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GENETIC AND METABOLIC REGULATION OF THE
PURINE CATABOLIC ENZYMES IN *Neurospora crassa*

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

William Robert Reinert, B.A.

The Ohio State University

1975

Reading Committee
G. A. Marzluf, Ph.D.
D. H. Ives, Ph.D.
G. A. Barber, Ph.D.

Approved by

George A. Marzluf
Advisor
Department of Biochemistry
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I gratefully acknowledge the financial support of the Department of Biochemistry.
VITA

1948......................Born - Springfield, Missouri

1970......................B.A., Denison University, Granville, Ohio

1971-1975...............Graduate Research Associate, The Department of Biochemistry, The Ohio State University, Columbus, Ohio

1971, 72, 73, 74........Teaching Associate, The Department of Biochemistry, The Ohio State University, Columbus, Ohio

PUBLICATIONS


"Genetic and Metabolic Regulation of Purine Metabolism in N. crassa." Genetics 77:s54 (1974)

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"Genetic and Metabolic Control of the Purine Catabolic Enzymes of Neurospora crassa." Mol. Gen. Genetics (accepted for publication)
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Introduction

A great deal is now known about genetic and metabolic regulation of gene expression in prokaryotes, but little is understood about the regulatory mechanisms present in higher organisms. The enormous increase in complexity of the simplest fungi over phage \( \lambda \), as judged by the number of base pairs in their respective genomes, is clearly one of the major reasons for the limited information available about control in eucaryotes. Genetic analysis of several regulatory systems in Neurospora has just begun to give us some insight into the control of gene action in higher forms (1).

**Neurospora crassa** is a fungus of the class Ascomycetes. Being a eucaryote, the organism possesses a defined nuclear membrane, seven chromosomes, and both sexual and asexual reproductive cycles. A very convenient genetic feature of the fungus is that during its asexual cycle, it contains only a haploid set of chromosomes, which facilitates isolation of various mutants. The life cycle of *N. crassa* is shown in Figure I.

In Neurospora, studies of the control of both anabolic and catabolic pathways are now receiving major emphasis, with at least two different types of regulation appearing.

The repression-versus-derepression type of control is best shown in the case of sulfur anabolism. Here, synthesis of an entire family of enzymes for sulfur metabolism in *Neurospora* has been shown to be
Figure 1. Life cycle of *N. crassa*

Haploid conidia are produced by hyphae; are of two mating types.

ASEXUAL:
- Conidia can grow vegetatively to produce mycelial mass.
- Conidia can fertilize protoperthecium of the opposite mating type.
- Ascospores can grow vegetatively to produce mycelial mass.
- Meiosis occurs to produce haploid ascospores.

SEXUAL:
- Male nuclei, conidium
- Female nuclei, ascogonium
- Trichogyne, mating tube
- Female nuclei produce ascogonium
- Male nuclei produce trichogyne
- Fusion of nuclei produces protoperthecium
- Diploid state in protoperthecium resulting from fusion of nuclei.
controlled by repression but not to require induction. Furthermore, this same group of enzymes is controlled by two regulatory genes which are unlinked to each other and to the structural genes which they control (2). One of these control genes acts in a positive way to activate the synthesis of the sulfur-controlled enzymes, while the second one appears to function in a negative manner.

A strikingly different type of control has been demonstrated by Giles for quinic acid metabolism in N. crassa (3). In the absence of catabolic repression, exerted by glucose or a similar favored carbon source, the synthesis of the quinate catabolic enzymes occurs only in the presence of a suitable inducer such as quinic acid. The structural loci for the three early catabolic enzymes are very tightly linked to qa-1, a positive regulatory gene, mutants of which have been found to display a pleiotrophic loss of the three enzymes while other qa-1 alleles are constitutive for these same activities (4). Since great differences appear to exist in the control of sulfur and quinate metabolism as well as for other pathways (1), additional studies of regulation in Neurospora may be informative. Towards this end, I have begun to examine the control mechanisms for nitrogen metabolism in Neurospora.

Neurospora crassa is routinely grown with ammonia as the nitrogen source, but many other compounds can also be used. Wolf (5) reported that the related species N. sitophila was quite selective in which nucleic acid derivatives it could utilize as a nitrogen source. In a more comprehensive study, Bollard (6) found that N. crassa could utilize most amino acids, amines, amides and purines, but not pyrimidines nor methylated urea compounds as nitrogen sources.
In order to study the control of nitrogen metabolism, I have examined purine catabolism, focusing attention on the control of the catabolic enzymes of this degradative pathway (Figure 2). The metabolism of hypoxanthine or xanthine requires six enzymes and results in the production of four equivalents of ammonia and one of glyoxylate. This work describes the regulation of the purine pathway by examination of enzyme activities after growth on various purine intermediates, isolation of mutants lacking specific enzymes in the pathway, the use of non-metabolizable purine analogues, and by studying the stability of the enzymes.

The synthesis of the first three regulated enzymes (uricase, allantoinase, allantoicase) was found to be controlled by both induction and repression whereas the last two enzymes (ureidoglycolate hydrolase and urease) are constitutive. Uric acid induces the synthesis of all three of the regulated enzymes while the next intermediate of the pathway, allantoin, was found to induce only allantoinase. Repression appears to be mediated by the end product, ammonia. Mutants blocked at steps 1, 3, 4 and 6 (Figure 2) have been isolated along with a special class of mutants which still possess all the enzymes but have lost their ability to regulate them. All of the mutants were characterized both genetically and biochemically. The structural genes for four enzymes of the pathway were found to be carried on several different chromosomes and thus are not clustered as is the case with the quinate pathway (7).

These new mutants have made it possible to study potential inducers for the enzymes of the pathway and thus better define the true inducer. Hypoxanthine can be metabolized to form the inducer but was shown not
Figure 2. The reactions involved in the degradation of purines to ammonia. The enzymes which catalyze these reactions are: xanthine dehydrogenase, uricase, allantoinase, allantoicase, ureidoglycolate hydrolase, and urease; they are indicated in the figure by the numbers 1-6, respectively.
Figure 2
to be itself an inducer of either uricase or allantoicase. Similarly, neither allantoin nor allantoic acid can induce these same two enzymes. The kinetics of induction and stability of the three regulated enzymes were examined. These studies showed that the activity of allantoicase and uricase is controlled coordinately and both are also stable enzymes. The activity of allantoinase, on the other hand, declines rapidly in vivo upon inhibition of protein synthesis, with a half-life of about 20 minutes. An independently isolated mutant, *arr*, allelic to *nit-2*, was found to possess pleiotrophic properties for the utilization of various nitrogen compounds. The possibility that this locus codes for a positive regulatory protein will be discussed.
Literature Review

In procaryotes, there are constitutive genes whose expression is continuous and their product, usually an enzyme, is present at essentially the same concentration regardless of the growth conditions. Regulated genes are not expressed constitutively, but their rate of synthesis is subject to genetic and metabolic signals which modulate transcription. Such regulated genes are often found to be clustered in operons—defined as a group of related genes that are coordinately regulated and transcribed into a single polycistronic mRNA. The operon is a long continuous DNA sequence which includes, in order: a promotor region, an operator region and a structural gene region. Control of an operon was perhaps best defined by Epstein and Beckwith (9) who stated that an operon was "a group of contiguous structural genes showing coordinate expression plus their closely associated controlling sites." The controlling sites are defined as elements which determine the expression of only those genes to which they are attached, i.e., they have 'cis dominant' effects. This definition describes certain features of the overall regulation of an operon, but not the actual mechanism of control nor whether it is positive or negative. The terms "positive control" and "negative control" will be frequently mentioned, thus an understanding of each type is necessary. Englesberg and Wilcox (10) stated that an operon is under negative control when the structural genes are free to be transcribed in the absence of additional regulatory
factors; this process is stopped when a repressor (defined as a regulatory protein with a negative effect) binds to an operator controlling site and thereby inhibits gene expression. A repressor, usually a protein, is specific for only a particular system and its absence allows unrestricted transcription. In a positive control system, transcription of the operon is dependent on the binding of a specific activator, usually a protein encoded by a separate specific regulatory gene, to the initiator controlling site. Thus, in the absence of this activator, the operon is turned off. The type of control, whether positive or negative, is not dependent on whether the operon is subject to induction or derepression.

Numerous examples have been reported in procaryotes where an operon is controlled in either a positive or negative fashion and there are even a few cases of mixed positively and negatively regulated operons. The lac operon (11-14) consists of a promoter and operator site, which regulate the transcription of a structural gene region which codes for enzymes involved in the utilization of lactose. The lac operon is under negative control; when no lactose is present in the cell, the lac-specific repressor molecule (a protein which is constitutively synthesized from another region of the bacterial genome) reversibly binds to the operator site and effectively inhibits transcription. If a suitable inducer, such as lactose, is added to the growth media, it will accumulate in the cell and reversibly bind to the lac repressor molecule. The inducer/repressor complex then dissociates from the operator site which thereby permits transcription of the adjacent structural genes. Many mutants with defective repressor regions (1\(^{-}\)), defective operator sites (0\(^{+}\)), and mutant or deleted promoter sites (\(P^{\alpha}\)) have been studied and have provided
evidence for the type of control found in the lac operon.

To demonstrate adequately that an operon is under positive control, Englesberg and Wilcox (10) stated that specific criteria must be met. They are (1) the occurrence of pleiotrophic negative mutants at a high frequency; (2) the isolation of a deletion mutation in the proposed regulatory gene; (3) the demonstration that the regulatory gene is not part of the operon it is proposed to control; (4) the isolation of constitutive mutants in the regulatory gene; (5) a test for dominance (that is, \( R^+ \) and \( R^C \) alleles being dominant to \( R^- \)); (6) the demonstration of cis-dominant mutants in the operon that affects the expression of the \( R^C \) and \( R^+ \) alleles; and (7) finding \( I^C \) cis-dominant (initiator) mutations within the controlled operon as "revertants" of a deletion of the regulatory gene.

The maltase operon (mal) is a good example of a system believed to be under pure positive control (14-18). The \( E. coli \) maltase gene-enzyme complex is actually a regulon comprising two unlinked regions on the bacterial genome, mal A and mal B. The mal A region consists of operon mal PQ, which are the structural genes, a controlling site, and mal T, the regulatory gene for the entire system which codes for an activator. The mal B region appears to consist of two operons with separate but linked controlling sites. This regulon is positively controlled because the product of mal T loci is necessary, in association with maltose, for expression of the two operons. Mutants of the mal T locus are pleiotrophic-negative mutations and recessive to mal T in merodiploids. In addition, alterations within the binding sites give rise to pleiotrophic-negative mutants as would be expected for a positive
However, malTc mutants have not yet been isolated so that dominance relationships between possible constitutive alleles and inducible alleles have not been tested.

The lac operon, which had been shown to be negatively controlled, was later found to be subject to positive control as well (20). Since the L-arabinase regulon of E. coli has also been shown to be positively and negatively regulated (10), it now seems clear that even relatively simple organisms can have complex systems of regulation.

Upon stimulation by various means, the enzyme adenyl cyclase produces more product (cyclic AMP) from ATP. Cyclic AMP in turn binds to a specific receptor protein. This protein has been variously referred to as CAP (catabolite activator protein) (21), CRP (cAMP receptor protein) (22), or CGA (catabolite gene activator) protein (23). This cAMP-CGA protein complex binds to the promotor region of the lac operon and promotes transcription of mRNA. The promotor binding site for cAMP-CGA protein is near but not the same site for binding of RNA polymerase nor the lac repressor protein. This same cAMP-CGA protein complex also stimulates the expression of numerous other operons which are subject to catabolite repression. Thus, in procaryotes, regulation of transcription is quite refined, with some operons being controlled in both a positive and negative fashion.

In eucaryotes, the existence of operons where the structural genes for either a catabolic or anabolic pathway are clustered and transcribed as a single mRNA is, at this time, the exception and not the rule. Structural genes coding for the histidine biosynthetic enzymes are scattered over seven different loci and four different chromosomes in
Neurospora (24). Those few operons in *N. crassa* in which two or more genes appear to be contiguous probably represent an "enzyme cluster", that is, a multi-enzyme aggregate containing all the activities encoded by the clustered genes (25-26). In addition, by definition, an operon contains an operator site and mutants in the operator region must be found to demonstrate an operon. No truly convincing examples of such operator constitutive mutants exist in eucaryotes. The possibility that the enzymes for quinic acid metabolism in *Neurospora* are localized in an operon is discussed below.

Metabolic pathways are regulated in *N. crassa*, but apparently not in the same manner as have been found in procaryotes. The more thoroughly studied pathways include those of sulfur metabolism (2, 27-34), the quinate catabolic system (3, 7, 35-40) and the leucine biosynthetic system (41-42). All three appear to be regulated by positive control, and the evidence for such control appears strongest for the first two systems.

In the sulfur system, depending on the external sulfur source and its concentration, a group of unlinked enzymes has been found to be simply regulated by repression and derepression. The enzymes involved are choline-0-sulfatase (27), two sulfate permeases (30), glucose-6-sulfate permease (31) and aryl-sulfatase. Either an excess of a poor sulfur source (glucose-6-sulfate or cysteic acid) or a limiting amount of a good sulfur source (inorganic sulfate or methionine) permits the synthesis of the above enzymes to be derepressed. The enzyme arylsulfatase shows the most dramatic increase upon derepression (500-2000 fold increase in specific activity). The increase in the amount of these
enzymes is not due to induction because simple starvation for a sulfur source causes an increase in synthesis. These same enzymes are repressed by high concentrations of either sulfate or methionine although neither is the true co-repressor of the sulfur system. Instead, the co-repressor is metabolically closer to cysteine.

