FORMATION OF AMMONIA FROM ALLANTOIN BY STREPTOCOCCUS ALLANTOICUS

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FORMATION OF AMMONIA FROM ALLANTOIN

BY STREPTOCOCCUS ALLANTOICUS

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

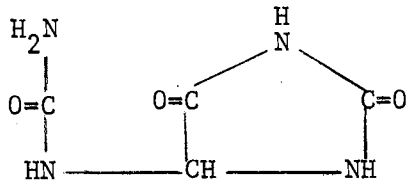
LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Allantoin occurs widely in nature where it arises partly from the degradation of purines and partly from synthesis by plants. In 1799 Buniva and Vauquelin (8) obtained, from amniotic fluid of cattle, a solution which on cooling yielded needle-shaped crystals of the substance at present known as allantoin. Grimaux (14) first succeeded in synthesizing allantoin from simple organic compounds. He heated one part of glyoxylic acid with two parts of urea for 8 to 10 hours at 100°C. He proposed the formula with the ring structure which is shown below.



The first experiments on allantoin degradation by microbes were made by Wohler (35). Vogels (32) summarized the ability of bacteria to degrade allantoin as reported by different authors. Species belonging to the genera Aerobacter, Alcaligenes, Arthrobacter, Escherichia, Flavobacterium, Nocardia, Pseudomonas, and Streptococcus were able to utilize allantoin for growth according to Meganathan (19).

Liebert (17) made an extensive study on microbial degradation of uric acid and allantoin. In a medium with uric acid as sole carbon and

nitrogen source, this acid was oxidized to allantoin by certain Pseudomonas strains. After separation of uric acid from the incubation mixture, allantoin was isolated by evaporation and precipitation with ethanol. Allantoin was proved to be broken down further by these bacteria with formation of two moles of urea and two moles of carbon dioxide according to the equation:

$$\underset{\text{allantoin}}{C_4H_6N_4O_3} + O_2 + H_2O \longrightarrow 2\ \underset{\text{urea}}{NH_2\text{-}CO\text{-}NH_2} + 2\ CO_2$$

Oxalic acid, identified as its calcium salt, proved to be an intermediate in this reaction. Furthermore, Liebert postulated glyoxylic acid as an intermediate. Fosse and Brunel (11) demonstrated the occurrence of allantoic acid as an intermediate during allantoin degradation in Soja hispida. The name allantoinase was suggested by these authors for the enzyme catalyzing the conversion of allantoin to allantoic acid.

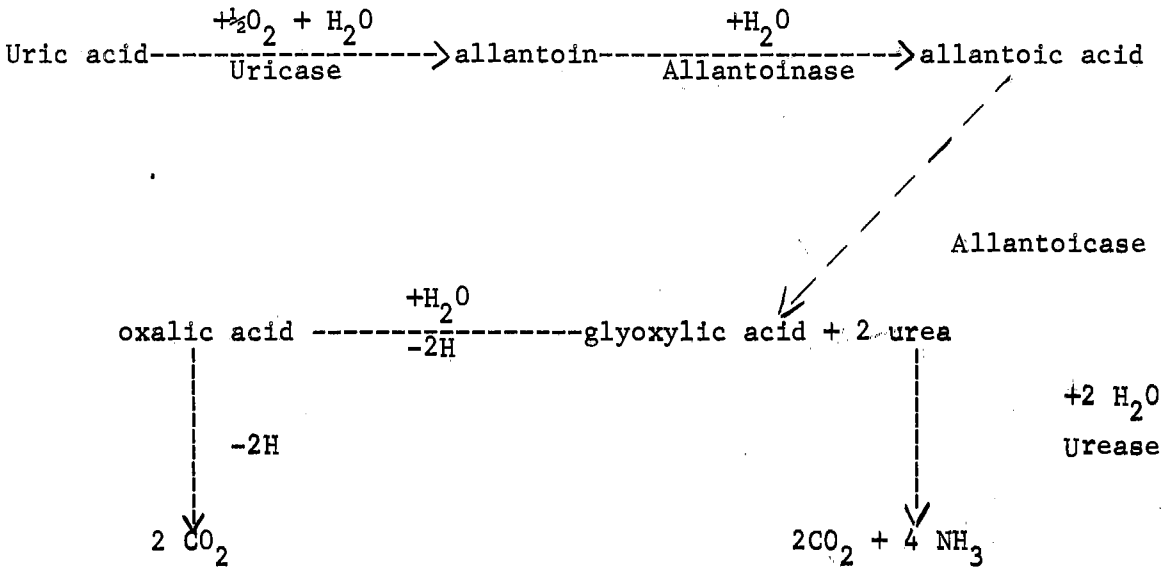
The enzyme which breaks down allantoic acid was found independently by Krebs and Weil (15) in frog's liver and by Brunel (7) in the mycelium of Aspergillus niger. The enzyme which catalyzed the reaction from allantoic acid to urea and glyoxylic acid was called allantoicase by these authors. Brunel (7) demonstrated the presence of allantoinase in 67 species of basidiomycetes and in 1936 found both allantoinase and allantoicase in Aspergillus niger and other ascomycetes.

Young and Hawkins (37) found that only Escherichia coli, Aerobacter aerogenes, and Proteus vulgaris rapidly removed allantoin from a complex growth medium. In Saccaromyces cerevisiae Hansen, Di Carlo et al (10) found that biotin was required for maximum growth on allantoin. The reason for the biotin requirement was not ascertained.

H. A. Barker first showed the ability of a bacterium to ferment allantoin under anaerobic conditions. Barker (1) isolated a new species of homofermentative lactic acid bacterium from San Francisco Bay mud in an allantoin-containing enrichment medium. The name Streptococcus allantoicus was proposed for this organism. He carried out fermentative studies and reported the concentrations of the following products in moles per mole of allantoin degraded: ammonia, 2.26; urea, 1.68; formate, 0.09; actate, 0.15; gylcolate, 0.14; and lactate, 0.01. Traces of glycine were also detected. Streptococcus allantoicus forms both ammonia and urea, but does not contain urease; therefore, Barker concluded that one ureido group is decomposed by a pathway not involving urea. In later studies, $^{15}NH_3$ was added to the medium and it was found that it was not incorporated into oxamic acid (3). Therefore, it was concluded that oxamic acid was formed directly from allantoic acid, without the intervention of free ammonia.

Campbell (9) isolated a Pseudomonas strain from San Francisco Bay mud using allantoin enrichments. Using cells grown on nutrient broth, glucose, or allantoin in manometric assays, he concluded that the enzymes of allantoin metabolism were adaptively formed. He showed that there was little difference in the ability of allantoin and allantoic acid-grown cells to degrade allantoin which would indicate that either compound can serve as inducer for the complete pathway. Campbell concluded that the aerobic and anaerobic pathways were different, but that the first steps were probably identical.

In view of the findings outlined above the following scheme may be given for the aerobic pathway of uric acid and allantoin degradation:



Valentine and Wolfe (24) reported that oxamic acid was formed from carbamyl oxamic acid by oxamic transcarbamylase in S. allantoicus. The carbamyl phosphate is further degraded to carbon dioxide and ammonia with the formation of adenosine triphosphate (25). The co-factors for formation of oxamate from allantoin or allantoic acid by a partially purified extract were identified as NAD, Mg^{++} and phosphate or arsenate (26). The following intermediates for the fermentation of allantoin by S. allantoicus were proposed by Valentine and Wolfe (27): allantoin----> allantoic acid---->glyoxylurea (ureidoglycolate)--->carbamyl oxamate---> oxamate. Gaudy (12) showed that the conversion of ureidoglycolate to glyoxylate and urea was mediated by the new enzyme glyoxylurease (ureidoglycolate synthetase). Gaudy and Wolfe (13) purified the ureidoglycolate synthetase of S. allantoicus 77-fold and showed the optimum pH to be 8.4 to 8.8. They also 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 in S. allantoicus was proposed by Valentine, et al (29). The pathway to oxamate in-

volves the decomposition of one ureido group before separation from the two-carbon moiety, as predicted by Barker (2). The "allantoicase" reaction involves two steps in S. allantoicus with the product, ureidoglycolate, serving as the point of digression of the energy-yielding and biosynthetic pathways. The degradation of allantoic acid may lead to the formation of two moles of urea and one mole of glyoxylate per mole of allantoin or one mole each of urea, oxamate, CO_2 and NH_3, depending upon the fate of the ureidoglycolate formed. Vogels (31,32) agreed that ureidoglycolate and allantoic acid are intermediates in the anaerobic degradation of allantoin. He also indicated that glyoxylic acid is an intermediate in the fermentation of allantoin and that glycine was to be found as one of the end-products of this fermentation.

In 1965 Vogels (33) reported that the degradation of allantoate to ureidoglycolate was a two-step reaction catalyzed by allantoate amidohydrolase and ureidoglycine aminohydrolase. The first step is catalyzed by allantoate amidohydrolase and allantoate is transformed to one mole of ammonia, one mole of carbon dioxide and one mole of ureidoglycine. In the second step, catalyzed by ureidoglycine aminohydrolase, the latter compound is converted to ammonia and ureidoglycolate. Cell-free extracts of Streptococcus allantoicus, Arthrobacter allantoicus, Escherichia coli, Escherichia coli var. acidilactici and Escherichia freundii exhibited only small activity in degrading allantoate. The activity of the extracts was enhanced several times by a short pretreatment with acid followed by rapid neutralization to pH 8.5, the pH optimum of the enzymic reaction. Acid-pretreatment was performed using citrate buffer (pH 2.0). Both crude extracts and those purified by chromatography on DEAE-cellulose contained a very active ureidoglycine

aminohydrolase which was not destroyed by acid-pretreatment. Vogels indicated that the sum of the amounts of ureidoglycolate and glyoxylate was equal to half the amount of ammonia formed. Ammonia was measured by NADH dehydrogenation with glutamate dehydrogenase and α-ketoglutarate in order to ascertain that the ammonia measured by the strongly alkaline Nessler's reagent (30) was not derived from hydrolysis of ureidoglycine, and it was reported that Nessler's reagent yielded the same values.

