar, after this had been cooled below 60° C.

C. Chemical Analyses

1. Allantoin

To a sample containing 0.2 to 1.0 μmole of allantoin, to which had been added water to a 5.0 ml volume, 1.0 ml of 0.5 N NaOH and 2 drops of phenylhydrazine hydrochloride (100 mg/30 ml) were added. The tubes were placed in boiling water for seven minutes. After this time they were cooled in tap water to room temperature. Then 1.0 ml of 0.65 N HCl and 1.0 ml of phenylhydrazine solution were added, after which the tubes were boiled for two minutes. They were cooled to room temperature again and 1.0 ml of potassium ferricyanide (500 mg/30 ml) and 4.0 ml of 10 N HCl were added. After standing at room temperature for 5 minutes, the tubes were read at 515 mμ on a Coleman Junior Spectrophotometer.

2. Allantoate

Allantoate was determined by addition of 1.0 ml phenylhydrazine solution and 1.0 ml of 0.15 N HCl to a tube containing 0.2 to 1.0 μmole of allantoate made up to a 6.0 ml

volume with water. The tubes were placed in boiling water for two minutes and the procedure continued as outlined for allantoin.

3. Glyoxylate

For the determination of 0.2 to 1.0 µmole glyoxylate, 1.0 ml of phenylhydrazine solution was added to the sample which had been brought to a 7.0 ml volume with water. The tubes were left at room temperature for 10 minutes, after which color development with hydrochloric acid and potassium ferricyanide was continued as previously described.

4. Protein Determination

The method of Sutherland, et al (17) was used for determination of the protein content of cell extracts. To 100 ml of 4% Na_2CO_3, 1.0 ml of 2% $CuSO_4 \cdot 5H_2O$ and 1.0 ml of 4% sodium-potassium tartrate were added. This mixture was prepared just prior to use. A 1.0 ml sample of appropriately diluted extract was added to 5.0 ml reagent and allowed to stand at room temperature for 45 minutes. Phenol reagent (Fisher Scientific Company) was diluted 1:2 with water and 0.5 ml was added to each tube and mixed immediately. After 15 minutes at room temperature, the color was read at 660 mµ.

D. Determination of Allantoin Uptake

A number of methods for measuring allantoin uptake were tested. These methods and the data obtained will be

described in Chapter III. The following method was adopted for determining the ability of mutant strains to remove allantoin from solution, since it yielded the most reproducible results.

Cells were grown overnight in 100 ml of 0.5% glucose in minimal salts and harvested in an RC-2 refrigerated centrifuge. They were then resuspended in 10 ml minimal salts with glucose added to a final concentration of 0.1% and placed on a shaker at 37° C for 30 minutes. At the end of the time, 9.0 ml of the cells were mixed with 1.0 ml allantoin solution containing sufficient allantoin to yield a final concentration in the cell suspension of 1.0 µmole allantoin/ml. A tube with a 1/10 dilution of the allantoin solution in water served as a control. A sample of 1.0 ml was removed from each tube at thirty minute intervals. Samples were centrifuged in the cold and the supernatant kept in ice until termination of the experiment.

E. Preparation of Cell-Free Extracts

1. Preparation of Extracts

The cells were grown overnight in 500 ml of minimal salts medium with 0.5% glucose and 0.08% yeast extract added to the medium. The yeast extract was autoclaved separately as a 2% solution. The cells were harvested by centrifugation in an RC-2 refrigerated centrifuge, washed with 0.85% NaCl, and then frozen overnight.

The cells were thawed by resuspension in 10 ml of 0.02 M potassium phosphate buffer, pH 7.0, and were broken by sonic oscillation, using five to six 15-second bursts, cooling in ice between each treatment. A cell-free extract was then obtained by centrifugation and discarding of the whole cells and cell debris.

2. Induction of Enzymes

After harvesting by centrifugation, the cells were resuspended in 20 ml minimal salts and sterile 10% glucose solution was added to a final concentration of 0.1%. The cells were placed on a shaker at 37° C for 30 minutes. At that time 18 ml of cells were added to 2.0 ml of minimal salts containing sufficient allantoin or allantoate to provide a final concentration of 1 μmole/ml allantoin or allantoate. A tube containing 10 ml of 0.4% allantoin medium and 0.1 ml of the cell suspension was placed on the shaker to check for growth as evidence of back-mutation. Samples of 1.0 ml were taken from the concentrated cell suspension at various time intervals along with a comparable dilution of the allantoin solution which served as a control. The samples were centrifuged in the cold and the supernatant was kept in ice until the appropriate chemical analysis could be made. At the end of the induction period (usually three hours) a final sample was taken and the remainder of the cells were washed with physiological saline, frozen overnight, and used to prepare cell-free extracts as

described above.

F. Assays of Enzyme Activity

The incubation mixture for the allantoinase determination contained 0.2 ml crude extract, 0.2 ml of 0.2 M potassium phosphate buffer, pH 7.0, 0.1 ml phenylhydrazine hydrochloride (13.6 mg/ml, neutralized), 0.4 ml allantoin (5 mg/ml), and distilled water to a volume of 1.0 ml. Two control tubes were included, one containing all components except enzyme, the other containing all except substrate. Incubation time was one hour at 30° C. A sample was then removed and assayed for allantoate and/or glyoxylate.

