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The aryl-beta-glucosidase and cellobiase have been compared in wild type and mutant strains of *Neurospora crassa*. The gluc-1 mutant failed to show significant induction by various concentrations of exogenous cellobiose. This strain also failed to show the usual endogenous induction exhibited by wild type *Neurospora* under a variety of experimental conditions. New techniques were developed to study the release of exogenous enzyme in the process of conidiation. Intact cells were also studied. The results were discussed in light of the Jacob and Monod model.

STUDIES ON THE BETA-GLUCOSIDASES
OF NEUROSPORA CRASSA

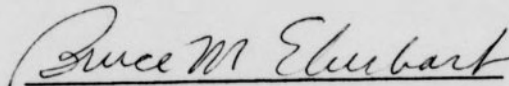
by

Catherine Ting Lee

A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Master of Arts

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Approved by


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INTRODUCTION

Enzyme induction in bacterial systems has been studied intensively (F. Jacob 1966, J. Monod 1966). Comparative studies have also been made in the late stage of development in Neurospora crassa. In this thesis, the induction of aryl-beta-glucosidase and cellobiase in *Neurospora* has been studied in the early stages of development. A mutant strain of *Neurospora* characterized by its low level of aryl-beta-glucosidase was used as well as wild type in an attempt to learn the function of the gluc-1 locus. In addition, the endogenous induction--an increase in enzyme activities during the process of differentiation--was studied. A comparison between endogenous induction and exogenous induction caused by external effectors has been discussed.

HISTORICAL BACKGROUND

It has been known for over sixty years that certain enzymes of micro-organisms are formed only in the presence of their specific substrate (Jacob and Monod 1961). This effect was later named "enzyme adaptation" by Karstrom in 1938, and the study of enzyme induction was initiated by the discovery of "diauxy"--two complete growth cycles which could be observed when bacteria grew in some varied mixtures of carbohydrates (Monod 1966). In 1946, it was shown that the adaptive enzyme systems are controlled in bacteria by genetic determinants (Monod and Audureau 1946). Now induced enzyme synthesis can formally be defined as the increase in the ratio of the rate of synthesis of a given enzyme to the rate of synthesis of total cell protein resulting from exposure of cells to compounds (inducers) which are identically or structurally related to the substrate of the given enzyme (Hogness 1959). A large number of inducible systems have been discovered and studied in bacteria. Among all of those, the "lactose" system (beta-galactosidase and related enzymes) of *Escherichia coli* has been studied most intensively by Monod et al. (Monod and Cohn 1952, Cohn 1957, Monod 1959, Jacob and Monod 1961). It has been proved by a combination of immunological and isotopic methods that the enzyme formed upon induction was derived from a complete de novo synthesis of enzyme molecules. It has also been proved that the beta-galactosidase was synthesized at the cellular level, that is, the response of all cells to induction is simultaneous and equal, if one meets the following conditions:

The first condition was the method of gratuity developed by Monod et al. (1952), in which the kinetics of enzyme formation should be studied under conditions where neither the presence of the enzyme itself, nor its inducer, influenced the general cellular metabolism. The inducer should induce only one new factor, namely, a supplementary process of specific synthesis which was precisely the phenomenon under investigation. For this purpose an inducer, thio-methyl-beta-D-glucoside (TMG), was used.

The second condition involved the use of non-resting cells. It was believed that the individuals of a resting cell population in a washed cell suspension, would have all levels of internal reserves and a wide variation in ability to make enzymes. The resultant kinetics, therefore, would not be applicable at the cellular level.

The last consideration was the inducer transport system. Rickenberg et al. (1956) had proved the presence of an induced permeation system in E. coli. Their evidence for this system was based on the observation of "cryptic" mutants that could not concentrate the inducer in the cell. These strains could, however, show induced beta-galactosidase activity when the external level of inducer was extremely high. The kinetics and specificity of the transport system was similar to those of typical enzymes and was given the name "permease."

Since 1953, some negative adaptations, i.e., specific inhibitions of enzyme synthesis have been discovered and observed (Monod and Cohen-Bazire, 1953; Cohn, Cohen and Monod 1953; Adelberg and Umberger 1953). Studies have revealed that the "repression" effect, as it was later named by Vogel (1957 a, b) is very closely analogous, albeit symmetrically

opposed, to the induction effect.

This was based on the observation that:

1. The specificity of induction or repression is not related to the structural specificity of the controlled enzymes.
2. It has also been shown that the rate of synthesis of different enzymes involved in the metabolism or synthesis of a single substance appears to be governed by a common system in bacteria.
3. This is so in the biochemical and genetic studies of a large number of mutants in both induction and repression systems.

(Jacob and Monod (1961, 1963) generalized and designed a model of the regulation of protein synthesis in bacteria.)

The occurrence of inductive and repressive effects in other microorganisms and in tissues of higher organisms have been observed in many instances, but it has not been possible to analyze any of these systems in detail, partly due to lack of adequate genetic systems. The fungus, Neurospora crassa, has been used for genetic studies for many years, and in many respects its biological system is between that of bacteria and higher organisms. Neurospora, therefore, seemed suitable for a study of enzyme induction.

The induction and genetic control of tyrosinase in Neurospora was studied by Horowitz et al. (1960). They found that there were three genes controlling the synthesis of tyrosinase. Of the three, only one gene has a structure-determining role in the synthesis of the enzyme. The other two genes influenced the synthesis indirectly through their control over an inductive mechanism that operates on the enzyme. In 1966, Nge et al. (1966) found an orthophosphate repressible alkaline phosphatase enzymes

in Neurospora. These are discussed in some detail by others (Urey 1966; Urey and Horowitz 1967).

Beta-glucosidase induction studies in Neurospora initiated by Singleton and Murphy (1958), continued by Belz and Zweiback (1959), and Kannwisher (1962) emphasized the later stages of the life cycle. Landmann (1954) observed that it was possible to induce lactase in resting mycelia and noted a diauxic effect on induction when mycelia were grown on sucrose and then induced in the same media with lactose. Washing the mycelia free of the sucrose and then inducing resulted in high levels of enzyme activity for as long as thirty-four hours. His work further showed that there were relatively large reserves available for the synthesis of enzyme in Neurospora. In the resting state the ability of the mycelia to produce the enzyme was dependent on the age of the mycelia at the time of induction. However, in the growing state, more factors were involved. The work of Kannwisher demonstrated the same possibility for beta-glucosidase induction in mycelia. He found also cellobiose was most suitable for beta-glucosidase induction in Neurospora.

The beta-glucosidase system studied before 1963 was found to contain two enzymes. The thermostable beta-glucosidase was named aryl-beta-glucosidase and the thermolabile one, cellobiase (Mahadeven and Eberhart 1964 a). These two enzymes were found to be under different regulatory gene control. Mutants of regulatory genes for both enzymes have been isolated. Gluc-1 and gluc-2 genes control the synthesis of aryl-beta-glucosidase only (Mahadeven and Eberhart 1964 b, Miller and Eberhart unpublished data). The cell-1 gene regulates the level of both cellobiase and cellulase (Myers and Eberhart 1966). However, aryl-beta-

glucosidase, cellobiase and cellulase can be induced in wild type strains simultaneously by cellobiose.

Zalokar and Cochrane (1956) observed that the metabolic patterns of the cells of *Neurospora* change with age and morphological differentiation. The most striking differentiation occurred at sporulation, and this differentiation was both of a morphological and a physiological nature. Studies by Zalokar (1959 a,b) in *Neurospora* again showed that specific activities of several enzymes were different in conidia, young hyphae, and mature mycelia. Succinic dehydrogenase was low in conidia and about ten times more active in mature mycelium. Aldolases increased about three times after the germination of conidia. Beta-galactosidase was formed in appreciable amounts only after prolonged growth and had a much higher specific activity in mycelia. The observations of Reinhardt (1892) confirmed that the pattern of the growth and differentiation of a *Neurospora* mycelium, as well as other fungi, is that the protoplasm migrates from older parts of the hyphae into the growing tips which brings the differentiation along the length of the mycelium. In 1964, Hill and Sussman studied the levels of trehalase and invertase found during the development of *Neurospora*. Invertase activity was highest in the mycelium after growth had been completed, whereas the greatest amount of trehalase activity was found in ungerminated conidia. Both enzymes showed the least activity in ascospores. The specific activity changed also, but not by the same order of magnitude. In 1966, Etten et al. did a more detailed study of changes in fungi with age. The decrease in the rate of respiration with age of *Rhizoctonia solani* and *Sclerotium baltaticola* was examined. Specific activities in cell free extracts were measured for

most of the enzymes in the hexose monophosphate shunt, Embden-Meyerhof-Parnas pathway, tricarboxylic acid cycle and terminal electron-transport system. In addition, glucose oxidase, isocitritase, and malic enzyme were measured.

According to Horowitz et al. (1960), the normal appearance and disappearance of tyrosinase activity during the life cycle of *Neurospora* is regulated by an inductive mechanism. The constitutive formation of the enzyme during growth of the organism is the result of self-induction, the inducer or inducers originating in the intracellular pool of aromatic amino acids. The changes of enzyme activities due to self-induction are considered as "endogenous induction" in this thesis.

MATERIALS AND METHODS

Selection of the Strains

A mutant strain, gluc-1, of *Neurospora* was detected by its reduced ability to cleave esculin (esculetin glucoside) in the growth media to esculetin and glucose. Esculetin then reacts with the ferric chloride also present in the media to produce a black precipitate that accumulates as a function of the amount of beta-glucosidase produced by the fungus. Strains containing the gluc-1 gene produce low levels of aryl-beta-glucosidase activity (Eberhart, Cross and Chase 1964). Another type of strain, gluc-2, shows still a lower degree of esculin destruction (Eberhart, Beck, and Miller unpublished data). The conidia from the wild type and mutant strains were induced by mM cellobiose for six hours. Differences in aryl-beta-glucosidase activity were detected. As indicated in Table 1, wild type strains have the highest inducibility, gluc-1 strains the next, and gluc-2 the least. Although the cellobiase can also be induced by cellobiose, there is no regular difference in cellobiase level among wild type, gluc-1, and gluc-2 strains.

Unless otherwise indicated, wild type St. Lawrence, 74 OR8-1a and gluc-1 strain CM25(4-5)A were used. For most of the induction studies, the conidia were used instead of mycelia in order to study the earliest stages of development.

Media and Growth of Cultures

For stock maintenance and conidial harvest, solid glycerol complete (GCP) media is used. This medium contains minimal medium (Vogel,

Table 1

Aryl-beta-glucosidase activities in conidia of
wild type, gluc-1 and gluc-2 strains^a

	Before induction 10X O.D. (410mu) ^b	After 6 hours induction 10X O.D. (410mu) ^b
StLa 74-0R8-1a(gluc+)	0.053	0.140
St A 4 <u>gluc+</u>	0.086	0.175
CM 75(9-5) <u>gluc-2</u>	0.004	0.012
CM 25(4-5)A <u>gluc-1</u>	0.017	0.037
CM 26(3-8)a <u>gluc-1</u>	0.010	0.030
30(2-3) <u>gluc-1</u>	0.012	0.073
27(2-5)A <u>gluc-1</u>	0.013	0.062
33(3-7) <u>gluc-1</u>	0.015	0.066

a. Enzyme assays are described in Materials & Methods (Preparation and determination of enzyme activity)

b. O.D. (600mu)

unpublished date) plus casitone (Difco) 1g, yeast extract (Difco) 2.5g; agar (Difco) 15g; glycerol (J. T. Baker Chemical Co., Phillipsburg, N. J.) 8 ml and vitamin stock solution 10 ml per 1000 ml of medium.

To increase the production of conidia, *Neurospora* was grown on 300 ml GCP solid media in a 2000 ml Erlenmeyer flask for 3 days, then the flask was inverted and allowed to grow for another 4 days.

For growing the mycelium liquid GCP media was used. Both conidia and mycelium were harvested and washed by filtration and centrifugation under aseptic conditions.

Cellobiose at a concentration of mM in phosphate buffer pH 6.0, 0.1 M was used as the basic induction media. Any modification will be mentioned in each specific experiment.

Preparation and Determination of Enzyme Activity

After the conidia or mycelia had been washed with distilled water at least three times by centrifugation or filtration, cells were suspended in phosphate buffer 0.1 M, pH 6.0, and ground by sonification at 2° to 5°C for 5 minutes. The suspension was centrifuged at 10,000 rpm, 0°C, for one hour, and the supernate was tested for enzyme activity. Beta-glucosidase activity was determined by the amount of chromogenic substance, p-nitrophenol, liberated by the hydrolysis of the substrate--p-nitrophenyl-beta-D-glucoside (PNPG). To 0.1ml of the 10mg./ml. solution was added 0.9ml. of the enzyme solution and the mixture was then left at room temperature for 10 minutes.

The reaction was stopped by adding 0.5ml of 1M tris buffer, shaking immediately. Results were read at 410mu with a colorimeter. Cellobiase activity was determined by using enzyme solution which had

been incubated in a 60°C water bath for 1 minute, then cooled down immediately. The enzyme activity of aryl-beta-glucosidase was obtained by subtracting the cellobiase activity from that of beta-glucosidase.

Protein content was determined by the method of Lowry (1951). A human serum albumin solution was used for the standard curve, and the specific activity of enzyme solution was calculated as units of activity per mg. of protein.

For the whole cell assay, a suspension of conidia was used instead of the enzyme solution. After adding the conidial suspension to the substrate, the mixture was kept shaking for 10 minutes. Before reading, centrifugation was used to remove the cells.

ENDOGENOUS INDUCTION

Method

A series of wild type and gluc-1 strains were inoculated into 250 ml Erlenmeyer flasks containing 25ml of media which consisted of 2% Vogel's minimal media and 2% sucrose. Approximately 60,000 conidia were placed in each flask, and all the cultures were incubated at room temperature. After the third day, one sample from both wild type and gluc-1 strains were taken each day. Mycelia and conidia were separated by filtration through a nylon net. The number of conidia was determined by counting under a light microscope. The wet mycelial pellet was weighed. Enzyme activities in conidia, mycelia, and media were determined.

Results

Following germination, the weight of cells increased steadily. The cultures then started to conidiate, and a decrease in the weight of mycelia was noticed. As the number of conidia produced increased, the weight of mycelia decreased. The relationship is shown in Figure 1.

The analysis of the total enzyme activities of the cultures is shown in Figure 2. The total units (units in mycelia, conidia and washings) of aryl-beta-glucosidase increased greatly in wild type, but not in the gluc-1 mutant. The pattern of increase in enzyme activities followed the pattern of the increase in conidia very closely. Cellobiase activities also increased, but to a lesser extent.



Figure 1. The change of the water level in the reservoir during the period of the flood.