Vogels (33) also pointed out that in the case of S. allantoicus the extent of activation is sharply determined by the pH used. At pH 4.5 no activation occurs, whereas at pH 4.1 the activation is nearly complete. At pH 1.9, activation reaches a maximum within 30 seconds at $0^{\circ}C$. On prolonged acid-pretreatment the activity decreases, probably owing to denaturation of the enzyme. Both activation and inactivation of the extracts occur in defined pH regions, and activation is encountered only at pH values below 4.3. The enzyme was inactivated rapidly at pH values between 5 and 7.5. Only about 10 percent of the original activity remained. There are two pH regions in which both activated and inactive forms of the enzyme remain unchanged, namely between 4.9 and 4.3 and above 8. Activation and inactivation are reversible processes; only a minor part of the enzyme is denaturated during these pretreatments. After the inactivation steps, a small activity remains, ranging from 5 to 10 percent of the total activity. The activation and inactivation are not connected with the dissociation of a low or high-molecular weight part of the molecule, and are probably due to intramolecular rearrangements in which the active site of the enzyme is involved.

Allantoate amidohydrolase is strongly activated by manganous ions, a maximal effect being reached at a concentration of about 10^{-4} M. At

concentrations higher than 10^{-3} M, manganous ions inhibit the reaction strongly. Ca^{++} and Co^{++} also stimulate the reaction but to a lesser extent than Mn^{++}. Zn^{++} and Cu^{++}, tested at a concentration of 10^{-4} M, inhibit the reaction strongly even in the presence of Mn^{++}. Purified allantoate amidohydrolase is only active in the presence of reducing substances, e. g. reduced glutathione; these compounds also enhance the activity several-fold in crude cell extracts. The enzyme is stable for several months when kept at -20^{o} C. Trijbels and Vogels (21) reported that allantoate amidohydrolase in crude extracts of Pseudomonas acidovorans is strongly inhibited by ureidoglycolate.

The organisms capable of degrading allantoate via this pathway, as reported by Trijbels and Vogels (21,33), are Pseudomonas acidovorans, Arthrobacter allantoicus, Streptococcus allantoicus, Escherichia coli, and E. coli var. acidilactici. Meganathan (19) reported that only Streptococcus allantoicus had allantoate amidohydrolase activity after testing Alcaligenes, Arthrobacter, E. coli, Nocardia, Pseudomonas aeruginosa, and Streptococcus allantoicus. All the above microorganisms have ureidoglycolate synthetase activity, according to Meganathan.

At the present time two pathways are known by which allantoate can be degraded. Valentine et al (29) and Vogels (32) assumed the allantoicase reaction to be a two-step reaction, each step yielding one mole of urea. Allantoate was converted to ureidoglycolate and urea. The enzyme catalyzing this reaction was called allantoicase. The second enzyme, catalyzing the conversing of ureidoglycolate to urea and glyoxylate, was called ureidoglycolase by Vogels and ureidoglycolate synthetase by Gaudy and Wolfe (13). The second pathway of allantoate degradation which was proposed by Vogels (33) has been described above. Trijbels (23) reported

that the degradation of allantoate in Pseudomonase acidovorans was catalyzed by allantoate amidohydrolase and not by allantoicase since two moles of ammonia and one mole of urea were produced per mole of allantoate, in the absence of urease. However, he also indicated that allantoate degradation by Pseudomonas aeruginose involved the enzyme allantoicase and not allantoate amidohydrolase, and allantoicase was demonstrated in cell-free extracts of Pseudomonas fluorescens, Penicillium notatum, Penicillium citreo-viride, frog liver and frog kidney. After these studies, Trijbels (23) stated that "the occurrence of ureidoglycine as an intermediate in allantoate degradation has not been confirmed so far, since this compound was not available as a substance nor could it be isolated from media, in which allantoate degradation occurred."

The three different pathways proposed for the degradation of allantoin in microorganisms are shown in Figure 1. These pathways differ in the number of reactions involved in the conversion of allantoic acid to glyoxylic acid. The first pathway proposed (9) 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 (28, 29) included one intermediate, ureidoglycolate, and included reactions 1, 3, 6 and 7 (Figure 1). Ureidoglycolate synthetase catalyzes the conversion of this intermediate to glyoxylate and urea (12,13). The second pathway, proposed by Vogels (32), included two intermediates between allantoic acid and ureidoglycolate and involves reactions 1, 4, 5, 6, 7 and 8, where allantoate amidohydrolase and ureidoglycine aminohydrolase are the enzymes involved in reactions 4 and 5.

It now seems likely that aerobic and anaerobic degradation of allantoin may involve identical reactions leading to the formation of urei-

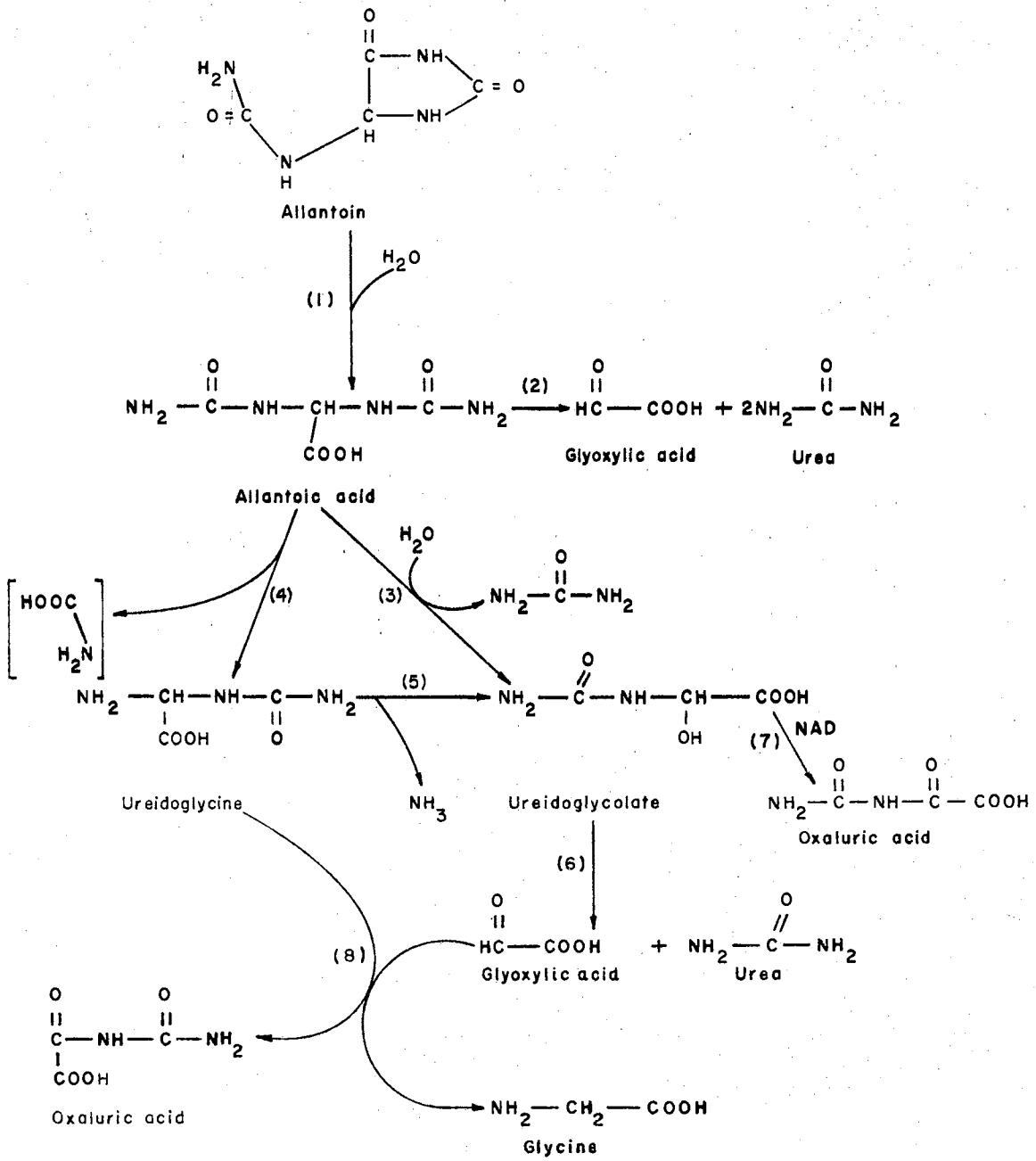


FIGURE I

PROPOSED PATHWAYS FOR ALLANTOIN DEGRADATION

doglycolate. In S. allantoicus, a portion of the ureidoglycolate is converted to oxaluric acid by reaction 7, according to Gaudy, or by both reactions 7 and 8, according to Vogels. The cleavage of oxaluric acid to oxamate and carbamyl phosphate and the further degradation of carbamyl phosphate to carbon dioxide and water is coupled with formation of adenosine triphosphate. This ATP is the major source of energy for the organism (5). This reaction sequence would not be required in an organism growing aerobically on allantoin.

Since the ammonia-forming enzymes (allantoate amidohydrolase and ureidoglycine aminohydrolase) are new enzymes for metabolism of allantoin according to Vogels, the following work was undertaken for the purpose of studying the ammonia-forming reactions in some detail. Also it was necessary to develop reliable techniques for these studies involving measurement of ammonia formation. Which of the three proposed schemes for allantoin degradation is correct will be the subject of continued study.

CHAPTER II

MATERIALS AND METHODS

Preparation of Crude Extract

Streptococcus allantoicus was grown at 30° C for 18 hours in 20-liter carboys in the medium described by Barker (1). The cells were harvested in a Sharples centrifuge, then frozen overnight at -20° C, and crushed in a Hughes press. The crushed cells were suspended in 50-60 ml of 0.02 M potassium phosphate buffer (pH 7.2) containing 0.05 per cent sodium thioglycolate, and 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.

Enzyme Assays

Without Acid-Pretreatment

Crude or purified extract containing an appropriate amount of protein in a volume of 0.6 to 1.8 ml was mixed with 7.2 to 8.4 ml of 0.1 M sodium barbital-HCl buffer (pH 8.5) which contained 15.0 to 16.0 μ moles per ml of allantoin or potassium allantoate, 11 μ moles per ml of reduced glutathione (GSH) and 0.1 μ mole per ml of manganous sulfate. The total volume was 9.0 ml. Tubes were incubated at 30° C for varying periods of time. Samples were diluted 1/10 for chemical analysis.