For the allantoicase assay, the incubation mixture contained the same components except that the substrate was potassium allantoate. Glyoxylate was assayed as the end product.

The incubation mixture for ureidoglycolate synthetase activity contained the same constituents except that the substrate was ureidoglycolate. The incubation time was only 10 minutes because of the rapid nonenzymic breakdown of the substrate. Glyoxylate was again the end product which was determined.

Vogels' procedure (22) was used for activation of allantoate amidohydrolase. The reaction mixture contained 0.6 ml enzyme and 1.2 ml 0.05 M sodium citrate-HCl buffer, pH 2.0. After 20 seconds at 25° C, the reaction was stopped immediately by addition of 7.2 ml of 0.1 M barbital buffer,

pH 8.5, which contained 21 µmole/ml potassium allantoate and 11 µmoles/ml glutathione. A blank which contained all the components except enzyme was prepared and both tubes were incubated at 30° C for 20 minutes. At that time, a dilution of 1.0 ml of the incubation mixture into 9.0 ml water was made. From the dilutions, a 0.2 ml sample from the blank, 0.1 ml and 0.2 ml samples from the tube with enzyme, and 0.1, 0.2, and 0.3 ml samples of the NH_4Cl standard (1.16 µmoles/ml) were taken. To each tube were added 1.0 ml of Nessler's reagent, and distilled water to a volume of 15.0 ml. After 10 minutes at 25° C, the per cent transmittance was read at 436 mµ.

G. Growth on Uric Acid

To differentiate between an enzyme block and a lack of permeability, growth on 0.15% uric acid in minimal salts was measured. Uric acid medium was adjusted to pH 7.0-7.2 and filtered through a Millipore filter. Thiamine at a concentration of 0.001 mg/ml was added, since no growth occurred on uric acid alone. A similar requirement for thiamine for growth on adenine was reported by Magasanik (15). The reason for this requirement is not known.

H. Chemicals

Allantoin was obtained from Sigma Chemical Company. Potassium allantoate was prepared in this laboratory by a modification of the procedure of Young and Conway (25).

Sodium ureidoglycolate was prepared by the method described by Gaudy (11).

CHAPTER III

EXPERIMENTAL RESULTS

A. Allantoate Amidohydrolase

The conditions under which Vogels determined allantoate amidohydrolase in P. acidovorans (18) were duplicated using P. aeruginosa. The measurement is based on the production of ammonia from allantoate by allantoate amidohydrolase.

There was no ammonia production in the wild type PA-1 non-induced cells. Also extracts of P. aeruginosa grown on allantoin and pretreated at pH 4.0 produced no ammonia (this assay was performed by C.H. Wu).

Using the same method, but with different volumes, i.e., 0.5 ml crude extract, 0.5 ml sodium citrate-HCl buffer, and 3.0 ml barbital buffer, measurement of ammonia production was also attempted with a different extract of PA-1 cells which had been grown on a one per cent solution of allantoin in minimal salts. Again there was no ammonia production.

B. Measurement of Allantoin Uptake

1. Method of Burrous and DeMoss

A method for measuring uptake described by Burrous and DeMoss (4) was used in an attempt to measure allantoin

uptake over short periods of time. It was desirable to be able to measure short-term uptake both to obtain measurements of rates which could be equated to permease activity and to follow induction of permease in mutant cells. The method of choice for such measurements involves the use of radioactive substrate, but no labeled allantoin is available. The methods were tested first with fully induced cells.

The cells used were grown at 37° C on 0.5% allantoin in minimal salts and at the time of removal from the shaker the per cent transmittance was read at 540 mu. The number of cells used was approximately 1.6×10^{10}, which was obtained by filtration of the appropriate volume of culture through a membrane (Millipore, HA, 0.45 u pore size) resting on a sintered glass support. The cells were washed with minimal salts directly on the membrane and the salts vacuumed off. No preincubation and a ten minute preincubation with glucose were tried to determine conditions for maximum allantoin uptake. If a preincubation period was used, the cells were not washed again before addition of allantoin. Varying amounts of time were allowed to elapse before the allantoin was vacuumed off. A 10 ml wash of minimal salts was used to rinse off residual allantoin and to serve as a diluent. An analysis was then made of the solution to determine allantoin remaining.

It was found that when a sintered glass support was used more than once, allantoin adhered to the glass; therefore the funnel was used only once and after each experiment

the funnels were cleaned with chromic acid.

Best results were obtained when using a ten minute preincubation with glucose and an allantoin concentration of 10 umoles/ml. Maximum per cent allantoin removed was 45% in five minutes using cells which had been grown on allantoin. However, it was apparent from repeated measurements of filtrates and washings that an undetermined and variable portion of the allantoin removed was adsorbed by the filter rather than by the cells.

