THE ROLE OF UREIDOGLYCINE IN THE METABOLISM OF ALLANTOIC ACID

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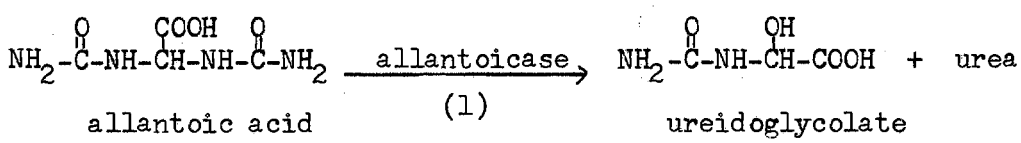
CHAPTER I

INTRODUCTION

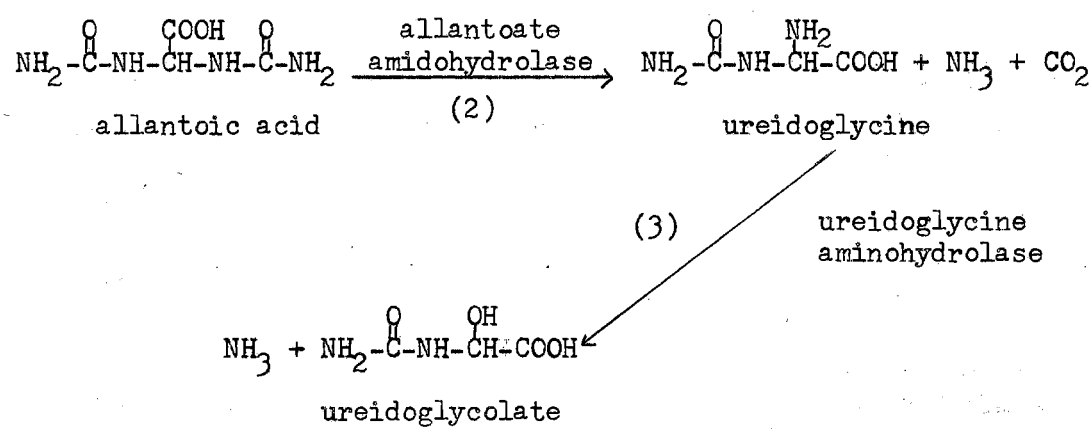
Allantoin occurs widely in nature where it arises partly from the degradation of purines by microorganisms and some animals and partly from synthesis by plants.

Allantoic acid is the product of the hydrolysis of allantoin by allantoinase. Two enzymes have been reported to degrade allantoate, i.e., both allantoicase and allantoate amidohydrolase. The two different proposed pathways for the conversion of allantoate to ureidoglycolate are as follows:

A.



B.



In reaction (1), allantoate is degraded by allantoicase to form ureidoglycolate and urea. In the alternate pathway (B), two enzymes (reactions 2 and 3) are involved in the degradation of allantoate. Ureidoglycine is a proposed intermediate in the production of ammonia, CO_2, and ureidoglycolate.

Reliable techniques had been developed in previous studies (1) for use in investigating the ammonia-forming reactions in the enzymatic degradation of allantoate. It was the purpose of this continuing investigation to determine whether both pathways may occur in the same microorganism or whether only one functions in any given organism. Possible approaches to the solution of this problem include isolation of mutants unable to degrade allantoate, isolation of urease-negative mutants, synthesis or isolation of the proposed intermediate—ureidoglycine, or purification of the enzymes involved.

If two different enzymes can be used in the same microorganism to degrade allantoate, it should be impossible to isolate allantoate-negative mutants in a single mutational step; if only one is used, mutants can be isolated. If urease-negative mutants still can grow on allantoate minimal medium, using allantoate as sole carbon, nitrogen and energy source, the pathway producing ammonia may be assumed to function physiologically. If urease-negative mutants cannot grow on allantoate minimal medium, then it may be concluded that urease is required for allantoate degradation and that ammonia is not produced <u>in vivo</u> from allantoate except through allantoicase and urease.

Based on the above reasoning, it seemed that the two most important steps toward a definitive answer to the choice of pathways would be isolation of one or both of the types of mutants described and synthesis

of ureidoglycine. Purification of all the enzymes involved could not be undertaken unless the substrate was available for ureidoglycine amino-hydrolase, and only isolation of mutants could offer proof of the requirement of a particular reaction for allantoate degradation in vivo. Therefore, both these approaches were attempted in the present study.

CHAPTER II

LITERATURE REVIEW

An enzyme which was reported to degrade allantoic acid to glyoxylic acid and urea was found independently by Krebs and Weil (2) in frog's liver and by Brunel (3) in the mycelium of Aspergillus niger. The enzyme was called allantoicase. Barker (4) was the first to report the anaerobic degradation of allantoate, using Streptococcus allantoicus which he had isolated from an allantoin enrichment. He determined the fermentation products but did not study individual enzyme reactions. Campbell (5) reported that allantoic acid was enzymatically decomposed to urea and glyoxylic acid by an aerobe, Pseudomonas sp., which he had also isolated from San Francisco Bay mud using allantoin enrichments.

The fermentation of allantoin was investigated further by Valentine et al. (6) in S. allantoicus. These authors separated the degradation of allantoic acid to glyoxylic acid and urea into two steps. Allantoate was degraded to ureidoglycolate and urea by the enzyme allantoicase. Ureidoglycolate was converted to urea and glyoxylic acid by a new enzyme glyoxylurease (ureidoglycolate synthetase).

The existence of ureidoglycolate as an intermediate in the degradation of allantoate was first proposed by Valentine and Wolfe (7). They described a method for the enzyme assay with cell-free extract from S. allantoicus to measure the degradation of ureidoglycolate. Gaudy (8) modified the procedure for the synthesis of ureidoglycolate in several

respects to obtain a pure crystalline product and showed that a new enzyme, ureidoglycolate synthetase, was involved in the degradation of ureidoglycolate to urea and glyoxylic acid. Gaudy (8) purified ureidoglycolate synthetase 135-fold from crude extracts of S. allantoicus by treatment with $MnCl_2$, fractionation on calcium phosphate gel, fractionation with ammonium sulfate and column chromatography on DEAE-cellulose.

The properties of this new enzyme were studied by Gaudy and Wolfe (9). The purified enzyme and recrystallized ureidoglycolate were used for these studies. The maximal activity of ureidoglycolate synthetase occurred at pH 8.4 to 8.8 and no cofactors were required for the reaction. Inhibitors of the enzyme were p-chloromercuribenzoate (at relatively high concentration), Hg^{++}, Zn^{++}, Cu^{++}, Fe^{++}, Fe^{+++}, and Ca^{++}. At 10^{-3} M concentration, Mg^{++} had no effect on enzyme activity and Mn^{++} at the same concentration increased enzyme activity slightly. The maximal velocity of the purified enzyme was 220 μ moles of glyoxylate formed per minute per mg of protein. The concentration of substrate required for half-maximal velocity was 3.3×10^{-2} M. The position of the equilibrium for the synthesis of ureidoglycolate was not affected by a pH change or by the presence of enzyme. The equilibrium constant for the reaction in the direction of synthesis was 7.6, which corresponded to a negative free energy change of 1,230 cal per mole.

The fact that S. allantoicus had been shown to convert allantoic acid to glyoxylic acid and urea through a two-step sequence by the enzymes allantoicase and ureidoglycolate synthetase suggested that the reaction generally attributed to the single enzyme allantoicase might also occur in two steps in other organisms and that ureidoglycolate

might act as an intermediate in the degradation of allantoate in all such organisms. The presence of the ureidoglycolate synthetase reaction in the yeasts Saccharomyces cerevisiae and Candida utilis was reported by Dommas (10). Independent genetic evidence which supported the pathway of allantoate degradation proposed by Valentine et al. (6) has been obtained by Gaudy and Bruce (11) for Pseudomonas aeruginosa. These studies confirmed the role of ureidoglycolate as the sole intermediate in the conversion of allantoate to glyoxylate and urea in this organism. Studies of the enzyme activities of allantoate-negative mutants of P. aeruginosa by Winter (12) gave further support to this conclusion.

The transduction data obtained by Bruce (13) indicated that the gene loci for the allantoin-negative mutants of P. aeruginosa which were studied were not linked. This conclusion was based on the fact that the numbers of transductants obtained using phage grown on the wild type as donor were no greater than those obtained with phage propagated on mutants with different enzyme lesions. However, Winter's studies indicated that there was coordinated control of these unlinked loci. These were the first data which were obtained for a Pseudomonas species in which both biochemical and genetic studies of a single pathway were made.

Meganathan (14) reported that bacteria belonging to the genera Aerobacter, Alcaligenes, Arthrobacter, Escherichia, Flavobacterium, Nocardia and Pseudomonas have the ability to grow on allantoin aerobically and species of Aerobacter, Escherichia, Streptococcus and Pseudomonas can ferment allantoin. Ureidoglycolate synthetase activity was demonstrated in cell-free extracts of representatives of each genus.

It seems certain, therefore, that ureidoglycolate is a commonly occurring intermediate in the degradation of allantoin, at least in microorganisms, and that the ureidoglycolate synthetase reaction is responsible for the conversion of ureidoglycolate to glyoxylate and urea.

Trijbels and Vogels (15, 16) have agreed that ureidoglycolate is the sole intermediate in the degradation of allantoate by Pseudomonas aeruginosa, Penicillium citreo-viride and Penicillium notatum. However, Vogels (17) has pointed out that, in the case of S. allantoicus, a reaction sequence which involved the degradation of allantoic acid by allantoicase could not be correct, because ammonia was formed in large quantities by S. allantoicus in the degradation of allantoate to ureidoglycolate. Since this organism does not contain urease and the quantity of urea found is much less than 2 moles per mole of allantoate used, Vogels concluded that S. allantoicus contains no allantoicase.

In 1966 Vogels (18) reported that the degradation of allantoate to ureidoglycolate in S. allantoicus was a two-step reaction. The first step was catalyzed by allantoate amidohydrolase and allantoate was converted to one mole of ureidoglycine, one mole of CO_2 and one mole of ammonia. In the second step, catalyzed by ureidoglycine aminohydrolase, ureidoglycine was transformed to ammonia and ureidoglycolate. The organisms capable of degrading allantoate via this pathway, as reported by Vogels (18), are Pseudomonas acidovorans, Escherichia coli, Escherichia coli var. acidilactici, Escherichia freundii, and Streptococcus allantoicus. Cell-free extracts of these organisms exhibited only small activity in degrading allantoate. The activity of the extract was enhanced several-fold by a short pretreatment with acid

followed by rapid neutralization to pH 8.5. Vogels reported that two moles of ammonia, one mole of ureidoglycolate and one mole of urea were formed from one mole of allantoate. Ammonia was measured by oxidation of NADH with glutamate dehydrogenase and α-ketoglutarate, and it was reported by Vogels that Nessler's reagent yielded the same values. He did not report studies of interference from other compounds in the incubation mixture, nor did he determine all nitrogenous products in the mixture. Wu (1) investigated the formation of ammonia by S. allantoicus and reported that different methods of ammonia determination affect the apparent ammonia production and also that different conditions affect the amount of ammonia formed. Studies showed that, of the methods tested, the microdiffusion method is the best for ammonia determination because apparently only the ammonium salt in the incubation mixture can react with the concentrated alkali and produce ammonia at the temperature used for incubation (37°C). Use of limited amounts of enzyme and rapid analysis of the reaction mixture were recommended for assay of the production of ammonia. These studies confirmed the presence, in extracts of S. allantoicus grown on allantoin, of enzymes which form ammonia from allantoic acid. Two pH optima were found for ammonia-forming enzymes, which may correspond to the optimum pH values for the two enzymes which form ammonia, allantoate amidohydrolase and ureidoglycine aminohydrolase.

Since allantoate amidohydrolase is a very interesting and unusual enzyme, Vogels and his associates have studied this reaction in considerable detail.

Vogels (18) reported that, in S. allantoicus, the extent of activation of allantoate amidohydrolase is sharply determined by the

pH used. At pH 4.5 no activation occurs, but at pH 4.1 the activation is almost complete. At pH 1.9, activation reaches a maximum within 30 seconds at 0°C. On prolonged pretreatment with acid, the activity decreases, probably due to denaturation. The enzyme was inactivated rapidly at pH values between 5 and 7.5. Both activation and inactivation of the extracts occurred in defined pH regions, and at two pH regions both activated and inactive forms of the enzyme remained unchanged; these were pH 4.3 to 4.9 and above 8.0. Activation and inactivation are reversible processes. According to Vogels, they are not connected with the dissociation of a low or high-molecular weight part of the molecule, and may be due to intramolecular rearrangements in which the active site of the enzyme is involved. The pH optimum for the enzymatic reaction is about 8.5 and the enzyme is activated by manganous ions and reducing substances, such as glutathione. Allantoate amidohydrolase from S. allantoicus has been purified 50-fold by acetone fractionation, DEAE-cellulose chromatography and gel filtration by van der Drift and Vogels (19).

A mechanism for the activation, inactivation and instability was proposed by van der Drift and Vogels (20, 21). "The extent and rate of activation depended both on the concentration of complexing substances and the pH value. Below pH 5.0, only H^+ ions were necessary to achieve activation. Above pH 5.0, activation took place only in the presence of complexing anions. A correlation was found between the rate and extent of activation by a particular anion on the one hand and the complex formation between the anion and Mn^{++} ions on the other hand. Activation was explained as a displacement of Mn^{++} ions from the active center of the enzyme by H^+ ions." Activation was presented by Vogels as follows.

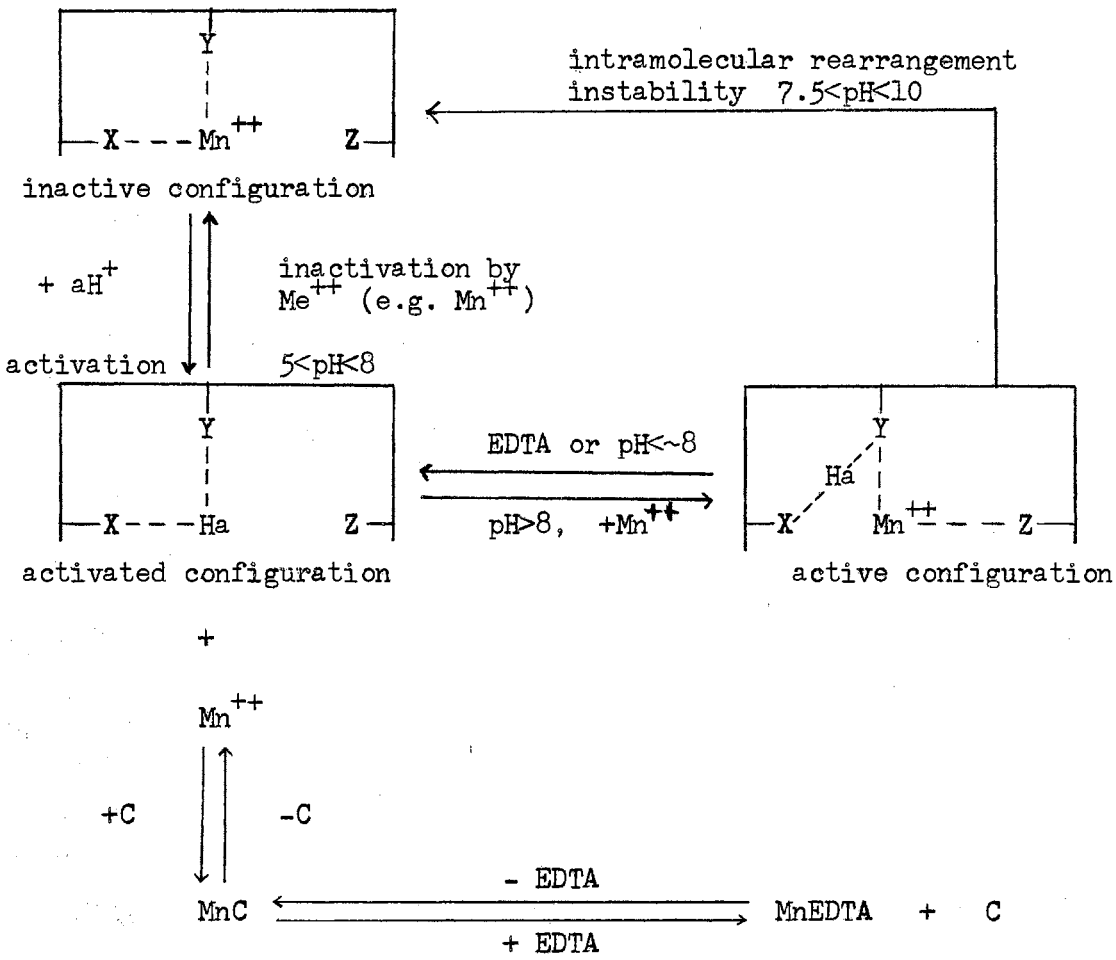
$$Ea^{-}\,Mn^{++} + aH^{+} \rightleftharpoons EH_a + Mn^{++}$$

inactive enzyme — activated enzyme

$$Mn^{++} + C \rightleftharpoons MnC, \quad \text{where C = complexing anion}$$

$$MnC + EDTA \rightleftharpoons MnEDTA + C$$

A decrease of the enzymic activity could be achieved by the addition of bivalent cations, especially Mn^{++} ions, at defined pH values and also by dilution of enzyme with EDTA-free buffer. The first process was called inactivation and the second one instability by van der Drift and Vogels. "The inactivation is pH-dependent and was observed between pH 5.3 and 8.0. In the absence of bivalent cations the enzyme rapidly lost activity above a pH of 8 due to instability. Loss of activity could be prevented by addition of EDTA and/or Mn^{++} ions (above pH 8) or below pH 8 by the addition of EDTA. Activity lost by inactivation or instability could be restored by a subsequent activation procedure." According to van der Drift and Vogels (21) the processes of activation, inactivation and instability and the reversibility of these processes was represented by the following reaction scheme.



Here C is the complexing anion and X, Y and Z represent three amino acid residues in the active center of the enzyme.

The activated configuration is active only above pH 8.0 in the presence of Mn^{++} ions and below pH 8.0 the active configuration is transformed into the inactive form in the presence of Mn^{++} and other bivalent cations. The activated configuration is not further converted into the inactive form in the presence of EDTA. The extent of activation is determined by the amount of H^{+} and Mn^{++} or some other bivalent cations in the medium. Mn^{++} is bound to other ligands and the active configuration of the enzyme is formed above pH 8.0. The enzyme was protected against loss of activity in this pH region by Mn^{++} ions above pH 8.0 and also by higher concentrations of EDTA. When added in a

sufficient amount, EDTA protected the enzyme against instability by transforming the active configuration into the activated form. Presumably, Mn^{++} ions can stabilize the enzyme above pH 8.0 by shielding one or more groups in the active center, e.g. X, and thus prevent the intramolecular rearrangement.

