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STUDY OF THE CHARACTERISTICS AND REGULATION
OF THE EXTRACELLULAR PROTEASES OF

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

Musetta Anne Hanson, B. S.

***********

The Ohio State University

1974

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I wish to thank Dr. George Marzluf for his patient assistance and guidance during my graduate training.

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PUBLICATIONS


"Control of a single enzyme in Neurospora crassa by multiple regulatory circuits." Proceedings of the National Academy of Science, USA. In press.
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<tr>
<td>ATEE</td>
<td>Acetyl tyrosine ethyl ester</td>
</tr>
<tr>
<td>BAEE</td>
<td>Benzoyl arginine ethyl ester</td>
</tr>
<tr>
<td>BLEE</td>
<td>Benzoyl leucine ethyl ester</td>
</tr>
<tr>
<td>BTEE</td>
<td>Benzoyl tyrosine ethyl ester</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CN</td>
<td>Carboxymethyl-</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethane</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylphosphofluoridate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloracetic acid</td>
</tr>
</tbody>
</table>
Introduction

*Neurospora crassa* is a fungus of the class Ascomycetes. It possesses seven distinct chromosomes, has a defined nuclear membrane, and undergoes both sexual and asexual reproduction. The life cycle of *N. crassa* is shown in figure 1.

Wild type *N. crassa* grows well when supplied with inorganic nitrogen, inorganic sulfur, trace salts, biotin, and sucrose as the carbon source. However, when deprived of either sulfur, nitrogen, or carbon, and given a protein the organism secretes an extracellular protease which enables it to use the protein as a source of the missing metabolite.

Extracellular proteases are produced by a large number of bacteria and fungi. In most studies of microbial proteases, attention has been focused on their isolation and characterization. The mechanism of control of these enzymes has received much less consideration. Matile (1) studied the *N. crassa* protease that appears during nitrogen limitation and stated that it was contained in cell vesicles. Drucker (2) studied the proteolytic species in *N. crassa* that he found to be under carbon control and induced by protein in medium with a limiting carbon source, but repressed by a readily available supply of carbon.
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 mycelia mass

**SEXUAL:**
- Male nuclei, conidium
- Female nuclei, ascogonium
- Trichogyne, mating tube

**Melosis occurs to produce haploid ascospores**

**Conidia can fertilize protopenteclum of the opposite mating type**

**Protopenteclum, type A**

**Diploid state in protopenteclum resulting from fusion of nuclei**

**Ascospores can grow vegetatively to produce mycelial mass**
Murakami, et al. (3), have reported a stimulative effect of proteins on protease formation by various species of Serratia.

This work describes the discovery and investigation of a protease of N. crassa produced under conditions of sulfur limitation and further compares this sulfur-controlled enzyme to the nitrogen-, and carbon-controlled ones.

Regulation over sulfur metabolism in N. crassa appears to control the entry of various sulfur-containing metabolites into the main pathway of sulfur assimilation. Good sulfur sources, such as methionine or inorganic sulfate, repress the synthesis of an entire family of related enzymes that function in the acquisition of sulfur, including aryl sulfatase, choline sulfatase, choline sulfate permease, two distinct sulfate permeases, and a specific methionine permease (4). None of the enzymes are inducible since they are formed even in the absence of their substrates. This same group of enzymes are regulated by two regulatory genes cys-3 and soon0. Cys-3 mutants fail to make any of the family of sulfur-repressible enzymes even under conditions of sulfur starvation. Cys-3 is not linked to any of the genes it controls. In heterocaryons, the cys-3 nuclei are recessive to the wild type nuclei. However, using electrophoretic variants of aryl sulfatase, it can be shown that the action of the cys-3+ allele is not limited to its own nucleus (4). Revertants of cys-3 are repressible. This is evidence which argues against a super-repressor
Interpretation of the data but favors the interpretation that the cys-3 gene makes a macromolecule needed to turn on a group of unlinked genes.

Scon⁰ (sulfur control, constitutive) is another control gene and is unlinked to cys-3 and the structural genes which it controls. All of the enzymes are not repressible in scon⁰ by the normal corepressors, methionine, cysteine, and sulfate. When scon⁰ and scon⁺ are present in a heterocaryon, the nucleus with scon⁰ is derepressed under all conditions while the scon⁺ is normally repressible. Again, the electrophoretic variants of aryl sulfatase were used to demonstrate this. Results suggest that the scon⁰ gene codes for a diffusible control element that is confined to the nucleus, but whether the control is positive or negative is inconclusive (5).

My work indicates that the protease gene is subject to this same sulfur control system, as well as some form of nitrogen and carbon control. If each of the three metabolic limitations should signal the synthesis of individually regulated and distinct protease species, the enzymes produced in the three cases should differ from one another. In contrast, consider the possibility in which a single structural gene for protease is regulated in a complex fashion and activated by each of the three distinct metabolic signals. In this case, the enzymes produced under any of the limiting conditions should be identical.

To examine these possibilities, I have compared the extra-
cellular proteases synthesized under the three contrasting conditions by a number of biochemical criteria. This work presents biochemical and genetic evidence which strongly suggests that the very same alkaline protease is indeed synthesized and secreted in response to a limitation of sulfur, nitrogen, or carbon.
A great number of investigations have been carried out on the characteristics of proteases from a wide range of organisms. Far fewer investigations have dealt with the control of these enzymes, although there is an extensive literature describing control processes which regulate other enzymes.

For the sake of conciseness, this survey will review the general characterization of microbial proteases, some of the better understood control models which have been postulated, and then more specifically describe the few studies of the control of fungal proteases.

Proteases have been isolated from a wide variety of microbial sources in recent years. Although these enzymes are predominantly extracellular, they range from endo- to exopeptidases, have various pH dependences, substrate specificities, and inhibitor responses. These enzymes have been divided into four main categories: (1) acid proteases, (2) DFP-sensitive alkaline proteases, (3) metal chelator-sensitive neutral proteases, and (4) thiol proteases (6).

In general, acid proteases (table 1) have a low content of basic amino acids and low isoelectric points, in the range of 2 to 6, while their pH optima lie between 2 and 5.
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Characteristics of enzyme</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aspergillus salitai</strong></td>
<td>Molecular weight, 35,000 Stable over pH range 2-5 pH optima (Proteins) 2.5-3.0 pH optima (peptides) 4.0-4.5 Amidase activity to Bz-Arg-NH$_2$</td>
<td>7-12</td>
</tr>
<tr>
<td><strong>Mucor Fusillis</strong></td>
<td>Molecular weight, 31,000 pH optima (hemoglobin) 3.8-4.0 pH optima (casein) 5.6 Stable in pH range 3-6</td>
<td>13-16</td>
</tr>
<tr>
<td><strong>Penicillium lantheinellum</strong></td>
<td>Molecular weight, 32,100 Stable in pH range 2-5 pH optima, 3-4 Inhibited by diazoacetyl norleucine methly ester</td>
<td>17-19</td>
</tr>
<tr>
<td><strong>Rhizopus chinensis</strong></td>
<td>Molecular weight, 35,000 Stable in pH range 2.8-6.5 pH optima 2.9-3.3 Isoelectric point, 5.2 esterolytic with TAAE</td>
<td>20-22</td>
</tr>
<tr>
<td><strong>Saccharomyces cerevisiae</strong></td>
<td>Molecular weight, 60,000 pH optima (hemoglobin) 3.0 Isoelectric point 3.8</td>
<td>23-27</td>
</tr>
<tr>
<td><strong>Aspergillus oryzae</strong></td>
<td>Stable in pH range 3-6 pH optima 3.0 no amidase activity Inhibited by Na lauryl sulfonate</td>
<td>12, 28-31</td>
</tr>
</tbody>
</table>
Most of the acid proteases seem to have molecular weights around 35,000. They show limited esterolytic activity, though some dipeptides can be used as substrates. Compounds such as DFP, thiol poisons and metal chelators do not inhibit the acid proteases, but diazo compounds, such as diazoacetyl-D,L-norleucine methyl ester, are active inhibitory agents. In some cases, fatty acid salts inhibit. Other acid proteases are rennin-like; that is, they will clot milk and their activity is determined as a function of this clotting ability.

