REGULATION OF THE ENZYMES OF PURINE CATABOLISM IN PSEUDOMONAS AERUGINOSA AND NEUROSPORA CRASSA

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Submitted to the Faculty of the Graduate College
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in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
July, 1970

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ACKNOWLEDGMENTS

My first and foremost thanks are due to Dr. Elizabeth Gaudy for her patient guidance, encouragement and assistance in many experiments. My thanks are also due to Drs. G. L. Barnes, L. A. Brinkerhoff, L. L. Gee and R. K. Gholson for serving on my advisory committee and to Dr. E. C. Noller for his advice on the nomenclature of organisms.

Appreciation is extended to Mr. Hank Heath for donating his mutant strain PA-1-707. I would like to thank my fellow graduate students, Mrs. Karen Brown, Mr. Ron Green, Mr. Hank Heath, Miss San-San Tsay, Mr. Floyd White and Dr. Chii-Huei Wu for their cordial friendship, association and many discussions throughout the course of this study.

Financial assistance provided in the form of a research assistantship from the National Institutes of Health, USPHS, research grant AI-05401 to Dr. Elizabeth Gaudy, is gratefully acknowledged. Financial assistance for preparing this thesis from the Watumull Foundation, Hawaii, is gratefully appreciated.

Finally, I would like to thank my father, mother and brothers for the confidence placed in me and their encouragement throughout my stay here. Special thanks are due to my wife, Ammi, for her patience and understanding.

Last, but not least, the author wishes to thank Mrs. Judi Shirazi for her excellent and efficient typing.

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CHAPTER I

INTRODUCTION

The early history of nucleic acid research was reviewed by Davidson and Chargaff (1955). During the period 1868-69, Friedrich Miescher discovered nucleic acids, and he is considered to be the father of the chemistry of the nucleus of the cell. The term nucleic acid was used for the first time in the year 1889 by Altmann. But it was not until the early part of this century that the existence of two types of nucleic acids in the cell, namely deoxyribonucleic acid and ribonucleic acid, was recognized.

Bendich (1955) has reviewed the history of purine bases. Fischer coined the term purine in the year 1907. Only two purines, adenine and guanine, are recognized as normal constituents of nucleic acids. Adenine was discovered and isolated by Kossel in the year 1885. He also succeeded in converting adenine to hypoxanthine. Magnus, in the year 1844, discovered guanine in the excreta of birds. During 1883-84, Kossel recognized guanine to be a constituent of nucleic acids. The conversion of guanine to xanthine was recognized first by Strecker in 1858. Scheele and Bergman discovered uric acid in the year 1776. Allantoin was first discovered in the amniotic fluid of cattle (Buniva and Vauquelin, 1799). Allantoic acid was found in nature for the first time in *Phaseolus vulgaris* (Fosse, 1928).

The catabolism of purines in living organisms and its evolutionary importance has received much attention. It is interesting, from an evolutionary standpoint, that primates, birds, uricolytic reptiles and insects other than Diptera excrete uric acid. Mammals (other than primates), insects (Diptera only), and gastropods excrete allantoin. Some teleost fishes excrete allantoic acid. Fishes (in general), Amphibia, and Lamellibranches (fresh water) excrete urea, and the Gephyrean worms, Lamellibranches (marine) and Crustacea excrete ammonia (Baldwin, 1957; Florkin, 1966).

The anaerobic degradation of purines has been thoroughly investigated (Barker, 1956; Barker, 1961). Here only the literature pertaining to the aerobic conversion of purines to allantoin will be reviewed. A summary of the reactions which have been studied in various organisms which degrade purines or their derivatives is shown in Figure 1.

The deamination of adenine and guanine results in the formation of hypoxanthine and xanthine, respectively. Roush, Questiaux and Domnas (1959) assumed the pathway for purine degradation in the yeast Candida utilis to be

$$\begin{tikzcd}
\text{Adenine} \arrow[r] & \text{Hypoxanthine} \arrow[d] & & \\
\text{Guanine} \arrow[r] & \text{Xanthine} \arrow[r] & \text{uric acid} \arrow[r] & \text{allantoin.}
\end{tikzcd}$$

Ammann and Lynch (1964) reported the pathway for adenine degradation in Chlorella pyrenoidosa as

Adenine → hypoxanthine → xanthine → uric acid → allantoin → end products.

LaRue and Spencer (1968) studied the utilization of various purines by 123 species of yeasts and concluded the pathway to be

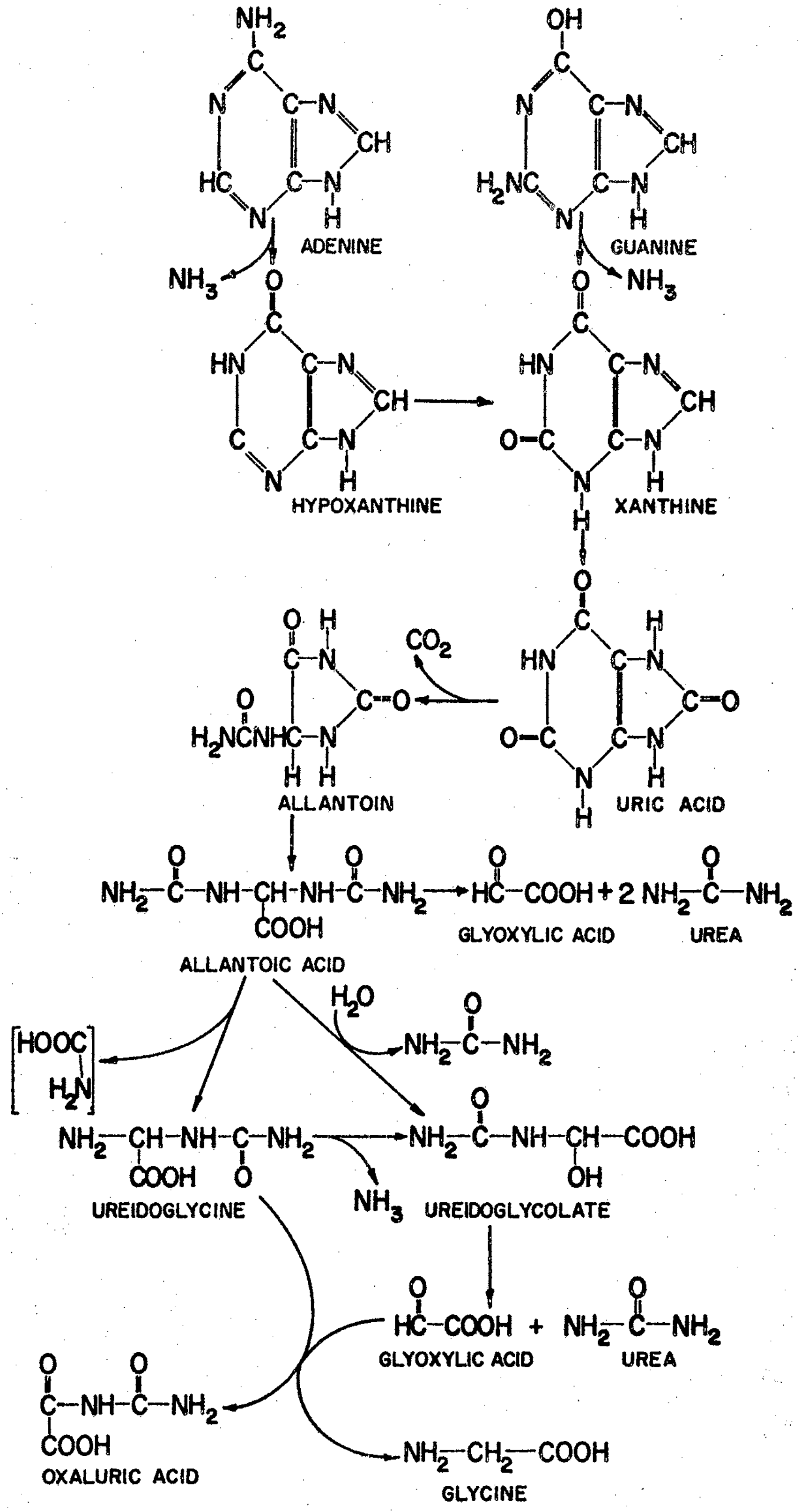

ADENINE
GUANINE
NH_3
NH_3
HYPOXANTHINE
XANTHINE
CO_2
ALLANTOIN
URIC ACID
ALLANTOIC ACID
GLYOXYLIC ACID
UREA
H_2O
UREIDOGLYCINE
NH_3
UREIDOGLYCOLATE
GLYOXYLIC ACID
UREA
OXALURIC ACID
GLYCINE

Figure I. Reactions in degradation of purines.

$$\begin{tikzcd} \text{adenine} \arrow[r] & \text{hypoxanthine} \arrow[d] \\ \text{guanine} \arrow[r] & \text{xanthine} \arrow[r] & \text{uric acid} \arrow[r] & \text{allantoin} \arrow[r] & \text{allantoic acid} \arrow[r] & \text{urea} + \text{glyoxylic acid.} \end{tikzcd}$$

None of these workers tested ureidoglycolate as an intermediate, although it had been proposed as such (Valentine and Wolfe, 1961b). Scazzocchio and Darlington (1968) showed the pathway of degradation of hypoxanthine in *Aspergillus nidulans* to be hypoxanthine → (xanthine) → uric acid → allantoin → allantoic acid → ureidoglycolate → urea + glyoxylate. Allam and Elzainy (1969) showed that the pathway of xanthine degradation in *Penicillium chrysogenum* was essentially the same except that they were unable to detect ureidoglycolate.

Adenine is converted to hypoxanthine by adenine deaminase (adenase). The tissues of many higher animals do not seem to contain this enzyme but it is present in microorganisms. It has been reported in *Escherichia coli* (Lutwak-Mann, 1936) and yeast (Roush, 1954). Adenine deaminase from *Azotobacter vinelandii* has been purified 35-fold. This purified preparation hydrolytically deaminates adenine but not guanine, cytosine, adenosine or many other related compounds (Heppel, Hurwitz and Horecker, 1957).

Guanine is deaminated to xanthine by the enzyme guanine deaminase (guanase). Unlike adenine, guanine can be deaminated by many higher animals (Schmidt, 1955). Guanase has been shown to be present in *Pseudomonas aeruginosa* (Clarke and Meadow, 1966) and in *Can. utilis* (Roush, Questiaux, and Domnas, 1959).

The enzyme which converts hypoxanthine and xanthine to uric acid is known as xanthine oxidase. The conventional and established concepts of enzyme specificity are weakened by the unique properties of this enzyme. It has been reported that more than 100 compounds can act as substrates for xanthine oxidase. Even though xanthine oxidase has been very widely studied, little is known about its biological function. Since xanthine and hypoxanthine are the most rapidly oxidized substrates, they are assumed to be the natural substrates. Some authors attempt to distinguish two closely related enzymes. One is known as xanthine oxidase which, in addition to many compounds, can use molecular oxygen as an electron acceptor. The other enzyme, known as xanthine dehydrogenase, uses molecular oxygen at a very low rate as an electron acceptor. Xanthine oxidase has been reported to be present in *Lactobacillus casei* (Villela, Affonso and Mitidieri, 1955) and *Pseudomonas* and *Vibrio* species (Dikstein, Bergman and Henis, 1957). Roush, Questiaux and Domnas (1959) reported that even though the yeast *Can. utilis* grows on xanthine or hypoxanthine as nitrogen source they were unable to detect xanthine oxidase or xanthine dehydrogenase activity. A similar observation was made by Roush and Shieh (1962) in *Torulopsis candida*. Allam and Elzainy (1969) reported that they could detect xanthine dehydrogenase activity by reduction of tetrazolium chloride in extracts of *Pen. chrysogenum*. When the formation of uric acid was measured, the approximate specific activity was only 0.2 mμmoles/min/mg protein.

Darlington and Scazzocchio (1968) were able to obtain mutants from *Asp. nidulans* which were unable to grow on hypoxanthine. However, when these mutants were tested on xanthine they were able to grow at a reduced rate. They were unable to obtain xanthine-negative mutants. Therefore, they proposed that there may be an alternate pathway for xanthine oxidation in this organism.

Pateman, et al. (1964) reported that xanthine dehydrogenase from

Asp. nidulans is unable to transfer electrons to molecular oxygen even though the enzyme can utilize methylene blue and various tetrazolium salts as electron acceptors. They also suggested that there may be a common co-factor for nitrate reductase and xanthine dehydrogenase in this organism since they isolated several mutants which had lost both the enzyme activities simultaneously.

The purification of xanthine dehydrogenase from *Clostridium cylindrosporum* (Bradshaw and Barker, 1960) and *Micrococcus lactilyticus* (Smith, Rajagopalan and Handler, 1967) has been reported. Substrate activation with xanthine oxidase from chicken liver was reported by Priest and Fisher (1969).

Xanthine oxidase (or xanthine dehydrogenase) converts xanthine and hypoxanthine to uric acid. The microbial decomposition of uric acid has been studied. Bachrach (1957) studied the aerobic breakdown of uric acid by pseudomonads. He reported the pathway to be uric acid ⟶ allantoin ⟶ allantoic acid ⟶ urea + glyoxylate. Schefferle (1965) reported that bacteria belonging to the genera *Corynebacterium*, *Nocardia*, *Streptomyces*, *Pseudomonas*, *Alcaligenes* and *Achromobacter* are capable of decomposing uric acid. Ammann and Reed (1967) reported that *Hydrogenomonas eutropha* can utilize uric acid or allantoin as the sole source of nitrogen.

The enzyme responsible for converting uric acid to allantoin is known as uricase (urate oxidase). Uricase has been purified from *Neurospora crassa* (Greene and Mitchell, 1957) and from *Arthrobacter pascens* (Arima and Nose, 1968; Nose and Arima, 1968). Roush and Domnas (1956) reported on the induced biosynthesis of uricase in *T. utilis*.

Barker (1943) first showed the ability of a bacterium to ferment allantoin under anaerobic conditions. He isolated a new species of homofermentative lactic acid bacteria from San Francisco Bay mud by using allantoin enrichment medium, and proposed the name Streptococcus allantoicus for this organism. The following fermentation balance was reported for Strep. allantoicus (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. This was the first reported instance of biological formation of oxamic acid. In addition to the above, traces of glycine were also detected. Barker (1961) also showed that the nitrogen of oxamic acid is not derived from ammonia by using N^{15}-labeled ammonia. Therefore he concluded that the oxamic acid must be formed directly by the oxidative cleavage of allantoic acid.

Campbell (1954, 1955) isolated a Pseudomonas strain from San Francisco Bay mud using an enrichment medium containing allantoin and studied the aerobic degradation of 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 observed 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, or 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.

Valentine and Wolfe (1960), in their studies on allantoin metabolism in Strep. allantoicus, showed that oxamic acid was formed from carbamyl oxamic acid by 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 (1961a) proposed the following intermediates for the fermentation of allantoin by Strep. 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 Strep. 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 Strep. 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 *Strep. 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) agreed that allantoic acid is formed from allantoin by *Strep. 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 postulated to involve intramolecular rearrangement. Later investigations (Trijbels and Vogels, 1966a, 1967; Vogels, 1966) supported the above findings. The organisms capable of degrading allantoate via this pathway, as reported by Vogels, are *Ps. acidovorans*, *Arth. allantoicus*, *Strep. allantoicus*, *E. 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) in an aerobic microorganism. They isolated mutants from a strain of *Ps. 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.

Meganathan (1968) showed that bacteria belonging to the genera *Aerobacter*, *Alcaligenes*, *Arthrobacter*, *Escherichia*, *Flavobacterium*, *Nocardia* and *Pseudomonas* have the ability to grow on allantoin aerobically and that species of *Aerobacter*, *Escherichia*, *Streptococcus* and *Pseudomonas* can ferment allantoin. All organisms which fermented allantoin formed oxamate. Allantoate amidohydrolase activity could not be detected in species of *Alcaligenes*, *Arthrobacter*, *E. coli*, *Nocardia*, or *Pseudomonas* or in *Ps. aeruginosa* even though the enzyme could be detected in *Strep. allantoicus*. However, ureidoglycolate synthetase activity was detected in all the organisms which were capable of growing on allantoin. Wu (1968) purified allantoate amidohydrolase from *Strep. allantoicus* approximately four-fold and found two pH optima, thus suggesting the possibility of the involvement of two enzymes in the formation of ammonia from allantoate.

Trijbels and Vogels (1966a, 1966b), using cell-free extracts of a urease-negative strain of *Ps. aeruginosa*, found no ammonia formation from sodium allantoate. They obtained similar results using *Ps. fluorescens*, *Pen. notatum*, and *Pen. citreo-viride*. Since no ammonia was detected, they concluded that the degradation of allantoate was not brought about by the allantoate amidohydrolase-ureidoglycine aminohydrolase reactions but by the allantoicase pathway. 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 Can. (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.

Trijbels and Vogels (1967) studied the degradation of allantoate and ureidoglycolate by Ps. aeruginosa. They reported that a single enzyme, allantoicase, is responsible for the conversion of allantoate to (-) ureidoglycolate. They also found that two enzymes are responsible for the degradation of ureidoglycolate. They reported that allantoicase degraded (+) ureidoglycolate three to four times faster than (-) ureidoglycolate and that (-) ureidoglycolase was specific for (-) ureidoglycolate. Van der Drift and Vogels (1969a) reported that (-) ureidoglycolate is formed by Strep. allantoicus and it is converted to glyoxylate and urea by (-) ureidoglycolase.

Vogels and his coworkers have devoted a considerable amount of effort to studies of the unusual properties of allantoate amidohydrolase. Van der Drift and Vogels (1967) partially purified this enzyme from Strep. allantoicus and studied the effect of pH, EDTA (ethylenediaminetetraacetate) and Mn^{++} upon its activation and inactivation. Van der Drift and Vogels (1969b) studied the dependence of allantoate amidohydrolase activation on pH and anions using Strep.

allantoicus extracts. Van der Drift and Vogels (1969c) reported on the inactivation and instability of allantoate amidohydrolase from *Strep. allantoicus* due to pH and metal ions. In all of the above studies partially purified extracts were used. It is well known that changes in pH and concentration of metal ions affect many proteins. Therefore, if partially purified extracts are used, it is possible that the effect on activity may not be entirely due to an effect on the enzyme itself.

Van der Drift and Vogels (1970) reported on the preparation of metal-free allantoicase from *Ps. aeruginosa* and the effect of hydrogen ions on activity and stability of the enzyme. They reported that thirteen bivalent cations restored catalytic activity with varying effectiveness.

Van der Drift, de Windt and Vogels (1970) studied the enzymatic and non-enzymatic reactions involved in the degradation of allantoate in *Strep. allantoicus*. On the basis of this study they concluded that "postulation of the enzymes ureidoglycine aminotransferase and ureidoglycine aminohydrolase appears to be not necessary, since the transamination reaction seemed to proceed non-enzymatically and the conversion of ureidoglycine to ureidoglycolate was most likely catalysed by allantoate amidohydrolase."

In summary, many of the reactions included in the pathway for purine degradation shown in Figure 1 have been studied individually in several organisms but some questions concerning several reactions remain. Adenase and guanase are responsible for the conversion of adenine and guanine to hypoxanthine and xanthine, respectively. Xanthine and hypoxanthine are converted to uric acid by xanthine oxidase or xanthine dehydrogenase. However, these reactions have not been detected in some organisms which degrade purines, and alternate pathways have been postulated. Uric acid is converted to allantoic acid by allantoinase. However, as shown in Figure 1, three different pathways have been postulated for the conversion of allantoic acid to urea and glyoxylic acid.

The purpose of this study was to determine the pathway of purine degradation in *N. crassa* and to study the regulation of the enzymes involved in purine catabolism in *N. crassa* and *Ps. aeruginosa*. *N. crassa* can utilize purines as the sole source of nitrogen but cannot use purines as carbon sources, whereas *Ps. aeruginosa* can utilize allantoin, an intermediate in purine degradation, as the sole source of carbon and/or nitrogen. Therefore, the choice of these two organisms for the present study offered several advantages. The pathway of purine degradation had not previously been studied in *Neurospora* and it was of particular interest to determine which of the proposed reactions for allantoate degradation is used by this organism. The allantoate amidohydrolase pathway would appear to be preferable to the allantoicase pathway in an organism which degrades purines only as nitrogen sources since by this pathway ammonia is formed directly and the additional action of urease is not required. However, the allantoate amidohydrolase pathway has been reported only in bacteria and the only two fungal species in which this segment of the pathway has been studied, both species of *Penicillium* (Trijbels and Vogels, 1966b), have been found to have the allantoicase pathway. The degradative pathway for allantoin in *Ps. aeruginosa* has been established as the allantoicase pathway by the studies of Bruce (1965), Winter (1967) and Trijbels and Vogels (1967), and Winter has studied the induction of enzymes in mutants of this organism. No previous studies of induction and repression of purine catabolism in Neurospora have been made nor has catabolite repression of the pathway been previously studied in either organism. Therefore, it was of interest to compare the control of the same pathway in two organisms, one a bacterium and the other a fungus, and, in particular, to study induction and repression of the same pathway in organisms which use the same compounds for different purposes.