Two regulatory genes called \textit{cys-3} and \textit{scon} have been extensively studied (2, 32-34) and found to be unlinked to the few structural gene mutants known for enzymes in the sulfur pathway. Mutants of the \textit{cys-3} loci have lost the ability to synthesize the sulfur-related enzymes under any condition and, in addition, are auxotrophs needing methionine to grow. The \textit{scon} mutants have lost the ability to repress synthesis of the sulfur enzymes and thus derepressed levels of the enzymes are found even under the usual repressing conditions.

The \textit{cys-3} locus is thought to code for a nuclear protein which (in the absence of repressor) is needed for the expression of the genes which encode the sulfur enzymes. Two other alternative explanations which have been satisfactorily eliminated are: (1) the \textit{cys-3} locus codes for a common polypeptide necessary for the activity of all the enzymes, and (2) the locus codes for a super-repressor. In the first case, temperature-sensitive mutants of \textit{cys-3} provided strong evidence; at 25° all the enzymes can be synthesized, but not at 35° (32). By studying the physical properties of aryl sulfatase, from the wild-type and \textit{cys-3} \textit{ts}, the enzyme was shown to have the same thermostability. If the \textit{cys-3} locus were making a common polypeptide, aryl sulfatase from \textit{cys-3} \textit{ts} should be labile at the non-permissive temperature, yet the enzyme was found to be stable. In the case of the super-repressor theory,
electrophoretically different, yet active, forms of aryl sulfatase (ars) were used (33). A heterocaryon was made which contained both the cys-3\(^+\) and cys-3 alleles; the mutant gene was recessive to its wild-type allele (32). When a heterocaryon was constructed consisting of cys-3, ars\(^F\) (one variant form) with cys-3\(^+\), ars\(^S\) (another variant), both aryl sulfatase forms were found under conditions of derepression (33). This implies that the product of cys-3\(^+\) does diffuse across the nuclear membrane and is dominant to cys-3 and thus strongly argues against the super-repressor theory. Therefore, the genetic loci for the family of sulfur enzymes appear to be unlinked and under positive control, with the product of the cys-3 locus being necessary for their expression.

Another positively regulated system in N. crassa involves the catabolic quinate pathway (3, 7, 35-40). The initial three enzymes degrading quinate to protocatechuic acid (PCA) are induced by quinate and repressed by glucose (or another good carbon source). In addition, (1) the enzymes are coordinately induced, and (2) the structural genes for the three non-aggregated enzymes are very tightly linked (3, 35-37). Thus, they may reside in an operon arrangement although more evidence is required to establish this possibility. Regulatory mutants located in the qa-1 locus which show a pleiotrophic loss of all three enzymes map very close to the three structural genes (3, 7). The evidence is strong that the qa-1 locus codes for a protein which, in the presence of quinate (and the absence of glucose), induced the synthesis of the enzymes (38). Studies with heterocaryons showed that qa-1\(^+\) is dominant to qa-1 and that a heterocaryon of qa-1 qa-2\(^+\) with qa-1\(^+\) qa-2 is able to grow on quinate. The common polypeptide theory was discounted by
thermostability studies on one of the enzymes from wild-type and several qa-1 revertants (3). The possibility that the qa-1 mutants were simple operator-constitutive mutants was dispelled by interallelic complementation of qa-1 mutants and the finding that qa-1+ must make a diffusible product, because the heterocaryon qa-1 qa-2+ + qa-1+ qa-2 grew on quinate. Thus, two pathways—one anabolic and the other catabolic—appear to be controlled in a positive manner, although major differences exist between the regulatory systems.

The pathway of purine catabolism and its control have been studied in both procaryotes and eucaryotes (43-47). In procaryotes, the pathway of allantoin degradation has been ascertained with numerous organisms. One basic pathway has been found, with about half of the organisms studied having one additional intermediate and having evolved an extra enzyme to catabolize it. In Pseudomonas acidovorans, Streptococcus allantoinus, Arthrobacter allantoinus and Escherichia species, the pathway is allantoin→allantoate→ureidoglycine→ureidoglycolate→glyoxylate→urea→ammonia + CO₂ (43-45). In Ps. aeruginosa, Ps. fluorescens, Pen. citro-viride and Pen. notatum, the pathway is less complex in that allantoin goes to allantoate, then directly to ureidoglycolate, then glyoxylate followed by urea to ammonia + CO₂ (46). Properties of allantoinase in the various organisms were examined although little effort was devoted to studying how the pathway was controlled. The active site of allantoinase was found to contain an essential thiol group in all of the organisms studied (47).

In eucaryotes, purine breakdown has been studied extensively in Aspergillus nidulans and Saccharomyces cerevisiae (48). The two
catabolic pathways are similar until the intermediate urea is produced. In *Aspergillus*, urea is simply broken down to ammonia and CO₂ by urease, but in *S. cerevisiae*, urea carboxylase produces allophanic acid from urea and a bound carboxyl group. Allophanic acid is then degraded to ammonia and CO₂ by allophanate hydrolase. Control of the purine degradative pathways is quite divergent since an early intermediate acts as the inducer in *Aspergillus* while the very last intermediate induces the enzymes in yeast.

Five enzymes are required to degrade allantoin to ammonia and CO₂ in yeast (48). Repression of the synthesis of two enzymes, allantoinase and allophanate hydrolase, was not by the end product, ammonia, but by serine or a number of other readily metabolized amino acids (49). The last intermediate in the pathway, allophanic acid, was shown to be the physiological inducer through studies with mutants and by using a chemically similar, yet non-metabolizable, compound, oxaluric acid (48, 50-52). The structural genes for allantoinase and allantoicase are closely linked in yeast but these two enzymes do not seem to be part of a multienzyme complex (53). Nor are the structural genes close enough to form an operon, and thus provide another example of two similarly regulated enzymes whose genes are separate from one another.

In *Aspergillus nidulans*, the pathway for catabolism of hypoxanthine and its control have been examined (54-60). Six enzymes are required to degrade hypoxanthine (or xanthine) to ammonia and CO₂ (54). The pathway is regulated in a very complex manner, with uric acid inducing both xanthine dehydrogenase and uricase, while both uric acid and
allantoin induce allantoinase, and allantoin induces allantoicase (55-56). All the inducible enzymes are repressed by high concentrations of ammonia. The last two enzymes of the pathway, ureidoglycollase and urease, appear to be synthesized constitutively. In addition to studying the intracellular enzymes responsible for catabolism, the transport systems for purines were investigated (57). Two separate uptake systems were shown: one which mediates the uptake of hypoxanthine, guanine and adenine, and the other transport system which is specific for xanthine and uric acid. By starting with an allantoinase-less mutant which was found to be very sensitive to purines, double mutants were isolated which had lost both their purine sensitivity and the ability to transport certain intermediates. One peculiar result reported with this system involved the utilization of xanthine (54-58). Xanthine induces both xanthine dehydrogenase and uricase in wild-type. The drug allopurinol prevents the utilization of hypoxanthine as a nitrogen source in Aspergillus by inhibiting xanthine dehydrogenase. It is most interesting that xanthine can still be used as a nitrogen source in the presence of allopurinol, even at a 100-fold greater concentration than needed to stop hypoxanthine utilization. This may indicate that there are two isoenzymes of xanthine dehydrogenase, one of which is relatively insensitive to allopurinol and able to oxidize xanthine. The structural genes for some of the purine catabolic enzymes have been located and were found to be scattered over several chromosomes in A. nidulans. A regulatory locus has been identified in Aspergillus which seems to specify a protein which acts in a positive fashion to stimulate the synthesis of a number of enzymes of nitrogen metabolism;
this positive regulatory protein seems to be inactive in the presence of ammonium (60). I will show that purine catabolism in *N. crassa* more closely resembles that in *A. nidulans* than in yeast, although many differences have also been found.
Methods and Materials

Organisms and growth conditions. Neurospora crassa wild-type strain 74-OR23-1A was used for growth studies, enzyme assays and mutant isolation. The mutant strains, with the exception of those which I isolated, were obtained from the Fungal Genetics Stock Center, California State University, Arcata, California. All stocks were routinely maintained on agar plates of Vogel's complete medium plus sucrose and any additional nutritional requirements. Conidial suspensions were made by removing both mycelia and conidia from plates inoculated seven days prior with a sterile platinum wire loop and placing this vegetative growth in a test tube with sterile water. This mixture was vigorously shaken and poured onto a small glass wool column through which only the conidia passed. For routine growth studies, Erlenmeyer flasks (125 ml) containing 20 ml of Vogel's medium (61) modified to contain no nitrogen, were supplemented with 1.5% sucrose and 10 mM concentration of various intermediates of the purine catabolic pathway. Since several purine intermediates and other organic nitrogen sources are destroyed by autoclaving, they were filter sterilized and then added aseptically to the flasks. These flasks were lightly inoculated with conidia (2000-3000 spores) and the cultures were allowed to grow at 30° for three days to stationary phase. The mycelial pads were then collected and dried to constant weight at 80°-90° overnight and data recorded as mg dry wt. per flask.
In the initial studies, larger quantities of mycelia for measurement of intracellular enzyme levels were cultured in 500 ml Erlenmeyer flasks containing 200 ml of modified Vogel's medium. These conditions were modified slightly for later experiments. It was found that growth on Vogel's medium containing a low concentration of NH$_4^+$ (as 2mM ammonium tartarate) produced the same uninduced level of the inducible enzymes, uricase and allantoicase, as did growth on only nitrate (5mM). Therefore, to induce the synthesis of these enzymes, hypoxanthine or uric acid (1mM) was added to the basal medium containing ammonium tartarate. Because all mutant strains can utilize NH$_4^+$ as a nitrogen source, the data from the various mutants were more easily interpretable. The flasks were inoculated with fresh conidia to an absorbance at 420 nm of 0.1 and allowed to shake (72 cycles/minute) on a New Brunswick reciprocating shaker at 25° for 20 to 24 hours. Some experiments required a change in the medium. These changes were accomplished by first allowing the mycelia to grow for a certain length of time and then carefully collecting the mycelial pad on a filter paper without allowing it to become dry. The pad was then placed in a flask of sterile water to dilute out any remaining traces of old medium and the mycelia were again collected as before. The pad was then resuspended in the appropriate new medium and the flask was returned to the shaker. At the termination of an experiment, the mycelia were collected on a filter, weighed, and ground with a pestle in a cold mortar using an equal weight of acid-washed sand and 3-5 fold volumes of 20 mM Tris-HCl buffer, pH 7.2. The homogenate was centrifuged at 15,000×g for 15 minutes at 4°. The clear supernatant solution was retained for enzyme assays.
**Enzyme assays:**

**Xanthine dehydrogenase** (xanthine-oxygen oxidoreductase EC1.2.3.2) This enzyme was assayed by a modification of the method of Yen and Glassman (62) and is based on the change in fluorescence when 2-amino-4-hydroxypteridine (AHP) is oxidized to isoxanthopterin. The reaction mixture contained 7 \( \mu \text{M} \) AHP, 20 \( \mu \text{M} \) methylene blue (MB) and 100 mM Tris-HCl, pH 8.0 (containing 1 mg bovine serum albumin per ml). These ingredients were placed in a glass fluorometer cuvette and then 50 \( \mu \text{l}-200 \mu \text{l} \) of enzyme extract was added to make the final volume 2.0 ml. The AHP is both insoluble and light sensitive; thus, adding a small volume of MB at a high concentration permitted preparation of a buffered AHP solution (in a light-free container) approaching the concentration desired. The cuvette was then placed in an Aminco-Bowman fluorospectrophotometer (model 4-8202) with the excitation wavelength set at 347 nm and the emission wavelength set at 405 nm. The sensitivity of the fluorometer was adjusted so that a solution of 1.6 \( \mu \text{M} \) quinine in 0.1 M \( \text{H}_2\text{SO}_4 \) read 100 on the transmission scale. The oxidation of AHP, as reflected in the increase in transmission, was followed automatically with a Sargent-Welch recorder (model XKR). One unit of xanthine dehydrogenase is defined as that amount of enzyme which converts 1 picomole of AHP to isoxanthopterin per minute. A change of 1 unit on the fluorometer scale corresponds to approximately 2 xanthine dehydrogenase units.

**Uricase** (urate: \( \text{O}_2 \) oxidoreductase, EC 1.7.3.3) This enzyme was assayed by the method of Greene and Mitchell (63) in which the decrease in absorbance, due to the loss of uric acid, was followed at 290 nm. Tris-HCl buffer (2.25 ml, 100 mM, pH 8.6 containing 200 \( \mu \text{g} \) uric acid/ml) was
pipetted into a 50 ml Erlenmeyer flask and the flask was placed in a New Brunswick Gyrotory Waterbath Shaker (model G76) at 30° ± 1°. At zero time, 0.25 ml of the enzyme extract was added to the flask and the mixture was allowed to shake vigorously for a predetermined amount of time—usually 10 to 15 minutes. The reaction was stopped by the transfer of 0.5 ml aliquots with an Eppendorf 500 µl pipette to 13 mm test tubes containing 4.5 ml of 12% HCl. The tubes were immediately shaken and the change in absorbance recorded. The control tube, stopped at zero time, contained 4.5 ml 12% HCl, 0.45 ml buffered substrate and 0.05 ml enzyme extract. The extinction coefficient for uric acid at 290 nm is 1.22 x 10^4 liters/mole (cm^2).