The DFP-sensitive proteases (table 2) in general have a serine residue at the active site making them susceptible to inhibition by organoflorophosphates such as DFP and PMSF. The reaction products of this inhibition are stable and the inhibition is irreversible. As a group, these proteases have a molecular weight between 20,000 and 25,000, an isoelectric point between 9 and 10.5, a broad alkaline pH optima and are stable over a pH range of 5-10. Furthermore these proteases are usually esterolytic and can often function as collagenases, gelatinases, or keratinases.

The thiol group of proteases include those which are metal-chelator-sensitive (table 3). These are generally metalloendopeptidases which have a neutral pH optima; thus they are sensitive to metal chelators such as EDTA and o-phenanthroline but insensitive to DFP and thiol reagents.
### TABLE 2

DFP-sensitive proteases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Characteristics of enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus flavus</em></td>
<td>Molecular weight, 19,000 Bromoacetone noninhibitory</td>
<td>22,32,33</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Molecular weight, 18,000 pH optima, 10.5 Inhibited by soybean trypsin inhibitor</td>
<td>28,33-38</td>
</tr>
<tr>
<td><em>Aspergillus sojae</em></td>
<td>Molecular weight, 25,000 Isoelectric point, 5.1</td>
<td>39-40</td>
</tr>
<tr>
<td><em>Penicillium cyaneo-fulvum</em></td>
<td>Molecular weight, 44,000 Broad specificity Elastase-like pH optima (polyglu), 4.4 pH optima (polylys), 10.7</td>
<td>41-44</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Alkaline pH optima Esterolytic p-Mercuribenzoate Inhibited Zymogen form in cell</td>
<td>21,23,45-46</td>
</tr>
</tbody>
</table>

### TABLE 3

Metal chelator-sensitive proteases

<table>
<thead>
<tr>
<th>Organism</th>
<th>Characteristics of enzyme</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Inhibition released by metal ions Stable in pH range 4-10 pH optima (casein) 8.0 Inactive in 2 min at 60°C</td>
<td>28,31,47-48</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Elastolytic Molecular weight, 39,500 Stable in pH range 6-10 Heat stable (85% active after 60 min at 70°C) pH optima (casein) 6.8</td>
<td>49-55</td>
</tr>
</tbody>
</table>
These neutral proteases rarely have esterolytic or amidase activity. The primary metal requirement of these enzymes is zinc although Cu\(^{++}\), Pb\(^{++}\), and Hg\(^{++}\) can replace the zinc. The molecular weights fall within the range of 35,000 to 40,000. A wide range of stabilities with regard to temperature and pH has been reported. In general, it has been reported that a hydrophobic amino acid is required at the point of cleavage. None of the neutral proteases have been shown to demonstrate esterolytic activity.

The thiol proteases are less common, with the streptococcal proteinase being the best characterized of this class. These enzymes are inhibited by thiol poisons such as \(\text{tioacetate}\) and p-mercuribenzoate, and have a sulfhydryl group in the active site.

Study of bacterial growth in response to different nutritional environments has shown that most organisms can adapt to a given situation by turning on or off the synthesis of particular needed or unneeded enzymes. Jacob and Monod (56) first postulated the "operon model" to explain how this regulation of enzyme synthesis could occur. Jacob and Monod based their model on studies of the \(\beta\)-galactosidase enzyme system in \(E.\ coli\). There are three genes which code for enzymes involved in the utilization of lactose. All three genes, called structural because they code for the structure of enzyme proteins, are sequentially clustered. Next to the structural genes is
a region of DNA called the operator. All three enzymes corresponding to the three genes appear rapidly and simultaneously when their common substrate is present (57-62). Mutants have been found that are not subject to the normal induction of enzymes by substrate; they produce all the enzymes at a high level regardless of the concentration of the substrate, lactose. These mutants have been shown to be of two types. The first type map at the z gene end of the operon and are called o° mutants (63-66). Such defective operator regions can not function to turn off the synthesis of the enzymes; in fact the defective o locus leaves the system permanently on. The second type of mutation maps some distance from the operon cluster. This other gene (i) is a regulatory gene and when defective, the structural genes are produced even in the absence of the inducer, lactose (67-75). All genes, both structural and regulatory, are transcribed into mRNA molecules. The product of the regulatory gene is a protein repressor which prevents the normal synthesis of the structural genes mRNA by preventing RNA polymerase from binding the operator region (76-79). The regulatory protein in the lactose system is inactivated by the inducer, lactose. However, in the histidine biosynthetic operon the regulatory protein is activated by a corepressor, histidine (80-87).

Since the original statement of the operon theory, studies of the lactose system have advanced to the point
that the operator DNA regions have been sequenced and the protein-nucleic acid interactions studied (88). In addition to the lactose systems, other operons have been found and studied. These include the arginine biosynthesis system (89-90), the arabinose system (91-96), and the tryptophan system (98-100).

Generally, higher organisms do not appear to possess the extent of functional grouping present in bacterial operons. Some gene clusters have been identified in fungi and it appears that in other systems a coordinate control is exerted over the synthesis of related enzymes despite the scattered nature of their genes. A conventional operon for the galactose system has been identified in yeast, but studies with *Neurospora crassa* have shown that gene clusters represent proteins of a multienzyme complexes.

It has been found in *N. crassa* that the genes for histidine biosynthesis are scattered over different chromosomes. However there is a locus which is functionally more diverse than a single gene; this comprises three genes which code for three enzymes of the pathway. Mutants of this locus show a polarized loss of all three of the enzymes. The results can be interpreted as due to the production of a polycistronic messenger which represents the cluster and is translated as such. It has been reported that these proteins are present in a multiple enzyme complex (101-102). There has also been found a cluster of five genes for the
aromatic amino acid pathway which converts dihydroquinololino acid to chorismic acid (103-109). Mutants resulting in the loss of one or more of the enzymes show polarity consistent with a polycistronic mRNA. The product of these genes form a complex. However no regulator or operator region has been found. Thus clustering may be necessary for some function concerned with multienzyme complex formation.

Another system of interest is that which enables *N. crassa* to grow on quinic acid as a carbon source (110-114). Enzymes needed are at low levels in wild type unless induction occurs through quinic acid. Three structural genes are clustered and linked to the regulatory gene. Mutants of the regulator can not be induced by quinic acid. Published data suggests that the regulatory gene codes for a regulatory protein needed for structural gene expression.