The mechanisms employed by microorganisms to control the synthesis and activity of many of their catabolic and anabolic enzymes have been reviewed frequently in recent years (e.g., Epstein and Beckwith, 1968) and will be discussed only briefly here. The discussion will be limited to mechanisms active in catabolic pathways.

Many microorganisms have the genetic capability of using any of a number of compounds as sources of carbon and energy or nitrogen. The metabolism of each of these compounds will require several enzymes. Consequently, each cell has the ability to make thousands of enzymes. But the continual synthesis of thousands of enzymes would amount to a tremendous waste of materials and energy if many of the enzymes are not continuously required. In order to conserve materials and energy, the cell is endowed with the genetic capability for a number of regulatory mechanisms, just as it is endowed with the capacity to metabolize a number of compounds and consequently to produce a number of enzymes. The regulatory mechanisms which are operative in catabolic pathways are repression, induction, catabolite repression and catabolite inhibition.

Repression is the prevention of the synthesis of an enzyme by a repressor. The repressor is a protein molecule synthesized under the direction of a regulator gene specific for the pathway. This specific repressor does not require activation by a co-repressor, a small molecule which is a product of the pathway concerned, but is synthesized in an active form. The active repressor combines with the operator region and prevents the synthesis of the enzyme by preventing transcription of the structural gene.

The enzymes for the degradation of many compounds are produced only when the compound in question (or a closely related compound) is added to the medium. When an enzyme is synthesized in response to the addition of a particular compound, the enzyme is said to be induced by the compound. An enzyme which can be induced is called an inducible enzyme, and the compound which induces an inducible enzyme is the inducer. The process whereby an inducible enzyme is formed is known as induction and is, in effect, the relief of repression. In induction, the active repressor is converted into an inactive repressor by combination with the inducer. Since now the repressor is inactive, it can no longer repress enzyme formation and consequently the enzyme is produced. An enzyme which is made whether or not its substrate is present is termed constitutive.

Catabolite repression is a process whereby a product of a catabolic pathway is responsible for the prevention of synthesis of catabolic enzymes required for utilization of another substrate. Here a catabolic product of the pathway acts as the co-repressor, i.e., the catabolite repressor is synthesized in an inactive form and is active only in combination with the catabolite. Catabolite repression can take place in the presence of the inducer, since the inducer does not inactivate this repressor.

Thus, repression and catabolite repression affect the rate of enzyme synthesis. In catabolic pathways, instances are known where the addition of one compound inhibits the utilization of another compound. Here the activity of the enzyme, rather than its synthesis, is affected. This process is known as catabolite inhibition. It was first reported by Gaudy, Gaudy and Komolrit (1963) and has been confirmed by Anthony and Guest (1968), McGinnis and Paigen (1969) and Stumm-Zollinger (1966).

At the time of starting this work, no report on the regulation of the enzymes of purine catabolism was available. Recently Scazzocchio and Darlington (1967, 1968) studied the regulation of the enzymes involved in hypoxanthine degradation in Asp. nidulans. They concluded, on the basis of their studies using mutants, that both xanthine dehydrogenase and urate oxidase were induced by uric acid. Hypoxanthine was ineffective in inducing uricase. However, they were unable to determine whether hypoxanthine could induce xanthine dehydrogenase. On the basis of their studies using the inhibitor allopurinol, which prevents the conversion of hypoxanthine to uric acid, they concluded that uric acid is the inducer. Ammonia was reported to be able to repress the formation of xanthine oxidase and urate oxidase. Allantoinase was reported to be induced maximally by the presence of both uric acid and allantoin. Ureidoglycolase was found to be constitutive.

CHAPTER II

MATERIALS AND METHODS

A. Organisms

The following organisms were used in this study:

Pseudomonas aeruginosa (PA-1) was obtained from B. W. Holloway, University of Melbourne.

Ps. aeruginosa strain 707 (PA-1-707) is a glucose-negative mutant derived from strain PA-1, which was kindly supplied by Harry E. Heath, III of this department.

Neurospora crassa FGSC #262, mating type A, also known as St. Lawrence standard 74A, vegetative reisolate by D. Newmeyer. The following inositol requiring mutants were also used. N. crassa FGSC #498, mating type a, also known as 89601, and N. crassa FGSC #497, mating type A, also known as 89601. The N. crassa cultures were obtained from the Fungal Genetics Stock Center, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire.

B. Media for Cultivation of Organisms

Ps. aeruginosa was grown in a minimal medium of the following composition:

$Na_2HPO_4 \cdot 7H_2O$	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	1000 ml

Unless otherwise stated, the carbon source was used at a concentration of 0.5 percent. The carbon source was either autoclaved or filter-sterilized separately.

N. crassa was grown in Frie's minimal medium or Vogel and Bonner medium for mutant isolation and in Vogel and Bonner medium for enzyme studies.

Frie's minimal medium (Hartman, Suskind and Wright, 1965) had the following composition:

Ammonium tartrate	5 gm
Ammonium nitrate	1 gm
KH_2PO_4	1 gm
$MgSO_4$	0.5 gm
NaCl	0.1 gm
$CaCl_2$	0.1 gm
Biotin	5 µg
Trace element solution	1 ml
Sucrose	20 gm
Distilled water	1000 ml

The trace element solution (Mandal, 1961) contained the following components:

H_3BO_3	30 mg
$CuCl_2$	125 mg
$FeCl_3 \cdot 6H_2O$	500 mg
$MnSO_4$	30 mg
Molybdic acid	170 mg
$ZnCl_2$	2000 mg
Distilled water	1000 ml

For plating of mutagenized conidia, ammonium nitrate was omitted.

Vogel and Bonner (1956) medium contained the following components:

Sodium citrate · $2H_2O$	131 gm
KH_2PO_4	250 gm
$MgSO_4$	4.9 gm
$CaCl_2$	3.8 gm
Trace element solution	5.0 ml
Biotin solution	2.5 ml

The components were added in order with stirring to 750 ml H_2O and the volume was made up to one liter. Chloroform (2 ml/1) was added as a preservative and the medium was stored at room temperature. This medium was diluted 50-fold for use with distilled water. Sucrose at a final concentration of 2.0 percent was added as a carbon source. When ammonium nitrate or ammonium tartrate was used as a nitrogen source, they were added to a final concentration of 0.2 percent. The purines, when serving as nitrogen source, were added at a concentration of 0.1 percent. The diluted medium was usually autoclaved after additions were made except when allantoin was used as a nitrogen source. Allantoin was dissolved and sterilized separately by passing through a Millipore filter (HA, 0.45 μ pore size). The pH of the medium was approximately 5.8.

The trace element solution contained the following components:

Citric acid·H_2O	5 gm
$ZnSO_4 \cdot 7H_2O$	5 gm
$FeSO_4 \cdot 7H_2O$	1 gm
$CuSO_4 \cdot 5H_2O$	0.25gm
$MnSO_4 \cdot 1H_2O$	0.05gm
H_3BO_3	0.05gm
$Na_2MoO_4 \cdot H_2O$	0.05gm

These were added in order to 95 ml H_2O. One ml of chloroform was added as preservative and the solution was stored at room temperature.

The biotin solution contained 10 mg biotin/100 ml of 50 percent (v/v) ethanol and was stored at 4°C.

For making solid medium, Frie's minimal medium or Vogel and

Bonner minimal medium was supplemented with 2 percent agar. For the induction of colonial paramorphs, sucrose was replaced by 1 percent sorbose and 0.1 percent sucrose (Tatum, Barratt and Cutter, 1949).

C. Maintenance of Stock Cultures and Growth Conditions

Stock cultures were maintained in 18 mm tubes containing 6 ml of solid medium. The cultures were grown under light. For use in mutagenesis, the organism was grown either on agar slants or in 125 ml flasks containing 20 ml medium solidified with agar. For growing inos$^-$ mutants, 100 μg of inositol were added per ml of medium.

D. Chemicals

Adenine, hypoxanthine and xanthine were obtained from Nutritional Biochemicals Corporation. Guanine hydrochloride was obtained from CalBiochem. Uric acid was obtained from CalBiochem or Mann Research Laboratories.

Allantoin was obtained from Sigma Chemical Company or from Nutritional Biochemicals Corporation. Potassium allantoate was prepared by a modification of the method of Young and Conway (1942) as follows: 1.0 M KOH was heated to a temperature of 80°C and allantoin was dissolved in it to a concentration of 10 percent (w/v). The temperature was maintained at 80°C for 45 minutes. The solution was cooled and ten volumes of 95 percent 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 consecutively 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). All other chemicals were of reagent grade, obtained commercially.

E. Mutagenesis

The conidia from the slants or 125 ml flasks were washed in distilled water and filtered through cheese cloth into a 125 ml Erlenmeyer flask. The conidia were washed in distilled water by centrifugation and resuspension at least three times before use in mutagenesis. Conidial concentration was determined by counting in a haemocytometer chamber and adjusted to 10^6 to 2×10^7/ml for mutagenesis and direct enrichment. For indirect enrichment approximate concentrations were used (estimated by optical density). Four different mutagenic agents were employed and a variety of procedures was used. These are described below:

1. Ultraviolet Light

The ultraviolet light source used was a 15 Watt Sylvania germicidal lamp. Usually 5 ml of a water or buffer suspension of conidia were irradiated at a distance of 20 cm. Two procedures were used:

a. A suspension of conidia in 0.067 M potassium phosphate buffer pH 7.0 was irradiated for 3 minutes.

b. A suspension of conidia in water was irradiated for 10 percent survival.

2. Ethylmethane Sulfonate (EMS)

Two procedures were used:

a. 10 ml of conidial suspension in 0.067 M potassium phosphate buffer pH 7.0 were treated for 18 hr with 0.13 ml EMS without shaking.

b. 20 ml of conidial suspension in 0.067 M phosphate buffer, pH 7.0 were shaken 5 hr with 0.25 ml EMS. This procedure is a modification of the procedure used by Malling (1966).

3. N-methyl-N′-nitro-N-nitrosoguanidine (NTG)

Two procedures were used:

a. 5 ml of conidial suspension in water were shaken for 30 min with 50 µg of NTG.

b. A 20 ml water suspension was shaken with 2 mg NTG. This procedure is a modification of the one used by Flavell and Fincham (1968). This procedure was used in three separate experiments.

4. Nitrous Acid Treatment

11.6 ml of conidial suspension in 0.1 M acetate buffer, pH 4.6, were shaken with 4.0 mg of $NaNO_2$ for 160 min. This procedure is a modification from De Serres, et al. (1967).

F. Mutant Enrichment

After mutagenesis by one of the methods listed above, the conidia were washed three times in distilled water and used for enrichment by one of the following procedures.

1. Filtration Enrichment

The mutagen-treated conidia either were directly used for enrichment by filtration or they were inoculated into a 125 ml Erlenmeyer flask containing 20 ml of medium with ammonium tartrate as the nitrogen source and allowed to sporulate. After sporulation, the conidia were harvested, washed three times and used in enrichment.

The concentration of the conidia was adjusted to 10^6/ml in 20-25 ml of medium containing allantoin as a nitrogen source. The flask was shaken at room temperature. Whenever visible growth occurred, the suspension was filtered through sterile cheese cloth into another sterile 125 ml flask. Shaking and filtration were continued for approximately 30 hr and the remaining conidia were plated. This procedure is a modification from Flavell and Fincham (1968).

2. Enrichment by Inositol-less Death

The mutagenized conidia were suspended in 28 ml of medium with allantoin as a nitrogen source and with 25 µg/ml inositol and shaken for 6 hr. The conidia were harvested and washed three times in distilled water by centrifugation and resuspension. After washing, the conidia were suspended in 20 ml of allantoin medium without inositol and shaken for five days. Then the conidia were harvested and suspended in 1 ml of water and 0.2 ml was plated on ammonium tartrate medium (Flavell and Fincham, 1968).

In some experiments, the mutagenized conidia were washed and shaken for 6 hr in allantoin medium with inositol. The conidia were then washed with water three times by centrifugation and resuspension. The conidia were plated on allantoin medium without inositol. After

4-5 days incubation, the plates were overlayed with 2.5 ml of ammonium tartrate medium with 100 µg/ml of inositol (Lester and Gross, 1959).

When hypoxanthine-negative mutants were sought, the allantoin in the medium was replaced by hypoxanthine. Two sets were plated with phenotypic expression and two sets without phenotypic expression. For plating, 5 ml of the spore suspension were mixed with 200 ml of hypoxanthine agar medium without inositol and poured into Pyrex dishes, 13 1/2" x 8 1/2" x 1 1/2" deep. The agar was overlayed with NH_4NO_3-inositol medium on the third or fifth day.

G. Testing of Mutants

The survivors from the plates were picked into tubes with ammonium nitrate as a nitrogen source and allowed to sporulate. The spores were tested by inoculating into 2 ml liquid medium (Flavell and Fincham, 1968) or into 6 ml of liquid medium with allantoin as nitrogen source. In some experiments, 20 ml of liquid medium in 125 ml Erlenmeyer flasks or solid medium in tubes with allantoin or hypoxanthine were used.

H. Chemical Analyses

1. 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 percent Na_2CO_3, 1.0 ml of 4 percent sodium-potassium tartrate and 1.0 ml of 2 percent $CuSO_4$ 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 optical density was read, after 15 minutes, at 660 mμ. Standards of bovine serum albumin, 30 to 90 μg, were run along with the samples.

2. 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 two 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 five minutes at room temperature, the optical density was read at 515 mμ using a Coleman Junior Spectrophotometer.

I. Preparation of Extracts

Ps. aeruginosa was grown in minimal medium on a shaker at 37°C (200 ml/1000 ml flask). For preparing cell-free extracts, cells were harvested in a refrigerated Servall Centrifuge, Model RC-2B. Cells were broken with a Bronson sonifier after resuspending in 0.02 M potassium phosphate buffer, pH 7.0. The extract was centrifuged at 12000 rpm for 15 min and the supernatant was used in enzyme assays.

N. crassa was grown in Vogel - Bonner minimal salts medium (200-250 ml/1000 ml flask). A 1 to 2 week old slant was washed with 10 ml distilled water and 4 ml of this spore suspension were added to each flask. The cultures were grown in the dark (to prevent sporulation) with shaking for 48 hr. The mycelium was harvested by pouring the culture through two layers of cheese cloth and was washed with twice the volume of chilled triple-distilled water, then pressed between folds of aluminum foil to remove excess moisture. The mycelium was kept frozen until used. The frozen mycelium was broken into bits in a mortar and placed in a pre-frozen X-press (Biochemical Processes, Inc.). The press was kept frozen for at least 12 more hours. The mycelium was broken using a Carver laboratory press, and suspended in an equal volume (w/v) of 0.02 M potassium phosphate buffer, pH 8.5. The debris was removed by centrifuging at 12000 rpm for 15 min and the clear supernatant was used in enzyme assays.

J. Enzyme Assays

1. Adenase

Five mg of adenine were dissolved in 0.2 ml of 0.5 N NaOH. A 0.1 ml volume of this solution was diluted to 10 ml with 0.2 M potassium phosphate buffer, pH 7.0, and used as substrate. The incubation mixture contained 2.8 ml of 0.2 M potassium phosphate buffer, 0.1 ml of substrate and 0.1 ml of extract. Decrease in optical density of this incubation mixture was followed at 265 mμ (Roush, 1954) in a Cary 14 recording spectrophotometer using a blank containing 2.9 ml of buffer and 0.1 ml of extract.

2. Guanase

Eight mg of guanine HCl were dissolved in 0.6 ml of 0.5 N NaOH and 0.15 ml of this solution was made up to 100 ml with 0.2 M potassium phosphate buffer, pH 7.0. To 2.9 ml of this substrate solution was added 0.1 ml of enzyme, and the decrease in optical density at 245 mμ was followed in a Cary 14 recording spectrophotometer (Roush and Norris, 1950) using a blank containing buffer rather than substrate.

3. Xanthine Dehydrogenase

Eight mg of xanthine were dissolved in 0.5 ml of 0.5 N NaOH and 7 mg of hypoxanthine were dissolved in 0.4 ml of 0.5 N NaOH. The substrates were made up to 100 ml with 0.2 M potassium phosphate buffer, pH 7.5. The incubation mixture contained 2.5 ml of xanthine or hypoxanthine solution, and 0.3 ml of a 1 mg/ml solution of 3(4,5-dimethyl thiazolyl 1-2) 2,5 diphenyl tetrazolium bromide (MTT); 0.1 ml of cell-free extract was added and mixed. Phenazine methosulfate (0.1 ml of a solution containing 3 mg/ml) was added as a mediator between the dehydrogenase and the tetrazolium dye (Ringler and Singer, 1958). The enzyme activity was measured by recording the reduction of the dye at 565 mμ using a Cary 14 recording spectrophotometer. The blank contained buffer rather than substrate.

4. Urate Oxidase

Three mg of uric acid were dissolved in 0.5 ml of 0.5 N NaOH, and 0.25 ml of this solution was made up to 100 ml with 0.1 M Tris buffer, pH 8.8 (Greene and Mitchell, 1957). For assay of the enzyme, 2.9 ml of the substrate were mixed with 0.1 ml of cell-free extract and the decrease in absorbance at 293 mμ was measured against a blank without substrate in a Cary 14 recording spectrophotometer (Kalckar, 1947).

5. 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 removed for determination of allantoate and/or glyoxylate (Winter, 1967).

6. 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.

7. 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.

8. Allantoate Amidohydrolase

The assay was performed according to Vogels (1966). The ammonia formed was determined by direct Nesslerization (Vogel , 1953).

CHAPTER III

EXPERIMENTAL RESULTS

A. Studies with Pseudomonas aeruginosa strain PA-1

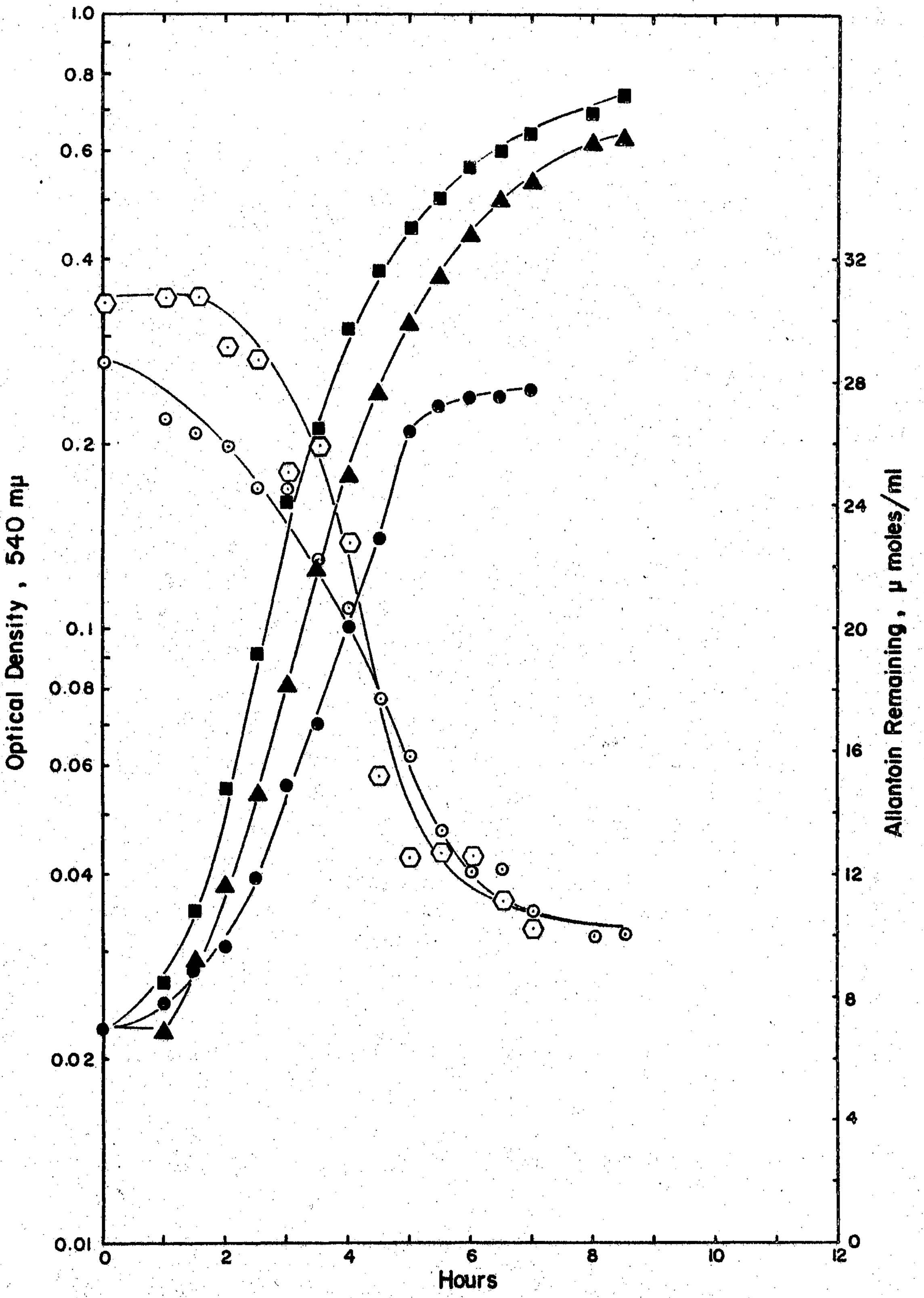
Three carbon sources which could possibly be expected to repress synthesis of allantoin degrading enzymes were used to study the control of this pathway. These were glucose, histidine and ammonium acetate.