2. Uptake of Allantoin Using a Syringe

The wild type PA-1 cells were washed with minimal salts, preincubated with glucose, and again centrifuged. The cells were resuspended in the allantoin solution and pulled into a syringe with a needle which was replaced with a Swinney Adapter (Millipore Filter Corporation) containing a Millipore filter (HA, 0.45 μ pore size). Samples were taken each three minutes; changing of the filter required at least this time interval.

Using cells which had been grown on allantoin, after 26 minutes 74% of the allantoin remained in solution; whereas, only 45% remained after five minutes using the method of Burrous and DeMoss. The cell concentration which could be used with this technique was limited by the small surface area of the filter, which rapidly became clogged. Since the rate which could be measured with fully-induced cells was so low, it was decided that this method would be

inadequate for measurement of permease induction.

3. Uptake of Allantoin Using a Metal Filter Tower

A metal filter tower was purchased from Tracerlab, Inc. A membrane filter (Millipore, HA, 0.45 u pore size-25 mm diameter) was used; this rested on a stainless steel fritted disc filter. The same procedure described above was employed, but when 1.6 X 10^{10} cells were used, short term exposure to allantoin was impossible because of the length of time involved in removing the allantoin by vacuum.

A slight modification was then made to allow use of high cell concentrations with rapid filtration. The cells were washed with minimal salts and collected by centrifugation. The allantoin solution was added and, when a sample was to be taken, a 1/100 dilution was made before filtration thereby enabling rapid removal of cells from the suspension.

A test was performed to determine the amount of allantoin adhering to the stainless steel disc with washes of either water or minimal salts. It was concluded that allantoin did not adhere to the metal. Therefore, this method was used in an attempt to measure induction of permease in glucose-grown wild type cells.

Cells were grown overnight in minimal medium containing 0.5% glucose and 0.08% yeast extract, harvested by centrifugation, washed once with minimal salts, and resuspended in minimal salts. The cell suspension was divided into three portions, one of which was preincubated with 0.1%

glucose for 30 minutes. Allantoin was added to one sample at a concentration of 10 umoles/ml. Samples of 0.1 ml were removed at ten minute intervals, added to 9.9 ml physiological saline and filtered. No reduction in allantoin concentration was observed after 60 minutes with or without preincubation with glucose. An increase in inducing concentration of allantoin to 50 umoles/ml resulted in no measurable uptake during 60 minutes.

Use of allantoin-grown cells in an identical experiment resulted in removal of 52% of allantoin (initial concentration 10 umoles/ml) in 60 minutes after preincubation with glucose and 49% in 40 minutes without preincubation.

Since the method was adequate for measurement of uptake by fully-induced cells, it was concluded that if a permease is induced by allantoin, its induction is quite slow and attempts to measure induction were discontinued.

4. Standard Method of Allantoin Uptake Measurement

The standard method adopted for determination of ability to accumulate allantoin was that described in Chapter II under "preparation of cell-free extracts". This method cannot be used to measure rates accurately because of the time required for centrifugation; thirty minutes is the shortest interval of sampling possible. However, this method gave the most consistent results. Figures 2 and 3 illustrate uptake measurements for the wild type, PA-1, and for one mutant, PA-1-120, which was capable of rapid accumulation.

Figure 2. Accumulation of allantoin by wild type cells grown on allantoin.
Cells were grown overnight in allantoin medium, harvested and washed by centrifugation, preincubated with glucose, and resuspended in minimal medium containing 1.0 umole allantoin per ml. Samples were removed at 30-minute intervals and centrifuged to remove cells. The allantoin concentration remaining in the supernatant was determined as described in the text.

