STUDIES ON THE METABOLISM OF ALLANTOIN BY BACTERIA

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CHAPTER I

INTRODUCTION

Allantoin was first discovered in the amniotic fluid of cattle (Buniva and Vauquelin, 1799). Grimaux (1876, 1877) was the first who succeeded in synthesizing allantoin from simpler organic compounds, using glyoxylic acid and urea. Allantoin is formed in the catabolism of purines through uric acid. The conversion of uric acid to allantoin is mediated by the enzyme uricase (Mahler, Baumand, and Hubscher, 1956). Wöhler (1853) showed the degradation of allantoin by an unidentified yeast. Laskowski (1951) described the conversion of allantoin to allantoic acid as catalyzed by the enzyme allantoinase and the degradation of allantoic acid as catalyzed by the enzyme allantoicase.

Vogels (1963) summarized the ability of bacteria to degrade allantoin as reported by different authors. Table I shows the list of organisms capable of degrading allantoin, as reported in the literature prior to 1963.

For an organism to be able to utilize allantoin as a sole nitrogen source, it should (based on presently proposed pathways) be able to convert allantoin to ammonia and to use ammonia as a sole nitrogen source. A number of organisms listed in Table I are known to be able to use ammonia as a

TABLE I

REPORTED ABILITY OF BACTERIA TO USE ALLANTOIN[1]

Organism	Nitrogen Source	Carbon Source
Pseudomonas aeruginosa	+	+
Pseudomonas fluorescens	+	+
Pseudomonas putida	+	+
Pseudomonas eisenbergii	+	+
Pseudomonas trifolii	+	
Pseudomonas sp.	+	+
Spirillum tenue	+	
Aerobacter aerogenes	+	
Aerobacter cloacae	+	
Paracolobactrum aerogenoides	+	+
Serratia marcescens	+	
Sarcina lutea	+	
Streptococcus allantoicus	+	+
Arthrobacter ureafaciens[2]		+
Bacillus megaterium	+	
Bacillus subtilis	+	
Bacillus polymyxa	+	
Bacillus guano	+	
Bacillus hollandicus	+	
Mycobacterium phlei[2]	+	
Mycobacterium smegmatis[2]		+
Mycobacterium fortuitum[2]		+
Mycobacterium thamnopheos[2]		+
Mycobacterium stercoris[2]		+
Mycobacterium butyricum[2]		+
Nocardia opaca	+	+

[1]Compilation of literature survey by Vogels (1963).

[2]Resting cell suspensions.

nitrogen source, but others are not reported, by Bergey's Manual at least, to be able to do so.

Barker (1943) has clearly shown that he could account for 99.5% of the nitrogen of allantoin as non-cellular nitrogen, i.e. as nitrogen-containing fermentation products. In spite of this clear-cut evidence, Vogels (1963) stated that Barker (1943) had shown that Streptococcus allantoicus could utilize allantoin as the sole source of nitrogen and carbon. Thus, in this case at least, the data were quoted mistakenly or were misinterpreted.

Vogels (1963) also tested a large number of stock cultures for ability to use allantoin as a sole source of carbon and/or nitrogen under anaerobic and aerobic conditions. He found only a few organisms capable of "good" growth on allantoin and failed to obtain even "feeble" growth with a number of genera reported by previous authors to be able to utilize allantoin. He criticized much of the early work on the basis that allantoin disappearance was not directly measured by chemical assay.

It was H.A. Barker, one of the outstanding microbiologists of our century, who first showed the ability of a bacterium to ferment allantoin under anaerobic conditions. Barker isolated a new species of homofermentative lactic acid bacteria from San Francisco Bay mud by using allantoin enrichment medium. He proposed the name Streptococcus allantoicus and left the decision as to the validity of the species to the future students of streptococci. After

studying the fermentation balance of the organism, the following fermentation balance was reported (in moles per mole of allantoin fermented): ammonia, 1.16; urea, 0.62; oxamic acid, 0.45; carbon dioxide, 1.68; formate, 0.09; acetate, 0.15; glycolate, 0.14; lactate, 0.01. In addition to the above, traces of glycine were also detected. Barker (1956, 1961) also showed that the nitrogen of oxamic acid is not derived from ammonia by using N^{15}-labeled ammonia. Therefore the oxamic acid had to be formed directly by the oxidative cleavage of allantoic acid.

Young and Hawkins (1944) showed that among ten intestinal bacteria tested only Escherichia coli, Aerobacter aerogenes, and Proteus vulgaris rapidly utilized allantoin dissolved in nutrient broth. They also showed that 3.3 moles of ammonia were formed from each mole of allantoin by E. coli. They concluded that the degradation of allantoin was not as simple as anticipated.

In spite of the reports that A. aerogenes can utilize allantoin as a nitrogen source (den Dooren de Jong, 1926; Mitchell and Levine, 1938) and that it can rapidly utilize allantoin (Young and Hawkins, 1944), Vogels proposed an "allantoin test" for the differentiation of the coli-aerogenes group (Vogels, 1963). According to him, only E. coli can utilize allantoin and A. aerogenes cannot. He went to the extent of suggesting the great importance of the "allantoin test" in water pollution control. He also concluded that this test has the same sensitivity as the

MacConkey test.

DiCarlo, Schultz, and Kent (1953), testing Saccharomyces cerevisiae, observed that this organism when utilizing urea, allantoic acid, and allantoin required increasing amounts of biotin. They concluded that the degradation of allantoin in this organism followed the pathway involving allantoinase and allantoicase.

Campbell (1954, 1955) isolated a Pseudomonas strain from San Francisco Bay mud using an enrichment medium containing allantoin. Using cells grown on nutrient broth, glucose, or allantoin in manometric assays, he concluded that the enzymes of allantoin metabolism were adaptively formed. He also showed that there was little difference in the ability of cells grown on allantoin and allantoic acid to degrade allantoin. With glyoxylate-grown cells, he was able to observe a long lag period before allantoin was degraded, even though glyoxylic acid and formic acid were oxidized immediately. Similarly a long lag period was required for the degradation of allantoin, allantoic acid, and glyoxylic acid when the cells were grown on formate, even though formate was degraded immediately. From these studies, Campbell concluded that the aerobic and anaerobic pathways are different even though the first steps are probably identical. He proposed the following sequence of reactions: allantoin ---→ allantoic acid ---→ glyoxylic acid + urea; glyoxylic acid ---→ CO_2 + HCOOH; HCOOH ---→ CO_2 + H_2O. Thiamine pyrophosphate and magnesium or manganese

were required for the oxidation of glyoxylate to formate and carbon dioxide.

Valentine and Wolfe (1960), in their studies on allantoin metabolism in S. allantoicus, showed that oxamic acid was formed from carbamyl oxamic acid by the mediation of the enzyme oxamic transcarbamylase. They further showed that the carbamyl phosphate derived from this reaction was decomposed to carbon dioxide and ammonia with the formation of adenosine triphosphate. Valentine and Wolfe (1961) also showed that for the formation of oxamate from allantoin or allantoic acid the partially purified enzyme required nicotinamide adenine dinucleotide, magnesium, and phosphate or arsenate.

Valentine and Wolfe (1961a) proposed the following intermediates for the fermentation of allantoin by S. allantoicus: allantoin ---> allantoic acid ---> glyoxylurea(ureidoglycolate) ---> carbamyl oxamate ---> oxamate. Valentine and Wolfe (1961b) described a method for the preparation of ureidoglycolate and demonstrated the ability of S. allantoicus extracts to degrade it. Vogels (1963) confirmed these findings.

Gaudy (1962) modified the procedure for the preparation of ureidoglycolate and showed that the conversion of ureidoglycolate to glyoxylate and urea was mediated by the enzyme glyoxylurease(ureidoglycolate synthetase). Gaudy (1962) and Gaudy, et al (1965) studied the conversion of ureidoglycolate to glyoxylate and urea. Gaudy (1962) and Gaudy and Wolfe

(1965) purified the ureidoglycolate synthetase of S. allantoicus 77-fold and showed the optimum pH to be 8.4 to 8.8. They further showed that the reaction catalyzed by the enzyme was reversible and determined the equilibrium constant to be 7.6.

A complete pathway for the degradation of allantoin was proposed by Valentine, et al (1962). The pathway for formation of oxamate was shown to conform to the prediction of Barker (1956, 1961). According to this pathway the "allantoicase reaction" involves two steps in S. allantoicus. The first product formed, ureidoglycolate, was shown to be the point of digression of the energy-yielding and biosynthetic pathways. Thus allantoic acid may lead to the formation of two moles of urea and one mole of glyoxylate per mole of allantoate or one mole of urea and one mole of carbamyl oxamate depending upon the fate of the ureidoglycolate formed.

Vogels (1963) agrees that allantoic acid is formed from allantoin by S. allantoicus, as proposed by Valentine, et al (1962). But according to him either ureidoglycine or ureidoglycolate can be formed from allantoate. The conversion of allantoate to ureidoglycine is mediated by the enzyme allantoate amidohydrolase. He also reported that this enzyme is activated by pretreating with acid and neutralization to pH 8.5. The process of activation has been claimed to be intramolecular rearrangement. In later investigations (Trijbels and Vogels, 1966a, 1966b; Vogels, 1963, 1966) he supported

the above findings. The organisms capable of degrading allantoate via this pathway, as reported by Vogels, are Pseudomonas acidovorans, Arthrobacter allantoicus, Streptococcus allantoicus, Escherichia coli, and E. coli var. acidilactici.

Gaudy and Bruce (1965) and Bruce (1965) obtained evidence in support of the pathway proposed by Valentine, Bojanowski, Gaudy, and Wolfe (1962). They isolated mutants from a strain of Pseudomonas aeruginosa by the use of ultraviolet light and ethylmethane sulfonate. These were blocked in different steps in the pathway. First, they used the ability of the mutants to grow on different intermediates as the criterion for the separation into different groups. The data were further strengthened by transduction studies. Winter (1967) obtained enzymatic data for the mutants.

Trijbels and Vogels (1966a, 1966b), using cell-free extracts of a urease-negative strain of Pseudomonas aeruginosa, found no ammonia formation from sodium allantoate. They obtained similar results using Pseudomonas fluorescens, Penicillium notatum, and Penicillium citreoviride. Since no ammonia was detected, they concluded that the degradation of allantoate was not brought about by the allantoate amidohydrolase-ureidoglycine aminohydrolase reactions. After these studies, Trijbels (1967) stated that "the occurrence of ureidoglycine as an intermediate in allantoin degradation has not been confirmed so far, since this compound was not available as a substrate nor could it

be isolated from media, in which allantoate degradation occurred."

Domnas (1962), using urease-negative strains of Saccharomyces cerevisiae and Candida (Torula) utilis, found both allantoicase and ureidoglycolate synthetase. He showed that the amount of enzyme found was affected by the nitrogen source upon which the organisms were grown. If the yeasts were grown on acetyl-urea or allantoin, the amount of glyoxylic acid formed from allantoic acid was quite significant. When grown on urea, no allantoicase was present. Both species contained ureidoglycolate synthetase. So he concluded that there seemed to be no pathway in these organisms for the degradation of urea through oxamic acid similar to that described by Barker (1961).

Darlington, et al (1965), using mutants of Aspergillus niger and the purine hypoxanthine as the sole nitrogen source, showed the path of purine breakdown to be hypoxanthine --→ xanthine ---→ uric acid ---→ allantoin ---→ allantoic acid ---→ urea ---→ ammonia. In their studies they had only one mutant which was blocked in the step allantoic acid ---→ urea, and thus no data were obtained which are pertinent to the controversial segment of the pathway.

Three different pathways which differ in the conversion of allantoic acid to glyoxylic acid have thus been proposed. They are shown in Figure 1.