1. Effect of Glucose on Allantoin Utilization

Since it is known that in a number of organisms glucose is capable of repressing the utilization of other substrates, it was thought that glucose might repress the utilization of allantoin. To verify this hypothesis, Ps. aeruginosa was inoculated into minimal medium containing a mixture of 0.5 percent allantoin and 0.2 percent glucose. The cells were pregrown on allantoin to eliminate the lag which usually occurs before utilization of allantoin is initiated. Allantoin and glucose alone served as individual controls. Optical density was recorded every 30 minutes and the rate of allantoin disappearance was followed by sampling from the allantoin control and from the mixture at 30 min intervals and determining the concentration of allantoin remaining in the medium after removal of cells by centrifugation. The results are shown in Figure 2. It can be seen from the figure that glucose did not cause diauxic growth in the mixture nor did it prevent the utilization of allantoin. The pattern of removal

Figure 2. Effect of glucose on utilization of allantoin by *Pseudomonas aeruginosa* strain PA-1.

Cells were grown on allantoin, washed by centrifugation and resuspension, and inoculated into 20 ml of minimal medium containing: (1) 0.5 percent allantoin; (2) 0.5 percent allantoin and 0.2 percent glucose; (3) 0.2 percent glucose. Growth on allantoin (●); growth on allantoin plus glucose (■); growth on glucose alone (▲); allantoin remaining in allantoin control (⬡); allantoin remaining in the mixture (○).


Optical Density , 540 mμ
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Allantoin Remaining , μ moles/ml
32
28
24
20
16
12
8
4
0
Hours
0
2
4
6
8
10
12

of allantoin from the control was essentially the same as in the mixture.

2. Effect of Histidine on Allantoin Utilization

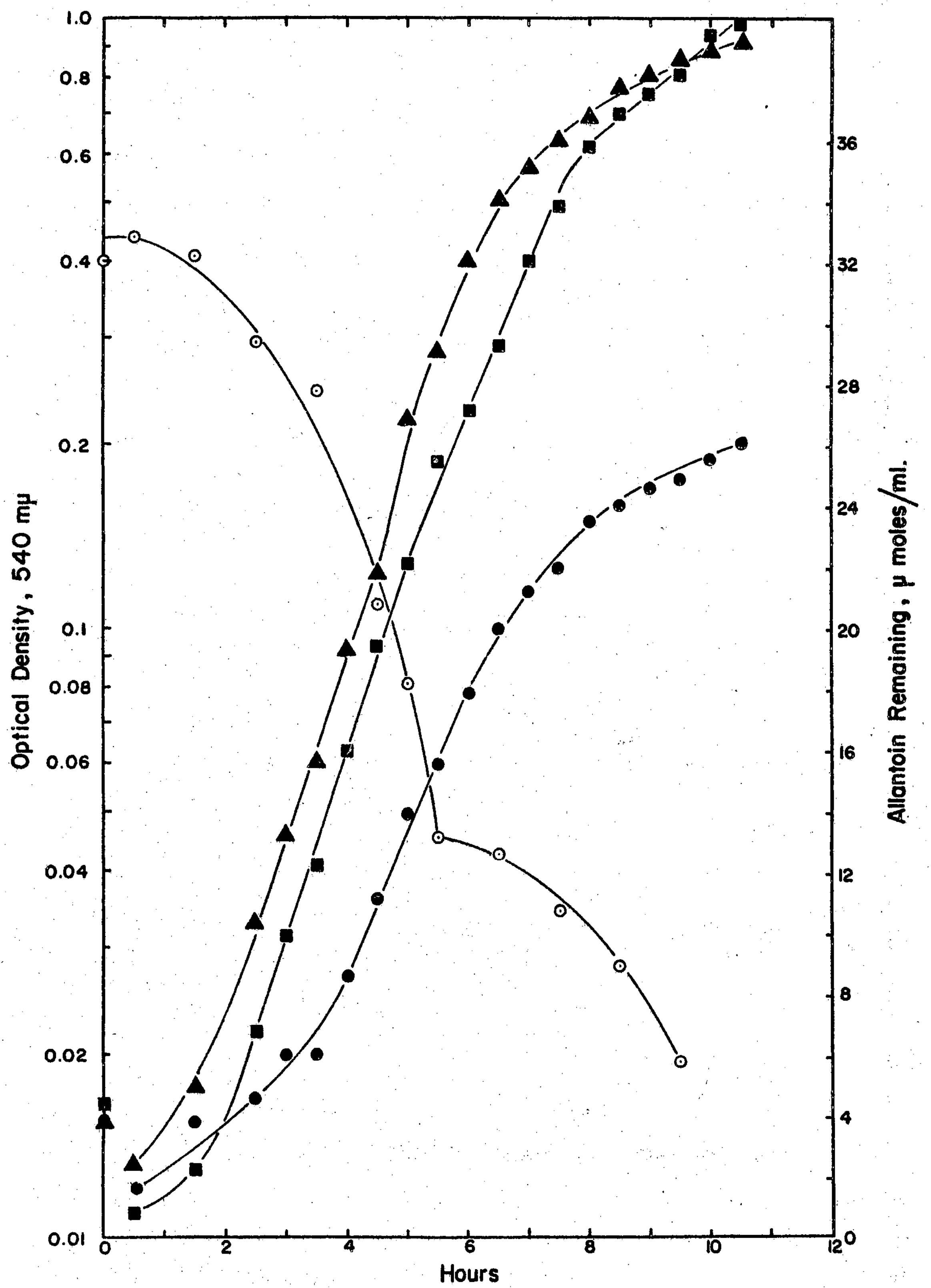
Since histidine is even more rapidly utilized as a carbon source by *Ps. aeruginosa* than is glucose, it was thought that histidine might be more effective than glucose in repressing utilization of allantoin. In order to test this, the organism was inoculated into a mixture of allantoin and histidine (0.5 percent of each). The cells were pre-grown on histidine to insure immediate rapid utilization of histidine and to provide optimum conditions for repression, since allantoin enzymes would have to be synthesized before allantoin could be used while those for histidine would be pre-formed. Allantoin and histidine separately served as controls. The optical density and the disappearance of allantoin from the mixture were followed at 30 minute intervals. The results are presented in Figure 3. It can be seen from the figure that there was no repression of allantoin utilization as defined by growth and substrate disappearance.

3. Effect of Acetate on Allantoin Utilization

Since acetate is an important two-carbon compound and the portion of allantoin utilizable as carbon source is also a two-carbon moiety, it was thought that acetate might repress allantoin utilization. *Ps. aeruginosa* was grown on nutrient broth and inoculated into a mixture of allantoin and ammonium acetate (0.2 percent each). Ammonium acetate and allantoin alone served as individual controls. Growth and the disappearance of allantoin from the mixture were followed by sampling

Figure 3. Effect of histidine on utilization of allantoin by Pseudomonas aeruginosa strain PA-1.

Cells were grown on histidine, washed by centrifugation and resuspension, and inoculated into 20 ml of minimal medium containing 0.5 percent each of the following carbon sources: (1) allantoin; (2) allantoin plus histidine; (3) histidine.
Growth on allantoin (●); growth on allantoin plus histidine (▲); growth on histidine alone (■); allantoin remaining in the mixture (○).


Optical Density, 540 mμ
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
0
2
4
6
8
10
12
Hours
Allantoin Remaining, μ moles/ml.
36
32
28
24
20
16
12
8
4
0

from the mixture and recording optical densities for all cultures at 30 min intervals. Figure 4 shows the results of the experiment. From the figure it can be seen that there was no diauxic growth and allantoin was utilized simultaneously with acetate. The rate of growth on the mixture of substrates was more rapid than on either alone, as had been the case to a lesser degree with the other substrate mixtures used.

B. Studies with Pseudomonas aeruginosa strain PA-1-707

1. Effect of Glucose on Growth on Allantoin

The glucose-negative mutant, strain PA-1-707, grown on nutrient broth, was inoculated into four tubes with the following substrates in minimal medium: Tube 1 contained allantoin; tube 2 contained glucose; tube 3 contained a mixture of allantoin and glucose; the fourth tube also contained allantoin, and glucose was added at five hr, i.e., after growth had started. Both allantoin and glucose were used at a concentration of 0.5 percent. The results are summarized in Figure 5. It can be seen from the figure that there was good growth on allantoin and no growth on glucose. There was very slight growth on the mixture with glucose added at zero time, i.e., less than one doubling of the population. In the tube where glucose was added after growth had been initiated, there was an immediate decrease in growth rate and there was only a slight increase in optical density thereafter (slightly more than one doubling).

2. Effect of Various Concentrations of Glucose on Growth on Allantoin

Figure 4. Effect of acetate on the utilization of allantoin by Pseudomonas aeruginosa strain PA-1.

Cells were grown in nutrient broth overnight, washed by centrifugation and resuspension and inoculated into 20 ml of minimal salt containing the following combinations: (1) 0.2 percent allantoin plus 0.3 percent NH_4Cl; (2) 0.2 percent allantoin plus 0.2 percent ammonium acetate; (3) 0.2 percent ammonium acetate.
Growth on allantoin (●); growth on allantoin plus acetate (■); growth on acetate alone (▲); allantoin remaining in the mixture (○).

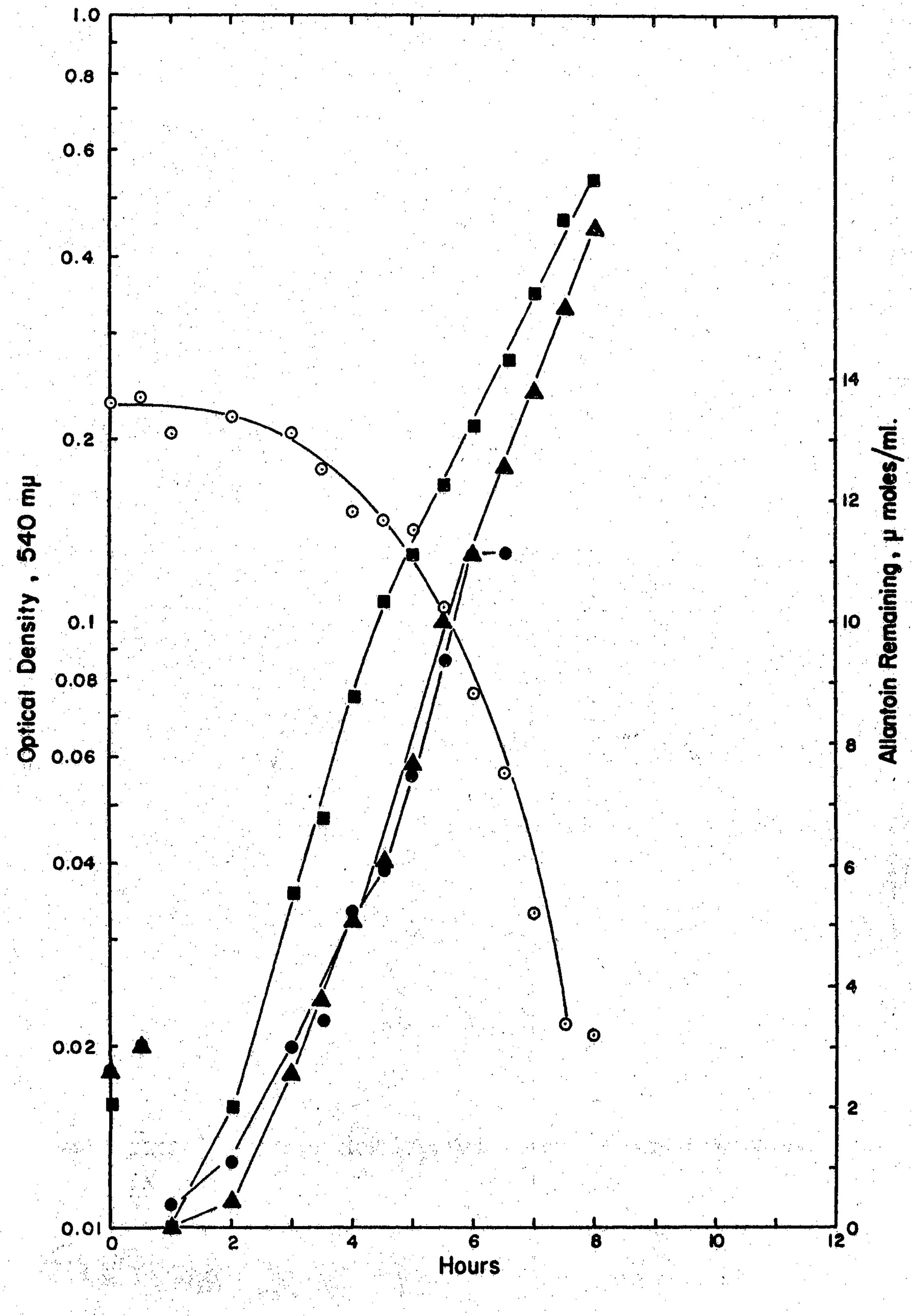
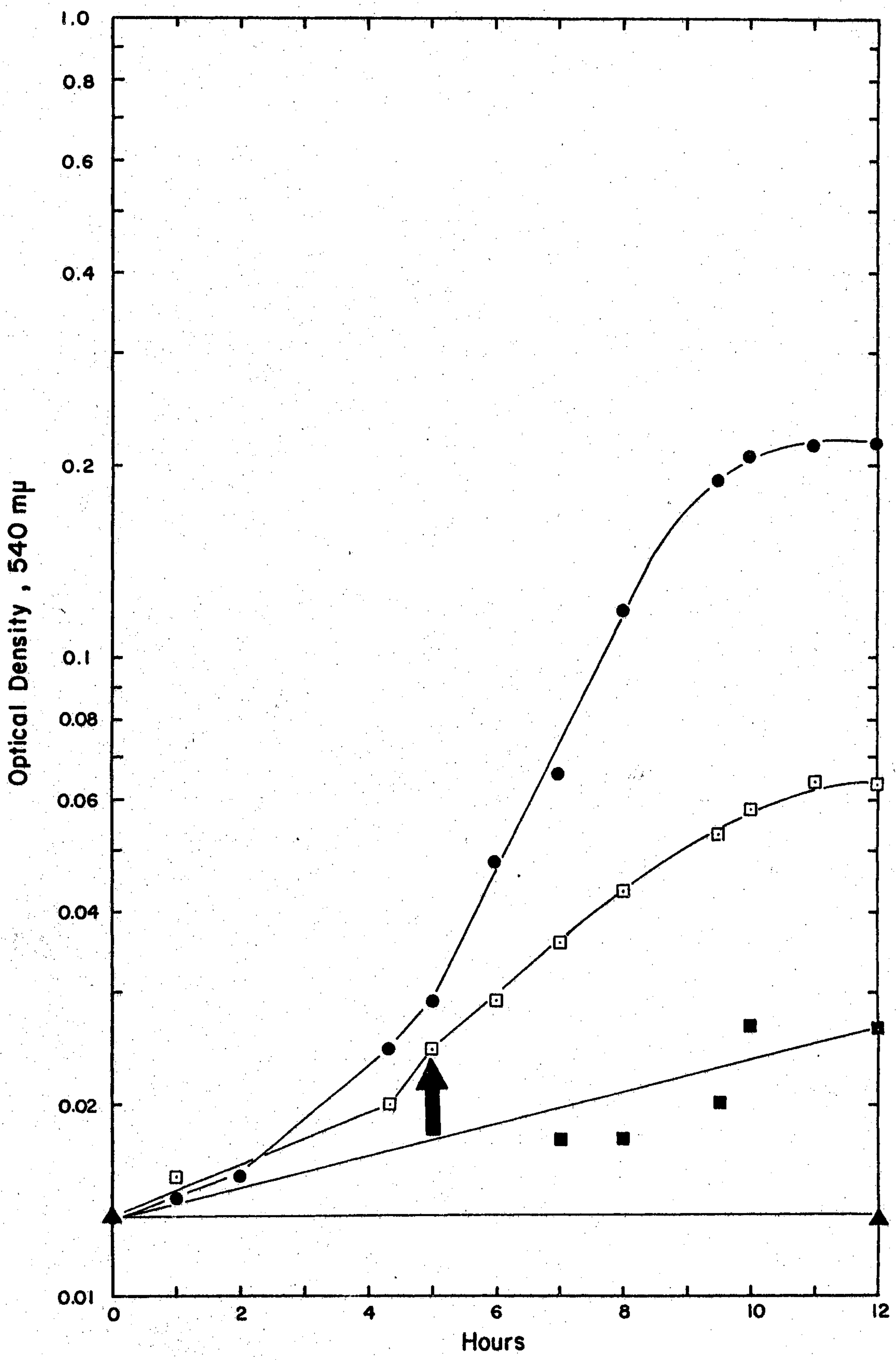

Optical Density , 540 mμ
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Allantoin Remaining, μ moles/ml.
14
12
10
8
6
4
2
0
Hours
0
2
4
6
8
10
12

Figure 5. Effect of glucose on growth on allantoin of *Pseudomonas aeruginosa* strain PA-1-707.

Cells were grown in nutrient broth, washed by centrifugation and resuspension, and inoculated into 6 ml portions of minimal medium containing 0.5 percent each of the following carbon sources: (1) allantoin; (2) allantoin plus glucose; (3) glucose. Growth on allantoin alone (●); growth on allantoin plus glucose (■); growth on glucose alone (▲); glucose added (to 0.5 percent) to cells growing on allantoin at the time indicated by arrow (□).


Optical Density , 540 mμ
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
0
2
4
6
8
10
12
Hours

Since in the previous experiment a high concentration of glucose was used, a second experiment was conducted to determine whether even low concentrations of glucose could prevent growth on allantoin. The results of an experiment in which allantoin was used at a concentration of 0.5 percent and glucose at concentrations of 0.01, 0.03, 0.05 and 0.1 percent are shown in Figure 6. The glucose control contained glucose at 0.5 percent. Only results obtained with the two lower concentrations of glucose are shown in the figure, but in all the four concentrations used there was essentially no growth.

3. Effect of Addition of Various Concentrations of Glucose after Initiation of Growth on Allantoin

Since there was slight growth when a high concentration (0.5 percent) of glucose was added after initiation of growth (Figure 5), it was thought that addition of smaller concentrations of glucose after initiation of growth might permit the growth of the organism. The results of such an experiment are shown in Figure 7. Again, only growth in the two lower concentrations of glucose was plotted. It can be seen from the figure that there was only a slight increase in optical density, equivalent to approximately one doubling, irrespective of the concentration of glucose added, after the effect of glucose was exerted. In all the four concentrations used there was the same amount of growth. It is interesting that with lower concentrations of glucose, the effect of its addition was delayed for several hours.

4. Effect of Glucose when Allantoin is Serving as the Sole Source of Carbon and Nitrogen

Figure 6. Effect of various concentrations of glucose on growth on allantoin of *Pseudomonas aeruginosa* strain PA-1-707.

Inoculum and media were as described in Figure 5 except that four different concentrations of glucose were used.
Growth on 0.5 percent allantoin (●); growth on 0.5 percent glucose alone (▲); growth on 0.5 percent allantoin plus 0.01 percent glucose (■); growth on 0.5 percent allantoin plus 0.03 percent glucose (□). When 0.05 percent and 0.1 percent glucose were added, the growth patterns were similar.