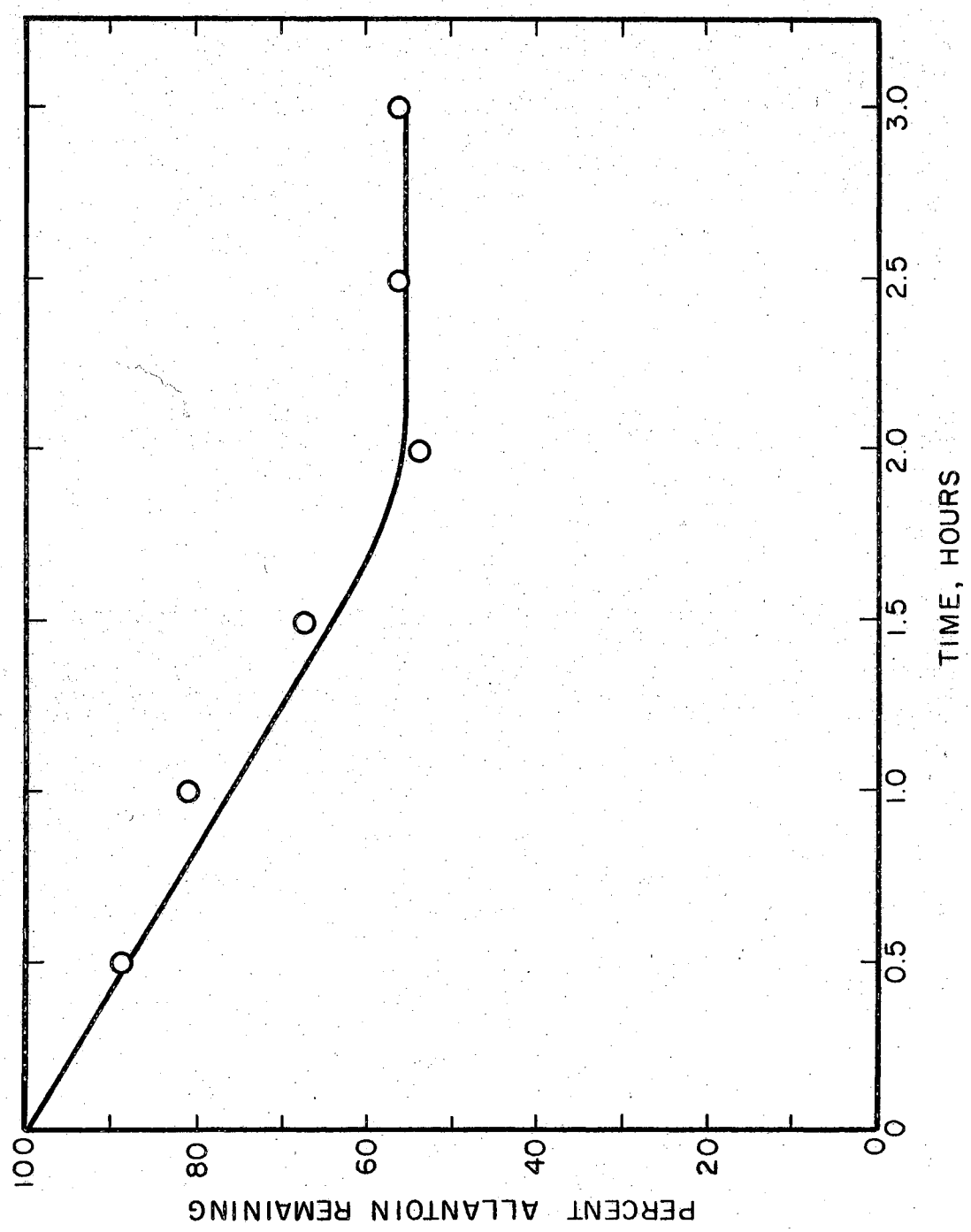
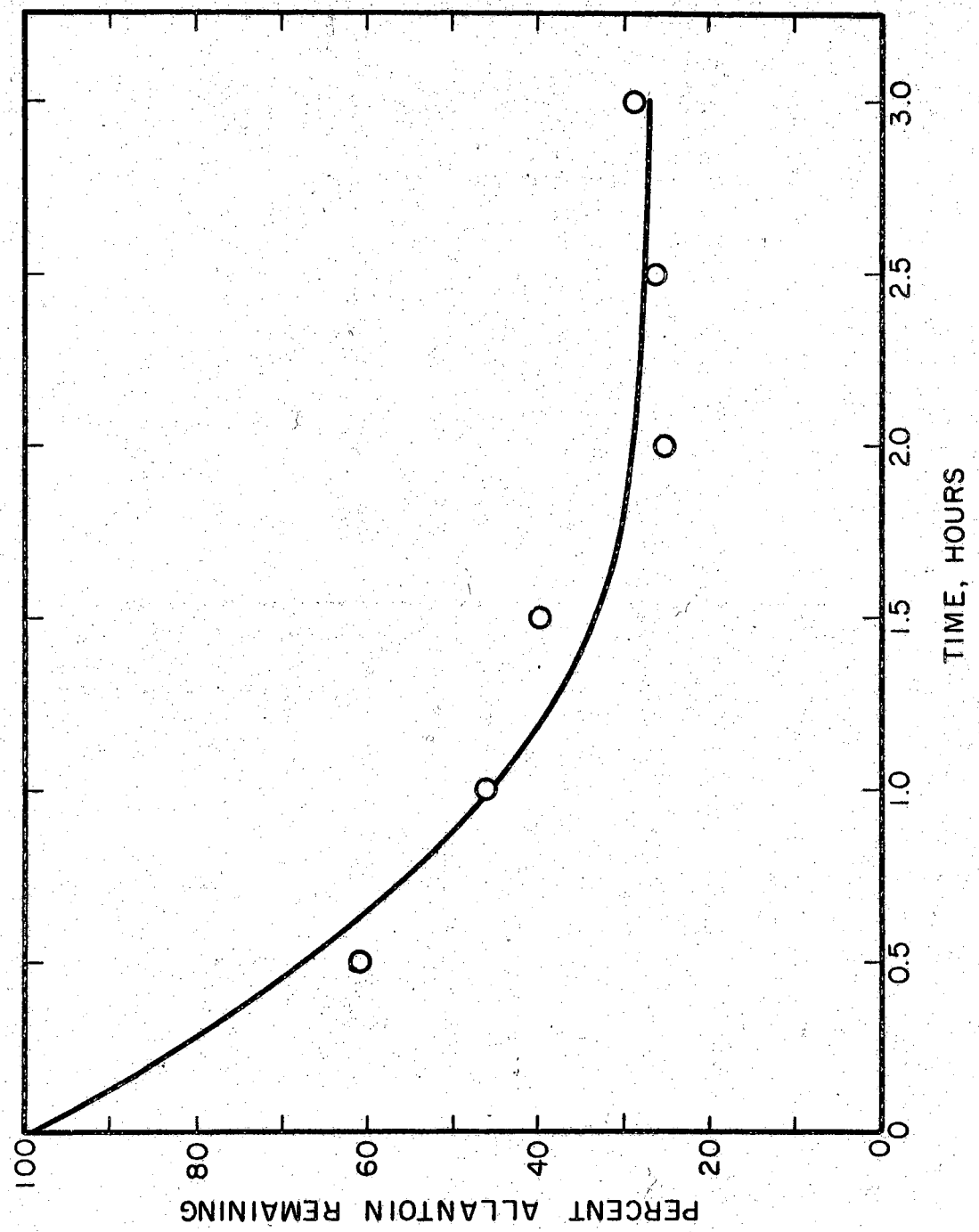