The second enzyme in the ammonia-forming pathway, ureidoglycine aminohydrolase, has not been studied. Its activity has been measured only as a part of the overall two-step reaction for conversion of allantoate to ureidoglycolate, ammonia and CO_2. No separate studies have been possible since the substrate, ureidoglycine, has not previously been synthesized or isolated.

CHAPTER III

MATERIALS AND METHODS

A. Strains of Bacteria

Three microorganisms were used in these studies. All were bacteria which can use allantoate as a sole source of carbon, nitrogen and energy; all are urease positive and are able to grow on minimal media. All have the ammonia-forming pathway B (see page 1) as shown by enzyme assays with cell-free extracts.

Pseudomonas acidovorans, den Dooren de Jong strain 14 (22), was used as a wild type in these studies; it was obtained from M. Doudoroff, University of California at Berkeley. Mutants were isolated as described below and were numbered in the order of isolation as 14-1, 14-2, 14-3, etc.

Aerobacter aerogenes, strain T1b, was used as a wild type in these studies; it was isolated from an allantoin enrichment and identified by R. Meganathan in this laboratory (14). Mutants were numbered in the order in which they were isolated. The following notation was used: T1b-1, T1b-2, T1b-3, etc.

Strain TW is an organism which was isolated on allantoate minimal medium in this study. It was identified as Aerobacter aerogenes and was used as a wild type in these studies. Mutants were isolated and were numbered in the order of isolation as TW-1, TW-2, TW-3, etc.

B. Cultivation of Bacteria

Since all three microorganisms produce ammonia when grown on allantoate, the medium must be highly buffered. The components of the minimal salts medium were as follows (amounts per liter): $Na_2HPO_4 .7\ H_2O$, 8.2 gm; KH_2PO_4, 2.7 gm; $MgSO_4 .7\ H_2O$, 0.4 gm; $FeCl_2$, 0.1 per cent solution, 0.5 ml; distilled water to volume. When a solid medium was required agar was added to a concentration of 2 per cent. The pH was 7.0 before autoclaving. Carbon and nitrogen sources, when used, were prepared as 10 per cent (W/V) solutions, passed through a Millipore filter (HA, 0.45 μ pore size), and added aseptically to the medium to a desired concentration. All incubations were at 30°C with aeration unless otherwise noted.

C. Measurement of Growth

Cell suspensions were read against appropriate blanks at 540 nm on a Coleman Junior Spectrophotometer. Culture tubes, 18 x 150 mm, were used for all optical density measurements.

D. Treatment with Mutagens

1. Ultraviolet Light (UV)

The UV source used was a 15 watt Sylvania germicidal lamp. This was placed 40 cm above the cell suspension. Six ml of a washed cell suspension in sterile saline (0.85 per cent solution of NaCl in distilled water) containing about 10^8 cells/ml were irradiated in a sterilized petri dish. The suspension was agitated during exposure. The cell suspension was added to an equal volume of nutrient broth and incubated for 6 hours in the dark to allow segregation of the mutant genomes.

2. Ethylmethane Sulfonate (EMS)

Cells were grown overnight in minimal medium containing 1.0 per cent allantoate as sole carbon and nitrogen source with aeration at 30°C. Cells were harvested by centrifugation, washed with sterile saline and resuspended in minimal salts medium containing no carbon or nitrogen source. To a 5.0 ml sample of this cell suspension (O.D. of 0.3 to 0.4) was added 0.1 ml of EMS. The cells were incubated at 30°C without shaking. Incubation time was 12 hours for P. acidovorans and 4 hours for A. aerogenes Tlb. The treated cells were centrifuged, washed with sterile saline and resuspended in 5.0 ml of minimal medium. A 0.5 ml portion of this cell suspension was inoculated into fresh minimal medium which contained 0.5 per cent succinate as carbon source and 0.1 per cent NH_4Cl as nitrogen source. After growth, the culture was centrifuged and the cells were washed twice with sterile 0.85 per cent NaCl solution.

3. N-Methyl-N'-Nitro-N-Nitrosoguanidine (NTG)

The procedure for treatment with NTG and subsequent isolation of the mutants produced was obtained from Adelberg, Mandel and Chen (23). The procedure was used with some modification in this laboratory to obtain mutants. Cells were grown on 1 per cent allantoate minimal medium (allantoate as sole carbon and nitrogen source) overnight with aeration at 30°C. Washed cells were resuspended in minimal salts. NTG was added as a sterile 2.0 mg/ml solution in minimal salts to 6.0 ml of this cell suspension (O.D. of 0.3 to 0.4); the final concentration of NTG was 100 µg/ml. This suspension was incubated 30 minutes at 30°C with aeration. The treated cells were washed with sterile saline and resuspended in 6.0 ml of minimal medium which contained 0.5 per cent

succinate or 0.5 per cent gluconate as carbon source and 0.1 per cent NH_4Cl as nitrogen source. After growth, the culture was centrifuged and the cells were washed twice with sterile saline.

4. Acriflavin

After overnight growth in 1 per cent allantoate minimal medium at 30°C with aeration, cells were washed and resuspended in minimal medium. Acriflavin was added to 6 ml of this suspension (O.D. 0.2 to 0.3). The final concentration of acriflavin was 2.5 µg/ml. This was incubated 48 hr at 30°C with shaking. In some experiments, the treated cells were washed with sterile saline and resuspended in 6 ml of minimal medium; 0.5 ml of this suspension was inoculated into fresh minimal medium containing 0.5 per cent succinate or 0.5 per cent gluconate as carbon source and 0.1 per cent NH_4Cl as nitrogen source. This step was included to eliminate auxotrophs prior to further selection. After growth, the culture was centrifuged and the cells were washed twice with sterile saline.

E. Isolation of Mutants

The mutagen-treated cells were resuspended in minimal medium containing carbon and nitrogen sources appropriate for the type of mutant to be selected. For the purpose of isolating nitrogen source mutants, cells were resuspended in minimal medium containing no nitrogen source and aerated at 30°C for 6 hours to deplete internal pools. Then 0.5 ml of this suspension was inoculated into the appropriate minimal medium containing 50,000 U penicillin/ml or 650 µg/ml D-cycloserine. The culture was incubated 18 hours on the shaker, then centrifuged, washed once with sterile saline and resuspended in minimal medium containing

0.5 per cent succinate or 0.5 per cent gluconate as carbon source and 0.1 per cent NH_4Cl as nitrogen source. This was incubated at 30°C on the shaker. After growth, the dilution giving between 50 and 100 colonies per plate was determined by preparing a series of dilution plates. The dilutions were stored at 4°C. Twenty plates of succinate minimal agar were inoculated by spreading 0.1 ml of the same dilution on the surface. Incubation was at 30°C until the colonies were 1 to 2 mm in diameter. The colonies were then replica-plated by the technique of Lederberg and Lederberg (24) to the appropriate selective plates and succinate minimal agar plates. Mutants were those which formed colonies on succinate but not on selective minimal agar plates. These were picked individually from succinate plates and were checked for growth on the appropriate fresh liquid minimal medium.

F. Tests for Carbon and Nitrogen Source Utilization

The minimal salts medium described above was used as the base for the growth media, with various compounds added as carbon and/or nitrogen sources. Cells were washed from a fresh nutrient agar slant with one ml of minimal salts medium and 0.1 ml of this suspension was added to the liquid minimal medium. Incubation was at 30°C with shaking. The following compounds were tested as carbon sources (at concentrations of 0.5 per cent): glucose, gluconate, succinate, histidine, allantoate, ureidoglycolate, ureidoglycine, glyoxylic acid, acetate, citrate. The following compounds were tested as nitrogen sources (at concentrations of 0.1 per cent): allantoate, ureidoglycolate, ureidoglycine, oxamate, urea, glycine, NH_4Cl.

G. Preparation of Crude Extract

The cells were grown overnight in 500 ml of minimal salts medium with 1 per cent allantoate or 0.5 per cent succinate plus 0.1 per cent allantoate. The cells were harvested by centrifugation in a Sorvall refrigerated centrifuge, washed with sterile saline and then frozen as a pellet overnight. The cells were thawed by resuspension in 10 to 15 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. The suspension was centrifuged for 20 minutes at 12,000 rpm in an RC-2B refrigerated centrifuge. A cell-free extract was obtained by discarding the whole cells and debris.

H. Enzyme Assays

1. Without Acid-Pretreatment

Crude extract containing an appropriate amount of protein in a volume of 0.1 to 0.6 ml was mixed with 5.8 to 7.2 ml of 0.1 M sodium barbital-HCl buffer (pH 8.5) which contained 15.0 to 20.0 µmoles per ml of potassium allantoate or ureidoglycine, 11 µmoles per ml of reduced glutathione (GSH) and 0.1 µmole per ml of manganous sulfate. The total volume was 7.5 or 9.0 ml. Tubes were incubated at 30°C for varying periods of time. Samples were used undiluted or were diluted 1/10 for chemical analysis.

2. Acid-Pretreatment

Crude extract containing an appropriate amount of protein in a volume of 0.1 to 0.4 ml was mixed with 1.2 ml of 0.05 M sodium citrate-HCl buffer (pH 2.0). After 30 seconds at room temperature, 5.8 or 7.2 ml of 0.1 M sodium barbital-HCl buffer, pH 8.5, were added. This buffer

contained 15.0 to 20.0 μmoles potassium allantoate or ureidoglycine, 11 μmoles reduced glutathione and 0.1 μmole manganous sulfate per ml. The total volume was 7.5 or 9.0 ml. The mixture was incubated at 30°C. Samples were used undiluted or were diluted 1/10 for chemical analysis.

I. Chemical Analyses

1. Allantoate

Allantoate was determined by addition of 1.0 ml of 0.15 N HCl and 1.0 ml of phenylhydrazine·HCl (100 mg/30 ml water) to a tube containing 0.2 to 1.0 μmole of allantoate made up to a 6.0 ml volume with distilled water. The tubes were immersed in a boiling water bath for 2.0 minutes. The tubes were cooled to room temperature and 1.0 ml of potassium ferricyanide (500 mg/30 ml water) and 4.0 ml of 10 N HCl were added. After standing 5 minutes at room temperature, the color was read at 515 nm on a Coleman Junior Spectrophotometer.

2. Glyoxylate

For the determination of glyoxylate, a sample containing 0.2 to 1.0 μmole of glyoxylate was made up to a 7.0 ml volume with distilled water. Then 1.0 ml of phenylhydrazine solution (100 mg/30 ml water) was added to each tube and, after thorough mixing, the tubes were kept at room temperature for 10 minutes. Consecutive additions of 1.0 ml of potassium ferricyanide (500 mg/30 ml water) and 4.0 ml of 10 N HCl were made with thorough mixing after addition of HCl. After standing 5 minutes at room temperature, the color was read at 515 nm on a Coleman Junior Spectrophotometer.

3. Urea

Urea was measured by the method of Jones, et al (25). A sample containing 0 to 1.25 µmoles of urea was made up to 4.25 ml with distilled water, and 2.0 ml of a 1:3 (V : V) mixture of sulfuric and phosphoric acids were added. Following the addition of 0.25 ml of 2,3-butanedione monooxime (30 mg/ml in water) solution, the tubes were placed in boiling water for 10 minutes in the dark. After cooling to room temperature in a water bath in the dark, the color was read at 490 nm in a Coleman Junior Spectrophotometer.

4. Ammonia

It has been found that the microdiffusion method for measuring ammonia (1) is the best method for use in studying the degradative pathway for allantoate. However, Nesslerization was used when a rapid assay was desirable.

a. Microdiffusion Method

The microdiffusion method was carried out as follows, using the 68 mm plastic Conway unit with liquid seal. Two ml of 45 per cent potassium carbonate, made up in 0.025 per cent NPX tergitol, were placed in the closing chamber. Three ml of 0.02 N sulfuric acid were placed in the inner chamber as absorbent. One ml of sample was then placed in the outer diffusion chamber and, immediately before closing, 2.0 ml of 45 per cent potassium carbonate in 0.025 per cent NPX tergitol were added to the outer diffusion chamber. After six hours incubation in the 37°C incubator, 1.0 ml of sulfuric acid was removed from the inner chamber and the ammonia content was determined by Nesslerization.

b. Nesslerization

A sample containing 0.1 to 1.0 µmole of ammonia was made up with distilled water to a volume of 15.0 ml. Samples from incubation mixtures often became turbid after addition of Nessler's reagent (26), interfering with measurement of color. The addition of 1.0 ml of 30 per cent sodium citrate before the addition of Nessler's reagent, prevented the formation of a precipitate. After addition of Nessler's reagent, the tubes were kept at room temperature for 10 minutes, and the color was read at 436 nm on a Coleman Junior Spectrophotometer.

5. Protein Content

The method of Sutherland, et al. (27) was used for determination of the protein content of cell extracts. A sample containing 0 to 0.1 mg protein was made up to 1.0 ml with water. To each tube, 5.0 ml of a 4 per cent sodium-potassium tartrate solution and 1.0 ml of 2 per cent $CuSO_4$ solution were added. The tubes were allowed to stand at room temperature for 40 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 nm in a Coleman Junior Spectrophotometer.

J. Chemicals

Potassium allantoate was prepared by a modification of the procedure of Young and Conway (28) developed in this laboratory (12). Reduced glutathione was obtained from Calbiochem Company and sodium barbital and glycine were obtained from Nutritional Biochemicals Corporation. Sodium glyoxylurea (ureidoglycolate) was prepared by the method described by Gaudy in 1962 (8). Glyoxylic acid was obtained from Fisher

Company and the disodium salt of succinic acid was obtained from Eastman Organic Chemicals. Ureidoglycine was synthesized in this laboratory.

K. Synthesis of Ureidoglycine

Since synthesis of ureidoglycine formed a significant portion of this study, the method of synthesis and analytical methods used in the study of the synthesized material will be described under "Results", Chapter IV.

CHAPTER IV

EXPERIMENTAL RESULTS

A. Evidence for Ammonia-forming Enzymes in Microorganisms

Three microorganisms were used in these studies. They are Pseudomonas acidovorans strain 14, Aerobacter aerogenes strain T1b and Aerobacter aerogenes strain TW. Cell-free extracts were prepared from allantoate-grown cells and checked for the presence of the ammonia-forming enzymes. Incubation mixtures were prepared (with the modifications described below) according to Vogels (18). Ammonia was determined, after incubation, by the microdiffusion method. The same incubation mixtures were also analyzed for allantoate, glyoxylate and urea.

The incubation mixture contained 16.0 µmoles/ml of potassium allantoate, 11 µmoles/ml of GSH and 0.1 µmole/ml of $MnSO_4$, all dissolved in 0.08 M barbital-HCl buffer, pH 8.5. Extracts used in the individual assays were: 0.3 ml of crude extract containing 8.6 mg protein/ml from A. aerogenes TW, 0.6 ml of dialyzed crude extract containing 5.5 mg protein/ml from A. aerogenes T1b, or 0.3 ml of crude extract containing 7.5 mg protein/ml from P. acidovorans. Total volume in all incubations was 9.0 ml. Duplicate incubations using each extract were carried out with and without acid pretreatment of the enzyme. Incubation time was 30 minutes at 30°C. Determinations were performed immediately after incubation.

Results are shown in Table I. Approximately 2 moles of ammonia were formed from each mole of allantoate used in the case of

TABLE I

PRODUCTS OF ALLANTOATE DEGRADATION BY CELL-FREE EXTRACTS

Cell-Free Extract		Allantoate Used	Glyoxylate	Urea	Ammonia	Nitrogen Recovery
		(μ moles/ml)				
P. acidovorans 14	1.	15.0	14.0	12.3	28.2	90%
	2.	9.0	8.4	6.9	17.5	88%
A. aerogenes TW	1.	16.8	18.8	12.6	34.5	90%
	2.	4.3	3.4	5.5	5.5	94%
A. aerogenes T1b	1.	9.6	9.6	6.8	21.2	90%
	2.	6.3	2.6	5.5	13.8	98%

1. Acid-Pretreatment

2. Nonacid-Pretreatment

acid-pretreated enzyme. The nitrogen recovery was calculated without the determination of oxamate since little or no oxamate is formed by aerobically-grown cells.

B. Characterization of Microorganism, Strain TW

Strain TW was isolated from a contaminated culture of P. acidovorans which had been treated with acriflavin. P. acidovorans can grow on minimal medium using gluconate as carbon source but cannot grow on glucose. Strain TW can use either glucose or gluconate as carbon source in minimal medium. Strain TW appears on an agar slant as slimy, thick, white, spreading growth and P. acidovorans as nonslimy, thin translucent spreading growth.

Strain TW is a gram-negative, catalase positive, methyl red negative, Voges-Proskauer positive microorganism. Colonies on EMB-lactose agar plates are mucoid, pale pink in color and devoid of a metallic sheen. Strain TW can use succinate, histidine, glucose, gluconate, citrate or allantoate as carbon source in minimal medium but P. acidovorans cannot use glucose or citrate as carbon source for growth.

Strain TW ferments litmus milk with production of acid (pale pink color in 48 hours). In fermentation broth, strain TW forms acid from glucose, arabinose, maltose, sucrose, raffinose, glycerol, mannitol, salicin, citrate and sorbitol. Gas is formed from glucose, lactose and sucrose. The other compounds were not checked for gas production. Nitrite is formed from nitrate. Growth occurs at 25°C and 37°C but growth rate is maximum at 30°C. According to the above data, strain TW has been identified as Aerobacter aerogenes.

C. Isolation of Mutants

1. Isolation of Mutants Which Cannot Use Allantoate as Carbon Source

a. Acriflavin and Penicillin G

A suspension of allantoate-grown cells of A. aerogenes strain TW was adjusted to an initial concentration of 10^4 to 10^5 cells/ml and treated with acriflavin as described previously. In order to allow selection of mutants unable to use allantoate either as a carbon or as a nitrogen source, the cells were collected, washed in sterile saline, and incubated for 6 hours with shaking at 30°C in minimal salts containing 0.5 per cent succinate but no source of nitrogen. The cells were then collected by centrifugation, washed with sterile saline, and resuspended at a concentration of approximately 1×10^9 cells/ml in 6 ml of 1 per cent allantoate minimal medium which contained 189 mg of penicillin G. Incubation with penicillin and preparation of plates for replication was carried out as described previously. Plates were replicated to allantoate minimal agar and to succinate-NH_4Cl minimal agar. Colonies growing on succinate-NH_4Cl but not on allantoate were picked off and tested for ability to use allantoate as carbon and/or nitrogen source.