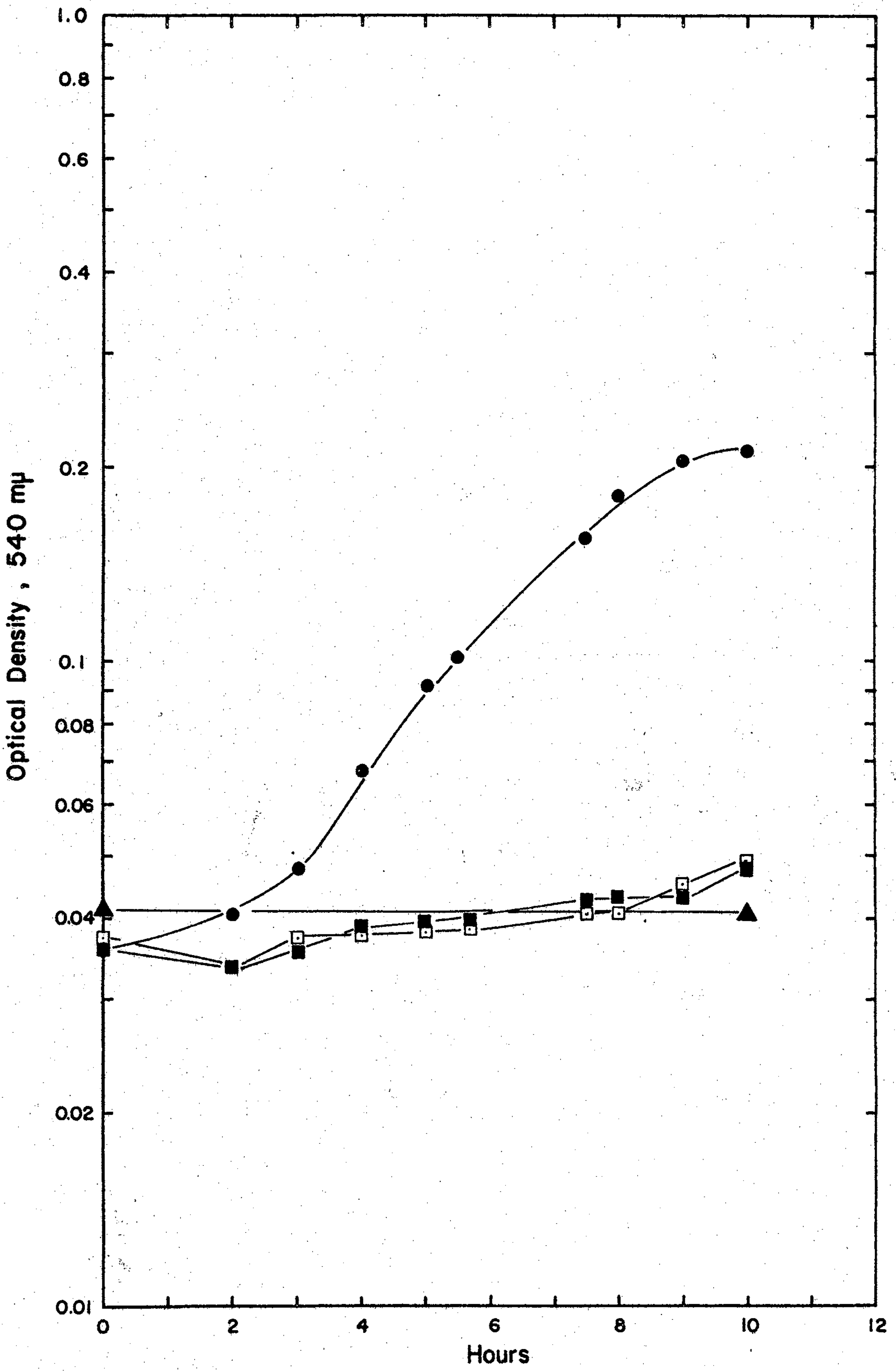
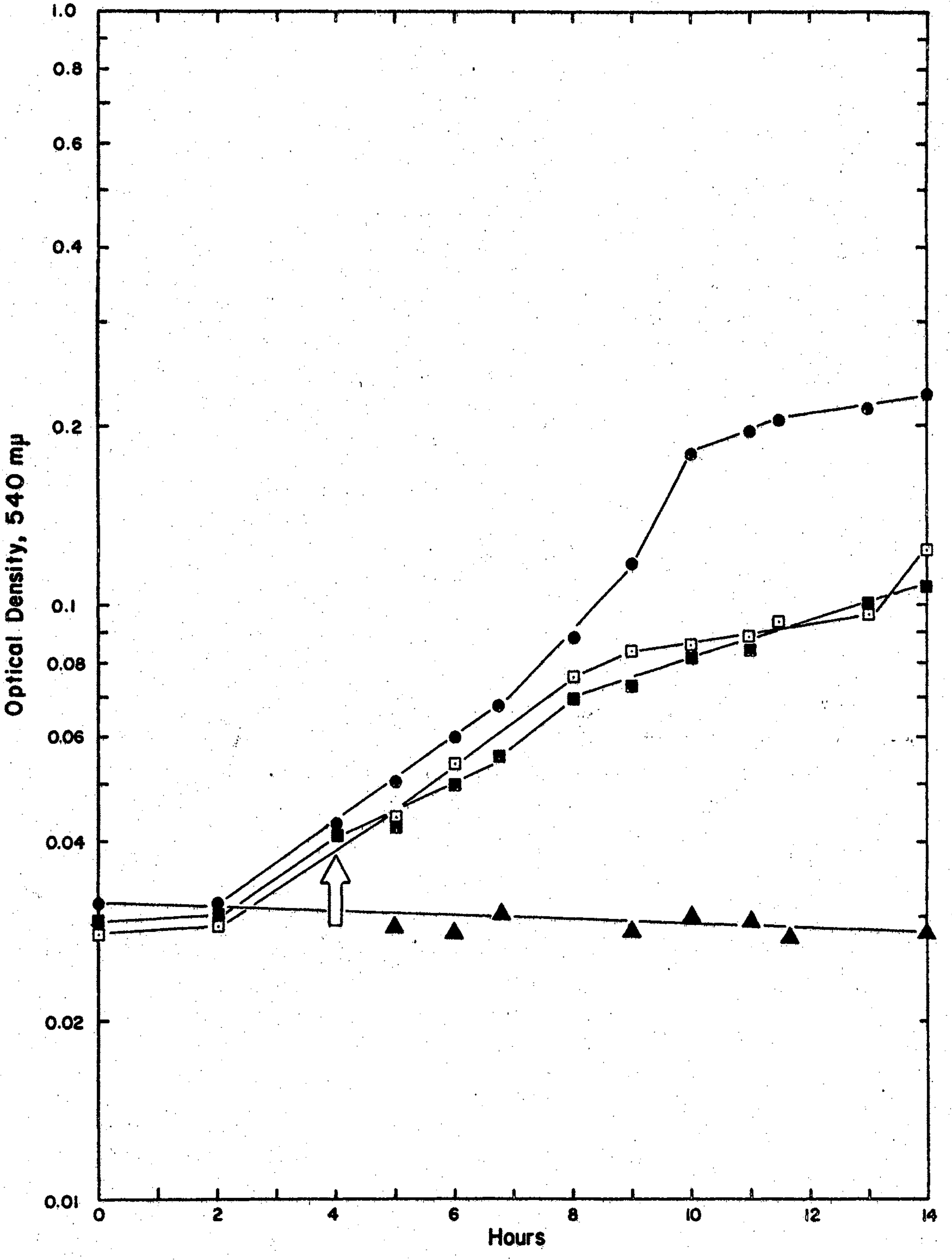

1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Optical Density , 540 mμ
0
2
4
6
8
10
12
Hours

Figure 7. Effect of addition of various concentrations of glucose after initiation of growth on allantoin by Pseudomonas aeruginosa strain PA-1-707.

Inoculum and media were as described in Figure 5 except that four different concentrations of glucose were added at the arrow.
Growth on 0.5 percent allantoin (●); growth on 0.5 percent glucose (▲); growth on 0.5 percent allantoin plus 0.01 percent glucose (■); growth on 0.5 percent allantoin plus 0.03 percent glucose (□). When 0.05 percent and 0.1 percent glucose were added, the growth patterns were similar.


Optical Density, 540 mμ
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
0
2
4
6
8
10
12
14
Hours

In all previous experiments, NH_4Cl had been added to the allantoin medium. It was thought that the organism might be able to grow if glucose were added when allantoin was serving as the sole source of both carbon and nitrogen. Neidhardt and Magasanik (1957) reported that utilization of histidine as source of both carbon and nitrogen relieved glucose repression of histidase. The result of this experiment is shown in Figure 8. The organism grew slightly better when allantoin was used as the sole source of carbon and nitrogen rather than as sole carbon source with an alternate source of nitrogen supplied. However, there was no difference in growth if glucose was added, whether allantoin was being used as the source of carbon or of both carbon and nitrogen.

5. Assays of Enzyme Activities in Cell-Free Extracts

Since no effect of glucose on enzymes of the allantoin pathway was detected in the wild type strain by examination of growth and substrate removal, more exact data on induction and repression were obtained by examination of enzyme levels in cell extracts.

Table I shows effects of exposure of lactate-grown cells to allantoin, allantoin plus glucose, and glucose on the synthesis of the enzymes of allantoin degradation in PA-1. From the table it can be seen that in glucose-exposed (uninduced) cells there were very low levels of allantoinase and allantoicase whereas the ureidoglycolate synthetase activity was relatively high. After induction with allantoin, there was a marked increase in the activities of all three enzymes, thus showing that the enzymes of allantoin degradation are inducible. When exposed to a mixture of allantoin and glucose, there

Figure 8. Effect of glucose when allantoin is serving as sole source of carbon and nitrogen in *Pseudomonas aeruginosa* strain PA-1-707.

Inoculum and media were as described in Figure 5 except that NH_4Cl was added or omitted.
Growth on allantoin plus NH_4Cl (●); growth on allantoin alone (○); growth on NH_4Cl plus glucose (▲); growth on allantoin plus glucose plus NH_4Cl (■); growth on allantoin plus glucose (□).

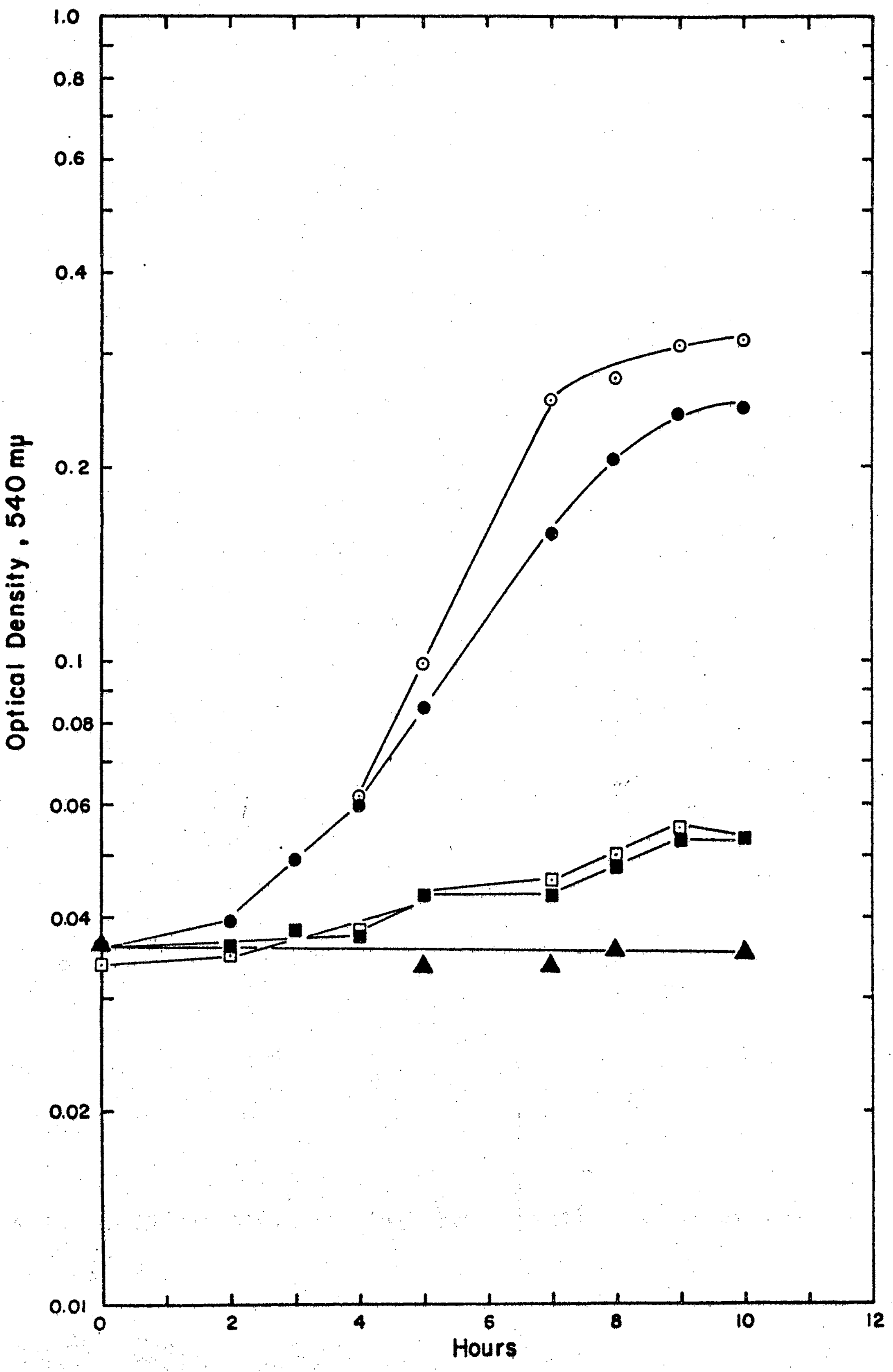

1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
Optical Density , 540 mμ
0
2
4
6
8
10
12
Hours

TABLE I

ALLANTOIN-DEGRADING ENZYMES IN STRAIN PA-1 EXPOSED TO ALLANTOIN AND/OR GLUCOSE

Cells exposed to	Specific activity, mμmoles/min/mg protein Allantoinase	Allantoicase	Ureidoglycolate synthetase
Allantoin	55.	74.	376.
Allantoin plus glucose	82.	49.	196.
Glucose	5.6	2.9	63.2

The cells were grown on minimal medium containing 0.5 percent lactate for 17 hr, washed, and exposed to the carbon sources indicated (0.5 percent each) for 4 hr. Extracts were then prepared as described in the text.

was no consistent effect on the levels of the three enzymes, but the enzyme data clearly show that enzymes of the allantoin pathway are not completely repressed by glucose.

Table II shows the enzyme activities in the mutant (PA-1-707). When induced with allantoin the organism had allantoinase, allantoicase and ureidoglycolate synthetase at high levels. When exposed to a mixture of allantoin and glucose, allantoinase activity was decreased, but allantoicase activity remained high. Ureidoglycolate synthetase activity was completely absent even though the enzyme was detected in cells exposed to glucose alone.

C. Studies with Neurospora crassa

1. Mutant Isolation

Figure 9 shows the ultraviolet survival curve obtained with the inositol-less mutant *N. crassa* FGSC #498. The curve shows a typical pattern with an initial "shoulder" resulting from the use of multinucleate conidia. This curve was used to determine the UV dosage required to obtain the desired proportion of survivors. None of the mutagens and enrichment techniques used in these studies yielded mutants deficient in metabolism of allantoin or hypoxanthine.

The following combinations of mutagenic, enrichment and selective techniques were used:

a. *N. crassa* #262

(1) Ultraviolet light for 3 min, followed by growth with ammonium tartrate and ammonium nitrate, enrichment, and plating on ammonium tartrate medium.

TABLE II

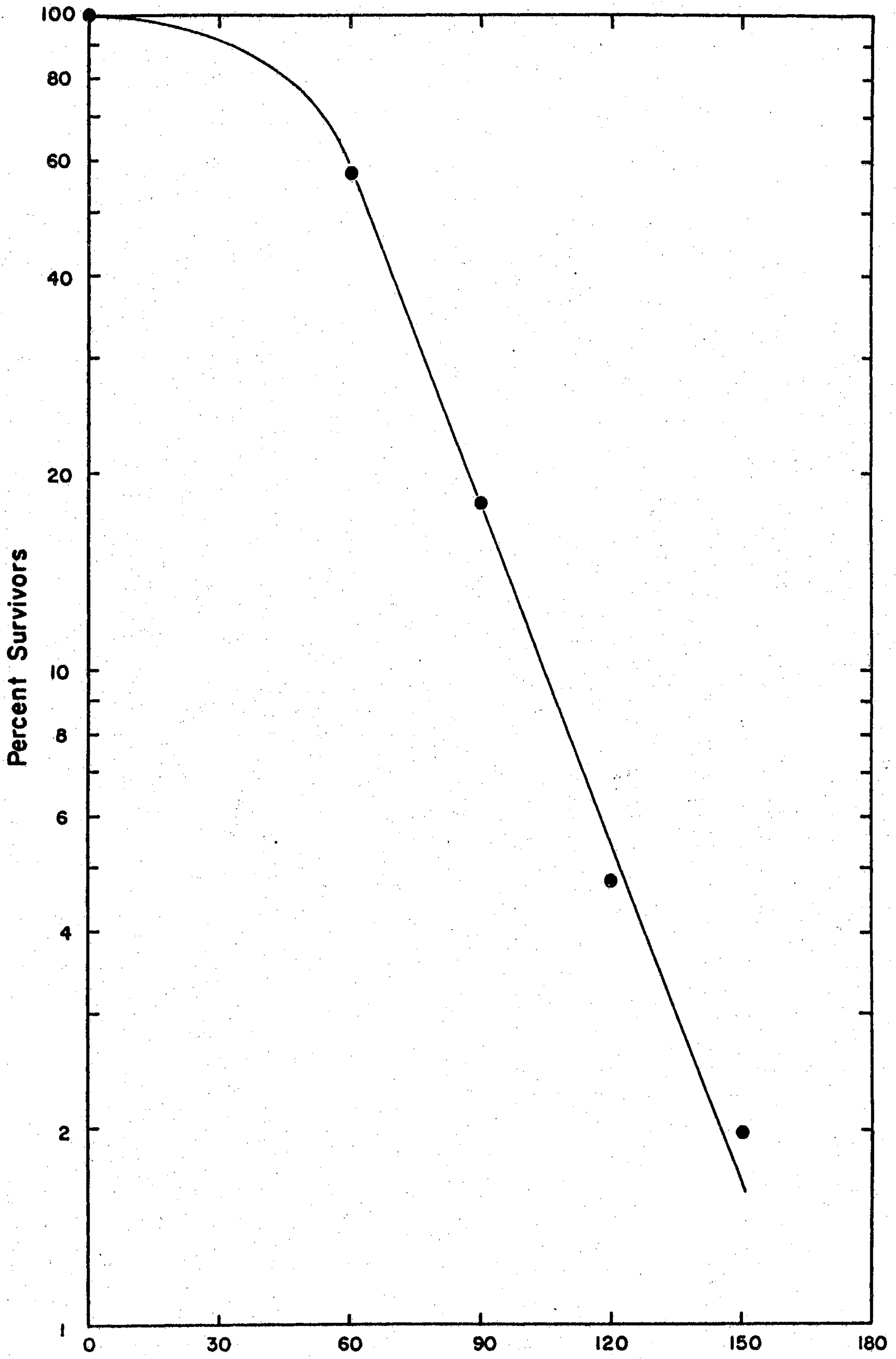
ALLANTOIN-DEGRADING ENZYMES IN STRAIN PA-1-707 EXPOSED TO ALLANTOIN AND/OR GLUCOSE

	Specific activity, mμmoles/min/mg protein		
Cells exposed to	Allantoinase	Allantoicase	Ureidoglycolate synthetase
Allantoin	83.	103.	296.
Allantoin plus glucose	30.	166.	0.0
Glucose	—	—	42.

Procedures as described for Table I.

Figure 9. UV survival curve for Neurospora crassa FGSC 498.

Five ml of a suspension of washed conidia in water were irradiated in a petri dish with shaking at a distance of 20 cm from the UV source (15 W germicidal lamp). At intervals, samples were withdrawn, diluted and plated on Vogel-Bonner medium with ammonium tartrate as the nitrogen source.


100
80
60
40
20
10
8
6
4
2
1
Percent Survivors
0
30
60
90
120
150
180
Seconds of Exposure

(2) EMS for 18 hr, followed by the same steps as for UV treatment.

(3) NTG (50 µg, 30 min), followed by filtration enrichment and plating on ammonium tartrate medium.

b. _N. crassa_ #498

All mutagenic treatments were followed by shaking for six hours with allantoin plus inositol to allow phenotypic expression, then by the further treatments listed below.

(1) Ultraviolet light to 10 percent survival, followed by shaking in liquid allantoin medium without inositol for 3 days or 5 days and plating on solid ammonium tartrate medium plus inositol.

(2) NTG (2 mg, 30 min), followed by plating on solid allantoin medium and overlaying after 5 days with ammonium tartrate plus inositol.

(3) NTG (2 mg, 30 min), followed by plating on solid allantoin medium and overlaying with ammonium tartrate plus inositol on the 6th day.

(4) EMS (0.25 ml, 5 hr), followed by plating on allantoin medium and overlaying with ammonium tartrate plus inositol on the 5th day.

c. _N. crassa_ #497

(1) NTG, (2 mg, 30 min), followed by incubation to allow phenotypic expression (2 samples) or plated immediately (2 samples),

and plated on trays by inoculating into agar. Overlayed with ammonium tartrate plus inositol on the 2nd day on one tray from each experiment and on the 5th day on one tray from each treatment.

The mutagenic treatments were effective in killing the conidia and the number of survivors after selective enrichment was quite low. A high proportion of morphological mutants was always obtained. These facts indicate that the mutagenic and selective treatments employed were effective.

