

PHANEROCHAETE CHRYSOSPORIUM  $\beta$ -GLUCOSIDASES: INDUCTION,  
CELLULAR LOCALIZATION, AND PHYSICAL CHARACTERIZATION

MARK HARRISON SMITH  
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The thesis "Phanerochaete Chrysosporium  $\beta$ -Glucosidases:  
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by Mark H. Smith has been examined and approved by the following  
Examination Committee:

---

Michael H. Gold, Thesis Advisor  
Associate Professor

---

G. Doyle Daves, Jr.  
Professor

---

Thomas M. Loehr  
Professor

---

Charles M. McInyre  
Assistant Professor

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To Debby

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## ABSTRACT

The white-rot basidiomycete Phanerochaete chrysosporium produces intracellular soluble and particulate  $\beta$ -glucosidases and an extracellular  $\beta$ -glucosidase. The extracellular enzyme is induced by cellulose but repressed in the presence of glucose. The molecular weight of this enzyme is 90,000. The  $K_m$  for p-nitrophenyl- $\beta$ -glucoside is  $1.6 \times 10^{-4}$  M; the  $K_i$  for glucose, a competitive inhibitor, is  $5.0 \times 10^{-4}$  M. The  $K_m$  for cellobiose is  $5.3 \times 10^{-4}$  M. The intracellular soluble enzyme is induced by cellobiose; this induction is prevented by cycloheximide. The presence of 300 mM glucose in the medium, however, had no effect on induction. The  $K_m$  for p-nitrophenyl- $\beta$ -glucoside is  $1.1 \times 10^{-4}$  M. The molecular weight of this enzyme is about 410,000. Both enzymes have an optimal temperature of 60° C and an  $E_{act}$  of 9.15 kcal/mole. The pH optima, however, were 7.0 and 5.5 for the intracellular and extracellular enzymes, respectively.

## INTRODUCTION

Cellulose is the major fraction of all vegetable matter on earth. The percentage of cellulose content varies among plant species and even within tissues of the same plant. Young leaves may contain only 10% cellulose, whereas wood may contain over 50% and cotton 90%. Overall, about 50% of vegetable carbon on earth is cellulose.

Lignin is the second most abundant natural polymer, being intimately associated with cellulose in woody tissues. Together, lignocellulosic matter constitutes a vast renewable resource.

The uses of wood fall roughly into three categories. It is used universally as a structural material for buildings, furniture, and artwork. It is the raw material for the pulp and paper industry, providing cellulose and cellulose derivatives for many applications. And wood is used as fuel for heating and cooking. It is the fuel of choice (or necessity) in much of the world. In some areas, extensive use has led to defoliation of large areas and an energy shortage comparable to that of the industrialized nations.

The building materials and pulp and paper industries provide the economic base for large regions and even entire countries. The nations of the Scandinavian peninsula are notably dependent upon their forests, and have initiated programs to increase their importance as raw material and energy reserves. Several areas of the United States are similarly

dependent upon forest reserves as essential components of their economies. In Oregon, for example, in 1976 the pulp and paper industry employed over 6,000 persons with a payroll of over \$133 million. The industry consumed over \$300 million in goods and services (freight, utilities, etc.) and paid \$21.7 million in state and local taxes (1).

There remains a great potential for further exploitation of existing resources. New, fast-growing trees promise to decrease the time between harvests. More important, however, is the potential use of the considerable wastes remaining after trees are harvested and processed. 40% of the biomass is left in the forest with present harvesting methods. Sawdust and planing chips are often under-utilized. Vast quantities of lignin wastes result from paper pulp manufacture.

Modern commercial pulping methods generally involve some form of sulfur treatment, such as with alkaline sodium sulfide or acidic calcium bisulfite. The remaining lignosulfonates, or kraft lignins, have at present little or no commercial value and are usually burned as a low-grade fuel for energy and to recover pulping chemicals. Burning of this high-sulfur material necessitates large expenditures for pollution abatement. Waste products also include a considerable amount of hemicelluloses, pectins, and other soluble carbohydrates which must be extensively treated.

Over 30% of the capital expenditures made by the pulp and paper industry in Oregon in the ten years ending in 1976 was spent on pollution control (1). Such expenditures, however aesthetically or philosophically pleasing, make less sense economically. The only returns in



investment that can be realized are the avoidance of fines, cleanup costs, and legal fees, which might otherwise be paid as a result of unchecked discharges.

It would be wise to utilize all of the tree. Marketable wastes are no longer wastes. Since the economic potential of kraft lignin is doubtful, the most promise may be seen in alternate pulping methods. Lignin is composed of aliphatic and aromatic subunits which, if easily separable, would find a ready market as alternatives to petroleum-based chemical feedstock. A different pulping process is required, one which would be less polluting and would salvage more of the biomass for effective consumption. One alternative may be biopulping, using microorganisms to separate the components of wood.

Biodegradation of wood is an extremely important natural process in light of the tremendous mass of lignocellulosic matter produced each year by the world's flora. The captured carbon must be recycled to be available for future generations. It is curious to note, therefore, that only a few microorganisms are known which efficiently degrade wood completely to CO<sub>2</sub> and water. A small number of Basidiomycete fungi, those responsible for "white rot" of wood, are efficient lignin degraders. Many other species of fungi degrade small amounts of lignin as they consume the carbohydrate components of wood. Recent work has shown that certain actinomycetes also have the ability to degrade lignin, but at severely restricted rates (8,12,38). The white rot fungi, however, have received the most attention as candidates for industrial processes. One of these is Phanerochaete chrysosporium Burds.

Burdsall and Eslin (11) proposed the name P. chryso sporium to identify several individual isolates which they found to share common biochemical and morphological characteristics, including a chryso sporium imperfect state (having a gold spore mat, from Greek chryso s = gold). Isolates possessing similar morphological and biochemical characteristics, which may be conspecific, have been referred to variously as Chryso sporium pruin osum, C. lign orum, Sporotrichum pruin osum, and S. pulverulentum. P. chryso sporium is ubiquitous in nature and has been isolated in many parts of the world. Many investigators have used this fungus to study the biodegradation of lignin, cellulose, and native wood (3,4, 23,30). Gold and Cheng have recently described methods to induce colony growth on solid media (17) and to harvest basidiospores (18). With these procedures, detailed biochemical genetic studies of the species are now possible.

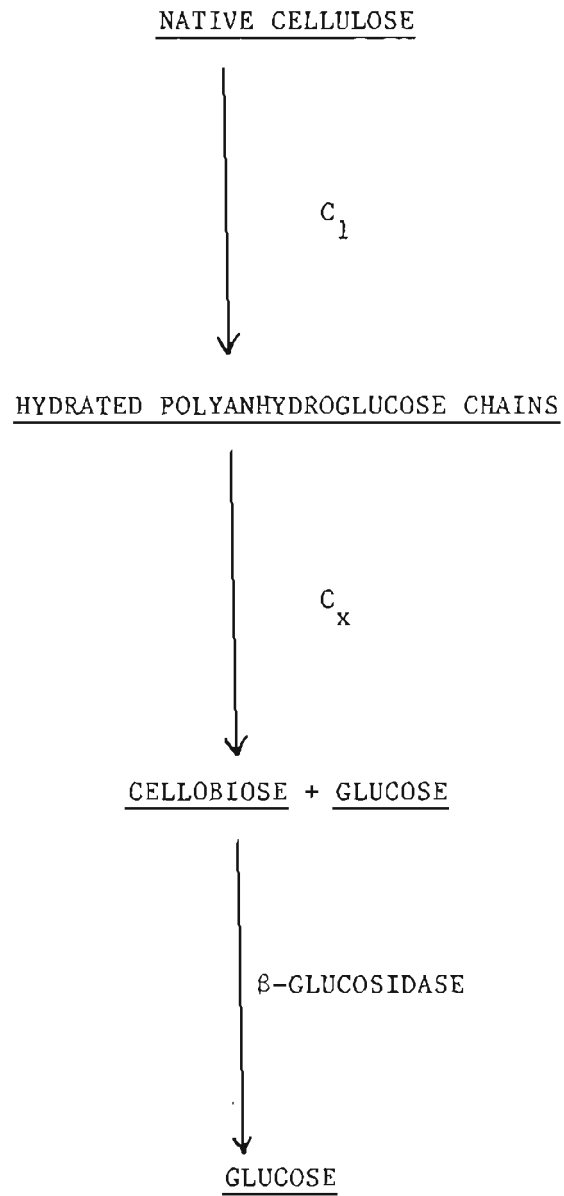
The biodegradation of cellulose is a very important aspect of the larger question of wood degradation. Cellulose is a long chain polymer of  $\beta(1-4)$  linked glucose units. The length of the chain varies depending on the source and the method of preparation, and probably is greatly variable within a given sample. A general scheme of enzymatic degradation as proposed by Reese et al. (34) is presented schematically in Fig. 1. It was observed that soluble derivatives of cellulose, such as carboxymethylcellulose, were more readily hydrolyzed than was native cellulose. The activity of an initiator enzyme,  $C_1$ , was presumed necessary but the actual function was unknown. It was believed to "disaggregate" the crystalline cellulose for more efficient attack by the actual

Figure 1

GENERALIZED ENZYMATIC CELLULOSE HYDROLYSIS SYSTEM.

After Reese et al. (34).

Highly crystalline native cellulose is first attacked by an initiating enzyme,  $C_1$ , to produce a modified cellulose which can be degraded by endo- and exoglucanases, together known as  $C_x$ , to cellobiose and glucose. Cellobiose is further degraded by  $\beta$ -glucosidase to glucose.



hydrolases. Recent work, however, has established the  $C_1$  from Trichoderma koningii as an exoglucanase, and that from T. viride as a cellobiohydrolase (9,20,43).

Several enzymes together constitute the  $C_x$  activity, which is activity directed toward solubilized cellulose. These enzymes are usually assayed by measuring the hydrolysis of carboxymethylcellulose. Two classes of  $C_x$  enzymes are recognized, the endoglucanases and the exoglucanases. Exoglucanases attack cellulose chains from the non-reducing end, removing successively glucosyl units. They are assayed by measuring the increase in reducing sugar content in the mixture (16). Endoglucanases can attack randomly within a chain, the terminal linkages being less susceptible to attack. This activity is measured as a reduction in the viscosity of a solution of carboxymethylcellulose (2). The end products of these enzymatic reactions are glucose and cellobiose, the latter being converted to glucose by the action of  $\beta$ -glucosidase.

Glycosidases, such as  $\beta$ -glucosidase, are usually specific for the sugar part of the substrate, allowing great variation in the aglycone portion of the molecule. For instance, cellobiase may be assayed by incubating it with p-nitrophenyl- $\beta$ -glucoside. The hydrolysis product p-nitrophenol is yellow in alkaline solution. Cellobiase may also be assayed by measuring the increase in free glucose resulting from the hydrolysis of cellobiose, but this often requires previous dialysis of the enzyme preparation to remove pre-existing glucose which would saturate the glucose detection system. Some cellobiases have been identified, however, which will not hydrolyze phenylglucosides (22).

