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The relationship of the extension of hyphal tips to secretion was examined in several strains of Neurospora crassa. Cellulase, an extracellular enzyme, was chosen as an index of protein secretion. The plan of this thesis was to examine the following points related to secretion:

1. Simplification of the assay of cellulase activity by improving existing methods and by originating other methods.

2. Determination of the number of cellulases present in induced mycelial and conidial cultures.

3. Study of factors which influence the rate of tip extension.

4. Determination if tip extension is necessary for secretion.

As part of the assay of cellulase activity, improvements were made in the Somogyi reducing sugar method. Several limitations of this method were identified including the inhibitory effect of high protein concentrations and the inhibitory effect of Vogel's and of Fries media.

The Worthington Glucostat reagent, in combination with excess β-glucosidase, was used to develop a spot test for cellulase activity and to determine the location of cellulase activity for thin-layer gel separations. An additional variation of the Glucostat method was developed using homovanillic acid rather than the standard Glucostat chromogen for a fluorescent determination of cellulase activity.

A qualitative cellulase assay was developed which employed the use of hydroxyethyl cellulose as substrate. The substrate solution was blended vigorously to yield a thick foam. Cellulase activity was indicated by the clearing of the cellulose foam.

Column gel filtration studies indicated the presence of one major cellulase in these enzyme preparations. Preliminary molecular weight studies with thin-layer gel filtration indicated this cellulase to have a molecular weight of less than 50,000 daltons.

Various cellulase induction experiments were conducted with both conidia and mycelia to determine the factors which influence the rate of tip extension and to determine if tip extension is necessary for secretion. Four systems of induction were employed to alter the rate of tip extension including the use of auxotrophs requiring components necessary for normal membrane synthesis, the use of various levels of a carbon source, the use of a Fries minimal medium, and the use of a colonial temperature-sensitive mutant. In addition, the phenomenon of agglutination of growth and its correlation with secretion was examined. However, no conclusive relationships were determined among secretion, tip extension, and agglutination.

INVESTIGATION OF THE RELATIONSHIP OF TIP EXTENSION AND CELLULASE SECRETION IN *NEUROSPORA CRASSA*

by

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APPROVAL PAGE

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INTRODUCTION

Cytological studies have been conducted to determine the mechanism of hyphal elongation in the filamentous fungi. These studies have led to a hypothesis that cytoplasmic vesicles fuse with the plasmalemma of the hyphal tips adding their surface area to the plasmalemma and emptying their contents into the wall region (McClure, Park, and Robinson, 1968; Calonge, Fielding, and Byrde, 1969; Brenner and Carroll, 1968; Chung and Trevithick, 1970; Grove and Bracker, 1970; Grove, Bracker, and Morré, 1970). Attempts have been made to ascertain the contents of these vesicles. Proposals are that they may contain cell wall components such as polysaccharides (Pickett-Heaps, 1968; Grove et al., 1970), enzymes which are responsible for a balance of lysis and synthesis of cell wall components (Grove et al., 1970; Matile, Cortat, Wiemken, and Frey-Wyssling, 1971; Bartnicki-Garcia and Lippman, 1972), and enzymes which may become extracellular or wall bound (Calonge et al., 1969; Chung and Trevithick, 1970; Moor and Girbardt separately as cited by Grove et al., 1970). The proposal that tip extension may be related to secretion of enzymes has been examined in this study. A review of the literature concerning hyphal tips and secretion is presented below.

In the apical zone of the filamentous fungi, there is an accumulation of unit membrane-bound cytoplasmic vesicles. These are usually present with the exclusion of all other organelles. These fungi are typically seen to have two types of vesicles which are known

only to differ in size (Grove, Bracker, and Morre, 1967; McClure et al., 1968). Smaller microvesicles are often clustered in a circular zone in the hyphal apex. Larger vesicles, termed cytoplasmic vesicles, are found outside this circular zone. It is thought that the Spitzenkörper of the septate fungi seen in light microscopy is equivalent only to the circular zone of microvesicles and not to the total accumulation of apical vesicles (Grove and Bracker, 1970).

Grove et al. (1970; Grove, Morré, and Bracker, 1967) have described the endomembrane system of hyphae of Pythium ultimum. They have observed the relationship between the nuclear membrane, the endoplasmic reticulum, the Golgi apparatus, and cytoplasmic vesicles. This system is apparently a three-dimensional network of vesicles and tubules, and is continuous both intracellularly and with the unit membrane of the plasmalemma. Cytoplasmic vesicles which were observed to bud off the cisternae of the Golgi apparatus were seen to fuse with the unit membrane of the plasmalemma and to liberate their contents into the wall region. Chung and Trevithick (1970) suggest that in Neurospora crassa these vesicles are involved in the formation of new cell wall and cell membrane and also in the discharge of secretory materials such as invertase and other extracellular enzymes.

An additional feature of Neurospora crassa hyphae is the presence of small pores embedded in the wall matrix which appear to consist of one or more pairs of lines bounded by protein (Manocha and Colvin, 1967). Chang and Trevithick (in preparation) propose that these pores are passages for transport of extracellular enzymes through the cell walls and that the large pores at the apex are continuously

made smaller by the addition of secondary substance allowing certain macromolecules to be trapped in the cell walls basipetal to the apex.

The purpose of this study was to examine possible relationships between secretion and extension of hyphal tips in several strains of Neurospora crassa. Cellulase, an extracellular enzyme, was chosen for study as an index for secretion. An extracellular enzyme is defined to be one which is liberated from the cells into the external medium during the process of germination and growth. Cellulase is included in the cellulase, β-glucosidase system previously studied in this laboratory (Eberhart, Cross, and Chase, 1964; Mahadevan and Eberhart, 1964a; Mahadevan and Eberhart, 1964b). Eberhart and Beck (unpublished) have shown cellulase levels of induced cultures to be greater in the extracellular medium than inside the induced cells.

Cellulase occurs as a complex system in fungi (King and Vessal, 1969). The complete cellulase system found in the cellulolytic fungi (fungi capable of hydrolyzing solid cellulose) is composed of three major parts: (1) a C_1 enzyme capable of hydrolyzing highly oriented solid cellulose, (2) a complex of β-1→4 glucanases (cellulases or C_x) capable of hydrolyzing soluble cellulose derivatives, and (3) β-glucosidases capable of cleaving smaller oligomers and dimers. The C_1 enzyme has not been found in the apparently non-cellulolytic Neurospora crassa. However, the other two components, β-1→4 glucanases and β-glucosidases, have been discovered in Neurospora crassa (Eberhart et al., 1964). In several fungi, the β-1→4 glucanases have been shown to exist as multi-component systems (Li, Flora, and King, 1965; Eriksson

and Rzedowski, 1969a; Eriksson and Rzedowski 1969b) and may also exist as a multi-component system in Neurospora crassa. Two cellulases were indicated in mycelial extracts of Neurospora crassa (Eberhart et al., 1964), but the exact number and physical properties of the cellulases in Neurospora crassa has not been determined. The activity of the cellulase of Neurospora crassa upon the substrate has been shown to be mainly on the internal linkages of the soluble cellulose poylmers indicating the endoenzyme nature of the cellulase (Chase, 1963; Eberhart et al., 1964). It was this incompletely defined group of extracellular β-1→4 glucanases with endocellulase activity which was chosen to be the index for levels of secretion.

The plan of this thesis was to examine the following points related to secretion and to this particular index of secretion:

1. Simplification of the assay of cellulase activity by improving existing methods and by originating other methods.

2. Determination of the number of cellulases present in induced mycelial and conidial cultures.

3. Study of factors which influence the rate of tip extension.

4. Determination if tip extension is necessary for secretion.

To investigate the last two points, induction experiments were conducted during these studies. Several strains were used in the course of these experiments. Four different systems were examined to find one which would show some measureable correlation between tip extension and cellulase secretion. These four systems varied in the mode of attempting to control tip extension. The systems and the questions to be examined are briefly described as follows:

1. What are the relationships among the levels of supplemental components for auxotrophs which require these components for normal membrane synthesis, the amount of tip extension, and the levels of secretion?

2. What are the relationships among levels of a carbon source, amount of tip extension, and levels of secretion?

3. What is the relationship of the media used for induction to the amount of tip extension and the levels of secretion?

4. What is the relationship between the number of actively elongating tips of the colonial mutant and the levels of secretion?

Time did not permit the completion of study of all of the above questions. A large portion of the work of this study was devoted to improving and to developing cellulase assays. Much remains to be done to establish the relationship between hyphal elongation and secretion.

MATERIALS AND METHODS

Chemicals

D (+) xylose, i-inositol, and homovanillic acid were purchased from Nutritional Biochemical Corporation. Carbowax - Polyethylene Glycol Compound 20-M was obtained from Union Carbide. Bio-Gel P-100 was from Bio-Rad Laboratories; N-Z Case from Sheffield Chemical; Bacto-Agar from Difco Laboratories; sucrose from Savannah Foods and Industries, Inc.; 4-methyl-umbelliferyl-β-D-glucopyranoside (umbelliferone) from Mann Research Lab; glacial acetic acid from Carco Chemical Company; Lyphogel from Gelman Instrument Company; and Glucostat from Worthington Biochemical Corporation. Trishydoxy-methylaminomethane (Tris) and β-glucosidase were products of Sigma Chemical Company. Glucose, potassium sodium tartrate, and sodium bicarbonate were purchased from Matheson Coleman and Bell. Bio Cert yeast extract, glycerol, ethylene glycol, and sodium sulfate were obtained from Fisher Scientific Company. Choline chloride and cupric sulfate were purchased from Merck and Company, Inc. Cellobiose and p-nitrophenyl-β-D-glucopyranoside (PNPG) were obtained from Calbiochem. Potassium phosphate, anhydrous sodium carbonate, and sodium arsenate were products of Mallinckrodt Chemical Company. Citric acid, ammonium sulfate, concentrated sulfuric acid, ether, and chloroform were from J. T. Baker Chemical Company.

Selection of Strains

The strains used in these studies were transferred from silica gel cultures except *chol-2* which was obtained from the Fungal Genetics Stock Center (FGSC). The colonial nutritional mutants used were found with auxanographic and minimal tests to be totally blocked in the synthesis of either inositol or of choline. Minimal tests were periodically conducted with these strains to insure that the strains had not reverted to wild type. Occasional transfers from silica gel insured culture continuity; however, there was no silica gel culture of the *chol-2* mutant. During the latter part of these studies the *chol-2* strain began to grow increasingly poorly on successive transfers and was abandoned. A colonial temperature-sensitive mutant was used in the latter part of these studies. The wild type strains often used as controls were STA-4 (St. Lawrence wild type) and 74-OR23-1A (Oak Ridge wild type). The strains and their origins are summarized in Table 1.

TABLE 1

STRAINS USED IN STUDY

FGSC No. or Most Common	Genotype	Origin of Stock
STA-4	St. Lawrence Wild Type	D. N. Perkins
74-OR23-1A	Oak Ridge Wild Type	D. D. Perkins
11-1A	*inos*	Rockefeller Institute
37401	*inos*	FGSC
5, 47904	*chol-2*	D. D. Perkins
33(2-6)A	*gluc-1*$^{+}$,*cot*,*ylo*	B. M. Eberhart

Growth of Strains for Transfer

All strains, both wild type and mutant, were maintained for weekly transfer at room temperature on slants of 8 ml glycerol sucrose complete medium supplemented with inositol and choline. This media contained 50 µg/ml of both inositol and choline, 0.1% N-Z Case, 0.35% Bio Cert yeast extract, 1.5% agar, 1% vitamin stock solution, 0.4% sucrose, and 0.1% Vogel's minimal salts (Vogel, 1956). The inositol- and choline-requiring strains had the appearance of wild type growth with these levels of inositol and choline. After the chol-2 mutant was abandoned, the extra choline was no longer added to the media for growth of the remaining strains.

Growth and Harvest of Conidia for Induction

Conidia for induction experiments were grown vegetatively in 250 ml Erlenmeyer growth flasks containing 50 ml medium or in 500 ml flasks containing 100 ml medium. During the course of these studies, two different media were used. These were the glycerol sucrose complete agar medium used for weekly transfers and Vogel's 1X minimal with 1.5% agar supplemented with 1.5 to 2% sucrose and 1% vitamin stock solution. For nutritional mutants, 50 µg inositol or choline per ml were added to both media. Conidia were grown for 7 to 8 days before harvest with three different conditions during these studies. Initially, cultures were maintained at room temperature in the dark for 7 to 8 days. Later, cultures were maintained at 30C in a dark incubator for 2 to 3 days, placed in light at room temperature for 12 to 24 hours, and then returned to the 30C incubator for the remaining time before harvest. An alternate method was to maintain cultures at room temperature

exposed to normal laboratory levels of light for the entire period of growth.

Sterile distilled water was added to the growth flasks which were shaken gently to suspend the conidia in the water. Usually 30 ml was added to the 250 ml flasks and 40 to 60 ml to the 500 ml flasks. The conidial suspensions were then filtered, initially through glass wool and later through four layers of sterile gauze. Conidial suspensions were refrigerated overnight before transferral to sterile centrifuge tubes for centrifugation. The pellet was resuspended in the induction medium.

Growth and Harvest of Mycelia for Induction

Mycelia for the initial induction experiments were grown at 25C in Vogel's 1X liquid medium containing 2% sucrose and 1% vitamin stock solution. Periods of 40 to 53 hours growth were used in the mycelial inductions of this study. The medium was supplemented with 50 µg inositol or choline per ml for the nutritional mutants. Cultures were shaken in 500 ml Erlenmeyer flasks containing 100 to 200 ml media on either an incubator rotary shaker (New Brunswick Scientific Company) at 150 rotations per minute or on a reciprocal shaker bath (New Brunswick Scientific Company) at approximately 100 cycles per minute. Sterile gauze filters were used for harvest of these shaken cultures and for washing the mycelia with sterile distilled water.

A second method of growth of mycelia was adopted later in this study for growth of the temperature-sensitive mutant. The medium used was identical to the above medium except for the addition of 0.1% agar to the medium for colonial growth of the mutant. The cultures were

maintained in 1500 ml flat-bottomed boiling flasks containing 750 to 1000 ml medium. Suspension of the mycelia was achieved by forcing sterile moist air into the flasks during the entire growth period. The temperature-sensitive mutant was grown colonially at approximately 31C in a medium containing agar and was grown with wild type morphology at temperatures ranging from 20 to 25C in a medium lacking agar. The 0.1% agar was added to keep the colonies separated during the aeration process. For harvesting, cultures were allowed to settle, and the growth medium was carefully decanted. They were washed two or three times with sterile distilled water in the same manner before transfer to the induction medium.

Induction of Conidia and Mycelia

Sterile conidia and mycelia were transferred aseptically to induction flasks. Conidia were suspended in either 500 ml Erlenmeyer flasks containing 200 to 250 ml or in 250 ml flasks containing 75 to 100 ml sterile medium and were shaken on either a reciprocal shaker bath or a rotary shaker. In most conidial induction experiments, the optical density of the inoculated induction flasks was determined photometrically at 600 nanometers (nm) at the initiation of induction. Initial absorbance values ranged from 0.500 to 0.530 except during the first conidial induction experiment in which the initial conidial optical density (OD) was only 0.054. Mycelia were suspended in 500 ml Erlenmeyer flasks containing 100 to 200 ml medium. Dry weights were obtained for the mycelial mass at the end of the induction period.