In two experiments using this technique, no mutants unable to use allantoate as nitrogen source were found. However, ten mutants unable to use allantoate as carbon source were isolated. These were strains TW-1, TW-2, TW-3, TW-10, TW-11, TW-12, TW-13, TW-14, TW-15 and TW-16.

b. Ethylmethane Sulfonate (EMS)

Treatment of P. acidovorans and A. aerogenes Tlb was carried out as described previously. No penicillin treatment was used. Cells were grown on succinate-NH_4Cl minimal medium after treatment with EMS and

plates for replication contained the same type of medium. Colonies were replicated on allantoate minimal agar and succinate-NH_4Cl minimal agar. Those growing on succinate but not on allantoate were picked from succinate plates and tested further.

Six mutants were isolated by this procedure. They were A. aerogenes strain T1b-2, P. acidovorans 14-1, 14-2, 14-3, 14-4 and 14-5.

2. Isolation of Mutants Which Cannot Use Allantoate as Nitrogen Source and Urease-Negative Mutants

Several different attempts to isolate mutants which could not use allantoate as nitrogen source or urease-negative mutants were unsuccessful. Presumably, the failure to isolate mutants of either type was due to the large amounts of ammonia formed from allantoate or urea by these microorganisms. Ammonia diffused throughout the replica plates and would have allowed growth of any mutants which might have been present, thus preventing their identification. In an attempt to minimize this interference, the minimum concentrations of allantoate and urea required to allow normal growth were determined for A. aerogenes TW in minimal medium containing glucose as carbon source. Figure 1 shows the variation in total growth with concentration of allantoate or NH_4Cl in glucose minimal medium for this organism. The concentration of nitrogen source in the plates used for replication was decreased to 0.02 per cent but it was still impossible to identify mutants. The methods used in attempts to isolate these mutants are described below.

a. Ultraviolet Light

It was shown by Kølmark (29, 30) that ultraviolet light was an effective mutagen for the isolation of urease-negative mutants. It was thought that ultraviolet light might be effective in isolating mutants

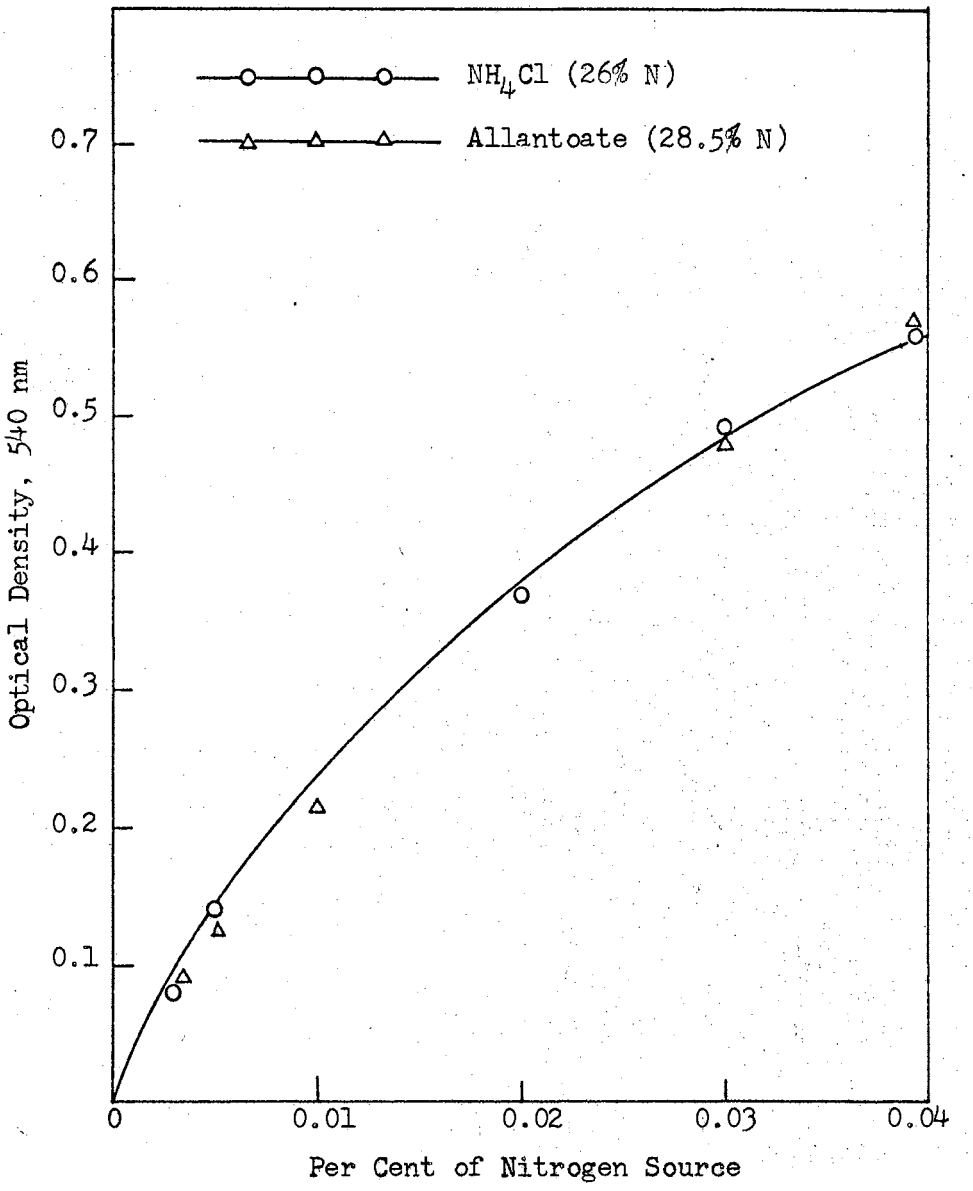


Figure 1. Concentration of Nitrogen Source and Cell Growth

Strain TW was incubated at 30°C with shaking for 18 hours. 0.02 ml of these cultures was transferred to 6 ml of fresh media and O. D. was read after 8 hours incubation.

unable to use allantoate as nitrogen source and for urease-negative mutants of the organisms used in this study. Figure 2 shows the ultraviolet survival curve determined for P. acidovorans from which the optimal UV dosage was chosen.

P. acidovorans was grown in 6 ml of nutrient broth at 30°C for 16 hours with shaking. Washed cells were resuspended in 6 ml of sterile saline at a concentration of approximately 10^8 cells/ml and were irradiated in a sterilized petri dish for 19 seconds which, according to the UV survival curve, should achieve 99.9 per cent killing. Irradiated cells were incubated in nutrient broth for 6 hours in the dark to allow for phenotypic lag. The surviving cells were then subjected to selection with D-cycloserine or penicillin G as described above. Replica plates contained allantoate or urea as nitrogen source at concentrations of 0.1 per cent to 0.02 per cent. No mutants were isolated.

b. Nitrosoguanidine and Ethylmethane Sulfonate

Cells were treated with nitrosoguanidine (NTG) or ethylmethane sulfonate (EMS) as described above. All EMS treatments were 12-hour exposures and 30 minute exposures were used for all NTG treatments. The following combinations of mutagen and selective agent were used:

(1) P. acidovorans, NTG, followed by penicillin

(2) P. acidovorans 14-1, NTG, followed by penicillin.

(3) P. acidovorans, EMS, followed by penicillin.

(4) P. acidovorans 14-1, EMS, followed by penicillin.

(5) P. acidovorans, EMS, followed by D-cycloserine.

(6) P. acidovorans 14-1, EMS, followed by D-cycloserine.

(7) P. acidovorans, EMS alone.

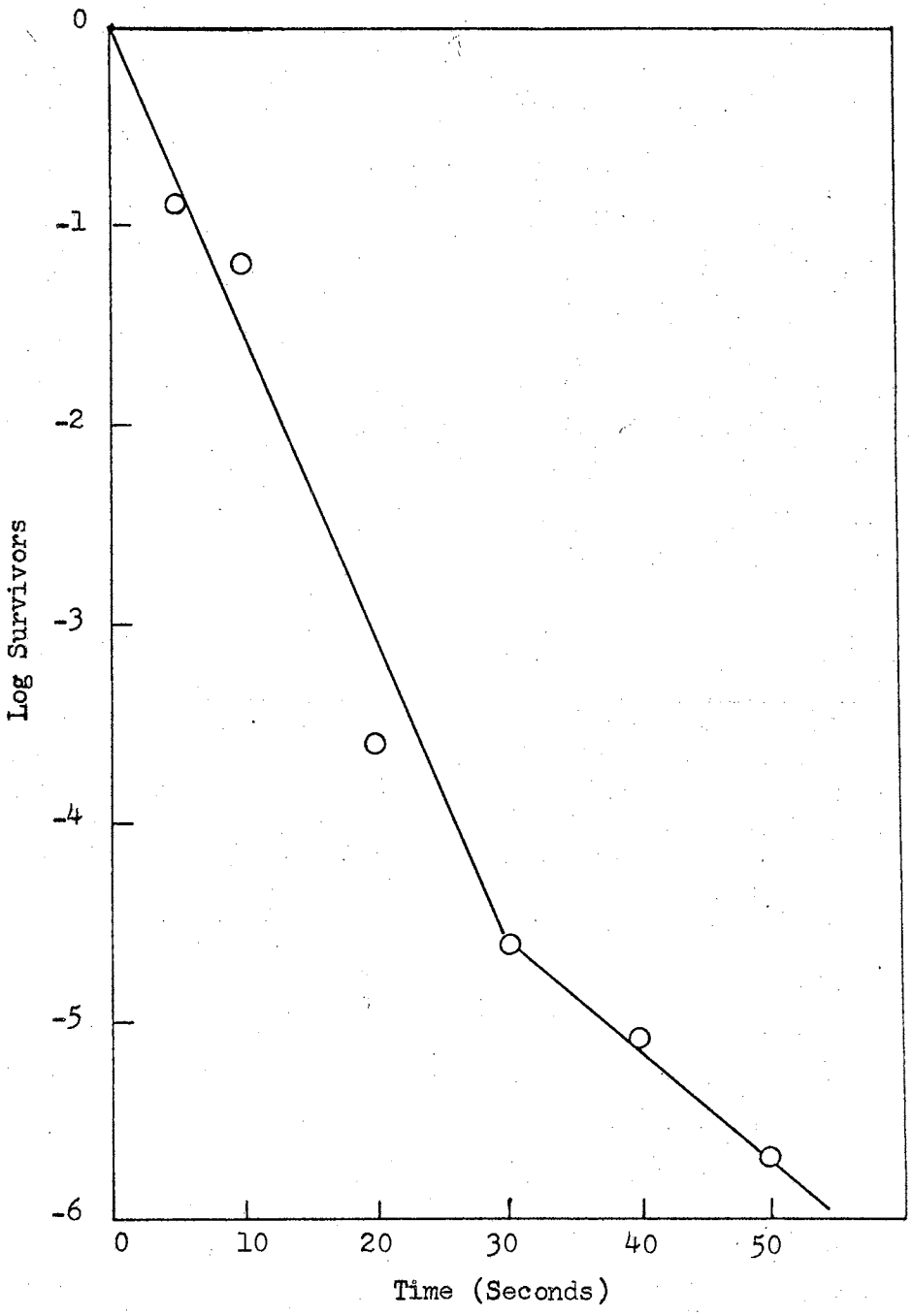


Figure 2. Ultraviolet Survival Curve for Pseudomonas acidovorans 14

6 ml cells were irradiated with UV source at 40 cm and samples were removed at times indicated. Surviving cells were determined by plate counts on nutrient agar.

(8) P. acidovorans, EMS followed by NTG.

(9) P. acidovorans 14-1, EMS followed by NTG.

In none of these experiments were mutants unable to use either allantoate or urea as nitrogen source isolated.

D. Characterization of Allantoate-Negative Mutants

1. Growth Studies

Cells from nutrient agar slants incubated overnight were suspended with 1 ml of minimal salts. One-tenth ml of this cell suspension was inoculated into 6 ml of minimal medium containing intermediates of allantoate degradation as carbon and/or nitrogen source. The initial optical density of these cultures was approximately 0.02. Cells were incubated at 30°C for 24 hours or more, with shaking except for Tlb which was grown in stationary culture. Data from these growth studies of wild types and mutants are shown in Tables II and III.

From the data shown in Tables II and III, we can conclude that strains 14-1, 14-2, 14-3, 14-4 and 14-5 derived from P. acidovorans and strains TW-10, TW-11, TW-12, TW-13, TW-14, TW-15 and TW-16, derived from A. aerogenes TW are mutants which are blocked at ureidoglycine aminohydrolase because they can use allantoate as nitrogen source and can use ureidoglycolate as sole source of carbon and nitrogen, but they are unable to use allantoate as sole carbon and nitrogen source. A. aerogenes Tlb-2 is apparently blocked at the same reaction since it can use ureidoglycolate or allantoate as nitrogen source but cannot use allantoate as carbon source. The evidence is not as clear-cut as for the other mutants since neither the wild type nor the mutant can use ureidoglycolate as carbon source. One also can conclude from the data

TABLE II

GROWTH OF WILD TYPES AND MUTANTS WITH INTERMEDIATES OF THE ALLANTOATE PATHWAY AS CARBON AND/OR NITROGEN SOURCE

Additions to Minimal Salts	Organism	Strains	O. D.
1% allantoate	P. acidovorans	14	0.30
	P. acidovorans	14-1, 2, 3, 4, 5	0.03
	A. aerogenes	TW	0.47
	A. aerogenes	TW-1, 2, 3	0.03
	A. aerogenes	TW-10, 11, 12, 13, 14, 15, 16	0.03
	A. aerogenes	T1b	0.18*
	A. aerogenes	T1b-2	0.02*
0.5% succinate + 0.1% allantoate	P. acidovorans	14	0.58
	P. acidovorans	14-1, 2, 3, 4, 5	0.60
0.5% gluconate + 0.1% allantoate	A. aerogenes	TW	0.87
	A. aerogenes	TW-1, 2, 3	0.86
	A. aerogenes	TW-10, 11, 12, 13, 14, 15, 16	0.80
	A. aerogenes	T1b	0.38
	A. aerogenes	T1b-2	0.39
0.5% ureidoglycolate	P. acidovorans	14	0.23*
	P. acidovorans	14-1, 2, 3, 4, 5	0.24*
	A. aerogenes	TW	0.30*
	A. aerogenes	TW-1, 2, 3	0.03*
	A. aerogenes	TW-10, 11, 12, 13, 14, 15, 16	0.15*
	A. aerogenes	T1b	0.03*
	A. aerogenes	T1b-2	0.03*
0.5% succinate + 0.1% ureidoglycolate	P. acidovorans	14	0.50
	P. acidovorans	14-1, 2, 3, 4, 5	0.47

TABLE II (Continued)

Additions to Minimal Salts	Organism	Strains	O. D.
0.5% gluconate + 0.1% ureidoglycolate	A. aerogenes	TW	0.88
	A. aerogenes	TW-1, 2, 3	0.85
	A. aerogenes	TW-10, 11, 12, 13, 14, 15, 16	0.88
	A. aerogenes	T1b	0.37*
	A. aerogenes	T1b-2	0.24*
0.5% glyoxylate + 0.1% NH_4Cl	A. aerogenes	TW	0.32*
	A. aerogenes	TW-1, 2, 3	0.04*
	A. aerogenes	TW-10, 11, 12, 13, 14, 15, 16	0.28*
0.5% gluconate + 0.1% urea	P. acidovorans	14	0.67
	P. acidovorans	14-1, 2, 3, 4, 5	0.65
	A. aerogenes	TW	0.80
	A. aerogenes	TW-1, 2, 3	0.78
	A. aerogenes	TW-10, 11, 12, 13, 14, 15, 16	0.82
	A. aerogenes	T1b	0.60
	A. aerogenes	T1b-2	0.56

*O. D. given is for 48 hr. growth. All others were read at 24 hr.

TABLE III

UTILIZATION OF INTERMEDIATES OF THE ALLANTOATE PATHWAY AS CARBON AND/OR NITROGEN SOURCE BY WILD TYPES AND MUTANTS

Compounds	Source of	Wild Types			Mutants			
		14	Tlb	TW	14-1-5	TW-1-3	TW-10-16	Tlb-2
Allantoate	C and N	+	+	+	-	-	-	-
	N	+	+	+	+	+	+	+
Ureidoglycolate	C and N	+	-	+	+	-	+	-
	N	+	+	+	+	+	+	+
Glyoxylate	C			+		-	+	
Urea	N	+	+	+	+	+	+	+

that the ammonia-forming enzymes are required in these organisms for complete degradation of allantoate and that only one of the two proposed pathways occurs in a single organism because mutants which are allantoate-negative can be isolated. Strains 14-1, 14-2, 14-3 of P. acidovorans are mutants which are blocked at a step subsequent to glyoxylate formation.

2. Formation of Ammonia by Nonproliferating Cells and Extracts

A. aerogenes TW and TW-16 were grown overnight in minimal medium containing 0.5 per cent succinate plus 0.1 per cent allantoate at 30°C with shaking. Cells were washed and resuspended in the same volume (6 ml) of minimal salts containing 0.1 per cent allantoate. The concentration of cells in the suspension was approximately 2×10^9 cells/ml. Cells were incubated at 30°C with shaking and 0.1 ml samples of the suspensions were removed at frequent intervals. Ammonia content was determined by Nesslerization. After 10 hours incubation, the cell concentration had not changed. Figure 3 shows the time course of ammonia formation by the wild type and the mutant, and indicates that the mutant has a greatly reduced ability to produce ammonia as compared to the wild type. If no ammonia is used by either type of cells and if allantoate amidohydrolase activity in the mutant is normal, one would expect the mutant to produce 25 per cent of the amount of ammonia produced by the wild type. The average for 12 sampling points was 21 per cent.