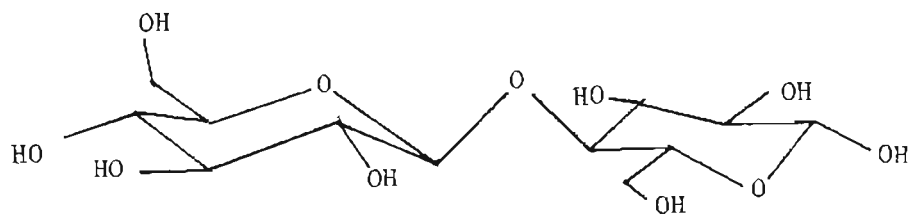
Many organisms possess the ability to hydrolyze modified cellulose such as carboxymethylcellulose or acid-swollen cellulose. The ability to degrade highly ordered native cellulose such as mature dried cotton fiber is less widely distributed. This function requires the activity of a  $C_1$  enzyme, whatever that activity might be.

Legler has extensively studied the mechanism of action of glycosidases. He has developed a series of active site-directed inhibitors, using epoxides of sugar analogs (28). For  $\beta$ -glucosidases, he used conduritol B epoxide, a derivative of myo-inositol (27) (Fig. 2). The equatorial hydroxyls about the cyclohexyl ring approximate the positions of the hydroxyls of glucose. The epoxide oxygen introduces a highly reactive functional group to the molecule. The substitution of a carbon in the cyclohexane ring for the oxygen in the corresponding pyran increases the stability of the inhibitor-enzyme bond (26).

Conduritol B epoxide was shown in several ways to be active site-specific (24,25). Competitive inhibitors protected the enzyme from inactivation. Other epoxides which had a greater general reactivity but less structural analogy to glucose had no effect. No inactivator was incorporated by denatured enzyme. The inactivator reacted with the enzyme with a 1:1 stoichiometry, and when the inhibitor was released by hydroxylamine, only (+)-inositol was recovered. Mercer et al. (32) showed that reaction occurs only with the D-enantiomer. Thus reaction occurs only when the epoxide oxygen mimics the ether oxygen of a  $\beta$ -glucoside. The rate of deactivation decreased with increasing pH. A sigmoidal response to pH indicated the participation of an acidic group

Figure 2

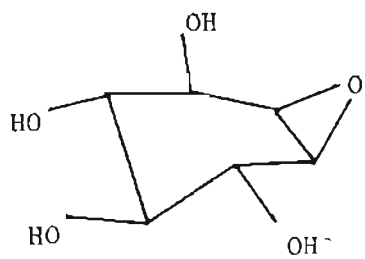
COMPARATIVE STRUCTURES OF CELLOBIOSE (A), MYO-INOSITOL (B),  
AND CONDURITOL B EPOXIDE (C).



A



B



C



with a pK of 6.1 for *Aspergillus* glucosidase and pK's of 5.6 and 7.3 for almond glucosidase A and B, respectively (26).

The release of (+)-inositol from the inactivated enzyme by hydroxylamine indicates the formation of an ester bond (25). Analysis of enzyme digests of *Aspergillus* glucosidase inactivated with radioactive conduritol B epoxide identified the site of esterification as a carboxyl group of an aspartic acid residue (7).

From this information, two possible mechanisms can be proposed (26) (Fig. 3). Mechanism A involves the formation of a carbonium ion intermediate, while mechanism B requires the formation of an ester bond between the enzyme and the sugar. The occurrence of an ester in the conduritol B epoxide inactivation reaction does not prove that an ester is formed during the hydrolysis of a glucoside.

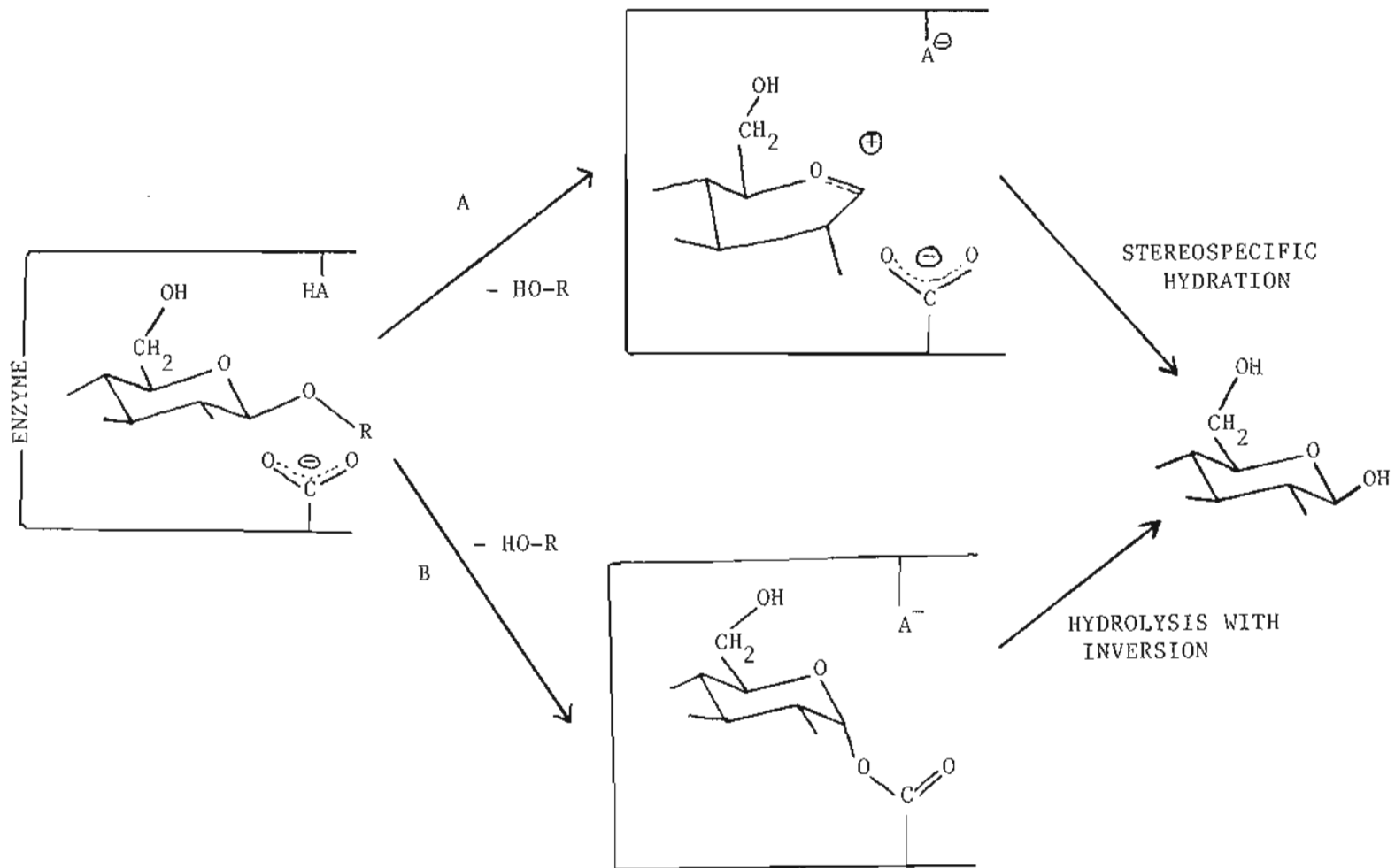
If mechanism B occurs, nucleophilic attack at C-1 of the glucosyl residue would be required to explain the stereochemistry of the products. This kind of hydrolysis is unusual for esters. Hydrolysis of most esters involves nucleophilic attack on the acyl carbon with cleavage of the O-acyl bond (33). The carbonium ion in mechanism A has a configuration similar to a lactone, thus a lactone inhibitor should be more efficient than an inhibitor which more resembles the substrate conformation (42). Indeed, D-glucono- $\beta$ -lactone is bound much more strongly than either p-nitrophenyl- $\beta$ -D-thioglucoside or  $\beta$ -D-glucose (26).

Eriksson and co-workers have extensively studied the cellulolytic enzymes released to the extracellular environment by *S. pulverulentum*,

Figure 3

PROPOSED MECHANISMS OF  $\beta$ -GLUCOSIDE HYDROLYSIS.

After Legler (26).



an isolate which may or may not be conspecific with the strain of P. chrysosporium used in this study. They have identified an exoglucanase (15), one or several  $\beta$ -glucosidases (16), a cellobiose oxidase (6), a cellobiose:quinone oxidoreductase (40,41), a cellobionolactonase (14), a glucose oxidase (14), and no less than five endonucleases (15). They have also presented evidence for  $C_1$  activity (16).

Figure 4 illustrates the role of these enzymes in cellulose hydrolysis. Regulation of this enzyme network is still open to speculation. From the previous discussion it would be expected that the lactones gluconolactone and cellobionolactone would act as competitive inhibitors of the glucosidases and of the endo- and exoglucanases. Gluconolactone has in fact been shown to be a potent inhibitor of the  $\beta$ -glucosidase (13).

Cellulose hydrolysis eventually produces glucose which is utilized by the cells as a source of energy. If the production of glucose exceeds its demand by the cells, and sufficient oxygen is present, then gluconolactone should be produced in sufficient quantities to inhibit the action of the  $\beta$ -glucosidases. The subsequent accumulation of cellobiose may affect the system in many ways. First, the endoglucanases act as transglucosylases at high cellobiose concentrations, so a synthesis of oligosaccharides would be expected. Water-insoluble cellodextrins will in fact precipitate if cellobiose is incubated with a crude cellulase preparation (16).