Of various buffers used for previous induction experiments, 0.1 M potassium phosphate buffer, pH 7.0, and 10^{-4} to 10^{-3} M cellobiose

produced the highest levels of cellulase (Eberhart and Beck, unpublished). Potassium phosphate buffer (hence referred to as phosphate buffer, was the standard buffer of these studies and was often used for induction.

Induction temperature was 25C or room temperature (19 to 21C) for all induction experiments. The inducer for cellulase production was cellobiose in concentrations of 10^{-4} M for the first three experiments and of 10^{-3} M for the other experiments. Cellobiose for induction was made sterile by filtering through sterile Swinnex-25 filter units with 0.22 µ pores and was maintained aseptically in the refrigerator at a concentration of 0.1 M. As attempts were made to establish a correlation between tip extension and cellulase secretion, various media were used for induction which are described later. Supplemental inositol or choline was added in the concentration of 50 µg/ml whenever required by the experimental design. This was also filter sterilized, and stock solutions were refrigerated at a concentration of 10 mg/ml. The sterile cellobiose, inositol, and choline were added to previously autoclaved media. This was done to insure that these components were not broken down or destroyed by the procedure of autoclaving. (Inositol and choline were added to growth media prior to the autoclaving of the entire media for convenience in dispensing the media into tubes or growth flasks.) The length of induction time ranged from 5 to 24 hours. At the end of the induction period, the conidia or mycelia were immediately separated from the induction media containing the extracellular cellulase either by centrifugation or by filtering through Whatman No. 1 filter paper.

Thirty to 60 ml samples of the induction media were also taken at various points during the 9- and 24-hour induction experiments. The conidial pellet or mycelia and the supernatant or filtrate were frozen separately.

Cellulase Assay by the Reducing Sugar Method

Cellulase activity was assayed by a modified form of the Somogyi (1953) adaptation of Nelson's reaction for testing the presence of reducing groups. The substrate used in these experiments was a substituted purified cellulose of medium viscosity, sodium carboxymethyl cellulose (CMC, Hercules Powder Company, Type 4MSP). In the reducing sugar assay which is quantitative under certain controlled conditions, one reducing group is liberated with every enzymatic cleavage of the β-1→4 glucosidic links of the substrate. During the assay, the oxidation of a reducing group occurs with the accompanying reduction of cupric ion to cuprous ion. Cuprous ion quantitatively reduces arsenomolybdic acid to the colored arsenomolybdous acid. The green or blue colors which are produced during the assay in the presence of reducing groups are determined photometrically at 540 nm in the Beckman/Spinco 151 Spectro-Colorimeter. Thus one obtains a direct correlation between enzymatic activity and number of reducing groups released.

Method. Viscosimetric cellulase assay indicated that cellulase activity was greatest at pH 6.0 (Eberhart and Beck, unpublished). To adjust the pH of the enzyme preparation to pH 6.0 for assay, the CMC solution was buffered between pH 5.6 and 6.0 with citrate phosphate or potassium phosphate buffer. The reaction mixture was composed of equal

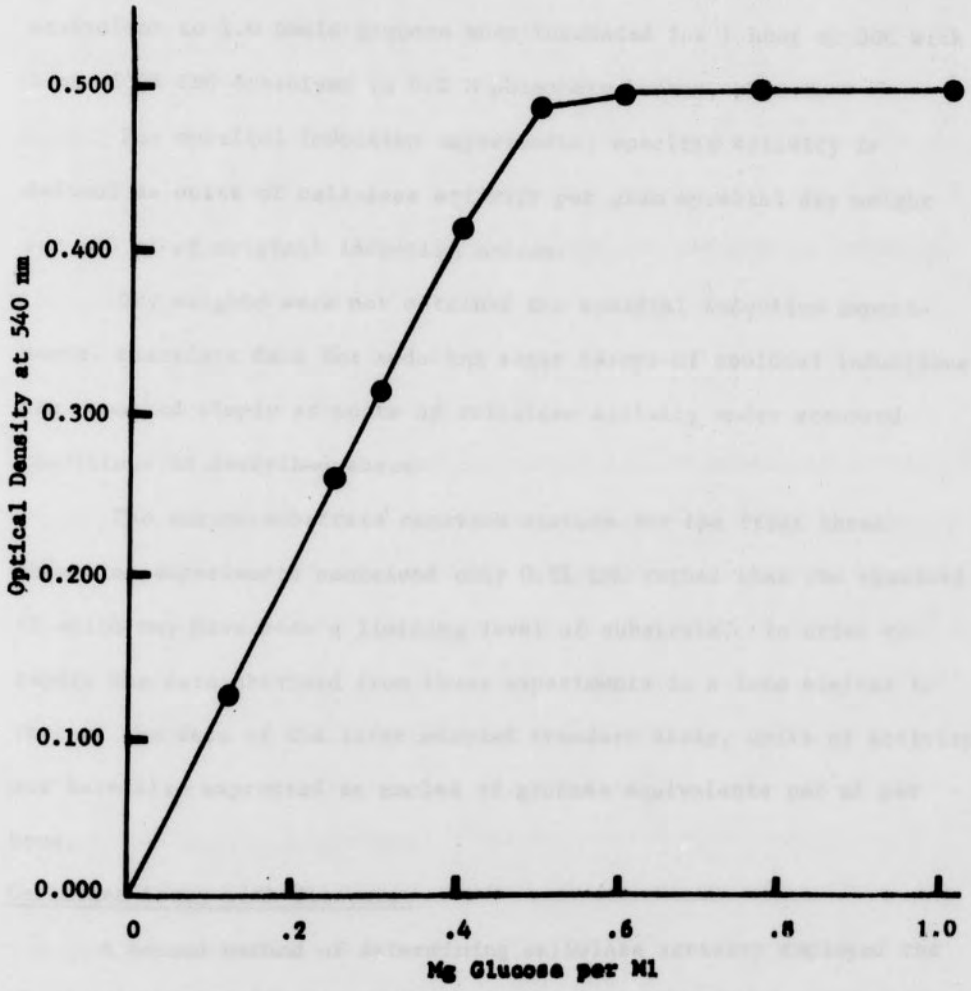
volumes of enzyme solution and the appropriately buffered sterile 2% CMC solution (usually buffered with 0.2 M potassium phosphate buffer, pH 6.0). The volumes used were determined by the numbers of samples to be taken during the assay for determination of reducing groups. The procedure of the assay was as follows. The desired volume of a buffered 2% CMC solution (usually 1.0 ml) was allowed to equilibrate in tubes in a water bath previously brought to 30C. Then an equal volume of the enzyme solution was added to complete the enzyme-substrate reaction mixture. The reaction was run for as long as desired. Two to 4 hours were usually sufficient to bring out an observable number of reducing groups. The reaction was stopped by the addition of a 0.2 ml sample of the mixture to an equal volume of the copper reagent in 16 x 150 mm test tubes. The standard procedure was to make reducing sugar determinations in duplicate. These tubes, with a marble placed on top to prevent evaporation, were placed in a boiling water bath for ten minutes. After cooling, 0.5 ml of the arsenomolybdate developing reagent was added and thoroughly mixed with a S/P Model S3220 mixer. The solution was diluted to a volume of 5 ml with 4.1 ml of distilled water. The tubes were then centrifuged for 5 minutes in the Sorvall Type SP/X angle-head desk centrifuge at approximately 450 x g to pellet coagulated protein precipitate. This speed was sufficiently low so that the assay tubes could be used for the centrifugation without breakage. Two controls were the buffer used in the CMC solution and a 1% CMC solution. Never were reducing groups seen to be liberated from a substrate solution containing no enzyme preparation. The control for reducing groups present in the enzyme preparations was a reducing group

determination of a sample of the enzyme-substrate mixture taken at the initial time of mixing. The spectrophotometric blank was always distilled water. All reducing sugar data appearing in this thesis were obtained by this method unless otherwise noted.

A standard curve for the presence of reducing groups was made by recording the absorbance of successive dilutions of a 1.0 mg/ml solution of glucose (Figure 1). Up to an optical density (OD) of at least 0.410, corresponding to 0.4 mg glucose per ml, the standard curve appears to be linear. At concentrations higher than 0.4 mg glucose per ml, the extinction of the copper and arsenomolybdate reagents is approached and the curve becomes asymptotic. With 1% CMC in the total enzyme-substrate reaction mixture, complete hydrolysis of the substrate to glucose would be equivalent to 10 mg glucose per ml; however, with the substituted cellulose, complete hydrolysis cannot be obtained but would probably yield glucose equivalent levels of above 0.4 mg/ml. A level of 0.5% CMC in the substrate mixture was found to be limiting of the enzyme reaction. Sterile CMC was therefore maintained at the higher level of 1% of the reaction mixture, although the 2% CMC stock solution was extremely viscous and difficult to pipette.

Standard Deviation of Duplicate Determinations. The standard procedure for cellulase assay by the reducing sugar method was to make all reducing sugar determinations in duplicate. The absorbance values obtained from these duplicate determinations were often not identical. To determine standard deviation for this method of assay, the absolute values of the differences between duplicate determinations

Figure 1. Glucose Standard Curve for Reducing Sugar Assay.



of each of 186 enzyme fractions was calculated. The average absolute difference for these duplicate determinations was 0.016. The standard deviation was calculated to be 0.036.

Units of Cellulase Activity. A unit of cellulase activity as determined by the reducing sugar method is defined as that activity shown by an amount of enzyme in 1 ml of the induction medium at the original dilution which will produce an amount of reducing sugar equivalent to 1.0 µmole glucose when incubated for 1 hour at 30C with 1 ml of 2% CMC dissolved in 0.2 M phosphate buffer, pH 6.0.

For mycelial induction experiments, specific activity is defined as units of cellulase activity per gram mycelial dry weight per 100 ml of original induction medium.

Dry weights were not obtained for conidial induction experiments, therefore data for reducing sugar assays of conidial inductions are reported simply as units of cellulase activity under standard conditions as described above.

The enzyme-substrate reaction mixture for the first three induction experiments contained only 0.5% CMC rather than the standard 1% which may have been a limiting level of substrate. In order to report the data obtained from these experiments in a form similar to that of the data of the later adopted standard assay, units of activity are here also expressed as µmoles of glucose equivalents per ml per hour.

Cellulase Assay with Glucostat

A second method of determining cellulase activity employed the Glucostat reagent for the colorimetric determination of glucose. The

substrate in these experiments was also CMC. Since the cellulase examined in these studies had endocellulase activity, an excess amount of β-glucosidase, an exoenzyme specific for β-1→4 linkages at the ends of molecules, was added to each enzyme-substrate reaction mixture. With every cleavage within a CMC molecule by the cellulase, an additional end was formed upon which the β-glucosidase could act to release glucose molecules.

The number of glucose molecules released during cellulase assays with Glucostat and β-glucosidase is therefore an indirect measure of the number of cleavages of the CMC polymer by the endocellulase. In addition, this β-glucosidase activity is continuous for the period of the assay with the subsequent continual release of glucose units. This makes it difficult to establish quantitative relationships between the number of the original cellulase cleavages and the final concentration of glucose.

The total Glucostat reaction consists of two separate enzymatic reactions. In the presence of oxygen, water, and glucose oxidase, glucose is oxidized to gluconic acid and peroxide. In the presence of peroxide and peroxidase, the Glucostat reduced chromogen is oxidized to a colored form. The red to yellow colors produced during the assay in the presence of the glucose liberated by the action of the celluase and β-glucosidase were determined photometrically at 410 nm with the Beckman 151 Spectro-Colorimeter.

<u>One-Step Assay</u>. Although the usual pH for the Glucostat reaction is pH 7.0, these reactions were run at pH 6.0 which seems to be the optimal pH for the cellulase. Curves of standards and cellulase

assays were compared for both of these pH values with few differences observed. The Glucostat reagent was prepared by mixing a solution of the Glucostat enzyme preparation in 49 ml of 0.1 M phosphate buffer, pH 6.0 and a solution of the Glucostat chromogen in 1 ml of distilled water. The substrate was a sterile solution of 1% or 2% CMC in 0.1 or 0.2 M phosphate buffer, pH 6.0. The β-glucosidase solution was at a concentration of 1 mg/ml of 0.1 M phosphate buffer, pH 6.0. The complete Glucostat reagent and the β-glucosidase were frozen and the CMC solution was refrigerated when not in use.

The usual procedure was a continuous assay in which the enzyme, substrate, β-glucosidase, and Glucostat were all added at the initial time of the assay. This was done by mixing 0.5 ml of a mixture of equal volumes of Glucostat, CMC, and β-glucosidase with 0.2 ml of a cellulase preparation. (This mixture of equal volumes of Glucostat, CMC, and β-glucosidase was also frozen when not in use.) The assay was conducted at room temperature for as long as desired and was stopped by the addition of 0.1 ml 2 N HCl. Usually 2 to 3 hours were sufficient to bring out enough color for photometric determination. Since the reagent becomes slowly colored as it stands as a result of a photosensitive reaction, one control included 0.5 ml of a mixture of equal volumes of Glucostat, CMC, and β-glucosidase, and 0.2 ml water. A second control was a mixture identical in proportions to the assay mixture but containing buffer instead of CMC. This control revealed levels of glucose present initially in an enzyme preparation. This continuous assay has the advantage of being a one-step procedure with the visible development of color by the Glucostat reagent in the

presence of glucose. Thus one may pick the time of assay which gives colors adequate for photometric determination.

Atypical Colors. During the continuous assay of the preparations obtained from the induction of ethylene glycol-treated cells from Experiment 6 (Results II), purple hues appeared in the reaction mixtures in addition to the typical reds. Attempts to relate the appearance of these unusual colors to the separate elements in the assay mixtures were unsuccessful. Although xylose crystals produced the usual red color within 10 minutes indicating glucose impurities in the xylose stock, concentrated solutions of xylose did not produce any color. Upon the addition of 0.1 ml 2 N HCl to spots of purple, the color immediately disappeared.

Two-Step Assay. A second procedure was a two-step assay in which only the enzyme, substrate, and β-glucosidase were mixed at the initial time of the assay. This was done by mixing 0.2 ml CMC, 0.1 ml β-glucosidase, and 0.2 ml enzyme at room temperature. After the desired length of time, usually 2 to 3 hours, 0.2 ml of the Glucostat reagent was added to the mixture. After 15 minutes, the Glucostat reaction was stopped by the addition of 0.1 ml 2 N HCl, and color was determined spectrophotometrically. The fact that this was a two-step assay was a disadvantage because one could not be sure that enough glucose had been liberated to be detected by the Glucostat reaction in 15 minutes.

Glucose Standard Curves. Standard assay curves for glucose were made by recording the absorbancies of successive dilutions of a 1.0 mg/ml glucose stock solution buffered at pH 6.0 with 0.2 M phosphate

buffer. Since the Glucostat reaction is specific for β-D-glucose, the glucose stock solutions were allowed to equilibrate before being frozen after which dilutions could be made without further equilibration. The slope of standard curves varied greatly according to the time when the reaction was stopped by the addition of HCl. Therefore, a standard time, 15 minutes, was chosen for stopping the glucose standards and the reaction mixtures of the two-step assay.