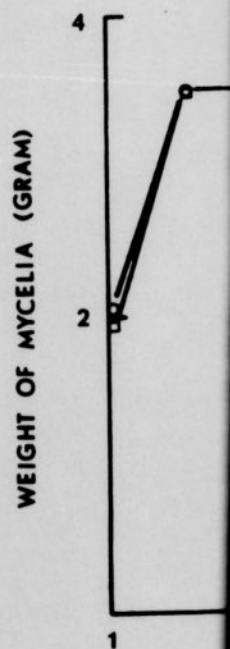
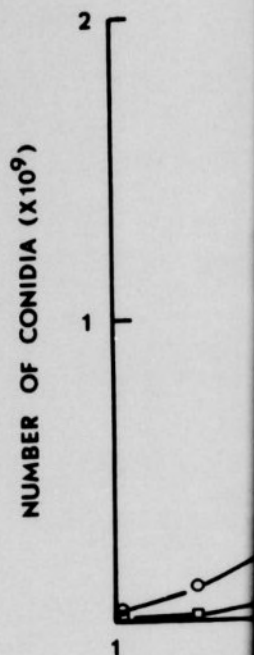


Figure 2

Figure 1. The change of the weight of mycelia and the number of conidia during endogenous induction

Wild type: -□-□-□-□-

Gluc-1: -○-○-○-○-



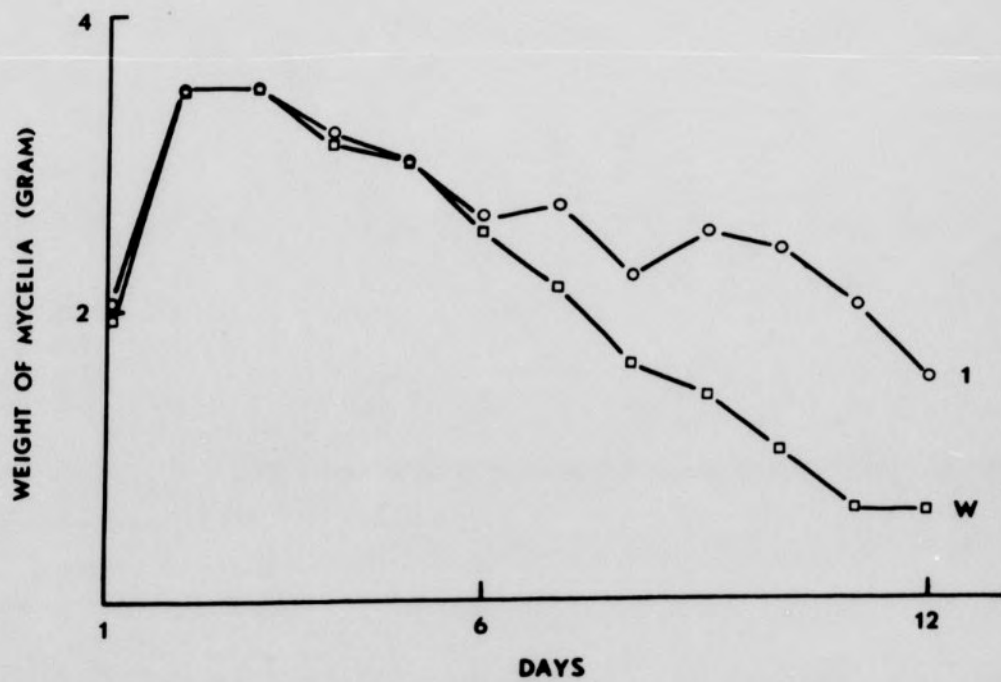
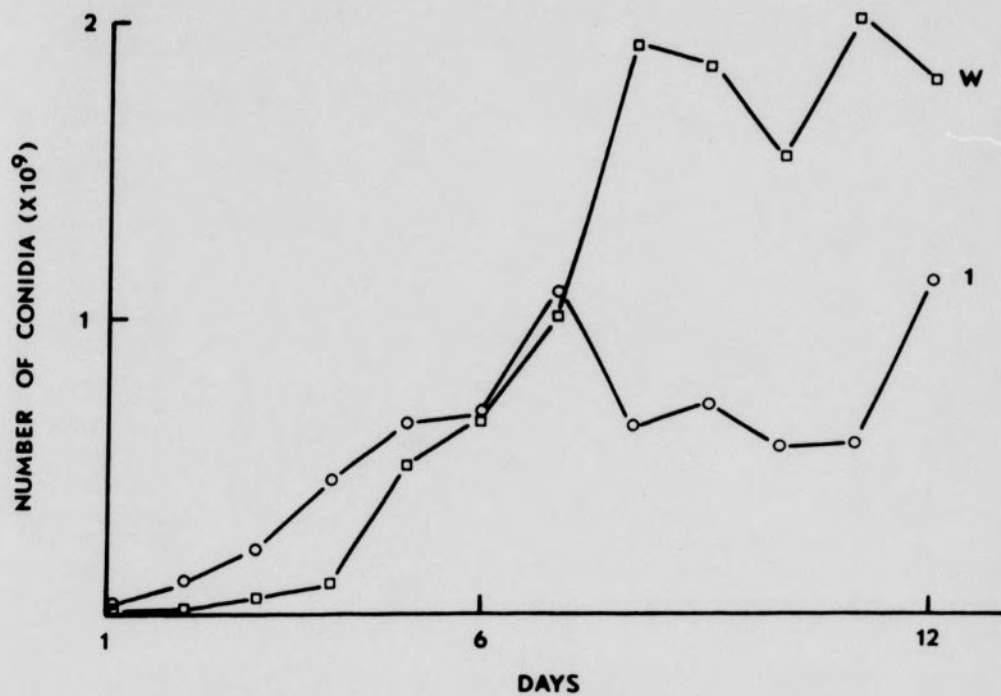
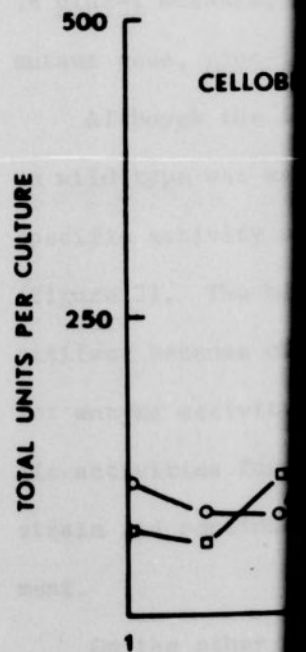
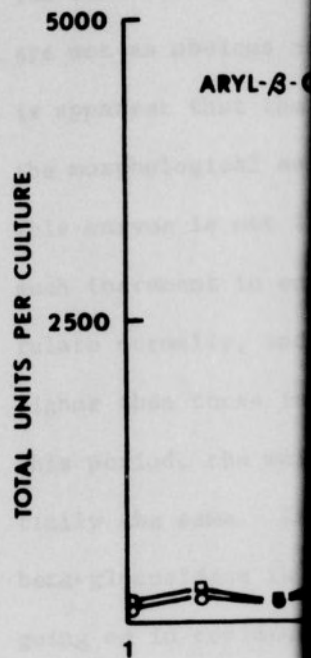
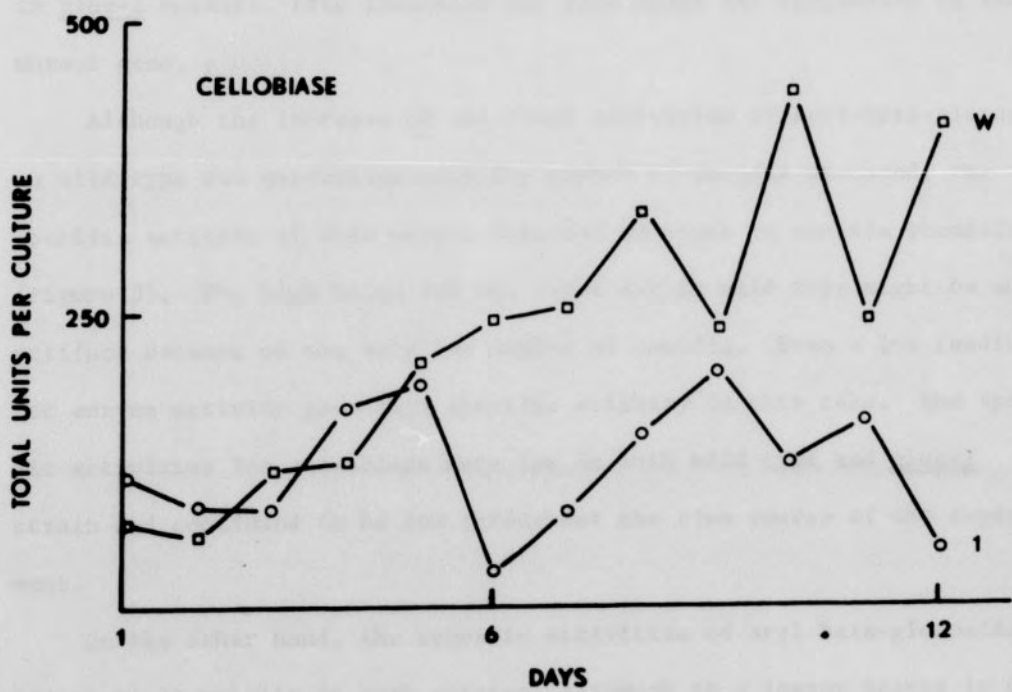
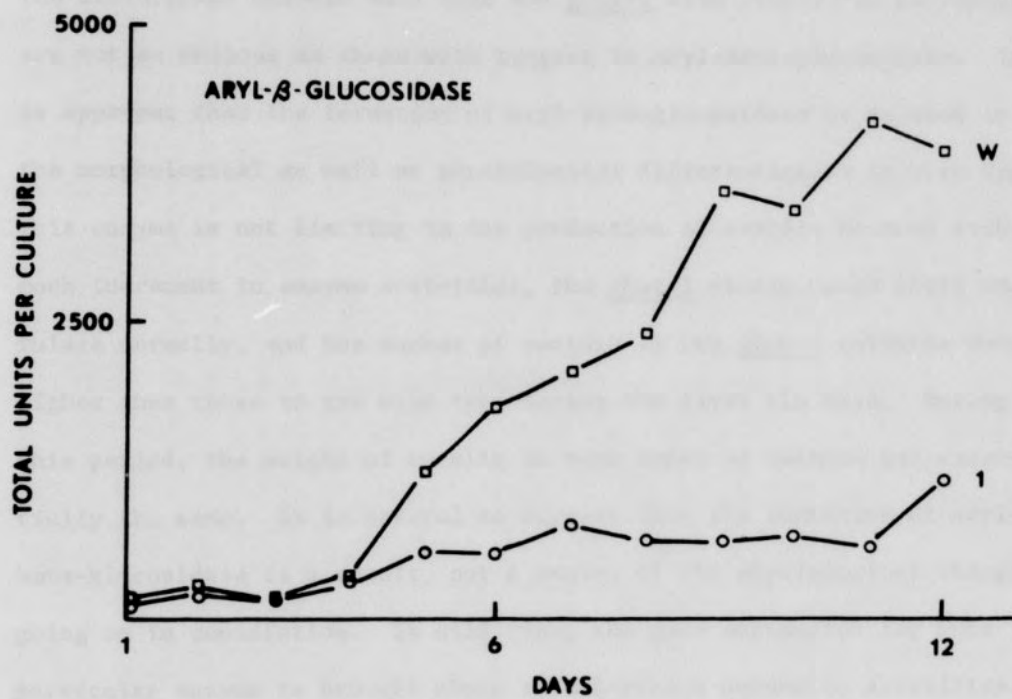


Figure 2. The change of total units of aryl-beta-glucosidase and cellobiase during endogenous induction

Wild type: -□-□-□-

Gluc-1: -○-○-○-





The differences between wild type and gluc-1 with respect to cellobiase are not as obvious as those with respect to aryl-beta-glucosidase. It is apparent that the formation of aryl-beta-glucosidase is related to the morphological as well as physiological differentiation in wild type. This enzyme is not limiting in the production of conidia because without much increment in enzyme activities, the gluc-1 strain could still sporulate normally, and the number of conidia in the gluc-1 cultures were higher than those in the wild type during the first six days. During this period, the weight of mycelia in both types of culture was essentially the same. It is natural to suggest that the formation of aryl-beta-glucosidase is a result, not a cause, of the physiological changes going on in conidiation. In wild type, the gene expression for this particular enzyme is brought about by endogenous metabolic activities. In gluc-1 mutants, this induction was less under the regulation of the mutant gene, gluc-1.

Although the increase of the total activities of aryl-beta-glucosidase in wild type was associated with the number of conidia produced, the specific activity of this enzyme remained constant in conidia themselves (Figure 3). The high value for the first day in wild type might be an artifact because of the very low number of conidia. Even a low reading for enzyme activity gave high specific activity in this case. The specific activities for cellobiase were low in both wild type and gluc-1 strain and continued to be low throughout the time course of the experiment.

On the other hand, the specific activities of aryl-beta-glucosidase increased in mycelia in both strains, although to a lesser degree in the