Laskowski (1951) stated that no attempts had been made

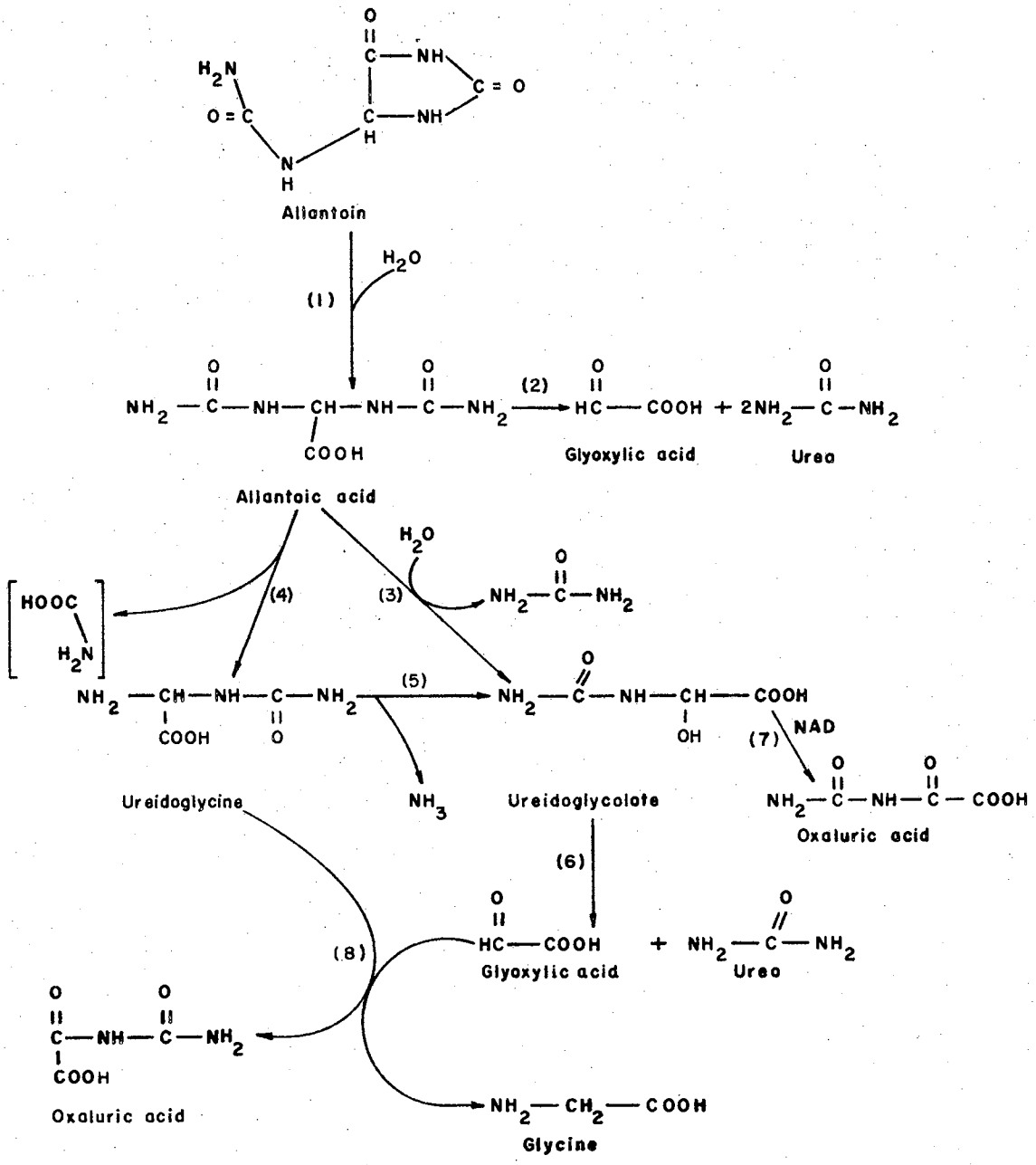


FIGURE I

PROPOSED PATHWAYS FOR ALLANTOIN DEGRADATION

to isolate allantoinase or allantoicase from S. allantoicus. Nagai and Funahashi (1961) purified allantoinase 65-fold from mung bean seedlings, by calcium phosphate gel treatment, followed by ammonium sulfate and acetone precipitation. Brunel (1936) did not succeed in purifying the allantoinase from Basidiomycetes due to the instability of the enzyme.

Vogels (1963) reported that the allantoinase activity in cell-free extracts of S. allantoicus and Arthrobacter allantoicus was completely destroyed by simple dialysis in distilled water or 0.1 M sodium phosphate buffer, pH 7. On storage at -20° C, the allantoinase activity of cell-free extracts of A. allantoicus was stable up to three months. Under the same conditions, the allantoinase activity of cell-free extracts of S. allantoicus was reduced by 75 per cent within two weeks.

Lee and Roush (1964) purified the allantoinase from S. cerevisiae six-fold. They reported that the enzyme is stable around pH 6 and has an optimum activity around pH 7-8. They also reported that the six-fold increase in the specific activity resulted in 58% loss of activity. The allantoinase from Candida utilis lost activity almost completely after ammonium sulfate treatment and dialysis.

Vogels, et al (1966) and Trijbels (1967) partially purified the allantoinases from S. allantoicus, A. allantoicus, E. coli, P. acidovorans, P. fluorescens, frog liver, goldfish liver, Phaseolus hysterinus, and Glycine hispida by ammonium sulfate fractionation and DEAE

column chromatography. They obtained different degrees of purification varying from 2.5 to 35-fold. They concluded that the allantoinases from higher plants and Pseudomonas species were rather stable whereas the enzymes from other bacteria, fungi, and yeasts were unstable.

In spite of the numerous reports regarding the ability of different bacteria to utilize allantoin as carbon and/or nitrogen source (Table I), Vogels (1963) was able to isolate only Streptococcus allantoicus and Arthrobacter allantoicus from allantoin enrichment cultures. He reported that among 126 stock cultures tested only 4 organisms belonging to the genus Pseudomonas and 3 belonging to the genus Escherichia could grow on allantoin. So he proposed the allantoin test for distinguishing Aerobacter and Escherichia.

In order to clarify the discrepancies existing between the many authors who reported their findings solely on the basis of growth on allantoin medium, with little or no idea as to the pathway, and the studies of Vogels (1963), a survey for organisms capable of utilizing allantoin under both aerobic and anaerobic conditions was carried out. In this study both stock cultures and numerous isolates from nature were included. The genus of each isolated strain was determined according to Bergey's Manual (1957), and species were determined in all cases where sufficient information is available for differentiation.

An additional objective of the survey for ability to use allantoin was to obtain a variety of organisms for use

in studies of the pathway of allantoin metabolism. Since three different pathways have been proposed, it is desirable to study these reactions in organisms belonging to different genera in order to determine whether one of the pathways is generally used by all bacteria or whether different organisms utilize different pathways. Enzyme assays, utilization of intermediates as carbon source and end-products of allantoin fermentation were employed as evidence of the pathways utilized.

At the time of the starting of this work, there were no reports on the purification and properties of allantoinase from S. allantoicus, and since Laskowski (1951) stated that S. allantoicus may prove to be an excellent source for this enzyme, it was decided to purify the enzyme from this organism.

CHAPTER II

MATERIALS AND METHODS

A. Cultures of Bacteria

The cultures of bacteria tested for their ability to utilize allantoin and its degradation products as carbon sources were obtained from the stock culture collection of the Department of Microbiology, Oklahoma State University, from individuals, or from allantoin enrichments. Allantoin enrichments were prepared by inoculating samples of soil or water from ponds, streams, or lakes into medium B (described below). These were incubated without aeration at room temperature or at 30° C.

After growth was obtained, the primary enrichment cultures were streaked on medium B solidified with 2% agar. Plates were incubated at 30° C, one plate from each enrichment aerobically and another in a N_2:CO_2 atmosphere. The procedure was repeated until pure cultures were obtained.

The organisms isolated from allantoin enrichments were identified according to Breed, Murray, and Smith (1957). Whenever the descriptions in the manual were inadequate for definite identification, it was limited to the level of genera. Identification tests for the isolates included: colonial morphology; morphology (wet mount and stained

preparations); motility and flagellation (wet mount, motility agar, Leifson's flagella stain); sugar fermentations; litmus milk reaction; Kligler's iron agar; SIM agar (Difco); citrate utilization (Koser's medium); starch hydrolysis; gelatin hydrolysis (tube and/or plate); nitrate reduction; indole formation; catalase; methyl red and acetyl methyl carbinol tests (MRVP broth); growth temperature; growth under aerobic and anaerobic conditions; requirement for organic nitrogen; fat hydrolysis (Nile blue agar), and Gram reaction. Pseudomonas aeruginosa strains were identified by phage typing. The names and sources of the organisms are presented in Appendix A.

B. Cultivation of Bacteria

The following media were used for the cultivation of organisms. The specific medium used depended upon the organism and the condition of growth (aerobic or anaerobic).

Medium A: $Na_2HPO_4 \cdot 7H_2O$, 8.2 gm; KH_2PO_4, 2.7 gm; $MgSO_4 \cdot 7H_2O$, 0.4 gm; NH_4Cl, 1.0 gm; $FeSO_4$, 0.1% solution, 0.5 ml; distilled water, 1 liter; allantoin was used at a concentration of 5 gm/l. Allantoin was dissolved in 500 ml water at 60° C and filtered through a Millipore filter (0.45 μ pore size). The rest of the salts were sterilized in 500 ml water and cooled to 60° before mixing with the allantoin solution.

Medium B: Medium B contained (per liter): 1.0 gm yeast extract (Difco); 1.0 gm K_2HPO_4; 4 ml of a 4% solution of

$MgSO_4$; 2 ml of a 2% solution of $MnSO_4$; 1.0 ml of a 1% solution of $CaCl_2$; 0.2 ml of a 0.1% solution of $FeSO_4$; 1.0 gm sodium mercaptoacetate; 4 gm allantoin and distilled water. Allantoin was dissolved and filtered separately as for Medium A.

Yeast extract and mercaptoacetate were added or omitted as required for individual organisms. Allantoin concentration was varied for certain experiments.

C. Chemicals

Allantoin was obtained from Sigma Chemical Company or from Nutritional Biochemical Corporation. Potassium allantoate was prepared by a modification of the method of Young and Conway (1942). 1.0 M KOH was heated to a temperature of 80° C and allantoin was dissolved in it to a concentration of 10% (w/v). The temperature was maintained at 80° C for 45 minutes. The solution was cooled and ten volumes of 95% ethanol were added. After overnight storage at 4° C, potassium allantoate crystals were harvested by filtration on a Buchner funnel. The crystals on the filter were washed with ethanol, acetone and ether and air-dried. The crystals were dissolved in a minimal volume of boiling distilled water and recrystallized. Ureidoglycolate was prepared according to Gaudy (1962).

D. Chemical Analyses

1. Allantoin

A sample containing 0.2 to 1.0 μmole of allantoin was made up to a volume of 5.0 ml with distilled water; 1.0 ml of 0.5 N NaOH and 2 drops of phenylhydrazine hydrochloride (100 mg/30 ml H_2O) were added. The tubes were immersed in a boiling water bath for seven minutes, then cooled in a water bath to room temperature. Then 1.0 ml of 0.65 N HCl and 1.0 ml of phenylhydrazine solution were added and the tubes were immersed in a boiling water bath for two minutes. The tubes were cooled to room temperature in a water bath, and 4.0 ml of 10 N HCl and 1.0 ml of potassium ferricyanide (500 mg/30 ml) were added. After 5 minutes at room temperature, the optical density was read at 515 mμ using a Coleman Junior Spectrophotometer.

2. Allantoate

A sample containing 0.2 to 1.0 μmole of allantoate was made up to 6.0 ml, 1.0 ml of 0.15 N HCl and 1.0 ml of phenylhydrazine solution were added and the tubes were placed for two minutes in a boiling water bath. The rest of the procedure was the same as that used for allantoin.

3. 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 and 1.0 ml of phenylhydrazine solution was added to the sample. The tubes were allowed to stand at room temperature for 10 minutes. The rest of the procedure was the same as for allantoin.

4. Oxamic Acid

Oxamic acid was estimated according to the method of Lipmann and Tuttle (1945). The sample was made up to a volume of 1.0 ml with distilled water, and 1.0 ml of a 28% solution of hydroxylamine hydrochloride (4M) neutralized with an equal volume of 14% NaOH (3.5M) to pH 6.4, was added. The tubes were placed in a boiling water bath for 15 minutes and cooled to room temperature in a water bath. After cooling, 1.0 ml of 0.1 M acetate buffer, pH 5.4, was added and the tubes were allowed to stand for 10 minutes at room temperature. After 10 minutes, 1.0 ml of 3 M HCl and 1.0 ml of a 12% solution of trichloroacetic acid were added, followed by 1.0 ml of a 5% solution of $FeCl_2 \cdot 6H_2O$ in 0.1 N HCl. The tubes were allowed to stand for 15 minutes at room temperature before reading at 540 mμ in a Coleman Junior Spectrophotometer.

5. Protein Content of Cell Extracts

Protein was determined according to the method of Sutherland, et al (1949). Cell extracts were diluted 1:20. Different volumes of this dilution (0.1 to 0.6 ml) were made up to 1.0 ml with water. To each tube, 5.0 ml of a reagent

containing 100 ml of 4% Na_2CO_3, 1.0 ml of 4% sodium-potassium tartrate and 1.0 ml of 2% $CuSO_4$ were added. The tubes were allowed to stand for 40 minutes at room temperature. Phenol reagent 2 N (Fisher Scientific Company) was diluted 1:2 with water and 0.5 ml of this reagent was added and mixed immediately. The color was read, after 15 minutes, at 660 mμ. Standards of bovine serum albumin, 30 to 90 μg, were run along with the samples.

E. Growth of Bacteria on Allantoin

1. Growth under Aerobic Conditions

Organisms which were capable of growing on minimal medium were grown in medium A for 18 hours at 30° or 37° C on a shaker in 18 mm tubes and the optical density was read at 540 mμ.

Organisms which required organic nitrogen were grown in medium B, without mercaptoacetate, and with and without allantoin to maximum growth. The optical density due to utilization of allantoin was calculated by difference.

For determining the relationship between growth and allantoin utilization, the organisms were grown in side-arm flasks in medium B with and without allantoin. The optical density was determined every 60 minutes and a sample was taken out each time the optical density was determined. The samples were centrifuged and the supernatants were frozen immediately and assayed later for allantoin content.

2. Growth under Anaerobic Conditions

Anaerobic growth was determined by growing the organisms in screw-cap tubes completely filled with medium B for 18 hours in a 95% N_2:5% CO_2 atmosphere at 30° C. The cells were removed by centrifugation and the allantoin and oxamic acid contents of the supernatants were determined. Reduction of allantoin concentration in the medium was used as a criterion for ability to ferment allantoin.

F. Pathway of Allantoin Degradation

1. Use of Allantoin and Intermediates

Medium B was modified by adding agar, reducing yeast extract to 0.05%, and omitting mercaptoacetate. 10 ml portions of this medium were cooled in a water bath to 46° C. A fresh nutrient agar slant was washed with 1.0 ml saline and 0.1 ml of this suspension was added to the medium at 46° C. The tubes were mixed and the suspension was poured into petri plates. After the plates had solidified, approximately 25 mg of each carbon source (allantoin, allantoate, ureidoglycolate, and glyoxylate) were placed on the surface of the plates at marked spots and growth was scored after 24-48 hours incubation at 30° C.

G. Growth of Cells and Preparation of Extracts

Cells were grown aerobically in medium A or B on a shaker (200 ml/1000 ml flask) or in 8 liter volumes aerated

in 20-liter carboys. For anaerobic growth, cells were grown in medium B in 250 ml flasks filled to the top or in filled 20 liter carboys.