PERCENT ALLANTOIN REMAINING
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
TIME, HOURS

Figure 3. Accumulation of allantoin by mutant PA-1-120.
The procedure was identical to that described for wild type cells (Figure 2) except that cells were grown in glucose medium.


PERCENT ALLANTOIN REMAINING
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
2.5
3.0
TIME, HOURS

These figures demonstrate that the induction conditions used were adequate.

Results obtained with all strains used in this study are shown in Table IV. Experiments with each strain were carried out several times and in most cases amounts removed were in agreement. The data given in the table (except those for 105 and 182) are for measurements made during induction of the cells used for preparing extracts, since these are most useful for correlation with enzyme measurements. Those mutants with 71% or more allantoin remaining after 3 hours were considered negative and those with 57% or less remaining were considered positive.

C. Enzyme Activities of Mutant Strains

In Table V the specific activities of the enzymes in the mutants studied are given. It should be explained that the conditions resulting in maximum specific activity were not sought, only a comparison of activities under identical conditions, demonstrating the presence or absence of enzymes in the pathway. All enzyme activities have been corrected for endogenous levels determined as described below.

In initial experiments, cells were grown on glucose minimal medium before induction with allantoin. Since several of the mutants grew rather slowly on minimal medium, 0.08% yeast extract was added to the growth medium to obtain the large amounts of cells needed for preparation of extracts. It was noted that endogenous levels of enzyme

TABLE IV

ACCUMULATION OF ALLANTOIN

STRAIN	% ALLANTOIN REMAINING
120	57
166	25
105	100
164	45
111	97
402	100
144	72
182	98
117	74
PA-1 (allantoin-grown)	57

Contact time was 3 hours except for PA-1-182, for which a 2-hour contact was used. Inducing concentration was 1.0 µmole/ml. Cells were removed by centrifugation and allantoin in the supernatant was measured. All cells except PA-1 were grown on glucose.

TABLE V

INDUCED ENZYME LEVELS IN ALLANTOIN-NEGATIVE MUTANTS

Strain	Inducer	Uptake	Specific Activity, mμmoles/min/mg protein		
			Allantoinase	Allantoicase	UG Synthetase
120	A	+	19.1	0.0	69.0
166	A	+	19.6	0.0	102.4
105	A	±	8.4	0.0	12.2
105	KA	-	0.0	0.0	44.4
164	A	+	0.0	0.1	0.0
111	A	-	0.0	0.8	0.0
402	A	-	0.1	0.0	0.0
144	A	±	4.9	3.1	10.0
144	KA	±	0.0	0.0	5.2
182	A	±	0.0	3.5	3.4
182	KA	±	0.0	4.8	0.0
117	A	±	0.0	0.0	0.0

Cells were grown on glucose and induced by exposure to allantoin as described in the text. Specific activities of extracts prepared from fully-induced cells, i.e., wild type cells grown on allantoin, were: allantoinase, 86.2; allantoicase, 30.7; ureidoglycolate synthetase, 411.

activity in extracts of wild-type cells were slightly higher if the cells had been grown in medium containing yeast extract. This presumably results from the presence of small amounts of purines in yeast extract which may induce low levels of enzyme activity. However, the "endogenous" levels in cells grown with added yeast extract seem to be more reproducible than those in cells grown on minimal medium. Table VI shows endogenous levels obtained in repeated measurements with the wild type and several mutants. Since it was not practical to measure endogenous levels in each batch of cells used for preparation of extracts, the average endogenous levels were used to correct activities measured in induced cell extracts. It may be noted from these data that allantoicase activity is completely absent in mutants 120, 105, and 301(a UV mutant derived from 105). These values and others which deviate significantly from the majority of measurements were omitted in calculating average activities.