2. Effect of Autoclaving on the Stability of Various Purines

Since most purines are relatively insoluble, it was impossible to filter sterilize them in the concentrations needed in the present study. Therefore, it was necessary to determine whether the purines were stable to autoclaving. The various purines were dissolved in Vogel-Bonner medium and checked for stability by recording the spectra of the same solutions before and after autoclaving (15 min at 15 psi) using the Cary 14 recording spectrophotometer. The results are summarized in Table III. There were no changes in the spectra due to autoclaving.

3. The Ability of Various Purines to Serve as Nitrogen Sources

In order to determine which purines can serve as nitrogen source, *N. crassa* was inoculated into liquid Vogel-Bonner medium containing the various compounds at a concentration of 0.1 percent and growth was scored visually after 48 hr as positive or negative. The results are presented in Table IV. From the table it can be seen that guanine, hypoxanthine, xanthine, uric acid and allantoin are capable of

TABLE III

EFFECT OF AUTOCLAVING ON THE STABILITY OF VARIOUS PURINES

Purine	Wave length of maximum absorption, mμ	
	Before autoclaving	After autoclaving
Adenine	265	265
Guanine	245-250	250
Hypoxanthine	250	250
Xanthine	268-269	268-269
Uric acid	293	293

2 mg of adenine, hypoxanthine, and uric acid were dissolved separately in 200 ml portions of Vogel-Bonner medium by heating to 60-70°C in a water bath. Spectra were recorded after diluting the solutions with the same medium, as required. 2 mg of guanine and xanthine were heated separately with 400 ml portions of Vogel-Bonner medium at 75°C. The undissolved material was removed and the solutions were used for recording spectra. Each solution was then autoclaved (undiluted) for 15 min at 15 psi and diluted as above, and spectra were again recorded.

A Cary 14 recording spectrophotometer was used to record spectra.

TABLE IV

ABILITY OF VARIOUS PURINES TO SERVE AS NITROGEN SOURCE FOR *NEUROSPORA CRASSA*

Purine	Growth
None	-
Adenine	-
Guanine	+
Hypoxanthine	+
Xanthine	+
Uric acid	+
Allantoin	+

The organism was inoculated into 250 ml of Vogel-Bonner sucrose medium with the respective purines as sole nitrogen sources, and growth was scored visually after 48 hr of incubation on a reciprocal shaker at room temp.

serving as the sole nitrogen source, whereas adenine is incapable of doing so.

4. Effect of Nitrogen Source on the Color of the Mycelium

When various compounds served as nitrogen source for growth in the dark, the colors of the mycelium were different. This was considered to be an interesting characteristic and worth recording. These observations are given in Table V. It can be seen from the table that the color of the mycelium was orange when grown on guanine, uric acid, allantoin or ammonium tartrate. When grown on hypoxanthine, xanthine or ammonium chloride the mycelium had a buff color.

5. Enzyme Activities and Induction of Enzymes

a. Adenase

N. crassa is unable to use adenine as a nitrogen source. This could be due to either the impermeability of the organism to this compound or to lack of adenase. Since other enzymes of purine degradation are inducible by products of the reaction (see below), it was thought that hypoxanthine (the product of adenase) might be able to induce adenase if the lack of growth is due to impermeability. Hypoxanthine-grown mycelial extract was tested for adenase. No enzyme activity could be detected.

b. Induction of Guanase

The ability of the various nitrogen sources to induce synthesis of guanase is summarized in Table VI. It can be seen from the table that, in addition to guanine, hypoxanthine, xanthine and uric acid are

TABLE V

EFFECT OF NITROGEN SOURCE ON THE COLOR OF MYCELIUM

Nitrogen source	Color of mycelium
Guanine	Orange
Hypoxanthine	Buff
Xanthine	Pale buff
Uric acid	Orange
Allantoin	Orange
NH_4 tartrate	Orange
NH_4Cl	Pale buff

Growth conditions were as described in Table IV.

TABLE VI

INDUCTION OF GUANASE BY VARIOUS NITROGEN SOURCES

Nitrogen source	Specific activity of guanase
Guanine	11.6
Hypoxanthine	19.3
Xanthine	22.3
Uric acid	29.9
Allantoin	2.3
NH_4 tartrate	3.4

Specific activity is defined as the mμmoles of substrate used/min/mg of protein. The calculations are based on an extinction coefficient of 10.35 for guanine (Beaven, Holiday and Johnson, 1955).

Mycelium was grown in Vogel-Bonner minimal medium with each of the compounds listed above as nitrogen source and sucrose as carbon source. Extract was prepared and the enzyme assayed as described in the text.

All nitrogen sources were used at a concentration of 0.1 percent in the growth medium except ammonium tartrate, which was used at a concentration of 0.2 percent.

capable of inducing guanase. Allantoin and ammonium tartrate did not induce the enzyme. The most efficient inducer of guanase was uric acid, while guanine itself was the least effective inducer.

c. Identification of the Product of Guanase as Xanthine

Since guanase was measured by determining the disappearance of substrate, it was thought necessary to show that xanthine is the product of the reaction. Since xanthine dehydrogenase in this organism requires an electron acceptor and an electron carrier (see below), it should accumulate xanthine if these are omitted from the incubation mixture. The extract was incubated with guanine and the spectrum of the incubation mixture was recorded after incubation. A single peak appeared with maximum absorption at 270 mµ. Similarly, when the extract was incubated with guanine the formation of xanthine could be observed by increase in absorbance at 270 mµ and the activities measured by both methods were comparable.

d. Assays for Xanthine Oxidase (dehydrogenase) Activity

Several methods have been used for measuring xanthine oxidase (dehydrogenase) activity. Since uric acid is the product of xanthine dehydrogenase, it should be possible to measure activity by following spectrophotometrically the formation of uric acid. Using both xanthine and hypoxanthine as substrates, the optical density of an incubation mixture containing buffer, extract and substrate was recorded at 293 mµ. No increase at 293 could be detected with either substrate. Similarly, a decrease at 250 mµ and 270 mµ using appropriately diluted solutions of hypoxanthine and xanthine, respectively, might be expected to result from disappearance of the substrates. No decrease could be detected with extract from either xanthine-grown or hypoxanthine-grown mycelium. These results indicated that the *N. crassa* enzyme might be a dehydrogenase, rather than an oxidase, and therefore might require addition of an electron acceptor for assay. The effects of addition of an electron acceptor and electron carrier are presented in Table VII. It can be seen from the table that when the tetrazolium dye, MTT, was added as electron acceptor, there was slight activity. But when MTT was supplemented with a mediator, phenazine methosulfate (PMS), the activity was increased more than 10-fold. The control, in which the extract was omitted, had no activity.

e. Effect of Storage on Xanthine Dehydrogenase Activity

In order to determine the stability of the extract with respect to activity toward two substrates, it was stored at -20°C, thawed, and assayed periodically. The results are summarized in Table VIII. The extract lost approximately one-third of its activity in 10 days and two-thirds in 21 days. Activity toward xanthine was lost slightly more rapidly than activity toward hypoxanthine.

f. Effect of Potassium Cyanide on Xanthine Dehydrogenase Activity

Cyanide has been used in the assay of dehydrogenases to enhance dye reduction by blocking electron transport through the cytochrome system. It was thought that addition of cyanide might further improve the assay for xanthine dehydrogenase activity. However, it was found that cyanide inhibited the enzyme activity. The results of experiments

TABLE VII

EFFECT OF VARIOUS METHODS OF MEASUREMENT ON XANTHINE DEHYDROGENASE ACTIVITY

Method	Substrate	Specific Activity
Increase at 293 mμ	Xanthine	0.0
	Hypoxanthine	0.0
Decrease at 250 mμ	Hypoxanthine	0.0
Decrease at 270 mμ	Xanthine	0.0
Additions (increase at 565 mμ)		
0.3 mg MTT	Xanthine	1.8
0.1 mg MTT plus 0.3 mg PMS	Xanthine	24.8
0.2 mg MTT plus 0.3 mg PMS	Xanthine	27.5
0.3 mg MTT plus 0.3 mg PMS	Xanthine	28.4
0.3 mg MTT plus 0.3 mg PMS (no extract)	Xanthine	0.0

For the UV measurements, the substrate solutions normally used were diluted. Xanthine was diluted 1:5 and hypoxanthine was diluted 1:7 with 0.2 M potassium phosphate buffer, pH 7.5.

Amounts given for MTT, 3(4,5-dimethyl thiazolyl 1-2) 2,5 diphenyl tetrazolium bromide, and PMS, phenazine methosulfate, are total weights of the compounds in the 3-ml reaction volume. Extract was from xanthine-grown mycelium. The calculations were based on an extinction coefficient of 10 for the formazan which is an average for the two values reported by Sowerby and Ottaway (1966). Specific activity is mμmoles/min/mg of protein.

TABLE VIII

EFFECT OF STORAGE ON ACTIVITY OF XANTHINE DEHYDROGENASE TOWARD HYPOXANTHINE AND XANTHINE

	Activity of xanthine dehydrogenase				
	Substrates				Activity Ratio
	Xanthine		Hypoxanthine		
Time of storage in days	Sp. act.	% of maximum activity	Sp. act.	% of maximum activity	$\frac{\text{Hypoxanthine}}{\text{Xanthine}}$
0	25.4	100	30.8	100	1.21
10	16.4	64	22.6	73	1.37
21	9.8	38	13.7	44	1.39

The mycelium was grown on xanthine as nitrogen source and extract was prepared and stored at -20°C.

using cyanide are summarized in Table IX. When the extract was prepared from mycelium grown on either xanthine or hypoxanthine, xanthine dehydrogenase activity toward both xanthine and hypoxanthine was inhibited. However, the inhibition was much more severe with extract from xanthine-grown mycelium.

g. Effect of Uric Acid on Xanthine Dehydrogenase Activity

Since cyanide was found to inhibit the reaction, it was thought that the cyanide inhibition might be due to inhibition of urate oxidase and accumulation of uric acid, leading to product inhibition of xanthine dehydrogenase. In order to test this hypothesis, the effect of addition of uric acid on the reaction was studied. The results are summarized in Table X. It can be seen from the table that the dehydrogenase activity toward both xanthine and hypoxanthine was inhibited in extracts from xanthine-grown mycelium. However, when the extract from hypoxanthine-grown mycelium was tested, the dehydrogenase activity toward xanthine was inhibited but not the activity toward hypoxanthine. In no case was the inhibition sufficient to account for the total inhibition found with cyanide.

h. Induction of Xanthine Dehydrogenase

The ability of various nitrogen sources to induce xanthine dehydrogenase synthesis is shown in Table XI. Xanthine and uric acid were found to be good inducers. Only the activity toward hypoxanthine seems to have been induced by guanine and hypoxanthine. Allantoin and ammonium tartrate did not induce the enzyme.

TABLE IX

EFFECT OF POTASSIUM CYANIDE ON ACTIVITY OF XANTHINE DEHYDROGENASE TOWARD XANTHINE AND HYPOXANTHINE

		Xanthine dehydrogenase activity			
		Substrates			
		Xanthine		Hypoxanthine	
Extract of mycelium grown on	Concentration of KCN	Specific activity	% of maximum activity	Specific activity	% of maximum activity
Xanthine	None	28.4	100	37.4	100
	10^{-2} M	5.4	19.0	4.0	10.6
	2×10^{-2} M	2.5	8.7	2.5	6.7
Hypoxanthine	None	2.9	100	5.2	100
	10^{-2} M	1.7	58.6	3.8	73.7
	2×10^{-2} M	0.6	20.6		

The enzyme was not pre-incubated with the inhibitor. The cyanide solution was added to the cuvette and recording was started immediately. The rate of dye reduction decreased during the first few minutes and became constant at 3 to 4 min. Rates were measured from that point. Specific activity is $m\mu$moles/min/mg of protein.

TABLE X

EFFECT OF URIC ACID ON XANTHINE DEHYDROGENASE ACTIVITY

Extract of mycelium grown on	Uric acid added, μmoles	Substrates			
		Xanthine		Hypoxanthine	
		Specific activity	% of maximum activity	Specific activity	% of maximum activity
Xanthine	-	28.4	100	37.4	100
	0.9	24.4	85.9	-	-
	1.8	22.4	78.8	-	-
	3.6	20.0	70.4	25.5	68.8
Hypoxanthine	-	2.9	100	5.2	100
	3.6	2.1	72.4	5.5	105

The uric acid solution was mixed with the incubation mixture and the recording was started immediately. The calculations are based on the rate of reduction after 3-4 minutes when the rate had become constant. Specific activity is mμmoles/min/mg of protein.

TABLE XI

INDUCTION OF XANTHINE DEHYDROGENASE BY VARIOUS NITROGEN SOURCES

Nitrogen source	Sp. act. of xanthine dehydrogenase Substrates Xanthine	 Hypoxanthine
Guanine	4.1	14.0
Hypoxanthine	3.3	7.8
Xanthine	28.4	37.4
Uric acid	13.0	19.9
Allantoin	2.0	3.0
NH_4 tartrate	3.1	4.1

Specific activity is mμmoles/min/mg of protein. Procedure was as described in Table VI. The optimum conditions described in Table VII were used for assay.

i. Induction of Uricase

The ability of various nitrogen sources to induce uricase synthesis is shown in Table XII. Guanine, hypoxanthine, xanthine and uric acid were good inducers. Allantoin was less efficient in inducing the enzyme. The organism apparently has a rather high endogenous level of uricase, or, alternatively, ammonium tartrate induces low levels of enzyme synthesis.

j. Induction of Allantoinase

Table XIII shows the induction of allantoinase. Uric acid was the best inducer; xanthine, guanine and allantoin were good inducers. However, when the organism was grown on hypoxanthine the activity was very low and was comparable to the endogenous level in ammonium tartrate-grown mycelium.

k. Induction of Allantoicase

Allantoicase apparently was induced equally well by guanine, hypoxanthine, xanthine, uric acid and allantoin. The enzyme activities are shown in Table XIV.

l. Induction of Ureidoglycolate Synthetase

Table XV shows the ureidoglycolate synthetase activities in extracts of mycelium grown on various nitrogen sources. The enzyme was present in relatively high levels when the organism was grown on ammonium tartrate. Hypoxanthine and allantoin induced the highest activities, followed by guanine and xanthine. There was no difference in activity between uric acid-grown and ammonium tartrate-grown extracts.

TABLE XII

INDUCTION OF URICASE BY VARIOUS NITROGEN SOURCES

Nitrogen source	Specific activity of uricase
Guanine	71.4
Hypoxanthine	81.7
Xanthine	84.7
Uric acid	82.6
Allantoin	27.7
NH_4 tartrate	14.5

The activities are based on an extinction coefficient of 12 (Kalckar, 1947). Specific activity is mμmoles/min/mg of protein. Procedure was as described in Table VI.

TABLE XIII

INDUCTION OF ALLANTOINASE BY VARIOUS NITROGEN SOURCES

Nitrogen source	Specific activity of allantoinase
Guanine	20.3
Hypoxanthine	5.7
Xanthine	26.4
Uric acid	36.2
Allantoin	19.2
NH_4 tartrate	6.7

The enzyme was assayed at a pH of 8.5. Incubation time was 20 min. Specific activity is mµmoles/min/mg of protein. Procedure was as described in Table VI.

TABLE XIV

INDUCTION OF ALLANTOICASE BY VARIOUS NITROGEN SOURCES

Nitrogen source	Specific activity of allantoicase
Guanine	25.3
Hypoxanthine	26.0
Xanthine	22.8
Uric acid	29.2
Allantoin	31.0
NH_4 tartrate	12.9

The enzyme was assayed at pH 7.0. Incubation time was 30 min. Specific activity is mμmoles/min/mg of protein. Procedure was as described in Table VI.

TABLE XV

INDUCTION OF UREIDOGLYCOLATE SYNTHETASE BY VARIOUS NITROGEN SOURCES

Nitrogen source	Specific activity of ureidoglycolate synthetase
Guanine	60.2
Hypoxanthine	105.
Xanthine	38.3
Uric acid	28.0
Allantoin	108.
NH_4 tartrate	26.0

The enzyme was assayed at pH 7.0. Incubation time was 10 min. Specific activity is mμmoles/min/mg of protein. Procedure was as described in Table VI.

m. Allantoate Amidohydrolase

This assay was performed on an extract prepared from allantoin-grown mycelium. No enzyme activity was detected.

n. Repression of Purine Catabolic Enzymes by Ammonium Tartrate

Since uric acid can induce all the enzymes for purine catabolism, the organism was grown on a mixture of uric acid and ammonium tartrate. The activities of the various enzymes are presented in Table XVI. It can be seen from the table that synthesis of guanase, xanthine dehydrogenase, uricase and allantoinase was repressed by ammonium tartrate even in the presence of inducer. Allantoicase and ureidoglycolate synthetase were not repressed.

6. Properties of the Enzymes of Allantoin Degradation

Since allantoin-degrading enzymes were determined by interdependent assays, some of the conditions for the assay of these enzymes were checked approximately before assaying them in extracts of *N. crassa*.

a. Allantoinase

This enzyme has an optimum pH range of 8.0 to 8.5 and pH 8.5 was chosen for the standard assay procedure. Only non-overlapping curves were obtained (Figure 10). Other buffers tried also gave non-overlapping curves. The other buffers used to check the effect of pH on allantoinase activity were: 2-(N-morpholino) ethane sulfonic acid (MES) for the pH range 6.0 to 7.0; N-Tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid (TES) 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;

TABLE XVI

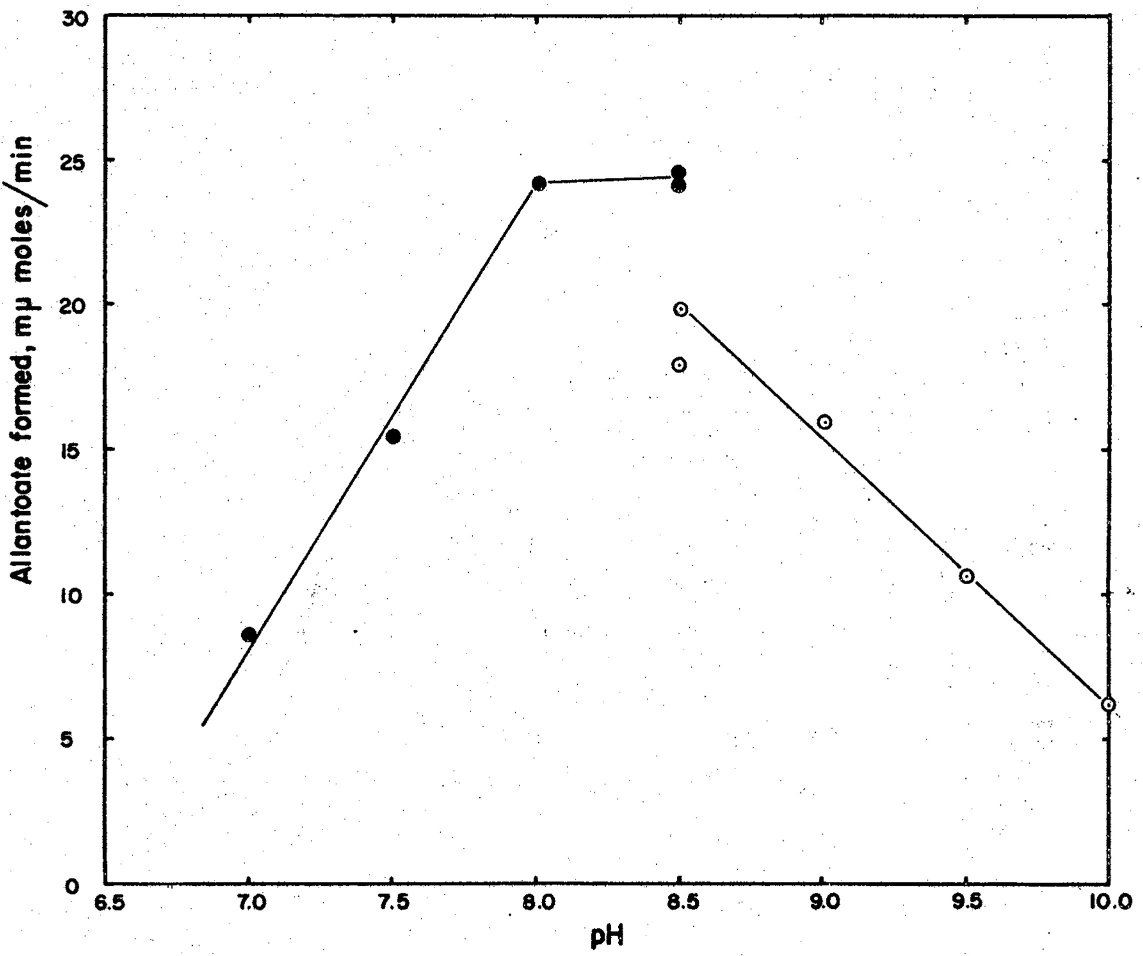
REPRESSION OF PURINE CATABOLIC ENZYMES BY AMMONIUM TARTRATE

	Nitrogen Source		
Enzymes	Uric acid	Uric acid + NH_4 tartrate	NH_4 tartrate
Guanase	29.9	6.1	3.6
Xanthine dehydrogenase			
(a. xanthine substrate)	13.7	6.2	3.1
(b. hypoxanthine substrate)	19.9	9.9	4.2
Uricase	82.6	34.2	14.5
Allantoinase	36.2	2.9	6.7
Allantoicase	29.2	28.5	12.9
Ureidoglycolate synthetase	28.0	100.	26.0

Specific activity is mμmoles/min/mg of protein. Procedure was as described in Table VI.