For preparing cell-free extracts, cells were harvested in a cooled Sharples centrifuge or in a refrigerated Servall centrifuge, model RC-2. Cells were broken with a Bronson sonifier after suspending in 0.02 M potassium phosphate buffer pH 7 or were frozen at -20° C, disintegrated in an X-press (Biochemical Processes, Inc.) and suspended in 0.02 M phosphate buffer, pH 7. In the case of Streptococcus allantoicus, the disintegrated cells were suspended in 0.02 M N,N-BIS(2-hydroxyethyl)glycine, pH 9. For suspending disintegrated cells of anaerobes, 0.05% mercaptoacetate was added to the buffer.

H. Enzyme Assays

1. Allantoinase

The incubation mixture contained 0.2 ml of buffer, 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized), 0.4 ml allantoin (5 mg/ml), an appropriate amount of enzyme and distilled water to 1.0 ml. The pH of the buffer depended upon the extract used. Tubes were incubated at 30° C and samples were removed for determination of allantoate and/or glyoxylate.

2. Allantoicase

The assay was similar to that for allantoinase except that an equal amount of potassium allantoate was used instead of allantoin, and glyoxylate was measured.

3. Ureidoglycolate Synthetase

The assay was similar to those for allantoinase and allantoicase except that 2 mg of sodium ureidoglycolate were used as substrate and the incubation time was 10 minutes. Glyoxylate formed was determined.

4. Allantoate Amidohydrolase

The assay was performed according to Vogels (1966). The ammonia formed was determined by direct Nesslerization or by a modified Conway method. The micro-diffusion dish contains three chambers. To the inner chamber, 3.0 ml of 0.02 N H_2SO_4 were added. To the outer chamber, 2.0 ml of 45% K_2CO_3 in 0.025% Tergitol NPX (non-ionic detergent) were added. To the middle chamber, 1.0 ml of the incubation mixture and 2.0 ml of 45% K_2CO_3 in 0.025% NPX were added and the dishes were incubated at 37° C for 6 hours. After 6 hours, 1.0 ml H_2SO_4 was removed from the center compartment and the ammonia content was determined by Nesslerization.

CHAPTER III

EXPERIMENTAL RESULTS

A. Isolation and Identification of Organisms

Fifty-two organisms, isolated from ponds, lakes, soil, sewage, and air, were identified using the methods and tests listed previously in Materials and Methods. The sources and identities of the organisms isolated in these studies are given in Appendix A.

The organisms isolated belonged to nine genera. Almost half the organisms isolated (25 strains) belonged to the genus Pseudomonas. Since information given in Bergey's Manual is insufficient for identification of most species of this genus, these organisms were separated into groups on the basis of 19 tests. Characteristics of the 11 groups of Pseudomonas are shown in Appendix B.

Stock cultures used in these studies, and the source from which each was obtained, are listed in Appendix C.

B. Growth on Allantoin

1. Aerobic Growth on Allantoin Minimal Medium

The organisms isolated from the different sources were tested for their ability to grow on glucose minimal medium.

Those organisms which were able to grow in 30 hours on glucose, using an ammonium salt as sole nitrogen source, were inoculated into medium A and growth was measured, after incubation for 18 hours, as optical density at 540 mμ. In the preliminary experiments, it was found that only organisms belonging to the genus Pseudomonas were able to grow on minimal medium. Among those organisms which were able to grow on allantoin minimal medium, four belonged to group III, and one to each of groups V, VI, X, and XI. Two of the stock cultures, Pseudomonas A and Pseudomonas C, which were reported by Bachrach (1957) to be able to grow on uric acid minimal medium, were tested but were found to be unable to grow on allantoin minimal medium. Altogether, five of the organisms showed feeble or no growth. Of the three isolates which grew poorly, one belonged to group III, one to group XI, and the other was Pseudomonas caviae. The data are presented in Table II.

2. Aerobic Growth on Allantoin with Organic Nitrogen

Other organisms, including stock cultures and all the isolates, were tested for their ability to grow on allantoin in medium B. An optical density of 0.100 or greater was arbitrarily chosen as a positive growth response. Bacteria belonging to seven different genera were capable of growing on this medium, including Aerobacter, Alcaligenes, Arthrobacter, Escherichia, Flavobacterium, Nocardia, and Pseudomonas species. Species belonging to 4 of these

TABLE II

AEROBIC GROWTH ON ALLANTOIN MINIMAL MEDIUM (A)

Organism		Group	O.D.
Soil-2	*Pseudomonas aeruginosa*	XI	0.264
LC-9	*Pseudomonas aeruginosa*	XI	0.102
T2b	*Pseudomonas caviae*	VIII	0.018
	Pseudomonas A		0.073
	Pseudomonas C		0.065
YL-2	*Pseudomonas* sp.	III	0.362
Sew-1	*Pseudomonas* sp.	III	0.099
SC-3	*Pseudomonas* sp.	III	0.317
Sew-2	*Pseudomonas* sp.	III	0.403
LC-19	*Pseudomonas* sp.	III	0.196
SC-1	*Pseudomonas* sp.	V	0.435
TP-2	*Pseudomonas* sp.	VI	0.367
Sew-3	*Pseudomonas* sp.	X	0.354

Cultures were incubated at 30° or 37° C on a shaker in 18 mm test tubes. Incubation time was 14-18 hours. An optical density of 0.1 or less was considered as poor growth.

genera and 6 other genera were found to be unable to grow even in the presence of organic nitrogen. These included species of Aerobacter, Arthrobacter, Bacillus, Brevibacterium, Escherichia, Micrococcus, Proteus, Pseudomonas, Sarcina, and Staphylococcus. These data are shown in Table III.

3. Growth vs. Allantoin Utilization

With the object of determining whether the organisms which grew on medium B were really using allantoin for growth, the optical density and the allantoin remaining were determined at 60-minute intervals during incubation. The results are shown in Figures 2 through 6. For all the five organisms tested, growth and optical density were closely correlated. There was a lag in the growth of the organism and in the uptake of allantoin. The length of the lag period varied from organism to organism. However, once growth started, the disappearance of allantoin and the increase in optical density were very rapid.

4. Anaerobic Growth on Allantoin

Facultative anaerobes were tested for their ability to ferment allantoin as measured by allantoin disappearance. No strict anaerobes were isolated or tested. All the isolates which were able to utilize allantoin anaerobically belonged to three genera. These data are shown in Table IV. The utilization of allantoin varied from 100% to 10% after

TABLE III

AEROBIC GROWTH IN MEDIUM B

Organism		OD
T1d1	Aerobacter aerogenes	0.316
LC-6	Alcaligenes sp.	0.132
LC-12	Alcaligenes sp.	0.247
LC-15	Alcaligenes sp.	0.247
13346	Arthrobacter pascens	0.337
LC-5	Arthrobacter sp.	0.327
LC-23	Arthrobacter sp.	0.327
LC-26	Arthrobacter sp.	0.357
T4a	Escherichia freundii	0.160
T4b	Escherichia freundii	0.224
	Flavobacterium sp.	0.196
LC-17	Nocardia sp.	0.327
K-2	Nocardia sp.	0.300
Soil-2	Pseudomonas aeruginosa XI	0.277
LC-9	Pseudomonas aeruginosa XI	0.383
T2b	Pseudomonas caviae VIII	0.128
	Pseudomonas fluorescens	0.265
	Pseudomonas B	0.161
T1a	Pseudomonas sp. II	0.396
T5b	Pseudomonas sp. II	0.372
T6	Pseudomonas sp. II	0.226
T7a	Pseudomonas sp. II	0.349
T7b	Pseudomonas sp. II	0.436
Soil-1	Pseudomonas sp. II	0.173
LC-16	Pseudomonas sp. II	0.509
Sew-1	Pseudomonas sp. III	0.167
Sew-2	Pseudomonas sp. III	0.245
YL-2	Pseudomonas sp. III	0.219
SC-3	Pseudomonas sp. III	0.327
LC-19	Pseudomonas sp. III	0.385
T1c2	Pseudomonas sp. IV	0.278
T1d2	Pseudomonas sp. IV	0.374
T3a	Pseudomonas sp. IV	0.303
SC-1	Pseudomonas sp. V	0.284
TP-2	Pseudomonas sp. VI	0.322
T2a	Pseudomonas sp. VII	0.116
Sew-3	Pseudomonas sp. X	0.198
T1b	Aerobacter aerogenes	0.075
6946	Arthrobacter simplex	0.000
T1c1	Arthrobacter sp.	0.000
YL-1	Arthrobacter sp.	0.000
LC-7	Arthrobacter sp.	0.000
	Bacillus cereus	0.006
	Bacillus megaterium	0.004

TABLE III (Continued)

Organism		OD
LC-1	*Bacillus* sp.	0.000
LC-2	*Bacillus* sp.	0.000
LC-3	*Bacillus* sp.	0.013
LC-4	*Bacillus* sp.	0.000
LC-8	*Bacillus* sp.	0.000
LC-10	*Bacillus* sp.	0.012
LC-20	*Bacillus* sp.	0.084
	Brevibacterium fuscum	0.000
23	*Escherichia coli*	0.000
B	*Escherichia coli*	0.000
316	*Escherichia coli*	0.000
F1	*Escherichia coli*	0.016
F2a	*Escherichia coli*	0.000
T3b	*Escherichia freundii*	0.000
T5a	*Escherichia freundii*	0.040
	Micrococcus roseus	0.000
	Proteus vulgaris	0.000
950	*Pseudomonas ovalis*	0.000
8209	*Pseudomonas ovalis*	0.053
	Pseudomonas A	0.000
	Pseudomonas C	0.000
F2b	*Pseudomonas* sp. I	0.013
LC-14	*Pseudomonas* sp. II	0.046
TP-1	*Pseudomonas* sp. IX	0.043
LC-18	*Sarcina hansenii*	0.009
SC-2	*Staphylococcus aureus*	0.039

Cultures were grown to maximum optical density at 30° or 37°C with shaking. Medium B containing 0.1% yeast extract and no mercaptoacetate was used with and without allantoin. Control cultures without allantoin were used to correct optical densities.

Figure 2. Growth vs. Allantoin Uptake for Strain TP-2, Pseudomonas sp. Group VI.
Cells were grown in medium B in flasks with side-arm tubes. Optical density was read hourly and samples were removed each hour, centrifuged, and the supernatants stored in the freezer for determination of the allantoin content. Controls were set up in medium without allantoin. Growth on allantoin alone was calculated by subtracting the optical density of the control culture grown without allantoin. These values were plotted.

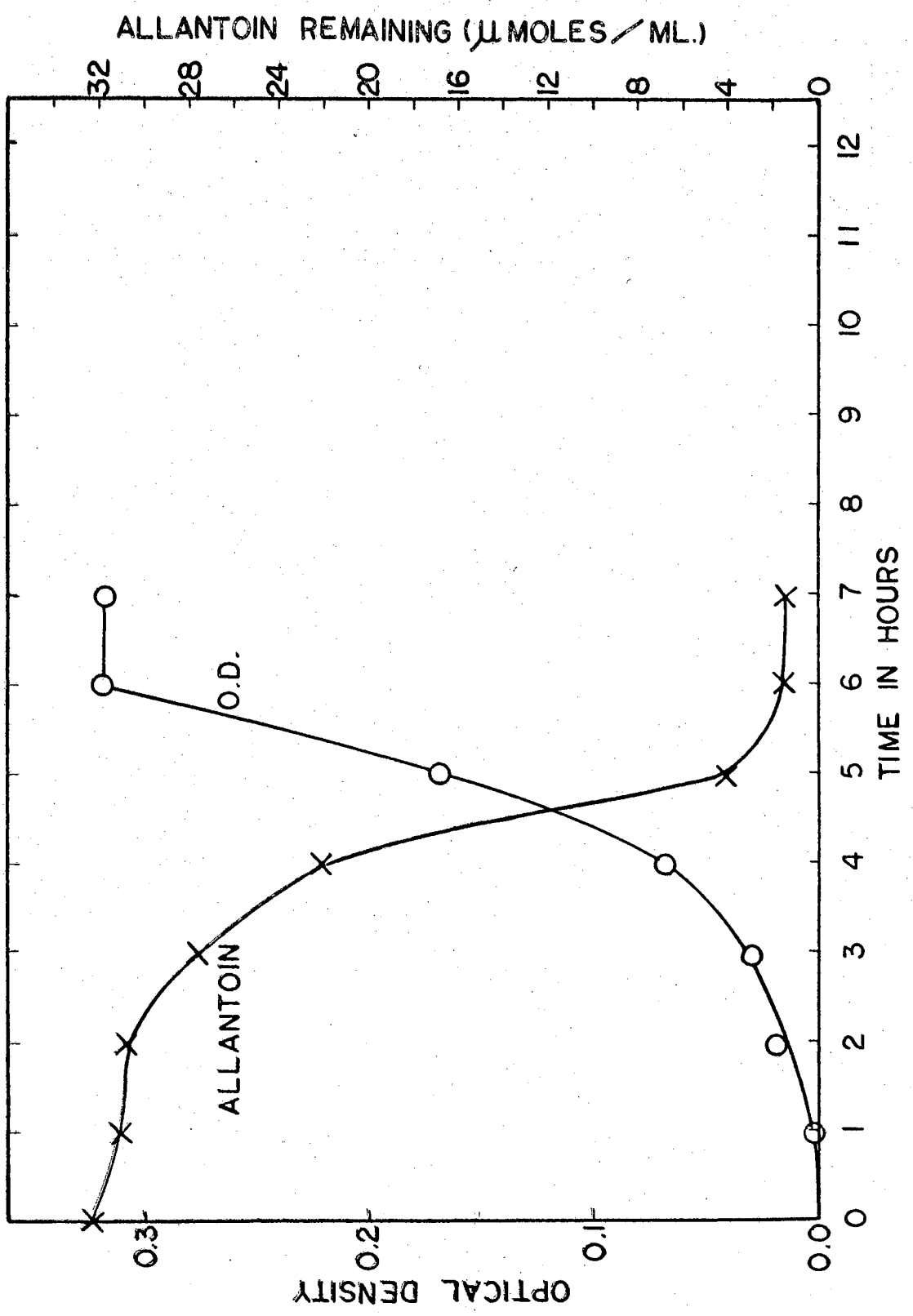

ALLANTOIN REMAINING (μ MOLES / ML.)
32
28
24
20
16
12
8
4
0
O.D.
ALLANTOIN
0.3
0.2
0.1
0.0
OPTICAL DENSITY
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN HOURS

Figure 3. Growth vs. Allantoin Uptake for Strain LC-17, Nocardia sp.
The procedure was identical to that described for Figure 2.