Extracts were prepared from one mutant of each organism (A. aerogenes TW-16, P. acidovorans 14-1, and A. aerogenes T1b-2) using cells grown on allantoate as nitrogen source. These extracts were assayed for allantoate amidohydrolase activity. In all cases, the formation of

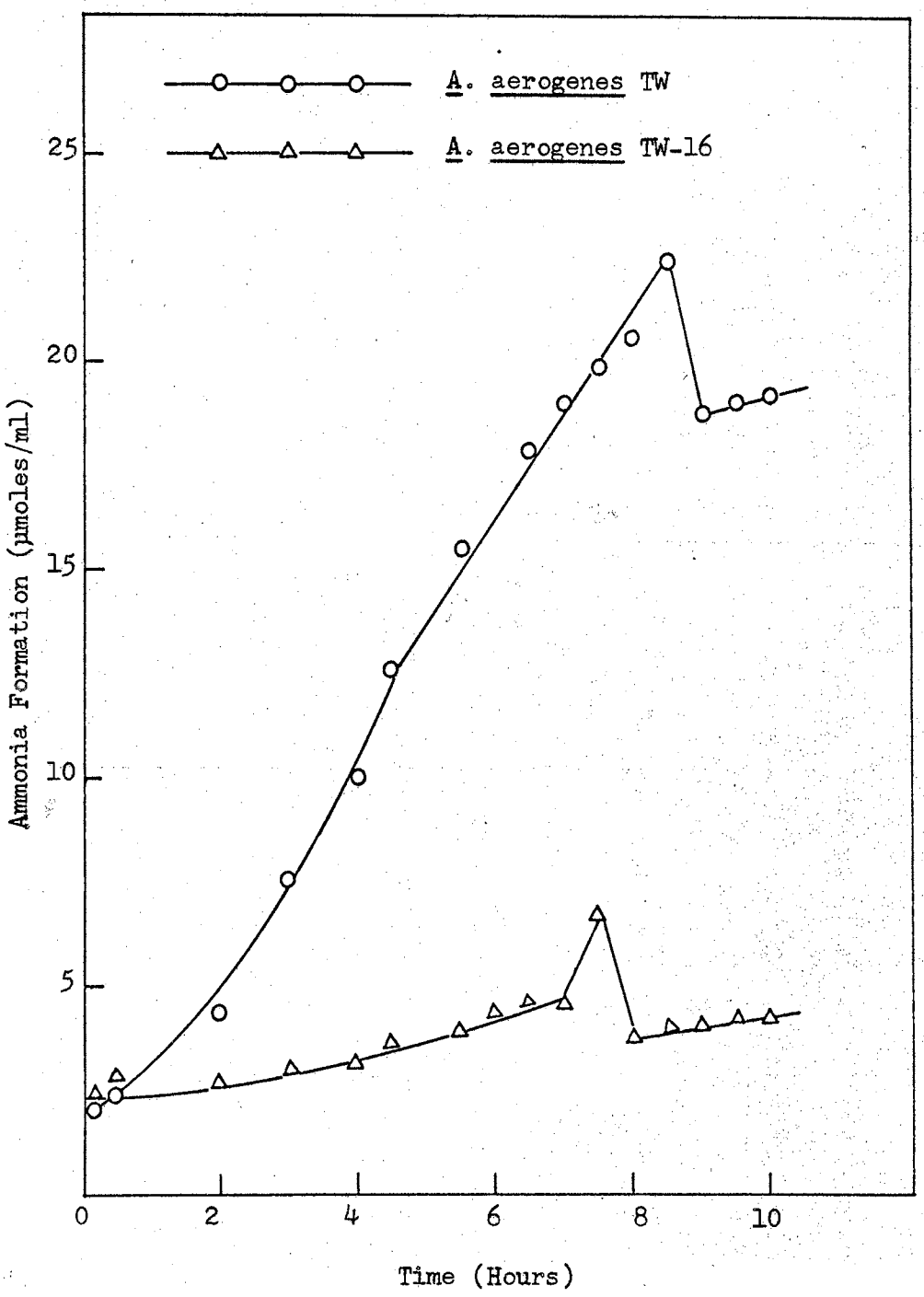


Figure 3. Formation of Ammonia from Allantoate by Non-Proliferating Cells

2×10^9 cells/ml were suspended in 0.1% allantoate minimal medium and 0.1 ml samples were taken for ammonia determination at times indicated.

ammonia was extremely slow and more ammonia was formed without acid pretreatment than with pretreatment.

E. Ureidoglycine

Ureidoglycine (NH_2-C(=O)-NH-CH(NH_2)-COOH) has been proposed as an intermediate in the degradation of allantoate by Vogels (17). This compound has never been synthesized or isolated and therefore has not actually been tested as an enzyme substrate. Vogels (17) attempted to isolate ureidoglycine from media in which enzymatic degradation of allantoate had been allowed, and from solutions in which allantoin was hydrolyzed under weakly alkaline conditions. These attempts failed, according to Vogels, because of the rapid hydrolysis of ureidoglycine to form ammonia, glyoxylate and urea.

1. Synthesis of Ureidoglycine

a. Oxaluric Acid

The procedure used for the preparation of oxaluric acid (NH_2-C(=O)-NH-C(=O)-COOH) was described by Biltz and Schrauder (31). Ten gm of alloxan·H_2O were dissolved in 20 ml of 30 per cent hydrogen peroxide. The mixture was heated in a boiling water bath for 10 minutes. The precipitate which formed was collected by filtration on a Buchner funnel and was washed on the filter with approximately 250 ml cold ethanol and 250 ml cold water in 50 ml portions. The compound was air-dried. The weight of the product obtained was approximately 4.6 to 5.0 gm in different preparations. This compound decomposed at 210°C. (Melting

points were determined using the Thomas-Hoover "Uni-Melt" capillary melting point apparatus, A. H. Thomas Co., Philadelphia.)

b. Oxaluric Oxime

A general procedure for the synthesis of oximes has been described by Shriner, Fuson and Curtin (32). This procedure was modified for synthesis of oxaluric oxime (NH_2-C(=O)-NH-C(=NOH)-COOH) as follows:

Ten gm of hydroxylamine hydrochloride were dissolved in 60 ml of distilled water; 40 ml of 10 per cent sodium hydroxide solution were then added. The mixture (pH 7.0) was warmed in a steam bath. When the temperature reached 80°C, 2.0 gm of oxaluric acid were added slowly with stirring. This solution was kept in the steam bath for another 10 minutes and was then cooled in an ice bath. In order to hasten crystallization, the sides of the beaker were scratched with a glass rod. Occasionally the addition of a few ml of cold distilled water and/or cold ethanol will assist in causing the oxime to separate. The precipitate was collected by filtration on a Buchner funnel and was washed with 200 ml cold ethanol in 40 ml portions and air-dried. The weight of material obtained (Fraction A) was approximately 1.0 gm. This compound decomposed at 268-272°C.

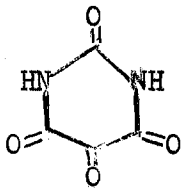
The mother liquor was stored at 4°C overnight. The precipitate was collected and washed as above. The weight of this material (Fraction B) was approximately 0.96 gm. This compound also decomposed at 268-272°C. Fraction A and Fraction B were combined and dissolved in 100 ml of hot water. Then 400 ml of 95 per cent ethanol was added to the solution and the mixture was stored at 4°C overnight for recrystallization. The crystals were collected and washed as described above and air-dried. This compound decomposed at 272°C.

c. Hydrogenation of Oxaluric Oxime

Catalytic hydrogenation of oximes may give primary, secondary or tertiary amines (33). The products from hydrogenation of oximes depend to an unusual degree on the solvent employed. Hydrogenation of oximes in neutral media usually leads to mixtures of primary, secondary and tertiary amines. Hartung (34) was the first to realize that formation of secondary amines could be prevented by carrying out the hydrogenation in acidic media. The procedure for hydrogenation of oxaluric oxime was carried out as follows:

One gm of recrystallized oxaluric oxime was dissolved in 500 ml of hot glacial acetic acid and 0.5 gm of 5 per cent Pd/C (palladium on carbon) was added to the solution. Mechanical stirring or shaking was provided and the temperature of the reaction mixture was maintained at 20 to 25°C. Hydrogen was supplied at a pressure of 20 psi. After 24 hours, 12 pounds of hydrogen had been absorbed by the reaction mixture. The catalyst was removed by filtration and washed with additional glacial acetic acid. The clear filtrate was evaporated to dryness *in vacuo* and the residue was collected (Fraction C). Fraction C (approximately 0.50 to 0.60 gm) was dissolved in 50 ml of minimal salts and 200 ml of 95 per cent ethanol were added. The mixture was stored at 4°C overnight. The precipitate was collected by filtration on a Buchner funnel and was washed several times with ethanol. The weight of Fraction D was approximately 0.2 to 0.25 gm. It decomposed at 255-258°C. The filtrate was evaporated to dryness *in vacuo* and the residue was designated as Fraction E. The weight of Fraction E was approximately 0.3 to 0.35 gm and it decomposed at 220-223°C.

The overall reaction for the synthesis of ureidoglycine can be represented as follows:

(1)  + H_2O_2 ⟶ $H_2N-\overset{O}{\overset{\|}{C}}-NH-\overset{O}{\overset{\|}{C}}-COOH$ + CO_2

alloxan — oxaluric acid

(2) $H_2N-\overset{O}{\overset{\|}{C}}-NH-\overset{O}{\overset{\|}{C}}-COOH$ + NH_2OH ⟶ $H_2N-\overset{O}{\overset{\|}{C}}-NH-\overset{NOH}{\overset{\|}{C}}-COOH$ + H_2O

oxaluric acid — oxaluric oxime

(3) $H_2N-\overset{O}{\overset{\|}{C}}-NH-\overset{NOH}{\overset{\|}{C}}-COOH$ + 2 H_2 $\xrightarrow{H^+}$ $H_2N-\overset{O}{\overset{\|}{C}}-NH-\overset{NH_2}{\overset{|}{CH}}-COOH$ + H_2O

oxaluric oxime — ureidoglycine

2. Characterization of Ureidoglycine

a. Tests for Utilization as Carbon or Nitrogen Source

Solutions were prepared by dissolving 0.2 gm of Fraction D or 0.2 gm of Fraction E in 100 ml of minimal salts. The solution containing Fraction D was designated as Solution A and that containing Fraction E was designated as Solution B. For testing Fraction E as carbon and nitrogen source, 6 ml of the solution were used without additions. For testing either solution for use as nitrogen source, 0.3 ml of a 10 per cent solution of the appropriate carbon source was added to 5.7 ml of the solution. NH_4Cl was included in the medium in controls with other carbon sources. Allantoate controls and uninoculated samples were also included. Cells used in this experiment were *P. acidovorans*, wild type and strain 14-1, and *A. aerogenes* TW, wild type and strain TW-16.

A volume of 6 ml of liquid medium was used in an 18 mm I. D. test tube and aeration was at 30°C. The incubation time was 24 hours unless otherwise indicated. Results are shown in Tables IV and V.

Data in Tables IV and V indicate that Fraction E is ureidoglycine. Since A. aerogenes TW-16 and P. acidovorans 14-1 are mutants which are blocked at ureidoglycine aminohydrolase, these mutants should not use ureidoglycine as carbon or nitrogen source. Both A. aerogenes TW and P. acidovorans can use Fraction E as carbon and nitrogen source but the mutants TW-16 and 14-1 cannot; therefore, Fraction E should be ureidoglycine.

b. Enzymatic Formation of Ammonia from Ureidoglycine

Figure 4 and Figure 5 show the formation of ammonia from Fraction E by a cell-free extract prepared from A. aerogenes TW. Enzymes were used with and without acid pretreatment. Ammonia was determined by Nesslerization.

In the experiment shown in Figure 4, the incubation mixture contained: 0.2 gm of Fraction E, 99 µmoles GSH, 0.9 µmole $MnSO_4$, 0.077 M barbital-HCl buffer (pH 8.5) and 0.2 ml of cell extract (7.3 mg protein/ml) in a total volume of 7.5 ml.

In the experiment shown in Figure 5, the incubation mixture contained: 0.2 gm of Fraction E, 99 µmoles GSH, 0.9 µmole $MnSO_4$, 0.077 M potassium phosphate buffer, pH 7.0, and 0.2 ml of cell extract (7.3 mg protein/ml) in a total volume of 7.5 ml.

In both cases, control tubes, which were identical to the experimental tubes except that no extract was added, were prepared and treated in the same way as the experimental tubes. No ammonia was formed in the control tubes.

TABLE IV

GROWTH OF WILD TYPES AND MUTANTS ON SOLUTION A
AS CARBON AND/OR NITROGEN SOURCE

Medium	Strain	O. D.
0.5% glucose + 0.1% NH_4Cl minimal medium	A. aerogenes TW	0.66
0.5% glucose + Solution A	"	0.82
1% allantoate minimal medium	"	0.32
0.5% glucose + 0.1% NH_4Cl minimal medium	A. aerogenes TW-16	0.70
0.5% glucose + Solution A	"	0.82
1% allantoate minimal medium	"	0.02
0.5% glucose + 0.1% allantoate minimal medium	"	0.90
0.5% succinate + 0.1% NH_4Cl minimal medium	P. acidovorans 14	0.60
0.5% succinate + Solution A	"	0.05
1% allantoate minimal medium	"	0.35
0.5% succinate + 0.1% NH_4Cl minimal medium	P. acidovorans 14-1	0.60
0.5% succinate + Solution A	"	0.07
1% allantoate minimal medium	"	0.02
0.5% succinate + 0.1% allantoate minimal medium	"	0.60
0.5% succinate + Solution A	No cells	0.00

TABLE V

GROWTH OF WILD TYPES AND MUTANTS ON SOLUTION B (UREIDOGLYCINE) AS CARBON AND/OR NITROGEN SOURCE

Medium	Strain	O. D.
1% allantoate minimal medium	A. aerogenes TW	0.32
Solution B	"	0.12*
0.5% glucose + Solution B	"	0.74
0.5% glycine minimal medium	"	0.01
0.5% glucose + 0.1% glycine minimal medium	"	0.60
1% allantoate minimal medium	A. aerogenes TW-16	0.02
0.5% glucose + Solution B	"	0.03
0.5% glycine minimal medium	"	0.02
0.5% glucose + 0.1% glycine minimal medium	"	0.49
1% allantoate minimal medium	P. acidovorans 14	0.32
Solution B	"	0.10*
0.5% succinate + Solution B	"	0.46
0.5% glycine minimal medium	"	0.01
0.5% succinate + 0.1% glycine minimal medium	"	0.70
1% allantoate minimal medium	P. acidovorans 14-1	0.02
0.5% succinate + Solution B	"	0.02
0.5% glycine minimal medium	"	0.01
0.5% succinate + 0.1% glycine minimal medium	"	0.63
Solution B	No cells	0.00

*48 hours incubation.

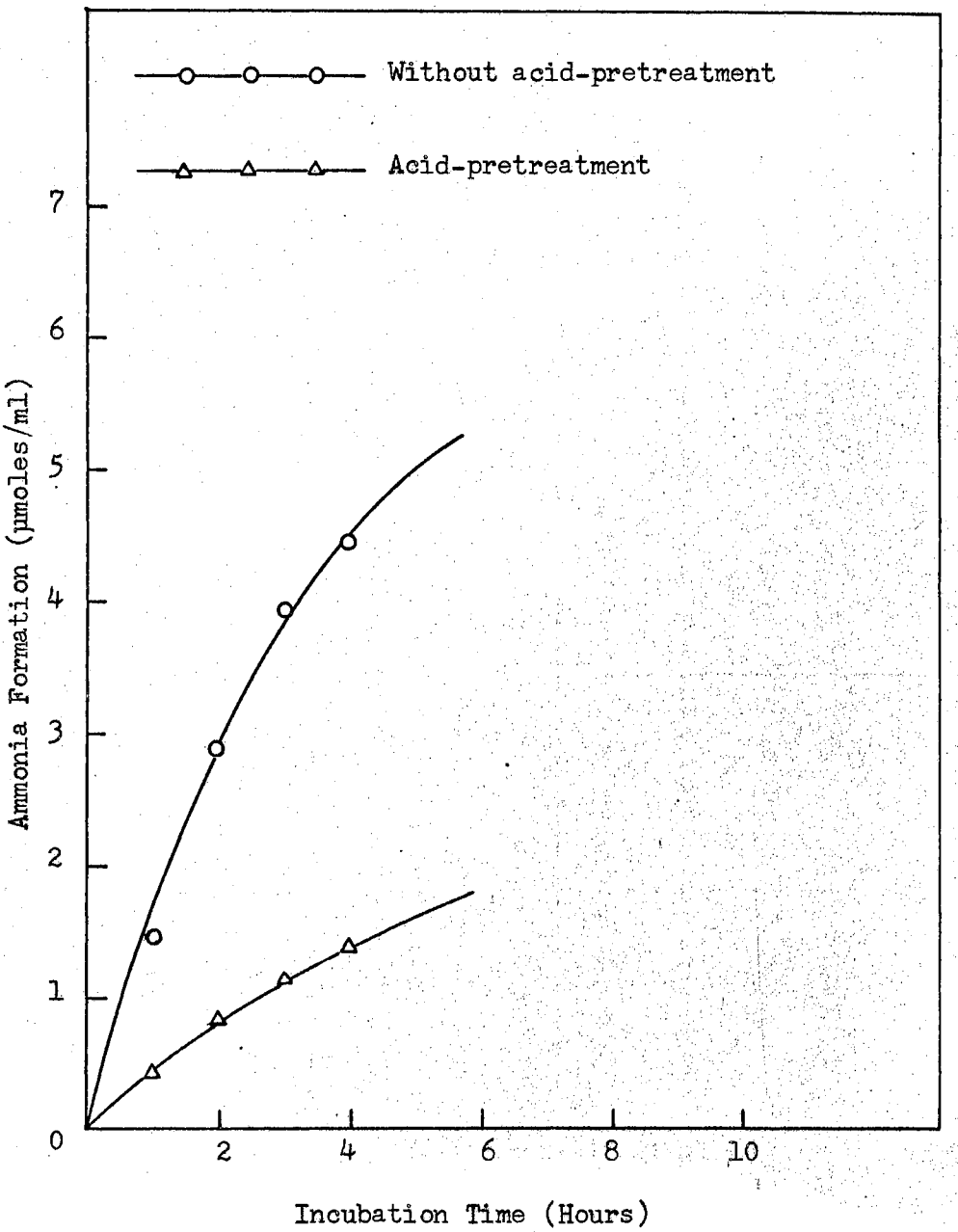


Figure 4. Formation of Ammonia from Ureidoglycine by Cell-Free Extract of Aerobacter aerogenes TW at pH 8.5.