Allantoinase (allantoin amidohydrolase, EC 3.5.2.5). This assay was a modification of the one presented by Van de Poll, Verwey and Koningsberger (64). This assay measures the amount of allantoate produced by converting it to glyoxylate by acid hydrolysis. The amount of glyoxylate was determined colorimetrically as described by Vogel and Van der Drift (65) who modified the Rimine-Schryver reaction (66). Each assay contained 7.0 µmoles of allantoin and 120.0 µmoles Tris-HCl, pH 7.2 in a total volume of 1.2 ml; to this 10-100 µliters of enzyme extract was added and thoroughly mixed. The mixture was then incubated at 30° for 10 minutes. The reaction was halted by addition of 0.1 ml HCl (11%) and 7.0 µmoles phenyl hydrazine-HCl. The contents were then mixed and placed in a boiling water bath for exactly 2 minutes and then rapidly cooled to 0°. Finally, 1.0 ml HCl (24%) and 15 µmoles K_2Fe(CN)_6 were added and mixed and the samples were allowed to stand at room temperature for 15 minutes before being read at 535 nm. The molar extinction coefficient
obtained by Vogel and Van der Drift (65) of $4.93 \times 10^4$ liters/mole·(cm²) was used in all calculations. The phenyl hydrazine-HCl and K₃Fe(CN)₆ had to be made fresh daily.

**Allantoicase** (allantoate amidinohydrolase EC 3.5.3.4). A convenient assay was devised in which the product, ureidoglycolate, was measured directly. To a test tube containing 5.7 μmoles of potassium allantoate and 100 μmoles Tris HCl pH 7.1 was added 50 to 200 μliters of enzyme extract in a total reaction volume of 1.0 ml. The tube containing the reaction mixture was incubated in a water bath at $30^\circ + 1^\circ$ for 10 minutes. The reaction was then halted with the addition of 0.5 ml of 0.5 N NaOH. The concentration of ureidoglycolate was determined according to the technique of Vogel and Van der Drift (65). A tube containing all reactants was stopped immediately with NaOH and the small amount of product observed was used as a correction value.

**Ureidoglycolase** (hydrolase). This assay was a modification of the one described by Scazzocchio and Darlington (55). One product, glyoxylate, could not be determined directly because the substrate contained some glyoxylate such that high background values were obtained. The other product, urea, was hydrolyzed by exogenously provided urease to yield ammonia which was then determined colorimetrically. The assay contained 10 μmoles potassium ureidoglycolate, 100 μmoles phosphate buffer, pH 7.2, 1 unit Sigma Chemical Company Type VII urease and 10 to 100 μliters enzyme extract in a total volume of 1.0 ml. The mixture was incubated at $30^\circ$ for 5 minutes when the reaction was stopped by addition of 0.5 ml 20% phenol in absolute ethanol. To the mixture was added 1.0 ml of the potassium hypochlorite solution as described by Muftic (67). The
absorbance of the samples was measured at 655 nm after 30 minutes.

**Urease** (urea aminohydrolase, EC 3.5.1.5). The assay mixture contained 10.0 μmoles urea and 0.1 ml extract in 0.1 M phosphate buffer at pH 7.2 in a total volume of 1.0 ml. After incubation at 30° for 5 minutes, the amount of ammonia produced was measured as described above.

**Protein Determination.** The amount of protein in a cell-free extract was ascertained by the method of Lowry et al (68) with serum albumin as the standard.

**Units.** One milliunit of activity was defined as the production of one nanomole of product/minute at 30° in the case of all of these enzymes. Specific activity is milliunits of activity divided by mg. protein.

**Mutant Selection.** The filtration enrichment technique of Catcheside (69) was used exclusively. Conidia from wild-type strain 74-0R23-1A were suspended in sterile water and filtered through glass wool to remove mycelial fragments. A volume of 5 ml of the conidial suspension, diluted to an absorbance of 1.0 at 420 nm, was placed in a petri plate and subjected to ultraviolet radiation to approximately 90% kill. After an hour in the dark, the suspension was placed in an 18x150 mm test tube with 5 ml of modified Vogel’s medium containing 2.0% sucrose and one of the purine intermediates (5mM) as the sole nitrogen source. In most mutant hunts, uric acid was the intermediate employed, but hypoxanthine had to be used to obtain hypoxanthine non-utilizers. The test tube was slowly aerated using filtered air through a 9-inch Pasteur pipette secured with cotton at the top of the tube. The suspension was filtered every 8 hours for the first day and then every 12 hours for the next 3 days. Any remaining ungerminated spores were then sedimented by centrifuging at 5,000 x g for 10 minutes, resuspended in sterile water and
spread over petri plates containing normal Vogel's medium supplemented with 1.5% sorbose and 0.1% sucrose. Any colony visible after 3 days' growth at 30° was isolated and a portion put into two 13x100 mm tubes, one containing 3.0 ml of Vogel's complete medium + 2.0% sucrose, and the other, modified Vogel's medium, 2.0% sucrose and uric acid (5mM). Colonies showing only slight or no growth in the uric acid containing medium were then further tested for growth on each of the purine intermediates as the sole nitrogen source, followed by an analysis for the activity of each of the purine catabolic enzymes.

Mapping Analysis. Those mutants exhibiting no growth on one or more purine intermediates and found to lack a corresponding enzymatic activity were crossed to wild-type. This was done to determine whether the lesion was due to a single or multiple mutation(s). Test tubes (18mm) containing slants of Bacto corn meal agar were inoculated with wild-type conidia and allowed to grow 2 to 3 days prior to addition of the mutant strains as the paternal parent. The crosses were maintained at 25° C and after 2 to 3 days, black perithecia formed. Approximately 10 days after fertilization, asci began to mature and to discharge ascospores. These ascospores lodged on the walls of the tube opposite the agar and after 21 to 24 days, the black ascospores had matured sufficiently to perform a random spore analysis. To do this, the black ascospores were harvested with a wet cotton swab and transferred to a test tube containing sterile water. The dormant ascospores were activated by "heat shock," whereby the tube was placed in an electric heating block (Bel-Art, Exacta-Heat, model 218) at 60° for 30 to 45 minutes. The spores, approximately 100 to 200, were then spread onto sorbose-agar
minimal plates (plus any nutritional requirements) and allowed to germinate for 1 to 2 days. Colonies containing two or more spores were identified with the aid of a microscope and destroyed. Colonies arising from a single ascospore were sampled twice with a sterile Pasteur pipette, with one "plug" placed in a 13 mm test tube containing 2.5 ml of Vogel's complete medium plus 2.0% sucrose, and the other plug in a second tube containing 2.5 ml of Vogel's medium minus nitrogen plus either 5 mM hypoxanthine or uric acid and 2.0% sucrose. The procedure was repeated for about 200 colonies for each cross. The inoculated tubes were put into a 30° constant temperature room for 3 to 5 days to allow growth of the colonies. At this time, the percentage mutant (non-growers) and normal phenotype was calculated. In most cases, there was approximately an equal number of each type, implying mutation of a single genetic locus.

Each mutant attributable to a point mutation was subsequently crossed with the triple reciprocal-translocation mutant strain, alcoy (FGSC no. 997). The alcoy strain is used for preliminary localization of mutated loci for six of the seven linkage groups. Alcoy was used as the protoperithecal parent as described by Perkins et al (70). The mature spores were heat-shocked and plated on sorbose minimal agar. The single spore colonies (at least 200 to 300 colonies) were sampled as described previously and allowed to germinate at 35° for two to three days. The cot progeny (those which have grown up at 34°) were sorted out and the remaining non-growers moved to 25° to allow growth of cot. After about six more days' growth, the markers albino, yellow and the unmapped mutation were scored. A table was constructed to tabulate the data (Table 7). If linkage (less than 39% recombination) of the mutant being
tested is found with one of the three translocations, this implies that the gene is located on one of two translocated chromosomes. The next step involves a cross of the new mutant with a double mutant strain which contains a single mutant gene on each of the two chromosomes involved in the prior translocation. After analysis of this cross, the chromosome containing the mutated locus can be assigned and the approximate map distance between the mutation of interest and the known mutation can be calculated. One can now proceed with fine structure analysis of the mutant because stocks of *N. crassa* with multiple mutations on each chromosome are available.

After any cross, the mating type of the progeny had to be determined before the next cross can be made because only opposite mating types (A and a) will cross. To ascertain the mating type of any colony, plates containing only Bacto corn meal agar were made and inoculated with either fluffy (fl) A or a and allowed to grow six days at 25°. The fluffy strain was used because it does not conidiate. A numbered grid was made on the back of each plate and a drop of each mutant conidial suspension was placed in equivalent grids of plates containing fl A and a. The plates were incubated at 25° for two weeks prior to determination of mating type.

**Heterocaryon Test for Complementation.** To determine if two similar mutants which map very close to each other (< 0.2 map units) are in fact allelic, their ability to complement in a heterocaryon can be tested. *N. crassa* has no vegetative diploid phase, but because the fungus is a coenocytic organism, it has more than one nucleus per cytoplasmic unit. Under normal conditions, all the nuclei (homocaryon) have the same
genetic background. The organism can be manipulated, however, such that two different types of nuclei exist in a common cytoplasm (heterocaryon). To construct a heterocaryon, the usual procedure is first to cross each of the two possible allelic mutants with mutants containing different known auxotrophic markers carried on linkage groups different from the possible allelic mutants. I wanted to determine if the nit-2 and I-7 mutants, both of which mapped on chromosome I, were non-complementary and thus possibly allelic. Nit-2 was crossed with his-6 (chromosome V) and I-7 with leu-2 (chromosome IV). Several double mutants of each (nit-2, his-6 and I-7, leu-2) were isolated and their mating type analyzed. There are several known heterocaryon-compatibility genes (7) whose products will interfere with the formation of a heterocaryon if different alleles are present at these loci. This is the reason for determining the mating type, because only strains with identical (a + a or A + A) mating types can form a heterocaryon.

The his-6 and leu-2 auxotrophic markers were included to be certain of the formation and growth of a heterocaryon when leucine and histidine are not present. Neither strain can grow alone on minimal medium, so any growth is due to formation of a heterocaryon. This is sometimes referred to as "forcing a heterocaryon." This test was performed under conditions which did not require activity of the genes of interest (I-7 and nit-2) by mixing a small number of conidia from each double mutant in the center of a petri plate containing only Vogel's minimal medium plus sucrose and agar. As the control, conidia from each strain were individually spread onto separate petri plates. If growth is found on the mixed conidial plate but none on the two control plates, a true
heterocaryon was made. Growth conditions were then altered (by using nitrate as the sole nitrogen source) such that both the auxotrophic markers and the two new mutant genes had to complement each other for growth of the heterocaryon to occur. The results of the heterocaryon analysis are given in the Results Section.

Chemicals. The substrates were purchased from Sigma. All other chemicals were of reagent grade. Both the ureidoglycolate (UG) supplied by Sigma and a sample which I synthesized were contaminated with free glyoxylate, preventing direct assay of ureidoglycolate hydrolase. UG was synthesized by the method of Valentine and Wolfe (72) in which urea is condensed with glyoxylate at pH 7.0 by the reduction of the aldehyde group to a hydroxyl group.
Results

Growth of Neurospora on various nitrogen sources. Wild-type Neurospora can grow on various purine catabolites as a nitrogen source almost as well as it does with ammonia (Fig. 3). Each of the compounds tested, except adenine, is as good a nitrogen source as ammonia when studied at a low concentration (below 2 mM). At higher concentrations, growth with the various intermediates was slower than on an equimolar concentration of ammonia. When these substances were tested at 5 mM and 10 mM, a definite pattern appeared in which the amount of growth achieved with each compound, with the exception of urea, was related to the number of steps required for its conversion into ammonia. Those intermediates of the degradative pathway closest to NH$_4^+$ clearly supported the best growth.

Several of the compounds employed are insoluble above 1.0 mM, and a precipitate forms in the bottom of the flask. In the case of uric acid, this precipitate disappeared with time, suggesting that as the mycelia transported the uric acid, its extracellular concentration was lowered, thus allowing more of the insoluble compound to dissolve.

The insert of Fig. 3 displays growth of wild-type on a fixed amount (200 μmoles) of allantoin with time. After three days, the culture reached about 66% of its final weight, which indicated that harvesting at that time provides a good measure of the growth rate of the fungus on any particular nitrogen source.
Figure 3. Growth of wild-type *N. crassa* on increasing concentrations of various purines or their intermediates as the sole nitrogen source. The mycelial pad was grown at 30° for three days, harvested, and dried overnight. The amount of growth with time on a constant amount of allantoin (10 μmoles/ml) is shown in the insert.
Growth tests with mutant strains. A number of mutants containing genetic blocks in various aspects of nitrogen metabolism or related pathways were tested for their ability to use four different compounds—ammonia, allantoin, arginine and urea—as nitrogen sources. Furthermore, the amount of growth achieved with allantoin was determined as a quantitative estimate of the ability of each mutant to utilize this particular compound as a nitrogen source. Table 1 shows that all but three of the mutants tested were able to use each of these compounds as the sole supply of nitrogen. The arginaseless mutant, arga, was specifically unable to utilize arginine for growth. This result implies that operation of the urea cycle is required to obtain ammonia from arginine and that transaminases cannot function in this role. The two urease-deficient mutants, ure-1 and ure-2, used ammonia as a nitrogen source but were incapable of utilizing allantoin, arginine or urea for this purpose. Davis (73) also noted similar results. Clearly, urease plays a key role in the metabolism of both purines and arginine.