Another example of positive control seems to be the leucine system of *N. crassa* (115-117). The three enzymes which catalyse pathway reactions are coded for by unlinked genes, and the level of the enzymes is controlled by the amount of leucine. Mutants which affect the first enzyme result in the loss of the other two activities. This has been explained by a model in which the first enzyme is directly controlled by leucine and its product induces the formation of the second two enzymes. A regulatory gene, *leu*-3 has been found whose mutants can not make the second
or third enzymes and levels of the first enzyme will not reach derepressed levels. This is explained as a gene for a positive control element that turns on structural gene expression.

Of more particular interest to this study are those few studies of control of proteases in fungi. Cohen (118-120) has studied the effect of nitrogen on the protease formation in Aspergillus. He has found that the regulation of extracellular protease synthesis and release appears to be a directly adaptive mechanism; when either carbon, nitrogen, or sulfur is limiting growth, protease synthesis is released from repression and continues until the deficient metabolite is again available. These results contrast with our own and those of Drucker (2, 121) which show that N. crassa requires not only derepression by limiting carbon, sulfur, or nitrogen, but also induction by the presence of a protein. Drucker describes the induction process as involving a zymoprotease which is cleaved to release a zymopeptide that acts as the specific inducer of greater amounts of protease, thus allowing the cells to use external protein. Matile (1) stated that the protease produced under nitrogen starvation in Neurospora is present in the cell in cell particles and is released as needed in a reverse pinocytosis process.
**Materials and Methods**

**Growth of organism.** *N. crassa* wild-type strain 74OR231A of the Oak Ridge genetic background was grown on either Fries minimal salts (122) with varying sulfur sources, or on Vogels minimal salts (123) which was modified by elimination of either the nitrogen or the sulfur sources. Sucrose at a concentration of 1.5% was added as the carbon source except where it was desired to have a carbon limitation for which 0.1% sucrose was employed. The growth medium was supplemented with bovine serum albumin to serve as the sulfur, nitrogen, or carbon source. The mutant strain *cys*−3 (allele P 22) was similarly grown with the addition of methionine. The mutant *inos*− (allele 89601) used in mutant hunt studies was grown on Vogels minimal salts supplemented with inositol. Erlenmeyer flasks (1000 ml or 250 ml) containing 250 ml or 40 ml respectively of medium were inoculated with a filtered suspension of conidia, so that a final optical density at 420 nm of 0.5 was obtained. The flasks were then shaken at 25°C for varying lengths of time, when the extracellular culture fluids were obtained by suction filtration on Whatman No. 1 filter paper to remove the mycelia.

In attempting to isolate a electrophoretic variant seventeen different wild type strains were used (Table 7),
all of which grew very well under inducing conditions and made a protease.

**Protease Assay.** Samples of cell-free filtrate were incubated with 1 ml 2% casein for 20 min at 37°C, when 2 ml of 5% trichloroacetic acid was added to stop the reaction and precipitate any undigested protein. A blank was prepared in the same way but the trichloroacetic acid was added prior to the enzyme. The precipitated protein was removed by centrifugation. The acid-soluble products of the enzymatic reaction were then determined with 1 ml samples of the supernatant fluid by the method of Lowry (124). Activity was found to be linear under the conditions described. Enzymatic activity is reported as the increase in the absorbance at 750 nm; specific activity is reported as the increase in the absorbance at 750 nm per milligram (dry weight) of the mycelial pad under the conditions described.

**Preparation of protease.** The extracellular growth medium was concentrated 20 - 50 fold at 4°C using an Amicon ultrafiltration apparatus with a PM-30 filter. The alkaline protease activity was retained and stable during the concentration process. This concentrated preparation will be referred to as concentrated crude protease. In order to obtain an enzyme preparation free of BSA, the crude protease was applied to a DEAE-Sephadex column (8.0 x 45.0 cm). The protease was eluted with 0.1 M Tris-HCl buffer, pH 8.0,
while the BSA remained bound to the column. The fractions containing protease were combined and concentrated approximately 10-fold. This preparation is completely free of BSA and will be referred to as purified protease, although its homogeneity has not been demonstrated.

**Gel-filtration and column chromatography.** A column (2.6 x 75 cm) of Sephadex G-75 (Pharmacia) was eluted with 0.05 M NaCl; its void volume was 105 ml. This column was used to determine the molecular weight of the protease produced in all three cases of nutrient limitation. Marker proteins used included cytochrome c (molecular weight 12,400), myoglobin (molecular weight 17,800), and hemoglobin (molecular weight 68,000). Concentrated protease was applied to a DEAE-Sephadex column (2.5 x 20 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, and eluted with several column volumes of the same buffer and then eluted with a 0.0 to 1.0 M gradient of NaCl. Protease was similarly eluted from a CM-Sephadex C-50 column (2.1 x 20 cm) using 0.1 M sodium phosphate buffer, pH 6.0, containing a gradient of 0.0 to 1.0 NaCl.

**Esterolytic activity.** The low-molecular-weight artificial substrates used to test for esterolytic activity were BAEE, BTEE, ATEE, and BLEE. A 15 ml sample of the substrate (10⁻³ M in tris(hydroxymethyl)aminomethane hydrochloride, 5 x 10⁻⁴ M) was incubated at 25°C with 0.1 ml of enzyme at various pH values. The volume (ml) of 9.93 x 10⁻³ N NaOH
titrated per minute was measured by using a Radiometer pH-stat assembly (Model TTT11, autotitrator; ABU 12, autoburette).

**Thermal inactivation and inhibitor studies.** Samples of the pure enzyme preparations were incubated in a water bath at 55°C with shaking. At various times, duplicate samples were withdrawn, cooled, and assayed for residual protease activity. For inhibition studies, samples of the pure enzyme were placed into each of two 10 ml beakers and stirred at room temperature. The potential inhibitor was added to one beaker and an equal volume of water was added to the other. Samples were removed from the beakers at various time intervals and assayed for protease activity.

**Electrophoresis.** Cellulose acetate strips (Gelman) were soaked in 0.05 M citrate buffer, pH 5.4, or in 0.05 M sodium barbital buffer, pH 8.0, and blotted with filter paper. A sample (10u1) of concentrated crude protease was applied to the origin at the center of each strip. Sufficient voltage was applied (usually about 300 volts) to obtain a constant current of 2.5 ma per strip. Electrophoresis was carried out in the Gelman apparatus at 0°C, using the buffer systems mentioned above, for varying times. Then the strips were removed and one set stained for protein with 2% Coomassie blue in methanol: glacial acetic acid: water (5:1:5). An identical set of strips was used to reveal protease activity by incubation on skim milk - agar sheets at 45°C for one hour (118). These sheets were prepared by melting 2% agar
In various buffers at either pH 4.0, 5.4, 6.0 (citrate buffer), or pH 8.0 (barbital), or pH 9.6 (glycine) with 1% dry milk. The melted agar-dry milk solution was poured onto glass plates to a depth of 1 mm and allowed to solidify. After incubation with the electrophoresis strips, these sheets were stained with 5% Amido Black 10B in methanol:glacial acetic acid: water (5:1:5) which resulted in clear bands at the site of enzyme activity on an otherwise dark blue background.