Figure 10. 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 of allantoin (12.6 µmoles); and 0.1 ml of extract. 0.2 M potassium phosphate buffer was used for the pH range 7.0 to 8.5 and 0.2 M sodium carbonate buffer was used for the pH range 8.5 to 10. Incubation time was 20 min.


Allantoate formed, mμ moles/min
30
25
20
15
10
5
0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH

glycine-NaOH for the pH range 9.0 to 10.5; sodium barbital-HCl buffer for the pH range 8.5 to 9.5.

The optimum time of incubation was found to be 10 minutes (Figure 11). However, a 20-minute incubation was used to allow formation of sufficient glyoxylate for accurate measurement. Figure 12 shows a Lineweaver-Burk plot for allantoinase (Lineweaver and Burk, 1934). Higher concentrations of substrate than were used would be required to saturate the enzyme.

b. Allantoicase

As in the case of allantoinase, a number of different buffers were used to determine the optimum pH for the allantoicase assay. With this enzyme, relative activities at the same pH values depended upon the buffer used. Therefore, different pH curves were obtained with different buffers. However, in almost all buffers used, maximum activity was obtained in the pH range 7.0 to 7.5. One such curve is shown in Figure 13 for the buffer used in the assay, i.e., potassium phosphate buffer. A pH of 7.0 was chosen for the standard procedure since the greatest reproducibility was found at this pH.

Figure 14 shows the accumulation of product with time of incubation. The curve shows that glyoxylate formation continued up to 40 min without a decrease in the reaction velocity. Thirty minutes were chosen as the standard incubation time. A Lineweaver-Burk plot for allantoicase activity (Lineweaver and Burk, 1934) is shown in Figure 15. The enzyme is not saturated by the substrate concentrations used.

Figure 11. Allantoinase activity vs. time.

The incubation mixture contained: 0.2 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); 0.8 ml of allantoin (12.6 µmoles); 0.4 ml of 0.2 M potassium phosphate buffer, pH 8.5; and 0.2 ml extract. The volume was made up to 2 ml with distilled water.

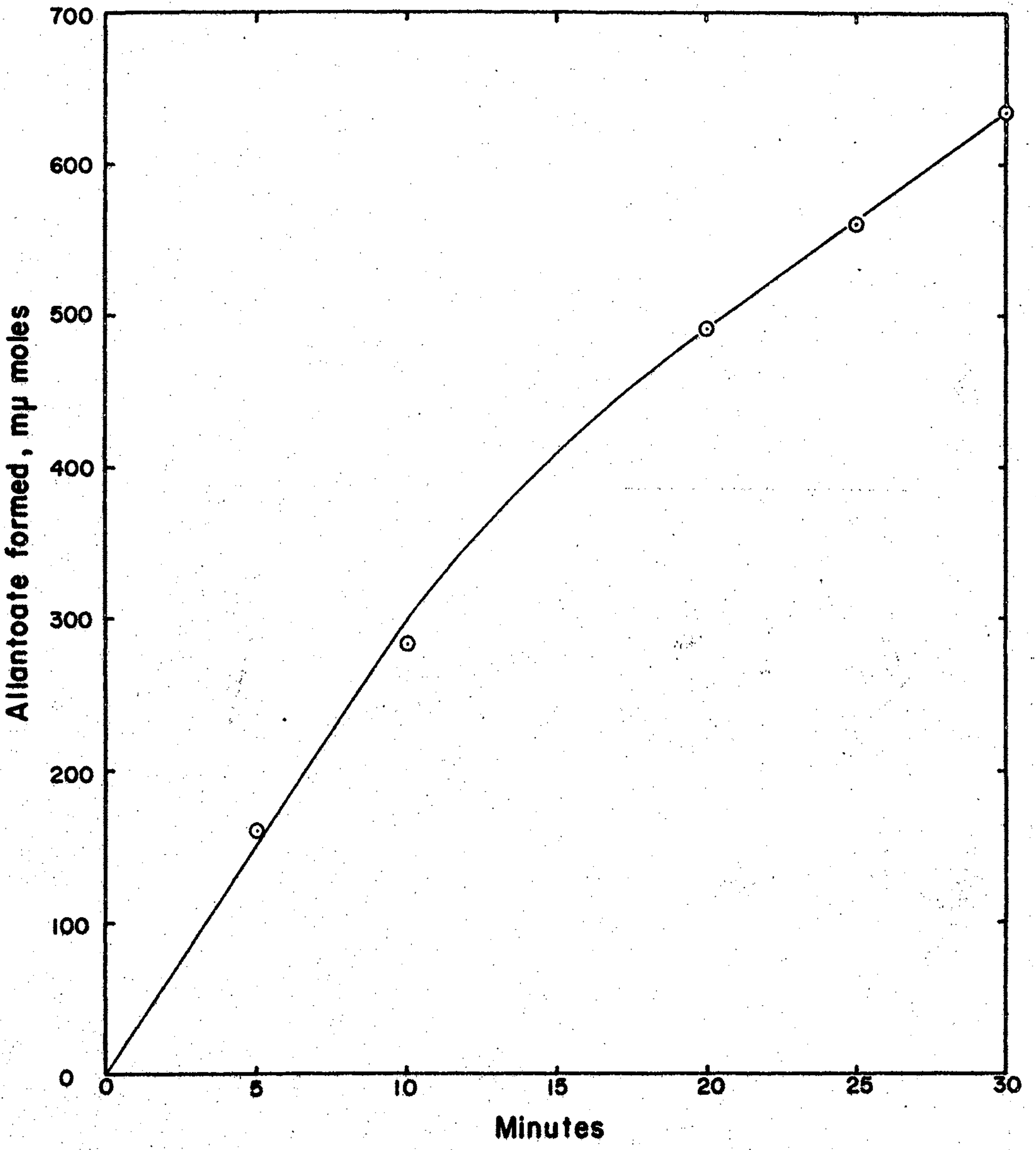

Allantoate formed, mμ moles
700
600
500
400
300
200
100
0
0
5
10
15
20
25
30
Minutes

Figure 12. Allantoinase activity vs. substrate concentration.

The incubation mixture contained: 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); variable amounts of allantoin; 0.2 ml of 0.2 M potassium phosphate buffer, pH 8.5; and 0.1 ml extract in a total volume of 1 ml. Incubation time was 20 min.

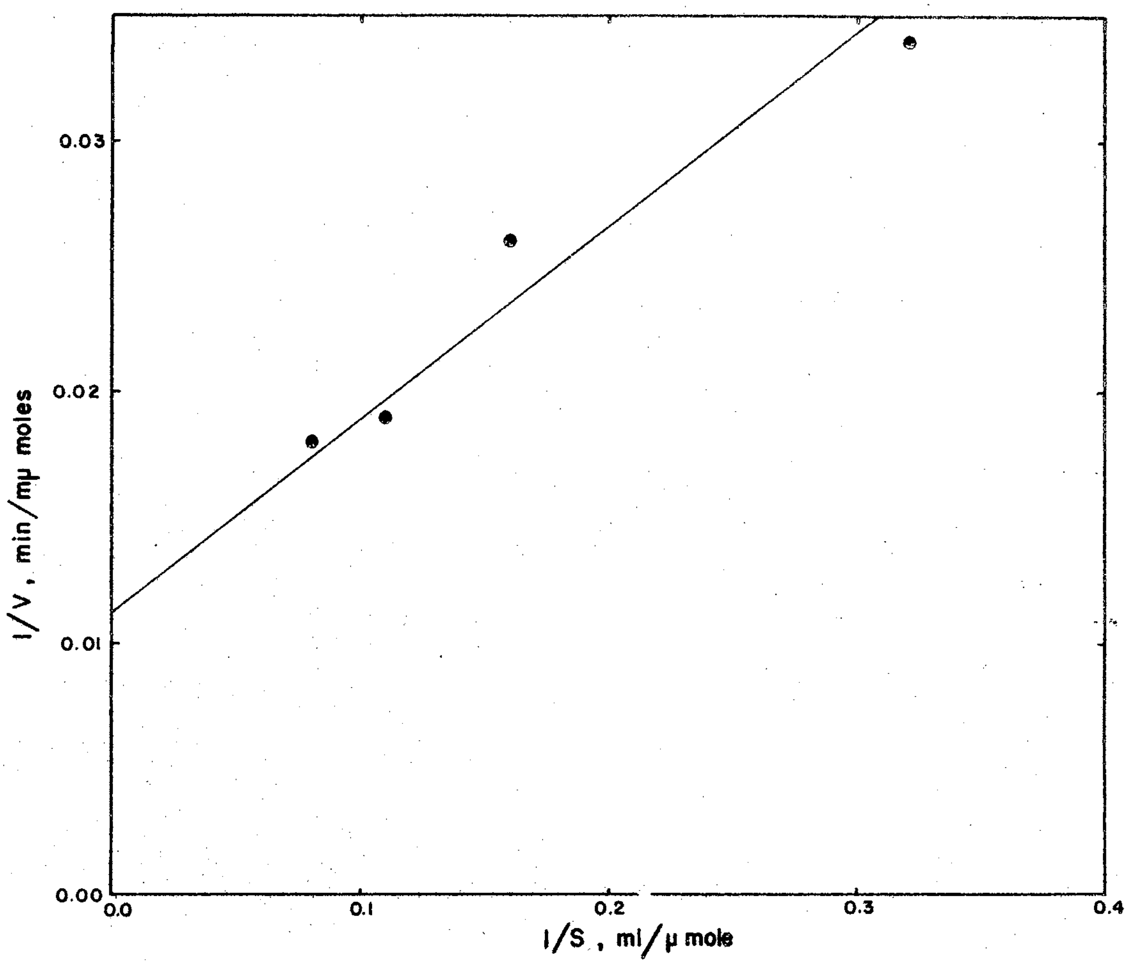

0.03
0.02
0.01
0.00
1/V , min/mμ moles
0.0
0.1
0.2
0.3
0.4
1/S , ml/μ mole

Figure 13. Optimum pH for allantoicase activity.

The incubation mixture contained 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); 0.2 ml of 0.2 M potassium phosphate buffer; 0.2 ml potassium allantoate (10 mg/ml); and 0.05 ml extract in a total volume of 1 ml.

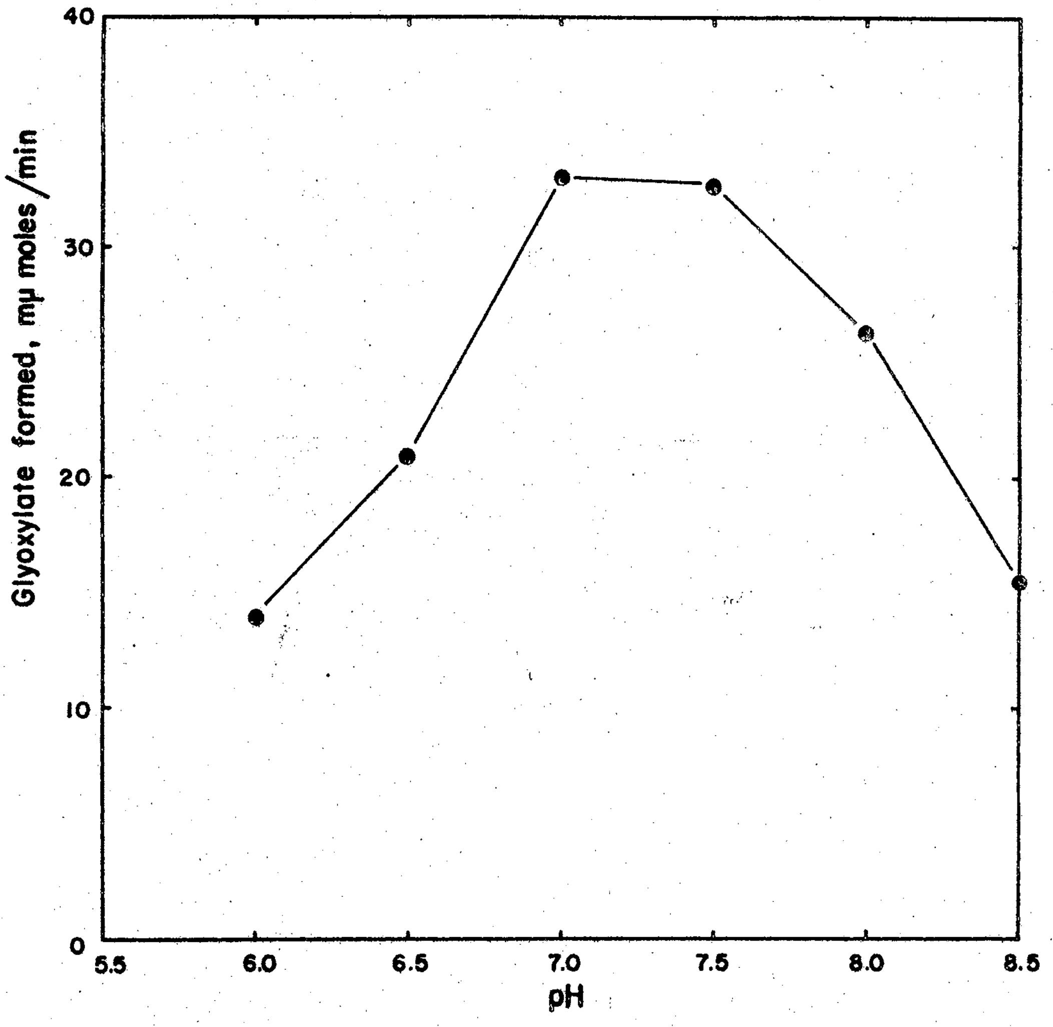

40
30
20
10
0
Glyoxylate formed, mμ moles/min
5.5
6.0
6.5
7.0
7.5
8.0
8.5
pH

Figure 14. Allantoicase activity vs. time.

The incubation mixture contained 0.2 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); 0.4 ml of potassium allantoate (10 mg/ml); 0.4 ml of 0.2 M potassium phosphate buffer, pH 7.0; 0.2 ml extract and water to a volume of 2 ml.

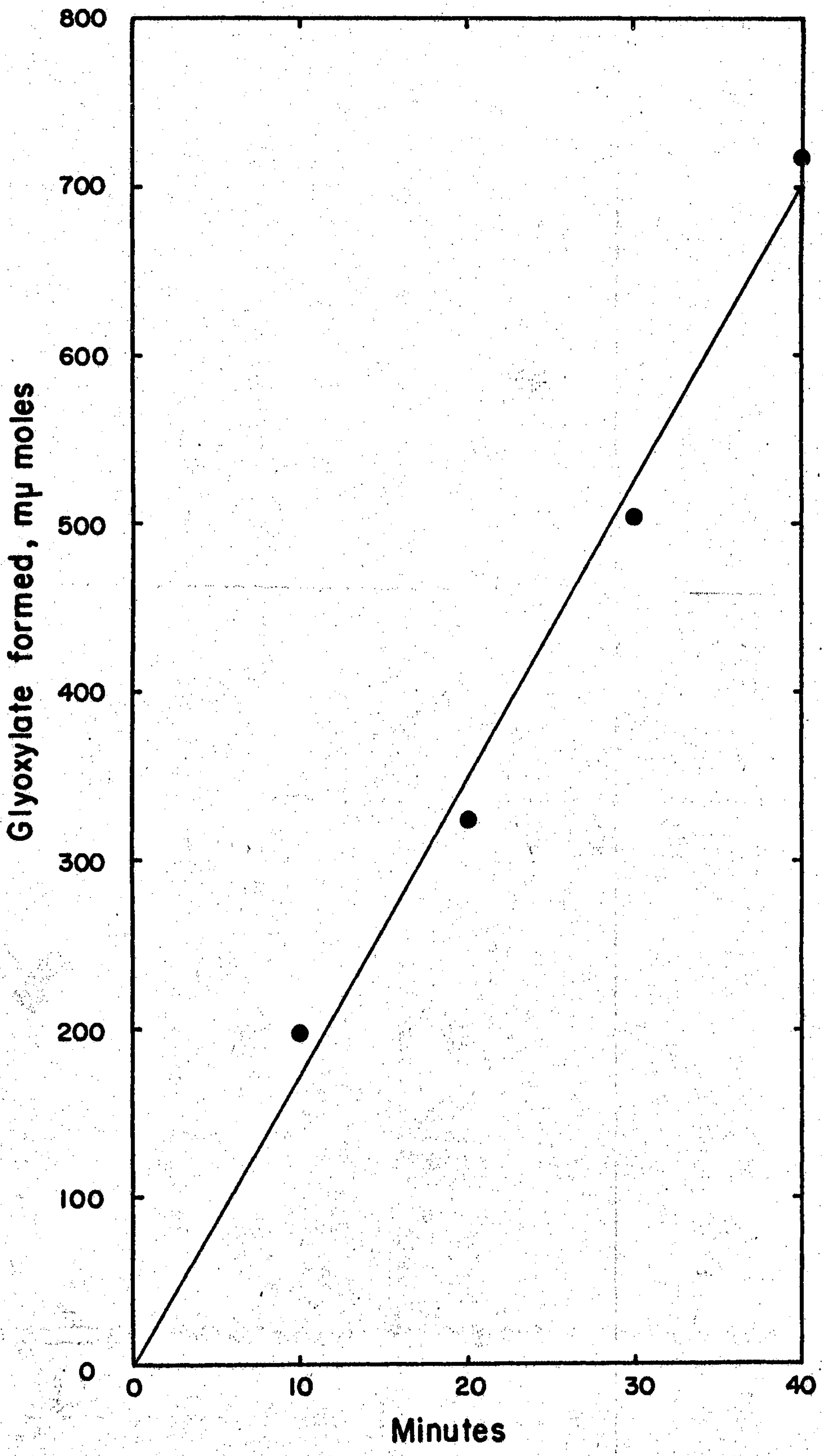
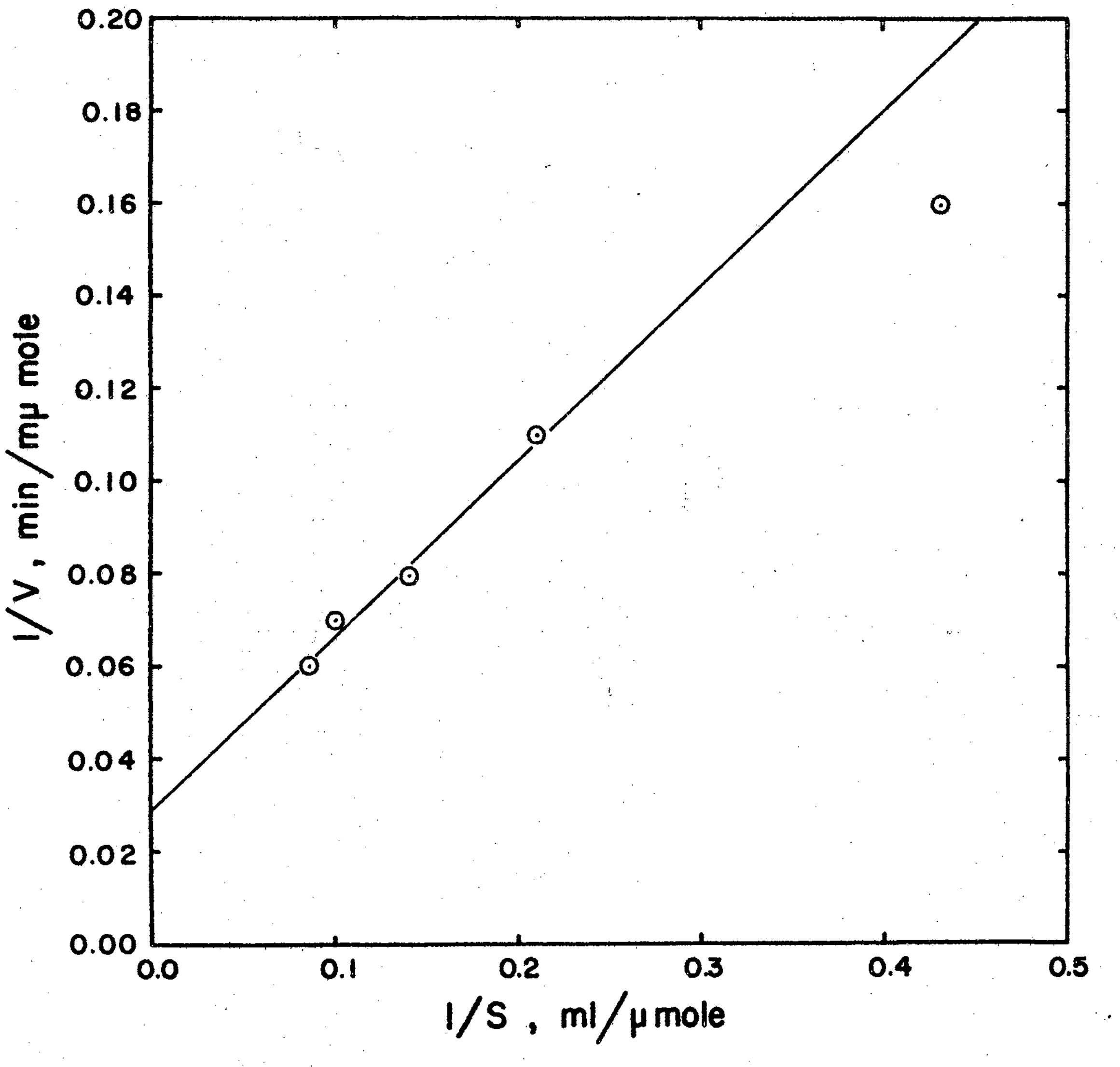

Glyoxylate formed, mμ moles
800
700
600
500
400
300
200
100
0
0
10
20
30
40
Minutes

Figure 15. Allantoicase activity vs. substrate concentration.