Five different mutants which are not able to use nitrate were studied; all of these mutants grew normally on the nitrogen sources being tested except the nit-2 mutant. Mutant strains with two different nit-2 alleles both grew normally with urea or ammonia but at a rate much slower than normal when furnished with allantoin. A more extensive growth experiment was undertaken using each intermediate in the purine pathway with the five different nit mutants (Table 5). Nit-3, nit-4 and nit-5 grew normally on all of the nitrogen sources tested, whereas nit-2, in addition to not utilizing allantoin, was unable to catabolize any purine product except urea. An unexpected finding was that nit-1 could grow
TABLE 1

GROWTH OF SEVERAL N. crassa MUTANTS ON VARIOUS NITROGEN SOURCES

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Allele</th>
<th>Growth Requirements</th>
<th>Nitrogen Source</th>
<th>Dry wt. c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ammonia</td>
<td>Arginine</td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>---------------------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>Ad-1</td>
<td>3254</td>
<td>adenine</td>
<td>(+)</td>
<td>(+)</td>
</tr>
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<td>Aga</td>
<td>UM096</td>
<td>orithine</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Am</td>
<td>32213</td>
<td>glutamate+ alanine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nit-1</td>
<td>34547</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nit-2</td>
<td>K31</td>
<td></td>
<td>+</td>
<td>+</td>
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<td>Nit-2</td>
<td>nr31</td>
<td></td>
<td>+</td>
<td>+</td>
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<td>Nit-3</td>
<td>14789</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
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<td>Nit-4</td>
<td>2</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nit-5</td>
<td>nr15</td>
<td></td>
<td>+</td>
<td>+</td>
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<td>Ure-1</td>
<td>9</td>
<td></td>
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<td>-</td>
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<td>Ure-2</td>
<td>47</td>
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<td>-</td>
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<td>Acu-1,</td>
<td>J148,</td>
<td>inositol</td>
<td>(+)</td>
<td>+</td>
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<tr>
<td>inos</td>
<td>R233</td>
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<td>Acu-5</td>
<td>J118</td>
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<td>+</td>
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<td>Acu-6</td>
<td>J131</td>
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<td>+</td>
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<td>Acu-7</td>
<td>J136</td>
<td></td>
<td>+</td>
<td>+</td>
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</table>

*Each strain was inoculated into test tubes containing the various nitrogen sources and allowed to grow, standing, for 3 days at 30°. At this time it was determined whether or not growth had occurred. ++ = growth; (+) = slow growth; -- = no growth.

bSupplements were added to a final concentration of 0.1 mM.

cAmount of growth in mg, with allantoin as sole nitrogen source.
with allantoin and uric acid, yet not with hypoxanthine. As will be discussed later, nit-2 appears to be a regulatory mutant displaying a multiple loss of several enzymes related to nitrogen metabolism and nit-1 appears to be a mutant lacking both nitrate reductase and xanthine dehydrogenase.

The catabolism of allantoin results in the formation of a molecule of glyoxylate and two molecules of urea. It was thought that mutants which were incapable of further metabolism of glyoxylate might accumulate a high level of this compound and display growth inhibition or perhaps be incapable of using purines as a nitrogen source because of product inhibition of ureidoglycolate hydrolase. However, as is evident in Table 1, all of the five different acu mutants tested, which are blocked in some step of acetate utilization, grew quite well on allantoin. These results would seem to exclude the possibilities that glyoxylate either serves a regulatory function or accumulates as a toxic intermediate. It should be noted that the extracellular growth medium was not assayed for the presence of free glyoxylate.

Metabolic regulation of catabolic enzyme levels. Wild-type mycelia were grown with shaking for 20-24 hours on the same compounds as used in the growth study. Under these conditions, the organism grew well on all the compounds used except xanthine and hypoxanthine. Table 2 describes the activity of the five enzymes required for the metabolism of uric acid to ammonia. The levels of the first three enzymes show significant variations, while the last two enzymes appear to be constitutive regardless of the nitrogen source utilized. Urease is a key enzyme because it is required to hydrolyze urea, the end product of several different pathways;
<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Urease</th>
<th>Allantoinase</th>
<th>Allantoinase</th>
<th>Ureidoglycolate Hydrolase</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium</td>
<td>8.9</td>
<td>0.26</td>
<td>10.9</td>
<td>17.3</td>
<td>38.3</td>
</tr>
<tr>
<td>Tartarate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ammonia + Uric Acid</td>
<td>20.0</td>
<td>0.8</td>
<td>13.4</td>
<td>22.5</td>
<td>41.0</td>
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<td>1.9</td>
<td>14.7</td>
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<td>Hypoxanthine</td>
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<td>14.0</td>
<td>8.7</td>
<td>66.9</td>
<td>43.7</td>
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<tr>
<td>Xanthine</td>
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<td>7.1</td>
<td>14.3</td>
<td>29.1</td>
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<tr>
<td>Uric Acid</td>
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<td>3.4</td>
<td>19.3</td>
<td>33.0</td>
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<td>Allantoin</td>
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<td>8.9</td>
<td>7.6</td>
<td>59.3</td>
<td>37.8</td>
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<td>Allantoic Acid</td>
<td>7.0</td>
<td>3.3</td>
<td>6.8</td>
<td>38.8</td>
<td>64.7</td>
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<tr>
<td>Ureidoglycolate</td>
<td>15.6</td>
<td>1.2</td>
<td>5.8</td>
<td>65.0</td>
<td>71.0</td>
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<tr>
<td>Urea</td>
<td>16.0</td>
<td>1.5</td>
<td>3.9</td>
<td>34.9</td>
<td>77.0</td>
</tr>
</tbody>
</table>

*Specific activity is reported as milliunits/mg. protein. The amount of nitrate utilized was 5 umoles/ml, which permits good growth and low enzyme activities. Ammonia concentration was 25 umoles/ml. N.T. = not tested. Details of the experiment are given in the Methods section.*
hence, its constitutive presence is not surprising. Since a high intracellular concentration of urea might be toxic, urease may also serve a protective function and thus display a continuous presence.

The first enzyme required for the catabolism of uric acid, uricase, appears to be controlled by both induction and repression (Table 2). The amount of uricase was maximal with hypoxanthine as the nitrogen source while xanthine and uric acid induced uricase synthesis somewhat less. Growth on allantoin or allantoic acid did not result in a clear increase in uricase activity. Ammonia caused repression of uricase synthesis, and the extent of repression increased with the extracellular ammonia concentration.

Allantoicase, the third enzyme of the pathway, seemed to follow the same general pattern as uricase. Ammonia resulted in repression, while uric acid appeared to induce allantoicase maximally. This differs from the control of allantoicase in *Aspergillus nidulans*, as was found by Scazzocchio and Darlington (55), in which case the enzyme was induced specifically by allantoin.

The level of allantoinase also varied widely, depending upon the nitrogen source available, which indicates that the activity of this enzyme is subject to metabolic regulation. However, it seems to be controlled somewhat differently than uricase and allantoicase in that maximal synthesis was found when either allantoin or uric acid was present in the external medium.

These results, showing significant changes in the levels of uricase, allantoicase and allantoinase, depending upon the nitrogen source being utilized, can be interpreted in at least two ways. The enzymes may
simply be subject to repression by ammonia, and those nitrogen sources which are only slowly metabolized to give NH$_4^+$ would then appear to cause induction. Alternatively, one or more substrates or intermediates of the pathway could actively induce the synthesis of the enzymes. Finally, induction and repression both may occur, and in this case, a compound might serve as an inducer but also be converted to NH$_4^+$, the likely corepressor. In the last instance, a rather complicated response in enzyme levels could result during growth of the wild-type strain. One particular purine catabolite might be the true inducer, so its presence would induce the synthesis of those regulated enzymes. At the same time, the catabolite could be degraded to form an intracellular pool of ammonia which in turn might repress the synthesis of these same enzymes. Thus, the observed activity of such a regulated enzyme in wild-type might lie somewhere between that of the fully induced and completely repressed level.

Derepression of allantoinase. The alternatives of regulation for the initial three controlled enzymes—derepression, induction or both—were more closely examined. Mycelia were first grown on ammonium tartrate medium for 20 hours, then washed and transferred to media either lacking all nitrogen or containing ammonium tartrate and incubated for an additional five hours. Under these conditions, end product repression could occur, but no purines or their catabolic intermediates which might serve as inducers were present.

The results of Table 3 show that the activity of four of the five catabolic enzymes did not change significantly in this experiment. Allantoinase, on the other hand, increased over eight-fold during the
### TABLE 3

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Growth Condition</th>
<th>Specific Activity</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uricase</td>
<td>Control</td>
<td>18.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No Nitrogen</td>
<td>22.6</td>
<td>20.</td>
</tr>
<tr>
<td>Allantoinase</td>
<td>Control</td>
<td>1.56</td>
<td>840.</td>
</tr>
<tr>
<td></td>
<td>No Nitrogen</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>Allantoinase</td>
<td>Control</td>
<td>5.57</td>
<td>21.</td>
</tr>
<tr>
<td></td>
<td>No Nitrogen</td>
<td>6.73</td>
<td></td>
</tr>
<tr>
<td>Ureidoo-lycolate</td>
<td>Control</td>
<td>9.7</td>
<td>8.</td>
</tr>
<tr>
<td>Urease</td>
<td>Control</td>
<td>38.9</td>
<td>28.</td>
</tr>
<tr>
<td></td>
<td>No Nitrogen</td>
<td>50.</td>
<td></td>
</tr>
</tbody>
</table>

*Mycelia were grown for 20 hours in Vogel's min. media as a shaking culture and then each pad was washed and resuspended into the same media or in Vogel's media lacking any nitrogen compounds. These cultures were allowed to shake for an additional 4 hours.*
same time period. This result suggested that the clear increase in allantoinase activity was due solely to derepression. To study this phenomenon more carefully, a series of mycelial pads were initially grown with nitrate as the nitrogen source and then transferred after 20 hours to media containing either allantoin, hypoxanthine or no nitrogen. The results displayed in Figure 4A show that in the absence of any nitrogen source, the activity of allantoinase rapidly increased for two hours, then started a slow decline. In the presence of hypoxanthine, the amount of allantoinase eventually increased to the same level found without nitrogen, but the increase occurred at a rate about 50% slower. With allantoin, the rate of enzyme increase was even slower and the final activity of allantoinase did not reach that found in the other two cultures.

Since the level of allantoinase increased when wild-type cells were transferred to medium lacking a nitrogen source and this increase was actually less when hypoxanthine or allantoin, potential inducers, were added, it seemed that the control of this enzyme might be mediated solely by repression. However, the possibility still existed that allantoinase was subject to both repression and induction and that potential inducers could also be metabolized to form the co-repressor. This possible interpretation could not be critically tested with the wild-type strain since metabolism of purines or their intermediates could quickly result in an intracellular pool of ammonia. To eliminate this problem, a urease-deficient mutant which cannot form ammonia, the presumed co-repressor, from intermediates of the purine catabolic pathway was used.
Figure 4. Development of allantoinase activity with time using various nitrogen sources after 20 hours of growth on 5 mM nitrate. In both cases, after 20 hours of growth, the mycelium was filtered, washed and transferred to the different nitrogen sources. In A, the increase in allantoinase activity from wild-type is shown. In B, enzyme activity with the urease mutant is shown.
SPECIFIC ACTIVITY OF ALLANTOINASE

WILD-TYPE

NO NITROGEN

HYPOXANTHINE

ALLANTOIN

NITRATE

AMMONIA

HOURS AFTER TRANSFER

SPECIFIC ACTIVITY OF ALLANTOINASE

ure-2

ALLANTOIN

URIC ACID

NO NITROGEN

NITRATE

HOURS AFTER TRANSFER
Unlike wild-type, maximum allantoinase activity was achieved with the \textit{ure-2} mutant in the presence of added uric acid or allantoin while cultures lacking a nitrogen source showed about 30% less of this enzyme. (Figure 4B) It is also noteworthy that the levels of the enzyme obtained by \textit{ure-2} are several times higher than found with wild-type under similar conditions. Thus, it appears that allantoinase is both an inducible and repressible enzyme and that in the case of wild-type, the potential inducers are metabolized to form the corepressor so that the fully induced enzyme level cannot be achieved. An apparent contradiction to this interpretation is that a considerable increase in the enzyme amount was found even in nitrogen-free medium which clearly contains no added inducer. However, internal induction of allantoinase synthesis may occur. This possibility is supported by the recent report by Mattoo, Joshi and Shah (74) that \textit{Neurospora} accumulates large intracellular pools of guanine and adenine when growing on a minimal medium. The metabolism of these endogenous purines would be expected to give rise to the actual inducers, uric acid or allantoin.