**Peptide mapping.** Insulin (2mg/ml) was incubated at 37°C for 20 hours with either pure sulfur, nitrogen, or carbon-controlled protease. The insulin digest was then lyophilized and resuspended in 200 ul of 0.2 M ammonium bicarbonate adjusted to pH 8.0; a 50 ul sample of the digest was applied to Whatman no 3MM filter paper (18.5 x 22 in). The paper was developed by descending chromatography for 17 hours in butanol: glacial acetic acid: water (60:10:40). The high voltage electrophoresis was performed at right angles to the direction of chromatography in pyridine acetate buffer, pH 3.7, for 1.5 hours at 3200 volts. (125). After complete drying, the chromatograms were dipped in a ninhydrin-collidine reagent and dried at 80°C for 10 min. The staining solution contained 600 ml absolute ethanol, 200 ml glacial acetic acid, 80 ml collidine, and 1.0 g ninhydrin (126). Other stains used included the Pauly stain for imidazoles and phenolic compounds. A solution prepared with one gram sulphanillic acid in 100 ml N HCl and an equal volume of 0.7%
aqueous sodium nitrate was sprayed on the paper; the paper
was dried and then sprayed with 10% sodium carbonate. For
detection of cystine, the paper was wetted lightly with a
solution containing 0.5 g sodium cyanide in 2 ml water
diluted to 25 ml with methanol. The paper was then sprayed
with nitroprusside solution prepared by dissolving 250 mg
sodium nitroprusside in 1 ml dilute sulphuric acid, and
then adding 2 ml concentrated ammonium hydroxide and
methanol to 25 ml (126).

**Isolation procedures for protease-negative mutants.** The
mutant enrichment technique, "inositol-less death" was used(127)
in this technique the starting material is an inositol-
requiring strain (inos, allele 89601) rather than wild type.
This strain dies quickly if incubated in minimal medium
without inositol. Mutations are selected efficiently if
they interfere with germination and growth and thus prevent
or forestall the suicidal process. Agar plates with medium
lacking sulfur or nitrogen and lacking inositol, but con-
taining all other nutrients plus a protein, were plated
with UV-irradiated inos- conidia and incubated several days
during which time a majority of the population died. These
plates were then overlaid with minimal agar medium containing
inositol and reincubated. Theoretically those cells which
had sustained a mutation in the protease gene could not
utilize the exogenous protein for growth and thus were
protected from the suicide process of the other inos- conidia.
Several of these hunts were done as described but incubated
at a higher temperature (37°C) in an attempt to find a temperature sensitive mutant. Colonies picked from the above described plates were grown in shaking flasks under conditions of induction. None tested were completely free of protease activity. There was evidence that there were at least two species of enzyme, a stable alkaline DFP-sensitive protease with pH optima at 8.0 and a neutral EDTA labile protease with pH optima at 7.0. Mutant selection procedures were carried out with pH carefully controlled. It was felt that if the neutral protease could be inactivated by pH then mutants of the alkaline protease gene would be selected for in the inositollesstreatment. Again colonies were picked from the plates and tested but none were found completely free of protease although several have been found in which protease production was reduced.
RESULTS

1. STUDIES OF THE SULFUR-CONTROLLED PROTEASE.

Induction-repression of the sulfur-controlled protease.
When wild-type conidia were germinated in Fries medium containing a limiting sulfur supply and a protein, exocellular proteases were found in the medium after 18 hours of growth. However, when a high concentration of sulfur was present in the medium in the form of sulfate or methionine, the proteases were not produced (fig. 2).

The presence of a high-molecular-weight proteinaceous substance was necessary for inducing the protease activity. Small-molecular-weight peptides, mixtures of amino acids, and various proteins were tested for their capacity to induce protease activity (table 4). Only the high-molecular-weight polypeptides were effective as inducers. The ability of a protein to induce proteolytic activity does not seem to correlate with its amino acid composition.

Easily metabolized sulfur sources such as sulfate and methionine repress protease production even in the presence of a protein, whereas poorer sulfur sources such as cysteic acid and S-ethyl cysteine permit enzyme synthesis.

The regulatory mutant, cys-3, does not produce the sulfur-controlled protease even under conditions of induction and repression, and this mutant is unable to
Figure 2. Induction and repression of sulfur-controlled protease.

Wild type conidia were inoculated into 40 ml of medium, and cells were grown as described in the Methods and Materials. Cells were collected at various times, the mycelial mat dried and weighed, and the extracellular media assayed for proteolytic activity.

(□) Fries salts - sulfur + BSA;
(◇) Fries salts + low sulfate (0.1 mM) + BSA;
(▲) Fries salts + high sulfate (2 mM) + BSA;
(○) Fries salts + low sulfate (0.1 mM), no protein.
FIGURE 3
PROTEASE ACTIVITY (ΔO.D.780)
# Table 4

**INDUCTION BY PROTEINS, PEPTIDES, AND AMINO ACIDS**

<table>
<thead>
<tr>
<th>INDUCER</th>
<th>PROTEASE SPECIFIC ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PROTEINS</strong></td>
<td></td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>13.49</td>
</tr>
<tr>
<td>Bovine Serum albumin</td>
<td>5.09</td>
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<tr>
<td>Insulin</td>
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<tr>
<td>Myoglobin</td>
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<tr>
<td>Casein</td>
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<tr>
<td>Hemoglobin</td>
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</tr>
<tr>
<td><strong>POLYAMINO ACIDS</strong></td>
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<td>Polyglutamate</td>
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<tr>
<td>Polyproline</td>
<td>4.58</td>
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<tr>
<td><strong>PEPTIDES</strong></td>
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</tr>
<tr>
<td>Met-Gly-Met-Met</td>
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</tr>
<tr>
<td>Tetrarglcyine</td>
<td>0</td>
</tr>
<tr>
<td>Triglycine</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>AMINO ACIDS</strong></td>
<td></td>
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<tr>
<td>Casamino acids</td>
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</tr>
<tr>
<td>amino acid mixtures</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>NO INDUCER</strong></td>
<td>0</td>
</tr>
</tbody>
</table>

*Conidia were inoculated into 40 ml of media containing low sulfate (0.1 mM) and a different potential inducer in each flask at a concentration of 1 mg per ml. After growth for 20 hours, the mycelial pads were harvested and the media assayed for proteolytic activity. Values are given as specific activity (change in absorbance per mg dry weight mycelial pad).*
utilize an exogenous protein as a sulfur source. Wild type and all other mutant strains tested will grow with their only available sulfur provided as an external protein.

**Inhibitor studies.** Some preliminary studies were undertaken to determine the number and types of extracellular proteases present during sulfur limitation. Inhibition studies were undertaken by using known inhibitors of the three major types of proteases. Diisopropylfluorophosphate (DFP) almost completely inhibited the protease activity (fig. 3). A high concentration (0.01 M) of ethylene-diaminetetracetate (EDTA) inhibited about 50% of the crude protease activity at pH 7.5 (table 5), whereas iodoacetate was not inhibitory, even at a 10mM final concentration. The EDTA inhibition could be reversed with an excess of Ca** ions. These results imply that the major protease species present is a serine protease and suggest that there may also be a minor species that requires a metal ion.