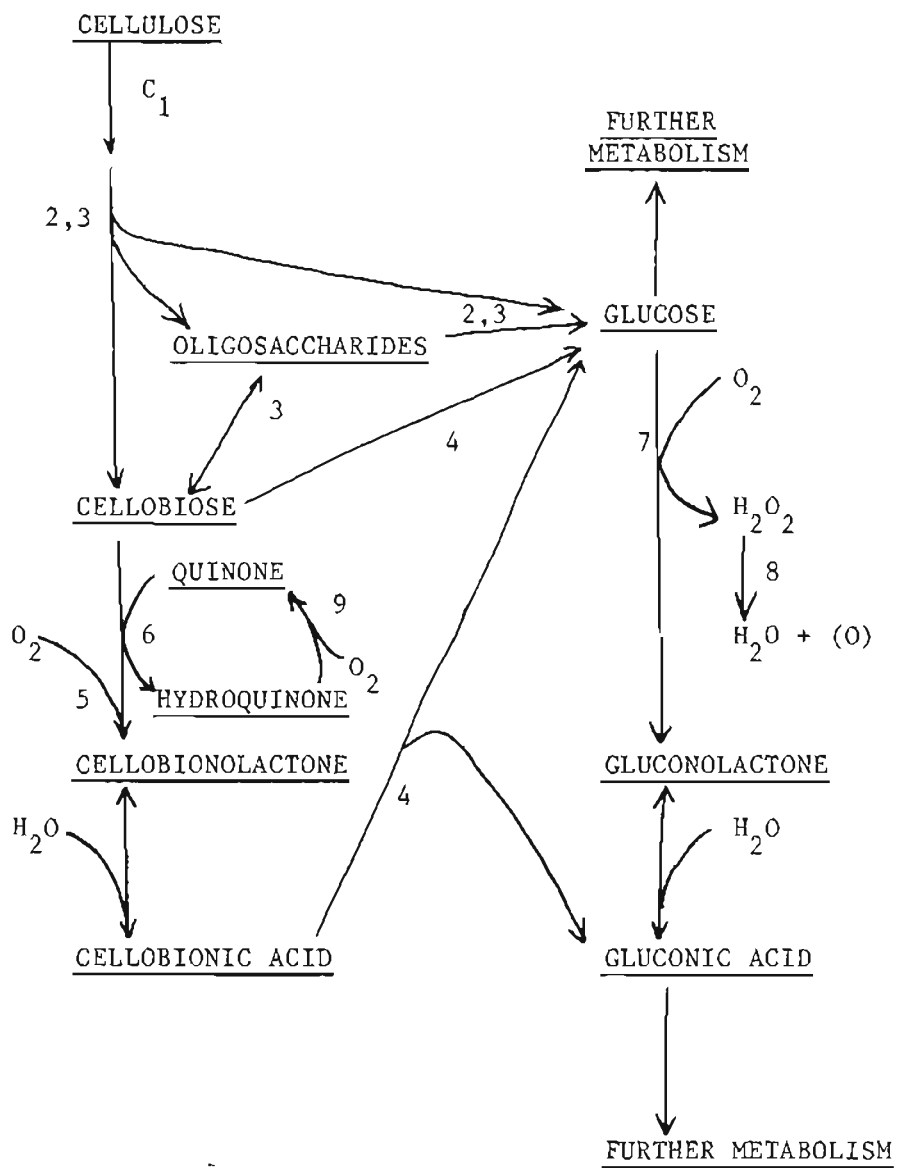
Accumulated cellobiose may be converted to cellobionolactone in the presence of oxygen and cellobiose oxidase or of quinone and cellobiose:

Figure 4

PATHWAYS OF CELLULOSE HYDROLYSIS.

After Eriksson (14).

The enzyme activities depicted are:  $C_1$  (1), endoglucanase (2), exoglucanase (3),  $\beta$ -glucosidase (4), cellobiose oxidase (5), cellobiose:quinone oxidoreductase (6), glucose oxidase (7), catalase (8), and laccase (9).



quinone oxidoreductase. The cellobionolactone may inhibit both the hydrolase and the transglucosylase activities of the endoglucanases. This effect has not been observed.

It can be seen from Figure 4 that cellobiose and its hydrolase hold a pivotal role in cellulose hydrolysis. The purpose of this study was to search for  $\beta$ -glucosidases in P. chrysosporium, characterize them, and investigate their localization, number of forms, regulation, and physical parameters.

## MATERIALS AND METHODS

Media and reagents. Growth media were obtained from Difco. Microcrystalline cellulose was from Baker Chemical Co. BioGel A-1.5m was purchased from BioRad. All other reagents were obtained from Sigma.

Growth of mycelia. A culture of P. chrysosporium ME446, obtained from the U.S. Forest Products Laboratory, Madison, Wis., was maintained on slants of Vogel medium N (39), with thiamine replacing biotin (modified Vogel), Table 1, and supplemented with 3% malt extract and 0.25% yeast extract.

Conidia were washed from slants, filtered through glass wool, and diluted with distilled water to a concentration of  $10^6$ /ml of medium. Cells were grown in Erlenmeyer flasks on a New Brunswick G-10 rotary shaker operating at a speed of 150 rpm and describing a 5 cm circle. The cells were grown at 28°C, in a medium consisting of modified Vogel salts and 2% glucose, or other carbon sources where indicated.

Intracellular enzyme induction and preparation. Cells grown for 48 hours in submerged culture containing 2% glucose were aseptically harvested by suction filtration, washed, and transferred to modified Vogel medium supplemented with an appropriate carbon source for enzyme induction. Samples of the mycelial suspension were then removed periodically, washed by suction filtration, and frozen. Frozen mycelium was ground with sand in a chilled mortar (19). All subsequent steps were



TABLE 1.  
Vogel's basal salts medium, modified (39).

<u>Salt</u>	<u>Final g/l</u>	<u>Final mM</u>
$\text{Na}_3$ Citrate. $2\text{H}_2\text{O}$	2.62	8.9
$\text{KH}_2\text{PO}_4$ , anhydrous	5.1	37
$\text{NH}_4\text{NO}_3$ , anhydrous	2.0	25
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2	0.81
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.1	0.68
Citric acid monohydrate	$5.0 \times 10^{-5}$	$2.4 \times 10^{-4}$
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	$5.0 \times 10^{-5}$	$1.7 \times 10^{-4}$
$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$	$1.0 \times 10^{-5}$	$2.5 \times 10^{-5}$
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	$2.5 \times 10^{-6}$	$1.0 \times 10^{-5}$
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	$5.0 \times 10^{-7}$	$3.0 \times 10^{-6}$
$\text{H}_3\text{BO}_3$ , anhydrous	$5.0 \times 10^{-7}$	$8.1 \times 10^{-6}$
$\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$	$5.0 \times 10^{-7}$	$1.9 \times 10^{-6}$
Thiamine HCl	$1.0 \times 10^{-4}$	$3.0 \times 10^{-4}$
Glass-distilled $\text{H}_2\text{O}$		
pH adjustment if necessary to 5.5 with HCl or NaOH		

performed at 0 to 4°C. The broken cells were extracted for 5 min in distilled water and centrifuged at 27,000 x g for 30 min. The supernatant was assayed for soluble enzyme activity and protein concentration. The supernatants from maximally induced cells were pooled, and solid ammonium sulfate (Mann, enzyme grade) was added to 90% saturation. The solution was stirred for 30 min and centrifuged at 27,000 x g for 20 min. The precipitate was suspended in distilled water, dialyzed until the addition of barium chloride failed to produce a precipitate from the dialysate, and lyophilized. The insoluble cellular material (walls and membranes) was washed 10 to 15 times with distilled water until the final wash showed no enzyme activity, then the the pellet was assayed for particulate enzyme activity and protein concentration.

Extracellular enzyme induction and preparation. Cells were grown in 1 liter of modified Vogel medium containing either 0.25% cotton or microcrystalline cellulose as the carbon source. Enzyme production was monitored by assaying 0.90 ml of culture fluid for activity. After 10 to 12 days, the culture fluid was filtered, and the filtrate was cooled to 0°C. Solid ammonium sulfate was added to bring the solution to 90% saturation. The solution was stirred for 30 min and centrifuged at 27,000 x g for 20 min. The precipitate was suspended in distilled water, dialyzed until sulfate free, and lyophilized.

Assays. Intracellular soluble and insoluble p-nitrophenyl- $\beta$ -glucosidase (PNPGase) activity was measured in a 1-ml reaction mixture containing 50 mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.4) buffer and 5 mM p-nitrophenyl- $\beta$ -D-glucoside (PNPG).

Reactions were started by the addition of 0.050 to 0.900 ml of enzyme solution and were carried out at 45°C for 10 min. After the reaction, 0.50 ml of 1 M NaOH in 1 M tris(hydroxymethyl)aminomethane (Tris) was added to develop the yellow color, and the tubes were cooled on ice. The p-nitrophenol produced was measured spectrophotometrically at 400 nm and compared to a standard curve.

Extracellular enzyme was assayed in a similar manner except that 50 mM 2-(N-morpholino)ethanesulphonic acid (MES) (pH 5.5) was used as the buffer.

Cellobiase activity was measured by the following method. Fifty ml of enzyme preparation was mixed with 0.150 ml of 83 mM cellobiose in HEPES or MES buffer as described above. The reaction mixture was incubated at 45° C for 10 minutes, at which time 0.300 ml of 0.2 M  $K_2HPO_4$  was added and the entire mixture was immersed in boiling water for five minutes. The glucose formed by the action of cellobiase was assayed by the following glucose oxidase procedure. A reagent solution was made of 30 mg o-dianisidine (1.2 mM in the reagent solution), 500 g peroxidase (Sigma type IX), and 0.10 ml glucose oxidase solution (about 10,000 units, Sigma type V) in 100 ml of a 0.10 M sodium phosphate buffer, pH 7.0. Two ml of this reagent were mixed with the 0.50 ml of solution to be tested and incubated at 30° C for 30 minutes, at which time the reaction was stopped and the purple color developed by the addition of 2.50 ml of 6 N HCl. The absorbance was measured at 540 nm and compared to a standard curve. The assay was performed on enzyme preparations which had been dialyzed free of glucose. Protein concentration was

measured by the method of Lowry et al. (29), using bovine serum albumin as the standard. Specific activity of PNPGase is defined as  $\mu\text{M}$  PNP released per minute per mg of protein. Specific activity of cellobiose is  $\text{M}$  glucose released per minute per mg of protein.

Gel filtration. Ammonium sulfate-fractionated, dialyzed, and lyophilized enzymes were dissolved in distilled water and gel filtered at  $4^{\circ}\text{C}$  on a column of BioGel A-1.5m (1.5 by 52 cm) equilibrated in 20 mM Tris chloride (pH 7.4) containing 100 mM NaCl. Fractions (1.5 ml) were collected, and samples were examined for enzyme activity. Enzymes partially purified by gel filtration were used to determine  $K_m$  and  $K_i$  values.

Determination of temperature optima and energies of activation. PNPGase activity was measured as described above at various temperatures between  $15^{\circ}\text{C}$  and  $80^{\circ}\text{C}$ . The Arrhenius activation energy was obtained from these data for the extracellular and for the intracellular soluble enzymes.

Heat inactivation of the enzymes was observed by incubating the enzymes at the test temperature for 5 min followed by storage in an ice bath until all samples were assayed at  $45^{\circ}\text{C}$  as described above.

Determination of pH optima. PNPGase activity was measured in a 1-ml reaction mixture containing 5 mM PNPG and 100 mM buffer adjusted to the proper pH. The following buffers were used: pH 7.4 to pH 8.4, HEPES; pH 7.0 to pH 7.4, morpholinopropanesulfonic acid (MOPS); pH 5.4 to pH 6.4, MES; pH 7.6 to pH 9.0, Tris; pH 4.0 to pH 6.0, succinic acid.

The reactions were started by the addition of 0.100 ml of enzyme solution, and were carried out at 45°C for 10 min, after which time the reaction mixtures were analyzed as described above.

Determination of  $K_m$  and  $K_i$ . The Michaelis-Menten constants ( $K_m$ ) were determined by the Lineweaver-Burke double reciprocal graphical method. The inhibition constants ( $K_i$ ) were determined by the graphical method of Dixon (35).

## RESULTS

The production of three intracellular enzyme activities is shown in Figure 5. When pregrown cells were transferred to a medium containing cellobiose, intracellular soluble PNPGase and cellobiase activities were induced quickly, reaching a maximum approximately 20 hours after the introduction of cellobiose. Insoluble PNPGase activity began to increase after 15 hours, reaching a maximum after approximately 33 hours. Particulate cellobiase activity could not be detected within this time period. The insoluble enzyme was not studied further. The presence of glucose in 0 to 300 mM concentration had a negligible effect on the induction of the intracellular enzymes by 1% cellobiose.