According to the Glucostat instruction manual, the Glucostat reaction goes to virtual completion after being incubated at 37C for 30 minutes or longer. Therefore, to establish standard curves for the continuous assay, the glucose standards were incubated for approximately 45 minutes at room temperature before being stopped with HCl. It was necessary to reduce the usual concentration of the glucose standard solutions so that the limit of the Glucostat reagents would not be reached by all standards in this time period.

Standard Deviation of the One-Step Assay. The standard procedure for cellulase assay by the Glucostat method was to make only one determination of activity per assay rather than two determinations as with the reducing sugar assay. It was found, however, that with duplicate determinations, the average absolute difference of such determinations was 0.068. The standard deviation was calculated to be 0.028.

Special Application and Variation of the Glucostat Method

Assay on Thin-Layer Gel. Cellulase activity chromatographed on thin-layer gel (TLG) plates was assayed by a Glucostat spot test. A TLG plate was placed surface up on a sheet of lined paper with dimensions of approximately 20 by 40 cm. A small stainless steel

spatula was then used to remove the gel from the plate in scrapes with widths correspondind to spaces of the notebook paper. Usually scrapes were taken across only half or three-fourths the width of the plate. These scrapes were placed in the depressions of a plastic spot plate, and 0.5 ml of the standard Glucostat mixture containing Glucostat, CMC, and β-glucosidase were added to each spot. This method was used only as a spot test to indicate the location of cellulase activity.

Fluorescent Determination. An additional variation of the Glucostat method was developed using homovanillic acid rather than the standard Glucostat chromogen. In the presence of peroxide and peroxidase, the non-fluorescent reduced form of homovanillic acid is oxidized to a fluorescent compound. The method used was to dissolve 2 mg homovanillic acid in 4 ml of the Glucostat enzyme (which was dissolved in 50 ml phosphate buffer, pH 6.0). It was discovered that fluorescence would not appear at pH 6.0. Upon the addition of 0.1 ml 1.0 M Tris buffer, an appreciable fluorescence appeared. This method was no more sensitive than the usual continuous Glucostat assay. Both detected, as a lower bound, approximately 0.01 mg glucose per ml within 10 minutes. Since this method was a two-step assay in which one could not see fluorescence until after the addition of Tris and since it was no more sensitive than the previously developed method, no other attempts were made to standardize the method.

Carboxymethyl Cellulose Viscosity Assay

A third measure of cellulase activity was based upon the change in viscosity of the substrate solution when mixed with a cellulase preparation (Levinson and Reese, 1949-50). This method was extremely

sensitive to cleavages of the CMC molecule by the enzyme; therefore, hundred-fold dilutions were made of preparations that showed activity with the reducing sugar method.

The substrate for this method was a 1% solution of CMC in 0.1 M phosphate buffer, pH 7.0 which had been blended for three minutes with a Waring blender. The procedure was to pipette 2.5 ml of the CMC solution into an Ostwald viscosimeter tube and to pipette 2.5 ml of the cellulase preparation into a separate test tube. These were both allowed to incubate in racks in a 40C bath. After 15 minutes, the cellulase sample was poured into the viscosimeter tube and mixed by gently forcing air through the solution. At the end of 30 minutes, the enzyme-substrate solution was forced into the measuring chamber of the tube. Flow rate from the first line to the second line of the measuring chamber was determined for each tube. Relative viscosity (n_{sp}) was determined by dividing the flow time of the mixture (t_x) by that of distilled water (t_{HOH}) for that tube.

$$n_{rel} = t_x / t_{HOH}$$

Specific viscosity (n_{sp}) is defined by $n_{sp} = n_{rel} - 1$ and fluidity by the reciprocal of specific viscosity. Fluidity constants ($1/n_{spk}$) for each tube were obtained using a control preparation containing buffer and CMC. The value for the final activity was found by the following calculation:

$$1000(1/n_{sp} - 1/n_{spk})$$

Tubes were cleaned by flushing with distilled water several times followed by rinsing with absolute or 95% ethanol and with ether and were dried by pulling a flow of air through the tubes.

Hydroxyethyl Cellulase Foam Qualitative Assay

A fourth method of determining cellulase activity was a spot test using the substituted cellulose, hydroxyethyl cellulose (HEC) prepared by the Hercules Powder Company. HEC or Natrosol of Type 250HR was made up as a 2% solution in 0.05 M phosphate buffer, pH 6.0. This was done by adding the HEC powder slowly to the buffer in a Waring blender container. This was blended for approximately 5 minutes and then allowed to sit for several hours until an extremely viscous layer formed in the bottom of the container. The mixture was then blended again to yield a thick foam.

HEC foam of this consistency was used for two types of spot tests for cellulase activity. One was to place approximately 0.5 ml of the foam in a depression of a spot plate and to add 0.2 ml of a cellulase preparation to the spot. Cellulase activity was indicated by the clearing of the foam as the bubbles burst.

A second spot test method was used to locate cellulase activity on a thin-layer gel plate. The foam was poured into a 30 x 50 cm aluminum pan to a depth of approximately 2 to 2.5 cm. The gel plate, to be assayed, was then placed on the foam, surface down. Clamps were placed on the ends of the plate which were of a size sufficient to allow the plate to rest on the top of the foam and not to settle to the bottom of the pan. This prevented dispersing of the gel beads by the foam. The bubbles were sufficiently long lasting to allow the plate to remain in this position for as long as 12 to 24 hours which was usually sufficient to show areas of clearing of the bubbles corresponding to cellulase activity. A disadvantage of the time sometimes involved in

this assay was that the cellulase could diffuse through the gel. Large areas of clearing resulted which would mask any separation of cellulases of similar but different molecular weights by TLG chromotography.

Thin-Layer Gel Filtration

The initial attempts to determine the number and molecular weight of cellulases in the cellulase preparations were with a Pharmacia thin-layer gel (TLG) apparatus and Bio-Gel P-100 (400 mesh). The gel was prepared by slowly suspending 5 g gel in approximately 200 ml of 0.05 M phosphate buffer, pH 6.0 and allowing this to equilibrate for at least 24 hours. Prior to pouring the gel, the excess buffer was decanted. The gel was then poured onto the 20 x 40 cm glass surface and was spread out evenly over the plate to a thickness of 0.6 mm with the Pharmacia TLG-spreader. The eluant used during this filtration was the same buffer as that used for preparing the gel with 60 ml placed in the upper reservoir and 40 ml in the lower of the apparatus. Paper bridges for connecting the gel layer with the developing buffer solution were strips of Whatman No. 3 (Pharmacia, 1971). The completed apparatus was sealed and allowed to equilibrate at a 10^{o} angle overnight. For application of samples of cellulase preparations, the TLG-chamber was placed in a horizontal position. At several points along the application slit, 10 to 20 λ samples of extracellular cellulase preparations were applied with micro-pipettes (Drummond Scientific Company). The plate was then set at either a 10^{o} or 15^{o} angle and was allowed to run for 4.5 hours. A sample of the aryl-β-glucosidase W_1 with a molecular weight of approximately 50,000 (Eberhart and Madden, unpublished) was run along with each plate as a reference molecular

weight. The W_1 preparation was obtained from the exotic strain P-212 collected by D. D. Perkins. W_1 activity was assayed by placing a strip of Whatman No. 1 soaked in umbelliferone on the plate. A fluorescent spot corresponding to the location of W_1 appeared on the paper and plate with degradation of the umbelliferone substrate by this enzyme.

Cellulase activity on TLG plates was assayed by the Glucostat method and the HEC foam method previously described.

Column Gel Filtration

A second procedure was followed to attempt to determine the number of cellulases in extracellular cellulase preparations. This was the use at room temperature of a K 50/60 "Jacketed" Sephadex column packed with Bio-Gel P-100 (50-100 mesh). The column had dimensions of approximately 5 x 44 cm. Five to 6.5 ml of concentrated cellulase preparations were placed on the column for filtration. A flow rate of 2 ml per minute was established by adjusting the height of the Mariotte flask containing the eluant which was 0.05 M phosphate buffer, pH 6.0. The first 200 to 250 ml which flowed through the column were collected in 50 ml fractions after which 100 to 136 fractions of 5 to 7 ml each were collected using an automatic fraction collector (Instrumentation Specialties Company, Model A). Fractions were maintained in a refrigerator until all were collected. A Glucostat continuous assay was then made of all samples. Those samples showing activity were frozen prior to further assay.

Photographic Procedures and Equipment

Development of Method. To determine the relationship between the secretion of an enzyme and the extension of hyphal tips, it was

necessary to devise a method of determining the presence or absence of hyphal tip extension in the liquid media induction cultures. With solid media, direct measurements of the extension of particular hyphal tips can be made. Thus one can examine relatively few hyphae for a statictically significant analysis of the culture's growth rates. In shaken or aerated liquid cultures, however, one can only examine a large number of hyphae to gain statistically significant data. The method devised was to induce conidia and to sample the liquid induction media at various points during induction and to examine these samples microscopically. Photomicrographs were taken of fields thought to be representative of each induction sample. In some experiments, additional 0.5 to 1.0 ml samples of the induction medium and conidia were mixed with an equal volume of Carnoy's fixative for observation at a later time. (Carnoy's fixative was prepared by mixing 5 ml glacial acetic acid, 30 ml 95% ethanol, and 15 ml chloroform.)

The main limitation of this method of sampling liquid media was asynchrony in germination and consequently in hyphal extension of the induced conidia. Attempts, which are described later in this thesis, to achieve synchronous germination were not successful. Other problems encountered were hyphal fusions and varying degrees of clumping which became more apparent with increasing length of time of induction. Since most cultures had to be induced for periods of 6 to 9 hours to obtain appreciable levels of extracellular cellulase, hyphae in the latter stages of induction were usually fused which made it difficult or impossible to determine the actual length of individual

hyphae by this visual method. Thus, even if synchrony of germination could have been achieved, statistical analysis of hyphal extension in the latter stages of induction would have been difficult. Therefore, only general statements of apparent relationships between the secretion of cellulase and the extension of hyphal tips have been made.

Method. Photomicrographs were taken using a Polaroid Land Instrument Camera, Model ED-10 and black and white Polaroid Type 107 Land Film Packs. The microscope used was a Wild M-11, equipped for phase contrast. All photomicrographs appearing in this thesis are of phase contrast. Both 10X and 40X objectives were used, always in combination with 10X oculars. A binocular head, used during visual examination, was exchanged for a monocular head equipped with either a photo or a wide-field compensating 10X ocular when photomicrographs were taken. Photomicrographs were taken at microscopic magnifications of 100X and 400X. Since the ED-10 camera factor is approximately 0.8, all photomicrographs of this study have a final scale of reproduction of either 80:1 or 320:1. With 100X microscopic magnification, exposures were of 2 to 4 seconds. With 400X, exposures were of 15 to 20 seconds. Since the film has a speed of 3000, the microscope bulb voltage had to be reduced to 2 volts to obtain exposures that could be controlled with a manual cable release.

The original Polaroid prints appear in the first copy of this thesis. All other thesis copies contain prints made by copying the Polaroid prints.

RESULTS I

Improvement of the Reducing Sugar Assay

Original Procedure. The original procedure of the assay was one used by Ballantine (1963) which was as follows (except for alterations of temperature and of buffer): 0.8 ml of a 1% solution were mixed with 0.4 ml of a 0.01 M phosphate buffer, pH 6.0, and with a 0.2 ml aliquot of enzyme solution. The assay was conducted at room temperature. At the time of the assay, 0.1 ml of this mixture was combined with 0.1 ml of the copper reagent, boiled for 10 minutes, cooled, mixed thoroughly with 0.2 ml of Nelson's developing solution, and diluted with 4.6 ml of distilled water for a total of 5.0 ml. With this assay, only very low absorbance values were obtained for enzyme assays. In addition, the pH of the enzyme-substrate reaction mixture was usually approximately pH 7.0 instead of the desired pH 6.0.

First Adaptation. The assay was adapted to increase cellulase activity and absorbance values. The new reaction mixture consisted of a 3 to 1 ratio of enzyme preparation to 2% CMC solution buffered at pH 5.4 with a citrate phosphate buffer. The less dilute assay mixture was as follows: 0.2 ml of the reaction mixture was combined with 0.2 ml of the copper reagent, boiled for 10 minutes, cooled, mixed thoroughly with 0.4 ml of the arsenomolybdate reagent and diluted with 4.2 ml water. This adaptation of the original assay gave absorbance values approximately three times greater than the old.

Appearance of Precipitate in Assay Mixture. During these early assays, a small amount of precipitate, which was thought to be protein, was found in most of the assay mixtures. Originally, the degree of precipitate was low and was carried as an experimental error through the assay since each aliquot of a particular experiment had approximately the same amount of protein present. Since the activities of these preparations were so low, these solutions were concentrated in dialysis tubes against Carbowax. Greater concentration of the preparations was correlated with a larger quantity of coagulated protein in the assay mixture. In addition to the protein precipitate, a small amount of the CMC substrate precipitated in the substrate control tubes. These deflecting materials were removed by centrifugation for the accurate determination of the reducing group concentrations. This centrifugation was done just prior to the spectrophotometric determination.

Enzyme Concentration Effects. A second problem encountered was masking of cellulase activity by concentrated preparations. In several experiments, an increase in concentration of enzyme solutions was often not correlated with a similar increase in apparent enzyme activity. With high concentration of some preparations and a correspondingly high amount of precipitate, indicated cellulase activity was usually quite less than that of the more dilute preparations. Various concentrations of a 74-OR23-1A preparation were assayed to obtain a better understanding of this concentration effect with one specific enzyme mixture. These concentrations were obtained by diluting this enzyme preparation which had been dialyzed and concentrated with

Carbowax 90 times the original dilution. Reducing sugar determinations were made after 4 hours of assay time. The results of this experiment are summarized in Table 2.

TABLE 2

ENZYME CONCENTRATION EFFECTS ON REDUCING SUGAR ASSAY

Degree of Concentration of Original Preparation	Units of Cellulase Activity
4.5X	0.020
9.0X	0.023
45.0X	0.010
90.0X	0.004

Concentrations of 4.5X and 9.0X gave similar rather then 2-fold different values for units of cellulase activity calculated for the original dilution. However, indicated cellulase activity was much lower for a 45-fold concentrate. This experiment indicates that concentration may interfere with the reducing sugar assay. To minimize interfering concentration effects, all aliquots from a given experiment were concentrated to approximately the same level.

Effect of Vogel's and of Fries Media. Other experiments indicated that both Vogel's and Fries media interfere with this cellulase assay. In glucose standard solutions, Vogel's medium at a concentration of 7.5X was found to be completely inhibitory of the detection of known glucose levels and was partially inhibitory at 5X concentration. Inhibition was small if any at 1X and 3X concentrations. The results

of this experiment with known glucose levels and various concentrations of Vogel's medium are summarized in Table 3. The effect of Vogel's medium upon actual cellulase assays was not determined.