\textbf{Catabolic enzyme levels in the ureaseless mutant.} The \textit{ure-2} mutant strain was grown with a relatively low concentration (5 mM) of sodium nitrate as the nitrogen source, since it has previously been found that the regulated enzymes would be neither fully induced nor repressed under these conditions. The addition of a purine metabolite to a \textit{ure-2} culture growing on a nitrate-containing medium should reveal whether it was an inducer since it could not be metabolized to ammonia. The enzyme levels observed with \textit{ure-2} growing on nitrate medium with and without possible inducers or ammonia, the co-repressor, are presented in Table 4. The
<table>
<thead>
<tr>
<th>Nitrogen Source</th>
<th>Enzyme</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uricase</td>
<td>Allantoínase</td>
<td>Allantoínsase</td>
</tr>
<tr>
<td>Ammonia</td>
<td>14.9</td>
<td>1.5</td>
<td>10.4</td>
</tr>
<tr>
<td>Ammonia + Nitrate</td>
<td>21.0</td>
<td>1.8</td>
<td>10.6</td>
</tr>
<tr>
<td>Nitrate</td>
<td>9.9</td>
<td>6.9</td>
<td>N.T.</td>
</tr>
<tr>
<td>Allantoin + Nitrate</td>
<td>8.6</td>
<td>40.2</td>
<td>13.5</td>
</tr>
<tr>
<td>Allantoin + Nitrate + Ammonia</td>
<td>12.5</td>
<td>3.5</td>
<td>19.3</td>
</tr>
<tr>
<td>Uric Acid + Nitrate</td>
<td>160.6</td>
<td>34.6</td>
<td>68.7</td>
</tr>
<tr>
<td>Uric Acid + Nitrate + Ammonia</td>
<td>51.8</td>
<td>5.8</td>
<td>21.2</td>
</tr>
</tbody>
</table>

*aThe amount of ammonia employed was 25 μmoles/ml, nitrate was 5 μmoles/ml, and the other two compounds, 10 μmoles/ml.*
enzyme activities of uricase, allantoinase and allantoicase in the wild-type and ure-2 mutant strains were comparable when grown with nitrate plus ammonia, a state of repression and non-induction. The addition of uric acid to the nitrate medium increased the amounts of all three enzymes in ure-2 to levels unattainable in wild-type, while allantoic acid resulted in increased levels of allantoinase alone.

The relatively high levels of these enzymes which occur in ure-2 only upon induction can be largely reversed by repression as demonstrated by the addition of uric acid plus ammonia to the basal medium containing nitrate (Table 4). Under these conditions of simultaneous induction and repression, allantoinase is present only at the fully repressed level while the level of uricase is still about five times higher than in the uninduced situation. These results with ure-2 strongly suggest that the synthesis of these three enzymes is controlled by both induction and ammonium-mediated repression.

Of the five enzymes necessary to degrade uric acid to ammonia, the last two—ureidoglycolase and urease—are constitutively synthesized and the first three enzymes—uricase, allantoinase and allantoicase—are regulated in a complex manner. Synthesis of these three enzymes appears to be repressed by the end product, ammonia. In addition, these same enzymes which were found to be induced by uric acid (Table 4) were also induced by hypoxanthine and xanthine. No reported mutants existed for enzymes of the purine pathway except in the case of urease. It seemed imperative to obtain mutants with genetic blocks which prevented conversion of one purine intermediate to another so that the identity of the true inducer of the pathway could be ascertained.
Isolation and Identification of Mutants. A number of mutants were isolated, as described in Methods, which could not use purines as a sole nitrogen source. After an initial screening for no growth on uric acid or hypoxanthine and strong growth on ammonium nitrate, conidia from each mutant were tested for growth on each of the intermediates in the purine catabolic pathway. The results are shown in Table 5. Four different classes of mutants were found; the first class (xdh) could not grow on hypoxanthine but grew well on any intermediate in the pathway beyond hypoxanthine. The second class (aln) could not utilize allantoin nor any intermediate prior to it, the third class (alc) could not catabolize allantoic acid and the fourth class (amr) grew very poorly on all the intermediates but grew normally on the end product, ammonia. All mutants were backcrossed to wild-type at least once, and all displayed an approximate 1:1 segregation (mutant to normal phenotype), indicating that each isolated mutant was the result of a single gene mutation. One mutant from each class was then further examined after growth on inducing media for the initial three inducible enzymes and the last constitutive enzyme in the pathway, urease. The results are shown in Table 6. The first class, xdh, possessed all of the assayed enzymes, although with hypoxanthine as the inducer, the levels of uricase and allantoicase found were those of the uninduced state. This result will be amplified in a later section on the nature of the inducer. The second class, aln, cannot grow on allantoin and was found to possess no allantoinase activity, but possessed induced levels of both uricase and allantoicase. The third class, alc, was found to lack the enzyme allantoicase. The last class, amr, grew very slowly on all of the purine intermediates,
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Allele</th>
<th>Nitrogen source&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>xdh-1 A</td>
<td>NMR 11</td>
<td>-</td>
</tr>
<tr>
<td>xdh-1 A</td>
<td>NMR 27</td>
<td>-</td>
</tr>
<tr>
<td>nit-1 A</td>
<td>34547</td>
<td>-</td>
</tr>
<tr>
<td>aln-1 A</td>
<td>U 3</td>
<td>-</td>
</tr>
<tr>
<td>alc-1 A</td>
<td>WRR 1</td>
<td>-</td>
</tr>
<tr>
<td>alc-1 A</td>
<td>WRR 5</td>
<td>-</td>
</tr>
<tr>
<td>amr A</td>
<td>I 7</td>
<td>-</td>
</tr>
<tr>
<td>nit-2 A</td>
<td>nr 37</td>
<td>-</td>
</tr>
<tr>
<td>ure-1 A</td>
<td>9-A</td>
<td>-</td>
</tr>
<tr>
<td>wild-type A</td>
<td>74 A</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sole nitrogen source added to modified Vogel's medium at 10 mM final concentration. Each allele represents an independently isolated mutant. 
+ = strong growth; (+) = poor growth; - = no growth
## TABLE 6

ENZYME ACTIVITIES OF MUTANTS FOLLOWING GROWTH ON DIFFERENT PURINE INTERMEDIATES

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Purine Intermediate</th>
<th>Uricase</th>
<th>Allantoinase</th>
<th>Allantoicase</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>xdh-1</td>
<td>hypoxanthine</td>
<td>30.</td>
<td>28.1</td>
<td>21.9</td>
<td>45.</td>
</tr>
<tr>
<td>aln-1</td>
<td>hypoxanthine</td>
<td>200.</td>
<td>0.0</td>
<td>40.8</td>
<td>40.</td>
</tr>
<tr>
<td>alc-1</td>
<td>hypoxanthine</td>
<td>150.</td>
<td>5.9</td>
<td>0.0</td>
<td>39.</td>
</tr>
<tr>
<td>amr</td>
<td>hypoxanthine</td>
<td>20.</td>
<td>1.56</td>
<td>8.2</td>
<td>42.</td>
</tr>
<tr>
<td>amr</td>
<td>uric acid</td>
<td>32.7</td>
<td>3.19</td>
<td>16.6</td>
<td>45.</td>
</tr>
<tr>
<td>ure-2</td>
<td>hypoxanthine</td>
<td>302</td>
<td>13.0</td>
<td>28.7</td>
<td>0.0</td>
</tr>
<tr>
<td>wild-type</td>
<td>hypoxanthine</td>
<td>160</td>
<td>6.4</td>
<td>60.</td>
<td>43.</td>
</tr>
</tbody>
</table>

\[ ^a \] Mycelia were grown for 20 hours in modified Vogel's medium containing 1.5 mM ammonium tartarate plus the indicated purine intermediate at a final concentration of 1 mM.

\[ ^b \] Nanomoles of product produced per minute per milligram of protein.
yet possessed measurable activity for all of the enzymes tested. However, the levels of the three inducible enzymes in amr (in the presence or absence of an inducer) were only those of the uninduced state. The amr mutant was found to have other special properties which will be described later.

**Mapping Data and Complementation Work.** A representative mutant from each of the four classes was subjected to genetic analysis. As stated previously, each mutant was first backcrossed to wild-type, and in each case, a roughly equal number of mutant and normal progeny was found. Several reisolates of the xdh-1 mutant (from cross with wild-type) showing strong growth in ammonia were analyzed for their mating type (A or a) as described in the Methods section. An xdh-1 A isolate was then crossed with alcoy a. The results are shown in Table 7. The xdh-1 gene revealed a low amount of recombination with the translocated albino marker, implying that the xdh-1 mutation was either on linkage group I or II. The xdh-1 mutant was then crossed to the appropriate "follow-up" strain which carries the markers aur (I) and pe (II) as described by Perkins et al (70). The results indicated that the xdh-1 locus is on linkage group II about 15 map units from pe. The same general procedure was followed for mapping aln-1 as shown in Table 8. The results of the alcoy cross suggested that aln-1 might be located on either linkage group III or VI. The follow-up cross with tryp (III), ylo (VI) indicated that aln-1 was not located in either of those linkage groups. Alcoy can only demonstrate linkage with six of the seven chromosomes since chromosome 7 is unmarked; thus aln-1 was crossed to a strain which had two markers on linkage group VII. The data showed aln-1 mapped to this chromosome.
### TABLE 7

**MAPPING DATA OF xdh-1**

Cross to wild-type: 94 colonies wild-type; 120 colonies mutant

44% wild-type vs 56% mutant

Conclusion: The xdh-1 mutation can be attributed to a single mutated locus.

Cross to alcov:

<table>
<thead>
<tr>
<th></th>
<th>+ cot -</th>
<th>+ al -</th>
<th>+ ylo -</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped +</td>
<td>18 97 10 114 3 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mutant -</td>
<td>22 80 75 17 31 54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

% recombination 45.2 13 50

Conclusion: Possible linkage to al, Group I or II.

Cross to aur(I), pe(II):

<table>
<thead>
<tr>
<th></th>
<th>+ aur -</th>
<th>+ pe -</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped +</td>
<td>19 14 9 13</td>
<td></td>
</tr>
<tr>
<td>mutant -</td>
<td>63 34 66 5</td>
<td></td>
</tr>
</tbody>
</table>

% recombination 41 15

Conclusion: Xdh-1 mutation is located on linkage Group II, 15 map units from pe.
TABLE 8

MAPPING DATA OF aln-1

Cross to wild-type: 90 colonies wild-type; 54 colonies mutant
62% wild-type vs 38% mutant

Conclusion: The aln-1 mutation can be attributed to a single mutated locus

Cross to alcov:

<table>
<thead>
<tr>
<th></th>
<th>cot</th>
<th>-</th>
<th>al</th>
<th>-</th>
<th>ylo</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped</td>
<td>34</td>
<td>67</td>
<td>42</td>
<td>60</td>
<td>23</td>
</tr>
<tr>
<td>mutant</td>
<td>34</td>
<td>73</td>
<td>46</td>
<td>60</td>
<td>37</td>
</tr>
<tr>
<td>% recombination</td>
<td>50</td>
<td>50</td>
<td>35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conclusion: Possible linkage to ylo, Group III or VI.

Cross to tryp(III), ylo(VI):

<table>
<thead>
<tr>
<th></th>
<th>tryp</th>
<th>-</th>
<th>ylo</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped</td>
<td>25</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>mutant</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>% recombination</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Conclusion: No linkage to either Group III or VI.

Cross to nic-3(VII), met-7(VII):

<table>
<thead>
<tr>
<th></th>
<th>nic-3</th>
<th>-</th>
<th>met-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped</td>
<td>7</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>mutant</td>
<td>35</td>
<td>5</td>
<td>35</td>
</tr>
<tr>
<td>% recombination</td>
<td>25</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

Conclusion: Aln-1 mutation is located on linkage Group VII, between nic-3 and met-7.
and since it was a three-point cross, the exact location of aln-1 with respect to nic-3 and met-7 was found. The order on the chromosome is nic-3, aln-1 and met-7, with aln-1 being 24 map units from nic-3 and 14 map units from met-7.

The alc-1 mutation, after crossing to alcoy, was located on either linkage group I or II (Table 9). The follow-up cross, again with aur (I) pe (II), showed that the alc-1 locus was on group II, 10.4 map units from pe. Since both xdh-1 and alc-1 are located on chromosome II and about the same distance away from pe, it was possible that they were clustered or at least tightly linked. However, a cross between xdh-1 and alc-1 showed 27% of recombination which demonstrates that the two loci are on opposite sides of the pe locus. The genetic localization of the amr mutant will be described later.

Nature of the Inducer. The work described in a previous section suggested that the inducer of uricase and allantoicase was uric acid or possibly some intermediate prior to uric acid. Hypoxanthine, xanthine and uric acid induced these two enzymes while allantoin was unable to induce either one. Since these earlier experiments were carried out with the wild-type strain, a major complication in interpreting the results was the possibility that a potential inducer might itself be inactive, but could be converted to a "genuine" inducer via metabolism. Indeed, by using two different mutant strains, xdh-1 and nit-1, I have demonstrated that hypoxanthine is not an inducer, although it can give rise to an inducer of uricase and allantoicase. The nit-1 mutant cannot utilize nitrate as a nitrogen source, although it can use nitrate. This mutant also fails to grow with hypoxanthine as a nitrogen source, as
TABLE 9

MAPPING DATA OF alc-1

Cross to wild-type: 63 colonies wild-type; 80 colonies mutant

44% wild-type vs 56% mutant

Conclusion: The alc-1 mutation can be attributed to a single mutated locus.

Cross to alcoy:

<table>
<thead>
<tr>
<th></th>
<th>cot</th>
<th>-</th>
<th>al</th>
<th>-</th>
<th>ylo</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped</td>
<td>+</td>
<td>20</td>
<td>45</td>
<td>0</td>
<td>56</td>
<td>0</td>
</tr>
<tr>
<td>mutant</td>
<td>-</td>
<td>31</td>
<td>53</td>
<td>80</td>
<td>1</td>
<td>41</td>
</tr>
<tr>
<td>% recombination</td>
<td>49</td>
<td>0.7</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conclusion: Possible linkage to al, Group I or II.