Acid-Pretreatment

Crude or purified extract containing an appropriate amount of protein in a volume of 0.6 ml was mixed with 1.2 ml of 0.05 M sodium citrate-HCl buffer (pH 2.0). After 30 seconds at room temperature, 7.2 ml of 0.1 M sodium barbital-HCl buffer (pH 8.5) were added. This buffer contained 15.0 to 16.0 μ moles allantoin or potassium allantoate, 11 μ moles reduced glutathione and 0.1 μ mole manganous sulfate per ml. The total volume was 9.0 ml. The mixture was incubated at 30° C. Samples were diluted 1/10 for chemical analysis.

Chemicals

Allantoin was obtained from Nutritional Biochemicals Corporation or from Sigma Chemical Company. Potassium allantoate was prepared by a modification of the procedure of Young and Conway (36) developed in this laboratory (34). Reduced glutathione was obtained from Calbiochem Company and sodium barbital was obtained from Nutritional Biochemicals Corporation. Potassium fluoride was obtained from Fisher Scientific Company and adenosine diphosphate (ADP) was obtained from Pabst Laboratories Division of Pabst Brewing Company.

Chemical Analyses

Allantoin

A sample containing 0.2 to 1.0 μ mole of allantoin was made up with distilled water to a volume of 5.0 ml; 1.0 ml of 0.5 N NaOH and 2 drops phenylhydrazine hydrochloride (100 mg/30 ml water) were added. The tubes were placed in boiling water for exactly 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 (100 mg/30 ml water) were added and the tubes were boiled in a water bath for 2 minutes. The tubes were cooled to room temperature again 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 mμ on a Coleman Junior Spectrophotometer.

Allantoate

Allantoin was determined by addition of 1.0 ml of phenylhydrazine solution (100 mg/30 ml water) and 1.0 ml of 0.15 N HCl to a tube containing 0.2 to 1.0 μ mole of allantoate made up to a 6.0 ml volume with distilled water. The tubes were immersed in a boiling water bath for 2 minutes, and the procedure continued as described for allantoin.

Glyoxylate

For the determination of glyoxylate, a sample containing 0.2 to 1.0 μ mole of glyoxylate was made up to a 7.0 ml volume. 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. The remainder of the procedure was the same as for allantoin.

Oxamic Acid

Oxamic acid was estimated according to the method of Lipmann and Tuttle (18). The sample containing 1.0 to 5.0 μ moles of oxamate was made up to a volume of 1.0 ml with distilled water, and 1.0 ml of 28 per cent neutralized hydroxylamine hydrochloride solution (3 volumes of

4M hydroxylamine hydrochloride : 1 volume of 14 per cent NaOH; pH 6.4) was added. The tubes were immersed in a boiling water bath for 15 minutes and cooled to room temperature in a water bath. After cooling, 1.0 ml acetate buffer (pH 5.4) was added and the tubes were allowed to stand at room temperature for 10 minutes. Then 1.0 ml of 3M HCl and 1.0 ml of a 12 per cent solution of trichloroacetic acid were added, followed by 1.0 ml of a 5 per cent solution of $FeCl_2 \cdot 6H_2O$ in 0.1 N HCl. The tubes were kept for 15 minutes at room temperature, then read at 540 mμ in a Coleman Junior Spetrophotometer.

Urea

Urea was measured by the method of Jones, et al (16). A sample containing 0 to 1.25 μ moles 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-diacetyloxime (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 mμ in a Coleman Junior Spectrophotometer.

Protein Content

The method of Sutherland, et al (20) 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 freshly mixed reagent containing 100 ml of 4 per cent Na_2CO_3, 1.0 ml of 4 per cent sodium-potassium tartrate and 1.0 ml of 2 per cent $CuSO_4$ 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 mμ in a Coleman Junior Spectrophotometer.

Ammonia

Since the development of a reliable method for measurement of ammonia in the incubation mixture formed a significant portion of this study, the methods used for ammonia determination will be described under "Results", Chapter III.

CHAPTER III

EXPERIMENTAL RESULTS

Analytical Methods and Nitrogen Balance

Vogels (33) measured ammonia formed by allantoate amidohydrolase by direct Nesslerization of the incubation mixtures. He did not report studies of interference from other compounds in the mixture, nor did he determine all nitrogenous products in the mixture.

Incubation mixtures were prepared according to Vogels and ammonia was determined by Nesslerization. The same incubation mixtures were also analyzed for allantoate, urea, oxamate and glyoxylate and the nitrogen recoveries were calculated. Recoveries as high as 150 per cent were found in repeated experiments. Therefore, before the enzyme could be studied, it was necessary to develop a reliable method for measurement of ammonia in the incubation mixture and also to check for possible interference in the methods used for other nitrogenous compounds.

Methods for Measuring Ammonia

Ammonia formed in the enzyme incubation mixture was measured by Nesslerization, microdiffusion, microkjeldahl distillation, the glutamate dehydrogenase method and the indophenol method.

1. Nesslerization

A sample containing 0.1 to 1.0 μ mole 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, interfering with measurement of color. The addition of 1.0 ml of 30 per cent sodium citrate before the addition of Nessler's reagent (30), prevented the formation of a precipitate. After addition of 1.0 ml of Nessler's reagent, the tubes were kept at room temperature for 10 minutes, and the color was read at 436 mμ on a Coleman Junior Spectrophotometer. Results of four different experiments are shown in Table I. It may be concluded from these data that the method gives very reproducible results.

2. 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. The incubation time required for complete recovery of ammonia was determined in a series of studies using standard ammonium chloride solution.

Results of four different experiments are shown in Table II. These data indicate that the method is as reproducible as is direct Nesslerization. The total amounts of ammonia measured and the ratios of ammonia to allantoate are much lower than those obtained by the direct Nessleri-

TABLE I

MEASUREMENT OF AMMONIA BY NESSLERIZATION

Allantoate Used (μmoles/ml)	Ammonia (μmoles/ml)	$\frac{\mu\text{moles Ammonia}}{\mu\text{mole Allantoate}}$
16.8	50.0	3.0
15.8	42.5	2.7
15.0	43.0	2.9
15.8	45.0	2.8

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and 0.6 ml of cell extract in a total volume of 9.0 ml. Enzymes were acid pretreated. Incubation time was 20 minutes at 30°C. Determinations were performed immediately after incubation.

TABLE II

MEASUREMENT OF AMMONIA BY MICRODIFFUSION

Allantoate Used (μmoles/ml)	Ammonia (μmoles/ml)	$\frac{\mu\text{moles Ammonia}}{\mu\text{mole Allantoate}}$
15.8	30.9	1.9
15.7	31.0	1.9
15.0	28.2	1.9
15.8	33.0	2.1

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and 0.6 ml of cell extract in a total volume of 9.0 ml. Enzymes were acid pretreated. Incubation time was 20 minutes at 30°C. Determinations were performed immediately after incubation.

zation method.

3. Microkjeldahl Method

A sample was placed in the chamber of the steam distillation apparatus which had previously been cleared of any contaminating ammonia by a blank distillation. Thirty per cent sodium hydroxide solution was then added to the sample in the chamber to more than neutralize the amount of acid present. The generation of steam in the boiler was started and the ammonia was collected in 0.1 N sulfuric acid in the receiving flask. The excess acid in the flask was titrated with 0.1 N NaOH using methyl red as indicator. Since steam distillation from concentrated alkali might lead to formation of ammonia from other nitrogenous compounds in the incubation mixture, this method was tested with standard solutions of allantoin, allantoate, oxamate and urea, as well as with the ammonia standard.

Table III shows that with this method, one mole of allantoin forms 1.5 moles ammonia, one mole of ammonia is formed from one mole of allantoate, or from one mole of urea, and 0.7 mole is formed from one mole of oxamate. This method therefore could not be used for ammonia determination in the present study.

4. Glutamate Dehydrogenase Method

Ammonia was determined in the following manner according to Vogels (33). In a one cm cuvette were mixed: 0.1 ml glutamate dehydrogenase (10 mg/ml), 2 ml of 0.1 M Tris-HCl buffer (pH 8.5), 0.05 ml NADH solution (10 μmoles/ml in 1 per cent $NaHCO_3$), 0.2 to 0.25 ml of α-ketoglutaric acid (50 μmoles/ml), varying amounts of standard NH_4Cl solution,

TABLE III

COMPOUNDS INTERFERING WITH MEASUREMENT
OF AMMONIA BY MICROKJELDAHL

Compound	μmoles	Ammonia (μmoles)
Allantoin	10	15.1
	10	15.1
Allantoate	10	10.2
	10	10.2
Urea	10	10.2
	10	10.2
Oxamate	15	10.0
	15	10.0
Ammonia	10	10.2
	10	10.2

A standard solution of each compound in distilled water was steam-distilled from alkaline solution as described in the text.

and distilled water to a total volume of 3.0 ml. Dehydrogenation of NADH was measured at 340 mμ on the Beckman DU Spectophotometer. Results are shown in Table IV and Figure 2. It may be seen from these data that neither the initial reaction rate nor the final optical density was proportional to the concentration of ammonia. Data showing that reaction rate is not proportional to concentration of ammonia have also been reported by Barratt and Strickland (4).

5. Indophenol Method

Spectrophotometric determination of ammonia as indophenol was carried out according to Bolleter, Bushman and Tidwell (6). Various amounts of samples from incubation mixtures were added to 25 ml of saturated boric acid solution in 50 ml volumetric flasks. Swirling the flask to ensure good mixing, 5 ml of chlorine water and 5 ml of 8 per cent phenol solution were added in order. The flask was placed in a steam bath for 3 minutes, removed and cooled rapidly. Five ml of 3 M NaOH were added, followed by distilled water to the 50 ml mark. After standing at room temperature for 5 minutes, the color was read at 625 mμ on a Coleman Junior Spectrophotometer.

Data obtained in four measurements are shown in Table V. Both total amounts of ammonia measured and the ammonia to allantoate ratios are comparable to those obtained by direct Nesslerization. Therefore, any compound being measured as ammonia in the reaction with Nessler's reagent is also reactive in this assay.