TABLE 3

EFFECT OF VOGEL'S MEDIUM UPON DETECTION OF KNOWN GLUCOSE

Glucose mg/ml	Concentration of Vogel's Medium	Indicated Glucose mg/ml
0.25	0	0.25
0.25	1.0X	0.25
0.25	3.0X	0.25
0.25	5.0X	0.18
0.25	7.5X	0.00
1.00	7.5X	0.00

Low levels of Fries medium also did not appear to inhibit the detection of known glucose; however, the presence of Fries medium in an eyzme-substrate reaction mixture was observed to affect the cellulase assay even with concentrations as low as 0.25X. A chol-2 cellulase preparation, to which this medium had been added to yield different concentrations, was assayed to discover the effect of Fries medium on the enzyme assay. Reducing sugar determinations were made after 4 hours of incubation. The results of this experiment are summarized in Table 4. Enzyme activity is recorded as glucose equivalents released per hour of incubation. With increasing concentrations of Fries medium in the assay reaction mixture there was increasing inhibition of the reducing sugar assay.

TABLE 4

EFFECT OF FRIES MEDIUM ON REDUCING SUGAR ASSAY

Concentration of Fries Medium	Cellulase Units per Hour
0	0.304
0.25X	0.222
1.00X	0.143
2.00X	0.076

In those induction experiments in which cellulase was secreted into Fries or Vogel's media, enzyme preparations were dialyzed against distilled water to eliminate inhibition by the media.

Effects of Cellular Contamination. Additional problems were discovered when attempts were made to determine a typical assay curve over a period of approximately 8 hours. In initial attempts, the curves ascended somewhat irregularly for a period of 4 to 5 hours after which the curves descended. The descending slope was often correlated with the degree of concentration of the cellulase preparation.

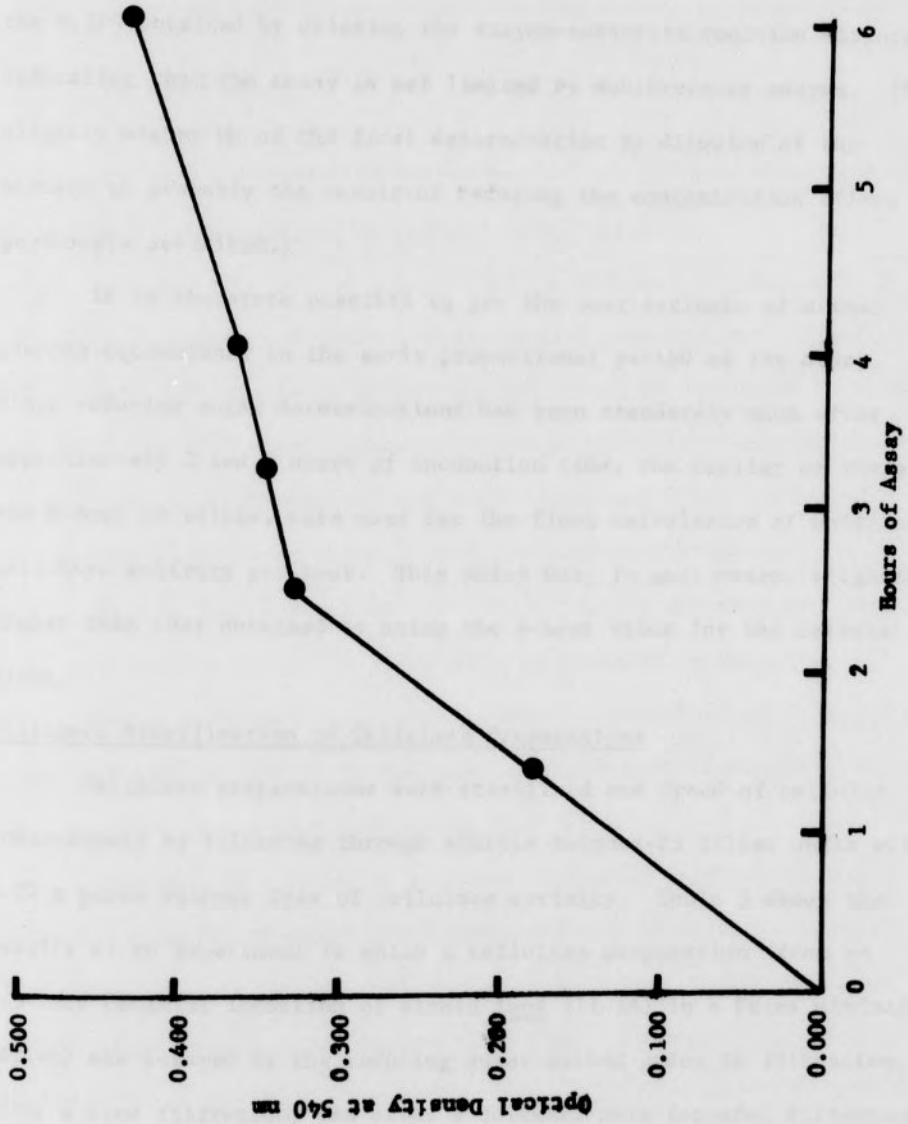
Two hypotheses derived to account for the decline and apparent removal of free reducing groups from the enzyme-substrate reaction mixtures were that removal might be the result of enzymatic destruction of reducing groups or the result of cellular contamination and uptake of the substrate products by the contaminants. Microscopic examination of a 3-day old refrigerated CMC solution revealed the presence of bacteria which appeared similar to the bacteria found in the enzyme-

substrate mixtures. Therefore, two sources of contamination of the enzyme-substrate mixtures were determined to be bacteria from substrate solutions and Neurospora conidia or fragments of mycelia present in enzyme preparations.

To eliminate cellular contaminants, buffered CMC solutions were routinely autoclaved for 7 minutes with only slight darkening of color, and enzyme preparations were centrifuged three times, 10 minutes each, at approximately 3000 x g with the Sorvall Type SP/X angle-head with careful separation of pellet and supernatant after each centrifugation. A procedure later adopted was filter-sterilization of enzyme preparations.

Assay with Sterile Substrate and Enzyme. A 6-hour assay curve with sterile substrate and enzyme is pictured in Figure 2. The assay curve is linear to an optical density of about 0.350 corresponding to 0.33 mg glucose equivalents per ml. After this, the curve levels off. This leveling off was found to be the result of limiting assay reagents, not limiting substrate or enzyme. It was shown that the assay did not measure total reducing groups present during the latter periods of the assay procedure by determining reducing groups for a 1:5 dilution of the 4-hour enzyme-substrate reaction mixture of Figure 2. The optical density thus obtained was multiplied by the 5X dilution factor to yield an OD value of 0.570 rather than the value of 0.363 shown in Figure 2 after 4 hours. This indicated that the assay reagents were limiting after 4 hours with this particular enzyme preparation.

Figure 2. Shape of Reducing Sugar Assay Curve with Sterile Substrate and Enzyme.



In addition, a projected OD value of 0.543 was obtained from Figure 2 for the 4-hour point by projecting the line determined by the points at 0 and 2.5 hours. This value closely corresponds with the 0.570 obtained by diluting the enzyme-substrate reaction mixture indicating that the assay is not limited by substrate or enzyme. (The slightly higher OD of the first determination by dilution of the mixture is probably the result of reducing the concentration effect previously described.)

It is therefore possible to get the best estimate of actual glucose equivalents in the early proportional period of the assay. Since reducing sugar determinations had been standardly made after approximately 2 and 4 hours of incubation time, the earlier of these, the 2-hour OD values, were used for the final calculation of units of cellulase activity per hour. This value was, in most cases, slightly higher than that obtained by using the 4-hour value for the calculations.

Millipore Sterilization of Cellulase Preparations

Cellulase preparations were sterilized and freed of cellular contaminants by filtering through sterile Swinnex-25 filter units with 0.22 μ pores without loss of cellulase activity. Table 5 shows the results of an experiment in which a cellulase preparation (from an 8.5-hour conidial induction of strain inos (11-1A) in a Fries minimal medium) was assayed by the reducing sugar method prior to filtration, after a slow filtration, and after a quicker, more forceful filtration. There was no appreciable loss of cellulase activity as there had been previously under similar conditions (Eberhart and Beck, unpublished).

TABLE 5

MILLIPORE STERILIZATION OF CELLULASE PREPARATIONS

Treatment of Preparations	Units of Cellulase Activity
unfiltered	0.013
filtered slowly	0.012
filtered quickly	0.012

Attempts at Determination of Number of Cellulases

Results from column gel filtration indicate the presence of one major cellulase in the extracellular enzyme preparations. Figures 3 and 4 show the results of assays for cellulase in fractions from two column filtration experiments. In each graph there is one major peak of activity corresponding to one major component. Any other cellulases present would be minor contributants to cellulase activity upon CMC.

Figure 3 is a graph of relative cellulase activity of fractions of a preparation of strain 74-OR23-1A as measured by the Glucostat and reducing sugar methods. The preparation was obtained from a 24-hour induction at 25C of 42-hour mycelia in a 0.05 M phosphate buffer, ph 7.0, induction medium containing 10^{-4} M cellobiose.

Figure 4 is a graph of activity from a preparation of the colonial temperature-sensitive mutant 33(2-6)A as measured by the Glucostat, reducing sugar, and viscosity methods. The preparation was obtained from a culture grown with wild type morphology at 21C for 45 hours which was then induced also with wild type growth conditions of 25C for 7 hours in a Fries medium containing 1.2×10^{-3} M cellobiose.

Figure 3. Column Gel Filtration I with Wild Type 74-OR23-1A.

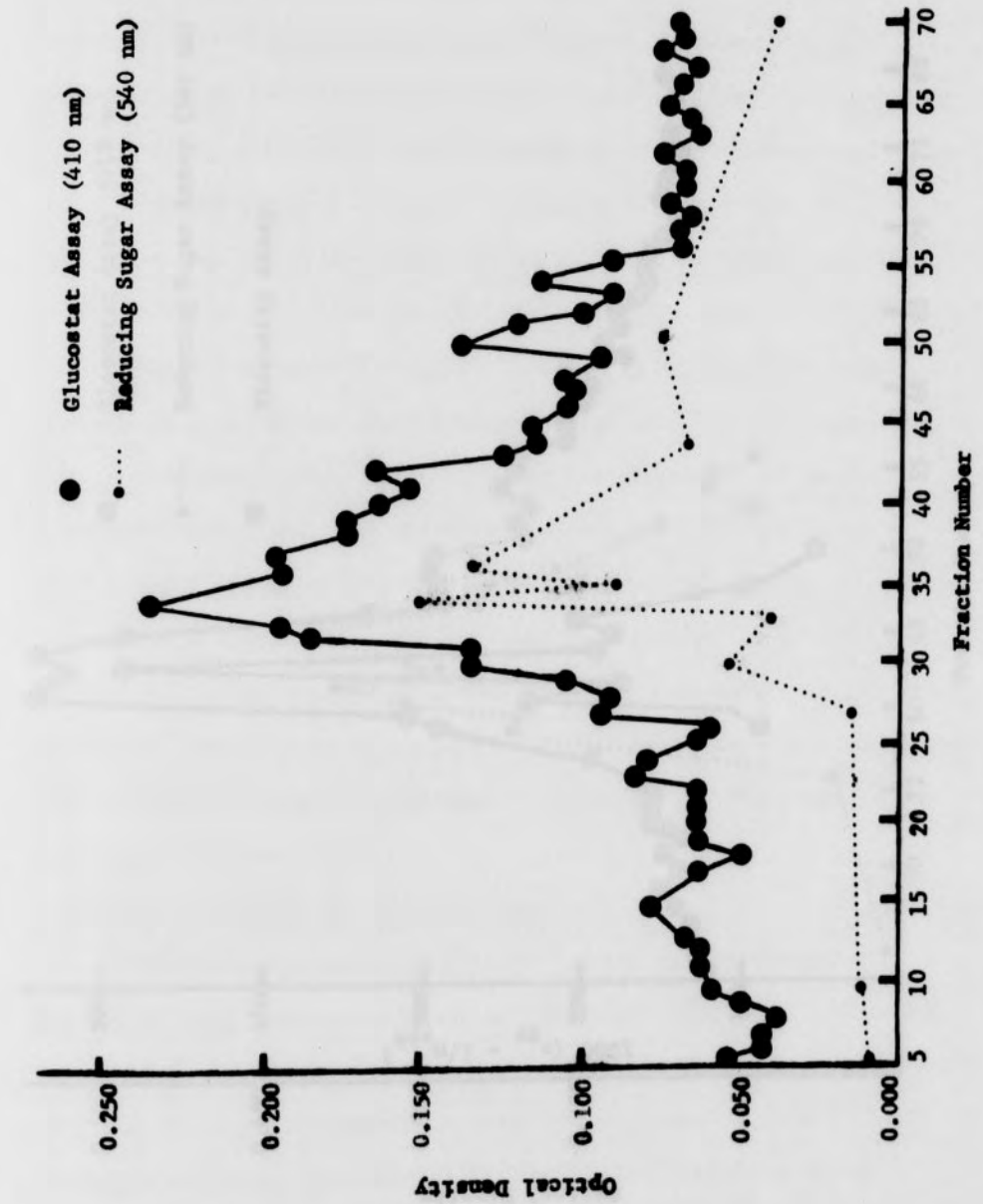
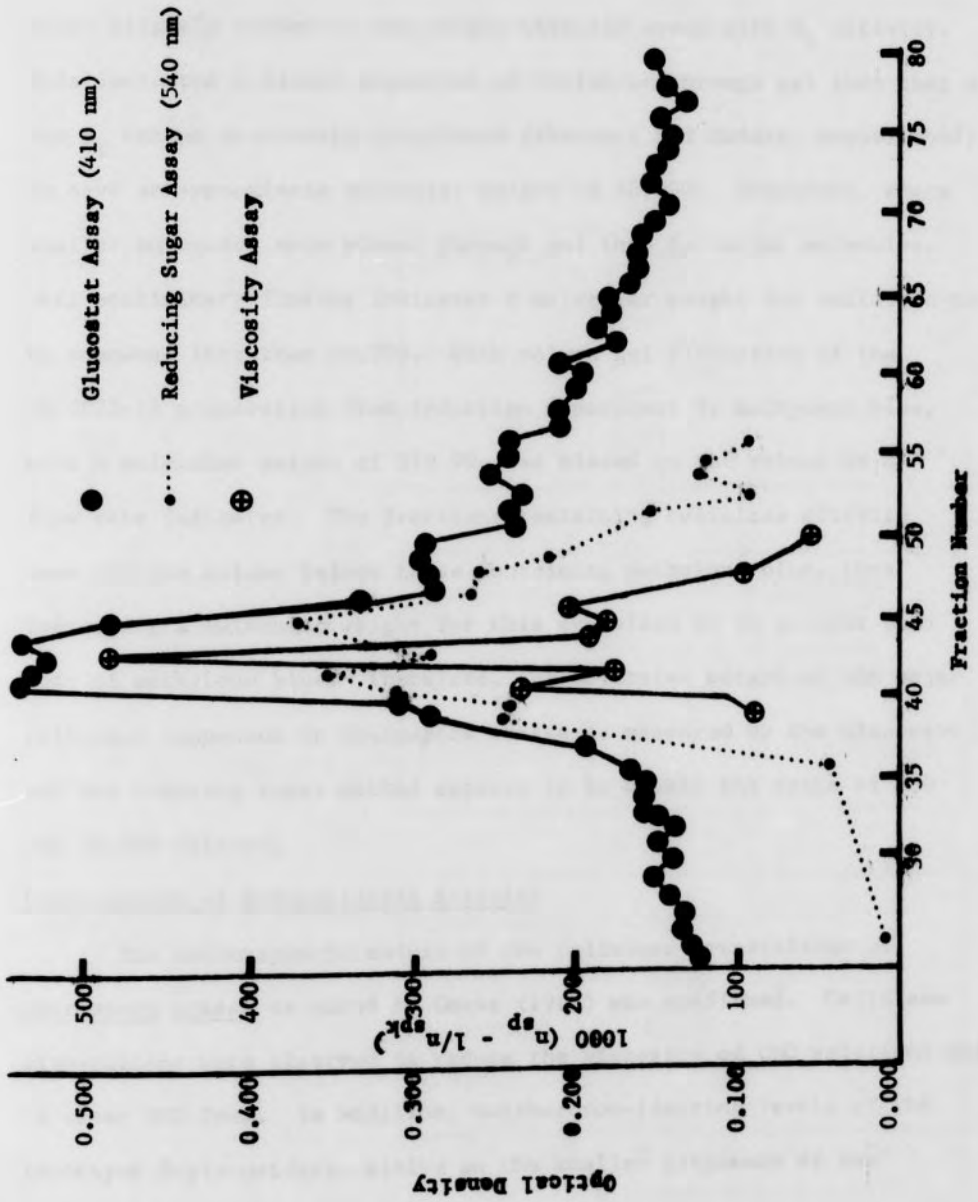


Figure 4. Column Gel Filtration II with Colonial Temperature-Sensitive Mutant 33(2-6)A.