The incubation mixture contained 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); varying concentrations of potassium allantoate; 0.2 ml of 0.2 M potassium phosphate buffer, pH 7.0; and 0.1 ml extract. The total volume was 1 ml. Incubation time was 30 min.


0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/V , min/mμ mole
0.0
0.1
0.2
0.3
0.4
0.5
1/S , ml/μ mole

c. Ureidoglycolate Synthetase

This enzyme has an optimum pH of 7.0 to 7.5 (Figure 16). Assays were routinely carried out at pH 7.0 to minimize the non-enzymatic hydrolysis of the substrate. The Lineweaver-Burk plot is shown in Figure 17.

Figure 16. Optimum pH for ureidoglycolate synthetase activity.

The incubation mixture contained 0.2 ml of 0.2 M phosphate buffer; 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); 0.2 ml of ureidoglycolate (5 mg/ml). Total volume was 1 ml. Incubation time was 10 min.

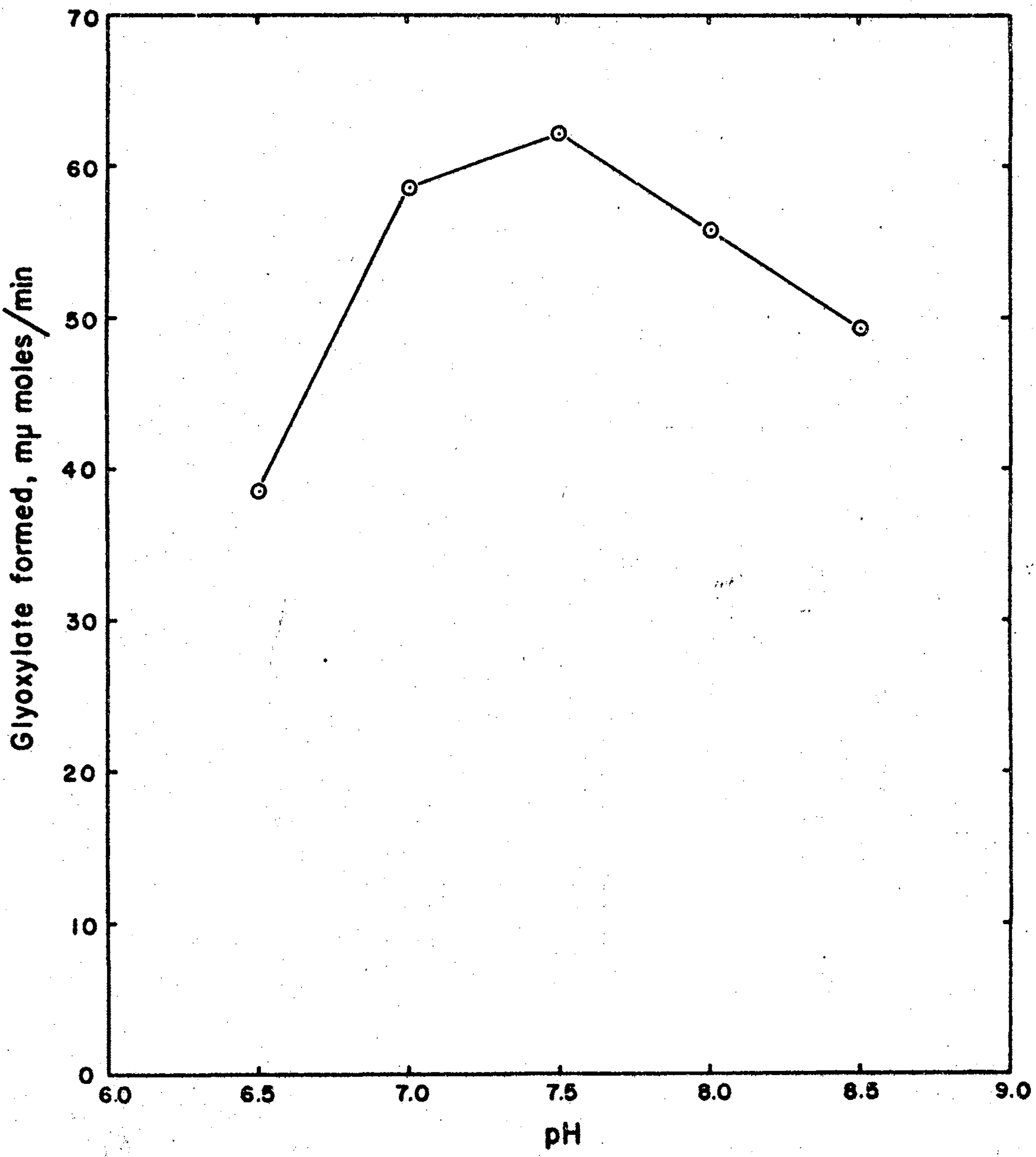
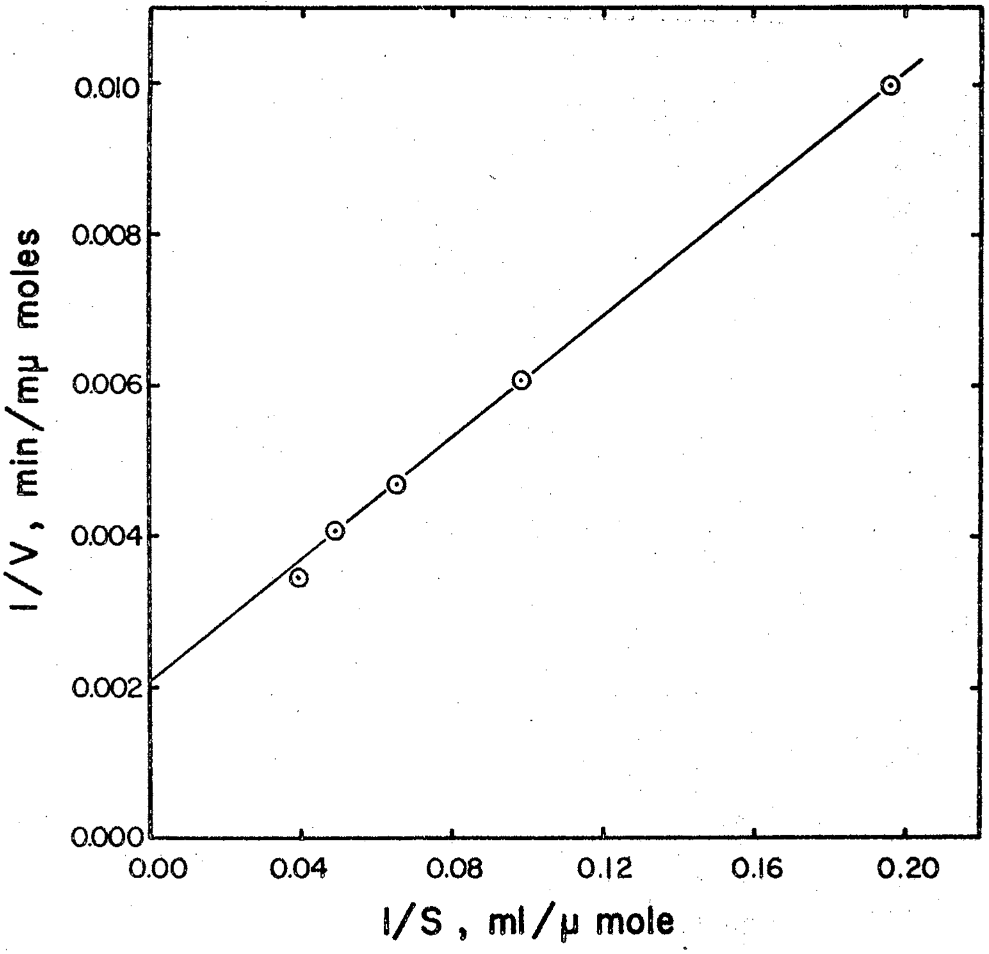

Glyoxylate formed, mμ moles/min
70
60
50
40
30
20
10
0
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

Figure 17. Ureidoglycolate synthetase activity vs. substrate concentration.

The incubation mixture contained 0.1 ml of phenylhydrazine hydrochloride (13.5 mg/ml, neutralized); varying amounts of ureidoglycolate; 0.2 ml of 0.2 M potassium phosphate buffer, pH 7.0; 0.1 ml extract. The total volume was 1 ml. Incubation time was 10 min.

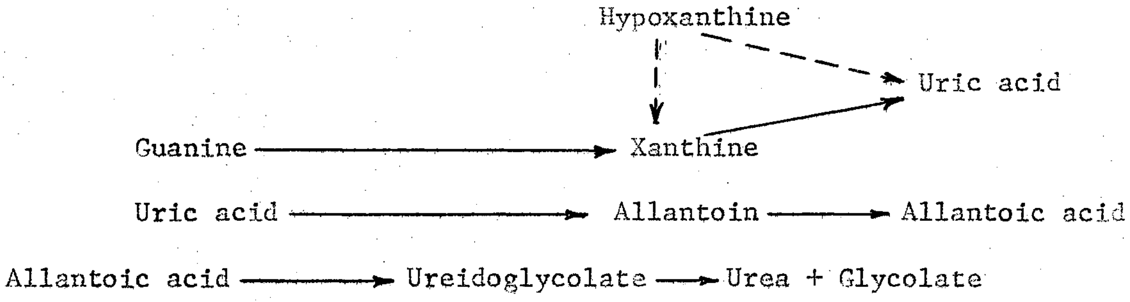

0.010
0.008
0.006
0.004
0.002
0.000
0.00
0.04
0.08
0.12
0.16
0.20
1/V, min/mμ moles
1/S, ml/μ mole

CHAPTER IV

DISCUSSION

A. The Catabolic Pathway for Purines in *N. crassa*

The various reactions which have been reported for the degradation of purines in microorganisms were summarized in Figure 1. In the present study, enzyme assays, using extracts of mycelia induced by a number of different compounds reported to be intermediates in the catabolism of purines, showed the pathway of *N. crassa* to be the following:


Hypoxanthine
Uric acid
Guanine
Xanthine
Uric acid
Allantoin
Allantoic acid
Allantoic acid
Ureidoglycolate
Urea + Glycolate

A summary of all enzyme measurements is given in Table XVII.

N. crassa lacks adenase, which converts adenine to hypoxanthine, and therefore cannot utilize adenine as a nitrogen source. Guanine is converted into xanthine by the enzyme guanase. Hypoxanthine and xanthine, the products of adenase and guanase, respectively, are converted to uric acid by the enzyme(s) xanthine dehydrogenase(s). The uric acid formed is converted to allantoin by uricase. Allantoinase is responsible for the conversion of allantoin to allantoic acid.

The reactions to this point appear to be common to all organisms

TABLE XVII

SUMMARY OF ENZYME ASSAY DATA

Enzyme	Specific activities, mμmoles/min/mg protein						
		Mycelium grown on					
	Endogenous	Guanine	Xanthine	Hypo-xanthine	Uric Acid	Allantoin	Uric acid + NH_4^+
Guanase	3.4	11.6	22.3	19.3	29.9	2.3	6.1
Xanthine dehydrogenase							
Xanthine substrate	3.1	4.1	28.4	3.3	13.0	2.0	6.2
Hypoxanthine substrate	4.1	14.0	37.4	7.8	19.9	3.0	9.9
Uricase	14.5	71.4	84.7	81.7	82.6	27.7	34.2
Allantoinase	6.7	20.3	26.4	5.7	36.2	19.2	2.9
Allantoicase	12.9	25.3	22.8	26.0	29.2	31.0	28.5
Ureidoglycolate synthetase	26.0	60.2	38.3	105	28.0	108	100

which degrade purines. However, three different pathways have been reported for the further degradation of allantoic acid in microorganisms (Figure 1, page 3). Only two of these require consideration, since ureidoglycolate is probably an intermediate in purine degradation in all microorganisms (Meganathan, 1968). One of these pathways involves the enzyme allantoicase, which converts allantoic acid to ureidoglycolate. Ps. aeruginosa is a typical example of an organism which uses this pathway (Bruce, 1965; Winter, 1967; and Trijbels and Vogels, 1967). The other pathway involves ureidoglycine as an intermediate between allantoic acid and ureidoglycolate. This intermediate was first proposed by Vogels (1963). In Strep. allantoicus, according to his proposal, allantoic acid could be degraded to ureidoglycolate but two intermediates might be involved. Allantoic acid, according to this pathway, would be converted to ureidoglycine by allantoate amidohydrolase and ureidoglycolate would be formed by ureidoglycine aminohydrolase. According to Vogels (1963, 1966), ureidoglycine is an intermediate in the degradation of allantoic acid in Ps. acidovorans, Arthrobacter allantoicus, Strep. allantoicus, E. coli, E. coli var. acidilactici and E. freundii. Recently van der Drift, de Windt and Vogels (1970) reported that it was unnecessary to postulate the existence of ureidoglycine aminohydrolase since a transamination reaction involving ureidoglycine and glyoxylate proceeds non-enzymatically.

However, by synthesis of ureidoglycine and by genetic studies using mutants (Wu, 1970; Wu, Eisenbraun and Gaudy, 1970), it was concluded that in Ps. acidovorans and in two strains of Aerobacter aerogenes the allantoate amidohydrolase pathway is the sole route for catabolism of allantoic acid and that ureidoglycine aminohydrolase activity is required for allantoate utilization by these organisms.

Since *N. crassa* uses purines as sole source of nitrogen, it would be advantageous for the organism to use allantoate amidohydrolase to produce ammonia rather than depending upon urease. However, this study indicates that the allantoicase route is used by *N. crassa* since allantoate amidohydrolase activity was not detected. The only other fungus, *Penicillium*, in which this portion of the pathway has been studied, also seems to have the allantoicase pathway (Trijbels and Vogels, 1966b). The ureidoglycolate formed by allantoicase is converted to urea and glyoxylate by ureidoglycolate synthetase (ureidoglycolase).

Xanthine dehydrogenase in *N. crassa* could not be measured by measuring uric acid formation. There are two factors which can be considered as possible explanations for this. First, uricase is very active in this organism. A comparison of relative specific activities of the enzymes of the pathway (Table XVII) shows that, under all conditions of growth, uricase activity is much higher than xanthine dehydrogenase activity. Therefore, uric acid should be converted to allantoin as rapidly as it is formed and would not accumulate sufficiently to allow measurement. However, if this were the reason that activity could not be detected by an increase in optical density at 293 mμ, it should still be possible to follow disappearance of substrate spectrophotometrically. But when substrate disappearance was measured, there was no apparent activity (Table VII). Therefore, it was concluded that the second possible explanation is the correct one, i.e., the xanthine dehydrogenase of *N. crassa* cannot transfer electrons to oxygen but requires an electron acceptor. When the tetrazolium dye MTT was added as an electron acceptor, there was only slight activity.

However, when phenazine methosulfate was added as a mediator of electrons between the enzyme and the electron acceptor, the activity increased more than 15-fold. To our knowledge, the use of phenazine methosulfate in combination with a tetrazolium dye in the xanthine dehydrogenase assay has not previously been reported in the literature although phenazine methosulfate has been used in conjunction with tetrazolium dye and potassium cyanide to measure L-α-glycerophosphate dehydrogenase (Lin, et al., 1962).

The failure of Roush, Questiaux and Domnas (1959) and Roush and Shieh (1962) to detect xanthine oxidase or xanthine dehydrogenase activity in Candida utilis and Torulopsis candida may have been due to the lack of a mediator for electrons. Apparently Roush, et al. tested phenazine methosulfate and neotetrazolium chloride separately in their attempts to measure activity.

Some of the properties of allantoinase, allantoicase and ureidoglycolate synthetase from N. crassa were studied in order to obtain, as far as possible, maximum reproducibility of assays with crude extracts. Allantoinase was assayed at pH 8.5 for 20 minutes, even though the optimum time was found to be 10 minutes, since the total amount of product formed at 10 minutes was below the optimum range for the colorimetric determination of glyoxylate after subsequent acid hydrolysis. A number of buffers were tried in attempts to obtain overlapping pH curves, but all of them gave only non-overlapping curves. However, the pH range for maximum activity was similar with different types of buffers.

Allantoicase can be measured only by coupling to ureidoglycolate synthetase. Since allantoicase is the rate-limiting reaction, as evidenced by the fact that ureidoglycolate synthetase activity is much higher in most cases and always at least equal to that of allantoicase (see Table XVII), the measurement is valid. Different buffers gave different optimum pH's for allantoicase. Phosphate buffer at pH 7.0 was chosen for maximum reproducibility.

Ureidoglycolate synthetase was assayed after 10 minutes incubation at pH 7.0 in order to reduce the spontaneous hydrolysis of the substrate. The substrate concentration used was in the range where **spontaneously hydrolyzed product was the smallest possible fraction of the total.**

The existence of separate enzymes (or pathways) for the oxidation of xanthine and hypoxanthine has been suggested on the basis of studies of the rate of oxidation of these compounds by whole cells of yeasts (Roush, Questiaux and Domnas, 1959; Roush and Shieh, 1962). Darlington and Scazzocchio (1968) were able to obtain hypoxanthine-negative mutants of Asp. nidulans. However, the mutants utilized xanthine, although at a slower rate than normal. On the basis of this finding they suggested that different pathways may be involved for the oxidation of these two purines. However, further proof was lacking since they were unable to obtain xanthine-negative mutants even after examining 37,000 colonies from mutagenized conidia.

Because of these previous reports, data relative to this question were obtained in the present study. Some evidence was obtained which suggests the existence of two xanthine dehydrogenases or, at least, different enzymes active against xanthine and hypoxanthine. Data taken from tables previously presented are summarized in Tables XVIII and XIX to allow easier comparison of data.

Whether the enzyme was induced with xanthine or hypoxanthine, MTT

TABLE XVIII

COMPARISON OF PROPERTIES OF XANTHINE DEHYDROGENASES INDUCED BY GROWTH ON XANTHINE AND HYPOXANTHINE

	Xanthine-grown		Hypoxanthine-grown	
	Substrates		Substrates	
	Xanthine	Hypoxanthine	Xanthine	Hypoxanthine
Requirement for MTT and PMS	+	+	+	+
Inhibition by KCN	81%	89%	42%	26%
Inhibition by uric acid	30%	31%	28%	0
Inactivation on storage, 21 days at -20°C	62%	56%		

TABLE XIX

COMPARISON OF INDUCTION PATTERNS RELEVANT TO DIFFERENCES IN METABOLISM OF XANTHINE AND HYPOXANTHINE

Induction of Xanthine Dehydrogenase (specific activities):

Inducer →	None	Guanine	Xanthine	Hypo-xanthine	Uric Acid	Repressed level, NH_4^+
Substrate:						
Xanthine	3.1	4.1	28.4	3.3	13.0	6.2
Hypoxanthine	4.1	14.0	37.4	7.8	19.9	9.9

Induction by Xanthine and Hypoxanthine (specific activities):

Enzyme →	Guanase	Uricase	Allan-toinase	Allan-toicase	Ureidoglycolate synthetase
Inducer:					
Xanthine	22.2	84.7	26.4	22.8	38.3
Hypoxanthine	19.3	81.7	5.7	26.0	105

and phenazine methosulfate were required for activity (Table XVIII). This may argue against the existence of two enzymes. However, some evidence of differences was obtained by the use of two inhibitors, cyanide and uric acid. When 10^{-2} M cyanide was used, the activity of xanthine-growth extract toward xanthine was inhibited 81 percent and that toward hypoxanthine was inhibited to an extent of 89 percent. However, in hypoxanthine-grown extracts, the activity toward xanthine was inhibited only 41 percent and the activity toward hypoxanthine was inhibited 26 percent. Thus, the hypoxanthine-induced activity is less sensitive to cyanide than is that induced by xanthine, and there is also a difference in the degree of inhibition which depends upon the substrate used with the hypoxanthine-induced enzyme. When 3.6 µmoles of uric acid were added, in xanthine-grown extracts the activities toward xanthine and hypoxanthine were inhibited to an extent of 30 and 31 percent, respectively, whereas in hypoxanthine-grown extracts the activity towards xanthine was inhibited 28 percent, but the activity towards hypoxanthine was not inhibited. Thus with uric acid, the only difference found was in the inhibition of the hypoxanthine-induced activities toward the two substrates. In summary, both cyanide and uric acid inhibit the enzyme activity toward both xanthine and hypoxanthine in xanthine-grown extracts to the same extent. In hypoxanthine-grown extracts, the activity towards xanthine is inhibited more than is the activity towards hypoxanthine.