Cross to aur(I), pe(II):

<table>
<thead>
<tr>
<th></th>
<th>aur</th>
<th>-</th>
<th>pe</th>
</tr>
</thead>
<tbody>
<tr>
<td>unmapped</td>
<td>+</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>mutant</td>
<td>-</td>
<td>47</td>
<td>28</td>
</tr>
<tr>
<td>% recombination</td>
<td>44</td>
<td>10.4</td>
<td></td>
</tr>
</tbody>
</table>

Conclusion: Alc-1 mutation is located on linkage Group II, 10.4 map units from pe.
shown in Table 5. The nit-1 mutant is thought to be defective in the production of a molybdenum cofactor which is an essential component of nitrate reductase (75-76). It was recently found that nit-1 lacks functional xanthine dehydrogenase and that the same molybdenum cofactor must be required for the activity of this enzyme (77). A second mutant, xdh-1, isolated during the course of this work, similarly fails to grow on hypoxanthine and lacks xanthine dehydrogenase activity. The nit-1 and xdh-1 mutants are clearly distinct because (1) they are not allelic and (2) unlike nit-1, the xdh-1 strain grows normally on nitrate. It is probable that the xdh-1 locus is the structural gene for xanthine dehydrogenase.

The intracellular levels of the three inducible enzymes were studied in wild-type and these two mutant strains after their growth in the presence or absence of various potential inducers. The results are shown in Table 10. In the case of wild-type grown in the absence of an inducer, only a low level (uninduced state) of uricase and allantoicase was found. The level of these two enzymes in wild-type was greatly increased (induced state) when either hypoxanthine or uric acid was added to the growth medium. However, hypoxanthine failed to induce either uricase or allantoicase in either nit-1 or xdh-1. Uric acid was still effective in inducing these two enzymes. It is noteworthy that a large intracellular pool of hypoxanthine was observed by the method of Kalkar (78) in both nit-1 and xdh-1, but not wild-type, following their growth in medium containing this compound. This result implies that the metabolism—but not the transport of hypoxanthine—is defective in these mutants and strongly suggests that hypoxanthine can be catabolized
### Table 10
ENZYME ACTIVITIES IN MUTANTS FOLLOWING GROWTH ON VARIOUS PURINE INTERMEDIATES

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Purine Intermediate</th>
<th>Specific activities b</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uricase</td>
<td>Allantoinase</td>
<td>Allantoicase</td>
<td></td>
</tr>
<tr>
<td>xdh-1</td>
<td>none</td>
<td>15.0</td>
<td>1.0</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>xdh-1</td>
<td>hypoxanthine</td>
<td>15.7</td>
<td>1.14</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
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a. Mycelia were grown in modified Vorrel's medium 1.5 mM ammonium tartrate plus purine intermediates (when present) at 1.0 mM.

b. Nanomoles product produced per minute per milligram of protein.
to an inducer but is not itself capable of induction. This also implies that neither adenine nor guanine is the true inducer.

Two other experiments support this conclusion: one, using an inhibitor of xanthine dehydrogenase (XDH) and the second, a non-metabolized analogue of uric acid. The drug allopurinol has been shown to be a potent inhibitor of XDH in Aspergillus nidulans (59) and many other organisms, yet does not affect the transport of hypoxanthine. We have found that Neurospora cannot utilize hypoxanthine as a nitrogen source in the presence of extremely low concentrations of allopurinol, although this compound does not interfere with growth on ammonium or uric acid. Significantly, allopurinol also prevents the induction by hypoxanthine of uricase and allantoicase in wild-type, reinforcing our belief that hypoxanthine is not itself an inducer (Table 11). Several non-metabolizable purine analogs were tested in the hope that one could be found which would mimic the true inducer and yield induced enzyme levels without giving rise to an intracellular pool of the repressor, ammonia. Such appears to be the case with the uric acid analogue, 8-azaxanthine, which caused the greatest induction of the enzymes (Table 11). Several other analogues gave rise to higher levels of the enzymes than found without any added inducer, but only 8-azaxanthine induced higher levels than did uric acid.

A curious situation exists since hypoxanthine apparently is not an inducer, yet the data of Table 10 show that growth of wild-type on hypoxanthine caused higher levels of uricase and allantoicase than did the true inducer, uric acid. A similar result is shown in Fig. 5A, where hypoxanthine again appears to be a more effective inducer than
<table>
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<th>Specific activities b</th>
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<td></td>
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<tr>
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</tr>
<tr>
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<td>43.7</td>
</tr>
<tr>
<td>2 methylthiocurate^111</td>
<td>42.4</td>
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</tbody>
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---

a. Mycelia were grown in modified Vogel's medium containing 1.5 mM ammonium tartarate plus supplements whose final concentrations were (i) 1 µM; (ii) 1 mM; (iii) 0.5 mM.

b. Nanomoles of product produced per minute per milligram of protein.
uric acid. This apparent paradox will be discussed later in the Results section.

The results presented earlier, using the wild-type and the ure-2 mutant, indicated that allantoin and later intermediates were not inducers of uricase or allantoicase. By the use of the newly-isolated mutants, aln-1 and alc-1, which lack, respectively, allantoinase and allantoicase activity, this idea was confirmed. Both uricase and allantoicase are inducible by hypoxanthine (or uric acid) in aln-1, and uricase is similarly induced in alc-1 (Table 6). Allantoin, which cannot be metabolized in aln-1, failed to induce either of these enzymes in this strain. Similarly, allantoic acid did not induce uricase in the alc-1 mutant (data not given). Since these compounds will accumulate in the respective mutants blocked for their metabolism, it is clear that neither allantoin nor allantoic acid can act as inducers.

Purine Toxicity and Xanthine Utilization. Darlington and Scassizzocchio (57) stated that an allantoinaseless strain of Aspergillus nidulans was severely inhibited on medium containing nitrate as a nitrogen source in the presence of an excess of any purine or allantoin. They suggested that the inhibition was due to the accumulation of allantoin or uric acid because of the genetic block. These workers used the allantoinaseless strain to isolate nitrate-utilizing mutants (in the presence of purine) which were later characterized to be purine transport defective. I wondered whether the similar mutants of N. crassa, aln-1 or alc-1, acted in the same manner. The results are shown in Table 12. Both mutants can utilize nitrate (10 mM) as a nitrogen source, but in the presence of an equal concentration of either xanthine or uric acid, no
<table>
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<th>Strain</th>
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<tr>
<td></td>
<td>aln-1</td>
<td>alc-1</td>
<td>w-t</td>
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<tr>
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<tr>
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<td>59.</td>
<td>96.</td>
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<td>67.</td>
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<td>N.G.</td>
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<td>30</td>
<td>55.</td>
</tr>
<tr>
<td>Xanthine+Ammonium tar.</td>
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<td>26</td>
<td>63</td>
</tr>
<tr>
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<td>N.G.</td>
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<td>25</td>
<td>91.</td>
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<tr>
<td>Uric Acid+Ammonium tar.</td>
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<td>115.</td>
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<tr>
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<td>N.G.</td>
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</tr>
<tr>
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<td>62</td>
<td>115</td>
</tr>
<tr>
<td>Allantoin+Ammonium tar.</td>
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<td>26</td>
<td>125</td>
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<tr>
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<td>72.</td>
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<tr>
<td>Nitrite&lt;sup&gt;e&lt;/sup&gt;</td>
<td>31</td>
<td>33</td>
<td>33.</td>
</tr>
<tr>
<td>Ammonium Tartrate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25</td>
<td>26</td>
<td>31.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Three day growth studies with each strain on the various nitrogen sources<br><sup>b</sup> 10 mM; <sup>c</sup> 10 mM; <sup>d</sup> 2 mM; <sup>e</sup> 5 mM; N.G. = No Growth
growth was found. Two other purine intermediates, hypoxanthine and allantoin, caused no inhibition of growth on nitrate with these Neurospora mutants, which contradicted the evidence found in Aspergillus. It appears that high intracellular concentrations of either xanthine or uric acid cause an inhibition of Neurospora growth on nitrate but no inhibition occurred with a high endogenous pool of allantoin as seen in Aspergillus. None of the purine intermediates inhibited growth when ammonia was used as the nitrogen source. Thus, the high concentration of xanthine or uric acid apparently specifically inhibits growth on nitrate by either preventing the transport of nitrate or some step in the reduction of nitrate to ammonia. To determine if possibly one or more catabolic steps were being inhibited by the purine products, growth on the first intermediate in the nitrate assimilatory pathway, nitrite, was tried. Both mutants (alc and aln) grew almost as well as the wild-type on nitrite (5 mM) and showed little growth inhibition in the presence of either xanthine or uric acid. This problem was not pursued because of other more relevant problems in the purine pathway, but this phenomenon is resolvable because both an assay for the nitrate transport and the enzyme nitrate reductase in N. crassa have been published (74-77, 79-82).

Another interesting result involved the utilization of xanthine as a nitrogen source. The xdh-1 mutant, which could not grow on hypoxanthine and lacked xanthine dehydrogenase activity, could nevertheless use xanthine as a nitrogen source at about 80% the rate of wild-type. In addition, allopurinol, the drug shown to inhibit xanthine dehydrogenase and hypoxanthine utilization, does not inhibit growth with xanthine as a
nitrogen source. This occurred even at a concentration of allopurinol equal to that of xanthine (1 mM) which was 1000-fold higher than that needed to stop growth on hypoxanthine. These two facts suggest that xanthine is oxidized to uric acid via a special system and the uric acid is then degraded normally because aln-1, alc-1 and ure-2 cannot grow on xanthine. This special system could involve a second allopurinol-resistant xanthine dehydrogenase or even a membrane oxidizing system which oxidizes xanthine to uric acid as the molecule is transported across the membrane. This phenomenon has also been observed in Aspergillus nidulans (58).

Induction Profile. The kinetics of induction of the initial three enzymes for uric acid catabolism was studied. Wild-type mycelia were grown in repressing conditions (high ammonia) and after 20 hours were transferred to media containing either hypoxanthine or uric acid as the sole nitrogen source. Figure 5 shows the increased synthesis of each enzyme which occurred after transfer to either of the purine intermediates. The three most important points to note concerning this experiment are the rate of enzyme increase, lag time (if any) before the increased rate of synthesis begins and the coordinate (or non-coordinate) increase of the three enzymes.

The increase in the observed amount of uricase with time (Fig. 5A) was somewhat different depending on which inducer was present; yet in both cases, a lag of about one hour occurred prior to the increase. The rate of increase of uricase after transfer to either intermediate was linear with time, although it was more rapid with hypoxanthine. This resulted in a higher specific activity of uricase after 20 hours'
Figure 5. Kinetics of induction of the three inducible enzymes in wild-type cells grown on 10 mM ammonia for 20 hours, then transferred to medium containing 1.0 mM hypoxanthine (---) or 1.0 mM uric acid (---) as sole nitrogen source and sampled at various times. 
A = uricase; B = allantoinase; C = allantoicase.
SPECIFIC ACTIVITY

TIME AFTER TRANSFER (hr)

(A) [Graph showing specific activity over time for different samples.]

(B) [Graph showing specific activity over time for different samples, with peaks at certain times.]

(C) [Graph showing specific activity over time for different samples, with a notable decline at a certain point.]
growth on hypoxanthine as compared to uric acid (Table 10). As noted earlier, this outcome was somewhat puzzling in light of the results given above, which indicated that uric acid is the true inducer and that hypoxanthine must be metabolized to form an inducer.

Allantoinase was previously shown to accumulate rapidly in either wild-type or \textit{ure-2} after transfer to various intermediates. Figure 5B shows that there was no noticeable time lag before increased synthesis of this enzyme occurred. This rapid increase in allantoinase continued for approximately two hours, followed by a slight but steady decline in activity. The cessation of allantoinase accumulation may be due to its susceptibility to turnover as demonstrated below. The third enzyme in this sequence, allantoicase, had also been found to be controlled by induction and repression. A brief lag, as observed with uricase, was observed before the increase in allantoicase occurred after transfer of mycelia to either intermediate; the amount of enzyme then increased linearly with time.

It is pertinent to inquire whether the increased levels of these three enzymes occurred in a coordinate or non-coordinate fashion. A plot of the amount of allantoinase versus uricase activity and also allantoicase versus uricase is shown in Figure 6. It is quite clear that the changes in allantoinase and uricase levels do not take place in a coordinate manner, since the points are scattered widely over the figure. However, the increase in allantoicase and uricase does appear to be coordinate.

Enzyme Stability \textit{in vivo}. It was desired to determine whether these enzymes were stable species \textit{in vivo} or were subject to dynamic turnover.
Figure 6. Uricase activity as a function of either allantoinase activity (●) or allantoicase activity (▲) in wild-type cells during induction by hypoxanthine. Each number represents a single culture that was assayed for all three enzymes. The progression of numbers follows the time points in the induction profile of Figure 5. A similar figure is obtained when enzyme induction with uric acid is plotted except that the slope of the line is decreased.
Figure 6
After the enzymes were induced to high levels, their possible turnover was examined following transfer of mycelia to repressing medium containing cycloheximide (10 μg/ml) to prevent any new synthesis. As shown in Figure 7, uricase and allantoicase were found to undergo very little turnover, even seven hours after the transfer. This result indicates that these two enzymes are relatively stable and that changes in the cellular concentration of either enzyme is primarily, if not solely, determined by changes in the rate of synthesis during mycelial growth. In addition, possible feedback inhibition of these two enzymes by various intermediates and the end product was studied. The results showed that no intermediate in the pathway or ammonia (up to 1 mM) caused significant inhibition of either uricase or allantoicase (data not shown).