**Esterolytic activity and pH optima.** The protease was tested for esterolytic activity against the low-molecular-weight artificial substrates ATEE, BTEE, BAEE, and BLEE. It was active with ATEE, but not with BLEE or BAEE, and only slightly so with BTEE. The pH optimum of the protease activity was determined by using both casein and ATEE (fig. 4). The activity curve obtained with casein is somewhat difficult to interpret; it likely represents two or more overlapping peaks, but could be one with a broad optimum. However,
Figure 3. DFP Inhibition.

Five ml samples of concentrated crude sulfur-controlled protease were put into each of two 10 ml beakers, and stirred at room temperature. DFP was added to one beaker to a final concentration of $5.1 \times 10^{-4} \text{M}$ and samples were then removed from each beaker at various time intervals. Those were assayed at 37°C with casein.

(○) control;
(□) DFP inhibition.
Table 5. INHIBITION OF PROTEASE ACTIVITY BY EDTA

<table>
<thead>
<tr>
<th>EDTA Concentration</th>
<th>Preincubation time with inhibitor</th>
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</thead>
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<tr>
<td></td>
<td>0</td>
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<tr>
<td>.0001 M</td>
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<td>.001 M</td>
<td>0</td>
</tr>
<tr>
<td>.01 M</td>
<td>30</td>
</tr>
</tbody>
</table>

Inhibition of protease activity

The enzyme was incubated at 37°C with the various concentrations of EDTA. At the indicated times, samples were withdrawn and assayed for proteolytic activity with casein as a substrate.
Figure 4. pH optima of the sulfur-controlled protease.

One ml samples of the crude protease were incubated for 15 min at 37°C at the given pH values, and casein was then added for proteolytic assay (○). Fifteen ml ATEE (10⁻³M in Tris-HCl, 5 x 10⁻⁴M) was incubated at 25°C with 0.1 ml enzyme at the indicated pH values (●). The volume (ml) of 9.93 x 10⁻³N NaOH titrated per min was measured using a Radiometer pH stat assembly (autotitrator model TTT11, auto burette ABU12).
FIGURE 4

PROTEASE ACTIVITY (Δ O.D. 780)

ESTEROLYTIC ACTIVITY (ml NaOH/min)
the narrow pH specificity of esterolytic activity observed with ATEE suggests that a single enzyme with a pH optimum at 8.5 is esterolytic, whereas another protease species without this esterolytic activity may have optima at lower pH values.

**Heat inactivation studies.** Heat inactivation studies showed that when crude enzyme was incubated for 15 min at various temperatures before assay at 37°C, a rapid decrease of activity occurred at temperatures higher than 45°C (fig. 5). The time dependent inactivation at 60°C showed that a 50% loss of activity occurred within 3 min.

**Time course of induction.** When wild type conidia were inoculated into Fries minimal medium containing low sulfate (0.1 mM) and a protein, protease activity began to appear at 18 hours in the extracellular medium, after which its appearance paralleled further growth (fig. 6). However, when conidia were germinated in medium with no sulfur source other than the exogenous protein, the protease activity appeared at much earlier times, approximately 6 to 8 hours (fig. 6). Under both conditions, the activity reached a maximum at 20 to 22 hours and then declined.
Figure 5. Heat inactivation of protease at 60°C.

Samples of the crude, concentrated enzyme were placed into a 60°C water bath. At various times, duplicate samples were withdrawn, cooled in ice, and casein was added to one tube while TCA and casein were added to the other. Protease activity was assayed at 37°C and is plotted as percent remaining activity. The insert shows the thermal inactivation of the protease as a function of temperature. Samples of the enzyme were incubated for 15 min in a water bath at the given temperatures, when they were cooled in ice, and then assayed with casein for protease activity.
Figure 5

% RESIDUAL ACTIVITY

TIME (min at 60°C)

% RESIDUAL ACTIVITY

20 30 40 50 60 70 80 90 100

20 30 40 50 60 70 80 90 100
Figure 6. Time course of protease induction.

Wild type conidia were inoculated into 40 ml of Fries salts minus sulfur + 1 mg/ml BSA (○) or Fries salts + 0.1 mM sulfate + 1mg/ml BSA (□). At the times indicated, the flasks were harvested as described and the proteolytic activities were determined. The dashed lines (dry weight) show the growth of the cultures.

(------) BSA only;
(--------) BSA + sulfate.
11. COMPARISON OF PROTEASES PRODUCED UNDER THREE DIFFERENT GROWTH CONDITIONS.

Enzyme stability and inhibition profiles. The pure extracellular alkaline protease synthesized in response to a limitation of sulfur, nitrogen, or carbon in each case shows good stability and retains nearly full activity for at least two weeks at 20°C. The enzymes showed an identical thermal lability at 55°C each with a half life of about 15 min and inactivation kinetics which indicated that a single protease species was present (fig. 7).

Inhibitors of the three major types of proteases were studied for their effect upon a pure preparation of the enzyme synthesized under each of the three different conditions. The inhibition profiles are identical and show 90% inhibition by 0.1 mM PMSF within 5 min (fig. 8) but no detectable inhibition by either EDTA (10mM) or iodoacetate (100mM).

Gel filtration and chromatography. The sulfur-controlled protease was concentrated by using an Amicon ultrafiltration cell and subjected to gel filtration with Sephadex G75. The proteolytic activity eluted as a single component and was separated from the BSA present in the medium as inducer. The column had been calibrated with marker proteins of known molecular weight. When all three crude protease preparations were subjected to gel filtration using Sephadex G75, the
Figure 7. Thermal Inactivation of the protease.

Samples of the pure enzyme were inactivated at 55°C for various times when duplicate samples were withdrawn and assayed for enzyme activity. The slight variability observed between samples is no greater than that obtained with multiple determinations using the same enzyme preparations.

(●) sulfur-controlled protease;
(□) nitrogen-controlled protease;
(○) carbon-controlled protease.
Log % Inactivation vs. Time at 55°C (min)

Figure 7
Figure 8. PMSF inhibition of the proteases.

Five ml samples of purified protease were put into each of two 10 ml beakers, and stirred at room temperature. PMSF was added to one beaker to a final concentration of 0.1 mM and samples were then removed from each beaker at various time intervals. These were assayed at 37°C with casein.

(□) control,
(○) sulfur-controlled enzyme,
(△) nitrogen-controlled enzyme,
(◊) carbon-controlled enzyme.
% REMAINING ACTIVITY

TIME (min)

FIGURE 8
enzyme in every case was eluted as a single peak (fig. 9) and exactly in the same position, corresponding to a molecular weight of 31,000 (fig. 10).

Identical chromatographic behavior of the proteases synthesized under the three different metabolic conditions was also obtained with both DEAE-Sephadex and CM-Sephadex. When a crude enzyme preparation was applied to a column of DEAE-Sephadex, all of the proteolytic activity was eluted as a single peak with 0.1 M Tris-HCl buffer, pH 8.0, while the BSA present in the medium as the inducer remained firmly bound to the column (fig. 11). Furthermore, a linear gradient of 0.0 to 1.0 M NaCl released the BSA at approximately 0.6 M NaCl but failed to release any additional peaks of protease activity. This pattern was observed in all three cases.

A mixture of the three pure protease preparations was applied to a CM-Sephadex column. All of the enzyme activity was eluted in a single peak with a linear gradient of NaCl (0.0 - 0.5M) (fig. 12).