Molecular Weight Determination

During thin-layer gel filtration studies, spots having cellulase activity from the indicated strains (Figure 5) were observed to occur slightly closer to the origin than did areas with W_1 activity. This indicated a slower migration of cellulase through gel than that of the W_1 enzyme previously calculated (Eberhart and Madden, unpublished) to have an approximate molecular weight of 50,000. Therefore, since smaller molecules move slower through gel than do larger molecules, this preliminary finding indicates a molecular weight for cellulase to be somewhat less than 50,000. With column gel filtration of the 74-0R23-1A preparation from induction Experiment 3, methylene blue, with a molecular weight of 379.90, was placed on the column as a flow rate indicator. The fractions containing cellulase activity came off the column before those containing methylene blue, thus indicating a molecular weight for this cellulase to be greater than that of methylene blue. Therefore, the molecular weight of the major cellulase component in Neurospora crassa as measured by the Glucostat and the reducing sugar method appears to be within the range of 380 and 50,000 daltons.

Confirmation of Endocellulase Activity

The endoenzymatic nature of the cellulase preparations of Neurospora crassa as noted by Chase (1960) was confirmed. Cellulase preparations were observed to reduce the viscosity of CMC solutions and to clear HEC foam. In addition, neither non-limiting levels of the exoenzyme β-glucosidase, active on the smaller oligomers of the

Figure 5. Filtration with Thin-Layer Gel.

Symbol	Method of Assay
▩	= Cellulase Assay by HEC Foam Method
⊠	= Cellulase Assay by Glucostat
⊡	= W_1 Assay with Fluorescent Umbelliferone

Plate 1.
15° angle for 4.5 hrs.

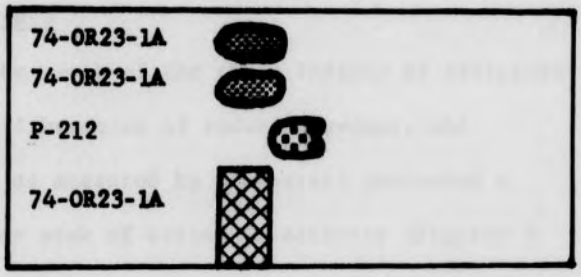


Plate 2.
10° angle for 6 hrs.

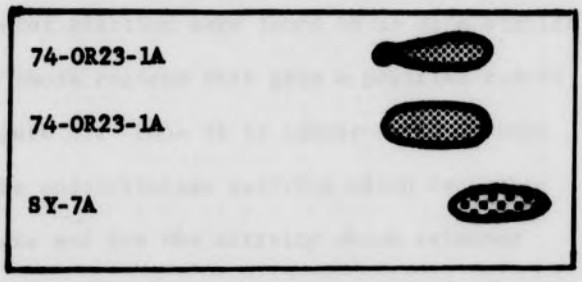
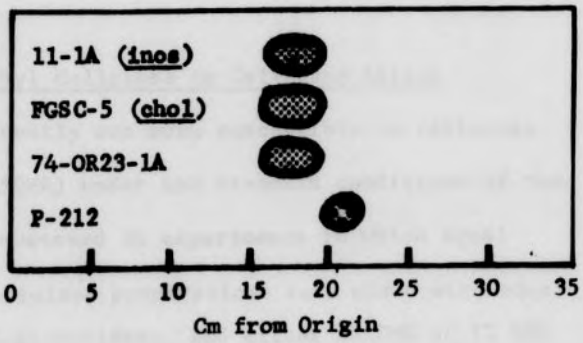


Plate 3.
10° angle for 5.5 hrs.



substrate, nor the cellulase alone could release observable levels of glucose units from the CMC polymer in the Glucostat assay. However, in the presence of limiting levels of cellulase and with excess levels of β-glucosidase, glucose release was indicated by a positive Glucostat reaction. This phenomenon can be explained by endocellulase cleavage of internal linkages of the substrate polymer and by the activity of the excess β-glucosidase upon these smaller oligomers.

Concurrence of Cellulase Assays

With column filtration, each of the three indices of cellulase activity (viscosity change, liberation of reducing groups, and liberation of glucose units as measured by Glucostat) indicated a similar location of the major peak of cellulase activity (Figures 3 and 4). In addition, scrapes of gel taken from TLG filtrations which gave a positive Glucostat reaction were found to be at a similar distance from the origin as those regions that gave a positive result with the HEC foam assay (Figure 5). Thus it is likely that the same enzyme is responsible for the endocellulase activity which decreases the viscosity of the substrate and for the activity which releases reducing groups. This allows study of the same enzyme with different types of assays.

Susceptibility of Hydroxyethyl Cellulose to Cellulase Attack

CMC (Type 4MSP) apparently was more susceptible to cellulase attack than was HEC (Type 250HR) under the standard conditions of the Glucostat assay. This was observed in experiments in which equal volumes of two different cellulase preparations were mixed with equal volumes of the Glucostat, β-glucosidase, and either 1% CMC or 1% HEC all prepared in 0.05 M phosphate buffer, pH 6.0.

Fries Medium as Enhancer of Cellulase Secretion

Two experiments were performed in which conidia were induced both in a buffer and in a Fries growth medium. All induction was with 10^{-3} M cellobiose. In both instances, the culture induced in the Fries medium produced greater levels (approximately 3-fold) of cellulase as measured by the reducing sugar assay of cellulase activity. The conditions and results of these two experiments are summarized in Table 6.

TABLE 6

EFFECT OF FRIES MEDIUM UPON CELLULASE SECRETION

Strain	Induction Medium	Conidial Inoculation	Hours Induction	Units of Cellulase
STA-4	0.05 M, pH 6 Phosphate	0.520 OD	6.5	0.027
STA-4	1X Fries Medium	0.500 OD	6.5	0.082
74-OR23-1A	0.1 M, pH 7 Phosphate	2 ml of suspension	6.0	0.005
74-OR23-1A	0.66X Fries Medium	2 ml of suspension	6.0	0.014

RESULTS II - INDUCTION EXPERIMENTS

Conidia and mycelia were induced in various media to determine the relationship between hyphal tip extension and cellulase secretion. Four systems were examined for this purpose:

1. Induction of auxotrophs requiring components necessary for normal membrane synthesis with various levels of these supplemental components. (For the remainder of this thesis, these auxotrophs are referred to as "membrane mutants".)

2. Induction with various levels of a carbon source.

3. Induction and comparison of cultures in both a Fries minimal growth medium and in a buffer.

4. Induction of a colonial temperature-sensitive mutant to examine the correlation of the number of tips and secretion.

Initial Conidial Induction - Experiment 1

An initial 6-hour conidial induction of the STA-4 wild type using 1.0 M phosphate buffer, pH 7.0, and 10^{-4} M cellobiose resulted in a low level of cellulase activity as measured by the reducing sugar assay. One ml of this enzyme preparation released 0.005 units of cellulase activity. The optical density of this conidial suspension at the beginning of induction was only 0.054; therefore, initial conidial concentrations of approximately 0.500 were used to increase secretion levels of later conidial induction experiments.

Initial Mycelial Induction - Experiment 2

A second preliminary induction experiment was conducted with

53-hour mycelia of the two "membrane mutant" strains chol-2 and inos (37401) in 0.05 M phosphate buffer, pH 7.0, 10^{-4} M cellobiose, and 50 µg choline or inositol per ml. Higher levels of cellulase activity were achieved in this second induction, although they were much lower than those of later inductions. Activity of these cellulase preparations was determined after 24 hours of induction by the reducing sugar assay in which the substrate was at a level of only 0.5% of the enzyme-substrate reaction mixture rather than the standard level of 1.0%. Specific activity was determined to be 0.085 for the chol-2 induction and 0.148 for the inos (37401) induction.

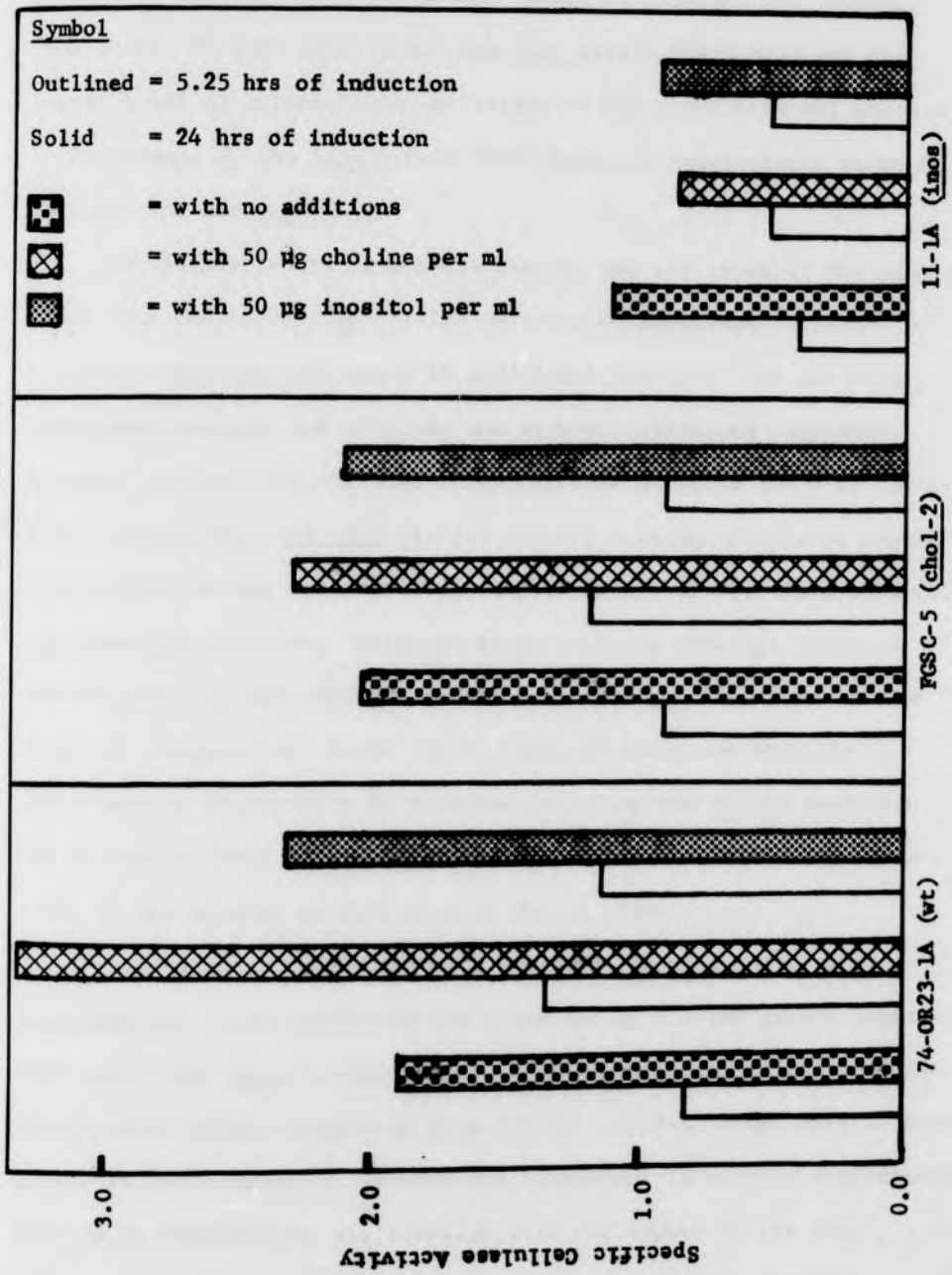
Strain inos (37401) secreted nearly twice as much cellulase into the extracellular medium during the 24-hour induction period as did cultures of chol-2 under these conditions. It must be noted in addition, however, that the cultures of inos (37401) had been maintained for several transfers on media containing only 1.2 µg of inositol per ml and had not appeared normal. Growth had consisted of long mycelia and few conidia with the media of the agar slants turning dark brown. This low level of inositol was supplied by the 1% vitamin stock solution. It was not until after this and the following induction experiment that it was calculated that the vitamin stock solution did not supply 32 to 50 µg inositol per ml required for wild type growth. Therefore, although optimal levels of inositol had been used in the growth media for the production of the actual mycelia used in these two experiments, the effects of the previous standard transfers on suboptimal media could not be determined.

System I - Induction of "Membrane Mutants"

Results of Experiment 3. Three different induction conditions were used to examine levels of cellulase secretion in mycelia of a wild type (74-OR23-1A) and two "membrane mutants"--inos (11-1A) and chol-2. The mycelia obtained from the growth flasks of each strain at the end of 42 hours were divided into approximate thirds and transferred to three flasks, each containing 100 ml medium, 0.05 M phosphate buffer, pH 7.0, for 24 hours. The three conditions of induction with 10^{-4} M cellobiose for each of the three strains were as follows: (1) no additional inositol or choline, (2) the addition of 50 µg choline per ml, and (3) the addition of 50 µg inositol per ml. Samples were taken after 5.25 and 24 hours of induction. Final dry weights for the mycelia of the nine induction flasks, each containing 100 ml of induction medium, ranged from 228 to 407 mg. Specific activity was determined for all samples by the reducing sugar method of assay. The results of this experiment are found in Figure 6. (The reaction mixture contained 0.5% CMC.)

Discussion of Experiment 3. It appears that additional choline and inositol enhanced cellulase secretion in the wild type. The relatively high cellulase levels produced by the wild type in the presence of choline was not explained. With chol-2, cellulase secretion appears to have been only slightly higher in the presence of optimal levels of choline. Perhaps this is an indication of large reserves of choline in the mycelia before transfer from the supplemented growth medium into the induction medium.