A 1.0 umole/ml concentration of allantoin in minimal salts was used for all inductions except for mutants 105 and 182, for which a 1% solution (63 µmoles/ml) of allantoin was used. Since these two mutants did not accumulate allantoin when exposed to a concentration of 1.0 µmole/ml (see Table IV), it was hoped that use of a higher concentration of allantoin would result in forcing sufficient allantoin into the cell to allow enzyme induction. The same technique was employed in induction of these two strains with allantoate.

TABLE VI

ENDOGENOUS ENZYME LEVELS

Cells grown with 0.08% yeast extract

Strain	Specific Activity, mμmoles/min/mg protein		
	Allantoinase	Allantoicase	UG Synthetase
PA-1	3.5*	2.7*	22.1*
PA-1	3.2*	3.7*	25.2*
PA-1	3.2*	2.2*	21.0*
PA-1	1.3*	3.4*	13.7
PA-1	3.0*	2.7*	19.4*
105	0.0	0.0	44.5
105	4.2*	0.0	19.3*
164	1.4*	0.9	9.9
111	4.7*	1.8*	17.7*
301	3.6*	0.0	19.5*
120	12.2	0.0	0.0
Average*	3.1	2.4	10.6
Cells grown without yeast extract			
PA-1	2.6	1.3	7.2
117	0.8	2.5	1.8

Cells were grown on medium containing 0.5% glucose, with or without yeast extract as indicated. Extracts were prepared as for induced cells. Specific activities marked with an asterisk were used in calculating average endogenous levels.

The technique appears to have been at least partially successful since low levels of induction were achieved (Table V).

From Table V it may be seen that mutants 120, 166, and 105 lack allantoicase activity. Both allantoinase and ureidoglycolate synthetase activity were present and were inducible by allantoin. None of the three enzymes were induced in mutant 164 although this strain actively accumulates allantoin. In mutants 111 and 402, which are impermeable to allantoin, no enzyme activity was induced. However, in strain 144, which accumulates allantoin very slowly, low levels of all three activities were found. Mutant 117 also accumulates allantoin at a very slow rate, and exposure to allantoin resulted in no enzyme induction. In mutant 182, when a high inducing level of allantoin or allantoate was used, no allantoinase activity was induced, but both allantoicase and ureidoglycolate synthetase were induced by allantoin and allantoicase by allantoate.

It would have been desirable to measure enzyme levels in wild type PA-1 induced with allantoin. However, difficulty was experienced in repeated attempts to induce PA-1. After allantoin had been introduced into the washed cell suspension of PA-1, lysis of the cells occurred, manifested after centrifugation by a gummy mass which was impossible to resuspend. Several causes were suggested, but all were found negative. Triple distilled, deionized water was used for all growth and resuspending media to eliminate the

possibility that impurities in the distilled water were causing the lysis, but lysis occurred within one hour during incubation.

To exclude the possibility of active phage a sample was removed from the medium after the cells had been sedimented. This sample was plated on PA-1 cells, but no plaques formed. It is suggested, however, that a defective phage could be present and no plaques would form. It is known from other work in our laboratory that PA-1 is lysogenic.

D. Growth on Uric Acid

The set of experiments designed to differentiate between a mutant with a permeability defect and one lacking a metabolic enzyme was that of growth on uric acid. Since uric acid is the direct precursor of allantoin, growth on uric acid indicates the ability to form all enzymes of the allantoin pathway. Mutants able to grow on uric acid, but not on allantoin or its metabolic products, must have a defect other than lack of a metabolic enzyme.

Previous attempts to grow PA-1 and other allantoin-utilizing pseudomonads on uric acid had been unsuccessful. Magasanik's report (15) that thiamine was required for growth of Aerobacter aerogenes, Escherichia coli, and Salmonella typhimurium on adenine or hypoxanthine suggested that thiamine might also permit growth of P. aeruginosa on uric acid. This was found to be the case.

The ability of PA-1 and various mutant strains to grow

on minimal medium plus thiamine, containing uric acid as sole carbon source, is shown in Table VII. Since uric acid is only slightly soluble, a low concentration (0.15%) was used and the amount of total growth, measured at 22 hours, was small. However, the difference was clear-cut. No change in optical density was observed in those mutants unable to grow on uric acid; i.e., the final optical density was the same as, or less than, the initial value. Data for three UV mutants derived from the original mutant strains are also shown.

Mutants previously shown to lack allantoicase activity (120, 166, and 105) were unable to grow on uric acid. Mutants 182 and 117 also were unable to grow on uric acid, while the remaining four strains, 164, 111, 402, and 144 grew as well as the wild type. Of the three UV mutants, the strain derived from 105 was unable to grow on uric acid, while those derived from 182 and 117 grew to the same extent as PA-1.

TABLE VII

GROWTH ON URIC ACID AS SOLE CARBON SOURCE

STRAIN	GROWTH, 22 HOURS
PA-1	+
120	-
166	-
105	-
164	+
111	+
402	+
144	+
182	-
117	-
301 (105-UV)	-
182-1 (182-UV)	+
117-2 (117-UV)	+

Growth was measured as change in optical density at 540 mμ after incubation for 22 hours at 37° C in minimal medium containing 0.15% uric acid and 0.01 μg/ml of thiamine.