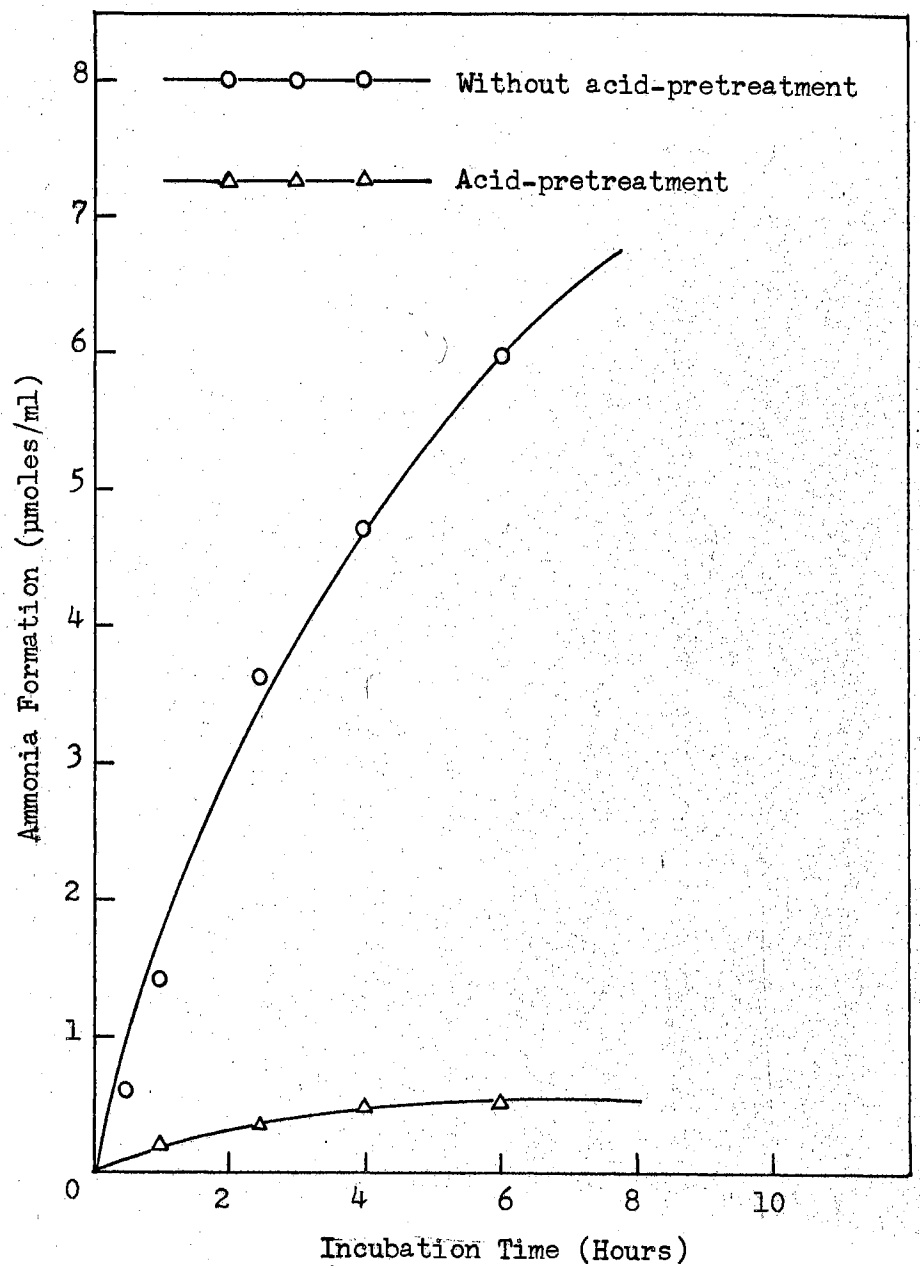


Figure 5. Formation of Ammonia from Ureidoglycine by Cell-Free Extract of Aerobacter aerogenes TW at pH 7.0.

c. Paper Chromatography and Color Reactions

(1) Tests with Ninhydrin

Amino acids, amines, and related compounds react with ninhydrin to form various colors. All α-amino acids react with ninhydrin reagent, 0.2 per cent in acetone (W/V), in the cold, usually within 3 hours and certainly overnight, giving a purple color in most cases although significant variations are obtained. If the chromatogram is heated for 2-3 minutes or more in an oven at 105°C, all compounds containing a primary amino group attached to an aliphatic carbon atom react to give a purple color in main but with significant variations. If a compound yields a color on heating but not on standing overnight in the cold, it is almost certainly not an α-amino acid (35).

Equal amounts of glycine, oxaluric oxime, 2,3-butanedione monooxime, Fraction D, and Fraction E were spotted on two strips of Whatman No. 1 filter paper. The papers were air-dried, then dipped through 0.2 per cent ninhydrin in acetone (W/V). Two per cent pyridine was added to the ninhydrin reagent immediately before use. One paper was left overnight at room temperature. After the acetone had evaporated, the second paper was heated for 2-3 minutes or more in an oven at 105°C.

After heating, spots containing glycine, oxaluric oxime, and Fraction D were purple, that for Fraction E was brownish-purple, and the 2,3-butanedione monooxime spot was reddish-purple. After standing overnight at room temperature, glycine reacted to yield a purple color and both Fraction D and Fraction E yielded a slightly purple color. No color reaction occurred with either oxime under these conditions. These results indicate that Fraction E and Fraction D contain α-amino acids.

(2) Tests with Ehrlich Reagent

Indoles yield a purple color with Ehrlich reagent (one volume of 10 per cent p-dimethylaminobenzaldehyde in concentrated HCl and 4 volumes of acetone), hydroxyindoles form a blue color and aromatic amines and ureido compounds appear yellow (35).

Equal amounts of oxaluric oxime, citrulline, Fraction D and Fraction E were spotted on Whatman No. 1 filter paper. After being air-dried, the paper was sprayed with Ehrlich reagent and colors began to develop at room temperature after 30 minutes. All compounds which were tested yielded yellow colors, indicating a ureido group.

(3) Descending Paper Chromatography

Two different solvent systems were used for chromatography. These were butanol:acetic acid:water (2:1:1) and butanol:pyridine:water (6:4:3). Results are shown in Figure 6.

Equal amounts of glycine, oxaluric acid, Fraction D and Fraction E were spotted on a Whatman No. 1 filter paper sheet. After the spots had dried, the papers were placed in the chromatographic chamber, previously equilibrated with solvent, and were developed 16 hours for the butanol : acetic acid : water system and 24 hours for the butanol : pyridine : water solvent. After being air-dried, the papers were sprayed with 0.5 per cent ninhydrin in acetone (with a few drops of pyridine) and heated in an oven at 105°C for several minutes. Glycine appeared as a purple spot, oxaluric oxime and Fraction D were slightly purple and Fraction E was brownish purple in color.

d. Infrared Spectra of Oxaluric Oxime and Ureidoglycine

The infrared spectra of oxaluric oxime and ureidoglycine are shown in Figures 7 and 8. About 1 mg of sample was mixed and ground well with

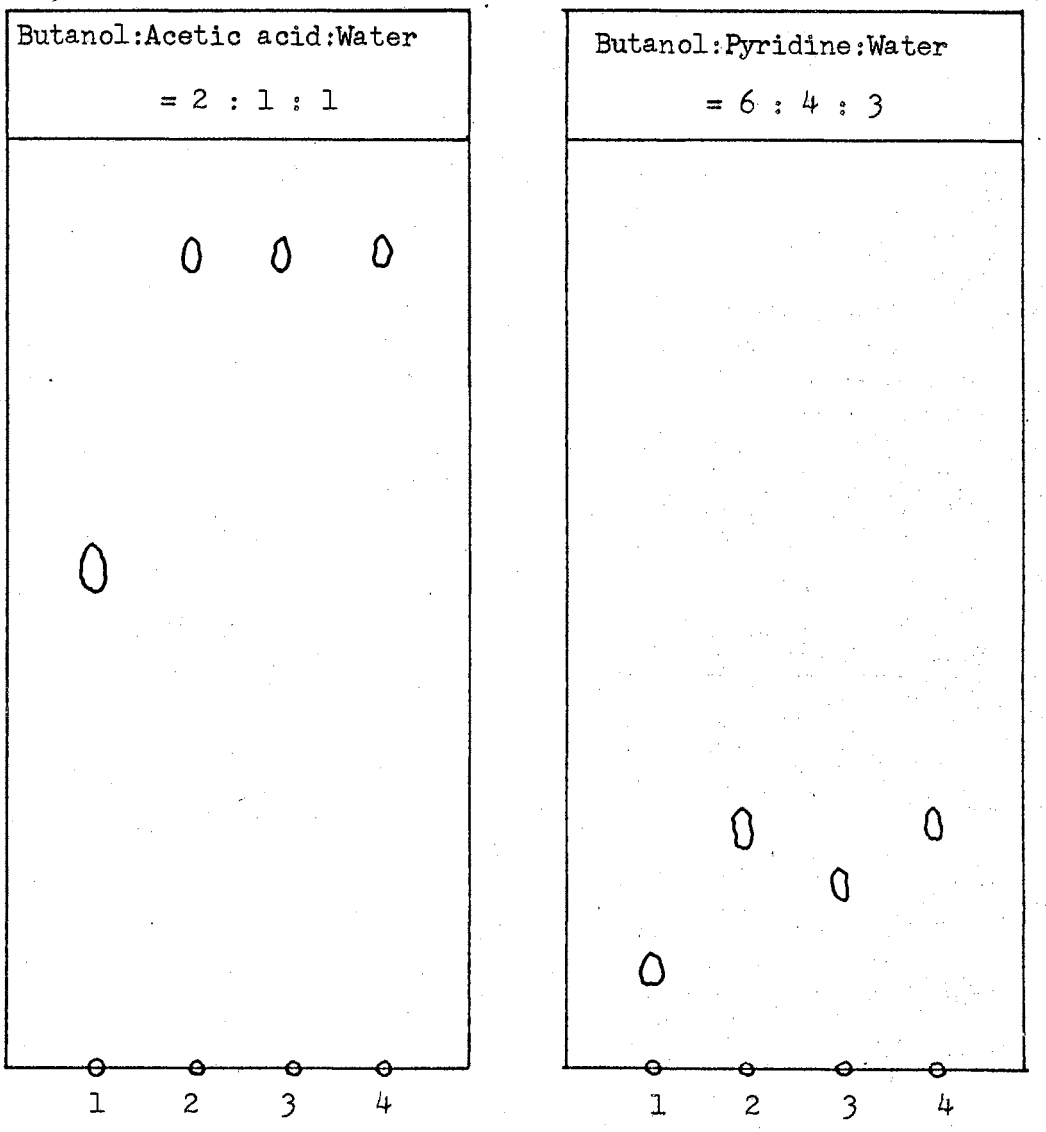


Figure 6. Paper Chromatography of Glycine, Oxaluric Oxime, Fraction D and Fraction E.

1. Glycine; 2. Oxaluric Oxime;
3. Fraction E (Ureidoglycine); 4. Fraction D.

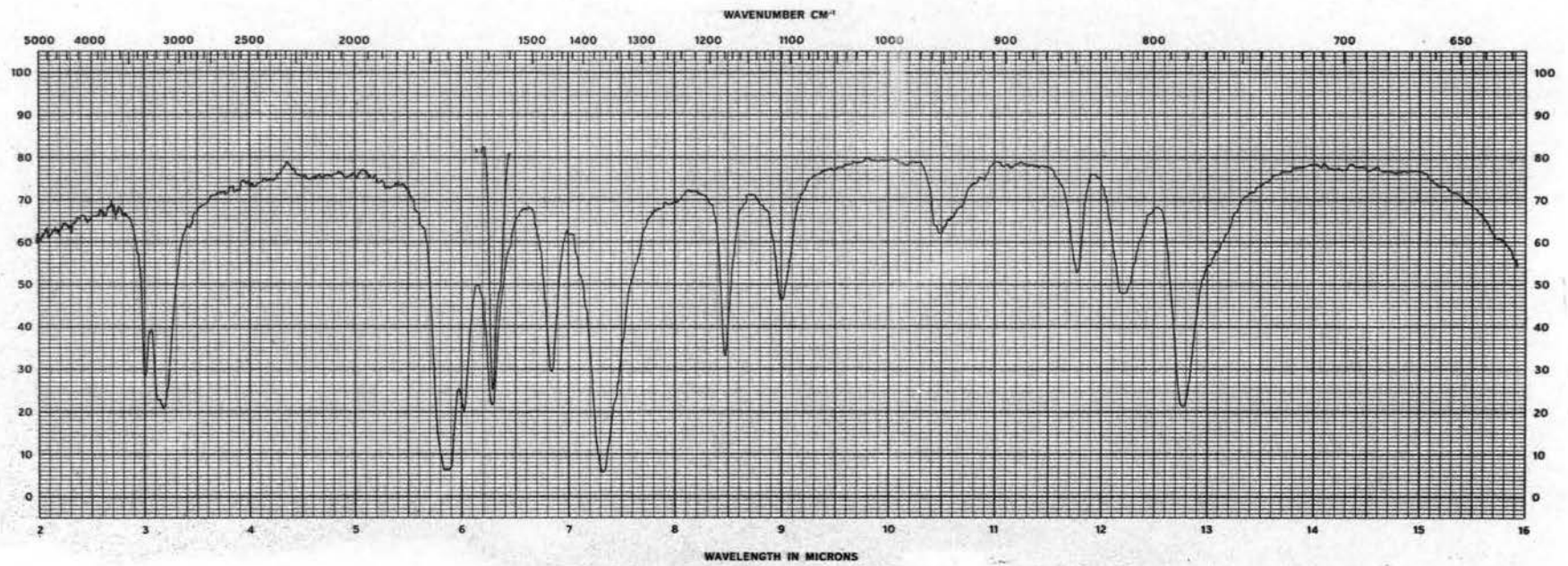


Figure 7. Infrared Spectrum of Oxaluric Oxime

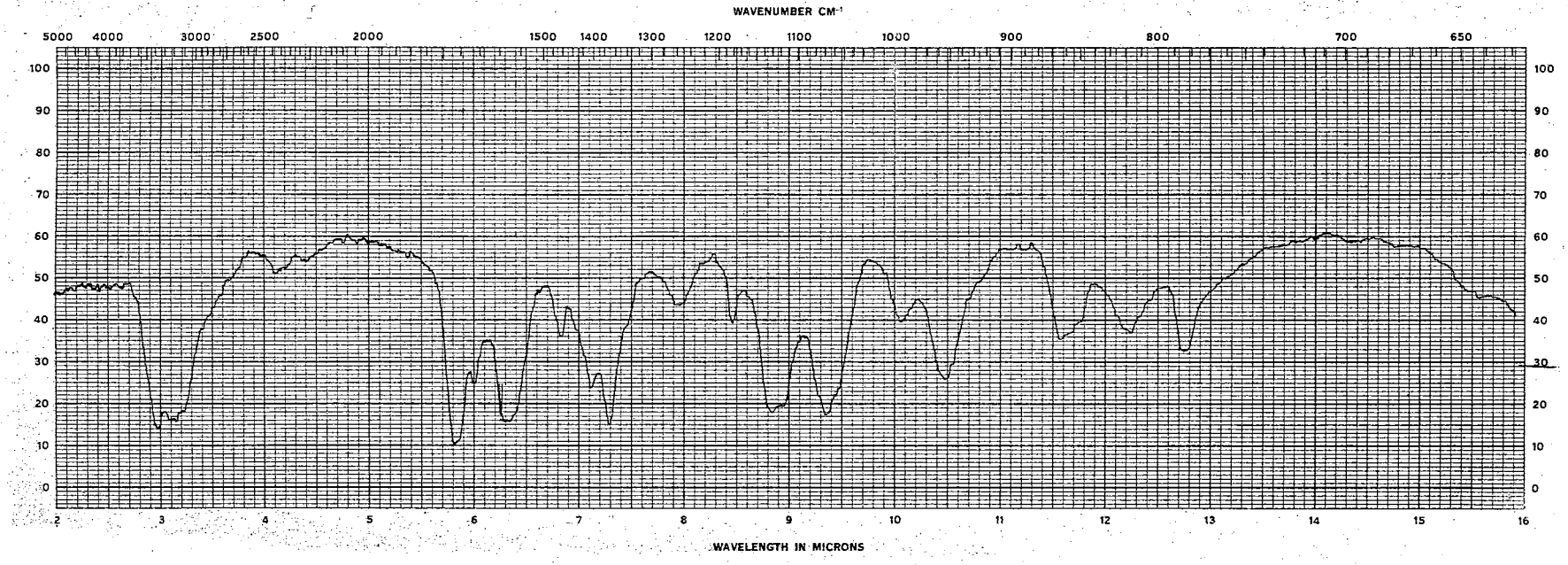


Figure 8. Infrared Spectrum of Ureidoglycine

100 mg of dried potassium bromide to form a pellet. Infrared spectroscopy was carried out using a Beckman IR-5A recording spectrophotometer, calibrated with polystyrene film at 6.2307 μ.

According to Silverstein and Bassler (36), free primary amino acids are characterized by the following absorptions: a medium N-H stretching band at 3130-3030 cm^{-1} (3.20-3.30 μ); a weak N-H stretching band at 1660 to 1610 cm^{-1} (6.03-6.22 μ) and a stronger N-H bending band at 1550-1485 cm^{-1} (6.46-6.74 μ); these bands are absent in N-substituted amino acids; the strongly ionized carboxyl absorbs at 1600-1560 cm^{-1} (6.25-6.40 μ) and more weakly at about 1400 cm^{-1} (7.15 μ). Primary amines are characterized by the following absorption: two absorption bands in the 3500-3300 cm^{-1} region (2.86-3.03 μ); the N-H vibrations in the region of 1650 to 1580 cm^{-1} (6.06-6.33 μ); broad N-H bending absorption at 900-650 cm^{-1} (11.1 to 15.4 μ) due to bending vibrations; and weak absorption bands for the unconjugated C-N linkage at 1220-1020 cm^{-1} (8.20-9.80 μ).

According to Colthup, Daly, and Wiberley (37), oximes absorb broadly at 3300-3150 cm^{-1} due to bonded OH stretch, at 1690-1620 cm^{-1} due to C=N stretch, and near 930 cm^{-1} due to N-O stretching.

e. Gas-Liquid Chromatography of Oxaluric Oxime and Ureidoglycine

A Hewlett-Packard series 5750 B gas chromatograph, equipped with a hydrogen flame detector and F & M Scientific Corporation Model 50 Automatic Attenuator was used for gas-liquid chromatography. A 1/8 in. x 6 ft. column packed with 10 per cent silicone gum rubber (F & M Part No. UC-W98) was used in this work.

Trimethylsilyl derivatives were prepared by treating 10 mg samples with 1 ml of anhydrous pyridine, 0.2 ml of hexamethyldisilazane, and 0.1 ml of trimethylchlorosilane. The reaction was carried out in a

plastic-stoppered vial and the mixture was shaken vigorously for a few minutes and was then allowed to stand at room temperature. The solutions became cloudy on addition of trimethylchlorosilane and no attempt was made to remove this precipitate, which in no way interfered with the subsequent gas chromatography. From 1.0 to 5.0 µl of the resulting mixture was used for injection into the column. The trimethylsilyl (TMS) derivatives of oxaluric oxime and ureidoglycine were chromatographed under identical conditions. The retention time of the TMS derivatives of oxaluric oxime were 20.2 minutes and 32.4 minutes (Figure 9). The retention time of the TMS derivative of ureidoglycine was 22.0 minutes and only one peak was obtained (Figure 10). Derivatization of ureidoglycine was complete in 1 to 2 hours at room temperature and no additional peaks were formed on standing. Figure 11 shows the chromatographic pattern obtained from a mixture of oxaluric oxime and ureidoglycine.

f. Ultraviolet Spectra

Ultraviolet spectra for oxaluric acid, oxaluric oxime and ureidoglycine were obtained on a Cary 14 recording spectrophotometer. For all compounds, standard 3 ml silica cells with 1 cm light paths were employed. Proper amounts of sample were dissolved in distilled water. Oxaluric acid absorbed maximally at 202 nm, oxaluric oxime at 199 nm and ureidoglycine at 193 nm. Figure 12 shows the absorption peaks for ureidoglycine and oxaluric oxime.