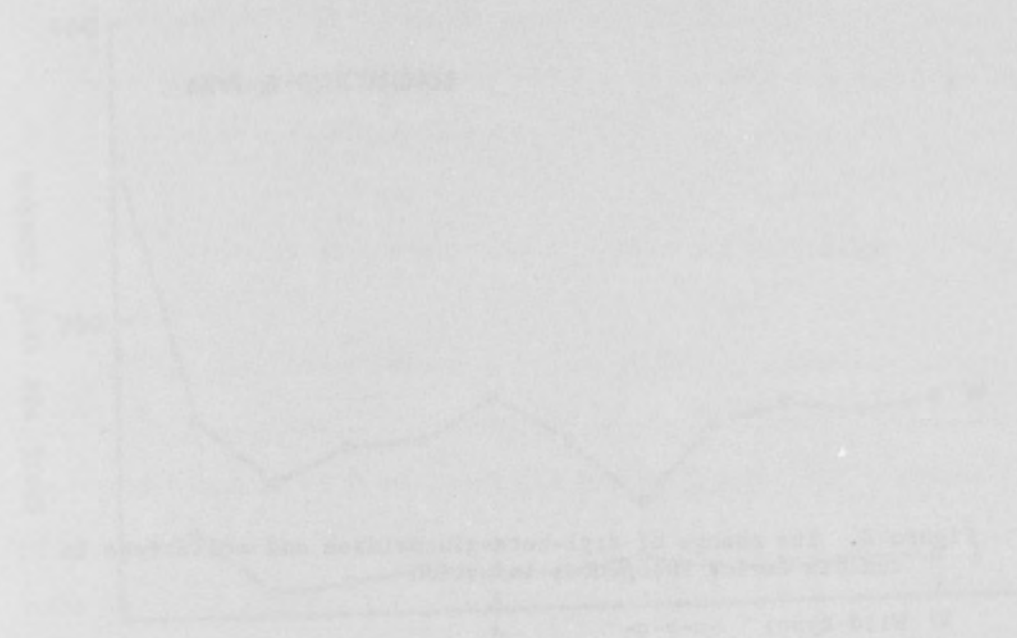
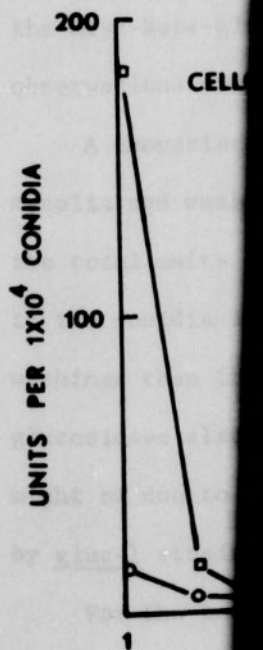
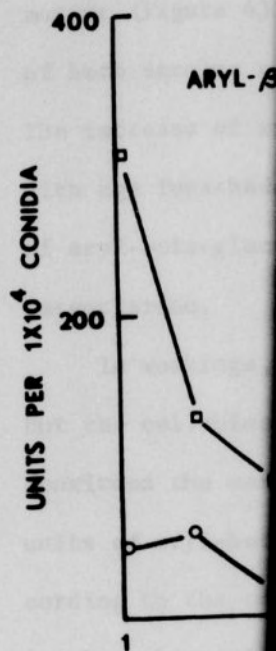
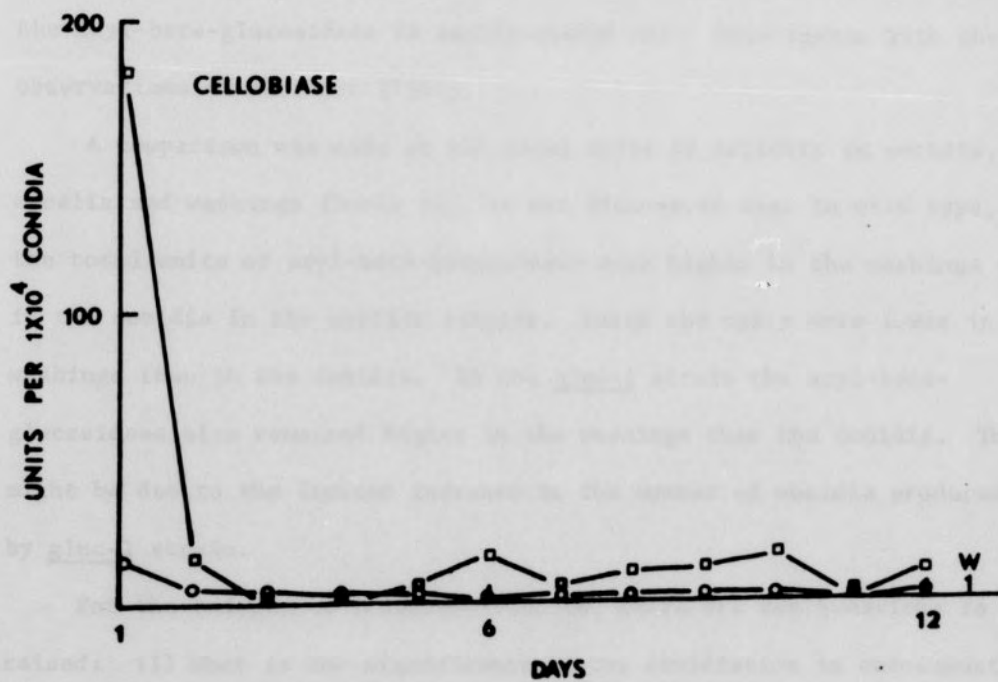
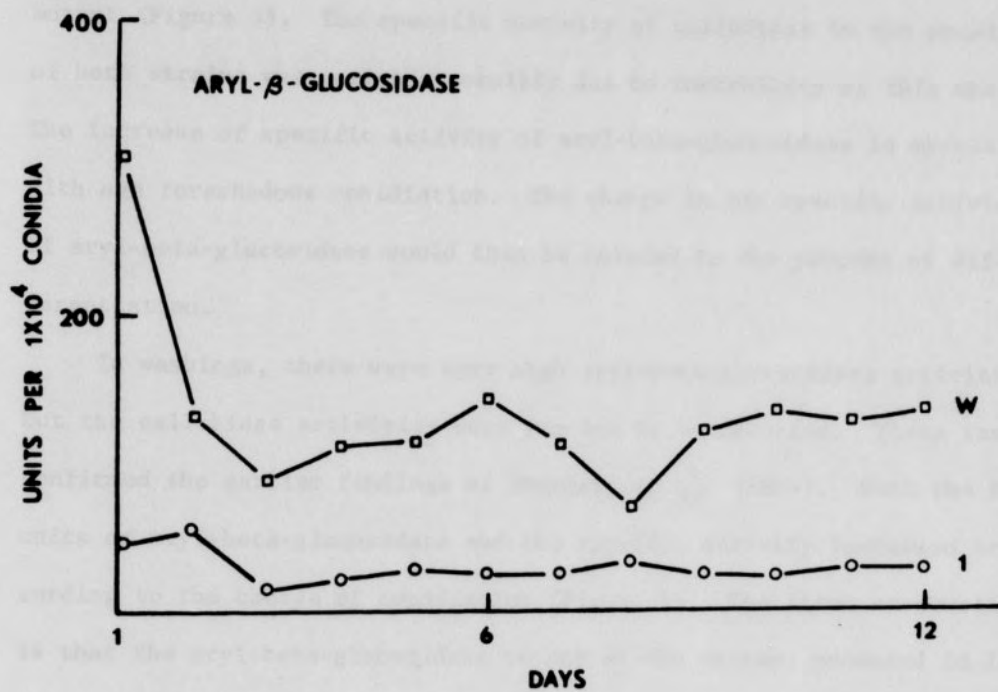


Figure 3. The change of aryl-beta-glucosidase and cellobiase in conidia during endogenous induction

Wild type: -□-□-□-

Gluc-1: -○-○-○-





llobiase in

mutant (Figure 4). The specific activity of cellobiase in the mycelia of both strains was variable possibly due to instability of this enzyme. The increase of specific activity of aryl-beta-glucosidase in mycelia with age foreshadows conidiation. The change in the specific activity of aryl-beta-glucosidase would then be related to the process of differentiation.

In washings, there were very high aryl-beta-glucosidase activities, but the cellobiase activities were too low to be detected. These results confirmed the earlier findings of Eberhart *et al.* (1964). Both the total units of aryl-beta-glucosidase and the specific activity increased according to the course of conidiation (Figure 5). The first observation is that the aryl-beta-glucosidase is one of the enzymes produced in large amounts prior to or during conidiation. The second observation is that the aryl-beta-glucosidase is easily washed off. This agrees with the observations of Eberhart (1961).

A comparison was made of the total units of activity in conidia, mycelia and washings (Table 2). It was discovered that in wild type, the total units of aryl-beta-glucosidase were higher in the washings than in the conidia in the earlier samples. Later the units were lower in washings than in the conidia. In the gluc-1 strain the aryl-beta-glucosidase also remained higher in the washings than the conidia. This might be due to the limited increase in the number of conidia produced by gluc-1 strain.

For the endogenous induction studies, there are two questions to be raised: (1) What is the significance of the conidiation in endoinduction? (2) What is the significance of enzyme distribution? These questions are discussed in more detail in the following sections.

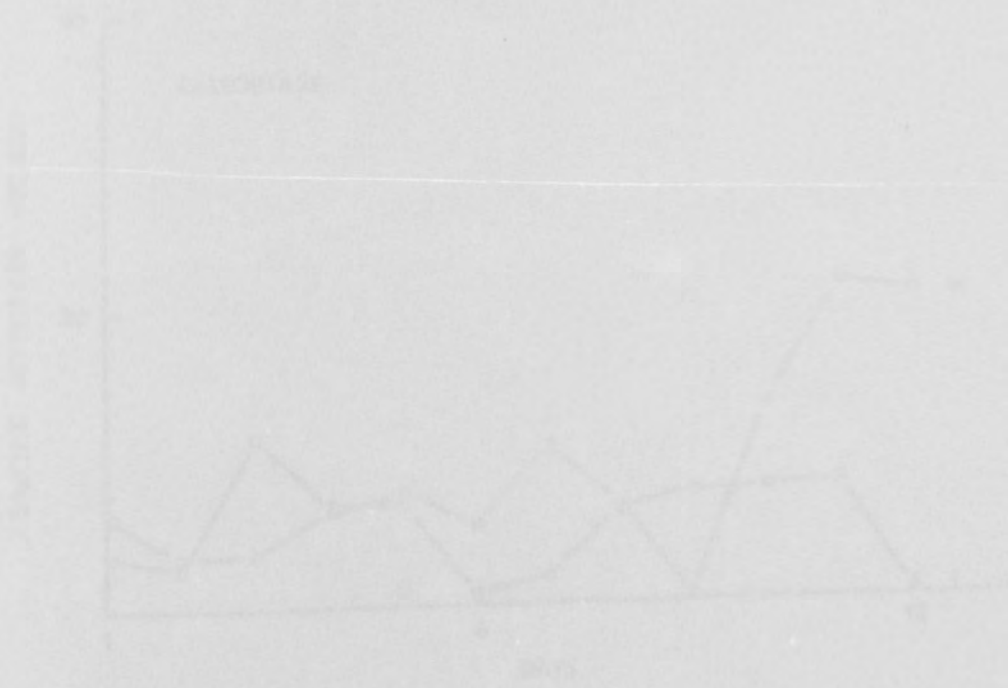
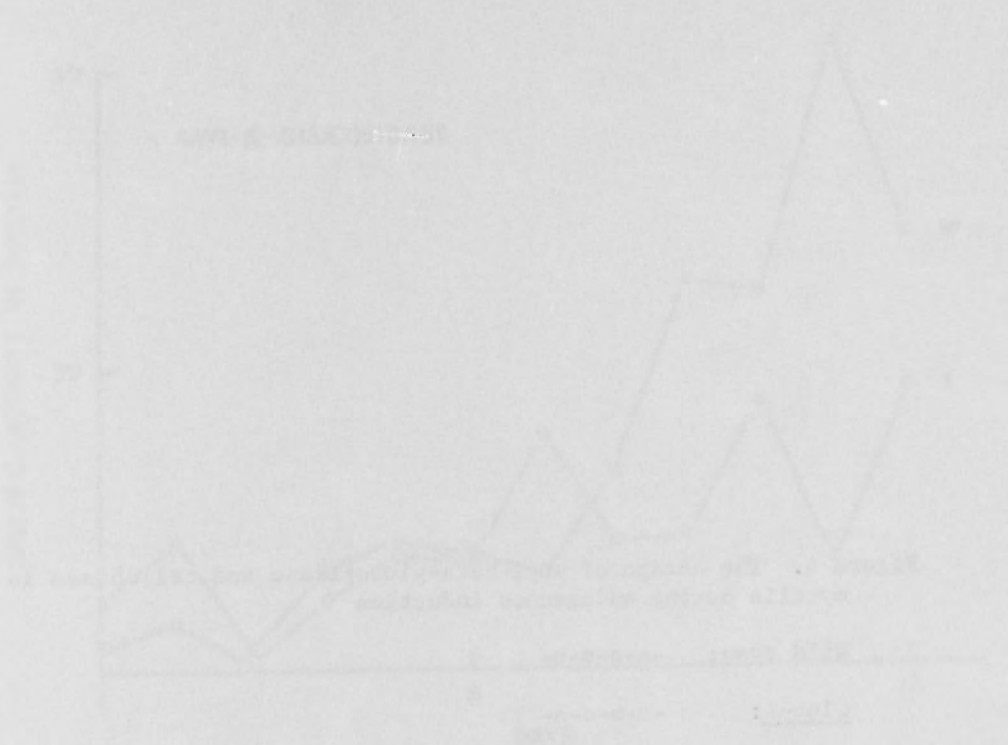
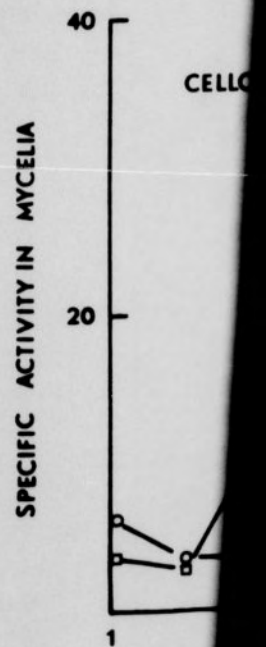
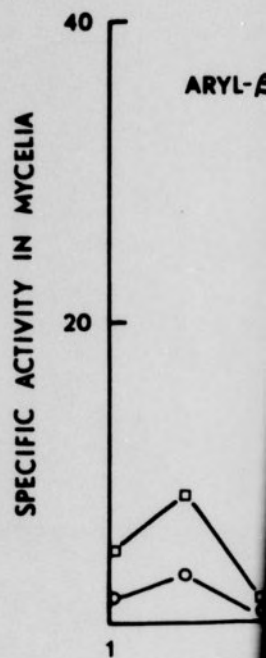
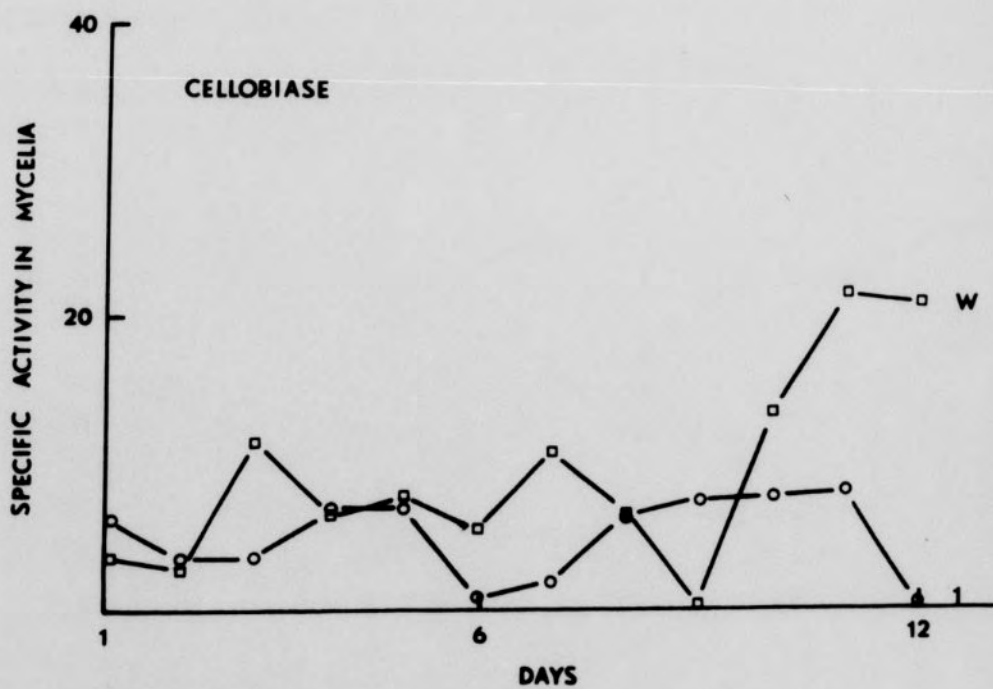
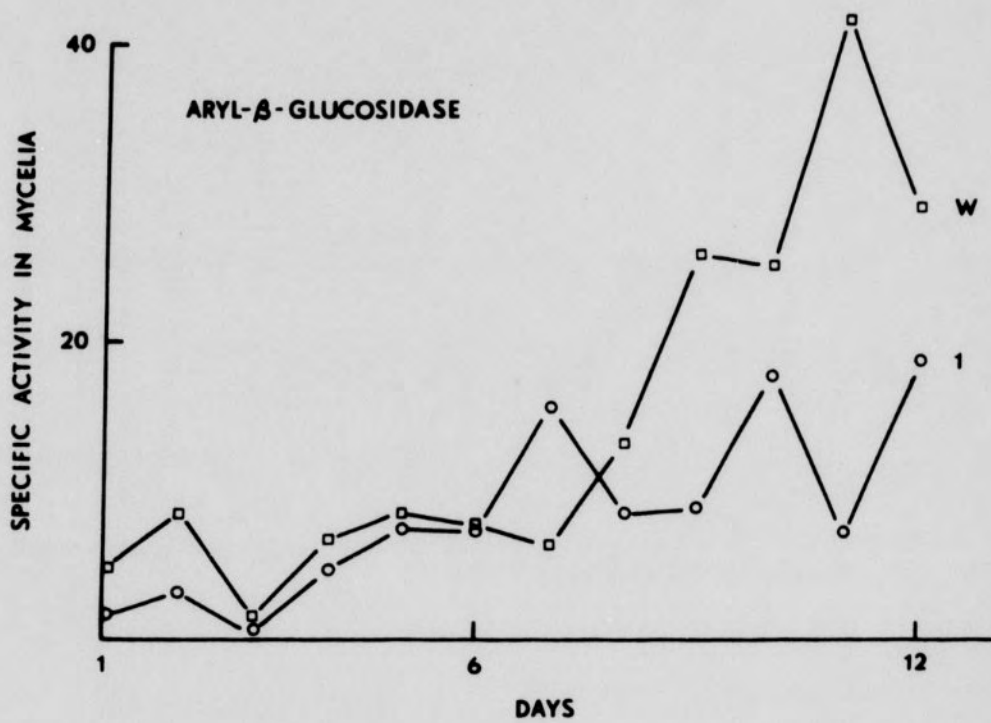


Figure 4. The change of aryl-beta-glucosidase and cellobiase in mycelia during endogenous induction