Interferences

Before choosing a method for determination of ammonia, based on

TABLE IV

AMMONIA DETERMINATION BY THE GLUTAMATE DEHYDROGENASE METHOD

Time (sec) / Tube / O.D.	0	15	30	45	60	75	90	105	120	135	150	160	180
1	0.09	0.065	0.1										
2	0.770	0.660	0.582	0.538									
3	0.860	0.75	0.70	0.665	0.642	0.612	0.596	0.583	0.570	0.560	0.552	0.549	0.542
4	0.800	0.664	0.572	0.581	0.471	0.439	0.413	0.391	0.379	0.366	0.358	0.358	0.346
5	0.855	0.617	0.497	0.410	0.342	0.296	0.261	0.238	0.216	0.198	0.190	0.181	0.175
6	0.840	0.495	0.333	0.230	0.162	0.120	0.094	0.082	0.076	0.074	0.074		

Tube 1 contained 0.5 μmole NH_4Cl, tubes 2 and 3 contained 0.1 μmole NH_4Cl, tube 4 contained 0.2 μmole NH_4Cl, tube 5 contained 0.3 μmole NH_4Cl, and tube 6 contained 0.4 μmole NH_4Cl. There was 0.1 ml of glutamic dehydrogenase in tubes 1 and 2 and 0.05 ml of the enzyme in tubes 3, 4, 5 and 6.

Figure 2. The Relation Between Ammonium Chloride Concentration and Reaction Rate.

The optical density readings listed in Table IV are plotted to show reaction rate.

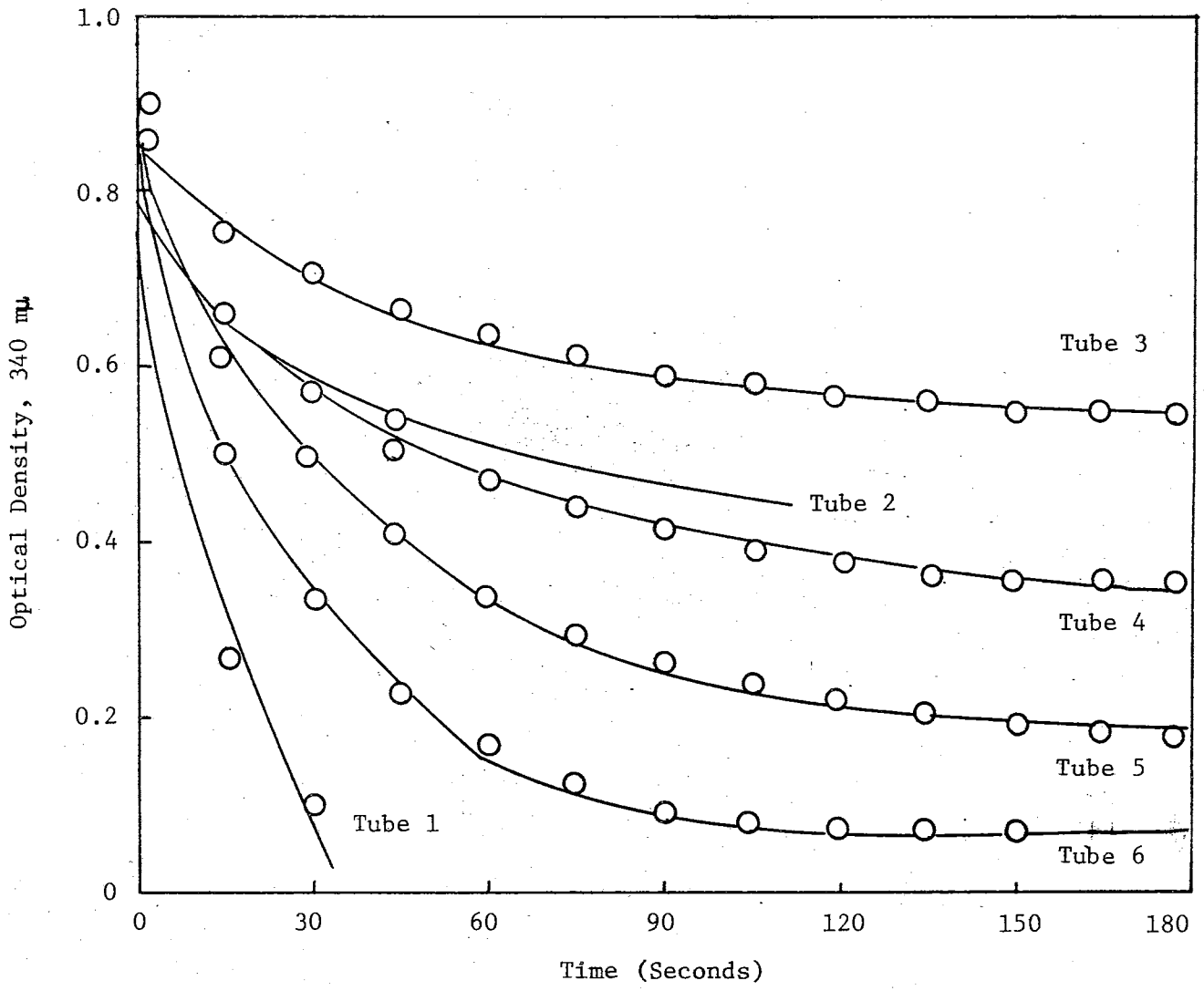

1.0
0.8
0.6
0.4
0.2
0
Optical Density, 340 mμ
0
30
60
90
120
150
180
Time (Seconds)
Tube 1
Tube 2
Tube 3
Tube 4
Tube 5
Tube 6

TABLE V

MEASUREMENT OF AMMONIA BY THE INDOPHENOL METHOD

Allantoate Used (μmoles/ml)	Ammonia (μmoles/ml)	$\frac{\mu\text{moles Ammonia}}{\mu\text{mole Allantoate}}$
15.8	42.4	2.7
15.8	40.4	2.6
15.8	39.6	2.5
15.8	38.8	2.5

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 3.5) and 0.6 ml of cell-free extract in a total volume of 9.0 ml. Enzymes were acid-pretreated. Incubation time was 30 minutes at 30°C. Determinations were performed immediately after incubation.

nitrogen recovery data, it was necessary to investigate interferences of all nitrogenous compounds known to be present in each of the assays used, since high nitrogen recoveries might be due to overestimates of other components of the incubation mixture. Mixtures of standard solutions of allantoin, allantoate, urea, oxamate, glyoxylate and ammonia were prepared, each mixture containing all except one of the compounds. Each mixture was then assayed by the procedure used for the determination of each component. Glutathione and barbital were also tested for interference in each assay, since both contain amino groups.

Only three assay methods were found to be subject to interference and these were checked for proportionality so that correction factors could be applied to these measurements. The data are shown in Tables VI, VII and VIII. Oxamate was found to produce a color with Nessler's reagent equivalent to one-fifth the amount of ammonia; i.e., five moles of oxamate would be measured as one mole of ammonia. Since amounts of oxamate formed in the incubation were always quite small compared to amounts of ammonia, this interference could not account for the high nitrogen recoveries or the excess ammonia measured by the Nessler's test.

As was previously known (12), allantoate reacted quantitatively in the assay for urea and urea measurements were therefore corrected for amounts of allantoate present. Both glutathione and barbital buffer react in the assay for oxamate, but the interferences are not additive. Therefore, blank incubation mixtures without enzyme were used to correct oxamate values.

Nitrogen Balance Using Nesslerization and Microdiffusion Methods

Table IX shows typical nitrogen balances calculated for a single

TABLE VI

INTERFERENCE OF OXAMATE IN AMMONIA DETERMINATION BY NESSLERIZATION

Oxamate (μmoles)	Ammonia (μmoles)
1.5	0.475
4.5	1.05
7.5	1.50
10.5	2.13
13.5	2.80
15.0	2.98

TABLE VII

INTERFERENCE OF ALLANTOATE IN DETERMINATION OF UREA

Allantoate (μmoles)	Urea (μmoles)
0.5	0.482
1.0	1.023
2.0	1.920

TABLE VIII

INTERFERENCE OF GSH AND BARBITAL-HCl BUFFER (pH 8.5) IN DETERMINATION OF OXAMATE

GSH (μmoles/ml)	Barbital-HCl Buffer (pH 8.5)	Oxamate (μmoles/ml)
-	0.08 M	3.6
11.0	-	10.7
11.0	0.08 M	10.2
11.0	0.08 M	9.9

TABLE IX

PRODUCTS OF ALLANTOIN DEGRADATION BY CELL-FREE EXTRACT

Allantoin Used (μmoles/ml)	Allantoate	Glyoxylate	Oxamate	Urea	NH_3	Nitrogen Recovery
		(μmoles/ml)				
					29.0[a]	82%[a]
23.6	2.2	11.2	0.76	19.2		
					70.0[b]	125%[b]

a. Microdiffusion Method
b. Nesslerization

The incubation mixture contained : 383.4 μmoles allantoin, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5), and 0.6 ml of cell-free extract (9.2 mg/ml protein) in a total volume of 9.0 ml. Enzyme was acid pretreated. Incubation time was 30 minutes at 30°C. Determinations were performed immediately after incubation.

incubation mixture in which ammonia was measured by both Nesslerization and microdiffusion. It was found that when data for Nesslerization were used, the nitrogen recovery was always over 100 per cent. If data from microdiffusion were used, the recovery was usually less than 100 per cent.

The nitrogen balance does not include ureidogylcolate and ureidoglycine. According to Trijbels and Vogels (21) the total amount of ureidoglycolate plus ureidoglycine was determined by adding 0.4 M NaH_2PO_4-Na_2HPO_4, pH 7.0, to the sample, heating in a boiling water bath for 5 minutes and measuring glyoxylate. This method was tested with ureidoglycolate and it was found that only 18.2 per cent of the ureidoglycolate was hydrolyzed. Therefore, ureidoglycolate and ureidoglycine are not included in the nitrogen balance and this may at least partially account for the low recovery obtained with the microdiffusion method for ammonia.