Figure 6. Specific Activity of Mycelial Induction - Experiment 3.



It is again noted that inos (11-1A) cultures used in Experiment 3 had been maintained on suboptimal levels of inositol for several transfers. In this experiment, the inos strain 11-1A produced the lowest level of extracellular cellulase of the three strains; whereas in Experiment 2, the inos strain 37401 produced nearly twice as much cellulase as did chol-2.

Cellulase levels were quite similar for all three of the samples taken from the three inos (11-1A) cultures of Experiment 3 after 5.25 hours of induction--the one with additional inositol, the one with additional choline, and also the one without additional supplement. However, the assay of the same three cultures after 24 hours of induction revealed that the inos (11-1A) culture lacking additional supplement in the medium, had relatively higher levels of cellulase than did the other two cultures. Although these cultures were not examined microscopically, this unsupplemented culture might have been in some phase of "inositolless death" by 24 hours of induction with the consequential destruction of membrane including the plasma membrane and an accompanying spill of intracellular contents, including cellulase, to the outside as described by Matile (1966).

Microscopic Examination of Similar Non-Induced Cultures of Experiment 4. This hypothesis was supported by a later growth experiment again with inos (11-1A). Microscopic examination of cells of two simultaneous growth studies of inos (11-1A) conidia, after only 12 hours growth in both minimal 1% sucrose and in minimal 1% sucrose supplemented with 50 µg inositol per ml, revealed that the hyphae of the non-supplemented culture were shorter than those of the supplemented

culture. At 400X magnification, examination revealed that the hyphae of the non-supplemented culture were less extended, highly vacuolated, and apparently necrotic. The hyphae of the supplemented culture appeared normal.

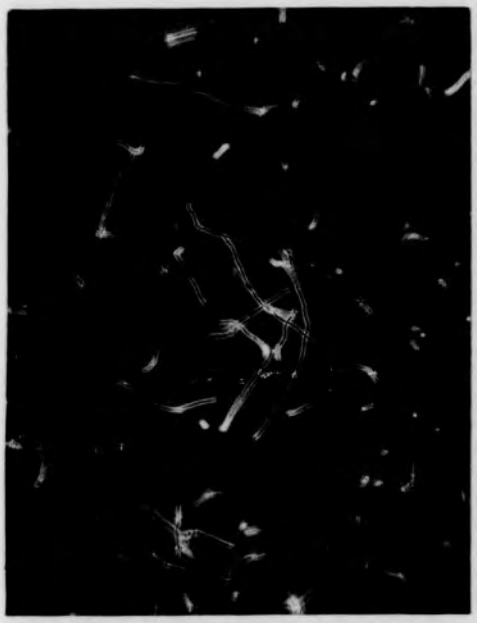
A similar growth study of chol-2 revealed that the hyphae of the non-supplemented culture at 12 hours were also shorter than those of the culture supplemented with 50 µg choline per ml; however, they appeared normal and not unusually vacuolated. Photographs (80:1) are found in Figure 7 showing both strains after 12 hours growth in both non-supplemented media and in media supplemented with inositol or choline. The relative lengths of hyphal extension are evident in these photographs although the internal conditions as described above are not visible.

Ethylene Glycol - Attempt to Gain Synchrony

The problems of asynchrony of conidial germination and the consequential complexity of statistical analysis of tip elongation rates within any one culture led to attemps to achieve greater synchrony of germination.

Conditions of Growth and of Dilution of Experiment 5. The method of Wilson and Bates (1972, 1974) of achieving approximately 75% germination within one hour with ethylene glycol treatment of conidia was attempted. In this first ethylene glycol study, induction was not attempted. Rather, cells of the chol-2 and inos (11-1A) strains were shaken for 88.5 hours in flasks of ethylene glycol medium. Each strain was shaken in 2 flasks, one containing 50 µg choline or inositol per ml and the other containing no additional supplement. Conidial enlarge-

Figure 7. Photographs of Growth of Experiment 4 (80:1).



A. inos with inositol

B. inos without inositol

C. chol-2 with choline

D. chol-2 without choline

ment occurred in all four flasks; however, a wide range of conidial sizes were found in all four cultures. No differences could be determined microscopically at 400X magnification between the supplemented and the non-supplemented inos (11-1A) cultures. In addition, no differences could be determined between the appearance of the enlarged of either of the chol-2 cultures; however, many of those conidia in the cholineless culture which were small appeared to be relatively highly vacuolated and necrotic.

Only the two minimal sucrose flasks of each of the two strains were diluted from 20% to 2% ethylene glycol for germination studies. The diluent was a minimal sucrose (1.5%) medium. After the 35-minute dilution process, half of each flask was removed and transferred into two additional flasks, each containing 50 µg of the appropriate supplement per ml.

Microscopic and Macroscopic Appearance of Growth of Experiment 5. A high level of synchrony of germination was not achieved. In addition no differences could be observed between the cultures of any of these four flasks by 2 hours and 20 minutes; however, by 46 hours, differences among the four flasks were quite pronounced. The supplemented chol-2 growth was in the form of a mycelial mass with long hyphae which were normal in appearance. Macroscopically, the chol-2 non-supplemented growth was of a much darker color. Microscopically, the growth was observed to consist mostly of small clusters of clumped hyphae. The growth which was in suspension consisted of short, stubby hyphae with the septa being close together.

The supplemented inos (11-1A) flask also contained a large mycelial mass with long hyphae, normal in appearance. However, all of the cells in the non-supplemented flask had the disorganized appearance of dead cells. Those conidia that had germinated appeared to have hyphae no longer than the hyphae had been at 2 hours 20 minutes.

Possible Correlates to Low Synchrony of Experiment 5. The lack of synchronous germination in this experiment may have been related to using cells treated with ethylene glycol for 88.5 hours rather than the recommended 48 hours and to using cells maintained in the non-supplemented ethylene glycol medium.

System II - Induction with Various Carbon Source Levels

A second ethylene glycol experiment was made using 48-hour cells taken from an ethylene glycol medium containing non-limiting levels of the supplemental membrane component. Induction of inos (11-1A) conidia was attempted by adding the inducer to the medium used for dilution of the ethylene glycol cells. The chol-2 strain was never used for this type of experiment.

Purposes of Experiment 6. With this experiment, it was hoped to attain a high level of synchrony and to determine if ethylene glycol-treated cells could be induced after dilution into a fresh medium. A carbon source was added to one flask containing induction medium to determine also if the presence of a carbon source affected the levels of cellulase secretion and of tip extension. A second induction flask lacked a carbon source. Both flasks were supplemented with the non-limiting level of 100 µg inositol per ml. Varying the levels of a carbon source was an alternate approach from the use of "membrane

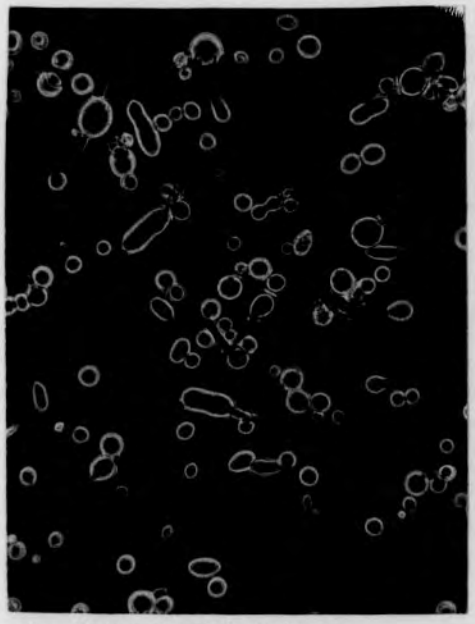
mutants" toward examining tip extension and membrane synthesis without the accompanying complexities of "inositolless death." It was supposed that when the enlarged ethylene glycol cells not supplemented with a carbon source had utilized their reserve of energy compounds, the rates of membrane synthesis and tip extension would be decreased and that a correlation between hyphal extension and extracellular cellulase levels could be determined.

Xylose as the Carbon Source in Experiment 6. Xylose was the carbon source chosen for this study since it had been found in earlier studies (Eberhart and Beck, unpublished) not to interfere with induction as had sucrose and glucose.

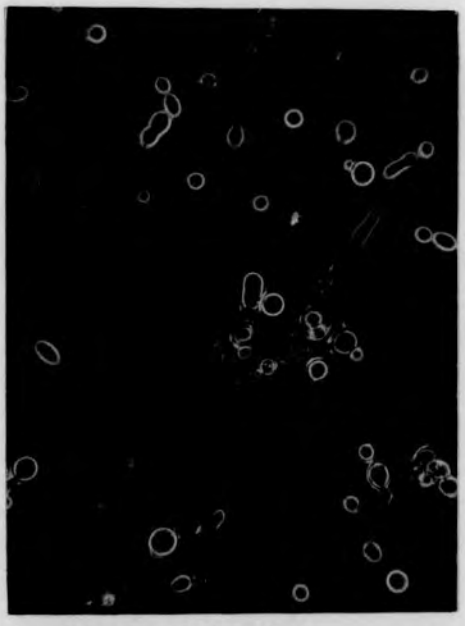
Conditions of Induction in Experiment 6. The diluent for this induction study consisted of Vogel's 1X minimal medium with 100 µg inositol per ml, 10^{-3} M cellobiose, and 2% xylose in one flask and in a second flask the same minus xylose. After the dilution process and 35 minutes into the induction period, the optical density of the conidial suspension in each flask was 0.530.

Microscopic Examination of Growth of Experiment 6. Samples were taken from both of these flasks periodically during the 9-hour induction period. Some were examined microscopically and others were frozen prior to assay of cellulase activity. Representative photographs of some of these samples appear in Figure 8. Again germination was not synchronous. The more greatly enlarged cells seemed to germinate earlier than did the smaller cells. A phenomenon noted was clumping of cells. Although both cultures exhibited clumping, it was noted by 3 hours that the culture lacking xylose had growth which was much more

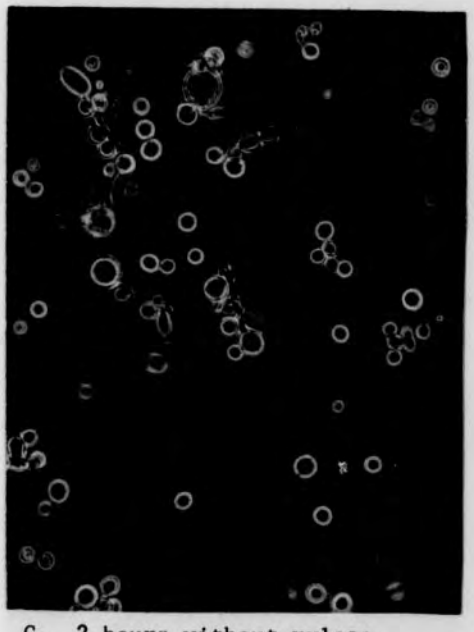
Figure 8. Ethylene Glycol-Treated Cells of Experiment 6 During Induction (320:1).



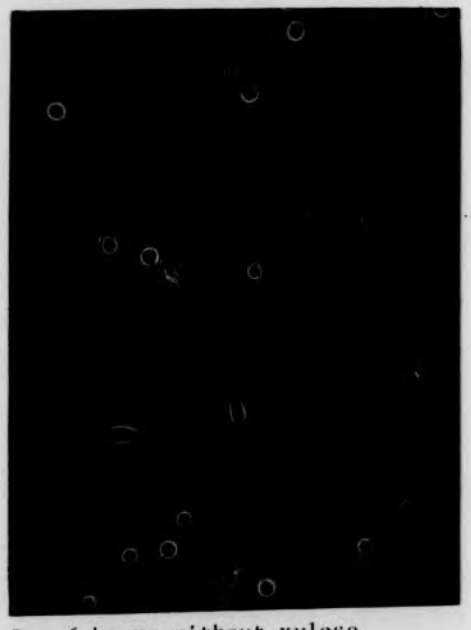
A. 2 hours with xylose



B. 6 hours with xylose

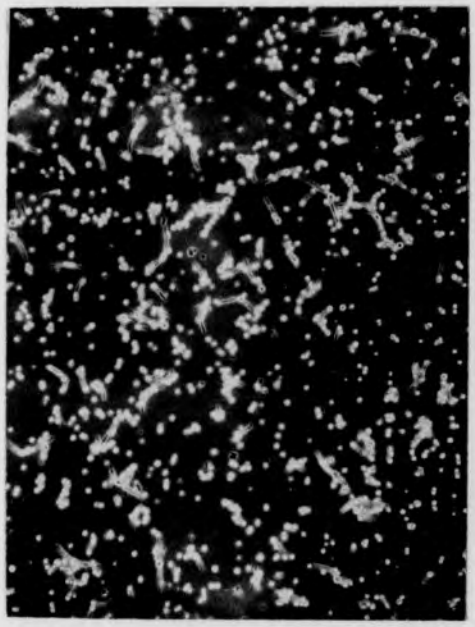


C. 2 hours without xylose

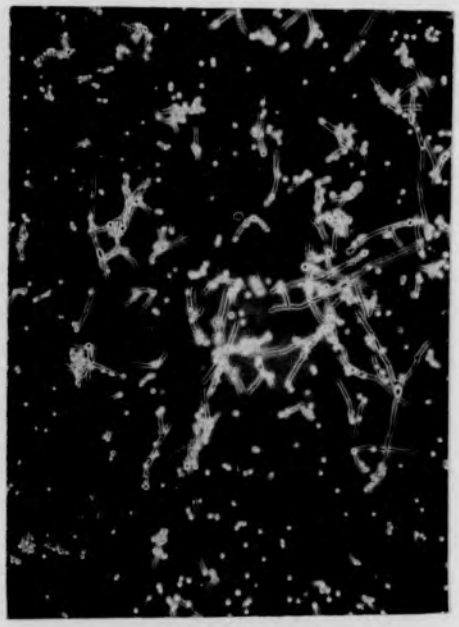


D. 6 hours without xylose

Figure 8 continued. (80:1).

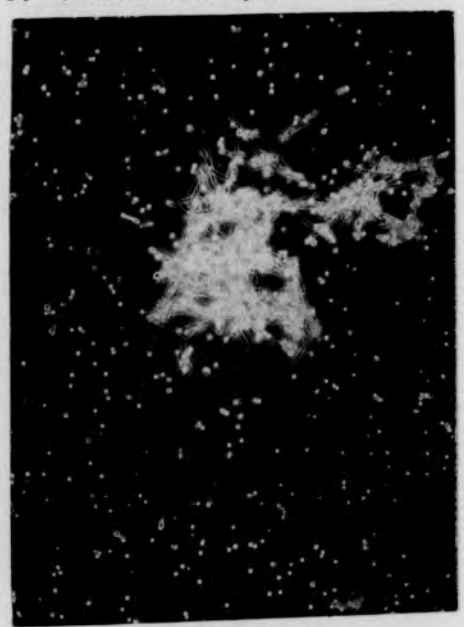


E. 3 hours with xylose



F. 6 hours with xylose

G. 3 hours without xylose



H. 6 hours without xylose

clumped. This trend continued for the 9 hours, except that clumping seemed to increase after 6 hours in the culture containing xylose. Photographs in Figure 8 are of fields chosen to be representative of the growth not included in the excessively clumped masses. It was noted that as the induction time progressed, agglutination of growth, and consequently clearing of the induction medium, increased especially in the culture lacking xylose.