The induction and subsequent repression of allantoinase are shown in Figure 8. The induction of allantoinase clearly required de novo protein synthesis, and most likely represents new synthesis of the enzyme. The activity of allantoinase at the time of the original transfer to inducing medium was at the usual low but easily-detectable level characteristic of repressed conditions. However, after two hours in the presence of the inducer plus cycloheximide, the cellular activity decreased nearly 20 times to a barely detectable level. Thus, the presence of inducer does not simply stabilize the enzyme, but the increased level which results from induction requires de novo protein synthesis. In cultures induced with hypoxanthine, the amount of allantoinase increased very rapidly and reached a maximum after two hours (see Fig. 5). The mycelia were then transferred back to media.
Figure 7. Stability of uricase and allantoinase in vivo. Cultures of wild-type cells were grown on modified medium containing 1.5 mM ammonium tartrate and 1.0 mM hypoxanthine. After 20 hours the cultures were transferred to medium containing 20 mM ammonia and cycloheximide (10 μg/ml). Cultures were harvested at designated times and assayed for uricase (O) and allantoinase (Δ).
Figure 8. Induction and repression of allantoinase in vivo. A large culture of wild-type cells was grown in medium with 10 mM ammonium tartrate for 20 hours then transferred to medium containing either 1.0 mM hypoxanthine as sole nitrogen source or 1.0 mM hypoxanthine plus cycloheximide (10 μgr/ml) for 2 hours. The hypoxanthine culture was then subdivided and transferred to 10 mM ammonium tartrate (Δ); 10 mM ammonium tartrate plus cycloheximide (10 μgr/ml) (Δ-); 10 mM hypoxanthine plus cycloheximide (○); or, no nitrogen source with cycloheximide (□) and sampled at various times as indicated.
Figure 8
containing repressing amounts of ammonia, or to media containing cyclo-
heximide (10 μg/ml) plus either hypoxanthine, ammonia or no nitrogen
source. The results, shown in the right half of Figure 8, demonstrated
that an almost 50% decrease of allantoinase occurred in just two hours
following renewed repression by ammonia. When the cells were instead
transferred to any medium containing cycloheximide, allantoinase activ-
ity declined so rapidly that less than 1% of the original activity re-
mained after just one hour; the in vivo half-life estimated for allan-
toinase in the presence of cycloheximide was 20 minutes. This enzyme
is either extremely labile or else subject to extensive intracellular
proteolysis. Allantoinase turnover was not prevented or slowed by
hypoxanthine nor was its degradation rate enhanced by ammonia (Fig. 8).
Furthermore, allantoinase activity is not subject to feedback inhibi-
tion by ammonia, since even 10 mM NH₄⁺ did not inhibit the enzyme in
vitro. Therefore, an increase in the cellular level of allantoinase
activity results from an increase in its rate of synthesis and not from
a change in its degradation rate.

Since allantoinase activity was found to turn over quite rapidly
in vivo, it was of interest to determine whether this enzyme was inacti-
vated in a cell-free extract. As shown in Figure 9, allantoinase was
indeed found to be labile in vitro. The presence or absence of cyclo-
heximide did not change its rate of degradation. Very little turnover
occurred at 0° but at 24° its rate of loss in vitro was probably suffi-
cient to explain its in vivo turnover. Thus, allantoinase undergoes
rapid and extensive turnover both in vivo and in vitro. If the lability
of this enzyme is due to proteolysis, then the responsible protease
Figure 9. Stability of allantoinase in vitro. Wild-type cells were grown in medium containing 1.5 mM ammonium tartrate and 1.0 mM hypoxanthine for 20 hours to induce allantoinase. A cell-free extract was prepared and samples were incubated for 0–4 hours at either 0° or 23.5° in the presence or absence of cycloheximide (10 μg/ml).

▲, extract only; ○, extract plus cycloheximide. At the indicated times, the extracts were assayed for allantoinase activity.
Figure 9

% MAXIMUM ACTIVITY

TIME (hr)

0 1 2 3 4 5

0 10 20 30 40 50 60 70 80 90 100

O 23.5°

O 0°
must already have been present in the cell before inhibition of protein
synthesis (by cycloheximide) and must itself be stable.

Genetic Regulation of Nitrogen Metabolism. The three regulated enzymes
of the purine catabolic pathway studied here have been found to be re-
pressed by ammonia and also induced, most likely, by uric acid. It
seemed probable that a regulatory protein would be required to interact
with these small molecular weight effectors and to participate in the
induction and repression events, probably at the level of transcription.
Such a regulatory protein could be positive or negative in its effect on
enzyme synthesis (or even both positive and negative). A mutational
loss of function of a positive control gene should result in a pleio-
tropic loss of enzymes and the ability to use the intermediates of the
purine degradative pathway. In contrast, the loss of the function of a
negative control gene would be expected to cause the various enzymes to
be constitutive.

A mutant, designated amr, which possesses the qualities character-
istic of a positive control gene, was isolated in this laboratory on the
basis of its inability to use purine intermediates and was found to have
a multiple enzyme loss, being deficient in allantoinase, allantoicase,
uricase and nitrate reductase. The mutant amr (allele 17) was repeatedly
back-crossed to wild-type, because its pleiotropic effects, characterized
by very slow growth on all purine intermediates as a sole nitrogen
source (Table 5) and only the uninduced, basal levels of the three regu-
lated enzymes during induction (Table 6) could result from multiple
mutant genes or from a single altered locus possibly coding for some type
of regulatory protein. While this work was in progress, Arst and Cove
reported on a genetic locus in *Aspergillus nidulans* (60), which was thought to code for a nitrogen regulatory protein. In a footnote in this report (6), Fincham and Coddington suggested that a similar locus might exist in *N. crassa* and that nit-2 (a strain originally isolated as a nitrate non-utilizer and shown to lack nitrate reductase) might represent such a mutant. The recombination frequency between amr and nit-2 (allele nr 37) showed that there was less than 0.2 map units separating the two mutations. Since they mapped so close, these two mutants, isolated as non-utilizers of two completely different nitrogen sources, might be allelic; if alleles, they would not be expected to complement one another in a heterocaryon. To be sure a viable heterocaryon was made, different auxotrophic markers were crossed into the amr and the nit-2 strains. Formation of a heterocaryon (nit-2 his-6 and amr leu-2) (mixed nuclei) was assured by forcing it to grow on minimal media, e.g.: in order for growth to occur, the nit-2 his-6 nuclei must provide the information necessary to synthesize histidine. Knowing that a heterocaryon had been constructed with the aid of these biochemical markers, I then tested for complementation between nit-2 and amr. The nit-2 and amr mutants failed to complement each other and could not utilize nitrate as a sole nitrogen source. This result implies that nit-2 and amr are allelic and affect the same protein. Complementation of the heterocaryon (nit-2 his-6 + amr leu-2) occurred for the amino acid requirements which indicated that the failure to produce a heterocaryon was not due to incompatibility, but rather was because nit-2 and amr do not complement.
The nit-2 mutant had previously been shown to lack nitrate reductase and to be unable to grow on hypoxanthine (75). More extensive growth studies showed that amr mutants were consistently unable to utilize a variety of nitrogen compounds which wild-type can employ. These results are shown in Table 13.

Several interesting facts concerning the catabolism of various amino acids can be derived from these data: (1) one group of amino acids is readily used as a nitrogen source by wild-type but cannot be utilized by the amr mutants (e.g., alanine, leucine, methionine, ornithine and phenylalanine), (2) certain amino acids (e.g., histidine, lysine and serine) are used poorly by wild-type but permit even less growth of the mutants, and (3) both wild-type and the mutant strains grow well on certain key amino acids (e.g., arginine, glutamine and aspartate). These results agree well with an earlier qualitative study of amino acid utilization by wild-type (5). I have not yet examined any of the enzymes involved in catabolism of the compounds listed in Table 13, but because of their diverse nature, the lack of any one enzyme clearly could not explain the inability of amr to utilize most of these compounds.
TABLE 13
GROWTH OF amr AND nit-2 MUTANTS ON VARIOUS NITROGEN SOURCES

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>Growth of mutants (^b)</th>
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<tbody>
<tr>
<td></td>
<td>nit-2 (nr 37)</td>
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<tr>
<td>alanine</td>
<td>13.8</td>
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<tr>
<td>arginine</td>
<td>90.4</td>
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<td>ammonia</td>
<td>67.3</td>
</tr>
<tr>
<td>nitrate</td>
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</tr>
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</table>

\(^a\)All amino acids were in the L form and were added to modified Vogel's medium to a final concentration of 10 mM as the sole nitrogen source.

\(^b\)Growth in milligrams dry weight after 3 days of growth.
Discussion

*Neurospora crassa* can utilize the purine bases as excellent sources of nitrogen. At least eight enzymes are required to fully degrade either guanine or adenine to ammonia. The last five steps of the purine catabolic pathway, which begins with uric acid, were most extensively examined in this study. The results showed that the last two enzymes in this sequence—ureidoglyoxylate hydrolase and urease—are constitutive. The first three enzymes—uricase, allantoinase and allantoicase—appear to be controlled by both induction and repression. The ureaseless mutant, *ure-2*, helped to show that the three regulated enzymes are controlled by repression and that the repressor is the end product, ammonia. The exact identity of the inducer is still uncertain and only by the isolation and identification of mutants in which each step of the purine degradative pathway is specifically blocked will the true inducer become known. Several mutants have been found which prevent interconversions of the intermediates.

Four intermediates of the purine pathway—hypoxanthine, uric acid, allantoin and allantoic acid—which could be inducers of uricase and allantoicase—were tested. The mutants *aln-1* and *alc-1*, which specifically block further metabolism of allantoin and allantoic acid, respectively, were used to show that an intermediate(s) prior to these two was the true inducer. Similarly, hypoxanthine itself was found not to cause induction, although it can be metabolized to the inducer. Hypoxanthine
failed to induce the enzymes in both the nit-1 and xdh-1 mutants, which were shown to lack xanthine dehydrogenase activity. The presence of uric acid resulted in good enzyme induction in the wild-type and all of these mutant strains. Thus, uric acid is a true inducer for uricase and allantoicase synthesis, although the possibility that an intermediate in the conversion of hypoxanthine to uric acid, perhaps xanthine, could also serve as an inducer cannot be excluded. Numerous unsuccessful attempts have been made to isolate uricase negative mutants and also mutants specifically unable to utilize xanthine as a nitrogen source. Such mutants, and double mutant strains constructed from them, would permit an unambiguous identification of the true inducer. The fact that exogenous xanthine and uric acid are toxic to the aln-1 and alc-1 strains suggests that the isolation of the desired mutants may be impossible with the filtration enrichment technique. Another possibility exists that there may be multiple forms of uricase and that no single gene mutation would completely stop growth on uric acid. This latter possibility does not seem likely because of the work of Greene and Mitchell (63). They purified uricase from N. crassa over 400-fold and found only one band of activity after ionophoresis; furthermore, the enzyme displayed only one pH optimum. Since no uricase mutants could be found, I attempted to find a compound which would strongly inhibit uricase activity because such a chemical block should yield equally valuable results. Several purine analogues inhibited in vitro activity about 90% but had little effect in vivo. Hashimoto (83) reported that potassium oxonate, a substituted s-triazine, completely inhibited uricase in vitro while not affecting XDH in liver cell
homogenates. This compound was found to completely inhibit *Neurospora* uricase in vitro at inhibitor concentrations less than 100 uM. However, oxonate did not stop growth of *Neurospora* on uric acid, even at exogenous concentrations greater than 1 mM. Thus, oxonate apparently cannot enter the cell and inactivate uricase.

The fact that 8-azaxanthine, a non-metabolizable analogue of uric acid, was found to be an excellent inducer of uricase and allantoicase supports the conclusion that uric acid is the inducer. Furthermore, allopurinol, a strong inhibitor of xanthine dehydrogenase, prevented hypoxanthine from being converted into an inducer in the wild-type strain. An interesting related result was the finding that xanthine utilization was not sensitive to allopurinol, even when the drug was present at equal molar concentrations. By contrast, growth on hypoxanthine was completely inhibited by a 1000-fold lower concentration of allopurinol. These results suggest that a different enzyme, or at least a second form of xanthine dehydrogenase, is involved in xanthine oxidation.

My findings with *nit-1* agree with those of Ketchum et al (77) who reported that this mutant lacks xanthine dehydrogenase activity and cannot use either hypoxanthine or nitrate as a nitrogen source. The *nit-1* locus has been implicated in the formation of a molybdenum-containing cofactor, which is common to both nitrate reductase and xanthine dehydrogenase (76). In *Aspergillus*, this dual function by a single metalloprotein (polypeptide) has been more extensively investigated by Cove et al (56, 59, 84-87). Five unlinked genes, designated the *cnx* series, led to the simultaneous loss of NADPH nitrate reductase and
xanthine dehydrogenase and hence inability to utilize either nitrate or hypoxanthine as sole nitrogen sources. It was thought that one or more of the cnx genes specified a molybdenum-containing cofactor necessary for both enzymes. Two alternatives seem possible for the five different cnx gene products. First, the cnx genes could specify an enzyme which catalyzes the formation of a cofactor absolutely required for activity, or second, the cnx genes could specify subunits which aggregate with other polypeptides to form either nitrate reductase or XDH. Temperature-sensitive mutations in three of the cnx genes proved that both alternatives were, in fact, true. Such mutants at two cnx loci showed no different half-life for nitrate reductase than wild-type at 35°, implying that the gene products were involved in the synthesis of the molybdenum cofactor. A temperature-sensitive mutant for the third cnx locus had a much shorter half life for nitrate reductase, suggesting that its gene product was a structural component of nitrate reductase.