F. Accumulation of Ureidoglycine by Mutant Lacking Ureidoglycine Aminohydrolase

P. acidovorans, strain 14-1, is a mutant which, according to growth data, is blocked at ureidoglycine aminohydrolase and therefore would be

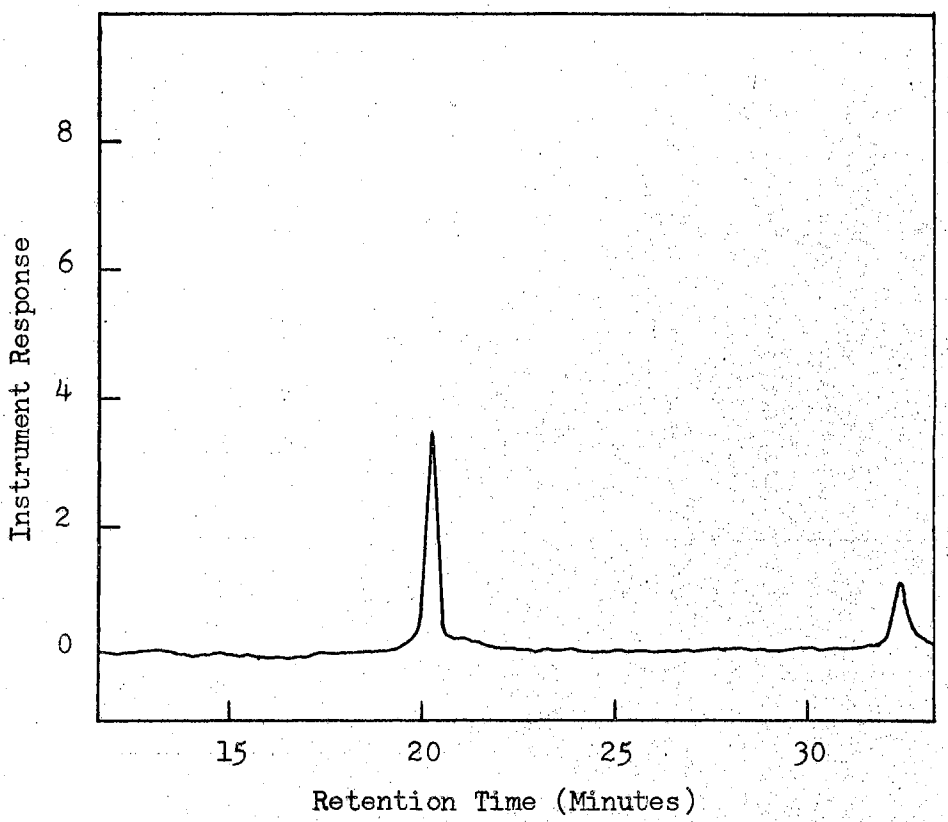


Figure 9. Gas Chromatography of Oxaluric Oxime

Conditions: 8 X attenuation, range 10^2, injector temperature 260°C, detector temperature 290°C, flow rate (Helium) 30 ml/min., chart speed 1"/3min. Sample size, 1-5 µl. Temperature program: 100°C isothermal for 10 min., 6°C/min to 200°C, hold at upper limit for 10 min.

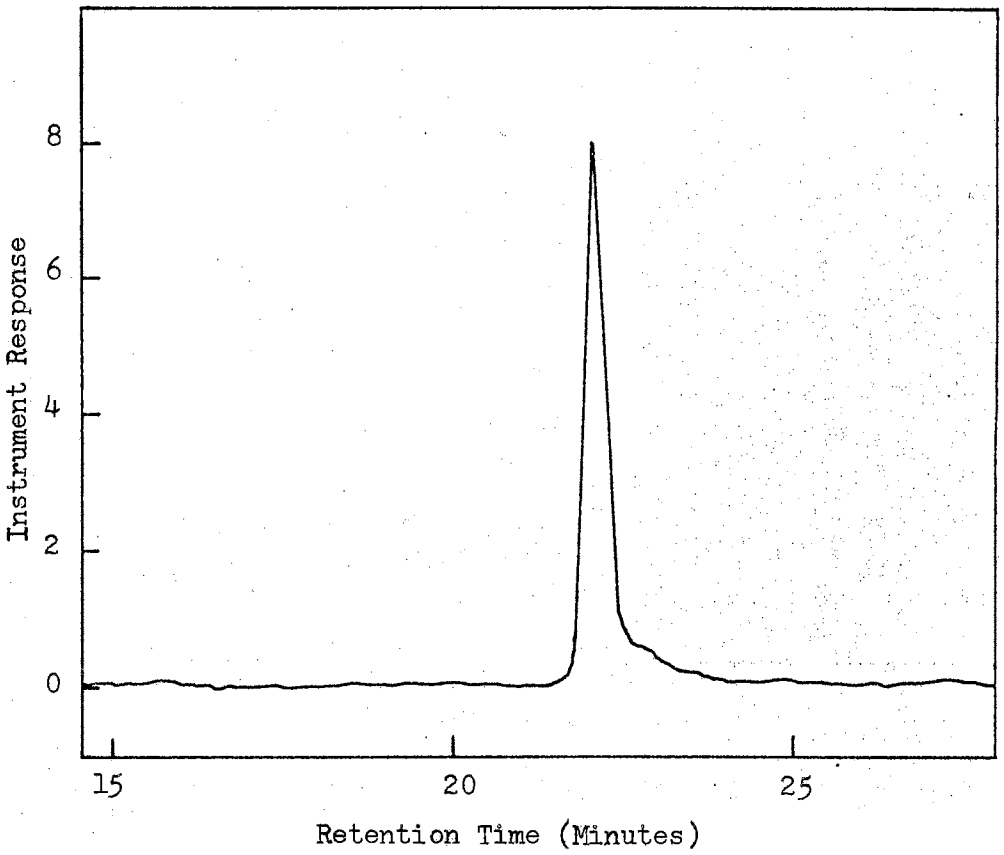


Figure 10. Gas Chromatography of Ureidoglycine

Conditions: 8 X attenuation, range 10^2, injector temperature 260°C, detector temperature 290°C, flow rate (Helium) 30 ml/min., chart speed 1"/3min. Sample size, 1-5 µl. Temperature program: 100°C isothermal for 10 min., 6°C/min. to 200°C, hold at upper limit for 10 min.

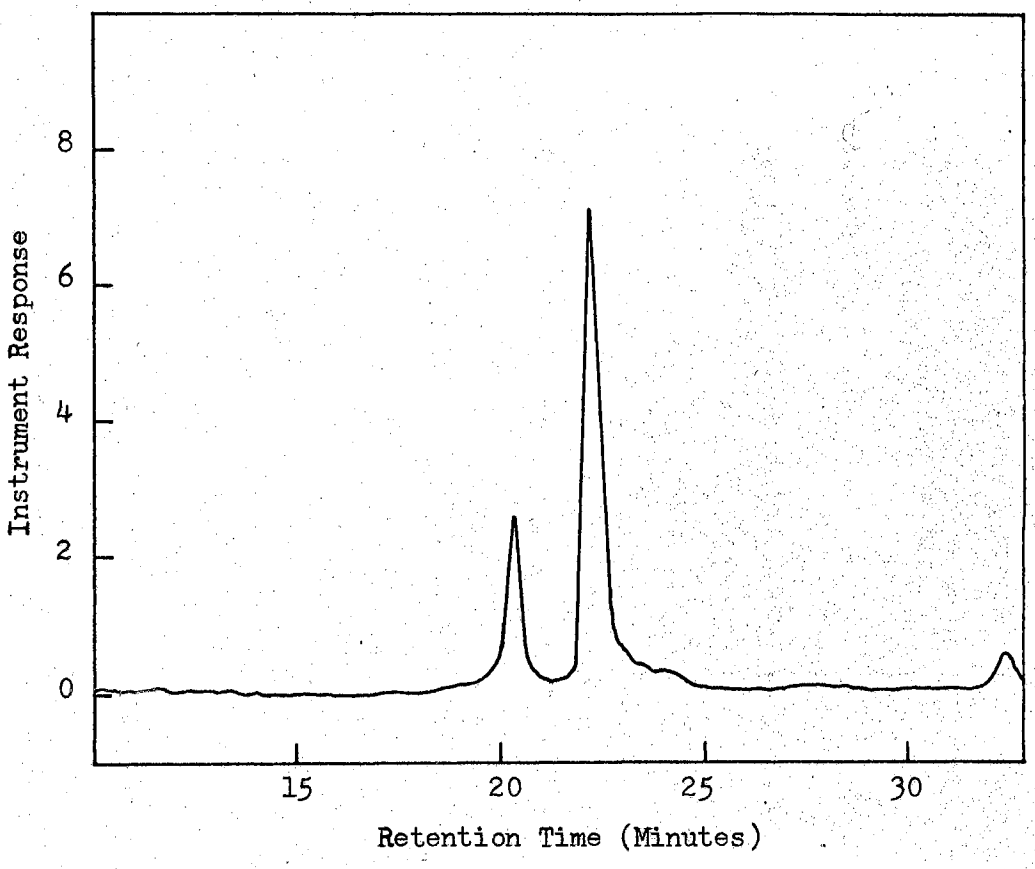


Figure 11. Gas Chromatography of Mixture of Oxaluric Oxime and Ureidoglycine

Conditions: 8 X attenuation, range 10^2, injector temperature 260°C, detector temperature 290°C, flow rate (Helium) 30 ml/min., chart speed 1"/3min. Sample size, 1-5 µl. Temperature program: 100°C isothermal for 10 min., 6°C/min. to 200°C, hold at upper limit for 10 min.

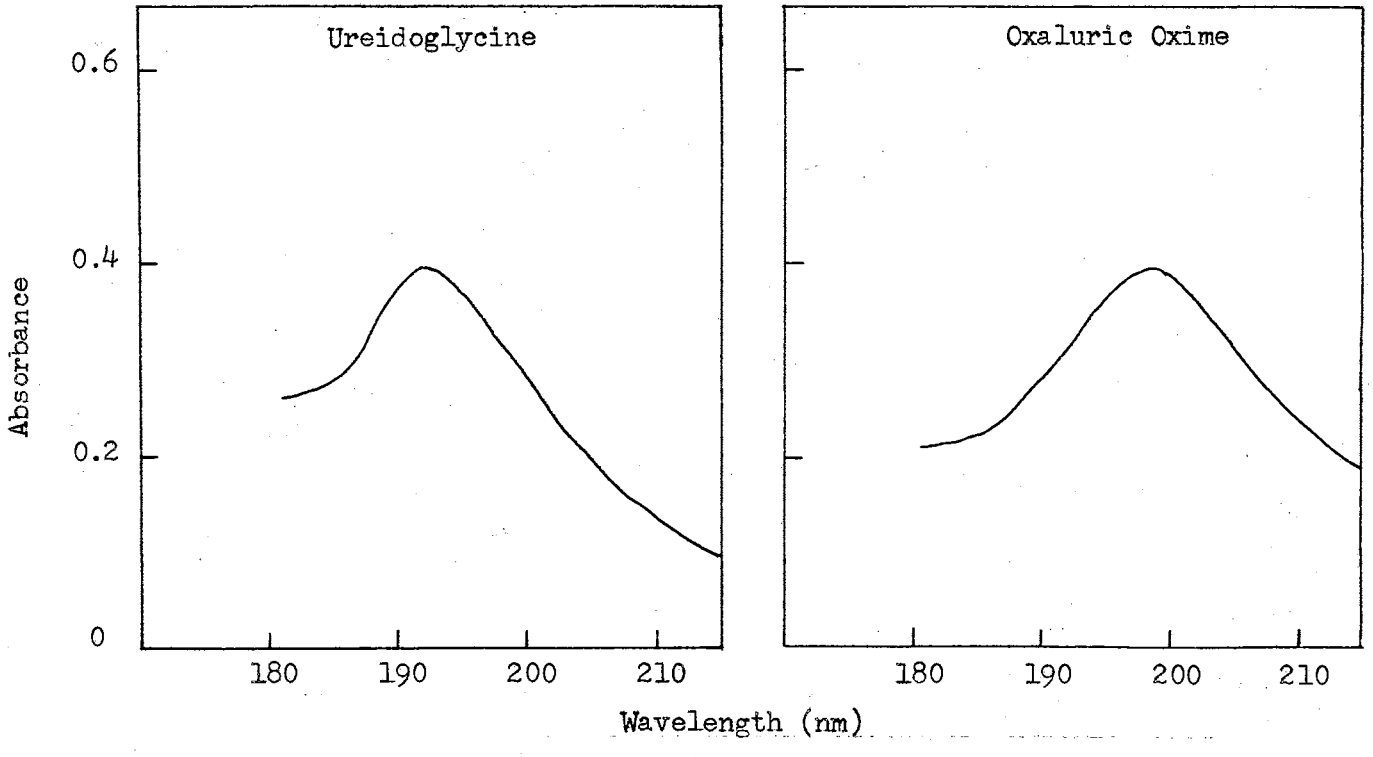


Figure 12. Ultraviolet Absorption Spectra of Oxaluric Oxime and Ureidoglycine

expected to accumulate ureidoglycine in growth medium containing allantoate. If ureidoglycine were accumulated in the growth medium, one would be able to detect the ureidoglycine by gas chromatography and by the ultraviolet absorption spectrum of the supernatant.

P. acidovorans 14-1 was grown overnight in 0.5 per cent succinate plus 0.1 per cent allantoate in minimal salts at 30°C with shaking. Cells were harvested by centrifugation and resuspended in minimal salts containing 0.1 per cent allantoate. The cell suspension (3×10^9 cells/ml) was incubated at 30°C with shaking. After 6 hours incubation, the supernatant was collected by filtration with a Millipore filter. A 0.1 ml sample of the supernatant was diluted to 15 ml with distilled water for determination of the ultraviolet absorption spectrum and the remainder of the supernatant was lyophilized for gas chromatography.

a. Ultraviolet Absorption Spectrum of Supernatant

One-tenth ml of 0.1 per cent allantoate was diluted to 15 ml with distilled water and 0.1 ml of the supernatant obtained as described above was diluted to 15 ml with distilled water. Ultraviolet spectra were recorded using a Cary 14 recording spectrophotometer. Standard 3 ml silica cells with 1 cm light paths were employed for all samples. Absorption peaks for allantoate were located at 213 nm (shoulder), 190 nm and 187 nm and the supernatant (6 hour sample) absorbed at 193 nm. Figure 13 shows the absorption spectra of allantoate and the supernatant. Comparison of Figure 12 and Figure 13 shows that the spectra for synthesized ureidoglycine and the material accumulated in the supernatant are essentially identical.

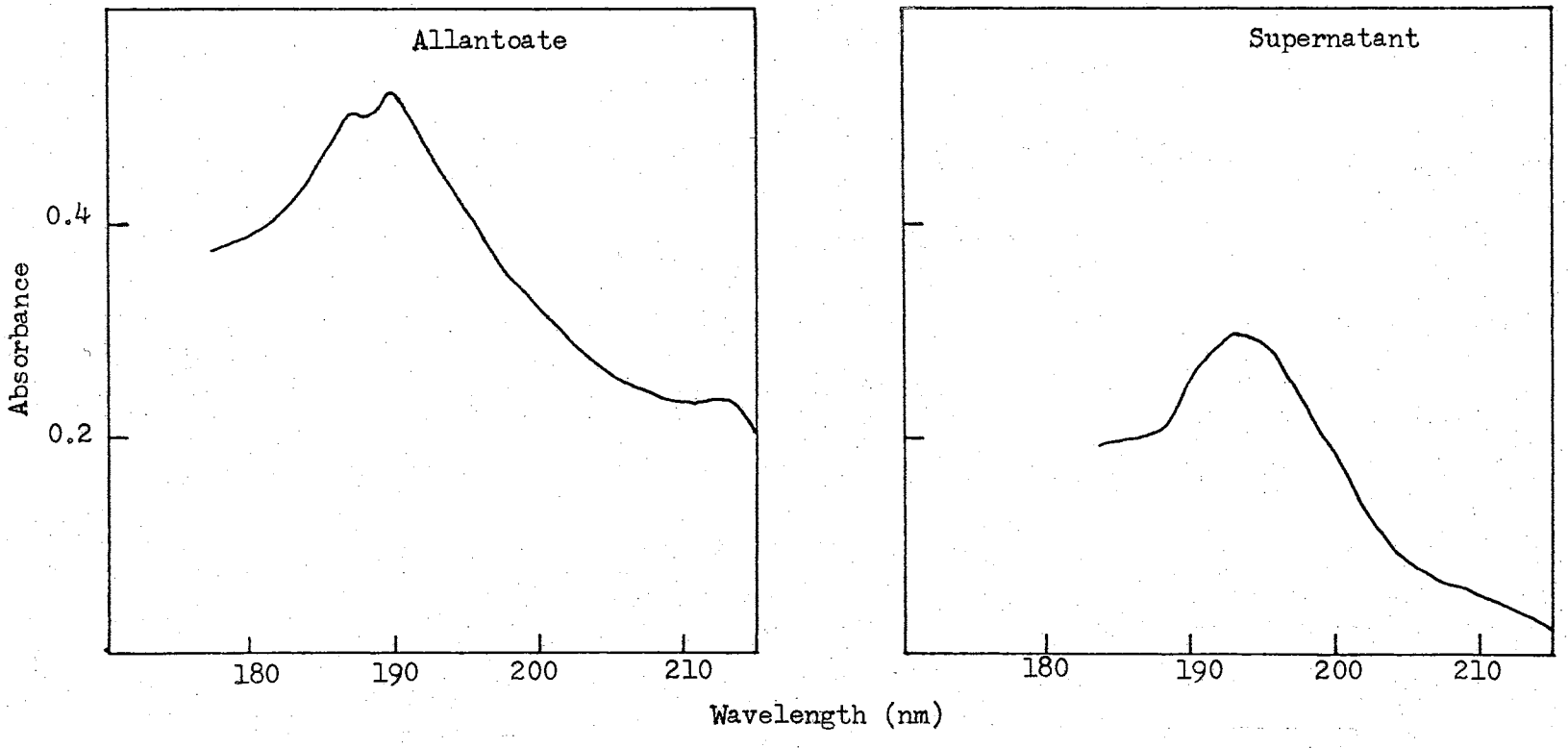


Figure 13. Ultraviolet Absorption Spectra of Allantoate and Supernatant of Pseudomonas acidovorans 14-1 Incubated with Allantoate for 6 Hours

b. Gas-Liquid Chromatography of Supernatant

Ten mg of allantoate and 10 mg of lyophilized supernatant were treated separately with 1 ml of anhydrous pyridine, 0.2 ml of hexamethyldisilazane, and 0.1 ml of trimethylchlorosilane. The reaction was carried out in plastic-stoppered vials and the mixtures were allowed to stand for 2 hours or longer at room temperature. A 1/10 dilution of the mixture containing supernatant was made with pyridine; the allantoate mixture was not diluted. One μ liter of the resulting mixture was injected into the gas chromatograph. The retention times of the TMS derivatives of allantoate were 11.0 minutes and 20.4 minutes. The retention time for the TMS derivative of the supernatant was 22.0 minutes and only one peak was obtained. Figures 14, 15 and 16 show the gas chromatography pattern for TMS-allantoate, TMS-supernatant and for a mixture of the TMS derivatives of supernatant and synthesized ureidoglycine (1/1 by volume). Only one peak was obtained from the mixture of synthesized ureidoglycine and supernatant.