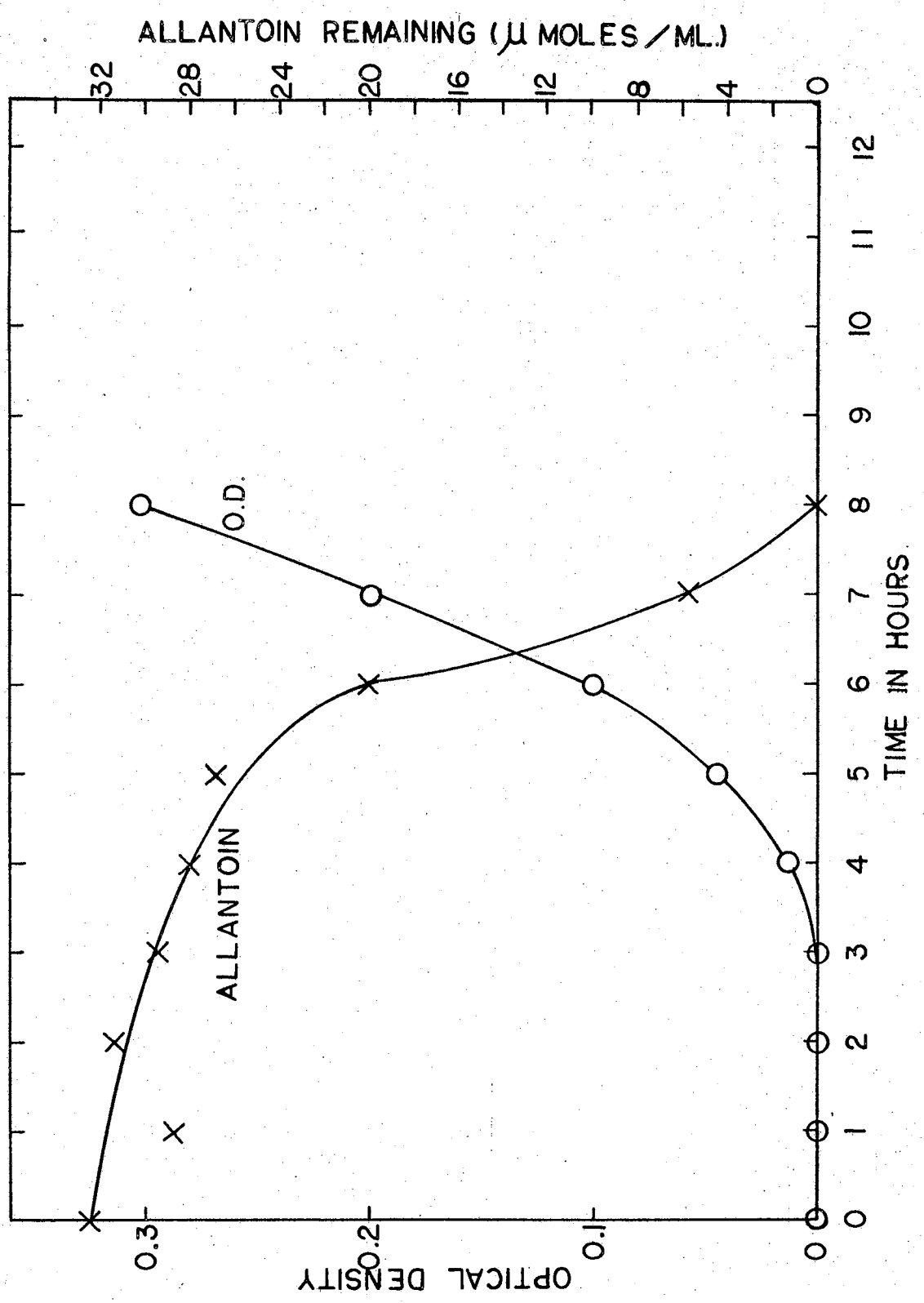

ALLANTOIN REMAINING (μ MOLES / ML.)
32
28
24
20
16
12
8
4
0
O.D.
ALLANTOIN
0.3
0.2
0.1
0
OPTICAL DENSITY
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN HOURS

Figure 4. Growth vs. Allantoin Uptake for Strain SC-1, Pseudomonas sp. Group V.
The procedure was identical to that described for Figure 2.

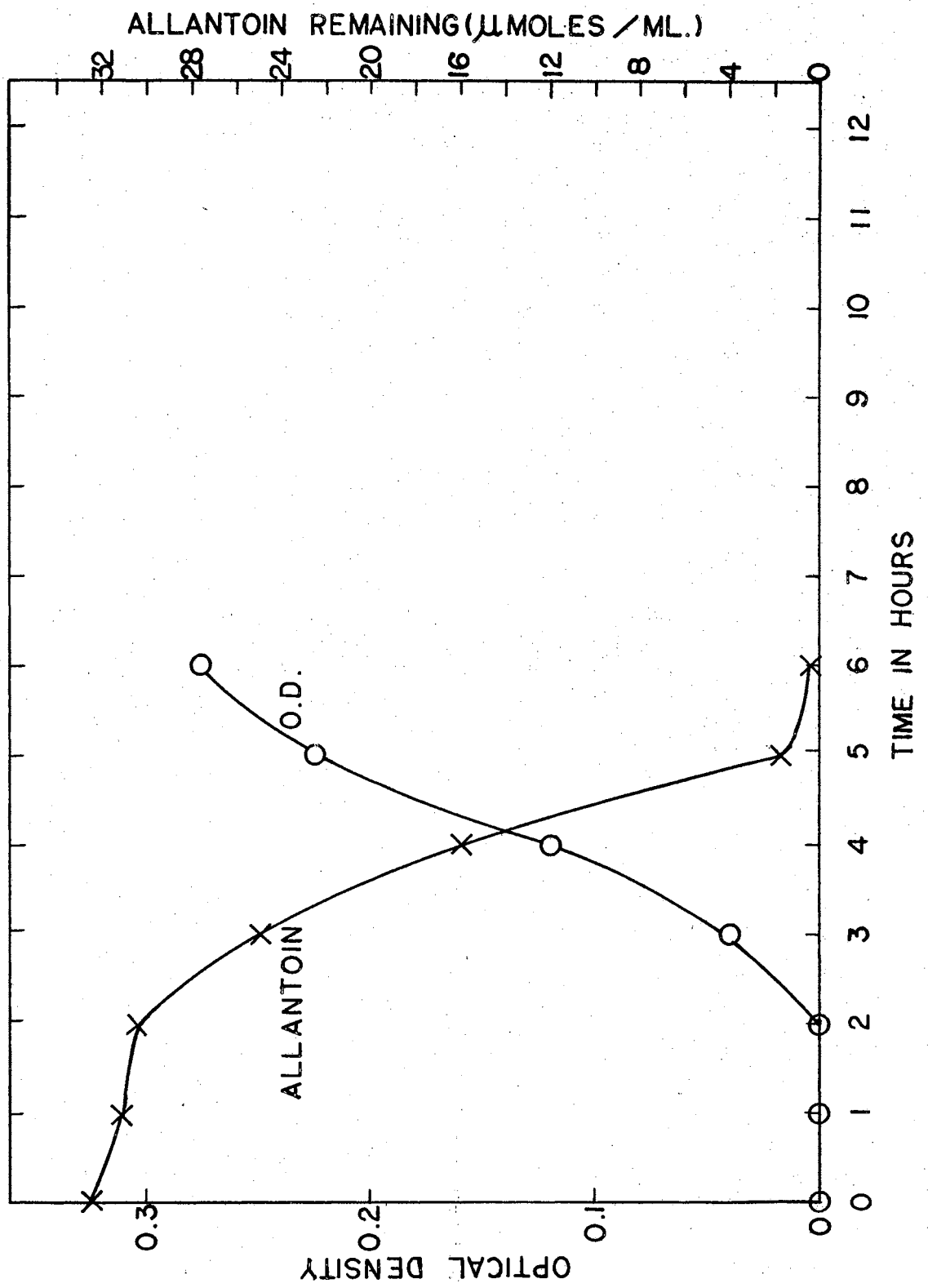

ALLANTOIN REMAINING (μ MOLES / ML.)
32
28
24
20
16
12
8
4
0
0.3
0.2
0.1
0.0
OPTICAL DENSITY
O.D.
ALLANTOIN
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN HOURS

Figure 5. Growth vs. Allantoin Uptake for Strain LC-5, Arthrobacter sp.
The procedure was identical to that described for Figure 2.

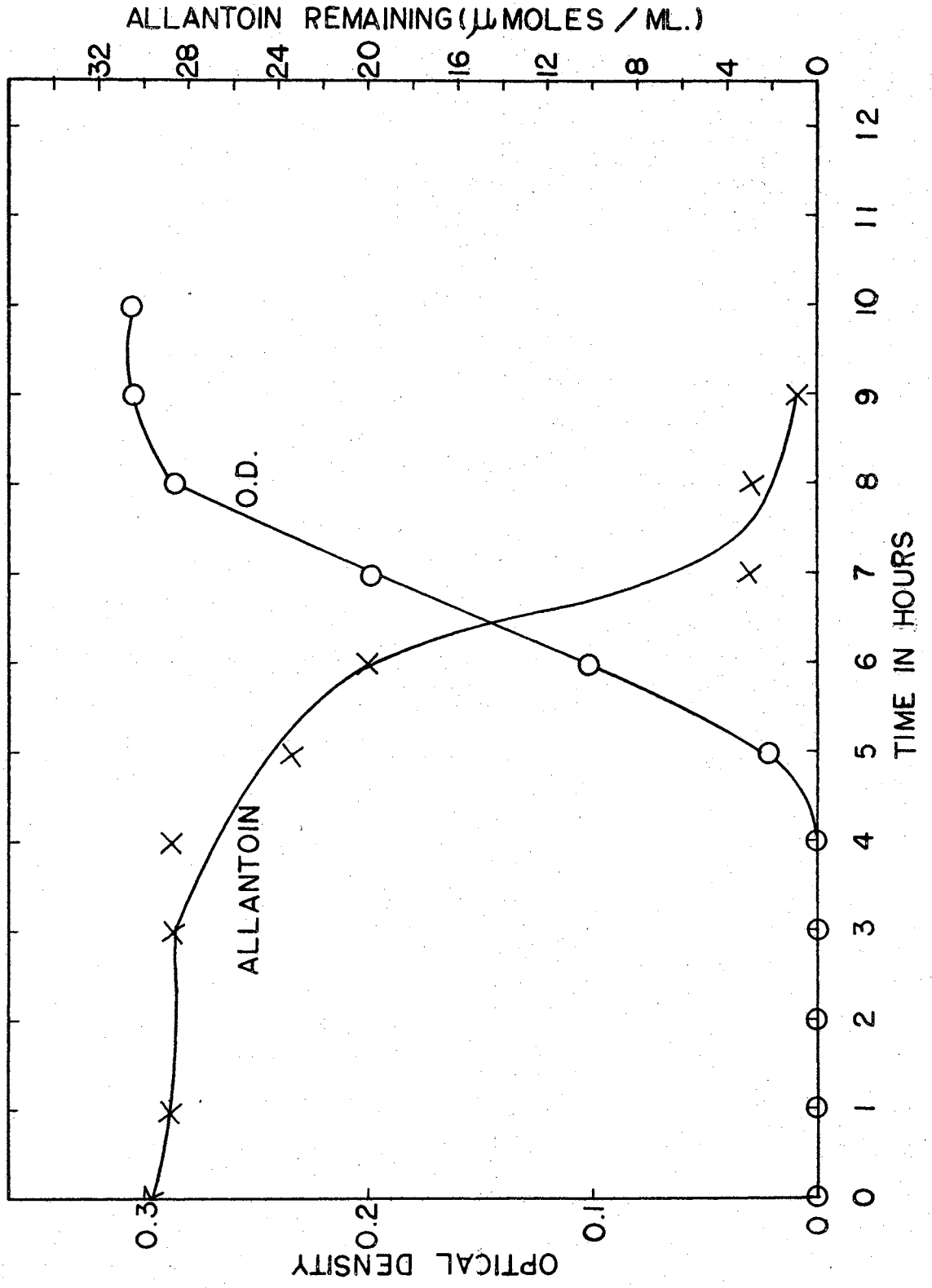

ALLANTOIN REMAINING (μ MOLES / ML.)
32
28
24
20
16
12
8
4
0
O.D.
ALLANTOIN
0.3
0.2
0.1
0
OPTICAL DENSITY
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN HOURS

Figure 6. Growth vs. Allantoin Uptake for Strain SC-3, Pseudomonas sp. Group III.
The procedure was identical to that described for Figure 2.