Electrophoresis. Electrophoresis at two different pH values (5.4 and 8.0) followed by the demonstration of protease activity at four pH values (4.0, 6.0, 8.0, and 9.6) revealed a single sharp band of protease activity in each case and precisely coincident migration of the enzyme formed under all three conditions (fig. 13). No activity could be demonstrated at pH 4.0 in agreement with the previously reported pH dependence of the sulfur-
Figure 9. Gel filtration of the protease.

A two ml volume of concentrated crude protease induced under each of the various conditions was placed on a Sephadex G75 column in each case. Fractions of 2.4 ml were collected and assayed for protease activity and for protein concentration. Myoglobin was added as a marker protein.

(-----) protease activity;
(----------) protein concentration;
peak A is BSA;
peak B is myoglobin.
Figure 10. Determination of molecular weight of protease.

Two ml of concentrated sulfur-controlled protease were placed on the G75 Sephadex column. Fractions of 2.4 ml were eluted with 0.05M NaCl and assayed for protein and protease activity. Marker proteins of known molecular weight were used to calibrate the column.
**Figure 10**

Log molecular weight plotted against elution fraction for hemoglobin, protease, S-controlled, myoglobin, and cytochrome C.
Figure 11. DEAE-chromatography of proteases

Pure protease was applied to a DEAE-Sephadex column (2.5x 20 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, and eluted with several column volumes of buffer and then eluted with a NaCl gradient (0.0-1.0 M).

(---) protease activity;
(--->--) protein.
FIGURE 11

PROTEASE ACTIVITY (ΔOD₇₅₀)

PROTEIN (mg/ml)

SULFUR

NITROGEN

CARBON

M CONC NaCl

PROTEIN (mg/ml)
Figure 12. CM-Sephadex chromatography

A mixture of purified proteases was applied to a CM-Sephadex column (2.1 x 20 cm) equilibrated with 0.1M sodium phosphate buffer, pH 6.0, and eluted with several column volumes of buffer. Then a gradient of NaCl (0.0 - 0.5 M) was applied.

(---) protease activity.
Figure 13. Electrophoresis of the extracellular protease.

Electrophoresis of concentrated crude protease samples was carried out at pH 5.4 (citrate buffer) and pH 8.0 (barbital buffer), and protease activity was demonstrated as described in the text.

Part A is a schematic diagram of the incubation pattern obtained after incubation of the strips at pH 6.0. Part B is a contact print of the agar-dry milk plate after incubation at pH 6.0, revealing the bands of protease activity for carbon-, nitrogen-, and sulfur-controlled proteases.
RUN AT pH 8.0  |  RUN AT pH 5.4
-------|-------|-------|-------|-------|-------|
INCUBATED AT pH 6.0 | INCUBATED AT pH 6.0
---|---|---|---|---|---|
Carbon | Nitrogen | Sulfur | Carbon | Nitrogen | Sulfur
---|---|---|---|---|---|
(-) Pole | Protease | Origin | BSA | (+) Pole
---|---|---|---|---|---
FIGURE 13 B: Contact print
controlled protease when assayed with casein (127).

**Catalytic activity of the protease.** The extracellular protease synthesized under the three metabolic conditions also shows an identical catalytic specificity. Using insulin as the substrate for hydrolysis by the protease, an identical and well resolved peptide map was obtained in each case. Figure 14 is a diagramatic representation of the peptide map obtained with the pure carbon-controlled protease and stained with the ninhydrin-collidine reagent. Figures 15 and 16 display the patterns obtained with the sulfur- and nitrogen-controlled proteases. An identical pattern in terms of the relative positions and distinctive colors of the major spots was obtained in fingerprints produced with each of the proteases. Similar peptide maps were instead more specifically stained so as to reveal only these peptides containing cysteine (nitroprusside reagent) or those with either histidine or tyrosine (Pauly reagent); exactly the same number and position of spots was found when the maps of the digestion products of the three enzymes were compared.

**Induction-repression of extracellular protease.** When an exogenous protein serves as the sole sulfur source for growth, the extracellular protease is synthesized and secreted into the medium beginning within 6 hours and follows a pattern, with time, of rise and decline in activity (127). A similar induction curve also occurs when
Figures 14, 15, 16. Peptide maps of insulin following protease hydrolysis.

Insulin digest of the three purified proteases were prepared and analyzed as described in the text. The following figures are diagrams of the peptide maps obtained with each of the protease preparations.

Figure 14, carbon-controlled enzyme,

Figure 15, sulfur-controlled enzyme,

Figure 16, nitrogen-controlled enzyme.
a protein in the medium constitutes either the sole nitrogen or carbon (fig. 17). In every case the extracellular protease appears only in the presence of an exogenous protein. It was not clear, however, whether the apparent induction evoked by the exogenous protein required de novo protein synthesis or was instead simply a release of pre-existing enzyme. Wild type cells were grown in medium containing a low concentration of inorganic sulfate for 15 hours and then transferred to medium completely lacking sulfur with no additions or containing BSA in the presence and absence of cycloheximide. It is obvious from the results shown in figure 18 that complete starvation in the absence of an exogenous protein does not result in protease production and also that protein synthesis is required for the appearance of the extracellular enzyme. This result suggests that induction involves de novo synthesis of the protease although a requirement for protein synthesis might exist in the secretion step.

The cys-3* control gene appears to produce a positive signal which is required for the synthesis of a number of enzymes of sulfur metabolism, cys-3 mutants having a pleiotropic loss of this entire family of enzymes (4). Cys-3 conidia were inoculated and allowed to grow in minimal media containing BSA but otherwise lacking either sulfur, nitrogen, or a carbon source. This mutant strain failed to grow or produce an extracellular protease during sulfur limitation but it did grow and formed the enzyme when
Figure 17. Induction of the three proteases.

Wild type conidia were inoculated into 40 ml of media. At the times indicated, the flasks were harvested as described and the proteolytic activities were determined.

(□) minimal media minus sulfur plus BSA;
(○) minimal media minus nitrogen plus BSA;
(△) minimal media minus carbon source plus BSA.
Figure 17
Figure 18. Time course of induction of protease.

Appearance of protease activity after wild-type conidia were grown in minimal medium containing 0.1 mM potassium sulfate for 15 hours before transferring cells to:

(○) minimal medium lacking sulfur,
(●) minimal medium plus BSA,
(□) minimal medium plus BSA plus cycloheximide.

Samples were removed at various times and assayed for protease activity.
limited for either nitrogen or carbon. When the \textit{cys-3} mutant is grown in medium containing a limiting amount of methionine plus an exogenous protein, it likewise fails to produce the protease although wild type cells do synthesize the enzyme under identical conditions (fig. 19).

The \textit{nit-2} locus of \textit{Neurospora} appears to be a regulatory gene involved in the control of nitrogen metabolism; a \textit{nit-2} mutant lacks nitrate reductase, allantoinase, and perhaps other related enzymes (Reinert and Marzluf, unpublished data). I have found that \textit{nit-2} is also incapable of synthesizing the extracellular protease or of utilizing an exogenous protein for growth when limited for nitrogen but grows normally and produces the protease when limited for either sulfur or carbon (fig. 20). Thus, the \textit{nit-2} mutant appears to be a regulatory gene analogous to \textit{cys-3} except that the former controls enzymes of nitrogen metabolism.