G. Crystallization of Ureidoglycine

Crystals of ureidoglycine were obtained from cold ethanol, by the following treatment:

Fraction E was dissolved in a minimal amount of distilled water. Ninety-five per cent ethanol (V/V) was added to a final concentration of 75 per cent. The solution was filtered through a Millipore filter (HA, 0.45 μ pore size). The crystals were formed after standing overnight at 4°C as macroscopically visible needles. The melting point of this material was 223°C. A photomicrograph of crystalline ureidoglycine is shown in Figure 17. Magnification is approximately 100 X.

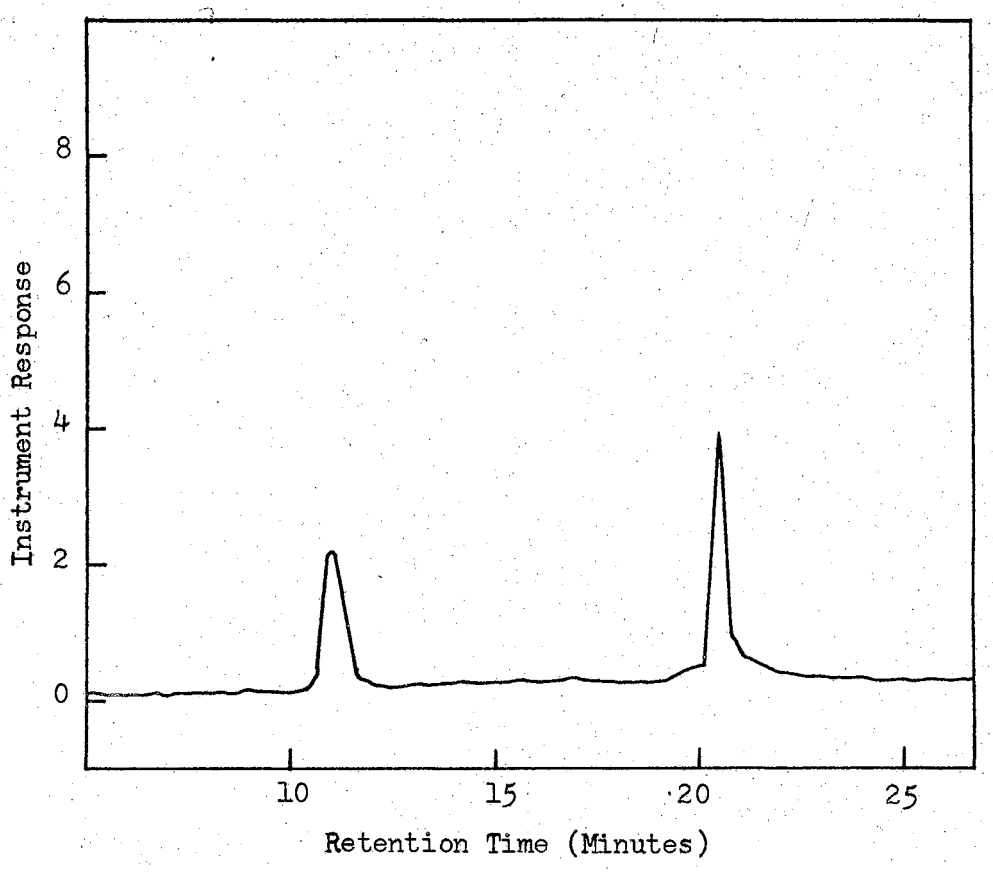


Figure 14. Gas Chromatography of Allantoate

Conditions: 8 X attenuation, range 10^2, injector temperature 260°C, detector temperature 290°C, flow rate (Helium) 30 ml/min., chart speed 1"/3 min. Sample size, 1-5 µl. Temperature program: 100°C isothermal for 10 min., 6°C/min. to 200°C, hold at upper limit for 10 min.

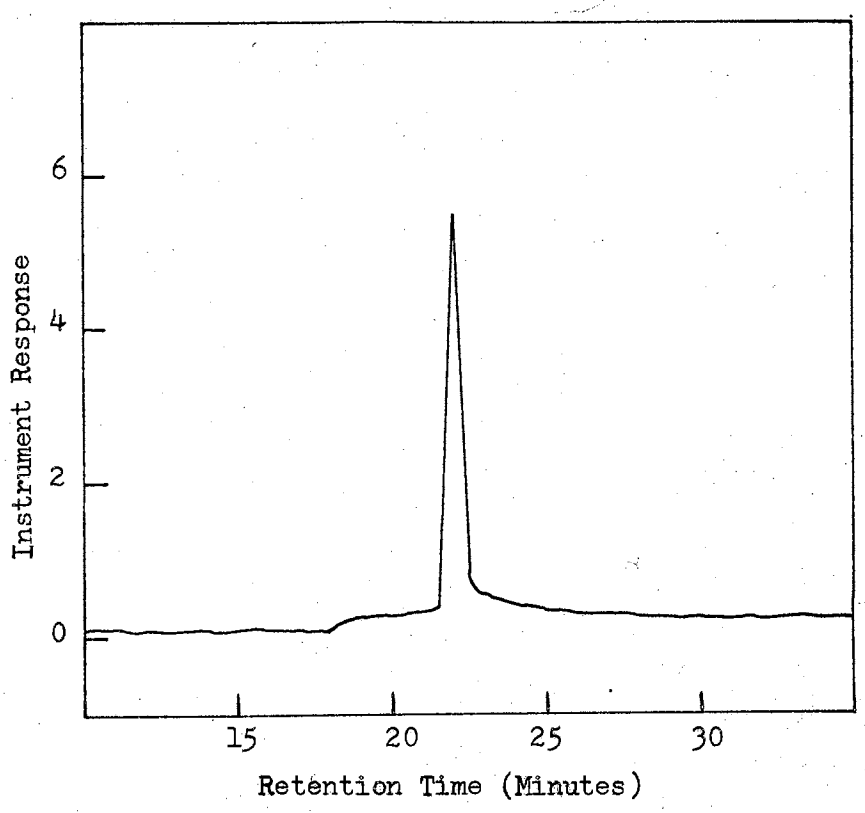


Figure 15. Gas Chromatography of Supernatant of Pseudomonas acidovorans 14-1 Incubated with Allantoate for 6 Hours.

Conditions: 8 X attenuation, range 10^2, injector temperature 260°C, detector temperature 290°C, flow rate (Helium) 30 ml/min., chart speed 1"/3 min. Sample size, 1-5 µl. Temperature program: 100°C isothermal for 10 min., 6°C/min. to 200°C, hold at upper limit for 10 min.

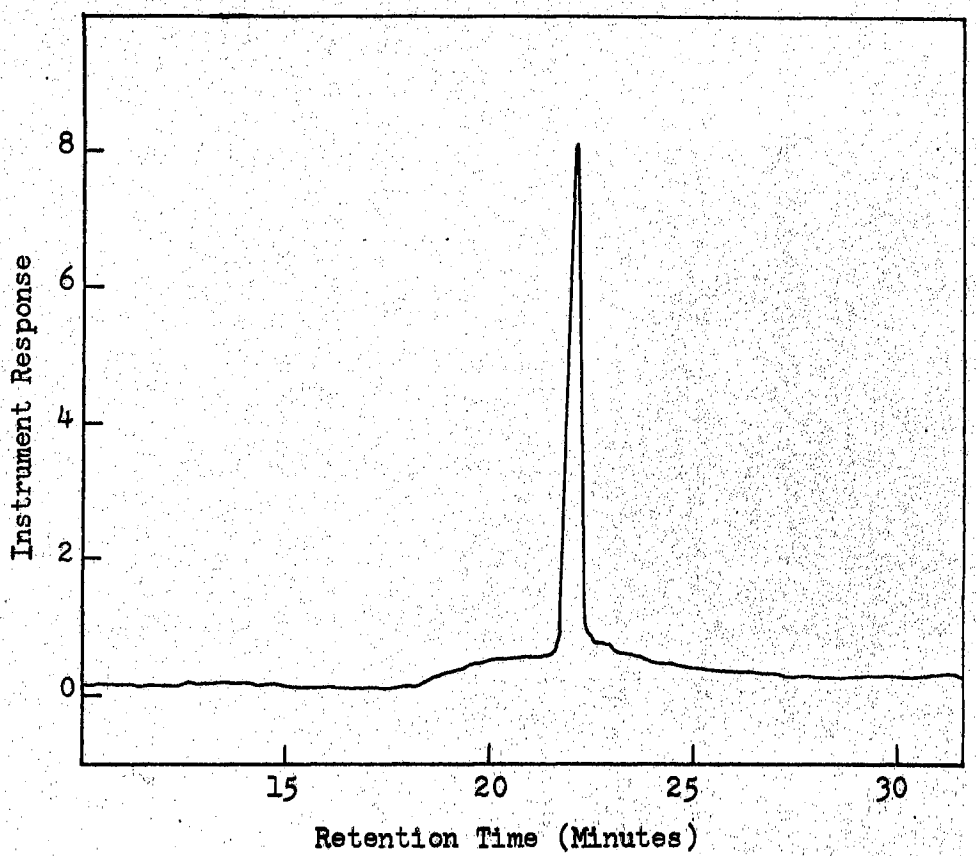


Figure 16. Gas Chromatography of Mixture of Supernatant and Synthesized Ureidoglycine.

Conditions: 8 X attenuation, range 10^2, injector temperature 260°C, detector temperature 290°C, flow rate (Helium) 30 ml/min., chart speed 1"/3 min. Sample size, 1-5 µl. Temperature program: 100°C isothermal for 10 min., 6°C/min. to 200°C, hold at upper limit for 10 min.

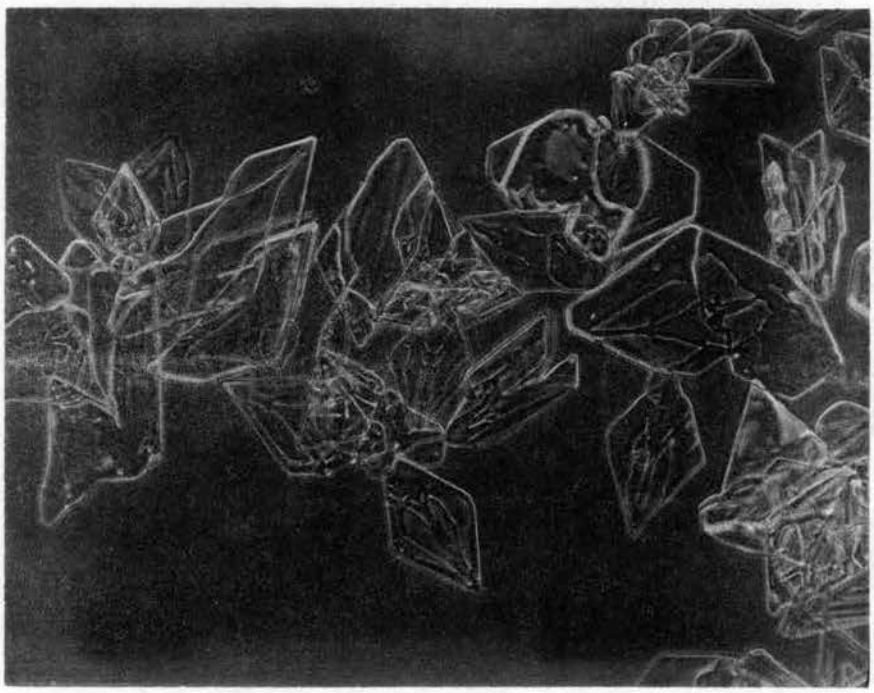


Figure 17. Photomicrograph of Crystals of Ureidoglycine

CHAPTER V

DISCUSSION

A. Allantoate-Negative and Urease-Negative Mutants

Two different pathways have been proposed for the conversion of allantoate to ureidoglycolate in microorganisms. Since a proposed intermediate in one pathway, ureidoglycine, had not been either synthesized or isolated from biological sources prior to the present study, proof of the occurrence of this compound and the enzyme postulated to catalyze its degradation was lacking. The enzyme which converts allantoate to ureidoglycine, ammonia and CO_2 had been studied in considerable detail but only ammonia had been measured specifically as a product of the reaction. Ureidoglycine had not been identified or isolated from the reaction mixture. The enzyme forming ureidoglycine, allantoate amidohydrolase, had properties which engendered considerable doubt as to its physiological importance. The necessity of activation by treatment at a fairly low pH suggested that its activity might be of little importance *in vivo*. If the formation of ammonia (and ureidoglycine) from allantoate were physiologically unimportant reactions, it would be expected that organisms capable of growing on purines or any of their degradation products, including allantoate, would necessarily possess the allantoicase pathway, whether or not the allantoate amidohydrolase pathway were also present. Indeed, it seemed possible that the allantoate amidohydrolase reaction might be an artefact resulting from acid treatment of

another enzyme, possibly allantoicase. Therefore, it was essential to determine whether both pathways could be present in the same organism. This aspect of the problem was approached through attempts to isolate allantoate-negative mutants based on the following analysis.

If both pathways exist and can function in the same organism, it should be impossible to isolate an allantoate-negative mutant of that organism by a single mutation. If only one pathway is used, such mutants can be isolated. Presumably, if a mutant which were blocked at allantoicase were selected and the organism had both pathways, allantoate amidohydrolase could still be used by the organism, or if a mutant blocked at allantoate amidohydrolase or ureidoglycine aminohydrolase were isolated, allantoicase could still be used by the organism. Therefore, it should be impossible to isolate allantoate-negative mutants if both pathways are available for use in the same microorganism.

Mutants which are unable to use allantoate as carbon source but can use allantoate as nitrogen source have been isolated. Growth data shown in Table III indicate that strains 14-1, 2, 3, 4 and 5 of P. acidovorans, strains TW-10, 11, 12, 13, 14, 15 and 16 of A. aerogenes TW, and strain Tlb-2 of A. aerogenes Tlb are mutants which are blocked at ureidoglycine aminohydrolase. Since the use of allantoate amidohydrolase would allow formation of one mole of ammonia from one mole of allantoate, these mutants can use allantoate as nitrogen source but not as carbon source. No other position for an enzyme block can be postulated which would explain the observed growth data for these mutants. The isolation of these mutants indicates that only one of the pathways can be used in the organisms studied; i.e., the pathway involving allantoicase is not present in these organisms.

The microorganisms which were used in this study are urease positive. If one could isolate a urease-negative mutant and this mutant still could use allantoate as nitrogen source for growth, this would offer additional proof that the ammonia-forming enzymes are used in allantoate degradation by these organisms. Unfortunately, repeated attempts to isolate urease-negative mutants were unsuccessful. These unsuccessful attempts may be explained as due to lack of a sufficiently selective enrichment procedure. Because of the large amounts of ammonia formed from allantoate by microorganisms, ammonia diffuses through the replicate plates and prevents identification of mutants which might be present. Ammonia excreted into the medium by wild type cells also would interfere with enrichment by the normal procedures which are dependent upon differential killing of non-growing (mutant) cells.

B. Synthesis of Ureidoglycine

Oxaluric acid is a known compound. Two methods for its synthesis have been reported, using different starting materials. Andrews and Sell (38) reported that oxaluric acid can be made from parabanic acid. A sample of parabanic acid was dissolved in the minimal amount of warm water, and concentrated base was added dropwise until a pH of about 10 was reached. On cooling the resulting solution, the salt crystallized out in thin needles. The free oxaluric acid was most easily prepared by acidification with concentrated hydrochloric acid of a warm water solution of the salt until a pH value of 2.0 was reached.

The method of Biltz and Schrauder (31), which used alloxan as starting material, was chosen for use in the synthesis of oxaluric acid in these studies. A yield of approximately 50 per cent was obtained. Oxaluric acid is only slightly soluble in water at room temperature and

decomposes at approximately 210°C. Oxaluric acid is decomposed by acid or alkali forming urea and oxalic acid. The synthesis of the oxaluric oxime was carried out at a pH of 7.0 to prevent the decomposition of oxaluric acid.

The synthesis of oxaluric oxime has not been reported to our knowledge. The compound which was synthesized in this study decomposed at 272° C. The infrared spectrum, gas chromatographic analysis and ultraviolet absorption spectrum for this compound were shown in Figures 7, 9 and 12, respectively.

Rylander (33) pointed out that "the course of oxime hydrogenation appears to be unusually sensitive to the catalyst, substrate, and reaction environment, making generalization about these reductions particularly tenuous." He also reported a comparison of palladium, platinum, rhodium and ruthenium in hydrogenation of acetoxime and 3-pentanone oxime; rhodium gave the best yield of primary amine. The products from hydrogenation of oximes depend to an unusual degree on the solvent employed. Hydrogenation of oximes in neutral media usually leads to a mixture of primary, secondary, and tertiary amines. Hartung (34) reported that formation of secondary amines could be prevented by carrying out the hydrogenation in acidic media. Several acidic solvents have been used for hydrogenation of oximes, such as acetic, sulfuric, phosphoric, and perchloric acids and acetic acid containing hydrogen chloride. In the present study, the synthesis of ureidoglycine was carried out in acetic acid. Five per cent palladium on carbon was used as catalyst for the hydrogenation of oxaluric oxime. The yield obtained was 30 per cent. The very slight solubility of oxime in acetic acid may explain the low yield. Other acidic media and catalysts might profitably

be tested for the purpose of obtaining higher yields in hydrogenation of oxaluric oxime.

C. Characterization of Oxaluric Oxime and Ureidoglycine

1. Identification of Hydrogenation Products by Biological Methods

P. acidovorans 14-1 and A. aerogenes TW-16 are mutants which are blocked at ureidoglycine aminohydrolase. P. acidovorans 14 and A. aerogenes TW are wild types which should use ureidoglycine as carbon and/or nitrogen source; the mutants should not. Data given in Tables IV and V show that the two wild type organisms can use Fraction E (ureidoglycine) as carbon and/or nitrogen source. The mutants cannot use Fraction E even as a nitrogen source in the presence of a readily used carbon source. This indicates that Fraction E is ureidoglycine.