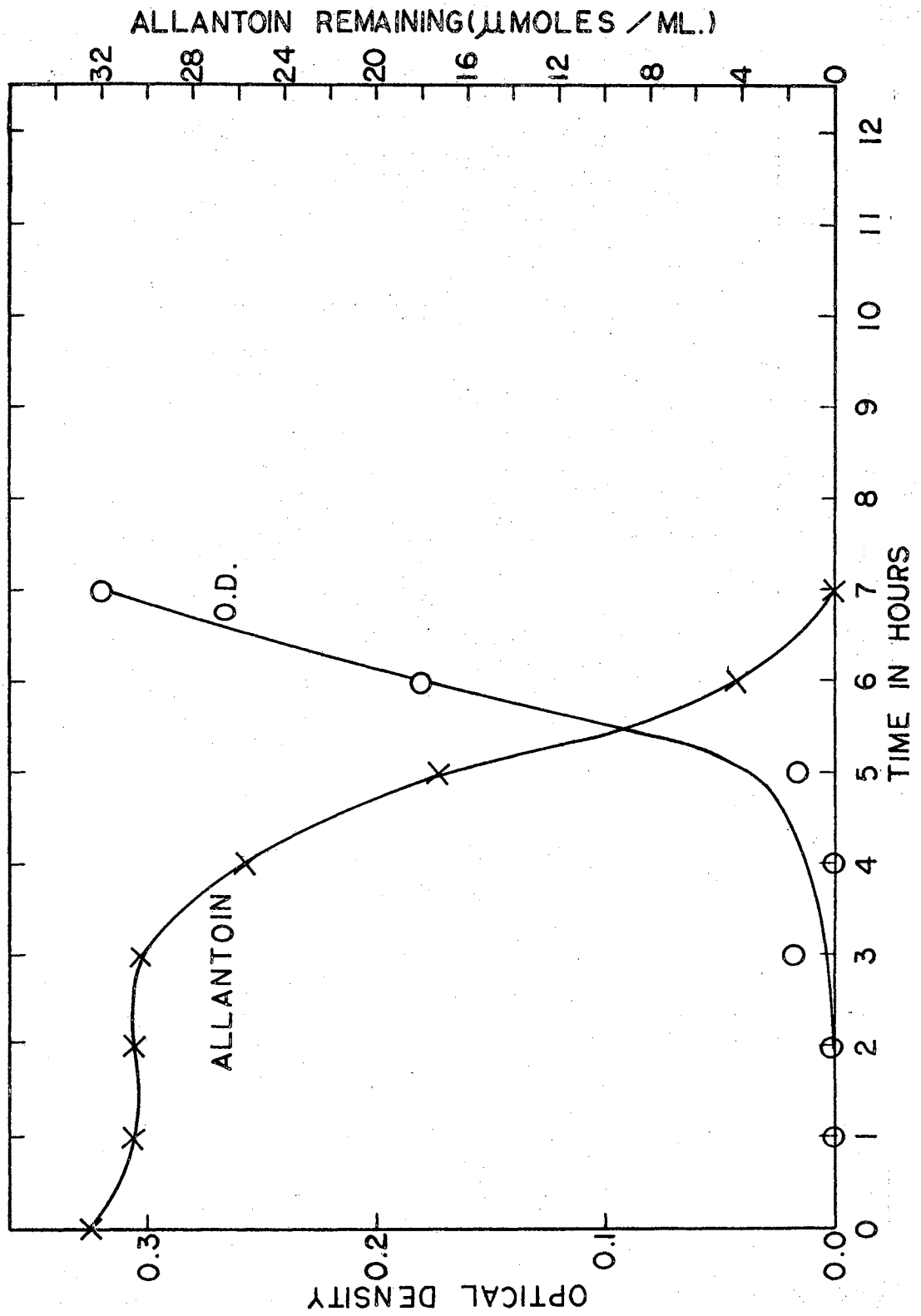

ALLANTOIN REMAINING(μMOLES / ML.)
32
28
24
20
16
12
8
4
0
O.D.
ALLANTOIN
0.3
0.2
0.1
0.0
OPTICAL DENSITY
0
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN HOURS

TABLE IV

ANAEROBIC UTILIZATION OF ALLANTOIN AND PRODUCTION OF OXAMATE

Organism	Hours of Incubation	% Allantoin Used	µmoles of oxamate per 100 µmoles allantoin
T1b *Aerobacter aerogenes*	18	100	53
T1d1 *Aerobacter aerogenes*	18	100	61
F2a *Escherichia coli*	18	36	77
T3b *Escherichia freundii*	18	100	55
T4a *Escherichia freundii*	18	84	62
	95	98	59
T4b *Escherichia freundii*	18	19	70
	95	99	55
T5a *Escherichia freundii*	18	10	98
	95	98	59
T2a *Pseudomonas* sp. VII	18	27	21
Streptococcus allantoicus	18	95	44

18 hours of incubation. Even within the same species, wide differences could be noted in ability to utilize allantoin under anaerobic conditions. All organisms tested produced oxamate from allantoin. The percentage of allantoin converted to oxamate also varied between wide limits.

C. Pathway of Allantoin Degradation

1. Growth on Allantoin and Its Intermediates

Twenty-seven bacteria belonging to seven different genera were tested for their ability to utilize allantoin and its intermediates as carbon sources. The patterns of growth are shown in Table V. The pattern of utilization varied from the ability to utilize all the four compounds to inability to utilize any compound. The organisms were divided into groups according to their ability to utilize allantoin and its intermediates. No pattern of utilization was characteristic of all species of a single genus.

2. Use of Allantoin as a Sole Nitrogen Source

According to the pathway proposed by Vogels (1963), ammonia is produced through the allantoate amidohydrolase reaction. Trijbels and Vogels (1966b) reported that allantoate amidohydrolase is present in Pseudomonas acidovorans. It was thought that any organism which has allantoate amidohydrolase should be able to grow using allantoin as the sole nitrogen source in the absence of urease. Therefore, 18 Pseudomonas species were tested for the ability to grow

TABLE V

GROWTH ON ALLANTOIN AND ITS INTERMEDIATES

Organism		A	KA	UG	G
LC-26	Arthrobacter sp.	+	±	±	±
T4a	Escherichia freundii	+	+	+	+
T5a	Escherichia freundii	+	±	±	+
T3b	Escherichia freundii	+	+	+	+
T5b	Pseudomonas sp. II	+	+	+	+
T1a	Pseudomonas sp. II	+	+	+	+
T7b	Pseudomonas sp. II	+	+	+	+
T6	Pseudomonas sp. II	+	+	+	+
T1d2	Pseudomonas sp. IV	+	+	+	+
T1c2	Pseudomonas sp. IV	+	+	+	+
LC-9	Pseudomonas aeruginosa XI	+	+	+	-
LC-17	Nocardia sp.	+	+	-	-
LC-5	Arthrobacter sp.	+	-	-	-
K-2	Nocardia sp.	+	-	-	-
23	Escherichia coli	-	±	+	+
T2a	Pseudomonas sp. VII	-	+	+	+
T22	Salmonella typhimurium	-	±	+	+
LC-23	Arthrobacter sp.	+	±	-	±
T4b	Escherichia freundii	+	+	-	+
T1b	Aerobacter aerogenes	-	-	±	±
T1c1	Arthrobacter sp.	-	-	±	±
F1	Escherichia coli	-	-	+	+
F2b	Pseudomonas sp. I	-	-	+	+
T1d1	Aerobacter aerogenes	-	-	±	-
T2b	Pseudomonas caviae VIII	-	-	+	-
	Proteus vulgaris	-	-	-	-

A = allantoin. KA = potassium allantoate. UG = ureidoglycolate. G = glyoxylate.
Cells were suspended in 10 ml of medium B without mercaptoacetate, containing 1.5% agar and 0.05% yeast extract. The medium was melted, cooled to 45° C, and cells added. The plates were poured and allowed to harden and 25 mg of each carbon source was placed at marked points on the surface.

on glucose minimal medium containing urea as the sole nitrogen source. Among 18 organisms tested, no organism could be found which was capable of growing on allantoin in the absence of urease. The organisms which have urease are capable of using allantoin as a carbon and nitrogen source as can be seen from Table VI.

3. Enzyme Activities of Cell Extracts

Cell-free extracts of twenty bacteria were tested for ureidoglycolate synthetase activity and extracts of eight organisms were tested for allantoate amidohydrolase activity. The data are presented in Table VII. The organisms belonged to 11 different genera. Among the twenty organisms, fifteen belonging to eight different genera had ureidoglycolate synthetase activity and five organisms belonging to four genera showed no activity. Allantoate amidohydrolase activity was tested in eight organisms belonging to six genera. Only one organism, Streptococcus allantoicus, possessed this enzyme activity.

4. Allantoin Degradation in Nocardia

The enzymes involved in the degradation of allantoin in Nocardia were assayed. The specific activities of these enzymes are presented in Table VIII. This organism had allantoinase, allantoicase, and ureidoglycolate synthetase activities, but no allantoate amidohydrolase activity was detected.

TABLE VI

AEROBIC GROWTH ON ALLANTOIN AS CARBON AND NITROGEN SOURCE

Organism			Urea + Glucose	Allantoin + NH_4Cl	Allantoin Only
T2b	*Pseudomonas*	*caviae* VIII	0.000	0.018	0.043
	Pseudomonas	A	0.000	0.073	0.013
	Pseudomonas	C	0.000	0.065	0.013
Sew-2	*Pseudomonas*	sp. III	1.071	0.403	0.382
SC-1	*Pseudomonas*	sp. V	1.083		0.382
Sew-3	*Pseudomonas*	sp. X	1.140	0.354	0.385
PA-1	*Pseudomonas*	*aeruginosa*	0.757		
PA-6	*Pseudomonas*	*aeruginosa*	0.545		
PA-7	*Pseudomonas*	*aeruginosa*	0.268		
PA-8	*Pseudomonas*	*aeruginosa*	1.022		
LC-9	*Pseudomonas*	*aeruginosa*	1.046		
Soil-2	*Pseudomonas*	*aeruginosa*	1.171		
	Pseudomonas	B	0.502		
SC-3	*Pseudomonas*	sp. III	0.854		
Sew-1	*Pseudomonas*	sp. III	0.699		
YL-2	*Pseudomonas*	sp. III	0.969		
LC-19	*Pseudomonas*	sp. III	0.959		
TP-2	*Pseudomonas*	sp. VI	0.979		

Medium A was modified by using urea as sole nitrogen source and glucose as carbon source. Numbers in table are optical density readings.

TABLE VII

UREIDOGLYCOLATE SYNTHETASE AND ALLANTOATE AMIDOHYDROLASE ACTIVITIES IN CELL-FREE EXTRACTS

Organism		Growth of Cells	UGS	AA
T1b	*Aerobacter aerogenes*	Anaerobic	+	
LC-6	*Alcaligenes* sp.	Aerobic	+	-
LC-23	*Arthrobacter* sp.	Aerobic	+	-
YL-1	*Arthrobacter* sp.	Aerobic	-	
	Bacillus cereus	Aerobic	-	
	Bacillus megaterium	Aerobic	-	
	Brevibacterium fuscum	Aerobic	-	
F1	*Escherichia coli*	Anaerobic	+	
F2a	*Escherichia coli*	Anaerobic	+	-
T4b	*Escherichia freundii*	Anaerobic	+	
	Flavobacterium sp.	Aerobic	+	
LC-17	*Nocardia* sp.	Aerobic	+	-
T1a	*Pseudomonas* sp. II	Aerobic	+	-
YL-2	*Pseudomonas* sp. III	Aerobic	+	
SC-1	*Pseudomonas* sp. V	Aerobic	+	
SC-3	*Pseudomonas* sp. III	Aerobic	+	
TP-2	*Pseudomonas* sp. VI	Aerobic	+	-
PA-1	*Pseudomonas aeruginosa*	Aerobic	+	-
	Streptococcus allantoicus	Anaerobic	+	+
LC-18	*Sarcina hansenii*	Aerobic	-	

UGS = ureidoglycolate synthetase. AA = allantoate amidohydrolase.

TABLE VIII

SPECIFIC ACTIVITIES OF ENZYMES OF ALLANTOIN DEGRADATION FROM A NOCARDIA SPECIES, LC-17

Enzyme	Sp. Act., μmoles/min/mg protein
Allantoinase	0.250
Allantoicase	0.138
Ureidoglycolate synthetase	0.990
Allantoate amidohydrolase	0.000

D. Purification of Allantoinase

1. $MnCl_2$ Treatment

Crude extract (Fraction A) was prepared according to the method described in Materials and Methods. Fraction A was placed on a magnetic stirrer in an ice bath and a 0.05 volume of 1.0 M $MnCl_2$ was added at the rate of one drop each 30 seconds with constant stirring. The extract was stirred for an additional 15 minutes. The precipitate was centrifuged out at 0° C. The clear extract was dialyzed against 4 liters of 0.02 M bicine buffer, pH 9.0, containing 0.05% sodium mercaptoacetate for 18 hours; the buffer was changed at 9 hours. This treatment removed about 2.3 mg protein/ml and resulted in 1.19 to 1.3-fold purification depending on the pH used for assaying the enzyme activity. The dialyzed extract was designated as Fraction B.

2. Ammonium Sulfate Fractionation

Fraction B was stirred slowly in an ice bath on a magnetic stirrer and cysteine hydrochloride was added slowly to bring the pH to 7.0. Then solid ammonium sulfate was added slowly with contant stirring. The precipitates formed at 0-50%, 50-60%, 60-70%, and 70-80% of saturation were collected separately by centrifugation. Each was dissolved in 5 ml of 0.02 M bicine buffer, pH 9.0, containing 0.05% sodium mercaptoacetate and dialyzed against 3 liters of the same buffer for 6 hours, changing the buffer at 3 hours. The

fractions which precipitated between 50-60% and 60-70% of saturation contained allantoinase activity. The fraction precipitated between 60 and 70% saturation, Fraction E, contained 2.3 mg of protein per ml. This fraction was purified 6.2 times, based on the assays performed at pH 8.0 for all fractions, or 4.3-fold if the assays for Fractions A and B were performed at pH 10.0. A summary of the purification procedure is given in Table IX. Fraction E was used for studying the properties of the enzyme.