Since all data except the ammonia measurements are identical, the discrepancy in the two calculations of nitrogen recovery must be due to errors in ammonia measurement. It must be concluded that the lower figure is more nearly correct since inclusion of ureidoglycolate and ureidoglycine could bring the recovery calculated from microdiffusion data closer to 100 per cent, while that calculated from Nesslerization data would be even further above 100 per cent. It is possible that ureidoglycine, or some other unknown product, may react with Nessler's reagent to produce the high values found for ammonia by this method.

Fermentation Balance

Streptococcus allantoicus was grown in the liquid medium described by Barker (1) at 30° C. After 18 hours, the supernatant was collected

by centrifugation. Allantoin, allantoate, oxamate, urea, glyoxylate, and ammonia in the supernatant were determined according to the methods described previously. Table X shows the products in μmoles per μmole of allantoin fermented, and the recovery of nitrogenous compounds.

Conditions Affecting Ammonia Production

Amount of Enzyme

Use of different amounts of enzyme in the incubation mixture affected ammonia formation. As shown in Figure 3, even at "zero time" incubation, with 0.6 ml of enzyme, almost the same amount of ammonia had been formed as at 10, 20, 30, 40, 50, and 60 minutes. With 0.6 ml of a 1/10 dilution of enzyme, the reaction proceeds slowly enough to allow measurement of the rate of the reaction.

It is also interesting to note that the ratio of ammonia to allantoate varied with the amount of enzyme used. With a greater amount of enzyme, the amount of ammonia formed per unit of allantoate degraded decreased. These data, from a different experiment, are shown in Table XI.

Acid- and Nonacid-Pretreatment with Different Amounts of Enzyme

According to Vogels (33), allantoate amidohydrolase requires acid-pretreatment to attain full activity. Table XII shows that with 0.6 ml of crude extract, there is little difference between acid- and nonacid-pretreatment of enzyme. However, with 0.1 ml or 0.2 ml of crude extract, the enzyme requires acid-pretreatment to exhibit activity.

TABLE X

FERMENTATION BALANCE

Compound	μmoles/ml	μmoles/μmole Allantoin	Nitrogen Recovery
Allantoin Used	26.3	1	
Allantoate	0.0	0	
Ammonia	51.0	1.9	98%
Glyoxylate	9.7	0.37	
Oxamate	20.0	0.76	
Urea	16.1	0.61	

Figure 3. Effect of Amount of Enzyme on Ammonia Production.

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5), and crude extract (13.3 mg/ml protein) as indicated. Enzymes were acid pretreated. Incubation was carried out for 60 minutes at 30°C and samples were removed and placed in an ice bath at the times indicated on the figure. Determinations were performed immediately after incubation.

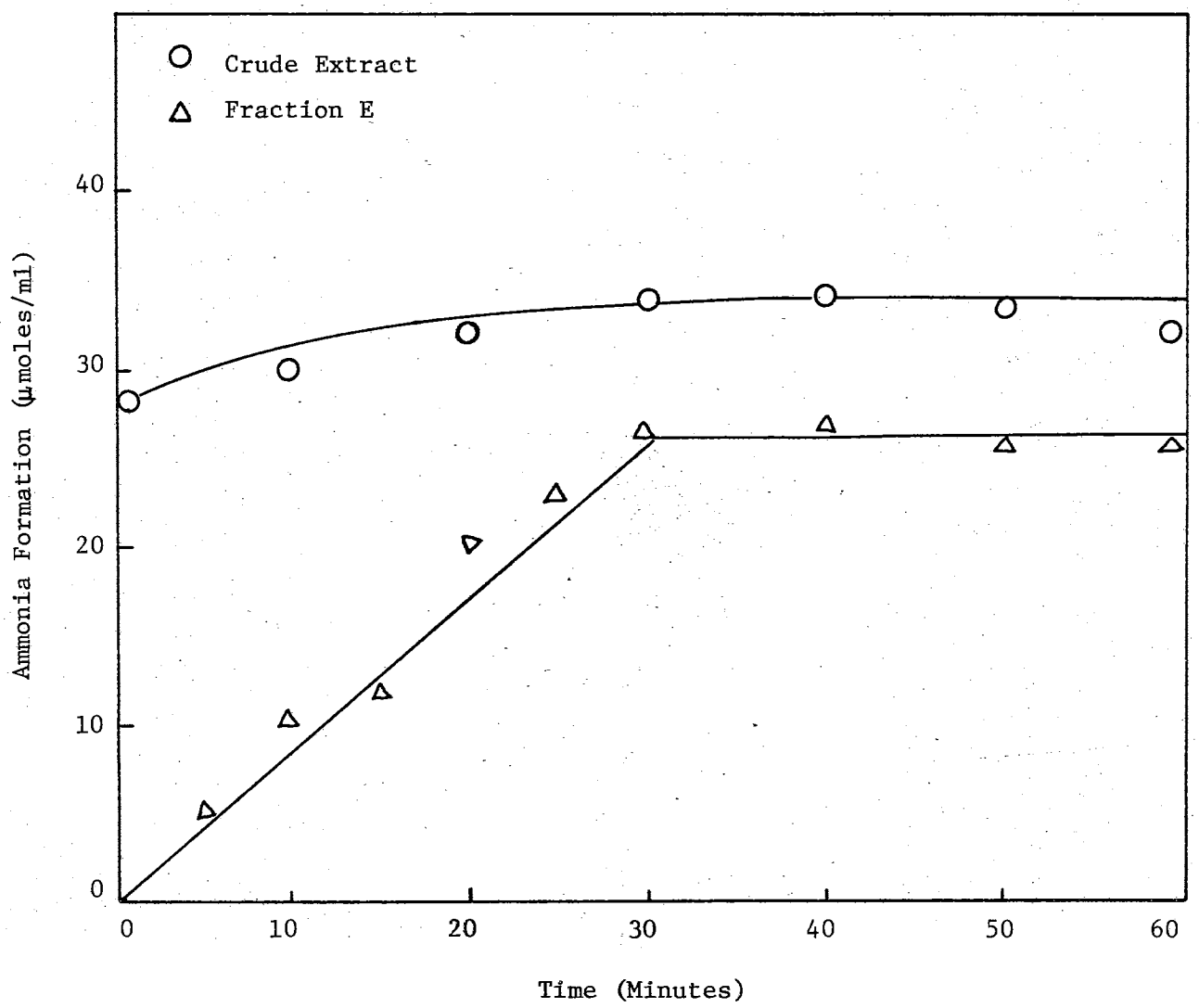

Crude Extract
Fraction E
Ammonia Formation (µmoles/ml)
0
10
20
30
40
0
10
20
30
40
50
60
Time (Minutes)

TABLE XI

SUBSTRATE USED AND AMMONIA PRODUCED
WITH DIFFERENT AMOUNTS OF ENZYME

Enzyme	Allantoate Used (μmoles/ml)	Ammonia Formation (μmoles/ml)	Ammonia/Allantoate
0.05 ml	0.0	2.7	0.0
0.2	3.0	11.1	3.7
0.4	9.3	23.4	2.5
0.6	13.0	24.0	1.8

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and enzyme as indicated. The enzyme preparation used was a 1/5 dilution of a dialyzed crude extract containing 11.1 mg protein per ml. Incubation time was 30 minutes at 30°C. Samples were frozen before being assayed.

TABLE XII

ACID AND NONACID-PRETREATMENT WITH DIFFERENT AMOUNTS OF ENZYME

Crude Extract (ml)	Pretreatment	Allantoate Used (μmoles/ml)	Ammonia Formation (μmoles/ml)
0.6	Nonacid	14.6	25.8
	Acid	15.8	33.0
0.2	Nonacid	0.0	0.0
	Acid	15.0	28.5
0.1	Nonacid	0.0	0.0
	Acid	5.3	18.5

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and crude extract (11-13 mg/ml protein) as indicated. Incubation was at 30°C for 30 minutes. Determinations were performed immediately after incubation.

Storage of Samples for Analysis

Since a number of analyses had to be performed for each incubation mixture, samples were removed, immediately frozen at -20°C and later thawed for analysis in some of the early experiments. In other experiments, analyses were carried out immediately and it was noted that fairly consistent differences in relative amounts of some products appeared when results obtained with frozen and unfrozen samples were compared. Several experiments were carried out using portions of the same incubation mixture to investigate these differences.

Results of a typical experiment are shown in Table XIII. Changes occurred during storage for one to three days at -20°C in concentrations of allantoin, glyoxylate and ammonia. Whether this is due to slow enzymatic activity, even at such a low temperature, or to spontaneous interactions is not certain. It was decided that samples should be diluted into ice water immediately upon removal from the incubation mixture and the analyses completed as rapidly as possible.

The overall effect of the changes occurring during storage in the freezer is a lessening of the difference between acid-treated and non-acid-treated enzyme activities.

Comparison of Dialyzed and Nondialyzed Extract

Twenty ml of crude extract were dialyzed for 24 hours against 4 liters of 0.02 M phosphate buffer (pH 7.0) containing 0.05 % sodium thioglycolate. Buffer was changed at 6-hour intervals. The enzymic activity (ammonia formation) was then compared with that of a non-dialyzed portion of the same extract. Results are shown in Table XIV. The

TABLE XIII

EFFECT OF STORAGE OF SAMPLES ON PRODUCTS OF ALLANTOIN DEGRADATION

Sample	Pretreatment of Enzyme	Allantoin Used (μmoles/ml)	Glyoxylate (μmoles/ml)	Ammonia (μmoles/ml)
Unfrozen	Acid	15.2	15.0	29.4
	Nonacid	10.0	5.8	16.5
Frozen	Acid	15.6	4.3	22.5
	Nonacid	15.4	3.1	18.0

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and 0.6 ml of crude extract (12.6 mg/ml protein). Incubation time was 30 minutes at 30°C.

TABLE XIV

AMMONIA FORMATION BY DIALYZED AND NONDIALYZED CRUDE EXTRACT

Extracts	Allantoate Used (μmoles/ml)	Ammonia (μmoles/ml)
Dialyzed	14.4	23.4
Nondialyzed	14.9	20.4
Dialyzed	14.2	19.5
Nondialyzed	14.7	18.3

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and 0.6 ml of cell-free extract in a total volume of 9.0 ml. Enzymes were acid pretreated. Incubation time was 30 minutes at 30°C. Determinations were performed immediately after incubation.

fact that the activities were essentially the same indicates that no dialyzable cofactors (other than Mn^{++}) are required for ammonia formation.