Data on the stability of the enzyme(s) failed to yield significant evidence. When xanthine-grown extract was stored at -20°C for 21 days, the loss in activity toward xanthine was 62 percent and that toward hypoxanthine was 56 percent. Since the hypoxanthine-induced enzyme had low initial activity, comparable data were not obtained for it. Such data might have shown differences similar to those found with the inhibitors.

Data for induction of all the enzymes by xanthine and hypoxanthine and for activities of xanthine dehydrogenase toward the two substrates in the mycelium grown in various media are summarized in Table XIX. Activity toward hypoxanthine was always greater than that toward xanthine, regardless of the inducer used. This was also true of the uninduced and repressed levels. Activity toward both substrates was induced maximally by growth on xanthine and to approximately 50 percent of the xanthine-induced level by uric acid. Both guanine and hypoxanthine failed to induce activity higher than the endogenous level toward xanthine, although both induced activity toward hypoxanthine. This difference suggests that there may be an enzyme specific for hypoxanthine in _N. crassa_. The fact that hypoxanthine does not induce activity toward xanthine may indicate that xanthine is not an intermediate in the conversion of hypoxanthine to uric acid. If it were, xanthine would be expected to accumulate in sufficient quantities to act as an inducer. It might be possible to obtain additional evidence concerning the existence of two "xanthine dehydrogenases" by further study of the enzyme induced by guanine or hypoxanthine. It is interesting that guanine is similar to hypoxanthine in this respect, since the product of guanine degradation is xanthine rather than hypoxanthine.

Induction of the other enzymes of the pathway by the two substrates, xanthine and hypoxanthine offers some additional evidence for different pathways of metabolism for the two compounds. The differences in levels of allantoinase and ureidoglycolate synthetase are probably related, as discussed below. The failure of hypoxanthine to induce levels of allantoinase greater than the endogenous level may indicate a further differentiation between the pathway of metabolism of hypoxanthine and that of the other compounds studied.

It was thought that mutants blocked in the various steps of allantoin degradation, in addition to enzyme data, might provide solid evidence as to which of the three possible pathways are operating in the degradation of allantoin. Therefore, repeated attempts were made to isolate allantoin-negative mutants using various mutagens and procedures. The mutagens were effective in reducing the number of survivors and a number of morphological mutants were obtained. Attempts were made to enrich for mutants by both filtration enrichment and inositol-less death. In the filtration enrichment procedure, it is possible for the wild type conidia to produce sufficient ammonia to be used by the mutants for growth and consequently they are lost. The inositol-less procedure may be selective only for particular types of mutants as has been observed by Lester and Gross (1958). They always obtained a high frequency of leucine-requiring mutants, but other mutations were found less frequently with this method. Further, there are only two steps from allantoin where mutants could be obtained theoretically. Only one attempt was made to isolate hypoxanthine-negative mutants. The particular strain used grew and sporulated only sparsely on hypoxanthine media.

These negative results do not indicate that it would be impossible to obtain mutants, if additional attempts were made using xanthine or hypoxanthine as substrates. The strains which were used in these studies were macroconidial strains. It might be possible to use a microconidial strain and the nylon net procedure, which was used successfully by Kølmark (1969) to isolate urease-negative mutants from *N. crassa*.

B. Control of Allantoin Metabolism in *Ps. aeruginosa*

In the case of a number of inducible catabolic enzymes, it has been shown that glucose and other readily available carbon sources can repress enzyme synthesis. Three carbon sources of different types, all of which are readily used by *Ps. aeruginosa*, were tested for ability to repress the synthesis of the allantoin-degrading enzymes. These were glucose, histidine and acetate. In all three cases, growth was more rapid on the mixture of carbon sources than on either alone, indicating utilization of both. Since the concentrations used were almost certainly too high to be rate-limiting, it is probable that the increased growth rate on the combinations of carbon sources reflects a difference in the number of reactions required to produce all the biosynthetic intermediates necessary for growth in minimal medium. Neither the shape of the growth curve nor the pattern of substrate removal indicated interference with allantoin utilization by any of the three compounds tested. This conclusion was supported by assays of enzyme activities in extracts of cells exposed to glucose, allantoin and a mixture of the two (Table I). A decrease in the activities of allantoinase and ureidoglycolate synthetase in cells exposed to allantoin in the presence of glucose (as compared to allantoin alone) may indicate partial repression but repeated assays would be required to determine the significance of the difference observed.

It has been pointed out by Mandelstam (1968) that a good carbon source represses the utilization of a poor carbon source. He defined a "good carbon source" as one giving a high rate of growth. He also pointed out that this effect is produced by "all carbon sources in a way that is inversely related to their 'goodness' as measured by the doubling time of cells, i.e., the better the growth on any particular compound, the more will that compound repress formation of inducible enzymes." Lessie and Neidhardt (1967) concluded that "the extent of repression by different organic compounds corresponded to their ability to promote rapid growth."

Inspection of the growth curves in Figures 2, 3 and 4 shows that at least glucose and histidine conform to the definition of a good carbon source and as such they should be able to repress allantoin utilization. The difference between rates of growth on histidine and allantoin was greater than that between glucose and allantoin. Furthermore, histidine was tested under the most favorable circumstances for exertion of repression. In spite of this there was no repression of growth and/or enzyme synthesis in the wild type strain.

Since repression in catabolic pathways is due to the accumulation of catabolite rather than to the repressing compound itself, it may be postulated that in this strain of Ps. aeruginosa there is never accumulation of a repressing intermediate inside the cell in sufficient concentrations to cause repression.

The above assumption was strengthened by studies on a mutant strain, PA-1-707. This glucose-negative mutant, when exposed to glucose, accumulates a compound which has been tentatively identified as gluconate (H. E. Heath, III, personal communication). This mutant is capable of normal growth on allantoin. However, when this strain was inoculated into a mixture of allantoin and glucose, growth was inhibited, irrespective of the concentration of glucose or whether glucose was added before or after initiation of growth. If glucose was added initially, growth was completely prevented. If glucose was added after initiation of growth, the growth rate was reduced appreciably. This growth may be due to the presence of enzymes synthesized before addition of glucose. When enzyme assays were performed in this strain, it was found that allantoin-induced cells had high levels of all enzymes. When cells were induced with allantoin in the presence of glucose, allantoinase activity was partially repressed, allantoicase activity was higher than normal and there was no detectable ureidoglycolate synthetase activity even though this enzyme was present in extract from cells exposed to glucose only. The absence of even endogenous levels of ureidoglycolate synthetase activity may be due to increased activity of glyoxylate carboligase which is responsible for utilization of glyoxylate. However, proof of this would require further study. It is difficult to explain how allantoicase activity is so high in the absence of ureidoglycolate synthetase even though this enzyme is measured by coupling to ureidoglycolate synthetase. However, it has been pointed out by Lessie and Neidhardt (1967) that in a mutant strain able to grow at 65 percent the normal rate on succinate or succinate plus histidine there was twice as much histidase as in the wild type. Hence, it might be possible that the actual activity of allantoicase is much higher than that measured. Since ureidoglycolate can hydrolyse spontaneously, it is possible to observe allantoicase activity to a lesser extent than its true value in the absence of ureidoglycolate synthetase. The repression of allantoin utilization in the mutant is not believed to be due to gluconate toxicity, since for the wild type gluconate is an excellent source of carbon for growth. The absence of ureidoglycolate synthetase is sufficient reason for the inability of the mutant to grow on allantoin in the presence of glucose. It is interesting that only this enzyme is completely repressed.

Neidhardt and Magasanik (1957) and Lessie and Neidhardt (1967) found that if ammonium salts were omitted from the medium and if the repressed compound was used as the sole source of carbon and nitrogen, the repression was relieved. This was tested with PA-1-707 by omitting ammonium chloride from the medium and using allantoin as the sole source of carbon and nitrogen. However, this did not relieve the repression. Grady, et al. (1969) found only slight derepression of lysine-degrading enzymes when lysine was used as sole source of nitrogen in the presence of glucose by a natural population, and pointed out that full derepression would not be required to allow use as a nitrogen source since the cell's requirement for nitrogen is much lower than that for carbon and energy.

C. Control of Enzymes in *N. crassa*

Reports on the induction of various enzymes involved in purine catabolism which have appeared in the literature are summarized in Table XX. It can be seen from the table that, except in *Candida utilis*, no complete study in any single organism has been reported, and in this yeast xanthine dehydrogenase was not studied because activity could not be measured. A possible reason for this has been discussed above.

KEY

Enzymes

Adase	Adenase
Gase	Guanase
XDH	Xanthine dehydrogenase
UOx	Urate oxidase
Alase	Allantoinase
Acase	Allantoicase
UGSase	Ureidoglycolate synthetase

Inducers

A	adenine
G	guanine
HX	hypoxanthine
X	xanthine
U	uric acid
Al	allantoin
AA	allantoic acid

NF - enzyme was not found in the organism
C - enzyme reported to be constitutive

References (see Bibliography for complete citation)

1. Franke, Taha, and Krieg (1952)
2. Roush (1954)
3. Roush and Domnas (1956)
4. Roush, Questiaux, and Domnas (1959)
5. Domnas (1962)
6. Lee and Roush (1964)
7. Scazzocchio and Darlington (1968)
8. Allam and Elzainy (1969)
9. Campbell (1954)
10. Clarke and Meadow (1966)
11. Winter (1967)
12. Fukumoto, Watanabe, and Yano (1968)
13. Kaltwasser (1969)

TABLE XX

COMPILATION OF PUBLISHED REPORTS ON THE INDUCTION OF PURINE-DEGRADING ENZYMES

Organism	Enzyme and inducers							
	Adase	Gase	XDH	UOx	Alase	Acase	UGSase	Ref.
Fungi:								
Aspergillus niger				C				1
Alternaria porri				C				1
Torulopsis utilis	A	A		A				2
Torulopsis utilis				U				3
Candida utilis	A,G,HX	A,G,HX,X	NF	A,G,HX,X,U				4
Candida utilis						Al	Al	5
Candida utilis					U,Al,AA	U,Al,AA		6
Saccharomyces cerevisiae						Al	Al	5
Aspergillus nidulans			U	U	U,Al	Al	C	7
Penicillium chrysogenum			C	C	X,Al			8
Bacteria:								
Pseudomonas sp.					Al,AA	Al,AA		9
Pseudomonas aeruginosa		G	G,X	G,X,U				10
Pseudomonas aeruginosa					Al	Al	Al,AA	11
Streptomyces sp.				U				12
Hydrogenomonas eutropha				U				13

It is difficult to determine whether enzymes reported as inducible by some investigators actually are so. Reports have been included in the table in which enzyme levels for cells grown on "inducers" were reported but endogenous, or uninduced, levels were not recorded. In any case, true inducers have not been determined in any organism by study of mutants (see discussion below).

It is not possible, without using mutants blocked in each step of the pathway, to determine whether any compound is a true inducer or is the precursor of an inducer. However, some tentative conclusions may be drawn from the data at hand. Examination of the specific activities in extracts prepared from mycelium grown on the various compounds (see Table XVII for summary) indicates that the true inducer for the enzymes which convert guanine to uric acid may be xanthine. The basis for this conclusion is shown in Table XXI. In mycelium grown on guanine, hypoxanthine or uric acid, the rate-limiting reaction (i.e., the lowest level of specific activity) is the conversion of xanthine to uric acid. Only in xanthine-grown mycelium are all the enzymes induced to comparable levels. Therefore, in growth on any other nitrogen source, xanthine would be expected to accumulate and could reach levels sufficient to induce the remaining enzymes of the pathway. This would be particularly important in preventing degradation of endogenously formed guanine which is required for synthesis of nucleic acids. The lack of ability to synthesize adenase prevents loss of adenine by degradation. The advantage to the cell of induction of catabolic enzymes for essential metabolites by the product of the first reaction, rather than by the metabolite itself, has been discussed by Palleroni and Stanier (1964).

TABLE XXI

LIMITING ACTIVITIES IN EXTRACTS OF MYCELIUM GROWN ON VARIOUS NITROGEN SOURCES

Mycelium grown on	Limiting reaction	Intermediate possibly accumulated from guanine
Guanine	Xanthine → uric acid	Xanthine
Xanthine	None	None
Hypoxanthine	Xanthine → uric acid	Xanthine
	Allantoin → allantoate	Allantoin
Uric acid	Xanthine → uric acid	Xanthine

An alternative conclusion is that product induction is a common phenomenon in the purine degradative pathway. Product induction has been reported in Ps. putida. In this organism, catechol oxygenase has been shown to be induced by cis, cis-muconate, the product of the enzyme (Canovas, Ornston and Stanier, 1967). Scazzocchio and Darlington (1968) reported that the xanthine dehydrogenase of Asp. nidulans is induced by uric acid, and other examples are given in Table XX.

Arguments similar to those used regarding induction by xanthine can be advanced concerning the induction of ureidoglycolate synthetase. As pointed out previously (Table XV), ureidoglycolate synthetase had maximal activity in mycelium grown in the presence of allantoin or hypoxanthine, followed by guanine and xanthine. These data can be explained if allantoin is assumed to be the inducer for this enzyme. Growth on allantoin resulted in maximal activity. If allantoin is the true inducer, then the lower the level of allantoinase activity, the higher should be the ureidoglycolate synthetase activity. This is found to be the case as shown in Table XXII, where the activity levels for the two enzymes are arranged to show the inverse order of activities. When allantoinase activity is low, allantoin will accumulate and will induce ureidoglycolate synthetase.

If the conclusions regarding inducers are correct, the pathway would appear to be composed of two regulons, i.e. blocks of enzymes which have a common inducer but whose loci are not known to be genetically linked (Stevenson and Mandelstam, 1965). Guanase, xanthine dehydrogenase and uricase would thus compose one regulon, with xanthine as the inducer. The product of this regulon, allantoin, would then induce the enzymes of the second regulon, allantoinase, allantoicase

TABLE XXII

COMPARISON OF ACTIVITIES OF ALLANTOINASE AND UREIDOGLYCOLATE SYNTHETASE

Mycelium grown on	Specific activities, mμmoles/min/mg protein	
	Allantoinase	Uredoglycolate synthetase
Hypoxanthine	5.7	105
Guanine	20.3	60.2
Xanthine	26.4	38.3
Uric acid	36.2	28.0

and ureidoglycolate synthetase. The fact that allantoin was the only compound tested which failed to induce synthesis of guanase and xanthine dehydrogenase, and also possibly of uricase, supports this idea. Definitive evidence can only be obtained by use of mutants which could not be obtained in this investigation. Further studies showing the accumulation of xanthine and allantoin in the mycelium under the conditions where their accumulation was postulated would provide additional supporting evidence.

Ammonium ion represses synthesis of guanase, xanthine dehydrogenase, uricase and allantoinase in *N. crassa* (Table XVI). Allantoicase and ureidoglycolate synthetase were not repressed. The derepression of ureidoglycolate synthetase to a level greater than that in mycelium grown on uric acid alone may be due to accumulation of allantoin, since uricase is not completely repressed while allantoinase is. If the postulated regulon organization is correct, repression of allantoinase might be expected not to occur in the absence of repression of the latter two enzymes. Scazzocchio and Darlington (1968) reported that in *Asp. nidulans* all the enzymes were repressed.

D. Comparison of Controls in the Two Organisms

Enzymes of the catabolic pathway for purines and their derivatives are inducible in both the organisms studied. The enzymatic steps involved in the conversion of allantoin to glyoxylate and urea are identical in the two organisms, although one, *Ps. aeruginosa,* is a bacterium which can use allantoin as sole source of carbon, nitrogen and energy, while the other, *N. crassa,* is a filamentous fungus, capable of using allantoin and its precursors only as a source of nitrogen.

Control mechanisms in neither organism have been studied extensively. Indeed, as pointed out by Gross (1969) in a recent review of genetic regulation in fungi, much more study of these mechanisms in fungi is needed. Demerec (1964) has stated a similar need for studies of *Pseudomonas*.

The present study has allowed comparison of controls of the same enzymes in organisms where they are used for different purposes. It is interesting that catabolite repression of the allantoin-degrading enzymes was not found in *Ps. aeruginosa* except under circumstances where accumulation of glucose catabolites is caused by a genetic block in glucose metabolism. On the other hand, in *N. crassa*, allantoinase synthesis is apparently controlled by "catabolite" repression since its synthesis is repressed by ammonia, which is the end product of degradation as a nitrogen source. As in *Ps. aeruginosa*, allantoicase and ureidoglycolate synthetase are apparently not subject to end-product control. However, the repression of allantoinase activity would be sufficient to prevent over-production of ammonia, which might be toxic to the organism, and perhaps this is a more important consideration than prevention of synthesis of two apparently unused enzymes. Ammonia does prevent synthesis of the enzymes of the first regulon, except possibly a small amount of uricase, and thus most of the unneeded enzymes of the pathway are controlled by the product.

CHAPTER V

SUMMARY AND CONCLUSIONS

The pathway for purine catabolism in *N. crassa* was shown to be:

$$\begin{tikzcd}
 & \text{Hypoxanthine} \arrow[d] \arrow[rd, dashed] & \\
\text{Guanine} \arrow[r] & \text{xanthine} \arrow[r] & \text{uric acid} \arrow[d] \\
 & & \text{allantoin} \arrow[d] \\
 & & \text{allantoic acid} \arrow[d] \\
 & & \text{ureidoglycolate} \arrow[d] \\
 & & \text{urea + glyoxylate}
\end{tikzcd}$$

There was some evidence that two enzymes are involved in the degradation of xanthine and hypoxanthine. Xanthine dehydrogenase required a mediator, phenazine methosulfate, to couple the enzyme to the tetrazolium dye used as electron acceptor. On storage the enzyme lost its activity toward both xanthine and hypoxanthine progressively, but the ratio of the two activities remained essentially constant. Potassium cyanide was found to inhibit xanthine dehydrogenase activity in both xanthine-grown and hypoxanthine-grown extracts. Uric acid was found to inhibit xanthine dehydrogenase activity toward both xanthine and hypoxanthine in xanthine-grown extracts. However in hypoxanthine-grown extracts, it inhibited only the activity toward xanthine.

Since mutants are important tools for the study of metabolic pathways and regulation, attempts were made to isolate mutants using a number of mutagens and enrichment techniques, but these efforts were unsuccessful.

Product induction may be a very common phenomenon in purine catabolism in _N. crassa_. This organism lacked adenase. Guanase was induced by growth on guanine, hypoxanthine, xanthine and uric acid. Uric acid was found to be a good inducer. Xanthine and hypoxanthine were moderate inducers and guanine was found to be a poor inducer.

Xanthine dehydrogenase activity toward both xanthine and hypoxanthine was found to be induced best by growth on xanthine. Uric acid was less effective. However, with growth on guanine or hypoxanthine, only the dehydrogenase activity towards hypoxanthine was induced but not the activity towards xanthine.

Uricase was found to be induced by growth on guanine, xanthine, hypoxanthine and uric acid. Allantoin was found to induce the enzyme partially.

Uric acid and xanthine were found to be good inducers for allantoinase. Guanine and allantoin were less effective inducers.

Allantoicase was found to be induced by growth on guanine, hypoxanthine, xanthine, uric acid and allantoin.

Allantoin appeared to be the true inducer of ureidoglycolate synthetase. An inverse relationship was found between the activities of allantoinase and ureidoglycolate synthetase, indicating that accumulation of allantoin might be responsible for induction of ureidoglycolate synthetase. However, the actual accumulation of allantoin in the mycelium should be demonstrated before any definite conclusions can be drawn.

Guanase, xanthine dehydrogenase(s), uricase and allantoinase were repressed by ammonium tartrate. Allantoicase and ureidoglycolate synthetase were not repressed.

Further studies using mutants are required before the true inducers in the pathway can be definitely established. Since the turnover of intracellular purines may play a definite role, the control of purine catabolism may be rather complex. However, mutants in conjunction with non-metabolisable analogs, should be used in further studies to clarify these relationships. Xanthine dehydrogenase should be purified to establish whether there are one or two enzymes present.

Studies with *Ps. aeruginosa* strain PA-1 showed that the utilization of allantoin is not repressed by acetate, glucose or histidine as measured by growth and substrate removal. Enzyme assays confirmed these results.

In a glucose-negative mutant of *Ps. aeruginosa*, addition of glucose at various concentrations and various times was found to inhibit growth. The inhibition of growth occurred even when allantoin was serving as the sole source of carbon and nitrogen. It is possible that in the wild type strain sufficient quantities of catabolites to cause catabolite repression did not accumulate. Enzymatic data for the mutant indicated that possibly the absence of ureidoglycolate synthetase activity in cells exposed to allantoin plus glucose is responsible for the prevention of growth.

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