The induction profile of the extracellular  $\beta$ -glucosidase is shown in Figure 6. Cells grown in the presence of cellulose produced maximal activity after about 7 days. When the cells were grown in the presence of 0.25% cellulose and 1% glucose, extracellular  $\beta$ -glucosidase did not appear until day 9. There was a 4-day lag between the disappearance of the exogenous glucose in the medium and the appearance of  $\beta$ -glucosidase activity. With 3% glucose in the medium,  $\beta$ -glucosidase did not appear. These results indicate that glucose or glucose metabolite(s) represses the formation of the extracellular  $\beta$ -glucosidase but not the formation of intracellular  $\beta$ -glucosidase. The mechanism of this repression is not known.

Figure 5

DEVELOPMENT OF THREE  $\beta$ -GLUCOSIDASE ACTIVITIES.

Enzyme specific activity is expressed as (micromoles of p-nitrophenol released per minute per milligram of protein)  $\times 10^3$  for intracellular soluble  $\beta$ -glucosidase ( $\odot$ ) and insoluble  $\beta$ -glucosidase ( $\bullet$ ) and as (micromoles of glucose released per minute per milligram of protein)  $\times 10^2$  for intracellular soluble cellobiase ( $\blacktriangle$ ).

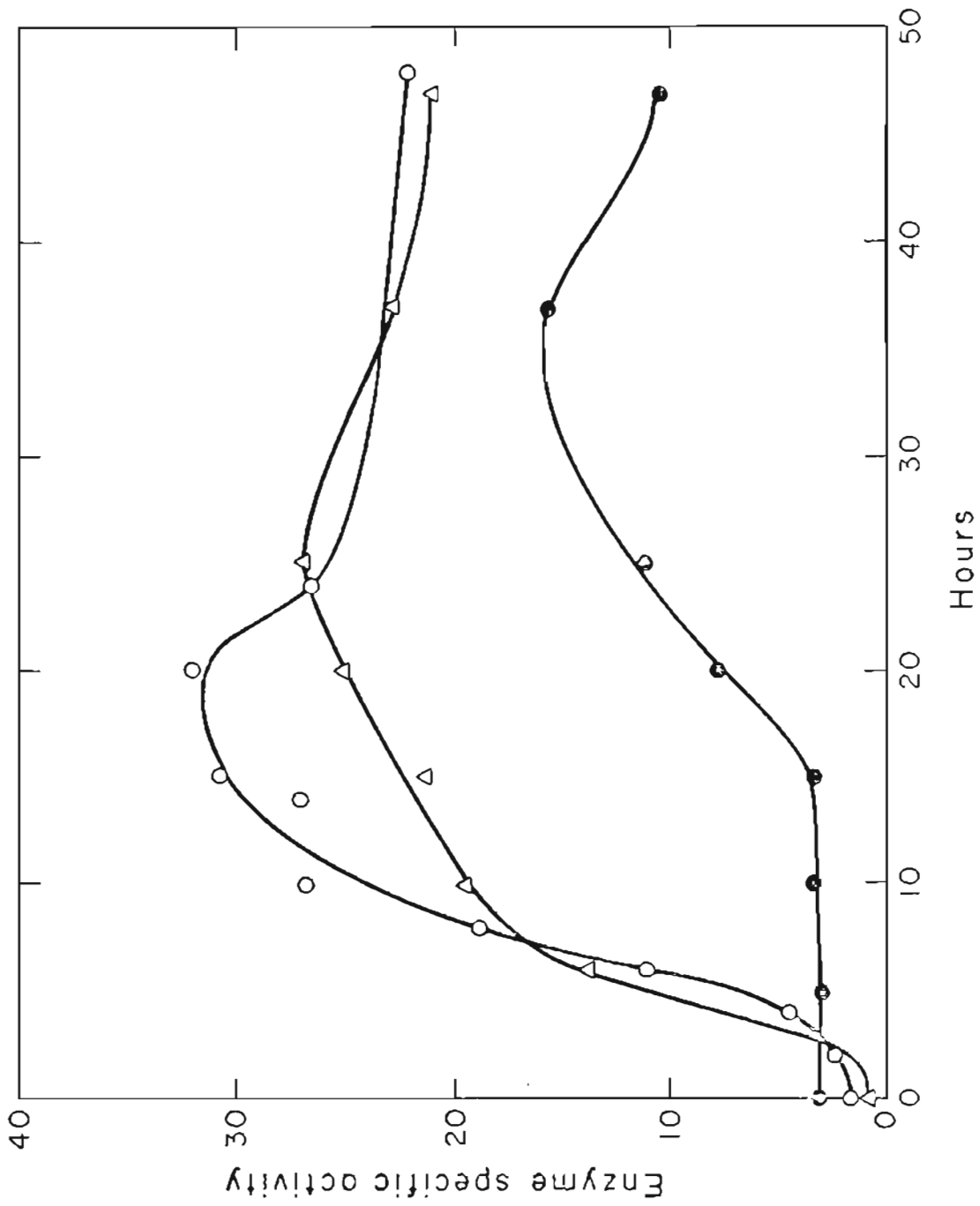
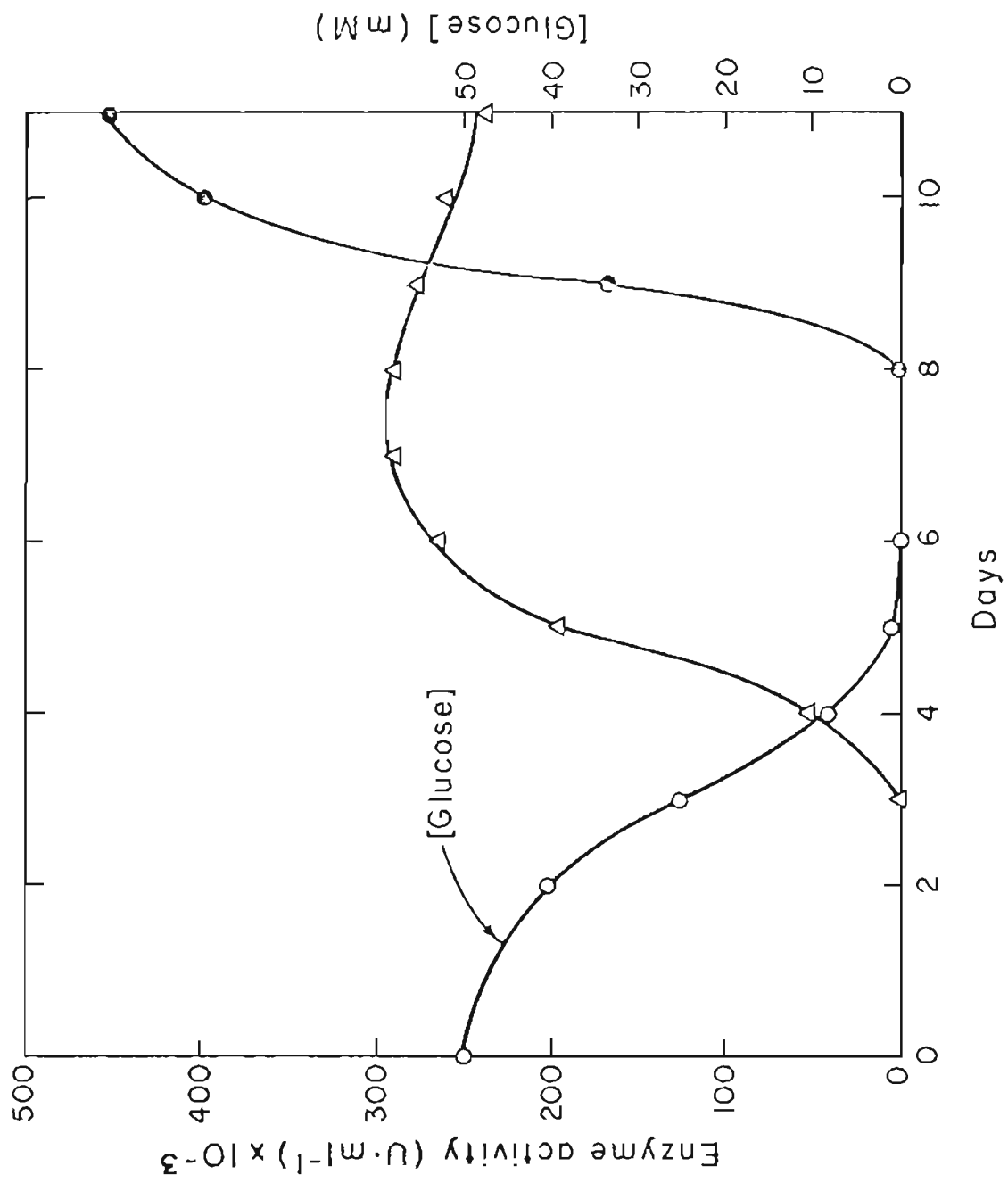




Figure 6

PRODUCTION OF EXTRACELLULAR  $\beta$ -GLUCOSIDASE  
AND ITS REPRESSION BY GLUCOSE.

Conidia were inoculated into flasks containing cotton in modified Vogel's medium with (●) or without (▲) glucose. When glucose was present initially, it was consumed rapidly (⊙).



The ability of various substrates to induce intracellular and extracellular  $\beta$ -glucosidase is shown in Table 2. The strongest inducers of the extracellular  $\beta$ -glucosidase were cellulosic polymers, whereas cellobiose was a poor inducer in comparison. Intracellular  $\beta$ -glucosidase, however, was maximally induced by cellobiose, and to a lesser extent by other substrates as shown.

The addition of cycloheximide ( $2.0 \times 10^{-5}$  M), a known protein synthesis inhibitor, to pregrown cells during the appearance of intracellular  $\beta$ -glucosidase halted any further increase in enzyme activity (Fig. 7). This result suggests that when cellobiose is introduced as the sole source of carbon, activation of pre-existing enzyme does not occur, but rather intracellular  $\beta$ -glucosidase is synthesized de novo.

Gel filtration of the intracellular and extracellular  $\beta$ -glucosidases indicated that they also differed with respect to molecular weight (Fig. 8). Whereas the intracellular enzyme eluted as a peak of molecular weight 410,000, the extracellular enzyme eluted as a single symmetrical peak with a molecular weight of 90,000 as calculated by the method of Andrews (5) (Fig. 8, insert).

PNPGase assays with both forms of the enzyme were linear with respect to time (up to 30 minutes) and protein concentration. The pH optimum for the intracellular enzyme was 7.0 (Fig. 9), whereas the pH optimum for the extracellular enzyme was 5.5 (Fig. 10). The temperature optimum for both enzymes was approximately 60°C (Fig. 11). When the temperature-activity data were plotted according to Arrhenius, straight lines were obtained (35). From this the energy of activation for both

Table 2.

Inducing ability of various carbon sources for the intracellular  
and extracellular enzymes.