E. Properties of Allantoinase

1. Stability of the Enzyme

In initial experiments, the enzyme was found to be highly unstable at pH 7 in the freezer. Therefore, the effects of different pH's and storage temperatures were investigated. The results are presented in Table X. Altogether, three different pH's (7.0, 8.0, and 9.0) and three different storage conditions (room temperature, 4° C, and -20° C) were investigated. Fraction A was found to be most stable in the freezer (-20° C) at pH 9.0 and somewhat less stable in the cold room (4° C) at pH 9.0. Fraction E was found to be stable at pH 9.0 in the cold room (4° C). No activity could be detected in Fraction E at the same concentration of enzyme when stored in the freezer at pH 9.0 or after storage at pH 7.0 in either the cold room or freezer.

TABLE IX

PURIFICATION OF ALLANTOINASE

Fraction	Total Units	% Recovery	Protein mg/ml	Specific Activity	Assay pH	Purification
A, Crude Extract	1615.05	100.0	9.7	11.1	8.0	1.00
B, Mn-Treated	1465.20	90.7	7.4	13.2	8.0	1.19
E, Ammonium Sulfate	792.35	49.0	2.3	68.9	8.0	6.30
A, Crude Extract	2269.80	100.0	9.7	15.6	10.0	1.00
B, Mn-Treated	2331.00	102.6	7.4	21.0	10.0	1.30
E, Ammonium Sulfate	792.35	35.0	2.3	68.9	8.0	4.30

Fresh Fractions A and B had an optimum pH of 10.0. Ammonium sulfate-treated extract was found to have a pH optimum of 8.0. After storage, the pH optimum of Fractions A and B drops to pH 8.0. Therefore, the purification of Fraction E was calculated in two ways. The upper portion of the table shows purification calculations based on the activity of Fraction A at pH 8.0, measured after storage for one month. The lower portion shows purification data based on the apparent optimum pH of each fraction, using fresh Fraction A.

TABLE X

EFFECT OF pH AND TEMPERATURE ON ENZYME STABILITY

Storage	Buffer	Storage pH	Storage Days	Assay pH	Specific Activity
1. Effect of pH on storage of Fraction A.					
Freezer	Phosphate	7.0	6	10	0.55
Freezer	Bicine	8.0	6	10	5.71
Freezer	Bicine	9.0	6	10	9.28
2. Effect of temperature on storage of Fraction A.					
None	Bicine	9.0	0	10	12.80
Room Temperature	Bicine	9.0	2	10	3.58
4° C	Bicine	9.0	13	10	7.55
Freezer	Bicine	9.0	13	10	12.10
3. Effect of temperature and pH on storage of Fraction E.					
4° C	Phosphate	7.0	11	8	0.00
Freezer	Phosphate	7.0	11	8	0.00
4° C	Bicine	9.0	11	8	30.17
Freezer	Bicine	9.0	11	8	0.00

2. Reactivation at Room Temperature

The effect of room temperature on Fraction B which had only 1/8 of the original activity after storage at pH 9.0 in a freezer is presented in Table XI. It can be seen that the enzyme regained one-half of its original activity.

3. Optimum pH for Allantoinase

The optimum pH for Fraction A was found to be 10.0 for fresh extract and 8.0 for old extract. The effect of pH on the allantoinase activity of Fraction E is shown in Figure 7. The optimum pH is 8.0-8.5. N-tris(hydroxymethyl)-methyl-3-aminoethanesulfonic acid (TES) buffer was used for the pH range 7.0 to 8.0 and N,N-bis(2-hydroxyethyl)glycine(bicine) was used for the pH range 8.0 to 9.0. Glycine-sodium hydroxide buffer was used for the pH range 9.0 to 10.5.

4. Enzyme Activity vs. Time

Figure 8 shows the rate of the allantoinase reaction vs. time. The rate is constant over a period of 30 minutes. Due to limiting substrate concentration, allantoic acid formation decreased after 30 minutes. The amount of allantoin which can be used in the incubation mixture is limited by its low solubility. The standard assay time of 10 minutes is within the linear response time for the enzyme.

5. Enzyme Activity vs. Protein Concentration

A range of 0 to 34.5 μg of protein was used in this

TABLE XI

REACTIVATION AT ROOM TEMPERATURE

Fraction	Storage	Time of Storage	Initial Activity	Final Activity
Fraction B	Freezer	1 day	---	16.80
Fraction B	Freezer	38 days	16.80	2.37
Fraction B	Room Temperature	4 days	2.37	7.38

Fraction A was prepared by suspending disrupted cells in bicine buffer, pH 9.0, and Fraction B was prepared by treating with $MnCl_2$ and dialyzing at pH 9.0 in bicine buffer. The initial activity was determined. Then this preparation was stored for 38 days in the freezer and the activity was again determined. The same extract was then stored at room temperature for 4 days and the activity was again determined.

Figure 7. Optimum pH for Allantoinase Activity. The incubation mixture contained: 0.4 ml of buffer, 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized), 0.4 ml allantoin (12.6 μmoles) and 0.1 ml of a 1/20 dilution of Fraction E. After 10 minutes incubation, the allantoate formed was assayed. N-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer was used for the pH range 7.0 to 8.0, N,N-Bis(2-hydroxyethyl) glycine (Bicine) buffer for the pH range 8.0 to 9.0, and glycine-sodium hydroxide buffer for the pH range 9.0 to 10.5.

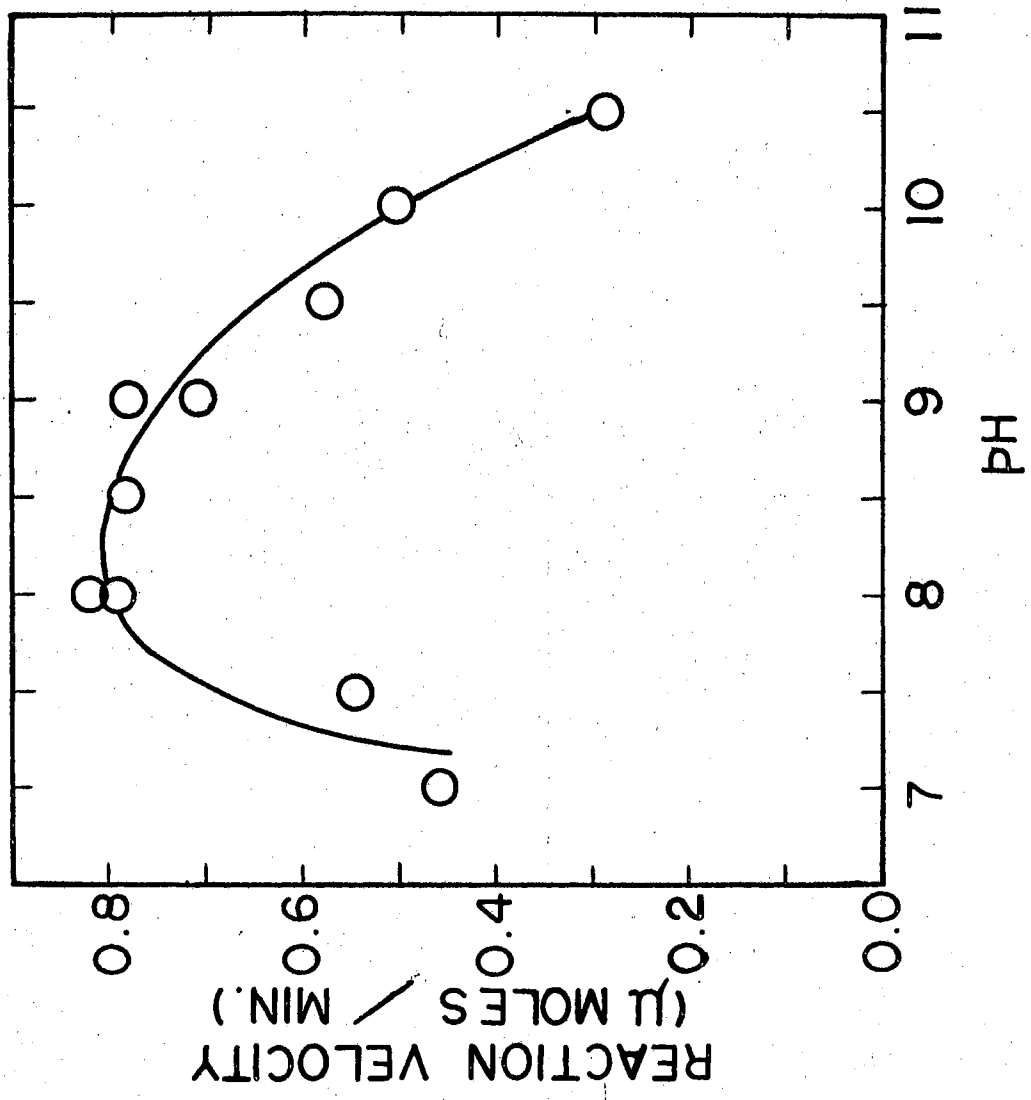
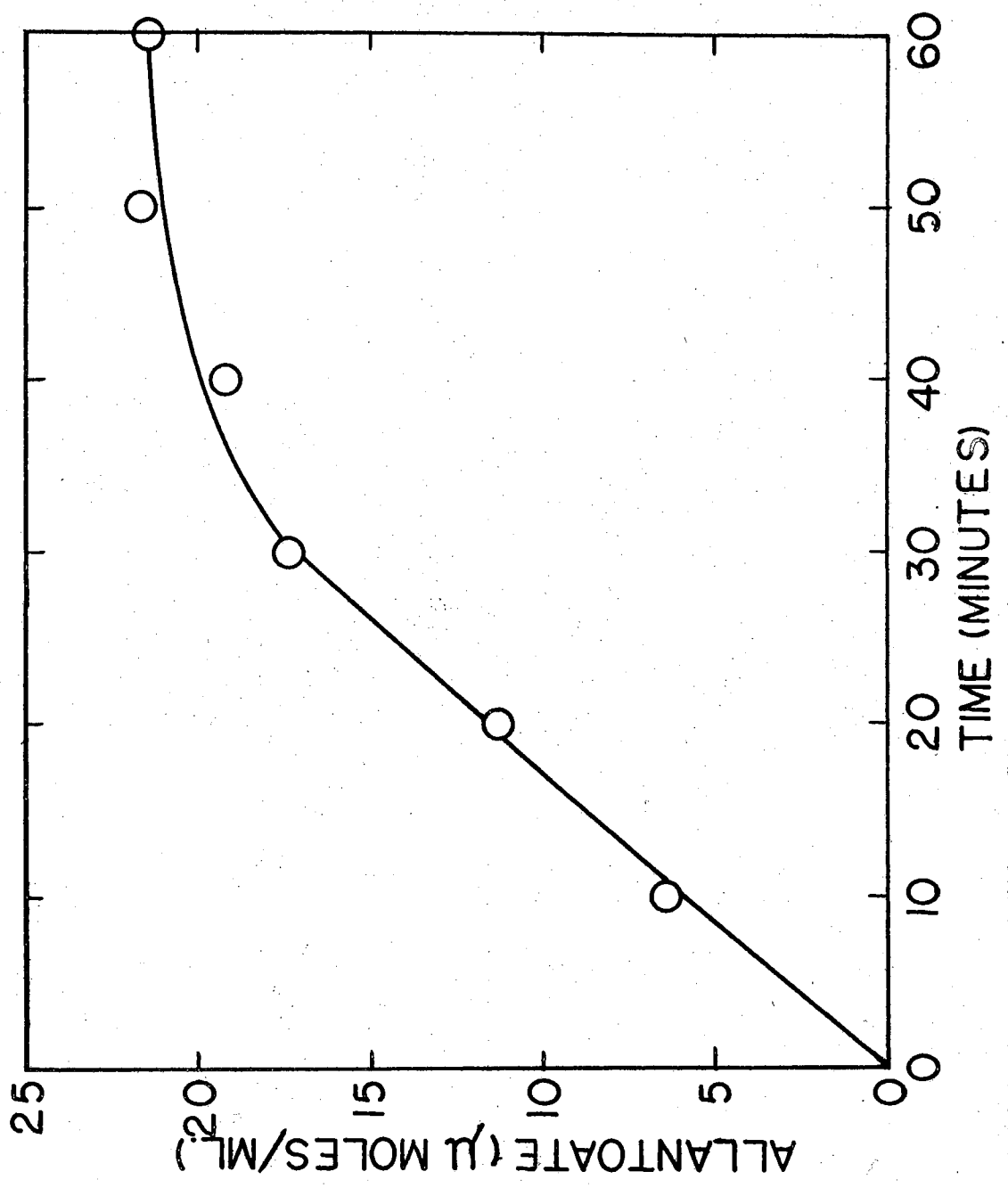

REACTION VELOCITY
(μ MOLES / MIN.)
0.0
0.2
0.4
0.6
0.8
7
8
9
10
11
pH

Figure 8. Enzyme Activity vs. Time.
The incubation mixture contained: 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized), 0.8 ml of allantoin dissolved in 0.1 M bicine buffer, pH 8.0, (25.3 μmoles) and 0.1 ml of a 1/20 dilution of Fraction E. Samples were taken at 10 minute intervals over a period of one hour.


ALLANTOATE (μ MOLES/ML.)
0
5
10
15
20
25
0
10
20
30
40
50
60
TIME (MINUTES)

assay. The results are shown in Figure 9. The reaction velocity was found to be proportional to enzyme concentration up to a total protein concentration of 11.5 µg/tube.