Wild type: -□-□-□-□-

Gluc-1: -○-○-○-○-



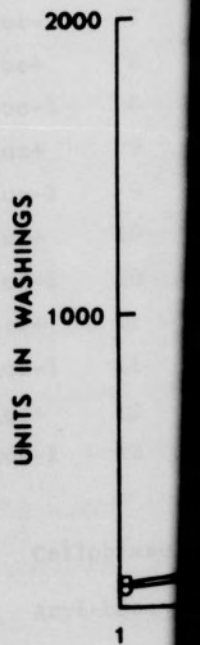
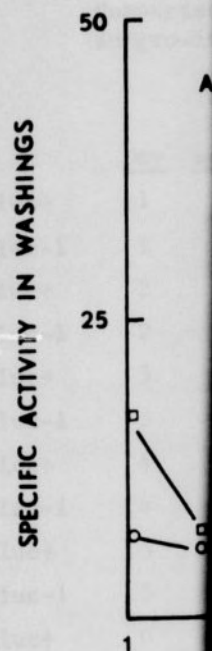


llobiase in

Figure 5. The change of aryl-beta-glucosidase activity in washings during endogenous induction

Wild type: -o-o-o-

Gluc-1: -o-o-o-



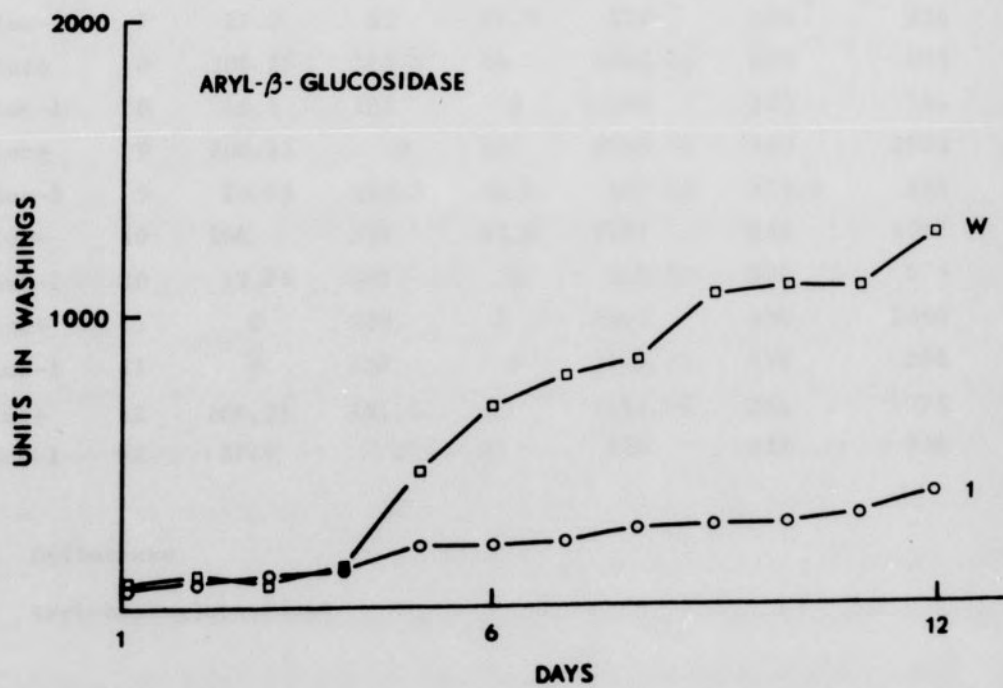
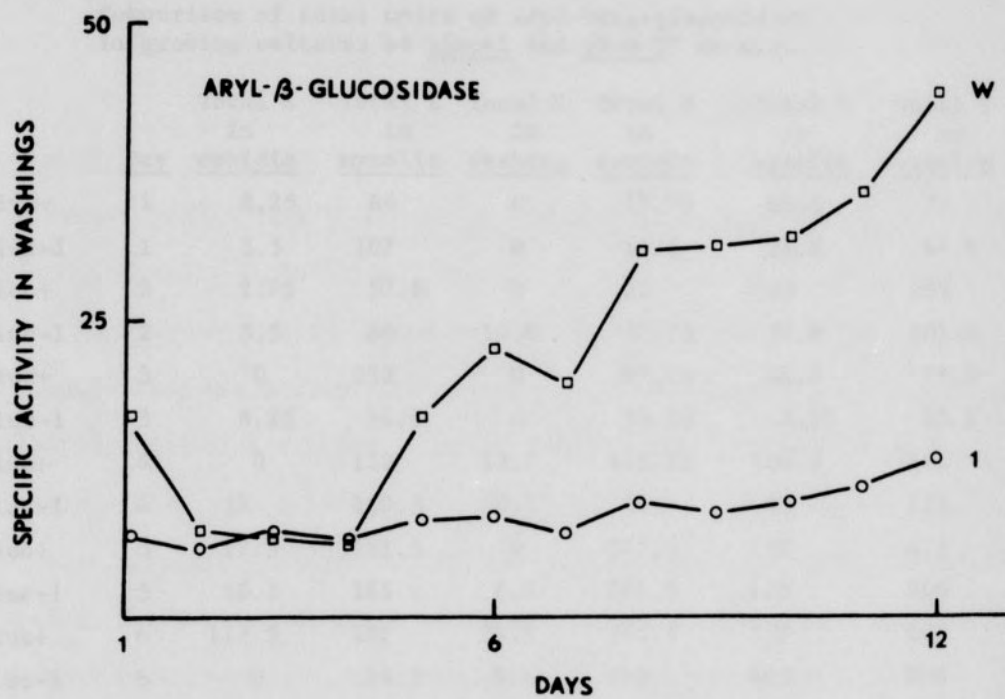


Table 2

Comparison of total units of aryl-beta-glucosidase
in growing cultures of gluc-1 and gluc-1⁺ strains

	Day	Total X in <u>conidia</u>	Total X in <u>mycelia</u>	Total X in <u>washing</u>	Total Y in <u>conidia</u>	Total Y in <u>mycelia</u>	Total Y in <u>washing</u>
gluc+	1	8.25	66	0	13.75	82.5	77
gluc-1	1	5.5	107	0	16.5	24.8	67.8
gluc+	2	2.75	57.8	0	22	165	105
gluc-1	2	5.5	66	13.8	57.75	57.8	101.5
gluc+	3	0	232	0	47.75	24.8	79.9
gluc-1	3	8.25	74.3	0	30.25	8.25	95.5
gluc+	4	0	132	13.2	118.25	104.5	141
gluc-1	4	11	140.5	20.8	99	99	132
gluc+	5	27.5	181.5	0	577.5	198	472
gluc-1	5	16.5	165	8.6	181.5	173	206
gluc+	6	115.5	107	21.5	973.5	157	685
gluc-1	6	0	16.5	8.6	165	165	206
gluc+	7	5.5	198	0	1210	115.5	792
gluc-1	7	27.5	33	17.5	275	264	236
gluc+	8	206.25	115.5	44	1361.25	223	835
gluc-1	8	16.5	132	0	198	183	264
gluc+	9	206.25	0	27	2268.75	330	1065
gluc-1	9	19.25	148.5	34.4	167.25	181.5	266
gluc+	10	264	132	42.9	2112	256	1092
gluc-1	10	13.75	107	0	123.75	264	279
gluc+	11	0	239	2	2640	480	1080
gluc-1	11	0	157	0	178.75	132	308
gluc+	12	206.25	181.5	22	2433.75	264	1275
gluc-1	12	27.5	0	22	330	322	396

X: Cellobiase

Y: Aryl-beta-glucosidase

EXOGENOUS INDUCTION

General Considerations

According to Cohn (1957) and Hogness, et al. (1959), the homogeneity of cells in induction referred to before could be established by using a nonmetabolizable inducer and cryptic cells. For this study, a nonmetabolizable inducer was not available. Cellobiose was used since Kannwisher (1962) had shown in his thesis that it was the best inducer in this system. It was suggested by Landmann (1954) that in *Neurospora* the resting state was more suitable than in the growing state. In the growing state there were more complicating factors involved. Enough reserves were present in resting cells to allow induction.

For most of our experiments, washed conidia were suspended in phosphate buffer containing mM cellobiose and shaken in a 25C water bath for induction studies.

Method and Results

1. The effect of the concentration of cellobiose on the induction of aryl-beta-glucosidase and cellobiase in wild type and gluc-1 strains. Strain 33(3-7) gluc-1 Cot and RB-1-(19) gluc-1+ cot were used in this experiment. According to Kannwisher (1962), cot strains were easier to handle and formed uniform cell suspensions. For a comparison to Kannwisher's result, these Cot strains were chosen for this particular experiment. They were grown in Vogel's media with 1% sucrose, 0.1% agar and 1% vitamin stock solution and aerated at 33°C. After incubation for 48 hours, the colonies were washed with sterile distilled water and

suspended in the induction media for 6 hours. After washing, proteins were released by sonication. Results are shown in Figure 6. Cellobiose of 10^{-4} M gave the highest induction of aryl-beta-glucosidase, and concentrations between 10^{-3} M and 10^{-4} M showed high efficiency for cellobiase. This result was different from that shown by Kannwisher, who indicated that 10^{-3} M cellobiose was the most efficient concentration for induction using 24 or 36 hour cells. It is certain that relative concentrations of cells as well as inducers were very critical for the results, also the age of the cell had a significant influence on the response of the cell to inducer.

2. Time course of induction of aryl-beta-glucosidase and cellobiase in wild type and gluc-1 conidia. Since most of the work done before on beta-glucosidase induction emphasized later stages, in this thesis, conidia were used instead of mycelia in order to study induction during the earliest stages of development. Since the concentration of inducer is very important in studying the kinetics of enzyme synthesis at a cellular level, 1mM cellobiose was used. In this experiment, conidia harvested and washed from GCP cultures were transferred and incubated at 25°C in phosphate buffer 0.1M, pH 6.0, containing 1mM cellobiose only. At different time intervals samples were taken by removing flasks from the water bath and harvesting the conidia by centrifugation (Figure 7). From this study, it was learned that after six hours induction, the culture showed the highest enzyme activity, both for aryl-beta-glucosidase and cellobiase in wild type. A longer time of induction resulted in lowered specific activity and total units. In gluc-1 strains, only the cellobiase was induced, but to a lesser extent. Kannwisher's data on

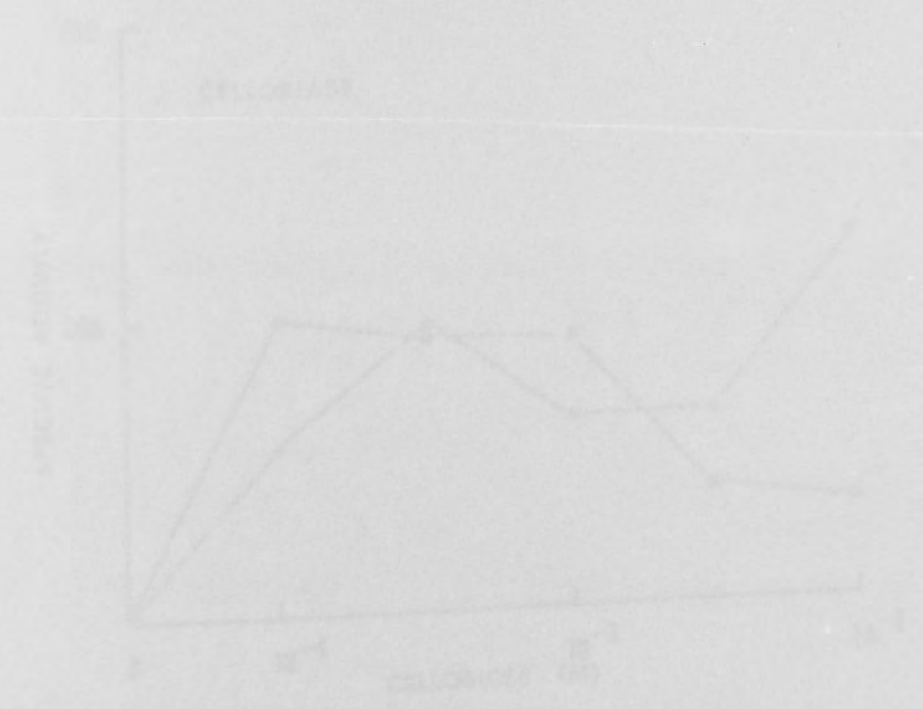
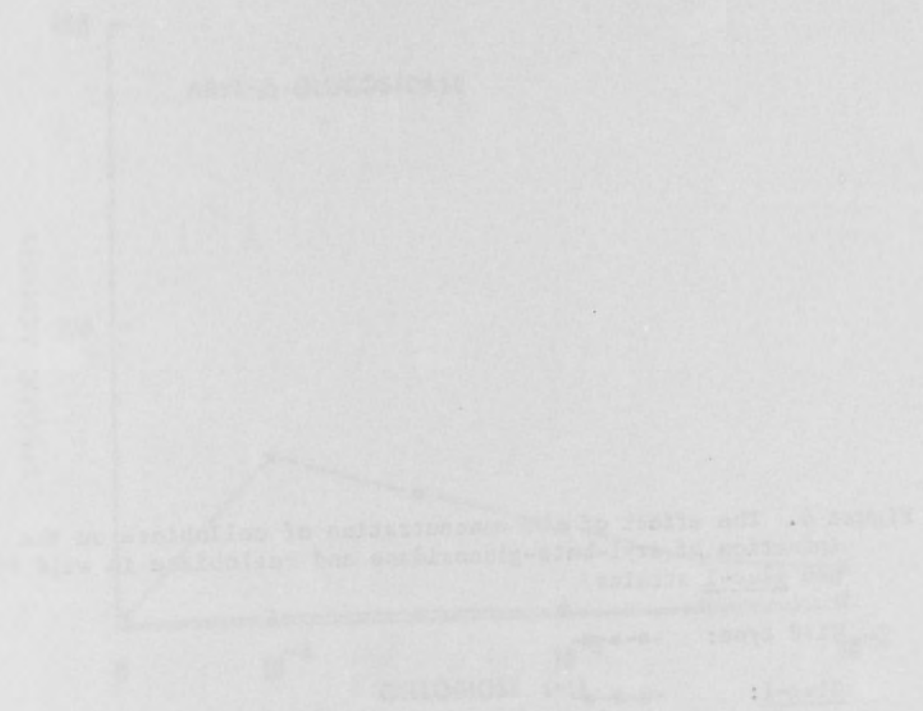
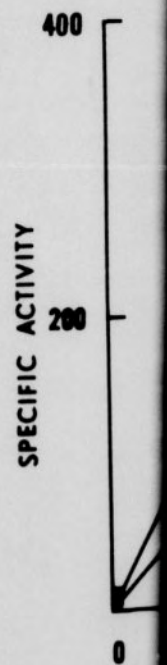
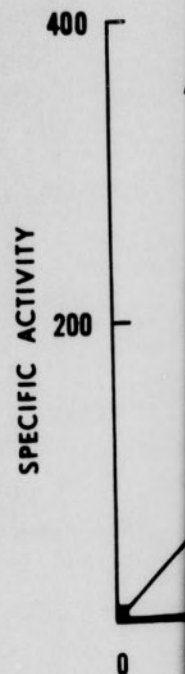