Carbon source <sup>a</sup>	Intracellu- lar $\beta$ - glucosidase <sup>a</sup> sp act (micromol/min per mg of protein)	Extracellu- lar $\beta$ - glucosidase <sup>b</sup> (U/ml) x 10 <sup>3</sup>
Cellobiose	27.5	0.5
Cotton	12.4	9.8
Walseth cellulose	11.8	3.2
Microcrystalline cellulose	11.3	14.9
Phenyl- $\beta$ -D-glucoside	11.0	c
Sucrose	8.9	c
Calcium cellobionate	7.6	c
<u>myo</u> -Inositol	7.3	c
Xylan	5.8	c
Glycerol	5.5	c
Xylose	5.0	c
Galactose	3.4	c
Maltose	3.3	c
No carbon	3.0	c
Spruce woodmeal	2.1	c
Mannose	1.1	c
Mannitol	0.8	c
Fructose	0.7	c
Glucose	0.5	c

<sup>a</sup>Soluble carbon sources are present in 1% concentration. Insoluble carbon sources are present at 1.25 g per 100 ml. Values shown are for 14 h induction.

<sup>b</sup>Conidia were inoculated into flasks containing the indicated carbon sources. A 0.90-ml sample of filtered culture fluid was assayed for enzyme activity as described in the text. Values shown are for 10 d of induction.

<sup>c</sup>Not done.

Figure 7

INHIBITION OF THE SYNTHESIS OF INTRACELLULAR  
 $\beta$ -GLUCOSIDASE BY CYCLOHEXIMIDE.

Normal production of enzyme induced with cellobiose ( $\bullet$ ) is completely prevented upon the addition of cycloheximide ( $2 \times 10^{-5}$  M) at 2 ( $\blacktriangle$ ) or 6 ( $\bullet$ ) hours after the addition of cellobiose.

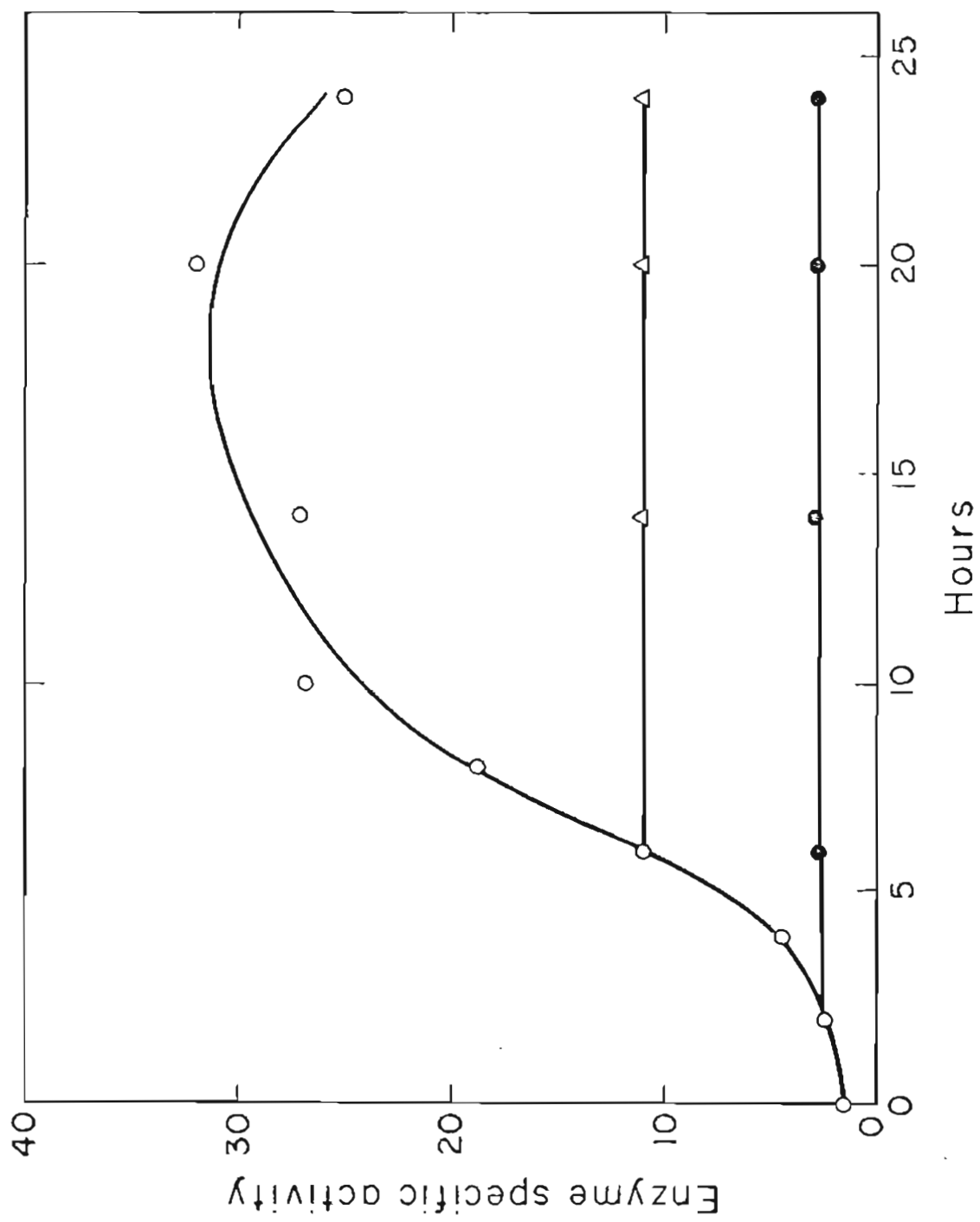


Figure 8

GEL FILTRATION OF THE SOLUBLE  $\beta$ -GLUCOSIDASES.

Gel filtration of the intracellular (●) and extracellular (○)  $\beta$ -glucosidases. A 2 ml sample of crude extract was applied to a column (1.5 by 52 cm) of BioGel A-1.5m equilibrated with 20 mM tris(hydroxymethyl) aminomethane-chloride in 100 mM NaCl (pH 7.4). Insert, log molecular weight versus  $V_e/V_o$  plot indicates that the molecular weight of the extracellular enzyme is 90,000, and that of the intracellular enzyme is 410,000.

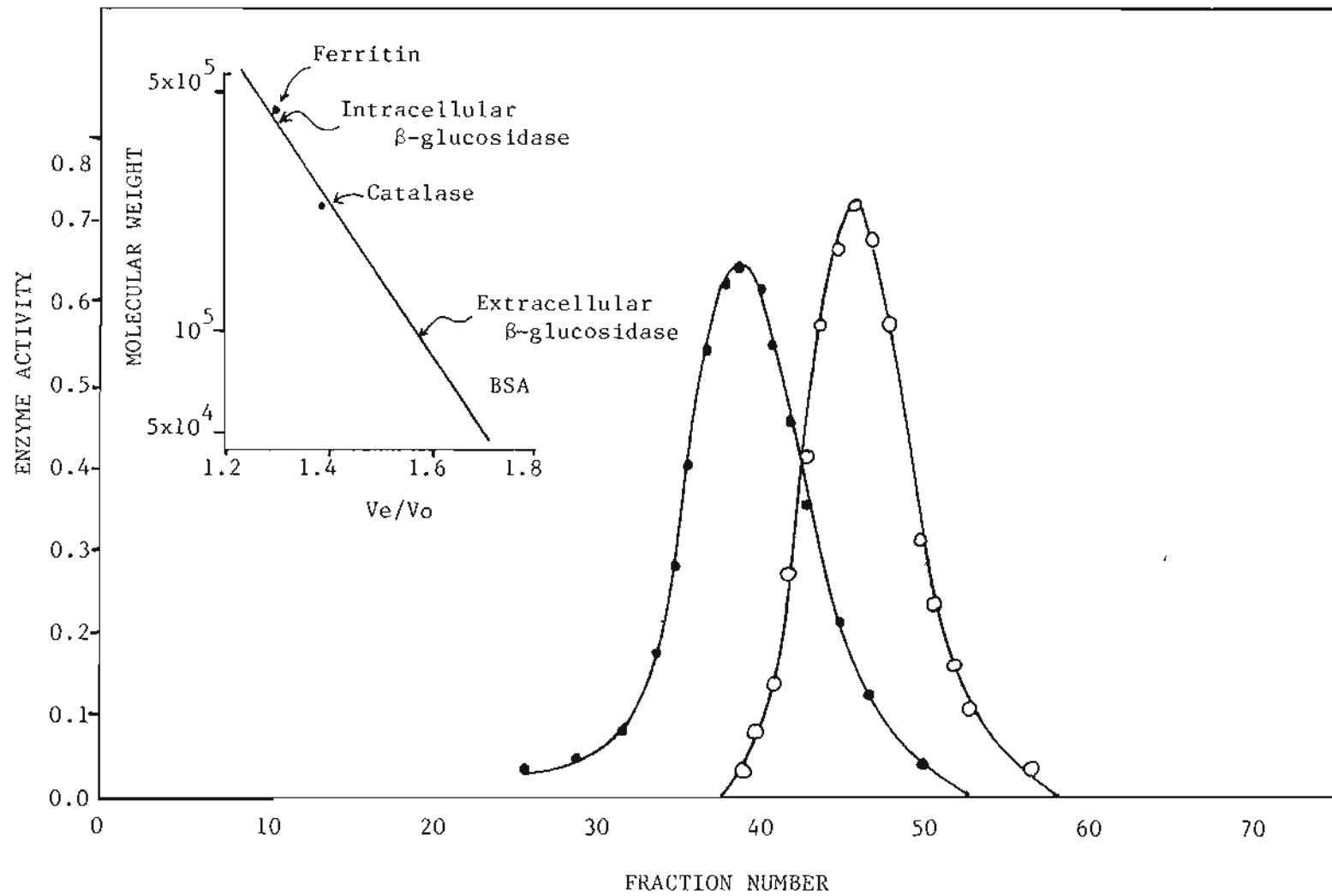




Figure 9

ACTIVITY VERSUS pH PLOT FOR THE INTRACELLULAR ENZYME.

Enzyme assay procedures were performed as described in Materials and Methods with the following buffers: (▲) Succinate. (■) MES. (■) MOPS. (●) HEPES. (●) Tris.