Enzyme Purification and Properties

Purification of Ammonia-Forming Enzymes

1. Streptomycin Treatment

Crude extract (Fraction A) was prepared as described in Chapter II. Fraction A was kept in a 0°C ice bath and 0.25 volume of 20 per cent streptomycin sulfate was added at a rate of 0.2 ml per minute with stirring. The extract was stirred for an additional 15 minutes. The precipitate was removed by centrifugation at 0°C. The clear extract was dialyzed for 18 hours against 4 liters of 0.02 M potassium phosphate buffer (pH 7.0, containing 0.05 per cent sodium thioglycolate) with changes of buffer at 6-hour intervals. This treatment removed about 20 to 34 per cent of the total initial protein and resulted in 1 to 1.04-fold purification. The dialyzed extract is designated as Fraction B.

2. Calcium Phosphate Gel Treatment

Fraction B was stirred slowly in an ice bath and 0.2 volume of calcium phosphate gel was added at a rate of 0.2 ml per minute. Stirring was continued slowly for an additional 15 minutes. The precipitate was removed by centrifugation at 0°C and discarded. The supernatant fraction is designated as Fraction C. Fraction C contained 45 to 59 per cent of the initial protein of Fraction B and yielded a 1.7 to 2.03-fold purification.

3. Ammonium Sulfate Fractionation

Fraction C was stirred slowly in an ice bath and 0.35 gm of solid ammonium sulfate was added per ml of extract (55 per cent saturation). Stirring was continued for 15 minutes and the precipitate was collected by centrifugation and dissolved in 10 ml of ice-cold 0.02 M potassium phosphate buffer, pH 7.0 (Fraction D-1). The supernatant was brought to 65 per cent saturation by addition of 0.066 gm of solid ammonium sulfate per ml and the precipitate collected in a similar manner (Fraction D-2). Fraction D-1 and Fraction D-2 were combined and dialyzed against 4 liters of 0.02 M potassium phosphate buffer (pH 7.0, containing 0.05 per cent sodium thioglycolate) for 18 hours; the buffer was changed at 6-hour intervals. The dialyzed fraction is designated as Fraction D. Fraction D contained 1.5 to 6.0 per cent of the total protein of Fraction A and yielded a 2.4 to 3.3-fold purification.

4. Second Calcium Phosphate Gel Treatment

Fraction D was treated as described in Section 2. The supernatant is designated as Fraction E. Fraction E contained 60 to 73 per cent of the total protein of Fraction D and yielded a 3.5 to 4.3-fold purification.

A summary of the complete purification procedure is given in Table XV. The final enzyme preparation contained 1.0 to 4.5 per cent of the total protein of the crude extract, and had a specific activity 3.5 to 4.3-fold that of the starting material.

5. Chromatography on DEAE Cellulose Column

In two separate attempts to achieve further purification by chroma-

TABLE XV

PURIFICATION OF AMMONIA-FORMING ENZYMES

Fraction	Total Units	% Recovery	Protein (mg)	Specific Activity	Purification
A	4760	100	594	8.0	1.0
B	3280	69	396	8.3	1.04
C	2440	51	178	13.7	1.7
D	1690	35	88.4	19.1	2.4
E	1490	31	52.6	28.3	3.5
A	1404	100	156	9.0	1.0
B	1561	111	130	8.9	1.0
C	1408	100	77.0	18.3	2.03
D	278	20	9.6	29.5	3.3
E	246	18	7.0	37.5	4.3

One unit of ammonia-forming enzyme activity is the amount of enzyme required for the formation of one micromole of ammonia per minute. Specific activity is expressed as units per mg protein. The activity of each fraction was determined by measuring ammonia formation by the microdiffusion technique. The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5), and 0.2 ml of each fraction. The enzymes were acid-pretreated. Incubation time was 30 minutes at 30°C. Samples were not frozen before analysis. Data are given for purification of two different crude extracts.

tography on DEAE, the activity could not be eluted from the column.

Properties of Ammonia-Forming Enzymes

1. Enzyme Activity in Precipitate after Acid-Pretreatment

It was noted that the enzyme preparations became turbid after acid pretreatment, and it seemed possible that some purification might be achieved by treating with acid.

1.2 ml of crude extract (7.6 mg/ml protein) was treated with 4.8 ml of 0.05 M sodium citrate buffer (pH 4.0). After centrifugation in the RC-2B at 12,000 rpm for 15 minutes, the precipitate and supernatant were separated and placed in an ice bath. The precipitate was dissolved in 6.0 ml of ice cold 0.02 M, pH 8.5 phosphate buffer (Fraction P). Enzyme activity was determined for both supernatant and Fraction P. Only Fraction P had activity in ammonia formation. However, almost all the protein of the extract was found in the precipitate, so that the treatment was not useful for purification. Results are shown in Table XVI.

2. Optimum pH for Enzyme Activity

The effect of pH on the ammonia-forming enzymes of Fraction A (crude extract) and Fraction E (partially purified) is shown in Figure 4. The optimum pH values are 7.9 and 8.7 for both Fraction A and Fraction E. The reasons for two pH optima are not known, but the two peaks may represent pH optima for two enzymes (allantoate amidohydrolase and ureidoglycine aminohydrolase) both of which form ammonia according to Vogels. It is not possible to test this explanation until ureidoglycine is available.

N-tris (hydroxymethyl)-methyl-3-aminoethane sulfonic acid (TES)

TABLE XVI

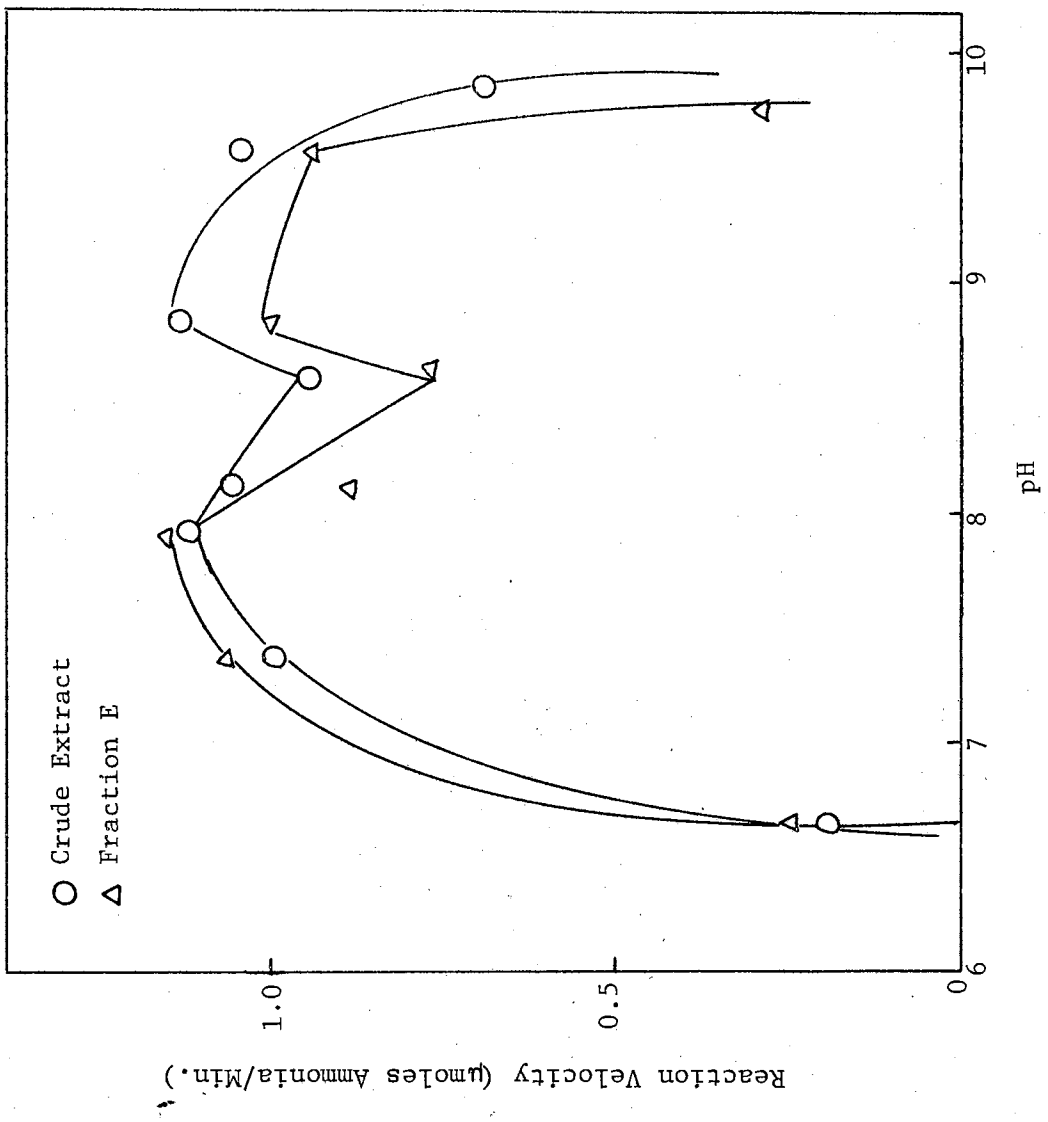
ENZYME ACTIVITY IN PRECIPITATE AFTER ACID-PRETREATMENT

Compound	Supernatant	Fraction P
Protein (mg/ml)	0.021	0.231
Allantoate Used (μmoles/ml)	0.0	49.5
Glyoxylate (μmoles/ml)	0.78	38.7
Ammonia (μmoles/ml)	0.0	57.2

The incubation mixture contained : 756 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5), and 1.5 ml of supernatant or 1.5 ml of Fraction P in a total volume of 9.0 ml. Incubation time was 60 minutes at 30°C. Determinations were performed immediately after incubation.

Figure 4. Optimum pH for Ammonia Forming Enzyme Activity.