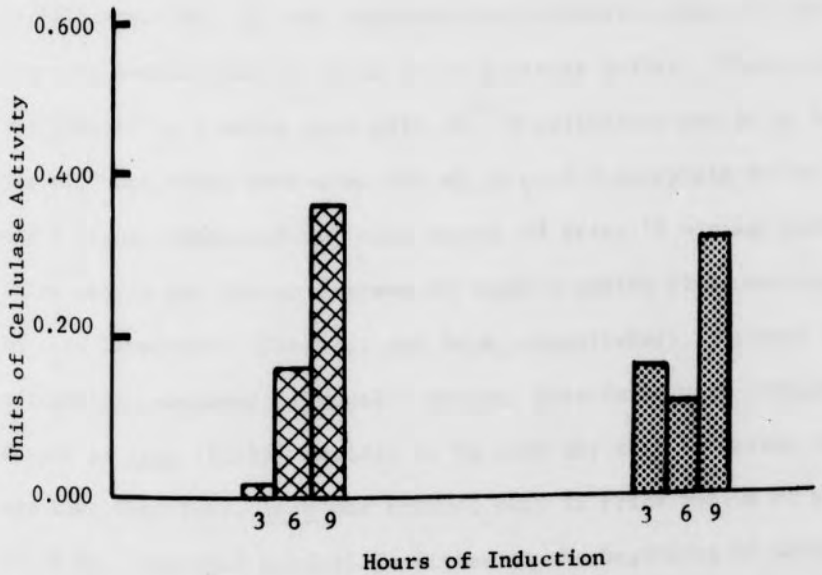
Assay of Cellulase Levels from Experiment 6. It was necessary to dialyze the samples of the cellulase preparations containing xylose to remove this reducing sugar which interfered with the reducing sugar assay. Therefore, all preparations--with and without xylose--were treated similarly with dialysis against distilled water for 12 to 24 hours and were concentrated with Carbowax approximately 3-fold prior to assay. Units of enzyme activity per ml were determined for the original dilution under standard assay conditions. Cellulase activity is summarized in Figure 9.

Discussion of Experiment 6. In Figure 9 there is a pronounced difference in extracellular levels of cellulase after 3 hours of induction. The visual difference apparent in Figure 8 between these two inos (11-1A) cultures with and without xylose after 3 hours were more related to different degrees of clumping than to different degrees of hyphal extension with the culture lacking xylose having a greater level of agglutination. There appeared to be a relationship between hyphal agglutination, suboptimal growth conditions, and extracellular levels of cellulase. Both agglutination and cellulase secretion had increased in the xylose-supplemented culture by 6 hours. The xylose

Figure 9. Cellulase Activity of Ethylene Glycol-Treated Cells of Experiment 6.

The plot shows cellulase activity of ethylene glycol-treated cells of strain inos (11-1A) induced in a medium containing 2% xylose and in a medium lacking xylose. Samples were taken from both of these flasks after 3, 6, and 9 hours of induction and were assayed for cellulase activity by the reducing sugar method. A unit of activity is one micromole glucose equivalents released per hour.

Symbol	Medium of Induction
⊠	= with 2% xylose
▩	= no xylose



concentration of this culture was not determined, but it is possible that the level could have been limiting by 6 hours.

System III - Induction of Cultures in Both a Buffer and in Fries Medium

Although relatively high levels of extracellular cellulase were achieved by induction of these ethylene glycol-treated cells, the lack of synchrony of germination did not seem to warrant the continuation of the more complex processes of ethylene glycol treatment without once again trying induction of untreated conidia.

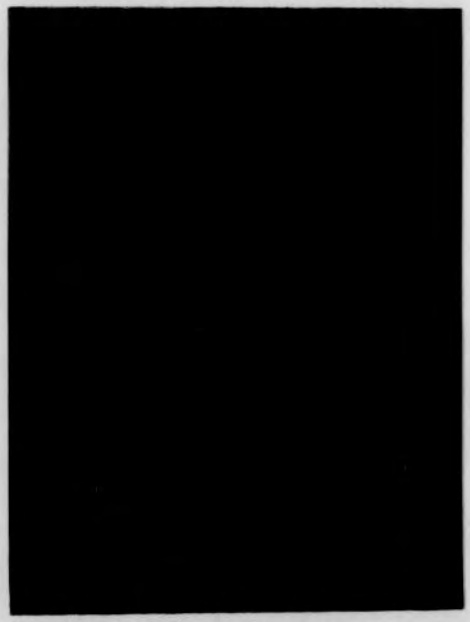
Conditions of Induction of Experiment 7. For this induction, conidia of the wild type (wt) strain STA-4 were induced for 9 hours and conidia of the inos strain 11-1A for 8.5 hours. With this experiment, the third approach was tried toward attempting to alter the rate of hyphal extension. It was supposed that extension might be greater in a growth medium than it would be in a simple buffer. Therefore, STA-4 was induced in 2 media each with 10^{-3} M cellobiose and 50 µg inositol per ml. One flask contained 200 ml of 0.05 M phosphate buffer, pH 6.0, and a second contained the same amount of Fries 1X minimal medium. Fries medium was chosen instead of Vogel's medium since earlier data of this laboratory (Eberhart and Beck, unpublished) indicated that citrate (a component of Vogel's medium) interferes with induction. Growth of inos (11-1A) conidia to be used for this induction experiment was low; therefore, inos was induced only in Fries medium at a volume of 75 ml. Conidial optical densities at the beginning of induction were 0.500 for STA-4 in Fries medium and 0.520 for both STA-4 in phosphate buffer and for inos (11-1A) in Fries medium. This was similar to the optical densities of the ethylene glycol-treated cells of the previous experiment.

Microscopic Examination of Growth of Experiment 7. Samples were taken periodically during the induction period for microscopic examination and for centrifugation and freezing prior to cellulase assay. Microscopic examination revealed no obvious difference in the levels of synchrony of germination between these conidia and the enlarged conidia of the previous ethylene glycol study. In addition, microscopic examination revealed no visual differences in hyphal lengths or in apparent extension rates between the STA-4 cultures in buffer and in Fries medium. Clumping did occur in both cultures of STA-4; however, there appeared to be a greater amount and an earlier appearance of agglutination in the culture induced in the buffer than in the one induced in Fries medium. Photographs in Figure 10 show asynchrony of germination and agglutination of cells. Agglutination occurred in both STA-4 cultures, although the degree of clumping, and hence the level of clearing of the induction medium was greatest in the buffer. It can also be seen that the level of germination of conidia of inos (11-1A) in Fries medium seems to have been less than the level of germination of STA-4 in either Fries medium or in buffer. The levels of agglutination, however, seem to have been quite similar for the inos culture in Fries medium after approximately 9 hours of induction and for the STA-4 culture in Fries medium.

Assay of Cellulase Activity of Experiment 7. Samples of the STA-4 preparation, taken after 3, 6.5, and 9 hours of induction, and samples of the inos (11-1A) preparation, taken only after 8.5 hours of induction, were frozen prior to further treatment. All samples were dialyzed against distilled water for 24 hours and were concentrated

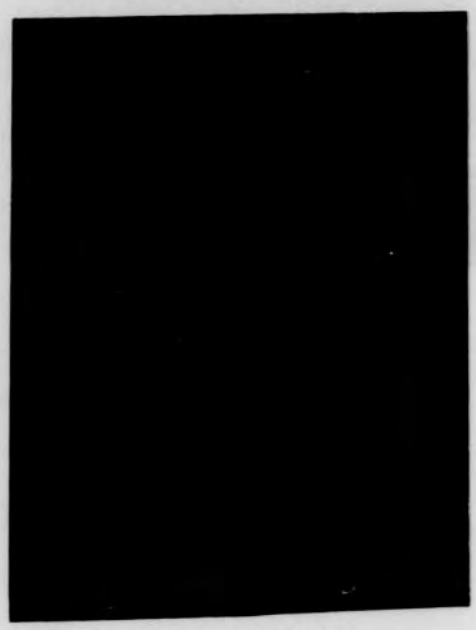
Figure 10. Conidial Cultures in Fries Medium and in Phosphate Buffer - Experiment 7 (320:1).

A. STA-4, 2 hours in Buffer



B. STA-4, 9 hours in Buffer

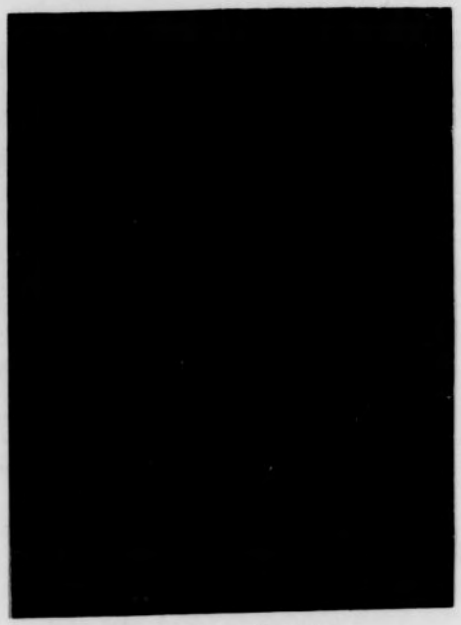
C. STA-4, 2 hours in Fries



D. STA-4, 9 hours in Fries

Figure 10 continued. (320:1).

E. inos, 2 hours in Fries



F. inos, 8.5 hours in Fries

approximately 3-fold before being assayed for cellulase activity. The reducing sugar assay data are summarized in Figure 11.

Discussion of Experiment 7. As shown in Figures 9 and 11, there was much less cellulase secretion by the conidia of Experiment 7 than by the enlarged conidia of the ethylene glycol induction of Experiment 6, both having initial conidial optical densities of approximately 0.500. In addition, secretion was greater for the STA-4 culture in Fries medium than for the same strain in buffer. Also, the levels of cellulase secretion were similar for both the inos (11-1A) culture in Fries medium and for the STA-4 culture in Fries medium after approximately 9 hours of induction.

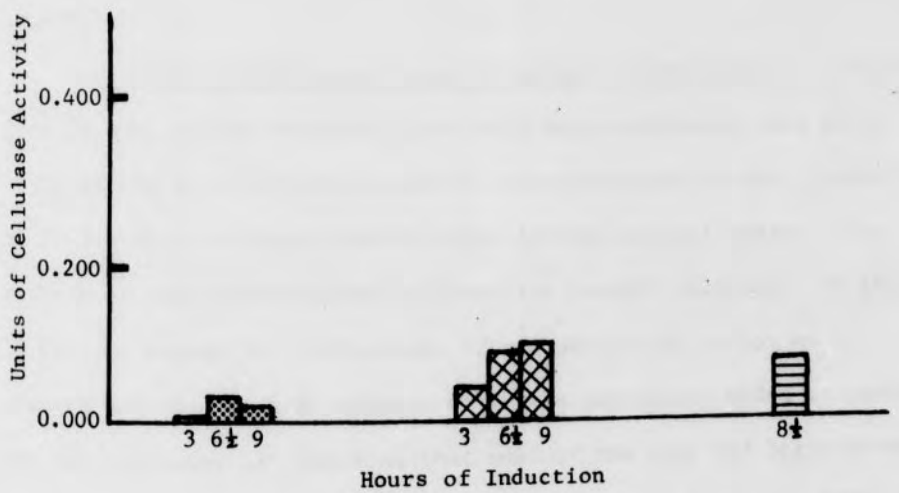
In Experiment 6 with the ethylene glycol-treated cells, there seemed to be greater secretion associated with greater levels of clumping. However, in Experiment 7 with untreated cells, there appeared to be less secretion associated with the cultures showing greater levels of clumping. Agglutination was greater and earlier in appearance in the Fries medium, yet cellulase activity was less and later in appearance in the buffer. Cellulase activity was approximately 3 times greater by 6 hours in the STA-4 culture induced in Fries medium than in the one in buffer. The correlation between agglutination and secretion was not apparent.

The original hypothesis being tested in Experiment 7 was that greater tip extension, and therefore, greater secretion would be found in the culture induced in a growth medium. However, no differences were observed in tip extension, although secretion was greater in the Fries medium culture than in the buffer.

Figure 11. Cellulase Activity of Untreated Conidia of Experiment 7.

The plot shows cellulase activity as measured by the reducing sugar method from 2 conidial cultures of strain STA-4 (wild type) which were induced in 0.05 M potassium phosphate buffer, pH 6.0 and in 1X Fries; and the activity of a conidial culture of strain inos (11-1A) induced in 1X Fries. Samples of both STA-4 cultures were taken after 3, 6.5, and 9 hours of induction for cellulase assay. The inos (11-1A) culture was assayed after 8.5 hours of induction.

Symbol		Strain and Condition of Induction
▩	=	STA-4 (wt) in potassium phosphate buffer
▩	=	STA-4 (wt) in 1X Fries medium
▤	=	inos (11-1A) in 1X Fries medium



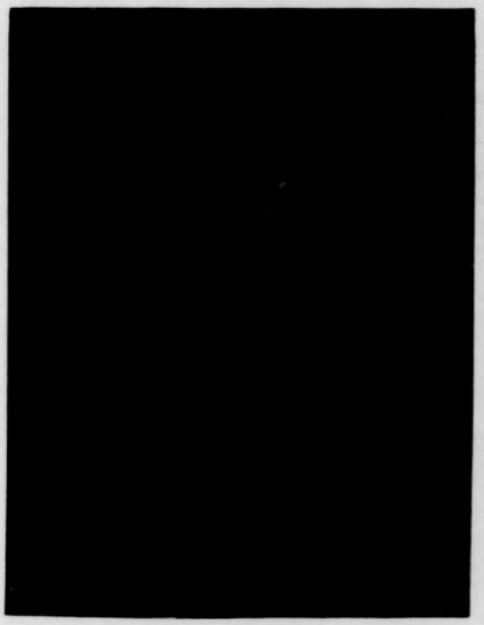
System IV - Induction with a Colonial Temperature-Sensitive Mutant

For a fourth approach toward attempting to establish the relationship between hyphal tips and cellulase secretion, the colonial mutant gluc-1^{+},cot,ylo was used for induction studies. Conditions for growth of the mutant in liquid medium in the form of separated, distinct colonies with numerous short hyphae had previously been established (Eberhart and Karibian, unpublished). It was proposed that colonies grown with these conditions could be transferred to a 25C induction medium, and that the numerous tips of the colonies would begin extension at a more rapid rate when subjected to optimal growth conditions. With this colonial mutant, there would be a greater number of tips per mass for colonially grown cultures than for the wild type growth.