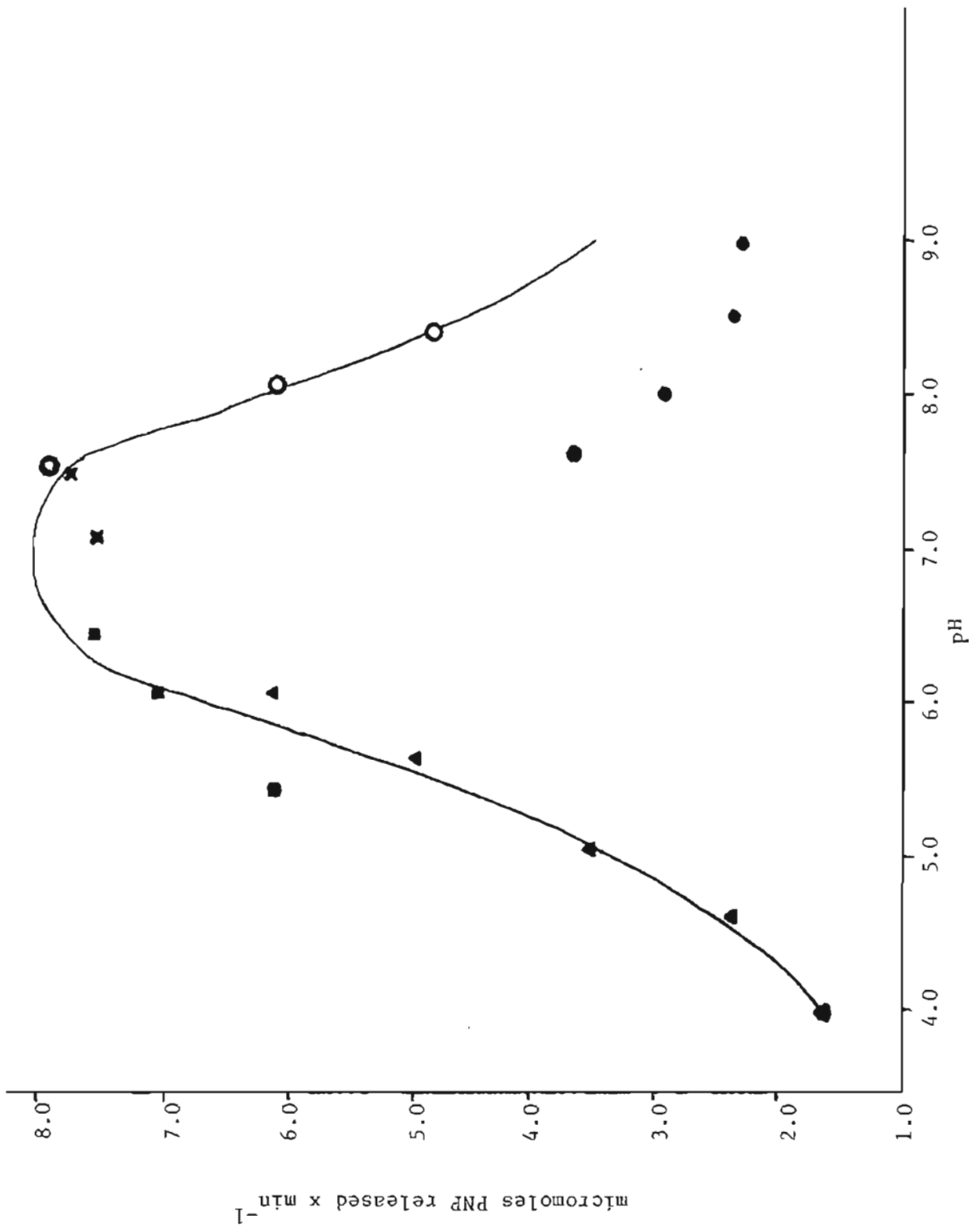


Figure 10

ACTIVITY VERSUS pH PLOT FOR THE EXTRACELLULAR ENZYME.

Enzyme assay procedures were performed as described in Materials and Methods with the following buffers: (▲) Succinate. (■) MES. (✕) MOPS. (⊙) HEPES. (●) Tris.

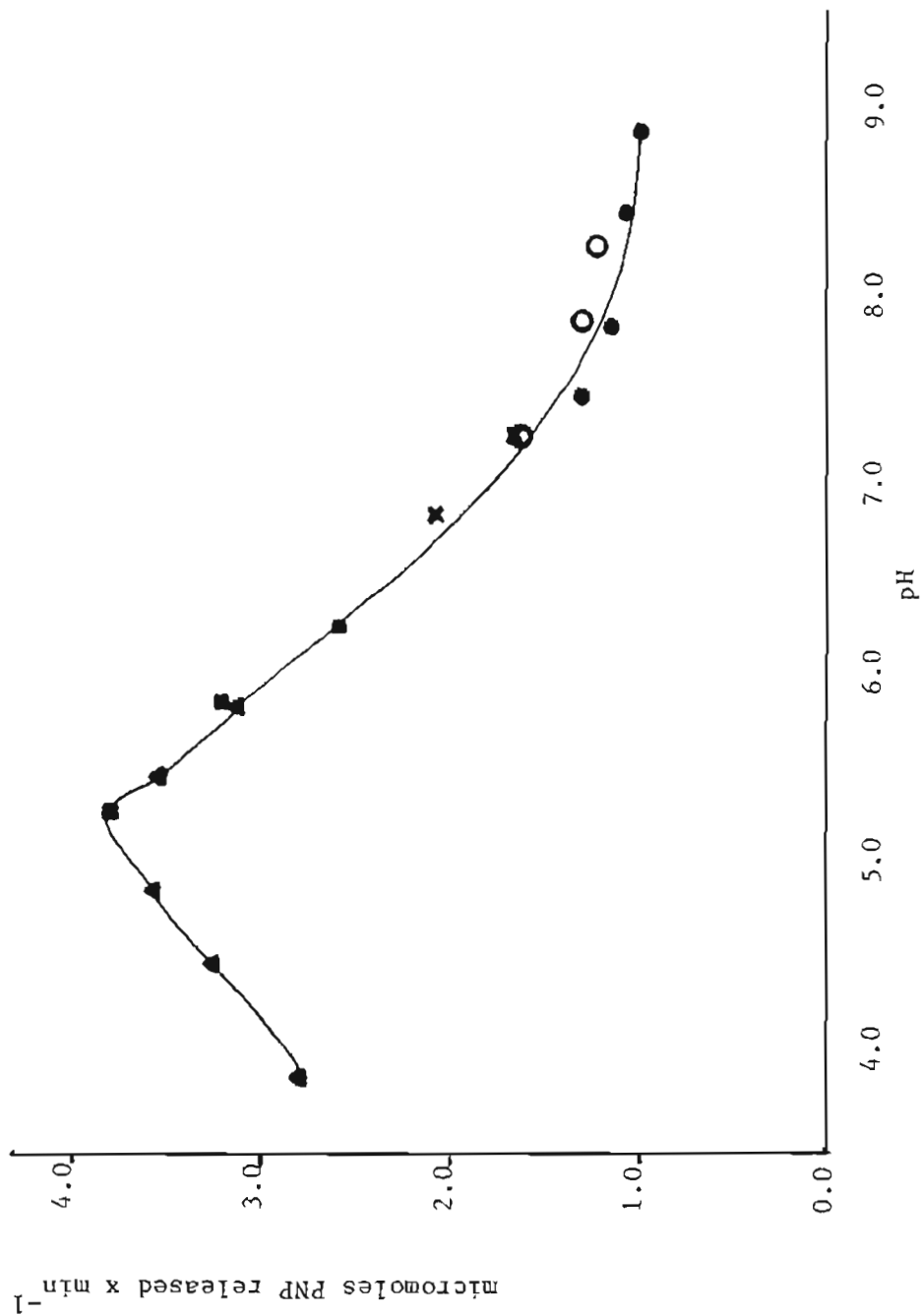
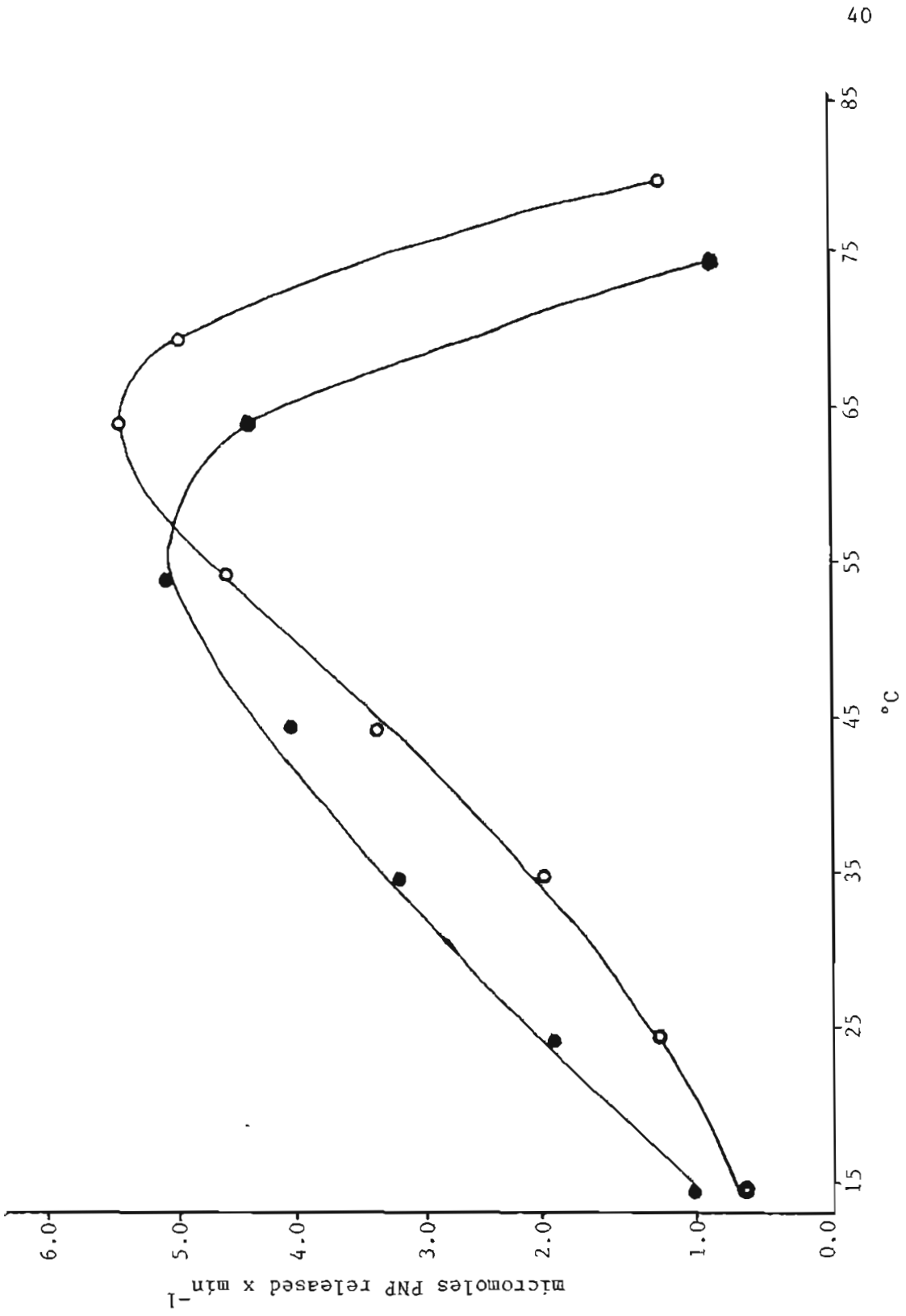


Figure 11  
TEMPERATURE RESPONSE CURVES FOR THE INTRACELLULAR (●) AND  
EXTRACELLULAR (○) ENZYMES.



enzymes was found to be 9.15 kcal/mole (ca.  $3.83 \times 10^4$  J/mole). PNPG saturation curves for both enzymes were hyperbolic. From Lineweaver-Burke plots,  $K_m$  values of  $1.1 \times 10^{-4}$  M and  $1.6 \times 10^{-4}$  M were calculated for the intracellular and extracellular PNPGases, respectively (Figures 12 and 13). The cellobiose saturation curve for the extracellular enzyme was hyperbolic, and the  $K_m$  for cellobiose was calculated to be  $5.3 \times 10^{-4}$  M (Fig. 14). Figure 15 shows a Dixon plot using extracellular  $\beta$ -glucosidase. The plot indicates that glucose is a competitive inhibitor with respect to PNPG. The  $K_i$  for glucose was calculated to be  $5.0 \times 10^{-4}$  M.