The incubation mixture contained : 141.3 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.1 ml of Fraction A (9.2 mg/ml protein) or Fraction E (1.8 mg/ml protein) and buffer at a concentration of 0.1 M. Enzymes were acid pretreated. Ammonia was determined after 30 minutes incubation at 30°C. Buffers were used as described in the text.


Crude Extract
Fraction E
Reaction Velocity (μmoles Ammonia/Min.)
1.0
0.5
0
6
7
8
9
10
pH

buffer was used for the pH range 7.0 to 8.0 and N, N-bis (2-hydroxyethyl) glycine (bicine) buffer for the pH range 8.0 to 9.0. Glycine-sodium hydroxide buffer was used for the pH range 9.0 to 10.0. All buffers at all pH values were tested separately in the incubation mixture without substrate to be certain that ammonia was not formed from the buffer itself.

3. Enzyme Activity vs. Time

The rates of ammonia formation vs. time for both Fraction A and Fraction E are shown in Figure 5. The rate is constant over a period of about 30 minutes. The 30 minute incubation time used in the assay is within the linear response time for the enzymes.

4. Enzyme Activity vs. Protein Concentration

A range of 0 to 150 μg of protein was used in the assay. The reaction velocity for ammonia formation was found to be proportional to enzyme concentration in the range of 0 to 135 μg per tube. The results are shown in Figure 6.

5. Inhibitors of Ammonia-Forming Enzymes

The effect on the ammonia-forming enzyme activity of three inhibitors of different types is shown in Table XVII. Potassium fluoride inhibits enzyme activity by 100 per cent at a concentration of 9×10^{-3} M. ADP inhibits enzyme activity by 30 per cent at a concentration of 1.9×10^{-8} M. ADP plus dibasic potassium phosphate has no effect on enzyme activity at a concentration of 1.9×10^{-8} M ADP and 0.2 M dibasic potassium phosphate. Sodium arsenate has no effect on enzyme activity at

Figure 5. Enzyme Activity vs. Time

The incubation mixture contained : 141.3 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and 0.2 ml of Fraction A (9.2 mg/ml protein) or 0.2 ml of Fraction E (1.8 mg/ml protein). Enzymes were acid pretreated. Samples were taken at 10 minute intervals over a period of one hour and determinations were made immediately after incubation.

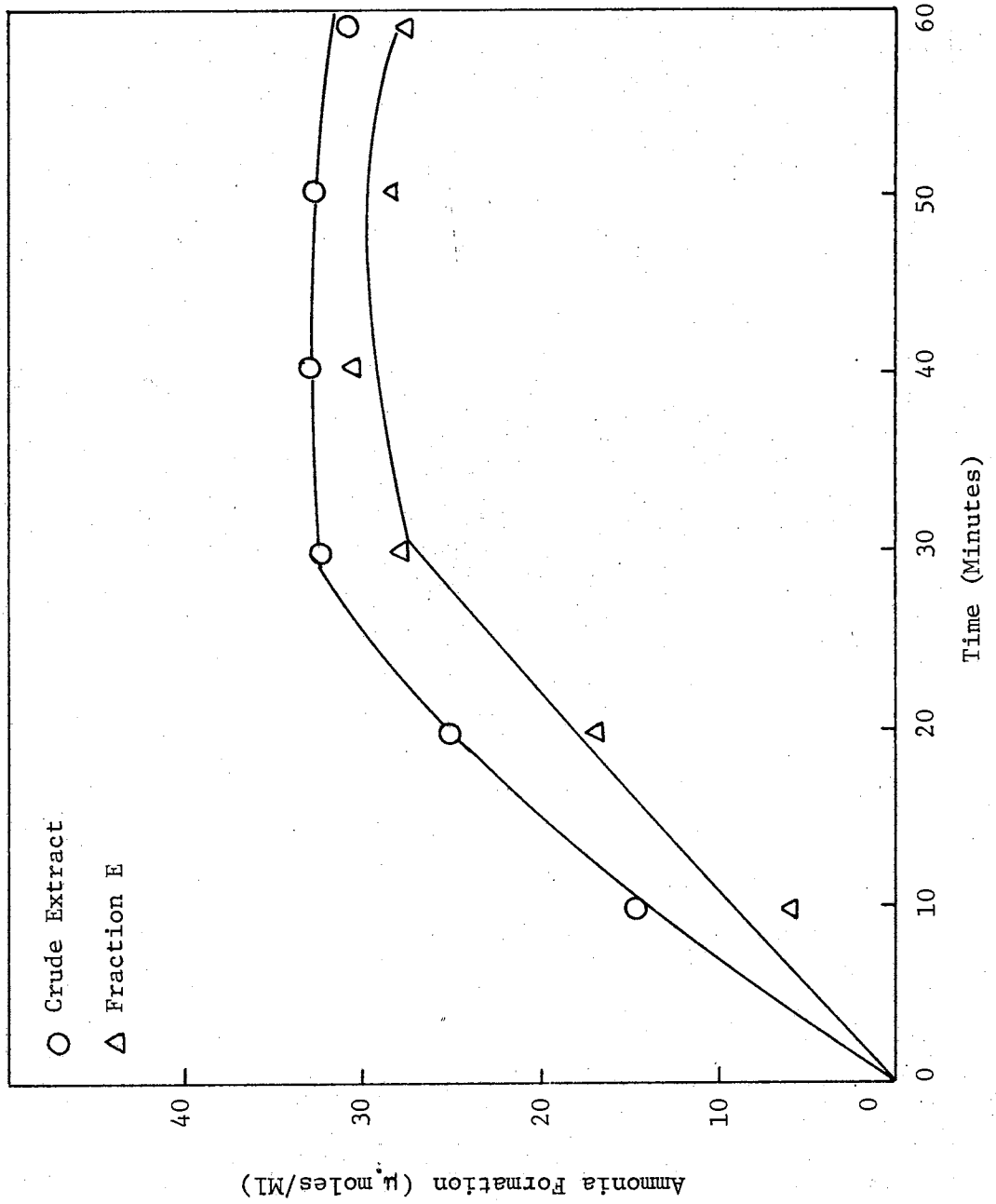

Crude Extract
Fraction E
Ammonia Formation (μ moles/Ml)
Time (Minutes)
0
10
20
30
40
50
60

Figure 6. Enzyme Activity vs. Protein Concentration.

The incubation mixture contained : 151.2 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5). A range of 0 to 150 μg of protein (crude extract) in the incubation tube was used and enzymes were acid pretreated. Incubation time was 30 minutes at 30°C. Ammonia was determined immediately after incubation.

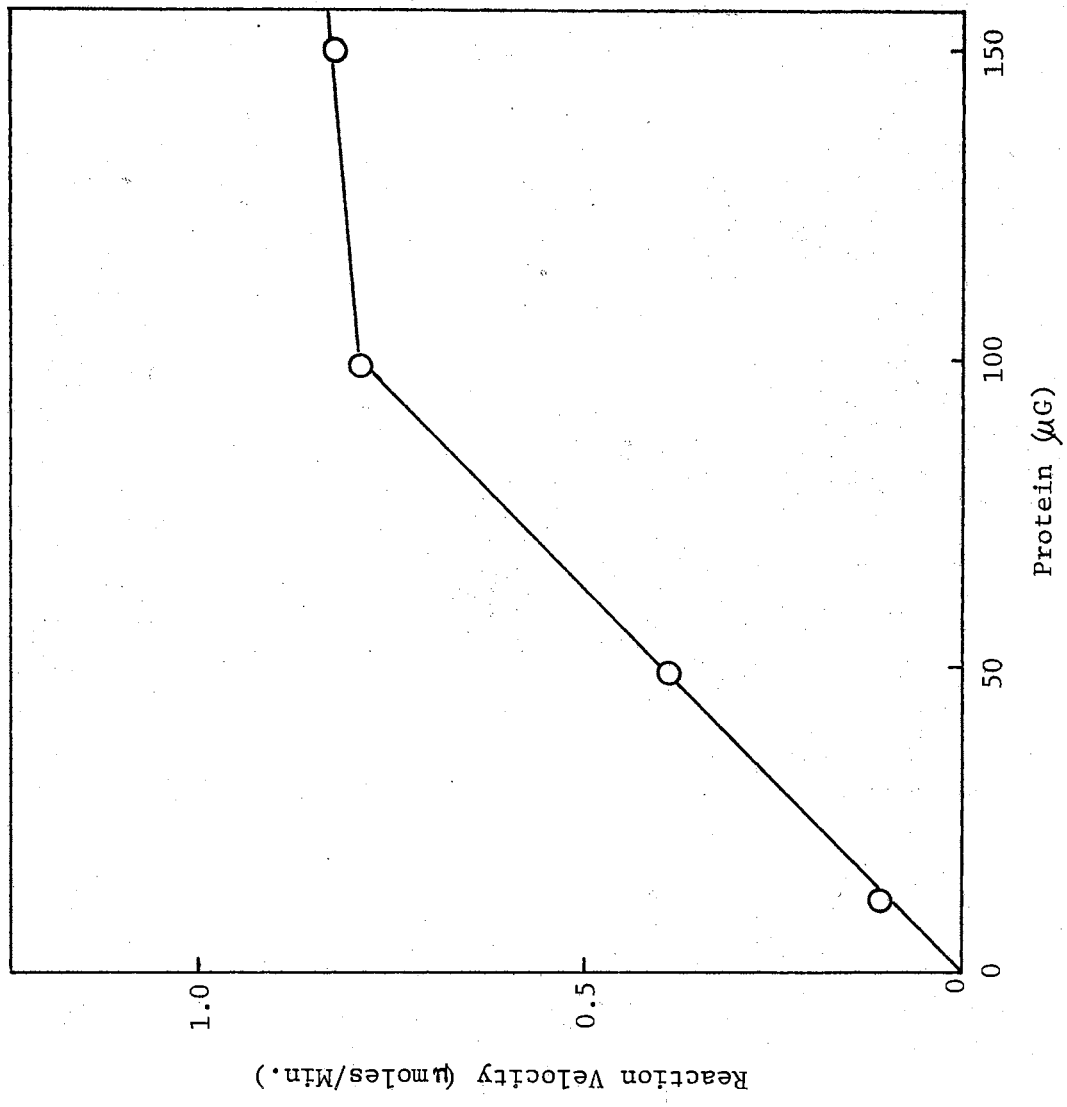

Reaction Velocity (μmoles/Min.)
1.0
0.5
0
0
50
100
150
Protein (μG)

TABLE XVII

EFFECT OF INHIBITORS ON AMMONIA FORMING ENZYMES

Inhibitor	Per Cent Inhibition			
	4×10^{-8} M	9×10^{-3} M	9×10^{-4} M	1.9×10^{-8} M
KF	-	100	100	-
Na_2HAsO_4	0	-	-	-
ADP	-	-	-	30
ADP + K_2HPO_4 (0.2 M)	-	-	-	0

The incubation mixture contained : 141.3 μmoles potassium allantoate, 99 μmoles GSH, 0.9 μmole $MnSO_4$, 0.08 M barbital-HCl buffer (pH 8.5) and 0.2 ml of crude extract (9.2 mg/ml protein). Enzymes were acid pretreated. Experimental tubes were incubated 30 minutes at 30°C and ammonia concentration was determined immediately after incubation.

a concentration of 4.0×10^{-8} M. (40 μmoles/ml).