\textbf{Isolation of protease mutants}

Various mutant hunt procedures were undertaken in order to find a structural gene mutation for the protease gene. It was felt that if such a mutant could be isolated based on selection under one type of growth condition such as sulfur limitation, and it did not make protease under the other two conditions of growth, it would provide strong evidence that there was indeed only one structural gene and not multiple genes, each under a different type of control. No mutants completely lacking the extracellular
Figure 19. Induction of protease in the cys-3 mutant.

Cys-3 conidia were inoculated into flasks containing 40 ml of media and permitted to grow for various times when the extracellular medium was collected and assayed for protease activity.

(●) Minimal medium minus nitrogen plus BSA and 0.25 mM methionine;
(□) Minimal medium minus sulfur plus BSA;
(○) Minimal medium minus any carbon source plus BSA and 0.25 mM methionine.
Figure 19

TIME OF GROWTH (hr)
Figure 20. Induction of protease in the *nit-2* mutant.

*Nit-2* conidia were inoculated into flasks containing 40 ml of media and permitted to grow for various times when the extracellular medium was collected and assayed for protease activity.

(□) minimal medium minus sulfur plus BSA,
(○) minimal medium minus nitrogen plus BSA,
(△) minimal medium minus carbon source plus BSA.
FIGURE 20

PROTEASE ACTIVITY ($\Delta OD_{750}$)

TIME (hr)

0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
protease were isolated; however several colonies were picked which in shaking culture showed a reduced amount of protease when compared to wild type grown under the same conditions (table 6). These mutants may be structural gene mutations of the protease gene. However, because of the partial protease activity remaining, perhaps because there are two proteases, it was not possible to map these mutants genetically. There was no way to test the progeny for the mutant gene because its effect was masked by the presence of the other protease. An alternative is to find a double protease mutant. At the time of this writing no such mutant has been isolated although some thirty different mutant hunts of various types have been done. Any mapping of the mutants will be complicated and involve some assay which permits a biochemical distinction of the two proteases for each of the progeny from various crosses.

**Electrophoretic variation of the alkaline protease among various wild type strains.** Seventeen different wild type strains (table 7) of *N. crassa*, all of which grow on minimal medium at 25°C, were grown under all three inducing conditions for protease; that is, with a sulfur, carbon, or nitrogen limitation plus a protein. The cells were harvested after 22 hours as previously described and the extracellular medium was electrophoresed at pH 8.0 and the protease activity was demonstrated as previously
<table>
<thead>
<tr>
<th>Mutant designation</th>
<th>OD750</th>
<th>Wet weight</th>
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<tbody>
<tr>
<td>Wild type</td>
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<td>1.107</td>
</tr>
<tr>
<td>2N</td>
<td>0.326</td>
<td>0.037</td>
</tr>
<tr>
<td>8N</td>
<td>0.305</td>
<td>0.036</td>
</tr>
<tr>
<td>13N</td>
<td>0.461</td>
<td>0.165</td>
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<tr>
<td>16N</td>
<td>0.567</td>
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<tr>
<td>18N</td>
<td>0.292</td>
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<td>25N</td>
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</tr>
<tr>
<td>30N</td>
<td>0.276</td>
<td>0.033</td>
</tr>
</tbody>
</table>

**Conditions of growth:** Conidia were inoculated into 40 ml of minimal media minus nitrogen plus BSA and grown in shaking culture. The extracellular media was collected as previously described and assayed for proteolytic activity.
<table>
<thead>
<tr>
<th>Strain</th>
<th>FGSC No</th>
<th>OD750</th>
<th>Dry weight</th>
</tr>
</thead>
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<tr>
<td>Abott</td>
<td>1228</td>
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<tr>
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<td>0.0944</td>
</tr>
<tr>
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<td>851</td>
<td>0.980</td>
<td>0.1175</td>
</tr>
<tr>
<td>Groveland-1c</td>
<td>1945</td>
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<td>0.1434</td>
</tr>
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<td>0.860</td>
<td>0.0813</td>
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<td>Tatum</td>
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<tr>
<td>Emerson</td>
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<td>0.1601</td>
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</table>

Growth conditions: Minimal salts minus nitrogen plus BSA (40 ml media in 250 Erlenmeyer) were inoculated with conidia and grown 20 hours in shaking cultures. The extracellular media was collected and assayed as described.
described. All of these wild type strains produce the protease and can utilize an exogenous protein for growth. The protease in every case had an identical electrophoretic mobility with one exception, Groveland-1c (FGSC 1945). This strain was found to possess an electrophoretic variant of the alkaline protease. The protease of Groveland-1c varies from that of the wild type strain 74-OR23-1A routinely used, by migrating toward the anode 3 mm more under the conditions employed. Furthermore, a difference in migration distance between the proteases of 1 cm was obtained by using 0.05 M acetate buffer, pH 5.4.

The electrophoretic mobility of the protease formed by Groveland-1c when limited for carbon, nitrogen, or sulfur was examined. The same electrophoretic variant was produced during the three different induction conditions (Fig 21). This result strongly implies that there is only one structural gene for the alkaline protease since if multiple copies existed one would clearly not expect the same type of mutation to have taken place in each of the three separate genes.
Figure 21. Electrophoretic variant of the alkaline protease.

Wild type FGSC 740R23 and wild type FGSC 1945 were grown under inducing conditions and the extracellular media collected as previously described. The media was electrophoresed using sodium barbital buffer (0.05 M) pH 8.0 and incubated on agar-dry milk sheet at pH 8.0 as described. A reproducible difference of 3mm could be seen between the proteases of the two strains. The figure is a diagram of the pattern obtained after incubation of the agar-dry milk sheets.
RUN AT pH 8.0
INCUBATED AT pH 8.0

CARBON SULFUR NITROGEN CARBON SULFUR NITROGEN
WT 740R23 WT 740R23 WT 1945 WT 1945 WT 1945

ORIGIN

PROTEASE

FIGURE 21

(+)

(-)
DISCUSSION

It is clear that wild type *Neurospora crassa* can synthesize extracellular proteolytic enzymes under conditions of limiting sulfur and in the presence of an inducing high molecular weight protein. The synthesis of this protease is repressed by adequate methionine or sulfate in the growth medium; protease synthesis is also regulated by the cys-3 control gene which is known to exert a positive control over the synthesis of a number of related enzymes of sulfur metabolism. Furthermore, I have demonstrated as Drucker and Matile have reported that protease activity appears in the extracellular media when the *Neurospora* cells are limited for carbon or nitrogen and given an inducing protein. This system of turning on the synthesis or release of the extracellular protease provides the cells with a means of overcoming their starvation by use of the protein for their carbon nitrogen, or sulfur needs. That this is not simply a general response to starvation conditions is shown by the fact that protease is not produced during starvation for other metabolites such as amino acids.

It seems that a mixture of three proteases is found during sulfur limitation. This is suggested by the pH curve that shows some activity below pH 4.0 and seems to have two peaks, one neutral and one alkaline.
Also, inhibition data show that there is an EDTA-sensitive protease and a DFP-sensitive protease. Considering what is known about protease types in general, it seems likely that the DFP protease has the esterolytic activity against ATEE at pH 8.0. Drucker also has suggested that three proteases are present in carbon limitation. The EDTA-sensitive neutral protease is extremely labile and its activity is completely lost during the concentration step. Thus the comparisons of the more stable alkaline proteases could be done without complicating presence of this second protease. Furthermore none of the conditions used would allow the acid protease to be active. Whether these other protease species are subject to the same controls remains to be demonstrated.