Figure 12

LINEWEAVER-BURKE PLOT FOR THE INTRACELLULAR  $\beta$ -GLUCOSIDASE.



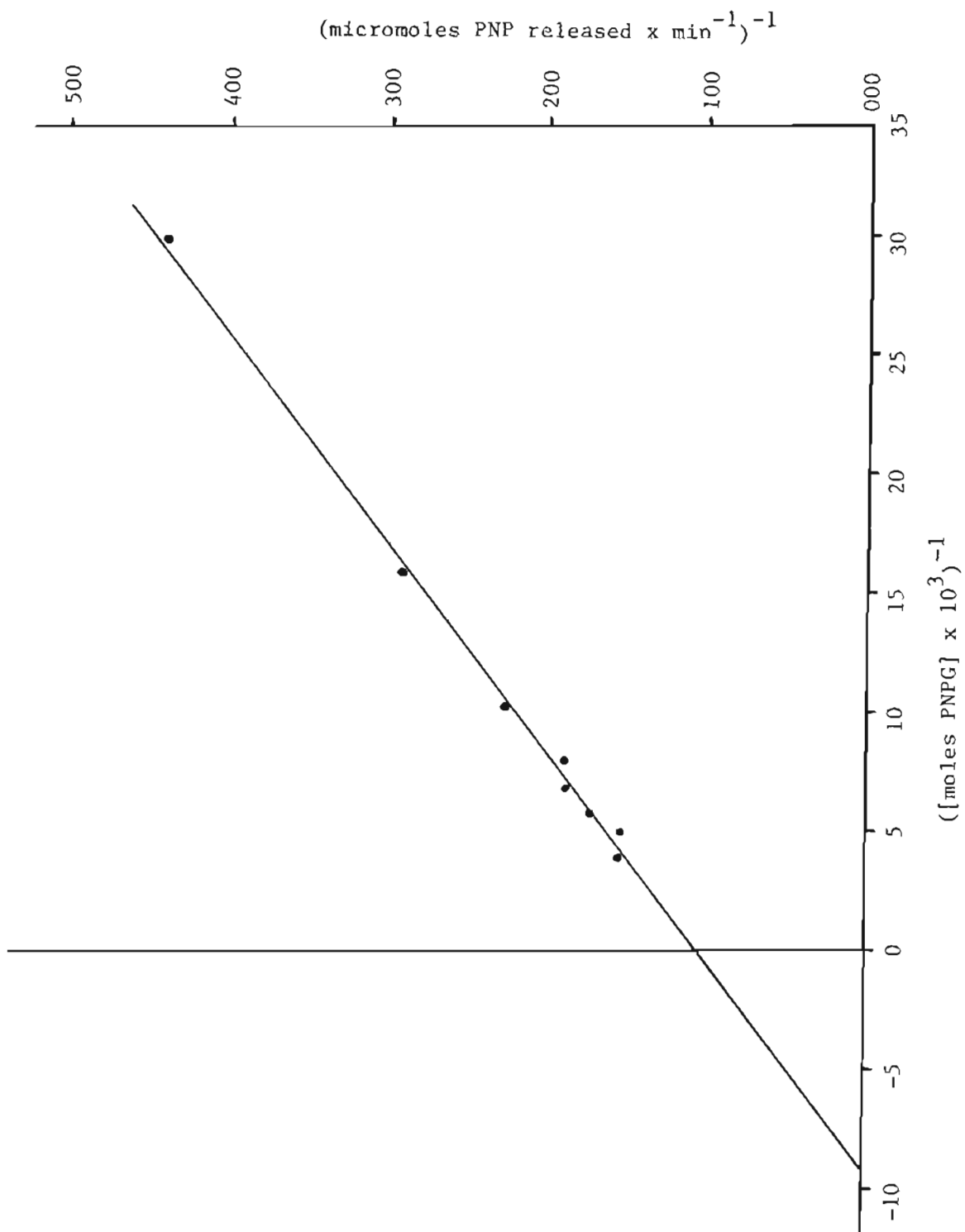


Figure 13

LINEWEAVER-BURKE PLOT FOR THE EXTRACELLULAR  $\beta$ -GLUCOSIDASE.

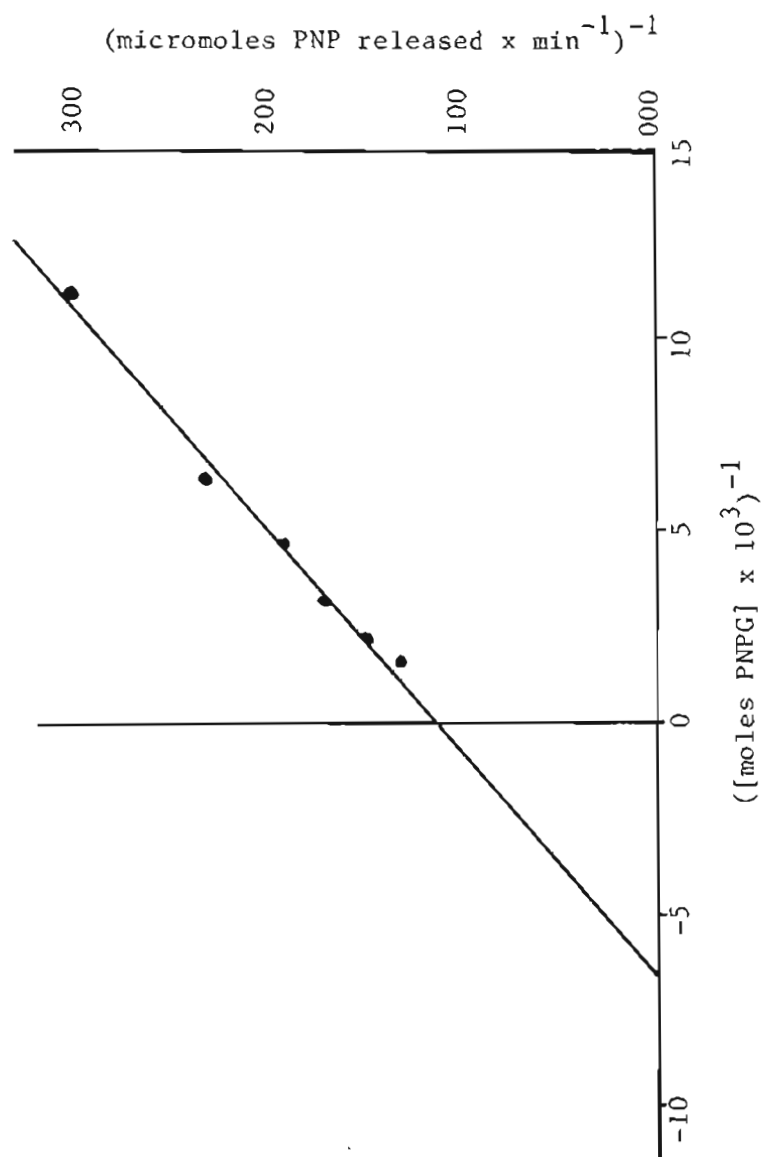


Figure 14

LINEWEAVER-BURKE PLOT FOR THE EXTRACELLULAR CELLOBIASE.

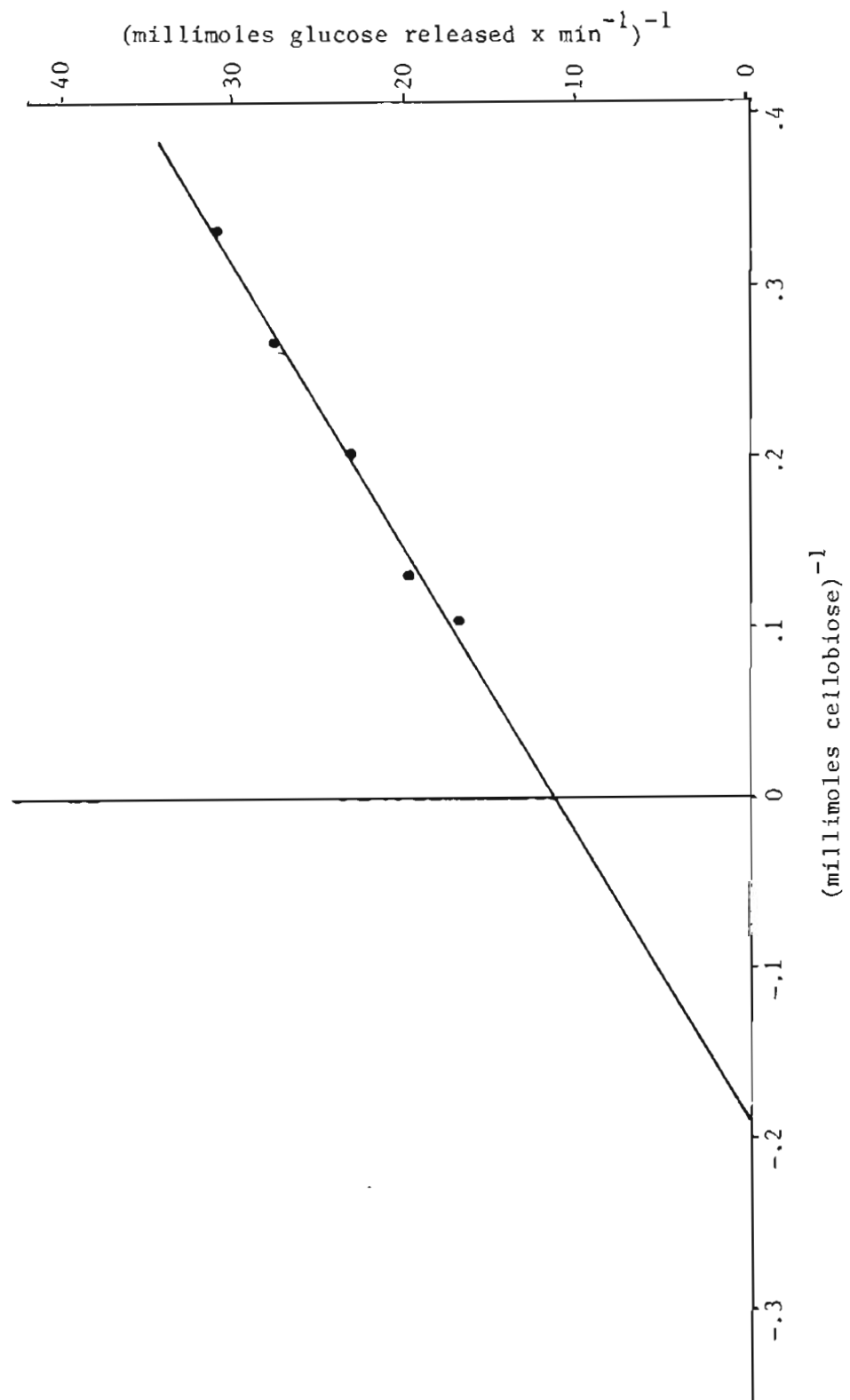
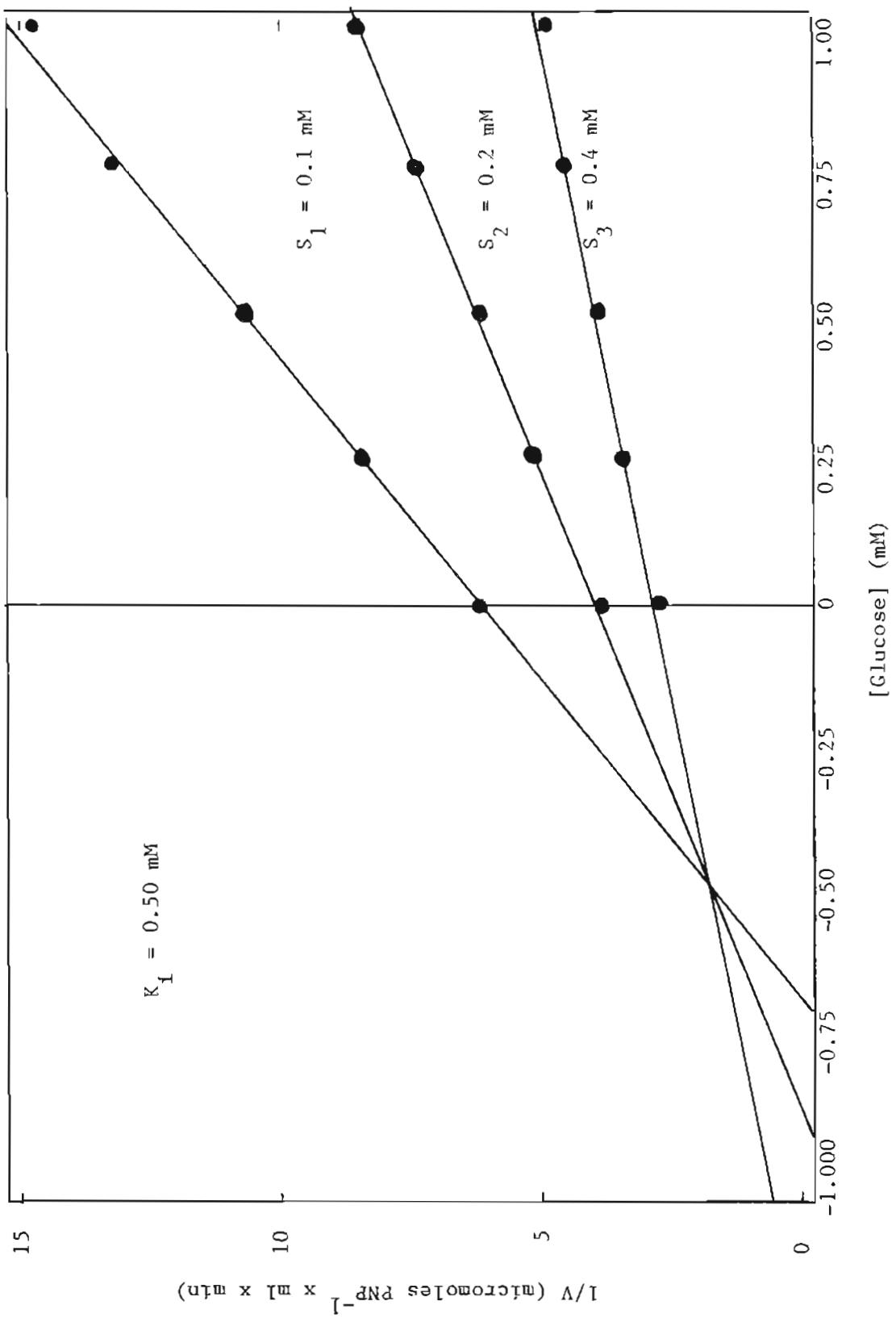