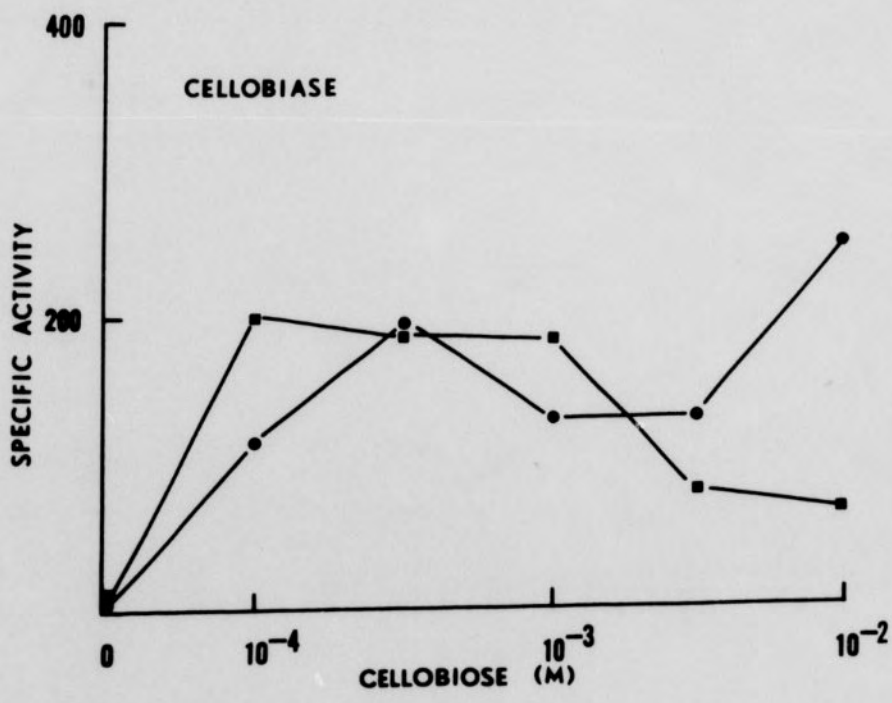
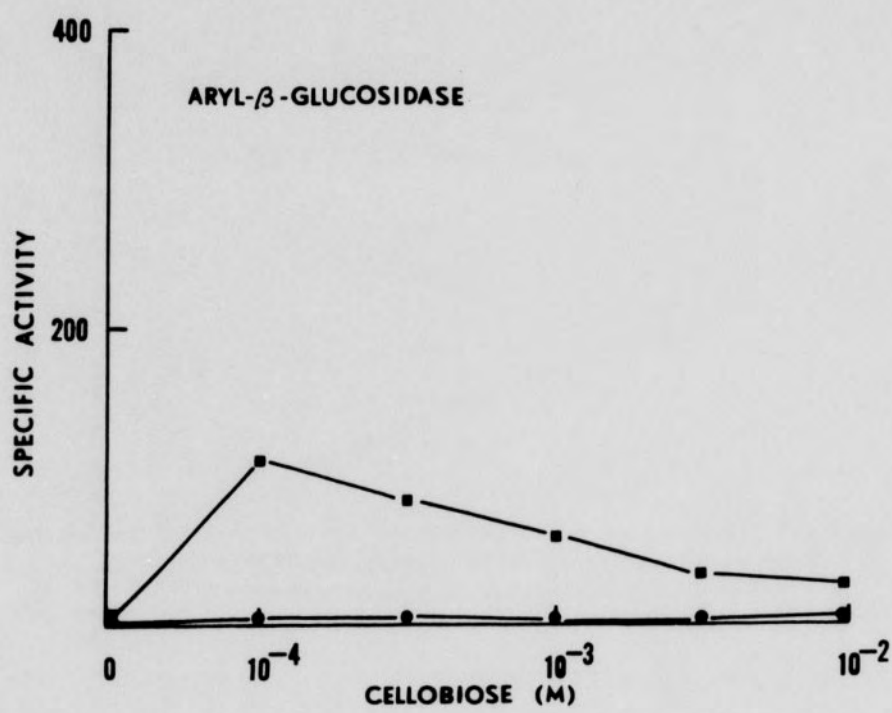
Figure 6. The effect of the concentration of cellobiose on the induction of aryl-beta-glucosidase and cellobiase in wild type and gluc-1 strains

Wild type: -■-■-■-

Gluc-1: -●-●-●-



se on the
in wild type



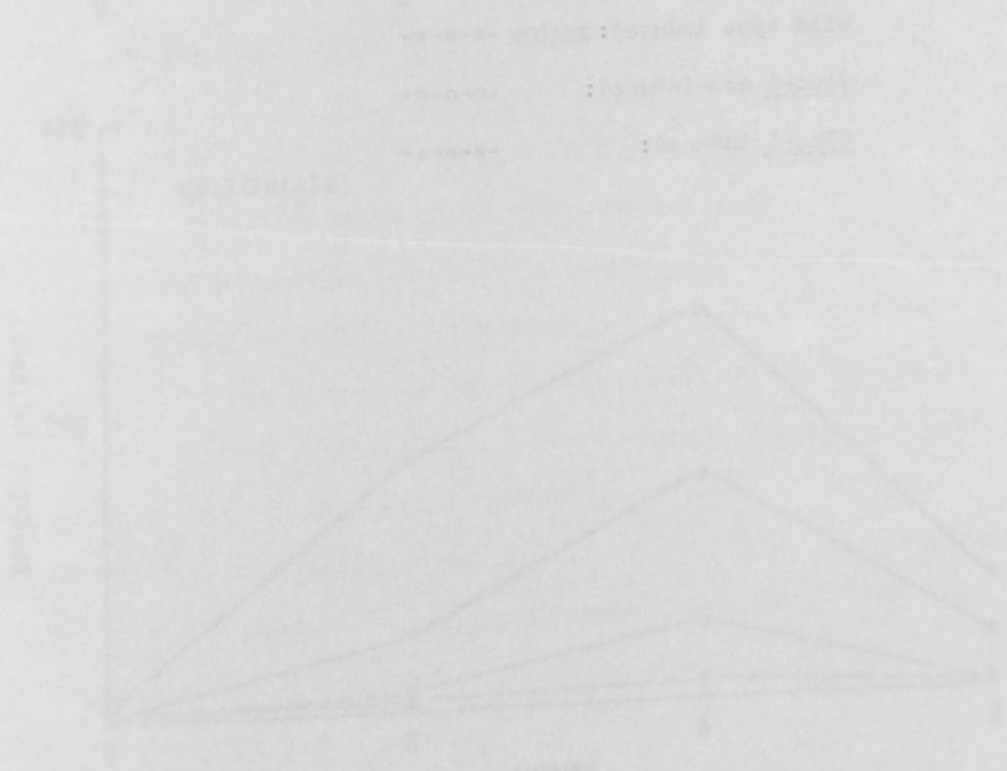
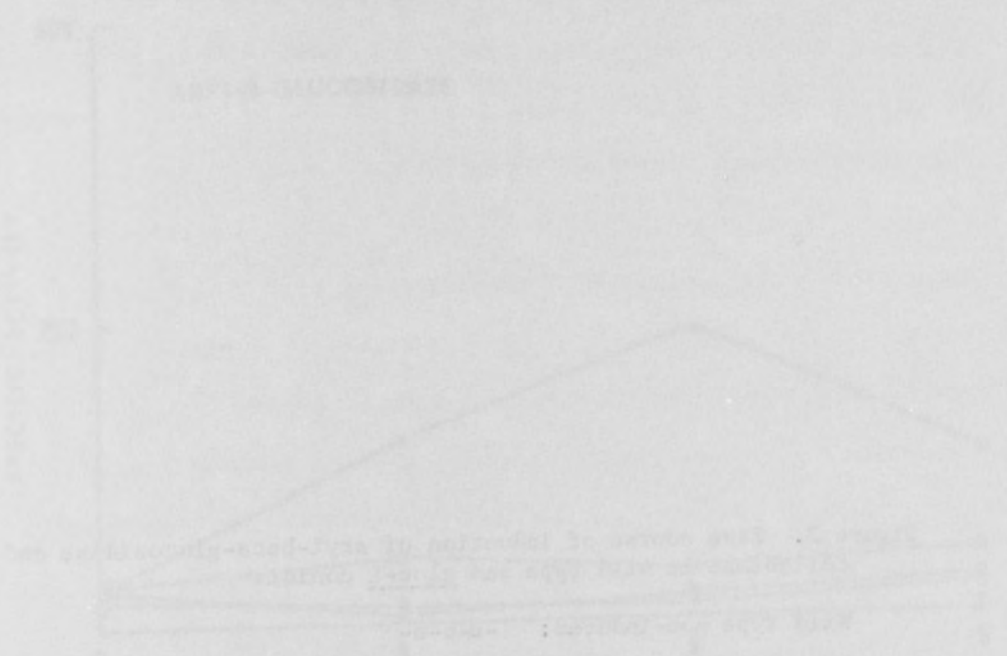


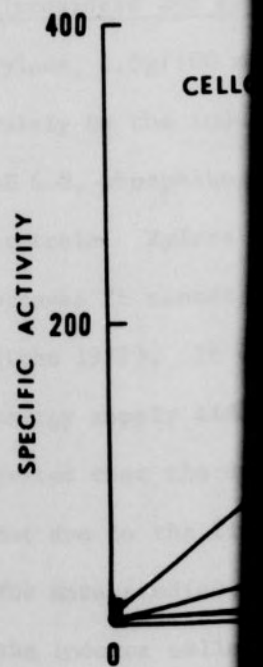
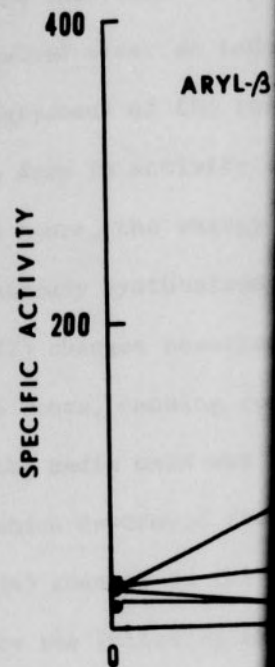
Figure 7. Time course of induction of aryl-beta-glucosidase and cellobiase in wild type and gluc-1 conidia

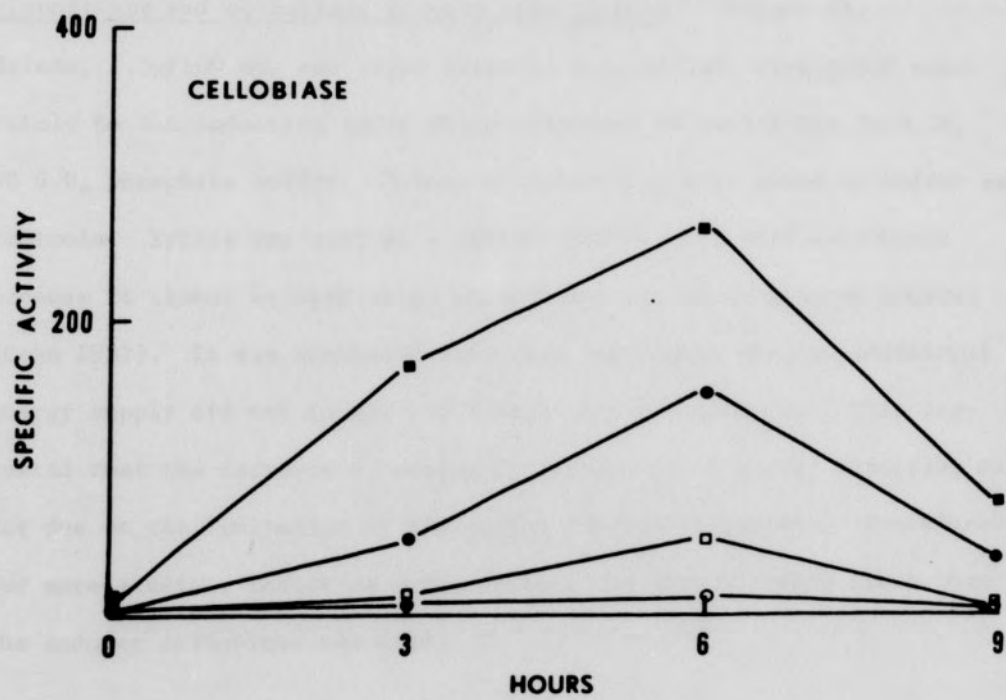
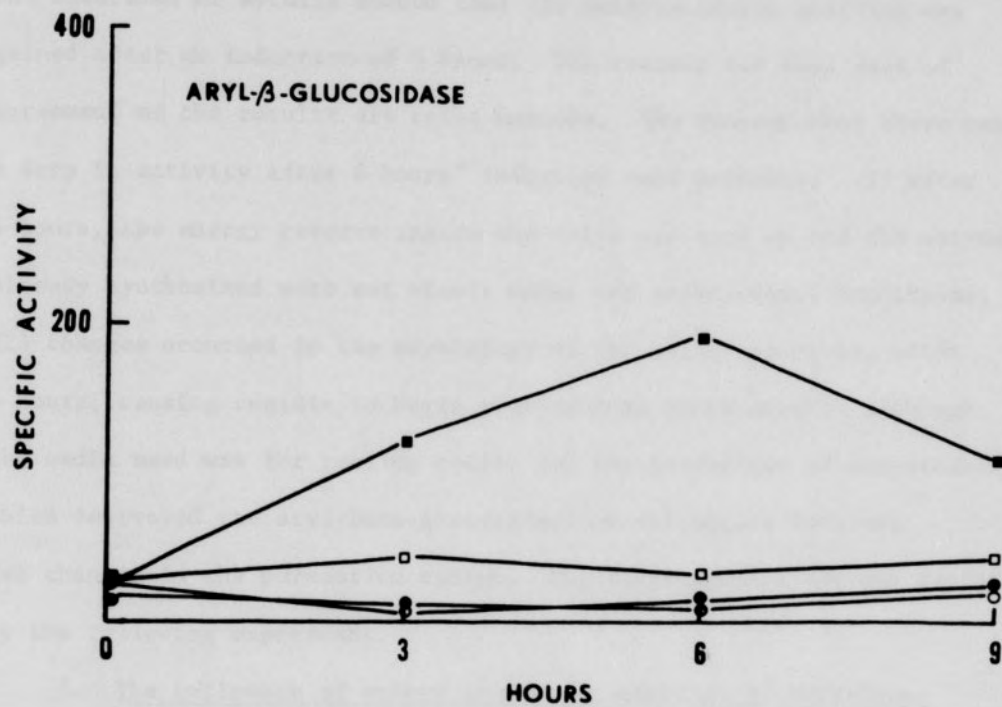
Wild type non-induced: -□-□-□-

Wild type induced: -■-■-■-

Gluc-1 non-induced: -○-○-○-

Gluc-1 induced: -●-●-●-





the induction of mycelia showed that the maximum enzyme activity was gained after an induction of 3 hours. The reasons for this lack of agreement of the results are still unknown. The reasons that there was a drop in activity after 6 hours' induction were probably: (1) after 6 hours, the energy reserve inside the cells was used up and the enzymes already synthesized were not stable under the experimental conditions; (2) changes occurred in the physiology of the cells, possibly, after 6 hours, causing conidia to begin growing into young mycelia although the media used was for resting cells; (3) the production of proteinase which destroyed the aryl-beta-glucosidase or cellobiase induced; (4) changes in the permeation system. The first possibility was tested by the following experiment.

3. The influence of energy source on induction of aryl-beta-glucosidase and cellobiase in wild type conidia. (Figure 8).

Xylose, 1.5g/100 ml, and yeast extract, 0.1g/100 ml, were added separately to the induction media which contained mM cellobiose in 0.1M, pH 6.0, phosphate buffer. Xylose or cellobiose were added to buffer as controls. Xylose was used as a carbon source other than cellobiose because it cannot be used as an inducer and can be considered minimal (Cohn 1957). It was concluded from this experiment that an additional energy supply did not change the time course of induction. This suggested that the decrease of enzyme activity after 6 hours' induction was not due to the limitation of the energy reserve in conidia. Therefore, for more studies, induction media without any energy supply other than the inducer cellobiose was used.