CHAPTER IV

DISCUSSION

A. Evidence Relating to Location of Mutations

Mutations in PA-1-120 and PA-1-166 were more easily defined than those in any of the other groups. These two mutants are grouped together on the basis of transduction studies, carbon source data, and enzyme information. They lack the enzyme allantoicase. From carbon source data they also had acquired the ability to grow on ureidoglycolate while the wild type Pseudomonas aeruginosa does not.

PA-1-105 and its ultraviolet mutant PA-1-301 present a slightly more complicated situation. Neither mutant will grow on uric acid; therefore, an enzyme block is indicated. Enzyme assays showed the presence of allantoinase and ureidoglycolate synthetase, but allantoicase activity was completely absent. With an absence of growth on allantoate and no allantoicase activity a mutation has occurred in the structural gene controlling production of this enzyme. PA-1-301 retained this block but has gained permeability to both ureidoglycolate and glyoxylate. This gain in permeability possibly is only a single mutation, as would be expected of UV, controlling permeability to both substrates. The enzyme data for this mutant support the

transduction data which showed a lesion of 105 identical to that in 120 and 166.

PA-1-111, -144, -145, -164, and -402 would be classified together on the basis of carbon source utilization because of growth only on allantoate. However, from transduction studies this group was divided into two groups-one containing PA-1-164 and PA-1-145(which was subsequently lost) and the other consisting of the three remaining mutants. The one significant biochemical dissimilarity is that PA-1-164 leaves only 45% allantoin in solution, whereas PA-1-111, -144, and -402 leave a minimum of 71.5%. Therefore, the allantoin accumulating mechanism of PA-1-164 is undamaged.

PA-1-164, although no enzyme activity is induced, can grow on uric acid, thereby disclosing that no enzyme block exists. The most reasonable explanation of these facts is that this is a super repressor mutant. That is, the mutation occurred in the regulator gene resulting in formation of a repressor insensitive to allantoin. Assuming that the enzymes are coordinately controlled, this mutation would prevent the synthesis of all enzymes in this regulon.

PA-1-144 is very inefficient in accumulation of allantoin, leaving 71.5% in solution, but PA-1-111 and -402, which are in the same genetic group as PA-1-144, are completely impermeable to allantoin, leaving 100% in solution. It is proposed that PA-1-144 is a "leaky" mutant permitting a sufficient quantity of allantoin to enter the cell to

result in a low level of enzyme induction. However, the rate of allantoin entrance into the cell, although high enough for an increase in enzyme activity, is not sufficient to allow for growth, since in carbon source utilization studies this organism would not grow on allantoin.

From their ability to grow on uric acid, it can be concluded that no enzyme block is present in this group.

Therefore, the inability of all three strains to grow on allantoin and the absence of induced enzyme activities in strains 111 and 402 can be attributed to lack of ability to accumulate allantoin. Although one of the three mutants (144) has a less severe defect than the other two (111 and 402), the function affected is the same in all three strains, as was predicted by transduction studies which placed them in a single genetic group.

PA-1-117 and -182 have mutations which are the most difficult to interpret. Transduction gave no information except that the differences in mutations were great enough that these mutants were not placed in any group nor seemingly were they related to each other. They were identical only in that there was no growth on any of the carbon sources.

In an effort to determine the position of these mutations, UV mutants were isolated from each parent by selection for ability to grow on ureidoglycolate. Every mutant from a single parent behaved identically, growing on allantoate, ureidoglycolate, and glyoxylate. From an

absence of growth on uric acid and from carbon source data on their UV mutants, an enzyme block is possibly located at the allantoinase step. This conclusion is supported, for strain 182 at least, by enzyme data.

After treatment with UV, the enzyme block was removed as evidenced by growth on uric acid and permeability to the three substrates. However, since no growth occurred with allantoin, it must be assumed that a permeability block for this substrate remained. Since, as was stated before, UV usually only elicits single site mutations, it is suggested that UV could have created a single-base deletion whereby in the genetic code the reading returns to normal with only a relatively small nonsense message occurring. This suppressor mutation would allow several repairs in the code to be made with a single point mutation (6). The probability that this might happen without extensive damage to other functions would depend upon control by an operon; in other words, the genes would have to be linked with no intervening, unrelated genes included. However, it has been proposed that, in general, P. aeruginosa genes are not linked and a "reading-frame" mutant, therefore, could not occur since the genes are scattered.

Alternatively, PA-1-182 and PA-1-117 may be impermeable to uric acid, allantoin, allantoic acid, ureidoglycolate, and glyoxylate. In this case, it would not be necessary to postulate an enzyme block, although the enzyme assays for 182 suggest that one is present. The UV-induced mutation

might then have restored permeability to all the subsrates except allantoin. Neither explanation is completely satisfactory but it can definitely be concluded that several functions were affected by EMS treatment in these two strains. These mutants offer interesting possibilities for study of multiple permeability effects and it is probable that only through such studies can the mutation pattern in these mutants be understood.