Figure 15

COMPETITIVE INHIBITION BY GLUCOSE OF  
EXTRACELLULAR  $\beta$ -GLUCOSIDASE ACTIVITY.

Dixon plot of the effect of glucose on the hydrolysis of PNPG.  $S_1$ ,  $S_2$ , and  $S_3$ , refer to the concentration of PNPG in the reaction mixture. PNP = p-nitrophenol.



## DISCUSSION

Several forms of  $\beta$ -glucosidase are produced by P. chrysosporium. An intracellular enzyme of molecular weight 410,000 is induced by cellobiose even in the presence of glucose. An extracellular enzyme of molecular weight 90,000 is induced by cellulose, but this induction is prevented in the presence of glucose. From these independent responses, it seems reasonable to speculate that the intracellular enzyme functions primarily in the assimilation of energy by producing glucose from cellobiose. Eriksson et al. showed that cellodextrins and cotton fibers treated with cellulase preparations from P. chrysosporium yielded cellobiose and glucose in the ratio 3:1 (16). Thus, cellobiose, being a major product of cellulose hydrolysis, may be the actual inducer of the intracellular enzyme. The cellulosic substrates, which also showed a high degree of inducing ability, may have been contaminated with significant quantities of cellobiose. Since glucose and cellobiose seem to appear together, repression of all  $\beta$ -glucosidase activity by glucose would result in accumulation of cellobiose. On the other hand, the appearance of glucose in the extracellular environment would be a signal that cellulolysis was proceeding, and repression would prevent the uneconomic production of excess cellulases. Indeed, Eriksson found that glucose repressed the production of all cellulolytic enzymes (14).



The extracellular enzyme activity was seen to be maximally induced by cellulose. The cellobiase activity of this enzyme may be just coincidental with a more general activity against polydextrans with  $\beta(1-4)$  linkages. The  $\beta$ -glucosidase from Lenzites trabea was found to have hydrolytic activity against cellodextrans up to five glucose units in length (21). Activity of the P. chrysosporium enzyme against these substrates was not investigated. Cellobiose appears to be easily assimilated by the cell to be hydrolyzed by the intracellular enzyme. The larger cellodextrans may be less easily assimilated, or not at all. The extracellular  $\beta$ -glucosidase may also function as an exoglucanase.

Deshpande et al. recently published the results of a study on the  $\beta$ -glucosidases of S. pulverulentum (13). They reported an extracellular enzyme which was maximally induced by cellulose. They separated the extracellular enzyme into five subfractions by a combination of isoelectric focusing and affinity chromatography on phenyl-Sepharose. These five enzymes were all found by sodium dodecylsulfate-polyacrylamide gel electrophoresis to have molecular weights of about 165,000 to 182,000, or roughly twice that found in this study.

Deshpande et al. also described a "cell wall-bound" enzyme which was maximally induced by cellobiose. Their method of analysis involved killing the cells with a one-hour treatment with 0.5% formalin and testing the killed and washed cells with PNPG. It is likely that they were actually observing the intracellular soluble enzyme which was leaking out of the dead cells. This is supported by their observations that this enzyme appeared very quickly (within 15-20 minutes after the

addition of cellobiose) and that when most of the nutrient carbon in the medium was cellobiose, the "cell wall-bound" enzyme predominated. In the study presented herein, cell wall-bound enzyme was not produced until about 15 hours after introduction of cellobiose, and intracellular soluble PNPGase reached a high specific activity very quickly after induction by cellobiose.

The  $K_m$  values for PNPG of the *P. chrysosporium* enzymes (about  $10^{-4}$  M) are similar to those observed for the enzymes from *Lenzites trabea* (21), *Trichoderma viride* (9), *Saccharomyces lactis* (37), and from the extracellular fluid of *Sporotrichum pulverulentum* (13). The molecular weights of the enzymes as determined by gel chromatography lie within the broad range of molecular weights found for various fungal  $\beta$ -glucosidases, from the relatively small enzyme from *T. viride* (m.w.  $\sim$  47,000) (9) to the comparatively large enzyme from *L. trabea* (m.w.  $\sim$  320,000) (21).

The inhibition of the extracellular enzymes by glucose was competitive, in contrast to the non-competitive inhibition by glucose of the *L. trabea* enzyme (21). The intracellular PNPGase and cellobiase activities reach their maximum levels within roughly the same time period after introduction of cellobiose, but cellobiase reached roughly 10 times the specific activity level of PNPGase.

This study has established the existence of at least two and probably three  $\beta$ -glucosidases which are characterized primarily by their cellular localization. They also differ in their induction parameters and in certain physical and chemical characteristics. The extracellular

enzyme activity has been shown to be specifically induced by polymeric glucose (cellulose) rather than its immediate substrate (cellobiose), and such production is prevented in the presence of glucose. The intracellular enzyme is produced de novo in response to cellobiose.

Several questions are raised by this study. The extracellular enzyme recovered by Deshpande and co-workers from the fungus they call S. pulverulentum was shown by them to have a molecular weight of roughly twice that of the extracellular enzyme described herein. Yet S. pulverulentum and P. chrysosporium are presumed to be conspecific. Two possibilities exist to explain the discrepancy. The two isolates may indeed be different organisms, or at least represent two phenotypes of the P. chrysosporium gene pool. More likely, different culture conditions have led to the dissimilarity. Of possible significance is the high concentration of citrate in the Vogel's medium, not present in the modified Norkrans medium used by Deshpande (13,15). Citrate, a very strong chelator, would severely restrict the availability of divalent cations, especially calcium and magnesium, in the medium. The addition of acid sodium citrate to blood at 15 mM concentration (36) is sufficient to inhibit blood clotting by sequestering calcium ions, preventing the activation of prothrombin. There is a higher concentration of calcium in blood than in Vogel's medium.

To investigate the relationship between cellulose hydrolysis and lignin degradation with a biochemical genetic approach, mutants should be isolated deficient in one or more of the hydrolytic activities involved.  $\beta$ -glucosidase activity might be monitored on differential media

by the hydrolysis of PNPG, or by the hydrolysis of methylumbelliferyl glucoside which releases the fluorescent methylumbelliferone upon hydrolysis of the glycosidic bond. The intracellular enzyme could be monitored specifically by adding no cellulosic substrates or including a small amount of glucose in the medium. The extracellular enzyme would be more difficult to specifically monitor, since it must be induced with cellulose, which also induces the intracellular enzyme to some extent.

The enzymes should be further characterized. The  $K_m$  for cellobiose of the intracellular enzyme should be determined. Inhibition of these enzymes by gluconolactone should be investigated and compared with the values reported by Deshpande. The hydrolytic activity of the partially purified extracellular enzyme on cellodextrins should be examined.

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## BIOGRAPHICAL NOTE

The author was born on January 26, 1954 in Boise, Idaho. He was raised on a small farm outside of Boise, and attended Meridian public schools. He was graduated valedictorian from Meridian High School in 1972. He attended Idaho State University from 1972 until his graduation in 1977, at which time he received Bachelor of Science degrees in both Chemistry and Microbiology.

In September 1977 the author enrolled in the graduate school of the Oregon Graduate Center for Study and Research at Beaverton, Oregon. In March, 1979, upon completion of the requirements for a Master of Science degree in Biochemistry, he left the Center to accept a position as Oral Biologist with Cooper Laboratories in Portland, Oregon, where he is presently employed.

The author is married to the former Debby Moyer. He is a member of the American Society for Microbiology, the American Chemical Society, and the American Association for the Advancement of Science.