CHAPTER IV

DISCUSSION

Measurement of Ammonia Formation

H. A. Barker (1) first reported that fermentation of allantoin by Streptococcus allantoicus can produce ammonia in growing cultures in the ratio of 2.26 moles of ammonia to 1.0 mole of allantoin used. In 1963 Vogels (32) reported that allantoate is degraded to ammonia, carbon dioxide and probably ureidoglycine by allantoate amidohydrolase in a crude extract of Streptococcus allantoicus or Arthrobacter allantoicus. Vogels (33) and Trijbels and Vogels (22, 23) reported that they also could detect allantoate amidohydrolase activity in Pseudomonas acidovorans, and E. coli var. acidilactici. They reported that ammonia was determined by Nesslerization and by the glutamate dehydrogenase method. The latter method was used primarily as a qualitative rather than as a quantitative assay.

The present study indicates that different methods of ammonia determination affect the apparent ammonia production and also that different conditions affect the amount of ammonia formed. Results show 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 concentrated alkali and produce ammonia at the temperature used for incubation (37°C). Ureidoglycine (as well as ammonia) may react with Nessler's reagent and the same reaction may

occur in the indophenol assay. The microkjeldahl method is complicated by too many interferences with intermediates and products of allantoin degradation. In the glutamate dehydrogenase method, the concentration of NH_4Cl is not the rate-determining factor for the reaction. It seems apparent from nitrogen recovery calculations that some compound other than ammonia is measured by the Nessler's reagent. Since this was the method used by Vogels, his ammonia determinations are probably in error.

Under different conditions, the amounts of ammonia formed are quite different. With a high concentration of enzyme, there is no difference between acid-pretreated and non-pretreated enzyme, but with a low concentration of enzyme, acid activation is required to produce ammonia. Storage of the incubation mixture (from acid-pretreated enzyme) at -20°C causes a decrease in glyoxylate and ammonia concentrations. For the non-acid-pretreated sample, storage at -20°C causes an increase in ammonia and a decrease in allantoin and glyoxylate concentrations. It was decided that further investigation of these changes was not warranted and all subsequent samples were analyzed as soon as possible after the end of the incubation period.

The results of this portion of the investigation indicated that considerable care must be exercised in measurements of the activity of enzymes which produce ammonia. In the present case, it was necessary to employ microdiffusion before Nesslerization, to control the total amount of enzyme carefully, and to carry out assays as rapidly as possible to prevent changes in concentration of substrate or products.

Purification and Properties of Ammonia-Forming Enzymes

Ammonia-forming enzymes of Streptococcus allantoicus have been

purified 3.5 to 4.3-fold. No allantoinase activity has been found in the partially purified enzymes. DEAE cellulose column chromatography was used but was not successful. Vogels (32) reported the specific activity of allantoate amidohydrolase was increased 20 and 12-fold for Streptococcus allantoicus and Arthrobacter allantoicus crude extracts, respectively, by DEAE cellulose column chromatography.

After treatment with 0.05 M sodium citrate buffer (pH 4.0), the activity of the ammonia forming enzymes was found in the precipitated fraction. Vogels (33) has reported that the activation and inactivation of the enzyme do not involve the dissociation of a low- or high-molecular weight part of the molecule, and are probably due to intermolecular rearrangements in which the active site of the enzyme is involved. He also reported that about 80 per cent of the total activity could be precipitated by addition of citrate-HCl buffer at pH 2.2 and that all of this material was in the active form.

In the present study it was found that the optimum pH for ammonia formation was either 7.9 or 8.7 for both Fraction A and Fraction E. Vogels (33) reported only one value, pH 8.5, as the optimum pH for ammonia formation. The pH curve was rechecked with all possible controls, with identical results. Since both the crude and the partially purified preparations exhibit the same behavior, the results appear to be valid. The only explanation which can be offered at present is that the two activity peaks represent the pH optima for the two enzymes producing ammonia, allantoate amidohydrolase and ureidoglycine aminohydrolase. Ammonia formation was found to be proportional to incubation time over a period of about 30 minutes and to the enzyme concentration in a range of 0 to 135 μg per tube.

Ammonia-forming enzymes are completely inhibited by potassium fluoride at a concentration as low as 9×10^{-4} M. This finding explains the report by Barker (1) that allantoate accumulates in the medium when fluoride is added to a culture of Streptococcus allantoicus in allantoin medium. Fluoride has been reported to have no effect on ureidoglycolate synthetase by Gaudy (12) and no effect on allantoinase by Meganathan (19). Since ammonia formation is inhibited by fluoride, which often inhibits phosphorylation reactions, and since the formamide group reported to be removed by allantoate amidohydrolase would yield an extra ATP if removed phosphorolytically as carbamyl phosphate, ATP and phosphate were added to the incubation mixture and the products determined. Both crude and partially purified preparations were inhibited, rather than stimulated, in the presence of ADP. The reason for the inhibition is not known. However, the data show that allantoate amidohydrolase does not carry out a phosphorolytic cleavage of allantoate. This conclusion is supported also by the finding that no dialyzable cofactors, other than Mn^{++}, are required for the reaction, and by the fact that arsenate has no effect on the reaction.

The Metabolism of Allantoin by Streptococcus Allantoicus

Three different pathways have been proposed for the degradation of allantoin in microorganisms (Figure 1). A complete pathway for allantoin fermentation in Streptococcus allantoicus was proposed by Valentine, Bojanowski, Gaudy, and Wolfe (29). Allantoic acid was an intermediate in allantoin degradation by allantoinase. Allantoicase degrades allantoic acid to yield glyoxylurea (ureidoglycolate) and urea, whereas cell free extracts, supplemented with DPN, $MgSO_4$, and phosphate, carry out an

oxidation of this compound. Ureidoglycolate is an intermediate in both the production of glyoxylate and urea (by the enzyme ureidoglycolate synthetase) and the oxidation to oxamate. Carbamyl oxamate, which accumulates in phosphate-deficient reaction mixtures, has been identified as an intermediate; DPN is a required cofactor for its formation. Carbamyl oxamate is phosphorolytically cleaved by oxamic transcarbamylase to yield carbamyl phosphate and oxamic acid. Glyoxylate is converted to carbon dioxide and tartronic semialdehyde.

Vogels (31, 32) agreed that allantoic acid and ureidoglycolate are intermediates in the fermentation of allantoin. However, Vogels (33) reported that allantoic acid is degraded in a two step reaction by allantoate amidohydrolase and ureidoglycine aminohydrolase. Uredoglycine is a proposed intermediate in both the production of ammonia and ureidoglycolate. Evidence obtained in the present study supports Vogels' conclusion that the ammonia-forming enzymes are present in Streptococcus allantoicus. However, two major questions remain unsolved. Vogels has concluded that Streptococcus allantoicus has no allantoicase. If this conclusion is correct, urea should be produced only from the ureidoglycolate synthetase reaction and the amount of urea produced should never exceed the amount of glyoxylate formed. However, in many analyses of reaction mixtures, the ratio of urea to glyoxylate is greater than 1.0. Therefore, it is possible that Streptococcus allantoicus has two enzymes capable of degrading allantoate, i.e., both allantoicase and allantoate amidohydrolase. The second problem arises in attempting to assign a physiological role to an enzyme (allantoate amidohydrolase) which is only activated when the pH is decreased to 4.0 or below and is inactivated again when the pH approaches neutrality, although its optimum pH

is 8.5. It does not seem possible that an enzyme with these peculiar properties could be responsible for an essential reaction in the sole energy-yielding pathway of the cell growing on allantoin. It seems more likely that this enzyme might play a specialized role in maintaining the intracellular pH.

Further study of this problem will be difficult unless "ureidoglycine", the proposed intermediate, can be obtained or prepared. Vogels (32) has failed to isolate ureidoglycine, perhaps because of the rapid hydrolysis of ureidoglycine into ammonia, glyoxylate and urea.

All the work that has been described above was based on chemical analysis of reaction mixtures. Genetic studies of the allantoin pathway sequence and the isolation or preparation of ureidoglycine will be the subjects of continued study.

CHAPTER V

SUMMARY AND CONCLUSIONS

The development of reliable techniques for measurement of ammonia-forming reactions in Streptococcus allantoicus and the study of some properties of the ammonia-forming enzymes were the major objectives of this investigation. It has been found that the microdiffusion method for measuring ammonia is the best method for the purpose of studying the ammonia-forming reaction. Use of limited amounts of enzyme and rapid analysis of the reaction mixture are recommended for assay of the production of ammonia.

The ammonia-forming enzymes of Streptococcus allantoicus were purified 3.5 to 4.3 times by treatment with streptomycin, adsorption on calcium phosphate gel and ammonium sulfate fractionation. The properties of the enzymes were investigated and two optimum pH values were found for the enzymes.

From the results of the present study, it may be concluded that the enzyme activity described by Vogels (33) is present in Streptococcus allantoicus. Further study of these enzymes, synthesis or isolation of the proposed intermediates, and study of mutants unable to degrade allantoate will be required before it can be determined whether the organism possesses one or two pathways for allantoin utilization.

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