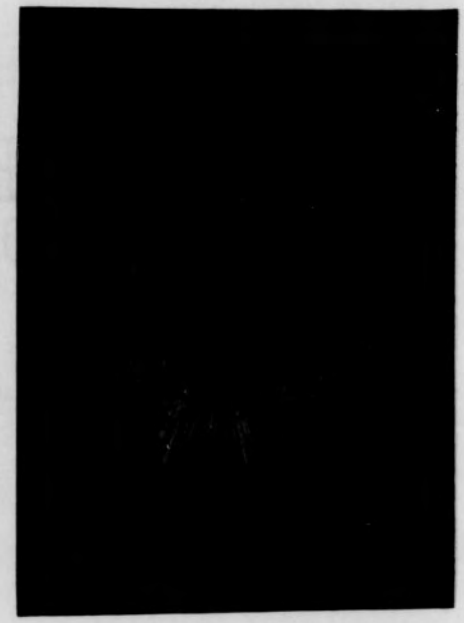
Pattern of Tip Extension of Colonies of Experiment 8. To establish if many of the numerous tips would begin extension soon after being placed in a 25C growth medium, colonies grown on agar plates at 31.7C for 48 hours were flooded with 1X Fries minimal medium. The pattern of tip extension was followed for several colonies. In Figure 12 are two sequential photographs of one particular colony at 15 minutes and at 1 hour 35 minutes after the medium was added to cover the agar surface. It appeared that most of the tips did begin extension soon after the growth conditions were altered to become more optimal.

Initial Induction - Experiment 9. An initial induction experiment was conducted with 42-hour colonies grown at 31.7C and induced in 0.1 M phosphate buffer, pH 7.0, for 5.5 hours. This induction yielded

Figure 12. Colonial Temperature-Sensitive Mutant Flooded with Fries Minimal Medium (80:1).



A. After 15 minutes



B. After 1 hour 35 minutes

an extracellular cellulase preparation with a specific activity of 0.326 as measured by the reducing sugar assay.

Second Induction - Experiment 10. A second experiment was conducted in order to compare cellulase secretion of mycelia grown at 21C with that of colonial growth. For this experiment, two flasks containing 1000 ml of medium were inoculated with similar levels of conidia. One culture was grown with colonial morphology and the other with wild type morphology, both for 40.5 hours. The growth of the two flasks were washed by decanting several washes of distilled water and were placed in two induction flasks containing 0.7X Fries minimal medium and 1.2×10^{-3} M cellobiose for 7 hours. The data for this experiment are summarized in Table 7.

TABLE 7

INDUCTION OF THE COLONIAL MUTANT - EXPERIMENT 10

Temperature of Growth	Type of Growth	Temperature of Induction	Specific Activity
31.7C	Colonial	25C	1.130
21.0C	Wild Type	25C	1.844

Discussion of Experiment 10. No simple relationship exists with these conditions between the ability to secrete amd the number of tips available. The colonial growth of 40.5 hours consisted of many more tips per weight than did the wild type growth; however, the specific activity of the extracellular cellulase was much less. It is possible that this lower level of secretion is related to a mucopolysaccharide which is discussed later in this work.

DISCUSSION

Discussion of Induction Experiments

System I - "Membrane Mutants". The attempts to establish relationships among levels of inositol or choline for the growth of the nutritional mutants, amount of tip extension, and the secretion of cellulase did not provide any conclusive results concerning this relationship. This system using nutritional "membrane mutants" is highly complex. The hypothesis which was the basis for trying these "membrane mutants" was that without the inositol or choline, there would be no membrane synthesis and no enzyme secretion. Provided that cell metabolism continued as normal, there would then be a build-up of cellulase in the interior of the cell. Upon the addition of the necessary nutrient, there would result a burst of cellulase activity as new membranes were synthesized and as tip extension reoccurred.

The worth of this hypothesis depends on the assumption that cell metabolism occurs as normal with limiting levels of inositol or choline, and that only membrane synthesis is altered. There is much evidence to the contrary, however. Matile (1966) has shown by density-gradient centrifugation three distinct fractions containing cellular membranes. One fraction contains mainly mitochondrial membranes, one most other cellular membranes called the light membranes, and a third fraction contains membranes of distinct particles found to contain proteases. In studies of cholineless and inositolless mutants, Matile discovered that inositol is incorporated in high levels in all three membrane

fractions, but that choline is incorporated only into the mitochondrial and light membrane fractions, and only very slightly into the protease particle membranes. Matile explains that in inositol-requiring mutants grown on suboptimal levels of inositol, the membranes of the protease particles degenerate releasing proteases into the interior of the cell resulting in autolysis of the cytoplasm. This would explain the active cellular destruction of "inositolless death" in contrast with the colonial growth of the cholineless mutants grown on suboptimal levels of choline.

This information may indicate that a study of a nutritional mutant as attempted during the course of these studies might have been more rewarding with a cholineless mutant than with the perhaps more complex system of the inositolless mutant. In additional studies of inositol-requiring strains, it has been observed that sulfate transportation systems (Marzluf, 1973) and sugar transportation systems (Scarborough, 1971) in Neurospora crassa did not function properly when the cells were depleted of inositol. It does seem that cell metabolism probably does not continue as normal in certain nutritional mutants deprived of their required nutrients. This imposes limitations on such a system of using "membrane mutants" to attempt to correlate tip extension with enzyme secretion.

System II - Carbon Source. The one attempt to try to establish the relationship among levels of a carbon source, tip extension, and cellulase secretion did not provide conclusive results. This system was attempted using ethylene glycol-treated cells in xylose-supplemented media. Significantly higher levels of cellulase were produced in both

cultures than in cultures from other induction experiments with untreated conidia all having a similar conidial optical density at the beginning of induction. This indicates that ethylene glycol-treated cells secrete higher levels of cellulase into the external medium. In a study by Lester and Byers (1965) certain glycols were found to enhance the activity of β-galactosidases. The phenomenon seen here of increased cellulase activity is probably not a case of glycol-enhancement since all the cellulase preparations were dialyzed against distilled water for 12 to 24 hours, removing most small molecules including glycols before assay of cellulase activity.

System III - Growth Medium. The third system did show greater levels of cellulase secretion with a Fries induction medium than with a simple buffered medium, however, no differences in tip extension could be distinguished.

Agglutination of Growth. In all these attempts at determining a relationship between tip extension and enzyme secretion, a phenomenon which kept reappearing was clumping of growth. It was not determined what effect this phenomenon had upon cellulase secretion, but it appeared in three experiments that agglutination is possibly related to suboptimal growth conditions. First, in the 46-hour mycelial cultures of the cholineless strain which had previously been treated with ethylene glycol, there was a greater amount of agglutination of growth in the growth medium lacking choline than in the one which had optimal levels of choline. When conidia of inos (11-1A), also treated with ethylene glycol, were later induced in both a xylose-supplemented medium and a non-supplemented medium, agglutination was more pronounced in the more simple and presumably less optimal buffer.

It was also observed in the same three experiments that the lower levels of clumping in the various supplemented media increased as time of induction progressed. This may have been associated with removal of the various supplements during growth, possibly resulting in limiting levels of these supplements.

Agglutination has been found by Reissig and Glasgow (1971) to be induced by the production by cells of a mucopolysaccharide (MP) which inhibits growth and causes vacuolation in addition to causing agglutination of cells. They identified the inducer of the restricted phase of growth in a colonial temperature mutant to be MP. They hypothesized that MP causes agglutination, and that agglutination causes restricted growth. In addition, they found the concentration of MP required to inhibit growth or to cause vacuolation and agglutination to be identical for both a wild type strain and for a colonial mutant. The main difference they observed between the mutant and the wild type appeared to be the timing of MP production and consequently of induction of the restricted phase of growth. Restricted growth appeared in the colonial strain by 10 to 12 hours and in the wild type only after about 20 hours. The agglutination appearing in the induction experiments of this study may also have been related to MP production.

System IV - Colonial Temperature-Sensitive Mutant. The fourth system employed the use of a colonial temperature-sensitive mutant. Induction of both wild-type growth and colonial growth at the temperature optimal for wild-type growth revealed a lower level of cellulase secretion with the colonially grown culture than with the wild-type grown culture.

It had been expected that with the larger numbers of tips present per mass in the colonial growth that there would be greater secretion from the colonial growth than from the wild-type--both induced at the temperature optimal for wild-type growth--if secretion were dependent upon tip extension and hence, upon the number of tips available. The results obtained with this preliminary experiment were in opposition to this hypothesis. It is possible that MP inhibition may have influenced the lower levels of cellulase secretion in the colonially grown culture. Reissig and Glasgow (1971) observed a protein doubling time of 2.5 hours for their colonial mutant during unrestricted growth. This protein doubling time was increased from 2.5 to 14 hours when MP was experimentally added to the culture to restrict growth. Conversely, it was observed that the dilution of a culture, already restricted in growth, into a fresh medium did not return the 14-hour doubling time to 2.5 hours, but rather only decreased the time from 14 hours to 9 to 10 hours. Apparently, dilution was not sufficient to remove MP and to eliminate totally the inhibition of protein systhesis and of growth by MP.

Similarly, when the colonially grown temperature mutant was diluted into a fresh induction medium, to be compared with unrestricted growth of the same strain as to levels of cellulase secretion, a lower level of cellulase secretion was revealed for the colonially grown culture. It is possible that MP, previously released by the culture during the period of colonial growth, was inadequately removed by the dilution process, and that the MP was responsible for partial inhibition of protein and cellulase synthesis and consequently of secretion.

Cellulase Number and Molecular Weight

The cellulase systems of many fungi have been shown to consist of a multiple-component complex of β-1-4 glucanases (C_x or cellulases); however, this multiple system has not been conclusively shown to exist in Neurospora crassa. Methods for investigation of cellulases in fungi (Eriksson, 1969) include purification by fractional precipitation, several electrophoretic techniques, paper chromatography, separation owing to differences in heat stability, and column chromatography including ion exchange chromatography and gel filtration.

Gel Filtration. Gel filtration of cellulolytic enzymes in fungi has been employed in many laboratories (Selby and Maitland, 1965; Eriksson and Pettersson, 1968; Eriksson and Rzedowski, 1969b). Culture filtrates from Myrothecium verrucaria were found (Selby and Maitland, 1965) to give three cellulolytic components with molecular weights of about 55,000, 30,000, and 5,300 daltons when fractionated on a Sephadex G-75 (200-375 mesh) column. The middle component was found to compose 90% of the total carboxymethyl cellulase activity. The Sephadex used by Selby and Maitland was filtered to yield a gel with a fractionation range of 3,500 to 67,000 daltons. Bio-Gel P-100 used in the studies of this thesis has a fractionation range of 5,000 to 100,000 daltons. With this gel, filtration studies indicated the presence of only one major cellulase in the extracellular enzyme preparations, and indicated that this cellulase had a molecular weight of less than 50,000 daltons. This result seems valid when compared with those of Selby and Maitland, both to molecular weight and number of the major components. Probably, any minor components in the preparations of these

studies were of too small a concentration to be observed by the assay methods used.

Historical Parallel to Number of Cellulases in Neurospora. Earlier studies of the β-glucosidase system of Neurospora crassa (Eberhart, et al., 1964) indicated two peaks of enzyme activity present in fractions precipitated from crude mycelial extracts by increasing ammonium sulfate concentrations in both the gluc-1 and the gluc-1^{+} strains. The mycelia used for these induction studies had been grown and induced for 6 days at 30C in minimal Vogel's medium with 1% CMC as inducer and with 1% xylose as a carbon source.

It is possible that this prolonged cultivation of 6 days may have resulted in an increasing heterogeneity of the enzyme or enzymes capable of cellulase activity. Bucht and Eriksson (1968) have discovered by isoelectric focusing that prolonged cultivation of cultures of Stereum sanguinolentum caused an increasing heterogeneity of the cellulolytic and related enzymes in the extracellular culture solution.

In the induction studies of this thesis, mycelia were grown no longer than 53 hours in a growth medium and were then washed and transferred to a fresh induction medium. Lengths of time of induction ranged from 6 to 24 hours. The influences of prolonged cultivation upon the cellulase of these studies would therefore probably have been minimal with these conditions of growth and induction and may possibly account for the indication of only one major cellulase rather than of two as in earlier studies of intracellular cellulases.

SUMMARY

The relationship of the extension of hyphal tips to secretion was examined in several strains of Neurospora crassa. Cellulase, an extracellular enzyme, was chosen as an index of protein secretion. The plan of this thesis was to examine the following points related to secretion:

1. Simplification of the assay of cellulase activity by improving existing methods and by originating other methods.

2. Determination of the number of cellulases present in induced mycelial and conidial cultures.

3. Study of factors which influence the rate of tip extension.

4. Determination if tip extension is necessary for secretion.

As part of the assay of cellulase activity, improvements were made in the Somogyi reducing sugar method. Several limitations of this method were identified including the inhibitory effect of high protein concentrations and the inhibitory effect of Vogel's and of Fries media.

The Worthington Glucostat reagent, in combination with excess β-glucosidase, was used to develop a spot test for cellulase activity and to determine the location of cellulase activity for thin-layer gel separations. An additional variation of the Glucostat method was developed using homovanillic acid rather than the standard Glucostat chromogen for a fluorescent determination of cellulase activity.

A qualitative cellulase assay was developed which employed the use of hydroxyethyl cellulose as substrate. The substrate solution was blended vigorously to yield a thick foam. Cellulase activity was indicated by the clearing of the cellulose foam.

Column gel filtration studies indicated the presence of one major cellulase in these enzyme preparations. Preliminary molecular weight studies with thin-layer gel filtration indicated this cellulase to have a molecular weight of less than 50,000 daltons.

Various cellulase induction experiments were conducted with both conidia and mycelia to determine the factors which influence the rate of tip extension and to determine if tip extension is necessary for secretion. Four systems of induction were employed to alter the rate of tip extension including the use of auxotrophs requiring components necessary for normal membrane synthesis, the use of various levels of a carbon source, the use of a Fries minimal medium, and the use of a colonial temperature-sensitive mutant. In addition, the phenomenon of agglutination of growth and its correlation with secretion was examined. However, no conclusive relationships were determined among secretion, tip extension, and agglutination.

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APPENDIX: LIMITATIONS OF REDUCING SUGAR ASSAY

Cellulase assay by the reducing sugar method was found to be a useful, quantitative method of assay if certain restrictions identified in this study were carefully observed. The most obvious limitations were careful pipetting and equal heating of all samples of a particular experiment. When using the Sampler micro-pipetting instruments (Oxford Laboratories), it was essential that the instruments be clean and well lubricated, and that the tips be accurately filled and completely emptied. Samples for assay of a particular experiment could be completed at different times as long as all samples were equally heated.

An additional requirement found to be necessary for quantitative results was to have low levels of protein in the enzyme-substrate reaction mixture. This could usually be achieved by assaying enzyme preparations at the original concentration. If activity was too low at the original concentration for assay by the reducing sugar method, it was possible to obtain useful data of relative activities by concentrating all samples of a given experiment to the same level.

Cellular contaminants in the substrate-reaction mixture were found to alter the shape of the assay curve, especially after 4 or 5 hours of assay. This undesirable effect can be avoided by mixing sterile CMC and enzyme, and by using clean methods of sampling for reducing sugar determinations.

It was found to be necessary to dialyze enzyme preparations containing Vogel's or Fries media since these solutions were found to be inhibitory either of cellulase activity or of the detection of known glucose.

The difficulties in pipetting the viscous 2% substrate solution were found to be decreased by allowing the solution to reach 30C before pipetting into the reaction tubes. The substrate solution was also allowed to reach 30C before the enzyme was added.