6. Enzyme Activity vs. Substrate Concentration

A Lineweaver-Burk (1936) plot of the variation in reaction velocity with the concentration of substrate is shown in Figure 10. The value of Km obtained graphically was 1.18×10^{-5} M. Maximum velocity for the allantoinase preparation was found to be 1.25 µM/min and, assuming the molecular weight of the protein to be 100,000, the turnover number was calculated as 1.09×10^{4} moles/minute/mole of protein.

Figure 9. Enzyme Activity vs. Protein Concentration. The incubation mixture contained: 0.2 ml of 0.1 M bicine buffer, pH 8.0, 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized), 0.4 ml of allantoin (12.6 µmoles), volumes of Fraction E containing 2.3 to 34.5 µg total protein, and distilled water to 1.0 ml. Incubation time was 10 minutes.

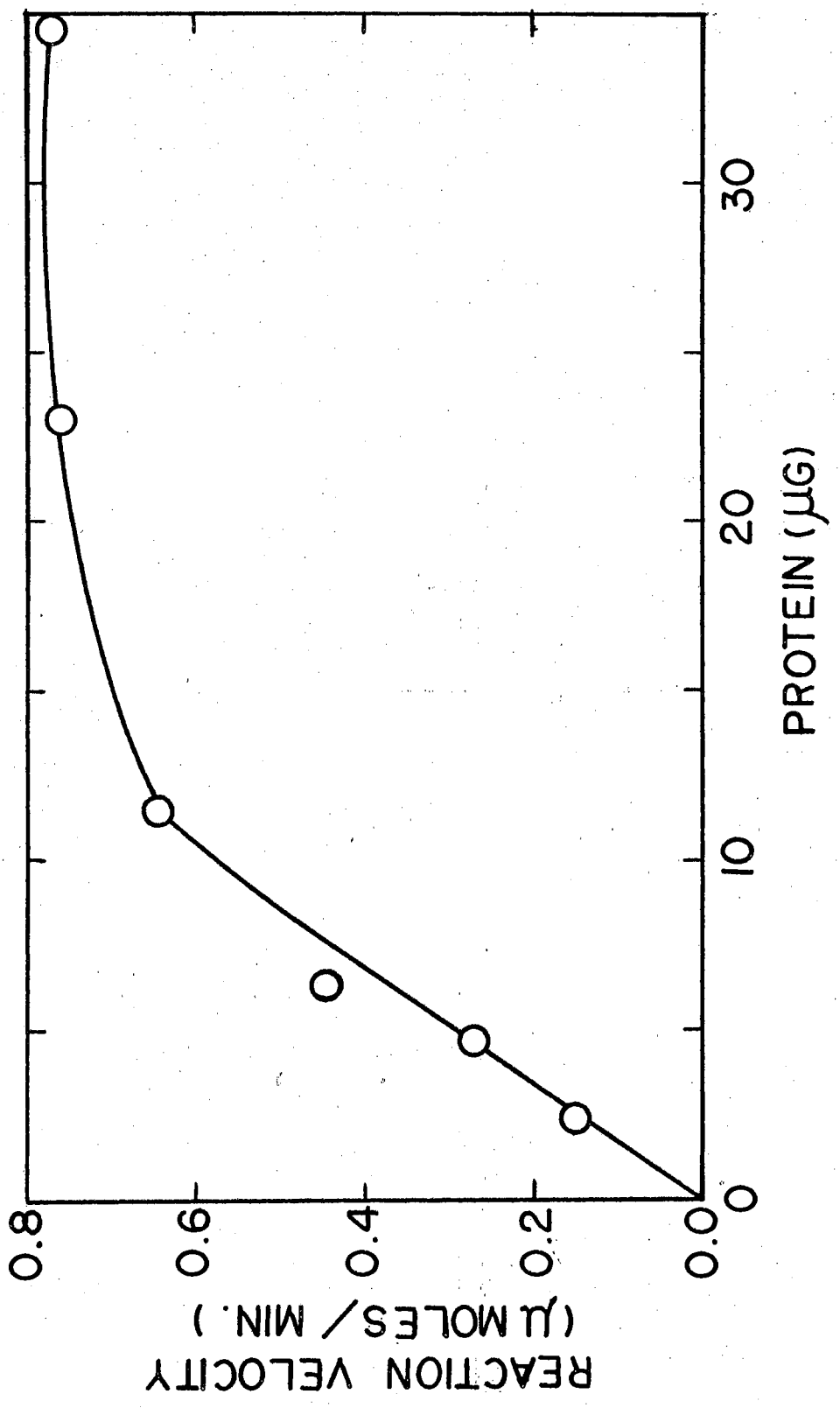
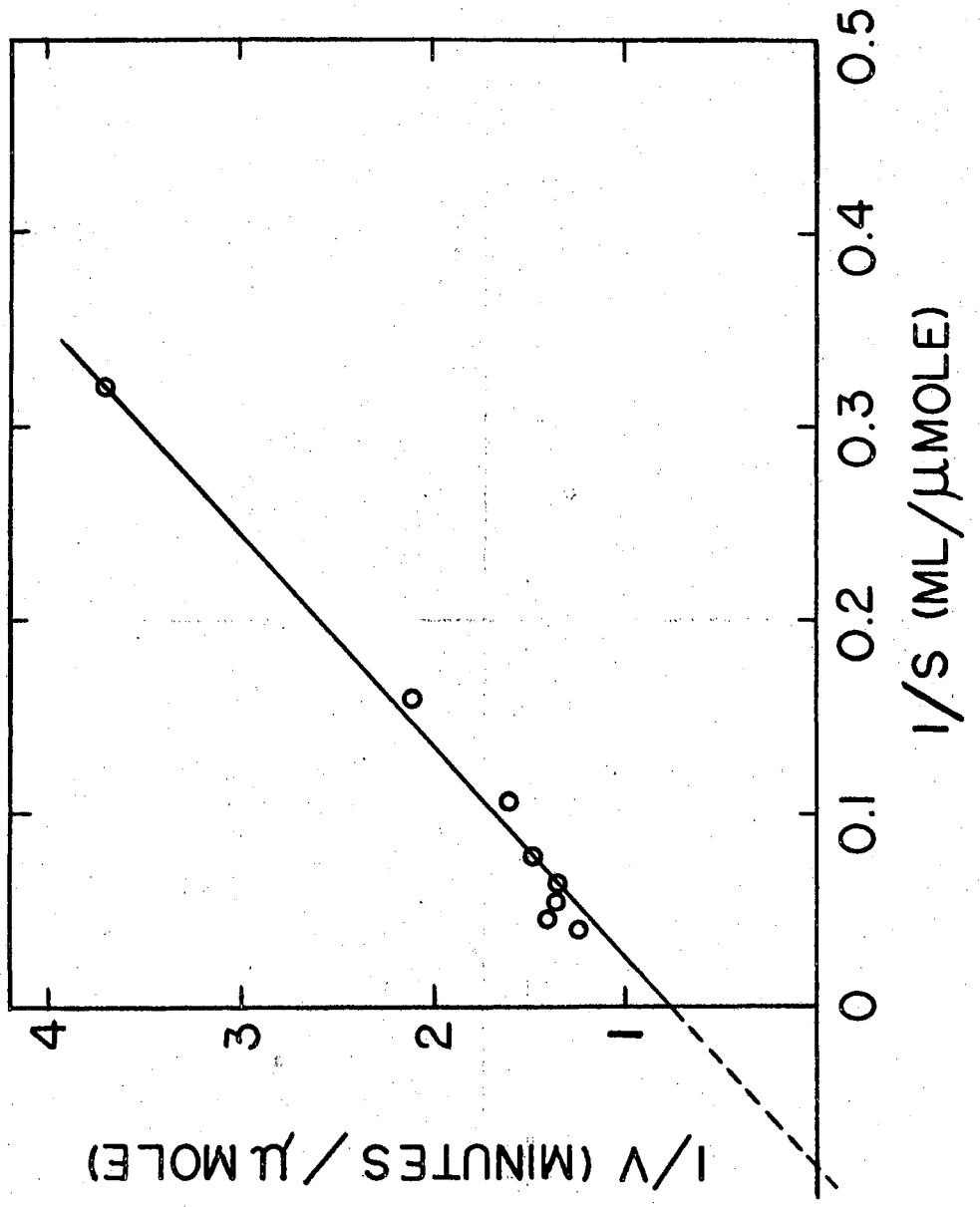

REACTION VELOCITY
(μ MOLES/ MIN.)
0.0
0.2
0.4
0.6
0.8
0
10
20
30
PROTEIN (μG)

Figure 10. Enzyme Activity vs. Substrate Concentration. The incubation mixture contained: 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized), 0.1 to 0.8 ml of allantoin (3.2 to 25.3 μmoles) dissolved in 0.1 M bicine buffer, pH 8.0, 0.1 ml of a 1/20 dilution of Fraction E and the required volume of 0.1 M bicine buffer, pH 8.0, to give a total volume of 1.0 ml. Incubation time was 10 minutes.


1/V (MINUTES / μ MOLE)
4
3
2
1
0
0.1
0.2
0.3
0.4
0.5
1/S (ML/μMOLE)

CHAPTER IV

DISCUSSION

A. The Metabolism of Allantoin by Bacteria

Studies of growth on allantoin done before 1963 showed that bacteria belonging to the genera Pseudomonas, Aerobacter, Spirillum, Bacillus, Mycobacterium, Nocardia, Arthrobacter, Streptococcus, Sarcina, and Serratia could use allantoin as a carbon and/or nitrogen source. But Vogels (1963) reported after testing a large number of stock cultures that, under aerobic conditions, good growth was obtained only with three organisms belonging to the genus Pseudomonas, and "feeble" growth was obtained with species of Pseudomonas, Arthrobacter, Flavobacterium, and Brevibacterium. He also reported that under anaerobic conditions good growth was obtained with organisms belonging to the genera Streptococcus, Arthrobacter, and Escherichia and feeble growth was obtained with two Pseudomonas species.

The discrepancies between results obtained by previous investigators and by Vogels may be explained in the light of results obtained in the present studies. These studies show that the metabolism of allantoin is neither confined to a few related genera nor is it universal in bacteria. Even within the same genus or species, some strains are able to

grow on allantoin and some are unable to do so.

The present study indicates that aerobic growth on allantoin is more common among Pseudomonas species than among any other organisms tested. Of the organisms studied, only those belonging to the genus Pseudomonas were capable of growing on allantoin minimal medium utilizing allantoin as the sole carbon and/or nitrogen source.

Bacteria belonging to the genera Aerobacter, Alcaligenes, Arthrobacter, Escherichia, Flavobacterium, Nocardia, and Pseudomonas were capable of growing on allantoin in the presence of yeast extract. Certain bacteria belonging to the genera Aerobacter, Arthrobacter, Bacillus, Brevibacterium, Escherichia, Micrococcus, Proteus, Pseudomonas, Sarcina, and Staphylococcus were unable to grow on allantoin even in the presence of yeast extract. Pseudomonas A and Pseudomonas C, which were reported by Bachrach (1957) to be able to grow on uric acid minimal medium, were unable to grow on allantoin medium even in the presence of yeast extract.

In general, it may be stated that a greater variety of genera are capable of growth on allantoin than would be realized by testing stock cultures. The discrepancy between the studies of Vogels (1963) and the present investigation may be due to the fact that Vogels used stock cultures and in the present study a large number of newly isolated organisms were used.

Evidence indicates that the enzymes of allantoin

degradation are probably inducible (Figures 2 through 6). This was previously reported by Campbell (1954) for a Pseudomonas species.

Ability to ferment allantoin, as determined by allantoin disappearance, was found in A. aerogenes, E. coli, E. freundii, and a species of Pseudomonas. Hence there is no basis for the allantoin test proposed by Vogels (1963) to distinguish Aerobacter and Escherichia.

Enzymatic data, as evidenced by the presence of ureidoglycolate synthetase shown in Table VII, indicate that in A. aerogenes, Alcaligenes sp., Arthrobacter sp., E. coli, E. freundii, Nocardia sp., and Pseudomonas sp. allantoin is degraded through ureidoglycolate.

It can be seen by comparing the enzymatic and growth data (Tables II, III, IV, and VII) that, among the organisms tested, only those which possess the enzyme ureidoglycolate synthetase were able to utilize allantoin. Those without this enzyme were unable to do so.

The production of oxamate under anaerobic conditions by bacteria belonging to the genera Aerobacter, Escherichia, and Pseudomonas, and the absence of allantoate amidohydrolase activities in cell-free extracts of these organisms show that allantoin fermentation in these organisms follows the pathway proposed by Valentine, Bojanowski, Gaudy, and Wolfe (1962).

In his studies, Vogels (Trijbels and Vogels, 1966a, 1966b; Vogels 1963, 1966) reported that he could detect

allantoate amidohydrolase activity in Pseudomonas acidovorans, Arthrobacter allantoicus, E. coli, and Streptococcus allantoicus.

Cell-free extracts of allantoin-grown cells of Alcaligenes sp., E. coli, Arthrobacter sp., Nocardia sp., and three Pseudomonas species tested in the present study had no allantoate amidohydrolase activity. Allantoate amidohydrolase activity could be detected only in S. allantoicus.

Eighteen species or strains of Pseudomonas were tested for ability to use urea as a nitrogen source in an effort to find organisms which might utilize the pathway of allantoin degradation proposed by Vogels (1963). Utilization of allantoin as sole nitrogen source in the absence of urease activity would indicate the possible presence of allantoate amidohydrolase, which forms ammonia from allantoate. Only three of the organisms tested lacked urease and none of these was able to grow on allantoin. Therefore, this attempt to locate organisms using the allantoate amidohydrolase pathway was unsuccessful. While it cannot be definitely stated, based on these results, that none of the fifteen organisms possessing urease possesses allantoate amidohydrolase activity, it can at least be stated that all would be capable of using allantoin as a sole nitrogen source in the absence of this enzyme.