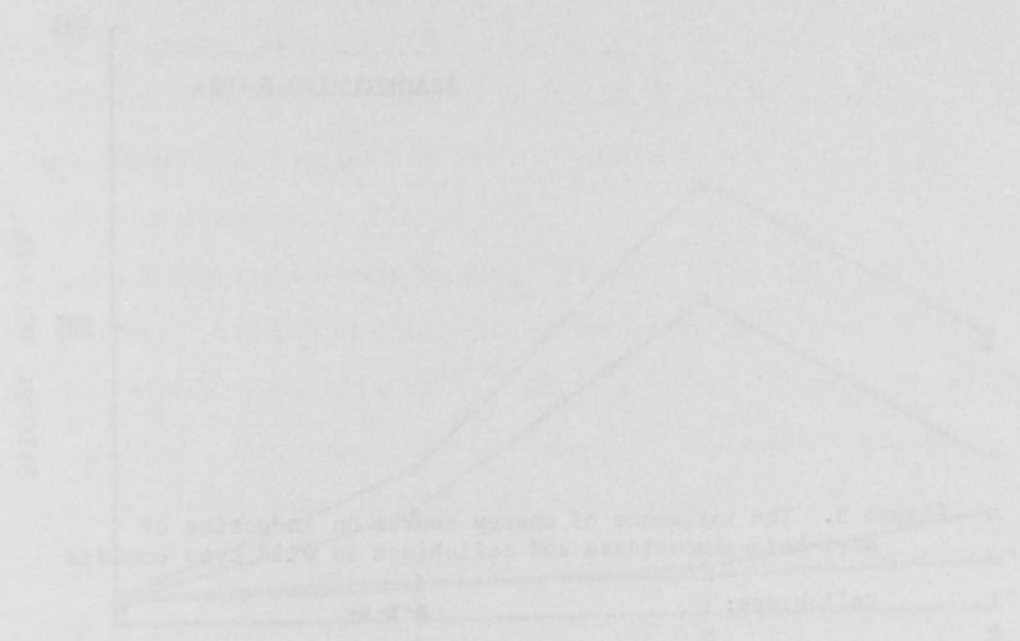


Fig. 1. The influence of energy content on the rate of... and... in... with... content.

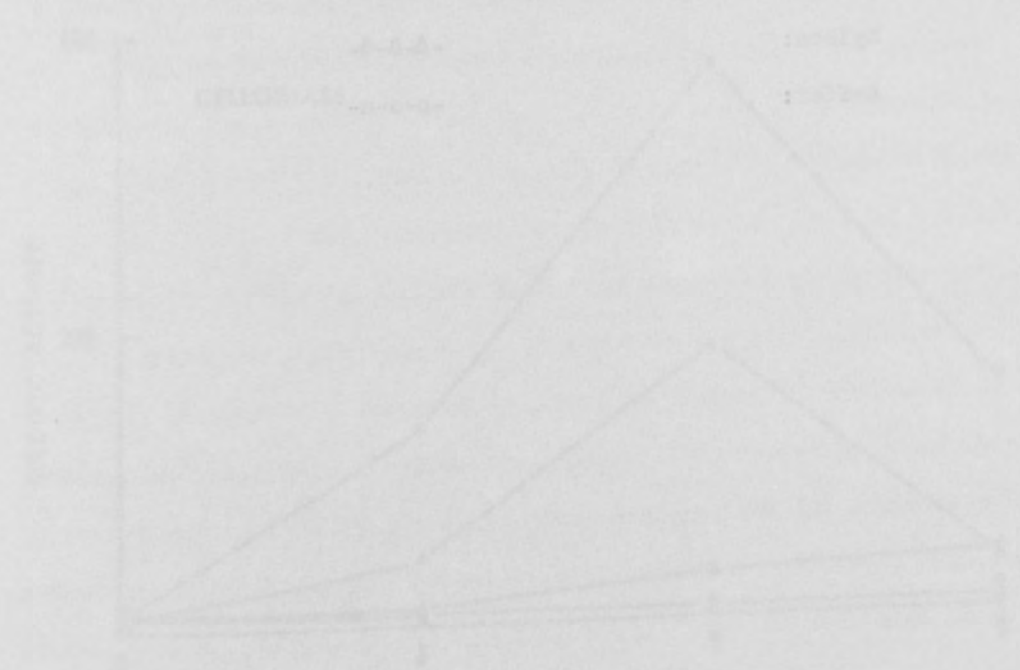
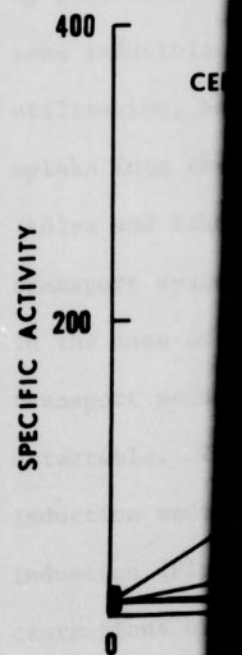
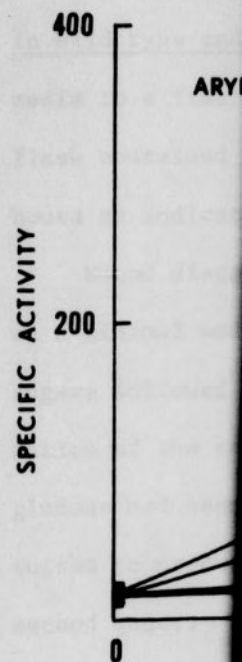
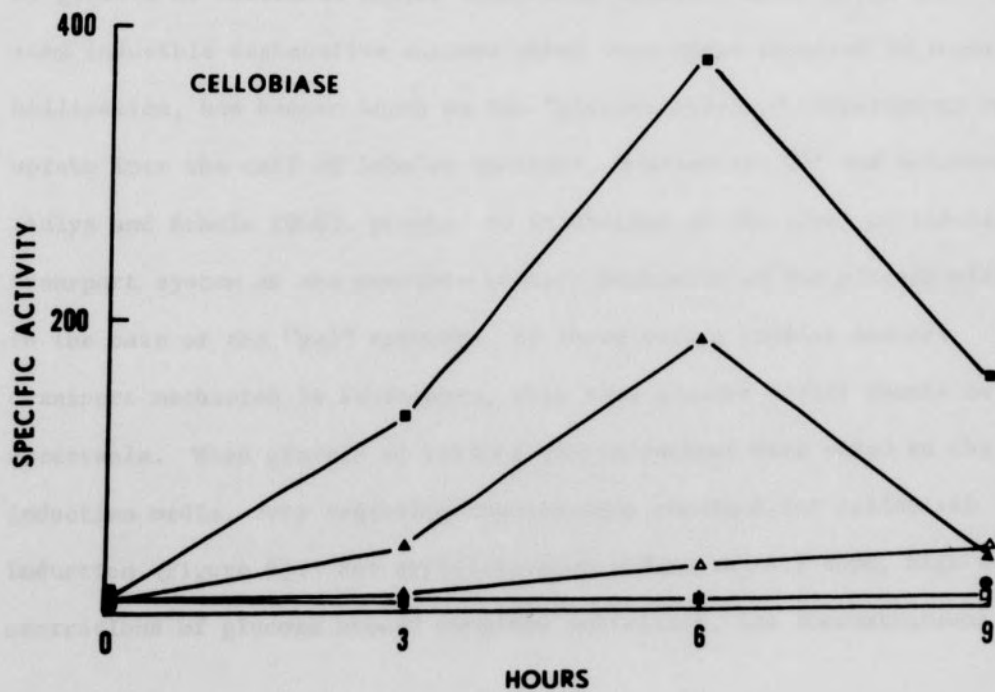
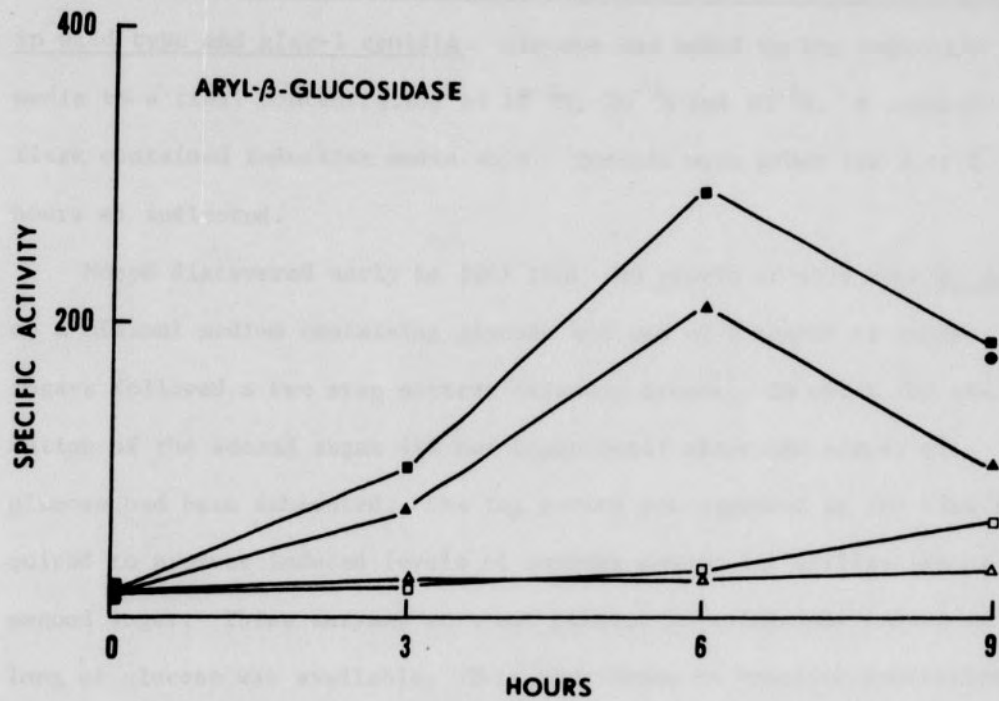


Fig. 2. The influence of energy content on the rate of... and... in... with... content.

Figure 8. The influence of energy source on induction of aryl-beta-glucosidase and cellobiase in wild type conidia

Cellobiose:	-■-■-■-
Cellobiose + yeast extract:	-●-●-●-
Cellobiose + xylose:	-▲-▲-▲-
Xylose:	-△-△-△-
Buffer:	-□-□-□-





4. The influence of glucose on the induction of beta-glucosidases in wild type and gluc-1 conidia. Glucose was added to the induction media to a final concentration of $10^{-4}M$, $10^{-3}M$ and $10^{-2}M$. A control flask contained induction media only. Conidia were grown for 3 or 6 hours as indicated.

Monod discovered early in 1941 that the growth of wild type E. coli on a minimal medium containing glucose and one of a number of other sugars followed a two step pattern (diauxic growth), in which the utilization of the second sugar did not begin until after the supply of glucose had been exhausted. The lag period corresponded to the time required to produce induced levels of enzymes needed for utilization of the second sugar. These enzymes were not present in sufficient amount so long as glucose was available. This phenomenon of specific inhibition by glucose of inducible enzyme formation, observed also in the case of some inducible degradative enzymes other than those involved in sugar utilization, had become known as the "glucose effect." Experiments on the uptake into the cell of labeled inducers, D-galactose C^{14} and D-fucose H^3 (Adlya and Echols 1966), pointed to inhibition at the level of inducer transport system as the possible primary mechanism of the glucose effect in the case of the "gal" enzymes. If there were a similar inducer transport mechanism in *Neurospora*, this same glucose effect should be detectable. When glucose of various concentrations were added to the induction media, very confusing results were obtained for cellobiase induction (Figure 9). For aryl-beta-glucosidase in wild type, high concentrations of glucose showed complete inhibition, low concentrations

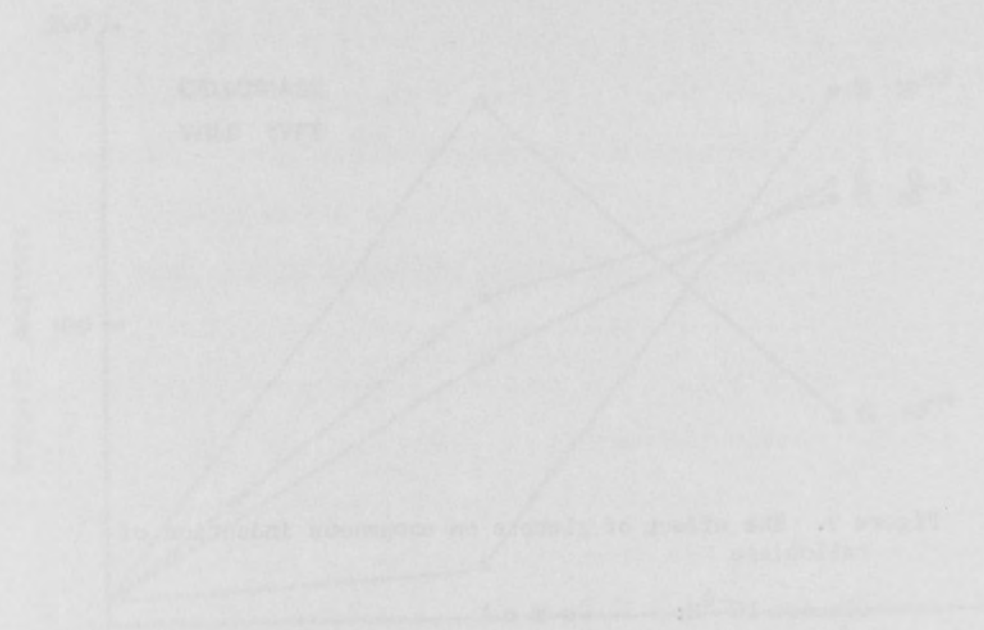


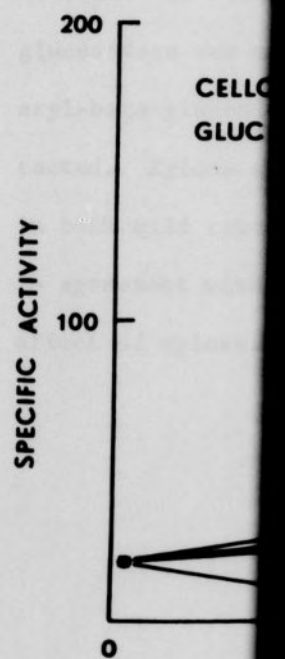
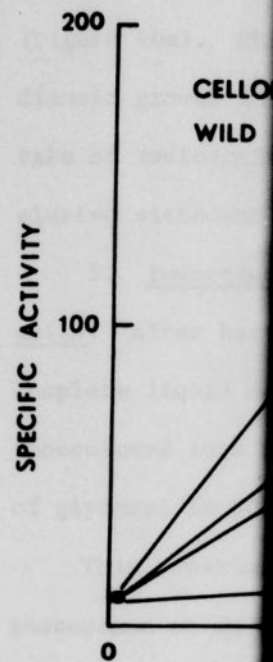
Figure 1. The effect of [illegible] on [illegible]