It cannot be concluded from the studies to date whether there is a permease, in the accepted sense, for allantoin. Permeability to allantoin and to its metabolites has been shown by Bruce and by the present studies to be genetically controlled. In several cases, it has been observed that a mutation, which can be assumed to be a single mutational event, affects the permeability of the cell to several substrates. However, multiple effects on permeability do not necessarily result from a single mutation, since in other cases permeability to only one substrate was affected. Attempts to measure permease induction indicated that no rapid induction occurred in wild type cells. However, several allantoin-negative mutants, grown on glucose, rapidly accumulated allantoin when exposed to a low concentration. The rate of uptake, determined by a method which only roughly approximated actual rates but which should allow fairly valid comparisons, was as rapid as that of the wild type cells grown on allantoin (Figures 2 and 3). Further study of this problem will be difficult

unless radioactive substrates can be obtained or prepared.

B. Evidence Relating to Control of Allantoin Metabolism

Fargie and Holloway (10) have reported that a number of gene loci for various biosynthetic pathways are unlinked in P. aeruginosa. However, in the related organism, P. fluorescens, evidence for coordinate control of degradative enzymes has been reported and the term regulon has been proposed to describe these units of control for which no genetic data are available (16). No single pathway in a Pseudomonas species has been studied both biochemically and genetically, and both types of data are needed before firm conclusions can be drawn concerning mechanisms of control in this organism.

The transduction data obtained by Bruce indicate that the gene loci for the allantoin-negative mutants studied thus far are not linked. This conclusion is based upon the same criterion employed by Fargie and Holloway; i.e., numbers of transductants obtained using phage grown on the wild type as donor were no greater than those obtained with phage propagated on mutants from other groups. However, in the present study evidence was obtained which indicates coordinated control of these unlinked loci.

The fact that the enzymes of the allantoin pathway are subject to metabolic control of some type is shown by their inducibility. This implies repression of enzyme formation in the absence of the substrate. In all such cases examined

in other organisms, repression has been shown to be genetically controlled and, according to current models, to be due to the action of one or more regulator genes responsible for production of a cytoplasmic repressor.

Additional evidence of repression of genetic origin is supplied by mutant PA-1-164. The only explanation for the behavior of this mutant, which is consistent with current theories of metabolic control, is the location of the mutation in a regulator gene which codes for a repressor specific for at least the three enzymes studied. The permease site cannot be subject to the same control since the ability of this strain to accumulate allantoin is unimpaired.

Evidence supporting coordinated control of the three enzymes studied was also obtained in these experiments. In strains 120, 166, and 105, all of which completely lack allantoicase activity, both allantoinase and ureidoglycolate synthetase were induced by allantoin, and ureidoglycolate synthetase was induced by allantoate in strain 105. The induction of ureidoglycolate synthetase in a cell completely lacking allantoicase is equivalent to gratuitous induction, i.e., induction by a non-metabolizable inducer. This type of induction is one criterion for control of the operon or regulon type (12,24). A second example of the same type of control is the induction of allantoicase and ureidoglycolate synthetase in the absence of allantoinase in strain 182. However, this example is not as convincing

as the previous one since the data for the allantoinase block are not conclusive.

Isolation of additional mutants, particularly mutants blocked at allantoinase and ureidoglycolate synthetase, will be necessary for complete elucidation of the controls operative in the allantoin pathway. However, these are the first data which have been obtained for a Pseudomonas in which both biochemical and genetic studies of a single pathway have been made.

CHAPTER V

SUMMARY

PA-120, PA-1-166, and PA-1-105, from transduction, carbon source data, and enzyme information only lack the enzyme allantoicase, which converts allantoic acid to ureidoglycolate.

PA-1-111, -144, -145, -164, and -402 are classified as a group based on carbon source data, but the group was divided into two genetically different groups on the basis of transduction studies. One group contained PA-1-164 and PA-1-145, but since PA-1-145 was lost only PA-1-164 was studied. PA-1-164 is proposed to be a super repressor mutant since allantoin can be accumulated by the cells, but enzyme levels are little changed from endogenous rates.

PA-1-144 is a "leaky" mutant, but insufficient amounts of allantoin are accumulated to allow growth. PA-1-111 and PA-1-402 are completely impermeable to allantoin. This group, which was shown to be genetically identical, is also identical biochemically since the same function, ability to accumulate allantoin, is affected in all three mutants. Their ability to grow on uric acid indicates the absence of an enzyme block.

From transduction data PA-1-117 and PA-1-182 could not be placed in any group and the resulting information from enzyme studies left the location of mutation uncertain. It is proposed that the mutation left these mutants impermeable to all the substrates, but UV mutants derived from them regained permeability to all substrates except allantoin.

It has been shown by transduction that the allantoin degradative pathway occurs as proposed by Valentine, et al. (20), that is, allantoin ---→ allantoic acid ---→ urea + ureidoglycolate ---→ urea + glyoxylic acid. Also, the enzyme studies just completed offer further proof of the same pathway.

Data have also been obtained which show that synthesis of the enzymes of this pathway is coordinately controlled, although the loci for the enzymes studied are not closely linked.

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