The distribution of the enzymes of allantoin degradation, as evidenced by carbon source utilization, is shown in Table V. The inability to grow on a particular

intermediate may be due to the absence of the enzyme responsible for the degradation or due to the lack of a permease. It has been shown previously (Bruce, 1965) that the inability of P. aeruginosa, strain PA-1, to grow on ureidoglycolate or glyoxylate is due to impermeability. In the present study, four organisms which were unable to use ureidoglycolate as a carbon source were shown to possess the enzyme for its degradation. These were Nocardia sp. LC-17, Arthrobacter sp. LC-23, Aerobacter aerogenes T1b, and Escherichia coli F1. Thus, it appears that impermeability to this compound may be fairly common even among bacteria in which it is used when formed internally from allantoin.

The enzyme data for a Nocardia sp. (Table VIII) show allantoate, ureidoglycolate, and glyoxylate to be intermediates in the degradation of allantoin in this organism.

B. Purification and Properties of Allantoinase

Allantoinase from Streptococcus allantoicus was purified 6.3 times, or 4.3 times, depending upon whether the calculations are based on fresh Fraction A or aged Fraction A. This discrepancy occurs because the optimum pH of fresh Fraction A was found to be 10.0; however, after storage the optimum pH drops to 8.0. It has been shown by Gaudy (1962) that, in fresh extracts of S. allantoicus, glyoxylate carboligase is very active, and the phenylhydrazine added to the incubation mixture to trap the glyoxylate formed does not do so completely. However, in old extracts, it was

shown that there was no glyoxylate carboligase activity. These results have been confirmed in the present study for stored Fraction A. There was no difference in glyoxylate formation in the presence and absence of phenylhydrazine when aged Fraction A was used; whereas, with fresh extract, no glyoxylate is detectible in the absence of phenylhydrazine. The high pH required for optimum specific activity in fresh extracts is due to the inhibition of glyoxylate carboligase at pH 10.0 and the consequent accumulation of glyoxylate.

The effect of pH and temperature of storage on allantoinase activity varied with the purity of the preparation. Crude preparations of allantoinase were found to be stable in the freezer at pH 9.0. But, with purification, the enzyme becomes more unstable at -20° C. It can be seen from Table X that Fraction E stored at pH 7.0 in the freezer or the cold room or the same fraction stored at pH 9.0 in the freezer loses activity completely within 11 days.

It was seen from Table XI that Fraction B retained only 1/8 of its original activity after storage in the freezer for 38 days and that it could be partially reactivated at room temperature. The enzyme regained approximately 50% of its original activity in four days. The exact extent of this reactivation could not be determined due to decomposition of the enzyme at room temperature. This property is similar to that reported by Burns (1962) for the tryptophanase of *E. coli*. He attributed this property to a

configurational change undergone by the enzyme at low temperatures which is reversed at room temperature.

Vogels, Trijbels, and Uffink (1966) have reported that the specific activity of allantoinase in crude cell-free extracts of Streptococcus allantoicus was 2.1 and that of the 5.5-fold purified extract was 11.6 µmoles/min/mg protein. They reported a recovery of 37% for their purification procedure, which involved fractionation on DEAE-cellulose and ammonium sulfate fractionation. The pH optimum of their purified extract was 7.5. In his earlier work, Vogels, (1963) had reported the pH optimum of allantoinase in crude extract to be 7.2-9.4. The Km of purified enzyme as reported by Vogels, et al (1966) was 5×10^{-3} M. They did not report on the maximum velocity or the instability of the enzyme except to state that the enzyme was relatively unstable in the freezer.

In the present study, considerably higher specific activities were obtained. The specific activity of fresh crude cell-free extracts was 15.6 at pH 10.0 and that of old extract was 11.1 at pH 8.0. The ammonium sulfate-treated fraction had a specific activity of 68.9 at pH 8.0, which was the optimum pH. The recovery was calculated to be 49%, if assays were made at pH 8.0, and 35% if the assays for Fractions A and B were made at pH 10.0. The Km obtained graphically 1.18×10^{-5} M, the maximum velocity was 1.25 µm/minute and the turnover number was calculated to be 1.09×10^{4} moles/minute/mole of protein.

CHAPTER V

SUMMARY AND CONCLUSIONS

The aerobic and anaerobic degradation of allantoin in bacteria belonging to fifteen different genera were studied. It has been found that, even though the degradation of allantoin among bacteria is not universal, it is not a character limited to a particular genus or family. Species belonging to the genera Aerobacter, Alcaligenes, Arthrobacter, Escherichia, Flavobacterium, Nocardia, Pseudomonas, and Streptococcus were able to utilize allantoin for growth. The ability to grow on allantoin minimal medium, using allantoin as the sole carbon and/or nitrogen source was found only among Pseudomonas species. The ability to degrade allantoin is more widespread in this genus than in any other studied.

Growth and/or enzymatic data showed that organisms belonging to the genera Aerobacter, Escherichia, Streptococcus, and Pseudomonas could utilize allantoin anaerobically and that all produced oxamate as an end product of fermentation.

In all the above organisms, except Streptococcus allantoicus, the pathway of degradation is the same as that proposed by Valentine, Bojanowski, Gaudy, and Wolfe (1962). In Streptococcus allantoicus, allantoate amidohydrolase

activity, as proposed by Vogels (1963), was detected.

The enzyme allantoinase, which is responsible for the conversion of allantoin to allantoic acid, was purified 6.3 times by $MnCl_2$ treatment and ammonium sulfate fractionation. The enzyme was found to be unstable at low pH values and at low temperatures. The higher the purity of the enzyme, the greater was the extent of inactivation. The partially purified enzyme was found to be fairly stable at pH 9.0 at 4° C. The inactivation due to cold could be at least partially reversed by storage at room temperature. The exact extent of reactivation could not be determined due to decomposition at room temperature. The properties of the enzyme were investigated.

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APPENDIX A

SOURCE AND IDENTITY OF ISOLATES

Strain	Source	Identity
T1b	Theta Pond	*Aerobacter aerogenes*
T1d1	Theta Pond	*Aerobacter aerogenes*
LC-6	Air	*Alcaligenes* sp.
LC-12	Air	*Alcaligenes* sp.
LC-15	Air	*Alcaligenes* sp.
T1c1	Theta Pond	*Arthrobacter* sp.
YL-1	Yost Lake	*Arthrobacter* sp.
LC-5	Air	*Arthrobacter* sp.
LC-7	Air	*Arthrobacter* sp.
LC-23	Air	*Arthrobacter* sp.
LC-26	Air	*Arthrobacter* sp.
LC-1	Air	*Bacillus* sp.
LC-2	Air	*Bacillus* sp.
LC-3	Air	*Bacillus* sp.
LC-4	Air	*Bacillus* sp.
LC-8	Air	*Bacillus* sp.
LC-10	Air	*Bacillus* sp.
LC-20	Air	*Bacillus* sp.
F1	Fire Station Pond	*Escherichia coli*
F2a	Fire Station Pond	*Escherichia coli*

Strain	Source	Identity
T3b	Theta Pond	*Escherichia freundii*
T4a	Theta Pond	*Escherichia freundii*
T4b	Theta Pond	*Escherichia freundii*
T5a	Theta Pond	*Escherichia freundii*
LC-17	Air	*Nocardia* sp.
Soil-2	Soil	*Pseudomonas aeruginosa* XI
LC-9	Air	*Pseudomonas aeruginosa* XI
T2b	Theta Pond	*Pseudomonas caviae* VIII
F2b	Fire Station Pond	*Pseudomonas* sp. I
T1a	Theta Pond	*Pseudomonas* sp. II
T5b	Theta Pond	*Pseudomonas* sp. II
T6	Theta Pond	*Pseudomonas* sp. II
T7a	Theta Pond	*Pseudomonas* sp. II
T7b	Theta Pond	*Pseudomonas* sp. II
LC-14	Air	*Pseudomonas* sp. II
LC-16	Air	*Pseudomonas* sp. II
Soil-1	Soil	*Pseudomonas* sp. II
Sew-1	Sewage	*Pseudomonas* sp. III
Sew-2	Sewage	*Pseudomonas* sp. III
YL-2	Yost Lake	*Pseudomonas* sp. III
SC-3	Stillwater Creek	*Pseudomonas* sp. III
LC-19	Air	*Pseudomonas* sp. III
T1c2	Theta Pond	*Pseudomonas* sp. IV
T1d2	Theta Pond	*Pseudomonas* sp. IV
T3a	Theta Pond	*Pseudomonas* sp. IV
SC-1	Stillwater Creek	*Pseudomonas* sp. V

Strain	Source	Identity
TP-2	Theta Pond	*Pseudomonas* sp. VI
T2a	Theta Pond	*Pseudomonas* sp. VII
TP-1	Theta Pond	*Pseudomonas* sp. IX
Sew-3	Sewage	*Pseudomonas* sp. X
LC-18	Air	*Sarcina hansenii*
SC-2	Stillwater Creek	*Staphylococcus aureus*

APPENDIX B

GROUPING OF ISOLATES IDENTIFIED AS PSEUDOMONAS SPECIES

Culture	Fl	LM	DB	LB	SB	St	G	F	S	I	N	Ci	P	A	MR	Ca	GM	37° C	42° C	Gr
F2b	M	Al	A	-	A	-	-	-	-	-	±	-	-	-	-	+	-	+	-	I
T1a	M	Al	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	II
T5b	M	Al	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	II
T6	M	Al	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	II
T7a	M	Al	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	II
T7b	M	Al	-	-	-	-	-	-	-	-	+	±	-	-	-	+	-	+	-	II
Soil-1	M	Al	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	II
LC-14	M	Al	-	-	-	-	-	-	-	-	+	+	-	-	-	+	-	+	-	II
LC-16	M	Al	-	-	-	-	-	-	-	-	±	+	-	-	-	+	-	+	-	II
Sew-1	M	Al	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	±	-	III
Sew-2	M	Al	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-	III
YL-2	M	Al	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-	III
SC-3	M	Al	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	+	-	III
LC-19	M	Al	-	-	-	-	-	±	-	-	+	+	+	-	-	+	+	+	-	III

Culture	Fl	LM	DB	LB	SB	St	G	F	S	I	N	Ci	P	A	MR	Ca	GM	37° C	42° C	Gr
T1c2	M	AR	A	-	A	-	-	-	-	-	+	+	-	-	-	+	-	+	-	IV
T1d2	M	AR	A	-	A	-	-	-	-	-	+	+	-	-	-	+	-	+	-	IV
T3a	M	AR	-	-	A	-	-	-	-	-	+	+	-	-	-	+	-	+	-	IV
SC-1	M	Al	A	-	-	-	-	±	-	-	+	+	-	-	-	+	+	+	-	V
TP-2	M	Al	-	-	-	-	-	+	-	-	+	+	+	-	-	+	+	+	+	VI
T2a	1	Al	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	+	VII
T2b	1	AC	A	A	A	+	+	-	-	+	+	-	-	-	+	+	+	+	+	VIII
TP-1	1	PA	A	-	A	+	+	±	-	+	+	+	-	+	±	+	-	+	+	IX
Sew-3	1-2	PA	-	-	-	-	+	+	-	-	+	+	+	-	-	+	+	±	-	X
Soil-2	1-2	P	-	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+	+	XI
LC-9	1-2	PA	-	-	-	-	+	+	+	-	+	+	+	-	-	+	+	+	+	XI

Key to Abbreviations Used

Fl, number of polar flagella

M, multiple

LM, litmus milk

Al, alkaline

AR, alkaline, reduction

AC, acid curd

P, peptonization

PA, alkaline, peptonization

DB, dextrose broth

LB, lactose broth

SB, sucrose broth

A, acid production

St, starch hydrolysis

G, gelatin hydrolysis

F, fat hydrolysis

S, H_2S production

I, indole production

N, nitrate reduction

Ci, citrate utilization

P, pigment production

A, acetoin production

MR, methyl red test

Ca, catalase production

GM, growth on glucose minimal medium

37°, growth at 37° C

Key to Abbreviations Used (Continued)

42° C, growth at 42° C

Gr, group number

APPENDIX C

STOCK CULTURES USED IN STUDY

Organism	Source
Arthrobacter pascens	ATCC 13346
Arthrobacter simplex	ATCC 6946
Bacillus cereus	O.S.U. 14
Bacillus megaterium	O.S.U. 18
Brevibacterium fuscum	E. A. Grula
E. coli B	Univ. of Illinois
E. coli 23	O.S.U.
E. coli 316	B. Magasanik
Flavobacterium sp.	N. N. Durham
Micrococcus roseus	O.S.U. 63
Nocardia sp. K-2	A. F. Gaudy
Proteus vulgaris	O.S.U. 73
Pseudomonas aeruginosa, PA-1	B. W. Holloway
Pseudomonas aeruginosa, PA-6	S. E. Burrous
Pseudomonas aeruginosa, PA-7	O.S.U. 76
Pseudomonas aeruginosa, PA-8	O.S.U. 78
Pseudomonas fluorescens	N. N. Durham
Pseudomonas ovalis	ATCC 950
Pseudomonas ovalis	ATCC 8209
Pseudomonas A	Bachrach via R.B. Cain

Organism	Source
Pseudomonas B	Bachrach via R.B. Cain
Pseudomonas C	Bachrach via R.B. Cain
Salmonella typhimurium T22	Univ. of Illinois
Streptococcus allantoicus	H.A. Barker

VITA

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Thesis: STUDIES ON THE METABOLISM OF ALLANTOIN BY BACTERIA

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