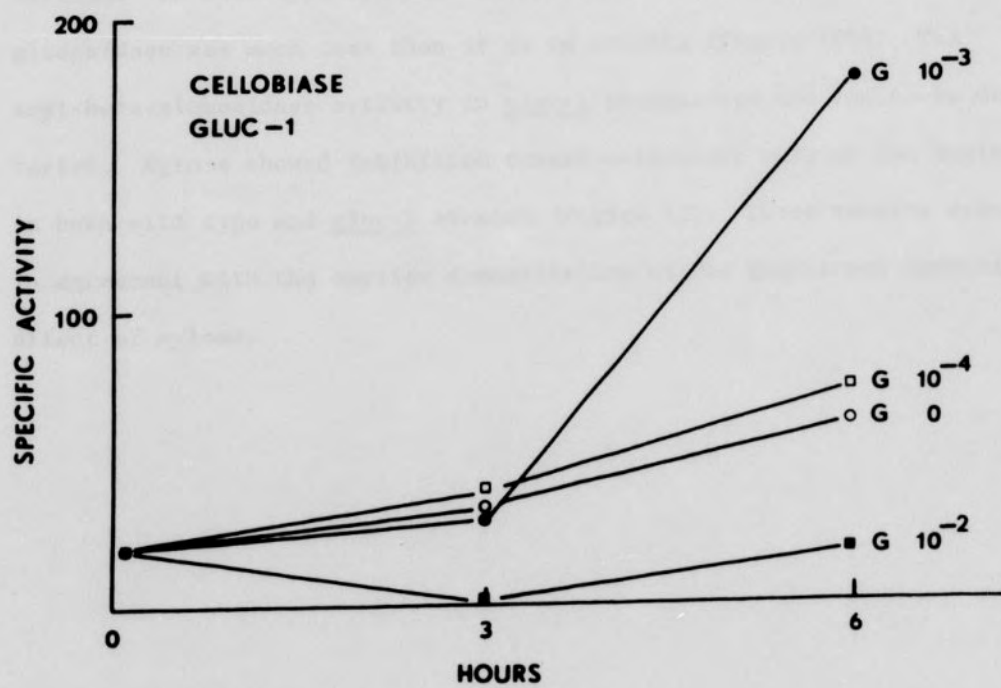
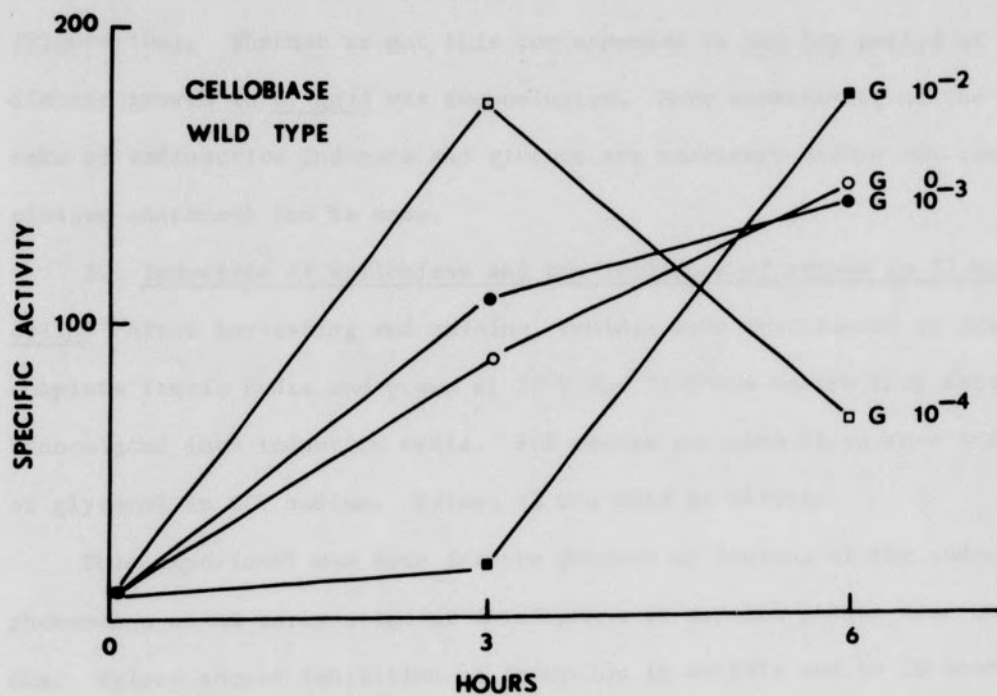


Figure 2. The effect of [illegible] on [illegible]

Figure 9. The effect of glucose on exogenous induction of cellobiase

Glucose 10^{-2} M: -■-■-■-
Glucose 10^{-3} M: -●-●-●-
Glucose 10^{-4} M: -□-□-□-
Glucose 0: -○-○-○-





showed inhibition at the beginning and slight stimulation later.

(Figure 10a). Whether or not this corresponded to the lag period of the diauxic growth in E. coli was inconclusive. More experiments on the uptake of radioactive inducers and glucose are necessary before any conclusive statement can be made.

5. Induction of cellobiase and the influence of xylose in 12 hour cells. After harvesting and washing, conidia were transferred to sucrose complete liquid media and grown at 25°C for 12 hours before they were inoculated into induction media. SCP medium contains 2% sucrose instead of glycerol in GCP medium. Xylose 2% was used as before.

This experiment was done for the purpose of looking at the induction phenomenon at an early stage of development in mycelia rather than conidia. Xylose showed inhibition on induction in conidia and in 12 hour mycelia. In wild type mycelia the specific activity of aryl-beta-glucosidase was much less than it is in conidia (Figure 10b). The aryl-beta-glucosidase activity in gluc-1 strains was too low to be detected. Xylose showed inhibition toward cellobiase only at the beginning in both wild type and gluc-1 strains (Figure 11). These results were not in agreement with the earlier demonstration of the gratuitous induction effect of xylose.

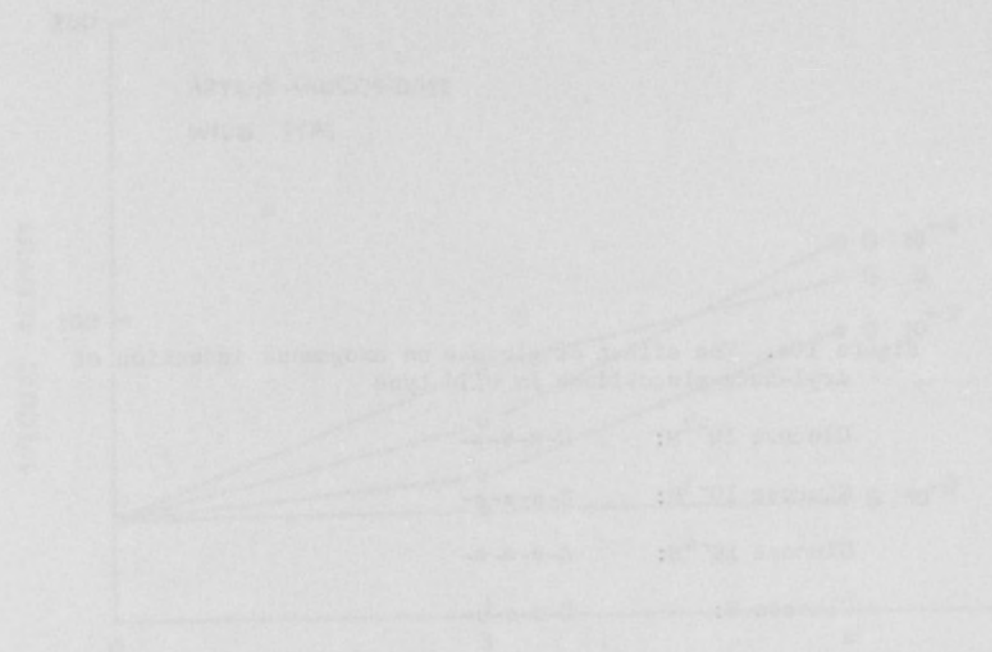


Figure 10. Influence of pH on the adsorption of lead(II) ions by the adsorbent.

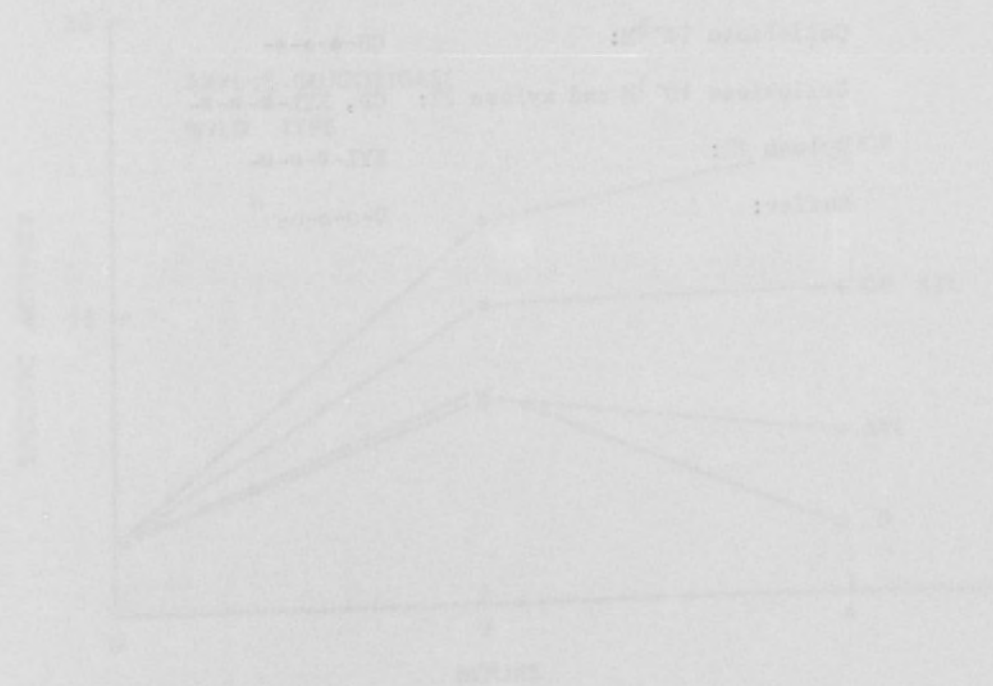


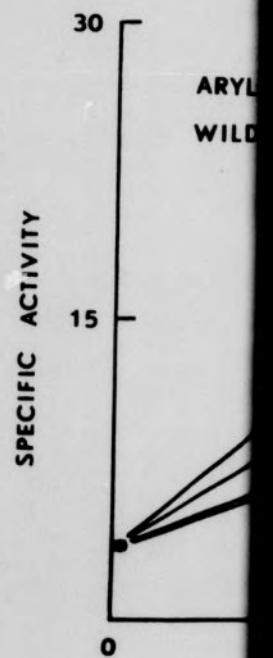
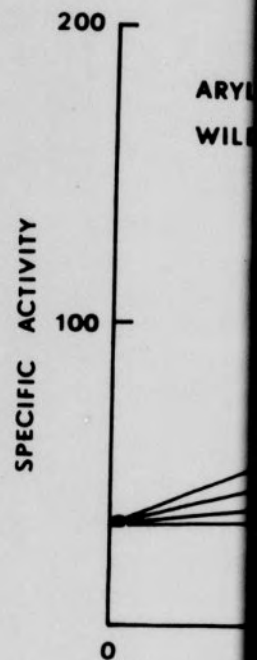
Figure 11. Influence of initial concentration of lead(II) ions on the adsorption of lead(II) ions by the adsorbent.

Figure 10a. The effect of glucose on exogenous induction of aryl-beta-glucosidase in wild type

Glucose $10^{-2}M$:	G-■-■-■-
Glucose $10^{-3}M$:	G-●-●-●-
Glucose $10^{-4}M$:	G-□-□-□-
Glucose 0:	G-o-o-o-

Figure 10b. Induction of aryl-beta-glucosidase and the influence of xylose in 12 hour wild type cells

Cellobiose $10^{-3}M$:	CB-●-●-●-
Cellobiose $10^{-3}M$ and xylose 2%:	CB, XYL-■-■-■-
Xylose 2%:	XYL-□-□-□-
Buffer:	0-o-o-o-