There was a possibility that oxaluric oxime and/or ureidoglycine might have decomposed to form urea and glycine under the very acidic conditions which were used for the hydrogenation. Both wild types and mutants can use glycine and urea as nitrogen sources but neither mutant could use Fraction E as nitrogen source. If Fraction E contained urea and/or glycine, both wild types and mutants should be able to use Fraction E as nitrogen source. Since only the wild types can use Fraction E as nitrogen source, it can be concluded that Fraction E does not contain urea or glycine.

It was shown in Figures 4 and 5 that ammonia is formed by a crude extract of A. aerogenes TW using Fraction E (ureidoglycine) as substrate at pH 7.0 or 8.5. This is additional evidence that Fraction E is ureidoglycine. Ammonia formation was quite slow and the reason for this is not known. Unfortunately, not enough ureidoglycine was available to

allow a complete study of the optimum conditions for enzyme activity. Since the reaction is a deamination, it is possible that a cofactor such as pyridoxal phosphate may be required. Substrate concentration was decreased to one-fourth of the concentration which was used in the assays shown in Figures 4 and 5 to prevent possible substrate inhibition, but the velocity of the reaction was not increased. Ammonia-forming enzyme activity was enhanced several-fold by acid-pretreatment of the extract when allantoate was used as substrate and the maximum specific activity is attained using incubation for 30 minutes at 30°C (1). Vogels has never determined which of the two ammonia-forming enzymes required acid-pretreatment. According to Figures 4 and 5, apparently allantoate amidohydrolase is the one which requires acid-pretreatment, since acid-pretreatment inhibited the activity of ureidoglycine aminohydrolase.

2. Identification of Ureidoglycine by Nonbiological Methods

Both oxaluric oxime and ureidoglycine are ninhydrin positive. Since oxaluric oxime yielded a color on heating but not on standing overnight in the cold and ureidoglycine gave a color reaction both on heating and on standing overnight in the cold, it may be concluded that oxaluric oxime is not an α-amino acid and that ureidoglycine is an α-amino acid. Paper chromatography (Figure 6) showed that Fraction E does not contain glycine; this confirms the conclusion based on growth studies (Table V). The R_f values for oxaluric oxime and Fraction E are sufficiently different in the butanol:pyridine:water system to show that Fraction E is not the starting material (the oxime). Only one spot was detected from Fraction E in two solvent systems in which the distance

traveled was quite different. If a secondary amine were present in Fraction E it should have been detected as a yellow spot with ninhydrin. No such spot was detected. Oxaluric oxime and Fraction E, when tested with Ehrlich's reagent yield a yellow color, indicating that both contain a ureido group as expected.

The infrared spectrum for oxaluric oxime showed broad absorption at 3300-3150 cm^{-1}, 1690-1620 cm^{-1} and 930 cm^{-1} due to OH stretch, and C=N stretch of oximes, indicating the synthesized oxaluric oxime has this functional group. Fraction E absorbed at 3130 cm^{-1} and 1640 cm^{-1} due to N-H stretching of the amino acid; at 1600 cm^{-1} and 1400 cm^{-1} due to the carboxyl group of the amino acid. A stronger N-H banding does not appear at 1550-1485 cm^{-1} for N-substituted amino acids. Since a band appeared at 1550 cm^{-1} for Fraction E, this compound is not an N-substituted amino acid. Two absorption bands appeared between 3500 and 3300 cm^{-1} for Fraction E and also at 1650-1580 cm^{-1}, 1220-1020 cm^{-1}, 900-650 cm^{-1}. These are characteristic absorptions for primary amines.

Trimethylsilyl derivatives of glycine, urea, allantoate, ureidoglycolate, oxaluric oxime, and Fraction E were prepared and subjected to gas-liquid chromatography. The retention times for these compounds were as follows:

glycine	23 minutes
urea	20 minutes
allantoate	11 and 20.4 minutes
oxaluric acid	20.2 and 32.4 minutes
oxaluric oxime	20.2 and 32.4 minutes
ureidoglycolate	20.5 and 21.4 minutes
Fraction E	22.0 minutes

Elution patterns for the TMS derivatives of oxaluric oxime, Fraction E and the mixture of Fraction E and oxaluric oxime were shown in Figures 9, 10 and 11. Since only one peak was detected with Fraction E, it may be

concluded that this fraction is not a mixture of primary, secondary and tertiary amines and is essentially pure. These data provide evidence that Fraction E is not and does not contain glycine, urea, allantoate, ureidoglycolate, oxaluric acid or oxaluric oxime.

Ultraviolet spectra of allantoate, ureidoglycolate, oxaluric acid, oxaluric oxime and Fraction E were recorded with a Cary 14 recording spectrophotometer. The absorption maxima of these compounds were as follows:

allantoate	187, 190 and 213 nm
ureidoglycolate	185 nm
oxaluric acid	202 nm
oxaluric oxime	199 nm
Fraction E	193 nm

The UV spectra also offer evidence that Fraction E is not and does not contain allantoate, ureidoglycolate, oxaluric acid or oxaluric oxime.

D. Accumulation of Ureidoglycine by Allantoate-Negative Mutant

P. acidovorans 14-1 is a mutant which is blocked at ureidoglycine aminohydrolase and therefore should accumulate ureidoglycine when exposed to allantoate. Gas-liquid chromatography of the TMS derivative of supernatant from mutant cells incubated with allantoate yielded only one peak at a retention time of 22.0 minutes, and a 1 : 1 mixture of TMS derivatives of the supernatant and Fraction E also yielded only one peak with a retention time of 22.0 minutes. The ultraviolet spectrum of the supernatant absorbed maximally at 193 nm and the UV absorption maximum of Fraction E was also at 193 nm. These results support the conclusion that Fraction E is ureidoglycine and P. acidovorans 14-1 is a mutant which is blocked at ureidoglycine aminohydrolase and can accumulate ureidoglycine in the medium.

E. Metabolism of Allantoate by Microorganisms

The three different pathways which had been proposed for the degradation of allantoin in microorganisms are shown in Figure 18. These pathways differ in the number of reactions involved in the conversion of allantoate to glyoxylic acid. The first pathway proposed (5) included reactions 1 and 2 and assumed the direct conversion of allantoate to glyoxylate and urea in a single reaction. The pathway proposed by Wolfe and co-workers (6, 7) included one intermediate, ureidoglycolate, and included reactions 1, 3, 6 and 7 (Figure 18). Ureidoglycolate synthetase catalyzes the conversion of ureidoglycolate to glyoxylate and urea (6, 7, 9). The second pathway, proposed by Vogels (17), included two intermediates between allantoic acid and ureidoglycolate and involved reactions 1, 4, 5, 6, 7 and 8. Allantoate amidohydrolase and ureidoglycine aminohydrolase are the enzymes which catalyze reactions 4 and 5. All the work described above was based on the chemical analysis of reaction mixtures.

Bruce (13) obtained proof by genetic studies with P. aeruginosa of the allantoin pathway sequence as proposed by Wolfe and co-workers (6, 7). The fact that Bruce was able to isolate mutants of this organism which lacked allantoicase and were unable to metabolize allantoin or allantoate indicated that only one pathway could be used in P. aeruginosa. Winter (12) also obtained proof by enzyme studies of allantoin-negative mutants of P. aeruginosa, of the pathway sequence as proposed by Wolfe and co-workers (6, 7). Winter (12) reported that synthesis of the enzymes of the allantoin pathway by P. aeruginosa is coordinately controlled, although the loci for the enzymes studied are not closely linked. Based on growth and/or enzymatic data,

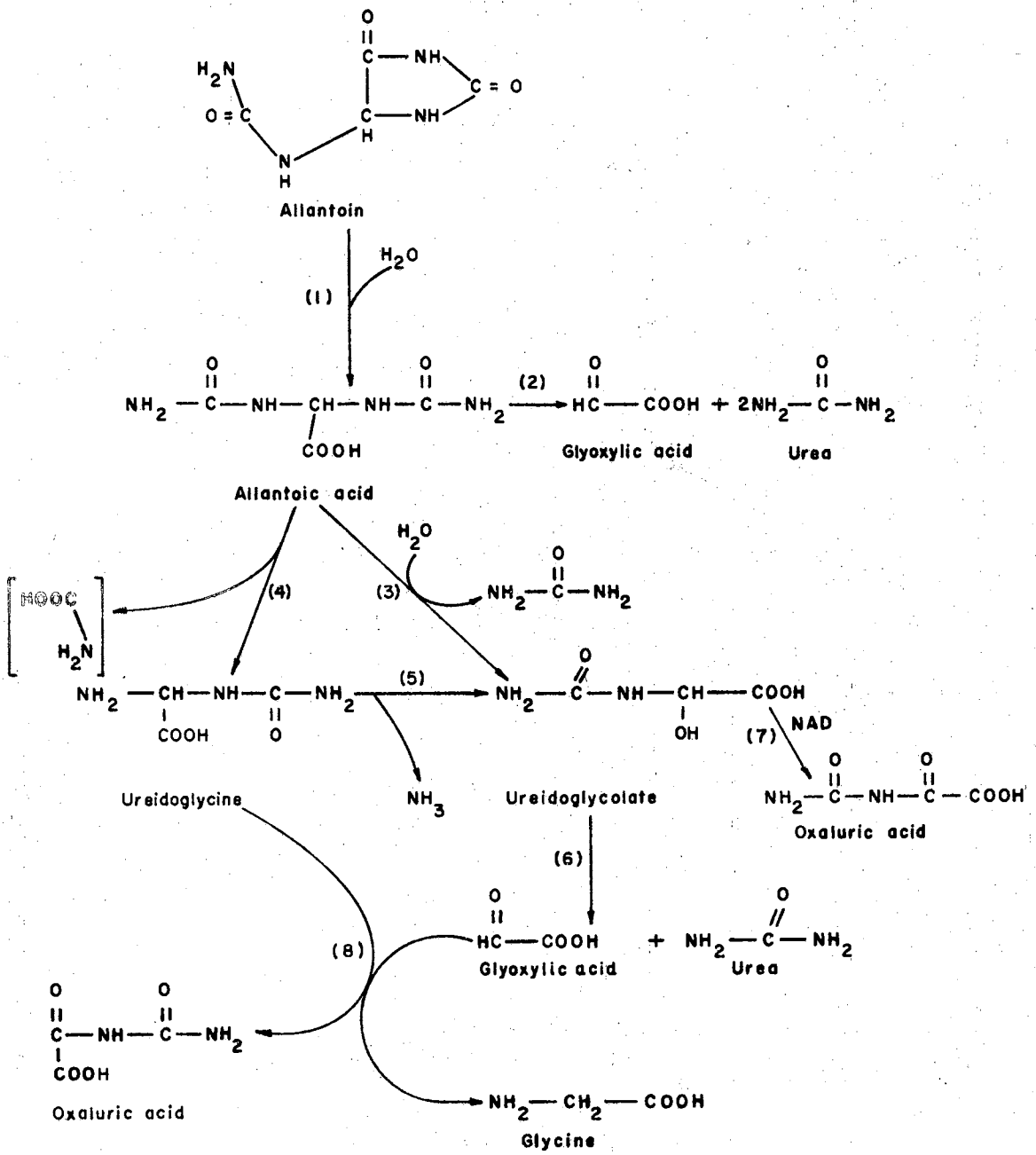


FIGURE 18

PROPOSED PATHWAYS FOR ALLANTOIN DEGRADATION

Meganathan (14) reported that in species belonging to the genera Aerobacter, Alcaligenes, Arthrobacter, Escherichia, Flavobacterium, Nocardia, and Pseudomonas, the pathway of degradation is the same as that proposed by Wolfe and co-workers (6, 7). In Streptococcus allantoicus, allantoate amidohydrolase activity, as proposed by Vogels (17) was detected.

Vogels (17, 18) reported that the degradation of allantoic acid to ureidoglycolate was mediated by allantoate amidohydrolase and ureidoglycine aminohydrolase, but not by allantoicase, in Pseudomonas acidovorans, Arthrobacter allantoicus, Streptococcus allantoicus, Escherichia coli, Escherichia coli var. acidilactici and Escherichia freundii. But Trijbels and Vogels (39) agreed that in certain microorganisms, e.g., P. aeruginosa, allantoate is degraded by allantoicase.

Recently, van der Drift, de Windt, and Vogels (40) reported that it seemed unnecessary to postulate the existence of ureidoglycine aminohydrolase since a transamination reaction involving ureidoglycine and glyoxylate seemed to proceed nonenzymatically. They concluded that the conversion of ureidoglycine to ureidoglycolate was most likely catalyzed by allantoate amidohydrolase. All the work by Vogels and his associates with allantoate amidohydrolase and ureidoglycine aminohydrolase has been based on the chemical analysis of enzymatic reaction mixtures.

In the present investigation, mutants which are blocked at ureidoglycine aminohydrolase have been isolated and ureidoglycine has been synthesized. The utilization of the synthesized ureidoglycine by wild type organisms capable of growing on allantoate, the inability of mutants lacking ureidoglycine aminohydrolase to utilize the synthesized compound, the formation of ammonia from the synthesized compound by extracts of allantoate-grown cells, and the formation from allantoate of

a compound identical to the synthesized ureidoglycine by mutants lacking ureidoglycine aminohydrolase offer the first concrete evidence that ureidoglycine is an intermediate in the catabolism of allantoin by some bacteria. The data obtained in the present study do not allow an unequivocal decision to be made concerning the existence of two distinct enzyme molecules for the allantoate amidohydrolase and ureidoglycine aminohydrolase activities.

Growth data using mutants indicate that growth on allantoate as nitrogen source occurs at the wild type rate. However, the allantoate amidohydrolase reaction would not be required to proceed as rapidly to provide ammonia for growth as to provide carbon for biosynthetic reactions and for energy. Assays of ammonia formation by whole, non-proliferating cells indicated that ammonia formation proceeded at slightly less than the expected rate based on comparison with wild type cells. However, the allantoate amidohydrolase reaction in extracts prepared from mutants has a greatly reduced velocity compared to that of the wild type enzyme. Since three mutants, derived from three different parental strains and therefore unquestionably independent, exhibited similar characteristics, it must be concluded that loss of ureidoglycine aminohydrolase activity necessarily affects allantoate amidohydrolase activity. This can be interpreted in two ways. Allantoate amidohydrolase could be very sensitive to product inhibition by ureidoglycine, so that accumulation of this compound in the reaction mixture would rapidly inhibit formation of ammonia. This explanation is consistent with the apparent difference in behavior of the enzyme in whole cells and in extracts. Whole cells apparently excrete ureidoglycine into the medium and it may therefore fail to accumulate in the cell

sufficiently to cause significant inhibition of allantoate amidohydrolase activity. Alternatively, a single enzyme molecule may be responsible for both enzyme activities. If this explanation is correct, it seems necessary to postulate that allantoate amidohydrolase is composed of subunits, most probably of two different polypeptides, one of which has the active site for allantoate degradation and the other the active site for ureidoglycine degradation. Normal configurations of both peptides could be required for full activity in either reaction. It would seem quite improbable that three different mutants would have defects in a single polypeptide which would eliminate ureidoglycine aminohydrolase activity completely while none of the three is sufficiently deficient in allantoate amidohydrolase activity to affect growth on this compound as a nitrogen source. The method of selection used in mutant isolation should have detected with equal probability mutants lacking either activity. If the two activities were mediated by two completely independent proteins, it is equally improbable that both activities could be affected by a single mutation. None of the mutants used in these studies reverted and therefore they may be assumed to be deletion mutants. If the loci for the two enzymes were adjacent, a single deletion could affect both activities even though the proteins had completely independent activity. However, the probability that this specific type of deletion would occur in three independent mutants is extremely low.

Based on the available data, it is concluded that normal ureidoglycine aminohydrolase activity is required for normal allantoate amidohydrolase activity. The basis for this requirement may be either removal

of an inhibitory product or the formation of an enzyme aggregate having both activities and requiring an unaltered configuration for both components for maximal velocity of either reaction.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Three microorganisms capable of using allantoate as the sole source of carbon and nitrogen were used in this investigation. These were Pseudomonas acidovorans 14, Aerobacter aerogenes TW and Aerobacter aerogenes T1b. All possess the ammonia-forming pathway for allantoate degradation, i.e., the enzymes allantoate amidohydrolase and ureidoglycine aminohydrolase. Mutants lacking ureidoglycine aminohydrolase were isolated from each wild type strain. These mutants are capable of using allantoate as nitrogen source but not as carbon source.

Ureidoglycine, a proposed intermediate in the pathway, has been synthesized. It has been characterized as follows:

1. Reaction with ninhydrin at room temperature indicates that the compound is an α-amino acid.

2. Reaction with Ehrlich's reagent to form a yellow color indicates the presence of a ureido group.

3. The infrared spectrum has absorption bands characteristic of a primary amine and an amino acid.

4. The compound has maximum UV absorption at 1930 angstroms.

5. Retention time for the trimethylsilyl derivative of the compound in gas-liquid chromatographic analysis was 22.0 minutes and a single, well-defined peak was obtained.

6. The wild type strains, P. acidovorans 14 and A. aerogenes TW, use the compound as a sole source of carbon and nitrogen.

7. Mutants lacking ureidoglycine aminohydrolase cannot use the compound as a source of either carbon or nitrogen.

8. Growth data, infrared and ultraviolet spectroscopy, melting points, and paper and gas-liquid chromatography were used to show that the compound is not, and does not contain, oxaluric oxime, glycine, urea, allantoate or ureidoglycolate.

9. Ammonia was formed from the compound by a cell-free extract of Aerobacter aerogenes TW.

10. Supernatant fluid from a suspension of Pseudomonas acidovorans 14-1, which lacks ureidoglycine aminohydrolase, was collected after exposure of the cells to allantoate for 6 hours. The supernatant contained a single compound which had an ultraviolet spectrum identical to that of the synthesized ureidoglycine and formed a TMS derivative inseparable from that of ureidoglycine by gas-liquid chromatography.

From these data, it is concluded that the synthesized compound is ureidoglycine. It is also concluded that ureidoglycine is an essential intermediate in the degradation of allantoate by the organisms used in this investigation and that ureidoglycine aminohydrolase activity is essential for the complete degradation of allantoate. If the organisms studied had possessed the allantoicase pathway in addition to the allantoate amidohydrolase pathway, or if ureidoglycine degradation could be accomplished solely by non-enzymatic transamination, mutants with the characteristics of those isolated in this investigation could not have been obtained. The effect of a mutation causing loss of ureidoglycine aminohydrolase activity upon the activity of allantoate amidohydrolase

indicates that these reactions may be catalyzed by a single protein molecule with two different active sites or, more likely, by an aggregate in which the structural integrity of both components is required for full activity in either reaction.

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