The purine catabolic pathway has been studied in Aspergillus nidulans and Saccharomyces cerevisiae by Scorzocchio, Darlington and others (54-59, 89-90) and Cooper, Lawther and Whitney (48-53, 91-94), respectively. In Aspergillus nidulans, six enzymes are required to degrade hypoxanthine to ammonia in a pathway identical to that which is found in Neurospora. A complicated pattern of induction and repression was reported for the first four enzymes of this sequence; various combinations of early intermediates maximally induced these enzymes while ammonia repressed their synthesis. The last two enzymes were found to be constitutive. A permease—required for transport of xanthine and uric acid—and uricase were found to be induced by uric
acid. Mutants lacking either the permease or uricase have been isolated and shown to be unlinked. It has recently been shown that the gene for each enzyme is under the same dual control, induction by uric acid and repression by the end product, ammonia. These authors suggest that uric acid binds to the product of the genetic locus, \textit{uaY}, which probably codes for a nuclear protein necessary for induction (in a positive manner) of the purine pathway. This inducer-protein complex is postulated to bind to a promoter region proximal to the two structural genes. In addition, there is another locus, \textit{are} \textit{A}, which is thought to code for a nuclear protein involved in ammonium repression. In the absence of ammonia, the \textit{are} protein is thought to bind to a site very near that for the uric acid-\textit{uaY} protein complex and only when both are present does initiation of transcription begin. There are now three lines of evidence for this model: (1) a mutation in the \textit{uaY} locus prevents induction of those uric acid regulated enzymes; (2) a mutation in the \textit{are} locus also prevents induction of those same enzymes and, in addition, other \textit{are} alleles prevent ammonium repression for those enzymes repressed by \( \text{NH}_4^+ \) and (3) a mutation has been obtained in what is thought to be the promoter region proximal to the structural gene for the uric acid permease. This mutant is an initiator constitutive with an "up-promotor" effect and is "cis" dominant (90). This mutation increases the maximally induced level of transport 2.5 fold and yet is still repressible by the end product, ammonia. Thus, this mutation is thought to be at the binding site for the uric acid-protein complex.

A very different pattern of regulation has been reported to occur in \textit{S. cerevisiae}. Yeast cannot grow on purines or uric acid because
they lack uricase, but can grow on allantoin. The degradation pathway is different in this organism at the final step, in which urea is carboxylated to allophanic acid, which is then cleaved to produce ammonia and carbon dioxide. This new intermediate, allophanic acid, was shown to induce the synthesis of all the enzymes participating in allantoin metabolism. Lawther and Cooper (95) are now studying this system to determine whether "induction" is due to an increase in (1) the rate of transcription, (2) the transport of the transcript out of the nucleus, or (3) the rate of translation of mRNA. Upon induction, an increase in the amount of allophanic hydrolase is found within three minutes, suggesting that induction involves transport of mRNA out of the nucleus.

From the kinetics of induction, it was concluded that (1) uricase and allantoicase are coordinately synthesized, while allantoinase is non-coordinately synthesized with these two enzymes in Neurospora and (2) hypoxanthine is a better inducer than uric acid for uricase in the wild-type strain. Coordinate synthesis means that the ratio of activities of two or more enzymes remain constant under all conditions of growth (96). This result suggests the possibility that the structural genes for uricase and allantoicase could be situated together in a single operon. However, coordinate synthesis of two enzymes is clearly insufficient evidence to presume that the corresponding structural genes do reside in the same operon. In this case, the fact that allantoinase, the enzyme which occupies an intermediate position in the catabolic pathway between uricase and allantoicase, is not coordinately synthesized with them, and is encoded by a gene unlinked to alc, indicates that an operon arrangement is unlikely. The presence of operons in Neurospora
is quite infrequent (1), although Chaleff (7, 37) has shown that the
three enzymes required for catabolism of quinate to protocatechuic
acid are all coordinately synthesized and that the structural genes
for these enzymes are tightly clustered and thus may constitute an
operon.

An apparent paradox was pointed out in the Results Section:
although hypoxanthine itself is not an inducer, it was found to lead to
a greater induction of uricase than did uric acid in the wild-type
strain. The suspected reason for this outcome is that when growth
occurs on uric acid, a large intracellular pool of this compound accumu­
lates and causes induction of uricase but also rapidly leads to the
formation of a substantial pool of ammonia, which at least partially
represses enzyme synthesis. Hypoxanthine is a poorer nitrogen source
than uric acid, which implies that its catabolism only slowly gives rise
to an intracellular pool of ammonia. Thus, it is postulated that hypo­
oxanthine is degraded slowly to give a sufficient pool of uric acid to
induce the synthesis of uricase but only slowly produces ammonia, so
that reduced growth occurs and enzyme synthesis is not repressed. In
agreement with this suggestion, it was shown (6) that a significant in­
crease above the usual level of uricase was induced by uric acid in the
ure-2 mutant, which is blocked in the formation of ammonia from uric
acid.

I would now like to consider the factors which regulate the amount
and activity of the three initial enzymes required for uric acid
catabolism. Uricase and allantoicase are inducible enzymes and appear
to be synthesized coordinately; these two enzymes do not display turnover
nor are they subject to feedback inhibition. Thus, changes in their cellular activity and concentration appear to result primarily, if not entirely, from changes in their rate of synthesis during mycelial growth. In marked contrast, allantoinase was found to undergo rapid and extensive turnover both in vivo and in vitro. When additional enzyme synthesis was inhibited with cycloheximide, the pre-existing allantoinase turned over in vivo with a half life of approximately 20 minutes. Its rate of turnover under these conditions was not altered by the presence of an inducer (hypoxanthine) or the repressor, ammonia. Allantoinase activity is not subject to feedback inhibition by ammonia. Thus, it appears that the cellular level (and activity) of allantoinase results from the steady state balance between its rate of synthesis, which can vary widely, and its apparent constant rate of degradation.

The extreme lability of allantoinase both in vivo and in vitro may be due to its sensitivity to an intracellular proteolytic enzyme, which itself is both constitutive and stable. North (97) reported that glycerol kinase (GK) was repressed at room temperature in the presence of a good carbon source such as sucrose. The enzyme was induced during growth with glycerol as the carbon source. The same increase in the amount of the kinase could also be attained in the presence of sucrose by decreasing the growing temperature to 4°C. The enzyme, induced by either method, was quite labile in vivo and upon inhibition of protein synthesis, activity was lost with a half life of approximately 15 minutes (98). In addition, raising the temperature of 4°C induced culture to 23°C inactivates the GK. Once the kinase activity had been lost, it was not possible to regain its activity, which implied that
the enzyme had undergone some type of irreversible inactivation. The degradation of tryptophan synthase in vitro has been shown to result from the action of a serine-type protease (99). This protease is different from the protease secreted into the growth medium by Neurospora in response to starvation for either carbon, nitrogen or sulfur starvation (100-102). An inactivation of allantoinase in vivo was also studied in Pseudomonas aeruginosa (103). It was found that the activity of the enzyme decreased in stationary phase when the cells were cultured in a medium containing allantoin or allantoate as the sole carbon, nitrogen and energy source. This inactivation was found to be due to the synthesis of a protein, which exhibited no proteolytic activity toward allantoinase. The addition of an inhibitor of protein synthesis was found to stop further loss of allantoinase and thus stabilizing any remaining activity. This protein was thought to bind specifically to allantoinase and irreversibly inactivate the enzyme. Thus, allantoinase is also unstable in Pseudomonas, but probably due to a different mechanism.

The factors regulating the stability of allantoinase seem to be quite different from those controlling nitrate reductase (NR) in Neurospora. The synthesis of nitrate reductase was found to be dependent on the presence of nitrate and was repressible by high concentrations of ammonia (104). After nitrate reductase was induced to maximal levels, the addition of ammonia or removal of nitrate from cultures caused a rapid inactivation of the enzyme. The loss of enzyme was slowed by the protein synthesis inhibitor, cycloheximide. Inactivation was dependent upon the presence of both subunits of nitrate reductase.
apparently because the inactivating agent, perhaps a protease, can recognize only the native conformation of the enzyme. In addition, the responsible agent is apparently itself labile (or must be synthesized de novo) because inhibition of protein synthesis slowed down the rate of disappearance of nitrate reductase. It was recently shown that the presence of nitrate protects the enzyme from degradation (105).

Is this turnover phenomenon found with these various enzymes in N. crassa really physiologically significant or just an artifact of the method of analysis? In the case of tryptophan synthase, the evidence suggests that inactivation is only found in vitro and hence is not meaningful in vivo. With allantoinase, glycerol kinase and nitrate reductase, the inactivation could be found without any drug being present, suggesting that these enzymes probably turn over naturally in the cell. A sensitive technique which would help determine if allantoinase were turning over naturally involves double-labelling the enzyme. The "double label" technique for a particular enzyme requires that a purification scheme or the exact distance of its migration during polyacrylamide gel electrophoresis has been previously ascertained. Then, after growth in the presence of either a $^{14}$C or $^3$H-labelled amino acid, the medium is changed such that the other radioactive form is present. If allantoinase is turning over in vivo, then the ratio of the two labels should drastically change with time. This technique has been used to study turnover of various enzymes in mammalian cells (106).

During the course of this work, a mutant designated as amr (for ammonium regulation) was isolated on the basis of its inability to utilize uric acid as a nitrogen source, and was subsequently found to
be allelic to nit-2 mutants, which were isolated as non-utilizers of nitrate. The amr mutant and two nit-2 alleles are completely or partially deficient in the utilization of a number of different nitrogen sources. These mutant strains cannot use nitrate, purines or their catabolic intermediates, some amino acids, or any exogenous proteins as a nitrogen source.

The amr mutants display a multiple enzyme loss. They lack or have only basal levels of nitrate reductase, uricase, allantoinase, allantoicase, xanthine dehydrogenase and an extracellular protease (75, 107). It also seems very likely that amr mutants lack additional ammonium-repressible enzymes related to nitrogen metabolism. The present knowledge of the amr locus suggests that it is a positive regulatory gene which mediates the repression of a number of enzymes related to nitrogen metabolism. It is suspected that this control gene may encode a regulatory protein which is required for the transcription of the structural genes for the various enzymes subject to ammonia repression. The postulated regulatory protein may be sensitive to ammonia, and in its presence may assume a conformation unable to bind to the predicted proximal promotor sites. The interference with the positive action of the nit-2 protein would facilitate repression of synthesis of this group of enzymes. The structural genes of those regulated enzymes which are both inducible and repressible—such as those of the purine pathway—are predicted to have a promotor region which contains at least two binding sites: one site specific for the amr protein and a second site for a regulatory protein which binds to it only in the presence of the inducer, e.g., uric acid. These considerations suggest
a model for the overall regulation of nitrogen metabolism in *Neurospora* (Figure 10). In this description I have focused attention on regulatory elements with a positive effect because of their predominance in the lower eucaryotes (1) and because of the characteristics of the relevant mutants which have been studied. According to this model, the positive action of the amr protein is reversed by ammonia whereas it is necessary for an inducer (e.g., uric acid) to bind to a second regulatory protein in order for it to serve as an activator. I suggest that the simultaneous presence of both of these regulatory proteins in their active conformation is necessary before transcription of the controlled structural genes can occur. In this regard, it is interesting to note that the amr locus seems to be a major control gene which may integrate the synthesis of many different enzymes of nitrogen metabolism. Inducible systems may have in addition a minor control gene which acts in concert with the amr gene product to induce just the enzymes of a particular pathway (such as the purine catabolic pathway). Some nitrogen-related enzymes may be ammonium-repressible but do not require induction. In this case, only the amr signal would be required for transcription of the pertinent structural loci. Finally, certain enzymes clearly involved in nitrogen metabolism may not be repressed by ammonia but simply require induction. Both ornithine transaminase and arginase may represent such enzymes which are inducible but not repressible.

In this last instance, transcription would likely require the presence of a relatively specific regulatory protein (or the absence of activity of a negatively-acting protein) but would be independent of the amr system. In this way, it is postulated that the many diverse reactions
Model Of Nitrogen Regulation:

Major Nitrogen Control Gene

- \( amr \)

- \( \text{NH}_4^+ \)

  Active Regulatory Protein

  Inactive Form

(+) Repressible System: General Amino Acid Permease

\( Pm\ G \)

Permease

Minor Nitrogen Control Genes

- \( pur-c \)

- \( nit-c \)

- \( arg-c \)

\( + \text{Inducer} \) (Arginine)

Active Regulatory Protein

Inactivating Protein

(+) Inducible/Repressible Genes: Purine Cat. Enzymes

\( aic \)

Allantoinase

Inducible Systems: Arginase

\( arg \)

Arginase

Figure 10
involved in nitrogen metabolism may be integrated and regulated as a group.
BIBLIOGRAPHY