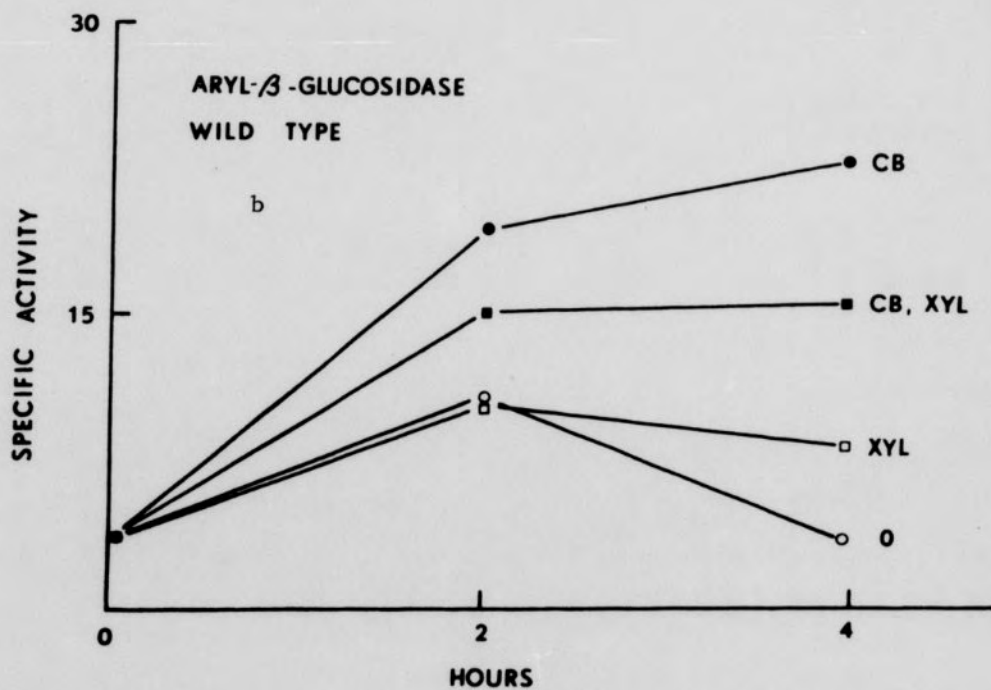
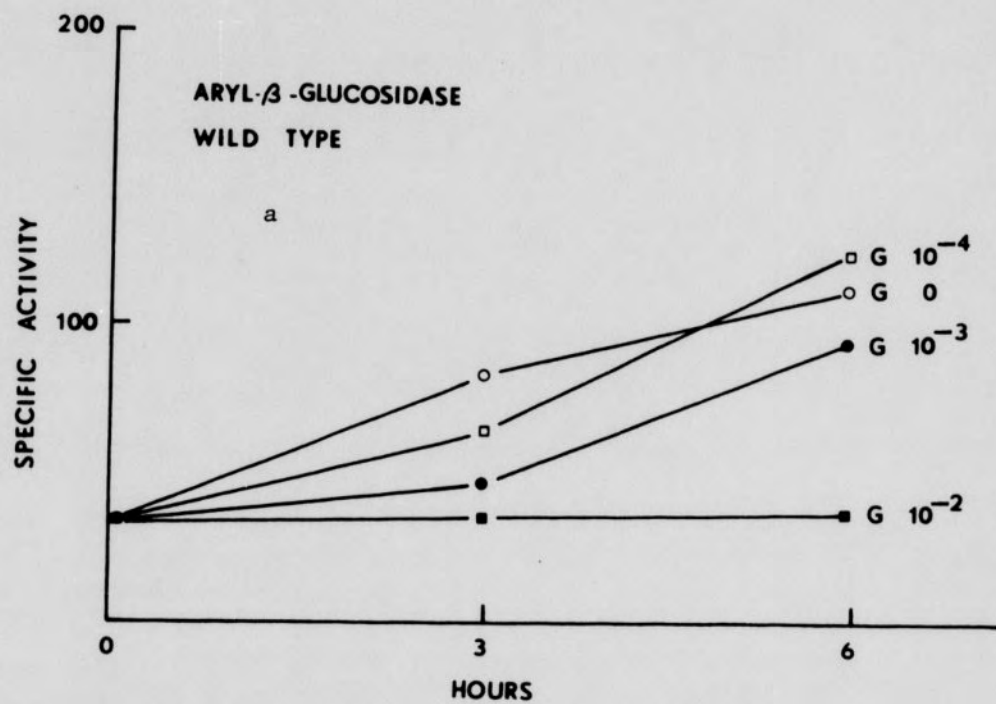
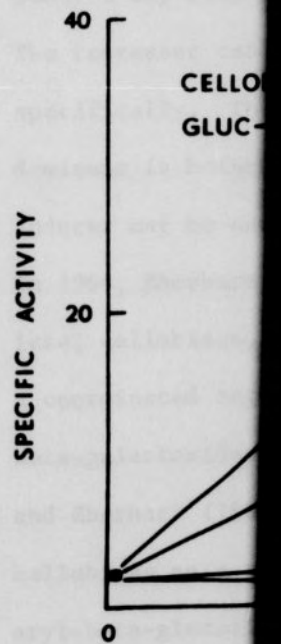
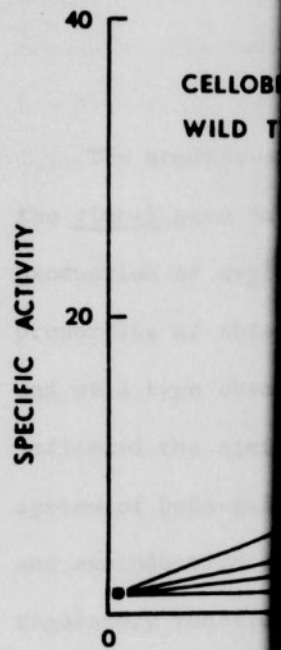
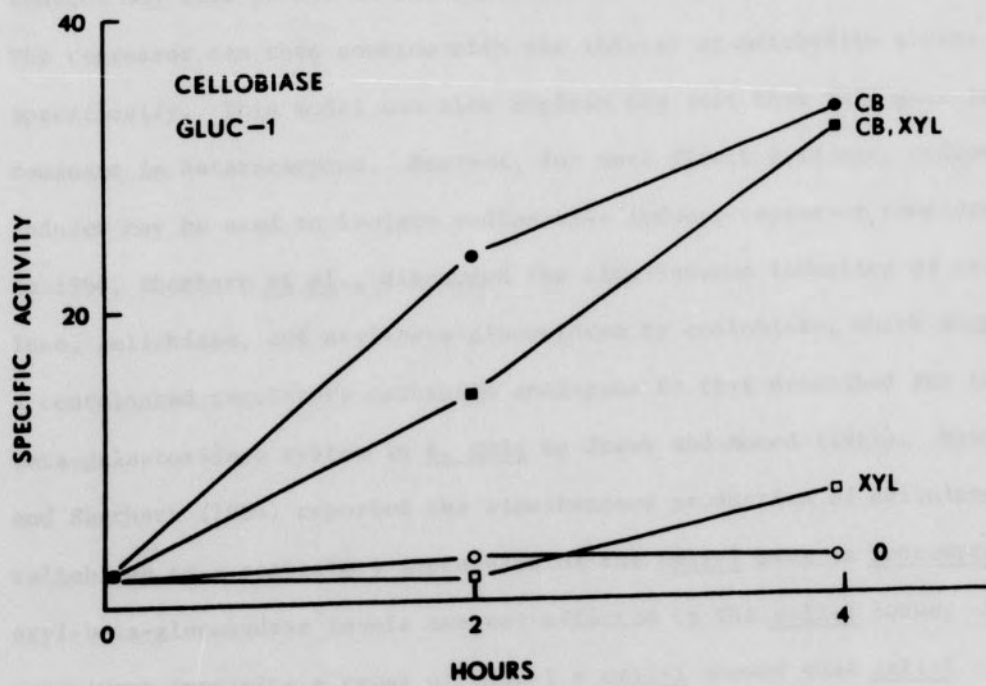
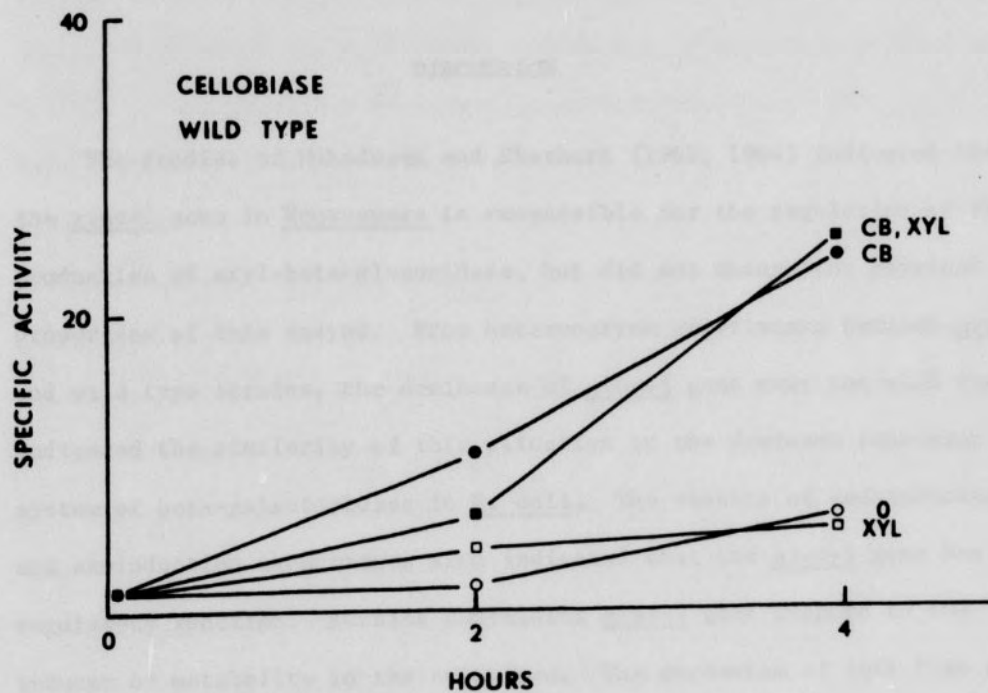


Figure 11. The induction of cellobiase and the influence of xylose in 12 hour cells

Cellobiose $10^{-3}M$:	CB-●-●-●-
Cellobiose $10^{-3}M$ and xylose 2%:	CB, XYL-■-■-■-
Xylose 2%:	XYL-□-□-□-
Buffer:	O-o-o-o-





DISCUSSION

The studies of Mahadeven and Eberhart (1962, 1964) indicated that the gluc-1 gene in Neurospora is responsible for the regulation of the production of aryl-beta-glucosidase, but did not change the physical properties of this enzyme. From heterocaryon experiments between gluc-1 and wild type strains, the dominance of gluc-1 gene over the wild type indicated the similarity of this situation to the dominant repressor system of beta-galactosidase in E. coli. The results of endoinduction and exoinduction experiments also indicated that the gluc-1 gene has the regulatory function. Strains containing gluc-1 gene respond to the inducer or metabolite in the cytoplasm. The mechanism of this type of control may also be due to the synthesis of a repressor by this gene. The repressor can then combine with the inducer or metabolite stereospecifically. This model can also explain the fact that this gene is dominant in heterocaryons. However, for more direct evidence, radioactive inducer may be used to isolate radioactive inducer-repressor complexes. In 1964, Eberhart et al., discussed the simultaneous induction of cellulase, cellobiase, and aryl-beta-glucosidase by cellobiose, which suggested a coordinated regulatory mechanism analogous to that described for the beta-galactosidase system in E. coli by Jacob and Monod (1961). Myers and Eberhart (1966) reported the simultaneous production of cellulase and cellobiase as a regulatory expression of the cell-1 gene in Neurospora; aryl-beta-glucosidase levels are not affected by the cell-1 locus. An experiment involving a cross of gluc-1 x cell-1 showed that cell-1

segregates independently of the gluc-1 character. The results in this thesis showed also the independent regulation of aryl-beta-glucosidase and cellobiase by either exoinduction or endoinduction. The gluc-1 mutant produces low aryl-beta-glucosidase, but the level of cellobiase remains the same. The addition of glucose, xylose or other energy sources indicates that the differences in enzyme level between wild type and mutant are not due to a difference in nutrient transport ability, therefore probably no permease system is involved since there was no glucose effect observed and the induction of cellobiase in both strains were very close in all circumstances. Endoinduction studies also support this suggestion. A mapping study of gluc-1, cell-1 and structural genes for corresponding enzyme may help to understand the mechanism of regulation and the significance of their relationship in utilization of cellulose in *Neurospora*.

Under the same experimental conditions, both the aryl-beta-glucosidase and cellobiase were induced to a different extent in conidia or young mycelia. The specific activities of these enzymes were always higher in conidial induction than mycelial. In their thesis work, Belz and Zweiback discovered the same phenomenon (Belz and Zweiback 1959). This difference must be due to the different responses of genes at various ages of development. In endogenous induction studies, there was no inducer added to the media prior to conidiation. The change in enzyme content must have been a result of change in endoenvironment of the cell. Studies by Zalokar (1959a, 1959b) on "growth and differentiation of *Neurospora* hyphae" and "enzyme activity and cell differentiation" in *Neurospora* also indicated variations in enzyme distribution at different ages of

cells and at different parts of a single cell. Thus, the endoinduction may be caused by a high local concentration or the lowering of repressor of a certain metabolite which can function as an inducer. However, the accumulation or disappearance of molecules in a cell must be under a control of gene function or a combination of functions of several genes.

Conidia of *Neurospora* were produced most abundantly when the environment did not favor growth. Results of this study, as well as an earlier report by Eberhart (1961), indicate that aryl-beta-glucosidase is an exocellular enzyme in conidia, but not in mycelia. Cellobiase is not an exocellular enzyme in any case. Although these two enzymes have overlapping substrate specificity, there may be some significance for Neurospora to produce more stable aryl-beta-glucosidase in conidia and to excrete it outside the cell under unfavored conditions. It would be interesting to study the cellobiase differentiation in cell-1 mutants which produce very high amounts of cellobiase in mycelia.

It has been well established that the mitotic process ensures the exact distribution of genes to each cell of the organism. The enzymatic and other differences between cells must arise from differences in the activity of the same set of genes in different cells. The starting and the stopping of the synthesis of specific proteins--differentiation at the molecular level--may occur by some process involving genic DNA, by the transcription of the DNA to form messenger RNA, by the combination of messenger RNA with the ribosome during protein synthesis, or by some transformation of the ultimate protein product (Viljee, 1966). Although much research has been conducted in this area, there is no definite answer yet for this complex problem. As a working hypothesis, many

investigators currently assume that cellular differentiation involves the activation of specific genetic sites, and there are many attempts to relate what is known of nucleic acid and protein metabolism to the study of differentiating cells. There are probably other mechanisms of control of the synthetic pathways that do not operate directly at the gene, but at the ribosomal level, or even at the cell surface where the transport of substances into and out of the cell is regulated (Villem, 1966). The situation in our system may be tested by adding actinomycin D or puromycin (which inhibit transcription and translation, respectively) to induction media. Cell extracts from conidia may be injected into mycelia to see whether any substance in conidia can cause a change in the induction behavior of mycelia. Fractionation and analysis of the cell extract might be able to give a clue to aryl-beta-glucosidase regulation.

A fungus is one of the simplest organisms which shows differentiation in its life cycle. With the long historical background in biochemical and genetic studies, Neurospora serves as a very good material for these newly developed studies of biochemical genetics.

SUMMARY

The aryl-beta-glucosidase and cellobiase have been compared in wild type and mutant strains of Neurospora crassa. The gluc-1 mutant failed to show significant induction by various concentrations of exogenous cellobiose. This strain also failed to show the usual endogenous induction exhibited by wild type Neurospora under a variety of experimental conditions. New techniques were developed to study the release of exogenous enzyme in the process of conidiation. Intact cells were also studied. The results were discussed in light of the Jacob and Monod model.

BIBLIOGRAPHY

- Adelberg, E. A. and Umbarger, H. E. 1953. *J. Biol. Chem.* 205: 475.
- Adhya, S. and Echols, H. 1966. *J. of Bact.* 92(3): 601.
- Belz, C. I. and Zweiback, E. M. 1959. Studies of growth-activity relationships in the beta-glucosidase system of *Neurospora crassa*. Senior Thesis, Princeton University, Princeton, New Jersey.
- Cohn, M. 1957. *Bacteriological Reviews* 21(3): 140.
- Cohn, M., Cohen, G. H. and Monod, J. 1953. *C. R. Acad. Sci. Paris* 236: 746.
- Eberhart, B. M., Cross, D. F. and Chase, L. R. 1964. *J. of Bact.* 87(4): 761.
- Eberhart, B. M. 1961. *J. Cell and Comp. Physiol.* 58(1): 11.
- Etten, J. L., Molitoris, H. P. and Gottlieb, D. 1966. *J. of Bact.* 91: 169.
- Hill, E. P. and Sussman, A. S. 1964. *J. of Bact.* 88(6): 1556.
- Hogness, D. S. 1959. *Biophysical Science - A study program*, 256.
- Horowitz, H. H., Fling, M., Macleod, H. L. and Sueoka, N. 1960. *J. Mol. Biol.* 2: 96.
- Jacob, F. 1966. *Science* 10: 1470.
- Jacob, F. and Monod, J. 1961. *J. Mol. Biol.* 3: 357.
- Kannwisher, S. A. 1962. A method for the induction of beta-glucosidase in *Neurospora crassa*. Senior Thesis, Princeton University, Princeton, New Jersey.
- Landman, O. E. 1954. *Arch. Biochem. and Biophys.* 52: 93.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. *J. Biol. Chem.* 193: 265.
- Mahadeven, P. R. and Eberhart, B. M. 1962. *J. Cell and Comp. Physiol.* 60(3): 281.
- Mahadeven, P. R. and Eberhart, B. M. 1964a. *Arch. Biochem. and Biophys.* 108: 22.

- Mahadeven, P. R. and Eberhart, B. M. 1964b. Arch. Biochem. and Biophys. 108: 30.
- Monod, J. 1966. Science 28: 475.
- Monod, J. and Andureau, A. 1946. Ann. Inst. Pasteur 72: 868.
- Monod, J. and Cohn, M. 1952. Advan. Enzymal. 13: 67.
- Monod, J. 1959. Angew. Chem. 71: 685.
- Monod, J. and Cohen-Bazire, G. 1953. C. R. Acad. Sci. Paris 236: 530.
- Monod, J., Changeaux, J. and Jacob, F. 1963. J. Md. Biol. 6: 306.
- Myers, M. G. and Eberhart, B. M. 1966. Biochem. and Biophys. Res. Communications 24(5): 782.
- Nye, J. F., Kadner, R. J. and Crocken, B. J. 1966. J. Mol. Chem. 241(7): 1468.
- Rickenberg, H. V., Cohen, G. N., Buttin, G. and Monod, J. 1956. Ann. Inst. Pasteur 91: 829.
- Reinhardt, 1892.
- Singleton, C. M. and Murphy, J. V. 1958. Preliminary studies of enzyme-substrate relationship in Neurospora crassa. Senior Thesis, Princeton University, Princeton, New Jersey.
- Urey, J. C. 1966. Enzyme Induction in Neurospora Crassa. Ph. D. Thesis, Univ. Microfilm (Ann Arbor, Mich.) Order No. 65-15, 435.
- Urey, J. C. and Horowitz, N. H. 1967. Bioch., Biophys. Acta 132: 300.
- Villee, C. A. 1966. Federation Proceedings 874.
- Vogel, H. J. 1957a. Proc. Nat. Acad. Sci., U. S. 43: 491.
- Vogel, H. J. 1957b. In the chemical Basis of Heredity, p. 276, Baltimore, Johns Hopkins Press.
- Zalokar, M. and Cochrane, V. W. 1956. American Journal of Botany 43(2): 107.
- Zalokar, M. 1959a. American Journal of Botany 46(7): 555.
- Zalokar, M. 1959b. American Journal of Botany 46(8): 602.