It is most interesting that an exogenous protein is required to induce the formation of the protease. It seems highly unlikely that such high-molecular-weight polypeptides are capable of entering the cells; furthermore, neither peptides nor amino acids are effective as inducers. Yet, the cells must somehow recognize the presence of the exogenous protein, because very little or no protease activity appears in its absence. The external protein may be required for the secretion of the protease rather than in its synthesis. Conceivably, the protease is membrane bound at the cell surface, perhaps in an inactive precursor state, and its release somehow involves the interaction of an exogenous protein. In this way, the protein could indirectly control the synthesis of the
protease by its involvement in the secretion step, provided that synthesis and secretion are coupled in some manner. Drucker also interpreted the induction of the carbon-controlled protease in this way. On the other hand, Matile has suggested that the nitrogen-controlled protease of Neurospora is localized within particles inside the cell. Another possible explanation as to why proteins, but not peptides, can induce protease is that the enzyme is secreted in both cases when sulfur is limited, but the exogenous protein serves to stabilize the enzyme, whereas peptides do not. This possibility is not considered to be likely. When the protease activity is separated from the exogenous inducer by using G-75 Sephadex, the protease component will remain active for several days.

The extracellular protease synthesized under each of the three different conditions appears to be identical by a number of biochemical criteria which include gel filtration, ion exchange chromatography, inhibition profiles, thermal inactivation, electrophoresis, and the pattern of its catalytic hydrolysis of insulin. If each of the three metabolic limitations were to signal the synthesis of an individually regulated and separate alkaline protease species, the enzymes produced in the three cases would almost certainly differ from one another. The results presented here imply rather that a single structural gene for the protease is controlled in a complex fashion and is activated by each of the three distinct metabolic signals. On the basis of information presently
available, we cannot yet exclude the alternative possibility that several nearly identical structural genes exist and are independently regulated; however, since such multiple genes should have diverged during evolutionary history and now encode protease species with different characteristics, this possibility is considered unlikely. In addition the existence of an electrophoretic variant of the protease that has the identical variation under all three conditions is strong evidence that there is only one structural gene. The probability that, in multiple copies of a gene, the very same mutation occurred in all three copies is very low. Figure 22 presents a model which can satisfactorily explain all of the available information and which we suspect may actually represent the correct mechanism for the regulation of this enzyme. In this model, a single structural gene, designated *prt*, encodes the extracellular protease. Adjacent to the structural gene are situated three receptor sites, possibly within a promoter region, for positive regulatory signals, one each for the sulfur, nitrogen, and carbon status of the cell. Unlinked to the structural gene, and most likely unlinked to each other, we postulate the existence of three independent positive regulatory genes each of whose products have affinity for one of the specific receptor sites as well as individual sensitivity to the pertinent repressor metabolite which reflects either the sulfur, nitrogen, or carbon status of the cell. These repressor metabolites are probably cysteine, $NH_4^+$, and either glucose or cyclic-AMP for the sulfur, nitrogen, and
Figure 22. Proposed model for regulation of extracellular protease in *Neurospora*.

"S" represents the *cys*-3 regulatory gene and "N" may be the *nit*-2 locus. The other genetic loci are as yet unidentified. $R^S$, $R^N$, and $R^C$ are the receptor sites postulated to exist in a promoter region adjacent to *prt*, the structural gene for the enzyme. Negative effectors are cysteine, $NH_4^+$, and either glucose or c-AMP for repression of sulfur, nitrogen, and carbon enzymes, respectively.
Figure 22
carbon signals, respectively. According to this mechanism, a regulatory macromolecule from any one of the three different control circuits would act at its specific receptor in the promoter region and would be sufficient to activate the structural gene for expression and subsequent synthesis of the protease. Accordingly, a strain with a loss of one regulatory signal by mutation, e.g., the sulfur signal, would still be capable of synthesis of the protease by another metabolic signal, e.g., the nitrogen signal. The expression "regulatory circuit" was chosen to permit a distinction between situations involving multiple regulatory signals which are an integral part of one complex control system and multiple signals which in fact arise from separate control systems or circuits. A single regulatory circuit could well include multiple control genes and effectors but they would all be closely interrelated and serve to control a group of related enzymes in a parallel manner. To illustrate, the regulatory system which controls the synthesis of enzymes for sulfur metabolism would constitute a regulatory circuit and is conceived to be independent of a second circuit responsible for the control of nitrogen metabolism, although some degree of interaction between the circuits would be possible. In this regard, it is important to note that the structural gene for the extracellular protease is not simply subject to multiple control signals but to multiple signals which arise from distinct regulatory circuits.
The available genetic evidence, although incomplete, also supports the concept of a single structural gene which is regulated in a complex fashion. The \textit{cys}-3 regulatory mutant, which has been studied in some detail, lacks a positive signal which controls the synthesis of a number of enzymes of sulfur metabolism which include aryl sulfatase, choline sulfatase, cholin-0-sulfate permease, and two sulfate permease species. This same \textit{cys}-3 mutant cannot synthesize the extracellular protease during sulfur limitation, but still produces the enzyme in response to either nitrogen or carbon starvation. Thus, the \textit{cys}-3 gene corresponds to the positive regulatory gene labeled "$S$" in the model, and in fact, is part of the basis for designing the model in this way. The regulatory macro-molecule produced by \textit{cys}-3 is presumed to activate the many unlinked structural genes which it controls, only one of which is \textit{prt}. The structural gene for the protease is distinctive, however, in that it also appears to be regulated by both a nitrogen and a carbon signal. The \textit{nit}-2 mutant has not been studied in as much detail but its characteristics suggest that it represents a positive control gene for nitrogen metabolism designated "$N$" in the model; the \textit{nit}-2 mutant lacks nitrate reductase and allantoinase and cannot synthesize the extracellular protease in response to nitrogen limitation. We predict that regulatory mutants analogous to \textit{cys}-3 and \textit{nit}-2 but specific for carbon regulation should exist. Finally, it should be noted that these major regulatory circuits
probably involve more than a single regulatory gene and a repressor metabolite and may require the interaction of several related control genes within any given circuit. In this regard, it is known that in addition to *cvs-3*, another control gene, known as *scon*, plays a significant role in the regulation of sulfur metabolism. It already seems clear that positive regulatory genes are common in *Neurospora*.

The picture which is emerging for the control of the extracellular protease and for other enzymes in *Neurospora* suggests that complex regulatory circuits exist in this organism and may point to their general presence in eukaryotes. This complexity in regulation may be related to the presence of repetitive DNA in higher forms - a regulatory role has often been suggested for repetitive DNA (128). Indeed, the theoretical regulatory mechanisms suggested for higher organisms by Britten and Davidson (129) were prompted largely as a result of their appreciation of repetitive DNA and include complex interacting systems for regulation with similarities to the model proposed here for the control of an enzyme in *Neurospora*. In this regard it is clearly of interest that the chromosomes of *Neurospora* possess both histones (130) and repetitive DNA (131). It can be anticipated that such complex control circuits may be vital to the time and tissue specific differentiation which occurs in higher organisms.
BIBLIOGRAPHY


